The transcription factor ThPOK acts late in helper T cell lineage specification and suppresses Runx-mediated commitment to the cytotoxic T cell lineage

Takeshi Egawa[1,*] and **Dan R. Littman**[1,2,*]

[1]Molecular Pathogenesis Program, The Helen and Martin Kimmel Center for Biology and Medicine at the Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY 10016

[2]The Howard Hughes Medical Institute, The Helen and Martin Kimmel Center for Biology and Medicine at the Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY 10016

Abstract

The transcription factor ThPOK has been shown to be required and sufficient for $CD4^+CD8^-$ thymocyte generation, yet the mechanism through which ThPOK orchestrates CD4 helper T cell lineage differentiation remains unclear. Here we utilized reporter mice to track expression of transcription factors in developing thymocytes. Distal promoter-driven Runx3 (*Runx3d*) expression was restricted to MHC class I-selected thymocytes. In ThPOK-deficient mice, Runx3d expression was de-repressed in MHCII-selected thymocytes, contributing to their redirection to the CD8 T cell lineage. In the absence of both ThPOK and Runx, redirection was prevented and cells potentially belonging to the CD4 lineage, presumably specified independently of ThPOK, were generated. Our results suggest that MHCII-selected thymocytes are directed towards the CD4 lineage independently of ThPOK, but require ThPOK to prevent Runx-dependent differentiation towards the CD8 lineage.

INTRODUCTION

Development of αβ T cells requires rearrangement of the *Tcra* and *Tcrb* loci and appropriate signals transduced via interactions between T cell receptors (TCR) on $CD4^+CD8^+$ double positive (DP) thymocytes and peptide in the context of major histocompatibility complex (MHC) molecules on thymic stromal cells. Only a small proportion of DP thymocytes is selected for differentiation into mature cells that home to peripheral secondary lymphoid organs. This positive selection process is accompanied or followed by coordinated activation and/or repression of genes that program differentiation of the selected thymocytes to specific lineages of mature T cells. $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells comprise a

*Correspondence should be addressed to T.E. (egawa@saturn.med.nyu.edu) or D.R.L. (littman@saturn.med.nyu.edu).

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majority of mature αβ T lymphocytes in mice and humans. After positive selection, DP cells expressing MHC class I- or MHC class II-restricted TCRs undergo differentiation into CD8^{+} and CD4^{+} lineage T cells, respectively; this differentiation involves transcriptional down-regulation of one or the other co-receptor1. CD4 expression is terminated in CD4^{−}CD8^{+} single positive (CD8SP) thymocytes through activation of the *Cd4* silencer, a negative *cis*-regulatory element in the *Cd4* locus; *Cd4* silencing is subsequently maintained by epigenetic mechanisms2,3. CD8 expression is transiently down-regulated in both MHCI- and MHCII-restricted thymocytes and is selectively reactivated in MHCI-restricted CD8SP cells through stage-specific enhancers4. In addition to *Cd4*, *Cd8a and Cd8b*, other genes are selectively activated or repressed in each mature T cell lineage, and a major goal has been to elucidate how lineage-specific gene regulation is coordinated.

During early hematopoiesis, a balance between distinct lineage-specific transcription factors determines fates of mature cells derived from common multipotent progenitors5. Similar gene regulatory networks may regulate the CD4 versus CD8 lineage decision. The C2H2 type zinc finger transcription factor ThPOK (http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=markerDetail&key=18709), encoded by the *Zbtb7b* gene, is required and sufficient for generation of CD4SP thymocytes following positive selection6,7. *hd/hd* (helper deficient) mice, which harbor a mutation in ThPOK, lack CD4^{+} helper T cells, and thymocytes selected by MHCII are redirected to the CD8^{+} T cell lineage. Forced expression of ThPOK results in redirection of MHCI restricted thymocytes to the CD4^{+} T cell lineage6,7.

Whereas ThPOK exerts a dominant influence over the differentiation of CD4SP thymocytes, it is not clear if differentiation of CD8SP thymocytes requires such a pivotal factor or occurs by default if ThPOK is not up-regulated. We previously identified the Runx family of transcriptional regulators as crucial for *Cd4* gene silencing associated with differentiation of the CD8^{+} cytotoxic T cell lineage8. Among the three Runx proteins that form heterodimers with the common subunit CBFβ (http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=markerDetail&key=15686), Runx3 (http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=markerDetail&key=45178) is the primary mediator of heritable *Cd4* silencing during development of the CD8SP lineage, and Runx1 (http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=markerDetail&key=45176) also contributes to partial *Cd4* silencing in the absence of Runx3 (ref. 8,9). Conditional inactivation of both Runx1 and Runx3 at the DP stage of thymocyte differentiation resulted in a complete loss of CD8SP thymocytes, although generation of CD4SP thymocytes was still observed10. Runx3 protein is detected in CD8SP but not CD4SP thymocytes, even though Runx3 mRNA was up-regulated in both lineages following positive selection10,11. These findings suggest that Runx3 expression is regulated at least in part via post-transcriptional mechanisms. Runx transcripts are initiated at two distinct sites; Runx3 expression from the distal promoter is detected exclusively in CD8SP thymocytes, whereas CD4SP cells express only the proximal promoter-derived transcript that is not sufficient for protein synthesis10. These results, together with our previous finding that Runx1 inactivation alone exerts little influence on development of the CD8^{+} lineage10, suggest that the CD8^{+} lineage-specific requirement for Runx function is satisfied primarily by Runx310,

and that activation of the Runx3 distal promoter (designated here as *Runx3d*) is a key event required for CD8 lineage differentiation of MHCI-restricted thymocytes. Even though it is not expressed in naïve peripheral CD4^{+} T cells, Runx3 protein is up-regulated in interferon (IFN)-γ–producing T helper type 1 ($T_H 1$) cells, where it contributes to repression of the interleukin (IL)-4 (*Il4*) gene, but the mechanism for its regulation in these cells is not known[12,13].

ThPOK expression in developing thymocytes is regulated by a *cis*-acting silencer element required to restrict ThPOK expression to the CD4^{+} lineage[14,15]. In the absence of this silencer, ThPOK is prematurely expressed in DP thymocytes, and all positively selected thymocytes differentiate into CD4^{+} lineage cells[14]. The ThPOK silencer is bound by Runx complexes, and ThPOK is prematurely expressed in pre-selection DP thymocytes in the absence of both Runx1 and Runx3 (ref. 14). This result implies that ThPOK activity is required for diversion of MHCI-selected Runx-deficient thymocytes to the helper T cell lineage, and suggests that Runx proteins continue to suppress ThPOK expression following positive selection of MHCI-restricted thymocytes. However, a requirement for Runx complexes in lineage-specific ThPOK silencing has been disputed[14,15].

To clarify the gene regulatory network governing lineage determination of αβ T cells, we generated knock-in reporter strains that facilitate single cell analysis of ThPOK and *Runx3d* expression in MHCI- and MHCII-selected thymocytes. By analyzing these reporter mice, we show here that ThPOK prevents up-regulation of Runx3 and other CD8 lineage-specific genes. We provide genetic evidence suggesting that ThPOK stabilizes the fate of MHCII-selected thymocytes that would otherwise be diverted to the CD8SP lineage due to constitutive Runx function. We also show that ThPOK is not required for the appearance of CD4SP cells in an environment in which MHCII-specific thymocytes are positively selected; this latter finding suggests that other factors act upstream of ThPOK to direct CD4 expression following positive selection.

RESULTS

Runx3d is required for Runx3 protein expression in CD8^{+} T cells

To visualize thymocytes expressing *Runx3d* mRNA, we generated a reporter allele (*Runx3d*[YFP]) by replacing the first coding exon utilized by the *Runx3* distal promoter with the yellow fluorescent protein (YFP) coding sequence (Supplementary Fig. 1a, online). In accordance with previous analyses of Runx protein and mRNA expression[10], YFP expression was detected in CD8^{+} T cells, but not in CD4^{+} T cells or B220^{+} cells in peripheral blood lymphocytes from *Runx3d*[YFP/+] mice (Supplementary Fig. 1b, online). To determine whether Runx3 distal promoter activity is required for Runx3 protein expression in CD8^{+} T cells, we generated homozygous *Runx3d*[YFP/YFP] mice, in which distal promoter-derived Runx3 expression was eliminated. Whereas germline deletion of both proximal promoter- and distal promoter-derived *Runx3* results in neonatal lethality in the 129 or C57BL6 genetic background[16,17], *Runx3d*[YFP/YFP] mice were viable and fertile, and showed no gross abnormality (data not shown). Runx3 protein was almost undetectable in CD8^{+} T cells, and was markedly reduced in T helper type ($T_H 1$)-polarized activated CD4^{+} T cells from *Runx3d*[YFP/YFP] compared to *Runx3d*[YFP/+] mice (Fig. 1a and Supplementary Fig. 1c,d,

online). Runx1 was up-regulated in Runx3-deficient CD8$^+$ T cells from both *Runx3*$^{F/F}$*Cd4-*cre$^+$ and *Runx3d*$^{YFP/YFP}$ mice (Fig. 1a and Supplementary Fig. 1d, online). This result indicates that most of Runx3 protein is derived from the distal promoter-driven transcript both in CD8$^+$ T cells and in T$_H$1-polarized CD4$^+$ T cells, and that only a small amount of Runx3 protein is expressed from the proximal promoter in T cells. In CD8$^+$ T cells from *Runx3d*$^{YFP/YFP}$ mice, *Cd4* silencing was incomplete and there was no up-regulation of CD103 (integrin αE) expression, which was previously shown to be dependent on Runx3 (ref. 18) (Fig. 1b,c). Thus, Runx3 expression from the distal promoter is required for *Cd4* silencing and CD103 expression in CD8SP thymocytes.

