Canonical and non-canonical Wnt signaling in hematopoiesis and lymphocyte development

Famili, F.

CHAPTER 5

TCF1 REGULATES T LYMPHOCYTE LINEAGE FIDELITY THROUGH ITS TARGET GENES GATA3 AND BCL11B

Farbod Famili[1]†, Laura Garcia Perez[1]†, Marja van Eggermond[1], Haoyu Wu[2], Martijn Brugman[1], Martijn Cordes[1], Machteld M. Tiemessen[3], Karin Pike-Overzet[1], Lucia Clemems-Daxinger[2], Frank J.T. Staal[1*]

†Equal contribution

[1.] Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, Netherlands

[2.] Department of Human Genetics, Leiden University Medical Center, Leiden, Netherlands

[3.] Jansen Discovery Center, Leiden, Netherlands

Submitted for publication

Abstract

T cell factor 1 (Tcf1) is the first T cell–specific protein induced in multipotent progenitors following Notch signaling in the thymus, leading to the activation of its two target genes, *Gata3* and *Bcl11b*. Tcf1 deficiency results in partial arrest in T cell development at various stages, high apoptosis, and increased development of B cells and myeloid cells. Phenotypically, fully T cell–committed Tcf1-deficient thymocytes have promiscuous gene expression and de-differentiate into immature thymocytes and non-T cells. Expressing Bcl11b in Tcf1-deficient cells rescues T cell development, but does not suppress the development of non-T cells; in contrast, expressing Gata3 suppresses the development of non-T cells, but does not rescue T cell development. These results reveal that T cell development is controlled by a minimal transcription factor network involving Notch signaling, Tcf1, and the subsequent division of labor between Bcl11b and Gata3, thereby ensuring a properly regulated T cell gene expression program.

Introduction

T cells are disease-fighting leukocytes that—similar to all blood cells—originate from hematopoietic stem cells (HSCs). However, whereas all other blood cell lineages develop in the bone marrow in specific niches , T cells develop in the thymus, a specialized organ located in the chest where progenitor cells migrate from the bone marrow and definitively commit to the T cell lineage, ultimately forming mature T cells [1]. The development of T cells within the thymus is a highly complex process involving successive stages in which the expression of CD4 and CD8 co-receptors occurs in distinct microenvironments [2]. Via a series of progressive developmental stages, T cell precursors (i.e., thymocytes) differentiate from double-negative (DN; $CD4^-CD8^-$) cells into intermediate immature single-positive (ISP; $CD8^+CD3^-CD4^-$) cells, then into double-positive (DP; $CD4^+CD8^+$) cells, and finally into single-positive (SP; $CD8^+CD4^-CD3^+$ or $CD4^+CD8^-CD3^+$) cells. In the DN stage, developing thymocytes can be further subdivided into four stages of differentiation based on their expression levels of CD44 and CD25: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$). Early stages are not committed to the T cell lineage (i.e., fate restricted), allowing alternate lineages to develop [3]. Indeed, B cells, dendritic cells, myeloid cells, and natural killer (NK) cells can all be generated from $CD44^+CD25^-$c-kit^{hi} early thymic progenitors (ETPs) [4,5], DN1 cells, and—albeit to a lesser extent—DN2 cells [6]. These multipotent cells, which can enter a number of differentiation programs, are directed towards the T cell lineage via a process called specification. The irreversible capacity to develop solely into T cells occurs somewhat later and is referred to as T lineage commitment; this process also involves the active repression of non-T cell lineages [7-9].

The microenvironment of the thymus provides a cellular context that drives T cell development. This process is initially driven by the expression of Notch ligands, particularly delta-like protein 4 (DLL4) [10], and later in the DP stage by providing the signals required to control positive selection (for self-MHC) and negative selection (against autoreactive T cell clones). The various stages in T cell development have been investigated in great detail using flow cytometry and genomic analyses; thus, T cell development serves as a paradigm for the molecular regulation of cell fate [11,12]. The fact that T cell development occurs in an anatomically separate niche has allowed researchers to study the detailed successive steps that underlie lineage specification and commitment. All of the events that establish the identity of T cell precursors are driven by Notch signalling [13], involving binding of the transcription factor RBP-J (also known as CBF1) to intracellular Notch ligands, thereby forming an active transcription factor complex in ETPs.

The subsequent stages in T cell development are governed by several key transcription factors that form an intricate gene regulatory network [14]. The core set of transcription

factors in the early phases of T cell development are Tcf1 (encoded by the gene confusingly termed *Tcf7*), Gata3, Bcl11b, and two members of the E2A family, Ikaros and Runx1 [14-17]. Importantly, the *Tcf7* gene is direct Notch signaling target and the first T cell–specific transcription factor induced by Notch signaling [18]; in contrast, Bcl11b drives T cell commitment by limiting the NK cell fate and activating the T cell developmental gene program at the DN2-DN3 stage [19], leading to expression of the fully rearranged TCR-beta gene in the DN3 stage. Rothenberg and colleagues showed that four transcription factors—Tcf1, Gata3, Notch/RBP-J, and to a lesser extent Runx1—are required for the timed expression of Bcl11b [14]. Of these four transcription factors, Tcf1 is the most complex, as it can act as both a transcriptional repressor (e.g., when bound by a co-repressor such as Groucho)or a transcriptional activator by binding β-catenin in order to respond to canonical Wnt signals [20]. Interestingly, Tcf1 also acts as a tumor-suppressor gene [21,22], and it can be functionally replaced—at least partially—by Lef1, a related transcriptional regulator expressed at approximately 50-fold lower levels than Tcf1 [23].

The precise role that Tcf1 plays in regulating T cell specification and commitment, and its interaction with other core regulatory factors in T cell development, is poorly understood. Therefore, we examined the role of Tcf1 in the earliest stages of T cell development, focusing primarily on fully committed DN3 cells. We found that Tcf1 is necessary for driving thymocytes down the T cell developmental path even after the T cell commitment stage, as Tcf1-deficient DN3 thymocytes can de-differentiate into DN1-like cells that can then develop into the myeloid and B cell lineages. In addition, we found that Tcf1 supports this "lineage fidelity" via two direct—and functionally complementary—target genes, *Gata3* and *Bcl11b*. An epistasis analysis using retroviral gene complementation in Tcf1-deficient stem cells revealed that the role of Gata3 in immature T cells is to repress B cell and myeloid fate, whereas Bcl11b establishes the T cell lineage program, and its expression can fully overcome the defect in T cell development inTcf1 deficient thymocytes.

Results

Tcf1 deficiency leads to several arrests in T cell development with increased non-T cells

Tcf1 deficiency results in multiple incomplete blocks in T cell development that vary from mouse to mouse (Fig 1A). While the block at the ISP stage is well described[24-26], the earlier blocks have not been well documented. We therefore set out to better characterize these blocks and noticed developmental arrests at DN1, DN2, DN3 and ISP stages. (Fig. 1A). Although the block at the ISP stage has been well described [24-26], arrests at the earlier stages have not been reported in detail.. In addition to the partial blocks in development shown in Fig. 1A, we also observed increased percentages of non-T cell lineages, most notably B cells and myeloid cells (Fig. 1B). In contrast, Tcf1 deficiency did not affect the development of NK cells (Fig. 1C), consistent with previous reports that the development of NK cells is independent of the effect of Tcf1 on T cell lineage [27,28]. Consistent with T cell developmental arrest, loss of Tcf1 significantly impaired αβ T cell development (Fig. 1C).

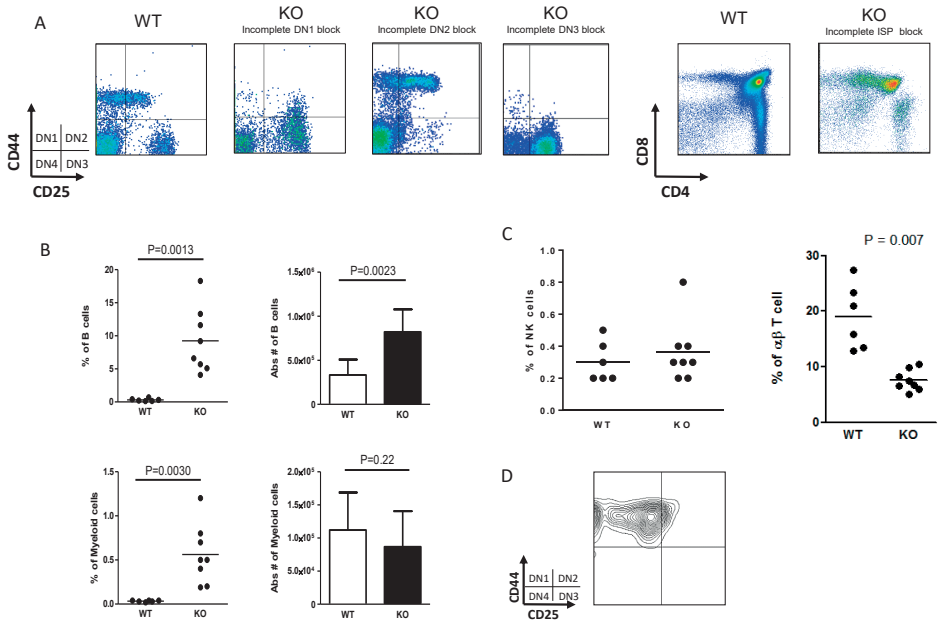

Figure 1. ***Tcf1 deficiency results in several incomplete blocks in T cell development and increased numbers of thymic B and myeloid cells***

A: Tcf1 deficient thymi can show different developmental arrests: DN1, DN2, DN3 or ISP. B: increased percentages of B cells and myeloid cells in Tcf1 -/- thymi. C: decreased numbers of αβT cells in Tc f1-/- thymi. D: Complete block at the DN1 to DN2 transition in the thymus of bone marrow chimeras generated from Tcf1 deficient stem cells.

