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Identification of a T Lineage-Committed Progenitor in Adult Blood

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SUMMARY

With help of a hCD25 reporter controlled by pre-T cell receptor α (Ptcra) regulatory elements, T cell precursors were identified in peripheral blood. Sca-1^{+}IL-7Rα[+]Flt3^{−} precursors that were c-kit[lo]Thy-1^{hi} generated T lineage cells when cultured on OP9-DL1 stromal cells and upon transfer into $Rag2^{-/-}Il2rg^{-/-}$ mice. No B cells were generated in vivo and only few in vitro. These cells, which we call circulating T cell progenitors (CTP), were found at the same frequency in Foxn1[nu/nu] thymus-deficient mice and wild-type mice, indicating that they were pre- rather than postthymic. Inhibition of Notch-dependent transcription in vivo reduced the frequency of intrathymic early T cell progenitors (ETP), but not CTP, indicating that the latter are less Notch dependent. Thus, CTP represent T lineage-committed T cell precursors linking extrathymic with intrathymic lymphopoiesis in adult mice.

INTRODUCTION

All blood-cell lineages are derived from self-renewing hematopoietic stem cells (HSC). HSC generate Fms-like tyrosine kinase receptor 3 (Flt3)-positive multipotent progenitors (MPPs), that are likewise lineage negative (Lin[−]), Sca-1^{+}, and c-kit[hi] (LSK) (Adolfsson et al., 2001), as well as RAG-1^{+} early lymphoid progenitors (ELPs) (Igarashi et al., 2002), L-selectin[+] progenitors (LSPs) (Perry et al., 2004), and common lymphoid progenitors (CLPs) (Kondo et al., 1997). In the adult thymus, progenitors from the bone marrow are recruited via the blood stream. However, the nature of thymic immigrants has remained elusive. Based on phenotypic similarity to the early T cell progenitors (ETPs), multipotent LSK cells in blood were assumed to represent the most effective thymic immigrants in adult mice (Allman et al., 2003; Benz and Bleul, 2005; Schwarz and Bhandoola, 2004). However, immigration of these cells into the thymus has not been directly shown, and ETPs unlike LSK cells are dependent on Notch signals (Sambandam et al., 2005; Tan et al., 2005).

CLP-2 cells that were identified in the BM of transgenic reporter mice that express human CD25 (hCD25) under control of the pre-T cell receptor α (Ptcra) promoter and enhancer may likewise constitute a population of thymic immigrants (Gounari et al., 2002; Martin et al., 2003). Such cells have a Lin[−]c-kit^{−/lo}B220[+] phenotype and originate from Lin[−]c-kit[+]B220[−]IL-7Rα^{+} CLP cells. CLP-2 efficiently enter the thymus upon intravenous transfer and have limited self-renewal capacity (Martin et al., 2003; Scimone et al., 2006). When cultured in the presence of Notch ligands, CLP-2 upregulate c-kit, downregulate B220, and quickly become DN2 cells (Krueger et al., 2006). Of note, early thymic immigrants after BM transfer are mostly c-kit[−] and enriched for B220^{+} cells (Mori et al., 2001). However, CLP-2 cells have not yet been detected in blood.

Homing of extrathymic progenitors to the thymus depends on P-selectin-PSGL-1 interactions, and P-selectin binding capacity was detected on LSK, CLP, and ETP cells (Rossi et al., 2005). In addition, the CCR9 chemokine receptor plays an important role, because $Ccr9^{-/-}$ BM-derived progenitors home poorly (Rossi et al., 2005; Scimone et al., 2006; Uehara et al., 2002).

Inside the thymus, the heterogeneous double-negative (DN) 1 thymocyte subset (CD44^{+}CD25^{−}) contains the most potent T cell precursors that were originally characterized as CD4^{lo}c-kit^{+} (Moore and Zlotnik, 1995; Wu et al., 1991). These cells have the potential to generate T, B, NK, and lymphoid dendritic cells. More detailed analysis of the DN1 subset led to the identification of LSK and IL-7Rα^{−/lo} ETPs (Allman et al., 2003) with high T, but only limited B and myeloid, potential. ETPs are heterogeneous with regard to Flt3 or CC chemokine receptor 9 (CCR9)-eGFP reporter gene expression (Benz and Bleul, 2005; Sambandam et al., 2005). Flt3^{+} ETPs are more immature, and loss of Flt3 or CCR9-eGFP expression coincides with loss of B cell potential. Subdivision of DN1 cells according to c-kit and heat-stable antigen (HSA) revealed the most potent T cell progenitors in the DN1a and b subsets that are likely linked by a precursor-product relationship (Porritt et al., 2004).

Progressive loss of B cell potential (Benz and Bleul, 2005; Sambandam et al., 2005) and clonal analysis of the most immature ETPs (Benz and Bleul, 2005) is best compatible with the notion that T and B lineages diverge intrathymically, although this is disputed (Balciunaite et al., 2005; Jenkinson et al., 2006; Lu et al., 2005). In contrast, T lineage commitment in fetal life has been claimed to occur prethymically. Of note, no CLPs have been identified in the fetus (Douagi et al., 2002; Kawamoto et al., 1997),

and in ontogeny, T cell progenitors appear to emerge earlier than B cell progenitors (Kawamoto et al., 2000). In addition, T cell progenitors lacking B or myeloid potential were found in fetal blood and were characterized either as c-kit$^+$IL-7R$^+$ (Ikawa et al., 2004), expressing paired immunoglobulin-like receptors (Masuda et al., 2005), or to be of the c-kit^{lo}Thy-1$^+$ phenotype (Rodewald et al., 1994).

Signaling from Notch transmembrane receptors has been implicated in lineage-fate decisions of cells in multiple developmental systems (Artavanis-Tsakonas et al., 1999). Specifically, conditional deletion of Notch1 in hematopoietic progenitors results in failure of T cell development and accumulation of B cells in the thymus (Radtke et al., 1999; Wilson et al., 2001). Inhibition of Notch-mediated transcription or interference with Notch-Notch ligand interactions has similar consequences (Koch et al., 2001; Maillard et al., 2004). Conversely, overexpression of intracellular Notch1 leads to aberrant T cell development in the BM (Pui et al., 1999). However, it has also been claimed that fetal thymic precursors are T lineage-committed, perhaps independently of Notch (Harman et al., 2005; Masuda et al., 2005).

Here we identified prethymic T cell progenitors circulating in blood of adult mice by means of transgenic reporter mice that express hCD25 under control of the Ptcra promoter. These cells have a c-kit^{lo}Thy-1$^+$ phenotype. In contrast to BM CLPs and CLP-2, these cells possess efficient T, but only very limited B and NK, potential. Of note, these progenitors are not affected by inhibition of Notch-dependent transcription, consistent with the possibility that extrathymic T lineage commitment is less dependent on Notch while Notch signals clearly can induce T lineage commitment and are essentially required at later stages of T cell development.

RESULTS

Lineage-Negative hCD25$^+$ Cells in Peripheral Blood of Adult Mice

T cell development in the thymus depends on continuous recruitment of hematopoietic progenitors from the BM via the blood. hCD25$^+$ T cell precursors have so far been identified in BM and thymus, and it has been shown that hCD25$^+$ CLP-2 can efficiently seed the thymus upon intravenous transfer. Here we have analyzed whether Lin$^-$hCD25$^+$ cells can be detected in the blood of mice under steady-state conditions. We found that approximately 0.06% of Lin$^-$ cells in peripheral blood expressed the hCD25 marker, whereas no background staining was detected in reporter-negative control mice (Figure 1A). This corresponds to a frequency of 4.1 ± 0.3 per 10^5 leukocytes or 330 ± 24 Lin$^-$hCD25$^+$ cells per mL blood. Further surface-marker characterization revealed that most of these cells are c-kit$^{-/lo}$ and some expressed B220. In both BM-derived CLP-1 and CLP-2 cells, expression of the hCD25 transgene corresponds to expression of pre-TCRα mRNA, whereas hCD25$^+$CD19$^+$ cells in BM, which are already committed to the B lineage, lack pre-TCRα

expression (Gounari et al., 2002). Thus, we analyzed pre-TCRα expression in peripheral Lin$^-$hCD25$^+$ cells as an indicator of T cell potential. Figure 1B shows that the pre-TCRα message is clearly present in circulating Lin$^-$hCD25$^+$ cells, suggesting that these cells might contain T lineage potential.

