# THE MOLECULAR MECHANISM OF APOPTOSIS AND ITS REGULATION BY BCL-2 IN THE T-CELL LINEAGE

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Apoptosis, or programmed cell death, is an evolutionarily conserved physiological process that ensures the elimination of unwanted or damaged cells from multicellular organisms (1,2). Although apoptosis can be initiated by diverse physiological and experimental stimuli, ultrastructurally apoptotic cells are characterized by plasma membrane reorganization and blebbing, cell shrinkage and nuclear fragmentation, suggesting the convergence of these signals on a common final effector pathway (1,3). In the last several years, unraveling the details of this molecular mechanism became one of the liveliest areas of molecular biology research.

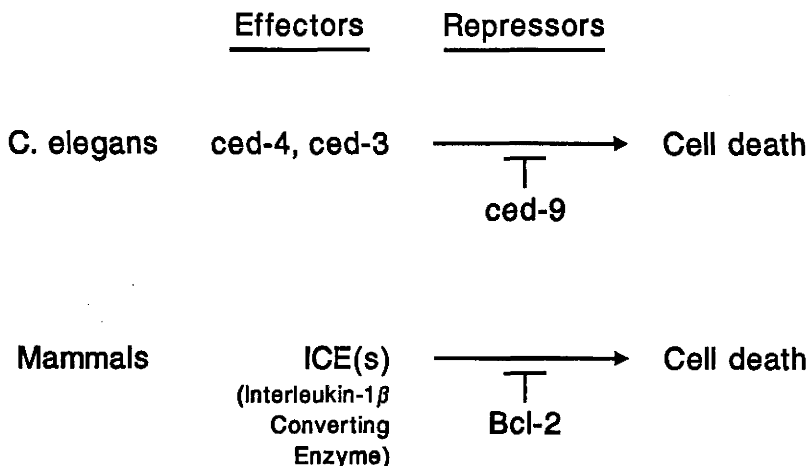


*Figure 1*

Insight into the molecular nature of this effector mechanism has been initially derived from genetic studies of a tiny worm, the nematode *Caenorhabditis elegans* (Figure 1). In this tiny worm of the original 1090 cells that are present in the developing animal 131 dies through apoptosis to form the 959 cell adult animal.


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In the nematode, two autosomal recessive death effector genes, *ced-3* and *ced-4* are required for the death of all 131 cells destined to die during worm development (2,4,5), whereas an autosomal dominant death repressor gene, *ced-9*, is essential for cell survival (6). Ced-3 is related to a family of mammalian protein degrading enzymes (caspases), such as ICE, (5,7), that are uniformly activated in mammalian apoptosis and required for certain aspects of cell death through the cleavage of a number of substrate proteins (3,8) (Figure 1). Ced-9 is a functional and structural homologue of Bcl-2 (9), the prototype member of the Bcl-2 protein family in vertebrates, that is able to inhibit the effect of many, but not all, apoptotic stimuli (10) (Figure 1). When expressed in mammalian cells, Ced-9, and the Bcl-2 functional homologue, Bcl-$x_L$ (11), can interact with (12-14) and inhibit the death inducing function of Ced-4 (12), while recruiting it from the cytosol to intracellular membranes (13). In addition, Ced-4 can simultaneously interact with, and presumably activate, Ced-3, or its mammalian counterparts, ICE (caspase 1), and FLICE (caspase 8), biochemically linking Ced-9 and the Bcl-2 family to Ced-3 and the mammalian caspases (12). These findings suggest that apoptosis is precipitated by the proteolytic cleavage of one or several critical substrates, and Bcl-2 may function by blocking the activation of caspases by inactivating the hitherto unidentified mammalian homologue (or analogue) of Ced-4 (12) (Figure 2).

**The Molecular Mechanism of Apoptosis**

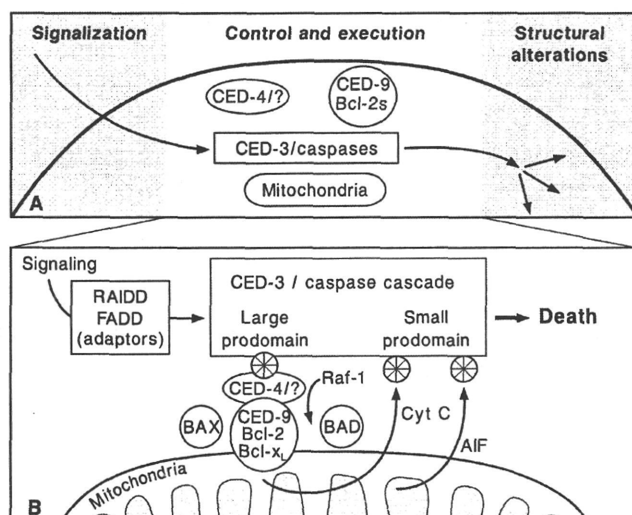



*Figure 2*

In mammalian cells, however, the control of apoptosis appears more complex than seen in *C. elegans*. For instance, mammals contain several genes encoding caspases and Bcl-2 homologues, while only a single essential copy of each type of

gene has been identified to date in the nematode (2,6). Also, Bcl-2 can prevent the mitochondrial release of cytochrome c (15,16) or an apoptogenic protease (AIF)(17), that are implicated in the initiation of some forms of cell death (17-19) (Figure 2). More importantly, in vertebrates two functional classes of Bcl-2 related proteins exist that share highly conserved Bcl-2 homology 1 (BH1), 2 (BH2), and 3 (BH3) domains: antiapoptotic members, including Bcl-2, Bcl-$x_L$, Mcl-1, and A1, that inhibit cell death, and proapoptotic members, including Bax, Bak, Bad, Bik, and Bid, that accelerate apoptosis and counter the death repressive function of Bcl-2 or Bcl-$x_L$ upon receiving a death signal (reviewed in (20,21))(Figure 3). Several *in vivo* studies confirm (22-26), that in vertebrates the balance between death-promoting and death-repressing members of the Bcl-2 family contributes a critical checkpoint that determines a cells's susceptibility to an apoptotic stimulus (27,28) (Figure 3).

