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Overlapping and Asymmetric Functions of TCR Signaling during Thymic Selection of CD4 and CD8 Lineages

Charles Sinclair¹ and Benedict Seddon

TCR signaling plays a central role in directing developmental fates of thymocytes. Current models suggest TCR signal duration directs CD4 versus CD8 lineage development. To investigate the role of TCR signaling during positive selection directly, we switched signaling off in a cohort of selecting thymocytes and followed, in time, their subsequent fate. We did this using an inducible Zap70 transgenic mouse model that allowed Zap70-dependent signaling to be turned on and then off again. Surprisingly, loss of TCR signaling in CD4⁺CD8⁻ thymocytes did not prevent their development into committed CD4 single positives (SPs), nor their continued maturation to HSA⁺ SPs. However, numbers of CD4 SPs underwent a substantial decline following loss of Zap70 expression, suggesting an essential survival role for the kinase. Termination of TCR signaling is considered an essential step in CD8 lineage development. Loss of Zap70 expression, however, resulted in the rapid death of CD8 lineage precursor thymocytes and a failure to generate CD8 SPs. Significantly, extending the window of Zap70 expression was sufficient for generation and export of both CD4 and CD8 T cells. These data reveal a parallel requirement for TCR-mediated survival signaling, but an asymmetric requirement for TCR-mediated maturation signals.


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Abbreviations used in this article: BM, bone marrow; CTV, CellTrace Violet; dox, doxycycline; dox.Id., fed dox for a single day; DP, double positive; met, methacycline hydrochloride; KO, knockout; RNA-seq, RNA sequencing; SP, single positive; Thalf, half-life; WT, wild-type.

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The online version of this article contains supplemental material.

Materials and Methods

Mice

C57BL/6J [wild-type (WT)] and congenic C57BL/6J.SJL-Pipra<sup>+</sup> (WT CD45.1 from here on) were used as control strains. b2m<sup>-/-</sup> and B6.129-H-2<sup>−/−</sup> (Mhc-II<sup>-/-</sup> from here on) (12) were bred with Rag1<sup>-/-</sup> mice to generate Rag1<sup>-/-</sup>b2m<sup>-/-</sup> and Rag1<sup>-/-</sup>Mhc-II<sup>-/-</sup> mice, respectively. Zap70<sup>-/-</sup> tetracycline-inducible Zap70 mice (TetZap70 from here on) and F5 Rag1<sup>-/-</sup>TetZap70 mice have been described previously (11). The Nur77-GFP reporter allele (13) was additionally bred onto the Nur77-GFP background to generate Nur77-GFP TetZap70 mice. All experiments with TetZap70, Nur77-GFP TetZap70, or F5 TetZap70 strains were performed with thymocytes obtained from bone marrow (BM) chimeric mice. Chimeras were generated by transferring 5 × 10<sup>6</sup> BM cells into sublethally irradiated (500 rad) Rag1<sup>-/-</sup>, Rag2<sup>-/-</sup>, Rag1<sup>-/-</sup>b2m<sup>-/-</sup>, or Rag1<sup>-/-</sup>Mhc-II<sup>-/-</sup> hosts, and allowing ≥4 wk for reconstitution. Mice were bred and housed in specific pathogen–free conditions at the Medical Research Council National Institute for Medical Research (London, U.K.), and experiments were performed in accordance with U.K. home office regulations. All mice were analyzed at 5–12 wk of age.

To induce Zap70 expression in different temporal windows, TetZap70 and F5 TetZap70 mice were fed a 3% (w/w) doxycycline (dox)–containing diet.
dist either continuously (dox) or overnight (doxId), or were given a single i.p. injection of 2 mg methacycline hydrochloride (met) (Vetranal [JLUKA]; Sigma-Aldrich) dissolved in distilled H2O and neutralized to pH 7 (met).

To constitutively inhibit thymic egress, Fty720 (Selleck Chemicals) was dissolved at 10 μg/ml in 10% specific pathogen-free mouse serum obtained from Parkes mice, and 2 mg/kg was injected i.p., bidurally for the duration of the experiment.

**Abs, flow cytometry, and cell sorting**

The following Abs were used in this study and purchased from eBioscience or BioLegend, unless otherwise indicated: unconjugated rabbit mAb against Egr1 (clone 44D5; Cell Signaling Technology); biotinylated Ab against CD45.1, CD45.2, and CD24 (HSA); FITC-conjugated Abs against CD5, HSA, CD45.1, and CD45.2; PE-conjugated Abs against Bcl-2, CD69, CD127 (IL-7Rα); Zap70 and rabbit-IgG (Jackson ImmunoResearch); PE Texas Red–conjugated Ab against CD4; PeCy7-conjugated Abs against CD5 and CD8; APC-conjugated Abs against TCR–β-chain and human/mouse Runx3 (clone 527327; R&D Systems); Efluor 450-conjugated Ab against CD4 and CD8; and Pacific Orange–conjugated Ab against CD8. Biotinylated Abs were detected with streptavidin conjugated to Pacific Orange (Invitrogen).

