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Thymocyte Negative Selection Is Mediated by Protein Kinase C- and Ca$^{2+}$-Dependent Transcriptional Induction of Bim$^1$

Kirsten Canté-Barrett,* Elena M. Gallo,† Monte M. Winslow,† and Gerald R. Crabtree$^{2,8†}$

The processes of positive and negative selection in the thymus both determine the population of T cells that will enter the peripheral immune system and eliminate self-reactive T cells by apoptosis. Substantial evidence indicates that TCR signal intensity mediates this cell fate choice: low-intensity signals lead to survival and differentiation, whereas high-intensity signals generated by self-Ag lead to cell death. The molecular mechanism by which these graded signals are converted to discrete outcomes is not understood. Positive selection requires the Ca$^{2+}$-dependent phosphatase calcineurin, whereas negative selection requires the proapoptotic Bcl-2 family member Bcl-2-interacting mediator of cell death (Bim). In this study, we investigated the regulation of Bim expression and the role of Ca$^{2+}$ in mediating negative selection. Our results show that transcription is necessary for both negative selection and Bim induction. Surprisingly, we also found that Ca$^{2+}$ is necessary for Bim induction. Induction of bim transcription appears to involve protein kinase C, but not calcineurin, JNK, p38 MAPK, or MEK. These results localize the decision point in positive vs negative selection to a step downstream of Ca$^{2+}$ signaling and suggest that negative selection signals induce Ca$^{2+}$-dependent bim transcription through PKC. The Journal of Immunology, 2006, 176: 2299–2306.

Developmental cell fate decisions are often made on the basis of signal intensity, yet relatively little is known about how these graded signals are converted to non-overlapping cell fates. For example, this analog-to-digital conversion is seen in sonic hedgehog and bone morphogenetic protein signaling, where genetic studies indicate that all components of these signaling pathways are required for all fates and that the mechanisms converting graded signals to discreet outcomes involves cross-regulation between transcription factors downstream of the pathway (1, 2). In contrast, our current understanding of T lymphocyte development is that the pathways for positive and negative selection appear to diverge, with a Bim-dependent pathway leading to negative selection and calcineurin- and ERK-dependent pathways leading to positive selection. Although other proteins are critical for negative selection, the present data indicate that Bim is a final common mediator of cell death in these pathways and is both necessary and sufficient for cell death (3–6).

Substantial evidence now supports the notion that self-Ags induce a strong TCR signal, leading to death of self-reactive thymocytes, whereas weaker signals produced by the binding of the self-MHC lead to cell survival, differentiation, and entry into the peripheral immune system (reviewed in Ref. 7). Thus, the currently accepted view is that the affinity and avidity between MHC/peptide and TCR determines the ultimate fate of the developing T lymphocyte.

One approach to understanding how signals of different intensity can produce radically different fates would be to identify the proteins critical for both positive and negative selection vs those needed for one cell fate but not the other. Signal transduction through the TCR involves a number of proteins common to both the positive and negative selection pathways, including the tyrosine kinases lck and Zap70, Tec kinases, the adaptor proteins Src homology 2 domain-containing leukocyte protein of 76 kDa and linker for activation of T cells, and the guanine nucleotide exchange factor Vav (8–13). Bouillet et al. (3) showed that Bcl-2-interacting mediator of cell death (Bim)$^3$ is required for negative selection of immature CD4/CD8 double-positive (DP) thymocytes, but does not play a role in positive selection. In contrast, thymocytes lacking calcineurin b1 have a complete block in positive selection but exhibit normal negative selection (14). Moreover, thymocytes lacking ERK or serum response factor accessory protein 1 function have a reduction in positive selection (15–20).

Thus, the current view is that low-intensity signals lead to positive selection via calcineurin and ERK, whereas high-intensity signals trigger the dominant-negative selection pathway via the proapoptotic proteins Bim and Bcl-2 antagonist/killer/Bcl-2-associated X protein (3, 21).

