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Expanding roles for ThPOK in thymic development

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Summary

The role of the zinc finger transcription factor ThPOK (T-helper inducing POZ-Kruppel like factor) in promoting commitment of $\alpha\beta$ T cells to the CD4 lineage is now well established. New results indicate that ThPOK is also important for development and/or acquisition of effector functions by other T cell subsets, including several not marked by CD4 expression, i.e. double negative invariant natural killer T (iNKT) cells, $\gamma\delta$ cells, and even memory CD8⁺ T cells. There is compelling evidence that ThPOK expression in most or all of these cases is dependent on T-cell receptor signaling and that differences in relative TCR signal strength/length may induce different levels of ThPOK expression. The developmental consequences of ThPOK expression vary according to cell type, which may partly reflect differences in ThPOK levels and/or in transcriptional networks between cell types.

Keywords

ThPOK; thymocyte; T-cell development; CD4; lineage choice; $\gamma\delta$ TCR

Introduction to thymic development

The majority of mature T cells arising in the thymus belong to the ‘conventional’ CD8⁺ and CD4⁺ $\alpha\beta$ T-cell subsets, which are restricted to major histocompatibility complex (MHC) class I and II ligands, respectively. Development of these cells from immature precursors can be readily tracked by expression of the CD4 and CD8 surface markers. The most immature precursor cells entering the thymus from the bone marrow lack expression of both CD4 and CD8 molecules [CD4[−]CD8[−], or double negative (DN)]. Within the DN compartment, development proceeds through four stages marked by differential expression of the CD44 and CD25 molecules, i.e. in order of maturity, CD44⁺CD25[−] (DN1), CD44⁺CD25⁺ (DN2), CD44[−]CD25⁺ (DN3), and CD44[−]CD25[−] (DN4). Productive T-cell receptor β (TCR β) rearrangement mediates traversal of the so-called β checkpoint at the DN3 stage, resulting in a massive burst of proliferation and progression to the CD4⁺CD8⁺ [double positive (DP)] stage. At the DP stage, engagement of MHC ligands by the $\alpha\beta$ TCR, so-called positive selection, mediates differentiation to the distinct SP CD4 and CD8 lineages. The CD4 and CD8 coreceptors play a key role in facilitating/enhancing MHC-mediated TCR signaling, as they interact directly with MHC class II or I molecules, respectively, and recruit important signaling factors to the TCR complex. Additional specialized T-cell lineages diverge from the main CD4/CD8 developmental pathway at the DN and DP stages. Thus, $\gamma\delta$ thymocytes separate from $\alpha\beta$ cells at the DN stage, while invariant natural killer T (iNKT) cells split off at the DP stage. $\gamma\delta$ thymocytes undergo further maturation (defined by CD24 downmodulation) and mostly maintain the DN phenotype, while iNKT cells diverge into DN and SP CD4 subsets.

Role of TCR signaling in CD4/CD8 lineage choice

The precise mechanisms governing divergence of different lineages in the thymus remain to be elucidated. However, considerable accumulating evidence points to a key role for TCR specificity and signaling in these processes. In this respect, the CD4⁺/CD8⁺ lineage control mechanism is the best studied. A key aspect of this process is that it results in a near-perfect correlation between adoption of the CD4⁺ or CD8⁺ lineage fates and TCR specificity towards MHC class II or I ligands, respectively, so that any model that seeks to explain CD4⁺/CD8⁺ lineage choice must account for this essential fact. Initially, two competing models were proposed: (i) the instructive model, which postulated that engagement of TCR by class I or II ligands results in qualitatively distinct TCR signals that directly induce development to the CD8⁺ or CD4⁺ lineages, respectively, and (ii) the stochastic selective model, which proposed that initial lineage choice is random and that the correlation between lineage choice and MHC specificity is achieved by subsequent elimination of cells whose coreceptor expression pattern does not match their MHC specificity (1–3). Experimental testing of these models, however, soon generated results that were inconsistent with either model. In particular, constitutive expression of one of the coreceptors failed to efficiently rescue putative ‘mismatched’ thymocytes, as would be predicted by the stochastic/selective model. Furthermore, under some experimental conditions, the correlation between MHC specificity and lineage choice broke down. For instance, class II-restricted thymocytes were found to develop quite efficiently to the CD8⁺ lineage in CD4-deficient mice, something that should not be permitted under a strict ‘qualitative’ instructive mechanism (4,5). The latter observation was critical in leading to a better understanding of lineage choice, as it indicated that CD8⁺ T-lineage commitment did not depend on a class I-restricted TCR or on CD8 but could be induced simply by diminishing the efficiency of TCR signaling. This realization led to two new and more sophisticated models. (i) According to the quantitative/instructive model, engagement of TCR by class I or II ligands results in quantitatively weaker or stronger TCR signals that directly induce development to the CD8⁺ or CD4⁺ lineages, respectively. Differences in TCR signal strength are postulated to reflect weaker or stronger affinity of the CD8 and CD4 cytoplasmic domains for the key signaling factor p56^{Lck} (4,6–8). (ii) The kinetic signaling model argues that relatively short or long duration of TCR signaling during thymic development directs development to the CD8⁺ or CD4⁺ lineages, respectively. Differences in TCR signal duration result from developmentally preprogrammed changes in coreceptor expression that occur at the DP>SP transition. In particular, most positively selected thymocytes seem to pass through a CD4⁺CD8^{lo} transitional stage, at which CD8 expression is selectively downmodulated, resulting in selective impairment of signaling through class I-restricted TCRs (9,10).

