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MULTI-AUTHOR REVIEW

Crossing paths: interactions between the cell death machinery and growth factor survival signals

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Abstract Cytokines and growth factors play a crucial role in the maintenance of haematopoietic homeostasis. They transduce signals that regulate the competing commitments of haematopoietic stem cells, quiescence or proliferation, retention of stem cell pluripotency or differentiation, and survival or demise. When the balance between these commitments and the requirements of the organisms is disturbed, particularly when it favours survival and proliferation, cancer may result. Cell death provoked by loss of growth factor signalling is regulated by the Bcl-2 family of apoptosis regulators, and thus survival messages transduced by growth factors must regulate the activity of these proteins. Many aspects of direct interactions between cytokine signalling and regulation of apoptosis remain elusive. In this review, we explore the mechanisms by which cytokines, in particular Interleukin-3 and granulocyte–macrophage colony-stimulating factor, promote cell survival and suppress apoptosis as models of how cytokine signalling and apoptotic pathways intersect.

Keywords Cytokines · Growth factors · Apoptosis · Haematopoiesis · Bcl-2 family · Tumorigenesis

Introduction

The average lifespan of a red blood cell in humans is 3–4 months, platelets live 3–5 days, and granulocytes 1 or 2 days, perhaps even less. Some lymphocytes may live many years, others only a few days. Yet, for the most part, in healthy individuals, the number of cells remains fairly constant. At times of stress, for example in response to infection or depletion of granulocytes and lymphocytes after cancer chemotherapy, vast numbers of new cells need to be generated. When infections are resolved or cells are adequately replaced, cells that are no longer required are removed and the rate of replacement scaled back. During these processes, haematopoietic stem cells (HSC) face decisions about self-renewal, to proliferate and to differentiate into various types of mature, functional blood cells, such as granulocytes, erythrocytes, megakaryocytes, monocytes and lymphocytes. Haematopoietic growth factors are a critical part of the mechanisms that maintain the HSC population and that regulate this homeostasis. For example, erythropoietin is required for normal development of red blood cell progenitors and increased levels are observed in response to anaemia (or in professional cyclists) to raise red cell numbers [1–3]. In contrast, studies in which the gene for granulocyte–macrophage colony-stimulating factor (GM-CSF) has been deleted suggest that this cytokine has a minor or redundant role in haematopoiesis under normal conditions [4, 5,] but does promote survival and self-renewal of granulocyte progenitors, enhances survival of mature neutrophils and induces proliferation and increases numbers of neutrophils, eosinophils and monocytes in response to stress [6–8].

Programmed cell death or apoptosis is one of the normal fates of HSC. HSC and their progeny rely on signals from growth factors for survival, and when those signals are lost, an endogenous cell death program is activated and cells rapidly die and are engulfed by phagocytes. Failure to die has a profound effect on normal homeostasis and may contribute to oncogenic transformation. Perhaps one of the

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most striking examples of this is when B lymphocytes acquire the t(14:18) translocation. As a result, cells overexpress Bcl-2, which blocks cell death in these lymphocytes. The outcome is the development of follicular lymphoma [9]. The identification of Bcl-2 as a transforming oncogene in follicular lymphoma, and subsequent demonstrations that Bcl-2 functions as an apoptotic inhibitor, established the paradigm that inhibition of cell death is part of the pathway to malignant transformation.

Growth factors such as GM-CSF and Interleukin-3 (IL-3) promote survival of haematopoietic cells, and depriving dependent cells of these signals results in apoptosis that can be blocked by Bcl-2. The clear implication is that GM-CSF and IL-3 signalling must, in some way, regulate the activity of Bcl-2 and related family members. As we shall discuss, there is substantial evidence to support this hypothesis but there also are suggestions that growth factor survival signalling exerts its effects independently of the Bcl-2 family.

Cytokines: IL-3, IL-5 and GM-CSF

IL-3, GM-CSF and interleukin-5 (IL-5) are related cytokines because their heteromeric receptors share a common ß chain. They are produced by several cell types, including activated T cells and mast cells, and have important roles in maintenance of stem cell populations, and immune and inflammatory responses. For example, IL-3 stimulates differentiation and proliferation of pluripotent stem cells and myeloid progenitor cells (which in turn may differentiate into erythrocytes, thrombocytes, granulocytes, monocytes and dendritic cells) and may influence growth and differentiation of T cells in immune responses [10–12]. GM-CSF induces the production of granulocytes (neutrophils, eosinophils and basophils) and monocytes that are capable of maturing into macrophages. IL-5 is primarily involved in the eosinophilic response observed following some infections [13–15].

