CD4-CD8 Lineage Differentiation: Thpok-ing into the Nucleus

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The mature αβ T cell population is divided into two main lineages that are defined by the mutually exclusive expression of CD4 and CD8 surface molecules (coreceptors) and that differ in their MHC restriction and function. CD4 T cells are typically MHC-II restricted and helper (or regulatory), whereas CD8 T cells are typically cytotoxic. Several transcription factors are known to control the emergence of CD4 and CD8 lineages, including the zinc finger proteins Thpok and Gata3, which are required for CD4 lineage differentiation, and the Runx factors Runx1 and Runx3, which contribute to CD8 lineage differentiation. This review summarizes recent advances on the function of these transcription factors in lineage differentiation. We also discuss how the “circuitry” connecting these factors could operate to match the expression of the lineage-committing factors Thpok and Runx3, and therefore lineage differentiation, to MHC specificity. The Journal of Immunology, 2009, 183: 2903–2910.

Setting the stage: positive selection and lineage choice

Most T lymphocytes carry TCRs made of α- and β-chains that recognize peptide Ags bound to MHC class I (MHC-I) or MHC class II (MHC-II) molecules. Peptides presented by these molecules differ in multiple respects, most notably by their origin (typically intracellularly synthesized molecules for MHC-I, endocytosed molecules for MHC-II). To this duality of Ag corresponds a dichotomy of T cells subsets that differ by the expression of the surface proteins CD8 and CD4, which serve as coreceptors for MHC-I and MHC-II, respectively; MHC-I-restricted T cells generally express CD8 whereas MHC-II-restricted T cells generally express CD4. Expression of CD4 and CD8 is mutually exclusive on mature T cells and is stably maintained throughout their postthymic life, thus distinguishing two distinct T cell lineages. These two lineages differentiate in the thymus from a population of precursors expressing both CD4 and CD8 molecules (double positive (DP) thymocytes).

The choice between CD4 and CD8 lineages occurs after thymocytes have rearranged their TCRα and TCRβ genes, and only in those cells that undergo positive selection, i.e., whose TCRs recognize MHC ligands of appropriate avidity in the thymic epithelium (1). In addition to setting Cd4 or Cd8 expression, lineage choice also affects the effector differentiation of T cells after Ag encounter, as CD8 effector cells are typically cytotoxic and express enzymes such as perforin and granzymes, whereas CD4 effector cells either help or suppress the function of other immune cells. Although the characteristic outcome of lineage choice, the matching of CD4-CD8 differentiation to MHC restriction, has been elucidated more than 20 years ago (2–4), the “nuts-and-bolts” have long remained enigmatic. The present review will focus on recent advances in our understanding of the transcriptional circuitry that controls CD4-CD8 lineage choice. Progress, recently discussed in depth (5), has also been made in the identification of environmental signals that direct thymocytes into either lineage; we will come back to this issue near the end of the present review.

Runx proteins and CD8 cell differentiation: an eloquent silencing

The first lead on the transcriptional control of lineage choice came from the identification of a cis-regulatory element, known as the Cd4 silencer, that dictates the lineage-specificity of Cd4 expression (6–8). This work opened the way to the breakthrough finding that silencer activity, and therefore the proper control of Cd4 expression, requires its recruitment of Runx transcription factors (9).

The evolutionary conserved Runx family is involved in many differentiation processes; in mammals, it includes three members (Runx1–3) that act as heterodimers with the structurally unrelated molecule Cbfβ (10). The defining feature of the family, the Runt homology domain, is located near the amino terminus of each member and mediates binding to DNA and association with Cbfβ. Runx-Cbfβ dimers either activate or repress transcription, depending on interactions with other factors and possibly post-translational modifications. Runx1 and Runx3, but not Runx2, have been implicated in T cell development (9, 11). Runx1 is expressed at and required for many steps of T cell differentiation (9, 12, 13), notably for the generation of DP thymocytes from their...
CD4^-CD8^- (double negative) precursors and for the survival of CD4 lineage cells (9, 13). Runx3 protein is not detected in DP thymocytes or resting CD4 cells, but it is up-regulated during the differentiation of CD8 cells in the thymus and remains expressed in postthymic CD8 cells (13–15) (Fig. 1); this expression pattern strongly correlates with that of mRNAs initiated at the most upstream (distal) of the two Runx3 promoters (13, 16) (Fig. 2A). Runx3 is also expressed in type 1 CD4 effector cells, where it promotes IFN-γ production (17, 18).

