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Running title: CBFs IN EARLY T AND NK CELL DEVELOPMENT
Abstract

CBFβ is the non-DNA binding subunit of the core binding factors (CBFs). Mice with reduced CBFβ levels display profound, early defects in T but not B cell development. Here we show that CBFβ is also required at very early stages of natural killer (NK) cell development. We also demonstrate that T cell development aborts during specification, as the expression of Gata3 and Tcf7, which encode key regulators of T lineage specification, is substantially reduced, as are functional thymic progenitors. Constitutively active Notch or IL-7 signaling cannot restore T cell expansion or differentiation of CBFβ insufficient cells, nor can overexpression of Runx1 or CBFβ overcome a lack of Notch signaling. Therefore the ability of the prethymic cell to respond appropriately to Notch is dependent on CBFβ, and both signals converge to activate the T cell developmental program.
Introduction

T cell development begins with colonization of the thymus by rare circulating bone marrow-derived progenitors \(^1\), which expand to generate a population of early T lineage progenitors (ETPs). ETPs then give rise in an orderly fashion to cells expressing both CD4 and CD8 (DP) via several intermediate CD4/CD8 double negative (DN) stages (DN2, DN3 and DN4) \(^2\). The earliest intrathymic stages of T lineage development, proliferation, and survival require signaling through Notch, c-kit, and the IL-7 receptor, plus the activity of several transcription factors including core binding factors (CBFs), Gata3, E2A, c-myb, Ikaros, TCF/LEF, and Ets family members \(^2\). Notch and the CBFs were shown to interact genetically in other contexts, although their genetic hierarchy during T cell development is unknown.

Natural killer (NK) cells develop in multiple sites including liver (fetal and adult), bone marrow, spleen, and thymus \(^3,4\). The first NK lineage committed progenitors can be identified through their expression of the IL-2/IL15R\(\beta\) chain (CD122) and the absence of lineage-specific and mature NK cell markers \(^3\). The differentiation of NKP into immature and mature NK cells is exquisitely dependent on IL-15 signaling \(^3\). NK cell development does not require signaling through the c-kit receptor \(^3\), and sustained Notch signaling inhibits NK cell differentiation \(^5-7\). Transcription factors required for NK cell development include Ets-1, MEF, Id2, TCF/LEF, and members of the Ikaros family \(^3\).

CBFs are heterodimeric transcription factors consisting of a DNA binding subunit (Runx1, Runx2, or Runx3) and a non-DNA binding CBF\(\beta\) subunit that increases the
affinity of the Runx subunits for DNA. Homozygous disruption of Runx1 results in a failure of hematopoietic stem cell (HSC) emergence in the conceptus ⁸, and in the adult Runx1 is required for megakaryocyte, B, and T cell development ⁹,¹⁰. Conditional deletion of Runx1 in bone marrow progenitors using Mx1-Cre blocked T cell development at the DN2 to DN3 transition ⁹,¹⁰, while deletion in DN3 cells with Lck-Cre modestly impaired the formation of DN4 and intermediate single positive cells ¹¹. Runx1 deletion in DP cells with Cd4-Cre reduced the number of mature CD4⁺ cells and eliminated a specialized subset of T cells with NK markers ¹¹,¹². However, an earlier, collective role for CBFs in T cell development was revealed by a hypomorphic Cbfb allele (Cbfb⁰⁰⁰) that, when carried over a nonfunctional Cbfb allele caused an 85% reduction in CBFβ protein levels ¹³. Although HSCs emerged in Cbfb⁰⁰⁰ fetuses and B cells were generated, there was a profound defect in T cell development with what appeared to be consecutive partially penetrant blocks in the generation of ETPs, DN2, and DN3 cells, and an almost complete absence of DN4 and DP cells ¹³.

Notch proteins (Notch 1-4) are transmembrane receptors, that upon binding the cell surface ligands Delta-like or Jagged, undergo two proteolytic cleavages to release the Notch intracellular domain (ICN) ¹⁴. ICN translocates into the nucleus, where it binds to the CSL/RBP-J (CBF1/RBP-J, Suppressor of Hairless, Lag-1) transcription factor, displacing corepressors and recruiting coactivators of the Mastermind-like (MAML) family. Disruption of Notch signaling either by conditional deletion of Notch1, by conditional deletion of CSL, or through expression of a truncated, dominant negative form of MAML1 completely blocks T cell development, and results in the generation of intrathymic B cells ¹⁴. Conversely, Notch is sufficient to drive T cell development, since
overexpression of a constitutively active form of Notch (ICN) leads to T cell at the expense of B cell development at extrathymic sites \(^{14}\). Exposure of hematopoietic progenitors to plate- or cell-bound Notch ligands of the Delta-like family can drive T lineage development in culture \(^{14-16}\).

In two well-characterized examples in hematopoiesis Notch signaling was shown to function genetically upstream of the CBFs. Inactivation of Notch or its ligand Serrate in *Drosophila* caused the loss of Lozenge (a Runx homologue) expression in hemocyte progenitors \(^{17}\), and mutations in Notch, Serrate, or Lozenge itself resulted in a failure to generate the subset of hemocytes called crystal cells \(^{17,18}\). In mice, both Notch1 signaling and Runx1 are required for hematopoietic cell emergence from the aorta/gonad/mesonephros (AGM) region \(^{8,19-22}\). Notch1 signaling defects in mice and zebrafish impair Runx1 expression in the AGM region \(^{19,20,23}\), and overexpression of Runx1 in Notch signaling mutants can rescue the emergence of hematopoietic cells from the AGM region, demonstrating that Runx1 is, at least in part, genetically downstream of Notch signaling \(^{23,24}\).

Here we characterized the molecular mechanism underlying the T cell defect caused by insufficient CBF\(\beta\) levels. We show that T cell specification does not occur, as its multiple early markers (*Gata3, Tcf7, Cd3e*) fail to be expressed. Notch signaling is not impaired, and although IL-7 signaling is decreased, it is not solely responsible for the T cell defect. Finally, we show that reduced CBF levels cause an early and profound block in NK cell development, which is the first demonstration that the CBFs play an essential role in the NK cell lineage.
Materials and Methods

Mice

Generation and genotyping of the \( Cbfb^{ss} (Cbfb^{im2.1Spe}) \) and \( Cbfb^{-} (Cbfb^{im1Spe}) \) alleles were described previously \(^{13,25}\). The animal protocols used in these studies were approved by our Institutional Animal Care and Use Committees.

Transplant analyses

C57BL/6 (B6.SJL-Ptprc<Pea3/BoyJ) x 129S1/SVImJ F1 mice (Ly5.1+/Ly5.2+) were subjected to two split doses of 550 Rads 3-4 hours apart. Each recipient received donor fetal liver (FL) and competitor bone marrow (BM) cells (2 \( \times 10^5 \) cells of each) via tail vein injection. All donor fetuses were of a mixed C57BL/6J and 129S1/SVImJ background and expressed the Ly5.2 (CD45.2) haplotype. Whole BM competitor cells were prepared from C57BL/6 (B6.SJL-Ptprc<Pea3/BoyJ) (Ly5.1+) mice.

Flow cytometry and cell sorting

Flow cytometric analyses were performed on a dual-laser FACSCalibur, FACSCanto, or on a four-laser LSRII (BD Biosciences). The following antibodies were purchased from PharMingen (San Diego, CA), Ebiosciences (San Diego, CA) or Biolegend (San Diego, CA): CD3ε (145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD25 (7D4), CD27 (LG.7F9), CD44 (IM7), CD45 (30-F11), B220 (RA3-6B2), Gr-1 (RB6-8C5), CD127/IL-7Rα (A7R34), TCRβ (H57-597), TCRγ (GL3), NK1.1 (PK136), CD49b (DX5), CD122 (TM-b1), CD132 (4G3),
NKG2D (CX5), CD45.1 (A20), CD45.2 (104), Thy1.2 (53.2.1), c-kit (2B8), and pStat5 (47). On the LSRII platform, doublets were excluded through their FSC-W and SSC-W characteristics and DAPI was used for dead cell exclusion. The data were analyzed using FlowJo (version 6.1.1, Tree Star, San Carlos, CA). Cells were sorted on a FACSaria (BD Biosciences).

**Cell cycle analysis**

Fetal thymocytes (17.5 dpc) were stained with FITC conjugated antibodies (CD8, TCRβ, TCRγ, CD11b, Ter119, B220) and subsequently cultured in DMEM with 10% FBS and pulsed with BrdU (BD Biosciences) for one hour. Cells were harvested and stained with anti-BrdU APC, CD45 PerCP-Cy5.5 and 7-AAD.

**In vitro IL-7 stimulation and intracellular staining for TCRβ, TCRγ, and pStat5**

Fetal thymocytes (17.5 dpc) were stained with FITC conjugated lineage antibodies (CD8, CD3, TCRβ, TCRγ, CD11c, B220, NK1.1, Mac1, Ter119) as described elsewhere. Thymocytes were then incubated at 37°C for 20 min in DMEM (Invitrogen, Carlsbad, CA) with 10% FBS, then treated with 0, 1, and 5 ng/ml IL-7 for an additional 20 min. At the end of the stimulation, cells were immediately fixed with 1.6% formaldehyde at room temperature for 10 minutes, and permeabilized in ice-old methanol for 20 minutes. Cells were stained with PE-conjugated anti-phospho-STAT-5 (Tyr694) plus antibodies to CD44, CD25, and CD45. The analysis of intracellular TCR staining was performed using the same protocol minus the incubation and cytokine stimulation.
Enrichment of FL progenitors and OP9 co-cultures

Lineage negative (Lin⁻) FL cells (E14.5–E17.5) were isolated by depletion of Lin⁺ (CD19, Gr1, Ter119, F4/80) cells using MACS LS columns (Miltenyi Biotech, Auburn, CA). An anti-IL-7Rα biotin labeled antibody was included in the lineage cocktail in experiments in which Notch signaling was inhibited by GSI.

OP9 and OP9-DL1 cells were cultured in Minimum Essential Medium Alpha supplemented with 20% FBS (Invitrogen). Co-cultures were performed in 24-well plates by adding 1-5 × 10⁵ Lin⁻ cells to confluent OP9-DL1 monolayers along with 5 ng/mL human Flt3L and 1 ng/mL murine IL-7 (PeproTech, Rocky Hill, NJ). IL-6 (1 ng/ml) and 25 ng/ml IL-15 were included in the cultures to enhance the generation of T, B and NK cells. Various concentrations of γ-Secretase Inhibitor X (GSI) (InSolution™, EMD Biosciences, San Diego, CA) in DMSO were added to the cultured cells. Co-cultured cells were harvested and analyzed weekly unless otherwise indicated.

Retroviral infection of hematopoietic progenitors

cDNAs encoding Runx1 (AML1b), the CBFβ heterodimerization domain (aa 1-141), full length CBFβ, Stat5a, and Stat5aF were subcloned into the bicistronic MigR1 retrovirus. MigR1 expressing the Notch ICN was previously described. Retroviruses were produced in Phoenix cells. One milliliter of viral supernatant, polybrene (2 ng/ml) and cytokines (IL-7 and Flt3L) were added to overnight co-cultures of Lin⁻ FL cells or thymocytes on OP9-DL1 in 24-well plates and
spinoculated at 1400 g at room temperature for 2 hours. The media was changed 24 hours post spinoculation and the co-culture continued as described above.

**Quantitative RT-PCR**

Total RNA was extracted from sorted or unsorted cells using the RNeasy Mini Kit and DNase I treatment (Qiagen, Valencia CA). RNA quality was assessed on agarose gels and quantified by Nano-Drop1000 (Nano-Drop, Wilmington, DE). First strand cDNA was generated using reverse transcriptase SuperscriptIII (Invitrogen) and oligo (dT)$_{20}$ primers. Real-time PCR was performed in triplicate on Applied Biosystems' 7500 Real-Time PCR System (Foster City, CA). Either Taqman probes or SYBR-Green (Applied Biosystems) were used to detect gene expression. The following pre-made mixture of primers and Taqman probes were used: Runx1 (Mm00486762_m1); Runx3 (Mm00490666_m1); Cbf (Mm00491551_m1); Jak1 (Mm00600614_m1); Jak3 (Mm00439962_m1); Stat3 (Mm00456961_m1); Stat5a (Mm00839861_m1); Stat5b (Mm00839861_m1); Hprt1 (Mm00446968_m1).

The following primers were used for SYBR Green detection: Dtx1
For TGAGGATGTGGTTCGGAGGT, Rev CCCTCATAGCCAGATGCTGTG; Hprt
For CTCCTCAGACCGCTTTTGTC, Rev TAACCTGGTTTCATCATCGCTAATC; Notch1
For CAGCTTGACACACCCAGACAGAC, Rev ACGGAGTACGGCCCATGTT. Primers for Pu.1, Cd3g and Cd3e were described previously$^{30,31}$. Absolute quantification of each gene was calculated by the standard curve method using 10-fold dilutions.
of a positive control (spleen cell cDNA). Expression of individual genes was normalized to \textit{Hprt} expression.

\textit{Western blot analysis}

GFP$^+$ cells were sorted from OP9-DL1 co-cultures (purity >99.9\%) and resuspended at $1 \times 10^5$ cells per ml in lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1\% Nonidet P-40, 0.5\% deoxycholate, 0.1\% SDS, 0.2 mM EDTA, 2.0 mM EGTA plus 1 $\mu$g/ml pepstatin A, 1 $\mu$M Pefablock, 2 $\mu$g/ml leupeptin, 2 $\mu$g/ml aprotinin). Lysates were boiled in SDS loading buffer, resolved by SDS-PAGE through 4 - 12\% Bis-Tris gels (Invitrogen), proteins transferred to nitrocellulose and the blot probed with a mouse monoclonal antibody to CBF$\beta$ ($\beta 141.2$) $^{25}$. The blots were developed with ECL reagents (Pico kit, Pierce, Rockford, IL).
Results

The T cell defect exhibited by Cbfbrss- cells is recapitulated ex vivo.

