ANNUAL REVIEWS

Annual Review of Immunology

CD4 Helper and CD8 Cytotoxic T Cell Differentiation

Ichiro Taniuchi

Laboratory for Transcriptional Regulation, RIKEN Center for Integrative Medical Sciences, Yokohama, Kanagawa 230-0045, Japan; email: ichiro.taniuchi@riken.jp

Annu. Rev. Immunol. 2018. 36:579–601

The *Annual Review of Immunology* is online at immunol.annualreviews.org

https://doi.org/10.1146/annurev-immunol-042617-053411

Keywords

thymus, lineage decision, T lymphocytes, transcription factors, TCR signals

Abstract

A fundamental question in developmental immunology is how bipotential thymocyte precursors generate both $CD4^+$ helper and $CD8^+$ cytotoxic T cell lineages. The MHC specificity of αβ T cell receptors (TCRs) on precursors is closely correlated with cell fate–determining processes, prompting studies to characterize how variations in TCR signaling are linked with genetic programs establishing lineage-specific gene expression signatures, such as exclusive CD4 or CD8 expression. The key transcription factors ThPOK and Runx3 have been identified as mediating development of helper and cytotoxic T cell lineages, respectively. Together with increasing knowledge of epigenetic regulators, these findings have advanced our understanding of the transcription factor network regulating the CD4/CD8 dichotomy. It has also become apparent that $CD4^+$ T cells retain developmental plasticity, allowing them to acquire cytotoxic activity in the periphery. Despite such advances, further studies are necessary to identify the molecular links between TCR signaling and the nuclear machinery regulating expression of ThPOK and Runx3.

1. INTRODUCTION

Development of T lymphocytes, defined as hematopoietic cells expressing either αβ or γδ T cell receptor (TCR) complexes, requires a primary lymphoid organ, the thymus, which provides a specific microenvironment essential for T lymphocyte differentiation (1). After homing of hematopoietic progenitors, referred to as early thymocyte progenitors (ETPs), possessing developmental potency to become non-T lymphoid cells, through guidance by CCR9/CCR7 expression (2, 3), ETPs initiate genetic programs directing their development into T cells through exposure to the thymic microenvironment, which provides Notch ligand stimulation (4). Classically, the process of T lymphocyte development in the thymus has been divided into four stages according to the expression of the surface markers CD4 and CD8 (5). ETPs do not express CD4 or CD8; hence, this earliest phase is referred to as the $CD4^{-}CD8^{-}$ double-negative (DN) stage, which is further divided into substages DN1 to DN4 based on the expression pattern of CD44 (or c-Kit) and CD25. Recent studies have revealed that full commitment to the T lymphoid lineage occurs at the DN2a-to-DN2b transition upon induction of the transcription factor Bcl11b (6–9). During this transition, DN thymocytes lose the potential to become non-T cells and undergo irreversible genome recombination at the *Tcrb* and *Tcrg* gene loci. Successful assembly of the V and DJ segments at the *Tcrb* locus producing the functional *Tcrb* chain results in formation of pre-TCR complexes, along with other T lineage–specific molecules, including the pTα chain (10). Signaling through pre-TCR complexes, which can occur without recognition of self-peptide (11), then activates expression of the *Cd4* and *Cd8* genes, driving DN thymocytes into the next developmental stage, referred to as the $CD4^{+}CD8^{+}$ double-positive (DP) stage. Signaling through pre-TCR complexes activates rearrangement of the V and J segments of the *Tcra* gene and inhibits additional rearrangement of the *Tcrb* locus (allelic exclusion) (12). Consequent coexpression of the TCRα and TCRβ chains results in generation of αβTCR complexes on the surface of $CD4^{+}CD8^{+}$ DP thymocytes.

CD4 and CD8 molecules are not merely markers that define thymocyte developmental stages; they are also essential to facilitating αβTCR recognition of peptides presented on MHC molecules, and in transmitting intracellular signals following recognition of self-peptide by αβTCR complexes (13). Thus, $CD4^{+}CD8^{+}$ DP thymocytes, for the first time during T cell development, express a complete antigen receptor, αβTCR, that can recognize self-peptide with the assistance of CD4/CD8 coreceptors. This signature feature forces $CD4^{+}CD8^{+}$ DP thymocytes into a process known as positive and negative selection, which selects cells based on appropriate reactivity of αβTCRs to self-peptides. Only a small proportion of DP thymocytes are positively selected (postselection thymocytes), and these cells are allowed to differentiate further, to become mature thymocytes (14, 15). The positive selection process is accompanied by changes in surface protein expression, including an increase in αβTCR expression and kinetic changes in expression of CD5, CD69, and the chemokine receptor CCR7 (16, 17). Although these changes occur equally in all postselection thymocytes, regardless of the type of MHC involved in TCR engagement, CD4 and CD8 coreceptors exhibit differential expression patterns among thymocytes selected according to MHC type, i.e., class I or class II. After temporal downregulation of the CD8 coreceptor, which occurs in all postselection thymocytes (18) and significantly influences differences in the duration of the initial positive-selection signals between MHC-I- and MHC-II-selected cells (19), CD4 expression is maintained specifically in MHC-II-selected cells that shut off CD8 expression, whereas MHC-I-selected cells reactivate the gene encoding CD8 and eliminate CD4 expression (5, 20). Thus, MHC-I- and MHC-II-selected thymocytes eventually, on completion of the maturation process, acquire $CD4^{-}CD8^{+}$ single-positive (CD8 SP) and $CD4^{+}CD8^{-}$ single-positive (CD4 SP) surface phenotypes, respectively. More

importantly, these CD8 SP and CD4 SP thymocytes are committed to becoming the functionally distinct effector subsets, i.e., cytotoxic and helper T cells, respectively (5, 20, 21).

In the field of T cell development research, since the molecular identity of the CD4/CD8 coreceptors was clarified in the mid-1980s (22, 23), a central question has been how this $CD4^+$ helper versus $CD8^+$ cytotoxic lineage dichotomy is regulated. Historically, two models were proposed to explain how CD4 and CD8 coreceptor expression patterns correlate with MHC restriction of αβTCRs on thymocytes as well as helper/cytotoxic lineage choice (24, 25). The "instructive" model proposed that differences in TCR signals activated a distinct genetic program leading to activation of either the *Cd4* or the *Cd8* gene (26). Another model, designated the stochastic/selection model, hypothesized that expression of the CD4 or CD8 coreceptor was random and that thymocytes expressing coreceptors that matched the specificity of their TCRs for MHC types were selected (25). Because this review focuses on recent advances, for summaries of the experimental challenges in discriminating between these two models please refer to reviews published in the 1990s (20, 27, 28). Since then, studies that aimed to unravel the molecular mechanisms regulating expression of the genes encoding the CD4 and CD8 coreceptors have identified essential *cis*-regulatory elements at these loci (5). These findings were followed by identification of transcriptional factors that are involved in *Cd4* and *Cd8* gene regulation, as well as in the separation of the helper and cytotoxic T cell lineages. Along with advances in understanding of epigenetic regulation in the 2000s, increasing information has become available on how *Cd4* gene expression is stably inherited (29). In the mid-2010s, a previously unappreciated plasticity, enabling cytotoxic function of helper lineage T cells, was discovered (30). In this review, I summarize current knowledge of the transcriptional regulation of the helper/cytotoxic lineage dichotomy (**Figure 1**) and provide my perspective on approaches to seek the missing pieces that link TCR signaling with transcriptional regulation, and on the importance of studies from an evolutional perspective.

2. KINETIC EXPRESSION OF THE CD4/CD8 CORECEPTORS

Before discussing the transcriptional regulation of the *Cd4* and *Cd8* coreceptor genes, I would like to emphasize the relevance of the distinct kinetic expression pattern of these coreceptors after positive selection. Given the precise match of the functionally distinct fates of T cells with the MHC specificity of their αβTCRs, it has been assumed that TCR signals induced by the engagement of self-peptide presented on the two types of MHC must differ in some way; however, intracellular components that associate with, or are proximal to, αβTCR/CD3 surface complexes are common to both CD4 and CD8 receptors, other than a stronger association of the nonreceptor tyrosine kinase Lck with the intracellular domain of the CD4 coreceptor relative to that of CD8 (31, 32). TCR engagement by self-peptides on MHC-II, then, would be expected to induce stronger activation of Lck kinase and thereby produce stronger signals in downstream intracellular signaling cascades, which would consequently instruct the cells to adopt the helper fate (the strength-of-signal model). Naturally, the influence of the strength of TCR signals on $CD4^+$ helper/$CD8^+$ cytotoxic fate has been investigated, for example, through modulation of Lck activity (33) and exchange of the intracellular domain between the CD4 and CD8 proteins (34). These approaches indicated a partial skewing of MHC-I-restricted cells into $CD4^+CD8^-$ helper T cells by enhanced Lck activity, indicating that signal strength alone cannot explain the differences in TCR signals that induce differential cell fates.

It has been pointed out that $CD4^+CD8^{lo}$ cells, which emerge after positive selection by either MHC-I or MHC-II, are bipotential for helper and cytotoxic lineages (35). Further analyses revealed that CD8 downregulation is mediated at the transcriptional level and that the *Cd8* gene is

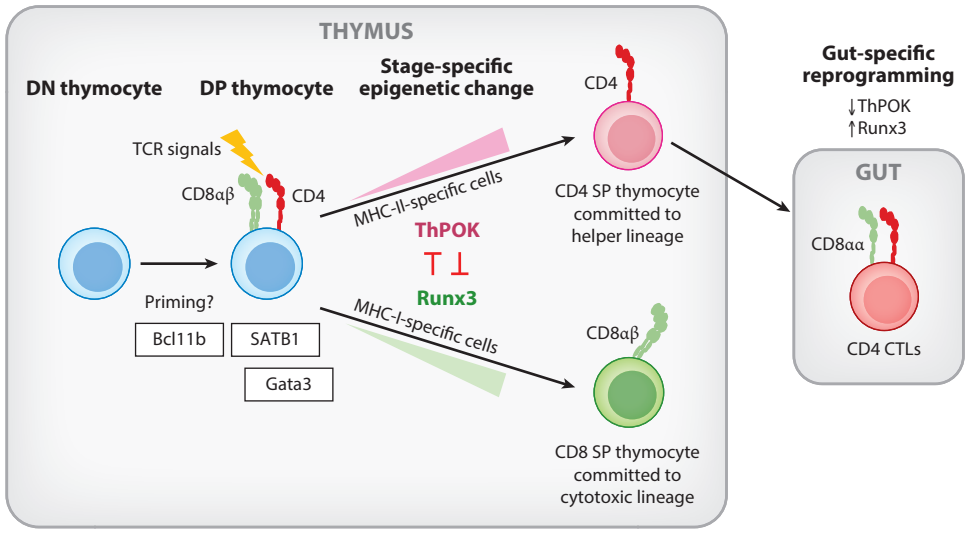

Figure 1

Schematic model of CD4 helper and CD8 cytotoxic T cell differentiation. Bipotential thymocyte precursors (DP thymocytes) expressing MHC-I- or MHC-II-specific TCRs differentiate into either CD8 SP or CD4 SP thymocytes, which are committed to cytotoxic and helper lineages, respectively. Expression of the transcription factors Runx3 and ThPOK in postselection thymocytes is essential for differentiation into CD8 SP or CD4 SP thymocytes, respectively. Antagonistic interplay between these two transcription factors is a key mechanism to separate cells of the two lineages. $CD4^+$ T cells retain developmental potential to become CD4 CTLs upon exposure to the gut-specific environmental cues that force the downregulation of ThPOK and induction of Runx3, which are required for the expression of CD8αα and for acquisition of cytotoxic activity. Upon expression of the T lineage commitment factor Bcl11b in the DN2 thymocyte subsets, the *Thpok* locus becomes primed for the T lineage type and is repressed by Bcl11b through both *Sth*-dependent and -independent mechanisms. Bcl11b is also involved in the regulation of enhancer activity during differentiation into helper lineage cells. SATB1 is also involved in regulation of the silencer and enhancer activity for the *Thpok* gene. Gata3 is essential for induction of the *Thpok* gene and plays a role in repression of the *Runx3* gene. Abbreviations: CTL, cytotoxic T lymphocyte; DN, double negative; DP, double positive, SP, single positive; *Sth*, *Thpok* silencer.