In agreement with these expression patterns, Runx1 represses Cd4 in double negative thymocytes whereas Runx3 is required for the proper silencing of Cd4 in CD8-differentiating cells (9, 13, 14). Although Runx3 disruption does not prevent CD8 cell differentiation, it results in reduced numbers of CD8 T cells, of which a substantial fraction maintains CD4 expression and therefore appears as CD4^+CD8^- (double positive). This phenotype reflects the functional redundancy between Runx1 and Runx3, as thymocytes lacking both molecules or the obligatory dimerization partner Cbfβ (both referred to as “Runx-deficient” hereafter) fail to differentiate into CD8 cells altogether (13, 16, 19). This compensatory effect is explained in part by the increased expression of Runx1 in Runx3-deficient cells (16), suggesting that Runx3 is the physiological effector of CD8 differentiation.

How do Runx factors promote CD8 lineage choice? Not only they repress Cd4, the CD4 lineage-defining gene in postthymic cells (9, 14, 20–22), but experiments in peripheral cells indicate that they promote the expression of genes characteristic of the CD8 lineage, including those that encode the cytotoxic enzymes perforin and granzyme B, the cytokine IFN-γ and the transcription factor Eomesoderm (23, 24). Furthermore, Runx3-deficient CD8 cells have reduced CD8 expression (13), and Runx3 binds to a CD8 enhancer specifically active in mature CD8 T cells (15). These observations indicate that Runx3 promotes the expression of CD8 lineage genes in mature T cells (13, 15) and suggest that it contributes to the establishment in the thymus of gene expression programs specific for the CD8 lineage, an activity often referred to as lineage specification (25).

The central role of Runx in CD8 lineage choice is underscored by the finding that Runx-deficient MHC-I-restricted thymocytes not only fail to become CD8 cells, but in fact become CD4 T cells (16, 19). Thus, Runx activity is necessary for CD8 lineage commitment. However, there is evidence that Runx is not the only activity necessary for CD8 commitment; enforced expression of Runx3 does not redirect MHC-II-restricted thymocytes into the CD8 lineage (21, 22), suggesting that CD8 lineage choice requires additional factors, expressed in MHC-I- but not in MHC-II-signalized cells, that would cooperate with Runx molecules. We will return to this issue in discussing the transcriptional circuitry of lineage choice.

The identity of these factors is a critical but as yet unanswered question for our understanding of lineage choice, and we will return to this issue in discussing the transcriptional circuitry of lineage choice. Other than Runx proteins and their known cofactors (26), the only nuclear molecule shown so far to be specifically required for CD8 cell development is the transcription factor IRF1 (IFN-regulatory factor 1). Although IRF1 acts in part indirectly through its ability to promote MHC-I expression by thymic stromal cells, it also promotes CD8 differentiation in a direct, thymocyte-intrinsic manner (27). The T-box

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**FIGURE 1.** Thpok, Gata3, and Runx3 expression and function during lineage differentiation. T cell differentiation stages are depicted on a schematic two-parameter plot of CD4 and CD8 expression. Expression levels of Gata3, Thpok, and Runx3 are denoted as undetectable (–), low (lo), intermediate (int), and high (hi). Checkpoints controlled by each factor are indicated (arrows). CD4-differentiating cells are shown in purple, and CD8-differentiating cells in green. There is no experimental distinction yet between specification and commitment steps during CD8 lineage differentiation. Although inactivation of both Runx1 and Runx3 is required to prevent CD8 cell development, this presumably reflects functional redundancy between these factors; thus, Runx3 is indicated as the “check-point keeper.”

