Central Tolerance to Tissue-specific Antigens Mediated by Direct and Indirect Antigen Presentation

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Abstract

Intrathymic expression of tissue-specific antigens (TSAs) by medullary thymic epithelial cells (Mtecs) leads to deletion of autoreactive T cells. However, because Mtecs are known to be poor antigen-presenting cells (APCs) for tolerance to ubiquitous antigens, and very few Mtecs express a given TSA, it was unclear if central tolerance to TSA was induced directly by Mtec antigen presentation or indirectly by thymic bone marrow (BM)-derived cells via cross-presentation. We show that professional BM-derived APCs acquire TSAs from Mtecs and delete autoreactive CD8 and CD4 T cells. Although direct antigen presentation by Mtecs did not delete the CD4 T cell population tested in this study, Mtec presentation efficiently deleted both monoclonal and polyclonal populations of CD8 T cells. For developing CD8 T cells, deletion by BM-derived APC and by Mtec presentation occurred abruptly at the transitional, $CD4^{high} CD8^{low} TCR^{intermediate}$ stage, presumably as the cells transit from the cortex to the medulla. These studies reveal a cooperative relationship between Mtecs and BM-derived cells in thymic elimination of autoreactive T cells. Although Mtecs synthesize TSAs and delete a subset of autoreactive T cells, BM-derived cells extend the range of clonal deletion by cross-presenting antigen captured from Mtecs.

Key words: thymus • tolerance • tissue-specific antigen • cross-presentation • AIRE

Introduction

Central tolerance is induced in the thymus, where developing thymocytes that recognize self-peptide–MHC complexes with too high affinity are deleted. For decades, immunologists have tried to understand the roles of thymic epithelial cells (tecs) and BM-derived cells in negative selection. Studies analyzing [Parent→F$_1$] BM chimeras or thymus-grafted animals conclusively demonstrated that BM-derived cells are strong inducers of thymic tolerance, whereas tolerance induction by tecs is incomplete (1, 2). Most of these experiments analyzed central tolerance to antigens that are widely expressed in many or all tissues.

Recent reports have renewed interest in the cell biology of negative selection by showing that in both human and mouse, medullary tecs (Mtecs) express a broad range of antigens (for example, insulin), whose expression was previously thought to be restricted to peripheral tissues (3–6). Importantly, there is growing evidence that Mtec expression of tissue-specific antigens (TSAs) leads to deletion of autoreactive T cells. Thymic expression of some TSAs is dependent on the autoimmune regulator protein (AIRE), and AIRE deficiency results in organ-specific autoimmune disease (7,

8). In fact, humans expressing mutations in AIRE develop multi-organ autoimmune disease (9). In addition, transgenic antigen, driven off tissue-specific promoters, results in thymic expression of antigen and elimination of T cells reactive against these antigens (10, 11). Interestingly, such T cells are rescued from deletion when they develop in AIRE-deficient thymuses (12). Taken together, these data demonstrate that there is strong central tolerance to TSA and that this process is required for the maintenance of self-tolerance.

The thymic APC that mediates tolerance to TSA remains unresolved. If Mtecs express TSAs yet are poor inducers of tolerance, then how are T cells reactive against TSAs eliminated? This problem is particularly acute for ectopic TSAs, which appear to be turned on in only 1–5% of Mtecs (3, 5, 13, 14). Based on these findings, we hypothesized that although Mtecs synthesize TSAs, they do not directly present antigen to delete TSA-reactive T cells. Rather, Mtecs could act as TSA reservoirs for professional BM-derived APCs, probably thymic DCs, which would capture TSAs from Mtecs, process the antigen, and cross-present the TSA

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epitopes to delete autoreactive thymocytes. The results from our studies show that DCs can indeed pick up TSAs from Mtecs and delete autoreactive T cells via cross-presentation. In addition, we find that Mtecs, surprisingly, can be strong inducers of tolerance to TSA, although these cells are limited in their capacity to induce tolerance to all TSA-reactive T cells tested in this study. Results from our studies provide a framework to understand the cellular interactions during negative selection.

Materials and Methods

Mice. CD45.1 congenics (B6.SJL–$Ptprc^a$Pep3[b]/BoyJ) were purchased from The Jackson Laboratory. RAG-1$^{-/-}$, OT-I, OT-I.K^{b}D$^{b-/-}$, OT-II, OT-II.IA$^{b-/-}$, B6, RIP-mOVA, OT-I.K14-K^b.K^{b}D$^{b-/-}$, OT-I.K14-K^b.RIP-mOVA.K^{b}D$^{b-/-}$, and K14-K^b.RIP-mOVA.K^{b}D$^{-/-}$ mice were bred and housed in pathogen-free facilities at the University of Washington. OT-I and OT-II mice have transgenic Vα2Vβ5 TCRs specific for OVA$_{257-264}$ in the context of H2-K^b, and OVA$_{323-329}$ in the context of IA^b, respectively (15, 16). RIP-mOVA mice express a membrane-bound form of OVA (residues$_{139-385}$) under control of the rat insulin promoter (RIP; 17).

To generate K14-K^b transgenic mice, H2-K^b cDNA was cloned into the BamHI site of the K14 promoter construct (provided by E. Fuchs, University of Chicago, Chicago, IL), followed by excision of the EcoRI fragment and injection into FVB $\times$ B6 embryos. Founder mice were crossed onto an MHC class I–deficient background, H2-K^{b}D$^{b-/-}$, and evaluated for their ability to positively select a polyclonal repertoire of CD8 as well as OT-I cells.

Purification of tecs. Thymuses from 25 mice, ages 3–12 wk, were diced and gently agitated with a magnetic stir bar in 50 ml RPMI 1640 plus 5% FCS at 25°C for 20 min to remove thymocytes. The thymic fragments were then incubated for 20 min with agitation at 30°C in 15 ml RPMI 1640 solution containing 20 mM Hepes, 2% FCS, 0.2 mg/ml collagenase I, and 25 μg/ml DNase I. After this step, the thymic fragments were saved and incubated for 20 min with agitation at 30°C in 15 ml RPMI 1640 solution containing 20 mM Hepes, 2% FCS, 0.2 mg/ml collagenase IV, 0.2 mg/ml dispase, and 25 μg/ml DNase I. This step was repeated twice, and the supernatants that contained dissociated tecs were saved, while the remaining thymic fragments were incubated for 25 min with agitation at 37°C in PBS containing 0.5 mM EDTA, 0.05% trypsin, and 50 μg/ml DNase I. The supernatants from this tyrpsin digest were combined with the supernatants from the collagenase/dispase digests and were washed twice in RPMI 1640/5% FCS, before being placed on a discontinuous percol density gradient for further tec enrichment. The low density fractions were saved and washed twice with RPMI 1640/5% FCS before staining with anti–CD45-PE, anti–Ep-CAM–Alexa Flour 647, and anti–CDR-1-DIG:anti-DIG FITC. Before cell sorting, cells were resuspended in buffer containing 7AAD (Molecular Probes) to identify dead or dying cells. Sorted Mtecs were 93% EP-CAM$^+$ and CD45/7AAD/CDR-1$^-$. Sorted cortical tecs (Ctecs) were 70% EP-CAM/CDR-1$^+$ and CD45/7AAD$^-$.

