Molecular Regulation of Early T-Cell Development in the Thymus

Moleculaire regulatie van vroege T-celontwikkeling in de thymus

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GENERAL INTRODUCTION

Parts of this chapter will be published as a review article in Leukemia

INTRODUCTION

The human body is under constant siege of pathogens - bacteria, viruses, fungi and parasites. We can only survive because these attackers are continuously fought off by our immune system. Important tasks within the immune system of vertebrates are performed by T lymphocytes, the executors of specific, cellular immunity.

The specificity of T lymphocytes lies in their T-cell receptor (TCR), through which they sense the presence of antigens in their environment. Each T cell expresses a TCR with a unique antigen-recognition site, so all T cells together can respond to an enormous variety of antigens. The highly diverse T-cell repertoire is generated by random recombination of discrete TCR gene segments. Via the TCR, T cells recognize peptide antigens that are displayed by antigen presenting cells (APCs), in the context of major histocompatibility complex (MHC) class I or class II molecules. Mature T cells carry out their function in cellular immunity as either $CD8^+$ cytotoxic T cells or as $CD4^+$ helper T cells. The humoral part of the specific immune system is supplied by B lymphocytes, which can secrete their antigen receptors in the form of antibodies.

T cells develop from multipotent precursors via a highly ordered, but complex differentiation pathway. A number of critical events occur during this T-cell development process. Cells proliferate, adopt a T-cell fate, and produce a TCR molecule via a strictly ordered process of gene rearrangements. Stringent selection processes make sure that the produced TCR molecule is self-MHC restricted but not reactive to self-antigens. Finally, the selected T cells are allowed to mature into functional effector T cells.

To become highly differentiated and thoroughly ‘educated’ cells, T cells need a specialized microenvironment for their development. The organ which has evolved to fulfill this task and which also gave T cells their name, is the Thymus. The hematopoietic cells that undergo T-cell development in the thymus are called thymocytes.

The importance of the thymus as essential microenvironment for T-cell development is illustrated by children afflicted by the DiGeorge syndrome, who sometimes completely lack a thymus. These children have severely reduced T cell numbers and suffer from recurrent life-threatening infections. DiGeorge syndrome also illustrates the fact that T cells are the only hematopoietic cell type that absolutely require the thymus for their development.

Although human T-cell development is the main theme of this thesis, most of the published knowledge about T-cell differentiation has been acquired by studies in the mouse. For that reason, this General introduction summarizes information about both human and murine thymocyte development. The main focus of this thesis is on the early ‘double negative’ stages of T-cell development in the thymus.

T-CELL DEVELOPMENT IN THE THYMUS

The thymus

The thymus is a bilobate organ located behind the sternum, just above the heart. It is remarkably large in newborns and children (Figure 1), reaching its maximum size (~25 cm^3) within the first 12 months of life. Thereafter the volume of true thymic tissue reduces progressively, and by adulthood the thymus is composed largely of adipose tissue[1]. This decline in thymus size and function during ageing is called thymic involution[2].

Organogenesis of the thymus in man starts at the end of the fourth week of gestation and in the mouse at day 10.5 of embryonic development (E10.5)[3,4]. Bilateral endodermal proliferations of the third pharyngeal pouch invade the underlying mesenchyme to form the thymic primordium or anlage. Between four and seven weeks (mouse E12.5), the primordia separate from the pharynx and migrate to their definitive location, where they fuse to form a single organ. The epithelial cells of the thymic rudiment develop through interactions with mesenchymal cells[5] and developing thymocytes[6,7] to finally generate a three-dimensional network architecture[8].

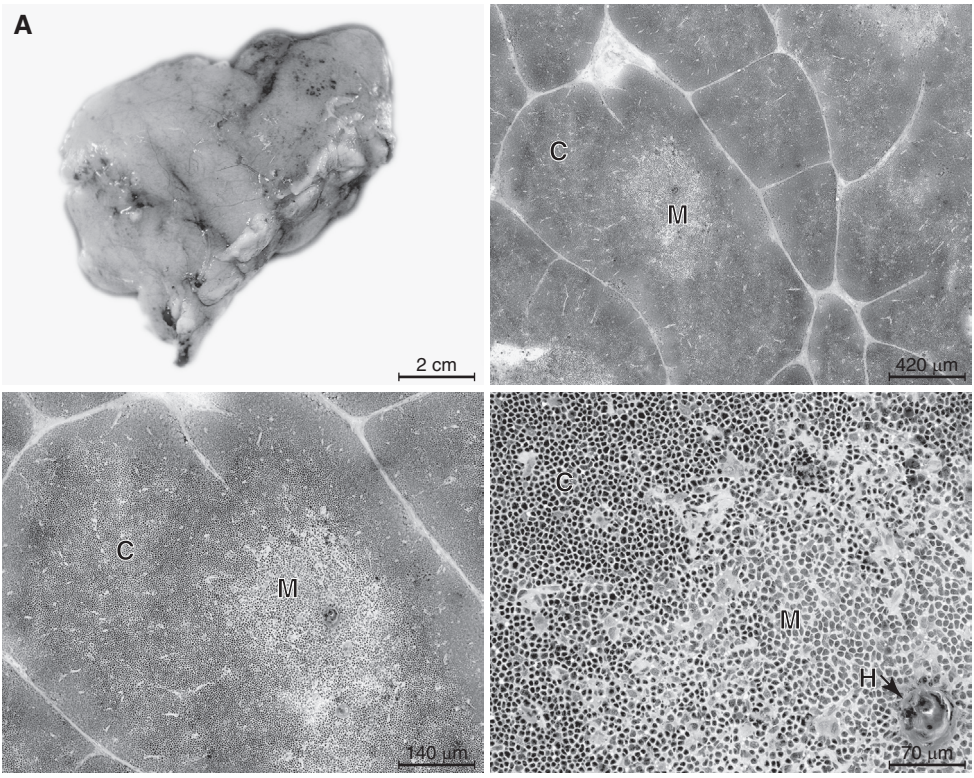
The bilobate structure of the thymus is surrounded by a thin fibrous capsule, invaginations of which form the septa that divide the lobes into many smaller pseudolobules and which carry blood vessels and nerves to the centre of the organ[8]. Every lobule comprises an outermost subcapsular region, a cortex, and an inner area, the medulla (Figure 1). Each of these regions contains several distinct types of thymic epithelial cells (TECs), creating a range of different microenvironmental niches, which provide signals for the developing thymocytes[9]. The cells that comprise the non-lymphocytic component of the thymus are together called the thymic stroma.

Origin of thymic progenitor cells

Shortly after the thymic anlage is formed, it is infiltrated by hematopoietic cells[10-12]. As the thymic rudiment is not yet vascularized, progenitor cells migrate into the anlage through the surrounding connective tissue[11,13], probably guided by chemotactic factors secreted from the thymic epithelium[14,15].

Like all blood cells, T cells are generated from a small cohort of pluripotent hematopoietic stem cells (HSCs). By definition, HSCs have self-renewal capacity and

Figure 1. Morphology of human and murine thymus.
(A) The right thymic lobe of a 6 month-old child operated for congenital heart disease. At this age, the thymus contains over $2x10^{10}$ thymocytes. HE stainings of tissue sections in three different magnifications show the lobular structure, the cortex (C) with high cellularity and the less cell-dense medulla (M). Hassall's corpuscles (H) are often found within the medulla. (B) The thymus of a 2.5 month-old (adult) mouse. A normal murine thymus can hold up to $2x10^8$ thymocytes. In the tissue section, cortical stromal cells are specifically stained (ER-TR4 antibody), and closely surround the thymocytes.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are pluripotent, i.e. they can give rise to cells of all hematopoietic lineages, including T and B cells, natural killer (NK) cells, dendritic cells (DCs), myeloid cells, erythrocytes and platelets[16,17]. The first adult-repopulating HSCs are generated in the aorta within the AGM (aorta-gonad-mesonephros) region at week four of gestation (mouse E10.5)[18,19]. However, umbilical and vitelline arteries, placental vasculature and the yolk sac may *de novo* generate HSCs as well. The mouse fetal liver is thought to be colonized by HSCs beginning at late E11 and serves as a reservoir for HSCs until the time of birth, when they migrate and settle in the bone marrow (BM)[20].

The progenitors that colonize the thymic anlage are probably not true HSCs, but may have a more restricted lineage potential. T-cell committed precursors have been detected in the murine AGM[21], fetal liver[22,23] and fetal blood[24]. Other studies describe the presence in fetal blood of T/K and T/NK/DC restricted progenitors, which are assumed to seed the thymus[25,26]. In any case, B cell potential has never been detected in the fetal thymus[27,28].

In adults, the hematopoietic stem cells that eventually give rise to thymocytes are located in the BM. Progenitors within the thymus can maintain thymocyte production only for short periods and seeding of the thymus with BM progenitors is therefore required to maintain thymopoiesis throughout adult life[29,30]. The number of thymus-colonizing cells is very low, probably only a few per day[29,31].

Also in the adult system, the identity of the thymus-seeding cell is unclear. In the mouse, several candidate thymus-colonizing progenitors have been proposed. These include lymphoid-restricted common lymphoid progenitors (CLPs)[32], early lymphoid progenitors (ELPs) that have limited myeloid potential[33] and multipotent $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells[34]. Even less is known about the identity of the thymus-seeding cell in man[35].

During the early stages of T-cell development, the multipotent progenitors that have entered the thymus lose the ability to differentiate into multiple lineages and their developmental potential becomes restricted to a T-cell fate. This process is called T-cell commitment[36].

Stages of T-cell development

Regardless of the identity of the seeding cell, it enters the thymus and starts T-cell development. The different events of T-cell development take place during a series of discrete phenotypic stages that can be recognized by expression of several key membrane molecules, mainly CD4 and CD8 (Figure 2). In both man and mouse, thymocytes are subsequently $CD4^{-}CD8^{-}$ (double negative, DN), $CD4^{+}CD3^{-}$ (human immature single positive, ISP) or $CD8^{+}CD3^{-}$ (mouse ISP), $CD4^{+}CD8^{+}$ (double positive, DP) and finally $CD4^{+}CD3^{+}$ or $CD8^{+}CD3^{+}$ (single positive, SP). As CD3 is part of the TCR complex, its expression indicates the presence of a functional TCR on the surface

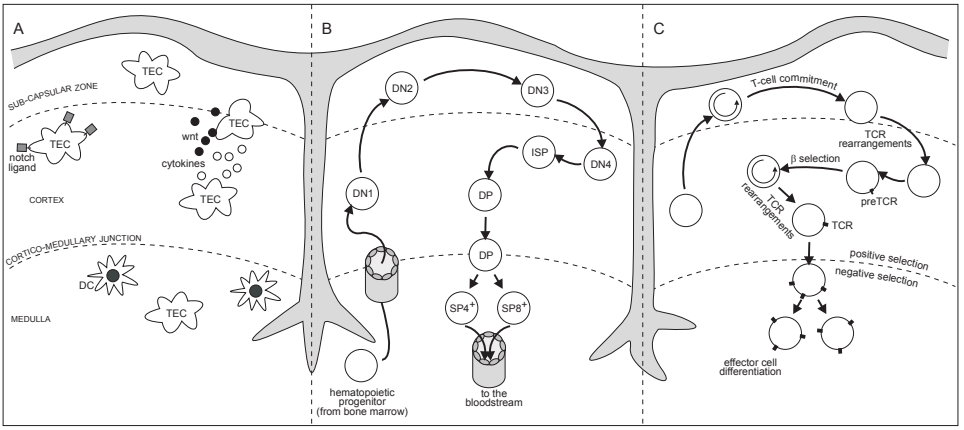

Figure 2. Schematic representation of three thymic lobules.
(A) The stromal compartments of the thymus. Each compartment contains specialized epithelial cells (TECs), which provide growth and differentiation factors for developing thymocytes. (B) The different thymocyte subsets and their migration through the anatomic niches. The earliest (DN) thymocytes enter the thymus at the cortico-medullary junction and migrate to the sub-capsular zone. Thymocytes differentiate into DP and SP cells while passing through the cortex and the medulla. (C) The primary events during T-cell development. Proliferation (indicated by a curved arrow), differentiation, TCR gene rearrangements and selection processes take place in different microenvironments.

membrane. In the mouse, the DN subset can be further subdivided into four stages: $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3) and $CD44^-CD25^-$ (DN4)[37]. In man, DN thymocytes are subsequently $CD34^+CD1a^-$ and $CD34^+CD1a^+$[38], and finally they loose expression of CD34, a marker characterizing immature cells[39].

The differentiation processes during T-cell development are in part guided by signals provided by the thymic stroma. The subsequent stages take place at defined anatomical sites within the thymus, which provide different external signals (Figure 2). Hematopoietic progenitor cells enter the thymus at the cortico-medullary junction[40]. As the numbers of progenitors that enter the thymus are very limited, a massive expansion takes place during the earliest stages of development. During these early DN stages, the thymocytes migrate to the outermost subcapsular region[40]. Here the first TCR gene rearrangements take place, which are described below. As thymocytes progress through the ISP and DP stages, they travel in opposite direction through the cortex, toward the medulla (Figure 2). Close to the cortico-medullary junction a series of selection processes ensues. The resulting immunocompetent medullary SP cells leave the thymus, at least in part via the blood[41].

Movements through the thymus are mediated by chemokines secreted by and adhesion molecules expressed on the thymic epithelium. Migration of the earliest

progenitors toward the outer cortex is dependent on chemokine receptors CCR7 and CCR9 on the thymocytes[42,43]. CCR7 is also involved in the migration of selected SP cells through the medulla[44]. In addition, adhesion molecules such as VCAM1 on the thymic epithelium are likely to be involved in thymocyte migration[45].

The generation of a T-cell receptor

The different steps of T-cell development all aim at the generation of mature T cells, each of which expresses a unique, self-MHC restricted and non-autoreactive TCR. This is achieved by random recombination of discrete TCR gene segments and subsequent shaping by intrathymic selection events.

TCR molecules consist of two disulfide-linked chains, either a TCRα and a TCRβ chain or a TCRγ and a TCRδ chain, each containing a constant and a variable domain[46,47]. The variable domain is responsible for the recognition of peptide/MHC complexes and is encoded by a combination of one of the many available V (variable), D (diversity, only for β and δ), and J (joining) gene segments. These gene segments are coupled via a tightly regulated series of TCR gene rearrangement steps, or V(D)J recombination, an example of which is depicted in Figure 3. Consequently, many different combinations

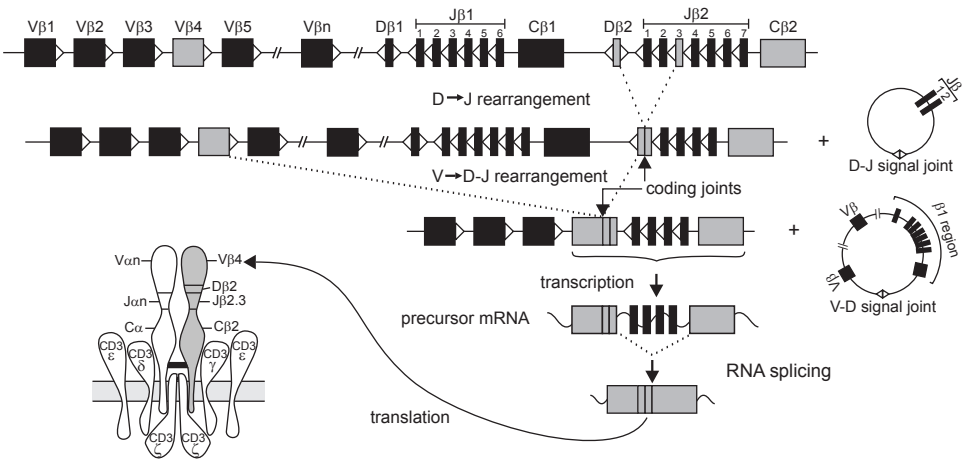

Figure 3. Schematic diagram of sequential rearrangement steps, transcription, and translation of the *TCRB* gene during T-cell differentiation.

In this example, first a Dβ2 to Jβ2.3 rearrangement occurs, followed by Vβ4 to Dβ2-Jβ2.3 rearrangement, resulting in the formation of a Vβ4Dβ2Jβ2.3 coding joint. The two extrachromosomal TCR excision circles (TRECs) that are formed during this recombination process are indicated as well; they contain the D-J signal joint and V-D signal joint, respectively. The rearranged *TCRB* gene is transcribed into precursor mRNA, spliced into mature mRNA, and finally translated into a TCRβ protein. The mature TCR, a dimer of a TCRα and a TCRβ chain, is expressed on the surface membrane together with the CD3 complex, consisting of CD3γ, CD3δ, CD3ε and CD3ζ proteins.

of gene segments can be made, which together represent the so-called combinatorial repertoire of TCR molecules.

The TCR gene segments are flanked by recombination signal sequences (RSS) consisting of conserved palindromic heptamer and nonamer sequences, separated by a spacer (12 or 23 nucleotides). The two recombination activating gene (RAG) proteins recognize the RSS sequence and initiate the rearrangement by introducing a DNA double strand break between the RSS and the rearranging gene segment. This can then be religated to another gene segment, forming a coding joint (Figure 3). Between the coupled gene segments nucleotides can be deleted or randomly inserted, leading to imprecise coupling of gene segments. This results in the so-called junctional diversity, which together with the combinatorial diversity guarantees an enormous repertoire of over 10^{12} different TCR molecules.

Rearrangements of the different TCR genes occur in a fixed order. During the DN stages, the *TCRD* locus rearranges first, followed by the *TCRG* genes[47,48] (Figure 2). Successful rearrangements may result in a functional γδ TCR and the development of a TCRγδ$^+$ T cell. Alternatively, a cell may enter by the αβ lineage by synthesizing a functional β-chain, which locus starts rearranging shortly after *TCRG*.

During the ISP stage, the functionality of the TCRβ chain is tested by expressing it on the cell surface together with the invariant pre-TCRα (pTα) chain (Figure 2), a process called β-selection[49]. Signaling by this pre-TCR complex induces the cells to enter the cell cycle and triggers differentiation into DP cells. Proliferation then ceases and *TCRA* gene rearrangements are initiated, starting with deletion of the *TCRD* gene, which is located between the Vα and Jα gene segments[48,50].

Productive rearrangements lead to the expression of a TCRαβ complex on the cell surface, which is then tested for the recognition of self-MHC molecules (positive selection) and absence of reactivity against self-antigens (negative selection)[51]. It has been estimated that 95% of developing thymocytes die by apoptosis[52]. The surviving cells become SP thymocytes that mature into either CD4$^+$ T helper cells or CD8$^+$ cytotoxic T cells, and migrate to the periphery as naïve recirculating T lymphocytes.

MOLECULAR REGULATION OF EARLY T-CELL DEVELOPMENT

β-selection and subsequent events are mediated by signals emanating from successively the pre-TCR and the TCR. The proliferation, commitment and rearrangement steps during early T-cell development are initiated and regulated by TCR-independent signals provided by the microenvironment. If a cell is receptive to a signal (by expressing the correct receptor), an intracellular signal transduction pathway is initiated and finally transcription factors will be activated. Transcription factors bind

to specific DNA sequences present in the core promoters or enhancers of genes, and thereby initiate gene-specific transcription. The coordinated expression of cohorts of genes will alter the phenotypical and the functional characteristics of the differentiating cell.

Regulatory signals provided by the thymic epithelium include chemokines and adhesion molecules, cytokines, Wnt proteins and Notch ligands. The molecular mechanisms of cytokines, Wnt proteins and Notch ligands, as well as their function during T-cell development, are described in the next paragraphs.

Cytokines

Cytokines are small soluble proteins secreted by one cell that can alter the behavior or properties of the same or another cell. A wide variety of soluble factors are produced by the thymic stroma[53], but surprisingly few cytokine or cytokine receptor mutant mice display alterations in thymocyte development[54]. Three cytokines that are likely to play important roles during the early stages of thymic differentiation are Interleukin 7 (IL-7), stem cell factor (SCF, the ligand for c-Kit (CD117)), and Fms-related tyrosine kinase 3 ligand (Flt3L, the ligand for fetal liver kinase 2 (Flk2, also called Flt3)).

IL-7 engages a receptor complex that consists of two chains: the IL-7 receptor α (IL-7Rα, CD127) and the common γ-chain (γc). The γc chain is also used by the receptors for IL-2, IL-4, IL-9, IL-15 and IL-21. Signals through the IL-7R are primarily transmitted by the Jak kinases Jak1 and Jak3 to the signal transduction and activator of transcription (STAT) 5[55]. Mice lacking IL-7, IL-7Rα, γc chain or Jak3 all exhibit a profound reduction in thymic cellularity. Nevertheless, all major thymocyte subsets (except for TCRγδ$^+$ cells) can be detected in such mice[56,57]. In remarkable contrast, human patients deficient for the same molecules completely lack T cells[58,59]. The principal function of IL-7 is supporting survival and proliferation of DN2 cells[60]. In addition, *in vitro* studies have suggested a (controversial) role for IL-7R signaling in promoting TCR rearrangements[61].

c-Kit is expressed in HSCs and very early progenitors. In the thymus, expression is high in the earliest thymocytes and is absent from DN3 onward[62]. Mutations of c-Kit or SCF result in a ten fold decrease in HSCs and blood leukocytes, and an even more severe reduction in DN1 thymocytes[63]. Together with data from *in vitro* studies, this suggests that SCF is necessary for the expansion of the earliest thymocytes[62].

The significance of Flt3L for T-cell development is controversial, as mice deficient for Flk2 did not show any abnormalities in their thymus[64], but adding Flt3L to cultures of developing thymocytes did increase thymocyte numbers[65].

Wnt signaling pathway

Wnt genes encode a large family of secreted glycoproteins that regulate cell-to-cell interactions in many different cell types and various species[66]. The central player in the Wnt-signaling cascade (described in detail in Figure 4) is the cytoplasmic protein

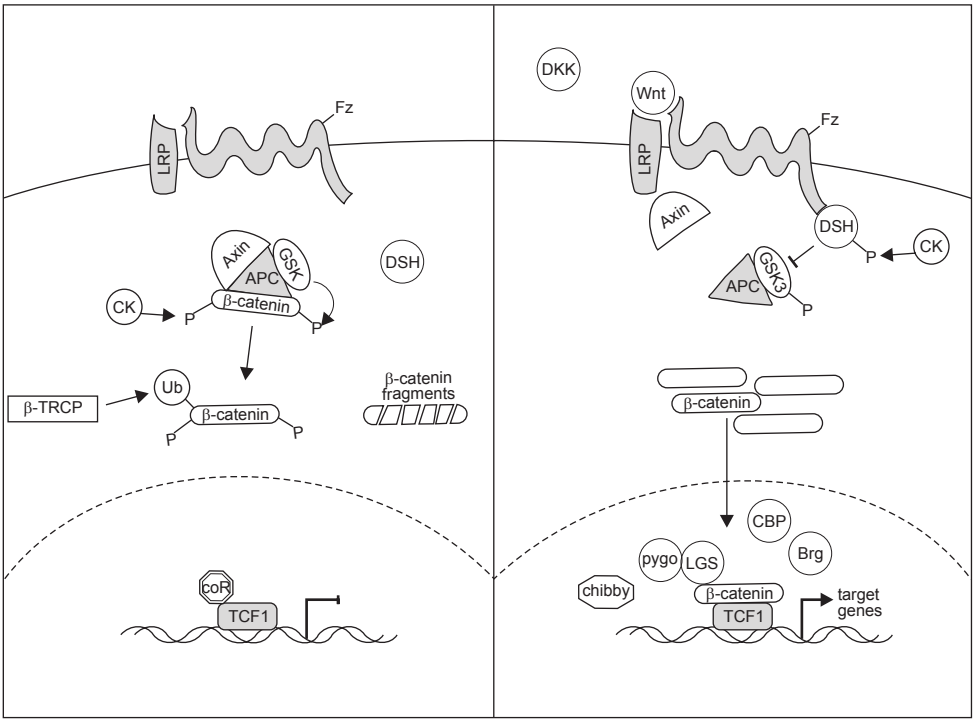

Figure 4. The canonical Wnt signaling pathway.
Left: In the absence of Wnt binding, β-catenin is sequestered in the cytoplasm by the tumor suppressor proteins Axin and adenomous polyposis coli (APC)[73]. While present in this 'destruction complex', β-catenin is phosphorylated by serine/threonine kinases casein kinase (CK) and glycogen-synthase kinase 3 (GSK3)[74]. Phosphorylated β-catenin is recognized by β-transducin-repeat-containing protein (β-TrCP), targeted for ubiquitination and degraded by the proteasome[75]. In the absence of β-catenin, members of the high mobility group (HMG) box containing T-cell factor/lymphocyte-enhancer-binding factor family (Tcf1, 3 and 4 and Lef1) are bound in the nucleus by transcriptional co-repressors (CoR) of the groucho family. Repression is mediated by recruitment of histon deacetylaces (HDAC)[76]. Right: Wnts bind to a receptor complex consisting of a member of the Frizzled (Fz) family of seven transmembrane proteins and the low-density lipoprotein receptor-related protein LRP5 or LRP6. How Wnt signals are transduced to the destruction complex is unclear. For sure, the Dishevelled (Dsh) protein is involved[77]. Dsh may directly interact with Fz[78]. In addition, LRP may directly interact with Axin[79]. Signaling results in inhibition of GSK3, which no longer can phosphorylate β-catenin, allowing accumulation of β-catenin and its translocation to the nucleus. Furthermore, various studies indicate that only dephosphorylated β-catenin is the transcriptionally active form[80,81]. β-catenin is targeted to the nucleus by Pygopus (pygo) and Legless (LGL, Bcl-9)[82]. Binding of β-catenin to Tcf recruits histon acetylace CBP/p300[83] and Brg-1, a component of the SWI/SNF chromatin remodeling complex[84]. Several factors can modulate Wnt signaling. For instance, interaction of Wnt with the receptor complex can be prevented by Dickkopf (Dkk), which binds to the LRP co-receptor[85]. Binding of β-catenin to Tcf/Lef can be inhibited by the nuclear protein Chibby[86]. Many other factors, known and yet unknown, may be involved in this pathway. Next to the canonical Wnt-signaling pathway, β-catenin independent mechanisms exist (reviewed in reference 87), but their implications for T-cell development have not been studied extensively.

β-catenin. In the absence of Wnt signaling, β-catenin is present in a cytoplasmic 'destruction complex' and is continuously phosphorylated and degraded. When a Wnt protein binds to the receptor complex consisting of a Frizzled (Fz) receptor[67] and a low-density lipoprotein (LDL) receptor-related protein (LRP5 or LRP6)[68], β-catenin is no longer phosphorylated and translocates to the nucleus, where it activates Tcf/Lef transcription factors[69,70], in thymocytes predominantly Tcf1[71,72].

In the thymus, Wnt proteins are mainly produced by the thymic epithelium, but to some extent also by the thymocytes[88-90]. Conversely, Fz receptors are expressed primarily by the thymocytes, but also by stromal cells[88,89]. Not much is known about which Wnts bind to which receptors. Biochemical studies are complicated by the fact that Wnts are palmitoylated and as a result hydrophobic and difficult to isolate[91]. Thus far, only Wnt3a has been produced as a recombinant protein[91]. This also complicates the direct analysis of the effects of Wnt proteins on developing T cells *in vitro*. Nevertheless, numerous studies have now revealed functional roles for several Wnt signaling components in T-cell development.

Using a Tcf reporter construct, in which expression of a reporter gene is turned on when Wnt signaling occurs in a cell, it was demonstrated that developing thymocytes can respond to Wnt signals in *in vitro* cultures[88]. Retroviral transduction of fetal thymocytes with Wnt1 and Wnt4 increased their proliferation in culture. Furthermore, blocking Wnt binding using soluble Fz receptors completely inhibited T-cell development at the DN and ISP stages[88]. The requirement of Wnt proteins for thymocytes was confirmed *in vivo*, by showing that mice deficient for both Wnt1 and Wnt4 have about 50% of the normal thymic cell numbers[92]. This relatively mild reduction, combined with the fact that thymocyte subset distribution was normal in these mice, points out that different Wnt proteins are functionally redundant. The significance of Fz receptors in thymocytes was also indicated by the accelerated thymic atrophy observed in mice deficient for Fz9[93]. But again the mild defect emphasizes the involvement of multiple Fz.

Of the downstream Wnt signaling components, Tcf1 has been most extensively studied. Two different Tcf1 mutant mice have been described, targeting either exon 5 (Tcf1(V)) or exon 7 (Tcf1(VII))[94]. In Tcf1(V) mutant mice, a low level of a truncated yet functional Tcf1 is still expressed, whereas the Tcf1(VII) mutation abolishes the DNA-binding activity of Tcf1 completely and is therefore regarded as a true Tcf1 null mutant. Young Tcf1 deficient mice have an incomplete block at the DN1, DN2 and ISP stages, while older mice display a complete block at the DN1 stage of thymocyte development[94,95].

Although Lef1 knock-outs have normal T-cell development[96], Lef1/Tcf1(V) double deficient mice display a complete block in T-cell differentiation at the ISP stage[97], showing the redundancy of these factors during thymocyte development.

The critical role of Tcf1 in T-cell development may also lie in Wnt independent

functions, for instance as transcriptional repressor. However, β-catenin binding to Tcf1 is essential for thymocyte differentiation, as only Tcf isoforms which contain the N-terminal β-catenin binding domain could rescue the thymic defect in Tcf1(VII) mice[88,98].

The importance of β-catenin for T-cell development is controversial. Presence of β-catenin mRNA was detected in all thymoycte subsets[89]. Mice with β-catenin deleted in all T-cells from DN3 onwards showed impaired T-cell development at the β-selection checkpoint[99], but mice transplanted with hematopoietic stem cells in which β-catenin had been inducibly deleted, did not show in any thymic defect[100]. As possible explanation, redundancy between β-catenin and its homologue plakoglobin (γ-catenin) has been suggested. Irradiated mice reconstituted with plakoglobin-deficient fetal liver cells did not display marked defects in T-cell development, only a slightly reduced survival of DN3 and DN4 thymocytes[101]. Nevertheless, mice overexpressing Axin, one of the components of the β-catenin destruction complex, exhibited reduced cell numbers and increased apoptosis in their thymus[102].

The question remains why the Wnt pathway is important for T-cell development. The above-described studies suggest that Wnt signaling affects those stages of thymocyte differentiation where proliferation occurs. A role for Wnt proteins in inducing proliferation would fit with the assumed function of Wnt signaling in stem cell self-renewal[103]. Wnt signaling does probably not directly induce a T-cell program, as transcription of T-cell specific genes and rearrangement of the *TCRB* genes is unaffected in Tcf/Lef deficient mice[97]. Furthermore, transplantation of irradiated mice with HSCs that overexpress a constitutively active form of β-catenin, lead to better reconstitution of all hematopoietic lineages, not preferentially T cells[104].

Additional roles for Wnt signaling have been reported in more mature stages of thymocyte development [98,99,101,105,106]. In these subsets, Wnts probably block apoptosis rather than inducing proliferation.

Wnt proteins are not only important for developing thymocytes, but also for thymic epithelial cells, in which expression of Fz receptors has been demonstrated as well[89]. Thus, Wnt proteins may provide part of the 'cross-talk' that exists between thymocytes and their microenvironment[7]. Interestingly, the expression in thymic epithelium of FoxN1, the transcription factor defective in a-thymic nude mice, is regulated by Wnt signaling[90].

Notch signaling pathway

The Notch signal transduction pathway is an evolutionary conserved mechanism that regulates cell fate determination during developmental processes[107]. The first indications that Notch might be involved in T-cell development, came from the finding that a part of the *NOTCH* gene (then termed *TAN-1*) is translocated to the *TCRB* locus in rare cases of human T-cell acute lymphoblastic leukemia (T-ALL)[108]. Later it was shown

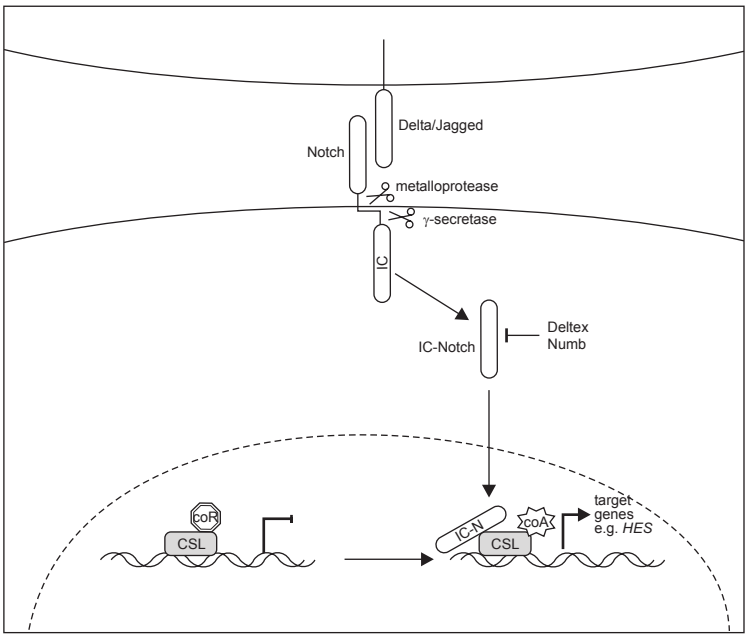

Figure 5. The Notch signaling pathway.
The Notch receptor consists of a large extracellular part containing multiple EGF-like repeats[110] and an intracellular part that includes several functional domains mediating Notch signal transduction. The glycosylation status of the Notch receptor can be modified by Fringe proteins (manic, lunatic and radical Fringe), thereby influencing ligand binding[111]. Ligands are of the Delta/Serrate/LAG-2 (DSL) family[107]. Interaction of Notch with a ligand can induce proteolytic cleavage of Notch, successively by metalloprotease and γ-secretase activities[112,113], releasing the intracellular part of the protein (intracellular (IC-) Notch). IC-Notch then translocates to the nucleus and binds to the nuclear transcription factor CSL (CBF/Suppressor of Hairless/Lag-1; in human usually called CBF1, in mouse RBP-Jκ)[114]. Deltex1[115] and Numb[116] are cytoplasmic regulators of Notch signaling.
Binding of IC-Notch to CSL induces the dislocation of co-repressors (coR) such as Msx2-interacting nuclear target protein (Mint[117]) and Notch-regulated ankyrin-repeat protein (Nrarp[118]), and recruitment of co-activators (coA), such as Mastermind (Maml[119]), and stimulates the transcription of Notch target genes. The best known Notch-target gene is *HES1* (Hairy-Enhancer of Split 1)[120], but undoubtedly many others exist. Several reports suggest alternative Notch signaling pathways which are CSL-independent and may involve Deltex as an effector protein[121].

that a large proportion of human T-ALLs have activating mutations in the *NOTCH1* gene[109]. The Notch family of transmembrane receptor consists of four members, Notch1 to 4, which can bind to the ligands Delta1, 3 and 4 and Jagged1 and 2. Signaling occurs via a unique mechanism, in which part of the receptor (intracellular Notch; IC-Notch) functions as a transcriptional activator. The pathway is described in detail in Figure 5.

In contrast to cytokines and Wnt proteins, the ligands that induce Notch signaling are not secreted, but rather expressed on the membrane of thymic epithelial cells. High levels of Notch ligands Jagged1 and 2 and Delta1 and 4 are expressed in the fetal and adult murine thymus[27,122-124]. Expression of Notch ligands has been reported in murine and human BM stroma (Jagged 1[125,126]) and murine fetal liver (mainly Delta1 and 4[27]) as well. Notch receptors are expressed by hematopoietic progenitor cells in the BM and fetal liver[123,127-129] and by DN thymocytes (predominantly Notch1 and 3[124,129,130]).

When Notch-expressing hematopoietic progenitor cells enter the thymic micro-environment and interact with Notch ligands on the thymic epithelium, they immediately start expressing Notch target genes[123]. This Notch signal is essential for the induction of a T-cell fate in these cells. This was first demonstrated in mice in which the *NOTCH1* gene was conditionally deleted, resulting in a complete block in T-cell development at the DN1 stage[131] and the emergence of ectopic B-cell development in the thymus[132]. These effects are mediated by Notch1 signaling through CSL, since CSL deficient mice display a similar phenotype[133]. Conversely, BM reconstitution of mice with cells transduced with IC-Notch instructed a T-cell fate in BM progenitors and inhibited B-cell development[134]. Similar results were obtained when mice were transplanted with human hematopoietic progenitors overexpressing IC-Notch1 or 4[127,135].

The expression of the different Notch ligands and their receptors in a stage- and tissue-specific manner raises the possibility of a unique function for each ligand[124]. However, the mechanisms by which specific ligands induce different cellular fates remain unclear.

Transplantation of mice with progenitors overexpressing Delta1 or 4 phenocopied the effect of overexpression of IC-Notch: DP cells emerged in the BM[136,137]. *In vitro*, this phenomenon can be mimicked using BM–derived stromal cell lines which otherwise promote myeloid and B-cell development of hematopoietic progenitor cells. Forced expression of Delta1 in these stromal cell lines induces murine and human progenitor cells to develop into T cells, in some studies up to DP and SP stages[138-141]. This co-culture system will be described in detail in the next paragraph. The striking T-cell generating effect of Delta could not be reproduced by expression of Jagged in stroma cell lines[138], although Jagged did inhibit development into B cells[140]. Furthermore, αβ T cell development was normal in Jagged2 deficient mice[142]. Signals induced by Jagged possibly promote NK cell or TCRγδ^{+} cell differentiation[140,142,143].

To further complicate matters, stable induction of a T-cell fate by Delta is dependent on contact time[144] and density of ligands, with lower doses of Delta also promoting B-cell development[145]. Furthermore, the interaction of Notch with the different ligands can be modulated by Fringe proteins[111]. Overexpression in the thymus of Lunatic Fringe, which is thought to inhibit Notch activation by Jagged and facilitate interaction with Delta[146], blocked T-cell commitment[147]. This finding suggested that Notch signals activated by

Jagged might be functionally more important for thymocytes than signaling through Delta.

The downstream mechanisms by which a Notch signal is translated into a T-cell program are still largely unclear. The best known Notch target genes encode Hairy-Enhancer of Split (Hes)1 and 5 and Hes-related repressor protein (Herp), basic-helix-loop-helix (bHLH) proteins that function as transcriptional repressors[120,148]. Indeed, overexpression of Hes1 and Hes5 in the BM partly inhibits B-cell development[149]. But although proliferation of early thymocytes is severely affected by Hes1 deficiency[150,151], thymocytes do develop in these mice. Hes1 can therefore not be the sole target of Notch signaling in the thymus.

Like Wnt signaling, the Notch pathway has also been implicated in later stages of T-cell development, mostly in the DN to DP transition[118,152-154]. Furthermore, mice in which Notch1 was deleted after the T/B-fate choice (Lck-cre x Notch1$^{lox/lox}$) show a complex phenotype which suggests that Notch1 contributes to both V to DJ rearrangement of the *TCRB* locus as well as to the physical elimination of cells which fail β-selection[155].

There are conflicting data on a function of Notch signalling in promoting αβ T cell development at the expense of γδ T cells[155-157] and in the selection between CD4 and CD8 SP thymocytes[153,154,158-160].

Other transcription factors

Next to Tcf1 and CSL, numerous other transcription factors have been described to be involved in T-cell development (reviewed by Rothenberg and Taghon[161]). Among the transcription factors that are crucial for the early stages of T-cell differentiation are GATA3 and bHLH factors.

GATA3 is a member of a transcription factor family that binds a GATA-consensus motif through a highly conserved zinc finger binding domain[162]. Within the hematopoietic system, GATA3 expression is confined to multipotent progenitors, T lymphocytes and NK cells[163-165]. Germline knock-out experiments show that GATA3 is critical for the generation of any identifiable T-cell precursors, even DN1 cells[164]. Furthermore GATA3 is necessary for normal β-selection, proliferation in the DN to DP transition, development of CD4 SP cells and for survival and differentiation of peripheral T cells[166-169].

The class I bHLH proteins, also known as E-box proteins, comprise E2A, HEB and E2-2, which bind DNA as homo- or heterodimers[170]. Of these transcription factors, E2A and HEB have been shown to be involved in T-cell differentiation. Overexpression of dominant negative mutants of HEB or any of the Id factors (HLH factors lacking the basic DNA-binding domain which heterodimerize with E-box proteins) completely blocks T-cell development at the DN stage[171-173].

T-CELL DEVELOPMENT *IN VITRO*

Studies of developmental processes, especially of human cells, call for the use of *in vitro* culture systems. B-cell development can be readily reproduced *in vitro* with the use of BM-derived stromal cell lines and the addition of defined sets of cytokines[174]. Myeloid and erythroid differentiation events can be studied in culture dishes that contain a mixture of defined cytokines in a semisolid medium (colony assays)[175]. However, until recently, the only way to induce T-cell development *in vitro* was to culture cells in a real thymic microenvironment by use of fetal thymic organ cultures (FTOCs)[176]. For this purpose, fetal thymic lobes of day 14 (E14) of gestation are isolated from mouse embryos. Around E14, the thymic anlage is colonized by progenitors from the hematopoietic tissues, implying that at this time-point the microenvironment is receptive for hematopoietic cells. E14 thymic rudiments are about one millimeter in diameter, demanding the use of a microscope. Lobes are depleted of endogenous thymocytes by treatment with deoxyguanosine[177] or by γ-irradiation[178] and the 'empty' lobes can be reconstituted with defined subsets of murine or human progenitor cells. This can be achieved using the 'hanging drop' method, although the inoculation of precursors via microinjection is more efficient[179]. The reconstituted lobes are maintained at an air/liquid interface by culturing them on polycarbonate filters.

FTOC is readily manipulable and allow analysis of the effects of reagents such as pharmacological inhibitors, neutralising antibodies and cytokines. Furthermore it facilitates study of the molecular regulation of T-cell development using retro- or lentiviral infection of precursors prior to their introduction into thymic lobes[178,180]. Finally, the roles of individual stromal cell components can be studied using a modified form of FTOC: reaggregate thymus organ cultures (RTOCs)[5]. For this purpose, the desired stromal cells are isolated and spun down together with thymocytes to form a pellet, which is cultured on a filter.

Disadvantages of the FTOC system are the cumbersome set up and the limited cellular yield. Recently, a culture system became available that demonstrated that a three-dimensional thymic architecture is not absolutely required for T-cell development. This culture system employs the OP9 stromal cell line that was originally derived from the BM of mice deficient in macrophage colony-stimulating factor (MCSF). The lack of MCSF expression contributed to the ability of OP9 cells to support B-cell differentiation from HSCs by limiting the clonal expansion of myeloid cells which interfere with lymphopoiesis[181]. By retrovirally introducing Notch ligand Delta1 in this cell line, the OP9 cells lost the ability to support B-cell development from HSCs and acquired the capacity to induce a normal program of T-cell differentiation from HSCs, including DP and $CD8^+$ SP stages[141]. No $CD4^+$ SP cells can be generated in this model because of the lack of MHC class II expression. Cultures are performed in the presence of IL-7 and

Flt3L. A range of different cell types has now been shown to be able to develop into T cells in this culture system, including mouse fetal liver and BM HSCs, embryonic stem cells and progenitors from human cord blood[139,141,182].

Comparable experiments have been performed with the BM stromal cell line S17[138]. However, although unmodified OP9 and S17 stromal cells appeared to function equally well in generating large numbers of B cells, OP9-Delta1 stromal cells were vastly superior in their ability to generate DP T cells compared to S17-Delta1[141]. This finding raises the important point that there is more to making T cells than the mere presence or absence of a Notch signal.

OUTLINE OF THE THESIS

The strictly controlled processes of T-cell development in the thymus are aimed at the generation of functionally mature T cells with a broad TCR repertoire, that can respond correctly and efficiently against invading pathogens. Errors occurring during certain events or stages of T-cell differentiation may lead to the development of diseases as diverse as leukemia, immunodeficiency or autoimmune disease. The objective of the research described in this thesis is to increase our knowledge of T-cell differentiation, with emphasis on human thymocyte development.

Chapter 2 describes the relative and absolute contribution of the different thymocyte subsets to the human thymus and how these change as children age. In Chapter 3 the thymocyte subsets are molecularly characterized in detail. Quantitative TCR gene rearrangement studies and gene expression profiling using microarrays are employed to obtain more insight into the initiation and regulation of TCR gene rearrangements. In Chapter 4, lineage potential and self-renewal capacity of two very immature thymocyte subsets are studied, with the goal of providing insight into the identity of progenitor cells that seed the thymus.

Chapters 5 to 8 go into the molecular regulation of T-cell development. As explained above, the Wnt and Notch pathways are crucial for the proliferation and differentiation of immature thymocytes. Chapter 5 examines, both in the human and the murine thymus, which thymocyte populations undergo Wnt signaling and how this is molecularly determined. In Chapter 6, target genes of the Wnt signaling pathway in human thymocytes are identified, which appear to be involved in proliferation and adhesion. The effect of the absence of a specific Wnt gene is investigated in Chapter 7, using a mouse deficient for Wnt3a. Wnt3a is demonstrated to be important not only for T-cell development, but for other hematopoietic lineages and stem cells as well. Chapter 8 is about the Notch signaling pathway: target genes of Notch signaling are determined in human progenitor cells and their contribution to T-cell development is evaluated.

Finally, Chapter 9 discusses the significance of the results described in chapters 2-8, with emphasis on T-cell commitment, and provides directions for future investigations.

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2

AGE-RELATED CHANGES IN THE CELLULAR COMPOSITION OF THE THYMUS IN CHILDREN

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ABSTRACT

Background: T-cell development in the thymus is an extensively studied subject, mainly in mice. Nevertheless, the normal composition and cell numbers of the non-involuted human thymus are largely unknown.

Objective: We aimed to gain insight into age related changes in different thymic subpopulations and to provide reference values for the distribution of thymocyte subsets. The composition of the normal thymus may serve as a reference for thymi in pathological conditions and may aid to diagnoses of immunodeficiency diseases.

Methods: Thymic lobes of 70 children (58 'immunologically normal' and 12 'diseased'), ranging in age from 8 days to 8 years old, were studied by four-color flowcytometric analysis. Detailed staining and gating strategies allowed us to dissect small subsets, including immature $CD4^{-}CD8^{-}$ populations and thymic B, NK and $TCR\gamma\delta^{+}$ cells.

Results: We demonstrate that distribution of thymocyte subsets changes with age and correlates with age-related fluctuations of T-lymphocyte counts in peripheral blood. Thymi of children 3 to 6 months of age appear to be the most active: they have high numbers of total thymocytes, the highest percentage of double positive cells, and large numbers of $CD34^{+}$ progenitors in their thymi. Furthermore, we show that the human thymus is a site for B-cell development, as all B-cell progenitor stages that can be found in the bone marrow are also present in the thymus.

Conclusion: We conclude that T-cell development in children is a dynamic process, answering to the demands of a maturing and expanding immune system.

INTRODUCTION

The thymus is a primary lymphoid organ that provides a unique microenvironment for the development of bone marrow derived progenitors into mature T cells[1]. In the thymus, thymocytes proliferate and differentiate, passing through a series of discrete phenotypic stages, during which they undergo T-cell receptor (TCR) gene rearrangements and positive and negative selection events, which form the TCR repertoire. A TCR molecule consists of either an α- and a β-chain or a γ- and a δ-chain. $TCR\alpha\beta^{+}$ cells are by far the most abundant T cells in the thymus and peripheral blood. The γδ lineage probably branches off early in the DN stage[2].

In man, the thymus is fully developed before birth in terms of the presence of all major subpopulations, including the most mature cells[3]. The rate of T-cell production by the thymus is greatest during childhood, when the peripheral TCR repertoire is being established due to antigenic pressure. After puberty, thymocyte numbers drastically decrease, although the thymus remains functional until old age[4-7].

Relatively little is known about the normal cell numbers and composition of the human thymus and how these parameters change during childhood. Apart from studies using TCR gene rearrangement excision circles (TRECs) to quantify recent thymic emigrants in peripheral blood both in adults and children[7-9], correlations between T cell numbers in peripheral blood and the cellular composition of the thymus in children have not been investigated.

We studied thymic lobes of 58 immunologically healthy children, ranging in age from 8 days to 8 years. Because of the considerable size of this group, we were able to give reference values, both for absolute numbers of thymocytes per lobe and for relative contributions of thymocyte subpopulations. These reference values may add to the diagnosis of complicated diseases, such as primary immunodeficiencies (e.g. SCID). A recent report[10] shows that a thymic biopsy may be a realistic tool for the diagnosis of such pathologies. Furthermore, detailed four-color flowcytometric analysis enabled us to investigate the normal existence of small subpopulations of great biological interest, like immature single positive cells (ISPs), TCRγδ$^+$ cells, immature T cell populations, and thymic B and NK cells. In addition to the description of healthy thymi, we show how the thymus differs in pathological conditions.

RESULTS

Thymic cellularity changes with age

We first investigated whether age dependent differences in thymic cellularity (here defined as total cell numbers) occurred. The absolute number of thymocytes per thymic lobe was plotted against the age of each child (Figure 1A) and the median and 10th and 90th percentiles were calculated for 6 age groups (Figure 1B). The number of cells per thymus appeared to be the lowest in the first two age groups, particularly in the neonates until 11 days, and increased gradually with age. The maximum cellularity was reached at 6 months of age, after which it again decreased. Significant differences were observed between the youngest age group and all other age groups over 3 months of age. Furthermore the 1 to 3 months age group had significantly lower cell numbers than the 6 to 9 month group ($p<0.01$).

The thymus of the patient suffering from protracted Rotavirus infection had extremely low thymocyte numbers. Children with Down syndrome tended to have lower thymocyte numbers: 5 out of 10 Down syndrome patients were below the 10th percentile of their age group.

No significant differences in cell numbers (nor in any subsequent flowcytometric assay) could be detected between boys and girls (data not shown).

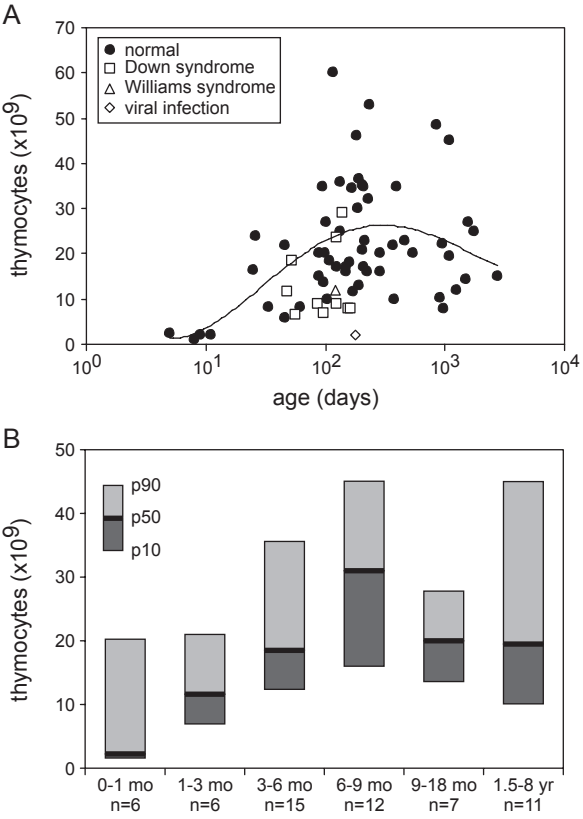

Figure 1. Thymic cellularity is age dependent.
(A) Total thymocyte numbers per thymic lobe in 'normal' (black dots) and 'diseased' (open symbols) children, plotted against the log age of each child. (B) Median total thymocyte numbers of healthy children in 6 age groups. Grey bars show 10th and 90th percentiles. Kruskal-Wallis test: p=0.011.

Distribution of major thymocyte subpopulations

To determine the relative distribution of thymocyte subsets and to investigate whether this changes with age, flowcytometric analysis of thymocyte populations was performed. Median values and ranges in six age groups for every subset described are listed in Table I. Thymocytes were stained for CD4, CD8 and CD3, to determine the 5 'classical' subsets: during thymocyte development cells are successively double negative (DN), immature single positive (ISP), double positive (DP) and CD4 or CD8 single positive (SP) (Figure 2A).

The percentage of DN cells was highest in the youngest age group, especially in the youngest neonates (Figure 2B and C). It was lowest in the 3-6 months group (p=0.013 versus 0-1 month group and p=0.001 versus 1.5-8 years group). The youngest children

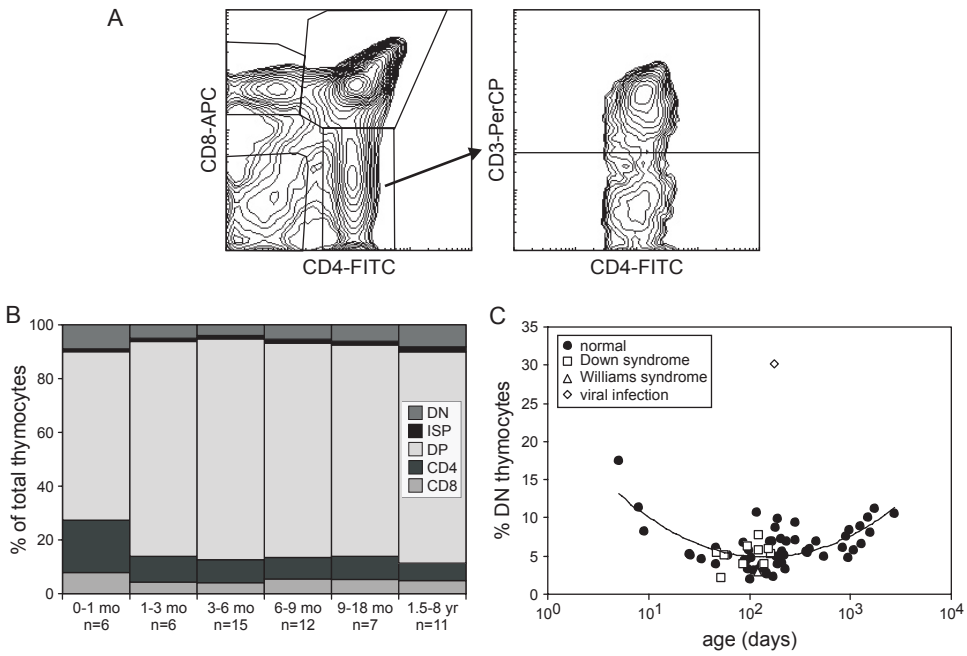

Figure 2. Distribution of 'classical' subsets is age dependent.
(A) Contourplot of a representative thymus sample (age 6 months). In the left panel $CD4^-CD8^-$ (DN) cells, $CD4^+CD8^+$ (DP) cells, and $CD8^+4^-$ (CD8 SP) cells can be distinguished. The $CD4^+8^-$ population can be further subdivided in $CD4^+3^-$ (ISP) cells and $CD4^+3^+$ (CD4 SP) cells (right panel). (B) Median values of percentage DN, ISP, DP and SP within the total thymocyte population in each age group. Significant differences between groups exist for DN cells (p=0.002), CD4 SP (p=0.03) and CD8 SP (p=0.037). (C) Percentages of DN cells within the total thymocyte population in 'normal' (black dots) and 'diseased' (open symbols) children, versus the log age.

had the lowest percentage of DP cells (median 57.4%), subsequently increasing until 76.3% in age group 3-6 months (Figure 2B). Variations in the percentage of DP cells were probably not stress-related, as in a different study no correlation was detected between the proportion of DP thymocytes and serum cortisol levels[12].

SP cells represent the most mature T cell population in the thymus. Both CD4 and CD8 SP populations were significantly higher in the youngest age group (Figure 2B). The ratio of CD4 SP versus CD8 SP gradually decreased with age (Figure 3), starting at 2.5 and reaching a ratio of 1.3 in the oldest age group. This decrease in CD4/CD8 ratio correlated remarkably well with that in peripheral blood of a different group of children (Figure 3, R=0.92).

Finally we determined whether the distribution of the 'classical' thymocyte subsets was influenced by disease conditions. Although the thymic composition of Down and

Table I. Percentages of thymocyte subsets in normal children.

