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Developmental Alterations in Thymocyte Sensitivity Are Actively Regulated by MHC Class II Expression in the Thymic Medulla

Steven C. Eck,* Peimin Zhu,† Marion Pepper,* Steven J. Bensinger,* Bruce D. Freedman,† and Terri M. Laufer‡*

Developing thymocytes are positively selected if they respond to self-MHC-peptide complexes, yet mature T cells are not activated by those same self-complexes. To avoid autoimmunity, positive selection must be followed by a period of maturation when the cellular response to TCR signals is altered. The mechanisms that mediate this postselection developmental tuning remain largely unknown. Specifically, it is unknown whether developmental tuning is a preprogrammed outcome of positive selection or if it is sensitive to ongoing interactions between the thymocyte and the thymic stroma. We probed the requirement for MHC class II-TCR interactions in postselection maturation by studying single positive (SP) CD4 thymocytes from K14/Aμb mice, in which CD4 T cells cannot interact with MHC class II in the thymic medulla. We report here that SP CD4 thymocytes must receive MHC class II signals to avoid hyperactive responses to TCR signals. This hyperactivity correlates with decreased expression of CD5; however, developmental tuning can occur independently of CD5, correlating instead with differences in the distribution of Lck. Thus, the maturation of postselection SP CD4 thymocytes is an active process mediated by ongoing interactions between the T cell and MHC class II molecules. This represents a novel mechanism by which the thymic medulla prevents autoreactivity. The Journal of Immunology, 2006, 176: 2229–2237.

Functional immune systems require that the T cell repertoire be honed to distinguish self from non-self. This self image is established in the thymus through the active selection and maturation of a randomly generated repertoire of precursor T cells. Positive and negative selection of T cells is dependent upon interactions of the clonotypic TCR on a developing thymocyte with its MHC-peptide ligand (1). Positive selection generates a repertoire of T cells that recognizes self-MHC-peptide complexes (2, 3). T cells with excessive reactivity toward self die by apoptosis during negative selection (4, 5).

Positively selected T cells by definition react with self-Ags; thus, negative selection is not sufficient to prevent autoaggression by the mature repertoire. Maturation in the thymus must also be accompanied by substantial changes in the cell’s responsiveness to TCR ligation so that mature peripheral lymphocytes are less responsive to self-peptides than are thymocytes undergoing selection. To understand how these changes are regulated, the differences between immature preselection double positive (DP) thymocytes and mature peripheral single positive (SP) lymph node (LN) cells have been examined (6–9). Early studies demonstrated that peptide and superantigen ligands that induced positive or negative selection could not activate mature peripheral LN cells, arguing that the sensitivity of the cells to these ligands changed during development. However, these studies compared two very different biological outcomes: selection and T cell activation. More recently, Davey et al. (7) and Lucas et al. (8) have demonstrated that mature LN T cells were less responsive to low-affinity ligands while retaining or increasing the response to high-affinity ligands. Both groups concluded that these sensitivity changes represent a mechanism for shifting the repertoire from self to foreign responsiveness during development.

The implication of these studies is that thymocytes must acquire the function and phenotype of peripheral lymphocytes after they have completed their selection and before their emigration to the periphery. Postselection SP T cells remain in the thymic medulla for 10–14 days, approximately one-half the lifespan of a thymocyte (10). Presumably, it is during this residency that the reactivity of the T cell to self ligands also changes. However, the medullary residency of SP thymocytes is one of the least studied areas of T cell development.

The thymic elements and molecular mechanisms that regulate SP maturation remain undefined. Specifically, it remains unknown whether positive selection initiates a developmental program that persists throughout thymic maturation to regulate developmental tuning or if the T cell setpoint is instead influenced by ongoing TCR-peptide (pMHC) interactions. In this study, we examine this question by analyzing the responsiveness of medullary SP CD4 T cells maturing in the presence or absence of MHC class II-TCR interactions. We find that the loss of interactions between MHC class II and TCR in maturing SP CD4 T thymocytes generates T cells that are considerably more responsive to anti-CD3-mediated stimulation. Both SP CD4 thymocytes and LN CD4 T cells developing in the absence of MHC class II express less CD5; however, differences in responsiveness were independent of CD5 expression. Instead, responsiveness correlated with the subcellular localization of Lck. Thus, setting T cell responsiveness during postselection...
maturity is subject to instruction mediated by MHC expression on thymic stroma.

Materials and Methods

Animals

K14/Aβ−/− (K14/Aβ−/−), Aβ−/−/− littermates (B6), and Aβ−/−/− mice (11) that have been backcrossed to at least 20 generations were maintained in our own animal facility and used between 6 and 14 wk of age. C57BL/6 (B6), B6.129, AND (12), and RAG-1-deficient mice were obtained from The Jackson Laboratory. AβEpi+ mice (13) were a gift from L. Ignatowicz (Medical College of Georgia, Augusta, GA). The University of Pennsylvania Institutional Animal Care and Use Committee approved all animal experiments.

