

# TOX Provides a Link Between Calcineurin Activation and CD8 Lineage Commitment

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## Abstract

T cell development is dependent on the integration of multiple signaling pathways, although few links between signaling cascades and downstream nuclear factors that play a role in thymocyte differentiation have been identified. We show here that expression of the HMG box protein TOX is sufficient to induce changes in coreceptor gene expression associated with  $\beta$ -selection, including *CD8* gene demethylation. TOX expression is also sufficient to initiate positive selection to the CD8 lineage in the absence of MHC–TCR interactions. TOX-mediated positive selection is associated with up-regulation of Runx3, implicating CD4 silencing in the process. Interestingly, a strong T cell receptor–mediated signal can modify this cell fate. We further demonstrate that up-regulation of TOX in double positive thymocytes is calcineurin dependent, linking this critical signaling pathway to nuclear changes during positive selection.

Key words: HMG box • T cell development • TCR signaling • gene regulation • Runx

## Introduction

The thymus generates a vast excess of cells that fail to complete maturation and die in situ as a result of stringent selection processes, including  $\beta$ -selection and positive selection (1).  $\beta$ -Selection refers to the requirement for productive rearrangement of TCR $\beta$  chain genes and expression of a pre-TCR to induce the survival, proliferation, and differentiation of precursor thymocytes. Positive selection operates at a subsequent stage of development, requiring productive rearrangement of TCR $\alpha$  chain genes and TCR-mediated recognition of self-MHC to induce cell survival and continued thymocyte maturation.

Both  $\beta$ -selection and positive selection elicit stable changes in gene expression. Among the best characterized of these changes is expression of the *CD4* and *CD8* coreceptor genes, with  $\beta$ -selection inducing a CD4<sup>–</sup>8<sup>–</sup> (double negative [DN]) to CD4<sup>+</sup>8<sup>+</sup> (double positive [DP]) phenotypic change in thymocytes and positive selection inducing a DP to CD4<sup>+</sup>8<sup>–</sup> or CD4<sup>–</sup>8<sup>+</sup> (single positive [SP]) transition. A great deal of work has focused on identifying the pre-TCR– and

TCR-mediated signals that are involved in  $\beta$ -selection and positive selection, respectively (2). In addition, there have been significant advances in understanding the regulation of coreceptor genes during thymic selection (3). In particular, members of the Ikaros and *Runt* domain transcription factor families have been demonstrated to bind regulatory regions of the *CD8* and *CD4* gene loci, respectively. Ikaros is thought to activate *CD8* gene expression by increasing the accessibility of other transcription factors to the locus (4), whereas Runx1 and Runx3 function to repress *CD4* gene expression by binding to a silencer regulatory element (5). In addition, components of mammalian SWI/SNF-like BAF chromatin remodeling complexes have been implicated in the control of *CD4* and *CD8* gene expression at various stages of T cell development (6–8).

Although the mechanistic details are still in dispute, data are accumulating that cell fate decisions associated with positive selection are regulated to some degree by the

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*Abbreviations used in this paper:* CD8ISP, CD4<sup>–</sup>CD8<sup>+</sup> immature single positive thymocyte; Cn, calcineurin; DN, CD4<sup>–</sup>8<sup>–</sup> double negative thymocyte; DP, CD4<sup>+</sup>8<sup>+</sup> double positive thymocyte; HMG, high mobility group; MAPK, mitogen-activated protein kinase; SP, CD4<sup>+</sup>8<sup>–</sup> or CD4<sup>–</sup>8<sup>+</sup> single positive thymocyte; Tg, transgenic; TOX, thymocyte selection-associated HMG box protein.

“length and strength” of intracellular signaling, with CD4 lineage commitment associated with stronger or more prolonged signaling than CD8 lineage commitment (9–17). The mitogen-activated protein kinase (MAPK) signaling pathway has been implicated in playing a central role in this process (11, 16–19). Ultimately, however, understanding lineage commitment necessitates knowledge of how quantitatively or qualitatively different signaling networks imprint distinct patterns of gene expression in the cell. One gene target of the MAPK pathway in the thymus, encoding the Egr-1 transcription factor (20), has been further linked to regulation of Id3 (21, 22), an inhibitor of E protein transcriptional activation that is required for positive selection (23). However, few other links between signaling pathways and transcriptional regulators of positive selection are known.

Calcineurin (Cn) is a calcium-activated serine/threonine phosphatase composed of catalytic (A) and regulatory (B) subunits. Two catalytic subunits, CnA $\alpha$  and CnA $\beta$ , are expressed in developing thymocytes. It has been known for more than a decade that positive selection is inhibited by the immunosuppressive agent and potent Cn inhibitor cyclosporin A (24, 25). More recently, genetic approaches have confirmed that Cn activation is required for positive selection. Although T cell development proceeds normally in mice that are deficient in CnA $\alpha$  (26), positive selection is inhibited in mice deficient in CnA $\beta$  (27). Conversely, expression of a constitutively active form of Cn in thymocytes has been reported to enhance positive selection (28). Activated Cn is known to bind and dephosphorylate NFATs in the cytoplasm, resulting in nuclear translocation of these transcriptional regulators (29). An active form of NFATc3 has been shown to substitute for calcium-mediated signals in the differentiation of a thymocyte cell line (30). However, the relevant targets of NFATs in the context of positive selection in the thymus are not known.

We have recently described a nuclear protein of the high mobility group (HMG) box family, thymocyte selection-associated HMG box (TOX), that is up-regulated during both  $\beta$ -selection and positive selection in the thymus (31). Transgenic mice that express TOX (TOX-transgenic [Tg]) in the majority of thymocytes show a significant increase in TCR<sup>+</sup> CD8SP thymocytes and a concomitant reduction in CD4 SP thymocytes. Moreover, the production of CD8SP thymocytes in these animals is class I MHC independent. In this study, we demonstrate that expression of TOX can elicit changes in coreceptor expression at both the DN and DP stages that mimic aspects of both  $\beta$ -selection and positive selection, respectively. Moreover, production of TCR<sup>+</sup> CD8SP thymocytes in TOX-Tg mice is independent of both class I and class II MHC, indicating that expression of this nuclear factor is sufficient to initiate a differentiation program to the CD8 lineage. The CD8SP thymocytes in TOX-Tg/MHC-deficient (MHC<sup>o</sup>) animals have down-regulated expression of the *CD4* gene and up-regulated expression of Runx3, a transcriptional regulator involved in *CD4* gene silencing. We also demonstrate that regulation of the TOX gene in DP thymocytes is downstream of Cn signaling, linking this signaling pathway to changes in coreceptor gene ex-

pression. Interestingly, development to the CD8 lineage in TOX-Tg mice can be overcome by TCR-mediated signaling in vivo. These results are discussed in the context of a model where lineage commitment during positive selection is regulated by an integration of MAPK and Cn signaling, the latter pathway involving up-regulation of TOX.

