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IL-4 augments anisomycin-induced p38 activation via Akt pathway in a follicular dendritic cell (FDC)-like line

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Abstract Follicular dendritic cells (FDCs) play pivotal roles in germinal center (GC) responses in secondary lymphoid follicles, and their functions are influenced by cytokines present in the GC. We investigated the functional effects of interleukin-4 (IL-4) using an established FDC-like line of HK cells. In spite of the activation of ERK1/2 and PI3K/Akt pathways by IL-4, which are implicated in the induction of cell proliferation and survival, IL-4 did not exhibit protective function on anisomycin-induced apoptosis of HK cells, but rather slightly enhanced it. This IL-4 effect correlated with the up-regulation of anisomycin-induced p38 signaling, which is attenuated by inhibition of the PI3K/Akt pathway. Expression of an active form of Akt increased anisomycin-elicited activation of p38 and its upstream kinase MKK3/6. Our data indicate a positive cross-talk between the p38 and PI3K/Akt pathways.

Key words: IL-4; Akt; p38; Follicular dendritic cell

1. Introduction

The germinal center (GC) of secondary lymphoid organs represents an anatomical site where B cells undergo somatic mutations, isotype switching and affinity selection, allowing the generation of memory B cells and plasma cells [1]. Follicular dendritic cells (FDCs) and T cells are required for normal GC development. FDCs are found closely associated with GC B cells. The interaction between these two cell types involves not only Ag-specific recognition but also a set of adhesion and co-stimulatory molecules which are necessary for GC B cells to survive and differentiate into memory cells [2]. This B cell process is also influenced by cytokines present in the GC microenvironment. Among the T cell-derived cytokines, interleukin-4 (IL-4) is required for the differentiation of B cells and controls the specificity of immunoglobulin class switching [3]. In vitro experiments mimicking GC reaction have indicated that IL-4 is a growth factor promoting GC B cell proliferation [4]. Apart from the effects of IL-4 on GC B cells, IL-4 can also influence the function of FDC in supporting GC reaction. FDC in vivo is shown to express IL-4 receptors [5]. Cultured human FDC lines respond to IL-4 by up-regulating the expression of vascular cell adhesion molecule-1 (VCAM-1) and CD23 [6,7]. However, intracellular pathways that are activated by IL-4 and that contribute to FDC function have not been established as yet.

Signal transduction through the IL-4 receptor involves three major pathways: JAK–STAT6, Raf–MEK–MAPK and the phosphatidylinositol-3′-kinase (PI3K)–Akt signaling pathways [8]. The two latter pathways are well recognized for their contribution to cellular proliferation and survival. Upon Ag stimulation, FDCs expand and differentiate to form web-like network structures. The FDC networks in the secondary lymphoid follicles are stably maintained for long periods of time until the Ag is cleared, and this maintenance appears to be regulated by appropriate cytokines that promote cell growth and survival [9]. Thus, having hypothesized that IL-4 is a cytokine that elicits the proliferation and survival signals for FDCs, we analyzed the intracellular signal transduction pathways and their effects by using an established FDC line of HK cells. HK cells exhibit functional and phenotypic characteristics of FDC in vivo, delaying apoptosis and stimulating growth and differentiation of GC B cells [10]. In addition, activated T cells augmented the proliferation of HK cells via contact and by soluble factor-dependent means [11], which is analogous to the in vivo observation that T cells are required for GC reaction [12] and FDC development [13]. HK cells also responded to TNF and soluble CD40 ligand (sCD40L) by activating ERK and p38 [14]. Here we show that IL-4 indeed acts as a growth factor for HK cells, activating ERK and PI3K/Akt pathways. Unexpectedly, despite the well-established function of Akt in protecting cells from apoptosis induced by various stimuli, including treatment of anisomycin, a protein synthesis inhibitor that leads to the activation of p38 [15,16], we found that IL-4-activated Akt sensitizes the anisomycin-induced p38 signaling pathway, which is responsible for the increased apoptosis induced by anisomycin.

