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Central Tolerance to Self-Antigen Expressed by Cortical Epithelial Cells[1]

Dita Mayerova and Kristin A. Hogquist[2]

The exposure of developing thymocytes to high-affinity self-Ag results in T cell tolerance. A predominant mechanism for this is clonal deletion; though receptor editing, anergy induction, and positive selection of regulatory T cells have also been described. It is unclear what signals are involved in determining different tolerance mechanisms. In particular, OT-I mice displayed receptor editing when the high-affinity self-Ag was expressed in cortical epithelial cells (cEC) using the human keratin 14 promoter. To test the hypothesis that receptor editing is a consequence of a unique instruction given by cEC presenting self-Ag, we created mice expressing the 2C and HY ligands under control of the keratin 14 promoter. Alternatively, we studied the fate of developing T cells in OT-I mice where Ag was presented by all thymic APC. Surprisingly, we found that the tolerance mechanism was not influenced by the APC subset involved in presentation. Clonal deletion was observed in 2C and HY models even when Ag was presented only by cEC; and receptor editing was observed in OT-I mice even when Ag was presented by all thymic APC. These results suggest that different TCRs show intrinsic differences in thymic tolerance mechanism. ***The Journal of Immunology*****, 2004, 172: 851–856.**

The exposure of immature T cells to self-peptide/MHC in the thymus is critical for immunological self-tolerance. The physical elimination of self-reactive progenitors via clonal deletion is one means by which this tolerance is achieved. Many TCR transgenic and nontransgenic models provide evidence that clonal deletion is a major tolerance mechanism (1). Indeed, recent evidence suggests that impaired clonal deletion in the thymus resulting from deficiency in the transcription factor AIRE, causes multiorgan autoimmune disease in humans and mice (2–5). Nonetheless, other mechanisms, including anergy induction (6, 7), receptor editing (8), and positive selection of regulatory T cells (9) have also been described. Recent studies of mice and humans deficient in the transcription factor, foxp3 showed that this transcription factor is critical for the induction of regulatory T cells and that animals with this deficiency also develop systemic autoimmunity (10–12). Therefore, it is clear that redundancy in central tolerance mechanisms is required for optimal immunological health.

Despite the importance of having multiple thymic tolerance mechanisms, it is unclear what signals are required for different fates: deletion, receptor editing, differentiation to a regulatory T cell, and development of anergy. A likely candidate could be the instruction given by different APCs in the thymus. While the vast majority of the cells in this organ are developing T cell progenitors themselves, the numerically small population of nonprogenitors have been shown to be highly critical for thymic developmental processes including tolerance induction (13, 14). Such "stromal" cells include the heterogeneous non-bone-marrow-derived thymic epithelial cells, which are divided into subcapsular, cortical, and medullary subsets; as well as bone marrow-derived dendritic cells and macrophages. Already, there is evidence suggesting a critical role for cell surface costimulatory molecules in the induction of clonal deletion (15, 16). These molecules are differentially expressed on the various thymic APC, implying one potential mechanism for differential instruction of cell fate.

Previous work in our lab showed that expression of a high-affinity self-Ag in cortical epithelial cells (cEC)[3] of the thymus did not lead to clonal deletion in the OT-I TCR transgenic model. Instead self-reactive progenitors continued to undergo endogenous TCRα gene rearrangement, suggesting receptor editing (17). This lack of a deletion response was consistent with previous experiments, which showed that cEC (2) do not induce deletion (18–20). Interestingly, cEC do not express costimulatory molecules known to be critical for endogenous superantigen mediated deletion, B7.1, B7.2, and CD40 (21). Thus, we hypothesized that receptor editing was a consequence of cEC presentation of self-Ag and that it would be used preferentially in tolerance to those Ags uniquely expressed by cEC and not by other thymic APC. To test this hypothesis, we used TCR transgenics that historically showed clonal deletion, and expressed their target Ag under the control of the keratin 14 (K14) promoter so it would be expressed in cEC. We found that cEC expression of self-Ag can cause either deletion or receptor editing, suggesting a receptor intrinsic difference in tolerance mechanisms and not an APC instruction mechanism.

Materials and Methods

Mice

C57BL/6 (B6) and C57BL/6.PL mice were obtained from The Jackson Laboratory (Bar Harbor, ME). OT-I mice express a transgenic receptor specific for the OVA 257–264 peptide (OVAp) in the context of the H-2K^b (22). For in vitro assays OT-I mice were bred to TAP-deficient background. 2C mice (23) express an alloreactive receptor that also has reactivity to a peptide (SIYRYYGL), in the context of H-2K^b (24, 25). HY mice express a transgenic receptor that recognizes male HY Ag in the context of H-2D^b (25). Transgenic strains expressing OT-I, 2C target Ags under the control of the human K14 promoter were generated as described (26, 27). They are referred to as K14-OVAp and K14-SIYp, respectively.

