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Bim-Mediated Apoptosis Is Not Necessary for Thymic Negative Selection to Ubiquitous Self-Antigens

Qian Hu, Alyssa Sader, Julia C. Parkman, and Troy A. Baldwin

T cell education in the thymus is critical for establishing a functional, yet self-tolerant, T cell repertoire. Negative selection is a key process in enforcing self-tolerance. There are many questions that surround the mechanism of negative selection, but it is currently held that apoptosis initiated by Bim and/or Nur77 is critical for negative selection. Recent studies, however, have questioned the necessity of Bim in maintaining both central and peripheral T cell tolerance. To reconcile these apparently contradictory findings, we examined the role of Bim in negative selection in the well-characterized, physiological HY<sup>C8</sup>-<sup>D8</sup> mouse model. We found that while Bim expression was required for CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocyte apoptosis, it was not required for negative selection. Furthermore, Bim deficiency did not alter the frequency or affinity of male reactive cells that escape negative selection in an oligoclonal repertoire. Collectively, these studies indicate that negative selection occurs efficiently in the absence of apoptosis and suggest that the current paradigm of negative selection requiring apoptosis be revisited.


Following expression of an αβ-TCR heterodimer at the CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) stage of development, thymocytes undergo rigorous selection processes designed to establish a T cell repertoire capable of responding vigorously to pathogen-infected cells but not healthy self-tissues (1). These selection processes appear to be controlled by the affinity of the TCR for self-peptide MHC (pMHC) complexes expressed on thymic stromal cells. If DP thymocytes fail to express a functional αβ-TCR or express an αβ-TCR that cannot interact with self-MHC, the thymocyte will die by neglect. A low- to moderate-affinity interaction between the TCR and self-pMHC complexes promotes thymocyte survival and differentiation (positive selection), while a high-affinity interaction results in the elimination of this specificity from the mature T cell pool (negative selection) (2). Research on the cause of the multigenic autoimmune disease autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) has recently highlighted the importance of negative selection in self-tolerance. APECED is caused by a mutation in the autoimmune regulator (AIRE) gene in humans (3, 4), and mice lacking AIRE develop a similar multiorgan autoimmune disease due to defects in the negative selection of T cells specific for tissue restricted Ags (TRAs) (5–7).

It is unclear at present how the same TCR can transduce a signal leading to either positive or negative selection, but differential MAPK signaling (kinetics, intensity, cellular location) appears to play a role (8–12). What is clear, however, is that this differential signal transduction results in changes in the gene expression profiles of thymocytes undergoing positive or negative selection (13–19). For example, negative selection induces the expression of proapoptotic molecules, including the Bcl-2 homology domain 3 (BH3) only Bcl-2 family member Bim and the orphan nuclear receptor Nur77. Because negative selection results in the induction of “suicide genes”, it is generally held that apoptosis is the primary mechanism used to enforce negative selection, with receptor editing and energy playing more limited roles under certain conditions (2). A large body of evidence supports the paradigm that Bim- and/or Nur77-mediated apoptosis induction is critical for negative selection. Bim deficiency results in the resistance of DP thymocytes to apoptosis in vitro and impairs negative selection in TCR transgenic and superantigen models of negative selection in vivo (20, 21). Furthermore, Bim-deficient mice develop a late-onset autoimmune disease (22). Similarly, overexpression of Nur77 is sufficient to induce apoptosis in DP thymocytes and inhibition of Nur77 activity inhibits negative selection in TCR transgenic models (23). Interestingly, while Bim operates at the level of the mitochondria to induce apoptosis, controversy exists as to how Nur77 induces apoptosis. The apoptotic activity of Nur77 was previously demonstrated to correlate with its transcriptional activity in the nucleus, suggesting that Nur77 functions to induce proapoptotic gene expression (24–26). However, it was recently reported that in DP thymocytes, Nur77 binds Bcl-2 and “converts” Bcl-2 from a normally antiapoptotic protein into a proapoptotic protein by exposing the BH3 domain in Bcl-2 (27). It is currently unclear if and/or how Bim and Nur77 cooperate during the induction of negative selection.

By its strictest definition, negative selection is an active process that prevents thymocytes bearing an autoreactive TCR from maturing in the thymus and entering the mature, peripheral T cell pool.

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Q.H. designed and performed research, analyzed data, and wrote the paper. A.S. designed and performed research and analyzed data. J.P. designed and performed research, analyzed data, and wrote the paper.

