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The thymus is seeded from the BM

T cells develop within the thymus (1). However, progenitors within the thymus can maintain thymocyte production only for short periods (2, 3). Hence, intrathymic or i.v. transfer of thymocytes results in transient thymopoiesis. BM transferred intrathymically also results in transient thymopoiesis. Only BM transferred i.v. results in sustained thymopoiesis (2, 3). These observations indicate that the thymus does not provide a suitable environment for self-renewal. Instead, seeding of the thymus with BM progenitors is required to maintain thymopoiesis throughout adult life (2, 3).

The thymus is only periodically receptive to seeding from the circulation, suggesting limiting numbers of niches whose occupation precludes further colonization until niche vacancy (4). Past estimates agree that the number of thymic seeding cells must be very small (5, 6), with perhaps 100–300 microenvironmental niches occupied by progenitors that are replaced at an average rate of 2–3% per day (7, 8). These findings highlight the difficulties in using direct approaches to identify and characterize thymus-settling progenitors. Instead, characterization of putative upstream BM progenitors and downstream thymic populations has been used to investigate T cell origins.

Immature thymocyte subsets

Early work demonstrated that immature progenitors within the thymus could be identified within the CD4\(^{-}\)CD8\(^{-}\) double-negative (DN) subset (9, 10). Expression of CD44 and CD25 was used to fractionate the DN population into further subsets that were proposed to constitute a developmental lineage for T cells (11–13). The most immature progenitors reside within the DN1 CD44\(^{+}\)CD25\(^{-}\) subset (11, 14), and possess multilineage potential (15). DN2 (CD44\(^{+}\)CD25\(^{-}\)) thymocytes lack B lineage potential (15) but are not fully committed to the T lineage, because they remain capable of dendritic cell development (16). Final commitment to the T lineage only occurs at the DN3 (CD44\(^^{-}\)CD25\(^{+}\)) stage, concomitant with extensive rearrangement of TCR \(\beta\), \(\gamma\), and \(\delta\) genes (17, 18). Productive rearrangement of a TCR\(\beta\) chain is necessary for differentiation of DN3 cells into CD4\(^{+}\)CD8\(^{-}\) double-positive (DP) thymocytes (19), which constitute the vast majority of thymocytes. These DP cells initiate rearrangement of the TCR\(\alpha\) locus (19), followed by \(\alpha\)\(\beta\)TCR-dependent selection (20, 21).

The DN1 population, which contains the most immature T lineage progenitors, is heterogeneous (15). A subset of this population, representing 0.05% of thymocytes (15), expresses high levels of c-Kit (22, 23) and low levels of CD4 (CD4low) (24), which may be due to passive acquisition (25). These progenitors possess short-term T, B, NKT, as well as NK and dendritic cell potential upon adoptive transfer into irradiated recipients (23, 26–28). A small degree of myeloid potential was also noted (23, 26). In line with their immature status, they were shown to lack TCR gene rearrangements (17, 29), although a low frequency of DJ1-JB1 rearrangements has been subsequently detected (30).

Further characterization of early T lineage progenitors (ETPs)

Recent work has re-examined the identity and origin of ETPs in adult B6 mice (31). Using an extensive lineage marker (Lin) gate (that lacked anti-CD4) (Table I) to remove differentiated cells, the thymic Lin\(^{-}\)CD44\(^{-}\)CD25\(^{-}\) population was shown to correspond to a minute 0.01% population. The majority of these Lin\(^{-}\)CD44\(^{-}\)CD25\(^{-}\} cells were c-Kit\(^{\text{high}}\), stem cell Ag-1 (Sca-1)\(^{\text{high}}\), and IL-7Ra\(^{\text{neg/low}}\), and were efficient T lineage progenitors. Other subpopulations of the Lin\(^{-}\)CD44\(^{-}\)CD25\(^{-}\} population lacked T progenitor activity (31). In this review,
Lin^−CD44^+CD25^+ c-Kit^{high} thymocytes are referred to as ETPs after past work (15). As CD44 and c-Kit can be concordantly regulated (32), staining for CD44 is not necessary for identification of ETPs (Table 1).

In surface phenotype, ETPs are nearly identical with the most immature BM progenitor pool, which is descriptively termed Lin^−Sca-1^{high}c-Kit^{high} (LSK) (Table 1) (33, 34). Differences between ETPs and BM LSKs exist at the population level for expression of cytokine receptors. A subset of ETPs expresses low but detectable levels of IL-7Rα by surface staining (31), whereas BM LSKs are IL-7Rα^− in adult mice (33, 35). Only a small subset of ETPs expresses fms-like tyrosine kinase 3 (Flt3) (36), whereas 60−70% of BM LSKs express Flt3 (33, 37).