specifically reactivated in MHC-I-selected cells, whereas *Cd8* is maintained in a repressive state during the differentiation of MHC-II-selected cells (18). This difference between the kinetics of CD4 and CD8 coreceptor expression results in variation in the duration of positive selection signals, i.e., persistence of TCR signals in MHC-II-selected cells due to constant CD4 expression, and disruption of TCR signals in MHC-I-selected cells due to temporal downregulation of CD8. Based on this "coreceptor reversal" (18), the kinetic signaling model was proposed (19), which would explain how helper/cytotoxic lineage choice is regulated by the difference in the duration of positive-selection signals between MHC-I- and MHC-II-selected cells. The kinetic signaling model was tested using several genetic approaches, involving genetic modulation of the expression pattern of CD4 (36, 37). Furthermore, CD4 upregulation following positive selection of MHC-II-selected cells is essential for appropriate (error free) differentiation into the $CD4^+$ helper lineage (38). These findings demonstrate that the unique expression kinetics of the CD4 and CD8 coreceptors are essential to the mechanisms that segregate helper/cytotoxic lineages. Hence, it is crucial to understand how *Cd4* and *Cd8* gene expression is regulated.

3. REGULATORY ELEMENTS FOR EXPRESSION OF CD4/CD8 CORECEPTORS

3.1. *Cd4* Gene Regulation

To understand the regulation of gene expression, it is essential to identify *cis*-regulatory regions, which may act on gene promoters from a distance, in some cases several hundred kilobases. The classical hallmark of such *cis*-regulatory regions was their hypersensitivity to DNase. Modern laboratories utilize this hallmark with an enzyme other than DNase and in combination with next-generation sequencing technology [i.e., ATAC-seq (39)], which enables the identification of genomic regions with open, or permissive, structures at the whole-genome level in small numbers of cells.

At the murine *Cd4* locus, on chromosome 6, several DNase hypersensitive sites were identified and functionally characterized using reporter transfection into cell lines and in vivo transgenic reporter expression assays (5). At the time of writing, four genomic regions that are functionally involved in *Cd4* gene regulation had been isolated. Initially, the approaches mentioned above identified a transcriptional enhancer located 13 kb upstream of the transcriptional start site (TSS) for the *Cd4* transcript, referred to as the *Cd4* proximal enhancer (*E4p*) (40). An *E4p* sequence of approximately 300 bp demonstrated capacity, in conjunction with the minimum *Cd4* promoter (*P4*), to drive reporter transgene expression in all αβ T cell subsets from the DN2/3 stage onward (41). Given the helper lineage–specific expression of the *Cd4* gene, this finding suggested the presence of another *cis*-regulatory element that could negatively regulate gene expression, often referred to as a transcriptional silencer. Sequential studies independently isolated such an element: the *Cd4* silencer (*S4*, in the first intron of the gene, 1.6 kb downstream of the TSS) (41, 42). Insertion of the 429-bp core sequence of *S4* into a transgenic reporter construct driven by *E4p* and *P4* was sufficient to restore helper-specific transgene expression and prevent premature transgene expression in the DN thymocyte subset. This result clearly indicated that the 429-bp *S4* element can repress transgene expression in both DN thymocytes and $CD8^+$ cytotoxic T cells. The essential requirement for *S4* in control of stage- and lineage-specific expression of the *Cd4* gene was confirmed by derepression of CD4 in DN2/3 thymocytes and $CD8^+$ T cells upon removal of the *S4* element from the murine *Cd4* gene (43, 44). This result indicates that a single regulatory element is responsible for repression of the *Cd4* gene in two cell subsets at distinct stages; however, the mode of *S4* action in *Cd4* repression differs in these two cell types. Conditional removal of *S4* from differentiated $CD8^+$ T cells revealed that *S4* is not necessary for maintenance of *Cd4* repression (43), whereas it is required to establish a repressed state at the *Cd4* locus during the transition into CD8 SP thymocytes. Thus, *Cd4* repression through *S4* in DN3 cells is reversible, whereas in $CD8^+$ T cells, it is stably inherited in the absence of *S4*. Mutation of *S4* exhibiting attenuated silencer activity generated distinct patterns of CD4 derepression, with uniform and variegated patterns in DN and $CD8^+$ T cells, respectively (45). These observations demonstrate the involvement of epigenetic regulation in *Cd4* repression in cytotoxic lineage T cells, leading to investigation of epigenetic modifications, including histone modifications and DNA methylation status, in the *Cd4* gene (46, 47). The results of such epigenetic studies suggested that modulation of DNA methylation status is important for both stable repression and stable expression of the *Cd4* gene (47). Unbiased screening of molecules involved in maintenance of *Cd4* repression using a short hairpin RNA (shRNA) library identified Dnmt1, a maintenance DNA methyltransferase (DNMT), as important in maintenance of *Cd4* silencing (47). This finding was consistent with the result of an experiment using the chemical compound 5-azacytidine (5-Aza) an inhibitor of Dnmt1. Although initial attempts did not indicate that CD4 could not be derepressed in $CD8^+$ T

cells by 5-Aza treatment (43), in a subsequent trial using an optimized dosage of 5-Aza, induction of CD4 expression was observed in $CD8^+$ T cells (48). These recent results have begun to provide molecular insights into how the outcome of different modes of *S4* activity could vary at the two developmental stages. However, how the stage- and lineage-specific activities of *S4* are regulated is yet to be fully elucidated, as discussed below in the context of the function of transcription factors involved in the helper/cytotoxic T cell lineage choice.

The physiological role of *E4p* was also examined using genetic experiments in mice. In mice with *E4p* deleted from both *Cd4* alleles ($Cd4^{\Delta E4p/\Delta E4p}$ mice), CD4 induction at the transition from the DN to the DP stage was severely reduced (46), whereas CD4 expression was induced after positive selection in the absence of *E4p*. These results not only confirm that *E4p* is responsible for initiation of CD4 expression but also suggest the presence of another enhancer(s), referred to as the *Cd4* maturation enhancer (*E4m*) (46), which would be predicted to become active in postselection thymocytes to restore CD4 expression from the $Cd4^{\Delta E4p}$ allele. Consistent with this finding, the *E4p/P4* combination alone failed to maintain transgene expression in activated $CD4^+$ T cells (49). Another study provided useful information regarding the position of the putative *E4m* enhancer by comparison of CD4 expression from two *Cd4* mutant alleles with deletion of genomic regions of different lengths 3′ of *S4*. The lack of a 1.0-kb region 3′ of *S4* led to instability of CD4 expression (48). Using the recent ATAC-seq database from the Immunological Genome Project (50), we identified another chromatin-accessible region in $CD4^+$ T cells, downstream from and proximal to *S4* (I. Taniuchi, unpublished data) to which the Runx/Cbfβ transcription factor complex and SATB1 bind (51). These observations predict that the *E4m* element is located approximately 1.0 kb downstream of *S4*. Removal of this putative *E4m* region together with *E4p*, in our laboratory (51) and by others (29), results in a complete loss of CD4 expression during T cell development. Thus, *E4p* and *E4m* are the main enhancers that dictate CD4 expression in αβ T cells. The roles of *E4m* in CD4 upregulation and in preventing errors in the choice of helper lineage by MHC-II-selected cells await further characterization in $Cd4^{\Delta E4m/\Delta E4m}$ mice.

3.2. *Cd8* Gene Regulation

The *Cd8* locus consists of the *Cd8a* and *Cd8b* genes and spans approximately 80 kb on mouse chromosome 6. Searches for DNase hypersensitive sites and conserved noncoding sequences (CNS) isolated at least six enhancers at the murine *Cd8* locus (*E8I-VI*) (5, 52, 53). The enhancer activity of each region was tested, primarily by reporter transgene expression assays and individual knockout of each region from the murine *Cd8* locus. Because other review articles have described the results of these approaches (5, 53), I do not discuss them in detail in this review. One important feature is that these enhancers are functionally redundant with one another (54, 55). Among the six enhancers, one, designated *E8I* and originally described as a 7.6-kb genomic region, has received attention because of its activity in driving transgene reporter expression in a CD8 lineage–specific manner when conjugated to the *Cd8a* promoter (56). The core sequence driving *E8I* enhancer activity is located within a 0.6-kb region at the 3′ end of the original 7.6-kb fragment, as removal of this 0.6-kb region resulted in reduced CD8 expression in $CD8^+$ T cells (51) and further reduced expression in CD8αα intraepithelial lymphocytes (IELs) (I. Taniuchi, unpublished data) to an extent similar to that observed in 7.6-kb *E8I*–deficient mice (54).

Similar to the *Cd4* gene, *Cd8* is subject to epigenetic regulation. In *E8I/II* double-deficient DP thymocytes, levels of histone H3 acetylation (H3Ac) and trimethylation on lysine 4 (H3K4me3) at the *Cd8* locus were reduced (57). In activated $CD8^+$ T cells lacking *E8I*, unstable CD8 expression was accompanied by a decrease in H3Ac and an increase in H3K27Me3 (52). Changes in DNA methylation status at the *Cd8* locus during thymocyte differentiation were also examined (58–61).

For example, DNA demethylation of a region within *E8V* occurs at the DN-to-DP transition, and the region undergoes further demethylation at the DP-to–CD8 SP transition (59). Intronic regions in the *Cd8a* gene also undergo specific DNA demethylation in $CD8^+$ T cells (51). At the *Cd8a* locus exhibiting variegated CD8 expression in DP thymocytes due to loss of both *E8I* and *E8II* enhancers, a high level of DNA methylation was retained in the *E8V* region, and removal of Dnmt1 restored variegated expression to some extent (57). Thus, DNA demethylation, which is regulated in part by enhancers activity, is correlated with CD8 expression during T lymphocyte development.

4. TRANSCRIPTIONAL FACTORS INVOLVED IN CD4/CD8 LINEAGE CHOICE

4.1. Runx Proteins

Isolation of *S4* as a key element regulating the helper lineage–specific expression of the *Cd4* gene accelerated the hunt for proteins that bind to its sequences. Several transcription factors and epigenetic regulators, including BAF (62) and Mi2-β (63), were identified as functional regulators of *S4* activity. This review focuses on the function of two of these transcription factors: Whereas Runx transcription factors (64) activate *S4*, T helper–inducing POZ/Krüppel-like factor (ThPOK) counteracts S4 activity (65, 66). Mapping of functional sites within the 429-bp *S4* sequences by in vitro transfection and in vivo mutagenesis approaches identified at least three functional sites (sites 1, 2, and 3) within the 134-bp core region (45). These sites exhibit redundancy with one another in activation of *S4*; combined mutations at each site further attenuated *S4* activity (45). Fine mapping of these functional sites revealed that site 2 sequences are identical to the Runx recognition motif (5′-PuACCACG/A-3′) (64). There is another Runx motif outside of the *S4* core, and targeted mutations that disrupt these two Runx motifs resulted in full CD4 derepression in $CD8^+$ T cells (64). In addition, Runx1 protein was isolated by yeast one-hybrid screening using the *S4* core sequence as bait (64), and its physiological binding to *S4* was later confirmed by chromatin immunoprecipitation (ChIP)-PCR (67) and ChIP-seq (51).

