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TOX is required for development of the CD4 T cell lineage gene program

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Abstract

The factors that regulate thymic development of the CD4⁺ T cell gene program remain poorly defined. The transcriptional regulator ThPOK is a dominant factor in CD4⁺ T cell development, which functions primarily to repress the CD8 lineage fate. Previously, we showed that nuclear protein TOX is also required for murine CD4⁺ T cell development. Here we sought to investigate if the requirement for TOX was solely due to a role in ThPOK induction. In apparent support of this proposition, ThPOK upregulation and CD8 lineage repression were compromised in the absence of TOX, and enforced ThPOK expression could restore some CD4 development. However, these “rescued” CD4 cells were defective in many aspects of the CD4⁺ T cell gene program, including expression of *Id2*, *Foxo1*, and endogenous *Thpok*, among others. Thus, TOX is necessary to establish the CD4⁺ T cell lineage gene program, independent of its influence on ThPOK expression.

Introduction

Transition of a multi-potent precursor cell to a fully differentiated cell requires induction of a gene program that both enables specialized cell functions and represses alternative cell fates. This is the case for a key cell fate bifurcation of developing T lymphocytes in the thymus, where immature CD4⁺CD8⁺ (double positive, DP) thymocytes are induced by TCR binding to MHC class I or class II molecules to differentiate into either the CD8 or CD4 T cell lineage, respectively. Lineage-specific transcriptional regulators, including ThPOK and GATA3 for the CD4 lineage and Runx3 and MAZR for the CD8-lineage, play essential roles in this process (1-3).

Runx3 and its closely related family member Runx1 function to silence expression of the *Cd4* gene in CD8 lineage cells (one aspect of alternative fate repression in this instance) and early CD4⁺CD8⁺ thymocytes, respectively (4). Despite these differential *in vivo* functional activities, Runx1 can partially compensate for loss of Runx3, as CD8 T cell development is most efficiently blocked if both *Runx1* and *Runx3* genes, or their shared protein partner Cbfb, are disrupted (5, 6). Misdirection of MHC class I specific cells to the CD4 lineage in mice deficient in both Runx 1 and 3 is observed, also consistent with a role for Runx activity in alternative fate repression (5, 7). However, expression of a Runx3 transgene is not sufficient to redirect MHC class II-restricted cells into the CD8 lineage (8), suggesting either that additional factors may be needed to activate CD8 lineage specification and/or to inhibit CD4 commitment or that timing of expression of this factor is critical. Although expression

of MAZR is not lineage-specific it, like Runx3 (7), can bind the *Thpok* silencer, helping to enforce the CD8 cell fate in MHC class I specific developing thymocytes (3). In addition to its CD4 silencing activity, Runx3 is known to positively regulate other aspects of the CD8 lineage program, including regulating expression of CD8 itself (4, 9), thus fulfilling roles as both an alternative fate repressor and a positive cell fate regulator. Interestingly, this dual activity correlates with Runx-mediated induced proximity of the *Cd4* and *Cd8* loci (10). Runx1, unlike Runx3, is also expressed in developing CD4 lineage thymocytes where it plays a role in IL-7R expression and cell survival (5).

For CD4 T lineage development, expression of ThPOK (encoded by the *Zbtb7b* locus, referred to as *Thpok* here) is key. ThPOK is a zinc-finger BTB-POZ domain containing transcriptional regulator that when absent leads to misdirection of MHC class II-specific cells into the CD8 lineage, and when ectopically expressed can misdirect MHC class I-restricted cells into the CD4 lineage, demonstrating the pivotal role of this nuclear protein in the CD4/CD8 lineage decision (11-14). GATA3 is more highly expressed in CD4 than CD8 lineage thymocytes and is similarly required for CD4 but not CD8 lineage development (15, 16). However, unlike ThPOK, the absence or overexpression of GATA3 leads to inefficient lineage misdirection of developing thymocytes (15, 16). The upregulation of GATA3 precedes that of ThPOK, and in the absence of GATA3, ThPOK fails to be induced (17, 18). Given that GATA3 binds the *Thpok* locus it may play a direct role in ThPOK regulation, although overexpression of GATA3 is not sufficient to induce ThPOK expression in DP thymocytes (19).

Commitment to the CD4 and CD8 lineages requires that ThPOK and Runx3 be ultimately expressed in a mutually exclusive fashion. This, at least in part, appears to be achieved by the cross-inhibitory activities of these proteins; each can repress expression of the other (6, 7, 20, 21). Moreover, ThPOK can antagonize the *Cd4*-silencing activity of Runx3 (22) and inhibits juxtaposition of the *Cd4* and *Cd8* loci (10). The question then arises as to whether ThPOK, like Runx3 for the CD8 lineage, is also a positive regulator of the CD4 T cell fate. In the absence of both ThPOK and Runx activity, the latter achieved by conditional knockout of *Cbfb*, thymocytes develop into the CD4 lineage (6). This argues against an obligatory role for ThPOK as a positive regulator of the CD4 lineage. As noted, GATA3 has been implicated as a ThPOK regulator. However, the fact that ThPOK cannot restore CD4 T cell development in the absence of GATA3 (17) suggests other functions for GATA3, including the possibility of a role in induction of CD4 lineage gene expression and/or cell survival. Over- and ectopic-expression of transgene-encoded GATA3 induces many changes in gene expression in MHC class II-specific TCR transgenic thymocytes, including downregulation of RAG genes and modulation of various molecules that might influence TCR-mediated signaling (15, 23). However, whether GATA3 is also a direct inducer of the CD4 T cell lineage gene program remains uncertain.

