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**COMPARABLE POTENCY OF IFN $\alpha$ 2 AND IFN $\beta$  ON IMMEDIATE JAK/STAT  
ACTIVATION BUT DIFFERENTIAL DOWN-REGULATION OF IFNAR2**

**Zrinka Marijanovic <sup>\*</sup>, Josiane Ragimbeau <sup>\*</sup>, José Van der Heyden <sup>†#</sup>, Gilles Uzé <sup>†</sup>,  
and Sandra Pellegrini <sup>\*1</sup>**

<sup>\*</sup>Unité de Signalisation des Cytokines, CNRS URA 1961, Institut Pasteur, Paris 75724;

<sup>†</sup>CNRS UMR 5124, Montpellier 34095, France.

<sup>#</sup>present address: Flanders Institute for Biotechnology, Department of Medical Protein Research, Ghent University, Ghent, Belgium

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Abbreviations used: CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; IFN $\alpha$ , interferon  $\alpha$ ; IFN $\beta$ , interferon  $\beta$ ; IFNAR1, interferon- $\alpha$  receptor 1 subunit; IFNAR2, interferon- $\alpha$  receptor 2 subunit; JAK, Janus kinase; STAT, signal transducer and activator of transcription; TrR, transferrin receptor.

<sup>1</sup>Address correspondence to: Sandra Pellegrini, Unité de Signalisation des Cytokines, Dept. of Immunology, Institut Pasteur, 25 rue du Docteur Roux, Paris 75724 cedex 15, France.

Tel: +33-1-40613305; Fax: +33-1-40613204; E-mail: [pellegrini@pasteur.fr](mailto:pellegrini@pasteur.fr)

## ABSTRACT

Type I interferons (IFN $\alpha/\beta$ ) form a family of related cytokines that control a variety of cellular functions through binding to a receptor composed of IFNAR1 and IFNAR2. Among type I IFN, the  $\alpha 2$  and  $\beta$  subtypes exhibit a large difference in their binding affinities to IFNAR1 and it was suggested that high concentration of IFNAR1 may compensate for its low intrinsic binding affinity to IFN $\alpha 2$ . We tested whether receptor-proximal signaling events are sensitive to IFNAR1 surface concentration by investigating the relationship between relative IFNAR1/IFNAR2 surface levels and IFN $\alpha 2$  vs IFN $\beta$  signaling potencies in several cell lines. For this, we monitored the activation profile of Jak/Stat proteins, measured basal and ligand-induced surface decay of each receptor subunit and tested the effect of variable IFNAR1 levels on IFN $\alpha 2$  signaling potency. Our data show that the cell surface IFNAR1 level is indeed a limiting factor for assembly of the functional complex, but an increased concentration of it does not translate into an IFN $\alpha/\beta$  differential Jak/Stat signaling nor it changes the dynamics of the engaged receptor. Importantly, however, our data highlight a differential effect upon routing of IFNAR2. Following binding of IFN $\alpha 2$ , IFNAR2 is internalized but, instead of being routed towards degradation as it is when complexed to IFN $\beta$ , it recycles back to the cell surface. These observations strongly suggest that the stability and the intracellular lifetime of the ternary complex account for the differential control of IFNAR2. Moreover, this work opens up the attractive possibility that endosomal-initiated signaling may contribute to IFN $\alpha/\beta$  differential bioactivities.

## INTRODUCTION

Type I IFNs are helical cytokines with pleiotropic activities that contribute to immediate defense against pathogens, development of adaptive immunity and protective antitumor responses [1]. Accordingly, type I IFNs regulate diverse activities of non immune and immune cells and are also regulators of bone homeostasis. A unique feature of this cytokine family is the existence of a multiplicity of ligands. In humans the family comprises 16 IFN subtypes, broadly referred to as IFN $\alpha/\beta$ , all binding the ubiquitously expressed type I IFN receptor, made of IFNAR1 and IFNAR2. These latter are single membrane-spanning proteins belonging to the class 2 cytokine receptor superfamily [2]. Upon IFN binding, membrane-proximal immediate signaling is initiated through catalytic activation of receptor-associated Tyk2 and Jak1 tyrosine kinases [3, 4]. Stat family members, such as the ubiquitously expressed Stat1/2/3, are then tethered to the activated receptor complex *via* specific phosphotyrosine recruitment motifs, undergo phosphorylation on tyrosine and translocate to the nucleus to drive gene expression. Non-Stat signaling pathways can also be activated by type I IFNs and are believed to modulate gene expression and to shape IFN-induced bioactivities in defined cellular contexts [5, 6]. IFN also activates membrane-proximal signaling events that promote receptor down-modulation and signal termination [7, 8].