**FIGURE 2.** Runx3 and Thpok loci. A, Schematic representation of the Runx3 locus showing the distal and proximal promoters (arrows), and the splicing of exon 1 (distal promoter) into the coding sequence of exon 2. As a result, Runx3 proteins translated from either mRNA only differ by their amino-terminal extremity (bottom graphs; cyan and yellow coloring). Dashed lines indicate splicing. Exons are shown as boxes, with coding sequences depicted as thicker rectangles. B, The Thpok locus is schematically depicted. Coding sequences are in exons 2 and 3 and are depicted as thick orange rectangles. Exons 1a and 1b are transcribed from alternative start sites. The proximal (3’) site is used preferentially in mature CD4 T cells, whereas the distal site is preferentially active in early thymocytes (57). Binding sites for Gata3, Runx3, and Thpok proteins are shown as boxes underneath the sequence. The cis-regulatory elements identified by knockout or transgenic reporter analyses are indicated at the bottom of the graph and include the silencer, part of the distal regulatory element (DRE), the general T lymphoid element (GTE) active throughout T cell development (57), and the proximal enhancer (or proximal regulatory element (PRE); Refs. 19 and 57). Knockout analyses have shown that the proximal enhancer is required for sustained Thpok transcription in mature CD4 lineage thymocytes and T cells (48). Deletion of the segment encompassing both Gata3 sites (and the proximal enhancer) severely reduced Thpok transcription in bacterial artificial chromosome reporter analyses (bracket) (40). Drawings are not to scale.
protein Eomesodermin promotes effector differentiation of mature CD8 cells and notably the expression of cytotoxic genes, but it is not required for their intrathymic differentiation (28, 29). Other transcription factors, including Mazr, NF-κB, and E-box binding proteins, as well as the Notch pathway, have been proposed to be involved, positively or negatively, in CD8 cell development (30–34); their role(s) in this process and, notably, whether they affect lineage differentiation per se, remain to be clarified.

Making a CD4 T cell

Thpok and Gata3 are required for CD4 cell development. Two CD4-differentiating transcription factors, Thpok (the product of a gene officially named Zbtb76 that will hereafter be referred to as Thpok for simplicity) and Gata3, were identified during the last few years. Thpok belongs to a large family of transcription factors that generally act as repressors and are characterized by a carboxyl-terminal DNA binding domain made of multiple zinc fingers (four in Thpok) and an amino-terminal BTB-POZ domain that mediates homodimerization (and possibly heterodimerization) (35). Thpok was identified as essential for CD4 differentiation after a patient quest to identify a spontaneous mutation (“helper deficient”) that causes a disruption of mouse CD4 cell development (36, 37). The culprit proved to be a single amino acid substitution in the second zinc finger of Thpok (38). In a separate study (39), Thpok (then named kRox) was identified in a microarray screen for genes up-regulated during positive selection and shown by gain-of-function analyses to inhibit CD8 differentiation and promote CD4 differentiation.

Two properties of Thpok deserve emphasis. First, although Thpok is expressed in a wide variety of cells, its expression in the thymus is highly lineage specific (38, 39): CD4 single-positive (SP) thymocytes (and all CD4 T cells) express Thpok, whereas DP and CD8 SP thymocytes do not. During MHC-II-induced selection, Thpok is up-regulated progressively as thymocytes down-regulate CD8 (Fig. 1). Second, both loss-of-function and gain-of-function experiments indicate that Thpok affects lineage choice but not positive selection. That is, Thpok-deficient, MHC-II-restricted thymocytes become CD8 instead of CD4 T cells. This analysis, initially performed in helper-deficient mice (37), was the first genetic demonstration that lineage choice and positive selection were independent, even if contemporaneous, processes. This was recently recapitulated in mice carrying a null allele of Thpok (16, 19, 40), demonstrating that lineage redirection does not result from an aberrant activity of the mutant protein. Conversely, transgenic expression of Thpok in MHC-I-restricted thymocytes prevents their CD8 differentiation and redirects them into the CD4 lineage (38, 39). The simplest interpretation of these experiments is that Thpok controls a key checkpoint after MHC-II-signalled thymocytes are rescued from programmed cell death, at which point it is required for CD4 lineage commitment and its absence redirects cells into the CD8 lineage. This Thpok-operated checkpoint occurs after the down-regulation of CD8 expression, as Thpok-deficient, MHC-II-signalled thymocytes are arrested as CD4+CD8low (16, 19, 40). Of note, both corepressor expression and functional differentiation are mismatched to MHC specificity in redirected thymocytes: Thpok-deficient, MHC-II-restricted cells express CD8 and cytotoxic markers, whereas Thpok transgenic MHC-I-restricted cells express CD4 and helper markers (16, 38, 39).

