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Abstract

T lymphocytes depend on the thymic microenvironment for initiation of the T cell developmental program. As the progenitors in the thymus have lost the capacity to self-renew, this process depends on the constant influx of hematopoietic progenitors that originate in the bone marrow. Nevertheless, thymic emigrants are heterogeneous and retain developmental plasticity for both the myeloid and lymphoid lineages. It is the role of the thymic microenvironment to steer these uncommitted progenitors towards a T cell fate. Still, the thymus also generates a unique population of thymic NK cells, thus raising the question of how the T versus NK lymphoid cell fate is determined intrathymically. Many factors have been implicated in the developmental pathways in the thymus and the processes are characterized by both subtle and not so subtle modifications in gene expression. In this review, we will consider the crucial factors governing lineage determination of T cells versus NK cells from bi-potent thymic NK/T precursors. Recent reports have shed new light on the complex interactions of cytokines and transcription factors at different cell fate decision branch points in thymopoiesis. We will discuss the implications of these findings and propose a model that may be applicable at this critical thymic NK/T juncture.

Introduction

T lymphocytes are a unique type of blood cells, as they require the thymus as a dedicated organ for their development. Although T cell development has been described outside the thymus (1-3), it is the thymic microenvironment that primarily supports early T cell development and guides the process of thymocyte positive and negative selection.

Thymopoiesis relies on a constant influx of hematopoietic stem cell (HSC)-derived progenitors from the bone marrow (BM). It is generally agreed that BM-resident HSC (defined as Lin$^-$Thy1.1^{lo}Sca-1$^+$c-Kit$^+$Flt3$^-$) give rise to multipotent progenitors (MPPs) that

have lost their self-renewal capacity before differentiating into more largely restricted common myeloid progenitors (CMP; defined as Lin⁻Sca-1⁻c-Kit⁺IL-7Rα⁻) (4) and common lymphoid progenitors (CLP; defined as Lin⁻Sca-1^{lo}c-Kit^{lo}IL-7R⁺) (5). Earlier work has shown that early thymic progenitors (ETP; Lin⁻Sca-1⁺IL-7Rα⁻CD25⁻c-Kit⁺), in addition to harboring T and NK cell potential, possessed some B lymphocyte potential (6). This led to early models proposing that BM-derived CLP can seed the thymus and subsequently generate ETP (5, 7, 8). More recent studies, however, revealed that thymic immigrants still possess myeloid potential (9, 10) leading to a new model in which the thymus is settled by lymphoid-myeloid progenitors (LMPs; Lin⁻Sca-1⁺c-Kit⁺Flt3⁺) with developmental potentials for myeloid, B, T and NK cell lineages (11-13). Given that more than one precursor may seed the thymus, the precise identity of the HSC-derived progenitor(s) that generate ETPs and their downstream progeny remains a subject of debate but clearly involves cells with developmental potentials for other (non-T cell) lineages.

The phenotype and developmental potential of intrathymic ETPs has been studied extensively in human and mouse. Obviously, T cell potential is the predominant finding in different subpopulations studied in the early thymic compartment, although potential for myeloid and other lymphoid lineages (B, NK) are also present (reviewed in (13). Concerning B cell potential, the murine thymus exports approximately 2×10^4 mature B cells to the periphery each day (14). As the majority of ETPs lack B potential (15-17), it is unclear how (or where) these B cell are generated. Early studies identified a bi-potent T/NK-committed progenitor in the murine thymus, characterized by expression of the natural killer marker NK1.1 (18-20). The corresponding human T/NK bi-potent thymic progenitor was also described (21, 22). These T/NK precursors lacked appreciable B cell and myeloid cell potential. While it is generally accepted that the BM is the primary site for NK cell development (23, 24), the discovery of bi-potent T/NK precursors suggests the possibility of a

distinct developmental pathway for NK cells in the thymus that could share properties with T cell development (25). Along these lines, a recent report demonstrated some T cell potential in the previously described population of BM-resident NK cell precursors (26, 27).

The existence of an evolutionarily conserved bi-potent T/NK progenitor in the thymus demonstrates that the thymic microenvironment is permissive for and can selectively foster the development of T lymphocytes and thymic NK cells. Lineage choice depends on two crucial mechanisms: the initiation of a developmental program for a certain lineage ("specification") and the final exclusion of any other fate ("commitment"). These processes involve a cascade of irreversible checkpoints that are required to shape the identity of the resultant lymphoid cell subset. Changes in gene expression, developmental potential and proliferation levels mark these phases, and this process is well described in the thymus. During the initial stages of development, T cell progenitors lack expression of CD4 and CD8 co-receptors and are therefore termed 'double negative (DN)'. DN thymocytes differentially express CD44 and CD25 surface makers (28). ETP have the $CD44^{+}CD25^{-}$ phenotype (DN1 stage) and acquire CD25 as they mature to become $CD44^{+}CD25^{+}$ DN2 cells. Both of these early thymocyte precursor stages show high levels of proliferation that are driven by external signals derived from growth factors, including stem cell factor and interleukin (IL)-7. Efficient T cell progenitors are found among DN1 cells bearing the corresponding receptor (c-Kit^{+}); this fraction is a very minor population (0.01%) of the young adult mouse thymus (17, 29). The DN2 population is further enriched with efficient T cell progenitors (30) and can be subdivided into a c-Kit^{high} DN2a population that maintains a potential to develop into DCs, mast cells, monocytes and NK cells (15, 31-33), while the c-Kit^{low} DN2b population has extinguished non T-cell potentials (34). Early thymocytes that reach the DN3 stage have down-regulated CD44 expression and are fully committed to the T cell lineage. While TCRβ D-J rearrangements may start before this stage (35), DN3 cells arrest their growth to allow for