Detection of surface Ags was performed with 2–5 × 10^6 cells, stained in 100 μl PBS containing 0.1% (v/v) BSA on ice in the dark for 1 h, as described previously (11, 14). Detection of Annexin V was performed using the Annexin V Apoptosis Detection Kit (eBioscience) according to the manufacturer’s instructions.

For subsequent detection of intracellular Zap70 expression, cells were fixed with IC Fixation Buffer (eBioscience), permeabilized for 3 min with 0.1% Nonidet P-40 (Igepal ca-630; Sigma-Aldrich), and stained for 12 h on ice against CD4 and CD8; and Pacific Orange–conjugated Ab against CD8. Biotinylated Abs were detected with streptavidin conjugated to Pacific Orange (Invitrogen).

Detection of Ags was performed with 2–5 × 10^6 cells, stained in 100 μl PBS containing 0.1% (v/v) BSA on ice in the dark for 1 h, as described previously (11, 14). Detection of Annexin V was performed using the Annexin V Apoptosis Detection Kit (eBioscience) according to the manufacturer’s instructions.

For subsequent detection of intracellular Zap70 and Runx3 expression, cells were fixed and permeabilized using the Foxp3 Fixation/Permeabilization Kit (eBioscience) according to the manufacturer’s instructions. Staining was performed in 1× permeabilization buffer for 1 h at room temperature (Runx3) or on ice (Egr1).

Flow cytometry was performed using a BD FACS Canto II (Becton Dickinson) or Cyan ADP (Beckman Coulter) analyzer. Cell sorting was performed using a BD FACSAria II (Becton Dickinson) or MoFlo XDP (Beckman Coulter) instrument. Data were analyzed using FlowJo software (v9.4.11, TreeStar).

**RNA sequencing**

Total RNA was prepared from cell-sorted populations with TRIzol according to the manufacturer’s instructions. RNA sequencing (RNA-seq) libraries were prepared using the mRNA Expression Seq-8 Sample Prep Kit (Illumina) and the Illumina duplex-specific nuclease protocol (15), according to the manufacturer’s instructions. Samples were sequenced in the Medical Research Council National Institute for Medical Research High Throughput Sequencing Facility, using an Illumina Genome Analyzer Ix. A total of 36 base-pair single-end reads were obtained using the Illumina pipeline, and aligned to the *Mus musculus* genome (mm9 assembly) using CLC Genomic Workbench (v5) with standard settings. Aligned reads were mapped to the RefSeq Database and DESeq normalized (16) using Avadis NGS software (v1.4.7). Gene expression was displayed as normalized reads per kilobase of exon per million reads.

**Intrathymic injections**

Intrathymic injection by blind injection into the thoracic cavity has been described (17, 18). Briefly, host mice were anesthetized via inhalation of isoflurane (Isoflow; Abbott). An incision ~1 cm was made along the midline overlying the lower cervical and upper thoracic region. Cells were suspended at 2–5 × 10^5 in air-buffered IMDM, and 10 μl suspension was injected into the anterior superior portion of each thymic lobe, using a Tridak Stepper pipette (Tridak). Mice were treated with the local anesthetic lidocaine for 10 min, using a 30-gauge needle through each of the incisions, resulting in a population comprising 90% DP1 cells.

**Measurement of thymocyte survival**

Thymocytes from Zap70^−/− mice were enriched for CD8α^+ cells, using MACS LS separation columns according to the manufacturer’s instructions, resulting in a population comprising ~90% DP1 cells. Enriched cells were labeled with CellTrace Violet (CTV; 4 mM) at a density of 10^7/ml in PBS containing 0.05% (v/v) BSA at 37°C for 10 min. Excess CTV was quenched by performing two washes with PBS containing 0.1% (v/v) BSA. Labeled cells were mixed with test populations of interest and intrathymically cotransferred to congenic B6 CD45.1 hosts for 2 d. The ratios of CTV− (test population)/CTV+ (CD8α-enriched Zap70^−/− “sensitive”) cells were determined before and after transfer by flow cytometry. Relative survival rates could be calculated as the (postinjection ratio) / (preinjection ratio).

