

Type I IFN induced IL1-Ra expression in hepatocytes is mediated by activating STAT6 through the formation of STAT2 : STAT6 heterodimer

Lei Wan ^{a, b, c, *}, Cheng-Wen Lin ^d, Ying-Ju Lin ^{c, e}, Jim J.C. Sheu ^{a, b}, Bing-Hung Chen ^f, Chiu-Chu Liao ^a, Yuhsin Tsai ^b, Wei-Yong Lin ^a, Chih-Ho Lai ^g, Fuu Jen Tsai ^{a, b, c, *}

^a Department of Medical Genetics and Medical Research, China Medical University Hospital, Taichung, Taiwan, Republic of China

^b Graduate Institute of Chinese Medical Science, China Medical University, Taichung, Taiwan, Republic of China

^c Department of Biotechnology and Bioinformatics, Asia University, Taichung, Taiwan, Republic of China

^d Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung, Taiwan, Republic of China

^e Graduate Institute of Acupuncture Science, China Medical University, Taichung, Taiwan, Republic of China

^f Faculty of Biotechnology, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China

^g Department of Microbiology, School of Medicine, China Medical University, Taichung, Taiwan, Republic of China

Received: July 30, 2007; Accepted: October 9, 2007

Abstract

The biological activities of type I interferons (IFNs) are mediated by their binding to a heterodimer receptor complex (IFNAR1 and IFNAR2), resulting in the activation of the JAK (JAK1 and TYK2)-STAT (1, 2, 3, 5 iso-types) signalling pathway. Although several studies have indicated that IFN- α and IFN- β can activate complexes containing STAT6, the biological role of this activation is still unknown. We found that exposure of hepatoma cells (HuH7 and Hep3B) to IFN- α or IFN- β led to the activation of STAT6. Activated STAT6 in turn induced the formation of STAT2:STAT6 complexes, which led to the secretion of IL-1Ra. The activation of STAT6 by type I IFN in hepatocytes was mediated by JAK1 and Tyk2. In addition, IFN- α or IFN- β significantly enhanced the stimulatory effect of IL-1 β on production of IL-1Ra. The present study suggests a novel function of IFN- α and IFN- β signalling in human hepatocytes. Our results provide evidence for the mechanism how IFN- α and IFN- β modulate inflammatory responses through activation of STAT6 and production of secreted IL-1Ra.

Keywords: type I interferon • STAT6 • STAT2 • IL-1Ra

Introduction

Interferons (IFN) are natural defence factors in vertebrates. This family of cytokines regulates antiviral,

anti-tumour and immunomodulatory activities [1]. There are two main types of IFNs: type I IFN, which includes IFN α and IFN β and type II, which comprises IFN γ . It is known that IFN α is mainly produced by certain leukocytes (such as dendritic cells and macrophages), IFN β by epithelial cells and fibroblasts and IFN γ by T cells [2]. Due to their multiple biofunctions, recombinant IFN α s have been approved in over 40 countries for treating more than 14 malignancies

*Correspondence to: Lei WAN, PhD. and Fuu Jen Tsai, MD, PhD.

Department of Medical Genetics and Medical Research, China Medical University Hospital, No. 2 Yuh-Der Road, Taichung, Taiwan.

Tel.: +88 6-4-22 05 21 21 Ext 20 07;

Fax: +88 6-4-22 03 32 95

E-mail: leiwan@www.cmuh.org.tw

and several virologic diseases [1]. Most notably, IFN α s are used in combination with chemotherapy to treat metastatic melanoma, condyloma acuminata, Kaposi's sarcoma and hepatitis B and C [3–5].

Type I IFN signalling in treated cells is mediated by dimerization of their receptors, IFNAR1 and IFNAR2. Janus kinases TYK2 and JAK1 are the key players in type I IFN signal cascades which can be activated by tyrosine auto-phosphorylation in response to receptor assembly and later phosphorylate the receptors [2, 6]. Biochemical studies have demonstrated that TYK2 and JAK1 constitutively and directly associate with IFNAR1 [2, 7, 8] and IFNAR2 [9], respectively. Accordingly, activated TYK2 and JAK1 can further recruit and activate the signal transducer and activator of transcription (STAT) proteins at the phosphotyrosine sites in the cytoplasmic tail of the receptor. Activated STATs, with a phosphorylated tyrosine at the carboxyl terminal portion, then form either homodimers or heterodimers, and translocate into the nucleus. IFN- α and IFN- β primarily activates STAT1, STAT2 and STAT3, and in certain cells can also activate STAT5 and STAT6 [2, 10–13]. After translocation into the nucleus, the STAT1:STAT2 heterodimer will associate with IFN regulatory factor 9 (IRF9, p48) to form a STAT1: STAT2: IRF9 trimer, also known as ISGF3-(interferon-stimulated gene factor 3); this trimer then binds to cis-acting IFN-stimulated response elements (ISREs) to induce transcription of many IFN-stimulated genes (ISGs). The homodimers STAT1:STAT1 and STAT3:STAT3, and the heterodimer STAT1:STAT3 bind to IFN- γ -activated sequence elements (GAS) directly in response to type I IFN stimulation [2, 14, 15].

IFN α treatment can interrupt virus propagation and has been shown to be very efficient in treating HBV and HCV patients. Recent studies further indicate that IFN not only contains anti-viral activities, but also modulates immune responses and cytokine networking. For example, IFN α has been shown to affect the balance between Th1/Th2 cytokines *in vivo* and *in vitro* [16–18]. In addition, IFN α stimulated the production of interleukin-1 receptor antagonist (IL-1Ra) *in vitro* [19, 20], which was also confirmed clinically in patients [21–23]. IL-1Ra has been characterized as an acute-phase protein [24–26] that contributes to both initiation and modulation of inflammatory responses. Maintenance of the balance between IL-1 and IL-1Ra is a key factor in preventing the development and progression of inflammatory diseases.

Although there is abundant evidence to suggest that IFN therapy is beneficial in the treatment of disease, the molecular mechanisms by which IFN stimulates the secretion of IL-1Ra remains poorly defined. In this study, we have found that exposure of hepatocytes to IFN- α or IFN- β led to the activation of STAT6, in addition to the activation of STAT1 and STAT2. Activated STAT6 in turn stimulated the formation of STAT2:STAT6 complexes, which led to the production of secreted IL-1Ra in hepatocytes.

Materials and methods

Reagents and cells

DMEM, Dulbecco's phosphate buffered saline (PBS), L-glutamine and antibiotic were obtained from Invitrogen. Foetal bovine serum (FBS) was purchased from Hyclone. Protein assay reagents were obtained from Bio-Rad Laboratories. X-tremeGENE siRNA transfection reagent was purchased from Roche Applied Science. The following antibodies were used: STAT1, STAT2, STAT3, STAT5, STAT6 and Tyk2 were from BD Transduction Laboratories; p-STAT1 (Tyr 701), p-STAT2 (Tyr 690), p-STAT3 (Tyr705), p-STAT5 (Tyr694), p-STAT6 (Tyr 641), JAK1 and β -actin were from Cell signalling Technology. For Chromatin immunoprecipitation (ChIP), the anti-STAT6 and anti-GAPDH were from Santa Cruz Biotechnology. Protease inhibitor cocktail for use with mammalian cell and tissue extracts and Phosphatase cocktail I were purchased from Sigma-Aldrich. Recombinant IFN α 2 was from Hoffmann-La Roche and recombinant IFN β was from Merck-Serono. IL-4 and IL-1 β were purchase from MDbio, Inc. The HuH7 (JCRB0403, Japanese Collection of Research Bioresources (JCRB), Osaka, Japan) and Hep3B (BCRC60434, Bioresource Collection and Research Center, HsinChu, Taiwan) hepatoma cell lines were maintained as adherent cultures in DMEM plus 10% FBS. Kinase inhibitors (JAK1: Pyridone 6; JAK3: WHI-p131) were obtained from Merck-Calbiochem.

Western blotting

Western blotting was performed on HuH-7 or Hep3B hepatoma cells which had been starved overnight in serum free medium and stimulated with IFN- α or IFN- β at a concentration of 400 IU/ml. Cells were then harvested, washed with 10 ml of PBS three times, and lysed with M-PER (Pierce) containing phosphatase inhibitor and protease inhibitor. Proteins were separated on a 7.5% SDS-PAGE gel

and blotted onto a polyvinylidene difluoride membrane. For visualization, membranes were probed with a species-specific secondary antibody conjugated to horseradish peroxidase; binding was detected by SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer's instructions (Pierce). To monitor protein loading on some of the immunoblots, membranes were stripped and re-probed with appropriate antibodies. Blots shown are representative of at least three individual experiments.

