Mcl-1 promotes survival of thymocytes by inhibition of Bak in a pathway separate from Bcl-2

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Abstract

The anti-apoptotic proteins Mcl-1 and Bcl-2 have been shown to be critical in T cell development and homeostasis, but the precise mechanism by which these proteins function in T cells and other cells of the body is unclear. Potential mechanisms have allowed both for overlapping and unique roles for these proteins due to their abilities to bind different pro-apoptotic Bcl-2 family members, but it is unclear which of these mechanisms are important in an *in vivo* context. By generation of various genetic mouse models, we have found that Mcl-1 deficient thymocytes die largely by a Bak-specific mechanism. *In vivo* deletion of Bak rescued the survival and developmental blocks of Mcl-1-deficient thymocytes at the double negative and single positive stages. Transgenic over-expression of Bcl-2 and *in vivo* deletion of Bax or Bim were unable to rescue Mcl-1-deficient thymocytes. Thus, Mcl-1 functions in a unique pathway from Bcl-2 in T lymphocytes, likely due to its specific ability to bind and sequester pro-apoptotic Bak. Together, these data provide an *in vivo* model for Mcl-1 activity and give us a greater understanding of the pathways that promote thymocyte survival.

Keywords

Mcl-1; Bcl-2; Bak; thymocyte survival

Introduction

T lymphocytes are a dynamic population that depends on careful regulation of apoptotic pathways for both its establishment and maintenance. In the thymus, hematopoietic precursors undergo a multi-stage developmental program, in which cells progress from the immature $CD4^-CD8^-$ double negative (DN) stage, through the $CD4^+CD8^+$ double positive (DP) stage, to $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) stages before exiting the thymus to the peripheral T cell pool. During this process, thymocytes undergo multiple selection steps to ensure a functional, non-self-reactive T cell receptor (TCR) is expressed on the cell surface. Classical apoptotic pathways play essential roles in thymic selection and peripheral responses, with critical implications for autoimmunity and homeostasis.[1]

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Bcl-2 family proteins, mediators of the intrinsic pathway of apoptosis, are defined by sharing one or more of four Bcl-2 homology (BH) domains and can be subdivided into three subgroups by structure and function: multidomain anti-apoptotic proteins, multidomain pro-apoptotic proteins, and pro-apoptotic BH3-only proteins.[2] The anti-apoptotic proteins, Bcl-2, Mcl-1, Bcl-x_L, Bcl-w, and A1, share multiple BH regions and inhibit pro-apoptotic family members via interactions between BH domains. Pro-apoptotic Bak and Bax, which contain BH1-3 regions, form homo-oligomeric pores in the mitochondrial membrane and are critical for release of cytochrome c and downstream events such as activation of Apaf-1 and effector caspases.[3, 4] BH3-only proteins act as sensors of apoptotic stimuli and induce apoptosis either by directly activating Bak and Bax ("activators") or by binding the anti-apoptotic proteins ("sensitizers"), although the extent to which they play these different roles *in vivo* is still unclear.[5–11]

Genetic mouse models have suggested distinct roles for the anti-apoptotic proteins in T lymphocytes. Bcl-2$^{-/-}$ thymocyte development is normal at early post-natal timepoints, but T cells in both the periphery and thymus are lost by 4 weeks of age, likely due to differences in Bcl-2-dependency between fetal liver and bone marrow derived hematopoietic progenitors.[12–15] Bcl-x, however, is not required for T cell development, effector function or memory, although Bcl-x$^{-/-}$ DP thymocytes have reduced survival under some conditions.[16–18] Mcl-1 is required for thymocyte progression past the DN stage and is also required for SP cells to reach maturation and for survival of naïve and activated peripheral T cells.[19, 20]

Mcl-1, Bcl-2 and Bcl-x_L are each expressed in a unique pattern in thymocytes. Mcl-1 is expressed in all thymocyte subsets.[19, 20] Bcl-2 is expressed in DN and SP stages but down-regulated in DP until positive selection.[21–23] Conversely, Bcl-x_L, the predominant splice product of the Bcl-x gene in thymocytes, exhibits high expression at DP but low expression at DN and SP.[16, 24] Given that Mcl-1 and Bcl-2 are expressed in DN and SP thymocytes, [20–23] it was intriguing that loss of Mcl-1 alone did not allow survival past these stages.[19, 20] Since Bcl-2$^{-/-}$ thymocytes display a similar defect,[12–15] it appears that both Bcl-2 and Mcl-1 are singly required for survival past the DN stage. This raised the question of whether Bcl-2 and Mcl-1 play distinct molecular roles in thymocytes. In order to assess this, we have generated genetic models to dissect the mechanisms of Mcl-1 activity. These studies will not only yield insight into mechanisms of thymocyte survival, but also have implications for understanding how the different types of Bcl-2 family members regulate apoptosis *in vivo*.

Results

Genetic models for dissecting the roles of Mcl-1

We previously developed an Mcl-1 deletion model in which exon 1 of the Mcl-1 gene was flanked by loxP recombination sites to create "floxed" mice (Mcl-1$^{f/f}$).[25] To achieve deletion at different stages in thymic development, these mice were crossed to mice in which Cre recombinase is expressed under the proximal Lck promoter, which allows efficient deletion by the double negative 3 (DN3) stage, or mice expressing Cre under the CD4 promoter/enhancer, which deletes by DP.[20, 26] Mcl-1-deficient lines were crossed to Bcl-2-

transgenic (Bcl-2[tg]),27 Bak^{−/−},28 Bax^{−/−},29 or Bim^{−/−} 30 mice to build a genetic pathway for Mcl-1 activity and determine the *in vivo* interactions of these proteins in thymocytes.

Over expression of Bcl-2 cannot rescue Mcl-1-deficient thymocytes

Previous observations from Mcl-1-deficient models implied that Mcl-1 and Bcl-2 play distinct roles at the DN stage. Alternatively, the possibility remained that endogenous levels of Bcl-2 were insufficient to rescue the loss of Mcl-1. To investigate these possibilities, we crossed Mcl-1[f/f]Cre mice to mice that over-express Bcl-2 as a transgene under the H-2K[b] promoter, yielding high expression in all hematopoietic cells.27 Flow cytometry confirmed expression of the Bcl-2 transgene in DN, DP and SP thymocytes in our system (data not shown).