efficient TCR rearrangement. Successful in-frame TCRβ rearrangements lead to expression of a functional pre-TCR, composed of the invariant pre-Tα chain, the TCRβ chain and the associated CD3 complex. Signals from the pre-TCR (β-selection) drive further thymocyte differentiation to the double-positive stage (36).

The cell surface phenotype of the aforementioned bi-potent T/NK progenitor (NK1.1^{+}CD117^{+}CD44^{+}CD25^{+}) strongly suggested that this population was contained amongst the DN2 subset of early thymocytes (30). DN2 cells uniformly express IL-7Rα (37) and a subset of the bi-potent T/NK precursors express IL-2Rβ (30). It is not clear whether IL-2Rβ expression varies amongst DN2a versus DN2b thymocyte subsets (38) or whether the T, NK or bi-potent T/NK potential of cells bearing IL-2Rβ differs from those lacking this cytokine receptor chain.

To acquire a T cell identity, thymocyte progenitors must first switch off other cell fates. The early stages of thymocyte development are marked by dramatic changes in gene expression and include key regulators of lymphocyte development. The complex interplay between external signals (cell-cell interactions, soluble factors of the microenvironment) and intrinsic signals (via transcription factors) determines lineage choice. The thymus creates a unique environment that strongly supports T cell development, but is also permissive for development of other cell types. In this review, we will consider some of the extrinsic and intrinsic regulators that condition the choice between T cell and NK cell development in the thymus from T/NK bi-potent precursors.

Regulators of the T and NK cell fate determination in the thymus

Interleukin-7

Interleukin-7 (IL-7) is an essential cytokine for T cell development in both mice and man (39, 40). IL-7 is produced by epithelial stromal cells in the thymus and bone marrow (41, 42) and by fibroblastic reticular cells in the lymph node (43).

IL-7 signals through the heterodimeric IL-7 receptor (IL-7R) composed of the IL-7Rα chain associated with the common cytokine receptor gamma chain (γ_c) (reviewed in (44). The IL-7R is expressed by early lymphoid precursors (including CLP, pro-B, pro-T and most naïve mature T cells) and therefore plays a critical role in the homeostasis of developing as well as mature T lymphocytes. IL-7 signaling impacts via several biochemical pathways. Activation of Janus kinases (JAK)-1 and -3 lead to subsequent phosphorylation and dimerization of signal transducer and activator of transcription 5 (STAT5) members that control gene expression. Parallel stimulation of phosphoinoside-3 kinases (PI3Ks) as well as Ras and mitogen-activated protein kinase (MAPK) pathways also impinge on gene transcription and survival (reviewed in (45).

Loss of IL-7 function in mice leads to a strong decrease in thymic cellularity, although TCR$\alpha\beta$ cells (but not TCR$\gamma\delta$ cells) are produced and enter the peripheral lymphoid tissues (46, 47). Interestingly, IL-7 or IL-7R-deficient mice show a relative increase of DN1 T cell precursors (39, 46), suggesting that homeostasis of post-DN1 thymocytes depend on IL-7 signals that mediate both survival and proliferative effects (40, 48, 49). IL-7Rα is clearly expressed during the DN1 and DN2 stage of T cell development, but is sharply down-regulated when cells progress to the DN3 stage (37). This expression pattern is consistent with a model whereby IL-7-driven DN1/DN2 proliferation generates the cellular substrate for TCR rearrangements that take place at the non-proliferating DN3 stage. This state of IL-7 non-responsiveness persists at the double positive stage but then IL-7 re-emerges as an important factor during the cell-fate decision at the later CD4 or CD8 single positive stage, as signals through IL-7Rα have been proposed to promote CD8 T cell differentiation (50-52). Finally,

IL-7 maintains naïve T cells in the periphery and can contribute to 'homeostatic' expansion and proliferation of peripheral T cells following insults that cause lymphopenia (53), reviewed in (54).

