The Activation Threshold of CD4⁺ T Cells Is Defined by TCR/Peptide-MHC Class II Interactions in the Thymic Medulla

Tom Li Stephen, Anastasia Tikhonova, Janice M. Riberdy and Terri M. Laufer

*J Immunol* 2009; 183:5554-5562; doi: 10.4049/jimmunol.0901104
http://www.jimmunol.org/content/183/9/5554

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2009/10/19/183.9.5554.DC1

**References**

This article cites 48 articles, 24 of which you can access for free at:
http://www.jimmunol.org/content/183/9/5554.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Activation Threshold of CD4⁺ T Cells Is Defined by TCR/Peptide-MHC Class II Interactions in the Thymic Medulla

Tom Li Stephen,* Anastasia Tikhonova,* Janice M. Riberdy,† and Terri M. Laufer²*‡

Immature thymocytes that are positively selected based upon their response to self-peptide-MHC complexes develop into mature T cells that are not overtly reactive to those same complexes. Developmental tuning is the active process through which TCR-associated signaling pathways of single-positive thymocytes are attenuated to respond appropriately to the peptide-MHC molecules that will be encountered in the periphery. In this study, we explore the mechanisms that regulate the tuning of CD4⁺ single-positive T cells to MHC class II encountered in the thymic medulla. Experiments with murine BM chimeras demonstrate that tuning can be mediated by MHC class II expressed by either thymic medullary epithelial cells or thymic dendritic cells. Tuning does not require the engagement of CD4 by MHC class II on stromal cells. Rather, it is mediated by interactions between MHC class II and the TCR. To understand the molecular changes that distinguish immature hyperactive T cells from tuned mature CD4⁺ T cells, we compared their responses to TCR stimulation. The altered response of mature CD4 single-positive thymocytes is characterized by the inhibition of ERK activation by low-affinity self-ligands and increased expression of the inhibitory tyrosine phosphatase SHP-1. Thus, persistent TCR engagement by peptide-MHC class II on thymic medullary stroma inhibits reactivity to self-Ags and prevents autoreactivity in the mature repertoire. The Journal of Immunology, 2009, 183: 5554–5562.

T

hymic selection generates a diverse repertoire of T cells that can respond to the universe of foreign Ags while maintaining tolerance to self. Positive selection generates a repertoire of T cells that recognize self-MHC-peptide complexes (1–3); negative selection purges thymocytes with strong reactivity to self (4). The peripheral repertoire is composed of T cells that recognize self-Ags, yet autoreactivity is quite rare. Thus, mechanisms for preventing the activation of T cells by self-ligands in the periphery must exist. We and others have previously termed this “developmental tuning” (5–8). Early studies by two different groups showed that developmental maturation of T cells decreases TCR reactivity to weak ligands without significantly changing the response to high-affinity cognate ligands (9, 10). We previously showed that interactions between CD4 single-positive (SP) thymocytes and MHC class II (MHC II) molecules on thymic medullary stroma are necessary to avoid hyper-reactivity of the mature repertoire (5). Thus, maturation during thymic medullary residency “developmentally tunes” the TCR to self-ligands, and this tuning is mediated by MHC II-positive thymic stroma.

Developmental tuning of CD4⁺ T cells is an active process requiring MHC II expression (5); yet, the molecular and cellular regulation of this process have not been characterized. Our previous results show that decreased responsiveness to TCR engagement is mediated by alterations in proximal TCR signaling pathways. We proposed that changes in the subcellular localization of the tyrosine kinase Lck regulate the threshold for activation of the TCR (5). However, developmental changes in the expression level or the activation state of other signaling molecules that regulate proximal TCR signaling may also occur during tuning. For example, increased expression of regulatory tyrosine phosphatases that inhibit Lck or its downstream targets would result in inhibition of TCR signaling; protection from the activity of phosphatases would enhance signaling (11). Neither the expression pattern of MHC II that regulates tuning nor the secondary molecular changes that occur are known.

In this study, we have used a series of bone marrow (BM) chimeras and genetically modified mouse models to further dissect mechanisms of developmental tuning. Our results show that MHC II expression on either medullary thymic epithelial cells (mTECs) or thymic dendritic cells (DCs) is sufficient to reduce TCR responsiveness to weak ligands. Moreover, we have found that tuning requires peptide-MHC II-specific interactions with the polymorphic TCR rather than the nonpolymorphic CD4 coreceptor. MHC II-specific interactions with the thymic medullary stroma inhibit ERK activation by weak ligands and elevate Src homology region 2 domain-containing phosphatase 1 (SHP-1) expression in the maturing SP thymocytes. These results show that developmental tuning by interactions with peptide-MHC II in the thymic medulla introduce negative regulatory mechanisms in the TCR signaling pathway and prevent self-reactivity in mature T cells.