Immunoprecipitation

Immunoprecipitation was performed on HuH7 cells which had been starved overnight in serum free medium, and stimulated with IFN- α or IFN- β at a concentration of 400 IU/ml or with IL-4 at a concentration of 10 ng/ml. Cells were washed with 10 ml of PBS three times and lysed with M-PER (Pierce) containing a protease inhibitor cocktail. Cell debris was removed by centrifugation. The lysates were then incubated with appropriate antibodies and Protein-A-Sepharose beads (Sigma). The antibody-protein complexes were collected by centrifugation, washed, eluted by boiling in sample buffer, and immunoblotted as described above.

Preparation of nuclear extracts and electrophoretic mobility shift assay

Nuclear extracts of HuH7 cells were prepared with a NE-PER nuclear extraction reagent (Pierce). IL-1Ra promoter STAT binding element 1 (IL-1Ra-SBE1): 5'-GCTCTTCTCCAGGAACTCAATG-3' and CD23b-GAS: 5'-GGGTGAATTTCTAAGAAAGGGACTA-3' were end-labelled by a biotin 3' end DNA labelling kit (Pierce). The binding reactions contained 3 μ l NE-PER nuclear extract protein, buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.05% Nonidet P-40, and 2.5% glycerol), 1 μ g of poly(dI-dC), and 2 nM of biotin-labelled DNA. The reactions were incubated at room temperature for 20 min. The reactions were electrophoresed on a 6% Tris-borate- ethylenediaminetetraacetic acid (EDTA) gel at 100 V for 1 hr in a 0.5x Tris-borate-EDTA buffer, and then transferred to a nylon membrane. The biotin-labelled DNA was detected with the LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce).

Chromatin immunoprecipitation

ChIP assays were according to Weinmann and Farnham (2002) [27] with modifications. A real-time PCR detection method was developed to avoid the use of isotope. Purified

immunoprecipitated DNA was analysed in 20 μ l PCR reactions and detected by Roche LightCycler 480 instrument using Taqman probe system. For IL1-Ra promoter, DNA fragments of 64 bp were generated by primer pairs IL1-Ra-F: 5'-gtatttcgcttctcgcagt-3' and IL1-Ra-R: 5'-aaagaaatagtcactaccaagc-3' and the detection of IL-1Ra promoter was using Roche Universal Probe 87. Cycling parameters were 95°C for 5 min, 45 cycles at 95°C for 10 sec, 50°C for 20 sec, 72°C for 1sec.

Gene knockdown using RNAi

STAT2, STAT6, JAK1 and Tyk2 were silenced by the validated Stealth™ RNAi DuoPak. The small interfering RNA (siRNA) sequences were as follows: STAT2: 1) 5'-GCAGUGGGAAAUGCUGCAGAAUCUU-3'; 2) 5'-GCAGGAUGUCUUCUGCUUCCGAUUAU-3'; STAT6: 1) 5'-GGGAGAAGAUGUGUGAAACUCUGAA-3'; 2) 5'-GAAUCCGGGAUCUUGCUCAGCUCAA-3'; JAK1: 1) 5'-GCACAGAAGACGGAGAAAUGGUUAU-3'; 2) 5'-GCCUUAAGGAAUAUCUCCAAAGAA-3'; Tyk2; 1) 5'-CCCAGAGAUGCAAGCCUGAUGCUAU-3'; 2) 5'-CCAUUCUGAAGACAGUCCAUGAGAA-3'. HuH7 cells were transfected with siRNA using X-tremeGENE siRNA transfection reagent. Briefly, 40 nM of siRNAs (*i.e.* 20nM of each siRNA) was diluted in 200 ml serum-free DMEM and mixed with 10 ml X-tremeGENE siRNA transfection reagent which had been diluted in 200 ml serum-free DMEM. The complexes were incubated at room temperature for 15 min. The mixture was added to 2 x 10⁵ HuH7 seeded in 6-well plates containing 1 ml opti-MEM (Invitrogen). Control cells were transfected with Stealth™ RNAi negative control. After incubating the cells at 37°C, 5% CO₂ for 12 hrs, siRNA complexes were washed out and replaced with DMEM containing 10% FBS. After 72 hrs post-transfection, transfected cells were harvested, and their ability to produce secreted IL-1Ra upon interferon treatment or the influence on the phosphorylation of STAT1, STAT2 and STAT6 was assessed. Gene knockdown was ascertained by Western blotting as described above, using specific antibodies and an anti- β -actin antibody as a loading control.

IL-1Ra enzyme-linked immunosorbent assay (ELISA)

HuH7 cells were seeded at 10⁴ cells/well in 96-well plates and transfected with STAT2, STAT6 or Stealth™ RNAi negative control. After 24 hrs post-transfection, cells were stimulated with 400 IU/ml IFN α , 400 IU/ml IFN β , 10 ng/ml IL4, 1 ng/ml IL-1 β or the combination of IL4 or IFN with IL-1 β for 48 hrs. IL-1Ra concentration was measured in culture

supernatant using a RayBio[®] human IL-1Ra ELISA kit. The sensitivity of the assay was less than 100 pg/ml.

Statistical analysis

The protein intensities detected by Western blotting were quantified by the Science Lab 2003 Multi Gauge Ver. 2.2 (Fujifilm) and the unstimulated protein samples were defined as basal. The results are expressed in relative expression concentration over basal and represented as the mean \pm S.D. of three independent experiments. Independent t-tests were used for the paired comparisons and a *P* value less than 0.05 was considered significant.

Results

Type I IFN induced STAT6 phosphorylation in HuH7 and Hep3B cells

To study the signalling pathways induced by type I IFN in hepatocytes, HuH7 and Hep3B cells were treated with 400 IU/ml IFN α or IFN β . We cannot detect any growth inhibition activities in HuH7 and Hep3B cells when treated with up to 3000 IU/ml IFN- α or IFN- β (data not shown). Cells were then harvested at different time points and Western blotted to study the activation of STAT proteins after treatments. As shown in Fig. 1, both IFN- α and IFN- β were able to activate STAT1, STAT2 and STAT3 in HuH7 (Fig. 1A) and Hep3B (Fig. 1B) cells, which are common pathways involved in type I IFN signalling. Interestingly, STAT5 and STAT6 were also activated in response to IFN- α and IFN- β , although STAT5 activation was much weaker than STAT6. IFN β induced stronger STAT5 activation than IFN α in both cell lines tested (Fig. 1). The time course study revealed that STAT2 and STAT3 activation prolonged longer time than STAT1 and STAT6 activation by detecting the content of phosphorylated tyrosine. In general, IFN β treatment induced more acute and stronger effects on STAT proteins than IFN α (Fig. 1A and B). It is notable that STAT6 activation exhibited kinetic patterns similar to those of STAT1 in IFN- α or IFN- β treated HuH7 (Fig. 1A and C) and Hep3B (Fig. 1B and D) cells, indicating that STAT6 plays a significant role in hepatocytes in response to type I IFNs.

The activation of STAT6 is mediated by the formation of STAT2:STAT6 heterodimer

Since IFN α -induced STAT6 activation in lymphocytes is accompanied by the formation of STAT2:STAT6 complex [10–12], immunoprecipitation was performed to determine whether a similar mechanism exists in hepatocytes (Fig. 2A). STAT6 protein in IFN- α - or IFN- β -treated HuH7 cells was pulled down by anti-STAT6 antibody and the protein complex was subjected to Western blotting with anti-STAT2 antibody and *vice versa*. It is known that STAT6 activation is the primary signalling mechanism involved in the IL-4 pathway [10, 28–30]. Hence, IL-4 treated cells in this experiment were utilized as the positive control. The data in Fig. 2A indicate that STAT6 protein directly associated with STAT2 after IFN- α or IFN- β treatments; this was not the case for cells treated with PBS control. siRNA against STAT2 was introduced into the cells to down-regulate the expression level of STAT2 protein (Fig. 2B). This treatment significantly reduced the level of phosphorylation of STAT6 protein at Tyr 641 by IFN α as compared to control RNAi treated cells (Fig. 2C and D), indicating that the formation of the STAT2:STAT6 heterodimer is required for STAT6 activation induced by type I IFNs.