In these and previous experiments, Mcl-1[f/f]LckCre mice exhibited a dramatic reduction (~90%) in total thymocyte number due to a block at DN, specifically, an accumulation at the CD44^{+}CD25^{+} DN2 and CD44^{−}CD25^{+} DN3 stages (Figure 1).20 A similarly profound loss in thymocyte number was observed in Mcl-1[f/f]LckCreBcl-2[tg] mice compared to both Bcl-2[tg] and wild type controls (Figure 1A). In spite of increased thymic cellularity in Bcl-2[tg] control mice, Mcl-1[f/f]LckCreBcl-2[tg] total cell numbers were not significantly different from those of Mcl-1[f/f]LckCre mice (Figure 1A). As previously observed, the thymic profile of Mcl-1[f/f]LckCre mice showed a skewing toward the DN compartment at the expense of DP (Figure 1B, upper panel). Although the Bcl-2 transgene alone increased DN percentage, the block at DN is also observable in Mcl-1[f/f]LckCreBcl-2[tg] mice (Figure 1B, upper panel). Additionally, the block at the DN2-DN3 stages is still observed in the Mcl-1[f/f]LckCreBcl-2[tg] mice (Figure 1B).

As different thymic subsets may have different apoptotic mechanisms, we examined whether Bcl-2 over-expression could rescue survival of Mcl-1-deficient SP thymocytes and peripheral T cells. We previously showed that Mcl-1[f/f]CD4Cre mice have normal cellularity in thymus and spleen, but a marked reduction in mature SP cells as defined by expression of TCRβ and the markers Qa2 and CD69 (mature SP cells = TCRβ^{+}Qa2^{+}CD69^{lo}) as well as both CD4^{+} and CD8^{+} T cells in the periphery.20 Similarly, Mcl-1[f/f]CD4CreBcl-2[tg] mice exhibited a loss of T cells in the spleen, although the percentage was consistently higher than Mcl-1[f/f]CD4Cre (Figure 1C-i). Like Mcl-1[f/f]CD4Cre mice, the thymic profile of Mcl-1[f/f]CD4CreBcl-2[tg] mice showed a reduction in the percentage of CD4^{+} and CD8^{+} SP cells (Figure 1C-ii) and in the percentage of TCRβ^{+} cells in the SP compartments (Figure 1C-iii [CD4^{+}SP] and –iv [CD8^{+}SP]). However, within the CD4^{+}TCRβ^{+} and CD8^{+}TCRβ^{+} SP compartments, Mcl-1[f/f]CD4CreBcl-2[tg] had higher proportions in the mature gate than Mcl-1[f/f]CD4Cre (Figure 1C-iii and -iv). Cell numbers of mature CD4^{+} and CD8^{+} SP cells in Mcl-1[f/f]CD4CreBcl-2[tg] mice were also increased, but were still significantly lower than the Bcl-2[tg] controls (Figure 1D). The same was true of both CD4^{+} and CD8^{+} T cells in the spleen (Figure 1E).

Because Bcl-2[tg] mice have elevated total cell numbers in both thymus and spleen, we compared the percentages of mature SP cells in the thymus and T cells in the spleen. For CD4^{+} SP cells, there was an increase in the mean percentage of mature SP cells in the thymus of Mcl-1[f/f]CD4CreBcl-2[tg] mice from that of Mcl-1[f/f]CD4Cre mice, although the

percentage was significantly lower than Bcl-2[tg] control (Figure 1F, left). For CD8^{+} SP cells, the percent of mature cells in the thymus was not significantly different from Mcl-1[f/f]CD4Cre (Figure 1F, right). The same respective results were observed for CD4^{+} and CD8^{+} T cells in the spleen (Figure 1G). These data show that while over-expression of Bcl-2 lends a modest improvement to survival of Mcl-1-deficient SP thymocytes and peripheral T cells, particularly CD4^{+} cells, there is still a significant impairment in survival of these cells as the transgene is unable to rescue percentages to wild type or Bcl-2[tg] control levels.

Bak^{−/−} partially rescues thymocyte defect in Mcl-1[f/f]LckCre mice while Bax^{−/−} cannot

One explanation for the non-redundancy of Mcl-1 and Bcl-2 is that these proteins have differential abilities to bind pro-apoptotic proteins. For example, the BH3-only protein Noxa binds Mcl-1 and A1 but not Bcl-2 or Bcl-x$_L$.[6, 9] Intriguingly, Bak has been shown to bind Mcl-1 at relatively high affinity, Bcl-x$_L$ at a lower affinity and did not bind Bcl-2 to a detectable level.[31] However, a different group recently showed that when Bak protein as opposed to Bak BH3 peptide[31] was used, Bcl-2 could bind Bak with similar or better affinity than Bcl-x$_L$, although this was somewhat dependent on the variant of Bcl-2,[32] so the *in vivo* specificity of Bak for Mcl-1 is unclear. To determine whether the death of Mcl-1-deficient thymocytes was occurring preferentially through Bak, we crossed Mcl-1[f/f]LckCre mice to Bak^{−/−} and Bax^{−/−} mice. While some groups but not others have observed a mild expansion in total cellularity of the Bax^{−/−} thymus,[29, 33] we observed no differences between wild type and Bak or Bax single knockouts (not shown) so all Cre-negative genotypes are represented in controls in our experiments.

Total thymocyte numbers in Mcl-1[f/f]LckCreBak^{−/−} mice were significantly increased over Mcl-1[f/f]LckCreBak^{+/−} although numbers did not reach control levels (Figure 2A). Mcl-1[f/f]LckCreBak^{+/−} mice were identical to age matched Mcl-1[f/f]LckCre mice (data not shown). Thymic cellularity of Mcl-1[f/f]LckCreBax^{−/−} mice was no different from Mcl-1[f/f]LckCre (Figure 2B). To determine which stages of thymocyte development were rescued, we compared cellularity in different thymic subsets. Mcl-1[f/f]LckCreBak^{−/−} mice had equivalent DN thymocyte numbers to control mice (Figure 2C, upper row), but in subsequent developmental subsets, cellularity in Mcl-1[f/f]LckCreBak^{−/−} mice did not reach that of controls. However, there was a significant increase in Mcl-1[f/f]LckCreBak^{−/−} cell numbers over Mcl-1[f/f]LckCreBak^{+/−} in all subsets except for CD8^{+} SP, indicating some persistence of rescued cells into later stages (Figure 2C, upper row). Cell numbers in Mcl-1[f/f]LckCreBax^{−/−} mice were not different from those in Mcl-1[f/f]LckCre mice for any subset (Figure 2C, lower row).

