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CD4-CD8 differentiation in the thymus: connecting circuits and building memories

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Abstract

The proper choice of the CD4-helper or CD8-cytotoxic lineages by developing T cells is crucial for the generation of an antigen-responsive and functionally fit T cell repertoire. Here we present a brief overview of the transcriptional control of this process, with emphasis on two issues. The study of *Cd4* expression, that had previously generated important paradigms for transcriptional regulation in eukaryotic cells, now brings new twists to the concept of 'epigenetic memory'. On the other hand, connections are emerging between transcriptional regulators critical for commitment to either lineage. The present review attempts to integrate these findings and discusses the still elusive mechanisms that match CD4-CD8 lineage differentiation to MHC specificity.

Introduction

The decision by developing T cells to adopt either of the lineages defined by the expression of CD4 or CD8 glycoproteins has captured the attention of immunologists and developmental biologists for years, because of its importance for immune response and of its paradigmatic value [1]. In addition to the mutually exclusive expression of CD4 or CD8, each lineage is characterized by distinct antigen specificities and functions. CD4 T cells are MHC-II restricted and pre-programmed for helper functions, whereas CD8 T cells are MHC I-restricted and pre-programmed for cytotoxic functions. CD4 and CD8 subsets constitute the bulk of αβ T cells and are the main component of T-mediated immune responses. They differentiate in the thymus from $CD4^+CD8^+$ 'double positive' (DP) precursors [2], and a critical aspect of this process is the matching of CD4 or CD8 lineage differentiation (and of helper vs. cytotoxic functions) to MHC-II or MHC-I specificity, respectively (Fig. 1). This highlight of the recent literature is focused on the control of *Cd4* expression and on the transcriptional mechanisms that underpin CD4-CD8 lineage differentiation in the thymus [3–5]. We refer the reader to recent reviews [1, 6] for a discussion of intrathymic signals that control lineage differentiation.

Cd4 gene expression

Previous studies of *Cd4* gene expression had spawned insights critical for our understanding of gene silencing [7], and the last two years have brought new thought-provoking results. Two *cis*-regulatory elements involved in *Cd4* expression had been identified earlier [7]: an upstream enhancer ('proximal', E4P) and an intronic silencer whose activity requires recruitment of repressor proteins Runx1 or Runx3 (Fig. 2). The conventional picture was that E4P is active throughout T cell development, whereas the silencer prevents *Cd4*

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expression in CD8 cells and in $CD4^{-}CD8^{-}$ (double negative, DN) thymocytes [8]. A first dent into this dichotomic view comes from the observation that E4P also contributes to *Cd4* repression in CD4-negative cells, by recruiting the transcriptional repressor AP4 [9], suggesting an unsuspected inter-dependence of activation and repression functions within the *Cd4* locus.

A stronger challenge to the conventional view, together with clarifications of an old controversy, come from experiments 'knocking-out' E4P to explore its functions [10]. The first surprise is that E4P has stage-specific activity: germline deletion shows the enhancer to be required for *Cd4* expression in DP thymocytes, but not in mature CD4 T cells or in CD4-differentiating thymocytes as CD4-expressing T cells develop despite germline E4P deletion. This was unexpected: if anything, the contrary could have been envisioned, because earlier experiments with transgenic reporters had suggested that an enhancer located downstream of the *Cd4* gene was active in DP thymocytes but not mature T cells [11]. In fact, the new report shows that this element is dispensable for *Cd4* expression at any stage of T cell development, but is active in lymphoid tissue inducer (LTi) cells, a subset of innate lymphoid cells that in mice express *Cd4* [12].

These observations imply that another *cis*-regulatory element (unknown enhancer, or the *Cd4* promoter itself) activates *Cd4* in CD4-lineage cells. However, things are not that simple. Strikingly, disrupting E4P in thymocytes affects *Cd4* expression in activated T cells [10]. Specifically, the CD4-expressing T cells that develop despite E4P deletion fail to sustain *Cd4* expression after activation, whereas *Cd4* expression is not affected, even in proliferating cells, by post-thymic deletion of a conditional ('floxed') E4P element [10]. These findings suggest that, in thymocytes, E4P 'deposits' activating epigenetic marks elsewhere on the *Cd4* locus, and that such marks contribute to stable *Cd4* expression in effector cells. What these marks are, and whether they target the promoter, a putative new enhancer or other components of the *Cd4* locus remain to be determined, and is further discussed in a review accompanying the original study [13].

