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Runx and ThPOK: A Balancing Act to Regulate Thymocyte Lineage Commitment

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ABSTRACT

CD4-positive helper T cells and CD8-positive cytotoxic T cells comprise the majority of T lymphocytes present in secondary lymphoid organs and are essential for acquired immunity. These two populations are derived from common precursors in the thymus and selected through interaction between their clonal T-cell receptors and major histocompatibility complex molecules. Although intensely studied as a model system for binary cell fate decisions, the mechanisms underlying the helper versus cytotoxic lineage choice in the thymus has been elusive. In the past few years, it has been demonstrated that the Runx family of transcription factors, particularly Runx3, is essential for the generation of cytotoxic lineage T cells, whereas the ThPOK zinc finger transcription factor that plays a crucial role in the differentiation of the helper lineage. Recent works have implied that a cross-regulation between Runx and ThPOK contributes to appropriate thymocyte lineage commitment. In this article, recent findings on the transcription factor networks governing thymocyte lineage decisions are discussed, focusing on the two factors, and provide insights into mechanisms of lineage-specific gene regulation in the process of T-cell commitments. J. Cell. Biochem. 107: 1037–1045, 2009.

KEY WORDS: Runx; ThPOK; THYMOCYTES; LINEAGE COMMITMENT; GENE SILENCING

Differentiation of hematopoietic progenitor cells to mature cells is tightly regulated. Immature progenitor cells undergo multiple steps of binary cell fate decision processes in which they give rise to more mature cells with limited differentiation potential. It has been suggested that each step involves two major layers of gene regulation. When precursor cells go through a binary fate decision, they activate genes which are specific for the lineage of choice. This process is called lineage specification. The developing cells then restrict their plasticity and secure the lineage choice by inactivating or repressing genes relevant to the alternative fate. This process is termed lineage commitment, which is presumably mediated by transcription factors induced during the specification phase. Although it is believed that complex gene regulation networks need to be orchestrated to achieve appropriate cell differentiation, it has not been clearly understood how lineage-specific transcriptional regulators contribute to lineage specification and commitment. Thymocyte differentiation is an ideal system to study such regulatory circuits.

CD4 expressing helper T cells and CD8 expressing cytotoxic T cells play crucial roles in acquired immune responses. These two major populations are derived from a common precursor population of thymocytes expressing both CD4 and CD8 co-receptors (called double positive, DP), which are required for recognition of major histocompatibility complex molecules (MHC) class II and MHC class I, respectively. Following TCRα rearrangements and surface expression of the TCRαβ complex, DP thymocytes are tested for affinity and avidity of their clonal TCRs to self-peptides presented in the context of either MHC class I or MHC class II on thymic epithelial cells [von Boehmer et al., 2003]. This process is called positive selection. Only a small fraction of DP thymocytes are selected and differentiate into the helper or cytotoxic lineage of mature T cells. During the selection process, DP thymocytes selected by MHC class I are directed to the cytotoxic lineage, whereas cells with MHC class II-restricted TCRs differentiate into the helper lineage. Although many models have been proposed, it is not clearly understood how TCR-MHC class I interaction and TCR-MHC class II interaction trigger the differential gene expression programs which direct selected thymocytes toward the cytotoxic and helper lineages, respectively [Singer et al., 2008].

In the past few years, several studies have shed light on the roles of key transcription factors in the specification and commitment of the individual lineages. In this article, we would like to highlight recent findings on the transcription factor networks governing helper versus cytotoxic T-cell lineage decisions and thus provide

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general insights into the regulation of binary fate decision processes in cell differentiation.