Next, we compared the surface phenotype of circulating Lin$^-$B220$^+$hCD25$^+$ and Lin$^-$B220$^-$hCD25$^+$ cells with that of Lin$^-$hCD25$^+$ CLP-1 (Lin$^-$hCD25$^+$B220$^-$c-kit$^+$) and CLP-2 (Lin$^-$hCD25$^+$B220$^+$c-kit$^{-/lo}$) cells from BM and hCD25$^+$ DN1 (Lin$^-$hCD25$^+$CD25$^-$CD44$^+$) cells from thymus, the majority of which were shown to be DN1a/b cells (Figure 1C; Krueger et al., 2006). CLP-1 cells were mostly c-kit$^+$IL-7Rα$^+$Flt3^{hi}Sca-1$^+$CD44^{hi}Thy-1.1$^-$, with a minor fraction of cells expressing the Thy-1.1 marker. CLP-2 cells were c-kit$^{-/lo}$IL-7Rα$^+$Flt3$^{-/lo}$Sca-1$^{-/lo}$CD44^{hi}Thy-1.1$^-$. In contrast, circulating Lin$^-$hCD25$^+$B220$^+$ cells were c-kit$^-$IL-7Rα$^-$Flt3$^-$Sca-1$^+$CD44$^+$ and heterogeneous for Thy-1.1$^+$ expression and thus did not correspond phenotypically to BM-derived CLP-2 cells. Circulating Lin$^-$hCD25$^+$B220$^-$ cells were c-kit$^+$IL-7Rα$^+$Flt3$^{-/lo}$Sca-1$^+$CD44^{hi}Thy-1.1$^+$, and thymic DN1 hCD25$^+$ cells were c-kit^{hi}IL-7Rα$^{-/lo}$Flt3^{lo}Sca-1$^+$CD44^{hi}Thy-1.1^{lo}. Approximately 4% of cells with a Lin$^-$B220$^-$c-kit^{lo}IL-7Rα^{lo}Flt3$^{-/lo}$Sca-1$^+$Thy-1.1$^+$ phenotype in blood expressed hCD25 and it is presently not known whether the hCD25-negative subset of these cells has similar functional properties as the hCD25$^+$ subset (see Figure S1 in the Supplemental Data available online). This is similar to the previous analysis of CLP-1 cells in bone marrow where only a fraction of these cells express hCD25 (Martin et al., 2003). Thus, blood-derived Lin$^-$hCD25$^+$B220$^-$ cells displayed a surface phenotype somewhat similar to CLP-1 cells, but differing with respect to expression of Flt3 and, markedly, by expression of Thy-1.1, suggesting that these cells may constitute an adult counterpart of previously described c-kit^{lo}Thy-1$^+$ T cell progenitors from fetal blood (Rodewald et al., 1994). In fact, these precursors in fetal blood were likewise found to express Ptcra (Bruno et al., 1995).

Developmental Potential of Lin$^-$hCD25$^+$ Cells

In order to assess the developmental potential of hCD25$^+$ cells from peripheral blood, we employed OP9-GFP and OP9-DL1 stromal cell cocultures (Schmitt and Zuniga-Pflucker, 2002). After 18 days of coculture on OP9-DL1 cells, CD4$^+$CD8$^+$ T cell progeny could be detected in cultures of hCD25$^+$c-kit^{lo}B220$^-$ cells, whereas no T cell progeny was found after coculture of hCD25$^+$B220$^+$ cells (Figure 2A). Coculture of hCD25$^+$c-kit^{lo}B220$^-$ cells with OP9-GFP cells showed that these cells also contained some NK and B cell potential (Figure 2A). No T cell development was observed in these cultures, as evident by the absence of Thy-1.1$^+$ cells, suggesting that T cell development from these precursors is Notch dependent (Figure 2A). Coculture of hCD25$^+$B220$^+$ cells on OP9-GFP cells again did not result in any detectable progeny (data not shown). In order to quantitate T, B, and NK lineage potential of blood-derived hCD25$^+$c-kit^{lo}B220$^-$ cells
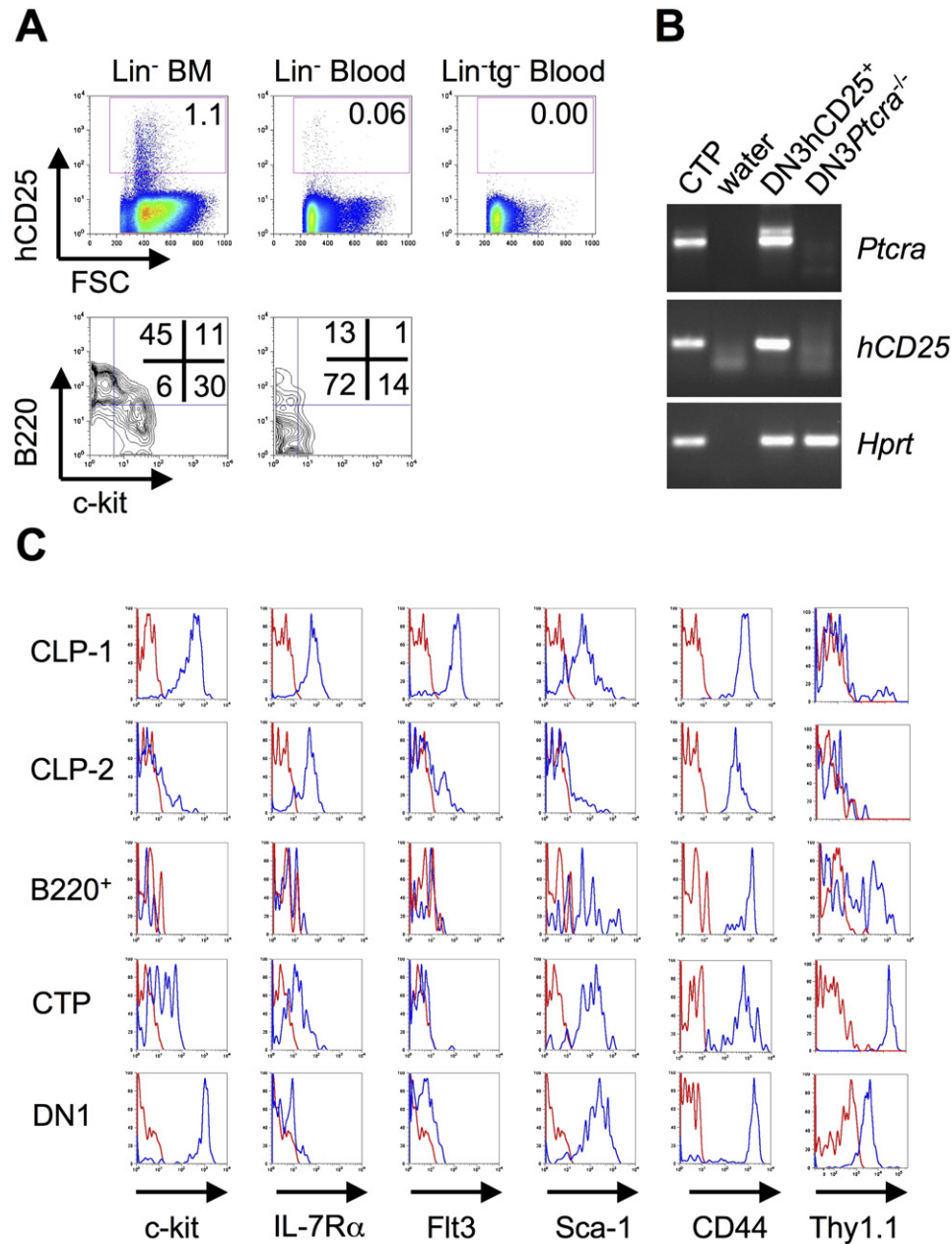
Figure 1. Circulating Lin⁻hCD25⁺ Precursors

(A) Lineage-depleted BM and blood cells from hCD25 transgenic and nontransgenic mice were stained for lineage markers, hCD25, c-kit, and B220. The lower panels show the expression of c-kit and B220 of electronically gated Lin⁻hCD25⁺ cells. Numbers in FACS plots indicate percentages of cells within gates or quadrants.

(B) Expression of pre-TCRα in circulating Lin⁻hCD25⁺ cells. RT-PCR was performed on 250 CTP cells. The same amount of cDNA from hCD25⁺ DN3 cells and DN3 cells from *Ptcra⁻/⁻* mice was used as positive and negative controls, respectively. One representative out of two independent experiments is shown.

(C) Expression of surface markers on different Lin⁻hCD25⁺ populations. BM CLP-1 (Lin⁻hCD25⁺c-kit⁺B220⁻) and CLP-2 (Lin⁻hCD25⁺c-kit⁻/loB220⁺) cells, blood Lin⁻hCD25⁺B220⁺ cells ("B220⁺"), and CTP (Lin⁻hCD25⁺B220⁻) and thymic hCD25⁺ DN1 (Lin⁻CD25⁻CD44hihCD25⁺) cells were stained for c-kit, IL-7Rα, Flt3, Sca-1, CD44, and Thy-1.1. Histograms show expression of the respective surface markers (blue histograms) or unstained controls (red histograms) of electronically gated populations as indicated above. One representative out of two independent experiments is shown.