The receptors for IL-3, IL-5 and GM-CSF consist of a heterodimeric complex of a specific ligand-binding alpha chain (αc) and a common beta chain (βc) [16, 17]. Activation of the receptor by GM-CSF is now understood at a structural level and currently serves as the model for signal activation for IL-5 and IL-3 [18, 19]. Following association of the ligand with α-chain, there is subsequent interaction between the ligand bound α-chain and the βc subunit leading ultimately to the assembly of a higher order, dodecameric signalling complex. Unlike other tyrosine kinase receptors, the βc has no intrinsic tyrosine kinase activity. Instead, each βc subunit is bound to a member of the Janus kinase family, JAK2. As the active signalling complex is assembled, the JAK2 molecules are brought into close proximity, allowing transphosphorylation of JAK2 and activation of signalling [20, 21].

The physiological roles of this family of cytokines have been established both by deleting the gene for the βc chain of the receptor in mice and by specific deletions of ligands. Deletion of βc had little if any impact on haematopoietic cell numbers but there were clear defects in mature cell function. The most prominent phenotype was delayed clearance of surfactant from the lungs resulting in an alveolar proteinosis, as a result of abnormal phagocyte function [22, 23]. When irradiated mice were transplanted with marrow from animals lacking βc, they were significantly slower to restore granulocyte population. In addition, GM-CSF and IL-5 were important in normal response to certain infections [4, 14, 24]. However, it is the established clinical utility of GM-CSF, and more recently G-CSF, which are used to treat significant granulocytopaenia, for example following chemotherapy [25], that underpins the importance of these signalling pathways.

GM-CSF, IL-3 and malignancy

In their much-cited paper in 2000, Hanahan and Weinberg suggested that most cancers have acquired, by mechanisms that vary from one cancer to another, a common set of functional capabilities [26]. The constitutive activation of growth factor signalling or abnormal expression of growth factors, such as IL-3 or GM-CSF, may contribute to the acquisition of some of these capabilities, namely proliferative signalling and protection against apoptosis. This is perhaps particularly so in haematopoietic malignancies. The link between GM-CSF and IL-3 signalling and cancer are highlighted in the following examples.

Approximately one-third of acute myeloid leukaemia (AML) and a lesser number of B-cell acute lymphoblastic leukaemia (B-ALL) overexpress the alpha subunit of the IL-3 receptor (IL-3Rα). Although a clear correlation between IL-3Rα expression and blast cells numbers exists, a correlation that has prognostic significance [27], less clear are the signalling consequences of IL-3Rα expression [28]. Constitutive IL-3 signalling alone is sufficient to induce lethal myeloproliferative disease in mice but does not result in a transplantable tumour [29]. An interesting clinical correlate of this experimental observation is juvenile myelomonocytic leukaemia (JMML). This disease is more properly classified as a myeloproliferative disorder, and one characteristic feature is a hypersensitivity to GM-CSF, meaning that monocytic cells can proliferate ex vivo in limiting doses of GM-CSF [30, 31]. However, activating mutations in GM-CSF are not found in this disease. Instead, JMML is typically associated with activating mutations of Ras or inactivating mutations of NF1

or PTPN11. PTPN11 mutations appear to induce hyperactivation of Ras and contribute to hypersensitivity of haematopoietic progenitors to GM-CSF [32].

It appears that oncogenic mutations that result in a phenotype of constitutive activation of growth factor signalling are most commonly identified in downstream signalling molecules. In spite of this, it is the case that IL-3 and GM-CSF receptors might still influence the behaviour of these cells. In Philadelphia chromosome positive (Ph^+ or Bcr-Abl^+) chronic myelogenous leukaemia (CML), elevated levels of GM-CSF and IL-3 and autocrine signalling in $CD34^+$ population can be inhibited by anti-IL-3, anti-GM-CSF or anti-IL3Rα antibodies [33]. Myeloproliferation in tissue culture of JMML can also be blocked by anti-GM-CSF antibodies [34].