We previously assessed the collective role of core binding factors in hematopoiesis by using a hypomorphic allele of the common non-DNA binding CBFβ subunit gene (Cbfbrss). Cbfbrss- fetal liver (FL) cells (expressing approximately 15% of normal CBFβ levels) contained HSCs that could contribute to the formation of myelo-erythroid and B lineage cells, but not to DN4 or DP T cells13. To further investigate the molecular basis of this defect, we cultured Lin- FL progenitors from 14.5 – 17.5 days post coitus (dpc) mice on OP9 stromal cells expressing the Notch ligand Delta-like1 (OP9-DL1 cells) to induce T cell differentiation ex vivo16. Within two weeks after establishing the OP9-DL1 co-cultures, Cbfbr+/+ progenitors underwent extensive proliferation (Fig 1A,B), and approximately half became CD4/CD8 double positive (DP) (Fig 1C,D) and gave rise to clear populations of TCRβ+ (not shown) and TCRγ+ cells (Fig 1E,F). In contrast, the in vitro expansion of Cbfbrss- cells was significantly depressed (Fig 1A,B). Cbfbrss- progenitors generated very few DP cells (Fig 1C,D), TCRγ+ cells (Fig 1E,F), or TCRβ+ cells (not shown), and the DN cells contained no detectable intracellular TCRβ (Fig 1G). Cbfbrrss/rss Lin- FL cells, which have higher CBFβ levels than Cbfbrss- cells13, also produced fewer cells and a significantly smaller percentage of TCRγ+ cells (Fig 1B,F), however the defects were not as pronounced as those of Cbfbrss- cells, indicating that T cell development was affected in a dose dependent manner ex vivo.
Cbfβ<sup>rss/-</sup> cells fail to undergo T lineage specification

We examined the DN populations to more precisely define the T cell developmental defect caused by reduced CBFβ dosage. Following seven days of culture on OP9-DL1, most Cbfβ<sup>+/+</sup> Lin<sup>-</sup> FL cells had progressed to the DN2 and DN3 stages, whilst the majority of Cbfβ<sup>rss/-</sup> cells were arrested prior to the DN2 stage (Fig 2A,B). Thymocytes from 16.5-17.5 dpc Cbfβ<sup>rss/-</sup> fetuses also exhibited an early T cell developmental arrest, but a higher percentage of thymocytes appeared to progress to the DN2 and DN3 stages (Fig 2C,D). We suspect that the lower numbers of phenotypic DN2 and DN3 cells generated ex vivo may be caused by differences in the quality or intensity of signaling provided by OP9-DL1 cells as compared to thymic stromal cells.

We examined Cbfβ<sup>rss/-</sup> DN cells for molecular markers of T cell differentiation. Thy1.2<sup>+</sup> DN1 thymocytes were present in Cbfβ<sup>rss/-</sup> 17.5 dpc fetuses, but in significantly reduced numbers (Fig 2E). Cbfβ<sup>rss/-</sup> DN thymocytes contained no intracellular TCRβ or TCRγ chains, which are normally found in DN3 and DN4 cells, respectively<sup>32</sup>, indicating that development arrested before the commitment stage (Fig 2F). The ex vivo defect was more pronounced, as Thy1.2<sup>+</sup> cells were absent in the DN1 population (Fig 2E), although most thymocytes that progressed to the DN2 stage did express Thy1.2 (Fig 2G). Cd3e expression, which can normally be found in both DN1 and DN2 cells<sup>33</sup> was essentially undetectable in Cbfβ<sup>rss/-</sup> DN2 cells purified from the OP9-DL1 cultures, and Cd3g mRNA levels were significantly reduced (Fig 2H). Gata3 and Tcf7 expression progressively
increased in wild type DN1 (Fig 2I) and DN2 (not shown) cells over a five-day culture period \(^3\), but remained low and unchanged in \(Cbfb^{rss/-}\) DN1 and DN2 cells. PU.1 expression was not elevated in the \(Cbfb^{rss/-}\) DN1 population, suggesting re-specification into myeloid lineage cells had not occurred \(^3\).

To determine whether \(Cbfb^{rss/-}\) fetuses contained functional thymic progenitors, we attempted to rescue T cell development by reintroducing CBF\(\beta\) into \(Cbfb^{rss/-}\) thymocytes. Although we could successfully rescue the formation of DP, TCR\(\gamma^+\), and DN2-DN4 cells following retroviral transduction of CBF\(\beta\) into Lin\(^-\) \(Cbfb^{rss/-}\) FL progenitors (Fig 2J,K), we could not efficiently rescue T cell development from \(Cbfb^{rss/-}\) fetal thymocytes (Fig 2L). We could transduce wild type thymocytes with GFP-expressing retroviruses and recover GFP\(^+\)CD45\(^+\) and GFP\(^+\)DP cells, thus our retroviral transductions were effective. However, we could recover only very few GFP\(^+\)CD45\(^+\) cells from \(Cbfb^{rss/-}\) thymocytes transduced with a bicistronic virus expressing both CBF\(\beta\) and GFP, although these included DP cells (Fig 2L). \(Cbfb^{rss/-}\) DN thymocytes were actively cycling (Fig 2M,N), thus it is unlikely that our failure to rescue T cell development is due to a lack of cells permissive for retroviral transduction. We conclude that \(Cbfb^{rss/-}\) thymi contain very few functional T cell progenitors. Altogether, these data indicate that \(Cbfb^{rss/-}\) cells have a global, early defect in T cell specification.

**IL-7 signaling and c-kit expression in \(Cbfb^{rss/-}\) cells**

We determined if CBF\(\beta\) insufficiency affected the expression or activity of one or more signaling pathways required at early stages of T cell development.
IL-7 signaling is essential at the DN1 and DN2 stages, and cell surface IL-7Rα levels become elevated at the DN2 stage 34-38. CBFs directly activate the *Il7ra* (IL-7Rα) gene at later stages of T cell differentiation, in DP and CD4+ cells 11,39. We found that the mean fluorescence intensity (MFI) of IL-7Rα (CD127) staining was comparable in *Cbfb*+/+ and *Cbfbrss/-* DN2 thymocytes, and that of the common gamma chain (γc/IL2RG/CD132) was approximately 2.5 fold higher (Fig 3A). However, the levels of mRNAs encoding several IL-7 signaling molecules, including Jak1, Stat5a, and Stat5b, were significantly (~2 fold) lower in *Cbfbrss/-* DN2 cells (Fig 3B) and intracellular pStat5 levels were decreased by ~7 fold (Fig 3C). Decreased JAK/STAT signaling, though, cannot be solely responsible for the T cell expansion or differentiation defects, as neither could be reversed upon expressing a wild type or constitutively active form of Stat5a 40 (Fig 3D).

c-kit signaling is also critical for the proliferation and differentiation of DN1 and DN2 cells 38,41. We found that neither the percentage of c-kit+ DN1 thymocytes, nor the MFI of c-kit staining was altered in *Cbfbrss/-* fetuses (Fig 3E). The percentage of c-kit+ cells and staining intensity was also similar in *Cbfb*+/+ and *Cbfbrss/-* DN1 cells derived from OP9-DL1 cultures (Fig 3F). Therefore the T cell defect does not appear to be caused by reduced cell surface c-kit expression, although deficiencies in c-kit signaling were not examined and cannot be ruled out.

*Notch signaling is active in Cbfbrss/- cells*
To examine the ability of Cbfbrss−/− progenitors to receive Notch signals, we purified the DN1 population from OP9-DL1 co-cultures at days 3, 4 and 5, and quantified the expression of Notch1 and several of its target genes by qRT-PCR (Fig 4A). Notch1 was expressed in Cbfbrss−/− cells at all time points examined, although its levels were reduced approximately 2 fold in freshly isolated “DN1” (i.e. Lin− CD45+ CD44+CD25−) FL cells (day 0) and in DN1 cells isolated after three days of culture on OP9-DL1 cells, and slightly more than two fold at days 4 and 5 of culture. Expression of the Notch1 target genes Hes1 and Hes5 were reduced ≤ 2-fold in Cbfbrss−/− DN1 cells at all time points examined (Fig 4A). Deltex1 (Dtx1) expression is known to be a very sensitive indicator of Notch signaling 42-44, and was reduced almost 10 fold in Cbfbrss−/− DN1 cells by days 4 and 5 of culture. Although this is a substantial decrease, Dtx1 levels did in fact increase in Cbfbrss−/− DN1 cells approximately 100-fold during the culture period. Thus, although there were significant reductions in the expression of Notch1 and several of its target genes, the fact that their expression was not completely absent and was significantly induced after exposure to Notch ligands suggested that Notch signaling was active in Cbfbrss−/− cells. Differences in the initial composition of the DN1 population that could change further over the culture period could account for the relative decreases in the levels of Notch1 and its targets, due to enrichment of the Cbfbr+/* (but not the Cbfbrss−/−) DN1 population for T lineage-specified cells.

To determine if Notch signaling was functionally intact, we treated OP9-DL1 co-cultures with a γ-secretase inhibitor (GSI) to block Notch signaling. When
Notch signaling is blocked in wild type progenitors, B cells will develop at the expense of T cells \(^{45,46}\). Since OP9-DL1 co-cultures of \(Cbfb^{rss/-}\) Lin\(^-\) cells did not contain CD19\(^+\) B cells (Fig 4B), nor were B cells detected in thymi of mice transplanted with \(Cbfb^{rss/-}\) FL cells \(^{13}\), this suggested that Notch signaling was actively repressing B cell development. As expected, inhibition of Notch signaling with 1 µM and 3 µM GSI resulted in the differentiation of CD19\(^+\) B cells from \(Cbfb^{+/+}\) Lin\(^-\) cells (Fig 4B). GSI even more efficiently induced the formation of B cells from \(Cbfb^{rss/-}\) Lin\(^-\) cells, confirming that Notch1 signaling was sufficiently active in \(Cbfb^{rss/-}\) cells to repress B cell fate (Fig 4B). An increase in NK1.1\(^+\) NK cells was also observed in \(Cbfb^{+/+}\) co-cultures treated with GSI, but not in \(Cbfb^{rss/-}\) co-cultures. Thus \(Cbfb^{rss/-}\) cells had a defect in NK cell development, which will be discussed in more detail later.

Although Notch signaling in \(Cbfb^{rss/-}\) cells was adequate to suppress B cell development, higher levels of Notch signaling are required to drive T cell development \(^7\). Therefore to confirm that the lower levels of Notch1, or other defects in Notch signaling were not the underlying cause of the T cell developmental defect, we transduced \(Cbfb^{rss/-}\) Lin\(^-\) FL cells with a retrovirus expressing the constitutively active intracellular form of Notch1 (ICN). \(Cbfb^{+/+}\) cells expressing high levels of ICN (based on the surrogate GFP marker) efficiently differentiated into DN2 and DN3 cells following seven days of culture on OP9 cells (Fig 4C). On the other hand, \(Cbfb^{rss/-}\) progenitors expressing ICN grew poorly, giving rise to a low percentage of GFP\(^+\) cells (1.7% ± 0.5% versus 66.6% ± 15.0% for \(Cbfb^{+/+}\) cells), and only a few of those cells differentiated to and
past the DN2 stage (Fig 4D). Thus, constitutively high levels of Notch signaling did not rescue T cell development, but instead selected against the growth or survival of $Cbfbrss^{-/-}$ cells. Since high levels of Notch signaling efficiently block B and NK cell development \(^{47}\), and T cell development from $Cbfbrss^{-/-}$ progenitors could not be rescued by ICN, the ICN-expressing $Cbfbrss^{-/-}$ cells could not differentiate into the T, NK or B cell lineages, and appeared to be selected against, thus yielding relatively few GFP\(^{+}\) cells in the cultures. Therefore the moderate decrease in Notch1 expression, or potential defects in ligand binding or cleavage of the Notch receptor are unlikely to be responsible for the $Cbfbrss^{-/-}$ early T cell defect but in fact may be selectively enriched to permit survival of the $Cbfbrss^{-/-}$ cells.

*Runx1, Runx3, and CBF\(\beta\) expression are not dependent upon Notch signaling.*

Runx1 expression is activated by Notch signaling during hematopoietic stem cell development \(^{19,20,23,24}\), thus we determined whether Runx1, Runx3, or Cbfb expression in T cells also required Notch signaling. Addition of 1 \(\mu\)M and 3 \(\mu\)M GSI to Cbfb\(^{+/+}\) OP9-DL1 co-cultures impaired the differentiation of DN2 and DN3 cells in a dose dependent manner (Fig 5A). Expression of the Notch1 targets Hes1 and Dtx1 was significantly decreased in DN1 cells, as was Gata3 and Tcf7 (Fig 5B), demonstrating that Notch signaling and T cell development were indeed inhibited at the 3 \(\mu\)M GSI concentration. However, Runx1, Runx3, and Cbfb expression did not significantly change in DN1 cells in the presence of GSI, consistent with the conclusion that none of these genes are downstream
activated targets of Notch1 in early T lineage progenitors (Fig 5B). Since heterogeneity of the DN1 population could obscure modest changes in gene expression, we purified c-kit⁺CD27⁺CD25⁻ lymphoid progenitors from the DN1 population and assessed Runx1 expression (Fig 5C). Inhibition of Notch signaling with 3 µM GSI resulted in a small but significant increase in Runx1 mRNA levels in c-kit⁺CD27⁺CD25⁻ cells, confirming that Runx1 expression in lymphoid progenitors does not require Notch signaling. Moreover, retroviral overexpression of neither Runx1 (Fig 5D) nor CBFβ (not shown) in Cbfβ+/+ progenitors treated with 3 µM GSI could rescue the perturbation in T cell development caused by inhibition of Notch signaling, supporting the hypothesis that blocking Notch signaling does not impair T cell development by affecting Runx1, Runx3, or Cbfβ expression. In summary, the activity of the Notch pathway does not depend on the CBFs, nor does CBF expression require Notch activity. Rather, Notch and CBF expression and activity are independently regulated in uncommitted thymic progenitors, and converge to specify the T cell lineage.

CBFβ is required for NK cell development

Although Cbfβ⁻⁻⁻⁻ Lin⁻ cells were capable of producing B cells, we observed a defect in NK cell development when Notch signaling was inhibited (Fig 4B). We examined this more directly by culturing Lin⁻ FL cells on OP9 stromal cells in the presence of Flt3L, IL-7, and IL-15. CD122 (IL2Rβ) is a subunit of the IL-15 receptor and is regulated by the CBFs. Cbfβ+/+ Lin⁻ FL cells generated a large number of CD122⁺ cells, including NK1.1⁺ CD122⁺ NK cells and NK1.1⁻ CD122⁺ NK
progenitors (NKP). Cbfb^{rss/-} Lin^- FL cells, on the other hand, produced very few CD122^+ cells (Fig 6A,B) that would be capable of responding to IL-15 signaling. These data indicate that ex vivo NK cell development from fetal liver cells was blocked very early, prior to the NKP stage.