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[3] Abbreviations used in this paper: cEC, cortical epithelial cells; DP, double positive; DN, double negative; K14, keratin 14.

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K14-HYp mice expressing HY (KCSRNRQYL) Ag under the control of the human K14 promoter were constructed as follows. The adenovirus E19/3K signal sequence-peptide expressing construct was generated using a multistep PCR procedure to produce insert oligomers coding for the adenovirus signal sequence (MRYMILGLLALAAVCSAA) and the HY peptide. The PCRs were performed with three oligomers: O1 (5′-GGG AAG CTT ACG ATG AAA TAC ATG ATC CTG GGC CTG CTG-3′) with a *Hin*dIII site at the 59 terminus; O2 (5′-GGC CTG CTG GCC CTG GCC GCC GTG TGC AGC GCT GCC-3′), and O3 for HY (5′-TTT CTC GAG TCA CAG GTA CTG CCT GTT CCT GCT GCA CTT GGC AGC-3′), with an *Xho*I site at the 5′ terminus. The terminal base pairs of O2 were complementary to O3 and permitted their joining thorough PCR. Similarly, the terminal base pairs of O1 were complementary to O2, permitting construction of the desired insert, including sequences from O1 to O2. Act-mOVA mice were obtained from B. Ehst and M. Jenkins (University of Minnesota, Minneapolis, MN). These mice express a transmembrane form of OVA under control of the actin promoter and CMV immediate-early enhancer (26). OVA could be detected on the surface of all white blood cells by flow cytometry with an OVA-specific Ab (Sigma-Aldrich, St. Louis, MO) and in all tissues by immunohistochemistry (data not shown). All mice were treated in accordance with federal guidelines approved by the University of Minnesota Institutional Animal Care and Use Committee.

Flow cytometric analysis of lymphoid organs

Thymi were harvested from the indicated mice between 6 and 8 wk of age and stained with Abs to CD4 (L3T4, RMA-5) and CD8 (53-6.7), both obtained from BD PharMingen (San Diego, CA). Vb5 (MR9.1)- and Va2 (B20.1.1)-specific Abs were used to detect the OT-I transgene, although it should be noted that these Abs also recognize endogenous Vb5 and Va2 TCR chains. A clonotype-specific Ab (1B2) was used to detect the 2C receptor along with an Ab to Vb8 (F23.1). A clonotype-specific Ab (T3.70) was used to detect the HY receptor along with an Ab to Vb8 (F23.1). Data were collected using a FACSCalibur (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (TreeStar, San Carlos, CA).

Isolation of thymic stromal cells

Subsets of cells from the thymus of K14-OVAp, K14-SIYp, K14-HYp, Act-mOVA, BALB/c, or C57BL/6 male mice were isolated as described previously (28). Briefly, thymi were harvested, minced in 5 ml/thymus of digestion buffer: 1× PBS with 1.2 U/ml dispase, 0.1% DNase, and 1.6 mg/ml collagenase (all Sigma-Aldrich), and incubated at 37°C for 30 min. Cells were filtered and washed, and single cell suspension was incubated with Abs to CD4 (GK1.5, TIB-207) (American Type Culture Collection (ATCC), Manassas, VA) at 4°C for 30 min. DP thymocytes and $CD4^+$ SP thymocytes were depleted by "panning". Cells were extensively washed and plated on plates coated with goat anti-rat IgG (Sigma-Aldrich) for 30 min at 4ºC. Nonadherent cells were collected and isolated using MACS (Miltenyi Biotec, Auburn, CA). FITC-conjugated N418 (HB-224, ATCC) was used to select dendritic cells, FITC-F4/80 to select macrophages (Caltag Laboratories, Burlingame, CA), FITC-conjugated CDR-1 to select cEC (HB-213, ATCC), and FITC-conjugated G8.8 (Developmental Studies Hybridoma Bank, University of Iowa, IA) to select mEC. FITC-conjugated magnetic beads were used (Miltenyi Biotec) and positively selected cells were passed over the selection column twice to increase purity. The negative fraction from the previous selection step was used as a source for selection for the next step and the purity was assessed by flow cytometry (data not shown).