During negative selection, DP thymocytes undergo apoptosis. The Bcl-2 family of proteins consists of both prosurvival and proapoptotic proteins and these proteins are crucial in regulating apoptosis by controlling mitochondrial damage and the subsequent activation of caspases. One subset of Bcl-2 family members contains only the third Bcl-2 homology (BH) domain and all of these BH3-only proteins discovered to date have a proapoptotic effect. Different apoptotic stimuli trigger apoptosis through activation of different BH3-only proteins (reviewed in Ref. 22). The proapoptotic BH3-only protein Bim is essential for apoptosis after T and B cell Ag-receptor cross-linking (23, 24), as well as for apoptosis after cytokine withdrawal in several cell types (25, 26). Importantly, as little as a 2-fold increase in Bim appears to be sufficient for cell death.

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for cell death, while bim heterozygosity reduces cell death and negative selection (4–6). Thus, Bim appears to be a highly dosage-sensitive and genetically dominant regulator of cell death, most likely acting downstream of other important regulators.

In this study, we explored the mechanism by which Bim is induced during negative selection upon strong TCR activation. In contrast to previous studies indicating the involvement of post-translational modifications (27, 28), we show that transcription of bim is necessary for negative selection of DP thymocytes. Consistent with the requirement of Ca\(^{2+}\) in negative selection (29, 30), we show that Ca\(^{2+}\) also plays a critical role in Bim induction. This provides evidence that the decision for positive or negative selection is made at or downstream of Ca\(^{2+}\). Finally, we present evidence that the Ca\(^{2+}\)-activated kinase protein kinase C (PKC) plays a role in regulating Bim expression and mediating negative selection. Based on these data, we present a model for cell-fate determination of DP thymocytes, in which Ca\(^{2+}\) plays a central role in both pathways and leads to induction of bim transcription in negative selection.

Materials and Methods

Mice

C57BL/6 mice between 6 and 10 wk of age were used in all experiments, unless otherwise noted. Mice were maintained in the animal facility of Stanford University in accordance with federal and institutional guidelines. The conditional calcineurin b1 (Cnb1) mice have been described (14). OT-I transgenic mice (31) on the TAP\(^{2}\) background were a gift from K. A. Hogquist (University of Minnesota, Minneapolis, MN).

Cells and reagents

Thymocytes were obtained by straining the whole thymus through a 70-μm nylon cell strainer. In all experiments, cells were incubated at 37°C/5% CO\(_2\) in RPMI 1640 containing 10% FBS supplemented with penicillin, streptomycin, 1-glutamine, sodium pyruvate, nonessential amino acids 2-ME, and HEPEs (pH 7.4). For peptide stimulation, EIINFEEKL and SL-INFEEKL peptides (Anaspec) were added to 2 μM with CH27-H2K\(^{b}\) cells (CH27 cell line transfected with H2K\(^{b}\)) (32), provided by M. M. Davis, Stanford University, Stanford, CA) for 2 h. Cells were then washed and fixed with 0.1% glutaraldehyde. The OT-I-TAP\(^{2}\) thymocytes were added in a 2:1 ratio to the fixed APCs. In vivo stimulation was done by i.p. injecting 50 μg of anti-CD3ε Ab (145-2C11; BD Biosciences), 24 and 44 h before thymocytes were harvested. For in vitro Ab stimulation, tissue culture plates were coated with 10 μg/ml anti-CD3ε (145-2C11; BD Biosciences) and 50 μg/ml anti-CD28 (37.51; BD Biosciences) in PBS overnight at 4°C. The Ab solution was removed before the addition of thymocytes. Incubations with PMA (25 or 50 ng/ml), ionomycin (0.5 μM), and brefeldin A (10 μg/ml; Calbiochem) were performed for 3 h. Cells were then stained with mitotracker green FM, fixed, and permeabilized with 0.1% Triton X-100 (15 min at room temperature). Intracellular Bim staining, cells were stained with anti-Bim (1:200; StressGen Biotechnology) antibodies and a secondary incubation of 30 min with (1:100) PE-conjugated anti-rabbit IgG (The Jackson Laboratory).