To investigate regulation of *Runx3d* expression in developing thymocytes, we next examined YFP expression in a series of intermediate thymocyte subsets defined by CD4, CD8, TCRβ, CD69 and CD24 (HSA) expression (Fig. 2a and Supplementary Fig. 2a,b, online). YFP$^+$ cells were not detected in pre-selected CD69$^-$ DP thymocytes (data not shown). Following positive selection, MHCI- and MHCII-selected thymocytes both transit through an intermediate CD69$^+$HSA^{hi}TCRβ^{int}CD4$^+$CD8^{lo} (referred to hereafter as CD4$^+$CD8^{lo}) stage (Supplementary Fig. 2c, online) 19–25. At this stage, we observed 2–5 % of cells expressing YFP (Fig. 2a and Supplementary Fig. 2d, online). These YFP$^+$ cells were absent in *Runx3d*$^{YFP/+}$ mice on a *B2m*$^{-/-}$ background, indicating that *Runx3d* transcription is activated only in positively selected MHCI-restricted thymocytes (Supplementary Fig. 2d, online). Following the CD4$^+$CD8^{lo} stage, MHCI-selected thymocytes become CD4$^+$CD8$^+$CD69$^+$TCRβ^{hi}, and such cells are absent in mice lacking β2-microglobulin, an essential component of MHCI molecules (*B2m*$^{-/-}$) (Supplementary Fig. 2e, online). In contrast, MHCII-restricted thymocytes continue to down-regulate surface CD8 expression to become CD4$^+$CD8$^-$ thymocytes before down-regulation of HSA. A majority of CD4$^+$CD8$^+$CD69$^+$TCRβ^{hi} thymocytes expressed YFP, and cells with the highest surface TCRβ expression were brightest for YFP (Fig. 2a). Mature HSA$^{lo/-}$ CD8SP thymocytes expressed a uniformly high amount of YFP (Fig. 2a). YFP expression was not detected in the MHCII-restricted CD4$^+$CD8$^-$HSA^{hi} population. These results indicate that *Runx3d*-YFP expression is highly restricted to MHCI-selected thymocytes, that it occurs largely following transit from the CD4$^+$CD8^{lo} stage, and that it specifically marks developing CD8SP thymocytes.

ThPOK expression specific to MHCII-restricted thymocytes

To visualize ThPOK expression in individual differentiating thymocytes, we generated a green fluorescent protein (GFP) knock-in allele of ThPOK (*Zbtb7b*^{GFP}) by replacing the entire exon 2 and exon 3 with GFP cDNA (Supplementary Fig. 3a, online). In the periphery, all CD4$^+$ T cells expressed high amounts of GFP, and a small proportion of CD8$^+$ T cells expressed GFP, although in approximately 10-fold lower quantities (Supplementary Fig. 4a, online)6,7. A large proportion of B220$^+$ cells also expressed low amounts of GFP (Supplementary Fig. 4a, online). In the thymus, GFP$^+$ cells were not detected in CD69$^-$ pre-selected DP thymocytes (data not shown). Following positive selection, approximately half of the CD4$^+$CD8^{lo} cells and all mature CD4SP thymocytes expressed high quantities of GFP (Fig. 2b). In the *B2m*$^{-/-}$ background, a larger proportion of the CD4$^+$CD8^{lo} thymocytes expressed GFP due to enrichment of MHCII-restricted cells (Fig. 2c). At the CD4$^+$CD8^{lo}

stage, distinct GFP^{hi} and GFP^{lo} populations were observed (Fig. 2b). We noted slightly higher TCRβ expression in GFP^{hi} cells, suggesting that MHCII-selected thymocytes undergo maturation while up-regulating ThPOK during the CD4$^+$CD8^{lo} stage (Fig. 2b). Thus, expression of ThPOK occurs earlier in differentiation than that of Runx3d. Consistent with little GFP expression in peripheral CD8$^+$ T cells, only a small subset of MHCI-selected thymocytes expressed low amounts of GFP. In *Zbtb7b*$^{GFP/+}$*H2-Ab1*$^{-/-}$ mice, which lack MHCII molecules, approximately 5% of CD4$^+$CD8^{lo}, CD4$^+$CD8$^+$CD69$^+$TCRβ^{hi} and CD4$^{lo/-}$CD8$^+$ populations expressed low quantities of GFP (Fig. 2c and data not shown). Approximately 4% of CD4$^+$CD8$^+$CD69$^+$TCRβ^{hi} and CD4$^{lo/-}$CD8$^+$ thymocytes co-expressed *Runx3d*-YFP and low amounts of *ThPOK*-GFP (Fig. 2d), but we could not detect cells expressing high amounts of both reporters. Thus, strong expression of *ThPOK* and *Runx3d* in positively selected thymocytes may be mutually exclusive.

We also generated mice with a null allele of ThPOK (Supplementary Fig. 3b, online) and examined *Zbtb7b*$^{GFP/-}$ mice that express no ThPOK protein. *Zbtb7b*$^{GFP/-}$ mice contained very few mature HSA$^{lo/-}$ CD4SP cells, but the total number of TCR^{hi}HSA$^{lo/-}$ thymocytes remained normal (Supplementary Fig. 4b, online). This phenotype is similar to that found in *hd/hd* mice[26]. As GFP expression in the CD69$^+$HSA^{hi}CD4$^+$CD8$^{lo/-}$ subpopulations was comparable between *Zbtb7b*$^{GFP/+}$ mice and *Zbtb7b*$^{GFP/-}$ mice, ThPOK up-regulation during positive selection appeared unlikely to be dependent on ThPOK expression itself (Fig. 3a). We observed a substantial amount of GFP expression in approximately two-thirds of CD8SP thymocytes and peripheral CD8$^+$ T cells in *Zbtb7b*$^{GFP/-}$ mice (Fig. 2e). GFP expression in CD8$^+$GFP$^+$ T cells in the periphery was approximately 3-fold lower compared to that in CD4$^+$ T cells (data not shown). All CD8SP thymocytes in *Zbtb7b*$^{GFP/-}$*B2m*$^{-/-}$ mice expressed GFP, indicating that they were MHCII-restricted cells redirected from the CD4SP lineage (Supplementary Fig. 4c). These findings suggest that MHCII-restricted positive selection is required and sufficient to turn on high ThPOK expression.

ThPOK blocks Runx3 expression

To determine whether there is cross regulation of ThPOK and Runx3 during thymocyte lineage specification, we examined expression of the ThPOK and *Runx3d* reporters in the absence of one or the other factor. *ThPOK*-GFP expression was unaffected by disruption of the distal promoter-derived Runx3 transcript or by transgenic overexpression of Runx3 (data not shown). In contrast, *Runx3d*-YFP was ectopically expressed in MHCII-restricted thymocytes in ThPOK-deficient mice, suggesting that ThPOK acts as an upstream negative regulator of Runx3 expression (Fig. 3b). Approximately 30–40% of CD4$^+$CD8$^-$ HSA^{hi} thymocytes expressed *Runx3d*-YFP in the absence of ThPOK; these YFP$^+$ cells expressed the highest amount of TCRβ (Fig. 3b). *ThPOK*-GFP was up-regulated despite the absence of ThPOK protein in MHCII-restricted CD4$^+$CD8$^-$HSA^{hi} thymocytes, which were present only in MHCII–sufficient mice and were eventually redirected to the CD8SP lineage (Fig. 3a, c). These findings suggest that *Runx3d*-YFP de-repression is an early indication of lineage redirection of ThPOK-deficient CD4SP "wannabe" cells to the CD8$^+$ T cell lineage.