CD69 up-regulation assay

Thymic APC were serially diluted in round-bottom 96-well plates. Control C57BL/6 cells were pulsed with 100 nM of OVAp or 1 μM or SIYp and incubated at 37°C for 60 min. Unbound peptide was washed away, and the cells were plated at serial dilution in round-bottom 96-well plates. A total of 5×10^4 thymocytes from OT-I TAP-deficient or 2C β_2 microglobulin-deficient mouse were added per well. Cells were spun together at 1000 rpm for 5 min and then placed at 37°C for 6 h. The cells were stained with PE-anti-CD69, PerCP-anti-CD4, and APC-anti-CD8. At least 20,000 $CD4^+CD8^+$ events were collected using FACSCalibur (BD Biosciences) and analyzed with FlowJo software (TreeStar, San Carlos, CA).

Bone marrow chimeras

Single-cell bone marrow suspensions from K14-OVAp or C57BL/6 mice was prepared from leg bones and cells were depleted of mature T cells by complement-mediated cytotoxicity using 30H12 (anti-Thy1.2; ATCC) as described (29). Bone marrow cells were then injected i.v. (10^7 cells/recipient) into lethally irradiated mice (900 rad).

Sorting of DN thymocytes and i.t. injections

Single cell suspensions were prepared from thymi of OT-I/PL mice and $CD4^+$ and $CD8^+$ cells were depleted by "panning" as described above. Remaining $CD4^+$ and $CD8^+$ cells were depleted by MACS separation using FITC-conjugated goat anti-rat IgG (Southern Biotechnology Associates, Birmingham, AL) and anti-FITC magnetic beads (Miltenyi Biotec). The resulting double-negative (DN) cells were enriched for $CD25^+$ cells using biotinylated anti-CD25 (BD PharMingen) and streptavidin-conjugated magnetic beads (Miltenyi Biotec). The positive fraction from the magnetic column was stained with FITC-anti-CD8, PE-anti-Vα2, Cychrome-anti-CD4, and APC-streptavidin (to bind biotinylated cell-bound anti-CD25). The cells were sorted into DN $CD25^+V\alpha2^+$ and DN $CD25^+V\alpha2^-$ cells using FACSort (BD Biosciences) at Cancer Center Flow Cytometry Core Facility, University of Minnesota. Cells were kept in 5% FCS/PBS medium until used for intrathymic injections. Cells were then washed with PBS and injected into each thymic lobe of lethally irradiated and bone-marrow reconstituted K14-OVAp or C57BL/6 host mice as previously described (30).

Results

Ag presentation by different thymic APC

Several TCR transgenic models display clonal deletion of CD4, CD8 double-positive (DP) progenitors when self-Ag is expressed in the thymus (31). Thus we were surprised to observe inefficient clonal deletion and subsequent receptor editing in the class I restricted OT-I TCR transgenic model, despite confirming that Ag was expressed in the thymus cortex, where DP progenitors reside (17). However, in this case the human K14 promoter was used to drive expression of the peptide Ag (OVAp) as a neo self-Ag. This promoter is active exclusively in cEC of the thymus (18, 20). To test whether cortical epithelial expression of self-Ag provides a unique microenvironmental instruction for receptor editing, we created additional transgenics that expressed the target Ags for the 2C and HY TCR transgenics under the control of the K14 promoter. Alternatively, we used transgenic mice that express OVA ubiquitously, to test if OT-I cells would undergo deletion when the neo self-Ag was presented by additional thymic APC.

The 2C target Ag we used was a nonnatural 8-aa peptide (SIYRYYGL, SIYp for short) (24). It efficiently stimulates $2C^+$ CD8 T cells when presented by K^b. The HY target Ag used was the natural 9-aa peptide (KCSRNRQYL, HYp for short) (32). It is derived from the *Smcy* gene product, encoded by the Y chromosome and expressed only in male mice. The final approach we used was to compare the effect of K14-OVAp on OT-I thymic development to that where the OVA was expressed broadly. For this we used Act-mOVA mice (26), which express a transmembrane form of OVA under the control of the chicken actin promoter and cytomegalovirus immediate early enhancer. This strain expresses OVA protein, as detected by immunohistochemistry, in virtually all tissues.

To test if Ag expression was compartmentalized as expected in the various strains, we purified thymic APC and performed an in vitro thymocyte stimulation assay using responders from the appropriate TCR transgenic. Thymic APC were purified as described previously (28), with slight modifications. Briefly, stromal cells were released from 5–8 adult thymii with collagenase, dispase, and DNase and enriched by elimination of $CD4^+$ and $CD8^+$ thymocytes by panning. Magnetic separation using Abs to DC (N418), macrophages (F4/80), cEC (CDR-1), or medullary epithelial cells (G8.8) was used to purify specific subsets. Purity was verified by staining with fluorochrome-conjugated Abs to the populations. To test for the presentation of specific peptide by APC subsets, we used an in vitro thymocyte stimulation assay. We measured CD69 up-regulation on Ag specific thymocytes cocultured for 6 h with the APC from Ag transgenic strains. The thymocytes were obtained from TCR transgenic animals on a nonselecting