Growth factors and Bcl-2 family members: the link for cell survival

It is self-evident that because GM-CSF and IL-3 maintain the viability and proliferation of some haematopoietic cell populations and cells default to apoptosis when growth factor is removed, the signals transduced by these cytokines suppress activation of apoptosis pathways. The main question is how. It is worth considering this question in a historical context as it throws some light onto the subsequent progress of research in this area. Bcl-2, the founding member of the Bcl-2 family of apoptosis regulators, was cloned at the breakpoint of the t(14:18) translocation associated with follicular lymphoma and demonstrated to be the oncogene responsible for this tumour [9, 35]. However, it was unclear how Bcl-2 functioned. Another line of research, directed to the identification and understanding of growth factors' characteristics, used haematopoietic progenitors derived from mouse bone marrow, serially passaged in conditioned media derived from the WEHI3B tumour line (which produces high amounts of IL-3). Several haematopoietic cell lines were derived, including FDCP-1 cells, and were dependent on conditioned media (IL-3) for proliferation and survival [36]. FDCP-1 cells proved a useful tool in assessing potential oncogenes. For example, overexpression of Bcr-Abl permitted these cells to survive and proliferate in the absence of IL-3 [37]. When Bcl-2 was overexpressed in FDCP-1 cells, it was evident that they were not able to proliferate in the absence of IL-3. However, strikingly, neither did these cells die when deprived of growth factor. Once IL-3 signalling was restored to these cells, proliferation recommenced. Bcl-2 thus maintained cell viability and regulated the cell death response to growth factor deprivation [38, 39]. It has since been demonstrated several times that Bcl-2 and related genes such as Bcl-x_L can block apoptosis in response to virtually all models of growth factor (or serum) deprivation. Direct regulation of the activity of the Bcl-2 family would be one obvious mechanism by which growth factors could regulate apoptosis (Fig. 1).

Bcl-2 family

Death of cells promoted by cytokine withdrawal is characterised by mitochondria membrane permeabilisation followed by release of cytochrome c, Smac/Diablo and other factors from the mitochondria to the cytoplasm and finally execution of the cells by activated caspases [40]. The Bcl-2 family of proteins function as central regulators of apoptosis by promoting or blocking changes in mitochondrial membrane permeability and, consequently, the release of pro-apoptotic factors and activation of caspases. The Bcl-2 family are so classified by the presence of one or more Bcl-2 homology (BH) domains, and members are divided into two major groups, the inhibitors of apoptosis and the inducers of cell death. The pro-apoptotic proteins required for initiation and execution of apoptosis includes the Bax death family and the BH3-only proteins [41]. The BH3-only members are activated by intracellular stresses

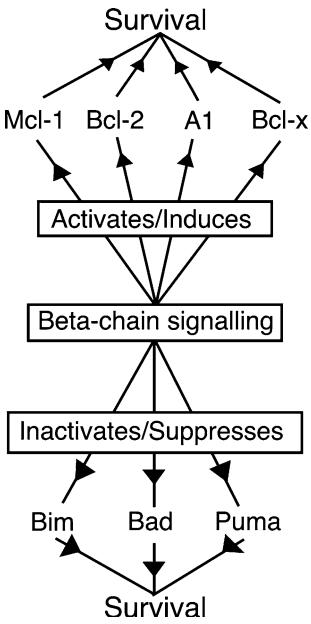

Fig. 1 Cytokine signalling and Bcl-2 family members. One of the mechanisms by which cytokine signalling promotes cell survival is through inhibition of apoptotic pathways. Bcl-2 family members are crucial for the regulation of cell death signalling. Activation of beta-chain signalling can suppress expression of BH3-only pro-apoptotic proteins, in particular, Bim, Puma and Bad. Induction and maintenance of anti-apoptotic proteins Mcl-1, Bcl-2, A1 and Bcl-x during activation of cytokine signalling is also crucial to prevent cell death induced by cytokine deprivation

that constitute a death stimulus and then function to directly suppress anti-apoptotic Bcl-2 proteins. The Bax-like proteins, in particular Bax and Bak, are essential to induce mitochondria disruption and apoptosis and do so by undergoing conformational changes [42–45]. Activation of these proteins is initiated by BH3-only proteins in two major ways, through neutralisation of anti-apoptotic Bcl-2 proteins and by direct activation of Bax and Bak [46, 47].

Expression and activation of Bax-like proteins play a central role in apoptosis mediated by growth factor deprivation. Withdrawal of IL-3 in IL-3-dependent cells results in translocation of Bax from the cytosol to the mitochondria, conformational change in Bax and induction of apoptosis [48]. However, even though IL-3-dependent cells from Bax-null mice fail to activate caspases in the first 24 h after IL-3 removal, apoptosis induced by IL-3 deprivation was only delayed in these cells, indicating that cell death mediated by growth factor withdrawal is not absolutely dependent on the expression and activation of Bax, although it is sufficient to induce apoptosis [49]. This is also true for IL-3-dependent cells lacking only Bak. However, in cytokine-dependent cells lacking both Bax and Bak, cell death mediated by growth factor withdrawal is completely abolished. Importantly, surviving cells retain the ability to proliferate and form colonies when IL-3 is restored, in much the same way as cells overexpressing anti-apoptotic Bcl-2 protein. Activation of Bax or Bak is therefore the key step in the commitment to apoptosis mediated by cytokine withdrawal [50, 51]. Thus, regulation of conformational changes and activation of Bax/Bak is the major checkpoint in governing whether cells live or die, which is determined by the interaction with other Bcl-2 family members, the Bax-like proteins and the BH3-only proteins.

