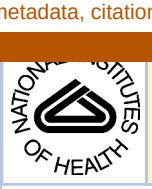

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Competition and collaboration: GATA-3, PU.1, and Notch signaling in early T-cell fate determination

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Abstract

T-cell precursors remain developmentally plastic for multiple cell generations after entering the thymus, preserving access to developmental alternatives of macrophage, dendritic cell, and even mast-cell fates. The underlying regulatory basis of this plasticity is that early T-cell differentiation depends on transcription factors which can also promote alternative developmental programs. Interfactor competition, together with environmental signals, keep these diversions under control. Here the pathways leading to several lineage alternatives for early pro-T cells are reviewed, with close focus on the mechanisms of action of three vital factors, GATA-3, PU.1, and Notch-Delta signals, whose counterbalance appears to be essential for T-cell specification.

Keywords

T cell development; Lineage commitment; Mast cells; Dendritic cells; Transcription factor

1. MODELS FOR HEMATOPOIETIC LINEAGE CHOICE

Hematopoietic stem cells can develop into any of at least ten different kinds of effector cells, and these can be grouped broadly into lymphoid, myeloid, erythroid, megakaryocytic, and other types. Stem cells become particular cell types by passing through sequences of partially restricted, but still pluripotent intermediates. The outcome of competing regulatory forces at work on the cells during the partially restricted intermediate stages ultimately forms the basis for the commitment decision and controls differentiation. In this review, we will discuss how this works as revealed in cells making the choice between the T-cell fate and its developmental alternatives.

Particular transcription factors can drive specific hematopoietic lineage choices. An example is the way erythro-megakaryocytic lineages separate from myeloid lineages (rev. by [1]). Here, the zinc finger factor GATA-1 apparently drives erythro-megakaryocytic fates in opposition to the E26 transformation-specific (Ets) family factor PU.1, whereas PU.1 plays a dominant role to direct myeloid fates in opposition to GATA family factors [2–6]. Downregulation of PU.1 in hematopoietic precursors provides one of the earliest indices that cells have undertaken an erythro-megakaryocytic lineage pathway [7,8]. In another dichotomy, Notch signals that activate the RBPJ transcription factor (also known as CSL, for CBF-1, Suppressor of Hairless, Lag-1) are pitted against the activity of the Paired-homeodomain transcription factor Pax5.

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Notch activation triggers and sustains the T-lymphocyte program while blocking the B-lymphocyte program, whereas Pax5 directs the B-cell program while blocking the T-cell program, in part by inhibiting Notch1 expression [9–11]. As these oppositions suggest, the dominance of each of these factors in a particular pathway is ultimately based not only on its ability to activate cell-type-specific genes, but also on its ability to repress or inhibit any factors important for alternative pathways (rev. in [12]).

The discovery of partially restricted progenitors, stable enough to identify and isolate prospectively, marked a profound advance toward understanding how hematopoietic lineage choice occurs [13–15]. The existence of these restricted progenitors revealed that when cells depart from the stem cell state they are not yet committed to adopt one particular developmental fate. Rather, they begin differentiating while equipped with a regulatory apparatus that could support any of several alternative fates. This suggests that fates may be grouped based on relatedness of the gene expression programs they activate, a grouping that ought to be reflected in the modes of action of their dominant transcription factors.

The exact relationships between these powerful regulators, in action, depend on whether lineage decisions are strictly hierarchical or not, with potential for more similar fates being combined longer than potential for more distinct fates. The notion that lymphocytes derive from a common lymphoid precursor and myeloid cells from a common myeloid precursor has been highly appealing. However, in a challenge to the hierarchical models, there is now abundant evidence that most T lymphoid precursors lose B lymphoid developmental potential earlier than they lose myeloid potential [16–19]. Most radically, T-cell development – like dendritic cell development – may not even be required to pass through a unique sequence of intermediates [20–25]. This can be explained by the fact that the essential T-lineage developmental program depends on a substantial overlapping of activities between transcription factors that are “T-cell specific” and factors capable of driving other fates (Figure 1). The specification of particular hematopoietic cell types by shifting, overlapping combinations of lineage-nonspecific factors has been noted for other lineages [26], and in all such cases it can only work if there is precise quantitative and stage-specific regulation of the factors involved to enable differentiation to progress. This overlap and mutual constraint is vividly illustrated in the relationships between three essential factors, Notch, PU.1, and GATA-3, during early T-cell development.

2. LANDMARKS FOR EARLY T-CELL DEVELOPMENT

Early-stage T-cell precursors are partially restricted cells that migrate to the thymus either just after or immediately before losing the ability to give rise to B lymphocytes, but before they shut off molecularly distinct developmental alternatives including macrophage or dendritic-cell fates. Differentiation of these cells occurs in the thymic cortex over a period of many days and is accompanied by a flexible degree of proliferation. Fig. 2 summarizes the cell-surface and molecular phenotypic criteria that distinguish mouse thymocytes in successive stages toward T-lineage commitment [27–34].

The earliest thymic subset, Early T-cell precursors (ETP) or “DN1” cells ($CD3^-$, $CD4^-$, $CD8^-$, c-Kit^{hi}, $CD44^+$, and $CD25^-$)[1], can undergo extensive intrathymic proliferation, especially in adult animals. But throughout this proliferation ETPs remain uncommitted to the T lineage, as they retain efficient competence to develop into natural killer, macrophage, and dendritic cells [16–19,35]. Transcription of *Rag1*, *Rag2*, and many T-lineage-specific genes is low or undetectable at the ETP stage. Then, a broad increase in T-lineage-associated gene

[1]“ETP” is used here instead of “DN1” because the classically defined DN1 thymocyte population ($CD44^+$ $CD25^-$) includes a majority of cells that are not T lineage precursors.

expression begins as the cells pass to the next stage, called DN2 ($CD3^-$, $CD4^-$, $CD8^-$, c-Kit^+, $CD44^+$, $CD25^+$). Still, many uncommitted cells remain in the DN2 population, exhibiting dendritic-cell, macrophage, and NK potential, and some cells in the DN2 population can even develop into mast cells [36]. However, these alternatives disappear completely as the cells progress further to the DN3 stage ($CD3^-$, $CD4^-$, $CD8^-$, c-Kit^{low}, $CD44^{low}$, $CD25^+$). DN3 cells express a full panoply of T-cell specific genes, indicating the maximal activation of positive regulators of T-lineage identity. At this stage, expression of *Rag1* and *Rag2* is fully upregulated, most cells slow or stop their proliferation, and the first complete rearrangements of TCRβ, TCRγ, and TCRδ genes occur. If successful, these rearrangements enable the cells to assemble a pre-TCR (rearrranged TCRβ + invariant pre-TCRα) or TCRγδ complex at the cell surface, and these complexes trigger signals that enable the cells to undergo β-selection or γδ-selection, respectively.

The stages of intrathymic development that lead up to β-selection (ETP to DN3) can be described as pro-T cell stages. T-cell development through these stages depends on a set of regulators that include both T-cell specific and shared transcription factors (Fig. 1)[30,32,37–42]. The T-cell specific transcription factors GATA-3 and TCF-1 are transcriptionally induced from a low level in prethymic precursors to a substantial level as early as the ETP stage, with further increase to the DN3 stage [43–47]. Another crucial transcription factor, the basic helix-loop-helix transcription factor E2A, is strongly upregulated through stabilization at the protein level, starting at the DN2 stage [48,49].