Unlike ThPOK and GATA3, the pattern of expression of the HMG-box nuclear protein TOX is not CD4 lineage biased (24). Nevertheless, TOX is required for CD4 T cell development, mediating progression of thymocytes from the CD4^{lo}CD8^{lo} (double dull, DD) to CD4⁺CD8^{lo} stages (25). Despite the absence of CD4⁺CD8^{lo} transitional cells in mice that lack TOX, CD8 T cell development is only modestly inhibited (25). In contrast to loss of ThPOK, however, there is little if any misdirection of MHC class II specific T cells into the CD8 lineage in the absence of TOX (25). Here we sought to investigate if the impaired development of CD4 T cells in TOX-deficient cells was solely due to an inability to induce ThPOK, or alternatively, if TOX might also be one aspect of the regulatory network required for establishment of CD4 lineage gene expression. To study this, we expressed a ThPOK transgene in TOX-deficient (*Tox*^{-/-}) thymocytes. While expression of ThPOK was sufficient to efficiently inhibit the CD8 lineage cell fate, thymic induction of many CD4 T

cell lineage genes, including *Thpok*, *Cd4*, *Id2*, and *Foxo1* as well as downstream gene targets remained impaired. These data implicate TOX as a key factor in establishment of the CD4 lineage gene program.

Materials and Methods

Mice

All mice were bred at the Cedars-Sinai Medical Center and kept under specific pathogen-free conditions. AND TCR-transgenic (Tg) (26), *Tox*^{-/-} (25), and ThPOK-Tg (13) mice were described previously. AND-Tg *Tox*^{-/-} mice were kept on a MHC^{b/b} genetic background and screened for the absence of mouse mammary tumor virus 3 and 13. All mouse studies have been reviewed and approved by the CSMC Institutional Animal Care and Use Committee.

Antibodies and flow cytometry

All antibodies used for flow cytometry were purchased from eBioscience or BD Biosciences. Anti-TOX (24) and anti-Runx (27) antibodies were described previously and anti-FOXO1 antibody was purchased from Cell Signaling Technologies. GATA3 intracellular staining was performed as per the manufacturer's recommendation (clone L50-823, BD Biosciences).

Activation of cells

CD4-prestained thymocytes or total splenocytes were stimulated with PMA (0.015 or 50 ng/ml, respectively) and ionomycin (0.3 or 500 ng/ml, respectively) *in vitro* for 4 hours and expression of CD154 or CD69 was assessed by flow cytometry.

Immunofluorescence

5 µm frozen sections from thymus were stained as indicated (along with DAPI for visualization of nuclei) and analyzed on a Leica TCS SP spectral confocal microscope using CytoView software (Infinicyte).

PCR

Real-time quantitative RT-PCR analysis was performed using the standard curve method, where samples were normalized based on *Gapdh* expression (and for enriched splenic samples, population purity as well) and analyzed using SDS 2.1 software (Applied Biosystems). Each gene expression determination was performed in at least duplicate, and at least two independent biological experiments were performed. Primers for real-time RT-PCR were purchased from QIAGEN, with the exception of the *Thpok* 3'-UTR pair: 5'-CCCTTCCGAGACCCCTCCTTCCTGGT and 5'-CAATCAGGCCACCCTGCCCTCCTCTT.

Statistics

The probability (*P*) associated with a Student's t-Test using a two-tailed distribution of equal variance is shown in some Figures.

Results

Impaired induction of ThPOK and poor CD8 lineage repression in TOX-deficient CD4 T cells

Although spleen cellularity is normal in *Tox*^{-/-} mice, the T cell developmental defect in these animals results in T cell lymphopenia, primarily due to loss of CD4⁺ T cells ((25) and

Fig. 1A). Despite this, TCR⁺CD4⁺ T cells can be detected in the spleen of these mice with age (Fig. 1A). In addition, we noted the presence of splenic CD4⁺CD8^{int} T cells in *Tox*^{-/-} mice that were not present in spleens from wildtype animals (Fig. 1A). Gene expression analysis in these subsets revealed that *Tox*^{-/-} CD4⁺ and CD4⁺CD8^{int} T cells express lower amounts of *Thpok* mRNA when compared to wildtype CD4⁺ T cells (Fig. 1B). Like their wildtype counterparts, CD8⁺ *Tox*^{-/-} T cells do not express *Thpok* (Fig. 1B). We also analyzed expression of the integrin CD103 on peripheral T cells. CD103 is normally expressed on approximately 30% of splenic CD8⁺ T cells, but few CD4⁺ T cells (28, 29) and Fig. 1C). However, in the absence of TOX, CD103 was expressed not only by CD8⁺ T cells, but by a similar proportion of CD4⁺ T cells; ~40% of CD4⁺CD8^{int} T cells and ~30% of CD4⁺CD8⁻ T cells expressed CD103 in these mice (Fig. 1C). The induction of CD103 on CD8 lineage thymocytes is regulated by Runx3 (30). As ThPOK has been shown to negatively regulate Runx3, the aberrant expression of the CD8 marker CD103 on the surface of *Tox*^{-/-} CD4⁺ T cells may be due to the impaired expression of ThPOK (and see below).