IFN- α - or IFN- β -induced phosphorylation of STAT6 is mediated by the JAK-STAT pathway

To determine the signalling cascade of STAT6 activation after type I IFN binding, a phosphorylation antibody array was performed to screen for receptor tyrosine kinases. Consistent with previous studies [2, 14], JAK1 and Tyk2 were signal transducers for IFN- α or IFN- β signalling in hepatocytes (Fig. 3A and B). Specific siRNAs were prepared and transfected into HuH7 cells to knockdown JAK1 and Tyk2 (Fig. 3B). However, neither JAK1 nor Tyk2 inhibition completely inhibited the phosphorylation levels of STAT1, STAT2 and STAT6 (Fig. 3C–E). When IFN- α - or IFN- β -treated cells were treated with a universal JAK kinase inhibitor (Pyridone 6) or with JAK1 + Tyk2 siRNAs, STAT6 phosphorylation was inhibited (Fig. 3G and F). As expected, treating cells with the

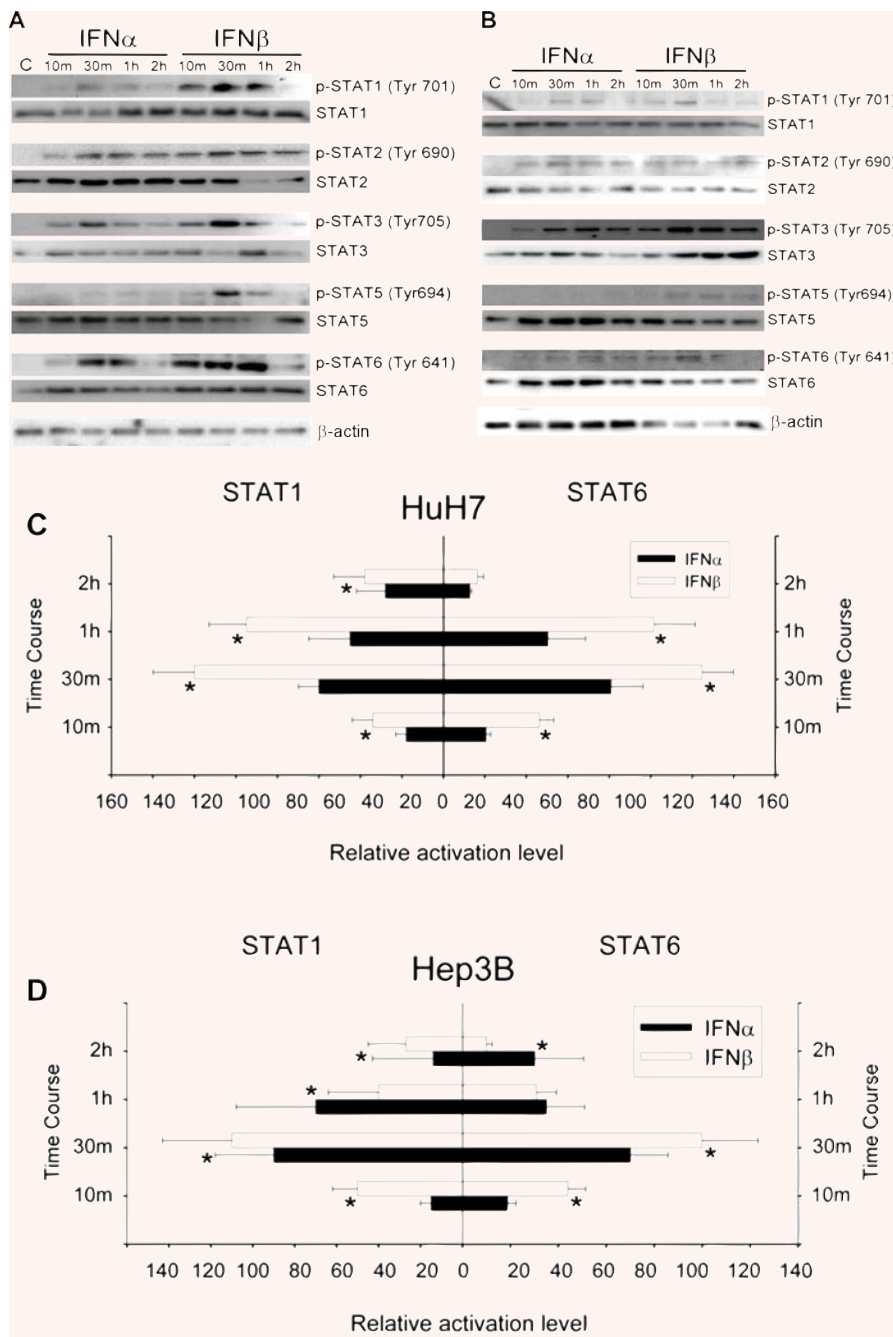


Fig. 1 STAT6 is tyrosine phosphorylated in response to IFN α and IFN β in HuH7 and Hep3B cells. **(A and B)** IFN- α and IFN- β were able to activate STAT1, STAT2, STAT3, STAT5 and STAT6 in HuH7 **(A)** and Hep3B **(B)** cells. HuH7 and Hep3B cells were either unstimulated or stimulated with 400 IU/ml IFN α or IFN β for the indicated time. 20 μ g cell extracts were resolved by 7.5% SDS-PAGE, and analysed by Western blotting using phosphoprotein specific antibodies (p-STAT1, p-STAT2, p-STAT3, p-STAT5, p-STAT6). The blot was later stripped and re-probed with STAT1, STAT2, STAT3, STAT5, STAT6 and β -actin antibodies to ensure equal loading of the cell extracts. **(C and D)** STAT6 activation exhibited similar kinetic patterns as STAT1 in IFN- α or β treated HuH7 **(C)** and Hep3B **(D)** cells. The amount of activated STAT1 and STAT6 of HuH7 **(C)** and Hep3B **(D)** cells treated with 400 IU/ml IFN α or IFN β was quantified and the results are expressed in relative expression level over basal; the results are represented as the mean \pm S.D. for three repetitions. Asterisks indicate the calculated P values for paired comparisons between IFN α and IFN β at various times; all were < 0.05 .

JAK3 inhibitor WHI-p131 did not influence the phosphorylation levels of STAT6 as compared to the non-relevant inhibitor control. These data indicate that both JAK1 and Tyk2 kinases were involved in IFN- α - or IFN- β -induced STAT6 activation in hepatocytes. These two kinases, therefore, may play redundant biofunctional roles, since down regulation of either of them did not block the signalling cascades.

Type I IFN induces activation of STAT6-triggered production of secreted IL-1Ra in HuH7 cells

To examine whether IFN-activated-STAT6 can bind to DNA fragments containing STAT6 binding element (SBE), an electrophoretic mobility shift assay was