At the same time, a rich legacy of transcription factor gene expression inherited from stem cell precursors continues throughout early stages of T-cell development [43,46,50]. Some prethymically expressed factors are maintained all the way through lineage commitment (E.-S. David-Fung et al., unpublished results), and several regulators crucial for T-cell differentiation have stem cell provenance; e.g. Myb, Runx1, Ikaros, and Gfi-1 [51]. Nevertheless, there are also parts of the stem cell legacy that must be downregulated in a precisely staged way in order to allow T-cell development to proceed. The ETP population retains strong expression of a number of stem cell associated factors, among them PU.1 and SCL, and at least in some ETP cells, GATA-2 and/or C/EBPα as well [52–55]. All of these genes are shut off by the DN3 stage. PU.1, C/EBPα, and GATA-2 can all promote non-T fates when overexpressed [36,53,54,56–59], and their precisely regulated repression is important for lineage commitment, as discussed below.

3. CONTROL OF EARLY T-CELL DEVELOPMENT BY EXTERNAL SIGNALS

T-lineage specification is driven and maintained by signals from the environment. The transition from each DN stage to the next depends on the existing internal transcription factor complement in the cell together with changes in transcription factor function induced by external environmental signals. The most important of these signals, on current evidence, are cytokine signals, signals through the Wnt-β-catenin-TCF pathway, and repeated signals from the Notch pathway. As for other hematopoietic lineages, receptors for trophic signals are highly regulated in T-lineage cells and only select receptors are expressed.

For survival and proliferation, the earliest T cell precursors may rely on signaling through the cytokine receptor c-Kit [60], but also from ETP stage through the end of DN2 stage they are dependent on IL-7/IL-7R signals [61]. Among the transcription factors required at these stages, recent evidence suggests that the Runx family factors may help establish appropriate levels of proliferation through their impact on IL-7R expression and function [62,63]. Proliferation may compete with differentiation, especially for adult-derived immature thymocytes, as high-level IL-7/IL-7R or c-Kit signaling is seen to retard progression from DN2 to DN3 stage even while

it promotes expansion [23,64,65]. However, these cytokine signals do not appear to promote differentiation, and are not sufficient to maintain viability without additional survival signals.

Wnts are suspected to be another part of the survival signal. This inference is based on the importance of the TCF-1 transcription factor and its relative LEF-1, which are the transcriptional effectors of the Wnt canonical signaling pathway. Both of these transcription factors are converted from repressors to activators by β-catenin, which is mobilized by signals from the canonical Wnt pathway. Activity of TCF-1 or LEF-1 is essential for passage through β-selection, and TCF-1 also plays a critical, nonredundant role in adult thymocytes in progression from ETP to DN2 [47,66,67]. TCF-1 target genes in thymocytes include many survival and proliferation genes [68]. It is supposed that most TCF/LEF functions are Wnt dependent, based on the severe developmental blocks caused by transgenic expression of Wnt inhibitory proteins, Dickkopf and ICAT [69,70]. However, the connections between the initial Wnt input, the effector function of TCF-1, and the β- or γ-catenin proteins that normally act as intermediates have remained somewhat difficult to parse. Forced activation of β-catenin in progenitor cells apparently causes them to arrest development in a multipotent stage and reverse commitment, rather than enhancing their commitment to the T-cell fate [71–73]. New data suggest that hematopoiesis and apparently normal T-cell development can occur even in the absence of both β- and γ-catenin [74], suggesting that some roles for TCF/LEF family transcription factors could depend on other mediators. This ambiguity raises the question of whether TCF-1 in DN2-stage cells could actually be playing an important role as a repressor rather than as an activator. If so, one potential repression target implicated at this stage is the PU.1 gene [75].

The most dominant environmental factor in T-cell specification is the availability of ligands for the transmembrane receptor, Notch. As reviewed elsewhere in detail, interaction between Notch1 and its ligands of the Delta-like class is a distinctive requirement for precursors entering and pursuing the T-cell pathway, from the generation and maintenance of ETPs all the way to the beginning of β-selection (see reviews [9,10,28,31,76–78]). Notch-Delta signaling contributes not only to T-lineage survival and growth but also to lineage choice. Notch-Delta signaling favors T-cell development and blocks B-cell development from multilineage prethymic precursors; then, even after B-lineage potential is permanently suppressed, it blocks ETP and DN2 cells from access to all other physiological alternatives to T-lineage choice [16,35,79,80].

4. CONTEXT-DEPENDENT MODULATION OF NOTCH SIGNALS

Notch signaling is not a Boolean operator that determines T-lineage fate at a stroke, but a quantitatively modulated participant in protracted lineage decisions. A sustained succession of interactions with Delta-class target ligands, through all the cell cycles from ETP throughout DN2 stages, is needed to elicit full commitment to the T lineage [35,36,45,81]. Notch/Delta interaction can drive many different starting populations of hematopoietic precursors into T-cell development [21,23,36], but the dosages required are subject to distinct thresholds at different stages [82–84], and different Notch-triggered effects require different signal intensities. Prethymic precursors from bone marrow require substantially higher levels of Notch signaling than even the earliest intrathymic precursors to initiate T-cell development in vitro [84]. However, only a low level of Notch signaling is needed to suppress and eliminate the B-cell option [35,81], while a stronger Notch signal intensity is needed to block the dendritic-cell and natural killer cell fate options [35,83,85]. ETP and the earliest fetal thymic immigrants begin T cell development in a Notch “primed” state, already showing upregulation of Notch target genes very early after entering the thymus [44,82,84,86,87]. Yet known Notch target gene expression peaks at the DN3 stage [43,88,89]. It is not clear yet which this

apparently amplified response reflects: stronger Notch-Delta signals, or interaction of constant-level Notch signals with a developmentally altered regulatory context.

The biochemical mechanisms through which cells detect and respond differentially to different levels of Notch pathway signals are still being investigated. Notch engagement triggers proteolytic release of the intracellular domain of Notch, which moves to the nucleus and provides an activation domain for the pre-existing transcription factor RBPJ (CSL). Recent data provide a possible mechanism to make certain target genes more sensitive than others to the strength of Notch signaling. Head to head CSL binding sites, called SPS (sequence paired sites), preferentially direct cooperative assembly of transcriptionally active complexes of RBPJ, intracellular Notch, and the coactivator MAML1 as compared to single sites for RBPJ [90]. This suggests a mechanism for translating strength of Notch signaling through to the target gene. Furthermore, such SPS sites may favor cooperation with basic helix-loop-helix E proteins bound at neighboring elements [91]. Such *cis*-regulatory elements could structurally underlie the responses that depend on Notch-E protein cooperation [92] at many early stages of T-cell development[93]. At the same time, Notch signaling is highly modulated by antagonistic transcription factors such as LRF, or Zbtb7a [94]; by antagonists of MAML1 assembly like Msx2-interacting protein (MINT)[95]; and by feedback from negative regulators of the cytoplasmic part of the cascade such as Deltex1 and Nrarp [96,97]. One further way in which the Notch signal could be regulated to achieve gradations of signal strength and duration is by diversity in the sites of S3 cleavage of Notch [98]. This generates two types of Notch intracellular domain, one more stable than the other, with a resulting effect on intensity of transcriptional response.