To determine if we could detect *Thpok* expression under conditions where there was extensive generation of CD4 lineage T cells, we crossed *Tox*^{-/-} mice with mice expressing the MHC class II-specific AND TCR (AND-Tg)(26). CD4⁺8⁻ (CD4SP) thymocytes failed to develop in AND-Tg *Tox*^{-/-} (Fig. 1D). The fact that few CD4⁺8⁺ (CD8SP) thymocytes develop in AND-Tg *Tox*^{-/-} mice is consistent with the lack of extensive lineage misdirection in *Tox*^{-/-} mice on an otherwise wildtype background (25). To verify that TOX-deficiency did not inhibit the initiation of positive selection in these animals, we analyzed expression of TCR and CD5. Both TCR and CD5 are expressed at low levels on DP thymocytes and are upregulated during initiation of positive selection as a consequence of TCR signaling ((31) and Fig. 1E). In AND-Tg thymocytes, however, CD5 is upregulated on most thymocytes, including both DP and CD4SP cells, due to global positive selection that results from uniform and early expression of the AND TCR (Fig. 1E). In AND-Tg *Tox*^{-/-} mice, the majority of cells also exhibited upregulated levels of CD5, although development of TCR^{hi} CD4SP cells was still impaired (Fig. 1E).

The differential effect of loss of GATA3 in development of the CD4 and CD8 T cell lineages is mirrored by the expression pattern of the protein, as GATA3 is more highly expressed in CD4SP than CD8SP thymocytes (15, 16). *Gata3* gene expression was indistinguishable in total thymocytes from *Tox*^{-/-} mice when compared to thymocytes from *Tox*^{+/+} or *Tox*^{+/-} mice (Fig. 1F). Thus, as observed using CD5 as a marker, the majority of DP thymocytes in ANDTg mice have received initial positive selection signals independent of TOX expression. *Gata3* and *Tox* are therefore independently regulated to a significant degree. While upregulation of *Tox* is initiated by TCR-mediated calcineurin signaling during positive selection (32), the regulation of GATA3 may instead be at least partially dependent on MAPK (15, 33). However, despite induction of GATA3, AND-Tg *Tox*^{-/-} thymocytes did not express *Thpok* (Fig. 1G). Thus, ThPOK fails to be induced in AND-Tg mice in the absence of TOX, despite initiation of positive selection and upregulation of GATA3.

ThPOK can repress the CD8 lineage independently of TOX, leading to CD4⁺ T cell development

These data raised the possibility that the failure to upregulate ThPOK was critical for TOX-dependent CD4 T cell development. To test this, we bred transgenic mice that express ThPOK (ThPOK-Tg) onto a *Tox*^{-/-} background. Analysis of surface expression of TCR and CD5 suggested that ThPOK-Tg *Tox*^{-/-} cells displayed a post-positive selection block identical to that observed in the thymus of *Tox*^{-/-} mice (Fig. 2A and (25)). Thus, while initiation of positive selection as assessed by presence of a CD5^{hi}TCR^{int} population was evident in both *Tox*^{-/-} and ThPOK-Tg *Tox*^{-/-} mice, CD5^{hi}TCR^{hi} thymocytes were markedly reduced in mice of either genotype.

Subset analysis based on coreceptor expression, however, presented a very different picture of the effect of enforced ThPOK expression. CD8SP thymocytes fail to develop in ThPOK-Tg mice as a result of misdirection of all positively selected thymocytes to the CD4 T cell lineage (13, 14). Similarly, CD4SP but not CD8SP thymocytes are present in ThPOK-Tg *Tox*^{+/-} mice (Fig. 2B, 2C). Although the frequency and number of DP thymocytes is not diminished in *Tox*^{-/-} mice, CD4SP thymocytes fail to develop and there is expansion of the post-selection DD subpopulation in these animals (Fig. 2B and (25)). This is in contrast to TCR⁺ CD8SP thymocytes, which develop in the absence of TOX (Fig. 2B and (25)). In *Tox*^{-/-} mice that also expressed the ThPOK transgene, however, TCR⁺ CD8SP cells were eliminated (Fig. 2B, 2C). In addition, although ThPOK-Tg *Tox*^{-/-} mice lacked CD4SP thymocytes like their *Tox*^{-/-} counterparts, a subset of cells that downregulated CD8 but failed to fully upregulate CD4 (CD4^{lo}SP) was present instead (Fig. 2B, 2C). Despite their unusual phenotype, CD4^{lo}SP thymocytes from ThPOK-Tg *Tox*^{-/-} mice expressed CD5 and GATA3 equivalently to that observed in CD4SP thymocytes from ThPOK-Tg *Tox*^{+/-} mice (Fig. 2D, 2E), although expression of TCR and CD69 was lower and similar to that of DD thymocytes (Fig. 2D and data not shown).

Enforced expression of ThPOK resulted in the return to a normal lineage-restricted expression pattern of CD103 in *Tox*^{-/-} mice, as ThPOK-Tg *Tox*^{+/-} CD4SP and *Tox*^{-/-} CD4^{lo}SP cells did not express CD103 (Fig. 3A). *Tox*^{-/-} CD8SP, and to a significantly lesser degree DD, thymocytes express Runx3 protein (25). However, like their *Tox*^{+/-} counterparts, DP and CD4^{lo}SP ThPOK-Tg *Tox*^{-/-} thymocytes do not express Runx3 (Fig. 3B).

Enforced expression of ThPOK resulted in normal splenic T cell numbers (Fig. 3C, 3E) but loss of the CD8 T cell subset due to shunting of these cells into the CD4 T cell lineage during thymic development (Fig. 3D, 3F). ThPOK-Tg *Tox*^{-/-} mice, in contrast, display T cell lymphopenia similar to that seen in *Tox*^{-/-} mice (Fig. 3D, 3E), although splenic CD8⁺ T cells were replaced by CD4⁺ T cells (Fig. 3D, 3F). As noted above, an abnormal subset of CD4⁺CD8^{int} T cells was found in *Tox*^{-/-} mice (Fig. 1A, Fig. 3D, 3G). These cells were diminished when ThPOK was also expressed (Fig. 3D, 3G). As expected, expression of *Prfl* was specific to the CD8 T cell lineage in wildtype mice, and CD4⁺ T cells from ThPOK-Tg *Tox*^{+/-} mice also failed to express this CD8 lineage marker (Fig. 3H). Expression of *Prfl* was also repressed in CD4⁺ T cells from ThPOK-Tg *Tox*^{-/-} mice (Fig. 3H). Together, these data demonstrate that repression of the CD8 lineage is ThPOK dependent but TOX independent.