Lineage commitment

The rest of this review will address the transcriptional control of the CD4-CD8 differentiation decision. Our discussion will be based on the idea that two conceptually distinct, even if possibly overlapping, processes contribute to this event: specification, the initiation of lineage-specific gene expression, and commitment, a biological event defined by the loss of the bi-potency characteristic of the precursor stage and of alternate differentiation potential.

The current perspective is that commitment results from the opposing activity of two transcriptional repressors, Thpok and Runx3 (Fig. 1) [3]. The zinc finger protein Thpok is up-regulated in MHC II-restricted thymocytes as they undergo CD4-lineage differentiation and is needed for CD4 lineage commitment. In contrast, MHC I-restricted cells up-regulate Runx3, which promotes CD8-lineage commitment redundantly with the related protein Runx1 [5]. Thpok represses CD8-lineage genes, including *Runx3* and *Cd8*, whereas Runx3 proteins repress *Thpok* and *Cd4*. Despite the apparent symmetry of this process, there is evidence for Thpok having a dominant effect on Runx3. Thpok antagonizes Runx3 functions in cells where both factors are expressed [14, 15]. In addition, transgenic Thpok expression prevents *Runx3* expression and CD8 differentiation, whereas transgenic Runx3 expression is insufficient to repress *Thpok* and CD4 differentiation [Refs. 16, 17, 18, and our unpublished results]. Transgene dosage effects, a typical caveat of gain-of-function studies, do not seem to account for these differences [18–20]. Rather, one possible interpretation is that additional CD8 lineage-specific factors cooperate with Runx3 to seal commitment, a possibility in line

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with the idea that non-Runx factors, so far unidentified, contribute to repress *Cd4* and *Thpok* in CD8 cells [21–23].

The repression of CD8-lineage genes by Thpok appears to operate, albeit with modifications, beyond lineage commitment in the thymus [24]. One notable variation is seen in IFNγ-producing (Th1) CD4 effectors, which express both *Thpok* and *Runx3* [25–27], yet do not re-express *Cd8* at least in part because of persistent *Thpok* expression [Ref. 25, and unpublished results from our laboratory]. Thus, the Thpok-Runx3 reciprocal repression has stage-specific characteristics that are not yet fully understood. In contrast, *Cd8* repression seems a general effect of Thpok. That is illustrated in iNK T cells, an αβ T cell population recognizing lipids presented by the MHC-like molecule CD1d, and that are $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{-}$ [28]. Thpok is expressed in both subsets at similar levels, and represses *Cd8* as in conventional T cells [29, 30]. Genome-wide analyses of Thpok binding will be helpful to determine the mechanistic bases of its distinct effects on *Cd8* and *Runx3* expression. Despite these remaining uncertainties, the Thpok-Runx3 mutual repression provides a robust mechanism explaining how cells embracing either lineage renounce the alternative fate.

Lineage specification

The question now hovering over the field is to decipher the initial lineage specification process: what sets this commitment machinery in motion by initiating expression of *Thpok* or *Runx3*, neither of which is expressed in DP thymocytes [16, 17, 31, 32]. Two factors potentially involved in Runx3 expression have been recently identified. Stat5, a target of IL-7 in the thymus, is critical for the generation of CD8 T cells; it acts redundantly with the related protein Stat6 and has been reported to promote *Runx3* expression [33]. It is not clear yet whether this effect is direct or indirect and how it integrates with other effects of IL-7 during CD8 T cell differentiation [34]. The implication of these observations is that IL-7, possibly redundantly with other cytokines [34, 35], promotes CD8-lineage specification. This provocative idea is a key tenet of the ‘kinetic signalling’ model of CD4-CD8 differentiation, a discussion of which is beyond the scope of this overview; for more information, the reader is referred to recent reviews [1, 6] and original studies [33, 36, 37]. The second *Runx3*-promoting factor, Ets1, is expressed at all stages of T cell development. Ets1 binds the *Runx3* locus and promotes *Runx3* expression (and *Cd4* silencing at least in part through its effect on *Runx3*) [20]. In addition, Ets1 contributes to IL-7 receptor expression [38]. While the latter finding provides another possible clue to its effect on *Runx3*, it also illustrates how arduous dissecting the pleiotropic functions of lineage specifying factors can become, an issue that we will encounter again with CD4 differentiation.