RNA Isolation and Real-Time PCR. RNA was purified (Rneasy Micro Kit; QIAGEN) from sorted tecs and made into cDNA (Sensiscript; QIAGEN) according to the manufacturer's instructions.

Real-time PCR was performed with the following primer and probe sets: HPRT: forward, 5′-TGGAAAGAATGTCTTGAT-TGTTGAA; reverse, 5′-AGCTTGCAACCTTAACCATT-TTG; probe, 5′-FAM-CAAACTTTGCTTTCCCTGGTTAA-GCAGTACAGCTAMRA; CAT L: forward, 5′-CTGTTG-CTATGGACGCAAGC; reverse, 5′-CAGAACCCCATGGT-CGAGG; probe, 5′-FAM-TTCAGGCATCTACTATGAAC-CCAACTGTAGCAG-TAMRA; CAT S: forward, 5′-GCCA-TTCCTCCTTCTTCTTCTACA; reverse, 5′-CAAGAACAC-CATGATTCACATTGC; probe, 5′-FAM AAGCGGTGTCT-ATGATGACCCCTCCTGTA-TAMRA; insulin: forward, 5′-GACCCACAAGTGGCACAA; reverse, 5′-ATCTACAATGC-CACGCTTCTG; probe, 5′-FAM-GCCCGGGAGCAGGTG-ACCTT-TAMRA; and OVA: forward, 5′-TCATGGTATGT-TGGCAAATATGG; reverse, 5′-TGATTAAAGGAACAAAA-GAGCACATT; probe, 5′-FAM-AATTGTTTTCCTTGTAC-CCATATGTAATGGGTCTTGTAMRA.

In a 25-μl reaction, cDNA was incubated with 0.5 μM forward and reverse primers (Invitrogen), 0.2 μM 5′,6-FAM-3′-TAMRA fluorogenic probe (Biosearch Technologies), and TaqMan 2$\times$ universal PCR Mastermix (Applied Biosystems). No template controls and no reverse transcriptase controls were included for each primer/probe set and cDNA set, respectively. Transcript levels were normalized to HPRT levels before determining the relative expression in the gene of interest.

Generation of BM Chimeras. To generate chimeric mice, 4–6-wk-old recipient mice were irradiated with 1,000 rads from a ^{137}Cs source and i.v. injected 1 d later with CD4- and CD8-depleted BM cells. Chimeric mice were maintained on antibiotic water containing neomycin sulfate and polymyxin B sulfate for 2 d before irradiation and 3 wk after irradiation. Mice were analyzed 8 wk after BM transfer. In experiments presented in Figs. 1, 1–6, BM was depleted of NK1.1$^+$ cells. In experiments presented in Figs. 5 and 6, recipient mice were depleted of NK1.1$^+$ cells (1 d before irradiation recipient mice were injected i.p. with 500 μg of the monoclonal antibody PK136).

Listeria Monocytogenes Infections. The recombinant *L. monocytogenes* strain engineered to secrete chicken OVA (rLmOva) was provided by H. Shen (University of Pennsylvania School of Medicine, Philadelphia, PA; reference 18). Frozen stocks of rLm-Ova were grown in brain–heart infusion broth. Bacteria culture samples were grown to mid-log phase, measured by OD (A600), and diluted in PBS for injection. Mice were infected with 2,000 CFUs of rLmOva (19).

Intracellular IFN-γ Staining. Intracellular IFN-γ staining was performed in accordance with the manufacturer's instructions (BD Biosciences). In 96-well plates, 1–2 $\times$ 10^6 cells/well were stimulated with medium alone (no peptide) or 10^{-7} M OVA peptide (SIINFEKL) for 5 h in the presence of 1 μg/ml brefeldin A. Cells were then washed, stained with anti–CD8 and anti–Ly5.2 (BD Biosciences), resuspended in permeabilization fixation buffer, and stained with anti–IFN-γ antibody. Labeled cells were washed in permeabilization buffer, resuspended in 4% paraformaldehyde buffer, and analyzed by flow cytometry.

Results

A Polyclonal Repertoire of CD8 T Cells Is Deleted in RIP-mOVA Thymuses. To study central tolerance to TSA, we used RIP-mOVA mice that express a membrane-bound form of OVA under the control of the RIP. In these mice, OVA is expressed in the pancreas, kidney, and thymus (specifically by Mtecs, see below; reference 17). The RIP-mOVA mouse was chosen as a model TSA for three main
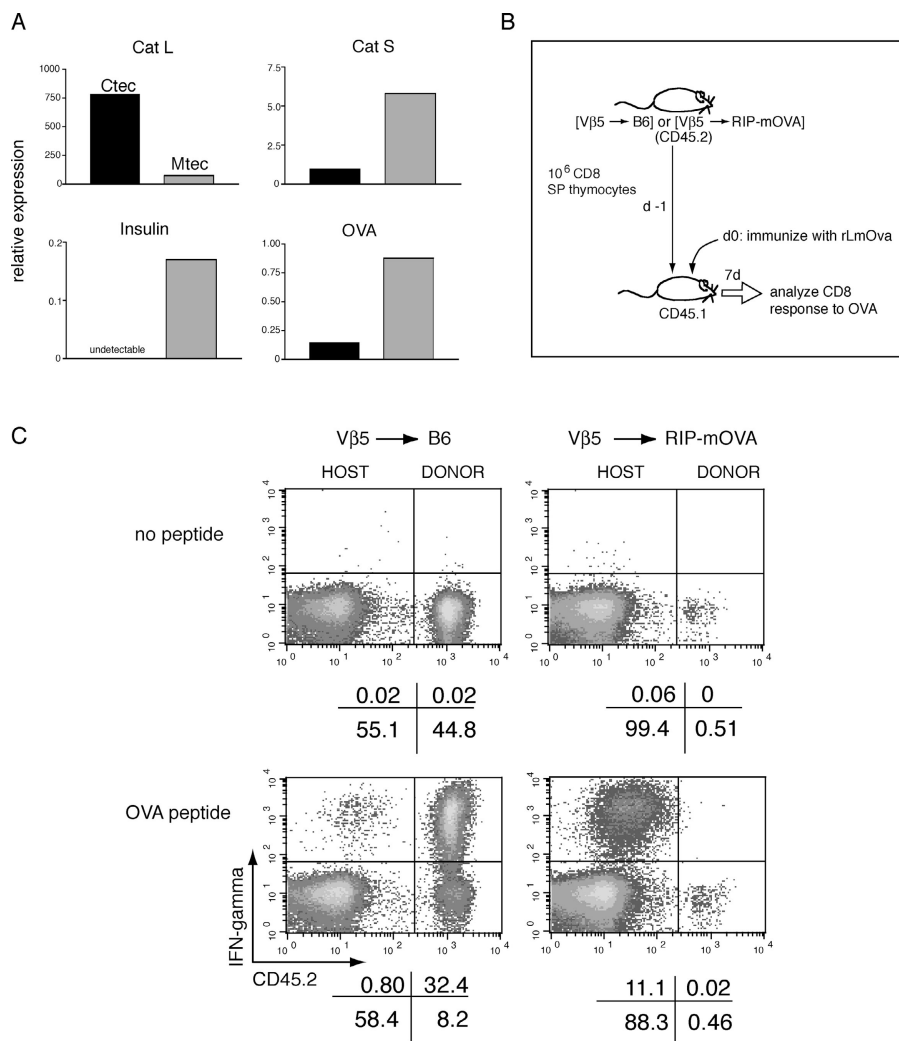
Figure 1. Expression of OVA by Mtecs leads to deletion of a polyclonal repertoire of OVA-specific CD8 T cells. (A) OVA is selectively expressed in Mtecs in RIP-mOVA thymuses. Ctecs and Mtecs from RIP-mOVA thymus were enriched and expression of OVA was investigated by real-time PCR. Cathepsin L (Cat L), cathepsin S (Cat S), and insulin were monitored as controls for cell purity. Ctecs expressed high levels of cathepsin L, low levels of cathepsin S, and no insulin. Meanwhile, Mtecs expressed low levels of cathepsin L, high levels of cathepsin S, and insulin (reference 3). (B) Grafting Vβ5 BM (CD45.2) into lethally irradiated B6 or RIP-mOVA recipients generated [Vβ5→B6] or [Vβ5→RIP-mOVA] BM-chimeric thymuses. 8 wk after BM transfer, 10^6 CD8 thymocytes were transferred into wild-type, congenically marked B6 mice (CD45.1). 1 d later, these mice were immunized with rLmOva. 7 d after immunization, splenocytes were isolated and their ability to produce IFN-γ was determined in response to a 5-h incubation with OVA peptide or medium alone. This experiment was performed twice with two mice per group. (C) IFN-γ and CD45.2 staining on gated CD8 T cells. Trasfer of thymocytes from [Vβ5→B6] is shown in the left panels. Transfer of thymocytes from [Vβ5→RIP-mOVA] is shown in the right panels. Numbers below indicate percentages of cells in each quadrant. Vβ5 transgenic donor-derived CD8 T cells are CD45.2+, whereas host-derived CD8 T cells are CD45.2−.