and BM-derived CLP-2 cells, we sorted different numbers of precursor cells onto OP9-DL1 and OP9-GFP cell-containing cultures and determined the frequency of lineage-positive outgrowth. This analysis revealed that the potential of hCD25⁺c-kitloB220⁻ cells to generate T cells was much higher than the potential to generate B or NK
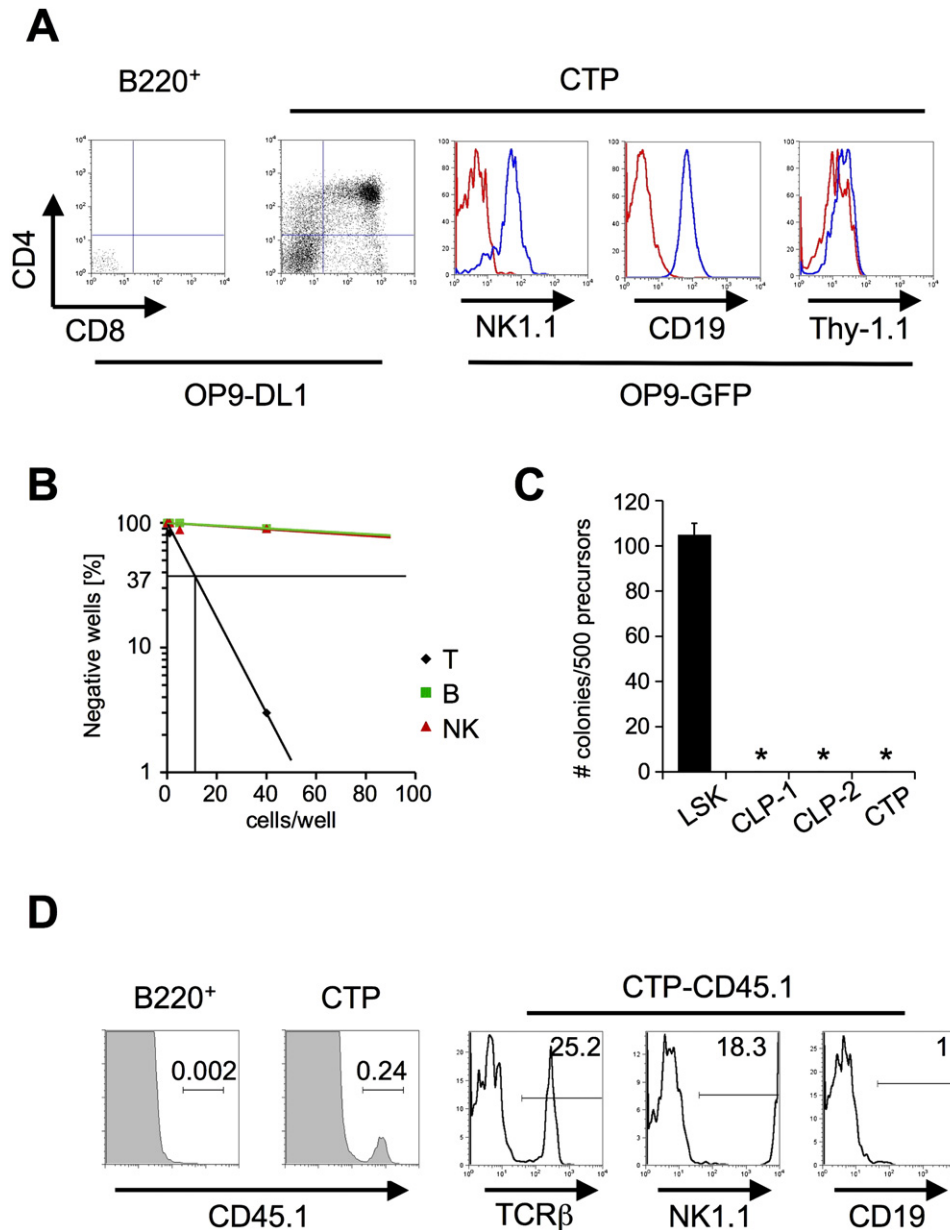
Figure 2. Developmental Potential of Circulating Lin⁻hCD25⁺ Cells

(A) Sorted Lin⁻hCD25⁺B220⁻ CTP and Lin⁻hCD25⁺B220⁺ cells were cocultured on OP9-DL1 or OP9-GFP cells for 18 days. Cells were stained for CD4, CD8, CD19, NK1.1, and Thy-1.1, and individual wells were analyzed by FACS. Blue histograms represent specific staining, red histograms represent unstained controls. 800 CD19⁺ (center) and 550 NK1.1⁺ (left) cells were recovered from starting cultures of 200 CTP. One representative out of three independent experiments is shown.

(B) Limiting dilution analysis of T, B, and NK potential of Lin⁻hCD25⁺B220⁻ cells (CTP) from peripheral blood. 1, 5, or 40 cells were directly sorted onto OP9-DL1 or OP9-GFP cells and analyzed by FACS after 18 days. Wells containing >50 (B and NK potential) or >100 (T potential) lineage-positive cells were scored positive.

(C) Analysis of myeloid potential of CTP cells. 500 CTP, BM-derived LSK, CLP-1, and CLP-2 cells were cultured in methylcellulose containing SCF, IL-3, IL-6, and Erythropoietin and colonies were counted microscopically. Data are shown as mean ± SEM (n = 4).

(D) 1000 sorted CTP or 50 Lin⁻hCD25⁺B220⁺ cells (CD45.1) were injected intravenously into irradiated $Rag2^{-/-}Il2rg^{-/-}$ recipients and spleens were analyzed 5 weeks after transfer by flow cytometry for expression of TCRβ, NK1.1, and CD19. One representative out of two independent experiments with 2 mice per group is shown.

cells (Figure 2B). Approximately 1 in 11 cells was able to give rise to T cells, whereas B or NK potential amounted to 1 in 390 or 1 in 330, respectively. In contrast, BM-derived CLP-2 cells had similar T and B cell potential (1 in 14 and 1 in 12, respectively), but lower NK potential (1 in 63) (Krueger et al., 2006). The relatively low NK lineage

potential in both assays might have been due to suboptimal conditions, i.e., assays were performed in the absence of IL-15. At concentrations of 200 cells per well (Figure 2A), we did not detect any wells containing cells of both lineages in the analysis of B and NK lineage potential on OP9-GFP cells. Thus, whereas lineage potential analysis of CLP-2 cells revealed a similar frequency of B and T lineage precursors (indicating that this assay was sufficiently sensitive to detect B lineage potential), the frequency of T cell precursors among hCD25+c-kit^{lo}B220$^-$ peripheral blood cells was much higher than that of B cell precursors. Therefore, we termed this population of cells "circulating T cell progenitors" (CTP).

Expression of the hCD25 transgene regulated by *Ptcra* promoter and enhancer elements is indicative of lymphoid commitment (Gounari et al., 2002; Martin et al., 2003). However, in order to formally test for myeloid potential, we performed colony-forming assays with methylcellulose cultures (Figure 2C). Whereas BM-derived LSK cells showed robust myeloid potential, no colonies were formed in cultures starting with BM-derived CLP-1, CLP-2 cells, or CTPs. In order to test the developmental potential of CTPs in vivo, we injected hCD25+B220+ and hCD25+c-kit^{lo}B220$^-$ cells (CD45.1) into irradiated *Rag2$^{-/-}$Il2rg$^{-/-}$* recipients (CD45.2). 5 weeks after transfer, we analyzed for donor-derived cells in the spleen. No donor-derived cells could be detected after transfer of hCD25+B220+ cells. However, we could not conclude from these experiments that hCD25+B220+ cells lack any precursor potential, because only rather limited numbers, which may have been too limited for a meaningful characterization of this population, could be isolated. Upon transfer of CTPs, we detected donor-derived T and NK cells, but no B cells (Figure 2D), in line with the results obtained in in vitro culture. A fraction of donor-derived cells were negative for all markers tested. These could constitute T cells of the γδ lineage. After 5 weeks, no donor-derived T cells could be detected in the thymus, suggesting a single wave of T cell development originating from CTPs, similar to what has been described previously for BM-derived CLP-2 cells (Martin et al., 2003).

Developmental Progression and Expansion of CTP

During the course of intrathymic differentiation, T cell precursors undergo extensive proliferation. In order to test whether CTPs have the potential to proliferate and thus represent potent T cell progenitors, we analyzed the expansion of CTP-derived cells in OP9-DL1 cocultures in comparison to blood-derived LSK cells, which have been suggested to constitute a source of T cell progenitors (Schwarz and Bhandoola, 2004). Both progenitor populations underwent proliferation during a culture period of 14 days, resulting in an approximately 1000-fold expansion (Figure 3A). Of note, the kinetics of expansion differed between LSK cells and CTPs: whereas LSK cell-derived cultures showed only moderate expansion during the first 4 days of cultures, CTPs expanded almost 100-fold during this period of time, which may be due to the differences in IL-7Rα expression and, thus, due to a faster
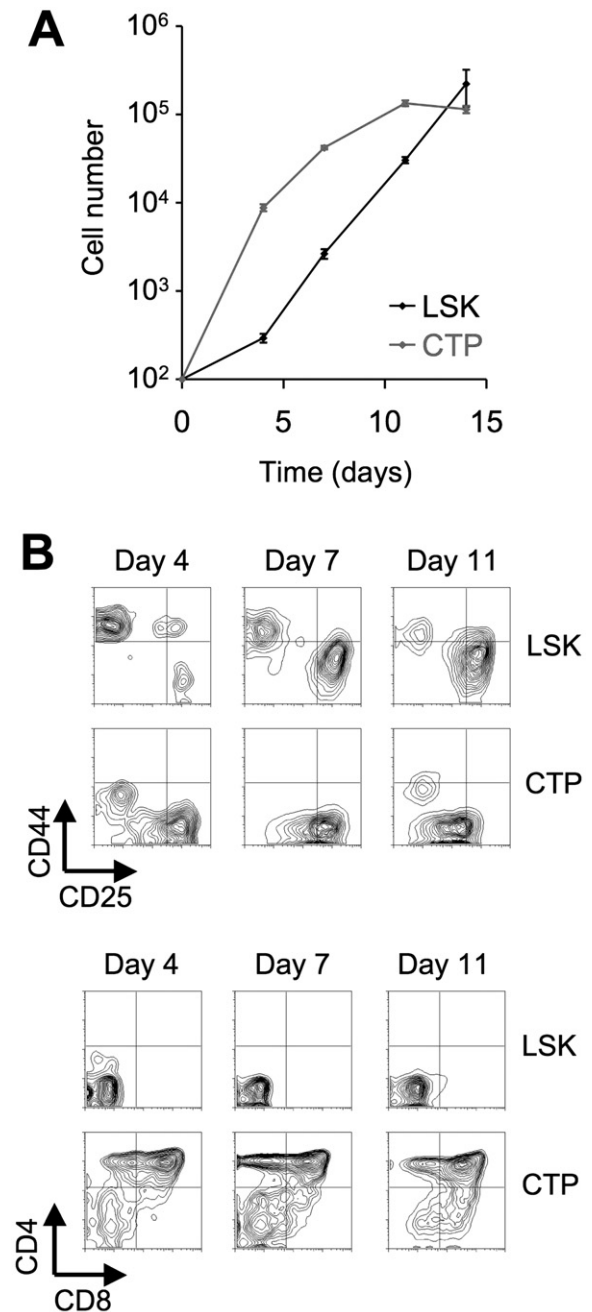

