

Proapoptotic Bcl-2 family member Bim is involved in the control of mast cell survival and is induced together with Bcl-X_L upon IgE-receptor activation

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Abstract

Mast cells play critical roles in the regulation of acute and chronic inflammations. Apoptosis is one of the mechanisms that limit and resolve inflammatory responses. Mast cell survival can be controlled by growth factors and activation of the IgE-receptor FcεRI. Members of the Bcl-2 protein family are critical regulators of apoptosis and our study provides evidence that the proapoptotic BH3-only family member Bim is essential for growth factor deprivation-induced mast cell apoptosis and that Bim levels increase upon FcεRI activation. Bim deficiency or Bcl-2 overexpression delayed or even prevented cytokine withdrawal-induced mast cell apoptosis in culture. The prosurvival protein Bcl-X_L and the proapoptotic Bim were both induced upon FcεRI activation. These results suggest that Bim and possibly also other BH3-only proteins control growth factor withdrawal-induced mast cell apoptosis and that the fate of mast cells upon FcεRI activation depends on the relative levels of pro- and antiapoptotic Bcl-2 family members.

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Abbreviations: BH, Bcl-2 homology; BMMC, bone marrow-derived mast cell; SCF, stem cell factor

Introduction

Mast cells are important effector and regulatory cells in inflammatory reactions. Upon activation, mast cells release and secrete inflammatory mediators, such as histamine, proteases, prostaglandin, leukotrienes and cytokines (e.g. TNF-α). Mast cells originate from hemopoietic stem cells in the

bone marrow and are dispersed throughout most tissues. Although mast cells have been studied mostly in the context of allergic diseases, it is now evident that these cells also play a critical role in host defense against certain pathogens.¹ Moreover, it has been demonstrated that mast cells can influence the onset and/or disease severity of chronic inflammatory reactions, for example in certain autoimmune diseases.²

In healthy individuals, the numbers of tissue mast cells are kept at a constant level, but during inflammation, such as allergy,³ allergic asthma⁴ or rheumatoid arthritis,⁵ their numbers are increased. When the inflammation has resolved, mast cell numbers decline, and there is evidence that apoptotic cell death is involved in this process. The regulation of mast cell homeostasis has not been studied in any great detail and the molecular mechanisms involved in mast cell apoptosis still need to be deciphered.

Mammals have two distinct apoptosis signaling pathways⁶ that converge upon activation of aspartate-specific cysteine proteases (caspases) which demolish cells by cleaving vital structural proteins and by activating latent enzymes that degrade DNA.⁷ The Bcl-2 family of prosurvival and proapoptotic proteins are critical regulators of cell survival⁷ and the balance between the two subgroups determines whether a cell will survive or undergo apoptosis. The prosurvival Bcl-2 family members include Bcl-2, Bcl-X_L, Bcl-w, A1, Mcl-1 and Boo/Diva and these proteins all share three or four regions of homology, called Bcl-2 homology (BH) regions. The proapoptotic family members can be further divided into two subgroups, the Bax/Bak-like proteins and the BH3-only proteins. The Bax/Bak subgroup consists of Bax, Bak, Bok/Mtd, Bcl-x_s, Bcl-G_L and Bflk and these proteins share two or three BH regions and, surprisingly, at least in the case of Bax also extensive structural similarity with their prosurvival relatives.⁷ The BH3-only proteins include Bik/Blk/Nbk, Bad, Hrk/DP5, Bid, Bim/Bod, Noxa, Bmf and Puma/Bbc3 and they share with each other and the Bcl-2 family only the BH3 region.⁸ The BH3 domain is essential for the binding of these proapoptotic proteins to the antiapoptotic Bcl-2 family members and for inducing apoptosis.⁸ Genetic and biochemical experiments have indicated that the BH3-only proteins initiate programmed cell death and stress-induced apoptosis and that the Bax/Bak-like proteins play an essential role in cell killing further downstream.^{9,10}

Knowledge about the role of Bcl-2 family members in the regulation of mast cell survival and apoptosis is limited. It has been shown that Bcl-2 and Bcl-X_L are expressed in mast cells,^{11–13} but very little is known about their function in these cells. It has been reported that *bax*–/– and *bcl-2*–/– mice have abnormal numbers of mast cells in certain tissues but no general difference with wt animals were observed.¹⁴ We have previously shown that the Bcl-2 family member A1 is critical for

the prosurvival stimuli triggered by crosslinking of the high-affinity IgE receptor, FcεRI.¹⁵

In this study, we provide the first evidence for an essential function of the proapoptotic BH3-only protein Bim in mast cells and that its expression can be regulated by FcεRI activation.

Results

Bim is an inducer of cytokine withdrawal-induced apoptosis in mast cells

To examine whether Bim has a function in the regulation of growth factor withdrawal-induced mast cell apoptosis, we compared the survival of *in vitro*-developed bone marrow-derived mast cells (BMMC) from *bim*^{-/-}¹⁶ and wt mice. In order to further study the role of Bcl-2 family members in the regulation of mast cell survival and apoptosis, mast cells were also generated from *vav-bcl-2* transgenic mice, which over-express Bcl-2 in all hemopoietic cells, including mast cells.¹⁷ The mast cells were produced in either IL-3, or in the presence of both IL-3 and stem cell factor (SCF). The BMMC developed from the wt, *bim*^{-/-} and *vav-bcl-2* transgenic mice all had typical mast cell morphology and staining of granula by toluidine blue. Flow cytometric analysis demonstrated that the different genotypes expressed the SCF-receptor Kit and the IgE-receptor FcεRI to similar extent (Figure 1).

It has previously been demonstrated that mast cells undergo apoptosis as a result of growth factor withdrawal.^{18,19} In our experiments, the wt, *bim*^{-/-} and *vav-bcl-2* transgenic mast cells were deprived of either IL-3, IL-3 and SCF, or of SCF alone. Cell death was determined by staining with propidium iodide followed by flow cytometric analysis. After

48 h of IL-3 deprivation from mast cells produced in IL-3 alone, only 13% wt cells had survived, whereas 42% of the *bim*^{-/-} cells were still alive (Figure 2). After 15 h of growth factor (IL-3 plus SCF) deprivation of mast cells produced in both IL-3 and SCF, only 8% of the wt cells remained alive, whereas 50% of the *bim*^{-/-} cells were still alive (Figure 3). When only SCF was withdrawn from BMMC produced in IL-3 plus SCF, 29% of the wt BMMC remained alive after 48 h, while 57% of the *bim*^{-/-} mast cells had survived (Figure 4). Higher concentrations of IL-3 delayed the cell death, but did not rescue the cells