BM NK cell development proceeds normally in the absence of IL-7 (55) and numbers and function of peripheral NK cell numbers in IL-7-deficient mice are unperturbed (46). In contrast, NK cells in the thymus appear highly dependent on IL-7 and this is correlated with a predominant expression of IL-7Rα on this subset of NK cells (56). As such, thymic NK cells, but not BM-derived NK cells, resemble T cells in their homeostatic requirement for IL-7. Additional similarities between thymic NK cells and T cells include their developmental requirement for GATA-3 (see below).

Considering their IL-7 dependency, one would predict that DN2 (and to a lesser extent DN1 cells) as well as thymic NK cells would localize in proximity to IL-7 producing thymic stromal cells. Using an IL-7 reporter mouse, IL-7-expressing MHC class II$^+$ thymic epithelial cells (TECs) could be identified (57). The majority of these IL-7$^+$ TECs expressed cortical markers and were localized as a band of cells at the cortico-medullary junction (CMJ). Petrie and Zúñiga-Pflücker reviewed the distinct microenvironments in the thymus, correlating the location of different thymocyte populations throughout development (58). ETPs enter the thymus at the CMJ, where DN1 and DN2 cells proliferate before migrating through the cortex. When T cell commitment is completed at the DN3 stage, the thymocytes are found in the sub-capsular zone where IL-7 expression is lowest. Although ETPs do not yet express IL-7Rα, its expression is increased as cells progress to the DN2 stage and gradually decreases as cells progress further during development. Interestingly, thymic NK cells have been found primarily at the CMJ (J. Di Santo, unpublished observations). Taken together, these findings suggest that IL-7 availability conditions not only early thymocyte survival and proliferation but also plays a role in development and/or maintenance of NK cells within the thymus.

Interleukin-15

Interleukin-15 (IL-15) was first discovered as an IL-2-like cytokine that promoted T cell activation in vitro (59, 60). The molecular basis underlying the biological similarities between IL-2 and IL-15 can be explained by fact that the receptors for these cytokines share multiple components including the IL-2Rβ and γ_c chains (44, 61). Nevertheless, these structurally related cytokines have unique biological roles in vivo in part due to the expression and function of their corresponding IL-2Rα and IL-15Rα chains (62, 63). Studies using mice deficient for IL-2Rα and IL-2Rβ suggested that IL-2 is required for regulation of peripheral T lymphocyte activation (via regulatory T cells), whereas IL-15 is a critically required for development and survival of NK cells (64, 65), as well as other innate T lymphocytes (reviewed in (66)).

The essential role for IL-15 in BM NK cell development begins with the triggering of IL-2Rβ chains expressed by NK cell progenitors (NKP) (26). NKP are Lin⁻ progenitors that lack most cell surface receptors expressed by peripheral NK cells (including NK1.1 and DX5) and upon IL-15 stimulation, proliferate and differentiate to generate phenotypically mature, and fully functional NK cells. IL-15 is therefore important for the development and maintenance of immature and mature NK cells, but not for the generation of NKPs (55). This last point is important as it indicates that other non-γ_c-dependent signals control NK cell lineage commitment.

IL-15 also plays an essential role in the development of thymic NK cells (56, 67). As thymic NK cell development also requires IL-7 (56), unique and/or redundant roles for IL-7 and IL-15 in the development, differentiation and maintenance of this unusual NK cell subset can be envisaged. The observation that both IL-7 and IL-15 are essential for NK thymopoiesis, indicates that these signals delivered by these cytokines can not compensate for

each other. Both IL-7 and IL-15 can promote cell survival by enhancing expression of anti-apoptotic members of the Bcl2 family (48, 65). Perhaps thymic NK cells receive limiting survival signals from each of these two cytokines and survival can only be ensured if both cytokines are present. An alternative explanation is that IL-7 and IL-15 act at distinct but sequential stages in thymic NK cell development. One probable scenario would include an important role for IL-7 in the development and/or maintenance of ETPs or T/NK bi-potent cells (that contain NK cell precursors), while IL-15 would act in a fashion similar to that of BM NK cell development, promoting survival, proliferation and differentiation of committed NKP once they have been fully specified. It is interesting to recall that before concluding the identification of the bi-potent T/NK progenitor, it was shown that cytokines influenced cell fate decisions in this precursor population with the levels of IL-2 and IL-15 controlling whether IL-2Rβ^+ TCR$^-$ cells develop into T or NK cells (62). As such, intrathymic IL-15 availability would dominantly promote (and control) thymic NK cell development, but might not impact on the initial T versus NK cell fate decision at the level of the T/NK bi-potent progenitor. This notion is also consistent with the lack of any obvious defects in early thymopoiesis in IL-15-deficient mice.