2. Materials and methods

2.1. Cell cultures and reagents

Cell culture media were purchased from Life Technologies (Rockville, MD, USA). An established FDC line [10–13], HK cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. HK cells with passages 13–17 were used for various experiments. Human IL-4 was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human TNF was obtained from

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Abbreviations: FDC, follicular dendritic cell; GC, germinal center; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol-3′-kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase

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Biotech Research Institute, LGCI, Korea. Anisomycin was purchased from Sigma. PD098059, SB203580, wortmannin, LY294002 and DMSO were purchased from Calbiochem (La Jolla, CA, USA). The rabbit anti-phospho-ERK, anti-phospho-p38 MAPK, anti-phospho-MKK3/6 and anti-phospho-Akt antibodies were purchased from New England Biolabs (Beverly, MA, USA).

2.2. Western blot analysis

After stimulation, cells were washed with cold phosphate-buffered saline, scraped and resuspended in lysis buffer containing 1% Nonidet P-40, 50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM EDTA, 1 mM pyrophosphate, 10 mM sodium orthovanadate, 3 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride and 100 mM sodium fluoride. Cell lysates were centrifuged at 15 000 rpm for 10 min at 4°C. The supernatants were electrophoresed through 10% SDS–PAGE. Proteins were transferred to nitrocellucose membrane (Bio-Rad, Richmond, CA, USA). The blots were blocked in Tris-buffered saline/Tween 20 supplemented with 5% bovine serum albumin for 1 h, incubated with various primary Abs for 1 h and then with 1:5000 diluted secondary Abs of horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz, CA, USA) for 1 h at room temperature. The blots were treated with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and detected by autoradiography.

2.3. In vitro JNK kinase assay

GST-c-Jun-(1–79) fusion protein was purified from bacterial lysates using glutathione-*S*-transferase (GST)–Sepharose beads (Amersham Pharmacia Biotech). After stimulation, HK cells were lysed in the lysis buffer described in the previous section. The lysates were mixed with 20 μl of GST-c-Jun bound to GST–Sepharose beads. The mixture was rotated at 4°C for 1 h and pelleted by centrifugation. The pelleted beads were washed twice with the lysis buffer and once with kinase buffer (20 mM HEPES, pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerol phosphate, 0.2 mM sodium orthovanadate, 10 mM sodium fluoride, and 0.2 mM dithiothreitol) and then resuspended in 50 μl of kinase buffer containing 50 μM ATP and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech). After 30 min of incubation at 30°C, the reaction was terminated by adding 2× Laemmli sample buffer and boiling for 3 min. Samples were resolved on 12% SDS–PAGE gel and subjected to autoradiography.

2.4. Measurement of apoptosis (nuclear staining with Hoechst 33258)

HK cells plated in 12-well plates were untreated or pretreated with 20 ng/ml of IL-4 for 15 min prior to anisomycin treatment. Cells given death-inducing stimuli were then fixed with 4% paraformaldehyde for 20 min at room temperature and stained with 50 ng/ml Hoechst 33258 (Sigma). For quantitation of apoptosis (presented as average ± standard error of the mean [S.E.M.]), cells were scored as apoptotic based on morphological criteria. A minimum of 250 cells was counted for each condition.

2.5. Adenoviral infection

Adenovirus expressing β-galactosidase and HA-tagged constitutively active myristylated Akt (myr-Akt) were kindly provided by Dr. Walsh (Tufts University, MA, USA) [17]. HK cells were infected with adenovirus with multiplicity of infection of 100 for 24 h, the condition in which uniform expression of the transgenes was observed in nearly 90% of the cells, as determined by staining the cells for β-galactosidase activity following infection with β-galactosidase virus. The virus was removed and cells were left to recover for 12 h in complete medium.