Materials and Methods

Animals

C57BL/6 (B6), B10.BR, AND TCR transgenic (Tg) (12), RAG-1-deficient, and H2-DM-deficient (H2-DM⁻/⁻) (13) mice were purchased from The Jackson Laboratory. H2-DM⁻/⁻ mice were backcrossed to B6 mice at least eight generations and used with H2-DM⁻/⁻ littermate controls. EA137/VA142 MHC II mutant (MUT) (EA137/VA142 A⁺ MUT) and control wild-type (WT) I-A¹b Tg (A⁺ WT Tg) mice were previously described (14). K14/A⁺b (2) and CD11c/A⁺b (15) transgenic mice were used together with...
Aβ+/− littermate controls. NG-BAC (RAG2/GFP) Tg reporter mice (16) were provided by Dr. Michel Nussenzweig (Rockefeller University, New York, NY). MHC II-deficient (Aβ−/−) mice (17) were backcrossed to B6 mice at least 26 generations. All animals were maintained and used in accordance with the institutional animal care and use guidelines of the University of Pennsylvania (Philadelphia, PA).

Abs and peptides

The fluorochrome-conjugated anti-mouse CD4, CD8, CD69, TCRβ, Vβ3, diphosphorylated ERK1/2 (pERK1/2) mAbs, and isotype controls were purchased form BD Pharmingen. Anti-CD3e (clone 145-2C11; American Type Culture Collection (ATCC)) mAb was purified from culture supernatant. Anti-SHP-1 (clone C19) Ab (18) was purchased from Santa Cruz Biotechnology. Rabbit IgG (staining control) and secondary donkey anti-rabbit IgG (allophycocyanin conjugated) Abs were from Jackson Immuno-Research Laboratories. Hybridomas for the anti-mouse CD4 (clone GK1.5), CD8 (clone 2.43), CD16/32 (clone 2-4G2), MHC II (clone M5114), and Thy1.2 (clone M1 Tri) mAbs used for blocking and cell depletion experiments were obtained from ATCC. Pigeon cytochrome c (PCC) 88–104 peptide was purchased from AnaSpec. The altered peptide ligand (APL) P99 (APL P99) (10, 19) has an Leu→Pro substitution at position 99 and was synthesized by the University of Pennsylvania Protein Chemistry Laboratory (Philadelphia, PA).

Cell isolation and intrathymic transfers

CD4 SP thymocytes were enriched by depleting CD8 and MHC II-positive cells using a previously described protocol (5). APCs from B10.BR or Aβ−/− mice were isolated from spleens by depleting T cells with anti-Thy1.2 mAb and rabbit complement (Cedarlane Laboratories). CD4 SP thymocytes (105) were injected into surgically exposed thymi (5). Transferred cells were distinguished from endogenous cells by either CFSE labeling or congenic markers. Mice were euthanized 4–5 days after the cell transfer.

Radiation BM chimeras

Mononuclear BM cells collected from the femurs and tibias of the donor mice were T cell depleted with anti-CD4 and anti-CD8 mAbs followed by sorting with anti-rat IgG-conjugated magnetic beads. T cell-depleted BM cells (1–2 × 106) were transferred i.v. into lethally irradiated (950 rad) recipients. Chimeras were given antibiotic-containing water for 2 wk and used for experiments at 6–8 wk.

T cell activation assays

Equal numbers (105) of CD4 SP thymocytes and Aβ+/− APCs were cocultured at 37°C in 96-well U-bottom tissue culture plates with graded concentrations of anti-CD3ε mAbs in RPMI 1640 medium supplemented with 10% (v/v) FBS, penicillin/streptomycin, 2-ME, and 1-glutamine. AND Tg CD4 SP (AND CD4 SP) thymocytes were stimulated in vitro with graded concentrations of PCC 88–104 or APL P99 peptides and B10.BR APCs. Cultured cells were harvested at 4 h to analyze TCR down-regulation or at 6–16 h to analyze CD69 up-regulation as described previously (5). Endogenous or intrathymically transferred cells that matured in the K14/Aβ+ (5) or Aβ−/− mice were used as positive controls for tuning.

Flow cytometry

Single-cell suspensions were incubated on ice for 20 min with anti-CD16/32 mAb (eCycR) followed by staining with fluorochrome-conjugated anti-mouse mAbs for 30 min. Cells were washed thoroughly and acquired using a FACSCalibur flow cytometer (BD Biosciences). Data was analyzed using FlowJo (Tree Star) software.

