Cytokines and Cell Death

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It has long been known that many cell types are dependent on specific cytokines that signal proliferation, regulate differentiation and suppress apoptosis. A detailed picture of the structure of several cytokine receptors has added greatly to our understanding of the molecular mechanism of receptor activation. At the other end, the explosion of knowledge of apoptosis pathways and the function of the Bcl-2 family has deepened the understanding of the effector arm of the programmed cell death pathway. However, the links between these two pathways are still unclear. I am reminded of a professional magician friend performing the levitation illusion. He drapes a member of the audience, usually a long-haired and beautiful woman, with a sheet that leaves her head and legs exposed. He then proceeds to 'levitate', her hair dangling free at one end, her legs in mid-air at the other. All the action is, of course, taking place beneath the sheet. In this article, my aim is to try and peek under the sheet and get a glimpse of the intersections between cytokine signalling pathways and apoptosis pathways, with particular reference to signalling by the haematopoietic cytokines interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF).

IL-3 and GM-CSF

IL-3 and GM-CSF are haematopoietic cytokines involved in normal haematopoiesis, including the maintenance and proliferation of myeloid progenitor cells and the regulation of myeloid differentiation (1). The receptor for each cytokine is a heterodimeric complex of a common beta chain (βc) and a cytokine-specific alpha chain. Loss of IL-3 or GM-CSF signalling in dependent cells results in apoptosis, characterised by mitochondrial outer membrane permeabilisation and displacement of cytochrome *c* and other factors from mitochondria to cytoplasm. Cytochrome *c* acts as a cofactor in the formation of a multimeric complex of the adaptor molecule Apaf-1 and procaspase-9, known as the apoptosome (2). This complex is the apex of the cascade that activates cell death proteases (caspases). Apoptosis in response to growth factor deprivation is probably a universal response in all cells when they lose essential survival signals. An ability to evade this response, acquired by mutation, contributes to malignant transformation. For example, the translocation associated with follicular lymphoma results in the overexpression of the pro-survival Bcl-2 protein in B-cells that blocks this apoptotic response (3), and in experimental models of malignancy, the inhibition of apoptosis cooperates with other oncogenes to accelerate the development of tumours (4).

Bcl-2 Family and Growth Factor Deprivation – a Historical Perspective

The WEHI3B cell line is a myeloid leukaemic cell line, in which the oncogenic changes arose from retroviral insertions leading to the abnormal expression of IL-3 and the homeobox gene HoxB8. Before IL-3 was eventually purified from this conditioned media, it was recognised that it could support the growth of bone marrow-derived cells. Continuous culture of bone marrow-derived haematopoietic cells in WEHI3B-conditioned media was used to derive the IL-3-dependent line FDC-P1 (5). FDC-P1 cells provided a useful tool in the analysis of oncogenes, such as Bcr-Abl, which permitted these IL-3-dependent cells to proliferate in the absence of IL-3 (6). It was in this model that a newly described proto-oncogene, Bcl-2, cloned from the breakpoint of follicular lymphoma, was tested. When David Vaux, then at the Walter and Elisa Hall Institute, over-expressed Bcl-2 in FDC-P1 cells, he found that cells were not able to proliferate in the absence of IL-3 (7). Astonishingly, however, these cells did not die either. They remained quiescent, arrested in the G0/G1 phase of the cell cycle, but were viable and able to proliferate again when IL-3 was restored. The implications were profound: Bcl-2 inhibited the cell death response to IL-3 deprivation and blocking this pathway contributed to oncogenesis. Further, IL-3 suppressed the activation of a death pathway (separate from proliferation pathways) that was primed to kill cells should they lose the IL-3 signal.

At One End of the Sheet – the Bcl-2 Inhibitible Pathway

The Bcl-2 family are subdivided into pro-survival and pro-apoptotic family members. It is the interaction between various Bcl-2 family members that determines whether a cell is configured for survival or death and these are described by Grant Dewson in this Showcase on Research. The question is: how do cytokines, such as IL-3 or GM-CSF, inhibit the activation of the death pathway to keep cells configured for survival, and what happens to cause a switch to death when cytokines are removed? One approach to this question is to identify the Bcl-2 family members involved in regulating apoptosis in response to cytokine degradation, and then determine how cytokine signalling regulates these proteins.