To assess whether there was also an in vivo NK cell defect, we examined thymuses from 15.5 dpc Cbfb^{rss/-} fetuses for NK cells. Bipotent T/NK progenitors and mature NK cells in the fetal thymus both express NK1.1. Cbfb^{rss/-} fetal thymuses contained very few NK1.1^+ (or NK1.1^+ CD122^+) cells (Fig 6C,D), indicating that almost no mature NK cells or bipotent T/NK progenitors were present. We also examined mice transplanted with Cbfb^{+/+}, Cbfb^{rss/rss}, and Cbfb^{rss/-} 17.5 dpc FL cells for the presence of donor-derived NK cells. As expected, there were no or very few donor-derived T cells in the bone marrow or spleen of mice transplanted with Cbfb^{rss/-} FL cells (Fig 6E,F). Donor-derived mature NK cells were present in the peripheral blood (not shown), bone marrow (Fig 6E), spleen (Fig 6F) and thymus (not shown) of transplant recipients of Cbfb^{+/+} and Cbfb^{rss/rss} FL cells. However Cbfb^{rss/-} donor-derived mature and immature NK cells (NK and iNK) were completely absent in recipients reconstituted with Cbfb^{rss/-} FL cells (Fig 6E,F). We also observed a decreased contribution of Cbfb^{rss/-} FL cells to CD19^+ B cells in the bone marrow, but this decrease was not observed in the spleen, suggesting peripheral compensation for a partial defect in bone marrow B cell development (Fig 6E,F). The precipitous decline in NK cell development that occurred when the CBFβ concentration dropped from 30% (Cbfb^{rss/rss}) to 15% (Cbfb^{rss/-}) of normal levels mirrored the abrupt and profound T cell defects we
observed at this same transition\textsuperscript{13}, indicating that both early T and NK cell development require a similar threshold level of CBF\(\beta\). We also examined the transplant recipients for committed NKP (Fig 6G). The percentage of donor-derived NK cells and their precursors in both bone marrow and spleen of the transplant recipients was lower than in the C57BL/6 (B6) control, perhaps because of the more advanced age of the transplant recipients. This precluded evaluation of donor-derived NKP in the bone marrow, however we were able to detect NKP in the spleen. Mice transplanted with Cbfb\textsuperscript{+/+}, Cbfb\textsuperscript{rss/rss}, and Cbfb\textsuperscript{rss/-} FL cells all contained a detectable population of NKP in their spleen (Fig 6G). Therefore the NK cell deficiency in transplanted adult mice occurred at the NKP to iNK transition, whereas in the fetal liver and thymus it preceded the emergence of NKP.

We (and others) examined the expression of core binding factor genes in iNK and NK cells purified from wild type mice to ascertain which members of the Runx family might be required in NK cells\textsuperscript{48}. Runx1, Runx3, and Cbfb mRNA were all found in Lin\textasciitildeSca-1\textasciitildec-kit\textasciitilde bone marrow progenitors, iNK, and NK cells, and both Runx3 and Cbfb mRNA levels increased during NK cell differentiation (Fig 6H). Runx2 mRNA levels were very low in Lin\textasciitildeSca-1\textasciitildec-kit\textasciitilde, iNK, and NK cells (not shown). Based on expression levels we predict that Runx1 and/or Runx3 are the Runx subunits most likely to be involved in NK cell development.
Discussion

The CBFs and Notch cooperate in early T cell development

We endeavored to define the molecular mechanisms underlying the requirement for CBFs in early T cell development by examining the expression of T cell specific genes, and signaling pathways important for T cell development in mice with reduced CBF\(\beta\) levels. A small percentage of \(Cbf\beta^{rss/-}\) cells reached the DP stage of T cell development when cultured on OP9-DL1 stromal cells, although the absolute number of cells that progressed past the DN1 stage was extremely low. The vast majority of \(Cbf\beta^{rss/-}\) cells expressed very little if any \(Cd3e\), \(Gata3\), or \(Tcf7\) mRNA, and no intracellular TCR\(\gamma\) or TCR\(\beta\) chains were present. Thus, \(Cbf\beta^{rss/-}\) cells lacked some of the earliest and most critical mediators of T-lineage development, indicating that T-lineage specification had essentially failed to occur. Although \(Cbf\beta^{rss/-}\) cells were competent to receive Notch signals and by virtue of that were restrained in their ability to undergo B cell development, they could not integrate the Notch signal in order to fully activate the T lineage program. Thus CBFs are needed to establish a T-lineage inducible state that is competent to respond to Notch signaling.

The mechanisms by which the CBFs and Notch signaling converge to specify T cells are unknown. One possibility is that they integrate at the level of common target genes in ETPs and at subsequent stages of DN thymocyte development. A few bona fide direct Notch targets are known in mammals, the best examples of which are the Hairy/Enhancer of SPLIT (HES) genes. Although no HES genes have been implicated as direct CBF targets, the mammalian HES-1 protein has...
been shown to physically interact with both Runx1 and Runx2, and HES-1 can potentiate Runx2 transactivation in reporter assays\textsuperscript{51}. Thus one mechanism by which Notch and CBFs may integrate their function is through the direct interaction of Runx and HES proteins. Genes such as \textit{Il2ra} that encodes the CD25 molecule could be directly and coordinately regulated by Notch and the CBFs\textsuperscript{39,43,52}.

Our observations during T cell development contrast with the initial stages of hematopoiesis in vertebrates, where Runx1 expression is dependent upon Notch1 signaling (Fig 7), and overexpression of the mammalian or zebrafish Runx1 protein could rescue hematopoiesis from the AGM region in Notch mutant animals\textsuperscript{19,20,23,24,53}. In contrast, neither Runx1 nor CBF\textsubscript{β} expression was decreased, nor could overexpression of Runx1 nor CBF\textsubscript{β} restore T cell development when Notch signaling was inhibited.

\textit{Defects in both early T and NK cell development implicate pathways essential for both lineages}

Although we could restore T cell development when we overexpressed CBF\textsubscript{β} in Cbf\textsubscript{β}\textsuperscript{LSS/−} Lin\textsuperscript{−} FL cells, we were unable to efficiently restore proliferation or T cell differentiation from Cbf\textsubscript{β}\textsuperscript{LSS/−} thymocytes. One possible explanation for this failure is that the defect may originate in a prethymic T cell progenitor, and as a result very few of them emigrate to the thymus, or they fail to proliferate or survive once they arrive. The majority of T cell progenitors in the fetal liver and thymus have both T and NK cell potential\textsuperscript{54-58}, and a defect within this population
could also account for the lack of NK cells we observed in OP9 cultures of fetal liver cells, and in the fetal thymus. Both the T and NK cell defects became pronounced when CBFβ levels were reduced from 30% to 15% of normal levels, consistent with the hypothesis that they be ultimately traced, in part, to perturbations in the expression of genes required in both lineages, or to a common progenitor. Preliminary data indeed suggest that 12.5 dpc Cbfβr-/- fetal livers contained 6 to 7-fold fewer phenotypic (c-kit+ CD127+ PIR-A+) trilineage T, NK, and dendritic cell (T/NK/DC) progenitors (not shown). Furthermore, fetal thymuses contained only very low numbers of NK1.1+ cells, which include all the bipotent T/NK progenitors and mature NK cells. The small number of Cbfβr-/- T/NK progenitors that do colonize the thymus may, in addition, be functionally defective, perhaps because they have undergone irreversible epigenetic changes, or were unresponsive to the cytokines (Flt3L and IL-7) that we used in the transduction cocktail.

The NK cell defect in the transplanted adult mouse appeared to occur somewhat later, at the transition between NKP and iNK cells. NKP differentiate from the HSC through intermediate precursors that have not been unambiguously identified, but may include the early and common lymphoid progenitors (ELP and CLP), both of which are distinct from the fetal liver T/NK progenitor. Since B cell development in Cbfβr-/- mice is only mildly affected, the ELP and CLP are presumably present, and are apparently able to give rise to NKP.

Other transcription factors are necessary for both T and NK cell development including members of the Ikaros, ETS, Id, and TCF7/LEF-1 families.
However, mutations in none of these genes selectively and profoundly affected both T and NK, but not B cell development in mice. The profound T and NK cell defects we observed in Cbfbrss/− mice most closely resemble those associated with the T− B+ NK− forms of severe combined immune deficiency syndrome in humans, which are caused by mutations that affect both the IL-7 and IL-15 signaling pathways. Molecules in both pathways are regulated by the CBFs. The CBFs regulate CD122 (Il2rb) expression in NK1.1+ cells, and Runx1 is required for full activation of the Il7ra gene in DN2, DN3, and DP (TCRβhi CD69+) thymocytes and in Foxp3− CD4+ cells. We found essentially no CD122+ cells in ex vivo cultures of Cbfbrss/− Lin− cells or in the thymus. IL-7Rα levels were unchanged in Cbfbrss/− DN2 cells, but IL-7 signaling was nonetheless compromised, as levels of phosphorylated Stat5 were lower. However, defective JAK/STAT signaling is unlikely to be the only defect in Cbfbrss/− T and NK cells. Neither wildtype nor constitutively active Stat5a could rescue the T cell abnormalities, indicating that defective JAK/STAT signaling alone cannot explain the T cell defect. Egawa et al. similarly concluded that defective IL-7 signaling was not the only factor limiting the number of Runx1-deficient CD4+ T cells. Furthermore, the defects in Cbfbrss/− cells are manifested before the IL-7 dependent stage of T cell growth, as their inability to express Cd3e, Gata3, or Tcf7 is consistent with a profound regulatory defect in T cell specification. In NK cells CBFs appear to regulate multiple genes including the Ly49 family in mouse and the killer cell Ig-like receptors in human. In the future, it will be interesting to explore whether abnormalities in Runx/CBFβ function might
account for a subset of SCID patients with as of yet no identified mutations.
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References


Figure legends

**Figure 1. T cell differentiation is blocked in a CBFβ dosage-dependent manner.**

A. Proliferation of Lin− (Ter119, CD19, Gr-1, F4/80) 17.5 dpc FL cells co-cultured on OP9-DL1 in the presence of Flt3L and IL-7, counted at weekly intervals. Each data point is averaged from at least nine fetal livers. Error bars in all figures represent standard error of the mean (SEM).

B. Bar graph depicting the fold expansion of Lin− FL cells from an allelic Cbfβ dosage reduction series, following 14 days of OP9-DL1 co-culture. Error bars represent SEM. The percentage of CBFβ protein relative to wild type levels is indicated below the bars. The number of independent animals analyzed was: Cbfβ+/+ = 13; Cbfβ+/− = 7; Cbfβrss/rss = 5; Cbfβrss/− = 10. Single asterisks (*) indicate significant (P < 0.05) differences relative to Cbfβ+/+ animals (unpaired, two-tailed Student’s t-test).

C. CD4 and CD8 expression following 14 days of co-culture on OP9-DL1.

D. Mean (± SEM) percentages of CD4+ CD8+ (DP) cells. Cbfβ+/+ = 17; Cbfβ+/− = 7; Cbfβrss/rss = 5; Cbfβrss/− = 16. Single asterisks (*) indicate significant (P < 0.05) differences relative to Cbfβ+/+ animals, and double asterisks (**) relative to Cbfβrss/rss animals.

E. Cell surface TCRγ expression in the bulk co-cultured cells.

F. Mean (± SEM) percentages of TCRγ+ cells after 14 days of culture. Cbfβ+/+ = 17; Cbfβ+/− = 7; Cbfβrss/rss = 5; Cbfβrss/− = 16.
G. **Intracellular TCRβ staining in DN cells (DN = CD8- Gr-1- since CD4 expression is inappropriately unregulated in Cbfbrss/- DN cells** \textsuperscript{13,72} following six and nine days of OP9-DL1 culture. \textit{Cbf}β\textsuperscript{+/-} = 4; \textit{Cbf}β\textsuperscript{rss/-} = 3.

**Figure 2. T cell specification fails to occur in Cbfbrss/- cells.**

A. Lin\textsuperscript{-} FL cells co-cultured on OP9-DL1 in the presence of Flt3L and IL-7 for seven days. CD8\textsuperscript{-} Gr1\textsuperscript{-} GFP\textsuperscript{-} cells (GFP gating was used to eliminate OP9-DL1) were analyzed for CD44 and CD25 expression.

B. Percentages of DN1, DN2, and DN3 cells (from A) averaged from 12 independent experiments. Error bars represent SEM. The difference between the percentages of \textit{Cbf}β\textsuperscript{+/-} and \textit{Cbf}β\textsuperscript{rss/-} cells was significant (*) at \( P \leq 0.01 \).

C. CD45\textsuperscript{+} Lin\textsuperscript{-} (Lin = CD8, CD3, TCRβ, TCRγ, CD11c, B220, Mac1, NK1.1, Ter119) 17.5 dpc fetal thymocytes analyzed for CD44 and CD25 expression.

D. Data from 6 animals of each genotype (from C). The difference between the percentages of \textit{Cbf}β\textsuperscript{+/-} and \textit{Cbf}β\textsuperscript{rss/-} cells was significant (*) at \( P \leq 0.03 \).

E. Expression of cell surface Thy1.2 on 17.5 dpc DN1 (Lin\textsuperscript{-}CD45\textsuperscript{+}CD44\textsuperscript{+}CD25\textsuperscript{-}) thymocytes, and on DN1 cells (CD8\textsuperscript{-} Gr1\textsuperscript{-}CD45\textsuperscript{+}CD44\textsuperscript{+}CD25\textsuperscript{-}) from day 6 OP9-DL1 cultures. The thymocyte data were averaged from 4 \textit{Cbf}β\textsuperscript{+/-} and 4 \textit{Cbf}β\textsuperscript{rss/-} fetuses, and the culture data from 4 \textit{Cbf}β\textsuperscript{+/-} and 3 \textit{Cbf}β\textsuperscript{rss/-} fetuses. Asterisks indicate significant differences between \textit{Cbf}β\textsuperscript{+/-} and \textit{Cbf}β\textsuperscript{rss/-} samples \( P = 0.01 \).

F. Percentage of 17.5 dpc DN thymocytes (CD45\textsuperscript{+} Lin\textsuperscript{-}, Lin=CD8, CD3, CD11c, B220, Mac-1, Ter119) expressing intracellular TCRβ (iTCRβ) and iTCRγ. Data are averaged from 3 \textit{Cbf}β\textsuperscript{+/-} and 3 \textit{Cbf}β\textsuperscript{rss/-} fetuses.
G. Percentage of Thy1.2⁺ DN2 cells following 7 days of OP9-DL1 culture.

H. *Cd3e* and *Cd3g* expression by qRT-PCR in DN2 cells sorted from day 7 OP9-DL1 cultures (culture conditions and staining as described in A). The purity of post-sort populations was > 95%. Expression of individual genes was normalized to *Hprt*. Data were derived from triplicate amplifications from three independent samples. Asterisks indicate significant differences between *Cbfβ⁺⁺⁺* versus *Cbfβrss⁻⁻⁻* values (*P ≤ 0.01*).

I. Lin⁻ FL cells cultured on OP9-DL1 cells, harvested at indicated time points. DN1 cells (Lin⁻ CD45⁺ CD44⁺ CD25⁻) were isolated by cell sorting (purity > 95%). The expression of individual genes was normalized to *Hprt* (note log scale for *Tcf7*) and displayed as relative to day 0 values of *Cbfβ⁺⁺⁺* DN1 cells. Values are averaged from triplicate samples isolated from three independent experiments. Asterisks indicate significant differences between *Cbfβ⁺⁺⁺* versus *Cbfβrss⁻⁻⁻* values (*P ≤ 0.05*).

J. Rescue of T cell development with CBFβ in 17.5 dpc *Cbfβrss⁻⁻⁻* FL cells. Lin⁻ FL cells (E17.5) were transduced with the indicated retroviruses and co-cultured on OP9-DL1. Cells were harvested after two weeks and GFP⁺ CD45⁺ cells analyzed for CD4 and CD8 expression (top panels) and TCRγ expression (middle panels). The bottom two plots are DN cells analyzed after one week of co-culture. Gated GFP⁺ Lin⁻ CD45⁺ cells were analyzed for expression of CD44 and CD25 (*n_experiments=11*).