Intracellular staining

SP thymocytes were fixed with 1.5% formaldehyde (Electron Microscopy Sciences) for 10 min at room temperature followed by surface staining with anti-CD4 and anti-CD8. Cells were then permeabilized with ice-cold methanol, washed thoroughly in staining buffer (PBS with 5% BSA and 0.01% sodium azide), and stained with either directly conjugated pERK1/2 mAbs or with anti-SHP-1 (C-19) Ab, followed by washing and staining with secondary donkey anti-rabbit IgG.

Quantitative RT-PCR

Total cellular RNA was extracted with the mirVana microRNA isolation kit (Applied Biosystem) and converted to cDNA with SuperScript II reverse transcriptase and random hexamers (Invitrogen). Quantitative PCR was performed on an Applied Biosystems PRISM 7500 sequence detection system. For SHP-1 and GAPDH, standard commercial TaqMan probes were used (Applied Biosystems). Samples were normalized to GAPDH and represented as fold change compared with double-positive (DP) thymocyte controls for SHP-1. Data are the mean ± SEM of three independent experiments done in triplicate.

Statistical analysis

Data were analyzed by a paired Student’s t test using GraphPad Prism 5.0 software. Statistical comparisons were made between individual mutant mice and their WT or heterozygous control groups only. Values of p < 0.05 (⋆) or p < 0.001 (⋆⋆) were considered significant.

Results

Interactions between CD4 SP thymocytes and MHC II on mTECs change TCR sensitivity

We previously asked whether developmental tuning of CD4+ T cells is an active event requiring TCR-MHC II interactions. To answer this question, the postselection maturation of CD4+ T cells was examined in K14/Aβ+ transgenic mice, in which MHC II expression is restricted to cortical thymic epithelial cells. Neither mTECs nor thymic DCs are MHC II positive in K14/Aβ+ mice. Therefore, CD4 SP T cells can be positively selected on MHC II+ cortical thymic epithelial cells but undergo no further interactions with MHC II in the thymic medulla. We found that postselection CD4 SP thymocytes maturing in the K14/Aβ+ thymic medulla were hyperresponsive to proximal TCR signaling (5). This suggested that interactions between maturing CD4 SP cells and MHC II-positive medullary stroma altered the TCR responsiveness to ligands. Multiple cell types express MHC II in the thymic medullary stroma. Our first set of experiments asked which MHC II-positive stromal cells mediate developmental tuning.

To study the requirement for MHC II expression on various medullary stromal cells for tuning, a series of BM chimeras (BMC) were generated. We compared the requirement for MHC II on radiation-resistant thymic epithelial cells and radiation-sensitive, BM-derived hematopoietic cells. To do this, lethally irradiated B6 WT recipient mice were injected with T cell-depleted Aβ−/− BM (Aβ−/− → WT). In these chimeras, radiation-resistant epithelial cells are the only MHC II-positive cells in the thymus. Control WT → WT and K14/Aβ+ → K14/Aβ+ chimeras were generated and analyzed in parallel. Positive selection is mediated by MHC II on thymic cortical epithelial cells (2), so CD4 SP thymocytes were generated in each of these chimeras (data not shown). CD4 SP thymocytes from the chimeras were enriched and stimulated with graded doses of anti-CD3ε mAb in the presence of Aβ−/− APCs (5). Up-regulation of the early T cell activation marker CD69 (20, 21) and down-regulation of surface TCR (5, 22, 23) were measured. When compared with CD4 SP cells from WT → WT BMC, CD4 SP thymocytes from K14/Aβ+ → K14/Aβ+ chimeras were hyper-responsive to stimulation with low concentrations of anti-CD3ε mAb with increased up-regulation of CD69 (Fig. 1, A and B) and down-regulation of TCR (Fig. 1C). In contrast, the responses of the CD4 SP thymocytes maturing in Aβ−/− → WT chimeras and the control WT → WT chimeras were the same (Fig. 1). Thus, MHC II expression restricted to radiation-resistant thymic epithelial cells is sufficient for the “developmental tuning” of maturing CD4 SP thymocytes.

MHC II expression on thymic DCs can reduce the TCR reactivity of CD4 SP thymocytes

Our results demonstrate that MHC II-positive mTECs could induce thymocyte tuning. We therefore asked whether this function is unique to thymic epithelial cells or whether interactions with MHC II-positive thymic DCs also alter TCR sensitivity. To address this question, we used CD11c/Aβ+ mice in which MHC II
expression is restricted to CD11c\(^+\) DCs (Ref. 15 and data not shown). CD11c promoter-driven MHC II expression in thymic DCs of CD11c\(\rightarrow\)K14/A\(\beta\) mice is lower than that of control A\(\beta\) mice (supplemental Fig. 1).\(^4\) In the absence of MHC II on thymic epithelium, there are no endogenous CD4 SP thymocytes in unstimulated cells. Data are representative of three experiments.

