CHAPTER 5

FKHR-L1 is a critical effector of cell death induced by interleukin-3 withdrawal: PKB-mediated inhibition of FKHR-L1 activity enhances cell survival by maintenance of mitochondrial integrity

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Abstract

Survival signals elicited by cytokines include activation of phosphatidyl inositol 3-kinase (PI3K), which in turn promotes activation of Protein Kinase B (PKB). Recently, PKB has been demonstrated to phosphorylate and inactivate Forkhead transcription factor FKHR-L1, which is a potent inducer of apoptosis. To explore the mechanisms underlying induction of apoptosis following cytokine withdrawal or FKHR-L1 activation, we have utilized a cell line in which FKHR-L1 activation can be specifically induced. Both cytokine withdrawal and FKHRL1 activation induce apoptosis as measured by Annexin-V binding and DNA laddering. The onset of apoptosis is preceded by an upregulation in $p27^{KIP1}$ and a concomitant decrease in cells entering the cell cycle. Induction of apoptosis by both cytokine withdrawal and FKHR-L1 activation correlates with disruption of mitochondrial membrane integrity and cleavage of effector caspases. This was preceeded by upregulation of the pro-apoptotic Bcl-2 family member Bim, suggesting a critical role for Bim in this process. Apoptosis does not correlate with cleavage of caspase-8, demonstrating that it is a death receptor independent process. Activation of PKB alone was sufficient to promote cell survival through maintenance of mitochondrial integrity and the resultant inhibition of effector caspases. Importantly, these data demonstrate that activation of FKHR-L1 alone is sufficient to induce the complete program of apoptotic events normally associated with survival factor withdrawal, suggesting it is a critical player in this process.

Introduction

In the absence of cytokines, hematopoietic cells stop proliferating and undergo programmed cell death, also known as apoptosis. This dependence on cytokines is necessary to maintain homeostasis in the immune system, and dysregulation of this process has been associated with autoimmune diseases, as well as malignancies (reviewed in [1]). The activation of cysteine proteases, caspases, leading to the cleavage of various substrates, including PARP (poly (ADP-ribose) polymerase) and the degradation of chromosomal DNA characterize a crucial step in the induction of apoptosis[2]. Caspases exist as inactive pro-enzymes in the cell, which themselves are activated through proteolytic cleavage upon induction of the apoptotic program (reviewed in [3]).

A well-characterized mechanism of initiating apoptosis is through ligand-mediated activation of cell surface death receptors, such as the TNF receptors and CD95 (APO-1/Fas) (reviewed in [4]). Caspase-8 is indispensable for transducing apoptotic signals initiated by death receptors, demonstrated by the observation that CD95 signaling is abrogated in *caspase-8 (-/-)* mice[5]. Caspase activation can also be triggered via a death receptor-independent mechanism, involving regulation of mitochondrial membrane permeability. Central to this "intrinsic" means of mitochondrial-initiated caspase activation is the release of cytochrome c from the intermembrane space of mitochondria into the cytosol.

Cytochrome c, together with Apaf-1 (apoptosis activating factor 1) promote activation of caspase-9[6,7], which activates downstream caspases, such as caspase-3 and 7[3]. Although loss of mitochondrial integrity can also be induced by death receptors, it is not essential for their induction of apoptosis[8].

Indispensable for the regulation of mitochondrial integrity are proteins of the Bcl-2 family. These consist of anti-apoptotic members, such as Bcl-2, Mcl-1 and Bcl-XL and pro-apoptotic members, such as Bad, Bim and Bid (reviewed in [9]). One mechanism by which cytokines are believed to promote survival is by inhibiting transcription[10,11] or activity[12,13] of pro-apoptotic members, as well as transcriptionally upregulating anti-apoptotic members[14,15].

The serine and threonine protein kinase, Protein Kinase B (PKB), also known as c-Akt, is involved in cytokine-mediated cell survival[12,16,17]. Anti-apoptotic signals from PKB include upregulation of Mcl-1[18] and inhibitory phosphorylation of Bad[12], although the relevance of Bad phosphorylation for the survival of hematopoietic cells remains unclear[19]. A recently identified mechanism by which PKB can promote rescue from apoptosis is through inhibitory phosphorylation of the Forkhead transcription factor FKHR-L1 (FOXO3a)[10,20]. Activity of this transcription factor has been linked to induction of apoptosis in hematopoietic cells[10,20]. Although PKB is a well-established player in rescue from apoptosis, it is not clear whether PKB exerts its anti-apoptotic effect upstream[21] or downstream[22] of mitochondria. Furthermore, little is known concerning the mechanisms by which FKHR-L1 can lead to activation of caspases, resulting in induction of apoptosis.

Here, we investigate the mechanisms of cytokine withdrawal and Forkhead-induced apoptosis and the role of PKB in rescue from apoptosis in cytokine-deprived cells. Our data demonstrate that FKHR-L1, as well as cytokine withdrawal, induces apoptosis through a death receptor-independent pathway. This involves upregulation of the pro-apoptotic Bcl-2 family member Bim, loss of mitochondrial integrity and caspase activation. Thus PKB can protect cells from cytokine withdrawal-induced apoptosis by inhibiting FKHR-L1, resulting in maintenance of mitochondrial integrity. These data shed new light on the mechanisms by which cytokines, through regulation of PKB activity, can modulate the survival of hematopoietic lineages.