The up-regulation of *Runx3* is part of a broader CD8-lineage specification program in the thymus, that includes genes encoding cytotoxic proteins (such as perforin). New evidence documents the role of Runx3 itself in this process [39], in addition to it repressing *Thpok* and *Cd4*. The few CD8 cells that develop in the absence of Runx3 (and depend on the redundant function of Runx1 [31]) have impaired expression of perforin [22, 31, 39], and of *Cd8* genes themselves. Even though the expression of CD8 is barely diminished in resting Runx3-deficient cells [32], it is strikingly reduced when these cells undergo effector differentiation and proliferation, consistent with the recruitment of Runx3 to *Cd8* enhancers [40, 41]. It is interesting a that similar difference between resting and activated CD8 cells was found in their usage of *Cd8 cis*-regulatory elements [41], and future studies will determine if, as for *Cd4* expression in CD4 cells [10], such differences result from epigenetic changes imprinted during intra-thymic differentiation.

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The CD4-lineage specification problem has been even more difficult to tackle. In contrast to Runx3 for cytotoxic genes, it is not yet clear to which extent Thpok itself contributes to the expression of CD4-lineage genes, including its own [15, 25, 42, 43]. However, genetic studies have identified several transcription factors important for CD4 T cell generation, and thus candidates for a specification function; these include Gata3, the DNA-binding protein Tox, and E proteins E2A and HEB [44–46]. Both Gata3 and Tox are up-regulated by TCR signaling, albeit possibly through distinct intra-cellular signaling pathways [47, 48]; in addition, Gata3 but not Tox is expressed higher in MHC-II than MHC I-restricted thymocytes [49]. Both factors are required for *Thpok* expression [19, 45], and Gata3 actually binds a region of *Thpok* important for its transcription [19] (Fig. 3). However, ectopic Thpok expression in Gata3- or Tox-deficient thymocytes fails to restore CD4 T cell differentiation, suggesting that each factor has a broader range of targets in developing CD4 cells [25, 50].

What are these targets is an important but yet unanswered question. It has been proposed that Tox serves to sustain or increase *Cd4* expression in MHC II-signaled cells [37, 50], an idea that would fit with the existence of a *Cd4 cis*-regulatory element specifically activated at this developmental stage [10]. However, this is unlikely to account for all Tox functions, as Tox inactivation severely disrupts the development of MHC II-restricted cells, whereas impaired CD4 expression redirects them to the CD8 lineage [36]. In addition, these analyses are complicated by the pleiotropic effects of these ‘CD4-specifying factors’: Tox affects late thymocyte maturation, and Gata3 TCR signal transduction [19, 50]. Such caveats apply to an even greater extent to E-proteins E2A and HEB: they are important for CD4-lineage differentiation and for the activity of the *Cd4* E4P, but also serve as gate-keepers of the DP to SP transition, most spectacularly by enforcing the requirement for MHC-induced T cell receptor (TCR) signaling during positive selection [44].

Matching lineage to MHC specificity

How does this specification machinery ensure expression of Thpok in CD4 cells and of Runx3 in CD8 cells? Because of the dominant effect of Thpok on Runx3 expression and function [14, 15, 25, 42], its up-regulation is thought to determine lineage choice, and there is strong evidence that it results primarily from the relief of repression [22, 23]. A *cis*-regulatory sequence called ‘distal regulatory element’ (DRE) or ‘silencer’ represses *Thpok* in DP thymocytes and CD8-lineage thymocytes (Fig. 3). Three transcriptional repressors bind the silencer and contribute to *Thpok* repression in DP thymocytes (and presumably in CD8-lineage cells) [22, 51, 52]: Runx proteins (Runx1 or Runx3), Mazr, a protein structurally related to Thpok, and Bcl11b, a molecule essential for early T lineage commitment [53]. For Runx proteins, there is evidence for direct repression through silencer binding [22]. While there is no such evidence for the other two factors, a similar mechanism is plausible for Mazr given its ability to associate with Runx1 [51]. The function of Bcl11b seems more complex as it may also contribute to repress *Runx3* [52].

This does not mean that positive regulation does not contribute to *Thpok* expression, and positive *cis*-regulatory elements (enhancers) have been identified in the *Thpok* locus [22, 23] (Fig. 3). However, a ‘silencer-less’ *Thpok* allele or reporter transgene is expressed equally well in MHC I- and MHC II-restricted cells, indicating that these enhancers are not by themselves responsible for the lineage specificity of *Thpok* expression [22, 23]. Thus, the key question is to understand how the silencer is inactivated, and therefore *Thpok* expressed, in MHC II but not MHC I-signaled cells. This could involve up-regulating transcriptional activators that overcome Runx-mediated repression. Gata3 is a prime candidate for such a function, as in mature T cells it antagonizes the functions of Runx proteins [54] and its expression is higher in MHC-II than MHC I-restricted thymocytes [49 and our own

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observations]. Other possible mechanisms, at present speculative, include (i) lineage-specific down-regulation of silencer-binding repressors, although there is no evidence that this is the case for Runx1, Mazr or Bcl11b, or (ii) post-translational modifications of critical members of this circuitry.