Western blotting

Total cell lysates from 1 to 5 × 10\(^{6}\) thymocytes were prepared in radioimmunoprecipitation assay buffer, typically after 3 or 4 h of stimulation and/or treatment in culture. For the experiment shown in Fig. 1D, CD4/CD8 DP thymocytes were sorted before lysates were prepared. For the experiment shown in Fig. 4A, thymocytes were enriched using CD8 MACS Dynal magnetic particles (Amersham) before cell lysates were prepared. Lysates were resolved on 4–12% gradient Bis-Tris NuPage gels (Invitrogen Life Technologies) and transferred to polyvinylidene difluoride. The Abs used were: rabbit anti-bim (1:1000; StressGen Biotechnologies), rabbit anti-Egr1 (1: 200; Santa Cruz Biotechnology), rabbit anti-phospho-ERK1/2 (1:1000; Cell Signaling Technology), goat anti-calcineurin b1 (1:1000; Santa Cruz Biotechnology), rabbit anti-actin (1:2500, Sigma-Aldrich), and mouse anti-heat shock protein (Hsp) 90 (1:2500; BD Biosciences). Signal was detected with ECL followed by exposure to autoradiograph film. For all Western blots, a longer exposure was necessary to visualize Bim\(_{b}\). In Fig. 3, a separate exposure for Bim\(_{b}\) as well as for Bim\(_{o}\), is shown.

RNase protection assay

Total RNA from nonstimulated and stimulated thymocytes was purified with an RNA isolation kit (Roche). One microgram of total RNA per sample was hybridized with bim and L32 probes, using the RPA III kit (Ambion). A 246-nt bim probe, which spans exons 5 and 6, and a 94-nt L32 probe against the large ribosomal subunit were synthesized using [\(\alpha\)-\(\beta\)-UTP (Amersham) in a transcription reaction using an RNA labeling kit (Roche). After the assay, samples were run on a 5.5% acrylamide/bisacrylamide (19:1) M urea gel. After drying, the gel was exposed overnight to a PhosphorImager screen (Molecular Dynamics). Quantitative analysis of band intensities was performed with the ImageQuant software, and the bim/L32 mRNA ratio was normalized to the 0-h time point. The protected bim fragment of 210 nt (exons 5 and 6) is common to the three main bim splice variants bim\(_{b}\), bim\(_{o}\), and bim\(_{b}\), and was used to calculate the bim/L32 ratio. The other bim fragment (111 nt) represents bim splice variants that contain exon 5, but lack exon 6 (33, 34).

Results

Both negative selection and Bim induction require transcription

Since Bim is both necessary and sufficient for negative selection of CD4/CD8 DP thymocytes (3), we tested whether transcription is required for negative selection, using the rapid and reversible transcription inhibitor DRB. To mimic negative selection, DP thymocytes were stimulated with plate-bound anti-CD3/anti-CD28, and DRB was applied to block transcription; apoptosis was measured by annexin V staining 3 h after stimulation (Fig. 1A). Stimulation of DP thymocytes increased apoptosis (10.4% compared with 4.1% for unstimulated cells), and this increase was completely blocked by DRB (4.5%). As a positive control, glucocorticoid-mediated apoptosis was induced with dexamethasone (35); dexamethasone increased apoptosis (19.5%), and this increase was completely blocked by DRB (4.5%).

We also tested the ability of DRB to maintain cell viability following Ab stimulation, using the mitochondrial dye CMX-Rosamine (Fig. 1B). Consistent with increased percentages of annexin V-positive cells, 3 h after stimulation DP thymocyte viability was reduced (Fig. 1B, open circles). DRB (gray lines) protected thymocytes from apoptosis, and this effect was reversed after DRB removal (right panel in Fig. 1B), providing further evidence that transcription is necessary for negative selection.

Having shown that transcription is required for negative selection in DP thymocytes, we examined whether Bim itself is induced upon stimulation. Samples from the experiment in Fig. 1A were used for Western blot analysis (Fig. 1C). Both plate-bound Ab stimulation and dexamethasone treatment caused increased levels of Bim\(_{b}\), and Bim\(_{b}\), these two Bim isoforms with the strongest apoptotic activity (4). Consistent with the in vitro induction of Bim, injecting anti-CD3 Ab as an in vivo model of negative selection also caused increased Bim levels (Ref. 3 and Fig. 1D). In contrast with what

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was reported recently (36), we found that Bim levels in DP thymocytes increased 24 and 44 h after i.p. injection of anti-CD3 Ab (Fig. 1D). As with apoptosis and cell viability, DRB completely blocked Bim induction following both Ab stimulation and dexamethasone treatment in vitro, suggesting that transcription of *bim* is necessary for apoptosis, and therefore negative selection. It is interesting to note that DRB also maintained the viability of unstimulated thymocytes (Fig. 1B) and reduced Bim levels compared with unstimulated cells in the absence of DRB (Fig. 1C), presumably because thymocytes in a suboptimal in vitro environment undergo apoptosis through transcriptional induction of Bim.

