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# Prevention of TGF-β-Induced Apoptosis by Interlukin-4 through Akt Activation and p70S6K Survival Signaling Pathways

Sue-Jane Lin[*‡], Chungming Chang[*‡], Ah-Kau Ng[‡¶], Shu-Han Wang[*], Jia-Je Li[†] and Cheng-po Hu[*†#]

Institute of Microbiology & Immunology, National Yang-Ming University[*], Division of Intramural Research Affairs, National Health Research Institute, Taipei[‡], Department of Applied Medical Sciences, University of Southern Maine, Portland, Maine, USA[¶], Department of Medical Research & Education, Taipei Veterans General Hospital, Taipei[†], Department of Life science, Tunghai University, Taichung[#], Taiwan, Republic of China

Running Title: IL-4 protection against TGF-β-induced apoptosis

Address correspondence to: Cheng-po Hu, Department of Medical Research & Education, Taipei Veterans General Hospital[†], No.201, Sec. 2, Shihpai Rd., Beitou District, Taipei City 112, Taiwan, Republic of China, Phone: 886-2-28757394, Fax: 886-2-28751562, E-mail: cphu@vghtpe.gov.tw

**In this study, we demonstrate that interleukin-4 (IL-4) protects human hepatocellular carcinoma (HCC) cell line Hep3B from apoptosis induced by transforming growth factor-β (TGF-β). Investigating the IL-4-transduced signaling pathways, we found that both insulin response substrate 1 and 2 (IRS-1/-2) and mitogen-activated protein kinase (MAPK) pathways were activated after IL-4 stimulation. The IRS-1/IRS-2 activation was accompanied by the activation of phosphotidylinositol-3-kinase (PI3K), leading to Akt and p70 ribosomal protein S6 kinase (p70S6K). Interestingly, a protein kinase C (PKC) inhibitor, Gö6976, inhibited the phosphorylation of Akt, suggesting that IL-4-induced Akt activation was PKC-dependent. Using specific inhibitors for PI3K or ERK, to investigate which pathways participate in the survival signalings, we demonstrated that the PI3K pathway, but not the MAPK pathway, was required. The role of PI3K activation was further supported by the observation that the constitutively active form of PI3K almost completely prevented TGF-β-induced apoptosis. Furthermore, a dominant negative Akt and/or Gö6976 only partially blocked the anti-apoptotic effect of IL-4. Similarly, rapamycin, which inhibited the activation of p70S6K, also only partially blocked the protective effect of IL-4. However, in presence of both rapamycin and dominant negative Akt with or without Gö6976, IL-4 almost completely lost the anti-apoptotic effect. Finally, we demonstrated that both Akt and p70S6K pathways were required for IL-4-induced phosphorylation of apoptotic molecule Bad. In summary, the protective effect of IL-4 against TGF-β-induced apoptosis is mediated mainly through PI3K activation, and is dependent on both of its downstream Akt and p70S6K signaling pathways.**

## Introduction

TGF-β has diverse biological effects on development, proliferation, differentiation, extracelluar matrix formation, immune functions and apoptosis (1-6). The induction of apoptosis by TGF-β has been investigated in many cell types, including T cells, B cells, primary oligodendrocytes, epithelial cells, endothelial cells, hepatocytes and various carcinoma cells (2). In the liver, TGF-β was found to suppress hepatocyte proliferation *in vitro* (7) and liver regeneration after partial hepatectomy. It also contributes to the progression of cirrhosis in the liver (8,9). Moreover, TGF-β-induced apoptosis was observed in fetal hepatocytes, primary hepatocytes and HCC cell lines (2).

IL-4 has been well-studied for its multiple roles in immune regulation; however, its effect on other biological systems has only become appreciated recently (10,11). In the liver, little is known about the mechanism of anti-apoptotic effects of IL-4, a product of $T_H2$ cells, basophils, mast cells and NKT cells (12). In mice, NKT cells, which account for

30-50% of resident intrahepatic T lymphocytes, can rapidly produce high levels of IL-4 after activation (13). IL-4 has been reported to protect the liver from ischemaia/reperfusion-induced injury through suppression of pro-inflammatory mediators (14,15). It also plays a protective role in regulating oxidative damage in the liver during schistosomiasis (16,17). Furthermore, IL-4 protects primary T, B, myeloid cells and tumor cells from apoptosis, and prevents human synovicytes from NO-induced apoptosis (18-21). These observations suggest that IL-4 has a direct or indirect protective effect on hepatocytes in the liver.

IL-4 stimulation has been linked to several known signaling pathways including the IRS pathway, the signal transducer and activation of transcription 6 (STAT6) pathway and the MAPK pathway. The IRS pathway is mediated by tyrosine phosphorylation of IRS-1/-2 and the activation of the downstream PI3K, which has consistently been shown to play a significant role in IL-4-mediated cell proliferation (22,23). Phosporylated and dimerized STAT6 widely regulates the expression of IL-4 responsive genes; however, the activation of MAPK pathway by IL-4 is only observed in certain cell types (24), suggesting that MAPK activation is not a universal signaling pathway in cellular response to IL-4.

In a pervious study, we have demonstrated that IL-4 binds with a high affinity to a HCC cell line Hep3B which expresses an IL-4 receptor complex of IL-4Rα, IL-13Rα1 and γc (25). In this study, we investigated the signaling pathways triggered by IL-4 in Hep3B cells, and examined their potential roles in the protection against apoptosis induced by TGF-β. Our results show that IL-4 protects Hep3B cells from TGF-β-induced apoptosis predominantly through the activation of IRS-PI3K pathway, and a concomitant activation of the downstream Akt and p70S6K signaling pathways. Furthermore, both pathways were involved in IL-4-induced phosphorylation of apoptotic molecule Bad.

**Materials and Methods**

*Cell culture and transient transfection:*

Hep3B is a HCC cell line. The stable transfectant clones, p110*-M, p110*-17, Akt (k-)-M and Akt (k-)-9, were generously provided by Ruey-Hwa Chen, Institute of Molecular Medicine, College of Medicine, National Taiwan University. p110*-M and p110*-17 express a constitutively active from of PI3K (26); Akt (k-)-M and Akt (k-)-9 express a dominant negative form of Akt (27). Parental Hep3B cells were cultured in a complete medium containing Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) , 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 2 mM L-glutamine and 1% non-essential amino acids at 37°C in 5% $CO_2$. All the stable clones were cultured in the complete medium containing 200 μg/ml G418. For transient transfection, 1 x $10^6$ Hep3B cells in 2.5-cm plates were washed three times with a serum-free (SF) medium and incubated for 3 h with 4 μg of plasmid DNA and 32 μl of Lipofectamine (Invitrogen Corporation, Carlsbad, CA, USA). The culture medium was then changed to fresh DMEM containing 10% FCS. After 18 h, the transfected cells were trypsinized, pooled and re-seeded. After overnight incubation, the cells were cultured in the SF medium for 1 day and then refreshed with SF medium with or without IL-4 for indicated time intervals.

*Reagents, antibodies and plasmids:*

Recombinant human IL-4 was purchased from R&D system (Minneapolis, MN, USA). PD98059, U0126, rapamycin, wortmannin, LY294002, Gö6950, Gö6976 and Gö6983 were purchased from Calbiochem (La Jolla, CA, USA). Anti-phosphotyrosine (4G10), anti-IRS-1, anti-IRS-2, anti-phospho-p70S6K (Thr-412), anti-Akt, anti-Akt (PH domain, agarose conjugated), anti-phospho-Akt (Ser-473), anti-phospho-Akt (Thr-308), anti-phospho-Bad (Ser-112) and anti-phospho-Bad (Ser-136) antibodies were purchased from Upstate (Charlottesville, VA, USA). Antibodies against ERK-2 (C-14) and Bad were from Santa Cruz (Santa Cruz, CA, USA). Antibodies against phospho-Bad (Ser-155), phospho-Elk-1 (Ser-383) and p70S6K, and Elk-fusion protein were from Cell Signaling Technology (Beverly, MA, USA). To generate

an expression plasmid for human *Bad*, phBad, the *Bam*HI-*Xba*I fragment containing the full length cDNA of *Bad* was cloned from Hep3B cells and then inserted into the *Bam*HI–*Xba*I site of the pcDNA3.1 expression vector (Invitrogen).

