

LLL12 Inhibits Endogenous and Exogenous Interleukin-6-induced STAT3 Phosphorylation in Human Pancreatic Cancer Cells

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Abstract. Pancreatic cancer is one of the most serious types of cancer, with a five-year survival rate at only 6%. There is a critical need to develop more effective treatments for pancreatic cancer. Growing evidence shows that chronic inflammation plays a crucial role in tumor initiation and progression. Here we demonstrated that the endogenous expression of the inflammatory cytokine interleukin-6 (IL-6) correlates with signal transducer and activator of transcription 3 (STAT3) phosphorylation in human pancreatic cancer cells. Inhibition of the endogenous IL-6/STAT3 pathway reduces cell viability. Exogenous IL-6 induces STAT3 phosphorylation, but differently induces phosphorylation of STAT3 upstream kinases, Janus kinase 1 (JAK1), JAK2, and tyrosine kinase 2 (TYK2). Interestingly, LLL12, a nonpeptide, cell-permeable small molecule, selectively blocked exogenous IL-6-induced STAT3 phosphorylation and nuclear translocation in both PANC-1 and ASPC-1 pancreatic cancer cell lines independently of the phosphorylation of JAK1, JAK2, and TYK2. These results suggest that the inhibition of endogenous and exogenous IL-6-mediated STAT3 signaling may be a potential therapeutic approach for pancreatic cancer.

The molecular mechanisms of pancreatic cancer remain unknown. In recent years, growing evidence has accumulated to show that chronic inflammation plays a crucial role in tumor initiation and progression (1). Interleukin-6 (IL-6), a pleiotropic cytokine, plays a critical role in inflammatory reactions (2). Recent studies have shown strong evidence that obesity may increase the risk of liver and other types of cancer due to the chronic inflammatory response caused by enhanced levels of IL-6/signal transducer and activator of transcription 3 (STAT3) and tumor necrosis factor (TNF)/STAT3 signaling (3). High levels of IL-6 have been detected in the serum of patients with various types of cancer, including pancreatic cancer (4).

IL-6 relays the signal through three major pathways: STAT3, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinases (PI3K) (5). STAT3 is considered an oncogene and is found to be constitutively activated in many types of human malignancy. When IL-6 binds to IL-6 receptor (IL-6R), glycoprotein 130 (GP130) molecules homodimerize and are subsequently phosphorylated by receptor-associated Janus kinases (JAKs). Phosphorylated GP130 provides docking sites for STAT3 molecules, which are then phosphorylated by JAKs. Phosphorylated STAT3 dimers translocate to the nucleus, where they bind to specific DNA elements and regulate gene expression (6).

Scholz *et al.* reported that phosphorylated STAT3 was overexpressed in pancreatic ductal carcinoma cells but not in ducts from chronic pancreatitis. Blocking STAT3 significantly reduced cell proliferation *in vitro* and tumor growth *in vivo* (7). Several small molecules have been found to effectively inhibit STAT3 activation in pancreatic cancer. However, most of them are not direct STAT3 inhibitors (8-11), and no candidates have been selected for clinical trials. Furthermore, few inhibitors have examined the inhibition of IL-6-mediated STAT3 phosphorylation in pancreatic cancer cells. LLL12, a novel small molecule, is able to inhibit

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Key Words: Interleukin-6, signal transducer and activator of transcription 3, pancreatic cancer.

constitutively activated STAT3 and causes apoptosis in a variety of human cancer cells (12).

Here we investigated the contribution of endogenous IL-6 to STAT3 activation in pancreatic cancer cells and the effect of LLL12 on exogenous IL-6-induced STAT3 phosphorylation and nuclear translocation in pancreatic cancer cells.

Materials and Methods

Small molecular compounds and antibody. LLL12 was synthesized in the laboratory of Dr. Pui-Kai Li. We purchased Stattic, a previously reported STAT3 inhibitor (13), from Calbiochem (San Diego, CA, USA) and anti-human IL-6 neutralizing antibody from R&D Systems (Minneapolis, MN, USA). Antibodies against P-STAT3 (Tyr705), STAT3, P-JAK1 (Tyr1022/1023), JAK1, P-JAK2 (Tyr1007/1008), JAK2, tyrosine kinase 2 (TYK2), P-TYK2 (Tyr1054/1055), P-AKT (Ser473), phosphorylated extracellular-signal-regulated kinase (P-ERK1/2) (Thr202/Tyr204), IL-6, IL-6R, interferon (IFN)- γ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as well as secondary antibody were from Cell Signaling Technology (Beverly, MA, USA).