	0-1 months		1-3 months		3-6 months		6-9 months		9-18 months		1.5-8 years	
	median	range	median	range	median	range	median	range	median	range	median	range
% DN	8.2	5.1-17.5	4.9	3.9-6.8	3.9	1.9-10.6	5.1	3.2-6.9	5.9	4.9-9.9	8.0	4.8-11.2
% ISP	0.97	0.57-1.14	1.18	0.71-2.32	1.22	0.40-3.44	1.41	0.51-3.27	1.36	0.53-2.56	1.82	0.82-3.18
% DP	57.4	46.7-77.8	78.5	64.5-83.3	79.6	64.8-88.9	76.3	63.4-82.7	76.4	73.6-80.1	76.6	63.8-82.9
% $CD4^+$ SP	17.8	8.8-27.5	9.4	6.5-15.0	8.3	5.3-12.9	7.7	5.3-14.6	8.4	5.6-10.7	6.3	1.7-11.9
% $CD8^+$ SP	7.2	4.7-9.6	4.2	2.4-5.3	4.0	2.2-6.5	5.2	2.8-6.8	5.2	3.7-6.0	4.8	1.7-5.7
% $CD34^+CD1a^{-1}$	4.7	2.3-12.3	6.2	3.2-10.1	7.3	1.9-21.3	6.5	2.2-15.9	4.8	2.3-6.6	4.7	2.9-8.5
% $CD34^+CD1a^{+1}$	5.0	0.85-7.9	13.2	6.9-29.4	13.8	3.9-23.9	11.1	5.8-21.7	6.7	2.6-14.3	8.0	1.4-20.2
% $TCR\gamma\delta^+$	0.22	0.05-0.37	0.31	0.14-0.48	0.22	0.03-0.46	0.29	0.09-0.67	0.24	0.07-0.46	0.27	0.07-0.59
% $TCR\alpha\beta^{+2}$	24.9	14.5-35.2	13.2	5.3-22.1	23.7	5.8-46.6	21.6	5.6-32.3	17.2	11.3-26.2	22.6	8.0-27.5
% B	0.30	0.10-0.64	0.27	0.16-0.36	0.31	0.17-0.66	0.31	0.14-0.69	0.51	0.31-1.10	0.49	0.19-1.08
% NK	0.28	0.05-0.66	0.10	0.04-0.20	0.08	0.03-0.13	0.07	0.04-0.19	0.09	0.04-0.28	0.09	0.0-0.16

[1] Percentage of DN thymocytes
[2] Percentage of DP thymocytes

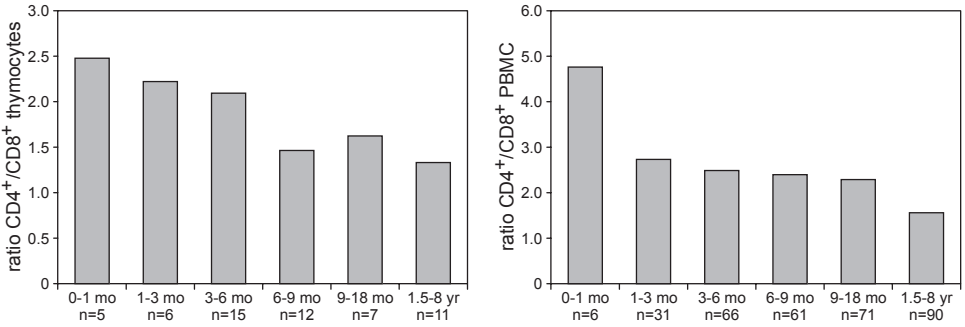

Figure 3. High correlation between CD4/CD8 ratios in thymus and blood.
Ratios of $CD4^+$ SP/$CD8^+$ SP cells in thymus (left panel) and $CD4^+$/$CD8^+$ T cells in peripheral blood (right panel) in 6 age groups.

Williams syndrome children did not differ in these aspects (Figure 2C), the patient with Rotavirus infection appeared to have a very high percentage of DN cells, associated with a low percentage of DP cells.

Early T-cell precursors

Since we found that the youngest children had the highest percentage of DN cells, the most immature 'classical' subset, we wanted to investigate whether the cells within this subset are also more immature in young children than at later ages. Therefore we stained thymocytes with CD34 and CD1a (Figure 4A). The most immature, 'stem cell like' cells in the thymus are CD34 positive and CD1a negative. One stage later, when the cells are committed to the T cell lineage, they become CD1a positive[13,14]. In the most

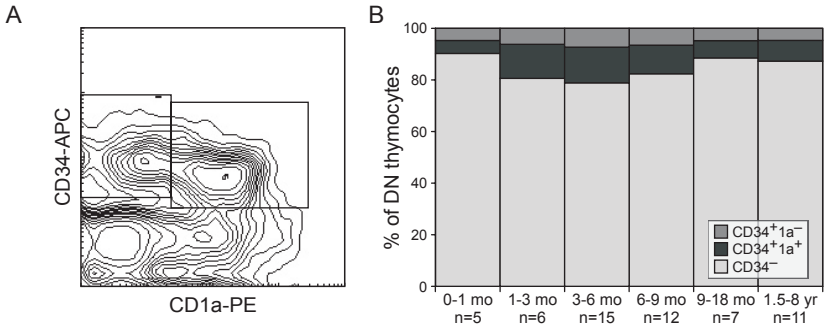

Figure 4. Distribution of early progenitors is age dependent.
(A) Contourplot of a representative thymus sample (age 6 months). CD34 and CD1a staining within the DN gate. (B) Median percentages of $CD34^+CD1a^-$, $CD34^+CD1a^+$ and total $CD34^-$ cells within the DN population in each age group. Significant differences between groups were found for $CD34^+CD1a^-$ ($p=0.039$) and $CD34^+CD1a^+$ ($p=0.001$).

mature DN stages, the cells lose expression of CD34, but they stay positive for CD1a.

Surprisingly, the percentage of CD34 positive cells within the DN population was not highest in the youngest age group, but instead significantly peaked at age 3-6 months (p=0.003 versus age group 0-1 month) (Figure 4B). After 6 months of age, the percentage of $CD34^+$ cells within the DN population tended to decline.

Expression of αβ and γδ T-cell receptors

DP T cells undergo positive and negative selection and only the surviving cells will start expressing high levels of TCRαβ molecules. Therefore we determined the percentage of DP cells that are $TCR\alpha\beta^+$ in thymi of different ages (Figure 5). No significant difference could be distinguished in expression of TCRαβ between the different age groups.

As it has been described that individuals with Down syndrome have lower numbers of $TCR\alpha\beta^+$ cells[15], we investigated whether this could be confirmed in our subject group as well. Surprisingly, the children with Down syndrome appeared to have high numbers of DP cells positive for TCRαβ (Figure 5). The same was true when regarding all thymocytes (data not shown).

The percentage of $TCR\gamma\delta^+$ cells was around 0.3% in all age groups (Figure 6A) and not significantly higher in the youngest age group. This was an unexpected finding, since $TCR\gamma\delta^+$ cells are the first T cells to appear during fetal development[16]. On the other hand, the relative contribution of $TCR\gamma\delta^+$ cells from thymic and extra-thymic origin is unknown, although an extra-thymic origin is controversial[17]. In any case, our finding corresponded with $TCR\gamma\delta^+$ cell numbers in blood, which were relatively low in the first age groups (Figure 6B).

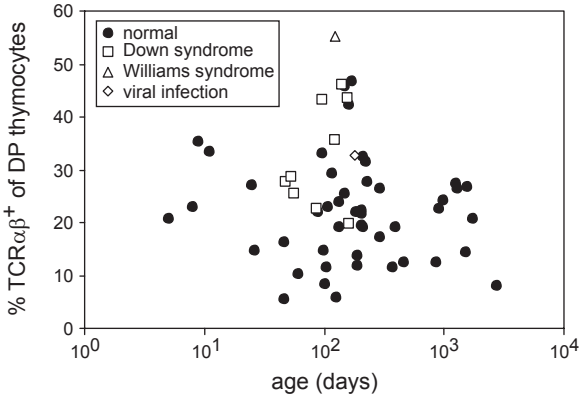

Figure 5. $TCR\alpha\beta^+$ cells in the thymus.
Percentages of $TCR\alpha\beta^+$ cells within the DP population in 'normal' (black dots) and 'diseased' (open symbols) children versus the age of each child.

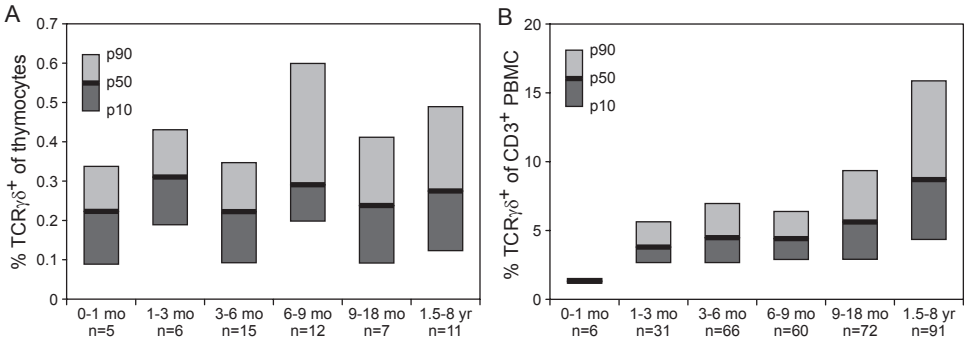

Figure 6. TCRγδ⁺ cells in the thymus.
(A) Median percentage of TCRγδ⁺ cells within the total thymocyte population was determined for healthy children divided into 6 age groups. Black lines represent median values for each age group and dotted bars show 10th and 90th percentiles. Differences between groups were not significant. (B) Percentage of TCRγδ⁺ within the CD3⁺ population in peripheral blood. Median percentages and 10th and 90th percentiles are indicated. Kruskal-Wallis test: $p<0.001$.

B and NK cells

To investigate whether B and NK cells are present in human pediatric thymus samples, thymocytes were stained with B cell marker CD19 and NK cell markers CD16 and CD56. Among total thymocytes, about 0.4% comprised B cells and 0.1% NK cells (Figure 7A). The percentage of B cells was significantly higher in the two eldest age groups, while the percentage of NK cells was highest in the youngest age group and more or less stable in the later age groups. The latter finding reflects the situation in the blood, where NK cell numbers are also highest in the youngest children [11]. In the thymus as well as in blood, the variation in NK cell numbers was very high in the first age group. The presence of thymic B cells was confirmed by immunohistochemistry of three different thymi. B cells were found to comprise about 25% of the thymic medullary cells, while the cortex contained only a few scattered B cells (Figure 7B).

The majority of the B and NK cells that are present in the thymus are not a result of blood contamination, as the estimated absolute number of blood derived B or NK cells (blood volume calculated by measuring the number of erythrocytes per thymic lobe), was always 50 to 100 times lower than the absolute number that we found. However, it does not indicate whether these cells have originated in the thymus or whether they have preferentially migrated to or expanded in the thymus.

To determine whether B-cell precursors are present in the thymus, we stained 3 thymi of different ages with markers for immature B cells. During B-cell development in the bone marrow, CD19 positive B cells are first CD10 positive, CD20 negative[18]. They then become positive for both CD20 and CD10 and finally they lose expression of CD10.

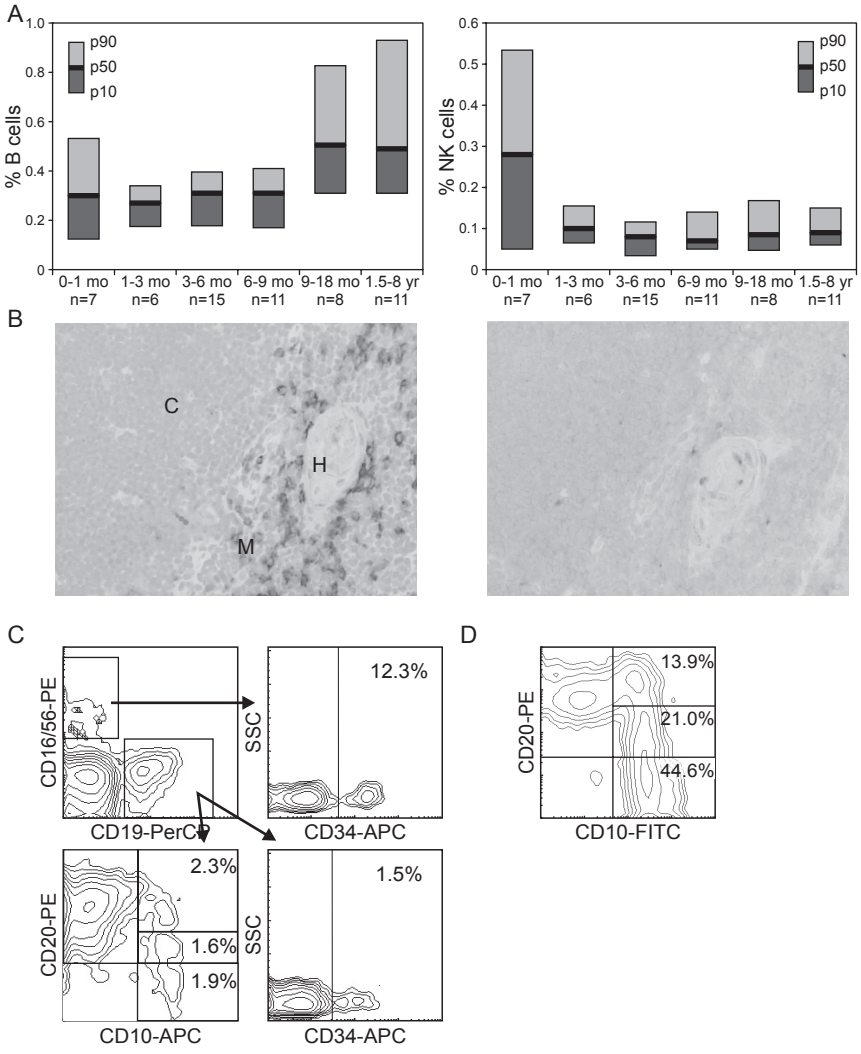

Figure 7. B and NK (precursor) cells in the thymus.
(A) Median percentage and 10th and 90th percentiles of B and NK cells within the total thymocyte population. Differences between groups were significant for B cells (p=0.005), but not for NK cells. (B) Thymic tissue sections stained for CD20 (left panel) or isotype control (right panel). Similar results were obtained with a CD19 antibody (not shown). C: cortex, M: medulla, H: Hassall's corpuscle. (C) Contourplots of a representative thymus sample stained with CD10 and CD20 within the $CD19^+$ gate (lower left panel) and with CD34 within both the $CD19^+$ gate (lower right panel) and the $CD16/56^+$ gate (upper right panel). (D) Representative contour plot of a normal pediatric bone marrow. Expression of CD10 and CD20 within the $CD19^+$ gate.

In the thymus all these stages could be distinguished within the CD19 gate (Figure 7C), displaying populations phenotypically similar to those in normal bone marrow (Figure 7D). By staining with CD34 and gating on CD19 or CD16/CD56 positive cells (Figure 7C), we showed that the human thymus contains stem cell like cells expressing lineage markers for B and NK cells. Together these data demonstrate that the thymus is a site for B and NK cell development.

DISCUSSION

The normal composition of the human pediatric thymus is a neglected subject, despite the large scientific interest in T-cell development. In this paper, we studied the thymic cellularity and used four-color flowcytometry to determine subset distribution of thymic lobes of 58 'immunologically normal' children, ranging in age from 8 days to 8 years. The considerable size of our subject group allows us to give reference values for a number of thymic subsets and to make statements that are of value both for general knowledge about T-cell development as well as for clinical purposes, although caution should be taken as the children in our control group are not healthy but suffer from congenital heart disease. Nevertheless, none of their cardiac defects are likely to have influenced the immune system. A part of these children were cyanotic before surgery. However, no differences in thymic subset distribution were observed between cyanotic and normally saturated children (data not shown).

Apart from studies examining post-mortem thymi[19,20], we know of no reports that determine total thymocyte numbers in thymi from healthy individuals. Two studies employ the elegant approach of analyzing cellular density per gram of thymus material[4,5], but this method does not take into account age related changes in total volume of the thymus, and therefore cannot determine total thymocyte numbers. Since we always received a complete thymic lobe after surgery, we were able to calculate the total cell number per thymic lobe.

In a few studies, thymic material from children was examined by flow cytometry[4,5,12], although from different viewpoints. Varas et al. compared thymus material of 9 neonates (age 1 to 20 days) and 11 children (age 1 month to 10 years)[12] and Marusic et al. included 11 children (age 1 to 14 years) in their study of 39, mostly adult, thymi[4]. Both these studies employed 2 or 3 color flow cytometry and mainly analyzed the large (e.g. DN, DP) thymocyte subsets.

Consistent with the study of Varas et al.[12], we found that thymi of infants until one month of age (neonates) differed from the other age groups, especially with respect to lower total cell numbers and relatively low percentage of DP cells. In particular the 8- to 11-day-old children were very similar to the neonates described by Varas et al.

However, these differences were not observed in all children below one month of age. Two children within this age group (25 and 26 days) fit more into the second age group, suggesting that the shift from a neonatal to a childhood thymic phenotype probably takes place around 3 weeks of age.

Unlike Varas et al.[12], we did not find increased numbers of very immature cells in neonates. In our study, the percentage $CD34^+$ cells is relatively low in neonates and increases at 3 months of age. This difference in observation may be due to the fact that Varas et al. do not subdivide the children over one month into age groups, and to the higher average age of the children in their study (23.4 months versus 13.1 months in our study).

Besides neonates, the age group of 3-6 months deviates most from all other groups. These children have high numbers of total thymocytes, the highest percentage of DP cells and the highest percentage of stem cells within the DN subset. Together these characteristics reflect a very active thymus of children encountering many antigens and establishing an extensive T cell receptor repertoire. This active thymus is mirrored by the high T cell numbers in peripheral blood of children of the same age[11].

Together, these results show that T-cell development during childhood is not merely a continuous process from a build up of T cells to the start of thymic involution, but rather a dynamic process, that fluctuates in response to the demands of the maturing and expanding peripheral immune system.

The bone marrow is the predominant site for B- and NK-cell development. However, data in mice have demonstrated that B cells can develop in the thymus[21] and it is known that NK cells can develop in the fetal thymic microenvironment[22,23]. We could detect low percentages of NK cells and B cells in the thymus in all age groups. Presence of B cells in the human thymus has been reported[24,25], but their origin remains controversial.

In our study, we demonstrate that B cell precursors can be found in the human thymus. These cells were not derived from the blood, as CD10 positive B cells are virtually absent in peripheral blood, with the sole exception of leukemia patients after chemotherapy cessation[26]. Thus, our study extends the finding of B-cell development in the murine thymus to the human situation. The presence of NK cell precursors suggests that the origin of (a part of) the thymic NK cells lies in the thymus as well.

Analysis of a large group of healthy thymi allows us to draw conclusions about the thymus in disease states. The dramatic effects that infections can have on the thymus, are exemplified by the patient in our study suffering from a protracted Rotavirus infection. This child's thymus had an extremely low cellularity and contained high percentages of DN and SP cells, reflecting very low numbers of DP cells. However, more samples from patients with ongoing infection would be needed to confirm this finding. In addition, we studied the thymus in children with Down and Williams syndrome, both mental retardation syndromes that often involve cardiac complications. Down syndrome

patients are known to have small and abnormal thymi[27], which is probably due to increased apoptosis caused by overexpression of copper-zinc superoxide dismutase[28]. In accordance with these studies, we find lower numbers of total thymocytes in Down syndrome patients (p=0.052 for age group 3-6 months). Consistent with the findings of Murphy and Epstein[15], we find no differences in subset distributions in thymi of children with Down syndrome. However we do not find the diminished percentage of TCRαβ$^+$ cells that they report. No data about the thymus in Williams syndrome have been reported. For our single Williams syndrome patient we show that subset distribution is within the normal range, but that total thymocytes counts are lower than in age matched controls.

Although unorthodox, a thymic biopsy is feasible as a recent paper demonstrates[10], and may add greatly to the diagnosis of immunodeficiency diseases. Differential presence of specific subpopulations as compared to our reference values can indicate the defects in T-cell development and may even give clues as to which gene defect would be the cause of the immunodeficiency.

In conclusion, our paper provides age dependent reference values for the normal composition of the human pediatric thymus. These are of value for *in vitro* studies of human T-cell development using fetal thymic organ culture and other stromal culture systems, as well as for clinical studies focusing on T-cell immunodeficiencies (SCID), HIV disease, development of allergies and lymphoid reconstitution after bone marrow transplantation.

MATERIALS AND METHODS

Thymus and blood samples

Thymic lobes were obtained from 70 children (90% Caucasian, 38 boys and 32 girls), operated for congenital heart disease. The thymus material was collected according to the informed consent guidelines of the Medical Ethics Committee of the Erasmus MC, Rotterdam. In all cases a complete thymic lobe was removed, for reasons of surgical approach. 57 children had septum defects, either isolated (30 children) or in combination with Fallot's tetralogy or a persistent ductus arteriosus (11) or in combination with another defect (16) and 13 had other cardiac defects, such as stenosis or hypoplasia. No children were diagnosed with DiGeorge syndrome.

In 58 children no additional disease was present: the patients presented without immunological or hematological disorders, were free of infections, did not receive prostaglandin treatment, and had no apparent cytological abnormalities. These children are referred to as 'immunologically normal'. In addition, we analyzed thymic lobes of 10 children with Down syndrome, one child with Williams syndrome and one child suffering from a protracted Rotavirus infection. All children below one year of age were born at term. The children ranged in age from 8 days to 8 years and were divided into 6 age groups (see Table II).

Peripheral blood data were obtained from a group of healthy children, that has been described previously[11].

Isolation and counting of thymocytes

Thymic lobes were stripped of the surrounding membrane and cut into small pieces. The fragments were subsequently disrupted by rubbing them gently through a stainless steel filter and washed with PBS containing 5% heat-inactivated fetal calf serum, until only stroma remained and all thymocytes were collected in the buffer. Thymocytes were spun down and resuspended in PBS containing 5% heat-inactivated fetal calf serum and 50 U/ml DNAseI.

The absolute leukocyte counts were determined in duplicate with a Coulter Counter model Z1 (Coulter Electronics), after lysis with ZAP-oglobin II (Beckman Coulter). Blood contamination of thymus samples was determined by measuring the number of erythrocytes per thymus. Therefore, a limited number of samples were counted both with and without lysing the cells.

Immunophenotyping

All monoclonal antibodies were directly conjugated and obtained from BD Biosciences, except for CD1a-RD1, which was from Beckman Coulter. All thymus samples were analyzed using a standard protocol of four tubes:

tube 1:	CD4-FITC	CD1a-RD1	CD3-PerCP	CD8-APC
tube 2:	CD4-FITC	CD1a-RD1	CD8-PerCP	CD34-APC
tube 3:	CD19-FITC	CD16- and 56-PE	CD3-PerCP	CD4-APC
tube 4:	TCRαβ-FITC	TCRγδ-PE	CD8-PerCP	CD4-APC

For characterization of B- and NK-cell subsets into further detail, a limited number of thymus samples was analyzed using the following antibodies: CD19-PerCP-Cy5.5, CD10-APC, CD20-PE, CD16-PE, CD56-PE and CD34-APC.

For the standard protocol, 100 µl aliquots with 10^6 thymocytes were incubated for 10 minutes at room temperature with combinations of antibodies. After washing, 10^5 cells were measured by flow cytometry. For the analysis of precursor B cells, 10^7 cells were stained and $1.5x10^5$ cells were measured within a $CD3^-$,$CD4^-$,$CD8^-$ gate. All analyses were performed with a FACSCalibur flowcytometer (BD Biosciences) that had been calibrated with CaliBRITE beads and Cell Quest Pro software.

Immunohistochemistry

6 µm tissue sections of 3 frozen human thymi were fixed in acetone and incubated with antibodies against CD20 (Dako) or CD19 (BD Biosciences) or an isotype matched IgG (R&D), followed by a biotinylated rabbit anti mouse antibody (Dako) and finally with avidin-biotin complex-horseradish peroxidase (Dako).

Statistical analysis

The Kruskal-Wallis test (a nonparametric alternative to one-way analysis of variance) was used to determine whether there were significant differences between groups. To test differences between two single groups, the nonparametric Mann-Whitney U test was used.

Table II. Composition of the 6 age groups.

	0-1 months		1-3 months		3-6 months		6-9 months		9-18 months		1.5-8 years	
	n	mean	n	mean	n	mean	n	mean	n	mean	n	mean
thymus	6	15	10	61	23	127	12	205	7	389	12	1350
blood	6	7	31	73	66	148	61	193	71	366	91	1378

n: number of children in each age group
mean: mean age (days)

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NEW INSIGHTS ON HUMAN T-CELL DEVELOPMENT BY QUANTITATIVE T-CELL RECEPTOR GENE REARRANGEMENT STUDIES AND GENE EXPRESSION PROFILING

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ABSTRACT

To gain more insight into initiation and regulation of T cell receptor (TCR) gene rearrangement during human T-cell development, we analyzed TCR gene rearrangements by quantitative PCR analysis in nine consecutive T-cell developmental stages, including $CD34^+$ lin^- cord blood cells as a reference. The same stages were used for gene expression profiling using DNA microarrays. We show that TCR loci rearrange in a highly ordered way (*TCRD-TCRG-TCRB-TCRA*) and that the initiating Dδ2-Dδ3 rearrangement occurs at the most immature $CD34^+CD38^-CD1a^-$ stage. *TCRB* rearrangement starts at the $CD34^+CD38^+CD1a^-$ stage and complete in-frame *TCRB* rearrangements were first detected in the ISP stage. *TCRB* rearrangement data together with the *PTCRA* (pTα) expression pattern show that human TCRβ-selection occurs at the $CD34^+CD38^+CD1a^+$ stage. By combining the TCR rearrangement data with gene expression data, we identified candidate factors for the initiation/regulation of TCR recombination. Our data demonstrate that a number of key events occur earlier than previously assumed and that human T-cell development is therefore much more similar to murine T-cell development than reported before.

INTRODUCTION

T cells develop from progenitors that migrate from the bone marrow into the thymus[1]. Thymocytes are roughly subdivided as either being double negative (DN), double positive (DP) or single positive (SP), based on the expression of the CD4 and CD8 co-receptors[1]. The DN stage is heterogeneous and can be subdivided into four distinct subsets in mice based on the expression of CD44 and CD25. In human, three clearly distinct DN stages can be recognized: a $CD34^+CD38^-CD1a^-$ stage, representing the most immature thymic subset and the consecutive $CD34^+CD38^+CD1a^-$ and $CD34^+CD38^+CD1a^+$ stages. Human DN thymocytes mature via an immature single positive (ISP $CD4^+$) and a DP stage into either $CD4^+$ or $CD8^+$ SP T cells that express functional T-cell receptors (TCR) and that exit the thymus[1].

A hallmark of T-cell development is the generation of T cells that express a functional TCR, either being TCRαβ or TCRγδ. During T-cell development, the variable domains of *TCRA, TCRB, TCRG,* and *TCRD* (located within *TCRA*) genes are assembled following rearrangement of variable (V), diversity (D), and joining (J) gene segments by a process called V(D)J recombination[2]. V(D)J recombination uses the RAG1 and RAG2 enzymes that selectively target recombination signal sequences (RSS) that flank V, D and J segments[2].

Studies in T-cell acute lymphoblastic leukemias (T-ALL) suggest that recombinations

of TCR genes are sequential between the different genes (*TCRD* > *TCRG* > *TCRB* > *TCRA*) as well as within a particular gene (e.g. *TCRD*: Dδ2-Dδ3, Dδ2-Jδ1, Vδ-Jδ1)[2,3], which is supported by limited data obtained from normal human T-cell subsets[4]. Therefore, the timing and efficiency of rearrangement of various TCR genes must be determined by the accessibility of gene segments to RAG enzymes. Evidence suggests that promoter and enhancer activity controlled by transcription factors regulate V(D)J recombination by modulating chromatin structures and rendering gene segments accessible to RAG cleavage[5,6].

T-cell development is for obvious reasons mainly studied in the mouse. Real-time quantitative PCR (RQ-PCR) and DNA microarray techniques allow careful analysis of small cell numbers. In this study we assessed the precise TCR gene configuration and the gene expression profiles of thymic subsets by RQ-PCR and Affymetrix DNA microarrays. By combining these two techniques we aimed for the identification of factors that play a role in regulating human TCR gene recombination.

RESULTS AND DISCUSSION

Definition of T-cell populations

From umbilical cord blood (UCB) $CD34^+$ lineage negative "stem cell-like" cells and from thymi $CD34^+CD38^-CD1a^-$, $CD34^+CD38^+CD1a^-$, $CD34^+CD38^+CD1a^+$, ISP $CD4^+$, DP $CD3^-$, DP $CD3^+$ and SP $CD4^+$ and SP $CD8^+$ subpopulations were obtained, representing consecutive stages of T-cell development (Figure 1). TCRαβ and TCRγδ expressing cells were obtained from thymic samples and peripheral blood. Cells from 5 donors

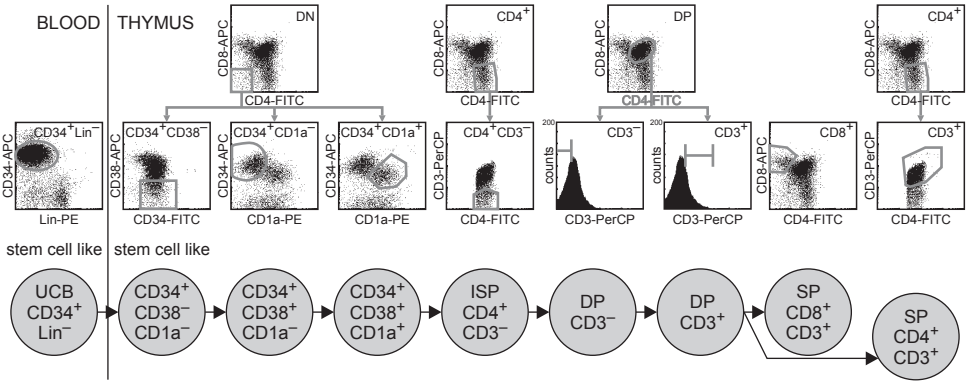

Figure 1. T-cell differentiation stages and gate settings for sorting.
The differentiation stages of human T-cell development are depicted schematically. FACS plots show the gating strategy used for sorting various populations.

were pooled to reduce intra-sample variation and all subsets were isolated twice from different donor pools.

Determination of TCR gene rearrangements by RQ-PCR and GeneScan analysis

TCR gene rearrangement analysis was performed in duplicate and on the two independently purified subsets (average is shown). The primers and TaqMan probes (listed at http://www.jem.org/cgi/content/full/jem.20042524/DC1) do not amplify germline DNA.

TCRD

The Dδ2-Dδ3 rearrangement was first detected in the earliest $CD34^+CD38^-CD1a^-$ thymic subset, much earlier during development than found in our previous Southern blotting-based report[4]. Dδ2-Dδ3 rearrangements reached maximum levels in the subsequent $CD34^+CD38^+CD1a^-$ stage and thereafter declined to low levels in the following subsets (Figure 2B). The initial Dδ2-Dδ3 wave was followed by Dδ2-Jδ1 rearrangements, which were detectable at low levels in $CD34^+CD38^-CD1a^-$ cells, increased in $CD34^+CD38^+CD1a^-$ and peaked in $CD34^+CD38^+CD1a^+$ cells (Figure 2C). Complete Vδ1-Jδ1 or Vδ2-Jδ1 rearrangements were first detected in $CD34^+CD38^+CD1a^-$ cells, increasing to peak levels in $CD4^+$ ISP cells after which they declined when the majority of thymocytes further differentiated into the TCRαβ lineage (Figure 2D). During TCRαβ T-cell lineage development, the *TCRD* gene is deleted from the *TCRA/D* gene complex[2,7] which results in the formation of a T-cell receptor excision circle (TREC) that may contain Vδ-Jδ1 rearrangements. TRECs do not replicate on cell division; consequently, they are diluted out in proliferating, developing T-cells[2]. Ki67 staining demonstrated high percentages of proliferating cells within the human ISP and DP $CD3^-$ thymic subsets (data not shown). This proliferation likely accounts for the observed decline in Vδ-Jδ1 rearrangements after the ISP stage. The Vδ1 gene segment appeared to be used preferentially in postnatal $TCR\gamma\delta^+$ thymocytes, which is in sharp contrast to the well-described preferential Vδ2 usage by peripheral $TCR\gamma\delta^+$ T cells (Figure 2D)[8]. We detected very low Vδ-Dδ3 levels in the thymic subsets, indicating that this represents a minor pathway to initiate *TCRD* gene rearrangement in postnatal thymus (Figure 2E).

TCRG

The first *TCRG* rearrangements (Vγ-Jγ1.1/2.1) were detected in the $CD34^+CD38^+CD1a^-$ thymic subset, earlier than we previously reported[4], and one stage after the initiation of *TCRD* rearrangement. These rearrangements peaked in $CD34^+CD38^+CD1a^+$ thymocytes, after which levels decreased to a relative constant level in subsequent subsets (Figure 3B). Vγ-Jγ1.3/2.3 rearrangements were first observed in $CD34^+CD38^+CD1a^-$ cells after which they rapidly increased and by far exceeded Vγ-

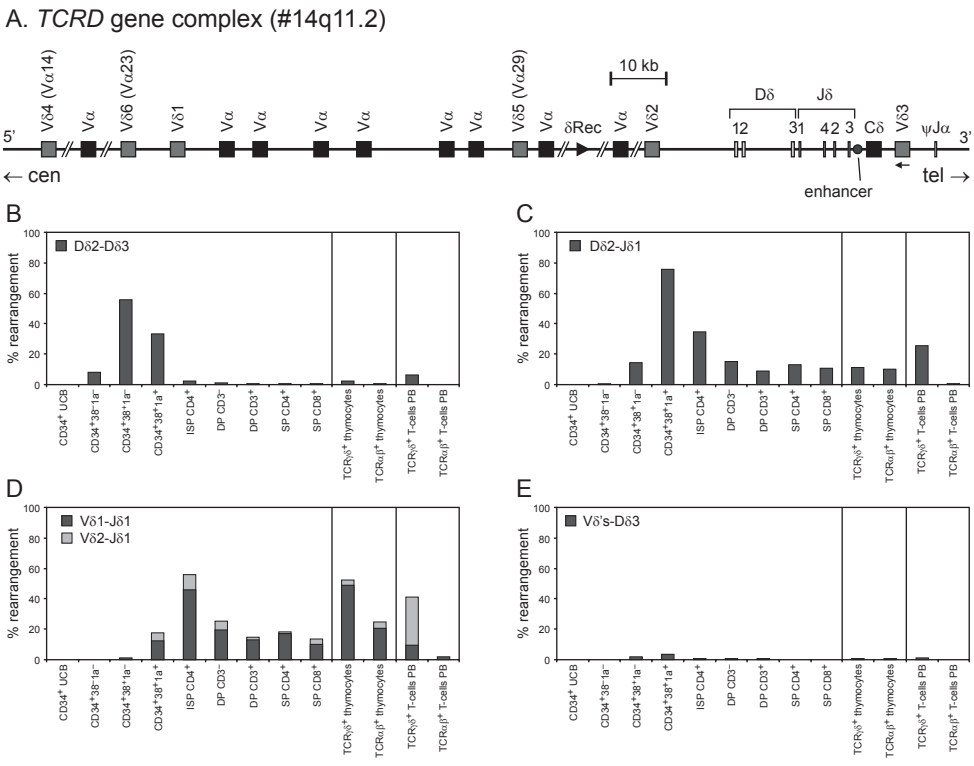

Figure 2. RQ-PCR analysis of *TCRD* gene rearrangements in human T-cell development and mature T cells.

(A) Schematic diagram of the human *TCRD* gene complex. The six 'classical' Vδ gene segments are scattered between Vα gene segments. As Vδ4, Vδ5, and Vδ6 are also recognized as Vα gene segments, their Vα gene code is given in parentheses (adapted from reference 9). (B) Analysis of Dδ2-Dδ3 rearrangement. (C) Analysis of Dδ2-Jδ1 rearrangement. (D) Analysis of Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements (E) Analysis of Vδ-Dδ3 rearrangements.

Jγ1.1/2.1 rearrangements from the ISP stage onwards (Figure 3C). Peripheral TCRγδ$^+$ and TCRαβ$^+$ T cells revealed lower Jγ1.3/2.3 usage than then their thymic counter parts (Figure 3C). In thymocytes Jγ1.2 was used at very low frequency, but massive positive selection for the Jγ1.2 segment occurred in peripheral TCRγδ$^+$ T cells (Figure 3D)[8].

TCRB

Dβ1-Jβ1 rearrangements were first detected at low levels in the CD34$^+$CD38$^+$CD1a$^-$ population and increased thereafter from CD34$^+$CD38$^+$CD1a$^+$ to CD4+ ISP cells (Figure 4B). Dβ2-Jβ2 rearrangements were first detected at low levels in CD34$^+$CD38$^+$CD1a$^+$ cells, one differentiation stage after Dβ1-Jβ1 rearrangements (Figure 4B). The seemingly

lower levels of Dβ-Jβ in DP CD3$^+$ are probably caused by variation within the lower range of detection of our assay and likely do not represent a true decrease.

Due to the complexity of the *TCRB* locus, Vβ-Jβ rearrangements were determined using non-quantitative GeneScan analysis[9]. The CD34$^+$CD38$^+$CD1a$^+$ thymocytes contained low levels of the first Vβ-Jβ rearrangements in which exclusively Jβ2 gene segments were used (Figure 4D), much earlier than described before[4]. From the ISP subset onwards Vβ-Jβ rearrangements were in-frame as shown by the triplet peaks (Figure 4E) and were retained throughout all subsequent stages of development (Figure 4E-H). Previous reports suggested that TCRβ-selection in humans is initiated at the ISP/DP stages of T-cell development[4,10,11], but the 3 nucleotide spacing of the peaks in our GeneScan analysis suggest that selection for in-frame *TCRB* already

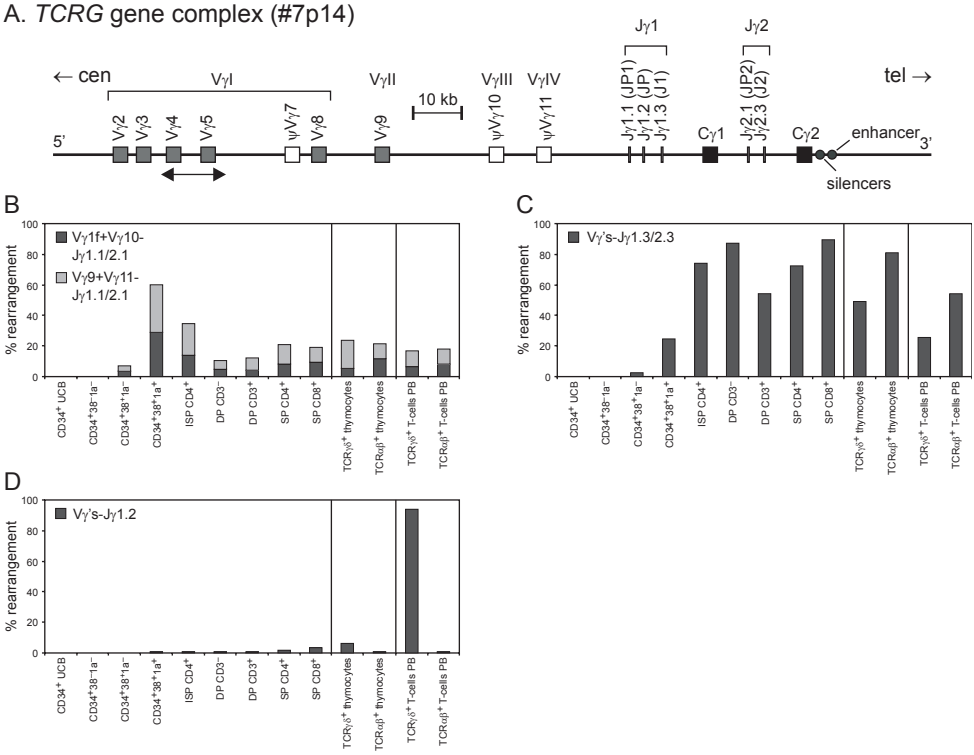

Figure 3. RQ-PCR analysis of *TCRG* gene rearrangements in human T-cell development and mature T cells.

(A) Schematic diagram of the human *TCRG* gene complex. Only the rearrangeable Vγ gene segments are depicted in black (functional Vγ) or in gray (non-functional Vγ). For the Jγ gene segments both nomenclatures are used (adapted from reference 9). (B) Analysis of Vγ to Jγ1.1 and Jγ2.1 rearrangements (C) Analysis of Vγ to Jγ1.3 and Jγ2.3 rearrangements (D) Analysis of Vγ to Jγ1.2 rearrangement.

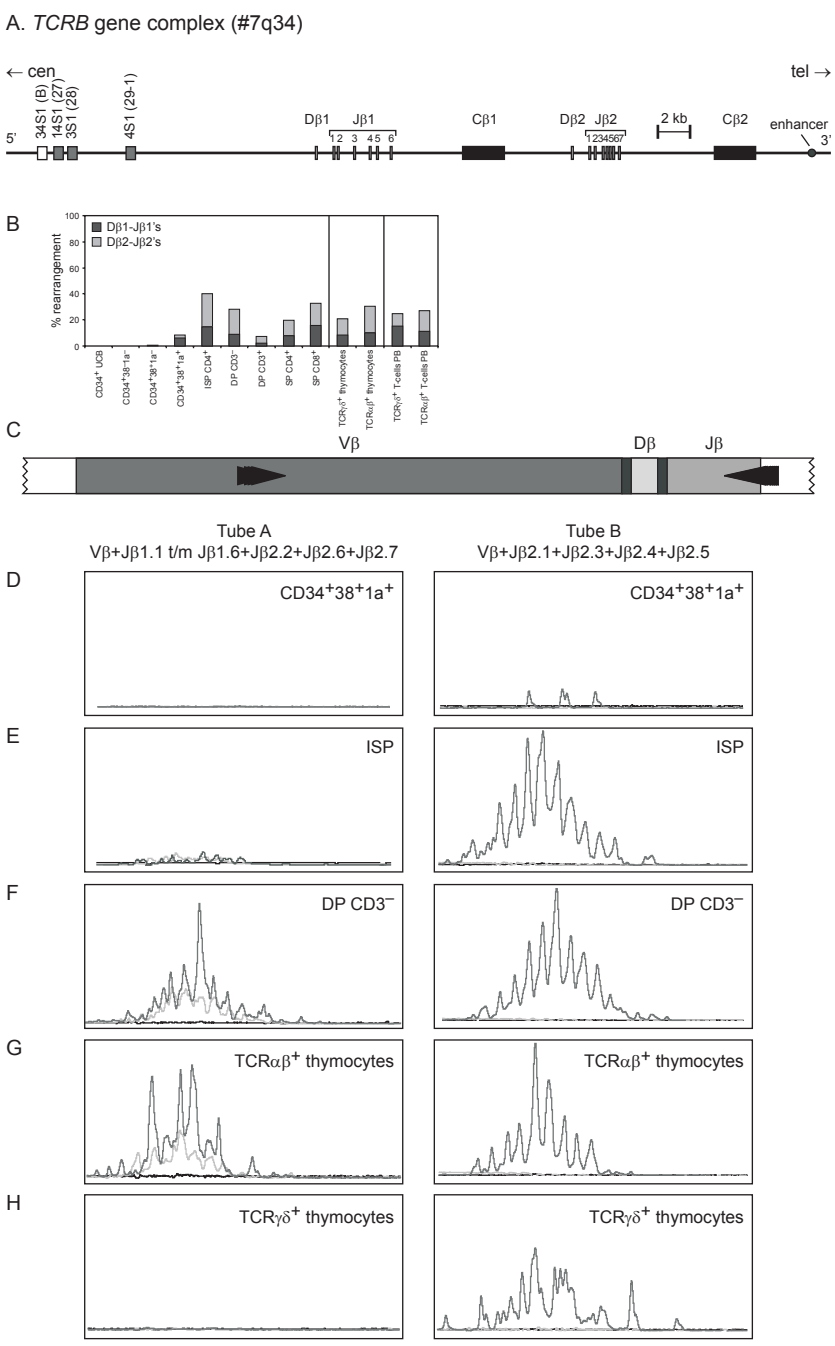
A. TCRB gene complex (#7q34)
← cen
tel →
5'
3'
34S1 (B)
14S1 (27)
3S1 (28)
4S1 (29-1)
Dβ1
Jβ1
1 2 3 4 5 6
Cβ1
Dβ2
Jβ2
1 2 3 4 5 6 7
2 kb
Cβ2
enhancer
B
% rearrangement
Dβ1-Jβ1's
Dβ2-Jβ2's
CD34+ UCB
CD34+38+1a+
CD34+38+1a+
CD34+38+1a+
ISP CD4+
DP CD3-
DP CD3+
SP CD4+
SP CD8+
TCRγδ+ thymocytes
TCRαβ+ thymocytes
TCRγδ+ T-cells PB
TCRαβ+ T-cells PB
C
Vβ
Dβ
Jβ
Tube A
Vβ+Jβ1.1 t/m Jβ1.6+Jβ2.2+Jβ2.6+Jβ2.7
Tube B
Vβ+Jβ2.1+Jβ2.3+Jβ2.4+Jβ2.5
D
CD34+38+1a+
CD34+38+1a+
E
ISP
ISP
F
DP CD3−
DP CD3−
G
TCRαβ+ thymocytes
TCRαβ+ thymocytes
H
TCRγδ+ thymocytes
TCRγδ+ thymocytes

Figure 4. RQ-PCR and GeneScan analysis of *TCRB* rearrangements in human T-cell development and in mature T cells.
(A) Schematic diagram of the human *TCRB* gene complex. (B) Analysis of Dβ to Jβ rearrangements. (C) Schematic diagram of Vβ-Jβ rearrangements as determined by GeneScan analysis. The primers for the Jβ1 cluster were HEX-labeled (solid lines), while primers for the Jβ2 cluster were FAM-labeled (dotted lines). (D-H) Analysis of Vβ -Jβ rearrangements in $CD34^+CD38^+CD1a^+$ cells (D), ISP cells (E), DP $CD3^-$ cells (F), $TCR\alpha\beta^+$ thymocytes (G) and $TCR\gamma\delta^+$ thymocytes (H).

occurs at the transition from $CD34^+CD38^+CD1a^+$ to ISP. From the DP CD3- fraction Vβ-Jβ1 rearrangements were also present, although less abundant than Vβ-Jβ2 rearrangements (Figure 4F; tube A). For Vβ-Jβ rearrangements usage of Jβ2 gene segments was preferred over Jβ1 (Figure 4D-H). $TCR\gamma\delta^+$ thymocytes contained no Vβ-Jβ1 rearrangements but did contain Vβ-Jβ2 rearrangements (Figure 4H).

TCRA

Due to large numbers of rearrangable Vα (~54) and Jα (61) gene segments[2], we could not design a multiplex RQ-PCR for reliable quantification of all Vα-Jα rearrangements. Instead, we aimed for an alternative approach in which we used different indirect measures to study *TCRA* recombination. *TCRA* recombination is initiated by the transcription of T-early alpha (TEA) in order to open the 5' site of the Jα cluster, which is followed by *TCRD* deleting rearrangements, particularly the δREC-ψJα rearrangement. These initiating events are then followed by multiple, consecutive Vα-Jα rearrangements[7,12].

To study initiation of *TCRA* rearrangement we determined the level of TEA-Cα transcripts as well as the occurrence of δREC-ψJα α rearrangements. TEA-Cα transcripts started to increase in $CD34^+CD38^+CD1a^+$ cells and reached peak levels in ISP and DP cells after which they declined again (Figure 5B). δREC-ψJα rearrangements were first detected in ISP cells and reached peak levels in SP and $TCR\alpha\beta^+$ thymocytes (Figure 5C). These data show that *TCRA* rearrangement has already started in the ISP cell population, but that there are still cells within the $CD3^+$ DP population that start rearrangement of the (most likely) second *TCRA* allele. Although TEA-Cα transcripts and δREC-ψJα rearrangements are good measures for initiation of *TCRA* rearrangement they cannot be used for quantification of the actual *TCRA* rearrangements. TEA-Cα is an mRNA product that cannot simply be extrapolated to the actual level of *TCRA* rearrangements. Quantification of δREC-ψJα is complex because it is strongly influenced by ongoing Vα- Jα rearrangements and the consequently produced TRECs (containing δREC-ψJα), whereas the amount of TRECs (and their dilution) is heavily dependent on the fraction of proliferating cells within specific subsets. Therefore, extra accumulation of TRECs may explain the relatively high δREC-ψJα levels in non-proliferating SP cells as compared to the preceding proliferating stages.

In an attempt to quantify *TCRA* recombination, we determined loss of germline

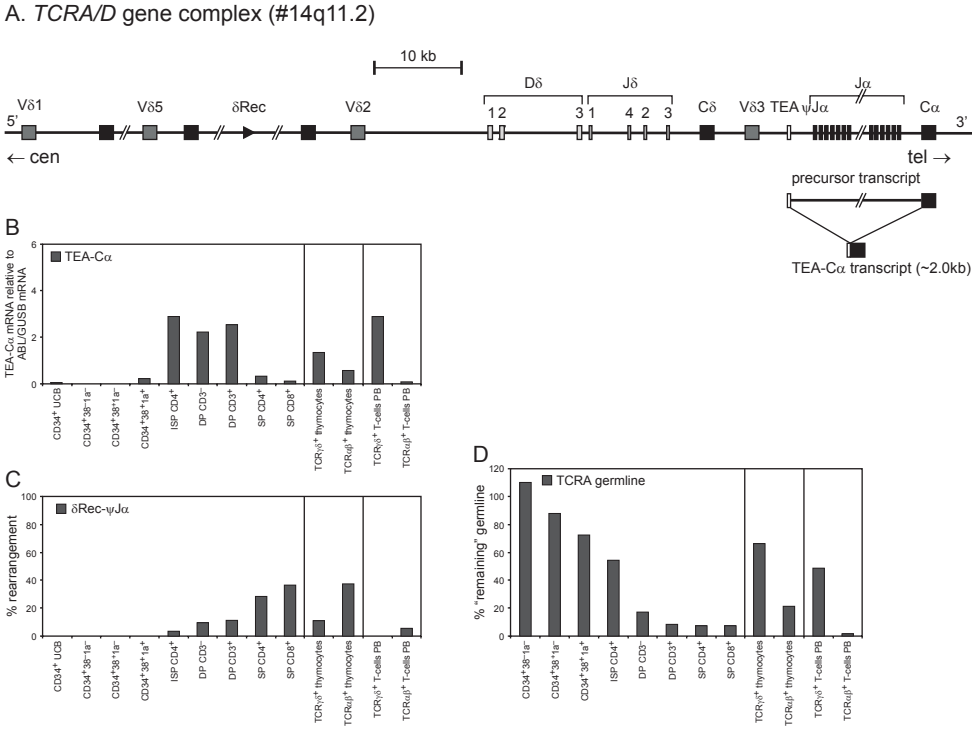

Figure 5. RQ-PCR analysis of initiating events around *TCRA* rearrangement in human T-cell development and mature T cells.
(A) Schematic diagram of the human *TCRA* gene complex. The TEA element forms a sterile mRNA with Cα sequences. The *TCRD* deleting elements δRec and ψJα are also indicated. (B) Analysis of TEA-Cα mRNA expression. Due to shortage of material, TEA expression was only determined once. (C) Analysis of the *TCRD* deleting rearrangement δRec-ψJα. (D) Analysis of "remaining" *TCRA* germline DNA.

TCRA DNA based on the disappearance of germline ψJα as indirect measure for *TCRA* rearrangements. For this approach we used DNA from CD34$^+$lin$^-$ UCB cells as 100% germline reference. Germline *TCRA* clearly declined from the CD34$^+$CD38$^+$CD1a$^+$ stage onwards with a major decline when ISP cells progress towards DP cells (Figure 5D). The low levels of germline *TCRA* in DP CD3$^-$ cells indicate that extensive *TCRA* rearrangement has occurred at this stage. The seemingly inconsistence with the relatively low levels of δREC-ψJα in these DP CD3$^-$ cells can be explained by the fact that TRECs containing δREC-ψJα are rapidly diluted-out in these heavily proliferating cells and that other *TCRD* deleting rearrangements can occur as well, such as δREC-Jα58 and Vδ-ψJα[7]. It should be noted that our approach is only suitable for quantification of major decreases in germline *TCRA*, which occur at early stages of T-cell development

(ISP/CD3$^-$ DP), but that it is much more difficult to accurately measure additional *TCRA* rearrangements at the later stages of T-cell development. The latter is because germline ψJα (for instance due to δREC-Jα58 rearrangements) remains detectable on TRECs and the difficulty of detecting further decreases within the 5-10% germline *TCRA* present in these stages.

We conclude that *TCRA* rearrangements are initiated when thymocytes progress from CD34$^+$CD38$^+$CD1a$^+$ towards the ISP stage, which is much earlier than previously reported[11], and is apparently ongoing up until the CD3$^+$ DP stage.

Microarray analysis

A total of 3848 probe sets underwent a significant change between any two successive stages of differentiation. The expression levels of these probe sets were used to calculate a correlation coefficient between all possible pairs of microarrays and revealed high correlation between biological repeats (not shown). This allowed us to use the average expression values of the two arrays performed per subset (obtained from 5 pooled thymi) for further analysis.

Expression data of the 3848 probe sets were used to perform hierarchical clustering using Genlab. Expression patterns of the 3848 probe sets were subdivided in k = 15 different clusters (A to O; Figure 6). This hierarchical clustering immediately revealed that the thymocyte subsets isolated based on the expression of surface markers have distinct gene expression profiles, although both SP subsets show highly similar expression profiles. A table with the members of each cluster including the expression data is provided at http://www.jem.org/cgi/content/full/jem.20042524/DC1. A large group of probe sets (580 in cluster B) is specifically expressed in CD34$^+$lin$^-$ UCB cells and shows a dramatic decrease in expression in the first thymic subset (e.g. *KIT* (CD117). Other non-T cell markers such as *BTK* and *PLCG2* show a slower decrease starting in the second and third thymic population (cluster H and F, respectively). One of the first probe sets directly related to T-cell differentiation to show increased expression represents *TCRD* (cluster G), followed shortly thereafter by *TCRB*, *TCRG* and *CD7* (cluster J). Increased expression of *RAG1*, *RAG2*, *DNTT* (TdT) and *PTCRA* (pTα) was detected immediately after T-cell commitment (cluster C). This particular combination of genes is specific for the T-cell lineage.

