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Decision checkpoints in the thymus

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Abstract

The development of T cells in the thymus involves multiple differentiation and proliferation events during which hematopoietic precursors give rise to T cells responding to antigen stimulation and ready for effector differentiation. This review addresses signaling and transcriptional checkpoints that control the intrathymic journey of T cell precursors. We focus on the divergence of αβ and γδ lineage cells and the elaboration of the αβ T cell repertoire, with special emphasis on the emergence of transcriptional programs that direct lineage decisions.

Introduction

The T cell arm of the immune system is essential for responses against infections. Multiple T cell subsets are distinguished based on the composition of their T cell antigen receptor (TCR) (αβ or γδ), their antigenic specificity and effector potential (Fig. 1). αβ T cells constitute the bulk of T cell populations in lymphoid organs and generally react against peptides presented by MHC-I or MHC-II molecules, whereas γδ T cells are generally not MHC-restricted and seem involved in the surveillance of microbial and non microbial tissue stress[1]. Unlike other immune cells, T cell develop in the thymus, through a process that can be separated into three broad steps (Fig. 1). The first spans from thymic colonization to T cell commitment, from where the second starts and leads to the divergence of αβ and γδ lineages. The third step sees αβ and γδ lineage cells complete their differentiation and acquire immunological properties, and in some cases effector functions. For most αβ lineage cells, this step is dominated by MHC-induced selection and results in the differentiation of thymocytes (‘single positive’, SP) that express either CD4 or CD8, two molecules that contribute to TCR recognition of MHC-II and MHC-I, respectively; such SP thymocytes are the direct precursors of mature T cells (Fig. 1).

This review discusses checkpoints that control the journey of T cell precursors in the thymus. After a brief overview of events that precede T cell commitment, we focus on the divergence of αβ and γδ lineages and the differentiation of αβ T cells. Work from many laboratories over the last few years has put the spotlight on transcriptional ‘circuits’ that control intrathymic checkpoints, and we have placed special emphasis on these emerging transcriptional ‘circuits’ and tried to connect them to intrathymic signals that direct lineage decisions. We refer the reader to recent reviews for important aspects of intrathymic development that are not covered here, including the mechanisms of antigen receptor rearrangement[2].

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From thymus settling to T cell commitment

While multiple types of progenitors can generate T cells under experimental conditions[3–6], recent evidence favors a model whereby the physiological thymic ‘settlers’, referred to as early thymic progenitors (ETP), are uncommitted cells that retain some myeloid but little if any B-lineage potential[7,8], although the intrathymic environment normally restrains their myeloid development[9]. Thymic colonization involves the chemokine receptor CCR9, probably redundantly with CCR7, and PSGL1, a ligand for P-selectin expressed on the thymic epithelium[10–12]. The loss of multipotency that defines T commitment is a gradual process. It occurs in ‘double negative’ (DN) thymocytes, that do not express CD4 or CD8, a subset itself separated into four sequential phenotypic stages (DN1 to DN4) based on expression of CD44 and CD25, and is not complete before the DN2 stage (Fig. 1).

T lineage commitment involves the sustained repression of alternate gene expression programs characteristic of other lineages. In early thymocytes (up to the DN2 stage), this requires signaling and transcriptional activation by Notch1 upon engagement by its ligand Delta-like 4 (DL4) expressed on the thymic stroma[13,14]. Such requirement for Notch1 in early precursors is demonstrated by both loss- and gain-of-function analyses[15,16]. However, even though a constitutively active version of Notch1 can promote extrathymic T cell differentiation, Notch1 is necessary but not sufficient for T cell commitment under physiological circumstances[17,18]. Other transcription factors, including Runx1, Gata3, and E-box proteins, cooperate with Notch1 to initiate T cell differentiation[19]. In addition, several factors impinge on Notch1 signaling activity, including the zinc finger transcription factor LRF that restrains Notch signaling in bone marrow progenitors[20], and post-translational modifiers of Notch1’s extra-cellular domain[21]. It is also important to note that Notch1 function is not limited to the exclusion of other developmental fates, as it promotes T lineage-specific gene expression and cell survival, and stimulates cell metabolism (see below)[17,22].

Whether and how Notch1 contributes to T cell commitment in DN2 cells is not yet known. In any case, its expression subsides during the late stages of T cell development[23,24], and it does not seem involved in maintaining lineage integrity in late thymocytes and mature T cells. Whether a single factor serves such a function in the T lineage, as Pax5 does in B cells[25], and its identity, are the focus of ongoing research.

The divergence of αβ and γδ lineages

β-selection and the choice of the αβ lineage

Although incomplete rearrangements at the TCRβ and γ loci occur in pre-commitment thymocytes, the critical rearrangement of variable gene segments occurs in T-committed DN3 cells[26]. Because of the deletion and untemplated addition of nucleotides during Rag-mediated recombination, most rearrangements are out-of-frame and fail to give rise to genes encoding a functional protein. The first checkpoint that committed thymocytes encounter, at the DN3 stage, precisely verifies proper TCR gene rearrangement.

For αβ precursors, this checkpoint is known as β-selection, and probes in-frame TCRβ gene rearrangement. It requires thymocytes to signal through a pre-TCR consisting of the product of a properly rearranged TCRβ gene, CD3 chains and pre-Tα, that binds TCRβ in the absence of rearranged TCRα[27,28]. Although the pre-TCR is not detectable at the cell surface, and is not believed to recognize any ligand[29], it signals, possibly through oligomerization[30], using intra-cellular intermediates similar to those triggered by TCR complexes in mature T cells. At least two additional signals contribute to β-selection: CXCR4, a receptor for the cytokine SDF[31,32], and Notch1. Notch1 promotes cell survival

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and metabolism through activation of the PI-3 kinase and Akt pathway, and contributes to overcome the differentiation block enforced in DN3 thymocytes by E2A proteins (see below)[33].