Lineage redirection due to a hypomorphic ThPOK allele

Our data imply that ThPOK expression following MHCII-restricted positive selection prevents expression of *Runx3d*. However, our analysis of *ThPOK*-GFP expression showed that a small proportion of CD8$^+$ T cells also expressed ThPOK but differentiated into the cytotoxic lineage with normal *Runx3d*-YFP expression. This observation suggests that there is a threshold level of ThPOK expression that is required for helper T cell lineage commitment. To study the effect of reduced ThPOK quantities, we employed a hypomorphic *Zbtb7b* allele, in which the *Pgk-neo*^{r} selection cassette was left in intron 1 of the *Zbtb7b* locus (*Zbtb7b*^{FN} allele) (Supplementary Fig. 3, online). CD4$^+$CD8$^-$ mature thymocytes and peripheral T cells were still present in *Zbtb7b*$^{FN/-}$ mice even though ThPOK expression was reduced by 80% compared to control CD4$^+$ T cells as determined by q-RT-PCR, by ThPOK protein expression, and by GFP expression from a *Zbtb7b*$^{GFP-neo}$ allele (the *Zbtb7b*^{GFP} allele with the *Pgk-neo*^{r} cassette in the same context as in the *Zbtb7b*^{FN} allele) (Fig. 4a,b and Supplementary Fig. 3, online). As compared to *Zbtb7b*$^{+/+}$, *Zbtb7b*$^{FN/+}$, *Zbtb7b*$^{+/-}$, or *Zbtb7b*$^{FN/FN}$ mice, *Zbtb7b*$^{FN/-}$ mice had fewer CD4$^+$CD8$^-$ cells and more CD4$^-$CD8$^+$ cells in HSA$^{lo/-}$ mature thymocyte and peripheral T cell populations, which resulted in an inverted CD4/CD8 ratio (Fig. 4c). CD4$^-$CD8$^+$ mature thymocytes and peripheral T cells, as well as CD4SP cells, were present in *Zbtb7b*$^{FN/-}$*B2m*$^{-/-}$ mice, indicating that some MHCII-selected cells were redirected to the CD8SP lineage in the presence of an attenuated amount of ThPOK (Fig. 4c). In addition to cells expressing CD4 or CD8, we observed atypical populations of mature thymocytes and T cells with a CD4$^+$CD8$^+$ phenotype in *Zbtb7b*$^{FN/-}$ and *Zbtb7b*$^{FN/-}$*B2m*$^{-/-}$ mice, suggesting that a reduced amount of ThPOK during thymocyte development affected the CD4 versus CD8 lineage decision (Fig. 4c). These DP cells and a fraction of CD4$^-$CD8$^+$ T cells expressed GFP in *Zbtb7b*$^{FN/GFP}$ mice, consistent with their being MHCII-restricted (Supplementary Fig. 5a, online). These results suggest that during CD4SP thymocyte development a threshold amount of ThPOK expression is needed to mediate CD4SP thymocyte differentiation, and that when ThPOK amounts fall below this threshold, MHCII-restricted cells are diverted to a CD8 lineage fate.

Unstimulated peripheral CD4$^+$ T cells do not normally express Runx3 protein. However, peripheral CD4$^+$CD8$^-$ T cells from *Zbtb7b*$^{FN/-}$ mice constitutively expressed Runx3, and CD4$^+$CD8$^-$ T cells from *Zbtb7b*$^{FN/-}$*Runx3d*$^{YFP/+}$ mice expressed YFP (Fig. 4d and Supplementary Fig. 5b, online). Runx3 protein expression was higher in CD4$^+$CD8$^+$ cells, and GFP expression from the *Zbtb7b*$^{GFP-neo}$ allele, which reflects ThPOK mRNA expression from the *Zbtb7b*^{FN} allele, was inversely correlated with Runx3 protein expression (Fig. 4d and Supplementary Fig. 5, online). These results indicate that a high quantity of ThPOK is required to block Runx3 up-regulation and diversion of MHCII-restricted thymocytes to the CD8SP lineage.

CD4SP cells can be generated in the absence of ThPOK

Our data indicated a correlation between Runx3 de-repression and redirection of MHCII-restricted thymocytes to the cytotoxic lineage. To determine whether Runx complexes are required for lineage redirection occurring in the absence of ThPOK, we generated mice doubly deficient for ThPOK and CBFβ. We used the *Lck*-cre transgene to conditionally

inactivate *Cbfb* because its later inactivation in DP thymocytes with *Cd4*-cre allows generation of CD8^{+} mature thymocytes (which de-repress CD4)12, most likely due to long half-life of the CBFβ protein. Conditional inactivation of *Cbfb* with *Lck*-cre allows thymocytes to proceed through beta selection and phenocopies loss of both Runx1 and Runx3 at the DP stage. HSA^{hi}CD69^{+} and HSA^{hi}TCRβ^{hi} positively selected thymocytes were reduced by approximately 5-fold in *Lck*-cre^{+}*Cbfb*[F/F] mice and *Lck*-cre^{+}*Cbfb*[F/F]*Zbtb7b*[GFP/−] mice as compared to littermate control or ThPOK-deficient mice (Fig. 5a,b). Similar to our previous observation in *Runx1*[F/F]*Runx3*[F/F]*Cd4*-cre^{+} mice10, CD4^{−}CD8^{+} mature thymocytes with intact CD4 silencing, that had escaped Cre-mediated inactivation of *Cbfb*, were present in *Lck*-cre^{+}*Cbfb*[F/F] mice. Although almost all of the mature thymocytes were redirected to the CD8SP subset in *Lck*-cre^{+} *Cbfb*^{+/+}*Zbtb7b*[GFP/−] mice, a majority of the mature HSA^{lo} thymocytes in *Lck*-cre^{+} *Cbfb*[F/F]*Zbtb7b*[GFP/−] mice were CD4^{+}CD8^{−} and were not redirected to the CD8 lineage (Fig. 5a). We also observed a CD4^{+}CD8^{−} T cell population not redirected to the CD8 lineage in lymph nodes of *Lck*-cre^{+}*Cbfb*[F/F]*Zbtb7b*[GFP/−] mice (Fig. 5a). The number of CD4^{+}CD8^{−} thymocytes and peripheral T cells were reduced compared to wild-type mice but were similar in *Lck*-cre^{+}*Cbfb*[F/F]*Zbtb7b*[GFP/+] and *Lck*-cre[+]*Cbfb*[F/F]*Zbtb7b*[GFP/−] mice (Fig. 5b). We do not believe that these cells represent a rare population of unconventional CD4SP thymocytes, as iNKT cells do not develop in the absence of Runx complexes27 and Foxp3^{+} regulatory T cells are severely reduced in both CBFβ-deficient mice and ThPOK-deficient mice (unpublished results). In addition, CD40L (CD154) was up-regulated upon stimulation of CD4^{+} T cells lacking both transcription factors, consistent with their being helper lineage cells (Supplementary Fig. 6, online). Thus these findings indicate that Runx complexes are required for redirection of MHCII-restricted thymocytes towards the CD8SP lineage in the absence of ThPOK, and ThPOK is not required for CD4 expression in mature MHCII-restricted thymocytes and CD4^{+} peripheral T cells.

ThPOK represses genes specific for the CD8SP lineage

The finding of Runx3d de-repression in ThPOK-deficient MHCII-restricted thymocytes raises the possibility that other CD8^{+} lineage-specific genes may also be de-repressed in these cells. To assess this possibility, we compared gene expression between CD4^{+}CD8^{−}HSA^{hi}GFP^{+} thymocytes from *Zbtb7b*[GFP/−] and *Zbtb7b*[GFP/+] mice. qRT-PCR analysis showed that *Zbtb7b*[GFP/−] cells expressed significantly higher amounts of transcripts that are normally expressed in the CD8SP lineage of wild-type mice, including *Cd8a*, *Itgae*, *Nkg7*, *Cd160* and *Prf1* (Fig. 6a). In addition, unlike wild-type CD4^{+} T cells, a majority of *Zbtb7b*[FN/−] CD4^{+} T cells produced IFN-γ when stimulated with anti-CD3 and anti-CD28 in the absence IL-12 (Fig. 6b). Expression of the CD8 lineage-specific IFN-γ–regulating transcription factor Eomes was significantly elevated in *Zbtb7b*[FN/−] compared to wild-type CD4^{+} T cells (Fig. 6c). These findings suggest that a high amount of ThPOK expression is required during development of CD4^{+} T cells to block the CD8SP lineage-specific gene expression program.