FACS profiling of the Mcl-1[f/f]LckCreBak^{−/−} thymus revealed increased DP percentage and decreased DN percentage compared to the Mcl-1[f/f]LckCreBak^{+/−} profile (Figure 2D). Consistent with a partial rescue, Mcl-1[f/f]LckCreBak^{−/−} had a higher percentage of CD44^{−}CD25^{−} DN4 cells than Mcl-1[f/f]LckCreBak^{+/−}, but the elevated percentage at the DN3 stage was not completely relieved (Figure 2D). Mcl-1[f/f]LckCreBax^{−/−}showed no relief in the DN block as observable by the percent DN/DP or the DN profile (Figure 2E). In fact, in most experiments, Mcl-1[f/f]LckCreBax^{−/−} contained higher percentages in the DN gate than Mcl-1[f/f]LckCreBax[+], even though Bax^{−/−} alone has no such increase.[33]

Finally, we examined Mcl-1 protein levels in thymocyte subsets to confirm that Mcl-1$^{f/f}$LckCreBak$^{-/-}$thymocytes have not retained Mcl-1 expression. We have previously shown that we are able to specifically detect Mcl-1 protein by flow cytometry.[20] Due to differences in background (isotype control) levels between thymic subsets, relative Mcl-1 levels are displayed as a ratio of the mean fluorescence intensity (MFI) of the Mcl-1 stain to that of the isotype control for each subset. Mcl-1$^{f/f}$LckCreBak$^{+/-}$ thymocytes contain wild type levels of Mcl-1, likely because surviving thymocytes have escaped deletion of Mcl-1 (Figure 2F). This is consistent with previous results demonstrated by Western blot or PCR in Mcl-1$^{f/f}$LckCre mice.[19, 20] Mcl-1$^{f/f}$LckCreBak$^{-/-}$ thymocytes have a detectable and statistically significant decrease in the Mcl-1 expression ratio in all major subsets, indicating that these cells have survived without Mcl-1 (Figure 2F). No such decrease is detectable in Mcl-1$^{f/f}$LckCreBax$^{-/-}$, indicating that most of the cells in these mice are those that have escaped deletion (Figure 2G).

Bak$^{-/-}$ but not Bax$^{-/-}$ rescues survival of Mcl-1$^{f/f}$CD4Cre mature SP thymocytes in a dose-dependent manner

Given the rescue of Mcl-1-deficient DN cells by Bak$^{-/-}$ and that we continued to detect increased cell numbers in later subsets, we sought to determine whether Bak$^{-/-}$ could rescue survival of Mcl-1-deficient mature SP cells. Thymus and spleen from Mcl-1$^{f/f}$CD4Cre mice had normal cellularity and this was unaltered by deletion of Bak or Bax(Figure 3A). The percentage of T cells in the Mcl-1$^{f/f}$CD4CreBak$^{-/-}$spleen returned to near control levels, and a partial increase over Mcl-1$^{f/f}$CD4Cre was observed in mice heterozygous for Bak (Figure 3B). We observed a return of Qa2$^+$CD69^{lo} cells in CD4$^+$ and CD8$^+$ SP compartments in both Mcl-1$^{f/f}$CD4CreBak$^{+/-}$ and Mcl-1$^{f/f}$CD4CreBak$^{-/-}$ mice (Figure 3B). Similar to what was observed with the Bcl-2 transgene, Mcl-1$^{f/f}$CD4CreBax$^{-/-}$ mice exhibited a modest improvement over Mcl-1$^{f/f}$CD4Cre in percentage of T cells in the spleen and percentage of Qa2$^+$CD69^{lo} cells within the already reduced SP compartments (Figure 3C). However, this did not match even the Bak$^{+/-}$ in the extent of rescue. Bak$^{-/-}$ rescued Mcl-1$^{f/f}$CD4Cre mature CD4$^+$ and CD8$^+$ SP cell numbers to wild type levels and a dose effect was observed in Bak$^{+/-}$ mice (Figure 3D). The same results were observed in the spleen, although the Bak$^{+/-}$ effect was not significant in CD8$^+$ T cells (Figure 3E). Although there was a statistically significant increase in the number of mature CD8$^+$ SP and CD4$^+$ and CD8$^+$ T cells in Mcl-1$^{f/f}$CD4CreBax$^{-/-}$, the absolute difference in cell numbers was minor, and Mcl-1$^{f/f}$CD4CreBax$^{-/-}$ was significantly different from Mcl-1-sufficient controls for all subsets (Figure 3F,G).

Loss of Bim does not rescue Mcl-1-deficient thymocytes

One of the proposed roles of the anti-apoptotic proteins is to inhibit BH3-only proteins, such as Bim, that are capable of directly activating Bak and Bax.[7, 8] Bim is known to be critical for preventing autoimmunity due to its roles in thymocyte negative selection and activated T cells.[30, 34, 35] Moreover, it has been shown that loss of Bim can rescue the survival and phenotypic defects of Bcl-2$^{-/-}$ mice, including thymocyte development.[36, 37] To determine whether Bim is important for apoptosis of Mcl-1-deficient thymocytes, we crossed Mcl-1$^{f/f}$CD4Cre mice to Bim$^{-/-}$ mice. Whereas there was no difference between Bim$^{+/+}$ and Bim$^{+/-}$ by our measures, Bim$^{-/-}$ and Mcl-1$^{f/f}$CD4CreBim$^{-/-}$ mice had higher total cell

numbers in spleen and thymus (Figure 4A). FACS profiling showed that $Bim^{-/-}$ control mice had normal T cell percentages in the spleen but elevated percentages of $Qa2^+CD69^{lo}$ mature cells within the SP compartments (Figure 4B, compare with Figures 1C,3B). However, $Mcl-1^{f/f}CD4CreBim^{-/-}$ mice showed a similar percentage of mature SP cells and peripheral T cells to $Mcl-1^{f/f}CD4Cre$ (Figure 4B, compare with Figures 1C,3B). In spite of increased total cellularity, $Mcl-1^{f/f}CD4CreBim^{-/-}$ mice displayed comparable cell numbers to $Mcl-1^{f/f}CD4CreBim^+$ in the mature SP (Figure 4C) and spleen T cell (Figure 4D) compartments. Thus, Bim does not appear to play a downstream role in promoting death of Mcl-1-deficient thymocytes.