Thymocytes that 'pass' β-selection enter the last proliferative burst they will encounter in the thymus. They initiate CD4 and CD8 expression (becoming 'double positive', DP) and TCRα gene rearrangement, resulting in the surface expression of TCRαβ complexes, whereas they cease to express receptors characteristic of hematopoietic cells, marking their 'coming of age' as immune cells. They also become unresponsive to cytokine signals, and specifically to IL-7 expressed by the thymic stroma and critical to early T cell development[34], due to their down-regulation of IL-7Rα and their expression of the inhibitor of cytokine signaling SOCS-1[35].

αβ vs. γδ lineage choice

Developing γδ cells proof-read their TCR rearrangement at a DN3 checkpoint similar to β-selection, although differing in two important respects. First, unlike for αβ cells, there is no 'pre-TCRγδ' and the DN3 checkpoint assesses signaling by complete TCRγδ complexes. Second, γδ lineage differentiation does not depend on Notch signaling[36]. How DN3 cells distinguish pre-TCR from TCRγδ signals, and make the corresponding developmental decisions, has attracted much attention[37]. In a simple perspective, TCRγδ signals would 'instruct' DN3 thymocytes to become γδ T cells, whereas pre-TCR signals would cause differentiation into DP cells. While the importance for β-selection of a proline-rich motif in the pre-Tα cytosolic tail agrees with this view[27,28], the 'instructive' perspective is challenged by the fact that each type of receptor can in certain circumstances direct development of the other lineage. Expression of αβ transgenic TCRs can 'redirect' cells into the γδ lineage[38], whereas disruption of the TCRβ gene, which prevents pre-TCR expression, results in the generation of small populations of γδ thymocytes that express CD4 and CD8, the hallmark of αβ precursors[39].

These findings have thrown the 'instructive' perspective into disfavor, and the current view is that γδ TCR signals are 'stronger' than pre-TCR signals, and that such 'strong' signals promote γδ lineage choice. Indeed, impairing TCRγδ signaling, through the expression of signaling-defective CD3ζ chains, causes the differentiation of TCRγδ-bearing DP thymocytes[40]. It is not yet clear why TCRγδ signals would be stronger than pre-TCR signals; notably, both receptors differ from TCRαβ as they do not require CD3δε to signal[41,42]. Two complementary hypotheses are that greater signaling by TCRγδ results from its higher expression compared to pre-TCR, and from engagement by intrathymic ligands. Indeed, thymocytes expressing a transgenic TCRγδ receptor develop into γδ T cells in mice expressing the receptor ligand, but adopt an αβ fate when the ligand is absent[43]. There is additional evidence that γδ T cell development requires engagement by an intrathymic TCR ligand[44], and it seems reasonable to assume this to be the rule, even though the identity of such ligands remains in most cases unknown.

Whether specific environmental signals direct thymocytes towards the γδ fate before the DN3 checkpoint (e.g. by affecting TCR gene rearrangement) has been a contentious issue[37,45]. The strong IL-7 requirement for TCRγ but not TCRβ gene rearrangement[26], and the remarkable emergence of waves of γδ T cells with mono- or oligo-clonal receptors carrying invariant rearrangements during embryonic development, are suggestive of 'induced' events. The complete loss of αβ but not γδ T cells caused by inactivation of the transcription factor Bcl11b is also consistent with the idea of an early divergence, as the block in αβ thymocyte development occurs at the DN2 stage before the completion of TCRβ rearrangement[46]. However, these findings are also consistent with distinct requirements for the survival or amplification of αβ vs. γδ T cells. Indeed, recent single cell

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analyses support the idea that γδ lineage commitment is solely the result of TCR signaling 'strength' at the DN3 checkpoint[47]. As recently highlighted[48], the heterogeneity of γδ T cells challenges the idea that they form a single lineage, and it is conceivable that distinct rules apply to distinct γδ subsets.

Transcriptional control

How TCR or pre-TCR signals impinge on the transcriptional circuitry in DN3 thymocytes is coming to light[49]. E-box binding proteins of the E2A family have emerged as a key target of these signals. This activity, contributed in developing T cells by the two molecules E47 and HEB, and that we will refer to as 'E2A', blocks development at the DN3 stage[50]. Cells use two complementary mechanisms to overcome this block: reduced expression of the genes encoding E47 or HEB, or increased expression of Id-family molecules, that inhibit E2A protein function[51]. A member of this family, Id3, is preferentially up-regulated in γδ lineage cells[52]. Although Id3 is also a target of pre-TCR signals, it has been proposed that the extent of Id3 up-regulation depends on signal strength, and distinguishes between pre-TCR and TCRγδ signals[52]. High Id3 expression in γδ cells would suffice to overcome the 'E2A block', whereas lower Id3 levels in pre-TCR signaled cells could do so only in cooperation with Notch1-induced down-regulation of E2A expression[36,43,52–54]. This model, which provides an elegant rationale for the differential Notch requirement of αβ vs. γδ lineage cell differentiation[53], predicts that Id3 disruption would impair γδ T cells development. While that is the case in fetal thymi, Id3 disruption unevenly affects adults γδ thymocyte subsets, and actually enhances the pool of γδ thymocytes displaying effector function[52,55]. Thus, additional studies will be needed to fully comprehend the role of Id3 in γδ lineage differentiation[52,55,56].

