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Interferon-α induces the growth inhibition of human T-cell leukaemia line Jurkat through p38α and p38β

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Interferon alpha (IFN-α) modulates the proliferation of different human tumour cell lines. It has been shown that IFN-α induces the growth inhibition of T-cell acute lymphoblastic leukaemia (T-ALL). However, its intracellular signalling mechanisms remain unknown. This study found that IFN-α inhibited the cell proliferation of human T-ALL cell line Jurkat in a dose- and time-dependent manner. A p38 inhibitor (SB203580), but not an extracellular signal-regulated kinase 1/2 inhibitor (PD98059) or c-Jun N-terminal kinase inhibitor (SP600125), eliminated IFN-α inhibition of Jurkat cell proliferation, indicating that p38 pathway is crucial for IFN-α-mediated growth inhibition. SB203580 targeted two p38 isoforms, p38α and p38β. The expression of p38α and p38β mRNA in Jurkat cells was examined by reverse transcriptase–polymerase chain reaction. The kinase activity of p38α and p38β was activated by IFN-α in Jurkat cells. To investigate the role of p38α and p38β isoforms in IFN-α-mediated growth inhibition, we generated stable clones that overexpressed the dominant-negative p38 isoform, p38α(AF) or p38β(AF), in Jurkat cells. Overexpression of p38α(AF) or p38β(AF) inhibited IFN-α-mediated p38 kinase activity and growth inhibition in Jurkat cells. Similarly, down-regulation of either p38α or p38β by isoform-specific small interference RNAs also reduced IFN-α-mediated growth inhibition. These results demonstrate that IFN-α can regulate growth inhibition of Jurkat cells through p38α and p38β.

Keywords: growth inhibition/IFN-α/Jurkat cells/p38α /p38β.

Abbreviations: CML, chronic myelogenous leukaemia; ERK1/2, extracellular signal-regulated kinase 1/2; IFN-α, interferon alpha; IFNAR, IFN-α receptor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription; T-ALL, T-cell acute lymphoblastic leukaemia.

Interferon alpha (IFN-α) belongs to the type I IFNs (IFN-α, -β and -ω) and is important in many biological functions, including antiviral, antiproliferation and immune response modulation (*1–4*). IFN-α also exerts antitumour activity in a range of haematological and non-haematological malignancies (*1*, *2*, *4*). Cell growth inhibitory activity of IFNs has been suggested as being one possible explanation for their antitumour action (*1–3*). It has been shown that IFN-α induces cell growth inhibition in the primary cells of T-cell acute lymphoblastic leukaemia (T-ALL) (*5*). However, its precise mechanisms remain unknown.

All type I IFNs bind to a common receptor composed of two subunits, IFN-α receptor (IFNAR)-1 and IFNAR-2. The interaction of IFN-α with its receptor components results in the activation of two tyrosine kinases of the Janus family, TYK-2 and JAK-1 (*6*, *7*). One downstream effect of JAK activation is the phosphorylation of the signal transducer and activator of transcription (STAT) proteins. Phosphorylated STAT proteins then translocate into the nucleus to regulate the transcription of specific genes (*6*, *7*). In addition to the STAT pathway, type I IFNs also activate mitogen-activated protein kinase (MAPK) family (*8*, *9*).

In many cell types, members of the MAPK family are key regulators of cell proliferation, differentiation and apoptosis. This family of proteins includes extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 (*10*, *11*). Recently it was reported that IFN-α inhibits the cell growth of chronic myelogenous leukaemia (CML) cells by activating p38 (*9*). This prompted us to study whether IFN-α inhibits cell proliferation of T-cell leukaemia line Jurkat, a malignancy established from human acute lymphoblastic leukaemia, by regulating MAPK family proteins. Jurkat cells possess the ability for unlimited proliferation used to study T-ALL (*12*, *13*). Our studies establish that the pharmacological inhibition of p38 with p38 inhibitor SB203580 reverses the suppressive effects of IFN-α on Jurkat cells, indicating that activation of this signalling cascade is important for anti-T-cell leukaemic effects of IFN-α. In other studies, we also identify the p38α and p38β of p38 isoforms as key modulators in IFN-α-inhibited cell proliferation of Jurkat cells. Taken together, our studies suggest that the inhibitory effect of IFN-α on the activation of the p38 pathway plays a critical role in the inhibition of T-cell leukaemia.