Runx transcription factor complexes are evolutionarily conserved heterodimeric complexes of a Runx protein and its mandatory partner, Cbfβ (68). Runx complexes can both activate and repress target gene expression in a context-dependent manner. In mammals, there are three Runx proteins, encoded by *Runx1*, *Runx2*, and *Runx3*, which map to different chromosomes (69). In addition to a Runt domain, which is essential for recognition of the Runx motif as well as for dimerization with Cbfβ protein (70, 71), a VWRPY pentapeptide at the C terminus of Runx proteins is well conserved among species (72), and it serves as a docking module to interact with the Grouch/TLE corepressor protein family (73). Removal of the VWRPY motif from both the Runx1 protein and the Runx3 protein (to generate $Runx1^{\Delta V/\Delta V}$:$Runx3^{\Delta V/\Delta V}$ mice) led to full CD4 repression in $CD8^+$ T cells (74), whereas $Runx3^{\Delta V/\Delta V}$ mice exhibited only partial CD4 derepression (75). Thus, there is functional redundancy between Runx1 and Runx3 in *Cd4* silencing. In contrast to the dominant function of Runx3 in $CD8^+$ T cells, Runx1 plays a major role in *Cd4* repression in DN2/3 thymocyte subsets (64). The differential requirements for Runx1 and Runx3 proteins stem from the differential expression pattern of the *Runx1* and *Runx3* genes during thymocyte differentiation. The two promoters distal-P1 and proximal-P2 are present in all vertebrate *Runx* genes (69). The *P1-Runx3* transcript is specific to CD8 SP thymocytes, whereas the *P2-Runx3* transcript is detected to some extent in other thymocyte subsets (76); however, in T cells, a noncanonical Kozak sequence in the *P2-Runx3* transcript results in inefficient translation of P2-Runx3 protein (77). Therefore, expression of Runx3 protein, or more specifically P1-Runx3

protein, is limited to CD8 SP thymocytes among thymocyte subsets (76). It is noteworthy that the presence of an antagonistic repression loop between Runx1 and Runx3 in hematopoietic cells (78) may cause aberrant induction of either protein when expression of the other is abrogated.

Runx1 plays an important role in efficient positive selection (76) and invariant natural killer T (iNKT) cell development (79). Runx1/Cbfβ complexes continue to occupy *S4* in DP thymocytes (67); however, *S4* does not repress *Cd4* gene expression in those cells. Thus, the mechanism that reverses the *S4* activity during the DN-to-DP transition functions beyond the level of Runx binding. It was reported that a chromatin loop is formed between *E4p* and *S4* in DN3 cells and released in DP thymocytes (80). Thus, modulation of the higher-ordered chromatin structure is one possible mechanism that allows DP thymocytes to escape *S4*-mediated *Cd4* repression. Interestingly, Mi-2β, one of the units forming NuRD ATPase chromatin remodeling complexes, was proposed to be recruited to *S4* via Ikaros and to be involved in *S4* inactivation (63). Fine-resolution analyses of interactions among genomic regions at different developmental stages and identification of molecules regulating chromatin architectures are necessary to further elucidate how the inactivation of *S4* at two stages is regulated. In addition to their function in *Cd4* repression, Runx proteins are also involved in activation of *Cd8* genes. Inactivation of *Runx1* in DN2/3 thymocytes using a Lck-Cre driver caused a delay in *Cd8* gene activation during the transition to the DP stage, as well as CD4 derepression, generating an immature CD4 SP thymocyte subset (64). Runx3 is essential for maintenance of CD8 expression in activated $CD8^+$ T cells (81) and induction of CD8αα expression during differentiation into $CD8\alpha\alpha^+$ IELs (82). Thus, upon induction of its expression through activation of the *P1-Runx3* promoter, Runx3 plays dual roles in establishment of *Cd4* silencing and reactivation of the *Cd8* gene (83) during differentiation of MHC-I-selected cells. How *P1-Runx3* expression is regulated is discussed below.

4.2. ThPOK

ThPOK (official name Zbtb7b) belongs to the BTB-POZ zinc finger transcription factor family. The name ThPOK originated from a report demonstrating its essential function in CD4 helper T cell development (84). In this review, I refer to this protein and its coding gene, *Zbtb7b* on mouse chromosome 3, as ThPOK and *Thpok*, respectively. The function of ThPOK was unraveled using two separate approaches. The first approach involved a search for the gene responsible for a helper-deficient phenotype found in a natural mutant mouse strain (85) and identified a point mutation that renders ThPOK nonfunctional by generating an amino acid substitution (R389G) in its second zinc finger domain (84). The second study isolated ThPOK as one of a number of molecules whose expression was specifically induced by positive-selection signals on MHC-II engagement (86). In both studies, ectopic expression of ThPOK from a transgene in preselection thymocytes resulted in CD4-skewed differentiation through redirected differentiation of MHC-I-selected thymocytes to the $CD4^+$ helper lineage (84, 86). Based on the redirection of MHC-II-selected cells into $CD8^+$ cytotoxic lineage cells by *hd* (R389G) mutation (87) or artificial elimination of functional *Thpok* (65), the presence or absence of ThPOK expression in postselection thymocytes is the most important determinant factor segregating the $CD4^+$ and $CD8^+$ phenotypes (88).

This critical role of ThPOK stems from its direct involvement in regulation of the *Cd4* and *Cd8* genes. ThPOK is recruited to the *S4* element and counteracts against *S4* function (65, 66). In a transfection assay, ThPOK could reverse *S4*-mediated repression, and ectopic expression of ThPOK in DN3 thymocytes led to significant *Cd4* derepression (66). In addition, CD4 repression by transgenic Runx3 expression in preselection thymocytes was reversed by transgenic ThPOK expression (66). The *Cd4* locus has been reported to be hypomethylated in ThPOK-deficient MHC-II-selected cells (47). This could reflect a failure of ThPOK-mediated *S4* inactivation. The

molecular mechanism underlying ThPOK antagonism of *S4* activity remains uncharacterized. Given the decrease in Runx binding to *S4* during differentiation into the CD4 lineage (67, 83), it is conceivable that ThPOK is involved in the release of Runx binding from *S4*.

4.3. Other Transcription Factors

In this section, I discuss other transcription factors involved in *Cd4/Cd8* gene regulation. The SWI/SNF-like nucleosome-remodeling BAF complex (62) and the transcription factor Ikaros (89) are also required to initiate *Cd8* activation at the transition to the DP stage, as well as for *Cd4* regulation. Given that Ikaros is a global regulator of chromatin architecture (63), in part through its association with NuRD complexes (90), modulating of chromatin structure may be key to activation of the *Cd8* locus. Recently, SATB1 (special AT-rich binding protein 1), a known genome organizer, was identified as involved in *Cd8* reactivation (51). SATB1 was first shown to bind to the putative negative regulatory elements within the *E8V* region of the *Cd8* gene, referred to as L2a (91). Based on the observation that knockdown of SATB1 enhanced variegated expression of a transgene driven by *E8V* (92), it was proposed that SATB1 can counteract L2a-mediated repression. ChIP-seq showed that SATB1 binds to multiple regions in the *Cd8* gene, including *E8I* and *E8II* (51). Loss of SATB1 function resulted in low and unstable CD8 expression in CD8 lineage cells, in part because of inefficient DNA demethylation at intronic regions during differentiation of thymocytes expressing OT-I MHC-I-specific transgenic TCR (51). When SATB1 deficiency was combined with transgenic ThPOK expression or *E8I* deficiency, CD8 expression in DP or mature thymocytes, respectively, was reduced (51). Thus, SATB1 is likely to regulate multiple enhancers in activation of the *Cd8* gene, via modulation of chromatin architecture. It is noteworthy that SATB1 is also involved in activation of the *E4m* element regulating CD4 expression in helper T cells (51) and CNS3 (51, 93) and CNS0 (93) for mediating *FoxP3* activation during differentiation of regulatory T cells in the thymus. The HMG transcription factor Tox is essential for development of $CD4^+$ T cells (94) and innate lymphoid cells (95).

5. TRANSCRIPTIONAL REGULATION OF *Thpok* AND *Runx3*

5.1. *Thpok* Gene Regulation

Helper lineage–specific expression of ThPOK and its dominating role in specifying the CD4 helper–lineage phenotype in postselection thymocytes indicates that unraveling the mechanism that links MHC-II-mediated positive-selection signals with *Thpok* induction should be a high priority for research focusing on the CD4/CD8 lineage choice. The *Thpok* gene is transcribed from two promoters, the distal (P1) and proximal (P2) promoters, in T lineage cells (84), whereas cells that are not of T lineage use only the P1 promoter. There are three *cis*-regulatory regions within the *Thpok* gene: a distal regulatory element (*DRE*) located 3.2 kb upstream from the P1-*Thpok* promoter, a proximal regulatory element (*PRE*) 1.8 kb downstream of the P2-*Thpok* promoter (96), and a general T lymphoid element (*GTE*) that resides between *DRE* and *PRE* (96). *DRE* consists of at least one enhancer and one silencer (96), which are designated the thymic enhancer (*TE*) (97) and the *Thpok* silencer (*Sth*) (96, 98, 99), respectively. Only enhancer activity, referred to as the proximal enhancer (*PE*) (65), has so far been detected within the *PRE*. Removal of *TE* and/or *PE* from a $Thpok^{gfp}$ reporter allele revealed their stage-specific functions (**Figure 2*a***). *TE* first acts after positive-selection signals are received (97), whereas *PE* is responsible for sequential increases in *Thpok* mRNA levels during differentiation of MHC-II-selected cells (65). The latter process is essential to generate a sufficient amount of ThPOK in MHC-II-selected cells for their full

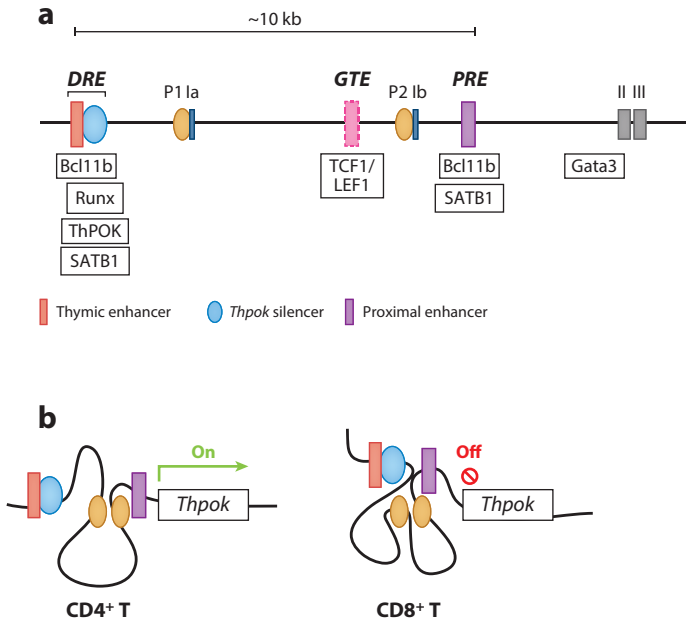

Figure 2

Structure of murine *Thpok* gene and its *trans*-acting proteins. (*a*) The *Thpok* gene is transcribed from two promoters: P1 (distal) and P2 (proximal). The latter is activated specifically in T lineage cells. There are three *cis*-regulatory regions within the *Thpok* gene: a distal regulatory element (*DRE*), a proximal regulatory element (*PRE*), and a general T lymphoid element (*GTE*). *DRE* consists of the thymic enhancer (*TE*) and the *Thpok* silencer (*Sth*), and *PRE* contains the proximal enhancer (*PE*). *TE* first acts after positive selection to initiate *Thpok* activation, whereas *PE* acts later for sequential increases in *Thpok* expression. Gata3 and TCF1/LEF1 are necessary for activation of *Thpok* through binding to regions upstream of exon II and *GTE*, respectively. Binding of Runx to *Sth* is essential for its silencer activity, whereas ThPOK counteracts *Sth* activity. Both Bcl11b and SATB1 are involved in regulation of both enhancers (*TE* and *PE*) and the silencer (*Sth*). (*b*) Topological regulation is possibly involved in *Thpok* gene regulation. Given the involvement of Bcl11b and SATB1 in the regulation of multiple *cis*-regulatory regions of the *Thpok* gene, assembly of regulatory regions onto the promoters is likely to depend on these two factors. It is conceivable that the *Sth* region is assembled in close proximity to the promoters specifically in $CD8^+$ T cells.

commitment to the helper lineage (65), mainly through activation of the P2 promoter. Inefficient induction of ThPOK through loss of *TE* also results in a redirected differentiation toward $CD8^+$ T cells in approximately 10% of MHC-II-selected cells (97). Expression of Thpok-GFP during T cell development was abrogated by removal of both *TE* and *PE* from the $Thpok^{gfp}$ reporter allele, whereas Thpok-GFP expression in B cells was unaffected (I. Taniuchi, unpublished data). This observation indicates that the other enhancer element, such as *GTE*, cannot compensate for lack of *TE* and *PE* activity, although *GTE* was capable of directing transgenic reporter expression in both $CD4^+$ and $CD8^+$ T cells (96). Because the effects of removal of *GTE* from the *Thpok* locus have not been tested, its physiological roles in *Thpok* regulation are less clear than those of the other two enhancers.