In contrast to these partial arrests in developing mice, transplanting Tcf1-deficient stem cells into adult recipient mice led to a complete block in T cell development at the DN1-

DN2 transition (Fig. 1D), presumably of an insufficient compensatory expression of Lef1 in these cells [29]. Moreover, we cultured Tcf1-deficient stem cells on OP9-DL1 cells and found a similar DN1-like arrest, with increased numbers and relative percentages of non-T cell lineages (data not shown, but see Figs. 5 and 6).

Tcf1 deficiency leads to high levels of apoptosis

Developmental arrest in the thymus is often accompanied by an increase in apoptosis. To examine the role of apoptosis in Tcf1-deficient thymocytes, we measured apoptosis and proliferation of various developmental stages in the thymus of Tcf1-deficient mice and wild-type littermates. Compared to wild-type cells, we found increased levels of apoptosis in Tcf1-deficient cells at nearly every stage (Fig. 2), as well as decreased cell proliferation in the DN2 and DN4 stages (not shown).Taken together, these results explain the decrease in T cells in Tcf1-deficient mice; however, they do not explain the increased numbers of non-T cells.

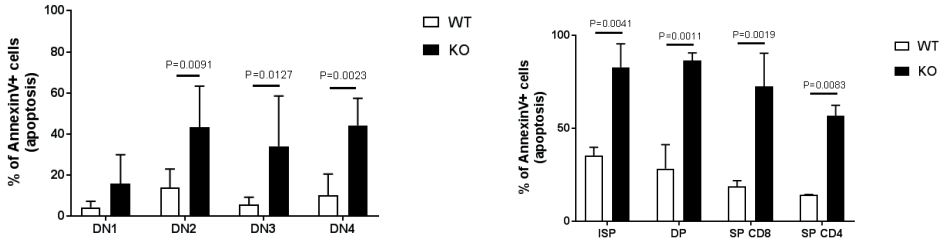

Figure 2: ***Increased apoptosis in Tcf1-/- thymocytes.***

Ex vivo Thymocytes were analysed by flow cytometry for various developmental subsets in combination with AnnexinV/7AAD. (n=4 or 5 per group)

Phenotypically, fully committed DN3 TCF1-deficient thymocytes have promiscuous gene expression and more open chromatin

Next, to better understand the role of Tcf1 in T cell commitment, we compared gene expression profiles between Tcf1-deficient thymocytes and wild-type thymocytes. The T cell commitment process starts at the DN2 stage and continues to the DN3a ($CD25^{+}CD44^{-}CD27^{-}$) stage in which a rearranged Tcrb gene is expressed in combination with pTA to form the pre-TCR complex in a process known as β-selection. After β-selection, the cells rapidly proliferate, express CD27, and are fully T cell committed based on expression of a functional, rearranged Tcrb gene [30]. We therefore performed whole-transcriptome RNA-Seq analysis on DN3b cells obtained from Tcf1-deficient and wild-type littermates (Fig 3A). For visualization the top 50 differentially expressed genes are shown and confirm absence of expression for Tcf7, but also that there were many fewer rearranged Tcrb genes than in wildtype control DN3b thymocytes, as indicated for the Trbj expressed gene segments(Fig. 3A). We used the genes differentially expressed

between Tcf1 deficient and wild type DN3b cells in a Gene Set Enrichment analysis (GSEA). We used published gene sets of T cell developmental stages to establish a DN2 signature [31]. The genes highly expressed in Tcf1-/- DN3b clustered strongly with DN2 cells, indicating that they share many characteristics of earlier developmental stages that are less T cell committed (Fig 3A). The RNA-seq data also indicated that many of the T cell commitment genes were low or not expressed while genes involved in for instance non T cell lineages (Pax5, Pu.1, Blc11a) were highly expressed. Based on these data we validated the expression of a number of important T cell developmental genes by Q-PCR on sorted DN1, DN2, DN3 and DN4 thymocytes. These results validated the RNA-Seq data and showed much lower expression of the T cell specific transcription factors Gata3 and Bcl11b (with higher expression of its functional counterpart Bcl11a) while the B cell commitment factor Pax5 and the myeloid associated factor Pu.1 were significantly higher expressed in the Tcf1 deficient thymocytes (Fig 3B). In addition, genes more associated with stem/progenitor cells (sometimes referred to as legacy genes[1]) such as c-kit were also significantly higher expressed(Fig 3B), while both Wnt and Notch target genes were decreased. Collectively, these data showed that while in some regards Tcf1-/- DN3 thymocytes were T cell committed, they also showed lineage infidelity, with expression of master regulatory genes from non-T cells. This notion was further substantiated by investigating the chromatin status of Tcf1 vs wildtype thymocytes using ATAC-Seq (Assay for Transposase-Accessible Chromatin sequencing) as discussed below.

Gata3 and Bcl11b are direct targets of Tcf1 and downregulated in Tcf1 deficient thymocytes

The downregulated mRNA expression levels of the transcription factors Gata3 and Bcl11b in various DN thymocyte stages in Tcf1 deficient mice, suggested that these factors may be direct target genes of Tcf1. In accordance, the Bcl11b and Gata3 enhancers contain conserved Tcf/Lef binding sites (see M&M). To check whether in DN thymocytes these promoters are regulated in a Tcf-dependent manner, we performed chromatin immune precipitation (ChIP) using a monoclonal antibody specific for Tcf1 (Fig 4A) followed by Q-PCR. This revealed binding of Tcf1 to the Gata3 and Bcl11b promoter sequences in wild type DN thymocytes but not in Tcf1 deficient thymocytes, showing that both genes are direct target genes of Tcf1. This finding was further substantiated by ATAC-Seq (Assay for Transposase-Accessible Chromatin) data which indicates chromatin accessibility Although in general, chromatin was less condensed in DN3b thymocytes lacking Tcf1 compared to wildtype DN3b cells, in certain specific areas the chromatin was more condensed. Focusing on the Bcl11b and Gata3 promoter/enhancer sequences, the chromatin in these promoters was less accessible compared to wild type littermate control DN3b cells (Fig 4B). Similarly, the TCRB loci were much less accessible in accordance with the RNA-Seq data. Interestingly, no major differences in chromatin accessibility were found at genes involved in alternative lineages, indicating that expression of these genes was not regulated at the level of chromatin opening.