Materials and Methods

Cell culture, reagents and antibodies

The T-ALL cell line Jurkat was cultured in a RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine,

100 U/ml penicillin and 100 μg/ml streptomycin. Recombinant IFN-α was purchased from R&D (Minneapolis, MN, USA). PD98059, SB203580 and SP600125 were purchased from Calbiochem (La Jolla, CA, USA). An antibody specific to Flag (M2) was purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-α-tubulin antibody (N 356) was purchased from Amersham Pharmacia Biotech (San Francisco, CA, USA).

Trypan blue exclusion assay

Cells were cultured in the indicated medium at a density of 1×10^5 cells/ml. To analyse proliferation activity, the number of viable cells was counted by trypan blue exclusion assay. Cells were stained with 0.2% trypan blue (Sigma-Aldrich) and cell counts were determined under a microscope at different time points.

Reverse transcriptase–polymerase chain reaction analysis

Total RNA was isolated from Jurkat cells using the Trizol reagent (Life Technology, Grand Island, NY, USA) according to the manufacturer's instructions. After purification, 1 μg of RNA was reverse transcribed at 42°C for 60 min with the primer, oligo dT_{18}, followed by enzyme inactivation at 70°C for 15 min. The resulting cDNA samples were amplified by PCR, using the following primers: p38α sense strand 5′-GTGCCCGAGCGTTACCAGACC-3′ and antisense strand 5′-CTGTAAGCTTCTGACATTTC-3′, which generates an ~313-bp product; p38β sense strand 5′-CACCCAGCCCT GAGGTTCT-3′ and antisense strand 5′-AATCTCCAGGCTGCCA GG-3′, which generates an ~365-bp product; β-actin sense strand 5′-GCATCCCCCAAAGTTCACAA-3′ and antisense strand 5′-AG GACTGGGCCATTCTCCTT-3′, which generates an ~150-bp product as the internal control. The PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide and photographed under ultraviolet light.

p38αAF and p38βAF stable clone generations

To establish Jurkat cell lines that stably overexpress the dominant-negative forms (AF) of p38α and p38β, Jurkat cells were transfected with an empty vector, pcDNA3, or pcDNA3 containing Flag-tagged inserts of p38αAF or p38β2AF by lipofection. Twenty-four hours after transfection, cells were diluted to ~1 cell/well in 96-well dishes and selected with 800 μg/ml G418. The stable clones derived from single cells were screened for the expression of Flag-p38αAF or Flag-p38βAF fusion protein by western blot analysis using an anti-Flag antibody. pcDNA3-Flag-p38αAF and pcDNA3-Flag-p38β2AF were kindly provided by Dr Jiahuai Han (*14*, *15*).

Western blot analysis

Total cell extracts were prepared as described (*16*). Protein lysate (50 μg) was resolved using sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) and then probed with primary antibodies. After binding with horseradish peroxidase-conjugated secondary antibodies, the blots were visualized with an enhanced chemiluminescence (ECL) detection system (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA).

In vitro *kinase assay*

The p38 *in vitro* kinase assay was performed using a p38 kinase assay kit (New England BioLabs, Beverly, MA, USA). Briefly, total cell extracts were prepared as described (*17*), and p38 kinases were immunoprecipitated with an immobilized phospho-p38 or Flag monoclonal antibody. After washing twice with lysis buffer and twice with kinase buffer [25 mM Tris–HCl (pH 7.5), 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4 and 10 mM $MgCl_2$], the immunoprecipitates were assayed for p38 kinase activity in a kinase buffer with 200 mM ATP and 2 mg of ATF2 fusion protein per reaction. The reaction was stopped with a SDS sample buffer and analysed by western blotting with a specific anti-phospho-ATF2 antibody. The pcDNA3-Flag-p38α and pcDNA3-Flag-p38β2 plasmids were kindly provided by Dr Jiahuai Han (*14*, *15*).

