

IL-4 protects the B-cell lymphoma cell line CH31 from anti-IgM-induced growth arrest and apoptosis: contribution of the PI-3 kinase/AKT pathway

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Interleukin-4 (IL-4) promotes lymphocyte survival and protects primary lymphomas from apoptosis. Previous studies reported differential requirements for the signal transducer and activator of transcription 6 (STAT6) and IRS2/phosphatidylinositol 3 kinase (PI-3K) signaling pathways in mediating the IL-4-induced protection from Fas-mediated apoptosis. In this study, we characterized IL-4-activated signals that suppress anti-IgM-mediated apoptosis and growth arrest of CH31, a model B-cell lymphoma line. In CH31, anti-IgM treatment leads to the loss of mitochondrial membrane potential, phospho-Akt, phospho-CDK2, and c-myc protein. These losses are followed by massive induction of p27^{Kip1} protein expression, cell cycle arrest, and apoptosis. Strikingly, IL-4 treatment prevented or reversed these changes. Furthermore, IL-4 suppressed the activation of caspases 9 and 3, and, in contrast to previous reports, induced the phosphorylation (deactivation) of BAD. IL-4 treatment also induced expression of BclxL, a STAT6-dependent gene. Pharmacologic inhibitors and dominant inhibitory forms of PI-3K and Akt abrogated the anti-apoptotic function of IL-4. These results suggest that the IL-4 receptor activates several signaling pathways, with the Akt pathway playing a major role in suppression of the apoptotic program activated by anti-IgM.

Keywords: IL-4, apoptosis, cell cycle, akt, PI-3 kinase

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Introduction

A key property of cancer cells is the capacity to avoid apoptosis, thereby promoting long-term survival and cellular expansion at the expense of surrounding normal cells.

Lymphoma cells may acquire resistance to apoptosis by genetic means or by alterations in signaling pathways that result in the abnormal regulation of the apoptotic process. Activation of these signaling pathways can be inherent to an individual signaling molecule or can be due to the unregulated production of or exposure to growth factors and cytokines.

The cytokine interleukin-4 (IL-4) is a potent survival factor for B and T lymphocytes and it protects primary lymphomas and lymphoma cell lines from apoptosis induced by a variety of stimuli. For example, IL-4 decreases the spontaneous apoptosis of cultured splenic B cells [1], a form of 'death by neglect'. On the other hand, crosslinking of the B-cell receptor (BCR) on either splenic B cells or on immature, model B-cell lymphomas results in cell death by a Fas-independent mechanism [2-6]. This form of 'death by activation' can be intercepted with T-cell helper

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Abbreviations: IL-4, interleukin 4; PI-3K, phosphatidylinositol 3 kinase; IRS, insulin receptor substrate; STAT6, signal transducer and activator of transcription 6; CytC, cytochrome C; mTOR, mammalian target of rapamycin; JAK, Janus kinase; PIP3, D3 phosphatidylinositol; BCR, B-cell receptor

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signals or with IL-4. However, the molecular mechanisms of protection have not been clearly defined.

B-cell antigen receptor (BCR) signaling is complex and can result in proliferation, differentiation, or apoptosis depending on the differentiation status of the cells and the extracellular environment. For example, B cells stimulated via CD40 will express Fas on their surface and become sensitive to FasL mediated death [5]. IL-4 treatment will reverse the FasL-mediated death [7]. These effects are not limited to normal cells. In fact, IL-4 can regulate the survival of B-cell chronic lymphocytic leukemia (B-CLL, [8, 9]). In this context, it is not surprising that a unique molecular signature of B-cell small lymphocytic lymphoma (SLL) is the elevated expression of the IL-4 receptor alpha chain [10]. The molecular details of how IL-4 promotes B-CLL survival have not been described. However, it has been shown that IL-4 slows proliferation of B-CLL and prevents the spontaneous and steroid-induced apoptosis of these cells likely by suppressing caspases [9].

Analyses of the mechanism by which IL-4 regulates cell survival have been performed with conflicting results. In the IL-3-dependent cell line 32D, the prevention of IL-3-withdrawal-induced apoptosis was dependent on the activation of the insulin receptor substrate (IRS)/phosphatidylinositol 3 kinase (PI-3K)/mammalian target of rapamycin (mTOR) pathway by IL-4 [11]. A pathway independent of IRS was also found to contribute to the IL-4-induced protection from apoptosis in this model; however, this pathway was independent of signal transducer and activator of transcription 6 (STAT6). Furthermore, pharmacologic inhibitors of PKC and PI-3K were able to block the IL-4-induced phosphorylation of Akt and the enhanced survival of human B-CLL [12]. On the other hand, activation of the STAT6 pathway was shown to play a role in the IL-4-induced suppression of Fas-induced apoptosis of primary CD40L-activated B cells, while the IRS2/PI-3K pathway appeared dispensable [13]. Furthermore, the STAT6 pathway was implicated in mediating the survival of classic Hodgkin's lymphoma cells in response to IL-13 [14]. In both cases, the responses were shown to be mediated by the induction of Bcl-xL via the activation of STAT6. However, this is not the case for resting B cells; IL-4 did not induce the expression of Bcl-xL (or Bcl-2) in resting B cells [3] and was able to protect B cells isolated from STAT6-deficient animals from spontaneous apoptosis [13, 15]. Thus, there is no general consensus on the mechanism by which IL-4 regulates cell survival in primary or transformed cells.

In this study, we characterized the signals activated by IL-4 that suppress apoptosis and growth arrest induced by anti-IgM in the model B-cell lymphoma line CH31. CH31 cells were derived from hyperimmunized mice and are thought to have arisen by antigen-driven selection and

lymphomagenesis [16]. They express IgM and CD5 on their surface, and crosslinking the BCR with anti-IgM antibodies leads to cell cycle arrest and apoptosis [17-19]. These events are associated with a loss of c-myc expression, loss of phospho-Akt, and loss of phospho-CDK2 (pCDK2), with a concomitant increase in p27^{Kip1} expression ([19-21] and results herein). Prevention of c-myc loss by mRNA stabilization prevented apoptosis induced by anti-IgM [22, 23]. We found that IL-4 treatment of CH31 cells prevented the growth arrest and apoptosis induced by anti-IgM in a dose-dependent manner. Additionally, we found that IL-4 completely suppressed the anti-IgM-mediated induction of caspase-9 and caspase-3 activities, induction of cytochrome C (CytC) release, and loss of mitochondrial membrane potential ($\Delta\Psi_m$). Strikingly, IL-4 treatment prevented the loss of c-myc protein, the losses of phospho-Akt and pCDK2, and the increase in p27^{Kip1} mediated by anti-IgM. However, it had no effect on the anti-IgM-mediated loss of phospho-MEK (pMEK). We found that pharmacologic inhibitors and dominant inhibitory forms of PI-3K and Akt abrogated the ability of IL-4 to protect cells from apoptosis, indicating an important role for the Akt pathway in the protection. These results suggest that the IL-4 receptor activates several signaling pathways that can suppress the apoptotic program activated by anti-IgM in B-cell lymphoma.

Materials and Methods

Cells and reagents

The B-cell lymphoma cell line CH31 was obtained from Dr David W Scott (Center for Vascular and Inflammatory Diseases, University of Maryland, Baltimore, MD). Single-lot and low-passage (< 20) CH31 cells were cultured in RPMI-1640 (Bio-Whittaker, Walkersville, MD), supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 1× MEM non-essential amino acids, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum, and maintained as described previously [16] at 37 °C in a humidified 7% CO₂ atmosphere. Recombinant mIL-4 was obtained from R&D Systems (Minneapolis, MN). Recombinant huIL-2 was a generous gift of Dr Steven Rosenberg, NCI. The PI-3K inhibitor LY294002, AG490, Calyculin-A, and the Akt inhibitor AktI (1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate) were obtained from Calbiochem/EMD Biosciences, San Diego, CA. Monoclonal anti-mouse IgM, clone B7.6 was a kind gift from Dr David W Scott. Propidium iodide and saponin were obtained from Sigma-Aldrich, St Louis, MO.