**Retroviral transduction BM chimeras**

Donor mice were treated with 100 mg/kg 5-fluorouracil i.p., and BM was harvested on day 5. BM cells (2.5 × 10^6) were cultured at 37°C/10% CO2 in complete DMEM (10% FCS, 20 mM HEPES, 1× nonessential amino acids, and 2-mercaptoethanol and antibiotics), along with stem cell factor (100 ng/ml), IL-3 (6 ng/ml), IL-6 (10 ng/ml), and human/mouse Runx3 (10 ng/ml) overnight to expand stem cells.

Transduction of BM, MSCV-Bcl-2-IRES-HuCD2 retroviral particles (1.25 × 10^7 in 300 μl, a kind gift from Dr. Owen Williams [Institute for Child Health, University College London]) were adsorbed to RetroNectin (Takara)-coated six-well tissue culture plates for 30 min at 37°C. Total BM cultures were then introduced to viral-adsorbed plates. The transduction procedure was repeated for 3 d consecutively, with the addition of stem cell factor (100 ng/ml), IL-3 (6 ng/ml), and IL-6 (10 ng/ml) on the second day. Transduced cells (2.5 × 10^6) were transferred to sublethally irradiated Rag1−/− hosts i.v., and host mice were analyzed after 6 wk.

**Statistics**

Statistical analysis and preparation of figures were accomplished using Graphpad Prism 6 (v6.0a). Half-lives (T_{1/2}) of thymocytes were estimated by nonlinear regression one-phase exponential decay curve fitting to data using Graphpad Prism 6 (v6.0a), using least squares fit. Per cell per day death rates were calculated as ln(2)/T_{1/2}.

**Results**

**Onset of thymocyte development following a pulse of Zap70 expression**

To evaluate the temporal requirement for TCR signaling during thymic selection, we used a previously described mouse model in which the kinase Zap70, critical for normal TCR signaling, is under control of tetracycline-inducible elements (TetZap70) (11). Thymic development in Zap70^−/− mice is arrested at the CD4^+CD8^− double positive (DP) stage (19). Following administration of inducing tetracycline derivatives dox or met, Zap70 expression is detectable at 8 h and within physiological range by 16 h, allowing restoration of positive selection (11). Dox and met both induce Zap70 expression in a comparable manner (14). To study how premature TCR signal termination influences thymic selection, we induced Zap70 expression in TetZap70 mice with a single injection of met and followed the developmental fates of thymocytes. Maximal Zap70 induction by met was observed at 24 h, was reduced by 48 h, and was lost by 72 h (Fig. 1A) (14). Measuring TCR and CD5 expression on thymocytes revealed restoration of thymic selection in dox-fed mice (Fig. 1B). Abundance of CD5^hi TCR^β^ cells increased with time, reaching plateau at day 6. In met-injected mice, the abundance of CD5^hi TCR^β^ cells increased in a similar manner until day 2, but reduced thereafter, coincident with loss of Zap70 expression. Previously, we showed that a population of CD4^+CD8^− thymocytes first appears ~24–48 h after dox administration and mature CD4 SPs thereafter. CD5, Nur77, Egr1, and Gata3 expression all depend on TCR signaling in thymocytes (13, 20–23). Therefore, to determine when TCR signaling had ceased in met-treated mice, we examined CD4 SP cells at different times for expression of these markers. At day 2, expression of these markers was comparable in both dox- and met-treated mice (Fig. 1C). At day 3, all three markers were already reduced in CD4 SPs from met-treated mice, and continued to fall thereafter (Fig. 1C). Analysis of gene expression at day 3 confirmed the loss of upstream inducing signals for these markers. CD5 and Nur77 mRNA had dropped to background levels of nonselecting (DP1) thymocytes, whereas Egr1 mRNA levels were substantially reduced. Significantly, ThPOK expression was maintained at similar levels in CD4 SPs from met-treated and dox-fed mice (Fig. 1D), confirming that CD4 SPs in met-treated mice
were lineage committed, despite loss of Gata3 expression at these time points, which is a key upstream signal (2, 23). Therefore, consistent with the absence of Zap70 protein at day 3, transcriptional targets downstream of TCR signaling were already reduced or at background levels by this time, showing that TCR signaling had been terminated.