*bim* mRNA levels increase following plate-bound Ab stimulation

For other cell types, posttranslation modifications or protein-protein interactions are believed to regulate Bim activity (27, 37). However, our results with DRB suggest that, in thymocytes, stimulation increases *bim* transcription. Therefore, increased *bim* message should be detectable following Ab stimulation. We used an RNase protection assay to measure *bim* message levels 1, 3, and 6 h following stimulation (Fig. 2). At all time points, stimulated thymocytes had higher levels of *bim* mRNA compared with unstimulated cells. As an internal control, *L32* mRNA was also measured, and used to normalize *bim* message levels (Fig. 2B). At 1 h, the *bim* mRNA level in stimulated thymocytes was ~2.5-fold that of unstimulated thymocytes, consistent with the increased Bim protein we observed at 3 h (Fig. 1C). A similar increase in *bim* message was seen when measured by real-time PCR (our unpublished data). We also observed increased *bim* mRNA in unstimulated thymocytes after 1 h in culture (Fig. 2), consistent with increased cell death in the absence of stimulation (Fig. 1, A and B).

Our results with DRB suggest that the increased *bim* mRNA in stimulated thymocytes is likely the result of increased *bim* transcription, and not simply increased stability of *bim* mRNA. This was confirmed by inhibiting transcription in unstimulated thymocytes and measuring *bim* mRNA at time points ranging from 15 min to 3 h. We found that under these conditions, *bim* mRNA levels were unchanged (our unpublished data). From this we conclude that increased *bim* mRNA (and thus protein) in stimulated thymocytes is not the result of *bim* mRNA stabilization, but represents an increase in *bim* transcription. Although the increases in *bim* mRNA and protein levels are modest (ranging from 2- to 3-fold), it is likely sufficient to account for cell death since previous studies have shown that cells are highly sensitive to Bim levels and that a 2-fold change can dramatically affect cell death (4, 5).

**Calcium is necessary for Bim induction in DP thymocytes**

In developing thymocytes, Ca\(^{2+}\) influx and calcineurin activation are necessary for positive selection, whereas calcineurin is not required for negative selection (14). To determine the role of Ca\(^{2+}\) in negative selection, we tested the effect of Ca\(^{2+}\) chelators on Bim induction following Ab stimulation. Bim levels were measured by both Western blot analysis and intracellular Bim staining followed by flow cytometry. The chelators EGTA (4 mM) and BAPTA-AM

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**FIGURE 1.** Both negative selection and Bim induction require transcription. A. Flow cytometric analysis of CD4/CD8 DP thymocytes with or without 3 h of stimulation by plate-bound anti-CD3/anti-CD28 or 100 nM dexamethasone, using annexin V staining as a marker for apoptosis. Stimulation-induced apoptosis was completely blocked by 125 \(\mu\)M DRB. B. Time course of viable DP thymocytes, measured by flow cytometry using the mitochondrial dye CMX-Rosamine and expressed as a percentage. Data were normalized to the 0-h time point, which was set as 100% viability. C, From the same experiment as in A, total thymocytes were lysed for Western blot analysis. In this and subsequent blots, actin was probed as a loading control, unless otherwise indicated. D, Western blot analysis of DP thymocyte lysates of mice that were i.p. injected 24 and 44 h earlier with anti-CD3. Hsp90 shows equal loading.
addition to playing a critical role in positive selection, Ca\(^{2+}\) for 1 h (our unpublished data). We therefore conclude that, in stimulated thymocytes treated with EGTA and BAPTA-AM completely blocked Bim induction (Fig. 3, respectively). Treatment with either EGTA or BAPTA-AM alone partially blocked Bim induction, while cotreatment with EGTA and BAPTA-AM blocked Bim induction following Ab stimulation of DP thymocytes. To test this, we used transgenic mice carrying the OT-I TCR (31) on a TAP0 background. Thymocytes in these mice express the OT-I TCR but are not positively or negatively selected in vivo due to TAP deficiency. Thus, isolated thymocytes were depleted of positively and negatively selected OT-I thymocytes using plate-bound Abs. Unstimulated or stimulated thymocytes, treated with calcium chelators as in Fig. 3, A and B. Moreover, chelating Ca\(^{2+}\) reduced Bim levels in unstimulated cells after 3 h in culture. These results implicate both intracellular and extracellular Ca\(^{2+}\) in Bim expression. Consistent with this notion, real-time PCR analysis revealed decreased bim mRNA levels in stimulated thymocytes treated with EGTA and BAPTA-AM for 1 h (our unpublished data). We therefore conclude that, in addition to playing a critical role in positive selection, Ca\(^{2+}\) is also necessary for inducing bim in thymocytes during negative selection.