Experimental Procedures

Cell culture

Ba/F3 cells were cultured in RPMI 1640 supplemented with 8% Hyclone serum (Gibco) and recombinant mouse IL-3 produced in COS cells[23]. Monoclonal Ba/F3 cells stably expressing FKHR-L1(A3):ER* and the polyclonal myrPKB:ER* Ba/F3 cell line have been described previously[10,24] and were cultured together with 500 μg/ml G418. For cytokine withdrawal experiments, cells were washed twice with PBS and resuspended in RPMI 1640 supplemented with 8% Hyclone serum and, in case of the polyclonal

myrPKB:ER* Ba/F3 cell line, cultured for two hours prior to adding 4-hydroxy tamoxifen (4-OHT).

Antibodies and reagents

Polyclonal antibodies against cleaved caspase-3 (#9661), cleaved caspase-7 (#9491S) and PARP (#9542) and phospho-Ser473 PKB (#9271S) were from New England Biolabs (Beverly, MA, U.S.A). Polyclonal caspase-8 antibody (559932) was from BD Pharmingen (San Diego, CA, USA). Bim polyclonal antibody was purchased from Affinity Bioreagents (Golden, CO, U.S.A.). $p27^{KIP1}$ and RACK1 mAb were purchased from Transduction Laboratories (Lexington, Kentucky, U.S.A.). Propidium iodide was from Sigma- Aldrich Chemie (Zwijndrecht, The Netherlands). Annexin V-FITC kit from Alexis (Kordia bv, The Netherlands). Rhodamine-123 was purchased from Molecular Probes (Leiden, The Netherlands). All other chemicals were reagent grade.

DNA laddering

10^7 cells were treated as indicated, lysed on ice for 10 minutes in buffer A (10 mM Tris-Cl pH 7.4, 10 mM EDTA, 0.2% TX-100, supplemented with 1 mM PMSF, 0.1 mM aprotinin and 1 mM leupeptin) and centrifuged at 14000 rpm 4°C for 10 minutes. The supernatant was added to an equal volume phenol:chloroform, rocked gently for 10 minutes, centrifuged and the upper phase was added to 1/10th volume sodium acetate (3 M, pH 5.4) and 2.5 volumes ethanol and incubated at -20°C for 15 minutes and subsequently spun down. The pellet was air-dried, resuspended in TE containing 2 μg/ml RNase A, incubated at 37°C for 30 minutes and run on a 2% agarose gel.

Western blotting

For the detection of all proteins, cells were lysed in ELB buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA together with inhibitors)[25]. Protein concentration was measured and equal amounts of protein were analyzed by SDS-PAGE. Blots were incubated overnight at 4°C with the appropriate antibodies (1:1000) and after hybridization with secondary antibodies developed utilizing Enhanced Chemiluminescence (ECL, Amersham).

FACS analysis of apoptosis

Preparation of cells for the analysis of cell cycle profiles has been described previously[10]. For the analysis of apoptosis by Annexin-V staining, cells were washed with ice-cold PBS, resuspended in binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin-V (Bender Medsystems, Vienna, Austria) for 10 min at room temperature, washed and resuspended in binding buffer containing 1 μg of propidium iodide (PI)/ml and fluorescence was analyzed by fluorescence-activated cell sorter (FACS).

Analysis of mitochondrial depolarization

For the analysis of changes in mitochondrial potential, $\Delta\psi_m$ [26], cells were incubated in RPMI together with 10 μg/ml Rhodamine-123 (Rh-123; Molecular Probes, Eugene, OR, U.S.A.) at 37°C for 30 minutes, washed twice with PBS and analyzed by FACS[10]. The percentage of cells falling within the range of Rh-123 fluoresence, indicative of depolarized cells, is shown.

Results

Induction of apoptosis correlates with upregulation of $p27^{KIP1}$ and Bim.

Cytokines of the interleukin (IL)-3, IL-5 and GM-CSF (granulocyte macrophage colony stimulating factor) family have a well-established function in transducing a proliferative and anti-apoptotic response in hematopoietic target cells and their precursors[27-29]. To examine the mechanisms underlying cytokine withdrawal induced apoptosis we utilized the mouse pre-B cell line, Ba/F3, which requires IL-3 both for proliferation as well as to overcome the default apoptotic program. Previously, we and others have shown that one mechanism by which cytokine-mediated rescue from apoptosis may be achieved is through inhibitory phosphorylation of the Forkhead transcription factor FKHR-L1 by PKB[20,24,30,31]. To specifically analyze the effect of FKHR-L1, we generated cell lines stably overexpressing an inducible form of active FKHR-L1, in which all 3 phosphorylation sites were mutated to alanine, FKHR-L1(A3):ER*. Addition of 4-hydroxy tamoxifen (4-OHT) to these cells results in the rapid induction of FKHR-L1 transcriptional activity, promoting induction of bona fide Forkhead targets[10].

Cells were either cytokine starved or treated with 4-OHT for 24 hours and apoptosis was measured by analyzing binding of Annexin-V-FITC. While cells that are Annexin-V positive represent early apoptotic cells; cells that are stained for both Annexin-V and PI represent cells that have initiated the apoptotic program for a longer period of time. Both cytokine withdrawal, as well as FKHR-L1 activity, induced apoptosis to a similar degree (Fig. 1A).

