The network of transcription factors that underlie the CD4 versus CD8 lineage decision

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Abstract

Virtually all mature T cells are CD4+CD8− or CD4−CD8+ and this not only is their most important surface-phenotype distinction but also has crucial functional consequences for the entire immune response. Both subsets arise from double-positive thymocytes, and much has been learned about the molecular events that govern this lineage bifurcation process. As detailed in this review, the signaling pathways and specific molecules that control this process are now being discovered. In particular, the transcription factors ThPOK (T-helper inducing POZ-Kruppel factor) and Runx3 have emerged as the crucial regulators of helper lineage commitment and the cytotoxic lineage, respectively. This article describes their antagonistic interaction that is an important mechanism of the lineage specification, as well as the hierarchy and importance of several other transcription factors and cytokine signals in the network of pathways that govern thymocyte helper/cytotoxic lineage commitment.

Keywords: cross-antagonism, cytokine signal, runx, TCR signal, ThPOK

Introduction

CD4-expressing helper lineage T cells and CD8-expressing cytotoxic lineage T cells are the two major lineages among mature T cells. Both of these lineages arise from the same pool of cells—CD4+CD8+ double-positive (DP) cells in the thymus. After positive selection, DP thymocytes choose either one of these lineages, which is consequently well correlated with their TCR specificity for MHC molecules (MHC Class II for CD4-expressing T cells and Class I for CD8-expressing T cells). This bifurcation to CD4 and CD8 lineages has been studied as a model of bi-modal lineage decisions for many years. Recent identification of transcription factors involved in lineage specification and commitment has greatly advanced our understanding of the genetic circuit underlying these processes. In this review, we summarize the transcriptional circuit operating in lineage decisions of T cells and place it in the context of regulation by signaling. For details on the signaling aspects of T-cell differentiation, please refer to another review (1).

'Master' regulator of lineage decision

The idea that there is a ‘master’ transcriptional regulator, which is necessary and sufficient to induce a specific cell type, is a popular one. One example that fits into such a model is a basic-helix-loop-helix transcription factor Myod (2). When Myod is expressed in non-muscle types of cells, such as fibroblast, it converts them into myoblasts. Conversely, genetic disruption of Myod results in total absence of skeletal muscle in mice, fulfilling the definition of master transcriptional regulator. In T cells, studies have identified many transcription factors that are important for their development (3), and some of these factors are crucial for the differentiation of a specific helper T-cell subset, such as T-bet in T Helpers 1 cell differentiation (4, 5) and Gata3 (GATA-binding protein 3) in T helpers 2 cells (6). Thus, the concept of ‘master transcription factor’ is applicable to helper T-cell subsets as well.

Although the progresses made in identification of factors regulating the development of helper T-cell subsets, researchers had not been able to identify factors that are absolutely required for the commitment to the helper or cytotoxic lineage in the thymus, partly because of experimental limitations. Thus, the transcriptional mechanism of helper versus cytotoxic lineage decision had remained a mystery for a long time.

ThPOK: the critical regulator of helper T-cell differentiation

Striking progress in this field was brought by the identification of ThPOK (T-helper inducing POZ-Kruppel factor; also known as Zbtb7b and cKrox) as a central transcription factor for helper T-cell development. In 1998, a mutant mouse strain lacking the differentiation of CD4 helper T cells was...
identified (7). This strain was therefore named the helper-deficient (HD) mutant. The HD phenotype was not due to the loss of MHC Class II molecule expression on cell surface, nor by a defect of cell surface CD4 expression in peripheral T cells, but was a result of the redirection of Class-II-restricted cells to CD8* cells (8). This finding not only is the first description of lineage redirection by a gene mutation but also demonstrates that positive selection and lineage decision are separate events.

