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Maintaining CD4-CD8 lineage integrity in T cells: where plasticity serves versatility

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Abstract

The divergence of the two $\alpha\beta$ T cell subsets defined by the mutually exclusive expression of CD4 and CD8 glycoproteins is an important event during the intrathymic differentiation of T lymphocytes. This review briefly summarizes the mechanisms that promote commitment to the CD4 or CD8 lineage in the thymus, and discusses the transcription factor circuits and epigenetic mechanisms that concur to maintain lineage integrity in post-thymic cells and how they adjust to allow expression of genes required for effector cell differentiation.

CD4 and CD8 T cells

The two T cell subsets defined by the expression of CD4 and CD8 form the bulk of T cells carrying an $\alpha\beta$ TCR and recognizing peptides bound to class I or class II Major Histocompatibility Complex (MHC) molecules (MHC-I or MHC-II, respectively). The differentiation of these two subsets in the thymus has been particularly well studied [1-3] and has important characteristics. Its outcome is binary and defines two distinct lineages: mature T cells express CD4 or CD8 but not both, and their expression of either coreceptor is stable and propagated through cell division. CD4-CD8 differentiation matches MHC restriction, so that MHC II-restricted cells express CD4 and MHC I-restricted cells CD8. It also corresponds with functional differentiation, such that CD4 cells are precursors of helper or regulatory cells, whereas CD8 cells are cytotoxic precursors. Analyses in mice genetically engineered to generate T cells in which coreceptor gene expression is mismatched to MHC specificity have shown that function segregates with coreceptor gene expression rather than MHC specificity [4,5]. This indicates that the same differentiation process determines coreceptor expression and functional differentiation, and that this process is normally set to match lineage differentiation to MHC specificity. Because of this coordinate matching of lineage and functional differentiation to MHC specificity, CD4-CD8 lineage 'choice' is thought to be central to the function of the immune system.

CD4-CD8 lineage differentiation is a late event during the intrathymic development of $\alpha\beta$ T cells (Fig. 1) [6]. It occurs in committed $\alpha\beta$ precursors that have rearranged both genes encoding their TCR chains (TCR β and TCR α) and started expression of both CD4 and CD8 coreceptors, thereby becoming 'double positive' (DP) thymocytes. In fact, most DP thymocytes carry TCRs unable to productively bind self-MHC peptide complexes expressed

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on the thymic stroma, and die in the thymic cortex by 'neglect' [7,8]. Only cells with appropriate avidity for such self ligands survive, a process called positive selection, and differentiate into mature T cells; the 'choice' of the CD4 and CD8 lineage is associated with that process. These selection and differentiation events complete the elaboration of the T cell repertoire, resulting in large populations of cells that are antigen specific, long lived and, for most of them, functionally dormant. Such cells populate peripheral lymphoid organs and are unique in that they are neither terminally differentiated nor post-mitotic. Upon antigen recognition, they proliferate and acquire effector properties that largely depend on environmental (notably cytokine) signals [9-11].

The basic CD4-CD8 commitment machinery in the thymus

This review briefly surveys the transcriptional control of the differentiation of CD4 and CD8 lineages in the thymus, and discusses how transcriptional components that enact this 'decision' are subsequently re-used during the differentiation of CD4 and CD8 effector T cells. We will refer the reader to recent reviews [1-3] for a discussion of the extra-cellular signals and transcription factors that control CD4-CD8 choice, and focus on the lineage commitment machinery, of which two key components have been identified.

The zinc finger transcription factor Thpok (also called cKrox, and encoded by a gene named *Zbtb7b* that we will refer to as *Thpok*) is expressed by all CD4 cells and is critical for CD4 lineage commitment [12-15]. Thpok is normally not expressed by DP thymocytes, and is up-regulated, through mechanisms that have yet to be elucidated, in MHC II- but not MHC I-signaled thymocytes [12,16]. The role of Thpok in CD4 T cell differentiation is demonstrated in *Thpok*-deficient mice, in which MHC II-restricted T cells are in normal or subnormal numbers but differentiate into the CD8 rather than the CD4 lineage [13-15,17]. Both loss- and gain-of-function analyses have shown that Thpok is a repressor of CD8-lineage gene expression, including of *Cd8* genes themselves [18,19] and of *Runx3*, a transcription critical for CD8-lineage differentiation (see below) [14,15,19]. Accordingly, enforced Thpok expression in developing MHC I-restricted thymocytes prevents their CD8-lineage differentiation [12,16]. Altogether, these studies indicate that Thpok, through its ability to repress CD8-lineage genes, is a key factor for CD4 commitment (biologically defined as the loss of CD8 developmental potential). Its contribution to the expression of CD4-lineage genes, which we will refer to its 'specification' function, remains to be fully evaluated.

On the other hand, Runx transcription factors are essential for CD8 T cell differentiation. In mammals, the Runx family includes three molecules (Runx1, Runx2, Runx3, of which Runx1 and Runx3 are expressed in T cells), with strong conservation across species and involvement in multiple differentiation processes [3,20]. Runx molecules can serve as transcriptional activators, often in synergy with other transcription factors, or as transcriptional repressors; in both instances, they bind DNA as obligate heterodimers with the unrelated factor Cbf β .