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7 Abbreviations used in this paper: DP, double positive; BH3, Bcl-2 homology domain 3; pMHC, peptide-MHC; SP, single positive; TRA, tissue restricted Ag.

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(2). Therefore, in situations where negative selection is truly prevented, an increased population of autoreactive T cells should be present in the mature thymocyte and T cell repertoire (7). Recently, Jorgensen et al. reexamined the role of Bim in negative selection in a superantigen model. In contrast to data published by the Strasser group (20), studies by Jorgensen et al. indicated that the absence of Bim did not impair negative selection, as evidenced by limited increases in superantigen reactive CD4 single-positive (SP) and CD8SP thymocytes (28). Progenitor apoptosis was not examined in this study. Therefore, conflicting evidence for the role of Bim in thymocyte negative selection exists in the literature.

To resolve this apparent contradiction, we examined selection events in the well-established, physiological HYcd4 mouse model in the presence or absence of Bim. The HYcd4 model has the advantages of TCR expression at the DN to DP transition, as is normally observed in nontransgenic thymocytes, and endogenous, ubiquitous selecting ligand expression: positive selection in female mice and negative selection in male mice (2, 29, 30). Our data demonstrated that while Bim was required for high-affinity Ag-induced DP thymocyte apoptosis, it was not required for thymocyte negative selection. Bim-independent negative selection was also observed in an oligochlonal T cell population. These data demonstrate that robust cell death-independent mechanisms exist for inducing negative selection and enforcing central tolerance.

Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from the National Cancer Institute. HYcd4 mice were previously described (29), Bim−/− mice (22) were provided by Dr. Bruce Blazar (University of Minnesota) and intercrossed with HYcd4 mice to generate HYcd4 Bim−/− mice. Vβ8 and Vβ8 Bim−/− mice were generated as a result of the HYcd4 Bim−/− crosses. All mice were bred and maintained in our colony at the University of Alberta, treated in accordance with protocols approved by the University of Alberta Animal Care and Use Committee and used between 4 and 12 wk of age for experiments.

Abs and flow cytometry

All fluorochrome-conjugated and biotinylated Abs were purchased from eBioscience, BD Biosciences, BioLegend, or Invitrogen, except for anti-actiive caspase 3 (Asp 175), which was purchased from Cell Signaling Technology. Cells were stained in FACS buffer (PBS, 1% FCS, and 0.02% sodium azide (pH 7.2)) with fluorochrome-conjugated or biotinylated Ab cocktails for 30 min on ice. Cells were washed twice in FACS buffer between staining cocktails. Intracellular staining for active caspase 3 (Asp 175), which was purchased from Cell Signaling Technologies, was performed using an eBioscience Fix/Perm kit (BD Biosciences). For pentamer staining, D7/s-mcy pentamers were purchased from Promemune and used as previously described (29). Cell events were collected on a FACScanto II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Cell sorting and quantitative RT-PCR

Thymocyte populations were sorted on a FACSAria (BD Biosciences), and total RNA was harvested using a Qiagen RNaseasy Mini kit. On-column DNase digestion was performed. cDNA was synthesized using the Invitrogen SuperScript III first-strand cDNA synthesis kit. Quantitative RT-PCR was performed using Applied Biosystems Power SYBR Green kit and the Applied Biosystems 7900 HT Fast real-time PCR system. For quantitation, the cycle threshold (Ct) value for the gene of interest was compared with that of β-actin and expressed as a percentage of β-actin.

In vitro stimulation

Thymocytes were harvested, labeled with CFSE, mixed with wild-type female splenocytes at a 4:1 ratio, and stimulated with the indicated concentration of s-mcy peptide. The cultures were harvested following 24 or 48 h of stimulation, and CD69 induction (24 h) or CFSE dye dilution (48 h) was measured.

Statistical analysis

Mean, SD, and p values were determined using Prism software (GraphPad Software). Values of p were calculated using a two-tailed unpaired t test with a 95% confidence interval.