Role of TCR signaling in γ δ T-cell development

In contrast to α β T cells, it has remained quite controversial whether TCR signaling plays a general role in the development of γ δ thymocytes. This is not because of strong evidence to the contrary, but mainly because possibilities for experimental manipulation of γ δ TCR signaling are relatively limited. In particular, most γ δ TCRs are not reactive with MHC molecules and their antigenic specificities remain largely unknown, and γ δ TCR signaling does not depend on CD4 or CD8 (or any other ‘coreceptor’ of similar function, as far as is known). Nevertheless, several observations suggest that TCR signaling plays a role in the development of at least some γ δ T cells. First, almost all mature γ δ thymocytes express high levels of CD44 and many express NK1.1, both of which are activation markers that are inducible on γ δ thymocytes by antibody-mediated TCR crosslinking (11). Secondly, mature (CD24[−]) γ δ thymocytes display a skewed TCR V region repertoire, in particular a larger proportion of mature than immature cells express V γ 1.1, suggesting that they have undergone selection based on their TCR specificity for intrathymic ligands (12). Third,

thymocytes expressing a transgenic $\gamma\delta$ TCR of defined specificity, the KN6 TCR, undergo maturation to the mature stage only in the presence of their specific intrathymic ligand, while in its absence they undergo alternative development to the $\alpha\beta$ lineage (13,14). TCR signal strength seems to influence $\gamma\delta$ development in at least two respects: (i) strong TCR signals seem to promote commitment to the $\gamma\delta$ lineage at the expense of $\alpha\beta$ commitment (14–17), and (ii) within the $\gamma\delta$ lineage, differentiation into specialized effector subsets seems to be controlled by differences in TCR signal strength. In particular, strong engagement by intrathymic ligands seems to promote development of IFN γ -producing $\gamma\delta$ cells (18). Although TCR signaling thus plays an important role in $\gamma\delta$ development, the downstream pathways and transcription factors that mediate alternate developmental signals remain to be defined.

Transcriptional regulation of coreceptor transcription

An important approach to revealing the underlying molecular pathways that control CD4⁺/CD8⁺ lineage choice has been the so-called ‘bottoms up’ strategy, which aims to elucidate these pathways by first defining the transcriptional control mechanisms of lineage specific genes, in particular CD4 and CD8 (19,20). Although technically challenging, due to its heavy reliance on mouse models, this approach has led to important insights. In particular, it was demonstrated that CD4 gene expression is controlled in a stage- and lineage-specific fashion by a transcriptional silencer (CD4 Silencer), that represses CD4 transcription in both DN and CD8⁺ thymocytes (21–24). CD8 gene expression, in contrast, is regulated in a more complicated manner by several enhancers with partly overlapping stage- and lineage-specific activities (25–30).

Conserved Runx binding sites were identified within the CD4 silencer, which were essential for repression of CD4 in the CD8⁺ lineage, indicating a critical function for Runx factors in lineage-specific CD4 regulation (31). Additionally, it has been reported that Runx factors are involved in the positive regulation of CD8 gene transcription (31,32). Runx3 protein is selectively induced in SP CD8 thymocytes, suggesting that it is the main Runx factor responsible for transcriptional repression of CD4 in these cells (31–33). The distal Runx3 promoter appears mainly responsible for this lineage-specific expression, as perturbing its function is sufficient to prevent production of Runx3 protein and normal CD4 derepression in SP CD8 cells (34,35). The mechanisms controlling lineage-specific regulation of the Runx3 distal promoter remain to be elucidated. Together, these results led to the conclusion that induction of Runx3 expression is a key event for promoting cytotoxic CD8⁺ T-cell development.

Some transcription factors important for CD4⁺ or CD8⁺ T-cell development

While Runx factors clearly play an important role in promoting the CD8⁺ lineage-specific pattern of gene expression, it remains controversial whether they play a role in the control of lineage commitment *per se*. Studies in which Runx factors are constitutively expressed in the T-cell lineage argue against an instructive role for Runx factors in promoting CD8⁺ commitment. Although constitutive Runx expression leads to substantial redirection of class II-restricted cells to the CD8⁺ lineage (32,36,37), simultaneous constitutive expression of CD4 corrects the defect, indicating that redirection is a secondary consequence of aberrant CD4 downmodulation (38). Indeed, similar redirection of class II-restricted thymocytes is observed in CD4-deficient mice (4,5). Conversely, Runx3^{-/-} mice do not exhibit redirection of class I-restricted thymocytes to the CD4⁺ lineage (35). Simultaneous ablation of both Runx1 and Runx3 in DP thymocytes leads to significant redirection of class I-restricted thymocytes to the CD4⁺ lineage (39). However, since no normal DP thymocytes ever lack

Runx expression, the physiological relevance of this observation for lineage commitment remains unclear (see below).

Another transcription factor that plays an important role in alternate lineage development is the zinc finger transcription factor Gata-3 (40,41). Gata-3 is first expressed in early thymocyte precursors and is required for their differentiation beyond the DN stage. Later in thymic development, Gata3 is preferentially upregulated in SP CD4 versus CD8 cells. Significantly, conditional deletion of Gata3 at the DP stage causes a specific block in CD4 development (41,42), while its constitutive expression selectively inhibits CD8⁺ T-cell development (40). Although Gata3^{-/-} mice exhibit some redirection of class II-restricted thymocytes to the CD8⁺ lineage, the efficiency of this process is very low (42). Conversely, constitutive Gata3 expression does not cause redirection of class I-restricted cells to the CD4⁺ lineage. These observations indicate that Gata3 is essential for supporting the CD4⁺ development program but is insufficient for directing CD4⁺ T-lineage choice. The exact role of Gata3 in CD4⁺ T-cell development remains unclear, but it appears to occur at a relatively early stage in the process. Thus, Gata3 is inducible by TCR signaling at the DP stage, and Gata3 deficiency severely impairs the generation of CD4⁺CD8^{lo} intermediate thymocytes (40–42). Interestingly, Gata3 deficiency also prevents appearance of CD4⁺CD8^{lo} intermediate cells in mice expressing a class I-restricted TCR, although development of SP CD8 cells otherwise proceeds normally (41). This is consistent with a general role of Gata3 in promoting development to the CD4⁺CD8^{lo} stage, which according to the kinetic signaling model may be an essential prerequisite for CD4⁺ but not CD8⁺ T-cell commitment, thus accounting for the selective defect in CD4⁺ development in Gata3^{-/-} mice. Overall, these observations suggest that Gata3 plays a critical role in supporting the CD4⁺ development program, perhaps antagonizing the CD8⁺ program, but is not a major regulator of lineage choice *per se*.