$\Delta\Psi_m$ detection

CH31 cells were cultured at initial densities of 0.25×10^6 live cells/ml. A 1-ml cell sample was quantitatively transferred to FACS tubes prior to staining. Mitochondrial membrane potential ($\Delta\Psi_m$) was measured with tetramethylrhodamine (TMR), which only binds to mitochondria membranes in the presence of high mitochondrial membrane potential ($\Delta\Psi_m$, [24]). The reagent was obtained from Molecular Probes/Invitrogen, Carlsbad, CA. Briefly, the cells were pulse labeled with 40 nM TMR for the final 20 min of the incubation,

then washed twice with cold phosphate-buffered saline (PBS), pH 7.40. The cells were finally resuspended in 300 μ l cold PBS, placed on ice, protected from light, and TMR fluorescence was analyzed within 30 min using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Data were analyzed using Cellquest™ software, Becton Dickinson. Negative controls for $\Delta\Psi_m$ included 20–40 μ M carbonylcyanide 3-chlorophenylhydrazone (CCCP, [25]), a $\Delta\Psi_m$ -uncoupling reagent (data not shown).

Apoptosis assays

Levels of apoptosis were analyzed using Annexin-V binding/PI exclusion or sub-diploid nuclei determination essentially by methods described in Carey and Scott [20]. To measure apoptosis by the Annexin-V method, cells were incubated at 0.25×10^6 /in FACS tubes, washed once with HEPES buffered saline (HBS: 20 mM HEPES, pH 7.4; 120 mM NaCl) and then once in Annexin-V binding buffer (AVBB), which consisted of HBS + 2.5 mM CaCl_2 . The cells were then resuspended in 300 μ l AVBB and the binding of Annexin-V directly conjugated to fluorescein isothiocyanate (FITC), or phycoerythrin (PE) was measured as per the manufacturer's protocols. For pMaxGFP-transfected cells, PS positivity was detected using biotinylated annexin-V (Biosource, Camarillo, CA). Detection was performed using streptavidin conjugated to peridinin chlorophyll protein (SA:PerCP). The cells were processed and analyzed by FACS as described above.

For determination of cellular DNA content and analysis of the cell cycle, cells were harvested as above, washed once with PBS and resuspended in 300 μ l of propidium iodide (PI) staining buffer containing 50 μ g/ml PI, 5 mM EDTA, 1 μ g/ml DNAase-free RNAase and 0.1% saponin in PBS essentially as described previously [20]. The samples were then incubated for 15–30 min at 37 °C and DNA content was analyzed on a FACScan cytometer (FACScan, Becton Dickinson). The apoptotic cells were defined as those with less than 2N DNA content.

Caspase assay

CH31 cells were cultured at 0.6×10^6 /ml complete RPMI in 12-well plates in triplicate. Cells were treated with 1 μ g/ml anti-mouse IgM in the presence or absence of 20 ng/ml mouse IL-4 or 50 μ M d-Boc-fmk. After 16 h, caspase 3 and caspase 9 activity were measured using CaspaTag Caspase Activity Kits as instructed by the manufacturer (Intergen company). Caspase activity was monitored using FACS and expressed as percent of cells positive for fluorescence.

Constructs and transient transfections

SH2-deleted p85 α PI-3K was originally constructed in the pcDNA3 mammalian expression vector in Dr Jonathan Ashwell's Laboratory (NIH) and was kindly provided by Dr Astrid Eder [26]. Kinase-dead Akt (K > A-Akt), originally constructed in pSG5 mammalian expression vector, was a kind gift from Dr Gerard Evan (Cancer Research Institute, UCSF, CA). Transient co-transfections were performed with 1 μ g of endotoxin-free pMaxGFP plasmid (Amaya, Inc., Gaithersburg, MD), with or without 5 μ g endotoxin-free construct or empty cassette (as noted in figure legends). CH31 cells were maintained in exponential growth phase and transfected using the Amaya Nucleofector System®, Amaya, Inc., Gaithersburg, MD. Transfections were optimized as per the manufacturer's protocols. pMaxGFP expression in CH31 was monitored by FACS. At 16 h post-transfection, $\geq 60\%$ of the surviving cells were observed to express pMaxGFP (data not shown). The cells were then treated

as described in the figure legends for the indicated times.

Phosphorylation analysis

Analysis of IL-4-induced phosphorylation was performed as previously described [27]. Briefly, CH31 cells were deprived of serum in RPMI for 2 h at 37 °C. After washing, 10^7 cells were resuspended in RPMI and incubated in the presence or absence of murine IL-4 (10 ng/ml) for 10 min at room temperature. The reaction was terminated by 10-fold dilution in ice-cold PBS. Cell pellets were lysed in HEPES lysis buffer (50 mM HEPES, 100 mM NaCl, 0.5% NP-40, 1 mM Na_3VO_4 , 50 mM NaF, 10 mM pyrophosphate, 1 mM PMSF, and protease inhibitor cocktail) and clarified. Protein determination was made using the BioRad kit according to the manufacturer. The soluble fraction was immunoprecipitated with a polyclonal rabbit anti-IRS2 or anti-p85 (Upstate Biotechnologies, Lake Placid, NY). The precipitates were washed in lysis buffer and solubilized in SDS sample buffer. The samples were separated on 7.5% SDS-polyacrylamide gels before transferring to a PVDF membrane. The membranes were then probed with a monoclonal anti-phosphotyrosine antibody, RC20-H (Transduction Labs, Lexington, KY) to detect tyrosine phosphorylated IRS2, or with anti-IRS-2 antibody. The bound antibody was detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Where indicated, the blots were stripped and probed with control antibodies.

Western blots

Typically, at least 2×10^7 cells at an initial density of 0.25×10^6 /ml were treated as indicated in the figure legends. Harvested cells were washed twice with cold PBS and excess PBS removed by aspiration. The cell pellets were then resuspended at 10^5 cells/ μ l of lysis buffer containing 1% IGEPAL CA-630 (NP40 replacement), 5 mM benzamidine, 2 mM β -glycerophosphate, 2 mM DTT, 2.5 mM EDTA, 2.5 mM EGTA, 10% glycerol, 5 mM $\text{Na}_4\text{P}_2\text{O}_7$, 2.5 mM Na_3VO_4 , 100 mM NaCl, 2.5 mM NaF, 20 mM Tris.HCl pH 8.0 (all from Sigma-Aldrich) and 1 \times protease cocktail inhibitor (Roche Diagnostics, Indianapolis, IN). Calyculin-A, a potent inhibitor of protein phosphatases 1 and 2A, was added to the lysis buffer just prior to adding the buffer to the cells, to attain a final concentration of 100 nM. The cell pellets were kept on ice and disrupted by sonication using a Branson Sonifier 250. The homogenates were then centrifuged for 10 min at $10\,000 \times g$ at 4 °C and the clarified supernatants removed and protein concentrations determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Lysates were adjusted to 2.5 μ g protein/ μ l in 1 \times Laemmli sample buffer containing 10% β -mercaptoethanol and heated at 95 °C for 5 min. Equal amounts of up to 50 μ g of protein were loaded per lane and resolved by SDS-PAGE. Resolved proteins were then transferred onto a PVDF membrane (Millipore, Bedford, MA) and processed for western blotting essentially as described previously [20]. Antibodies included the following: anti-phospho and total CDK2, Akt and MEK obtained from Cell Signaling, Beverly, MA; anti-Bid, Bcl-xL, caspase 9, and Bim antibodies obtained from the same source; and anti-p27^{Kip1}, C-Myc and cytochrome C antibodies obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Densitometric analysis was performed using UnScanit™ Software (Silk Scientific, Orem, UT).

Cytosolic fractions

Cells were seeded and treated as described for western blotting. The samples were split into two. One was lysed as described above and the other was processed for preparation of cytosolic fractions. For

the latter method, IGEPAL detergent was excluded and replaced with 0.005% saponin, a mild detergent that, in this concentration range, selectively permeabilizes plasma membranes [28]. The cells were vortexed gently and incubated on ice for 20 min, then gently vortexed again. Following ultracentrifugation at $100\,000 \times g$ for 1 h at 4 °C, the supernatant designated the cytosolic fraction was collected. CytC protein level in this fraction was compared with that in homogenates from cells sonicated in IGEPAL630-containing lysis buffer.