Results

Thymic profile of HYcd4 Bim−/− mice

Given the existing controversy over the requirement for Bim in thymic negative selection, we wanted to examine the role of Bim in T cell development using the well-established HYcd4 model. Previously, the role of Bim in thymocyte selection was studied in classical TCR transgenic mice where the TCR αβ heterodimer is expressed in the DN stage of development, resulting in premature negative selection at the DN to DP transition or in a superantigen-driven selection system where superantigen-MHC recognition by the TCR is mechanistically different than pMHC recognition by the TCR (20, 28). Neither of these model systems is reflective of pMHC-mediated negative selection at the DP stage (2, 30). The HYcd4 model utilizes a Cre/loxP system to conditionally express the HY TCRα chain at the DN to DP transition, which mimics endogenous TCRα expression (29). We bred HYcd4 mice to Bim−/− mice to create HYcd4 Bim−/− mice. This allowed us to specifically determine the role of Bim in pMHC-mediated positive and negative selection of DP thymocytes in female and male mice, respectively. Thymocytes expressing the male Ag-reactive TCR were identified using the T3.70 mAb. Bim deficiency did not alter the percentage of T3.70+ thymocytes in HYcd4 Bim−/− female mice compared with HYcd4 female mice, but HYcd4 Bim−/− male mice contained an elevated percentage of T3.70+ cells compared with HYcd4 male mice (data not shown). Additionally, by staining the thymocytes with anti-Thyl.2, we did not detect an increase in non-T cell populations (i.e., Thyl.2−) in Bim-deficient compared with Bim-sufficient mice (data not shown). We next examined the CD4/CD8 profiles of T3.70+ thymocytes from the different mouse strains. Bim deficiency did not appear to dramatically alter the CD4/CD8 profile in either female or male mice (Fig. 1A). Both HYcd4 and HYcd4 Bim−/− male mice contained both DP thymocytes and a substantial population of CD8SP thymocytes, while both HYcd4 and HYcd4 Bim−/− male mice contained mostly DP thymocytes and lacked a substantial CD8SP population (Fig. 1A). We consistently observed an increase in CD4 and CD8 “dulling” in HYcd4 Bim−/− male DP thymocytes compared with HYcd4 male DP thymocytes, resulting in a higher percentage of DN phenotype cells (Fig. 1A). We hypothesize the exaggerated “DP dull” phenotype and higher percentage of DN phenotype thymocytes arises from prolonged high-affinity TCR signaling resulting from an increased lifespan due to an inhibition in apoptosis in the absence of Bim (see below). Alternatively, these DN cells could originate from DP thymocytes that truly failed negative selection.

To ensure that Bim deficiency did not alter pMHC recognition, we compared the levels of CD69, CD5, CD2, and PD-1 expression on T3.70+ DP thymocytes. As previously observed, all of these markers were up-regulated in HYcd4 male mice compared with B6 and HYcd4 female mice (29, 31). We observed an increased proportion of DP thymocytes expressing CD69 from HYcd4 Bim−/− female mice compared with HYcd4 female mice, suggesting enhanced positive selection efficiency, but no difference in CD5 or CD2 expression levels (Fig. 1B and data not shown). No difference in expression of CD69, CD5 or CD2 on DP thymocytes was found in HYcd4 Bim−/− male mice compared with HYcd4 male mice (Fig. 1B and data not shown). Interestingly, the percentage of DP thymocytes expressing PD-1, but not the level of PD-1 expression, was increased in HYcd4 Bim−/− male mice (Fig. 1C). This further supports an increased lifespan for DP thymocytes in the absence of Bim.
Bim since we know that PD-1 expression on DP thymocytes is induced maximally 48 h following TCR stimulation (31). Normally, PD-1 expression on DP thymocytes from wild-type mice is difficult to detect (32); however, in nontransgenic Bim−/− mice, a significant population of PD-1+ DP thymocytes was observed (data not shown). Given that PD-1 is expressed on DP thymocytes following a high-affinity signal, the PD-1+ DP thymocytes from nontransgenic Bim−/− mice may have received a high-affinity signal in vivo.

**FIGURE 1.** The phenotype of thymocytes undergoing positive and negative selection in HYcd4 Bim−/− mice. A, CD4 by CD8 profile of total thymocytes from Vβ8 and Vβ8 Bim−/− mice and CD4 by CD8 profiles of T3.70+ thymocytes from HYcd4 and HYcd4 Bim−/− female and male mice. Data are representative of at least eight mice per strain. B, The expression of CD69 and CD5 on total (B6) or T3.70+ thymocytes from HYcd4 Bim−/− male and female mice. Data are representative of at least eight mice per strain. C, The expression of PD-1 by side scatter (SSC) profile of T3.70+ DP thymocytes from the indicated strain in female and male mice. Data are representative of four independent experiments.