Growth factors and anti-apoptotic Bcl-2 family members: partners in life

The anti-apoptotic Bcl-2 family members include Bcl-2, Bcl-x_L, Mcl-1, A1 and Bcl-w. Studies in knock-out mice emphasise the crucial role that anti-apoptotic Bcl-2 family members play in normal development. Bcl-2 is required for survival of mature lymphocytes and melanocytes, and mice lacking Bcl-2 develop severe kidney disease [52]. Bcl-x_L is important for neuronal and erythroid cells, and mice lacking Bcl-x_L die during embryonic development [53, 54]. Mcl-1-deficient mice suffer a similar fate but even earlier in development, since Mcl-1 is essential for early haematopoiesis and later stages of granulocyte differentiation [55]. A1 is required for the survival of mature B cells and neutrophils whereas Bcl-w is necessary for the normal development of sperm progenitors in adult mice [56, 57].

What evidence is there that cytokine signalling regulates the levels or function of anti-apoptotic Bcl-2 family members? Overexpression of Bcl-2 family members in cytokine-dependent cells can take one only so far in considering this question as induced expression of the anti-apoptotic Bcl-2 family members will block apoptosis. But this does not indicate that growth factors block apoptosis by upregulating the levels of Bcl-2 family members [58, 59]. However, in the case of at least one Bcl-2 family member, Mcl-1, there is substantial evidence to suggest that growth factors, in particular GM-CSF, critically regulate Mcl-1 levels particularly in haematopoietic cells.

GM-CSF signalling pathways tightly control Mcl-1 expression in TF-1 myeloid progenitor cells. Upon deprivation of GM-CSF from these cells, Mcl-1 protein levels dramatically decline as a result of proteosomal degradation followed by loss of cell survival. Restimulation of TF-1 cells with GM-CSF immediately induced Mcl-1 mRNA followed by resynthesis of the protein. Downregulation of Mcl-1 by antisense constructs antagonised, in part at least, cell survival signalling transduced through the GM-CSF receptor. Moreover, truncation mutants of the GM-CSF receptor revealed that a region between amino acids 573 and 755 of the receptor β chain was required for Mcl-1 induction [60]. Other studies have shown that addition of GM-CSF to neutrophils is able to delay apoptosis of these cells by stabilising and increasing intracellular levels of Mcl-1 [61, 62]. Together, these analyses suggested that Mcl-1 is an immediate-early gene activated by the GM-CSF signalling pathway and may therefore be one of the major components of the mechanism by which GM-CSF and similar cytokines maintain survival.

Clearly, there is a transcriptional upregulation of Mcl-1 by growth factors but it remains unclear what transcription factors are required. Another remaining question is how does loss of GM-CSF result in Mcl-1 degradation? Mcl-1 is phosphorylated by GSK-3, leading to Mcl-1 ubquitinylation and degradation. In the presence of GM-CSF or IL-3 signalling, GSK-3 activity is suppressed by PI3K/AKT. Once the signal is lost, GSK becomes activated and capable of phosphorylating Mcl-1 which is the target for degradation [63]. It seems likely that, once GM-CSF signalling is lost, several parallel pathways rapidly lead to Mcl-1 inactivation and degradation. Other studies suggests that inhibition of Mcl-1 activity may be independent of its degradation. Antagonism by BH3-only proteins and disruption of Mcl-1 binding to Bak or Bax may be sufficient to inactivate Mcl-1 [64, 65] and induce cell death. Degradation of Mcl-1 is not absolutely required for its inactivation [65].

Bcl-2 and Bcl-x_L may also be regulated by phosphorylation mediated by kinases activated by cytokine signalling or its loss. Phosphorylation of Bcl-2 by PKC-α or ERK1/2

appears to be required for survival mediated by IL-3 signalling [66–68]. However, the functional significance of phosphorylation or dephosphorylation of Bcl-2 and Bcl-x_L in the control of apoptosis responses remains controversial. We will discuss the kinases and their role in growth factor-mediated survival and death more specifically later in the review.

BH3-only: opposing survival factors signalling

To date, at least ten mammalian BH3-only proteins have been described, among them, Bad, Bid, Bim, Bmf, Noxa, Puma and Hrk. Using knockout mice and cell lines derived from these mice, roles for each in a range of apoptotic stimuli, including cytokine deprivation, antigen receptor signalling, oncogenes activation and chemotherapeutic drugs, have been described [51, 69–72]. From these studies, it has been established that the BH3-only proteins Bad, Bim and Puma are involved in cell death mediated by loss of growth factor signals, particularly in haematopoietic cells.