It is well documented that IFN $\alpha$  and IFN $\beta$  engage the receptor components with different efficiencies [9-11]. Recent work has extended previous studies and determined the biophysical parameters of binding (association and dissociation rate constants) of type I IFN subtypes to the extracellular domains of the two receptor subunits immobilized on an artificial lipid support [12]. These *in vitro* analyses showed that affinities and rate constants of IFN $\alpha$ 2 and IFN $\beta$  interaction with the receptor ectodomains are different. IFN $\alpha$ 2 exhibits nanomolar binding affinity and IFN $\beta$  exhibits ~100 pM binding affinity towards IFNAR2. Larger differences were observed in the ligand binding affinity to IFNAR1. IFN $\alpha$ 2 exhibits micromolar binding affinities to the IFNAR1 ectodomain, while IFN $\beta$  exhibits ~50 nM binding affinity towards IFNAR2. It was demonstrated that the kinetics of dissociation of IFN $\alpha$ , but not of IFN $\beta$ , from the immobilized complex is strongly affected by the concentration of IFNAR1 [12, 13]. Moreover, it was proposed that the differential potency of IFN subtypes to elicit an antiproliferative response lies in their different ability to bind IFNAR1 [14]. Overall, these studies suggested that recruitment of IFNAR1 is a rate limiting

step in the assembly of the ternary ligand-receptor complex and that a sufficiently high concentration of IFNAR1 may compensate for its low intrinsic affinity to IFN $\alpha$ 2.

Since IFNAR1 is the low affinity component in the formation of the ternary complex, one would predict that immediate receptor-initiated signaling events may be sensitive to IFNAR1 surface concentration. Indeed, the level of cellular IFNAR1 appears to be tightly regulated. We have shown that IFNAR1 is a relatively short lived protein that matures to the cell surface and is rapidly internalized and degraded, unless stabilized through its association with Tyk2 [15]. Upon IFN $\alpha$  binding, IFNAR1 is targeted to degradation through a biochemical cascade that has been in part elucidated and requires Tyk2 catalytic activity [8, 16].

Based on these premises, our primary goal was to investigate the relationship between IFNAR1 and IFNAR2 surface levels and immediate-early signaling events, *ie* activation of Jak/Stat molecules and receptor down-modulation, induced by IFN $\alpha$ 2 or IFN $\beta$ . For this, we have measured relative levels of IFNAR1 and IFNAR2 expressed in different human cell lines and monitored herein early kinetics and dose dependence of Jak/Stat activation. We have also studied basal and ligand-induced surface decay of IFNAR1 and IFNAR2. To directly test the effect of variable IFNAR1 levels, we have artificially modulated its concentration and analyzed possible effects on IFN signaling potency.

Despite the heterogeneity in the levels of the two subunits in different cell lines, a unifying concept emerges from this comprehensive analysis that points to comparable level of activation of Jak/Stat molecules in response to the two IFN subtypes and to a differential effect on traffic of the IFNAR2 receptor subunit.

## EXPERIMENTAL PROCEDURES

### *Cell culture and transfection procedures*

293T and HeLa cells were cultured in DMEM (Dulbecco's modified Eagle's medium) and Daudi and Jurkat cells in RPMI supplemented with 10% foetal calf serum at 37°C in 6% CO<sub>2</sub>. Human amnion-derived WISH cells were transfected using the calcium phosphate DNA precipitation technique. To over-express IFNAR1, a plasmid encoding IFNAR1 and pSV2neo were co-transfected at a ratio of 10/1. Clones were selected in 1.2 mg/ml of G418 (Geneticin, Invitrogen).

To silence IFNAR1, a 19 nt siRNA was designed to target the 3'UTR mRNA sequence of IFNAR1. The sequence ATACGGGCAAGCTCTTAAC was inserted into the RNAi vector

pSUPERretro (OligoEngine, Seattle) as indicated by the manufacturer. This puro<sup>R</sup>-siRNA plasmid was co-transfected with pSV2neo at molar ratio of 10/1. Cells were first selected in G418 to favour clones with highest amount of plasmid DNA. G418<sup>R</sup> clones were then kept in puromycin to maintain pressure for the siRNA plasmid. Clones were screened for IFNAR1 levels by flow cytometry using mAb AA3. For the experiment shown in Fig. 8, 293T cells were transiently transfected using Lipofectamine (Invitrogen) with an expression plasmid encoding either IFNAR1 or the IFNAR1 specific RNAi described above. All transfections included an EGFP expression plasmid at a ratio of 1/50 of total DNA. Transfection mixtures were normalized for the same concentration of total DNA with the corresponding empty vector. Two days after transfection, cells were treated for 2 hrs with 200 pM IFN $\alpha$ 2 or  $\beta$  or left untreated and receptor levels were analyzed by flow cytometry.

