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Differential Roles for Bim and Nur77 in Thymocyte Clonal Deletion Induced by Ubiquitous Self-Antigen

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Negative selection, primarily mediated through clonal deletion of self-reactive thymocytes, is critical for establishing self-tolerance and preventing autoimmunity. Recent studies suggest that the molecular mechanisms of negative selection differ depending on the thymic compartment and developmental stage at which thymocytes are deleted. Using the physiological HY^d4 TCR transgenic model of negative selection against ubiquitous self-antigen, we previously found that one of the principal mediators implicated in clonal deletion, Bim, is required for caspase-3 activation but is ultimately dispensable for negative selection. On the basis of these data, we hypothesized that Nur77, another molecule thought to be a key mediator of clonal deletion, could be responsible for Bim-independent deletion. Despite comparable Nur77 induction in thymocytes during negative selection, Bim deficiency resulted in an accumulation of high-affinity–signaled thymocytes as well as impairment in caspase-mediated and caspase-independent cell death. Although these data suggested that Bim may be required for Nur77-mediated cell death, we found that transgenic Nur77 expression was sufficient to induce apoptosis independently of Bim. However, transgenic Nur77-induced apoptosis was significantly inhibited in the context of TCR signaling, suggesting that endogenous Nur77 could be similarly regulated during negative selection. Although Nur77 deficiency alone did not alter positive or negative selection, combined deficiency in Bim and Nur77 impaired clonal deletion efficiency and significantly increased positive selection efficiency. Collectively, these data shed light on the different roles for Bim and Nur77 during ubiquitous Ag-mediated clonal deletion and highlight potential differences from their reported roles in tissue-restricted Ag-mediated clonal deletion.

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Q.N.H. designed and performed research, analyzed data, and wrote the paper. T.A.B. designed and performed research, analyzed data, and wrote the paper.

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caspases 6 and 7 have not been detected in thymic tissue during negative selection (13). Among the proteins induced by high-affinity TCR signaling, the Bcl-2 family member Bim and members of the orphan nuclear receptor Nrl4a family (Nur77 and Nrl-1) have been associated with proapoptotic functions (14). Through interactions with pro- and antiapoptotic Bcl-2 family members at mitochondria, Bim promotes release of cytochrome c and subsequent caspase activation. Unlike the critical role of Bim in TRA-mediated clonal deletion (15, 16), Bim is not required for deletion in the HYcd/ model or other UbA-driven models (12, 15, 17). However, we found that Bim was essential for caspase-3 activation in thymocytes, suggesting that clonal deletion in HYcd/ Bim−/− mice was mediated by a caspase-independent cell death mechanism. Although caspase-independent negative selection has been previously reported in the literature, others have made opposing conclusions as well (18, 19). Caspase-independent cell death in mature T cells also has been observed (20). Little insight into the mechanism of caspase-independent cell death in thymocytes or T cells is currently available.

Nur77 (also known as Nrl4a1) has long been associated with T cell death, with early experiments showing that inhibition of Nur77 in T cell hybridomas reduces TCR-induced death (21, 22). Furthermore, overexpression of Nur77 results in dramatic decreases in thymocyte numbers and an increased frequency of thymocytes with DNA fragmentation (23). Two mechanisms of Nur77-mediated death have suggested: induction of proapoptotic genes through its role as a transcription factor in the nucleus and conversion of Bcl-2 to a proapoptotic form via exposure of its Bcl-2 homology domain 3 (BH3) at mitochondria (24–27). Previous studies have concluded that deficiency in Nur77 alone does not impair UbA-mediated clonal deletion, perhaps due to redundant functions of its family member Nrl-1 (also known as Nrl4a3) (23, 28, 29). However, recent data suggest that Nur77 deficiency is sufficient to impair TRA-mediated clonal deletion (27), arguing against complete redundancy by Nrl-1 in some contexts.

Given that Nur77 has been implicated in thymocyte apoptosis and is also linked to caspase-independent death in other cell types (30–32), we considered Nur77 a strong candidate for mediating clonal deletion in HYcd/ Bim−/− mice. In this present study, we found that Nur77, like Bim, was not required for UbA-mediated deletion. Although Bim deficiency resulted in an accumulation of high-affinity–signaled thymocytes, likely because of inhibition of caspase-mediated and caspase-independent cell death pathways, Nur77 deficiency had no such effects. Although transgenically expressed Nur77 was able to activate caspase-3 in DP thymocytes independently of Bim, TCR-induced Nur77 was unable to activate caspase-3 during Bim-independent negative selection in HYcd/ mice. Because we observed that the provision of TCR signaling impairs the ability of transgenic Nur77 to activate caspase-3, we suggest that the proapoptotic potential of Nur77 is inhibited during TCR-driven negative selection. Although we found the role of Nur77 in clonal deletion to be minimal, our data suggest that Nur77 does play a role in modulating positive and negative selection. The contribution of Nur77 to thymic selection processes was revealed only when Bim was additionally deleted. Collectively, these data highlight the different roles of Bim and Nur77 in UbA-mediated negative selection and suggest key differences from their roles in TRA-mediated negative selection.

Materials and Methods

Mice

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). HYcd/ mice were generated as described previously (5). Bim−/− mice have been described previously (33). Nur77−/Fl mice (23) were a gift from Dr. A. Winoto (University of California, Berkeley, CA). Nur77−/− mice (28) were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-I mice (34) were a gift from Dr. M. McGargill (St. Jude Children’s Research Hospital, Memphis, TN). Mice were bred to other backgrounds to generate the strains used in this study. 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.