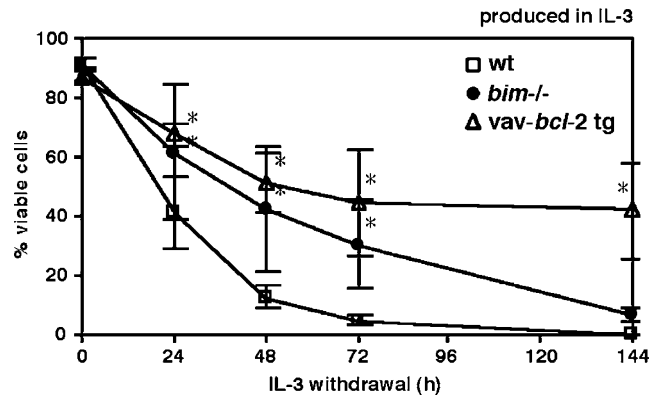


Figure 2 Bim deficiency and *bcl-2* transgene expression inhibit IL-3 withdrawal-induced mast cell death. Wt, *bim*^{-/-} and *vav-bcl-2* transgenic BMMC produced in IL-3 were deprived of IL-3, and cell viability was analyzed at the time points indicated. Cell viability was determined by propidium iodide staining and flow cytometric analysis. Data are presented as the mean ± S.D. (*n* = 4). Significant differences compared to wt were obtained as indicated (**P* < 0.05)

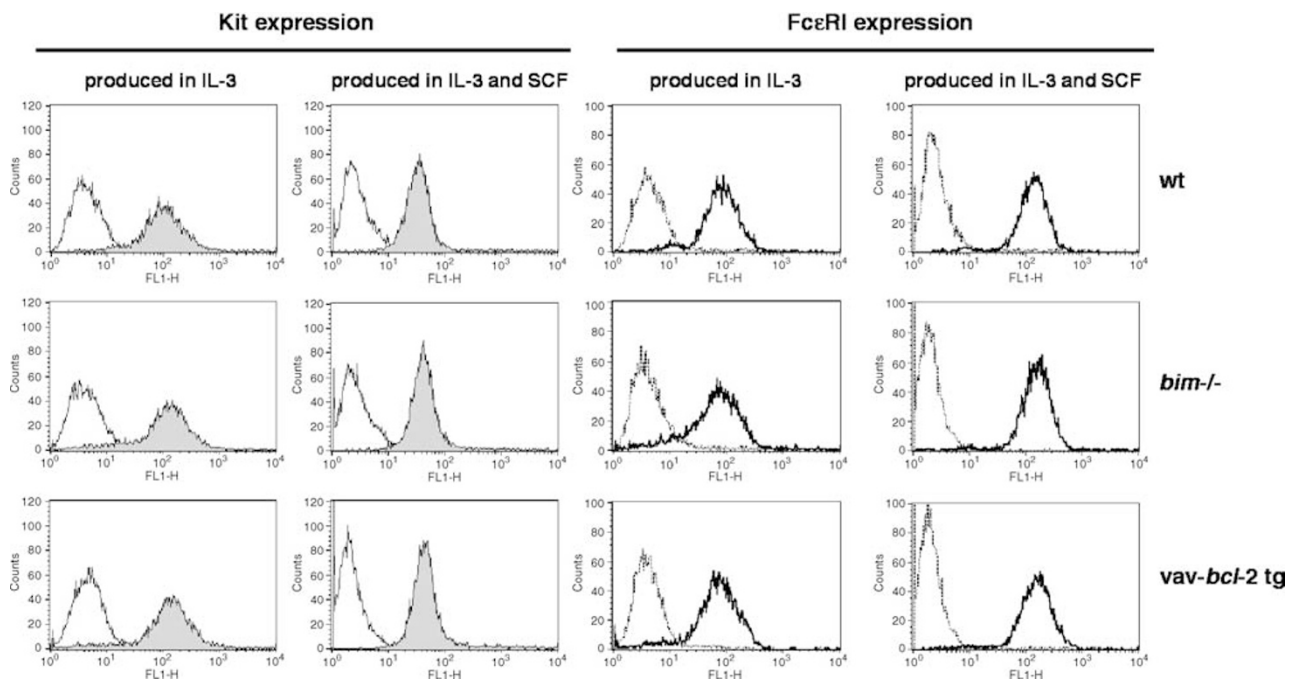


Figure 1 Wt, *bim*^{-/-} and *vav-bcl-2* transgenic BMMC express the receptors Kit and FcεRI to similar extent. By flow cytometric analysis, the receptor expression was analyzed on BMMC produced in IL-3 and on BMMC produced in a combination of IL-3 plus SCF. Left panel: filled peaks = Kit expression, empty peaks = isotype control. Right panel: thick line = FcεRI expression, dotted line = isotype control. The results shown are representative of three independent experiments

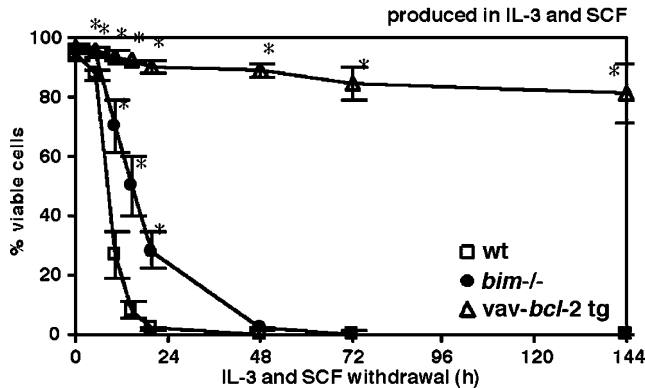


Figure 3 Bim deficiency and *bcl-2* transgene expression inhibit mast cell death induced by removal of IL-3 and SCF. Wt, *bim*^{-/-} and *vav-bcl-2* transgenic BMMC produced in IL-3 and SCF were deprived of both IL-3 and SCF, and cell viability was analyzed at the time points indicated as described in Figure 2. Data are presented as the mean \pm S.D. ($n = 6$). Significant differences compared to wt were obtained as indicated (* $P < 0.05$)

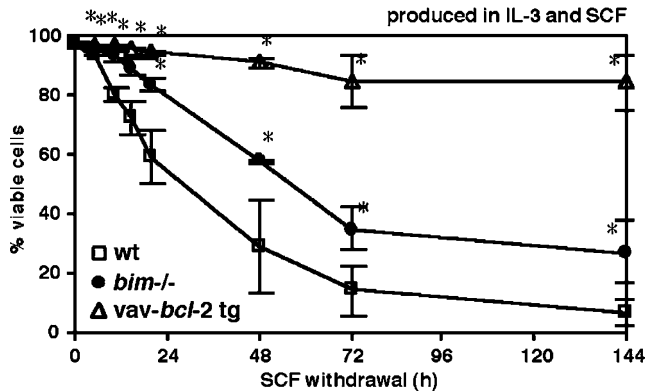


Figure 4 Bim deficiency and *bcl-2* transgene expression inhibit SCF withdrawal-induced mast cell death. Wt, *bim*^{-/-} and *vav-bcl-2* transgenic BMMC produced in IL-3 and SCF were deprived of SCF, and cell viability was analyzed at the time points indicated as described in Figure 2. Data are presented as the mean \pm S.D. ($n = 4$). Significant differences compared to wt were obtained as indicated (* $P < 0.05$)

from undergoing apoptosis (data not shown). The results demonstrate for the first time that Bim is involved in the induction of mast cell death following growth factor withdrawal. Our results also show that IL-3 alone is not sufficient to promote survival of mast cells produced in both IL-3 plus SCF.