In young adult B6 mice, ETPs represent slightly less than 0.01% of unfractionated thymocytes (1 per 15,000−20,000 thymocytes), and undergo 20,000- to 50,000-fold peak expansion upon intrathymic injection, with the peak occurring ~3 wk after transfer (31). In addition to sufficient proliferative ability to generate the number of DP cells that is present in the mouse thymus, the ETP population possesses all lineage potentials known to reside within the DN1 compartment (Ref. 31; our unpublished data). However, ETPs are too numerous to be the earliest thymus-settling progenitors, given past estimates of the very low number of progenitors that seeds thymus from blood (8). It instead seems likely that the majority of ETPs arise by expansion of small numbers of thymus-settling cells.

BM progenitors for ETPs

The lymphoid restriction and lack of self-renewal ability of early progenitors within the thymus has suggested that they may derive from similar lymphoid-restricted and nonrenewing progenitors within the BM. The identification of lymphoid-restricted common lymphoid progenitor (CLP) in BM supported this view (35). Identification of CLPs relied on the demonstrated importance of IL-7 in early B and T cell development (35, 38, 39). CLPs were originally identified as Lin^−, IL-7Rα^−, c-Kit^{low}, and Sca-1^{low} (Table I). CLPs have also been identified using an alternative protocol in which c-Kit is replaced with AA4.1 (40), a mAb that recognizes a protein homologous to C1qR, because CLPs are AA4^+ (41). However, defined, CLPs can be seen to differ by multiple phenotypic criteria from BM LSKs (31 Table I). CLPs have been suggested to represent a common progenitor for T and B lineages (35) (Fig. 1A). However, CLPs have never been shown to seed the normal thymus, and lymphoid-restricted progenitors with the full panoply of surface markers that define CLPs cannot be detected in normal adult thymus (13, 31, 35). In addition to CLPs, the BM contains other progenitors that will efficiently make T cells in the thymic environment. Most relevant is the BM LSK population, which is upstream of CLPs and contains both Flt3^− self-renewing HSCs, as well as multipotent nonrenewing LSKflt3^− progenitors (33, 37) (Table I; Fig. 1).

Functional comparison of ETPs and CLPs

At first glance, ETPs and CLPs appear functionally similar in that they are both lymphoid-restricted nonrenewing progenitors (31, 35, 40). However, unlike CLPs, ETPs possess a weak myeloid differentiation potential (31). In addition, ETPs produce more DP thymocytes for longer periods of time when directly injected into the thymus of sublethally irradiated recipients (31). Conversely, ETPs are inefficient at making B lineage cells compared with CLPs (31). Only small numbers of CD19^+ B lineage cells appear upon i.v. transfer of ETPs, after protracted periods.

ETPs are not downstream of CLPs

A functional comparison of CLPs and ETPs is difficult to reconcile with a progenitor-successor relationship linking CLPs and ETPs. Instead, a picture emerges of parallel processes of lymphoid differentiation, one in the thymus that results in loss of B lineage potential, and the other in the BM that results in loss of T lineage potential. Differentiation in both sites is linked to loss of myeloid potential, perhaps by down-regulation of necessary cytokine receptors for myeloid development (30, 41).

The phenotypic and functional comparison of ETPs and CLPs suggests that ETPs are more proximal than CLPs to BM LSK progenitors. However, the inefficiency of ETPs in generating B cells suggests that only a small fraction of ETPs possess B lineage potential. Consistently, the limiting number of ETPs necessary to detect B lineage progeny in stromal cell cultures was estimated at 150 (30), whereas the limiting number of CLPs was estimated at 10 (41). The low frequency of B lineage progenitors within the ETP compartment could have two explanations: 1) a small number of thymus-settling progenitors with multilineage potential generates a relatively large pool of progeny that loses B lineage potential; or 2) the majority of thymus-settling cells lose B lineage potential before thymic seeding.

If ETPs and CLPs are not obligatorily linked by a progenitor-successor relationship but instead arise in parallel from LSK progenitors in BM, the requirements for their differentiation may be distinct. Adult Ikaros^−/− mice make T cells but no B
cells (42). An examination of early progenitors in Ikaros$^{-/-}$ mice revealed that they possess ETPs but lack CLPs (31). These findings indicate ETPs develop via a CLP-independent pathway.