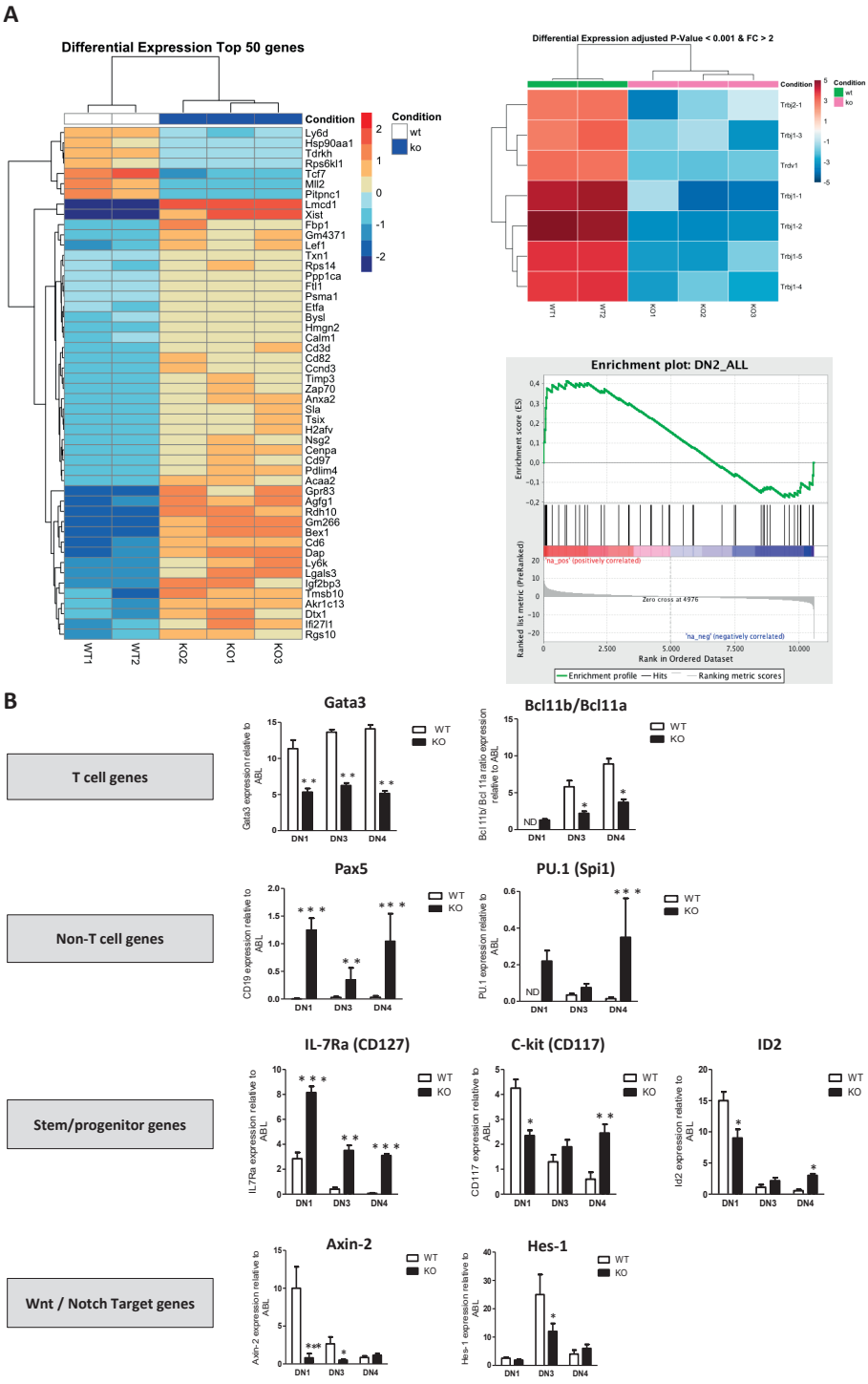
A
Differential Expression Top 50 genes
Condition
wt
ko
Ly6d
Hsp90aa1
Tdrkh
Rps6kl1
Tcf7
Mll2
Pitpnc1
Lmcd1
Xist
Fbp1
Gm4371
Lef1
Txn1
Rps14
Ppp1ca
Ftl1
Psma1
Etfa
Bysl
Hmgn2
Calm1
Cd3d
Cd82
Ccnd3
Timp3
Zap70
Anxa2
Sla
Tsix
H2afv
Nsg2
Cenpa
Cd97
Pdlim4
Acaa2
Gpr83
Agfg1
Rdh10
Gm266
Bex1
Cd6
Dap
Ly6k
Lgals3
Igf2bp3
Tmsb10
Akr1c13
Dtx1
Ifi27l1
Rgs10
WT1
WT2
KO2
KO1
KO3
Differential Expression adjusted P-Value < 0.001 & FC > 2
Trbj2-1
Trbj1-3
Trdv1
Trbj1-1
Trbj1-2
Trbj1-5
Trbj1-4
Enrichment plot: DN2_ALL
Enrichment score (ES)
'na_pos' (positively correlated)
Ranked list metric (PreRanked)
Zero cross at 4976
'na_neg' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
B
T cell genes
Non-T cell genes
Stem/progenitor genes
Wnt / Notch Target genes
Gata3
Gata3 expression relative to ABL
Bcl11b/Bcl11a
Bcl11b/ Bcl 11a ratio expression relative to ABL
Pax5
CD19 expression relative to ABL
PU.1 (Spi1)
PU.1 expression relative to ABL
IL-7Ra (CD127)
IL7Ra expression relative to ABL
C-kit (CD117)
CD117 expression relative to ABL
ID2
Id2 expression relative to ABL
Axin-2
Axin-2 expression relative to ABL
Hes-1
Hes-1 expression relative to ABL
WT
KO
DN1
DN3
DN4
ND

Figure 3: *Tcf1 deficient DN3b cells show promiscuous gene expression and more open chromatin loci compared to wild type littermate controls (continued)*

A: Heat map of the top 50 differentially expressed gene as determined by RNA-SEq of sorted DN3b cells from wild type and Tcf1 deficient thymi, with an extra focus on rearranged TCR products on the right. GSEA of the differentially expressed genes (Tcf1-/- over wild type for DN3b) are enriched for DN2 genes. B: Q-PCR validation of RNA-Seq data for selected T cell specific genes, genes expressed in non-T cells and legacy genes whose expression is inherited from stem cells/ multipotent progenitors

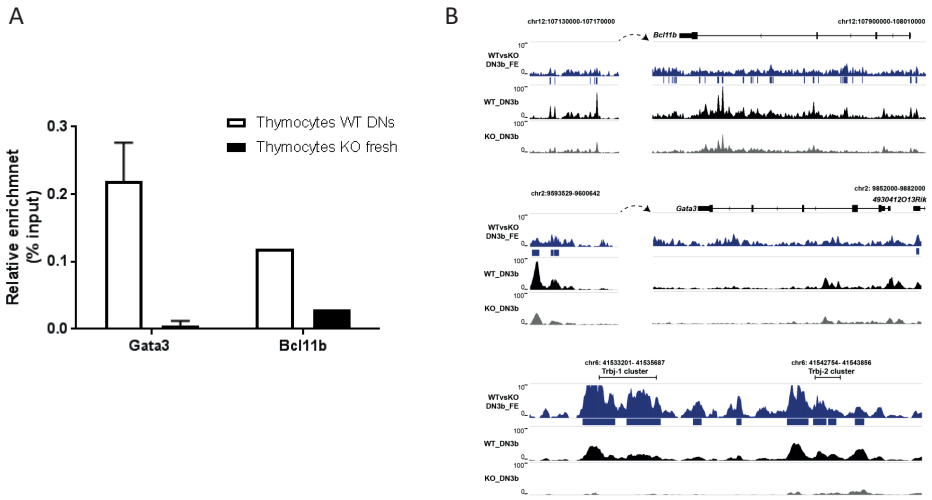

Figure 4: *Chromatin analysis in Tcf1 deficient vs. wild type DN thymocytes.*

A. Chromatin immune precipitation with an antibody specific for Tcf1 revealed that the Gata3 and Bcl11b promoters are occupied by Tcf1 in vivo, whereas in Tcf1-/- DN thymocytes no binding can be detected. Negative controls with IgG instead of anti-Tcf1 showed no enrichment. B. ATAC-Seq data mined for the Bcl11b, Gata3 and TRB-J genomic regions. Per locus the relative abundance of transposase accessible regions is indicated. In blue the fold enrichment(FE) of wildtype over Tcf1-/- DN3b cells are indicated, blue bars underneath show the significant peaks called by MACS2. In black the individual ATAC-seq profile from each genotype is shown. Data are shown as normalized read density.

Phenotypically, fully committed DN3 Tcf1-deficient thymocytes de-differentiate into DN1 thymocytes, B cells, and myeloid cells

Based on the hypothesis derived from these results, that Tcf1 deficient DN3 thymocytes may not be T cell committed, we sought to better investigate the differentiation capacity of Tcf1-/- DN3 thymocytes. Therefore, DN3 cells were sorted and cultured under conditions with strong T cell inducing capacity. Indeed the majority of wild type DN3 thymocytes differentiated further into DN4 cells, with a smaller part remaining DN3 (Fig 5A). Unexpectedly, most Tcf1-/- DN3 thymocytes dedifferentiated into DN1 and DN2 cells, with extensive B and myeloid development while only a minority of cells remained DN3 without any further

development along the T cell lineage (Fig 5A). Especially development into B cells was extensive, with up to 60% of DN3 thymocytes developing into B cells (Fig 5B).These DN1 and DN2 cells were not a contaminating fraction that grew out, as intracellular staining for Tcrb revealed high Tcrb expression in these DN1/2 cells at similar levels as wild type DN3 and DN4 cells (Fig 5B). We conclude that Tcf1KO cells dedifferentiate to less committed cells and exhibit lineage infidelity with significant development into alternative (non-T) lineages. When ETP cells rather than DN3 cells were seeded on OP9-DL1, as expected Tcf1 deficient cells were arrested in development at DN1, with abundant B and myeloid development, whereas wild type stem cells differentiated along the T cell lineage with many fewer non-T cells (Suppl Fig 1)