Figure 3. Developmental Progression and Expansion of CTP

(A) 100 blood-derived LSK cells and 100 CTP were sorted and cocultured on OP9-DL1 cells. Cell numbers were assessed by FACS. Data are shown as mean ± SEM (n = 4).

(B) 100 blood-derived LSK cells and 100 CTP were cocultured on OP9-DL1 cells. After 4, 7, and 11 days, cells were analyzed for the expression of CD4 and CD8 (bottom) and electronically gated CD4$^-$CD8$^-$ DN cells for the expression of CD44 and CD25 (top). One representative out of two independent experiments is shown.

response to IL-7 present in the cultures. To analyze whether this difference was reflected by differences in developmental progression, we analyzed the surface
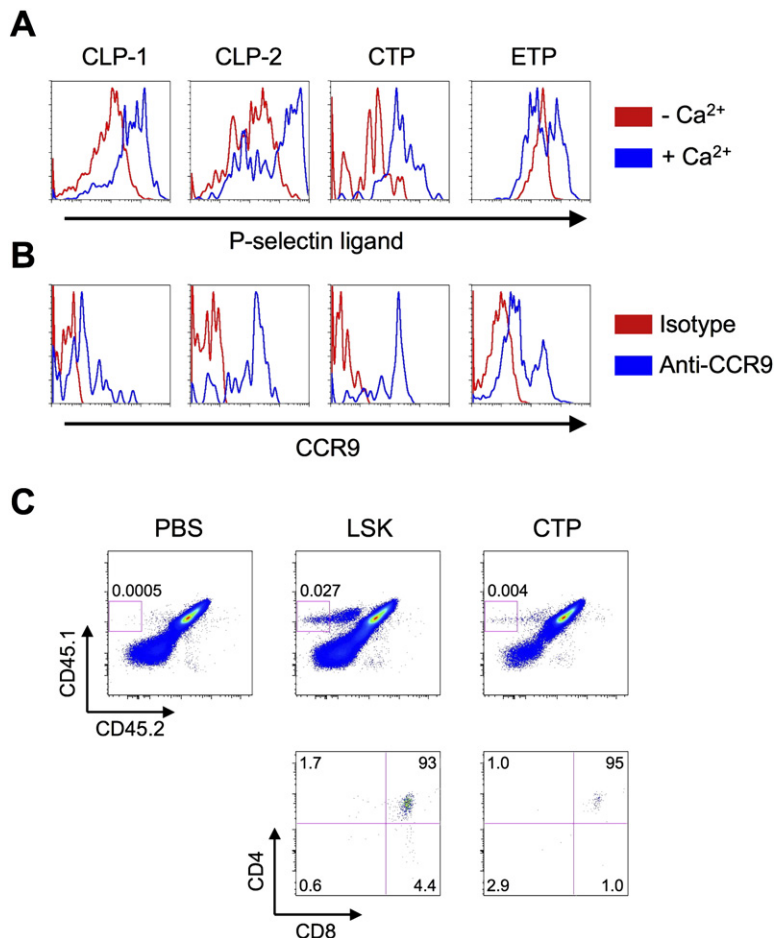
Figure 4. CTP Express Thymus Homing Markers and Home to the Thymus

(A) BM-derived CLP-1 and CLP-2 cells, CTP (identified as described in Figure 1), and thymic ETP (hCD25$^+$CD44$^+$c-kit^{hi}CD25$^-$) were stained with P-selectin-Ig fusion proteins in the presence (blue histograms) and absence (red histograms) of free Ca^{2+}.

(B) BM-derived CLP-1 and CLP-2 cells, CTP, and thymic ETP were stained with CCR9 antibodies (blue histograms). Red histograms represent staining with an isototype control. One representative out of three independent experiments is shown.

(C) 1.5 × 10^3 CTP or LSK cells from blood (CD45.1$^+$CD45.2$^-$) were injected intravenously into sublethally irradiated (FVB × C57BL/6)F1 mice (CD45.1$^+$CD45.2$^+$). Thymi were analyzed 2 weeks after transfer by flow cytometry for expression of CD45.1, CD45.2, CD4, and CD8. Numbers in FACS plots indicate percentages of cells within gates or quadrants.

phenotype of LSK cell and CTP-derived cultures at various time points (Figure 3B). After 4 days, most LSK-derived cells displayed a CD44$^+$CD25$^-$ DN1 phenotype that predominantly reached the CD44$^-$CD25$^+$ DN3 stage after 11 days. No DP cells were detected during this culture period. In contrast, CTP-derived cultures contained a high proportion of DP cells already after 4 days of cultures, with the remaining DN cells mainly being of the DN3 and CD44$^-$CD25$^-$ DN4 phenotypes. These results indicate that the accelerated initial expansion of CTPs correlates with more rapid differentiation when compared to blood-derived LSK cells.

CTP Express Thymic Homing Molecules and Home to the Thymus

The signals that mediate thymus homing of circulating progenitors are still largely unknown. However, there is evidence that the CCL25-CCR9 chemokine receptor system and P-selectin-PSGL-1 interactions are involved in thymus homing (Rossi et al., 2005; Scimone et al., 2006; Uehara et al., 2002). Therefore, we addressed the question whether CTPs expressed P-selectin ligands and CCR9 on their surface. BM-derived CLP-1 and CLP-2, CTPs, and thymic hCD25$^+$CD44$^+$c-kit^{hi}CD25$^-$ ETP were stained with P-selectin-Ig fusion proteins in the presence and absence of calcium. Calcium-dependent binding was detected on all populations, indicating that CLP-1, CLP-2, and CTP cells express similar amounts of P-selectin ligands (Figure 4A). ETP displayed heterogeneous expression of P-selectin ligands, consistent with previously published results (Scimone et al., 2006). CTP exhibited CCR9 surface expression to a similar extent as CLP-2 cells, whereas CLP-1 cells expressed somewhat lower amounts of CCR9 (Figure 4B). Expression of CCR9 on ETPs was heterogeneous, which is consistent with data from previous studies and the implication that loss of CCR9 expression on ETPs correlates with progressive differentiation (Benz and Bleul, 2005; Scimone et al., 2006). These data indicate that CTPs have a similar pattern of homing receptors as CLP-2 cells that efficiently home to the thymus (Martin et al., 2003; Scimone et al., 2006).

Because of the limited number of CTPs, only a few thymic homing experiments were carried out after intravenous injection of CTPs or LSK cells from blood. Analysis of donor-derived cells was performed at 2 weeks after transfer. As shown in Figure 4C, donor-derived thymocytes at this stage mostly exhibited the CD4$^+$CD8$^+$ DP phenotype, with LSK cells producing 7 times more DP cells when

compared to CTPs. This confirms previous results with CTPs from fetal blood (Rodewald et al., 1994), but leaves open the question whether the difference in LSK and CTP-derived DP cells reflects differences in thymic homing or differences in the kinetics of generating DP thymocytes.