Flow cytometry

Single-cell suspensions were incubated with anti-CD16/32 (2.4G2; American Type Culture Collection) before staining with fluorochrome-conjugated Abs to CD3, CD4, CD5, CD8, CD69, CD24, CD25, CD45.1, CD45.2, Qa-2, and TCRb (BD Biosciences) and were analyzed on a FACS Calibur with CellQuest Pro (BD Biosciences) and FlowJo (Tree Star) software.

Cell enrichment and sorting

Enriched SP CD4 thymocytes were obtained by treating with anti-CD8 mAb (2.43; American Type Culture Collection) followed by magnetic sorting using goat anti-rat IgG conjugated beads (Qiagen). Purities of >75% were routinely obtained. Additional purification by positive selection with anti-CD4 beads (AutoMacs; Miltenyi Biotec) yielded SP CD4 cell purities >94%. For lymph node CD4 T cell purification, Abs to CD8 (2.43), B220 (RA-3), and CD16/32 (24G2) (American Type Culture Collection) were used with anti-rat IgG beads as above to purities >75%. To generate APCs, spleens were depleted of T cells with mAb to the Thy1 Ag (M1T1; American Type Culture Collection) and rabbit complement (Cedarlane Laboratories).

T cell activation

Purified SP CD4 thymocytes or spleen and LN T cells were labeled with CFSE (Molecular Probes) according to established protocols (14). Labeled cells were cultured at 10⁵ cells/well in 96-well round-bottom polystyrene plates containing an equivalent number of T-depleted APCs (from Aβ−/− mice unless otherwise indicated) and anti-CD3e (2C11; American Type Culture Collection). Cultures were harvested after 62–66 h for FACS analysis. Responder frequency, defined as the proportion of the plated CD4+ cells that proliferated, and proliferative capacity, defined as the number of daughter cells generated by the average responder T cell, were calculated as previously reported (15). Alternatively, cultures were harvested after 4–16 h to analyze CD69 up-regulation. For peptide stimulation of AND or AND/RAG TCR-transgenic (Tg) cells, B10.BR (I-Ek−/− mice) and anti-CD3e (2C11; American Type Culture Collection). Cultures were harvested after 62–66 h for FACS analysis.

Results

SP CD4 thymocytes have enhanced sensitivity to TCR stimulation when MHC class II is absent in the thymic medulla

We hypothesized that SP CD4 thymocyte responsiveness is sensitive to interactions between TCRs and self-peptide-loaded MHC class II molecules in the thymic medulla. Thus, we predicted that depriving maturing thymocytes of tonic interactions would alter this developmental program. To test this, we examined TCR-dependent responses of maturing SP CD4 thymocytes from K14/Aβ−/− mice. K14/Aβ−/− mice have MHC class II, I-Aβ, expression limited to cortical thymic epithelium (18). This expression pattern permits the positive selection of CD4 T cells, but positively selected thymocytes have no further interactions with MHC class II cells. This also results in defective negative selection and a CD4 T cell repertoire with significant “auto”reactivity to I-Aβ-positive APCs (18). Therefore, we used αCD3e mAb presentation by class II-deficient (Aβ−/−/−) APCs to bypass K14/Aβ−/− autoreactivity as well as repertoire differences in TCR specificity and avidity. To examine proliferative responses, SP CD4 cells were labeled with the intracellular dye CFSE and stimulated with graded doses of αCD3. As shown in Fig. 1A, the percentage of K14/Aβ−/− SP CD4 cells that proliferated (responder frequency) as well as the average number of divisions that proliferating cells underwent (proliferative capacity) in response to αCD3 were increased when compared with those of wild type (WT) thymocytes. These differences were especially evident at the lowest concentrations of Ab, where K14/Aβ−/− SP cells proliferated but WT SP cells did not. Maximal stimulation elicited similar responses from K14/Aβ−/− and WT cells. As shown in Fig. 1B, these differences were maintained in mature CD4 LN T cell responses, suggesting that differences initiated in the thymus are retained by mature T cells. Importantly, CD8 cell responses from the two mice were not different (data not shown). Thus, K14/Aβ−/− SP CD4 cells were markedly more responsive to limiting αCD3 stimulation than were their WT counterparts.
mixing the two populations did not argue against an effect of differential regulatory T cell development or function.

Proliferation is a distal event in the response to TCR stimulation, which integrates proximal TCR signaling, costimulation, cytokine signaling, and cell cycle progression. Therefore, we examined events more proximal to TCR stimulation, including TCR internalization, Ca2+ flux, and CD69 up-regulation. CD69 expression increases rapidly after TCR signaling (20–23) and is also developmentally regulated during thymocyte maturation (24). Young SP thymocytes express high levels of CD69 and lose expression during maturation (25, 26). Expression of CD69 was not significantly different between SP CD4 thymocytes from K14/Ab and B6 mice (data not shown and Fig. 2A). This suggests that the maturational down-regulation of CD69 is independent of tonic pMHC/TCR interactions and allowed us to assay the increase in CD69 expression that follows TCR stimulation. Fig. 2A shows that K14/Ab SP CD4 thymocytes require less TCR stimulation to up-regulate CD69 expression than do WT SP CD4 cells. As with proliferation, individual responses were not altered by coculture of the K14/Ab and WT cells (data not shown), and each had similar maximal responses. Thus, the biochemical differences leading to enhanced proliferation are cell intrinsic and present during G1.