## Materials and Methods

**Animals.** All mice were bred at the Scripps Research Institute and kept under specific pathogen-free conditions. The mice used in this study were RAG-1 deficient (32), RAG-2 deficient (33), TCR $\alpha$  deficient (34), MHC deficient (35), 2C TCR-Tg (36) on a B6 background, AND TCR-Tg (37) on an H-2<sup>b</sup> or H-2<sup>b/k</sup> background, DO11.10 TCR-Tg (38) on an H-2<sup>d</sup> background, and CnA $\beta$  deficient (27).

**Flow Cytometry.** Thymocytes were stained as described previously (31) and analyzed on a FACScan or FACSsort using CellQuest software (Becton Dickinson). Typically, 5,000–20,000 viable cells were acquired based on their forward and side light scatter, and the log fluorescence is shown. For isolation of specific thymocyte subsets, cell populations were sorted using FACStar Plus or FACS<sup>®</sup> Vantage DiVa I cell sorters (Becton Dickinson).

Fluorescein isothiocyanate-conjugated anti-CD8 $\alpha$  (53–6.7; eBioscience), phycoerythrin-conjugated or peridinin chlorophyll protein-conjugated anti-CD4 (L3T4; eBioscience), phycoerythrin-conjugated anti-CD8 $\beta$  (53–5.8; BD Biosciences), phycoerythrin-conjugated anti-heat stable antigen (HSA) (30-F1; eBioscience), phycoerythrin-conjugated anti-CD44 (1M7; eBioscience), allophycocyanin-conjugated anti-CD25 (PC61; eBioscience), biotinylated or allophycocyanin-conjugated anti-TCR $\beta$  (H57–597; BD Biosciences), phycoerythrin-conjugated or biotinylated anti-CD69 (H1.2F3, BD Biosciences) mAbs were used for flow cytometry.

**RT-PCR.** Total RNA was isolated from sorted DP and CD8<sup>+</sup> CD4<sup>lo/-</sup> thymocytes from TOX-Tg/MHC<sup>o</sup> mice using the RNeasy RNA kit (QIAGEN). Primer pairs (Invitrogen) used were: CD8 $\alpha$  5′-TGCCATGAGGGACACGAATAATAAG-3′ and 5′-TAAATATCACAGGCGAAGTCCAATC-3′; CD8 $\beta$  5′-TTCTTGGTTGGGGCAGTTGTAGGAA-3′ and 5′-TGGCTCTGGCTGGTCTTCAGTATGA-3′; CD4 5′-CTGATGTGGAAGGCAGAGAAGGATTC-3′ and 5′-CAGCAGCGAAGCAGGAACACTGTCT-3′; TCR $\alpha$  5′-TGGGGCCATTGCCTGGAGCAACCAGA-3′ and 5′-CACAGCCTCAGCGTCATGAGCAGGT-3′; Thy-1 5′-CGTACCCTCCCGCGTCACCCTCTCC-3′ and 5′-GCAGGCTTATGCCGCCACACTTGACCA-3′; and  $\beta$ -actin 5′-GGCAACGAGCGGTTCCGATGCCCTGA-3′ and 5′-GCCAGGATGGAGCCACCGATCCACA-3′.

**DNA Methylation Assay.** Genomic DNA was isolated from sorted thymocytes using the DNeasy tissue kit (QIAGEN) and digested with HhaI or NcoI. 100 ng of DNA template was used for PCR amplification of the sequence flanking the HhaI and NcoI sites in the CD8 locus with the primer pair 5′-GCC-CCAGCCTGCACACCTGGGCTACA-3′ and 5′-GATGTTCACAGGACCCTGCTGGCCAAA-3′.

**In Vitro Activation and Culture of Thymocytes.** Thymocytes from TCR $\alpha$  chain-deficient (TCR $\alpha$ <sup>o</sup>) or CnA $\beta$ -deficient mice were isolated and cultured in the absence or presence of 0.2 ng/ml PMA (Calbiochem) and/or 0.05–0.2  $\mu$ g/ml ionomycin (Calbiochem), with and without inhibitors cyclosporin A (5  $\mu$ g/ml) (Sigma-Aldrich) or U0126 (10  $\mu$ M) (Calbiochem) for 6–8 h at

37°C. Cells were analyzed by flow cytometry and/or by Western blot. In other experiments, sorted DP thymocytes from TOX-Tg/MHC<sup>o</sup> mice or whole thymocytes from MHC<sup>o</sup> mice were cultured for 44 h at 37°C and 10% CO<sub>2</sub> in DMEM medium supplemented as described previously (18) and used for flow cytometric analysis.

**Immunoblotting.**  $3 \times 10^5$  thymocytes were lysed in SDS sample buffer and subjected to Western blot as described previously (31). Primary antibodies used were affinity purified rabbit anti-TOX antibody (150–300 ng/ml) (31), polyclonal anti-Runx (1:3,000) (39), or anti- $\beta$ -actin (1:5,000) (Ab-1; Oncogene Research Products). Identification of Runx proteins was confirmed by Western blot analysis of transfected cells expressing Runx1 or Runx3 (unpublished data).