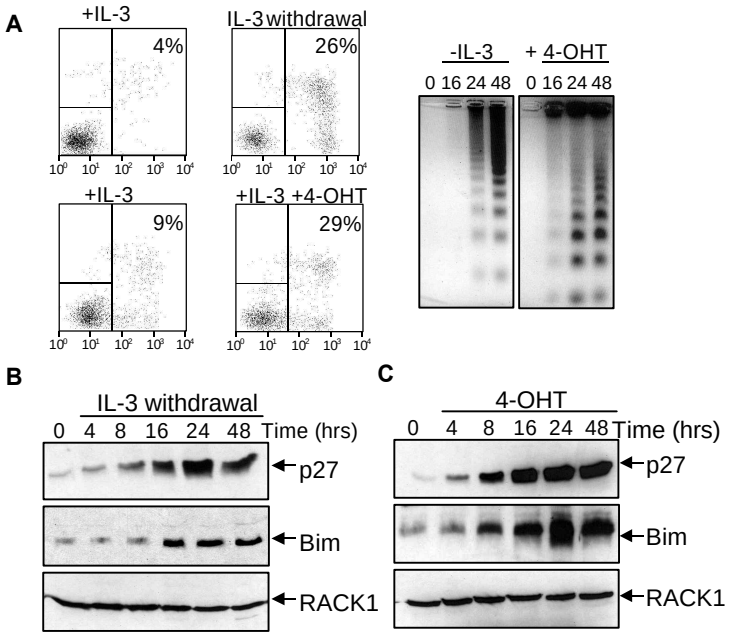

Figure 1. Induction of apoptosis by cytokine withdrawal or FKHR-L1 activity.
(A) Left: Ba/F3 cells were cultured in the presence or absence of cytokines and FKHR-L1(A3):ER* cells were cultured with IL-3 without or with 4-OHT (100 nM) for 24 hours and analyzed for Annexin-V binding and PI-staining as described in Experimental Procedures. Right: Ba/F3 cells were IL-3 starved (left) or FKHR-L1(A3):ER* expressing cells were treated with 4-OHT (100 nM, right) for the times indicated. DNA laddering was analyzed as described in Experimental Procedures. (B) Ba/F3 cells were cytokine-starved for the indicated times, lysed and equal amounts of protein were analyzed for levels of $p27^{KIP1}$ (upper panel) or Bim (middle panel). Samples were analyzed with a RACK1 antibody to verify equal protein loading (lower panel). (C) Ba/F3 cells stably expressing FKHR-L1(A3):ER* were treated with 4-OHT for indicated times, lysed and analyzed as above.

Next, we analyzed the kinetics by which apoptosis was induced utilizing DNA laddering, a measure for the final events characterizing apoptosis. Both cytokine withdrawal, as well as FKHR-L1 activity, induced apoptosis within a similar time frame (Fig. 1B). Recently, we have demonstrated that both $p27^{KIP1}$ and Bim (Bcl-2 interacting mediator of cell death) transcriptional targets of FKHR-L1[10,24]. We examined whether the kinetics of upregulation of $p27^{KIP1}$ and Bim protein correlated with induction of apoptosis. Both cytokine withdrawal and FKHR-L1 activation resulted in an upregulation of $p27^{KIP1}$, and Bim (Fig. 1B, C). These events occur relatively early and precede the cleavage of DNA observed in Fig. 1B.

Cytokine withdrawal and FKHR-L1 activity induce cell cycle arrest followed by apoptosis.

p27^{KIP1} is involved in cell cycle arrest in G_1 through inhibition of cyclin-CDK complexes[32,33], but has also been described to function in the induction of apoptosis through a yet unidentified mechanism[10,34]. Bim is a potent inducer of apoptosis through binding to and thus inhibiting anti-apoptotic Bcl-2 members[35]. To see whether upregulation of p27^{KIP1} and Bim reflected an altered distribution of cells in the cell cycle, we analyzed the cell cycle profile of cells at various times. Upon cytokine withdrawal, cells stop initiating cell division and accumulate in G_1, within the first 8 hours of starvation (Fig. 2A). After 16 hours of cytokine deprivation cells start to undergo apoptosis, as measured by a DNA content less than 2N chromosomes, the sub-G_1 peak. (Fig. 2A). By 48 hours, a majority of cells has initiated a program of apoptotic cell death. Similar findings were observed in 4-OHT-treated FKHR-L1(A3):ER* cells (Fig. 2B), suggesting that indeed the presence of a G_1 arrest before undergoing apoptosis is related to the initial upregulation of p27^{KIP1} and Bim (Fig . 1B and 1C).