We next asked whether increased intracellular Ca\(^{2+}\) is sufficient to induce Bim, using the endoplasmic Ca\(^{2+}\)-ATPase inhibitor thapsigargin to induce increased cytosolic [Ca\(^{2+}\)]. Even at 50 nM, well above its IC\(_{50}\), thapsigargin failed to increase Bim levels after 3 h (Fig. 3C). Thus, simply raising cytosolic [Ca\(^{2+}\)] does not appear to be sufficient to induce Bim.

**Calcineurin is not required for Bim induction**

As mentioned above, calcineurin does not play a role in negative selection. Thus, calcineurin should not be necessary for Bim induction following Ab stimulation of DP thymocytes. To test this, we used a conditional knockout mouse in which the calcineurin b1 subunit is specifically deleted in the thymus upon lck-cre expression (14). Control (Cnb1\(^{+/+}\); lck-cre\(^{-/-}\)) and Cnb1 deficient (Cnb1\(^{-/-}\); lck-cre\(^{-/-}\)) thymocytes showed a similar increase in Bim level 4 h after plate-bound Ab stimulation (Fig. 4, A and B) Unstimulated Cnb1-deficient thymocytes had slightly increased Bim levels compared with unstimulated control thymocytes, consistent with the moderate decrease in viability of these thymocytes in culture (14). Consistent with the fact that calcineurin does not play a role in negative selection, these results show that calcineurin is not required for Bim induction.

**Bim is induced by a negative-, but not positive-, selecting ligand in OT-1 thymocytes**

If transcription of bim is critical in the differentiation between positive and negative selection then we would predict that positively selecting stimuli would not induce Bim, whereas negatively selecting stimuli would. To test this, we used transgenic mice carrying the OT-1 TCR (31) on a TAP\(^{0}\) background. Thymocytes in these mice express the OT-1 TCR but are not positively or negatively selected in vivo due to TAP deficiency. Thus, isolated thymocytes can be driven to either positive or negative selection by

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**FIGURE 2.** bim mRNA levels increase following plate-bound anti-CD3/anti-CD28 stimulation. A, RNase protection assay of bim mRNA using a 246-nt probe which protects two fragments (210 and 111 nt; see Materials and Methods). The upper bim band (210 nt) represents the protected fragment common to the three main bim splice variants bim\(_{EL}\), bim\(_L\), and bim\(_S\). The lower bim band (111 nt) represents other bim splice variants. A 94-nt probe which protects a 78-nt fragment of the large ribosomal subunit L32 was used as a loading control. Negative and positive controls included yeast RNA treated with or without RNase after the hybridization step, respectively. –, unstimulated; +, anti-CD3/anti-CD28 stimulation for the times indicated. B, The ratio of 210-nt protected bim bands to their respective protected L32 bands in A was determined, and normalized to the 0-h time point.

(100 μM) were used to capture extra- and intracellular Ca\(^{2+}\), respectively. Treatment with either EGTA or BAPTA-AM alone partially blocked Bim induction, while cotreatment with EGTA and BAPTA-AM completely blocked Bim induction (Fig. 3, A and B). Moreover, chelating Ca\(^{2+}\) reduced Bim levels in unstimulated cells after 3 h in culture. These results implicate both intracellular and extracellular Ca\(^{2+}\) in Bim expression. Consistent with this notion, real-time PCR analysis revealed decreased bim mRNA levels in stimulated thymocytes treated with EGTA and BAPTA-AM for 1 h (our unpublished data). We therefore conclude that, in addition to playing a critical role in positive selection, Ca\(^{2+}\) is also necessary for inducing bim in thymocytes during negative selection.