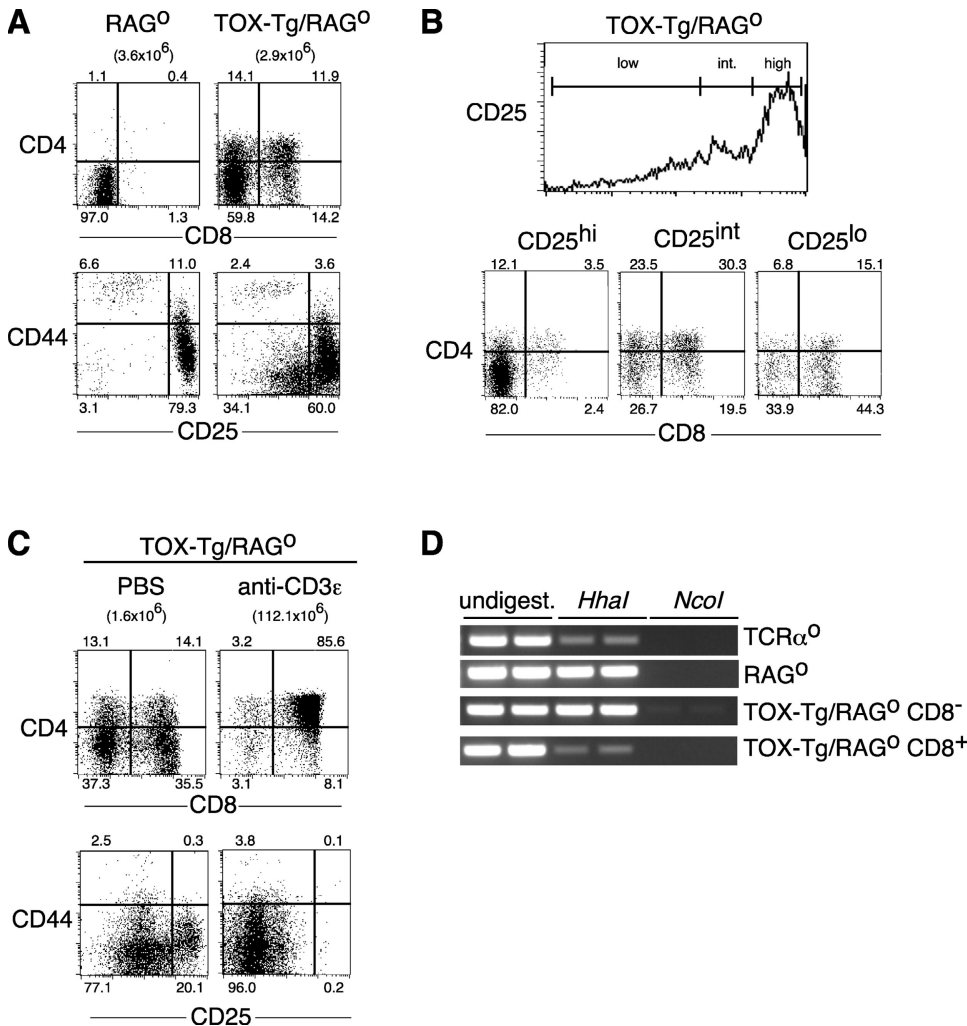
## Results

*TOX Is Sufficient to Initiate Coreceptor Changes Associated with  $\beta$ -Selection.* DN thymocytes progress through distinct developmental stages that can be monitored by the expression of CD44 and CD25; the stages are termed DN1 (CD44<sup>+</sup>CD25<sup>-</sup>), DN2 (CD44<sup>+</sup>CD25<sup>+</sup>), DN3 (CD44<sup>-</sup>CD25<sup>+</sup>), and DN4 (CD44<sup>-</sup>CD25<sup>-</sup>) (40). Transition of thymocytes from

the DN3 to the DN4 stage during  $\beta$ -selection requires signaling by the pre-TCR complex (41).

We have reported previously that TOX is up-regulated in the DN3 blast population as a consequence of  $\beta$ -selection and subsequently down-regulated before the DP stage (31). To determine the role of TOX during  $\beta$ -selection, we generated TOX-Tg mice on a RAG<sup>o</sup> background. RAG<sup>o</sup> thymocytes have a developmental block at the DN3 stage and severely reduced thymic cellularity due to the failure to undergo  $\beta$ -selection (33). Although the thymic cellularity of TOX-Tg/RAG<sup>o</sup> mice was similar to that of RAG<sup>o</sup> mice, the expression of the TOX transgene resulted in a complex pattern of coreceptor expression (Fig. 1 A). Four distinct cell populations, DN, CD4<sup>lo</sup>CD8<sup>-</sup>, DP, and CD8SP thymocytes, were present in these mice. An identical staining pattern was obtained when cells were stained for CD4 and CD8 $\beta$ , indicating that the CD8<sup>+</sup> thymocytes expressed a CD8 $\alpha\beta$  heterodimer, as do normal DP and CD8SP thymocytes (unpublished data).

Expression of TOX on a RAG<sup>o</sup> background also resulted in the appearance of CD44<sup>-</sup>CD25<sup>lo</sup> thymocytes, consistent with initiation of the DN3 to DN4 transition in some cells (Fig. 1 A). To determine the developmental re-

