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Minireview

Closer to the Source: Notch and the Nature of Thymus-Settling Cells

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Questions regarding T cell development have recently received much attention, but the earliest intrathymic differentiation steps in adult mice have remained controversial. Three new papers together show that for at least some thymus-settling precursors, the loss of B lineage potential occurs in the thymus, and Notch acts on multipotent progenitors early after thymic entry.

The development of mature blood cell types from hematopoietic stem cells occurs via a series of intermediate stages with progressive loss of alternate lineage potentials. Although much is known about the discrete differentiation steps taken by developing thymocytes and the importance of Notch signaling in this process, the initial intrathymic events remain elusive. The earliest population of intrathymic T cell progenitors is a subset of the DN1 (Lineage-marker-negative CD44$^+$ CD25$^-$) population that expresses high levels of the cytokine receptor c-Kit (Shortman and Wu, 1996; Allman et al., 2003). These cells are termed early T lineage progenitors (ETPs), and although they comprise less than 0.01% of the adult thymus, emerging evidence indicates that they are functionally and phenotypically heterogeneous. Understanding this heterogeneity should provide answers to several controversial questions in T cell development, which include the identity of thymus-settling progenitors, whether loss of non-T lineage potentials occurs in progenitors prior to thymic entry, and the precise role of Notch signaling at these early developmental stages.

Previous studies indicated that T/NK-restricted progenitors are present in the fetal circulation, even in athymic *nu/nu* mice (Rodewald et al., 1994; Carlyle and Zuniga-Pflucker, 1998). Further, the earliest thymus-colonizing cells in day 11–12 fetal mice are restricted to the T, NK, and DC lineages, and lack B potential (Harman et al., 2005; Masuda et al., 2005). Hence, loss of B lineage potential and restriction to the T/NK/DC lineages occurs prethymically in fetal mice. Whether this is true in adult mice is unknown. Cells in the most immature thymocyte populations of adult mice possess non-T lineage potentials, including myeloid, B, NK, and DC potential (Shortman and Wu, 1996), but it is uncertain whether these alternate potentials derive from multipotent T lineage progenitors or from separate non-T progenitor populations. The presence of B potential in the ETP population has been particularly controversial. Recent work suggests that the ETP population in thymus of adult mice contains progenitors with B lineage potential (Allman et al., 2003). Upstream of the thymus, several multipotent progenitor populations possessing T and B potential have been identified in the bone marrow, including cells with a Lineage-marker-negative Sca-1$^+$ c-Kit^{hi} (LSK) phenotype, c-Kit^{lo} lymphoid-restricted common lymphoid progenitors (CLP), and B220$^+$ c-Kit$^-$ progenitors (CLP-2) (Kondo et al., 1997; Adolfsson et al., 2001; Martin et al., 2003). The LSK population has been isolated from blood of adult mice and thus has access to the thymus (Schwarz and Bhandoola, 2004), whereas the CLP-2 population has been shown to efficiently settle in the thymus after intravenous delivery (Martin et al., 2003). These data are consistent with thymic colonization by multipotent progenitors that contain at least T and B potential, and perhaps additional lineage potentials (hereafter referred to as T/B multipotent progenitors). However, other recent data have questioned whether T/B multipotent progenitors can be found in the thymus, instead suggesting that only non-ETP populations within the DN1 subset contain B potential (Porritt et al., 2004; Balciunaite et al., 2005). Thus, it is unclear if the adult mouse thymus is settled by cells possessing B and T progenitor potential or by a mixture of T potent and B potent cells.

Regardless of the exact properties of adult thymus-settling cells, the importance of Notch signaling in promoting T cell development is clear. Previous work has shown that conditional ablation of Notch1 signals leads to a loss of T cell production and to the development of B cells in the thymus (Radtke et al., 1999). Conversely, overexpression of activated Notch1 results in a loss of B cell production and the appearance of CD4$^+$CD8$^+$ developing T cells in the bone marrow (Pui et al., 1999). These results are consistent with two possible scenarios: one in which a multipotent precursor chooses the T or B lineage depending on the presence or absence of Notch signals, and another in which the presence or absence of Notch signals selects for the outgrowth of T- or B-committed progenitors.

Deciphering the B lineage potential of thymus-settling T progenitors is thus key to understanding how and where Notch signals induce T lineage development. Recently, three papers have isolated the most immature subset of ETPs in the adult thymus and tested their lineage potentials. Further, the role of Notch signaling in these early populations has been examined (Benz and Bleul, 2005; Sambandam et al., 2005; Tan et al., 2005). The following paragraphs discuss these recent data, focusing first on early progenitors and then on their requirement for Notch.