THYMOCYTE LINEAGE CHOICES AND CO-RECEPTOR EXPRESSION

During differentiation of helper or cytotoxic T cells from DP thymocytes, either the CD8 or CD4 co-receptor is selectively turned off [Ellmeier et al., 1999]. The tight link between fate choices and co-receptor expression implies the existence of co-regulation between the two processes. Regulation of genes encoding these co-receptors has therefore been intensively studied through genetic analyses [Kioussis and Ellmeier, 2002]. The *Cd8a/Cd8b* genes, encoding components of the CD8 co-receptor, have multiple enhancer elements, and utilize stage-specific enhancers at different stages of thymocyte differentiation. Transgenic reporter studies have demonstrated that one of the *Cd8* enhancers, the $E8_{III}$ enhancer, is sufficient to activate CD8 expression at the pre-selected DP thymocyte stage [Feik et al., 2005]. Such DP stage-specific enhancer activity is down-regulated following positive selection regardless of TCR specificity to MHC class I or class II, leading to a loss of CD8 expression and the generation of an intermediate subpopulation with a $CD4^+CD8^{lo}$ phenotype [Singer et al., 2008]. Subsequently, MHC class I-selected thymocytes reactivate CD8 expression through the activation of mature stage-specific enhancers, including the $E8_I$ enhancer, while MHC class II-selected thymocytes continue to lose surface CD8 expression to acquire the CD8-negative phenotype [Ellmeier et al., 1997, 1998]. In contrast, the *Cd4* gene utilizes both positive and negative *cis* elements for its expression in the thymus [Sawada et al., 1994; Ellmeier et al., 1999]. Stage- and lineage-specific CD4 expression depends on the activity of the negative *cis* element, the *Cd4* silencer, antagonizing the activity of positive regulatory elements, such as the proximal enhancer, which is active in all thymocytes subpopulations including cytotoxic lineage cells [Sawada et al., 1994]. Following positive selection, *Cd4* silencer activity is selectively up-regulated in the MHC class I-selected thymocytes. Thus, MHC class I-selected thymocytes actively regulate co-receptor expression through reactivation of CD8 and silencing of CD4 expression, and it is therefore conceivable that transacting factors involved in the regulation of CD4 and CD8 expression in MHC class I-restricted thymocytes regulate development of the cytotoxic lineage. Interestingly, conditional deletion of the *Cd4* silencer revealed that the silencer element is required only for establishment of the silencing, but not for the maintenance of the silenced states of the *Cd4* locus [Zou et al., 2001]. In addition, partial inhibition of the *Cd4* silencer through mutations caused a variegated pattern of CD4 expression in mature $CD8^+$ T cells [Taniuchi et al., 2002b]. These results suggest that *Cd4* silencing is maintained by epigenetic gene silencing in the cytotoxic lineage. Because epigenetic gene regulation has been implicated in cell fate decision processes in general, regulators contributing to the establishment of *Cd4* silencing may potentially be key players for the thymocyte lineage commitment.

THE Runx PROTEINS IN CO-RECEPTOR REGULATION AND THYMOCYTE DEVELOPMENT

Through intensive deletion and mutagenesis analyses, several key sequences within the 430 bp *Cd4* silencer were identified [Taniuchi et al., 2002b]. Runx proteins have been shown to bind two of the key sequences, and are required for stage- and lineage-specific *Cd4* silencing [Taniuchi et al., 2002a]. Runx proteins are characterized by their evolutionarily conserved DNA binding motif called the *runt* domain, which is homologous to that of a *Drosophila* segmentation gene, *runt* [Gergen and Butler, 1988]. The Runx family consists of three genes in the mammalian system, and each Runx protein is essential for cell differentiation in various lineages during development, including hematopoietic, mesenchymal, and neuronal lineages [Okuda et al., 1996; Wang et al., 1996a; Komori et al., 1997; Levanon et al., 2002]. Runx proteins form heterodimers with the non-DNA binding subunit, CBFβ, for DNA binding as well as protection from degradation [Sasaki et al., 1996; Wang et al., 1996b; Huang et al., 2001]. Among the three members, Runx1 is essential for *Cd4* silencing in immature thymocytes, while Runx3 is required for *Cd4* silencing in the cytotoxic lineage of T cells, although the loss of *Cd4* silencing in Runx3-deficient cytotoxic lineage T cells is attenuated by compensatory up-regulation of Runx1 [Taniuchi et al., 2002a; Woolf et al., 2003]. It has also been suggested that Runx3 is important for the reactivation of CD8 expression following MHC class I-restricted thymocyte selection through binding to CD8 enhancers [Sato et al., 2005]. Runx3 protein is necessary for normal cytotoxic lineage development [Egawa et al., 2007]. Following conditional *Runx3* inactivation at the DP stage, the number of $CD8^+$ mature thymocytes is reduced and the expression of some cytotoxic lineage-specific genes is down-regulated. Furthermore, conditional inactivation of both *Runx1* and *Runx3* prior to positive selection eliminates functional redundancy between Runx1 and Runx3, and results in complete loss of $CD8^+$ cytotoxic lineage cells, although helper lineage cells can still be generated. Thus, Runx complex activity, primarily mediated by Runx3 expression, is required for the cytotoxic lineage development.