Knockdown of p38α and p38β by small interference RNAs

Jurkat cells (1×10^6 cells) were transfected with 1 μg of control small interference RNA (siRNA), siRNA-p38α or siRNA-p38β. After 48 h, reverse transcriptase–polymerase chain reaction (RT–PCR) was performed. To analyse proliferation activity, Jurkat cells (1×10^6 cells) were transfected with 1 μg of siRNA and treated without or with IFN-α. The cells were incubated for 72 h and then trypan blue exclusion assay was performed. The siRNA-p38α and siRNA-p38β as well as a control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analysis

Qualitative data are presented as the mean and standard error of the mean (SEM). Statistically significant differences between groups were analysed with Student's *t*-test. A P value of <0.05 was considered significant.

Results

IFN-α inhibited cell proliferation of Jurkat cells in a dose- and time-dependent manner

In the present study, we used the T-ALL cell line Jurkat to investigate the signal transduction mechanism after treatment with IFN-α. We first studied the effect of IFN-α on cell proliferation, examining the proliferation activity of Jurkat cells cultured with various concentrations of IFN-α for 72 h. As shown in Fig. 1, IFN-α reduced the number of viable cells in a dose-dependent (Fig. 1A) and time-dependent manner (Fig. 1B).

IFN-α inhibited cell proliferation of Jurkat cells by the p38 pathway

MAPK signalling pathways are pivotal for cell activation and proliferation. To examine the role of MAPK signal pathways in the cell proliferation of Jurkat cells treated with IFN-α, we used specific inhibitors against p38, ERK1/2 and JNK. The p38 inhibitor, SB203580,

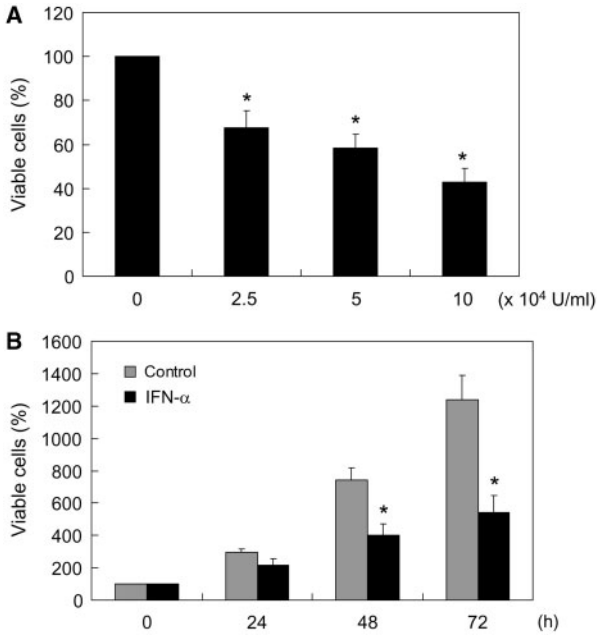

Fig. 1 IFN-α inhibited the growth of Jurkat cells. (A) Growth inhibition of Jurkat cells by IFN-α was assessed by a trypan blue exclusion assay after exposure to the indicated concentrations of IFN-α for 72 h (B) and to 10×10^4 U IFN-α for up to 72 h. Data represent the mean ± SEM of experiments in quadruplicate. *$P < 0.05$ comparing IFN-α treatments to the untreated control.

eliminated IFN-α effects of antiproliferation in Jurkat cells (Fig. 2A). As shown in Fig. 2B and C, neither the ERK1/2 inhibitor, PD98059, nor JNK inhibitor, SP600125, affected IFN-α-inhibited cell proliferation of Jurkat cells compared with IFN-α treatment alone.