Creation of mixed bone marrow chimeras

To study the effects of the Nur77 transgene, wild-type (WT) (CD45.1+) and Nur77−/Fl (CD45.2+) bone marrow (BM) were mixed 50:50 and transferred into lethally irradiated WT (CD45.1+) recipients. To study positive and negative selection, HYcd/ HYcd Nur77−/−, or HYcd/ Bim−/− female (F) BM was mixed with non-TCR transgenic F or male (M) BM at a ratio of 40:60 and transferred into lethally irradiated WT F or M recipients (sex-matched based on WT donor BM). For other chimeras, we transferred HYcd/ F (CD45.1+CD45.2+), HYcd/ Bim−/− Nur77−/− F (CD45.1+CD45.2+), and WT F or M (CD45.1+CD45.2+) in a 20:20:60 ratio into WT (CD45.1+CD45.2+) F or M recipients. T cells were depleted from BM donors by i.p. administration of 100 μg anti-Thy1.2 (clone 30H12) 1 and 2 d prior to BM harvest and additionally on day +1 in HYcd/ WT and Bim−/− Nur77−/− mixed recipients. The resulting chimeras were given water containing neomycin trisulfate (40 mg/l) and polymixin B sulfate (15 mg/l), both purchased from Sigma-Aldrich, for the first 4 wk post-transplantation. Chimeras were harvested 8–12 wk later for analysis.

Abs and flow cytometry

All fluorochrome conjugated and biotinylated Abs were purchased from eBioscience, BD Biosciences, BioLegend, or Invitrogen, except for anti-cleaved caspase 3 (Asp175) from Cell Signaling Technology and anti-Bcl-2 (BH3 domain specific) from Abgent. Annexin V (AV)-allophycocyanin was purchased from eBioscience. Cells were stained in FACS buffer (PBS, 1% FCS, and 0.02% sodium azide (pH 7.2)) with Abs or reagents, according to manufacturer’s instructions. Intracellular staining for cleaved caspase-3, BH3 Bcl-2, and total Bcl-2 was performed using the BD Fix/Perm kit. Staining for BH3 Bcl-2 and total Bcl-2 additionally involved incubation with 5% goat serum as described previously (24). Intracellular staining for Nur77, Helios, and Foxp3 was performed using the eBioscience Foxp3 Staining Buffer Set. Cell events were collected on a BD FACSCanto II and analyzed with FlowJo software (Tree Star).

Statistical analysis

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

Results

Delayed deletion of DP thymocytes in the absence of Bim despite Nur77 expression

Although the low number of Ag-specific (T3.70+ CD8SP thymocytes in HYcd/ Bim−/− M mice indicates that Bim was ultimately not required for negative selection (Fig. 1A) as previously shown (12), the number of T3.70+ DP thymocytes in HYcd/ Bim−/− M mice remained high relative to HYcd/ WT F and M mice. Using the Ikara family member Helios as a marker of high-affinity TCR signaling, an accumulation of high-affinity–signaled cortical (CRR7 CD44CD68m) thymocytes was recently reported in polyclonal Bim−/− mice (35). Likewise, using the HYcd/ model to examine Ag-specific negative selection, we found an accumulation of Helios+ T3.70+ DP thymocytes in HYcd/ Bim−/− M compared with HYcd/ M mice. Specifically, the proportion of T3.70+ DP that expressed Helios to those that did not was ∼2:1 in HYcd/ Bim−/− M mice compared with 1:1 in HYcd/ M mice (Fig. 1C, top panel). Similar results were observed in the CD8SP compartment (Fig. 1C, bottom panel). Furthermore, HYcd/ Bim−/− F mice exhibited an increased frequency of T3.70+ Helios+ DP compared with HYcd/ F mice, suggesting an accumulation of DP thymocytes of other TCR specificities as well. Although caspase-3 activation does not occur during Bim-independent negative selection (12), phosphatidyserine exposure on HYcd/ Bim−/− M DP thymocytes was
evident (Fig. 1D), as indicated by AV binding. These data suggest that Ag-specific thymocytes in HYcd4 Bim<sup>2−/2</sup>M mice are ultimately deleted but in a delayed manner compared with Bim-sufficient counterparts. Because studies suggest that caspase-3 is the predominant executioner caspase in thymocytes (13,36), we hypothesized that clonal deletion in HYcd4 Bim<sup>2−/2</sup>M mice occurs via a caspase-independent cell death mechanism. Given that Nur77 is implicated in UbA-mediated negative selection in classical TCR transgenic models (23, 29) as well as caspase-independent death in other cell types (30–32), we considered Nur77 a strong candidate for mediating Bim-independent clonal deletion in HYcd4 mice. Indeed, we found that Nur77 induction during negative selection was comparable in the presence and absence of Bim (Fig. 1E, 1F). To support these ex vivo data, we used in vitro stimulation with plate-bound anti-CD3ε, anti-CD28, and anti-CD2 (27), with or without the pan-caspase inhibitor z-V AD-FMK, to assess the relative contributions of Bim and Nur77 to TCR-induced DP death. Importantly, in this in vitro model allowed us to synchronize the duration of TCR stimulation that WT and Bim<sup>−/−</sup> thymocytes received. Consistent with in vivo events, Nur77 induction was normal in Bim<sup>−/−</sup> DP (Supplemental Fig. 1A), but TCR-induced caspase-3 activation did not occur in the absence of Bim (Supplemental Fig. 1B). In contrast, Nur77 deficiency had no impact on TCR-induced caspase-3 activation (Supplemental Fig. 1C). Importantly, z-VAD-FMK was still largely able to inhibit caspase-3 activation after 24 h in culture.

