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The Tcrb locus is subject to a host of regulatory mechanisms that impart a strict cell and developmental stage-specific order to variable (V), diversity (D), and joining (J) gene segments. The Tcrb locus is also regulated by allelic exclusion mechanisms, which restrict functional rearrangements to a single allele. The production of a functional rearrangement in CD4+CD8– double-negative (DN) thymocytes leads to the assembly of a pre-TCR and initiates signaling cascades that allow for DN to CD4+CD8– double-positive (DP) differentiation, proliferation, and feedback inhibition of further Vβ to DJβ rearrangement. Feedback inhibition is believed to be controlled, in part, by the loss of Vβ gene segment accessibility during the DN to DP transition. However, the pre-TCR signaling pathways that lead to the inactivation of Vβ chromatin have not been determined. Because activation of the MAPK pathway is documented to promote DP differentiation in the absence of allelic exclusion, we characterized the properties of Vβ chromatin within DP thymocytes generated by a constitutively active Raf1 (Raf-CAAX) transgene. Consistent with previous reports, we show that the Raf-CAAX transgene does not inhibit Tcrb recombination in DN thymocytes. Nevertheless, DP thymocytes generated by Raf-CAAX signals display normal down-regulation of Vβ segment accessibility and normal feedback inhibition of the Vβ to DJβ rearrangement. Therefore, our results emphasize the distinct requirements for feedback inhibition in the DN and DP compartments. Although MAPK activation cannot impose feedback in DN thymocytes, it contributes to feedback inhibition through developmental changes that are tightly linked to DN to DP differentiation. The Journal of Immunology, 2006, 176: 6824–6830.

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3 Abbreviations used in this paper: RSS, recombination signal sequence; DN, double negative; Cad, carbamyltransferase dihydrotase; DP, double positive; LAT, linker for activation of T cells; PKC, protein kinase C; SLP-76, Src homology 2 domain-containing leukocyte protein.


demonstrated to promote DP differentiation in pre-TCR-deficient mice (11, 19, 22, 23). In addition, the LckF505 and PKCα-CAT transgenes provide allelic exclusion signals as shown by the inhibition of Vβ to DJβ rearrangements at the endogenous Tcrb locus (11, 19). However, developing thymocytes containing Ras<sup>12</sup> or Raf-CAAX transgenes displayed normal levels of endogenous Vβ to DJβ rearrangement (22, 23). Thus, Ras<sup>12</sup> and Raf-CAAX are thought to promote DN to DP differentiation but not contribute to allelic exclusion. Because deletion of Lcp<sup>3</sup> (encoding SLP-76) results in a loss of thymocyte differentiation and a loss of allelic exclusion (24), it was concluded that the signaling pathways involved in differentiation and allelic exclusion diverge downstream of SLP-76 but upstream of Ras (4, 5).

To better understand the relationship between pre-TCR signaling and the developmental changes at the Tcrb locus associated with allelic exclusion, we analyzed the properties of DP thymocytes generated by a constitutively active Raf-CAAX transgene. Consistent with previous publications, we show that the Raf-CAAX transgene does not extinguish Tcrb recombination in DN thymocytes. However, DP thymocytes generated by Raf-CAAX signals display normal down-regulation of Vβ segment transcription and accessibility and normal feedback inhibition of Vβ to DJβ rearrangement. Thus, although the MAPK pathway is not sufficient to enforce Tcrb allelic exclusion, it clearly contributes to the feedback inhibited state in DP thymocytes. Our data suggest distinct requirements for Tcrb feedback inhibition in DN and DP thymocytes and indicate that a component of the feedback mechanism is tightly linked to DP differentiation.

**Materials and Methods**

**Mice**

C57BL/6 (B6), 129, Rag2<sup>-/-</sup> mice, Rag2<sup>-/-</sup> × Tcrb transgenic mice (Rxβ) (25), Raf-CAAX transgenic mice (23), and Lat<sup>-/-</sup> mice (17) were housed at the Duke University Vivarium. Lat<sup>-/-</sup> mice were a gift from W. Zhang (Duke University, Durham, NC). Raf-CAAX transgenic mice were a gift from R. Perlmutter (Amgen, Thousand Oaks, CA). All mice were used in accordance with protocols approved by the Duke University Institutional Animal Care and Use Committee.