*Apoptosis assay*

Apoptosis was determined using the terminaldeoxynucleotidyl ransferase-mediated dUTP nick end labeling (TUNEL) assay and the Cell-Death Detection ELISA assay. For the TUNEL assay, the In Situ Death Detection Kit (Roche Applied Science; Penzberg, Germany) was used. In brief, $3 \times 10^5$ Hep3B cells seeded in 6-well plates were serum-starved for 48 h and then treated with IL-4 and/or TGF-β for 30 h, 36 h or 42 h. Cells were fixed with 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. The fluorescein-labeled cells were then analyzed by flow cytometry. For the Cell-Death Detection ELISA assay (Roche Applied Science), cells were seeded in 48-well plates at a density of $3 \times 10^4$ cells/well and various kinase inhibitors were added to the cells after 2 days of serum-starved. After 2 h, cells were incubated with IL-4 and/or TGF-β in the presence or absence of various kinase inhibitors for 18 h. The presence of soluble histone-DNA complex as a result of DNA fragmentation was measured according to the manufacturer's instructions.

*Immunoprecipitation*

Hep3B cells were washed twice with ice-cold Hank's balanced salt solution (HBSS), and lysed in the lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride [PMSF], 2 μg/ml aprotinin, 1 μM leupeptin, 1 mM $Na_3VO_4$, 80 mM pyrophosphate and 50 mM NaF) on ice. After centrifugation at 12,000 g, the pellet was discarded and 1 mg of protein was reacted with 1 μg of various antibodies at 4°C for 16 h. The immunoprecipitates were mixed with 30 μl of protein A-Sepharose beads at 4°C for 1 h. The beads were then washed three times with the lysis buffer and added with 30 μl of boiled 2X sample buffer (50 mM Tris-HCl; pH 6.8, 2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 12.5 mM EDTA, 0.02 % bromophenol blue). The mixture was then boiled at 100°C for 5 min. The supernatant was subjected to SDS-PAGE.

*Western blotting*

After SDS-PAGE, the proteins in the acrylamide gel were transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad, CA, USA) in the transfer buffer containing 48 mM Tris, pH 8.3, 192 mM glycine, 20% methanol, and 0.0375% SDS. The transfer was conducted under constant current (1 mA per square cm of nitrocellulose membrane) for a period of 1.5-2 h. The membrane was blocked in 5% skimmed milk in phosphate-buffered saline (PBS) at 4°C for overnight, incubated with diluted antibodies in 1% milk/PBS at 37°C for 1 h, washed 3 times with PBS containing 0.05% Tween-20, and reacted with peroxidase-conjugated secondary antibodies at RT for 45 min. The signal was then visualized by ECL system according to the manufacturer's instructions (Pierce Biotechnology, Inc; Rockford, IL, USA).

*Phosphatidylinositol 3-kinase (PI3K) assay*

For the PI3K assay, $2.5 \times 10^6$ cells were lysed in 0.5 ml of the lysis buffer used for immunoprecipitation described before. After centrifugation at 12,000 g, the pellet was discarded and 1 mg of protein was pre-incubated with 30 μl protein A-Sepharose at 4°C for 1 h and the protein A-Sepharose was discarded after centrifugation. The supernatant was reacted with 1 μg of anti-phosphotyrosine antibody (4G10) and 30 μl of protein A-Sepharose beads at 4°C for overnight. After the immunoprecipitates were washed three times with the lysis buffer, twice with LiCl buffer (100 mM Tris, pH 7.6, 0.5 M LiCl), and twice with TNE buffer (10 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA), 10 μl of phosphatidylinositol (Sigma, 2 mg/ml in 20 mM HEPES, pH 7.4) were incubated with the beads for 10 min on ice. The kinase reaction was then initiated by the addition of 40 μl of the reaction buffer (20 mM HEPES, pH 7.4, 10 mCi [$\gamma$-$^{32}$P] ATP, 20 mM ATP, 5 mM $MgCl_2$) at RT for 15 min and stopped by an addition of 100 μl of 1 N HCl. The mixture was then added with 200 μl of chloroform/methanol with a 1:1 ratio. The

extracted lipid in the organic phase was spotted onto a thin-layer chromotography plate and developed in chloroform/ methanol/4M $NH_4OH$ (9:7:2). The labeled phosphatidylinositol 3-phosphate (PI3-P) was then visualized by autoradiography.

*Mitogen-activated protein kinase (MAPK) assay*

Hep3B cells were cultured with or without PD98059 or U0126 after 2 days of serum starvation. After 2 h, cells were incubated with IL-4 (100 ng/ml) for 15 minutes, washed twice with cold HBSS and then lyzed in the buffer containing 20 mM Tris, pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM NaF, 1 mM $Na_3VO_4$, 1 μg/ml leupeptin, 2 mM PMSF and 1mM β-glycerolphosphate on ice for 5 min. After centrifugation at 12,000 g, the pellet was discarded and 200 μg of protein were reacted with 2 μg of anti-ERK-2 antibody at 4°C for 3 h. The immunoprecipitates were mixed with 30 μl of protein A-Sepharose beads for 1 h, washed twice with the lysis buffer, twice with the kinase buffer (25 mM Tris, pH7.5, 0.1 mM $Na_3VO_4$, 5 mM β-glycerolphosphate, 2 mM DTT and 10 mM $MgCl_2$), and suspended in 48 μl of the kinase buffer containing 200 μM ATP and 2 μg of the substrate Elk-fusion protein. The kinase reaction was carried out at 30°C for 30 min and terminated by the addition of 12.5 μl of 5X sample buffer. After boiling, 30 μl of the sample was subjected to SDS-PAGE and immunoblotting analysis with anti-phospho-Elk-1 (Ser-383) antibody.

**Results**

**IL-4 protects Hep3B cells from TGF-β-induced apoptosis**

To investigate the anti-apoptotic effect of IL-4 on HCC cells, Hep3B cells were treated with TGF-β in the presence or absence of IL-4. As measured by the TUNEL assay, Fig. 1A shows that TGF-β induces apoptosis at 30 h, 36 h and 42 h, and IL-4 almost completely rescues Hep3B cells from apoptosis (53% tunnel positive cells for the TGF-β treatment vs. 1.92% tunnel positive cells for the combined treatment of TGF-β and IL-4). We further verified the anti-apoptotic ability of IL-4 using a cell death detection ELISA kit which measures the soluble histone-DNA complex resulting from DNA fragmentation (Fig. 1B). These results convincingly demonstrate that IL-4 prevents TGF-β-induced apoptosis in Hep3B cells.

**Activation of IRS/PI3K pathway is critical to the anti-apoptotic effect of IL-4**

To investigate the mechanisms underlying the reversing effect of IL-4 on apoptosis, we attempted to characterize the signaling pathways transduced by IL-4 in Hep3B cells. As shown in Fig. 2A, we found that IL-4 activated IRS-1 and IRS-2 in Hep3B cells at 5 and 15 min after incubation. To examine the contribution of various signaling pathways to the anti-apoptotic effect of IL-4, we measured the direct activation of signaling molecules in the presence of specific inhibitors. Our data demonstrated that IL-4 stimulation activated PI3K in Hep3B cells, as measured by a standard PI3K assay, and this activation was blocked by 5 μM wortmannin, a specific inhibitor of PI3K (Fig. 2B). These results prompted us to investigate whether PI3K activation was involved in the anti-apoptotic effect of IL-4. In Hep3B cells treated with 0.2, 2, 5 μM wortmannin (Fig. 2C) or 0.5, 2, 10 μM LY294002, a specific inhibitor of PI3K (Fig. 2D), the IL-4-mediated prevention of TGF-β-induced apoptosis was blocked in a dose-dependent manner. To further verify the involvement of PI3K in the anti-apoptotic effect of IL-4, we used two stable clones of Hep3B cells expressing constitutively activated PI3K, p110*-M and p110*-17, wherein the iSH2 domain of the regulatory subunit was fused to the catalytic subunit of the kinase. The results indicated that the p110*-M cells were completely resistant to TGF-β-induced apoptosis (Fig. 2E). However, the p110*-17 clone, which expressed a lower amount of the constitutively activated PI3K (data not shown), was less resistant. Collectively, these findings provide evidences that IL-4-mediated PI3K activation plays an important role in the prevention of TGF-β-induced apoptosis in Hep3B cells.

**MAPK pathway is not involved in the anti-apoptotic effect of IL-4**

Fig. 3A shows that ERK-2 in MAPK pathway is also activated by IL-4 treatment, and this activation of ERK-2 can be blocked

by its inhibitors, PD98059 or U0126. However, neither PD98059 nor U0126 inhibited the anti-apoptotic effect of IL-4 (Fig. 3B), suggesting that IL-4-induced activation of MAPK does not transduce an anti-apoptotic signaling against TGF-β-induced apoptosis in Hep3B cells.