#### *Antibodies and other reagents*

Recombinant IFN $\alpha$ 2b was a gift of D. Gewert (Wellcome, U.K.) or G. Adolf (Ernst Boehringer Institute, Vienna) and IFN $\beta$  was from L. Runkel (Biogen Idec, Boston). IFN were purified to specific activities > 10<sup>8</sup> IU/mg of protein. Cycloheximide (CHX) (Sigma) was used at 20  $\mu$ g/ml on HeLa and 293T cells and at 10  $\mu$ g/ml on Jurkat and Daudi cells. Monensin (Sigma) was solubilized in absolute ethanol and used at 25  $\mu$ M. Human transferrin (Sigma) was solubilized in cell culture medium and was used at 50  $\mu$ g/ml. Phospho-Ser535-specific IFNAR1 Abs, described in [17], was a gift of S. Fuchs (University of Pennsylvania, Philadelphia, PA). Tyk2 phospho-specific Abs was from Calbiochem. Tyk2 mAb T10-2 was described in [18]. Phospho-tyrosine Stat1 and Stat2 Abs were from Upstate and phospho-tyrosine Stat3 Abs from BioLabs. Stat1, Stat2, Stat3 Abs and 4G10 anti-phosphotyrosine mAb were from Upstate. Ubiquitin FK2 mAb was from Biomol; IFNAR1 mAbs (AA3, EA12) were a gift from L. Runkel and 64G12 from P. Eid (CNRS, Villejuif, France). IFNAR2 mAb CD118 was purchased from PBL Biochemical Laboratories. IFNAR2 mAbs H10 and D5 were from L. Runkel.

#### *Flow cytometric analysis*

Transiently transfected 293T cells (Fig. 8) were stained with mAbs AA3 (IFNAR1) and D5 (IFNAR2) and signals were amplified with biotinylated rat anti-mouse IgG (Jackson Immunochemicals) and APC-conjugated streptavidin (Becton Dickinson) and analyzed with a FACScalibur. IFNAR1 and IFNAR2 expression levels were determined by the Geometric mean APC fluorescence values within subpopulations of transfected cells identified by the intensity of EGFP fluorescence. To assess the effect of monensin in Fig. 9A, down-regulation

of the transferrin receptor from the cell surface was studied. Briefly, HeLa cells were starved for 1 hr, preincubated for 15 min with monensin (25  $\mu$ M) before addition of transferrin (50  $\mu$ g/ml) for 30 min. In Fig. 9B, cells were preincubated with monensin 15 min before addition of IFN $\alpha$  or IFN $\beta$ . Surface IFNAR1 was monitored by incubating cells with 10  $\mu$ g/ml mAb AA3 and surface IFNAR2 was monitored with 10  $\mu$ g/ml of mAb CD118 for 30 min on ice, followed by 30 min incubation with 10  $\mu$ g/ml biotinylated anti-mouse IgG and with streptavidin-phycoerythrin (Jackson ImmunoResearch Laboratories, Inc.). The level of surface transferrin receptor (TrR) was monitored with OKT9 mAb (a gift from A. Alcover, Institut Pasteur, Paris). Cells were analyzed using a FACscan (Becton Dickinson), using CellQuest software.

#### *Protein analysis by immunoblot*

Cell lysates were prepared in modified RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP40, 0.5% DOC, 0.03% SDS) with 1 mM Na<sub>3</sub>VO<sub>4</sub> and a cocktail of antiproteases from Sigma. For the experiment in Fig. 5, cells were lysed in NP40 buffer (50 mM Tris/HCl pH 8, 150 mM NaCl, 1% NP40, 0.5 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM N-ethylmaleimide and antiproteases). IFNAR1 was immunoprecipitated with 1  $\mu$ g of mAb EA12 and detected with mAb 64G12. IFNAR2 was immunoprecipitated with 1  $\mu$ g of mAb CD118 and detected with a mixture of H10 and D5 mAbs.

#### *Antiproliferative effect*

HeLa cells were plated in 96-well plates at a density of 2500 cells/well. Twenty hours later cells were left untreated or treated with 1-10000 pM of either IFN $\alpha$  or IFN $\beta$  for additional 72 h. Cells were then washed with PBS, fixed with paraformaldehyde and stained with 0.5% crystal violet for 5 min. After removing the solution, cells were washed with deionized water and air-dried. Crystal violet was solubilized with 1% Triton-X-100. Optical density was analyzed at 570 nm.