The expression of Runx1 and Runx3 in T cells follows different patterns. Runx1 is expressed at most stages of T cell development. Its expression is lower in CD8- than in CD4-lineage cells [21-23]. accordingly, while disruption of *Runx1* in the $\alpha\beta$ lineage (i.e. at the DP stage using *Cd4*-Cre excision of a conditional *Runx1* allele) has no known consequence on CD8 cell generation, it has pleiotropic effects in CD4 T cells [23,24]. In contrast, Runx3 expression in thymocytes is largely limited to CD8-lineage cells and 'non conventional' subsets (e.g. $\gamma\delta$ or NK T cells) [15,23-26]. *Runx3* disruption in the $\alpha\beta$ T lineage impairs several aspects of CD8 T cell differentiation [23-25]. *Runx3*-deficient CD8-lineage cells are in reduced numbers and fail to properly silence *Cd4*, so that some appear

CD4⁺CD8⁺ in peripheral lymphoid organs. Deletion of one *Runx1* and both *Runx3* alleles causes all remaining CD8 cells to express CD4, whereas complete disruption of Runx activity prevents CD8 T cell development [15,25,27]. This indicates that the subdued phenotype of Runx3-deficient cells is due to compensation by Runx1; indeed, the potential for functional redundancy between the two genes is increased by the enhanced expression of Runx1 in Runx3-deficient CD8 cells [23]. Altogether, these findings implicate Runx3 as critical for the generation of CD8 cells in the thymus.

Runx activity both promotes expression of genes characteristic of the CD8 lineage (a 'specification' function), including those encoding CD8 α and CD8 β subunits and cytotoxic enzymes such as perforin and Granzyme B [28], and the commitment of T cell precursors to the CD8 lineage. The committing function has been exceptionally well documented at the molecular level. Runx3 binds and represses the two defining CD4-lineage genes, *Cd4* and *Thpok*. In the *Cd4* locus, Runx-binding sites are critical components of the *Cd4* silencer, an intronic element required to restrain the expression of *Cd4* to CD4-lineage cells (Fig. 2) [24,29,30]. In addition to its effect on *Cd4*, Runx3 is thought to contribute to *Thpok* repression in CD8-differentiating thymocytes. The repressive effect of Runx3 on *Thpok* has similarities and differences with that on *Cd4*, and its analysis is also complicated by its functional redundancy with Runx1 [27]. Similar to *Cd4*, Runx binding sites have been identified within a silencer (itself part of a 'Distal Regulatory Element' [DRE]) located upstream of the *Thpok* promoter and needed to prevent *Thpok* gene expression in DP thymocytes and CD8 cells (Fig. 3)[27,31]. The current evidence indicates that Runx1 and Runx3 physiologically contribute to *Thpok* repression at distinct developmental stages. However, the developmental 'timing' of repression is different from *Cd4*: in DP thymocytes, Runx1 binds the *Thpok* silencer and this binding contributes to prevent premature transcription [27]. Biochemical and genetic evidence suggest that Runx3 performs the same function in CD8-differentiating thymocytes in a manner redundant with Runx1 (as only the complete disruption of Runx activity, not that of Runx1 or Runx3 separately) 'redirects' MHC I-restricted thymocytes into the CD4 lineage [23,24,27].

These findings have led to a model in which the commitment engine in the thymus is a dual negative regulatory loop involving both Runx1 and Runx3, and *Thpok* (Fig. 3). One prominent characteristic of this loop is to produce a binary developmental outcome where cells become CD4 or CD8, respectively expressing either *Thpok* or *Runx3* but not both, thereby preventing 'partial' differentiation status. Indeed, analyses in mice carrying hypomorphic *Thpok* alleles, that express reduced levels of a wild-type *Thpok* protein, indicate that even in such a situation the outcome of differentiation remains binary, with generation of cells expressing CD4 or CD8 but not both [15,19]. Similar regulatory modules have been shown to operate in other biological systems and have long been identified for their ability to unambiguously separate binary fates [32,33].

Important regulatory components are probably missing from this simple model. Notably, Runx proteins are necessary but not sufficient for *Thpok* repression as enforced expression of *Runx3* in DP thymocytes fails to prevent *Thpok* expression and CD4 T cell differentiation [34]. Accordingly, genetic analyses of *Thpok* cis-regulatory elements have identified motifs aside from Runx-binding sites important to restrain *Thpok* expression to MHC II-restricted thymocytes [31]. This suggests that other factors cooperate with Runx molecules to promote *Thpok* repression. The BTB-POZ zinc finger transcription factor Mazr, recently shown to bind the *Thpok* silencer in DP thymocytes and to contribute to *Thpok* repression in DP thymocytes [35], is one such molecule, and others may remain to be discovered. Because Runx1 is normally expressed in CD4-differentiating thymocytes and post-thymic CD4 cells, it is likely that repressing the expression of such additional factors, or overcoming their

activity, is essential for *Thpok* up-regulation during the differentiation of CD4 T cells in the thymus.

Given the importance of the *Thpok*-*Runx* dual negative regulatory loop for CD4-CD8 commitment in the thymus, the question arises whether it continues to operate in post-thymic T cells. As we will see, although naïve post-thymic T cells express only either *Thpok* or *Runx3*, that is not the case of differentiating effector cells, which can express both factors and yet retain their defining *Cd4* or *Cd8* gene expression. These observations raise the question of how CD4-CD8 lineage integrity is maintained in post-thymic T cells, which we will now discuss.