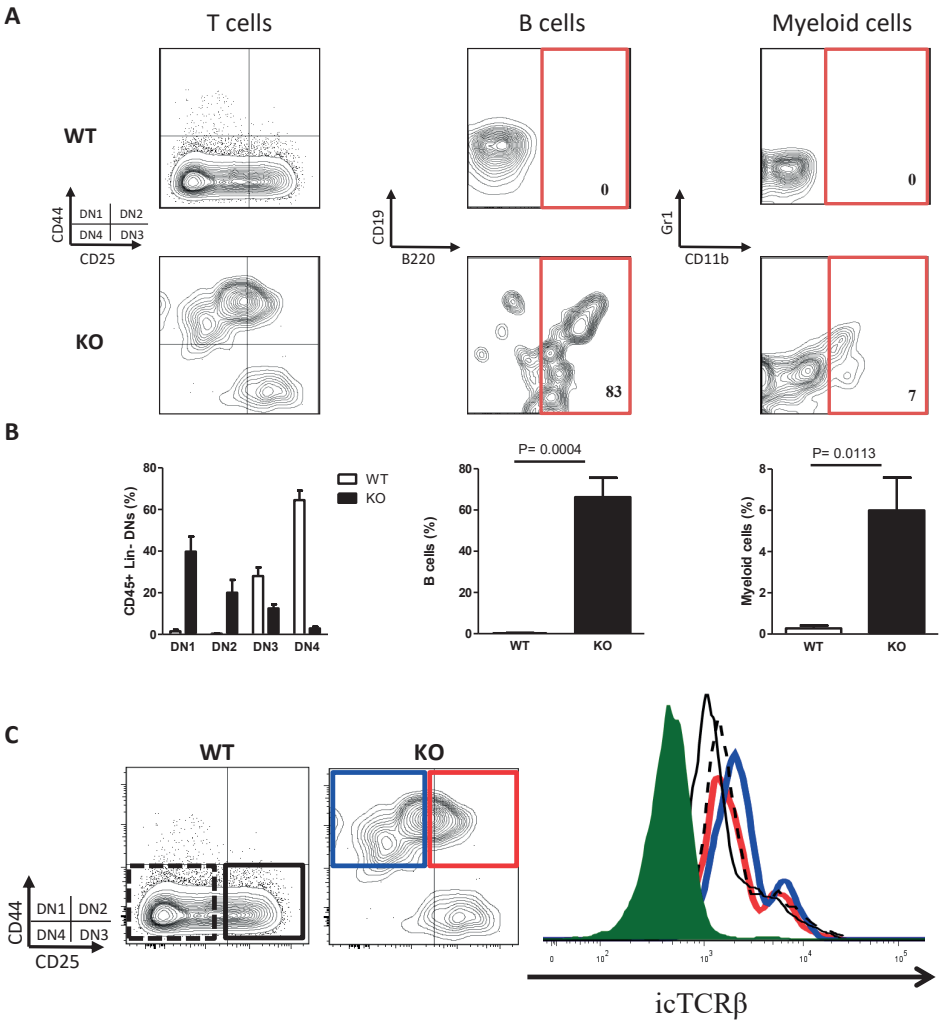

Figure 5: ***Tcf1 deficient DN3 cells de-differentiate into DN1/2 cells with multipotent lineage capacity (continued)***

A: wild type DN3 cells sorted and seeded on OP9-Dl1 cells develop largely further into DN4 or remain DN3, while Tcf1 deficient cells develop into DN1 and DN2 cells with prominent B cell and myeloid cell development B: Quantification of the developmental plasticity nad de-differentaion effcts of DN3 Tcf1 deficient thymocytes into DN1, DN2, myeloid and B cells. C: Intracellular TCRβ staining reveals the dedifferentiated DN1 and DN2 cells to be derived from DN3 cells

De-differentiation into alternate lineages can be prevented by expressing Gata3 in Tcf1 deficient thymocytes

Epistasis analysis is a powerful genetic tool, often used in model organisms such as Drosophila to investigate hierarchical relationships between genes[32]. It can be more complex to perform in mammals such as mice, where not only expression per se but also gene dosage is important. For instance, while complete loss of Gata3 blocks T cell development at the earliest stages, transgenic overexpression of Gata3 can lead to development of mast cells in the thymus[33-36]. We therefore expressed Gata3 and Blc11b using recombinant retroviruses as they have a broad range of expression that would allow different phenotypes to be selected under the strong developmental pressure of the thymic microenvironment. We used retroviruses encoding GFP and Gata3 or Bcl11b together with GFP to investigate complementation of the Tcf1 phenotype by either Gata3 or Bcl11b(Fig 6A,7A). We used retroviruses solely encoding GFP as negative controls.

Expression of Gata3 could partially rescue the development of Tcf1-/- thymocytes from a DN1 arrest to DN2 but not further(Fig 6B). Strikingly, Gata3 strongly suppressed the enhanced development of B and myeloid cells (granulocytes as well as monocytes) from Tcf1-/- as well as the less prominent non T development from wild type thymocytes (Fig 6C). The suppression of B cell development was also observed in vivo when Gata3 completed Tcf1-deficient stem cells were transplanted in irradiated recipient mice (Fig 6D, right and supplemental Fig 2A). However, thymic T cell development again was arrested at a DN1/2 transition, barely different than GFP control transduced cells (Fig 6D, left; supplemental fig 2B). Thus, the major role of Gata3 in earliest DN development is the suppression of non T cell development with only a minor feed forward role into the T cell program.

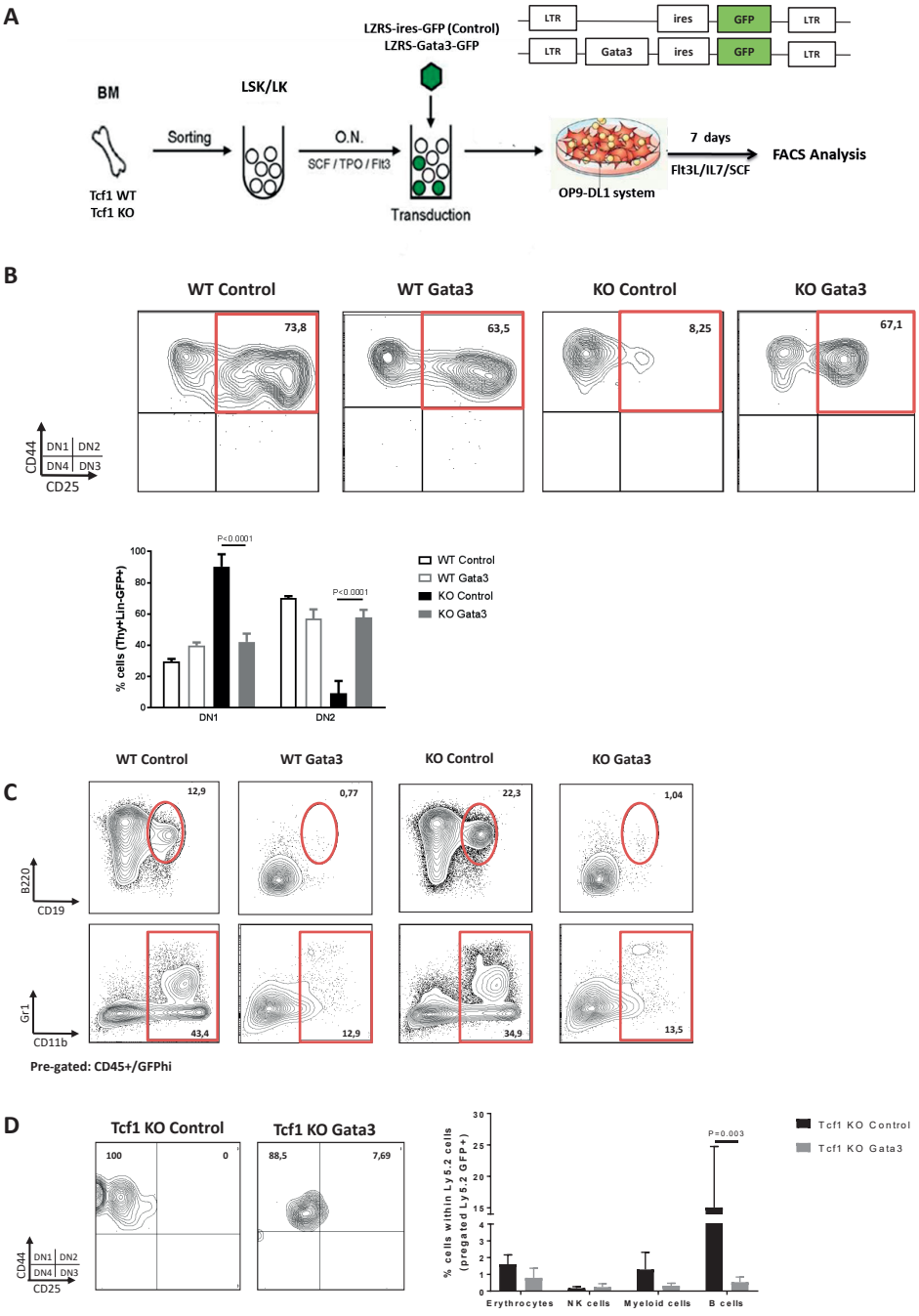
A
LZRS-ires-GFP (Control)
LZRS-Gata3-GFP
LTR
ires
GFP
LTR
LTR
Gata3
ires
GFP
LTR
BM
LSK/LK
Sorting
O.N.
SCF / TPO / Flt3
Transduction
7 days
Flt3L/IL7/SCF
FACS Analysis
OP9-DL1 system
Tcf1 WT
Tcf1 KO
B
WT Control
WT Gata3
KO Control
KO Gata3
73,8
63,5
8,25
67,1
CD44
CD25
DN1
DN2
DN4
DN3
% cells (Thy+Lin-GFP+)
P<0.0001
P<0.0001
DN1
DN2
WT Control
WT Gata3
KO Control
KO Gata3
C
WT Control
WT Gata3
KO Control
KO Gata3
12,9
0,77
22,3
1,04
B220
CD19
Gr1
CD11b
43,4
12,9
34,9
13,5
Pre-gated: CD45+/GFPhi
D
Tcf1 KO Control
Tcf1 KO Gata3
100
0
88,5
7,69
CD44
CD25
DN1
DN2
DN4
DN3
% cells within Ly5.2 cells (pregated Ly5.2 GFP+)
P=0.003
Erythrocytes
NK cells
Myeloid cells
B cells
Tcf1 KO Control
Tcf1 KO Gata3