Proliferation assay

Cells were maintained as described above and treated as indicated in the accompanying figure legends. Comparative DNA synthesis, an indicator of relative proliferation, was determined by the tritiated thymidine assay as described previously [20]. Briefly, 200 µl of cells were seeded onto 96-well microtiter plates at an initial density of 0.25×10^6 /ml and treated as described in the accompanying figure legend. For the last 4 h of the incubation, the cells were pulse labeled with 2.5 µCi/ml ^3H -deoxythymidine (New England Nuclear/Perkin-Elmer, Boston, MA). The cells were harvested onto glass fiber filters using a Packard Filtermate 196™. The filters were washed three times with water and once with 20 ml 70% ethanol. Radioactivity incorporation was determined using a Packard beta counter.

Results

Effect of IL-4 on anti-IgM-induced growth arrest and apoptosis

Cross-linking the BCR on CH31 cells using anti-IgM

antibodies leads to growth inhibition, cell cycle arrest and apoptosis ([17-19], illustrated in Figures 1 and 2); therefore, these cells have been studied extensively as a model for self-tolerance by clonal deletion. In keeping with previous studies, we found that the addition of recombinant IL-4 prevented the anti-IgM-induced growth arrest and apoptosis (Figure 1). It has been shown that IL-4 can slow the proliferation of several tumor cell lines, a point confirmed in Figure 1A for CH31 cells [8, 9, 29]. Importantly, we observed that IL-4 reversed the potent growth inhibition caused by anti-IgM treatment (Figure 1). Using nuclear staining with propidium iodide as an indicator of nuclear DNA content, we found that IL-4 suppressed the induction of sub-diploid DNA, a measure of apoptosis, by anti-IgM (Figure 1B and 1C). This suppression of apoptosis correlated with an enhancement of cell survival as determined by trypan blue dye exclusion (data not shown). Furthermore, we found that IL-4 dramatically antagonized the effect of anti-IgM on the cell cycle (Figure 2). Treatment with anti-IgM caused a >3-fold reduction in the percentage of CH31 cells in S-phase (from 22% to 7%) and a >2-fold reduction in G2/M phase (from 22% to 10%). The addition of IL-4 completely reversed these trends, reaching control levels at > 1 ng/ml IL-4. Together, these results suggest that IL-4-activated signaling pathways abrogate the growth arrest

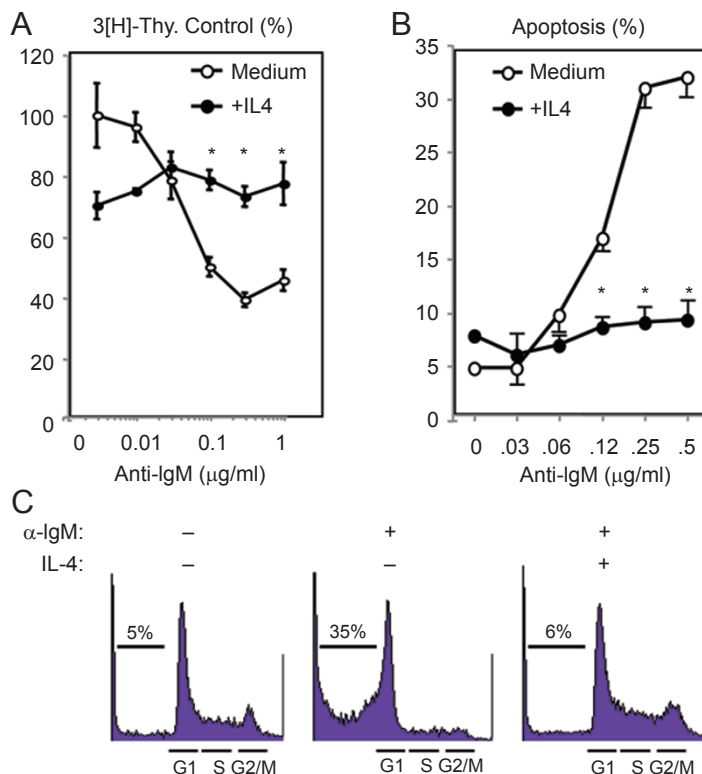


Figure 1 Effect of IL-4 on anti-IgM-induced growth arrest and apoptosis. CH31 cells were plated in fresh medium and treated with or without IL-4 (10 ng/ml) in the presence or absence of various concentrations of monoclonal anti-IgM for 16 h. **(A)** The cells were pulsed with ^3H -thymidine for the last 4 h of culture as described in Materials and Methods. Relative uptake is expressed as percent of untreated controls \pm SEM. The data are representative of more than three separate, independent experiments. The Student's *t*-test was used to determine the significance of the differences \pm IL-4 at each anti-IgM concentration ($*p < 0.05$). **(B)** Cells were analyzed for nuclear DNA content following permeabilization and propidium iodide staining as described in Materials and Methods. The average percentage of apoptotic cells, defined as those cells containing less than 2N DNA content, is shown \pm SEM. The Student's *t*-test was used to determine the significance of the differences \pm IL-4 at each anti-IgM concentration ($*p < 0.05$). **(C)** Representative FACS histograms of nuclear DNA content are shown. The percentage of apoptotic cells, defined as those cells containing less than 2N DNA content, is indicated. The DNA content peaks indicating cells in the G1, S, or G2/M phase of the cell cycle are marked.

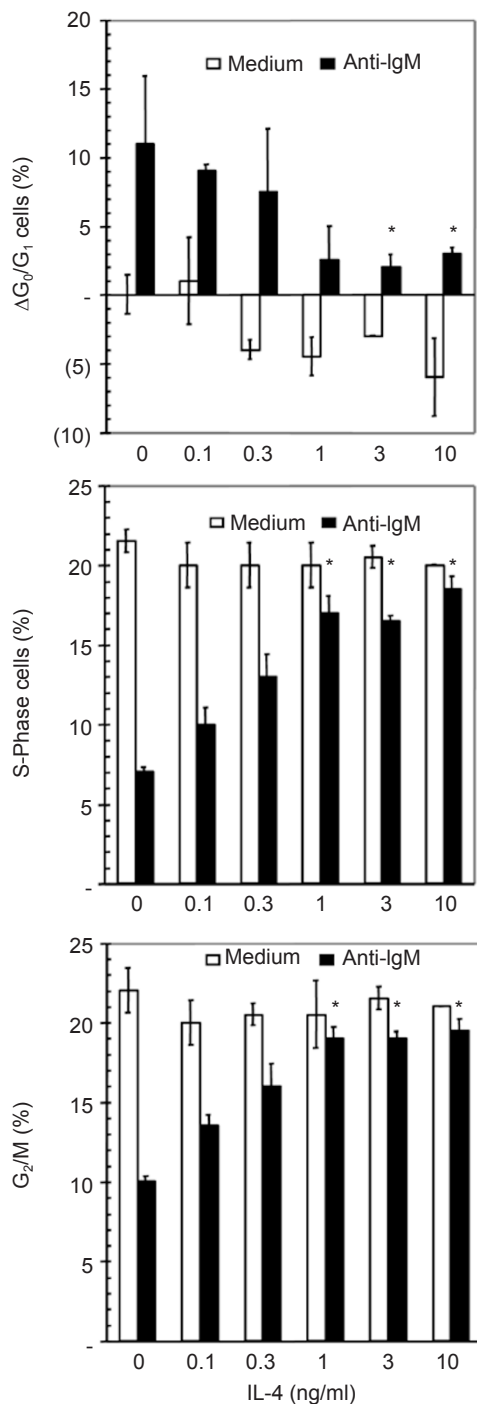


Figure 2 Effect of IL-4 on cell cycle. CH31 cells were plated in fresh medium and treated with or without various concentrations of IL-4 in the presence or absence of anti-IgM (1 μ g/ml) for 16 h. Analysis of the nuclear DNA content following permeabilization and propidium iodide staining was performed as described in Materials and Methods and illustrated in Figure 1C. The changes in percentages of cells in G₀/G₁, S-phase, and G₂/M were calculated by gating on the DNA-content curves. Average values \pm SEM are shown. The Student's *t*-test was used to determine the significance of the differences \pm IL-4 in the presence of anti-IgM at each IL-4 concentration ($*p < 0.05$).