Discussion

Despite extensive effort over the past two decades, the mechanism by which DP cells undergoing positive selection commit to either the CD4$^+$ or CD8$^+$ lineage is not yet understood. The discovery of the *hd* mouse, which harbors a mutation in the *Zbtb7b* gene that encodes ThPOK, clearly demonstrated that positive selection could occur independently of lineage commitment[26,28]. Subsequent studies have suggested that ThPOK serves as a "master regulator" that directs the differentiation of helper T cells[6,7]. This conclusion was based on findings that forced expression of ThPOK redirected MHCI-selected thymocytes to the CD4$^+$ lineage, and that MHCII-selected cells adopted the CD8$^+$ lineage fate in the absence of functional ThPOK. Furthermore, a defect in a Runx-dependent silencer within the *Zbtb7b* gene resulted in de-repression of ThPOK in DP cells and in redirection of MHCI-selected thymocytes[14]. Here we showed that ThPOK is up-regulated in positively selected CD4$^+$CD8^{lo} thymocytes before induction of Runx3 in cells destined to become CD8$^+$ T cells, that ThPOK represses expression of Runx3 in MHCII-selected cells transiting from the CD4$^+$CD8^{lo} to the CD4$^+$CD8$^-$ stage, and, intriguingly, that MHCII-selected thymocytes can become CD4SP cells even in the absence of ThPOK and, hence, may be specified to the helper lineage independently of ThPOK. These results argue that ThPOK may not be a "master regulator" but rather that it reinforces the CD4 lineage decision, at least in part by repressing *Runx3* and thus preventing cells from expressing CD8$^+$ lineage-specific genes.

Among the three Runx proteins, Runx3 is the only one specifically expressed in the CD8 lineage, where it is transcribed from its distal promoter. We previously demonstrated that conditional inactivation of *Runx3* resulted in de-repression of the *Cd4* gene in CD8 lineage T cells, but not in loss of CD8SP thymocytes or in redirection of MHCI-restricted thymocytes to the CD4SP lineage [10]. This is most likely explained by functional redundancy achieved upon compensatory up-regulation of Runx1, and, accordingly inactivation of both Runx1 and Runx3 in DP thymocytes resulted in complete loss of CD8 lineage cells and in lineage redirection[10,14]. The results thus imply that, in the absence of genetic manipulation, activation of the *Runx3* distal promoter is critical for initiation of CD8SP development. In the current study, we showed that ThPOK suppresses *Runx3d* expression in MHCII-selected cells. Following positive selection, a large proportion of MHCII-restricted thymocytes within the CD4$^+$CD8^{lo} population expressed the *ThPOK*-GFP reporter, but the *Runx3d*-YFP reporter was not expressed by MHCI-restricted thymocytes until they differentiated further. ThPOK-deficient MHCII-restricted thymocytes, in contrast, de-repressed *Runx3d* after they up-regulated *ThPOK*-GFP reporter expression in CD4$^+$CD8$^{lo/-}$ subsets. This observation suggests that CD4$^+$ T cell differentiation requires high ThPOK expression that is initiated prior to Runx3 up-regulation. In the presence of insufficient amounts of ThPOK, MHCII-restricted thymocytes fail to block Runx3 expression and are redirected to the CD8SP lineage. These findings suggest that *Runx3d* transcription can be activated regardless of MHC restriction but is normally blocked by ThPOK in a CD4$^+$ lineage-specific manner. Analysis of regulation of the *Runx3* distal promoter may clarify the potential cross regulation between these two transcription factors.

In mice lacking both ThPOK and Runx complexes, lineage redirection of MHCII-restricted CD4SP cells no longer occurred, suggesting that the CD4 lineage can be specified even in

the absence of ThPOK. Consistent with this interpretation, in preliminary studies we found that CD40L (CD154), which is expressed on the surface of wild-type activated CD4^{+} but not CD8^{+} T cells, was up-regulated in CD4^{+}CD8^{−} T cells lacking both ThPOK and CBFβ. This result appears at first to be inconsistent with observations of previous studies on lineage regulation by ThPOK and Runx. For example, de-repression of ThPOK in the absence of Runx complexes in DP cells resulted in diversion of MHCI-restricted cells to the CD4^{+} lineage14. However, this effect was not demonstrated to be dependent on ThPOK. Similar lineage diversion observed upon targeted deletion of the ThPOK silencer may have resulted in higher-than-normal amounts of ThPOK in MHCI-selected cells; this elevated ThPOK expression may thus have effectively phenocopied forced ThPOK expression in transgenic mice, and may have bypassed the transcriptional mechanisms that normally precede ThPOK up-regulation during CD4 lineage specification14.

Our results therefore suggest that signals initiated in DP thymocytes upon TCR interaction with peptide-loaded MHCII molecules specify differentiation to the CD4^{+} lineage prior to the activity of ThPOK, which is expressed in this lineage upon release of its silencing and, possibly, activation of lineage-restricted positive regulatory elements14. The signals and factors involved in specification of the CD4^{+} lineage and in induction of ThPOK remain to be defined. GATA3 another transcription factor known to be required for the differentiation of CD4^{+} lineage cells, is up-regulated earlier than ThPOK, at the CD69^{+}TCR^{lo} DP stage29. In the absence of GATA3, MHCII-selected thymocytes are arrested at the CD4^{+}CD8^{lo} stage30. GATA3 or other unknown CD4 specification factors may be required for ThPOK up-regulation, although a GATA3 consensus binding sequence in the *ThPOK* silencer appears to be dispensable15.

These findings are consistent with the notion that there is reciprocal regulation of ThPOK and Runx complexes. Runx1 and Runx3 are required for ThPOK silencing in DP cells, although their role in ThPOK regulation following positive selection remains unclear. Specific expression of Runx3 in the CD8SP lineage raises the possibility that ThPOK silencing is maintained by Runx3 in MHCI-restricted thymocytes, and that ThPOK silencing is 'released' in MHCII-selected thymocytes that do not express Runx3. However, as ThPOK up-regulation in MHCII-selected thymocytes occurs earlier than Runx3 up-regulation in MHCI-selected thymocytes, it is unlikely that Runx3 determines lineage-specific ThPOK expression. Alternatively, it is possible that Runx1 may modulate *ThPOK* silencer activity following positive selection signals either by acting alone or in cooperation with other CD4SP specifying factors, such as GATA3. Indeed it has been demonstrated that Runx and GATA factors potentially interact with each other and synergistically regulate blood cell lineage decisions in Drosophila31,32.

The roles of ThPOK and Runx factors in the helper versus cytotoxic T cell lineage decision appear to be asymmetric. Whereas ThPOK represses Runx3 expression following positive selection, the Runx proteins do not appear to prevent ThPOK expression except at the DP stage. In contrast to the effect of forced expression of ThPOK, transgenic overexpression of Runx proteins fails to redirect MHCII-specific cells to the CD8 lineage. Therefore, following induction of ThPOK in CD4 lineage thymocytes, its expression appears to become resistant to Runx-mediated repression. This may be due to epigenetic changes or to active

inhibition of the repressive effect of Runx complexes, which have been reported to remain associated with the *ThPOK* silencer even in CD4^{+} lineage cells[14].

ThPOK is likely to function not only to repress Runx3 expression, but also to repress other factors involved in cytotoxic lineage commitment or to insulate genes involved in CD4^{+} lineage commitment from the effects of Runx expression. Thus, in the absence of ThPOK, not only Runx proteins but also other ThPOK target genes are likely involved in the diversion to the CD8^{+} lineage. Following intrathymic differentiation, ThPOK and Runx3 protein can be co-expressed in activated peripheral CD4^{+} T cells under T_H1 polarizing conditions[12,13] (data not shown). The mechanism which permits Runx3 expression in mature cells in the presence of ThPOK is unknown, but may involve a dominant role for the transcription factor T-bet, which is required for Runx3 expression in T_H1 cells[13]. Effector helper T cell functions may depend on expression of both transcription factors.

The "kinetic signaling" model of CD4 vs. CD8 lineage choice[33,34] proposes that, during the post-positive selection CD4^{+}CD8^{lo} stage, extended signaling due to interaction of CD4 with MHCII results in helper lineage specification. Our results are potentially consistent with this model, and are the first to demonstrate that the CD4^{+}CD8^{lo} thymocyte subpopulation is heterogeneous with respect to expression of ThPOK. It is hence possible that only those cells with prolonged signaling up-regulate ThPOK. These may also be cells that preferentially up-regulate GATA3 in response to MHCII[29].

In summary, we have provided genetic evidence suggesting that ThPOK may not be essential for specification of the CD4SP lineage, while Runx complexes are required for lineage redirection in the absence of ThPOK. ThPOK plays an essential role to prevent commitment to the CTL lineage following MHCII-restricted selection, at least in part by inhibiting up-regulation of Runx3 from the distal promoter. Future studies are needed to identify factors necessary for CD4^{+} lineage specification prior to ThPOK induction, to determine if there is a basal differentiation state following positive selection by either MHCI or MHCII, and to elucidate the gene regulatory network governing thymocyte lineage diversification.

