Antigen Challenge Inhibits Thymic Emigration

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Antigen Challenge Inhibits Thymic Emigration 1

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T cell development in the thymus involves a series of TCR-mediated control points including TCR-β selection and positive and negative selection. Approximately half of the thymic sojourn is spent in the medulla, where thymocytes undergo final maturation before emigrating to the periphery. Although it is acknowledged that thymic emigration is an active process, relatively little is known about how this is regulated, why it takes so long, and whether TCR-mediated signaling can influence this step. Using wild-type and TCR transgenic mice, we found that Ag injected i.v. or intrathymically led to a striking reduction in the number of recent thymic emigrants (RTE) in the periphery. This was caused by inhibition of T cell export rather than peripheral deletion, because a cohort of RTE that was already released before in vivo Ag challenge was not depleted, and similar results were observed in Bim-deficient mice, which have impaired T cell deletion. Within the thymus, the loss of RTE was associated with retention of medullary thymocytes rather than increased negative selection. In addition to Ag-specific inhibition of export, some TCR-independent suppression of emigration was also observed that appeared to be partly the result of the inflammatory cytokine TNF. Thus, in addition to its accepted role in intrathymic selection events, TCR signaling can also play an important role in the regulation of thymic emigration. *The Journal of Immunology, 2006, 176: 4553–4561.

The TCR has a pivotal role in several key stages of T cell development. The first of these, known as TCR-β selection, sees triple-negative (CD4+/CD8+/CD3−) thymocytes undergo cell-autonomous signaling before transitioning into double-positive (DP) thymocytes (1, 2). In a second checkpoint, DP thymocytes expressing a self MHC-restricted TCR are positively selected by thymic cortical epithelial cells to mature into either CD8+ or CD4+ single-positive (SP) thymocytes, depending on whether the selecting signal is delivered by MHC class I or class II, respectively (3). And third, if TCR signaling strength to self MHC/peptide is too high, auditioning thymocytes are purged from the repertoire through negative selection to guard against potential auto-reactivity (3, 4). Thymocytes that survive positive and negative selection migrate into the thymic medulla, where, in mice, they spend ~2 wk (approximately half of their time in the thymus) before being exported to the periphery (5, 6). During this time, they down-regulate heat-stable Ag (HSA/CD24) and CD69, and up-regulate CD62L and Qa2 (7, 8).

The export of naive T cells from the thymus arguably constitutes the final checkpoint in T cell development. Recent studies have indicated a putative role for a number of G̅ protein-coupled factors in thymocyte emigration, including CCR7 (9), CXCR4 (10–12), and sphingosine-1-phosphate receptor 1 (S1P1) (13–15), which collectively imply that it is an actively regulated process. An important and controversial question is whether the cells that leave the thymus, termed recent thymic emigrants (RTE), more closely resemble those in the thymic medulla or those in the periphery. Phenotypically, RTE are distinct from most medullary thymocytes (8), yet as a group they also do not fully resemble peripheral naive T cells, as they collectively retain low levels of HSA and have not yet fully up-regulated Qa2 like their peripheral counterparts (8, 16). Some RTE derived from neonatal mice may also express elevated levels of CTLA4 (17), which could suggest reduced functional capacity. In terms of function, an early study showed that the adult RTE proliferative and cytotoxic response to stimulation in vitro was similar to that of peripheral T cells (18). This is consistent with findings that resistance to negative selection is acquired at a time in the medulla coincident with HSA down-regulation among SP thymocytes (19). Another study, however, suggested that RTE, identified using a novel system involving the expression of GFP under the control of the RAG-2 promoter, were less able to up-regulate CD25 in response to stimulation and displayed an attenuated proliferative response (20). Similarly, RTE derived from fetal thymus organ culture were unable to elicit acute graft-vs-host disease when transferred into allogeneic mice (17), although it is not clear whether the behavior of these in vitro RTE is equivalent to freshly isolated adult RTE. Collectively, these studies suggest that the thymic medullary sojourn sees positively selected thymocytes make the transition from being susceptible to deletion by TCR signaling, to acquiring the functional capacity to respond to TCR signals by undergoing activation, although this may not be fully complete until after they reach the periphery. This, alongside the fact that some RTE undergo a TCR-mediated proliferative burst immediately before export (21–23), poses the