Notch1

Notch1 is a transmembrane signaling receptor required at multiple stages of T cell development. Notch1 signaling is essential for the initiation of T cell development in the thymus, as Notch1-deficiency completely ablates further T cell development from thymic ETPs (68, 69). Furthermore, Notch1 is critical for suppression of B cell fate from thymic progenitors as Notch1 deficiency leads to thymic B cell development. Ectopic activation of Notch1 signaling in hematopoietic progenitors resulted in emergence of T lymphocyte development at the expense of B cell development in the bone marrow that was independent of the thymic microenvironment (70). Furthermore, Notch1 signaling influences later stages

of T cell development, including the αβ versus γδ T cell fate (71), the choice between CD4 and CD8 T cell fate (72), as well as the choice between Th1 versus Th2 cell fates (73).

In mammals, Notch signaling is activated by binding to one of four known ligands: Jagged1 (74), Jagged2 (75), Delta-like-1 (Dll1) or Delta-like-4 (Dll4) (76, 77). Ligand binding leads to a series of proteolytic cleavages of the Notch molecule, resulting in the membrane release and transport of the intracellular Notch domain to the nucleus where it behaves as a transcriptional activator (reviewed in (78)). While *in vitro* studies indicated that both Dll1 and Dll4 promoted Notch1-dependent T cell development (79), thymic epithelial cells (TECs) only express Dll4 (80, 81). Furthermore, inactivation of Dll4 expression in TECs leads to a complete block in T cell development (82) indicating that Dll4 is the essential Notch1 ligand provided by the thymic microenvironment that is required for initiation of T cell development. This strengthens the view that the thymus creates a special niche that provides Notch ligands, guiding hematopoietic progenitors towards the initiation of the T cell developmental program.

Does Notch1 signaling impact on NK cell development in the thymus? Notch1 is expressed by ETP and triggering of this receptor plays a critical role in extinguishing B cell fate in this precursor population. Given the presence of thymic NK cells and the abundance of Dll4 expressed by cortical TEC (including those at the CMJ; (80)), Notch1 signals must either be neutral for development of thymic NK cells or else a fraction of ETPs must develop into thymic NK cells before encountering a Dll4-expressing TEC. Bhandoola and colleagues showed that Notch signals are required for the generation of ETPs, while the population of phenotypically identical $Lin^-Sca-1^+c-Kit^+$ cells in the BM and in the circulation was not affected (83). Notch1 deletion in HSC does not significantly impact on subsequent BM NK cell development or on the overall numbers, phenotype or function of splenic NK cells (84). Recently, the effect of Notch1 deletion on thymic NK cell development was investigated

(Ribiero et al, submitted). While Notch1-deficiency completed abrogated ETP and subsequent T cell development as expected (68-70), the number of thymic NK cells that developed in the absence of Notch1 was normal. Collectively, these results indicate that Notch1 signals neither inhibit nor promote NK cell development in the thymus.

GATA-3

GATA-3 is a zinc finger transcription factor recognizing a consensus WGATAR motive that is involved in multiple types of tissue development (85, 86). Germ-line GATA-3-deficiency leads to lethality by embryonic day 11.5 (87). In the hematopoietic system, GATA-3 plays critical roles in the development of T and thymic NK cells as GATA-3-deficient hematopoietic progenitors fail to generate these cells. Concerning T cell development, few cells progress past the DN2 stage (82, 88, 89), and using conditional gene ablation techniques, GATA-3 was also shown to be required at later stages of T cell development, including β-selection (90), the generation of CD4$^+$ SP thymocytes (90) and for CD4$^+$ T-helper-2 cell (T$_H$2) differentiation (91, 92). GATA-3 dependency correlates with expression levels that are highest in DN2 and DN3, diminish in DP and rises again during generation of CD4 SPs and T$_H$2 cells (88).

Although GATA-3 is essential for T cell development, overexpression of GATA-3 in T cell progenitors completely blocks T cell development and diverts their development towards the mast cell lineage (93). In this case, Notch acts as an antagonist of GATA-3 instead of as a collaborator in T cell development, demonstrating that transcription factors can have multiple effects, based on tightly regulated expression levels. GATA-3 is essential for development for thymic NK cells that express IL-7Rα and seed peripheral lymph nodes (56), while GATA-3 facilitates NK cell maturation (promoting IFN-γ production) and homing of liver-resident NK cells (94).

Both Notch1 and GATA-3 are involved in T cell specification, although it remains unclear whether they play specific, redundant or synergistic roles in this process. Initial work by Hoflinger and colleagues using Pax5-deficient B cell progenitors cultured on Dll1-expressing stroma showed that GATA-3 expression was up-regulated after 24 hours of culture in a Notch1-dependant fashion (95). Flavell and colleagues demonstrated that Notch1 could directly regulate the expression of GATA-3 in developing Th2 cells through direct binding of RBP-Jκ to the GATA-3 promoter (73). Together, these studies suggest that Notch1 signals might directly control GATA-3 expression in the murine T cell lineage. Interestingly, studies using human thymocyte precursors did not find GATA-3 as a Notch1 target (96, 97).