**Flow cytometric analysis and thymocyte isolation**

Surface staining was performed on thymocytes according to standard staining protocols. All Abs were purchased from BD Pharmingen. In all instances, cells were initially incubated for 5 min with a mAb specific for CD16 and CD32 (clone 2.4G2) to block nonspecific staining. Analysis and sorting of DN and DP thymocytes was conducted using FITC-conjugated anti-CD4 (clone GK1.5) and PE-conjugated anti-CD8 (clone 53-6.7). The exclusion dye 7-aminoactinomycin D was included in all cell-sorting experiments. To perform intracellular TCRβ staining, thymocytes were surface stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8. The thymocytes were then permeabilized and stained intracellularly with CyChrome-conjugated anti-TCRβ (clone H57-597) according to the Cytofix/Cytotox kit (BD Pharmingen). TCRβ staining was evaluated in the CD4<sup>+</sup>CD8<sup>+</sup> population. Cell sorting and analysis were conducted using a FACSort<sup>TM</sup> (BD Biosciences) or FACSVantage SE (BD Biosciences) and CellQuest software.

**RT-PCR**

RNA was extracted from cells using TRIzol (Invitrogen Life Technologies) according to the manufacturer’s instructions. For germline transcription analysis, contaminating genomic DNA was removed with DNA-free (Ambion) according to the manufacturer’s instructions, and cDNA synthesis was performed with Transcriptor (Roche) and random hexamer primers (Roche) according to the Roche protocol. For analysis of R<sub>ag1</sub> and R<sub>ag2</sub> expression, cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) and an oligo(dT) primer (Invitrogen Life Technologies) according to the manufacturer’s protocol. PCR analysis of germline transcription was performed on 3-fold serial dilutions of cDNA using a “touchdown” PCR strategy: 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at annealing temperature, and 1 min at 72°C, and a 10-min extension at 72°C. Annealing temperature was held at 68, 65, and 62°C for 5 cycles each and at 58°C for 17 cycles. Amplicons were electrophoresed through agarose gels and visualized by ethidium bromide staining. Vβ primers used for germline transcription were positioned in the leader sequence and downstream of the RSS, respectively. Primers for Vαβ11, Vβ12, DJβ1, and Act-in (9) and Tcrγ (26) were previously described. Tcrα primers were: 5′-AGAACCTGCTGTTACAGTATTA-3′ and 5′-GAGTCAGGGCTCTGACGGCTT-3′.

**Chromatin immunoprecipitation**

Thymocytes were harvested and mononucleosomes were prepared as previously described (27). Mononucleosomes (25 μg) were immunoprecipitated with 5 μg each of Abs against diacytethylated histone H3, tetracyactylated histone H4, dimethylated histone H3K4, and control rabbit IgG (Upstate Biotechnology). The bound and input fractions were quantified by real-time PCR using SYBR green and a Light Cycler (Roche). Ratios of input:bound were calculated and were normalized to those for carbamoyltransferase dihydroorotase (Cd) in each sample. Primers for Vβ12, Vβ13, T4/5, and Cad have been previously described (9). DJβ1 primers were: 5′-GATCCAGAATGGTCATTACGG-3′ and 5′-CTGATCATCTTTTGTCGTTTA-3′.