K. Western blot showing CBFβ(p22) protein levels resulting from retroviral expression relative to endogenous protein levels, in whole cell extracts prepared
from GFP* CD45+ cells purified from the OP9-DL1 cultures of Lin- FL cells (CD45+ cell purity ≥ 99.9%). The blot was probed with a monoclonal antibody to CBFβ. The two endogenous CBFβ isoforms generated as a result of alternative splicing (p21.5 and p22) are both visible on this gel. Samples were normalized for actin expression, and the relative amounts of CBFβ determined from a dilution series (not shown). CBFβ levels in retrovirally-transduced cells were 5-fold higher than endogenous levels. Lanes: 1, Cbfb<sup>rss/-</sup> + MigR1; 2, Cbfb<sup>rss/-</sup> + CBFβ; 3, Cbfb<sup>+/+</sup> + MigR1; 4, Cbfb<sup>+/+</sup> + CBFβ

L. Inefficient rescue of T cell development upon restoring CBFβ expression in 17.5 dpc Cbfb<sup>rss/-</sup> thymocytes. Thymocytes were transduced with the indicated retroviruses and co-cultured for 7 days on OP9-DL1. Gated GFP+CD45+ cells were analyzed for CD4 and CD8 expression in the plots below. GFP+CD45- cells are predominantly OP9-DL1 cells.

M. Cell cycle status of CD45+ Lin- (Lin=CD8, TCRβ, TCRγ, CD11b, Ter119, B220) 17.5 dpc fetal thymocytes.

N. Summary of cell cycle data from 5 individual samples per genotype. The differences between Cbfb<sup>+/+</sup> and Cbfb<sup>rss/-</sup> cells in G0/G1 and G2/M were significant at $P \leq 0.01$.

Figure 3. JAK/STAT and c-kit signaling in Cbfb<sup>rss/-</sup> T cells.

A. Expression of IL-7Rα (CD127) and γc (IL2RG/CD132) on the DN2 thymocytes from Figure 2C (17.5 dpc). Data are representative of four experiments.
B. DN2 cells were sorted from OP9-DL1 cultures (culture conditions and staining as described in Figure 2A). The purity of post-sort populations was >95%. Expression of individual genes was normalized to *Hprt*. Values are averaged from triplicate samples from three independent experiments. Asterisks indicate significant differences (*P* ≤ 0.05).

C. pStat5 levels in 17.5 dpc DN2 thymocytes upon ex vivo stimulation with 0, 1 and 5 ng/ml IL-7 for 20 minutes. Shown is the MFI of the difference between pStat5 under IL-7 stimulated and non-stimulated conditions. The data are averaged from 5 *Cbfb*+/+ and 5 *Cbfbrss/-* fetuses. Differences are significant at *P*<0.0001.

D. Rescue of T cell development from *Cbfbrss/-* Lin- FL cells with CBFβ, but not wild type or constitutively active Stat5a (S711F; Stat5a<sup>F</sup>), or MigR1 alone (-) (n=5). Cells were harvested after two weeks. The expansion of *Cbfbrss/-* cells (left graph) is calculated by dividing the number of GFP<sup>+</sup> cells on day 14 by that on day 7 of culture. Only CBFβ expression significantly (*, *P* ≤ 0.0001) increased cell numbers compared to MigR1-transduced *Cbfbrss/-* cells. The right hand graph shows the percentage of GFP<sup>+</sup> cells expressing CD4 and CD8.

E. Expression of cell surface c-kit on 17.5 dpc DN1 cells. Bar graph on left is the percentage of c-kit<sup>+</sup> DN1 (Lin<sup>-</sup> as in Fig 2B; CD45<sup>-</sup>CD44<sup>-</sup>CD25<sup>-</sup>) thymocytes, and on right is the MFI of c-kit staining on c-kit<sup>+</sup> DN1 thymocytes. The data are averaged from 4 *Cbfb*+/+ and 4 *Cbfbrss/-* fetuses.
F. Percentage of DN1 (CD44+ CD25− CD45+ Lin−) cells expressing surface c-kit and MFI of c-kit staining on c-kit+ DN1 cells after six days of OP9-DL1 culture. The data are averaged from 4 Cbfb+/+ and 3 Cbfb−/− fetuses.

Figure 4. Notch signaling is active in Cbfb−/− cells and NK cell development is defective.

A. Lin− FL cells cultured on OP9-DL1 cells, harvested at indicated time points and stained with antibodies as described in Figure 2A. DN1 cells (Lin− CD45+ CD44+ CD25−) were isolated by cell sorting (purity > 95%). The expression of individual genes was normalized to Hprt (note log scale for Dxt1) and displayed as relative to day 0 Cbfb+/+ DN1 cells. Values are averaged from 9 samples (triplicate samples from three independent experiments). Asterisks indicate significant differences between Cbfb+/+ versus Cbfb−/− values (P ≤ 0.05).

B. Flow cytometric analysis of Lin− FL cells cultured on OP9-DL1 (+Flt3L, IL-7, IL-6, IL-15) in the absence and presence of the gamma secretase inhibitor (GSI) inhibitor X for 7 days. GFP− CD45+ cells were analyzed for expression of NK1.1 (NK cells) and CD19 (B cells). n_experiments = 7.

C. Lin− Cbfb+/+ 14.5 dpc FL cells transduced with either MigR1 or a retrovirus expressing the Notch1 intracellular domain (ICN) and cultured for 7 days on OP9 stromal cells in the presence of Flt3L and IL-7. CD8− CD19− Gr1− cells were gated for GFP expression in the left hand panels, and GFP+ cells analyzed for CD44 and CD25 expression in the right hand panels.
D. Lin<sup>-</sup> Cbfb<sup>rss/-</sup> FL cells transduced with MigR1 or ICN, and analyzed as in panel C.

**Figure 5.** Inhibition of Notch signaling does not affect Runx1, Runx3, or Cbfb expression.

A. Flow cytometric analysis of Lin<sup>-</sup> FL cells cultured on OP9-DL1 in the absence and presence of the indicated concentrations of GSI for 7 days. CD8<sup>-</sup> CD19<sup>-</sup> Gr1<sup>-</sup> GFP<sup>-</sup> cells were analyzed for CD44 and CD25 expression (n<sub>experiments</sub> = 8).

B. Gene expression profile of DN1 cells sorted from day 7 OP9-DL1 co-cultures treated with DMSO (white bars), 1.0 µM GSI (gray bars), or 3.0 µM GSI (black bars). Cell sorting, RNA preparation, and real-time PCR were performed as described in Figure 4A. Taqman probes were used for the quantification of Runx1, Runx3, Cbfb and Hprt, and SYBR green was used for the remainder of the genes. Expression of each gene was quantified in comparison to a standard curve prepared with dilutions of spleen cDNA. The expression of individual genes is displayed relative to Hprt. Data are averaged from four independent experiments. Error bars represent SEM and asterisks indicate significant differences between GSI treated and untreated cells (P ≤ 0.05).

C. Runx1 expression in lymphoid progenitors (c-kit<sup>-</sup>CD27<sup>-</sup>CD25<sup>-</sup>) isolated from day 3 OP9-DL1 cultures in the absence and presence of 3 µM GSI (averaged from triplicate samples from three independent experiments). The increase in Runx1 expression in GSI-treated cultures was significant at P < 0.01.
D. Ectopic expression of Runx1 in Cbfb+/+ FL cells cultured on OP9-DL1 in the absence and presence of GSI at 3 µM. Analysis was performed as in panel A.

**Figure 6. CBFβ is required at an early stage of NK cell development in vivo.**

A. Representative scatter plots of Lin- 15.5 dpc FL cells grown on OP9 cells in the presence of Flt3L, IL-7, and IL-15. Gr-1-CD19- cells were analyzed for CD122 and NK1.1 expression.

B. Percentage and absolute number of CD122+ cells (±SD) harvested from OP9 cultures established from 3 Cbfb+/+ and 4 Cbfb/rss/rss fetuses (n_experiments = 3).

C. NK cells in 15.5 dpc fetal thymuses.

D. Total number of thymic NK1.1+ and NK1.1+ CD122+ cells averaged from 3 Cbfb+/+ and 3 Cbfb/rss/- fetuses (n_experiments = 3).

E-G. Lethally irradiated CD45.1 x CD45.2 F1 recipients were reconstituted with wild-type CD45.1+ bone marrow and CD45.2+ FL cells from Cbfb+/+, Cbfb/rss/rss or Cbfb/rss/- fetuses (expressing 100%, 30% and 15% of normal CBFβ levels, respectively).

E. Bone marrow of recipient mice analyzed 10 months after reconstitution. The data are from gated FL-derived (CD45.2+) progenitors. A representative example is shown in each group (Cbfb+/+, n=4; Cbfb/rss/rss, n=4; Cbfb/rss/-, n=5). Bone marrow from a 10 week old C57BL/6 (B6) mouse is shown as control. CD19-CD3-TCRβ-NK1.1+DX5+ cells are mature NK cells, while immature NK lineage cells (iNK) have a NK1.1+Dx5- phenotype.
F. The spleen of recipient mice analyzed in a similar way, illustrating the absence of immature and mature NK cells among cells derived from Cbfbsr/- progenitors.

G. Primitive NK lineage committed progenitors (NKP) (CD122/IL2Rβ+NKG2D+ NK1.1−Dx5−LinT−) (LinT = CD3 CD4 CD8α TCRβ TCRγ) are preserved in the spleen of recipient mice. NKP express the IL-2/IL-15Rβ chain and have an NK1.1′Dx5′ CD19′CD3′CD4′CD8a′TCRβ′TCRγ′ phenotype. A fraction of these cells expresses NKG2D 3.

H. Expression Runx1, Runx3, and Cbfb by qRT-PCR in LSK (Lin=CD4, CD8, TCRβ, TCRγ, DX5, CD19, Mac-1, Ter119), iNK (CD4′ CD8′ TCRβ’ TCRγ’ CD19′ CD49b' NK1.1+ CD122+), and mature NK (CD4’ CD8’ TCRβ’ TCRγ’ CD19’ CD49b+ NK1.1+ CD122+) cells sorted from bone marrow of 8 – 12 week old wild type mice. The purity of post-sort populations was > 98%. Expression of individual genes was normalized to Hprt. Asterisks indicate significant differences from LSK cell values (P ≤ 0.01).

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Figure 7. Genetic interactions between the CBFs and Notch signaling in the specification of HSCs, T, and NK cells in the fetus.

Notch receptors and ligands are expressed on the aortic endothelial cells in the AGM region that give rise to HSCs 20. Notch signaling is required for Runx1 expression in endothelial cells and for the formation of HSCs 19,20,23,24, and thus
Notch is genetically upstream of Runx1 in HSC formation. (Solid lines indicate molecular interactions, and dashed lines represent cell migrations. PB, peripheral blood.)

HSCs and progenitors are released into the circulation from their sites of formation and colonize the fetal liver. We speculate that Runx1 and/or Runx3 plus CBFβ are required to generate NKP from either a bipotent T/NK or another progenitor in the fetus, and perhaps in T/NK progenitors themselves. CBFs are also required for the NKP to iNK transition, based on data from the adult.

Circulating progenitors expressing all three CBF complexes colonize the thymus where they encounter high levels of Notch ligands. CBFs confer upon these progenitors the ability to respond to Notch signaling, which results in T cell specification and progression to the ETP/DN2 stage accompanied by the expression of a suite of T cell specific genes. Not shown are NKT cells, which differentiate from DP T cells in a Runx1-dependent manner.
Figure 1

A

B

C

D

E

F

G

Cell expansion (fold)

Cbfβ^{+/+}

Cbfβ^{%-/-}

CD4

CD8

Cell counts

TCRγ

iTγR^{+} cells (%)

Cbfβ^{+/+}

Cbfβ^{%-/-}

day 6

day 9
Figure 2

A. Cbfb$^{-/-}$, Cbfb$^{+/+}$

B. Cell counts (%)

C. Cbfb$^{-/-}$, Cbfb$^{+/+}$

D. Cell counts (%)

E. Thymocytes, OP9-DL1 cultures

F. ITGAP, DN thymocytes

G. Cbfb$^{-/-}$ DN2, Cbfb$^{+/+}$ DN2

H. mRNA levels (fold)

I. Relative expression

J. Cbfb$^{+/+}$ + MigR1, Cbfb$^{+/+}$ + CBFβ

K. CD4, CD8

L. CD45, GFP

M. Cbfb$^{-/-}$, Cbfb$^{+/+}$

N. Cell numbers (%)
Figure 3

A

Isotype

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

Cell counts

CD127

CD132

B

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

mRNA/FRP

Jak1

Jak3

Stat3

Stat5a

Stat5b

IL-7 (ng/ml)

1

1

5

5

C

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

MFI of pStats

D

Cell expansion (Fold)

\(Cbf\beta\)

Stat5a

Stat5b

\(Cbf\beta\)

Stat5a

Stat5b

E

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

% c-kit DN thymocytes

MFI of c-kit

F

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

\(Cbfb^{+/+}\)

% c-kit DN cells

MFI of c-kit
Figure 4

A

![Graph showing expressions of Notch1, Hes1, Hes5, and Dtx1 over days of co-culture for Cbf^{+/+} and Cbf^{ss-}.]