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presentation of peptides on class I MHC molecules; presentation of the agonist peptide SIINFEKL induces negative selection of these thymocytes, while a synthetic variant with the sequence EIINFEKL (expressing functional calcineurin) and Cnb1fl; lck-cre− (calcineurin-deficient) thymocytes have comparable Bim induction after anti-CD3/anti-CD28 stimulation for 4 h. B, Flow cytometric analysis of DP thymocytes from the same experiment as in A, C. Western blot analysis of OT-I/TAP3 thymocytes after incubating 4 h with APCs presenting no peptide (no selection), SIINFEKL (negative selection), or EIINFEKL (positive selection). Bim is induced only when SIINFEKL is presented. Egr-1 induction is shown as a positive control for both peptides.

FIGURE 4. Calcineurin is not required for Bim induction and a negative-, but not positive-, selecting ligand induces Bim. A, Western blot analysis of OT-I/TAP3 thymocytes. Cnb1fl; lck-cre− (expressing functional calcineurin) and Cnb1fl; lck-cre− (calcineurin-deficient) thymocytes have comparable Bim induction after anti-CD3/anti-CD28 stimulation for 4 h. B, Flow cytometric analysis of DP thymocytes from the same experiment as in A, C. Western blot analysis of OT-I/TAP3 thymocytes after incubating 4 h with APCs presenting no peptide (no selection), SIINFEKL (negative selection), or EIINFEKL (positive selection). Bim is induced only when SIINFEKL is presented. Egr-1 induction is shown as a positive control for both peptides.

PKC inhibitors block anti-CD3/anti-CD28-dependent Bim induction and apoptosis

Because our thapsigargin experiments suggested that increased cytosolic [Ca2+] is not sufficient to induce Bim, some other factor(s) might be necessary for inducing Bim and negative selection. The phorbol ester PMA is commonly used in combination with the Ca2+ ionophore ionomycin to activate T cells (41). As with T cells, Egr-1 and pERK1/2 were induced in thymocytes after treatment with PMA and ionomycin (Fig. 5A). We therefore hypothesized that cotreatment with PMA and ionomycin would mimic negative selection and induce Bim in thymocytes. PMA treatment alone caused moderate Bim induction, while ionomycin only slightly induced Bim (Fig. 5). However, treatment with both PMA and ionomycin increased Bim to a comparable or even greater level than stimulation with anti-CD3/anti-CD28 (Fig. 5B), suggesting that PMA and ionomycin treatment is sufficient to induce negative selection.

FIGURE 5. PMA and ionomycin induce Bim in thymocytes. A, Western blot analysis of total thymocytes treated with PMA (25 ng/ml) and/or ionomycin (0.5 μM). Cotreatment with PMA and ionomycin strongly induces Bim. Egr-1 and pERK1/2 are shown as positive controls, and Hsp90 is shown as a loading control. B, Flow cytometric analysis of DP thymocytes stimulated with plate-bound Abs or treated with PMA (50 ng/ml) and/or ionomycin (0.5 μM).
PMA is a diacylglycerol analog commonly used to activate PKC, and thus the ability of PMA to induce Bim in thymocytes implicates PKC in the negative selection pathway. Therefore, we tested the effect of three selective PKC inhibitors (42–44) on Bim induction and apoptosis during plate-bound Ab stimulation. The inhibitor Go 6976 blocked Bim induction in a dose-dependent manner, with 1 μM sufficient for a complete block (Fig. 6, A and B). Similar results were obtained with 1 μM Go 6850 and Go 6983. In contrast, Egr-1 induction was not fully blocked by these inhibitors, consistent with its selective role in positive selection. To examine the role of other kinases in Bim induction, we tested inhibitors of JNK, p38 MAPK, and MEK, and found that blocking each of these kinases had no effect on Bim induction following Ab stimulation (Fig. 6B). Consistent with the block in Bim induction, all three PKC inhibitors also blocked thymocyte apoptosis after plate-bound Ab stimulation, whereas JNK, p38 MAPK, and MEK inhibitors had no effect on cell death (Fig. 6C and data not shown).