reasons. First, in multiple RIP transgenic lines, this promoter was shown to be on in rare Mtecs (5). Second, OVA is membrane bound, which should limit the thymic presentation of antigen to OVA-expressing Mtecs or cells that acquire OVA by interacting with such Mtecs. A major goal of our studies is to understand if thymic DCs acquire TSA directly from Mtecs. For this reason, use of a secreted antigen would greatly limit interpretation of our experiments, given the possibility that thymic DCs, or other cells, could acquire the antigen through diffusion and endocytosis. The final reason we chose RIP-mOVA as a model TSA is because thymic deletion of OVA-specific T cells in these mice is known to be dependent on the thymic expression of OVA and is not the result of OVA reaching the thymus from the periphery, either via blood or from a migrating APC (20).

To understand if OVA is regulated in the same manner as insulin or other TSAs, we purified Ctecs and Mtecs from RIP-mOVA thymuses and assessed the expression of OVA by real-time PCR. As is the case for other TSAs, we found that Mtecs are the primary producers of OVA in the RIP-mOVA thymus (Fig. 1 A). To determine if a polyclonal

repertoire of OVA-specific CD8 T cells is deleted in RIP-mOVA thymuses, we made use of Vβ5 transgenic mice (21). T cells from these mice use the TCR-β chain of the OVA-specific OT-I TCR and have a high precursor frequency of OVA-specific CD8 T cells (~1–2%; reference 21). Importantly, OVA-specific CD8 T cells in Vβ5 transgenic mice have diverse TCR Vα usage, indicating that OVA-specific CD8 T cells in these mice have a polyclonal TCR repertoire (21).

To ask whether polyclonal OVA-specific CD8 T cells are deleted during development in RIP-mOVA thymuses, we determined whether Vβ5 transgenic CD8 single positive (SP) thymocytes from RIP-mOVA mice were capable of responding to OVA. CD45.1 congenic hosts received 10^6 CD8 SP thymocytes from CD45.2+ [Vβ5→B6] or [Vβ5→RIP-mOVA] BM-chimeric mice, and were then immunized with 2,000 *L. monocytogenes*–expressing OVA (rLmOva) 1 d later (Fig. 1 B). As expected, we detected a robust response to OVA from the adoptively transferred Vβ5 transgenic CD8 T cells isolated from B6 thymuses amounting to >30% of total CD8 cells in the spleen 7 d after immunization (Fig. 1 C, left panels). In this case, the re-

sponse elicited from 10^6 donor cells is 40 times greater than the response of the entire host CD8 compartment. In contrast, we were unable to detect any CD8 response to OVA from Vβ5 transgenic CD8 T cells isolated from RIP-mOVA thymuses. In this case, only host CD45.2$^-$ CD8 T cells responded (Fig. 1 C, right panels). These results show that a polyclonal repertoire of OVA-specific CD8 T cells is efficiently deleted in the RIP-mOVA thymus.

Deletion of CD8 T Cells in RIP-mOVA Thymus Occurs Late in Development. Use of Vβ5 transgenic mice in the previous experiments demonstrated that OVA-specific CD8 T cells are efficiently deleted when they develop in RIP-mOVA thymuses. To study the timing of thymic deletion more precisely, we monitored the development of TCR transgenic OT-I T cells in deleting RIP-mOVA or nondeleting B6 thymuses. Cellularities differed only modestly between these thymuses (B6: $83.0 \times 10^6 \pm 2.6$ vs. RIP-mOVA: $63.7 \times 10^6 \pm 6.4$), suggesting that a minor population of thymocytes is eliminated in the RIP-mOVA thymus. Gating on the principal populations of OT-I thymocytes, we found that the number of double positive (DP) thymocytes (DP bright and DP dull) was comparable between RIP-mOVA and B6 (Fig. 2 A). We consistently found a modest reduction in the percentage of CD4 SP T cells in RIP-mOVA compared with B6 thymuses. Most of these cells have been shown to be transitional cells and pre-

cursors to CD8 SP thymocytes in the OT-I system (22). In addition, we found a threefold reduction in the percentage of cells in the CD8 SP gate in RIP-mOVA thymuses. The remaining CD8 SP thymocytes in the RIP-mOVA thymus were immature because they expressed high levels of CD24 (heat stable antigen) and low levels of transgene-encoded TCR (Vα2; Fig. 2 B). The bulk of SP CD8 cells in RIP-mOVA thymuses are therefore precursors to DP cells. Overall, there is close to a 100-fold reduction in the number of phenotypically mature CD8 T cells in the RIP-mOVA thymus compared with the B6 thymus.