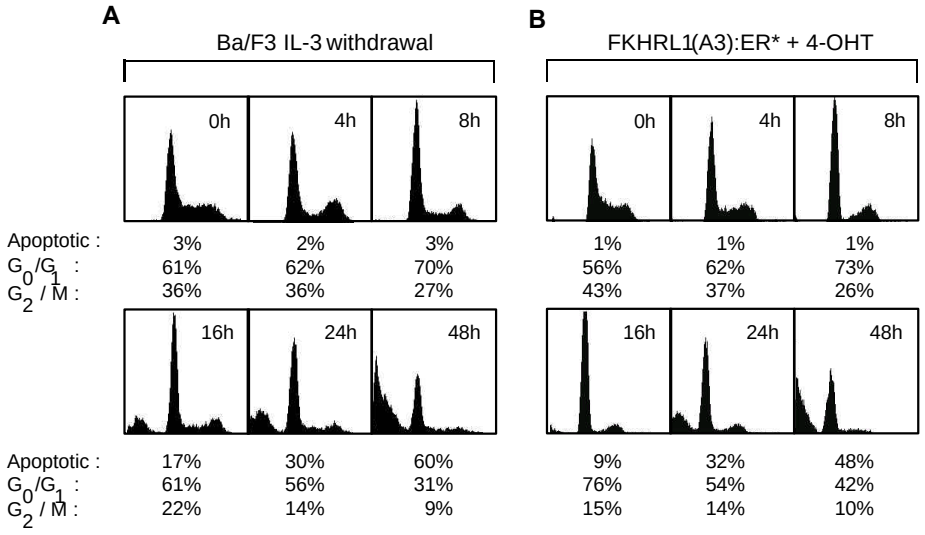

Figure 2. Cell cycle analysis of cytokine-starved cells and cells in which FKHR-L1 activity is induced.

(A) Ba/F3 cells were cytokine-starved for indicated times, fixed, stained with propidium iodide and analyzed by FACS. (B) Ba/F3 cells stably expressing FKHR-L1(A3):ER* were treated with 4-OHT (100 nM) for the times indicated and processed as in (A). Data depicted are representative of at least three independent experiments.

Caspase activation and PARP cleavage follow cytokine withdrawal and FKHR-L1 activation.

Very little is known of the mechanisms by which cytokine withdrawal promotes caspase activation and cleavage of apoptotic substrates[36,37]. Whether this occurs through a death receptor-dependent or -independent pathway and whether the function of FKHR-L1 is implicated therein remains to be established. We have recently reported that Bim is an important mediator of FKHR-L1-induced apoptosis[10]. Furthermore, Forkhead-mediated upregulation of Fas ligand has been proposed in T cells[20]. This suggests that both death receptor-dependent, as well as death receptor-independent mechanisms may be involved in Forkhead-mediated induction of apoptosis. We sought to investigate this in more detail, examining both cytokine withdrawal and FKHR-L1 activity. Utilizing antibodies specific for cleaved caspase-3 and caspase-7, their activation was analyzed following cytokine withdrawal. Caspase-3 (Fig. 3A, upper panel) and caspase-7 (Fig. 3A, middle panel) were both cleaved after cytokine withdrawal. This occurred approximately 16 hours after removal of cytokine. Analysis of 4-OHT-treated FKHR-L1(A3):ER* cells showed similar kinetics of caspase-3 and caspase-7 cleavage (Fig. 3B, upper and middle panel). A well-characterized caspase substrate that is cleaved when cells undergo apoptosis is PARP (poly (ADP-ribose) polymerase)[38], an enzyme involved in DNA repair (reviewed in [39]). To examine whether PARP is cleaved in either cytokine withdrawal or Forkhead-induced apoptosis, lysates of cytokine-starved cells or 4-OHT-treated FKHR-L1(A3):ER* cells were again analyzed. Both IL-3 withdrawal (Fig. 3C, left), as well as Forkhead activity (Fig. 3C, right) resulted in PARP cleavage with kinetics similar to caspase activation (Fig. 3A,B). These data suggest that similar mechanisms are involved in both cytokine withdrawal and FKHR-L1-induced apoptosis. Furthermore, the activation of caspases proceeds the upregulation of both $p27^{KIP1}$ and Bim (Fig. 1B,C).

Cytokine withdrawal and FKHR-L1 activity promote mitochondrial depolarization.

Mitochondria play a critical role in the initiation of apoptosis, which is accompanied by a loss of mitochondrial transmembrane potential (Ψ_m) and leakage of cytochrome c[40]. This, together with Apaf-1 is required for the onset of the activation of the "caspase-cascade"[6,7] and is indispensable for death receptor-independent induction of apoptosis. To analyze mitochondrial integrity in apoptotic Ba/F3 cells we utilized rhodamine-123 (Rh-123), a dye that binds to mitochondria in a membrane potential-dependent way[26,41]. In cytokine-starved Ba/F3 cells (Fig. 4A), as well as in 4-OHT-treated FKHR-L1(A3):ER* cells (Fig. 4B), loss of Ψm was observed after 16 hours, and increased dramatically over time. The kinetics are similar to those of caspase activation (Fig. 3A and 3B). This suggests that both cytokine withdrawal, as well as FKHR-L1 activation result in loss of mitochondrial transmembrane potential, subsequently resulting in cytochrome c release and activation of caspases.

