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Molecular and Cellular Basis of T cell Lineage Commitment

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Abstract

The thymus forms as an alymphoid thymic primordium with T cell differentiation requiring the seeding of this anlage. This review will focus on the characteristics of the hematopoietic progenitors which colonize the thymus and their subsequent commitment/ differentiation, both in mice and man. Within the thymus, the interplay between Notch1 and IL-7 signals is crucial for the orchestration of T cell development, but the precise requirements for these factors in murine and human thympoeisis are not synonymous. Recent advances in our understanding of the mechanisms regulating precursor entry and their maintenance in the thymus will also be presented.

Keywords

Thymus; early thymus precursor; human; intrathymic transfer; Notch; IL-7

1. Introduction

During embryogenesis, the generation of the prospective thymic epithelium in the third pharyngeal pouch endoderm and the vascularization of the resulting thymus anlagen is dependent upon the activity of the Foxn1 transcription factor [1,2]. The expansion and maturation of this embryonic thymic epithelium is regulated by neural crest-derived mesenchyme [3]. Immigration of the first T precursors occurs only upon proper fabrication of this thymic anlage; their differentiation within the thymus results in the establishment and maintenance of a mature peripheral T cell pool with a wide self MHC-restricted non-autoimmune TCR repertoire.

The crucial role of the thymus in T cell generation was demonstrated by seminal studies conducted by the Australian scientist Francis Albert Pierre Miller [4]. The thymus was already acknowledged by the Greeks and its name may be derived from the Greek word “thymos” which denoted life force or soul. Despite this rich history and even though Miller’s pioneering work on the thymus was performed in 1961, it was long thought that the thymus was obsolete with its function having become redundant during the course of evolution. Indeed, in 1963, Sir Peter Medawar, recipient of the 1960 Nobel prize for his work on graft rejection and the acquisition of immune tolerance, stated; “We shall come to

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regard the presence of lymphocytes in the thymus as an evolutionary accident of no very great significance" (Medawar, 1963) [5]. During the past half century, remarkable progress has been made, and it is no longer possible to study T cell development without recognizing the critical importance of the thymus in this process.

At what point in its development does a hematopoietic precursor cell become destined to pursue a T lineage fate? The phenotype and characteristics of hematopoietic progenitors have been extensively studied during the past decade, resulting in the identification of multiple bone marrow and peripheral blood subsets that are capable of differentiating into T lineage cells in the thymus. Despite enormous advances in the field, there are still considerable issues that remain unresolved. The phenotypes of hematopoietic precursor cells in mice and humans differ significantly, adding yet another layer of complexity to research aimed at understanding the regulation of T lineage commitment. Further complexities are the result of recent findings showing that the destinies of apparently identical hematopoietic precursors differ based on the milieu in which they are localized; i.e. bone marrow vs. thymus. Here, we review recent findings arising from studies of hematopoietic precursor phenotypes in mice and humans, regulation of thymocyte differentiation and thymic importation of hematopoietic precursors.

2. Thymocyte Differentiation: Which cells are competent to pursue a T lineage fate?

In the murine thymus, the immature early T-lineage progenitor (ETP) has high T and only limited B potential [6]. These thymocyte precursors, characterized as lin–CD44+CD25–Sca-1+CD117c-kit+ (LSK) with only low CD127 (IL-7Rα) expression, are derived from hematopoietic stem cells (HSC) in the bone marrow. However, HSC themselves do not appear capable of seeding the thymus under physiological conditions. The most immature progenitors in the thymus do not harbor stem cell potential as shown by their inability to support long-term thymopoiesis; intrathymic transfer of progenitors already present in the thymus results in only a single wave of thymopoiesis [7-9]. Based on these experimental data, it has been concluded that long-term thymocyte differentiation requires an on-going migration of donor progenitors from the BM to the thymus. However, the precise nature of the precursor or precursors responsible for seeding the thymus under physiological conditions has not yet been well elucidated [10].