The BH3-only protein Bad is able to bind and inhibit the function of Bcl-x_L, Bcl-2 and Bcl-w but not of Mcl-1 or A1 [73]. In studies using IL-3-dependent cells overexpressing Bad, it was evident that the ability of Bad to bind and neutralise Bcl-x_L was being regulated during growth factor signalling. Bad was phosphorylated at two critical serine residues, in a manner that could be blocked by PI3K inhibitors, and when phosphorylated formed a complex with the chaperone protein 14-3-3 in the cytosol [74, 75]. Bad bound to 14-3-3 was unable to bind to Bcl-x_L. The sequestering of Bad by 14-3-3 was reversed in the absence of IL-3, as a consequence of PI3K inactivation, Bad was no longer phosphorylated and was thus free to bind and suppress Bcl-x_L. Surprisingly, IL-3-dependent cells derived from Bad null mice remained susceptible to apoptosis induced by IL-3 deprivation indicating that Bad was redundant for IL-3 deprivation-induced apoptosis, at least in this model [51]. Even though deletion of Bad has been associated with malignant transformation, resistance to cytokine deprivation does not seem to be the mechanism by which tumour development occurs.

Bim-mediated apoptosis plays an important role in the regulation of haematopoetic cell numbers since Bim-deficient mice accumulate abnormally high numbers of lymphoid and myeloid cells [72]. These animals are particularly susceptible to lymphoid tumours in the presence of another oncogenic stimuli, such as the overexpression of c-myc [76]. In vitro, several compelling lines of experimental evidence suggest an important role for Bim in apoptosis mediated by cytokine deprivation in different cell types. Bim expression is necessary for apoptosis induced by IL-2 deprivation in human T cell lymphoblasts, since downmodulation of Bim expression by siRNA in these cells increased cell survival in the absence of IL-2 [77]. In mice, Bim levels increased in activated T cells deprived of IL-2 and apoptosis is delayed and partially inhibited in activated T cells derived from *bim*$^{-/-}$ mice [78]. IL-3-dependent mast cells derived from bone marrow of Bim-deficient mice also survive IL-3 deprivation [79]. However, the protective effect of Bim deletion is not observed in all models of IL-3 deprivation [51].

There are several potential mechanisms by which Bim is activated when growth factor signalling is lost. In sympathetic neurones deprived of nerve growth factor (NGF), Bim expression increased and could be suppressed by a dominant negative of c-Jun. This suggests a possible pathway in which c-Jun, activated by NGF signalling, transcriptionally represses Bim expression [80]. In haematopoietic cells, Bim may be transcriptionally regulated by the forkhead transcription factors (FoxO) [81]. In another models, lack of cytokine signalling promoted dephosphorylation of FoxO3a, as a result, loss of PI3K/AKT activity, leading to FoxO3a translocation to the nucleus and transcriptional upregulation of Bim and Puma [81, 82]. Deletion of FoxO3a did not completely abolish expression levels of these BH3-only proteins but partially prevented apoptosis induced by growth factor withdrawal [83]. Clearly, other transcription factors regulate Bim expression in response to cytokine deprivation and other apoptotic stimuli, and transcriptional regulation of Bim is more complex than our current understanding.

Bim is also regulated post-translationally by sequestration to cytoskeletal structure of the cells. In normal conditions, Bim isoforms Bim_{EL} and Bim_L are sequestered into dynein motor complex through a direct interaction with LC8. In IL-3 deprivation, the localisation of Bim to microtubules by virtue of the interaction with LC8 is disrupted by cytoskeletal changes that occur early (prior to caspase activation) in cytokine deprivation [84]. This mechanism is not confined to IL-3 deprivation and appears to be a more general mechanism by which Bim senses cytoskeletal alteration as an early indicator that a cell is sufficiently compromised and should now activate the apoptotic program.

Bim protein levels and function can be regulated by post-translational modification, in particular phosphorylation [85, 86]. Serum stimulation induces a MAPK-dependent (ERK1/2) phosphorylation of serine residues in exon 2, in particular serine 65 targets Bim for degradation but does not substantially alter Bim interactions with anti-apoptotic Bcl-2 family members. JNK-dependent phosphorylation at threonine 112 (T112), in response to UV irradiation for example, appears to be important for Bim-Bcl-2 binding, and mutation of this residue to alanine

diminishes the proapoptotic activity of Bim [86]. This may be because Bim phosphorylated at T112 is no longer bound to the dynein motor complex. Cytokine deprivation results in phosphorylation of Bim at T112 as part of the mechanism of Bim activation. The presence of a survival signal activates kinases that phosphorylate BimEL as a means of reducing the total input of Bim in the cell (Fig. 2). It remains to be determined how true this is in the context of IL-3 and GM-CSF signalling.