**Akt is required for IL-4-mediated prevention of TGF-β-induced apoptosis**

To understand how PI3K signaling contributes to the IL-4-mediated prevention of TGF-β-induced apoptosis, we investigated whether its downstream Akt was involved. Our results demonstrated that IL-4 induced the phosphorylation of Akt at Ser-473 at 15 min, and the phosphorylation was completely blocked by 5 μM wortmannin or 10 μM LY294002 (Fig. 4A). To address the role of Akt in the anti-apoptotic effect of IL-4, two stable Hep3B transfectants Akt (k-)-M and Akt (k-)-9, which expressed dominant negative Akt, were employed. As shown in Fig. 4B, the expression of dominant negative form of Akt partially suppressed the preventive effect of IL-4 on the TGF-β-induced apoptosis (enhancing cell death from 20% to 43% and 46% in Akt (k-)-9 and Akt (k-)-M clones, respectively). This observation implies that other anti-apoptotic mechanisms may also be involved.

**PKC is involved in the anti-apoptotic effects of IL-4**

To address whether PKC participates in the downstream signaling of PI3K, we investigated the effect of IL-4 on TGF-β-induced apoptosis in the presence of three PKC inhibitors; Gö6976 (inhibits PKCα, β1, μ), Gö6983 (inhibits PKCα, β, γ , δ ) and Gö6850 (inhibits PKCα, β1/2, δ, γ, ε). We found that 2.5 μM of Gö6976 partially reversed the anti-apoptotic effect of IL-4, (Fig. 5A), increasing cell death from 30% to 66%, whereas Gö6850 and Gö6983 had no effect on the IL-4 dependent protection (data not shown). To examine whether PKC is related to the Akt pathway, we studied the effect of PKC inhibitor, Gö6976, on the anti-apoptotic effect of IL-4 in Akt (k-)-M cells expressing dominant negative Akt. Interestingly, Gö6976 did not further reverse IL-4's protective effect in Akt (k-)-M cells (Fig. 5B, 56.5% cell death for the control vs. 62% cell death for the treated, $p = 0.16$). (Fig. 5B) These results suggest that the anti-apoptotic signalings induced by Akt and PKC may originate from a common pathway. Furthermore, the IL-4-induced phosphorylation of Akt at Ser-473 and Thr-308 could not be detected in Akt (k-)-M cells after the treatment of Gö6976 (Fig. 5C). These results indicate that PKC may be an upstream signaling molecule of Akt, and PKC-mediated Akt activation is involved in the anti-apoptotic effect of IL-4.

**p70S6K pathway is involved in IL-4-mediated prevention of apoptosis**

To further elucidate whether the p70S6K pathway, a downstream signaling molecule of PI3K, is activated by IL-4, Western blotting with an anti-phospho-p70S6K antibody was performed. As shown in Fig. 6A, IL-4 treatment results in the phosphorylation of p70S6K at Thr-412 at 15 min. Rapamycin which interferes with the activation of p70S6K also blocks the phosphorylation of p70S6K. Furthermore, two PI3K inhibitors, 5 μM wortmannin or 10 μM LY294002, also inhibit the phosphorylation of p70S6K. These results indicated that p70S6K is also a downstream molecule of PI3K after IL-4 stimulation. To test whether p70S6K participates in the anti-apoptotic function of IL-4, various concentrations (2, 10 and 50 nM) of rapamycin were employed. Results in Fig. 6B demonstrate that 50 nM of rapamycin partially reverses the prevention of IL-4 on TGF-β induced apoptosis (raising cell death from 24% to 44%), indicating that p70S6K pathway may also participate in the anti-apoptotic effect of IL-4. To discern whether the p70S6K pathway runs in parallel to the Akt pathway, we measured the effect of rapamycin in Akt (k-)-M cells which expressed a dominant negative Akt. As shown in Fig. 6C, 50 nM of rapamycin blocked the basal level and the IL-4-induced activation of p70S6K in Akt (k-)-M cells. Similarly, rapamycin impaired the anti-apoptotic effect of IL-4 in Akt (k-)-M cells, enhancing cell death from 55% to 78% (Fig. 6D). These results indicate that p70S6K is activated through a PI3K-dependent manner and the

PI3K-dependent p70S6K pathway is involved in the anti-apoptotic effect of IL-4 on TGF-β-induced apoptosis in Hep3B cells. To further demonstrate the participation of PKC-mediated Akt and p70S6K as survival signaling molecules in IL-4's anti-apoptotic effect, we studied the combined effect of Gö6976 and rapamycin on the IL-4 action. These two inhibitors completely reversed the anti-apoptotic effect of IL-4 on TGF-β-induced apoptosis in Akt (k-)-M cells (Fig. 6E, increasing cell death from 58% to 94%) and in Hep3B cells (Fig. 6F, increasing cell death from 32% to 91%). Thus, these results demonstrate that together IL-4-mediated PKC-transduced Akt and p70S6K survival pathways are both necessary and sufficient for the inhibition of TGF-β-induced apoptosis.

**Both Akt and p70S6K pathways contribute to IL-4-induced phosphorylation of Bad at Ser-136.**

It has been reported that Akt and p70S6K phosphorylate in Bad at Ser-136, leading to prevent the cell death (28-31). We found that IL-4 stimulation resulted in the phosphorylation of Bad at Ser-112 and Ser-136 (Fig. 7A). To explore the molecular link between IL-4-induced survival signaling and Bad phosphorylation, we first examined the effect of PI3K inhibitors, wortmannin, LY294002 on IL-4-induced phosphorylation of Bad. As shown in the Fig. 7B, phosphorylation of Bad at Ser-112 and Ser-136 was blocked by wortmannin or LY294002, suggesting that the PI3K pathway is required for IL-4-induced phosphorylation of Bad at Ser-112 and Ser-136. Next, we attempted to understand the involvement of p70S6K and Akt in the phosphorylation of Bad. The results in Fig. 7B show that p70S6K inhibitor, rapamycin, also blocks the phosphorylation of Bad at Ser-112 and Ser-136. However, Fig. 7C shows that IL-4 failed to induce the phosphorylation of Bad at Ser-136, but still induced the phosphorylation of Bad at Ser-112 in Akt (k-)-M cells. Thus, both Akt and p70S6K pathways seem to be required for IL-4-induced phosphorylation of Bad at Ser-136, whereas only the later pathway participates in the phosphorylation of Bad at Ser-112.

**Discussion**

IL-4 regulates a wide variety of biological functions. Other than its well studied function as a $T_H2$ cytokines, IL-4 acts as a survival factor in a number of cell types, including T cells, B cells, myeloid cells, synoviocytes, edothieal cells, fibroblasts and cancer cells (19,21,32-37). In the liver, IL-4 has been reported to inhibit ischemaia/reperfusion induced liver injury, through the suppression of pro-inflammatory mediators (15). IL-4 also protects against TNF-α-mediated oxidative damage and apoptosis during schistosomiasis (17). However, it remains uncertain whether IL-4 has a direct or an indirect protective effect on the apoptosis of hepatocellular caicinoma cells. Unlike IL-4, TGF-β, on the other hand, serves as a negative mediator of liver homeostasis by promoting apoptosis, and inhibiting proliferation or regeneration in liver cells. Previous studies have been reported that several pro-apoptotic processes, including production of reactive oxygen species (ROS), activation of caspase-3, -8 and -9, downregulation of Bcl-2 and Bcl-$X_{L,}$ and activation of Smad-2, -3 and -4 dependent pathways, are involved in TGF-β-mediated apoptotic death in cultured hepatocytes and HCC cells (2). To gain insight into liver combined effects of liver cytokines in diseases, this study has focused on elucidating the survival signaling pathways mediated IL-4, using the model of TGF-β-induced apotopsis in Hep3B cells.

The IRS-PI3K, STAT6 and MAPK signaling pathways delivered through IL-4 receptors (IL-4R) have been extensively studied in various cell types. Our results demonstrated that IRS-1/-2 (Fig. 2A), MAPK (Fig. 3A) and STAT6 (data not shown) pathways were activated by IL-4 in Hep3B cells. We further demonstrated that IL-4 activation of PI3K, a downstream signaling molecule of IRS-1/-2, was also activated proved in an *in vitro* PI3K assay and that the anti-apoptotic effect of IL-4 was mainly mediated through the PI3K pathway (Fig. 2, C, D and E). However, IL-4-induced activation of MAPK pathway was not involved in the prevention of TGF-β-induced apoptosis (Fig. 3, A and B).

Akt, a downstream signaling molecule of PI3K, plays an important role in survival mechanisms mediated by various growth factors (38,39). However, dominant negative Akt only partially blocked the anti-apoptotic effect of IL-4 (Fig. 4B). These findings suggest that other signaling pathways may be also involved. We found that Gö6976, an inhibitor for PKCα, β and μ isoforms, also partially blocked the anti-apoptotic effect of IL-4. However, it did not further decrease the anti-apoptotic effect of IL-4 in Akt (k-)-M cells which expressing dominant negative Akt. Interestingly, Gö6976 also inhibited the IL-4-induced phosphorylation of Akt (Fig. 5C). These results suggest that Akt lies in the downstream of PKC. Pervious reports have shown that PKCα was involved in the induction of Akt phosphorylation (40,41). Our results indicated that IL-4 could not induce the membrane translocation of PKCα, β and μ in Hep3B cells (data not shown). Thus, it is possible that other PKC isoforms are activated by IL-4 stimulation.