Within the BM, two subsets of hematopoietic precursors cells harbor the potential to generate thymocytes; multipotent progenitors (MPP) as well as the more committed common lymphoid progenitors (CLP). There is still some controversy concerning which cell subsets are the immediate precursors to thymocytes. MPP, retaining both myeloid and lymphoid potential, are generated from HSC and are characterized by the upregulation of the Fms-like tyrosine kinase receptor 3 (Flt3/CD135; resulting in LSK/CD135+ cells) [11]. Subsets of MPP, characterized by expression of molecules such as RAG-1 [12] and P-selectin [13] can also give rise to thymic precursors. This is also the case for the more committed CLP that, in contrast to MPP subsets, lack myeloid potential [14]. At least two major CLP subsets have been identified; CLP-1 (lin–Sca-1+CD117+/loCD127+CD135+) [15] and CLP-2 (lin–Sca-1+CD117–CD127+CD135+B220+ (CLP-2) [16], with the latter thought to be the most differentiated population with T cell potential before commitment to the B cell lineage [16]. The recent finding that immature thymocytes retain myeloid potential [17,18] supports a model wherein MPP directly enter the thymus. Furthermore, MPP have been shown by numerous laboratories to be responsible for a more extended thymopoiesis than CLP [12,13,19-22]. Nevertheless, recent reports have raised new questions as they implicated CLPs as the only immediate source of ETPs, with MPPs allowing an extended thymopoiesis because of their renewal in the BM before further

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differentiation into CLPs [23,24]. Thus, further experiments are necessary to elucidate the stages directly linking HSC in the bone marrow to a T cell-committed progenitor in the thymus.

3. Of mice and men: Phenotypes of human thymocyte precursors and subsets

In humans, the phenotype of the primitive hematopoietic precursor capable of giving rise to differentiated lineages is distinct from that detected in mice. In contrast to the lineage-Sca-1+c-Kit+ (LSK) progenitors found in mice, human haematopoietic precursors harbor the CD34 marker. The CD34+ cells that seed the thymus can differentiate into multiple lineages, giving rise not only to T, B and NK cells of the lymphoid compartment, but also to myeloid cells such as DCs and erythrocytes [25,26]. T cell commitment thus occurs after entry of precursors into the thymus. It is generally agreed that human HSC differentiate into either myeloid or lymphoid lineage restricted precursors (CLP). Notably, a CD34+ subset with a CD10+CD24-phenotype has recently been shown to possess the characteristics of a CLP, retaining the ability to generate B, T and NK lineages but having lost myeloid potential [27]. These progenitors are present in the cord blood and in the BM but can also be found in the blood throughout life. As such, they may constitute the proximate thymic precursors in man.

In the human thymus, precursors with the $CD10^{+}CD7^{-}$ phenotype express genes that are common to both the T and B cell differentiation pathways but they appear to be committed to T cell differentiation [27]. Thus, in contrast to early murine thymocyte progenitors that have been reported to maintain myeloid differentiation potential (as discussed above, see [17,18]), this does not appear to be the case for human precursors. In humans, thymocyte differentiation of early precursors proceeds by the stepwise acquisition of CD7 followed by CD1a, a member of the CD1 family of MHC-like glycoproteins. $CD34^{+}CD1a^{+}$ pre T cells, in contrast to their upstream $CD34^{+}CD1a^{-}$ precursors, are rearranged at the TCRβ, γ and δ loci [28], and are unable to develop into non-T lineages (see [25,26,29].

The phenotype of human thymocytes undergoing β-selection has not been completely explicited, with several lines of evidence indicating that β-selection can occur in distinct populations of cells differing in their CD4/CD8 expression profiles. A very low frequency of productive TCRβ V-DJ rearrangements has been detected in the double negative (DN) $CD34^{+}CD1a^{+}$ population, suggesting that a few cells may undergo β-selection before the expression of either CD4 or CD8 [30]. Directly downstream of the $CD34^{+}CD1a^{+}$ phase, cells express CD4, but not yet CD8, and they are referred to as $CD4^{+}$intermediate single positive (ISP) cells. Intracytoplasmic (ic) $TCR\beta^{-}$ populations have been found in $CD4^{+}$ISP cells and in the $CD4^{+}CD8\alpha^{+}\beta^{-}$ early double positive thymocytes, thus demonstrating that not all populations beyond the $CD34^{+}CD1a^{+}$ stage are post β-selection cells [28,31]. Blom *et al* reported that β-selection can occur at the $CD4^{+}$ISP stage, based on their detection of TCRβ V-D-J recombination in a low level of $CD4^{+}$ISP cells and cytoplasmic expression of TCRβ protein in 5% of $CD4^{+}$ISP cells [28]. The group of Toribio, however, located the β-selection checkpoint at a later stage, namely, in $CD4^{+}CD8\alpha^{+}CD8\beta^{+}$ DP cells that express a complex of TCRβ and pTα on the cell membrane [31]. On the basis of these studies, it appears that expression of a TCRβ protein and subsequent β-selection occurs within a certain developmental window and is not tightly coupled to regulation of CD4, CD8α and CD8β expression. In contrast, it appears that β-selection in mice occurs at a precise stage of differentiation, within DN3 ($CD25^{+}CD44^{-}$) thymocytes [32]. The timing of the rearrangement has a functional consequence; In mice, it is those DN4 cells that have undergone a productive TCRβ rearrangement that show a proliferative burst [33-35], whereas in humans, we have found that it is the CD4ISPs and $CD4^{hi}$DP immature thymocytes (characterized as $TCR\alpha\beta^{lo}$) thymocytes that are proliferating and express high