METHODS

Mice

Cbfb, *Runx3*, and *Runx1* conditional knockout mice were described previously[8,12]. *B2m*^{−/−} mice[35], *H2-Ab1*^{−/−} mice[36], *Cd4*-cre and *Lck*-cre transgenic mice[37] were purchased from Taconic Farms, and EIIa-cre transgenic mice[38] were from the Jackson Laboratory. For targeted insertion of YFP into the *Runx3* locus, genomic fragments for homologous arms were PCR amplified from a BAC clone (RP24-309N18) encompassing the *Runx3* locus. A targeting vector was constructed such that a 46 nt Runx3 coding sequence starting with an initiating codon from the distal promoter transcript was replaced with the YFP coding sequence. The *Not*I linearized targeting vector was electroporated into embryonic stem cells and G418 and gancyclovir double resistant colonies were screened by PCR for homologous recombination at the 3' end. Positive clones were validated by Southern blot with *Bam*HI digestion. Genomic fragments encompassing the *Zbtb7b* locus were excised from a BAC

clone (RP23-126P10) and cloned into pBluescript. For targeted deletion or conditional targeting of *Zbtb7b*, a targeting vector was constructed to flank exon 2 and exon 3, containing the entire coding sequence of *Zbtb7b*, with loxP sites. The neomycin resistance cassette was inserted approximately 1.1 kb upstream of exon 2 in a forward orientation with an additional loxP site at its 5' end. *Sac*II-linearized targeting vector was transfected into ES cells by electroporation. G418 and gancyclovir double resistant colonies were screened by Southern blotting. For generation of *Zbtb7b*^F/+ mice, *Zbtb7b*^FN/+ ES cells were transiently transfected by pMC-Cre to remove the neomycin resistance gene. The *Zbtb7b*^− allele was obtained by crossing *Zbtb7b*^FN/+ mice to EIIa-cre transgenic mice. All mice were maintained under specific pathogen free conditions in the Skirball Institute Animal Facility. All experiments were performed in accordance with the protocol approved by the IACUC at the NYU School of Medicine.

Flow cytometry

All monoclonal antibodies were purchased from eBioscience or BD Bioscience. Clone names for individual antibodies used in this study are as follows: anti-CD4 (RM4-5), anti-CD8α (53–6.7), anti-TCRβ (H57–597), anti-HSA (M1/69), anti-CD69 (H1.2F3), anti-CD103 (2E7), anti-CD154 (MR1). anti-IL-4 (11B11), anti-IFN-γ (XMG1.2). Single cell suspensions were stained with the antibodies and DAPI, and analyzed with a LSRII flow cytometer (BD Biosciences) equipped with 355 nm, 405 nm, 488 nm and 633 nm lasers. 510/20, 545/35, 495LP, and 525LP filters were used to separate GFP and YFP signals. Data were analyzed using Flowjo software (Tree Star). Cell sorting was performed with a FACS Aria (BD Biosciences). The purity of sorted samples was higher than 99%.

Real time PCR for gene expression analysis

Total RNA was prepared from sorted thymocytes or lymphocytes using Trizol (Invitrogen). cDNA was synthesized with Superscript reverse transcriptase (Invitrogen). Real time PCR analysis was performed as described previously[10]. Primers and Taqman probes for *Gata3* and *Zbtb7b* were described previously[6,39]. Other primer sequences are listed in Supplementary Table 1, online. Gene expression was examined using 3 independent samples and statistical difference was tested using two-tailed T test with unequal variance assumption. *P* values smaller than 0.05 were considered significant.

Immunoblotting

FACS purified cells were washed once with PBS and lysed in buffer containing 150 mM NaCl, 2 mM EDTA, 20mM Tris-HCl (pH7.5), 10% glycerol and 1% NP-40. The lysate was cleared by centrifugation, denatured in $1 \times$ Laemmli buffer and separated by SDS-PAGE. Anti-pan-Runx recognizing the C-terminus of Runx1 and Runx3 was described previously[10]. Anti-ThPOK was generated by immunizing rabbits with a recombinant GST-fusion protein containing amino acids 423–543 of ThPOK. Immunoblot was performed using crude serum. Anti-HMG1 was used to estimate loading.

T cell stimulation and intracellular staining

Purified CD4$^+$CD8$^-$CD25$^-$CD62L$^+$CD44^{lo} naïve T cells were stimulated with plate-bound anti-CD3 (145-2C11, 1 μg/ml) and anti-CD28 (37.5.1, 5 μg/ml) for 3 days in the presence of recombinant mouse IL-2 (20 U/ml) and in the absence of IL-12. The cells were restimulated with PMA and ionomycin for 4 hours in the presence of GolgiPlug (BD Biosciences), fixed with 2% paraformaldehyde, permeabilized with 0.3% saponin, and intracellularly stained for IFN-γ and IL-4 in the presence of 0.03% saponin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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a

c

b
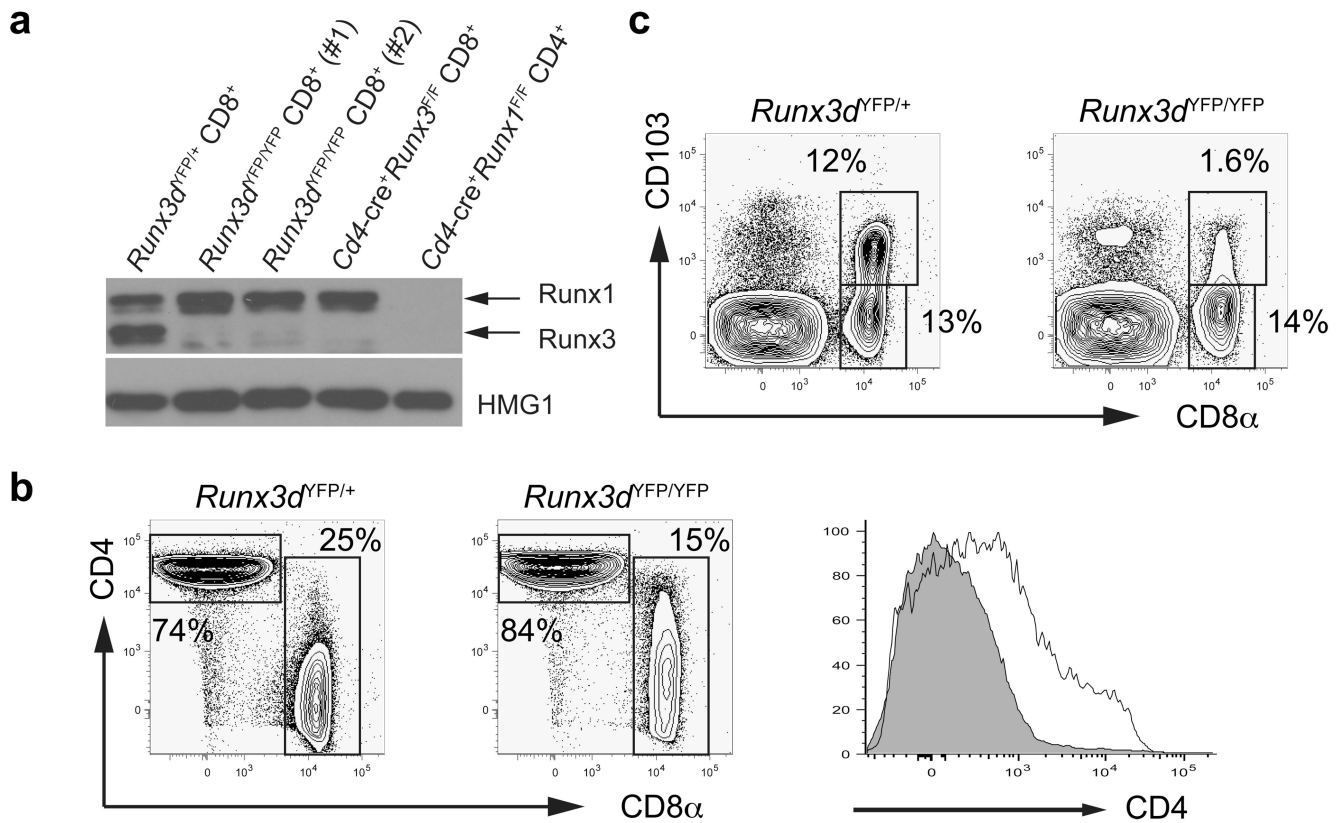
Figure 1. CD8SP lineage-specific Runx3 expression from its distal promoter is required for *Cd4* silencing and CD103 expression in CD8$^+$ T cells