FIGURE 1. MHC II expression on thymic epithelial cells tunes maturing CD4 SP thymocytes. CD4 SP thymocytes were purified from A\(\beta\) \(\rightarrow\) WT, WT \(\rightarrow\) WT, or K14/A\(\beta\) \(\rightarrow\) K14/A\(\beta\) BMCs. Purified CD4 SP thymocytes were stimulated in vitro with an equal number of A\(\beta\) splenic APCs and increasing doses of anti-CD3e mAb for 16 h followed by surface staining and FACS analysis for relative CD69 expression. A, Representative histograms of CD69 expression at two different doses of anti-CD3e. B, Cumulative data of CD69 expression at multiple doses of anti-CD3e stimulation. Shown are representative data from four experiments. C, TCR down-regulation was quantified by stimulating CD4 SP thymocytes from the chimeras with A\(\beta\) splenic APCs conjugated with 100 or 1000 ng/ml anti-CD3e mAb for 4 h, followed by surface staining for TCR\(\beta\). To calculate the percentage of initial TCR expression, the mean fluorescence intensity of stimulated cells was divided by that of unstimulated cells. Data are representative of three experiments.

The reduced tuning of CD4\(^+\) SP cells transferred into CD11c\(\rightarrow\) A\(\beta\) thymi suggests that DCs are less efficient than mTECs at tuning. However, we were concerned about whether lower levels of MHC II on the transgenic DCs, when compared with WT DCs (supplemental Fig. 1), resulted in reduced tuning in the CD11c\(\rightarrow\)A\(\beta\) mice. To rule out this possibility, we generated WT \(\rightarrow\) A\(\beta\) or A\(\beta\) \(\rightarrow\) WT chimeras in which only thymic hematopoietic cells express MHC II. In these chimeras, thymic DCs have WT levels of MHC II expression. Control A\(\beta\) \(\rightarrow\) A\(\beta\) and WT \(\rightarrow\) WT chimeras were generated and analyzed in parallel. Intrathymically transferred AND CD4 SP thymocytes that matured in the different chimeric thymi were stimulated in vitro with PCC 88–104 or APL P99 and analyzed for surface CD69 levels. As shown in Fig. 2C, the response to the agonist peptide PCC 88–104 was the same in all of the chimeras. Similar to the changes mediated by DCs in the CD11c\(\rightarrow\)A\(\beta\) thymus, interactions with MHC II on thymic hematopoietic APCs in WT \(\rightarrow\) A\(\beta\) BMCs reduced the TCR reactivity of maturing AND CD4 SP cells to APL P99. However, a larger percentage of transferred thymocytes that matured in the WT \(\rightarrow\) WT BMCs lost their responsiveness to weak stimulation by APL P99. Thus, interactions with thymic DCs expressing a WT level of MHC II alone are also not sufficient to tune all the maturing CD4 SP thymocytes, and DCs are therefore less efficient than mTECs in developmental tuning.

Developmental tuning is peptide dependent

Interactions between SP thymocytes and MHC II on thymic stromal cells could be mediated by either the polymorphic TCR or the nonpolymorphic CD4 coreceptor. We first asked whether changes in TCR reactivity are dependent upon interactions between the TCR and self-peptide-MHC complexes on thymic stromal cells. MHC II molecules in H2-DM\(\rightarrow\) mice predominantly present one self-peptide, the invariant chain-derived CLIP (13, 24, 25). Polyclonal WT or monoclonal TCR Tg CD4 T cells are deprived of self-peptide-specific MHC II-TCR interactions after transfer to H2-DM\(\rightarrow\) mice (26, 27). To define the requirement for self-peptide-MHC II-TCR interactions in tuning, CFSE-labeled polyclonal WT or monoclonal AND CD4 SP thymocytes were transferred intrathymically to H2-DM\(\rightarrow\), K14/A\(\beta\), or control H2-DM\(\rightarrow\) thymi. CD4 SP thymocytes were enriched 5 days after thymic transfer and stimulated in vitro. CD69 levels were analyzed by FACS. Results are summarized in Fig. 3. To ensure that H2-DM deficiency does not inherently disrupt tuning, we first examined the
endogenous CD4 SP cells. Endogenous CD4 SP thymocytes from H2-DM<sup>−/−</sup> and H2-DM<sup>+/−</sup> thymi responded equally to stimulation with anti-CD3ε mAb and were less responsive than hyperactive K14/A<sub>p</sub><sup>−/−</sup> CD4 SP cells (Fig. 3A). Thus, H2-DM deficiency did not affect the TCR reactivity of endogenous CD4 SP thymocytes.