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1 Abbreviations used in this paper: DP, double positive; HSA, heat-stable Ag; i.t., intrathymic; OVAp, OVA peptide (SIINFEKL); pLN, peripheral lymph node; RTE, recent thymic emigrant; S1P1, sphingosine-1-phosphate receptor 1; SEB, staphylococcal enterotoxin B; SP, single positive; WT, wild type.

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4 Abbreviations used in this paper: DP, double positive; HSA, heat-stable Ag; i.t., intrathymic; OVAp, OVA peptide (SIINFEKL); pLN, peripheral lymph node; RTE, recent thymic emigrant; S1P1, sphingosine-1-phosphate receptor 1; SEB, staphylococcal enterotoxin B; SP, single positive; WT, wild type.
important question of how Ag encounter will affect the process of thymic emigration itself. In this study, we have determined the impact of TCR ligation on thymic emigration and RTE by challenging mice i.v. or intrathymically (i.t.) with Ag, and quantifying and characterizing RTE via i.t. FITC labeling. This approach has allowed us to directly measure the effect of Ag on peripheral RTE, mature thymocytes, and T cell export from the thymus.

Materials and Methods

Mice

C57BL/6J (BL/6) mice were obtained from the Baker Medical Research Unit Privetc Animal Centre and the Walter and Eliza Hall Institute Central Animal House, and C3H/HeJ, C57BL/6.Bim+/− (24), and C57BL/6.OT-I (25) (Line 243-2) mice were obtained from the Walter and Eliza Hall Institute Central Animal House. C57BL/6.TNF-α−/− (26) (TNE−/−) mice were obtained from the Peter MacCallum Cancer Centre. Bim−/− gene-targeted mice were originally generated from targeted W9.5 embryonic stem cells (129sv background) and were then backcrossed >10 generations to C57BL/6 mice before use. TNE−/− mice were generated using C57BL/6-derivcyclic stem cells. Mice were housed in the Baker Medical Research Unit Precinct Animal Centre under specific pathogen-free conditions. All mice used in this study were aged between 6 and 10 wk, and were housed for at least 2 wk after transport, before experimentation. Techniques performed in this study received approval from the Baker Medical Research Unit Precinct Animal Centre Ethics Committee.

Intrathymic injection

Animals were anesthetized by i.p. injection of ~0.3 mg of xylazine hydrochloride (lium xylazil; Troy Laboratories) and 1.5 mg of ketamine hydrochloride (Ketal; Parke-Davis) in 300 µl of PBS, and subsequently administered with 50 µg/10 g (0.5 µg/kg) body weight of the anagelcic carprofen (Ramipril; Pfizer) s.c. The upper part of the thoracic cavity was opened to expose the thymic lobes, which were each injected with ~10 µl of a 1 mg/ml solution of FITC (in sterile PBS) (Molecular Probes), which typically resulted in the random labeling of 30–70% of the thymocyte population. In some experiments, this solution also contained Staphylococcal enterotoxin B (SEB; between 1 ng and 5 µg) (Toxin Technology) or 100 nM (10−5 M) OVA peptide (OVA, SIINFEKL) (Minotopes; Clay- ton). The wound was closed with surgical staples, and the mice were kept warm until fully recovered. Mice were killed 24 h after injection, and lymphoid organs were removed for analysis. Care was taken to avoid cross-contamination between FITC-labeled thymocytes with other organs during experiments.

Thymectomy

The thymi of anesthetized adult mice was exposed, as described above. Both lobes were removed by suction, and the wound was closed with two surgical staples. At the completion of the experiment, the pleural cavity was always checked to ensure complete absence of thymic tissue.