Although the spotlight has recently been on Thpok, the first transcription factor identified as necessary for CD4 but not CD8 cell differentiation is Gata3, a member of a distinct zinc finger protein family (41–43). Gata3 is expressed at and required for multiple steps of T cell development, from early T lineage specification to effector differentiation of mature CD4 cells (44). Positive selection is accompanied by asymmetric changes in Gata3 expression (Fig. 1), which is low but detectable in DP thymocytes and up-regulated in CD4-differentiating thymocytes, but goes down during CD8 differentiation (42, 45). Loss-of-function analyses using conditional deletion of Gata3 in DP thymocytes or retroviral knockdown short hairpin RNA transduction, showed that Gata3 is required for the development of CD4 but not CD8 cells (41, 42).

Gata3, Thpok, and CD4 lineage differentiation. A recent study compared the functions of Gata3 and Thpok during CD4 differentiation (40). Similarly as for Thpok, Gata3 is required before CD4 lineage commitment; that is, Gata3-deficient, MHC-II-restricted thymocytes can be redirected to the CD8 lineage. However, when assessed on thymocytes with a diverse endogenous TCR repertoire, this redirection is not as efficient as that of Thpok-deficient cells (40), which probably explains why it is observed with some but not all TCR specificities (40, 41, 46). Analyses of Thpok and Gata3 expression in thymocytes undergoing positive selection in the absence of the other molecule defined the sequence of intervention of these factors. The normal up-regulation of Gata3 was observed in Thpok-deficient, MHC-II-restricted thymocytes; in contrast, no Thpok was detected in Gata3-deficient thymocytes. Thus, Gata3 is required for the expression (and presumably acts upstream) of Thpok, which does not exclude the possibility that Gata3 also promotes the terminal differentiation or survival of CD4 cells as originally proposed (41).

These findings raised the tantalizing possibility that Gata3 is needed for CD4 cell differentiation simply to promote Thpok expression. However, this is not the case: enforcing Thpok expression in Gata3-deficient thymocytes (using a Thpok transgene) does not restore their CD4 differentiation (40), indicating that Thpok requires Gata3 to promote CD4 differentiation. Thus, Gata3 is required before commitment to the CD4 lineage and serves to promote the expression of Thpok and other factors required for CD4 lineage differentiation.

Strikingly, although Gata3 is required for Thpok to promote CD4 differentiation, Gata3 is not required for Thpok to inhibit CD8 differentiation, as transgenic expression of Thpok blocked the CD8 differentiation of Gata3-deficient cells (40). Clues to interpret this observation came from studies of Thpok function in postthymic T cells. When retrovirally transduced into mature CD8 cells, Thpok represses the expression of CD8 lineage genes, including those encoding CD8 and cytotoxic effectors perforin and granzyme B (47). Conversely, conditional Thpok disruption in mature CD4 cells causes CD8 re-expression and promotes the expression of granzyme B (23). In contrast, gain-of-function and loss-of-function analyses suggest a lesser role for Thpok in promoting expression of CD4 or CD4 lineage genes (23, 47). Another key observation comes from mice carrying an hypomorphic Thpok allele that directs the expression of insufficient levels of a wild-type Thpok protein. Although such low-level
Thpok expression supports the generation of CD4 SP thymocytes and T cells, these cells inappropriately express CD8 lineage genes (16, 23, 48).

It is useful at this point to come back to the distinction between specification and commitment (25). The idea is that lineage-specific gene expression programs are progressively specified in response to external cues in precursors that are initially bipotent and have been primed into a nonpolarized gene expression pattern. Such lineage divergence remains reversible until the cells undergo commitment, which is biologically defined as the loss of alternate development fates (25). In this context, the findings summarized above suggest that the main function of Thpok is not to promote CD4 lineage gene expression but to prevent the onset of CD8 lineage gene expression; that is, to promote CD4 lineage commitment; they also indicate that this committing activity would not require Gata3 (40). Conversely, the fact that Thpok fails to rescue the CD4 differentiation of Gata3-deficient thymocytes indicates that Gata3 is required for CD4 lineage specification (40).