It has recently been suggested that B220+CD19+ pre-TCRα gene (pTα)+ progeny of CLPs, termed CLP-2s, may seed thymus (43, 44). Cells with a CLP-2 phenotype can be identified as evidenced by expression of RAG, whereas other LSKflt3$^+$ progenitors are RAG-. CLPs are proposed to give rise to lymphoid-restricted CLPs that in turn give rise to B cells in the BM. ETPs and therefore T cells do not arise from CLPs, but instead arise from progenitor populations upstream of CLPs. These upstream BM progenitors include ELPs, but may also include some HSCs as well as RAG- LSKflt3$^+$ progenitors (designated by the lighter shading around these progenitors). Hence, the split between T and B lineages occurs earlier than had been previously appreciated.

A common progenitor population for T and B cells

As ETPs and CLPs are not linked by a progenitor-successor relationship, the question arises as to the identity of the common progenitor of T and B cells. ETPs are similar in surface phenotype to the BM LSK compartment, which includes HSCs (45, 46) as well as multipotent but nonrenewing progenitors that can be distinguished from HSCs by their expression of Flt3 and CD27, and in Thy1.1-expressing strains only as being Thy1.1$^-$ (33, 37, 45, 47). In this review, the multipotent nonrenewing LSK population is designated LSKflt3$^+$ (33). CLPs are now known to arise from the LSKflt3$^+$ population, via a differentiation process that requires Flt3 ligand (Flt3L) (33, 48). LSKflt3$^+$ progenitors are therefore intermediates between HSCs and CLPs (Fig. 1).

The LSKflt3$^+$ population in BM is itself heterogeneous. This was discovered through the analysis of BM progenitors in mice which had green fluorescent protein knocked into the lymphoid-specific gene recombinase-activating gene (RAG)-1 (34). Hemopoietic progenitors that expressed RAG and were green fluorescent protein-positive in these mice were shown to be of two types: 1) an LSK progenitor that is IL-7Rα$^-$ and Flt3$^+$ (and therefore LSKflt3$^+$), termed the early lymphoid progenitor (ELP); and 2) a more mature c-KittlowSca-1low cell in which a large fraction express IL-7Rα and are probably CLPs (34). ELPs possess efficient lymphoid potential but inefficient myeloid potential as compared with RAG$^-$ LSK cells (34). Sca-1lowc-KittlowRAG$^+$ progenitors possess only lymphoid potential (34).

A fraction of ELPs possesses D-J IgH rearrangements (34), indicating that RAG is functional at this early stage. ETPs and CLPs also possess D-J IgH rearrangements (31), consistent with an origin from ELPs. This provides one explanation for the presence of D-J IgH rearrangements in mature T cells (49). ELPs therefore a plausible progenitor population for both ETPs and CLPs (Fig. 1B).

Lymphoid specification in ELPs

The expression of RAG by ELPs indicates that lymphoid specification initiates earlier than previously appreciated (34). Expression of RAG is currently the earliest marker for hemopoietic progenitors undergoing lymphoid specification, because it precedes the expression of IL-7Rα (34).

Lymphopoiesis in RAG-deficient mice arrests in T lineage cells at the pre-TCR checkpoint, and in B lineage cells at the pre-BCR checkpoint (13). Early expression of RAG in the BM is therefore unlikely to be required for lymphoid development. Furthermore, when HSCs are directly injected into the thymus, T cell development ensues (7, 33), indicating that early lymphoid specification in the BM is not necessary for development of T cells in the thymus. At this time, it remains mysterious as to why RAG is expressed so early in hemopoietic development. Early expression of RAG may reflect coordinate regulation of RAG with other genes important for early steps of lymphoid development in the BM. Further study of the signals that turn on RAG, as well as identification of coordinately regulated genes, may provide an understanding of this earliest known step in lymphoid specification.

Regulation of RAG is complex, and different locus control elements are responsible for RAG expression in B vs T lineage cells (50, 51). Whether different control elements are responsible for RAG expression in different progenitor populations such as CLPs, ETPs, and ELPs or ELP subsets has not been determined. In this regard, ELPs possess robust T and B lineage potential at the population level (34), but single-cell assays have not yet been performed. Such assays have been performed in neonatal fetal liver (52). The surprising result is that progenitors