Cell suspensions

Mice were killed by CO₂ asphyxiation, and organs were removed for analysis. To obtain a single-cell suspension, organs were gently pressed between two glass slides into two glass slides into two glass slides into two glass slides into two glass slides into two glass slides into two glass slides into two glass slides. The suspension was filtered through 70 µm nylon mesh. Spleen cell suspensions were depleted of RBC by incubation with RBC lysis buffer (Sigma-Aldrich). Cell concentration and viability were determined using an automated cell counter (Z Series Dual; Coulter Electronics). The cell suspensions were then washed by centrifugation (420 g, 5 min, 4°C), and the cell pellet was resuspended in FACS buffer.

Flow cytometry

For flow cytometric analysis, FITC-conjugated anti-CD45.2 (clone 104), CD8 (53-6.7), CD25 (7D4), and CD49 (H1.2F3); PE-conjugated anti-CD4 (RM4-5), CD8 (53-6.7), CD24 (M1/69), CD25 (PC61), and CD69 (H1.2F3); biotinylated anti-Vβ6 TCR (R4-17) and Vβ8.1/2 TCR (MR5-2); PerCP-conjugated anti-CD4 (RM4-5) and CD8 (53-6.7); allophycocyanin-Cy7-conjugated CD4 (GK1.5); and allophycocyanin-conjugated anti-CD8 (53-6.7) and CD25 (PC61) Abs were purchased from BD Pharmingen. Streptavidin coupled to PerCP, PE-Cy7, or allophycocyanin was purchased from BD Pharmingen. In all experiments, FcR block (clone 2.4G2, grown in house) was used to prevent nonspecific binding of Abs. Cell suspensions were stained in 96-well U-bottom plates (3 × 10⁶ cells/test) by gently resuspending in 30 µl of appropriate mAb or secondary conjugate and incubating for 25 min at 4°C in the dark. Between incubations, cells were washed twice by the addition of 200 µl of FACS buffer and centrifuged (320 × g, 3 min, 4°C). Flow cytometric acquisition was performed on a FACSCalibur or LSR-II (BD Biosciences). Typically, data were collected on 1 × 10⁶ viable cells for each sample, and, where necessary, additional acquisition was performed on electronically gated populations (e.g., FITC−/T). Nonviable lymphocytes were excluded on the basis of forward light scatter vs side light scatter. Analysis was performed using CellQuest and FACS Diva software (BD Biosciences).

Quantification of RTE

For detection of RTE, the thymus was injected with FITC, and lymphoid organs were removed 16 or 24 h later. Samples were counted and stained with anti-CD8 PE and anti-CD4 PerCP Abs, and analyzed by flow cytometry. RTE were defined as FITC+ cells expressing either CD4 or CD8 (to exclude autofluorescent cells and doublets). In each experiment, a non-FITC-injected control mouse was included to control for background fluorescence. RTE were quantified by analyzing the percentage of thymocytes stained FITC+, total cell counts for the spleen or peripheral lymph nodes (pooled inguinal, axial, and brachial), and the proportion of T cells within these organs that were FITC+.

Statistical analysis

A Mann-Whitney rank sum U test was applied to relevant data to determine statistical significance. A value of p < 0.05 was considered statistically significant.

Results

Peripheral SEB challenge results in a reduced number of RTE

To assess the impact of Ag encounter on RTE, we injected mice i.v. with 50 µg of the superantigen SEB. At the same time, mice were injected i.t. with FITC to allow for the subsequent detection of FITC+ RTE in the periphery. SEB specifically activates T cells expressing particular TCR Vβ chains by bridging MHC class II on APCs to the relevant TCR-β chain. These include Vβ8+ TCR, but not others, such as those expressing the Vβ6+ TCR. The interaction between SEB and the TCR is independent of CD4 and CD8 coreceptor expression or TCR specificity (27). Thus, analysis of the non-SEB-reactive Vβ6+ T cell compartment alongside Vβ8+ T cells can serve as an important internal control for the specific activity of SEB.