Puma/Bbc3 was identified as a BH3-only protein transcriptionally regulated by p53. This BH3-only protein is essential for hematopoietic cell death triggered by several stimuli such as ionising radiation (IR), deregulated c-Myc expression and cytokine withdrawal [87, 88]. Puma-deficient hematopoietic cells derived in a variety of ways resist growth factor deprivation both in short-term and clonogenic assays, indicating Puma functions to ensure cells commit to apoptosis in the absence of cytokines [51, 87, 88]. Inhibition of Puma expression by treatment of cells with shRNA attenuated cell death mediated by IL-3 withdrawal [89]. Puma, like Bim (but unlike Bad), can bind and inhibit all anti-apoptotic Bcl-2 family members, and Puma and Bim may cooperate to induce apoptosis in some instances, since *puma*$^{-/-}$ or double knockout *puma*$^{-/-}$ *bim*$^{-/-}$ mast cells or activated T cells appear to be more resistant than *bim*$^{-/-}$ to cytokine withdrawal. On the other hand, in a model of IL-3 deprivation, *puma*$^{-/-}$ cells were clonogenically protected from IL-3 deprivation whereas *bim*$^{-/-}$ cells behaved like wild-type cells [90].

Most evidence suggests that Puma is transcriptionally regulated in response to cytokine deprivation. Protein and mRNA levels increase in myeloid progenitors after IL-3 deprivation [90]. Several transcription factors appear to be involved in Puma regulation in response to different death stimuli. p53 and the SNAIL family member Slug regulate Puma expression following gamma irradiation. In response to cytokine deprivation, Puma is regulated, in part, by FoxO3a, which may also regulate Bim expression. Whilst cytokine deprivation is not typically thought of as a p53-dependent death stimulus, experimental evidence indicates that p53$^{-/-}$ myeloid progenitor cells have increased survival in limiting cytokine concentration [91] and that shRNA knockdown of p53 increases survival and decreases Puma expression in IL-3-starved FL5.12 cells [89]. It

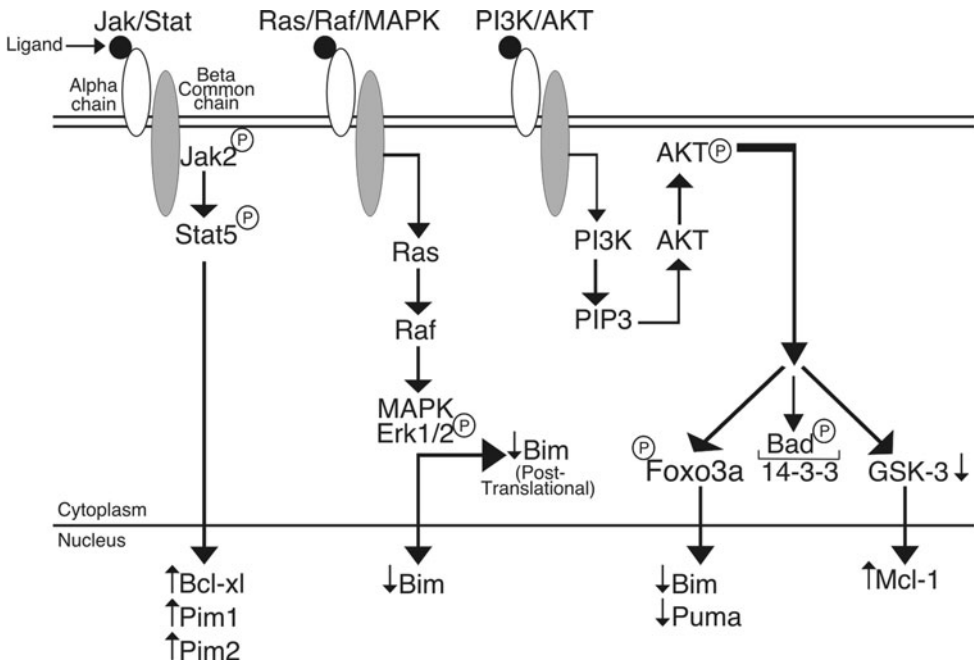

Fig. 2 Downstream signalling events after receptor activation. Cytokine stimulation results in initiation of several signalling cascades influencing the transcription and post-translational modification of certain downstream targets. Three well-examined signalling pathways that are activated by IL-3 or GM-CSF via beta-chain activation are briefly illustrated here. *Jak/Stat* pathway: receptor activation leads to JAK2 phosphorylation, in turn phosphorylating the Stat family proteins which translocates to the nucleus to control genes responsible for survival and proliferation, such as Bcl-x_L, Pim1 and Pim2. *Ras/Raf/MAPK* pathway: receptor activation induces phosphorylation and activation of Ras, which in turn activates Raf and the MAP kinases. Post-translational as well as possible transcriptional regulation of Bim is controlled in part by the Ras/Raf/MAPK pathway. *PI3K/AKT* pathway: activation of PI3K through cytokine signalling induces activation and phosphorylation of AKT that in turn phosphorylates and inactivates Bad by binding to 14-3-3. FoxO3a is also phosphorylated, in turn negatively regulating the BH3 only proteins, Bim and Puma. Phosphorylated AKT also regulates GSK-3, indirectly maintaining Mcl-1 levels

remains unknown whether Puma, like Bim, is also subject to post-translational regulation. It is clear however, that unlike Bim, Puma is always localised to mitochondria [92].