background. This assay requires very low numbers of APC and has a high sensitivity to Ag dose (low picomolar range) (33). The up-regulation of CD69 does not require a dedicated costimulus such as B7, so heterogeneity in the costimulatory molecules expressed was expected not to be an issue. The control cultures were thymic APC purified from nontransgenic mice, but pulsed with OVAp or SIYp peptide ex vivo. Fig. 1*A* shows that cEC express the specific peptide/MHC in K14-OVAp transgenic mice. cECs stimulated CD69 up-regulation, while macrophages and medullary epithelial cells did not. This was observed for K14-SIYp transgenic mice as well (Fig. 1*C* and data not shown). DC also presented the Ag in K14-OVAp transgenic mice (Fig. 1*A*), albeit very weakly. DC presentation was not observed in K14-SIYp mice (Fig. 1*C*). It is possible that DC do not directly synthesize the peptide transgene, but rather "cross-present" the Ag synthesized by cEC. This possibility was tested in bone marrow chimeras where bone marrow from C57BL/6 mice was used to reconstitute lethally irradiated K14-OVAp or control mice. Presentation by cEC was used as a positive control for the assay (Fig. 1*F*) since epithelial cells are radioresistant. Thymic DC from B6→K14-OVAp bone marrow chimeras were also able to stimulate OT-I thymocytes in vitro (Fig. 1*E*). This suggests that DC cross-present Ag synthesized by other cells in K14-OVAp mice. In contrast, all thymic APC presented Ag in Act-mOVA mice (Fig. 1*B*). This was also observed in BALB/c mice for the 2C Ag (Fig. 1*D* and data not shown). Thus, the presentation pattern confirms that the K14 promoter restricted ligand expression to specific thymic APC.

Thymic phenotype does not vary with different presenting cells

We measured the thymic cellularity (Table I) and cell surface phenotype (Fig. 2) of thymii from 4 to 6 wk old TCR transgenic animals crossed to Ag expressing strains. OT-I TCR transgenic animals have, on average, 40×10^6 DP thymocytes. As reported previously (17), introduction of the Ag under control of the K14 promoter did not result in dramatic reduction of this population (Fig. 2, OT-I/K14-OVAp). The observed reduction was ~2-fold (Table I). Instead the number of "edited" DP cells-those that express a high level of Vβ5, but low level of Vα2 increased 3-fold.