The *Sth* element was identified by searching for the region responsible for the restriction of reporter transgene expression driven by *Thpok* enhancers/promoters in helper lineage cells (96) and was found to be one of Runx-binding regions in the *Thpok* gene (98). Removal of core *Sth* sequences from the $Thpok^{gfp}$ reporter allele caused derepression of ThPOK-GFP expression in

both preselection DP thymocytes and CD8$^+$ T cells (98). As expected, aberrant expression of ThPOK due to loss of *Sth* from the *Thpok* locus led to abrogation of CD8$^+$ T cell generation by enforced redirection of MHC-I-restricted cells into the CD4$^+$ T cell lineage (98). Loss of function of Runx complexes also caused *Thpok* derepression in CD8$^+$ T cells (74, 98), and targeted mutation of two Runx-binding motifs within *Sth* abrogated its silencer activity (100), indicating that binding of Runx complexes to *Sth* is essential for *Sth* to exert silencer function, similar to *S4* activation (64). However, the mode of Runx-mediated repression differs between the *Sth* and *S4* silencers, in terms of dependency on the VWRPY motif. In *Runx1*$^{\Delta V/\Delta V}$:*Runx3*$^{\Delta V/\Delta V}$ mice lacking the VWRPY motif of both the Runx1 and Runx3 proteins, the *S4* silencer became nonfunctional; however, *Sth* could still repress *Thpok* to some extent in CD8$^+$ T cells (74). In addition, in preselection thymocytes, although mutation of two Runx sites caused *Thpok* derepression, VWRPY deficiency had almost no impact (100). Presumably, other corepressor molecules, such as HDAC and Sin3A, which associate with Runx proteins (101), compensate for the lack of VWRPY-mediated Groucho/TLE recruitment to *Sth*. Thus, in addition to differences in stage specificity between *S4* and *Sth* (only *Sth* is active in DP thymocytes), other motifs in Runx proteins, or other proteins binding to *Sth*, could compensate for the VWRPY-mediated *Thpok* expression.

Overall, helper lineage–specific expression of *Thpok* is achieved by *Thpok* repression in MHC-I-restricted cells through the Runx-dependent silencer, *Sth*, and vice versa; ThPOK plays an essential role in repression of *Runx3* expression during differentiation of MHC-II-restricted cells (65, 102, 103). Thus, antagonistic interplay between two major lineage-specifying factors serves as a central mechanism to segregate the CD4 helper and CD8 cytotoxic phenotypes (104). Therefore, it is crucial to understand how the repression of *Thpok* is released upon receiving MHC-II-mediated TCR signaling. Although there is less Runx binding to *Sth* in CD4$^+$ T cells than in CD8$^+$ T cells (105), Runx complexes still occupy the *Sth* in CD4$^+$ T cells (98), in which *Sth* activity is canceled. Thus, whether Runx complexes bind to *Sth* or not does not constitute an on or off switch to *Sth* activity. This observation suggests that identification of other *Sth*-binding proteins is important. ThPOK itself also associates with the *Sth* element and interferes with *Sth*-mediated gene repression in transfection assays (65). This function of ThPOK could contribute to the increase in ThPOK levels during MHC-II-restricted cell differentiation, through formation of an autoamplification loop (104), although such a loop cannot be involved in the initial inactivation of *Sth* for *Thpok* induction.

MAZR, another member of the BTB-POZ zinc finger protein family, is necessary for *Sth* activity. MAZR was first identified as a negative regulator of the *Cd8* enhancer *E8II* (57). Similar to other BTB-POZ members, MAZR associates with N-CoR corepressor complexes and counteracts *Cd8* activation at the DN and DP stages (57). Conditional inactivation of *Mazr* by a *Cd4-cre* driver caused *Thpok* derepression and partial redirection of MHC-I-restricted cells into CD4$^+$ lineage cells (106). These phenotypes were further enhanced by a combination of MAZR deficiency with mutations of Runx family genes (107).

Bcl11b is another zinc finger transcription factor essential for *Thpok* repression. Complete arrest of early thymocyte development at the DN2a-to-DN2b transition due to loss of Bcl11b characterized Bcl11b as a T lineage–commitment factor (6, 7); however, subsequent studies revealed that Bcl11b plays multiple roles during T cell development after the DN2 stage (108–110) and is also essential for the differentiation (111, 112) and function (113) of group 2 innate lymphoid cells (ILC2s). Inactivation of *Bcl11b* by *Cd4-cre* caused derepression of *Thpok* in preselection thymocytes (114) and impaired generation of mature thymocytes (115). Regarding *Thpok* regulation by Bcl11b, Bcl11b binds to *Sth* independently of Runx binding, while ThPOK binding to *Sth* requires Runx binding (105). Interestingly, aberrant *Thpok* expression in preselection thymocytes (114) is driven only by the P1 promoter (105), as observed in non–T lymphoid lineage cells. Hence, the

use of T cell–specific regulatory regions for *Thpok* expression in Bcl11b-deficient thymocytes was tested by using a unique reporter allele, $Thpok^{gfp:\Delta TESPE}$, generated by removal of the *TE*, *Sth*, and *PE* sequences from the $Thpok^{gfp}$ allele. Although expression of ThPOK-GFP from $Thpok^{gfp:\Delta TESPE}$ was undetectable in Bcl11b-sufficient preselection thymocytes, it was observed in Bcl11b mutant cells (105). This indicates the presence of an *Sth*-independent mechanism for *Thpok* repression by Bcl11b. The repressive function of Bcl11b requires its last zinc finger motif at the C terminus of the protein (105). *Thpok* expression is marginally induced in $CD8^+$ T cells after TCR stimulation, albeit only from the *P1-Thpok* promoter (100). This release of *Thpok* repression was inhibited by artificial enhancement of *Sth* activity through increasing its copy number from one to three (100). Removal of Bcl11b from activated $CD8^+$ T cells enhanced *Thpok* derepression, and a combination of Bcl11b haploinsufficiency and *Runx* mutations resulted in an increased proportion of $CD8^+$ T cells in which *Thpok* was derepressed (105). These observations indicate that Bcl11b is also essential for *Sth* function; therefore, Bcl11b can repress *Thpok* both in an *Sth*-dependent manner and in an *Sth*-independent manner. Importantly, the expression level of ThPOK-GFP was significantly decreased in Bcl11b-deficient $CD4^+CD8^-$ T cells (105), indicating that Bcl11b is also necessary for activation of enhancer(s) of *Thpok* gene expression.

SATB1 was also isolated as an *Sth*-binding protein by biochemical purification using core *Sth* sequences (51). Levels of *Thpok* in MHC-II-restricted cells, such as cells expressing MHC-II-specific transgenic OT-II TCR, are severely reduced in response to loss of SATB1, resulting in a partial redirection to $CD8^+$ T cells (51). Testing of the effect of SATB1 deficiency on several mutant $Thpok^{gfp}$ alleles revealed that SATB1 can regulate not only *TE* and *PE* activity, but also *Sth* activity (51). Thus, similar to Bcl11b, SATB1 is involved in regulation of multiple regulatory regions at the *Thpok* locus. SATB1 is essential for the formation of chromatin loops at *Th2* cytokine loci (116). Moreover, Bcl11a, a counterpart molecule for Bcl11b in non–T lymphoid hematopoietic cells, is involved in switching of developmental enhancers in the β-globin gene through modulation of chromatin looping (117). Thus, it is conceivable that both Bcl11b and SATB1 play important roles in regulation of the assembly of multiple regulatory regions at the *Thpok* locus (**Figure 2*b***). This could explain why Bcl11b and SATB1 influence both positive and negative regulation of the *Thpok* gene.

Gata3 is another transcription factor that has been shown to be essential for *Thpok* induction (118). Gata3 is required for the development of early thymocytes (119) and $CD4^+$ T cells (120, 121). Induction of *Thpok* was significantly impaired during differentiation of Gata3-deficient thymocytes (118), and Gata3 binding to a region upstream and proximal to exon II, which was essential for driving expression from a BAC transgene construct, was observed (118). However, as the physiological roles of this Gata3-bound region are not well characterized, it remains unclear how Gata3 activates the *Thpok* gene. Interestingly, transgenic ThPOK expression failed to rescue $CD4^+$ T cell development in a Gata3-deficient background, whereas inhibition of $CD8^+$ T cell differentiation by transgenic ThPOK was Gata3 independent (118). Thus, Gata3 has a ThPOK-independent function that is essential for $CD4^+$ T cell development, and it is important to unravel the details of this role. Recently, it was reported that double deficiency of the additional HMG transcription factors TCF1 and LEF1 results in a reduction of the $CD4^+$ helper lineage (122), caused by partial redirection of MHC-II-selected cells into the $CD8^+$ T lineage, probably due to inefficient induction of *Thpok* by impaired *GTE* activation (122).

The ten-eleven translocation (TET) protein family is essential for active DNA demethylation through enzymatic activity converting 5-methyl cytosine (5mC) to 5-hydroxymethyl cytosine (5hmC); the latter is subsequently converted to 5-formylcytosine and 5-carboxylcytosine (123–125). There are three TET family members in mammals (TET1–3), and inactivation of *Tet2* and *Tet3* in mice results in low *Thpok* expression levels during thymocyte differentiation (126). This

suggests that DNA demethylation by recruitment of TET2/3 through enhancers, such as *TE* and *PE*, is likely involved in *Thpok* activation.