IFN-α inhibited cell proliferation of Jurkat cells by both p38α and p38β

The specific p38 inhibitor, SB203580, targeted both the p38α and p38β isoforms of p38 proteins (*18*, *19*). Jurkat cells expressed both p38α and p38β genes according to the RT–PCR analysis (Fig. 3A). To determine whether IFN-α induces the activation of p38α and p38β isoforms, Jurkat cells were transiently transfected with plasmid encoding Flag-tagged p38α or p38β and treated without or with IFN-α for 72 h. To monitor the kinase activity of each p38 isoform, expressed p38 was immunoprecipitated using an anti-Flag antibody. The ability of the active p38 to phosphorylate its substrate, ATF2, was then examined by a kinase assay and the kinase product was detected by western blot with phospho-ATF2 specific antibody (P-ATF2). The results showed that both p38α and p38β activities increased after IFN-α treatment (Fig. 3B). We assessed which p38 isoforms, p38α and p38β, were involved in the IFN-α inhibition of Jurkat cell proliferation. To approach this, Jurkat cells were stably transfected with an empty vector (Jurkat/mock) or vectors encoding the dominant-negative p38α and p38β isoforms (AF) (Jurkat/p38αAF and Jurkat/p38β2AF).

The AFs are p38 mutants that cannot be phosphorylated since Thr-Gly-Tyr (TGY) dual phosphorylation sites have been changed to AGF (*20*). The expression level of two AF forms of Flag-tagged p38 proteins were detected in stable cell lines by western blot analysis using a Flag antibody (Fig. 4A). To confirm

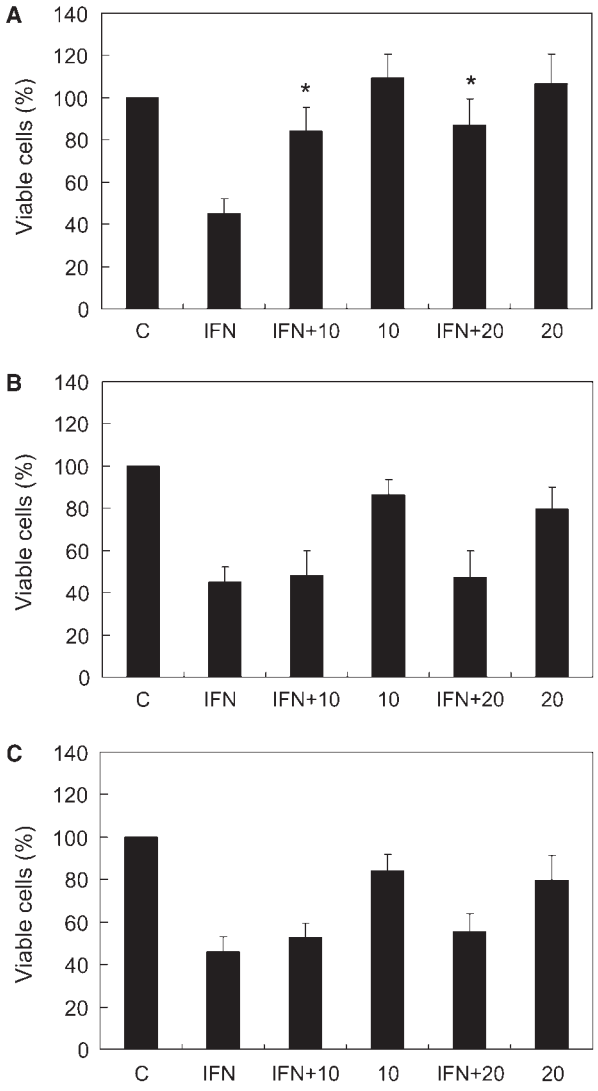

Fig. 2 Inhibition of the p38 pathway eliminated the antiproliferative effects of IFN-α in Jurkat cells. Either SB203580 (A), PD98059 (B) or SP600125 (C) was added 30 min before 10×10^4 U IFN-α was added. The numbers 10 and 20 refer to 10 and 20 μM inhibitors. Cells were analysed to determine viability with a trypan blue exclusion assay at 72 h. Cell numbers are expressed relative to untreated cells (100%). The data are the mean of four independent experiments. The difference between the effect of IFN-α and that of IFN-α plus the inhibitor was statistically significant ($P < 0.05$).