In mature thymocytes, Runx3 protein expression is restricted to the cytotoxic lineage, and is not detected in the helper lineage, although Runx3 mRNA expression is observed in both lineages [Hayashi et al., 2001; Taniuchi et al., 2002a; Egawa et al., 2007]. These findings suggest lineage-specific differential regulation of Runx3 expression between the two lineages, which may involve post-transcriptional regulation. All three Runx genes have two major promoters [Bangsow et al., 2001]. In the mouse *Runx3* locus, the two promoters are separated by a ~30 kb long intron and transcribe mRNAs which encode proteins harboring distinct N-termini. Distal promoter activity is specifically detected in thymocytes in the cytotoxic lineage following MHC class I-restricted selection and is required for Runx3 protein expression [Egawa et al., 2007; Egawa and Littman, 2008]. When Runx3 expression from the distal promoter is inactivated, the vast majority of Runx3 protein expression is lost in $CD8^+$ T cells, which causes de-repression of CD4 expression. In wild-type mice, the proximal promoter-derived transcript is detected in both helper and cytotoxic lineages as well as

in other tissues, such as dorsal root ganglia (DRG). However, it is not efficiently translated in mature thymocytes and T cells, while Runx3 protein is expressed in DRG from the proximal transcript [T. Egawa, unpublished work]. Thus, the cytotoxic lineage-specific Runx3 activity is regulated at the transcriptional level through its promoter usage as well as at the translation level.

ThPOK AND THE CD4$^+$ T-HELPER LINEAGE

A critical transcription factor for the helper lineage T-cell differentiation was identified through analysis of a spontaneous mutant strain of mice. Kappes and coworkers identified a mutant mouse strain, helper-deficient (*hd*), which lacks helper T cells [Dave et al., 1998]. The mutation was mapped to the *Zbtb7b* locus, which encodes the ThPOK transcription factor [He et al., 2005]. ThPOK was also independently identified through microarray analysis by Bosselut and coworkers as a gene up-regulated following positive selection [Sun et al., 2005]. ThPOK is expressed selectively in the helper lineage CD4$^+$CD8$^-$ mature thymocytes, but not in cytotoxic lineage CD4$^-$8$^+$ thymocytes or pre-selected DP thymocytes [He et al., 2005; Setoguchi et al., 2008]. ThPOK belongs to the BTB-POZ (bric-a-bric, tramtrack, broad complex, poxvirus zinc finger) family of transcriptional regulators, which is characterized by an N-terminal BTB domain and tandem C2H2 type of zinc finger motifs at the C-terminus. This family consists of approximately 40 proteins in mammals, and some BTB-POZ proteins are implicated in lineage decisions and lymphocyte development, including Bcl6, LRF/Pokemon, MAZR, and PLZF [Dent et al., 1997; Ye et al., 1997; Bilic et al., 2006; Maeda et al., 2007; Raberger et al., 2008; Savage et al., 2008]. The BTB domain is predicted to be required for homo-dimerization and recruitment of co-repressors, whereas the zinc finger motifs serve as the DNA binding domain. The *hd* mutation results in a single amino acid substitution in one of the zinc finger motifs of ThPOK, and was predicted to cause the loss of DNA binding [He et al., 2005]. In *hd/hd* mice, both MHC class I- and MHC class II-restricted thymocytes develop into the cytotoxic lineage [He et al., 2005]. In contrast, forced expression of ThPOK in all thymocytes prior to positive selection causes exclusive generation of helper lineage cells. This is caused by the expense of the cytotoxic lineage due to re-direction of MHC class I-restricted thymocytes toward the helper lineage [He et al., 2005; Sun et al., 2005]. These results together indicate that ThPOK expression in the selected thymocytes is required and sufficient for development of helper lineage cells. Because lack of functional ThPOK expression does not significantly affect the total number of mature thymocytes, ThPOK is not required for positive selection of DP thymocytes. It also suggests that specificity of a TCR to either MHC class I or class II is not a sufficient determinant for the lineage choice.

RECIPROCAL AND ASYMMETRIC CROSS-REGULATION BETWEEN Runx AND ThPOK

Runx3 and ThPOK are specifically expressed in and important for the development of cells directed toward cytotoxic and helper lineages, respectively. After positive selection, their expression is mutually exclusive in the majority of thymocytes differentiating into the two conventional T lineages [Egawa and Littman, 2008]. Provided that binary lineage determinations in blood cell development or lymphocyte differentiation is regulated by antagonistic interplay of lineage-specific transcription factors important for opposing lineages [Laslo et al., 2006; Reynaud et al., 2008; Zhou et al., 2008], it is possible that ThPOK and Runx cross-regulate each other's expression or activity (Fig. 1).