5.2. *Runx3* Gene Regulation

Along with understanding *Thpok* regulation, an understanding of the mechanisms regulating cytotoxic lineage–specific expression of *P1-Runx3* is clearly crucial. However, in contrast to what we know about the *Thpok*, *Cd4*, and *Cd8* genes, scarce information is available regarding regulatory regions in *Runx3*. Recent studies have begun to identify regulatory regions in the *Runx3*. A combinational activation of three regulatory regions, present as a cluster between −80 and −60 kb upstream of the *P1-Runx3* promoter, is crucial for *P2-Runx3* expression in TrkC sensory neurons (127). Regarding *Runx3* expression in T cells, the ChIP-seq approach detected that at least two regions, −39 and −21 kb upstream of the *P1-Runx3* promoter, are occupied by several transcription factors, including Runx/Cbfβ and SATB1 (51), Bcl11b, and ThPOK (105). Individual removal of the −39 kb or −21 kb region (105) from the $Runx3^{tdTomato}$ reporter allele resulted in a reduction of the Runx3-tdTomato level in $CD8^+$ T cells, and combined removal of both regions further reduced Runx3-tdTomato levels (105). The presence of residual Runx3-tdTomato expression after removal of both the −39 kb and −21 kb regions suggests the presence of another enhancer(s) that can compensate for the −39/−21 enhancers. Indeed, one conserved region at −18 kb upstream of *P1-Runx3* is also involved in *Runx3* expression in $CD8^+$ T cells, as removal of the −18 kb region results in halving of *Runx3* expression (I. Taniuchi, unpublished data). These observations indicate that at least three functional enhancers cooperatively regulate expression of the *P1-Runx3* transcript in $CD8^+$ T cells (**Figure 3*a***).

How is the activity of these enhancers regulated? IL-7 signaling is essential for differentiation of CD8 SP thymocytes and induction of *P1-Runx3* expression (128). A severe reduction in the number of CD8 SP thymocytes induced by conditional removal of the common cytokine γ chain (γ_c) confirmed the importance of γ_c cytokines for generation of CD8 SP thymocytes (129). The role of SOCS1, an inhibitor of multiple cytokine signals, has also been investigated. Although transgenic ThPOK expression prevented emergence of CD8 SP thymocytes, removal of SOCS1 partially restored differentiation of Runx3-expressing CD8 SP thymocytes in ThPOK-transgenic mice (130). Conversely, transgenic expression of SOCS1 restored CD4 SP thymocytes in ThPOK-deficient mice (130). Therefore, it appears that an important function of ThPOK in directing $CD4^+$ helper development involves induction of SOCS1. However, it remains unclear how ThPOK induces *Socs1*, and a molecular link between the γ_c cytokine stimulation and *Runx3* induction has not been established. Since signal transducer and activation of transcription 5 (Stat5) is a common transcription regulator downstream of γ_c cytokine stimulation (131), it will be interesting to examine whether Stat5 binds to the three functional enhancers described above in $CD8^+$ T cells upon IL-7 stimulation.

Expression levels of *P1-Runx3* were decreased on loss of SATB1 (51). Importantly, $CD8^+$ T cell–specific expression of *P1-Runx3* was abrogated by loss of Bcl11b (105). Using the dual reporters $Thpok^{gfp}$ and $Runx3^{tdTomato}$, most peripheral αβ T cells coexpressing the *Thpok* and *Runx3* genes were found to develop in $Bcl11b^{F/F}$:*Cd4-cre* mice. This result indicates that Bcl11b is essential for establishment of the lineage-specific expression patterns of both *Thpok* and *Runx3*. Conversely, the chaotic expression of *Thpok* and *Runx3* in response to loss of Bcl11b function induces a redirected differentiation in both MHC-I- and MHC-II-selected cells in $Bcl11b^{F/F}$:*Cd4-cre* mice (105). In Bcl11b-deficient $CD4^+$ T cells, ThPOK can still bind to *Runx3* enhancers, and *Socs1* and *Socs3* are expressed at levels comparable to those observed in control $CD4^+$ T cells (105). These observations suggest that Bcl11b may have a role in modulating

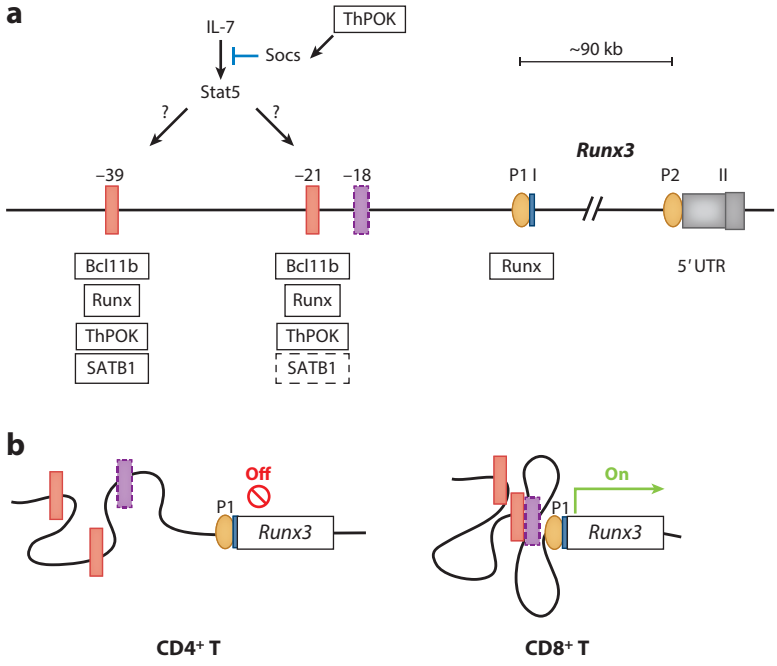

Figure 3

Structure of murine *Runx3* gene and its *trans*-acting proteins. (*a*) The *Runx3* gene is transcribed from two promoters, the distal (P1) and the proximal (P2) promoters. Expression of *P1-Runx3* transcripts is specific to the CD8 single-positive thymocyte subset during thymocyte differentiation. There are at least three *cis*-regulatory regions, at −39 kb, −21 kb, and −18 kb upstream of the *P1-Runx3* promoter. The −39 kb and −21 kb regions are occupied by several transcription factors, including Runx/Cbfβ, Bcl11b, and SATB1. Removal of the −39 kb or −21 kb regions results in a reduction of *P1-Runx3* expression in $CD8^+$ T cells, indicating that these regions are functional enhancers to drive *Runx3* expression in these cells. Expression of *Runx3* is induced by IL-7 stimulation, which is inhibited by ThPOK through induction of Socs, which is an inhibitor of multiple cytokine signals. ThPOK binding to the −39 kb and −21 kb regions in the *Runx3* gene also suggests an inhibitory mechanism for ThPOK on the enhancers' activity through its direct association with them. (*b*) Topological regulation is possibly involved in activation of the *Runx3* gene. Given the functional redundancy between the three enhancers, these three regions in the *Runx3* gene are likely to associate with the *P1-Runx3* promoter, specifically in $CD8^+$ T cells, through formation of chromatin loops.

chromatin loop formation in ThPOK-mediated *Runx3* repression after ThPOK binding to *Runx3* enhancers (**Figure 3*b***). Regarding *Runx3* repression, Gata3 can also repress *Runx3* in $CD4^+CD8^{lo}$ postselection thymocytes before ThPOK is induced (132).

6. MODULATION OF CD4/CD8 LINEAGE IDENTITY IN THE PERIPHERY

An important advance made in the last decade in the study of CD4/CD8 lineage choice was the finding that $CD4^+$ T cells retain plasticity and are capable of activating a cytotoxic program. It had previously been thought that commitment to the $CD4^+$ helper or $CD8^+$ cytotoxic lineage in the thymus remained stable in the cells once they migrated to the periphery. Ample evidence on epigenetic mechanisms also supported this conventional idea by providing molecular insight into how stable gene silencing is established and is inherited (29, 133). Expression of the *Cd4* and *Thpok*

genes (specifically the *P2-Thpok* transcript) is stably silenced in $CD8^+$ T cells, even in the absence of the silencer elements *S4* and *Sth*, which are essential for establishing the silenced state of the genes in the thymus (43, 100). In contrast, *Cd8* expression can be induced in $CD4^+$ T cells after in vitro TCR stimulation with TGF-β and is accompanied by a reduction in ThPOK expression levels (134). Consistent with this finding, in vivo conditional ablation of ThPOK function in mature $CD4^+$ T cells induces *Cd8* expression (103). Thus, the *Cd8* gene in $CD4^+$ T cells is not as firmly silenced as the *Cd4* gene in $CD8^+$ T cells and can be induced upon ThPOK downregulation. Of note, during in vitro differentiation into the Th1 subset, the *Runx3* gene is also temporally induced (135, 136). A logical question, then, is whether or not reduction of *Thpok* expression in $CD4^+$ T cells occurs in physiological settings.

Identification of the $CD4^+CD8\alpha\alpha^+$ IEL subset, and the process leading to its development, not only demonstrated that modulation of *Thpok* expression contributes to the generation of this unique subset but also revealed a previously unappreciated plasticity in $CD4^+$ T cells. A proportion of $CD4^+CD8^-$ T cells injected into lymphopenic host mice acquired CD8αα expression specifically in the gut (137). $CD4^+CD8\alpha\alpha^+$ IELs are present in the steady state and lose *Thpok* expression, instead expressing *Runx3*. In vivo lineage tracing confirmed that $CD4^+CD8\alpha\alpha^+$ IELs are progeny of cells that had experienced *Thpok* expression or, more accurately, had inactivated *Sth* function (137). Total gene expression signatures demonstrated that cytotoxicity-related molecules, such as GzmB and CRTAM, are also induced in $CD4^+CD8\alpha\alpha^+$ IELs (137, 138) and, importantly, that $CD4^+CD8\alpha\alpha^+$ IELs can acquire cytotoxic activity (137). Thus, similar to differentiation into several effector Th subsets by exposure to specific environmental cues (139), $CD4^+$ T cells retain the developmental potency to acquire cytotoxic functions and become $CD4^+$ CTLs upon antigen stimulation in the context of the gut-specific environment. A retrospective review of the literature indicates that some studies reported cytotoxic functions of $CD4^+$ T cells (140–142). It has now become apparent that reprogramming is involved in the functional modulation that endows $CD4^+$ T cells with cytotoxic activity (30). Although other transcription factors, such as T-bet, are also crucial (143), downregulation of *Thpok* and induction of *Runx3* are key to mediation of this reprogramming at the mucosal borders (137, 144, 145). Since Bcl11b appears to function as a guardian to maintain *Thpok* levels and repress *Runx3* expression in conventional $CD4^+$ T cells (105), it will be interesting to examine whether Bcl11b is involved in the generation of $CD4^+$ CTLs in the gut.

7. CONCLUSIONS AND PERSPECTIVE

Although the number of molecules known to have functional roles in CD4 helper/CD8 cytotoxic lineage choice has increased, how the cellular signals initiated on TCR engagement by self-peptides on distinct MHCs are converted into either *Thpok* or *Runx3* expression is not yet fully understood. Regarding *Thpok* regulation, the mechanism regulating *Sth* activity is a key to determining lineage-specific *Thpok* expression and should be a nuclear target of TCR signaling. The new players, SATB1 and Bcl11b, which are necessary for both *Sth* and enhancer activity, presumably via topological control of their assembly, were recently identified. However, whether any stage- and lineage-specific topological changes occur at the *Thpok* locus remains to be examined. Clearly, measurement of chromatin looping, as well as nuclear positioning of the *Thpok* locus, should be investigated. Given the compact structure of the *Thpok* gene, it will be important to develop technology that enables investigation of the interaction of genomic regions located within a range of a few kilobase pairs in a reliable manner.