## RESULTS

### *Lack of correlation between relative R2/R1 ratios and Jak/Stat activation*

In order to analyze early signaling events in cells expressing different surface levels of IFNAR1, we first measured the relative ratio of IFNAR1 and IFNAR2 present at the surface of four human cell lines. The steady state level of IFNAR1 and IFNAR2 was measured by flow cytometry, using two mAbs (AA3 and CD118) and an identical staining protocol (Experimental procedures). Daudi (Burkitt's lymphoma B lymphocytes), Jurkat (leukemia T

lymphocytes), HEK293T (embryonic kidney cells) and HeLa (carcinoma-derived epithelioid cells) were analyzed. As shown in Fig. 1A, the relative expression of the two subunits (referred as the R2/R1 ratio) was remarkably dissimilar, with two extreme profiles represented by Jurkat (R2/R1 around 4) and HeLa cells (R2/R1 around 0.5).

Based on these profiles, we chose Daudi and Jurkat for further studies. These cells express different R2/R1 ratios (around 2 in Daudi and 4 in Jurkat) and, being both of lymphoid origin, are best amenable to direct comparison. We first analyzed early kinetics of Jak/Stat phosphorylation induced by a single dose (500 pM) of either IFN subtype. Within 15 min of stimulation, phosphorylation of examined proteins was close to maximal level (Fig. 1B and C). At around 30 min, phosphorylation started to decline, with the exception of Stat2 which persisted for at least 4 hr. The profiles of IFN $\alpha$ 2 and IFN $\beta$  stimulated signals in each cell line were remarkably similar. However, phosphorylated Tyk2 and Stat2 were more abundant in Daudi cells, while phosphorylated Stat3 was more abundant in Jurkat cells. Notably, the extent of phosphorylation of these proteins closely reflected their total content in each cell line (Fig. 1B and C).

Next, we analyzed dose response profiles of Tyk2, Stat1/2/3 phosphorylation after 30 min of treatment with IFN $\alpha$ 2 or IFN $\beta$  (Fig. 1D). In both cell lines, phosphorylation of Tyk2, Stat1/3 was dose-dependent, reaching saturation at around 100 pM of either IFN subtype. Surprisingly, phosphorylation of Stat2 appeared not to be dose-dependent within the range of cytokine dose tested (Fig. 1D). In conclusion, the magnitude and duration of Jak/Stat activation by IFN $\alpha$  and IFN $\beta$  were virtually identical in each of the two cell lines and similar conclusions were reached from analyses of phosphorylation profiles in 293T and HeLa cells (data not shown). Moreover, we could not directly correlate R2/R1 ratios neither with  $\alpha/\beta$  differential signaling nor with sensitivity to IFN $\alpha$ 2.

#### *IFNAR1 is limiting for ligand-induced Jak/Stat activation*

Comparison of signaling in different cell types presents obvious limitations, one being the variable content of signaling effectors. Thus, we decided to monitor  $\alpha/\beta$  signaling in sister clones expressing different levels of IFNAR1. For this, we chose human WISH cells, well known for their  $\alpha/\beta$  differential antiproliferative response [13]. As shown in Fig. 2, the comparative analysis performed in these cells did not reveal  $\alpha/\beta$  differential potency in Jak/Stat signaling and confirmed the distinctive profile of Stat2 activation with respect to Stat1/3.

Three stable transfectants were derived from WISH (parental) cells: a control clone (R23) expressing IFNAR1 to the same level as parental cells, a clone that over-expressed IFNAR1 (R16) and a clone with low IFNAR1 (CPO2) which was obtained by RNAi technology (see Experimental procedures). The relative surface level of the two receptor subunits in these clones is shown in Fig. 3A. IFNAR1 was approximately 7 fold higher in R16 cells and 2 fold lower in CPO2 cells with respect to R23 or parental WISH. As expected, IFNAR2 levels were comparable in all clones. In western blot analysis, IFNAR1 level was barely detectable in CPO2 cells and was strongly increased in R16 cells as compared to endogenous IFNAR1 in R23 cells (Fig. 3B, top panels).