**CELL AUTONOMOUS RHEOSTAT OF PROGRAMMED CELL DEATH**

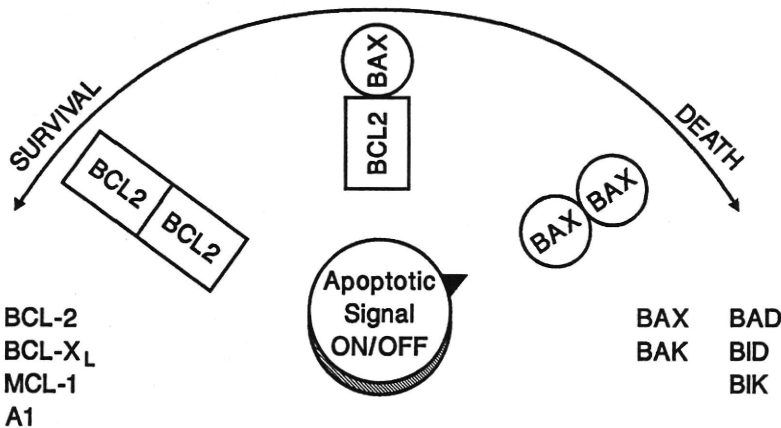



*Figure 3*

The molecular mechanism(s) by which Bax and its homologues exert their death promoting function at physiological expression levels is not clear. Inducible overexpression of Bax in yeast or in mammalian cells can trigger cell death in the absence of additional apoptotic stimuli (29-33). Of note, this Bax induced apoptosis proceeds even when caspase activation is inhibited (32). Similarly, mutations within the BH1, BH2 and BH3 domains of Bcl-2 and Bcl-$x_L$ that abrogate their function also cause loss of heterodimerization with Bax in mammalian cells (34-36). These data suggest that at high expression levels proapoptotic Bcl-2 re-

lated proteins possess the capacity to be directly cytotoxic and that Bcl-2 and Bcl-$x_L$ may have to form a protein complex with them to counter cell death. However, selected BH1 and BH2 mutants of Bcl-$x_L$ can exert their death-repressing activity even in the absence of heterodimerization with Bax (37). In mammalian cells, Bax can also directly compete with Ced-4 for association with Bcl-$x_L$, together implying that at physiological expression levels Bax-like proteins may merely act as inert competitive inhibitors of Bcl-2 and its functional homologues (12)(Figure 2).

In a recent study (38), we attempted to assess the antiapoptotic mechanism of Bcl-2 in relation to its capacity to dimerize with Bax and its homologues in a physiological context. The main findings of this study is the topic of this presentation.

## Bax-like activity in splenic T-cells, but not in thymocytes

To examine the role of Bax, or Bax-like activity, on thymocyte and splenic T-cell apoptosis, we first examined the effect of zVAD-fmk on their spontaneous cell death. As initial controls, Fas- mediated apoptosis of Jurkat cells in the presence or absence of 100 uM zVAD-fmk was tested. As previously described (32), Jurkat cells treated with anti-Fas antibody died rapidly, a process that was completely prevented by simultaneous treatment with zVAD-fmk (Figure 4A).

To test the effect of caspase inhibition on the spontaneous apoptosis of thymocytes and splenic T-cells, suspensions of these cells from non-transgenic littermates were placed *in vitro* in RPMI 1640 medium supplemented with 5% fetal calf serum in the presence or absence of 100 uM zVAD-fmk. The spontaneous apoptosis of thymocytes was significantly reduced by zVAD-fmk, almost to the same extent as provided by Bcl-2 mI-3 (Figure 4B). Spontaneos apoptosis of splenic T-cells, however, was essentially unaffected by the addition of identical concentration of zVAD-fmk (Figure 4C). To ensure that zVAD-fmk was equally functional in both cell types, thymocytes and splenic T-cells were also treated with anti-Fas antibody. Of note, murine thymocytes and splenic T-cells are known to present Fas receptor on the cell surface (39,40). Anti-Fas antibody treatment accelerated the rate of spontaneous cell death in both cell types, and simultaneous treatment with 100 uM zVAD-fmk completely inhibited this Fas induced acceleration of cell death in both thymocytes and splenic T-cells (Figure 4B,C). Thus, while zVAD-fmk proved functional in both cell types, it did not alter the rate of spontaneous apoptosis in splenic T-cells, an observation identical to that seen with Bax-induced cytotoxicity (32).