TCR activation, which drives downstream activation, causes rapid down-regulation of the receptor (27). This down-regulation is mediated by activation of proximal tyrosine kinases and is maximum within 5 h (28, 29). To examine TCR down-regulation, SP CD4 cells were stimulated via the CD3 complex using Ab-loaded, Ab+/-, adherent macrophage monolayers as APCs. Importantly, Fig. 2B shows that resting SP CD4 thymocytes from K14/Ab and Ab+/- mice express equivalent surface levels of TCR. At various time points after stimulation, TCR levels on SP CD4 thymocytes were determined by staining with an Ab to the TCR surface was significantly greater in SP K14/Ab than WT SP CD4 cells to reach peak Ca2+ flux after receptor cross-linking. Increases in the magnitude and duration of Ca2+ signaling were also enhanced in K14/Ab SP CD4 cells but were more variable (compare Figs. 2C and 6E). Thus, the increased response to stimulation of K14/Ab SP CD4 cells is associated with accelerated Ca2+ response kinetics.

CD69 up-regulation, TCR down-regulation, and Ca2+ flux responses all suggest that proximal TCR signaling mediates the differences between SP CD4 thymocytes from K14/Ab and WT mice. If TCR-mediated activation differences are restricted to proximal events, bypassing these events should negate any observed differences. Indeed, K14/Ab and WT SP CD4 thymocytes do up-regulate CD69 (Fig. 2E) and proliferate equivalently (data not shown) when stimulated with PMA and ionomycin. Thus, the increased responsiveness of K14/Ab cells is secondary to differences in early TCR signal transduction and bypassing early signaling events is sufficient to eliminate downstream differences. Collectively, these results demonstrate that maturation of SP CD4 cells in the absence of pMHC class II-T cell interactions promotes the generation of mature T cells with enhanced sensitivity to TCR activation.

We wished to determine whether the proliferative differences were cell intrinsic as opposed to mediated by soluble factors or dominant regulatory populations. Therefore, K14/Ab and WT congenic B6-SJL SP CD4 cells were mixed together before activation. As shown in Fig. 1C, mixing the two populations did not alter the proliferation of the individual repertoires. Thus, the differences are intrinsic to the individual cells. These results also argue against differential production of cytokines. Indeed, K14/Ab and WT SP CD4 thymocytes produce equivalent, albeit small, amounts of IL-2 (data not shown). More importantly, consistent with our prior findings that regulatory CD4+CD25+ T cells are present and functional in K14/Ab and B6 mice (19), these mixing studies argue against an effect of differential regulatory T cell development or function.

We also examined Ca2+ flux after TCR cross-linking, as this response is initiated within the first minutes after TCR signal transduction (30). As shown in Fig. 2D, K14/Ab SP CD4 thymocytes consistently fluxed Ca2+ more rapidly, requiring about 30 fewer seconds than did WT SP CD4 cells to reach peak Ca2+ flux after receptor cross-linking. Increases in the magnitude and duration of Ca2+ signaling were also enhanced in K14/Ab SP CD4 cells but were more variable (compare Figs. 2C and 6E). Thus, the increased response to stimulation of K14/Ab SP CD4 cells is associated with accelerated Ca2+ response kinetics.

Collectively, these results demonstrate that maturation of SP CD4 cells in the absence of pMHC class II-T cell interactions promotes the generation of mature T cells with enhanced sensitivity to TCR activation.
Maturation of SP CD4 cells after intrathymic transfer

We have hypothesized that the enhanced sensitivity to TCR ligation in K14/Aβb SP CD4 cells is a result of postpositive selection maturation in the absence of TCR/pMHC interactions. However, our analyses compared the responses of SP CD4 thymocytes that were positively selected in two different thymi. Thus, it was important to verify that the differences were not secondary to differences in positive selection. We first examined the responses of DP thymocytes in the two strains. To avoid the confounding issue of selection by MHC class I molecules, DP thymocytes were purified from K14/Aβb or WT mice; CD69 up-regulation after stimulation with anti-CD3ε was analyzed. Fig. 3A shows that activation of the two populations was equivalent. Additionally, there was no difference in cell death as measured by 7-aminoactinomycin D staining (data not shown). Thus, immature DP thymocytes in the two populations have the same sensitivity.

If tuning occurred during SP residency, then positively selected WT SP thymocytes should mature differently in class II-sufficient and class II-deficient medullae. To test this, WT SP CD4 thymocytes were purified and transferred via intrathymic injection into the thymi of either WT or K14/Aβb mice for 5 days. The reverse thymic transfer (K14/Aβb into WT) could not be interpreted because the postselection maturation is disrupted by the response of the K14/Aβb class II-reactive CD4 cells. Recovery of transferred WT thymocytes was similar from the two environments with 1011–1012 SP donor thymocytes recovered after transfer of 107 cells. Maturation of the SP thymocytes was equivalent in B6 and K14/Aβb recipients; donor cells transferred into both thymi became Qa2high and heat-stable antigen-low and did not proliferate in vivo (data not shown).