Next, we sought to determine at what stage OVA-specific T cells are deleted in the RIP-mOVA thymus. We gated on the principal thymocyte populations and used TCR (Vα2) expression level as a maturity marker (Fig. 2 C). DP bright T cells were comparable in number and phenotype in B6 and RIP-mOVA thymuses. Next, we looked at DP dull cells that have undergone an early stage of positive selection and down-regulated CD4 and CD8. Vα2 staining revealed two distinct populations of DP dull cells in the B6 thymic environment, including a relatively immature population that was TCR intermediate (DP dull TCR++) and a more mature population that was TCR high (DP dull TCR++++). In striking contrast, only the immature DP dull TCR++ population was present in RIP-mOVA thymuses, whereas the DP dull TCR++++ population was absent. TCR levels
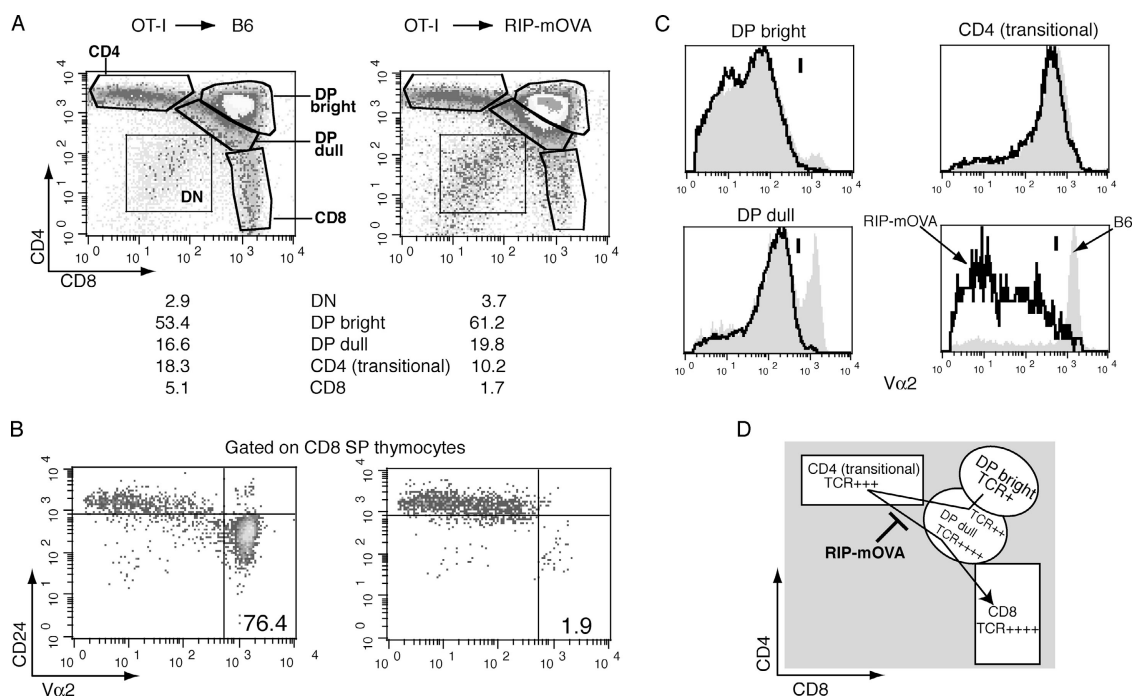
Figure 2. OT-I T cells are deleted late in development in RIP-mOVA thymuses. (A) OT-I transitional cells and CD8 SP thymocytes are reduced in RIP-mOVA thymuses. Grafting OT-I BM into lethally irradiated B6 or RIP-mOVA recipients generated [OT-I→B6] or [OT-I→RIP-mOVA] BM-chimeric thymuses. Thymocytes from the indicated mice were analyzed for expression of CD4 and CD8 by flow cytometry. The numbers below indicate the percentages of cells in each gate. (B) Remaining CD8 SP thymocytes in RIP-mOVA mice are immature. CD24 and TCR (Vα2) expression after gating on CD8 SP thymocytes. Numbers in the quadrants indicate the percentage of mature phenotype cells. Results presented here are representative of three independent studies with two to four mice per group. (C) Vα2 expression by thymocyte populations gated as shown in A. Solid black line represents thymocytes from RIP-mOVA mice, and shaded gray histograms represent thymocytes from B6 mice. The bar indicates Vα2 levels on transitional thymocytes from B6 mice. (D) Schematic representation of OT-I thymocyte development and postulated stage of deletion in RIP-mOVA thymuses.

on transitional cells (CD4+ TCR+++) in both B6 and RIP-mOVA thymuses were comparable. The level of TCR on these transitional thymocytes is intermediate to that on the two DP dull thymocyte populations found in B6, and is marked by a bar in Fig. 2 C. Finally, the CD8 SP gated cells in the B6 thymus contain a majority of TCR++++ cells, whereas the cells in the RIP-mOVA thymus contain very few mature cells (Fig. 2, B and C).

The pathway of T cell development in control mice and the stage of deletion in the RIP-mOVA thymus that we deduce from these results are presented schematically in Fig. 2 D. We conclude that in both B6 and RIP-mOVA thymic environments, DP bright TCR+ T cells are positively selected and become DP dull TCR++ T cells. Up to this point, there is no difference between control and deleting thymuses. The cells then up-regulate CD4 expression and develop into transitional cells (CD4+ TCR+++). Next, in the B6 thymus, transitional cells become DP dull TCR++++ T cells and eventually give rise to mature CD8 SP T cells (CD8 TCR++++). In contrast, DP dull TCR++++ T cells are undetectable in the RIP-mOVA thymus, suggesting that deletion occurs just before this stage of development. Evidence for deletion at the transitional cell stage comes from the observation that there is a modest yet consistent reduction in the percentage of transitional (CD4 TCR+++) cells in the RIP-mOVA thymus (Fig. 2 A).

Efficient Deletion of CD8 T Cells in RIP-mOVA Thymus Does Not Require Antigen Presentation by BM-derived Cells. To determine if antigen presentation by BM-derived cells was responsible for the deletion of CD8 T cells in RIP-mOVA thymuses, we investigated both Vβ5 and OT-I transgenic thymocytes developing in environments where BM-derived cells did not express MHC class I and therefore could not present OVA to developing thymocytes. We generated BM chimeras by grafting MHC class I–deficient Vβ5 BM (Vβ5.MHC I$^{-/-}$) into lethally irradiated RIP-mOVA recipients ([Vβ5.MHC I$^{-/-}$→RIP-mOVA] BM chimeras) and asked whether Vβ5 transgenic CD8 SP thymocytes from these chimeric mice were capable of responding to OVA. NK1.1-depleted RAG-1$^{-/-}$ hosts received 5×10^5 CD8 SP thymocytes and were immunized with 2,000 rLmOva 1 d later (Fig. 3 A). We detected robust responses to OVA from Vβ5 thymocytes that matured in B6 thymic environments ([Vβ5→B6] and [Vβ5.MHC I$^{-/-}$→B6] BM chimeras; Fig. 3 B). In contrast, we did not detect any OVA-specific responses from Vβ5 CD8 T cells that matured in RIP-mOVA thymic environments in which BM-derived cells expressed MHC class I ([Vβ5→RIP-mOVA] BM chimeras) and, surprisingly, when BM-derived cells did not express MHC class I ([Vβ5.MHC I$^{-/-}$→RIP-mOVA] BM chimeras).