**Analysis of V(D)J recombination products**

VDJβ and DJβ coding joints were analyzed by touchdown PCR (as described above) using a template genomic DNA prepared from thymocytes. To analyze signal end intermediates, thymocyte genomic DNA was digested and linker ligation was performed as previously described (28). Linker-ligated DNA was then used to amplify signal end intermediates by touchdown PCR (as described above). CD14 was amplified by PCR as follows: 94°C for 5 min, followed by 20 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 1 min, and a 10-min extension at 72°C. Amplicons were electrophoresed through agarose gels and analyzed by Southern blot using 32P-labeled oligonucleotide probes. The signal end primer for Vβ6 was: 5′-GTCG TAGC AACTGTTTTCAGTC-3′. Primers and probes for Vβ11, Vβ12, Vβ13, DJβ2, and CD14 (9) and the linker and primer (29) were described previously. For sequence analysis, amplified Vβ12 coding joints were purified through agarose gels, cloned using a TOPO TA Cloning kit for Sequencing (Invitrogen Life Technologies), and sequenced using a model 3730 DNA Analyzer (Applied Biosystems).

**Results**

**Analysis of gene expression and chromatin structure in Raf-CAAX-derived DP thymocytes**

To analyze the properties of DP thymocytes generated by MAPK activation, we bred the Raf-CAAX transgene onto a Raf2<sup>-/-</sup>-background (RxRC) and examined the expression of various genes. Expression of the Raf-CAAX transgene is driven by the Ick proximal promoter and Igk chain enhancer and is primarily restricted to immature lymphocytes (23). This Raf protein is constitutively active due to a farnesyl signal (CAAX) which provides targetting to the cell membrane, mimicking normal Ras activation. Consistent with a previous publication (23), Raf-CAAX signals generated DP thymocytes with normal CD4 and CD8 expression. However, we observed a delay in thymocyte differentiation (Fig. 1A) and variable thymic cellularity between RxRC littersmates (10–40 × 10<sup>6</sup> in 4- to 7-wk-old mice). RT-PCR was performed on purified RxRC DP thymocytes as well as DN (Rag2<sup>-/-</sup> × Tcrβ<sup>-/-</sup>) and DP (Rag2<sup>-/-</sup> × Tcrb transgene, Rxβ) thymocyte control populations.

This analysis showed that RxRC DP thymocytes were similar to Rxβ with respect to the level of Tcrγ, Tcrα, Vβ11, and Vβ12 germline transcription (Fig. 1B). The only discrepancy was that RxRC DP thymocytes displayed reduced germline Dβ1 transcripts as compared with the DP (Rxβ) control. The significance of this observation is unknown.

Thymocyte differentiation from DN to DP is also accompanied by a loss in histone H3 and H4 acetylation and histone H3K4 dimethylation within germline Vβ gene segments. Chromatin immunoprecipitation was performed using Abs specific for diacytethylated histone H3, tetracyactylated histone H4, and dimethylated histone H3K4 in DN (Rag2<sup>-/-</sup>), DP (Rxβ), and unfractonated RxRC thymocytes (69 and 77% DP). Immunoprecipitated chromatin was analyzed by real-time PCR at various sites within the Vβ gene segments. Chromatin immunoprecipitation was performed using Abs specific for diacytethylated histone H3, tetracyactylated histone H4, and dimethylated histone H3K4 in DN (Rag2<sup>-/-</sup>), DP (Rxβ), and unfractonated RxRC thymocytes (69 and 77% DP). Immunoprecipitated chromatin was analyzed by real-time PCR at various sites within the Vβ gene segments.
The Tcrb locus. Levels of histone acetylation and H3K4 dimethylation were high at the promoters (P) and RSSs of Vβ gene segments in the DN control but were decreased in both Raf-CAAX and control Rxβ DP thymocytes. Nevertheless, all samples analyzed displayed equivalent levels of histone H3 and H4 acetylation and histone H3K4 dimethylation at control sites DB1 and trypsinogen (T4/T5) (Fig. 2). Thus, DP thymocytes generated by the Raf-CAAX transgene display gene expression patterns and Vβ chomatin changes similar to those seen in DP thymocytes generated by a complete pre-TCR signaling cascade.