Fig. 3B documents the functional responses of the transferred thymocytes. WT SP CD4 cells that matured in the class II-deficient environment expressed substantially less CD69, indicating reduced TCR ligation.

**Figure 2.** K14/Aβb hyperproliferation correlates with differences in proximal signaling. A, Purified K14/Aβb (solid line histograms) and Aβb+/− (shaded histograms) SP CD4 thymocytes were stimulated with an equal number of Aβb+/− APCs and anti-CD3 mAb as indicated for 16 h before determination of relative CD69 expression. Representative histogram overlays are shown on the right. Mean and SD of three individual mice are shown. Data representative of 10 experiments. B, CD3 expression on K14/Aβb and Aβb+/− littermates was determined by staining whole thymocyte cell preparations with Abs to CD3, CD4, and CD8. Representative CD3 histograms of SP CD4 gated cells for individual mice are shown. MFI and SD for three separate mice were 327 ± 25 for WT and 318 ± 21 for K14/Aβb. C, To measure TCR down-regulation, Aβb+/− macrophage monolayers were pulsed with 1 μg/ml anti-CD3 mAb for 45 min and washed, and purified SP CD4 thymocytes were added. Nonadherent thymocytes were removed at the indicated times and stained with FITC-labeled anti-TCRmAb (H57). Percent of initial TCR expression was calculated by dividing the geometric MFI for stimulated cells by that of unstimulated cells. Representative data of three separate experiments are shown. D, Ca2+ flux was measured on fura 2-AM-loaded SP CD4 thymocytes from K14/Aβb and B6 mice by digital imaging microscopy and expressed as the ratio of fluorescence emission ratio at 510 nm from cells sequentially excited at 340 nm and 380 nm. Cells were treated with biotinylated anti-CD3 mAb (1 μg/ml) and cross-linked with streptavidin (1 μg/ml) at the indicated time. Mean of responding cells as well as individual cell tracings are shown. Representative results of three separate experiments.

**Maturation of SP CD4 cells after intrathymic transfer**

We have hypothesized that the enhanced sensitivity to TCR ligation in K14/Aβb SP CD4 cells is a result of postpositive selection maturation in the absence of TCR/pMHC interactions. However, our analyses compared the responses of SP CD4 thymocytes that were positively selected in two different thymi. Thus, it was important to verify that the differences were not secondary to differences in positive selection. We first examined the responses of DP thymocytes in the two strains. To avoid the confounding issue of selection by MHC class I molecules, DP thymocytes were purified from β2-microglobulin-deficient K14/Aβb or WT mice; CD69 up-regulation after stimulation with anti-CD3ε was analyzed. Fig. 3A shows that activation of the two populations was equivalent. Additionally, there was no difference in cell death as measured by 7-aminocanomycin D staining (data not shown). Thus, immature DP thymocytes in the two populations have the same sensitivity.
K14/Ah b medulla exhibited enhanced CD69 activation responses to αCD3 stimulation when compared with the identical WT cells maturing in a class II-sufficient environment. Thus, transfers of SP CD4 cells to a K14/Ah b thymus recapitulates the phenotype of K14/Ah b SP CD4 cells, confirming that the hypersensitivity of the K14/Ah b SP CD4 cells is secondary to attenuated postselection maturation, rather than abnormal positive selection.

Like the SP CD4 thymocytes, mature CD4 T cells from K14/Ah b mice exhibit enhanced sensitivity to αCD3 stimulation, prompting us to ask whether transferred WT thymocytes retain their altered responsiveness after leaving the thymus. B6 SP CD4 thymocytes transferred to K14/Ah b or B6 thymus that had emigrated from the thymus could be found in the LNs of recipient mice, and those in the K14/Ah b host were more responsive than were those from the WT host (Fig. 3C). Thus, the alterations in responsiveness observed in thymocytes were retained outside the thymus.

**SP CD4 cells can adjust responsiveness independently of CD5 expression**

The signaling function of the proximal TCR can be modulated by several associated cell surface receptors. Surface levels of many of these molecules are regulated in turn by TCR-MHC interactions. CD5 is one putative inducible negative regulator of TCR signal transduction (31). Immature DP thymocytes and peripheral LN cells alter their expression of CD5 in response to ongoing TCR-MHC-peptide interactions with decreased TCR-MHC interactions correlating with decreased levels of CD5 and relatively increased TCR sensitivity (32). We first characterized the surface expression of CD5 on SP thymocytes in different thymic environments. CD5 levels were substantially lower on SP CD4 thymocytes in K14/Ah b mice than on WT thymocytes (Fig. 4A), potentially reflecting differences in the level of selecting MHC class II. Importantly, WT thymocytes transferred intrathymically adapt CD5 surface expression to mirror that of the host mouse (Fig. 4B). Thus, CD5 levels are not fixed during selection; instead, postmaturation SP CD4 thymocytes regulate expression of CD5 to reflect the MHC class II expression of the immediate milieu.