**FIGURE 1.** Past (A) and revised (B) models of early steps in lymphoid differentiation in BM (beige) and thymus (blue). A, Lymphoid-restricted CLPs arise from self-renewing and multipotent HSCs via a nonrenewing but multipotent LSKflt3$^+$ intermediate. CLPs were previously suggested to represent a common progenitor of T and B lineages. B, The LSKflt3$^+$ population is heterogeneous. A subset of the LSKflt3$^+$ population termed ELP is lymphoid specified, as evidenced by expression of RAG, whereas other LSKflt3$^+$ progenitors are RAG-. ETPs are proposed to give rise to lymphoid-restricted CLPs that in turn give rise to B cells in the BM. ETPs and therefore T cells do not arise from CLPs, but instead arise from progenitor populations upstream of CLPs. These upstream BM progenitors include ELPs, but may also include some HSCs as well as RAG- LSKflt3$^+$ progenitors (designated by the lighter shading around these progenitors). Hence, the split between T and B lineages occurs earlier than had been previously appreciated.
with only T/B potential cannot be identified. Instead, T lineage-committed cells appear to arise from multipotent progenitors via an intermediate with T and myeloid lineage potential (52). These results suggest ELPs may turn out to be a heterogeneous progenitor pool, with separate progenitors possessing T or B lineage potential. Consistent with such a perspective, recent work (in Thy1.1 mice only) has identified a Thy1.1− BM LSK population that possesses efficient T progenitor activity but inefficient B and myeloid progenitor activity (53).

*From BM to blood*

Rescue of lethally irradiated mice with blood leukocytes was demonstrated over 40 years ago, indicating that HSCs must circulate in blood (54). More recent work has confirmed that para-biased mice (in which the circulation of two mice is anastomosed) exchange HSCs (55). Therefore, it appears that HSCs normally circulate in search of vacant stem cell and perhaps thymic niches (56). Progenitors in blood are present at a frequency too low to allow direct identification, but CFU-cell are present in blood at a frequency of 1:10⁸ WBCs, and day 12 CFU-spleen in blood have been estimated at a frequency of 0.3:10⁸ WBCs (57). Because HSCs are also present in blood, the identity and lineage restriction of additional progenitors is difficult to address using in vivo experiments, because the huge proliferative capacity of HSCs means that their progeny dominate in such experiments (7).

*From blood to thymus*

Given that HSCs are likely to circulate in blood (54, 55), one must ask whether such HSCs also seed the adult thymus. Intravenous transfer of ETPs or other thymic progenitor populations does not result in sustained multilineage reconstitution in recipient mice (3, 31), suggesting that self-renewing HSCs at most constitute a very small fraction of ETPs. In recent work, irradiated mice were inoculated i.v. with lineage-depleted BM, and thymus-settling progenitors were found to be largely restricted to the lymphoid lineage (58). However, it remains possible that thymic colonization is effected by a small number of HSCs and/or multipotent progenitors, if such colonization is accompanied by a rapid loss of self-renewal capability and nonlymphoid lineage potentials (15). More work is needed to determine which progenitors physiologically seed the adult thymus.

CD25 and c-Kit expression have been used to characterize progenitor migration from the blood into the thymus (59). In this work, c-Kit+/CD25− cells were shown to enter in a narrow zone in the perimedullary cortex, and migrate via the cortex toward the subcapsular area (59). There are substantial opportunities for interaction of T lineage progenitors with thymic stromal cells (59, 60) that likely play a key role in guiding early steps of intrathymic T lineage specification.

*Environmental signals*

Cytokines as well as Notch signals are critically required at the earliest stages of T lineage development. Cytokines are thought to function by supporting survival and proliferation (38, 61–63), and a role in cellular trafficking has also been suggested (60), whereas Notch is thought to play a key role in commitment to the T cell lineage (64). However, the stage at which development arrests in the absence of cytokines or Notch signals, and whether development arrests at a single defined stage, are less well characterized than for downstream RAG-dependent checkpoints (20).

*Cytokines*

Stem cell factor and IL-7 are cytokines known to be important in early T cell development (63, 65), and the thymus in c-Kit+/− mice is essentially alymphoid (63). A viable c-Kit+/− variant (Vickid) that survives to adulthood and displays severe blocks in very early T cell development has been reported (66). IL-7 or IL-7R knockouts show a 100-fold reduction in thymic cellularity (38, 65), but all stages of development of αβ lineage cells are present, whereas γδ cells fail to develop. IL-7 plays an important role in differentiation of DN2 thymocytes to T lineage-committed DN3 cells (38). Whether IL-7 plays an additional role in adult thymopoiesis before the DN2 stage remains to be determined. No alterations in thymopoiesis have been reported in Flt3−/− mice (67), but a modest reduction in DN2 but not DN3 or DP thymocytes has been reported in Flt3+−/− mice (48). Hence, Flt3 and Flt3L may play a role at early stages of intrathymic T lineage development, although such a role may be obscured in Flt3−/− and Flt3L−/− mice by compensatory mechanisms.