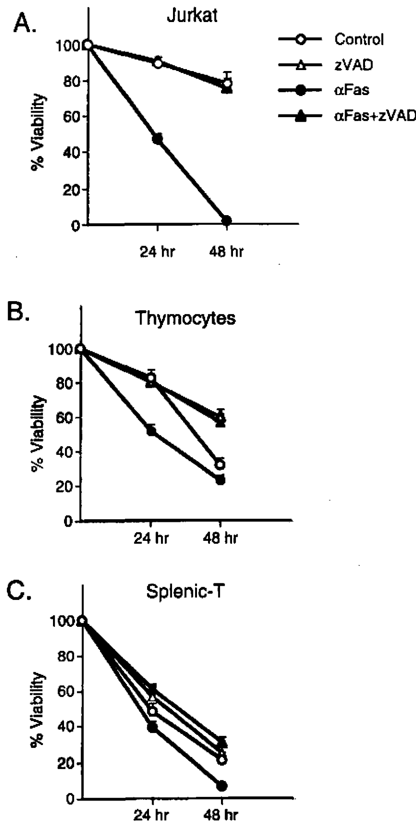


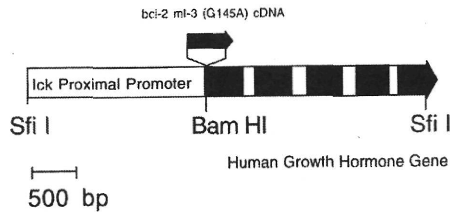

Figure 4

## Generation of transgenic mice expressing BH1 mutant Bcl-2 in thymocytes and peripheral T-cells

As thymocyte and splenic T-cell apoptosis differed in their sensitivity to caspase inhibition, this lineage appeared suitable to study the importance of Bcl-2's heterodimerization capacity for its antiapoptotic function. Therefore, we developed a transgenic mouse model to assess the effects of a BH1 domain substitution mutant of Bcl-2 (mI-3, $G_{145}A$) (34) upon T-cell development and T-cell death. This well-characterized Bcl-2 mutant fails to counter apoptosis in FL5.12 cells following IL-3 withdrawal, and does not heterodimerize with proapoptotic members of the Bcl-2 family, such as Bax (34), or Bid (21). A transgenic construct was generated by inserting a human *bcl-2 mI-3* cDNA downstream of the $lck^{pr}$ (41)(Figure 5A). The 3' untranslated portion of this construct provided introns, exons, and the poly(A) addition site from the human growth hormone gene hGH. Seven founder animals bearing the *lckpr-bcl-2 mI-3* construct were identi-

fied. Five lines were established, and each line was examined for human Bcl-2 mI-3 expression in the thymus (Figure 5B, top panel) and spleen (Figure 5B, bottom panel) by Western immunoblot analysis utilizing the human Bcl-2 specific monoclonal antibody (moAb), 6C8 (42). The tissue specificity of the *lckpr-bcl-2 mI-3* transgene was examined by Western blot analysis which failed to show transgene expression in non-lymphoid tissues, including the brain, heart, kidney, liver, or lung (data not shown). The thymus of transgenic animals contained a distinct cortex and medulla and was normal in size. The distribution of splenic red and white pulp was similar in transgenic and control littermate mice (data not shown). The two lines, 67 and 72, with the highest levels of human Bcl-2 mI-3 expression in thymocytes (Figure 5B, top panel) and splenocytes (Figure 5B, bottom panel) were further characterized, and compared to the previously established *lckpr-bcl-2 wild-type* (wt) transgenic model (43).

A




B

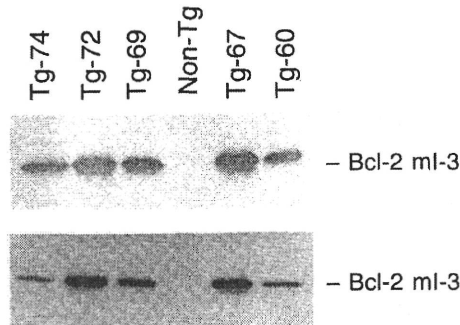



*Figure 5*

**Bcl-2 wt, but not Bcl-2 mI-3, heterodimerizes with Bax both in thymocytes and peripheral T-cells**

To confirm the inability of overexpressed Bcl-2 mI-3 to heterodimerize with endogenous proapoptotic Bcl-2 family members, such as Bax, co-immunoprecipitation experiments in *bcl-2 wt* and *bcl-2 mI-3* thymocytes and splenic T-cells were

performed. When [$^{35}$S] methionine labeled *bcl-2 wt* thymocytes were immunoprecipitated with the human Bcl-2 specific 6C8 moAb, a low amount of endogenous p21 protein was coprecipitated with human Bcl-2 (lane 2, Figure 6A, top panel). Immunostaining of a Western blot of the same immunoprecipitate with the murine Bax specific polyclonal antibody, 651 (21), confirmed the identity of p21 as murine Bax (lane 2, Figure 6A, bottom panel). Identical immunoprecipitations on [$^{35}$S] methionine labeled *bcl-2 mI-3* thymocytes revealed a lack of heterodimerization between Bcl-2 mI-3 and endogenous Bax. (lane 3, Figure 6A, top panel).

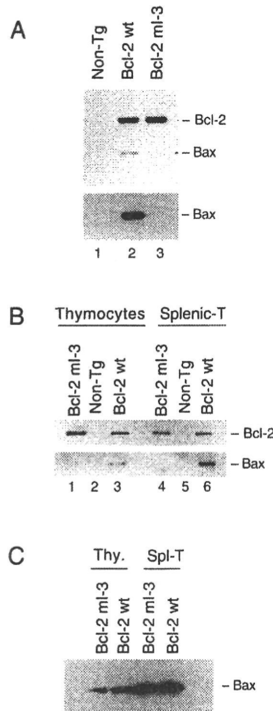



*Figure 6*

As metabolic labeling of splenic T-cells with [$^{35}$S] methionine was not very effective; co-immunoprecipitation experiments on lysates of unlabeled thymocytes and splenic-T cells were performed (Figure 6B). Equal amount of protein lysates of *bcl-2 wt* and *bcl-2 mI-3* thymocytes and splenic T-cells were immunoprecipitated with the human Bcl-2 specific 6C8 moAb, and Western blots of the immunoprecipitates were immunostained with either biotinylated 6C8 moAb, for human Bcl-2 (Figure 6B, top panel), or with the 651 polyAb, for murine Bax (Figure 6B, bottom panel). Thymocytes and splenic T-cells of both *bcl-2 wt* and *bcl-2 mI-3* transgenics expressed comparable amount of human Bcl-2, although the expression levels in thymocytes were again somewhat higher (Figure 6B, top

panel). Bcl-2 immunoprecipitated either from *bcl-2 wt* thymocytes (Figure 6B, lane 3) or splenic T-cells (Figure 6B, lane 6) demonstrated heterodimerization with endogenous murine Bax, but the amount of Bcl-2/Bax heterodimers were about five fold higher in splenic T-cells compared to that seen in thymocytes. Immunostaining of 6C8 immunoprecipitates from *bcl-2 mI-3* thymocyte (Figure 6B, lane 1) or splenic T-cell lysates (Figure 6B, lane 4) demonstrated no association between Bcl-2 and Bax in either cell types.