CTP Are Present in Blood of *Foxn1*$^{nu/nu}$ Mice

T cell precursors have been proposed to emigrate from the thymus (Lambolez et al., 2006). Because the expression of thymus homing markers does not rule out the possibility that CTPs might originate in the thymus rather than the BM, we tested this possibility by generating athymic hCD25 transgenic *Foxn1*$^{nu/nu}$ mice and analyzing lineage-negative hCD25$^+$ cells in both BM and peripheral blood (Figure 5). FACS analysis revealed that the frequency of Lin$^-$hCD25$^+$ cells in BM and peripheral blood of *Foxn1*$^{nu/nu}$ mice was very similar to that of wild-type (wt) littermates (Figure 5A). In addition, the ratio of reporter-positive B220$^+$ cells and CTPs was virtually the same in *Foxn1*$^{nu/nu}$ mice when compared to wt littermates, and CTPs from *Foxn1*$^{nu/nu}$ mice did express Thy-1.1. In order to functionally analyze CTPs from *Foxn1*$^{nu/nu}$ mice, we assessed their developmental potential in OP9-DL1 cocultures. As shown in Figure 5B, *Foxn1*$^{nu/nu}$ mouse-derived CTPs were able to generate DP cells to a similar extent as their wt-derived counterparts. *Foxn1*$^{nu/nu}$-derived CTP also displayed similar kinetics of developmental progression as their wt counterparts (Figure 5C). These results indicate that CTPs are not derived from the thymus.

CTP in the Absence of Notch-Induced Transcription

As shown in Figure 2, CTPs display a clear bias toward the T lineage in terms of developmental potential. Notch signaling plays a major role in T lineage commitment, and we have shown in Figure 2A that CTPs are dependent on Notch signals to further differentiate into T cells. However, it was not clear whether the observed T cell bias depended on Notch-dependent transcription. This was analyzed by adoptive transfer experiments of lineage-negative BM cells from hCD25 transgenic mice that were retrovirally transduced to express either a dominant-negative form of the coactivator mastermind-like fused to eGFP (DN-MAML) or eGFP alone (MigR1). 6 to 8 weeks after transfer, BM, peripheral blood, and thymus from recipient mice were analyzed by FACS, and the frequency of eGFP-expressing cells in different populations was determined. eGFP expression was detectable at similar amounts in all populations of control-transduced mice (Figure 6A, top; Figure 6C, black bars). DN-MAML-transduced cells were completely absent among thymic DN3 cells and almost completely absent among DN2 cells, indicating the functionality of the construct used. Interestingly, the frequency of DN-MAML-transduced ETP cells was only reduced by 80%, suggesting that this population contained precursors that did not require Notch-dependent transcription (Figure 6A, bottom; Figure 6C, red bars). Although we detected more Thy-1.1$^-$ cells among the reporter-positive Lin$^-$B220$^-$c-kit^{lo} cells in the adoptive transfer experiment when compared to the direct ex vivo

analysis (irrespective of whether cells were mock-transduced or transduced with DN-MAML) (Figure 1C), the frequency of eGFP$^+$Thy-1.1$^+$ CTP in DN-MAML-transduced cells was identical to control-transduced cells (Figure 6B), indicating that CTPs do not require Notch-dependent transcription.

DISCUSSION

The identity of thymus-colonizing T cell progenitors in the adult mouse remains largely elusive, although a number of different candidate populations have been described. By using a hCD25 reporter gene under control of *Ptcra* regulatory elements, our laboratory had previously identified a Lin$^-$c-kit$^{-/lo}$B220$^+$ common lymphoid progenitor "CLP-2" in BM, which efficiently enters the thymus upon intravenous transfer (Martin et al., 2003; Scimone et al., 2006) and progresses developmentally along the T lineage in vitro with kinetics similar to ETPs (Krueger et al., 2006). In the adult organism, T cell progenitors most likely enter the blood prior to thymic colonization. We therefore searched for reporter-positive cells in blood. In fact, such cells can be found in blood, but their phenotype differs from reporter-positive cells in BM in that the majority is B220$^-$ and expresses low amounts of c-kit and IL-7Rα as well as high amounts of Thy-1.1. The few reporter-positive B220-positive cells from blood differ phenotypically from BM-derived B220-positive CLP-2 cells in that the former, but not the latter, lack expression of IL-7Rα and are heterogeneous for expression of the Thy-1.1 marker. These cells could not be functionally analyzed, i.e., no progeny was detected when these cells were injected intravenously or cultured on OP9-DL1 feeders. However, we cannot exclude that BM-derived CLP-2 circulate in blood in numbers that are below our limits of detection. The B220$^-$ cells produced T cells when injected intravenously and produced CD4$^+$CD8$^+$ DP thymocytes in culture and in vivo. The estimated frequency of these cells in the circulation of approximately 330 per ml of blood is similar to that reported for LSK cells (Schwarz and Bhandoola, 2004). In addition, we observed a similar capacity of CTP and LSK cells to undergo a proliferative burst in vitro. Considering that a thymus may contain 100–300 progenitor niches of which 2% to 3% are replenished per day (Donskoy and Goldschneider, 1992; Spangrude and Scollay, 1990) and considering that CTPs have the potential of extensive expansion, it is well conceivable that these cells alone could be capable of generating all T lineage cells. This would also be compatible with the finding that CTPs express CCR9 and PSGL-1, which are critical for homing of progenitors to the thymus (Rossi et al., 2005; Scimone et al., 2006; Uehara et al., 2002). Subfractionation of the thymic DN1 population according to expression of c-kit and HSA led to the identification of five DN1 subsets, of which DN1a and DN1b correspond to ETPs (Porritt et al., 2004). Thy-1$^+$ cells were found within the heterogeneous DN1c, d, and e subfractions. However, in contrast to CTPs, these populations did not exhibit a proliferative burst capacity, which is characteristic for ETPs. When
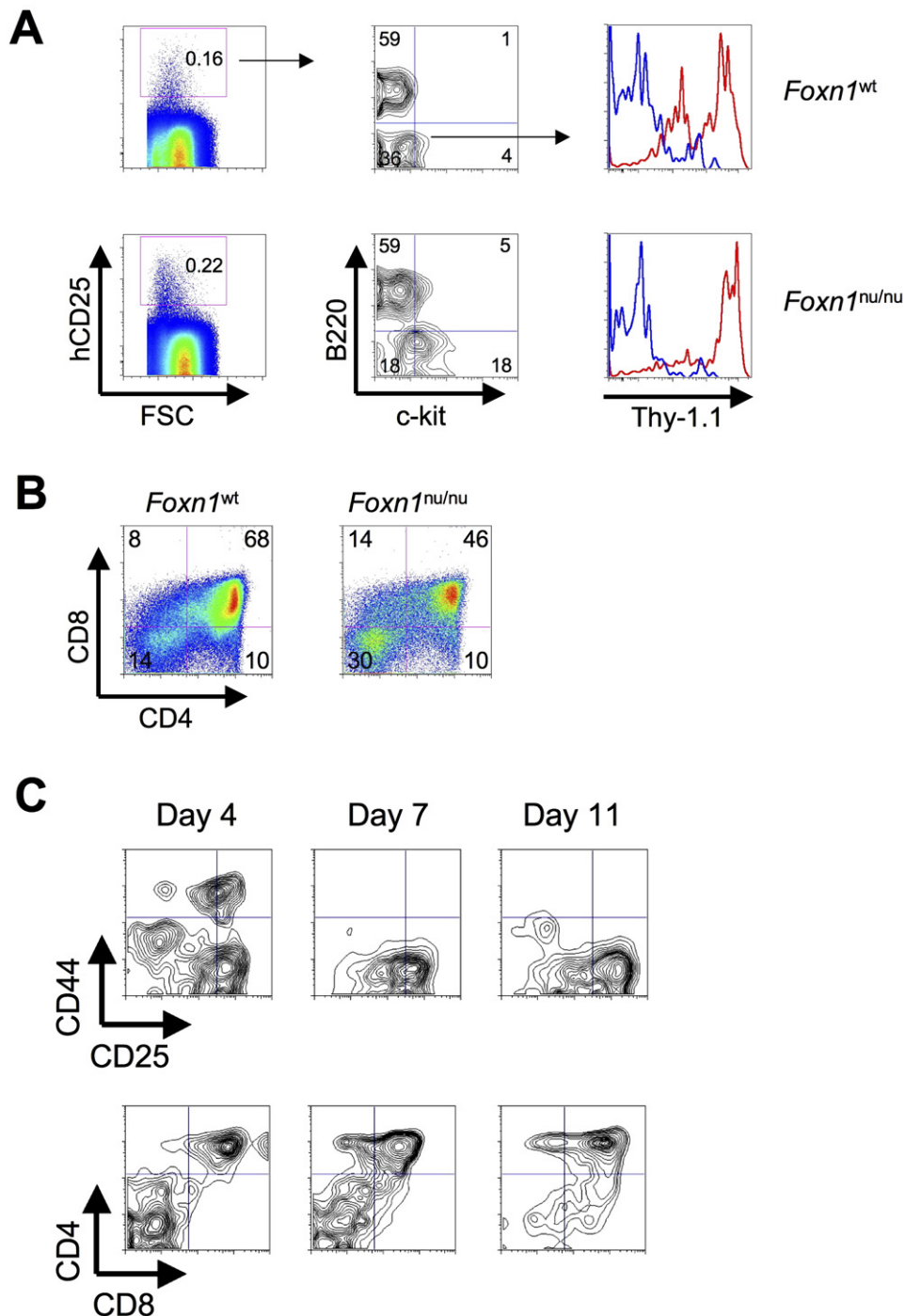
Figure 5. CTP Are Present in Peripheral Blood of *Foxn1*[nu/nu] Mice

(A) CTP from hCD25 transgenic *Foxn1*[nu/nu] mice and hCD25 transgenic *Foxn1*[wt] littermates were analyzed by FACS. Cells were stained with antibodies against lineage markers, hCD25, c-kit, B220, and Thy-1.1 to reveal the frequency of Lin^{−}hCD25^{+} CTP. Numbers in FACS plots indicate percentages of cells within gates or quadrants. One representative out of four independent experiments is shown.