We also investigated the effect of overexpressing the prosurvival gene *bcl-2* on growth factor withdrawal-induced mast cell apoptosis. Overexpression of Bcl-2 protected mast cells even more potently against cytokine deprivation-induced apoptosis than did loss of Bim (Figures 2–4). Hence, additional Bcl-2 inhibitable death inducers besides Bim, most likely other BH3-only proteins, may be involved in the mast cell death triggered by growth factor withdrawal.

When growth factors were withdrawn from the *vav-bcl-2* transgenic BMMC that had been produced in both IL-3 and SCF, there was an almost complete rescue from cell death even after 6 days, the latest time point studied (Figures 3 and 4). When IL-3 was withdrawn from the BMMC produced in only IL-3, overexpression of Bcl-2 also provided substantial

protection from apoptosis, but not as much as for the cells produced in both IL-3 and SCF (Figure 2).

An interesting observation was that mast cells produced in both IL-3 and SCF are much more sensitive to growth factor withdrawal than BMMC produced in IL-3 alone (Figures 2 and 3). For wt mast cells produced in IL-3, it took 3 days of growth factor withdrawal before most cells were dead (Figure 2). However, when wt mast cells that had been produced in both IL-3 plus SCF were starved of their growth factors, almost all cells were already dead after 20 h (Figure 3). Together with the observation that upon cytokine withdrawal Bcl-2 overexpression can almost completely rescue BMMC produced in IL-3 and SCF but only partially rescue those grown in IL-3, these results indicate that there are significant differences in the mechanisms regulating apoptosis following growth factor withdrawal, depending on whether mast cells have been produced in IL-3 alone or by the combined stimulation with IL-3 and SCF.

The prosurvival effect of IgE-receptor activation is not due to inactivation of Bim

We also investigated whether inhibition of Bim could be involved in activation-induced survival of mast cells. In order to examine this, wt, *bim*^{-/-} and *vav-bcl-2* transgenic BMMC were deprived of growth factors with or without prior IgE-receptor crosslinking. If inactivation of Bim was essential for the prosurvival stimuli induced by IgE-receptor crosslinking, one would expect no additional prosurvival effect in Fc ϵ RI-stimulated *bim*^{-/-} mast cells compared to nonstimulated *bim*^{-/-} cells. However, both the wt and the *bim*^{-/-} mast cells obtained a clear prosurvival stimulus upon Fc ϵ RI activation, indicating that inhibition of Bim alone cannot be the sole prosurvival activity of Fc ϵ RI stimulation (Figure 5a,b,d and e).

Fc ϵ RI stimulation does not promote survival in SCF-deprived or Bcl-2 overexpressing mast cells

As already described above, the mechanisms of growth factor withdrawal-induced BMMC death depends on the cytokines in which they have been produced. Similarly, we found that the method of mast cell production affected Fc ϵ RI activation-induced BMMC survival. When only SCF was withdrawn from BMMC produced in IL-3 plus SCF, no prosurvival effect was obtained as a result of IgE-receptor crosslinking (Figure 5c,f and i). This differed from the experiments in which both growth factors (IL-3 plus SCF) were withdrawn from mast cells (Figure 5b and e), and from the experiments in which IL-3 was withdrawn from mast cells produced in IL-3 alone (Figure 5a,d and g). In addition, we found that *vav-bcl-2* transgenic mast cells produced in IL-3 plus SCF did not survive better upon cytokine withdrawal when activated through their Fc ϵ RI compared to cells not stimulated through the Fc ϵ RI (Figure 5h and i).

Bim and Bcl-X_L proteins are upregulated upon IgE-receptor crosslinking

We next examined the expression of several pro- and antiapoptotic proteins of the Bcl-2 family in BMMC and in