It was shown previously that activation of the ERK1/2 pathway phosphorylates and degrades BimEL (45, 46). Indeed, we found that BimEL was phosphorylated upon stimulation (see Figs. 4, A and C, 5A, and 6A). This phosphorylation was blocked by the MEK inhibitor U0126 (our unpublished data), while Bim induction was unaffected by this inhibitor (Fig. 6B). This suggests that while phosphorylation of BimEL through the ERK1/2 pathway does occur in thymocytes upon stimulation, it does not affect Bim levels.

Discussion

The currently accepted view of positive and negative selection is that weak TCR signals lead to positive selection and cell survival, while strong signals lead to negative selection by apoptosis. In the present study, we investigated the mechanism of negative selection, focusing on the regulation of the proapoptotic protein Bim. Our data are consistent with a model in which strong TCR activation triggers a Ca2+-dependent pathway involving PKC activation and subsequent transcriptional induction of Bim (Fig. 7). Surprisingly, we found that cells undergoing negative selection have only a modest increase in Bim levels, ~2- to 3-fold. However, this small increase in Bim protein appears to be sufficient to induce cell death, suggesting that thymocytes are extremely sensitive to apoptotic signals. Two previous observations support this conclusion. First, mice haploid for bim have reduced negative selection (6), and secondly, overexpression studies have shown that only a small increase in Bim protein levels induces cell death (4, 5). Therefore, we conclude that, in DP thymocytes, transcriptional induction of bim is the primary mechanism leading to increased levels of Bim.

Previous studies have reported protein-protein interactions or posttranslational modifications as a means of controlling Bim levels. Association of Bim with the dynein motor complex through binding the dynein L chain (LC8) has been suggested (27); however, we found no evidence of such an interaction in thymocytes (our unpublished data). Additionally, several studies have called attention to phosphorylation of BimEL by ERK1/2 after survival signals, which in turn results in ubiquitination and proteosome degradation, thereby decreasing Bim protein levels and protecting the cell from apoptosis (28, 37, 45, 46). Even though this mechanism keeps Bim levels low in other cell types such as neurons, we have not observed proteolytic regulation in thymocytes (our unpublished data). Thus, withdrawal of survival signals leads to apoptosis by both transcriptional regulation of Bim, as well as Bim accumulation due to reduced proteolysis. In thymocytes, however, negative selection appears to use a different pathway induced by Ag-receptor cross-linking and requires bim transcription. Our data are in agreement with microarray analysis in NOD mice, which...
Weak TCR signals activate calcineurin, leading to positive selection; strong signals additionally induce Ca\textsuperscript{2+}-dependent PKC activation, Bim, and cell death, which is dominant over positive selection. The PKC response in negative selection may be the result of enhanced Ca\textsuperscript{2+} influx in terms of duration and/or frequency reported to correlate with negatively selecting stimuli (55–57). If the levels of Ca\textsuperscript{2+} required to activate PKC were higher than for calcineurin, this could be a mechanism for discriminating the stronger negatively selecting stimuli from weaker positively selecting signals. Alternatively, conformational changes in the TCR or associated CD3 chains induced by negatively selecting peptides (58) might lead to PKC activation by an unidentified mechanism; this is supported by the finding that conformational changes in specific CD3 chains following interaction with Nck might discriminate positive vs negative selection (59). However, the CD3e-Nck interaction is not required for negative selection (60) and negatively selecting peptides or strong agonists do not appear to induce specific conformational changes in the TCR (61). In addition, the conformational changes induced by weak or strong agonists are indistinguishable and not indicative of signal strength (62). Thus, we favor a model in which the decision point in thymocyte selection is the differential requirement for Ca\textsuperscript{2+}-induced PKC activation vs calcineurin and ERK activation.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


**FIGURE 7.** Model for signal discrimination in negative vs positive selection, with Ca\textsuperscript{2+} as a key player in both pathways. The two pathways diverge downstream of proximal signaling molecules required for both positive and negative selection. A high-intensity signal through the TCR leads to negative selection of thymocytes through Ca\textsuperscript{2+}-dependent PKC activation and subsequent bim transcription. This pathway is dominant over the calcineurin and ERK pathways for positive selection. PKC activation and subsequent Bim induction may require higher Ca\textsuperscript{2+} levels than are necessary for calcineurin activation. Alternatively, negative selection could induce PKC activation through a conformational change at the TCR (faint dashed arrow). Our model allows for both possibilities. A putative transcription factor X is believed to drive bim transcription upon negative selection. In addition to Bim, misshapen/Nck-interacting kinase-related kinase and growth factor receptor-bound protein 2 have been implicated in negative selection (63, 64).

showed defective bim mRNA induction correlating with impaired negative selection in these mice (47, 48).