ThPOK cannot induce the CD4 lineage program in the absence of TOX

To determine if the CD4 lineage program was induced in ThPOK-Tg *Tox*^{-/-} CD4⁺ T cells, we surveyed some hallmarks of CD4 lineage commitment, including expression of the coreceptor itself. The DD phenotype is a reflection of coreceptor gene expression. Thus, the *Cd4* gene is poorly expressed by *Tox*^{-/-} DD thymocytes, when compared to DP thymocytes (Fig. 4A). However, despite expression of ThPOK, ThPOK-Tg *Tox*^{-/-} CD4^{lo}SP cells do not reactivate transcription of the *Cd4* gene to normal levels (Fig. 4B). This is consistent with the TOX-dependence of the DD to CD4⁺8^{lo} transition (25), a developmental progression that normally precedes ThPOK expression (13, 14).

One defining marker of the CD4 lineage is expression of ThPOK itself. To analyze this in ThPOK-Tg mice, we utilized a primer pair specific for the 3'UTR of *Thpok*, a region of the gene not included in the transgene. CD4SP thymocytes and CD4⁺ T cells in both *Tox*^{+/-} and ThPOK-Tg mice expressed endogenous *Thpok* (Fig. 4C, 4D). Expression of transgene-encoded ThPOK in the latter animals, therefore, does not inhibit endogenous *Thpok* locus activation during CD4 lineage commitment. Indeed, expression of ThPOK may inhibit its

own silencing in a positive feedback loop (21). In sharp contrast, however, ThPOK-Tg *Tox*^{-/-} CD4^{lo}SP thymocytes and CD4⁺ T cells poorly expressed endogenous *Thpok*, despite expression of transgene-encoded ThPOK in the cell (Fig. 4C, 4D).

Positive selection in the thymus requires a decrease in E protein transcriptional activity. This is regulated by upregulation of Id (inhibitor of DNA binding) proteins that lack DNA binding capability and form non-functional dimers with E proteins, resulting in dominant negative inhibition of E protein activity. Loss of Id3 partially inhibits development of both CD4 and CD8 SP thymocytes (34), while direct loss of E protein expression, via dual conditional deficiency in HEB and E2A, promotes some changes in gene regulation shared with positive selection, even in the absence of TCR signaling (35). Like Id3, Id2 is also upregulated during positive selection (data not shown), although positive selection is not inhibited in Id2-deficient mice (36, 37). To address whether loss of TOX altered the normal induction of Id proteins, we compared CD4SP and CD4^{lo}SP thymocytes from ThPOK-Tg *Tox*^{+/-} and ThPOK-Tg *Tox*^{-/-} mice, respectively. In ThPOK-Tg *Tox*^{-/-} CD4^{lo}SP thymocytes, expression of *Id2* was significantly decreased in the absence of TOX (Fig. 4E), while no significant change in *Id3* was detected (Fig. 4F).

A reduction in *Id2* expression would be predicted to result in failure to fully downregulate E protein activity. As a consequence, one might expect loss of expression of genes that are repressed by E proteins and induced by the decrease in E protein activity during positive selection. To determine if this was the case, we took advantage of gene expression data from analysis of doubly deficient E2A and HEB mice (35). We noted that expression of the Tescalcin gene (*Tesc*), encoding an EF-hand calcium binding protein implicated as a regulator of plasma membrane-type Na⁺/H⁺ exchangers (38), cell differentiation programs (39), and possibly calcineurin activity (40) was highly upregulated in DP thymocytes upon loss of E protein activity. Moreover, we found that *Tesc* was highly upregulated at the CD4^{8lo} transitional stage of positive selection (data not shown). Expression of *Tesc* was reduced in CD4^{lo}SP thymocytes from ThPOK-Tg *Tox*^{-/-} mice when compared to CD4SP thymocytes from ThPOK-Tg *Tox*^{+/-} mice (Fig 4G). Together, these results are consistent with a failure to fully repress E protein activity.

In order to extend these results to another gene target for which there was better functional characterization in the context of CD4 T cells, we analyzed expression of *Foxo1*, also upregulated upon loss of E proteins in DP thymocytes (35). FOXO1 is a transcriptional regulator that is induced during positive selection (41) and plays a key role in regulating cell migration and maintaining quiescence of mature T cells (42, 43). FOXO1 was significantly decreased at the level of both mRNA (Fig. 5A) and protein (Fig. 5B, 5C) in ThPOK-Tg *Tox*^{-/-} CD4^{lo}SP thymocytes in comparison to ThPOK-Tg *Tox*^{+/-} CD4SP thymocytes. One target of FOXO1 is *Klf2* (44). Consistent with reduction in FOXO1, *Klf2* failed to be fully expressed in the absence of TOX when these same cell populations were compared (Fig. 5D). FOXO1 and KLF2 have many gene targets including *Slpr1*, *Il7r*, *Ccr7* and *Sell* (encoding L-selectin). Indeed, *Slpr1* was significantly decreased in ThPOK-Tg *Tox*^{-/-} CD4^{lo}SP thymocytes in comparison to ThPOK-Tg *Tox*^{+/-} CD4SP thymocytes (Fig. 5E). In addition, surface expression of CD127 (IL-7R α), CCR7 and CD62L were all decreased in the absence of TOX (Fig. 5F). ThPOK-Tg *Tox*^{-/-} CD4^{lo}SP thymocytes were also smaller than ThPOK-Tg *Tox*^{+/-} CD4SP thymocytes (Fig. 5G) and more similar in size to DD thymocytes (not shown), suggesting a differentiation or maturation defect.