Despite comparable level of Bcl-2 wt expression in thymocytes and splenic T-cells, the amount of endogenous Bax that coprecipitated with Bcl-2 wt in splenic T-cells was substantially higher than in thymocytes (Figure 6B, compare lane 3 and 6). Thus, we were interested to determine if this difference was due to varying expression level of endogenous Bax, or represented differential dimerization capacity between Bcl-2 and Bax within the two cell types. Consequently, equal amount of protein lysates of thymocytes and splenic T-cells from *bcl-2 wt* and *bcl-2 mI-3* transgenic mice were assessed for the expression level of endogenous Bax with the murine Bax specific 651 polyclonal Ab. No difference in endogenous Bax expression was seen between *bcl-2 wt* and *bcl-2 mI-3* thymocytes or splenic T-cells (Figure 6C). However, endogenous Bax expression was about five fold higher in splenic T-cells compared to that seen in thymocytes in both the *bcl-2 wt* and *bcl-2 mI-3* transgenics (Figure 6C). Of note, Bcl-2 wt or Bcl-2 mI-3 expression did not alter endogenous Bax levels compared to non-transgenic controls (data not shown).

## The effect of Bcl-2 mI-3 on T-lineage maturation

To assess the functional effect of enforced Bcl-2 mI-3 expression in the T-cell lineage thymic maturation was first studied. Expression of Bcl-2 mI-3 in the thymus did not substantially modify the number of thymocytes, yet it altered the distribution of thymocyte subsets. *bcl-2 mI-3* transgenics uniformly demonstrated an ~ 4 fold increase in CD4$^-$8$^+$ single positive thymocytes (Figure 7, top panel). Some animals also displayed an ~ 1.4 fold increased percentage of CD4$^+$8$^-$ single positive cells. The increase in CD4$^-$8$^+$ thymocytes changed the average ratio of CD4$^+$8$^-$/CD4$^-$8$^+$ cells from 5:1 in control mice to 2:1 in transgenic mice. Immunoblot analysis revealed no difference in the amount of Bcl-2 mI-3 protein in CD4$^+$8$^-$ versus CD4$^-$8$^+$ cells (data not shown). This phenotypic effect of expressing *bcl-2 mI-3* in the thymus is similar to that seen with the *bcl-2 wt* transgene (43,44), namely an increase in mature thymocytes predominantly skewed towards the CD8$^+$ subset. Similarly, all transgenic animals had an increased percentage of CD3$^{hi}$/TCR$^{hi}$ thymocytes (~30%) compared with control littermates (~15%) (Figure 7, bottom panel). Transgenics displayed a reciprocal decrease in CD3$^{int-lo}$ cells (Figure 7, bottom panel). Thymocytes that have successfully com-

pleted thymic selection demonstrate increased CD3 expression, whereas $CD3^{int\text{-}lo}$ cells represent immature thymocytes, most of which are believed to die while undergoing thymic selection. Of note, transgenic thymocytes also contained increased numbers of cells with an intermediate level of TCR and CD3. This $TCR/CD3^{med}$ population is thought to represent a transitional intermediate stage following positive selection (45,46).

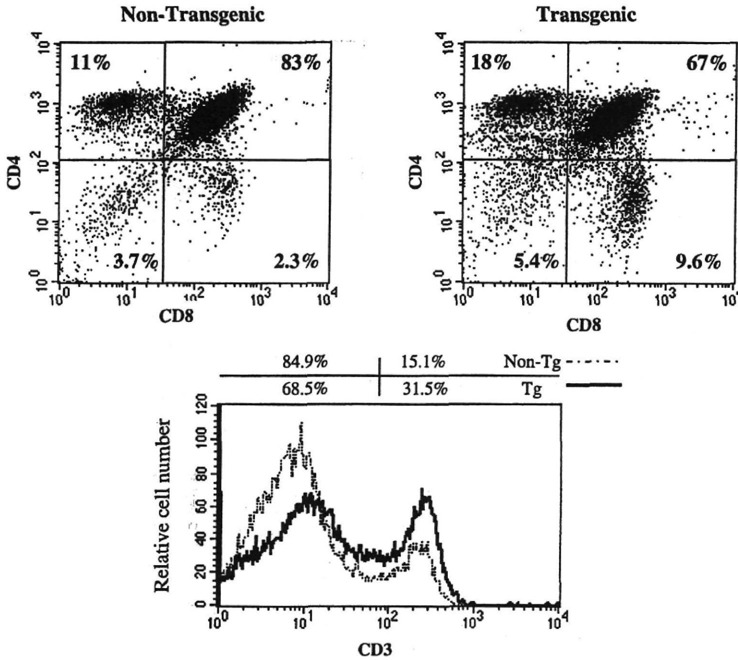



*Figure 7*

Flow cytometric analysis of splenocytes from six week old transgenic mice revealed an increase in both the number of T-cells ($Thy1^+$, $CD3^+$ cells) and the percentage of $CD8^+$ cells (Figure 8). The percentage of $CD8^+$ T-cells increased ~1.5 fold in young 6 week old transgenic animals (11% to 17%) (Figure 8, top panel), but decreased to a ~1.1 fold increment by 12 weeks of age (data not shown). The ratio of $CD4^+/CD8^+$ T-cells in the splenocytes of six week old transgenic mice averaged 1.2 compared with 1.5 in control littermate mice. Similar ratios of $CD4^+/CD8^+$ T-cells in the lymph node cells were seen (data not shown). Similarly, all six week old transgenic animals had an increased percentage of $CD3^{hi}/TCR^{hi}$ splenocytes (~45%) compared with control littermates (~30%) (Figure 8, bottom panel). By twelve weeks of age the differences in CD3 expression level of transgenic and control animals diminished to the same extent as seen in $CD4^+$, $CD8^+$ expression (data not shown). While the percentage of B cells

in six week old transgenic spleens was decreased by 15%, the absolute number of B cells was comparable in transgenic and control spleens. This phenotypic effect of expressing *bcl-2 mI-3* in splenocytes is significantly weaker to that seen with the *bcl-2 wt* transgene where an increase in the CD8$^+$ subset is more pronounced (43,44).