**FIGURE 2.** Bim is required for DP thymocyte apoptosis in vivo. A, Representative active caspase-3 by side scatter (SSC) profile of T3.70+ DP thymocytes from HYcd4 and HYcd4 Bim−/− male and female mice. B, Quantification of active caspase-3 levels in T3.70+ DP thymocytes from the indicated strain. Data are compiled from at least five mice per strain in at least five independent experiments. C, Number of T3.70+ DP thymocytes recovered from the indicated strains. Data are compiled from at least seven mice per strain in at least seven independent experiments. Data depict the means ± SD. **p < 0.0002; ***p = 0.0004; n.s., not significant.

**Bim is required for DP thymocyte apoptosis**

Previous experiments examining the requirement for Bim in negative selection indicated that Bim performed an essential role in negative selection by inducing apoptosis in DP thymocytes (20, 21). Therefore, we examined the consequence of Bim deficiency on DP thymocyte apoptosis using an Ab that recognizes only the cleaved and thus activated form of caspase 3. T3.70+ DP thymocytes from the indicated mice were electronically gated and the percentage of cells containing activated caspase-3 was determined. As previously reported, we found that compared with B6 or HYcd4 female mice, HYcd4 male mice contained a 6-fold higher percentage of active caspase-3+ T3.70+ DP thymocytes (Fig. 2, A and B) (31). Compared with HYcd4 male mice, HYcd4 Bim−/− male mice displayed a 16-fold reduction in the percentage of active caspase-3+ T3.70+ DP thymocytes (Fig. 2, A and B). No statistical difference in the percentage of cells containing active caspase-3 was observed between HYcd4 Bim−/− female and male mice (Fig. 2, A and B), suggesting that the high-affinity signal required to induce apoptosis normally was unable to do so in the absence of Bim. These data support Bim as an essential mediator of high-affinity Ag-induced apoptosis. Additionally, the few T3.70+ CD8SP thymocytes from HYcd4 Bim−/− male mice contained virtually no active caspase-3+ cells, while the same thymocyte population in HYcd4 male mice contained a substantial fraction of active caspase-3+ cells (supplemental Fig. 1).5 Given the critical role of Bim in DP thymocyte apoptosis, we next determined the influence of Bim deficiency on DP thymocyte numbers. As previously published, we observed a 3-fold decrease in the number of T3.70+ DP thymocytes in HYcd4 male mice compared with HYcd4 female thymocytes (Fig. 2C) (29). However, there was no difference in the number of T3.70+ DP thymocytes in HYcd4 Bim−/− male mice compared with HYcd4 Bim−/− female mice. We also

5 The online version of this article contains supplemental material.
observed a decrease in the number of T3.70+ DP thymocytes in HY<sup>cd4</sup> Bim<sup>−/−</sup> female mice compared with HY<sup>cd4</sup> female mice (Fig. 2C). This difference is likely explained by a reduction in proliferation at the β-selection checkpoint resulting from Bim deficiency as reported by Hutcheson and Perlman (33) and not impairment in DN thymocyte survival (34). Collectively, these data suggest that Bim is a critical mediator of DP thymocyte apoptosis during negative selection and that Bim-mediated apoptosis is responsible for the 3-fold decrease in T3.70+ DP thymocytes in HY<sup>cd4</sup> male mice compared with HY<sup>cd4</sup> female mice.