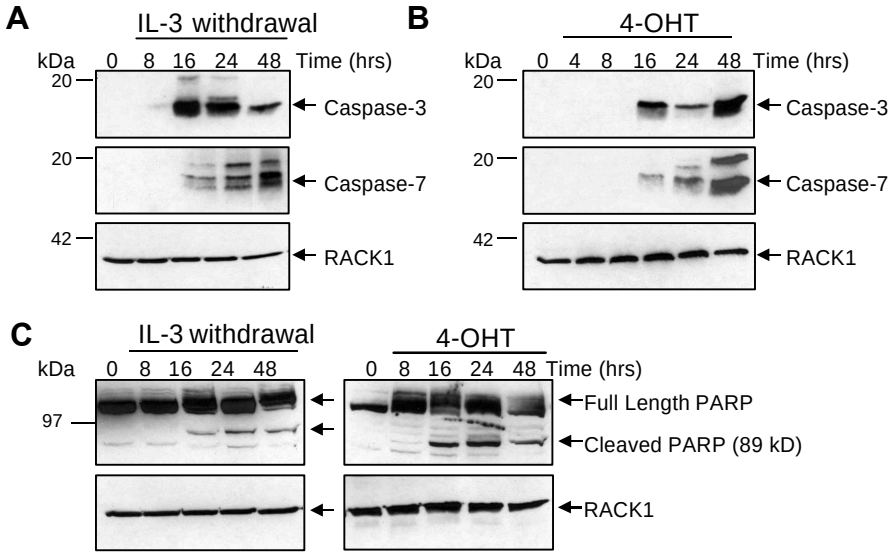

Figure 3. Analysis of caspase activation and PARP cleavage in cytokine-starved cells and 4-OHT-treated FKHR-L1(A3):ER* cells.

(A) Cells were IL-3-starved for the times indicated and caspase-3 and 7 activation was analyzed using cleavage-specific antibodies. Equal protein loading was verified by analyzing samples for RACK1 expression. (B) 4-OHT-treated FKHR-L1(A3):ER* cells were analyzed as in (A). (C) PARP cleavage was examined in cytokine-starved cells (left) or 4-OHT-treated FKHR-L1(A3):ER* cells (right).

PKB-mediated rescue from apoptosis correlates with maintenance of mitochondrial integrity.

PKB is involved in inhibition of apoptosis by cytokines[12,16,17], however, a role for this kinase in the regulation of mitochondrial integrity remains unclear. PKB has been proposed to rescue cells from apoptosis by maintenance of mitochondrial transmembrane potential and preventing cytochrome c release[21]. However, others have observed rescue from apoptosis downstream from cytochrome c release[22]. To investigate the role of PKB in maintenance of mitochondrial integrity in more detail, we made use of a polyclonal Ba/F3 cell line stably expressing a 4-OHT-inducible, active form of PKB, myrPKB:ER*[10]. Treatment of these cells with 4-OHT resulted in a dramatic phosphorylation of myrPKB:ER* (Fig. 5A, upper panel), allowing us to analyze the effect of PKB activation following cytokine withdrawal. Indeed, activation of PKB in the absence of cytokines was sufficient to rescue cytokine-starved myrPKB:ER* cells from apoptosis (Fig. 5B), as measured by Annexin-V-FITC staining. To further elucidate a role for PKB

upstream or downstream of mitochondria, we analyzed whether PKB was capable of abrogating cytokine withdrawal induced loss of mitochondrial transmembrane potential. Ba/F3 cells and myrPKB:ER* cells were cultured with or without IL-3 in the presence or absence of 4-OHT. In Ba/F3 cells, loss of mitochondrial transmembrane potential in the absence of cytokines could not be rescued by 4-OHT (Fig. 5C, upper panel), excluding aspecific effects of 4-OHT. In myrPKB:ER* cells however, addition of 4-OHT substantially decreased the loss of Ψm upon cytokine withdrawal (Fig. 5C, lower panel). This suggests that PKB exerts its anti-apoptotic activity by maintaining mitochondrial integrity. Caspase activity was subsequently analyzed. Activity of both caspase-3 and caspase-7 was substantially reduced in 4-OHT-treated compared to untreated myrPKB:ER* cells. Our findings of a partial rescue from apoptosis (Fig. 5B) and caspase activity (Fig. 5D) may be explained by the fact that the myrPKB:ER* cell line is a polyclonal cell line, potentially expressing heterogeneous levels of myrPKB:ER*, the lower levels being insufficient to rescue cells from cytokine withdrawal-induced apoptosis. Taken together, these findings demonstrate that PKB-mediated rescue from apoptosis correlates with maintenance of mitochondrial potential, resulting in a reduction of caspase activity.

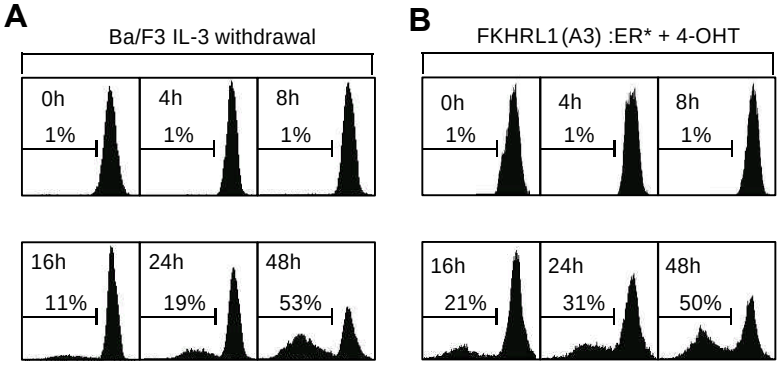

Figure 4. Induction of mitochondrial transmembrane depolarization by cytokine starvation or FKHR-L1 activity.