Overall, the withdrawal of IL-3 or GM-CSF from dependent cell lines has the net effect of diminishing levels of at least one anti-apoptotic Bcl-2 protein whilst increasing levels and activation of pro-apoptotic family members, in particular the BH3-only proteins. The balance is tipped in favour of activation of Bax and Bak and cell death.

Tracing the pathway back: from Bcl-2 family members to the receptor

At the level of Bcl-2 family members, there is now a relatively detailed understanding of interactions between family members and how the nature of these interactions can favour cell survival or cell death. Although several mechanisms that might regulate Bcl-2 family members in response to cytokine signalling or cytokine deprivation have been described, it is this connection between growth factor signalling and regulation of apoptotic pathways that remains poorly understood. Whether there is a specific signalling kinase or pathway activation during GM-CSF or IL-3 signalling that regulates apoptosis or whether suppression of cell death is the net effect of many signalling pathways activated by GM-CSF and IL-3 remains to be determined. However, the bulk of evidence would suggest that multiple signalling pathways contribute to interaction between cytokine and apoptosis signalling (Fig. 2). We will discuss the evidence supporting a role for some of these kinases below.

In contrast to other tyrosine receptor kinases, IL-3 and GM-CSF receptors do not have intrinsic tyrosine kinase activity and are dependent on the Janus kinase JAK2 to activate and initiate the multiplicity of signalling cascades that occur after the ligand binds the receptor. Following GM-CSF stimulation and multimerization of the receptor, JAK2 is activated by transphosphorylation and in turn may phosphorylate other tyrosine residues on βc resulting in the recruitment of signalling molecules to the βc and activation of several signalling cascades [21, 93, 94]. Activation of JAK2 leads to βc phosphorylation of several tyrosine residues some of which act as docking sites for signal transduction and activators of transcription (STATs). Phosphorylation of STATs by JAK results in its dissociation from the receptor and translocation to the nucleus to activate gene transcription. In the case of IL-3 and GM-CSF signalling, STAT5 activation appears important since constitutively active mutants of βc result in constitutive STAT5 activation [95]. Several extensive mutational analyses of βc chain have defined regions and residues required for JAK2 activation and downstream STAT activation [96–98]. It is evident that JAK2 activation is required for all signalling outcomes transduced by βc, including proliferation, differentiation and survival. Disruption of STAT5 phosphorylation, by mutation of various tyrosine residues in βc chain, also diminishes proliferative, differentiation and survival signals, although the nature and magnitude of these effects vary with the model used [99, 100]. Likewise, increased apoptotic cell death of haematopoietic cells is observed in STAT5-deficient mice [101]. Several transcriptional targets of STAT5 key to survival signalling have been suggested, including c-fos, pim-1 and Bcl-x_L [102–104].

The Pim serine/threonine kinases, Pim-1 and Pim-2, are important mediators of cytokine signalling and may contribute to the development of solid tumours and certain types of leukaemia [105, 106]. Pim-2 protein levels rapidly decline following IL-3 withdrawal in FL5.12 cells and are induced again after IL-3 restoration. Constitutive expression of Pim-2 maintains haematopoietic cell survival in the absence of IL-3 at a similar level to constitutively active AKT but importantly independent of AKT [107, 108]. This indicates that AKT activation and Pim-2 activation after IL-3 signalling transduce independent survival signals. Exactly how Pim-2 promotes cell survival is unclear because, even though exogenously expressed Pim-2 can phosphorylate the BH3-only protein Bad [107, 108], Bad is redundant for IL-3 withdrawal-induced cell death. Another possible mechanism is suggested by the data that demonstrates NF-κB activation by Pim-2 [109]. Canonical NF-κB signals cell survival through several mechanisms, including regulation of Bcl-2 members [110].

Pim-1, when exogenously expressed, can maintain the survival of haematopoietic cells in the absence of IL-3 and can phosphorylate Bad at serine 112 [111]. Activation of STAT-3 and -5 by GM-CSF signalling may induce Pim-1 expression in human eosinophils and suppresses apoptosis of these cells [104]. Studies using pharmacological inhibitors of Pim kinases and overexpression of dominant-negative of Pim have demonstrated that Pim kinase activity is required for IL-3-mediated survival of basophils [112]. Once again, it remains to be determined precisely how Pim-1 can mediate factor independent cell survival.