In addition to their mechanistic interest, an important objective of these studies is to link silencer inactivation to the intrathymic signals that promote CD4-lineage differentiation and *Thpok* expression [1, 6, 36, 55, 56]. There is strong evidence, including from a recent 'knock in' study [36], that TCR signaling needs to persist longer in thymocytes for CD4 than for CD8 lineage choice, another tenet of the 'kinetic signaling' perspective [1]. One would then expect such 'long' signals to inactivate the *Thpok* silencer and promote *Thpok* expression, and there is indirect support for that possibility [23]. Conversely, it is possible that TCR signaling contributes to *Runx3* repression: a recent study proposes that IRF4, a target of TCR signals in the thymus, could serve such a function by binding the *Runx3* locus, among broader effects than cannot be attributed to *Runx3* down-regulation [57]. However, additional analyses will be needed to fully appreciate the function of IRF4 in thymocytes, as IRF4 disruption did not affect CD4-CD8 differentiation [58].

Conclusions and perspectives

The last two years have seen connections emerging between transcription factors involved in the choice of the CD4 or CD8 lineage, bringing us a step closer to understanding the transcriptional circuits controlling that process. Important challenges lie ahead. The underpinnings of epigenetic control of gene expression and silencing will certainly be an important area of investigation for the coming years; clues may emerge from studies of non-coding RNAs and histone or DNA modifications [59]. Imaging studies are entering the field with provocative questions. Notably, the possibility that transcription at *Cd4* and *Cd8* loci are mechanistically coupled by Runx proteins has emerged from analyses showing close approximation of both loci in cells that express CD8 [60]. This effect has specific requirements, both in *cis* (*Cd4* silencer and *Cd8* enhancers) and in *trans* (Runx activity). The puzzling question is that, even when close, *Cd4* and *Cd8* loci remain separated by micrometer-range distances; elucidating the mechanistic bases of the association should provide insight into changes in subnuclear architecture that accompany and possibly affect lineage specific gene expression. Solving the maze of CD4-lineage differentiation is also a pressing challenge, notably in light of the clinical importance of CD4 cell deficiencies. An important objective of future research will be to determine whether this process results from the combined activities of multiple transcription factors, or whether a single factor, yet to be identified, serves as a major CD4-specifying activity.

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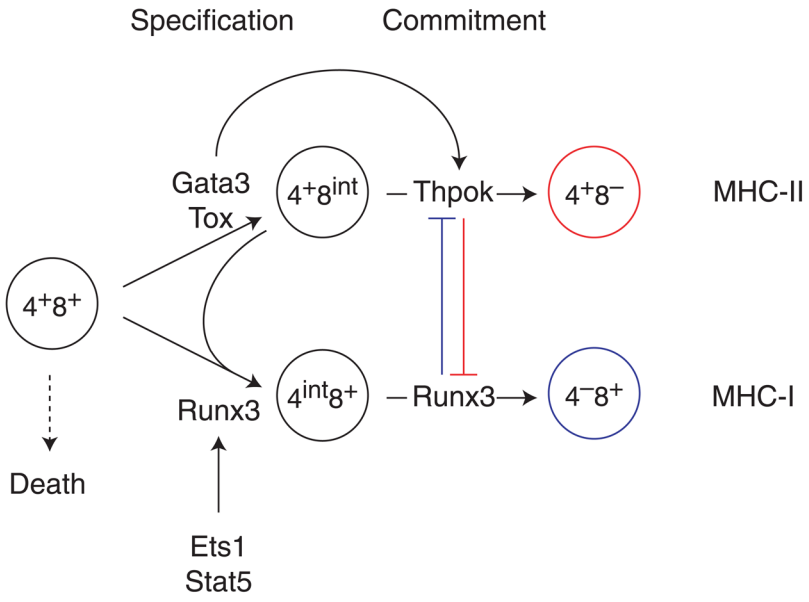

Figure 1. Specification and commitment to the CD4 and CD8 lineages

DP thymocytes have rearranged genes encoding TCRα and TCRβ chains and express surface TCR complexes. These cells are programmed to undergo apoptotic cell death in the thymic cortex unless their TCR is productively engaged by MHC molecules expressed by the thymic epithelium, an event referred to as positive selection. Rescued thymocytes differentiate into CD4 or CD8 T cells, depending on whether they are MHC II- or MHC I-restricted, respectively. Lineage differentiation includes two conceptually distinct steps, specification and commitment. For the CD4 lineage, specification involves Gata3, Tox and E-proteins E2A and HEB (not shown), whereas commitment requires Thpok, which represses CD8-lineage genes including *Runx3*. For CD8 cells, specification involves Runx3, which also contributes to commitment by repressing *Thpok* and *Cd4*. Stat5 and Ets1 contribute to Runx3 expression, the latter binding the *Runx3* locus. Note that the $CD4^{+}CD8^{int}$ cells has precursor activity for both CD4 and CD8 lineages and is thought to include truly bi-potent cells [1]. In contrast the $CD4^{int}CD8^{+}$ subset only has CD8 precursor activity.