Eventually, the Zbtb7b gene was identified as being responsible for the HD phenotype (9). The Zbtb7b gene (referred as the ThPOK gene in this review) encodes a zinc-finger transcription factor, ThPOK, which belongs to the BR-C, ttk and bab (BTB)/poxvirus zinc finger (POZ) family. The HD mutation was found to be a point mutation within the second zinc finger, causing substitution of an Arg residue that contacts DNA directly. Around the same time, ThPOK was independently isolated through the screening of genes that are up-regulated upon positive selection (10). Expression of the ThPOK gene is only detected in thymocytes after positive selection and is further up-regulated in cells developing into the helper lineage, whereas it is silenced in mature CD4*CD8* single-positive (SP) thymocytes (9, 10).

Strikingly, forced expression of ThPOK causes total inhibition of CD8* cell development because of a re-directed differentiation of MHC Class-I-selected cells into CD4*CD8* SP T cells (9, 10). In contrast, targeted deletion of ThPOK gene in mice causes phenocopies of HD mice—a lack of CD4* T cells due to redirection of Class-II-selected cells into CD4*CD8* T cells (11, 12). These results clearly indicate that ThPOK is an essential and sufficient factor to imprint the helper fate in postselection thymocytes regardless of their TCR specificities for MHC molecules.

**Runx antagonizes ThPOK and induces a cytotoxic lineage gene program**

Another important finding in this field was the identification of Runx transcription factor complexes as essential repressors for the Cd4 gene as well as the ThPOK gene. Runx complexes function as heterodimers between Runx proteins—which contain the evolutionarily conserved RUNT domain for DNA binding—and core-binding factor β (Cbfβ), which itself does not bind DNA but enhances Runx’s ability to bind DNA. There are three Runx genes in mammals, namely Runx1, Runx2 and Runx3.

The role of Runx1 (its human homologue is also known as acute myeloid leukemia 1) in definitive hematopoiesis has been known from the germ line knockout studies (13, 14); however, its more specific function in T cells was discovered relatively recently through the study of mechanisms underlying helper lineage-specific CD4 expression, which is regulated by the activity of the intronic silencer within the Cd4 gene (15, 16). There are two Runx-binding sites within the 434 bp of the Cd4 silencer. Targeted mutations of these two sites cause loss of the silencer activity (17); furthermore, decreased activity of Runx complexes by loss of expression of Runx1, Runx3 (18) or Cbfβ (19) during thymocyte differentiation causes derepression of the Cd4 gene in CD8* cytotoxic lineage cells.

Subsequent study showed that Runx complexes are required not only for Cd4 gene regulation but also for development of CD8-lineage cells. Overexpression of Runx3 results in an increase of mature CD8 SP cells partly through redirection of Class-II-restricted cells (20, 21). Conversely, perturbed Runx activity causes a decrease in the CD8* cells due to redirection of MHC Class I-selected cells into CD4* cells (22–24).

Importantly, ablation of Runx complexes causes derepression of the ThPOK gene in preselection DP thymocytes where usually ThPOK is not expressed (25). Also, a much higher level of ThPOK expression is observed in Runx-depleted CD4*CD8* peripheral T cells, which are MHC Class-I-selected cells that have failed to silence CD4 and to maintain an appropriate CD8 level; furthermore, chromatin immunoprecipitation assays detected a direct binding of Runx complexes to the ThPOK locus through the region that exhibits a transcriptional silencer activity in a reporter transgene assay (25) (Fig. 1A). These observations indicate that Runx complexes actively repress ThPOK expression by activating the silencer element via direct association.

Interestingly, the expression of a yellow fluorescent protein (YFP) reporter gene under the control of the distal promoter of the Runx3 gene (dRunx3YFP), which is active only in CD8-lineage T cells, is up-regulated in CD4 lineage cells when ThPOK functions are compromised, indicating that ThPOK is necessary for repression of the dRunx3 gene during differentiation of CD4 helper lineage T cells (24); furthermore, conditional inactivation of the Cbfβ gene in DP thymocytes allows the emergence of CD4*CD8* mature T cells with helper characteristics even under a ThPOK-deficient background (24). These results suggest that the one critical role of ThPOK is to repress the expression of Runx3, thereby preventing cells from committing to the cytotoxic lineage. The cross-antagonism between ThPOK and Runx complexes is thus the most important part of the mechanisms that controls CD4 versus CD8 cell fate decision, as we further discuss later.