In the past two years, Notch signaling has been suggested to affect T-lineage gene expression through yet additional pathways, and this is through gene-specific modulation of the effects of other transcription factors. Although the biochemical mechanisms remain to be determined, Notch pathway mediators can also modify the activities of GATA-3 and PU.1 as discussed below, effectively restraining their abilities to promote non-T fates.

5. ESSENTIAL ROLES FOR GATA-3 AND PU.1 IN EARLY T CELL DEVELOPMENT

Both GATA-3 and PU.1 are essential for T-cell development. GATA-3, a double zinc finger (C4-type) transcription factor, is expressed in an almost T lineage-specific pattern among hematopoietic cells. It is turned on among the earliest genes activated by Notch-Delta signaling in hematopoietic precursors, and is used throughout T cell development from the ETP stage all the way to post-thymic responses to antigen. The ETS-family transcription factor PU.1 is almost the reverse, with virtually no expression in most thymocytes or mature T cells. PU.1 is most prominent in B cells, multipotent hematopoietic progenitors, and myeloid cells of various lineages, where it has well-documented positive gene regulatory activities. The competitive antagonism between PU.1 and GATA family factors is thought to control myeloid vs. erythro-megakaryocytic fate decisions as already noted, and so a simple expectation would be that GATA-3 might dictate T-lineage fate while PU.1 should oppose it. Nevertheless, substantial expression of GATA-3 and PU.1 RNA overlaps in ETP and DN2 stage thymocytes.

The loss of GATA-3 at the germline or stem-cell stage eliminates all recognizable T-cell precursors at least as early as the DN2 stage [99–101]. If GATA-3 is conditionally deleted later, at different times after thymic entry, powerful effects are seen at multiple later stages of T-cell development (rev. by [38]). Similarly, germline disruption or early conditional deletion of PU.1 eliminates most or all T cell development [102–105]; a severe hypomorphic allele also causes profound inhibition of fetal T-cell development [106]. Because PU.1 expression in T-lineage cells is confined to the earliest DN stages, the knockout phenotype is also abnormal in

early stages. PU.1 is needed for generation of precursors with the ability to migrate to the thymus; it is not yet clear for how long after their arrival intrathymic intermediates require PU.1 as well. However, throughout the extensive intrathymic proliferation by ETPs, these cells maintain a level of PU.1 expression similar to those found in multilineage prethymic precursors and stem cells [7,8,43,107], arguing for a continuing intrathymic role.

The target genes that GATA-3 and PU.1 are needed to regulate at these early stages are not clearly identified, in part because so little is known about the requirements of cells so close to the stem-cell stages. The blocks caused by losses of these factors long precede the blocks that are seen at β-selection due to lack of TCR rearrangements or any of the signaling components that transduce TCR signals. Thus, most T-lineage genes that have been suggested as targets for GATA-3, including the *Rag* recombinases, TCR genes, CD5, and the Th2 cytokine complex, are unlikely to account for its early essential role (see [38] for review). Both GATA factors and PU.1, however, can positively or negatively affect expression of the two most important growth factor receptors used in ETP and DN2 cells, namely c-Kit and IL-7Rα [36, 108–112]. These results raise the possibility that balanced activity of GATA-3 and PU.1 is primarily needed in early thymocytes to support survival and growth.

The paradigm of competition between PU.1 and GATA factors in erythromyeloid development might make this cooperative action seem surprising, but it is not exceptional. First, the mutual interference that occurs between PU.1 and GATA factors is at the level of protein/protein interaction. This kind of mechanism should depend on diffusion, and should not benefit from specific co-recruitment to neighboring DNA sites to accelerate interaction. Therefore, interference should be highly concentration-dependent, and as long as both PU.1 and GATA protein concentrations are below a certain threshold, they may not interfere in fact. Second, there may be steric constraints to the antagonism between PU.1 and GATA factors at target genes where both factors engage DNA simultaneously. For example, the inhibition of PU.1 transactivation by GATA-1 depends on an interaction between the DNA binding domains of the factors, the Ets β3-β4 region of PU.1 with the C-terminal zinc finger of GATA-1 [4,113], whereas the inhibition of GATA-1 DNA binding by PU.1 requires polarized binding of the PU.1 N-terminus to the GATA-1 C-terminal finger [114]. There is clearly no antagonism at some mast-cell lineage target genes that depend on simultaneous GATA and PU.1 binding [115]. Thus, balanced activity of GATA-3 and PU.1 could selectively promote expression of a particular subset of target genes that respond to both factors, while inhibiting expression of targets that respond to one or the other exclusively.

6. GATA-3-DRIVEN LINEAGE INFIDELITY: DOSAGE AND NOTCH SENSITIVITY

With its T-lineage specific expression and recurrent T-lineage roles, GATA-3 has been an appealing candidate for a factor conferring T-lineage identity in early thymocytes (Fig. 1B). However, gain of function of GATA-3, in the presence or absence of Notch signals, fails to enhance either appearance or developmental progression of the earliest T-lineage cells [36, 111,116,117]. Only around the time of lineage commitment, from the DN3 stage or its human equivalent onward, does high-level GATA-3 become compatible with modulation of fates within the T lineage [38,116,118–120]. Thus, high-level GATA-3 is inhibitory for some of the same stages of T-cell development that require normal levels of GATA-3. GATA family factors can activate a cell-cycle arrest mechanism [121,122], and so one possibility would be that excessive GATA-3 simply causes loss of cellularity. Indeed, high-level GATA-3 can inhibit survival of later-stage thymocytes, and a conserved KRR motif within GATA-3 specifically mediates this cytostatic activity [123]. However, this is not the only cause of interference with early T-cell precursors. High-level GATA-3 actually promotes an alternative, non-T lineage choice. The effects of high-level GATA-3 are context-dependent: whereas it can promote a

megakaryocytic fate from bone marrow stem cells [117], it specifically drives a mast-cell development program when expressed in early T-lineage cells [36] (Figure 3).

This lineage diversion is interesting for three reasons. First, the mast-cell program has not previously been shown to be related to any lymphoid program [124–126], and so its activation within T-lineage precursors by a "T-lineage-specific" transcription factor is remarkable. Second, the ability of thymocytes to respond to GATA-3 in this way is developmentally regulated, observing the same commitment boundary as other non-T fates. While ETP and DN2 stage cells can respond to GATA-3 in this way, DN3 cells cannot. This suggests that the diversion depends on additional intrinsic features of the earliest-stage cells which may be lost with commitment. Finally, the mast-cell pathway is effectively blocked in thymocytes by Notch signaling. When Notch-Delta interaction is also occurring, high-level GATA-3 simply blocks survival, and the mast-cell program cannot be completed [36].

Respecification is a slow, multistep process [36], as diagrammed in Fig. 3. Overexpression of GATA-3 in fetal thymocytes causes a number of early gene regulatory effects within the first 40 hr, but it does not materially alter the expression of most T-cell differentiation markers or regulatory genes. Among the most pronounced early changes are a strong, temporary downregulation of PU.1 (in the ETP and DN2 cells) and upregulation of the mast-cell transcription factor MITF (microphthalmia associated transcription factor) and the two other hematopoietic GATA factors, GATA-1 and GATA-2. These effects occur concurrently with a strong upregulation of c-Kit expression and can be detected, more or less, whether Notch signaling is present or not. Over the next days, further changes occur to activate an increasing set of mast-cell genes, including those encoding transcription factors SCL (*Tal1*) and Gfi-1b, while T-lineage-specific gene expression becomes increasingly broadly inhibited. The emergence of viable converted cells now depends on removal from Notch-Delta signals. It is likely that the additional steps depend on the newly induced (or reinduced) transcription factors as well as on GATA-3 itself (Fig. 3). The distinctness of this stage is reflected in restored expression of PU.1, to levels appropriate for the mast-cell fate [127]. However, there is still a substantial "hangover" of expression of a few T-cell genes, such as CD3ε and CD3γ, even in the cells that have undergone complete phenotypic conversion to c-Kit^{high} Thy-1^{low} CD27^{-} mast-cell precursors. Full maturation to a surface FcεRI^{+} state depends on further culture in mast-cell cytokines for at least another week (Fig. 3).