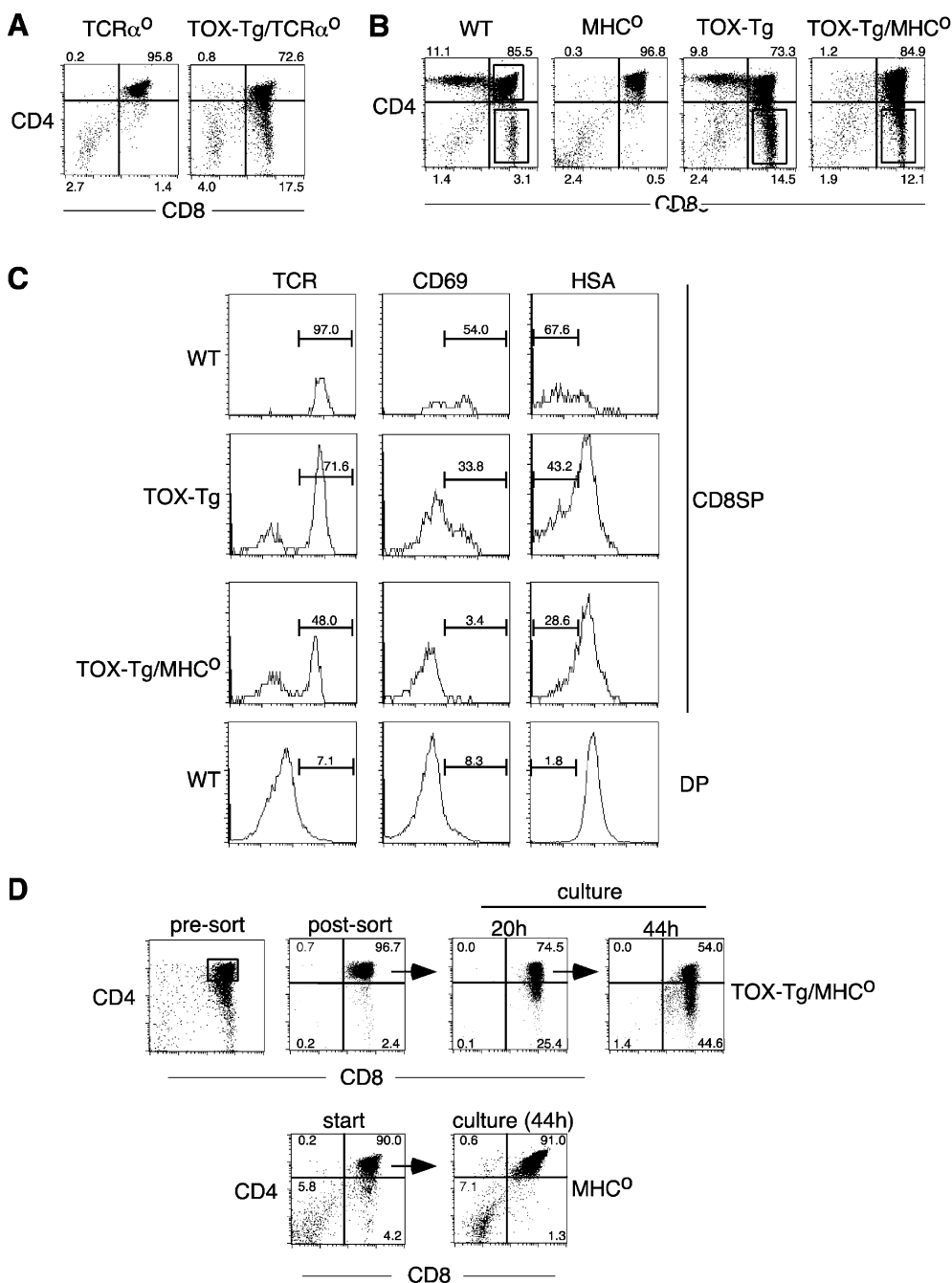


**Figure 1.** TOX induces development of coreceptor positive thymocytes in RAG<sup>o</sup> mice. (A) TOX-Tg/RAG<sup>o</sup> thymocytes were stained for expression of CD4, CD8 $\alpha$ , CD44, and CD25 and analyzed by flow cytometry. Percentages of total thymocytes in each quadrant and total thymic cellularity are indicated. (B) CD4 and CD8 $\alpha$  expression (dot plots) on CD25 gated thymocytes (histogram) in a TOX-Tg/RAG<sup>o</sup> mouse. (C) TOX-Tg/RAG<sup>o</sup> mice were injected i.p. with PBS or anti-CD3 $\epsilon$  antibody, and the thymocytes were analyzed 5 d later for expression of CD4 and CD8 $\alpha$  or CD44 and CD25 as in A. (D) Methylation status of the CD8 locus in thymocytes from mice with the indicated genotype as analyzed by restriction digest and PCR. CD8 $\alpha$ <sup>+</sup> and CD8 $\alpha$ <sup>-</sup> thymocyte cell subpopulations were purified by cell sorting.

relationship between different cell populations, thymocytes from TOX-Tg/RAG<sup>o</sup> mice were four-color stained for CD4, CD8, CD25, and CD44. The TOX-Tg/RAG<sup>o</sup> DN and some CD4<sup>lo</sup>CD8<sup>-</sup> thymocytes were CD44<sup>-</sup>CD25<sup>hi</sup>, similar to the majority DN3 population found in RAG<sup>o</sup> thymocytes (Fig. 1 A). In contrast, the majority of DP and some CD4<sup>lo</sup> cells displayed intermediate levels of CD25, consistent with a post-DN3 stage of development (Fig. 1 B). Thymocytes with a CD8SP phenotype are found both as a DN4 to DP transitional population (CD4<sup>-</sup>CD8<sup>+</sup> immature single positive thymocyte [CD8ISP]) and as a product of DP thymocytes after positive selection. Surprisingly,

the CD8SP thymocytes in TOX-Tg/RAG<sup>o</sup> mice were CD25<sup>lo</sup> when compared with DP thymocytes in these same animals, most consistent with a post-DP stage rather than a CD8ISP stage of development (Fig. 1 B).

Expression of TOX in transgenic animals on a wild-type background does not block  $\beta$ -selection, nor does it result in aberrant expression of CD25 on DP or CD8SP thymocytes (31). Thus, it seems likely that the reduction in CD25 in CD8SP compared with DP cells in TOX-Tg/RAG<sup>o</sup> mice represents a developmental progression. The failure of CD25 to be fully down-regulated in these cells may reflect the absence of proliferation and/or pre-TCR



**Figure 2.** TOX induces development of CD8SP thymocytes in the absence of TCR-MHC interactions. (A and B) Expression of CD4 and CD8 $\alpha$  on thymocytes from mice with the indicated genotype. Percentages of total thymocytes in each quadrant are indicated. (C) Expression of TCR $\beta$ , CD69, and HSA on CD8SP thymocytes gated as in B from mice with the indicated genotypes. DP thymocytes from wild-type mice were used as a control to set appropriate gates. The percentage of TCR<sup>hi</sup>, CD69<sup>+</sup>, and HSA<sup>lo</sup> cells are shown. (D) Total MHC $^0$  thymocytes or sorted DP thymocytes from TOX-Tg/MHC $^0$  mice were cultured ex vivo and analyzed for CD4 and CD8 $\alpha$  expression by flow cytometry after 20 or 44 h. Percentages of total thymocytes in each quadrant are shown. Viable yield of TOX-Tg/MHC $^0$  thymocytes was 73% after 20 h of culture.



signals normally associated with  $\beta$ -selection. In support of this, anti-CD3 $\epsilon$  treatment of TOX-Tg/RAG $^{\circ}$  mice led to increases in thymic cellularity and loss of CD25 on DP and CD8SP cells (Fig. 1 C) as it does in RAG $^{\circ}$  mice (42).

Transcriptional activation of the *CD8* gene locus during the transition from the DN to DP stage of thymocyte development is associated with DNA demethylation of the gene (43). Since expression of TOX was sufficient to induce up-regulation of both CD8 $\alpha$  and CD8 $\beta$  on DN thymocytes, we asked whether TOX also induced a change in the methylation status of these loci. A CpG sequence that forms part of an HhaI restriction site is found between the linked *CD8 $\beta$*  and *CD8 $\alpha$*  genes and is a site of DNA methylation (43, 44). Methylation status of this site was assessed by restriction digest of genomic DNA followed by PCR. In RAG $^{\circ}$  thymocytes, the CD8 locus is transcriptionally silent and this site is methylated, as evidenced by resistance to digestion with the methylation-sensitive enzyme HhaI and successful PCR with primers that flank this site (Fig. 1 D). However, PCR fails after digestion of the DNA with the control enzyme NcoI that recognizes an adjacent sequence that does not include a CpG methylation site (Fig. 1 D). In contrast, this site is partially demethylated in DP thymocytes (total thymocytes from TCR $\alpha$ -deficient mice) and is therefore sensitive to HhaI digestion (Fig. 1 D).