Of special interest for TCRβ-selection and initiation of *TCRA* rearrangement is the fact that *PTCRA* (pTα) expression increased in the CD34$^+$CD38$^+$CD1a$^-$ stage and peaked in the CD34$^+$CD38$^+$CD1a$^+$ and ISP stages, after which it declined. Mouse microarray data[13] have shown a similar expression pattern of pTα, with a minor peak at DN3 and a larger peak at the DP CD3$^-$ stage. Experiments with pTα mutant mice indicate that TCRβ-selection in the mouse occurs at DN3[14], and that *TCRA* recombination is initiated after TCRβ-selection has occurred[14]. Here we show that initiation of *TCRA* recombination

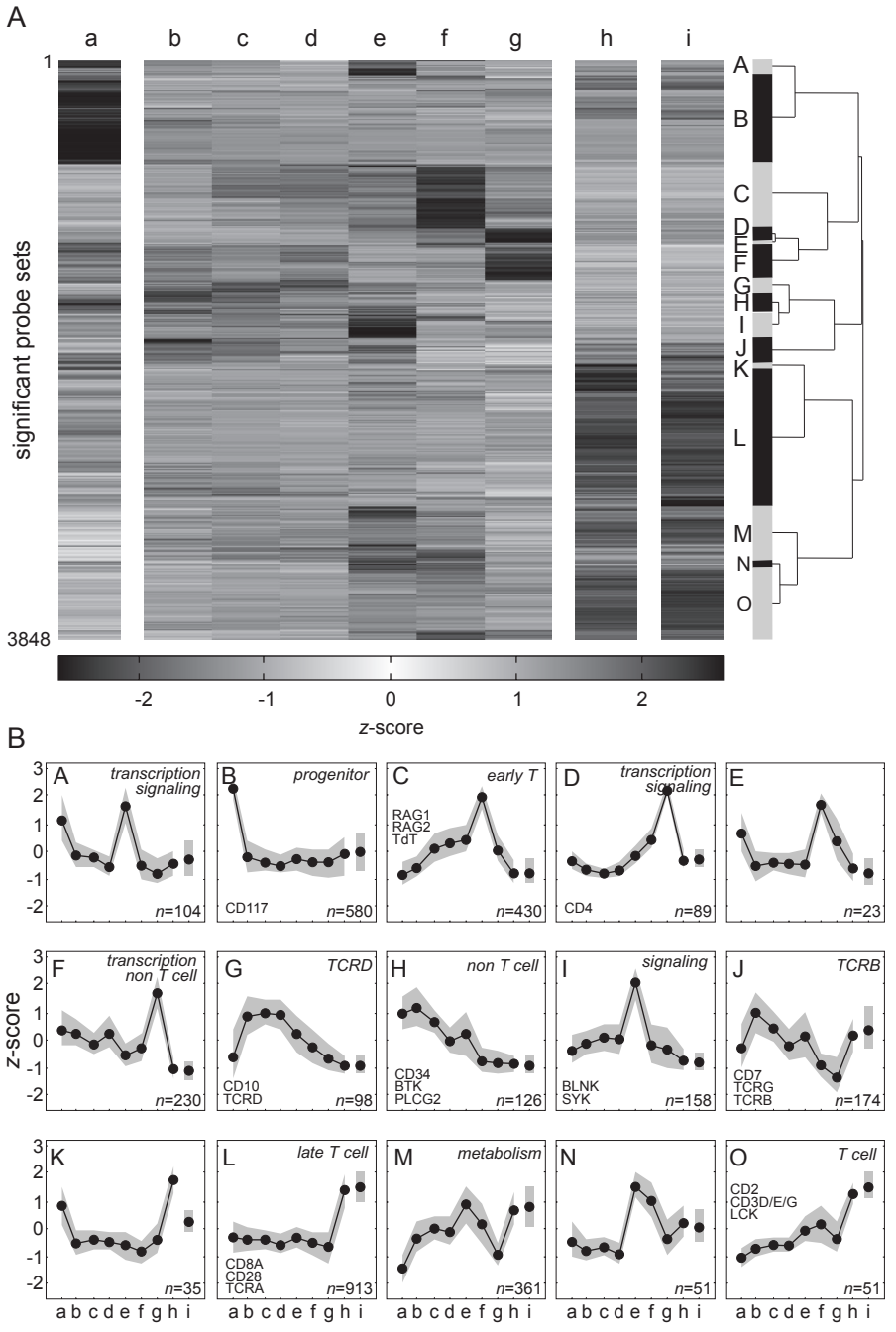
A
a b c d e f g h i
1
3848
significant probe sets
A B C D E F G H I J K L M N O
-2 -1 0 1 2
z-score
B
A transcription signaling n=104
B progenitor CD117 n=580
C early T RAG1 RAG2 TdT n=430
D transcription signaling CD4 n=89
E n=23
F transcription non T cell n=230
G TCRD CD10 TCRD n=98
H non T cell CD34 BTK PLCG2 n=126
I signaling BLNK SYK n=158
J TCRB CD7 TCRG TCRB n=174
K n=35
L late T cell CD8A CD28 TCRA n=913
M metabolism n=361
N n=51
O T cell CD2 CD3D/E/G LCK n=51
z-score
a b c d e f g h i

Figure 6. Hierarchical clustering of the 3848 differentially expressed probe sets into 15 clusters.
(A) Probe sets shown in rows. Analyzed subsets are shown in columns (a: $CD34^+lin^-$ UCB, b: $CD34^+CD38^-CD1a^-$, c: $CD34^+CD38^+CD1a^-$, d: $CD34^+CD38^+CD1a^+$, e: ISP, f: DP $CD3^-$, g: DP $CD3^+$, h: SP $CD4^+$, i: SP $CD8^+$). Grey values correlate with the value of the z-score. On the right side of the figure the 15 clusters are depicted (A-O). (B) z-score trend representation shown for each cluster. Clusters codes (A-O) are displayed in the top left corner of each graph. The z-score is shown on the y-axis, the thymic subsets (labels are the same as in A) on the x-axis. The gray area represents 1 SD. In the bottom left corner of the graphs some representative cluster members are mentioned. n (bottom right corner) indicates the number of probe sets per cluster.

starts in $CD34^+CD38^+CD1a^+$ cells. The analogous pTα expression between mice and men, together with our *TCRB* GeneScan and our *TCRA* recombination data, indicates that human TCRβ-selection occurs at the $CD34^+CD38^+CD1a^+$ stage instead of the previously suggested ISP/DP stage[4,10,11].

Some probe sets that represent genes involved in double strand (ds)DNA break repair, such as *G22P1* (*KU70*), *XRCC5* (*KU80*), *PRKDC* (*DNAPK*), *DCLRE1C* (*ARTEMIS*), *XRCC4* and *LIG4* (*DNA ligase IV*), showed no significant change in expression between any of the subsequent developmental stages. Probe sets representing genes that take part in the TCR complex, such as *CD3D*, *CD3E*, *CD3G* and *ZAP70* are relatively over-represented starting in the ISP $CD4^+$ population (Figure 6; cluster O), although expression of *CD3D*, *CD3E* and *ZAP70* mRNA is already detectable in the first thymic subset that we analyzed. Transcription of the *TCRA* locus (cluster L) is first seen in the DP $CD3^-$ stage, after TCRβ-selection has already occurred. Surface markers such as CD5, CD8α and β1, CD27 and CD28 (cluster L) that are generally seen on more mature T cells, were indeed found to be upregulated in the latest stages of T-cell development. Cclusters A and I, harboring genes involved in signaling, showed a peak in expression in the ISP stage of T-cell development.

Identification of factors regulating TCR gene rearrangements

To identify candidate transcription factors involved in regulating TCR recombination the list of 3848 probe sets was filtered based on Gene Ontology (GO) annotation (transcriptional activity and DNA binding) yielding a final list of 446 probe sets encoding a total of 361 genes (available at http://www.jem.org/cgi/content/full/jem.2004 2524/DC1). Expression of genes associated with T-cell commitment/differentiation and/or V(D)J recombination, such as *NOTCH1*, *HES1*, *GATA3*, *BCL11B*, *RAG1*, *RAG2*, and *DNTT* (TdT) strongly increased in early T-cell differentiation.

To determine which genes may have a role in regulating TCR rearrangement, hierarchical cluster analysis was performed (Figure 7A). The 446 probe sets were divided in 15 clusters, of which the prototypic expression patterns are depicted in Figure 7B. Clusters 3, 5, 6, 7, 14 and 15 (indicated by * in Figure 7B) contain genes

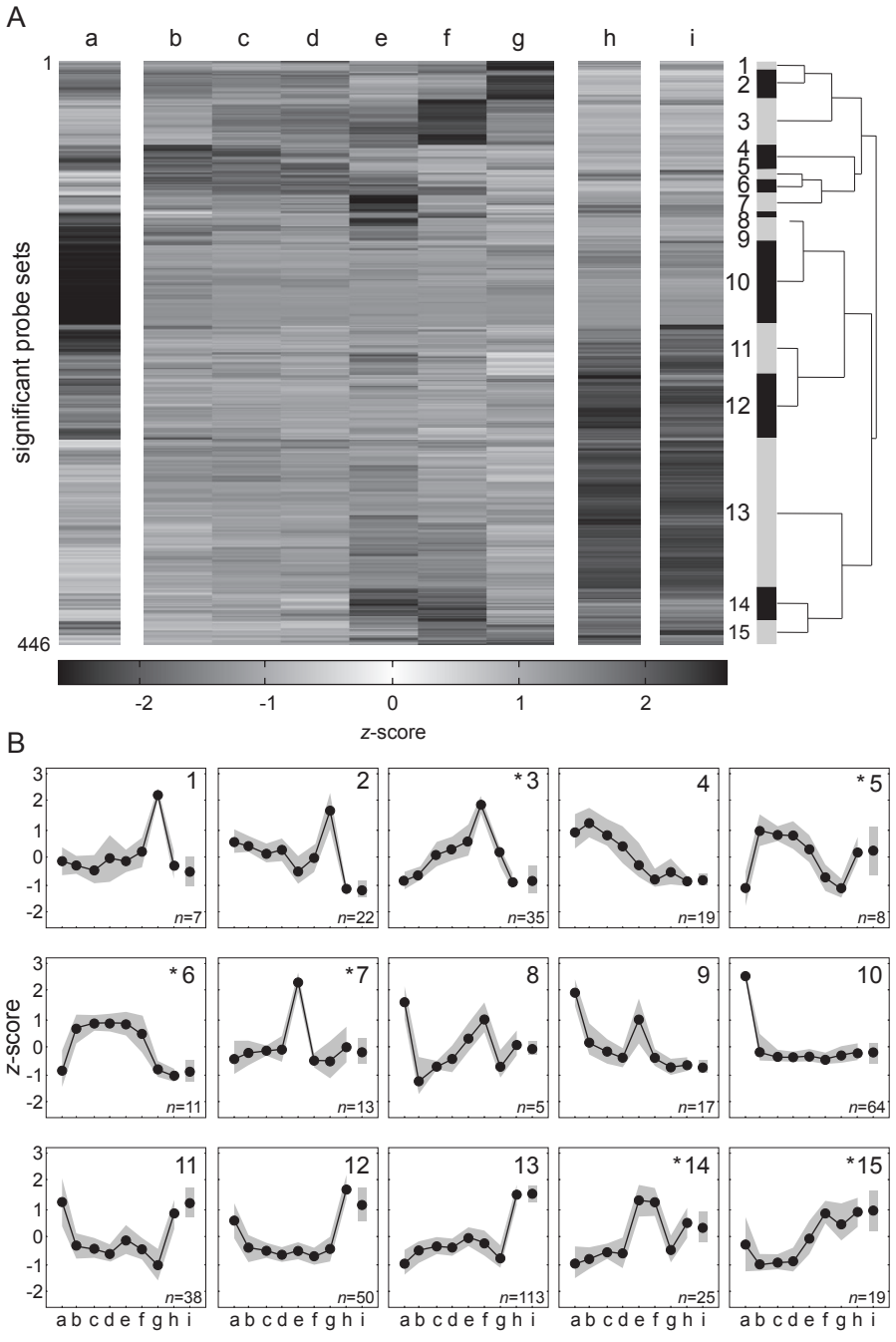
A
a b c d e f g h i
1
446
significant probe sets
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
-2 -1 0 1 2
z-score
B
1 2 *3 4 *5
*6 *7 8 9 10
11 12 13 *14 *15
n=7 n=22 n=35 n=19 n=8
n=11 n=13 n=5 n=17 n=64
n=38 n=50 n=113 n=25 n=19
z-score
a b c d e f g h i

Figure 7. Analysis of probe sets related to transcriptional regulation.
(A) Hierarchical clustering of 446 probe sets differentially expressed over the various stages and related to transcriptional regulation and DNA binding. Labels of the thymocyte subsets are the same as in Figure 6. (B) z-score trend representation for each cluster deduced from the 446 probe sets. Cluster codes (1-15) are shown in the top right corner, z-score on the y-axis, subsets on the x-axis. The gray area represents 1 SD. The asterisk in the upper right corner indicates clusters that contain genes that are potentially important in regulating TCR rearrangements. n indicates the number of probe sets in a cluster.

of which expression levels increase concomitantly with episodes of active TCR gene rearrangement, as determined above, and which may therefore encode factors that initiate and/or regulate TCR rearrangements. Genes present in clusters 3, 5, 6, 7, 14 and 15 are presented in Table I.

We propose that clusters 3, 5 and 6 contain genes common for the rearrangement of all four TCR genes. Cluster 5 likely contains genes important for the regulation of *TCRD*, *TCRG* and *TCRB* rearrangements while cluster 3 likely contains genes that need to be expressed at a higher level when *TCRA* rearranges. These clusters include *DNTT* (cluster 6), *RAG 1*/*RAG 2* (cluster 3) as well as genes encoding factors such as NOTCH1 (cluster 6), RORC (cluster 3), SMARCA4 (BRG1) (clusters 6 and 3), H2AFX (cluster 3) that have previously been linked to regulation of *TCRG*, *TCRB* or *TCRA* rearrangements[15-18].

Genes in cluster 7, 14 and 15 such as *SPIB*, *ICSBP1*, *TCF4*, *CREB1*, *ETS1* and *LEF1*, with high expression in ISP and DP subsets, may encode factors involved in regulating *TCRA* rearrangements, as well as allelic exclusion of the *TCRB* locus. CREB1 in conjunction with factors such as Ets1 and Lef1 activates the *TCRA* enhancer[19]. Recently, Ets1 has been identified as a critical factor for allelic exclusion at the *TCRB* locus[20]. Genes present in cluster 7 show a specific expression peak in the ISP stage. Among these genes *ICSBP1* (IRF8) and *SPIB* are likely candidates for initiating *TCRA* rearrangements, as they encode factors that have been implicated in the regulation of Ig light-chain transcription and recombination in developing B cells[21,22], while involvement of *TCF4* (E2-2) is supported by the finding that E2-2 deficient mice exhibit reduced transition from the DN to the DP stage[23].

Novel insights into human T-cell development

We confirm that TCR loci rearrange in a highly ordered way (*TCRD*-*TCRG*-*TCRB*-*TCRA*) and defined sequential rearrangement steps of *TCRD*, *TCRG*, *TCRB*, and initiation of *TCRA* recombination to specific human thymic subsets. Importantly, our data show that recombination of the TCR genes occurs earlier during human T-cell development than previously reported[4,11]. Given that in mice *TCRD* rearrangement starts at DN1, followed by *TCRG* in DN2 and *TCRB* in DN2 but especially DN3[1,24], the human

Table I. Transcription and DNA binding related genes with expression profiles that correlate with TCR gene rearrangements in human T-cell development.

	common *TCR*	common *TCR* *D,G,B>A*	common *TCR* *A>B,G,D*	*TCRA* / allelic exclusion *TCRB*	*TCRA* / allelic exclusion *TCRB*	*TCRA* / allelic exclusion *TCRB*
catergory	cluster 6	cluster 5	cluster 3	cluster 7	cluster 14	cluster 15
transcription factor/ coregulator of transcription	*BAZ1B*	*BAZ2B*	*AEBP1*	*ACYP2*	*BRD8*	*ABCA7*
	ETS2	*FOS*	*CBFA2T3*	*BCL11A*	*CNOT3*	*ARNTL*
	HMGB3	*HES1*	*E2F1*	*CD86*	*CREB1*	*BCL6*
	KLF4	*IRF1*	*FOXM1*	*CUTL2*	*FALZ*	*CENTB1*
	MYB	*STAT4*	*GFI1*	*ICSBP1* (IRF8)	*IFI16*	*ELF1*
	NOTCH1	*TCEAL1*	*MYBL1*	*IRF7*	*ILF3*	*ETS1*
		TCFL1	*MYBL2*	*MNDA*	*LEF1*	*GATA3*
			NFATC3	*MYCL1*	*LEREPO4*	*GMEB2*
			NOTCH3	*SPIB*	*M96*	*HTATIP*
			ORC6L	*TCF4*	*NFYA*	*LEF1*
			RORC		*NR2C1*	*LRRFIP1*
			SAP30		*PWP1*	*MXD4*
			TFDP2		*SOLH*	*TCF7*
			TMPO		*TAF5*	*ZNF297B*
			TRIP13		*TBDN100*	
			TTF1		*TCF12* (HEB)	
			WHSC1		*TCF8*	
			ZNF423		*TFAM*	
					ZNFN1A1 (IKAROS)	
recombination	*DNTT* (TdT)		*RAG1*			
			RAG2			
chromatin-remodelling	*H2AFY*		*CHAF1A*		*HDAC4*	*HDAC7A*
	SMARCA4		*EZH2*		*YY1*	*SATB1*
			H2AFX			
			HMGB2			
			HMGN2			
			SMARCA4			
DNA repair	*MCM2*		*BRCA1*		*BTG2*	*BTG2*
	MCM6		*MCM7*			
			PTTG1			

$CD34^+CD38^-CD1a^-$, $CD34^+CD38^+CD1a^-$ and $CD34^+CD38^+CD1a^+$ subsets resemble murine early DN1 ($CD44^+CD25^-CD117^-$), late DN1+DN2 ($CD117^+$ DN1; $CD44^+CD25^+$), and DN3 ($CD44^-CD25^+$) stages, respectively. However, the relative frequency of DN1 cells in mice is higher than that of the corresponding human subset ($CD34^+CD38^-CD1a$). We also demonstrate that TCRβ-selection and initiation of *TCRA* rearrangements do not occur at the ISP/DP stages of human T-cell development[4,10,11], but instead already at the $CD34^+CD38^+CD1a^+$ stage. This is similar to the mouse, in which TCRβ-selection occurs in DN3 thymocytes[14].

Based on the TCR rearrangement data and the expression of key recombination and differentiation genes (e.g., *RAG1, RAG2* and *PTCRA*), we show that human and mouse T-cell development are much more similar than previously assumed. In addition candidate factors for regulation of TCR recombination are identified. We propose an up-dated human T-cell differentiation model, as shown in the Discussion of this thesis. These novel data help to bridge gaps in our understanding of human T-cell development, and should provide insight into the development of T cell acute lymphoblastic leukemia and SCID.

MATERIALS AND METHODS

Isolation of cell samples from thymus, umbilical cord blood and peripheral blood

Thymi were obtained as surgical tissue discards from children aged 7 weeks to 3 years (median of 6 month) undergoing cardiac surgery at the Erasmus MC Rotterdam, with informed consent from the parents. The children did not have immunological abnormalities. Thymocytes were isolated by cutting the thymic lobes into small pieces and squeezing them through a metal mesh, and stored at -80ºC until further analyses. Mononuclear cells were isolated by Ficoll-Paque (Amersham Biosciences) density centrifugation from human umbilical cord blood (UCB) obtained from full-term normal deliveries and from peripheral blood of healthy volunteers. All samples were obtained according to the guidelines of the Medical Ethical Committee of the Erasmus MC that also approved the human studies.

Purification of subpopulations

Total mononuclear cells or thymocytes from 5 donors were pooled to reduce intra-sample variation. After thawing, pooling and Ficoll density separation, thymocytes were labeled with fluorochrome-conjugated monoclonal antibodies. In order to isolate the $CD34^+$ lineage $(lin)^-$ UCB cells, $CD34^+CD38^-CD1a^-$, $CD34^+CD38^+CD1a^-$ and $CD34^+CD38^+CD1a^+$ thymocytes, magnetic beads (Myltenyi Biotech) were used to enrich for $CD34^+$ cells. For isolation of the ISP population, thymocytes were depleted of $CD3^-$ expressing cells using magnetic beads. For isolation of $TCR\gamma\delta^+$ thymocytes, magnetic beads were used to enrich for $TCR\gamma\delta^+$ cells. All magnetic beads were used according to the manufacturers protocol. After MACS isolation, the enriched cells were labeled with fluorochrome-conjugated monoclonal antibodies for further purification by high speed cell sorting. In order to isolate mature $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T-cells, peripheral blood mononuclear cells were labeled with fluorochrome-conjugated monoclonal antibodies for cell sorting. All cell sorting was performed on a FACS DiVa cell sorter (BD Biosciences). Monoclonal antibodies used, with the clone in brackets: CD4-FITC (SK3), CD38-FITC (HB7), TCRαβ-FITC (WT31), CD1a-RDI (T6), CD3-PE (SK7), CD16-PE (B73.1), CD19-PE (4G7), CD56-PE (M431), TCRγδ-PE (11F2), CD3-PerCP (SK7), CD8-PerCP (SK1), CD19-PerCP (SJ25C1), CD3-APC (SK7), CD8-APC

(SK1) and CD34-APC (8G12) (all from BD Biosciences), CD13-RDI (MY7) and CD33-RDI (906) (from Beckman Coulter). Purity of sorted population was determined on the FACS Calibur (BD Biosciences) and shown to be over 95% for all populations. All populations were sorted twice using different donors for each sort.

DNA isolation, and RQ-PCR analyses of TCR gene rearrangements

DNA from thymic subsets, $CD34^+$ lin^- UCB cells and peripheral $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T cells was extracted with the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich), according to the manufacturers protocol. DNA was extracted from control cell lines using the QIAamp Blood Midi kit (Qiagen) according to the manufactures protocol. RQ-PCR for quantification of different TCR recombinations was essentially performed as described previously using ABI Prism 7700 equipment (Applied Biosystems)[25]. The following *TCRD* recombinations were determined. Dδ2-Dδ3; using Dδ2 and Dδ3 specific primers, Dδ2-Jδ1; using Dδ2 and Jδ1 specific primers, Vδ1-Jδ1 and Vδ2-Jδ1; were performed in two independent PCR reactions using either a Vδ1or a Vδ2 primer in combination with a Jδ1 primer, Vδ-Dδ3; using six specific Vδ primers (Vδ1-Vδ6) in combination with a Dδ3 primer. The cell lines/diagnostic samples that were selected as clonal control DNA for *TCRD* rearrangements are the following: Dδ2-Dδ3 (Nalm 16), Dδ2-Jδ1 (Loucy), Vδ1-Jδ1 (Peer), Vδ2-Jδ1 (T-ALL sample), all Vδ's-Dδ3 (Nalm 16 with Vδ2-Dδ3). *TCRG* rearrangements were determined as follows: Vγ to Jγ1.1 and Jγ2.1 rearrangements were determined in two independent PCR reactions using a Jγ1.1/2.1 consensus primer (recognizing Jγ1.1 and Jγ2.1) in combination with either a VγI family primer (VγIf; recognizing Vγ2, Vγ3, Vγ4, Vγ5, ψVγ7 and Vγ8) and a Vγ10 primer or in combination with a Vγ9 and a Vγ11 primer. Analysis of Vγ to Jγ1.3 and Jγ2.3 rearrangement which was performed using a Jγ1.3/2.3 consensus primer (recognizing Jγ1.3 and Jγ2.3) in combination with VγIf, Vγ9, Vγ10 and Vγ11 primers. Analysis of Vγ to Jγ1.2 rearrangement which was performed using a Jγ1.2 primer in combination with VγIf, Vγ9, Vγ10 and Vγ11 primers. The cell lines/diagnostic samples that were selected as clonal control DNA for *TCRG* rearrangements are the following: Vγ1f+Vγ10-Jγ1.1/2.1 (HUT 78 with Vγ8-Jγ1.1), Vγ9+Vγ11-Jγ1.1/2.1 (ARR with Vγ9-Jγ1.1), all Vγ's-Jγ1.3/2.3 (RPMI 8402 with Vγ10-Jγ2.3 and Vγ4-Jγ2.3), all Vγ's-Jγ1.2 (T-ALL sample with Vγ9-Jγ1.2). *TCRB* Dβ to Jβ rearrangements were performed in two independent PCR reactions containing either a Dβ1 primer in combination with six primers for the Jβ1 cluster (one specific primer for each Jβ1 gene segment) or a Dβ2 primer in combination with seven primers for the Jβ2 cluster (one specific primer for each Jβ2 gene segment). As a clonal control for Dβ1-Jβ1 and Dβ2-Jβ2 gene rearrangements we used HUT78 which contains a Dβ1-Jβ1.2 and Dβ2-Jβ2.3 rearrangement. Furthermore, in order to examine initiation of *TCRA* rearrangement, we determined the TCRD deleting δREC-ψJα rearrangement using a δRec primer in combination with a ψJα specific primer and for which we used H-SB2 as clonal control. In addition we determined the decrease of *TCRA* germline DNA in all thymic subsets and peripheral $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T cells by using a primer located just upstream of ψJα in combination with a primer located just downstream of ψJα. For this purpose $CD34^+lin^-$ UCB cells were used as a hundred percent *TCRA* germline control. All applied forward/reverse primers and TaqMan probes are available at http://www.jem.org/cgi/content/full/jem.2004 2524/DC1; most have been described previously[5,9,26,27].

Control cell lines and T-ALL controls were serially diluted in a background of un-rearranged (germline) DNA in order to generate a standard curve ranging from 100% to 0.1% of specific template DNA (specific rearrangement) in a non-template background. An albumin RQ-PCR was performed to normalize the amount of input DNA of non-diluted control DNA and DNA from thymocyte subsets, $CD34^+lin^-$ UCB cells and peripheral T-cells, using serially diluted reference DNA. A typical RQ-PCR mixture of 25 µl contained TaqMan Universal MasterMix (Applied Biosystems), 540-900 nM of each primer, 100 nM of FAM-TAMRA labeled probe, 0.4 µg of bovine serum albumin and 50 ng of genomic DNA. Positive controls were considered to be clonal and to contain hundred percent of the specific rearrangement. C_T values of thymic subsets, $CD34^+lin^-$ UCB cells and peripheral T cell DNA for a particular TCR RQ-PCR were subsequently plotted on the corresponding standard curve, and expressed as relative rearrangement levels as compared to the corresponding clonal control DNA. The sensitivity of each

assay was at least 1%. All RQ-PCR experiments were performed in duplicate on two independently purified sets of all isolated subsets.

GeneScan analysis for complete and in-frame Vβ-Jβ gene rearrangements

In order to analyze Vβ-Jβ rearrangements and selection of the TCRβ chain (i.e. in-frame *TCRB* gene rearrangements) in the isolated populations, complete *TCRB* V-J rearrangements were amplified with HEX-labeled primers for the Jβ1 cluster and FAM-labeled primers for the Jβ2 cluster. This was done by two multiplex PCR reactions as described by the BIOMED-2 Concerted Action BMH4 CT98-3936: tube A containing primers for each Jβ1 segment as well as Jβ2.2, Jβ2.6, and Jβ2.7 primers in combination with Vβ primers and tube B containing Jβ2.1, Jβ2.3, Jβ2.4, and Jβ2.5 primers in combination with Vβ primers[9]. The obtained PCR products were subsequently subjected to GeneScan analysis as described before[9].

TEA expression by real time quantitative (RQ)-PCR analyses

In order to determine the expression of the sterile T-early alpha (TEA) transcript in the isolated populations, RNA was reverse transcribed into cDNA as described previously[28]. Subsequently the cDNA was diluted by adding 80 µl of milli Q water yielding a final concentration of 10 ng/µl. As positive control for TEA expression the immature T-cell line SUPT3 was used and as a negative control the immature T-cell line H-SB2, which has both *TCRD* alleles deleted and therefore expected to be TEA negative, was used as well as the non-hematopoietic cell line HeLa. RQ-PCR was performed as previously[29], and the expression of TEA was defined by calculating the ratio of TEA to the average value of the control genes Abelson (*ABL*) and beta-glucuronidase (*GUSB*)[29].

Microarray analysis

Microarray analysis was essentially done as described previously[30] and according to MIAME guidelines [31]. RNA was isolated using RNeasy columns (Qiagen) according to the manufacturers protocol and RNA quality was assessed using the Agilent 2100 BioAnalyzer. Two to five µg of total RNA from the $CD34^+CD38^+CD1a^-$, $CD34^+CD38^+CD1a^+$, ISP, DP $CD3^-$, DP $CD3^+$, SP $CD4^+$ and SP $CD8^+$ populations was used to generate double-stranded cDNA using SuperScript reverse transcriptase and a T7-oligo(dT) primer. An ENZO kit (ENZO-Life Sciences Inc) was used to convert cDNA into biotinylated cRNA utilizing T7 RNA polymerase and biotinylated ribonucleotides. Biotinylated cRNA was separated from enzymes and unincorporated nucleotides using RNeasy 'clean-up' spin columns (Qiagen). For the smaller, earlier populations, $CD34^+lin^-$ UCB cells, $CD34^+CD38^-CD1a^-$ thymocytes and for control purposes the $CD34^+CD38^+CD1a^+$ thymocytes, the GeneChip Eukaryotic Small Sample Target Labeling Assay Version II (Affymetrix) was used to generate biotinylated cRNA. Eleven µg of cRNA was fragmented for 35 min at 95 ˚C. 10 µg of fragmented cRNA was then hybridized to HG-U133A human genome microarrays for 16 hours at 45 ˚C followed by washing, staining and scanning at 570 nm (Affymetrix). All raw microarray data are freely available at http://franklin.et.tudelft.nl/.

Statistical analysis

Probe intensity background was removed using robust multichip analysis (RMA[32]). The intensity levels were then quantile normalised[33]. Array groups (two biologically independent arrays per group) corresponding to the development stages were compared based on the perfect match (PM) probe intensity levels only[32], by performing a per-probe set two-way analysis of variance (ANOVA, with factors "probe" and "stage"). This resulted in average expression levels over the two biological repeats for each probe set in each stage, as well as a *p*-value for the significance of the difference between the stages. As data resulting from the standard protocol ("non-amplified") are not directly comparable to those from the small sample protocol ("amplified"), the expression levels measured for probe sets under the small sample protocol were scaled, according to the results in the $CD34^+CD38^+CD1a^-$ population, which was analyzed by both protocols. For each individual probe set, the scaling factor used was the ratio between the average expression level measured for that probe set in the $CD34^+CD38^+CD1a^-$ population, under

the standard protocol and the small sample protocol.

The *p*-values were adjusted for multiple testing using Šidák step-down adjustment[34] and all differences with adjusted *p*-values <0.05 were considered significant. All probe sets that underwent a significant change between any two successive stages of differentiation ($CD34^+lin^-$ UCB vs $CD34^+CD38^-CD1a^-$, $CD34^+CD38^-CD1a^-$ vs $CD34^+CD38^+CD1a^-$, $CD34^+CD38^+CD1a^-$ vs $CD34^+CD38^+CD1a^+$, $CD34^+CD38^+CD1a^+$ vs ISP, ISP vs DP $CD3^-$, DP $CD3^-$ vs DP $CD3^+$, DP $CD3^+$ vs SP $CD4^+$ and DP $CD3^+$ vs SP $CD8^+$) were selected.

The OmniViz software package was used to construct and display a Pearson correlation matrix between the individual microarrays, based on the expression values of significant probe sets. Before calculating correlations, all expression values lower than 30 were set to 30. Expression values were then $\log_2$-transformed and the per probe set geometric mean was subtracted. Further analysis was performed using the Genlab software toolbox, running in the Matlab programming environment (http://www.genlab.tudelft.nl). After per-probe set normalization to zero mean and unit standard deviation (*z*-score), a hierarchical clustering (complete linkage) was calculated based on Pearson correlation. The number of clusters *k* was determined by looking for a local minimum of the Davies-Bouldin index calculated for $k=1, \ldots, 30$[35].

For further analysis the list of significant probe sets was filtered, using the annotation found in the Affymetrix NetAffx analysis database (http://www.affymetrix.com/), for probe sets related to transcriptional activity based on the following Gene Ontology (GO) Consortium designations: nucleic acid binding (GO: 3676), DNA binding (GO: 3677), transcription factor activity (GO: 3700), transcription cofactor activity (GO: 3712), transcription co-activator activity (GO: 3713), transcription co-repressor activity (GO: 3714), transcription (GO: 6350), transcription, DNA dependent (GO: 6351), regulation of transcription, DNA dependent (GO: 6355), transcription from Pol II promoter (GO: 6366), transcription initiation from Pol II promoter (GO: 6367), transcription factor complex (GO: 5667), negative regulation of transcription (GO: 16481), transcriptional activator activity (GO: 16563), transcriptional repressor activity (GO: 16564) and positive regulation of transcription (GO: 45941). Less relevant genes obtained from these categories (e.g., RNA editing enzymes/polymerases, nucleosome assembly proteins, histones, etc) where manually removed from this list. The resulting 446 probe sets were then hierarchically clustered as described above.

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THE HUMAN THYMUS CONTAINS MULTIPOTENT PROGENITORS WITH T/B LYMPHOID, MYELOID AND ERYTHROID LINEAGE POTENTIAL

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Submitted

ABSTRACT

It is a longstanding question which bone marrow-derived cell seeds the thymus and to what level this cell is committed to the T-cell lineage. We sought to elucidate this issue by examining gene expression, lineage potential and self-renewal capacity of the two most immature subsets in the human thymus, namely $CD34^+CD1a^-$ and $CD34^+CD1a^+$ thymocytes.

DNA microarrays revealed the presence of several myeloid and erythroid transcripts in $CD34^+CD1a^-$, but not in $CD34^+CD1a^+$ thymocytes. Lineage potential of both subpopulations was assessed using *in vitro* colony assays, bone marrow stroma cultures and *in vivo* transplantation into NOD/SCID mice. The $CD34^+CD1a^-$ subset contained progenitors with lymphoid (both T and B), myeloid and erythroid lineage potential. Remarkably, development of $CD34^+CD1a^-$ thymocytes towards the T-cell lineage, as shown by T-cell receptor delta gene rearrangements, could be reversed into a myeloid cell-fate. In contrast, the $CD34^+CD1a^+$ cells yielded only T cell progenitors, demonstrating their irreversible commitment to the T-cell lineage. Both $CD34^+CD1a^-$ and $CD34^+CD1a^+$ thymocytes failed to repopulate NOD/SCID mice.

Together, these observations suggest that the human thymus is seeded by multipotent progenitors with a much broader lineage potential than previously assumed. These cells resemble hematopoietic stem cells, but lack sufficient self-renewal capacity.

INTRODUCTION

The thymic microenvironment is exceptional in its ability to sustain production of T cells[1]. However, hematopoietic stem cells (HSCs) that will eventually give rise to T cells, are derived from the bone marrow (BM). The nature of the thymus-seeding cell and its relation to several BM progenitors has remained elusive, despite being the subject of intense investigation. Furthermore, it is controversial whether cells commit to the T-cell lineage pre- or intrathymically[2,3].

Aided by modern cell sorting techniques, many studies in the mouse have recently readdressed these issues. Adult murine BM has been demonstrated to contain precursors with a restricted T/B lymphoid potential, so-called common lymphoid progenitors (CLPs)[2,4]. However, the earliest thymic immigrants were shown to differ from CLPs in several aspects[5]. In peripheral blood, T-lineage potential appeared to be restricted to $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) progenitor populations, rather than to CLPs[6]. Recently, two detailed analyses of the earliest subpopulations in the murine thymus showed variable lineage potential of different subsets[7,8]. Together, these studies point towards a model in which a range of BM-derived progenitors colonize the thymus, probably including

multipotent progenitors and more lineage-restricted precursor cells[9]. For the human system, comparable experimental data are lacking. Determining lineage potential of early thymocytes will help to unveil the identity of the thymus-seeding cell in man.

Both in man and mouse, the most immature cells in the thymus do not express CD4 and CD8 and therefore are called double negative (DN). In humans, the DN stage can be further subdivided by staining for CD34 and CD1[10,11]. CD34 is a marker for hematopoietic stem cells and progenitors in all hematopoietic organs. Also in the thymus, the most immature cells are highly positive for CD34 and levels decline as the cells mature[10,11]. Concomitant with the decrease of CD34, thymocytes acquire expression of CD1[12]. This is usually detected by staining for CD1a, but CD1b, c, d and e are also expressed. This order of developmental steps is supported by the rearrangement status of the T-cell receptor (TCR) loci in the consecutive stages. The $CD34^+CD1a^-$ cells start to rearrange their *TCRD* genes, but mostly have their *TCRG* and *TCRB* loci still in germline position, whereas expression of CD1a is accompanied by rearrangements of *TCRD* (V to DJ), *TCRG* and *TCRB* (D to J) loci[13]. The immature single positive (ISP) cells have lost CD34 expression and contain mature *TCRB* (V to DJ) rearrangements[13]. The $CD34^+CD1a^-$ and $CD34^+CD1a^+$ subsets comprise about 0.4% and 0.6% of all thymocytes respectively[11].

Because of their TCR gene rearrangement status, $CD34^+CD1a^+$ thymocytes are thought to be irreversibly committed to the T-cell lineage. Nevertheless, their potential to differentiate into other lineages has not been studied in detail. Lineage potential of $CD34^+CD1a^-$ is generally thought to be less restricted, but the range of progeny they can generate is under debate. The capability of human $CD34^+$ thymocytes to develop into natural killer (NK) cells and dendritic cells (DCs) has been demonstrated by several studies, both in fetal[14,15] and adult[15,16] thymus. B-cell precursor potential of $CD34^+CD1a^-$ thymocytes has, to our knowledge, never been reported. However, we and others have shown the presence of significant numbers of B cells in the human thymus[11,17,18]. Furthermore, in the thymus we could detect the presence of all BM B-cell progenitor stages, indicating that B cells develop in the thymus, albeit at much lower frequencies than in BM[11].

Myeloid potential of $CD34^+$ thymocytes was investigated by a number of studies several years ago, yielding contradictory results. While one study could generate myeloid colonies from $CD34^+$ as well as from $CD34^-$ thymocytes[19], others could not confirm any myeloid potential in $CD34^+$ thymocytes[20]. Toribio and co-workers demonstrated that myeloid DC precursors can be generated from $CD34^+$ thymocytes in cultures containing M-CSF[21].

In this study, we investigated the potential of $CD34^+CD1a^-$ and $CD34^+CD1a^+$ thymocytes to develop into all hematopoietic lineages, as well as their ability to self-renew.

RESULTS

CD34$^+$CD1a$^-$ but not CD34$^+$CD1a$^+$ thymocytes express genes of the myeloid and erythroid/megakaryocyte lineages

Previously, gene expression profiles have been generated of eight thymocyte subsets corresponding to consecutive differentiation stages[13]. We here studied the expression profiles of two populations in greater detail: the most immature CD34$^+$CD1a$^-$ subset that that at least retains NK and DC potential[10] and the CD34$^+$CD1a$^+$ thymocytes that are presumed to be T-cell committed (Figure 1).

As expected, CD34$^+$CD1a$^-$ cells expressed high levels of progenitor marker transcripts such as *CD34* and *CD133* (Figure 2A), reflecting their immature phenotype. In the CD34$^+$CD1a$^+$ population upregulation of T-cell specific transcripts (e.g. *CD3*, *PTCRA* (the gene for pTα) and *LAT*) and genes involved in TCR rearrangements (e.g. *RAG*, *DNTT* (the gene for TdT), sterile *TCRB* transcripts) occurred (Figure 2B). To our surprise, we found that concurrently transcription of many myeloid-associated genes, including *myeloperoxidase* (*MPO*), *CD13* and *CD33* was downregulated during development from CD34$^+$CD1a$^-$ to CD34$^+$CD1a$^+$ cells (Figure 2C). In addition, the gene for the erythrocyte/megakaryocyte specific transcription factor *NF-E2* (*nuclear factor erythroid-derived 2*) was transcribed at significantly lower levels in the CD34$^+$CD1a$^+$ cells (Figure 2C).

Our microarray data were validated using flow cytometry. The percentage of cells positive for CD13/33 and MPO proteins was higher in the CD34$^+$CD1a$^-$ population than in the CD34$^+$CD1a$^-$ cells (Figure 2D).

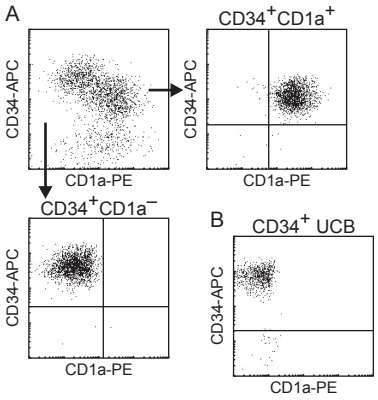

Figure 1. Purification of cell subsets.
(A) Total CD34$^+$ cells were isolated from human thymus using AutoMACS and subsequently FACS sorted into CD34$^+$CD1a$^-$ (lower panel) and CD34$^+$CD1a$^+$ (right panel) fractions. (B) CD34$^+$ cells were isolated from human UCB using AutoMACS and FACS sorting.

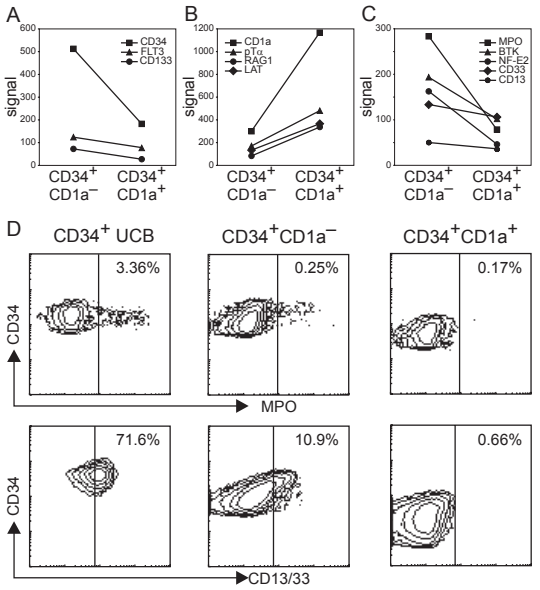

Figure 2. Gene expression of CD34⁺CD1a⁻ and CD34⁺CD1a⁺ thymocytes.
Expression levels (in arbitrary fluorescence units) of several genes were extracted from previously performed Affymetrix microarrays[13]. (A) 'Stem cell-like' genes, (B) T-cell specific genes, (C) 'non-T lineage' genes. (D) Expression patterns of MPO and CD13/CD33 were validated at the protein level using flow cytometry. CD34⁺ cells from thymus and UCB were purified using AutoMACS and gated for the indicated populations. Expression of CD13/33 in mature granulocytes was at least 10 fold higher than in CD34⁺ UCB cells.

These data suggest that CD34⁺CD1a⁻ thymocytes are not only immature and not yet committed to the T-cell lineage, but may in addition still possess myeloid and erythroid potential.

CD34⁺CD1a⁻ but not CD34⁺CD1a⁺ thymocytes have myeloid and erythroid potential *in vitro*

To study the myeloid and erythroid lineage potential of the two CD34⁺ thymocyte populations, cells were sorted and cultured in *in vitro* assays that allow outgrowth of either granulocyte/monocyte colony forming units (CFU-GM) or erythrocyte burst forming units (BFU-E). As a positive control, CD34⁺ cells from human umbilical cord blood (UCB) were used. The CD34⁺CD1a⁺ thymocytes did not give rise to any colonies in our assays, but the CD34⁺CD1a⁻ thymocytes yielded clearly detectable colonies, both CFU-GM and BFU-E (Table I and Figure 3A and B). The efficiency of CD34⁺CD1a⁻ thymocytes to yield myeloid colonies was about 30 times lower than that of CD34⁺ UCB cells, while erythroid potential was about 100 times less efficient (Table I).

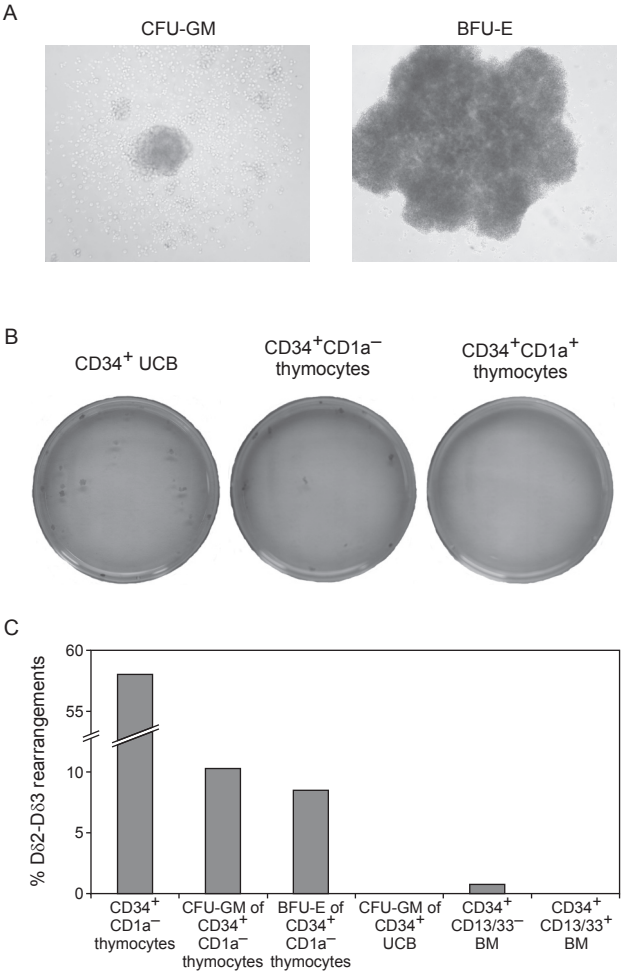

Figure 3. *In vitro* colony assays.
Sorted $CD34^+CD1a^-$ and $CD34^+CD1a^+$ thymocytes and $CD34^+$ UCB cells were plated in semi-solid cultures containing appropriate cytokines to generate myeloid (CFU-GM) or erythroid (BFU-E) colonies. (A) Typical myeloid (left) and erythroid (right) colonies generated from $CD34^+CD1a^-$ thymocytes. (B) Overview of BFU-E dishes in which 10^5 $CD34^+CD1a^-$ or $CD34^+CD1a^+$ thymocytes or 10^3 $CD34^+$ UCB cells were plated. No colonies were detected in dishes with $CD34^+CD1a^+$ thymocytes. (C) RQ-PCR analysis of immature *TCRD* gene rearrangements (Dδ2-Dδ3) in original $CD34^+CD1a^-$ thymocytes, myeloid and erythroid colonies generated from $CD34^+CD1a^-$ thymocytes, myeloid colonies generated from $CD34^+$ UCB cells and $CD34^+$ cells from children's BM sorted into either $CD13/33^-$ (mostly B cell progenitors) and $CD13/33^+$ (myeloid progenitors) fractions. Percentages of rearranged alleles in each sample are shown.

Table I. *In vitro* colony assays.

	CFU-GM[1]	BFU-E[1]
$CD34^+$ UCB	3267 ± 176	533 ± 233
$CD34^+CD1a^-$	108 ± 3	4.5 ± 1.5
$CD34^+CD1a^+$	0	0

[1] Numbers of myeloid (CFU-GM) and erythroid (BFU-E) colonies calculated per 10^5 input cells. Means and standard errors of triplicate wells. Results are representative of three independent experiments.

Rearrangements of the *TCRD* locus were quantitatively determined in the acquired colonies. While colonies derived from $CD34^+$ UCB cells did not contain any rearranged *TCRD* genes, the most immature *TCRD* rearrangements (Dδ2-Dδ3) were present in about 10% of the alleles in the $CD34^+CD1a^-$ thymocyte-derived colonies, both CFU-GM and BFU-E (Figure 3C). The proportion of Dδ2-Dδ3 found in the original $CD34^+CD1a^-$ thymocytes was approximately 60%[13], while $CD34^+CD13/33^+$ myeloid progenitor cells from children's BM did not contain any *TCRD* rearrangements (Figure 3C). $CD34^+CD13/33^-$ BM cells, which represent mainly B-cell precursors, did have (very low) levels of *TCRD* rearrangements (Figure 3C), consistent with earlier reports[22]. These data demonstrate that the developmental fate of cells that have initiated their differentiation into the T-cell lineage can still be reversed towards the myeloid and erythroid lineages.

Lineage capacity and frequency of $CD34^+CD1a^-$ and $CD34^+CD1a^+$ thymocytes

To assess the ability of $CD34^+CD1a^-$ and $CD34^+CD1a^+$ thymocytes to develop into different hematopoietic lineages in more detail, we performed cultures on OP9 BM stromal cells expressing either GFP alone (OP9-GFP) or Notch ligand Delta1 (DL) in combination with GFP (OP9-DL). Culturing of $CD34^+$ UCB cells on OP9-GFP has been previously shown to induce myeloid and B lymphoid development, while OP9-DL allows the development of T cells[23]. Both $CD34^+CD1a^-$ and $CD34^+CD1a^+$ thymocytes efficiently developed into $CD4^+CD8^+$ double positive cells when cultured for 14 days on OP9-DL (Figure 4A), although $CD34^+CD1a^-$ cells yielded four fold higher cell numbers (Figure 4B), probably because they have higher proliferative capacity.

As was expected from the colony assays (Table I), there were large differences between $CD34^+CD1a^-$ and $CD34^+CD1a^+$ thymocytes when cultured on OP9-GFP. The $CD34^+CD1a^-$ cells were able to proliferate on OP9 cells lacking DL, although they yielded significantly lower cell numbers than when cultured with DL (Figure 4B). In contrast, $CD34^+CD1a^+$ cells did not grow on OP9-GFP (Figure 4B), illustrating their irreversible commitment to the T-cell lineage and thereby need for continuous Notch signaling[24].

After culturing $CD34^+CD1a^-$ thymocytes on OP9-GFP for 14 days, a subset of cells was found to be negative for CD7 (Figure 4C) and had developed into myeloid and B cells (Figure 4D). In some cultures both myeloid and B lymphoid cells were detected,

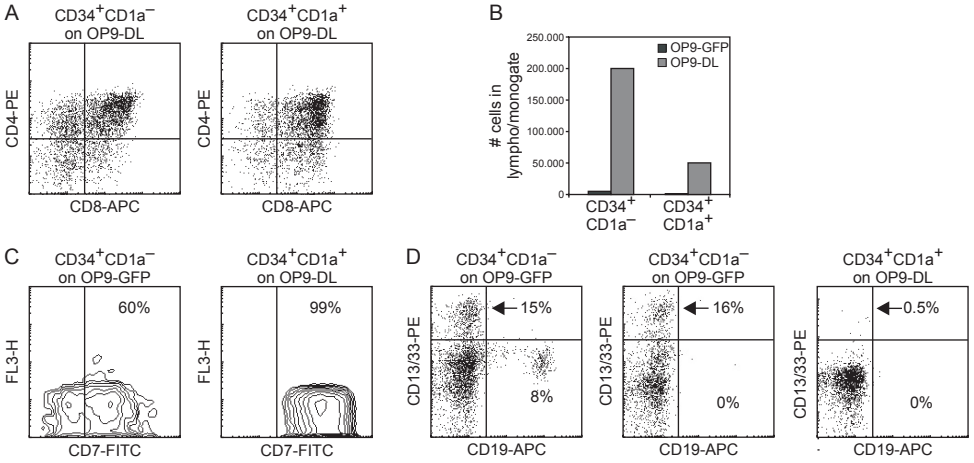

Figure 4. OP9 co-cultures.
(A) Development of $CD4^+CD8^+$ double positive cells from $CD34^+CD1a^-$ (left panel) and $CD34^+CD1a^+$ (right) thymocytes cultured for 14 days on OP9-DL; 1000 cells per well were seeded. (B) Cell numbers per well after 14 days of culturing 1000 $CD34^+CD1a^-$ or $CD34^+CD1a^+$ thymocytes on OP9-GFP or OP9-DL. (C) CD7 staining of 300 $CD34^+CD1a^-$ thymocytes cultured on OP9-GFP (left panel) or OP9-DL (right) for 14 days. (D) Presence of $CD19^+$ B cells and/or $CD13/33^+$ myeloid cells in 14-day co-cultures of 300 $CD34^+CD1a^-$ thymocytes on OP9-GFP (left and middle panel) or on OP9-DL (right panel).

whereas in others cells from a single lineage were observed (Figure 4D). On the contrary when cultured on OP9-DL, all $CD34^+CD1a^-$ thymocytes developed into CD7 positive cells (Figure 4C) and only minute percentages (<1%) of cells representing non T-cell lineages were detected (Figure 4D right panel).

To determine the frequencies by which $CD34^+CD1a^-$ thymocytes can develop into the different lineages, we performed limiting dilution cultures on OP9-GFP. The frequency of progenitors with B lineage potential was approximately 1 in 540 and with myeloid potential approximately 1 in 160 (Figure 5). In the presence of GM-CSF, the frequency of myeloid progenitors was increased to 1 in 48 $CD34^+CD1a^-$ thymocytes (Figure 5).

As a control, the progenitor frequency of $CD34^+CD1a^-$ thymocytes that can give rise to T cells was determined by culturing cells on OP9-DL for 14 days and staining for CD4 and CD8. The T-cell progenitor frequency was found to be only 1 in 27 $CD34^+CD1a^-$ thymocytes (Figure 5, right panel). A similar frequency was found when single cells were seeded on OP9-DL: after 14 days growing cells were found in 7 out of 192 wells. As it can be presumed that in an *in vivo* thymus environment the vast majority of $CD34^+CD1a^-$ cells would develop into T cells, the OP9 system appears to be rather inefficient for culturing human cells. The real frequencies of myeloid and B lineage progenitors within

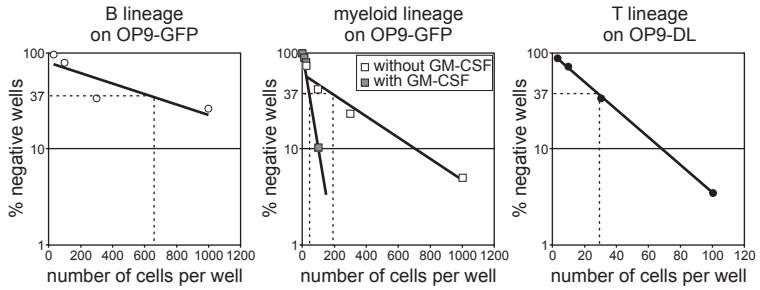

Figure 5. Frequencies of myeloid, B- and T-cell lineages.
Precursor frequencies within the CD34$^+$CD1a$^-$ thymocyte population were determined by limiting dilution assays and calculated using the method of maximum likelihood. Left panel: CD19$^+$ B cells on OP9-GFP. Frequency 1 in 644 (720 and 270 in subsequent experiments). Middle panel: CD13/33$^+$ myeloid cells on OP9-GFP. Frequency 1 in 193 (126 in a second experiment) in the absence of GM-CSF (open squares) and 1 in 48 in the presence of GM-CSF (solid squares). Right panel: CD8$^+$ T cells on OP9-DL. Frequency 1 in 29.

CD34$^+$CD1a$^-$ thymocytes are therefore probably also a factor 30 higher than calculated from our experiments.

CD34$^+$CD1a$^-$ and CD34$^+$CD1a$^+$ thymocytes lack *in vivo* repopulating capacity, inherent to HSCs

The finding that CD34$^+$CD1a$^-$ thymocytes have T/B lymphoid, myeloid, and erythroid developmental capacity, together with the previously demonstrated NK cell and DC potential, raises the possibility that these cells either behave as very immature multi-lineage progenitors or as true HSCs. A hallmark of HSCs is self-renewal capacity, of which NOD/SCID repopulating ability is the most commonly accepted read-out for the human system[25].

Consequently, we performed transplantations of CD34$^+$CD1a$^-$ and CD34$^+$CD1a$^+$ thymocytes into sublethally irradiated immunodeficient NOD/SCID mice. As a control we transplanted 10^5 sorted CD34$^+$ UCB cells and showed that these efficiently repopulated BM and spleen (but not the thymus) of all mice tested (4-54% of BM cells positive for human CD45; mean 26%) and gave rise to B-cell and myeloid lineages (Figure 6A). In contrast, transplantation with CD34$^+$CD1a$^-$ or CD34$^+$CD1a$^+$ thymocytes did not yield any cells positive for human CD45 above background levels (non-transplanted mice) in any organ, even when administered in a very high dose (5x10^6 CD34$^+$ thymocytes per mouse) (Figure 6B). These results suggest that CD34$^+$ thymocytes do not have self-renewal capacity.

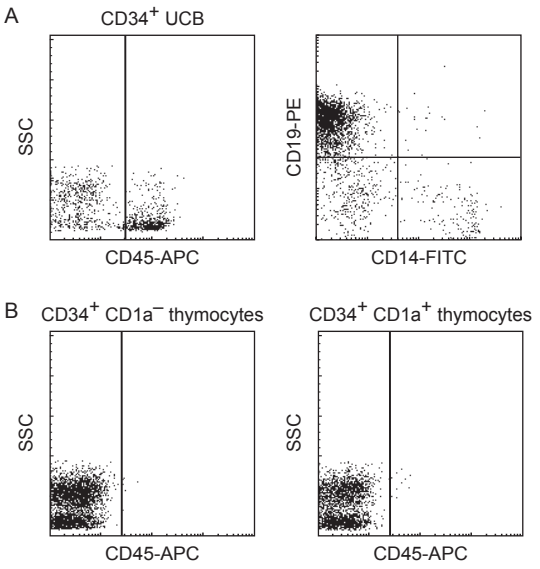

Figure 6. Repopulation of NOD/SCID mice.
(A) Human $CD34^+$ UCB cells efficiently repopulate BM of NOD/SCID mice. Left panel: expression of human CD45 within the lympho/monogate. Right panel: expression of CD19 and CD14 within the CD45 gate. (B) No human $CD45^+$ cells were detected in BM of mice transplanted with $CD34^+CD1a^-$ thymocytes (left panel) or with $CD34^+CD1a^+$ thymocytes (right panel).