B

![Graphs showing NK1.1 and CD19 expression in Cbf^{+/+} and Cbf^{ss-} cells treated with different concentrations of GSI.]
Figure 6

A

\(\text{Gr-1} \quad \text{CD19} \quad \text{CD122} \quad \text{NK1.1}\)

\(\text{Cbf}\beta^{+/+} \quad \text{Cbf}\beta^{+-}\)

B

\[\text{\% CD122+ Cells} \quad \# \text{CD122+ Cells (K x 10^6)}\]

\[\text{Cbf}\beta^{+/+} \quad \text{Cbf}\beta^{+-}\]

C

\[\text{SSC} \quad \text{FSC} \quad \text{CD45} \quad \text{CD117} \quad \text{CD122}\]

\(\text{Cbf}\beta^{+/+} \quad \text{Cbf}\beta^{+-}\)

D

\[\# \text{NK1.1+ Cells} \quad \# \text{NK1.1+ CD122+ Cells}\]

\[\text{Cbf}\beta^{+/+} \quad \text{Cbf}\beta^{+-}\]

E

\[\text{Ly5.2+ Bone Marrow}\]

\[\text{B6 Control} \quad \text{Cbf}\beta^{+/+} \quad \text{Cbf}\beta^{ss/\text{rss}} \quad \text{Cbf}\beta^{ss/-}\]

\[\text{CD19} \quad \text{CD3 + TCR}\]

\[\text{DXS} \quad \text{NK1.1}\]

\[\text{IL2R}\gamma\]

F

\[\text{Ly5.2+ Spleen}\]

\[\text{B6 Control} \quad \text{Cbf}\beta^{+/+} \quad \text{Cbf}\beta^{ss/\text{rss}} \quad \text{Cbf}\beta^{ss/-}\]

\[\text{CD19} \quad \text{CD3 + TCR}\]

\[\text{DXS} \quad \text{NK1.1}\]

G

\[\text{Ly5.2+ Spleen}\]

\[\text{B6 Control} \quad \text{Cbf}\beta^{+/+} \quad \text{Cbf}\beta^{ss/\text{rss}} \quad \text{Cbf}\beta^{ss/-}\]

\[\text{CD19} \quad \text{IL2R}\gamma\]

\[\text{NK1.1}\]

\[\text{NKP}\]

\[\text{NKG2D}\]

H

\[\text{mRNA/ipt}\]

\[\text{Runx1} \quad \text{Runx3} \quad \text{Cbf}\beta\]

\[\text{LSK} \quad \text{INK} \quad \text{NK}\]
Figure 7
Core binding factors are necessary for natural killer cell development, and cooperate with Notch signaling during T cell specification

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Running title: CBFs IN EARLY T AND NK CELL DEVELOPMENT
Abstract

CBFβ is the non-DNA binding subunit of the core binding factors (CBFs). Mice with reduced CBFβ levels display profound, early defects in T but not B cell development. Here we show that CBFβ is also required at very early stages of natural killer (NK) cell development. We also demonstrate that T cell development aborts during specification, as the expression of Gata3 and Tcf7, which encode key regulators of T lineage specification, is substantially reduced, as are functional thymic progenitors. Constitutively active Notch or IL-7 signaling cannot restore T cell expansion or differentiation of CBFβ insufficient cells, nor can overexpression of Runx1 or CBFβ overcome a lack of Notch signaling. Therefore the ability of the prethymic cell to respond appropriately to Notch is dependent on CBFβ, and both signals converge to activate the T cell developmental program.
Introduction

T cell development begins with colonization of the thymus by rare circulating bone marrow-derived progenitors, which expand to generate a population of early T lineage progenitors (ETPs). ETPs then give rise in an orderly fashion to cells expressing both CD4 and CD8 (DP) via several intermediate CD4/CD8 double negative (DN) stages (DN2, DN3 and DN4). The earliest intrathymic stages of T lineage development, proliferation, and survival require signaling through Notch, c-kit, and the IL-7 receptor, plus the activity of several transcription factors including core binding factors (CBFs), Gata3, E2A, c-myb, Ikaros, TCF/LEF, and Ets family members. Notch and the CBFs were shown to interact genetically in other contexts, although their genetic hierarchy during T cell development is unknown.

Natural killer (NK) cells develop in multiple sites including liver (fetal and adult), bone marrow, spleen, and thymus. The first NK lineage committed progenitors can be identified through their expression of the IL-2/IL15Rβ chain (CD122) and the absence of lineage-specific and mature NK cell markers. The differentiation of NKP into immature and mature NK cells is exquisitely dependent on IL-15 signaling. NK cell development does not require signaling through the c-kit receptor, and sustained Notch signaling inhibits NK cell differentiation. Transcription factors required for NK cell development include Ets-1, MEF, Id2, TCF/LEF, and members of the Ikaros family.

CBFs are heterodimeric transcription factors consisting of a DNA binding subunit (Runx1, Runx2, or Runx3) and a non-DNA binding CBFβ subunit that increases the
affinity of the Runx subunits for DNA. Homozygous disruption of Runx1 results in a failure of hematopoietic stem cell (HSC) emergence in the conceptus \(^8\), and in the adult Runx1 is required for megakaryocyte, B, and T cell development \(^9,10\). Conditional deletion of Runx1 in bone marrow progenitors using Mx1-Cre blocked T cell development at the DN2 to DN3 transition \(^9,10\), while deletion in DN3 cells with Lck-Cre modestly impaired the formation of DN4 and intermediate single positive cells \(^11\). Runx1 deletion in DP cells with Cd4-Cre reduced the number of mature CD4\(^+\) cells and eliminated a specialized subset of T cells with NK markers \(^11,12\). However, an earlier, collective role for CBFs in T cell development was revealed by a hypomorphic Cbfb allele (Cbfbrss\(^{ss}\)) that, when carried over a nonfunctional Cbfb allele caused an 85% reduction in CBF\(\beta\) protein levels \(^13\). Although HSCs emerged in Cbfbrss\(^{-/-}\) fetuses and B cells were generated, there was a profound defect in T cell development with what appeared to be consecutive partially penetrant blocks in the generation of ETPs, DN2, and DN3 cells, and an almost complete absence of DN4 and DP cells \(^13\).

Notch proteins (Notch 1-4) are transmembrane receptors, that upon binding the cell surface ligands Delta-like or Jagged, undergo two proteolytic cleavages to release the Notch intracellular domain (ICN) \(^14\). ICN translocates into the nucleus, where it binds to the CSL/RBP-J (CBF1/RBP-J, Suppressor of Hairless, Lag-1) transcription factor, displacing corepressors and recruiting coactivators of the Mastermind-like (MAML) family. Disruption of Notch signaling either by conditional deletion of Notch1, by conditional deletion of CSL, or through expression of a truncated, dominant negative form of MAML1 completely blocks T cell development, and results in the generation of intrathymic B cells \(^14\). Conversely, Notch is sufficient to drive T cell development, since
overexpression of a constitutively active form of Notch (ICN) leads to T cell at the expense of B cell development at extrathymic sites. Exposure of hematopoietic progenitors to plate- or cell-bound Notch ligands of the Delta-like family can drive T lineage development in culture.

In two well-characterized examples in hematopoiesis Notch signaling was shown to function genetically upstream of the CBFs. Inactivation of Notch or its ligand Serrate in Drosophila caused the loss of Lozenge (a Runx homologue) expression in hemocyte progenitors, and mutations in Notch, Serrate, or Lozenge itself resulted in a failure to generate the subset of hemocytes called crystal cells. In mice, both Notch1 signaling and Runx1 are required for hematopoietic cell emergence from the aorta/gonad/mesonephros (AGM) region. Notch1 signaling defects in mice and zebrafish impair Runx1 expression in the AGM region, and overexpression of Runx1 in Notch signaling mutants can rescue the emergence of hematopoietic cells from the AGM region, demonstrating that Runx1 is, at least in part, genetically downstream of Notch signaling.

Here we characterized the molecular mechanism underlying the T cell defect caused by insufficient CBFβ levels. We show that T cell specification does not occur, as its multiple early markers (Gata3, Tcf7, Cd3e) fail to be expressed. Notch signaling is not impaired, and although IL-7 signaling is decreased, it is not solely responsible for the T cell defect. Finally, we show that reduced CBF levels cause an early and profound block in NK cell development, which is the first demonstration that the CBFs play an essential role in the NK cell lineage.
Materials and Methods

Mice

Generation and genotyping of the Cbfβ^{ss} (Cbfβ^{tm2.1Spe}) and Cbfβ^{−} (Cbfβ^{tm1Spe}) alleles were described previously\textsuperscript{13,25}. The animal protocols used in these studies were approved by our Institutional Animal Care and Use Committees.

Transplant analyses

C57BL/6 (B6.SJL-Ptprc\textsuperscript{a}>Pep3\textsuperscript{b}/BoyJ) x 129S1/SVImJ F1 mice (Ly5.1\textsuperscript{*}/Ly5.2\textsuperscript{*}) were subjected to two split doses of 550 Rads 3-4 hours apart. Each recipient received donor fetal liver (FL) and competitor bone marrow (BM) cells (2 X 10\textsuperscript{5} cells of each) via tail vein injection. All donor fetuses were of a mixed C57BL/6J and 129S1/SVImJ background and expressed the Ly5.2 (CD45.2) haplotype. Whole BM competitor cells were prepared from C57BL/6 (B6.SJL-Ptprc\textsuperscript{a}>Pep3\textsuperscript{b}/BoyJ) (Ly5.1\textsuperscript{*}) mice.

Flow cytometry and cell sorting

Flow cytometric analyses were performed on a dual-laser FACSCalibur, FACSCanto, or on a four-laser LSRII (BD Biosciences). The following antibodies were purchased from PharMingen (San Diego, CA), Ebiosciences (San Diego, CA) or Biolegend (San Diego, CA): CD3\textsubscript{ε} (145-2C11), CD4 (RM4-5), CD8\textsubscript{α} (53-6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD25 (7D4), CD27 (LG.7F9), CD44 (IM7), CD45 (30-F11), B220 (RA3-6B2), Gr-1 (RB6-8C5), CD127/IL-7R\textsubscript{α} (A7R34), TCR\textsubscript{β} (H57-597), TCR\textsubscript{γ} (GL3), NK1.1 (PK136), CD49b (DX5), CD122 (TM-b1), CD132 (4G3), NKG2D...
(CX5), CD45.1 (A20), CD45.2 (104), Thy1.2 (53.2.1), c-kit (2B8), and pStat5 (47). On the LSRII platform, doublets were excluded through their FSC-W and SSC-W characteristics and DAPI was used for dead cell exclusion. The data were analyzed using FlowJo (version 6.1.1, Tree Star, San Carlos, CA). Cells were sorted on a FACSARia (BD Biosciences).

Cell cycle analysis

Fetal thymocytes (17.5 dpc) were stained with FITC conjugated antibodies (CD8, TCRβ, TCRγ, CD11b, Ter119, B220) and subsequently cultured in DMEM with 10% FBS and pulsed with BrdU (BD Bioscience) for one hour. Cells were harvested and stained with anti-BrdU APC, CD45 PerCP-Cy5.5 and 7-AAD.

In vitro IL-7 stimulation and intracellular staining for TCRβ, TCRγ, and pStat5

Fetal thymocytes (17.5 dpc) were stained with FITC conjugated lineage antibodies (CD8, CD3, TCRβ, TCRγ, CD11c, B220, NK1.1, Mac1, Ter119) as described elsewhere 26,27. Thymocytes were then incubated at 37°C for 20 min in DMEM (Invitrogen, Carlsbad, CA) with 10% FBS, then treated with 0, 1, and 5 ng/ml IL-7 for an additional 20 min. At the end of the stimulation, cells were immediately fixed with 1.6% formaldehyde at room temperature for 10 minutes, and permeabilized in ice-old methanol for 20 minutes. Cells were stained with PE-conjugated anti-phospho-STAT-5 (Tyr694) plus antibodies to CD44, CD25, and CD45. The analysis of intracellular TCR staining was performed using the same protocol minus the incubation and cytokine stimulation.
Enrichment of FL progenitors and OP9 co-cultures

Lineage negative (Lin⁻) FL cells (E14.5–E17.5) were isolated by depletion of Lin⁺ (CD19, Gr1, Ter119, F4/80) cells using MACS LS columns (Miltenyi Biotech, Auburn, CA). An anti-IL-7Rα biotin labeled antibody was included in the lineage cocktail in experiments in which Notch signaling was inhibited by GSI.

OP9 and OP9-DL1 cells were cultured in Minimum Essential Medium Alpha supplemented with 20% FBS (Invitrogen). Co-cultures were performed in 24-well plates by adding 1-5 × 10⁵ Lin⁻ cells to confluent OP9-DL1 monolayers along with 5 ng/mL human Flt3L and 1 ng/mL murine IL-7 (PeproTech, Rocky Hill, NJ). IL-6 (1 ng/ml) and 25 ng/ml IL-15 were included in the cultures to enhance the generation of T, B and NK cells. Various concentrations of γ-Secretase Inhibitor X (GSI) (InSolution™, EMD Biosciences, San Diego, CA) in DMSO were added to the cultured cells. Co-cultured cells were harvested and analyzed weekly unless otherwise indicated.

Retroviral infection of hematopoietic progenitors

cDNAs encoding Runx1 (AML1b), the CBFβ heterodimerization domain (aa 1-141), full length CBFβ, Stat5a, and Stat5aF were subcloned into the bicistronic MigR1 retrovirus. MigR1 expressing the Notch ICN was previously described. Retroviruses were produced in Phoenix cells. One milliliter of viral supernatant, polybrene (2 ng/ml) and cytokines (IL-7 and Flt3L) were added to overnight co-cultures of Lin⁻ FL cells or thymocytes on OP9-DL1 in 24-well plates and spinoculated at 1400 g at room
temperature for 2 hours. The media was changed 24 hours post spinoculation and the
co-culture continued as described above.

**Quantitative RT-PCR**

Total RNA was extracted from sorted or unsorted cells using the RNeasy Mini Kit
and DNase I treatment (Qiagen, Valencia CA). RNA quality was assessed on agarose
gels and quantified by Nano-Drop1000 (Nano-Drop, Wilmington, DE). First strand cDNA
was generated using reverse transcriptase SuperscriptIII (Invitrogen) and oligo (dT)$_{20}$
primers. Real-time PCR was performed in triplicate on Applied Biosystems' 7500 Real-
Time PCR System (Foster City, CA). Either Taqman probes or SYBR-Green (Applied
Biosystems) were used to detect gene expression. The following pre-made mixture of
primers and Taqman probes were used: Runx1 (Mm00486762_m1); Runx3
(Mm00490666_m1); Cbfb (Mm00491551_m1); Jak1 (Mm00600614_m1); Jak3
(Mm00439962_m1); Stat3 (Mm00456961_m1); Stat5a (Mm00839861_m1); Stat5b
(Mm00839861_m1); Hprt1 (Mm00446968_m1).

The following primers were used for SYBR Green detection: Dtx1
For TGAGGATGTGGTTCGGAGGT, Rev CCCTCATAAGCCAGATGCTGTG; Hprt
For CTCCTCAGACCGCTTTTTCG, Rev TAACTGGGTCCATCATCGCTAATC; Notch1
For CAGCTTGCACAACCAGACAGAC, Rev ACGGAGTACGGCCCATGTT. Primers for
Pu.1, Cd3g and Cd3e were described previously $^{30,31}$. Absolute quantification of each
gene was calculated by the standard curve method using 10-fold dilutions of a positive
control (spleen cell cDNA). Expression of individual genes was normalized to Hprt
expression.
Western blot analysis

GFP+ cells were sorted from OP9-DL1 co-cultures (purity >99.9%) and resuspended at 1x 10^5 cells per ml in lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.2 mM EDTA, 2.0 mM EGTA plus 1 μg/ml pepstatin A, 1 μM Pefablock, 2 μg/ml leupeptin, 2 μg/ml aprotinin). Lysates were boiled in SDS loading buffer, resolved by SDS-PAGE through 4 - 12% Bis-Tris gels (Invitrogen), proteins transferred to nitrocellulose and the blot probed with a mouse monoclonal antibody to CBFβ (β141.2) 25. The blots were developed with ECL reagents (Pico kit, Pierce, Rockford, IL).
Results

The T cell defect exhibited by Cbfbrss/- cells is recapitulated ex vivo.