Maintaining CD8 lineage integrity: the case for rigidity

Epigenetic silencing of CD4-lineage genes in CD8 cells

The studies that addressed how mature T cells maintain their CD4-CD8 lineage differentiation have reached contrasting results for CD4 and CD8 lineage cells. In CD8 cells, *Runx3* is needed for cytotoxic effector differentiation, including expression of cytotoxic enzymes perforin and granzyme B, and of $\text{IFN}\gamma$, a prototypical CD8 cytokine [28]. In contrast, there is evidence that CD8 cells epigenetically silence CD4-lineage genes independently from *Runx3* expression [36]. This idea first emerged from studies of *Cd4* expression, using mice carrying a 'floxed' *Cd4* silencer element, whose excision by the Cre recombinase leaves a 'silencer-less' *Cd4* allele [37]. When the silencer is deleted in the germline or in DP thymocytes, all CD8 lineage cells maintain *Cd4* expression and therefore appear as $\text{CD4}^+\text{CD8}^+$ in peripheral lymphoid organs [37,38]. This demonstrates the critical role of the silencer, as both CD4- and CD8-lineage cells express transcription factors required for *Cd4* gene expression. In contrast, deletion of the silencer in post-thymic CD8 cells (that have developed with an intact silencer and are thus $\text{CD4}^-\text{CD8}^+$) fails to restore *Cd4* expression [37]. The simplest interpretation of these results is that epigenetic modifications of the *Cd4* locus, established during the late stages of intrathymic differentiation, irreversibly silence *Cd4* expression. Such 'silencing' is maintained and propagated through cell division even in the absence of the transcription factors (and cis-regulatory elements) that installed it in developing thymocytes, and is therefore distinct of 'active repression' that requires the continued recruitment of repression factors to relevant cis-regulatory elements [30,37,39]. Further supporting the idea of a stable epigenetic repression of CD4-lineage genes, enforced expression of *Thpok* in CD8 cells, by retroviral transduction, fails to cause *Cd4* gene reexpression, or expression of the CD4-lineage marker *Cd40lg*, although enforced expression of *Thpok* in thymocytes counteracts the *Cd4*-repressive function of *Runx3* [18,40].

Whether *Thpok* is also subject to epigenetic silencing in CD8-lineage cells remains to be determined, although the current evidence suggests this would not be to the same extent as *Cd4*. While *Thpok* expression is not detected in *ex-vivo* CD8 cells (whether naïve or memory) [12-16], analyses of mice carrying a 'silencer-less' *Thpok* reporter allele have suggested that CD8 cells have all transcription factors necessary for *Thpok* expression [27]. However, it remains to be determined, using conditional deletion analyses, if this reflects true epigenetic silencing of *Thpok*, similar to that seen for *Cd4*, or active repression mediated by the *Thpok* silencer in mature CD8 cells. Supporting the second possibility, and suggesting greater plasticity at the *Thpok* than at the *Cd4* locus, it was recently demonstrated that *in vitro* or *in vivo* activated CD8 cells express the *Thpok* gene [41,42]; this expression results in detectable *Thpok* protein expression and is important for the clonal expansion of CD8 effectors and their proper expression of cytotoxic enzymes upon rechallenge [41]. Thus, *Cd4* and *Thpok* repression in CD8 cells appear to involve distinct mechanisms on which future studies will shed light.

Silencing mechanisms: facts and hypotheses

How epigenetic silencing is enforced remains enigmatic, and mechanistic investigations of *Cd4* epigenetic silencing have yet to provide decisive clues. Imaging analyses of CD8-differentiating thymocytes have shown that *Cd4* loci are relocated to nuclear heterochromatin, an area typically concentrating silenced genes [43,44]. Whether this is a cause or a consequence of *Cd4* silencing remains to be determined; notably, *Cd8* genes undergo a similar relocation to heterochromatin in CD4-differentiating thymocytes, even though they are not subject to the same epigenetic silencing as *Cd4* (see below). In addition to subnuclear localization, covalent modifications of histones and DNA, namely methylation of histone H3 on lysine residues 9 and 27 (H3K9 and H3K27, respectively) and methylation of cytosines in CpG dinucleotides have been implicated in gene silencing [45-47]. Chromatin immunoprecipitation analyses have reported an enrichment in trimethyl H3K9 and H3K27 at the *Cd4* promoter in CD8 cells relative to CD4⁻CD8⁻ thymocytes (the precursors of DP thymocytes, in which transient silencer activity prevents premature *Cd4* expression [30]). Because *Cd4* is transiently repressed in CD4⁻CD8⁻ thymocytes but irreversibly silenced in CD8 cells, this finding raised the hypothesis that silencing is caused by these histone modifications [48]. Multiple enzymes can contribute to H3K9 methylation, often in a stepwise fashion, complicating genetic analyses of this process; as a result, it is too early to formulate conclusions on its role in *Cd4* silencing [49,50]. The role of H3K37 methylation has been evaluated by disrupting the gene encoding Ezh2, the enzymatic component of the Polycomb Repressive Complex 2 (PRC2), and main H3K27 methyl transferase activity in T cells [51]. These analyses did not find any CD4 re-expression by Ezh2-deficient CD8 cells, although it cannot at present be excluded that this is due to redundancy with the related Ezh1 molecule [52,53] or to the extended half life of H3K27 trimethylation, that would long survive the disruption of the *Ezh2* gene [54].

The role of DNA methylation in maintaining *Cd4* silencing has been addressed genetically, in mice carrying a conditional allele of the gene encoding the DNA methyl transferase Dnmt1 that propagates CpG DNA methylation during cell division (but does not contribute to *de novo* DNA methylation) [55]. Disrupting Dnmt1 activity in mature T cells promotes expression of IL-4 in CD8 cells, but was not reported to promote their expression of CD4 [55,56]. Furthermore, inhibition of DNA methylation by the inhibitor 5 aza-deoxycytidine does not cause CD4 re-expression by CD8 cells made 'silencer-less' by post-thymic excision of the *Cd4* silencer [37]. These results suggest that DNA methylation is not required to maintain *Cd4* silencing. One limitation inherent to such experiments is that DNA methylation has pleiotropic effects, notably on cell proliferation and survival. Indeed, enforced expression of the anti-apoptotic molecule Bcl-x_L rescues at least in part the development of thymocytes made Dnmt1-deficient before the DP stage of development [55]. As a result, it is conceivable that post-thymic cells made Dnmt1-deficient die prior to the point where removal of putative epigenetic marks on the *Cd4* gene would result in re-expression.