Discussion

By generating various genetic models, we have shown that Mcl-1 and Bcl-2 play separate molecular roles at two critical points in thymic development. Over-expression of Bcl-2 was unable to rescue the survival of Mcl-1-deficient thymocytes at either the DN or SP stage. Additionally, while it has been shown that $Bim^{-/-}$ can rescue Bcl-2-deficient thymocytes,[37] $Bim^{-/-}$ was unable to rescue Mcl-1-deficient thymocytes and thus Bim does not appear to be the downstream effector of cell death in the absence of Mcl-1. Intriguingly, $Bak^{-/-}$ but not $Bax^{-/-}$ could rescue Mcl-1-deficient thymocytes at both the DN and SP stages, yielding evidence that specific antagonism of Bak may be the major role of Mcl-1 in thymocytes.

An *in vivo* model for Mcl-1 activity in T cells

Two opposing but not necessarily exclusive models have emerged for the mechanism of the anti-apoptotic molecules Bcl-2, Mcl-1 and $Bcl-x_L$. The "direct-activation model" suggests that the anti-apoptotic proteins function to inhibit the activity of BH3-only activator proteins (Figure 5A).[8–10] By this model, certain BH3-only molecules, namely Bim, Bid and possibly Puma, directly activate Bak and Bax and binding of the anti-apoptotic proteins to these BH3-only activators inhibits apoptotic pathways.[8–10] Alternatively, under the "Bak/Bax sequestration" model, the anti-apoptotic proteins function to bind Bak and Bax directly to prevent their oligomerization and/or activation (Figure 5B).[5] By this model, all BH3-only proteins function upstream to affect the ability of anti-apoptotic proteins to bind Bak/Bax and the distinction between "activators" and "sensitizers" really lies in the differential abilities of the BH3-only proteins to bind the anti-apoptotic proteins.[5, 6, 31]

One limitation of the studies leading to these models is that specific interactions between the Bcl-2 family members have been largely defined using BH3-peptides and were performed in cell lines,[6, 31] and further studies are needed to examine the *in vivo* consequences of these interactions. Recent data from knock-in mice expressing mutant forms of Bim suggest that both its ability to bind the anti-apoptotic proteins (indirect activation) and its ability to bind Bax (direct activation) are important for its full apoptotic effects.[11] The data we have presented here allow us to build an *in vivo* pathway of the roles of Mcl-1 in thymocytes (Figure 5C). Our data supports previous biochemical data showing specificity at the level of the Mcl-1-Bak interaction.[31] Together, these results are supportive of a model in which Mcl-1 directly sequesters Bak in thymocytes. We have shown that loss of the direct activator Bim does not rescue Mcl-1-deficient thymocytes, although this does not rule out that other

BH3-only "activators" are not downstream of Mcl-1. However, Bid and Puma have both been shown to activate Bax as well as Bak,[3, 10] and our data shows that Mcl-1-deficient thymocytes die by a Bak-specific mechanism. Release of BH3-only proteins upon deletion of Mcl-1 may still contribute to the death of thymocytes, particularly in DN cells where rescue by Bak$^{-/-}$ was incomplete. It remains possible that one of these BH3-only proteins could preferentially activate Bak over Bax, either directly or indirectly, by a mechanism that is yet to be appreciated. Future studies should continue to explore which other Bcl-2-family proteins contribute to the death of Mcl-1-deficient cells. However, the robust rescue by Bak$^{-/-}$ is suggestive of a major role of Mcl-1 in thymocytes being to directly antagonize Bak.

The role of Mcl-1 appears to be slightly different in each thymic subset. In DN cells, Mcl-1 sequesters Bak, and loss of Mcl-1 leads to Bak activation (Figure 5C, left). As rescue by Bak$^{-/-}$ is only partial at this stage, it is likely that Mcl-1 also inhibits other apoptotic signals that are less selective for Bak, such as BH3-only proteins that directly activate Bak/Bax (as shown in Figure 5C). Although our data does not directly address the role of Bcl-2, published data places Bim genetically downstream of Bcl-2, and suggests that the major role of Bcl-2 in thymocytes is to inhibit the activation of Bax and/or Bak by Bim (Bax activation displayed in Figure 5C). It is likely that Bim functions both directly to activate Bax as well as indirectly because mutant forms of Bim that cannot bind Bax partially alleviate the Bcl-2$^{-/-}$ phenotype but do not entirely recapitulate the rescue observed with the Bim$^{-/-}$.[11] In data not presented, we observed that Bim$^{-/-}$ does not appear to rescue Mcl-1$^{f/f}$LckCre thymocytes, so Bim alone is unlikely responsible for the incomplete rescue of Mcl-1-deficient cells at DN. In DP cells, both Mcl-1 and Bcl-x$_L$ can sequester Bak (Figure 5C, middle), as suggested by biochemical data[31] and our previous data in which DP cells were lost when both Mcl-1 and Bcl-x were deleted.[20] It is unclear whether Mcl-1 and Bcl-x$_L$ also inhibit Bax in DP thymocytes or whether Bax is inactive at this stage. In SP cells, direct inhibition of Bak appears to be a more dominant role of Mcl-1 due to the extent of rescue by deletion of Bak, even at the heterozygous level (Figure 5C, right). As Bcl-2^{tg} and Bax$^{-/-}$ yielded a slight improvement in SP thymocyte survival, Mcl-1 likely also plays a minor cooperative role with Bcl-2 in inhibiting Bax (Figure 5C, right). Since this was not observed in Bim$^{-/-}$, it could suggest that direct inhibition Bax by Mcl-1 is responsible, although Mcl-1 could inhibit one of the other activator BH3-only proteins for this effect. Although not displayed in Figure 5C for simplicity, upstream BH3-only proteins play important roles in affecting the ability of the anti-apoptotic proteins to perform the functions displayed.

Non-redundancy of Bcl-2 family members

The rescue of Mcl-1 deficient thymocytes by the Bak knockout was somewhat surprising considering that Bak and Bax are presumed to be largely redundant.[28] Neither Bak or Bax single knockout mice display a significant phenotype while the double knockout mouse has profound defects in several systems, including thymocytes.[28, 29, 33] However, there is some evidence that Bak and Bax, while performing similar functions, may be regulated differently. Under resting conditions, Bak is localized on the mitochondrial membrane while Bax can be found in the cytosol until induction of apoptosis when it relocates to the mitochondrial membrane, coincident with a conformational shift to the active form and

multimerization.38–40 While hard evidence is still needed, on a conceptual level, this could allow for Bak and Bax to be subject to different regulatory mechanisms. Additionally, while our work focuses on the roles of the Bcl-2 family members, other proteins may affect the activity of Bak and/or Bax. Mitochondrial protein VDAC2 has been shown to bind monomeric Bak in the mitochondrial membrane and contribute to the susceptibility of cells to apoptosis through Bak.41, 42