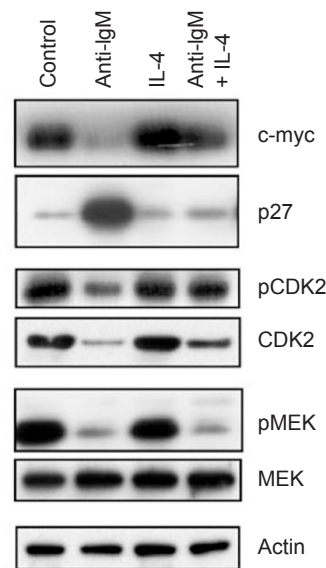


Figure 3 IL-4 reverses many, but not all, of the anti-IgM-induced molecular effects. 1.5×10^7 cells were treated with or without anti-IgM (1 μ g/ml) in the presence or absence of mL-4 (5 ng/ml) for 16 h. The cells were harvested, washed and extracted as described in Materials and Methods. C-Myc, p27 (p27), activation-specific (phosphoThr160) CDK2, total CDK2, activation-specific (phospho Ser²¹⁷/Ser²²¹)-MEK_{1&2}, total MEK, and β -actin were analyzed by western blotting. The presented data are representative of at least three separate independent experiments.

and apoptosis effects of anti-IgM stimulated pathways.

CH31 lymphoma cells display constitutively elevated c-myc expression and activated CDK2 [19, 30]. These changes are known to confer proliferative and survival advantages in several B-lymphoma lines [20, 23, 31-33]. Previous results from the Scott lab demonstrated that treatment of CH31 with anti-IgM results in the loss of c-myc [30] and inactivation of CDK2 [19], an enzyme that promotes cell cycle progression and suppresses accumulation of the cyclin-dependent kinase inhibitor p27^{Kip1} through phosphorylation and proteasomal targeting [34]. Phosphorylation of CDK2 on Threonine 160 (pCDK2) is required for CDK activity and, therefore, the measurement of T¹⁶⁰ phosphorylation is an excellent indicator of its activation [35]. Consistent with previous results, we confirmed that treatment of CH31 cells with anti-IgM resulted in a loss of c-myc expression, loss of pCDK2 and a rise in expression of the CDK inhibitor p27^{Kip1} (Figure 3). To test whether IL-4 interfered with these pathways, we cultured CH31 cells in the presence or absence of IL-4 and anti-IgM. We found that IL-4 potently prevented the anti-IgM-induced loss of c-myc protein (Figure 3). This is in contrast to older studies analyzing the effects of IL-4 on

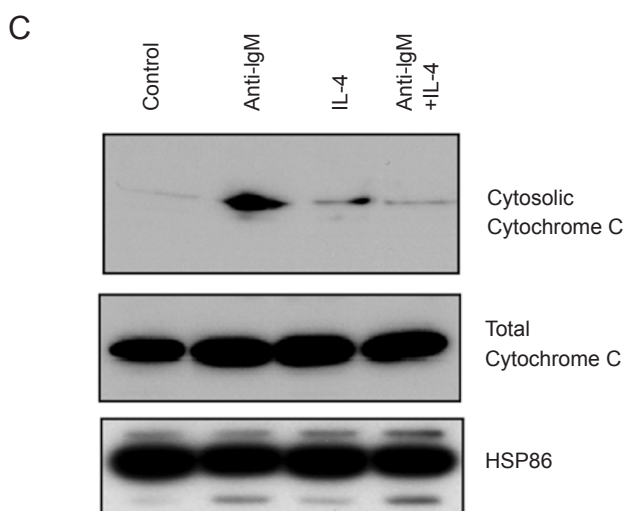
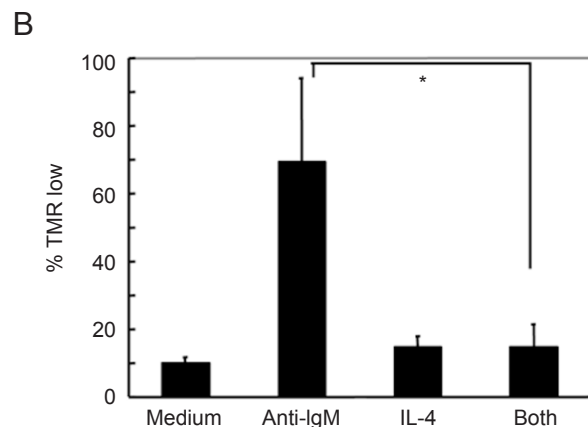
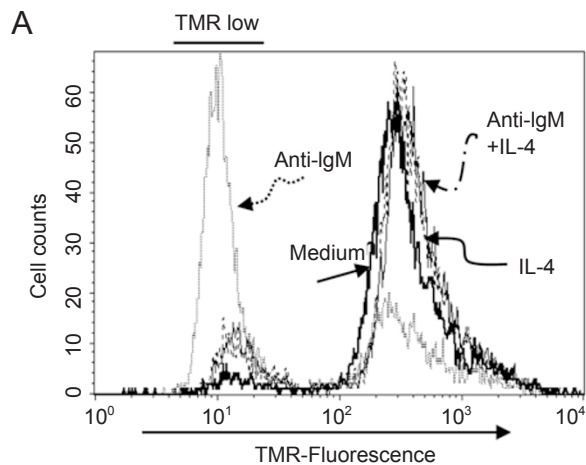


Figure 4 IL-4 prevents the loss of mitochondrial membrane potential induced by anti-IgM. CH31 cells were treated with or without anti-IgM (1 μ g/ml) in the presence or absence of mIL-4 (5 ng/ml) for 16 h. **(A)** The cells were stained with tetramethylrhodamine (TMR) for the last 15 min of the incubation and analyzed by FACS to determine mitochondrial membrane potential ($\Delta\Psi$ m). **(B)** Cells with mean TMR fluorescence of 160 arbitrary units were designated as ‘TMR-Low’. The average values for percent TMR-low cells and the SEM from three separate experiments are shown. The Student’s *t*-test was used to determine the significance of the differences +/- IL-4 in the presence of anti-IgM (**p* < 0.05). **(C)** CH31 cells were cultured (0.6×10^6 /ml) in complete RPMI in the presence or absence of 1 μ g/ml anti-mouse IgM in the presence or absence of 20 ng/ml mouse IL-4 before cell lysis. The cytosolic fraction was isolated as described in Materials and Methods. The cytosolic fractions and total cell lysates were analyzed by western blotting for cytochrome C. The cytosolic fractions were blotted with anti-HSP86 as control.

c-myc mRNA expression [6]. Furthermore, we observed that IL-4 prevented the anti-IgM-induced loss of pCDK2 and it also prevented the induction of p27^{Kip1} (Figure 3). Banerji *et al.* established that WEHI-231, another extensively documented, model B-lymphoma line, contained constitutively activated ERK, which was completely inactivated following BCR crosslinking [21]. Furthermore, Guilbault and Kay established that anti-IgM stimulated the loss of Ras effector signaling [36]. Therefore, we also examined the effect of IL-4 on Ras effector via monitoring activation-specific phosphorylation of the extensively characterized Ras/Raf target, MEK. Our results show that proliferating CH31 cells contained constitutive, robustly phosphorylated MEK (pMEK, Figure 3). Anti-IgM treatment resulted in its near-complete dephosphorylation, which was not prevented by IL-4 treatment. These data indicate that IL-4 does not interfere with all anti-IgM-mediated signaling events and

further suggest that maintenance of MEK/ERK signaling is not required for IL-4 mediated protection from anti-IgM-induced growth arrest or cell death. Notably, actin expression was not affected by these treatments, indicating that protein loading had been equalized. All together, these data clearly show that, in the presence of anti-IgM, IL-4 treatment results in the stabilization of c-myc protein and pCDK2 and the dramatic reduction of p27^{Kip1}.