Previous studies have reported that p70S6K is a downstream signaling molecule of PI3K, but it is not required for IL-4-promoted survival of myeloid cells (40). Our results showed that phosphorylation of p70S6K was detected in IL-4-treated Hep3B cells, and it could be blocked by PI3K inhibitors wortmannin and LY294002, as by p70S6K inhibitor rapamycin, indicating that p70S6K was at the downstream of PI3K, and that it partially contributed to the anti-apoptotic effect of IL-4 (Fig. 6, A and B), and blocking p70S6K activation by rapamycin could further decrease the anti-apoptotic effect of IL-4 in Hep3B cells expressing dominant negative Akt (Fig. 6D). In addition, treatment with Gö6976 and rapamycin almost completely blocked the anti-apoptotic effect of IL-4 in parental Hep3B cells and Hep3B cells expressing dominant negative Akt (Fig. 6, E and F). Thus, Akt and p70S6K concertedly achieved full maximal anti-apoptotic effect of IL-4. Several mechanisms have been proposed for the anti-apoptotic effect of activated Akt, including the inhibition of the apoptotic protein Bad via its phosphorylation by survival kinase molecules (41). Our reservation with IL-4 is in agreement with other studies demonstrating that growth factor suppression of apoptosis could in part be attributed to Bad inactivation by phosphorylation of its multiple Serine residues (42,43).

Activated Akt or p70S6K could phosphorylate a apoptotic molecule Bad at Ser-136 (28,31); however, some studies reported that IL-4-activated Akt did not induced Bad phosphorylation (44,45). We found that IL-4 induced phosphorylation of Ser-112 and Ser-136 (Fig. 7A), but not at Ser-155 (data not shown) in Hep3B cells overexpressing Bad. Furthermore, treatment of wortmannin, LY294002 (PI3K inhibitors) and rapamycin (p70S6K inhibitor) resulted in the inhibition of Bad phosphorylation at Ser-112 and Ser-136. We also found that IL-4 could not induce the phosphorylation of Bad at Ser-136 in Akt (k-)-M cells. Thus, the phosphorylation of Ser-136 seemed to be dependent on both Akt and p70S6K pathways. Pervious studies demonstrated that Bad phosphorylation at Ser-112 is through the activation of ERK-1/2 (30). However, IL-4 could induce the activation of ERK, but this pathway did not contribute to prevent cell death by IL-4 (Fig. 3, A and B). Thus, it will be interesting to explore p70S6K is direct responsible for phosphorylating Bad at Ser-112 in Hep3B cells.

Although the PI3K/Akt, STAT3, MAPK and p38 pathways have been reported that to be activated by various survival factors, including cytokines, which suppress TGF-β-induced apoptosis by different survival factors (26),(27),(46,47), these evidence and our findings suggested that survival factors used various the survival pathways to prevent TGF-β-induced apoptosis in Hep3B cells. On the other hand, discrepancies exist among various studies including ours; the anti-apoptotic effect of IL-4 appears to be mediated through different survival signaling pathways in various cell types in response to different death signals. For example, the activation of STAT6 is required for the protective effect of IL-4 in B cells, but not STAT6 and IRS-2 in T cells (44). Recently, the crosstalk between of insulin and

TGF-β signaling pathways was found to be linked by Akt and Smad-3 pathways in Hep3B cells (47,52). To elucidate how IL-4 might counteract with cellular response to TGF-β, we began by examining the direct effect of IL-4 on Smad-3-induced activation and transcription using the 3TP-Lux plasmid system or Smad-mediated gene expression. Our result demonstrated that IL-4 has no effect on Smad-3-regulated transcription (data not shown) or on Smad-mediated expression of Death-associated protein kinase (DAPK) (data not shown). These results suggest that the anti-apoptotic effect of IL-4 might not be attributable to its direct interference with cellular responses to TGF-β stimulation.

This study with HCC has yielded several helpful hints to elucidate the anti-apoptotic mechanism of IL-4 in HCC cells and the finding may be applicable to hepatocytes. Our results indicated that either the Akt pathway or the p70S6K survival pathway alone was only partially effective in IL-4-mediated protection of hepatocytes from TGF-β-induced apoptosis. In conclusion, the two pathways appear able to function cooperatively to achieve the maximal anti-apoptotic effect of IL-4 in Hep3B cells (Fig. 8). It remains to be explored, however, if the anti-apoptotic effect *in vitro* of IL-4, a product of T, B and NKT cells in liver, serves any physiological role *in vivo*. All these studies have provided strong evidences to suggest that IL-4 could protect hepatocytes from cellular programming that leads to apoptosis.

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**Footnotes**

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4. Address orrespondence and reprint request to Cheng-po Hu, Department of Medical Research and Education, Taipei Veterans General Hospital, No.201, Sec. 2, Shihpai Rd., Beitou District, Taipei City 112, Taiwan, Republic of China, Email address: cphu@vghtpe.gov.tw
5. Special abbreviations used in this paper: IL-4; Interleukin-4, HCC; hepatocellular carcinoma, TGF-β; transformining growth factor- beta, IRS-1/-2; insulin receptor substrate 1 and 2, PI3K; phosphatidylinositol 3-kinase, MAPK; mitogen-activated protein kinase, PKC; protein kinase C, p70S6K; p70 ribosomal protein S6 kinase, (P110*-M and P110*-17): transfectants with constitutively active from of PI3K, 〔Akt (k-)-M and Akt (k-)-9〕: transfectants with dominant negative Akt, STAT6; signal transducer and activation of transcription 6, TUNEL; terminaldeoxynucleotidyl ransferase-mediated dUTP nick end labeling.

**Figure legends**

Fig. 1. IL-4 prevents TGF-β-induced apoptosis. (A) Hep3B cells were seeded in 6 well-plates at a density of 3 x $10^5$ cells /well. After 2 days of serum starvation, cells were fed with fresh SF medium without or with IL-4 (100 ng/ml), TGF-β (5 ng/ml) or IL-4 plus TGF-β. Apoptotic cells were measured by TUNEL assay at 30 h, 36 h and 42 h by flow cytometry ( ■ Negative control, incubated with label solution, in the absence of terminal transferase, □Test sample, incubated with TUNEL reaction mixture) or (B) Hep3B cells were seeded in 48 well-plates at a density of 3 x $10^4$ cells /well and were cultured as described above. Apoptotic cells were measured by a Cell-Death Detection ELISA at 18 h.

Fig. 2. Activation of IRS/PI3K pathway is required for the anti-apoptotic effect of IL-4. (A) Hep3B cells were treated with IL-4 at indicated times. Cell lysates with equal amounts of protein were immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibody. The immunoprecipitates were subjected to SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody (4G10). The membranes were then stripped and reused for immunoblottings with anti-IRS-1 or anti-IRS-2 antibody to verify equal amounts of IRS-1 or IRS-2 in all samples. (B) Hep3B cells were treated with IL-4 for 5 min. Lysates of Hep3B cells treated with IL-4 alone or in the presence of PI3K inhibitor, wortmannin, were immunoprecipitated with anti-phosphotyrosine antibody (4G10). The immunoprecipitates were incubated with phosphatidylinositol (PI) and $^{32}$P-γATP. The lipid products were thenanalyzed by thin-layer chromatography and autoradiography. (C) and (D) Hep3B cells were cultured as described in Fig 1B. PI3K inhibitors wortmannin (C) or LY294002 (D) were added to the cells after 2 days of serum starvation. After 2 h, cells were incubated with IL-4 (100 ng/ml), TGF-β (5 ng/ml) or IL-4 plus TGF-β. Apoptotic cells were measured as described in Fig 1B. (E) Hep3B cells or stable clones (p110*-M, p110*-17) expressing the constitutively active from of PI3K were treated with TGF-β (5 ng/ml), and apoptotic cells were measured described in Fig.1B ( *p* value was analyzed by t test).

Fig. 3. The MAPK pathway is not involved in the anti-apoptotic effect of IL-4. (A) Hep3B cells were treated with IL-4 for 15 min alone or in the presence of MAPK inhibitor. Lysates of Hep3B cells treated with IL-4 alone or in the presence of MAPK inhibitor, PD98059 or U0126, were immunoprecipitated with anti-ERK-2 antibody. The immune complexes were resuspended in the kinase buffer containing ATP and Elk-1 fusion protein substrate. After reaction, the samples were subjected to SDS-PAGE and immunoblotting with anti-phospho-Elk-1 (Ser-383) antibody or anti-ERK-2 antibody. (B) Apoptotic cells were measured as described in Fig 1B. Various concentration of ERK-1/2 inhibitor, PD98059 or U0126 was added into the cells after 2 days of serum starvation. After 2 h, cells were incubated with IL-4 (100 ng/ml), TGF-β (5 ng/ml) or IL-4 plus TGF-β.