Under normal circumstances in the bone marrow, the crucial transcription factor for initiating mast-cell development is GATA-2 [125,128]. GATA-3 overexpression has similar or identical effects to forced expression of GATA-2 in early thymocytes, consistent with the extensive structural similarities between these related factors [36]. Thus GATA-3 probably opens up access to the mast-cell pathway by activating genes that are normally regulated in bone-marrow precursors by GATA-2. But the interesting point is that GATA-3 is normally a participant in the T-cell program, not the mast-cell program, and a collaborator with Notch signaling, not an antagonist. In the unstable developmental context of early T-lineage cells, its entire developmental impact on the cells depends on its level of expression. Thus avoiding excessive induction of GATA-3 in the ETP and DN2 stages is as important for T-cell development as guaranteeing that some GATA-3 will be present.

It remains to be determined how the committed state of DN3 cells blocks access to the mast-cell program. But how does Notch signaling prevent this lineage choice in the ETP and DN2 stages? All of the short-term effects of GATA-3 are slightly stronger when Notch signals are removed [36]. High-level GATA-3 actually appears to blunt Notch1 expression itself as well as the expression of certain Notch target genes, suggesting a competitive mechanism, but most T-cell transcription factors are minimally affected by GATA-3 overexpression, with or without Notch-Delta signaling. The largest Notch-dependent difference in GATA-3 effects that can be

noted at this early point is that TCF-1 is repressed only when Notch signals are absent. Thus, Notch signaling may indirectly be acting either to maintain the transcriptional effector of the Wnt-β-catenin pathway, or else to preserve a repressive function of TCF-1 which may be important for T-lineage fidelity in this case. TCF-1 and increasingly LEF are then severely repressed in later stages of the conversion to a mast-cell precursor state [36]. If these factors are serving in thymocytes as repressors of PU.1 expression [75](see above), then their own downregulation as the cells become fully converted mast-cell precursors may help to explain why PU.1 expression resumes [36](Fig. 3, dashed lines). These results suggest that GATA-3 is linked with Notch signaling, TCF/LEF factors, and PU.1 in a network of dose-dependent, competitive interactions that determine access of early T-cell precursors to the mast-cell developmental pathway.

7. PU.1 AND MYELOID ALTERNATIVES TO THE T-LINEAGE PROGRAM

Although needed to start T-cell development, PU.1 also helps to maintain a progenitor-like developmental plasticity in the ETP and DN2 cells. During commitment, expression of PU.1 and SCL (Tal1) precipitously shuts off [129,130]. Forced gain of PU.1 function, beyond the stage when it is normally shut off [53,54,56–58,111], provides evidence that endogenous PU.1 expression in earlier T-cell precursors is probably rate-limiting for their developmentally regulated access to macrophage and dendritic-cell fates.

Forced PU.1 expression can undo the commitment of DN3 thymocytes and convert them to myeloid dendritic cell phenotype, passing through dual-lineage phenotypic intermediates that clearly show the transdifferentiation in progress [54,56–58](Figure 4). High-level, dysregulated PU.1 expression can inhibit growth under “lymphoid” cytokine conditions, but the converted cells thrive if myeloid growth factors are supplied [54] or if the cells express a Bcl2 transgene [57,58]. The mechanism PU.1 uses to promote these alternative fates appears to be intrinsically Notch-sensitive. Notch-Delta signaling can restrain exogenous PU.1 from causing respecification of DN2 or DN3 cells [54,58](Fig. 4), even when forced expression of PU.1 reaches levels more than ten times higher than normal ETP or DN2 levels. The important feature of this antagonism is that Notch signaling does not directly inhibit expression of PU.1 itself, while PU.1 also does not appear to inhibit Notch1 or Notch3 expression directly, at least not when overexpressed in DN2 or DN3 stage cells [58]. Thus PU.1 as expressed in DN2 cells should be a conditional mediator of lineage plasticity, which ideally positions the cells in a regulatory state where responses to microenvironmental Notch signals can dictate their fates.

Even in the DN3 stage, the committed state remains intrinsically rather flimsy with respect to myeloid and dendritic-cell alternatives. Other exogenous genes can also undo the commitment of DN3 cells. Forced expression of the myeloid factor C/EBPα or its relative C/EBPβ can also respecify DN3 cells to myeloid lineage cells very efficiently, to an inflammatory macrophage fate rather than a myeloid dendritic cell fate [54](Fig. 4). Furthermore, surprisingly, DN3 cells that have downregulated PU.1 express peak levels of the PU.1 relative Spi-B, and yet if artificially expressed at sufficiently high levels, Spi-B itself can also cause conversion of the cells to a dendritic-cell phenotype [56]. Thus the commitment of normal DN3 cells may be a composite of the recent repression of PU.1, absence of C/EBP factors, quantitative limits on the expression of Spi-B, and the relatively weaker myeloid-promoting potency per molecule of the Spi-B which they do express. Spi-B expression here may provide the cells with continuation of any T-lineage promoting functions that PU.1 formerly may have served; for example, PU.1 (and probably Spi-B) may actually upregulate expression of the T-lineage markers *Cd25* and *Ptcra* [57,111,131].

Somewhat like PU.1, C/EBPα effects on DN3 cells are blunted by Notch signaling [54]. However, C/EBPα has a much more direct antagonistic effect on expression of Notch1 itself.

Preliminary evidence suggests also that Notch-Delta signaling may actively inhibit C/EBPα expression in immature fetal thymocytes, implying a more direct binary opposition than that of Notch and PU.1 [36,58]. Thus, sustained C/EBPα expression would be incompatible with passage through the Notch-dependent early stages of T-cell development. Indeed, endogenous C/EBPα may very well contribute to the robust myeloid potential of many ETPs, but its expression is already very low by the DN2 stage [54,132](E.-S. David-Fung & E. V. R., unpublished results). However, even in DN2 cells reinduction of C/EBPα can be triggered by ectopic growth factor receptor stimulation [59], especially in the absence of Notch signaling. It is likely to be the combination of this upregulated C/EBPα with endogenous PU.1 that explains why ectopic growth factor receptor signaling is sufficient to drive ETP and DN2 cells into myeloid lineage conversion [79,133].