**Plasticity of genetic program regulated by ThPOK**

Given the crucial role of ThPOK in cell fate determination to the helper lineage, it is of interest whether ThPOK is continuously required to maintain the helper identity or it becomes dispensable once genetic programming for the helper lineage is completed. Transfer of CD4 T cells after conditional inactivation of the ThPOK gene (using the causes recombination–LoxP system) into lymphopenic host mice results in a re-expression of CD8 in some of these cells (26). In addition, CD4 T cells that developed under insufficient amount of ThPOK were found to ectopically express proteins associated with the cytotoxic lineage, such as Eomes, perforin and Granzyme B (26). Interestingly, when these cells are cultured under non-polarizing Th condition, their differentiation was heavily skewed toward IFN-γ production, which is usually associated with cytotoxic T cells. This is presumably in part caused by Runx3 derepression since the retroviral transduction of dominant-negative form of Runx protein quenched the CD8 phenotype, whereas Runx3 transduction into wild-type CD4 cells induced GzmB expression (26).
It is intriguing that the helper T-cell program enforced by ThPOK expression is reversible to a certain degree since this may contribute to ensure a correct lineage decision during the lineage specification phase as we discuss later.

**Transcription factors upstream of ThPOK**

Since the expression of ThPOK is critical for CD4 lineage commitment, the next obvious question is: what are upstream factors that initiate ThPOK gene expression?

One such candidate is the Gata3 transcription factor. The Gata3 gene is expressed throughout the T-cell differentiation. In addition to its well-characterized function in Th2-cell differentiation, current studies unraveled that Gata3 plays an essential role in helper versus cytotoxic lineage choice. The level of Gata3 expression goes down at the transition from the double negative (DN) to the DP stage and goes up again only after Class-II-mediated positive selection (27). Interestingly, specification to the CD4 lineage was impaired in the absence of Gata3 (27, 28), whereas overexpression of Gata3 inhibits CD8 differentiation (27).

Since both Gata3 and ThPOK are important for CD4 cell differentiation, the hierarchical order of these factors in lineage decision process was examined. Whereas ThPOK deficiency did not affect the expression of Gata3 protein, Gata3 deficiency results in a lack of ThPOK-expressing cells in the CD69+ postselection thymocyte subset. Together with a binding of Gata3 at two regions in the ThPOK locus (12) (Fig. 1A), it is likely that Gata3 directly regulates ThPOK expression in postselected thymocytes; however, it was also shown that transgenic ThPOK expression can not rescue CD4 cell development under a Gata3-deficient background, indicating that Gata3 has more functions than just inducing ThPOK transcription during the specification/commitment to the helper lineage (Fig. 2).

Myb also plays an important role in CD4 specification. T-cell-specific conditional inactivation of Myb causes defects in recombination at both Tcrb and Tcra loci, decreased survival of preselection DP cells and defective differentiation toward the CD4 T lineage while minimally affecting CD8 T cells (29, 30). A recent study showed that the defect in CD4 differentiation in Myb knockout lies before the induction of Gata3 (31). The study also showed that Myb binds to the Gata3 promoter (Fig. 1B) and activates it, placing Myb upstream of Gata3 and ThPOK (Fig. 2).

Another transcription factor that acts early in postselected thymocytes is thymocyte selection-associated high mobility group box (TOX) (32). Tox knockout leads to a complete block of CD4+ cell differentiation while preserving CD8+ cells (33). In the CD4+CD8- population, which consists of postselected cells that are in transition to mature T cells, the expression of ThPOK is absent. This suggests that TOX acts as an upstream molecule of ThPOK induction for driving CD4 lineage development, although it is equally possible that there is an essential CD4 specification pathway regulated by TOX, and acting in parallel to the ThPOK-dependent one (Fig. 2).