Upon validating the in vitro stimulation model, we examined two features of cell death reported to occur in the absence of caspase activity: loss of mitochondrial membrane potential (20) and phosphatidylserine exposure (37), as indicated by the inability of cells to retain tetramethylrhodamine ethyl ester (TMRE) and positive labeling with AV, respectively. Although these processes also occur during caspase-mediated apoptosis, measuring these features in the context of pan-caspase inhibition would permit characterization of TCR-induced caspase-independent death. Inhibition of caspase activity with z-VAD-FMK tended to reduce the frequency of TMRE<sup>lo</sup> and AV<sup>+</sup> WT DP observed after 6 h of stimulation (Supplemental Fig. 2A), but impairment was no longer observed after 24 h (Supplemental Fig. 2B), consistent with the idea that caspase-independent death is delayed. Bim deficiency significantly inhibited loss of mitochondrial membrane potential and phosphatidylserine exposure, even when mediated by caspase-independent mechanisms (compare WT with z-VAD and Bim<sup>−/−</sup> with z-VAD). This highlights a distinction between Bim deficiency and loss of caspase activity and places Bim as a critical regulator of both caspase-mediated and caspase-independent death pathways. However, increases in TMRE<sup>lo</sup> and AV<sup>+</sup> DP were still observed upon TCR stimulation in the absence of Bim (1.4- and 1.5-fold over unstimulated, respectively, after 24 h), supporting the existence of a Bim- and caspase-independent cell death mechanism following TCR stimulation. Thus, these in vitro data strengthen our conclusion that clonal deletion in HYcd4 Bim<sup>−/−</sup>M mice was delayed but ultimately intact.

No apparent conversion of Bcl-2 during negative selection

In contrast to Bim deficiency, we found no impairment in TCR-induced caspase-mediated or caspase-independent death in the
absence of Nur77 at either time point (Supplemental Fig. 2). Furthermore, we know that Nur77 was induced upon TCR stimulation in vivo and in vitro (Fig. 1E, Supplemental Fig. 1A) but could not compensate for Bim deficiency in activating caspase-3 (Supplemental Fig. 1B) (12). Although Nur77 has long been associated with T cell death (21, 22), its mechanism of action remains contentious. One reported mechanism involves TCR-induced nuclear export of Nur77 and subsequent binding of Nur77 to Bcl-2, resulting in exposure of the BH3 domain of Bcl-2 and conversion into a proapoptotic form (24). Importantly, the conclusion that Bcl-2 was converted is dependent on data demonstrating that total Bcl-2 expression was unchanged. Because this was shown in the classical HY TCR transgenic model, we measured Bcl-2 conversion in the HY<sup>cd4</sup> model to assess the possibility that this is the mechanism by which HY<sup>cd4</sup> Bim<sup>−/−</sup> DP thymocytes are eliminated. Although there was a modest increase in binding of the BH3 BH3 domain–specific Ab in HY<sup>cd4</sup> WT and Bim<sup>−/−</sup> M T3.70<sup>+</sup> DP compared with T3.70<sup>+</sup> DP in F mice, we also found increased total Bcl-2 expression (Fig. 2A). This is consistent with previous studies in various models showing increased Bcl-2 mRNA upon high-affinity TCR stimulation (38–40). Therefore, in our hands, BH3 Bcl-2 Ab binding appears to correlate with increased Bcl-2 expression rather than a bona fide “conversion” event. When we calculated BH3 Bcl-2 mean fluorescence intensity (MFI) relative to total Bcl-2 MFI (arbitrary units), there was no significant difference between M and F DP thymocytes, regardless of Bim deficiency (Fig. 2B). In fact, this ratio tended to be lower in M mice compared with F mice, supporting a lack of Bcl-2 conversion during negative selection. These observations are in line with the apparent inability of Nur77 to mediate caspase-3 activation in HY<sup>cd4</sup> Bim<sup>−/−</sup> M DP thymocytes and argue against an important role for Nur77 in mediating Bim-independent clonal deletion against UbA.

**FIGURE 2.** No apparent conversion of Bcl-2 during negative selection in vivo. Abs specific for the BH3 domain of Bcl-2 or another part of Bcl-2 (“total Bcl2”) were used to stain ex vivo thymocytes. (A) For each of the HY<sup>cd4</sup> strains, MFI for BH3 Bcl-2 or total Bcl-2 from T3.70<sup>+</sup> DP is depicted as fold change over MFI from total DP in WT mice. (B) The same data are depicted as a ratio of the MFI for BH3 Bcl-2 to the MFI of total Bcl-2 within each mouse. Data represent a minimum of four replicates in each strain from four independent experiments.

Transgenic Nur77 is sufficient to induce caspase-3 activation

We considered the possibility that Nur77-mediated apoptosis requires Bim—for example, by directly or indirectly inducing Bim expression (27). To characterize the functional relationship between Nur77 and Bim, we examined the effect of transgenic Nur77 expression in Bim-sufficient and Bim-deficient thymocytes. In Nur77-FL mice, expression of the full-length Nur77 transgene is driven by the p56<sup>leuk</sup> promoter at the DN2/DN3 transition (41). By examining the contribution of WT versus Nur77-FL progenitors in mixed BM chimeras, we found that thymocytes first became sensitive to Nur77-mediated death between the DN3 and DN4 stages (Fig. 3A). Consequently, Nur77 overexpression resulted in a severe reduction in the frequency and number of DP and SP thymocytes, which occurred regardless of Bim expression (Fig. 3B, 3C). Direct examination of caspase-3 activation showed that transgenic Nur77 was sufficient to activate caspase-3 in DP thymocytes independently of Bim (Fig. 3D). Therefore, dependency on Bim cannot explain the lack of Nur77-mediated caspase-3 activation during negative selection in HY<sup>cd4</sup> Bim<sup>−/−</sup> M mice.