Both C/EBPα and PU.1 drive pro-T cells to myeloid identities through paths of several distinct steps over a period of days (Fig. 4). Each of these steps represents a potential opportunity for regulatory intervention, either by Notch signals or by other signaling pathways. Strikingly, and in contrast to GATA-3 promoted lineage diversion, both myeloid factors start by undermining T-lineage identity, through downregulation not only of differentiation genes but also of multiple DN3-expressed regulatory genes. C/EBPα represses Notch1, GATA-3, and E2A, while PU.1 represses Myb, TCF-1, Gfi-1, E proteins, certain other Ets family transcription factors, and Ikaros [54,57,58]. Some myeloid gene expression also begins. Then, to complete the transformation, each of these factors can reactivate the other's expression after several days in the absence of Notch signals [54,58](Fig. 4). These cross-activations of myeloid regulators do not precede, but rather follow, the downregulation of T-cell regulatory factors. When Notch signaling neutralizes the effects of high-level PU.1, its effects are mostly to protect the expression of these T-lineage transcription factors rather than to block the earliest increases in myeloid-associated gene expression [58](Fig. 4, "+DL"). Later, C/EBPα reactivation may be inhibited in thymocytes receiving Notch signals [36,58], but it is not clear whether the effect is direct. This raises the possibility that among the T-cell factors that are initially targeted, there are Notch-dependent "gatekeeper" functions that would otherwise restrain the cells from the myeloid program, and that these functions need to be disabled before trans-differentiation can proceed.

PU.1 and C/EBPα do not reach the point of positive cross-regulation by a common route. Because these two factors have divergent initial effects on Notch, GATA-3, and Myb [54, 58], the early intermediates in lineage conversion should be quite different (Fig. 3A). This is strikingly reminiscent of a recent report that forced sequential expression of GATA-2 and C/EBPα in common lymphoid progenitors, a "tabula rasa" cell type, leads to highly biased generation of either eosinophils or basophils, with the whole developmental outcome depending on the order in which these factors act [125]. The C/EBPα-driven program is therefore a different lineage alternative for DN3 cells than the pathway activated by PU.1. When artificially overexpressed, C/EBP factors can even force DN3 thymocytes to adopt a form of myeloid fate after their endogenous PU.1 genes have been excised [54]. The fact that these different myeloid pathways are just one gene away for DN3 cells shows that these newly-committed cells differ in relatively few respects from the regulatory state and gene accessibility pattern of multipotent precursors.

8. GATA-3 AS ANTAGONIST OF MYELOID LINEAGE OPTIONS

The functions mobilized by Notch to preserve T-lineage identity before lineage commitment need to be identified. GATA-3 is an obvious candidate for one of the T-lineage gatekeeper functions that antagonizes myeloid fates under normal conditions. Myeloid lineage conversion of thymocytes by either C/EBPα or PU.1 is inhibited if GATA-3 is co-expressed [54,134]. Furthermore, GATA-3 may be suspected of a role in lineage commitment itself, as

overexpression of GATA-3 in early fetal thymocytes efficiently downregulates PU.1 RNA expression as already noted [36,111,118]. GATA-3 itself is encoded by one of the genes that is turned on earliest in progenitors as they begin the T-cell developmental program in response to Notch-Delta signaling. GATA-3 is also a direct target of Notch induction in the context of T helper 2 cells [135,136]. Therefore GATA-3 might be implicated in aspects of the Notch-dependent protection of developing T cells from myeloid diversion by PU.1 or C/EBPα.

In reality, the interplay between GATA-3 and myeloid determination is probably more subtle. Whether or not GATA-3 is able to protect cells from myeloid differentiation is very much dependent on cellular context and probably also dependent on its exact level of expression. Indeed, in non-T lineage cells GATA-3 can even trigger myeloid development, as observed in Pax5-deficient pro-B cells [137]. The simplest case of GATA-3 blocking the myeloid fate may be when C/EBPα expression is imposed on DN3 cells [54]. Here, one of the earliest effects of C/EBPα or C/EBPβ seems to be downregulation of GATA-3. Adding back GATA-3 can therefore be seen as an epistasis test, in which exogenous GATA-3 can restore the missing function. Coexpressed GATA-3 can block Mac-1 (CD11b) upregulation by C/EBPα, an aspect of the C/EBPα response that appears to reflect cross-induction of PU.1, and this inhibition is consistent with the ability of high GATA-3 to block PU.1 expression [36,111,118]. Even here, however, there is not clear evidence how much of the T-cell program GATA-3 can restore. For example, it is not clear whether GATA-3 can prevent C/EBPα from downregulating Notch and E protein expression [54], which would independently be required for full T-cell development.

As an antagonist for PU.1, the possible role of GATA-3 is more complex. High-level PU.1, unlike C/EBPα, has little immediate effect on expression of GATA-3 itself or of Notch1. When PU.1 effects are neutralized by Notch signaling, again there is little if any impact on levels of GATA-3 RNA; instead, the strongest protective effects are seen on RNAs encoding a completely different set of T-lineage regulators: Myb, E proteins, Gfi-1, and Bcl11b [58]. TCF-1, also downregulated by PU.1, is also partially protected by Notch signaling. Thus, when GATA-3 is co-expressed with PU.1, it is probably antagonizing PU.1 in a supraphysiological, gain-of-function mode. Thus, it is possible that GATA-3 coexpression with PU.1 does not really restore the T-lineage program but simply provides antagonism for PU.1 by titrating it at the protein level, preventing PU.1 from transactivating the myeloid genes. However, the biochemical basis for these effects remains unresolved. Many of the most immediate impacts of PU.1 on pro-T cells are the repression of critical T-cell genes. Yet GATA factors are not thought to block PU.1 binding to DNA, but simply PU.1 transactivation; it is completely unclear what effect they would have on any target genes where PU.1 acts as a direct repressor.

Ultimately, GATA-3 may only be one part of the mechanism antagonizing myeloid fates in developing T cells. Under normal circumstances, GATA-3 could also play a role in the programmed downregulation of PU.1 itself (D. D. S.-A., unpublished results), although current published evidence suggests that the normal control of PU.1 levels in developing thymocytes may be more affected by inputs from TCF/LEF family and Runx family transcription factors [75,138]. On the other hand, the abilities of the myeloid transcription factors to repress *Gata3* expression or block GATA-3 DNA binding could play a substantial role in the timing of T-cell developmental progression, as discussed in the final section.

9. TRANSDIFFERENTIATION, NOT DEDIFFERENTIATION, IN RESPONSE TO REGULATORY PERTURBATION

Neither myeloid nor mast-cell programs are thought to be closely related to lymphocyte developmental programs, and indeed, conversion of thymocytes to dendritic cells by PU.1, inflammatory macrophages by C/EBPα, and mast cells by excess GATA-3 all involve a repression of T-lineage specific gene expression. Their diversionary activity is even more

remarkable in view of the positive roles that two of these diversion-inducing factors normally play in T-cell development, when expressed at different times and levels. This raises the question of whether the effects of these factors are simply to cause T-cell precursors to revert to a more primitive, multipotent progenitor state, from which all these diverse pathways naturally emerge.

The question is more interesting because recent data indicate that in vivo, PU.1 downregulation coincides with the main onset of T-lineage specific gene expression from the DN2 to the DN3 stage, linking a major positive regulatory event in the T-cell program with the loss of alternative choices. T-lineage genes appear to be upregulated first in that subset of DN2 cells that have already begun to downregulate PU.1 [129,139]. PU.1 acts as a transcriptional repressor of genes encoding T-lineage transcription factors including TCF-1 and E proteins [58], and through protein-protein interactions it could antagonize GATA-3 functions needed for progression to the DN3 stage (D. D. S.-A. and E. V. R., unpublished results) as long as it is expressed. Conceivably, some of these effects could be strong enough even in the presence of Notch signaling to enable endogenous PU.1 to play a timing role for the triggering of particular aspects of normal T cell development that depend on levels of E proteins, TCF-1, and GATA-3.