No lineage diversion of CD4 SP thymocytes upon termination of TCR signaling

We first examined the consequence of losing TCR signaling in newly generated CD4+CD8lo thymocytes. Termination of TCR signaling in this subset is thought to be a key event in directing cells into a CD8 lineage fate. To test this, we analyzed thymic development in mice treated with either continuous dox feeding or a single injection of met, using a previously characterized temporal scheme of DP maturation (11). Briefly, three populations of DP thymocytes at distinct temporal stages of development can be identified using TCR and CD5 markers. TCRloCD5lo DP1 thymocytes constitute preselection thymocytes; TCRintCD5hi DP2 thymocytes consist of class I– and class II–restricted thymocytes between 12 and 48 h of development; and TCRhiCD5int DP3 thymocytes are exclusively CD8 lineage cells and develop by ~60 h after onset of selection (Supplemental Fig. 1). Following met treatment, the DP2 population increased in size, similar to dox controls, until day 2, but subsequently underwent a rapid contraction in the absence of continued Zap70 expression (Fig. 2A).
No thymocytes falling into a CD4 SP gate were evident in TetZap70 mice until day 2 after Zap70 induction and could represent either uncommitted CD4⁺CD8⁺ cells or fully committed CD4 lineage T cells (Supplemental Fig. 1). Development of CD4 SP in met-treated mice was similar to that in dox-treated controls until day 3. Thereafter, the compartment underwent a sustained contraction (Fig. 2B). To see whether loss of CD4 SPs was because CD4⁺CD8⁺ cells were diverted to the CD8 lineage in the absence of TCR signaling, we examined the development of DP3 and CD8 subsets. Like the CD4 SP compartment, the DP3 subset grew in met-treated mice until day 3, but also underwent a rapid contraction in subsequent days (Fig. 2C). Surprisingly, very few CD8 SP thymocytes were observed in met-treated mice at later time points (Fig. 2D). Finally, to explicitly seek evidence of lineage diversion among thymocytes expressing class II-restricted TCRs, we examined development in BM chimeras of 

\[ \text{Rag}^{1/-} \text{b2m}^{1/-} \text{deficient hosts reconstituted with BM from TetZap70 donors [MhcI knockout (KO) TetZap70], compared with control chimeras using MHC-sufficient \text{Rag}^{1/-} \text{hosts. The absence of class I MHC expression had no detectable impact on the generation of CD4 lineage thymocytes. CD4 SP development proceeded with identical kinetics and magnitude to that in b2-microglobulin–expressing control chimeras (Supplemental Fig. 2). Analysis of CD4 SP and CD8 SP subsets in dox-fed and met-treated MhcI KO TetZap70 chimeras confirmed results obtained in intact mice (Fig. 2E, 2F). The contraction of the CD4 SP compartment in met-treated mice could not be accounted for by successful diversion and development to the CD8 lineage.} \]

**CD4 lineage thymocytes mature, but do not survive, in the absence of continued Zap70 expression**

Although the CD4 SP compartment contracted in met-treated mice, there remained a significant population of cells until at least day 7. Phenotypic analysis of CD4 SP thymocytes revealed a remarkably normal maturation process in the absence of Zap70 expression. Although CD4 SPs were exclusively immature HSA⁺ at day 3, the following days, cells acquired a mature HSA⁺ phenotype and progressively lost CD69 expression (Fig. 3A). Given their apparently normal maturation, we asked whether the decline in CD4 SPs was simply due to egress of mature cells. To assess this, we blocked S1P-dependent thymic egress in TetZap70 mice with the S1P-receptor agonist Fty720. In dox-fed mice, the effects of Fty720 were not apparent until day 6, in line with previous work showing that T cells egress ~6 d after onset of selection (11, 24). Significantly, Fty720 treatment of met-injected mice did not result in an increase of the CD4 SP compartment between days 3 and 6, when the greatest contraction was observed (Fig. 3B). At day 7 and later, slightly more CD4 SPs were found in Fty720-treated hosts, suggesting some egress in met-treated mice at these time points. However, these results indicate that the substantial decline in CD4 SP thymocytes (Thalf of 1.28 d) between day 3 and day 6 was not due to their egress. Having excluded lineage diversion and egress as the causes of observed contraction in the CD4 SP compartment, we finally asked whether the death of CD4 SP thymocytes in the absence of Zap70 expression could account for the cell loss. Analyzing markers of apoptosis on CD4 SPs from met-treated mice revealed increasing frequencies of apoptotic cells that correlated with the decline in CD4 SPs (Supplemental Fig. 3A). Therefore, to directly measure cell survival in vivo, purified CD4 SP thymocytes were isolated from dox-fed and met-injected TetZap70 mice at day 3, when the CD4 SP compartment of both groups are comparable in size. Purified populations were then injected intrathymically into Fty720-treated WT hosts, together with a dye-labeled tracer cohort of Zap70⁻/⁻ thymocytes as controls. Recipients of thymocytes from dox-fed donors were also fed dox to maintain Zap70 expression in the donor population. Phenotypic analysis of donor populations 2 d later confirmed that thymocytes remained CD4 SPs (Fig. 3C) and did not give rise to long-lived CD8 SP thymocytes. However, there was ~4-fold reduction in the recovery of donor thymocytes from met-treated donors compared with controls (Fig. 3D), a comparable reduction to that observed in met-treated mice between days 3 and 5. Bcl2 expression is upregulated during positive selection (18). Analyzing Bcl2 protein levels in CD4 SP from dox- and met-treated mice revealed increased frequencies of apoptotic cells that correlated with the decline in CD4 SPs (Supplemental Fig. 3A). Therefore, to directly measure cell survival in vivo, purified CD4 SP thymocytes were isolated from dox-fed and met-injected TetZap70 mice at day 3, when the CD4 SP compartment of both groups are comparable in size. Purified populations were then injected intrathymically into Fty720-treated WT hosts, together with a dye-labeled tracer cohort of Zap70⁻/⁻ thymocytes as controls. Recipients of thymocytes from dox-fed donors were also fed dox to maintain Zap70 expression in the donor population. Phenotypic analysis of donor populations 2 d later confirmed that thymocytes remained CD4 SPs (Fig. 3C) and did not give rise to long-lived CD8 SP thymocytes. However, there was ~4-fold reduction in the recovery of donor thymocytes from met-treated donors compared with controls (Fig. 3D), a comparable reduction to that observed in met-treated mice between days 3 and 5. Bcl2 expression is upregulated during positive selection (18). Analyzing Bcl2 protein levels in CD4 SP from dox- and met-treated mice revealed that Bcl2 upregulation occurred normally in these cells (Supplemental Fig. 3B), suggesting that a defect in Bcl2 expression was not responsible for the loss of cells in met-treated mice. Consistent with this view, attempts to rescue SP cell development in met-treated mice by overexpressing Bcl2 were unsuccessful (Supplemental Fig. 3C).
Continuous Zap70 expression is essential for generation of CD8 SP thymocytes