We also investigated CD4 lineage functional maturation by assessing CD40L (CD154) induction. Although activated ThPOK-Tg CD4SP thymocytes upregulated CD154 in response to PMA and ionomycin, their CD4^{lo}SP *Tox*^{-/-} counterparts did not (Fig. 5H). Splenic CD4⁺ T cells in ThPOK-Tg *Tox*^{-/-} mice also had a defect in induction of CD154

upon activation (Fig. 5I, 5K). However, this was not due to loss of a downstream signaling response, as the MAPK-dependent activation marker CD69 was upregulated upon activation even in the absence of TOX (Fig. 5J, 5L). A higher frequency of ThPOK-Tg *Tox*^{-/-} CD4⁺ T cells were CD44^{hi} (Fig. 5N) and expressed CD69 (Fig. 5J, 5L) (also CD44^{hi}, data not shown) in the absence of stimulation when compared to ThPOK-Tg *Tox*^{+/-} CD4⁺ T cells (Fig. 5J, 5L). Furthermore, there was a reduction in *Foxo1* expression (Fig. 5M) when compared to CD4⁺ T cells from ThPOK-Tg *Tox*^{+/-} mice, mirroring effects in the thymus. Together, these markers indicated that a significant proportion of CD4 T cells in ThPOK-Tg *Tox*^{-/-} mice were in an activated state. Upregulated expression of both CD44 and CD69 on naïve CD4⁺ T cells has also been reported in mice lacking FOXO1 (42). Thus, the reduction in FOXO1 in the absence of TOX is likely causative of the activated phenotype.

The decrease in expression of FOXO1 gene targets included factors responsible for thymocyte survival and migration. We had previously demonstrated that loss of TOX is not associated with increased apoptosis in the thymus (25). Nevertheless, since *bcl2* is a downstream gene target of IL-7R signaling in the thymus (45), and there was diminished expression of IL-7R in the absence of TOX, we asked whether expression of a *bcl2* transgene might overcome the loss of TOX. Expression of BCL2 did not rescue the CD4 T cell developmental defect of *Tox*^{-/-} mice, although it did result in increased frequencies of DD and CD8SP subsets (Fig. 6A and (25)). Consistent with the latter thymic phenotype, expression of BCL2 rescued the splenic T cell lymphopenia of *Tox*^{-/-} mice by increasing the frequency of CD8⁺ T cells (Fig. 6B). Although expression of CCR7, which functions in migration of single positive thymocytes from the cortex into the medulla, and S1PR1, required for thymic egress, were impaired in ThPOK-Tg *Tox*^{-/-} mice, CD4⁺ T cells were still able to populate the spleen, although in reduced numbers (Fig. 5D, 5F). Impaired but not absent CD4 T cell maturation in these animals was also reflected by small medullary regions that nonetheless contained CD4SP thymocytes (Fig. 7).

In sum, despite CD8 lineage repression and the development of CD4⁺ T cells in ThPOK-Tg *Tox*^{-/-} mice, induction of the full CD4⁺ T lineage gene program, including those encoding proteins involved in cell differentiation, maturation and function, failed to be established in the absence of TOX.

Discussion

We had previously found that CD4 T cell development is severely impaired in the absence of TOX (25), a nuclear protein predicted to be a structure-dependent but sequence-independent DNA binding factor (46). The minor CD4 T cell population that escapes the thymus in the absence of TOX had lower than normal expression of *Thpok*, supporting a role for TOX in regulation of ThPOK expression. In addition, a population of splenic CD4⁺8^{int} T cells was present in *Tox*^{-/-} mice, and the normal lineage bias of CD103 expression was eliminated. CD4⁺8^{int} cells have also been reported in mice that express a hypomorphic allele of *Thpok* (6, 21), consistent with presence of these cells in *Tox*^{-/-} mice due to misregulation of ThPOK during thymic development. Indeed, CD8 repression in peripheral CD4⁺ T cells has been demonstrated to require continued expression of ThPOK (6, 47).

Whether regulation of ThPOK by TOX is a direct or indirect effect remains to be determined. In terms of the latter, it is possible that TOX influences TCR signaling, likely needed for ThPOK induction (14). Although we have not observed any gross perturbations in TCR-mediated signaling in *Tox*^{-/-} thymocytes (25), reduced expression of CD4 and the TCR could be a factor. Alternatively, TOX could act independently on the ThPOK locus or renders the locus more accessible to GATA3 (17) and/or other factors. Indeed, the fact that TOX is also expressed in cells developing to the CD8 lineage indicates that cell context and

additional inputs likely modify the effects of TOX expression. Enforced expression of ThPOK eliminates expression of CD8, CD103 and *Prfl* by *Tox*^{-/-} CD4⁺ T cells. ThPOK also repressed expression of Runx3 even in the absence of TOX, likely preventing CD103 expression (30). ThPOK can inhibit its own silencing, possibly by inhibition of Runx3, thus initiating a positive feedback loop (21). Nevertheless, ThPOK and the lack of Runx3 expression were not sufficient to fully activate the *Thpok* locus in the absence of TOX. We conclude from these data that TOX plays a role in alternative fate repression solely as a modulator of ThPOK induction.