(A) Ba/F3 cells were IL-3 starved for the times indicated and mitochondrial transmembrane depolarization was measured using rhodamine-123 staining as described in Experimental Procedures. (B) 4-OHT-treated FKHR-L1(A3):ER* cells were analyzed as in (A). Data are representative of several independent experiments.

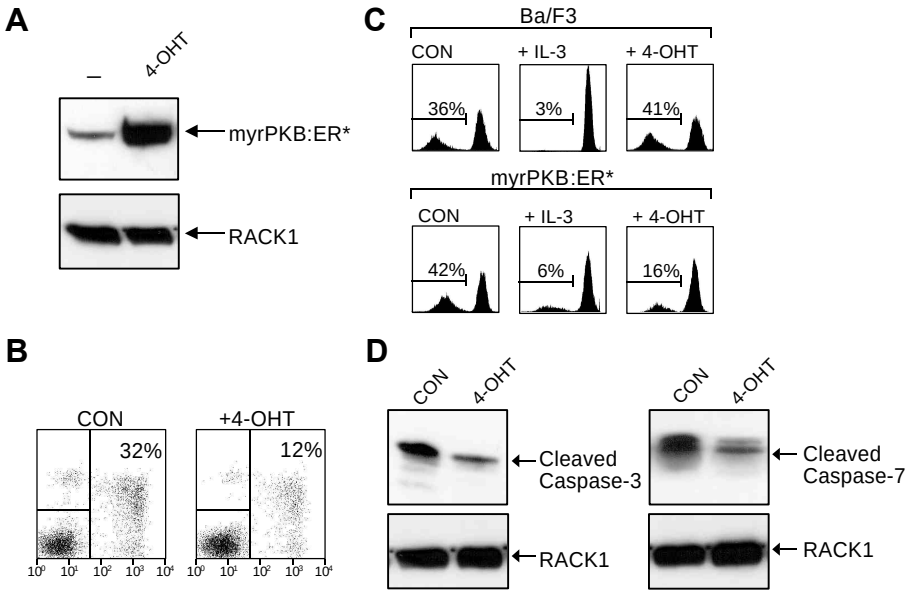

Figure 5. Activation of PKB rescues cells from apoptosis and maintains mitochondrial transmembrane potential.

(A) Ba/F3 cells stably expressing myrPKB:ER* were left untreated or treated with 4-OHT (100 nM) for 36 hours and PKB phosphorylation was measured using a PKB phospho ser-473-specific antibody. (B) myrPKB:ER* cells were cytokine-starved in the absence (left) or presence (right) of 4-OHT (100 nM) for 36 hours and the percentage of Annexin-V-FITC positive cells was determined as describe in the Experimental Procedures. (C) Ba/F3 cells or Ba/F3 cells stably expressing myrPKB:ER* were cytokine-starved in the absence or presence of 4-OHT (100 nM) or cultured with IL-3 for 36 hours and mitochondrial integrity was examined using rhodamine-123 as described in Experimental Procedures. (D) Caspase-3 (left) and caspase-7 (right) activation was measured in myrPKB:ER* cells that were cytokine-starved for 36 hours in the absence or presence of 4-OHT (100 nM) using activation-specific antibodies and reprobed with RACK1 antibody to verify equal protein loading.

Cytokine withdrawal and FKHR-L1 activity induce apoptosis independent of death receptor activation.

FKHR-L1 has been proposed to induce Fas ligand (FasL) in T cells[20], which could contribute to FKHR-L1-mediated induction of apoptosis. Fas/FasL signaling induces cleavage and activation of caspase-8 cleavage which is an indispensable and specific downstream event of death receptor induced apoptosis[5,42]. Analysis of caspase-8 cleavage is thus a means to discriminate between death receptor-dependent or death receptor-inde-

pendent induction of apoptosis. As Ba/F3 cells express Fas, albeit at very low levels[43,44], Forkhead-mediated induction of Fas ligand could potentially trigger apoptosis by inducing caspase-8 activation. We analyzed a potential role for Fas/FasL signaling in the induction of apoptosis by cytokine withdrawal or Forkhead activation by measuring caspase-8 cleavage. While Ba/F3 cells expressed caspase-8, neither cytokine withdrawal (Fig. 6A) nor Forkhead activity (Fig. 6B) resulted in caspase-8 cleavage. As a positive control Jurkat T-cells were treated with a cross-linking anti-Fas antibody which clearly induced cleavage and activation of caspase-8 (Fig. 6C). This argues against a role for Fas/Fas ligand signaling in FKHR-L1-mediated apoptosis.