Identification of ThPOK as a key regulator of CD4⁺/CD8⁺ T-lineage commitment

More than 10 years ago, we identified a spontaneous mutant mouse strain in our colony that exhibited a selective absence of mature CD4⁺ T cells and increased representation of CD8⁺ cells, a phenotype we referred to as ‘helper-deficient’ (HD) (43). The HD defect was caused by a single autosomal recessive mutation of an unknown gene, unlinked to either CD4 or MHC class II. The phenotype was only observed in homozygous mutant animals, while heterozygotes were normal. The responsible mutation was eventually mapped to the gene encoding the zinc finger transcription factor ThPOK (T-helper inducing POZ-Kruppel like factor), also known as Zfp67, cKrox or Zbtb7b (44). ThPOK belongs to the POK family of transcription factors, which encode two functional domains, a Kruppel-like zinc finger domain that is responsible for DNA binding and a BTB-POZ domain, which is implicated in homodimerization and recruitment of other factors (45). ThPOK was originally cloned as a binding factor and negative regulator of collagen gene promoters (46). This negative regulatory role is consistent with the known function of the POZ domain in other POK family members, like Bcl-6 and PLZF (47). In these cases, the POZ domain mediates chromatin-remodeling and transcriptional repression by interacting with histone deacetylases and corepressor molecules. The point mutation of ThPOK in HD mice alters a residue in the zinc finger domain predicted to directly interact with DNA, so that the HD phenotype seems to reflect the inability of ThPOK to bind important DNA targets. Targeted knockouts of ThPOK yield the same phenotype as HD mice, demonstrating that it represents a loss-of-function allele (34,48).

Initial studies revealed two intriguing features of the mutant HD phenotype. First, class II-restricted cells were not blocked in development but instead underwent highly efficient

redirection to the CD8⁺ lineage, indicating that ThPOK is necessary for mediating CD4⁺ commitment and preventing CD8⁺ commitment (49). Importantly, redirected thymocytes in HD mice do not just adopt the CD8 coreceptor expression pattern but show induction of other CD8 markers, such as perforin and CD103, indicating that ThPOK regulates the overall commitment process not just the coreceptor expression pattern. Secondly, all other aspects of $\alpha\beta$ T-cell development were unaffected in HD mice. In particular, development of class I restricted cells to the CD8⁺ lineage as well as negative selection proceeded normally (44,49). Even positive selection of class II-restricted thymocytes, as assessed by the proportion of CD69⁺TCR β ⁺ thymocytes generated, was unaffected. These observations were of considerable interest, in that they provided the first evidence that lineage commitment and positive selection of $\alpha\beta$ thymocytes were genetically separable and thus mechanistically distinct (49). They also suggested that the HD mutation did not cause a defect in TCR signaling, as the HD phenotype is markedly different from mice with a TCR signaling defect, such as those deficient in Lck or Erk activity/expression (6,50). Consistent with this view, biochemical assays of TCR signaling in T cells from HD mice all proved normal.

Two observations suggest that ThPOK is a key component of a TCR-mediated mechanism that controls lineage commitment. (i) ThPOK expression is induced at the DP>SP transition. While ThPOK expression is essentially undetectable at the CD69⁻ DP stage, i.e. prior to receipt of a TCR signal, substantial expression is found in intermediate CD4⁺CD8^{lo} cells, i.e. immediately after positive selection. Of greater significance, class II-restricted CD4⁺CD8^{lo} thymocytes express much higher ThPOK levels than class I-restricted cells, and levels increase further in SP CD4 thymocytes, while diminishing to background levels in SP CD8 thymocytes (44,51). ThPOK expression is maintained in the peripheral CD4⁺ compartment, implying that it has other significant functions after full maturation. Indeed, terminating ThPOK expression in mature CD4⁺ cells leads to aberrant derepression of CD8 (42). 2) In mice expressing a constitutive ThPOK transgene, all thymocytes develop to the CD4⁺ lineage, including class I-restricted cells (44,51). Redirection of class I-restricted thymocytes to the CD4⁺ lineage in ThPOK transgenic mice indicates that ThPOK is sufficient for CD4⁺ T-cell commitment. Together with observations from HD mice, these results imply that CD8⁺ commitment is a default pathway that occurs in the absence of ThPOK and is unlikely to require a specific mediator analogous to ThPOK.

These results are consistent with either of the currently favored models of lineage commitment, i.e. quantitative/instructive or stochastic/selective. The striking accumulation of CD4⁺CD8^{lo} thymocytes in HD compared to wildtype mice is particularly consistent with the idea that lineage choice is determined at this stage, as proposed by the kinetic signaling model.

Role of ThPOK in iNKT development

We have recently identified an important role for ThPOK in differentiation and acquisition of effector functions by iNKT cells (52). iNKT cells develop from DP precursors in the thymus but express a relatively limited TCR repertoire characterized by predominant usage of V α 14 and recognition of lipid antigens in the context of CD1d (53). A large proportion of mature iNKT thymocytes express CD4, suggesting the possibility that they are dependent on ThPOK for their normal maturation. Consistent with this notion, ThPOK-GFP reporter mice show GFP expression in α GalCer-labeled iNKT cells, although somewhat surprisingly in both DN and CD4⁺ subsets. Examination of HD mice shows a striking shift in coreceptor expression pattern by iNKT cells, in that SP CD4 iNKT cells are essentially absent, while a novel SP CD8 iNKT population develops instead. Mice, in contrast to humans, do not normally possess SP CD8 iNKT cells. These results indicate a key role of ThPOK in