Effects of IL-4 on the apoptotic pathway

Unlike TNF α - (tumor necrosis factor alpha) and Fas-induced death, the anti-IgM-induced apoptosis in B-cell lymphoma cells is independent of caspase 8 and involves the dysregulation of the mitochondrion [37-39]. This mitochondria-facilitated, intrinsic or ‘Type 2’ pathway is started by the initial release of small amounts of CytC from mitochondria [40]. In the cytoplasm, CytC complexes

with caspase-9 and Apaf-1, forming the apoptosome and beginning the intrinsic apoptotic process [41, 42]. Recently, Eldering *et al.* demonstrated that anti-IgM-mediated apoptosis of Ramos cells invoked a type-2 pathway which was closely associated with the collapse of the mitochondrial membrane potential ($\Delta\Psi_m$), a *bona fide* measure of overall mitochondrial health [38, 40]. Therefore, we first examined whether IL-4 influenced the BCR-mediated loss of $\Delta\Psi_m$. Indeed, we found that anti-IgM treatment induced the loss of $\Delta\Psi_m$, which was completely blocked by co-administration of IL-4 (Figure 4A and 4B). We next examined the effect of IL-4 on anti-IgM-stimulated CytC release. Like other investigators [38, 40], we found that anti-IgM treatment of CH31 induced the release of CytC into the cytosol (Figure 4C). Strikingly, IL-4 completely blocked this release. These results indicate that IL-4 signaling interferes with mitochondrial changes induced by anti-IgM treatment that otherwise can lead to mitochondrial dysfunction and apoptosis.

Mitochondrial disruption and apoptosome formation result in the activation of the initiator caspase-9, which in turn can activate the effector caspase 3 [41]. Therefore, we analyzed the effect of IL-4 on caspase activation in response to anti-IgM treatment. Anti-IgM treatment induced the activation-specific cleavage of pro-caspase 9 as detected by western blotting; IL-4 completely prevented this cleavage (Figure 5A). Further, anti-IgM induced the activation of caspases 9 and 3 as detected by cleavage of selective intracellular caspase substrates (Figure 5B); IL-4 suppressed this cleavage to the levels observed in cells treated with the pan caspase inhibitor D-Boc-fmk. Taken together, these results suggest that IL-4 protects CH31 cells from anti-IgM-induced apoptosis by preventing mitochondrial dysregulation and subsequent caspase activation.

Effects of IL-4 signaling on the PI-3K/Akt pathway and protection from apoptosis

IL-4 is known to induce the activation of several signal transduction pathways in Janus kinase (JAK)-dependent manners [43]; both the IRS/PI-3K and the STAT6 pathways have been linked to suppression of apoptosis in other cell types [11-13]. Thus, we first analyzed the ability of IL-4 to induce these pathways in CH31 (Figure 6A). IL-4 stimulated the tyrosine phosphorylation of IRS2, its association with the p85 subunit of PI-3K, and the tyrosine phosphorylation of STAT6 in CH31. We next examined the ability of IL-4 to regulate proteins involved in mitochondria-regulated apoptosis downstream from IRS2/PI-3K or from STAT6 (Figure 6). Active Akt has been termed a master regulator of cell survival [44]. Consistent with this notion, we found that CH31 cells contain Akt constitutively phosphorylated on S⁴⁷³ (pAkt), a marker of its activation (Figure 6, [45]).

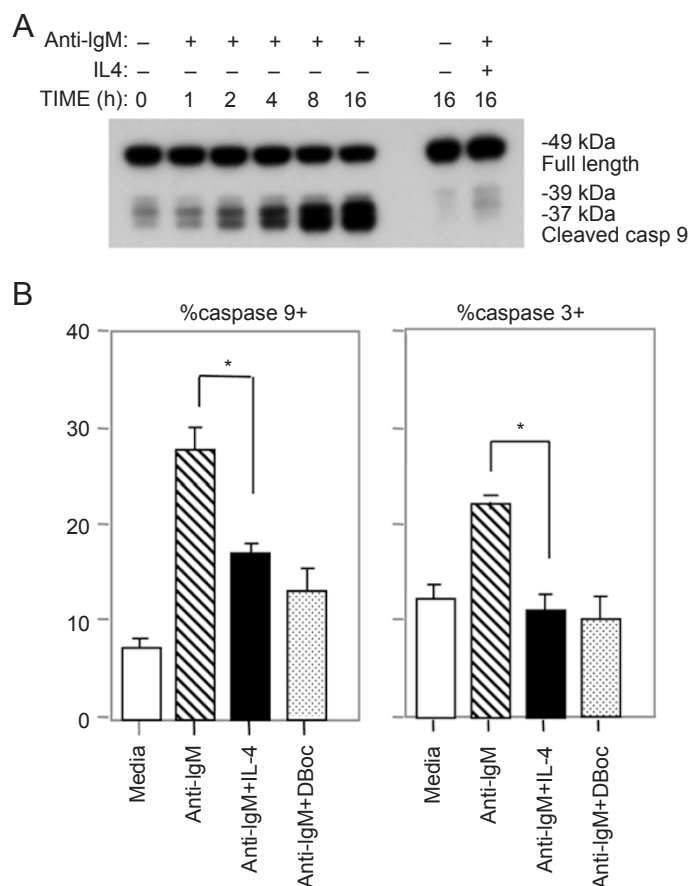


Figure 5 IL-4 suppresses the activation of caspases by anti-IgM. **(A)** CH31 cells were cultured ($0.6 \times 10^6/ml$) in 2 ml of complete RPMI in 12-well plates. Cells were treated with 1 $\mu g/ml$ anti-mouse IgM for various times in the presence or absence of 20 ng/ml mouse IL-4 as indicated. The cells were harvested, washed and extracted as described in Materials and Methods. Caspase 9 was analyzed by western blotting. The presented data are representative of at least three separate independent experiments. **(B)** CH31 cells were cultured in triplicate ($0.6 \times 10^6/ml$) in 2 ml of complete RPMI in 12-well plates. Cells were treated with 1 $\mu g/ml$ anti-mouse IgM in the presence or absence of 20 ng/ml mouse IL-4 or 50 μM D-Boc-fmk. After 18 h, caspase 3 and caspase 9 activity were measured using the CaspaTag Caspase Activity Kits (Intergen company). Cells expressing active caspase were detected by FACS. The average value for percent positive \pm SEM are shown. The Student's *t*-test was used to determine the significance of the differences \pm IL-4 in the presence of anti-IgM ($*p < 0.05$). These results are representative of three independent experiments.

Treatment of these cells with anti-IgM resulted in a loss of pAkt; this loss was abrogated by IL-4 treatment (Figure 6B). pAkt activity controls both the expression and activities of multiple pro-apoptotic proteins such as BAD, GSK-3 β , IKK, caspase-9 and the forkhead (FKHR) transcription