Is developmental tuning regulated by these changing levels of CD5? We directly determined the requirement for CD5 by examining the postselection maturation of CD5-deficient SP CD4 thymocytes. CD5-deficient SP CD4 cells were purified from CD5-deficient B6 thymi and transferred into either WT B6 or K14/Ah b thymi. Equivalent numbers of CD5-deficient SP CD4 cells were obtained from the two host thymi 5 days after transfer, and the CD69 response to increasing doses of αCD3ε was assayed. As summarized in Fig. 4C, CD5-deficient cells transferred into WT thymi remain less reactive than those transferred into K14/Ah b thymi. Thus, CD5 is not required for SP CD4 thymocytes to adjust their responsiveness.

We found that SP CD4 cells positively selected on WT levels of I-A b did not tune appropriately in the MHC class II-deficient K14/Ah b thymic medulla. However, K14/Ah b mice express less I-A b on cortical epithelium than do A b+/+ mice (18). SP CD4 cells developing in Ah b+/+ and Ah b+/- mice respond identically to stimulation via the TCR in our studies (data not shown) despite the different levels of class II expressed in the two strains. Thus, considerable differences in the level of class II mediating positive selection do not appear to alter the responsiveness of the mature SP CD4 cells.

To verify that altered responsiveness of K14/Ah b mice was not secondary to selection on decreased levels of class II, we examined the maturation of SP CD4 thymocytes in a second line of mice in which MHC class II expression is substantially reduced but has a normal anatomical distribution (33). A bEpII + mouse express a transgenic Ah b molecule covalently bound to the antigenic peptide Ee52-63 (13). With invariant chain sufficiency, peptide exchange occurs and a diverse class II-associated peptide repertoire is maintained. Nevertheless, transgene-dependent MHC class II expression is substantially lower than normal (33). We found that SP CD4 thymocytes from A bEpII + thymi have decreased levels of CD5 (Fig. 4D), which is consistent with selection on low levels of MHC class II. Indeed, the surface level of CD5 closely matched that of K14/Ah b SP CD4 thymocytes. However, the responses of A bEpII + SP CD4 thymocytes to TCR stimulation were similar to those of B6 SP CD4 cells rather than K14/Ah b SP CD4 cells when assessed by either CD69 up-regulation or TCR internalization (Fig. 4, E and F). These results confirm that CD5 levels are sensitive to the density of MHC molecules available for TCR interactions. However, the level of CD5 does not define the thymocyte responsiveness to stimulation via the TCR. Thus, maturation in a thymic medulla devoid of MHC class II is significantly different from that occurring where MHC class II expression is reduced but not anatomically restricted.

**Lck availability and association with CD4 correlate with thymocyte responsiveness**

Thymocytes maturing in the absence of pMHC/TCR interactions have hyperactive responses that are negated by stimulation with PMA and ionomycin. This suggests alterations in signaling pathways proximal to the TCR. We focused on the expression and

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**FIGURE 3.** The responses of SP CD4 cells are not fixed by positive selection. A, Thymocytes from K14/Ah b (•) or Ah b+/- (■) that had been bred onto a B2-microglobulin-deficient B6 background were stimulated with an equal number of Ah b+/- APCs and αCD3 mAb as indicated for 3.5 h before determination of relative CD69 expression. DP cells were identified by staining with anti-CD4 and -CD8 mAb. B and C, 10^5 purified SP CD4 thymocytes from WT mice (B6.SJL or B6) were transferred into the thymi of either B6 or K14/Ah b mice. Five days later, purified SP CD4 thymic (A) and CD4 LN cells (B) were obtained, cultured with anti-CD3 and Ah b+/- APCs, and followed for CD69 expression 16 h later as in Fig. 2A. Transferred cells were identified by congenic markers (B6.SJL donors) or CFSE staining before transfer (B6 donors). Representative results of six (B) and three (C) experiments shown. Similar results were obtained after 4 h of stimulation (data not shown).
subcellular localization of Lck in developing SP CD4 cells. The Src family kinase, Lck, associates with the cytoplasmic domain of CD4. Coligation of the TCR and CD4 by MHC class II molecules recruits Lck into the TCR signaling complex where it phosphor-ylates the ITAMs of CD3 and initiates the principal signal cascade leading to T cell activation (reviewed in Ref. 34). Work by Holdorf et al. (35) has shown that Lck activation requires active recruit-ment of the CD4 coreceptor into the pMHC/TCR signaling com-plex. As shown in Fig. 5, A and B, TYRO75 expressions are negated by recruitment of CD4. Ca2+ flux was measured as in Fig. 2. Fura 2-AM-loaded SP CD4 thymocytes were cross-linked by addition of streptavidin (1 μg/ml; solid lines) alone or together with biotinylated anti-CD4 (GK1.5; 1 μg/ml; dotted lines) and were cross-linked by addition of streptavidin (1 μg/ml) at the indicated time. Overlays of the mean response comparing CD3 cross-linking alone to that with CD4 for K14/Athp cells (C) and B6 cells (D) are shown. E and F are overlays of data from C and D and compare the K14/Athp and B6 cell responses to CD3 cross-linking alone (E) and with CD4 (F). Data are representative of two separate experiments.

TCR complex, Lck could be more limiting in WT as compared with K14/Athp SP CD4 thymocytes.