In contrast, intrathymically transferred WT CD4 SP thymocytes

![Diagram](image.png)

**FIGURE 2.** MHC II-positive thymic DCs alter the reactivity of CD4 SP thymocytes. CFSE-labeled polyclonal WT or AND CD4 SP thymocytes (10<sup>7</sup>) were intrathymically transferred to CD11c/A<sub>p</sub><sup>−/−</sup>, K14/A<sub>p</sub><sup>−/−</sup>, or A<sub>p</sub><sup>−/−</sup> APCs and increasing concentrations of anti-CD3ε mAb. After 16 h, CD69 expression was determined by FACS. A, Transferred polyclonal CD4 SP cells were stimulated in vitro with an equal number of A<sub>p</sub><sup>−/−</sup> APCs and increasing concentrations of anti-CD3ε mAb. After 16 h, CD69 expression was determined by FACS. B, Transferred AND CD4 SP thymocytes were stimulated in vitro with equal numbers of B10.BR splenic APCs and increasing concentrations of agonist PCC 88–104 or partial agonist APL P99 peptides for 16 h and then assayed by FACS for CD69 expression. Data represent five (A) and six (B) experiments. *p < 0.05; **p < 0.001 between CD11c/A<sub>p</sub><sup>−/−</sup> and A<sub>p</sub><sup>−/−</sup> control groups.

![Diagram](image.png)

**FIGURE 3.** Developmental tuning is peptide dependent. CFSE-labeled WT or AND CD4 SP thymocytes (10<sup>7</sup>) were intrathymically transferred to H2-DM<sup>−/−</sup>, K14/A<sub>p</sub><sup>−/−</sup>, or control H2-DM<sup>+/−</sup> mice. CD4 SP thymocytes were purified 5 days after the transfer. WT CD4 SP cells were stimulated in vitro with A<sub>p</sub><sup>−/−</sup> splenic APCs and anti-CD3ε mAb for 16 h and assayed for CD69 surface expression by FACS. A, CD69 expression on endogenous CD4 SP cells (CFSE<sup>+</sup>). B, CD69 expression on intrathymically transferred WT cells (CFSE<sup>+</sup>). Data represent four experiments. C, AND CD4 SP thymocytes purified 5 days after intrathymic transfer into the indicated hosts were stimulated with B10.BR APCs and increasing concentrations of PCC 88–104 or APL P99 peptides and assayed for CD69 surface expression. Data are representative of six independent experiments. *p < 0.05; **p < 0.001 between H2-DM<sup>−/−</sup> and control H2-DM<sup>+/−</sup> groups.)
maturing in both H2-DM\(^{-/-}\) and K14/A\(_{b}^{b}\) thymi were activated by much lower doses of anti-CD3e stimulation than transferred cells recovered from control H2-DM\(^{+/+}\) thymi (Fig. 3B). Similarly, AND CD4 SP cells transferred into H2-DM\(^{-/-}\), K14/A\(_{b}^{b}\), and control H2-DM\(^{+/+}\) thymi responded equally to the strong cognate peptide PCC 88–104 (Fig. 3C; left). However, the same transferred cells maturing in H2-DM\(^{-/-}\), but not in control H2-DM\(^{+/+}\) thymi, retained their reactivity to the weak ligand APL P99. This enhanced responsiveness to APL P99 was similar to that of transferred cells maturing in K14/A\(_{b}^{b}\) thymi that completely lack MHC II expression in the thymic medulla (Fig. 3C, right). In parallel with increased CD69 expression, AND CD4 SP thymocytes maturing in H2-DM\(^{-/-}\) thymi also had increased down-regulation of the TCR following APL stimulation (data not shown). Thus, when maturing thymocytes are unable to interact with peptide-MHC complexes in the thymic medullary stroma, tuning does not proceed normally.

**CD4 coreceptor engagement with MHC II is not required for tuning**

These results suggest that the tuning of CD4\(^{+}\) T cells depends on TCR-peptide-MHC II interactions. We next asked whether CD4-MHC II interactions are also required for tuning. To do so, we used EA137/VA142 A\(_{b}\) MUT mice, which have two amino acid mutations in the \(\beta\)-chain of the MHC II I-A\(_{b}\) molecule that disrupt the CD4 binding site (14). Control A\(_{b}\) WT Tg mice with matched MHC II expression were examined in parallel (14). CFSE-labeled CD4 SP thymocytes (10\(^{7}\)) from A\(_{b}\) WT Tg mice were intrathymically injected into EA137/VA142 A\(_{b}\) MUT or control A\(_{b}\) WT Tg mice. Five days after transfer, CD4 SP thymocytes were recovered and stimulated in vitro with A\(_{b}\) \(^{-/-}\) APCs and increasing concentrations of anti-CD3e mAb. After 16 h, the relative surface expression of CD69 was assayed by FACS. Data represent four experiments.