While Notch1 signals are critical in suppressing B cell fate in ETPs (98), Notch triggering alone is not sufficient to complete this process (99) indicating that additional signals are required. We recently found that GATA-3 plays an important role in sealing T cell commitment fate initiated by Notch1 signals. GATA-3-deficient DN2 cells showed clear molecular signs of T cell specification, but unlike their WT counterparts, were able to develop into mature B cells when cultured *in vitro* with OP9 stroma and IL-7 (García-Ojeda, Klein Wolterink et al, unpublished results). These results point to an important role for GATA-3 cooperating with Notch1 in order to extinguish B cell fate that completes at the DN2 stage.

Concerning molecular mechanisms of GATA-3 action in thymocyte development, few transcriptional targets have been identified. The enhancers of the CD3δ and the TCRα, -β, -γ, and -δ genes contain GATA binding sequences (100-102) consistent with the lack of functional TCR rearrangements in GATA-3-deficient DN3 cells (90). Using microarray analysis of WT and GATA-3-deficient DN2 cells, we found that Bcl11b expression was markedly decreased in the absence of GATA-3 (García-Ojeda, Klein Wolterink et al, unpublished results). The strong increase in Bcl11b expression during the transition from

DN2 to DN3 stages suggests that GATA-3 may play a role in up-regulation of Bcl11b, which as described below, is a critical determinant of T cell commitment.

Id2

Inhibitor of DNA binding 2 (Id2) is one of four related helix-loop-helix (HLH) proteins that inhibit transcriptional activity of basic HLH E-box transcription factors, such as E2A, E2-2 and HEB (reviewed in (103). The balance between E-box and Id proteins clearly influences cell fate decisions within the hematopoietic system: for example, Id2 or Id3 over-expression efficiently inhibits B and T cell developmental programs from hematopoietic precursors (104), whereas E2A-deficiency results in a B cell developmental arrest (105). In contrast, Id proteins favor generation of NK cells under these conditions and Id2 is essential for BM NK cell development *in vivo* (106).

The role of Id2 in the generation of thymic NK cells is not fully defined. Id2 plays an important role in NK cell lineage commitment from thymic bi-potent T/NK cell precursors is evidenced in the mouse by the fact that early thymocytes can be directed to the NK lineage by ectopic expression of Id2 and that Id2-deficiency appears to eliminate thymic NK cell precursors (107). Nevertheless, the residual population of NK cells that has been documented in the spleens of Id2-deficient mice bears IL-7Rα (108). As thymic NK cells express IL-7R (56), this led Boos and colleagues to propose that these few NK cells in Id2-deficient mice were the product of a thymic pathway of NK cell development that was Id2-independent (108). While this remains possible, we have observed that thymic NK cells are strongly decreased in the absence of Id2 and that the athymic (Foxn1-deficient) nude mice reconstituted with Id2-deficient BM HSC can still generate a a similar small population of IL-7Rα$^+$ splenic NK cells (Hasan et al, unpublished observations). Interestingly, immature BM NK precursors (that normally express low levels of IL-7Rα; (55)) appear increased in the

absence of Id2 (Hasan et al., unpublished observations) and may contribute to this unusual population found in the spleens of Id2-deficient mice.

Recent observations in human NK cells demonstrated that Id2 expression in CD34^{+} CD1a^{-} T/NK cell precursors inhibited T cell development and dramatically increased NK cell development 45-fold. The Id2-induced NK developmental potential could be further enhanced by addition of IL-15 (109). These results suggest a model whereby Id2 plays a critical role in the cell fate decision that generates NK cells from bi-potent T/NK precursors that is then sustained and promoted by intrathymic IL-15.

Nfil3

Nfil3 (nuclear factor interleukin-3 regulated), also known as E4bp4 (E4-binding protein 4) is a basic leucine zipper (bZIP) transcriptional activator and was initially described for its ability to limit viral transcription and for promoting IL-3 mediated survival of pro-B cells (110). The involvement of bZIP proteins in the control of developmental processes both in and outside the hematopoietic system suggested that Nfil3 might also serve a role in lymphoid development. Although Nfil3-deficiency did not lead to gross hematopoietic abnormalities (erythroid, myeloid and T, B and NKT cell development appeared unaffected), three independent laboratories showed that Nfil3-deficient mice almost completely lacked mature NK cells (111-113). These observations identify Nfil3 as an essential transcription factor required for NK cell lineage development. The NK cell defects caused by Nfil3 deficiency are cell-intrinsic as they are recapitulated following transfer of Nfil-3-deficient HSC. In addition, retroviral transduction of Nfil3 into HSC promoted NK cell (111).