While Id3 emerges as a possible 'sensor' converting signal strength into distinct transcriptional outcomes[50], how it is linked to 'effector' transcription factors that are selectively required for the differentiation of DN3 cells into αβ or γδ lineages remains to be determined. Three effector activities specifically or preferentially contribute to the generation of DP thymocytes from 'β-selected' cells. Runx1 is necessary for the proliferative burst that follows β-selection, but not for the generation of γδ T cells[57]. RORγt, encoded by the *Rorc* gene, is a hallmark of DP thymocytes in the thymus (in addition to its other functions in the immune system) and notably promotes their survival by up-regulating expression of the anti-apoptotic protein Bcl-XL[58]. However, there is evidence that *Rorc* expression depends on E2A and inhibits thymocyte proliferation[59], suggesting that it may not directly be induced by pre-TCR signals, which reduce E2A activity and promote proliferation, or that its induction by pre-TCR signals is delayed by the concurrent up-regulation of the transcription factor Egr3[59]. The HMG protein TCF1 (encoded by the *Tcf7* gene, and partly redundant with the related factor LEF) is needed for the generation of DP thymocytes, whereas the role of its 'conventional' partner β-catenin is not yet fully delineated[60–64].

Reciprocally, Sox13, another HMG transcription factor, inhibits the generation of αβ T cells and is important for the development of at least some γδ T cells[65], in part by antagonizing the function of TCF1, even though it remains unclear whether Sox13 contributes to γδ lineage 'choice' per se. Members of the Egr transcription factor family (Egr1, Egr2 and Egr3) are triggered by both pre-TCR and TCRγδ signals; high expression of these factors promotes γδ T cell development, possibly by repressing RORγt expression[59] and as intermediates for Id3 up-regulation[43,52]. Whether such factors, and others preferentially expressed in γδ T cells[66], contribute to γδ lineage differentiation *per se* or promote the survival or expansion of γδ T cell subsets remains to be determined.

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The elaboration of the αβ T cell repertoire

Positive and negative selection of αβ lineage cells

The rest of this review discusses the differentiation of αβ lineage cells, that emerge from β-selection as DP thymocytes and are the precursors of conventional CD4 and CD8 cells. Three key events mark the developmental progression of these cells: (i) positive selection, the rescue from programmed cell death of DP thymocytes whose TCRαβ productively interacts with self MHC peptide complexes (MHCp) expressed by the thymic epithelium (or with other MHC or MHC-like molecules)[67] (ii) negative selection, the elimination of self-reactive cells, and (iii) acquisition of functional competence, notably marked by the termination of either CD4 or CD8 expression ('lineage differentiation') and its matching to MHC specificity.

The need for positive selection is a direct consequence of the random nature of TCR rearrangement and of the high diversity of MHC alleles. As a result, most DP thymocytes in a given individual fail to productively interact with MHCp and die by 'neglect' within a few days, even though evolutionary pressure has resulted in a 'germline-encoded' MHC reactivity of TCR variable regions, thereby increasing the yield of positive selection[68,69]. CD4 and CD8 molecules provide an additional guard against the selection of non-MHC reactive cells, as they sequester the tyrosine kinase Lck required to initiate TCR signaling, thereby restricting its activity to TCRs engaged by MHCp, which, unlike non MHC-ligands, co-engage CD4 or CD8[70].

The avidity of T cells for MHC-bound self peptides, that underpins positive selection, is a correlate of their reactivity against MHC-bound foreign peptides. Thus, mechanisms have evolved to prevent the development of T cells with overt reactivity against MHC-bound self peptides, or to redirect such cells towards immune suppression. Thymocytes carrying receptors with the highest avidity for MHCp undergo TCR-induced programmed cell death (negative selection), a process essential for central tolerance[71]. It notably involves the exposure of thymocytes to tissue-specific antigens ectopically expressed by medullary epithelial cells in a manner dependent on the transcription factor Aire[72]. In addition, there is evidence that Foxp3 expressing thymic-derived Treg cells, another essential facet of central tolerance, express receptors with higher avidity for self than conventional MHC II-restricted cells[73,74]. We will not address further these issues, despite their obvious importance for the elaboration of the T cell repertoire, as they have been recently discussed[71]. Instead, the rest of this section will focus on how positively selected thymocytes 'sense' low avidity MHCp, and on the transcriptional circuitries that convert TCR signals into lineage decisions.

Setting the signaling threshold

Thymocytes that undergo positive selection, including those that are not subsequently eliminated by negative selection, undergo phenotypic changes that resemble those of mature T cells upon stimulation by their cognate antigen. While these changes in thymocytes do not lead to cell proliferation and effector differentiation, this raises the question of why MHCp that promote positive selection do not cause a similar 'abortive' activation program in mature T cells. One obvious possibility is that the set of peptides generated by thymic epithelial cells, that present positively selecting MHCp, differs from that of professional bone marrow derived APCs, which post-thymic T cells interrogate in their quest for antigen. There is evidence that this is the case, and that may contribute to the unique reactivity of thymocytes (and to reduce the footprint of negative selection over the positively selected repertoire)[75–77]. However, DP thymocytes are intrinsically more sensitive than mature T cells to low-avidity MHCp[78–80], raising the question of what lowers the threshold to TCR engagement in DP thymocytes.

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In addition to CD5, which is thought to 'tune' the sensitivity of mature and immature T cells to TCR signals[81], recent studies have highlighted the potential role of MicroRNA (miR) 181a in DP thymocytes[82]. MiR181a is highly expressed in immature thymocytes, and rapidly down-regulated after positive selection, and both gain and loss-of-function analyses supports its role in raising thymocyte sensitivity to TCR engagements. MiR repress gene expression by targeting messenger RNA (mRNA) for degradation or translational repression: while the full set of targets of miR181a remains to be identified, it impairs expression of phosphatases that inhibit TCR signaling, and notably that de-phosphorylate Erk kinases. Erk kinases have been proposed to have increased responsiveness to TCR engagement in DP thymocytes, as a result of calcineurin-mediated signaling in cells undergoing β-selection[83]; it is possible that this is due to miR181a, although its expression is not increased by β-selection[82]. It will be important to confirm the role of miR181a genetically, and to examine how disruption of miRNA generation affects positive selection[84–86]. Nonetheless, miRNAs are required for the generation of Treg cells[87] and the terminal differentiation of CD8 cells[86,87], suggesting that the repertoire of miRNA functions in the thymus is just being discovered[88].