DISCUSSION

Our study demonstrates that $CD34^+CD1a^-$ thymocytes have potential to generate T, B, myeloid and erythroid cells *in vitro*. Megakaryocytic potential was suggested by the expression of NF-E2 in $CD34^+CD1a^-$ thymocytes and was confirmed by culturing thymocytes in suspension in the presence TPO and SCF. While $CD34^+CD1a^+$ thymocytes did not survive, $CD34^+CD1a^-$ thymocytes could be cultured in this condition for at least 14 days and developed into large cells resembling megakaryocytes, although definitive flow-cytometric characterization (e.g. by staining for CD41) was hindered by high autofluorescence (data not shown). As previous studies have already provided evidence for development into NK and DC cells from $CD34^+CD1a^-$ thymocytes[14-16], precursors for the full range of hematopoietic cells appear to be present in the human thymus. Although we did not formally prove that all these lineages can be attributed to a single progenitor cell, our data are evocative of the existence of an immature, stem cell like progenitor in the human thymus. Also studies in the murine thymus have shown that the DN1 subset (the earliest DN subset in the mouse) contains multilineage progenitors[7,8,26]. These

included precursors for T, B and NK cells and DC and macrophages, but erythrocyte development has not been shown before.

Frequencies of myeloid and erythroid potential, as determined by *in vitro* colony assays, were markedly lower in the $CD34^+CD1a^-$ thymocytes than in $CD34^+$ UCB cells, probably because UCB contains higher numbers of more mature progenitors. Also in the OP9 co-cultures we found low frequencies of myeloid and B lineage progenitors in the $CD34^+CD1a^-$ subset. As frequencies of the different lineages did not overlap, we could not provide definitive evidence for a thymic pluripotent progenitor. Instead, the $CD34^+CD1a^-$ population may contain committed precursors for alternative (non-T) lineages. However, we think it highly unlikely that, for instance, a committed erythroid progenitor would enter the thymus. More efficient single cell assays will be needed to definitely solve the question of pluripotent progenitors versus mixtures of lineage-committed progenitors. In our hands, single cell cultures were technically difficult and inconclusive, probably because the OP9 system is not optimal for human cells. La Motte-Mohs et al.[23] have demonstrated that the OP9-DL culture system allows T-cell development from human $CD34^+CD38^-$ UCB cells, but in this study large numbers of cells (>10,000 per well) were seeded and no limiting dilutions were performed.

Although seeding efficiencies on OP9-GFP were too low to draw definitive conclusions, we did find wells containing both B and myeloid cells, also among wells seeded with limited numbers of cells (e.g. 10 or 30). Furthermore, the frequency of myeloid progenitors, when cultured on OP9-GFP in the presence of GM-CSF, was only two times lower than the frequency of T-cell progenitors. Together these data point towards the existence of multilineage progenitors in the human thymus.

The remarkable finding that normal human thymocytes that have undergone immature *TCRD* rearrangements can still be induced to develop into myeloid and erythroid cells, while these rearrangements are absent in $CD13/33^+$ myeloid progenitors in BM, demonstrates that the distinction between lymphoid, myeloid and erythroid development is not as rigid as often assumed. A close lineage relationship between myeloid cells and T cells has also been suggested by experiments in the fetal mouse, by Katsura and co-workers[27].

The range of lineage potentials of early thymocytes may give clues about the identity of the BM progenitor that seeds the thymus. In the mouse, several candidate thymus-colonizing progenitors have been proposed. These include lymphoid-restricted common lymphoid progenitors (CLPs)[2], early lymphoid progenitors (ELPs) that have limited myeloid potential[28], multipotent $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) cells[6] and bipotent T/myeloid progenitors[29]. Experimental data in the mouse argue against the CLP as a likely candidate. Although CLPs were detected in murine BM[2], cells with the same surface markers could not be detected in the thymus[5,6]. Furthermore, BM contains other progenitors upstream of CLPs (e.g. LSK cells) that can efficiently generate T

cells[6,30]. Our finding that the earliest subset in the human thymus (as a population) has a very broad lineage potential, suggests that also the human thymus is not seeded by lymphoid restricted CLPs, but probably by more stem-cell like progenitors. This view is strengthened by our observation in OP9-GFP co-cultures that the frequency of myeloid potential within $CD34^+CD1a^-$ thymocytes was higher than that of B cells, as is the case for normal human HSCs. Adolfsson and co-workers recently identified a population in the LSK compartment of murine BM that has lymphoid and myeloid, but not erythroid and megakaryocytic potential[31]. The fact that we find cells with erythroid potential within the $CD34^+CD1a^-$ population suggests that at least part of the thymus-seeding cells are true HSCs.

The hypothesis that that the human thymus is seeded by a stem-cell like cell is supported by the fact that HSCs circulate in adult peripheral blood, both in mouse[32] and human[33] and can repopulate the thymus of irradiated mice[6]. However, this suggestion is contradicted by our finding that $CD34^+CD1a^-$ thymocytes lack sufficient self-renewal capacity, as they could not repopulate NOD/SCID mice. In the mouse it has been shown that proliferation of a precursor that enters the thymus is limited to several weeks and that no permanent endogenous stem cell exists[34]. Nevertheless, it remains possible that the thymus is seeded by a HSC that rapidly looses self-renewal capacity and reduces non-T cell potential after entering the thymus and interacting with molecules on the thymic epithelium, for instance Notch ligands. Another possibility is that only a minute fraction of the cells that seed the thymus retains self-renewal and that this population is too rare to be detected with currently employed assays.

An alternative explanation for the fact that $CD34^+CD1a^-$ thymocytes could not repopulate NOD/SCID mice is that they lack BM homing capability. One of the adhesion molecules necessary for BM homing is CXCR4[35,36]. However, using flow cytometry we could not detect any difference in CXCR4 expression between $CD34^+$ UCB cells, which efficiently repopulate NOD/SCID BM, and $CD34^+$ thymocytes (data not shown). This suggested that the BM homing capacity through CXCR4 is not affected, although absence of other homing signals in $CD34^+$ thymocytes cannot be excluded. Poor homing capability can be considered as an additional argument against the existence of a thymic HSC, as murine HSCs were shown to have high BM-seeding efficiency[37].

In contrast to the immature characteristics of the $CD34^+CD1a^-$ cells, the $CD34^+CD1a^+$ thymocytes showed a T-cell specific gene expression pattern and did not yield any non-T-lineage cells in *in vitro* cultures. We did not test NK and DC potential, but previous studies have shown a very limited NK and no DC precursor activity of $CD34^+CD1a^+$ cells[10]. Together these findings suggest that human $CD34^+CD1a^+$ thymocytes are the equivalents of murine DN3 cells, in which final commitment to the T-cell lineage occurs[38]. This confirms our recently proposed scheme in which the stages of human T-cell development are highly similar to those in the mouse[13].

In summary, we here characterized the $CD34^+CD1a^-$ and $CD34^+CD1a^+$ thymic populations by gene expression profiling and functional studies with respect to lineage potential and self-renewal capacity. We show that lineage potential of the $CD34^+CD1a^-$ thymocytes is much broader than previously thought and includes erythrocytic and B-lymphocytic capacity. Although clonal assays with a higher seeding efficiency than those used here are necessary to prove whether all lineages can be attributed to a single progenitor, our data strongly suggest that the human thymus is seeded by multipotent progenitors. These progenitors are distinct from CLPs and may be pluripotent HSCs, which immediately loose self-renewal capacity upon contact with the thymic epithelium.

MATERIALS AND METHODS

Thymus and umbilical cord blood material

Human thymus, UCB and BM material was obtained according to the informed consent guidelines of the Medical Ethical Committee of Erasmus MC, Rotterdam. Thymus material was obtained from children operated for congenital heart disease. All children were between 8 days and 2 years of age and did not suffer from hematological or immunological diseases. Thymocytes were isolated by disrupting the thymus on an iron filter and frozen until further use. After thawing, viable thymocytes were isolated using Ficoll density centrifugation.

BM material was obtained from remaining graft cells of BM transplantation procedures. UCB and BM mononuclear cells were isolated using Ficoll density centrifugation and frozen down until further use. Over 90% of UCB mononuclear cells were viable after thawing.

Cell purification

For each sorting experiment, frozen thymocytes or UCB mononuclear cells from three to five different human donors were used. $CD34^+$ progenitor cells were pre-purified by AutoMACS (Milteny Biotec) before FACS sorting. Cells were stained with CD1a-RD1 (Beckman Coulter) and/or CD34-APC (BD Pharmingen) for 30 minutes on ice, washed and sorted on a FACS Digital Vantage (DiVa) cell sorter (BD Biosciences) (Figure 1).

BM mononuclear cells were not MACS pre-purified but immediately stained with CD3-FITC, CD20-FITC, CD34-APC (all from BD Pharmingen) and CD13-RD1 and CD33-RD1 (Beckman Coulter) and sorted on a DiVa cell sorter.

Microarray analysis

RNA was isolated from purified UCB and thymocyte subsets and processed to cDNA and cRNA according standard methods for microarray analysis. Gene expression profiles were generated using Affymetrix U133A microarrays and have been described previously[13].

Flow-cytometric validation of microarray results

For all flow-cytometric analyses, $CD34^+$ cells were purified using AutoMACS (Myltenyi Biotec). For surface markers, cells were stained with CD34-APC (BD Pharmingen), CD1a-RD1 (Beckman Coulter), CXCR4-FITC (R&D Systems) or CD13-RD1 and CD33-RD1 (Beckman Coulter) for 10 minutes at room temperature. For intracellular staining of MPO, cells were stained first with surface markers, then fixed and permeabilized using IntraPrep reagent (Beckman Coulter) and stained with MPO-FITC (DakoCytomation). Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and Cell Quest Pro software.

I*n vitro* colony assays

Purified thymocyte and UCB subsets and chimeric mouse BM samples were assayed for the presence of human granuloctye-macrophage colony-forming units (CFU-GM) and erythroid burst-forming units (BFU-E) by *in vitro* colony formation in viscous methylcellulose culture medium as described previously[39]. After 14 days of culture, the number of colonies was counted under a microscope.

Real-time quantitative PCR (RQ-PCR) of TCR gene rearrangements

DNA was isolated from cell samples using a GenElute mammalian genomic miniprep kit (Sigma). Gene rearrangements in the *TCRD* locus were quantitatively determined using TaqMan-based RQ-PCR as described previously[13]. Albumin DNA was determined in each sample to normalize for cDNA input. Nalm16 DNA was used as clonal control and reference sample in each experiment.

Stroma cultures

OP9 cells (BM stromal cell line, obtained from the ATCC) that either expressed human Delta1 (DL) in combination with GFP (hereafter called OP9-DL) or GFP alone (OP9-GFP) were generated by retroviral transduction. OP9-GFP and OP9–DL were grown in confluent layers in 96 well plates in αMEM (Cambrex) containing 20% fetal calf serum. $CD34^+CD1a^-$ and $CD34^+CD1a^+$ cells were FACS sorted onto the OP9 layers using a FACS Digital Vantage (DiVa) cell sorter (BD Biosciences). $CD34^+CD1a^-$ thymocytes were seeded on OP9-GFP at 1000, 300, 100, 30, 10 and 3 cells per well (48 wells per condition per experiment) and on OP9-DL at 1000, 300, 100 and 30 cells per well (12 wells per condition) or 100, 30, 10 and 3 cells per well (48 wells per condition) or 1 cell per well (192 wells). $CD34^+CD1a^+$ cells were seeded on OP9-GFP and OP9-DL at 1000 cells per well (12 wells). Cells were cultured for 14 days in the presence of 5 ng/ml human IL-7 and 25 ng/ml human SCF (both R&D Systems) and in one experiment with 80 ng/ml human GM-CSF (PeproTech).

Transplantations of NOD/SCID mice

10-15 weeks old NOD/LtSz-scid/scid (NOD/SCID) mice received a sublethal dose of 3.5 Gy of total body irradiation. Mice were intravenously injected with 10^5 FACS sorted $CD34^+$ UCB cells or 10^6 (1^{st} experiment) or $5x10^6$ (2^{nd} experiment) sorted $CD34^+CD1a^-$ or $CD34^+CD1a^+$ thymocytes in 250 µl of PBS. In the first experiment, five mice per group were used. In the second experiment, two mice per thymocyte group were used, because of the difficulty of obtaining sufficient cell numbers. To prevent infections, mice received 100 mg/l ciprofloxacin (Bayer) in their drinking water.

Thirty-five days after transplantation, the mice were killed and femurs, spleen and thymus were isolated. In each organ, total cell numbers were determined and the percentage of human cells and their subset distribution was assayed by flow cytometry using the following antibodies: CD8-PE (Sanquin) and CD4-FITC, CD14-FITC, CD19-PE, CD3-PerCP, CD45-APC (all from BD Pharmingen). Cell samples of non-transplanted mice were stained as negative controls.

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5

WNT SIGNALLING IN THE THYMUS IS REGULATED BY INTRACELLULAR SIGNALING MOLECULES

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ABSTRACT

Wnt signaling is essential for T-cell development in the thymus, but the stages in which it occurs and the molecular mechanisms underlying Wnt-responsiveness have remained elusive. Here we examined Wnt signaling activity in different thymocyte populations by determining β-catenin levels, Tcf-reporter activation and expression of Wnt target genes. We demonstrate that Wnt signaling occurs in all thymocyte subsets, including the more mature populations, but most prominently in the double negative (DN) subsets. This differential sensitivity to Wnt signaling was not caused by differences in the presence of Wnts or Wnt receptors, as these appeared to be expressed at comparable levels in all thymocyte subsets. Rather, it can be explained by high expression of activating signaling molecules in DN cells, e.g. β-catenin, plakoglobin and long forms of Tcf1, and by low levels of inhibitory molecules. By blocking Wnt signaling from the earliest stage onwards using overexpression of Dickkopf, we show that inhibition of the canonical Wnt pathway blocks development at the most immature DN stage. Thus, Wnt signaling occurs most prominently in the DN thymocytes, which appear to be preprogrammed to respond to Wnt signals.

INTRODUCTION

The thymus is a primary lymphoid organ that provides a unique microenvironment for the development of bone marrow- or fetal liver-derived progenitors into mature T cells. This process involves a series of discrete phenotypic stages that can be recognized by expression of several key membrane molecules[1]. In both human and mouse, thymocytes are subsequently $CD4^-CD8^-$ (double negative, DN), $CD4^+CD3^-$ (human immature single positive, ISP) or $CD8^+CD3^-$ (mouse ISP), $CD4^+CD8^+$ (double positive, DP) and finally $CD4^+CD3^+$ or $CD8^+CD3^+$ (single positive, SP). In the mouse, the DN subset can be further subdivided into four stages: $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3) and $CD44^-CD25^-$ (DN4). In the human thymus, $CD34^+CD1a^-$ cells correspond to murine DN1+2 thymocytes and $CD34^+CD1a^+$ cells are homologous to DN3[2]. The proliferation, differentiation and T-cell receptor (TCR) rearrangement steps that occur in the thymus are tightly regulated by a number of molecular pathways. One such pathway that is increasingly recognized to be important for T-cell development is the Wnt signaling cascade[3]. Several Wnt transduction routes have been described (reviewed by Veeman et al.[4]). The focus of this report is on the canonical (β-catenin dependent) Wnt signaling pathway.

Wnt proteins are secreted molecules that regulate cell-to-cell interactions in many different cell types and various species. In the thymus, Wnts are produced mainly

produced by the thymic epithelium[5] . A role for Wnt proteins in T-cell development has been deduced from mice deficient for both Wnt1 and Wnt4[6]. These mice show decreased thymocyte numbers, but no change in developmental pattern, suggesting a principally proliferative function for these Wnts.

Wnts bind to a receptor complex consisting of a member of the Frizzled (Fz) family of seven transmembrane proteins and the LDL-receptor-related proteins LRP-5 or LRP-6. Knock-out mice have been generated for a number of Fz, but, possibly partly due to their often early lethal phenotypes, defects in hematopoiesis have only been found in the Fz9 null mice[7]. The most notable defects in Fz9 null mice are partial blocks in pro- and pre-B-cell development and accelerated thymic atrophy in aging animals.

As the high redundancy of the different Wnts and Fz in the thymus[3] obscures their importance when studied in straight knock-outs, overexpression of negative regulators of the Wnt pathway may be more informative. For instance, soluble Fz receptors have been shown to block early thymocyte development in fetal thymic organ culture (FTOC) at the DN to DP transition[8]. These studies have indicated an important role for the membrane proximal components of the Wnt pathway, but the exact contribution of the different Wnts, Fz and LRPs that are expressed by thymic stroma and thymocyte subsets in human and mouse are unknown.

After binding of a Wnt protein to a Fz receptor, a complex cascade of events is initiated, finally leading to the inhibition of the negative regulatory kinase GSK-3β[9] which, in a complex together with Axin and APC, phosphorylates β-catenin. Inhibition of β-catenin phosphorylation prevents its ubiquitylation and proteasomal breakdown. Furthermore, various studies indicate that only dephosphorylated β-catenin is the transcriptionally active form[10,11]. Overexpression of a constitutively active form of β-catenin has been shown to induce self-renewal of hematopoietic stem cells[12], but the role of β-catenin in T-cell development is controversial. Mice with β-catenin deleted in all T cells from DN3 onwards showed impaired T-cell development at the β-selection checkpoint[13], but mice transplanted with hematopoietic stem cells in which β-catenin had been inducibly deleted did not show in any thymic defect[14]. However, the normal expression of both total and dephosphorylated β-catenin in the different thymocyte subsets under physiological conditions has not been studied.

Dephosphorylated β-catenin is targeted to the nucleus by Pygopus and Legless/BCL9[15], where it binds to Tcf/Lef transcription factors[16], in thymocytes predominantly Tcf1. Young Tcf1 deficient mice have an incomplete block at the DN1, DN2 and ISP stages, while older mice display a complete block at the DN1 stage of thymocyte development[17,18].

In the absence of β-catenin, Tcf1 functions as a repressor of transcription[19]. Binding of β-catenin to Tcf/Lef recruits chromatin-modifying and -remodelling complexes to transcribe Wnt target genes[20], which in the most immature thymocytes regulate

proliferation and cell-adhesion[21].

Although the phenotype of the Tcf1 deficient mouse suggests a role for Wnt signaling in the early stages of T-cell development, definite proof for a role of Wnt signaling is not provided by such mice, as the block might be a result of abolishment of the repressive functions or other Wnt independent functions of Tcf1. Additional roles for Wnt signaling have been reported in more advanced stages of thymocyte development, including differentiation into DP cells[22], survival of DP cells[23] and generation of mature CD8+ thymocytes[24] have been reported. It remains unclear during which stages of T-cell development Wnt signalling occurs and is functionally important.

In this study we set out to answer a number of important questions with respect to Wnt signaling in the thymus (Figure 1). First we investigated at which stages in the thymus Wnt signaling occurred and which cells were able to respond to Wnt. Second, we carefully analyzed the expression of Wnts, Fz and LRPs throughout mouse and human T-cell development. Third, we studied the molecular mechanisms underlying the differential Wnt responsiveness between subsets. Finally, we performed functional studies blocking the canonical Wnt pathway from DN1 onwards to assess the earliest stage in the thymus where Wnt signaling might be required.

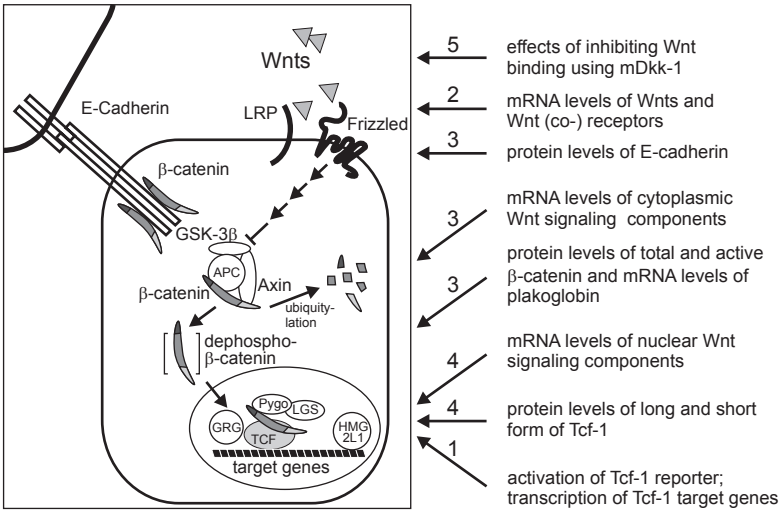

Figure 1. Experimental dissection of critical steps in the Wnt signaling pathway in thymocyte subsets.

Arrows indicate the different levels of Wnt signaling that were studied in human and murine thymocyte subsets. First, it was determined in which thymocyte subsets Wnt signaling occurs (1). Subsequently, levels of membrane proximal (2), cytoplasmic (3) and nuclear (4) Wnt signaling components were measured by various methods, as indicated. Finally, the effect of blocking Wnt binding in the thymus was studied (5).

RESULTS

Wnt signalling occurs mainly in the early DN stages

We have used three read-outs for active Wnt signaling, namely levels of β-catenin, the activation of a Tcf reporter and the transcription of known thymic Wnt target genes (Figure 1).

Levels of β-catenin in thymocyte subsets

As dephosphorylated β-catenin is highly stable and not degraded in the proteosome, elevated levels of total β-catenin are an indication that Wnt signaling occurs in a cell. We have developed a flow-cytometric assay to determine the presence of β-catenin in thymocyte subsets. This assay was validated by treating Jurkat cells with lithium chloride, which inhibits GSK-3β and leads to a profound increase in β-catenin levels (Figure 2A).

In the human thymus, the highest levels of β-catenin were observed in the DN cells and especially in the most immature DN $CD34^+$ fraction, which corresponds to murine DN1-3 cells[2] (Figure 2B). This finding suggested that Wnt signaling is active preferentially in these cells.

Similarly, in the adult murine thymus, β-catenin was present predominantly at the DN and ISP stages (Figure 2C). In fetal thymic lobes of embryonic day 14, 15 and 16, high levels of β-catenin were present. Levels were higher at the DN1+2 stages than at the DN3+4 stage and clearly decreased in the DP cells that arose on embryonic day 16 (Figure2D).

An antibody against active (dephosphorylated) β-catenin has been developed[10]. Unfortunately, we were unable to detect dephosphorylated β-catenin by flow cytometry (data not shown), presumably because of its strong nuclear localization, which precludes access to the antibody. We have, however, detected signals by Western blotting (see below).

Activation of a Tcf/Lef reporter

The next vital step in the Wnt signaling cascade is the binding of dephosphorylated β-catenin to Tcf/Lef high mobility group (HMG) box transcription factors. To study the transcriptional activation of the Tcf/β-catenin complex at sequential stages of T-cell development, we used a lentiviral Tcf reporter system, in which expression of GFP is driven by a Tcf/Lef-responsive promoter (TOP-GFP)[12]. As a negative control, we used a vector containing mutated Tcf binding sites (FOP-GFP).

Hematopoietic stem cells from human cord blood were infected with this these lentiviruses and induced to develop into T cells *in vitro* in fetal thymic organ cultures (FTOC). After two days of transduction, none of the cells expressed GFP (Figure 3A),

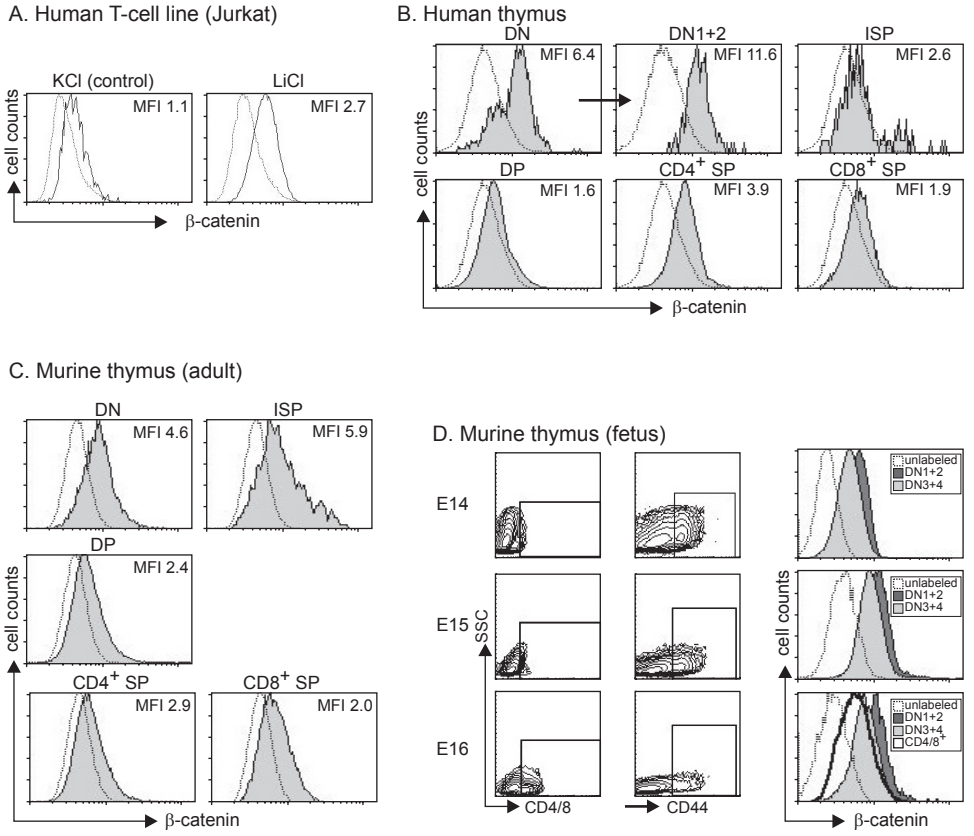

Figure 2. Differential expression of β-catenin in thymic subsets.
Intracellular β-catenin levels were determined by flow cytometry in (A) Jurkat cells stimulated for 24 hrs with either KCl or LiCl, (B) freshly isolated human thymocytes, (C) freshly isolated adult murine thymocytes and (D) murine thymic lobes from 14, 15 and 16 day old embryo's. All thymus samples were counterstained to distinguish different subsets. As CD25 antibodies failed in this protocol, only DN1+2 versus DN3+4 distinctions could be made. Dotted lines represent isotype (human) or unstained (mouse) controls. Mean fluorescence intensity (MFI) of the β-catenin signal - background signal is indicated in each graph.

indicating that *in vitro* cultured HSC do not undergo active Wnt signaling. However, after inducing T-cell development in FTOC, a population of GFP^{high} cells could be observed that was absent from the FOP-GFP control (Figure 3B). GFP levels were analyzed in DN, ISP, DP and SP thymocyte subsets (Figure 3C). Surprisingly, Tcf-dependent signaling was detected in all thymocyte subsets, including the more mature populations. However the mean fluorescence of the GFP-positive fraction was 2-3 times higher in the DN cells, consistent with the high levels of β-catenin in these cells.

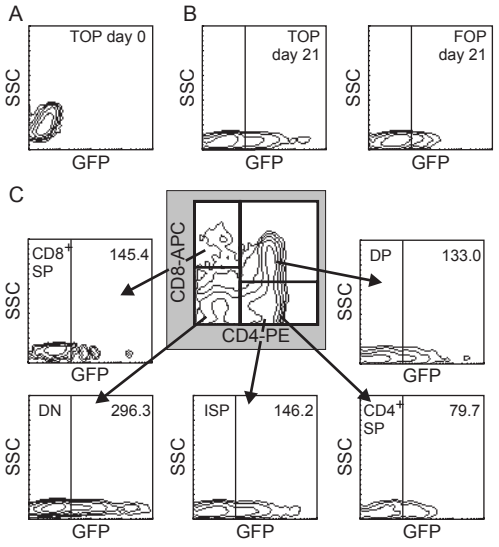

Figure 3. Tcf-reporter expression during human thymocyte development.
CD34⁺ precursor cells from human cord blood were transduced with a lentiviral vector encoding a Tcf reporter (TOP) or a reporter containing mutated Tcf binding sites (FOP) and cultured in FTOC. (A) Tcf dependent GFP expression after two days of transduction and (B) after three weeks of FTOC. (C) Tcf-dependent GFP expression in the different thymocyte subsets after three weeks of FTOC. ISP and CD4⁺ SP populations were distinguished by gating on CD3 negative and positive cells, respectively. The mean fluorescence of the GFP positive population is indicated in each graph. Mean fluorescence of GFP positive cells transduced with FOP was similar in all subsets (around 60).

Expression of Wnt target genes

Cells undergoing Wnt signaling are expected to induce expression of Tcf dependent target genes. We have previously identified a core set of seven Wnt target genes in human CD34⁺ DN thymocytes, namely c-fos, fosB, c-jun, CL100, integrin p150,95, hsp70 and NRPB[21]. Using RQ-PCR we studied the expression of these target genes in all human thymocyte subsets and found that c-fos, CL100 PTPase and hsp70 were expressed preferentially in the most immature DN populations, while fosB and c-jun were higher expressed in the more mature stages[21].

Microarray studies of the murine thymocyte subsets have been performed by Petrie and co-workers[25]. By mining these data for thymic Wnt target genes, we found that p150,95 was expressed at high levels at the DN1 stage and rapidly decreased in the more mature stages (Figure 4A). For c-fos, fosB and c-jun this differential expression was less clear. However, Petrie and co-workers studied the expression of fos and jun also by RQ-PCR and found that they were expressed preferentially at the DN1 stage and were virtually absent in the other stages[25].

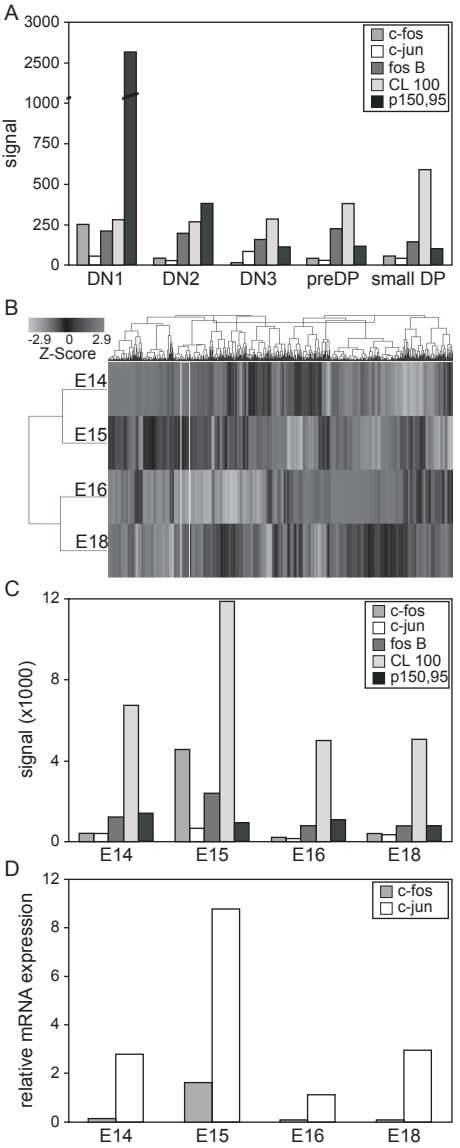

Figure 4. Expression of thymic Wnt target genes during thymocyte development.
(A) Thymic Wnt target genes in murine thymocyte subsets as determined by microarray analysis by Tabrizifard et al.[25] (B) Hierarchical clustering of gene expression profiles generated from murine thymic lobes of embryonic day 14, 15, 16 and 18. (C) Expression of Wnt target genes in embryonic thymi determined by microarray analysis. Fluorescence units were normalized over the whole experiment. A signal over 100 indicates presence of a gene. (D) RQ-PCR validation of c-fos and c-jun expression in embryonic thymi, relative to house-keeping gene GAPDH.

During ontogeny, the DN subset is the first subpopulation to arise in the embryonic thymus and is the only population at day 14, followed by the appearance of ISP at day 15, DP at day 16 and CD4 and CD8 SP at day 17. We generated expression profiles of thymic lobes of 14, 15, 16 and 18-day-old mouse embryos using DNA microarray technology (Figure 4B). In these expression profiles, we investigated the expression of the same core set of target genes. While p150,95 was expressed at all embryonic stages at similar levels, all other genes were expressed most prominently at day 15 (Figure 4C), when thymocyte numbers are rapidly increasing but all cells still are very immature. For c-fos and c-jun this finding was confirmed using RQ-PCR (Figure 4D). The lower expression of Wnt target genes from day 16 onwards, likely reflects the increasing contribution of DP and SP cells and the reduced levels Wnt signaling in those subpopulations.

Taken together these results point to a high level of Wnt signaling preferentially in the most immature DN, and to a lesser extend ISP, thymocyte subsets.

Wnts and receptors are present in all thymocyte subsets

To investigate which factors determine whether cells are receptive to Wnt signaling, we examined the presence of several Wnt signaling components in the different thymocyte subsets. First we determined the expression of the membrane-associated factors: expression of Wnt, Fz and LRP genes. As no reliable antibodies for flow cytometry are available against these components, we used TaqMan RQ-PCR to determine mRNA levels in sorted thymocyte populations in both human and murine thymus, as well as in embryonic thymic lobes (Figure 5A-I and Supplementary Figure 1).

Both in man and in mouse, most Wnts investigated appeared to be expressed in all thymocyte subsets, but at different levels. Both in human and murine thymus, Wnt5 and Wnt10 were the most prominent Wnts (Figure 5A and D). Both in human and mouse, Wnt expression was most marked in the SP subsets, although in mice Wnt5b was expressed at high levels in the DP subset as well. In the thymic stroma, high levels of Wnt5a (human) and Wnt4, Wnt5b and Wnt10 (mouse) were detected, generally at levels significantly higher than in thymocytes (Supplementary Figure 1 and data not shown).

Of the Frizzled receptors, Fz6 was by far the most prominent in the human thymus (Figure 4B) and Fz5 and Fz6 in the murine thymus (Figure 5E). Levels of Fz varied between thymocyte subsets and tended to be lowest in the DP fraction. LRP5 and 6 were expressed at similar levels, both in mouse and in human, and expression was more or less equal between the different thymocyte subsets (Figure 5C and F).

Interestingly, during fetal development a different set of Wnts was expressed than in the mature thymus: here Wnt3a and Wnt4 were most prominent, although Wnt 5b was also present at moderate levels (Figure 5G). While expression of Wnt4 decreased

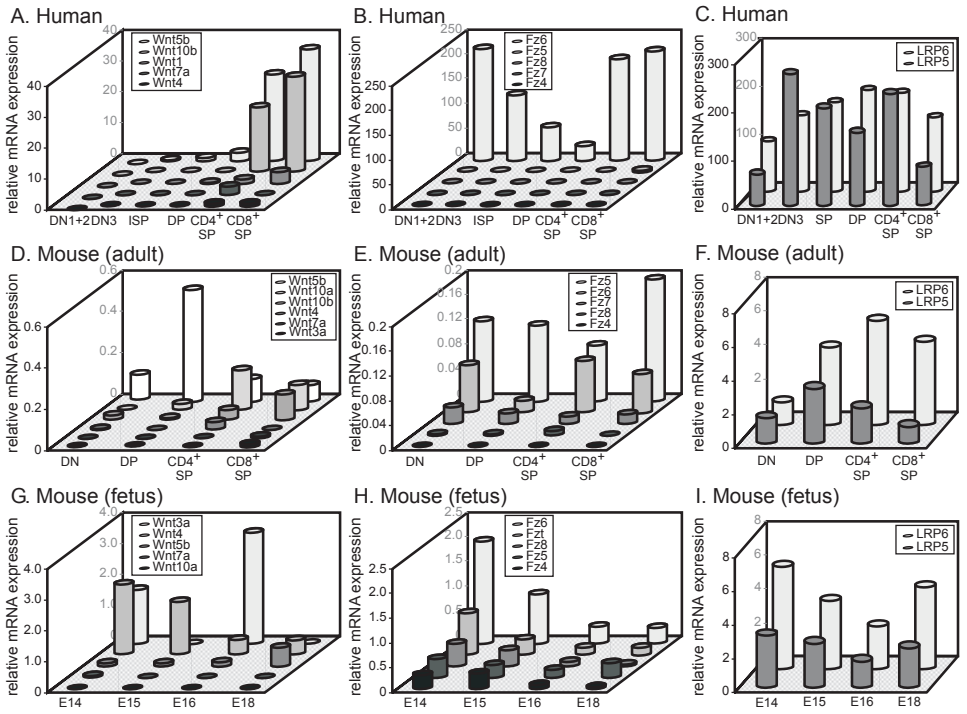

Figure 5. Expression of Wnts, Fz and LRPs in thymic subsets.
mRNA levels of different Wnts and Fz were determined by RQ-PCR. (A) Human Wnts, (B) human Fz, (C) human LRPs, (D) murine Wnts, (E) murine Fz, (F) murine LRPs, (G) murine Wnts in embryonic thymi, (H) murine Fz in embryonic thymi, (I) murine LRPs in embryonic thymi. All graphs show results of a single representative experiment out of 2-5 performed, as replicate experiments always showed the same trend, but absolute expression levels varied. Expression levels are relative to GAPDH expression in the same sample.

gradually in time, Wnt5b seemed to increase to levels detected in the mature thymus, possibly reflecting a decreased contribution of stromal cells in more mature thymic lobes. Also, in contrast to the mature thymus, all Fz investigated were present at readily detectable levels, although in accordance with the mature thymus, Fz6 was the most prominent (Figure 5H). The Fz tended to be expressed highest at embryonic day 14 and gradually decreased with time, presumably reflecting diminishing contribution of very immature cells to the total thymocyte population, and therefore the need for Wnt signaling. LRP5 and 6 were expressed at equal levels during all examined days of embryonal development (Figure 5I).

The expression pattern of the Wnts (mainly by the stroma and the more mature SP thymocytes), Fz (variable levels in thymocyte subsets) and LRPs (constitutively

expressed), cannot account for our finding that active Wnt signaling occurs predominantly in the DN and ISP subsets. Other mechanisms must therefore regulate differential Wnt signaling.

Expression of intracellular signaling molecules predisposes DN thymocytes to respond strongly to Wnt signals

We studied the expression of several intracellular Wnt signaling components using Western blot and microarray analysis. As there is no antibody against murine Tcf1 available[26], these experiments were performed using sorted human thymocyte subsets.

Protein levels of intracellular Wnt signaling components

Western blotting showed presence of total β-catenin in all thymocyte populations, but mainly in the $CD34^+$ DN1-3 populations and somewhat lower in the ISP subset (Figure 6A), confirming our results obtained by flow cytometry (Figure 2B). The same distribution was detected for active (dephosphorylated) β-catenin, although this was completely absent from the DP stage onwards. Staining of human thymus tissue slides with the same antibody against dephosphorylated β-catenin, showed a pattern of nuclear foci in thymocytes (Figure 6B), confirming a function as an active transcription factor in these cells.

In many cell types, β-catenin is bound to E-cadherin at the cell membrane and functions in cell adhesion (membrane bound β-catenin pool), rather than as a signaling component (cytoplasmic β-catenin pool)[27]. In human thymocytes, high levels of β-catenin were not caused by elevated expression of E-cadherin in the same subsets, as E-cadherin levels were relatively constant and low in all subsets (Figure 6A).

Presence of Tcf/Lef proteins is obviously a prerequisite for Wnt signalling to occur and Tcf1 is the most important family member expressed in mature thymocytes[3]. Expression of Tcf1 protein was detected in all populations at similar levels (Figure 6A). Interestingly, however, the ratio between expression of the long (activating) and short (repressing) splice-isoforms of Tcf1 differed between the different thymocyte subsets. This ratio was highest in the $CD34^+CD1a^-$ (DN1+2) cells (Figure 6C), allowing the final transduction of Wnt signals to occur preferentially in these immature cells.

mRNA levels of intracellular Wnt signaling components

Presence of other intracellular Wnt signaling components was extracted from gene expression profiles of human thymocytes subsets[2]. Plakoglobin (γ-catenin), another armadillo protein that can activate Tcf/Lef transcription factors[28], showed an expression pattern highly similar to that of β-catenin (Table I), also peaking in the $CD34^+$ (DN1-3) thymocytes. Conversely, expression of Axin, one of the molecules that functions to

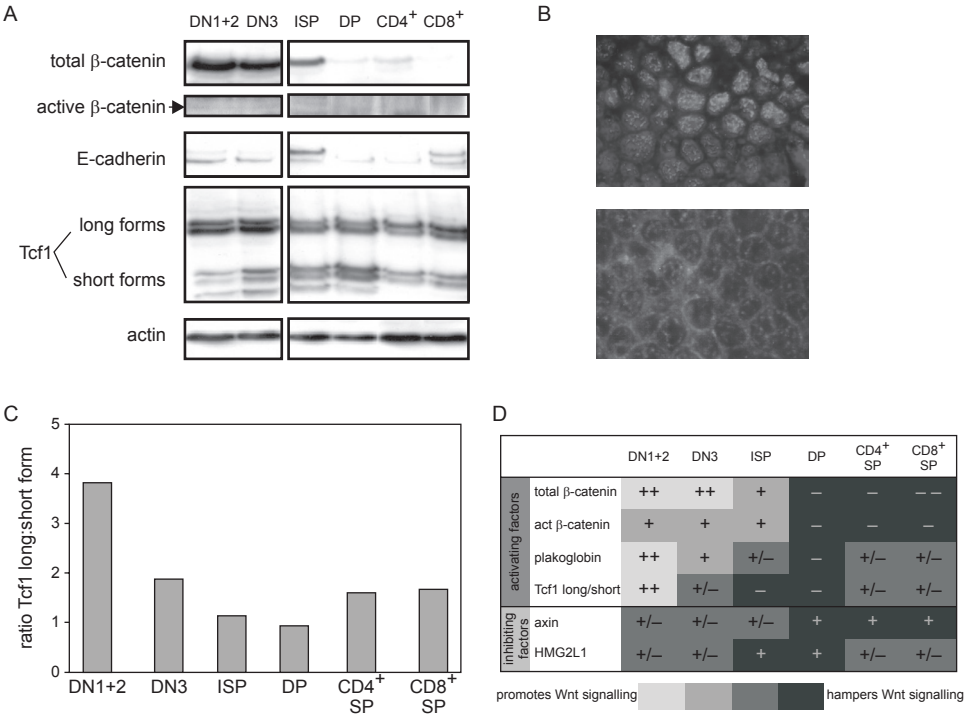

Figure 6. Expression of intracellular Wnt signaling components in the human thymus.
DN1/2 thymocytes were defined as $CD34^{+}CD1a^{-}$ and DN3 as $CD34^{+}CD1a^{+}$. (A) Protein levels in sorted thymocyte subsets determined by Western blot. An arrow indicates the very faint, but visible band of dephosphorylated β-catenin, present in the early subsets. (B) Immunofluorescent staining of dephosphorylated β-catenin in thymic tissue. Upper panel: A focal pattern of dephosphorylated β-catenin is found in the nuclei (stained with Dapi). Lower panel: total β-catenin stains brightly, mainly at the membranes, while dephosphorylated β-catenin is present only in the nuclei. (C) Ratio of long versus short form of Tcf1 in sorted thymocyte subsets (quantification of Western blot). (D) Summary of intracellular Wnt signaling components. Expression levels of Axin, plakoglobin and HMG2L1 were determined in human thymocyte subsets by microarray analysis.

retain β-catenin in the complex in the cytoplasm, increased gradually during thymocyte maturation. Also HMG2L1, a nuclear negative regulator of Wnt signaling[29], was expressed at lower levels in the most immature (and also the SP) populations (Table I). Levels of APC, Groucho and transcriptional activators Pygopus and Legless/Bcl9[15] did not change during thymocyte differentiation (Table I).

Together these results suggest that active Wnt signaling in the early thymocyte subsets is determined by elevated levels of activating and lower levels of inhibiting factors in the Wnt signaling cascade, as summarized in Figure 6D. The most immature thymocytes therefore appear to be preprogrammed to respond to Wnt signals.

Table I. Expression levels of intracellular Wnt signaling molecules[1].

	DN1+2	DN3	ISP	DP	$CD4^+SP$	$CD8^+SP$
JUP	327.2	184.3	112.8	88.5	108.4	156.7
APC	84.0	65.0	57.7	70.5	65.8	57.3
Axin1	182.9	165.2	173.9	205.2	217.4	227.5
TLE3	192.3	213.7	206.9	200.6	177.0	208.5
Pygo1	39.2	40.7	35.3	39.9	32.7	33.2
Bcl9	93.8	103.6	80.4	94.0	118.5	130.7
HMG2L1	144.5	146.8	185.1	202.6	161.7	147.1

[1]Relative mRNA expression levels of different Wnt signaling components in human thymocyte subsets determined by microarray analysis. TLE3 is the only Groucho homologue that was found to be expressed at significant levels in thymocytes. JUP: junction plakoglobin.

Wnt signaling is crucial for early thymocyte development

The data presented above show that Wnt signalling is preferentially active in the most immature DN subsets. However, the importance of Wnt signalling during the DN stages of thymocyte differentiation has not been studied, largely because conditional deletion of Wnt components during thymocyte development relies on Cre-transgenic mice that fail to show significant expression in DN1 and DN2 subsets (e.g. the Lck or CD4 promoter). To examine the effect of inhibited Wnt signaling on early T-cell development, we employed a retroviral vector encoding the gene for mouse Dickkopf-1 (mDkk-1). This protein binds to the LRP co-receptor for Wnt and thereby potently inhibits binding of Wnt and activation of the Wnt signaling cascade[30]. We transduced murine bone marrow and fetal liver cells with the mDkk-1 retrovirus and studied the ability of these cells to develop into T cells in FTOC. Although the results of six different experiments were variable, in all cultures of mDkk-1 transduced cells a strong selection against high mDkk-1 expression (i.e. high GFP signals) was detectable (Figure 7A). In experiments in which a meaningful number of GFP expressing cells survived, the GFP^+ cells exhibited a complete block at the DN1 stage (Figure 7B). This block was less severe for the cells expressing intermediate amounts of GFP. These results show that Wnt signaling is crucial for developing thymocytes, already at the DN1 stage. The striking similarity between the Tcf1 deficient mice and the block induced by inhibition of Wnt binding, strongly suggest that Wnt signaling is the sole or main inducer of Tcf1/β-catenin dependent transcription in the thymus.

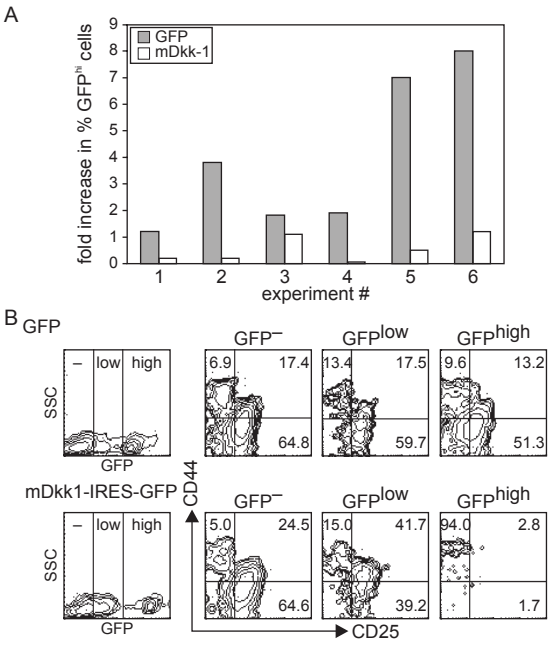

Figure 7. Overexpression of mouse mDkk-1 blocks T-cell development at the DN1 stage.
(A) Murine bone marrow cells (experiment #1,2 and 4) or fetal liver cells (#3, 5 and 6) were transduced with a vector containing the mDkk-1 gene in combination with GFP (mDkk-1) or a control vector containing only GFP (GFP). Fold increases in the percentage of GFPhigh cells from day 0 to day 9 of FTOC are shown for 6 different experiments. (B) After 9 days of culture in FTOC, thymocyte subset distribution was determined in cells gated for absent, low and high GFP expression, both for cells transduced with the control vector (upper panels) and with mDkk-1 (lower panels). Percentages of DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$) and DN3 ($CD44^-CD25^+$) subsets are indicated in the quadrants. Experiment #3 is shown.

DISCUSSION

Although various reports have indicated a functional role for Wnt signaling in the thymus[6,8,17,31], several important aspects have remained elusive. These include the nature of Wnt ligands and Fz receptors expressed by the various thymocyte subsets and during thymic ontogeny, the stages in which Wnt signaling takes place and why it takes place in those subsets.

Both levels of β-catenin and GFP expression from a Tcf reporter construct were found to be highest in the DN1-3 subsets, in humans characterized by expression of CD34. After these stages, cells stop proliferating and start rearranging their TCR genes. Although ISP cells had higher levels of total β-catenin, Tcf-dependent transcription was

not increased in these cells. Presumably, proliferation from DN3 onwards is mainly driven by (pre-) TCR signaling rather than by Wnt signals.

It was unexpected to find that also the more mature populations in the thymus undergo Wnt signaling, albeit at low levels, since Wnts are thought to give mainly proliferative signals[21,32] and since in the Tcf1 knock-out mouse only the DN and ISP stages are affected[18]. However, a number of reports have demonstrated a role for Wnt signaling in more mature stages of T-cell development[22-24]. The function of Wnt signaling in the more mature subsets is probably directed towards prevention of apoptosis rather than towards proliferation.

To investigate which factors determine whether cells can respond to Wnt signals, we made an inventory of Wnt signaling components in different stages of T-cell development in human and mouse. Because of the lack of suitable antibodies against Wnts and Fz, assessment of proximal Wnt signaling components has thus far relied largely on non-quantitative PCR detections and Northern blot experiments[8]. We confirm the finding of Jenkinson and co-workers[5] that the thymic epithelial cells (TECs) comprising the stromal compartment are the major source of Wnt factors in the thymus. TECs are the main source of growth factors for developing thymocytes, not only of classical cytokines such as Il-7 and SCF, but apparently also of Wnt proteins.

Surprisingly, the SP thymocyte subsets also expressed significant levels of Wnt genes. As Wnt proteins are highly insoluble and presumably are unable to diffuse throughout the thymus, the Wnts produced by more mature medullary thymocytes are unlikely to reach the cortical immature cells and most likely represent autocrine signals. High concentration of Wnts surrounding the more mature cells may explain why they undergo (low level) Wnt signaling, while expressing many negative regulatory factors of the Wnt signal transduction pathway. Alternatively, these Wnts may bind to neighbouring stroma cells, which also express Fz and LRP receptors, to provide 'cross-talk'. It would be interesting to study whether Wnts are produced in specific stromal niches, e.g. subcapsular or at the corticomedullary junction.

All thymocyte subsets express Fz receptors and LRP5 and 6 at high levels and are therefore theoretically able to respond to exogenous Wnt proteins. However, Tcf1 dependent transcription was detected mainly in the early DN cells, indicating that other positive factors essential for Wnt signaling were missing in more mature cells. We found that β-catenin levels were significantly higher in the most immature DN subsets and the same was true for mRNA levels of the β-catenin homologue plakoglobin. The role of β-catenin during T-cell development has been addressed in two different conditional knockout mouse models, which have generated conflicting results. The study by Sen and co-workers indicated that β-catenin-mediated signals are required for normal T cell development[13], while Radtke and colleagues reported that hematopoiesis including T-cell development in the thymus proceeded normally in absence of β-catenin[14]. As a

possible explanation for their negative results, Cobas *et al.* suggested that expression of plakoglobin may compensate for the absence of its homologue β-catenin[14]. The parallel expression of β-catenin and plakoglobin in our experiments provides evidence that this is a plausible explanation and may account for the relatively mild or absent phenotype of β-catenin deficient mice[13,14].

In addition to elevated β-catenin, we show that the ratio between the long and short splice-isoforms of Tcf1 is highest in the DN subsets. Different isoforms of Tcf1 have been described[33], but their function remains unclear. Some of these isoforms lack the β-catenin binding domain and therefore probably function as dominant negative proteins with regard to Wnt signaling. The various molecular forms of Tcf1 arise by both alternative splicing and alternative promoter usage. It would be of obvious interest to study regulation of these processes. We propose that the short Tcf1 isoforms have regulatory functions in the thymus and that a critical balance between long and short isoforms determines whether Wnt signals are finally transduced into transcription of target genes. Likewise, it is of interest to understand how expression of Wnt, Tcf and other components of the pathway are transcriptionally regulated, as their differential expression during thymocyte development is likely controlled at the level of gene expression.

Our results show that Wnt signalling mainly occurs in the most immature DN stages. However, the role of Wnt signaling in these subsets has not been conclusively studied. The Tcf1(VII) knock-out mouse has a severe phenotype in the early stages of T-cell development[17,18], but this might be due to the loss of repressive functions of Tcf1. In the conditional β-catenin knock-out employed by Sen and co-workers, the effect on early DN cells can not be studied as a consequence of the later expression of Lck (DN3)[13], while the complete β-catenin knock-out has no phenotype at all[14]. Finally, targeted mutation of Wnts or Fz receptors would not give a clear answer, since they are highly redundant as we show in this study and has been observed in Wnt1Wnt4 double deficient mice. To circumvent these problems, we overexpressed the mDkk-1 gene to block binding of Wnt proteins to the LRP co-receptor in stem cells. When induced to develop into T cells in FTOC, mDkk-1 expressing cells were blocked in the DN1 stage. This effect was dose dependent, with the cells expressing low levels of GFP still being able to develop into DN2 cells. These findings are consistent with a function of mDkk-1 as competitive inhibitor of Wnt proteins.

During the DN1 and 2 stages of T-cell development, thymocytes undergo massive proliferation to expand the thymocyte pool before the TCR rearrangement and selection processes are initiated[34]. The strong complete block of T-cell development in DN1 when Wnt signaling is inhibited, demonstrates the importance of Wnts as proliferation factors for these immature cells.

In summary, we demonstrate that all thymic subpopulations can undergo low levels

of Wnt signaling, but the highest levels of active Wnt signaling were detected in the most immature DN cells. This differential responsiveness is not determined by expression of membrane associated factors, but rather by the balance between activating and inhibiting intracellular components of the Wnt signaling pathway, as shown in Figure 6D. The high levels of β-catenin expression in the DN subsets, coupled with high expression of the Wnt-responsive long Tcf1 isoforms compared to the shorter inhibitory isoforms, as well as low levels of factors that negatively control Wnt signaling such as Axin and HMG2L1, predetermine a high responsiveness to Wnt signaling in DN thymocytes.

The regulation of responsiveness to developmental clues by differential expression of intracellular regulators rather than by abundance of receptors or ligands provides a previously unrecognized mechanism to regulate signal transduction events. To our knowledge this is the first demonstration that only cells preprogrammed to respond to external stimuli do so during differentiation. It seems likely that such mechanisms operate during other developmental processes in other tissues.

Since Wnt signals are important for survival and expansion of lymphocyte progenitors, it has been suggested that deregulated Wnt signaling may be a mechanism underlying leukemogenesis. Indeed, several reports have now indicated that in various hematological malignancies, aberrant Wnt signaling constitutes a key oncogenic event; these include AML, CML, CLL, precursor B-ALL and multiple myeloma (reviewed by Staal and Clevers[35]). For the malignant counterparts of normal T-cell progenitors, i.e. T-cell acute lymphoblastic leukemias (T-ALL), such indications have not been found. Given the immature ($CD3^-$) phenotype of most T-ALL and the high level of Wnt signaling in the normal immature thymocytes, as we report here, simple detection of elevated levels of (dephosporylated) β-catenin in T-ALL may not suffice to indicate a causative role for Wnt signaling in the leukemogenic process. It may well be that mutations in the Wnt pathway, for instance activating mutations in β-catenin, will lead to high levels of Wnt signaling in T-ALL, thereby contributing to leukemogenesis. A comparable role has been documented for another pathway important in DN thymocytes development, namely the Notch pathway[36].

MATERIALS AND METHODS

Isolation of cells

Human thymus material was obtained from children operated for congenital heart disease, according to the informed consent guidelines of the Medical Ethical Committee of Erasmus MC, Rotterdam. All children were between 8 days and 2 years of age and did not suffer from hematological or immunological diseases. Thymocytes were isolated by disrupting the thymus on an iron filter. The remaining stroma was carefully washed to remove all remaining thymocytes and lysed in RLT buffer (Qiagen) for RNA isolation. Cells were either directly used for flow-cytometric analysis or frozen until further use. After thawing, viable thymocytes were isolated using Ficoll density centrifugation.

All animal experimentation was done in accordance with legal regulations in The Netherlands, which include approval by the local Animal Experiments Committee. Mouse thymi were obtained from 2 and 4 weeks old female C57Bl/6 mice. Thymocytes were isolated by disrupting the thymi through a 70 µm cell strainer (Falcon) and immediately used for flow cytometry or sorting. The remaining stroma was washed thoroughly to remove all thymocytes, then snap-frozen and grained into small particles that were dissolved into RLT buffer (Qiagen) for RNA isolation.