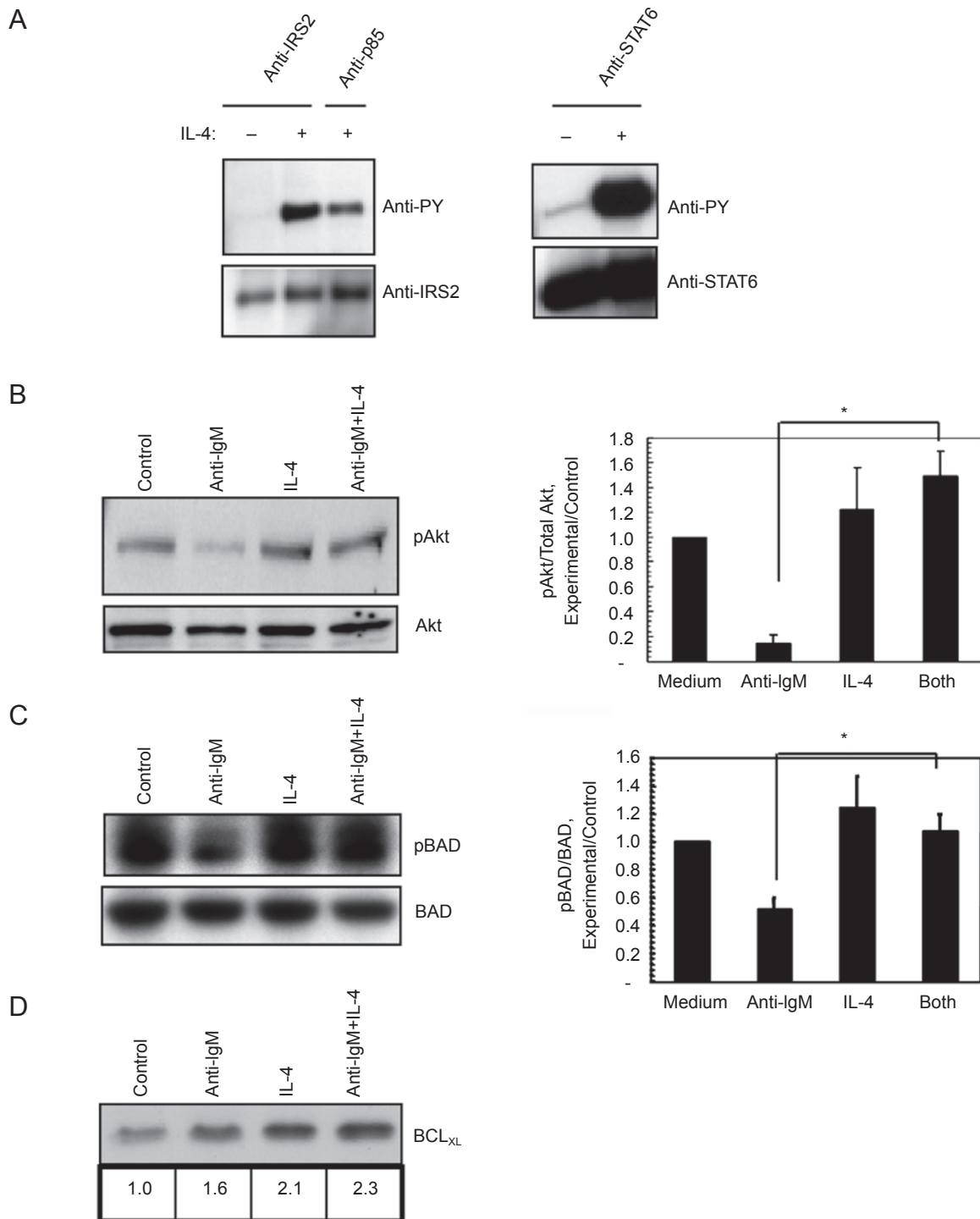


Figure 6 IL-4 signaling in CH31. **(A)** CH31 cells were treated with IL-4 for 30 min and cell lysates were immunoprecipitated with anti-IRS2, anti-p85, or anti-STAT6 as indicated. Western blots were probed with anti-phosphotyrosine. The blots were stripped and re-probed with anti-IRS2 or anti-STAT6 as appropriate. **(B-D)** Cells were treated as described for Figure 3. Total lysates were prepared and activation-specific phosphorylation of Akt (phospho Ser473), total Akt **(B)**, deactivation-specific phosphorylation of BAD (phospho Ser136), total BAD **(C)**, and total Bcl-xL **(D)** were examined by western blotting. Three independent experiments were analyzed by densitometry to calculate the average ratio of phosphorylated Akt to total Akt or phosphorylated BAD to total BAD protein. The average ratios and the SEM are shown as bar graphs. The Student's *t*-test was used to determine the significance of the differences +/- IL-4 in the presence of anti-IgM ($*p < 0.05$). The blots for Bcl-xL were analyzed by densitometry, and the levels of expression relative to the untreated control are shown. These data are representative of at least three separate, independent experiments.

factors [44-46]. BAD can perturb the mitochondrion by antagonizing the survival functions of Bcl-2 and Bcl-xL, and phosphorylation of S¹³⁶ on BAD (pBAD) by Akt suppresses BAD activity [47, 48]. Intriguingly, we found that, in addition to inducing the dephosphorylation of Akt, anti-IgM caused a reduction in the phosphorylation of BAD. IL-4 treatment alone enhanced the phosphorylation of BAD and, importantly, maintained BAD phosphorylation in the presence of anti-IgM (Figure 6C). These results are in contrast to studies on IL-3-dependent cell lines where IL-4 did not induce the phosphorylation of BAD [49]. Interestingly however, anti-IgM treatment induced a modest (1.6 ×) increase in expression of Bcl-xL over untreated control CH31 (Figure 6D), but did not induce cleavage of BID or Bim (not shown), two classic targets of the caspase-8 signaling

paradigm [47]. IL-4 induced a stronger increase in Bcl-xL (2.1 ×) expression and slightly enhanced its expression in the presence of anti-IgM (from 1.6 × to 2.3 ×, Figure 6D). Thus, IL-4 may antagonize anti-IgM-mediated death signals that converge on the mitochondrion, by suppressing the pro-apoptotic function of BAD and by increasing Bcl-xL beyond a minimal threshold.

The activation of all signaling pathways by IL-4 is dependent on the activation of JAK family members and, indeed, we found that AG490, an extensively utilized, selective JAK inhibitor [50], abrogated the ability of IL-4 to protect CH31 cells from anti-IgM-induced apoptosis (Figure 7A). The IL-4-induced activation of the IRS2/PI-3K pathway plays a significant role in the protection of 32D cells from IL-3-withdrawal-induced apoptosis and