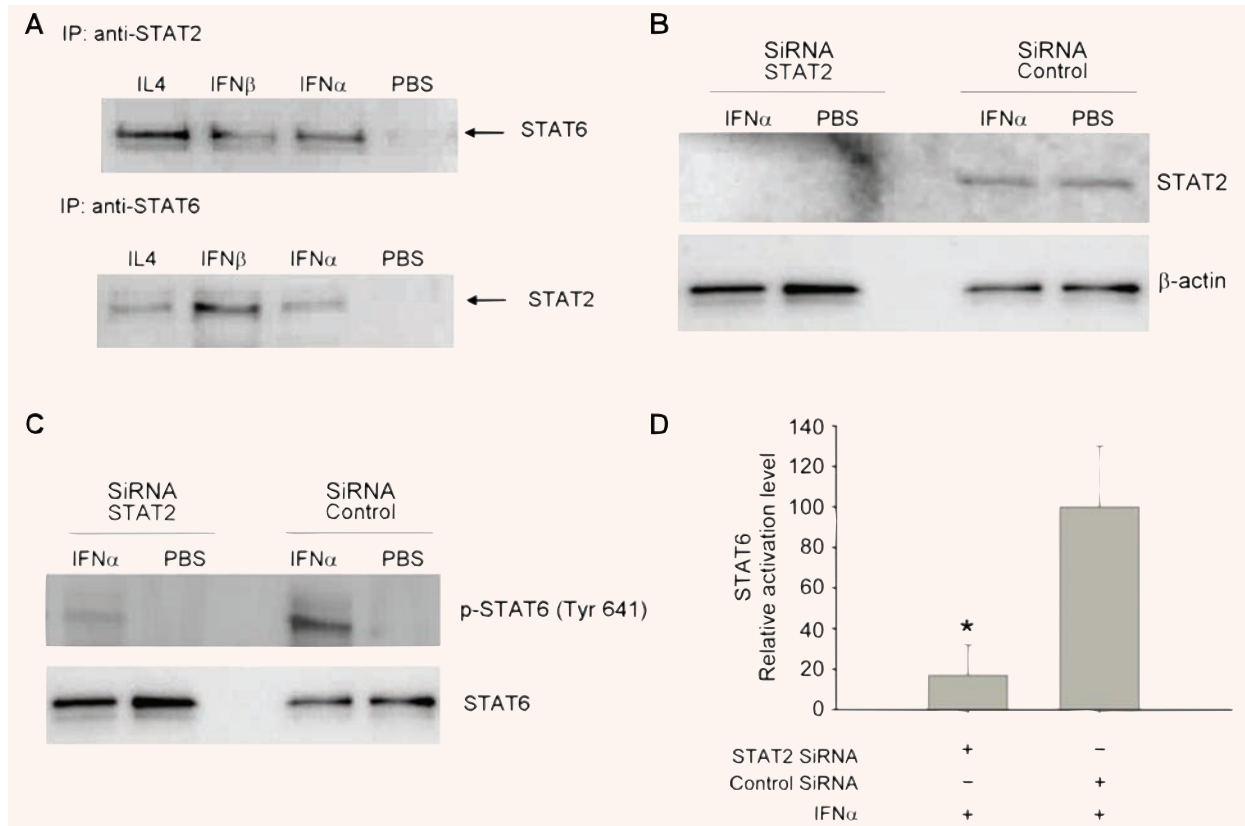
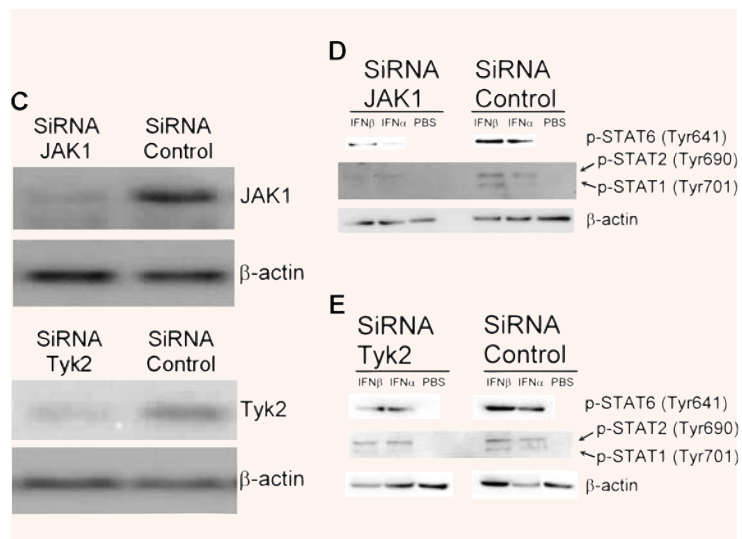
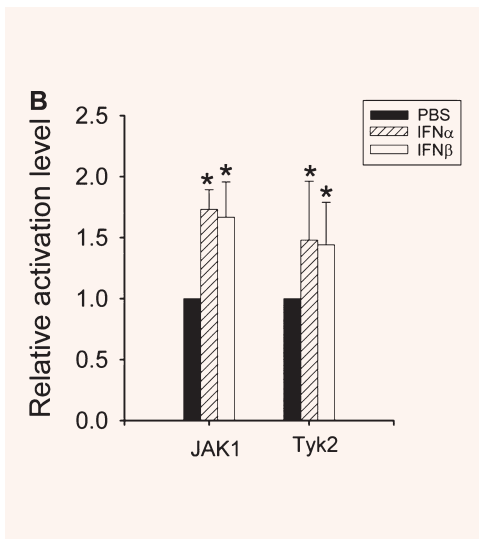
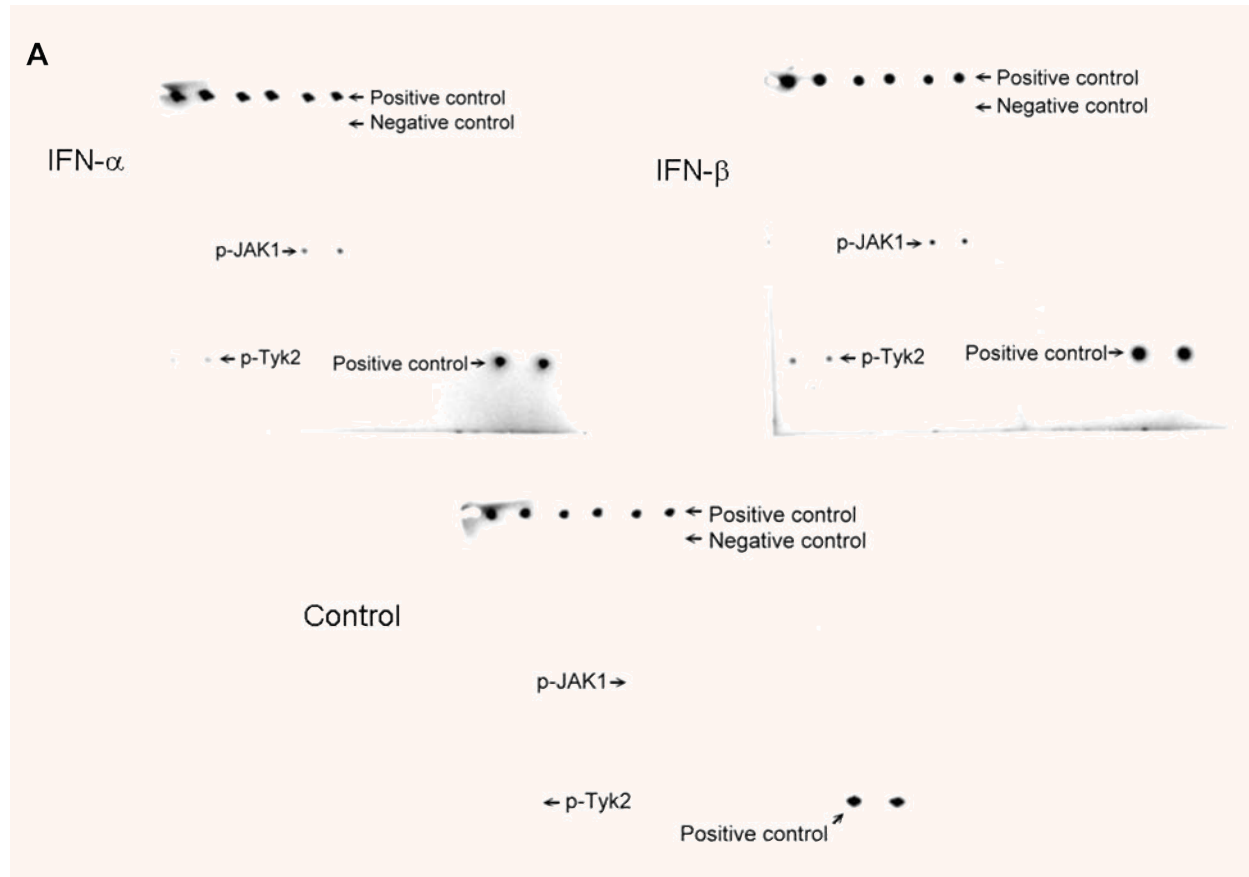


Fig. 2 The activation of STAT6 is mediated by the formation of STAT2:STAT6 heterodimer. **(A)** STAT6 formed a heterodimer with STAT2. HuH7 cells either were unstimulated (PBS) or were stimulated with IFN α (400 IU/ml), IFN β (400 IU/ml) and IL-4 (10 ng/ml) for 30 min. Extracts were then immunoprecipitated (IP) with either a STAT2 antibody or a STAT6 antibody, resolved by 7.5% SDS-PAGE, and analysed by Western blotting using either an anti-STAT6 (top panel) or an anti-STAT2 antibody. Extracts from IL-4-stimulated HuH7 cells were included as a control for STAT6 activation. **(B)** SiRNA against STAT2 was introduced into the cells to down-regulate the expression level of STAT2 protein. STAT2 inhibition was checked by Western blotting using anti-STAT2 antibody. The blot was later stripped and re-probed with β -actin antibody to ensure equal loading of the cell extracts. **(C)** STAT6 phosphorylation was inhibited by down-regulating STAT2. Extracts were prepared from HuH7 cells with or without a 30-min stimulation at 37°C with either PBS or IFN α (400 IU/ml). The activation of STAT6 after STAT2 knockdown was determined by Western blotting. The blot was later stripped and re-probed with STAT6 antibody to ensure equal loading of the cell extracts. **(D)** Phospho-STAT6 was quantified and the results are expressed in relative expression level over basal and represented as the mean \pm S.D. for three repetitions. Asterisks indicate the calculated *P* values for paired comparisons between IFN α and PBS; all were <0.05.