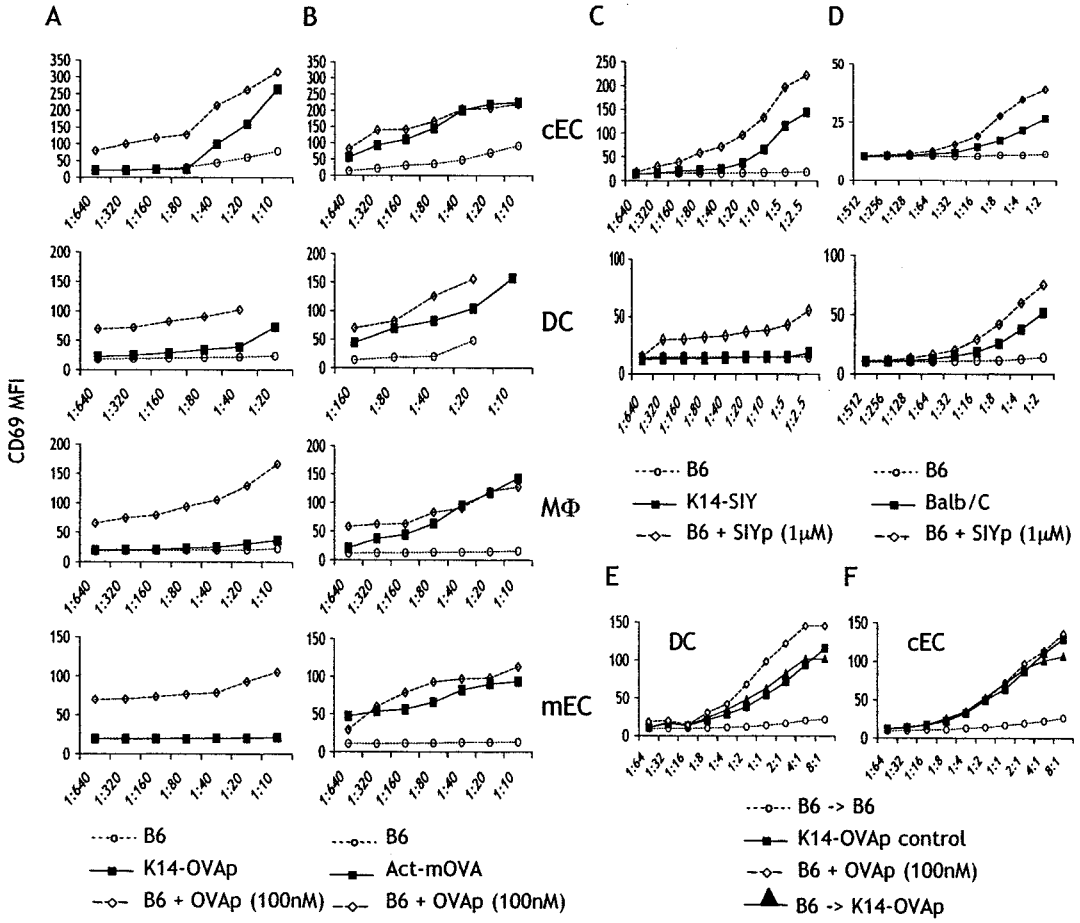

FIGURE 1. Ag presenting function of thymic APC from various strains. *A*, Both cEC and dendritic cells from K14-OVAp thymus are able to present Ag to OT-I cells. Dendritic cells, cEC, macrophages, and medullary epithelial cell (mEC) were isolated from thymi of B6 mice (○) and K14-OVAp mice (■) and incubated with OT-I TAP-deficient thymocytes at the indicated ratio. As a positive control B6 cells were pulsed with 100 nM OVA (◇). *B*, All thymic APC from Act-mOVA mice were able to present Ag to OT-I cells. Dendritic cells, cEC, macrophages, and mEC were isolated from thymi of B6 mice (○) and Act-tmOVA mice (■) and incubated with OT-I thymocytes. As a positive control B6 cells were pulsed with 100 nM OVA (◇). *C*, Only cEC but not dendritic cells from K14-SIYp mice are able to present Ag to 2C thymoctytes. cEC and dendritic from K14-SIYp and control B6 mice were isolated and incubated with 2C β_2 microglobulin-deficient thymocytes. *D*, Both cEC and dendritic cells from 2C/CB6F1 mice are able to present Ag to 2C thymocytes. Stromal cells were isolated and incubated with 2C thymocytes as described in *C*. The cells were harvested 6 h later and stained with Abs to CD8, CD4, and CD69, and 20,000 $CD8^+CD4^+$ events were collected and analyzed for CD69 expression. The total MFI for CD69 was determined. One of three representative experiments for K14-OVAp and K14-SIYp mice is shown. *E* and *F*, cEC and dedritic cells were isolated from B6→K14-OVAp, B6→B6 bone marrow and control K14-OVAp mice 4 wk after reconstitution. Stromal cells were incubated with OT-I TAP-deficient thymocytes and analysis was performed as described for *A* and *B*.

Table I. *Thymocyte cellularity in various strains of mice (10^6)*[a]

Strain	Total Thymocytes	DP	Edited DP
OT-I	68 ± 49.4	39.5 ± 31.2	2
OT-I/K14-OVAp	37 ± 10	21 ± 7	6
OT-I/Act-mOVA	32 ± 20.6	17 ± 13	6
2C	41.8 ± 19	16.3 ± 10.9	0.09 ± 0.11
2C/K14-SIY	14.4 ± 9.9	0.31 ± 0.27	0.06 ± 0.11
2C/Balb/c	3.8 ± 3	0.26 ± 0.23	NA
HY (F)	70 ± 30.8	39 ± 19.3	0.06 ± 0.04
HY/K14-HY (F)	14 ± 7.5	1.4 ± 2	0.15 ± 0.2
HY (M)	10 ± 6	0.29 ± 0.13	0.02 ± 0.03

[a] Total cell numbers of thymocytes were calculated after exclusion of dead cells by trypan blue. Surface staining was performed as described in *Materials and Methods*, using indicated mice. Number of double positive (DP) cells was calculated as a percentage of CD8, and CD4-positive cells of total cells. The number of edited DP cells was calculated as a percentage of DP cells that express transgenic TCRβ chain but did not stain with clonotypic Ab to the transgenic TCRα chain, i.e., V$\beta5^+$V$\alpha2^-$ DP cells in OT-I mice, V$\beta8^+$1B2$^-$ DP cells in 2C mice, and V$\beta8^+$T3.70$^-$ in HY mice. These numbers indicate average cell numbers and standard deviation from 16 OT-I, 12 OT-I/K14-OVAp, 13 OT-I/Act-mOVA, 11 2C, 10 2C/K14-SIYp, 3 2C/BALB/C, 6 HY females, 6 HY/K14-HY females, 8 HY males.