Negative selection occurs in the absence of Bim

If Bim deficiency truly impaired negative selection, one might predict this impairment to manifest itself as an increase in the mature CD8SP thymocyte population. Therefore, we performed a detailed analysis of the thymic CD8SP compartment in HY<sup>cd4</sup> and HY<sup>cd4</sup> Bim<sup>−/−</sup> mice. HY<sup>cd4</sup> female and HY<sup>cd4</sup> Bim<sup>−/−</sup> female mice both contained a substantial population of CD8SP thymocytes (Fig. 3A). Interestingly, there was a 2-fold increase in the percentage of T3.70+ CD8SP thymocytes in HY<sup>cd4</sup> Bim<sup>−/−</sup> female mice compared with HY<sup>cd4</sup> female mice, suggesting enhanced positive selection in the absence of Bim. In both HY<sup>cd4</sup> male and HY<sup>cd4</sup> Bim<sup>−/−</sup> male mice, there was a dramatic reduction in the percentage of T3.70+ CD8SP thymocytes (Fig. 3A). To determine whether Bim deficiency enhanced the development of mature (CD24<sup>low</sup>) T3.70+ CD8SP thymocytes, we examined CD24 expression on T3.70+ CD8SP thymocytes. In HY<sup>cd4</sup> and HY<sup>cd4</sup> Bim<sup>−/−</sup> female mice, most T3.70+ CD8SP thymocytes were CD24<sup>low</sup> while in HY<sup>cd4</sup> and HY<sup>cd4</sup> Bim<sup>−/−</sup> male mice, about half of the few T3.70+ CD8SP thymocytes were CD24<sup>low</sup> (Fig. 3B). Overall, there was a 40-fold reduction in the number of CD24<sup>low</sup> T3.70+ CD8SP thymocytes in HY<sup>cd4</sup> male compared with female mice and a 34-fold reduction in HY<sup>cd4</sup> Bim<sup>−/−</sup> male compared with female mice (Fig. 3C). Although not statistically significant, there was a 2-fold increase in the number of CD24<sup>low</sup> T3.70+ CD8SP thymocytes in HY<sup>cd4</sup> Bim<sup>−/−</sup> female mice compared with HY<sup>cd4</sup> female mice, again supporting enhanced positive selection or CD8SP survival in the absence of Bim. A compilation of the T3.70+ thymocyte numbers from the HY<sup>cd4</sup> and HY<sup>cd4</sup> Bim<sup>−/−</sup> female and male mice is presented in Table I. Therefore, it appears that in the HY<sup>cd4</sup> mouse model, Bim deficiency does not impair thymocyte negative selection.

The fact that Bim<sup>−/−</sup> mice do develop autoimmune disorders suggests that these mice may contain functional peripheral T cells that escape negative selection. To determine the impact of Bim on the peripheral CD8 compartment in HY<sup>cd4</sup> mice, we examined the lymph node and spleen of HY<sup>cd4</sup> and HY<sup>cd4</sup> Bim<sup>−/−</sup> male and female mice. As previously reported (29), HY<sup>cd4</sup> female mice contain relatively few T3.70+ T cells in the lymph node and spleen (supplemental Fig. 2 and data not shown). These T3.70+ cells are mostly CD8<sup>+</sup> and display a naive, CD44<sup>low</sup> phenotype (supplemental Fig. 2). Bim deficiency does not appear to influence this phenotype. HY<sup>cd4</sup> male mice contain an elevated percentage and number of peripheral T3.70+ T cells in comparison to female mice (supplemental Fig. 2). Most of the T3.70+ cells in male mice are CD8<sup>+</sup>, albeit with lower CD8 levels than in female mice, with only a small percentage bearing a DN phenotype. This is in contrast to the peripheral T3.70+ cells in conventional HY male mice that mostly display a DN phenotype (data not shown). The reason for the high number of peripheral T3.70+ cells in the HY<sup>cd4</sup> male mice given the low number of thymic T3.70+ CD8SP is currently unclear, but it is likely due to the expansion of the few T3.70+ cells.

Table I. Bim is required for DP thymocyte clonal deletion but not negative selection

<table>
<thead>
<tr>
<th>Strain</th>
<th>HY&lt;sup&gt;cd4&lt;/sup&gt; F</th>
<th>HY&lt;sup&gt;cd4&lt;/sup&gt; Bim&lt;sup&gt;−/−&lt;/sup&gt; F</th>
<th>HY&lt;sup&gt;cd4&lt;/sup&gt; M</th>
<th>HY&lt;sup&gt;cd4&lt;/sup&gt; Bim&lt;sup&gt;−/−&lt;/sup&gt; M</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3.70+ DP</td>
<td>58.7 ± 29.3</td>
<td>47.4 ± 25.8</td>
<td>19.6 ± 7.07</td>
<td>44.9 ± 38.4</td>
</tr>
<tr>
<td>T3.70+ CD8SP</td>
<td>3.04 ± 1.13</td>
<td>4.93 ± 2.54</td>
<td>0.142 ± 0.054</td>
<td>0.383 ± 0.262</td>
</tr>
<tr>
<td>T3.70+ CD24&lt;sup&gt;low&lt;/sup&gt; CD8SP</td>
<td>1.95 ± 0.91</td>
<td>4.19 ± 2.56</td>
<td>0.048 ± 0.046</td>
<td>0.123 ± 0.048</td>
</tr>
</tbody>
</table>