performed using nuclear extracts from HuH7 cells cultured in the presence of IFN α , IFN β and IL-4. Previously IL-4 was also shown to activate STAT6 in the treated cells, the nuclear extract from IL-4 treated cells was utilized as the positive control. DNA fragments containing CD23 promoter (located at bases -230 ~ 214 region) and IL-1Ra-SBE1 (bases -254 ~ -231), promoter regions previously identified as the binding elements for activated STAT6 in IL-4 treated cells, were mixed with these nuclear extracts. As shown in Fig. 4A, treatment with IFN α , IFN β and

IL-4 significantly enhanced STAT6 binding to CD23 promoter as compared to the PBS treatment control. We also found that IFN- α - or IFN- β -activated STAT6 was able to bind to IL-1Ra-SBE1 (IL-4 positive control) (Fig. 4B and C). Moreover, a ChIP experiment was also performed to confirm the binding of IFN-activated-STAT6 to IL-1Ra promoter. As shown in Fig. 4D, we can detect IL-1Ra promoter in IFN α and IFN β treated HuH7 cells' chromatin co-immunoprecipitated with anti-STAT6 antibody but not in chromatin co-immunoprecipitated with



anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody. The crossing point (Cp) value for anti-STAT6 antibody co-immunoprecipitated chromatin were 35.5, 25.55 and 24.82 of PBS-, IFN α - and IFN β -treated HuH7 cells.

To further confirm whether the binding of STAT6 to the promoter leads to activation of IL-1Ra gene transcription, the protein levels of IL-1Ra were measured from the culture media of the cells treated with IFN α , IFN β and IL-4, respectively. Compared with the PBS-treated control cells, IFN α , IFN β and IL-4 treatments of hepatocytes increased the production of IL-1Ra from 20 pg/ml to 92 pg/ml (IFN α), 260 pg/ml (IFN β) and 43 pg/ml (IL-4) (Fig. 5B).

It has been shown that IL-4 co-stimulated with IL-1 β has a synergistic effect on production of secreted IL-1Ra protein and mRNA in hepatocytes [31]. Accordingly, the IL-1Ra protein levels of IL-1 β -treated cells were measured with and without the combination of IFN α , IFN β and IL-4 (Fig. 5B). The results showed that IFN- α or IFN- β also significantly enhanced the stimulatory effect of IL-1 β on the production of secreted IL-1Ra (Fig. 5B).

The formation of the STAT2:STAT6 heterodimer was required for STAT6 activation (Fig. 2A) siRNA of STAT2 was transfected into HuH7 cells to test the production of secreted IL-1Ra. As shown in Figure 5C, the production of secreted IL-1Ra was reduced in HuH7 cells treated with IFN α or IFN β (data not shown) but not in those treated with IL4, IL-1 β or a combination of IL4 and IL-1 β . These data indicate that the activation of STAT6 depends on STAT2, which requires formation of the STAT2:STAT6 heterodimer. In addition, cells pre-treated with STAT6 siRNA secreted much less IL-1Ra (Fig. 5A and B). These data support that the production of secreted IL-1Ra was induced by activated STAT6 in response to type I IFNs treatments.

Discussion

It has been shown that IFN- α and β have pleiotropic effects on various target cells; however, the molecular mechanisms by which IFN- α and β exert such diverse effects have not been fully elucidated. IFN- α and β stimulate the cytolytic capacity and function of NK cells, the phagocytic functions of macrophages and their production of cytokines, and the expression of major histocompatibility complex molecules in



Fig. 3 IFN- α - or IFN- β -induced phosphorylation of STAT6 is mediated by the JAK-STAT pathway. (A) JAK1 and Tyk2 were signal transducers for IFN α or IFN β signalling in hepatocytes. A phosphorylation antibody array detecting relative levels of phosphorylation of 71 different receptor tyrosine kinases was incubated with extracts prepared from HuH7 cells; the extracts were either unstimulated (PBS), or stimulated with IFN α (400 IU/ml) or IFN β (400 IU/ml) for 30 min. (B) The normalized activation level of JAK kinases (JAK1 and Tyk2) is presented. (C) SiRNAs against JAK1 and Tyk2 were introduced into the cells to down-regulate their expression levels. JAK1 and Tyk2 inhibition were checked by Western blotting using anti-JAK1 and TYK2 antibodies. The blot was later stripped and re-probed with β -actin antibody to ensure equal loading of the cell extracts. (D and E) Neither JAK1 (D) nor Tyk2 (E) inhibition completely inhibited the phosphorylation levels of STAT2 and STAT6. Extracts were prepared from HuH7 cells with or without a 30-min stimulation at 37°C with PBS, IFN α (400 IU/ml), and IFN β (400 IU/ml). The activation of STAT2 and STAT6 by IFN α and IFN β after JAK1 and Tyk2 knockdown were determined by Western blotting. The blot was later stripped and re-probed with STAT2 or STAT6 antibodies to ensure equal loading of the cell extracts. (F) STAT2 and STAT6 activation was inhibited by both JAK1 and Tyk2 knockdown. (G) STAT6 activation was not inhibited by JAK3 inhibitor. HuH7 cells were pretreated with 50 μ M Pyridone 6, WHI-p131 or with DMSO as control for 6 hrs. The medium was replaced with fresh medium containing 400 IU/ml IFN α , IFN β or PBS as control for 30 min. STAT6 inhibition were checked by Western blotting using anti-phospho-STAT6 antibodies. The blot was later stripped and re-probed with STAT6 antibody to ensure equal loading of the cell extracts.