One piece of evidence supporting this possibility was demonstrated by analyzing regulation of ThPOK expression during thymocyte differentiation. Like CD4 expression, ThPOK expression is partly regulated by an inhibitory *cis* acting element, designated the ThPOK silencer or distal regulatory element (DRE). This silencer element was identified independently by two studies. One study mapped the silencer as one of the two regions at the *Zbtb7b*/ThPOK locus, which are bound by the Runx complexes through a ChIP-on-chip assay [Setoguchi et al., 2008]. The other study identified the silencer though mapping of DNaseI hypersensitive sites at the *Zbtb7b*/ThPOK locus [He et al., 2008]. The ThPOK silencer is active in pre-selection DP thymocytes and cells of the cytotoxic lineage. Deletion of the silencer in the context of a knocked-in ThPOK-GFP reporter allele results in GFP expression in DP and CD4$^-$8$^+$ thymocytes of the cytotoxic lineage [Setoguchi et al., 2008]. In the absence of the ThPOK silencer, ThPOK is prematurely expressed in DP thymocytes, which, similar to transgenic ThPOK expression, presumably results in re-direction of MHC class I-restricted thymocytes to the helper lineage [Setoguchi et al., 2008]. These results suggest that ThPOK expression is actively repressed in the pre-selection DP thymocytes and cytotoxic lineage selected thymocytes through ThPOK silencer activity. In the absence of Runx1, ThPOK mRNA is up-regulated, indicating that Runx1 is required for blocking premature ThPOK expression before positive selection, and may thus preserve the unbiased differentiation potential of pre-selected thymocytes [Setoguchi et al., 2008]. Conditional inactivation of both *Runx1* and *Runx3* in DP thymocytes results in higher ThPOK expression than *Runx1* inactivation alone, suggesting that Runx3 can also contribute to ThPOK silencing in Runx1-deficient DP thymocytes, although little Runx3 protein expression is detected at the DP stage [Setoguchi et al., 2008]. In the absence of both Runx1 and Runx3, differentiation of cytotoxic lineage cells is completely abrogated [Egawa et al., 2007]. This could be due partly to re-direction of MHC class I-selected thymocytes to the helper lineage caused by loss of ThPOK silencer activity. Indeed, CD4-positive mature thymocytes are generated in MHC class II-deficient mice in the absence of Runx1 and Runx3 activity. These class I-restricted CD4$^+$ T cells exhibit some characteristics of the helper T-cell, such as expression of CD154 (CD40 ligand) and interleukin 4 after in vitro TCR stimulation [Setoguchi et al., 2008]. Because Runx complex binding to the ThPOK silencer is observed both in helper and cytotoxic lineage cells [Setoguchi et al., 2008], it is possible that additional factors specifically expressed in the cytotoxic lineage may direct lineage-specific ThPOK silencing after positive selection.

Other pieces of data have suggested ThPOK-dependent inhibition of Runx3 expression and of other cytotoxic lineage-specific gene expression. In normal thymocytes, Runx3 expression from its distal