If accessibility of Lck regulates the setpoint of maturing thymocytes, then K14/Athp thymocytes should respond better when CD4 is not incorporated into the TCR signaling complex, as is the case when anti-CD3ε is presented by Athp−/− APCs. Conversely, activation of WT thymocytes should require recruitment of the CD4 coreceptor into the signaling complex. We examined this in three different ways. We first compared Ca2+ flux in K14/Athp and WT SP CD4 cells in response to co-cross-linking CD4 and CD3. Specifically, CD4 SP thymocytes were treated with biotinylated

FIGURE 4. MHC class II availability supersedes CD5 expression in tuning the SP CD4 thymocyte setpoint. A, CD5 profiles of unmanipulated SP CD4 cells from K14/Athp (solid line) and B6 mice (shaded histogram) shown. B, CD5 expression changes in transferred thymocytes. A total of 10^7 purified, CFSE-labeled B6 SP CD4 cells were transferred into thymi of B6 or K14/Athp mice. Five days later, transferred cells were recovered from B6 (top) or K14/Athp mice (bottom). Relative expression of CD5 was determined as in A; mean CD5 expression on host B6 and K14/Athp cells is also shown. Results representative of three separate experiments. C, Purified SP CD4 thymocytes from B6/C57BL/6 mice were CFSE-labeled, transferred, and recovered 5 days later from thymi of B6 or K14/Athp mice as in B. CD69 responses to anti-CD3 stimulation in the presence of Athp−/− APCs were determined as in Fig. 3. D, CD5 expression on SP CD4 Athp+ thymocytes was determined as in A. E and F, Purified SP CD4 thymocytes from the indicated donor mice were stimulated with Athp+/− APCs and anti-CD3 (2C11). After 16 h, TCR expression (D) and CD69 expression (E) were determined. CD69 values for Athp+ cells are mean and SD of four mice.

FIGURE 5. Developmental tuning is dependent on Lck localization and recruitment. A, Whole cell lysates of K14/Athp and B6 SP CD4 thymocytes were serially diluted, run on polyacrylamide gels, and probed with anti-Lck mAb. B, Total SP CD4 cell lysates were unmanipulated (lanes 1 and 2) or immunoprecipitated with anti-CD4 mAb (GK1.5) and protein G Sepharose (lanes 3–6). Whole cell lysate (lanes 1 and 2), resuspended pellet (lanes 3 and 4), and supernatant (lanes 5 and 6) fractions were probed as above. Relative densitometry was determined by pixel analysis in Photoshop (Adobe) on scanned films and is shown below the samples for comparison. Results are representative of three and four separate experiments, respectively. C–F, Thymocyte differences are negated by recruitment of CD4. CD5 profiles of unmanipulated SP CD4 thymocytes from K14/Athp (gray) and B6 mice (black) were treated with biotinylated anti-CD3 mAb (2C11; 1 μg/ml; solid lines) alone or together with biotinylated anti-CD4 (GK1.5; 1 μg/ml; dotted lines) and were cross-linked by addition of streptavidin (1 μg/ml) at the indicated time. Overlays of the mean response comparing CD3 cross-linking alone to that with CD4 for K14/Athp cells (C) and B6 cells (D) are shown. E and F are overlays of data from C and D and compare the K14/Athp and B6 cell responses to CD3 cross-linking alone (E) and with CD4 (F). Data are representative of two separate experiments.
mAbs to CD4 and CD3; Ca^{2+} flux was measured after the addition of streptavidin. Fig. 5, C-F, shows that cross-linking CD4 with CD3 modestly decreased the lag time preceding the Ca^{2+} response of K14/A_{b}^{b} SP CD4 cells. In contrast, Ca^{2+} flux in WT CD4 SP cells was accelerated by ~30 s to closely match the timing of the K14/A_{b}^{b} CD4 SP cell response. Thus, incorporation of CD4-associated Lck altered the response of WT thymocytes so that they resembled K14/A_{b}^{b} SP CD4 cells.

To further test our hypothesis, we examined the response of SP CD4 thymocytes to peptide Ags. PCC-specific AND CD4+ TCR Tg Tg T cells can be positively selected on I-A^{b} molecules but respond to PCC on I-E^{k}-positive APCs. Additionally, partial agonist peptides for PCC have been identified; we used a peptide with proline substituted at position 99 (P99) (8). Maturation of thymocytes is characterized by loss of the response to partial agonists but maintenance of the response to full agonist ligands (7, 8). Moreover, the difference between full and partial agonist responses may be secondary to the recruitment of CD4 and activation of Lck (36, 37).

A total of 10⁷ SP CD4 thymocytes from AND TCR Tg mice were transferred into WT or K14/A_{b}^{b} thymi. Responses to CD3 cross-linking and agonist PCC 88-104 or partial agonist peptides were examined 5 days later. Similar to our observations with polyclonal cells, AND SP CD4 cells recovered from K14/A_{b}^{b} thymi were more sensitive to low concentrations of anti-CD3 presented by class II-deficient APCs than were those AND SP CD4 cells recovered from WT thymi (Fig. 6A). Indeed, limiting the specificity of the TCR exaggerated the differences between the two populations, indicating that maturational differences in the sensitivity of SP CD4 cells are independent of TCR specificity.