If excess GATA-3, PU.1, and C/EBPα each caused reversion to a multipotent progenitor state before promoting redifferentiation, then the intermediate stages of the reconversion process should go through a common stage. Furthermore, this common stage should be similar to the phenotype of a natural multipotent progenitor. The transcription factor profile of natural multipotent progenitors is marked by high-level coexpression of at least four informative transcription factors: GATA-2, PU.1, SCL (Tal1), and Myb [43,140]. Indeed, forced high-level GATA-3 turns on GATA-2 and SCL in thymocytes that no longer express these factors, and it preserves the high-level Myb expression that thymocytes naturally share with progenitors. However, it sharply downregulates PU.1, in contrast to normal progenitors. On the other side, high-level expression of PU.1 in thymocytes fails to turn on GATA-2 (C. Franco & E. V. R., unpublished data) and actively represses Myb expression. Although the two perturbations have in common an ability to downregulate TCF-1 (encoded by *Tcf7*), this too may go through different pathways, as TCF-1 is almost completely protected from GATA-3 effects by Notch signaling, while it is still inhibited by PU.1 in the presence of Notch-Delta signals. Thus the intermediate regulatory states of the conversion process are completely different, and in both cases they differ markedly from natural multipotent progenitors, and even from ETP thymocytes. This shows that the paths to lineage diversion do not require backtracking to a common multipotent state, but instead involve alternative, direct reprogrammings of thymocytes from the midst of their T-lineage differentiation process.

10. CONCLUSIONS

The results reviewed here show that T-cell precursor specification is not only a protracted process but a remarkably precarious one at the regulatory level. This makes sense of much biology that could otherwise seem counterintuitive. An increasingly rich literature confirms that most T-cell precursors naturally retain access to non-T and non-lymphoid programs well beyond the stage when they have lost B-lineage potential [16–19,81,141–143]. The myeloid developmental choices can be triggered even more readily in immature thymocytes before the DN3 stage by manipulations that might have been imagined to leave the basic regulatory state intact, such as stimulation through ectopic cytokine receptors [79,133]. Although the divergence in gene expression program is less dramatic, until the DN3 stage these cells also have access to the distinct natural killer pathway, which can be enhanced through antagonism of E protein activity and/or removal of Notch-Delta signals [35,83,85,144–147]. This simultaneous access to multiple distinct developmental programs can now be seen as the consequence of a tug of war among regulatory forces acting normally within the cells between

the ETP and the DN3 stage (Figure 5). Among the T, B, NK, mast, and dendritic-cell or myeloid fates, the only one prohibited to T-cell precursors at an early stage is the B-cell fate, due to the extreme incompatibility of initial B-cell specification with Notch signals. The other fates are actually promoted by factors which also continue to have important roles in the T-lineage pathway itself.

In this review, we have looked in detail at the interaction among three of these factors, PU.1, GATA-3, and Notch, and to a lesser extent at roles of C/EBP factors and TCF-1/LEF family members. The alternatives made available to early T-cell precursors by selective dysregulation of these factors require several steps of reprogramming, but not a return to a stem-cell condition. This is important because it shows that the dynamic balancing of these regulatory forces maintains a degree of true multipotentiality that is inherent in T-cell precursor states throughout the onset of T-lineage gene expression. Even in DN3 cells, where ectopic cytokine receptor signaling, withdrawal of Notch signals, and GATA-3 overexpression are no longer capable of causing respecification, reintroduction of C/EBP factors or PU.1 are sufficient to overcome the fragile new state of commitment. Yet later, the lineage identity of a mature T cell in the periphery is remarkably robust over arbitrarily large numbers of cell generations and through many storms of activation-induced regulatory change, including high-level GATA-3 expression [38,148], intense MAP Kinase activation [149–151], frequent deprivation of Notch signals, and even in some cases reinduction of PU.1 [152]. The contrast reveals that another level of commitment must exist for T-cell precursors, possibly through an epigenetic mechanism, which must occur after the DN3 stage and remains to be discovered and dissected.

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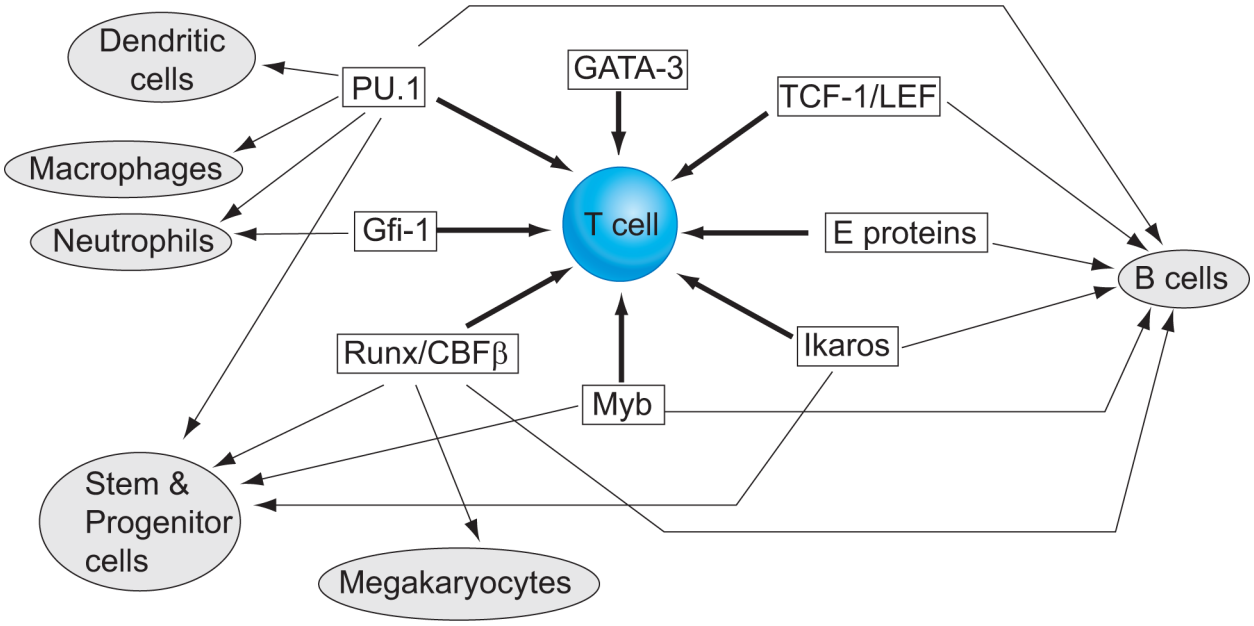

Figure 1.
T cell regulatory requirements in relation to other hematopoietic pathways. Schematic summary of the transcription factor requirements for T-lineage specification from hematopoietic stem cells (thick arrows), indicating alternative hematopoietic pathways that are naturally promoted by the same factors (thin arrows). For reviews and updated references, see [1,39,154–162]. Note that although GATA-3 is T-lineage specific under normal conditions shown here, it is also capable of driving a non-T differentiation program even in early T-lineage cells if expressed at an elevated level.