Figure 6: ***Re-expression of Gata3 suppresses B and myeloid development in Tcf1 deficiency. (Continued)***

A:layout of retroviral complementation experiments with GFP and/or Gata3

B: Gata3 expression partially overcomes the DN1 thymocyte block and suppressed the enhanced non-T cell lineages after 7 days in the OP9-Dl1 culture system (Two-way ANOVA) Error bars represent the SD from two independent experiments. C: in vivo complementation reveals suppression of B cell development also in the spleen, but minimal and partial rescue of T cell development in the thymus (D)(two-way ANOVA) Error bars represent the SD from 3-4 individual mice per group.

The T cell lineage-specific defects caused Tcf1 deficiency can be rescued by expressing Bcl11b

Bcl11b(Fig 7A) in contrast rescued the T cell development of Tcf1 deficient cells virtually completely. Bcl11b transduced Tcf1 deficient stem cells developed readily into Thy1 positive (Fig 7B) cells, and could develop into DN2 and DN3 thymocytes to a similar degree as wild type thymocytes (Fig 7C). However, overexpression of Bcl11b did not influence B and myeloid development from Tcf1 deficient cells.

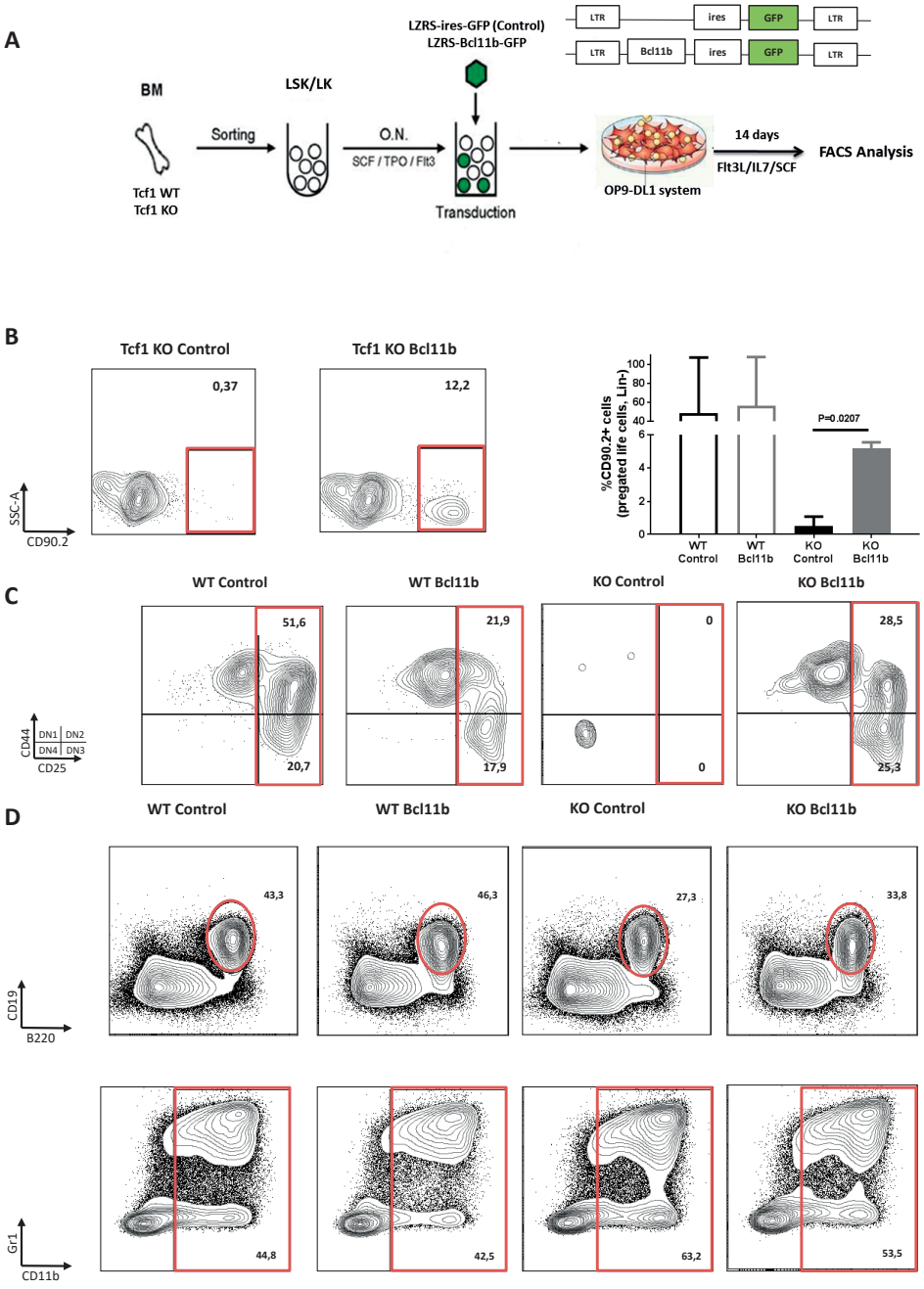

Figure 7: ***Re-expression of Bcl11b rescues T cell development from Tcf1 deficient stem cells.***

A: layout of retroviral complementation experiments with Bcl11b. B: Thy1 expression is rescued in Tcf1 deficiency by expression of Bcl11b after 14 days in OP9-Dl1 culture. C: Bcl11b fully rescues T cell development from Tcf1 -/- stem cells that otherwise are arrested in DN1. D: Bcl11b overexpression does not affect myeloid and B cell development. (Two-way ANOVA) Error bars represent the SD from three wells in two independent experiments.

Discussion

T cell development has been used as a classic example of a relatively ordered pathway to study cell fate determination [16], thereby giving the impression that transcriptional regulation during T cell development is a well-understood process. Despite this general belief, however, and compared to other developmental processes (for example, B cell development, which has similar requirements in terms of proliferation, lineage restriction, immune receptor rearrangement, and checkpoints for premature and mature immune receptors), the roles of the major transcription factors in T cell development are rather poorly understood. In B cell development, a clearly defined linear hierarchical relationship exists between E2A, EBF1, and Pax5 [37-44]. However, with respect to early T cell development, whether the Notch (RBP-J), Gata3, Bcl11b, Runx1, E2A, Tcf1/Lef1, Ikaros, and/or Hox genes play unique, redundant, or synergistic roles remains unclear and is the subject of intense research that focuses largely on either individual factors or the collective activity of these factors using computational biology. Considering that Notch signaling is required for T cell development, and given that the first T cell–specific target gene is *Tcf7* [18], which encodes Tcf1, we investigated the process of T cell lineage commitment in Tcf1-deficient mice.

Although Tcf1-deficient mice have been studied extensively, a comprehensive description of developmental arrest in these mice is currently lacking. Here, we report that developing thymocytes in adult Tcf1-deficient mice have incomplete arrest at several stages, including the previously reported ISP block [26].