The fact that Thpok is not required for CD4 specification was actually established by assessing the development of thymocytes deficient for both Thpok and Cbfb (16). These cells carry a germline Thpok disruption and delete a conditional Cbfb allele (thereby losing Runx activity altogether) in pre-DP thymocytes. Whereas Thpok-deficient, MHC-II-restricted thymocytes are redirected into the CD8 lineage, double-deficient (Thpok and Runx) cells adopt a CD4 fate. Thus, Thpok is not needed to specify the CD4 lineage, but it prevents Runx-directed CD8 lineage differentiation, supporting the idea that it serves to promote CD4 commitment. The presence of CD4 lineage cells in Thpok-Runx double-deficient mice, but not in Thpok single-deficient mice, suggests that Runx inhibits the expression or function of one or more CD4 lineage-specifying factors that direct Runx-deficient cells toward the CD4 lineage. Gata3 is an interesting candidate for this function, as analyses in peripheral T cells suggest that Runx activity represses Gata3 expression (49), and the same may be true in thymocytes.

**Gata3 promotes CD4 lineage specification.** The role of Gata3 in CD4 lineage specification has yet to be fully explored. There is evidence that Gata3 directly activates CD4 lineage genes, as chromatin immunoprecipitation (ChIP) experiments documented binding of Gata3 to two distinct sites within a region of the Thpok locus important for its expression (Fig. 2B) (40). Although the contribution of each of these sites to Thpok expression is not yet known, these findings suggest that Gata3 serves as a direct activator of Thpok transcription. In addition (or alternatively) to such a direct effect on CD4 lineage genes, it is possible that Gata3 promotes the expression of genes that are not lineage specific but are nonetheless required for CD4 lineage choice and thereby brings MHC-II-restricted thymocytes to a stage where they are competent for CD4 lineage commitment. Specifically, in line with the concept that TCR signals of greater duration are required for CD4 differentiation than for CD8 differentiation (5), it is possible that Gata3 promotes the expression of genes that are important for TCR signal transduction. The absence of an obvious TCR signaling defect in Gata3-deficient DP thymocytes (41) does not exclude this possibility, as the phenotypic manifestations of Gata3 gene disruption depend on the half-life of Gata3 molecules and the products of their target genes, all of which are largely unknown at present.

In addition, it is conceivable that thymocytes undergoing positive selection, in which the expressions of TCR and Gata3 are higher than in DP cells, are more stringently dependent on Gata3 for TCR signaling. Supporting such possibilities, the expression of targets of TCR signaling (including TCR itself and the adhesion molecule CD69) is lower in Gata3-deficient than in Gata3-sufficient intrathymically signaled thymocytes (40, 41). Reduced TCR signaling in the absence of Gata3 would also explain the small numbers of CD8-directed MHC-II-restricted cells in Gata3-deficient thymi, as these cells may fail to complete their differentiation in the absence of appropriate signaling.

**A transcriptional network promoting CD4 cell differentiation.** Two other transcription factors, Myb and Tox, have been shown to promote CD4 cell differentiation. Myb, the product of the proto-oncogene c-myb, is highly expressed in DP thymocytes (unlike Gata3 or Thpok), down-regulated during the DP to SP transition, but remains higher in CD4- than in CD8-differentiating thymocytes. Inactivation of Myb in DP thymocytes impairs CD4 but not CD8 cell development; conversely, a constitutively active form of Myb (vMyb) inhibits CD8 T cell development without redirecting MHC-I-restricted thymocytes into the CD4 lineage (50, 51). Analyses of gene expression in Myb-deficient cells indicate that Myb is important for Gata3 transcription; accordingly, transient transfection experiments and ChIP assays suggest a direct activation of the Gata3 promoter by Myb (51). Such an effect would explain why Myb is important for CD4 cell differentiation, but not the strong inhibition of CD8 cell development by vMyb, because enforced Gata3 expression only modestly inhibits CD8 lineage differentiation (Ref. 52; K. Wildt and R. Bosselut, unpublished observations).