3. Results

3.1. IL-4 activates ERK1/2 and PI3K/Akt pathways in HK cells

Since IL-4 has a growth-promoting effect on hematopoietic cells, including B cells, T cells and mast cells [4,18], we examined whether this cytokine elicits such function in HK cells. Cells maintained in 10% FCS-containing medium were stimulated with various doses of IL-4 for 24 h. The effect of IL-4 on the proliferation of HK cells was measured by [^{3}H]thymidine incorporation during the last 16 h chasing period. Fig. 1A shows that IL-4 increased the [^{3}H]thymidine uptake dose-dependently, suggesting that IL-4 is a growth factor for this cell line. We next examined whether IL-4 activates ERK1/2, a molecular hallmark of proliferation of cells. Cells that were serum-starved by maintaining them in 0.25% FCS-containing medium for 24 h were stimulated with IL-4 for the indicated time and the IL-4-induced activation of ERK1/2 was determined by Western blot analysis using antibody detecting phosphorylated ERK. Fig. 1B shows that ERK1/2 activation by IL-4 was evident after 15 min stimulation. The level of phosphorylation was sustained until 60 min and then decreased at 120 min after IL-4 treatment. This IL-4-induced ERK1/2 activation is MEK-1 and -2 dependent, because cells pretreated with 50 μM PD098059, an activation inhibitor of ERK1/2, 2 h prior to stimulation did not show ERK1/2 activation upon treatment with IL-4. Experiments examining

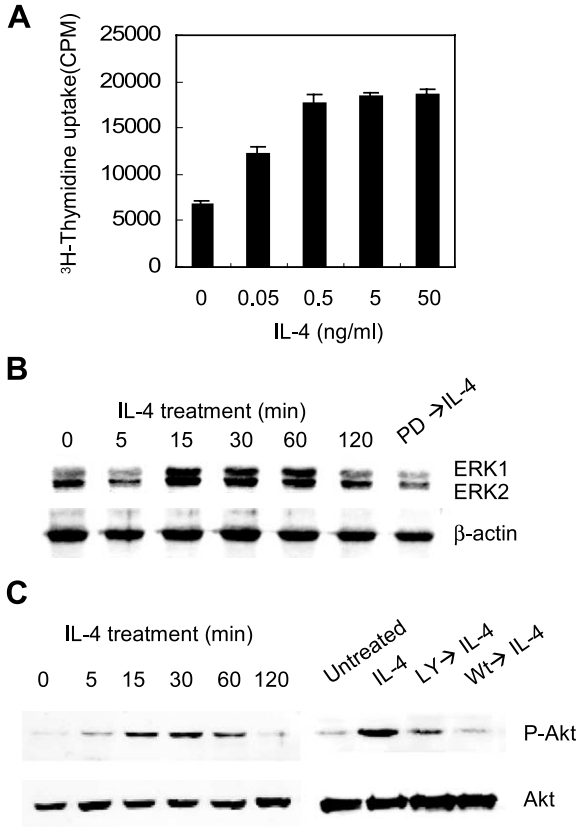

Fig. 1. IL-4 stimulates HK cell proliferation and activates ERK1/2 and PI3K/Akt. A: HK cells were seeded in a 96-well plate at about 5000 cells/well. After 24 h incubation, the cells were treated with IL-4 at indicated concentrations. The cells were pulsed with 0.5 μCi of [^{3}H]thymidine during the last 16-h period, and harvested onto filter. The incorporated [^{3}H]thymidine in DNA was measured by liquid scintillation counter. B: Confluent HK cells maintained in low serum-containing medium (0.25% FCS) for 24 h were stimulated with IL-4 (20 ng/ml) for the indicated times. For inhibition of ERK1/2 activation, HK cells were pretreated with 50 μM PD098059 for 2 h and subsequently stimulated with IL-4 for 15 min. Cell lysates were immunoblotted using the anti-phospho-ERK antibody. C: Serum-starved HK cell cultures were stimulated with IL-4 for the indicated times. For inhibition of Akt activation, HK cells were pretreated with 10 μM LY294002 (*LY*) or 100 nM wortmannin (*Wt*) for 2 h and subsequently stimulated with IL-4 for 15 min. Akt activation was examined by immunoblotting cell lysates with antibodies to phospho-Akt or Akt.

whether IL-4 activates other members of the MAPK family, JNK and p38, revealed no activation of such MAPKs (data not shown).