The study of Tcf1-deficient mice is generally complicated by three factors. First, in the absence of Tcf1, the HMG box transcription factor Lef1—which is expressed in the thymus, albeit at much lower levels than Tcf1—plays a compensatory role [23,29,45]. This low-level expression of Lef1 causes incomplete penetrance of the Tcf1-deficient phenotype. However, if Tcf1-deficient stem cells are either transplanted into recipient mice or cultured on OP9-DL1 cells to induce T cell differentiation, complete block occurs at the DN1 stage (see Fig. 1D). Therefore, in our experiments we used bone marrow-derived cells obtained from Tcf1-deficient mice. Second, Tcf1-deficient mice are prone to developing T cell lymphomas in the thymus [22], which is similar to T-cell acute lymphoblastic leukemia (T-ALL) in patients. As discussed above, this issue can be overcome by using Tcf1-deficient stem cells instead of thymocytes. The third issue associated with studying Tcf1-deficient mice is that Tcf1 functions as both a transcriptional repressor and a transcriptional activator (for example, when bound to the Wnt mediator β-catenin). Indeed, when Tcf1-dependent promoters were tested using *in vitro* reporter systems, transcription occurred only when β-catenin was also expressed [46,47]. Consistent with this notion, Tcf1 binds to the promoter/enhancer regions of the target genes *Gata3* and *Blcl11b*, and we found evidence that Tcf1 binds to

β-catenin at these promoter regions (data not shown). In addition, DN stages of T cell development show high canonical Wnt signaling, which is driven by β-catenin and Tcf/Lef [48]. On the other hand, some of Tcf1's functions in the earliest stages of T cell development are independent of β-catenin [18], possibly due to the redundant role of Lef1.

A seminal study by Busslinger and colleagues revealed that Pax5 is a major lineage commitment factor in the development of B lymphocytes [42,43,49]. Thus, B cells that lack Pax5 can de-differentiate into multipotent progenitor cells that can replenish all hematopoietic lineages, even *in vivo*. In this respect, our findings are somewhat analogous, as Tcf1-deficient DN3 cells—which should be fully committed—have promiscuous gene expression and can de-differentiate into immature cells that can give rise to non-T cell lineages, including B cells and myeloid cells. Indeed, key transcription factors that drive alternate lineages (e.g., the transcription factors Bcl11a, Pax5, and Pu.1) are robustly expressed in Tcf1-deficient DN3 and DN4 cells, but not in wild-type cells. In contrast to wild-type cells—in which global chromatin accessibility is restricted in order to ensure that only T cell lineage genes are expressed—Tcf1-deficient cells have many more accessible loci, thereby explaining their functional role as multipotent progenitor cells. In contrast with Pax5-deficient cells, however, only a small number of Tcf1-deficient cells survive the de-differentiation process, which is likely due to the high level of apoptosis in Tcf1-deficient thymocytes.

Given that both *Bcl11b* and *Gata3* are key target genes for Tcf1, we expressed these transcription factors in Tcf1-deficient cells in an attempt to rescue the thymic phenotype. Similar analyses of epistasis have been used previously in model organisms (e.g., *Drosophila*) to delineate both hierarchical and functional relationships. The expression of exogenous *Gata3* has been shown to suppress B cell development in the wild-type thymus [50]; however, we found that Gata3 also suppresses myeloid fate in DN thymocytes. Interestingly, Gata3 does not suppress myeloid fate in the bone marrow, even though the effect on B cell development occurs outside of the thymus.

Our finding that the constitutive expression of Bcl11b in Tcf1-deficient cells fully rescued T cell development suggests a division of labor between Bcl11b and Gata3, with Gata3 suppressing non-T cell lineages and Bcl11b inducing the expression of T cell–specific genes. Taken together, the data from our group and others indicate a gene network in which Notch signaling via RBP-Jk drives the expression of Tcf1, which in turn activates Gata3 and Bcl11b, most likely in collaboration with Notch signals that can also act directly on these genes' promoters. Interestingly, in addition to its requirement for initiating the T cell commitment process, Tcf1 expression is also required to maintain lineage fidelity. In skin stem cells, lineage infidelity increases the likelihood of malignancy [51]. Thus, given that loss of Tcf1 leads to the rapid development of T cell lymphomas, lineage infidelity may also serve as a previously unrecognized factor in leukemogenesis.

Materials and Methods

Mice

C57Bl/6 TCF-1 -/- ΔVII/ΔVII mice were originally described by Verbeek et al (1995) and C57Bl/6-Ly5.1 mice were purchased form Charles Rivers Laboratories. Mice were bred and maintained in the animal facility of Leiden University Medical Center. All animal experiments were performed in accordance with legal regulations in The Netherlands and with approved protocols of the Dutch animal ethical committee.

Mice used for transplantation assay were kept in specified pathogen-free section and were fed with special food and antibiotic water. Genotyping assay of newborn Tcf1 mice was performed with DNA samples from earpieces using GoTaq Flexi DNA polymerase kit (Promega) according to manufacturer's instructions.

Flow cytometry and cell sorting

Single cell suspensions from thymus, spleen, BM and blood were stained with monoclonal antibodies against CD3e, CD4, Cd8a/Ly-2, CD11b/Mac-1, CD19, CD25, CD27, , CD44/Ly-24, CD45.1/Ly-5.1, CD45.2/Ly-5.2, B220/CD45R, CD90.2/Thy1.2, CD117/c-kit, CD135/Flt3, Gr1/Ly-6G-6C, NK1.1, Sca1/Ly-6A, TCRβ, and Ter-119/Ly-76 (See STAR Table_ Antibodiesfor additional antibody information). All antibodies used were directly conjugated to biotin, fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin Chlorophyll-a Protein (PerCP), PE-Cy7, allophycocyanin (APC), APC-Cy7 or efluor450. Biotinylated antibodies were revealed with streptavidin conjugated antibodies (PE, efluor450, APC-Cy7, APC or Pe-Cy7) (all antibodies were purchased from BD or eBioscience).

Cells were blocked with normal mouse serum (NMS, Invitrogen) for 10min at room temperature and subsequently cell surface staining was performed in two steps. Firstly, cells were incubated for 30min at 4°C in the dark with the antibody-mix solution including directly conjugated antibodies at the optimal working solution in FACS buffer (PBS pH7.4, 0.1% azide, 0.2% BSA). After washing with FACS buffer, a second 30min incubation step at 4°C was performed with the streptavidin conjugated antibodies mix.

Cell apoptosis was assessed by AnnexinV and 7AAD staining, which was performed following the PE AnnexinV Apoptosis detection Kit protocol (BD Pharmingen) after the cell surface staining. Proliferation assay was done by intracellular Ki67 staining (mIgG as control) with PE Mouse anti-human Set protocol (BD Pharmingen). For that purpose, cells were initially stained for cell surface markers as described previously and subsequently fixated and permeabilized by using fixation/permeabilization buffer (eBiosience) for an

hour at 4°C. Cells were then washed with permeabilization (eBiosience) buffer with 2% NMS and stained with Ki67 or IgG1 solution for 30min at 4°C in the dark.

Double positive CD4&CD8 cells before DN cell sorting and lineage positive cells before LSK/LK sorting were depleted using magnetic-activated cell sorting, autoMACS (Miltenyi Biotec). For DNs sorting, thymocytes were first stained with anti-CD4 and CD8-biotin, following by Streptavidin microbeads staining according to manufacturer instruction (Miltenyi Biotec). For LSK/LK cell sorting, lineage depletion kit (Miltenyi Biotec) was used according to manufacturer instruction. Subsequently, depleted cells were stained again for DNs or LSKs as described before. Cell sorting was performed on FACSAria II (BD Biosciences) or stained cells were measured with FACS-CantoII (BD Bioscience). Data was analysed using FlowJO (Tree Star). All different hematopoietic populations were defined as described in Table S2_Appendix.

Cell culture

Bone-marrow-derived stromal cell line OP9 and OP9-DL1 cells which ectopically express the Notch ligand Delta-Like 1 (DL1) were used as described by J.C. Zuñiga-Pflucker. Sorted DN cells were cultured on OP9 or OP9 WT/OP9-DL1 (10:1) confluent monolayers in αMEM (Lonza)-10%FCS, 1% P/S (Life Technologies) and GlutaMAX (Life Technologies) medium complemented with 50 ng/ml rmFlt3L, 50 ng/ml rmSCF, 10 ng/ml rmIL-7, and 50μM β-mercaptoethanol (β-ME; Sigma-Aldrich). (all cytokines purchased from R&D). Cells were harvested after 7 to 14 days of coculture and were analysed by flow cytometry.

Transduced LSK and LK with LZRS-ires-eGFP (control), LZRS-Gata3-eGFP or LZRS-Bcl11b-GFP vector were cultured on OP9-DL1 monolayer for 6 to 14 days in αMEM-10%FCS complemented with rmIL7 (10 ng/ml),rm Flt3L (50 ng/ml), rmSCF (10 ng/ml) and β-ME (50μM). Harvested cells were analysed by flow cytometry or sorted.

Retroviral production

LZRS-Gata3 and Bcl11b plasmids were obtained from Addgene and cloned into LZRS-ires-eGFP vector (Addgene, control vector). Control, Gata3 and Bcl11b retroviruses were generated using Phoenix ecotropic packaging cell line (ATTC). Cells were cultured in IMDM (Lonza)-10%FCS-1% Penicillin/Streptomycin -1%Glutamine and transfected with plasmids using X-treme Gene9 DNA transfection reagent (Roche) protocols. Selection of transfected cells was performed with 1mg/mL puromycin (Sigma-Aldrich) for a week and viral supernatant was harvested at 24h and 48h.