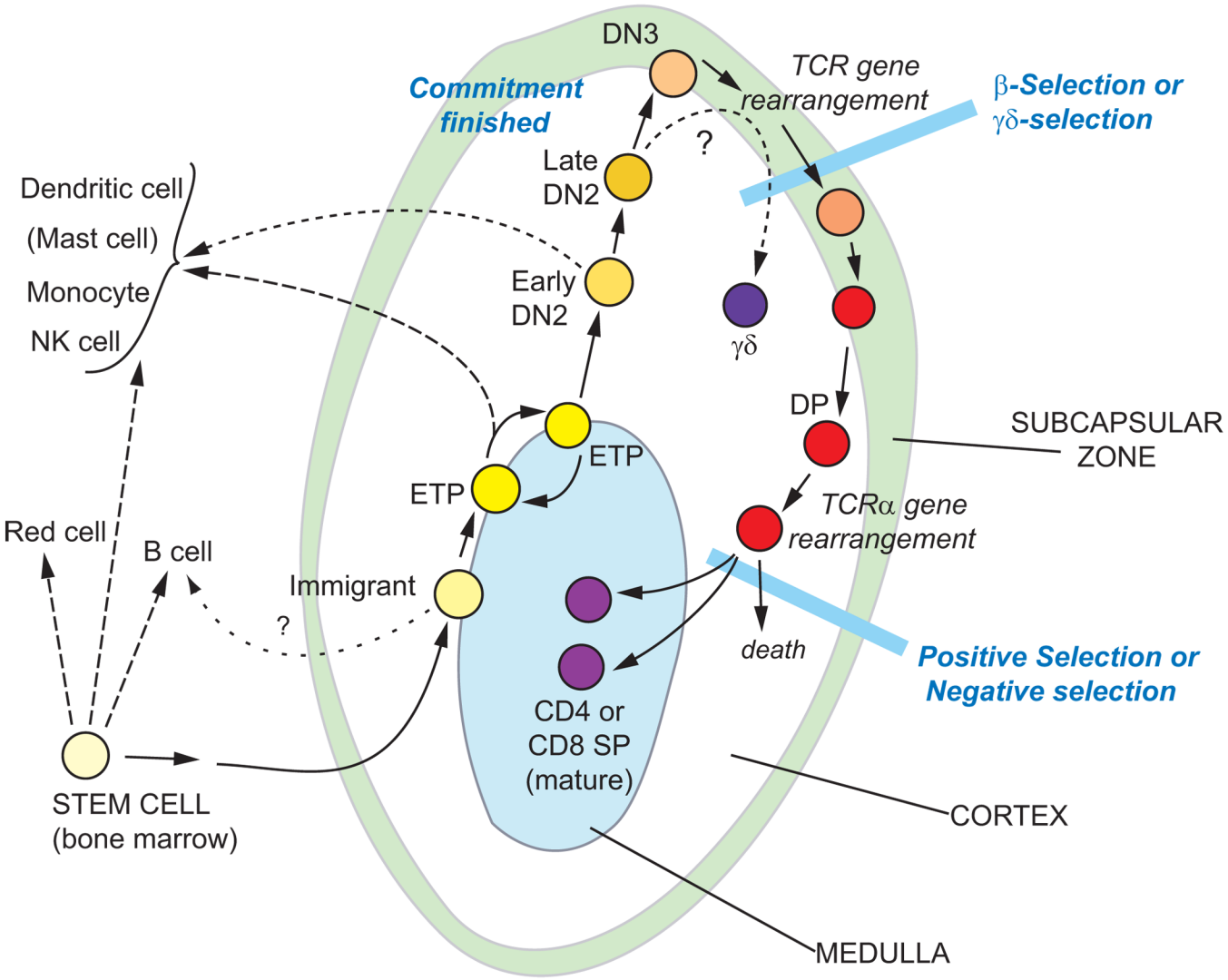

Figure 2.

T cell developmental stages. Stages of early T-cell development in the young postnatal mouse are shown in the context of their ordered migration through different compartments of the thymus (rev. in [28,153]). Cells in the ETP and DN2 stages undergo extensive proliferation (>10 cell cycles in all), and then the only other major phase of proliferation is immediately following β-selection. To the left are shown the developmental fates that cells at each stage can display if they are removed from the thymic microenvironment at the indicated stages.

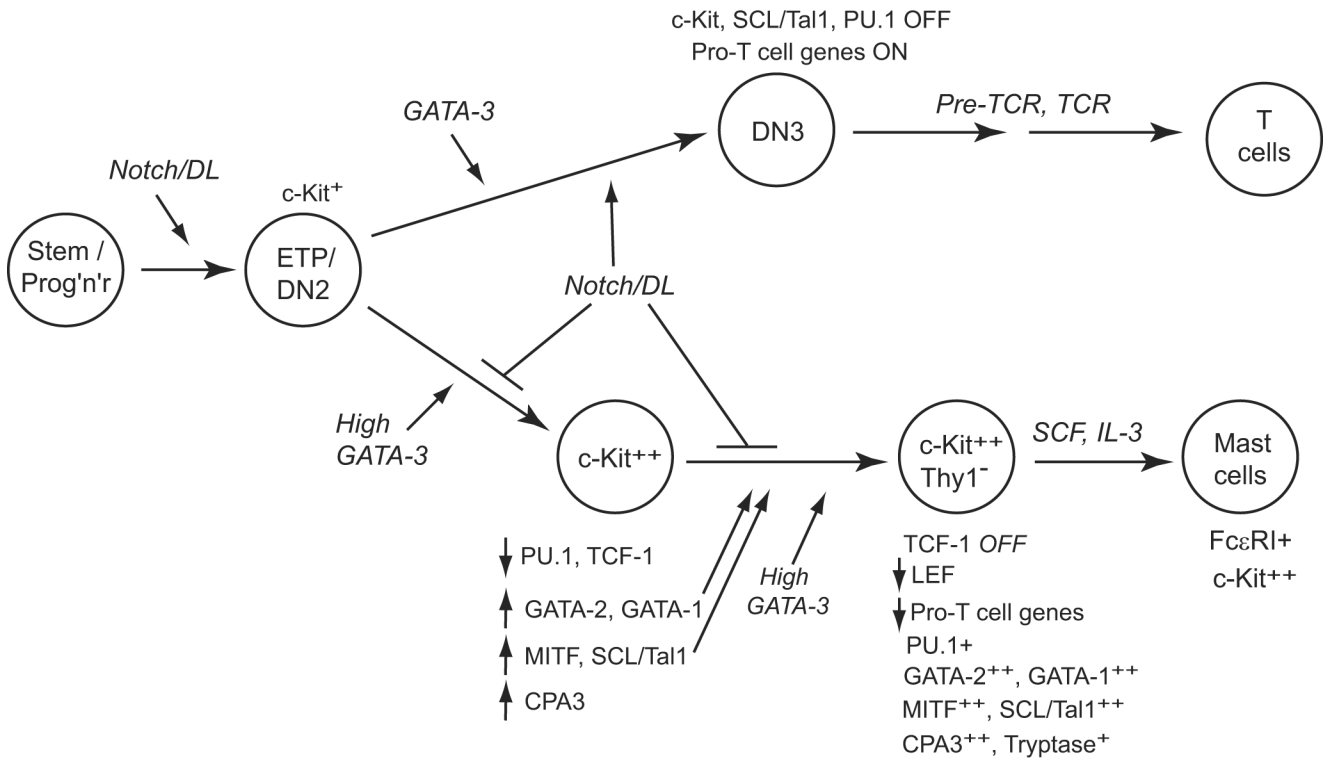

Figure 3.