Mouse fetal thymic lobes were obtained from embryos from 14, 15, 16 and 18 days pregnant C57Bl/6 mice. Thymic lobes were disrupted by gently passing them through a 24G syringe.

Antibodies

All monoclonal antibodies against human and mouse proteins were obtained from BD Pharmingen, except for human CD1a-RD1, which was from Beckman Coulter.

Cell sorting

For each sorting experiment, frozen thymocytes of five different human donors or fresh thymocytes of four different mice were used. $CD34^+$ human thymocytes were pre-purified by AutoMACS (Milteny Biotec) before FACS sorting. Cells were stained for 30 minutes on ice, washed and sorted on a FACS Digital Vantage (DiVa) cell sorter (BD Biosciences). Purity of the recovered subpopulations was checked and was always greater than 95%.

Intracellular staining of β-catenin

Freshly isolated thymocytes were immediately fixed in 2% paraformaldehyde at 37°C and permeabilized in PBS containing 0.5% saponin, 4% fetal calf serum, 1mM glycerolphosphate and phosphatase inhibitor cocktails I and II (Sigma). Cells were stained for one hour with an antibody against β-catenin (clone 8E4 (Upstate), FITC labeled according to standard procedures) at 4°C. Cells were washed, resuspended in PBS containing 0.5% BSA and stained with cell surface markers for 1 hour at room temperature. After washing, cells were analysed using a FACSCalibur flow cytometer (BD Biosciences). Unstained and isotype-control stained samples showed similar patterns.

TaqMan-based real-time quantitative-PCR (RQ-PCR)

All primer and probe sequences were designed with the Primer Express version 1.5 software (Applied Biosystems) and are listed in Table II. One µg RNA isolated from sorted subpopulations was treated with 1U of DNase I and subsequently transcribed to cDNA with Superscript II Reverse transcriptase (200 U), oligo(dT) and random hexanucleotide primers. A 1/20 cDNA mixture was used for RQ-PCR for each primer/probe set and performed in duplicate. The RQ-PCR mixture of 25 µl contained TaqMan Universal mastermix, 900 nM primers and 100 nM probe and was run on the PRISM 7700 sequence detection system containing a 96 well thermal cycler (Applied Biosystems).

For each primer/probe set, a dilution curve was made using cell line DNA as a target. The relative amount of Wnt/Fz/LRP cDNA in every thymocyte subset was read from the corresponding dilution curve. The amount of input cDNA was normalized to GAPDH expression (human kit from Applied Biosystems, mouse kit from Biosource) in the same sample.

Efficiencies of most RQ-PCRs were similar, and therefore it was possible to compare expression levels of the different Wnts, Fz and LRPs. As no good dilution curve was obtained for human Wnt1 and Fz8 and mouse Wnt4 and Wnt10b, these were read from another representative curve. No successful primer/probe sets could be developed for mouse Wnt1 and Wnt5a.

Microarrays

RNA was isolated using RNeasy columns as described by the manufacturer (Qiagen). The integrity of the RNA was tested on an agarose gel. 5 µg of RNA was used to generate ds cDNA using superscript reverse transcriptase and a T7-oligo dT primer. The resulting cDNA was used in an *in vitro* cRNA reaction using T7 RNA polymerase and biotinylated ribonucleotides employing an ENZO kit (ENZO). The biotinylated cRNA was cleaned up using RNeasy spin columns (Qiagen) and quantified

Table II. All primer/probe sets.

	forward primer	reverse primer	probe
human Wnt1	CTGTCGAGAAACGGCGTTTATC	GATGGAACCTTCTGAGCAGGAG	CGCTATCACCTCCGC
human Wnt4	CATTGAGGAGTGCCAGTACCAGT	CGGGCAAGGAGTCGAGTG	AACCGGCGCTGGAACTGCTCC
human Wnt5a	ACTCGCCCACCACACAAGA	AGCCGGTGCTCTCATTGC	CGACCCCAGCCCTGACTACTGCGT
human Wnt7a	CTCCTCAGCAGAAAAGACAAGCTC	CCCGCTGCAAGTCAGATTG	CCCGCAGCTTGGAAACGGTCC
human Wnt10b	GCGTCAAGCACACGGTGTT	TCTCCTGTTCCTGGCGTTGT	CAGGCAACTTCAGGCCCAGAATCTCA
human Fz4	ACGCTGTGAACCCGTCCTG	GGCACCTCTTCATCACCTGG	TTCCCACCACAGAACGACCACAACC
human Fz5	CGCGAGCACAACCACATC	AGAAGTAGACCAGGAGGAAGACGA	CCACGGGCCCTGCACTGTGC
human Fz6	CACCCCCAGGTTAAGAGAACAG	AGAGAGTCTGGAGATGGATGCTG	ACTGTGGTGAACCTGCCTCGCCA
human Fz7	CCTCGACGCTCTTTACCGTT	AGGAAGATGATGGGCCGC	ATGCGGCGCTTCAGCTACCCAG
human Fz8	CTGGATCGGCCTGTGGTC	GAAGCGCTCCATGTCGATAAG	CCACCTTCGCCACCGTCTCCA
human LRP5	CGTGATTGCCGACGATCTC	GAATGCTGTGCAGATTCCAGTC	CACCCGTTCGGTCTGACGCAGTACA
human LRP6	CAACAGAGCCCCGACATCAT	ATAGAGTTGCTTGTCCAGTGGGTC	AGCCTTCGGAATGTCCGGGCC
mouse Wnt3a	TGGCCCTGTTCTGGACAAAG	CTACTCCAGCGGAGGCGAT	CCCGGGAGTCAGCCTTTGTCCA
mouse Wnt4	ACAACAACGAGGCTGGCAG	CCCGTGACACTTGCACTCC	AGGCCATCTTGACACACATGCGGG
mouse Wnt5b	ACCGAGGTTGTGGACCAGTATG	CGGAGAGGAGCGGCCT	CTGTAAGTGACTGCACCACACGGCCT
mouse Wnt7a	CGCCAAGGTCTTCGTGGAT	CGACCCGCCTCGTTATTG	TGCCCGGACGCTCATGAACTTACA
mouse Wnt10a	CGACCTGGTCTACTTTGAGAAATCT	ACAGTGCCTGCCGAGTCC	CCGACTTCTGTGAGCGCGAGCC
mouse Wnt10b	CGGGACATCCAGGCGAG	TCCGCTTCAGGTTTTCCGT	TCCACAACAACAGGGTGGGACGC
mouse Fz4	TGACCCTAGCAATCCATCCC	AGTTGTACGAGAATGTGCGGAA	CCCGGGCTCCGGACGTCTG
mouse Fz5	CAGTACTTCCACCTTGCTGCCT	CCGTCCACCGAGCTCAGT	CCCAGTGTCAAGTCCATTACGGCGC
mouse Fz6	GGACGAGCAGACCGAGCA	TCCGGTTCCCAGAGAGCTT	ACACTCCCAGCGCCAAAGTCGG
mouse Fz7	CCGGCCACCATCGTGTT	CAGGTGCGTTCCCAGTGC	CCTGCTACTTTTATGAGCAGGCCTTCCGA
mouse Fz8	GGGAGGCCACGCACAACT	CCCGATGTGATGCCCACTAC	CGGACCAGGCTCGCAGGCC
mouse LRP5	GTGATGTGCCAGCGCTACAC	TGAAGTTGAGAGGCACATGAGG	TGGGCCCTTTCCCCACGAGTATGTT
mouse LRP6	CCCGAGGCTTTCCTTCTGTT	GCCACATTGTTGTTGTTTGTTTC	TATGCGTCTGATATCCGCTCTCCTCGA
mouse c-fos	GGAGGTCTGCCTGAGGCTTC	CAACGACCCTGAGCCCAAGCCAT	CACGTTGCTGATGCTCTTGAC
mouse c-jun	GACGGACCGTTCTATGACTGC	TGGAAACGACCTTCTACGACGATGCC	GGAGGAACGAGGCGTTGA

by spectrophotometric methods. Fragmentation of 20 µg cRNA was performed at 95°C for 35 min. 10 µg of fragmented cRNA was subsequently hybridized for 16 hrs to MG-U74Av2 microarrays (Affymetrix) at 45°C. After staining with Streptavidin-PE and washing, the arrays were scanned in an HP/Affymetrix scanner at 570 nm. For all experiments the 5'/3' ratios of GAPDH (0.82-0.88), scaling factor, noise and presence calls were comparable. Scanned images were analyzed using Affymetrix Micorarray Suite 5.0 software and Rosetta Gene Resolver System Biosoftware. All expression data are available as Supplementary Information and can be found at our website http://franklin.et.tudelft.nl.

Western blot

Sorted cells were directly lysed in SDS sample buffer. Proteins were separated on a denaturing 12.5% polyacrylamide gel for 1 hour and transferred to a nitrocellulose membrane. After blocking, blots were probed with the following antibodies: anti-β-catenin (clone 14, Transduction Laboratories), anti-dephosphorylated β-catenin (clone 8E7, Upstate), anti-E-cadherin (clone 36, Transduction Laboratories), anti-β-actin (Abcam Ltd) and rat anti-mouse-HRP (Pierce) and visualized by enhanced chemiluminescence (Amersham). Tcf1 bands were quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

Immunofluorescence

6 µm frozen sections from human thymus were fixed with methanol and stained for 1-2 hours with anti-dephosporylated β-catenin (clone 8E7, Upstate), labelled with Alexa fluor 594 (Molecular Probes) and anti-total β-catenin (clone 14, Transduction Laboratories), labelled with Alexa Fluor 488 (Molecular Probes). Sections were embedded in vectashield containing DAPI (Vector Laboratories). Specimens were viewed on a Zeiss AxioPlan2 microscope.

Retroviral and lentiviral transduction and FTOC

The retroviral mDkk-1 containing plasmid LZRS-mDkk1-IRES-eGFP (mDkk-1) and control plasmid LZRS-IRES-eGFP (GFP) were transfected into Phoenix Ecotropic packaging cells. Stable high-titer producer clones were selected with puromycin and virus was frozen down until use. Lentiviral Tcf1 reporter vectors have been described[12]. Virus was produced by transfection of 293T cells using 3rd generation packaging system plasmids[37]. Viral supernatant was collected for two days and frozen down until use.

Bone marrow cells from 16-26 weeks old C57Bl/6 mice were passed over a Percoll density gradient. Fetal liver cells were isolated from day 14 embryos. Cells were cultured over night in serum free enriched Dulbecco's modified Eagle's medium[38] supplemented with 50 ng/ml human Flt3-L, 50 ng/ml human TPO and 100 ng/ml murine SCF. $CD34^+$ cells were isolated from human cord blood using AutoMACS (Milteny Biotec) and cultured over night in RPMI with 10% FCS, 10 ng/ml human Flt3L, 10 ng/ml human TPO and 50 ng/ml human SCF. $0.5\text{-}3x10^6$ cells were transduced with viral supernatant in the presence of the same cytokines.

After 2 days of transduction, cells were harvested, counted and transduction efficiency was measured using flow cytometry. 30,000 human $CD34^+$ cells or 50,000 murine cells were transferred into irradiated day 14 fetal thymic lobes using the hanging drop method[39]. After 24-72 hours, the lobes were transferred to a filter (Whatman) floating on medium. After 9 or 21 days of culturing, cells were harvested and stained for flow cytometry.

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WNT TARGET GENES IDENTIFIED BY DNA MICROARRAYS IN IMMATURE CD34$^+$ THYMOCYTES REGULATE PROLIFERATION AND CELL ADHESION

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ABSTRACT

The thymus is seeded by very small numbers of progenitor cells that undergo massive proliferation before differentiation and rearrangement of T-cell receptor genes occurs. Various signals mediate proliferation and differentiation of these cells, including Wnt signals. Wnt signals induce the interaction of the cytoplasmic co-factor β-catenin with nuclear Tcf transcription factors. We identified target genes of the Wnt/β-catenin/Tcf pathway in the most immature ($CD4^-CD8^-CD34^+$) thymocytes using Affymetrix DNA microarrays in combination with three different functional assays for *in vitro* induction of Wnt signaling. A relatively small number (~30) of genes changed expression, including several proliferation-inducing transcription factors such as c-fos and c-jun, protein phosphatases and adhesion molecules, but no genes involved in differentiation to mature T-cell stages. The adhesion molecules likely confine the proliferating immature thymocytes to the appropriate anatomical sites in the thymus. For several of these target genes, we validated that they are true Wnt/β-catenin/Tcf target genes using real-time quantitative PCR and reporter gene assays. The same core set of genes was repressed in Tcf1-null mice, explaining the block in early thymocyte development in these mice. In conclusion, Wnt signals mediate proliferation and cell adhesion, but not differentiation of the immature thymic progenitor pool.

INTRODUCTION

The Wnt protein family consists of 18 secreted factors that regulate cell fate, polarity and cell growth in receptive cells[1]. Many of the Wnt genes in the mouse have been investigated by targeted mutation, leading to very specific developmental defects[2]. In addition, deregulated Wnt signaling is involved in oncogenesis of several different types of human cancers, including tumors of the colon, kidney, liver, prostate and ovarium[3]. Current models of Wnt signaling assume that secreted Wnt proteins bind to receptors of the Frizzled family on the cell surface. Via several different proteins, the signal is transduced to cytoplasmic β-catenin, which then enters the nucleus and forms a complex with members of the Tcf family of transcription factors to activate transcription of Wnt target genes[4].

The founding members of the Tcf family are T cell factor 1 (Tcf1) and Lymphoid Enhancing Factor 1 (Lef1)[5]. In adult mammals, Tcf1 is expressed uniquely in T lymphocytes, whereas Lef1 is expressed at lower levels in T cells, as well as in B cells and other cell types[6]. The functions of both these genes have been investigated by targeted mutation experiments. Tcf1 null mice have an incomplete, but marked block in thymocyte development[7], and fail to generate *de novo* thymocytes after transplantation

of hematopoietic precursors in irradiated hosts[8]. Two different Tcf1 mutant mice have been generated[7]. In Tcf1(V) mutant mice, a low level of a truncated yet functional Tcf1 is still expressed, whereas the Tcf1(VII) mutation abolishes the DNA binding activity of Tcf1 completely and these mice are regarded as true Tcf1 null mutants. Young Tcf1(VII)$^{-/-}$ mice have an incomplete block at the CD4^{-}CD8^{-} double negative (DN) and a more prominent block at the immature single positive (ISP) stages of thymocyte differentiation, whereas older mice display a complete block at the DN1 stage of development. Lef1 null mice lack hair, teeth, mammary glands and trigeminal nuclei and die around birth[9], but have normal thymocyte development. Lef1 can partially compensate for the Tcf1 defect, especially in the neonatal and fetal thymus. While Tcf1(V) mutant mice are viable and have modestly impaired thymocyte development, Lef1/Tcf1(V) double mutant mice display a complete block in thymocyte development at the ISP stage[10]. Lef1/Tcf1(VII) double mutant mice die at day 10 of embryogenesis[11] with dramatic developmental defects affecting the limb buds and neural tube. Interestingly, the phenotypic abnormalities of these double-mutant mice are highly reminiscent of those in Wnt3a mutant mice[11].

Both Tcf1 and Lef1 function as nuclear effectors of Wnt signals[12,13]. Both nuclear proteins are activated by binding to β-catenin, which supplies a powerful transactivation domain to the DNA binding activity of Tcf family members. β-catenin is subject to complex regulation via several key components of the Wnt pathway, including the tumor suppressor genes Axin and APC (adenomatous polyposis coli) and the negative regulatory protein kinase GSK-3β[1] (see Figure 1). In absence of Wnt signaling, GSK-3β is actively inducing phosphorylation of β-catenin and its subsequent degradation

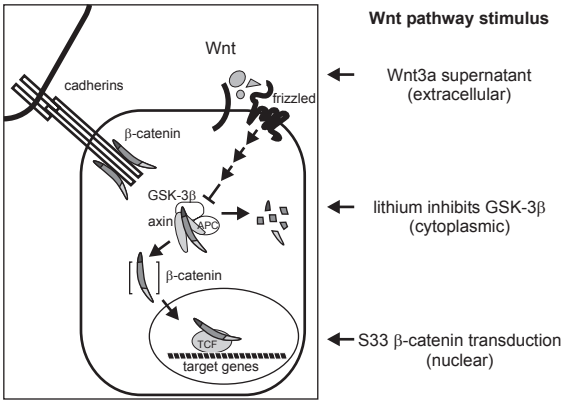

Figure 1. Simplified overview of Wnt signaling and stimuli used in this study.
The Wnt pathway can be activated at multiple levels, either by stimulation of a Wnt ligand binding to a Frizzled receptor, by pharmacological inhibition of the GSK-3β kinase, or by providing the activated form of β-catenin. All three ways lead to the formation of an active β-catenin-Tcf transcription factor complex. The target genes of this pathway in the thymus were unknown until this study.

via the ubiquitin/proteasome pathway. In the absence of β-catenin in the nucleus, Tcf proteins may recruit members of the TLE/Grg family, which act as global repressors of transcription[14]. Upon Wnt signaling, GSK-3β is inactivated leading to accumulation of dephosphorylated β-catenin. Lithium can strongly inhibit GSK-3β activity and can therefore be used to mimic Wnt signaling[15]. Lithium treatment can activate transcription of Tcf reporter genes in fibroblasts and epithelial lines[15], and to a lesser extend in T cells[16].

Wnt signaling has been shown to occur in hematopoietic stem cells and developing T and B cells[17-20]. Several reports indicate that Wnts can act in synergy with cytokines as hematopoietic growth factors. For instance, Wnts induce expansion of murine fetal liver stem cells and induce proliferation of human $CD34^+$ hematopoietic stem cells[17]. In addition, Reya et al. demonstrated that Wnts can act as growth factors for pro-B cells[18]. We have shown that Wnt signaling induces proliferation of fetal DN thymocytes[19]. Moreover, retroviral expression of soluble Wnt receptor mutants that block Wnt signaling impairs thymocyte development, indicating that Wnt signaling is required for normal T-cell development[19]. Finally, Wnt signaling is important in the regulation of survival of DP thymocytes[20].

To further understand the role of Wnt signaling in T-cell development, it is imperative to identify the target genes of the β-catenin/Tcf transcription factor complex in thymocytes. The recent development of DNA microarrays allows the rapid and unbiased identification of genes expressed under different conditions. We have chosen to purify human $CD34^+$ DN thymocytes instead of murine early DN thymocytes for several reasons. First, many more thymocytes can be obtained from human thymi than from the mouse. Given that the most immature DN thymocytes (DN1 and DN2) comprise only a small (~1%) subset of adult thymocytes, the choice of human thymocytes allows more readily the isolation of sufficient amounts of RNA for use on DNA microarrays. Second, the currently available microarrays for humans generally contain more genes than the murine chips. Third, human immature thymocytes can easily be purified based on the presence of the CD34 stem/progenitor cell marker on their cell surface**.** Here we have used Affymetrix microarrays to identify the transcriptional response the Wnt/β-catenin/Tcf pathway in the most immature thymocytes.

RESULTS

The experimental system

It is well established that recombinant Wnt proteins are difficult to produce in soluble form *in vitro*, with the notable exception of murine Wnt3a, which can be made in biologically active form by transfected L cells, a system which very recently has become available (http://www.stanford.edu/~rnusse/assays/wntproteins.html). In almost all

studies, researchers have therefore relied on pharmacological or genetic ways to induce Wnt signaling *in vitro.* Here we have used three functional assays to induce Wnt signaling (see Figure 1): 1. treatment with LiCl, which inhibits GSK-3β, a key step in activating the Wnt pathway; 2. retroviral expression of an activated form of β-catenin; 3. supernatant from L cells transfected with a Wnt3a expression vector. Treatment with LiCl has the advantage of its rapid action, allowing time course investigations of Wnt signaling, but lacks some specificity, because GSK-3β also is involved in regulation of Rel/NF-AT transcription factors[21,22]. The retroviral system is highly specific, but is not suited for time course experiments, because of the nature of the transduction process.

To isolate sufficiently high numbers of $CD4^-CD8^-$ immature thymocytes that can be manipulated to induce Wnt signaling and yield sufficient RNA for microarray experiments, we isolated human $CD34^+$ thymocytes. $CD34^+$ immature thymocytes were isolated by magnetic beads to >96% purity (Figure 2A) and treated with LiCl and KCl for various lengths of time. LiCl is a well-known inducer of Wnt signaling, but its induction of Tcf-dependent transcription (the ultimate nuclear consequence of the Wnt cascade) is not as strong in mature T lymphocytes as in other cell types (e.g. fibroblasts, epithelial cells[23]), but using sensitive reporter assays a 5- to 10-fold induction of Tcf-dependent

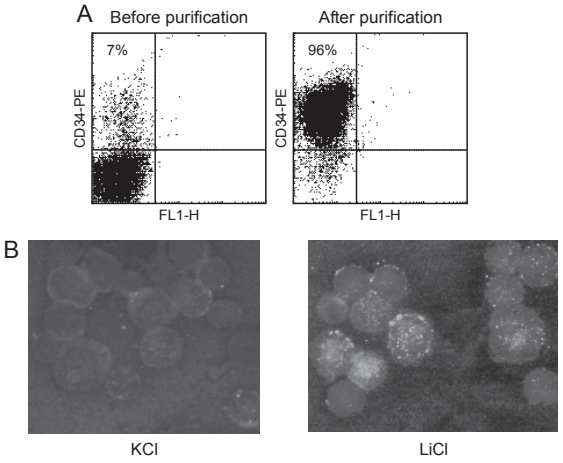

Figure 2. Lithium activates Wnt signaling in $CD34^+$ immature thymocytes.
(A) Purification of immature $CD34^+$ thymocytes. Thymocytes were purified by magnetic-bead isolation using the CD34 marker. In fresh thymocytes this $CD34^+$ population constitutes ~1% of the cells, but in frozen and subsequently thawed thymocytes 3-7%, because of the selective loss of DP thymocytes after freeze/thawing. After MACS purification cells were >96% $CD34^+$. (B) Lithium activates Wnt signaling in $CD34^+$ immature thymocytes. Purified $CD34^+$ thymocytes were treated for 6 hours with 20 mM LiCl or KCl. Cytospins were made and analyzed by immunofluorescence using an antibody specific for dephosphorylated β-catenin, followed by a FITC-labeled secondary antibody. The clear increase in nuclear β-catenin indicates that Wnt signaling has been induced in these cells.

transcription can be observed in Jurkat T cells (data not shown). We therefore first evaluated whether LiCl could induce Wnt signaling in primary prothymocytes. These primary cells are not transfectable with reporter gene plasmids and therefore we used a recently developed antibody that specifically recognizes the activated, dephosphorylated form of β-catenin[24,25]. LiCl indeed induced an increase in the amount of dephosphorylated β-catenin (Figure 2B).

Wnt signaling induced by lithium treatment

We isolated RNA from $CD34^+$ thymocytes treated for 3, 6 or 18 hours with 20 mM LiCl, using KCl as control treatment. Microarray analysis on Affymetrix U95 GeneChips (containing 12,600 genes) revealed that approximately ~15 genes were upregulated and ~10 downregulated more than 3 fold at the 3 and 6 hour time points (Table I). During the shorter time points (3 and 6 h) cycloheximide (CHX) was added to inhibit *de novo* protein synthesis, thereby allowing identification of primary target genes. For the 18-hour timepoint this was not possible due to cytotoxicity of longer CHX treatment. Most genes were slightly more upregulated after 6 hours compared to 3-hour treatment. The upregulated genes included proliferation-inducing transcription factors such as *c-fos*, *fosB* and other nuclear proteins (*AHNAK*, *GILZ*, *cysteine-rich protein* (*CRP)1* and *CRP2*), cell cycle regulators (*Checkpoint suppressor 1*, *CDK15 kinase*, *cdc-like kinase 2* and *EB3* which binds mitotic spindles) and signaling molecules such as the *CL100 dual specificity phosphatase*. Downregulated genes also concerned nuclear targets such as *BTG1*, *BRCA2*, *nuclear pore complex proteins* (*Nup88*), *c-myb* and signaling molecules such as *PTPase2A*. In addition, the pro-apoptotic gene *TRAIL* was downregulated. Several other genes were induced albeit at a low level (2-2.5 fold), including the T-box transcription factor *Brachyury*, a known Wnt target gene in other cell types.

In order to identify those genes that were statistically significantly changed, we performed the 18-hour treatment with biological triplicates (6 microarrays). Significance analysis of microarrays (SAM) is a statistical test specifically developed for identification of such genes[26]. A tuning parameter δ can be set corresponding to the number of false positive genes included in the set of genes identified as significantly changing in expression. We chose to have less than one false positive gene included and performed SAM analysis on the three 18-hour treatment experiments. In doing so, we identified 9 transcripts that significantly changed, all upregulated after LiCl treatment (see Table II). Four of these genes were also found at the earlier time points, suggesting that they are direct target genes. These are the *CRP1* and *CRP2* genes, which are highly conserved nuclear proteins with a LIM/ double Zn finger motif, the large (700 kD) nuclear protein *AHNAK* (meaning 'giant' in Hebrew), and the anti-apoptotic, lymphoid specific Zn finger protein GILZ (glucocorticoid induced lymphoid Zinc-finger). The other genes are apparently induced with slower kinetics or may not be direct target genes.

Another way to identify relevant genes in the wealth of microarrays data is clustering analysis. We performed hierarchical clustering on the three time points to identify clusters of genes that behaved similarly in terms of induction or repression (Figure 3A). In general, similar sets of genes were found as those described in Table I and II. As expected, the *c-fos* and *fosB* genes were most highly induced after 3 and 6 hours, but not after 18 hours. To further validate the differential expression of the identified genes, we performed TaqMan-based RQ-PCR analysis for several transcripts. In general these

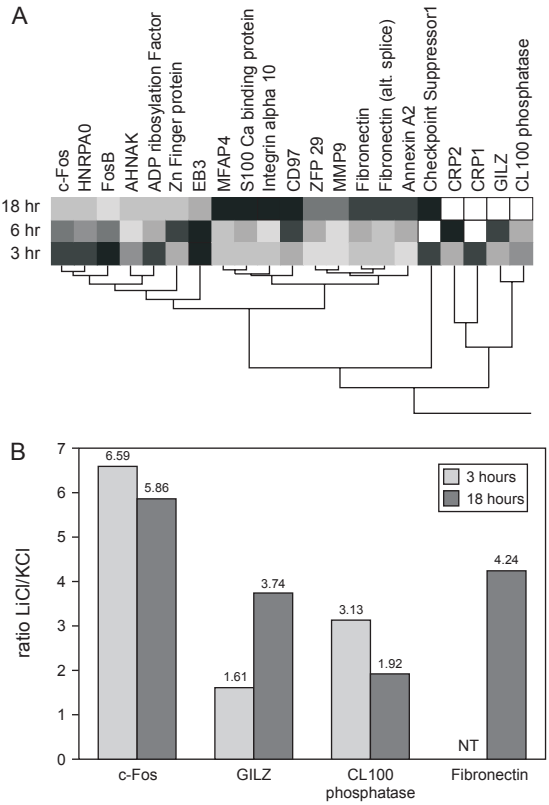

Figure 3. Target genes of the Wnt/b-catenin/Tcf pathway induced by LiCl.
(A) Cluster analysis of Wnt target genes at 3, 6 and 18 hours after stimulation with LiCl. The data from the genes found to be differentially induced or repressed with Microarray Suite software were used to perform hierarchical clustering using GeneLinker Gold program. Although this cluster analysis does not indicate statistical significance, similar sets of genes were found as by SAM analysis. (B) Independent validation of Wnt target genes by TaqMan RQ-PCR. RNA was extracted from KCl and LiCl treated immature thymocytes. RQ-PCR was done for c-fos, GILZ, CL100 phosphatase and fibronectin. Data shown are the average of triplicate measurements of one experiment out of three done. NT= not tested (because fibronectin is not induced at earlier time points).

data correlated well with the microarray findings (Figure 3B) for both the earlier and later time points.

Table I. Induced and repressed genes in CD34$^+$ thymocytes after treatment with LiCl[1].

Annotation induced genes	Fold change (3 h)	Fold change (6 h)
CDK15 kinase	4.3	1.3
CL100 phosphatase	3.2	2.0
EGR	1.7	3.0
HrpA0	4.4	3.0
hyp protein with Ankyrin repeats	1.6	3.2
rhinovirus receptor (HRV)	2.7	3.4
α-tubulin	2.6	3.4
ADP ribosylation factor-like protein	4.6	3.6
GILZ	2.2	3.9
FosB	5.7	5.4
c-Fos	6.8	4.5
EB3 protein	4.7	6.3
Cys rich protein (CRP)	3.9	10.1
AHNAK	5.7	13.2
Check point suppressor	4.0	13.8
Annotation repressed genes	Fold change (3 h)	Fold change (6 h)
diacylglycerol kinase	-2.3	-3
ISGF-3,	-1.9	-3
CD8 β-chain	-4.6	-3.1
ganglioside expression factor 2-like	-1.7	-3.2
PP2A	-4.5	-3.3
RNA polymerase II largest subunit	-2.6	-3.4
phosphodiesterase	-2.5	-3.6
TRAIL	-2.1	-3.6
SLP-76	-2.1	-3.2
CD53	-1.8	-4.1
BTG nuclear protein	-3.8	-4.6
Nup88	-1.8	-4.8

[1] CD34$^+$ thymocytes were treated with LiCl or KCl for 3 or 6 hours after which RNA was isolated and probed on U95A GeneChips. Only genes that were expressed and showed reliable upregulation or downregulation of 3-fold or more at at least one time point are shown.

Table II. SAM analysis of triplicate microarrays[1].

Annotation	Average fold change (LiCl/KCl)
CRP1	9.1
AHNAK	6.5
Fizzy related cell cycle regulator	5.3
GILZ	6.2
cysteine protease	4.7
cellular fibronectin	5.5
S100 Calcium bindingprotein	5.3
CRP2	6.5
Fibronectin, alternative splice	4.0

[1]Eighteen-hour treatment of $CD34^+$ thymocytes with LiCl and KCl. Three independent experiments were performed (six U95A microarrays). Average fold change in LiCl treated $CD34^+$ thymocytes as compared to control (KCl treated)

Wnt signaling induced by retroviral transduction with constitutively active β-catenin

As a second treatment to induce Wnt signaling in $CD34^+$ immature thymocytes, we transduced the cells either with a mouse CD8 (Lyt2a) marker virus or a retrovirus encoding a constitutively active form of β-catenin linked via an IRES sequence to the Lyt2a marker gene. The S33-β-catenin mutant is a specific activator of Wnt signaling and provides a strong transactivation domain for its nuclear partner Tcf1, whilst not being under negative control of GSK-3β because one of the serine residues phosphorylated by GSK-3β (S33) is mutated.

Immature $CD34^+$ thymocytes were transduced with the IRES-Lyt2a marker gene or S33-β-catenin-IRES-Lyt2a with very similar efficiencies (Figure 4A) making them comparable for microarray analysis. After transduction, RNA was isolated and used for microarray analysis. Similar to the experiments described above, only a limited number of genes (N=14) was found to be differentially regulated between the two treatments (Figure 4B). Wnt target genes identified after transduction with S33-β-catenin include *c-fos*, *fosB*, the *CL100 dual specificity phosphatase* and *β7* and *p50,95 integrins* which were also found in the lithium experiments, as well as *JWA* and *NRPB*. Down regulated were the structural proteins *laminin* and the pro-apoptotic gene *Requiem* (*HREQ*) (both low, 2-fold). Repeat experiments revealed similar clusters of genes, although the fold induction or repression varied. We reasoned that this could be caused by differences in kinetics in the retroviral transduction experiments. The complexities of transduction experiments, including integration, expression of β-catenin and induction of Wnt/Tcf target genes, do not allow direct assessment of the kinetics of induction and consequently

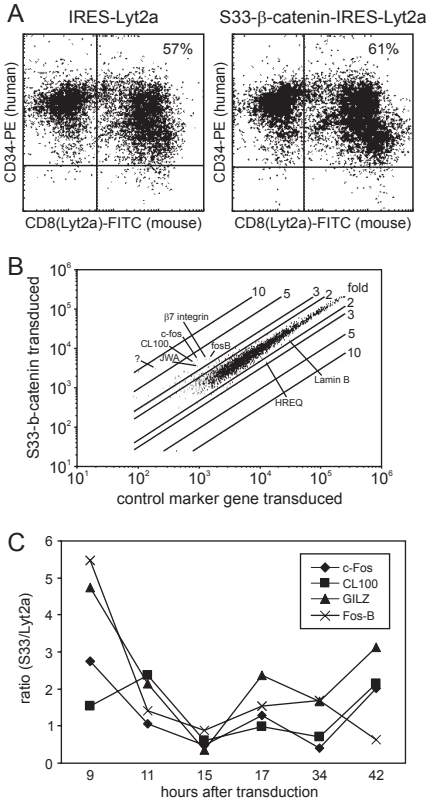

Figure 4. Wnt target genes identified after transduction of CD34⁺ immature thymocytes with a retrovirus encoding constitutively active β-catenin.
(A) Transduction of CD34⁺ cells with a retrovirus encoding the marker gene Lyt2a (murine CD8), or the S33-β-catenin-IRES-Lyt2a gene. Cells were transduced in the presence of IL-7 for 2 days and stained with antibodies against CD34 and murine CD8. (B) Genes induced or repressed by induction of Wnt signaling as identified by Affymetrix microarray analysis of Lyt2a vs. S33-β-catenin-IRES-Lyt2a transduced immature thymocytes. Shown is a dot plot for all genes expressed after 2 days of transduction. Gene expression level is indicated in arbitrary fluorescence units. Genes that do not change in expression (the vast majority) follow the line under a 45° angle. The other lines indicate 2-, 3-, 5- and 10-fold changes in gene expression. (C) TaqMan RQ-PCR validation of differential expression of Wnt target genes after induction of Wnt signaling by transduction of S33-β-catenin. RNA extracted from Lyt2a- or S33-β-catenin-IRES-Lyt2a-transduced cells was used for quantitative PCR analysis using GAPDH as control gene. Results are shown as a ratio of S33-β-catenin vs. Lyt2a signals. A time course was done at various time points (9-48 hours) after initiation of transduction. Shown are data for c-fos, fosB, CL100 phosphatase, and GILZ.

may cause variations in the target genes detected. We therefore performed a time course experiment for many time points after transduction of immature thymocytes with S33-β-catenin using RQ-PCR as the read-out for several Wnt target genes (Figure 4C). These kinetic experiments revealed a rapid induction of *c-fos* and *GILZ* (presumably by transcription of β-catenin from the unintegrated proviral DNA) followed by a lag time and a 2- to 2.5-fold induction at later time points (presumably expression of β-catenin from the integrated retroviral LTR). This induction of Wnt target genes in the time course experiments therefore confirms the microarray experiments.

Wnt signaling induced by stimulation with supernatant from Wnt3a transfectants

During the course of our studies, L-cells transfected with murine Wnt3a were shown to produce biologically active Wnt3a. For two reasons this system may also be able to induce Wnt signaling in human $CD34^+$ immature thymocytes. First, murine and human Wnt3a are highly homologous (85% amino acid identity) and second, Wnt3a is expressed in the thymus (Chapter 5).

Immature thymocytes were incubated with supernatant from Wnt3a transfected L cells and from parental L cells (negative control) and analyzed on Western blot using the above described antibody against the active (dephosphorylated) form of β-catenin. Wnt3a containing supernatant was able to induce active β-catenin in immature thymocytes, indicating that Wnt signaling was active in these cells (Figure 5). We next used similar conditions for microarray experiments. Genes that were induced or repressed over 2.5 fold are listed in Table III. The genes for Fos and Jun and for several other transcription factors were induced, such as LMO2, NRPB, CREM and at lower levels of significance, CL100 phosphatase, β7 integrin and AHNAK. *Cytochrome P450*, which was modestly induced after β-catenin transduction, was more clearly induced after Wnt3a stimulation. Interestingly, the most highly induced gene after Wnt3a stimulation encodes an ubiquitin

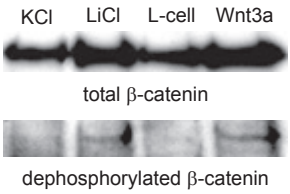

Figure 5. Murine Wnt3a activates Wnt signaling in immature $CD34^+$ thymocytes.
$CD34^+$ DN thymocytes were isolated and incubated with supernatant from parental L cells or L cells stably transfected with a Wnt3a expression construct. As a positive control purified $CD34^+$ thymocytes were also treated for 6 hours with 20 mM LiCl or KCl. Cells were lysed in boiling SDS sample buffer and analyzed by Western blotting with anti-ABC, an antibody specific for the activated form of β-catenin (bottom) and with an antibody recognizing the total β-catenin pool (top). The expected increase in total β-catenin levels after LiCl treatment, were also seen after stimulation with Wnt3a.

Table III. Stimulation of CD34$^+$ immature thymocytes with Wnt3a[1].

Annotation induced genes	Fold change (Wnt3a vs L cell)
Ubiquitin ligase E2	9
GPNMB glycoprotein	8
c-jun	5.7
CYBB	4.6
LMO2	4.3
HRY	3.7
ENC1 nuclear matrix protein	2.8
EST (?)	2.6
MIR-7	2.6
Dual-specificity PTPase 6	2.6
CREM	2.6
c-fos	2.5
TSH-R	2.5
cytP450	2.5
IFI30	2.5
TIEG	2.5
Annotation repressed genes	**Fold change (Wnt3a vs L cell)**
NK4	-5.8
MMP7 matrilysin	-4.8
PDCL	-4.3
IL1beta	-3.8
tryptase1	-3.1
TNFRSF4	-2.9
GRO1	-2.9
MAP4K2	-2.5

[1]Induced and repressed genes (> 2.5 fold) after stimulation with parental L cell supernatant vs Wnt3a-containing supernatant for 4 hrs. A 2.5-fold threshold is set, as this is the lowest difference that can be reliably validated by RQ-PCR.

ligase, known to be involved in degradation of β-catenin, thereby providing a negative feedback on Wnt signaling. Among the downregulated genes several proteases that modify extracellular matrix (ECM) components stand out (*MMP7*, *tryptase1*), potentially influencing cell adhesion to ECM components.

We again validated the microarray results using RQ-PCR for the Wnt target genes *c-fos*, *CL100 phosphatase* and *β7 integrin*. For unknown reasons the differential expression

as detected by RQ-PCR was significantly higher than detected by microarray, namely *c-fos*: 5 fold, *CL100 phosphatase*: 4.8 fold and *β7 integrin*: 2.2 fold (data not shown), more in line with the fold induction in the retroviral transduction experiments.

TCF-reporter gene assays

To validate the identification of target genes of the Wnt/β-catenin/Tcf pathway, we performed two different types of experiments on selected target genes. First, we tested the promoters of several genes identified by different treatments (*fibronectin*, *c-fos*, *brachyury*, and *β7 integrin*) for induction of Tcf dependent transcription in transient transfection assays with luciferase reporters. Second, we checked expression of core target genes in thymocyte subsets of Tcf1(VII)$^{-/-}$ mice.

Reporter gene experiments reveal much smaller differences with natural promoters (containing binding sites for many transcription factors in addition to the one being tested) than with artificial promoters consisting of multimerized binding sites for the tested transcription factors. Nevertheless, we could demonstrate a small induction of promoter activity for fibronectin, brachyury, and β7 integrin (Figure 6) whereas for fos the effect was minimal, probably because our 600-bp fos promoter construct contained only suboptimal Tcf binding sites. Most likely other promoter/enhancer sites around the *c-fos* gene contain additional Tcf sites. Functional identification of such sites is a daunting task, given that enhancers can lie >50 kb up- and downstream of their target genes. Our reporter assays are consistent with the microarray and RQ-PCR data in identification of Wnt target genes, but show a smaller induction. For instance, although the induction of

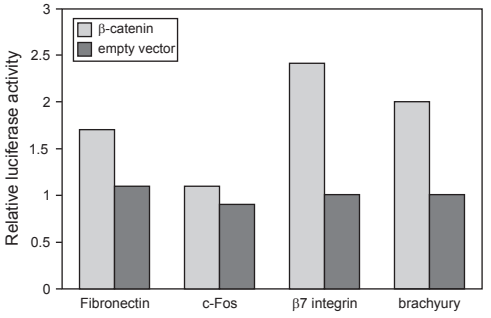

Figure 6. TCF-reporter gene analysis of several Wnt target genes.
Promoters of c-fos, fibronectin and brachyury were cloned in front of a luciferase reporter gene and used in transient transfection assays together with co-transfection of S33-β-catenin or vector control. For reference the optimal Tcf reporter construct (TOP), consisting of multimerized binding sites for Tcf was included as well (>50 fold activation; not shown). The brachyury and β7 integrin promoter constructs contain one optimal Tcf binding site, the fibronectin promoter two, whereas the c-fos promoter used only contains two suboptimal sites. Data shown are the average of duplicate measurements of one experiment out of three done. The luciferase values obtained with vector control were set as 1.0.

fibronectin by LiCl was approximately 4-fold as assessed by microarray, the induction of fibronectin promoter activity in the luciferase reporter gene assay was barely 2-fold. A likely explanation for this difference is that the fibronectin reporter construct lacks important Tcf sites that are activated by Wnt signals *in vivo*. Similarly low induction of the fibronectin promoter by Wnt signals has recently been reported for *Xenopus* fibronectin[27]. Our reporter gene data also show that genes identified by only one stimulus, such as *brachyury* and *fibronectin*, behave as target genes in isolated reporter gene assays, whereas in functional assays they may not be bona fide Wnt targets in prothymocytes because of the lack of induction by Wnt3a or S33-β-catenin. Thus, we regard *c-fos* as a true Wnt target gene, because it was found in all three conditions to induce Wnt signaling (including those under cyclohexamide treatment), even though in the reporter gene asasys with the short c-fos promoter we could not demonstrate induction by Wnt signals. In contrast, genes such as fibronectin or brachyury were only found after lithium treatment and are Wnt targets in other cell types, but probably not in thymocytes.

We checked the expression of the core set of seven Wnt target genes by RQ-PCR throughout T-cell development (see Table IV). Interestingly, many of these genes were expressed at the highest level in $CD34^+$ thymocytes (*c-fos*, *CL100 PTPase*, *hsp70*), suggesting that their expression is significantly regulated by Wnt signals. Undoubtedly other pathways also regulate expression of Wnt target genes, in particular those found to be highly expressed at later stages of development (*c-jun*, *fosB*).

Expression of Wnt target genes in Tcf1 null mice ($Tcf1(VII)^{-/-}$)

It is likely that important Wnt target genes are evolutionary conserved in the same types of cells. We therefore checked the expression of three prominent target genes, *c-fos*, *c-jun* and *CL100 phosphatase* in mice that lack the Tcf1 transcription factor

Table IV. Expression of core Wnt target genes during thymocyte development as measured by RQ-PCR[1].

Stage	*c-fos*	*fosB*	*c-jun*	*CL100*	*NRPB*	*P150,95*	*HSP70*
$CD34^+CD1a^-$	92	1	1	466	< 1	< 1	522
$CD34^+CD1a^+$	93	0.4	10	367	< 1	< 1	561
DN ($CD34^-$)	19	6	22	49	< 1	< 1	11
DP	30	9	32	64	< 1	< 1	15
CD4 SP	55	11	16	37	< 1	< 1	4
CD8 SP	80	35	26	110	< 1	< 1	5

[1]Data are presented as 1000 times the level of a particular transcript compared to the level of GAPDH as housekeeping gene (i.e. 1000 corresponds to the level of GAPDH in a particular subsets). Subpopulations were sorted from the human thymus to >95% purity.

(hereafter referred to as Tcf1(VII)$^{-/-}$). These mice are not able to induce Wnt target genes because of the lack of nuclear integration of Wnt signals. Young Tcf1(VII)$^{-/-}$ (<12 weeks) mice have an incomplete block in T-cell development at the ISP stage, later (around 6 months of age) followed by a more complete block at the DN stage. We used thymi from 7-week-old wild type control mice and Tcf1(VII)$^{-/-}$ mice, sorted the DN and ISP cell populations and checked the levels of Wnt target genes by RQ-PCR (Figure 7A). While the levels of *c-fos*, *c-jun* and *CL100 phosphatase* were not dramatically different in the DN stage (where cells still develop normally in Tcf1(VII)$^{-/-}$ mice of this age), the levels of *c-fos* and *c-jun* were dramatically reduced in ISP cells (Figure 7B). In addition, the levels of CL100 phosphatase were 3-fold reduced. These experiments underscore that these genes function as Wnt target genes and confirm the microarray experiments.

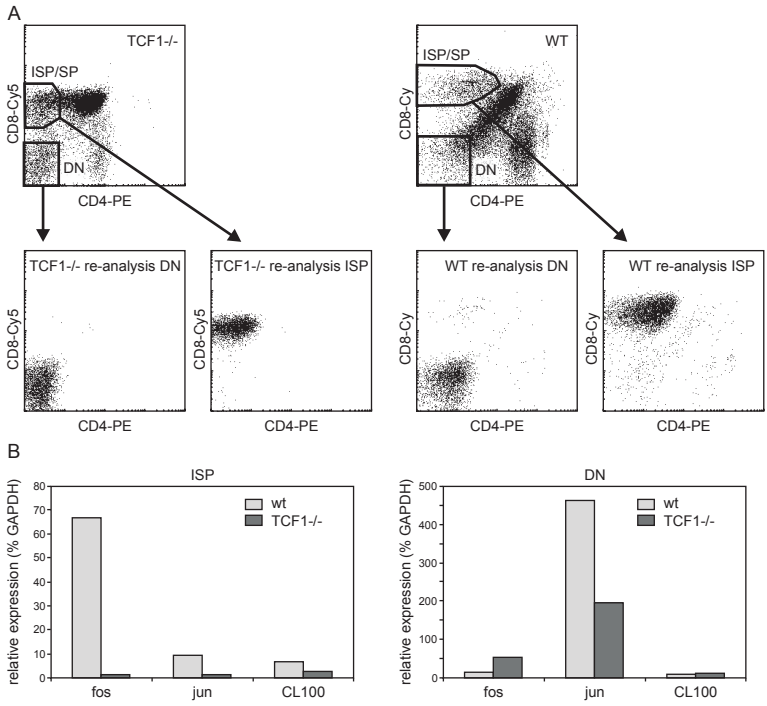

Figure 7. Expression of Wnt target genes is decreased in Tcf1(VII)$^{-/-}$ mice compared to wild type mice.

(A) Thymi from wild type and Tcf1(VII)$^{-/-}$ mice were sorted in DN and ISP populations based on staining with CD4, CD8 and CD3. DN cells were defined as CD4$^-$CD8$^-$CD3$^-$, ISP cells as CD8$^+$CD3$^-$. Upper panels depict starting populations, lower panels show populations after sorting. All sorted populations were >98% pure and CD3$^-$ (not shown). (B) RNA was extracted and the expression of c-fos, c-jun and CL100 phosphatase was analyzed in DN and ISP cells by RQ-PCR, with GAPDH as control. Values are given as percentage of GAPDH x 1000.

DISCUSSION

Target genes of the Wnt/β-catenin/Tcf pathway in thymocytes have remained elusive. Here we have investigated the target genes induced by Wnt signals that activate the β-catenin/Tcf complex in $CD34^{+}DN$ thymocytes (prothymocytes) using three different functional assays to activate Wnt signaling *in vitro*. Remarkably, no markers of T-cell differentiation were induced; we instead found that markers of proliferation and cell-cell adhesion were upregulated. This is consistent with the notion that Wnt proteins can provide proliferative signals for stem cells in various tissues, such as crypt cells in the colon, hematopoietic stem cells, pro-B cells and pro-T cells.

In general, a relatively small number of Wnt target genes (in total approximately 30) was found. LiCl, although widely used as a pharmacological agent to mimick Wnt signaling, is somewhat nonspecific, as is reflected by the much larger number of genes found after LiCl treatment than with the other two stimuli (Figure 8). By definition, genes identified by transduction with S33-β-catenin or Wnt3a stimulation are Wnt target genes. A core set of seven genes is induced by both specific stimuli: *c-fos*, *fosB*, *CL100 phosphatase*, *integrin p150,95*, *c-jun*, *hsp70* and *NRPB*; most of these genes are induced by LiCl as well. Because the three functional stimuli differ in modes of action, signal strengths, and kinetics of induction, it is not surprising that some differences in gene expression were found, but the functional clusters were highly comparable. The

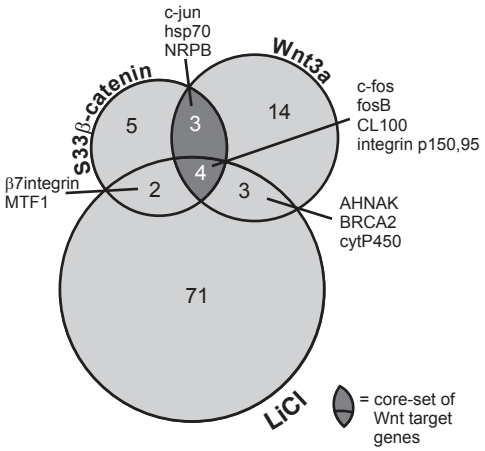

Figure 8. Venn diagram of Wnt target genes in prothymocytes identified by three different functional stimulations.
The area of each circle is proportional to the number of target genes identified by each stimulus. Note that not all genes induced by LiCl are bona fide Wnt targets. A core set of 7 genes is identified among the 31 Wnt target genes defined.

identified target genes can be clustered into several groups: genes encoding transcription factors and other nuclear proteins *(c-fos*, *fosB*, *AHNAK*, *GILZ*, *NRPB*, *LMO*), together forming a cluster of genes involved in cell proliferation; signaling molecules in particular phosphatases (*CL100 phosphatase*); molecules involved in apoptosis (*GILZ*, *TRAIL*); cell adhesion molecules (*fibronectin*, *laminin*, *p150,95*, *β7 integrin*) and ECM modifiers ((*MMP7*, *tryptase1*).

Some genes found only after lithium treatment may be true Wnt targets in thymocytes as well, because they have been identified as Wnt targets in other species, including the T box transcription factors brachyury in murine ES cells[40] and cellular fibronectin in *Xenopus*[27]. Promoters of these genes are responsive to β-catenin (see Figure 5). However, because they were not significantly induced by S33-β-catenin transduction nor Wnt3a stimulation, we have excluded them in the cluster of 31 target genes.

It should be noted that a number of known Wnt target genes in other tissues are apparently not induced by Wnt signaling in $CD34^+$ thymocytes. Of note, *CyclinD1* and *c-myc* expression were not changed, even though both genes are represented on the arrays used. In addition, expression of anti-apoptotic gene *Bcl-x* did not change, even though this gene has been proposed to play role in Tcf-induced protection against apoptosis in DP thymocytes[20]. Apparently, Wnt signaling differs between immature and more mature stages of T-cell differentiation.

The lack of known T-cell differentiation genes among the identified target genes seems at variance with data from Gounari et al.,[28] who showed that transgenic mice over expressing constitutively active β-catenin seem to by-pass signals transmitted via the pre-TCR and differentiate into DP cells. However, *in vivo* differentiation experiments cannot evaluate the effects of constitutively active β-catenin per differentiation stage. For example, the pre-TCR provides proliferative signals as well. These proliferative signals superimposed on other differentiation signals given in the context of the thymus might be sufficient to induce differentiation. However, the DP and SP cells generated by somatic activation of β-catenin lack rearranged TCRβ chains and do not survive properly[28], indicating that the proliferative signals provided by ectopic activation of Wnt signaling do not mimic normal proliferation and differentiation signals emanating from the pre-TCR. In conclusion, our results support the idea that Wnt signals induce proliferation, but not differentiation of the earliest progenitor cell compartment in the thymus. This is consistent with a recent report by Sen and colleagues showing a proliferative defect in thymocytes of Wnt4/Wnt1 null mice[29].

c-fos is one of the target genes induced the strongest (>5 fold) and found in all three types of experiments, whereas *c-jun* is found in the Wnt3a and S33-β-catenin experiments. Both c-fos and c-jun are well-known transcription factors that mediate cell cycle progression and proliferation[30,31]. c-fos null mice show a reduced cellularity in the thymus[32], without any strong blocks at a particular stage, consistent with a proliferative

defect at various stages of development and somewhat reminiscent of thymi from young Tcf1(VII)$^{-/-}$ mice. FOS family members (fos, fosB, fra1) form the so-called Activator Protein 1 complexes by heterodimerization with JUN family members (jun, junB, junD). Because redundancy between these factors (e.g., between fos and fosB) can affect the consequence of gene targeting experiments, dominant negative molecules may better reveal the importance of fos/jun in biological processes. Mice with a dominant negative c-jun have been made[33] and display a defect in cell cycle progression during the transition of DN to DP thymic development, indicating that fos/jun transcription factors promote proliferation at DN and ISP stages. These are the same stages of development affected in Tcf1(VII)$^{-/-}$ mice. Given that fos/jun transcription factors are strongly decreased in expression in those thymic subpopulations that are affected in Tcf1(VII)$^{-/-}$ mice (Figure 7), lack of Wnt-induced fos/jun-mediated proliferation explains the defect in Tcf1(VII)$^{-/-}$ mice. Thus our findings provide a molecular mechanism for the developmental defect in Tcf1(VII)$^{-/-}$ mice.

Interestingly, in a differential screen between genes expressed in CD34^{+}CD38^{-} hematopoietic stem cells (HSC) versus CD34^{+}CD38^{+} progenitor cells in the human bone marrow, Eaves and colleagues have identified *Fos* and *Jun* as genes highly expressed in HSCs (CD34^{+}CD38^{-}) compared with more mature progenitor cells (CD34^{+}CD38^{+}), consistent with a role of these factors in proliferation of stem cells[35].

Among the several signaling molecules found, *CL100 dual specificity phosphatase* was highly induced in all three types of experiments. This gene encodes a MAPK3 phosphatase implicated in cell proliferation[36,37]. Interestingly, this gene is preferentially expressed in CD34^{+}CD38^{-} HSCs compared to CD34^{+}CD38^{+} cells[38], similar to *c-fos* (see above), suggesting that Wnt signaling in early progenitor cells in the thymus induces similar target genes as those expressed in HSCs.

Several genes involved in cell-cell adhesion, including *fibronectin* and *integrins β7* and *p150,95* were found as target genes as well. These proteins are involved in compartmentalization and cell migration within the cortical region of the thymus and perhaps in attracting common lymphoid progenitors to the thymus. Petrie and coworkers[39] have found that DN1 cells are confined to the perimedullary cortex, while DN2 and DN3 cells are found in the outer cortical regions. At the transition of DN1 to DN3 cell (CD34^{+} pro-T cell to pre-TCR^{+} T cell) thymocytes upregulate β7 integrin expression (β7 heterodimerized with α4 or αE integrins) and become more adhesive to fibronectin. Thus, Wnt signals may assure a proper migration of precursors along this stromal matrix or keep cells localized to a certain region of the cortex where cells receive proliferative signals before pre-TCR expression and TCRβ selection.

Based on these findings, we propose that Wnt induced β-catenin/Tcf target genes in immature thymocytes are not directly involved in inducing differentiation, but instead provide proliferative signals (for instance via c-fos and c-jun) to induce cell division in

the precursor cell compartment of the thymus (Figure 9). In fact, because only very few progenitor cells from bone marrow or fetal liver seed the thymus, a vigorous proliferation of the most immature cells in the thymus is required. This has been recognized for over two decades by various groups and is still studied[41-43]. The nature of this proliferative signal is provided by cytokines such as IL-7 and SCF, but apparently also by Wnt proteins[19]. Additionally, Wnt signals can be involved in the migration of immature thymocytes through thymic cortex or in confinement of precursor cells to those cortical regions where proliferation can occur (Figure 9). This may help preventing TCR gene rearrangement at proliferative stages of development or occurrence of selection processes at the corticomedullary junction at inappropriate stages of development. Apparently, the Wnt/β-catenin/Tcf pathway provides both proliferative signals for immature thymocytes in order to maintain the thymic precursor cell compartment and adhesion signals that assure that T-cell development proceeds at the appropriate anatomical sites in the thymus.