Recently, MAZR, a BTB-POZ domain-containing transcription factor encoded by Patz1, was also identified as a negative regulator of ThPOK (34). The expression of MAZR is high in DN and DP and lower in CD4 and CD8 SP thymocytes as well as T cells in spleen (35). MAZR binds to the ThPOK silencer in DP thymocytes (Fig. 1A), and Patz1 knockout mouse shows partial derepression of ThPOK in positively selected thymocytes as well as mature CD8 T cells, and partial redirection of Class-I-selected T cells into CD4 lineage (34). At the moment, its position within the hierarchy of lineage specification factors is not yet clear, and further studies on its regulation as well as its mechanism of action are awaited.
Cytokine signals as inducers of Runx3 and a cytotoxic cell fate

Compared with the CD4 lineage, the pathway that leads to Runx3 expression and the CD8 T-cell lineage was rather obscure; however, recent studies have provided an unexpected link between dRunx3 induction and resumption of cytokine responsiveness to cytokines, most likely IL-7, upon CD8 cell lineage commitment, which depends on the cessation of the TCR signal (21). The mechanism that ensures a proper lineage choice matching to the MHC specificity of the TCR has been a subject of intense debate for years, but now the ‘kinetic signaling’ model is a widely accepted one that can explain most observations made in the past (1). After positive selection, CD4+CD8+ DP cells eventually turn off either CD4 or CD8 to become CD4−CD8+ SP or CD4+CD8− SP, respectively; however, before reaching the SP stage, positively selected thymocytes initially become CD4+CD8lo cells by down-regulating CD8 regardless of the MHC classes they were selected by (36). This asymmetric coreceptor down-regulation provides the cue governing the cell’s lineage commitment. When the cells express a TCR that recognizes MHC Class I, down-regulation of the CD8 coreceptor results in a short-lasting TCR signaling, which instructs CD8 lineage commitment and the resumption of CD8 expression (‘coreceptor reversal’). Conversely, the signaling mediated via a TCR that recognizes peptide-MHC Class II complex would not be affected by CD8 down-regulation, and a long-lasting TCR signal directs these cells to become CD4 SP.

It has recently been proposed that the reactivation of signal transducer and activator of transcription-mediated cytokine signals is crucial for CD8 T-cell commitment after perturbation of the TCR signal (21). After positive selection, DP cells regain responsiveness to γc cytokine signals (37), which is only temporal in the presence of continuous TCR signaling (36); however, when the TCR signal is aborted, such as in Class-I-selected CD4+CD8lo− cells, they maintain responsiveness to signals by γc cytokines. In postselected

Fig. 2. The hierarchy and interactions of transcription factors that control the CD4+ versus CD8+ programs. DP thymocytes are positively selected by interaction with cells expressing MHC Class I or Class II. Cells that commit to the CD4+ program to become the CD4+CD8− helper lineage express transcription factors such as Tox, c-Myb, Gata3, which induces ThPOK expression but also regulate CD4 lineage commitment in ThPOK-independent manner (uncertain interactions indicated by dashed lines); cytokine signaling is important for the cytotoxic lineage differentiation, and its inhibition by suppressor of cytokine signaling 1 is released upon positive selection. However, the signaling becomes active only after the cessation of the TCR signaling. ThPOK enhances its own expression and inhibits Runx3, which is the crucial transcription factor for the CD8+ program; conversely, Runx3 and the cytokine signal that are crucial for the CD8+ program inhibit ThPOK. Note that the molecules depicted also affect other gene programs and act at other stages of T-cell development.
thymocytes, γc cytokine signals, most likely by IL-7, induce dRunx3 expression as well as providing a survival signal (21) (Fig. 2).

Early events leading to ThPOK or Runx3 induction and lineage specification

Now we have an emerging picture of the transcription factor network governing the T-cell helper/cytotoxic lineage decision (Fig. 2). ThPOK and Runx3 are the key players in this network and can antagonize one another. This antagonism is believed to be necessary to maintain lineage integrity once a cell is committed to a specific type.