Control of gene expression in DP thymocytes

Identifying the gene expression programs that 'orchestrate' the differentiation of DP thymocytes into mature T cells has proven a difficult endeavor. This is due in part to our incomplete understanding of the underlying effector mechanisms. Thus, although rescue from cell death defines positive selection, how TCR signals prevent thymocyte apoptosis is not yet well understood. TCR signaling increases expression of the anti-apoptotic molecule Bcl-2, but Bcl-2 is not required for T cell development[89,90], presumably because of functional redundancy with other members of the Bcl-2 family, including Mcl1[91,92]. Another complicating factor is the multiplicity of events induced by TCR signaling in thymocytes, in addition to positive selection *per se*. These include (i) the expression of the chemokine receptor CCR7, that promotes the migration of thymocytes from the cortex to the medulla[93–95], (ii) the acquisition of mature T cell gene expression programs, notably the expression of IL-7Rα[96], and (iii) the differentiation into the CD4 or the CD8 lineage[97,98]. While it could be conceived that specific signal transduction pathways trigger these events in DP thymocytes, there is little or no evidence that this is the case. Rather, the signaling cascades immediately downstream of TCR appear similar to those in mature T cells, including the Erk kinase and the calcineurin-NFAT pathways that are both required for positive selection[99–101].

Evidence is emerging that these cascades initially target a set of 'sensor' transcription factors that, most likely indirectly[101], control the expression of effectors molecules involved in the generation of mature αβ T cells, including IL-7Rα and CCR7. As in DN3 thymocytes, E2A is a primary target of these sensors, reflecting its keystone role in the transcriptional circuitry of DP cells (Fig. 2A). E2A activity promotes TCR gene rearrangement and expression of genes characteristic of immature thymocytes (including CXCR4)[102], and contributes to repress genes characteristic of mature thymocytes, including Foxo1, Klf2, IL-7Rα and CCR7[103]. The upregulation of Id3, through a cascade involving the Erk pathway, its direct target Elk4, and Egr proteins[104,105], is an early consequence of TCR signaling (Fig. 2A); the ensuing reduction in E2A activity appears as a key step in the transition from pre-selection DP to post-selection SP thymocyte. Of special interest are mechanisms that restrain Foxo1 and Klf2 expression in DP thymocytes, given the role that these factors play in T cell survival and homeostasis. How E2A contributes to such repression[103], and whether positive factors induced by TCR signals contribute to Foxo1 up-regulation, remain to be determined (Fig. 2B).

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CD4-CD8 lineage differentiation

In addition to being rescued from cell death (positive selection *per se*) TCR signaled thymocytes undergo functional differentiation into mature T cells. One key aspect of this process is the differentiation into the CD4 or CD8 lineage. This includes the termination of expression of either coreceptor, and the initiation of gene expression programs characteristic of helper (CD4) or cytotoxic (CD8) cells, two events that have long been recognized as being mechanistically coupled[106,107]. Because a functional immune system requires that this lineage 'decision' be matched to MHC specificity, so that MHC II-restricted thymocytes become helper CD4 cells, and MHC I-restricted thymocytes cytotoxic CD8 cells, its mechanisms have attracted much attention over the last two decades. The last few years have seen significant progress in the elucidation of the transcriptional circuits that promote CD4 or CD8 differentiation, that we will discuss first.

Two transcription factors, Thpok and Runx3, specifically expressed in CD4 and CD8 differentiating thymocytes, respectively, are important for this process[108–111]. Thpok is required for CD4 commitment and acts at least in part by repressing expression of CD8 lineage genes, including *Runx3*[110,112–114]. Runx3 is important for the silencing of *Cd4* in CD8 cells[108,109], and the complete disruption of Runx activity (that is of Runx3 and of the partly redundant factor Runx1) prevents CD8 cell development[57,113,115]. While this effect is due in part to unrestrained *Thpok* expression in Runx-deficient thymocytes[115], cells lacking both Runx and Thpok activities fail to become CD8, indicating a specific role of Runx proteins in CD8-lineage differentiation[113]. These findings have led to the proposal that a dual negative regulatory loop involving Thpok and Runx3, which mutually prevent expression of each other, results in lineage commitment (Fig. 3A).

A critical question is how thymocytes initiate expression of *Runx3* and *Thpok*, neither of which is expressed in preselection DP cells. There is little information yet available for *Runx3*. The transcription factor Ets1 binds *Runx3* and promotes its expression in CD8-lineage cells, but Ets1 is not CD8-lineage specific and its function in CD8-lineage commitment remains to be clarified[116]. Our understanding of *Thpok* gene regulation is more advanced. *Thpok* expression is limited to CD4-lineage cells by an upstream regulatory element with both positive and negative ('silencing') functions[115,117]. The binding of Runx molecules to this element is required, although not sufficient, for its silencing activity in DP thymocytes[115,117], and ongoing efforts search for additional factors that contribute to prevent *Thpok* expression in DP and MHC I-restricted thymocytes, including the zinc finger protein Mazr[118]. The impaired CD4-differentiation of thymocytes deficient for E2A and HEB could suggest that this activity is required for *Thpok* expression[103]. However E2A activity is important for *Cd4* expression in DP thymocytes[103]. Thus, it is possible that the reduced CD4 expression in E2A-deficient DP cells, rather than an intrinsic E2A requirement for *Thpok* expression, accounts for the impaired CD4-differentiation of E2A-HEB deficient thymocytes[103].