Fig. 4. Akt is involved in the anti-apoptotic effect of IL-4. (A) Hep3B cells were treated with IL-4 for 15 min alone or in the presence of PI3K inhibitor, wortmannin or LY294002. Cell lysates with equal amounts of protein were subjected to immunoblottings with anti-phospho-Akt (Ser-473) antibody. The membrane was then stripped and immunoblotted with anti-Akt antibody. (B) Apoptosis of Hep3B cells and stable clones Akt (k-)-M; Akt (k-)-9 expressing a dominant negative Akt were measured as described in Fig. 1B.

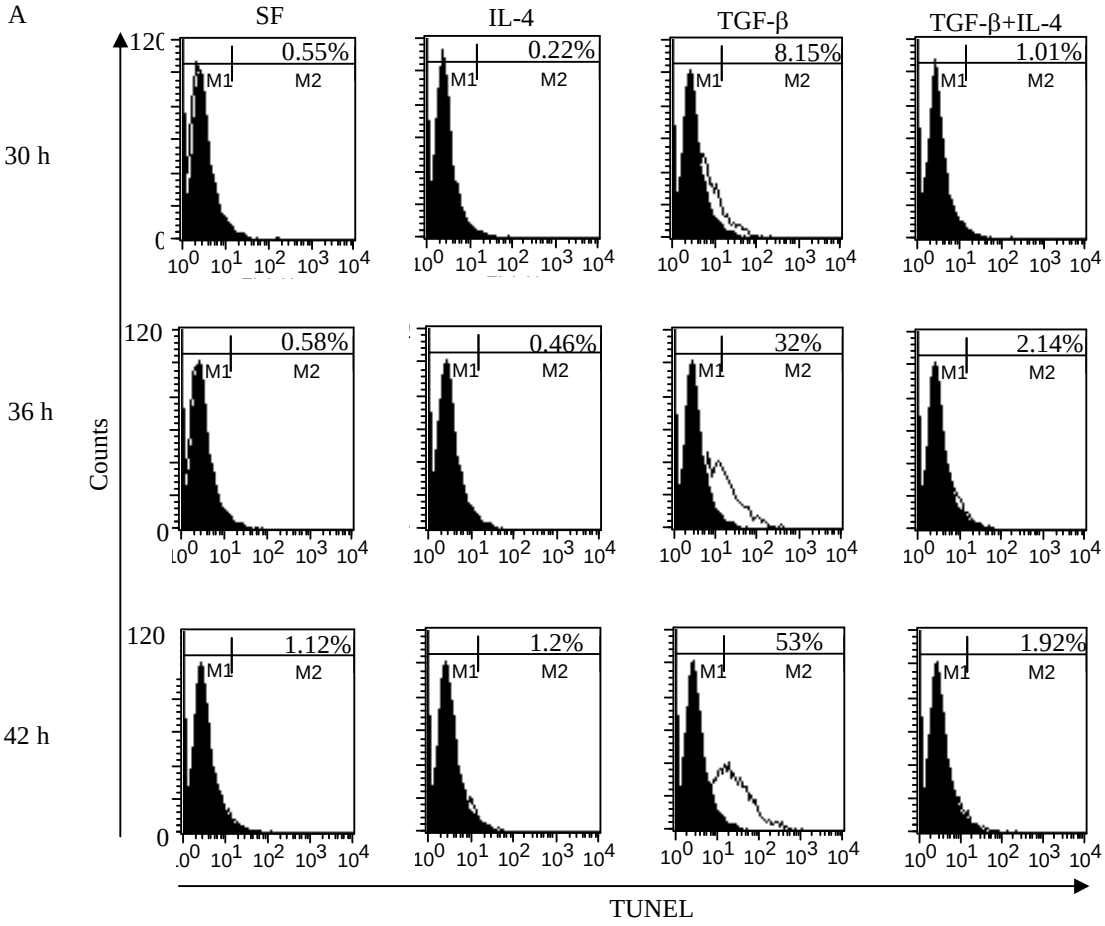
Fig. 5. PKC inhibitor, Gö6976, inhibits the anti-apoptotic effect of IL-4 and the activation of Akt. Apoptosis of (A) Hep3B cells and (B) stable clones Akt (k-)-M cells (B) were measured as described in Fig 1B. PKC inhibitor, Gö6976 was added to the cells after 2 days of serum starvation. After 2 h, cells were incubated with IL-4 (100 ng/ml), TGF-β (5 ng/ml) or IL-4 plus TGF-β. (C) Hep3B cells were treated with IL-4 for 15 min alone or in the presence of Gö6976. Lysates of IL-4 treated Hep3B cells with equal amounts of protein were immunoprecipitated with anti-Akt (PH domain, agarose conjugate). The immunoprecipitates were subjected to SDS-PAGE and immunoblotting with anti-phospho-Akt (Ser-473) or anti-phospho-Akt (Thr-308) antibodies. The membrane was then stripped and immunoblotted with anti-Akt antibody.

Fig. 6. The p70S6K pathway is involved in the anti-apoptotic effect of IL-4. (A) Hep3B cells were treated with IL-4 for 15 min alone or in the presence of various kinase inhibitors. Lysates of IL-4 treated Hep3B cells were subjected to SDS-PAGE and immunoblotting with anti-phospho-p70S6K (Thr-412) and anti-p70S6K antibodies. (B) Apoptosis of Hep3B cells was measured as described in Fig. 1B. Rapamycin was added to the cells after 2 days of serum starvation. (C) Akt (k-)-M cells were treated with IL-4 for 15 min alone or in the presence of rapamycin. Lysates of IL-4 treated Akt (k-)-M cells were subjected to SDS-PAGE and immunoblotting with anti-phospho-p70S6K (Thr-412) and anti-p70S6K antibodies. (D), (E) and (F) Apoptosis of Akt (k-)-M cells (D and E) and Hep3B cells (F) were measured as described in Fig. 1B. Rapamycin and Gö6976 were added to the cells after 2 days of serum starvation. After 2 h, cells were incubated with IL-4 (100 ng/ml), TGF-β (5 ng/ml) or IL-4 plus TGF-β.

Fig. 7. Both Akt and p70S6K pathways are required for IL-4-induced phosphorylation of Bad at Ser-136. The plasmid containing human Bad, phBad, was transfected to Hep3B cells and Akt (k-)-M cells. After 18 h, the cells were pooled, re-seeded and cultured in SF medium for 1 day. IL-4 (100 ng/ml) was added into the culture for indicated times. Cell lysates were prepared and subjected to immonoblottings with anti-phospho-Bad (Ser-112) and anti-phospho-Bad (Ser-136) antibodies. (A) Bad transfected Hep3B cells treated with or without IL-4. (B) Bad transfected Hep3B cells treated with IL-4 together wortmannin, LY294002 or rapamycin. (C) Bad transfected Akt (k-)-M cells treated with or without IL-4. The membranes were then stripped and immunoblotted with anti-Bad antibody.

Fig. 8. Proposed model of IL-4 survival signaling pathways in Hep3B cells. Following the activation of IRS/PI3K pathway through IL-4 and IL-4R complex interaction, activated PI3K contributed to the PKC-mediated Akt activation and p70S6K signaling pathways. While both pathways could phosphorylate apoptotic molecule, Bad, at Ser-136, only p70S6K participated in the phosporylation of Bad at Ser-112. In summary, Akt and p70S6K signaling pathways were both necessary and sufficient for the inhibition of TGF-β-induced apoptosis.