Tox, an high mobility group domain-containing nuclear protein, is required for the development of CD4 cells and acts upstream of Thpok (53). Tox disruption also modestly impairs CD8 cell development, suggesting that it is important for a late positive selection step in addition to, or rather than, CD4 lineage differentiation per se. It is difficult at present to locate the checkpoint controlled by Tox. The requirements for Tox, Gata3, and Thpok during the differentiation of NK T cells suggest that Tox acts upstream of Gata3 and Thpok. NK T cells recognize CD1d-bound lipid Ags through aβ TCRs with limited diversity (54) and normally are either CD4 CD8 - or CD4 CD8-. Tox is more stringently required than Gata3 or Thpok for the differentiation of NK T cells (Refs. 53 and 55; L. Wang, A. Bendelac, and R. Bosselut, unpublished observations), which is consistent with the idea that Tox affects a developmental step independent of and preceding Gata3 and Thpok up-regulation and CD4 lineage differentiation. However, arrested Tox-deficient conventional thymocytes, which display a unique CD4lowCD8low surface phenotype, have up-regulated Gata3 (53), suggesting that Tox is not simply upstream of Gata3 in a linear cascade of transcription factors promoting CD4 differentiation.

Although additional studies will be needed to connect Tox, Gata3, and Thpok transcription factors, recent findings have established significant links between Runx factors and Thpok, on which the rest of this review will focus.
CD4 and CD8 cells, suggesting that, similarly as for which the silencer had been deleted. GFP was expressed in both lineage cells (19, 57). The importance of this element was demonstrated in mice carrying hypomorphic Thpok alleles and displaying aberrant CD8 lineage gene expression (16, 23, 48). Thus, as had been proposed early on (56), Thpok represses Runx3 expression, and future studies will determine whether this activity of Thpok is direct or not.

Reciprocally, Runx molecules repress Thpok expression, as shown notably by the aberrant expression of Thpok in Runx-deficient DP thymocytes (19). There is evidence that this repression is direct (19) and is carried out through the recruitment of Runx complexes to a silencer located 3.1-kbp upstream of the Thpok gene (Fig. 2B). The silencer, part of a distal regulatory element that displays enhancer activity in some contexts (57), was shown by transgenic reporter analyses to prevent inappropriate expression of Thpok in DP thymocytes and CD8 lineage cells (19, 57). The importance of this element was demonstrated in mice carrying a knock-in Thpok-GFP allele from which the silencer had been deleted. GFP was expressed in both CD4 and CD8 cells, suggesting that, similarly as for Cd4, transcriptional repression is essential to restrict the expression of Thpok to the CD4 lineage (19). ChIP analyses have identified two Runx binding sites within the silencer (19), and one study found that these sites are required for proper Thpok repression in CD8 cells (19). However, another study mapped active sites of the silencer to a 80 bp core located upstream of, and thus not including, the Runx binding sites (57). Detailed silencer mutagenesis by homologous recombination will be useful to delineate the respective contributions of these regulatory elements to Thpok expression.

Initiating Thpok and Runx3 expression. The picture emerging from these findings is that of a negative regulatory loop connecting Thpok and Runx3 and leading to a “winner take all” expression of one of these factors only (Fig. 3). Indeed, analyses with knock-in fluorescent reporter alleles suggest that there is no high-level coexpression of Thpok and Runx3 in wild-type thymocytes (16). Such a scenario, which has been proposed as a general mechanism for lineage divergence (58), raises two questions: what represses expression of these factors in DP thymocytes (in which neither is appreciably expressed), and what promotes the expression of either of them (and thereby the repression of the other) in cells that undergo positive selection.

Although cis-regulatory analyses of Thpok and Runx3 genes are not yet advanced enough to answer these questions, we think two important notions emerge from analyses of the Thpok silencer (19). First, the silencer serves two successive functions, similar but not identical, during T cell development. As discussed above, it restricts Thpok expression to the CD4 lineage by preventing its up-regulation in MHC-I-restricted thymocytes, an activity presumably involving Runx3. In addition, the silencer represses Thpok in preselection DP cells (and thereby differs from the Cd4 silencer that is inactive in DP thymocytes). This function of the silencer depends at least in part on Runx, because low-level Thpok expression can be detected in Runx-deficient preselection DP thymocytes (19), unlike in their wild-type counterparts (38, 39). As Runx3 is not expressed in DP cells, these findings indicate that Thpok is kept in check in preselection thymocytes by silencer-recruited complexes, presumably nucleated around Runx1. The second notion stems from the observation that Thpok expression in silencer-deficient DP thymocytes remains much lower than in mature T cells (19). Thus, activating factors acting on additional Thpok cis-regulatory elements (Fig. 2B) (19, 48, 57) must promote Thpok transcription in cells undergoing selection.