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levels of the ubiquitous glucose transporter Glut1 [36]. In mice, it is known that β-selection and subsequent proliferation correlate with a reversal in the polarity of thymocyte migration from the subcapsullar zone back into the cortex [37,38], and this requires an adherence to the extracellular matrix [39]. In humans, the data indicate that it is the $Glut1^{+}CD4^{hi}TCR\alpha\beta^{lo}$ DP population that is associated with this critical phase of migration within the thymus [36]. It will therefore be important to study the functionality of the corresponding murine and human thymocyte populations based on their differentiation stage rather than based solely on the presence of cell surface markers.

4. Of mice and men: Notch and IL-7

Within the thymic microenvironment, the interplay between the Notch1 transcription factor and the IL-7 cytokine are crucial in supporting and orchestrating T cell development. However, the precise requirements for these factors in murine and human thympoeisis, as monitored by differentiation and proliferation, are not synonymous. The role of Notch1 in commitment to the T cell lineage was elegantly demonstrated by two ground-breaking studies showing that a constitutively active Notch1 results in αβ T cell development in the bone marrow while conversely, the conditional ablation of Notch1 expression causes an early block in T cell development with the development of phenotypically normal immature B cells in the thymus [40,41]. Indeed ETP, in contrast with BM HSC (LSK), are dependent on Notch signals for their generation and/or maintenance [42,43]. The transduction of Notch signals in the murine thymus has recently been shown to be mediated by the delta-like 4 (DL4) ligand; interaction of thymic progenitors with DL4-expressing thymic epithelial cells (TEC) suppresses B lineage potential and is an essential and nonredundant step in T cell lineage commitment [44,45].

While the transduction of Notch signals is crucial for both human and murine T lineage commitment, the role of this transcription factor in further thymocyte differentiation appears to differ significantly between humans and mice. In marked discrepancy with murine thymocyte differentiation, high Notch activation appears to inhibit human TCR-αβ differentiation, skewing progenitors toward the γδ lineage. In order to rescue human αβ lineage differentiation, the level of Notch activation must be reduced [46]. This is particularly intriguing as in mice, it has been reported that high Notch levels at the DN3 stage are required for TCR-αβ differentiation whereas TCR-γδ-expressing cells can survive and expand in the absence of Notch [47]. Moreover, while the requirement for Notch at the β-selection checkpoint is absolute in mice [48], it is not essential for human thymopoiesis. In the absence of Notch, human thymocyte precursors, including immature CD34+ cells can efficiently proceed to a mature DP stage, albeit with a significantly reduced proliferation [49,50]. The differentiation of these human thymocytes is dependent on IL-7, contrasting with the murine system wherein IL-7 signaling is not required at this stage [48,51,52].

The significant disparities in the requirements for Notch in murine and human thymopoiesis clearly warrant further investigation. One bias that may account for some of these discrepancies is related to the experimental system; while murine progenitors are studied in both *in vitro* and *in vivo* systems, it is much more difficult to use the latter for human studies. Nevertheless, it should be noted that some of the Notch-related differences described above are based on *in vitro* studies, with culture of both murine and human thymic precursors on OP9 stroma expressing the Notch1 ligand DL1 [53]. It will be of interest to determine whether some of the dissimilarities discussed above reflect differences in the natural expression profile of the Notch ligand DL4, as regards its level and distribution (ie. cortex and medulla) in the murine versus human thymus.

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The differential responsiveness of human and murine thymocytes to IL-7 likely reflects distinct expression profiles of the cytokine receptor itself (IL-7Rα) as well as the stage at which β-selection occurs (see section 3 above). Of note, the earliest human thymocyte progenitors, but not their mouse counterpart [6,54], have been reported to express the IL-7Rα chain [25,27]. Moreover, while IL-7Rα is not expressed on murine DP thymocytes [55,56], it is easily detectable on the human DP subset, at both the protein [36,57-59] and mRNA levels (our unpublished observations). In agreement with these data, murine DP thymocytes fail to phosphorylate Stat5 or upregulate Bcl-2 [56,60] following IL-7 stimulation, while human DP thymocytes show an induction of Stat5 phosphorylation in response to this cytokine (Louise Swainson and N.T., unpublished observations). The outcome of IL-7 signaling in human ETP as well as DP thymocytes is unclear and will require further study. This is in an important issue because, at least in mice, an IL-7-insensitive stage between the β-selection and positive selection checkpoints is considered to be essential for the death of unsignaled thymocytes (death by neglect). In this regard, it is notable that while IL-7 stimulates Stat5 phosphorylation in human DP cells, a “full” IL-7 signaling response is not detected: Whereas IL-7 stimulation of human SP thymocytes results in activation of the PI3K pathway with cell surface upregulation of Glut1, this response is not detected in human DP cells ([61] and L.S., unpublished observations). Thus, these results suggest that even in the presence of an IL-7R complex, IL-7-mediated signals are dampened at the DP stage of human thymocyte differentiation.