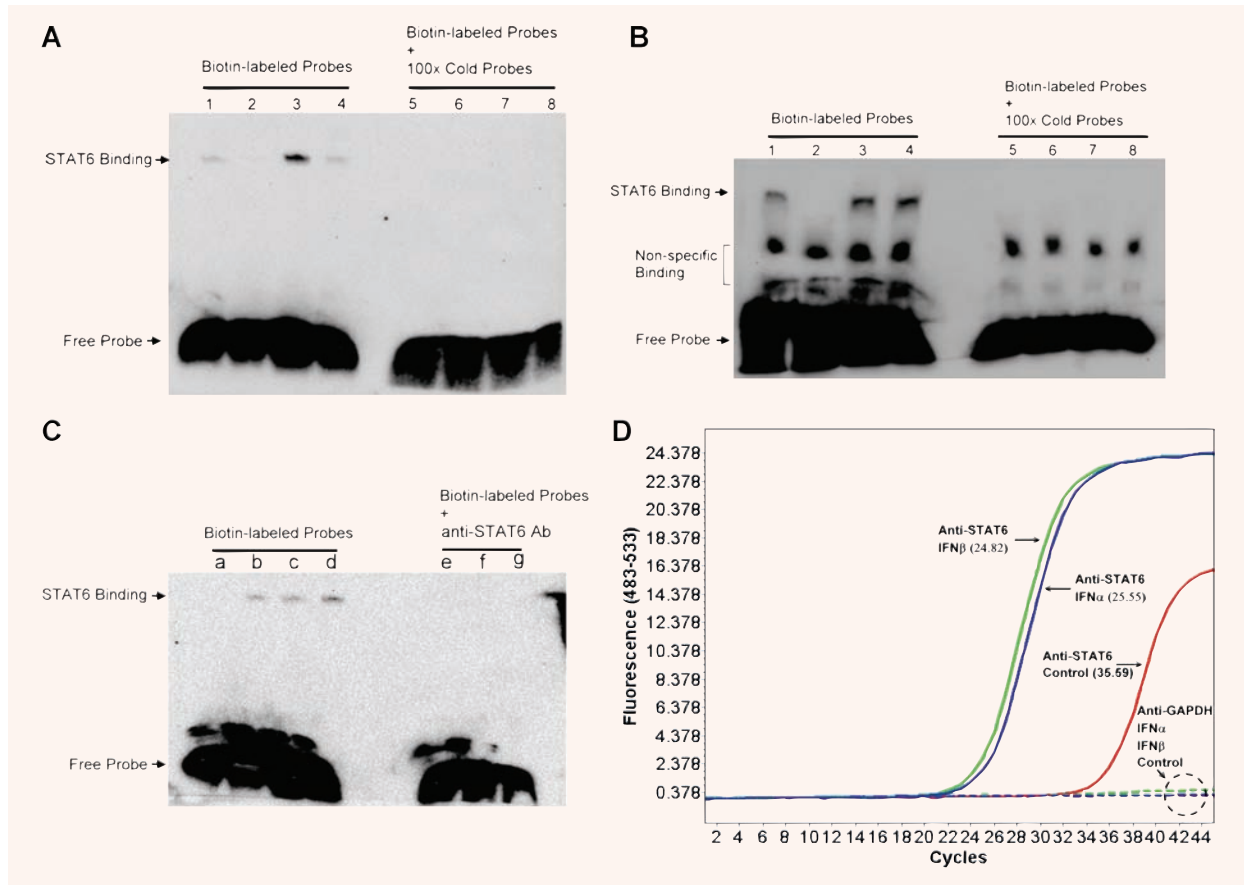


Fig. 4 Treatment of HuH7 cells with IFN α , IFN β , and IL-4 significantly enhanced STAT6 binding to CD23 and IL-1Ra promoter. Extracts were prepared from Huh7 cells with or without a 30-min stimulation at 37°C with IL-4 (10 ng/ml) (lane 1 and 5), phosphate buffered saline (lane 2 and 6; red lines for ChIP), IFN α (400 IU/ml; blue lines for ChIP) (lane 3 and 7; blue line for ChIP), and IFN β (400 IU/ml; green lines for ChIP) (lane 4 and 8). The nuclear extracts were examined by electrophoretic mobility shift assay (EMSA) using either biotin-labelled CD23 (**A**) and IL-1Ra (**B**) GAS probes alone or together with 100-fold molar excess of non-biotin labelled probes. The antibodies against STAT6 inhibited STAT6 binding to IL-1Ra GAS probes (**C**). Activated STAT6 is bound to the IL-1Ra promoter in native chromatin determined by ChIP assays (**D**). DNA fragments recovered from ChIP assays with anti-STAT6 (solid lines) or anti-GAPDH (dashed lines) were analysed by quantitative PCR using Roche universal probe 87. The Cp values were shown in parentheses.

most immune cells and many other types of somatic cells [1]. In contrast to the well-established functions in innate immunity, less is known about the functions of IFN- α and β in adaptive immunity. Some studies have indicated that IFN- α and β promotes Th1 differentiation and inhibits IL-4-induced IgE production and CD23 expression, indicating that IFN- α and β is a Th1 cytokine similar to IFN- γ [32]. It is a central dogma in immunology that IL-4 and IFN- γ have opposing effects on the regulation of adaptive immune responses. IFN- α and β share some of the same signalling mechanisms associated with IFN- γ . It was therefore expected that IL-4 and IFN- α/β have

opposing effects in adaptive immune responses. However, according to the results reported by Gupta *et al.* [11], Fasler-Kan *et al.* [12], and the present study, the phosphorylation of STAT6 by IFN- α and β is indistinguishable from that by IL-4. Interleukin-4 is a pleiotropic cytokine that plays an important role in modulating the immune-inflammatory reactions of growth, differentiation, cytokine production and surface molecule expression in lymphocytes, macrophages, stromal cells, and liver cells. The effect of IL-4 is primarily anti-inflammatory [10, 28]. IL-4 reduces the production of IL-1, TNF- α , and other pro-inflammatory mediators. IL-4 has been shown to increase the

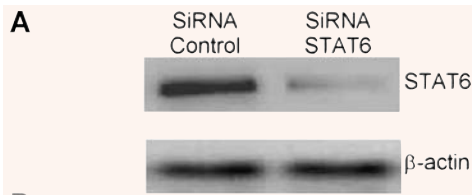
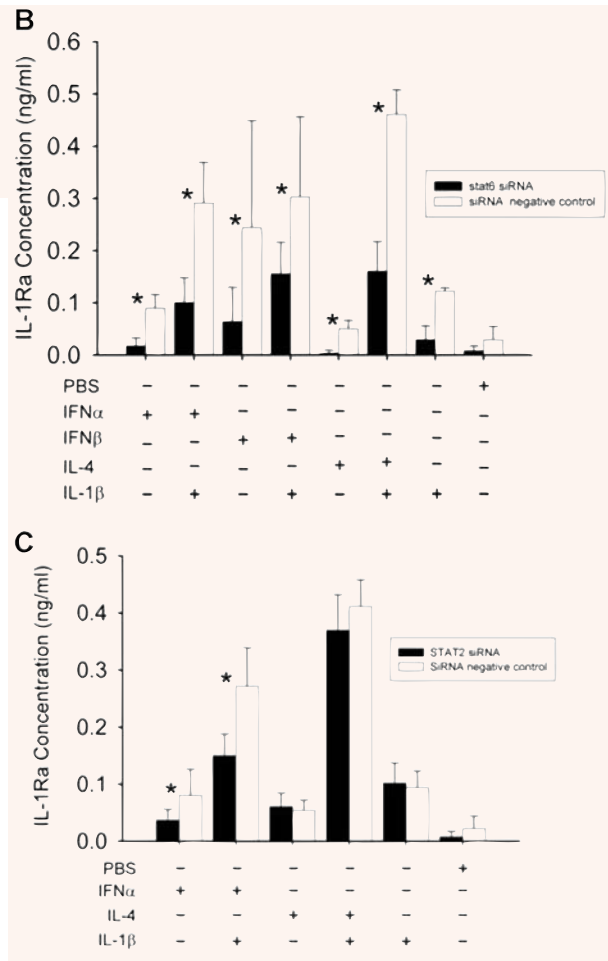


Fig. 5 Type I IFN-induced activation of STAT6 mediated the production of IL-1Ra. **(A)** SiRNA against STAT6 was introduced into the cells to down-regulate the expression level of STAT6 protein. STAT6 inhibition was checked by Western blotting using anti-STAT6 antibody. The blot was later stripped and re-probed with β -actin antibody to ensure equal loading of the cell extracts. **(B)** STAT6 is required for secreted IL-1Ra production in HuH7 cells activated by IFN α or IFN β . Culture supernatant were prepared from HuH7 cells with or without a 48 hour stimulation period at 37°C with phosphate buffered saline (control), IFN α (400 IU/ml), IFN β (400 IU/ml), IL-4 (10 ng/ml), and IL-1 β (1 ng/ml) as indicated. Secreted IL-1Ra concentration was assessed in supernatants of triplicate independent experiments and is presented as mean \pm S.D. Asterisks indicate the calculated *P* values for paired comparisons between STAT6 siRNA and siRNA negative control; all *P* values were <0.05. **(C)** Secreted IL-1Ra production is down-regulated by inhibiting the expression of STAT2 in HuH7 cells activated by 400IU/ml IFN α . Assay procedures are the same as above.



production of IL-Ra in monocytes [33], macrophages [34] and liver cells [31]. Moreover, IL-4 enhanced IL-1 β -induced production of IL-Ra in liver cells, indicating that the anti-inflammatory activity of IL-4 was partly mediated by the production of IL-1Ra.

Immune-mediated liver damage is thought to play an important role in the pathophysiology of chronic hepatitis C. Pro-inflammatory cytokines stimulate local immune activation by increasing the expression of adhesion molecules by endothelial cells and production of cytokines and chemokines, thereby inducing the local recruitment of circulating immune cells. The natural antagonists of pro-inflammatory cytokines soluble tumor necrosis factor receptor (sTNFRs) and IL-1Ra have an opposite effect. Previously reports have shown that injecting healthy individuals and chronic hepatitis C patients with IFN α induced a sustained and dramatic increase in circulating levels of IL-1Ra [22, 23]. IFN α can simultaneously decrease the viral replication and the inflammatory process caused by hepatitis C infection. The anti-inflammatory activity of IFN may be partly from the production of IL-1Ra.