Proapoptotic function of Nur77 is inhibited on TCR transgenic backgrounds

The ability of transgenic Nur77 to induce robust caspase-3 activation in DP thymocytes is a stark contrast to the inability of endogenous Nur77 to activate caspase-3 during negative selection in HY<sup>cd4</sup> Bim<sup>−/−</sup> M thymocytes. However, a limitation of using Nur77-FL mice is that transgenic Nur77 activity is generally not subject to regulation by TCR signaling, given that expression of the Nur77 transgene precedes that of the TCR at the DP stage and that many DP thymocytes do not experience productive TCR signaling in a polyclonal repertoire. In order to determine whether TCR signaling affects the function of Nur77, we crossed Nur77-FL mice onto the OT-I and HY<sup>cd4</sup> TCR transgenic backgrounds. Like the Nur77 transgene, the OT-I TCR is expressed at the DN stage, whereas the HY TCR is expressed at the DP stage in the HY<sup>cd4</sup> model. On a polyclonal background, transgenic Nur77 expression resulted in severely reduced thymic cellularity (Fig. 4A). Compared with polyclonal Nur77-FL mice, thymic cellularity was modestly but significantly rescued in HY<sup>cd4</sup> Nur77-FL F and M mice and was ~10-fold higher in OT-I Nur77-FL mice. Despite the increase in total thymocyte number on the OT-I background, development into Ag-specific CD8<sup>+</sup>SP thymocytes was still largely inhibited by overexpression of the Nur77 transgene (Fig. 4B). Next, we examined activation of caspase-3 to assess the apoptotic function of Nur77 in precursor DP thymocytes that had received a TCR stimulus. Consistent with increased cellularity, we found decreased frequencies of cleaved caspase-3<sup>+</sup> DP in TCR transgenic Nur77-FL mice compared with polyclonal Nur77-FL mice (Fig. 4C). The decrease in Nur77-mediated death was not due to reduced Nur77 transgene expression because the amount of Nur77 expressed was similar or higher in TCR transgenic compared with polyclonal Nur77-FL DP, whereas caspase-3 activation was disproportionally low. Specifically, the frequency of cleaved caspase-3<sup>+</sup> DP from all TCR transgenic Nur77-FL strains was significantly lower compared with polyclonal Nur77-FL mice, and this could not be attributed to decreased Nur77 transgene expression. This observation was most dramatic in OT-I Nur77-FL mice, consistent with the idea that earlier TCR expression, in particular at a time in development similar to when Nur77-FL was expressed, resulted in increased protection from apoptosis and rescue of thymocyte numbers. Furthermore, among polyclonal Nur77-FL DP thymocytes, those that received TCR stimulation (CD69<sup>+</sup>) exhibited a significantly lower frequency of caspase-3
activation compared with CD69− Nur77-FL DP despite similar Nur77 expression (Fig. 4D). These data suggest that TCR signaling during negative selection inhibits the proapoptotic function of Nur77 either directly or indirectly.

Nur77 is not required for, but can modulate, negative selection against UbA

Several lines of evidence thus far suggest that Nur77 plays a minimal role in clonal deletion against UbA. To directly assess the contribution of Nur77 to clonal deletion, we generated mixed BM chimeras in which HYcd4 F or HYcd4 Nur77−/− F BM was mixed with WT F or M BM and transplanted into irradiated WT F or M recipients to model positive and negative selection, respectively (6). We mixed HYcd4 F or HYcd4 Nur77−/− F BM with WT

FIGURE 3. Transgenic Nur77 expression induces thymocyte death independent of Bim. (A) CD45.1+ WT and CD45.2+ Nur77 transgenic (Nur77-FL) BM were mixed at a ratio of 50:50 and transferred into lethally irradiated WT recipients. The contribution of WT versus Nur77-FL BM (referred to as percent chimerism) in each T cell subset was calculated by dividing the ratio of %CD45.1+ cells to %CD45.2+ cells by the %CD45.1+/CD45.2− ratio from the CD19+ B cell reference population. Chimeras were generated from two independent sets of donors in two independent experiments. (B and C) Native Nur77-FL mice on Bim+/+ or Bim−/− backgrounds and their non–Nur77-transgenic counterparts were phenotyped ex vivo by flow cytometry. The frequency of each thymocyte subset was used to calculate absolute cell numbers. (D) Frequency of DP thymocytes with cleaved caspase-3 from each strain. For nonchimeric mice, data were obtained from a minimum of three mice per strain from three independent experiments and is depicted as mean ± SD. ***p < 0.001. n.s., not significant.