Similarly, analysis of OT-I thymocytes that developed in RIP-mOVA thymic environments in which BM-derived cells did not express MHC class I ([OT-I.MHC I$^{-/-}$→RIP-mOVA] BM chimeras), revealed that OT-I thymocytes were efficiently deleted in this setting. Thus, the percentages of CD8 SP thymocytes and phenotypically mature CD8 SP thymocytes were comparable to that seen in control RIP-mOVA thymuses in which BM-derived cells expressed MHC class I (Fig. 4, A and B). These data demonstrate that antigen presentation by BM-derived cells is not required for the deletion of OVA-specific polyclonal or monoclonal CD8 thymocytes in the RIP-mOVA thymic environment, and suggest that Mtec presentation is sufficient to delete OVA-specific CD8 T cells. One problem with this interpretation is the possibility that host MHC
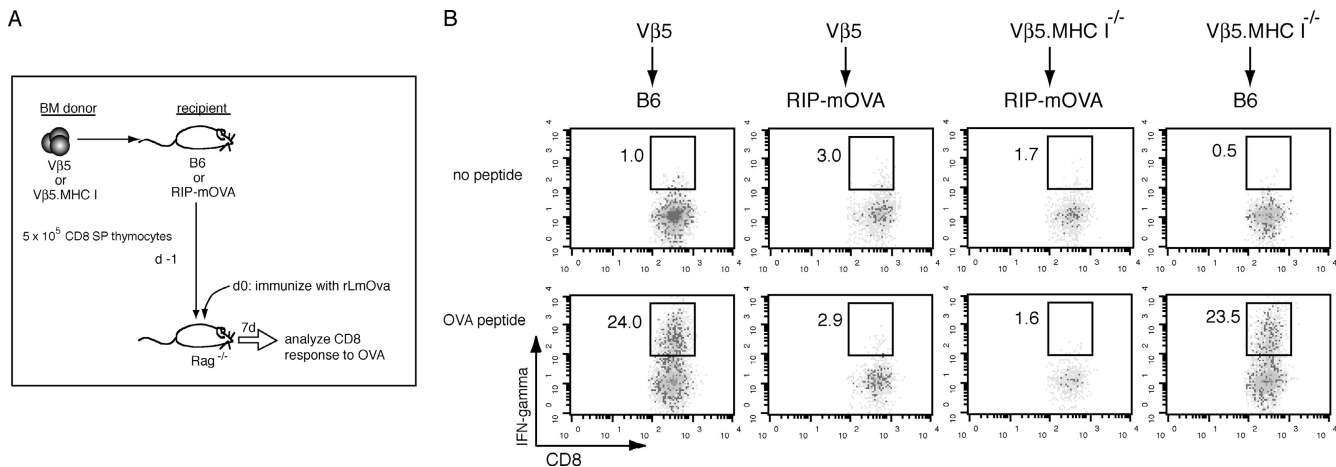
Figure 3. Deletion of OVA-specific polyclonal CD8 thymocytes does not require antigen presentation by BM–derived cells. (A) Grafting Vβ5 or Vβ5.MHC I$^{-/-}$ marrow into lethally irradiated B6 or RIP-mOVA recipients generated [Vβ5→B6], [Vβ5→RIP-mOVA], [Vβ5.MHC I$^{-/-}$→B6], and [Vβ5.MHC I$^{-/-}$→RIP-mOVA] BM-chimeric thymuses. 2–4 mo after BM transfer, 0.5×10^6 Vβ5 CD8 thymocytes matured in the indicated thymic environments were transferred into NK1.1-depleted (i.p. injection of 500 μg of the monoclonal antibody PK136) RAG-1$^{-/-}$ mice. 1 d later, these mice were immunized with rLmOva. To eradicate remaining rLmOVA, on day 4 after immunization, mice were injected i.p. with 1 mg ampicillin and were given ampicillin in their drinking water (5 mg/ml) for the remainder of the experiment. 7 d after immunization, splenocytes were isolated and their ability to produce IFN-γ was determined in response to a 5-h incubation with OVA peptide or medium alone. (B) IFN-γ and CD8 staining on gated CD8 T cells from the indicated transfers. Numbers indicate percentages of cells within the gate.
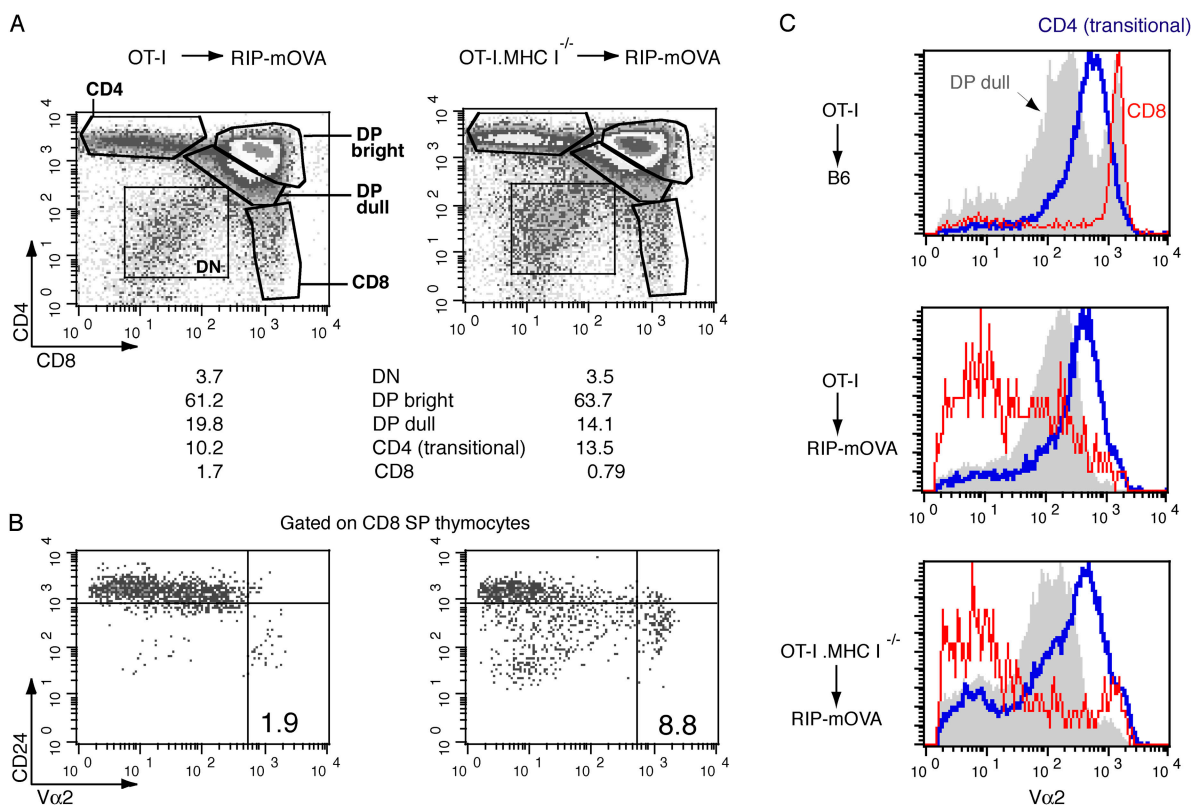
Figure 4. Deletion of OT-I/RIP-mOVA thymocytes does not require antigen presentation by BM-derived cells. Lethally irradiated RIP-mOVA mice were reconstituted with either OT-I or OT-I.MHC I$^{-/-}$ BM. Results presented here are representative of three independent studies with two to four mice per group. (A) Thymocytes from the indicated mice were analyzed for CD4 and CD8 expression by flow cytometry. The numbers indicate the percentage of cells in each gate. (B) CD24 and Vα2 expression after gating on CD8 SP thymocytes. Numbers in the quadrants indicate the percentage of mature phenotype cells. (C) Vα2 expression was analyzed on DP dull, CD4, and CD8 thymocytes taken from the indicated mice. The data analyzed are from the gated populations shown in Fig. 2 A for [OT-I→B6] BM chimeras and from the gated populations shown in A for [OT-I→RIP-mOVA] and [OT-I.MHC I$^{-/-}$→RIP-mOVA] BM chimeras. Shaded gray histogram represents the DP dull population, and blue and red lines represent CD4 SP (transitional) and CD8 SP populations, respectively.

class I–sufficient, BM-derived cells were not completely replaced by MHC class I–deficient BM grafts. This is not the case, however, because we were unable to detect any MHC class I–bearing thymic DCs from chimeric thymuses ([MHC I$^{-/-}$→WT] BM chimeras). This indicates that antigen presentation leading to clonal deletion in chimeric thymuses is mediated exclusively by Mtecs.