To investigate whether surface IFNAR1 concentration affected sensitivity to IFN $\alpha$ 2, we compared the Jak/Stat activation profile of these clones. Cells were incubated with 500 pM IFN $\alpha$ 2 for 5, 15 and 30 min and protein phosphorylation was monitored (Fig. 3B). Phosphorylation of all proteins, with the exception of Stat2, was reduced in CPO2 cells as compared to the other clones. Interestingly, Stat2 phosphorylation was comparable in the three clones and did not decay with time. We further compared CPO2 and R23 cells to assess whether the weak response of CPO2 cells could be augmented further by increasing IFN $\alpha$ 2 dose. As shown in Fig. 4A, even at the highest dose tested (2500 pM), the response of CPO2 cells remained low, approaching that of control R23 cells stimulated with 10 pM.

Despite the remarkable difference of IFNAR1 levels in R16 and R23 cells, their Jak/Stat phosphorylation profiles by IFN $\alpha$  were quite comparable (Fig. 3B). To explore the possibility of an increased IFN $\alpha$ 2 sensitivity of R16 cells, we compared response profiles of the two clones to low ligand doses (Fig. 4B). If anything, R16 cells were slightly less responsive than R23 to low cytokine doses. Altogether, these data showed that a mere 2 fold decrease in surface IFNAR1, as in CPO2 cells, had a considerable impact on the magnitude of signaling and on the IFN $\alpha$  sensitivity threshold. Conversely, a surplus of IFNAR1 over endogenous level did not translate into increased signaling nor in increased sensitivity to IFN $\alpha$ . These results demonstrate that cell surface IFNAR1 concentration is indeed a critical factor for assembly of a functional signaling complex, but its increased concentration does not augment IFN $\alpha$ , nor IFN $\beta$  (data not shown), signaling potency. Moreover, these results imply that assembly of the trimeric ligand-receptor complex is the step determining the extent of downstream Jak/Stat activation.

*IFN $\beta$ , but not IFN $\alpha$ 2, induces IFNAR2 down-regulation*

Along with Jak/Stat engagement, binding of IFN also triggers membrane-proximal signals which promote internalization and drive endosomal sorting of the engaged receptor. Numerous studies have implicated ubiquitination of ligand-activated receptors as a signal for endosomal sorting towards degradation [19-21]. Recent data showed that, upon IFN $\alpha$  binding, IFNAR1 becomes serine phosphorylated and consequently ubiquitinated prior to lysosomal degradation [8]. Much less is known about ligand-induced post-translational modification and traffic of IFNAR2. We therefore tested whether IFN $\alpha$ 2 and IFN $\beta$  led to differential modification of IFNAR1 and/or IFNAR2. As shown in Fig. 5 (left panels), IFNAR1 was inducibly phosphorylated on tyrosine and on Ser535 and heavily ubiquitinated. IFNAR2 was inducibly phosphorylated on tyrosine but was not ubiquitinated (Fig. 5, right panels). All modifications were induced to a similar extent by IFN $\alpha$ 2 and IFN $\beta$ . Similar conclusions were reached upon analysis of HeLa cells (data not shown).

Jaitin et al. [13] recently showed that a 2 hr stimulation of 293T cells with IFN $\beta$ , but not IFN $\alpha$ , causes down-regulation of surface IFNAR2. We thus asked whether this  $\beta$ -specific effect could be generalized to other cell types and whether different surface R2/R1 ratios (as in cell lines shown in Fig. 1A) influenced this  $\alpha/\beta$  differential trait. In order to thoroughly assess the effect of ligand binding on receptor dynamics, we first determined in the four cell lines the surface stability of each receptor subunit in the absence of stimulus. Cells were treated with cycloheximide (CHX) to prevent new protein synthesis and the decay of each subunit was followed by flow cytometry (Fig. 6). Interestingly, IFNAR2 displayed an apparent slow basal turnover, with 90% of the initial content present at 3 h. On the other hand, surface IFNAR1 decayed rapidly in all but Daudi cells, with between 55 to 60% of the initial values remaining at 3 h (Fig. 6). The different stability of the two receptor subunits was also confirmed by western blot analyses (data not shown). Overall, these results showed that the two receptor subunits have different basal turnovers, with IFNAR1 being considerably more dynamic than IFNAR2.