Cell culture. Human pancreatic cancer cell lines (ASPC-1, PANC-1, and SW1990) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Mediatech Inc, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY, USA) and 1% penicillin/streptomycin (Mediatech Inc). The cells were maintained at 37°C with 5% CO₂.

RT-PCR. RNA was extracted using RNeasy kits (Qiagen, Valencia, CA, USA). Reverse transcription was performed using Omniscript reverse transcription kit (Qiagen). Polymerase chain reaction (PCR) amplification was carried out under the following conditions: 5 minutes at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 60 seconds at 72°C, with a final extension of 10 minutes at 72°C. 4 genes were detected and the primers listed below (14): GAPDH: 5'-TGATGACATCAAGAAGGTGGTGAAG-3', and 5'-TCCTTGGAGGCCATGTGGGCAT-3' (240 bp); IL-6: 5'-GAGAAAGGAGACATGTAACAAGAGT-3', and 5'-GCGCAGAATGAGATGAGTTGT-3' (193 bp); GP130 IL-6R gene primers were purchased from SA Biosciences (Frederick, MD, USA) and the sizes are 103 bp and 150 bp, respectively. The sequences were not available.

Western blot analysis. To examine whether endogenous IL-6 contributed to STAT3 activation, SW1990 cells were treated with DMSO, 2.5 μ g/ml of anti-human IL-6 antibody, 10 μ M of Stattic or 5 μ M of LLL12 for 24 hours, protein expressions of P-STAT3 (Tyr705) and STAT3 were tested.

To investigate the effect of exogenous IL-6, ASPC-1 and PANC-1 cells were cultured in serum-free medium overnight and were then treated with different concentrations (0-25 ng/ml) of IL-6 for 30 minutes. After treatment, protein expressions of P-STAT3, STAT3, P-JAK1, JAK1, P-JAK2, JAK2, TYK2, P-TYK2, P-AKT, P-ERK1/2 were analyzed.

To examine whether and how LLL12 inhibits exogenous IL-6-induced STAT3 phosphorylation, ASPC-1 and PANC-1 cells were cultured in serum-free medium overnight and were then pretreated with different concentrations (0-5.0 μ M) of LLL12 for 2 hours, or same concentration (5 μ M or 10 μ M) of LLL12 for different times (0-2.0 hours), followed by 25 ng/ml of IL-6 or IFN- γ for 30

minutes; protein expressions of STAT1, P-JAK1 and others listed above were then analyzed.

Western blot procedure: Cells were lysed in cold radio-immunoprecipitation assay (RIPA) lysis buffer containing proteasome inhibitor cocktail and phosphatase inhibitor cocktail. Total lysates were resolved by SDS polyacrylamide gel electrophoresis (PAGE) and were transferred to PVDF membrane. Membranes were probed with specific primary antibody (1:1,000) and horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000). Immunoreactive bands were visualized using electrochemiluminescence (ECL) solution (Cell Signaling) and premium autoradiography film (Denville Scientific Inc, Metuchen, NJ, USA).

Cell viability assay. Viability was assessed using CyQUANT NF Kit (Molecular Probe, Invitrogen, USA) according to the manufacturer's manual. Briefly, SW1990 cells were seeded into a 96-well plate. The cells were then treated with DMSO, anti-human IL-6 antibody (2.5 μ g/ml), Stattic (10 μ M) and LLL12 (5 μ M), respectively. After 24-hour treatment, the medium was removed and 100 μ l of dye binding solution was added to each well. The plate was incubated at room temperature for 45 minutes. The fluorescence was measured at an excitation wavelength range of 485 nm and an emission wavelength range of 530 nm.

Apoptosis assay. Apoptosis was measured using caspase3/7 assay (Promega, Madison, WI, USA) according to the manufacturer's manual. SW1990 cells were seeded into a 96-well plate and treated as indicated in cell viability assay. After the treatment, 100 μ l of Apo-One Caspase3/7 reagent were added to each well and the plate was incubated at 37°C for 45 minutes. The fluorescence was measured at an excitation wavelength range of 485 nm and an emission wavelength range of 530 nm.