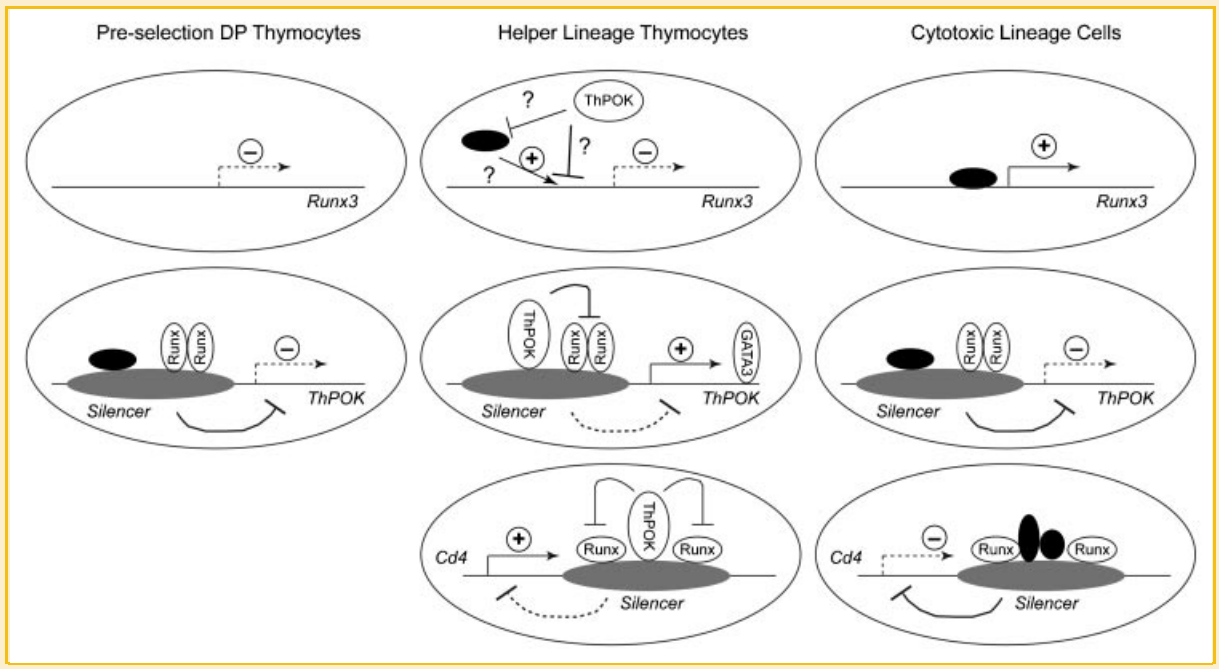

Fig. 1. A model for antagonistic interplay between ThPOK and Runx during thymocyte lineage decisions. Top and middle: Lineage-specific Runx3 and ThPOK expression is regulated by reciprocal antagonistic actions of Runx complexes and ThPOK. In developing helper lineage cells, Runx3 up-regulation is blocked in the presence of ThPOK, while ThPOK expression is repressed both in pre-selection DP thymocytes and cytotoxic lineage cells by Runx complexes. This repression may require additional factors (shown as a filled circle). In helper lineage cells, it was suggested that ThPOK is up-regulated through release of the repression and GATA3 recruitment to the enhancer. Once ThPOK is expressed, it binds to its own silencer to "lock" the silencer to the "off" state. Bottom: ThPOK also binds to the *Cd4* silencer in helper lineage cells, which may protect expression of Cd4 from Runx-mediated silencing.

promoter is restricted to cells in the cytotoxic lineage [Egawa and Littman, 2008]. Its expression is up-regulated as MHC class I-selected cells silence CD4 expression and reactivate CD8 expression, whereas MHC class II-restricted cells do not up-regulate Runx3 during development. Although MHC class II-selected thymocytes lacking ThPOK are finally re-directed to the cytotoxic lineage, ThPOK-deficient MHC class II-restricted thymocytes transiently exhibit the $CD4^+CD8^-$ phenotype by extinguishing CD8 expression similarly to wild-type cells, which is not observed in MHC class I-restricted thymocytes in mice lacking MHC class II [Egawa and Littman, 2008]. ThPOK-deficient MHC class II-restricted thymocytes also turn on ThPOK mRNA expression, suggesting that ThPOK is dispensable for the initiation of differentiation toward the helper lineage or initial ThPOK up-regulation [Egawa and Littman, 2008]. In contrast to wild-type cells, ThPOK deficient $CD4^+CD8^-$ thymocytes ectopically activate distal promoter-derived Runx3 expression, indicating that Runx3 up-regulation in MHC class II-selected thymocytes is blocked by ThPOK [Egawa and Littman, 2008]. It remains unclear, however, if ThPOK directly suppresses Runx3 expression. This is consistent with another finding in mice expressing reduced amounts of ThPOK. When ThPOK expression is reduced by deletion of an enhancer or by insertional mutations, a proportion of MHC class II cells are re-directed toward the cytotoxic lineage, resulting in a reduced number of $CD4^+$ helper lineage cells [Egawa and Littman, 2008; Muroi et al., 2008; Wang et al., 2008a]. In addition, the remaining $CD4^+CD8^-$ cells express Runx3 protein and exhibit some cytotoxic characteristics, including increased expression of the T-box transcription factor Eomes and a high-level of interferon gamma [Egawa and Littman, 2008; Muroi et al., 2008; Wang et al., 2008a]. These findings suggest that ThPOK antagonizes Runx3-dependent gene regulation, which directs selected thymocytes, regardless of their MHC specificity, toward the cytotoxic lineage, and that this antagonistic action is mediated in part through blocking Runx3 up-regulation from the distal promoter. Consistent with this interpretation, ThPOK deficient MHC class II-selected thymocytes are able to differentiate into the helper lineage in the absence of Runx complex activity through conditional inactivation of CBFβ [Egawa and Littman, 2008]. Therefore, ThPOK is required to prevent Runx-dependent programming of MHC class II-selected thymocytes toward the cytotoxic lineage to secure the initial lineage choice determined by TCR-MHC specificity. Once thymocytes are specified toward the helper lineage by positive selection, ThPOK may no longer be required for the continuous helper lineage differentiation in the absence of Runx complex activity.

MECHANISMS OF Runx- AND ThPOK-DEPENDENT THYMOCYTE LINEAGE COMMITMENT

Currently, it is unknown how Runx3 or ThPOK regulates thymocyte lineage commitment at the molecular level. Transgenic Runx3 expression cannot re-direct thymocytes to the cytotoxic lineage, indicating that Runx3 is not sufficient for the differentiation pathway, and suggesting that Runx3-dependent gene expression is