In addition to activating the ERK1/2 pathway, IL-4 is shown to activate the PI3K/Akt pathway that promotes cell survival in various cell types [19–23]. Thus, we investigated whether Akt is activated in response to IL-4 in HK cells. As shown in Fig. 1C, phosphorylation of Akt occurred 5 min after cells were exposed to IL-4, reached a maximum at 30 min and gradually decreased to the basal level by 120 min. Akt is an established downstream target of PI3K that is activated following the stimulation of cells with growth factors. Upon treatment of HK cells with inhibitors of PI3K (LY294002 or wortmannin) prior to IL-4 addition, the phosphorylation of Akt was blocked, confirming that IL-4-induced Akt activation is mediated through the PI3K pathway. TNF and sCD40L were shown not to activate Akt in this cell type (data not shown).

3.2. *IL-4 does not play a role in the protection of HK cells from apoptosis induced by anisomycin*

Considerable attention has been focused on the role of the PI3K/Akt pathway as a source of anti-apoptotic signal. Before investigating whether IL-4-activated Akt plays a protective role in apoptosis, we initially determined what stimuli can induce HK cell death. Among the death-inducing stimuli that were tested, treatment of HK cells with 1 μg/ml of anisomycin, a ribotoxic drug that inhibits cellular protein synthesis, was most effective in inducing apoptosis, causing 25.5% of cells to undergo apoptosis (Fig. 2). Anisomycin is a strong activator of stress-induced MAPKs, p38 and JNK [15,16,24,25]. Blockage of p38 activation by pretreating cells with a specific p38 inhibitor SB203580 caused the complete inhibition of anisomycin-induced apoptosis, indicating that apoptosis by anisomycin is p38-dependent. In this death-inducing system, IL-4 pretreatment slightly augmented the apoptotic process, leading to 29% of apoptosis in cells pretreated with IL-4 for 15 min. Activation of PI3K/Akt has been shown to inhibit apoptosis induced by treatment of anisomycin in other cell types [15,16]; however, in HK cells IL-4-activated PI3K/Akt did not exhibit such protective function.

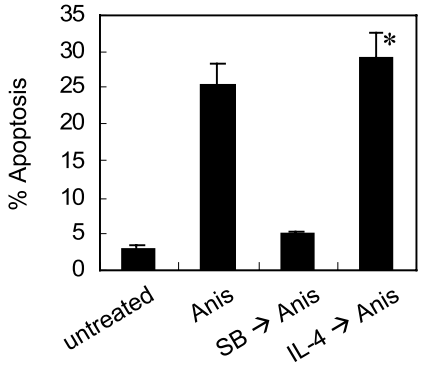

Fig. 2. IL-4 effect on anisomycin-induced HK cell apoptosis. Unprimed HK cells or cells exposed to 20 ng/ml IL-4 for 15 min were treated with 1 μg/ml anisomycin (Anis) for 24 h. For inhibition of p38 activation, HK cells were pretreated with 10 μM SB203580 (SB) for 2 h. Cells were fixed, stained with Hoechst dye and counted for apoptosis. The data presented are the results of five independent experiments. The mean and S.E.M. are presented with an asterisk where increase in percentage of apoptosis is statistically significant ($P < 0.05$), when compared to Anis alone.