We previously assessed the collective role of core binding factors in hematopoiesis by using a hypomorphic allele of the common non-DNA binding CBFβ subunit gene (Cbfbrss). Cbfbrss/- fetal liver (FL) cells (expressing approximately 15% of normal CBFβ levels) contained HSCs that could contribute to the formation of myeloid-erythroid and B lineage cells, but not to DN4 or DP T cells13. To further investigate the molecular basis of this defect, we cultured Lin- FL progenitors from 14.5 – 17.5 days post coitus (dpc) mice on OP9 stromal cells expressing the Notch ligand Delta-like1 (OP9-DL1 cells) to induce T cell differentiation ex vivo16. Within two weeks after establishing the OP9-DL1 co-cultures, Cbfb+/+ progenitors underwent extensive proliferation (Fig 1A,B), and approximately half became CD4/CD8 double positive (DP) (Fig 1C,D) and gave rise to clear populations of TCRβ+ (not shown) and TCRγ+ cells (Fig 1E,F). In contrast, the in vitro expansion of Cbfbrss/- cells was significantly depressed (Fig 1A,B). Cbfbrss/- progenitors generated very few DP cells (Fig 1C,D), TCRγ+ cells (Fig 1E,F), or TCRβ+ cells (not shown), and the DN cells contained no detectable intracellular TCRβ (Fig 1G). Cbfbrrss/rss Lin- FL cells, which have higher CBFβ levels than Cbfbrss/- cells13, also produced fewer cells and a significantly smaller percentage of TCRγ+ cells (Fig 1B,F), however the defects were not as pronounced as those of Cbfbrss/- cells, indicating that T cell development was affected in a dose dependent manner ex vivo.
*Cbfβ* β<sup>−/−</sup> cells fail to undergo T lineage specification

We examined the DN populations to more precisely define the T cell developmental defect caused by reduced CBFβ dosage. Following seven days of culture on OP9-DL1, most *Cbfβ* β<sup>+/+</sup> Lin<sup>−</sup> FL cells had progressed to the DN2 and DN3 stages, whilst the majority of *Cbfβ* β<sup>−/−</sup> cells were arrested prior to the DN2 stage (Fig 2A,B). Thymocytes from 16.5-17.5 dpc *Cbfβ* β<sup>−/−</sup> fetuses also exhibited an early T cell developmental arrest, but a higher percentage of thymocytes appeared to progress to the DN2 and DN3 stages (Fig 2C,D). We suspect that the lower numbers of phenotypic DN2 and DN3 cells generated *ex vivo* may be caused by differences in the quality or intensity of signaling provided by OP9-DL1 cells as compared to thymic stromal cells.

We examined *Cbfβ* β<sup>−/−</sup> DN cells for molecular markers of T cell differentiation. Thy1.2<sup>+</sup> DN1 thymocytes were present in *Cbfβ* β<sup>−/−</sup> 17.5 dpc fetuses, but in significantly reduced numbers (Fig 2E). *Cbfβ* β<sup>−/−</sup> DN thymocytes contained no intracellular TCRβ or TCRγ chains, which are normally found in DN3 and DN4 cells, respectively<sup>32</sup>, indicating that development arrested before the commitment stage (Fig 2F). The *ex vivo* defect was more pronounced, as Thy1.2<sup>+</sup> cells were absent in the DN1 population (Fig 2E), although most thymocytes that progressed to the DN2 stage did express Thy1.2 (Fig 2G). *Cd3e* expression, which can normally be found in both DN1 and DN2 cells<sup>33</sup> was essentially undetectable in *Cbfβ* β<sup>−/−</sup> DN2 cells purified from the OP9-DL1 cultures, and *Cd3g* mRNA levels were significantly reduced (Fig 2H). *Gata3* and *Tcf7* expression progressively increased in wild type DN1 (Fig 2I) and DN2 (not shown) cells over a five-day culture period<sup>30</sup>, but remained low and unchanged in *Cbfβ* β<sup>−/−</sup> DN1 and DN2 cells. *PU.1* expression was not elevated in the *Cbfβ* β<sup>−/−</sup> DN1 population, suggesting re-
specification into myeloid lineage cells had not occurred 30.

To determine whether Cbfbrss/- fetuses contained functional thymic progenitors, we attempted to rescue T cell development by reintroducing CBFβ into Cbfbrss/- thymocytes. Although we could successfully rescue the formation of DP, TCRγ⁺, and DN2-DN4 cells following retroviral transduction of CBFβ into Lin⁻ Cbfbrss/- FL progenitors (Fig 2J,K), we could not efficiently rescue T cell development from Cbfbrss/- fetal thymocytes (Fig 2L). We could transduce wild type thymocytes with GFP-expressing retroviruses and recover GFP⁺CD45⁺ and GFP⁺DP cells, thus our retroviral transductions were effective. However, we could recover only very few GFP⁺CD45⁺ cells from Cbfbrss/- thymocytes transduced with a bicistronic virus expressing both CBFβ and GFP, although these included DP cells (Fig 2L). Cbfbrss/- DN thymocytes were actively cycling (Fig 2M,N), thus it is unlikely that our failure to rescue T cell development is due to a lack of cells permissive for retroviral transduction. We conclude that Cbfbrss/- thymi contain very few functional T cell progenitors. Altogether, these data indicate that Cbfbrss/- cells have a global, early defect in T cell specification.

**IL-7 signaling and c-kit expression in Cbfbrss/- cells**

We determined if CBFβ insufficiency affected the expression or activity of one or more signaling pathways required at early stages of T cell development. IL-7 signaling is essential at the DN1 and DN2 stages, and cell surface IL-7Rα levels become elevated at the DN2 stage 34-38. CBFs directly activate the Il7ra (IL-7Rα) gene at later stages of T cell differentiation, in DP and CD4⁺ cells 11,39. We found that the mean fluorescence intensity (MFI) of IL-7Rα (CD127) staining was comparable in Cbfb⁺/+ and
Cbfbrss/− DN2 thymocytes, and that of the common gamma chain (γc/IL2RG/CD132) was approximately 2.5 fold higher (Fig 3A). However, the levels of mRNAs encoding several IL-7 signaling molecules, including Jak1, Stat5a, and Stat5b, were significantly (~2 fold) lower in Cbfbrss/− DN2 cells (Fig 3B) and intracellular pStat5 levels were decreased by ~7 fold (Fig 3C). Decreased JAK/STAT signaling, though, cannot be solely responsible for the T cell expansion or differentiation defects, as neither could be reversed upon expressing a wild type or constitutively active form of Stat5a 40 (Fig 3D).

c-kit signaling is also critical for the proliferation and differentiation of DN1 and DN2 cells 38,41. We found that neither the percentage of c-kit+ DN1 thymocytes, nor the MFI of c-kit staining was altered in Cbfbrss/− fetuses (Fig 3E). The percentage of c-kit+ cells and staining intensity was also similar in Cbfbr+/+ and Cbfbrss/− DN1 cells derived from OP9-DL1 cultures (Fig 3F). Therefore the T cell defect does not appear to be caused by reduced cell surface c-kit expression, although deficiencies in c-kit signaling were not examined and cannot be ruled out.

Notch signaling is active in Cbfbrss/− cells

To examine the ability of Cbfbrss/− progenitors to receive Notch signals, we purified the DN1 population from OP9-DL1 co-cultures at days 3, 4 and 5, and quantified the expression of Notch1 and several of its target genes by qRT-PCR (Fig 4A). Notch1 was expressed in Cbfbrss/− cells at all time points examined, although its levels were reduced approximately 2 fold in freshly isolated “DN1” (i.e. Lin− CD45+ CD44+CD25+) FL cells (day 0) and in DN1 cells isolated after three days of culture on OP9-DL1 cells, and slightly more than two fold at days 4 and 5 of culture. Expression of
the Notch1 target genes Hes1 and Hes5 were reduced ≤ 2-fold in Cbfbrss/- DN1 cells at all time points examined (Fig 4A). Deltex1 (Dtx1) expression is known to be a very sensitive indicator of Notch signaling, and was reduced almost 10 fold in Cbfbrss/- DN1 cells by days 4 and 5 of culture. Although this is a substantial decrease, Dtx1 levels did in fact increase in Cbfbrss/- DN1 cells approximately 100-fold during the culture period. Thus, although there were significant reductions in the expression of Notch1 and several of its target genes, the fact that their expression was not completely absent and was significantly induced after exposure to Notch ligands suggested that Notch signaling was active in Cbfbrss/- cells. Differences in the initial composition of the DN1 population that could change further over the culture period could account for the relative decreases in the levels of Notch1 and its targets, due to enrichment of the Cbfb+/+ (but not the Cbfbrss/-) DN1 population for T lineage-specified cells.

To determine if Notch signaling was functionally intact, we treated OP9-DL1 co-cultures with a γ-secretase inhibitor (GSI) to block Notch signaling. When Notch signaling is blocked in wild type progenitors, B cells will develop at the expense of T cells. Since OP9-DL1 co-cultures of CbfbRSS/− Lin− cells did not contain CD19+ B cells (Fig 4B), nor were B cells detected in thymi of mice transplanted with CbfbRSS/− FL cells, this suggested that Notch signaling was actively repressing B cell development. As expected, inhibition of Notch signaling with 1 μM and 3 μM GSI resulted in the differentiation of CD19+ B cells from Cbfb+/+ Lin− cells (Fig 4B). GSI even more efficiently induced the formation of B cells from CbfbRSS/− Lin− cells, confirming that Notch1 signaling was sufficiently active in CbfbRSS/− cells to repress B cell fate (Fig 4B). An increase in NK1.1+ NK cells was also observed in Cbfb+/+ co-cultures treated with GSI, but not in
Cbfbrs/- co-cultures. Thus Cbfbrs/- cells had a defect in NK cell development, which will be discussed in more detail later.

Although Notch signaling in Cbfbrs/- cells was adequate to suppress B cell development, higher levels of Notch signaling are required to drive T cell development. Therefore to confirm that the lower levels of Notch1, or other defects in Notch signaling were not the underlying cause of the T cell developmental defect, we transduced Cbfbrs/- Lin- FL cells with a retrovirus expressing the constitutively active intracellular form of Notch1 (ICN). Cbfb+/+ cells expressing high levels of ICN (based on the surrogate GFP marker) efficiently differentiated into DN2 and DN3 cells following seven days of culture on OP9 cells (Fig 4C). On the other hand, Cbfbrs/- progenitors expressing ICN grew poorly, giving rise to a low percentage of GFP+ cells (1.7% ± 0.5% versus 66.6% ± 15.0% for Cbfb+/+ cells), and only a few of those cells differentiated to and past the DN2 stage (Fig 4D). Thus, constitutively high levels of Notch signaling did not rescue T cell development, but instead selected against the growth or survival of Cbfbrs/- cells. Since high levels of Notch signaling efficiently block B and NK cell development, and T cell development from Cbfbrs/- progenitors could not be rescued by ICN, the ICN-expressing Cbfbrs/- cells could not differentiate into the T, NK or B cell lineages, and appeared to be selected against, thus yielding relatively few GFP+ cells in the cultures. Therefore the moderate decrease in Notch1 expression, or potential defects in ligand binding or cleavage of the Notch receptor are unlikely to be responsible for the Cbfbrs/- early T cell defect but in fact may be selectively enriched to permit survival of the Cbfbrs/- cells.
Runx1, Runx3, and CBFβ expression are not dependent upon Notch signaling.

Runx1 expression is activated by Notch signaling during hematopoietic stem cell development, thus we determined whether Runx1, Runx3, or Cbfβ expression in T cells also required Notch signaling. Addition of 1 μM and 3 μM GSI to Cbfβ+/− OP9-DL1 co-cultures impaired the differentiation of DN2 and DN3 cells in a dose dependent manner (Fig 5A). Expression of the Notch1 targets Hes1 and Dtx1 was significantly decreased in DN1 cells, as was Gata3 and Tcf7 (Fig 5B), demonstrating that Notch signaling and T cell development were indeed inhibited at the 3 μM GSI concentration. However, Runx1, Runx3, and Cbfβ expression did not significantly change in DN1 cells in the presence of GSI, consistent with the conclusion that none of these genes are downstream activated targets of Notch1 in early T lineage progenitors (Fig 5B). Since heterogeneity of the DN1 population could obscure modest changes in gene expression, we purified c-kit⁺CD27⁺CD25⁻ lymphoid progenitors from the DN1 population and assessed Runx1 expression (Fig 5C). Inhibition of Notch signaling with 3 μM GSI resulted in a small but significant increase in Runx1 mRNA levels in c-kit⁺CD27⁺CD25⁻ cells, confirming that Runx1 expression in lymphoid progenitors does not require Notch signaling. Moreover, retroviral overexpression of neither Runx1 (Fig 5D) nor CBFβ (not shown) in Cbfβ+/− progenitors treated with 3 μM GSI could rescue the perturbation in T cell development caused by inhibition of Notch signaling, supporting the hypothesis that blocking Notch signaling does not impair T cell development by affecting Runx1, Runx3, or Cbfβ expression. In summary, the activity of the Notch pathway does not depend on the CBFs, nor does CBF expression require Notch signaling.
activity. Rather, Notch and CBF expression and activity are independently regulated in uncommitted thymic progenitors, and converge to specify the T cell lineage.

**CBFβ is required for NK cell development**

Although Cbfbrss/−Lin− cells were capable of producing B cells, we observed a defect in NK cell development when Notch signaling was inhibited (Fig 4B). We examined this more directly by culturing Lin− FL cells on OP9 stromal cells in the presence of Flt3L, IL-7, and IL-15. CD122 (IL2Rβ) is a subunit of the IL-15 receptor and is regulated by the CBFs. Cbfbrss/− FL cells generated a large number of CD122+ cells, including NK1.1+CD122+ NK cells and NK1.1−CD122+ NK progenitors (NKP). Cbfbrss/− Lin− FL cells, on the other hand, produced very few CD122+ cells (Fig 6A,B) that would be capable of responding to IL-15 signaling. These data indicate that ex vivo NK cell development from fetal liver cells was blocked very early, prior to the NKP stage.

To assess whether there was also an in vivo NK cell defect, we examined thymuses from 15.5 dpc Cbfbrss/− fetuses for NK cells. Bipotent T/NK progenitors and mature NK cells in the fetal thymus both express NK1.1. Cbfbrss/− fetal thymuses contained very few NK1.1+ (or NK1.1+CD122+) cells (Fig 6C,D), indicating that almost no mature NK cells or bipotent T/NK progenitors were present. We also examined mice transplanted with Cbfbrss/+, Cbfbrss/rss, and Cbfbrss/− 17.5 dpc FL cells for the presence of donor-derived NK cells. As expected, there were no or very few donor-derived T cells in the bone marrow or spleen of mice transplanted with Cbfbrss/rss FL cells (Fig 6E,F). Donor-derived mature NK cells were present in the peripheral blood (not shown), bone marrow (Fig 6E), spleen (Fig 6F) and thymus (not shown) of transplant recipients of Cbfbrss+/+
Cbfβ<sup>rss/rss</sup> FL cells. However Cbfβ<sup>rss/-</sup> donor-derived mature and immature NK cells (NK and iNK) were completely absent in recipients reconstituted with Cbfβ<sup>rss/-</sup> FL cells (Fig 6E,F). We also observed a decreased contribution of Cbfβ<sup>rss/-</sup> FL cells to CD19<sup>+</sup> B cells in the bone marrow, but this decrease was not observed in the spleen, suggesting peripheral compensation for a partial defect in bone marrow B cell development (Fig 6E,F). The precipitous decline in NK cell development that occurred when the CBFβ concentration dropped from 30% (Cbfβ<sup>rss/rss</sup>) to 15% (Cbfβ<sup>rss/-</sup>) of normal levels mirrored the abrupt and profound T cell defects we observed at this same transition<sup>13</sup>, indicating that both early T and NK cell development require a similar threshold level of CBFβ. We also examined the transplant recipients for committed NKP (Fig 6G). The percentage of donor-derived NK cells and their precursors in both bone marrow and spleen of the transplant recipients was lower than in the C57BL/6 (B6) control, perhaps because of the more advanced age of the transplant recipients. This precluded evaluation of donor-derived NKP in the bone marrow, however we were able to detect NKP in the spleen. Mice transplanted with Cbfβ<sup>++/-</sup>, Cbfβ<sup>rss/rss</sup>, and Cbfβ<sup>rss/-</sup> FL cells all contained a detectable population of NKP in their spleen (Fig 6G). Therefore the NK cell deficiency in transplanted adult mice occurred at the NKP to iNK transition, whereas in the fetal liver and thymus it preceded the emergence of NKP.