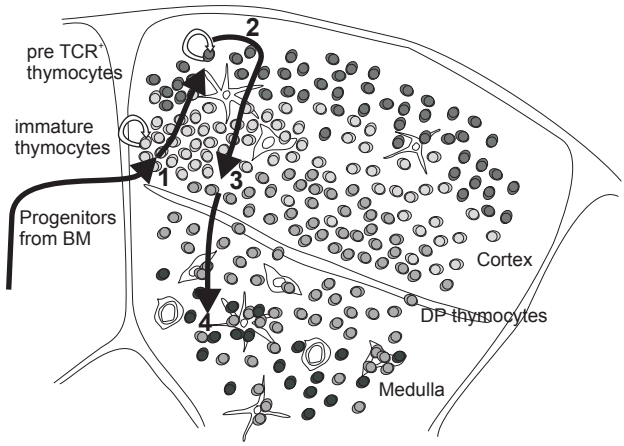

Figure 9. Model: Wnt signals induce proliferation and cell adhesion of immature CD34+ thymocytes.
HSC or common lymphoid progenitors are seeding the thymus from the bone marrow and undergo proliferation, partially driven by Wnt signals, but also by cytokines such as IL-7 and SCF (1). Cells migrate to the outer cortex via different integrins and extracellular matrix components, some of which are Wnt targets (β7 integrin, fibronectin). After this proliferative stage, rearrangement of TCRb occurs, leading to expression of the pre TCR (TCRβ/pTα) on the cell membrane. This generates another stage of proliferation (2) where Wnt signaling may play a role after which cells start expressing CD4 and CD8 to become DP thymocytes (3), followed by rearrangement of TCRa and positive and negative selection at the corticomedullary junction (3). Mature SP thymocytes subsequently end up in the medulla (4).

MATERIALS AND METHODS

Isolation of immature human thymocytes

Thymic tissue was obtained from children undergoing cardiac surgery who did not have any immunological abnormalities. All tissues were obtained according to the guidelines of the Medical Ethical Committee (IRB) of Erasmus MC, Rotterdam, The Netherlands. Thymocytes were isolated by mechanical disrupture of the tissue and squeezing through a mesh. Thymocytes were frozen in liquid nitrogen in RPMI medium containing 10% FCS and 10% DMSO. Thymocytes from 5-10 donors were pooled to reduce individual variation. After thawing, pooling and Ficoll density centrifugation, $CD34^+$ thymocytes were purified by MACS (Miltenyi), according to standard protocols. The degree of purification was assessed by flow cytometry.

Isolation of murine thymocytes

Thymi were removed from 7 weeks old C57Bl/6 and Tcf1(VII) null mice (backcrossed to C57Bl/6). Cell suspensions were made and stained with antibodies specific for mouse CD4, CD8 and CD3. DN and ISP subpopulations were sorted on a FACS Digital Vantage (DiVa) cell sorter (BD Biosciences) to greater than 98% purity and immediately used for RNA isolation.

Methods for *in vitro* induction of the Wnt/β-catenin/Tcf pathway

Treatment with pharmacological agents. $10x10^6$ $CD34^+$ thymocytes were treated with 20 mM LiCl or KCl as control for the indicated length of time. In some experiments cells were also treated with the protein synthesis inhibitor cycloheximide (CHX, 1 µg/ml). These treatments were done in the presence of 10 ng/ml human IL-7 to make the results comparable to the retroviral transduction data. LiCl induced Wnt signaling was assessed by staining of dephosphorylated β-catenin[24].

Retroviral transduction. The retroviral plasmids LZRS-S33-β-catenin-IRES-Lyt2a and LZRS-IRES-Lyt2a (as control) were constructed and transfected into Phoenix amphotropic packaging cell lines using Fugene-6 (Roche). Stable high titer producer clones were selected with puromycin (1 µg/ml). $CD34^+$ thymocytes ($30\text{-}50x10^6$) were pre-stimulated with IL-7 (10 ng/ml) for one day, and subsequently transduced using Retronectin coated petri dishes (Takara) and recombinant retrovirus containing supernatant for two days, with daily replenishing of retroviral supernatant. The efficiency of the retroviral transduction was evaluated by flow cytometry. Cells were stained with antibodies against human CD34 and mouse CD8 (Lyt2a) and analyzed on a Calibur flowcytometer using Cell Quest-pro software (Becton Dickinson).

Stimulation with Wnt3a containing supernatant. For stimulation with Wnt3a, cells were incubated with conditioned medium from L cells stably transfected with a pcDNA-Wnt3a construct. The supernatant was filtered through a 0.22 µm filter and incubated with immature thymocytes for 4 hrs, using supernatant from parental L cells as negative control.

Induction of Wnt signaling in $CD34^+$ thymocytes by Wnt3a was assessed by Western blotting. In short, immature thymocytes were isolated, treated with Wnt3a or control supernatant and lysed in boiling 2x SDS sample buffer as described. Lysates were run on a 10% SDS-PAGE gel, blotted and stained with an antibody against the activated form of β-catenin (anti-ABC)[24,25] or a pan-β-catenin antibody (Transduction Laboratories) and visualized by Enhanced Chemiluminescence (Amersham). Treatment with LiCl or KCl was used as extra positive or negative control for detection of activated β-catenin

Microarray analysis

RNA was isolated using RNeasy columns as described by the manufacturer (Qiagen). The integrity of the RNA was tested on 1% formaldehyde containing agarose gels. 5-8 µg of RNA was used to generate ds cDNA using superscript reverse transcriptase and a T7-oligo dT primer. The resulting cDNA was used in an *in vitro* cRNA reaction using T7 RNA polymerase and biotinylated ribonucleotides employing an ENZO kit. The biotinylated cRNA was cleaned up using RNeasy spin columns (Qiagen) and quantified by spectophotometric methods. An adjusted cRNA yield was calculated to reflect carryover of unlabeled

total RNA. Fragmentation of 20 µg cRNA was performed at 95°C for 35 min. 5 µg of cRNA was hybridized to a Test3 microarray (Affymetrix) to check the quality of the procedure. 10 µg of fragmented cRNA was subsequently hybridized for 16 hrs to U95Av2 microarrays (Affymetrix) at 45°C. After washing and staining, the arrays were scanned in an HP/Affymetrix scanner at 570 nm. For all experiments reported here the 5'/3' ratios of GAPDH were 1.5 or less (usually 0.9-1.1). In comparison experiments, care was taken that the scaling factor, noise and presence calls were comparable.

Bioinformatics

Data reported here are extracted from a total of 16 different U95Av2 GeneChips used under the various conditions described. The scanned images were analyzed using Affymetrix Microarray suite 4.2 software, using the KCl treated samples or the Lyt2a transduced sample or the sample treated with L cell supernatant as baseline. Further analysis was done using microDB and desktop Mining Tool 3.0 software. Statistical significance of the overnight LiCl treatment was tested using the freeware program SAM (Significance Analysis of Microarrays) version SAM 1.12. The false discovery rate (FDR) was 9% for the SAM analysis. Hierarchical clustering was done on the three time points analyzed using GeneLinker Gold 2.0 (Molecular Mining Corporation).

Transcription factor binding sites in promoter sequences were identified using Alibaba 2.1 and Transfac 4.0 (Gene regulation web site www.gene-regulation.com).

TaqMan-based real-time quantitative-PCR (RQ-PCR)

The mRNA expression of several target genes was tested using TaqMan-based RQ-PCR. The isolated RNA was treated with 1U of DNase I and subsequently 1 µg RNA was transcribed to cDNA with AMV-RTase (5 U), oligo(dT) and random hexanucleotide primers. 1/20 cDNA mixture was used for RQ-PCR for each primer/probe set and performed in duplicate. The cDNA was normalized to GAPDH (Applied Biosystems). The RQ-PCR mixture of 25 µl contained TaqMan Universal mastermix, 900 nM primers and 100 nM probe and was run on the ABI PRISM 7700 Sequence Detection System containing a 96 well thermal cycler (Applied Biosystems). All primer and probe sequences were designed with the Primer Express version 1.5 software (Applied Biosystems). Primer and probe sequences used were: CFOS (forward) 5'-AACCTGTCAAGAGCATCAGCAG-3', CFOS (reverse) 5'-GAGCGGGCTGTCTCAGAGC-3', CFOS (probe) 5'-AGCTGAAGACCGAGCCCTTTGATGACTT-3', CL100 (forward) 5'-GCA GAGGCGAAGCATCATCT-3', CL100 (reverse) 5'-CAGCACCTGGGACTCAAACTG-3', CL100 (probe), 5'- CCAACTTCAGCTTCATGGGCCAGC-3', GILZ (forward) 5'-GCGTGAGAACACCCTGTTGA-3', GILZ (reverse) 5'-GGCTCAGACAGGACTGGAACTT-3', GILZ (probe) 5'-ACCCTGGCAAGCCCAGAGCAGCT-3', Brachyury (forward) 5'-TCCTTCCTGCTGGACTTTGTG-3', Brachyury (reverse) 5' CATTCCCCGTTCGCGTACT-3', Brachyury (probe) 5'-CGGCGGATAACCACCGCTGG-3', Fibronectin (forward) 5'-TGAGGAACAT GGTTTTAGGCG-3', Fibronectin (reverse) 5'-CTTGGCCTATGCCTTATGGG -3', Fibronectin (probe) 5'-CACCGCCCACAACGGCCAC-3'. For murine genes we used CL100 (forward) 5'-TGTTGGATTGTCGCTCCTTCT–3', CL100 (reverse) 5'-TGAAGCGCACGTTCATCGAG-3' and CL100 (probe) 5'- TCAACGCCGGCCACATCGC-3' , c-fos (forward) 5'- GGAGGTCTGCCT GAGGCTTC-3', c-fos (reverse) 5'- CACGTTGCTGATGCTCTTGAC-3', c-fos (probe) 5'-CAACGACCCTGAGCCCAAGCCAT-3', c-jun (forward) 5'- GACGGACCGTTCTATGACTGC-3', c-jun (reverse) 5'-GGAGGAACGAGGCGTTGA-3' , c-jun (probe) 5'-TGGAAACGACCTTCTACGACGATGC C-3'.

Reporter Gene assays

293T cells were transfected with the luciferase reporter genes TATA-TOP (Tcf-optimal), TATA-FOP (mutated constructs) and natural promoter constructs from Wnt target genes together with a pCI-S33-β-catenin expression construct or a pCI-neo negative control construct. For natural promoters we constructed pGL3-fibonectin by cloning the fibronectin 1.7 kb promoter from FN-CAT (kindly provided by Dr. T. Gorogh, University of Kiel, Germany), into the pGL3 basic luciferase vector (Promega). The brachyury-promoter-luc was generated by cloning the brachyury promoter from brachy-LacZ into pGL3-

basic-luciferase (brachyury promoter kindly provided by Dr. B. Herrmann, Germany). The Fos-luc reporter construct was kindly provided by Dr. T. Moeroey (University of Essen, Germany) and the β7 integrin promoter-luc by Dr. G. Krissansen (University of Auckland, New Zealand). Luciferase assays were essentially done as described previously[19].

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7

SEVERE HEMATOPOIETIC DEFECTS IN WNT3A DEFICIENT MICE

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To be submitted

ABSTRACT

Wnt proteins are considered to be essential for proliferation of hematopoietic progenitor cells, but direct genetic evidence for this notion is lacking. Support for this hypothesis comes in part from *in vitro* studies employing soluble Wnt3a. Nevertheless, the physiological function of Wnt3a and other Wnt proteins during hematopoiesis remains elusive. Because of the early embryonic lethality of the mutation, our studies were restricted to day 12 fetal thymic lobes and fetal liver stem cells. Using culture systems and reconstitution of sublethally irradiated mice, we found a hematopoietic stem cell defect with severely disturbed myelopoiesis, erythropoiesis and T-lymphopoiesis. Cultured Wnt3a deficient thymic lobes presented with diminished thymocyte numbers and an over time decreasing percentage of double positive cells. Wnt3a deficient fetal liver stem cells normally developed into B and T cells in culture systems with wild-type stromal support, but yielded reduced numbers of myeloid and erythroid cells in colony assays. A hematopoietic stem cell defect was indicated by significantly reduced numbers of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells in Wnt3a deficient fetal livers and severely impaired *in vivo* reconstitution, with complete absence of T cells and myeloid cells.

Thus, we show that targeted mutation of a Wnt gene results in various severe defects in hematopoiesis.

INTRODUCTION

Wnt proteins are secreted factors that regulate proliferation and cell fate determination in many different cell types and organisms[1]. In mice and man, 19 Wnt family members are recognized. Upon binding of a Wnt protein to a receptor of the Frizzled family on the cell surface, a complex cascade is initiated, eventually (in the canonical Wnt pathway) leading to the accumulation and activation of cytoplasmic β-catenin[2,3]. Subsequently, β-catenin enters the nucleus and forms a complex with members of the Tcf family of transcription factors (comprising Tcf1, 3 and 4 and Lef1) to activate transcription of Wnt target genes.

Accumulating evidence suggests a role for Wnt proteins in the hematopoietic system[4]. This was initially recognized by studies in mice that have targeted mutations of Tcf family members. In adult mammals, Tcf1 is expressed uniquely in T lymphocytes, whereas Lef1 is expressed at lower levels in T cells, as well as in B cells and other cell types[5]. Tcf1 null mice have an incomplete, but marked, block in thymocyte development[6,7]. Lef1 mutant mice have normal T-cell development[8], but mild defects in B-cell development in the bone marrow (BM)[9]. Later it was found that Tcf1/Lef1 double mutant mice display a complete block in the thymus at the immature single positive (ISP) stage of T-cell

development[10], indicating functional redundancy of these two transcription factors.

Subsequent studies analyzed upstream components of the Wnt signaling pathway in the hematopoietic system, most extensively so in T-cell development. Until recently, it has been unclear at which stages of T-cell development Wnt signaling occurs. Using a Tcf-GFP reporter we have shown that especially the earliest double negative (DN1 and 2) thymocytes undergo Wnt signaling, although later stages still show some Wnt activity (Chapter 5). Several Wnt and Frizzled family members are expressed in the thymus, some at high levels[11]. Blocking Wnt binding in *in vitro* fetal thymic organ cultures (FTOCs) using soluble Fz receptors completely inhibited early thymocyte development[12]. In addition, mice deficient for both Wnt1 and Wnt4 as well as mice transgenic for Axin, an inhibiting factor in the Wnt pathway, showed reduced thymic cellularity[13,14].

The main function of Wnt signaling in the thymus probably is to induce proliferation, as Wnt1 and Wnt4 have been shown to be growth factors for early T cells in FTOCs[12]. Furthermore, we have shown that most Wnt target genes in human $CD34^+$ thymocytes are involved in proliferation[15].

Despite this large body of evidence, the importance of Wnt signaling for early T-cell development has recently been challenged by studies that examined two different conditional β-catenin deficient mice. Mice with β-catenin deleted from DN3 onwards showed impaired T-cell development at the β-selection checkpoint[16], but mice transplanted with hematopoietic stem cells in which β-catenin had been inducibly deleted, did not show any thymic effect[17]. The effect of β-catenin deficiency in the latter system is probably limited because of redundancy with β-catenin homologue plakoglobin, which is expressed preferentially at the early stages of T-cell development (Chapter 5).

Proliferation probably is the main function of Wnt signaling in developing B cells as well. Addition of Wnt3a-conditioned medium to fetal liver pro-B cells induced cell cycling in culture[9]. In Lef1 mutant mice, reduced numbers of developing B cells were found in the fetal liver and in perinatal BM, caused by diminished proliferation and increased apoptosis[9]. In addition, mice deficient for Wnt receptor Frizzled 9 were found to have lower numbers of developing B cells in the BM, probably because of reduced clonal expansion prior to Immunoglobulin light chain rearrangement in pre-B cells[18].

Besides functioning during lymphocyte development, Wnt signaling has been shown to be important for hematopoietic stem cell (HSC) biology. Tcf-GFP reporter studies have demonstrated Wnt signaling in HSCs *in vivo* as well as *in vitro* after stimulation with purified Wnt3a[19]. Here again, a role for Wnt signaling in self-renewal is likely, as murine Wnt1, 5a and 10b[20] and Wnt3a[21] and human Wnt2b, 5a and 10b[22] induced *in vitro* expansion of cells closely related to HSCs. Retroviral transduction of murine HSCs with a constitutively active form of β-catenin promoted proliferation and maintenance of immature phenotype in cultures, and improved reconstitution of all lineages in irradiated hosts, while transduction with Wnt-signaling inhibitor Axin yielded exactly the opposite results[19].

The function of several Wnt genes has been investigated by targeted mutations in the mouse (reviewed by Moon[23]), which lead to very specific developmental defects. Most of these mutant mice have severe problems, e.g. in limb formation or neural development, and die during embryogenesis or shortly after birth.

Mice carrying a null allele of Wnt3a have been generated by McMahon and co-workers[24] and have been studied extensively. Wnt3a homozygous mutant embryos die between fetal day 10.5 and 12.5. These embryos lack caudal somites[24-26], have a disrupted notochord[24] and exhibit dysmorphology of the central nervous system[24,27].

The hematopoietic system of the Wnt3a knock-out mouse has not been examined, presumably because studies are complicated by the lethality of this mutation early *in utero*. Nevertheless, the high expression of Wnt3a in thymic stroma (Chapter 5) and the fact that it is a growth factor for pro-B cells and HSCs *in vitro* make it of high interest. In addition, Wnt3a deficient embryos are morphologically highly similar to Tcf1/Lef1 double deficient embryos[28], suggesting comparable defects also in the hematopoietic system of both strains. Here we used cultures of fetal thymi and fetal liver stem cells as well as *in vivo* reconstitutions to study the effect of the Wnt3a mutation on hematopoiesis. As Wnt3a is the only Wnt currently available as recombinant protein, it is commonly used as a prototype Wnt to study effects of Wnt proteins in culture[15,19,21,29,30]. Here we address the physiological role of Wnt3a in the development of the different hematopoietic lineages.

RESULTS

T-cell development in Wnt3a deficient mice

Wnt3a deficiency causes embryonic lethality between gestational day 10 and 13[24]. Mice heterozygous for the Wnt3a deletion were bred to generate Wnt3a deficient and wild type/heterozygous day 12 embryos (Figure 1A). No differences were observed between wild type and heterozygous embryos in any of our assays nor have been reported in literature. Therefore, wild type and heterozygous embryos were considered to be similar and will from here on be referred to as ‘wild type’.

First we studied T-cell development in these embryos. In the developing mouse embryo, the thymic rudiment is first colonized by immature cells at approximately day 10-11 of gestation[31,32]. At embryonic day 12 (E12), thymic lobes contain very few thymocytes, most of which are in the DN1 stage, making flow-cytometric analysis not very informative at this time point. Therefore, we cultured the E12 lobes on a filter floating on medium for 9-17 days, to allow further T-cell development.

E12 thymic lobes are not yet well-defined structures, complicating reliable isolation. In general, however, Wnt3a deficient thymi were found to contain lower cell numbers

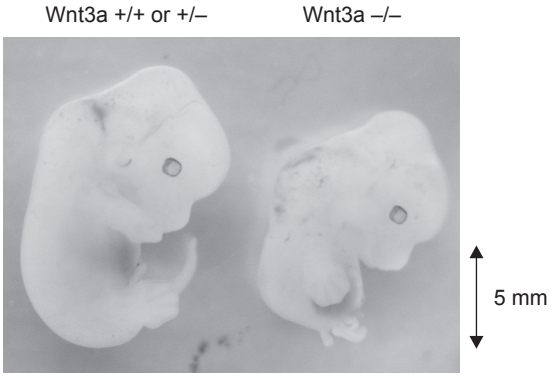

Figure 1. E12 embryos.
Photograph of E12 embryos that are wild type or heterozygous (left) or homozygous (right) for the mutated Wnt3a gene. Deficient embryos lack caudal limbs and tail, although a small tail-rudiment is often visible.

(usually about three fold lower) than wild type embryos (Figure 2A), irrespective of the duration of culturing.

After 9 days of culturing, all stages of T-cell development could be distinguished, including mature single positive (SP) $CD4^+$ and $CD8^+$ cells, both in wild type and Wnt3a deficient embryos (Figure 2B). At this time-point, no differences were detected in subset distribution between Wnt3a deficient and wild type thymi. However, when lobes were cultured for prolonged times (e.g. 14 or 17 days), T-cell development in Wnt3a deficient thymi appeared to deteriorate (Figure 2C). This presented mainly as a decrease in the percentage of double positive (DP) cells and an accumulation of ISP thymocytes, suggesting a block at the ISP stage (Figure 2C).

Since thymic lobes could not always be reliably distinguished in all E12 embryos (knock-out as well as wild type), we confirmed the presence of thymic lobes in Wnt3a deficient embryos using immunohistochemistry. Tissue sections of E12 embryos were prepared and stained with HE to visualize different tissues. Presence of a thymic anlage could be demonstrated in all four Wnt3a deficient embryos examined (Figure 3). This was confirmed by staining with ER-TR4, which identifies thymic epithelial cells[33] (Figure 3 lower panels).

These data demonstrate that in Wnt3a deficient animals the thymic anlage is normally developed and is seeded with hematopoietic cells, but that T-cell development is affected, especially with respect to cell numbers. To investigate whether this defect is intrinsic to the hematopoietic cells that seeded the thymus, we cultured wild type and Wnt3a deficient E12 fetal liver cells in irradiated thymic lobes from wild type

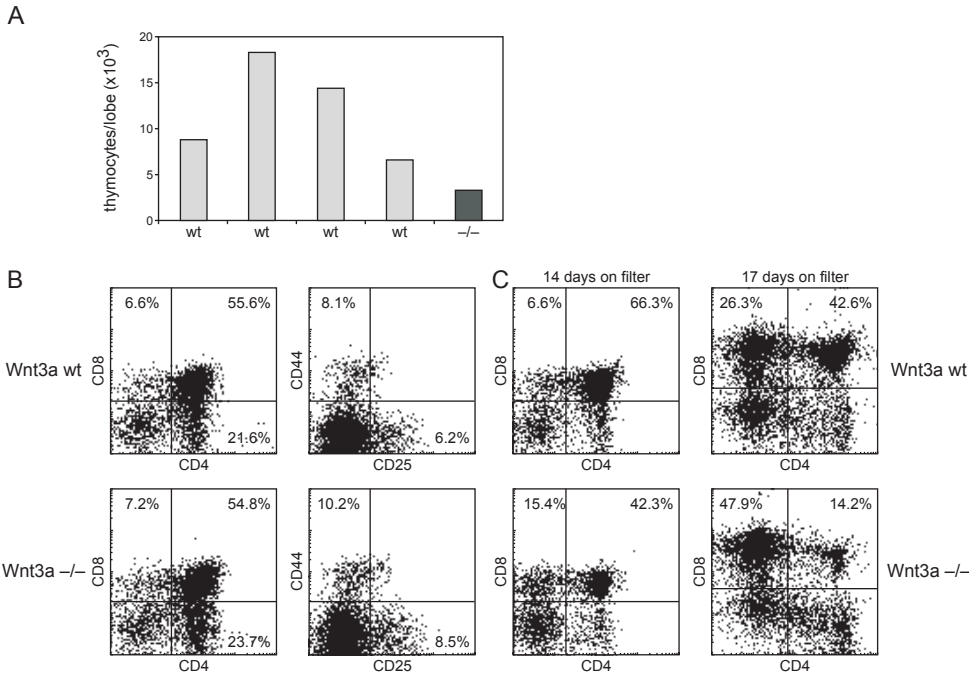

Figure 2. Wnt3a deficiency affects fetal T-cell development.
E12 thymic lobes from wild type and Wnt3a deficient E12 embryos were cultured in FTOC to allow T-cell development to proceed, and harvested at different time-points. (A) Total thymocyte numbers per thymic lobe after 13 days of culture. Similar results were obtained in all experiments, irrespective of the duration of culturing. (B) After 9 days of culture no differences between wild type and Wnt3a deficient lobes were observed. Thymocytes were stained for CD4 and CD8 to distinguish DN, DP and SP subsets (left panels) and within a $CD4^-CD8^-$ gate with CD25 and CD44 to distinguish DN1-4 subsets (right panels). (C) After 14 (left panels) and 17 days (right panels) on filter, Wnt3a deficient thymic lobes contain fewer DP cells.

E14 embryos. After 14-21 days of culture, lobes were stained for flow cytometry. No differences were observed between FTOCs using wild type or Wnt3a deficient fetal liver cells, with respect to cell numbers or thymocyte subset distribution (Figure 4). The reduced T-cell development in Wnt3a deficient embryos must therefore be attributed to absence of Wnt3a production by the thymic stroma.

Development of other lineages from Wnt3a deficient fetal liver cells

As Wnt3a has been shown to be a growth factor for pro-B cells when provided as a prototype Wnt *in vitro*[9], we were interested in the capacity of Wnt3a deficient fetal liver cells to develop into B cells. This would test the function of Wnt3a in a more physiological setting. Wnt3a deficient fetal liver cells were co-cultured with BM stromal cell lines Sys-

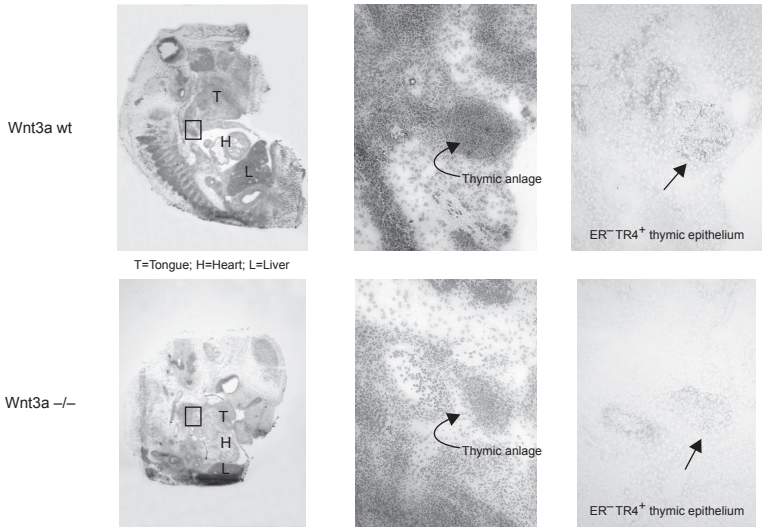

Figure 3. Normal thymic anlage in Wnt3a deficient embryos.
Tissue sections of wild type (left) and Wnt3a deficient embryos. Left panels: HE staining of complete embryos. T: tongue, H: heart, L: liver. The region of the thymic anlage is indicated and magnified in the middle panels (HE staining) and right panels (ER-TR4 antibody which stains thymic epithelium). Isotype control for ER-TR4 was completely blank. Stainings shown are representative for four wild type and four knock-out embryos examined.

1 and OP9. Both wild type and Wnt3a deficient fetal liver cells efficiently developed into CD19 positive B cell progenitors (Figure 5A). Most cells were in the pro-B cell stage (Figure 5A), but after prolonged culturing expression of intracellular IgM could be detected both in wild type and knock-out cultures (data not shown). Surprisingly, after 9 days of culturing, the percentage of $CD19^+$ cells was significantly higher in the Wnt3a deficient cultures (Figure 5A and B). This increase, however, was not reflected by an absolute increase in cell numbers. Rather it could be attributed to the marked reduction in a population of side-scatter-high cells, that on further examination appeared to be MAC1 positive myeloid cells (Figure 5B), suggesting that Wnt3a deficiency negatively effects myelopoiesis.

To further examine the effect of Wnt3a deficiency on myeloid development, we performed *in vitro* colony assays with E12 fetal liver cells. Wnt3a deficient fetal liver cells gave rise to significantly fewer myeloid colonies (Figure 5C), confirming our results on BM stromal cultures. Surprisingly, the effect on erythroid colony formation was even more striking: Wnt3a deficient fetal liver cells yielded six fold fewer erythroid colonies than wild type cells (Figure 5C).

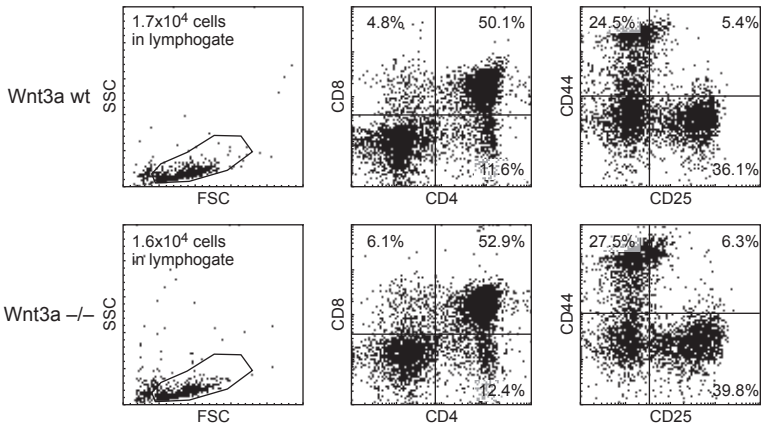

Figure 4. Normal T-cell development from Wnt3a deficient fetal liver stem cells.
Wild type (upper panels) and Wnt3a deficient (lower panels) E12 fetal liver cells were cultured in irradiated E14 wild type thymic lobes. After 14 days, lobes were harvested and analyzed using flow cytometry. FTOCs from wild type and Wnt3a deficient fetal liver cells yielded similar cell numbers (left), similar percentages of DN, DP and SP subsets (middle) and similar percentages of DN1-4 cells (right panels). Data are representative of five experiments.

Stem cell defect in Wnt3a deficient fetal liver

Differences in T-lymphoid, myeloid and erythroid development suggest that Wnt3a deficiency affects very immature hematopoietic lineage decisions and/or aspects of HSC differentiation or self-renewal. To investigate this, we analyzed hematopoietic progenitor cells in the fetal liver of wild type and Wnt3a deficient embryos. Total cell numbers and total percentage of hematopoietic cells (determined by CD45 expression) did not differ between wild type and Wnt3a deficient fetal livers (data not shown), nor did the percentage of lineage positive cells and the total population of c-Kit positive cells. However, the percentage of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells, which contain HSCs[34], was significantly reduced in Wnt3a KO fetal livers (Figure 6A and B).

This finding suggested that Wnt3a deficient embryos have a problem in the HSC compartment. To test whether stem cell function is defective in these embryos, we performed transplantations of Wnt3a deficient fetal liver cells into sublethally irradiated wild type mice. 10^6 fetal liver cells (pooled samples of three Wnt3a deficient or three wild type embryos) containing the CD45.2 congenic marker were intravenously injected into $CD45^{+}$ sublethally irradiated recipients. After five, eight and ten weeks, chimerism was analyzed in the blood of the recipient mice. In a number of mice, no donor cells were detected at any time point, probably due to failing intravenous injection. However, the proportion of mice in which no $CD45.2^{+}$ cells were detected was higher in mice

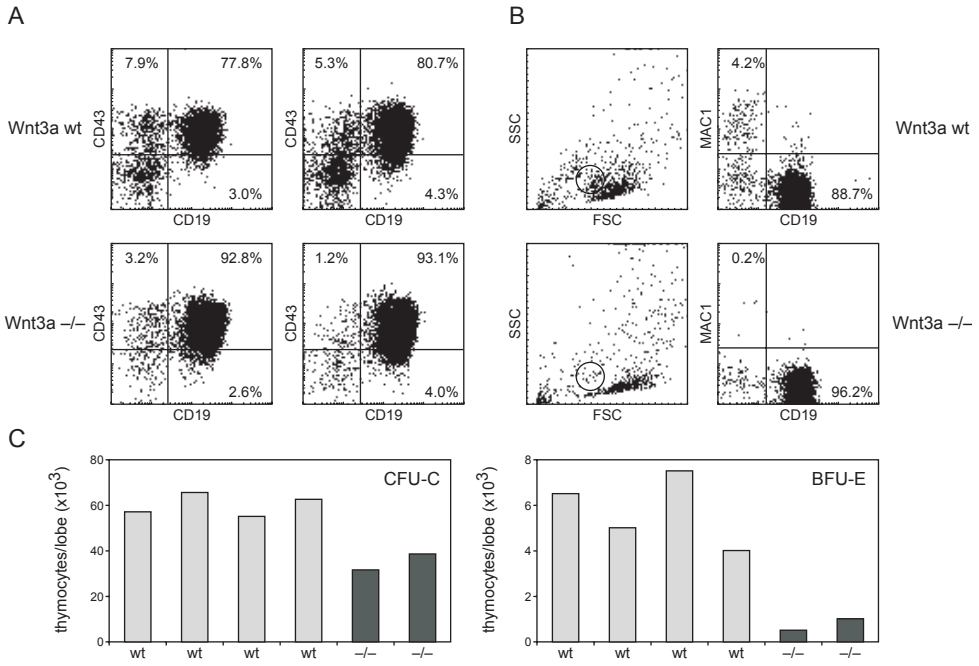

Figure 5. Decreased myelopoiesis from Wnt3a deficient fetal liver cells in BM stromal co-cultures.

Wild type and Wnt3a deficient E12 fetal liver cells were cultured for 7 (Figure A) or 9 (Figure B) days on confluent layers of OP9 BM stromal cells, which induces development in both myeloid and B-lymphoid lineages. (A) Wnt3a deficient fetal liver cells yield increased percentages of B cells. Most cells are $CD19^+CD43^+$ pro-B cells. (B) A population of side-scatter high cells is lacking in cultures from Wnt3a deficient fetal liver cells. These are $MAC1^+$ myeloid cells. (C) *In vitro* colony assays. Wild type and Wnt3a deficient E12 fetal liver cells were plated in semi-solid cultures containing appropriate cytokines for 10 days to generate myeloid (CFU-C) and erythroid (BFU-E) colonies. Numbers of colonies (derived from 50,000 cells for CFU-C and from 10,000 cells for BFU-E) from individual fetal livers are shown. For BFU-E, only large red colonies were counted. Results representative of two experiments.

transplanted with Wnt3a deficient fetal liver cells (two out of three) than in mice receiving wild type cells (two out of five) (Table I). Furthermore, the percentage of $CD45.2^+$ cells was much lower in the $Wnt3a^{-/-}$-transplanted mouse (0.2% after ten weeks) than in the wild type-transplanted mice (10-59%) (Table I). In a second experiment, in which a suboptimal number of fetal liver cells was injected, none of the six mice transplanted with Wnt3a deficient fetal liver cells showed any reconstitution, while four out of six mice transplanted with wild type fetal liver cells had a (small) population of $CD45.2^+$ cells in their blood, spleen, BM and thymus (data not shown). Together these results indicate that Wnt3a deficient fetal liver cells much less efficiently repopulate sublethally irradiated mice and confirm a likely stem cell defect caused by Wnt3a deficiency.

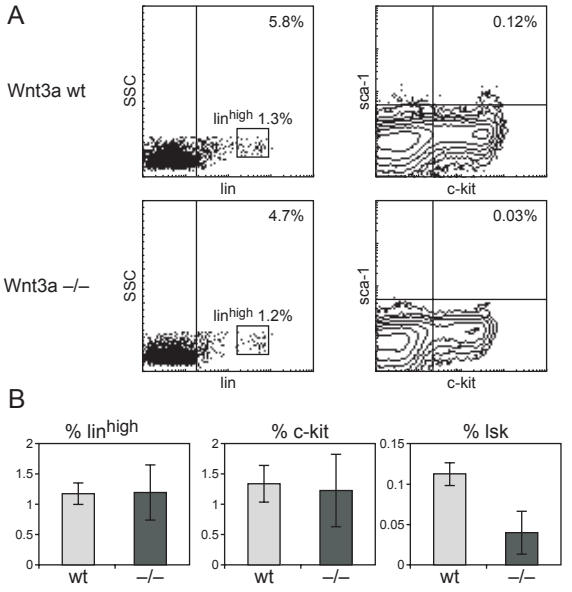

Figure 6. Decreased HSCs in Wnt3a deficient fetal livers.
(A) Flow-cytometric analysis of wild type (upper panels) and Wnt3a deficient (lower panels) E12 fetal liver cells. Cells were stained for lineage markers (left panels) and for c-Kit and Sca-1 within the Lin^- gate (right panels). The $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ (LSK) population is assumed to contain HSCs. (B) Percentage of cells highly positive for lineage markers, $c\text{-}Kit^+$ cells and LSK cells within fetal livers. Mean and SD of 7 wild type and 3 Wnt3a deficient fetal livers.

Table I. Chimerism after transplantation.

mouse transplanted with	% CD45.2 cells
wt fetal liver	53.7
wt fetal liver	58.7
wt fetal liver	10.2
wt fetal liver	0.0
wt fetal liver	0.0
$Wnt3a^{-/-}$ fetal liver	0.2
$Wnt3a^{-/-}$ fetal liver	0.0
$Wnt3a^{-/-}$ fetal liver	0.0
not transplanted	0.0

Percentage of CD45.2 (Ly5.2) positive cells in the blood, 10 weeks after transplantation with wild type or Wnt3a deficient E12 fetal liver cells.

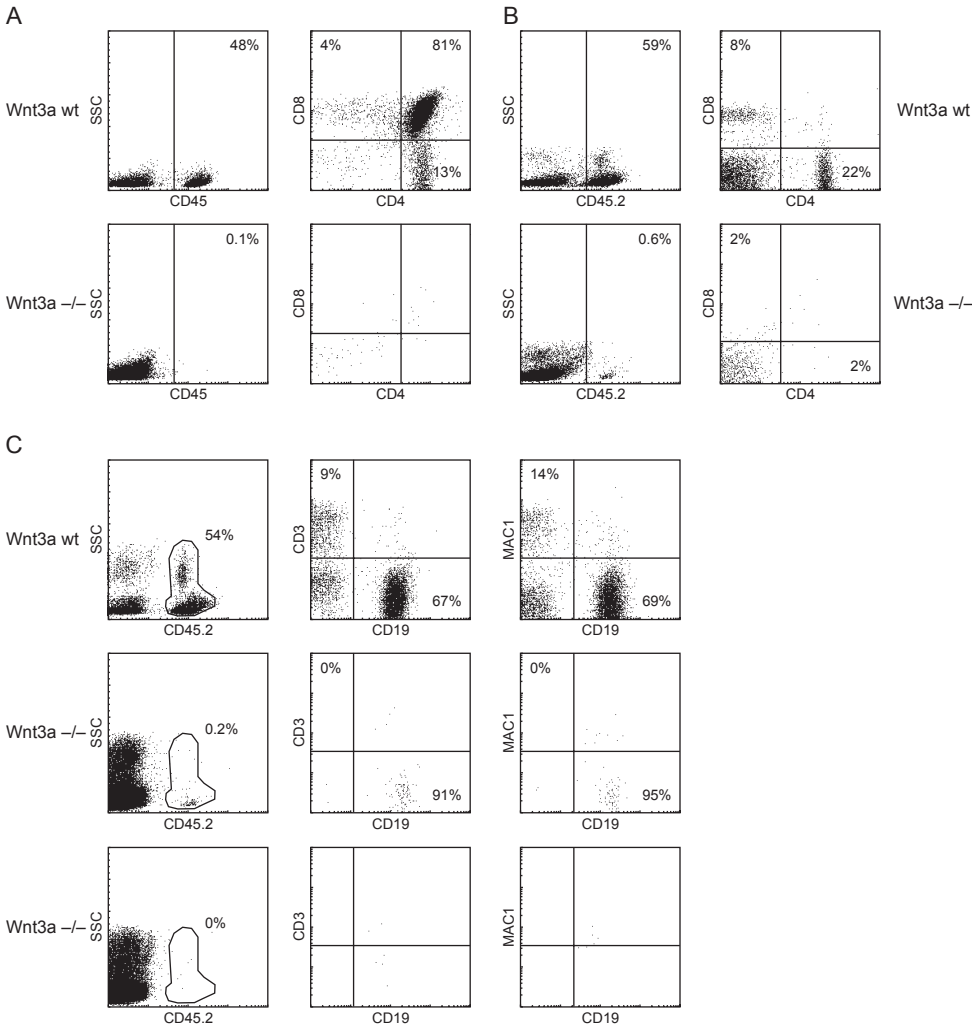

Figure 7. Repopulation of sublethally irradiated mice.
Results shown are from the one mouse transplanted with Wnt3a deficient fetal liver cells and one out of three mice reconstituted with wild type fetal liver cells that showed reconstitution with donor ($CD45.2^+$) cells. (A) Reconstitution of thymus. Left: CD45.2 staining, right: T-cell development within the $CD45.2^+$ gate. (B) Reconstitution of spleen. Left CD45.2 staining, right: mature $CD4^+$ and $CD8^+$ T cells within the $CD45.2^+$ gate. (C) Reconstitution of blood. Left: CD45.2 staining, middle: B cells and T cells, right: B cells and myeloid cells.

Reconstitution of hematopoietic lineages

To monitor the ability of Wnt3a deficient fetal liver cells to reconstitute different hematopoietic lineages, we analyzed the thymus, spleen, BM and blood of transplanted mice. While wild type fetal liver cells efficiently repopulated the thymus of sublethally irradiated mice (15, 48 and 80% of thymocytes of donor origin), no CD45.2$^+$ cells could be detected in the thymus of mice transplanted with Wnt3a deficient fetal liver cells (Figure 7A). The absence of thymocyte development was reflected by the complete lack of CD45.2$^+$ mature CD4$^+$ and CD8$^+$ T cells in the spleen (Figure 7B).

In accordance with the *in vitro* cultures of fetal liver cells on BM stroma, much higher percentages of B cells were detected within CD45.2$^+$ population in blood, spleen and BM of mice transplanted with Wnt3a deficient fetal liver cells (Figure 7C, only blood is shown). Again, this increased percentage of B cells was caused by the absence of other populations, most noteworthy a complete lack of side scatter^{high}, MAC1$^+$ myeloid cells (Figure 7C). These results confirm that Wnt3a deficiency leads to defects in myelopoiesis.

Due too very low CD45.2$^+$ cell numbers in the mouse transplanted with Wnt3a deficient cells, small cell populations (e.g. LSK cells and myeloid subsets) in BM could not be reliably analyzed (data not shown).

DISCUSSION

Despite a large body of evidence pointing towards an important role for Wnt signaling during the differentiation of various hematopoietic lineages, little genetic evidence from specific Wnt knock-out mice exists to support this hypothesis. As Wnt3a is the only Wnt available as recombinant protein, it is commonly used in *in vitro* studies and has been shown to be a proliferative factor for HSCs and thymocytes[15,19,21]. In this report we aimed to elucidate the *in vivo* functions of Wnt3a in hematopoiesis by studying Wnt3a deficient mice.

We found that the thymic defects resulting from Wnt3a deficiency were relatively mild. In all embryos surviving until E12, a structure consisting of thymic epithelium could be detected by immunohistochemistry. This organ appeared to be seeded by hematopoietic cells, which on further culturing were able to develop into all thymocyte subsets.

However, lower thymocyte numbers were detected in most cultures of Wnt3a deficient thymi, in accordance with a proliferative role for Wnt proteins in the most immature thymocyte subsets (Chapter 5, references 12, 14). The decreased cell numbers in Wn3a deficient thymi are reminiscent of both Tcf1 knock-out mice and Wnt1xWnt4 double deficient mice[6,7,14]. Wnt1xWnt4 deficiency resulted in a 50-70% decrease in thymocyte

numbers in E15-16 fetuses[14]. The only partially reduced cellularity in both Wnt1xWnt4 and Wnt3a deficient mice, as opposed to the complete absence of mature thymocytes in the Tcf1/Lef1 double knock-out[10], illustrates the high functional redundancy of different Wnt proteins in the thymus. Apart from Wnt1, Wnt4 and Wnt3a, we have shown high expression of Wnt5b, Wnt10a and Wnt10b in the murine thymus (Chapter 5).

While the Wnt1xWnt4 double deficient mice showed no defects in the pattern of T-cell development, we found a reduction in DP cells in Wnt3a deficient thymi, mainly after prolonged culturing. This is very similar to the phenotype of Tcf1 knock-out mice, in which defective T-cell development became more pronounced as mice aged[7]. The decline in DP cells observed in Tcf1 deficient mice is presumably caused by decreased proliferation at the DN4 and ISP stages.

The defects in T-cell development were mainly caused by the absence of Wnt3a in the thymic stroma, as fetal liver cells cultured in wild type lobes developed identical to wild type cells. This finding fits with the expression pattern of Wnt3a, with high mRNA levels in fetal thymic lobes already at E13 (Chapter 5), at which day stroma still is the main constituent of the lobes. Furthermore we have shown expression of Wnt3a in adult thymic stroma but not in thymocytes (Chapter 5).

In contrast to the FTOC experiments, Wnt3a deficient fetal liver cells transplanted into sublethally irradiated animals did not yield any T cells at all, indicating a cell-autonomous defect. The seemingly contradictory results of the cultures and *in vivo* experiments can be explained by the large differences between both assays. For FTOC, large numbers of progenitor cells are directly transferred into an environment which strongly induces T-cell development and in which no competitor cells are present, whereas for reconstitution of sublethally irradiated mice, cells have to home to the BM, proliferate, differentiate into progenitors that are able to seed the thymus, leave the BM and migrate to the thymus. As only very few cells home to the thymus, massive expansion of the earliest thymocytes is crucial. Wnt-driven proliferation (and possibly Wnt-induced adhesion molecules necessary for homing[15]) may be required at several of these steps and the absence of thymocytes and T-cells in the transplanted mice probably is the result of these cumulative defects.

Wnt3a has been shown to be a growth factor for pro-B cells *in vitro*[9], but *in vivo* evidence for a function for Wnt3a in B cells is lacking. As B220 positive cells were not yet present in E12 fetal livers of both wild type and Wnt3a deficient embryos, we studied B lymphopoiesis from fetal liver stem cells. We found markedly reduced numbers of B cells after *in vivo* transplantation of Wnt3a deficient fetal liver cells, but in contrast to T and myeloid cells, B cells were not completely absent. In stromal cultures, B cells appeared not to be affected at all. The mildly decreased B cell numbers in the BM of both the Lef1 knock-out and the Frizzled 9 knock-out mouse suggest a role for Wnt signaling in B-cell development[9,18], but other Wnts than Wnt3a are probably more

important. We could not detect expression of Wnt3a in total adult BM or in BM stromal lines (data not shown), although expression of Wnt3a in BM cells (but not stroma) has been reported[9].

Myeloid populations were strongly decreased, both in stromal cultures and colony assays, and appeared to be absent after transplantation. These findings suggested a possible role for Wnt signaling in myelopoiesis. Thus far, no experimental evidence exists for a role of Wnt proteins in hematopoietic compartments other than T and B lymphocytes and HSCs. However, elevated expression of plakoglobin has been reported in acute myeloid leukemia (AML)[35] and increased Wnt signaling during blast crisis in chronic myeloid leukemia (CML)[36], suggesting a role for Wnts in (subsets of) myeloid cells as well. The question remains whether Wnt3a deficiency causes myeloid alterations by affecting a myeloid progenitor cell, or rather by influencing HSC differentiation or self-renewal.

The reduced *in vivo* repopulation and the fact that multiple lineages were affected by Wnt3a deficiency, including severely reduced erythroid development, suggested a stem cell problem to us. Indeed we found a significant reduction of LSK cells in Wnt3a deficient fetal livers. LSK cells are heterogeneous, containing true HSCs as well as downstream populations of multipotent progenitors that have lost the capacity to self-renew[34]. Reduced numbers of HSCs or downstream precursors containing myeloid and erythroid potential may explain the reduced myeloid and erythroid development observed in Wnt3a deficient embryos.

A role for Wnt signaling in stem cell self-renewal and inhibition of differentiation has been reported not only for HSCs[19,20], but also for embryonic stem cells[29] and stem cells in the gut[37], skin[38] and neuronal system[39,40]. Interestingly, the markedly reduced reconstitution of sublethally irradiated animals by Wnt3a deficient fetal liver cells indicates autocrine production of Wnt3a by progenitor cells. This is in contradiction with the often-assumed function of Wnts as being growth factors secreted by the microenvironment. Using quantitative PCR we could not detect Wnt3a transcripts in total E14 fetal liver, but this does not exclude Wnt3a expression in purified progenitor populations.

We conclude that Wnt3a deficiency leads to a mild thymic phenotype, no defects in B-cell development, severe defects in myelopoiesis and erythropoiesis and a marked decrease in LSK cell numbers. For the first time we demonstrate a physiological function for a specific Wnt in HSC biology. Although further studies are necessary to examine the exact identity of the decreased progenitor populations and the extend and functional consequences of their reduction, these results are highly encouraging for studies into the use of Wnt3a for clinical purposes. For instance, Wnt proteins could possibly be used to expand HSCs *in vitro* before they are genetically altered and transferred back into the patient during gene therapy. Additionally, Wnts could be used *in vivo* to aid the reconstitution of the immune system of patients after BM transplantation.

MATERIALS AND METHODS

Mice

All animal experimentation was done in accordance with legal regulations in The Netherlands, which include approval by the local ethical committee. Mice were kept in the animal facility of the Erasmus MC. C57Bl/6 mice were obtained from Harlan, C57Bl/6-CD45.1 (Ly5.1) mice from the Jackson Laboratory. Mice heterozygous for the Wnt3a mutation were kindly provided by T. Yamaguchi (National Cancer Institute-Frederick, Frederick, MD). Genotyping was performed as described[24].

Tissues and cells

Mice heterozygous for the Wnt3a mutation were bred and embryos of gestational day 12 (E12) were collected. A tissue sample of each embryo was used for genotyping. Fetal thymic lobes and fetal livers were isolated. Thymic lobes were cultured on a filter (Whatman) floating in medium for 9-17 days. Fetal livers were passed over a filter to obtain a single-cell suspension. Cells were either frozen down until further use or directly used for flow-cytometric analysis or culture experiments.

E14 fetal thymic lobes from normal C57Bl/6 mice were isolated and irradiated at 14.4 Gy using a γ-source.

Immunohistochemistry

6 µm tissue sections of frozen E12 embryos were fixed in acetone. For visualization of the different organ structures, sections were stained with haematoxylin-eosin (HE). To envisage thymic epithelium, sections were incubated with concentrated supernatant from a hybridoma producing ER-TR4 antibody[33] followed by a biotinylated rabbit anti-rat antibody (DakoCytomation) and finally with avidin-biotin complex-horseradish peroxidase (DakoCytomation). Sections were incubated with 3-amino-9-ethylcarbazole (AEC) substrate (DakoCytomation) to detect peroxidase.

Fetal thymic organ culture (FTOC) using fetal liver cells

Fetal liver cells were cultured for 24-48 hrs in RPMI containing 10% fetal calf serum, 10 ng/µl recombinant murine Interleukin 7 (IL-7) and 50 ng/µl recombinant murine Stem Cell Factor (SCF) (both from R&D), after which 50,000 cells were transferred into irradiated E14 fetal thymic lobes using the hanging drop method[41]. After 24-72 hours, the lobes were transferred to a filter (Whatman) floating on medium. After 14-21 days of culturing, cells were harvested and stained for flow cytometry with CD25-FITC, CD4-PE, CD8-PerCP and CD44-APC antibodies (all from BD Pharmingen). All flow-cytometric analyses were performed using a FACSCalibur flow cytometer (BD Biosciences) and Cell Quest Pro software.

BM stroma co-cultures of fetal liver cells

Fetal liver cells were cultured for 24-48 hrs in RPMI containing IL-7 and SCF. 10,000-50,000 cells were cultured in 12 wells plates on confluent monolayers of BM stromal cell lines, either Sys-1 or OP9 in the presence of IL-7 and SCF. Medium and cytokines were refreshed every 3 days.

After 7-16 days cells were harvested and stained for flow cytometry using the following antibodies: CD45-FITC, B220-FITC, CD11c-FITC, CD19-PE, CD43-PE, IgM-PE, GR1-PE, TER119-PE, B220-APC, CD19-APC, MAC1-APC (all from BD Pharmingen) and IgM-FITC (H chain specific, from Jackson ImmunoResearch).

***In vitro* colony assays**

Fetal liver cells were cultured over night with IL-7 and SCF and assayed for the presence of granuloctye-macrophage colony-forming units (CFU-C) and erythroid burst-forming units (BFU-E) by *in vitro* colony formation in viscous methylcellulose culture medium containing cytokines: for BFU-E 4U/ml human erythropoietin (EPO, Behringwerke AG) and 100 ng/ml murine SCF (R&D); for CFU-C 30 ng/ml murine IL-3, 100 ng/ml SCF and colony stimulating factor (CSF, 300x dilution of ConA adsorbed fraction

of pregnant mouse uterus extract). 10,000 and 50,000 cells were plated in duplicate dishes. The number of colonies was determined after 10 days of culture.

Analysis of progenitor populations in fetal liver

Single cell suspensions of fetal liver cells were prepared and directly stained with Sca-1-FITC, B220-PE, MAC1-PE, GR1-PE, TER-119-PE and c-Kit-APC (all from BD Pharmingen) and analyzed using flow cytometry.

Fetal liver transplantations

8-11 week-old C57Bl/6 mice harboring the CD45.1 (Ly5.1) allele as a congenic marker, received a sublethal dose of 6 Gy total body irradiation. Mice were intravenously injected with 10^6 freshly isolated fetal liver cells ($CD45.2^+$) in 250 µl of PBS or remained untransplanted as negative control. In a second experiment, $2.5x10^5$ thawed fetal liver cells were transplanted. To prevent infections, mice received 100 mg/l ciprofloxacin (Bayer) in their drinking water.

Five, eight and ten weeks after transplantation, blood was drawn and analyzed for cells expressing the CD45.2 marker. Ten weeks after transplantation, mice were killed and BM, spleen and thymus were isolated. In each organ, total cell numbers were determined and the percentage of $CD45.2^+$ cells and their subset distribution was assayed by flow cytometry using the following antibodies: CD45.2-FITC, CD25-FITC, IgM-FITC, CD11c-FITC, CD4-PE, TCRγδ-PE, TER119-PE, Sca-1-PE, CD45.2-PerCP, CD8-PerCP, MAC1-PerCP, Lin-PerCP (CD3-biotin, B220-PerCP, NK1.1-biotin, MAC1-PerCP, GR1-biotin, TER119-biotin, Streptavidin-PerCP), CD3-APC, CD44-APC, CD19-APC, MAC1-APC, c-Kit-APC, NK1.1-biotin, and Streptavidin-APC (all from BD Pharmingen). Cell samples of non-transplanted mice were stained as negative controls.

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IDENTIFICATION OF NOTCH TARGET GENES IN HUMAN T-CELL DEVELOPMENT: NO DIRECT INDUCTION OF A T-CELL SPECIFIC GENE PROGRAM

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To be submitted

INTRODUCTION

Of the many signals that are involved in T-cell development, Notch signaling is probably most conspicuous[1]. The Notch signal transduction pathway is an evolutionary conserved mechanism that regulates cell fate determination during developmental processes[2]. Four family members of the transmembrane Notch receptor are recognized, named Notch1 to 4. Signaling is initiated when the large extracellular domain of the Notch receptor binds a membrane-bound ligand on a neighboring cell. The five Notch ligands in mammals are Delta1, 3 and 4 and Jagged1 and 2. Binding of a ligand induces proteolytic cleavage of the intracellular part of the Notch protein (IC-Notch), which translocates to the nucleus and binds to the transcription factor CSL (in human referred to as CBF1, in mouse as RBP-Jκ), activating transcription of Notch target genes[3].

T cells develop from multipotent progenitor cells that seed the thymus from the bone marrow or fetal liver[1]. The thymus is rich in expression of Notch ligands[4] and after entering the thymic microenvironment, progenitors immediately start expressing Notch target genes[5]. These newly generated thymocytes do not express CD4 and CD8 and are therefore called double negative (DN). In humans, these most immature DN thymocytes are characterized as $CD34^+CD1a^-CD38^-$ and are homologous to murine DN1 cells[6]. In the next DN stage, thymocytes are $CD34^+CD1a^-CD38^+$ (DN2). This subset still contains (at the population level) potential to develop into all hematopoietic lineages (Chapter 4). In the subsequent stage ($CD34^+CD1a^+$, DN3), thymocytes become irreversibly committed to the T-cell lineage[6]. Finally, thymocytes lose expression of CD34 and proceed into the immature single positive (ISP) stage.

The importance of Notch signaling for the induction of a T-cell fate was first demonstrated in mice in which the Notch1 gene was conditionally deleted, resulting in a complete block in T-cell development at the DN1 stage[7] and the emergence of ectopic B-cell development in the thymus[8]. Conversely, overexpression of IC-Notch in the bone marrow instructed a T-cell fate in bone marrow progenitors and inhibited B-cell development[9]. The same effect ensued when Delta4 was overexpressed in the bone marrow[10]. *In vitro*, this phenomenon can be mimicked by expression of Delta1, but not Jagged1, in bone marrow stromal cell lines. Using this co-culture system, T cell precursors can be efficiently generated from murine and human hematopoietic progenitors[11-14].