Cells lacking both of the two key pro-apoptotic Bcl-2 family members, Bax and Bak, are completely resistant to IL-3 withdrawal, and like cells overexpressing Bcl-2, proliferate again when growth factor is restored (8,9). Regulation of the activation of Bax and Bak is therefore a key step in the commitment to apoptosis. The BH3-only proteins (pro-apoptotic members of the Bcl-2 family containing a single Bcl-2 homology domain) function as sensors of cellular stresses. They initiate apoptosis by repressing the anti-apoptotic proteins like Bcl-2, Bcl-x_L and Mcl-1, and may also, in certain cases, directly bind and activate Bax and Bak. One hypothesis is that the activity of BH3-only proteins is regulated by cytokine signalling pathways and this is the link between cytokine signalling and the apoptosis pathway.

Pick a BH3-only Protein, Any BH3-only Protein?

Experiments in IL-3-dependent cells suggest that three of the ten mammalian BH3-only proteins, Bad, Bim and Puma, are involved in detecting loss of growth factor signals. Of these, the experimental evidence for Bad is probably the weakest. Co-immunoprecipitation experiments demonstrated that Bad binds the chaperone protein 14-3-3 in a manner dependent on the phosphorylation of Bad at two critical serine residues by protein kinase B (PKB or AKT). Furthermore, dephosphorylated Bad was no longer able to bind 14-3-3 and was free to bind and inhibit the prosurvival protein Bcl-x_L (10,11). This suggests a model whereby IL-3 signalling, by activating AKT, represses apoptosis by inactivating Bad. One of the problems with this model is that IL-3-dependent cells derived from Bad-deficient mice remained susceptible to apoptosis provoked by IL-3 deprivation (8). Clearly, deletion of Bad can contribute to malignant transformation (12), but resistance to growth factor deprivation may not be the mechanism.

In contrast, prolonged survival after cytokine deprivation is observed in several cell types derived from both *Bim*$^{-/-}$ and *Puma*$^{-/-}$ mice (8,13-15). In the peripheral blood and spleens of Bim-deficient mice, there are increased populations of most haematological cell types, presumably as a result of diminished apoptosis. Activated thymocytes from *Bim*$^{-/-}$ mice survive interleukin-2 (IL-2) deprivation and Bim-deficient mast cells survive IL-3 deprivation. Puma-deficient myeloid precursor cells or mast cells survive cytokine deprivation and proliferate again when cytokine is restored. The data suggest that both Bim and Puma 'sense' the loss of cytokine signalling. How does this happen? Not surprisingly, evidence exists for several potential mechanisms.

Post-translational modification, more specifically phosphorylation, of Bim by kinases activated in cytokine signalling may regulate Bim activity (reviewed in 16). One Bim isoform, Bim_{EL} (Bim 'extra-long'), is phosphorylated on specific serine residues by ERK1/2 and by JNK. The effect of such post-translational modifications of Bim is to regulate Bim turnover and its interactions with other proteins. Cytokine signalling activates kinases that, among other substrates, phosphorylate any Bim that may be present and prevent it from promoting apoptosis. This may explain how Bim may exist at detectable levels in some cells without inducing apoptosis. However, the true physiological role of Bim regulation by phosphorylation *in vivo* is yet to be established. To date, there is no unequivocal evidence to support or refute post-translational modification of Puma, although there are some potential phosphorylation sites in the protein.

Bim and Puma are transcriptionally upregulated after cytokine deprivation, suggesting that cytokine signalling represses this response. Perhaps the best established model of this transcriptional regulation involves the Forkhead transcription factor, FoxO3a (15,17). This model also has a central role for AKT because FoxO3a is a substrate for AKT and is inactivated when phosphorylated. When cytokine signalling is lost, dephosphorylated FoxO3a translocates to the nucleus to transcriptionally upregulate Puma and Bim. The prediction that FoxO3a-deficient cells would be resistant to apoptosis provoked by cytokine deprivation was directly tested in IL-3-dependent mast cells (15). Surprisingly, both Puma and Bim protein levels increased after IL-3 deprivation in FoxO3a-deficient cells, but nevertheless, some resistance to IL-3 deprivation was observed. The resistance was, however, less than when either Puma, Bim or both were deleted. Clearly, transcription factors other than FoxO3a regulate Bim and Puma expression following cytokine deprivation. On this note, although p53 is primarily involved in the DNA damage response, deletion of p53 does appear to protect some myeloid precursor cells from apoptosis at limiting cytokine concentrations (18) and Puma was first identified as a p53-inducible protein. The mechanism by which deletion of p53 protects against growth factor deprivation is, however, unknown.

What Other Bcl-2 Family Members are Hidden Up the Sleeve?