Bcl11b can also repress *Thpok* through an *Sth*-independent mechanism and is essential for *Runx3* repression in ThPOK-expressing $CD4^+$ T cells (105). Therefore, it is possible that

modulation of Bcl11b function acts as a convertor of TCR signals. In this regard, it is noteworthy that Bcl11b possesses at least 26 serine (S)/threonine (T) residues that can be phosphorylated by PMA/ionomycin stimulation in a T cell line (146). However, alanine (A) substitution of 20 of the S/T residues encoded by exon IV of the murine *Bcl11b* gene did not result in apparent dysregulation of *Thpok* or *Runx3* expression (I. Taniuchi, unpublished data); although this observation does not exclude the possibility that phosphorylation at the other six S/T residues is important in modulating Bcl11b function, it suggests that phosphorylation levels of the Bcl11b protein are unlikely to serve as an important regulatory mechanism for *Thpok* and *Runx3* expression. Rather, it has become clear that regulatory mechanisms acting through the C terminus zinc finger motif of the Bcl11b protein are crucial for *Thpok* and *Runx3* regulation (105). Given that *Foxp3* induction is also abrogated by lack of the C-terminal zinc finger motif of the Bcl11b protein (105), it will undoubtedly be essential to isolate molecules that interact with Bcl11b through the C-terminal zinc finger motif. In addition, to further characterize the mechanisms regulating *Sth* activity, it would be ideal to develop technologies that can identify molecular complexes, including genome regions, long and short RNA, and proteins with their posttranslational status, specifically on *Sth* or at least in close proximity to *Sth*, in a nonbiased manner.

It has not been examined whether any antecedent process is required to couple TCR signals with the transcriptional program governing lineage decisions. Recently, it was demonstrated that the pioneering enhancer CNS3 (147) confers a poised state to the *Foxp3* promoter in precursor cells in response to TCR stimulation (148). Prior binding of SATB1 to CNS0 (93), followed by its sequential binding to CNS3 (51, 93), is essential for *Foxp3* induction upon receiving TCR signals. Similarly, Bcl11b primes the *Thpok* locus to the T lineage type at the DN-to-DP transition and generates T cell–specific regulatory regions (*Sth* and *TE/PE* enhancers) prepared to receive TCR signals. The relevance of such antecedent processes in downstream regulation should be clarified in future studies.

The identification of $CD4^+$ CTLs led me to ponder why CD4 and CD8 lineages require separation during a primary developmental process. In mammals and birds, these two types of $\alpha\beta$ T cells are present as finely separated subsets; however, it is unclear whether the helper or cytotoxic lineage appeared first, and how the genetic program that separates the two lineages was exploited, during evolution. Comparative genomics has demonstrated that genes encoding MHC-II, CD4, and the invariant chain are not retained in the Atlantic cod (*Gadus morhua*) genome (149), whereas most other teleosts have these genes. In the elephant shark (*Callorhinchus milii*), a set of cytotoxic lineage–related genes (*MHC-I*, *Runx3*, *Cd8*, *Gzn*, and *Infg*) are present; however, although *Thpok* and *MHC-II*-related genes were acquired, the *Cd4* gene is not yet present in this species (150). Thus, it was proposed that a primordial helper system, which may be geared toward a Th1-type response, may function together with cytotoxic lineage T cells in the elephant shark (150). Interestingly, *Sth*-related sequences are present in the elephant shark *Thpok* gene (I. Taniuchi, unpublished data). There are two *Cd4* genes, *Cd4-1* and *Cd4-2*, in some teleost genomes, including zebrafish and rainbow trout (151), presumably as a result of gene duplication. Although *Cd4-1* encodes CD4 protein with four immunoglobulin domains, as observed in tetrapod CD4, *Cd4-2* encodes a protein with only two immunoglobulin domains (151, 152). Cells expressing either CD4-1 or CD4-2 did not express CD8a in the spleen (152), and an *S4*-homologous element is present in zebrafish and medaka *Cd4* genes (I. Taniuchi, unpublished data). Information obtained from genomic studies indicates that related genes and regulatory regions are likely present; however, this does not always mean that the genes are expressed or that the regulatory regions are functional. Combinational studies in mice and other species, in particular fish, will provide novel insights into how processes that segregate the CD4 helper and CD8 cytotoxic lineages were exploited and refined during evolution.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The work in the laboratory of I.T. is supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS).

LITERATURE CITED

1. Boehm T, Bleul CC. 2006. Thymus-homing precursors and the thymic microenvironment. *Trends Immunol.* 27:477–84
2. Zhang SL, Bhandoola A. 2014. Trafficking to the thymus. *Curr. Top. Microbiol. Immunol.* 373:87–111
3. Liu C, Saito F, Liu Z, Lei Y, Uehara S, et al. 2006. Coordination between CCR7- and CCR9-mediated chemokine signals in prevascular fetal thymus colonization. *Blood* 108:2531–39
4. Yui MA, Rothenberg EV. 2014. Developmental gene networks: a triathlon on the course to T cell identity. *Nat. Rev. Immunol.* 14:529–45
5. Ellmeier W, Sawada S, Littman DR. 1999. The regulation of CD4 and CD8 coreceptor gene expression during T cell development. *Annu. Rev. Immunol.* 17:523–54
6. Li L, Leid M, Rothenberg EV. 2010. An early T cell lineage commitment checkpoint dependent on the transcription factor *Bcl11b*. *Science* 329:89–93
7. Ikawa T, Hirose S, Masuda K, Kakugawa K, Satoh R, et al. 2010. An essential developmental checkpoint for production of the T cell lineage. *Science* 329:93–96
8. Li P, Burke S, Wang J, Chen X, Ortiz M, et al. 2010. Reprogramming of T cells to natural killer–like cells upon *Bcl11b* deletion. *Science* 329:85–89
9. Kueh HY, Yui MA, Ng KK, Pease SS, Zhang JA, et al. 2016. Asynchronous combinatorial action of four regulatory factors activates *Bcl11b* for T cell commitment. *Nat. Immunol.* 17:956–65
10. von Boehmer H, Fehling HJ. 1997. Structure and function of the pre-T cell receptor. *Annu. Rev. Immunol.* 15:433–52
11. Yamasaki S, Saito T. 2007. Molecular basis for pre-TCR-mediated autonomous signaling. *Trends Immunol.* 28:39–43
12. Krangel MS. 2009. Mechanics of T cell receptor gene rearrangement. *Curr. Opin. Immunol.* 21:133–39
13. Gao GF, Rao Z, Bell JI. 2002. Molecular coordination of αβ T-cell receptors and coreceptors CD8 and CD4 in their recognition of peptide-MHC ligands. *Trends Immunol.* 23:408–13
14. Klein L, Kyewski B, Allen PM, Hogquist KA. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat. Rev. Immunol.* 14:377–91
15. Nitta T, Murata S, Ueno T, Tanaka K, Takahama Y. 2008. Thymic microenvironments for T-cell repertoire formation. *Adv. Immunol.* 99:59–94
16. Cowan JE, Parnell SM, Nakamura K, Caamano JH, Lane PJ, et al. 2013. The thymic medulla is required for Foxp3$^+$ regulatory but not conventional CD4$^+$ thymocyte development. *J. Exp. Med.* 210:675–81
17. Ueno T, Saito F, Gray DH, Kuse S, Hieshima K, et al. 2004. CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *J. Exp. Med.* 200:493–505
18. Brugnera E, Bhandoola A, Cibotti R, Yu Q, Guinter TI, et al. 2000. Coreceptor reversal in the thymus: signaled CD4$^+$8$^+$ thymocytes initially terminate CD8 transcription even when differentiating into CD8$^+$ T cells. *Immunity* 13:59–71
19. Singer A. 2002. New perspectives on a developmental dilemma: the kinetic signaling model and the importance of signal duration for the CD4/CD8 lineage decision. *Curr. Opin. Immunol.* 14:207–15
20. Singer A, Adoro S, Park JH. 2008. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat. Rev. Immunol.* 8:788–801

21. Germain RN. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat. Rev. Immunol.* 2:309–22
22. Littman DR, Thomas Y, Maddon PJ, Chess L, Axel R. 1985. The isolation and sequence of the gene encoding T8: a molecule defining functional classes of T lymphocytes. *Cell* 40:237–46
23. Maddon PJ, Littman DR, Godfrey M, Maddon DE, Chess L, Axel R. 1985. The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. *Cell* 42:93–104
24. Chan SH, Cosgrove D, Waltzinger C, Benoist C, Mathis D. 1993. Another view of the selective model of thymocyte selection. *Cell* 73:225–36
25. Davis CB, Killeen N, Crooks ME, Raulet D, Littman DR. 1993. Evidence for a stochastic mechanism in the differentiation of mature subsets of T lymphocytes. *Cell* 73:237–47
26. Robey EA, Fowlkes BJ, Gordon JW, Kioussis D, von Boehmer H, et al. 1991. Thymic selection in CD8 transgenic mice supports an instructive model for commitment to a CD4 or CD8 lineage. *Cell* 64:99–107
27. Robey E. 1994. Introduction: commitment to CD4 and CD8 lineages—stochastic or instructive? *Semin. Immunol.* 6:207–8
28. Chan SH, Benoist C, Mathis D. 1994. In favor of the selective model of positive selection. *Semin. Immunol.* 6:241–48
29. Issuree PD, Ng CP, Littman DR. 2017. Heritable gene regulation in the CD4:CD8 T cell lineage choice. *Front. Immunol.* 8:291
30. Cheroutre H, Husain MM. 2013. CD4 CTL: living up to the challenge. *Semin. Immunol.* 25:273–81
31. Veillette A, Zuniga-Pflucker JC, Bolen JB, Kruisbeek AM. 1989. Engagement of CD4 and CD8 expressed on immature thymocytes induces activation of intracellular tyrosine phosphorylation pathways. *J. Exp. Med.* 170:1671–80
32. Wiest DL, Yuan L, Jefferson J, Benveniste P, Tsokos M, et al. 1993. Regulation of T cell receptor expression in immature $CD4^+CD8^+$ thymocytes by p56lck tyrosine kinase: basis for differential signaling by CD4 and CD8 in immature thymocytes expressing both coreceptor molecules. *J. Exp. Med.* 178:1701–12
33. Hernandez-Hoyos G, Sohn SJ, Rothenberg EV, Alberola-Ila J. 2000. Lck activity controls CD4/CD8 T cell lineage commitment. *Immunity* 12:313–22
34. Bosselut R, Feigenbaum L, Sharrow SO, Singer A. 2001. Strength of signaling by CD4 and CD8 coreceptor tails determines the number but not the lineage direction of positively selected thymocytes. *Immunity* 14:483–94
35. Suzuki H, Punt JA, Granger LG, Singer A. 1995. Asymmetric signaling requirements for thymocyte commitment to the $CD4^+$ versus $CD8^+$ T cell lineages: a new perspective on thymic commitment and selection. *Immunity* 2:413–25
36. Sarafova SD, Erman B, Yu Q, Van Laethem F, Guinter T, et al. 2005. Modulation of coreceptor transcription during positive selection dictates lineage fate independently of TCR/coreceptor specificity. *Immunity* 23:75–87
37. Adoro S, McCaughtry T, Erman B, Alag A, Van Laethem F, et al. 2012. Coreceptor gene imprinting governs thymocyte lineage fate. *EMBO J.* 31:366–77
38. Sarafova SD, Van Laethem F, Adoro S, Guinter T, Sharrow SO, et al. 2009. Upregulation of CD4 expression during MHC class II-specific positive selection is essential for error-free lineage choice. *Immunity* 31:480–90
39. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. 2013. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat. Methods* 10:1213–18
40. Sawada S, Littman DR. 1991. Identification and characterization of a T-cell-specific enhancer adjacent to the murine CD4 gene. *Mol. Cell. Biol.* 11:5506–15
41. Sawada S, Scarborough JD, Killeen N, Littman DR. 1994. A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. *Cell* 77:917–29
42. Siu G, Wurster AL, Duncan DD, Soliman TM, Hedrick SM. 1994. A transcriptional silencer controls the developmental expression of the CD4 gene. *EMBO J.* 13:3570–79