How does Nfil3 fit among the network of transcription factors and cytokines that have been implicated in NK cell development? As stated earlier, IL-15 is a crucial factor for NK cell development and survival (64) and the expression patterns of Nfil3 correlates with the dependency on IL-15 at the different stages of NK cell development (55): Nfil3 is detected in

NKPs, is up-regulated in immature NK cells and is maintained in mature NK cells. Stimulation of progenitor cells with IL-15 did not induce NK cell development in the absence of Nfil3, however, ectopic expression of Nfil3 in IL-15Rα-deficient HSCs or in the presence of IL-15 blocking antibodies improved NK cell development, indicating that Nfil3 may function downstream of IL-15 (111). Gene expression analysis revealed that Id2 and Gata3 levels were reduced in the absence of Nfil3, while over-expression of Nfil3 in Nfil3-deficient HSCs enhanced Id2 and GATA-3 levels compared to non-transfected control cells. Moreover, retroviral transduction of Id2 into Nfil3-deficient cells improved NK cell generation. Together these data suggest that Id2 and GATA-3 function downstream of Nfil3 (111).

A role for Nfil3 in the development of thymic NK cells is unknown. It is possible that the consequences Nfil3- and Id2-deficiency for thymic NK cell generation should largely overlap as Nfil3 can influence Id2 levels in the NK cell lineage (111). Still, development of lymph nodes and Peyer's patches (that require Id2) appears normal in the absence of Nfil3 (H. Brady, personal communication), indicating that the control of Id2 expression by Nfil3 might be cell-specific, or that other transcription factors can compensate for the loss of Nfil3 in the development of lymphoid tissues. Analysis of Nfil3 expression in early thymocyte precursors and an assessment of thymic NK cell development in Nfil-3-deficient mice should provide important information in this regard.

Bcl11b

Bcl11b and the related Bcl11a are Krüppel-like transcription repressors that play essential and opposing roles in lymphocyte development. Whereas Bcl11a has mainly been implicated in B cell development (114), Bcl11b plays a major role in early T cell development (115). Bcl11b acts as a stimulator of TCRβ gene rearrangement, necessary for αβ but not γδ T cell development, and also plays an important role in early thymocyte survival and expansion

via regulation of Bcl-X$_L$ (115, 116). Recent studies further characterized the role of Bcl11b in the stepwise differentiation of early thymocyte precursors (117, 118). In the absence of Bcl11b, a profound developmental block at the CD44$^+$CD25$^+$ DN2 stage could be documented. Bcl11b-deficient DN2 cells appeared T-lineage specified as they expressed normal levels of Gata3, Tcf7 and Ets1 (119). In contrast, stem cell-like genes (Tal1, Sfpi1, Lyl1, Gfi1b, Erg) and genes that are normally associated with a NK cell identity (Id2, Il2rb, Ncr1 and Nfil3) were de-repressed in Bcl11b-deficient DN2 cells. These results place Bcl11b as a major regulator of T cell identity in multipotent thymocyte progenitors that acts to extinguish alternative cell fates (including the NK cell lineage).

What signals regulate Bcl11b expression at the DN2 stage? DN2 cells can be maintained *in vitro* using immobilized Dll4 in the presence of IL-7, SCF and Flt3L (117) and retain potentials to develop into αβ T cells, NK cells, dendritic cells and macrophages. By simply reducing IL-7 levels, these DN2-dominated cultures demonstrate brisk and robust T cell differentiation. This observation suggested that IL-7 acts as a rheostat to regulate further T cell development. Ikawa and colleagues further showed that Bcl11b expression was inversely related to IL-7 levels thereby linking this cytokine/transcription factor pair in control of T cell commitment (117).

The Liu laboratory reported that induced ablation of Bcl11b in either immature or mature T cells leads to the adoption of a NK cell phenotype (118). Bcl11b loss led to a decrease in the expression of T cell-related genes (Gata3, Ets1, Tcf1, Hes1) and a corresponding up-regulation of NK-associated genes (Id2, Il2rβ, Nfil3). The resultant Induced T-to-Natural Killer (ITNK) cells expressed the NK cell marker NKp46 and lysed tumors *in vivo* and *in vitro*. Even mature CD8$^+$ T cells could be 'reprogrammed' to ITNKs by deletion of Bcl11b, suggesting that Bcl11b is represses 'NK cell-like' qualities, long after commitment to the T cell lineage.

These studies identify Bcl11b as a crucial factor for induction and maintenance of T cell identity and are an important contribution to our understanding of the T cell commitment. Although Notch1 signals are crucial for initiation of T cell specification, these signals alone cannot suppress B, NK or myeloid cell fates. Additional transcription factors are thus required to advance (and maintain) the T cell instruction program. These recent studies demonstrate that Bcl11b is essential to repress myeloid and NK cell potentials in DN2 cells. A sequential cascade of T cell specification and commitment emerges, whereby Notch1, GATA-3 and Bcl11b act in concert to extinguish stem cell, B cell, myeloid and NK cell potentials. The inter-relationships between these three transcription factors are not fully defined, although Notch signaling has been suggested to regulate both GATA-3 and Bcl11b expression (118, 120). Critical targets of GATA-3 (that are either activated or repressed) are not known, although Bcl11b now emerges as a potential candidate.