determining the coreceptor expression pattern of the CD4⁺ iNKT subset. The most likely explanation for the appearance of CD8⁺ iNKT cells in HD mice is that they arise through 'redirection' of precursors that would normally adopt the CD4⁺ phenotype, although this remains to be directly demonstrated. It therefore appears that CD8⁺ development is default pathway for CD4⁺ iNKT cells, which occurs in the absence of ThPOK, as is the case for conventional CD4⁺ thymocytes. These findings have implications for the absence of CD8⁺ iNKT cells in normal mice. Specifically, it had been proposed that CD8 might function as a coreceptor for CD1d recognition, causing heightened TCR signaling by SP CD8 iNKT cells, which in turn could cause their negative selection. However, the appearance of CD8 iNKT cells in HD mice is hard to reconcile with such a view, unless ThPOK were also necessary for negative selection. There is no evidence in favor of such a role, however, and indeed ThPOK is not required for negative selection in other contexts (44). While these results demonstrate that the choice between CD4 and CD8 coreceptor expression by iNKT CD4⁺ cells is determined by ThPOK expression, this does not, however, seem to be the case for the DN iNKT subset. First, DN iNKT cells fail to express CD4, even though these cells express similar levels of ThPOK mRNA to CD4⁺ iNKT cells. Secondly, many or all DN iNKT cells do not upmodulate CD8 in the absence of functional ThPOK, as the proportion of DN iNKT cells is not diminished in HD mice. This observation suggests an important difference in transcription factor-mediated or epigenetic control of the CD4 and CD8 loci between CD4⁺ and DN iNKT cells. The developmental basis for divergence of iNKT cells into DN and CD4⁺ subsets is unclear. It is possible that they may exhibit distinct TCR repertoires, and there is some evidence that they may be functionally distinct, in that DN iNKT cells exhibit a more rapid and pronounced interleukin-17 (IL-17) response (54). ThPOK deficiency also has a significant effect on iNKT effector functions. A key feature of iNKT cells is their ability to rapidly produce multiple cytokines upon antigenic stimulation, including IL-4 and interferon- γ (IFN γ). IL-4 secretion in response to α GalCer-CD1d is severely diminished in HD mice for both DN and CD8⁺ iNKT subsets. The fact that ThPOK deficiency causes a similar defect in cytokine production in both subsets, implies that ThPOK is functionally important and active in all iNKT cells. This supports the view that the different coreceptor expression patterns of CD4⁺ and DN iNKT subsets are determined not by availability of functional ThPOK but by some other mechanism, e.g. subset-specific differences in chromatin conformation of these loci. Although production of cytokine proteins by iNKT cells requires antigenic stimulation, production of IL-4 and IFN γ mRNA occurs constitutively, i.e. even in the absence of antigenic stimulation. ThPOK-deficient mice show a severe decrease in constitutive IL-4 transcripts and a significant decrease in IFN γ transcripts, indicating that ThPOK controls cytokine production at the level of transcription, not by post-transcriptional mechanisms that control cytokine protein levels. It will be interesting to explore whether ThPOK controls cytokine transcription by direct binding to cytokine gene regulatory elements or indirectly via intermediary factors. Recently, Plzf, another member of the POK family of transcription factors, has been shown to play a key role in iNKT development. In contrast to ThPOK deficiency, which does not affect iNKT cell numbers, Plzf deficiency results in a severe decrease of iNKT cells, indicating that it represents a 'master regulator' of this lineage (55,56). In contrast to ThPOK, Plzf is not expressed in conventional T cells and thus has no role in their differentiation. Residual iNKT cells in Plzf-deficient mice, like ThPOK-deficient iNKT cells, exhibit impaired primary cytokine production, although the defect seems more severe, as it strongly reduces both IL-4 and IFN γ production. Whether ThPOK and Plzf control cytokine production by overlapping or distinct mechanism remains to be determined. The fact that they both belong to the POK family of transcription factors could suggest functional overlap or even functional interaction via the BTB domain, as this has been implicated in homo- and heterodimerization between POK factors.

Role of ThPOK in $\gamma\delta$ development

The above results indicate that the role of ThPOK in T-cell development is not confined to promoting SP CD4 development and that additional functions might therefore exist in other T-cell subsets. While previous reverse transcriptase polymerase chain reaction (RT-PCR) analyses of ThPOK mRNA levels in total DN thymocytes had not detected significant expression, recent availability of ThPOK-GFP reporter mice has allowed this issue to be reexamined in more detail. Surprisingly, GFP expression was detected in a significant fraction of $\gamma\delta$ thymocytes, particularly within the CD24⁺ subset (57,58). $\gamma\delta$ thymocytes can be divided into two major subsets according to expression of CD24 and CD44, i.e. 'immature' CD24⁺CD44⁺ and 'mature' CD24⁺CD44⁺ subsets [also referred to as 'cluster A' and 'B' fractions, respectively (59)]. Functional maturity of the CD44⁺CD24⁺ $\gamma\delta$ thymocytes is demonstrated by their capacity to proliferate and secrete cytokines in response to TCR engagement, which is not the case for CD44⁺CD24⁺ $\gamma\delta$ thymocytes (14,60). The developmental fate of immature CD44⁺CD24⁺ $\gamma\delta$ thymocytes is incompletely understood. A minority of these cells apparently undergo final maturation to the CD24⁺CD44⁺ stage in the thymus. However, the vast majority of immature $\gamma\delta$ thymocytes appear to remain CD24⁺, and their ultimate fate remains controversial. Interestingly, at least some of these cells are able to reach the periphery, since the bulk of $\gamma\delta$ TCR⁺ recent thymic immigrants (RTEs) exhibit a CD24⁺ phenotype (61,62). However, most of these CD24⁺ RTEs seem short-lived (62), and their contribution to the long-term peripheral $\gamma\delta$ compartment is therefore unclear. Thus, it remains unknown whether those CD24⁺ $\gamma\delta$ thymocytes which fail to progress to the mature CD24⁺ stage in the thymus represent a developmental dead end or are part of an alternate developmental pathway that is completed in the periphery. In ThPOK-GFP reporter mice, about 10–15% of immature $\gamma\delta$ TCR⁺ thymocytes express low green fluorescence protein (GFP) reporter levels, while the majority of mature $\gamma\delta$ thymocytes express high GFP levels, suggesting that the GFP^{lo} immature cells are the major precursors of mature $\gamma\delta$ thymocytes (58). Importantly, GFP⁺ cells express endogenous ThPOK transcripts, indicating that the transgene accurately tracks ThPOK transcription in these $\gamma\delta$ cells. The increase in the proportion of GFP⁺ $\gamma\delta$ cells during the transition from immature to mature stages suggests that ThPOK may play a physiological role in $\gamma\delta$ maturation. Indeed, in HD mice, the absolute number of mature $\gamma\delta$ thymocytes is substantially reduced (by 50–70%), demonstrating a key role for ThPOK in commitment/maturation of $\gamma\delta$ thymocytes and/or in proliferation/survival of mature $\gamma\delta$ thymocytes (Fig. 1A).