We (and others) examined the expression of core binding factor genes in iNK and NK cells purified from wild type mice to ascertain which members of the Runx family might be required in NK cells<sup>48</sup>. Runx1, Runx3, and Cbfβ mRNA were all found in Lin<sup>-</sup>Sca-1<sup>+</sup>c-kit<sup>+</sup> bone marrow progenitors, iNK, and NK cells, and both Runx3 and Cbfβ mRNA levels increased during NK cell differentiation (Fig 6H). Runx2 mRNA levels
were very low in Lin\(^{-}\)Sca-1\(^{+}\)c-kit\(^{+}\), iNK, and NK cells (not shown). Based on expression levels we predict that Runx1 and/or Runx3 are the Runx subunits most likely to be involved in NK cell development.
Discussion

*The CBFs and Notch cooperate in early T cell development*

We endeavored to define the molecular mechanisms underlying the requirement for CBFs in early T cell development by examining the expression of T cell specific genes, and signaling pathways important for T cell development in mice with reduced CBFβ levels. A small percentage of Cbfbrss/- cells reached the DP stage of T cell development when cultured on OP9-DL1 stromal cells, although the absolute number of cells that progressed past the DN1 stage was extremely low. The vast majority of Cbfbrss/- cells expressed very little if any Cd3e, Gata3, or Tcf7 mRNA, and no intracellular TCRγ or TCRβ chains were present. Thus, Cbfbrss/- cells lacked some of the earliest and most critical mediators of T-lineage development, indicating that T-lineage specification had essentially failed to occur. Although Cbfbrss/- cells were competent to receive Notch signals and by virtue of that were restrained in their ability to undergo B cell development, they could not integrate the Notch signal in order to fully activate the T lineage program. Thus CBFs are needed to establish a T-lineage inducible state that is competent to respond to Notch signaling.

The mechanisms by which the CBFs and Notch signaling converge to specify T cells are unknown. One possibility is that they integrate at the level of common target genes in ETPs and at subsequent stages of DN thymocyte development. A few bona fide direct Notch targets are known in mammals, the best examples of which are the Hairy/Enhancer of SPLIT (HES) genes. Although no HES genes have been implicated as direct CBF targets, the mammalian HES-1 protein has been shown to physically interact with both Runx1 and Runx2, and HES-1 can potentiate Runx2
transactivation in reporter assays. Thus one mechanism by which Notch and CBFs may integrate their function is through the direct interaction of Runx and HES proteins. Genes such as Il2ra that encodes the CD25 molecule could be directly and coordinately regulated by Notch and the CBFs.

Our observations during T cell development contrast with the initial stages of hematopoiesis in vertebrates, where Runx1 expression is dependent upon Notch1 signaling (Fig 7), and overexpression of the mammalian or zebrafish Runx1 protein could rescue hematopoiesis from the AGM region in Notch mutant animals. In contrast, neither Runx1 nor CBFβ expression was decreased, nor could overexpression of Runx1 nor CBFβ restore T cell development when Notch signaling was inhibited.

*Defects in both early T and NK cell development implicate pathways essential for both lineages*

Although we could restore T cell development when we overexpressed CBFβ in Cbfbrss/− Lin− FL cells, we were unable to efficiently restore proliferation or T cell differentiation from Cbfbrss/− thymocytes. One possible explanation for this failure is that the defect may originate in a prethymic T cell progenitor, and as a result very few of them emigrate to the thymus, or they fail to proliferate or survive once they arrive. The majority of T cell progenitors in the fetal liver and thymus have both T and NK cell potential, and a defect within this population could also account for the lack of NK cells we observed in OP9 cultures of fetal liver cells, and in the fetal thymus. Both the T and NK cell defects became pronounced when CBFβ levels were reduced from 30% to 22%.
15% of normal levels, consistent with the hypothesis that they be ultimately traced, in part, to perturbations in the expression of genes required in both lineages, or to a common progenitor. Preliminary data indeed suggest that 12.5 dpc Cbfβ^{rss-} fetal livers contained 6 to 7-fold fewer phenotypic (c-kit^{+} CD127^{+} PIR-A^{+}) trilineage T, NK, and dendritic cell (T/NK/DC) progenitors \(^{55}\) (not shown). Furthermore, fetal thymuses contained only very low numbers of NK1.1^{+} cells, which include all the bipotent T/NK progenitors and mature NK cells \(^{49}\). The small number of Cbfβ^{rss-} T/NK progenitors that do colonize the thymus may, in addition, be functionally defective, perhaps because they have undergone irreversible epigenetic changes, or were unresponsive to the cytokines (Flt3L and IL-7) that we used in the transduction cocktail.

The NK cell defect in the transplanted adult mouse appeared to occur somewhat later, at the transition between NKP and iNK cells. NKP differentiate from the HSC through intermediate precursors that have not been unambiguously identified, but may include the early and common lymphoid progenitors (ELP and CLP), both of which are distinct from the fetal liver T/NK progenitor \(^{3}\). Since B cell development in Cbfβ^{rss-} mice is only mildly affected, the ELP and CLP are presumably present, and are apparently able to give rise to NKP.

Other transcription factors are necessary for both T and NK cell development including members of the Ikaros, ETS, Id, and TCF7/LEF-1 families \(^{59-68}\). However, mutations in none of these genes selectively and profoundly affected both T and NK, but not B cell development in mice. The profound T and NK cell defects we observed in Cbfβ^{rss-} mice most closely resemble those associated with the T⁻ B⁺ NK⁻ forms of severe combined immune deficiency syndrome in humans, which are caused by mutations that
affect both the IL-7 and IL-15 signaling pathways. Molecules in both pathways are regulated by the CBFS. The CBFS regulate CD122 (Il2rb) expression in NK1.1+ cells, and Runx1 is required for full activation of the Il7ra gene in DN2, DN3, and DP (TCRβ hi CD69+) thymocytes and in Foxp3- CD4+ cells. We found essentially no CD122+ cells in ex vivo cultures of Cbfbrss/- Lin- cells or in the thymus. IL-7Rα levels were unchanged in Cbfbrss/- DN2 cells, but IL-7 signaling was nonetheless compromised, as levels of phosphorylated Stat5 were lower. However, defective JAK/STAT signaling is unlikely to be the only defect in Cbfbrss/- T and NK cells. Neither wildtype nor constitutively active Stat5a could rescue the T cell abnormalities, indicating that defective JAK/STAT signaling alone cannot explain the T cell defect. Egawa et al. similarly concluded that defective IL-7 signaling was not the only factor limiting the number of Runx1-deficient CD4+ T cells. Furthermore, the defects in Cbfbrss/- cells are manifested before the IL-7 dependent stage of T cell growth, as their inability to express Cd3e, Gata3, or Tcf7 is consistent with a profound regulatory defect in T cell specification. In NK cells CBFS appear to regulate multiple genes including the Ly49 family in mouse and the killer cell Ig-like receptors in human. In the future, it will be interesting to explore whether abnormalities in Runx/CBFβ function might account for a subset of SCID patients with as of yet no identified mutations.
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References


Figure legends

Figure 1. T cell differentiation is blocked in a CBFβ dosage-dependent manner.

A. Proliferation of Lin− (Ter119, CD19, Gr-1, F4/80) 17.5 dpc FL cells co-cultured on OP9-DL1 in the presence of Flt3L and IL-7, counted at weekly intervals. Each data point is averaged from at least nine fetal livers. Error bars in all figures represent standard error of the mean (SEM).

B. Bar graph depicting the fold expansion of Lin− FL cells from an allelic Cbfb dosage reduction series, following 14 days of OP9-DL1 co-culture. Error bars represent SEM. The percentage of CBFβ protein relative to wild type levels is indicated below the bars. The number of independent animals analyzed was: Cbfb+/+ = 13; Cbfb+/− = 7; Cbfb−/− = 5; Cbfb−/− = 10. Single asterisks (*) indicate significant (P < 0.05) differences relative to Cbfb+/+ animals (unpaired, two-tailed Student’s t-test).

C. CD4 and CD8 expression following 14 days of co-culture on OP9-DL1.

D. Mean (± SEM) percentages of CD4+ CD8+ (DP) cells. Cbfb+/+ = 17; Cbfb+/− = 7; Cbfb−/− = 5; Cbfb−/− = 16. Single asterisks (*) indicate significant (P < 0.05) differences relative to Cbfb+/+ animals, and double asterisks (**) relative to Cbfb−/− animals.

E. Cell surface TCRγ expression in the bulk co-cultured cells.

F. Mean (± SEM) percentages of TCRγ+ cells after 14 days of culture. Cbfb+/+ = 17; Cbfb+/− = 7; Cbfb−/− = 5; Cbfb−/− = 16.
G. Intracellular TCRβ staining in DN cells (DN = CD8- Gr-1- since CD4 expression is inappropriately unregulated in Cbfβ<sup>rss/-</sup> DN cells<sup>13,72</sup>) following six and nine days of OP9-DL1 culture. Cbfβ<sup>+/+</sup> = 4; Cbfβ<sup>rss/-</sup> = 3.

Figure 2. T cell specification fails to occur in Cbfβ<sup>rss/-</sup> cells.

A. Lin<sup>-</sup> FL cells co-cultured on OP9-DL1 in the presence of Flt3L and IL-7 for seven days. CD8<sup>-</sup> Gr1<sup>-</sup> GFP<sup>-</sup> cells (GFP gating was used to eliminate OP9-DL1) were analyzed for CD44 and CD25 expression.

B. Percentages of DN1, DN2, and DN3 cells (from A) averaged from 12 independent experiments. Error bars represent SEM. The difference between the percentages of Cbfβ<sup>+/+</sup> and Cbfβ<sup>rss/-</sup> cells was significant (*) at \( P \leq 0.01 \).

C. CD45<sup>+</sup> Lin<sup>-</sup> (Lin = CD8, CD3, TCRβ, TCRγ, CD11c, B220, Mac1, NK1.1, Ter119) 17.5 dpc fetal thymocytes analyzed for CD44 and CD25 expression.

D. Data from 6 animals of each genotype (from C). The difference between the percentages of Cbfβ<sup>+/+</sup> and Cbfβ<sup>rss/-</sup> cells was significant (*) at \( P \leq 0.03 \).

E. Expression of cell surface Thy1.2 on 17.5 dpc DN1 (Lin<sup>-</sup>CD45<sup>+</sup>CD44<sup>+</sup>CD25<sup>-</sup>) thymocytes, and on DN1 cells (CD8<sup>-</sup> Gr1<sup>-</sup>CD45<sup>+</sup>CD44<sup>+</sup>CD25<sup>-</sup>) from day 6 OP9-DL1 cultures. The thymocyte data were averaged from 4 Cbfβ<sup>+/+</sup> and 4 Cbfβ<sup>rss/-</sup> fetuses, and the culture data from 4 Cbfβ<sup>+/+</sup> and 3 Cbfβ<sup>rss/-</sup> fetuses. Asterisks indicate significant differences between Cbfβ<sup>+/+</sup> and Cbfβ<sup>rss/-</sup> samples (\( P = 0.01 \)).

F. Percentage of 17.5 dpc DN thymocytes (CD45<sup>-</sup> Lin<sup>-</sup>, Lin=CD8, CD3, CD11c, B220, Mac-1, Ter119) expressing intracellular TCRβ (iTCRβ) and iTCRγ. Data are averaged from 3 Cbfβ<sup>+/+</sup> and 3 Cbfβ<sup>rss/-</sup> fetuses.
G. Percentage of Thy1.2+ DN2 cells following 7 days of OP9-DL1 culture.

H. Cd3e and Cd3g expression by qRT-PCR in DN2 cells sorted from day 7 OP9-DL1 cultures (culture conditions and staining as described in A). The purity of post-sort populations was > 95%. Expression of individual genes was normalized to Hprt. Data were derived from triplicate amplifications from three independent samples. Asterisks indicate significant differences between Cbfb+/+ versus Cbfbrss/- values (P ≤ 0.01).

I. Lin- FL cells cultured on OP9-DL1 cells, harvested at indicated time points. DN1 cells (Lin- CD45+ CD44+ CD25-) were isolated by cell sorting (purity > 95%). The expression of individual genes was normalized to Hprt (note log scale for Tcf7) and displayed as relative to day 0 values of Cbfb+/+ DN1 cells. Values are averaged from triplicate samples isolated from three independent experiments. Asterisks indicate significant differences between Cbfb+/+ versus Cbfbrss/- values (P ≤ 0.05).

J. Rescue of T cell development with CBFβ in 17.5 dpc Cbfbrss/- FL cells. Lin- FL cells (E17.5) were transduced with the indicated retroviruses and co-cultured on OP9-DL1. Cells were harvested after two weeks and GFP+ CD45+ cells analyzed for CD4 and CD8 expression (top panels) and TCRγ expression (middle panels). The bottom two plots are DN cells analyzed after one week of co-culture. Gated GFP+ Lin- CD45+ cells were analyzed for expression of CD44 and CD25 (n_experiments=11).

K. Western blot showing CBFβ(p22) protein levels resulting from retroviral expression relative to endogenous protein levels, in whole cell extracts prepared from GFP+ CD45+ cells purified from the OP9-DL1 cultures of Lin- FL cells (CD45+ cell purity ≥ 99.9%). The blot was probed with a monoclonal antibody to CBFβ. The two endogenous CBFβ isoforms generated as a result of alternative splicing (p21.5 and
p22) are both visible on this gel. Samples were normalized for actin expression, and the relative amounts of CBFβ determined from a dilution series (not shown). CBFβ levels in retrovirally-transduced cells were 5-fold higher than endogenous levels. Lanes: 1, \( Cbfb^{rss/-} + \text{MigR1} \); 2, \( Cbfb^{rss/-} + \text{CBFβ} \); 3, \( Cbfb^{+/+} + \text{MigR1} \); 4, \( Cbfb^{+/+} + \text{CBFβ} \).