**LAT is required for allelic exclusion**

We sought to analyze the potential for ongoing Tcrb locus recombination in DP thymocytes generated solely by a Raf signal. Expression of the Raf-CAAX transgene on a wild-type background would not allow us to distinguish between DP thymocytes arising from Raf signals and those arising from a full complement of pre-TCR signals. For this reason, we attempted to eliminate pre-TCR signaling potential upstream of Ras in recombinase-sufficient thymocytes. We predicted that if we bred Raf-CAAX onto a Lat−/− background (LxRC), the resulting DP thymocytes would be generated by Raf-CAAX signals alone. Lat is a proximal pre-TCR signaling protein required for normal thymocyte development (17). Thymocytes deficient in Lat are completely blocked at the DN to DP transition, despite efficient VDJβ recombination. To determine whether this adaptor molecule is essential for propagating allelic exclusion signals, we bred a rearranged Tcrb transgene onto a Lat−/− background and analyzed the endogenous Vβ to DJβ rearrangement. Predictably, the Tcrb transgene did not rescue the block in DN to DP differentiation in Lat−/− thymocytes (Fig. 3A). Southern blot analysis of PCR-amplified coding joints revealed DJβ to Jβ rearrangement irrespective of LAT or Tcrb transgene expression (Fig. 3B). However, whereas Vβ to DJβ rearrangement was dramatically inhibited in Lat−/− thymocytes containing a Tcrb transgene, the Tcrb transgene had no apparent effect on Vβ to DJβ rearrangement in Lat−/− thymocytes. Therefore, we conclude that the adaptor protein LAT is required to efficiently propagate feedback inhibition signals emanating from the pre-TCR.

**Feedback inhibition in Raf-CAAX-derived DP thymocytes**

We next examined recombinase activity at the Tcrb locus in LxRC DN and DP thymocytes by using ligation-mediated PCR to detect signal end intermediates indicative of ongoing recombination. To serve as control samples, genomic DNA was extracted from purified DP thymocytes from Lat−/− mice in the presence or absence of the Raf-CAAX transgene. Signal end intermediates were easily detected at Vβ6 and Vβ11 in LxRC DN thymocytes but were dramatically reduced in all DP samples (Fig. 4A). One explanation for a lack of signal end intermediates would be defective Rag expression in LxRC DP thymocytes. To address this, we isolated RNA from purified LxRC DN and DP thymocytes and performed RT-PCR for Rag1 (Fig. 4B) and Rag2 (data not shown). Expression of Rag1 and Rag2 in LxRC DN and DP thymocytes was comparable to the Lat−/− DP control samples. A second explanation would be a lack of available DJβ substrate, due to high level Vβ to DJβ recombination in the DN compartment. However, PCR
analysis of genomic DNA from unfraccionated (Fig. 4C) or DP fractionated (Fig. 4D) LxRC thymocytes revealed levels of DJβ2 recombination substrates that were equivalent to the \( \text{Lat}^{+/+} \) controls. Thus, the Raf-CAAX transgene fails to suppress Vβ to DJβ rearrangement on either a \( \text{Lat}^{+/+} \) or \( \text{Lat}^{-/-} \) background as measured in total thymocytes or fractionated DN thymocytes (Fig. 4, A and C). However, DP thymocytes generated by the Raf-CAAX transgene display normal inhibition of \( Tcrb \) locus recombination, despite ongoing \( \text{Rag} \) expression and available DJβ2 substrates.

**Enforcement of β selection in \( \text{Lat}^{-/-} \) Raf-CAAX thymocytes**

Because MAPK activation can induce DP differentiation on a \( \text{Rag}2^{-/-} \) background (Fig. 1A), it would be expected that the DN to DP transition in LxRC thymocytes could occur in the absence of TCRβ expression and as a consequence without β selection. Therefore, we evaluated the presence of functional VDJβ rearrangements in DP thymocytes using intracellular TCRβ staining. Under the constraints of β selection, all DP thymocytes must express a functional TCRβ chain. If these constraints are removed, only 55%, reflecting the probability of an in-frame rearrangement on either of the two alleles, would be expected to do so. Surprisingly, essentially all DP thymocytes in LxRC mice expressed a functional TCRβ protein, suggesting that β selection was intact. Therefore, we evaluated the presence of functional VDJβ rearrangement on either of the two alleles, would be expected to do so. Surprisingly, essentially all DP thymocytes in LxRC mice expressed a functional TCRβ protein, suggesting that β selection was intact.
(Fig. 5A). To verify this result, genomic DNA was isolated from purified Lat⁺/⁺ and LxRC DP thymocytes and Vβ12DJβ2 coding joints were amplified by PCR. Assuming no allelic exclusion and no β selection, only 33% of the rearrangements should be in-frame. In contrast, functional β selection with no allelic exclusion predicts that 60% of rearrangements should be in-frame, whereas functional β selection with allelic exclusion predicts that 72% of rearrangements should be in-frame. Sequence analysis of cloned coding joints revealed similar percentages of in-frame rearrangements in both DP samples (Fig. 5B, Lat⁺/⁺ 78% and LxRC 72%).