CD4⁺ T cells can develop even in the absence of ThPOK as long as the activity of Runx factors is also eliminated (6). This observation suggests that additional factors likely play a positive role in development of a CD4 lineage program. Our data suggest that TOX is one such factor. CD4⁺ T cells replaced CD8⁺ T cells in *Tox*^{-/-} mice when ThPOK was expressed. Despite this, CD4 lineage development in the thymus was compromised in these animals, including failure to fully re-express CD4 and poor induction of CD154 on SP thymocytes and CD4 T cells. The former observation is consistent with the TOX-dependence (25) but ThPOK independence (6, 12) of the DD to CD4⁺CD8^{lo} transition. Thus, one aspect of the requirement for TOX in mediating the development of CD4⁺CD8^{lo} cells may be regulation of the *Cd4* locus itself.

Despite expression of CD4 on both DP thymocytes and CD4SP thymocytes, there are significant differences between these immature and mature cells in terms of accessibility, occupancy and histone modification of the promoter, enhancer and silencer elements (48-50). While the DD subpopulation in particular has not been studied in this context, there is a report of silencer activity in these cells as assessed using marker transgenic mice (48). Runx3 protein can be detected at low levels in DD thymocytes (25). CD8 lineage T cells, in contrast, express high levels of Runx3 coincident with a switch to the distal promoter (5). It is possible that TOX plays a role in relief of this silencing leading to full reexpression of CD4 and acquisition of the CD4⁺CD8^{lo} phenotype. If so, this effect would have to be cell context dependent, since expression of TOX in DP thymocytes can lead to *Cd4* silencing (32). Alternatively, TOX may influence other regulatory elements, including that of a yet to be identified *Cd4* enhancer of mature T cells (50). In addition, modulation of E protein activity (see below), implicated in *Cd4* gene regulation (51) might also play a role in misregulation of CD4 in the absence of TOX.

The basic helix-loop-helix E protein transcriptional regulators E2a/E47 and HEB play multiple roles in the thymus (reviewed in (52)). In terms of the DP to SP transition, inhibition of E protein activity mediated by TCR induction of Id3 plays a non-redundant role, particularly for CD4SP development (34). The normal expression of Id3, which is rapidly induced as a consequence of TCR activation of the MAPK pathway during positive selection (53), in *Tox*^{-/-} mice is consistent with unperturbed initiation of positive selection in the absence of TOX (25). In contrast, Id2 expression peaks at the later TOX-dependent CD4⁺CD8^{lo} transitional stage (data not shown), and expression of Id2 was diminished in ThPOKtg *Tox*^{-/-} CD4SP thymocytes.

A functional role for Id2 in the thymus has not been defined, but one might predict that upregulation would further diminish E protein activity. That the DP to SP transition is inhibited in Id3-deficient but not Id2-deficient mice, may reflect the differences in kinetics of expression of these proteins. Since Id2 is upregulated after induction of Id3 during positive selection (data not shown), it would not be expected to compensate for loss of Id3, while Id3 may have compensatory activity when Id2 is absent. The finding of reduced Id2 in ThPOKtg *Tox*^{-/-} CD4SP thymocytes led us to ask whether there would be consequences for expression of genes that might normally be repressed by E protein activity. This was

indeed the case as expression of both *Tesc* and *Foxo1*, genes previously shown to be upregulated in E2a/HEB doubly deficient thymocytes (35) and in the case of *Foxo1*, downregulated upon expression of E47 in a thymic lymphoma cell line (54), were poorly induced in the absence of TOX. The role of tescalcin in the thymus remains to be determined, although there is the potential to modulate cell signaling and/or cell differentiation (39, 40, 55). Better defined is the function of FOXO1 in CD4⁺ T cells (42). Expression of FOXO1 primary and downstream gene targets, including *Klf2*, *Slpr1*, *Il7r*, *Ccr7* and *Sell*, was compromised in the absence of TOX. ThPOKtg *Tox*^{-/-} CD4SP thymocytes were still able to localize to medullary regions of the thymus, albeit inefficiently, and exit to the periphery, indicating that downregulation of these factors did not result in a complete block in chemotaxis or migration. Moreover, decreased FOXO1 expression in the absence of TOX was also associated with increased frequency of CD44^{hi} (and CD69^{hi} on a subset of these) CD4⁺ T cells in ThPOKtg *Tox*^{-/-} mice, as also observed in FOXO1-deficient mice (42).

It is intriguing that *Tox*^{-/-} mice, like *Id2*-deficient mice, have blocks in development of NK cells and LTi cells (56, 57). *Tox*^{-/-} bone marrow NK cells also have lower expression of *Id2*, similar to that reported in the thymus here (58). Nevertheless, ectopically expressed *Id2* does not rescue development of *Tox*^{-/-} NK cells *in vitro*, pointing to additional roles for TOX in this process (58). Together, our data are consistent with TOX as an upstream modulator of *Id2* expression, both in developing T cells and in other cell lineages. Expression of the E protein E47 in an E2a-deficient thymic lymphoma cell line leads to modest reduction in *Tox* expression (54), suggesting that E proteins could be an upstream repressor of TOX expression in some contexts. However, TOX and *Id3* are induced by different signaling pathways (32, 53) and TOX expression precedes that of *Id2* (data not shown). These data argue against the idea that a reduction in E protein expression at the initiation of positive selection is key to TOX induction. An alternate possibility is that there is a feed-forward regulatory loop where TOX-dependent upregulation of *Id2* and ensuing downregulation of E protein activity enhances TOX expression. Indeed, TOX and *Id2* expression both peak at the CD4⁺8^{lo} stage.