To investigate the consequences of premature TCR signal termination for generation of mature CD8 SPs, we analyzed class I–restricted thymocyte development both in chimeras of Rag1<sup>−/−</sup> Mhc-II<sup>−/−</sup> hosts reconstituted with BM from TetZap70 donors [MhcII KO TetZap70] and in class I–restricted TCR transgenic F5 Rag1<sup>−/−</sup> TetZap70 mice. Importantly, development of CD8 SPs was similar between MhcII KO TetZap70 and control TetZap70 chimeras (Supplemental Fig. 2). Therefore, we saw no evidence of a detectable population of class II–restricted cells that were also class I restricted and could supplement the CD8 SP subset in the absence of MhcII expression; there was also no suggestion that the presence of CD4 SPs influenced CD8 SP development. Next, MhcII KO TetZap70 chimeras were injected with met or fed dox continuously as control. By day 3, the DP3 subset increased in abundance similarly in thymi from both met- and dox-treated mice. Following continued Zap70 expression in dox-fed chimeras, CD8 SP thymocytes appeared at day 4 and accumulated thereafter. In met-treated mice, the DP3 subset contracted after day 3, and was lost by day 5 (Fig. 4A). Contraction of the DP3 subset was not a consequence of onward development toward a CD8 lineage fate, as no corresponding increase in CD8 SP cell abundance was observed. Rather, the CD8 SP compartment underwent a small increase at day 4 that was not sustained in the following days. To look more closely at CD8 lineage development, we performed similar experiments using TCR transgenic F5<sup>Rag1<sup>−/−</sup></sup> TetZap70 mice in which class I–restricted cells are present at a much higher frequency than in thymi with polyclonal repertoires. Met treatment of F5<sup>Rag1<sup>−/−</sup></sup> TetZap70 mice resulted in a wave of F5 thymocyte development (Fig. 4B). DP1 subset contracted with a corresponding short-lived increase in DP2 population. DP3 development peaked at day 3 but underwent a rapid contraction thereafter. Cells falling in the CD8 SP gate were more evident in met-treated F5<sup>Rag1<sup>−/−</sup></sup> TetZap70 mice than in polyclonal chimeras and, interestingly, they appeared largely as
CD4loCD8+ phenotype (Fig. 4B, Supplemental Fig. 4), peaking in abundance by day 5. Thereafter, this population underwent a rapid contraction in size.