Next, we determined whether the timing of OT-I deletion in the absence of MHC class I–bearing BM cells was similar to that seen when BM cells expressed MHC class I (Fig. 2, C and D). We examined expression of Vα2 on DP dull, transitional, and CD8 SP subsets developing in RIP-mOVA thymuses in which antigen presentation is mediated exclusively by Mtecs ([OT-I.MHC I$^{-/-}$→RIP-mOVA] BM chimeras). Deletion of OT-I thymocytes occurred between the transitional TCR+++ and DP dull TCR++++ stages of development at precisely the same stage observed for cells developing in thymuses in which both BM and Mtecs expressed MHC class I ([OT-I→RIP-mOVA] BM chimeras; Fig. 4 C). DP dull TCR++++ cells were absent whether or not MHC was expressed on DCs. In addition, the percentage of transitional cells in [OT-I.MHC I$^{-/-}$→RIP-mOVA] BM chimeric thymuses was modestly reduced compared with nondeleting B6 thymuses, similar to the reduction seen in [OT-I→RIP-mOVA] BM chimeric thymuses (Figs. 2 A and 4 A, respectively). In summary, deletion of OT-I cells in the RIP-mOVA thymus occurs at the transitional stage of development whether Mtecs mediate deletion exclusively, or Mtecs and DCs both have the potential to mediate deletion.

BM-derived Cells in the RIP-mOVA Thymus Delete CD8 Thymocytes. The data presented in Fig. 4 demonstrate that antigen presentation by BM-derived cells is not required for deletion of OT-I cells developing in RIP-mOVA thymuses. Next, we wanted to determine whether BM-derived cells could acquire OVA from Mtecs and process and present it to delete OT-I thymocytes. This required a system in which Mtecs do not express MHC class I, whereas BM-derived cells express MHC class I. Deletion of OT-I thymocytes in this setting could only be attributed to antigen presentation by BM-derived cells. However, because positive selection requires MHC expression on Ctecs, we generated mice in which thymic expression of MHC class I is limited to Ctecs. This was accomplished using the keratin 14 promoter to drive the expression of H2-K^b in the Ctecs of K^{b}D^b $^{-/-}$ mice (K14-K^b.MHC I$^{-/-}$ mice; 23–25). Posi-

OT-I.MHC I $^{-/-}$ OT-I.K14-K^b.MHC I $^{-/-}$ OT-I.K14-K^b.RIP-mOVA.MHC I $^{-/-}$

CD4

DP

DN

CD8

CD4

CD8

DN	1.7	1.9	1.9
DP	89.5	75.3	77.4
CD4	6.0	14.8	14.8
CD8	0.33	1.0	0.86

B

Gated on CD8 SP thymocytes

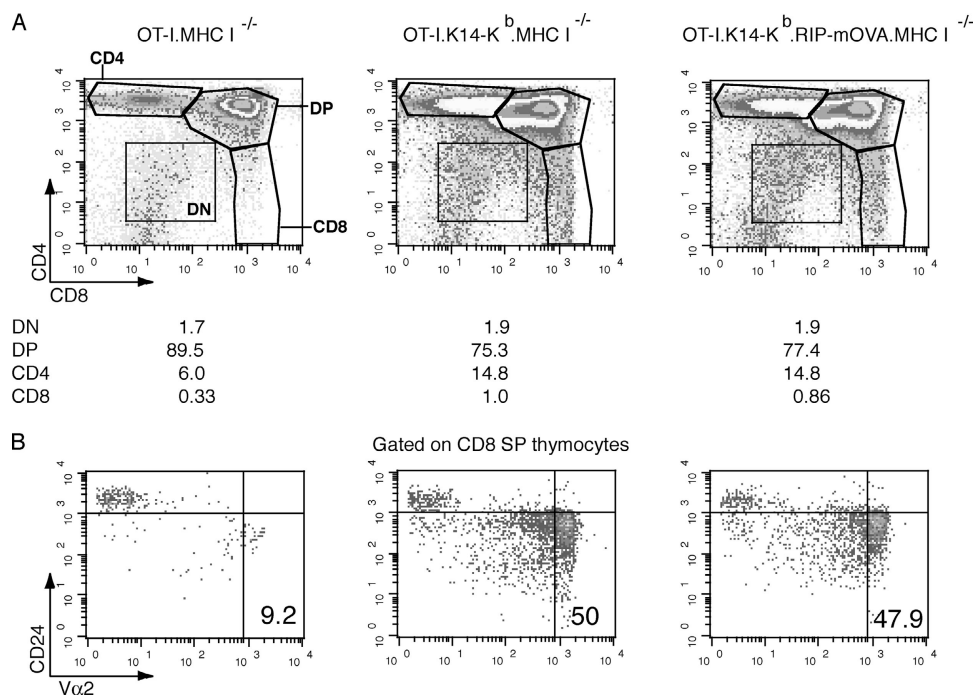CD24

Vα2

9.2   50   47.9

Figure 5. OT-I CD8 T cells are positively selected in K14-K^b thymuses and are not negatively selected when RIP-mOVA is also expressed. Results presented here are representative of two studies with one to three mice per group. All mice are littermates. (A) Thymocytes from the indicated mice were analyzed for CD4 and CD8 expression. The numbers below indicate the percentage of cells in each gate. (B) CD24 and Vα2 expression after gating on CD8 SP thymocytes. Numbers in the quadrants indicate the percentage of cells with a mature phenotype.

tive selection of OT-I thymocytes is supported in K14-K^b transgenic thymuses because there is an increase in the percentage of mature (CD8$^+$ Vα2$^+$ CD24^{low}) CD8 thymocytes in K14-K^b transgenic mice (OT-I.K14-K^b.MHC I$^{-/-}$) compared with K14-K^{b-} mice (OT-I.MHC I$^{-/-}$; Fig. 5, A and B). Importantly, when OT-I.K14-K^b.MHC I$^{-/-}$ mice were crossed to MHC class I–deficient RIP-mOVA mice (OT-I.K14-K^b.RIP-mOVA.MHC I$^{-/-}$ mice), OT-I thymocytes were not deleted (Fig. 5, A and B, right panels). In these mice, OT-I DP T cells are positively selected by K^b-expressing Ctecs, but are not negatively selected by Mtecs (which express OVA) or BM-derived cells because neither of these cell types express MHC class I.

Given this result, we next sought to determine whether MHC class I expression only on DCs could negatively select OT-I thymocytes. We grafted MHC class I–expressing OT-I BM into recipient mice that expressed both K14-K^b and RIP-mOVA ([OT-I→K14-K^b.RIP-mOVA.MHC I$^{-/-}$] BM chimeras). Compared with chimeras in which neither BM-derived cells nor Mtecs expressed MHC class I ([OT-I.MHC I$^{-/-}$→K14-K^b.RIP-mOVA.MHC I$^{-/-}$] BM chimeras), mature OT-I thymocytes were deleted (Fig. 6, A and B). There was a reduction in the percentage of cells in the CD8 SP T cell gate and a further reduction in the percentage of mature CD24^{low} Vα2^{high} thymocytes within this population. These results demonstrate that BM-derived cells, most likely thymic DCs, are able to acquire OVA from Mtecs and cross-present it to mediate deletion of OT-I thymocytes.