Retroviral transduction

LSK and LK sorted cells were stimulated overnight in StemSpan serum-free expansion medium (StemCell Technologies) supplemented with 10ng/ml rmTPO (R&D) , 50ng/ml rmFlt3L (R&D) and 100ng/ml rmSCF (R&D). Antibiotic mix with polymixine B sulphate, kanamycin, penicilin/streptomycin and amphotericin B was added to prevent bacterial infections. Hematopoietic progenitors were transduced using RetroNectin (Takara Bio Inc) coated wells according to the manufacturer's instructions. Non-tissue culture plates were coated with RetroNectin overnight at 4ºC and then blocked with 2% bovine serum albumin (BSA) in PBS for 30min. Retroviral supernatant (24h or 48h) was centrifuged at 1500xg for 2h at 32ºC and incubated an extra hour at 37ºC. After coating, viral supernatant was removed and stimulated cells were immediately added on the virus-coated plates. Cells were cultured in StemSpan medium supplemented with rmTPO (10 ng/ml), rmFlt3L (50 ng/ml) and rmSCF (100 ng/ml) and transduced overnight at 37ºC. LZRS-ires-eGFP,LZRS-Gata3-ires-eGFP and LZRS-Bcl11b-ires-eGFP transduced cells were used for *in vitro* and *in vivo* approaches.

Quantitative real time (rt)-PCR

RNA from sorted cells was purified using Micro RNeasy kit (Qiagen) and reverse transcribed into cDNA using Superscript III kit (Invitrogen). RT-PCR was performed using TaqMan Universal Master Mix II in combination with specific probes for indicated genes from Universal Probe Library (Roche). Specific primers for ABL-2, Bcl11a, Bcl11b, Gata3, Pax5, PU.1/Spfi1, IL-7Ra, CD117/c-kit, ID2, Axin-2 and Hes1. were designed and purchased from Sigma-Aldrich (See specific gene sequences on Table 3_Appendix). Samples were analyzed by StepOnePlus RT-PCR system (Life Technologies). Relative transcript abundance was determined by ΔCt and expression levels were normalized for the endogenous reference gene ABL-1. All samples were run in at least in duplicates.

RNA-SEQ

RNA from sorted DN3b cells ($Lin^-CD25^+CD44^-CD27^+$) from Tcf1-/- and wild type littermates thymi was isolated using the Mini RNeasy Kit (Qiagen) The integrity (scores > 9.0) of the RNA was determined on the Agilent 2100 Bioanalyzer (Agilent). Total RNA enrichment for sequencing poly(A) RNAs was performed with the TruSeq mRNA sample preparation kit (Illumina). 1µg of total RNA for each sample was used for poly(A) RNA selection using magnetic beads coated with poly-dT, followed by thermal fragmentation. The fragmented poly(A) RNA enriched samples were subjected to cDNA synthesis using Illumina TruSeq preparation kit. cDNA was synthesized by reverse transcriptase (Super-Script II) using poly-dT and random hexamer primers. The cDNA fragments were then blunt-ended through an end-repair reaction, followed by dA-tailing. Subsequently, specific double-stranded bar-coded adapters were ligated and library amplification for 15 cycles was performed. The pooled cDNA library

consisted of equal concentration bar-coded samples. The pooled library was sequenced in one lane, 36 bp single read on the HiSeq2500 (Illumina).

RNaseq data were aligned to the mm10 genome using the STAR aligner (Dobin et al., 2013) and quantified using featureCounts . The raw counts data were processed using the "voom" function (Law et al., 2014) in the limma R package which normalizes the data and assigns a weight for each measurement for subsequent linear model fitting.. Differential expression was assessed using the limma moderated T statistic.. Normalization for replicate number and technical parameters was also applied directly to the voom result to obtain "normalized counts," which were used for data visualization. Geneset enrichment was performed using the "RankSumWithCorrelation" function in the limma R package, which automatically corrects enrichment statistic inflation due to correlation among genes

ATAC-SEQ

15,000 sorted DN3b Cells were washed 1 time with cold PBS. Pellets were spun down at 500 g for 5 min at 4°C, and the supernatant was removed carefully. 20 µl of transposase mix (10µl 2xTD buffer, 1 µl TDE (Nextera DNA Library Prep Kit; Illumina), 0.2 µ digitonin (G9441, Promega), 8.8 µl nuclease-free water) was added to the cells. Reactions were incubated at 37°C for 30 min. Transposed DNA was purified using the MinElute Reaction Cleanup Kit (28204, Qiagen), amplified, and again purified according to published protocols (Buenrostro et al 2015, CurrProtocMolBiol). Size selection was done using Low Range Ultra Agarose (161-3107, Bio-Rad). Fragments between 150-600 bp in size were used for further analysis. Quality and quantity of the libraries was assessed by Bioanalyzer High Sensitivity DNA Analysis Kit (Agilent) before sequencing. Libraries were sequenced 50 bp, paired-end, on a HiSeq4000.

Reads were mapped to mm10 using bwa mem(Li H et al 2010, Bioinformatics) with default settings. Only reads with high mapping quality (Q>10) were used for further analysis, and DNA duplicates were removed using samtools(Li H et al 2009, Bioinformatics). Data were aggregated by each unique genotype and subsampling was done to correct for sequencing depth(about 23million reads). Differential peaks(in bed file format) between WT and Tcf1 KO cells were called using MACS2 with the following parameters: -g mm -B –nomodel (q-value <0.05). BigWig-tracks were generated by MACS2 using WT as input and Tcf1 KO as control. The track of Fold Enrichment (WT over KO) was generated using the bdgcmp function in MACS2 with the following settings: -m FE.

Chromatin immunoprecipitation

DN thymocytes ($CD8^-CD4^-$) from Tcf1-/- and wildtype littermates were sorted and subsequently crosslinked with formaldehyde (Sigma). Crosslinking was quenched with Glycine and after cell lysis chromatin was sonicated into fragments. Sonicated chromatin was precleared

and incubated with antibodies. TCF-1 (C46C7; #2206 Cell Signalling Technologies). Immuno precipated chromatin complexes were purified and quantified by real-time PCR using Fast-start Universal Sybr Green Master mix (Roche)

Competitive transplantation assay

Competitive transplantation assay is used to determine HSC development and functionality in vivo by measuring multi-lineage reconstitution of hematopoiesis in irradiated transplanted mice. This assay was performed with the Ly5.1/Ly5.2 (CD45.1/CD45.2) system. 12.5000 transduced LSK and 40.000 transduced LK cells from Ly5.2 mice were transplanted into lethally irradiated (8.07Gy) Ly5.1 mice (8-12 weeks), together with 300.000 spleenocytes (Ly5.1) as support cells. Chimerism and peripheral T cell were analysed at week 6 after transplantation in peripheral blood. Mice were sacrificed for analysis 7 weeks after transplantation to evaluate hematopoietic system repopulation. Mice were considered repopulated when ≥1% multi-lineage Ly5.2 cells could be detected. Single cell suspension from the thymus, spleen and bone marrow (BM), as well as lysate blood were analysed by flow cytometry as described previously.

Statistical methods

All statistics were calculated and all graphs were generated using GraphPad Prism6 (GraphPad Software). Statistical significance was determined by standard two-tailed Student t test t student (*$p<0.05$) or ANOVA.

Acknowledgements

Authors are indebted to Dr. Sjef Verbeek for critically reading the manuscript and many useful suggestions. This work was supported in part by a TOP grant from ZonMW to FJTS, a H2020 grant SCID-Net to FJTS, KPO and LGP and a ZonMW E-RARE grant to FJTS, MC and KPO.

Author contributions

Conceptualization: FF, LGP, FJTS.; Methodology, FJTS, KPO, LCD.; Investigation: FF, LCP, MvE, HW,MB, MC, MMT.; Writing – Original Draft, FJTS.; Writing – Review & Editing, FF, LCP, MB, MC, MMT, KPO, LCD, FJTS Resources, MB, MC.; Supervision, FJTS, KPO, LGP. Authors have no conflict of interest to report.