Mature $\gamma\delta$ thymocytes in adult mice can be further subdivided into two major subsets based on surface marker and cytokine expression pattern. One subset, preferentially expresses IFN γ upon stimulation, and predominantly expresses the V γ 1.1 segment and the NK1.1 surface marker (and are therefore sometimes referred to as NKT $\gamma\delta$ cells) (12,60,63). A second $\gamma\delta$ subset preferentially secretes IL-17, preferentially utilizes V γ 2, and expresses the CCR6 surface marker (60,63) (Fig. 1). In the thymus, NK1.1⁺ and CCR6⁺ $\gamma\delta$ subsets are found exclusively within the mature CD24⁺ fraction, each subset contributing 30–40% of this fraction. The upstream signals and transcriptional pathways that promote alternate development into these two functionally distinct $\gamma\delta$ subsets are poorly understood. However, it has been reported that antigen-experienced $\gamma\delta$ cells develop preferentially into the IFN γ -producing subset (18). Comparison of NK1.1⁺ and CCR6⁺ $\gamma\delta$ thymocytes from ThPOK-GFP reporter mice shows that the former expresses higher levels of GFP, suggesting a more important role for ThPOK in their development. Indeed analysis of adult HD mice showed a severe (4–5 fold) reduction in absolute numbers of NK1.1⁺ $\gamma\delta$ thymocytes, but only a mild (<twofold) reduction of CCR6⁺ cells. Given its role in CD4⁺/CD8⁺ commitment, we considered whether higher ThPOK expression might favor development of $\gamma\delta$ thymocytes to the NK1.1⁺ $\gamma\delta$ 'lineage' and whether this might occur at the expense of development to the CCR6⁺ lineage. To test the first point, we examined

whether proportions of NK1.1⁺ and CCR6⁺γδ subsets were altered in mice expressing a constitutive ThPOK transgene (ThPOK^{const} mice), in which ThPOK expression is initiated at the DN2 stage, i.e. prior to γδ TCR expression by most γδ thymocytes. Strikingly, the proportion of mature γδ thymocytes in these mice was substantially increased and most of these cells expressed NK1.1, indicating that ThPOK in fact promotes development and/or expansion of the NK1.1⁺γδ subset (Fig. 1B). To test whether expansion of the NK1.1⁺ subset occurred at the expense of development to the CCR6⁺ fraction, we further examined whether V region usage was altered in NK1.1⁺ and CCR6⁺γδ subsets from HD or ThPOK^{const} mice. However, no such shift in V region usage was detected. In particular, the CCR6⁺γδ cells generated in HD mice still uniformly utilized Vγ 2, indicating that ThPOK deficiency did not result in aberrant development of Vγ 1.1⁺ thymocytes to the CCR6⁺ lineage. Conversely, most NK1.1⁺ thymocytes generated in ThPOK^{const} mice still utilized Vγ 1.1. Further, CCR6⁺ and NK1.1⁺γδ subsets from HD and ThPOK^{const} mice showed similar preferential production of IL-17 and IFNγ, respectively, as is the case in wildtype mice. Hence, ThPOK appears to selectively promote development/expansion of the NK1.1⁺ Vγ 1.1⁺γδ subset but does not seem to control the choice between NK1.1⁺ and CCR6⁺ fates. To assess whether ThPOK might selectively promote proliferation of NK1.1⁺γδ thymocytes, we carried out *in vivo* BrdU labeling of thymocytes from wildtype, HD, and ThPOK^{const} mice. Mature (CD24⁻) γδ thymocytes, regardless of strain, showed no incorporation of BrdU after short pulse times and much less incorporation than immature γδ thymocytes even after 1 week of BrdU treatment, indicating that these are long-lived cells with slow turnover. Importantly, there was no difference in BrdU incorporation for mature γδ thymocytes from HD or ThPOK^{const} mice, compared to wildtype controls, suggesting that proliferation of these cells is unaffected by presence or absence of functional ThPOK (58). The most likely explanation for the altered frequencies of mature γδ cells in HD and ThPOK^{const} mice seems therefore to be altered homeostasis, i.e. a change either in the rate of entry of immature precursors into this subset or in the rate of exit due to emigration or death.

We suspect that ThPOK may, in fact, promote selection of immature γδ precursors to the mature stage, because the subset of immature γδ thymocytes that express ThPOK exhibits a similar skewed pattern of V region usage. In adult wildtype animals, Vγ 1.1⁺ cells comprise only 15% of immature but 40% of mature γδ thymocytes, indicating significant selection for Vγ 1.1⁺ cells during maturation. In mice expressing a ThPOK-GFP reporter, GFP⁺ immature (DN3 and DN4) γδ cells already exhibit 40% Vγ 1.1 usage, strongly suggesting that ThPOK expression marks these cells for maturation to the CD24⁻ stage. It is interesting that mice lacking the helix-loop-helix (HLH) transcriptional regulator Id3, exhibit a massive selective increase in the proportion and absolute number of Vγ 1.1⁺ cells, similar to ThPOK^{const} mice, so that similar mechanisms may be involved (64,65). At least two mechanisms operating at different developmental stages have been suggested to mediate this effect in Id3^{-/-} mice. First, it has been shown that Id3^{-/-} mice exhibit an increase in Vγ 1.1 rearrangement, suggesting that one normal function of Id3 may be to repress Vγ 1.1 rearrangement, thereby limiting the size of the Vγ 1.1⁺ precursor pool (65). This is consistent with previous observations that E protein targets of Id3 are involved in regulating TCR rearrangement (66,67). Secondly, Id3 seems to have a role in selection of γδ TCR⁺ thymocytes based on their antigen specificity, as revealed by analysis of Id3^{-/-} mice expressing the KN6 transgene (64). The KN6 TCR recognizes the non-classical MHC product T-10^b with higher affinity than the T-10^d ligand, resulting in negative selection of KN6⁺ thymocytes on the H-2^b but not the H-2^d background. Importantly, in the absence of Id3, negative selection of KN6⁺ thymocytes is markedly diminished, indicating a role for Id3 in γδ TCR-mediated selection (64). Given that Vγ 1.1⁺ thymocytes are thought to be highly enriched for autoreactive specificities (12,64), increased generation of Vγ 1.1⁺ cells in Id3^{-/-} mice may also partly reflect rescue from negative selection. In support of such a