Surface IFNAR1 and IFNAR2 levels were then monitored in cells treated with IFN $\alpha$ 2 or IFN $\beta$ , in the presence of CHX (Fig. 7A). Within the first hour, IFNAR1 decreased more rapidly in cells treated with either ligand than in CHX only treated cells shown in Fig. 6. This ligand-induced acceleration was observed in all cell types (Fig. 7A, left panels). At 90 min, the rate of decay decreased, approaching the basal rate seen in Fig. 6. Importantly, a clear difference in down-regulation of IFNAR2 by the two ligands was observed for 293T and

HeLa cells. IFN $\beta$  caused a substantial decrease (approaching 50% at 90 min) in surface IFNAR2, whereas IFN $\alpha$ 2 had a minimal effect (Fig. 7A, right panels). The differential effect of IFN $\alpha$ 2 and IFN $\beta$  on IFNAR2 down-regulation was less pronounced in Daudi cells and appeared negligible in Jurkat cells. Since these cells express higher level of IFNAR2 relative to IFNAR1 (high R2/R1 ratio), the percentage of mobilized IFNAR2 may be small and uncomplexed IFNAR2 may remain at the cell surface. Indeed, a differential effect between IFN $\alpha$ 2 and IFN $\beta$  was evident upon plotting arbitrary IFNAR2 internalization units instead of percentages of initial values (insets in Fig. 7A). Western blot analyses of total receptor content confirmed the ligand-induced decrease of IFNAR1 in the four cell lines. The decrease of IFNAR2 was detectable in IFN $\beta$ -treated 293T and HeLa cells, but not in Jurkat or Daudi cells (Fig. 7B).

To study the effect of varying IFNAR1 level on IFNAR2 down-regulation, we depleted or over-expressed IFNAR1 in 293T cells (see Experimental procedures) and monitored by FACS receptor levels after a 2 hr-treatment with IFN $\alpha$ 2 or IFN $\beta$ . As seen in Fig. 8, in response to IFN $\alpha$ 2 the surface decay of IFNAR2 was minimal even in cells with a surplus of IFNAR1 as compared to control cells (endogenous IFNAR1 level taken as 1). Thus, the failure of IFN $\alpha$ 2 to promote IFNAR2 down-regulation is not due to limiting amount of IFNAR1. In response to IFN $\beta$ , IFNAR2 was down-regulated in control cells as expected. The amount of down-regulated IFNAR2 diminished in cells depleted of IFNAR1, but did not proportionally increase in cells with a surplus of IFNAR1.

Overall, these results demonstrate a differential effect on post-binding receptor dynamics, with solely IFN $\beta$  being able to down-modulate IFNAR2. While this IFN $\beta$  specific effect can be generalized to different cell types, the extent by which it is experimentally detectable appears to depend on the relative expression level of the two chains and hence on the proportion of IFNAR2 which is engaged in the trimeric complex.

#### *IFN $\alpha$ 2 induces IFNAR2 recycling*

We have shown above that IFN $\alpha$ 2 failed to down-regulate surface IFNAR2, although it efficiently accelerated IFNAR1 down-regulation. We reasoned that the constant level of IFNAR2 measured at the cell surface could either reflect stability at the plasma membrane or it could result from the dynamic equilibrium between IFN $\alpha$ -induced internalization and recycling. In order to distinguish between these two possibilities, we used monensin, a drug that blocks recycling without affecting the internalization process [22]. The toxic effect of

monensin on the viability of different cell lines was tested. HeLa cells were chosen as they were minimally affected by the drug. Moreover, in these cells a 2 log difference in the antiproliferative potencies of IFN $\beta$  and IFN $\alpha$ 2 could be measured (Fig 9A).

To control the action of the ionophore, we monitored the surface level of the transferrin receptor (TrR), which undergoes continuous recycling in the presence of transferrin [23]. As expected, TrR level decreased approximately 50% in monensin-treated cells. In the same condition, basal IFNAR2 turnover was unaffected (Fig. 9B). HeLa cells were then treated with IFN $\alpha$ 2 or IFN $\beta$  in the presence or absence of monensin, and surface decay of IFNAR1 and IFNAR2 was measured by flow cytometry. Monensin did not affect IFNAR1 dynamics (Fig. 9C, top panels) nor it affected the disappearance of surface IFNAR2 induced by IFN $\beta$  (bottom right panel). However, monensin led to a substantial decrease of surface IFNAR2 in cells treated with IFN $\alpha$  (bottom left panel). Thus, following binding of IFN $\alpha$ 2 to the receptor complex, IFNAR2 is indeed internalized but, instead of being routed towards a degradation pathway, it appears to recycle back to the cell surface.