In contrast, AND SP CD4 cells recovered from K14/A_{b}^{b} or WT thymi responded equally to the strong agonist peptide, PCC 88-104, presented by I-E^{k}-positive B10.BR APCs. However, the same AND CD4 SP thymocytes recovered from K14/A_{b}^{b} thymi remained responsive to the weak agonist peptide, P99; the AND TCR Tg thymocytes maturing in class II-deficient thymi appropriately retain an immature phenotype.

To confirm a role for the recruitment of Lck in thymocyte maturation, the response to PCC was re-examined in the presence of CD4 blockade. AND SP CD4 cells recovered from WT mice were more sensitive than those from K14/A_{b}^{b} mice to blockade of CD4 (Fig. 6D). Thus, the presence of medullary MHC class II expression during the maturation of SP CD4 T cells correlates with differential sensitivity to full and partial agonist ligands. This sensitivity, in turn, correlates with the subcellular location of Lck and the requirement for recruitment of the CD4 coreceptor into the TCR signaling complex.

**Discussion**

Understanding the biology of thymic development requires the resolution of multiple paradoxes. Chief among them is that lymphocytes are positively selected by self-peptide/MHC complexes that they will again encounter in the periphery. Negative selection removes those cells with the highest affinity for self. However, changes in the signaling properties of the TCR also prevent self-complexes from fully activating mature LN T cells. Recent data suggest that this maturation is characterized by the selective loss of sensitivity to low-affinity, positively selecting ligands with either no change or increases in the response to high-affinity (foreign) ligands. The mechanisms that mediate these maturation changes remain poorly characterized. We set out to ask whether this developmental tuning is an automatic response initiated by positive selection or if maturation requires ongoing TCR-MHC interactions.

By examining the maturation of SP CD4 thymocytes in an MHC class II-deficient thymic medulla, we found that developmental tuning is not an inevitable consequence of positive selection. Thus, before positive selection, DP thymocytes in K14/A_{b}^{b} and WT mice are similar in responsiveness. After positive selection, the cells become more responsive. This is unlikely to simply reflect differences in positive selection as thymocytes positively selected in WT thymi mature differently in the presence or absence of medullary class II expression. Similarly, A_{b}^{b}EpII+ SP CD4 cells have normal setpoints despite being selected by and maturing on reduced levels of class II. Thus, developmental tuning is actively regulated by TCR-pMHC interactions and requires a normal anatomic distribution of MHC class II expression.

There has been much previous work examining the “tuning” of mature peripheral T cells deprived of constitutive MHC-TCR interactions. Some groups have observed enhanced reactivity of cells in the class II-deficient environment (38) (39), whereas others have argued that loss of class II stimulation decreases the setpoint of the resting T cell (40). It could be argued that the current results simply extend this earlier work by showing that SP thymocytes are sensitive to the loss of MHC class II signals. We suggest that, at the stage of SP maturation, there is a developmental requirement for MHC class II-TCR interactions that defines the setpoint of the T cell before emigration to the periphery. Thus, it will be important in future experiments to determine whether the tuning mechanism we describe here is restricted to thymocytes and whether, once set, it is relatively fixed or reversible, for example, in cases in which SP CD4 thymocytes tuned in MHC class II-deficient environments encounter normal MHC class II in the periphery.

Changes in TCR responsiveness that accompany thymocyte maturation correlated with differences in the relative expression of...
CD5. Why consider a role for CD5 in the process of postselection maturation? During thymocyte development, CD5 levels are modulated in response to TCR-MHC interactions; high-affinity or multiple TCR-pMHC interactions increase CD5 levels on DP thymocytes (32). Smith et al. (38) previously showed that the level of CD5 remains responsive to the strength of ongoing TCR-MHC interactions in mature peripheral T cells in reaggregation cultures. Similarly, we found that postselection SP thymocytes modulate CD5 levels after intrathymic transfer to mimic the CD5 expression on host SP CD4 cells. Thus, CD5 levels are defined quite specifically by peptide/MHC-TCR interactions and can be modulated throughout thymic development. In many previous studies, the level of CD5 correlates with changes in the sensitivity of the TCR (31, 32, 38, 41). In most cases this correlation has not been demonstrated to be causative. However, deletion of CD5 through homologous recombination clearly increases the avidity of the stromal cell-DP thymocyte interaction during positive and negative selection so that thymocytes that would normally be positively selected become negatively selected (31, 32). Also, Hawiger et al. (42) used anti-CD5 Abs and CD5-deficient mice to suggest that dendritic cell-induced tolerance is dependent on changes in the expression of CD5. In contrast, comparison of the responses of K14/A<sup>b</sup> and K14/A<sup>b</sup> SP CD4 thymocytes shows that TCR reactivity need not correlate with the surface level of CD5. More importantly, by using transfers of CD5-deficient thymocytes, we found that expression of CD5 is not required to establish the set-point of the TCR in the medulla. Although it is likely that modulation of TCR signaling by CD5 does contribute to the (overall) response of the maturing CD4<sup>+</sup> T cell in maturing SP CD4 cells, the role of CD5 is secondary to other mechanisms mediated by thymocyte-stromal cell interactions.