IL-4-induced expression of secreted IL-1Ra is mediated by the activation of STAT6. The phosphorylation of STAT6 has been linked to the activation of JAK3 in human hepatocytes. Day *et al.* [35]. reported that the JAK3 mutant strongly suppressed IL-4-stimulated tyrosine phosphorylation of STAT6; however, in our study, the inhibition of JAK3 kinase activity by WHI-p131 did not inhibit the IFN- α β -induced phosphorylation of STAT6. Type I IFN activate receptor associated JAK1 and Tyk2 kinases. The activated receptor-JAK complex, in turn, phosphorylates STAT1, STAT2, STAT3 and STAT6 with no substrate specificity [2]. The activation of STAT1 and STAT2 has been clearly shown to play an essential role in the anti-viral and anti-tumour activities of IFN- α and β in STAT1- [36, 37] and STAT2- [38] deficient mice. The activation of STAT3 by IFN- α and β is also implicated in the anti-viral activity of both IFN- α and β . Therefore, the activation of STAT1, STAT2, and

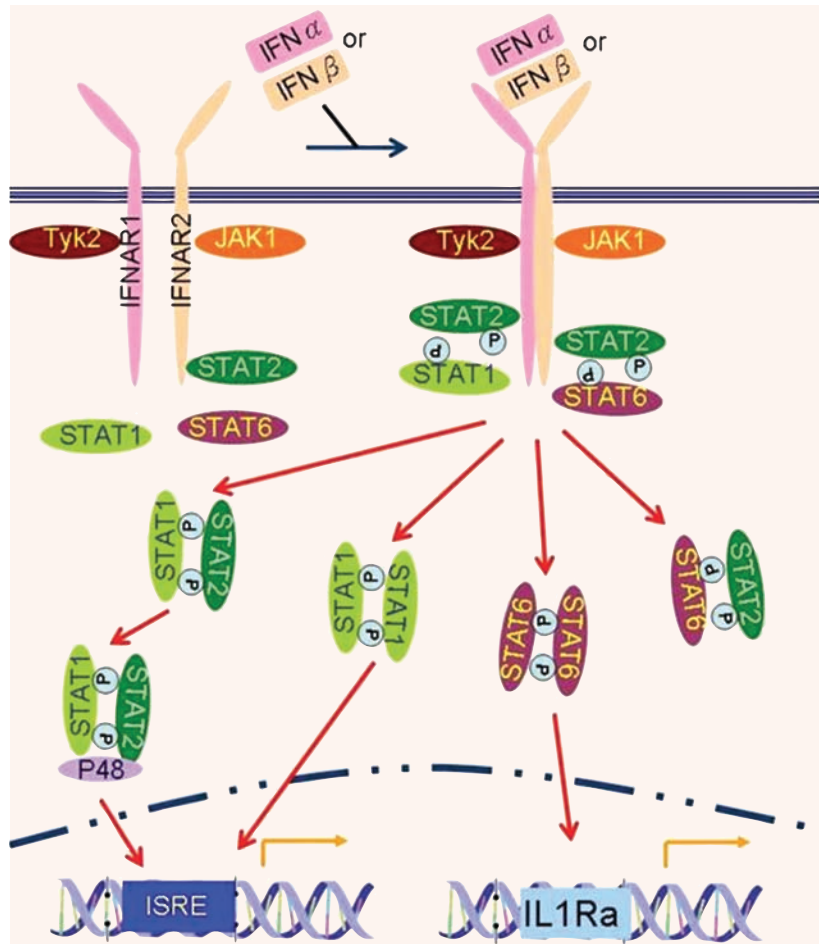


Fig. 6 A model for STAT6 activation on IFN α/β signalling. Tyk2 is pre-associated with IFNAR1, while JAK1, STAT2 are pre-associated with IFNAR2c. IFN α and IFN β induce the receptor heterodimerization and subsequently the IFNAR1 is phosphorylated and binds to STAT2, which is then phosphorylated at Tyr690. The phospho-STAT2 binds to IFNAR2 and recruited STAT1 or STAT6 to the receptor. Subsequently, STAT1 or STAT6 is then phosphorylated at Tyr701 or Tyr641, respectively, by JAK1 or Tyk2. The phosphorylated STAT6 will form a homodimer and bind to the IL-Ra promoter, thereby inducing the transcription of IL-Ra.

STAT3 by IFN- α and β in human hepatocytes contributed in part to the effectiveness of IFN- α and β anti-viral therapy hepatitis C patients.

Activated STAT6 plays a central role in mediating the biological functions of cytokines IL-4 and IL-13, which are tightly connected to the T_H2 branch of the immune system. Expressions of many cytokines responsible for organizing the infiltration of TH2 cells and eosinophils into sites of allergic inflammation are regulated by STAT6. Activated STAT6 is one of the molecules induced by IL-4 which may contribute to the immunomodulatory activities. The molecular mechanism by which IFN- α/β induce the phosphorylation of STAT6 were previously shown to be associated with IFN resistance [11]. STAT6 activation was markedly more reduced in IFN-resistant Daudi cell sub-clones than in IFN-sensitive Daudi cells. In the present study, we found that the activation of STAT6 in hepatocytes was associated with modulation of

inflammatory responses by the induction of secreted IL-1Ra expression. Therefore, the function of IFN- α and β is similar in some respect to that of IL-4.

The IFN- α or IFN- β activation pathway, in which ligands trigger the activation of STATs, begins by inducing dimerization of IFNAR1 and IFNAR2, which leads to the activation of JAK1 and Tyk2 kinases. It has been demonstrated that the phosphorylation of STAT2 at Tyr 690 is required for efficient activation of STAT1 [39–41]. In U6A cells, a mutant cell line which lacks STAT2, the phosphorylation of STAT1 is weak. We have also demonstrated that the down-regulation of STAT2 by RNAi reduced the phosphorylation of STAT1 (data not shown) [40]. The stimulation of HuH7 cells with IFN- α or IFN- β results in the association between STAT2/STAT6. Moreover, the activation of STAT6 and the expression of IL-1Ra were inhibited by inhibiting STAT2 protein expression (Fig. 2C and 5B). These observations prompt us to

suggest a mechanism through which STAT6 is activated by IFN- α or IFN- β (Fig. 6). According to this mechanism, binding of IFN- α or IFN- β to their high-affinity receptors leads to the dimerization of IFNAR1 and IFNAR2. The activated JAK1 and Tyk2 kinases then activate STAT2 by phosphorylating Tyr 690. The phosphorylated-STAT2 seems to provide a docking site for STAT1 [39] and STAT6, where they are subsequently activated following phosphorylation at Tyr 701 and 641, respectively. Phosphorylated-STAT6 then dissociates from the docking site, homodimerizes, and binds to the promoter of the IL-Ra gene, thereby initiating transcription of IL-Ra mRNA. Such activation mechanism may also occur with the formation of the STAT1:STAT1 homodimer, which is known to be activated by IFN γ . Indeed, IFN α has been shown to contribute to efficient IFN γ signalling through the homodimerization of STAT1. However, whether the STAT2:STAT6 heterodimer binds to DNA and activates gene expression remains unknown.

The most important findings in the present study are the observations that IFN- α or IFN- β induce the phosphorylation of STAT6 in hepatocytes and the expression of the STAT6 target gene, IL-1Ra. At the same time, the activation of STAT6 is mediated by binding of STAT6 to phosphorylated-STAT2 which is associated with IFNAR2. We also found that IL-1Ra is up-regulated by IFN- α , IFN- β , IL-4, and L1 β . Furthermore, IFN- α , IFN- β and IL-4 amplify the inducing effect of IL-1 β on production of IL-1Ra, suggesting that IFN- α and β may exert their anti-inflammatory effects through the production of IL-1Ra.

Acknowledgements

We thank Yu-Huei Liang for preparing this manuscript. This study was supported by grants from the National Science Council (94-2320-B-039-042- and 95-2320-B-039-045-), Taipei, Taiwan and grants from China medical University (CMU95-061 and CMU95-062), Taichung, Taiwan.