* Absolute number of T3.70+ thymocytes from the indicated strains. Data were compiled from between 4 and 10 mice (mean ± SD × 10<sup>6</sup>).
CD8SP that are able to escape negative selection once in an environment expressing their cognate Ag. In support of this idea, the T3.70+ CD8+ cells from HY^{cd4} male mice are CD44^{high}, suggesting Ag-driven expansion (supplemental Fig. 2). While Bim deficiency does not influence the percentage of peripheral T3.70+ cells in HY^{cd4} male mice, it does influence their phenotype since there are approximately equal percentages of CD8+ and DN phenotype cells in the absence of Bim (supplemental Fig. 2). Similar to T3.70+ CD8+ cells in HY^{cd4} male mice, T3.70+ CD8+ cells from HY^{cd4} Bim^{-/-} male mice are CD44^{high}, while the T3.70+ DN phenotype cells from HY^{cd4} Bim^{-/-} male mice are CD44^{low} (supplemental Fig. 2). The T3.70+ cells from the HY^{cd4} and HY^{cd4} Bim^{-/-} male mice are CD69^{-}, suggesting that they are not overtly activated (data not shown). The reason for the change in phenotype in peripheral T3.70+ cells in HY^{cd4} Bim^{-/-} mice is currently unclear, but given the fact that HY^{cd4} male mice do not appear to develop autoimmune diseases despite high numbers of T3.70+ CD8+ peripheral cells, we do not think these or the HY^{cd4} Bim^{-/-} T3.70+ cells are pathogenic. Because of the complexities of the peripheral T cell population in HY^{cd4} and HY^{cd4} Bim^{-/-} male mice, we also examined the role of Bim in negative selection in V\beta8 TCR transgenic mice (see below).

**Bim deficiency does not affect negative selection in an oligoclonal repertoire**

To this point, we have examined the requirement for Bim in the negative selection of a monoclonal thymocyte population. We next wanted to determine whether Bim was required for negative selection in an oligoclonal repertoire. To do this, we utilized the V\beta8 TCR transgenic mouse that expresses the TCR\beta-chain derived from the HY TCR. In female V\beta8 TCR transgenic mice, there is an elevated frequency of male Ag-reactive thymocytes and T cells with variable affinities for s-mcy peptide in the context of H-2D\alpha, making it possible to examine selection events in an oligoclonal repertoire (35). By using pentamers of H-2D\alpha/s-mcy complexes, we can specifically identify male Ag-reactive thymocytes and T cells. Compared with female B6 mice, we found an elevated percentage of CD8+ T cells and thymocytes specific for D\alpha/s-mcy in the spleen, lymph node, and thymus of female V\beta8 mice (Fig. 4, A and B, and data not shown). We found a similar frequency of D\alpha/s-mcy-specific T cells in V\beta8 Bim^{-/-} female mice compared with V\beta8 female mice (data not shown). As previously reported, negative selection mostly eliminates D\alpha/s-mcy specificities in the peripheral CD8 T cell and CD8SP thymocyte compartment of V\beta8 male mice (29, 35), and Bim deficiency does not affect this elimination (Fig. 4, A and B, and data not shown). Since negative selection is not perfect and low-affinity clones are able to escape this process (36), we determined whether Bim deficiency influenced the affinity of the T cell clones that escape negative selection. Using a strategy similar to Zehn and Bevan (36), we CFSE-labeled bulk thymocytes from B6, V\beta8 female and male and V\beta8 Bim^{-/-} male mice to assess the response of the CD8SP thymocytes to increasing concentrations of s-mcy peptide. At 24 h following stimulation, a population of CD8SP thymocytes from V\beta8 female mice began to induce CD69 at a peptide concentration of 10^{-8} M, while CD8SP thymocytes from male V\beta8 and V\beta8 Bim^{-/-} mice did not begin to induce CD69 until an s-mcy concentration of 10^{-6} M (Fig. 4C). This indicates that the CD8SP thymocytes that escape negative selection in male mice have a 100-fold reduction in the affinity for s-mcy compared with female mice and that Bim deficiency does not influence the affinity of the escapees. Similar results were also obtained by measuring CFSE dilution 48 h poststimulation (data not shown). Therefore, it appears that mechanisms independent of Bim-mediated apoptosis can enforce negative selection in an oligoclonal repertoire.