43. Zou YR, Sunshine MJ, Taniuchi I, Hatam F, Killeen N, Littman DR. 2001. Epigenetic silencing of CD4 in T cells committed to the cytotoxic lineage. *Nat. Genet.* 29:332–36
44. Leung RK, Thomson K, Gallimore A, Jones E, Van den Broek M, et al. 2001. Deletion of the CD4 silencer element supports a stochastic mechanism of thymocyte lineage commitment. *Nat. Immunol.* 2:1167–73
45. Taniuchi I, Sunshine MJ, Festenstein R, Littman DR. 2002. Evidence for distinct CD4 silencer functions at different stages of thymocyte differentiation. *Mol. Cell* 10:1083–96
46. Chong MM, Simpson N, Ciofani M, Chen G, Collins A, Littman DR. 2010. Epigenetic propagation of CD4 expression is established by the *Cd4* proximal enhancer in helper T cells. *Genes Dev*. 24:659–69
47. Sellars M, Huh JR, Day K, Issuree PD, Galan C, et al. 2015. Regulation of DNA methylation dictates *Cd4* expression during the development of helper and cytotoxic T cell lineages. *Nat. Immunol.* 16:746–54
48. Henson DM, Chou C, Sakurai N, Egawa T. 2014. A silencer-proximal intronic region is required for sustained CD4 expression in postselection thymocytes. *J. Immunol.* 192:4620–27
49. Manjunath N, Shankar P, Stockton B, Dubey PD, Lieberman J, von Andrian UH. 1999. A transgenic mouse model to analyze $CD8^+$ effector T cell differentiation in vivo. *PNAS* 96:13932–37
50. Heng TS, Painter MW. 2008. The Immunological Genome Project: networks of gene expression in immune cells. *Nat. Immunol.* 9:1091–94
51. Kakugawa K, Kojo S, Tanaka H, Seo W, Endo TA, et al. 2017. Essential roles of SATB1 in specifying T lymphocyte subsets. *Cell Rep*. 19:1176–88
52. Sakaguchi S, Hombauer M, Hassan H, Tanaka H, Yasmin N, et al. 2015. A novel *Cd8-cis*-regulatory element preferentially directs expression in $CD44^{hi}CD62L^+$ $CD8^+$ T cells and in $CD8\alpha\alpha^+$ dendritic cells. *J. Leukoc. Biol.* 97:635–44
53. Kioussis D, Ellmeier W. 2002. Chromatin and *CD4*, *CD8A* and *CD8B* gene expression during thymic differentiation. *Nat. Rev. Immunol.* 2:909–19
54. Ellmeier W, Sunshine MJ, Losos K, Littman DR. 1998. Multiple developmental stage-specific enhancers regulate CD8 expression in developing thymocytes and in thymus-independent T cells. *Immunity* 9:485–96
55. Hostert A, Garefalaki A, Mavria G, Tolaini M, Roderick K, et al. 1998. Hierarchical interactions of control elements determine $CD8\alpha$ gene expression in subsets of thymocytes and peripheral T cells. *Immunity* 9:497–508
56. Ellmeier W, Sunshine MJ, Losos K, Hatam F, Littman DR. 1997. An enhancer that directs lineage-specific expression of CD8 in positively selected thymocytes and mature T cells. *Immunity* 7:537–47
57. Bilic I, Koesters C, Unger B, Sekimata M, Hertweck A, et al. 2006. Negative regulation of CD8 expression via *Cd8* enhancer–mediated recruitment of the zinc finger protein MAZR. *Nat. Immunol.* 7:392–400
58. Carbone AM, Marrack P, Kappler JW. 1988. Demethylated CD8 gene in $CD4^+$ T cells suggests that $CD4^+$ cells develop from $CD8^+$ precursors. *Science* 242:1174–76
59. Harland KL, Day EB, Apte SH, Russ BE, Doherty PC, et al. 2014. Epigenetic plasticity of *Cd8a* locus during $CD8^+$ T-cell development and effector differentiation and reprogramming. *Nat. Commun.* 5:3547
60. Hamerman JA, Page ST, Pullen AM. 1997. Distinct methylation states of the CD8 beta gene in peripheral T cells and intraepithelial lymphocytes. *J. Immunol.* 159:1240–46
61. Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, et al. 2001. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* 15:763–74
62. Chi TH, Wan M, Zhao K, Taniuchi I, Chen L, et al. 2002. Reciprocal regulation of CD4/CD8 expression by SWI/SNF-like BAF complexes. *Nature* 418:195–99
63. Naito T, Gomez-Del Arco P, Williams CJ, Georgopoulos K. 2007. Antagonistic interactions between Ikaros and the chromatin remodeler Mi-2 β determine silencer activity and *Cd4* gene expression. *Immunity* 27:723–34
64. Taniuchi I, Osato M, Egawa T, Sunshine MJ, Bae SC, et al. 2002. Differential requirements for Runx proteins in *CD4* repression and epigenetic silencing during T lymphocyte development. *Cell* 111:621–33
65. Muroi S, Naoe Y, Miyamoto C, Akiyama K, Ikawa T, et al. 2008. Cascading suppression of transcriptional silencers by ThPOK seals helper T cell fate. *Nat. Immunol.* 9:1113–21

66. Wildt KF, Sun G, Grueter B, Fischer M, Zamisch M, et al. 2007. The transcription factor zbtb7b promotes CD4 expression by antagonizing runx-mediated activation of the CD4 silencer. *J. Immunol.* 179:4405–14
67. Yu M, Wan M, Zhang J, Wu J, Khatri R, Chi T. 2008. Nucleoprotein structure of the CD4 locus: implications for the mechanisms underlying CD4 regulation during T cell development. *PNAS* 105:3873–78
68. Speck NA. 2001. Core binding factor and its role in normal hematopoietic development. *Curr. Opin. Hematol.* 8:192–96
69. Levanon D, Groner Y. 2004. Structure and regulated expression of mammalian RUNX genes. *Oncogene* 23:4211–19
70. Kagoshima H, Shigesada K, Satake M, Ito Y, Miyoshi H, et al. 1993. The Runt domain identifies a new family of heteromeric transcriptional regulators. *Trends Genet.* 9:338–41
71. Daga A, Tighe JE, Calabi F. 1992. Leukaemia/*Drosophila* homology. *Nature* 356:484
72. Ito Y. 2008. RUNX genes in development and cancer: regulation of viral gene expression and the discovery of RUNX family genes. *Adv. Cancer Res.* 99:33–76
73. Levanon D, Goldstein RE, Bernstein Y, Tang H, Goldenberg D, et al. 1998. Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. *PNAS* 95:11590–95
74. Seo W, Tanaka H, Miyamoto C, Levanon D, Groner Y, Taniuchi I. 2012. Roles of VWRPY motif-mediated gene repression by Runx proteins during T-cell development. *Immunol. Cell Biol.* 90:827–30
75. Yarmus M, Woolf E, Bernstein Y, Fainaru O, Negreanu V, et al. 2006. Groucho/transducin-like Enhancer-of-split (TLE)-dependent and -independent transcriptional regulation by Runx3. *PNAS* 103:7384–89
76. Egawa T, Tillman RE, Naoe Y, Taniuchi I, Littman DR. 2007. The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. *J. Exp. Med.* 204:1945–57
77. Kim B, Sasaki Y, Egawa T. 2015. Restriction of nonpermissive RUNX3 protein expression in T lymphocytes by the Kozak sequence. *J. Immunol.* 195:1517–23
78. Spender LC, Whiteman HJ, Karstegl CE, Farrell PJ. 2005. Transcriptional cross-regulation of RUNX1 by RUNX3 in human B cells. *Oncogene* 24:1873–81
79. Egawa T, Eberl G, Taniuchi I, Benlagha K, Geissmann F, et al. 2005. Genetic evidence supporting selection of the Vα14i NKT cell lineage from double-positive thymocyte precursors. *Immunity* 22:705–16
80. Jiang H, Peterlin BM. 2008. Differential chromatin looping regulates CD4 expression in immature thymocytes. *Mol. Cell. Biol.* 28:907–12
81. Hassan H, Sakaguchi S, Tenno M, Kopf A, Boucheron N, et al. 2011. Cd8 enhancer E8I and Runx factors regulate CD8α expression in activated $CD8^+$ T cells. *PNAS* 108:18330–35
82. Pobezinsky LA, Angelov GS, Tai X, Jeurling S, Van Laethem F, et al. 2012. Clonal deletion and the fate of autoreactive thymocytes that survive negative selection. *Nat. Immunol.* 13:569–78
83. Sato T, Ohno S, Hayashi T, Sato C, Kohu K, et al. 2005. Dual functions of Runx proteins for reactivating CD8 and silencing CD4 at the commitment process into CD8 thymocytes. *Immunity* 22:317–28
84. He X, Dave VP, Zhang Y, Hua X, Nicolas E, et al. 2005. The zinc finger transcription factor *Th-POK* regulates CD4 versus CD8 T-cell lineage commitment. *Nature* 433:826–33
85. Dave VP, Allman D, Keefe R, Hardy RR, Kappes DJ. 1998. HD mice: a novel mouse mutant with a specific defect in the generation of $CD4^+$ T cells. *PNAS* 95:8187–92
86. Sun G, Liu X, Mercado P, Jenkinson SR, Kypriotou M, et al. 2005. The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. *Nat. Immunol.* 6:373–81
87. Keefe R, Dave V, Allman D, Wiest D, Kappes DJ. 1999. Regulation of lineage commitment distinct from positive selection. *Science* 286:1149–53
88. Kappes DJ, He X. 2006. Role of the transcription factor Th-POK in CD4:CD8 lineage commitment. *Immunol. Rev.* 209:237–52
89. Harker N, Naito T, Cortes M, Hostert A, Hirschberg S, et al. 2002. The CD8α gene locus is regulated by the Ikaros family of proteins. *Mol. Cell* 10:1403–15
90. Sridharan R, Smale ST. 2007. Predominant interaction of both Ikaros and Helios with the NuRD complex in immature thymocytes. *J. Biol. Chem.* 282:30227–38