Reference

- Pfeffer LM, Dinarello CA, Herberman RB, Williams BR, Borden EC, Borden R, Walter MR, Nagabhushan TL, Trotta PP, Pestka S.** Biological properties of recombinant alpha-interferons: 40th anniversary of the discovery of interferons. *Cancer Res.* 1998; 58: 2489–99.
- Caraglia M, Marra M, Pelaia G, Maselli R, Caputi M, Marsico SA, Abbruzzese A.** Alpha-interferon and its effects on signal transduction pathways. *J Cell Physiol.* 2005; 202: 323–35.
- Moriyama M, Arakawa Y.** Treatment of interferon-alpha for chronic hepatitis C. *Expert Opin Pharmacother.* 2006; 7: 1163–79.
- Tagliaferri P, Caraglia M, Budillon A, Marra M, Vitale G, Viscomi C, Masciari S, Tassone P, Abbruzzese A, Venuta S.** New pharmacokinetic and pharmacodynamic tools for interferon-alpha (IFN-alpha) treatment of human cancer. *Cancer Immunol Immunother.* 2005; 54: 1–10.
- Eggermont AM.** Current status of interferon-alpha in the treatment of melanoma. *Cancer Chemother Biol Response Modif.* 2005; 22: 729–37.
- Caraglia M, Vitale G, Marra M, Budillon A, Tagliaferri P, Abbruzzese A.** Alpha-interferon and its effects on signaling pathways within cells. *Curr Protein Pept Sci.* 2004; 5: 475–85.
- Domanski P, Fish E, Nadeau OW, Witte M, Plataniotis LC, Yan H, Krolewski J, Pitha P, Colamonici OR.** A region of the beta subunit of the interferon alpha receptor different from box 1 interacts with Jak1 and is sufficient to activate the Jak-Stat pathway and induce an antiviral state. *J Biol Chem.* 1997; 272: 26388–93.
- Colamonici O, Yan H, Domanski P, Handa R, Smalley D, Mullersman J, Witte M, Krishnan K, Krolewski J.** Direct binding to and tyrosine phosphorylation of the alpha subunit of the type I interferon receptor by p135tyk2 tyrosine kinase. *Mol Cell Biol.* 1994; 14: 8133–42.
- Novick D, Cohen B, Rubinstein M.** The human interferon alpha/beta receptor: characterization and molecular cloning. *Cell.* 1994; 77: 391–400.
- Wurster AL, Tanaka T, Grusby MJ.** The biology of Stat4 and Stat6. *Oncogene.* 2000; 19: 2577–84.
- Gupta S, Jiang M, Pernis AB.** IFN-alpha activates Stat6 and leads to the formation of Stat2:Stat6 complexes in B cells. *J Immunol.* 1999; 163: 3834–41.
- Fasler-Kan E, Pansky A, Wiederkehr M, Bategay M, Heim MH.** Interferon-alpha activates signal transducers and activators of transcription 5 and 6 in Daudi cells. *Eur J Biochem.* 1998; 254: 514–9.
- Eriksen KW, Sommer VH, Woetmann A, Rasmussen AB, Brender C, Sveigaard A, Skov S, Geisler C, Odum N.** Bi-phasic effect of interferon (IFN)-alpha: IFN-alpha up- and down-regulates interleukin-4 signaling in human T cells. *J Biol Chem.* 2004; 279: 169–76.
- Leonard WJ.** Role of Jak kinases and STATs in cytokine signal transduction. *Int J Hematol.* 2001; 73: 271–7.
- Kotenko SV, Pestka S.** Jak-Stat signal transduction pathway through the eyes of cytokine class II receptor complexes. *Oncogene.* 2000; 19: 2557–65.

16. **Ariyasu T, Tanaka T, Fujioka N, Yanai Y, Yamamoto S, Yamauchi H, Ikegami H, Ikeda M, Kurimoto M.** Effects of interferon-alpha subtypes on the TH1/TH2 balance in peripheral blood mononuclear cells from patients with hepatitis virus infection-associated liver disorders. *in vitro Cell Dev Biol Anim.* 2005; 41: 50–6.
17. **Feng J, Misu T, Fujihara K, Misawa N, Koyanagi Y, Shiga Y, Takeda A, Sato S, Takase S, Kohnosu T, Saito H, Itoyama Y.** Th1/Th2 balance and HTLV-I proviral load in HAM/TSP patients treated with interferon-alpha. *J Neuroimmunol.* 2004; 151: 189–94.
18. **Hempel G, Galle PR, Lohr HF.** Quantitative analysis of specific Th1/Th2 helper cell responses and IgG subtype antibodies in interferon-alpha-treated patients with chronic hepatitis C. *J Med Virol.* 2001; 64: 340–9.
19. **Coclet-Ninin J, Dayer JM, Burger D.** Interferon-beta not only inhibits interleukin-1beta and tumor necrosis factor-alpha but stimulates interleukin-1 receptor antagonist production in human peripheral blood mononuclear cells. *Eur Cytokine Netw.* 1997; 8: 345–9.
20. **Huang Y, Blatt LM, Taylor MW.** Type 1 interferon as an antiinflammatory agent: inhibition of lipopolysaccharide-induced interleukin-1 beta and induction of interleukin-1 receptor antagonist. *J Interferon Cytokine Res.* 1995; 15: 317–21.
21. **Cotler SJ, Craft T, Ferris M, Morrissey M, McCone J, Reddy KR, Conrad A, Jensen DM, Albrecht J, Taylor MW.** Induction of IL-1Ra in resistant and responsive hepatitis C patients following treatment with IFN-con1. *J Interferon Cytokine Res.* 2002; 22: 549–54.
22. **Ohno Y, Fujimoto M, Nishimura A, Aoki N.** Change of peripheral levels of pituitary hormones and cytokines after injection of interferon (IFN)-beta in patients with chronic hepatitis C. *J Clin Endocrinol Metab.* 1998; 83: 3681–7.
23. **Naveau S, Emilie D, Borotto E, Portier A, Lazizi Y, Giraud V, Grangeot-Keros L, Capron F, Galanaud P, Chaput JC.** Interleukin-1 receptor antagonist plasma concentration is specifically increased by alpha-2A-interferon treatment. *J Hepatol.* 1997; 27: 272–5.
24. **Dinarello CA.** Induction of interleukin-1 and interleukin-1 receptor antagonist. *Semin Oncol.* 1997; 24: S9-81–93.
25. **Arend WP, Gabay C.** Physiologic role of interleukin-1 receptor antagonist. *Arthritis Res.* 2000; 2: 245–8.
26. **Gabay C, Gigley J, Sipe J, Arend WP, Fantuzzi G.** Production of IL-1 receptor antagonist by hepatocytes is regulated as an acute-phase protein *in vivo*. *Eur J Immunol.* 2001; 31: 490–9.
27. **Weinmann AS, Farnham PJ.** Identification of unknown target genes of human transcription factors using chromatin immunoprecipitation. *Methods.* 2002; 26: 37–47.
28. **Schroder AJ, Pavlidis P, Arimura A, Capece D, Rothman PB.** Cutting edge: STAT6 serves as a positive and negative regulator of gene expression in IL-4-stimulated B lymphocytes. *J Immunol.* 2002; 168: 996–1000.
29. **Kraus J, Borner C, Holtt V.** Distinct palindromic extensions of the 5'-TTC...GAA-3' motif allow STAT6 binding *in vivo*. *FASEB J.* 2003; 17: 304–6.
30. **Hebenstreit D, Wirnsberger G, Horejs-Hoeck J, Duschl A.** Signaling mechanisms, interaction partners, and target genes of STAT6. *Cytokine Growth Factor Rev.* 2006; 17: 173–88.
31. **Gabay C, Porter B, Guenette D, Billir B, Arend WP.** Interleukin-4 (IL-4) and IL-13 enhance the effect of IL-1beta on production of IL-1 receptor antagonist by human primary hepatocytes and hepatoma HepG2 cells: differential effect on C-reactive protein production. *Blood.* 1999; 93: 1299–307.
32. **So EY, Park HH, Lee CE.** IFN-gamma and IFN-alpha posttranscriptionally down-regulate the IL-4-induced IL-4 receptor gene expression. *J Immunol.* 2000; 165: 5472–9.
33. **Crow AR, Song S, Semple JW, Freedman J, Lazarus AH.** A role for IL-1 receptor antagonist or other cytokines in the acute therapeutic effects of IVIg? *Blood.* 2007; 109: 155–8.
34. **Ohmori Y, Smith MF Jr, Hamilton TA.** IL-4-induced expression of the IL-1 receptor antagonist gene is mediated by STAT6. *J Immunol.* 1996; 157: 2058–65.
35. **Day RM, Soon L, Breckenridge D, Bridges B, Patel BK, Wang LM, Corey SJ, Bottaro DP.** Mitogenic synergy through multilevel convergence of hepatocyte growth factor and interleukin-4 signaling pathways. *Oncogene.* 2002; 21: 2201–11.
36. **Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D, Carver-Moore K, DuBois RN, Clark R, Aguet M, Schreiber RD.** Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell.* 1996; 84: 431–42.
37. **Horvath CM, Darnell JE Jr.** The antiviral state induced by alpha interferon and gamma interferon requires transcriptionally active Stat1 protein. *J Virol.* 1996; 70: 647–50.
38. **Park C, Li S, Cha E, Schindler C.** Immune response in Stat2 knockout mice. *Immunity.* 2000; 13: 795–804.
39. **Li X, Leung S, Kerr IM, Stark GR.** Functional subdomains of STAT2 required for preassociation with the alpha interferon receptor and for signaling. *Mol Cell Biol.* 1997; 17: 2048–56.
40. **Leung S, Qureshi SA, Kerr IM, Darnell JE Jr, Stark GR.** Role of STAT2 in the alpha interferon signaling pathway. *Mol Cell Biol.* 1995; 15: 1312–7.
41. **Qureshi SA, Leung S, Kerr IM, Stark GR, Darnell JE, Jr.** Function of Stat2 protein in transcriptional activation by alpha interferon. *Mol Cell Biol.* 1996; 16: 288–93.