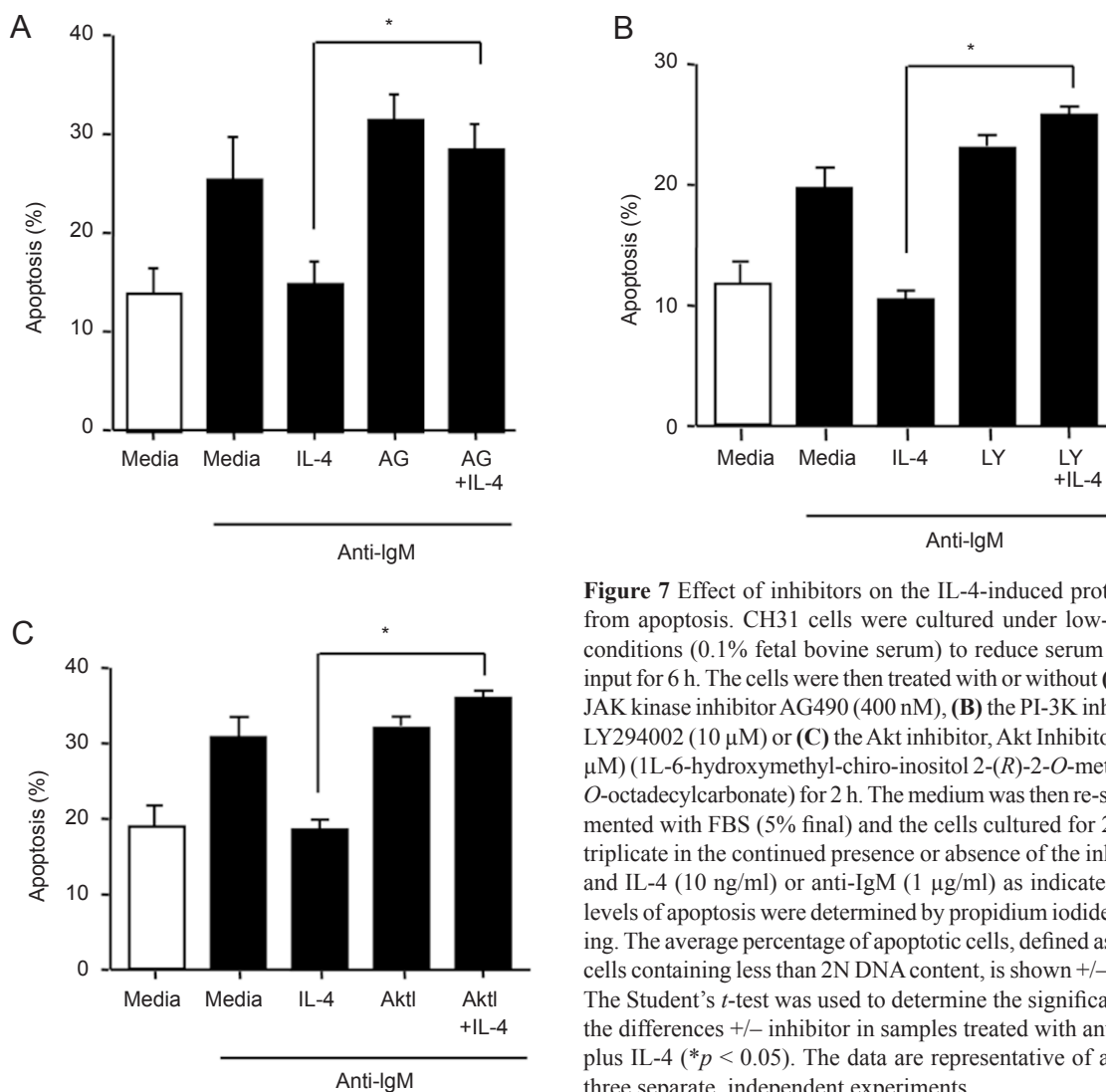


Figure 7 Effect of inhibitors on the IL-4-induced protection from apoptosis. CH31 cells were cultured under low-serum conditions (0.1% fetal bovine serum) to reduce serum signal input for 6 h. The cells were then treated with or without (A) the JAK kinase inhibitor AG490 (400 nM), (B) the PI-3K inhibitor, LY294002 (10 μM) or (C) the Akt inhibitor, Akt Inhibitor I (10 μM) (1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate) for 2 h. The medium was then re-supplemented with FBS (5% final) and the cells cultured for 24 h in triplicate in the continued presence or absence of the inhibitor and IL-4 (10 ng/ml) or anti-IgM (1 μg/ml) as indicated. The levels of apoptosis were determined by propidium iodide staining. The average percentage of apoptotic cells, defined as those cells containing less than 2N DNA content, is shown ± SEM. The Student's *t*-test was used to determine the significance of the differences ± inhibitor in samples treated with anti-IgM plus IL-4 (**p* < 0.05). The data are representative of at least three separate, independent experiments.

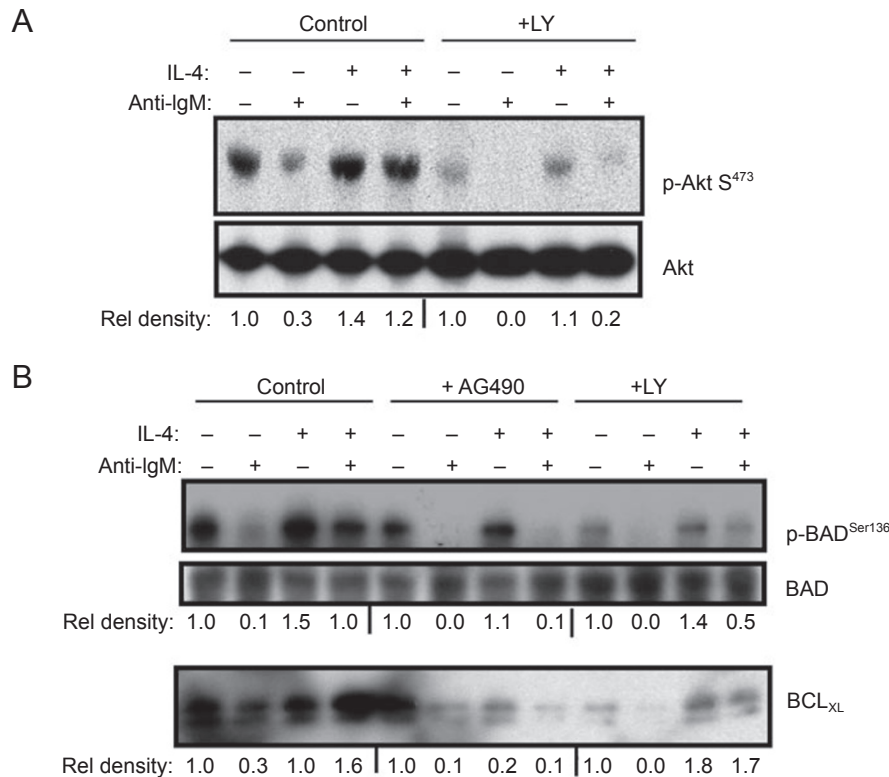


Figure 8 Effect of inhibitors on the IL-4-induced phosphorylation of Akt and Bad, and induction of Bcl-xL. CH31 cells were cultured under low-serum conditions (0.1% fetal bovine serum) to reduce serum signal input for 6 h. The cells were then treated with or without the JAK kinase inhibitor AG490 (400 nM) or the PI-3K inhibitor, LY294002 (10 μ M) as indicated for 2 h. The medium was then re-supplemented with FBS (5% final) and the cells cultured for 24 h in the continued presence or absence of the inhibitor and IL-4 (10 ng/ml) or anti-IgM (1 μ g/ml) as indicated. Total lysates were prepared and activation-specific phosphorylation of Akt (phospho Ser473), total Akt (**A**), deactivation-specific phosphorylation of BAD (phosphor Ser136), total BAD, and total Bcl-xL (**B**) were examined by western blotting. The blots were analyzed by densitometry as described in Figure 6 and the levels of expression relative to the untreated control in each inhibitor group are shown. These data are representative of two separate, independent experiments.

expression of p85 α in primary B cells is required for protection from apoptosis in the presence of anti-IgM plus IL-4 treatment [11, 51]. Therefore, we tested the role of PI-3K activity in the ability of IL-4 to reverse the anti-IgM-induced apoptosis of CH31 cells by using the PI-3K inhibitor LY294002 [52]. We found that LY294002 pre-treatment abrogated the ability of IL-4 to protect cells from apoptosis induced by anti-IgM (Figure 7B). These results suggest that PI-3K and molecules downstream of it are involved in the IL-4-mediated protection. To test whether Akt function is directly involved with the IL-4-mediated protection from apoptosis, we cultured CH31 cells in the presence of the Akt inhibitor AktI, which prevents binding of Akt to D3 phosphatidylinositol (PIP3) [53]. We observed that this inhibitor also abrogated the ability of IL-4 to protect cells from anti-IgM-induced apoptosis (Figure 7C), suggesting that Akt activity and its downstream sequelae contribute to

the anti-apoptotic activity of IL-4 in this model.

In normal cells, the phosphoinositol 3 phosphatase (PTEN) antagonizes PI-3K [54]. However, PTEN protein expression was not detectable in CH31 cells (Carey, unpublished). Thus, it was possible that classic targets of PI-3K action could remain active even in the presence of the PI-3K inhibitor. Therefore, we analyzed the phosphorylation status of Akt in the presence or absence of LY294002. LY294002 treatment alone suppressed the levels of endogenous pAkt in keeping with previous studies showing that CH31 cells have constitutive PI-3K signaling that is highly sensitive to LY294002 [20]. In addition, LY294002 abrogated the ability of IL-4 to maintain the levels of pAkt in anti-IgM-treated cells (Figure 8A). Thus, the ability of IL-4 to protect CH31 cells from anti-IgM-induced apoptosis correlated highly with its ability to mediate Akt phosphorylation via a PI-3K-dependent

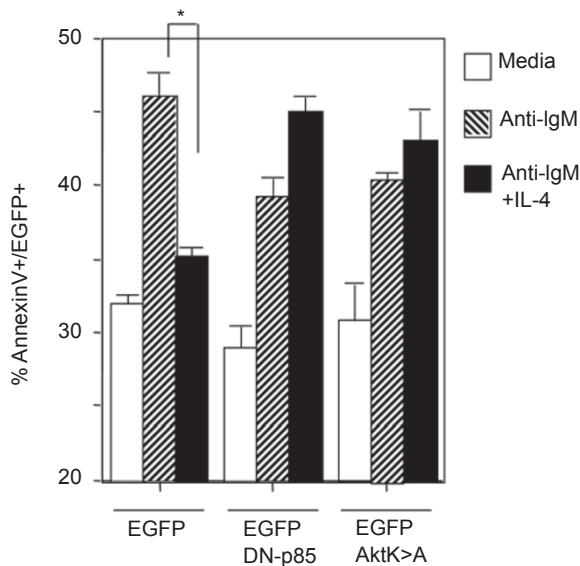


Figure 9 Transient transfection analysis. CH31 cells were transiently transfected with pMAXGFP in combination with or without mammalian expression vector constructs encoding either SH2-deleted p85^{PI-3K} (DN-p85) or a kinase-dead mutant form of Akt/PKB (AktK > A). The cells were washed 6 h post-transfection, seeded at 0.25×10^6 live cells/ml, and treated with or without IL-4 (10 ng/ml) or anti-IgM (1 μ g/ml) for 16 h. Apoptosis was determined by assessing Annexin-V binding on GFP+ cells by FACS as described in Materials and Methods at 16 h post-stimulation. Average values of three experiments \pm SEM are shown. The Student's *t*-test was used to determine the significance of the differences \pm IL-4 in the presence of anti-IgM (**p* < 0.05).

pathway. We further analyzed the effects of LY294002 and AG490 on the phosphorylation of Bad and expression of Bcl-xL (Figure 8B). As shown in Figure 6, CH31 cells displayed phosphorylation of Bad that was reduced by anti-IgM. IL-4 treatment increased the phosphorylation of BAD and maintained BAD phosphorylation in the presence of anti-IgM. However, in the presence of AG490, IL-4 did not induce the phosphorylation of Bad over the untreated control. LY294002 treatment suppressed the basal levels of phosphorylated Bad and Bcl-xL. Even so, we observed that the ability of IL-4 to induce or maintain pBad was reduced in the presence of LY294003, while its ability to induce Bcl-xL over control was relatively unaffected. In light of the effects of these inhibitors on apoptosis (Figure 7), these results suggest that the IL-4-activated PI-3K/Akt pathway plays a dominant role in the protection of these cells from anti-IgM-induced apoptosis.