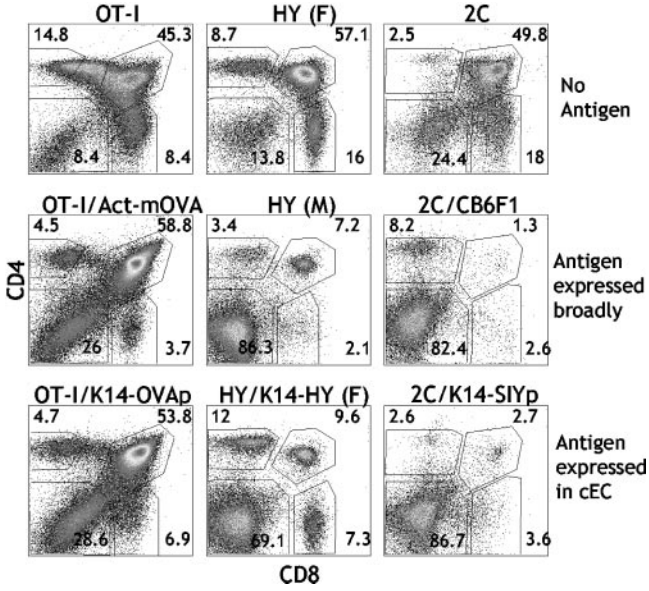

FIGURE 2. Thymic selection outcome is independent of Ag-expression pattern in different TCR transgenics. Thymocytes from OT-I, OT-I/Act-tmOVA, and OT-I/K14-OVAp mice, HY female, HY male, and HY/K14-HY female and from 2C, 2C/CB6F1, and 2C/K14-SIYp mice were stained with Abs to CD8 and CD4 and analyzed by FACS. The numbers represent percentages of cells in respective gates. The plots are representative of 16 OT-I, 12 OT-I/K14-OVAp, 13 OT-I/Act-mOVA, 11 2C, 10 2C/K14-SIYp, 3 2C/CB6F1, 6 HY females, 6 HY/K14-HY females, and 8 HY males analyzed.

Surprisingly, this was also observed in OT-I mice where OVA was presented by all thymic APC (Fig. 2, OT-I/Act-mOVA). Again there was not a dramatic reduction in DP numbers, and the number of "edited" DP increased 3-fold (Table I). The possibility that DC presentation of the Ag in K14-OVAp was contributing to receptor editing was also addressed by analysis of bone marrow chimeras made by reconstituting lethally irradiated K14-OVAp mice with OT-I/Kb^o/Db^o/RAG^o bone marrow. In these mice the OVAp Ag cannot be presented by bone marrow derived DC due to the Kb deficiency. The thymic phenotype of such chimeras was indistinguishable from intact transgenics (data not shown), further suggesting that presentation of OVAp by bone marrow-derived DC does not change the tolerance outcome. Altogether these results rule out the hypothesis that exclusive presentation of Ag by cEC causes poor clonal deletion, and subsequent receptor editing in OT-I/K14-OVAp mice. One might have predicted that clonal deletion was not efficient in OT-I/K14-OVAp mice because of limited Ag availability (34). This apparently is not the case, since OT-I/Act-mOVA mice also show inefficient clonal deletion and the level of Ag presentation by various thymic APC is quite high in Act-mOVA mice (Fig. 1*B*).

For the 2C TCR transgenic strain, we studied the effect of broad vs cortical epithelial Ag expression by comparing B6 mice that express the SIYp peptide under control of the human K14 promoter, to those expressing the classic ligand for 2C, L^d, which is broadly expressed in vivo. Fig. 2 shows the greatly reduced numbers of DP cells observed in L^d expressing animals (2C/CB6F1), as reported previously (23). Interestingly, when Ag expression was limited to cEC, a similar extent of deletion was observed, and there was no accumulation of edited DP cells (Fig. 2, 2C/K14-SIYp and Table I). Like the previous result, this suggests that cEC Ag presentation does not provide unique instruction for developing thymocytes regarding clonal deletion. It is possible that the K^b/SIYp and Ld/self peptide ligands may be subtly different in the way they stimulate the TCR. Thus, we generated and compared the thymic effect in a model system where the stimulating complex was identical, i.e., HY. We compared the thymic phenotype of HY TCR transgenic females to that of HY males and HY/K14-HY females. HY female mice have, on average, 40×10^6 DP thymocytes. In males, the SMCY protein and the target male Ag are expressed widely in all tissues (35). The DP numbers in male mice are reduced ~40-fold in male mice (Table I), as originally reported for this strain (25), suggesting a clonal deletion mechanism. We found that HY female mice that expressed the male Ag under control of the K14 promoter also showed a similar extent of deletion (Fig. 2, HY/K14-HY, and Table I). Altogether, these results allow us to conclude that cortical epithelial presentation of self-Ag can support either clonal deletion or receptor editing. This also suggests that the factors favoring one response or the other are likely to be model-intrinsic or TCR-intrinsic.