Specific roles for Mcl-1 in different cell types

It was interesting that we observed a more complete rescue of Mcl-1-deficient thymocytes by Bak$^{-/-}$ in SP cells than in DN cells, indicating that even between different thymic subsets, the dominant activity of Mcl-1 is different. Similarly, the minor rescue observed in Bcl-2^{tg} and Bax$^{-/-}$ SP thymocytes was more evident in CD4$^+$ cells than CD8$^+$ cells, indicating that CD4$^+$ and CD8$^+$ cells may have a different balance between Mcl-1-specific and Mcl-1/Bcl-2-shared pathways. Recently, a mouse model was published in which specific promoter regions of Mcl-1 were mutated leading to reduced expression of Mcl-1 in thymocytes and peripheral T cells.43 The phenotype in this hypomorphic mouse was more severe in CD8$^+$ than CD4$^+$ T cells, correlating well with our data that CD8$^+$ cell survival was not improved by Bax$^{-/-}$ or Bcl-2^{tg}. These data suggest that CD8+ T cells are more exclusively dependent on Mcl-1 than CD4$^+$ T cells. In addition to T cells, Mcl-1 is required for neutrophil survival.25 Interestingly, it was recently published that the Bak/Bax double knockout rescued Mcl-1-deficient neutrophil survival, but unlike what we observe in thymocytes, the authors stated that neither Bak or Bax knockout alone rescued.44 Mcl-1-deficient neutrophils were not rescued by Bim$^{-/-}$, but Bim$^{-/-}$ could rescue activated Mcl-1-deficient macrophages in their system.44

The reasons for the differences in the roles of Mcl-1 between different cell types are not yet clear. However, because the balance between anti- and pro-apoptotic factors are what ultimately determine cell fate, a likely contributor would be differences in expression and activity of other Bcl-2 family members between cell types. Upstream BH3-only proteins inhibit the anti-apoptotic proteins and can combine to sensitize the cell towards certain stimuli. For example, while neither Noxa or Bad alone could cause apoptosis in certain experimental systems, the co-expression of these proteins or their BH3-regions led to cell death.6, 31 Bad has been shown to bind Bcl-2 and Bcl-x$_L$ but not Mcl-1 directly, while Noxa binds Mcl-1 and A1 but not Bcl-x$_L$ or Bcl-2.6 Thus, Bad could indirectly influence the role of Mcl-1 in that the more Bad activity there is in a cell, the less Bcl-2 and Bcl-x$_L$ are able to perform shared roles with Mcl-1 and the more susceptible the cell becomes to loss of Mcl-1 protein or function. Additionally, the activity of Noxa, which inhibits Mcl-1, is likely to play a large role in determining the availability of Mcl-1 to perform its specific roles as well as shared roles with Bcl-2. As the biochemical pathways are further defined, it will be important to consider that each cell type and condition may have a different balance of Bcl-2 family members. Given the importance of the Bcl-2 family in the lymphoid and other systems and the current interest in this family as targets of cancer therapy, it will be critical to continue to delineate the roles of the anti-apoptotic proteins *in vivo* in different cell types.

Materials and Methods

Mice

Generation of Mcl-1[f/f] mice was described previously.[25] Mcl-1-floxed mice were back-crossed 7 generations to C57BL/6. LckCre and CD4Cre mice[26] were obtained from Taconic (Hudson, NY, USA). Bak,[28] Bax,[29] and Bim[30] knockout mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Bcl-2-transgenic mice[27] were provided by Dr. Motonari Kondo. With the exception of Bcl-2[tg] lines, all mice were on C57BL/6 background. For each experiment, littermate controls were used or age-matched controls substituted as necessary. All mice were analyzed between 4–8 weeks of age. Mice were maintained in barrier, specific pathogen free facilities. All work was performed under guidelines approved by the Duke University Institutional Animal Care and Use Committee.

Cell Counts and Flow Cytometry

Thymus and spleen were harvested into FACS buffer (PBS containing 2% FBS and 0.2% NaN_3) and kept on ice. Single cell suspensions were made by smashing organs with a plunger and passing cells through nylon mesh. Red blood cells were lysed using ACK buffer. Cells were enumerated on a hemacytometer in 0.1% Trypan Blue. Fc receptors were blocked by 10–15 minute incubation with 24G2 supernatant. Cells were stained for 20–30 minutes with antibodies to various surface markers (CD4, CD8, CD69, Qa2, TCRβ, B220, CD44, CD25) directly conjugated to the fluorochromes FITC, PE, PE/Cy5, PE/Cy7, APC or APC/Cy7. All antibodies were obtained from eBioscience (San Diego, CA, USA) or BioLegend (San Diego, CA, USA) and were used at a concentration of 0.125–0.5μl per 10^6 cells in 100μl. For analysis, cells were washed and resuspended in FACS buffer containing 2μg/ml propidium iodide for exclusion of dead cells and run on a BD FACSCanto™ cytometer (BD Biosciences, San Jose, CA, USA). All analyses were performed on FloJo software (Tree Star, Inc. Ashland, OR, USA).

Intracellular Staining for Mcl-1

Cells were fixed after surface staining in 2% PFA for 20–60 minutes, washed and stored in FACS buffer at 4°C. Fixed cells were permeablized for 20 minutes in FACS Buffer with 0.1% Saponin, 5% Normal Donkey Serum (Jackson Immunoresearch, West Grove, PA, USA), and 10% 24G2 supernatant. Cells were stained with 0.1μg/10^6 cells in 100μl with rabbit polyclonal anti-Mcl-1 (Rockland, Inc. Gilbertsville, PA, USA) or 0.1μg Rabbit IgG (Rockland) for 30–60 minutes in the same buffer. Cells were washed with 0.1% Saponin in FACS Buffer and stained with 1μl/10^6 cells FITC-donkey-anti-rabbit (Jackson Immunoresearch) for 30 min in 0.1% Saponin. Cells were run immediately after washing on a BD FACSCanto™ cytometer.

Statistical Analyses

Data were further analyzed and graphed using Prism (Graphpad Software, San Diego, CA, USA). Statistical significance was defined by p values less than 0.05 as calculated by unpaired two-tailed t tests.