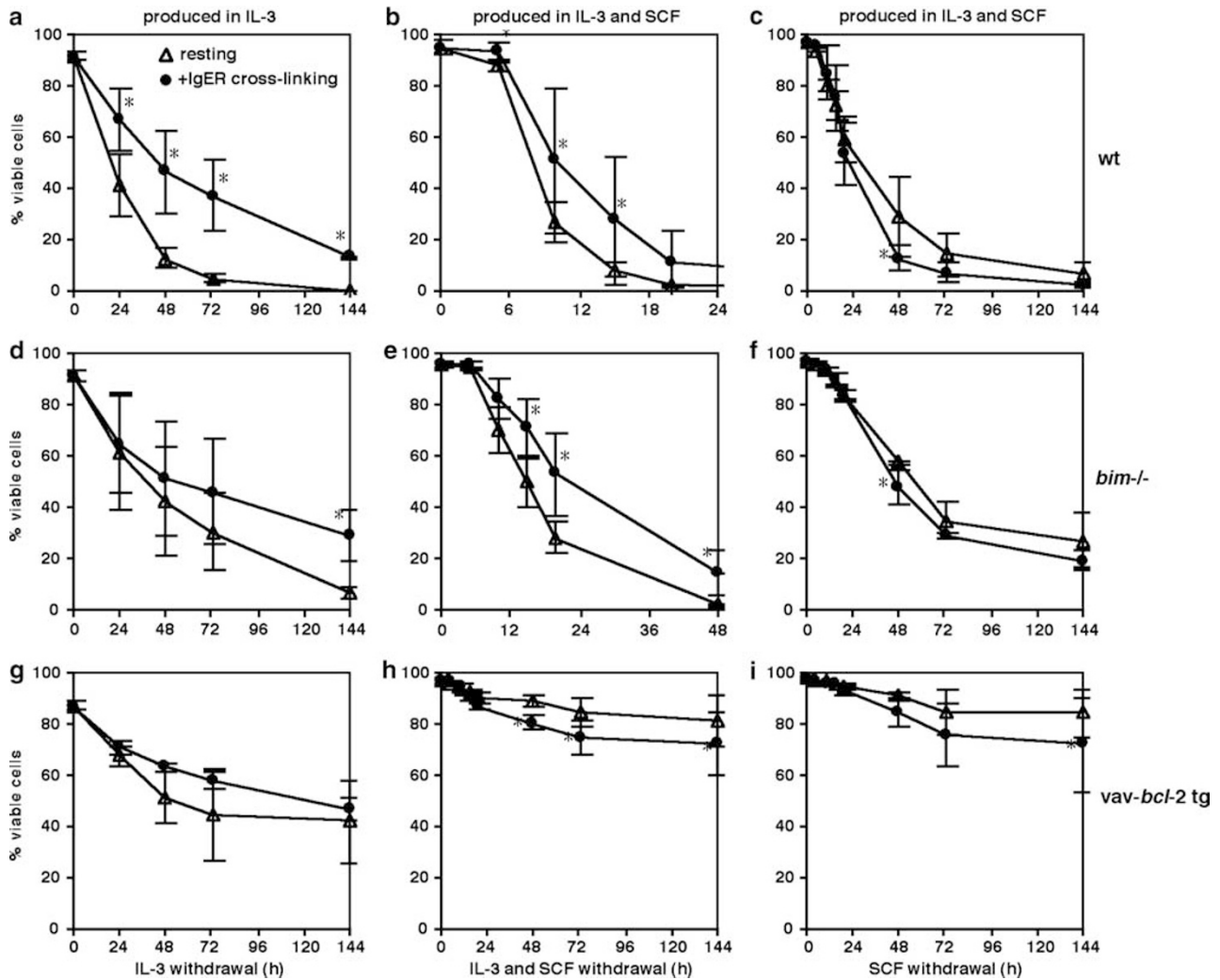


Figure 5 Absence of Bim does not affect the prosurvival stimulus from IgE-receptor crosslinking. In the experiments shown (a–i) wt, *bim*^{-/-} or *vav-bcl-2* transgenic BMMC were activated by crosslinking of the FcεRI by IgE, followed by withdrawal of growth factors. (a, d and g) BMMC produced in IL-3 were deprived of IL-3, with or without IgE-receptor activation prior to growth factor withdrawal. (b, e and h) BMMC produced in IL-3 and SCF were activated and deprived of IL-3 and SCF. (c, f and i) BMMC produced in IL-3 and SCF were activated, and SCF was withdrawn. In all experiments, cell viability was analyzed as described in Figure 2. Data are presented as the mean ± S.D. (n = 4–6). Significant effects compared to resting cells were obtained as indicated (*P < 0.05)

the C57 mast cell line (Figure 6). The C57 mast cell line was used to compare primary growth factor-dependent mast cells with a growth factor-independent cell line. Cell lysates were prepared from resting and FcεRI-activated BMMC grown for 10 h in the presence or absence of IL-3 and SCF (Figure 6a). The C57 mast cell line is growth factor independent, therefore these cells were deprived of serum instead of growth factors, and the cells were harvested and lysed 72 h after activation and/or serum deprivation (Figure 6b). All of the Bcl-2 family members analyzed were readily detectable in BMMC and C57 cells (Figure 6). These included the prosurvival proteins Bcl-X_L, Bcl-2 and Bcl-w, the proapoptotic multi BH domain protein Bak and the proapoptotic BH3-only proteins Bim, Bmf and Bad.

In agreement with our previous mRNA expression analysis,¹⁵ we could detect upregulation of Bcl-X_L protein levels upon IgE-receptor crosslinking, both in BMMC and in C57 cells (Figure 6). We also observed that withdrawal of IL-3 and

SCF caused a shift in migration of the Bcl-X_L and Bcl-2 bands on the Western gel (Figure 6a). The Bcl-X_L and Bcl-2 proteins were found as distinct, slow-migrating bands in lysates from the growth factor-deprived cells, whereas lysates from cells grown in the presence of cytokines also contained faster-migrating proteins (Figure 6a). This shift in migration could be due to differences in phosphorylation status. Both Bcl-X_L and Bcl-2 are known to be phosphorylated and dephosphorylated, and it has been reported that this can affect their function.^{20,21}

It has been reported that Bim protein levels are increased upon growth factor withdrawal in several cell types.^{22,23} Surprisingly, we found that both Bim_{EL} and Bim_L were strongly increased following IgE-receptor activation (Figure 6). We also analyzed the expression of the Bcl-2 family members Bim, Bcl-X_L and Bcl-2 in BMMC produced in IL-3. A similar expression pattern could be observed in BMMC produced in IL-3 as for those produced in IL-3 plus SCF (Figure 6c). Bim and Bcl-X_L were induced after IgE-receptor activation,