Consistent with earlier reports (28–30), we found that 16 h after SEB injection, Vβ8+ peripheral T cells were activated and had up-regulated CD25 and CD69, relative to PBS-injected control animals (Fig. 1). A small (but statistically significant; p < 0.05) increase in CD25 and CD69 expression by Vβ6+ T cells was also observed, which is likely to reflect TCR-independent, bystander (probably cytokine-driven) T cell activation (31, 32). In support of the previous findings showing very little division or apoptosis occurs within the first 16 h of SEB challenge (29, 33–35), SEB did not cause a change in the number of resident T cells in either the peripheral lymph nodes (pLN) or spleen (Fig. 1Bi).

In contrast to resident peripheral T cells, analysis of FITC−/T RTE revealed a strikingly different result. In mice treated with SEB, there was a proportional drop in Vβ8+, but not Vβ6+ RTE after 16 h compared with PBS-treated controls (p < 0.05). This suggested that SEB encounter caused a specific loss of Ag-reactive RTE, particularly in the pLN (Fig. 1Bi). The overall number of RTE was also clearly reduced (Fig. 1Bi). Factoring in data on total cellularity demonstrated that this reduction was not solely restricted to SEB-reactive RTE subsets, as in addition to Vβ8+ RTE, there was also a statistically significant reduction in the number of Vβ6+ RTE (Fig. 1Bii) despite their normal to increased frequency. The reduction in RTE numbers appeared to be occurring to a similar extent within both the CD4+ and CD8− subsets.
Therefore, while the numbers of RTE appear to be nonspecifically reduced, the loss of Vβ8⁺ RTE was greater, as indicated by the relative decrease in Vβ8⁺ RTE proportions. These data suggested that while resident (mature) T cells were activated by i.v. SEB injection, there was a specific loss of Ag-reactive RTE. This was superimposed over a nonspecific decline in all RTE that was independent of direct TCR signaling. Of note, there was evidence that SEB injected i.v. had entered the thymus, because there was specific up-regulation of CD25 on Vβ8⁺ thymocytes (Fig. 1A), consistent with previous findings (36).

RTE are resistant to SEB-mediated deletion in vivo

Given that, in addition to peripheral T cells, thymocytes were also being activated by i.v. injected SEB, it was difficult to delineate whether the loss of RTE in the periphery was the result of a peripheral event, such as deletion or relocation of RTE, or the consequence of a reduction in thymic output in the period that followed antigenic challenge. To distinguish between these possibilities, we examined the effect of SEB stimulation on a population of RTE already established in the periphery. For this study, mice were injected i.t. with FITC as before, but in this experiment, the thymus was removed 24 h later, before the administration of Ag. This created a cohort of FITC⁺ peripheral RTE, but removed any further thymic output. These mice were challenged i.v. with either SEB (50 μg) or PBS at the time of thymectomy, and RTE and resident T cell populations were quantified 16 h later. We reasoned that if RTE were susceptible to deletion in the periphery, this approach should still lead to a reduction in RTE numbers. However, if disrupted thymic export was responsible for the loss of RTE, then it should result in no overall change to RTE numbers. As shown in Fig. 2A, SEB injection had no effect on RTE numbers in either the pLN or spleen of thymectomized mice, indicating that it affects thymic export, but not survival of RTE in the periphery. Interestingly, in contrast to in vitro studies, suggesting that RTE express less CD25 in response to antigenic stimulation (20), Vβ8⁺ RTE exhibited an activated CD25⁺ phenotype that appeared to be comparable to resident T cells in vivo (Fig. 2B). This indicated that RTE are not susceptible to SEB-mediated deletion in the periphery, nor do they immediately migrate out of secondary lymphoid tissues upon stimulation. Consistent with these in vivo findings and an earlier report (18), RTE were also resistant to deletion mediated by anti-CD3 and anti-CD28 mAbs in vitro (data not shown). Taken