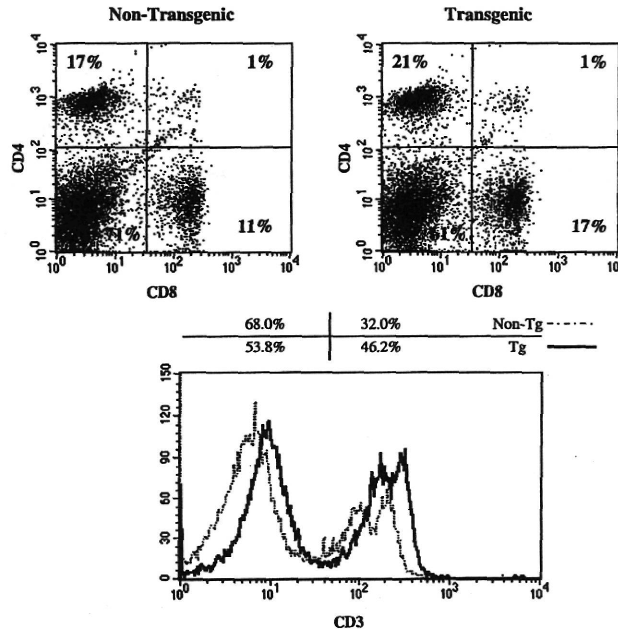



*Figure 8*

**Increased survival of *bcl-2 mI-3* transgenic thymocytes, but not transgenic peripheral T-cells in-vitro and in-vivo**

Immature CD4$^+$8$^+$ cortical thymocytes, as well as peripheral T-cells die rapidly in culture (47), an effect that is countered by overexpression of Bcl-2 (43,44), or Bcl-$x_L$ (48,49). To assess the effects of *bcl-2 mI-3* on the viability of these cells, suspensions of thymocytes, splenic T-cells, and lymph node cells from *bcl-2 mI-3, bcl-2 wt,* and control littermates were placed *in vitro* in RPMI 1640 medium supplemented with 5% fetal calf serum (Figure 9). Western immunostain on equal amounts of protein lysates of each sample demonstrated comparable Bcl-2 expression in thymocytes, lymph node cells and splenic T-cells from *bcl-2 mI-3* and *bcl-2 wt* mice (Figure 9A), although Bcl-2 expression was slightly higher in thymocytes compared to peripheral T-cells.

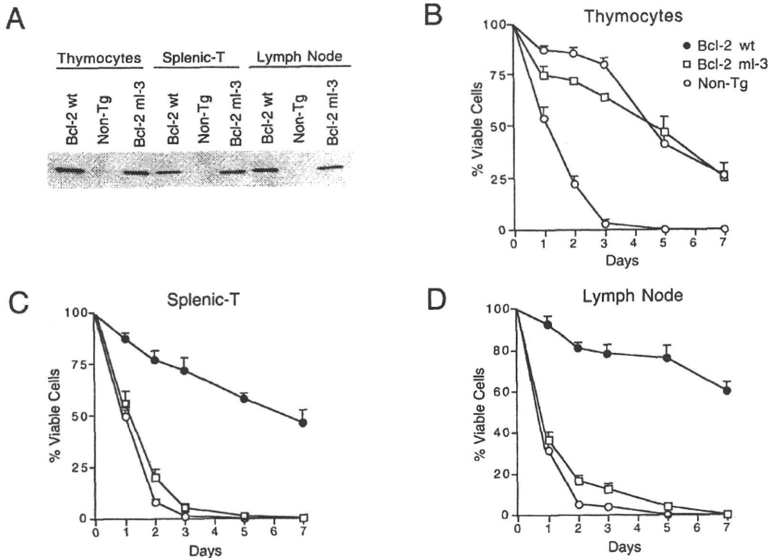



*Figure 9*

Thymocytes from *lckpr-bcl-2 wt* mice demonstrated improved survival, while the majority of control thymocytes died within the first three days, as previously described (43,44). Remarkably, the *bcl-2 mI-3* thymocytes demonstrated similar survival rates to that seen with Bcl-2 wt expression (Figure 9B). After 7 days, ~25% of *bcl-2 mI-3* and *bcl-2* wt thymocytes were still viable whereas <1% of the normal thymocytes survived. Flow cytometric analysis at day 7 revealed the persistence of DP and SP thymocytes from both *bcl-2 mI-3* and *bcl-2 wt* transgenic mice (data not shown). Contrary to that seen with thymocytes, peripheral T-cells of *bcl-2 mI-3* mice exhibited no increased survival *in vitro* (Figure 9C,D). Despite comparable Bcl-2 mI-3 expression to that seen in thymocytes (Figure 9A), splenic T-cells and lymph node cells from *bcl-2 mI-3* mice died at a rate similar to that of control littermates when placed in culture (Figure 9C,D). At the same time, peripheral T-cells from *bcl-2 wt* mice were remarkably resistant, as previously described (43,44); After 7 days, ~36 % of splenic T-cells and ~ 59 % of lymph node cells from *bcl-2 wt* mice were still viable (Figure 9C,D).

As Bcl-2 mI-3 was able to block the spontaneous apoptosis of thymocytes *in vitro*, it was of interest to determine whether Bcl-2 mI-3 could also extend thymocyte survival *in vivo*. Triggering thymocytes with dexamethasone or anti-CD3 both *in vivo* and *in vitro* has been shown to induce apoptosis of primarily the immature $CD4^+CD8^+$ DP cell population (50-53). Consequently, mice were treated with dexamethasone or anti-CD3, and thymocytes were evaluated 48 hours later (Figure 10).