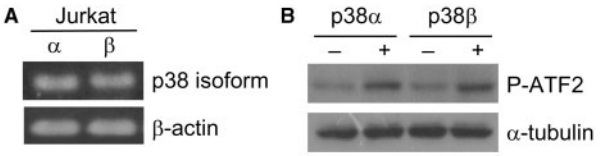

Fig. 3 p38α and p38β are the IFN-α-responsive p38 isoforms in Jurkat cells. (A) p38α and p38β mRNA expressions in Jurkat cells. Total RNA was amplified using RT–PCR. Each PCR included primers specific for the p38α and p38β and primer for β-actin as an internal control. (B) Jurkat cells were transfected with plasmids encoding Flag-tagged p38α or p38β and treated without (–) or with (+) 10×10^4 U IFN-α for 72 h. Cell lysates were immunoprecipitated with anti-Flag antibody. The immunoprecipitates were then subjected to *in vitro* kinase assay for p38 as described in 'Materials and Methods' section. Phospho-ATF2 (P-ATF2) is the product of the kinase reaction determined by western blotting using anti-phospho-ATF2 antibody. The α-tubulin level was monitored as a loading control.

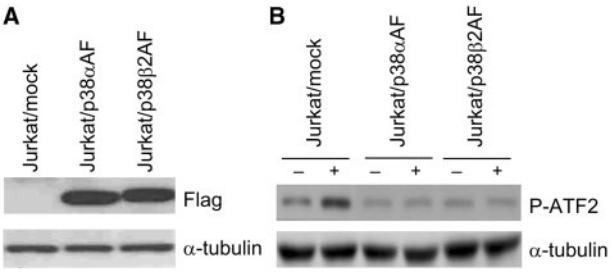

Fig. 4 Overexpression of the dominant-negative p38α and p38β isoforms, p38αAF and p38β2AF, in separate Jurkat stable cell lines. (A) Protein cell lysates from each Jurkat stable cell line were separated on SDS–PAGE. Protein levels of different p38 isoforms were detected by western blotting using an anti-Flag antibody. The same membrane was stripped and reblotted with an anti-α-tubulin antibody. (B) Cells were treated without (–) or with (+) 10×10^4 U IFN-α for 72 h. Cell lysates were immunoprecipitated with an anti-phospho-p38 antibody. The immunoprecipitates were then subjected to an *in vitro* kinase assay for p38 as described in 'Materials and Methods' section. Phospho-ATF2 (P-ATF2) is the product of the kinase reaction of p38 kinase. The α-tubulin level was monitored as a loading control.

the inactive kinase activity of p38 (AF) mutants, cells were treated without or with IFN-α for 72 h. Then, cell extracts were prepared and p38 was immunoprecipitated using an anti-phospho-p38 antibody. The precipitated kinase was assayed for its ability to phosphorylate its substrate protein, ATF2. The kinase activity of p38 was induced by IFN-α in Jurkat/mock cells, indicating that p38 was activated after IFN-α stimulation. In contrast, p38αAF and p38β2AF blocked IFN-α-induced activation of p38 in Jurkat/p38αAF and Jurkat/p38β2AF cells (Fig. 4B). Taken together, the above data confirm that p38αAF and p38β2AF are inactive kinases expressed in Jurkat cells that are able to block the p38 pathway.

We further examined the roles of p38α and p38β in the IFN-α-inhibited cell proliferation of Jurkat cells. As shown in Fig. 5, with cell proliferation efficiency without IFN-α set to 100%, IFN-α inhibited cell proliferation of Jurkat and Jurkat/mock cells by ~40–50%. In Jurkat/p38αAF and Jurkat/p38β2AF cells, the antiproliferative effects of IFN-α were eliminated despite the separate overexpression of p38αAF and p38β2AF ($*P<0.05$) (Fig. 5).