To investigate the nature of early intrathymic precursors, Tan et al. used transgenic mice expressing Lunatic Fringe (L-Fng) under the control of the thymus-specific proximal *lck* promoter (Tan et al., 2005). L-Fng is a Golgi-localized glycosyltransferase that modifies the extracellular domains of Notch receptors and alters the ability of Notch1 to interact with its Delta-like ligand. It

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was previously shown that the L-Fng transgene decreased the ability of precursors to activate Notch1 in a non-cell-intrinsic manner, resulting in impaired T cell development and accumulation of intrathymic B cells (Koch et al., 2001). Thus, the L-Fng transgenic thymus likely represents an environment in which progenitors receive weakened Notch1 signals, although the mechanism by which this occurs requires further clarification. Tan et al. now report that primitive (Lineage-marker-negative) bone marrow progenitors are affected by this altered environment but more mature committed progenitors are not. Lineage-marker-negative bone marrow progenitors gave rise to more B cells after injection into the L-Fng thymus compared to the wild-type thymus. In contrast, intrathymic maturation of committed bone marrow pro-B cells was similar after injection into L-Fng and wild-type thymi. These data indicate that the increased numbers of B cells developing in unmanipulated L-Fng transgenic thymi do not derive from committed B cell progenitors. Instead, these B cells likely derive from more primitive multipotent progenitors that make the B versus T decision after entry into the L-Fng thymus.

Benz and Bleul examined whether multipotent progenitors with T and B potential could be identified within the adult thymus (Benz and Bleul, 2005). They began by fractionating ETPs to isolate the most immature subset and enrich for putative thymus-settling cells. Using a heterozygous CCR9-EGFP knockin reporter mouse, they found that ETPs could be divided into CCR9-EGFP^{hi} and CCR9-EGFP^{lo} subsets. Further, the earliest thymus-colonizing cells in the E11.5 fetus were CCR9-EGFP^{hi}, and a portion of adult bone marrow LSK cells, CLPs, and c-Kit^{+} circulating progenitors also expressed the reporter. From these data, they reasoned that CCR9-EGFP^{hi} ETPs may be an immature subset of ETPs, and they confirmed this idea by examining the kinetics of differentiation of each ETP subset in fetal thymic organ cultures. CCR9-EGFP^{hi} ETPs were delayed in their differentiation to the DN3 stage compared to CCR9-EGFP^{lo} ETPs, whereas CCR9-EGFP^{hi} circulating progenitors had slower kinetics than either ETP subset, but similar to bone marrow progenitors.

Sambandam et al. fractionated ETPs based on expression of Flt3 (CD135), a cytokine receptor that is expressed on the nonrenewing fraction of bone marrow LSK cells, CLPs, and CLP-2 cells, but on only 5%–20% of the thymic ETP population. In this work, the same strategy of comparing differentiation kinetics was used to show that Flt3^{+} ETPs are less mature than their Flt3^{lo} counterparts. Moreover, Flt3^{+} ETPs had greater expansion potential than Flt3^{lo} ETPs upon intrathymic injection.

Both groups then assessed the B lineage potential of each ETP subset. Benz and Bleul used an in vitro system of coculture with OP9 stromal cells to induce B cell differentiation. B potential was found in the CCR9-EGFP^{hi} ETP subset. Others have not been able to detect the B lineage potential of ETPs using OP9 coculture, possibly reflecting variation in the stromal cell lines used (Porritt et al., 2004; Balciunaite et al., 2005; Sambandam et al., 2005; Tan et al., 2005). Sambandam et al. used in vivo assays to show that B lineage potential was restricted to the Flt3^{+} ETP subset. Flt3 expression was functionally relevant in that Flt3 ligand was required for B cell development from ETPs. However, Flt3 expression was not sufficient to confer B potential on ETPs, since limiting dilution analyses indicated that only a fraction of Flt3^{+} ETPs contained B potential. These data suggest a scenario whereby progenitors entering the thymus express Flt3, are CCR9-EGFP^{hi}, and contain B potential. As they expand and differentiate, they lose B potential and expression of CCR9-EGFP and Flt3, generating a population of downstream ETPs that lacks B progenitor potential (Figure 1).

This model requires that incoming progenitors contain both T and B potential at the single-cell level, rather than at the population level. Benz and Bleul showed that single progenitors with T and B potential are present in the thymus, using a clever strategy of mixing OP9 stromal cells with OP9 cells expressing the Notch ligand Delta-like-1 (OP9-DL1), creating a culture system that supports B and T cell development from single progenitors in the same well. Although colony generation by single CCR9-EGFP^{hi} cells isolated from the thymus was less efficient in this system than on OP9-DL1 cultures (one-tenth of wells plated gave rise to a colony), about 20% of colonies contained CD19^{+} B cells and Thy1^{+} T cells.