**Figure 1**


A
SF
IL-4
TGF-β
TGF-β+IL-4
30 h
36 h
42 h
Counts
TUNEL
M1
M2
0.55%
0.22%
8.15%
1.01%
0.58%
0.46%
32%
2.14%
1.12%
1.2%
53%
1.92%

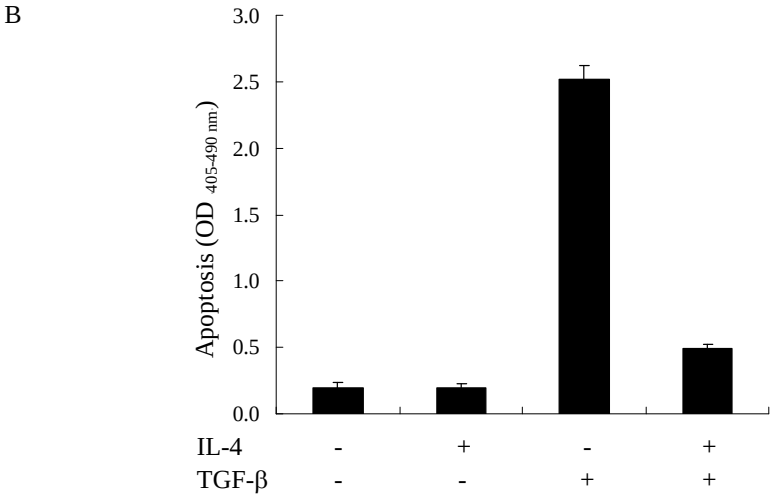
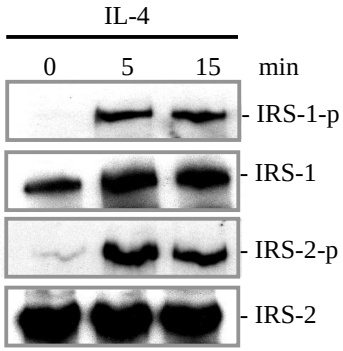
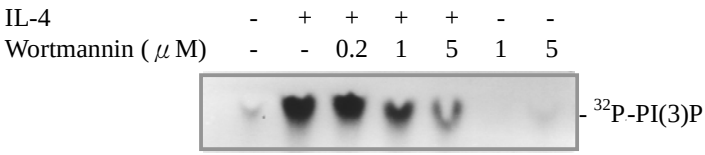

B
Apoptosis (OD 405-490 nm)
IL-4
TGF-β

**Figure 2**

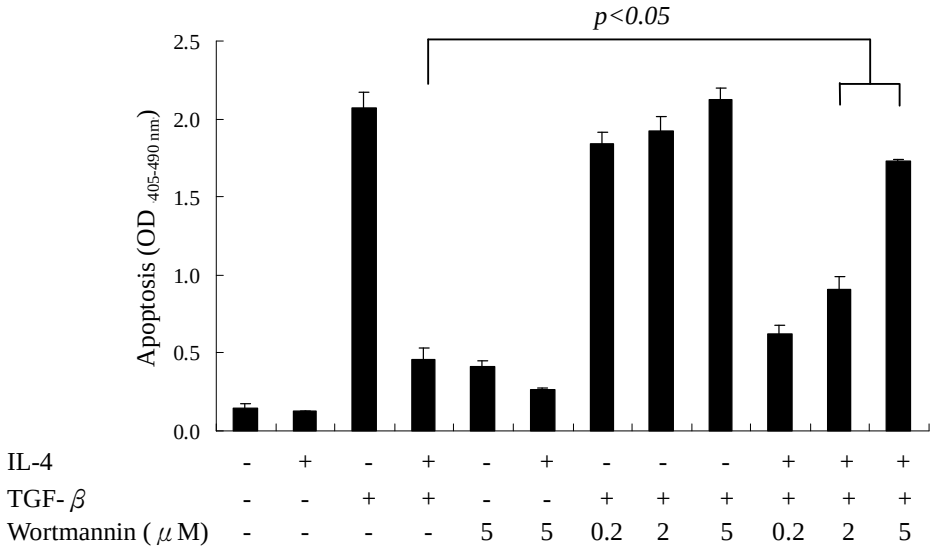
A


IL-4
0
5
15
min
- IRS-1-p
- IRS-1
- IRS-2-p
- IRS-2

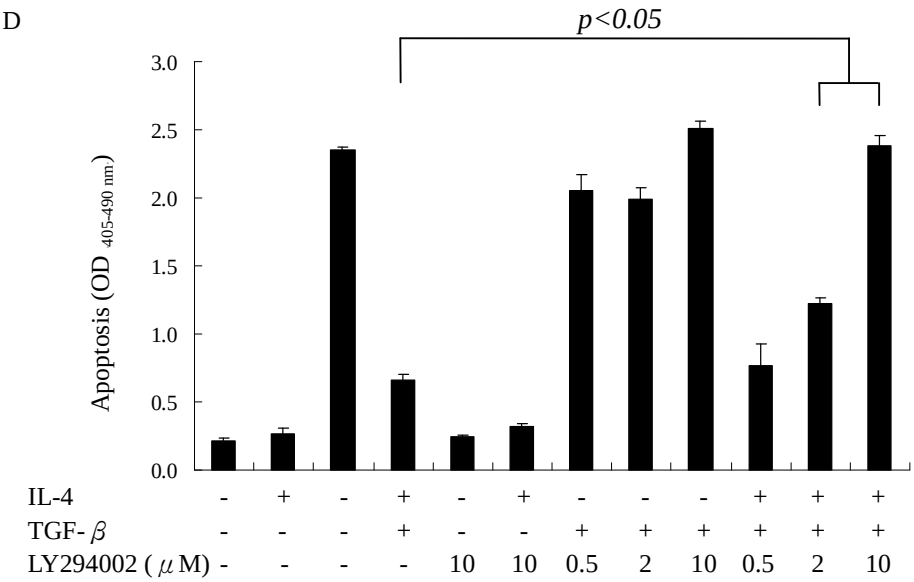
B


IL-4 - + + + + - -
Wortmannin (μM) - - 0.2 1 5 1 5
- 32P-PI(3)P

C


p<0.05
Apoptosis (OD 405-490 nm)
2.5
2.0
1.5
1.0
0.5
0.0
IL-4 - + - + - + - - - + + +
TGF-β - - + + - - + + + + + +
Wortmannin (μM) - - - - 5 5 0.2 2 5 0.2 2 5

D


p<0.05
Apoptosis (OD 405-490 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
IL-4 - + - + - + - - - + + +
TGF-β - - - + - - + + + + + +
LY294002 (μM) - - - - 10 10 0.5 2 10 0.5 2 10

E

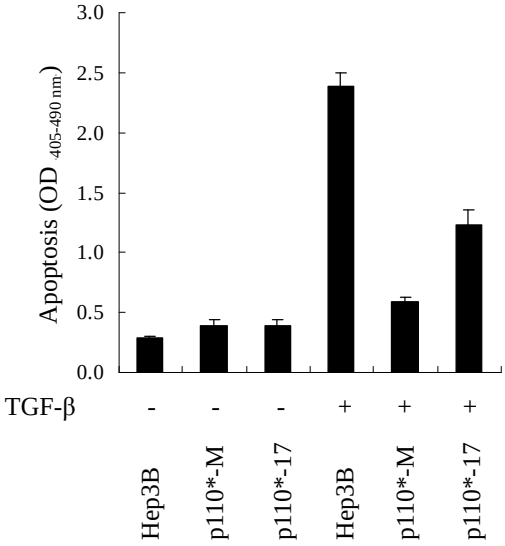
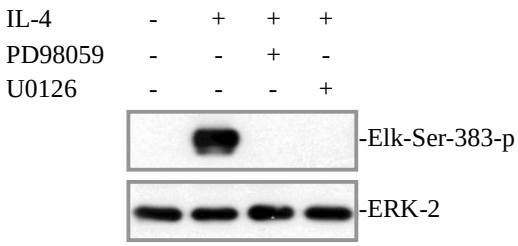
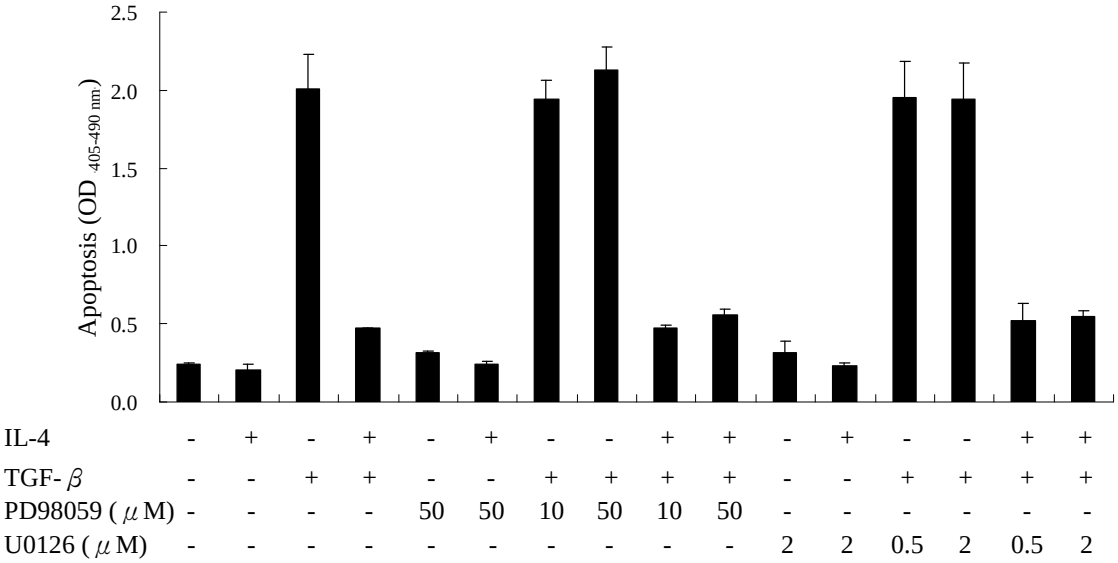