(B) 200 CTP from hCD25 transgenic *Foxn1*[nu/nu] mice and hCD25 transgenic *Foxn1*[wt] littermates were sorted and cocultured on OP9-DL1 cells. After 14 days, cells were analyzed for the expression of CD4 and CD8. Numbers in FACS plots indicate percentages of cells within quadrants. One representative out of three independent experiments is shown.

(C) 100 CTP from hCD25 transgenic *Foxn1*[nu/nu] mice were sorted and cocultured on OP9-DL1 cells. After 4, 7, and 11 days, cells were analyzed for the expression of CD4 and CD8 (bottom) and electronically gated CD4^{−}CD8^{−} DN cells for the expression of CD44 and CD25 (top). One representative out of two independent experiments is shown.
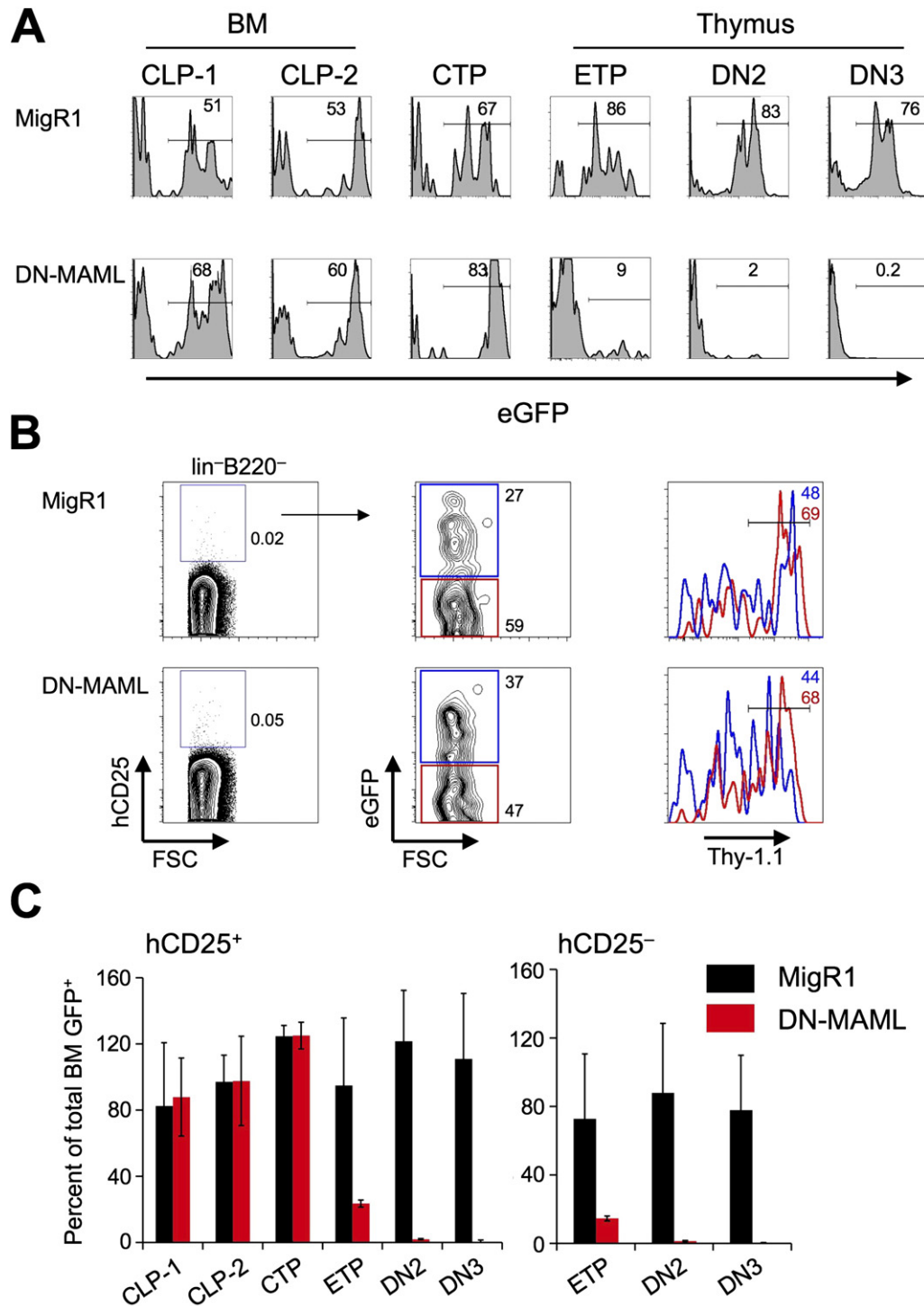
Figure 6. CTP Do Not Require Notch-Induced Transcription

Sorted lineage-negative cells from hCD25 transgenic BM were infected with DN-MAML or MigR1 control retrovirus and transferred into irradiated hosts. BM, peripheral blood, and thymus were analyzed by FACS 6–10 weeks after transfer. Numbers in histograms indicate the frequency of eGFP$^+$ cells.

(A) eGFP expression in different populations of one representative experiment of six mice per group.

(B) Analysis of Thy-1.1 expression in DN-MAML or MigR1-transduced blood-derived Lin$^-$B220$^-$c-kit^{lo} cells. Numbers in FACS plots indicate percentages of cells within gates or quadrants. Blue gates and histograms indicate eGFP$^+$ cells; red histograms indicate eGFP$^-$ cells.

(C) Statistical analysis of four independent experiments. The percentage of eGFP-positive cells was normalized to the percentage of eGFP-positive cells of total BM for each independent experiment. Data are shown as mean ± SEM.

analyzing reporter-positive DN1 cells, which are enriched in the DN1a and b ETP populations (Krueger et al., 2006), we found low Thy-1.1 expression, which appears to be in contrast to the original characterization of DN1a and b cells. This may have been due to differences in mouse strains expressing different Thy-1 alleles as has been described for HSC (Spangrude and Brooks, 1992).

Interestingly, intravenous injection did not yield any B cells, and the frequency of B cell precursors in this subset in OP9 cultures was much lower than the frequency of T cell precursors. Thus, we termed these cells circulating T cell progenitors (CTPs). In fact, given the absolute number of CTPs in the bloodstream, the degree of B lineage potential detected in vitro can probably be considered negligible. Although we were able to detect NK potential in vivo, the in vitro assay revealed a very low NK precursor frequency, similar to that for B cells. This may have been due to the fact that the in vitro assay was performed without addition of IL-15, i.e., under conditions that are suboptimal for NK cell differentiation. The absence of B lineage potential in CTPs is in stark contrast to LSK cells and other extrathymic subsets with T cell progenitor potential such as MPPs, ELPs, and LSPs as well as from CLPs and CLP-2, although a certain T lineage bias has also been described for LSPs (Perry et al., 2004). In contrast, different circulating fetal thymic progenitors have been demonstrated to be T lineage restricted prior to thymic colonization (Ikawa et al., 2004; Masuda et al., 2005; Rodewald et al., 1994). Notably, one population (initially characterized by Rodewald et al.) also phenotypically resembles the adult CTPs population by being c-kit^{lo}Thy-1$^+$ and expressing pre-TCRα mRNA (Bruno et al., 1995; Rodewald et al., 1994). In fact, these cells, like their equivalents in adult blood, could migrate into and differentiate in the thymus. Thus, thymus-seeding CTPs are apparently present in both fetal and adult blood. Our isolation of adult CTPs was based on expression of a hCD25 reporter gene driven by regulatory elements of *Ptcra*. Isolation of CTPs from wt mice could be attempted from a Lin$^-$Thy-1.1$^+$Sca-1$^+$c-kit^{lo}IL-7Rα^{lo} population, which includes all Lin$^-$B220$^-$Flt3$^-$ reporter-positive cells in hCD25 transgenic mice. Conversely, only about 4% of these Lin$^-$Thy-1.1$^+$Sca-1$^+$c-kit^{lo}IL-7Rα^{lo} cells express hCD25, raising the possibility that only a fraction of CTPs express the reporter, much like only a fraction of CLP-1 cells in BM is reporter positive (Martin et al., 2003).