Integrating T/NK cell fate decisions during early thymopoiesis

The thymus is considered as the "cradle" for T cell development since this organ provides the unique microenvironment that simultaneously promotes T cell development and suppresses many residual cell fates in the developing hematopoeitic precursors that enter into it. How is it then possible that NK cells (and to a lesser extent DC, myeloid cells and B cells) develop within this "hostile" environment? One simple model would propose that these non-T cell fates would result only upon failure to successfully complete the T cell program. Nevertheless, experimental evidence for signs of "T cell failure" (i.e. out of frame TCRβ chain VDJ rearrangements) is generally lacking in non-T cells in the thymus or outside of it. We are therefore left with the model whereby early thymocyte progenitors may have an equally good chance of becoming a T cell or any one of the non-T cell fates. Let's examine such a model in more detail.

With the assumption that immature thymic progenitors have equal potential to develop into cells of any lineage, cell-cell interactions within the thymic microenvironment, together with the directed or stochastic expression of cytokine receptors, might provide the signals that then lead to changes in the expression of specific transcription factors (121, 122). These transcription factors (acting alone or in concert) control gene expression and subsequent lineage specification and at the same time the repression of genes from alternative cells fates. Ultimately, the successful commitment and differentiation to the T cell lineage will depend on the interplay of several different transcription factors (including Notch1, GATA-3, Bcl11b and others) that are expressed at early stages of T cells development. Recent data demonstrated the necessity for continued expression of some of these factors to maintain "T cell identity", suggesting that permanent suppression of other cell fates (at least for the NK cell lineage) may operate even in mature T cells (118).

Initiation of T cell differentiation depends on Notch1 signalling. Without Notch1 signals, T cell precursors fail to become specified, and instead give rise to B cells (68). This function of Notch1 is mediated by the conversion of RBPSuh, a repressor by default into an activator as a result of translocation of the intracellular domain of Notch to the nucleus (123, 124). However, DN1 cells that have experienced Notch signals (for even extended periods) have not fully excluded their B cell potential (125), indicating that there is (at least) one additional factor necessary that acts in concert with Notch1 to extinguish B cell potential as cells progress to the DN2 stage (where B cell potential is completely suppressed, (38)). As discussed above, we recently identified GATA-3 as one such factor (García-Ojeda, Klein Wolterink et al.). Interestingly, GATA-3 suppresses B cell but not myeloid cell potential in most DN2 cells, leading Kawamoto and colleagues to use the term 'DN2mt cell' to describe this characteristic (10). More recently, this group reported that the further commitment of

DN2mt cells to the T cell lineage was associated with Bcl11b-mediated transcriptional repression of myeloid gene expression (117).

The fact that DN2 cells also possess NK potential in various experimental systems further strengthens the heterogeneity within this developmental stage. Recent work has shed some light on this heterogeneity as it was possible to associate the NK cell potential of this subset with a particular cell surface phenotype. Yiu et al. demonstrated that the NK potential of DN2 cells selectively resides in the population of DN2 cells expressing high levels of CD117 (termed DN2a), while CD117^{lo} cells (denoted DN2b) lack NK potential and are committed to the T cell lineage (38). Intuitively one would predict that DN2a cells are the precursors of DN2b cells, although this has yet to be experimentally demonstrated leaving open the possibility that both subpopulations derive independently. Again, Bcl11b is likely playing a central role in this transition, as Bcl11b acts to repress NK cell lineage traits (118) and can also modulate CD117 (c-Kit) expression (along with other cytokine receptors including CD122 and CD127; (118)). Sustained Notch1 signalling at the DN2 stage may have a role in the transition to the DN2b stage as Bcl11b has been proposed as a direct Notch target (93).

All of the above-mentioned signals are clearly focusing the DN2 cells towards the T cell lineage. Despite this overwhelming T-cell-orienting influence, a subset of cells apparently manages to develop along the NK cell pathway. Here again modulation in cytokine receptor and transcription factor expression likely play determinant roles. The transcription factor Runx3 is highly expressed in the DN1 subset and has been shown to control IL-2Rβ expression in developing NK cells (126). A fraction of DN2 cells can apparently up-regulate IL-2Rβ expression possibly due to sustained Runx3 expression. Accordingly, these cells would become sensitive to locally produced IL-15 in the thymus. How could IL-15 signalling help specify the NK cell lineage program? Nfil3 can be induced in hematopoietic precursors

following exposure to IL-15 (111). Still, additional intrathymic signals would be necessary for commitment to the NK cell lineage as Nfil3 is also expressed by non-NK cells (including DC and myeloid cells) and can be induced upon stimulation in B cells (112, 113). One possibility would involve Nfil3-induced Id2 expression (111). Moreover, Nfil3 may also be involved in regulating GATA-3 expression (127). As such up-regulation of Nfil3 might go a long way in selectively promoting intrathymic NK cell development through inhibiting B cell potential (via GATA-3 in concert with Notch1), and blockade of T (and B) cell potential through Id2-mediated squelching of E-box protein activity. In this model, Nfil3, Id2, and GATA-3 might act in concert to allow intrathymic NK cell development to occur.