In summary, although genetic studies offer strong support to the idea of epigenetic silencing of CD4-lineage genes in CD8 T cells, the mechanistic bases for such silencing remain unclear.

Post-thymic enforcement of commitment in CD4 cells: the case for plasticity

The Thpok-Runx3 circuitry in post-thymic CD4 cells

Do CD4 cells use similar epigenetic mechanisms to prevent expression of CD8-lineage genes? Several observations suggest otherwise. First, although they do not re-express CD8,

effectors CD4 cells can express genes typical of cytotoxic effectors such as perforin or granzyme B. While the expression of such genes is typically much lower than in CD8 effector cells, it is 100-1000 times higher than in naïve CD4 cells [19]. Furthermore, it can be induced in CD4 cells by enforced expression of Eomesodermin, a transcription factor important for the proper differentiation of CD8 cells into cytotoxic effector or memory cells [57-59], suggesting greater plasticity of gene expression in CD4 than in CD8 cells. Another important point came from studies of CD4 cell effector differentiation. CD4 T cells can adopt multiple effector profiles, characterized by specific cytokine production and transcription factor expression patterns (Fig. 1). Notably, they can differentiate into Th1 effectors that make IFN γ and promote responses against intra-cellular pathogens, or into Th2 cells (that express IL-4, IL-5 and IL-13 and are essential for defenses against multicellular parasites) [9]. Quite strikingly, Th1 effector differentiation requires *Runx3*, and differentiating Th1 effector express as much Runx3 protein as CD8 cells, despite continued expression of *Thpok* (and *Cd4*) [15,19,60,61].

Thus, whereas CD8 cells silence or keep at low level the expression of *Thpok* and genes typical of the CD4 lineage, at least some CD4 effector displays high-level expression of genes typical of the CD8 lineage. This raises two reciprocal questions, that we will now address: how do such Runx3-expressing CD4 cells maintain *Cd4* and *Thpok* expression, and what role, if any, does *Thpok* have in post-thymic CD4 cells to control expression of Runx3 and CD8-lineage genes ?

First, why is it that expression of Runx3 in Th1 CD4 cells (or more generally enforced Runx3 expression in CD4 cells [34,62]) fails to repress *Cd4*, whereas it does so in DP thymocytes [34,63]? To some extent, similar issues are raised from the persistent *Runx1* expression in CD4-lineage thymocytes and T cells. Analyses in cell lines, and *in vivo*, have shown that in cells that co-express *Thpok* and Runx3, the former counteracts the *Cd4* silencing effects of Runx3 and thereby ensures *Cd4* expression [40]. Two hypotheses have been advanced to account for this observation (Fig. 2). First, *Thpok* has been shown to bind the *Cd4* silencer, and it has been proposed that such binding opposes the recruitment of Runx molecules or their ability to mediate repression [14]. Analyses of Runx1 binding to the silencer are consistent with this idea: although Runx1 is expressed in both DP thymocytes and CD4 T cells, it associates with the *Cd4* silencer in DP thymocytes that do not express *Thpok*, but not in mature CD4 T cells that do [48]. Second, experiments in cell lines assessing the activity of a *Cd4* reporter construct indicated that, paradoxically, the ability of *Thpok* to counteract Runx3-mediated *Cd4* repression required the activity of histone deacetylases, a family of enzymes that contribute to gene repression [40]. This suggests that *Thpok* counteracts Runx-mediated repression by inhibiting the expression of so far unknown genes whose products would be required for Runx3 to repress *Cd4*, so that their absence in *Thpok*-expressing CD4 cells would make *Cd4* insensitive to Runx. Further work will be needed to distinguish between these two possibilities, which are not mutually exclusive.

Results suggesting that the *Cd4* locus is subject to positive epigenetic control during positive selection shed yet another light on the persistent *Cd4* expression in Th1 effectors despite high levels of Runx3 [36]. Conditional deletion of the *Cd4* 'proximal enhancer' required for *Cd4* expression in thymocytes (Fig. 2) demonstrates that post-thymic deletion of the enhancer does not affect *Cd4* expression by effector T cells, suggesting that an 'open' epigenetic status was installed in the thymus; it is possible, although speculative at this point, that in this new epigenetic status *Cd4* is no longer sensitive to *Runx3* expression, even in the absence of *Thpok*. This idea would fit with the observation that activated CD4 cells maintain *Cd4* expression despite *Thpok* disruption [19] (see below). However, there is evidence for differential control of *Cd4* in activated and resting T cells [36,64]. Thus, it is possible that *Thpok*, even if not needed to maintain *Cd4* expression in Th1 effectors, is

necessary to protect *Cd4* expression from Runx1-mediated repression in resting CD4 cells [14].

The issues raised by the lack of *Thpok* repression in Runx3-expressing Th1 effectors are fundamentally similar to those we just encountered with *Cd4*. However, a few differences are worth noting and point to the possibilities the *Cd4* and *Thpok* silencer may not be regulated through the same mechanisms. Specifically, chromatin immunoprecipitation analyses in resting cells indicate that Runx complexes bind the *Thpok* silencer in both CD4 and CD8 T cells [27], and it seems reasonable to propose that the same would be true in Th1 effectors. Thus, even though Thpok molecules bind the *Thpok* silencer [14], such binding does not prevent the recruitment of Runx complexes, but would counteract their repressive activity. This interesting perspective raises the question of how Thpok, which belongs to a family of proteins primarily characterized as transcriptional repressors [65], could act to antagonize the repressive activity or Runx3 at the *Thpok* (or *Cd4*) silencer. Thpok was recently shown to bind and serve as a substrate for the p300 acetyl-transferase co-activator, which conceivably could convert Thpok into a transcriptional activator. However, acetylation was reported to increase Thpok stability and repressive activity [66], and further analyses will be needed to investigate how Thpok could antagonize gene repression.