**FIGURE 1.** RTE are decreased after an i.v. challenge with SEB. Mice were injected i.t. with FITC and simultaneously challenged with SEB (50 μg i.v.). Sixteen hours later, cells were analyzed by flow cytometry for the presence of RTE (FITC⁺ RTE) and resident (FITC⁻) T cells in the pLN and spleen. A, Depicts CD25 and CD69 expression on lymphocytes in the pLN and thymic CD4 and CD8 SP thymocytes, for both Vβ8⁺ and Vβ6⁺ subsets following injection with SEB or PBS. Bi, Illustrates the effect of SEB on the overall cell number of resident T cells in pLN and spleen. The effect of SEB on the proportions of Vβ8⁺ (SEB-reactive) and Vβ6⁺ (SEB-nonreactive) subsets among RTE, for both CD4⁺ and CD8⁺, is shown in Bii, while iii and iv show the total numbers of total RTE and Vβ subsets, respectively. *n = 7 for PBS and n = 6 for SEB-treated mice. Error bars represent SEM; *, p < 0.05 and **, p < 0.01 using a Mann-Whitney rank sum U test.
Intrathymic injection of SEB inhibits thymic emigration.

To directly examine whether i.t. antigenic encounter could disrupt thymic emigration, SEB and FITC were coinjected i.t. and RTE numbers were measured in the periphery 24 h later. In this model, only low doses of SEB (2–200 ng) were required to induce effects (~100 times lower than those required for i.v. challenge). As was the case with peripheral antigenic challenge, i.t. SEB administration led to reduced RTE numbers, and in this system, the Ag-specific effects were far more pronounced, such that the proportion of V\textsuperscript{8+} cells among RTE was specifically reduced with exposure to SEB in pLN (dose response shown in Fig. 3A), as well as spleen (Fig. 3D) and blood (Fig. 3E). The proportion of V\textsuperscript{8+} RTE increased accordingly. The relative proportions of V\textsuperscript{8+} and V\textsuperscript{8+} T cells among RTE suggest that SEB administered directly into the thymus can affect export in a TCR-specific manner. However, again some nonspecific inhibition of T cell export also occurred, as illustrated by the reduced overall number of V\textsuperscript{8+} RTE, despite their increased frequencies (Fig. 3Aiv). One possibility to explain the general reduction in RTE was the possible presence of contaminating LPS in the SEB preparation, although the SEB used was the highest quality grade (<5 EU/mg) and certified to be largely free of contaminants. To directly investigate this possibility, we also examined C3H/HeJ mice, which lack the TLR4 pathway required for LPS signaling. Similar data with Ag-specific inhibition superimposed over nonspecific inhibition of RTE were obtained in these experiments (Fig. 3B), demonstrating that it is unlikely that contaminating LPS was responsible for the observed effects. Taken together, these data suggest that activation of thymocytes, both directly through SEB-TCR interactions, and indirectly, possibly through effects downstream of SEB-specific T cell activation, such as cytokine production, leading to bystander cell responses, is capable of suppressing T cell export from the thymus.

A possible explanation for the TCR-independent reduction in RTE following SEB challenge was the systemic production of the cytokine TNF, which is known to be responsible for toxic shock in SEB-sensitized mice (28) and can lead to apoptosis of immature thymocytes (37). Therefore, we tested the impact of SEB injected i.t. on RTE in TNF\textsuperscript{−/−} mice (which have normal T cell development (38)). As for wild-type (WT) mice, there was a specific loss of the V\textsuperscript{8+} T cell subset among RTE after SEB encounter (Fig. 3C). However, in contrast to WT mice, the decline in V\textsuperscript{8+} RTE was not observed for CD4\textsuperscript{+} RTE and was less pronounced for CD8\textsuperscript{+} RTE, with a decrease only seen at the highest dose (50% loss in TNF\textsuperscript{−/−} compared with 80% loss in WT mice). These observations suggest that TNF production, presumably by SEB-activated V\textsuperscript{8+} T cells and thymocytes, is largely responsible for the nonspecific inhibition of T cell export in WT mice. This is despite the fact that we still detected signs of bystander cell activation (CD25 and CD69 up-regulation) in TNF\textsuperscript{−/−} mice, particularly at the highest dose of SEB (200 ng) (data not shown). Interestingly, the overall number of RTE in untreated TNF\textsuperscript{−/−} mice appeared marginally reduced compared with WT mice (~2- to 3-fold) (Fig. 3, A and C), despite the mice having normal thymocyte numbers (~2 × 10\textsuperscript{8} cells). This phenomenon has not been reported previously and may suggest a TNF-dependent element in the process of thymic export.