Runx3-deficient mice have a reduced CD8 SP compartment, and cells exhibit a CD4loCD8+ phenotype (25), similar to that observed in met-treated mice. We therefore asked whether alterations in Runx3 protein levels following manipulation of Zap70 expression could account for defective CD8 lineage development in met-treated TetZap70 mice. Upregulation of Runx3 protein was readily detectable in WT DP3 thymocytes (Fig. 4C). Interestingly, both DP3 and CD8 SPs from dox-fed mice had reduced levels of Runx3 compared with WT controls, consistent with the previously reported reduction in CD8 SP development in TetZap70 mice (11). In met-treated TetZap70 mice, Runx3 abundance in DP3 thymocytes
was lower than in either dox-fed or WT controls. Although differences in Runx3 protein between met- and dox-treated mice were small, similar differences were also observed when RNA levels were also measured in these populations, which correlated well with observed protein expression (Fig. 4D). The few CD8 SP thymocytes present in these mice did express higher Runx3 than did DP3 thymocytes, but it was reduced compared with that expressed in CD8 SPs from either WT or dox-fed control TetZap70 mice (Fig. 4E). Therefore, the extent to which Runx3 expression was induced in WT and TetZap70 mice correlated with onward development to the CD8 SP compartment.

Extending the temporal window of Zap70 expression restores both development and egress of CD4 and CD8 T cells

As we could detect no CD8 SPs and little egress of CD4 SPs in met-treated TetZap70 mice, we asked what minimal window of Zap70 expression was required for development and egress of mature T cells. To find out, we fed dox for a single day (dox1d) to TetZap70 mice. Because dox has a longer half-life than met in vivo, Zap70 expression in these mice was maintained until day 2 before expression was lost by day 4 (Fig. 5A), a day longer than in mice treated with met. The increased duration of Zap70 expression resulted in an increased expansion of both CD4 SP and DP3 populations that peaked at day 5, before both populations contracted (Fig. 5B). Significantly, the CD8 SP compartment now also underwent a sustained increase in size, peaking at day 6, before contracting in size. Finally, to determine whether SP thymocytes generated in dox1d treatment were capable of egress, mice were additionally treated with Fty720 to prevent SP emigration. Significantly, Fty720 treatment resulted in increases in both CD4 SP and CD8 SP compartments, which reached a plateau maintained until day 8, after which both subsets contracted with T half of 1.14 d and 0.9 d respectively (Fig. 5C). Analysis of HSA expression revealed that CD8 SPs generated in dox1d-treated mice continued to mature and lose HSA in the absence of continued Zap70 expression (Fig. 5D), as was observed among CD4 SP thymocytes (Fig. 3A).

TCR signaling tunes Runx3 expression in vivo

In dox-fed TetZap70 mice, induction of Runx3 expression in DP3 and CD8 SP thymocytes was suboptimal compared with that in WT. Furthermore, expression was even lower in met-treated TetZap70 mice that only transiently express Zap70 and fail to develop mature CD8 SPs. Because dox1d-treated TetZap70 mice gave rise to CD8 SP thymocytes that subsequently lose Zap70 expression, we also analyzed Runx3 expression to see if continued expression was dependent on continued Zap70 expression. Comparing Runx3 expression by thymocytes from dox- and dox1d-treated mice revealed similar levels in DP3 thymocytes at day 4, but slight reduction in CD8 SPs (Fig. 6A, 6B). Measuring expression over the following 3 d revealed that Runx3 expression in DP3 thymocytes is gradually lost with time. Similarly, although Runx3 expression was stably expressed in dox-fed mice, Runx3 protein levels decreased with time until day 7, preceding the subsequent contraction in the CD8 SP compartment. These data suggest that continued Zap70 expression plays a role in maintaining Runx3
expression in CD8 SP thymocytes and, therefore, CD8 lineage identity.

Discussion

In this study, we investigated the temporal requirements for Zap70-dependent TCR signaling during thymopoiesis. Although we found no evidence that premature termination of TCR signaling controlled thymic SP lineage decisions, we instead found that Zap70 expression is essential for survival of all stages of thymocyte development of both CD4 and CD8 lineages. A short-pulse Zap70 expression was sufficient to generate a wave of selecting cells, including CD4 SP thymocytes by day 2 of protein induction. Surprisingly, these CD4 SPs were already committed within ~48 h because they expressed high levels of ThPOK independent of Zap70 expression. In addition, abrogation of TCR signaling in CD4 SPs thereafter did not result in their development into CD8 SP or precursor DP3 populations. The absence of detectable CD8 SP development in met-treated mice also suggests that TCR signal termination either failed to divert less mature DP2 thymocytes to a CD8 lineage fate, or if diversion was a potential fate, cells did not survive long enough in the absence of TCR signaling for redirection to become apparent or detectable. Whatever is the case, it was clear that generation of CD8 SP thymocytes was reliant on intact TCR signaling.