mechanism, defects in other genes involved in TCR surface expression and/or signaling, i.e. Itk and CD3 δ , also selectively promote development of V γ 1.1 $^{+}$ cells (58,68,69). Our observation that ThPOK is highly upmodulated in KN6 $^{+}$ thymocytes in the presence of the strong T10 b but not the weak T10 d ligand and other evidence (see below) support the view that ThPOK expression by $\gamma\delta$ thymocytes is induced primarily by strong TCR signaling. This implies that the major role of ThPOK in promoting commitment/development of V γ 1.1 $^{+}$ thymocytes and other high affinity $\gamma\delta$ cells to the NK1.1 $^{+}$ subset occurs after V γ rearrangement and $\gamma\delta$ TCR surface expression, i.e. affects post-rearrangement selection. Consistent with this view, analysis of ThPOK-GFP reporter transgenic mice reveals little if any GFP expression in DN subsets that do not express surface $\gamma\delta$ TCR (58).

ThPOK induction is regulated by TCR signaling

There is accumulating evidence that ThPOK induction in T lymphocytes is regulated by strength and/or duration of TCR signaling. In the context of $\alpha\beta$ development, we and others have shown that the level of ThPOK mRNA expressed by transitional CD4 $^{+}$ CD8 lo thymocytes correlates closely with MHC specificity, such that class II-restricted cells express much higher ThPOK levels than class I-restricted cells. Importantly, class II-restricted CD4 $^{+}$ CD8 lo cells from HD mice still express high ThPOK levels, even though these cells are actually undergoing commitment to the CD8 $^{+}$ T-cell lineage (44). These results indicate that ThPOK induction at the CD4 $^{+}$ CD8 lo stage correlates with TCR specificity rather than ultimate lineage choice. We have further demonstrated that antibody-mediated TCR stimulation induces ThPOK expression by class I-restricted CD4 $^{+}$ CD8 lo thymocytes *in vivo* (70). Taken together, these observations argue strongly for a causal link between strength or length of TCR signaling and ThPOK induction in CD4 $^{+}$ CD8 lo intermediate cells. Interestingly, DP thymocytes show essentially no ThPOK expression in normal mice or in response to antibody-mediated TCR stimulation, either *in vitro* or *in vivo*, suggesting that the ThPOK locus is insensitive to TCR stimulation at this stage, or that there is a considerable lag between the inductive signal and resulting transcription at the ThPOK locus (51,70).

Further compelling evidence for a causal relationship between TCR signaling and ThPOK induction arises from analysis of $\gamma\delta$ cells. First, the highest levels of ThPOK in $\gamma\delta$ thymocytes are found among the NK1.1 $^{+}$ IFN γ -producing subset. NK1.1 is known to be an activation marker for $\gamma\delta$ thymocytes that can be induced by antibody-mediated TCR stimulation (71), and development of IFN γ -producing $\gamma\delta$ cells has been shown to depend on ligand exposure (18). Secondly, in the KN6 $\gamma\delta$ TCR transgenic model, ThPOK expression levels correlate with the relative affinity of the KN6 TCR for its intrathymic ligands, i.e. ThPOK levels are much higher in the presence of the strong T10 b than the weak T10 d ligand (58). Finally, *in vitro* antibody-mediated TCR engagement leads to ThPOK induction in $\gamma\delta$ thymocytes, as well as in a DN cell line that expresses a $\gamma\delta$ TCR (58). Further, it has recently been shown that even mature SP CD8 T cells can be induced to reexpress ThPOK in response to strong antibody-mediated TCR stimuli in combination with CD28 ligation *in vitro* or upon *in vivo* stimulation with viral antigen (39). Overall, these observations argue for a common TCR-dependent mode of ThPOK induction in a variety of T-cell lineages and developmental stages. Nevertheless, there are clearly important differences in the level of induction in different settings, i.e. both $\gamma\delta$ thymocytes and CD8 $^{+}$ T cells show lower induction than class II-restricted CD4 $^{+}$ CD8 lo thymocytes, likely due to differences in other transcription factors or in accessibility of the ThPOK locus between these cell types.

Transcriptional regulation of ThPOK

To begin deciphering the mechanism by which TCR signaling leads to ThPOK induction, we and others have characterized the *cis*-acting elements that control ThPOK transcription, with the ultimate goal of identifying the upstream factors/pathways that control them (39,70). We first employed bacterial artificial chromosome (BAC) complementation to define the genomic region that is sufficient to confer proper regulation of ThPOK expression and lineage commitment. To demonstrate normal lineage-specific control, we introduced a series of BAC subclones into HD/HD mice and into mice expressing a class I-restricted TCR transgene. Normal regulation of lineage commitment was demonstrated by restoration of CD4 development in HD/HD mice, but not in mice expressing a class I restricted TCR. These experiments showed that a 20 kb genomic region extending from 17 kb upstream to 500 bp downstream of the ThPOK coding exons contains all elements required for normal regulation of ThPOK expression.