antagonized, possibly by ThPOK, to allow differentiation of the helper lineage [Grueter et al., 2005]. These findings suggest that key genes for thymocyte differentiation may be targets of both ThPOK and Runx. Additional data suggest that ThPOK may inhibit Runx protein functions directly through a protein–protein interaction (Fig. 1). For example, both Runx complexes and ThPOK bind to the *Cd4* silencer, which is a critical *cis* element determining differential co-receptor expression [Muroi et al., 2008]. When Runx3 is overexpressed in DP thymocytes, CD4 expression is silenced presumably through activation of the *Cd4* silencer by Runx3 [Grueter et al., 2005; Kohu et al., 2005]. However, when ThPOK is co-expressed, CD4 down-regulation is blocked, suggesting that ThPOK inhibits Runx3-mediated silencing [Wildt et al., 2007]. In DP thymocytes and cytotoxic lineage cells, ThPOK expression is inhibited by activity of the ThPOK silencer, whose function requires Runx complexes [Setoguchi et al., 2008]. Interestingly, the ThPOK silencer is also bound by ThPOK itself in the helper lineage, whereas Runx complexes appear to bind to the silencer constitutively [Muroi et al., 2008]. This finding implies that a recruitment of ThPOK to Runx-bound silencer elements inhibits Runx-dependent gene silencing. In this particular case, ThPOK may regulate its own expression through a positive feed forward loop that protects from Runx-dependent silencing. This may be a global mechanism which would account for the dominant effects of ThPOK. ThPOK and Runx would normally compete for regulation of target genes. Because ThPOK overexpression in mature cytotoxic T cells down-regulates some lineage-specific genes, such as *Cd8a*, *Ifng* and *Eomes*, and de-represses some helper lineage genes, such as *Il4* and *Gata3*, antagonistic effects of ThPOK on Runx-dependent gene regulation may involve both gene silencing and activation [Jenkinson et al., 2007].

Alternatively, ThPOK-dependent inhibition of Runx-mediated gene regulation could be indirect. Runx proteins regulate their target genes as either activators or repressors. Recruitment of co-activator p300/CBP has been linked to Runx-dependent gene activation, whereas the Groucho/TLE family of co-repressors plays an important role in Runx-dependent gene repression [Levanon et al., 1998; Yamaguchi et al., 2004]. Although it remains elusive what regulates the recruitment of different sets of co-factors to Runx complexes, it is possible that other transcription factors juxtaposing with Runx proteins at their target binding sequences may contribute to bringing in a specific set of co-factors and specify Runx-dependent gene regulation. Indeed, deletion and mutagenesis analyses of the *Cd4* silencer have revealed that Runx proteins are required, but not sufficient for *Cd4* silencer functions [Taniuchi et al., 2002a,b]. *Cd4* silencing requires additional factors that are co-recruited to the silencer elements. Because the ThPOK silencer is bound by Runx complexes in both helper and cytotoxic T cells, as well as in DP thymocytes, recruitment of additional unknown transcription factors may determine if ThPOK expression is silenced. Since such additional factors would be likely expressed specifically in the cytotoxic lineage, in which both CD4 and ThPOK are silenced, overexpressed ThPOK or endogenous ThPOK expression in the helper lineage may down-regulate these factors and thus inhibit silencing of CD4 and ThPOK independently of Runx proteins. This hypothesis is consistent with an in vitro finding that the antagonistic action of ThPOK against Runx-mediated *Cd4* silencing is sensitive to treatment with histone deacetylase inhibitors [Wildt et al., 2007].

ROLES OF ADDITIONAL TRANSCRIPTION FACTORS IN THYMOCYTE LINEAGE SPECIFICATION

In addition to Runx3 and ThPOK, a few other transcription factors are implicated in regulation of thymocyte lineage choices. Among those factors, GATA3 is a critical regulator of helper lineage differentiation [Pai et al., 2003]. GATA3 has been shown to be important at many stages during T-cell development, including early T-cell lineage development and the polarization of activated helper T cells toward the interleukin 4 producing T_H2 lineage [Ting et al., 1996; Zheng and Flavell, 1997]. When *Gata3* is conditionally inactivated at the DP stage, differentiation of CD4SP mature thymocytes is severely compromised, whereas differentiation of cytotoxic lineage mature thymocytes is not affected. This is consistent with another finding that GATA3 expression is up-regulated immediately after positive selection in MHC class II-restricted thymocytes, whereas GATA3 expression remains constant and low in MHC class I-selected thymocytes [Hernandez-Hoyos et al., 2003]. Differentiation of MHC class II-restricted cells toward the helper lineage is arrested at the $CD4^+CD8^{lo}$ stage in the absence of GATA3 [Pai et al., 2003]. In normal mice, a substantial fraction of $CD4^+CD8^{lo}$ intermediate thymocytes expresses ThPOK, which is required for their continued differentiation to the helper lineage [Egawa and Littman, 2008]. Interestingly, GATA3-deficient MHC class II-selected thymocytes lack ThPOK expression, suggesting that up-regulation of GATA3 may be required for ThPOK expression specifically in MHC class II-selected thymocytes [Wang et al., 2008b]. GATA3 binds to two DNase hypersensitive regions located in the first intron of the ThPOK/*Zbtb7b* gene [Wang et al., 2008b]. The intronic fragment containing the GATA3-bound regions is required for ThPOK-BAC reporter gene expression, suggesting that GATA3 acts as an upstream regulator of ThPOK expression following MHC class II-restricted positive selection [Wang et al., 2008b]. Because forced expression of ThPOK, which re-directs GATA3-sufficient MHC class I-selected thymocytes to the helper lineage, fails to rescue helper lineage differentiation in the absence of GATA3 [Wang et al., 2008b], GATA3 likely regulates genes, other than ThPOK, which are also important for helper lineage differentiation. Alternatively, GATA3 itself, which is also expressed in MHC class I-selected thymocytes at a low level, may cooperate with ThPOK to regulate target genes for the helper lineage differentiation.