Signaling through Notch receptors has been suggested to mediate T versus B lineage decisions. Thus, constitutive Notch signaling leads to extrathymic T cell development, whereas abrogation of Notch signaling results in accumulation of B cells in the thymus (Koch et al., 2001; Maillard et al., 2004; Pui et al., 1999; Radtke et al., 1999; Wilson et al., 2001). Inhibition of Notch signaling by a dominant-negative mutant of the Notch coactivator Mastermind-like did not reduce the frequency of CTPs in peripheral blood and did not abrogate Thy-1 expression on those cells, which correlates with T lineage commitment. In contrast, further development along the T lineage was clearly Notch dependent, because DP thymocytes could be gen-

erated only on Notch ligand-expressing OP9-DL1 feeder cells, but not on OP9-GFP control feeder cells. These data suggest that terminal differentiation of CTPs into T lineage cells was Notch dependent, whereas early T lineage commitment was less dependent on Notch. This is consistent with a recent study postulating that fetal thymic precursors are T lineage restricted prior to receiving intrathymic Notch signals (Harman et al., 2005). Another study that used mice deficient in the Notch target gene Hes1 and showing that fetal thymic precursors, which are characterized by the expression of paired-immunoglobulin like receptors, are independent of this transcription factor could further support the idea of Notch-independent early T lineage commitment (Masuda et al., 2005). Thus, our results and results by others are consistent with a Notch-independent early T lineage commitment step, which is further enforced by intrathymic Notch signals. This indicates that, at least for certain precursors such as CTPs, adult T lineage commitment parallels fetal T cell development. However, additional studies are required to address molecular mechanisms of Notch-independent T lineage commitment.

EXPERIMENTAL PROCEDURES

Mice
hCD25 transgenic mice (FVB, Thy-1.1, CD45.1) have been described (Gounari et al., 2002; Martin et al., 2003). C57BL/6 *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ and NCR-*Foxn1*$^{<nu>}$ mice were purchased from Taconic Farms (Germantown, NY). NCR-*Foxn1*$^{<nu>}$ were crossed with hCD25 transgenic mice for two generations to generate hCD25 transgenic *Foxn1*$^{nu/nu}$ mice. hCD25 transgenic *Foxn1*$^{nu/nu}$ mice homozygous for the Thy-1.1 allele were used in experiments. (FVB × C57BL/6)F1 mice (CD45.1$^+$CD45.2$^+$) were generated by crossing hCD25 transgenic mice with C57BL/6 mice (CD45.2) for one generation. All mice were maintained in the specific-pathogen-free animal facilities of the Dana-Farber Cancer Institute, and all animal procedures were done in compliance with the guidelines of the DFCI Animal Resources Facility, which operates under regulatory requirements of the US Department of Agriculture and Association for Assessment and Accreditation of Laboratory Animal Care.

Cell Lines and Cell Preparations
OP9 bone marrow stromal cells expressing the Notch ligand delta-like ligand 1 (OP9-DL1) and OP9-control cells (OP9-GFP) were provided by J.C. Zúñiga-Pflücker (University of Toronto, Toronto, Canada) and maintained in αMEM supplemented with 55 μM 2-mercaptoethanol, 10 mM HEPES (pH 7.5), 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 50 μg/ml gentamycine, and 20% heat-inactivated fetal bovine serum (FBS) and passaged as described (Schmitt and Zuniga-Pflucker, 2002). Blood was obtained from anesthetized mice through the retro-orbital venous sinus or cardiac puncture with identical results. Between 0.6 and 1 ml of blood were obtained per mouse, and coagulation was prevented through addition of 20 U/mL heparin (Abbott Labs, IL). Red blood cells were removed by centrifugation over Ficoll-Paque (Amersham). Typically, blood from 20–30 mice was used, allowing the isolation of approximately 500 Lin$^-$hCD25$^+$ cells by cell sorting. BM cells and thymocytes were obtained as described previously (Martin et al., 2003).

Flow Cytometry and Cell Sorting
Monoclonal antibodies specific for CD4 (RM4-5, GK1.5), CD8 (53-6.7), CD25 (PC61), CD44 (IM7), TCRβ (H57-597), TCRγδ (GL3), Gr-1 (RB6-8C5), erythroid cell marker (Ter-119), CD19 (1D3), CD11c

(HL3), CD11b (M1/70), pan-NK (DX5), NK1.1 (PK136), CD45.1 (A20), B220 (RA3-6B2), c-kit (2B8), Sca-1 (E13-161.7), Thy-1.1 (CD90.1, OX-7), and human CD25 (M-A251) were purchased from BD Biosciences and were used as biotin, fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), PerCP-Cy5.5, PE-Cy7, allophycocyanin (APC), or APC-Cy7 conjugates. Monoclonal antibodies specific for Flt3 (A2F10) and IL-7Rα (A7R34) were purchased from eBioscience. Anti-CCR9 (rat IgM) and P-selectin-Ig were provided by U. von Andrian. FITC-conjugated anti-human Fc was from Caltag. PE-Texas red or PerCP conjugated streptavidin was used to reveal staining with biotinylated mAb. Four-color flow cytometry was performed on a FACSCalibur (BD, San Jose, CA). Six-color and seven-color flow cytometry was performed on a FACSAria (BD). Data were analyzed with FlowJo software (Treestar). For analysis, dead cells and debris were excluded by appropriate gating of forward and sideward scatter. Lineage-negative cells were isolated from total thymocytes by staining cell suspensions with a biotinylated lineage-specific antibody cocktail, followed by incubation with streptavidin-conjugated magnetic beads (Dynal) and magnetic bead depletion of mature lineages. Enriched cell suspensions were surface stained with streptavidin-PE-Texas red or streptavidin-PerCP. Cells were sorted with a FACSAria (BD). All populations were resorted; sorted cells were of ≥ 99% purity, as determined by postsort analysis.

OP9 Cocultures
OP9 coculture assays were essentially performed as described (Schmitt and Zuniga-Pflucker, 2002). Precursors were plated at an initial density of 1–5 × 10² cells onto subconfluent OP9-GFP or OP9-DL1 monolayers at 5 × 10⁴ cells/well in a 24-well plate. All cocultures were performed in the presence of 1 ng/ml IL-7 and 5 ng/ml Flt3 ligand (Flt3L) for OP9-DL1 T cell differentiation assays and 5 ng/ml IL-7 and 5 ng/ml Flt3L for OP9-GFP cocultures. In certain experiments, Flt3L was replaced by 100 ng/ml SCF as indicated. At day 4 of differentiation, the culture medium was exchanged. Contaminating OP9 cells were eliminated by filtering the harvested cocultured cells through a 70 μm cell strainer prior to flow cytometric analysis. For cultures of less than 10² precursors, cells were plated directly onto 96-well plates containing 10⁴ γ-irradiated OP9-GFP or OP9-DL1 cells (15 Gy) by a FACSAria cell sorter.

Methylcellulose Cultures
To determine erythroid and myeloid potential, cells were cultured in Methocult M3434 (StemCell Technologies, Vancouver, Canada) containing rmSCF, rmIL-3, rhIL-6, and rhErythropoietin according to the manufacturer's instructions. Plates were inspected and numbers of colonies were determined 8–10 days after the start of cultures.

Retroviral Infections
The retroviral construct encoding a truncated N-terminal fragment of mastermind-like 1 fused to eGFP (DN-MAML) and the control vector MigR1 were provided by J.C. Aster (Brigham and Women's Hospital, Harvard Medical School, Boston, MA) and have been described (Maillard et al., 2004; Weng et al., 2003). Retroviral supernatants were generated by transient transfections of 293T cells with these retroviral constructs and appropriate packaging plasmids (Ory et al., 1996). Lineage-negative BM cells from hCD25 transgenic mice were retrovirally transduced as described (Aifantis et al., 2002) and intravenously injected into irradiated (8 Gy) syngeneic hosts. The resultant chimeric mice were analyzed after 6–10 weeks.

Adoptive Transfers
50 to 1500 sorted lineage-negative hCD25⁺ cells from peripheral blood were intravenously injected into irradiated $Rag2^{-/-}Il2rg^{-/-}$ (5 Gy) mice or (FVB × C57BL/6)F1 (6.5 Gy). Mice were analyzed 2 or 5 weeks after transfer by flow cytometry. Donor cells were distinguished from host cells by expression of CD45.1 and absence of expression of CD45.2.

RT-PCR
Cells were sorted and mRNA was extracted by means of the High Pure total mRNA isolation kit (Roche, Basel, Switzerland). cDNA was prepared with Superscript II RT kit (Invitrogen) and PCR was performed according to standard procedures. Oligonucleotide primer sequences were: hCD25-5′, 5′-TGAGAACTTCAGGCTCCTGGGC-3′; hCD25-3′, 5′-TGGCTTTGAATGTGGCGTGTGG-3′; pTa-5′, 5′-GGCACCCCCTTT CCGTCTCT-3′; pTa-3′, 5′-GTCCAAATTCTGTGGGTGGGA-3′; HPRT-5′, 5′-CACAGGACTAGAACACCTGC-3′; HPRT-3′, 5′-GCTGGTGAAA AGGACCTCT-3′.

Supplemental Data
One Supplemental Figure can be found with this article online at http://www.immunity.com/cgi/content/full/26/1/105/DC1/.

REFERENCES

Adolfsson, J., Borge, O.J., Bryder, D., Theilgaard-Monch, K., Astrand-Grundstrom, I., Sitnicka, E., Sasaki, Y., and Jacobsen, S.E. (2001). Up-regulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity 15, 659–669.

Aifantis, I., Borowski, C., Gounari, F., Lacorazza, H.D., Nikolich-Zugich, J., and von Boehmer, H. (2002). A critical role for the cytoplasmic tail of pTalpha in T lymphocyte development. Nat. Immunol. 3, 483–488.