We propose a model where TOX is required for *Cd4* reexpression at the DD to CD4⁺8^{lo} transition as well as to create a permissive state for ThPOK induction, resulting in CD8 fate repression independently of the activity of TOX. In addition, suppression of E protein activity may occur in discrete stages during positive selection. TOX would play a role in *Id2* upregulation and full E protein inhibition at the CD4⁺8^{lo} stage, necessary for induction of multiple aspects of the CD4 T cell lineage gene program as well as to enhance TOX expression. The fact that TOX is not CD4 lineage specific points to the influence of other factors, potentially GATA3, that likely influence the biological function of TOX. Nevertheless, our data demonstrate that the 'pulse' of TOX expression during positive selection is a key regulatory event in CD4 lineage differentiation.

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Abbreviations used in this article

DP CD4⁺CD8⁺ double positive thymocyte

CD4SP or CD8SP	CD4⁺CD8⁻ or CD4⁻CD8⁺ single positive thymocyte
Tg	transgenic

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Figure 1.

Lineage confusion in TOX-deficient T cells.

A, Flow cytometry of splenocytes from *Tox*^{+/+} and *Tox*^{-/-} mice stained for expression of TCRβ, CD4 and CD8α. Numbers indicate percentage of gated populations. Data are representative of at least ten experiments. B, Quantitative RT-PCR analysis of expression in purified splenic T cells from *Tox*^{+/+} and *Tox*^{-/-} gated as in (A). Results are represented as relative to expression of *Gapdh*. Two independent experiments are shown. C, Flow cytometry of splenic T cells gated for expression of CD4 (top) or CD8 (bottom) from *Tox*^{+/+} and *Tox*^{-/-} mice co-stained for CD103. Numbers indicate percentage of gated populations. Data are representative of three experiments. D-E Thymocytes from mice of the indicated genotype were immunostained for (D) CD4 and CD8 or (E) CD5 and TCRβ and analyzed by flow cytometry. Numbers indicate frequency (%) of cells in the adjacent gates. Data is representative of four animals. F, Comparison of *Gata3* expression in AND-Tg *Tox*^{-/-} and control (AND-Tg *Tox*^{+/+} or AND-Tg *Tox*^{+/-}) thymocytes (data from three experiments are

pooled). *G*, Comparison of *Thpok* expression in AND-Tg *Tox*^{-/-}, control (AND-Tg *Tox*^{+/+} or AND-Tg *Tox*^{+/-}), and *Tox*^{-/-} thymocytes (results from three experiments are shown).

Figure 2.

Complementation of *Tox*-deficient T cells with ThPOK. *A-B*, Flow cytometry of total thymocytes from *Tox*^{+/+}, *Tox*^{-/-}, *ThPOK-Tg Tox*^{+/+} and *ThPOK-Tg Tox*^{-/-} mice stained for CD5, TCR β , CD4 and CD8. Numbers indicate percentage of gated populations. Data are representative of four experiments. *C*, Absolute cell numbers of thymocyte populations from *Tox*^{+/+}, *Tox*^{-/-}, *ThPOK-Tg Tox*^{+/+} and *ThPOK-Tg Tox*^{-/-} mice are plotted. Each symbol represents one mouse. Data represent at least four experiments. *D*, Histogram overlays of surface CD5, TCR β , and CD69 expression in *ThPOK-Tg Tox*^{+/+} CD4SP (red) and *ThPOK-Tg Tox*^{-/-} CD4^{lo}SP (blue) thymocytes as gated in (*B*). Data is representative of four experiments. *E*, Intracellular staining for GATA3 in DP (black), TCR⁺CD8SP (blue) and CD4SP or CD4^{lo}SP (red) thymocyte subsets as gated in (*B*). The median fluorescence intensity for the GATA3 antibody signal is indicated for the CD4SP and CD4^{lo}SP populations in each mouse strain. Data are representative of four experiments.

Figure 3.

Repression of the CD8 gene program by ThPOK is TOX-independent.

A, Flow cytometry of total thymocytes from *Tox*^{+/+}, *Tox*^{-/-}, *ThPOK-Tg Tox*^{+/+} and *ThPOK-Tg Tox*^{-/-} stained for CD4, CD8 and CD103. Surface expression of CD103 was determined on CD4^{SP} or CD4^{lo}SP (red), CD8^{SP} (black) and DD (green) thymocyte subsets. Numbers indicate percentage of gated populations. Data are representative of three experiments. **B**, Expression of Runx proteins was determined by Western blot in sorted thymocyte lysates and compared to wild-type (WT) CD8⁺ T cells. Expression of actin was determined and used as a loading control. Data are representative of two experiments. **C**, Absolute spleen cellularity in *Tox*^{+/+}, *Tox*^{-/-}, *ThPOK-Tg Tox*^{+/+} and *ThPOK-Tg Tox*^{-/-} is plotted. Bars represent average values and standard deviations are represented by error bars (n=4). Data are representative of at least four experiments. **D**, Flow cytometry of total splenocytes stained for expression of TCRβ, CD4 and CD8. Numbers indicate percentage of gated populations. **E-G**, Average frequencies of TCRβ⁺ splenocytes (**E**), as well as the CD4⁺ and CD8⁺ subsets within the TCRβ⁺ subset (**F**), as gated in (**D**) are shown (n=4). The frequency of CD4⁺8^{int} T cells in the spleen is plotted (**G**) (n=4). *P = 0.011 **P = 0.0008. Error bars represent standard deviations. Data are representative of four experiments. **H**, Quantitative

RT-PCR analysis of *Prfl* expression in purified splenic T cells from mice with the indicated genotypes. Results are represented as relative to expression of *Gapdh*. Two independent experiments are shown, except in the case of wild-type (WT) CD8⁺ T cells where one experiment is plotted.