While Thpok and Runx are necessary for lineage commitment, they are not sufficient. Notably, both the transcription factor Gata3 and the HMG protein Tox are required for CD4 cell differentiation and *Thpok* expression[112,119,120]. Although Gata3 binding to the *Thpok* locus[112] and its preferential expression in MHC II-signaled thymocytes[121] make it a good candidate as an 'inducer' of *Thpok* expression, whether Gata3 acts by relieving Runx-dependent *Thpok* repression remains to be elucidated. Of note, a *Thpok* transgene fails to rescue Gata3-deficiency in CD4-differentiating cells, indicating a role for Gata3 beyond its ability to promote *Thpok* expression[112].

How intrathymic signals set this transcriptional circuitry in motion so that it matches lineage differentiation with MHC specificity has attracted much interest[122–124]. Although it was

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initially envisioned that specific signals induced by MHC-I or MHC-II molecules, and transduced through CD4 and CD8 could 'instruct' lineage differentiation[125,126], recent evidence favors the possibility that the kinetics of TCR signals play a decisive role in lineage 'choice'[98]. Genetic analyses supports the concept that 'longer' TCR signals are required for CD4- than for CD8-lineage commitment[98,127,128], specifically that CD4-lineage commitment requires TCR signals to persist until the cessation of CD8 expression, whereas no reciprocal requirement exists for CD8-lineage commitment. The 'kinetic signaling' model of lineage choice (Fig. 3B) proposes that asymmetric changes in CD4 and CD8 expression induced by TCR signaling in DP thymocytes cause MHC II-induced TCR signals to persist longer than those induced by MHC-I[128–130], and ongoing research is directed at determining how TCR signals affect *Thpok* or *Runx3* expression. In addition, the transcription factor Stat5, a key messenger of IL-7 signals, is important for CD8 but not CD4 T cell development and, unexpectedly acts in a manner redundant with the related protein Stat6[131]; these findings underscore the unique role of IL-7 in the generation of CD8 cells[131,132].

Terminal maturation

In addition to CD4-CD8 differentiation, the functional differentiation of thymocytes includes aspects that appear common to both lineages, notably controling thymic egress and cell quiescence. The zinc finger transcription factor Klf2 is essential to both phenomena, as it promotes expression of surface molecules involved in T cell trafficking, including L-selectin and the receptor for sphingosine 1 phosphate that is required for mature thymocytes to enter the bloodstream[133,134] and restrains effector cytokine production in naïve T cells[135]. Foxo1 is also essential for in the survival and homeostasis of mature T cells, notably because it promotes expression of IL-7Rα [136,137] and of Klf2[137,138] (Fig. 4). While redundancy among Foxo factors has so far prevented an exhaustive study of their role in the thymus[139], it is possible that they act similarly in mature thymocytes, and preliminary evidence suggests that this is the case[133,134]. The post-translational control of Foxo1 activity adds a critical layer of regulation[139]. In mature T cells, IL-7 signaling inhibits Foxo1 nuclear translocation and therefore its activity in a PI3-kinase dependent manner (Fig. 4). While it is possible that the same happens in thymocytes, TCR signals also activate PI3 kinase[140], suggesting a scenario whereby TCR signals would act in a dual fashion to control Foxo1 and its targets: they would promote Foxo1 expression, but need to subside to allow it to stimulate gene expression. While hypothetical, such a mechanism would prevent Foxo1 activity in, and therefore thymic egress of, self-reactive thymocytes undergoing continued TCR signaling in the medulla.

Invariant iNK T cell differentiation

While the vast majority of αβ T cells are restricted by MHC-I or MHC-II molecules, small subsets are selected on other MHC or MHC-like molecules. The most abundant of these, invariant NK T cells (iNK T) recognize lipid-bound MHC I-like CD1d molecules[141,142]. These cells carry TCRs made of a nearly invariant TCRα chain (Vα14Jα18 in mice) associated to a small set of TCRβ partners (Vβ8.2, Vβ7 or Vβ2). Although NK T cells account for a minor fraction of mature thymocyte and T cell populations in the spleen or LN, they accumulate in non lymphoid tissues, including the gut and liver where they form a large fraction of resident T cells and are stimulated by macrophages[143,144]. Like all αβ T cells, NK T cells are generated in the thymus from DP thymocytes[145]. Unexpectedly, although they are selected by MHC I-like CD1d, they fail to express CD8 and often retain CD4 expression (although a subset will eventually lose CD4 to become DN).

The selection of iNK T cells differs from that of conventional αβ T cells in three key respects[142,146]. First, CD1d selecting molecules are expressed on DP thymocytes, not on the

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cortical epithelium. Second, there is an additional requirement for signaling through homotypic interactions of SLAM family receptors, requiring activity of the SAP adaptor and the Fyn tyrosine kinase. Although the potential redundancy between SLAM family members and their close genetic linkage complicates genetic analyses, SLAMF1 and SLAMF6-deficient thymocytes demonstrate partial defects in iNKT cells implying a role in the development and function of these cells[147]. Third, these cells expand in the thymus, presumably as a consequence of TCR and SAP-mediated signaling and subsequently acquire effector properties, including expression of NK receptors and production of cytokines such as IL-4 and IFNγ.