FIGURE 4. Proapoptotic ability of transgenic Nur77 is reduced on TCR transgenic backgrounds. (A) Thymic cellularity of Nur77-transgenic and non–Nur77-transgenic mice from various backgrounds. Data depict a minimum of five mice of each strain from at least two independent experiments, shown as mean ± SD. *p < 0.05, ***p < 0.001. (B) Representative phenotypes of OT-I and OT-I Nur77-FL thymi. (C) The frequency of cleaved caspase-3 in total DP (polyclonal), T3.70+ DP (HYcd4), and Vα2+ DP (OT-I) was overlaid on Nur77 MFI from the same populations. Nur77 MFI from Nur77-FL is not significantly different from any TCR transgenic Nur77-FL strains. Frequency of cleaved caspase-3 is significantly different between Nur77-FL and TCR transgenic Nur77-FL strains: p < 0.01 for HYcd4 F Nur77-FL and OT-I Nur77-FL, p < 0.001 for HYcd4 M Nur77-FL. Data depict a minimum of four mice of each strain from at least two independent experiments. (D) The frequency of caspase-3 activation in CD69+ and CD69− DP from polyclonal mice was overlaid on Nur77 MFI from the same populations. Nur77 MFI is not significantly different from any TCR transgenic Nur77-FL strains: p < 0.001 between CD69+ and CD69− Nur77-FL DP.
BM at a ratio of 40:60 to reduce the frequency of TCR transgenic thymocytes, thereby providing a more physiological selection environment. Both HY<sup>cd4</sup> → F and HY<sup>cd4 Nur77<sup>−/−</sup></sup> → F chimeras showed robust generation of T3.70° CD8SP thymocytes (Fig. 5A), the vast majority of which were mature (CD24<sup>lo</sup>) (Fig. 5B). In contrast, both HY<sup>cd4</sup> → M and HY<sup>cd4 Nur77<sup>−/−</sup></sup> → M chimeras largely lacked T3.70° CD8SP thymocytes. Among the very few T3.70° CD8SP that were generated in M chimeras, a smaller proportion exhibited the mature CD24<sup>lo</sup> phenotype compared with those in F recipients (Fig. 5B). Across all experiments, the frequency of CD24<sup>lo</sup> cells among T3.70° CD8SP was comparable, if not lower, in HY<sup>cd4 Nur77<sup>−/−</sup></sup> → M compared with HY<sup>cd4</sup> → M chimeras. When we accounted for precursor frequency by comparing the number of T3.70° DP to T3.70° CD8SP, both within each chimera (Fig. 5C) as well as across all experiments (Fig. 5D), we found no significant difference in negative selection efficiency in the absence of Nur77. In further support of Nur77 deficiency having little impact on clonal deletion, there was no impairment in caspase-3 activation (Fig. 5E), consistent with our in vitro stimulation data (Supplemental Fig. 1C). With respect to positive selection, there was likewise no significant difference associated with Nur77 deficiency. Rather, positive selection efficiency was affected by the frequency of Ag-specific precursors. Both HY<sup>cd4</sup> → F and HY<sup>cd4 Nur77<sup>−/−</sup></sup> → F chimera sets contained chimeras that exhibited reduced establishment of donor BM and corresponding enhanced positive selection, as represented by a higher frequency of CD8SP compared with DP (Fig. 5C). This may be because of decreased competition for positive selection ligands among precursors. Overall, the lack of impact on positive selection and UbA-mediated negative selection in Nur77-deficient mice is consistent with conclusions made from older TCR transgenic models (23, 28, 29).

**FIGURE 5.** Nur77 is not required for positive or negative selection. HY<sup>cd4</sup> F or HY<sup>cd4 Nur77<sup>−/−</sup></sup> → F BM plus WT BM was transplanted into WT F or M recipients. The resulting chimeras are labeled WT → F, KO → F, WT → M, and KO → M, where WT or KO refers to Nur77 status of HY<sup>cd4</sup> donor BM. Thymocyte development in the chimeras was assessed by CD8 by CD4 profiles of T3.70° thymocytes (A) and CD24 expression among T3.70° CD8SP thymocytes (B). The absolute numbers of T3.70° DP and T3.70° CD8SP were calculated and depicted as before-and-after (C) and scatter dot (D) plots. (E) The frequency of T3.70° DP thymocytes exhibiting cleaved caspase-3 (C3) was quantified. (F) Representative expression of Helios and PD-1 in T3.70° DP thymocytes. (G) Helios MFI among Helios<sup>+</sup> T3.70° DP thymocytes was quantified over all experiments. Representative CD69 expression (H) and frequency (I) of CD69 induction among T3.70° DP thymocytes across all experiments. Data depict a minimum of five chimeras of each type derived from at least three independent BM donors, with the exception of WT → M chimeras of which there were four generated from two independent BM donors. Data are depicted as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant.
Although Nur77 was not required for clonal deletion, we observed a number of changes in the high-affinity TCR signaling phenotype in the absence of Nur77. Helios induction has been shown to specifically occur upon high-affinity TCR signaling (35) (Fig. 1C), and we have previously demonstrated that PD-1 is induced during negative selection in the HYcd4 model (38). Helios and PD-1 induction were evident in HYcd4 and HYcd4 Nur77+/− → M T3.70+ DP thymocytes, although the frequency of Helios+ cells and the MFI of Helios were lower in the absence of Nur77 (Fig. 5F, 5G). In contrast, the frequency and amount of CD69 expression were increased in HYcd4 Nur77+/− → M compared with HYcd4 → M T3.70+ DP (Fig. 5H, 5I). Therefore, although Nur77 deficiency has minimal impact on Uba-mediated clonal deletion, Nur77 may function to alter expression of proteins induced by TCR signaling. Importantly, the altered TCR signaling phenotype observed in HYcd4 Nur77+/− → M chimeras is distinct from the patterns in HYcd4 Bim+/− M. Specifically, in the absence of Bim, we observed an increased frequency of Helios+T3.70+ DP (Fig. 1C) but no difference in Helios MFI. Furthermore, unlike negative selection in the absence of Nur77, the frequency of CD69+T3.70+ DP was unaffected, whereas the amount of CD69 was decreased in HYcd4 Bim+/− M mice (Supplemental Fig. 3).