Allman, D., Sambandam, A., Kim, S., Miller, J.P., Pagan, A., Well, D., Meraz, A., and Bhandoola, A. (2003). Thymopoiesis independent of common lymphoid progenitors. Nat. Immunol. 4, 168–174.

Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. (1999). Notch signaling: cell fate control and signal integration in development. Science 284, 770–776.

Balciunaite, G., Ceredig, R., and Rolink, A.G. (2005). The earliest subpopulation of mouse thymocytes contains potent T, significant macrophage, and natural killer cell but no B-lymphocyte potential. Blood 105, 1930–1936.

Benz, C., and Bleul, C.C. (2005). A multipotent precursor in the thymus maps to the branching point of the T versus B lineage decision. J. Exp. Med. 202, 21–31.

Bruno, L., Rocha, B., Rolink, A., von Boehmer, H., and Rodewald, H.R. (1995). Intra- and extra-thymic expression of the pre-T cell receptor alpha gene. Eur. J. Immunol. 25, 1877–1882.

Donskoy, E., and Goldschneider, I. (1992). Thymocytopoiesis is maintained by blood-borne precursors throughout postnatal life. A study in parabiotic mice. J. Immunol. 148, 1604–1612.

Douagi, I., Colucci, F., Di Santo, J.P., and Cumano, A. (2002). Identification of the earliest prethymic bipotent T/NK progenitor in murine fetal liver. Blood 99, 463–471.

Gounari, F., Aifantis, I., Martin, C., Fehling, H.J., Hoeflinger, S., Leder, P., von Boehmer, H., and Reizis, B. (2002). Tracing lymphopoiesis with

the aid of a pTalpha-controlled reporter gene. Nat. Immunol. *3*, 489–496.

Harman, B.C., Jenkinson, W.E., Parnell, S.M., Rossi, S.W., Jenkinson, E.J., and Anderson, G. (2005). T/B lineage choice occurs prior to intra-thymic Notch signaling. Blood *106*, 886–892.

Igarashi, H., Gregory, S.C., Yokota, T., Sakaguchi, N., and Kincade, P.W. (2002). Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. Immunity *17*, 117–130.

Ikawa, T., Masuda, K., Lu, M., Minato, N., Katsura, Y., and Kawamoto, H. (2004). Identification of the earliest prethymic T-cell progenitors in murine fetal blood. Blood *103*, 530–537.

Jenkinson, E.J., Jenkinson, W.E., Rossi, S.W., and Anderson, G. (2006). The thymus and T-cell commitment: the right niche for Notch? Nat. Rev. Immunol. *6*, 551–555.

Kawamoto, H., Ohmura, K., and Katsura, Y. (1997). Direct evidence for the commitment of hematopoietic stem cells to T, B and myeloid lineages in murine fetal liver. Int. Immunol. *9*, 1011–1019.

Kawamoto, H., Ikawa, T., Ohmura, K., Fujimoto, S., and Katsura, Y. (2000). T cell progenitors emerge earlier than B cell progenitors in the murine fetal liver. Immunity *12*, 441–450.

Koch, U., Lacombe, T.A., Holland, D., Bowman, J.L., Cohen, B.L., Egan, S.E., and Guidos, C.J. (2001). Subversion of the T/B lineage decision in the thymus by lunatic fringe-mediated inhibition of Notch-1. Immunity *15*, 225–236.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661–672.

Krueger, A., Garbe, A.I., and von Boehmer, H. (2006). Phenotypic plasticity of T cell progenitors upon exposure to Notch ligands. J. Exp. Med. *203*, 1977–1984.

Lambolez, F., Arcangeli, M.L., Joret, A.M., Pasqualetto, V., Cordier, C., Di Santo, J.P., Rocha, B., and Ezine, S. (2006). The thymus exports long-lived fully committed T cell precursors that can colonize primary lymphoid organs. Nat. Immunol. *7*, 76–82.

Lu, M., Tayu, R., Ikawa, T., Masuda, K., Matsumoto, I., Mugishima, H., Kawamoto, H., and Katsura, Y. (2005). The earliest thymic progenitors in adults are restricted to T, NK, and dendritic cell lineage and have a potential to form more diverse TCRbeta chains than fetal progenitors. J. Immunol. *175*, 5848–5856.

Maillard, I., Weng, A.P., Carpenter, A.C., Rodriguez, C.G., Sai, H., Xu, L., Allman, D., Aster, J.C., and Pear, W.S. (2004). Mastermind critically regulates Notch-mediated lymphoid cell fate decisions. Blood *104*, 1696–1702.

Martin, C.H., Aifantis, I., Scimone, M.L., von Andrian, U.H., Reizis, B., von Boehmer, H., and Gounari, F. (2003). Efficient thymic immigration of B220+ lymphoid-restricted bone marrow cells with T precursor potential. Nat. Immunol. *4*, 866–873.

Masuda, K., Kubagawa, H., Ikawa, T., Chen, C.C., Kakugawa, K., Hattori, M., Kageyama, R., Cooper, M.D., Minato, N., Katsura, Y., and Kawamoto, H. (2005). Prethymic T-cell development defined by the expression of paired immunoglobulin-like receptors. EMBO J. *24*, 4052–4060.

Moore, T.A., and Zlotnik, A. (1995). T-cell lineage commitment and cytokine responses of thymic progenitors. Blood *86*, 1850–1860.

Mori, S., Shortman, K., and Wu, L. (2001). Characterization of thymus-seeding precursor cells from mouse bone marrow. Blood *98*, 696–704.

Ory, D.S., Neugeboren, B.A., and Mulligan, R.C. (1996). A stable human-derived packaging cell line for production of high titer retro-virus/vesicular stomatitis virus G pseudotypes. Proc. Natl. Acad. Sci. USA *93*, 11400–11406.

Perry, S.S., Wang, H., Pierce, L.J., Yang, A.M., Tsai, S., and Spangrude, G.J. (2004). L-selectin defines a bone marrow analog to the thymic early T-lineage progenitor. Blood *103*, 2990–2996.

Porritt, H.E., Rumfelt, L.L., Tabrizifard, S., Schmitt, T.M., Zuniga-Pflucker, J.C., and Petrie, H.T. (2004). Heterogeneity among DN1 pro-thymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. Immunity *20*, 735–745.

Pui, J.C., Allman, D., Xu, L., DeRocco, S., Karnell, F.G., Bakkour, S., Lee, J.Y., Kadesch, T., Hardy, R.R., Aster, J.C., and Pear, W.S. (1999). Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity *11*, 299–308.

Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H.R., and Aguet, M. (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity *10*, 547–558.

Rodewald, H.R., Kretzschmar, K., Takeda, S., Hohl, C., and Dessing, M. (1994). Identification of pro-thymocytes in murine fetal blood: T lineage commitment can precede thymus colonization. EMBO J. *13*, 4229–4240.

Rossi, F.M., Corbel, S.Y., Merzaban, J.S., Carlow, D.A., Gossens, K., Duenas, J., So, L., Yi, L., and Ziltener, H.J. (2005). Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. Nat. Immunol. *6*, 626–634.

Sambandam, A., Maillard, I., Zediak, V.P., Xu, L., Gerstein, R.M., Aster, J.C., Pear, W.S., and Bhandoola, A. (2005). Notch signaling controls the generation and differentiation of early T lineage progenitors. Nat. Immunol. *6*, 663–670.

Schmitt, T.M., and Zuniga-Pflucker, J.C. (2002). Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. Immunity *17*, 749–756.

Schwarz, B.A., and Bhandoola, A. (2004). Circulating hematopoietic progenitors with T lineage potential. Nat. Immunol. *5*, 953–960.

Scimone, M.L., Aifantis, I., Apostolou, I., von Boehmer, H., and von Andrian, U.H. (2006). A multistep adhesion cascade for lymphoid progenitor cell homing to the thymus. Proc. Natl. Acad. Sci. USA *103*, 7006–7011.

Spangrude, G.J., and Brooks, D.M. (1992). Phenotypic analysis of mouse hematopoietic stem cells shows a Thy-1-negative subset. Blood *80*, 1957–1964.

Spangrude, G.J., and Scollay, R. (1990). Differentiation of hematopoietic stem cells in irradiated mouse thymic lobes. Kinetics and phenotype of progeny. J. Immunol. *145*, 3661–3668.

Tan, J.B., Visan, I., Yuan, J.S., and Guidos, C.J. (2005). Requirement for Notch1 signals at sequential early stages of intrathymic T cell development. Nat. Immunol. *6*, 671–679.

Uehara, S., Grinberg, A., Farber, J.M., and Love, P.E. (2002). A role for CCR9 in T lymphocyte development and migration. J. Immunol. *168*, 2811–2819.

Weng, A.P., Nam, Y., Wolfe, M.S., Pear, W.S., Griffin, J.D., Blacklow, S.C., and Aster, J.C. (2003). Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. Mol. Cell. Biol. *23*, 655–664.

Wilson, A., MacDonald, H.R., and Radtke, F. (2001). Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. J. Exp. Med. *194*, 1003–1012.

Wu, L., Scollay, R., Egerton, M., Pearse, M., Spangrude, G.J., and Shortman, K. (1991). CD4 expressed on earliest T-lineage precursor cells in the adult murine thymus. Nature *349*, 71–74.