Interestingly, Bcl11b deficiency leads to increased levels of Nfil3 in early thymocyte precursors, indicating that a cross-inhibitory regulatory loop might exist between these two factors (Figure). Up-regulation of Bcl11b at the DN2b stage might inhibit Nfil3 and Id2 expression, resulting in the suppression of the NK fate, while up-regulation of Nfil3 (and subsequently Id2) might counter Bcl11b-induced T cell potential by suppressing E-box activity (118) and thereby promoting NK fate. The relative levels of Bcl11b versus Nfil3 in DN2a cells would therefore be major determinants of the T *versus* NK cell fate at this critical juncture. Interestingly, DN2a and DN2b cells express similar amounts of *Bcl11b* mRNA (38). While this might not necessarily translate into similar levels of Bcl11b protein in these populations, it could indicate that the promotion of the T cell fate and the suppression the NK cell fate might necessitate additional signals. However, once the T cell fate is established Bcl11b appears to be sufficient to assure this as deletion of only Bcl11b in mature, peripheral T cells results in the conversion of these cells into NK cells (ITNK; (118)).

In line with this model in which relative expression levels of Nfil3/Bcl11b determine DN2 NK/T cell fate decisions, we found that Nfil3 was also expressed by all DN subsets (Vosshenrich et al., unpublished results). Although Nfil3 levels are lower than in NK cells

(including thymic NK cells), highest expression levels are found among the DN2 subset and decline during subsequent stages of T cell differentiation. A similar phenomenon may explain the expression of Bcl11b by a fraction of thymic NK (118) that might hint at their 'DN2 origins' and would be extinguished as these cells mature further.

In conclusion, much of the recent data appear to indicate a decisive split between T cell and thymic NK cell fate at the DN2 stage. Does this imply that all thymic NK cells transit via the DN2 intermediates? We think that this is not likely to be the case, as thymic NK cells can develop normally in absence of Notch signalling that lack ETP and DN2 cells (Ribeiro et al., unpublished). Thus, the DN2 stage probably does not represent an obligatory stage in the development of thymic NK cells. Alternative sources for DN2-independent thymic NK cells include other BM-derived multi-potent hematopoietic precursors or NKP themselves. Elevated Nfil3 expression in any of these precursors would promote their NK potential given a sufficient amount of IL-15 in the tissue microenvironment. The proportion of thymic NK cells that express Bcl11b (as a possible indictor of passage through the DN2 stage) could provide an indicator of the relative contribution of these different pathways.

Table 1

	T	NK	Thymic NK	References
IL-7	essential	not required	essential	(39, 40, 46, 47, 55, 56)
IL-15	not required	essential	essential	(55, 56, 59, 60, 67)
Notch	essential	not required	not required	(70, 80, 81, 84)
Gata3	essential	not required	essential	(56, 82, 88, 89, 94)
Id2	not required, redundancy with	essential	essential	(104, 107-109)

	other Id factors ?			
Nfil3/E4bp4	not required	essential	?	(111, 112)
Bcl11b	essential	limits development ?	?	(117, 118, 120)

Figure 1
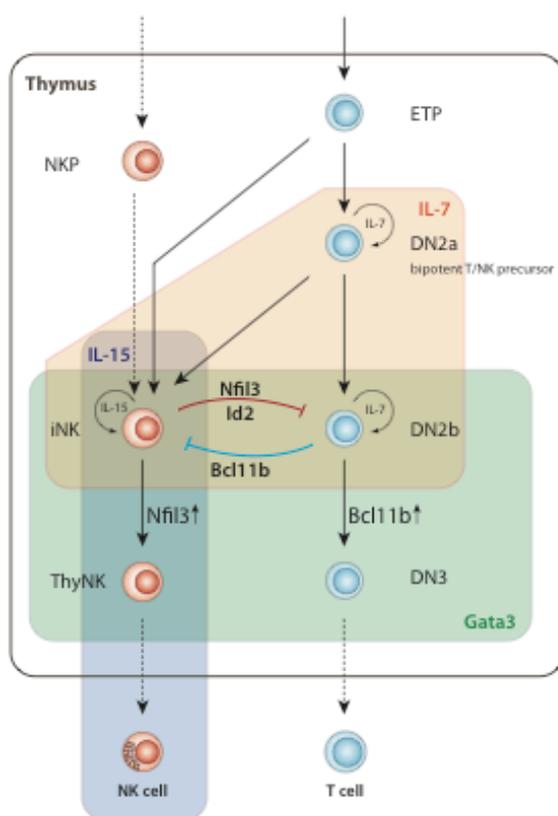
Acknowledgements

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