**FIGURE 4.** Bim is not required for thymocyte negative selection in an oligoclonal repertoire. A, Splenocytes from the indicated strain were stained with PE-conjugated D\alpha/s-mcy pentamer followed by staining with anti-CD4, anti-CD8, and anti-CD19. The CD8 by D\alpha/s-mcy pentamer profile on CD19- cells is depicted. B, Compilation of percentage of pentamer binding CD8+ splenocytes from four mice in four independent experiments. C, CFSE-labeled thymocytes from the indicated mice were cultured with B6 female splenocytes and the indicated concentration of s-mcy peptide for 24 h. The cells were harvested and the induction of CD69 on the CD8SP thymocytes was measured by flow cytometry. The percentage of CD8SP thymocytes that induced CD69 is depicted in the graph as the means ± SD. Data are compiled from two to three mice per strain in three independent experiments.

**Discussion**

It is currently thought that apoptosis is the primary mechanism by which negative selection is achieved. The current paradigm states that a high-affinity TCR signal received by DP or semimature SP thymocytes induces the expression of proapoptotic molecules, namely Bim and Nur77, resulting in cell death. In the present study, we demonstrated that while Bim appears to be essential for DP thymocyte apoptosis, the loss of Bim does not impair negative selection. These data support the original findings of the Strasser group where they described Bim as an essential protein for thymocyte apoptosis (20) and the Marrack group’s data that demonstrated Bim was not required for negative selection (28). Based on our findings, it can be concluded that in addition to Bim-mediated apoptosis, other mechanisms exist for enforcing negative selection. We think our data also suggest that the current dogma surrounding negative selection be revisited.

If negative selection is still occurring in the absence of Bim-mediated apoptosis, then what mechanisms are responsible for enforcing negative selection independent of Bim? Two obvious candidate proteins are the death receptor Fas and the orphan nuclear
receptor Nur77. While the Fas pathway was recently shown to cooperate with Bim in preventing autoimmunity (37–39), evidence supporting a role for Fas (or any other death receptor) in negative selection to ubiquitous self-Ags is lacking (40). Furthermore, activation of caspase-3 is downstream of both the extrinsic pathway (Fas) and intrinsic pathway (Bim) and we do not detect an increase in active caspase-3 in HYcd4 Bim−/− male over female mice as would be expected if the Fas pathway was compensating for the loss of Bim. Based on previous experiments, one might predict Nur77 and Bim to operate in parallel pathways leading to clonal deletion. However, all of these experiments were performed in Bim-expressing thymocytes. Our data suggests that Bim is required for Nur77-mediated apoptosis, since the level of apoptosis is equivalent in HYcd4 Bim+/− female and male DP thymocytes while Nur77 expression is still elevated in HYcd4 Bim−/− male DP over female DP (data not shown). Therefore, without Bim expression, high-affinity Ag-mediated induction of Nur77 is unable to induce apoptosis over background levels, suggesting an apoptosis-independent mechanism of negative selection in Bim-deficient mice. Data in the literature support the idea that Bim and Nur77 cooperate to induce apoptosis during negative selection. Inhibition of Nur77 activity is able to inhibit apoptosis in F5 transgenic mice injected with NP peptide even though Bim should still be expressed in these mice (23). Furthermore, thymocyte apoptosis is inhibited in Bim-deficient mice even though Nur77 expression should be unaffected (20). It is unclear at this point at what level Bim is required for Nur77-mediated apoptosis. Examining apoptosis induction in Bim-deficient Nur77 transgenic thymocytes and negative selection in the absence of both Bim and Nur77 activity will be critical for determining the relationship between Bim and Nur77 in negative selection.

In addition to apoptosis, receptor editing and anergy have been described as mechanisms employed to induce negative selection (Fig. 5) (2). Since receptor editing does not appear to be a mechanism utilized to induce negative selection in male Ag-reactive thymocytes (41), we do not favor this as the alternative mechanism to apoptosis. Interestingly, in HYcd4 Bim−/− male mice, we find that most of the T3.70+ DP thymocytes express PD-1 and given the role of PD-1 expression in peripheral T cell anergy (42), it is tempting to speculate that anergy induction is the apoptosis-independent mechanism of negative selection employed in this situation. Additional experiments examining the role of PD-1 in thymic negative selection in the presence and absence of Bim will be required to determine the consequence of PD-1 expression on DP thymocytes following high-affinity stimulation.