5. Getting In: Regulating access of hematopoietic precursors to the thymus

Irrespective of the precise nature of the cell(s) gaining access to the thymus (see section 2 above), it is important to understand the processes regulating the migration and colonization of these hematopoietic progenitors. The murine thymus is apparently not continually receptive to the import of hematopoietic progenitors, alternating between refractory and responsive periods [62]. During refractory periods, donor progenitor cells differentiate into T cells only if they are directly injected into the thymus [62,63]. The gated entry of thymocyte precursors has been reported to be regulated by the size of the immature pool, and particularly by the number of DN3 cells [63].

The emergence of the thymus occurred approximately 470 million years ago in fish with jaws (known as gnathostomes) with the evolution of the adaptive immune system. Jawless vertebrates, with the living representatives being lamprey and hagfish, lack a thymus but they appear to have evolved a B cell-like system for adaptive humoral responses [64]. In contrast, in jawed vertebrates, including the zebrafish, a thymic organ is present. The thymus forms as an alymphoid thymic primordium with the entry of hematopoietic progenitors clearly driven by chemoattractants. In zebrafish, recent technological advances have allowed the journey of individual hematopoietic progenitors to be visualized; CD41+ hematopoietic precursors traced for over 12 hours traverse through the mesenchyne and undergo directional migration to the nascent thymus from as far as 350 μm [65].

The data presented above clearly point towards the primordial role of chemoattractants in regulating progenitor entry into the thymus. In an exciting recent study, Boehm and colleagues analyzed the genetic phylogeny of species spanning the 500 million years of thymus evolution and found that orthologues for CCR9 and its ligand CCL25 receptor first appeared in cartilaginous fishes, coinciding with the evolutionary emergence of the thymus. Intriguingly, the emergence of the CXCR4/CXCL12 pair antedates this period [66]. Using zebrafish as a model, Bajoghli et al. showed that the homing of hematopoietic precursors to the thymus is completely dependent on the cooperation between this new chemokine/chemokine receptor pair and the evolutionary more ancient CXCR4/CCL12 pair (already present in the thymus-lacking lamprey) [66].

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In mice, there is a high level of redundancy in the ability of chemokine receptor/ligand pairs to regulate the entry of hematopoietic progenitors into the thymus. Knockout mice models have highlighted the roles of signals mediated by CCL19-CCL21/CCR7, CCL25/CCR9 and P-selectin/PSGL-1 interactions in the seeding of the thymus by extra-thymic hematopoietic precursors but abrogation of any one of these receptor-ligand interactions only modestly affects thymus colonization [20,21,38,67-69]. As the entry of thymic progenitors is “gated” rather than continuous, it is interesting to note that the expression of ligands such as CCL25 and P-selectin on thymic epithelium have recently shown to be regulated as a function of thymic availability for precursor importation, with high levels corresponding to increased receptivity [70].

Previous important work suggested that the signals regulating progenitor cell entry into the thymus differ in the fetal (pre-vascularisation) and postnatal periods: In the absence of CCR7/CCR9, there is a severely impaired colonization of the thymus in the fetus but thymic cellularity is restored by postnatal day 1 [71]. These data raised significant questions as to the differences in the mechanisms regulating the colonization of the thymus pre- and post-thymus vascularisation. Two recent studies have shed light on this issue demonstrating that even during the post-natal period, the absence of CCR7/CCR9 severely impacts on thymic settling with a >2-log reduction in early thymic progenitors. However, postnatally, a compensatory expansion of early thymic progenitors, or possibly of more committed progenitors, results in the recovery of thymic cellularity by the DP stage of differentiation [72,73]. The ensemble of these studies shows that a coordinated action of the receptors on thymic precursors (including CCR7, CCR9 and PSGL-1) with their respective ligands on thymic epithelium regulates precursor entry. However, even when these migratory signals are severely compromised, a dynamic and robust proliferation of early thymic progenitors may compensate for the entry defect.