The PI3K and AKT kinase pathway has been the focus of considerable attention with regard to cytokine-mediated survival signalling, and it has been previously mentioned in this review. In the presence of IL-3 or GM-CSF, PI3K is activated and generates PIP_3 which recruits AKT to the cell membrane promoting its phosphorylation and activation [113]. Once activated, AKT phosphorylates many different molecules that contribute to the suppression of apoptosis (Fig. 2). The principal line of evidence that support the role of PI3K/AKT survival signals are that PI3K inhibitors cause apoptosis in IL-3 or GM-CSF-dependent cells and

overexpression of constitutively activated AKT promotes growth factor-independent cell survival [114]. As previously mentioned, AKT can directly phosphorylate and inactivate Bad, phosphorylate and inactivate FoxO3a (which in turn may regulate Bim and Puma) and suppress GSK-3 activity, and so maintain Mcl-1 levels. It seems likely that all such effects of PI3K/AKT activation, and perhaps many others, contribute to the survival effect of AKT, and much remains to be learned. There are three isoforms of AKT, AKT1, AKT2 and AKT3, and little is known about which isoform phosphorylates which targets to contribute to cell survival. Interestingly, deletion of single isoforms of AKT has no impact on haematopoietic cell survival. PI3K inhibitors and AKT-specific inhibitors may have therapeutic utility in the treatment of several malignancies, including AML. Whilst neither PI3K nor AKT are often the subject of activating mutations in AML, both are often constitutively activated in the disease, even in the absence of mutation [115].

Overexpression of constitutively active AKT can partially mimic the survival effects but not the proliferative effects of IL-3 signalling. Also, like IL-3 signalling, AKT maintains the expression of nutrient transporter proteins and uptake of many essential nutrients into cells, an effect in large part mediated via activation of mTOR [116]. Indeed, in IL-3-dependent FL5.12 cells, AKT-dependent survival in the absence of IL-3 was completely dependent on the presence of glucose in the media [117]. Such observations clearly indicate that AKT regulates survival by maintaining adequate substrates for vital intracellular processes, quite independently of any effect on the expression or activation of Bcl-2 family members or other apoptosis proteins. By extension, the manner in which cytokines maintain cell viability may also be mediated by similar metabolic effects. Equally clear, however, is the fact that apoptosis pathways are activated in the absence of IL-3 or following the deprivation of metabolic substrates such as glucose. Even if the primary effect of cytokine deprivation is diminished intracellular levels of nutrients, this is still a trigger for the activation of apoptotic pathways. Indeed, the elevated Puma expression that follows IL-3 deprivation can be blunted by enforced expression of the glucose transporter Glut1 or the provision of a freely diffusible glucose substrate, methyl-pyruvate [89]. The question then becomes not which survival kinases regulate apoptosis pathways, but how are apoptosis pathways activated by limited metabolic substrates? It is perhaps most likely that cytokine signalling both maintains cellular nutrient uptake and directly regulates the propensity of apoptosis pathways to activate.

In addition to activation of Jak/STAT and PI3K/AKT pathways, IL-3 signalling may also activate the Ras/Raf/MAPK pathway to promote cell survival, as previously mentioned in post-translational modification of Bim. Amplification of *ras* proto-oncogene and activating mutations leading to expression of constitutively active form of Ras and/or Raf are found in 30% of human cancers, such as melanomas and some leukaemias. These mutations are strongly associated with the ability of cells to proliferate and survive in the absence of growth factors. Overexpression of an active mutant of Ras (Ras G12V) prevented apoptosis mediated by IL-3 withdrawal, although it did not induce a significant IL-3-independent proliferation [118]. Furthermore, inhibition of MAPK activity decreased viability in limiting doses of IL-3 [119]. The mechanisms by which MAPKs mediate cell survival remain to be determined. Although, like other signalling kinases, some evidence supports regulation of expression or activation of Bcl-2 family members such as Bim and Bcl-x_L [120].

The activation of several kinases and different signalling pathways by cytokines leads to multiple synchronised change in a cell to control the balance between life and death. The exact mechanisms and signalling pathways that are activated remain controversial. Although Bcl-2 family members appear to be one of the key points for regulation of survival mediated by growth factors, cells lacking Bax and Bak, still succumb eventually to IL-3/substrate deprivation, indicating that activation of apoptotic pathways is critical in situations where rapid and efficient execution of apoptosis is required. However, in the long term, many other signalling pathways may also contribute to the suppression of apoptosis. Understanding the multiple signals and identifying specific protein modifications in cytokine signalling will add to our knowledge of molecular interactions involved in maintenance of haematopoiesis and growing tumours.

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