Acknowledgments

Abbreviations

DN	double negative
DP	double positive
SP	single positive
TCR	T cell receptor
BH	Bcl-2 homology
MFI	mean fluorescence intensity

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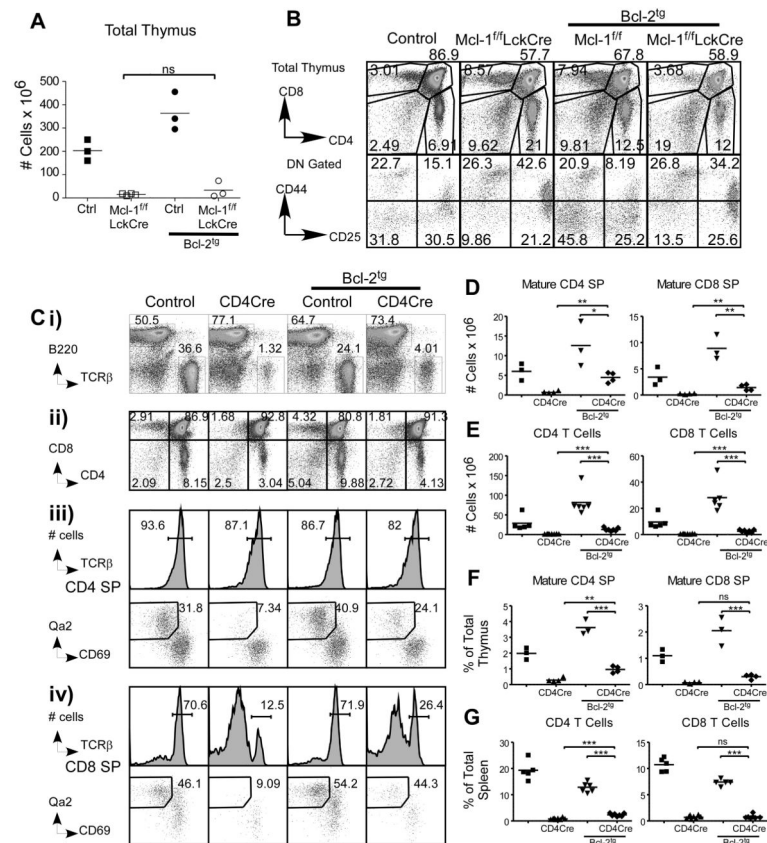
Figure 1. Effect of over-expression of Bcl-2 in Mcl-1$^{f/f}$LckCre and Mcl-1$^{f/f}$CD4Cre mice

(**A**) Total thymocyte number in control (ctrl), Mcl-1$^{f/f}$LckCre, Bcl-2^{tg}, and Mcl-1$^{f/f}$LckCreBcl-2^{tg} mice. Control is Mcl-1$^{f/f}$ or Mcl-1$^{+/+}$. (**B**) Representative FACS plots of control, Mcl-1$^{f/f}$LckCre, Bcl-2^{tg}, and Mcl-1$^{f/f}$LckCreBcl-2^{tg} mice. Top panel: CD4 vs. CD8 staining of total thymus. Numbers represent percent of total. Lower panel: CD44 and CD25 expression in DN cells. Numbers represent percent of DN. (**C**) Representative FACS plots of control (Mcl-1$^{f/f}$ or Mcl-1$^{f/+}$), Mcl-1$^{f/f}$CD4Cre, Bcl-2^{tg} (Mcl-1$^{f/f}$ or Mcl-1$^{f/+}$), Mcl-1$^{f/f}$CD4CreBcl-2^{tg} mice from spleen (**i**), thymus (**ii**), CD4$^+$ SP gated thymocytes (**iii**) and CD8$^+$ SP thymocytes (**iv**). Qa2 vs CD69 SP plots were pre-gated on TCRβ$^+$. Numbers represent percent of total (**i, ii**) or pre-gated populations (**iii, iv**). (**D,E**) Total number of mature (TCRβ$^+$Qa2$^+$CD69^{lo}) CD4$^+$ and CD8$^+$ SP cells in the thymus (**D**) and CD4$^+$ and CD8$^+$ T cells (TCRβ$^+$) in the spleen (**E**) of control, Mcl-1$^{f/f}$CD4Cre, Bcl-2^{tg}, Mcl-1$^{f/f}$CD4CreBcl-2^{tg} mice. (**F,G**) Percent mature (TCRβ$^+$Qa2$^+$CD69^{lo}) CD4$^+$ and CD8$^+$ SP cells in total thymus (**F**) and percent CD4$^+$ and CD8$^+$ T cells (TCRβ$^+$) in total spleen (**G**) of control, Mcl-1$^{f/f}$CD4Cre, Bcl-2^{tg}, Mcl-1$^{f/f}$CD4CreBcl-2^{tg} mice. For **C–G**, "CD4Cre" represents Mcl-1$^{f/f}$CD4Cre. All mice were 5–8 weeks of age and data represents 2 (LckCre) or 5 (CD4Cre) separate experiments. n = 3–4 (LckCre) and 5–8 (CD4Cre), Qa2 vs CD69 not available for all experiments. For all graphs, line represents mean total/percentage. P value is illustrated as *p<0.05, **p<0.01, ***p<0.001, or not significant "ns". Mcl-1$^{f/f}$CD4Cre and Mcl-1$^{f/f}$LckCre were significant from control by all measures displayed.
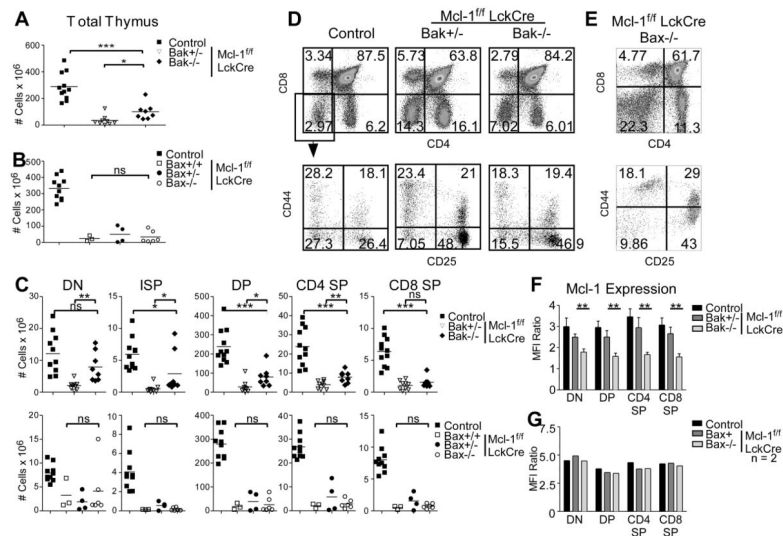
Figure 2. Thymic phenotype of Bak$^{-/-}$ or Bax$^{-/-}$ mice crossed to Mcl-1$^{f/f}$LckCre
(**A,B**) Total thymic cellularity of control, Mcl-1$^{f/f}$LckCreBak$^{+/-}$, and Mcl-1$^{f/f}$LckCreBak$^{-/-}$
(**A**) or control, Mcl-1$^{f/f}$LckCre, Mcl-1$^{f/f}$LckCreBax$^{+/-}$, and Mcl-1$^{f/f}$LckCreBax$^{-/-}$ (**B**).
Control = Mcl-1$^{f/f}$ or Mcl-1$^{f/+}$ with Bak$^{+/+}$, Bak$^{+/-}$, or Bak$^{-/-}$ (**A**) or Bax$^{+/+}$, Bax$^{+/-}$ or
Bax$^{-/-}$ (**B**). (**C**) Total numbers of thymic subsets: DN, immature single positive (ISP =
CD4$^-$CD8$^+$TCRβ$^-$), DP, CD4 SP, CD8 SP (CD4$^-$CD8$^+$TCRβ$^+$). (**D,E**) Representative
FACS plots of control, Mcl-1$^{f/f}$LckCreBak$^{+/-}$, Mcl-1$^{f/f}$LckCreBak$^{-/-}$ (**D**) and
Mcl-1$^{f/f}$LckCreBax$^{-/-}$ (**E**). (**F,G**) Mcl-1 expression in thymic subsets. Mcl-1 expression
measured by the MFI ratio of anti-Mcl-1 to Rabbit IgG control. Bars are mean + standard
deviation (SD). All mice were 4–7 weeks old and represent 5 separate experiments each for
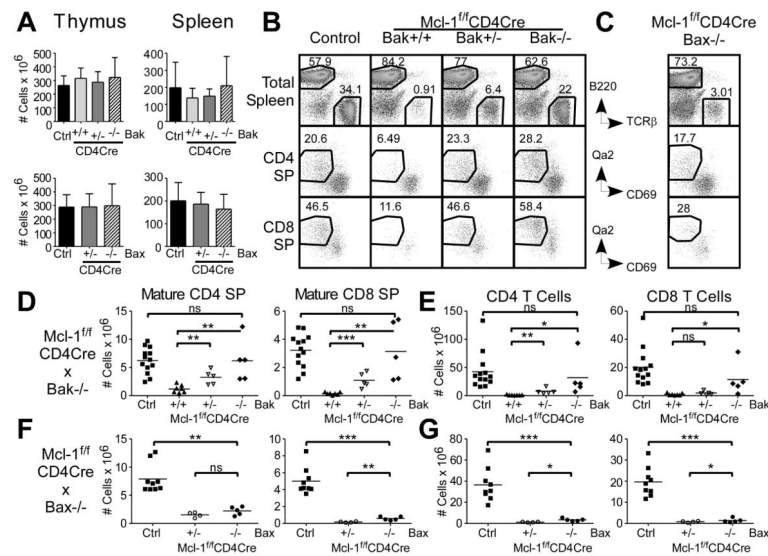Bak$^{-/-}$ and Bax$^{-/-}$. n = 8–11 (Bak) and 3–10 (Bax).