## DISCUSSION

The evolutionary conservation of multiple type I IFN subtypes in all eutherian mammals evokes distinct physiological roles despite shared receptor usage. Here, we have investigated whether IFN $\alpha$ 2 and IFN $\beta$ , well described for their different mode of engagement of the receptor, differ in membrane-proximal signaling potency and in receptor down-regulation processes. In particular, we have studied cell lines that express endogenous IFNAR1 and IFNAR2 at different relative ratios and evaluated potential contribution of this ratio to early Jak/Stat signaling by the two IFN subtypes. Our major observations can be summarized as follows : (i) within each cell line, the phosphorylation profiles of Jak/Stat molecules induced by IFN $\alpha$ 2 or IFN $\beta$  are virtually identical within the first four hours of stimulation; (ii) the extent of tyrosine phosphorylation of Jak/Stat components appears to be proportional to the amount of protein in the cell, rather than to the R2/R1 ratio; (iii) the activation profile of Stat2 differs from that of Tyk2, Stat1 and Stat3 and this in all cellular contexts analyzed; (iv) most remarkably, IFN $\alpha$ 2 and IFN $\beta$  lead to differential down-regulation and routing of IFNAR2.

Previous studies have reported some differences in the phosphorylation profile of Jak/Stat proteins by IFN $\alpha$  and IFN $\beta$ . In human myocardial fibroblasts selective IFN $\beta$ -induced activation of Jak1 and higher Stat1/2 phosphorylation were observed, along with a 120-fold higher sensitivity to the antiviral effect of IFN $\beta$  as compared to IFN $\alpha$  [24]. In human vascular

endothelial cells, a 2-3 fold higher Stat1 phosphorylation was observed with IFN $\beta$ , whereas a 2-3 log difference in  $\alpha/\beta$  potency was measured in functional assays [25]. In monocyte-derived immature dendritic cells, levels of activation of Stat1/2 by IFN $\alpha$ 2 and  $\beta$  were found to be comparable [26], as in the four human cell lines described in the present work. In sum, these results suggest that cell type differences in the potency of the two IFN subtypes to activate membrane-proximal signaling events do exist. However, when present, these differences are relatively modest, do not reflect ligand binding affinities to IFNAR1 and are unlikely to account for the large differences in potencies measured in functional assays. Indeed, in spite of identical phosphorylation potency, we did measure a 2 log difference in the antiproliferative potencies of IFN $\alpha$ 2 and IFN $\beta$  on HeLa cells (Fig. 9A).

It has been previously shown that IFNAR1 contributes to enhance the affinity of the cellular receptor complex for the ligand and also modulates ligand selectivity [9, 27]. Studies of the assembly of the trimeric complex on artificial surfaces argued against a substantial interaction between the receptor subunits in the absence of ligand. Instead, the local concentration of IFNAR1 was shown to influence the assembly of the IFN $\alpha$  ternary complex [12, 13]. Not surprisingly, we found that the relative surface expression level of the two IFN receptor subunits varies in different cell lines. Yet, within each cell line and regardless of the R2/R1 ratio, IFN $\alpha$ 2 and IFN $\beta$ -induced phosphorylation profiles were comparable. Furthermore, IFNAR1 does indeed represent a critical factor for both IFN $\alpha$ 2 and IFN $\beta$ -driven activation of the Jak-Stat signaling complex. In fact, depletion of endogenous IFNAR1 in WISH cells led to a significant reduction of signal activation which could not be compensated by increased ligand dose. On the other hand, a surplus of IFNAR1 in WISH cells did not change the IFN sensitivity profile nor the kinetics of Jak/Stat activation (Fig. 3, data not shown). Thus, in spite of the ability of IFNAR1 to compensate for weak IFN $\alpha$  binding, increased surface concentration of IFNAR1 does not change signaling potency.

Comparison of IFN signaling in Jurkat and Daudi cells indicated that the basal cellular content of Stat1/3, rather than receptor levels, impacts on the extent of their phosphorylation and thus, most likely, on downstream gene induction profiles. While this may contribute to cell type specific differences in IFN action, it also evokes the notion that changes in the content of Stats, as upon cell activation or differentiation, may tune IFN responses and gene induction programs [28-31]. Interestingly, Stat2 phosphorylation appears quite unique in all cellular contexts tested: it is saturated more readily and at lower IFN doses, is more sustained and barely affected by IFNAR1 depletion. We previously showed that Stat2, as opposed to

Stat1/3, is not engaged at all upon induced dimerization of the IFNAR1 cytoplasmic domain [16]. Thus, the unique Stat2 activation profile may, at least in part, be consequent to its constitutive association to the IFNAR2 chain [32].