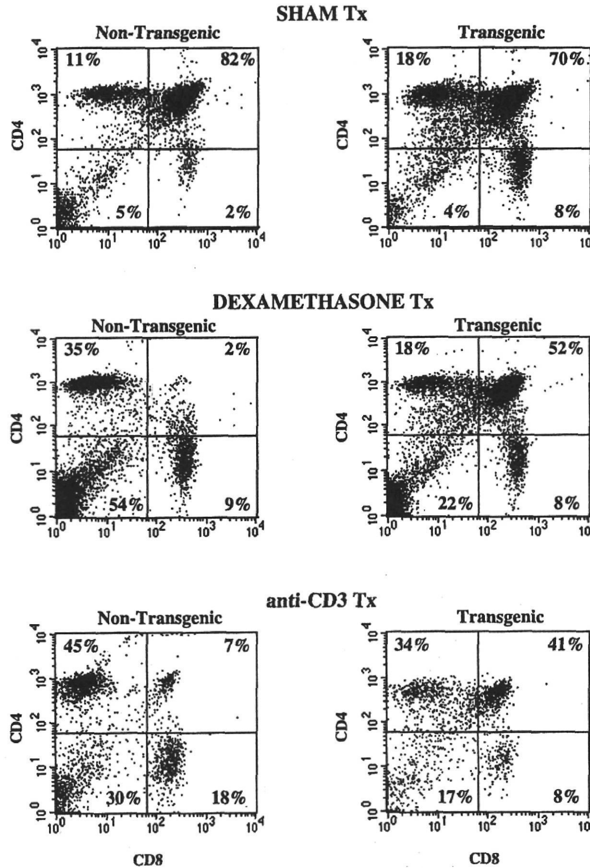



*Figure 10*

Intraperitoneal injection of 0.5 mg dexamethasone depleted a mean of 98% of thymocytes in control mice, as compared with vehicle treatment alone. However, *bcl-2 mI-3* mice were markedly resistant with an average decrease of only ~25% with this dose. Flow cytometric analysis of the surviving thymocytes indicated that dexamethasone only slightly reduced the $CD4^+8^+$ thymocytes in transgenic mice, but almost completely eliminated the $CD4^+8^+$ population from control mice (Figure 10, middle panel). Similarly, 48 hours following the intraperitoneal injection of 50 ug of affinity-purified anti-CD3 monoclonal antibody, the number of $CD4^+8^+$ thymocytes was decreased substantially (~ 85%) in control mice. The *bcl-2 mI-3* mice were again resistant to *in vivo* anti-CD3 treatment, although a slight decrease in the number of $CD4^+8^+$ thymocytes could be observed with an average decrease of ~40% with this dose (Figure 10, bottom panel). Thus, Bcl-2 mI-3 proved capable of countering apoptosis of thymocytes, but not of peripheral T-cells, both *in vitro* and *in vivo*.

## Concluding remarks

In mammalian cells, death-promoting and death-repressing members of the Bcl-2 family readily form heterodimers with each other (reviewed in (20,21)), but the significance of this physical interaction to their respective function is controversial. Inducible overexpression of Bax in mammalian cells can in itself induce apoptosis that is countered by Bcl-2 (31-33). Also, selected mutations within the BH1, BH2 and BH3 domains of Bcl-2 and Bcl-$x_L$ that disrupt their heterodimerization with proapoptotic Bcl-2 family members, such as Bax or Bid (21,34,35), can also result in the abrogation of their function (34-36). These data argue that a critical level of Bax homodimers activates downstream effector molecules and that antagonists, such as Bcl-2, prevent apoptosis by inactivating Bax through heterodimerization (34). However, recent experimental evidence has challenged and partially invalidated this model. Selected BH1 mutants of Bcl-$x_L$, that are unable to dimerize with Bax, can still counter cell death (37). Also, Bcl-$x_L$ can interact with mammalian caspases through Ced-4, a physical association that is negatively influenced by Bax (12). Moreover, *bcl-2* can functionally substitute for *ced-9* in *C. elegans* (54), an organism in which no *bax* homologue have been identified. These data offer an alternative hypothesis in which Bcl-2 and its functional homologues exert their action by preventing the activation of caspases through the formation of inactive Bcl-2/ Ced-4 homologue/ caspase complexes. In turn, the death repressor function of such complexes may be neutralized by competition with an inert Bax molecule (12).

The data presented in this paper provide evidence for the validity of both models within the T-cell lineage. Our results demonstrate that depending on the physiological context Bcl-2 exerts its antiapoptotic function by one of two separate mechanisms; one that requires heterodimerization with pro-apoptotic Bax-like molecules, and one in which such physical interaction is not required. To determine the antiapoptotic function of Bcl-2 in relation to its capacity to dimerize with Bax or its homologues at their physiological expression levels, we generated transgenic mice expressing a non-dimerizing BH1 mutant of Bcl-2 (Bcl-2 mI-3)($G_{145}A$) in the T-cell lineage. Previous studies demonstrated that this mutant Bcl-2 is unable to counter apoptosis induced by growth factor deprivation or glucocorticoid treatment in cell lines possessing high levels of endogenous Bax (34). Yet, a similar BH1 mutation of *ced-9* enhanced its survival promoting function in *C. elegans* (55), suggesting a dichotomy between the mammalian and nematode cell death machinery. Similarly, while in thymocytes spontaneous cell death was countered by zVAD-fmk, the spontaneous apoptosis of splenic T-cells remained unaffected by this caspase inhibitor (Figure 4). Inasmuch as resistance to zVAD-fmk represents a hallmark of Bax or Bax-like activity (32), these results implicate an active apoptosis inducing function of Bax or its homologues in peripheral T-cells, but a lack of such activity in thymocytes. Thus, thymocytes appeared a rea-

sonable candidate for a cell type in which Bcl-2 may function in a heterodimerization-independent fashion.