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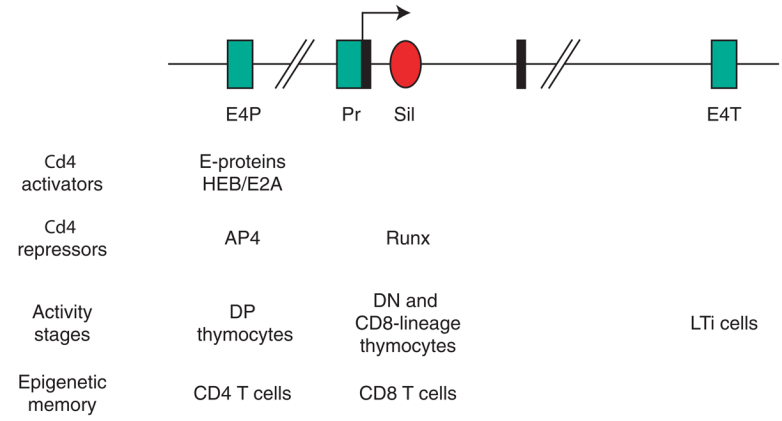

Figure 2. *Cd4 cis*-regulatory elements

Schematic representation (not on scale) of the *Cd4* locus, with exons 1 and 2 (black boxes), the silencer (red-filled oval) and positive regulatory elements (green-filled rectangles), including the proximal enhancer (E4P), promoter (Pr) and a downstream enhancer known as 'thymic enhancer' (E4T) even though it is now known to be active in LTi cells, not in thymocytes. Transcription factors important for the activity of each element are indicated, as are cell subsets in which each element is active, or determines 'epigenetic memory' despite having no intrinsic activity in the subset. Note that while AP4 does not bind the silencer, it interacts with Runx molecules and could therefore 'bridge' that element with E4P. Factors distinct from Runx proteins are thought to contribute to *Cd4* silencing because the silencer contains functionally important motifs in addition to Runx binding sites [21].

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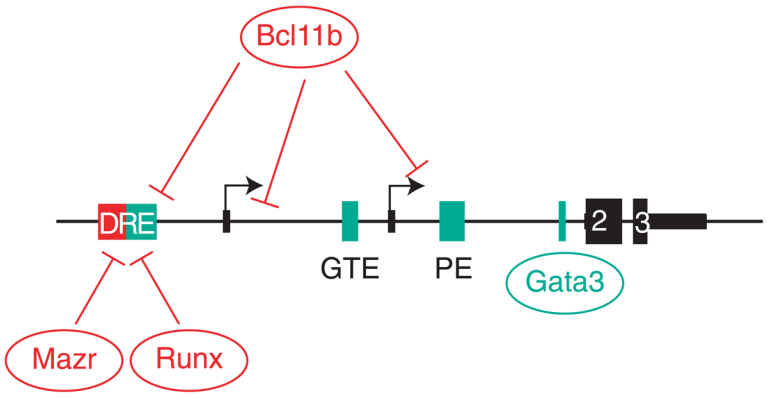

Figure 3. *Thpok cis*-regulatory elements

The *Thpok* locus is schematically represented (not on scale). The two transcription start sites, defined by alternate exons 1a and 1b, are shown as arrows and exons as black boxes; thick areas depict coding regions in exons 2 and 3. Boxes (top) indicate the distal regulatory element (DRE, including the silencer), the general T lymphoid element (GTE), the proximal enhancer (PE), and a Gata3 binding site, using the same color code as in Fig. 2. Binding areas of repressors (Runx molecules, Mazr and Bcl11b) and activators (Gata3) are schematically depicted. While the DRE is depicted as including distinct activating (green) and silencing (red) elements, it is not clear whether these activities are physically separated [19, 22, 23]. Note that Runx molecules also bind the proximal enhancer [22], although it is not know whether that binding serves an activating or repressive function. In CD4-lineage cells, which express *Thpok*, Thpok molecules bind the silencer [15].