Thpok represses CD8-lineage gene expression in post-thymic CD4 cells

Thus, the ability of Runx molecules to repress *Thpok* (and *Cd4*) is developmental stage- and cell type-dependent. On the other side of the same coin, the expression of Runx3 and cytotoxic genes in Th1 effectors suggests that Thpok does not serve to repress these genes in post-thymic CD4 cells, and that the antagonism between Thpok and CD8-lineage genes is unique to developing thymocytes. In fact, that is not the case, as demonstrated by eliminating Thpok from mature CD4 cells that have developed as Thpok-sufficient, using Cre-induced deletion of a conditional *Thpok* allele [19]. The most striking result of these experiments was that post-thymic expression of Thpok is required to prevent CD8 re-expression [19, and L.W. and R.B., unpublished data]. Reciprocally, gain-of-function analyses show that enforced Thpok expression in CD8 cells represses *Cd8* expression and notably targets the activity of the *Cd8E8(I)* enhancer, an element preferentially active in mature CD8 cells [18,39,67,68]. The repression of *Cd8* by Thpok fits with the idea that this factor, similar to the related proteins Bcl6, PLZF or LRF, would primarily serve as a transcriptional repressor, possibly by recruiting chromatin modifying complexes [65].

These initial observations in Thpok-deficient CD4 cells observations make two points. First, unlike *Cd4* which in CD8 cells is epigenetically silenced independently of Runx3, active repression is critical to maintain *Cd8* repression in CD4 cells. Current investigations are exploring the mechanistic bases of *Cd8* repression, and notably whether it involves direct Thpok recruitment to *Cd8* genes. Indeed, the second point is that such active repression relies at least in part on Thpok. Additional investigations, in *Thpok*-conditional mice and in mice carrying hypomorphic *Thpok* alleles, have shed light on the range and mechanisms of this repression. *Cd8* genes are not the only Thpok targets in CD4 cells: Thpok-deficient CD4 T cells express Runx3 [14,19], and specifically the isoform produced from the *Runx3* distal promoter that is normally characteristic of CD8 cells [23]. Their expression of perforin and granzyme B genes was increased relative to that of wild-type CD4 cells [14,19]. Such cytotoxic gene expression is Runx-dependent, as up-regulation of granzyme B in Thpok-deficient CD4 cells is inhibited by a dominant-negative version of Runx3 [19]. This suggests that a key function of Thpok in post-thymic CD4 cells is to repress Runx3, and this conclusion is further supported by preliminary observations that the *Cd8* re-expression by Thpok-deficient CD4 cells is partly dependent on Runx3 (L.W. and R.B., unpublished results). However, these observations should not mask the importance of Runx3-

independent mechanisms as wild-type Th1 effectors do not re-express CD8, despite their high Runx3 expression, indicative of an Runx3-independent Thpok repression of *Cd8*.

In vitro analyses suggest that Thpok-disruption in CD4 cells has functional consequences aside from *Cd8* or cytotoxic gene expression. Because Runx3 promotes Th1 at the expense of Th2 differentiation [60,61], the inappropriate expression of Runx3 by Thpok-deficient CD4 cells raised the possibility that their functional differentiation would be skewed towards the Th1 fate. Indeed, Thpok-deficient CD4 cells preferentially give rise to Th1 effectors when activated *in vitro* in the absence of polarizing cytokine signals [19]. Such Th1 differentiation is dependent on Runx activity. In addition, it is associated with (and largely dependent on) increased expression of the transcription factor Eomesodermin [19], which normally contributes to expression of IFN γ and of the cytotoxic program in CD8 effectors [57-59]. In contrast, it does not require T-bet, a transcription factor related to Eomesodermin and necessary to the Th1 differentiation of wild-type CD4 cells [69,70] (R.B. and L.W., unpublished observations). Such Th1 skewing does not result from the loss of Th2-differentiating potential, as, unlike CD8 T cells, *Thpok*-deficient CD4 cells become Th2 effectors if activated in the presence of the proper Th2-polarizing cytokine environment [19]. This finding, together with the persistent expression of CD4 and *Cd40lg* by Thpok-deficient CD4 cells [19, Y.X. and R.B., manuscript in preparation], are consistent with the hypothesis that the key function of Thpok in CD4 cells is to prevent inappropriate expression of *Runx3* and CD8-lineage genes, rather than to promote expression of CD4-lineage specific genes. By constraining Runx3 expression, Thpok would prevent 'preemptive' Th1 effector differentiation upon activation, and therefore keep open a wider range of effector differentiation potential for CD4 cells than there is for CD8 cells.

Why plasticity ?

The distinct mechanisms used by CD4 and CD8 cells to maintain lineage integrity correlate with their distinct effector differentiation potentials, specifically with the need for Runx3 during Th1 effector differentiation of CD4 cells. Whether such need reflects truly distinct biochemical 'functions' of Runx3 that cannot be performed by Runx1 (such as distinct sets of interaction partners), or simply distinct expression patterns (e.g. that Runx1 expression cannot reach levels needed for Th1 differentiation) remains to be determined. Regardless, the need for Runx3 during Th1 differentiation implies that CD4 T cells do not irreversibly silence *Runx3* during intrathymic CD4-lineage differentiation. In fact, plasticity is a recurring theme during CD4 effector cell differentiation, as highlighted by recent studies on the 'reversibility' of CD4 effector fates [11].