The unique properties of iNK T cells are dependent on the transcription factor PLZF[148,149]. Unlike CD1d or SLAM-SAP signals, PLZF is not required for iNK T cell development, as iNK T cells can be detected in PLZF-deficient mice; however, such cells are in vastly reduced numbers, they fail to exhibit the high-level expression of NK receptors and cytokines that is typical of their wild-type counterparts, and, similar to conventional T cells, they display a naïve phenotype and home to lymph nodes and spleen.

Future challenges

Signals and circuits in developing thymocytes

The preceding pages have highlighted the considerable progress made over the last few years in our understanding of the signals and circuits that control thymocyte development. These years have also seen the emergence of new questions, including the role of pre-senilins, a class of enzymes notably involved in Notch signaling, in positive selection[150], and the discovery of Themis[151–155], the prototype a newly identified class of molecules. Themis is required for positive selection and the generation of a normal T cell repertoire, and that requirement appears quite specific as Themis-deficient mice do not display other overt phenotypic defects. While there is no consensus yet on whether Themis directly promotes TCR signal transduction, or serves downstream in the cytosol or nucleus, its disruption impairs TCR signaling during the DP to SP transition. In fact, the developmental arrest of Themis-deficient thymocytes resembles that of thymocytes engineered to cease TCR signaling during the DP to SP transition[156], suggesting that Themis functions in the kinetic integration of TCR signals over time, or in tuning thymocyte sensitivity to low-avidity ligands during positive selection.

Molecular bases of commitment

A recurring theme in the preceding pages is that circuitries operating at binary checkpoints promote the expression of ‘commitment’ factors that seal lineage fate, with Runx3 and Thpok at the CD4-CD8 checkpoint illustrating this thinking. While the identification of such factors, most notably of a putative T commitment factor, is a major objective of current research, a broader question is how they maintain lineage ‘integrity’, i.e. prevent the re-emergence of alternate gene expression programs in committed cells. CD4-CD8 differentiation offers a striking example, where the circuitry that decides commitment in the thymus is ‘recycled’ during CD4 cell effector differentiation[157]. While Thpok is important to prevent re-expression of CD8-lineage genes in post-thymic CD4 cells[158], epigenetic mechanisms also contribute to maintaining mature T cell CD4 or CD8 identity, including the silencing of either coreceptor gene[158,159]. Much is expected from current investigations that aim at determining what underpins epigenetic control, including histone methylation, nuclear localization, and at delineating the respective role of epigenetic and direct transcription control, especially in light of recent progress in cell ‘reprogramming’[160].

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The biology of quiescence

Aside from these mechanistic issues, the past years have brought new tools to tackle unanswered 'biological' questions. One key issue is what controls quiescence in thymocytes and T cells. Following the discovery that PLZF is critical for the acquisition of effector properties by iNK T cells, it was soon realized that its expression is not limited to that lineage. Indeed, subsets of $\gamma\delta$ T cells express PLZF, and transgenic expression of PLZF seems to confer an 'innate-like' effector-type phenotype to developing thymocytes[55,161,162]. As recurrent themes are emerging, notably that such cells often react against low-diversity foreign antigens or self determinants released by stress tissues[163], and that their development involves homotypic SLAM-SAP signals and cytokine signaling[164,165], it will be important to examine whether this extends to other effector subsets in the thymus. The potential impact of such 'innate-like' populations is illustrated by the recent discovery that their increased number in mice with impaired activity of Tec-family tyrosine kinases[164,165] promotes in *trans* the effector differentiation of conventional CD8 thymocytes (Kristin Hogquist, personal communication). The other side of the question is to identify the specific factors that maintain quiescence in 'conventional' thymocytes and mature T cells, with Klf2, Foxp1, a transcription factor distantly related to Foxo1, and Slfn2, a protein of unknown biochemical function, appearing as likely candidates[135,137,166,167].

Integrating at the organ level: cross talk in the thymus

The homotypic SLAM interactions in iNK T cell development illustrate the importance of the 'cross-talk' between thymocytes in intrathymic development. While it has long been known that such cross talk, or that between thymocytes and stroma, affects thymus biology[168,169], recent studies have started to dissect the signals involved[66,170]. Interactions between SP thymocytes and medullary epithelial cells have received particular attention: newly generated SP thymocytes spend several days in the medulla before exiting the thymus[170], where their exposure to tissue antigens expressed in medullary epithelial cells prevents the generation of self-reactive T cells[76]. Feed-back signaling from SP thymocytes is important for this effect, as it induces the expression of the autoimmune regulator Aire in medullary epithelial cells, through interactions between thymocyte-expressed RANK (receptor activator of NF-κB), and its ligand RANKL on epithelial cells, in a manner partly redundant with CD40-CD40L interactions[171–173]. The ongoing investigations of these and other cell-cell interactions build the foundation of an 'organ biology' approach to T cell development.

In summary, T cell development has generated immense interest for more than two decades, both because of the key immunological questions it raises, and as model system for cell differentiation processes, from programmed cell death to epigenetic silencing. The time now appears ripe for new harvests on these two fronts. New genetic and investigative tools tackle outstanding immunological questions, including the emergence of the distinct T lineages and the ligands that promote selection, whereas analyses of signaling and transcriptional mechanisms define new paradigms for gene expression and cell differentiation. In addition, emerging data on human T cell development highlight similarities and differences with mouse experimental systems[174]. These are exciting times for thymus biology !