Combined deficiency in Bim and Nur77 results in impaired negative selection and enhanced positive selection

Although deletion appeared to be delayed (Fig. 1) because of impaired cell death (Supplemental Figs. 1, 2) (12), Bim deficiency did not ultimately impair clonal deletion against Uba. We also noted that Nur77 was not required for clonal deletion and Nur77 deficiency did not result in any apparent delay in deletion as seen in Bim-deficient mice (Fig. 5, Supplemental Figs. 1, 2). However, given the major role of Bim in mediating thymocyte apoptosis, Bim expression during negative selection in HYcd4 Nur77+/− mice may have masked a contribution for Nur77 in clonal deletion. To examine this possibility, we transferred a mix of CD45 congenic BM from HYcd4 F, HYcd4 Bim+/− Nur77+/− (double knockout [DKO]) F, and WT F or M donors in a 20:20:60 ratio into WT F or M recipients. With respect to the frequency of CD8SP among T3.70+ thymocytes, there was some variation among the four M recipients we analyzed; two of the chimeras are depicted in Fig. 6A to demonstrate this variability. However, in three of four M recipients, the frequency of T3.70+ CD8SP of HYcd4 DKO origin was higher than those of HYcd4 WT origin. This suggests that combined deficiency in Bim and Nur77 resulted in impaired clonal deletion. This conclusion is further evidenced by examining the maturity of the T3.70+ CD8SP thymocytes in particular. By calculating the ratio of the number of T3.70+ DP to T3.70+CD24hi CD8SP, we found that relative clonal deletion efficiency between thymocytes of DKO and WT origin was similar in all four M chimeras, with a 3- to 4-fold impairment associated with the absence of Bim and Nur77 (Fig. 6B). In fact, even in M chimera number 1, which contained a low frequency of T3.70+ CD8SP of HYcd4 DKO origin (Fig. 6A), those T3.70+ CD8SP clearly show increased CD24 downregulation compared with WT-derived counterparts (Fig. 6C). We found that increased CD24 downregulation on WT-derived T3.70+ CD8SP correlated with an increased frequency of T3.70+ CD8SP (compare M chimera number 1 to M chimera number 2), whereas DKO-derived T3.70+ CD8SP were uniformly CD24hi (Fig. 6C). These trends were also true for the other two chimeras that are not depicted. Importantly, in three of four HYcd4 WT + DKO mixed M chimeras, the frequency of HYcd4 WT–derived T3.70+ CD8SP was considerably higher than what we typically observe in HYcd4 M, HYcd4 Bim+/−, or HYcd4 Nur77+/− M mice (compare M number 2 in Fig. 6A to Fig. 5A and Ref. 12). This raises the possibility of thymocyte-extrinsics effects on clonal deletion when DKO hematopoietic cells are present. We also examined the proportion of T3.70+ DP expressing Helios and found that it was higher among cells of HYcd4 DKO origin compared with HYcd4 WT origin (Fig. 6D).

This is reminiscent of the phenotype in HYcd4 Bim+/− M compared with HYcd4 M mice (Fig. 1C), suggesting delayed deletion of high-affinity–signaled thymocytes. The less dramatic difference between WT and DKO in these chimeras may be because of lower T3.70+ frequency and more efficient selection than in native mice; nevertheless, the same trend was apparent. This altered frequency of Helios+T3.70+ DP was independent of the frequency of T3.70+ CD8SP (Fig. 6A, 6D). We then examined the expression of Helios in WT and DKO-derived T3.70+ CD8SP thymocytes. Similar to what was observed in Fig. 1C, when clonal deletion was efficient (i.e., M chimera number 1), about half of the few remaining WT T3.70+ CD8SP were Helios+ (Fig. 6E). As with CD24 downregulation, WT-derived T3.70+ CD8SP showed increased downregulation of Helios in chimeras with a higher frequency of T3.70+ CD8SP (Fig. 6E). Compared with WT-derived T3.70+ CD8SP, the proportion of DKO-derived T3.70+ CD8SP expressing Helios was consistently lower. Along with increased CD24 downregulation, this suggests that DKO-derived T3.70+ CD8SP thymocytes are of a more mature stage. Similar to WT-derived cells, downregulation of Helios on DKO-derived T3.70+ CD8SP correlated with increased frequencies of T3.70+ CD8SP (Fig. 6E). In summary, although there is evidence for an intrinsic impairment in clonal deletion among DKO thymocytes, the atypical increase in T3.70+ CD8SP of WT origin in these mixed chimeras, as well as variations in their phenotype between chimeras, suggests that other factors resulting from Bim and Nur77 deficiency also affected thymocyte selection.

Last, we examined positive selection efficiency in the absence of Bim, Nur77, or both proteins using F recipients. HYcd4 Bim+/− → F chimeras generally showed an increased frequency of T3.70+ CD8SP thymocytes compared with HYcd4 WT → F chimeras (Fig. 6F), recapitulating what we see in native HYcd4 Bim+/− F mice (12). In support of this, the ratio of T3.70+ DP to T3.70+ CD8SP was mildly, although not significantly, lower in the absence of Bim (Fig. 6G). As shown in Fig. 5, Nur77 deficiency alone did not appreciably alter the frequency of T3.70+ CD8SP thymocytes or the ratio of T3.70+ DP to T3.70+ CD8SP. In comparison with either single KO, we observed a dramatic increase in the frequency of HYcd4 DKO T3.70+ CD8SP as well as a significantly higher number of T3.70+ CD8SP relative to T3.70+ DP precursors (Fig. 6F, 6G). These data suggest that Nur77 normally has an inhibitory effect on positive selection, albeit a minor one because it was only revealed when Bim was additionally deleted. In light of this, the increased frequency of HYcd4 DKO T3.70+ CD8SP in M chimeras also could be partly because of an increase in positive selection as well as a modest impairment in clonal deletion. Taken together, our results demonstrate that although Nur77 deficiency alone has minimal effects on thymocyte selection, a role for Nur77 in modulating negative and positive selection can be revealed when both Bim and Nur77 are absent.