L. Inefficient rescue of T cell development upon restoring CBFβ expression in 17.5 dpc \( Cbfb^{rss/-} \) thymocytes. Thymocytes were transduced with the indicated retroviruses and co-cultured for 7 days on OP9-DL1. Gated GFP⁺CD45⁺ cells were analyzed for CD4 and CD8 expression in the plots below. GFP⁺CD45⁻ cells are predominantly OP9-DL1 cells.

M. Cell cycle status of CD45⁺ Lin⁻ (Lin=CD8, TCRβ, TCRγ, CD11b, Ter119, B220) 17.5 dpc fetal thymocytes.

N. Summary of cell cycle data from 5 individual samples per genotype. The differences between \( Cbfb^{+/+} \) and \( Cbfb^{rss/-} \) cells in G0/G1 and G2/M were significant at \( P \leq 0.01 \).

Figure 3. JAK/STAT and c-kit signaling in \( Cbfb^{rss/-} \) T cells.

A. Expression of IL-7Rα (CD127) and γc (IL2RG/CD132) on the DN2 thymocytes from Figure 2C (17.5 dpc). Data are representative of four experiments.

B. DN2 cells were sorted from OP9-DL1 cultures (culture conditions and staining as described in Figure 2A). The purity of post-sort populations was > 95%. Expression of individual genes was normalized to \( Hprt \). Values are averaged from triplicate samples.
from three independent experiments. Asterisks indicate significant differences \((P \leq 0.05)\).

C. pStat5 levels in 17.5 dpc DN2 thymocytes upon ex vivo stimulation with 0, 1 and 5 ng/ml IL-7 for 20 minutes. Shown is the MFI of the difference between pStat5 under IL-7 stimulated and non-stimulated conditions. The data are averaged from 5 \(Cbfb^{+/+}\) and 5 \(Cbfb^{rss/-}\) fetuses. Differences are significant at \(P<0.0001\).

D. Rescue of T cell development from \(Cbfb^{rss/-}\) Lin\(^{-}\) FL cells with CBF\(\beta\), but not wild type or constitutively active Stat5a (S711F; Stat5a\(^F\)), or MigR1 alone (-) (n=5). Cells were harvested after two weeks. The expansion of \(Cbfb^{rss/-}\) cells (left graph) is calculated by dividing the number of GFP\(^+\) cells on day 14 by that on day 7 of culture. Only CBF\(\beta\) expression significantly (*) \((P \leq 0.0001)\) increased cell numbers compared to MigR1-transduced \(Cbfb^{rss/-}\) cells. The right hand graph shows the percentage of GFP\(^+\) cells expressing CD4 and CD8.

E. Expression of cell surface c-kit on 17.5 dpc DN1 cells. Bar graph on left is the percentage of c-kit\(^+\) DN1 (Lin\(^{-}\) as in Fig 2B; CD45\(^+\)CD44\(^+\)CD25\(^-\)) thymocytes, and on right is the MFI of c-kit staining on c-kit\(^+\) DN1 thymocytes. The data are averaged from 4 \(Cbfb^{+/+}\) and 4 \(Cbfb^{rss/-}\) fetuses.

F. Percentage of DN1 (CD44\(^+\) CD25\(^-\) CD45\(^+\) Lin\(^{-}\)) cells expressing surface c-kit and MFI of c-kit staining on c-kit\(^+\) DN1 cells after six days of OP9-DL1 culture. The data are averaged from 4 \(Cbfb^{+/+}\) and 3 \(Cbfb^{rss/-}\) fetuses.

**Figure 4. Notch signaling is active in Cbfb\(^{rss/-}\) cells and NK cell development is defective.**
A. Lin\textsuperscript− FL cells cultured on OP9-DL1 cells, harvested at indicated time points and stained with antibodies as described in Figure 2A. DN1 cells (Lin\textsuperscript− CD45\textsuperscript+ CD44\textsuperscript+ CD25\textsuperscript−) were isolated by cell sorting (purity > 95\%). The expression of individual genes was normalized to \textit{Hprt} (note log scale for \textit{Dxt1}) and displayed as relative to day 0 \textit{Cbbf}/\textit{b}\textsuperscript+/+ DN1 cells. Values are averaged from 9 samples (triplicate samples from three independent experiments). Asterisks indicate significant differences between \textit{Cbbf}/\textit{b}\textsuperscript+/+ versus \textit{Cbbf}/\textit{b}\textsuperscript/rss/− values (\(P \leq 0.05\)).

B. Flow cytometric analysis of Lin\textsuperscript− FL cells cultured on OP9-DL1 (+Flt3L, IL-7, IL-6, IL-15) in the absence and presence of the gamma secretase inhibitor (GSI) inhibitor X for 7 days. GFP\textsuperscript− CD45\textsuperscript+ cells were analyzed for expression of NK1.1 (NK cells) and CD19 (B cells). \(n_{\text{experiments}} = 7\).

C. Lin\textsuperscript− \textit{Cbbf}/\textit{b}\textsuperscript+/+ 14.5 dpc FL cells transduced with either MigR1 or a retrovirus expressing the Notch1 intracellular domain (ICN) and cultured for 7 days on OP9 stromal cells in the presence of Flt3L and IL-7. CD8\textsuperscript− CD19\textsuperscript− Gr1\textsuperscript− cells were gated for GFP expression in the left hand panels, and GFP\textsuperscript+ cells analyzed for CD44 and CD25 expression in the right hand panels.

D. Lin\textsuperscript− \textit{Cbbf}/\textit{b}\textsuperscript/rss/− FL cells transduced with MigR1 or ICN, and analyzed as in panel C.

\textit{Figure 5. Inhibition of Notch signaling does not affect Runx1, Runx3, or Cbbf expression.}

A. Flow cytometric analysis of Lin\textsuperscript− FL cells cultured on OP9-DL1 in the absence and presence of the indicated concentrations of GSI for 7 days. CD8\textsuperscript− CD19\textsuperscript− Gr1\textsuperscript− GFP\textsuperscript− cells were analyzed for CD44 and CD25 expression (\(n_{\text{experiments}} = 8\)).
B. Gene expression profile of DN1 cells sorted from day 7 OP9-DL1 co-cultures treated with DMSO (white bars), 1.0 μM GSI (gray bars), or 3.0 μM GSI (black bars). Cell sorting, RNA preparation, and real-time PCR were performed as described in Figure 4A. Taqman probes were used for the quantification of Runx1, Runx3, Cbfb and Hprt, and SYBR green was used for the remainder of the genes. Expression of each gene was quantified in comparison to a standard curve prepared with dilutions of spleen cDNA. The expression of individual genes is displayed relative to Hprt. Data are averaged from four independent experiments. Error bars represent SEM and asterisks indicate significant differences between GSI treated and untreated cells (P ≤ 0.05).

C. Runx1 expression in lymphoid progenitors (c-kit⁺CD27⁺CD25⁻) isolated from day 3 OP9-DL1 cultures in the absence and presence of 3 μM GSI (averaged from triplicate samples from three independent experiments). The increase in Runx1 expression in GSI-treated cultures was significant at P < 0.01.

D. Ectopic expression of Runx1 in Cbfb⁺/+ FL cells cultured on OP9-DL1 in the absence and presence of GSI at 3 μM. Analysis was performed as in panel A.

Figure 6. CBFβ is required at an early stage of NK cell development in vivo.

A. Representative scatter plots of Lin⁻ 15.5 dpc FL cells grown on OP9 cells in the presence of Flt3L, IL-7, and IL-15. Gr-1⁻CD19⁻ cells were analyzed for CD122 and NK1.1 expression.

B. Percentage and absolute number of CD122⁺ cells (±SD) harvested from OP9 cultures established from 3 Cbfb⁺/+ and 4 Cbfb⁰⁰⁻⁻ fetuses (n_experiments = 3).

C. NK cells in 15.5 dpc fetal thymuses.
D. Total number of thymic NK1.1+ and NK1.1+ CD122+ cells averaged from 3 Cbfb+/+ and 3 Cbfb<r>s<s/> fetuses (n_experiments = 3).

E-G. Lethally irradiated CD45.1 x CD45.2 F1 recipients were reconstituted with wild-type CD45.1+ bone marrow and CD45.2+ FL cells from Cbfb<+/+>, Cbfb<rss/rss> or Cbfb<rss/-> fetuses (expressing 100%, 30% and 15% of normal CBFβ levels, respectively).

E. Bone marrow of recipient mice analyzed 10 months after reconstitution. The data are from gated FL-derived (CD45.2+) progenitors. A representative example is shown in each group (Cbfb<+/+>, n=4; Cbfb<rss/rss>, n=4; Cbfb<rss/->, n=5). Bone marrow from a 10 week old C57BL/6 (B6) mouse is shown as control. CD19<CD3<TCR<β> NK1.1<DX5> cells are mature NK cells, while immature NK lineage cells (iNK) have a NK1.1<DX5< phenotype.

F. The spleen of recipient mice analyzed in a similar way, illustrating the absence of immature and mature NK cells among cells derived from Cbfb<rss/-> progenitors.

G. Primitive NK lineage committed progenitors (NKP) (CD122/IL2Rβ<+> NKG2D<+) NK1.1< DX5< LinT<+) (LinT = CD3 CD4 CD8α TCRβ TCRγ) are preserved in the spleen of recipient mice. NKP express the IL-2/IL-15Rβ chain and have an NK1.1<DX5< CD19< CD3<CD4<CD8α TCRβ TCRγ< phenotype. A fraction of these cells expresses NKG2D.</g>  

H. Expression Runx1, Runx3, and Cbfb by qRT-PCR in LSK (Lin=CD4, CD8, TCRβ, TCRγ, DX5, CD19, Mac-1, Ter119), iNK (CD4< CD8< TCRβ TCRγ CD19< CD19< CD49b< NK1.1< CD122< ) and mature NK (CD4< CD8< TCRβ TCRγ CD19< CD19< CD49b< NK1.1< CD122< ) cells sorted from bone marrow of 8 – 12 week old wild type mice. The purity of post-sort populations was > 98%. Expression of individual genes was normalized to Hprt. Asterisks indicate significant differences from LSK cell values (P ≤ 0.01).
Figure 7. Genetic interactions between the CBFs and Notch signaling in the specification of HSCs, T, and NK cells in the fetus.

Notch receptors and ligands are expressed on the aortic endothelial cells in the AGM region that give rise to HSCs. Notch signaling is required for Runx1 expression in endothelial cells and for the formation of HSCs, and thus Notch is genetically upstream of Runx1 in HSC formation. (Solid lines indicate molecular interactions, and dashed lines represent cell migrations. PB, peripheral blood.)

HSCs and progenitors are released into the circulation from their sites of formation and colonize the fetal liver. We speculate that Runx1 and/or Runx3 plus CBFβ are required to generate NKP from either a bipotent T/NK or another progenitor in the fetus, and perhaps in T/NK progenitors themselves. CBFs are also required for the NKP to iNK transition, based on data from the adult.

Circulating progenitors expressing all three CBF complexes colonize the thymus where they encounter high levels of Notch ligands. CBFs confer upon these progenitors the ability to respond to Notch signaling, which results in T cell specification and progression to the ETP/DN2 stage accompanied by the expression of a suite of T cell specific genes. Not shown are NKT cells, which differentiate from DP T cells in a Runx1-dependent manner.
Figure 2

A

B

C

D

E

F

G

H

I

J

K

L

M

N
Figure 3

A. Isotype

B. Cbfb<sup>+/+</sup> vs Cbfb<sup>-/-</sup>

C. Cbfb<sup>+/+</sup> vs Cbfb<sup>-/-</sup>

D. Cell expansion (N=6)

E. MFI of c-kit

F. MFI of c-kit
Figure 4

A

![Bar graphs showing relative expression of Notch1, Hes1, Hes5, and Dtx1 over days of co-culture for Cbfb<sup>+/+</sup> and Cbfb<sup>−/−</sup> genotypes.](image)

B

![Flow cytometry plots showing NK1.1 and CD19 expression for Cbfb<sup>+/+</sup> and Cbfb<sup>−/−</sup> genotypes with 0, 1, and 3 μM GSI treatment.](image)

C

![Flow cytometry plots for Cbfb<sup>+/+</sup> + MigR1 and Cbfb<sup>+/+</sup> + ICN showing GFP<sup>+</sup> CD44<sup>+</sup> and GFP<sup>+</sup> CD25<sup>+</sup> cells.](image)

D

![Flow cytometry plots for Cbfb<sup>−/−</sup> + MigR1 and Cbfb<sup>−/−</sup> + ICN showing GFP<sup>+</sup> CD44<sup>+</sup> and GFP<sup>+</sup> CD25<sup>+</sup> cells.](image)
Figure 5

A

Cbfb<sup>+/+</sup>

0 µM GSI

1 µM GSI

3 µM GSI

Cbfb<sup>rss/-</sup>

CD44

CD25

B

Hes1

Dtx1

Gata3

Tcf1

Runx1

Runx3

C

0 µM GSI

3 µM GSI

C-kit

CD27

Cell count

CD25

D

0 µM GSI

+ MigR1

+ Runx1

3 µM GSI

+ MigR1

+ Runx1

CD44

CD25
Figure 6

(A) Flow cytometry analysis of Cd122 expression in Cbfβ+/+ and CbfβΔΔT cells. Gr-1, CD19, CD117, and NK1.1 were used as markers.

(B) Bar graphs showing the percentage of CD122+ cells and the number of CD122+ cells in Cbfβ+/+ and CbfβΔΔT cells.

(C) Schematic representation of the cell populations in Cbfβ+/+ and CbfβΔΔT cells, showing the percentage of NK1.1+ cells.

(D) Bar graphs depicting the NK1.1+ CD122+ cell population in Cbfβ+/+ and CbfβΔΔT cells.

(E) Flow cytometry analysis of Ly5.2+ bone marrow, showing CD19, CD3 + TCRβ, and NK1.1 expression in B6 Control, Cbfβ+/+, CbfβΔΔT, and CbfβΔΔT cells.

(F) Flow cytometry analysis of Ly5.2+ spleen, showing CD19 and NK1.1 expression in B6 Control, Cbfβ+/+, CbfβΔΔT, and CbfβΔΔT cells.

(G) Flow cytometry analysis of Ly5.2+ spleen, showing IL2Rβ and CD122 expression in B6 Control, Cbfβ+/+, and CbfβΔΔT cells.

(H) Bar graph showing the mRNA expression of Runx1, Runx3, and Cbfβ in LSK, INK, and NK populations.
Figure 7

AGM Region
Notch1 → Runx1 → HSC

PB
Runx1
Runx3
CBFβ

Fetal Liver
Runx1
Runx3
CBFβ
NK
NKP
Runx1
Runx3
CBFβ
INK
NK

PB
T/NK

Thymus
ETP/DN2
T cells
TCF7
GATA3
CD25
JAK1
Stat5

Runx1
Runx2
Runx3
CBFβ

Runx1
Runx2
Runx3
CBFβ

Runx1
Runx2
Runx3
CBFβ

Runx1
Runx2
Runx3
CBFβ

Runx1
Runx2
Runx3
CBFβ