Two other scenarios that may explain negative selection in the absence of apoptosis both involve a block in positive selection of DP thymocytes (Fig. 5). In the first scenario, only a low-affinity signal would be able to induce a factor required for positive selection. We have ruled out Id3 and Runx3 as possible candidate proteins in this scenario because the expression level of Id3 and Runx3 is similar in HYcd4 Bim−/− female and male DP thymocytes (supplemental Fig. 3). Since our molecular understanding of positive selection is incomplete, there could be other unidentified positive selection factors only induced by a low-affinity signal, leaving this model as a viable possibility. In the second scenario, a high-affinity signal could induce a protein that interferes with the positive selection process. We favor the second scenario over the first since negative selection is dominant over positive selection in the HYcd4 model, and T3.70+ DP thymocytes theoretically should encounter both the positively selecting and the negatively selecting ligand in male mice.

One of the other questions raised by our data relates to the relative contributions of the apoptotic and nonapoptotic mechanisms of negative selection in wild-type mice. In HYcd4 male mice, we find that Bim-dependent apoptosis is able to reduce the number of DP thymocytes by approximately two-thirds. This leaves approximately one-third of the DP thymocytes possibly subject to nonapoptotic mechanisms of negative selection. Determining the relative importance of each “arm” of negative selection will require an understanding of the nonapoptotic arm and ways to inhibit this mechanism.

With respect to positive selection, we found that there was an increase in the percentage and absolute number of T3.70+ CD8SP thymocytes in HYcd4 Bim−/− female mice compared with HYcd4 female mice. This could occur as a result of enhanced survival of positively selected Bim−/− CD8SP thymocytes; however, we did not observe any differences in apoptosis in T3.70+ CD8SP from HYcd4 or HYcd4 Bim−/− female mice. Instead, a reduction in the number of T3.70+ DP thymocytes, but an increase in the number of T3.70+ CD8SP thymocytes, in HYcd4 Bim−/− female mice suggests that the efficiency of positive selection is enhanced in the absence of Bim. We hypothesize that in the absence of Bim, DP thymocytes could survive longer, thereby increasing the probability of encountering their positively selecting ligand. Since we know that CD69 expression is gradually increased over time during positive selection (31), increased levels of CD69 on T3.70+ DP thymocytes from HYcd4 Bim−/− female mice supports this hypothesis.

Collectively, our data provide new insight into the molecular mechanism of thymocyte negative selection. They indicate that while apoptosis is a robust mechanism to induce negative selection and the proapoptotic protein Bim plays a crucial role in clonal deletion, other potent nonapoptotic mechanisms can also be employed. This study was limited to negative selection in response to a ubiquitous, MHC class I-presented peptide. However, we know that negative selection to TRAs is critical for self-tolerance. Since negative selection to TRAs requires positive selection and trafficking to the medulla (43), it is possible that Bim deficiency can rescue TRA-specific thymocytes from negative selection. Based on our work, we hypothesize that the autoimmune disorders observed in Bim-deficient mice could result from incomplete negative selection of TRA-specific T cells rather than ubiquitous self-Ag-specific T cells. Understanding how negative selection to ubiquitous self-Ags as well as TRAs is governed will provide insight into how central tolerance is enforced and autoimmunity is prevented.
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Disclosures
The authors have no financial conflicts of interest.

References
Figure S1: Bim is required for caspase 3 activation in CD8SP thymocytes. Representative active caspase 3 by side scatter (SSC) profile of CD8SP thymocytes from B6 mice and T3.70+ CD8SP thymocytes from HY^{cd4} and HY^{cd4} Bim^{--} female and male mice.

Figure S2: Bim-deficiency influences the peripheral T cell phenotype in HY^{cd4} male mice. Top Row: Representative T3.70 by side scatter profile of Thy1.2+ lymph node cells from the indicated mice. Middle Row: CD4 by CD8 profile of T3.70+ lymph node cells from indicated mice. Bottom Row: CD44 expression on CD8+ T3.70+ lymph node cells from indicated mice. The HY^{cd4} Bim^{--} T3.70+ DN population from male mice is also included.

Figure S3: The expression level of Id3, Runx3 and CCR7 during selection. The indicated thymocyte subsets from the indicated mouse strain were examined for the expression of Id3 (A), Runx3 (B) and CCR7 (C) by qRT-PCR. The data are expressed as a percentage of the control gene β-actin. For the HY^{cd4} Bim^{--} female and male DP the mean ± S.D. for two replicates of two independent sorts is shown. For all other populations, four replicates of a single sort are shown. The trend observed for HY^{cd4} female and male DP thymocytes was similar to our previous gene array study where mRNA expression levels from three independent sorts were examined.