Immunofluorescence. PANC-1 cells were seeded on glass slides and then pretreated with 5 μ M of LLL12 for 2 hours, followed by 25 ng/ml of IL-6 for 30 min. After the treatment, cells were fixed with cold methanol and blocked in blocking buffer. The slides were then incubated overnight with specific primary antibody against p-STAT3 (1:100) at 4°C. After washing with PBS plus 0.1% Tween-20 solution (PBS-T), the slides were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (green; 1:100) (Molecular Probe, Invitrogen) for one hour at room temperature. The cells were mounted with Vectashield HardSet mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Images were captured by Leica Microsystems (Bannockburn, IL, USA).

Statistical analysis. Statistical significance was calculated by Student's *t*-test. A *p*-value of <0.05 was considered significant.

Results

Endogenous IL-6 contributes to STAT3 activation and blockade of P-STAT3 causes apoptosis. To investigate whether human pancreatic cancer cells overexpress IL-6 and IL-6R and express high levels of phosphorylated STAT3, we evaluated their RNA or protein levels in ASPC-1, PANC-1, and SW1990 cells. IL-6 mRNA and phosphorylated STAT3 were enhanced in SW1990 cells (Figure 1A and B); ASPC-

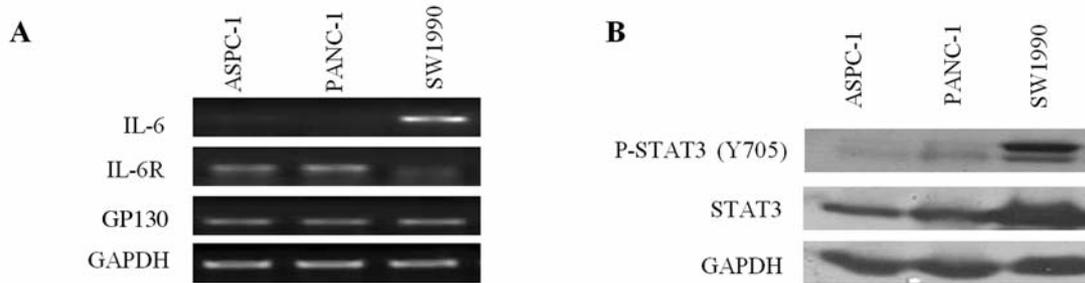


Figure 1. Endogenous IL-6 contributes to STAT3 activation. The mRNA levels of IL-6, IL-6R, and GPI30 in pancreatic cancer cells were analyzed by RT-PCR (A). The level of P-STAT3 in pancreatic cancer cells was evaluated by Western blot (B).

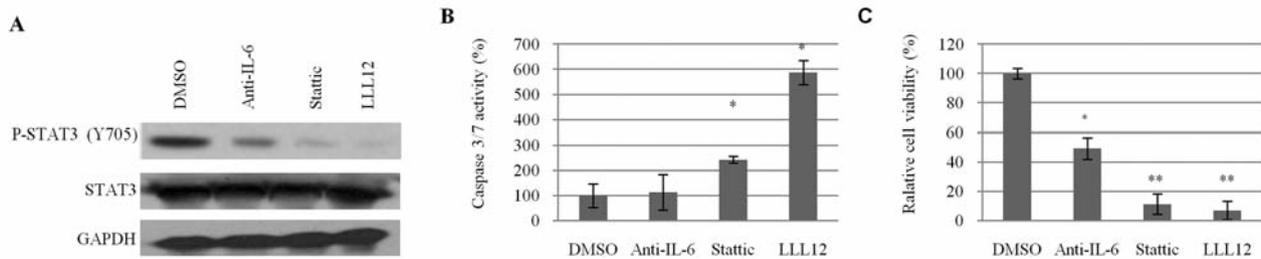


Figure 2. Blockade of P-STAT3 causes apoptosis. SW1990 cells were treated with anti-IL-6 antibody (2 μ g/ml), Stattic (10 μ M) or LLL12 (5 μ M). P-STAT3 expression, apoptosis and viability were examined by Western blot (A), apoptosis assay (B), and viability assay (C), respectively. Significantly different from the control at * $p < 0.05$, ** $p < 0.01$.

1 and PANC-1 cells overexpressed IL-6R mRNA. We also examined the mRNA levels of GPI30 and did not observe significant changes in the three cell lines.