Figure 4.

Development of the CD4 lineage program and E protein activity are TOX dependent.

A-B, Expression of the *Cd4* gene was assessed by quantitative real-time RT-PCR in sorted thymocyte populations from *Tox*^{-/-} mice (A) and *ThPOK-Tg* mice on a *Tox*^{+/-} or *Tox*^{-/-} background (B). C-D, Expression of the endogenous *Thpok* 3'UTR was assessed by quantitative real-time RT-PCR in sorted thymocyte populations (C) or in enriched CD4⁺ T splenocytes (D). Results are expressed relative to *Gapdh* (A-D). Two (B-C) and three (D) independent experiments are plotted. E-G, Expression of the *Id2* (E), *Id3* (F) and *Tesc* (G) genes was assessed by quantitative RT-PCR in sorted thymocyte populations from *ThPOK-Tg* mice on a *Tox*^{+/-} or a *Tox*^{-/-} background. Averages of three independent experiments are plotted and error bars show standard deviations (E-F). * *P* = 0.005 (E). Two independent experiments are plotted (G). Results are expressed relative to *Gapdh*.

Figure 5.

Induction of CD4⁺ T cell maturation and function is dependent on TOX. A, Quantitative RT-PCR analysis of expression of *Foxo1* in purified CD4SP (from *ThPOK-Tg Tox*^{+/-} mice) or CD4^{lo}SP (from *ThPOK-Tg Tox*^{-/-} mice) thymocytes. Averages of three independent experiments are plotted and error bars show standard deviations. * *P* = 0.02. B, Western blot showing expression of FOXO1 levels in sorted DP and CD4SP (from *ThPOK-Tg Tox*^{+/-} mice) or CD4^{lo}SP (from *ThPOK-Tg Tox*^{-/-} mice) thymocytes. C, Quantitation of FOXO1 protein levels from (B), normalized to expression of β-actin in the same samples. Averages of three independent experiments are plotted and error bars show standard deviations. * *P* = 0.005. D-E, Quantitative RT-PCR analysis of expression of *Klf2* (D) and *S1pr1* (E) in purified CD4SP (from *ThPOK-Tg Tox*^{+/-} mice) or CD4^{lo}SP (from *ThPOK-Tg Tox*^{-/-} mice) thymocytes. Averages of three independent experiments are plotted and error bars show standard deviations. * *P* = 0.007 (D) and **P* = 0.02 (E). Results are expressed relative to

Gapdh. *F-G*, Histogram overlays showing expression of CD127, CCR7 and CD62L (*F*) and cell size as assessed by forward scatter (*G*) on CD4SP (from *ThPOK-Tg Tox^{+/-}* mice) (grey) or CD4^{lo}SP (from *ThPOK-Tg Tox^{-/-}* mice) (black) thymocytes. Data are representative of three experiments. *H-I*, Flow cytometry of expression of CD154 on resting (grey) or *ex vivo* activated (black) CD4SP (from *ThPOK-Tg Tox^{+/-}* mice) or CD4^{lo}SP (from *ThPOK-Tg Tox^{-/-}* mice) thymocytes (*H*) and CD4⁺ T cells from *Tox^{+/-}*, *Tox^{-/-}*, *ThPOK-Tg Tox^{+/-}* and *ThPOK-Tg Tox^{-/-}* (*I*) mice is shown. Data are representative of two experiments. *J*, Flow cytometry of expression of CD69 on resting (grey) or *ex vivo* activated (black) CD4⁺ T cells from *ThPOK-Tg Tox^{+/-}* and *ThPOK-Tg Tox^{-/-}* (*I*) mice is shown. Data are representative of three experiments. *K-L*, Quantitation of data in (*I*) and (*J*), respectively. Black bars represent activated CD4⁺ cells, while grey bars represent resting CD4⁺ cells. * $P = 1.2 \times 10^{-4}$ (*K*), * $P = 6.4 \times 10^{-8}$ (*L*). *M*, Expression of *Foxo1* was assessed by quantitative RT-PCR in sorted CD4⁺ T cells from *ThPOK-Tg* mice on a *Tox^{+/-}* or a *Tox^{-/-}* background. Averages of three independent experiments are plotted and error bars show standard deviations. * $P = 0.009$. *N*, Flow cytometry for expression of CD44 on CD4⁺ T from *ThPOK-Tg Tox^{+/-}* (grey) and *ThPOK-Tg Tox^{-/-}* (black) mice is shown. Data is representative of four experiments.

Figure 6. Expression of cell survival factor BCL2 fails to rescue CD4⁺ T cell development in the absence of TOX. *A*, Thymocytes from mice of the indicated genotype were immunostained for CD4 and CD8 and analyzed by flow cytometry. *B*, Expression of CD4 and CD8 was analyzed on TCRβ⁺ splenocytes. *A-B*, Numbers indicate frequency (%) of cells in the adjacent gates. Data are representative of two animals.

Figure 7. Small thymic medullary regions form, and contain CD4⁺SP cells, in ThPOK-Tg *Tox*^{-/-} mice. A-D, Frozen thymic sections from ThPOK-Tg *Tox*^{+/-} (A,C) and ThPOK-Tg *Tox*^{-/-} (B,D) mice, stained for CD4 (green) and CD8 (red). DP cells appear as yellow. Medullary thymic epithelial cells are identified by staining with the lectin *Ulex europaeus* agglutinin 1 (blue). In (D), the arrows point to examples of CD4SP cells in a medullary region. Original magnification of 20x (A,B) and 63x (C,D). Data are representative of two animals.