Zap70 expression was required for generation of CD4 SP and CD8 SP thymocytes. Of interest, once SPs had developed, their continued maturation no longer appeared to require Zap70 signaling. CD4 SPs in met-treated mice, and CD8 SPs generated by extending the window of Zap70 expression in dox1d-treated mice, both continued to mature by downregulating HSA expression. These data suggest that the characteristic maturation program of SPs operates independently of TCR signaling (26). Maturation of SP thymocytes involves NF-κB signaling (27, 28), and induction of KLF2 to promote thymic egress (29). Our data clearly indicate that TCR-independent mechanisms control these late SP maturation events. In contrast, continued survival of SPs remained strictly dependent on Zap70, regardless of maturation stage. The T\textsubscript{nat} of HSA\textsuperscript{hi} SPs or more mature HSA\textsuperscript{lo} SPs were all similar (∼1 d). Significantly, death rates of HSA\textsuperscript{hi} CD4 SPs, HSA\textsuperscript{hi} CD4 SPs, and HSA\textsuperscript{lo} CD8 SPs in the absence of Zap70 expression were 0.54, 0.6, and 0.78/cell/day, respectively. These rates are >10 times higher than death rates estimated for Zap70-expressing SPs (18), which specifically include negative selection. Therefore, the death of Zap70-deficient SPs we observed most likely represents loss of positively selected thymocytes, rather than perturbation to ongoing negative selection, which would account for only ~10% of observed cell loss. Consistent with this view, recoveries of Zap70-deficient CD4 SPs from met-treated mice following their adoptive transfer were also substantially lower than those of Zap70-expressing CD4 SPs from dox-fed donors, whose recovery will reflect the combination of both survival and death by negative selection.

In contrast to CD4 lineage thymocytes, a short window of Zap70 expression was insufficient to generate CD8 SPs. We found evidence that Runx3 expression was reduced when Zap70 signaling was cut off in met-treated mice. Furthermore, prior to maximal contraction of CD8 SPs in met-treated F5 Rag1\textsuperscript{−/−}TetZap70 mice at day 5, thymocytes resembled CD8 lineage cells found in Runx3-deficient mice that have a CD4\textsuperscript{lo}/CD8\textsuperscript{hi} phenotype (25). Although loss of Runx3 coincided with contraction of the CD8 SP compartment in dox1d-treated mice, it is unclear whether this was a causal event, and further studies should investigate whether Runx3 regulates CD8 SP survival. Although previous studies have implicated a role for IL-7 signals in Runx3 induction in vitro (4), Runx3 expression is maintained when either IL-7R or γ-chain cytokine receptors are ablated in vivo (30, 31). Our data are, to our knowledge, the first demonstration that Runx3 may be also regulated by TCR signaling during late-stage CD8 SP differentiation.

Surprisingly, we found that in addition to impaired CD8 SP maturation, the survival of both CD4 and CD8 SP thymocytes was profoundly reduced in the absence of continuous Zap70 expression. Thus, although CD4 and CD8 SPs have asymmetric requirements for Zap70-dependent maturation signals, they appear to share a parallel necessity for Zap70-dependent survival signaling, mirroring the survival requirements of peripheral naive T cells of both lineages (32). The observed requirement for TCR-dependent thymic survival signals places specific constraints on how the TCR directs development. Although cessation of TCR signaling was evoked as a mechanism to direct developing thymocytes to a CD8 lineage (6, 33), our data show that a complete cessation of signaling results in rapid death of CD8 SPs and their progenitors. If complete loss of TCR signaling does redirect cells to a CD8 fate, such an outcome is superseded by a more acute need for Zap70-dependent survival signals, and experimental redirection of thymocytes was not detectable in this study, either in intact mice or among cohorts of adoptively transferred CD4\textsuperscript{+}CD8\textsuperscript{+} thymocytes. Furthermore, in met-treated mice Zap70 expression was lost between day 2 and day 3, whereas mRNA levels of immediate early genes CD5 and Nr4a1 had returned to background by day 3, and mRNA for Egr1 took an extra day to reach baseline. However, protein expression for these genes took considerably longer to decay. Thus, although transcriptional targets of TCR signaling can...
be acutely regulated, the downstream consequences for protein expression are likely to be far longer lived. This observation implies that coreceptor modulation is incompatible with acute control of the CD8 lineage decision per se, although conceivably it could be interpreted in terms of more subtle quantitative change in TCR-regulated gene expression. Weak TCR signaling could play a permissive role in differentiation by promoting CD8 lineage cell survival while failing to trigger a CD4 lineage fate. Indeed, a dual role of TCR signaling to promote survival, coupled with a reliance on quantitatively distinct TCR signaling to distinguish selection of different lineages, may be expected to specifically disadvantage those cells reliant on weaker signals, specifically CD8 SPs (13). Of note, although similar proportions of class I– and class II–restricted thymocytes actively participate in development, we recently reported that death is far greater among selecting class I– than class II–restricted cells (18), and this may be accounted for by reduced TCR survival signaling delivered to class I–restricted repertoire.