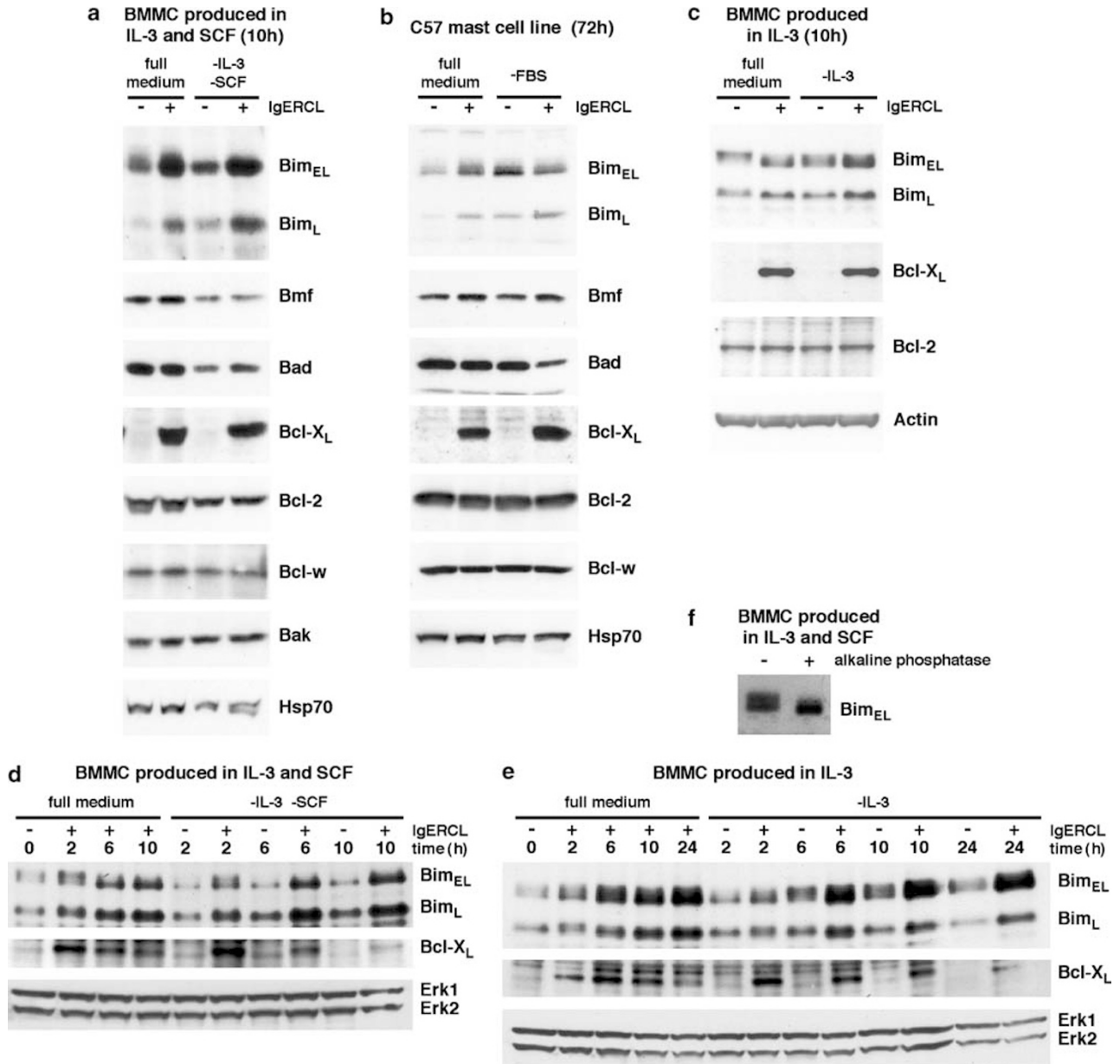


Figure 6 Crosslinking of Fc ϵ RI by IgE increases the levels of Bim and Bcl-X_L proteins. (a) Wt BMMC produced in IL-3 and SCF were activated by IgE-receptor crosslinking, deprived of IL-3 and SCF, or subjected to both stimuli. After 10 h, the levels of different Bcl-2 family members were analyzed by Western blotting using specific polyclonal or monoclonal antibodies. (b) C57, a cytokine-independent mast cell line, was stimulated by IgE-receptor crosslinking and/or deprived of FBS. After 72 h, protein levels were analyzed as in (a). (c) Wt BMMC produced in IL-3 were activated by IgE-receptor crosslinking and/or deprived of IL-3. After 10 h, protein levels were analyzed as in (a). (d and e) BMMC produced in IL-3 plus SCF or in IL-3 alone were activated by IgE-receptor crosslinking and/or deprived of growth factors for varying periods of time. Protein levels were analyzed as in (a). (f) Cell lysates from BMMC produced in IL-3 plus SCF were treated with alkaline phosphatase, and then analyzed by Western blotting as in (a). The probing with anti-Hsp70, anti-Actin, or anti-Erk Abs was used as control for the amount of proteins loaded. The results shown are representative of two independent experiments

whereas Bcl-2 levels were unaffected. This is the first observation that FcR activation can lead to upregulation of a proapoptotic BH3-only protein.

We further analyzed the kinetics of the IgE-receptor activation-induced upregulation of Bim and Bcl-X_L. Both Bim and Bcl-X_L were upregulated 2 h after IgE-receptor crosslinking (Figure 6d and e). This was observed in the BMMC produced in IL-3 plus SCF as well as in the cells produced in IL-3 alone.

Growth factor deprivation changed both the level and the migration pattern of the Bim protein. In BMMC grown in the presence of IL-3 and SCF, Bim_{EL} consists of proteins migrating with slightly varying mobilities on SDS-PAGE giving rise to a blurry band (Figure 6a). However, following deprivation of IL-3 and SCF, only fast-migrating Bim_{EL} was found. As for Bcl-X_L and Bcl-2, also the Bim protein has been reported to be a target for phosphorylation²⁴ and this is thought to regulate its degradation through the proteasome

pathway.^{25,26} In order to analyze whether the differences in migration mobility of Bim_{EL} on the Western gel is due to differences in phosphorylation status of Bim_{EL}, cell lysates from BMMC cultured in IL-3 plus SCF were treated with alkaline phosphatase before loading onto a Western gel. This treatment abolished the slower migrating portion of the Bim_{EL} band (Figure 6f), demonstrating that this upper band represents a phosphorylated form of Bim_{EL}.

Discussion

Bim has been shown to be involved in the regulation of apoptosis in several cell types, including B lymphocytes, mature T lymphocytes, thymocytes, monocytes, granulocytes, osteoclasts and neurons.^{16,23,26–28} Our studies demonstrate for the first time that Bim is involved in the induction of mast cell death following growth factor withdrawal. Since we only observed a delay in the induction of death in *bim*^{-/-} mast cells compared to wt cells, and not a total rescue, other factor(s) in addition to Bim most likely are involved in cytokine deprivation-induced mast cell death. It is possible that other BH3-only proteins expressed in mast cells play an important role in this cell death and this needs to be investigated further by using knockout mice lacking, for example, Bad, Blk, Puma or Bid. Puma is of particular interest since it has recently been shown that Puma plays a critical role in cytokine withdrawal-induced apoptosis of thymocytes and myeloid progenitors.^{29,30} The finding that overexpression of Bcl-2 protected mast cells even more potently against cytokine withdrawal-induced apoptosis than did loss of Bim provides further evidence that other Bcl-2 inhibitable death inducers besides Bim, most likely other BH3-only proteins, are involved in the mast cell death caused by growth factor deprivation.

In our experiments, we could observe some differences depending on how the BMMC were produced – in IL-3 alone or by costimulation of IL-3 plus SCF. For example, we found that Bcl-2 protected more potently from growth factor deprivation-induced apoptosis in BMMC produced in IL-3 plus SCF compared to in BMMC produced in IL-3 alone. This may indicate that in mast cells Bcl-2 can compensate more efficiently for SCF-receptor signaling than for IL-3 signaling. A possible explanation for this finding may be that Bcl-2 noninhibitable death inducers contribute to the growth factor withdrawal-induced death of mast cells produced in only IL-3. Alternatively, Bcl-2 may be more efficiently neutralized in mast cells produced in IL-3 alone compared to those produced in IL-3 plus SCF.

We also observed that the BMMC produced in both IL-3 plus SCF proliferated much more rapidly than did the BMMC produced in IL-3 alone (data not shown). This is consistent with what has been observed by others.³¹ This may indicate that increased level of cytokine stimulation and increased cell cycling render mast cells (and perhaps also other cell types) more dependent on growth signals.

Another interesting finding was that mast cells produced in both IL-3 plus SCF undergo apoptosis much more rapidly upon growth factor withdrawal than does BMMC produced in IL-3 alone. A similar rapid decrease in mast cell viability upon SCF withdrawal from cells maintained in SCF has been

reported earlier.¹⁹ Taken together, our results demonstrate that depending on whether mast cells have been produced in IL-3 alone or by the combined stimulation with IL-3 and SCF, the cells differ significantly in their response to growth factor deprivation.