How this circuitry operates to restrict Thpok expression to MHC-II-signaled thymocytes is obviously a key question. Two distinct but nonmutually exclusive frameworks must be considered when addressing this question, depending on whether external committing signals are delivered to MHC-II- or MHC-I-restricted thymocytes. First, it is possible that signals specific to MHC-II-restricted thymocytes cause Thpok up-regulation (and therefore decide CD4 commitment). The simplest perspective would be that such signals up-regulate Thpok activators that bind a positive cis-regulatory element within the locus, thereby overcoming silencer activity. A significant obstacle to this perspective is that expression of the silencer-deficient Thpok-GFP allele is as high in CD8 as in CD4 SP Thpok-sufficient thymocytes (19). Thus, in the absence of silencer activity, similar Thpok expression levels can be reached in MHC-I- and MHC-II-signaled cells, and Thpok activators are not specific to MHC-II-signaled cells.
findings suggest that silencer activity is dominant over Thpok activators induced by positive selection and is the key restraint on Thpok expression; they lead to a variation on this first theme, whereby signals specific to MHC-II-restricted thymocytes would antagonize silencer activity and allow Thpok up-regulation (19). Such signals could conceivably target the activity of Runx1 molecules or the expression or activity of other factors important for silencing. Along this line, Thpok molecules were shown to bind the Thpok silencer by ChIP analyses, suggesting that Thpok counteracts silencer activation and therefore contributes to a positive feed back loop promoting its own expression (48). Although this mechanism may be important for sustained Thpok expression in mature CD4 cells, it is unclear whether it contributes to initial Thpok up-regulation, as analyses with a Thpok-GFP reporter allele indicate that high-level Thpok expression in the thymus does not require expression of Thpok molecules (Refs. 16 and 48; L. Wang and R. Bosselut, unpublished observations).

From the point of view of the circuitry, the reciprocal framework, namely that the lineage specificity of Thpok expression results from CD8-committing signals delivered to MHC-I-restricted thymocytes, is equally possible. The idea here is that sustained silencer activation in response to such signals keeps Thpok activators in check and prevents its up-regulation in these cells. Although it is possible that such signals would act by up-regulating Runx3, it is also possible that they activate the silencer by extending or increasing the repressive activity of Runx1-nucleated complexes. That second possibility would fit with the observations that the activity of the Runx3 distal promoter does not require MHC-I-specific signals, as it is expressed in Thpok-deficient, MHC-II restricted thymocytes, and that Thpok is kept silent in MHC-I-restricted thymocytes before detectable Runx3 up-regulation (16).

**Plugging-in environmental cues**. It is time to let environmental signals enter this discussion. This has been a controversial subject, and the reader is referred to previous reviews for a full perspective on the topic (5, 56, 59, 60). Within the last few years, however, a strong case has emerged that the duration of TCR signaling during selection determines lineage choice, with persistent TCR signaling promoting CD4 lineage choice and transient TCR signaling promoting CD8 lineage choice (5).

The kinetic signaling model offers an elegant rationale as to why TCR signaling would persist longer in MHC-II- than in MHC-I signaled thymocytes (61, 62). It proposes that TCR signaling in DP thymocytes (regardless of MHC specificity) down-regulates the expression of the Cd8 gene and, therefore, CD8 surface proteins, but not the expression of Cd4. In this perspective, the down-regulation of the coreceptor necessary for MHC-I-induced signaling generates a signaling asymmetry that is the core engine of lineage choice (62). This basic idea is supported by analyses of T cell development in mice engineered to prematurely terminate TCR signaling or CD4 expression in response to positive selection signals (63, 64).