To determine if TOX was sufficient to alter the methylation pattern of the CD8 locus, CD8 $^{+}$  and CD8 $^{-}$  thymocytes were purified by cell sorting from TOX-Tg/RAG $^{\circ}$  mice and analyzed as above. Although the HhaI site was methylated in CD8 $^{-}$  thymocytes and resistant to digestion, the CD8 $^{+}$  thymocytes showed partial demethylation, consistent with activation of the *CD8* gene locus (Fig. 1 D).

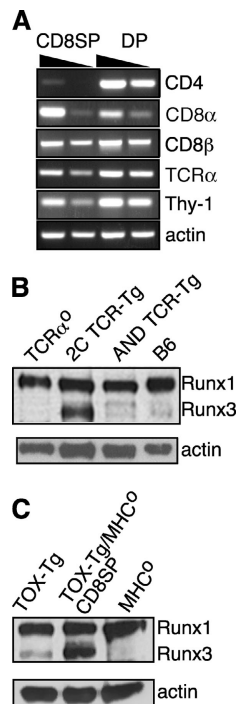
*TOX Is Sufficient to Initiate Coreceptor Changes Associated with Positive Selection to the CD8 Lineage.* We have reported previously that TOX-Tg mice have increased numbers of CD8SP thymocytes and reduced numbers of CD4SP thymocytes (31). Production of CD8SP thymocytes in these mice is not dependent on expression of class I MHC. The fact that TOX-Tg/RAG $^{\circ}$  mice also had CD8SP thymocytes and that these cells had down-regulated expression of CD25 when compared with DP thymocytes in these same animals, suggested that TOX might be sufficient to initiate positive selection and  $\beta$ -selection.

To test this, we bred TOX-Tg mice onto a TCR $\alpha^{\circ}$  background. Thymocytes from TCR $\alpha^{\circ}$  mice are unable to undergo positive selection and are blocked at the DP stage of development. However, expression of TOX resulted in appearance of a pronounced CD8SP subpopulation, even in the absence of a functional TCR complex (Fig. 2 A). In contrast, no CD4SP thymocytes were detected in these mice (Fig. 2 A). The CD8SP thymocytes from TOX-Tg/TCR $\alpha^{\circ}$  mice were neither functionally nor phenotypically mature (not depicted). However, in the absence of the TCR as a marker it was not clear whether these cells were CD8ISP thymocytes or CD8SP thymocytes that had failed to undergo complete maturation.

This issue was addressed using MHC class I and II doubly deficient (MHC $^{\circ}$ ) mice, since the thymocytes in these animals are unable to undergo positive selection but express the TCR. Thus, CD8ISP and CD8SP thymocytes can be distinguished by expression of the TCR on the latter. In contrast to MHC $^{\circ}$  mice, TOX-Tg/MHC $^{\circ}$  mice had a pronounced CD8SP population, similar to that seen in TOX-Tg/TCR $\alpha^{\circ}$  mice (Fig. 2 B). A significant proportion (48.0%) of these CD8SP thymocytes also expressed the TCR, indicating that they are not CD8ISP cells (Fig. 2 C). No TCR $^{+}$  CD4SP thymocytes were present in these mice (Fig. 2 C and not depicted).

CD69 is a marker of TCR-mediated signaling associated with positive selection (45). In a normal thymus, approximately half of the CD8SP thymocytes express CD69 (Fig. 2 C). TOX-Tg mice on a wild-type background also contained CD69 $^{+}$  CD8SP thymocytes (Fig. 2 C). However, few CD8SP thymocytes in TOX-Tg/MHC $^{\circ}$  mice expressed CD69, consistent with TCR-independent development of these cells. Down-regulation of HSA occurs during the later stages of SP thymocyte maturation (46). Approximately 70 and 40% of CD8SP thymocytes in normal animals and TOX-Tg mice, respectively, were HSA $^{lo}$  (Fig. 2 C). HSA $^{lo}$  cells were also present among TOX-Tg/MHC $^{\circ}$  CD8SP thymocytes, although the extent of down-regulation was reduced compared with wild-type cells (Fig. 2 C).

To definitively demonstrate that DP cells can give rise to CD8SP cells in the presence of TOX and absence of positive selection, DP thymocytes were purified by cell sorting and cultured *in vitro* (Fig. 2 D). A significant proportion of TOX-Tg/MHC $^{\circ}$  DP thymocytes spontaneously down-regulated CD4 over a 44-h culture period, giving rise to CD8SP cells (Fig. 2 D, top row). In contrast, DP thymocytes



**Figure 3.** *CD4* gene silencing in TOX-Tg mice. (A) RT-PCR analysis for expression of the indicated genes in purified CD8SP or DP thymocytes isolated from TOX-Tg/MHC $^{\circ}$  mice. (B and C) Western blot analysis for expression of Runx proteins in whole cell lysates prepared from total thymocytes or isolated CD8SP cells as indicated. Expression of  $\beta$ -actin was used as a loading control.