TOX is another transcription factor required for helper lineage thymocyte differentiation [Aliahmad and Kaye, 2008]. In TOX deficient mice, the number of mature thymocytes of the helper lineage is severely reduced, whereas the cytotoxic lineage thymocyte number is less severely affected. Because TOX deficient thymocytes and GATA3 deficient thymocytes similarly lack ThPOK expression after positive selection, and GATA3 is normally up-regulated in positively selected thymocytes deficient for TOX, TOX may directly regulate ThPOK expression in cooperation with GATA3. However, it is also possible that TOX is required for

normal CD4 expression following MHC class II-restricted positive selection. In TOX deficient mice, MHC class II-selected thymocytes accumulate as a $CD4^{lo}CD8^{lo}$ (or DP-dull) population, suggesting that TOX may be necessary to maintain or reactivate CD4 expression after positive selection.

E proteins (also known as basic helix-loop-helix proteins) may also play important roles in thymocyte selection and lineage determination. In the normal thymus, DP thymocytes rearrange the TCRα locus, and only positively selected thymocytes up-regulate surface TCRαβ expression and migrate to the periphery. However, when E proteins E2A (encoded by *Tcfe2a*) and HEB (*Tcf12*) are conditionally inactivated, a substantial number of $CD4^{-}CD8^{+}$ T cells lacking surface TCRαβ expression are detected in the mature thymocyte fraction and in the peripheral T-cell pool, suggesting essential functions of E2A and HEB at the positive selection check point [Jones and Zhuang, 2007]. Interestingly, E2A/HEB deficient cells which bypassed positive selection develop exclusively into the cytotoxic lineage, but not into the helper lineage, suggesting that E2A/HEB may be required for helper lineage differentiation, or may block cytotoxic lineage development. Alternatively, similar to the case of TOX deficient mice, the lack of differentiation of cells of the helper lineage in E2A/HEB deficient mice could be secondary to dysregulation of CD4 expression. HEB binds to the *Cd4* enhancer that is required for CD4 expression in DP thymocytes, and HEB/E2A-deficient DP cells express CD4 at a lower level compared to wild-type thymocytes [Sawada and Littman, 1993; Jones and Zhuang, 2007]. Helper lineage differentiation may require instructive signals during positive selection. The instructive signals appear to be mediated through TCR-CD4-MHC class II interaction. It has been suggested that high CD4 expression needs to be maintained for helper lineage differentiation [Sarafova et al., 2005]. At least a fraction of MHC class II-restricted thymocytes differentiate into the cytotoxic lineage when CD4 expression is absent [Tyznik et al., 2004]. Future studies will be necessarily to determine if these E proteins and TOX regulate thymocyte lineage decisions independently of *Cd4* expression.

MULTIPLE STEPS DURING THYMOCYTE LINEAGE DECISIONS

Several lines of evidence have suggested that binary cell fate decisions involve multiple steps, in which mixed gene expression relevant to both of the progeny fates is initially induced by gene activation and then resolved by selective gene silencing as one fate of a developing cell is determined and fixed. In MHC class II-restricted thymocyte selection, selected cells initially up-regulate GATA3, which acts as a lineage specifying factor and then turns on ThPOK transcription at the $CD4^{+}CD8^{lo}$ stage. Although activation of ThPOK transcription is specifically observed following MHC class II-restricted selection and ThPOK mRNA (+) cells appear to initiate a series of genetic programs directing full lineage commitment, the fate of these cells has not yet been determined because these cells are re-directed to the cytotoxic lineage in the absence of ThPOK protein [He et al., 2005; Egawa and Littman,