It has been reported that crosslinking of the high-affinity IgE-receptor Fc ϵ RI elicits a prosurvival response in mast cells.^{15,32} We have previously shown that mRNA levels for the prosurvival genes A1 and Bcl-X_L are increased following IgE-receptor crosslinking and that A1 is critical for the activation-induced survival of mast cells.¹⁵ We investigated whether inhibition of Bim function is the most critical prosurvival stimulus elicited by IgE-receptor crosslinking. Our results do not support this hypothesis, but instead indicate that inhibition of Bim alone cannot be the sole prosurvival activity of Fc ϵ RI stimulation. Since Fc ϵ RI stimulation did not further increase the survival of cytokine-deprived mast cells overexpressing Bcl-2, it is likely that this antiapoptotic signal prevents activation of certain BH3-only proteins, perhaps Puma or Bad, since they have been implicated in growth factor withdrawal-induced apoptosis.^{29,30,33}

Not only were there differences in the mechanisms regulating apoptosis following growth factor withdrawal depending on the cytokines used for cell growth but we could also observe differences in the IgE-receptor activation-induced prosurvival stimuli. When BMMC produced in both IL-3 plus SCF were IgE-receptor activated followed by withdrawal of SCF alone, the mast cells did not survive better than did mast cells that had not been Fc ϵ RI stimulated. In contrast, IgE-receptor stimulation elicited a prosurvival activity in mast cells deprived of both IL-3 and SCF. Moreover, in Bcl-2-overexpressing mast cells produced in IL-3 plus SCF, there was no prosurvival response from IgE-receptor activation compared to resting mast cells, when depriving the cells of cytokines following the Fc ϵ RI stimulation. The protection of the cells may, in these experiments, already be maximal due to the overexpression of Bcl-2, which could explain why no further prosurvival effect is seen after Fc ϵ RI activation.

In a previous study, we analyzed the mRNA levels of several Bcl-2 family members in resting and IgE-receptor-activated cells, and found that the prosurvival members A1 and Bcl-X_L were upregulated.¹⁵ However, at the protein level, it has not yet been thoroughly analyzed which Bcl-2 family members might be involved in the regulation of apoptosis following IgE-receptor crosslinking or growth factor withdrawal. It has previously been reported that SCF withdrawal leads to downregulation of the Bcl-2 and Bcl-X_L protein levels in human mast cells.¹³ In some other cell types (e.g. lymphoid or neuronal cells), the proapoptotic BH3-only protein Bim has been reported to be upregulated upon growth factor withdrawal,^{22,23,34} but the expression of Bim has not yet been studied in mast cells. In this study, we found that the prosurvival Bcl-X_L and, to our surprise, the proapoptotic Bim proteins were both strongly upregulated following IgE-receptor activation. This is the first time that it has been shown that FcR activation can result in upregulation of a proapoptotic BH3-only protein. We have recently shown that activation of the T-cell receptor induces upregulation of Bim and that this is critical for the deletion of autoreactive thymocytes.²⁷ The signaling pathways responsible for

Fc ϵ RI-mediated upregulation of Bim needs to be further investigated. Mast cell activation through Fc ϵ RI has been reported to stimulate the Akt signaling pathway.³⁵ Activation of Akt has in several cell types been shown to result in phosphorylation, and hence inactivation, of forkhead transcription factors, such as FOXO3a.³⁶ Since FOXO3a, when in an active state, is considered as an important transcription factor for Bim,²² the observed Fc ϵ RI-mediated upregulation of Bim was somewhat unexpected. Studies on other transcription factors besides forkhead transcription factors might shed light on signaling molecules that are responsible for the upregulation of Bim following IgE-receptor activation.

The finding that both prosurvival and proapoptotic members of the Bcl-2 protein family are upregulated in response to IgE-receptor activation might explain why this type of activation in some experimental settings results in decreased cell death, while no prosurvival effect is seen in other conditions. Cell fate is likely to be determined by the balance between the prosurvival and the proapoptotic molecules.

An interesting observation was that deprivation of IL-3 and SCF from mast cells can alter the phosphorylation state of several Bcl-2 family members, including Bim, Bcl-2 and Bcl-X_L. Bim becomes dephosphorylated following withdrawal of IL-3 and SCF, while Bcl-2 and Bcl-X_L appear to be phosphorylated. Several previous studies have demonstrated that the phosphorylation status can affect the function of these proteins.^{20,21,25}

Although Bim is clearly an initiator of cytokine deprivation-induced mast cell death, our results indicate that other proteins are also involved in this process. Since overexpression of Bcl-2 protected mast cells against cytokine withdrawal more potently than loss of Bim, other Bcl-2 inhibitable proteins, quite likely other BH3-only proteins, are probably activated by growth factor deprivation. Our expression analysis shows that neither Bmf nor Bad protein levels are increased upon IL-3/SCF deprivation, but these proteins might still be involved in this process if their proapoptotic activity is activated by a post-translational mechanism. Moreover, there exist several other BH3-only proteins that remain to be studied for their role in regulating mast cell apoptosis.

Collectively, our results show that Bim, together with other Bcl-2 inhibitable proteins, is involved in the regulation of apoptosis and survival of mast cells. In Figure 7a, a model is presented on how cytokine withdrawal could lead to mast cell apoptosis by activating Bim and other BH3-only proteins, thereby inhibiting the prosurvival effect of Bcl-2 and other prosurvival homologs. We have also found that IgE-receptor crosslinking upregulates the protein levels of both prosurvival and proapoptotic Bcl-2 family members, namely, Bcl-X_L and Bim. Considering these results and previously published data regarding the involvement of A1 in IgE-receptor activation-induced mast cell survival,¹⁵ we present a model for how the fate of mast cells following IgE-receptor crosslinking could be fine-tuned by prosurvival and proapoptotic Bcl-2 family members (Figure 7b). The balance between proapoptotic and prosurvival members of the Bcl-2 protein family depends on the amounts of different family members present in a cell. Circumstances that could affect the prosurvival/proapoptotic balance include whether the proteins are in active or inactive

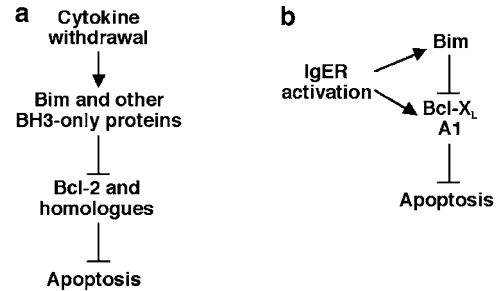


Figure 7 Two possible models for the role of Bim in the regulation of mast cell survival. (a) A mechanism by which cytokine withdrawal induces mast cells to undergo apoptosis could be that Bim and activation of other BH3-only proteins hinder prosurvival Bcl-2 homologues from performing their antiapoptotic function. (b) Crosslinking of the Fc ϵ RI by IgE leads to upregulation of both prosurvival and proapoptotic members of the Bcl-2 family. The balance between prosurvival and proapoptotic proteins following IgE-receptor activation of mast cells might determine if the net result of the receptor activation is increased survival or apoptosis

forms, the subcellular localization of these proteins and the mode of interaction between the different family members.³⁷

Materials and Methods

Mice

The mice used were Bim-deficient (266/266del) mice backcrossed for more than 12 generations onto a C57BL/6J genetic background,¹⁶ *vav-bcl-2-69* transgenic mice, expressing human Bcl-2 under control of the *vav* promoter in all hemopoietic cells, on an inbred C57BL/6J genetic background¹⁷ and wt C57BL/6J mice (from the Walter and Eliza Hall Institute mouse breeding facility, Kew, Australia). All experiments with animals were performed according to the guidelines of the Royal Melbourne Hospital Research Foundation Animal Ethics Committee.