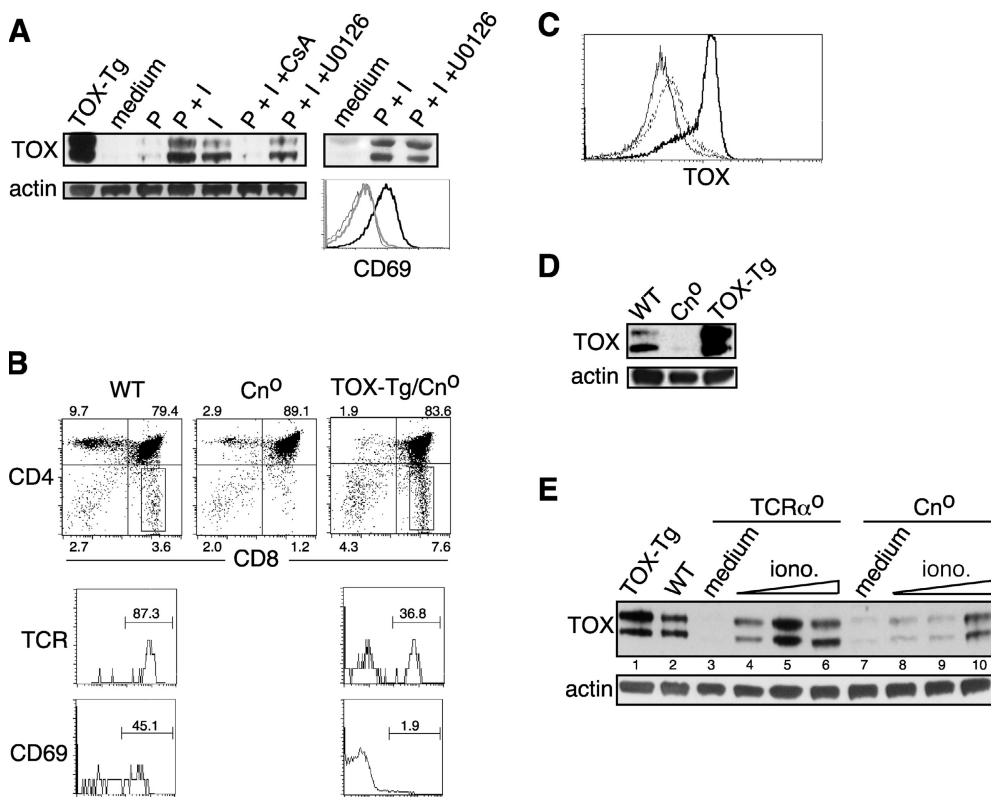
from MHC<sup>o</sup> mice maintained expression of both CD4 and CD8 (Fig. 2 D, bottom row). Together, the data demonstrate that expression of TOX in DP thymocytes is sufficient to induce down-regulation of CD4 and partial maturation in the absence of normal positive selection signals.

**TOX and Coreceptor Gene Expression.** Loss of CD4 expression during the DP to CD8SP thymocyte transition is controlled by activation of a silencer element in the *CD4* gene locus (47). To determine whether loss of cell surface CD4 on TOX-Tg/MHC<sup>o</sup> CD8SP thymocytes reflected a change in gene expression, RT-PCR was performed on purified cell populations. No significant difference in expression of *TCR $\alpha$* , *CD8 $\alpha$* , or *CD8 $\beta$*  genes was observed when TOX-Tg/MHC<sup>o</sup> CD8SP and DP thymocytes were compared (Fig. 3 A). However, *CD4* gene expression was markedly reduced in the CD8SP subpopulation (Fig. 3 A). Interestingly, some diminution of *Thy-1* gene expression was also apparent in the CD8SP population (Fig. 3 A), another indication of partial maturation of these cells (48).

Recently, the Runx1 and Runx3 proteins have been demonstrated to regulate the expression of the *CD4* gene by binding to the CD4 silencer element (5). Moreover, CD8SP thymocytes were absent in mice that were deficient in Runx3 and were heterozygous for a deletion mutant of Runx1 (49). Conversely, the overexpression of Runx1 in vivo resulted in an increase in the total number of CD8SP thymocytes (39).

As ectopic expression of TOX also led to an increase in CD8SP thymocytes, we assessed the levels of Runx proteins in thymocytes from different mouse strains used in this study (Fig. 3 B). Runx3 is more highly expressed by CD8SP thymocytes than CD4SP thymocytes (5, 39), consistent with its role in CD4 silencing. Thus, in circumstances where almost all the thymocytes were DP or CD4SP including TCR $\alpha^o$ , AND TCR-Tg, and MHC<sup>o</sup> mice, no Runx3 was detectable (Fig. 3, B and C). In contrast, Runx1 was expressed at similar amounts in thymocytes from these mice (Fig. 3, B and C) as expected (5, 50). Conversely, in animals where CD8SP thymocytes were present including normal mice and especially in class I MHC-restricted 2C TCR-Tg mice, Runx3 was up-regulated (Fig. 3 B).

To assess the changes induced by expression of TOX in vivo, we then investigated the levels of Runx proteins in TOX-Tg thymocytes. TOX-Tg thymocytes expressed both Runx1 and Runx3, consistent with the production of CD8SP thymocytes in these animals (Fig. 3 C). Most interestingly, however, CD8SP thymocytes that were produced in the absence of positive selection in TOX-Tg/MHC<sup>o</sup> mice had up-regulated Runx3 and maintained expression of Runx1 (Fig. 3 C). This indicates that expression of TOX is sufficient to initiate a sequence of events in developing thymocytes, including *CD4* gene silencing and the associated up-regulation of the CD4 silencer binding protein Runx3.



**Figure 4.** Expression of TOX is regulated by Cn activation. (A) Expression of TOX in TCR $\alpha^o$  thymocytes activated with PMA and ionomycin in the absence or presence of inhibitors of Cn (cyclosporin A, CsA) or MEK (U0126). Also shown is CD69 expression on cells cultured in medium (thin line), with PMA and ionomycin (thick black line), or with PMA/ionomycin and U0126 (thick gray line). Expression of TOX in thymocytes derived from a TOX-Tg mouse is shown as a control. (B) Expression of CD4 and CD8 $\alpha$  on thymocytes derived from various animals as indicated (dot plots). Percentage of total thymocytes in each quadrant is shown. Expression of TCR $\beta$  and CD69 was also analyzed on gated CD8SP thymocytes, and the percentage of TCR<sup>hi</sup> or CD69<sup>+</sup> cells is indicated. Expression of TOX was determined by intracellular staining and flow cytometry (C) in WT (dashed line), Cn<sup>o</sup> (thin line), and TOX-Tg (thick line) thymocytes or by Western blot of whole cell lysates (D). (E) Thymocytes from

TCR $\alpha^o$  and Cn<sup>o</sup> mice were cultured in medium (lanes 3 and 7) or 0.2 ng/ml of PMA and 0.05 (lanes 4 and 8), 0.1 (lanes 5 and 9), or 0.2 (lanes 6 and 10)  $\mu$ g/ml of ionomycin. TOX-Tg thymocytes were analyzed as a control (lane 1).