Functions of Notch in later stages of T-cell development and in other hematopoietic compartments have been reported as well and are reviewed elsewhere[15,16].

To develop into a T cell, Notch signaling is clearly indispensable, but the downstream mechanisms by which a Notch signal is translated into a T-cell program are still largely unclear. The best known Notch target genes are Hairy-Enhancer of Split (*HES*)*1* and *HES5* and Hes-related repressor protein (*HERP*). Hes and Herp are basic-helix-loop-helix

(bHLH) proteins that function as transcriptional repressors[17,18]. Indeed overexpression of Hes1 and Hes5 in the bone marrow partly inhibits B-cell development[19]. But although proliferation of early thymocytes is severely affected by Hes1 deficiency[20,21], thymocytes still develop in these mice. Hes1 can therefore not be the sole target of Notch signaling responsible for inducing a T-cell fate.

PTCRA (the gene for pre-T cell receptor α (pTα)) was found as a Notch target using Representational Difference Analysis (RDA) in murine thymoma cell lines with or without retroviral transduction of IC-Notch[22]. The pTα promoter was demonstrated to contain a CSL binding site and could be activated by IC-Notch *in vitro*[23]. Nevertheless, the physiological role of Notch signaling for pTα expression during T-cell development remains controversial, as pTα expression was not affected in mice in which Notch1 was conditionally deleted from the DN3 stage onwards[24]. A role for Notch1 in opening of the T-cell receptor-β (*TCRB*) locus has been shown, but only for the complete *TCRB* rearrangements (V to DJ)[24,25].

Other Notch target genes identified in the thymoma cell line were *DTX1* (gene for Deltex1), *Ifi-202*, *Ifi-204*, *Ifi-D3*, *ADAM19* (Meltrinβ)[22]. A number of other genes have been reported as being Notch targets, including *NOTCH1* itself[26], *NRARP* in Xenopus embryos[27], *BCL2* in thymoma cells[28], *CCND1* (gene for cyclin D1) in a kidney cell line[29], *CDKN1A* (gene for cyclin-dependent kinase inhibitor 1A (p21, Cip1)) in keratinocytes[30] and *TCF3* (gene for E2A)[9,31]. It is unknown whether these genes are *in vivo* targets in the earliest thymocytes and whether they function in T-cell commitment.

In this study we investigated the downstream mechanisms of Notch signaling in human hematopoietic progenitor cells. We induced Notch signaling using two different methods and studied the transcription of known and newly recognized Notch target genes. Up- or downregulation of genes upon Notch signaling was compared to expression levels in the relevant thymocyte subpopulations, to examine whether these genes have a physiological role in T-cell commitment. We demonstrate that Notch signaling by itself does not induce a T-cell program, but we found several novel Notch target genes, some of which we have tested for their contribution to T-cell development.

RESULTS

Induction of Notch signaling

To identify target genes of Notch signaling, gene expression profiles were generated of human hematopoietic progenitors that did or did not undergo Notch signaling. We provided Notch signals in two different ways: by transfection of IC-Notch or by co-culturing cells on Delta1 expressing stroma.

A strong Notch signal was induced by transfecting $CD34^+$ cells from human cord

blood (UCB) with a truncated Notch gene, encoding the intracellular, constitutively active part of the Notch protein (IC-Notch). It has been shown previously that human CD34$^+$ cells expressing IC-Notch develop into T/NK progenitors when co-cultured on bone marrow stroma[32]. CD34$^+$ UCB cells were transfected with an expression vector containing the *IC-Notch* gene or as a control with the empty expression vector. A separate GFP vector was co-transfected in 1/10 concentration in order to monitor transfection efficiency. Six hours after transfection, the percentage of GFP expressing cells was already high and comparable between IC-Notch transfected and control cells (Figure 1). The percentage of cells positive for the *IC-NOTCH* gene was probably even higher, as 88% of cells transfected with the same DNA amount of the GFP vector only were positive for GFP (data not shown). These results suggested that the *IC-NOTCH* gene was also expressed at this time point. 24 hours after transfection GFP expression had slightly decreased (Figure 1).

Another way to induce the Notch signaling cascade is to allow progenitor cells to interact with their cognate ligand responsible for inducing T-cell development. To this end, human CD34$^+$ UCB cells were cultured on the bone marrow stromal cell line S17, which was retrovirally transduced with either human Delta1 in combination with GFP (S17-DL) or with GFP only (S17-GFP)[11]. Other groups have shown that CD34$^+$ UCB cells cultured

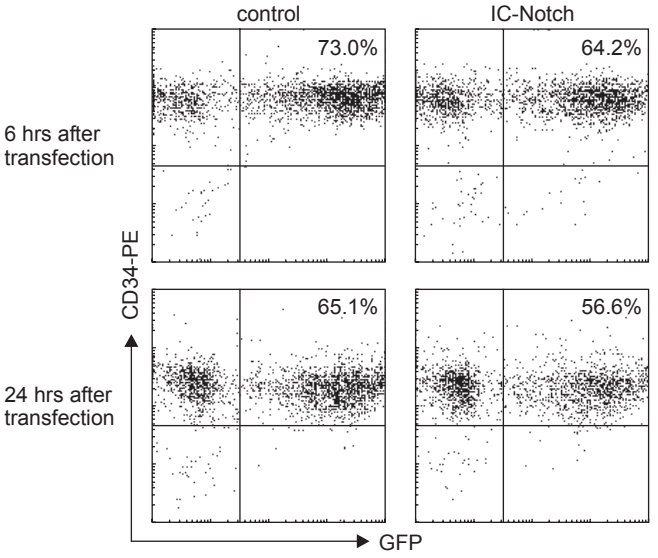


Figure 1. Transfection of hematopoietic progenitor cells.
CD34$^+$ progenitors from human UCB were transfected with a control vector (left panels) or a construct containing the gene for constitutively active Notch (IC-Notch, right panels), together with a GFP vector. After 6 hours (upper panels) and 24 hours (lower panels) the percentage of GFP$^+$ cells was determined using flow cytometry.

in this and similar systems develop into double positive (DP) thymocytes[11,14] and also in our hands CD34$^+$ progenitor cells efficiently developed into CD7$^+$ T/NK precursors cells after 2 weeks of culturing on S17-DL (data not shown). CD34$^+$ progenitor cells were cultured on S17-DL for 3, 6 and 24 hours, as kinetics of translocation of Notch-IC to the nucleus and induction of target RNA transcription are unknown.

From all samples RNA was isolated and gene expression profiles were generated using Affymetrix technology. Pair-wise comparisons were made between samples with or without Notch signaling. Numbers of significantly differentially expressed genes (≥ 2 fold difference, $p<0.003$) are listed in Table I. Both up- and downregulated genes were found, indicating that Notch functions as an activator as well as a repressor of transcription in hematopoietic progenitors.

Expression profiles of human thymocyte subsets have been previously generated and described in detail[6]. The differential expression of genes induced by Notch signaling in hematopoietic progenitors was compared to the relevant UCB and thymocyte subsets: CD34$^+$ UCB versus DN1 (CD34$^+$CD1a$^-$CD38$^-$) and DN1 versus DN2 (CD34$^+$CD1a$^-$CD38$^+$). DN1 cells have just entered the thymus and are therefore expected to upregulate Notch target genes[5]. The DN2 cells should also express Notch target genes, as they have contacted Notch ligands for prolonged periods and as Notch-ligand interactions are necessary throughout the DN1 and 2 stage for irreversible T-cell commitment[33]. In addition, during the DN2 stage, commitment to the T-cell lineage is initiated and cells start rearranging T-cell receptor β (*TCRB*) genes[6].

Expression of previously reported Notch target genes

Comparison files were mined for the expression of known Notch target genes, to determine whether these changed after Notch activation in hematopoietic progenitors as well. In all samples in which Notch signaling was activated, transcription of *HES1*, the universal Notch target[2], was clearly increased (Figure 2A). This was most pronounced in the progenitor cells transfected with IC-Notch for 6 hours, reflecting the strong Notch signal induced in this way. 24 hours after transfection, *HES1* was still upregulated, but to a lesser extend. This might indicate negative feed-back mechanisms that are initiated after receiving a strong Notch signal. Alternatively it may reflect the fact that transfection

Table I. Numbers of significantly up- or downregulated genes (≥2 fold, p<0.003).

	6 hr ICN[1]	24 hr ICN[1]	3 hr S17-DL[2]	6 hr S17-DL[2]	24 hr S17-DL[2]
U133A array	70↑ 12↓	17↑ 44↓	10↑ 87↓	13↑ 14↓	137↑ 162↓
U133B array	35↑ 5↓	13↑ 0↓	ND[3]	ND[3]	ND[3]

[1]ICN: progenitor cells from human UCB transfected with pcDNA-IC-Notch versus control pcDNA.
[2]S17-DL: progenitor cells from human UCB cultured on S17-DL versus S17-GFP.
[3]ND: microarray not performed.

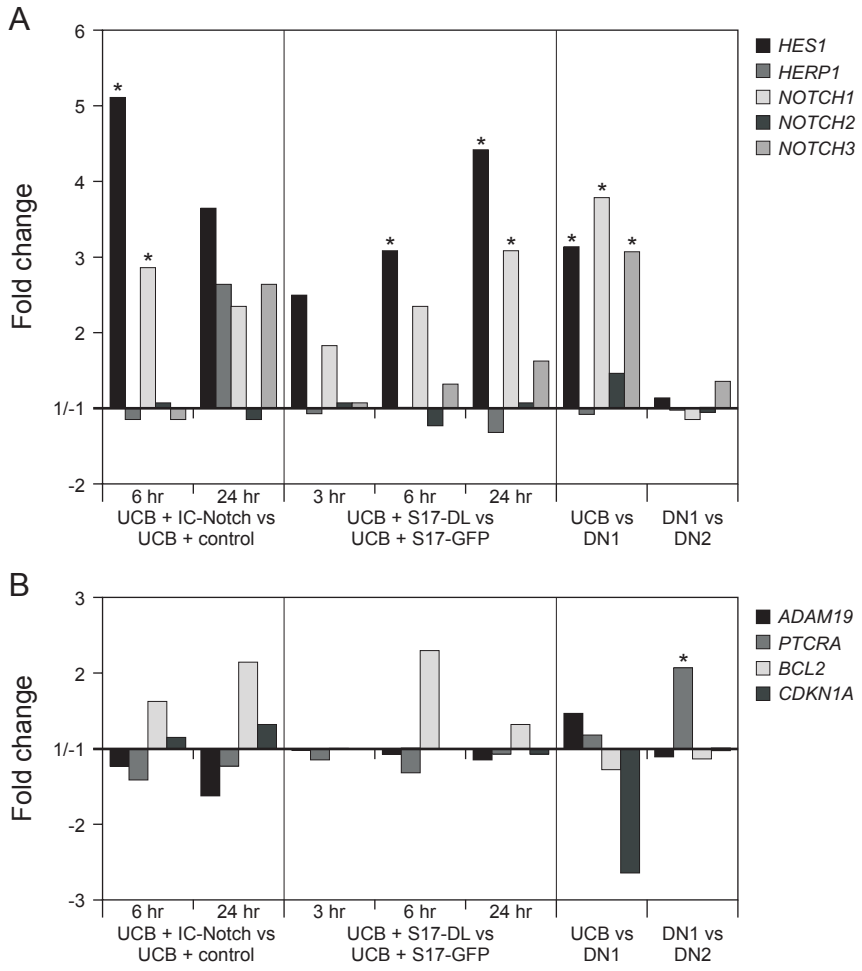


Figure 2. Differential expression of putative Notch target genes.
(A and B) Fold changes in expression levels extracted from gene expression profiles generated from hematopoietic progenitor cells with or without induced Notch signaling, as well as previously performed gene expression profiles from human UCB and thymocyte subsets[6]. Left: comparisons of progenitors transfected with pcDNA-IC-Notch versus control pcDNA. Middle: comparisons of progenitors cultured on S17-DL versus S17-GFP. Right: comparisons of $CD34^+$ UCB cells versus $CD34^+CD1a^-CD38^-$ (DN1) thymocytes and comparisons of $CD34^+CD1a^-CD38^-$ (DN1) thymocytes versus $CD34^+CD1a^-CD38^+$ (DN2) thymocytes. Asterisks indicate a significant difference in expression levels (fold change >1.8, $p<0.003$).

was transient, consistent with the slightly diminished GFP expression found after 24 hours (Figure 1).

In the cells cultured on S17-DL, *HES1* expression was most prominent after 24 hours (Figure 2A). *HES1* was also highly upregulated in the DN1 thymocytes as compared to $CD34^+$ UCB cells (Figure 2A), demonstrating that the artificially induced Notch signals in our experiments mimic the relevant processes *in vivo*.

HERP1 and *2* were not expressed in any of the samples, neither before nor after inducing a Notch signal (Figure 2A). *NOTCH1* itself is also a Notch target[26] and was found to be significantly upregulated after 6 hours of transfection and after 24 hours of culturing on S17-DL (Figure 2A). This was reflected by high expression in the DN1 subset as compared to UCB (Figure 2A). Also *NOTCH3* was transcribed at high levels in the DN1 cells as compared to UCB, but Notch signaling *in vitro* induced *NOTCH3* transcription to a lesser extend and with slower kinetics than *NOTCH1* (Figure 2A). *NOTCH2* (Figure 2A) and *NOTCH4* (not shown) were not differentially expressed in any of the samples.

Of the other previously reported Notch target genes mentioned above, only *NRARP* was significantly upregulated after induction of Notch signaling (3.5 fold increased 6 hours after transfection of IC-Notch (not shown)). The other genes were either not differentially expressed or not expressed at all (Figure 2B and data not shown), indicating that they are not Notch target genes in early hematopoietic progenitor cells. This finding is especially remarkable for Deltex1 and pTα, which are often assumed to be Notch targets during T-cell development[12,22,23]. In the thymocyte subsets, significantly increased mRNA expression of *PTCRA* was only found at the DN2 stage, but not at the DN1 stage (Figure 2B), while genuine Notch target genes (*HES1*, *NOTCH1*) were expressed already in DN1 thymocytes (Figure 2A). PTα may be a Notch target gene at later stages of T-cell development, but is not immediately upregulated when cells enter the thymus.

For *BCL2* a consistent, but not significant, trend of upregulation was observed in most comparisons, which was nevertheless not reflected in the thymocyte subsets (Figure 2B). Conversely, *CDKN1A* (p21) was significantly decreased in the DN1 subset, but did not show any differential expression after Notch activation (Figure 2B).

Notch does not induce a T-cell program

The mechanisms by which Notch signaling induces or promotes a T-cell fate still warrant elucidation. To investigate whether Notch signaling can trigger T-cell commitment by directly inducing TCR gene rearrangements or transcription of ‘classical’ T-cell specific genes, we mined our dataset for relevant genes.

No induction of *RAG1*, *RAG2* and *DNTT* (gene for TdT) and germline transcripts of *TCRB*, *TCRD* and *TCRG* were found after Notch signaling (Figure 3A and not shown),

suggesting that Notch has no direct function in opening of the TCR loci or the initiation of TCR gene rearrangements.

In addition, we found no differential expression (mostly no expression at all) of T-cell specific genes after activation of Notch signaling, while these genes were highly upregulated in the DN1 thymocytes (including *CD7* and *CD2*) or in the DN2 subset (including *CD1a*, *LAT* and *CD3γ*) (data not shown).

Next, we investigated differential expression of a number of transcription factors that are known to be important for T-cell development[1]. Tcf1 and GATA3 are among the first transcription factors to show up when a cell initiates a T-cell program[34], which we confirmed in our DN1 subset (Figure 3B). Although it has been suggested that Notch1 acts upstream of Tcf1 and GATA3[25], we did not find induction of these genes after Notch signaling (Figure 3B).

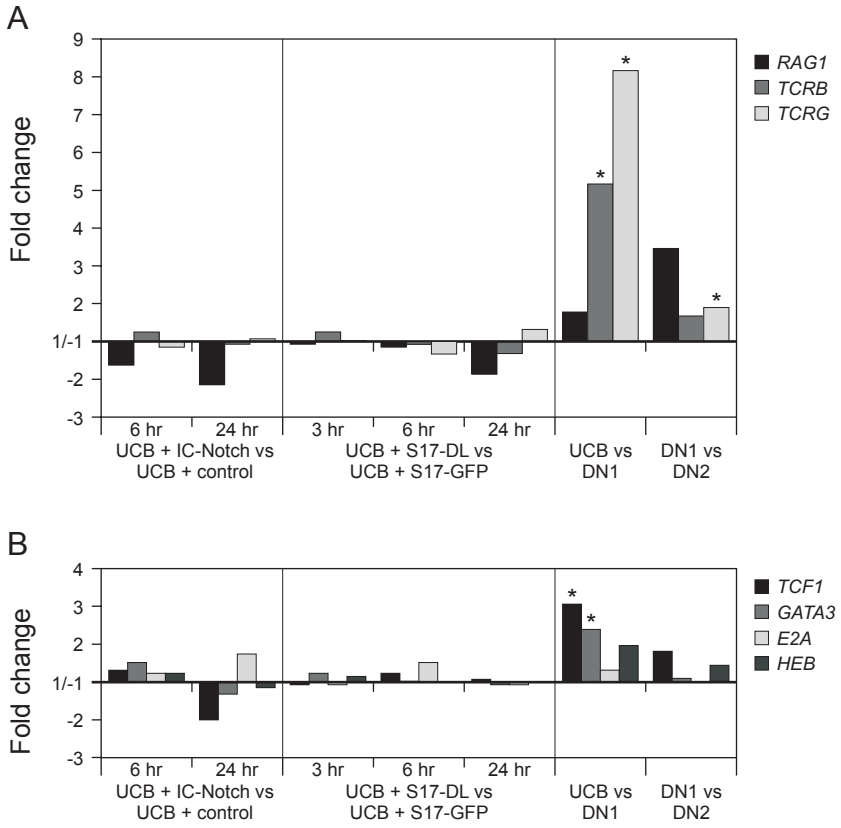


Figure 3. Differential expression of T-cell associated genes.
Determined by microarray analysis as indicated for Figure 2. (A) TCR gene rearrangement-associated genes. (B) Transcription factors involved in T-cell development.

Some reports have proposed that Notch inhibits B-cell development through inhibition of bHLH factor E2A[9,31], although E2A is clearly important for T-cell development as well, both as a homodimer and as dimerization partner for HEB[1]. In our experiments, we found no upregulation of either *E2A* or *HEB* after Notch signaling (Figure 3B).

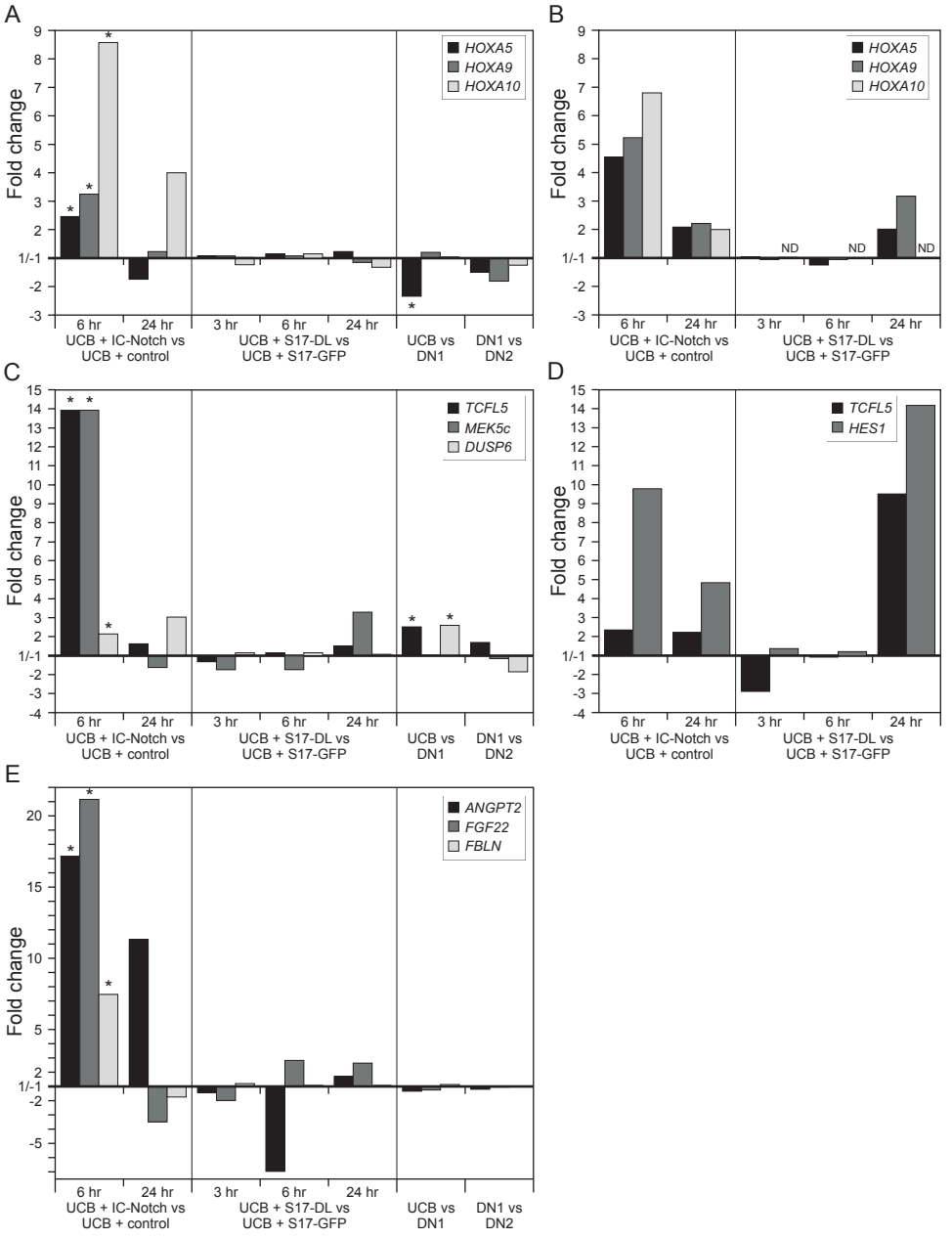
Novel Notch target genes in hematopoietic progenitors

Next to the known Notch target genes *HES1* and *NOTCH1*, we discovered significant differential expression of a number of previously unknown Notch target genes. Six hours after transfection with IC-Notch, high induction of *HOXA5*, *HOXA9*, *HOXA10* and to a lesser extend *HOXA7* mRNA was detected (Figure 4A and B). Upregulation was markedly decreased after 24 hours. In S17-DL cultures, a small increase in HOXA transcripts could be detected after 24 hours by RQ-PCR but not by microarray analysis (Figure 4A and B). *HOXA5*, *A9* and *A10* were previously shown to be expressed in the human thymus[35], but we did not observe upregulation of these genes in DN1 and DN2 thymocytes (Figure 4A).

Among the highest upregulated genes 6 hours after IC-Notch transfection were *TCFL5* (Transcription Factor Like 5), *MEK5c* and *DUSP6* (Dual-specificity phosphatase 6, also termed MAP kinase phosphatase 3) (Figure 4C). TCFL5, a transcription factor of the bHLH family, was first identified in spermatocytes[36,37]. Both MEK5c and Dusp6 can influence MAP kinase activity and interactions between the Notch and MAP kinase pathways have been described[38]. Our microarray results showed limited upregulation of *MEK5c* and *TCFL5* transcripts after 24 hours of culturing on S17-DL, while for unknown reasons RQ-PCR validation of *TCFL5* showed high induction in this condition as well (Figure 4D). *TCFL5* and *DUSP6* were significantly increased in the DN1 thymocytes as compared to $CD34^+$ UCB (Figure 4C), supporting a functional role of these genes in T-cell differentiation.

Lastly, we found high upregulation of a number of genes associated with vasculogenesis, including *ANGPT2* (gene for angiopoietin), *FGF22* and *FBLN* (gene for fibulin) (Figure 4E). These genes were detected only after transfection with IC-Notch and not after co-culture on S17-DL or in thymocyte subsets, suggesting that their induction is not related to thymic function, but rather to other biological processes. These vasculogenesis genes may be physiologically induced by Notch ligands other than Delta1, for instance by Jagged. Interestingly, Notch signaling has been implicated in vasculogenesis and angiogenesis[39-41]. Furthermore, hemangioblasts (bipotent

Figure 4. Differential expression of novel Notch target genes.
(A) HoxA transcripts determined by microarray analysis as indicated for Figure 2. (B) HoxA transcripts determined by RQ-PCR. ND: not detected. (C) TCFL5, MEK5c and Dusp6 transcripts determined by microarray analysis. (D) Hes1 and TCFL5 transcripts determined by RQ-PCR. (E) Transcription of angiogenesis genes determined by microarray analysis.


A
Fold change
HOXA5
HOXA9
HOXA10
6 hr
24 hr
3 hr
6 hr
24 hr
UCB + IC-Notch vs UCB + control
UCB + S17-DL vs UCB + S17-GFP
UCB vs DN1
DN1 vs DN2
B
ND
C
TCFL5
MEK5c
DUSP6
D
HES1
E
ANGPT2
FGF22
FBLN

progenitors for endothelial and hematopoietic cells) are present among $CD34^+$ UCB cells[42]. This intriguing finding warrants further research, but is beyond the scope of this report.

Functional validation of novel Notch target genes *TCFL5* and *HOXA5*

Perhaps the most striking novel Notch target genes identified in our studies were *TCFL5* and the *HOXA* genes. The relevance of these genes is substantiated by the finding of putative CSL binding sites upstream of *TCFL5* exon 1 and in the human *HOXA5-HOXA4* intergenic region (data not shown). Nevertheless, functional studies are necessary to understand the significance of TCFL5 and HoxA proteins for hematopoiesis and T-cell development in particular.

First we data-mined previously performed microarrays of all major thymocyte subsets[6] for the expression of *TCFL5*. We found a progressive increase in *TCFL5* mRNA from DN1 up to the early DP stage (Figure 5A). Levels sharply dropped when thymocytes started expressing CD3, suggesting that TCFL5 functions mainly during the early stages of T-cell development (Figure 5A).

To test whether TCFL5 is functionally important during T-cell development, we retrovirally expressed the *TCFL5* gene together with the *GFP* gene in human $CD34^+$ UCB hematopoietic progenitors. As a control, $CD34^+$ cells were transduced with the same construct containing only the *GFP* gene. After transduction, cells were cultured in fetal thymic organ cultures (FTOC) to allow them to differentiate into T cells. After 9 days in FTOC, thymic lobes were harvested and stained for flow-cytometric analysis. The TCFL5 transduced GFP^+ cells showed an acceleration of T-cell development: the percentage of DP cells was almost doubled as compared to control transduced GFP^+ and GFP^- cells and TCFL5 transduced GFP^- cells. (Figure 5B and data not shown). In addition, the percentage of $TCR\gamma\delta^+$ cells was markedly increased in the TCFL5 transduced GFP^+ cells (Figure 5C).

We found a remarkable increase in HoxA5, A9 and A10 in progenitor cells transfected with IC-Notch (Figure 4A), suggesting a role for these genes in hematopoiesis. Mice deficient for HoxA9 have previously been shown to display defects in myeloid, B-cell and T-cell development[43,44], while on the other hand overexpression of HoxA10 inhibited lymphoid development[45]. HoxA5 deficient mice were generated some years ago[46], but their hematopoietic system has not been studied. We now analyzed the bone marrow, spleen and thymus of HoxA5 knock-outs and heterozygous and wild type littermates of 2.5, 4 and 25 weeks old. No consistent abnormalities were detected in populations of B, T, NK and myeloid cells in the thymus, bone marrow and spleen of HoxA5 deficient mice (data not shown). As the thymic defects of HoxA9 deficient mice were much more pronounced in fetal mice than in adults, we also analyzed HoxA5 deficient embryos. We found normal T-cell development in fetal thymic lobes of HoxA5 deficient embryos and in

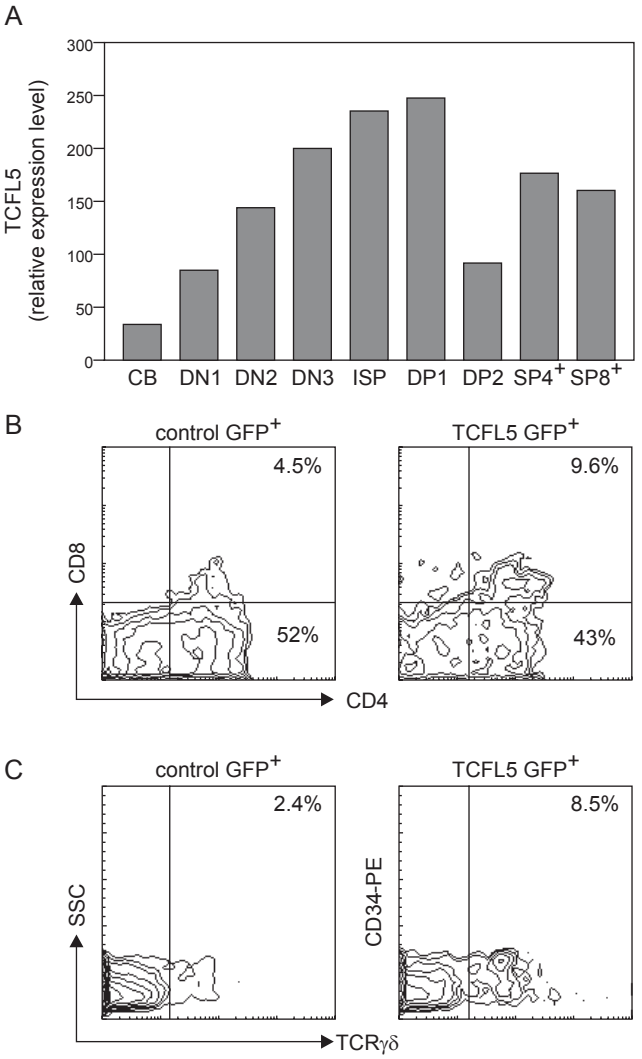


Figure 5. Functional validation of a role for TCFL5 in T-cell development.
(A) Relative amounts of TCFL5 mRNA in thymocyte subpopulations. Values extracted from previously performed gene expression profiles[6]. CB: $CD34^+$ UCB, DN1: $CD34^+CD1a^-CD38^-$, DN2: $CD34^+CD1a^-CD38^+$, DN3: $CD34^+CD1a^+$, ISP: $CD4^+CD3^-$, DP1: $CD4^+CD8^+CD3^-$, DP2: $CD4^+CD8^+CD3^+$, $SP4^+$: $CD4^+CD3^+$, $SP8^+$: $CD8^+CD3^+$. (B and C) Human $CD34^+$ UCB cells were retrovirally transduced with a vector containing only the gene for GFP (left panels) or TCFL5 and GFP (right panels) and allowed to develop into T-cells in FTOC. After 9 days of culture, cells were harvested and examined by flow cytometry. (B) Expression of CD4 and CD8 within the GFP^+ gate. (B) Expression of TCRγδ within the GFP^+ gate.

FTOCs of HoxA5 deficient fetal liver cells (Figure 6A). Furthermore, no differences were found in numbers of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells between the fetal livers of HoxA5 deficient and normal embryos (Figure 6B), indicating that hematopoietic stem cells are normally present in these mice. Colony assays were performed to determine the frequency of erythroid and myeloid progenitors in HoxA5 deficient bone marrow: no abnormalities were detected (data not shown). To investigate whether HoxA5 deficient bone marrow progenitors can reconstitute the immune system under competitive conditions, bone marrow cells were transplanted into sublethally irradiated recipients. After five, eight and ten weeks, the percentage of donor cells in the blood was identical between mice transplanted with wild type or HoxA5 deficient bone marrow cells (Figure 6C). Furthermore, all hematopoietic lineages were normally present among donor cells in thymus, bone marrow and spleen of the recipients.

DISCUSSION

Despite the unmistakable role of Notch signaling for T-cell lineage specification, studies to identify downstream Notch target genes in relevant primary cell types have not been undertaken.

In the present study we took advantage of the recently introduced AMAXA nucleofection system, which allows transient transfection of primary hematopoietic progenitor cells with a high efficiency and relatively low mortality. Using this technique, we were able to induce a rapid and strong Notch signal. Furthermore, the S17-DL stromal cell co-culture system allowed us to more physiologically mimic the Notch signal encountered by progenitor cells that enter the thymus. We chose to use human $CD34^{+}$ UCB cells as source of progenitor cells, as these are readily available, unactivated, multipotent, and efficiently develop into T-cells when cultured in murine fetal thymic lobes or on bone marrow stroma expressing Delta1[11,12,32]. Progenitor cells start expressing Notch target genes only after entering the thymus[5], which made them preferable over $CD34^{+}$ thymocytes which have already contacted Notch ligands. Using $CD34^{+}$ UCB cells and either IC-Notch transfection or the S17-DL co-culture system in combination with Affymetrix microarray technology, we found upregulation of the generally acknowledged Notch target genes *HES1*, *NOTCH1* and *NRARP*, which validated our procedures. The delayed upregulation of target genes in cells cultured on S17-DL as compared to transfection with IC-Notch indicates that cells need prolonged or multiple contacts with the Delta1 ligand to get accumulation of significant amounts of IC-Notch.

Our assays did not show induction of a number of genes previously reported to function as Notch targets during T-cell development, including *PTCRA*, *E2A* and *DTX1*[9,22,31]. This is especially critical for Deltex1, as many studies use expression of

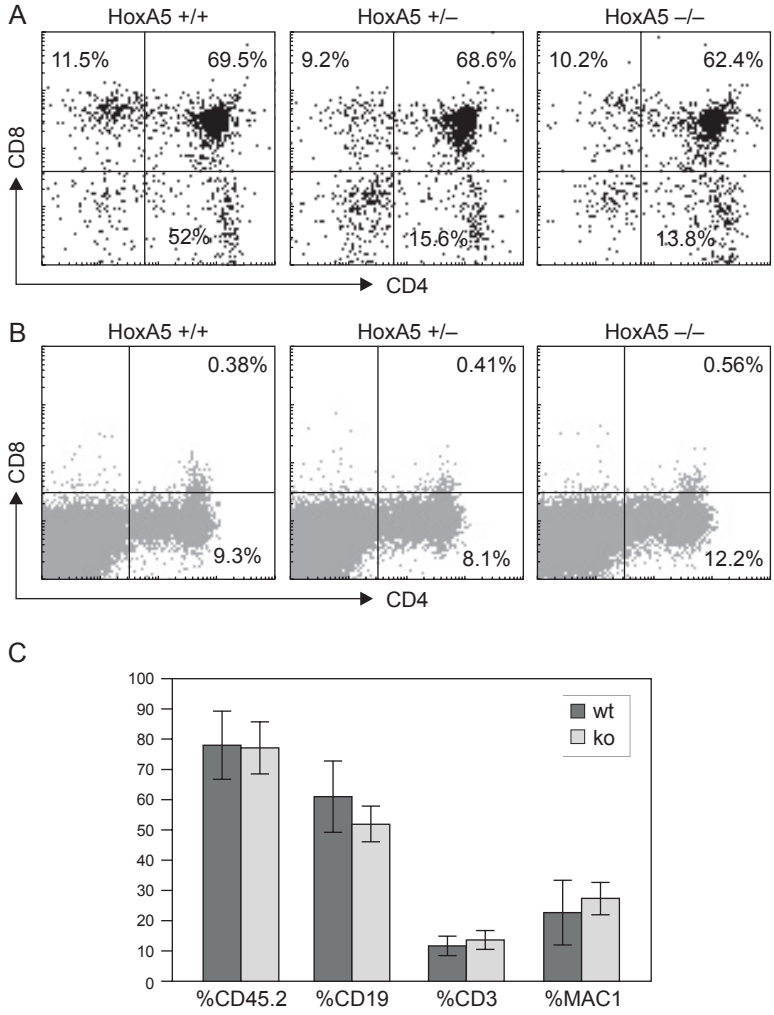


Figure 6. Hematopoiesis in HoxA5 deficient mice.
(A) Thymic lobes of normal (left and middle panels) and HoxA5 deficient (right) embryos of day 14 of gestation (E14) were cultured on filters. After 16 days, T-cell development in the lobes was analysed using flow cytometry. (B) Hematopoietic progenitor populations were determined in fetal livers of normal (left and middle panels) and HoxA5 deficient (right) E12 embryos. Percentages of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells are shown in the upper right quadrant. (C) Sublethally irradiated mice were transplanted with wild type or HoxA5 deficient bone marrow cells. After ten weeks, the percentage of $CD45^{+}$ donor cells and the percentage of $CD19^{+}$ B cells, $CD3^{+}$ T cells, and $MAC1^{+}$ myeloid cells within a $CD45^{+}$ gate were determined in the blood of the recipients.

Deltex1 as the read-out for active Notch signaling, mostly in combination with Hes1[12,47], but in some cases as sole evidence[48]. The main proof for *PTCRA* and *DTX1* as Notch targets in T-cell development comes from studies by Deftos et al., in which IC-Notch was retrovirally transduced into a murine DP thymoma cell line[22,28]. Apart from the fact that Notch signaling and downstream events may be dysregulated in malignantly transformed cells, retroviral transduction takes several days and identified genes may represent indirect targets or may have resulted from compensatory mechanisms. Upregulation of genes identified in the thymoma line was confirmed in the thymi of mice transgenic for IC-Notch[22], but may again result from indirect activation. Nevertheless, it possible that *PTCRA* is a Notch target gene in more differentiated T-cell precursors. This would fit with our finding that *PTCRA* is upregulated at the DN2 stage and with the fact that Notch signaling continues throughout the DN2 stage[33].

We did not find any evidence for direct induction of a T-cell program by Notch signaling. This is in contrast with a recent study by Höflinger et al., in which a Notch signal was induced by culturing multipotent progenitor cells on OP9-DL[25]. These cells rapidly upregulated *PTCRA, DTX1, GATA3* and *TCF1,* but not *HES1* transcripts. However, these results should be interpreted with caution, as this study used murine Pax5$^{-/-}$ pro-B cells expanded on the ST2 cell line as source of uncommitted progenitors: non-physiological cells that express many B cell specific genes and have already started TCR gene rearrangements (Vγ-Jγ, Vδ-Jδ and Dβ-Jβ)[25].

Our findings are consistent with experiments of Taghon et al. in which murine fetal liver progenitors were cultured on OP9-DL[47]. This study showed that *GATA3*, *TCF1* and *PTCRA* began to be expressed after three days of culture on OP9-DL, while high *HES1* transcription was detected already after one day. These findings indicate that either Notch signaling induces T-cell genes in more differentiated thymocytes, or Notch signaling stimulates the expression of other transcription factors, which then in turn activate or repress lineage differentiation genes. TCFL5 is a likely candidate for such a function.

TCFL5 is a member of the group B bHLH proteins and presumably binds to the same non-canonical E-box as Hes1[36]. It was first found in spermatocytes, but was not detected in the thymus by Northern-blot analysis[36,37]. This might be explained by the fact that *TCFL5* levels decrease in the more mature stages of T-cell development (Figure 5A), which comprise the larger part of the thymus. Also during spermatogenesis, TCFL5 is expressed in a highly cell-type and stage-specific pattern[36,37].

By overexpressing TCFL5 in FTOC, we obtained preliminary clues that TCFL5 indeed positively regulates T-cell development. The observed effects were mild, presumably because TCFL5 is expressed already during the early stages of T-cell development (Figure 5A), and enhanced expression is not likely to induce remarkable changes. Our ongoing siRNA studies will further show the significance of TCFL5 function.

The finding of the *HOXA* cluster genes as Notch targets is novel and intriguing. Notch induced *HOXA* transcripts may be important for T-cell development, as HoxA9 deficient mice have a partial block at the DN1 stage of thymocyte development[44]. Furthermore, overexpression of *HOXA* genes is a cause of human T-ALL[49]. We did not find upregulation of *HOXA* genes in DN1 cells. Possibly, *HOXA* genes are induced only in a tiny subset of DN1 cells that have just contacted Delta, and are rapidly downregulated afterwards. However, we did not detect any defects in T-cell differentiation or hematopoiesis in general in HoxA5 deficient mice. The high functional redundancy of the different *HoxA* genes probably obscures the importance of HoxA5 for hematopoiesis. It would be highly interesting to study T-cell development from progenitors deficient for more than one *HoxA* gene.

Other than a role in T-cell development, the Hox genes may be involved in alternative Notch-regulated processes in hematopoietic stem cells. Notch signaling is clearly important for self-renewal of hematopoietic progenitors (reviewed in reference 15). Interestingly, *HOXA5*, *A9* and *A10* were found to be part of the 'stem cell profile'[50]. Without doubt, the relation between Notch signaling and HoxA expression should be studied in greater detail.

The mechanism by which Notch signaling initiates a T-lineage differentiation program in hematopoietic progenitors remains a fascinating issue for immunologists. Here we demonstrate that Notch does not directly activate a T-cell specific transcription program, nor does it directly induce TCR gene rearrangements. For sure, Hes1 executes part of the downstream Notch effects. In addition, TCFL5 and HoxA proteins may prove to be major player in Notch-regulated mechanisms during T-cell development.

MATERIALS AND METHODS

Isolation of cells

Human umbilical cord blood (UCB) material was obtained according to the informed consent guidelines of the Medical Ethical Committee of Erasmus MC, Rotterdam. UCB mononuclear cells were isolated using Ficoll density centrifugation and frozen down until further use. Over 90% of UCB cells were viable after thawing. For each experiment, frozen UCB cells of at least four different donors were used. $CD34^+$ progenitor cells were purified using immunomagnetic beads (Miltenyi Biotec). Purity of the recovered subpopulations was checked by staining with CD34-PE (BD Pharmingen) and flow cytometry and was always greater than 95%.

Transfection of $CD34^+$ UCB cells

Human cDNA encoding the intracellular domain of Notch1 (amino acids 1770-2555[51]) was cloned into the multiple cloning site of eukaryotic expression vector pcDNA3 (Invitrogen). $CD34^+$ UCB cells were cultured for one hour in the presence of 10 ng/ml recombinant human IL-7 and 50 ng/ml recombinant human SCF (both from R&D Systems). Subsequently, $4x10^6$ cells were transfected with 4 µg pcDNA3-IC-Notch or empty pcDNA3 using an AMAXA human CD34 Nucleofector kit and an AMAXA Nucleofector Device, according to the manufacturers protocol. 0.1 µg of pEGFP-C1 (BD Clontech) was co-transfected

with each sample. After transfection, cells were cultured on confluent layers of the murine bone marrow stromal cell line S17, in the presence of 10 ng/ml IL-7 and 50 ng/ml SCF. After 6 and 24 hours, cells were carefully harvested without disturbing the S17 monolayer and RNA was isolated. A small aliquot of cells was taken from each sample to monitor transfection efficiency: GFP expression was determined by flow-cytometry (FACS Calibur, BD Biosystems).

Co-culture of CD34$^+$ UCB cells on S17

S17 bone marrow stromal cells retrovirally transduced with either Delta1-IRES-eGFP (S17-DL) or control LZRS-IRES-eGFP (S17-GFP) were kindly provided by dr. L. Parreira (Faculdade de Medicina de Lisboa, Portugal) and have been described previously[11]. S17 cells were grown in confluent monolayers in 24 wells plates. 1x10^5 CD34$^+$ UCB cells were co-cultured with S17 in the presence of 10 ng/ml IL-7 and 50 ng/ml SCF. After 6 and 24 hours cells were carefully harvested and processed for RNA isolation.

Microarray analysis

Affymetrix microarray analysis of the samples transfected with pcDNA or pcDNA-IC-Notch was performed as described previously[52]. For the samples co-cultured on S17, an extra cycle of cDNA and cRNA synthesis was performed. The starting amount of RNA for the first round of cDNA synthesis was 30-60 ng.

For the transfected samples, Affymetrix U133A and B microarrays were used, for co-cultured samples only U133A arrays. In comparison experiments, care was taken that the 5'/3' GAPDH ratio, scaling factor, noise, and percentage of presence calls were comparable. Scanned images were analyzed using Affymetrix Microarray Suite 5.1 software, and pair-wise comparisons were generated using the appropriate control samples (without induced Notch signaling) as baseline. Differential expression was considered significant when the gene received a 'present call' for at least one of the microarrays used for the comparison, the fold change was >1.8 and change-p value <0.003. Differential expression of genes after Notch induction was compared to expression profiles of CD34$^+$ UCB cells and the relevant thymocyte subsets: comparisons of CD34$^+$ UCB cells versus CD34$^+$CD1a$^-$CD38$^-$ (DN1) and CD34$^+$CD1a$^-$CD38$^-$ versus CD34$^+$CD1a$^-$CD38$^+$ (DN2)) (data generated previously[6]).

Probes for *DTX1* and *NRARP* were present only on the U133B array. *HES5* was not represented on the microarray used (U133). Of the Ifi family, only *Ifi16* was present on the microarrays.

Real-time quantitative-PCR (RQ-PCR)

The expression of several target genes was tested using TaqMan-based RQ-PCR. 1 µg cRNA generated in the microarray analysis procedure was treated with 1U of DNase I and subsequently reverse transcribed to cDNA with avian myeloblastosis virus-reverse transcriptase (5 U), oligo(dT) and random hexanucleotide primers.

Mixes containing the appropriate pre-designed primer/probe sets were obtained from Applied Biosystems. For *TCFL5*, *Hes1*, *HoxA9* and *HoxA10* these were Assays-on-Demand, for *HoxA5* an Assay-by-Design. A 1/20 cDNA mixture was used for RQ-PCR for each primer/probe set and performed in duplicate. The RQ-PCR reaction was performed using TaqMan Universal mastermix (Applied Biosystems) and was run on a PRISM 7700 sequence detection system containing a 96 well thermal cycler (Applied Biosystems). RQ-PCR results were normalized to *GAPDH* expression (kit from Applied Biosystems) in the same sample.

Functional testing of TCFL5 in fetal thymic organ culture (FTOC)

Full-length cDNA for human TCFL5 was obtained from Invitrogen, cloned into the retroviral vector LZRS-IRES-eGFP and high titer retrovirus was produced in the Phoenix packaging cell line. CD34$^+$ UCB cells were cultured for one day and retrovirally transduced for two days in the presence of 50 ng/ml SCF, 10 ng/ml recombinant human TPO and 50 ng/ml recombinant human Flt3L (all from R&D Systems). The percentage of GFP positive cells was determined using flow cytometry and cells were transferred into irradiated murine embryonic day 14 thymic lobes. After two days, lobes were cultured on filters floating

on medium. After an additional nine days, lobes were harvested, single cell suspensions were made and T-cell development was analyzed using a FACS Calibur flowcytometer (BD Biosystems). Monoclonal antibodies against human antigens were CD1a-RD1 (Beckman Coulter) and TCRγδ-PE, CD3-PerCP, CD8-PerCP, CD4-APC and CD34-APC (all from BD Pharmingen).

HoxA5 KO mice

Mice deficient for HoxA5 have been described in detail[46]. Mice heterozygous for the mutated *HoxA5* gene were bred to generate deficient, heterozygous and wild type littermates. HoxA5 deficient mice were viable and did not display evident abnormalities. In mice of 2.5, 4 and 25 weeks old, bone marrow, spleen and thymus were analyzed for presence of different hematopoietic lineages using flow cytometry. Embryonic thymic lobes of day 16 were directly stained for flow-cytometry. Embryonic thymic lobes of day 13 and 14 were cultured on a filter for 16 days, after which T-cell development was analyzed using flow-cytometry. Fetal livers of embryonic day 13 and 14 were either directly used for flow-cytometry to analyse progenitor populations or transferred into irradiated wild type day 14 thymic lobes for FTOC experiments. Chimeric thymic lobes were cultured for 14 days on a filter, after which T-cell develoment was analyzed using flow-cytometry.

HoxA5 deficient BM cells were assayed for the presence of granuloctye-macrophage colony-forming units (CFU-C) and erythroid burst-forming units (BFU-E) by *in vitro* colony formation in viscous methylcellulose culture medium containing cytokines: for BFU-E 4U/ml human erythropoietin (EPO, Behringwerke AG) and 100 ng/ml murine SCF (R&D); for CFU-C 30 ng/ml murine IL-3, 100 ng/ml SCF and colony stimulating factor (CSF, 300x dilution of ConA adsorbed fraction of pregnant mouse uterus extract). 40,000 and 100,000 cells were plated in duplicate dishes. The number of colonies was determined after 10 days of culture.

HoxA5 deficient BM cells were assaysed for their ability to reconstitute the immune system of sublethally irradiated mice. 8-11 week-old C57Bl/6 mice harboring the CD45.1 (Ly5.1) allele as a congenic marker, received a sublethal dose of 6 Gy total body irradiation. Mice were intravenously injected with $2x10^6$ thawed bone marrow cells ($CD45.2^+$) in 250 µl of PBS. To prevent infections, mice received 100 mg/l ciprofloxacin (Bayer) in their drinking water. Five, eight and ten weeks after transplantation, blood was drawn and analyzed for cells expressing the CD45.2 marker. Ten weeks after transplantation, mice were killed and BM, spleen and thymus were isolated. In each organ, total cell numbers were determined and the percentage of $CD45.2^+$ cells and their subset distribution was assayed by flow cytometry.

Monoclonal antibodies against mouse antigens were ER-MP20-FITC (anti Ly6c, own culture), CD25-FITC, IgM-FITC, Sca1-FITC, CD45.2-FITC, CD4-PE, CD43-PE, TER119-PE, NK1.1-PE, CD127-PE, Sca-1-PE, CD8-PerCP, Lin-PerCP (CD3-biotin, B220-PerCP, NK1.1-biotin, MAC1-PerCP, GR1-biotin, TER119-biotin, Streptavidin-PerCP), CD44-APC, B220-APC, CD19-APC, MAC1-APC and c-kit-APC (all from BD Pharmingen).

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9

GENERAL DISCUSSION

Parts of this chapter will be submitted as an invited review to Trends in Immunology

T-cell development in the thymus is a complex and highly regulated process. During the process of differentiation from multipotent stem cells to mature T cells, proliferation, restriction of lineage potential, TCR gene rearrangements and selection events occur, all accompanied by changes in gene expression. Many extracellular cytokines, adhesion molecules, intracellular signal transduction pathways and transcription factors are involved and a comprehensive understanding of thymocyte differentiation remains to be established. Two key issues into which the research presented in this thesis has provided insight are highlighted in this General discussion: detailed phenotypic and genetic description of the different stages of T-cell development, and cellular and molecular aspects of T-cell commitment.

HUMAN THYMOCYTE SUBPOPULATIONS

Before studying the molecular regulation of T-cell development, it is imperative to know the characteristics of the normal thymic subpopulations in which the different processes take place. In Chapters 2-4, our basic knowledge about the stages of human T-cell development was updated. The classification of thymocyte subsets based on expression of CD4, CD8, CD34 and CD1a is commonly used and TCR rearrangements in human thymocytes have been studied previously[1]. We used multi-color flow cytometry, high-speed cell sorting, quantitative PCR and microarray technology to perform more detailed and sensitive analyses. These findings are summarized in Figure 1.

Based on the TCR gene rearrangement status of the various subpopulations, the stages of human T-cell development appear to be highly similar to the stages in the mouse[2], allowing the use of mouse nomenclature for human thymocytes as well. For example, human $CD34^{+}CD38^{-}CD1a^{-}$ cells correspond to murine $CD44^{+}CD25^{-}$ thymocytes and can therefore be called DN1. One exception exists: the human ISP subset is more similar to the murine DN4 subpopulation in that these are the rapidly cycling cells that have undergone β-selection. The murine ISP population is extremely small and usually ignored in studies of thymocyte differentiation.

The DN $CD34^{-}$ (DN4) subpopulation confronted us with some problems. This subset was omitted in Chapter 3, because no high quality mRNA could be obtained from these cells. There may be several reasons for this. First, the DN4 population is defined only by exclusion markers, possibly leading to the isolation of an impure fraction, containing dead or dying cells, stromal components and debris. Second, this population is heavily rearranging *TCRB* genes and has largely shut down proliferation, greatly reducing mRNA transcription. And finally, a large part of the DN4 cells may be undergoing apoptosis, after failing to produce a functional TCRβ chain. Nevertheless, TCR gene rearrangement and gene scan analyses were performed in these (suboptimally sorted)

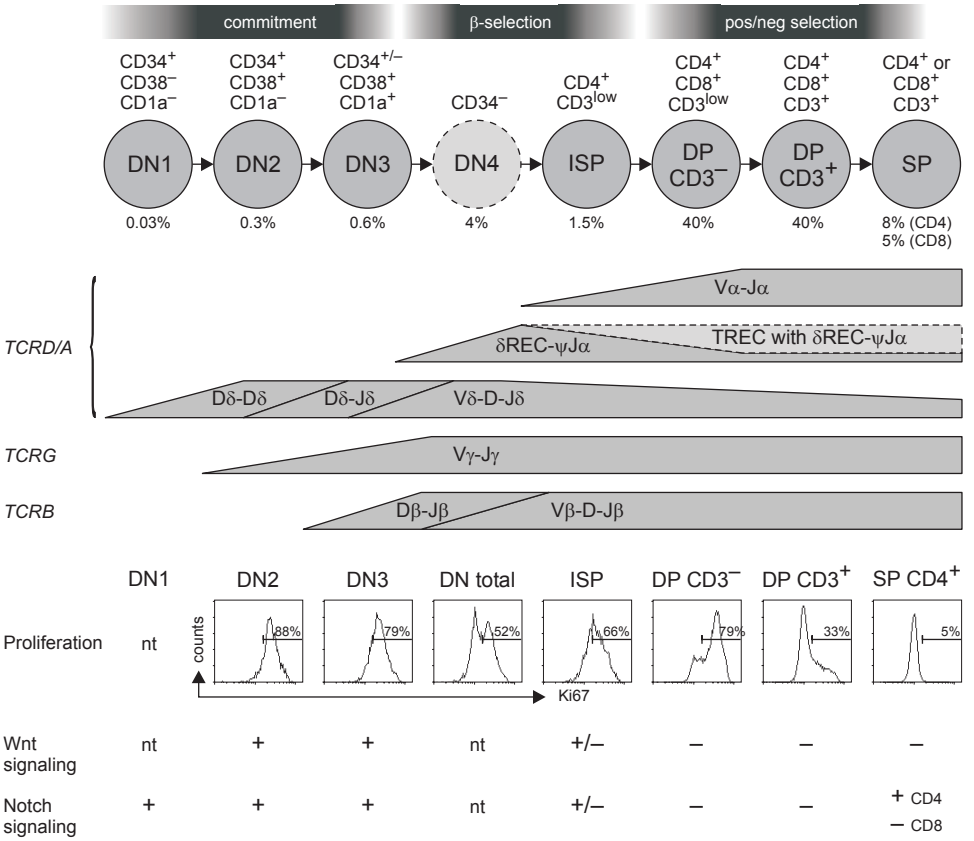

Figure 1. Characteristics of human thymocyte subpopulations.
Percentages of the different subsets were determined in Chapter 2. Numbers shown are typical for the thymus of a 6 to 9-month old child. Percentages of CD3- and CD3+ DP subsets are similar, but the exact contribution of each is difficult to determine, as a gradual increase in CD3 expression occurs during the DP stage. TCR gene rearrangements were quantitatively determined in Chapter 3 and were used to pinpoint β-selection to the DN4 to ISP transition. Chapter 4 demonstrates that T-cell commitment (with respect to loss of erythroid, myeloid and B-lymphoid potential) is completed before the DN3 stage. The percentage of proliferating cells in each subset was analysed by flow cytometry, using intracellular Ki67 staining. Of the CD8+ SP cells 2% was positive for Ki67. Presence of Wnt signaling in each subset was studied in Chapter 5, using Tcf reporter expression, β-catenin levels and Wnt target gene transcription. Presence of Notch signaling was deduced from the level of Hes1 transcripts, as extracted from the gene expression data generated using microarrays in Chapter 3. nt: not tested.

cells and showed the presence of complete, in-frame *TCRB* genes (data not shown). The presence of a functional TCRβ-chain in combination with preTα (of which high transcript levels are present already in the CD34$^+$CD1a$^+$ stage) would imply that β-

selection takes place in the DN CD34$^-$ stage. This hypothesis is supported by the fact that *TCRA* rearrangements start in the ISP stage. However, it is somewhat later than in the mouse, in which β-selection occurs at the DN3 stage[3].