To examine whether endogenous IL-6 contributed to STAT3 activation, SW1990 cells were treated with anti-IL-6 antibody or STAT3 inhibitors Stattic and LLL12 for 24 hours. We observed that all the treatments reduced STAT3 phosphorylation, indicating that endogenous IL-6 contributed to STAT3 phosphorylation and that STAT3 inhibitors were able to block endogenous IL-6-induced STAT3 phosphorylation (Figure 2A). Decreases in P-STAT3 were concomitant with increased apoptosis (Figure 2B) and lowered cell viability (Figure 2C).

Exogenous IL-6 induces STAT3 phosphorylation but differently induces phosphorylation of STAT3 upstream kinases. The levels of serum IL-6 have been shown to be elevated in patients with pancreatic cancer (4). To investigate whether exogenous IL-6 can induce STAT3 phosphorylation in pancreatic cancer cells with low levels of endogenous IL-6, ASPC-1 and PANC-1 cells were treated with increased concentrations of IL-6. After treatment with IL-6, P-STAT3 expression was induced in both cell lines in a dose-dependent manner, whereas the levels of STAT3 did not change (Figure 3A). To further explore which tyrosine kinase mediated the

IL-6-induced STAT3 phosphorylation, we examined JAK1, JAK2, and TYK2. In ASPC-1 cells, P-JAK1, P-JAK2, and P-TYK2 were all up-regulated with the treatment of IL-6, whereas only P-JAK1 was induced in PANC-1 cells (Figure 3B). In contrast to P-STAT3, P-AKT and P-ERK were not enhanced by the treatment of IL-6 (Figure 3C).

LLL12 specifically inhibits exogenous IL-6-induced STAT3 phosphorylation. To examine whether LLL12 may inhibit exogenous IL-6-induced STAT3 phosphorylation in pancreatic cancer cells, ASPC-1 and PANC-1 cells were pre-treated with LLL12 followed by IL-6. P-STAT3 expression was induced in IL-6 treated cells, whereas IL-6 did not activate STAT3 in LLL12 pre-treated cells (Figure 4A). The selectivity of LLL12 for STAT3 inhibition was evident when compared with that for STAT1. IFN- γ treatment resulted in phosphorylation of STAT1, whereas LLL12 pre-treatment had no effects on the extent of STAT1 phosphorylation (Figure 4B). Furthermore, LLL12 did not affect JAK1, JAK2, and TYK2 phosphorylation (Figure 4C).

We then examined whether LLL12 pre-treatment may block IL-6-induced P-STAT3 nuclear accumulation and STAT3 nuclear translocation. PANC-1 cells were pre-treated with LLL12 followed IL-6 for. After the treatment,

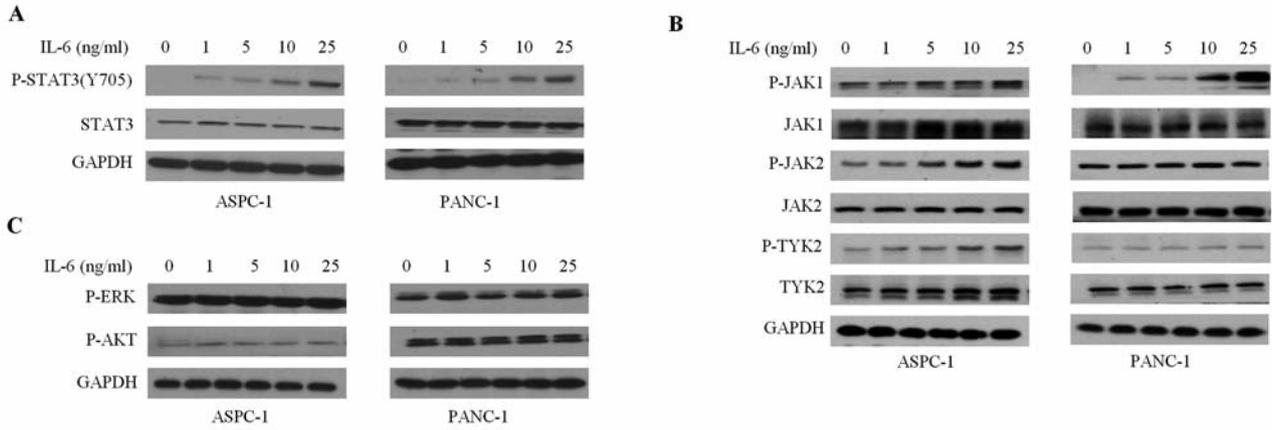


Figure 3. *IL-6 induces STAT3 phosphorylation but differently induces phosphorylation of STAT3 upstream kinases. ASPC-1 and PANC-1 cells were cultured in serum-free medium overnight and were then treated with different concentrations of IL-6 for 30 minutes. After treatment, protein expressions of STAT3 (A), P-JAK1, JAK1, P-Jak2, JAK2, P-TYK2, TYK2 (B), P-AKT, and P- ERK (C) were analyzed by Western blot.*