(**a**) Runx3 and Runx1 protein expression in purified CD8$^+$ cells from *Runx3d*$^{YFP/YFP}$ and *Runx3d*$^{YFP/+}$ mice is shown with anti-HMG1 blot as loading control. Lysates from *Cd4*-cre$^+$*Runx1*$^{F/F}$ CD4$^+$ T cells and *Cd4*-cre$^+$*Runx3*$^{F/F}$ CD8$^+$ T cells were used as negative controls for Runx1 and Runx3 expression, respectively. (**b,c**) CD4 and CD8 (**b**) or CD8 and CD103 (**c**) expression in TCRβ$^+$ lymph node (LN) cells from *Runx3d*$^{YFP/YFP}$ and *Runx3d*$^{YFP/+}$ mice. In the right panel in (**b**), CD4 expression in CD8$^+$ T cells from *Runx3d*$^{YFP/YFP}$ (open histogram) and *Runx3d*$^{YFP/+}$ (shaded histogram) mice is shown. Mean fluorescent intensity: *Runx3d*$^{YFP/+}$ 308, *Runx3d*$^{YFP/YFP}$ 1767. Data shown are representative of more than 3 independent experiments.
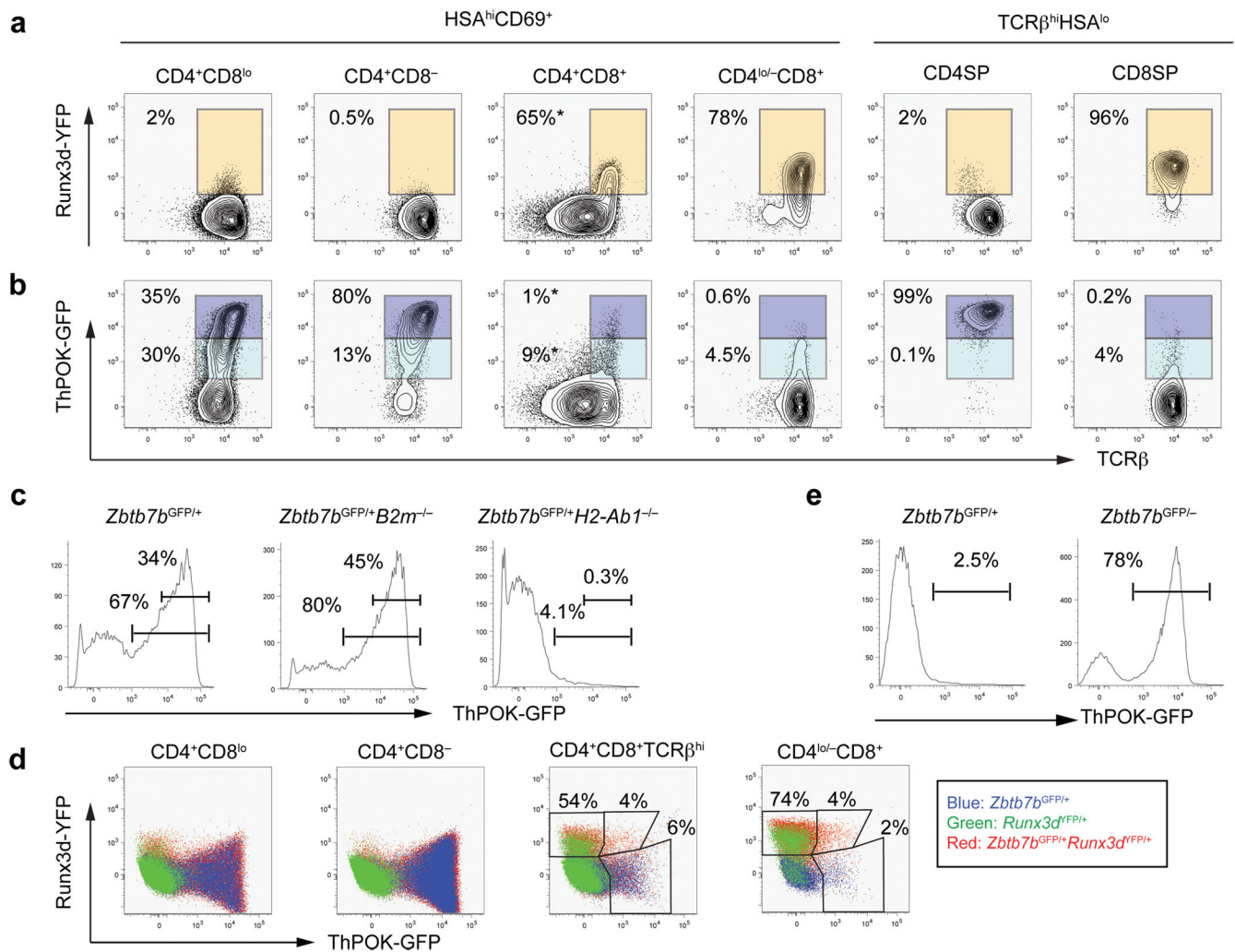
Figure 2. *Runx3d* and *ThPOK* reporter expression in developing thymocytes

(**a**) Distal promoter-derived Runx3 expression determined by the *Runx3d*-YFP reporter in thymocyte subpopulations as defined in Supplementary Fig. 2, online. YFP+ cells are gated with orange rectangles. (**b**) *ThPOK*-GFP reporter expression in developing thymocytes. GFP^{hi} and GFP^{lo} populations are gated with dark and light blue rectangles, respectively. Percentages indicate frequencies of YFP+, GFP^{hi}, and GFP^{lo} cells among thymocyte subsets marked at top, except for those in the CD4+CD8+ subpopulation, for which percentages correspond to frequencies of gated cells among CD4+CD8+TCRβ^{hi} thymocytes (marked with asterisks). (**c**) *ThPOK*-GFP reporter expression in MHCI- and MHCII-restricted CD4+CD8^{lo} thymocyte subpopulations. The percentages of total GFP+ cells (bottom gate) and GFP^{hi} cells (top gate) are shown. (**d**) Mutually exclusive high *ThPOK*-GFP and *Runx3d*-YFP expression during thymocyte differentiation. *ThPOK*-GFP and *Runx3d*-YFP expression in developing thymocytes from indicated mice. Numbers indicate percentages of cells within gates. (**e**) Continued *ThPOK*-GFP expression in redirected MHCII-restricted ThPOK-deficient CD8+ T cells. *ThPOK*-GFP expression in CD8+ T cells from *Zbtb7b*$^{GFP/+}$ or

Zbtb7b$^{GFP/-}$ mice. Numbers indicate percentages of GFP$^+$ cells. Data shown are representative of more than 3 independent experiments.

a

b

CD69+ HSAhi


c

HSAhi CD69+ gated thymocytes
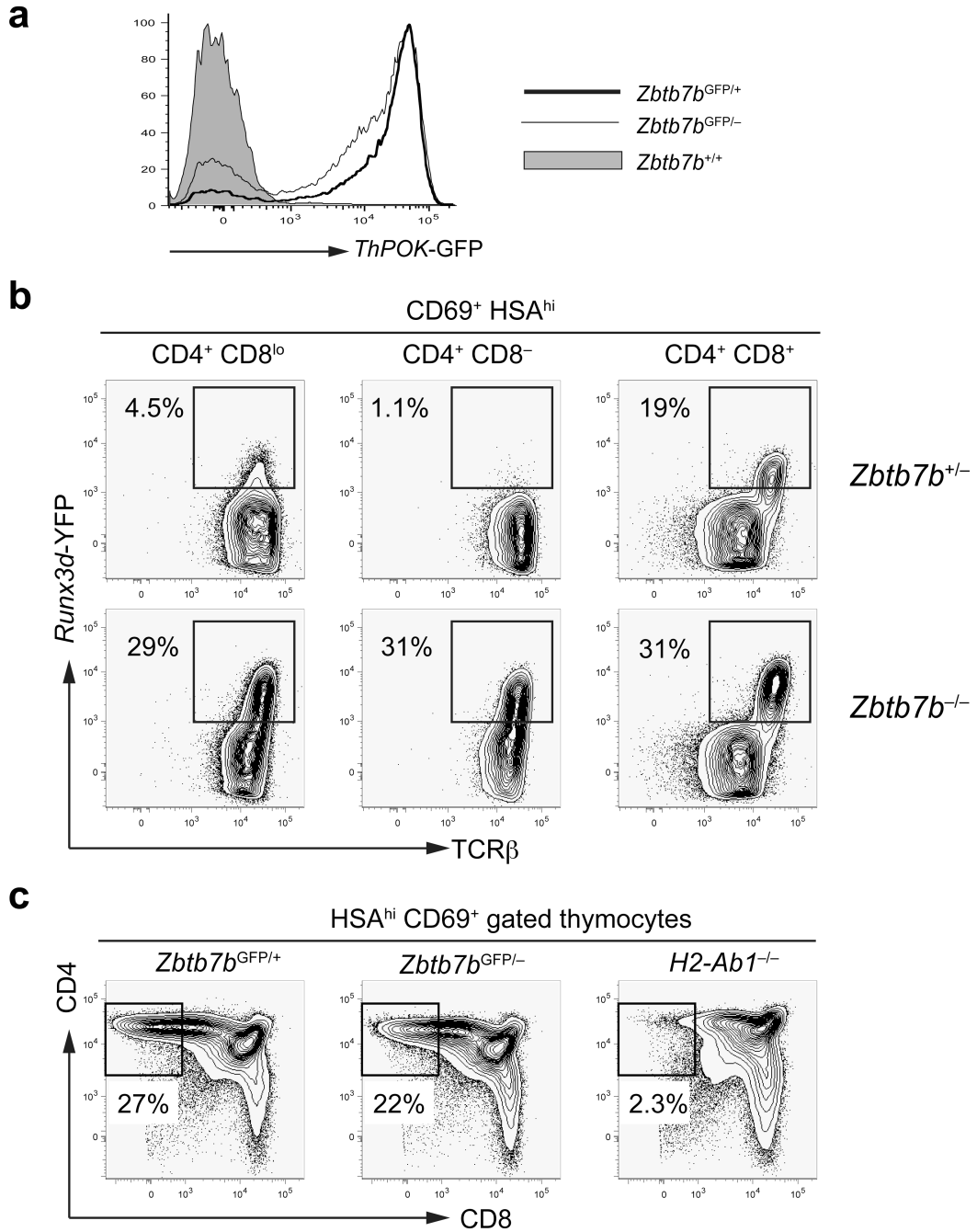
Figure 3. CD8+ lineage-specific *Runx3d*-YFP expression is de-repressed in ThPOK-deficient MHCII-restricted thymocytes