**Developmental tuning alters ERK activation**

Our results shows that interactions between CD4 SP thymocytes and self-peptide-MHC II complexes on mTECs and thymic DCs decrease TCR responsiveness in maturing thymocytes to weak ligands such as APLs without affecting the response to high-affinity ligands. Previous studies of mature peripheral CD4\(^{+}\) T cells have shown that the loss of TCR responsiveness to weak ligands is mediated by decreased activation of ERK, a positive regulator of the proximal TCR signaling molecule Lck (11). We therefore asked whether MHC II-dependent changes in CD4 SP thymocyte responsiveness were also associated with the loss of ERK activation by weak ligands. ERK activation was assayed by flow cytometry in AND CD4 SP thymocytes maturing in different thymic environments. Representative results are shown in Fig. 5. Basal levels of ppERK1/2 were the same in AND CD4 SP thymocytes
that matured in the MHC II-negative thymic medulla of K14/Aβb and the control MHC II-positive Aβb+/− thymic medulla (data not shown). Following stimulation with the high-affinity cognate peptide PCC 88–104, CD4 SP cells recovered from K14/Aβb and control Aβb−/− thymi had equivalent phosphorylation of ERK. In contrast, AND CD4 SP cells that matured in the MHC II-negative K14/Aβb thymic medulla had increased levels of phosphorylated ERK in response to low affinity APL P99 stimulation compared with cells maturing in control Aβb+/− thymi. Indeed, in untuned SP thymocytes in K14/Aβb thymi, ERK activation in response to APL P99 persisted at least 30 min following stimulation (data not shown). Similarly, AND CD4 SP thymocytes maturing in H2-DM−/− thymi, but not in control H2-DM+/− thymi, phosphorylated ERK following APL P99 stimulation (supplemental Fig. 2). Thus, interactions between the maturing CD4 SP thymocytes and peptide-MHC II on thymic medullary stromal tissues that mediate tuning prevent ERK activation by weak ligands.

Developmental tuning is associated with up-regulation of SHP-1 expression

The decrease in TCR responsiveness that accompanies stimulation could be regulated by diminished activation of positive regulators such as ERK or by increasing negative regulators such as tyrosine phosphatases (11, 18). We focused on the tyrosine phosphatase SHP-1, as the lack of SHP-1 results in hyperphosphorylation of both the proximal TCR complex and the downstream signaling molecules that couple the activated TCR to the Ras/MAPK response pathway (28, 29). Interestingly, CD4+ T cells from SHP-1 heterozygous mice can respond to weak ligands such as MHC variant peptides (30), a phenotype reminiscent of immature untuned thymocytes. We therefore used flow cytometry to track the level of SHP-1 during thymocyte maturation. Mature CD4 SP thymocytes express more SHP-1 than do immature DP cells (Fig. 6A) (10). We next asked whether the induction of SHP-1 expression on maturing CD4 SP thymocytes depended upon TCR-peptide-MHC II interactions in the thymic medulla. To address this question, we first analyzed the SHP-1 expression levels in CD4 SP thymocytes from K14/Aβb and the control Aβb+/− mice. As can be seen in Fig. 6B (left), CD4 SP thymocytes from K14/Aβb mice express less SHP-1 than control Aβb+/− CD4 SP thymocytes. To verify this observation, SHP-1 expression was assayed in intrathymically transferred AND CD4 SP thymocytes maturing in H2-DM−/−, K14/Aβb, or control H2-DM+/− thymi. CD4 SP thymocytes maturing without peptide-MHC II interactions in either H2-DM−/− or K14/Aβb thymi had less SHP-1 than transferred cells maturing in control WT thymi (Fig. 6B, right). We observed the same results with intrathymically transferred polyclonal WT CD4 SP thymocytes (data not shown). Thus, the up-regulation of SHP-1 expression in maturing SP thymocytes is mediated by TCR-peptide-MHC II interactions with thymic medullary stroma.