To further address the roles of p38α and p38β in IFN-α-mediated growth inhibition, siRNAs specific for p38α or p38β was used to knock down p38α and p38β expressions in Jurkat cells. Knockdown of p38α or p38β expressions were demonstrated by suppression of p38α or p38β mRNA expressions in Jurkat cells (Fig. 6A). Either p38α or p38β knockdowns reduced IFN-α-mediated growth inhibition ($P<0.05$) (Fig. 6B). The results suggest that the inhibition of Jurkat cell proliferation by IFN-α involves both p38α and p38β.

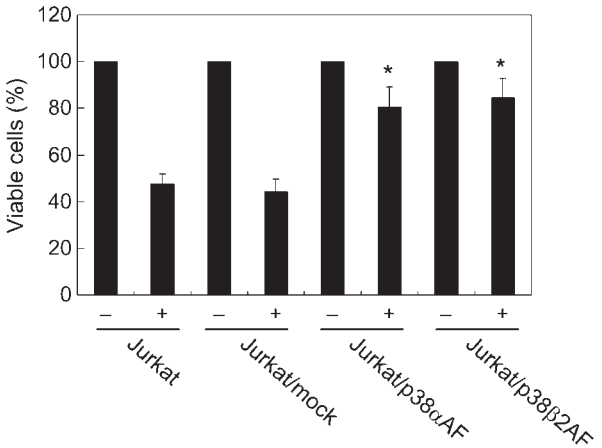

Fig. 5 The IFN-α-mediated antiproliferative effect was eliminated in Jurkat cell lines expressing the dominant-negative p38 mutants, p38αAF and p38β2AF. The different Jurkat stable cell lines in the presence (+) or absence (−) of 10×10^4 U IFN-α were analysed from four independent experiments. Cells were analysed to determine their viability with a trypan blue exclusion assay at 72 h. The results are presented as the mean ± SEM, and are shown as a percentage of the untreated condition. $*P<0.05$ comparing Jurkat/p38αAF + IFN-α or Jurkat/p38β2AF + IFN-α to Jurkat + IFN-α or Jurkat/mock + IFN-α.

Discussion

It is known that IFN-α is a regulatory factor which inhibits cell growth in T-ALL (*5*); hence, it is critical to characterize its intracellular signalling mechanisms. In this study, our data provide evidence that IFN-α inhibits cell growth by activating the p38 pathway in the T-ALL cell line, Jurkat cells. Furthermore, the p38 isoforms, p38α and p38β, play critical roles in the growth inhibitory effect of IFN-α in Jurkat cells. This was revealed by the finding that the pharmacological inhibition and the dominant-negative mutants or siRNAs of p38 eliminated the biological effects of IFN-α in IFN-α-sensitive Jurkat cells.

IFN-α not only plays a suppressive role in the haematological tumour cells but also in normal haematopoiesis (*21–25*). Previous studies also showed that p38 played an important role in the antiproliferative effects of IFN-α on normal erythroid and myeloid progenitors (*26*). Therefore, the p38 pathway may participate in antileukaemic and antihaematopoietic effects of IFN-α.p38 activation is well known for mediating cell growth inhibition. The p38 subfamily is generally activated in response to environmental stresses (*14*, *27*, *28*). Depending on the context, four isoforms of the p38 subfamily (p38α, p38β, p38γ and