**TOX Is Downstream of Cn Signaling.** The *Tox* gene was isolated from DP thymocytes which were cultured with PMA and ionomycin under conditions that induce cell differentiation (31). Both MAPK and Cn signaling are required for positive selection (as described in Introduction). To address whether these signaling pathways are also involved in regulation of the *Tox* gene, we asked whether one or both pharmacologic activators needed to be present to up-regulate TOX expression. Cultured TCR $\alpha^{\circ}$  (primarily DP) thymocytes expressed little TOX as assessed by Western blotting (Fig. 4 A). As expected, PMA and ionomycin induced up-regulation of TOX in these cultured cells (Fig. 4 A). However, PMA alone was a poor activator of TOX expression, whereas ionomycin alone fully up-regulated the protein in DP thymocytes (Fig. 4 A). Consistent with these results, cyclosporin A, an inhibitor of Cn, but not U0126, a MEK antagonist, inhibited up-regulation of TOX in this system (Fig. 4 A). In contrast, induction of the MAPK-dependent activation marker CD69 (20, 51) was inhibited by U0126 (Fig. 4 A).

We also took a genetic approach to analyze the dependence of TOX up-regulation on Cn. The reduction in Cn signaling in Cn $\beta$ -deficient (Cn $^{\circ}$ ) mice results in severe inhibition of positive selection evidenced by a decrease in TCR $^{+}$  CD4SP and CD8SP thymocytes (27) (Fig. 4 B). The incomplete block in positive selection in these animals is likely due to compensation by Cn $\alpha$ . Cn $^{\circ}$  thymocytes express lower amounts of TOX than wild-type thymocytes when analyzed by flow cytometry (Fig. 4 C) or by Western blot (Fig. 4 D). Moreover, the efficacy of induction of TOX by ionomycin is severely reduced in Cn $^{\circ}$  thymocytes compared with TCR $\alpha^{\circ}$  thymocytes (Fig. 4 E). The residual TOX induction is presumably mediated by Cn $\alpha$ , consistent with a reduction but not complete inhibition of positive selection in these animals (Fig. 4 B).

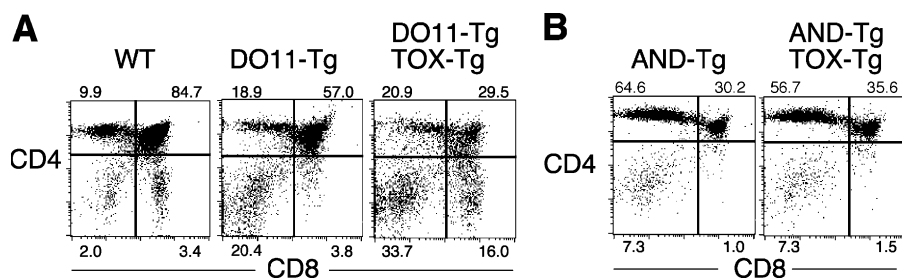
If TOX is downstream of Cn signaling one would predict that loss of the *Cn $\beta$*  gene would have no effect on the production of CD8SP thymocytes in TOX-Tg mice. To test this, we bred TOX-Tg mice onto a Cn $^{\circ}$  background. Although expression of TOX did not rescue CD4SP thymocyte development in Cn $^{\circ}$  mice, there was a fivefold increase in the frequency of TCR $^{+}$  CD8SP thymocytes in TOX-Tg/Cn $^{\circ}$  mice (Fig. 4 B). In addition, few CD8SP thymocytes in TOX-Tg/Cn $^{\circ}$  mice expressed CD69, consistent with their production in the absence of TCR signaling normally associated with positive selection (Fig. 4 B).

These results demonstrate that expression of TOX induces development of a post-DP CD8SP thymocyte population in the absence of MHC-mediated positive selection. The question arises as to why expression of TOX skews development to the CD8 lineage. CD4SP thymocytes are present in TOX-Tg mice, although somewhat reduced in number (31). As shown here, the development of these CD4SP thymocytes is dependent on expression of TCR and MHC and on Cn (Fig. 2, A and B, and Fig. 4 B). In addition, CD4SP thymocytes in TOX-Tg mice are indistinguishable phenotypically from CD4SP in wild-type mice and show normal up-regulation of TCR, CD5, and CD69 (31). Thus, TOX is not sufficient to initiate CD4 lineage commitment, and development of the CD4 lineage maintains a requirement for positive selection even in TOX-Tg mice. Therefore, we reasoned that CD4SP cells would develop as a result of TCR-mediated signals that could overcome the ability of TOX to initiate CD8 lineage development.

We have reported previously that expression of TOX promoted the appearance of CD8SP cells in class II MHC-restricted DO11 TCR Tg mice (31) (Fig. 5 A). However, CD4SP thymocytes were also present in these double transgenic animals, raising the possibility that DO11 TCR signaling was only partially effective in competing with TOX. To test this, we bred TOX-Tg mice to AND TCR-Tg mice on a selecting background. AND TCR-Tg mice exhibit more pronounced skewing to the CD4 lineage than DO11 TCR-Tg mice, presumably because of increased affinity of the AND TCR for class II MHC expressed in the thymus. Importantly, when the TOX transgene was expressed in conjunction with the AND TCR transgene, few CD8 SP thymocytes were observed (Fig. 5 B).

## Discussion

Expression of TOX is transiently up-regulated during both  $\beta$ -selection and positive selection of thymocytes. In this study, we demonstrated that enforced TOX expression was sufficient to induce the differentiation of cells through these developmental checkpoints as assessed by changes in coreceptor expression but failed to induce cellular proliferation normally associated with the DN to DP transition. This shows a clear split in the downstream effectors that are sufficient for changes in coreceptor gene expression and those that initiate cell proliferation during  $\beta$ -selection. The failure of TOX to induce proliferation of DN thymocytes also indicates that the vast majority of DP thymocytes that



**Figure 5.** TCR signaling in vivo competes with TOX activity. (A and B) Expression of CD4 and CD8 $\alpha$  on thymocytes from indicated mice was analyzed by flow cytometry. Percentage of total thymocytes in each quadrant is shown.

develop in TOX-Tg mice on a wild-type background are the product of  $\beta$ -selection. Although expression of TOX was sufficient to induce subpopulations of DP and CD8SP thymocytes in RAG<sup>o</sup> mice, these cells maintained intermediate levels of CD25 on the cell surface. This is an additional indication that TOX is an incomplete mimic of pre-TCR signaling. Similarly, the expression of a stable form of  $\beta$ -catenin results in development of DP thymocytes in RAG<sup>o</sup> mice, with limited proliferation and only partial down-regulation of CD25 (52).