*Notch*

Four Notch receptors (Notch 1–4), and five Notch transmembrane ligands (Jagged1, Jagged2, and Delta-like 1, 3, and 4) have been described in mice (64). Inducible inactivation of Notch1 results in an early block in intrathymic T lineage differentiation at the DN1 stage, and accumulation of increased numbers of B lineage cells in the thymus (68). Conversely, overexpression of constitutively active Notch1 in hematopoietic progenitors results in development of T lineage cells to the DP stage in the BM, whereas B lineage development is abrogated at an early stage (69). In these reports, myeloid development in BM appears unaffected (68, 69). Additional work has verified and extended these findings (70, 71).

Extrathymic T lineage development in the gut is known to be Notch1 dependent (72). T lineage-committed progenitors have been demonstrated in fetal liver (73) as well as spleen of irradiated mice given BM i.v. (74), but it is unknown whether commitment to the T lineage in these circumstances is Notch dependent. Compellingly, when BM stromal cells expressing a retrovirally encoded Notch ligand Delta-like 1 are cultured with multipotent progenitors from fetal liver, T lineage cells are generated (Ref. 75); also see Ref. 76), whereas the parental cell line supports B lineage development only.

Known target genes for Notch signaling in T lineage cells include hairy/enhancer of split (Hes)1, Hes5, Delta1, and Notch1 itself (64, 77). pTα is a plausible T lineage-specific target of Notch (78). Both Hes1 and Hes5 inhibit B lineage development from multipotent progenitors when retrovirally overexpressed in hematopoietic progenitors (79). Hes1-deficient mice have a partial defect in early T lineage development (80). In fetal mice, early progenitors have been identified both adjacent to as well as within the developing thymus. Examination of these progenitor populations reveals expression of known Notch target genes in progenitors within the thymus, but little expression in progenitors that have not yet entered the fetal thymus (81).

Taken together, these data suggest that one distinguishing (but not unique) feature of the thymic environment may be the
ability to send strong Notch signals into colonizing progenitors, possibly due to expression of key Notch ligands on thymic stromal cells. All other features of the thymic and BM environments appear permissible for both T and B lineage development.

The T vs B decision

The Notch data have been interpreted as strong genetic evidence for a T/B lineage commitment decision that is controlled in a bipotent progenitor by the presence of signals downstream of Notch1 (68, 69). The simplest model based on these data is that progenitors with T and B potential colonize the thymus and subsequently receive Notch1 signals, resulting in loss of B lineage potential. The same progenitors in the BM that avoid Notch1 signals commit to the B lineage. This is an attractive model but may be an oversimplification. The known data are compatible with the possibility that a range of progenitors colonizes the thymus, including multipotent progenitors as well as lineage-restricted progenitors such as ELPs. T lineage progression in all of these progenitors would depend on Notch signaling. Loss of nonlymphoid potentials in multipotent progenitors that colonize the thymus may occur via Notch-dependent mechanisms that operate within the thymic context, by Notch-independent mechanisms, or by both.

T cell origins

Recent work makes it appear unlikely that T cells derive from CLPs as previously proposed (Fig. 1A). ETPs may instead derive from the BM LSK compartment, which is upstream of CLPs (Fig. 1A). The ELP subset of BM LSKs is undergoing a process of lymphoid specification, as evidenced by RAG expression and D-J IgH rearrangements. D-J IgH rearrangements are present in ETPs and CLPs, suggesting that ELPs represent a common progenitor population for T and B cells.

Of the progenitors in the BM LSK compartment, HSCs also circulate in blood. RAG+ ELPs and RAG- LSKflt3+ progenitors may also circulate in blood. Small numbers of these circulating progenitors likely seed the thymus, where they undergo cytokine-mediated expansion overlapping with Notch-mediated progenitor down the T lineage pathway. Therefore, it is suggested that ETPs derive from a heterogeneous pool of progenitors. The earliest steps of lymphoid specification in BM are beginning to be explored, and the mechanisms by which Notch plays such a critical role in T lineage development are beginning to be unraveled. The small number of thymus-colonizing progenitors makes it likely that their identification in normal mice will elude direct approaches in the near future. Where and how early progenitors integrate signals from their environment, resulting in loss of alternative lineage potentials and progression down the T lineage pathway, all remain to be elucidated. The challenge is to develop techniques that allow more sophisticated and quantitative inquiry into the earliest steps of T lineage specification and subsequent commitment.

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