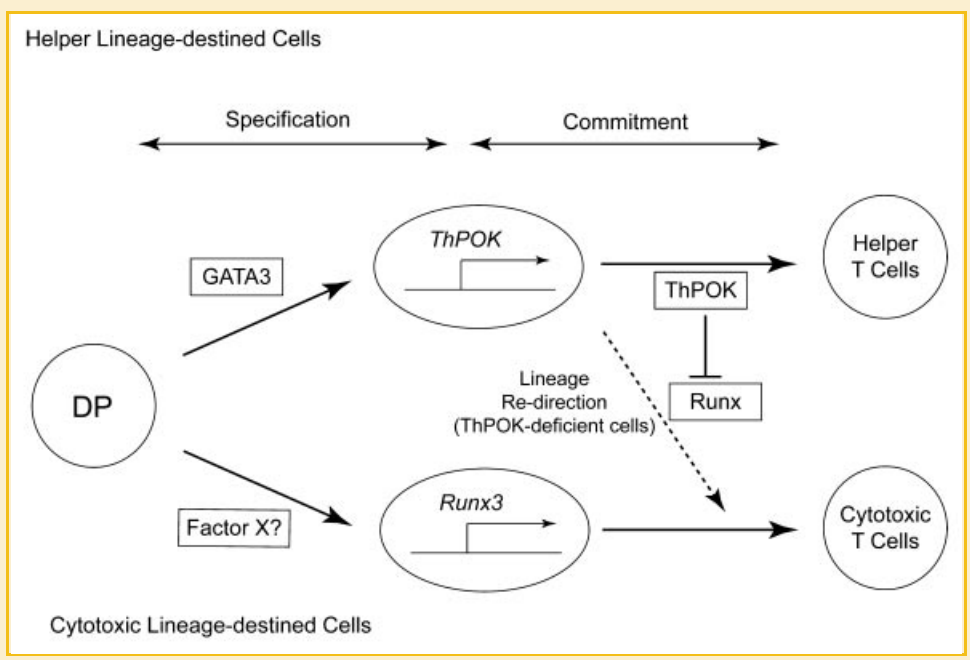

Fig. 2. The Roles of Runx complexes and ThPOK in thymocyte cell fate decisions. During normal thymocyte differentiation, ThPOK expression is detected in the helper lineage, which is dependent on GATA3, whereas Runx3 expression, driven by its distal promoter, is specifically observed in the cytotoxic lineage. In the absence of ThPOK, helper lineage-destined cells with active ThPOK transcription turn on Runx3 expression and are re-directed to the cytotoxic lineage. Such re-direction does not occur in the absence of both ThPOK and Runx complexes. These observations suggest that ThPOK functions as a helper lineage commitment factor by repressing the Runx-dependent cytotoxic lineage differentiation potential of the helper-destined cells.

2008]. ThPOK protein likely secures the helper lineage fate of MHC class II-restricted thymocytes by eliminating Runx-mediated cytotoxic lineage potential. Thus, the helper lineage differentiation requires at least two layers of a transcription factor network involving GATA3 for lineage specification and ThPOK for lineage commitment (Fig. 2).

In contrast, the cytotoxic differentiation pathway has not been as well characterized as the helper lineage pathway. Currently, there are no known factors that are both necessary and sufficient for the cytotoxic lineage differentiation, raising the possibility that in the presence of Runx complex activity cytotoxic lineage differentiation may not require GATA3 or ThPOK-equivalent lineage specifying/commitment factors. Conversely, provided that regulators of the *Cd4* and ThPOK silencers regulate cytotoxic lineage specification, that the *Cd4* silencer can be activated by Runx3 overexpression, and that the ThPOK silencer is functional already in the DP thymocytes, key transcription factors for the cytotoxic lineage may already be expressed prior to positive selection. Because Runx3 up-regulation is observed specifically in the cytotoxic lineage and may well be regulated by such factors, analysis of Runx3 gene regulation may contribute to identification of these factors.

CONCLUSIONS AND FUTURE DIRECTIONS

Recent findings suggest that ThPOK is required late in helper lineage determination after cells have already been specified toward the lineage by other transcription factors, such as GATA3. ThPOK likely secures the initial fate choice of MHC class II-restricted thymocytes in part by blocking up-regulation of Runx3 and antagonizing Runx complex activity, such as *Cd4* gene silencing. However, a number of questions remain to be answered. Most studies so far have relied on the expression of a small number of genes, including *Cd4* and *Cd8a/Cd8b*, as a readout for the lineage choices. It is important to more broadly define the genetic signature of the helper and cytotoxic lineages. It would be intriguing to determine whether the cytotoxic lineage differentiation occurs by default, or it requires specification factors which correspond to GATA3 in the helper lineage and function as upstream regulators for Runx3 expression. Finally, it is crucially important to explore the mechanisms of how different positive selection signals through either TCR-CD8-MHC class I or TCR-CD4-MHC class II are translated into distinct gene expression programs in order to understand the initiation of genetic programs governing thymocyte lineage choices.

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