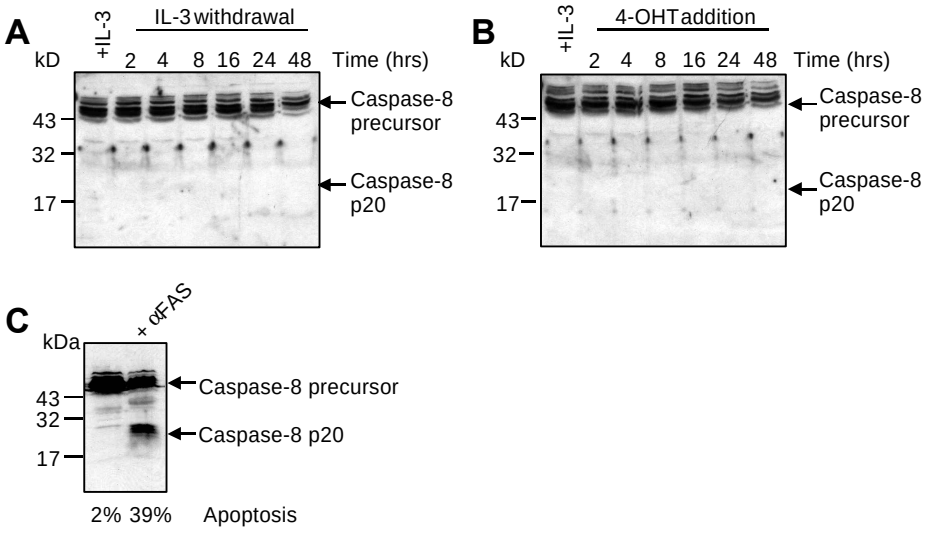

Figure 6. Caspase-8 is not activated in response to cytokine starvation or FKHR-L1 activation. (A) Caspase-8 cleavage was analyzed in Ba/F3 cells that were cytokine-starved for the times indicated. (B) Analysis of capase-8 cleavage in 4-OHT (100 nM) treated FKHR-L1(A3):ER* cells. (C) Jurkat cells were treated with or without αFAS18 (1 μg/ml). After 24 hours samples were harvested and protein concentration measured. Equal amounts of protein were analyzed by SDS-PAGE and anti-caspase-8 western blotting.

Discussion

In this report we analyzed the mechanisms of cytokine withdrawal and FKHR-L1-induced apoptosis, as well as PKB-mediated rescue from apoptosis. Utilizing cells expressing FKHR-L1(A3):ER* allowed us to uniquely analyze the effects of FKHR-L1 activation. Interestingly, no differences between cytokine withdrawal and FKHR-L1-mediated apoptosis were observed. Importantly, our results suggest that FKHR-L1 alone could account for induction of the apoptotic program triggered by cytokine withdrawal. In both cases, cells were first arrested in G_1 and then underwent apoptosis (Fig. 2). This was accompanied by a sequential upregulation of $p27^{KIP1}$, which is involved in arresting cells in G_1 and the induction of apoptosis[10,32,33], and Bim, which can induce apoptosis by binding to anti-apoptotic members of the Bcl-2 family[35] (Fig. 1B and 1C). Bim appears to be essential for the induction of apoptosis in lymphocytes, since cytokine-deprived lymphocytes from *Bim (-/-)* mice fail to undergo apoptosis[45]. Furthermore, lymphocytes derived from *$p27^{KIP1}$ (-/-)* mice undergo apoptosis at a significantly decreased rate compared to those from wildtype mice[10]. This suggests that the upregulation of both $p27^{KIP1}$ and Bim may play critical roles in the induction of the apoptotic program initiated by cytokine withdrawal.

We also analyzed whether Fas/Fas ligand signaling may be involved in induction of apoptosis upon cytokine withdrawal as previously proposed[20]. Neither cytokine withdrawal nor FKHR-L1 activity resulted in cleavage of caspase-8, an event specific for death receptor signaling[5,42]. This suggests that apoptosis either by cytokine withdrawal or FKHR-L1 activity is initiated through a death receptor-independent mechanism. In support of this, overexpression of anti-apoptotic Bcl-2 members, which rescue death receptor-independent apoptosis, but not death receptor-dependent apoptosis in lymphocytes[46,47] are able to rescue both cytokine withdrawal, as well as FKHR-L1-induced apoptosis[14,24].

PKB has been demonstrated to negatively regulate members of a subfamily of Forkhead transcription factors: AFX, FKHR and FKHR-L1 (reviewed in [48]). Recently, members of the SGK (serum- and glucocorticoid-induced kinases) family, phosphorylating consensus sequences similar to PKB were found to be required for full phosphorylation of FKHR-L1 *in vivo* and IL-3-mediated survival[49,50]. This suggests that both kinases may be required for phosphorylation-mediated inactivation of FKHR-L1. This may explain why PKB was unable to completely inhibit cytokine withdrawal-induced apoptosis (Fig. 5). However, PKB was capable of significantly abrogating cytokine withdrawal induced loss of mitochondrial potential (Fig. 5C). Thus we can conclude that PKB exerts its anti-apoptotic effect at a pre-mitochondrial level, preventing intracellular release of cytochrome c. A potential role for PKB in rescue from apoptosis and prevention of cytochrome c leakage has also been proposed in apoptosis induced in Rat1 fibroblasts by UV-irradiation[21], as well as in epithelial cells by detachment from extracellular matrix[51]. However, PKB has also been previously shown to inhibit ceramide-induced apoptosis in

hybrid neuron motor 1 cells downstream of cytochrome c release[22]. These findings may be explained by differences in apoptotic stimuli in different cell types suggesting that PKB has the potential to act at multiple levels. Furthermore, difference in species could be an explanation for the differential contribution of PKB to rescue from apoptosis. PKB promotes rescue from apoptosis by inhibitory phosphorylation of caspase-9 in human cells[52], but not in mouse or rat cells since the PKB phosphorylation site in caspase-9 is not present[53]. PKB has also been linked to the upregulation of anti-apoptotic Bcl-2 member Mcl-1[18], which is essential in cytokine-mediated rescue from apoptosis[14]. This regulation of an anti-apoptotic Bcl-2 member, involved in the maintenance of mitochondrial integrity also supports a role for PKB upstream of cytochrome c leakage in cytokine-mediated rescue from apoptosis.