Potential explanations for the TCR-specific loss of RTE following SEB treatment included Ag-induced i.t. negative selection of mature thymocytes, and/or retention and accumulation of activated mature thymocytes in the thymic medulla. Following i.t. SEB challenge, V\textsuperscript{8+} SP thymocytes increased in cell size (forward scatter), and up-regulated CD25 and CD69, consistent with a partially activated state (Fig. 4). These activation markers were not up-regulated to the same extent when compared with mice in which SEB was administered systemically, possibly due to the much lower doses of SEB used in the i.t. injections. Similar to the experiments involving a peripheral challenge with SEB (Fig. 1), CD69 was also partially up-regulated on V\textsuperscript{8+} thymocytes, again presumably indicative of mild bystander cell activation. There was no significant change in the number of DP or SP thymocytes, nor in the proportion of V\textsuperscript{8+} thymocytes in the SP compartment, despite the dramatic effect on RTE (Fig. 5). Given the loss of RTE from the periphery following SEB challenge, we predicted that this might be associated with a concomitant accumulation of the most mature subset (HSA\textsuperscript{high}) of SP thymocytes. Indeed, this was the case for the V\textsuperscript{8+} fraction (Fig. 5), which was significantly increased in number after i.t. SEB treatment (p < 0.05), although surprisingly, there was no significant change to the number of V\textsuperscript{8+} cells. This suggested that (non-SEB-reactive) V\textsuperscript{8+} mature SP thymocytes were accumulating after SEB treatment, consistent with the hypothesis that emigration was being inhibited by SEB, although the same could not be said for V\textsuperscript{8+} cells. This might be
because Vβ8+ T cells can transiently up-regulate HSA following SEB stimulation (39), thus making them resemble semimature SP thymocytes. Although there was an apparent decrease in the mean number of semimature (HSAhigh) CD4 SP Vβ8+ thymocytes, this was not statistically significant. This contrasts with previous observations that this population is susceptible to negative selection in response to SEB (19, 40), but this discrepancy is probably due to the earlier time of analysis (24 h compared with 48 h) and/or the much lower dose of SEB used in our study. Our results suggested that the SEB-mediated loss of RTE is not simply a consequence of the deletion of their precursors within the thymus, but rather a direct disruption of T cell export. To test this hypothesis more directly, we injected SEB into bim−/− mice, which have a defect in clonal deletion of immature as well as mature T cells (34, 40–42). Following an i.t. challenge with SEB, Vβ8+ RTE were specifically lost to a similar extent from the pLN (Fig. 6), spleen, and
blood (data not shown) of bim−/− and WT mice, indicating that this process is not due to i.t. deletion of SEB-reactive thymocytes. Collectively, these findings indicate that TCR stimulation of thy-
mocytes in vivo does not promote T cell export, but rather leads to its profound inhibition.

**Intrathymic OVAp injection causes a loss of RTE in OT-I mice**

To extend these studies to a conventional antigenic challenge, similar experiments were conducted using anti-OVA TCR transgenic mice. The vast majority (>95%) of CD8 SP thymocytes in these mice expressed a Vα2+ TCR (data not shown). We injected 2 ng (1 ng per lobe in 10 µl, 0.002 nmol) of agonist peptide (OVAp, SIINFEKL) i.t. into OT-I OVAp/H-2Kb-specific CD8+ TCR transgenic mice together with FITC to identify RTE. Based on previous in vivo OVAp titration experiments performed by others (43), and the lack of noticeable peripheral T cell activation when this dose of OVAp was injected i.t. (see below), we reasoned that 2 ng was a relatively low concentration of Ag that should not induce systemic T cell activation (typically other studies used between 10 and 100 µg of OVAp to activate peripheral CD8+ TCR transgenic T cells in vivo (37, 43, 44).