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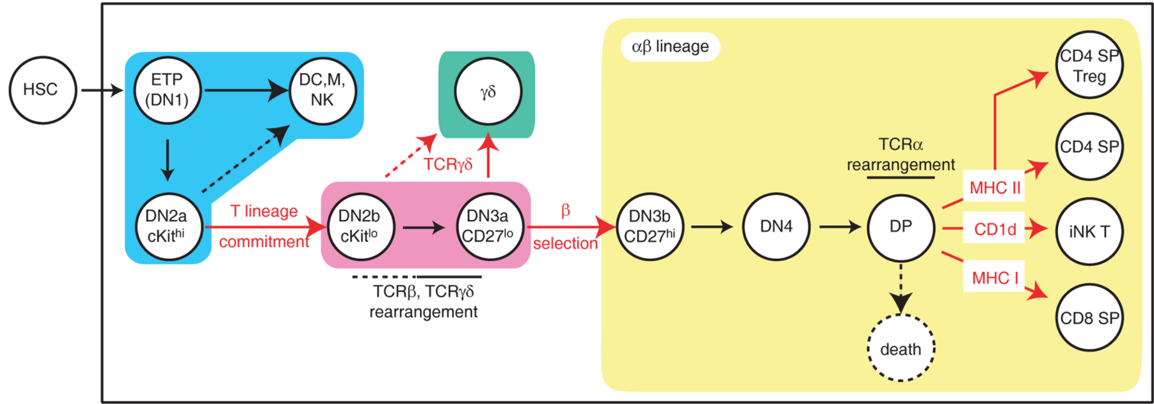

Figure 1. overview of T cell development

Thymic developmental stages are depicted. Expression of CD4 and CD8 separates $CD4^{-}CD8^{-}$ double negative (DN), $CD4^{+}CD8^{+}$ double positive (DP) and cells expressing either coreceptor (single positive, SP), whereas the expression of CD44 and CD25 defines four DN subsets: $CD44^{+}CD25^{-}$ [DN1], $CD44^{+}CD25^{+}$ [DN2], $CD44^{-}CD25^{+}$ [DN3], and $CD44^{-}CD25^{-}$ [DN4]. The earliest precursors, known as ETP, that enter the thymus from the bone marrow are part of an heterogeneous DN1 subset that includes both subsequent intermediates in the T differentiation pathway and cells belonging to other lineages[175]. The DN2 and DN3 subsets are themselves divided into two stages based on the expression of the receptor cKit and of CD27, respectively. Critical checkpoints addressed in the text are shown in red. Rounded rectangles group cell subsets according to the key developmental step they belong to: early uncommitted progenitors (blue), T committed progenitors before the separation of αβ and γδ lineages (purple) and committed αβ (yellow) or γδ (green) lineage cells.

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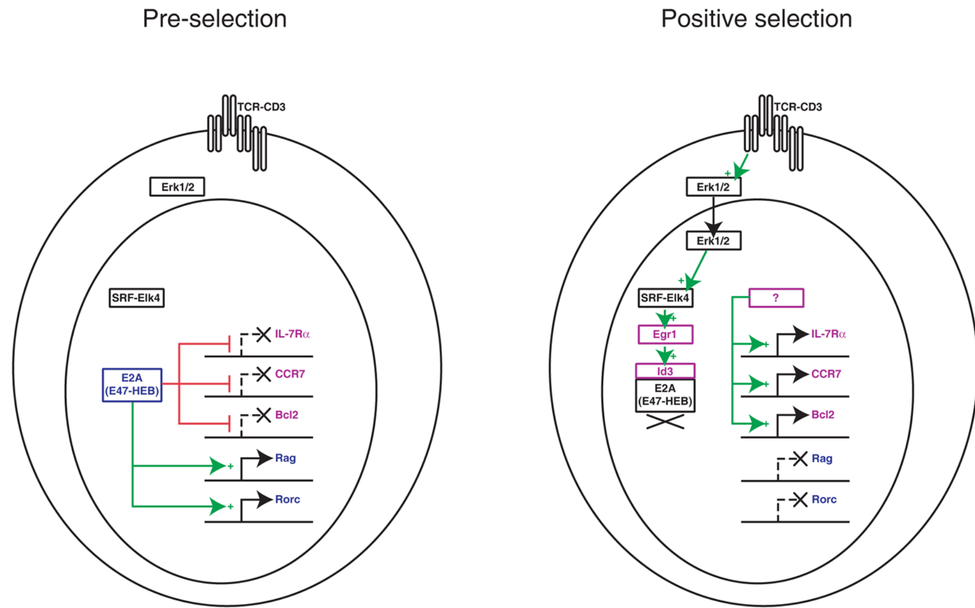

Figure 2. transcriptional circuitries in differentiating αβ T cells

In pre-selection DP thymocytes (left), E2A is thought to promote expression of *Rorc* and *Rag* genes, thereby ensuring TCR gene receptor expression and TCRα locus accessibility. In addition, E2A restrains expression of IL-7Rα, Bcl-2 and CCR7, although it is not clear whether such effects are direct or indirect (e.g. through effects on Foxo1 expression). Positive selection signals (right) reduce E2A activity by increasing Id3 expression, indirectly through Erk-dependent up-regulation of Egr proteins. Positively selected thymocytes have ceased expression of DP-stage genes and up-regulated Bcl-2, CCR7 and IL-7Rα, presumably as a result of the termination of E2A activity and of the induction of activators that may include Foxo1. Arrows or block signs do not necessarily indicate direct effects.