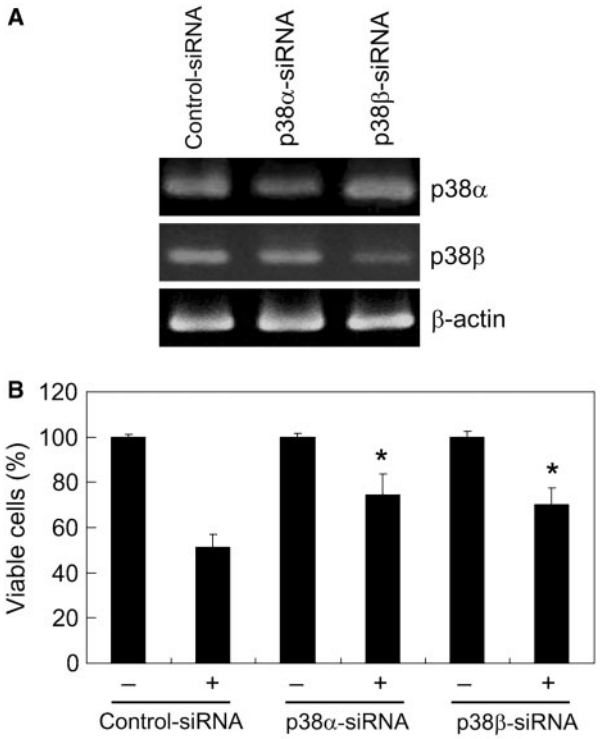

Fig. 6 Knockdown of p38α or p38β expression reduced IFN-α-mediated growth inhibition. (A) Jurkat cells were transfected with control siRNA or siRNA specifically directed against p38α or p38β. After 48 h, p38α or p38β expressions were analysed by RT–PCR. β-actin was used as a loading control. (B) Jurkat cells were transfected with siRNAs and treated without (−) or with (+) 10×10^4 U IFN-α for 72 h. Cells were analysed to determine their viability with a trypan blue exclusion assay. The results are presented as the mean ± SEM from three different experiments, and are shown as a percentage of the untreated condition. $*P < 0.05$ comparing Jurkat/p38α-siRNA + IFN-α or Jurkat/p38β2-siRNA + IFN-α to Jurkat/control siRNA + IFN-α.

p38δ) have different roles within cells (*29*). The SB203580 inhibitor of the p38 pathway used in our study and others has been previously shown to exhibit specificity for p38 (*17*, *18*). It acts by binding to the ATP site and inhibiting kinase activity of p38 (*30*). SB203580 targets both the p38α and p38β isoforms that have verified the important roles of p38 proteins in many cell activities (*31*–*33*). Using this approach, we observed that SB203580 eliminated the antiproliferative effect of IFN-α, which suggests that this IFN-α response occurs via a p38-dependent pathway in Jurkat cells. These results are consistent with a previous report on p38 involvement in which treatment with SB203580 leads to the inhibition of antileukaemic activity of IFN-α in CML cells (*9*). Recent findings indicate that SB203580 directly inhibits thromboxane synthase and cyclooxygenases-1 and -2; however, such instances may not be specifically caused from p38 kinase inhibition (*34*). To interfere with this pathway, we therefore utilized an alternative approach to overexpress dominant-negative mutants of p38 isoforms, p38αAF and p38βAF or to knock down p38α and p38β expressions. The results showed that each of the two isoforms of the p38 subfamily member contributed to the inhibitory effect of IFN-α on Jurkat cell proliferation.

Including the MAPK pathway, IFN-α activates various other pathways such as JAK–STAT pathway. It will be interesting to understand whether other pathways are involved in IFN-α-mediated cell growth inhibition.

Several previous studies demonstrated the effectiveness of treatment of adult T-cell leukaemia/lymphoma (ATLL), a malignancy of peripheral T lymphocytes caused by human T-cell leukaemia/lymphoma virus type-1 (HTLV-1) with IFN-α (*35*, *36*). A combination of IFN-α and other drugs increases survival rate *in vivo* (*35*) and induces apoptosis in HTLV-1-infected cells *in vitro* (*36*), which suggests that other drugs can enhance the IFN-α's effects. Our data showed that the effects of IFN-α are weak in Jurkat cells, yielding about 40–50% inhibition. Therefore, further investigation is necessary to determine whether other drugs synergize with IFN-α in inducing Jurkat cell growth inhibition. Of note, however, our data showed that the IFN-α treatment of Jurkat cells decreased the number of viable cells. Clarification is required whether cell apoptosis was increased or cell cycle progression was arrested.

In summary, we report for the first time that with participation of p38α and p38β isoforms, IFN-α can inhibit proliferation activity of the T-cell leukaemia cell line, Jurkat.

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Conflict of interest

None declared.

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