As seen in the experiments using SEB, there was a profound (~95%) reduction in the number of CD8+ SP RTE in both the pLN and spleen 24 h after peptide challenge, with no change in the number of peripheral resident CD8+ T cells (Fig. 7A). Peptide-mediated activation was restricted to the thymus, as illustrated by the up-regulation of CD69 and CD25, and increase in forward light scatter on CD8 SP cells in the thymus, but not those found in the pLN (Fig. 7C) or spleen (data not shown). The partially activated phenotype of CD8+ SP thymocytes demonstrated that peptide injected i.t. was presented to medullary thymocytes. Interestingly, and similar to an i.t. challenge with SEB, the absolute number of DP thymocytes and the CD4 and CD8 profiles was unaffected (Fig. 7, B and C), indicating that no overt negative selection was occurring, at least when using this low dose of peptide within this time range. OVAp challenge resulted in a bias toward lower HSA expression on OT-I CD8 SP thymocytes. There was roughly a

**FIGURE 4.** T cell activation status following i.t. challenge with SEB. A 10 µl solution containing SEB (200 ng) or PBS was injected i.t. into the thymuses of C57BL/6J mice. Twenty-four hours later, thymocytes were analyzed by flow cytometry. Histograms depicting CD25, CD69, and forward light scatter are shown for CD4 SP and CD8 SP Vβ8+ and Vβ6+ thymocyte populations. Data are representative of n = 5 mice per group.
3-fold increase in the number of mature (HSA\textsuperscript{low}) CD8 SP thymocytes, whereas no significant change was observed for semimature (HSA\textsuperscript{mod}) CD8 SP thymocytes (Fig. 7B). This is consistent with retention of more mature cells as a result of activation. Although it is well established that OT-I DP thymocytes are susceptible to OVAp-mediated deletion (25, 45), it is important to note that, in the present protocol, only very low doses of OVAp were used to avoid systemic T cell activation, and the analysis was restricted to 24 h to minimize loss of FITC-labeling intensity. Taken together, these findings demonstrate that TCR ligation in vivo leads to the inhibition of thymic emigration of T cells.

**Discussion**

This study has revealed that antigenic challenge inhibits T cell export from the thymus. Using SEB to target Vβ\textsuperscript{8} \ T cells, the results show that Ag-reactive (Vβ\textsuperscript{8}) cells were most potently affected and were clearly reduced in frequency within the RTE population. The Ag-specific inhibition of T cell export did not appear to be associated with negative selection, because potentially sensitive thymocyte populations were not depleted. Moreover, export was also similarly inhibited in bim\textsuperscript{−/−} mice, which have defective T cell negative selection (40, 42). Importantly, in addition to the Ag-specific reduction in thymic emigration, there was also a more general, non-Ag-specific reduction in thymic emigration, as indicated by analysis of non-SEB-reactive Vβ\textsuperscript{6} cells and total RTE numbers. The nonspecific inhibition was most pronounced following i.v. SEB injection, but it was also observed to a lesser extent after i.t. SEB injection. This suggests that the difference may be due to the use of much lower doses of SEB in the i.t. injection experiments, resulting in a more localized response with less systemic T cell activation.