Conversely, the presence of Runx3 molecules in Th1 CD4 effectors requires active mechanisms to counteract their repression of genes essential for CD4 cells, including *Cd4* and *Thpok*, and its potential positive effect on *Cd8* genes. Both in resting cells and during effector differentiation, Thpok is an essential contributor to such mechanisms, but it is not the only one. Notably, the transcription factor Gata3, which is essential for Th2 differentiation, counteracts the effects of Runx3 during the differentiation of Th2 cells and specifically prevents its activation of IFN γ and Eomes expression [71].

A similar situation where cells epigenetically silence genes that have become useless (or potentially detrimental) and actively repress those for which there is a future need is encountered at the lineage separation checkpoint between T and NK cells [72]. These two lineages share many properties, notably the cytotoxicity and effector cytokine production patterns common to NK and CD8 T cells. Thus, it was of particular interest that the transcriptional repressor Bcl11b, recently shown to be required for T cell lineage commitment in the thymus, notably serves to avoid 'drifting' towards NK-like gene

expression [73-75] (even though it is also important for CD8 cells to undergo cytotoxic effector differentiation [76]). That is, disruption of *Bcl11b* in committed $\alpha\beta$ T cells (from the DN3 stage to post-thymic cells) results not only in a reduced expression of T cell specific genes, but in the appearance of NK-specific genes and the generation of NK-like cells with cytotoxic function [75]. In contrast, although T cell precursors maintain the potential to revert to a myeloid fate until the *Bcl11b*-operated commitment checkpoint, disruption of *Bcl11b* in committed T cells did not give rise to re-expression of typical myeloid genes, including *PU.1* and *C/EBP α* [73,74]. These findings are consistent with the possibility that T cells maintain an active and reversible repression of genes characteristic of the NK program, as some of these are part of the cytotoxic effector program used by CD8 effector T cells, whereas genes characteristic of the myeloid lineage would be epigenetically silenced.

Conclusions and perspectives

Three conclusions emerge from this comparison of processes that establish and maintain the divergence of CD4 and CD8 lineages. First, CD4 and CD8 cells use distinct mechanisms to maintain their lineage integrity, understood as the persistent repression of genes characteristic of the other lineage. In CD4 cells, the same molecule, *Thpok*, is instrumental to establish commitment (in the thymus) and maintain lineage integrity (in post-thymic cells). To the contrary, whereas CD8 cells use *Runx3* to establish commitment in the thymus, they seem to rely on *Runx3*-independent epigenetic silencing to repress CD4-lineage genes. Second, the co-expression of *Runx3* with *Cd4* and *Thpok* in Th1 CD4 effectors indicates that, although CD4-lineage integrity is maintained by an active *Thpok*-based circuitry, this circuitry differs from that operating in CD4-differentiating thymocytes. Third, the control of *Thpok* and *Cd4* expression is unlikely to follow similar rules as *Thpok* but not *Cd4* is expressed by activated CD8 cells, whereas *Cd4* but not *Thpok* is expressed in DP thymocytes. In addition, although both genes have silencer elements that recruit *Runx* proteins and can bind *Thpok*, *Runx* complexes bind the *Thpok* silencer in both immature thymocytes and mature T cells, whereas they bind the *Cd4* silencer in preselection thymocytes but not in CD4 cells.

The opposition between active repression and epigenetic silencing should not be overemphasized, as epigenetic mechanisms and active repression can cooperate to control gene expression. To stay within the scope of this review, *Cd8* repression in CD4 cells seems to epitomize such cooperation, as re-expression of *Cd8* by *Thpok*-deficient cells is variegated, suggesting an additional layer of epigenetic control [19]. However, the fact that no *Cd8* re-expression is observed in *Thpok*-sufficient cells indicates that such epigenetic control is subordinate to *Thpok* activity. More broadly, although there is evidence for self-maintained epigenetic silencing or activation that has become independent of cis-regulatory elements that installed them in the first place, there is also evidence of 'epigenetic' marks that are removed in response to differentiation signals [77-79]. This suggests that in many circumstances there is an interplay between active transcription factors networks and epigenetic marks. One attractive possibility is that signals remove methylation marks by recruiting histone de-methylases [80-82], whereas in the absence of such signals methylation marks are self-propagated.

Understanding which chromatin modifications promote epigenetic silencing and activation, and how they are installed in cells that undergo lineage commitment, are important challenges for current research. It is possible that transcriptional repressors recruit epigenetic marking complexes, including histone deacetylases and methylases, and thereby contribute to install epigenetic silencing [83], but studies in yeast and higher eukaryotes suggest additional mechanisms [84]. Notably, the possibility that non-coding RNAs would be

involved in gene silencing (and more generally in the control of gene expression) has recently attracted much interest [85,86]. Analyses in several systems, including X-chromosome inactivation, Hox gene expression and cell-cycle related genes have pointed to the ability of non-coding RNAs to repress transcription both in *cis* (on the gene they are transcribed from) and in *trans* [87,88]. Several mechanisms have been proposed to underlie such effects, including the repression of sense transcription by anti-sense transcription non-coding transcripts. In the case of the tumor-suppressor p15 gene, encoding a cell-cycle inhibitor, gain-of-function analyses suggest that non-coding anti-sense transcripts give rise to epigenetic silencing presumably mediated by heterochromatin formation [89]. Indeed, non-coding RNAs have been shown to recruit repressive or activating chromatin-modifying complexes [87], and future investigations will lift the veil on this important, yet poorly understood, aspect of epigenetic gene expression control.