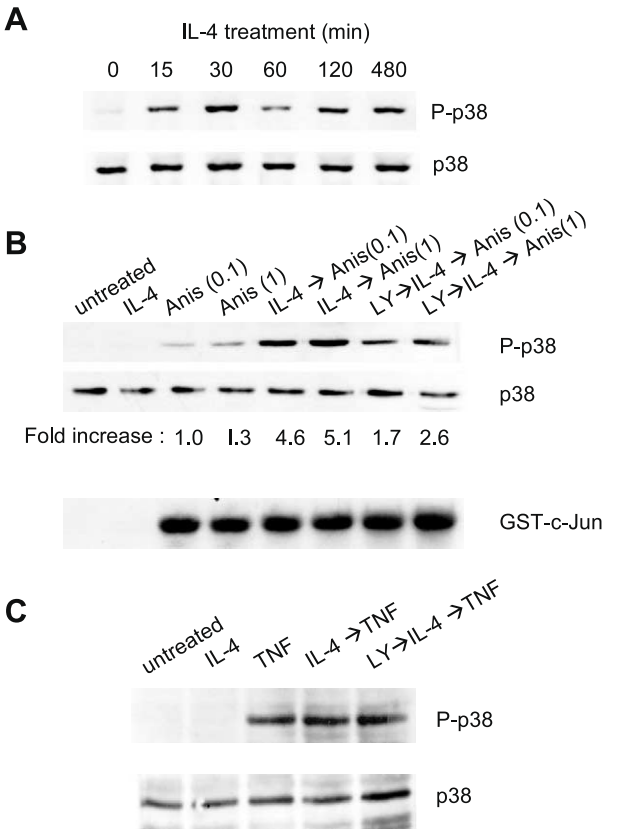

Fig. 3. A: Persistent p38 activation by anisomycin. Serum-starved HK cell cultures were stimulated with IL-4 (20 ng/ml) for the indicated times. p38 activation was examined by immunoblotting cell lysates with antibodies to phospho-p38 or p38. B,C: IL-4 pretreatment augments p38 activation by anisomycin (panel B), but not that by TNF (panel C). HK cells either unexposed or exposed to IL-4 for 15 min were stimulated with a suboptimal dose (0.1 μg/ml) [Anis(0.1)] or a death-inducing dose (1 μg/ml) of anisomycin, [Anis(1)] or with TNF (20 ng/ml) for 30 min. For inhibition of Akt activation, 10 μM LY294002 (LY) was treated for 2 h prior to the indicated stimuli. Cell lysates were subjected to immunoblot analysis for p38 activation. Quantification of bands was performed by densitometry and the signal intensity of phosphorylated p38 in each lane was normalized to the cprresponding p38 band in that lane prior to calculation of fold increases. The same lysates were also incubated with GST-c-Jun fusion protein, and JNK activity was measured by the phosphorylation of c-Jun.

3.3. *IL-4 pretreatment augments anisomycin-induced p38 activation, which is inhibited by the blockage of the PI3K/Akt pathway*

To understand the mechanism by which IL-4 pretreatment promotes the anisomycin-induced apoptotic process, we examined the effects of IL-4-activated signals on anisomycin-induced p38 activation because this stress-induced kinase is involved in the induction of the apoptotic process in a variety of cell types [15,16,24,25] as well as in HK cells. Investigation of the activation pattern of p38 in response to anisomycin treatment for various lengths of time revealed that HK cells exhibited a sustained biphasic activation of p38 (Fig. 3A). 15 min exposure to IL-4 prior to treatment of anisomycin with a suboptimal dose (0.1 μg/ml) or with a death-inducing dose (1 μg/ml) for 30 min resulted in more than four-fold increase in anisomycin-induced p38 phosphorylation as compared with that of IL-4 unprimed cells (Fig. 3B). Somewhat unexpect-