6. Forcing entry of progenitors into the thymus: Long-term thymopoiesis

The concept that the early thymic precursors (characterized as CD44+CD25–Sca-1+CD117(c-kit)+) are the source for all subsequent thymocyte differentiation is widely accepted, but this has not been formally proven. It is clear that the thymic environment can modulate the function and phenotype of precursors. While BM HSC and ETP share many phenotypic markers, only the latter are dependent on Notch signals for their generation and maintenance [42,43]. Moreover, exposure of bone marrow-derived CLP to a Notch ligand or to the thymic environment itself results in the rapid acquisition of a more mature phenotype [74] [21]. Based on these data, it is not surprising that the transfer of wild type bone marrow progenitors into the thymus of an immunodeficient mouse results in a more rapid and diverse T cell reconstitution than that observed following the intravenous transfer of these cells [75].

As ongoing thymocyte differentiation requires a sustained periodic colonization of the thymus by precursors from the bone marrow [7-9], the current thinking is that stem cells with self-renewing capacity cannot take up residence in the thymus. Indeed, the absence of hematopoietic stem cells in the thymus has been one of the holy grails in the field of thymocyte differentiation. It is likely that the absence of “true” HSC in the thymus is not due to the modulation of their phenotype within the thymus environment itself (as described above), but rather to an absence of chemokine receptors (see section 5 above) fostering their migration into the thymus. Previous conclusions that the thymus cannot sustain the maintenance of a progenitor cell with self-renewal capacity, even after its forced entry into the thymus, were based on experiments wherein thymic or BM precursors were injected into the thymus in a non-competitive setting, and in most cases, the thymus was perturbed by irradiation prior to transplantation [6-9,13,22,76]. Even in the absence of irradiation,

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intrathymically injected BM progenitors do not sustain long-term thymopoiesis in WT mice where there is a normal importation of precursors [21]. However, in the context of an immunodeficient mice (ZAP-70-deficient) where there is an available precursor niche, the intrathymic administration of hematopoietic progenitors promotes and sustains long-term thymopoiesis [77].

Long-term donor thymopoiesis in the intrathymically transplanted mice described above was not due to the continued recruitment of progenitors from the BM and was specifically due to the intrathymic presence of BM precursors that do not naturally migrate to the thymus. Specifically, WT precursors that had naturally entered the thymus were not able to reproduce the effect of WT BM precursors but the precise phenotype/characteristics of the cell capable of maintaining long-term thymopoiesis has yet to be determined. Irrespective of the phenotype, the data indicate that it is the forced entry of a WT BM precursor into a thymic niche, not accessible to circulating host precursors, that allows for long-term thymocyte development. We hypothesized that in the ZAP-70-deficient mouse, WT progenitors are able to fill an available stromal niche, thereby sustaining long-term thymopoiesis. Indeed, in the absence of ZAP-70, the numbers of c-Kit+ ETP were significantly lower than that detected in WT mice. Following the intrathymic transfer of WT HSC, the numbers of ETPs increased dramatically. Notably, the increase in this early thymic precursor pool following IT transplantation was associated with a change in the thymic architecture [78], with a striking formation of demarcated medullary and cortical regions [77]. Given the lower levels of P-selectin and CCL25 in the thymus of ZAP-70-deficient mice (as compared to WT), it will be important to assess whether the thymic organ structure itself is a factor in the expression of the CCL25/P-selectin chemoattractants. Moreover, it will be vital to determine the interplay between precursor niches in the thymus and the receptivity of the thymus to importation of “natural” extrathymic precursors.

As in healthy individuals, it is thought that long-term thymocyte differentiation following hematopoietic stem cell transplantation requires an on-going migration of donor progenitors from the BM to the thymus. In the case of transplanted severe combined immunodeficiency patients, who are often not conditioned prior to stem cell administration, this long-term thymopoiesis may, in fact, not occur. This conclusion is based on several findings: 1) Only few naïve T cells are detected 10 years after transplantation, correlating with a skewing of the TCR repertoire [79]; 2) The number of TRECS, representing thymic export, decreases to extremely low levels within 18 years following transplantation whereas in normal individuals, this process occurs over 80 years [80]; and 3) T cell function declines at late time points following transplantation [81]. We have recently shown that thoracoscopy can be easily and safely used to achieve intrathymic transfer in anesthetized macaques, with accurate targeting of the thymus requiring a total procedure time of less than 15 min [82]. Thus, it becomes of significant interest to determine whether an intrathymic, as compared to intravenous, administration of hematopoietic progenitors to SCID patients promotes an increased donor occupancy of thymic precursor niches with an associated augmentation in long-term T cell differentiation.

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