91. Banan M, Rojas IC, Lee WH, King HL, Harriss JV, et al. 1997. Interaction of the nuclear matrix-associated region (MAR)-binding proteins, SATB1 and CDP/Cux, with a MAR element (L2a) in an upstream regulatory region of the mouse *CD8a* gene. *J. Biol. Chem.* 272:18440–52
92. Yao X, Nie H, Rojas IC, Harriss JV, Maika SD, et al. 2010. The L2a element is a mouse CD8 silencer that interacts with MAR-binding proteins SATB1 and CDP. *Mol. Immunol.* 48:153–63
93. Kitagawa Y, Ohkura N, Kidani Y, Vandenbon A, Hirota K, et al. 2017. Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment. *Nat. Immunol.* 18:173–83
94. Aliahmad P, Kaye J. 2008. Development of all CD4 T lineages requires nuclear factor TOX. *J. Exp. Med.* 205:245–56
95. Seehus CR, Aliahmad P, de la Torre B, Iliev ID, Spurka L, et al. 2015. The development of innate lymphoid cells requires TOX-dependent generation of a common innate lymphoid cell progenitor. *Nat. Immunol.* 16:599–608
96. He X, Park K, Wang H, He X, Zhang Y, et al. 2008. CD4-CD8 lineage commitment is regulated by a silencer element at the ThPOK transcription-factor locus. *Immunity* 28:346–58
97. Muroi S, Tanaka H, Miyamoto C, Taniuchi I. 2013. Cutting edge: fine-tuning of *Thpok* gene activation by an enhancer in close proximity to its own silencer. *J. Immunol.* 190:1397–401
98. Setoguchi R, Tachibana M, Naoe Y, Muroi S, Akiyama K, et al. 2008. Repression of the transcription factor Th-POK by Runx complexes in cytotoxic T cell development. *Science* 319:822–25
99. Hedrick SM. 2008. Thymus lineage commitment: a single switch. *Immunity* 28:297–99
100. Tanaka H, Naito T, Muroi S, Seo W, Chihara R, et al. 2013. Epigenetic *Thpok* silencing limits the time window to choose $CD4^+$ helper-lineage fate in the thymus. *EMBO J.* 32:1183–94
101. Durst KL, Hiebert SW. 2004. Role of RUNX family members in transcriptional repression and gene silencing. *Oncogene* 23:4220–24
102. Egawa T, Littman DR. 2008. ThPOK acts late in specification of the helper T cell lineage and suppresses Runx-mediated commitment to the cytotoxic T cell lineage. *Nat. Immunol.* 9:1131–39
103. Wang L, Wildt KF, Castro E, Xiong Y, Feigenbaum L, et al. 2008. The zinc finger transcription factor Zbtb7b represses CD8-lineage gene expression in peripheral $CD4^+$ T cells. *Immunity* 29:876–87
104. Taniuchi I. 2009. Transcriptional regulation in helper versus cytotoxic-lineage decision. *Curr. Opin. Immunol.* 21:127–32
105. Kojo S, Tanaka H, Endo TA, Muroi S, Liu Y, et al. 2017. Priming of lineage-specifying genes by Bcl11b is required for lineage choice in post-selection thymocytes. *Nat. Commun.* 8:702
106. Sakaguchi S, Hombauer M, Bilic I, Naoe Y, Schebesta A, et al. 2010. The zinc-finger protein MAZR is part of the transcription factor network that controls the CD4 versus CD8 lineage fate of double-positive thymocytes. *Nat. Immunol.* 11:442–48
107. Sakaguchi S, Hainberger D, Tizian C, Tanaka H, Okuda T, et al. 2015. MAZR and Runx factors synergistically repress ThPOK during $CD8^+$ T cell lineage development. *J. Immunol.* 195:2879–87
108. Avram D, Califano D. 2014. The multifaceted roles of Bcl11b in thymic and peripheral T cells: impact on immune diseases. *J. Immunol.* 193:2059–65
109. VanValkenburgh J, Albu DI, Bapanpally C, Casanova S, Califano D, et al. 2011. Critical role of Bcl11b in suppressor function of T regulatory cells and prevention of inflammatory bowel disease. *J. Exp. Med.* 208:2069–81
110. Albu DI, VanValkenburgh J, Morin N, Califano D, Jenkins NA, et al. 2011. Transcription factor Bcl11b controls selection of invariant natural killer T-cells by regulating glycolipid presentation in double-positive thymocytes. *PNAS* 108:6211–16
111. Walker JA, Oliphant CJ, Englezakis A, Yu Y, Clare S, et al. 2015. Bcl11b is essential for group 2 innate lymphoid cell development. *J. Exp. Med.* 212:875–82
112. Yu Y, Wang C, Clare S, Wang J, Lee SC, et al. 2015. The transcription factor Bcl11b is specifically expressed in group 2 innate lymphoid cells and is essential for their development. *J. Exp. Med.* 212:865–74
113. Califano D, Cho JJ, Uddin MN, Lorentsen KJ, Yang Q, et al. 2015. Transcription factor Bcl11b controls identity and function of mature type 2 innate lymphoid cells. *Immunity* 43:354–68
114. Kastner P, Chan S, Vogel WK, Zhang LJ, Topark-Ngarm A, et al. 2010. Bcl11b represses a mature T-cell gene expression program in immature $CD4^+CD8^+$ thymocytes. *Eur. J. Immunol.* 40:2143–54

115. Albu DI, Feng D, Bhattacharya D, Jenkins NA, Copeland NG, et al. 2007. BCL11B is required for positive selection and survival of double-positive thymocytes. *J. Exp. Med.* 204:3003–15
116. Cai S, Lee CC, Kohwi-Shigematsu T. 2006. SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. *Nat. Genet.* 38:1278–88
117. Xu J, Sankaran VG, Ni M, Menne TF, Puram RV, et al. 2010. Transcriptional silencing of γ-globin by BCL11A involves long-range interactions and cooperation with SOX6. *Genes Dev.* 24:783–98
118. Wang L, Wildt KF, Zhu J, Zhang X, Feigenbaum L, et al. 2008. Distinct functions for the transcription factors GATA-3 and ThPOK during intrathymic differentiation of $CD4^+$ T cells. *Nat. Immunol.* 9:1122–30
119. Hosoya T, Maillard I, Engel JD. 2010. From the cradle to the grave: activities of GATA-3 throughout T-cell development and differentiation. *Immunol. Rev.* 238:110–25
120. Hernandez-Hoyos G, Anderson MK, Wang C, Rothenberg EV, Alberola-Ila J. 2003. GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. *Immunity* 19:83–94
121. Pai SY, Truitt ML, Ting CN, Leiden JM, Glimcher LH, Ho IC. 2003. Critical roles for transcription factor GATA-3 in thymocyte development. *Immunity* 19:863–75
122. Steinke FC, Yu S, Zhou X, He B, Yang W, et al. 2014. TCF-1 and LEF-1 act upstream of Th-POK to promote the $CD4^+$ T cell fate and interact with Runx3 to silence *Cd4* in $CD8^+$ T cells. *Nat. Immunol.* 15:646–56
123. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, et al. 2009. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 324:930–35
124. Kriaucionis S, Heintz N. 2009. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* 324:929–30
125. Ito S, Shen L, Dai Q, Wu SC, Collins LB, et al. 2011. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* 333:1300–3
126. Tsagaratou A, Gonzalez-Avalos E, Rautio S, Scott-Browne JP, Togher S, et al. 2017. TET proteins regulate the lineage specification and TCR-mediated expansion of iNKT cells. *Nat. Immunol.* 18:45–53
127. Appel E, Weissmann S, Salzberg Y, Orlovsky K, Negreanu V, et al. 2016. An ensemble of regulatory elements controls Runx3 spatiotemporal expression in subsets of dorsal root ganglia proprioceptive neurons. *Genes Dev.* 30:2607–22
128. Park JH, Adoro S, Guinter T, Erman B, Alag AS, et al. 2010. Signaling by intrathymic cytokines, not T cell antigen receptors, specifies CD8 lineage choice and promotes the differentiation of cytotoxic-lineage T cells. *Nat. Immunol.* 11:257–64
129. McCaughtry TM, Etzensperger R, Alag A, Tai X, Kurtulus S, et al. 2012. Conditional deletion of cytokine receptor chains reveals that IL-7 and IL-15 specify CD8 cytotoxic lineage fate in the thymus. *J. Exp. Med.* 209:2263–76
130. Luckey MA, Kimura MY, Waickman AT, Feigenbaum L, Singer A, Park JH. 2014. The transcription factor ThPOK suppresses Runx3 and imposes $CD4^+$ lineage fate by inducing the SOCS suppressors of cytokine signaling. *Nat. Immunol.* 15:638–45
131. Leonard WJ. 2001. Role of Jak kinases and STATs in cytokine signal transduction. *Int. J. Hematol.* 73:271–77
132. Xiong Y, Castro E, Yagi R, Zhu J, Lesourne R, et al. 2013. Thpok-independent repression of Runx3 by Gata3 during $CD4^+$ T-cell differentiation in the thymus. *Eur. J. Immunol.* 43:918–28
133. Rodriguez RM, Lopez-Larrea C, Suarez-Alvarez B. 2015. Epigenetic dynamics during $CD4^+$ T cells lineage commitment. *Int. J. Biochem. Cell Biol.* 67:75–85
134. Konkel JE, Maruyama T, Carpenter AC, Xiong Y, Zamarron BF, et al. 2011. Control of the development of $CD8\alpha\alpha^+$ intestinal intraepithelial lymphocytes by TGF-β. *Nat. Immunol.* 12:312–19
135. Naoe Y, Setoguchi R, Akiyama K, Muroi S, Kuroda M, et al. 2007. Repression of interleukin-4 in T helper type 1 cells by Runx/Cbfβ binding to the *Il4* silencer. *J. Exp. Med.* 204:1749–55
136. Djuretic IM, Levanon D, Negreanu V, Groner Y, Rao A, Ansel KM. 2007. Transcription factors T-bet and Runx3 cooperate to activate *Ifng* and silence *Il4* in T helper type 1 cells. *Nat. Immunol.* 8:145–53
137. Mucida D, Husain MM, Muroi S, van Wijk F, Shinnakasu R, et al. 2013. Transcriptional reprogramming of mature $CD4^+$ helper T cells generates distinct MHC class II–restricted cytotoxic T lymphocytes. *Nat. Immunol.* 14:281–89

138. Takeuchi A, Badr Mel S, Miyauchi K, Ishihara C, Onishi R, et al. 2016. CRTAM determines the CD4$^+$ cytotoxic T lymphocyte lineage. *J. Exp. Med.* 213:123–38
139. Nakayamada S, Takahashi H, Kanno Y, O'Shea JJ. 2012. Helper T cell diversity and plasticity. *Curr. Opin. Immunol.* 24:297–302
140. Appay V, Zaunders JJ, Papagno L, Sutton J, Jaramillo A, et al. 2002. Characterization of CD4$^+$ CTLs ex vivo. *J. Immunol.* 168:5954–58
141. Brown DM. 2010. Cytolytic CD4 cells: Direct mediators in infectious disease and malignancy. *Cell Immunol.* 262:89–95
142. Marshall NB, Swain SL. 2011. Cytotoxic CD4 T cells in antiviral immunity. *J. Biomed. Biotechnol.* 2011:954602
143. Reis BS, Hoytema van Konijnenburg DP, Grivennikov SI, Mucida D. 2014. Transcription factor T-bet regulates intraepithelial lymphocyte functional maturation. *Immunity* 41:244–56
144. Reis B, Rogoz A, Costa-Pinto F, Taniuchi I, Mucida D. 2013. Mutual expression of the transcription factors Runx3 and ThPOK regulates intestinal CD4$^+$ T cell immunity. *Nat. Immunol.* 14:271–80
145. Sujino T, London M, Hoytema van Konijnenburg DP, Rendon T, Buch T, et al. 2016. Tissue adaptation of regulatory and intraepithelial CD4$^+$ T cells controls gut inflammation. *Science* 352:1581–86
146. Vogel WK, Gafken PR, Leid M, Filtz TM. 2014. Kinetic analysis of BCL11B multisite phosphorylation–dephosphorylation and coupled sumoylation in primary thymocytes by multiple reaction monitoring mass spectroscopy. *J. Proteome Res.* 13:5860–68
147. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. 2010. Role of conserved non-coding DNA elements in the *Foxp3* gene in regulatory T-cell fate. *Nature* 463:808–12
148. Feng Y, van der Veeken J, Shugay M, Putintseva EV, Osmanbeyoglu HU, et al. 2015. A mechanism for expansion of regulatory T-cell repertoire and its role in self-tolerance. *Nature* 528:132–36
149. Star B, Nederbragt AJ, Jentoft S, Grimholt U, Malmstrom M, et al. 2011. The genome sequence of Atlantic cod reveals a unique immune system. *Nature* 477:207–10
150. Venkatesh B, Lee AP, Ravi V, Maurya AK, Lian MM, et al. 2014. Elephant shark genome provides unique insights into gnathostome evolution. *Nature* 505:174–79
151. Laing KJ, Zou JJ, Purcell MK, Phillips R, Secombes CJ, Hansen JD. 2006. Evolution of the CD4 family: teleost fish possess two divergent forms of CD4 in addition to lymphocyte activation gene-3. *J. Immunol.* 177:3939–51
152. Takizawa F, Magadan S, Parra D, Xu Z, Korytar T, et al. 2016. Novel teleost CD4-bearing cell populations provide insights into the evolutionary origins and primordial roles of CD4$^+$ lymphocytes and CD4$^+$ macrophages. *J. Immunol.* 196:4522–35