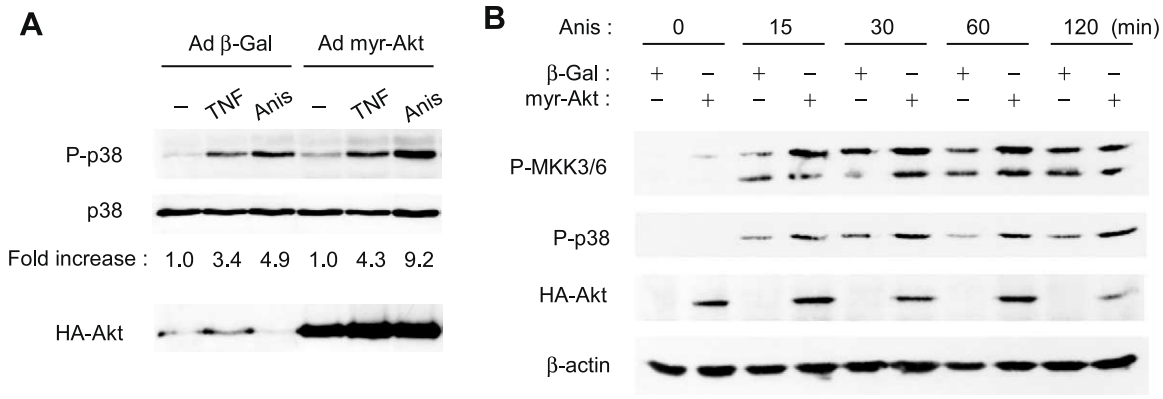

Fig. 4. Akt up-regulates the activation of p38 signaling by anisomycin. A: HK cells were infected with adenoviruses for β-galactosidase (Ad β-Gal) and HA-tagged myr-Akt (Ad myr-Akt). After 24 h incubation in complete medium, cells were serum-starved and treated with TNF (20 ng/ml) or anisomycin (1 μg/ml) for 30 min. Cell lysates were subjected to immunoblot analysis with antibodies specific to phospho-p38, p38, or HA. A representative result of three independent experiments is shown. Intensity of phospho-p38 bands in each lane was quantified by scanning densitometry of immunoblot. Fold increases in the phospho-p38 levels were calculated after normalizing the levels to p38 levels in each lane. B: HK cells were infected with adenoviruses as indicated. Cells were serum-starved and stimulated with anisomycin for the times indicated. Cell lysates were processed for immunoblot analysis with antibodies to phospho-p38, phospho-MKK3/6, HA, or β-actin.

edly, we repeatedly observed that this IL-4-induced enhancement of p38 activation by anisomycin was attenuated when the PI3K/Akt pathway was blocked by treatment of cells with LY294002 before consecutive addition of IL-4 and anisomycin, suggesting that IL-4-activated PI3K/Akt is involved in the up-regulation of anisomycin-induced p38 activation. This IL-4 effect appeared to be specific for p38 activation, because no significant change was seen in c-Jun phosphorylating activity of JNK induced by anisomycin (Fig. 3B, bottom panel). Furthermore, TNF-induced p38 activation was not affected by IL-4 pretreatment (Fig. 3C), suggesting a selective IL-4 effect on the regulation of anisomycin-elicited p38 activation.

3.4. *Akt up-regulates anisomycin-induced p38 activation pathway*

To seek further evidence that the increased anisomycin-induced p38 phosphorylation by IL-4 is mediated by Akt activation, recombinant adenovirus-mediated gene transfer method was employed to facilitate ectopic expression of Akt proteins in HK cells. Fig. 4A shows that infection of HK cells with a constitutively active form of Akt (myr-Akt, HA-tagged) increased the anisomycin-induced phosphorylation of p38 9.2-fold compared to control cells infected with β-galactosidase adenovirus which showed 4.3-fold increase. However, TNF-induced p38 phosphorylation was not significantly affected by expression of the myr-Akt. These data confirm the results shown in Section 3.3, demonstrating that IL-4-activated Akt is responsible for the sensitization of the anisomycin-triggered p38 signaling pathway.

To examine whether Akt can up-regulate p38 upstream activators MKK3/6, HK cells infected with adenovirus expressing the β-galactosidase or myr-Akt were treated with anisomycin for various time periods and processed for immunoblot analysis using the phosphotyrosine-specific MKK3/6 antibody (Fig. 4B). Compared to cells infected with control β-galactosidase adenovirus, cells infected with the myr-Akt adenovirus showed stronger band intensities of phosphorylated MKK3/6 by anisomycin treatment. The increase in the phosphorylation of MKK3/6 paralleled the phosphorylation of p38 for the time periods examined. Infection with the myr-Akt virus itself phosphorylated MKK3/6 (the second lane in the panel marked by P-MKK3/6). These results indicate that Akt cross-talks with the p38 signaling pathway at the level or upstream of MKK3/6 in HK cells.

4. Discussion

In the present study, we report that an established FDC line of HK cells responded to IL-4 by activating ERK1/2 and PI3K/Akt pathways. Akt activated by IL-4 did not contribute to the prevention of anisomycin-induced HK cell death, it rather facilitated the apoptotic process by up-regulating the p38 signaling pathway. IL-4 alone did not induce the activation of p38 in this cell line, but it assisted the anisomycin-elicited p38 activation through Akt activation, suggesting a positive cross-talk between the p38 MAPK and PI3K/Akt pathways.