References

1. Rothenberg EV, Moore JE, Yui MA. Launching the T-cell-lineage developmental programme. *Nature reviews Immunology* 2008; **8**(1): 9-21.
2. Weerkamp F, Pike-Overzet K, Staal FJ. T-sing progenitors to commit. *Trends in immunology* 2006; **27**(3): 125-31.
3. Jenkinson EJ, Jenkinson WE, Rossi SW, Anderson G. The thymus and T-cell commitment: the right niche for Notch? *Nature reviews Immunology* 2006; **6**(7): 551-5.
4. Hollander G, Gill J, Zuklys S, Iwanami N, Liu C, Takahama Y. Cellular and molecular events during early thymus development. *Immunological reviews* 2006; **209**: 28-46.
5. Wu L, Antica M, Johnson GR, Scollay R, Shortman K. Developmental potential of the earliest precursor cells from the adult mouse thymus. *The Journal of experimental medicine* 1991; **174**(6): 1617-27.
6. Luc S, Luis TC, Boukarabila H, et al. The earliest thymic T cell progenitors sustain B cell and myeloid lineage potential. *Nature immunology* 2012; **13**(4): 412-9.
7. Bhandoola A, von Boehmer H, Petrie HT, Zuniga-Pflucker JC. Commitment and developmental potential of extrathymic and intrathymic T cell precursors: plenty to choose from. *Immunity* 2007; **26**(6): 678-89.
8. Keefe R, Dave V, Allman D, Wiest D, Kappes DJ. Regulation of lineage commitment distinct from positive selection. *Science* 1999; **286**(5442): 1149-53.
9. Rothenberg EV, Telfer JC, Anderson MK. Transcriptional regulation of lymphocyte lineage commitment. *Bioessays* 1999; **21**(9): 726-42.
10. Koch U, Fiorini E, Benedito R, et al. Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *The Journal of experimental medicine* 2008; **205**(11): 2515-23.
11. Rothenberg EV. Stepwise specification of lymphocyte developmental lineages. *Current opinion in genetics & development* 2000; **10**(4): 370-9.
12. Rothenberg EV. Transcriptional control of early T and B cell developmental choices. *Annual review of immunology* 2014; **32**: 283-321.
13. Deftos ML, Bevan MJ. Notch signaling in T cell development. *Current opinion in immunology* 2000; **12**(2): 166-72.
14. Kueh HY, Yui MA, Ng KK, et al. Asynchronous combinatorial action of four regulatory factors activates Bcl11b for T cell commitment. *Nature immunology* 2016; **17**(8): 956-65.
15. Engel I, Johns C, Bain G, Rivera RR, Murre C. Early thymocyte development is regulated by modulation of E2A protein activity. *The Journal of experimental medicine* 2001; **194**(6): 733-45.
16. Rothenberg EV. Negotiation of the T lineage fate decision by transcription-factor interplay and microenvironmental signals. *Immunity* 2007; **26**(6): 690-702.
17. Georgopoulos K, Moore DD, Derfler B. Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. *Science* 1992; **258**(5083): 808-12.
18. Weber BN, Chi AW, Chavez A, et al. A critical role for TCF-1 in T-lineage specification and differentiation. *Nature* 2011; **476**(7358): 63-8.
19. Li L, Leid M, Rothenberg EV. An early T cell lineage commitment checkpoint dependent on the transcription factor Bcl11b. *Science* 2010; **329**(5987): 89-93.
20. Staal FJ, Meeldijk J, Moerer P, et al. Wnt signaling is required for thymocyte development and activates Tcf-1 mediated transcription. *European journal of immunology* 2001; **31**(1): 285-93.

21. Staal FJ, Clevers H. Tales of the unexpected: Tcf1 functions as a tumor suppressor for leukemias. *Immunity* 2012; **37**(5): 761-3.
22. Tiemessen MM, Baert MR, Schonewille T, et al. The nuclear effector of Wnt-signaling, Tcf1, functions as a T-cell-specific tumor suppressor for development of lymphomas. *PLoS biology* 2012; **10**(11): e1001430.
23. Yu S, Zhou X, Steinke FC, et al. The TCF-1 and LEF-1 transcription factors have cooperative and opposing roles in T cell development and malignancy. *Immunity* 2012; **37**(5): 813-26.
24. Schilham MW, Wilson A, Moerer P, Benaissa-Trouw BJ, Cumano A, Clevers HC. Critical involvement of Tcf-1 in expansion of thymocytes. *Journal of immunology* 1998; **161**(8): 3984-91.
25. Schuijers J, Clevers H. Adult mammalian stem cells: the role of Wnt, Lgr5 and R-spondins. *The EMBO journal* 2012; **31**(12): 2685-96.
26. Verbeek S, Izon D, Hofhuis F, et al. An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature* 1995; **374**(6517): 70-4.
27. Held W, Clevers H, Grosschedl R. Redundant functions of TCF-1 and LEF-1 during T and NK cell development, but unique role of TCF-1 for Ly49 NK cell receptor acquisition. *European journal of immunology* 2003; **33**(5): 1393-8.
28. Huang Z, Xie H, Ioannidis V, et al. Transcriptional regulation of CD4 gene expression by T cell factor-1/beta-catenin pathway. *Journal of immunology* 2006; **176**(8): 4880-7.
29. Okamura RM, Sigvardsson M, Galceran J, Verbeek S, Clevers H, Grosschedl R. Redundant regulation of T cell differentiation and TCRalpha gene expression by the transcription factors LEF-1 and TCF-1. *Immunity* 1998; **8**(1): 11-20.
30. Gravestein LA, van EW, Ossendorp F, Borst J. CD27 cooperates with the pre-T cell receptor in the regulation of murine T cell development. *The Journal of experimental medicine* 1996; **184**(2): 675-85.
31. Yui MA, Feng N, Rothenberg EV. Fine-scale staging of T cell lineage commitment in adult mouse thymus. *Journal of immunology* 2010; **185**(1): 284-93.
32. van de Wetering M, Cavallo R, Dooijes D, et al. Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* 1997; **88**(6): 789-99.
33. Hendriks RW, Nawijn MC, Engel JD, van Doorninck H, Grosveld F, Karis A. Expression of the transcription factor GATA-3 is required for the development of the earliest T cell progenitors and correlates with stages of cellular proliferation in the thymus. *European journal of immunology* 1999; **29**(6): 1912-8.
34. Hosoya T, Kuroha T, Moriguchi T, et al. GATA-3 is required for early T lineage progenitor development. *The Journal of experimental medicine* 2009; **206**(13): 2987-3000.
35. Taghon T, Yui MA, Rothenberg EV. Mast cell lineage diversion of T lineage precursors by the essential T cell transcription factor GATA-3. *Nature immunology* 2007; **8**(8): 845-55.
36. Ting CN, Olson MC, Barton KP, Leiden JM. Transcription factor GATA-3 is required for development of the T-cell lineage. *Nature* 1996; **384**(6608): 474-8.
37. Allman D, Li J, Hardy RR. Commitment to the B lymphoid lineage occurs before DH-JH recombination. *The Journal of experimental medicine* 1999; **189**(4): 735-40.
38. Bain G, Robanus Maandag EC, te Riele HP, et al. Both E12 and E47 allow commitment to the B cell lineage. *Immunity* 1997; **6**(2): 145-54.
39. Enver T. B-cell commitment: Pax5 is the deciding factor. *Curr Biol* 1999; **9**(24): R933-5.
40. Hagman J, Lukin K. Transcription factors drive B cell development. *Current opinion in immunology* 2006; **18**(2): 127-34.

41. Lin H, Grosschedl R. Failure of B-cell differentiation in mice lacking the transcription factor EBF. *Nature* 1995; **376**(6537): 263-7.
42. Mikkola I, Heavey B, Horcher M, Busslinger M. Reversion of B cell commitment upon loss of Pax5 expression. *Science* 2002; **297**(5578): 110-3.
43. Nutt SL, Heavey B, Rolink AG, Busslinger M. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5 [see comments]. *Nature* 1999; **401**(6753): 556-62.
44. Rolink AG, Nutt SL, Melchers F, Busslinger M. Long-term in vivo reconstitution of T-cell development by Pax5- deficient B-cell progenitors [see comments]. *Nature* 1999; **401**(6753): 603-6.
45. Okamura R, Sigvardsson M, Galceron J, Verbeek S, Clevers H, Grosscedl R. Overlapping functions of Tcf-1 and Lef-1 in T lymphocyte development. *Immunity* 1998; **8**: 11-20.
46. Roose J, Molenaar M, Peterson J, et al. The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* 1998; **395**(6702): 608-12.
47. Van de Wetering M, Castrop J, Korinek V, Clevers H. Extensive alternative splicing and dual promoter usage generate Tcf-1 protein isoforms with differential transcription control properties. *Molecular and cellular biology* 1996; **16**(3): 745-52.
48. Luis TC, Naber BA, Roozen PP, et al. Canonical wnt signaling regulates hematopoiesis in a dosage-dependent fashion. *Cell stem cell* 2011; **9**(4): 345-56.
49. Urbanek P, Wang ZQ, Fetka I, Wagner EF, Busslinger M. Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP [see comments]. *Cell* 1994; **79**(5): 901-12.
50. Garcia-Ojeda ME, Klein Wolterink RG, Lemaitre F, et al. GATA-3 promotes T-cell specification by repressing B-cell potential in pro-T cells in mice. *Blood* 2013; **121**(10): 1749-59.
51. Ge Y, Gomez NC, Adam RC, et al. Stem Cell Lineage Infidelity Drives Wound Repair and Cancer. *Cell* 2017; **169**(4): 636-50 e14.