What factor(s) might regulate bim transcription in thymocytes? One candidate is the forkhead transcription factor FOXO3A, which responds to IL-3 or neurotrophin withdrawal (25, 26). However, our preliminary studies with the Bim promoter, which contains one FOXO3A-binding site and drives expression in HEK-293T cells (49), suggest that it does not respond to negative selection stimuli in thymocytes (our unpublished data). Therefore, in thymocytes, additional transcription factor(s) or regulatory elements (which we call factor X in Fig. 7) may be necessary to induce bim transcription.

In addition to requiring transcription, we found that both Bim induction and thymocyte apoptosis require PKC activity, as demonstrated by three different PKC inhibitors. All three inhibitors (Gö 6976, Gö 6850, and Gö 6983) block at least PKCα and β, conventional isomers that are activated by diacylglycerol and Ca\textsuperscript{2+}. Previous work has indicated that Gö 6850 and Gö 6976 (partially) inhibit thymocyte death induced by anti-CD3 and anti-CD28 Abs (50). Furthermore, experiments with PKC knockout mice have ruled out PKCβ and PKCe in T cell development and signaling (51, 52), leading us to propose that PKCα is the most likely isoform involved in Bim induction. However, we cannot exclude the possibility that other PKC isomers, such as PKCθ (50, 53, 54), are involved in negative selection.

Consistent with the requirement for a Ca\textsuperscript{2+}-dependent PKC isoform, we found that Bim induction is Ca\textsuperscript{2+}-dependent. Moreover, the Ca\textsuperscript{2+}-dependent phosphatase calcineurin, which is necessary for positive selection, is required neither for negative selection (14) nor Bim induction. Thus, Ca\textsuperscript{2+} is common to both pathways and may serve as the final decision point sorting signals to distinct cell fates.
Bim REGULATION IN THYMOCYTE NEGATIVE SELECTION


CORRECTIONS


Figure 8 is incorrect. The corrected figure is shown below.

![Corrected Figure 8](image)


The second author’s middle initial was omitted. The correct name is Robert L. Ferris.


The eighth author’s last name was misspelled. The correct name is Leda Q. Vieira.

The ninth author’s last name was misspelled. The correct name is Hideaki Nakajima.


In Figure 2A, the three left hand dot plot panels from Ly9+/+ cells were mistakenly duplicated in the three right hand dot plot panels of Ly9−/− cells. The numbers in each of the quadrants are correct and the error does not change any interpretation in the article. The corrected figure is shown below.

![Corrected Figure](image-url)


One of the first author’s affiliations was omitted. The corrected list of authors and affiliations is shown below.

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In Figure 1B, the WT Ca flux data line is missing from the Ca flux graph. The corrected figure is shown below.

In **Discussion**, the last reference in the paper is incorrect. The corrected sentence and reference are shown below.

It is known that the cytoplasmic domains of several components of the TCR complex tend to homo-oligomerize at high concentrations (41); perhaps ligand-induced clustering of the TCR drives the cytoplasmic domains of proximal receptors to rearrange, exposing the Nck binding epitope and propelling other signaling cascade processes.


In **Discussion**, in the second sentence of paragraph six, 10S-HDNA should have been 10S-HDHA. The corrected sentence is shown below.

Recently, classic steric analysis of 10S-HDHA and the formation of 10,20-diHDHA and 17-H(p)DHA were reportedly optimized for the plant LOs (49).


The title of the article is incorrect. The corrected title is shown below. The error has been corrected in the online version, which now differs from the print version as originally published.

Thymocyte Negative Selection Is Mediated by Protein Kinase C- and Ca ≈ 2+-Dependent Transcriptional Induction of Bim