Bcl-2 mI-3 did not heterodimerize with Bax either in thymocytes or in peripheral T-cells, in agreement with previous findings (34). (Figure 6). Yet, enforced thymic expression of Bcl-2 mI-3 protected immature $CD4^+8^+$ thymocytes from spontaneous, glucocorticoid and anti-CD3-induced apoptosis (Figure 9,10). Bcl-2 mI-3 also altered thymocyte maturation and increased the percentages of $CD3^{hi}$ and $CD4^-8^+$ thymocytes (Figure 7), both patterns being similar to that seen with wild-type Bcl-2 (43,44). Thus, within immature thymocytes, the antiapoptotic function of Bcl-2 is apparently independent of its capacity to heterodimerize with Bax or its homologues. In contrast, Bcl-2 mI-3 could not counter apoptosis of peripheral T-cells while wild-type Bcl-2 remained effective (Figure 9). The loss of antiapoptotic activity of Bcl-2 mI-3 correlated with a higher Bax expression level in peripheral T-cells, and the amount of Bax that co-precipitated with wild-type Bcl-2 proved significantly increased compared to that seen in thymocytes (Figure 6). Thus, within peripheral T-cells the antiapoptotic function of Bcl-2 apparently requires a capacity to heterodimerize with Bax or its homologues.

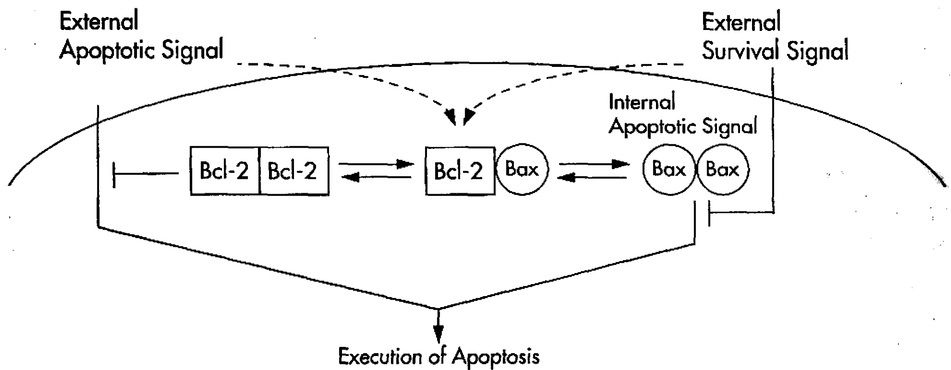



*Figure 11*

These data together suggest a dual effector function for both death-promoting and death- repressing members of the Bcl-2 family (Figure 11). As shown here within the T-cell lineage, Bcl-2 can counter apoptosis induced by external apoptotic stimuli, such as TCR engagement or glucocorticoid treatment, independent of its association with Bax or its homologues. While thymocytes do express a low amount of endogenous Bax (Figure 6), apoptosis induced by gamma-irradiation or dexamethasone treatment is not altered in bax -/- mice (56). Yet, the same apoptotic stimuli in conjunction with enforced expression of Bax results in increased thymocyte cell death (25). Thus, a threshold level of Bax expression is apparently required for its death-accelerating function in thymocytes. Of note,

external death signals themselves can alter the inherent Bcl-2 to Bax ratio (57). As spontaneous *in vivo* thymocyte apoptosis can be delayed by zVAD-fmk (Figure 4), Bax in thymocytes seems to act as an inert competitive inhibitor, perhaps by competing for the Bcl-$x_L$ expressed in immature $CD4^+8^+$ thymocytes (49). In contrast, spontaneous cell death of peripheral T-cells is apparently initiated by the active cytotoxicity of either Bax or one of its functional homologues, as it is not affected by zVAD-fmk (Figure 4). Inactivation of Bax or its homologues in these cells can be achieved either by external survival signals or by heterodimerizing Bcl-2. Several mechanistic possibilities may account for the antiapoptotic effect of Bcl-2 in this case. In one scenario, Bcl-2 might simply act as an inert competitive inhibitor through disrupting the formation of Bax-like homodimers. Alternatively, Bcl-2/Bax heterodimers could possess a biochemical function that is diametrically opposed to Bax homodimers. The identification of loss-of-function Bcl-2 mutants with intact Bax dimerization capacity (58) favours this latter possibility. In either case, whether Bax and its homologues are directly cytotoxic or only act as inert competitive inhibitors appears to depend on the presence of additional mediator molecules, such as proposed for the role of Bid (21).

Cell suicide is present in a variety of unicellular organisms suggesting that apoptosis in multicellular organisms may have very primitive evolutionary origins. For instance, some strains of *E. coli* will activate the expression of bacterial toxins that trigger cell death to insure plasmid maintenance or as a response to external events by suicidal pore or channel formation through their plasma membrane (59-61). The core structure of Bcl-$x_L$ that resembles the membrane-insertion domain of pore-forming bacterial toxins (62), together with its demonstrated ion-channel function in synthetic lipid bilayers (63), strongly implicate the evolutionary conservation of this mechanism in multicellular organisms. Yet, in the nematode *C. elegans* Ced-9 appears to function by an alternative mechanism that involves blocking the activation of Ced-3 through the formation of inactive Ced-9/ Ced-4/ Ced-3 complexes (12).

Our data suggest the preservation of both pathways of cell death initiation in mammalian cells (Figure 11); one that is initiated by Bax and its homologues and that may operate on the principles of unicellular pore-forming cell suicide systems; and one in which the activation of caspases plays a central role as seen in *C. elegans*. Bcl-2 can counter apoptosis when either pathway is involved, but must heterodimerize with Bax and its homologues when cell death is initiated by their active cytotoxic function.

## FIGURE LEGENDS

**Fig. 1.**
The genetic regulation of programmed cell death

In *C. elegans*, two genes, *ced-3* and *ced-4* are required for the death of all 131 cells destined to die during worm development, whereas another gene, *ced-9*, is essential for cell survival. Ced-3 is homologous to mammalian caspases, such as ICE. Ced-9 is a homologue of mammalian Bcl-2.