Mast cell cultures

BMMC were obtained by culturing mouse bone marrow cells in RPMI medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (TRACE Scientific), 50 μ M 2-ME, 50 μ g/ml penicillin, 25 μ g/ml streptomycin and 1000 U/ml of IL-3 (produced by X63/0 cells transfected with a mouse IL-3 expression construct). Alternatively, BMMC were produced in medium supplemented with 1000 U/ml IL-3 and 25 ng/ml SCF (rmSCF, produced in *Pichia pastoris* and purified from culture supernatants by reverse phase chromatography). In one experiment for analysis of protein levels, BMMC were obtained by culturing in RPMI medium (Sigma) supplemented with 10% heat-inactivated FBS (Gibco), 10 mM HEPES buffer, 1 \times MEM nonessential amino-acid solution, 1 mM sodium pyruvate, 2 mM L-glutamine (all from Sigma), 50 μ M 2-ME, 100 IU/ml penicillin G, 50 μ g/ml streptomycin and 5 ng/ml IL-3 (rmlIL-3, PeproTech). All bone marrow-derived mast cells were cultured for a minimum of 3 weeks before they were used, and no experiments were performed on cells older than 9 weeks. The maturity and purity of the cells were examined by toluidine blue staining. Expression of Kit and Fc ϵ RI was analyzed by flow cytometric analysis using FITC-anti-mouse CD117 (c-Kit) (2B8), FITC-conjugated rat IgG_{2b} isotype control (both from Pharmingen), FITC-conjugated anti-mouse Fc ϵ RI- α (MAR-1) and FITC-conjugated Armenian Hamster IgG isotype control (both from eBioscience).

The mast cell line C57³⁸ (a gift from Dr. SJ Galli, Stanford University, Stanford, CA, USA) was cultured in RPMI medium (Gibco) supplemented

with 10% heat-inactivated FBS, 50 μ M 2-ME, 50 μ g/ml penicillin and 25 μ g/ml streptomycin.

Mast cell activation

For activation through crosslinking of the IgE-receptor, mast cells were sensitized for 90 min at 37°C with a monoclonal mouse anti-TNP IgE-antibody (IgE1-b4, ATCC), used as a 15% v/v hybridoma supernatant (corresponding to 1.5 μ g/ml IgE). After two washings in PBS, the cells were resuspended in medium and activated by addition of 100 ng/ml TNP-BSA (coupling ratio: 9/1) (Biosearch Technologies). To the control cells neither IgE nor TNP were added, but the cells were subjected to the washing and incubation steps.

Cell death assay

To monitor cell death, cells were stained with propidium iodide (2 μ g/ml) and analyzed in a FACScan (Becton Dickinson). Values presented are the means \pm S.D. ($n = 4-6$). For statistical analysis, we used an analysis of variance (ANOVA) followed by multiple comparison by Fisher's method. Differences were considered significant at $P < 0.05$.

Western blotting

Cells were lysed in buffer containing 0.05 M Pipes-NaOH, 0.05 M Hepes, pH 7.0, 2 mM MgCl₂, 1 mM EDTA, 1% Triton-X 100 and Complete Protease Inhibitor Cocktail tablets (Roche). The nuclei were pelleted, and the soluble fraction was used for analysis. Proteins were size-separated under reducing conditions by SDS-PAGE (NOVEX) and transferred onto nitrocellulose membranes (Amersham Biosciences). The membranes were probed with either of the following antibodies: rabbit polyclonal anti-Bim, anti-Bad, anti-Bcl-w (all from Stressgen Biotechnologies, the Bim antibody detects both Bim_{EL} and Bim_L), anti-Bim (Affinity BioReagents, detecting both Bim_{EL} and Bim_L), anti-Bmf,³⁹ anti-Bak (Sigma), anti-Bcl-2, anti-Erk, (both from Santa Cruz Biotechnology), mouse monoclonal anti-Bcl-X (clone 7B2.5, a gift from Professor C Thompson, Abramson Institute, Pennsylvania, PA, USA), anti-Hsp70 (a gift from Dr. R Anderson, Peter MacCallum Cancer Institute, Melbourne), rat monoclonal anti-Bcl-w (clone 16H12⁴⁰), hamster monoclonal anti-mouse Bcl-2 (clone 3F11, a gift from Professor S Korsmeyer, Dana-Farber Cancer Institute, Boston, MA, USA), HRP-conjugated goat polyclonal anti-Actin (Santa Cruz Biotechnology). The HRP-conjugated secondary antibodies used were either sheep anti-rabbit Ig, sheep anti-mouse Ig (both Silenus Labs), goat anti-rabbit Ig (Cell Signaling Technology), sheep anti-mouse Ig (Amersham Life Science), goat anti-rat Ig or goat anti-hamster Ig (both Southern Biotechnology Associates). Enhanced chemiluminescence system was used for detection. Each experiment was performed twice independently. For the experiments performed on BMMC, the cells were derived from separate mice.

Treatment with alkaline phosphatase

Cell lysates were divided into two fractions. One fraction was incubated in dephosphorylation buffer and 200 U/ml of alkaline phosphatase (both from Roche) at 40°C for 1 h. The other fraction (control) was treated in the same way, but without the addition of alkaline phosphatase. After treatment, the lysates were analyzed by Western blotting.