Figure 3. Phenotype of thymus and spleen from Bak$^{-/-}$ or Bax$^{-/-}$ mice crossed to Mcl-1$^{f/f}$CD4Cre

(**A**) Total cell numbers in thymus and spleen of Mcl-1$^{f/f}$CD4Cre mice crossed to Bak$^{-/-}$ and Bax$^{-/-}$. Bars are mean + SD. Control (Ctrl) mice are Mcl-1$^{f/f}$, Mcl-1$^{f/f}$Bak$^{+/-}$, Mcl-1$^{f/f}$Bak$^{-/-}$ or Mcl-1$^{f/f}$, Mcl-1$^{f/f}$Bax$^{+/-}$, and Mcl-1$^{f/f}$Bax$^{-/-}$. (**B,C**) Representative FACS plots for control, Mcl-1$^{f/f}$CD4Cre, Mcl-1$^{f/f}$CD4CreBak$^{+/-}$, and Mcl-1$^{f/f}$CD4CreBak$^{-/-}$ (**B**) and Mcl-1$^{f/f}$CD4CreBax$^{-/-}$ (**C**). Upper row shows percent T cells (TCRβ$^+$) and B cells (B220$^+$) in the spleen. Middle and bottom rows show percent Qa2$^+$CD69^{lo} mature cells in CD4$^+$TCRβ$^+$ SP compartment (middle) and CD8$^+$TCRβ$^+$ SP compartment (bottom) in the thymus. (**D,E**) Total cell numbers of mature TCRβ$^+$Qa2$^+$CD69^{lo} SP cells in thymus (**D**) and CD4$^+$ and CD8$^+$ T cells in spleen (**E**) of Mcl-1$^{f/f}$CD4CreBak mice. (**F,G**) Total cell numbers of mature TCRβ$^+$Qa2$^+$CD69^{lo} SP cells in thymus (**F**) and CD4$^+$ and CD8$^+$ T cells in spleen (**G**) of Mcl-1$^{f/f}$CD4CreBax mice. Mice were 5–7 weeks of age and represent 7 (Bak) or 4 (Bax) separate experiments. n = 5–13 (Bak) or 5–9 (Bax) per group.
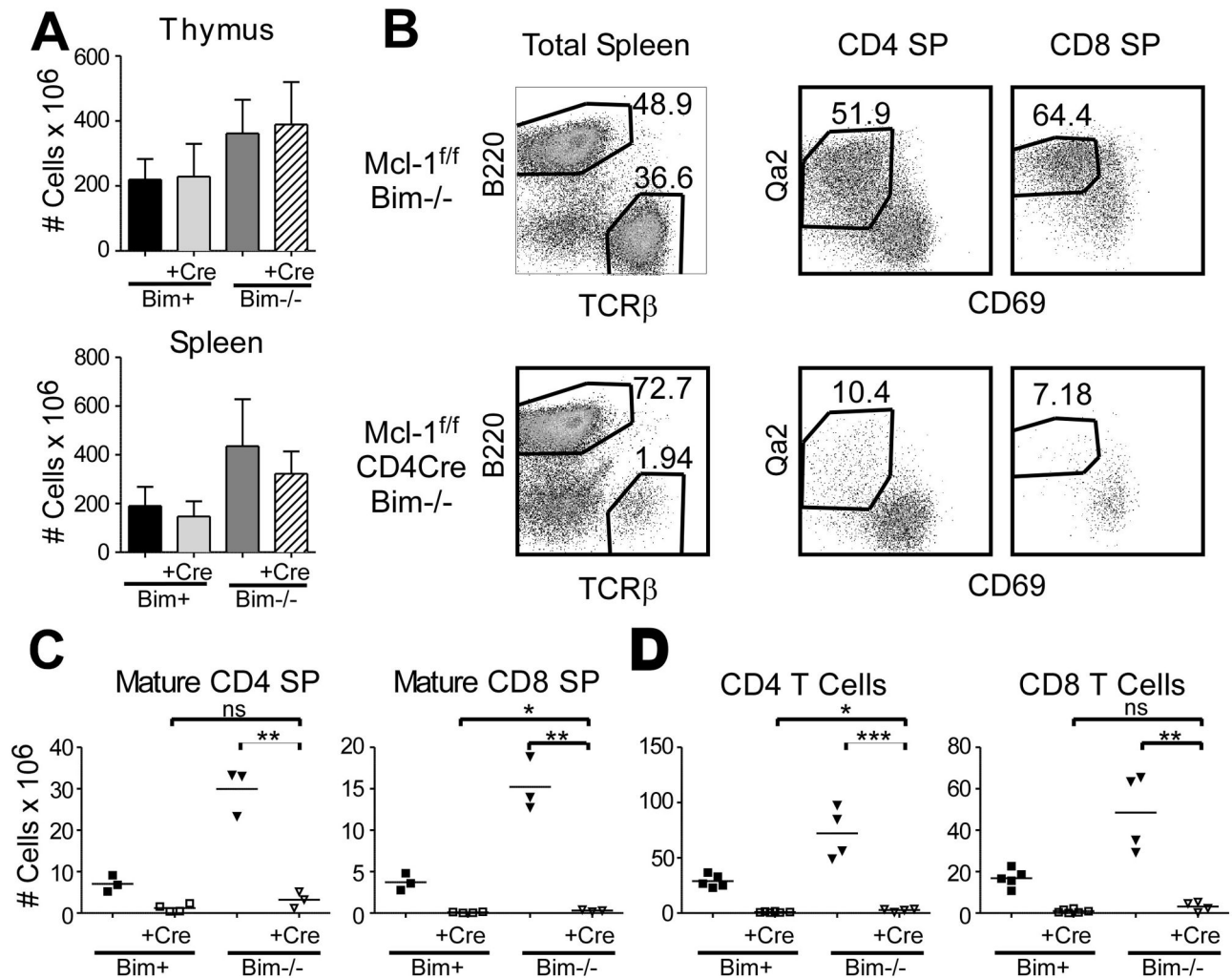
Figure 4. Phenotype of thymus and spleen of Mcl-1$^{f/f}$CD4CreBim$^{-/-}$
(**A**) Total cell number in thymus and spleen of Mcl-1$^{f/f}$CD4Cre mice crossed to Bim. Bars are mean + SD. Cre-negative controls are Mcl-1$^{+/+}$, Mcl-1$^{f/+}$ or Mcl-1$^{f/f}$ and Bim$^+$ (Bim$^{+/+}$ or Bim$^{+/-}$) or Bim$^{-/-}$ as indicated. All Cre$^+$ mice are