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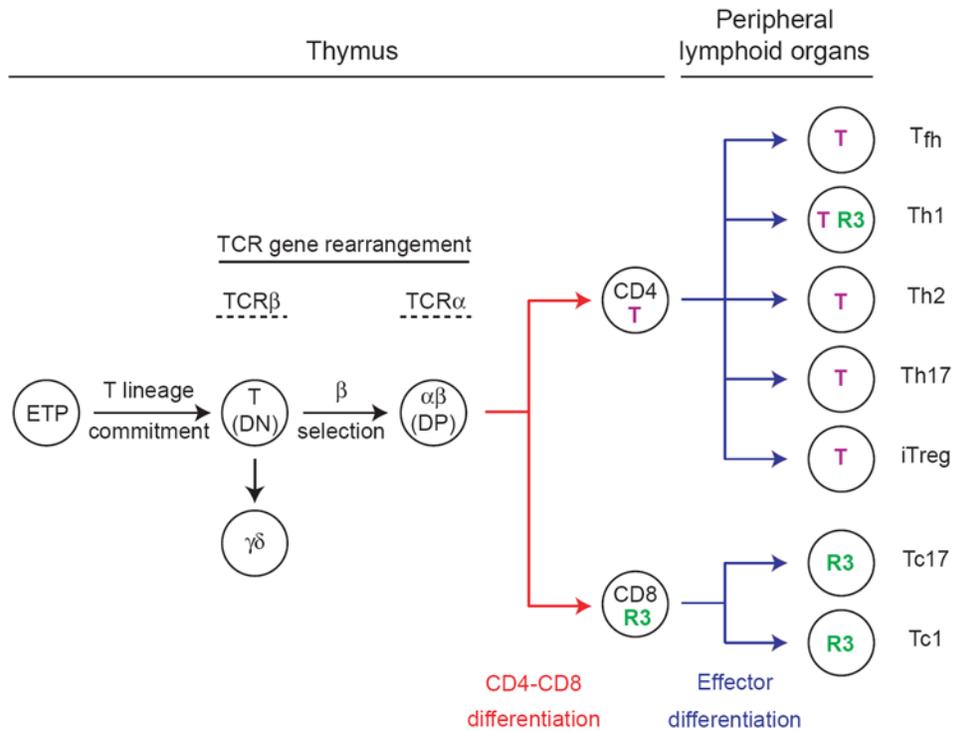


Figure 1. Outline of T cell differentiation

Key intermediates in T cell development are represented, including uncommitted thymic precursors (Early thymic progenitors, ETP), T-lineage committed cells (T) rearranging TCR genes prior to the onset of CD4 or CD8 expression ('double negative' cells, DN), $\alpha\beta$ lineage precursors (DP thymocytes) and $\gamma\delta$ T cells [for review, see Refs. 6,90,91]. Key developmental checkpoints are shown by arrows: commitment of early intrathymic precursors (at the DN2 stage) to the T lineage, commitment to DN cells to the $\alpha\beta$ (β -selection) or $\gamma\delta$ lineage, CD4-CD8 and effector differentiation. The developmental windows for TCR β and TCR α rearrangement are indicated. The progeny of DP thymocytes, CD8 and CD4 cells are shown, as is a non-exhaustive list of the effector type they generate upon activation. Subsets with high-level expression of *Thpok* (T, purple) or *Runx3* (R3, green) are schematically depicted. Effector CD8 cells that make IL-17 (Tc17) have been reported both *in vitro* and *in vivo*, and display reduced cytotoxic activity with reduced expression of cytotoxic genes perforin, granzyme B and of the transcription factor Eomes [92-94] but nonetheless continue expressing *Runx3* (Y.X, L.W. and R.B, unpublished results).