To directly test the contribution of the PI-3K/Akt signaling module on the protection of the cells from apoptosis

using molecular techniques, we transiently transfected CH31 with a plasmid encoding pMaxGFP in the presence or absence of cDNA encoding a dominant inhibitory form of p85 α or a kinase-dead mutant Akt (Figure 9). The cells were treated with anti-IgM in the presence or absence of IL-4. Annexin V staining of the pMaxGFP⁺ cells was then analyzed as an indicator of apoptosis in the transfected cells. We found that the DN-p85 α and the K > A mutant Akt abrogated the ability of IL-4 to suppress anti-IgM-induced apoptosis. Thus, the IRS2/PI-3K/Akt pathway plays an important role in the anti-apoptotic effect of IL-4 in these cells.

Discussion

IL-4 is known to be a potent survival factor for many cell types [1]. It acts to prevent apoptosis induced by a number of stimuli and thus could contribute to the abnormal outgrowth of cells *in vivo*. Indeed, high IL-4 levels have been associated with reduced apoptosis of non-Hodgkin's lymphoma [55]. Interestingly, primary mediastinal large B-cell lymphomas (PMBL) have been shown to contain constitutive activation of STAT6 without evidence of cytokine production [50]. On the other hand, classical Hodgkin's lymphoma Reed–Sternberg cells produce IL-13 and display IL-13-dependent STAT6 activation and enhanced survival [14]. These effects are thought to be regulated by the STAT6-mediated induction of Bcl-xL [14, 50, 55]. In contrast, IL-4 was able to substantially protect B cells isolated from STAT6-deficient animals from spontaneous apoptosis [13, 15], indicating the contribution of another signaling pathway. Therefore, a detailed understanding of the mechanism(s) by which IL-4 and IL-13 regulate cell cycle and suppress apoptosis could provide important targets for cancer therapy.

Although IL-4 treatment alone reduced the proliferation of CH31 cells by ~20%, IL-4 reversed anti-IgM-mediated inhibition of cell proliferation. The slowing of proliferation appeared to be beneficial for cell health since the induction of apoptosis was completely blocked and the percentage of G₀/G₁-arrested cells was reduced virtually to control under these conditions. That IL-4 maintains a net proliferative advantage is shown by the data presented in Figure 1 and is supported by those presented in Figure 3 where IL-4 maintained CDK2 activation (pCDK2, [35]), suppressed p27^{Kip1} protein expression, and prevented the down-regulation of c-Myc. In a recent profile of genes using the GS320 systemTM (Capital Genomix, Gaithersburg, MD), we observed that anti-IgM treatment down-regulated CDK2 mRNA in both ECH408 and WEHI-231 cells (both undergo apoptosis in response to anti-IgM treatment). Furthermore, we found that CDK2 protein was also down-

regulated in these cells following treatment with anti-IgM (data not shown). Consistent with those observations, the data presented here show that anti-IgM selectively reduces CDK2 expression in CH31, since the expression of actin and MEK are not similarly affected. It is important to note that, although CDK2 expression was only partially restored in the presence of IL-4, pCDK2 levels were completely restored to control. This restoration could help explain the observed suppression of p27^{Kip1} protein accumulation [21, 46] and continued cell cycle progression. Since it has been shown that active Akt maintains active CDK2 and prevents p27^{Kip1} accumulation in WEHI-231 [21], it is reasonable to propose that the IL-4-induced maintenance of active Akt leads to the preservation of pCDK2 and suppression of p27^{Kip1} accumulation in CH31 cells.

While IL-4 maintained Akt and CDK2 phosphorylation in the presence of anti-IgM stimulation, it did not maintain MEK phosphorylation. Thus, these results clearly show that IL-4 does not influence all signaling events stimulated by anti-IgM in these cells, and also that maintenance of pMEK is not required for protection from apoptosis. Typically, IL-4 signaling activates Akt but does not stimulate the Ras, Raf, MEK pathway [43]. Thus, its ability to maintain the phosphorylation of Akt and its inability to influence the regulation of the Ras, Raf, MEK pathway by anti-IgM are consistent with known IL-4 effects.

In contrast with studies in other systems, the results presented here establish the PI-3K/Akt pathway as a critical mediator of the protection from anti-IgM-induced apoptosis by IL-4. PI-3K has been firmly established as a critical regulator of apoptosis in primary lymphocytes. Deletion of the p85 α regulatory subunit resulted in massive anti-IgM-stimulated apoptosis of B cells; this apoptosis was not prevented by IL-4 treatment [51]. Interestingly, LPS treatment stimulated cell cycle progression in both wild-type and p85^{-/-} cells. However, p85^{-/-} cells could not progress beyond the G₁ phase of the cell cycle and defaulted to apoptosis. Thus, p85 α was required for both continued cell cycle progression and protection from apoptosis. Our results using the PI-3k inhibitor LY294002 and transient overexpression of an inhibitory p85 α deletion mutant are in agreement with these data. Furthermore, we found that treatment with the Akt inhibitor AktI and overexpression of the kinase-dead Akt mutant K > A, blocked the IL-4-mediated protection of CH31 cells from anti-IgM-induced apoptosis. These results indicate that Akt lies at the nexus of signals generated by IL-4 that protect these cells from growth arrest and apoptosis.

Akt is a major regulator of cellular survival since its activity is directed toward suppressing the activities and/or expression of multiple pro-apoptotic proteins [44, 45]. Phosphorylation of S¹³⁶ on BAD (pBAD) by Akt sup-

presses BAD activity so that pBAD cannot antagonize the pro-survival functions of Bcl-2 and Bcl-xL [47, 48]. In this report, we found that IL-4 prevented anti-IgM-mediated deactivation (S⁴⁷³ dephosphorylation) of Akt (Figure 6) and maintained pBAD at levels seen in controls. Interestingly, anti-IgM or IL-4 treatment alone induced an increase in expression of Bcl-xL. However, clearly, Bcl-xL elevation alone (as seen in anti-IgM-treated cells) was not sufficient to counter anti-IgM-mediated death signals. These results would argue that IL-4 protection is routed through the maintenance of Akt activation by preserving pS⁴⁷³ Akt and the suppression of BAD activity by maintaining pBAD phosphorylation rather than simply by regulating Bcl-xL protein expression. Since the regulation of Bcl-xL expression by IL-4 is known to be dependent on the STAT6 signaling pathway [13, 14, 56], it is reasonable to propose that IL-4 protects a variety of cell types from apoptosis by activating at least two independent signaling pathways leading to elevated Bcl-xL (STAT6 pathway) and suppression of BAD activity (IRS2/PI-3K/Akt pathway).

The activation of multiple pathways leading to prevention of apoptosis in T-cells has been described for IL-7 [57]. The relative contribution, and thus importance, of a particular pathway in preventing apoptosis may be dependent on the type of cell, its transformation status, and its environment. Therefore, the precise mechanism by which IL-4 suppresses apoptosis (PI-3 K/Akt, or STAT6, or both pathways) may also differ depending on the cell type, the differentiation or transformed status of the cell, and the method of inducing apoptosis. This could have profound implications for the design of molecular strategies to block the effects of IL-4 or IL-13 in patients. Thus, targeting of the IRS/PI-3K/Akt and STAT6 pathways may be necessary to prevent abnormal outgrowth of cells *in vivo*.

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