Mcl-1$^{f/f}$ and Bim$^+$ (Bim$^{+/+}$ or Bim$^{+/-}$) or Bim$^{-/-}$ as indicated. (**B**) Representative FACS plots of Bim$^{-/-}$ control and Mcl-1$^{f/f}$CD4CreBim$^{-/-}$. Left side shows percent T cells (TCRβ$^+$) and B cells (B220$^+$) in the spleen. Right side shows percent Qa2$^+$CD69^{lo} mature cells in CD4$^+$TCRβ$^+$ SP compartment (middle) and CD8$^+$TCRβ$^+$ SP compartment (far right). (**C, D**) Numbers of mature TCRβ$^+$Qa2$^+$CD69^{lo} SP cells in thymus (**C**) and CD4$^+$ and CD8$^+$ T cells in spleen (**D**) of Mcl-1$^{f/f}$CD4CreBim. Mice were 4–8 weeks of age and represent 4 separate experiments. n = 4–7.
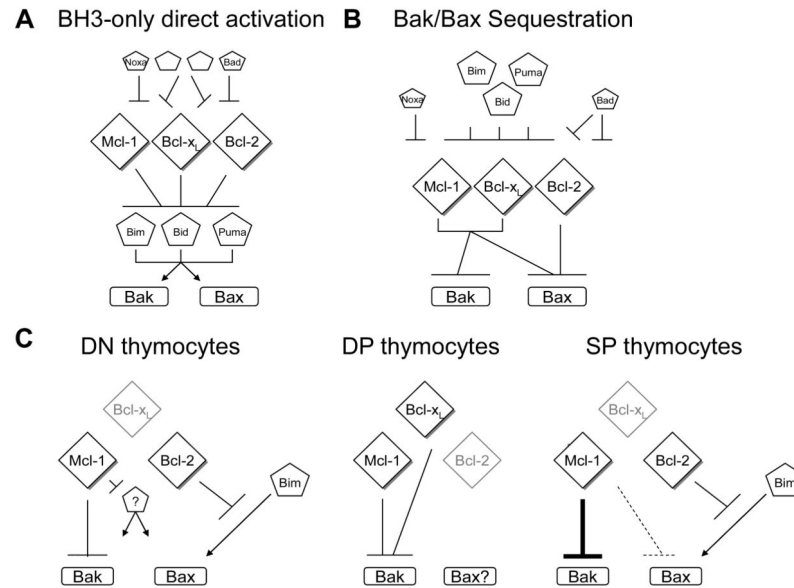
Figure 5. Models for the activity of the anti-apoptotic proteins

Anti-apoptotic proteins are displayed as diamonds, BH3-only proteins are pentagons with larger symbols representing putative "activators" and smaller symbols representing "sensitizers," and Bak and Bax are symbolized as rectangles. (**A**) "Direct activation model" The anti-apoptotic proteins Mcl-1, Bcl-2 and Bcl-x_L inhibit activator BH3-only proteins Bim, Bid and possibly Puma which can directly activate Bak and Bax. Sensitizer BH3-only proteins, such as Noxa and Bad, have varying specificities and affect availability of anti-apoptotic proteins. (**B**) "Bak/Bax sequestration model" The anti-apoptotic proteins directly inhibit activation of Bak and Bax by binding and sequestering them from activation and/or homo-oligomerization. BH3-only proteins effect the interaction and have varying strengths due to ability to neutralize the different anti-apoptotic proteins. (**C**) Diagram of the major roles of the anti-apoptotic proteins throughout thymic development.