One mechanism of thymocyte maturation, epitomized by alterations in CD5 levels, is that accessory molecules and/or coreceptors dominantly alter TCR signaling. Alternatively, proximal signaling molecules linked to the TCR itself could change function or localization during development. Our results suggested differences in early signal transduction between SP CD4 thymocytes that matured in the absence or presence of interactions with class II molecules. Particularly interesting were the differences in the kinetics of Ca<sup>2+</sup> flux. The more rapid Ca<sup>2+</sup> flux observed in K14/A<sup>b</sup> SP CD4 cells is consistent either with a reduced requirement for the number of triggered TCRs needed to successfully induce a Ca<sup>2+</sup> flux or with differences in the availability of second messengers to the TCR complex. Many lines of evidence suggest that secondary messengers are more available in K14/A<sup>b</sup> SP CD4 cells. First, AND SP CD4 cells maturing in the K14/A<sup>b</sup> thymus retain their responsiveness to partial agonist peptides. Second, a greater proportion of cellular Lck is found free of CD4 in K14/A<sup>b</sup> cells. Finally, cross-linking CD3 with CD4 accelerated the kinetics of Ca<sup>2+</sup> flux in WT cells to parallel those of K14/A<sup>b</sup> SP CD4 cells. Together, our results suggest that differences in the relative distribution of Lck play a major role in the response differences between K14/A<sup>b</sup> and WT SP CD4 cells. Indeed, the phenotype of K14/A<sup>b</sup> thymocytes is identical with that of CD4<sup>+</sup> T cell clones generated by Filipp et al. (43) that express a mutant Lck that is unable to associate with CD4. When Lck cannot associate with CD4, T cells have enhanced responsiveness to TCRβ cross-linking but WT responses to Ag-dependent stimulation.

Lck signaling regulates multiple stages of T cell function. It directs the double negative to DP transition, is central to the CD4/CD8 lineage commitment decision (44), and mediates the normal homeostasis of peripheral T cells (45). Wiest et al. (44) previously suggested that low TCR levels in DP thymocytes are tied to limiting expression of Lck; the total level of Lck increases in the SP thymocyte in parallel with increases in TCR levels. We postulated that medullary maturation was regulated by the relative accessibility of this newly expressed Lck to different surface receptors. Our results show that SP CD4 cells maturing in a class II-deficient environment expressed normal levels of Lck. However, more of this Lck was found disassociated from CD4. This is especially intriguing in light of the observation by Lucas et al. (8) that more Lck is associated with the TCR in DP thymocytes than in mature T cells, where Lck is predominantly associated with CD4. Together, these results suggest a model in which postselection “tuning” is controlled in part by the subcellular distribution of Lck. Our results conclusively demonstrate that this is an active process requiring the presence of MHC class II-peptide complexes in the medulla.

What are the functional consequences of altering the subcellular localization of Lck during maturation? The localization of Lck did not affect the response of AND SP CD4 cells to stimulation with APC plus agonist peptide, a stable interaction that recruits ample CD4 costimulation (36). In contrast, AND thymocytes maturing in a class II-deficient medulla remain responsive to partial agonist P99 peptide. Thus, the relocalization of Lck increases the dependency of the mature T cell response on CD4 costimulation. This limits responses to TCR peptide/MHC interactions that are sufficiently long lived to permit secondary CD4 engagement—a requirement that prevents activation by low affinity self-peptides.

We do not believe that the changes documented here preclude the involvement of other mechanisms in postselection maturation. For example, Starr et al. (46) have shown that sialylation of O-linked glycans contributes to maturational tuning of CD8 thymocytes. It is of note that in preliminary analyses we do not see differences in peanut agglutinin staining between WT and K14/A<sup>b</sup> SP CD4 thymocytes (data not shown); however, transcriptional profiling of thymocyte subpopulations has highlighted multiple changes that occur between the DP and SP stages, including expression of CD5 and other regulatory receptors such as CD53 and VLA-4, chemokine receptors, signaling molecules, and transcription factors (1, 32, 47, 48). In the future, the K14/A<sup>b</sup> system should prove extremely useful in dissecting which of these changes follows from positive selection and which are regulated during postselection maturation by TCR signals.

The thymic medulla has a unique role in the molding of the T cell repertoire to prevent autoimmunity. Dendritic cells localized to the corticomedullary junction and medullary epithelium are ideally positioned to mediate the deletion of autoreactive thymocytes. Similarly, the transcription factor AIRE is specifically expressed in medullary epithelial cells (49). There, it regulates the ectopic expression of tissue-specific Ags to increase the proportion of the autoreactive repertoire that is deleted (49, 50). In this study, we have demonstrated that MHC class II expression in the thymic medulla also regulates the final setpoint of the maturing thymocyte. Thus, the thymic medulla prevents autoreactivity in two distinct ways: through deletion of autoreactive T cells and maturational tuning of the surviving repertoire.

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Disclosures
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