To identify potential *cis*-acting elements within the minimal 20 kb ThPOK locus, we next mapped DNase hypersensitive (DHS) sites in primary thymocytes. DHS sites represent accessible regions of chromatin that are associated with DNA binding factors and often mark regulatory elements. We identified six separate DHS sites, A–F, of which B and D coincide with the alternate distal and proximal promoters, while the others fall within introns or in the upstream flanking region. Functional analysis of these putative regulatory elements was carried out using a series of reporter transgenics containing different fragments of the 20 kb minimal ThPOK locus in association with a GFP reporter cassette (70). Strikingly, deletion of DHS site A, led to promiscuous reporter expression in both CD4⁺ and CD8⁺ lineages, indicating that this region, now referred to as the distal regulatory element (DRE), functions as a lineage specific silencer. Deletion of the DRE from a BAC transgene encoding ThPOK resulted in derepression of ThPOK in class I restricted cells and severe reduction of CD8 T cells. Germline deletion of this element results in a similar phenotype, establishing the essential physiological role of this element in lineage-specific transcriptional control of ThPOK (He and Kappes, unpublished) (39). In addition to its lineage-specific silencer activity, the DRE also encodes enhancer activity. This is apparent in the context of both the minimal human CD2 and murine distal ThPOK promoters (neither of which are sufficient by themselves to mediate expression in T cells). In both cases, insertion of the DRE upstream of the promoter results in CD4⁺ lineage-specific reporter expression. Hence, the DRE silencer restricts activity of both its own enhancer and of associated promoters to the CD4⁺ lineage. It will be important to establish whether the DRE silencer and enhancer activities are encoded by distinct motifs within the 500bp DRE element, or whether alternate functions are mediated by binding of different transacting factors to the same motifs.

The observation that the DRE was necessary to suppress ThPOK expression in the CD8⁺ lineage implied the existence of another *cis* element with promiscuous enhancer activity. Indeed, further reporter gene analysis has identified a strong enhancer near DHS site C with activity in both CD4⁺ and CD8⁺ T cells, as well as a large fraction of CD4⁺CD8^{lo} and DP thymocytes, which is termed the general T-cell element (GTE). Activity of the GTE in non-CD4⁺ cells is repressed in the presence of the DRE. Finally, a third enhancer, which mediates CD4-lineage specific expression, maps near DHS site E (proximal regulatory element, PRE). Importantly, the PRE by itself shows no activity outside of CD4⁺ T cells, so that promiscuous transgene expression in the absence of the DRE seems entirely attributable to the GTE. Reporter transgenes containing only the GTE still show expression in the CD4⁺ subset, demonstrating directly that the GTE is promiscuous, i.e. not DP and CD8⁺ specific. Because of its substantial activity in the CD4⁺ lineage, the GTE may play an important physiological role in supporting high level expression of ThPOK. The PRE appears to

encode only enhancer not silencer activity, as it cannot suppress GTE-dependent reporter expression in CD8⁺ cells in the context of transgenes that include both elements. Reporter transgenics that include only the PRE in the context of the proximal promoter show preferential expression in peripheral CD4⁺ T cells (70), while reporter transgenics that include only the DRE in the context of the distal promoter show preferential expression in CD4⁺ thymocytes (70). This suggests a division of labor between these elements, whereby the DRE is particularly important for the earliest wave of ThPOK induction in class II-restricted thymocytes, which likely initiates CD4⁺ commitment, while the PRE is responsible for subsequent ThPOK expression by more mature CD4⁺ cells. In support of the latter point, targeted deletion of the PRE affects ThPOK expression more severely in mature CD4⁺ T cells than CD4⁺ thymocytes and leads to aberrant downmodulation of CD4 by a substantial reaction of peripheral class II-restricted T cells (48).

Since the DRE seems primarily responsible for initiation of ThPOK expression early during the DP>SP transition and since its ablation completely blocks development of class I-restricted cells to the CD8⁺ lineage, we propose that the DRE serves to discriminate between class I- and class II-restricted TCR signals in uncommitted cells and essentially acts as a molecular switch to control ThPOK expression and lineage choice in response to these alternate TCR signals. In particular, we would suggest that strong or long TCR signals antagonize silencing activity of the DRE and convert it into an enhancer. Of note, ThPOK expression in $\gamma\delta$ thymocytes seems to be similarly controlled by the DRE, as a DRE-dependent reporter transgene faithfully tracks ThPOK expression in $\gamma\delta$ thymocytes. The putative upstream factors that regulate DRE activity remain largely unknown. Given the role of Runx factors in coreceptor regulation, it is noteworthy that the DRE contains two conserved Runx binding motifs. Deletion of these sites partly derepresses reporter expression in CD8⁺ T cells, indicating that Runx factors are important for CD8 lineage specific silencing of ThPOK (39,70). On the other hand, it seems unlikely that Runx factors normally play an instructive role in ThPOK regulation and lineage choice. Thus, induction of Runx3 in CD8⁺ thymocytes seems to occur too late to actually initiate CD8⁺ lineage choice, i.e. it occurs after the CD4⁺CD8^{lo} stage when lineage choice is believed to be decided (34,35). Furthermore, Runx3 deficiency does not cause redirection of class I-restricted cells to the CD4⁺ lineage (35), nor does constitutive Runx3 expression impair ThPOK induction by class II-restricted thymocytes or promote their redirection to the CD8⁺ lineage (34,38). Ongoing functional dissection of the DRE indicates that other conserved segments support significant lineage-specific silencing activity in isolation, arguing that DRE function is likely to be controlled by multiple factors besides Runx. Identifying these factors and determining the temporal order in which they are recruited to the DRE will be critical to unraveling the pathway by which TCR signaling is linked to ThPOK induction. Although, lineage-specificity of ThPOK transcription may primarily be controlled through the DRE element, the overall level of ThPOK expression is likely to depend on other *cis* elements as well, e.g. two additional enhancers, and two alternate promoters (39,70). Interestingly, two conserved Gata consensus motifs have been identified near DHS site F, i.e. upstream of the first ThPOK coding exon, which could be important for Gata3-mediated regulation of ThPOK transcription (42). Of note, a constitutive ThPOK transgene does not rescue CD4⁺ development in Gata3^{-/-} mice, indicating that the role of Gata3 in supporting CD4⁺ development cannot solely involve promoting ThPOK expression.