Discussion

In this study, we examined the contribution of Bim and Nur77 to thymocyte selection. Because of their high level of induction during negative selection as well as their reported roles in apoptosis, Bim and Nur77 were thought to be critical mediators of clonal deletion (14). As previously reported, HYcd4 Bim+/− M mice ultimately lack Ag-specific CD8SP thymocytes despite abrogation of caspase-3 activation (12). It has been shown that impaired clonal
deletion because of lack of CD28/B7 costimulation can result in diversion of self-reactive clones to the DN subset, which subsequently become precursors for CD8αα intraepithelial lymphocytes (42). Although polyclonal Bim−/− mice have an increased frequency of TCRβ+ DN thymocytes (43), clonal diversion does not appear to be a dominant fate for self-reactive thymocytes in HYcd4 Bim−/− M mice because HYcd4 Bim−/− M mice lack a substantial T3.70+ DN compartment. Furthermore, T3.70+ DP thymocytes exhibit phosphatidylserine exposure, suggesting that death remains the fate of self-reactive clones in the absence of Bim.

Given the importance of caspase-3 in thymocyte apoptosis, we favor elimination of self-reactive clones by a caspase-independent mechanism in HYcd4 Bim−/− M mice. Although we cannot rule out activation of executioner caspases other than caspase-3 in vivo, others have reported no activation of caspase-6 or caspase-7 upon antigenic stimulation of thymocytes (13). Furthermore, our in vitro data indicate that caspase-independent death can occur following TCR stimulation. Although there is precedent for caspase-independent negative selection in vivo (18), the matter has been contentious (19), perhaps owing to differences in model systems. In the HYcd4 model where timing of TCR expression and selection are physiological, we conclude that Bim deficiency hinders UbA-mediated deletion but is not ultimately required. This is supported by an increased frequency of high-affinity–signaled T3.70+ DP thymocytes in HYcd4 Bim−/− M mice, similar to the accumulation of high-affinity–signaled cortical thymocytes in polyclonal Bim−/− mice (35, 44).

By using z-VD-FMK to block the activity of all caspases in vitro, we found that Bim deficiency still impaired mitochondrial

**FIGURE 6.** Nur77 modulates positive and negative selection. CD45 congenic HYcd4 F and HYcd4 Bim−/−Nur77−/− (DKO) F were mixed with WT BM in a ratio of 20:20:60 and transferred into lethally irradiated F or M recipients. (A) Thymic profile of T3.70+ populations from each donor compartment within two M chimeras. (B) The ratio of the number of T3.70+ DP thymocytes to the number of T3.70+CD24+ CD8SP thymocytes of HYcd4 WT or HYcd4 DKO origin in each M chimera. The accompanying numbers indicate the fold change in this ratio for HYcd4 WT/HYcd4 DKO. (C) Expression of CD24 and T3.70 among total CD8SP thymocytes from the two M chimeras depicted in (A). (D and E) Expression of Helios and T3.70 among total DP and total CD8SP of HYcd4 WT versus HYcd4 DKO origin from the two M chimeras depicted in (A). (F and G) HYcd4 WT and Bim−/− chimeras were generated in the same manner as HYcd4 Nur77−/− chimeras in Fig. 5. Representative profiles of T3.70+ thymocytes from each chimera strain (F) and ratio of the number of T3.70+ DP to T3.70+ CD8SP across all chimeras (G) are depicted. For HYcd4 WT + DKO → F and M chimeras, data depict four chimeras of each type, derived from one BM donor. For single KO chimeras, data depict a minimum of five chimeras of each type derived from a minimum of two independent BM donors. Data are depicted as mean ± SD. *p < 0.05.
The Journal of Immunology

almost never observe an increase in T3.70+ CD8SP to this degree. This is postulated to lead to release of apoptosis inducing factor, a known mediator of caspase-independent death. To our knowledge, the role of Bim in regulating mitochondrial membrane potential, particularly in the context of TCR-induced apoptosis, has not been previously examined. Although not completely understood, mitochondrial depolarization and cytochrome c release are not necessarily concomitant or mechanistically linked. This would be consistent with our observation of a low level of TCR-induced mitochondrial depolarization despite abrogation of caspase-3 activation in Bim−/− DP thymocytes. Our conclusion that Bim is a key regulator of caspase-mediated and caspase-independent thymocyte apoptosis is consistent with the role of the Bcl-2 family as gatekeepers of mitochondrial integrity.

In contrast to Bim deficiency, Nur77 deficiency did not impair deletion of DP thymocytes in vivo or upon TCR stimulation in vitro. Despite being implicated in caspase-independent cell death (30–32), we did not find any inhibition of cell death in Nur77−/− DP thymocytes even when caspase activity was abrogated with z-VAD-FMK. That Nur77 was dispensable for UbA-mediated clonal deletion is an interesting difference from a model of TRA-mediated negative selection in which Nur77 deficiency was sufficient to impair deletion of CD4SP thymocytes (27). Bim was identified as a Nur77 transcriptional target in that model, which likely explains why no further impairment in deletion was observed in a DKO. In contrast to TRA-mediated deletion of OT-II thymocytes, our data suggest that Bim has a greater role than Nur77 in the context of UbA-mediated clonal deletion, implying that Nur77 would be but one regulator of Bim (if at all). A different role for Nur77 in DP versus SP thymocytes is not implausible. In support of this, phosphorylation and subcellular localization of Nur77 have been shown to be different between stimulated DP thymocytes and mature CD8+ T cells (48). Likewise, in contrast to its lesser impact on UbA-mediated clonal deletion, Bim deficiency is known to block TRA-mediated deletion of CD8SP thymocytes (15, 16). Given these context-dependent differences in the mechanism of clonal deletion, it would be of interest to assess the role of Nur77 in TRA-mediated deletion of CD8SP thymocytes as well.