The p21ras-activated protein kinase MEK has also been proposed to rescue cells from apoptosis[54-56], potentially through activation of downstream targets that phosphorylate Bad[55]. Furthermore, MEK initiated signals can result in the phosphorylation of anti-apoptotic members of the Bcl-2 family[56], thereby enhancing their stability[57]. However, utilizing the myrPKB:ER* cell line we have demonstrated that PKB alone is sufficient to protect cells from programmed cell death (Fig. 5B,C). Our data do not however rule out the possibility that MEK plays a role in these events.

Increased PKB activity can result in cellular transformation[58-60]. Although the exact mechanisms by which PKB is capable of promoting oncogenesis remains to be established, inhibitory phosphorylation of FKHR-L1 could very well contribute to this process, leading to a decrease both Bim and $p27^{KIP1}$ levels. This is supported by the observation that a decrease in $p27^{KIP1}$ levels is associated with a poor prognosis in cancer[61-63].

Taken together, our data suggests that cytokine-induced signaling can inhibit cells from apoptosis through activation of PKB (or SGK), which inhibits FKHR-L1 and Bad through phosphorylation, and transcriptionally upregulates Mcl-1. In the absence of cytokines, PKB is inactive, resulting in dephosphorylation and activation of Bad and transcription of FKHR-L1 targets $p27^{KIP1}$ and Bim. This results in induction of the apoptotic program through loss of mitochondrial integrity, leakage of cytochrome c, subsequent activation of caspases and cleavage of substrates. These events are summarized in a model (Fig. 7).

Our findings provide a greater insight into the mechanisms regulating induction of apoptosis in lymphocytes, and probably other hematopoietic cells, upon cytokine withdrawal. PKB alone is sufficient to inhibit apoptosis through the maintenance of mitochondrial transmembrane potential. This is likely to be due to the inhibition of FKHR-L1, thus preventing transcription of the pro-apoptotic Bcl-2 family member Bim. A greater understanding of the mechanisms by which cytokines regulate cellular survival will help towards the design of novel pharmacological agents for therapeutic intervention in a variety of proliferative and degenerative disorders of the immune system.

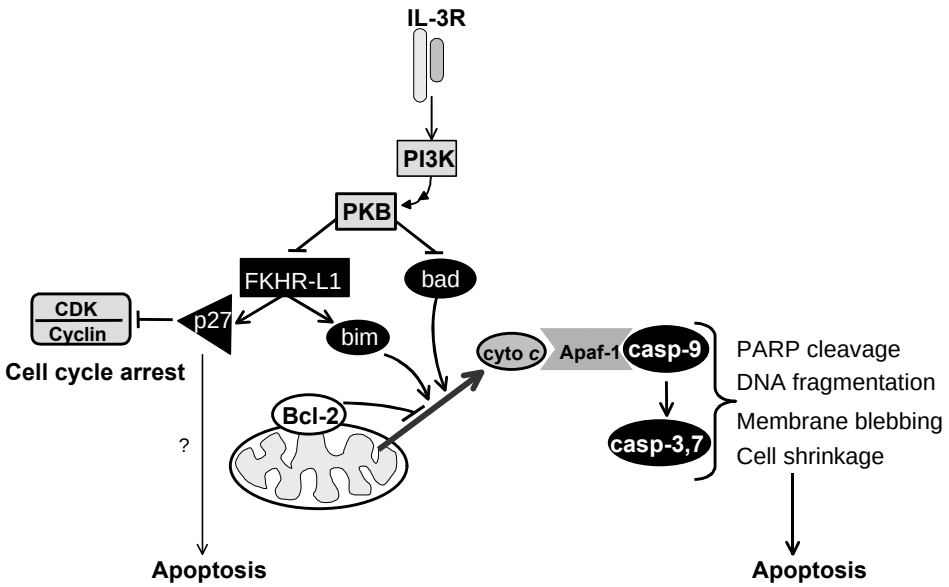

Figure 7. A model for cytokine withdrawal-induced apoptosis.

In the absence of cytokines, PKB and SGK are inactive, resulting in dephosphorylation and subsequent activation of pro-apoptotic Bcl-2 member Bad and the FKHR-L1 transcription factor. Transcriptional activity of FKHR-L1 elevates levels of Bim and $p27^{KIP1}$. $p27^{KIP1}$ inhibits cell cycle progression and helps to promote apoptosis in an as yet unidentified manner. Bim, possibly together with Bad, promotes loss of mitochondrial integrity and leakage of cytochrome c. This triggers activation of caspases and subsequent cleavage of downstream targets, resulting in apoptosis.

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