Defining potential ThPOK target genes

Given the expanding role of ThPOK in diverse T-cell subsets, it seems likely that there are multiple important target genes for ThPOK, which may be partly shared or distinct between different developmental stages and lineages. In the context of CD4⁺/CD8⁺ T-cell commitment, strong candidate target genes are those involved in establishing the phenotypic

and functional distinctions between the CD4⁺ and CD8⁺ lineages, most obviously the CD4 and CD8 genes themselves. Indeed, several studies provide evidence that ThPOK regulates these genes, perhaps directly. Thus, enforced expression of ThPOK in CD8⁺ T cells leads to reduced expression of CD8⁺ lineage genes, including CD8, GzmB, and Perforin, while in DN thymocytes it causes derepression of CD4 (48, 72, He and Kappes, unpublished data). Decreased CD8 expression seems to result in part from reduced activity of the E8I CD8 enhancer (72), while CD4 derepression results from impaired function of the CD4 silencer (73). Conversely, impairment of ThPOK expression in peripheral CD4⁺ cells leads to upmodulation of CD8⁺ lineage genes and loss of CD4 expression by many cells (42,48). In this case, the defect in CD4 expression is corrected by deletion of the CD4 silencer, demonstrating that ThPOK acts to antagonize CD4 silencer function. ChIP-on-Chip experiments indicate that ThPOK binds near the CD4 silencer, although the exact binding site and causal effect of such binding remain to be established (48).

As mentioned above, there is also evidence that ThPOK plays a significant role in controlling cytokine transcription in a variety of T-cell lineages. Strong support for such a role comes from analysis of iNKT cell function in HD mice. Thus, liver Vα 14 iNKT cells isolated from these mice following *in vivo* stimulation with α GalCer tetramers show a severe reduction in IL-4 transcripts, as well as a moderate reduction in IFNγ transcripts compared to wildtype control mice (52). Unstimulated Vα 14 iNKT cells are known to express some IL-4 transcripts constitutively, i.e. even in the absence of TCR stimulation, and this basal transcription was also dramatically reduced in HD mice. Hence, it seems that ThPOK is important for establishing the transcriptional activity of the IL-4 locus in iNKT cells before the TCR stimulus is received by these cells. Furthermore, it has been shown, using ThPOK conditional knockout mice, that ThPOK-deficient CD4⁺ T cells exhibit a severe defect in IL-4 protein production under ThN stimulation conditions (42). The requirement for ThPOK was abolished under Th2 stimulation conditions, presumably because its role was superseded by Th2-specifying factors produced under these conditions. Finally, it has been reported that long-lived memory CD8 cells from HD mice, exhibit a substantial reduction in IL-2 production upon rechallenge (39). Conversely, transgenic expression of ThPOK results in increased IL-4 and IL-2 production by CD8⁺ T cells (72). In all these cases, it remains to be determined whether the requirement for ThPOK reflects direct control of cytokine transcription by ThPOK or more indirect influence on the cells' transcriptional regulatory network, e.g. by repression of Runx.

Concluding remarks

Although ThPOK is widely transcribed in many non-lymphoid tissues, its role in T lymphocytes has so far appeared limited to regulation of CD4⁺/CD8⁺ lineage choice. This was consistent with its expression pattern in T lymphocytes, which seemed to be largely restricted to conventional CD4⁺ lineage cells. Only recently has its wider expression pattern in diverse T-cell lineages and its multiple functions in these subsets become apparent (Fig. 2). Indeed, ThPOK expression has now been documented in most T-cell lineages in which its transcript levels have been carefully assessed, ranging from very low levels in antigen-stimulated peripheral CD8⁺ cells to maximal levels in developing SP CD4 thymocytes. In each case, the major initiating event in ThPOK induction appears to be TCR signaling. Indeed, for at least two cell types, i.e. γ δ thymocytes and CD4⁺CD8^{lo} thymocytes, TCR dependency seems to be mediated by the same transcriptional element, i.e. the DRE. However, precise quantitative control in different subsets is likely to involve other *cis* elements as well. The downstream events mediated by ThPOK expression appear to differ significantly depending on T-cell lineage and developmental stage, leading to a variety of outcomes, including specification of CD4⁺ lineage fate, promotion of γ δ thymocyte maturation, and acquisition of effector functions by iNKT cells. The precise expression

levels of ThPOK and/or the cellular context in which it is expressed are presumably key factors in determining the functional consequences of ThPOK expression in different T-cell subsets. It is evident that many important questions regarding the developmental role of ThPOK remain to be addressed. In particular, it will be important to determine the key target genes of ThPOK and whether these differ between different T-cell stages and lineages. It is possible that ThPOK regulates distinct genes in different cell types, depending on chromatin accessibility or availability of important coregulatory factors. Alternatively, ThPOK may regulate the same genes in all lineages, with different developmental outcomes reflecting quantitative differences in ThPOK-mediated control and/or different functions of these target genes in different cellular contexts. Given the widening roles of ThPOK in T lymphocytes future studies to distinguish these possibilities should provide important insights into many aspects of T-lymphocyte development and function.

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Fig. 1. ThPOK promotes $\gamma\delta$ thymocyte maturation

(A) FACS analysis of indicated gated $\gamma\delta$ thymocyte subset from HD^{+/-} and HD^{-/-} littermates stained with antibodies against CD44, CD24, NK1.1, and CCR6. (B) Absolute cell numbers of mature (CD24⁻) $\gamma\delta$ thymocytes from HD^{+/-}, HD^{-/-}, and ThPOK^{const} mice. Total mature cells or NK1.1⁺ and CCR6⁺ subsets are plotted separately, as indicated.

Fig. 2. ThPOK controls development of multiple T-cell lineages

Schematic representation of thymic development, showing major pathways and thymocyte subsets. Red arrows or bars indicate developmental steps that are affected by ThPOK deficiency. Gray and red circles represent relative cell number of particular subsets in wildtype or HD^{-/-} mice, respectively.