(**a**) ThPOK up-regulation independently of ThPOK following positive selection. *ThPOK*-GFP reporter expression in HSAhiCD69+CD4+CD8lo/− thymocytes from *Zbtb7b*+/+, *Zbtb7b*GFP/+ and *Zbtb7b*GFP/− mice. (**b**) *Runx3d*-YFP expression in thymocyte subpopulations in the presence or absence of ThPOK. YFP+ cells are gated as indicated by rectangles. (**c**) CD8 down-regulation following thymocyte positive selection in the absence of ThPOK. CD4 and CD8 expression in HSAhiCD69+ thymocytes from *Zbtb7b*GFP/+,

Zbtb7b[GFP/−] and *H2-Ab1*^{−/−} mice is shown. CD4^{+}CD8^{−} cells are gated as shown within rectangles and their frequencies are indicated. Data shown are representative of more than 3 independent experiments.
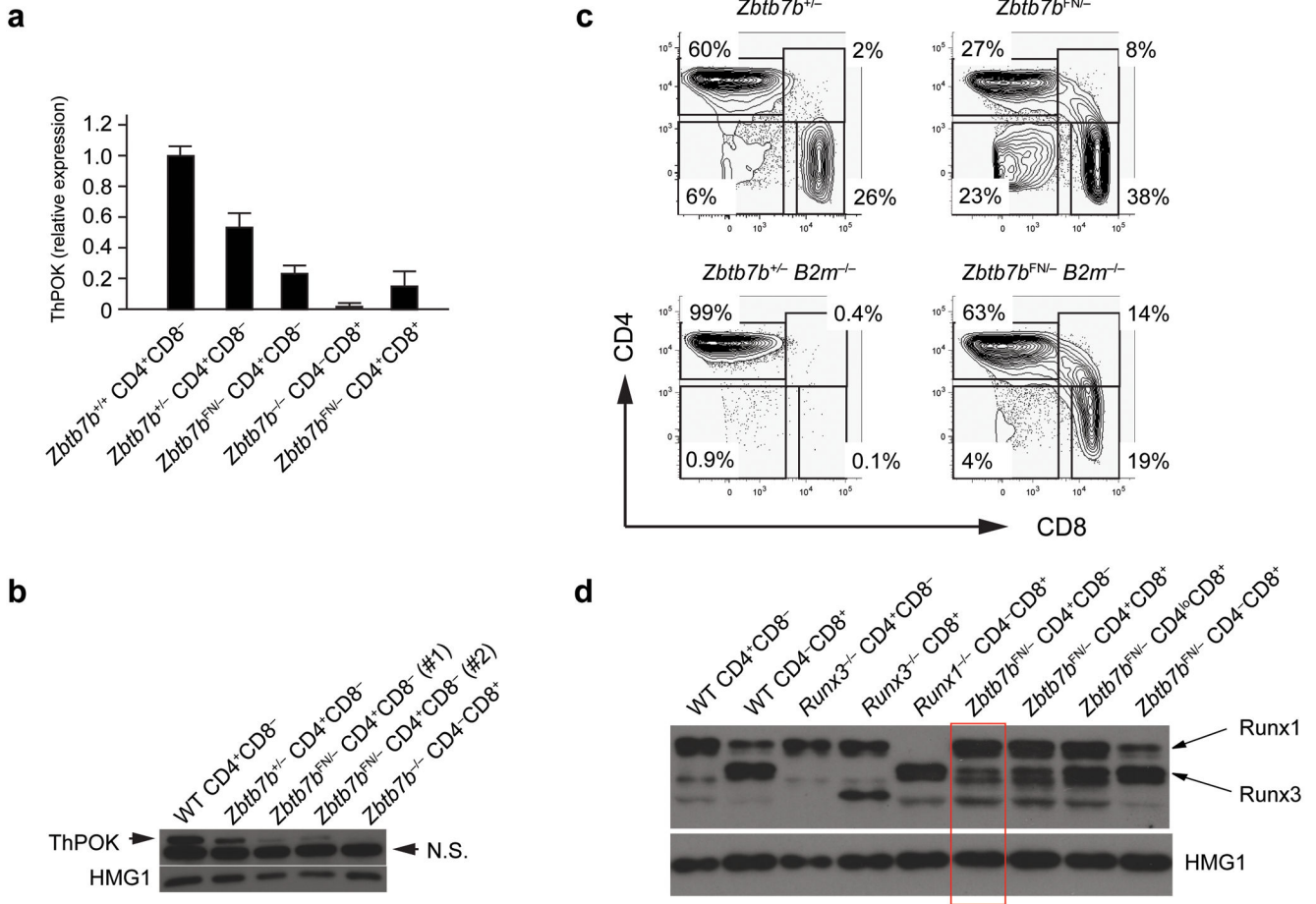
Figure 4. CD4$^+$ T cell differentiation in the presence of reduced amounts of ThPOK
(**a,b**) ThPOK expression in CD4$^+$CD8$^-$ T cells from *Zbtb7b*$^{FN/-}$ mice. ThPOK expression
was examined by qRT-PCR (**a**) and immunoblotting (**b**). qRT-PCR data are shown as
averages and standard deviations from three independent samples. (**c**) Lineage redirection of
MHCII-restricted T cells to the CD8 lineage in the presence of reduced amounts of ThPOK.
CD4 and CD8 expression in gated HSA$^{lo/-}$TCRβ^{hi} mature thymocytes from *Zbtb7b*$^{+/-}$,
Zbtb7b$^{FN/-}$, *Zbtb7b*$^{+/-}$*B2m*$^{-/-}$ and *Zbtb7b*$^{FN/-}$*B2m*$^{-/-}$ mice is shown. Percentages of
CD4SP, CD8SP, CD4$^+$CD8$^+$ and CD4$^-$CD8$^-$ cells are indicated. (**d**) De-repressed Runx3
protein expression in CD4$^+$CD8$^-$ T cells from *Zbtb7b*$^{FN/-}$ mice. Runx1 and Runx3
expression (indicated by arrows) was examined by immunoblotting with anti-pan-Runx.
Anti-HMG1 was used as loading control. Data shown are representative of more than 3
independent experiments.
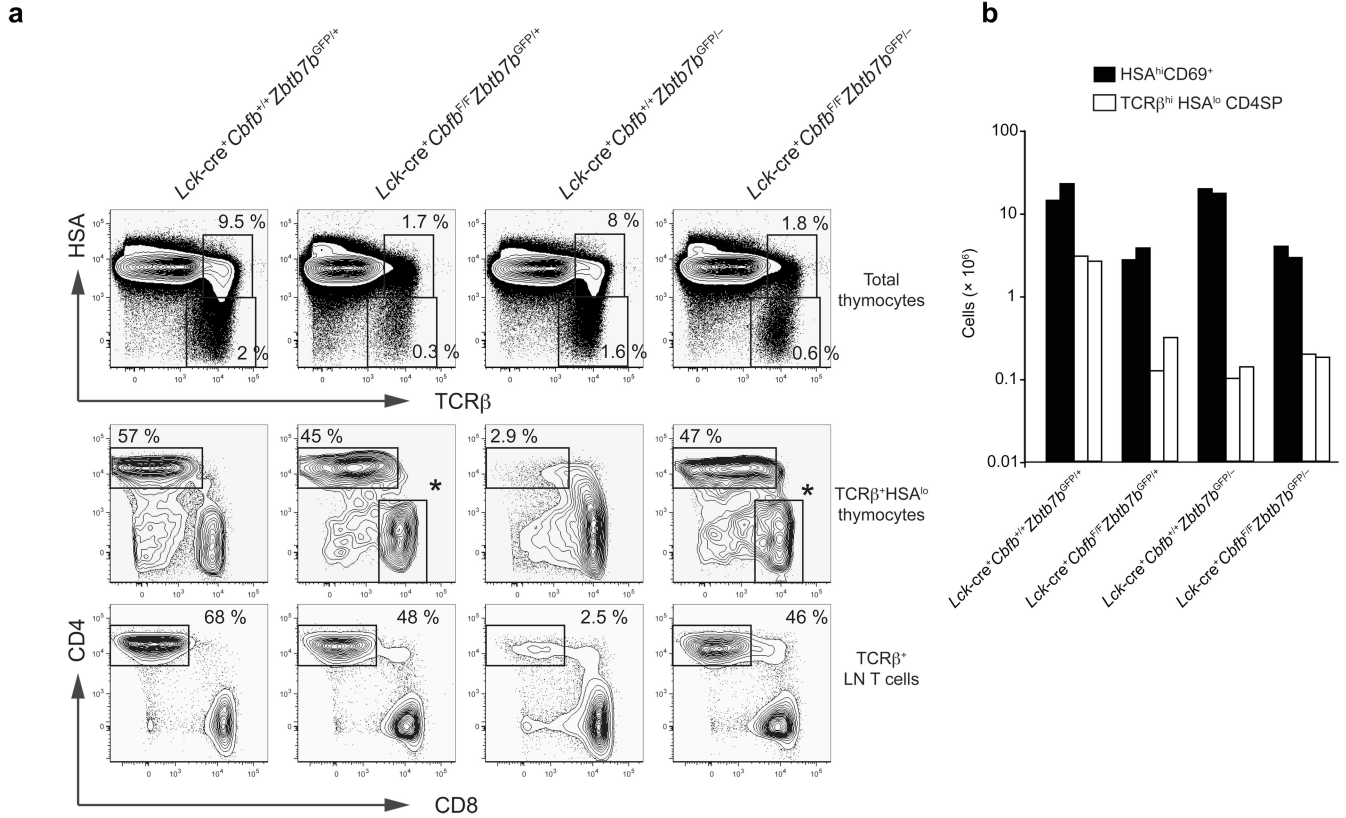
Figure 5. ThPOK is dispensable for differentiation of CD4SP thymocytes