These data imply that there are functional differences between early ETPs and late ETPs. Two of the recent reports also examined the role of Notch signals on thymic populations and found that they are required for the generation of both early and late ETPs. Unmanipulated L-Fng transgenic thymi have reduced numbers of ETPs and DN2 (Lin^{-} CD44^{+} CD25^{+}) thymocytes, suggesting that Notch signals drive the expansion of these populations. Sambandam et al. used retroviral transduction of hematopoietic stem cells with a dominant-negative Mastermind-like-1 (DNMAML1) construct that ablates signaling from all four Notch receptors. Almost no DNMAML1^{+} ETPs were generated, but circulating DNMAML1^{+} LSK cells were intact, indicating that Notch signals are required upstream of ETPs but within the thymus (Figure 1). Expression analysis in wild-type thymi revealed that both the Flt3^{+} and the Flt3^{lo} ETP subsets expressed Notch target genes, although at higher levels in the Flt3^{lo} subset. Flt3 expression was rapidly downregulated from the cell surface when high levels of Notch signaling were induced in ETPs, consistent with the idea that Notch signals may drive the transition between Flt3^{+} ETPs and Flt3^{lo} ETPs. In addition to potentially driving the transition from early to late ETPs, the requirement for Notch signals in early thymocytes may reflect a prosurvival function of Notch. Consistent with this idea, Tan et al. show that in *Notch1*$^{+/+}$*:Notch1*$^{+/-}$ mixed bone marrow chimeras, the ability of *Notch1*$^{+/-}$ cells to compete declines with each progressive developmental step in the thymus (Tan et al., 2005).

Although Notch signals are important for driving ETP generation and T commitment, they are not completely incompatible with B lineage development. The L-Fng transgenic system has an intermediate phenotype, where weak Notch signals in the L-Fng thymus are insufficient for suppression of B lineage development from multipotent progenitors and insufficient for normal ETP/DN2 generation, although both processes occur to

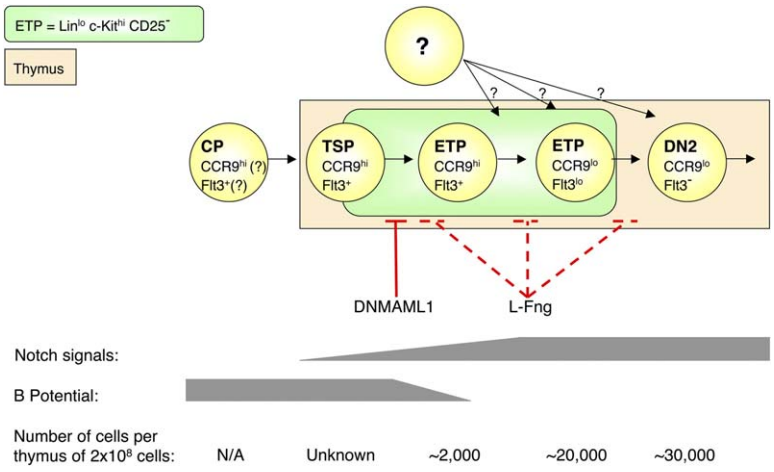

Figure 1. Model of Early T Cell Development

Circulating progenitors (CP) that include LSK (Lineage^{lo} Sca-1^{+} c-Kit^{hi}) progenitors with multilineage potential enter the thymus as thymus-settling progenitors (TSP). Weak or short-duration Notch signals cause the loss of B potential in these precursors. It is also possible that progenitors lacking B potential enter the thymus at downstream points. Increased intensity or duration of Notch signals induce the TSP population to expand and lose the ability to produce B cells, although some of these progenitors may still express high surface levels of CCR9-EGFP and Flt3. Further Notch signaling induces a large expansion of ETPs, which downregulate expression of CCR9-EGFP and Flt3. These Flt3^{lo} CCR9-EGFP^{lo} cells represent the vast majority of the ETP population. Notch signals are also required for the transition to the DN2 stage and subsequent stages of early thymocyte development. Abbreviations: CP, circulating progenitor; TSP, thymus-settling progenitor; ETP, early T lineage progenitor; DN2, CD4/CD8 double-negative 2; DNMAML1, dominant-negative Mastermind-like-1; L-Fng, Lunatic Fringe transgene. Question marks denote speculative aspects of this model. Solid red lines denote complete inhibition and dashed red lines denote partial inhibition.