The emergence of CD4+CD8− helper-like cells in the absence of both ThPOK and Cbfb suggests that the default differentiation pathway of postselected thymocytes could be the helper T-cell lineage—at least in these circumstances—and that the role of ThPOK in helper cell specification is in part achieved through repressing the activity of the Runx complexes. It is thus important to understand how their activities are modulated to achieve proper lineage specification and commitment, especially before cells finally commit to each lineage. The lineage specification process starts after positive selection, which induces ThPOK; however, positive selection has a different effect on ThPOK induction, inducing much higher ThPOK expression in MHC Class-II-selected CD4+CD8α cells than the same population selected by MHC Class I molecules (9–11, 24).

In addition, ThPOK potentially acts to sustain its own expression both by direct inhibition of Cd4 silencer activity, which results in a lasting MHC Class-II-restricted TCR signal, and by direct antagonism of the ThPOK silencer during differentiation of Class-II-restricted cells (11) (Fig. 1A). This self-amplification loop of ThPOK could be important to efficiently guide Class-II-selected cells toward the correct helper T-cell pathway at the start of the specification process.

It is not clear yet whether and how much ThPOK expression is induced in Class-I-selected cells; however, if it is expressed, these cells need to reverse the helper lineage fate by silencing ThPOK expression. The reversibility of the expression status of the ThPOK gene during certain developmental window will thus be crucial during lineage correction in which helper-leaning cells are reoriented to the cytotoxic lineage. In this regard, it is worth mentioning that lineage commitment imposed by ThPOK is reversible to a certain degree during the lineage specification process in the thymus (11) as well as in mature CD4+ T cells (26), suggesting that some target genes of ThPOK retain reversibility throughout cell development.

In the normal setting, signaling by γc cytokines, e.g. IL-7, after the cessation of the TCR signal induces dRunx3; however, since dRunx3 is immediately induced after positive selection in the absence of ThPOK (24), dRunx3 is likely to be repressed by ThPOK even in Class-I-selected cells. This implies that, during coreceptor reversal, cytokine signals may silence ThPOK expression to interrupt the feed-forward genetic loop that will otherwise result in a helper cell fate. Then, the induced Runx3 represses ThPOK and secures the cytotoxic gene program. Consistent with this notion, forced cessation of TCR signaling ex vivo, presumably followed by cytokine signaling, can induce differentiation of CD8+ SP cells from ThPOK-expressing precursors (11). It is important to clarify whether cytokine signals may repress residual ThPOK activity to induce dRunx3 in the physiological setting as well.

Conclusions and further questions

Although our knowledge of the lineage decision process is richer than ever, there are still important questions to be addressed besides the ones that we mentioned above.

The first issue is when lineage decision is finalized and what factor finalizes the decision. The study by Muroi et al. (11) shows an inverse correlation between the ThPOK expression level and the potential to become CD8+ SP cells. A certain amount of accumulated ThPOK may thus be necessary to irreversibly commit to helper T cells. But it is also possible that repression of the cytotoxic program by ThPOK could provide enough time to seal the helper T-cell fate by other factors regulated by Gata3 or Tox. A more quantitative analysis of the expression kinetics of the involved factors as well as their relative activities will be necessary to understand how the initial plastic status resolves into mutually exclusive gene expression pattern.

The other question is, what is the mechanism of ThPOK repression? Since the Runx complex binds to the ThPOK silencer even in cells expressing ThPOK (25), the activity of the silencer is regulated beyond the Runx binding. Identification of other proteins that bind to the silencer and the potential modifications of Runx complexes would help to further understand the molecular mechanisms regulating ThPOK gene expression.

It is also important to identify factor(s) beside ThPOK that are crucial for helper cell development and whose expression or function is regulated by Gata3. Knowing how transcription of Runx3 from the distal promoter is regulated is also critical for our understanding of lineage commitment into the cytotoxic lineage; the mechanisms that coordinate these factors and signaling events are largely unknown. Through studies to address these questions, unexpected and exciting findings will advance our understanding of the mechanism of the T-cell lineage decision processes.

References

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