(**a**) HSA and TCRβ expression in total thymocytes (top), and CD4 and CD8 expression in HSA$^{lo/-}$ TCRβ^{hi} mature thymocytes (middle) and TCRβ$^+$B220$^-$ peripheral T cells (bottom) in the absence of ThPOK, CBFβ, or both. The CD4$^-$CD8$^+$ cells shown with asterisks in *Lck*-cre$^+$*Cbfb*$^{F/F}$ and *Lck*-cre$^+$ *Cbfb*$^{F/F}$*Zbtb7b*$^{GFP/-}$ panels are those that escaped Cre-mediated inactivation of *Cbfb* and hence have normal *Cd4* silencing. Data shown here are representative from two independent experiments with similar results. (**b**) Absolute numbers of HSA$^+$CD69$^+$ positively selected thymocytes and HSA$^{lo/-}$ mature CD4SP thymocytes in the absence of ThPOK, CBFβ, or both. Each column shows cell numbers in a mouse with each of the genotypes.

a

b

c
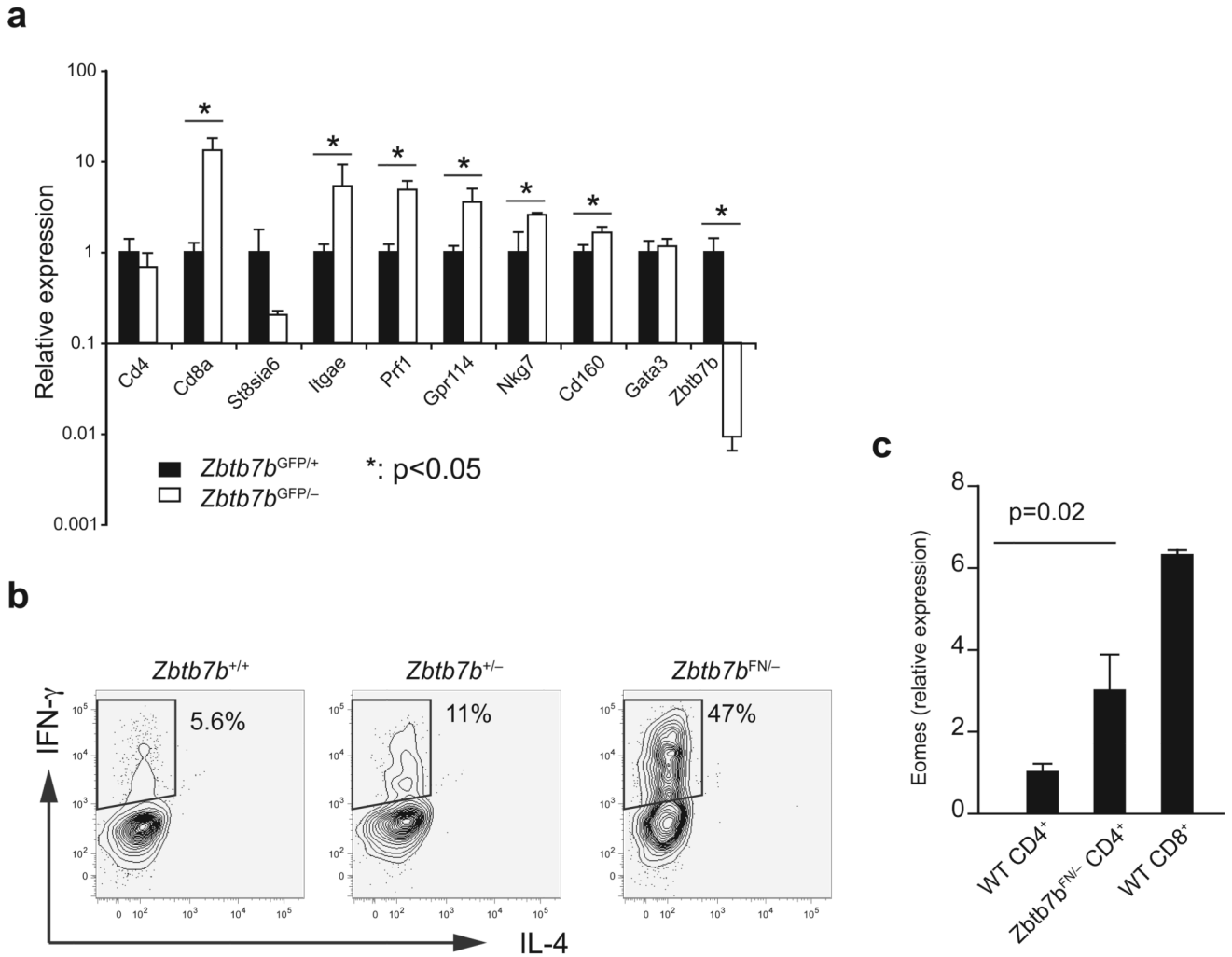
Figure 6. De-repression of CD8+ lineage-specific genes in MHCII-restricted cells in the absence of ThPOK or in the presence of insufficient amount of ThPOK

(**a**) MHCII-restricted HSA^{hi}CD69$^+$CD4$^+$CD8$^{lo/-}$ *ThPOK*-GFP^{hi} thymocytes from *Zbtb7b*$^{GFP/+}$ (black columns) or *Zbtb7b*$^{GFP/-}$ (white columns) were purified using gates as shown in Fig. 3, and expression of CD4$^+$ lineage-specific genes (*Cd4*, *St8sia6, Zbtb7b* and *Gata3*) and CD8$^+$ lineage-specific genes (*Cd8a*, *Itgae*, *Prf1*, *Gpr114*, *Nkg7* and *Cd160*) was examined by qRT-PCR. mRNA expression of individual genes was normalized against *Hprt1* expression and average expression in *Zbtb7b*$^{GFP/+}$ cells was set as 1. (**b**) Intracellular staining for IFN-γ and IL-4 in *Zbtb7b*$^{FN/-}$ CD4$^+$CD8$^-$ T cells following 3 days of stimulation with anti-CD3 and anti-CD28 in the absence of IL-12. (**c**) Expression of the CD8$^+$ lineage-specific IFN-γ regulator Eomes in CD4$^+$ T cells from wild-type and *Zbtb7b*$^{FN/-}$ mice and in wild-type CD8$^+$ T cells, as quantified by q-RT-PCR. *Hprt1*-normalized *Eomes* mRNA expression is shown as average and standard deviations from three independent samples. Statistical difference was tested by two-tailed T test with assumption of unequal variance. Genes that showed a *P* value smaller than 0.05 are marked with asterisks in (**a**). Actual *P* values for individual genes were as follows: *Cd4*: 0.28,

St8sia6: 0.13, *Zbtb7b*: 0.01, *Gata3*: 0.5, *Cd8a*: 0.04, *Itgae*: 0.04, *Prf1*: 0.01, *Gpr114*: 0.09, *Nkg7*: 0.04, *Cd160*: 0.02.