Stepwise respecification of ETP and DN2 cells by GATA-3 overexpression. Early, intermediate, and late stages in the reprogramming of ETP or DN2 cells by forced expression of GATA-3. The stages where Notch signaling exerts inhibition are shown. Adapted from Supplementary Figure 6, ref. [36]. Note that susceptibility of cells to these interactions is limited to the stages before DN3, when an unidentified commitment function blocks access to the mast-cell pathway.

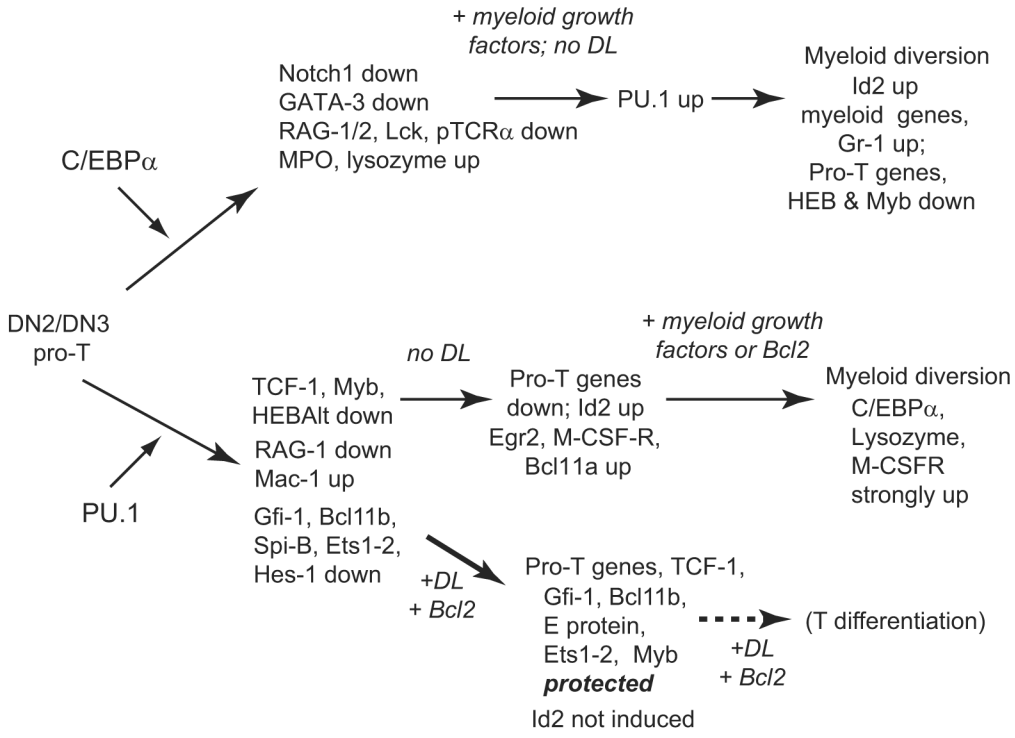

Figure 4.

Myeloid diversion pathways for advanced pro-T cells by re-introduction of C/EBPα or PU.1. Comparison of the sequences of gene expression changes induced by transduction of pro-T cells with PU.1 or with C/EBPα, from first effects (day 1–2) through lineage conversion (4–9 days). Effects summarized in the figure were measured on purified DN3 pro-T cells from adult mouse thymus, transduced with C/EBP factors [54] or on DN2/3 stage fetal thymocytes, transduced with PU.1 [58]. “Pro-T genes” include *Rag1*, *Lck*, *LAT*, and *ZAP70*, the products of which are needed for TCR function. Continued progress along the myeloid pathway depends on the lack of Notch-Delta signals in PU.1 overexpressing cells. The pathway branch indicated by “+DL” shows how reintroduction of Notch-Delta signaling can stall or reverse the reprogramming of fetal pro-T cells by PU.1. Although high-level PU.1 is inhibitory for proliferation and progression of T-lineage cells beyond the DN3 stage, Notch signaling and neutral viability support (a Bcl2 transgene) can restore substantial T-lineage differentiation even beyond β-selection. Major gene targets that are differentially affected by PU.1 in the presence or absence of Notch-Delta signaling are shown (data from ref. [58]).

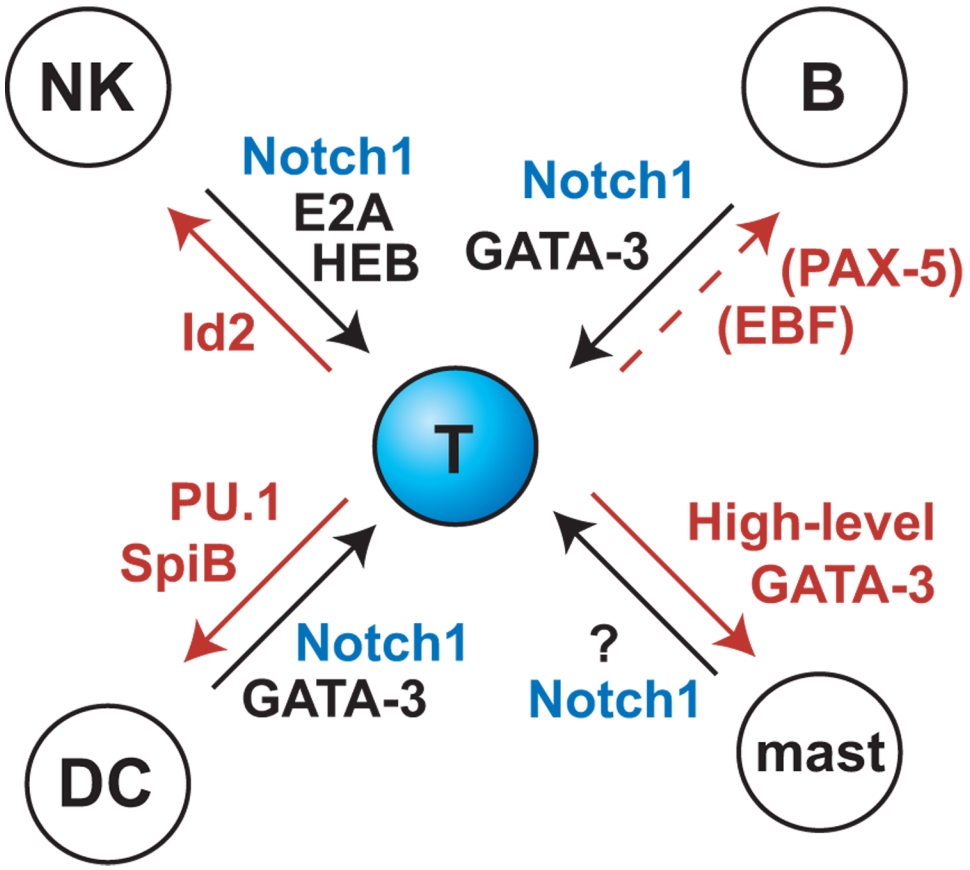

Figure 5.
Schematic of counterbalancing and dose-dependent regulatory forces that act on T-cell precursors through the ETP and DN2 stages. Regulators named in red denote forces diverting cells from the T-cell pathway. Of these, only EBF and Pax5 are not normally active in the thymus during these stages. Notch signaling acts as a constraint on all of these alternatives. This dynamic equilibrium is normally resolved at the DN3 stage, in part by the downregulation of PU.1, but almost certainly through other regulatory changes as well [163].