The fraction of thymocytes exiting the thymus per day as RTE is generally <1% of the total thymic cellularity (46, 47). We found a statistically significant increase in the number of mature HSA\textsuperscript{low} Vβ\textsuperscript{6} thymocytes following SEB treatment that supported the notion that mature medullary thymocytes were being retained within the thymus. Further analysis (data not shown) revealed that the number of accumulated Vβ\textsuperscript{6} cells in the thymus correlated with the number of Vβ\textsuperscript{6} RTE that were missing from the periphery. Surprisingly, there was no significant change in the number of Vβ\textsuperscript{8} (SEB-reactive) HSA\textsuperscript{low} thymocytes in either WT or bim\textsuperscript{−/−} mice, although interestingly, analysis of OT-I mice revealed an increase in this population in response to i.t. OVA challenge. The reason for this puzzling discrepancy is unclear, although HSA can be up-regulated on T cells in response to SEB-mediated activation, which complicates interpretation of experiments in which it is used as a differentiation marker (39).

Our finding that antigenic challenge inhibits T cell export seems to contradict an earlier report that found that T cell export from the thymus is unaffected by immunization with OVA (48). However, that study was measuring export following peripheral immunization of non-TCR transgenic animals, in which TCR-specific effects may have been overlooked due to the minute contribution of Ag-specific T cell clones among RTE, and nonspecific effects might not have been brought on by the small frequency of responder cells. Another study assessing the effect of immunization with mouse mammary tumor virus superantigen (Mtv-7)-expressing spleen cells on the emigration of Ag-specific cells from the thymus also failed to demonstrate any effect in adult mice (49). However, that study was more focused on the long-term effects over several days. Furthermore, as thymic emigration was not measured following an i.t. challenge with Ag, it is unlikely that the peripherally injected cellular Ags entered the thymus in significant numbers.

The inhibitory effects on emigration resulting from Ag encounter provide some insight into the requirements for the delivery of newly formed T cells from the thymus to the peripheral T cell pool. Previous reports have shown that RTE appear to undergo a TCR-mediated proliferative burst immediately before export (21–23), which raised the possibility that TCR engagement might augment T cell export from the thymus. Although our data showing that high-affinity TCR ligation inhibits thymic export might seem inconsistent with the earlier studies (21–23), it is possible that lower affinity self peptide/MHC interactions play a positive regulatory role in thymic emigration (23), whereas higher affinity interactions have an opposing effect.

We surmise that the retention of activated, mature thymocytes might have a number of consequences. For example, it is known that CD4\textsuperscript{−}CD25\textsuperscript{+} regulatory T cells develop in the thymus and recognition of cognate Ag at this site may facilitate this cell fate decision (50, 51). Thus, it is conceivable that antigenic challenge may retain mature Ag-specific cells in the thymus and promote their transformation into Ag-specific regulatory T cells. Indeed,
some preliminary experiments found significantly increased numbers of thymic CD4+CD25+ cells in i.t. SEB-injected mice, but a definitive conclusion cannot be drawn at this stage due to the up-regulation of CD25 on activated conventional T cells (Fig. 4 and data not shown). Clarification of this issue may await further analysis using other markers such as the Foxp3 transcription factor (52) to identify regulatory cells, and testing i.t. Ag-induced CD4+CD25+ cells for suppressive activity.

Although the precise mechanism responsible for inhibiting thymic export in response to antigenic challenge was not identified, one explanation might involve the G_i protein-coupled receptor S1P_1, a receptor recently described to be critical for T cell export (13). It is possible that the inhibition of thymic emigration is due to down-regulation of S1P_1 on thymocytes, as other studies have shown that TCR stimulation of peripheral CD4+ T cells can result in down-regulation of this receptor and loss of chemotactic responsiveness to its ligand S1P (53). Several other factors have also been implicated in the regulation of thymic egress, such as CD69, which inhibits thymic emigration when overexpressed (54, 55), or chemokines including CCR7 (9), CXCR4 (10–12). We attempted to monitor the cell surface expression of S1P_1 and CCR7 on thymocytes by flow cytometry using commercially available Abs, but were unable to detect clear and specific staining with these reagents (data not shown).

In summary, our data show a previously unrecognized influence of TCR signaling in the process of T cell production by the thymus. These findings are potentially important, as they suggest that thymic output may be compromised in association with sustained antigenic challenge, such as would occur with chronic infection. This finding will be important to consider in terms of what role the thymus has in an ongoing immune response.

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