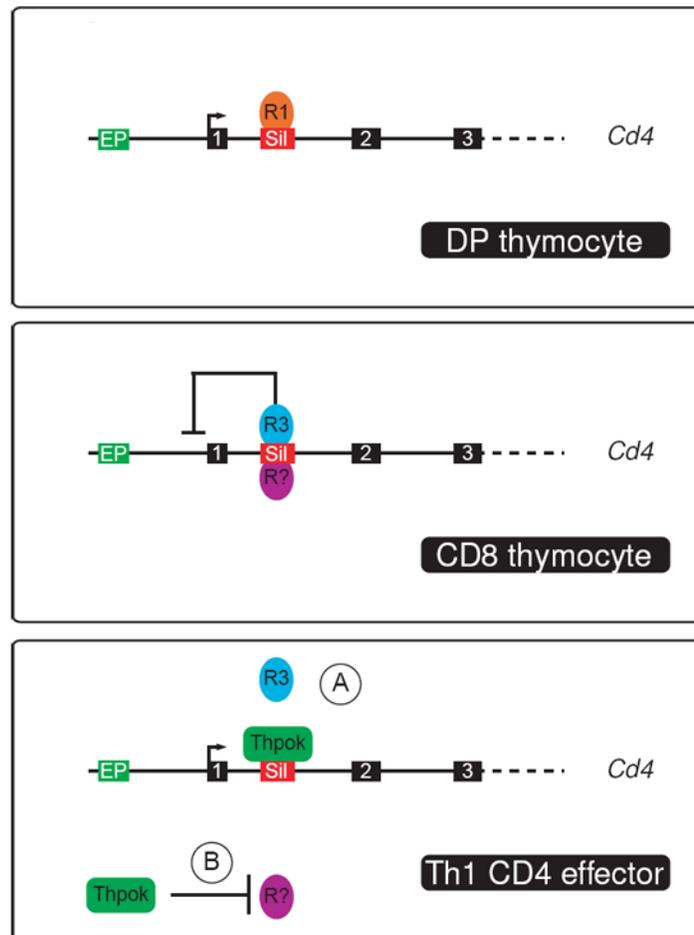


Figure 2. Thpok and Runx3 antagonism at the *Cd4* locus

The *Cd4* locus is schematically depicted with the transcription start site shown as an arrow and the first three exons as numbered black boxes. The *Cd4* silencer (Sil) and proximal enhancer (EP) are shown as red and green boxes, respectively. In CD8-lineage thymocytes (middle), Runx3 (R3) and putative additional repressors (R?) binds the silencer, resulting in *Cd4* repression and eventual silencing. In DP thymocytes (top), Runx1 (R1) binds the silencer, but fails to repress *Cd4*. Although this may be because of the absence of additional repressors (as depicted), enforced Runx3 expression in DP thymocytes similarly represses *Cd4* repression, consistent with the possibility that the dosage of Runx proteins affects their ability to repress *Cd4*. In Th1 CD4 effectors (bottom), Runx3 fails to repress *Cd4*. Two mechanisms have been proposed to explain this effect: (A) Thpok binds the *Cd4* silencer, suggesting that it could prevent recruitment of Runx3 (as depicted) or of required co-repressors, or antagonize their repressive activity; (B) Thpok could repress the expression of required co-repressors.

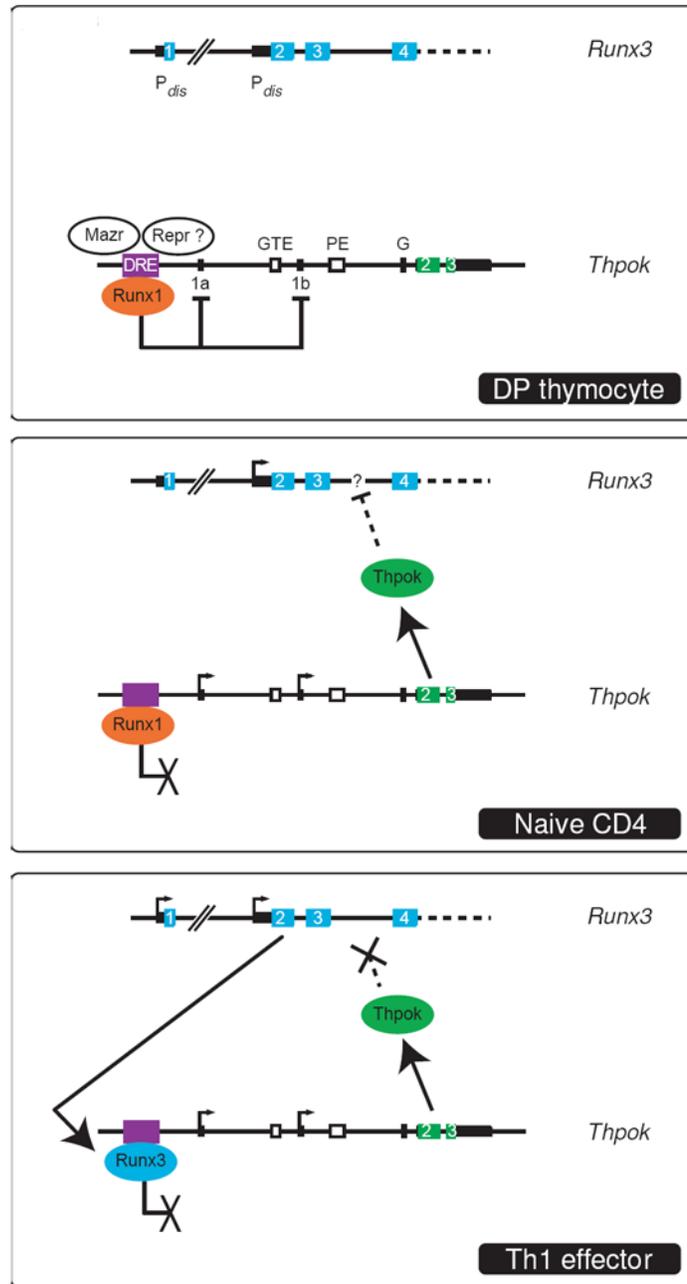


Figure 3. Transcriptional plasticity at the *Thpok* locus

The *Runx3* and *Thpok* loci are schematically represented in three separate boxes. For each gene, transcription start sites are shown as arrows and exons as numbered cyan or green boxes; non-coding exonic sequences are depicted as thinner black rectangles. Note the two alternative promoters and first exons (1a and 1b) of *Thpok*. Boxes (top) indicate the distal regulatory element (DRE, which includes or overlaps with the silencer [27,31]) the general T lymphoid element (GTE), the proximal enhancer (PE), and a conserved sequence (G) that includes a Gata3 binding site [13]. The distal (P_{dis}) and proximal (P_{prox}) *Runx3* promoters are indicated upstream of exons 1 and 2, respectively. In DP thymocytes (top), *Thpok* is not transcribed because of reversible repression involving Runx1, Mazr and potentially unknown other repressors (Repr ?). The *Runx3* distal promoter, that gives rise to protein-

producing transcripts, is not active. In CD4 SP thymocytes and naïve CD4 T cells (middle), *Thpok* is expressed and represses *Runx3* through so far unknown mechanisms (dotted line); note that this does not prevent transcription from the proximal promoter [23]. Although Runx1 is expressed and bound to the silencer, it does not repress *Thpok*, possibly because of the absence of the other repressors (see text for discussion). In Th1 effectors (bottom), *Thpok* is expressed but no longer represses the *Runx3* distal promoter. Conversely, Runx3 fails to repress *Thpok*; it is shown as bound to the silencer by analogy with Runx1 in resting cells (see text for discussion). Drawings are not on scale.