As previously noted (5, 65), the core kinetic signaling concept, namely that signaling persists longer in MHC-II- than in MHC-I-signalized cells, fits with the idea that such persistent signals trigger Thpok expression in MHC-II-restricted thymocytes. Indeed, there is evidence that TCR signaling promotes Thpok expression (57), although the absence of appropriate in vitro conditions for CD4 lineage differentiation complicates these analyses. The kinetic signaling model also proposes that IL-7 cytokine signaling contributes to CD8 lineage choice (62). In line with this possibility, it has been proposed that IL-7 activates the Thpok silencer by inducing Runx3 (5), highlighting that the two frameworks discussed above are complementary rather than mutually exclusive.

Much remains to be done to identify intermediates between environmental signals and the core Runx-Thpok loop. The best candidate to date has been Gata3, owing to its greater up-regulation in MHC-II- than MHC-I-signalized thymocytes (42, 45). Furthermore, the potential positive effect of Gata3 on TCR signal transduction would place Gata3 at the center of a positive feedback loop amplifying differences in TCR signaling and converting them into qualitatively distinct outcomes (66). However, enforced expression of Gata3 in developing thymocytes does not significantly affect lineage choice (Refs. 42 and 52; K. F. Wildt and R. Bosselut, in preparation), suggesting that up-regulation of Gata3 expression is not the key link between TCR signals and Thpok expression.

Although the identification of the negative feedback loop between Thpok and Runx is a major progress in our understanding of CD4-CD8 differentiation, there is considerable evidence that this loop requires other factors to operate properly, and such factors are potential targets for environmental signals. First, Runx activity is not sufficient for Thpok repression; Runx binds the Thpok silencer not only in DP thymocytes and in CD8 cells but also in CD4 cells that express Thpok (19), and there is evidence that additional segments of the silencer are required for its function (57). Accordingly, as has been alluded to previously, Runx activity is not sufficient to prevent CD4 differentiation, because enforcing Runx1 or Runx3 expression in thymocytes does not per se impair CD4 lineage differentiation (21, 22, 67) or Thpok expression (L. Wang, K. F. Wildt, and R. Bosselut, manuscript in preparation). Although Runx3-transgenic mice have reduced CD4 SP cell populations, this is due to Runx3-mediated inhibition of CD4 expression in DP thymocytes, as normal numbers of CD4 cells develop in Runx3-transgenic mice lacking the Cd4 silencer in which CD4 expression is insensitive to Runx3 (21). Altogether, these findings make a strong case that Runx activity represses Thpok and promotes CD8 commitment in cooperation with other rate-limiting factors expressed in MHC-I- but not in MHC-II-signalized cells. It is possible that these factors are similar or identical to those proposed to bind the Cd4 silencer and to cooperate with Runx to repress Cd4 (68), although the composition or regulation of Cd4- and Thpok-repressing complexes likely differs because Cd4 but not Thpok is expressed in DP thymocytes.

**Conclusions and perspectives**

The last few years have seen substantial progress in our understanding of CD4-CD8 lineage differentiation. Key transcription factors promoting CD4 (Thpok, Gata3) or CD8 (Runx3) lineage differentiation have been identified, setting the foundation for the transcriptional regulatory network controlling lineage differentiation. These new findings have brought new challenges and placed older ones under a new spotlight. Among the latter is connecting transcription factors with signals that contribute to lineage choice, including determining whether
and how Thpok is induced by TCR signals. Another area of investigation concerns the unknown factors that promote CD8 lineage differentiation and may contribute to the repression of Thpok or Cd4 in CD8 lineage cells and possibly to the lineage-specific activity of some Cd8 enhancers (8). Perhaps most critically, the progress made in identifying DNA binding proteins that control lineage choice has yet to be matched in understanding the mechanisms of gene activation or silencing. Although conditional deletion of the Cd4 silencer indicates that Cd4 silencing is epigenetically maintained in postthymic CD8 cells (69), it remains to be determined how the Cd4 locus evolves from reversible repression in CD8-differentiating thymocytes to epigenetic silencing in mature T cells, and more generally whether and how the transcription factors that control lineage choice in thymocytes deposit epigenetic marks that restrain gene expression in postthymic cells (70).

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