Deletion of CD4 T Cells in RIP-mOVA Thymuses Requires Antigen Presentation by BM Cells. The data in Figs. 3 and 4 show that Mtecs are potent inducers of tolerance to developing OVA-specific CD8 T cells. We investigated the ability of Mtecs to delete OVA-specific CD4 T cells from

MHC class II–restricted OT-II TCR transgenic mice. In the nondeleting [OT-II→B6] BM chimeric thymuses, normal numbers of mature CD4 SP thymocytes (CD4$^+$ Vα2^{high} CD24^{low}) are detected (Fig. 7, A–C). Analysis of the deleting [OT-II→RIP-mOVA] BM chimeric thymuses revealed a reduction in the percentage of CD4 SP thymocytes and a further reduction in the percentage of these cells that were mature. In contrast, analysis of RIP-mOVA thymuses in which only Mtecs, and not BM cells, expressed MHC class II ([OT-II.MHC II$^{-/-}$→RIP-mOVA] BM chimeras), demonstrated that OT-II thymocytes were not deleted in this setting. Normal numbers of mature phenotype CD4 SP T cells were present as shown by a compilation of numbers of mature phenotype CD4$^+$ Vα2^{high} CD24^{low} OT-II cells from many mice (Fig. 7 C). This result demonstrates that Mtecs cannot serve as APCs to delete OT-II thymocytes in RIP-mOVA thymus. Deletion in this case is absolutely dependent on DCs picking up OVA from Mtecs for presentation to developing OT-II thymocytes. This is in contrast to the situation with OVA-specific CD8 T cells, in which either direct presentation by Mtecs or cross-presentation by DCs can cause deletion.

Discussion

Mtecs express a broad range of TSA and thymic tolerance to TSA can be remarkably efficient. The fact that only rare Mtecs actually express a given TSA (~1-5% of Mtecs in cases that have been analyzed; references 3, 5, 11, 13, and 14), plus the fact that epithelial cells might be poor at presenting antigen for T cell deletion, led us to propose that indirect or cross-presentation of Mtec-derived TSA by DCs would be key in inducing efficient central tolerance to TSA.
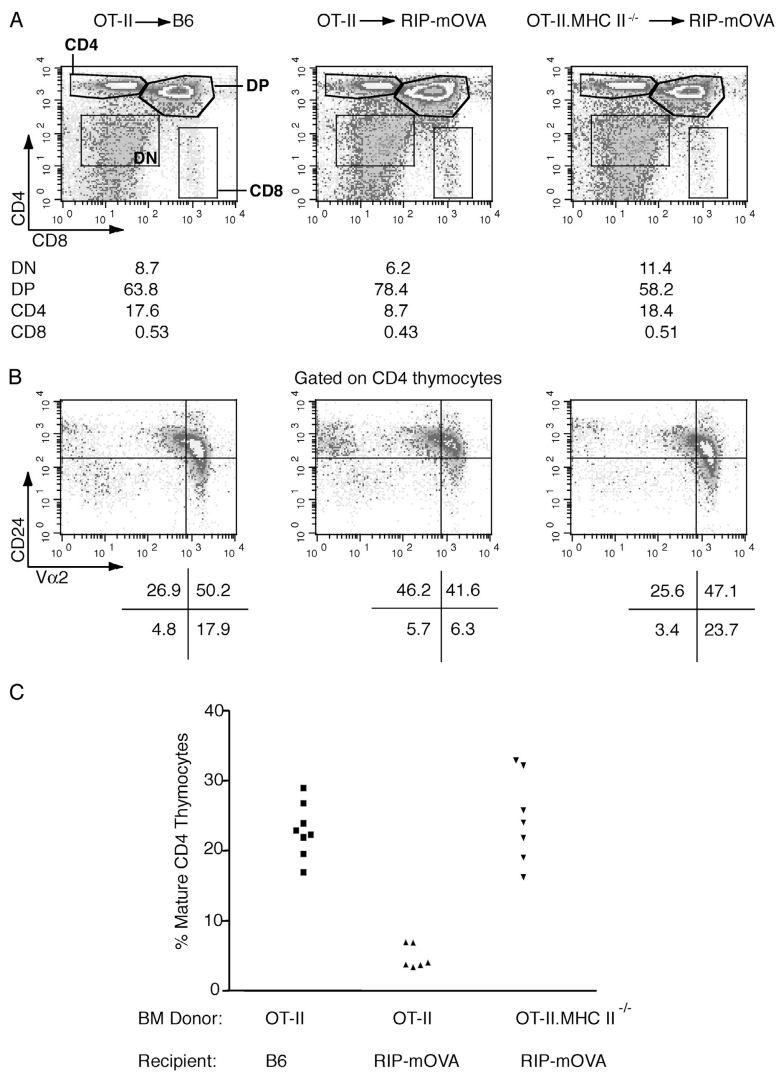
BM Donor = OT-I.MHC I$^{-/-}$ OT-I

Recipient = K14-K^b.RIP-mOVA.MHC I$^{-/-}$ K14-K^b.RIP-mOVA.MHC I$^{-/-}$

A

1.3	DN	1.9
79.1	DP	87.7
13.2	CD4	5.7
1.8	CD8	0.66

B   Gated on CD8 thymocytes

Figure 6. BM-derived cells in RIP-mOVA thymus can efficiently delete OT-I thymocytes. OT-I.MHC I$^{-/-}$ or OT-I BM was grafted onto lethally irradiated K14–K^b.RIP-mOVA.MHC I$^{-/-}$ recipients. (A) Thymocytes from the indicated mice were analyzed for CD4 and CD8 expression. The numbers below indicate the percentage of cells in each gate. (B) CD24 and Vα2 expression after gating on CD8 SP thymocytes. Numbers in the quadrants indicate the percentage of mature phenotype cells. Results presented here are representative of two studies with two mice per group.

This proposal was certainly borne out in the analysis of CD4 T cells expressing the OT-II TCR, where we show that OVA has to be acquired by DCs to tolerize (Fig. 7).

At this stage we do not know whether this requirement for antigen presentation by DCs is representative of central tolerance induction for all developing CD4 thymocytes, or whether it is peculiar to the OT-II TCR transgenic model. From older studies of central tolerance induction to ubiquitous antigen (such as allogeneic MHC), there is evidence that CD4 T cells can be tolerized by tecs. However, in these cases the tolerizing antigen was expressed by 100% of epithelial cells and there is only one report that has addressed whether TSAs, possibly synthesized by only 1–5% of Mtecs, could be directly presented to induce CD4 tolerance. In this analysis, CD4 TCR transgenic thymocytes did not require antigen presentation by BM-derived cells to be deleted (13, 26). However, in this report the investigators analyzed tolerance to a secreted antigen, making it difficult to know how many Mtecs or Ctecs were actually presenting this antigen, as it was probable that the secreted TSA could diffuse throughout the thymus and be taken-up and presented by Mtecs and Ctecs. It is likely that the deletion in this study was mediated by Ctecs, and not Mtecs, because deletion targeted pre–positively selected DP thymocytes that are known to reside in the cortex and not the medulla (13, 26).