TCR$^+$ DN progenitors have a poor ability to give rise to DP

It was somewhat surprising to observe robust clonal deletion in the HY and 2C systems when Ag was presented predominantly by cEC. cEC do not express B7 ligands (36), yet DP thymocyte deletion was shown to be costimulus dependent (15, 16). However, given that TCR transgenic models express the TCR$\alpha\beta$ earlier in development than normal mice, it was possible that engagement of the TCR before the generation of DP cells led to elimination of the progenitor. This was previously suggested to be the case for the HY model (37). Indeed, these three TCR transgenic models do differ in the extent to which the $\alpha\beta$ TCR is expressed on CD25$^+$ DN progenitors. Virtually all CD25$^+$ DN progenitors express the $\alpha\beta$ TCR in 2C mice, 85% do in HY mice, and 59% do in OT-I mice (30). This was interpreted to mean that the OT-I receptor comes on "later" in development than 2C or HY. Thus, we considered the possibility that only those progenitors that encounter Ag for the first time at the DP stage would undergo receptor editing, whereas those that encounter Ag at the DN stage would undergo deletion. To address this, we sorted OT-I TCR transgenic CD25+ DN progenitors that did or did not express the OT-I receptor on the cell surface and intrathymically injected them into K14-OVAp or control recipients. Analogous experiments in the HY system are not reported, as we were unable to sort sufficient

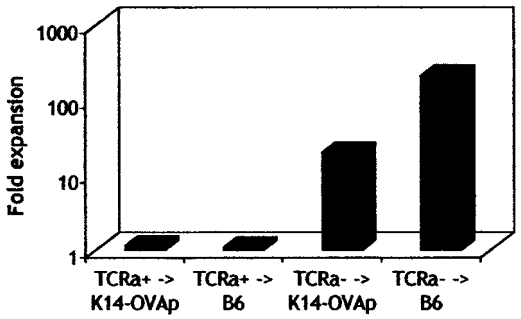

FIGURE 3. Only TCR-DN progenitors give rise to edited DP. DN thymocytes from adult OT-I/PL (Thy1.1$^+$) mice were sorted into CD25$^+$TCR$^+$ or CD25$^+$TCR$^-$ pools and intrathymically injected into B6 or K14-OVAp (Thy1.2) mice. Nine days later thymocytes were collected and analyzed by flow cytometry. Thy1.1$^+$ DP cells were enumerated and the fold expansion (relative to the number of DN input cells) was calculated. One of three experiments with similar outcome is shown.

numbers of TCRα^-CD25$^+$ DN progenitors from HY mice. Consistent with the above possibility, we noted that only TCR$^-$CD25$^+$ DN progenitors gave rise to DP cells in K14-OVAp recipients (Fig. 3), and such cells had an "edited" phenotype only in K14-OVAp hosts (data not shown). In contrast, very few cells could be recovered from K14-OVAp recipients that received TCR$^+$CD25$^+$ DN progenitors. However, TCR$^+$CD25$^+$ DN cells transferred into non-Ag expressing thymi also did not give rise to DP cells, suggesting an inherent inability of these progenitors to divide and differentiate into $\alpha\beta$ lineage cells, consistent with previously published results (30, 38). This suggests that DP cells largely arise from the small population of TCRα^-CD25$^+$ DN in OT-I transgenic. We cannot exclude the possibility that the observed effect is specific only to OT-I transgene since data from 2C and HY transgenic were not obtained. Nevertheless, finding lessens the concern that ectopic or early expression of TCRα in DN cells underlies the differences we observed between these TCR transgenic models.