The *CD8* locus is demethylated in TOX-Tg/RAG<sup>o</sup> thymocytes that express CD8 at the cell surface (Fig. 1 D). In some contexts, demethylation appears to be causative for *CD8* gene expression. Thus, mice lacking the maintenance DNA methyltransferase *Dnmt1* abnormally express CD8 $\alpha\beta$  heterodimer in the  $\gamma\delta$  T cell subset (53). Demethylation may also play an important role in regulation of *CD8* gene expression at the DN to DP transition, although remethylation is not required for loss of CD8 expression in CD4 T cells (43, 53). In addition to the activation of the *CD8* gene, we also observed CD4<sup>lo</sup>-expressing thymocytes in TOX-Tg/RAG<sup>o</sup> mice (Fig. 1 A). Based on the expression of CD44 and CD25 (Fig. 1 B), it seems most likely that these cells arise from derepression of *CD4* gene expression in the DN3 population. The fact that ectopic expression of a single nuclear factor can elicit such a complex collection of cell phenotypes suggests that timing and cellular context are critical for the function of TOX.

Changes in *CD4* gene expression during T cell development are regulated by a silencer element located in the first intron of the *CD4* gene (47). The loss of *CD4* gene expression in TOX-Tg thymocytes subsequent to the DP stage is consistent with activation of this silencer. TOX is highly expressed in CD4CD8 double dull thymocytes in normal mice (31), a post-DP stage at which silencer activity is also observed (54). Moreover, Runx3 is up-regulated in CD8SP thymocytes from TOX-Tg/MHC<sup>o</sup> mice (Fig. 5 C). Runx3 binds the CD4 silencer element and plays a role in extinguishing CD4 expression in thymocytes committed to the CD8 lineage (5, 49), further implicating TOX in CD4 silencing. TOX contains an HMG box, a protein domain that bends and unwinds DNA by primarily contacting the minor groove (55). The crystal structure of Runx1, a closely related protein to Runx3, revealed that DNA bending is required in order for this transcription factor to bind to its recognition sequence (56). Thus, TOX could be involved in both induction of Runx3 and in changing the conformation of DNA targets to allow Runx factors to bind and exert their function.

Regulation of coreceptor expression in the thymus is at least partly regulated by changes in chromatin. Ikaros, a nuclear factor that is essential for fetal lymphoid development and that plays multiple roles during adult T cell development (57, 58), has been reported to act as both a transcriptional activator and repressor through interactions with proteins that can alter chromatin structure (59). The repressor activity of Ikaros is associated with packaging of genes into

transcriptionally silent heterochromatin (60). Interestingly, the effect of loss of Ikaros on DN thymocytes has some similarities with the effects of expression of TOX. In both instances, there is pre-TCR-independent production of DP thymocytes in the absence of proliferation (58). However, Ikaros has been demonstrated to be required for the full activation of the *CD8 $\alpha$*  locus (4). BAF57, a component of a SWI/SNF-like chromatin remodeling complex, is an HMG box protein with a single HMG box motif that is predicted to bind DNA in a sequence-independent fashion (61) as is TOX (62). Thymocytes from mice lacking wild-type BAF57, or its associated ATPase Brg-1, also exhibited changes in coreceptor expression including derepression of *CD4* in DN thymocytes and impairment of *CD8* gene activation (6–8). Thus, the common thread in the ability of TOX to induce activation and epigenetic modification of the *CD8* locus in DN cells and activation or silencing of the *CD4* locus in DN or DP cells, respectively, could be the induction of architectural changes in chromatin, as reported for Ikaros and members of the SWI/SNF-like complex. This is certainly consistent with the known and proposed functions for HMG box proteins in transcriptional regulation and nucleosome remodeling (55, 63).

The loss of *CD4* gene expression on a subpopulation of thymocytes in TOX-Tg mice was independent of normal TCR and MHC-mediated positive selection. This argues against the possibility that ectopic expression of TOX alters T cell development as a result of changes in expression of the TCR or associated signaling proteins (3). We had reported previously a decrease in the CD4SP to CD8SP thymocyte ratio in TOX-Tg mice (31). This phenotype is now understood by the ability of TOX to promote CD8SP development in the absence of positive selection. The reduction of CD4SP thymocytes in these mice is likely due to a preemption of normal positive selection by early expression of the TOX transgene in DP thymocytes.

Positive selection requires TCR-mediated activation of MAPK and Cn signaling pathways. How these pathways are integrated by the cell and how they regulate lineage commitment remains unknown, although a number of lines of evidence suggest that the MAPK pathway can have an overriding deterministic effect on the CD4 T cell fate (11, 16–19). We have shown here that up-regulation of TOX in DP thymocytes is Cn dependent, providing a link between this signaling pathway and CD8 lineage commitment. Expression of TOX induced differentiation of CD8SP thymocytes independent of expression of MHC or TCR or activation of Cn. However, TOX is not sufficient to induce CD4SP development under these same conditions, suggesting a requirement for additional downstream effectors for CD4 T cell development. One such factor, GATA-3, has been shown recently to promote differentiation of thymocytes to the CD4 fate while inhibiting CD8SP development (64). Moreover, GATA-3 expression has been linked with “strong” TCR signaling (64), correlating with previous findings that the strength of TCR signaling can bias lineage commitment decisions.



These data lead to a model where development of CD4 and CD8 lineages each require both MAPK- and Cn-mediated signals. The strength and balance between these signaling pathways determines cell fate. Cn-mediated signaling during the initiation of positive selection to either T cell lineage results in up-regulation of TOX. Unopposed, TOX plays a role in CD4 silencing and CD8 lineage commitment, possibly via up-regulation of Runx3 and/or changes in chromatin structure. However, in the presence of sufficient MAPK signaling and possibly additional Cn-mediated signals (17) the effect of TOX is negated, leading to CD4 lineage commitment. This model explains why CD4SP development is not affected by expression of TOX in circumstances where there is a potent TCR signal and where timing of TCR and TOX expression are coincident, such as in TOX/AND TCR double Tg mice. We predict that one function of increased/prolonged signaling during positive selection to the CD4 lineage is induction or activation of an inhibitor of TOX. One intriguing possibility is a role for GATA-3 in this activity. However, since sustained expression of GATA-3 is not sufficient to divert class I MHC-restricted developing T cells into the CD4 lineage (64), it seems unlikely that GATA-3 alone would function in this regard. Nevertheless, one might predict that the threshold of signaling that is necessary to compete with TOX will also be the set point for CD4 lineage commitment (16).

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