**Fig. 2.**
The molecular mechanism of apoptosis

(A) The three main stages of apoptosis.

(B) The control and execution stage. Members of the Bcl-2 family (shown here Bcl-2, Bcl-$x_L$, Bax, and Ced-9) can interact with themselves on mitochondrial surface. Antiapoptotic Bcl-2-like proteins can further interact with the Ced-4/Ced-3 complex. See text for further details.

**Fig. 3.**
Schematic model of the Bcl-2 cell death checkpoint

In vertebrates the balance between death-repressing (left panel) and death-promoting (right panel) members of the Bcl-2 family contributes a critical check-point that determines a cells's susceptibility to an apoptotic stimulus.

**Fig. 4.**
zVAD-fmk delays spontaneous apoptosis of thymocytes but not of splenic T-cells

(A) Jurkat cells treated with 100ng/ml of anti-human Fas moAb in the presence or absence of zVAD-fmk (100 uM).

(B) FL5.12 cells were deprived of IL-3 in the presence or absence of zVAD-fmk (100 uM).

(C,D) Thymocytes (C), and splenic T-cells (D) from non-transgenic mice were initially plated in RPMI/ 5% FCS at 6 x $10^5$ cells per ml in the presence or absence of zVAD-fmk (100 uM) and with or without 100ng/ml of anti-human Fas moAb. Cell viabilities of triplicate cultures were assessed by annexin V-FITC/ PI staining and plotted as the mean±SD. Legends shown in (A) is applicable to all panels.

**Fig. 5.**
*lckpr-bcl-2 mI-3* transgene construct and analysis of human Bcl-2 mI-3 expression

(A) A 0.75 kb of human *bcl-2 mI-3* (closed box) was inserted at the BamHI site 3' to the 3.2 kb $lck^{pr}$ (open box). Introns and exons (hatched boxes) of hGH constitute the 3' untranslated region.

(B) Western blot analyses of cell lysates with the 6C8 moAb. Twenty-five micrograms of protein of cell lysates from thymocytes (top panel) and splenocytes (bottom panel) was analyzed. Tissues were isolated from transgenic lines 60, 67, 69, 72 and 74, and non-transgenic littermates.

**Fig. 6.**
Analysis of Bax expression and its heterodimerization with Bcl-2

(A,B) Cell lysates of (A) [$^{35}$S] methionine-labeled or (B) unlabeled control (non-Tg) or transgenic thymocytes expressing human wild-type Bcl-2 (Bcl-2 wt) or human Bcl-2 mI-3 (Bcl-2 mI-3) were immunoprecipitated with the 6C8 anti-human Bcl-2 moAb. All immunoprecipitated proteins were resolved by SDS-Polyacrylamide gel electrophoresis. Gels were processed for fluorography (A) or electrotransferred and immunostained for Bcl-2 with biotinylated 6C8 moAb (B, top panel) or for Bax with the 651 polyAb (B, bottom panel).

(C) Western blot analyses of cell lysates for Bax expression. Twenty five micrograms of protein of cell lysates from thymus and splenic T-cells was stained for endogenous murine Bax with the 651 polyAb.

**Fig. 7.**
CD3, CD4 and CD8 expression in transgenic (Tg) and non-transgenic (non-Tg) thymocytes

(Top panel) Representative histograms of thymocytes stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 moAb-s. The percentage of cells of each phenotype is indicated.

(Bottom panel) Single-cell suspension of thymocytes from transgenic (solid line) and non-transgenic (dotted line) were stained with FITC-conjugated anti-CD3 moAb. The percentage of cells of that are $CD3^{lo\text{-}int}$, and $CD3^{hi}$ is indicated. Similar results were obtained in both lines 67 and 72.

**Fig. 8.**
CD3, CD4 and CD8 expression in transgenic (Tg) and non-transgenic (non-Tg) splenocytes

(Top panel) Representative histograms of splenocytes stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 moAb-s. The percentage of cells of each phenotype is indicated.

(Bottom panel) Single-cell suspension of splenocytes from transgenic (solid line) and non-transgenic (dotted line) were stained with FITC-conjugated anti-CD3 moAb. The percentage of cells of that are $CD3^{hi}$ is indicated. Similar results were obtained in both lines 67 and 72.

**Fig. 9.**
Bcl-2 expression and in-vitro survival of thymocytes and peripheral T-cells

(A) Western blot analyses of cell lysates with the 6C8 moAb. Twenty five micrograms of protein of cell lysates from thymocytes, splenic T-cells and lymph node cells of control (non-Tg) or *lckpr-bcl-2 wt* (Bcl-2 wt) and *lckpr-bcl-2 mI-3* (Bcl-2 mI-3) transgenics was analyzed.

(B,C,D) Viability of thymocytes (B), splenic T-cells (C), and lymph node cells (D). Cells were initially plated in RPMI/ 5% FCS at 6 x $10^5$ cells per ml in 96 well flat-bottomed plates and cell viabilities of triplicate cultures were assessed by annexin V-FITC/ PI staining and plotted as the mean±SD. Legends shown in (B) is applicable to panels (C,D) as well.

**Fig. 10.**
Dexamethasone and anti-CD3 depletion of thymocytes in-vivo

Representative two-color immunofluorescence dot plots of CD4 and CD8 expression of surviving thymocytes. Thymocytes recovered from *lckpr-bcl-2 mI-3* transgenic (Tg) and non-transgenic (non-Tg) mice 48 hrs following intraperitoneal injection of RPMI 1640 (sham), 0.5 mg dexamethasone, or 50 ug of affinity-purified anti-CD3 moAb, were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 moAb-s. The percentage of cells of each population is indicated.

**Fig. 11.**
Model for the dual function of Bcl-2 and Bax

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