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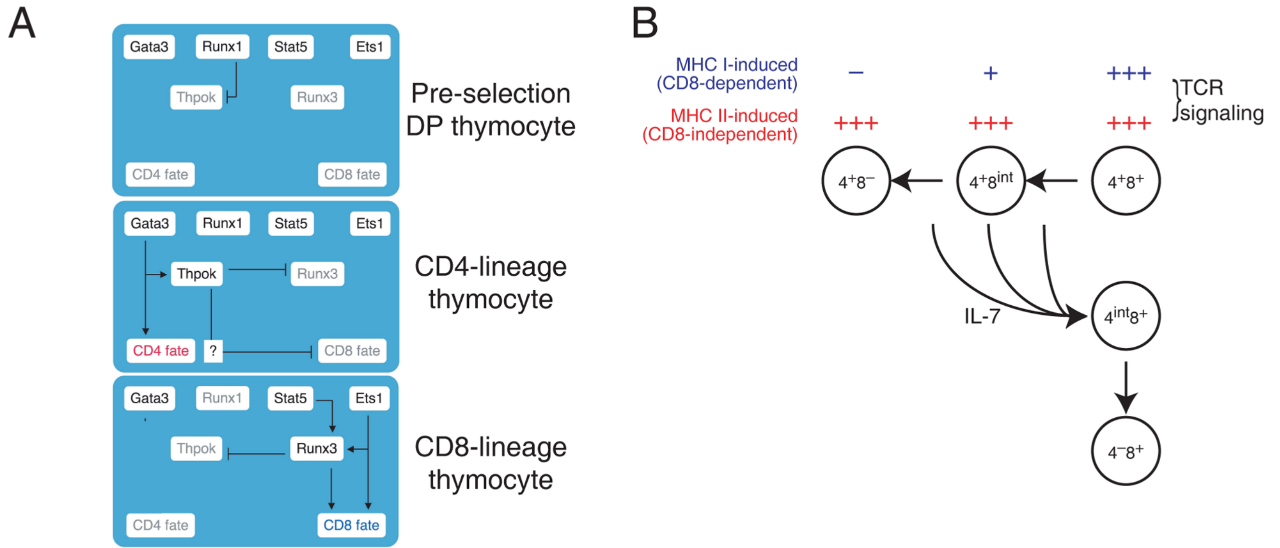

Figure 3. CD4-CD8 lineage differentiation

(A) Components of the transcriptional circuitry that promotes CD4-CD8 differentiation are schematically depicted and interconnected at three stages of T cell development. In preselection cells, Runx1-nucleated activities repress *Thpok* expression. In CD4-differentiating cells, Runx1-mediated *Thpok* expression is relieved, although Runx1 is still expressed in CD4 cells in which it binds the *Thpok* gene. Gata3 promotes both *Thpok* expression and additional developmental events required for CD4 cell differentiation. Thpok prevents *Runx3* up-regulation and CD8 differentiation. In CD8-differentiating cells, *Thpok* repression is maintained, presumably through Runx3. Ets1 promotes *Runx3* expression, and binds the *Runx3* locus, whereas Stat5 has been reported to relay IL-7 signaling to *Runx3*[131]. Grey lettering indicates factors not expressed at a particular stage. Other factors (including Tox) are omitted for clarity. Arrows or block signs do not imply direct effects.

(B) The 'kinetic signaling' model of lineage differentiation posits that intrathymic TCR signaling, regardless of MHC specificity, represses *Cd8* expression, causing thymocytes to adopt a $CD4^{+}CD8^{int}$ surface phenotype. TCR signaling in MHC II-restricted thymocytes is not affected by CD8 down-regulation, and its persistence eventually seals CD4 commitment. In contrast, TCR signaling in MHC I-restricted thymocytes is impaired by CD8 down-regulation, and its cessation causes 'coreceptor reversal' i.e. the cessation of *Cd4* expression and the resumption of *Cd8* expression.

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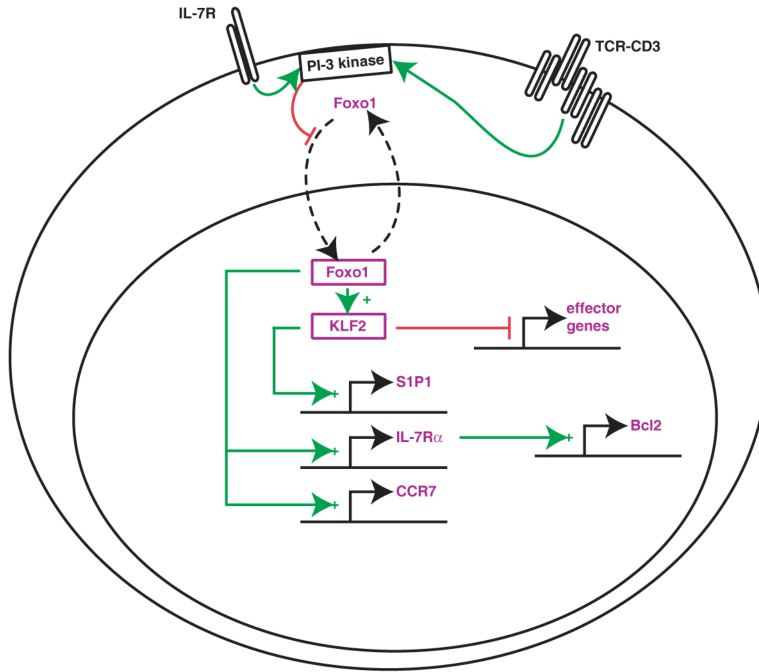

Figure 4. An hypothetical transcriptional network in mature thymocytes and T cells

Based on analyses in mature T cells, a transcriptional circuitry is proposed in mature thymocytes, that enables expression of IL-7Rα, CCR7 and Klf12, which itself controls thymic egress by increasing expression of the receptor for sphingosine 1-phophate (S1P1), T cell trafficking and quiescence. Foxo1 activity is inhibited by PI-3 kinase-dependent phosphorylation, that promotes its sequestration in the cytosol, contributing to the self-limiting IL-7Rα expression characteristic of mature T cells[176]. It may also act as a 'licensing' factor in the thymus to prevent the release of self reactive thymocytes due to their persistent TCR signaling.