Although Nur77 deficiency alone did not alter positive or negative selection efficiency, combined Bim and Nur77 deficiency appeared to exacerbate effects on thymocyte selection seen in HYcd4 Bim-deficient mice. For one, positive selection of thymocytes of HYcd4 DKO origin was dramatically increased compared with that of HY−/− Bim−/− origin. In M chimeras, we also observed an increase in Ag-specific CD8SP in the absence of Bim and Nur77. Because T3.70 CD8SP of HYcd4 WT origin, in addition to those of HYcd4 DKO origin, tended to be more prevalent than in native HY−/− M mice, it appears that thymocyte-extrinsic factors were partly responsible for altering thymocyte development. This may be a result of combined Bim and Nur77 deficiency affecting other immune cell populations because we almost never observe an increase in T3.70 CD8SP to this degree in native HYcd4 WT, HYcd4 Bim−/−, or HYcd4 Nur77−/− M mice. For example, Nur77−/− macrophages exhibit a more inflammatory phenotype than WT macrophages upon phagocytosis of apoptotic thymocytes (49) or activation with LPS (50). Furthermore, Nur77−/− mice lack a population of monocytes thought to be involved in resolution of inflammation (51). The increase in Ag-specific CD8SP thymocytes in HYcd4 WT + DKO mixed chimeras is also partly due to thymocyte-intrinsic effects of Bim and Nur77 deficiency because T3.70 CD8SP of DKO origin tended to be more prevalent than WT counterparts within a given M chimera. This may indicate that Nur77 has a minor role in clonal deletion that was only uncovered when Bim was additionally deleted. Alternatively, the increased frequency of DKO-derived CD8SP thymocytes could be because of other thymocyte-intrinsic changes caused by Bim and Nur77 deficiency such as enhanced positive selection. The key issue is whether Nur77 is contributing to active cell death programs induced by high-affinity TCR signaling and/or other aspects of thymocyte development following low-affinity TCR signaling. Although we cannot rule out either scenario, our results suggest that Nur77 may not play as large a role in clonal deletion as previously thought, at least in the context of UbA-mediated deletion of DP thymocytes. For example, we found that Nur77 is induced upon high-affinity TCR signaling but cannot activate caspase-3 in the absence of Bim. This is not due to a requirement for Bim in Nur77-mediated apoptosis because Bim expression is not required for transgenic Nur77-mediated caspase-3 activation. Rather, TCR signaling appears to inhibit the apoptotic potential of Nur77.

Regulation of Nur77 by TCR signaling has been established in the literature. For example, studies have reported TCR-induced translocation of Nur77 to mitochondria, although whether this occurs in thymocytes is contentious (24, 25, 48). Nuclear export of Nur77 could be a protective or prodeath mechanism because Nur77 has been proposed to mediate apoptosis via its transcriptional activities in the nucleus or through protein interactions at mitochondria (24–26, 48). Our findings do not support the proposed conversion of Bcl-2 into a proapoptotic form by Nur77. On the contrary, we found that TCR signaling limits the apoptotic potential of Nur77, even though Bcl-2 is upregulated upon stimulation (38). Furthermore, transgenic Bcl-2 expression impairs deletion in classical TCR transgenic mice (52) and non-TCR transgenic mice (35), which is not congruent with conversion by Nur77. Last, despite an increase in the total amount of BH3 domain exposure, there is no caspase-3 activation during negative selection in HYcd4 Bim−/− M mice (12). With respect to the localization of Nur77, Fassett et al. (27) have identified a host of transgenic mice (35), which is not congruent with conversion by Nur77. Last, despite an increase in the total amount of BH3 domain exposure, there is no caspase-3 activation during negative selection in HYcd4 Bim−/− M mice (12). With respect to the localization of Nur77, Fassett et al. (27) have identified a host of TCR-induced genes whose transcription is regulated by Nur77. Furthermore, it was recently demonstrated that Nur77 and other Nur77 family members can directly transactivate Foxp3 expression (53). These findings are difficult to reconcile with complete export of Nur77 from the nucleus. Our results do not rule out nuclear export of Nur77 but suggest that wherever it is localized, its proapoptotic role is limited during negative selection against UbA.

We propose that another potential role for Nur77 during thymocyte selection is modulation of TCR signaling. For example, the enhanced positive selection of HYcd4 DKO F thymocytes over HYcd4 Bim−/− F thymocytes that we observed may be indicative of increased TCR signaling strength and consequent rescue of DP thymocytes from death by neglect. Although Nur77 deficiency alone does not enhance positive selection, altered TCR signaling in the absence of Nur77 in combination with extended thymocyte lifespan because of Bim deficiency may facilitate the accumulation of sustained TCR signaling required for positive selection (54). In support of increased TCR signaling in the absence of Nur77, we found an elevated frequency and, to a lesser extent, amount of CD69 expression on HYcd4 Nur77−/− DP thymocytes compared with HYcd4 WT. Other indications of modulated TCR signaling include differences in Helios and PD-1 expression in HYcd4 Nur77−/− M DP thymocytes. Notably, expression of these

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TCR signaling markers were different in HY\textsuperscript{cd4} Nur77\textsuperscript{-/-} M compared with HY\textsuperscript{cd4} Bim\textsuperscript{-/-} M mice—the latter exhibiting an increased frequency of Helios\textsuperscript{+} and PD-1\textsuperscript{+} DP (12) that is consistent with delayed deletion. It is interesting to speculate that a lowered TCR signaling threshold could also manifest as increased agonist selection of T\textsubscript{Eff} that is observed in Nur77\textsuperscript{-/-} mice (27). In an apparent paradox, combined deficiency of Nur77 and Nor-1 results in limited development of T\textsubscript{Eff} because of their role in transactivation of the Foxp3 promoter (53). These observations can be reconciled if Nor-1 is still inducing Foxp3 expression in the context of stronger TCR signaling in Nur77\textsuperscript{-/-} thymocytes. Collectively, our study reveals new insights on the role of Bim and Nur77 in thymocyte selection, contributing to a picture that is more complex than just a matter of life versus death.

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Disclosures

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References