Apoptosis (OD 405-490 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
TGF-β - - - + + +
Hep3B
p110*-M
p110*-17
Hep3B
p110*-M
p110*-17

**Figure 3**

A

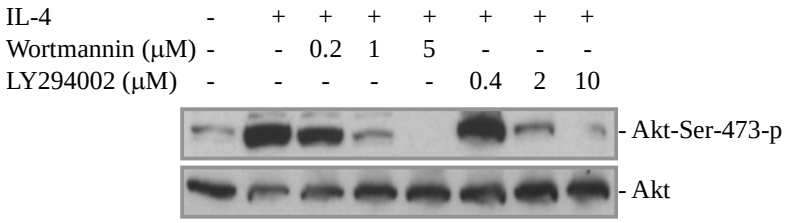

IL-4 - + + +
PD98059 - - + -
U0126 - - - +
-Elk-Ser-383-p
-ERK-2

B


Apoptosis (OD 405-490 nm)
2.5
2.0
1.5
1.0
0.5
0.0
IL-4 - + - + - + - - + + - + - - + +
TGF-β - - + + - - + + + + - - + + + +
PD98059 (μM) - - - - 50 50 10 50 10 50 - - - - - -
U0126 (μM) - - - - - - - - - - 2 2 0.5 2 0.5 2

## Figure 4

A


IL-4 - + + + + + + +
Wortmannin (μM) - - 0.2 1 5 - - -
LY294002 (μM) - - - - - 0.4 2 10
- Akt-Ser-473-p
- Akt

B

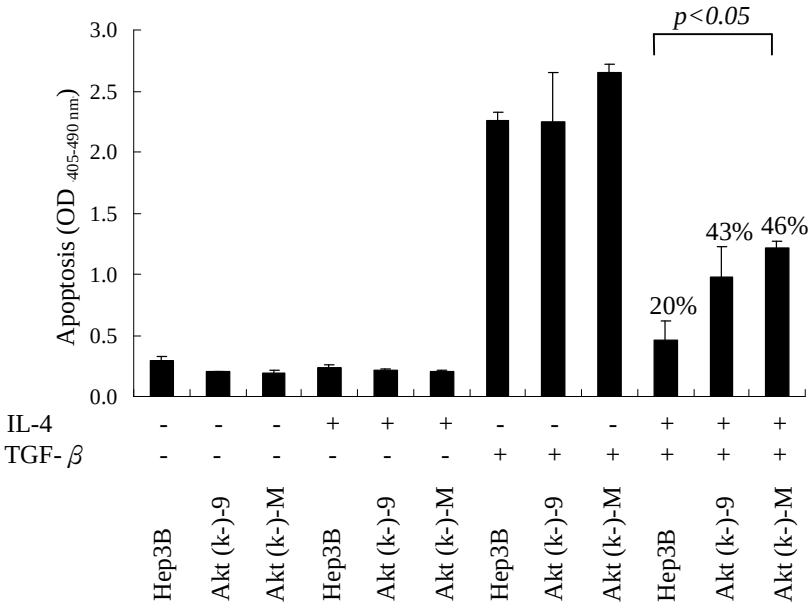
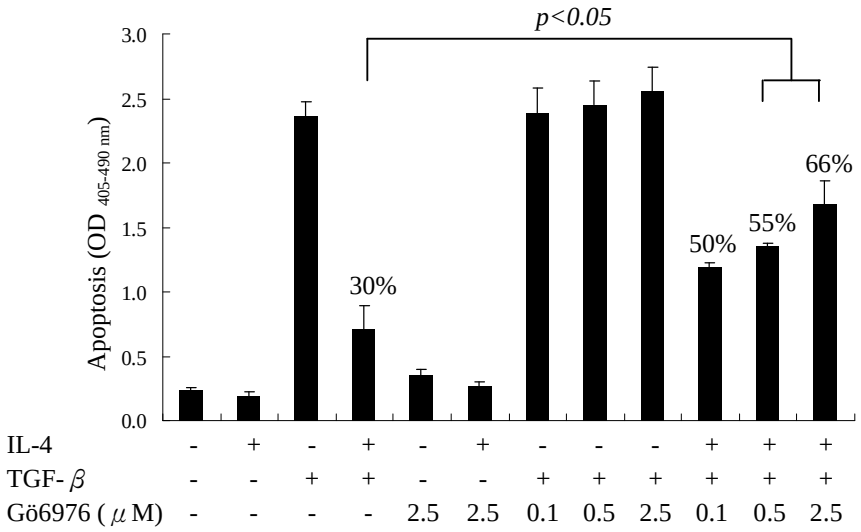
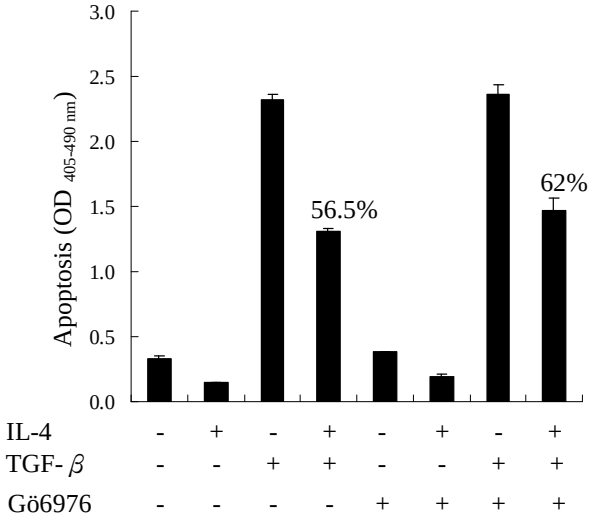

p<0.05
Apoptosis (OD 405-490 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
20%
43%
46%
IL-4 - - - + + + - - - + + +
TGF-β - - - - - - + + + + + +
Hep3B
Akt (k-)-9
Akt (k-)-M
Hep3B
Akt (k-)-9
Akt (k-)-M
Hep3B
Akt (k-)-9
Akt (k-)-M
Hep3B
Akt (k-)-9
Akt (k-)-M

**Figure 5**

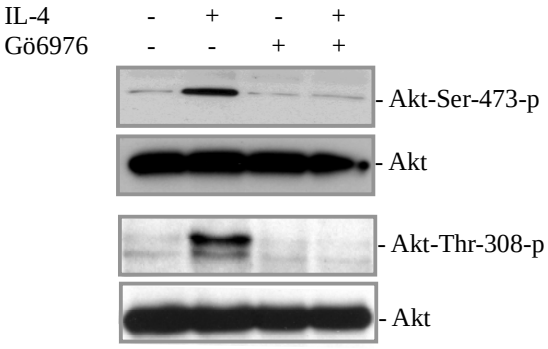
A


p<0.05
Apoptosis (OD 405-490 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
30%
50%
55%
66%
IL-4 - + - + - + - - - + + +
TGF-β - - + + - - + + + + + +
Gö6976 (μM) - - - - 2.5 2.5 0.1 0.5 2.5 0.1 0.5 2.5

B

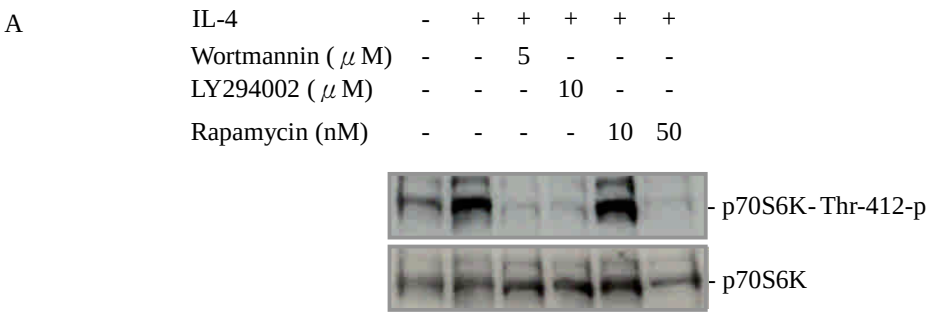

Akt (k-)-M cells
Apoptosis (OD 405-490 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
56.5%
62%
IL-4 - + - + - + - +
TGF-β - - + + - - + +
Gö6976 - - - - + + + +