Deletion of Puma or Bim does not provide the same survival and retention of clonogenic potential as deletion of both Bax and Bak (8). Deletion of Puma and Bim has an additive effect (15). In contrast, overexpression of anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-x_L or Mcl-1 does 'phenocopy' Bax/Bak double-knockout cells. This suggests that although cytokine signalling might repress the activation of certain BH3-only proteins, maintenance of anti-apoptotic Bcl-2 protein levels above a certain threshold is an efficient way to neutralise all active BH3-only proteins and prevent the activation of Bax and Bak. Protein levels of Bcl-2 or Bcl-x_L do not change dramatically in response to cytokine deprivation, but GM-CSF signalling has been shown to maintain levels of the anti-apoptotic Bcl-2 family member, Mcl-1, and withdrawal of GM-CSF results in rapid degradation of Mcl-1 (19). Maintenance of Mcl-1 levels may therefore be another part of the mechanism by which GM-CSF and similar cytokines maintain survival (20).

At the Other End of the Sheet – Receptor Activation

Although many cytokine receptors have intrinsic tyrosine kinase activity, IL-3 and GM-CSF receptors do not. Instead, a Janus kinase, JAK-2, is bound to the βc and becomes phosphorylated following ligand-receptor binding. In turn, JAK-2 phosphorylates tyrosine residues on βc and initiates several signalling cascades. Recently, the structure of the GM-CSF receptor has been solved by an Australian group and this has provided insights into the activation of the receptor by ligand binding (21). The receptor consists of a hexameric complex of the four domains of a common β chain and the two domains of a GM-CSF receptor α chain. However, a higher order, dodecameric complex is the active signalling conformation. Signalling from this conformation is critically dependent on the formation of an interaction surface by two βc and one α chain (termed site 4). Mutations to this surface disrupt dodecamer formation, but not hexamer formation, and significantly block proliferative signalling. It will be interesting and informative to determine how such mutants might affect survival signalling.

Under the Sheet

The phosphorylated tyrosines in βc act as docking sites for adaptor molecules involved in the initiation of other signalling pathways, such as activation of PI3K/AKT, RAS/RAF/ERK or JAK/STAT pathways. Mutational analyses of

the βc have mapped regions and specific tyrosine residues required for the activation of specific signalling pathways and cellular responses (22-24) (**Fig. 1**). Mutations that abolish JAK-2 binding or activation prevent proliferative and survival signalling, and mutation of all tyrosine residues to phenylalanine also abolish proliferation. Tyrosines Y577, Y612 and Y695 are sufficient to signal proliferation. Using this mutational approach, mutants were identified that appeared to separate proliferation from survival signalling, although it is legitimate to question whether, in the context of normal signalling, these signals are ever separate. One answer to this question is suggested from work by Mark Guthridge and Angel Lopez at the Hanson Institute in Adelaide. Using human GM-CSF-responsive cell lines, they showed that phosphorylation of a conserved serine residue, S585, was critical to suppressing apoptosis, particularly at limiting concentrations of cytokine (25). At low doses, cells survived, but did not proliferate and βc was phosphorylated at S585, but not at Y577. With increased GM-CSF doses, Y577 phosphorylation was detected and cells began dividing, but S585 phosphorylation was lost. Limited GM-CSF concentrations may be sufficient to maintain survival of a haematopoietic stem cell, but proliferation only occurs when abundant cytokine is present, perhaps at times of infection or in response to neutropenia. Several interesting questions immediately present themselves. Is the receptor in a different conformation at low dose GM-CSF compared to high dose GM-CSF? If it is the same, why is there no tyrosine phosphorylation? If different, how does S585 get phosphorylated? Which serine threonine kinase is responsible and what signalling pathways downstream of the serine phosphorylation are activated?

Several cytosolic signalling pathways are activated when IL-3 or GM-CSF binds to the receptor (**Fig. 2**). Although represented separately, there is considerable cross-talk between these pathways, which makes assigning specific biological responses to particular signalling cascades challenging. For example, Ras activation can activate PI3K signalling, and PI3K/AKT and Ras pathways activate downstream targets such as mTOR. Most focus has centred on the activation of the PI3K/AKT pathway as the principal 'survival pathway'. Several potential substrates have already been mentioned, including BH3-only proteins and FoxO3a. Other potential prosurvival functions of AKT may be mediated via effects on maintaining nutrient uptake and protein synthesis through the activation of proteins including mTOR and S6 kinase. However, it may be a significant overstatement to describe the PI3K/AKT pathway as the 'survival pathway'. Enforced expression of constitutively active AKT does prolong the survival of myeloid cells in the absence of IL-3; however, at least half such cells die in the first days of the experiment (26). In our hands, AKT overexpression delays, but does not prevent, apoptosis after IL-3 withdrawal. Interestingly, activating mutations in AKT in tumours are relatively rare, but mutations in PI3K or the PIP3 phosphatase PTEN are much more common, suggesting mutations upstream of AKT are a more 'efficient' way to produce a tumour and that kinases other than AKT are important for the survival signal. For example, Pim2 is a serine threonine kinase that is transcriptionally downregulated following IL-3 withdrawal. Much like AKT, enforced expression of Pim-2 results in prolonged survival of at least some cells after IL-3 deprivation (26). The related kinase Pim1 may also transduce an IL-3 dependent survival signal in basophils (27).