some degree. This finding is consistent with in vitro data from other laboratories. Schmitt et al. titrated a Notch signaling inhibitor into cocultures of fetal liver hematopoietic progenitors and OP9-DL1 stroma (Schmitt et al., 2004). Their data showed that as Notch signals became stronger (decreasing concentration of inhibitor), B cell development was lost and T cell development increased, but intermediate concentrations of inhibitor were permissive for B and T development. The Bernstein group showed that providing low-density plate bound Notch ligand (DL1) encouraged both B and T development (Dallas et al., 2005). Unfortunately, it is not possible to compare levels of Notch signals across these different experimental systems.

From these recent data, it is clear that Notch signals are critical at very early steps of intrathymic differentiation and that Notch signals act on T/B multipotent precursors within the thymus. However, loss of B potential may not occur immediately after exposure of progenitors to Notch signals. This idea of lagging commitment has been recently studied in detail using OP9 and OP9-DL1 cultures (Taghon et al., 2005). Fetal liver progenitors that had received Notch signals for a week and then were switched to OP9 coculture still retained limited B potential. These data indicate that the duration of Notch signaling may be a critical factor in the loss of alternative lineage potentials. In this regard, much remains to be learned about where and how Notch signals are delivered in the thymus. As thymocytes differentiate, they migrate through the thymus, presumably through changing microenvironments. It is not known if these microenvironments contain variable levels or types of Notch ligand.

These recent papers advance several aspects of our knowledge of early T cell development; however, many uncertainties remain. Although Benz and Bleul have demonstrated the presence of T/B multipotent precursors in the thymus, such data do not preclude the possibility that progenitors lacking B lineage potential also enter the thymus. Indeed, assessment of thymus-homing cells 24 hr after intravenous injection of bone marrow showed that c-Kit^{hi} progenitors settling in the thymus contained T and NK potential, but failed to give rise to B lineage cells in vitro (Porritt et al., 2004). It is possible that loss of B potential in thymus-settling progenitors occurred rapidly, within the 24 hr between administration of bone marrow progenitors and analysis of thymic-settling cells. However, a more interesting possibility is that T/NK-restricted cells enter the thymus downstream of the Flt3^{+} CCR9-EGFP^{hi} ETP subset (Figure 1). Such T/NK-restricted progenitors have not yet been identified in bone marrow or blood of adult mice, but substantial heterogeneity exists within bone marrow and blood LSK progenitor populations previously characterized as multipotent (Adolfsson et al., 2001; Igarashi et al., 2002; Perry et al., 2003; Schwarz and Bhandoola, 2004). Investigation using single-cell assays of progenitor potential may reveal T/NK-restricted progenitors within various multipotent progenitor populations in bone marrow of adult mice. However, the present demonstration of T/B multipotent progenitors within the thymus does indicate that prethymically T/NK restricted progenitors are unlikely to represent the only pathway of T cell development in adult mice. Instead, at least some T/B multipotent progenitors must settle in the adult thymus.

The identity of multipotent thymus-settling progenitors will likely be further clarified by increasingly sophisticated analyses of early thymocyte progenitor populations at the single-cell level. In this regard, a diverse range of bone marrow progenitors with multilineage potential is able to undertake T cell development when placed in the thymus, including multipotent LSK cells as well as lymphoid-restricted CLP and CLP-2 cells (Adolfsson et al., 2001; Allman et al., 2003; Martin et al., 2003). The ability of the thymus to impose the T lineage fate upon a diverse assortment of progenitors provides a potential insight into why the site of T cell development is anatomically isolated from the developmental site of all other blood cells. Such isolation may be necessitated by the ability of Notch signals to instruct a broad range of bone marrow progenitors down

the T cell developmental pathway. Hence, it is tempting to speculate that physiological Notch signals that drive intrathymic T cell development would override development of other lineages if they occurred in the bone marrow. It follows that the ability to traffic to and settle in the thymus may separate physiologically relevant T progenitors from other bone marrow progenitors with T lineage potential.

Recent work has begun to localize fate choices and the delivery of Notch signals to specific early thymic progenitor populations. This information is required to further study the molecular underpinnings of T cell development, and as the field moves closer to the earliest events occurring in the bone marrow, blood, and the entry gate of the thymus, additional work will begin to focus on the necessary steps that precede commitment. Translating observations made in mice to humans is another tremendous challenge for the future. So although recent work has given greater definition to the thymic development model of cellular differentiation, we are left with an area ripe for research.

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