Indeed, a recent report demonstrated that thymocytes were deleted mostly in the cortex when the antigen was soluble, whereas deletion occurred mostly in the medulla when the same antigen was membrane bound (27).

The inability of Mtecs to directly present antigen to delete OT-II thymocytes contrasted with the efficiency of DC-mediated deletion via antigen capture and could have a number of explanations. First, DC presentation delivers a stronger (deleting) signal to the immature thymocyte based on their superior battery of costimulatory and adhesion molecules. Although no single accessory molecule has been shown to be crucial for negative selection, a full investigation comparing thymic DC and Mtec expression has not been undertaken (28). Second, in the case of many TSAs, expression in the thymus medulla might be limited to a small fraction, <5%, of Mtecs. DCs that capture antigen from Mtecs may therefore outnumber the number of Mtecs presenting antigen. Also, DCs may cover a larger area of the medulla and they may carry antigen to specific areas. DCs are known to be especially common in the cortico–medullary junction, and this area might be specialized to allow DCs to present self-antigen to positively selected cortical thymocytes as they traverse this junction en route to the medulla. The ability of DCs to capture antigen from Mtecs and efficiently delete all dangerously self-reactive thy-

Figure 7. Antigen presentation by BM-derived cells is required for deletion of OT-II thymocytes in RIP-mOVA thymus. Lethally irradiated B6 or RIP-mOVA mice were grafted with either OT-II or OT-II.MHC II$^{-/-}$ BM. (A) Thymocytes from the indicated mice were analyzed for CD4 and CD8 expression. The numbers indicate the percentage of cells in each gate. (B) CD24 and Vα2 expression after gating on CD4 SP thymocytes. Numbers in the quadrants indicate the percentage of mature phenotype cells. (C) Graphic representation of results from three studies. Each symbol represents data from one mouse.

mocytes is of key importance in the prevention of autoimmune disease. It is likely that a specialized mechanism, which forces positively selected T cells to run a gauntlet of cells efficiently presenting self-antigen, would evolve to enforce deletion of autoreactive T cells.

In contrast to the absolute requirement for professional APCs to acquire antigen from Mtecs to induce central tolerance in the MHC class II–restricted OT-II system, we find that MHC class I–restricted, OVA-specific thymocytes can be efficiently deleted by antigen presented directly by Mtecs as well as by DC cross-presentation (Figs. 3, 4, and 6). The ability of Mtecs to present MHC class I–restricted OVA and efficiently delete both polyclonal and monoclonal OVA-specific CD8 thymocytes might be due to high TCR affinity and/or efficient antigen presentation. Although no information exists on the TCR affinities of the OVA-specific CD8 T cells in Vβ5 transgenic mice, one might predict that these cells represent a range of TCR affinities, as they are polyclonal and use multiple TCR-α chains. The affinity of the OT-I TCR for its ligand has been measured (29) and might be higher than that of OT-II for its ligand, though no

information exists on the OT-II TCR affinity. Another possibility to explain Mtec deletion of CD8 but not CD4 thymocytes is that Mtecs that synthesize membrane-bound OVA might be more efficient at processing and presenting MHC class I/OVA compared with class II/OVA, though one would expect that this membrane-bound form of OVA would readily enter the class II antigen processing and presentation pathway (30). Finally, the stability of the peptide–MHC complexes, K^b/OVA$_{257-264}$ and IA^b/OVA$_{323-329}$, are likely to differ, with the class I complex being much more stable than the class II complex.

Two other surprising findings came out of our analysis of CD8 T cell deletion. One is how abrupt and early after positive selection deletion occurred. The second is the fact that direct antigen presentation by Mtecs alone can induce the same pattern of abrupt, early deletion (Fig. 4). OT-I T cell development from the DP to the SP stage follows a similar pattern to that of other CD8 T cells (22, 31). After positive selection, both CD4 and CD8 expression are down-regulated (DP dull), then CD4 is up-regulated (transitional), and then CD4 is down-regulated (DP dull) as

these cells finally mature to CD8 SP cells. In step with this differentiation, the selecting TCR is gradually up–regulated. This "dance" allowed us to pinpoint the stage of deletion in RIP-mOVA thymuses. Surprisingly, deletion occurs at the transitional stage when the developing thymocyte expresses 10–30-fold lower levels of the CD8 coreceptor, and 2–3-fold lower TCR levels than expressed on CD8 SP cells (Figs. 2 and 4). Therefore, deletion of TSA-specific T cells at this stage is remarkably efficient, particularly when we consider that there are extremely low levels of antigen present in the medulla. Other studies on Nurr77 expression have suggested that transitional thymocytes are primary targets for negative selection (32).

This pattern of abrupt, early deletion would be consistent with the idea of a "gauntlet" of DCs mediating self-tolerance at the cortico–medullary junction as positively selected transitional cells transit from the cortex into the medulla, except for our finding that direct antigen presentation by Mtecs alone (when DCs do not express MHC class I) results in the same pattern of deletion (Fig. 4). We must assume in this case that transitional cells enter the thymic medulla, and then "zoom" around the medulla at high speed. Recent studies using multiphoton microscopy of T cells in lymph node and thymic organ cultures have shown that they travel at an average velocity of 14 μm per min, and can reach peak velocities of 40 μm per min (33–36). If newly generated medullary T cells also locomote like this they could potentially interact with 1,000–5,000 Mtecs in 1 d. Therefore, medullary thymocytes would have plenty of chances to find and be deleted by rare TSA-expressing Mtecs.

We show for the first time that professional, BM-derived DCs in the thymus can acquire TSAs from Mtecs and present epitopes to developing CD4 and CD8 T cells to enforce self-tolerance. For OT-II CD4 T cells, this professional uptake and presentation is absolutely essential for tolerance induction. On the other hand, CD8 T cell tolerance was induced directly by Mtecs as well as indirectly by DCs. At this point we do not propose a general rule where Mtecs can delete CD8 but not CD4 T cells. Instead, we favor the hypothesis that Mtecs can induce tolerance in T cells with high TCR–self-peptide/MHC avidity and the CD8 T cells we analyzed, but not the OT-II T cells, fall within this range. DCs, on the other hand, delete T cells that fall into a broader avidity range. How do thymic DCs acquire antigen from Mtecs? In the periphery, CD8αα DCs are adept at phagocytosing dead or dying cells and cross-presenting foreign antigen to T cells (37). The same subset of DCs also presents cell-associated self-antigen produced by islet cells in the lymph nodes draining the pancreas (38). This subset of DCs seems designed to present cell-associated antigen, and thymic DCs belong to this same subset. Thymic DCs may acquire TSAs by engulfing apoptotic Mtecs, or from healthy Mtecs in a process referred to as "nibbling," whereby browsing DCs take membrane-enclosed blebs from the surface of viable cells (39). Finally, Mtecs may release small vesicles called exosomes that could be taken up by DCs (40).

Our results provide a snapshot of a dynamic and sound thymic environment where Mtecs are uniquely suited to synthesize TSA, and DCs are designed to capture and present TSA to delete autoreactive T cells. This relationship between Mtecs and DCs has evolved to maximize the impact of negative selection.

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