The potential for ERK1/2 to phosphorylate Bim has already been described, however, other signalling pathways may also mediate cytokine-signalled survival. Overexpression of an activated form of Ras did block IL-3 withdrawal-induced apoptosis without inducing significant IL-3-independent proliferation in an IL-3-dependent cell line (28). It should not be forgotten that the activating Ras mutations resulting in juvenile myelomonocytic leukaemia are characterised by the ability of leukaemic cells to proliferate (and survive, of course) in culture in the absence of growth factors. Inhibition of MAP kinase (MAPK) activity inhibited IL-3-dependent proliferation and also decreased viability at limiting doses of IL-3 (29). Activated forms of MAPK permit cells to proliferate independently of IL-3, indicating that the MAPK pathway may not signal survival independently of proliferation.

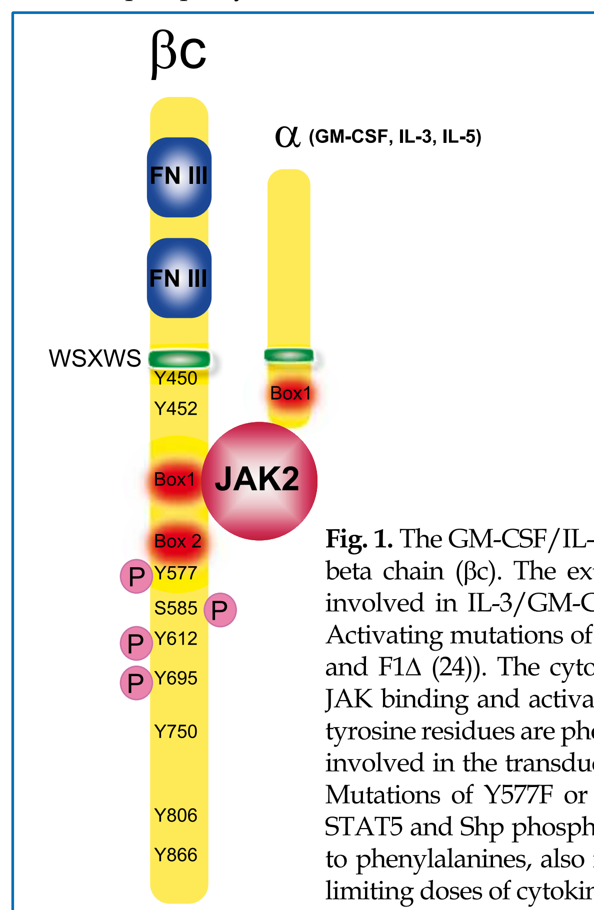

Fig. 1. The GM-CSF/IL-3 receptor is a heterodimer of a specific alpha chain and a common beta chain (βc). The extracellular domain of the βc has two fibronectin type III repeats involved in IL-3/GM-CSF binding. The βc also has a WSXWS transmembrane domain. Activating mutations of βc have been identified in the transmembrane domain (e.g. V449E and F1Δ (24)). The cytosolic portion of βc contains Box 1 and Box 2 motifs, involved in JAK binding and activation. Deletion of these domains abolishes signalling. A number of tyrosine residues are phosphorylated after ligand binding. Tyrosines 577, 612 and 690 are all involved in the transduction of proliferative signals (in addition to some survival signals). Mutations of Y577F or Y612F abolish signalling and each residue alone is sufficient for STAT5 and Shp phosphorylation. The other tyrosine residues, when individually mutated to phenylalanines, also result in diminished proliferation. Serine 585 is phosphorylated at limiting doses of cytokine and transduces a survival signal.

The fact that cytokines signal for cell survival independently of proliferation is no sleight of hand, and this survival signalling is essential in the normal function of cytokines and contributes to the pathogenesis of diseases such as acute myeloid leukaemia (25). It also appears that many cytoplasmic signalling pathways contribute to the suppression of apoptosis and it would be näive to think that regulation of survival by cytokines will depend on a single signalling pathway or a sole Bcl-2 family member. The sheer complexity of these interacting pathways will mean that there are still many secrets to be revealed before we understand the trick.