Discussion

The efficient deletion induced by cEC in the 2C and HY models in our study was surprising given that previous work suggested that cEC do not support clonal deletion (18, 20). In fact, these studies used the same K14 promoter to drive expression of MHC class I or II molecules in MHC null mice, thereby specifically restoring class II expression in cEC. While such expression was sufficient to restore positive selection of CD8 and CD4 T cells, it did not support negative selection in response to superantigen, endogenous self-Ag, or exogenous foreign Ag (18). Furthermore, such T cells were overtly self-reactive in vitro and in vivo (18, 20). One difference between these studies and ours is the use of MHC ligands vs peptide Ag. MHC ligands must be recognized on the cells that synthesize the MHC gene, while peptide Ags can, in theory, be transferred or cross-presented by other APC. Thus it is possible that dendritic cell cross-presentation of Ag in K14-peptide transgenic mice (Fig. 1*E*) could cause deletion. However, DC presentation was observed in K14-OVAp mice, but not K14-SIYp mice. And this did not correlate to observed deletion, which was found in K14-SIYp mice, but not K14-OVAp mice. Alternatively, the efficiency with which cEC mediate deletion could reflect how costimulus dependent clonal deletion is in that particular model. cEC do not express B7 or CD40 costimulatory molecules and have little costimulatory capacity (36). And clearly some forms of clonal deletion are dependent on this costimulus (15, 16). It is possible that 2C and HY are less dependent on a costimulus for deletion than OT-I or the peptide, superantigen, and allo-reactive models used in the above studies.

Previous work suggested that 2C and HY mice express the TCR transgene earlier in development than OT-I mice (30). We considered the possibility that this could explain the differences we observed between transgenic models. $\alpha\beta$TCR expression at the DN stage is an unphysiologic property of TCR transgenic mice, since normal mice do not acquire surface $\alpha\beta$TCR expression until the DP stage, after TCRα locus rearrangement. Because DN thymocytes traverse the cortex en route to the subcapsular region (39), it is possible that TCR transgenic DN progenitors die upon encounter with Ag expressing cEC, preventing their development to the DP stage (37). Whereas, in the OT-I model if the receptor was not expressed until the DP stage, engagement may not lead to death as efficiently. However, intrathymic transfer experiments, (Fig. 3 and Ref. 30) as well as other experiments (40), suggest that the DP cells in TCR transgenics probably arise from the small population of TCR negative DN progenitors. Thus we think it is unlikely that differences in transgene expression timing can explain the difference in deletion efficiency.

We were unable to identify specific factors that led to clonal deletion vs receptor editing. Nonetheless our study supports a receptor intrinsic difference in tolerance mechanisms and not a difference in APC instruction. The role of APC instruction has also been studied in the context of dictating clonal deletion vs positive selection of regulatory T cells. In the 6.5 TCR transgenic system, both epithelial and hemopoetic Ag expression could induce regulatory T cells (19, 41, 42). Indeed in other TCR transgenic systems, the generation of regulatory T cells occurs concurrently with deletion (43–45). One possibility is a stochastic model, where some progenitors are deleted and other are positively selected to have regulatory function. The relative efficiencies of these processes may be dictated by the APC type, but both would occur and be advantageous to the animal.

An alternative to the APC instruction theory is that intrinsic properties of the specific receptor could influence thymic outcome. Indeed studies where a single receptor displayed different deletion efficiencies with different ligands clearly establishes a precedent for this (46, 47). Certainly different affinities of the TCRs for peptide/MHC may underlie the differences in thymic outcome. Perhaps a lower affinity TCR interaction does not trigger apoptosis as efficiently, and results in receptor editing instead of clonal deletion. We considered this unlikely in this particular case since two of the receptors used in this study, OT-I and 2C, have been extensively characterized in terms of affinity. When the same technique was used to study each (in this case surface plasmon resonance) both the overall affinity and the off-rate were similar between OT-I and 2C (48, 49). Other possible intrinsic factors are receptor level, extent to which the receptor is internalized (signal duration), and coreceptor dependence. These factors could all contribute to varying degrees of deletion efficiency. Those receptors that do not efficiently induce deletion allow the induction of additional tolerance mechanisms such as positive selection of regulatory T cells and receptor editing. Furthermore, receptor editing (certainly) and positive selection of regulatory T cells (possibly) may be limited to those interactions with self-Ag that occur at the immature DP stage. However, clonal deletion can also occur later in development after positive selection, when the cell resides in the medulla, since thymocytes remain susceptible to clonal deletion for some time after positive selection (50). Therefore, inefficiencies in clonal deletion in response to self-Ags expressed exclusively by medullary cells may not qualify for the other central tolerance mechanisms, such as receptor editing and positive selection of regulatory T cells.

In our study the outcome of thymic selection was independent of Ag-expression pattern in the thymus. These data support a model in which mechanisms of central tolerance are determined by intrinsic differences in the TCR rather than signals provided by specific APC during T cell development. Also, it raises an intriguing possibility that for particular TCR transgenic models clonal deletion may be sufficiently mediated by encounter with high-affinity Ag on cEC. The exact properties that predispose for clonal deletion efficiency remain to be established.

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