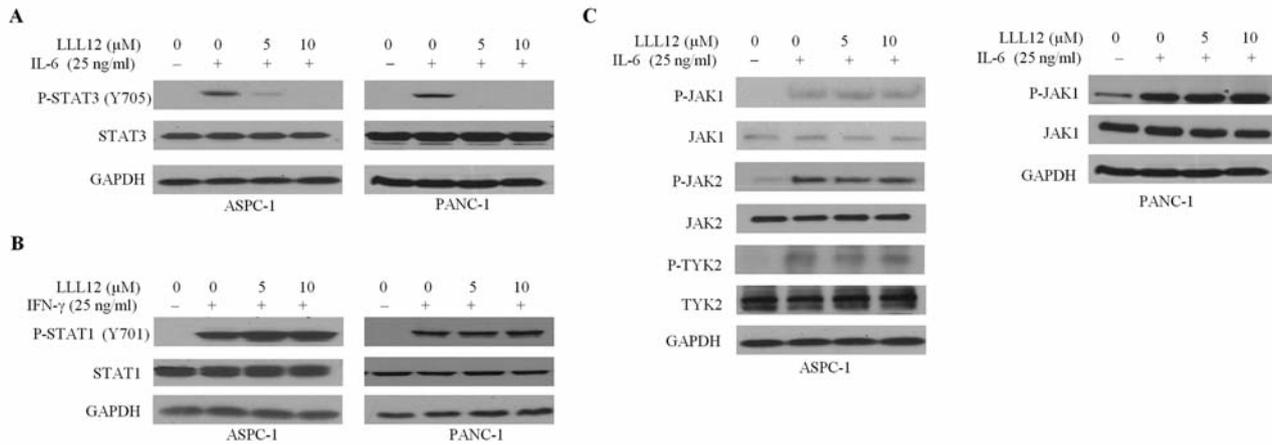


Figure 4. *LLL12 specifically inhibits exogenous IL-6-induced STAT3. ASPC-1 and PANC-1 cells were cultured in serum-free medium overnight and then were pre-treated with LLL12 for 2 hours followed by 25 ng/ml of IL-6 or IFN- γ for 30 minutes. After treatment, protein expressions of P-STAT3 and STAT3 (A), P-STAT1 and STAT1 (B), P-JAK1, JAK1, P-Jak2, JAK2, P-TYK2, and TYK2 were examined by Western blot (C).*

immunofluorescence was performed to analyze the localization of P-STAT3 and STAT3. IL-6 induced the accumulation of P-STAT3 in the nucleus, whereas the pre-treatment with LLL12 blocked this process (Figure 5).

LLL12 inhibits IL-6-induced STAT3 phosphorylation in a dose- and time-dependent manner. To examine whether LLL12 may inhibit IL-6-induced STAT3 phosphorylation in a dose and time-dependent manner, ASPC-1 and PANC-1 cells were pre-treated with increased concentrations of LLL12 for two hours or with 5 μ M of LLL12 for different times followed by IL-6 treatment. As expected, LLL12 inhibited P-STAT3 in a dose- (Figure 6A) and time-dependent fashion (Figure 6B).

Discussion

Pancreatic cancer is estimated to have caused 36,800 deaths in the United States in 2010 (15). The present work was prompted by the following facts: high levels of IL-6 have been detected in the serum of patients with pancreatic cancer (4); aberrant activation of STAT3 is found in a large percentage of patients with pancreatic cancer (16); targeting STAT3 has been shown to effectively inhibit cancer cell viability (17).