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References

1. Wedemeyer J, Tsai M and Galli SJ (2000) Roles of mast cells and basophils in innate and acquired immunity. *Curr. Opin. Immunol.* 12: 624-631
2. Benoist C and Mathis D (2002) Mast cells in autoimmune disease. *Nature* 420: 875-878
3. Viegas M, Gomez E, Brooks J and Davies RJ (1987) Changes in nasal mast cell numbers in and out of the pollen season. *Int. Arch. Allergy. Appl. Immunol.* 82: 275-276
4. Gibson PG, Allen CJ, Yang JP, Wong BJ, Dolovich J, Denburg J and Hargreave FE (1993) Intraepithelial mast cells in allergic and nonallergic asthma. Assessment using bronchial brushings. *Am. Rev. Resp. Dis.* 148: 80-86
5. Godfrey HP, Ilardi C, Engber W and Graziano FM (1984) Quantitation of human synovial mast cells in rheumatoid arthritis and other rheumatic diseases. *Arthritis Rheum.* 27: 852-856
6. Strasser A, Harris AW, Huang DC, Krammer PH and Cory S (1995) Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *EMBO J.* 14: 6136-6147
7. Marsden VS and Strasser A (2003) Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more. *Annu. Rev. Immunol.* 21: 71-105
8. Huang DC and Strasser A (2000) BH3-only proteins - essential initiators of apoptotic cell death. *Cell* 103: 839-842
9. Zong WX, Lindsten T, Ross AJ, MacGregor GR and Thompson CB (2001) BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev.* 15: 1481-1486
10. Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T and Korsmeyer SJ (2001) BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol. Cell* 8: 705-711
11. Cervero C, Escribano L, San Miguel JF, Diaz-Agustin B, Bravo P, Villarrubia J, Garcia-Sanz R, Velasco JL, Herrera P, Vargas M, Gonzalez M, Navarro JL and Orfao A (1999) Expression of Bcl-2 by human bone marrow mast cells and its overexpression in mast cell leukemia. *Am. J. Hematol.* 60: 191-195
12. Yeatman II CF, Jacobs-Helber SM, Mironsef P, Gillespie SR, Bouton LA, Collins HA, Sawyer ST, Shelburne CP and Ryan JJ (2000) Combined stimulation with the T helper cell type 2 cytokines interleukin (IL)-4 and IL-10 induces mouse mast cell apoptosis. *J. Exp. Med.* 192: 1093-1103
13. Mekori YA, Gilfillan AM, Akin C, Hartmann K and Metcalfe DD (2001) Human mast cell apoptosis is regulated through Bcl-2 and Bcl-XL. *J. Clin. Immunol.* 21: 171-174
14. Maurer M, Tsai M, Metz M, Fish S, Korsmeyer SJ and Galli SJ (2000) A role for Bax in the regulation of apoptosis in mouse mast cells. *J. Invest. Dermatol.* 114: 1205-1206
15. Xiang Z, Ahmed AA, Moller C, Nakayama K, Hatakeyama S and Nilsson G (2001) Essential role of the pro-survival bcl-2 homologue A1 in mast cell survival after allergic activation. *J. Exp. Med.* 194: 1561-1569
16. Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F, Adams JM and Strasser A (1999) Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 286: 1735-1738
17. Ogilvy S, Metcalf D, Print CG, Bath ML, Harris AW and Adams JM (1999) Constitutive Bcl-2 expression throughout the hematopoietic compartment affects multiple lineages and enhances progenitor cell survival. *Proc. Natl. Acad. Sci. U.S.A.* 96: 14943-14948

18. Mekori YA, Oh CK and Metcalfe DD (1993) IL-3-dependent murine mast cells undergo apoptosis on removal of IL-3. Prevention of apoptosis by c-kit ligand. *J. Immunol.* 151: 3775–3784
19. Iemura A, Tsai M, Ando A, Wershil BK and Galli SJ (1994) The c-kit ligand, stem cell factor, promotes mast cell survival by suppressing apoptosis. *Am. J. Pathol.* 144: 321–328
20. Poruchynsky MS, Wang EE, Rudin CM, Blagosklonny MV and Fojo T (1998) Bcl-xL is phosphorylated in malignant cells following microtubule disruption. *Cancer Res.* 58: 3331–3338
21. Haldar S, Jena N and Croce CM (1994) Antiapoptosis potential of bcl-2 oncogene by dephosphorylation. *Biochem. Cell Biol.* 72: 455–462
22. Dijkers PF, Medemadagger RH, Lammers JW, Koenderman L and Coffey PJ (2000) Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr. Biol.* 10: 1201–1204
23. Putcha GV, Moulder KL, Golden JP, Bouillet P, Adams JA, Strasser A and Johnson EM (2001) Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. *Neuron* 29: 615–628
24. Shinjyo T, Kuribara R, Inukai T, Hosoi H, Kinoshita T, Miyajima A, Houghton PJ, Look AT, Ozawa K and Inaba T (2001) Downregulation of Bim, a proapoptotic relative of Bcl-2, is a pivotal step in cytokine-initiated survival signaling in murine hematopoietic progenitors. *Mol. Cell. Biol.* 21: 854–864
25. Ley R, Balmanno K, Hadfield K, Weston C and Cook SJ (2003) Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. *J. Biol. Chem.* 278: 18811–18816
26. Akiyama T, Bouillet P, Miyazaki T, Kadono Y, Chikuda H, Chung UI, Fukuda A, Hikita A, Seto H, Okada T, Inaba T, Sanjay A, Baron R, Kawaguchi H, Oda H, Nakamura K, Strasser A and Tanaka S (2003) Regulation of osteoclast apoptosis by ubiquitination of proapoptotic BH3-only Bcl-2 family member Bim. *EMBO J.* 22: 6653–6664
27. Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, Puthalakath H, Pellegrini M, Cory S, Adams JM and Strasser A (2002) BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature* 415: 922–926
28. Villunger A, Scott C, Bouillet P and Strasser A (2003) Essential role for the BH3-only protein Bim but redundant roles for Bax, Bcl-2, and Bcl-w in the control of granulocyte survival. *Blood* 101: 2393–2400
29. Villunger A, Michalak EM, Coultas L, Mullauer F, Bock G, Ausserlechner MJ, Adams JM and Strasser A (2003) p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 302: 1036–1038
30. Jeffers JR, Parganas E, Lee Y, Yang C, Wang J, Brennan J, MacLean KH, Han J, Chittenden T, Ihle JN, McKinnon PJ, Cleveland JL and Zambetti GP (2003) Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* 4: 321–328
31. Itakura A, Miura Y, Hikasa Y, Kiso Y and Matsuda H (2001) Interleukin-3 and stem cell factor modulate cell cycle regulatory factors in mast cells: negative regulation of p27Kip1 in proliferation of mast cells induced by interleukin-3 but not stem cell factor. *Exp. Hematol.* 29: 803–811
32. Yoshikawa H, Nakajima Y and Tasaka K (1999) Glucocorticoid suppresses autocrine survival of mast cells by inhibiting IL-4 production and ICAM-1 expression. *J. Immunol.* 162: 6162–6170
33. Ranger AM, Zha J, Harada H, Datta SR, Danial NN, Gilmore AP, Kutok JL, Le Beau MM, Greenberg ME and Korsmeyer SJ (2003) Bad-deficient mice develop diffuse large B cell lymphoma. *Proc. Natl. Acad. Sci. U.S.A.* 100: 9324–9329
34. Bouillet P and Strasser A (2002) BH3-only proteins – evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. *J. Cell. Sci.* 115: 1567–1574
35. Kitaura J, Asai K, Maeda-Yamamoto M, Kawakami Y, Kikkawa U and Kawakami T (2000) Akt-dependent cytokine production in mast cells. *J. Exp. Med.* 192: 729–740
36. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J and Greenberg ME (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96: 857–868
37. Adams JM (2003) Ways of dying: multiple pathways to apoptosis. *Genes Dev.* 17: 2481–2495
38. Young JD, Liu CC, Butler G, Cohn ZA and Galli SJ (1987) Identification, purification, and characterization of a mast cell-associated cytolytic factor related to tumor necrosis factor. *Proc. Natl. Acad. Sci. U.S.A.* 84: 9175–9179
39. Puthalakath H, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, Cheney RE, Huang DC and Strasser A (2001) Bim: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* 293: 1829–1832
40. O'Reilly LA, Print C, Hausmann G, Moriishi K, Cory S, Huang DC and Strasser A (2001) Tissue expression and subcellular localization of the pro-survival molecule Bcl-w. *Cell Death Differ.* 8: 486–494