Activation of PI3K/Akt functions as a source of survival and anti-apoptotic signals, and often cross-talks with the p38 signaling pathway. Berra et al. [26] have shown that simple inhibition of ERK activation by PD098059 in HeLa cells cultured in serum-free medium causes sustained p38 activation, leading to apoptosis induction. This effect can be blocked by the presence of serum through the PI3K/Akt signaling pathway, suggesting that PI3K/Akt signaling antagonizes p38 activation and subsequent apoptosis induction. Similarly, in endothelial cells, vascular endothelial growth factor activates the PI3K/Akt pathway to protect cells from apoptosis by down-regulating the p38 signaling pathway [27]. However, in other instances PI3K/Akt up-regulates the activation of p38. According to the work done by Madrid et al. [28], in the context of IL-1β-induced Akt activation, treatment of cells with IL-1β or expression of myr-Akt enhanced the phosphorylation and activation of p38, and this increased p38 activity was shown to increase the transcriptional potential of NF-κB. Furthermore, it has been demonstrated that Akt forms a stable complex with p38 in human neutrophils [29]. Therefore, it is likely that the PI3K/Akt pathway regulates the activation of p38 in a positive or negative manner. Since activation of p38 appears to be critically associated with the anisomycin-induced HK cell death (Fig. 2), the slight death-promoting effect on anisomycin-induced cell death in HK cells appears to be mediated

by the up-regulation of p38 activity by Akt that is activated by IL-4.

Positive regulation of the p38 pathway by Akt in HK cells involves the utilization of the p38-activating kinase MKK3/6, as shown by the fact that over-expression of activated Akt alone can induce the phosphorylation of MKK3/6, and increases the anisomycin-induced MKK3/6 phosphorylation (Fig. 4). In addition, it has been suggested that down-regulation of p38 signaling by Akt also involves the modulation of MKK3/6 activity by phosphorylating MKK upstream kinase MEKK3 in endothelial cells, whose phosphorylation reduces its activity to activate the MKK3/6–p38 pathway [27]. How Akt activates the MKK3/6–p38 pathway in HK cells is presently unclear, but we showed that IL-4-activated Akt increased p38 phosphorylation by anisomycin, but not that by TNF. Since MKK3/6 are also critical components of the TNF-stimulated p38 signaling pathway [30] and shown to be phosphorylated by TNF treatment in HK cells (data not shown), positive cross-talk between IL-4-induced Akt and anisomycin-induced p38 signaling pathways must occur upstream of MKK3/6. Recently it has been demonstrated that anisomycin-stimulated signaling to p38 activation originates further upstream of MKK family enzymes and utilizes components which are not involved in TNF receptor-mediated kinase activation [25]. Further studies will be required to identify the molecular basis of this cross-talk event.

Although activation of the PI3K/Akt pathway is required and sufficient for cell survival, this does not seem to be always the case. For example, inhibition of the PI3K/Akt pathway does not induce apoptosis in cerebellar granule neurons maintained in serum and potassium-rich medium [31], and in superior cervical neurons [32]. Furthermore, a recent study has indicated that activation of Akt is pro-apoptotic under certain circumstances [33]. It showed that when caveolin-1, the main structural component of caveolae that modulates the activities of molecules involved in growth factor signaling, was overexpressed in 293 and HeLa cells, there was a significant activation of the PI3K/Akt pathway and the resulting elevated activity of Akt sensitized cells to the stress-induced apoptosis. Although a precise causal effect of IL-4-activated Akt on the enhanced anisomycin-induced p38 signaling and apoptosis of HK cells has to be determined, our present study provides additional evidence that elevated Akt activity may promote cell death through the up-regulation of the p38 signaling pathway.

So far, studies published to date do not point out when and why FDCs in vivo undergo apoptosis. However, evaluation of the FDC network for a long period of time after induction of GC responses reveals that the FDC networks degenerate in the resolution stage of GC [34]. Whether the major population of FDC in the involuting follicles of normal lymphoid tissues undergoes programmed cell death is an open question. The fate of FDCs in vivo may be the result of a complex interplay between signals that are delivered by GC cytokines which include IL-4.

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