Developmental tuning is not complete until late during medullary residency

Maturing CD4 SP thymocytes spend ~1–2 wk in the thymic medulla (23, 31). Thus, SP thymocytes are a heterogeneous population of immature, intermediate, and fully mature CD4 cells. We therefore asked whether tuning occurs throughout thymic medullary residency or at a particular point during medullary residency. Hogquist and colleagues have recently shown that, as SP thymocytes in RAG2/GFP reporter mice (16) mature, they slowly lose expression of GFP (31). CD4 SP thymocytes can be divided into GFPhigh immature, GFPintermediate, and GFPhlow mature populations (31). As shown in the experimental outline in Fig. 7A, thymocytes from RAG2/GFP reporter mice were enriched for CD4 by CD8 depletion. CD4-enriched cell populations were then FACS sorted into GFPhigh, GFPintermediate, and GFPhlow populations. The sorted cells were transferred separately into K14/Aβb or control Aβb+/− (WT) mice (left) or AND CD4 SP cells purified five days after intrathymic transfer to H2-DM−/−. CD4 SP thymocytes that matured in K14/Aβb, or H2-DM−/− (WT) mice (right) were stained for intracellular SHP-1 and analyzed by flow cytometry. Data represent three experiments.

FIGURE 6. Thymocyte tuning correlates with increased SHP-1 expression. A, Thymocytes from WT B6 mice were stained for SHP-1, CD4, and CD8 and analyzed by FACS. Left, Gate for CD4+CD8− DP and CD4 SP thymocytes. Right, SHP-1 expression on DP and CD4 SP thymocytes. B, Endogenous CD4 SP thymocytes from K14/Aβb or control Aβb+/− (WT) mice (left) or AND CD4 SP cells purified five days after intrathymic transfer to H2-DM−/−, K14/Aβb, or H2-DM−/− (WT) mice (right) were stained for intracellular SHP-1 and analyzed by flow cytometry. Data represent three experiments.
We then asked whether changes in SHP-1 expression correlate developmentally with susceptibility to tuning. We again used RAG2/GFP reporter mice to permit analysis of CD4 SP thymocytes of different ages. Fig. 7 shows that the up-regulation of the SHP-1 level in maturing CD4 SP thymocytes parallels maturation. Thus, the highest level of SHP-1 expression occurs in the GFPlow mature CD4 SP thymocytes (Fig. 7C). As shown in Fig. 7D, the maturational change in SHP-1 expression is regulated transcriptionally. Thus, TCR-MHC II-mediated interactions that occur during the early stages of CD4 SP medullary residency up-regulate SHP-1 expression to increase negative regulation of the TCR signaling machinery.

**Discussion**

We have examined the mechanisms that regulate the reduction in TCR responsiveness accompanying the maturation of CD4 SP thymocytes. We previously showed that developmental tuning is an active process requiring the expression of MHC II on thymic stroma. In this study, we report that decreases in TCR reactivity
during medullary maturation require interactions between the self-peptide-MHC II expressed on mTECs and thymic DCs with the TCR, but not with CD4. Tuning is associated with inhibition of ERK activation and up-regulation of SHP-1 phosphatase levels, resulting in enhanced negative regulation of TCR signaling.

Tuning can be initiated by MHC II expression limited to either mTECs or thymic DCs; however, the effect mediated by mTECs is complete whereas DCs are less efficient. Why are MHC II-positive mTECs more efficient in mediating tuning than thymic DCs? MHC II expression on thymic DCs in the CD11c/Aβb mouse is lower than that of control Aβ b/+ DCs (supplementary Fig. 1) (15). The TCR threshold may be different when a T cell selected on WT peptide-MHC II molecules encounters medullary stroma expressing less class II. However, a fraction of CD4 SP thymocytes were not tuned (Fig. 2C) in WT→Aβ b−/− BMCs wherein thymic DCs express the WT level of MHC II. Thus, even with WT levels of MHC II, thymic DCs were not able to tune the whole maturing CD4 SP thymocytes when mTECs were MHC II negative. Multiple studies have demonstrated that peptide-MHC complexes can be transferred from mTECs to thymic DCs in vivo, whereas DC to epithelial cell transfer does not occur (32–34). Indeed, this transfer may contribute to negative selection of thymocytes specific for peripheral tissue Ags expressed by mTECs. If thymic DCs participate in tuning via peptide-MHC II complexes acquired from mTECs, then tuning in CD11c/Aβb mice and WT→Aβ b−/− BMCs in which mTECs are MHC II-negative would be reduced. It is interesting to note that the MHC II expression levels on mTECs are lower than those of thymic DCs (supplemental Fig. 1). Therefore, in a WT thymus wherein both mTECs and thymic DCs are MHC II positive, DC acquisition of peptide-MHC II complexes from mTECs may, in fact, enhance overall tuning efficiency. Our data indicate the possibility of a functional collaboration between mTECs and thymic DCs in tuning the maturing SP thymocytes and the induction of central tolerance. However, at present we do not know whether the CD4 SP thymocyte populations tuned by mTCEs and thymic DCs are the same or different. Finally, the autoimmune regulator AIRE regulates the expression of a wide array of tissue restricted Ags (35–38) in mTECs that may enhance their efficiency.