Okada *et al.* reported that 30 out of 55 pancreatic cancer patients had detectable levels of IL-6. Only 1 out of 20 healthy adults and 2 out of 25 patients with chronic pancreatitis had detectable levels of IL-6 (4). Ebrahimi *et al.* analyzed circulating levels of IL-6 in 50 patients with

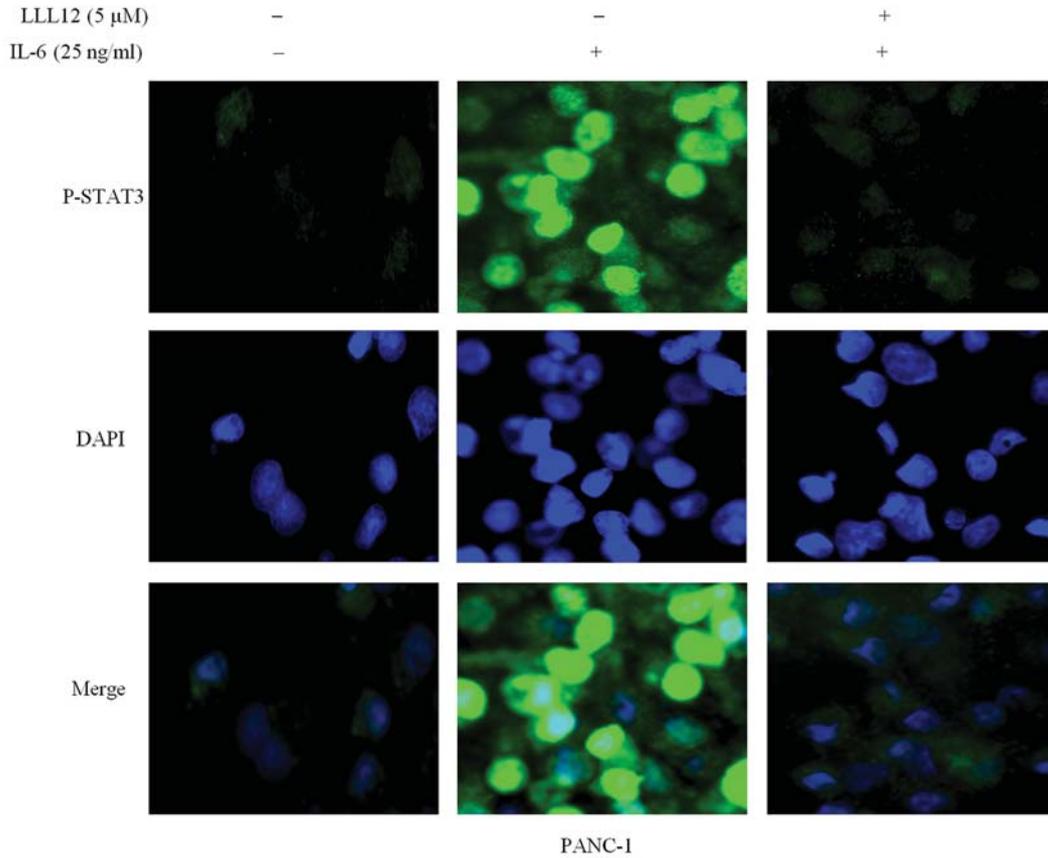


Figure 5. LLL12 blocks IL-6-induced P-STAT3 nuclear accumulation. PANC-1 cells were cultured on coverslips in serum-free medium overnight and were pre-treated with 5 μ M of LLL12 for 2 hours followed by 25 ng/ml of IL-6 for 30 minutes. After treatment, the localization of P-STAT3 was analyzed by immunofluorescence.