Allelic inclusion in Lat⁻/- Raf-CAAX DN thymocytes

The above experiments imply that on a Lat⁻/- background, the Raf-CAAX transgene synergizes with the pre-TCR to promote DN to DP transition with β selection. We wondered whether the same synergy could lead to effective feedback inhibition of Tcrb recombination in DN thymocytes and, thus, allelic exclusion. Our previous analysis of LxRC mice (Fig. 4) could not address this possibility because in that model ongoing Tcrb recombination in DN thymocytes is required to assemble a pre-TCR. Therefore, we bred both Raf-CAAX and Tcrb transgenes onto the Lat⁻/- background and used a PCR strategy to analyze the endogenous Vβ to DJβ2 rearrangement (Fig. 6). As shown previously, neither the Raf-CAAX transgene alone nor a Tcrb transgene alone could block Vβ to DJβ2 rearrangement on a Lat⁻/- background. Moreover, the two transgenes were no more effective when tested together. Thus, although Raf-CAAX can synergize with the pre-TCR in Lat⁻/- mice to enforce feedback inhibition in the DP compartment, it is unable to do so in the DN compartment. These results imply that there are distinct requirements for feedback inhibition in the DN and DP compartments. In addition, the pre-TCR signaling requirements for feedback inhibition in DN thymocytes appear distinct from those involved in β selection and DP differentiation.

Discussion

Previous studies of constitutively active Raf1 and Hras1 transgenes indicated that they could promote DN to DP differentiation on a recombinase (and hence pre-TCR-) deficient background, but could not feedback inhibit Tcrb recombination on a recombinase (and pre-TCR-) sufficient background. Feedback inhibition of Tcrb recombination associated with allelic exclusion must be enforced in both DN and DP thymocytes. However, the previous studies could not address the status of feedback inhibition in DP thymocytes generated solely by the MAPK pathway, since in the genetic models used, recombinase-sufficient DP thymocytes were likely produced in response to the complete panoply of signals downstream of the pre-TCR. Our examination of LxRC mice addressed this shortcoming, because the recombinase-sufficient DP thymocytes generated are absolutely dependent on Raf signaling. Similarly, the DP thymocytes of RxRC mice are absolutely dependent on Raf signaling. Our analysis of these DP thymocyte populations revealed changes in Tcrb locus germline transcription, histone modifications, and recombinese activity that are typical of DP thymocytes generated by a complete pre-TCR signaling cascade. Thus, feedback inhibition at the Tcrb locus is intact in DP thymocytes generated by Raf signaling.

Unexpectedly, we found that β selection was preserved in LxRC DP thymocytes. We expected a substantial fraction of these DP thymocytes to contain nonfunctional Tcrb rearrangements due to constitutive MAPK activation. Instead we found high levels of intracellular TCRβ staining, and sequence analysis of VDJβ coding joints revealed a similar percentage of functional rearrangements in both Lat⁺/⁺ and LxRC DP thymocytes. The maintenance of β selection suggests that thymocytes expressing both pre-TCR and Raf-CAAX at the cell surface have a selective advantage over those expressing Raf-CAAX alone. This occurs despite the fact that
Raf-CAAX is capable of inducing DN to DP differentiation when expressed without a pre-TCR in Rag2-/- thymocytes. One possibility is that the pre-TCR may serve a scaffold function to allow for more efficient signaling by Raf-CAAX. Alternatively, Raf-CAAX may synergize with currently unknown LAT-independent pre-TCR signaling pathways to promote more efficient differentiation and survival signals. To date, the only known TCR signaling pathway documented to be activated independently of LAT is the MAPK pathway itself (30). MAPK activation under these circumstances involves the direct recruitment of the adaptor proteins Grb2 and Sos to phosphorylated CD3 chains, thus bypassing the need for LAT. Therefore, it is possible that additive Raf signals in pre-TCR expressing LxRC mice may lead to the β selection observed.