Developmental tuning requires the expression of H2-DM and, presumably, peptide-dependent TCR-MHC II interactions. In contrast, tuning does not require interactions between nonpolymorphic CD4 and MHC II. This is surprising, as TCR signaling in mature T cells depends on the CD4 coreceptor (39, 40). Similarly, the change in responsiveness of CD8+ T cells that occurs with maturation is mediated, in part, by increased sialylation of the CD8 coreceptor and decreased CD8-MHC class I interactions (41–43). Moreover, we previously proposed that tuning leads to an increased dependence on CD4 engagement for TCR signaling (5). However, MHC class II-dependent reorganization of proximal signaling during tuning is regulated by the TCR, not by CD4. In fact, we observed increased tyrosine phosphorylation of Lck, the key signaling molecule associated with proximal signaling (44) when there is no TCR-peptide-MHC II interaction in the thymic medulla (T. L. Stephen and T. M. Laufner, unpublished data). Even though productive MHC II-CD4 interactions are not required for developmental tuning, the coreceptor may still participate in reorganization of the proximal signaling by peptide-MHC II-TCR interactions. Evaluation of tuning in CD4-deficient SP thymocytes may provide more information about the requirements for a CD4 molecule in developmental tuning with the caveat that altered distribution of CD4-associated molecules such as Lck in CD4-deficient cells will make it difficult to interpret the results.

We previously proposed that MHC II-dependent postselection tuning is controlled in part by regulation of the subcellular localization of Lck; the association of Lck with the CD4 coreceptor in mature SP thymocytes increases the dependency on CD4 ligation for TCR signaling (5). Now, we report that MHC II-dependent tuning also regulates the expression of the inhibitory tyrosine phosphatase SHP-1. The negative regulation of TCR signaling by SHP-1 is probably mediated via dephosphorylation of the activation tyrosine of Lck, Y394 (44, 45). Although, SHP-1-deficient mature T cells are hyperactive, the absence of SHP-1 does not alter the basal phosphorylation of Lck (29). Taken together, these data suggest that SHP-1 may have more access to CD4-associated Lck present in mature T cells than to the CD4 nonassociated “free” Lck present in immature SP thymocytes (5). It will be important to examine this possibility by using biochemical and ultrastructural analyses. Nonetheless, the changes in both SHP-1 expression and Lck localization result in increasing the TCR threshold in mature peripheral T cells.

Germain and colleagues have proposed a “seesaw” model of TCR regulation in which SHP-1 and ERK determine the response to high vs low affinity ligands. Weakly binding ligands induce recruitment of SHP-1, which inactivates Lck. Strong agonist ligands activate ERK, which protects Lck from SHP-1 binding during signaling (11, 46–48). In agreement with this model, our data shows that peptide-MHC II signals to T cells during medullary residency are associated with increased SHP-1 phosphatase levels and decreased ERK activation by weak ligands. Together, these changes contribute to the increased TCR signaling threshold of mature CD4+ T cells.

Our results suggest that tuning has been completed between the intermediate (GFPlow) and late (GFPhigh) stages of medullary residency. These experiments also suggest that interactions of immature SP thymocytes with peptide-MHC II result in nonreversible changes in the TCR signaling properties, as mature GFPhigh cells transferred into MHC II-deficient thymi did not alter their responsiveness. Identification of the T cell developmental stage in which tuning occurs will guide future experiments to the biochemical changes that prevent T cell autoreactivity.

This study is a first step toward unraveling the complex cellular interactions during thymic medullary residency that set the TCR response to ligands. We identified the importance of TCR interactions with peptide-MHC II on thymic medullary stroma in establishing regulatory mechanisms to maturing thymocytes. The establishment of such regulatory check points increases the T cell threshold for ligands in such a way that self-Ags cannot activate the TCR in mature T cells and thereby avoid autoreactivity in the healthy immune system.

Acknowledgments

We thank Gregory F. Wu for critical reading of the manuscript and Eric J. Allenspach and A. Bhandoola for discussions.

Disclosures

The authors have no financial conflict of interest.

References


0. Stefanova, I., B. Hemmer, M. Vergelli, R. Martin, W. E. Bidddison, and R. N. Germain. 2003. TCR ligand discrimination is enforced by competing ERK signaling by TCR antagonists or partial agonists.