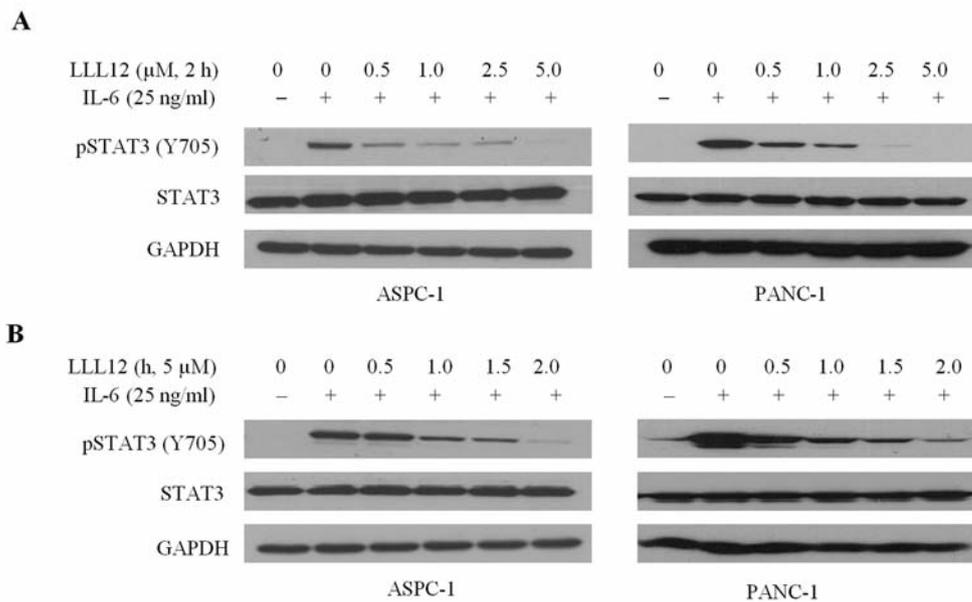


Figure 6. LLL12 inhibits IL-6-induced P-STAT3 in a dose- and time-dependent manner. ASPC-1 and PANC-1 cells were pre-treated with different concentrations of LLL12 for 2 hours (A) or with 5 μ M of LLL12 for different times (B) followed by 25 ng/ml of IL-6 for 30 minutes. Protein expressions of P-STAT3 and STAT3 were analyzed by Western blot.

pancreatic carcinoma. They found that the IL-6 range in patients is 0.7-38 pg/ml, whereas the range in normal controls was 0.7-4.3 pg/ml (18). Another group analyzed blood samples from 13 patients with advanced pancreatic cancer and 6 healthy adults. Serum levels of IL-6 were significantly higher in cancer patients (19). Elevated levels of IL-6 are detected in several types of cancer and appear to play an important role in the oncogenesis of these cancer types (20-23). Clinical studies demonstrated that elevated levels of serum IL-6 may correlate with tumor size and the presence of liver metastases in pancreatic adenocarcinoma patients (24). Autocrine IL-6 signaling is an important pathway to activate oncogenic STAT3 in cancer cells (20).

Here we investigated the expression of IL-6 and STAT3 phosphorylation in human pancreatic cancer cell lines. We found that the expression of IL-6 correlated with the expression of STAT3 phosphorylation. The blocking of autocrine IL-6 signaling in pancreatic cancer cells secreting elevated levels of IL-6 inhibited STAT3 phosphorylation and subsequently caused a reduction of cell viability.

The addition of exogenous IL-6 to two different pancreatic cancer cell lines revealed the different activation of the upstream protein kinases of STAT3. In PANC-1 pancreatic cancer cells, only JAK1 phosphorylation was increased. In ASPC-1 pancreatic cancer cells, however, phosphorylation of JAK1, JAK2, and TYK2 were all up-regulated. This suggests that the activation of STAT3 by IL-6 signaling may not act through the same upstream kinases in different pancreatic cancer cell lines.

The effect of a newly developed compound LLL12 against IL-6-induced STAT3 activation in pancreatic cancer has also been evaluated. Computer models with docking simulation showed that LLL12 binds directly to the phosphorylated tyrosine 705 binding site of the STAT3 monomers (12), and this is confirmed in this study in that LLL12 did not inhibit the activation of STAT3 upstream kinases, JAK1, JAK2 and TYK2 (Figure 4C). These results suggest that LLL12 directly acts on STAT3 but not on JAK1, JAK2 and TYK2. The effects of LLL12 on the activation of STAT1, a tumor suppressor, were also evaluated. LLL12 did not affect IFN- γ -induced STAT1 activation (Figure 4B), suggesting that LLL12 selectively inhibited STAT3 phosphorylation.

In summary, IL-6/STAT3 signaling is important for the survival of pancreatic cancer cells. The inhibition of autocrine IL-6/STAT3 signaling by small molecular inhibitor such as LLL12 may provide a novel therapeutic approach for pancreatic cancer treatments.

Acknowledgements

This research was funded by an NIH R21 grant (R21CA133652-01A1) and AACR Pancreatic Cancer Action Network to Dr. Jiayuh Lin.

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Received March 3, 2011

Revised May 11, 2011

Accepted May 12, 2011