Finally, thymic TCR-dependent survival signaling may play an important role in selecting the mature TCR repertoire. Cortical thymic epithelial cells that initiate positive selection present a distinct array of self-peptides because they express unique proteolytic machinery, including cathepsin L, thymus-specific serine protease, and the β5, proteosomal subunit (34–36). Given that maturing SP thymocytes undergo corticomedullary migration, our results suggest a novel selective mechanism imparting broad peptide reactivity to the peripheral T cell compartment. We predict that a continuous thymic requirement for TCR signaling will selectively purge cells restricted exclusively to cortically limited self-peptide. This may represent a third form of thymic deletion, distinct from negative selection or death by neglect. Rather, thymocytes are “selected then neglected” upon reaching the thymic medulla.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplementary material

Figure S1 - Restoration of thymic development in dox and met treated TetZap70 mice. TetZap70 mice were continuously fed dox (A) or given a single injection of met i.p. (B) to induce Zap70 expression, and phenotype of cells analysed by flow cytometry on days 1-7. Pseudo-color plots show expression of CD4 versus CD8 on total thymocytes, CD5 versus TCR on DP thymocytes, and CD4 and CD8 on total TCR^{hi}CD5^{hi} mature thymocytes. Data are representative of ≥3 independent experiments.
Figure S2 - Loss of MhcI or MhcII expression does not perturb remaining MHC restricted selection

*Rag1<sup>−/−</sup> b2m<sup>−/−</sup> IAb<sup>−/−</sup> and *Rag1<sup>−/−</sup> mice were irradiated (500 rads) and reconstituted with bone marrow from TetZap70 donors to generate MhcI KO TetZap70, MhcII KO TetZap70 and Mhc+ control TetZap70 chimeras respectively. Following reconstitution, MhcI KO TetZap70, MhcII KO TetZap70 or Mhc+ control TetZap70 chimeras were fed dox for 0-10 days to induce Zap70 expression. Line graphs show the frequency of CD4 or CD8 SP thymocytes from indicated strains.
Figure S3 - Increased markers of apoptosis in CD4 SP from met treated mice but failure of Bcl2 over-expression to rescue cell loss.

Tet Zap70 mice were continuously fed dox or given a single injection of met i.p. to induce Zap70 expression, and phenotype of cells analysed by flow cytometry on days 2-7. (A) Graph is of frequency of CD4 SP thymocytes that were stained +ve with annexin V and also had sub-diploid DNA content as determined by 7AAD DNA staining. (B) Histograms are of Bcl2 expression by CD4 SP from met treated mice (red line) compared with CD4 SP from dox fed mice (black line), DP1 thymocytes (grey fill) as compared with isotype negative control staining (dotted line). (C) Bone marrow from 5-FU treated donor Tet Zap70 mice was transduced in vitro with a MSCV-Bcl2-IRES-HuCD2 expressing retrovirus. Irradiated Rag1⁻/⁻ hosts were reconstituted with transduced bone marrow cells and left to analyzed after six weeks. Mice were given a single injection of met. Thymi of mice were
then analysed at days 3 and 5 after injection. Density plot shows Bcl2 vs huCD2 expression by whole thymus from chimeras 3 days after injection of met. Boxes indicate gates used to identify huCD2_{hi} transduced cells vs huCD2_{lo} non-transduced thymocytes. Histograms are of Bcl2 expression by DP, CD4 SP, CD8 SP thymocytes by huCD2_{hi} (black lines) vs huCD2_{lo} (grey fills) cells. Density plots (right) are of CD4 vs CD8 expression by huCD2_{hi} and huCD2_{lo} cells from met treated chimeras at days 3 and 5 after injection.
Figure S4 - Restoration of thymic development in dox and met treated F5 \( \text{Rag}^{1/-} \) TetZap70 mice. F5 \( \text{Rag}^{1/-} \) TetZap70 mice were continuously fed dox (A) or given a single injection of met i.p. (B) to induce Zap70 expression, and the phenotype of cells was analysed by flow cytometry on days 1-7. Pseudo-color plots show expression of CD4 versus CD8 on total thymocytes, CD5 versus TCR on DP thymocytes, and CD4 versus CD8 on total TCR\textsuperscript{hi}CD5\textsuperscript{hi} mature thymocytes. Data are representative of ≥3 independent experiments.