C

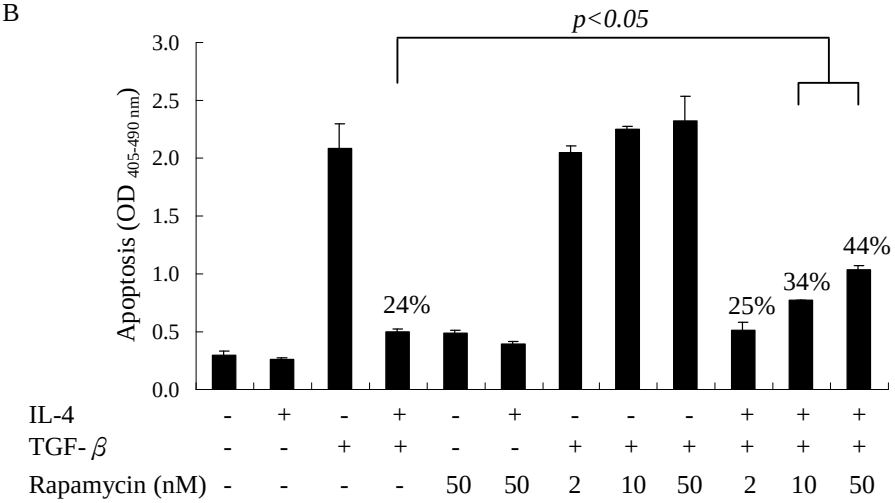


## Figure 6

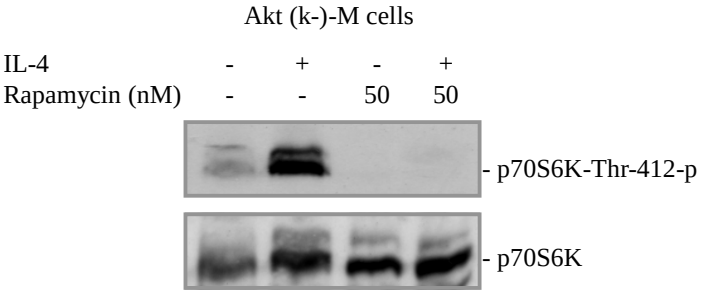
A


IL-4 - + + + + +
Wortmannin (μM) - - 5 - - -
LY294002 (μM) - - - 10 - -
Rapamycin (nM) - - - - 10 50
p70S6K-Thr-412-p
p70S6K

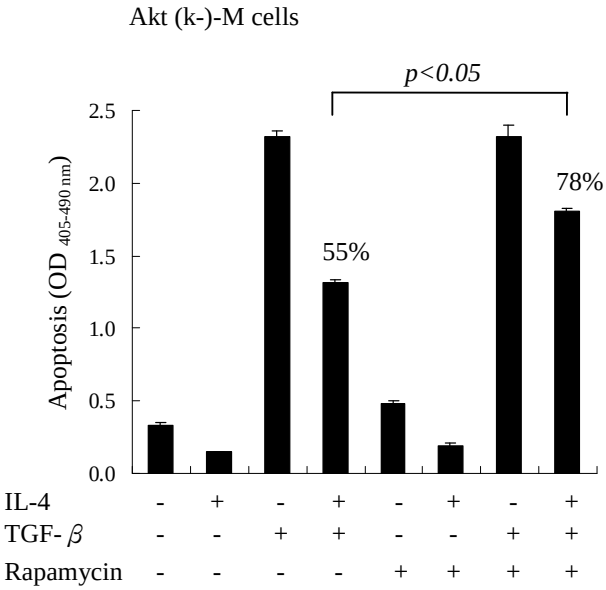
B


p<0.05
Apoptosis (OD 405-490 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
24%
25%
34%
44%
IL-4 - + - + - + - - - + + +
TGF-β - - + + - - + + + + + +
Rapamycin (nM) - - - - 50 50 2 10 50 2 10 50

C

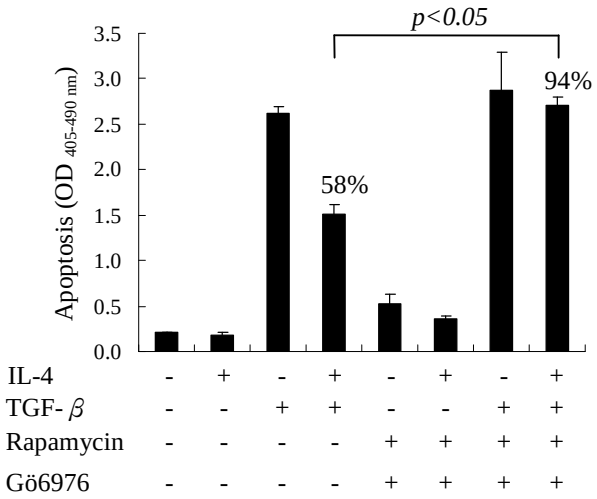

Akt (k-)-M cells
IL-4 - + - +
Rapamycin (nM) - - 50 50
- p70S6K-Thr-412-p
- p70S6K

D


Akt (k-)-M cells
p<0.05
Apoptosis (OD 405-490 nm)
2.5
2.0
1.5
1.0
0.5
0.0
55%
78%
IL-4 - + - + - + - +
TGF-β - - + + - - + +
Rapamycin - - - - + + + +

E

Akt (k-)-M cells




F

Hep3B cells

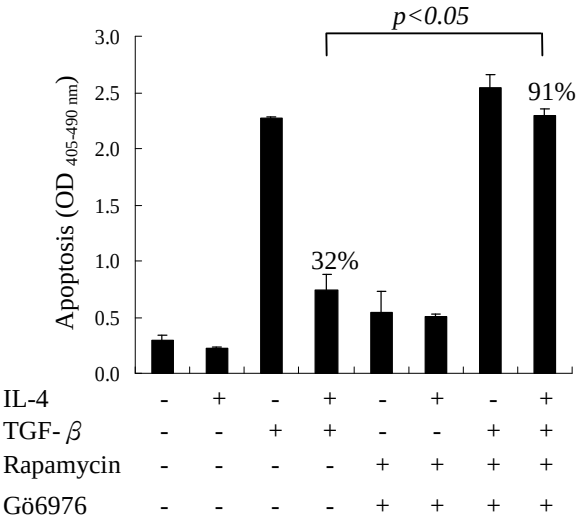
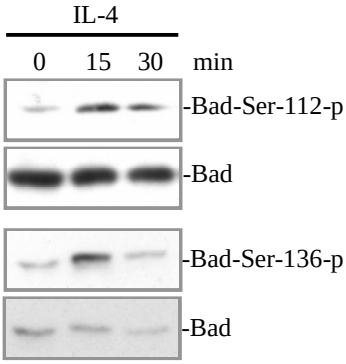
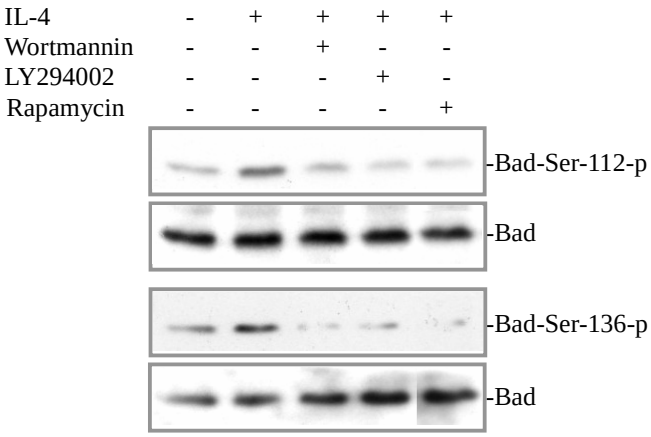


**Figure 7**

A


IL-4
0 15 30 min
-Bad-Ser-112-p
-Bad
-Bad-Ser-136-p
-Bad

B


IL-4 - + + + +
Wortmannin - - + - -
LY294002 - - - + -
Rapamycin - - - - +
-Bad-Ser-112-p
-Bad
-Bad-Ser-136-p
-Bad

C


Akt (k-)-M cells
SF IL-4
-Bad-Ser-112-p
-Bad
-Bad-Ser-136-p
-Bad

**Figure 8**

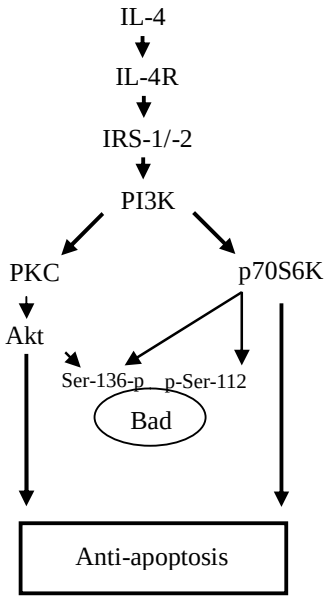



Figure 8. Proposed model of IL-4 survival signaling pathways in Hep3B cells.