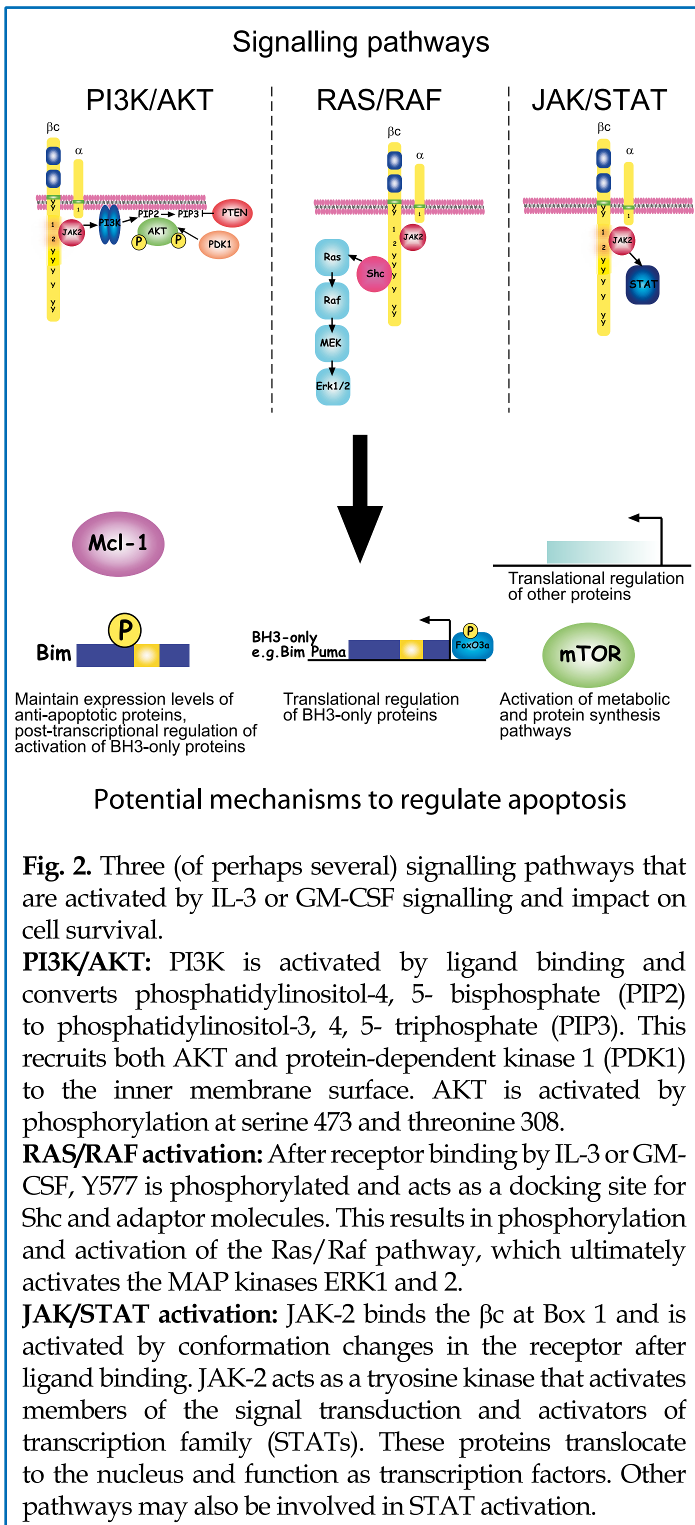

Fig. 2. Three (of perhaps several) signalling pathways that are activated by IL-3 or GM-CSF signalling and impact on cell survival.

PI3K/AKT: PI3K is activated by ligand binding and converts phosphatidylinositol-4, 5- bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5- triphosphate (PIP3). This recruits both AKT and protein-dependent kinase 1 (PDK1) to the inner membrane surface. AKT is activated by phosphorylation at serine 473 and threonine 308.

RAS/RAF activation: After receptor binding by IL-3 or GM-CSF, Y577 is phosphorylated and acts as a docking site for Shc and adaptor molecules. This results in phosphorylation and activation of the Ras/Raf pathway, which ultimately activates the MAP kinases ERK1 and 2.

JAK/STAT activation: JAK-2 binds the βc at Box 1 and is activated by conformation changes in the receptor after ligand binding. JAK-2 acts as a tryosine kinase that activates members of the signal transduction and activators of transcription family (STATs). These proteins translocate to the nucleus and function as transcription factors. Other pathways may also be involved in STAT activation.

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