All thymocyte subpopulations described in Figure 1 were isolated and gene expression profiles were generated using microarray technology (described in Chapter 3), resulting in a huge data-set which has already proven valuable. The thymocyte expression profiles were used to identify transcription factors associated with particular TCR gene rearrangements. In addition, the gene expression data helped to answer specific questions in Chapter 4, 5 and 8 as well as in other studies[4,5]. We are convinced that also in further studies regarding T-cell development these expression profiles will prove to be a rich source of information. Furthermore, gene expression in thymocytes can be compared to other cell types, e.g. to find genes functioning in similar processes in both T- and B-cell development, and may be used as a 'base-line' for comparisons with corresponding populations in deregulated T-cell development (e.g. T-ALL or SCID), to find genes involved in these diseases.

Dangers of phenotypically defined subsets

Although the thymocyte expression profiles and the data presented in Figure 1 are useful and have provided new insights, our studies have, perhaps above all, demonstrated that this division into subsets is incomplete, and call attention to the limitations of surface immunophenotypes. Two potential 'threats' are being encountered: the sorted population might be functionally impure and a functionally uniform population might be spread over multiple phenotypical stages.

Most of the studied thymocyte populations are functionally heterogeneous, first of all because they all contain proliferating and non-proliferating cells, as determined by Ki67 staining (Figure 1), perhaps with the sole exception of the SP subsets, which appear essentially resting. Each subpopulation should reasonably contain non-cycling cells, as TCR rearrangements take place in every subset as well. It is generally accepted that proliferation and gene rearrangements do not occur simultaneously in the same cell, as this would bring about the danger of unwanted gene translocations which are frequently observed in T-ALL[6].

The massive pre-TCR independent expansion that occurs in the DN1, 2 and 3 subsets, appears to be interrupted by rearrangements of the *TCRD*, *TCRG* and *TCRB* genes. Whether there is a dynamic alternation of proliferation and rearrangement in the different stages or rather a large heterogeneity in the timing of rearrangement initiation remains to be examined. For example, some cells may start rearranging *TCRD* already in the DN1 stage, while others keep cycling until DN3 and then start rearrangements. Another example of a functionally impure population is the ISP subset: in this stage pre-TCR driven expansion occurs, as well as termination of proliferation

and initiation of *TCRA* rearrangements. In addition, some cells are still rearranging *TCRB* genes, probably because earlier attempts at producing an in-frame TCRβ-chain were unsuccessful.

Exemplary for heterogeneous surface marker expression on a functionally uniform population are the cells in which *TCRA* rearrangements occur. *TCRA* rearrangements are stretched over a number of subsets, starting in ISP and being completed only in the SP stages. If opening of the *TCRA* locus would be studied by sorting for instance DP $CD3^-$ cells, only a proportion of the target population would be isolated, and the conclusions drawn may not hold for the entire population.

Obviously, the surface markers we use now do not suffice to isolate functionally pure cell populations. Many of the currently employed antibodies stain surface antigens that do not necessarily have a direct relation to the process they are intended to represent. For example, upregulation of CD1a seems to correlate nicely with the point of T-cell commitment, but probably purely coincidental. The use of antibodies against proteins that are functionally involved in specific processes, e.g. proliferation or pre-TCR signaling, would be of great help. Our microarray data may help to identify such markers. Unfortunately, many of such newly-identified proteins will not be expressed at the cell surface, and intracellular staining is at present not compatible with RNA-based gene expression profiling.

T-CELL COMMITMENT

At some point during T-cell development, a thymocyte will find itself on the track towards becoming a T cell, while all other options are closed. This thymocyte is committed to the T-cell lineage. The process of T-cell commitment in a multipotent progenitor is an intriguing issue of which many questions remain unanswered. Several processes may contribute to T-cell commitment, including loss of alternative lineage gene expression, loss of alternative lineage potential, expression of a T-cell specific gene program and initiation of T-cell specific gene rearrangements. Finally the production of a functional TCRβ chain will induce signaling through the pre-TCR, an event that is absolutely specific for T cells. However, final T-cell commitment likely precedes pre-TCR signaling. Furthermore, the loss of alternative lineage potential does not happen at a single instant; in stead the moment may vary with respect to the lineage that is excluded. For example, differentiation into the erythroid lineage is presumably shut down at an earlier stage than the NK-cell lineage.

The thymus-seeding cell

The issue of T-cell commitment starts with the question to what level the cells that seed the postnatal thymus are committed to the T-cell lineage. The difficulty in answering this question is that cells will immediately be influenced by the thymic microenvironment as soon as they enter the thymus. Progenitors that have T-cell developmental capacity but differ from HSCs have been identified in bone marrow (BM) and blood[7-10], but it is

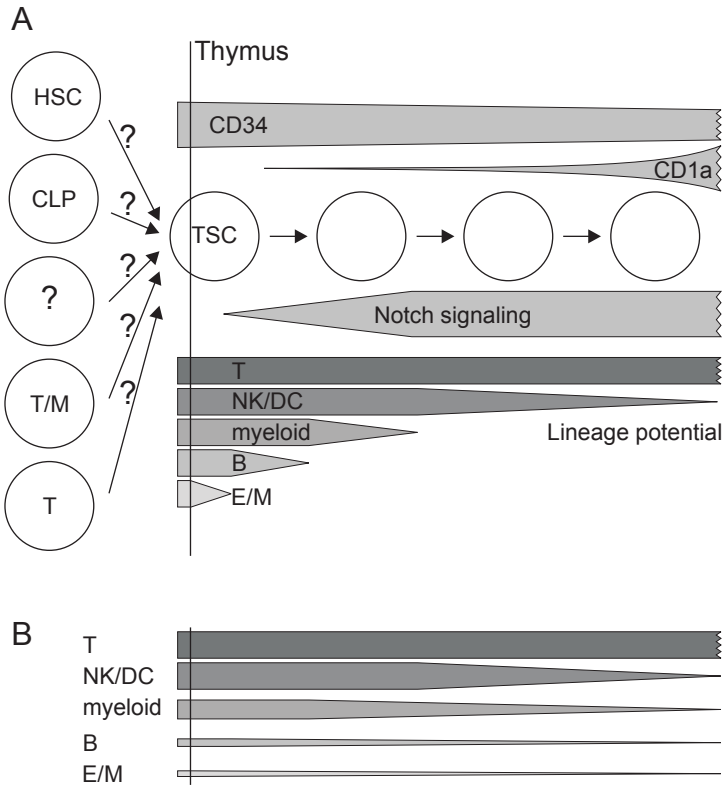

Figure 2. The earliest stages of T-cell development.
(A) Several candidate thymus-seeding cells (TSC) have been proposed, ranging from HSCs or non-renewing multipotent progenitors to committed T-cell precursors (CLP: common lymphoid progenitor; T/M: T/myeloid bipotent progenitor, T: T-cell committed progenitor). Cells that enter the thymus receive Delta-induced Notch signals via the thymic stroma and start their development into T cells. As they develop, thymocytes gradually lose expression of CD34 and acquire CD1a. In addition, lineage potential (either of individual cells or on the population level) declines. It remains to be demonstrated whether thymus-seeding cells have equal potential for each lineage with rapid loss of non-T-cell potential upon thymic entry (depicted in A), or whether alternative lineage potential is already reduced before cells arrive in the thymus (depicted in B). E/M: erythroid/megakaryocytic.

unknown whether these cells seed the thymus physiologically (Figure 2). Harman et al.[11] have elegantly addressed this question by isolating the cells that are present in the mesenchyme surrounding the fetal thymus but have not yet entered. These cells were shown to be committed T-cell precursors[11]. However, the cells that initially populate the fetal thymus and contribute to the establishment of the thymic microenvironment may be entirely different from the few postnatal thymus-seeding cells that predominantly serve to keep the thymocyte counts at level.

In the mouse, identification of the thymus-seeding cell has lately been approached by sorting minute DN populations from the thymus and by studying their lineage potential and other characteristics, hoping to find the cells that have contacted the thymic stroma for a minimally short period. The thymus-seeding cell is generally thought to lie within the ETP (early T-cell progenitor) population. Classic ETPs are defined as being DN1, Lin^- and c-Kit^{hi} [12] and are supposedly highly heterogeneous themselves. Additional markers, varying per research group, were included to subdivide ETPs and other DN populations into several fractions, which appeared to have different T- and B-cell developmental capacities. This also depended on the *in vivo* and *in vitro* tests used to delineate lineage potential[13-15]. The question remains whether these different subpopulations all arise from the same thymus-seeding cell or whether diverse BM precursors (some of which may not have T-cell potential) enter the thymus[16].

Interestingly, a recent study indicates that ETPs derive from the true thymus-seeding cell, which is not found within the ETP population itself[17]. The lineage potential of ETPs may therefore not reflect the full range of potentials of the thymus-seeding cell. This finding demonstrates that sorting of minute populations is not always advantageous, for two reasons. Firstly, the target subset may be entirely missed, simply because it is not included in any of the isolated populations. Secondly, the pure target population may fail to develop in subsequent *in vivo* or *in vitro* assays, because it might need accessory cells. It is, for instance, possible that the thymus-seeding cell needs additional thymocyte subsets for its survival or differentiation.

The limited set of markers available for the studying immature human thymocytes can be regarded as a disadvantage, but this also created a lucky side effect (Chapter 4). By taking the entire $CD34^+CD1a^-$ population, we were sure to include the thymus-seeding cells, even though they may represent a tiny fraction of this subset. Because of the large cell numbers in the human thymus and the easy purification of $CD34^+$ cells, we were able to culture large numbers of these cells in colony assays. This may have been the reason why we could detect the low erythroid (and presumably megakaryocytic) potential in thymocytes (Chapter 4), which was never found in the mouse. We could not demonstrate that all different lineages can develop from a single precursor in the thymus. Nevertheless, the full range of hematopoietic lineage potentials in the $CD34^+CD1a^-$ population, together with the fact that we found precursors for B and

NK cells in the human thymus (Chapter 2), suggested that the thymus is seeded by a HSC or a non-self renewing multipotent progenitor, or a mixture of progenitors with the collective potential to develop into all blood cell lineages (Figure 2).

Alternative lineage potential in the human thymus

As erythroid potential within the $CD34^+CD1a^-$ subset is very low, it is probably lost soon after multipotent progenitors have entered the thymus (Figure 2). Alternatively, an erythroid cell fate is not rapidly inhibited in $CD34^+CD1a^-$ thymocytes, but has for most cells already been excluded in the BM (Figure 2). It will be challenging to experimentally prove either of these options.

Restriction of myeloid lineage potential in the thymus appears to be less stringent than of erythroid potential, as the frequency of myeloid progenitors within the $CD34^+CD1a^-$ population was relatively high: in the presence of GM-CSF only two-fold lower than the frequency of precursors with T-lineage potential (Chapter 4). This would suggest that erythroid potential is excluded at an earlier stage than myeloid potential and that this might be regulated by different signals from the thymic microenvironment.

B-lineage potential within the $CD34^+CD1a^-$ cells was much lower than myeloid developmental capacity (Figure 2). This finding would argue against the CLP as the thymus-seeding cell and is in favor of a T/myeloid bipotent progenitor. However, it may also be that the thymus is colonized by HSC-like progenitors and that the thymic microenvironment more strictly inhibits a B-cell fate than other lineages, presumably by Notch signaling (see below), thereby allowing the residual myeloid potential to become more prominent.

In any case, potential to differentiate into erythroid, myeloid and B-lymphoid cells was completely abolished in the $CD34^+CD1a^+$ thymocytes, placing T-cell commitment before the DN3 stage (Figure 2). This is in accordance with previous findings that the capacity to develop into DC and NK cells is also lost in $CD34^+CD1a^+$ thymocytes[18]. Also in the mouse, DN3 thymocytes are thought to be irreversibly committed to the T-cell lineage[19]. As pre-TCR signaling occurs in the $CD34^-$ (DN4) subset and commitment is completed at the $CD34^+CD1a^+$ stage, signaling through the pre-TCR is most likely not part of the T-cell commitment process.

While we have shown that a proportion of $CD34^+CD1a^-$ cells still retains a broad lineage potential, another part of these cells may already be committed to the T-cell lineage. Again additional markers are needed to distinguish between committed and multipotent cells. Single cell differentiation assays will be needed to determine the proportion of T-lineage committed cells within each stage, as well as the range of lineage potentials within a single cell. Apart from the fact that single cell assays using human cells so far did not work well in our hands, no assays exist in which T- and B-cell differentiation from a single progenitor can be studied simultaneously. The difficulty in

developing a culture system in which both T cells and other lineages can be generated lies in the fact that T-cell development requires the presence of Notch ligand Delta, while Delta suppresses development into B cells, and potentially other lineages.

Loss of alternative lineage gene expression

In Chapter 4 we have demonstrated the expression of non-T cell genes in the $CD34^+CD1a^-$ subset and their downregulation in the $CD34^+CD1a^+$ stage. Why are these alternative lineage genes expressed in early thymocytes? Some genes may reflect the actual development of an alternative lineage in the thymus. We have shown the presence of $CD34^+$ precursors for B and NK cells in the human thymus (Chapter 2). We did not study the presence of myeloid precursors in the thymus, but we did confirm protein expression of MPO, CD13 and CD33 within the $CD34^+CD1a^-$ subset (Chapter 4). Physiological development of small numbers of myeloid cells in the thymus could well be envisaged, for instance to play a role in positive or negative selection.

On the other hand, physiological development of erythrocytes and platelets in the thymus is unlikely and *in vitro* erythroid potential of $CD34^+CD1a^-$ thymocytes was low (Chapter 4). Nevertheless, a relatively high expression of the erythroid/megakaryocytic transcription factor NF-E2 was detected in the $CD34^+CD1a^-$ subpopulation (Chapter 4). Several other studies have shown 'promiscuous' expression of lineage-specific genes by multipotent cells[20-22]. It has been suggested that low level transcription of multiple genes is necessary to prime differentiation down several lineages[23]. Alternatively, promiscuous expression may be the result of the wide-open chromatin state of stem cells and multipotent progenitors, rather than of their active transcription directed at protein production. Decondensed chromatin does not necessarily mean expression, but for certain genes which need active repression to be turned off, it may lead to low level transcription. Once lineage commitment has been achieved, the genes expressed in the excluded lineage pathways are permanently silenced[24]. Thus, alternative lineage gene expression in $CD34^+CD1a^-$ thymocytes may just mean that these cells are 'keeping their options open', another sign that the $CD34^+CD1a^-$ subset contains multipotent progenitors.

For sure, epigenetic events occur during T-cell development[25]. We found differential expression of several regulators such as histon deacetylases between the $CD34^+CD1a^-$ and $CD34^+CD1a^+$ subsets. Another recently identified regulatory mechanism is represented by microRNAs, a class of small non-coding RNA species, which presumably act by controlling mRNA translation[26]. Recent work has demonstrated a cell-type specific distribution of microRNAs in human tissues and tumors, suggesting that microRNAs are involved in establishing a lineage specific gene program[27]. It would be interesting to study the microRNA profiles of the different stages of T-cell development.

Expression of T-cell specific genes

Many classical 'T-cell genes', for instance pTα, are expressed in the $CD34^+CD1a^-$ subset, some (including CD3ε) as early as the $CD38^-$ stage (Chapters 3 and 4), i.e. in cells that are not yet committed to the T-cell lineage. Furthermore, many of these alleged T-cell genes are not specific for the T-cell lineage, for instance CD4 can be expressed on myeloid cells[28], pTα on DCs[29], and cytoplasmic CD3 can be detected in NK cells[30], again indicating that their expression is no evidence for T-cell commitment. Obviously, the transcription of T-cell specific genes in uncommitted T-cell precursors is an indication that a T-cell program is being established, but the program is not yet definitive. The collection of positive regulators for a certain lineage has been termed 'lineage specification'[19] (Figure 3).

The specificity of these specification genes for the T-cell lineage probably lies in combinations that are exclusive for T cells, e.g. expression of pTα together with a functional TCRβ chain. Furthermore, T-cell commitment is associated with high-level expression of these specification genes: we found massive upregulation of these genes in the $CD34^+CD1a^+$ stage (Chapter 4).

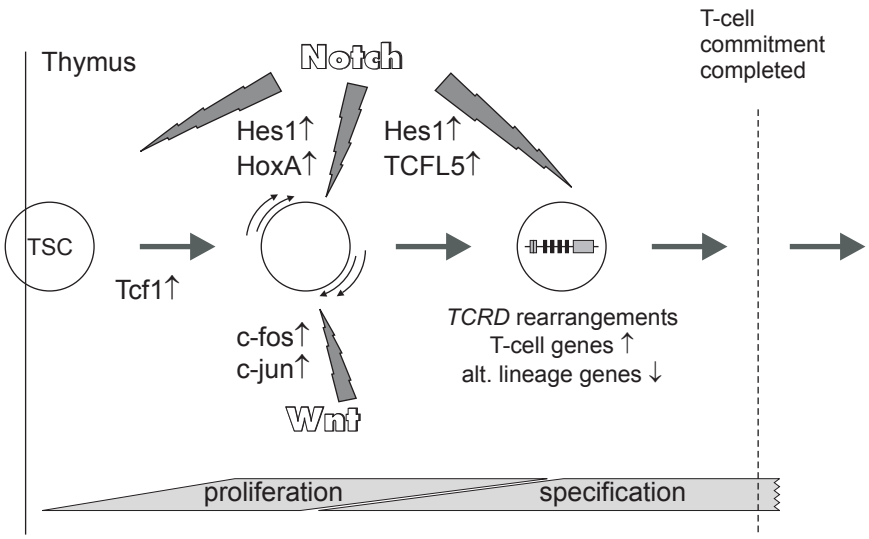

Figure 3. Hypothetical order of events during the earliest stages of T-cell development.
The thymus-seeding cells encounter Delta ligands on the thymic stroma and start their development into the T-cell direction. These immature thymocytes rapidly start expressing Tcf1, which is subsequently activated by Wnt signals. Wnt target genes (e.g. *c-fos* and *c-jun*) and Notch target genes (e.g. *Hes1* and *HoxA*) together induce massive proliferation of early thymocytes. Only after sufficient cell cycles have been undergone, specification can occur, which is dependent on Notch target genes (e.g. *TCFL5*) and perhaps also on Wnt signals. Specification comprises the TCR gene rearrangements and initiation of gene expression programs that will ultimately lead to a cell that is irreversibly committed to the T-cell lineage[19].

It is largely unknown which transcription factors regulate the expression of T-cell genes. We did not find any T-cell specific genes when inducing either Wnt signaling in $CD34^+$ thymocytes (Chapter 6) or Notch signaling in $CD34^+$ UCB cells (Chapter 8), although Tcf1 has been shown to interact with sequence motifs in CD3 and TCR enhancers[31,32] and CSL with binding sites in the pTα enhancer[33]. In addition, E-box elements are important for pTα transcription[34]. However, no transcription factor has been shown to directly induce a T-cell transcriptional program single-handedly. Presumably, simultaneous action of several transcription factors, as well as other factors involved in chromatin accessibility, are necessary for T-cell specification.

TCR gene rearrangements

The hallmark of a T cell is the expression of a TCR. Do TCR rearrangements indicate or even induce T-cell commitment? Components of the rearrangement machinery (RAGs, TdT) are already present in the uncommitted $CD34^+CD1a^-$ subset (although, like the T-cell genes, they are highly upregulated at the $CD34^+CD1a^+$ stage) and rearrangements of the *TCRD* locus (Dδ2 to Dδ3) can be detected at this stage (Chapter 3 and Figure 1). However, Dδ2 to Dδ3 rearrangements are not T-cell specific: they are detected in B cells as well[35]. We (Chapter 4) and others[35] could not detect immature *TCRD* rearrangements in myeloid cells (with the exception of myeloid leukemias, in which these and more mature rearrangements are probably caused by deregulated and prolonged RAG expression), suggesting that Dδ2 to Dδ3 rearrangements are lymphoid specific. The fact that Dδ2 to Dδ3 rearrangements are present in B cells and not in myeloid cells might indicate that B and T cells derive from a common bi-potent precursor. More likely is the explanation that in early B cells that are rearranging their B-cell receptor heavy chains, the D segments of the *TCRD* locus are not yet fully inaccessible to the rearrangement machinery. Another argument for the fact that Dδ2 to Dδ3 rearrangements do not induce commitment to T-cell or lymphoid lineages in general, is the finding that cells containing Dδ2 to Dδ3 rearrangements can still be induced to develop into myeloid and erythroid lineages (Chapter 4). Thus, the expression of rearrangement machinery and the occurrence of early *TCRD* rearrangements in the $CD34^+CD1a^-$ subset presumably indicate T-cell specification rather than T-cell commitment (Figure 3), similar to the expression of T-cell specific genes.

Dδ to Jδ and Dβ to Jβ rearrangements appear to be T-cell specific (leaving aside B-cell and myeloid leukemias) and occur during the $CD34^+CD1a^+$ stage, concurrently with T-cell commitment. But whether a causal relation exists between TCR rearrangements and T-cell commitment remains to be tested.

How TCR gene rearrangements are induced and regulated by transcription factors is not completely understood. IL-7R signaling[36] and E-box proteins[37] have been implicated in initiation of TCR rearrangements, but both are not T-cell specific. In Chapter 3 we

identified a number of transcription factors the expression of which correlates with the initiation of rearrangements on the different TCR genes. Whether these factors have a physiological function in opening of TCR loci has to be tested in rearrangement assays using non-lymphoid cells[38] and by overexpression and inhibition (siRNA) of these genes in FTOC.

Molecular regulation of lineage potential

In contrast to the emerging picture of a highly complex regulation of T-cell development, the molecular regulation of B-cell commitment appears to be relatively straight-forward[39]. During B-cell development, lineage specification is induced by the transcription factors E2A and EBF, which also induce Pax5 expression. Pax5 is necessary for the induction of crucial B-cell genes and represses alternative lineage fates[40,41]. Interestingly, Pax5 inhibits T-cell development by active repression of Notch1 expression[42].

For T-cell development, a master regulator comparable to Pax5 is not known. Notch signaling appears to be able to repress a B-cell fate, as overexpression of IC-Notch induces T-cell development in the BM, at the expense of B cells. However, direct inhibition of Pax5 by Notch signaling has not been described in literature. We could not study downregulation of Pax5 in our thymocyte microarrays, because Pax5 is not expressed in any subset, including $CD34^+$ UCB. Other studies have shown that Pax5 is rapidly downregulated when cells developing into the B-cell lineage are cultured on the T-lineage supporting stromal cell line OP9-DL[43]. Nevertheless, Notch signaling is not specific for the induction of a T-cell fate: it is also involved in self-renewal of HSCs[44], in induction of myeloid cell fates[45], and in the choice between erythroid and megakaryocytic differentiation[46].

Studies in human progenitor cells suggested that Notch signaling also inhibits a myeloid cell fate[47], but the molecular mechanism is unknown. Perhaps stringent molecular inhibition of myeloid development is not necessary during thymocyte differentiation. Development of myeloid lineages is strongly dependent on myeloid cytokines such as M-CSF and GM-CSF[48]. These cytokines are in all probability absent in the thymus, and myeloid development is thereby automatically excluded and does not have to be actively repressed (Figure 4). This might also explain why myeloid potential was greatly enhanced by addition of GM-CSF to OP9 cultures (Chapter 4).

In contrast, CLPs in BM (which are actually B cell progenitors[12,49]), completely lack myeloid potential[8]. Because of the abundant presence of myeloid growth factors in the BM, myeloid fates have to be actively repressed in CLPs (Figure 4). Similar reasoning explains why T-cell potential is still present in CLPs. As T cells will normally not develop in the BM because of the absence of Delta ligands (they will develop as soon as Delta1 or Delta4 are overexpressed[50]), T-cell development does not have to be actively repressed (Figure 4).

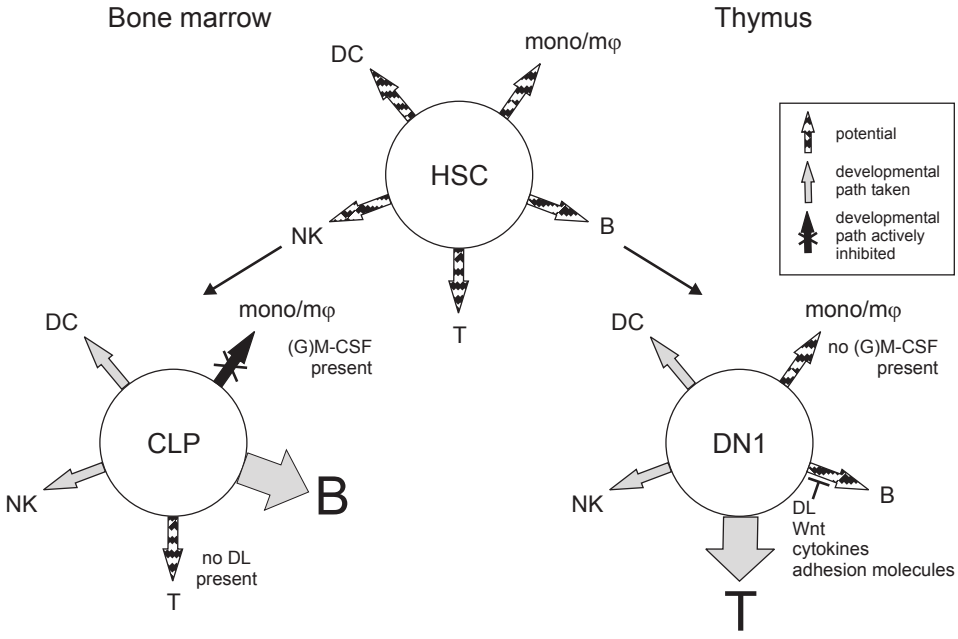

Figure 4. Hypothetical model of B- and T-cell commitment.
As multiple lineages develop in the BM (left), non-B-cell lineages have to be actively suppressed in CLPs. T cells do not develop in the BM because of the absence of Delta, and therefore do not need active suppression. Conversely in the thymus (right), T-cell oriented microenvironmental signaling makes sure that only T cells develop. Therefore, active repression of alternative lineages is not necessary in the thymus.

Development of DC and NK cells appears to be more 'tolerant' to Notch signaling[47,51-53]. Other mechanisms should therefore exist to exclude these lineages during T-cell development. Spi-B, a member of the Ets family of transcription factors involved in plasmacytoid DC development[54] was shown to be downregulated from the $CD34^{+}CD1a^{-}$ to the $CD34^{+}CD1a^{+}$ subset[55]. The inhibitory bHLH proteins of the Id family are thought to induce an NK-cell fate at the expense of T-cell development[1,56], although we found an increase of Id transcripts in $CD34^{+}CD1a^{+}$ thymocytes and subsequent stages rather than the expected downregulation (data not shown).

Notch as a T-cell commitment factor

If a quintessential T-cell commitment factor should exist, Notch would be the most likely candidate. Notch signaling, as measured by Hes1 expression, is absent from BM LSK cells, but present in the earliest thymocytes that can be identified, both in mouse[13,17] and human (Figure 1), although this may be different during fetal development[11]. Loss of Notch1 function in lymphoid progenitors results in B lymphopoiesis in the thymus, at the

expense of T-cell development[57], while overexpression of a constitutively active form of Notch induces ectopic T-cell development in the BM and inhibits B-cell development that would normally take place there[47,58]. However, in Chapter 8 we show that up to 24 hrs after Notch induction, although Hes1 is highly expressed, no T-cell genes or T-cell specific transcription factors (Tcf1, GATA3, HEB) are induced. Recent work in the mouse by Taghon et al. confirmed this finding[43]. In this study, murine fetal liver precursors were cultured on OP9-DL. After one day of culturing, Hes1 was already highly upregulated, but the induction of T-cell genes started only at day 3. Similarly, in OP9-control cultures, Pax5 began to be expressed only at day 3. Furthermore, this study found the presence of uncommitted cells both in OP9-DL and OP9-control cultures up to day 6 of culture. Thus, binding of Delta by a progenitor cell is indispensable for becoming a T cell and in the presence of Delta no other lineages will develop, but Notch signaling does not directly induce T-cell commitment itself. The presence of a Notch signal appears to permit other positive regulators to set up a T-cell program.

What happens during the time span when cells receive a Notch signal, but do not yet start a T-cell program? Of course, Hes1 is rapidly induced. Hes is required at the earliest stage of T-cell development[59], but overexpression of Hes1 or Hes5 is not sufficient to promote ectopic T-cell development from BM lymphoid progenitors[60]. We have identified a number of novel factors that are induced immediately after Notch signaling, of which we consider the HoxA cluster (mainly HoxA5, A9 and A10) and TCFL5 the most interesting ones (Chapter 8; Figure 3). Although we find no *HOXA* upregulation in the early thymocyte subpopulations, it is possible that *HOXA* genes are only induced briefly in the small fraction of true thymus seeding cells that initially encounter the Delta ligand on thymic stroma. In such a scenario, HoxA induces a transcriptional program and then is rapidly downregulated itself, so that we cannot detect its expression in the heterogeneous pool of $CD34^{+}CD38^{-}$ cells. Interestingly, a recent study has shown that dysregulated HoxA expression can cause T-ALL[61], suggesting a proliferative function of *HOXA* genes in thymocytes. It is an appealing thought that the earliest function of Notch signaling in the thymus might be to induce proliferation (see also 'Proliferation before specification?'). Indeed, far larger cell numbers are generated when progenitor cells are cultured on OP9-DL than on control OP9 (Chapter 4), although this finding might reflect the higher proliferative capacity of T-cell progenitors compared to B-cell progenitors, rather than be a consequence of Notch signaling *per se*. Additional experiments are necessary to prove that *HOXA* genes are direct targets of Notch signaling and to unravel their function in T-cell development. The HoxA5 deficient mouse studied in Chapter 8 did not show any thymic defects, probably because of functional redundancy between different HoxA proteins. Mice deficient for several *HOXA* genes are likely to suffer from severe developmental defects during embryogenesis, necessitating difficult experiments such as performed for the Wnt3a knock-out (Chapter 7). A more feasible approach

would be to knock down several *HOXA* genes in FTOC using siRNA. Overexpression studies may elucidate whether HoxA genes indeed induce proliferation.

In contrast to *HOXA* genes, *TCFL5* was found to be upregulated in the early thymocyte subsets (Chapter 8), suggesting an extended function of TCFL5 at multiple points of T-cell development, like that of Hes1. Indeed, overexpression of TCFL5 seemed to positively affect thymocyte differentiation in FTOC. Again loss- and gain-of-function experiments will be useful to further clarify the role of TCFL5 in T-cell development. Furthermore, the working mechanism of TCFL5 needs to be elucidated. TCFL5 is thought to bind DNA through an E-box sequence[62]. Can TCFL5 induce TCR rearrangments? Does TCFL5 inhibit B-cell development, for instance by interfering with E2A function?

Wnt signaling and T-cell commitment

Wnt proteins generally are not considered to be T-cell commitment factors. However, in Chapter 5 we show that Wnt signaling is mainly active at the $CD34^+$ stages: before and around T-cell commitment (Figure 1). The strong blocks seen in $Tcf1(VII)^{-/-}$ and $Tcf1^{-/-}/Lef1^{-/-}$ mice demonstrate that Wnt signals are involved in T-cell development, but this has been interpreted as a failure in expansion of early subpopulations rather than a differentiation defect[63, 64]. We confirmed a predominantly proliferative function for Wnt signaling in Chapter 6: target genes of Wnt signaling in $CD34^+$ thymocytes appeared to be mainly involved in proliferation. No T-cell genes were found to be upregulated. Furthermore, Wnt3a deficiency (Chapter 7) and Wnt1/Wnt4 double deficiency[65] lead to decreased thymocyte numbers, but only to mild defects in T-cell development. To block binding of all possible Wnts, we overexpressed the Dickkopf protein in hematopoietic progenitors (Chapter 5). When cultured in FTOC, these cells expanded less than untransduced cells, but in addition showed a complete block at the DN1 stage. Because of the low numbers of cells expressing mDkk1, we were not able to further phenotype these arrested DN1 cells. It would be interesting to investigate whether these cells, like Notch deficient thymocytes[66], exhibit characteristics of B cells or other lineages.

Interestingly, $Tcf1^{-/-}$ hematopoietic progenitor cells failed to reconstitute the thymus of irradiated mice, even when injected intrathymically[64]. Apparently, the initial expansion of progenitors after entering the thymus is critical for the progression into T-cell development. Wnt3a deficient progenitors also failed to reconstitute the thymus after transplantation (Chapter 7), but whether these cells fail to expand in the thymus or whether they never reach the thymus because of extrathymic stem cell problems remains to be elucidated.

In conclusion, Wnts are probably not classic commitment factors, in that they induce a T-cell program and inhibit alternative lineage genes. Rather, they are necessary for proliferation of the early thymocyte subpopulations (Figure 3). But apparently, in the absence of Wnt signals and hence proliferation, T-cell differentiation is arrested. The

Wnt-driven expansion of the earliest thymocytes can therefore be considered as an essential part of the T-cell development process.

Induction of T-cell commitment

Having explored the different processes and factors that might play a role in T-cell commitment, we may now draw some conclusions. T-cell commitment is probably not induced by the expression of T-cell specific genes or by TCR gene rearrangements, as these indicate specification rather than commitment (Figure 3). For sure, lineage potential becomes restricted during the course of T-cell development. Nevertheless, no presently known factor plays a decisive lineage-specific commitment role through direct or indirect repression of alternative lineage genes, a role that in B-cell development is played by Pax5.

It is a tantalizing thought that there is no need for active suppression of alternative lineages in the thymus, as the thymic microenvironment only supports T-cell development (Figure 4). This is exemplified by the rapid increase of T-cell precursors and the almost complete absence of alternative lineages when hematopoietic progenitors are cultured on OP9-DL. Many lineages develop in the BM: B cells, NK cells, various myeloid lineages, erythrocytes and platelets. The stroma therefore provides growth and survival factors for all these lineages. Once cells start developing in one direction, other options should be foreclosed, to prevent any confusion of molecular programs (Figure 4). This might be the reason why pro-B cells, unlike DN1 thymocytes, do not develop into myeloid cells in colony assays after ectopic expression of the IL-2R or GM-CSF receptor[67]. The thymus provides an excellent environment for the development of early thymocytes: a high density of Notch ligand Delta, an abundance of Wnt proteins, presence of cytokines like IL-7, SCF and Flt3L and appropriate adhesion molecules. Development of any other lineage than T-cells simply does not stand a chance (Figure 4).

But does the thymic microenvironment actively induce T-cell commitment? Binding of Delta is clearly important for T-cell development, but may support other functions, such as proliferation or survival, rather than T-cell commitment. My conviction is that T-cell commitment is a not a process that is actively aimed for, but rather a spin-off of T-cell development. To become a T lymphocyte, a cell has to rearrange its TCR genes and induce a T-cell program. As a result of these changes, transcriptional and epigenetic modifications take place. Finally the cell will have arrived at a stage where it can only become T cell and nothing else.

Timing of events

In what order are the different environmental signals received and responded to? Unless they have a completely unexpected phenotype (e.g. $CD34^-$), the true thymus seeding cells should be contained within the $CD34^+CD38^-$ fraction. This subset (as a

whole) already expresses Hes1 (Figure 1). The $CD34^{+}CD1a^{-}$ population, containing the $CD38^{-}$ cells, has high levels of Wnt signaling (Chapter 5). In fact, expression of c-fos, although not necessarily only the target of Wnt signaling, peaks in the $CD38^{-}$ population (our microarray data, not shown).

These data demonstrate that both Notch and Wnt signals are received rapidly after entering the thymus (Figure 3). As the $CD34^{+}CD38^{-}$ population is heterogeneous and as yet no markers are available to further delineate this subset, we do not know whether either the Notch or the Wnt signal is received first. The OP9-DL co-culture system is a useful tool to study such questions. In Chapter 5 we have described a lentiviral Tcf-GFP reporter. Although this reporter needs some optimization, it can be used to determine how quickly cells receive a Wnt signal after starting OP9-DL culture. We are currently developing a similar Notch reporter, in which the DsRed gene is under control of CSL binding sites. If we succeed in transducing both reporters into a single progenitor cell, culture on OP9-DL will demonstrate whether cells first receive a Wnt or a Notch signal and whether these signals can simultaneously occur in a single cell or whether they are mutually exclusive. In even more sophisticated experiments, more reporters are added, to visualize signaling through E-box proteins, GATA3, STAT5 or other transcription factors.

The OP9-DL system can also be used to determine how long it takes a cell to induce a T-cell program after the initial Wnt and Notch signals, e.g. by quantitatively determining T-cell genes and TCR gene rearrangements at different time-points. The characteristics (TCR rearrangements, gene expression profile, reporter activation) of cells at certain time-points after culturing on OP9-DL can be correlated to a detailed phenotypic analysis of the thus obtained subset, to enable discrimination of the same subpopulation within the normal thymus or FTOC.

Proliferation before specification?

The initial Notch and Wnt signals apparently do not (directly) induce a T-cell program. What is their function? For Wnt signals, this probably is proliferation. As argued above (see ‘Notch as a T-cell commitment factor’), Notch signals may be involved in inducing expansion of the earliest thymocytes as well.

During the selection processes that are part of T-cell development, a large proportion of the thymocytes die from apoptosis and death by neglect. In order to get a reasonable number of mature T cells to reach the finish line, it is imperative that plenty of precursors start the T-cell development process. It is possible that progenitors entering the thymus are obliged to go through a fixed number of cell cycles before differentiation may start (Figure 3). In the previously mentioned article by Taghon et al.[43], the authors propose the existence of a ‘factor x’, which determines whether a cell, after receiving a Notch signal, starts to express T-cell genes. This would explain why cells cultured on OP9-DL

differentiate a-synchronously[43]. It may well be possible that 'factor x' in fact represents a certain degree of expansion.

Do Wnt and Notch signals combine forces to induce proliferation of the earliest thymocytes (Figure 3)? It is generally thought that Wnt and Notch fulfill opposing functions in the thymus: Wnt in proliferation, Notch in differentiation, and that these processes are mutually exclusive. However, as indicated in Figure 1, the patterns of Wnt and Notch signaling seem highly correlated: they occur in the same subsets. Nevertheless, as argued in the first paragraphs of this General discussion, presumably all subsets are heterogeneous and include both proliferating and differentiating cells. Reporter assays as described above should sort this out.

Both Wnt[68] and Notch[44,69] signaling have been implicated in HSC self-renewal. We have confirmed a role for Wnt3a in HSC maintenance in Chapter 7. A recent report has suggested molecular cooperation between the Notch and Wnt pathways: Notch would keep HSCs in an undifferentiated state, allowing Wnt to induce proliferation[69]. Furthermore, Wnt signaling in HSCs upregulated Notch target genes, although no molecular explanation was provided[69].

Positive interaction between Wnt and Notch signaling has also been shown outside the hematopoietic system[70-72], but other studies have indicated opposing functions for both pathways. For instance in skin, Notch signalling induces differentiation[73,74] and acts as a tumor suppressor by inhibiting β-catenin-mediated signalling[75].

Differential effects of Notch signaling (e.g. proliferation or differentiation) may depend on the ligand that is encountered. In the BM, Notch signaling through Jagged may inhibit differentiation of HSCs. Inhibition of differentiation may also be the role of Jagged in the thymus, as it has been shown that a Jagged signal is able to block differentiation into the B- cell lineage[76], although this is controversial[52]. In any case, differentiation into the T-cell lineage is dependent on Delta[50,52,76].

FUTURE CHALLENGES

The remarkable role of Notch in T-cell development was discovered only six years ago[66]. It may therefore be possible that the quintessential T-cell commitment factor will be found in the future. Nevertheless, it is my conviction that the major molecular players in T-cell development are now known. Major break-throughs with regard to T-cell development will more likely be achieved in other areas. Three subjects will be pivotal for future research.

First, the thymus seeding cell and its immediate progeny will have to be identified by detailed immunophenotyping and subsequently characterized in terms of lineage potential. The best chance to find these extremely rare cells is probably in the human

thymus, because of the huge cell numbers it contains. But, especially for the human thymocyte subsets, new markers will have to be identified. A number of markers currently used in the mouse (e.g. CD24, CD27, CD117, Flt3[13-15,77] should also be tested for expression in the human thymus. In addition, microarray studies will be helpful to identify novel markers, that preferentially reflect key processes.

Second, the different features of the thymic microenvironment should be further characterized. The thymic microenvironment has long been known to be extraordinary[78] and I have hypothesized that it so strongly supports T-cell development that in fact a real T-cell commitment signal might not be needed. Preferentially using confocal microscopy (because of the 3D architecture of the thymus) and good antibodies, different microenvironmental niches (with presence of particular Wnts, high or low levels of certain Notch ligands, etc.) may be identified. The ultimate goal would be to identify the migration pattern of developing thymocytes through the putative sequential microenvironmental niches and to delineate how this correlates with their differentiation.

Finally, future research should focus less on individual factors and pathways and more on the integration of the different signals. Not only interactions between Wnt and Notch signaling should be further investigated, but also those involving other microenvironmental factors (cytokines, adhesion molecules) and other transcription factors (GATA3, E-box proteins and many others). How do they all cooperate to set up the T-cell program? Furthermore, studies into the establishment of lineage specific gene programs will increasingly concentrate on novel regulatory mechanisms like microRNAs and epigenetic phenomena.

Step by step such studies will lead to a complete understanding of T-cell development in all its aspects and to the final goal: development of better diagnostics and therapies for diseases involving the early stages of T-cell development, such as T-ALL and SCID. Flow-cytometric assays will be central for fast and accurate diagnosis of patients into specific subcategories, associated with different prognoses and therapies. For such studies, development of new, highly specific high affinity antibodies against key molecules involved in lymphocyte development is necessary.

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ABBREVIATIONS

APC	Adenomatous Polyposis Coli
BFU	burst forming unit
bHLH	basic-helix-loop-helix
BM	bone marrow
CFU	colony forming unit
CLP	common lymphoid progenitor
CSL	CBF/Suppressor of Hairless/Lag-1
DC	dendritic cell
DL	Delta
DN	double negative
DP	double positive
E	day of embryonic development
FLT3L	Fms-related tyrosine kinase 3 ligand
FTOC	fetal thymic organ culture
Fz	Frizzled
GFP	green fluorescent protein
ISP	immature single positive
GSK3	Glycogen synthase kinase-3
Hes	Hairy-enhancer of split
HSC	hematopoietic stem cell
IL	Interleukin
IC-Notch	intracellular domain of Notch
Lef	Lymphoid enhancing factor
LRP	Low-density lipoprotein-receptor-related protein
LSK	lineage$^-$ Sca-1$^+$ c-Kit$^+$
NK	natural killer
pTα	pre-T-cell receptor α
RAG	recombination activating gene
TPO	thrombopoietin
SCF	stem cell factor
SCID	severe combined immunodeficiency disease
SP	single positive
T-ALL	T-cell acute lymphocytoblastic leukemia
TCR	T-cell receptor
TCRA/B/G/D	gene for T-cell receptor α/β/γ/δ
TdT	Terminal deoxynucleotidyl Transferase
TEA	T-early alpha
TEC	thymic epithelial cell
Tcf	T-cell factor
UCB	umbilical cord blood

SUMMARY

All cells of the immune system are derived from hematopoietic stem cells (HSCs) located in the bone marrow (BM). While all other hematopoietic lineages develop in the BM, this is not the case for T cells. For the complicated differentiation events that comprise T-cell development, such as rearrangements of the T-cell receptor (TCR) genes, positive and negative selection events and effector cell differentiation, a specialized environment is required: the thymus.

Precursor cells from the BM seed the thymus and start developing into T cells. The various steps of thymocyte differentiation are guided by proteins produced by the thymic stroma. Many aspects about the consecutive stages of T-cell development and the proteins involved are still incompletely understood. Better understanding of these processes will contribute to the improvement of diagnostics and therapies for diseases in which T-cell development is affected, such as T-cell acute lymphoblastic leukemia and SCID.

In Chapter 2 of this thesis, we determined the numbers and relative contributions of the different thymocyte subpopulations in the human thymus and how these values change as children age. The thymus appeared to be most active in children 6 to 9 months of age. In addition, we demonstrated that B-cell development takes place in normal human thymus.

To gain more insight into the regulation of TCR gene rearrangement processes, we studied the several thymocyte subpopulations in more detail in Chapter 3. In each subpopulation, we quantitatively determined the amount of rearranged alleles of each TCR locus. Furthermore we used DNA microarray to study up- and downregulation of genes in each subset, in relationship to the TCR gene rearrangements.

It remains elusive which progenitor cells from the BM seed the thymus. Are these cells already committed to the lymphoid or T-cell lineage? Or do these cells retain potential to develop into any hematopoietic cell type and do they develop into T cells only because of signals emanating from the thymic stroma, which induce T-cell development? In Chapter 4 we attempted to resolve this issue by studying the most immature populations in the thymus. Using several culture systems, we investigated whether these thymocytes are able to develop into different hematopoietic lineages. We conclude that the thymus-seeding cells are very immature and possibly are true HSCs.

Two signal transduction pathways that are considered to be crucial for T-cell development were studied in Chapters 5 to 8: the Wnt and the Notch cascade. Both signal transduction routes are activated by extracellular molecules: the Wnt pathway by proteins of the Wnt family that are secreted by the thymic stroma as well as by thymocytes themselves and the Notch pathway by Delta and Jagged proteins that are expressed on the cell membrane of stroma cells.

In Chapter 5 we demonstrate that Wnt signaling predominantly occurs in the most immature thymocyte subpopulations. This is not determined by membrane proximal factors, as Wnt proteins are mainly produced by the more mature thymocytes, while Wnt receptors are expressed at equal levels in all subsets. Whether cells are able to respond to a Wnt signal appears to be determined by the presence of activating factors and the absence of inhibiting factors in a cell.

Which genes are induced or repressed after a thymocyte receives a Wnt signal? This question was answered in Chapter 6, using three different methods to induce Wnt signaling in combination with DNA microarrays. Most of the identified genes appeared to be involved in proliferation and in adhesion to the thymic stroma.

To investigate the function of a specific Wnt gene during T-cell development and hematopoiesis in general, we studied mice deficient for Wnt3a in Chapter 7. This mutation is embryonically lethal, which complicates experimental approaches. By culturing fetal thymi and hematopoietic progenitor cells from fetal liver *in vitro* and by transplantations into irradiated mice, we were able to study the immune system of these mice. From our results we can conclude that Wnt3a plays a role in T-cell but not B-cell development and is critical for HSC maintenance.

Chapter 8 focuses on the Notch signal transduction pathway. Similar to the experiments described in Chapter 6 for the Wnt pathway, we here investigated which genes are induced or repressed by a Notch signal. For this purpose, we activated Notch signaling in progenitor cells from human umbilical cord blood. Although Notch signaling is essential for the development of T cells from multipotent precursors, we did not find upregulation of T-cell specific genes. Apparently Notch signaling does not directly induce a T-cell program in progenitor cells. We identified a number of novel Notch target genes, which may be important for T-cell development.

Finally, the results of the different chapters are integrated in Chapter 9, with emphasis on T-cell commitment. I suggest that alternative lineages are not actively repressed during T-cell development, as the thymus provides an excellent microenvironment for the preferential development of T cells.

Why is the thymic microenvironment so special that T cells develop only in this organ? The thymic stroma provides many signals that promote T-cell development, such as cytokines, adhesion molecules, Wnt proteins and Notch ligands. However, neither Wnt nor Notch signaling directly induces a T-cell program. My hypothesis is that Wnt and Notch signals cooperate to induce massive proliferation of the thymus-seeding cells. Only when enough thymocytes have been generated, a subsequent Notch signal is able to effectively initiate T-cell differentiation.

SAMENVATTING VOOR NIET-IMMUNOLOGEN

Het immuunsysteem zorgt voor de afweer tegen ziekteverwekkers (bijvoorbeeld virussen en bacteriën) die het lichaam binnendringen. Een belangrijke rol in het immuunsysteem wordt vervuld door T-cellen. Dit zijn afweercellen die circuleren door het bloed en de lymfeklieren, op zoek naar geïnfecteerde cellen, met als doel deze te doden. Bovendien kunnen ze andere afweercellen aanzetten tot een adequate afweerreactie. T-cellen zijn dus zeer gespecialiseerde cellen.

T-cellen dragen op hun celoppervlak zogenaamde T-celreceptoren, moleculen waarmee ze andere lichaamscellen aftasten. Iedere T-cel draagt één type T-celreceptor. Omdat een mens miljarden T-cellen heeft, kunnen alle T-cellen van een mens tezamen in principe iedere vreemde indringer herkennen. Al die miljarden verschillende T-celreceptoren worden gevormd door het willekeurig aan elkaar plakken (‘recombineren’) van verschillende gensegmenten die coderen voor delen van de T-celreceptoren. De aldus ontstane T-celreceptoren worden uitgebreid getest: ze moeten goed in elkaar zitten, maar ze mogen geen lichaamseigen structuren herkennen. Alleen cellen die er in slagen om een goede T-celreceptor te produceren, mogen uitgroeien tot volwassen (‘rijpe’) T-cellen.

Alle afweercellen in het lichaam ontstaan uit bloedstamcellen die zich bevinden in het beenmerg. Ook de ontwikkeling van bloedstamcellen tot rijpe afweercellen vindt plaats in het beenmerg. Dit geldt echter niet voor T-cellen! Het recombineren van de T-celreceptor gensegmenten, het testen van de T-celreceptoren en het uitrijpen tot gespecialiseerde T-cellen zijn ingewikkelde processen, die in principe alleen plaatsvinden in de thymus (zwezerik).

Voorlopercellen uit het beenmerg reizen naar de thymus en beginnen daar aan hun ontwikkeling tot T-cel. De zich ontwikkelende cellen in de thymus worden thymocyten genoemd. De thymusomgeving (het ‘stroma’) maakt signaalstoffen die ervoor zorgen dat de thymocyten de verschillende ontwikkelingsstappen doormaken. Bepaalde signaalstoffen zetten thymocyten aan tot celdeling, andere zorgen bijvoorbeeld dat de recombinatie van de T-celreceptor gensegmenten begint. Er is nog veel onduidelijk over de opeenvolgende ontwikkelingsstappen die plaatsvinden in de thymus en de precieze rol van verschillende signaalstoffen daarbij. Een beter begrip van deze verschillende processen zal bijdragen aan de verbetering van diagnostiek en therapie voor ziekten waarbij de T-celontwikkeling is aangedaan, bijvoorbeeld T-cel leukemie en aangeboren immuundeficiënties.

De verschillende ontwikkelingsstadia van thymocyten (‘subpopulaties’) zijn te onderscheiden door thymocyten te kleuren met fluorescerende markers. In Hoofdstuk 2 van dit proefschrift onderzochten wij in welke aantallen en verhoudingen de verschillende subpopulaties aanwezig zijn in de humane thymus, en hoe deze waarden veranderen

naarmate kinderen ouder worden. De thymus blijkt het meest actief in baby's van 6 tot 9 maanden oud. Ook tonen wij in Hoofdstuk 2 aan dat een kleine populatie van B-cellen, afweercellen die normaalgesproken uitrijpen in het beenmerg, zich ontwikkelt in de thymus.

Om meer inzicht te krijgen in de regulatie van de T-celreceptor recombinatieprocessen, kijken wij in Hoofdstuk 3 nog gedetailleerder naar de verschillende populaties van thymocyten. Wij bestudeerden in welke subpopulaties de recombinaties van de verschillende T-celreceptor gensegmenten plaatsvinden en welke genen hierbij 'aan' en 'uit' gezet worden.

Het is onduidelijk welk type voorlopercellen uit het beenmerg naar de thymus reizen. Zijn deze cellen voorbestemd om T-cel te worden? Of zijn het cellen die nog alle kanten op kunnen, maar die zich louter tot T-cel zullen ontwikkelen omdat ze in een thymusomgeving terechtkomen en signalen krijgen die hen tot T-celontwikkeling aanzetten? In Hoofdstuk 4 proberen wij deze vraag te beantwoorden door de meest onrijpe subpopulaties in de thymus uitgebreid te bestuderen. Wij onderzochten met behulp van verschillende kweeksystemen of deze thymocyten nog in staat zijn zich te ontwikkelen tot andere afweercellen dan T-cellen. Dit blijkt het geval te zijn, waardoor wij kunnen concluderen dat de cellen die binnenkomen in de thymus zeer onrijp en mogelijk zelfs echte bloedstamcellen zijn.

De signaalstoffen die worden geproduceerd door het thymusstroma binden aan receptoren op het celoppervlak van de thymocyten. Hiermee zetten ze in de thymocyten een reeks van gebeurtenissen in gang (een 'signaaltransductiecascade') die uiteindelijk leidt tot het 'aan' en 'uit' zetten van bepaalde genen, bijvoorbeeld genen die zorgen voor celdeling of voor recombinatie van gensegmenten. Twee signaaltransductiecascades worden bestudeerd in de Hoofdstukken 5 t/m 8: de Wnt-cascade en de Notch-cascade. De Wnt-cascade wordt aangezet door signaalstoffen van de Wnt-familie, die worden uitgescheiden door het thymusstroma en de thymocyten zelf; de Notch-cascade wordt aangezet door de signaalstoffen Delta en Jagged, die op het celoppervlak van de stromacellen zitten.

In Hoofdstuk 5 bestudeerden wij in welke subpopulaties van thymocyten de Wnt-cascade actief is en tonen aan dat dit voornamelijk in de meest onrijpe populaties is. Wnt-eiwitten blijken echter vooral te worden geproduceerd door de rijpere thymocyten en Wnt-receptoren zijn in gelijke mate aanwezig in alle subpopulaties. Of cellen in staat zijn te reageren op een Wnt-signaal, blijkt te worden bepaald door de aanwezigheid van factoren in de cel die de Wnt-cascade stimuleren en de afwezigheid van remmende factoren.

Welke genen 'aan' en 'uit' worden gezet nadat een thymocyt een Wnt-signaal heeft gekregen, hebben wij onderzocht in Hoofdstuk 6. Wij vonden voornamelijk genen die zorgen voor celdeling en voor het plakken ('adhesie') van de thymocyten aan het stroma.

De Wnt-cascade is actief in sommige subpopulaties van thymocyten en zet bepaalde genen ‘aan’. Maar zijn Wnt-eiwitten werkelijk onmisbaar voor T-celontwikkeling? In Hoofdstuk 7 bestuderen wij muizen die een specifiek Wnt-eiwit, Wnt3a, missen. Omdat Wnt-eiwitten belangrijk zijn tijdens de foetale ontwikkeling, gaan Wnt3a knock-out muizen als embryo dood, hetgeen experimenten bemoeilijkt. Door foetale thymocyten en bloedstamcellen uit de foetale lever in kweekschaaltjes te laten uitrijpen, kunnen wij toch het afweersysteem bestuderen. Uit onze resultaten kunnen wij concluderen dat Wnt3a een rol speelt in T-celontwikkeling maar niet in B-celontwikkeling, en vooral belangrijk is voor bloedstamcellen.

De Notch-signaaltransductiecascade staat centraal in Hoofdstuk 8. Net als in Hoofdstuk 6 voor de Wnt-cascade, wordt hier gekeken welke genen ‘aan’ en ‘uit’ worden gezet door een Notch-signaal. Hier hebben wij een kunstmatig Notch-signaal gegeven aan bloedstamcellen uit humaan navelstrengbloed. Hoewel Notch-signalen onontbeerlijk zijn voor een bloedstamcel om zich te ontwikkelen tot T-cel, werden er geen genen actief die specifiek zijn voor T-cellen. Wel vonden wij een aantal nieuwe Notch-gereguleerde genen, die mogelijk belangrijk zijn voor T-celontwikkeling.

De resultaten uit de voorgaande hoofdstukken worden in een breder verband beschouwd in Hoofdstuk 9, waarbij de nadruk ligt op T-cel ‘commitment’, het proces dat zorgt dat een cel op een bepaald moment alleen nog T-cel kan worden en niets anders. Ik suggereer dat de thymus een zo gunstige omgeving vormt voor T-celontwikkeling, dat het actief remmen van andere mogelijke lotsbestemmingen onnodig is.

Wat maakt de thymusomgeving zo speciaal, dat T-cellen zich alleen daar ontwikkelen? Het thymusstroma produceert grote hoeveelheden signaalstoffen, bijvoorbeeld Wnt- en Notch-signalen, die T-celontwikkeling bevorderen. Maar Wnt- noch Notch-signalen zetten direct T-celgenen ‘aan’. Mijn hypothese is dat Wnt- en Notch-signalen samen zorgen voor een enorm aantal celdelingen in de meest onrijpe thymocyten. Pas als er voldoende thymocyten zijn gegenereerd, kan een volgend Notch-signaal ervoor zorgen dat de T-celontwikkeling werkelijk in gang wordt gezet.

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Floor

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