We have definitively shown that Raf signals can promote reduced Vβ accessibility and down-regulation of Vβ to DJβ rearrangement within the DP compartment. Thus, at least some developmental changes that are associated with the allelic exclusion program are induced by the MAPK pathway. Interestingly, a recent examination of Ets-1-deficient (Ets-1-/-) thymocytes also suggested a role for the MAPK pathway in Tcrb allelic exclusion (31). Ets-1 is a transcription factor essential for T cell and B cell development. Ets-1 is activated via phosphorylation downstream of calcium signaling or the Ras-Raf-MAPK pathway. Ets-1-/- thymocytes display impaired DN to DP differentiation, decreased proliferation, and an increase in apoptosis. Moreover, they display a loss of allelic exclusion as judged by surface expression of two distinct TCRβ proteins and the inability of a Tcrb transgene to inhibit Vβ to DJβ recombination. The MAPK pathway may therefore contribute to Tcrb allelic exclusion via the activation of Ets-1.

Despite the enforcement of feedback in DP thymocytes, the inability of the Raf-CAAX transgene to extinguish Tcrb recombination suggests that MAPK activation alone is insufficient to fully signal for Tcrb allelic exclusion. This result is consistent with the results of previous studies (23, 32). Our finding that feedback inhibition is maintained in LxRC DP thymocytes argues that the failure of Raf signals to promote allelic exclusion must map to the DN compartment. The delay we observed in the development of DP thymocytes is consistent with inefficient DN to DP differentiation, which could possibly disrupt feedback inhibition in the DN compartment. Alternatively, this phenotype could reflect a defect in proliferation in the context of normal DN to DP differentiation. We note that a constitutively active Ras(ON) transgene was previously shown to promote DP differentiation with thymic cellularity identical to that of controls but, like Raf-CAAX, did not extinguish Tcrb recombination in the DN compartment (22). This result suggests that the disruption of allelic exclusion may be independent of either the efficiency of differentiation or the extent of proliferation.

It is now thought that allelic exclusion of Ag receptor loci may be regulated through multiple mechanisms. Reduced accessibility of Vβ gene segments in DP thymocytes likely contributes to Tcrb feedback inhibition, but evidence suggests it is not the only requirement since feedback inhibition is maintained even for accessible Vβ segments (9, 10). Additional studies suggest changes in locus conformation or subnuclear positioning as possible mechanisms for allelic exclusion at Ig loci (33–39). However, these mechanisms are still not sufficient to explain the maintenance of feedback inhibition on an already rearranged Tcrb allele (40). Therefore, enforcement of allelic exclusion likely involves several layers of control in addition to those already described. The fact that any of these events may be enforced in DN thymocytes, in a pre-TCR-dependent but MAPK-independent manner, may explain the failure to block Tcrb recombination in this compartment in the Raf-CAAX model.

Despite this failure, we find that differentiation directed by Raf-CAAX leads to a phenotypically normal DP population displaying the expected inactivation of Vβ gene segments. Our inability to detect continued Vβ to DJβ rearrangements within the DP compartment in LxRC mice suggests that the MAPK pathway contributes to both differentiation and feedback inhibition of Tcrb recombination. We suggest that at least a component of the feedback mechanism is tightly linked to DN to DP differentiation and is itself sufficient to suppress Vβ to DJβ recombination in the DP compartment. Additional studies will be required to clarify the distinct components of feedback inhibition that are operative in DN and DP thymocytes.

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