The lack of correlation between ligand-IFNAR1 affinity and Jak/Stat activation potency suggests that  $\alpha/\beta$  differential bioactivities must be achieved through mechanisms other than immediate Jak/Stat activation. Moreover, these mechanisms are likely to be influenced by ligand binding affinity. Our present analysis of ligand-induced receptor traffic provides some interesting clues to this issue. Despite the heterogeneity in the expression level of the two subunits in different cell lines, consistent differences were observed in the dynamics of the two receptor subunits. With the exception of Daudi cells, where IFNAR1 is highly expressed and stable, in all other cell lines studied IFNAR1 exhibits a relatively short half-life and is down-regulated similarly by IFN $\alpha$ 2 and IFN $\beta$ . Interestingly, IFNAR2 exhibits a longer half-life which is differentially affected by the two IFN subtypes: following binding of IFN $\beta$ , surface IFNAR2 levels decrease, whereas following binding of IFN $\alpha$ 2 surface IFNAR2 remains constant as the internalized pool predominantly recycles back to the cell surface. This notion was supported by an experiment that tested the effect of monensin on receptor dynamics. In the presence of this well characterized inhibitor of recycling, IFN $\alpha$ 2 led to significant down-regulation of IFNAR2 (Fig. 9). These data evoke previous biochemical demonstrations that IFNAR1 and IFNAR2 can be co-immunoprecipitated after stimulation with IFN $\beta$ , but not IFN $\alpha$  [33-36]. Moreover, in one such study performed on Daudi cells, active IFN $\beta$ , but not IFN $\alpha$ , could be recovered in the immunoprecipitated complex [37].

Our results extend to several human cell lines the  $\alpha/\beta$  differential down-regulation of IFNAR2 originally reported in 293T cells [13]. Moreover, our studies in these latter cells demonstrate that the IFN $\beta$ -induced down-regulation of IFNAR2 indeed depends on formation of the ternary complex and that increasing IFNAR1 does not modify ligand capacity to down-regulate IFNAR2. These observations strongly suggest that it is the strength of ligand binding and/or the lifetime of the ternary complex which may account for the differential control of IFNAR2.

That the strength of ligand binding may be critical for receptor routing is well established. A more stable ligand-receptor interaction at the moderately acidic conditions of early endosomes, may promote receptor degradation and prevent recycling [38, 39]. Recent studies have implicated ubiquitination of activated receptors as a signal for endosomal sorting towards degradation [19] and the absence of ubiquitination was shown to divert internalized

receptors towards the recycling pathway [20, 21, 40]. While ligand-induced ubiquitination of IFNAR1 promotes its efficient sorting towards degradation [8, 16], we showed here that IFNAR2 does not undergo ligand-induced ubiquitination (Fig. 5). Hence, differential ubiquitination is unlikely to underlie the  $\alpha/\beta$  differential effect on IFNAR2 routing. An alternative possibility is that IFN $\beta$ , through efficiently bridging IFNAR2 to ubiquitinated IFNAR1, drives the complex towards a degradative pathway. While the molecular steps by which IFN $\alpha$ 2 and IFN $\beta$  lead to differential intracellular routing of IFNAR2 remain to be elucidated, our results imply that neither ligand properties nor receptor traffic impacts on immediate-early activation of Jak/Stat.

Divergent endosomal sorting of activated receptors has emerged as a dynamic process that can tune and diversify intracellular signaling [41]. For example, it was shown that TGF- $\beta$  receptors, residing in different membrane microdomains, can internalize through distinct endocytic compartments, which in turn mediate either signal activation or receptor degradation [42]. Interestingly, it has been reported that a fraction of IFNAR1 can be found in lipid rafts in HeLa cells and mouse embryo fibroblasts [43, 44]. This observation raises the possibility that membrane accessibility of (competent) receptor subunits may control their basal and ligand-induced dynamics. In this scenario, the intrinsic binding property of the ligand combined with cell type differences in availability of competent receptors would contribute to the  $\alpha/\beta$  differential IFNAR2 routing. Whether divergent traffic routes of the engaged receptors may shape IFN subtype-specific signaling and/or contribute to Stat-independent signaling [6] remains an appealing and open question.

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## FIGURE LEGENDS

*Figure 1. Surface levels of IFNAR1 and IFNAR2 and profiles of IFN-induced Jak/Stat phosphorylation in four cell lines.*

(A) Steady-state levels of IFNAR1 and IFNAR2 at the cell surface of the indicated cell lines were measured by flow cytometry. (B and C) Kinetics of IFN $\alpha$  and IFN $\beta$ -induced Tyk2 and Stat1/2/3 phosphorylation in Daudi and Jurkat cells. Cells were incubated with 500 pM IFN $\alpha$ 2 or IFN $\beta$  for the indicated time. Total lysates (40  $\mu$ g) were resolved by SDS/PAGE and the levels of phosphorylated or total Tyk2 and Stat1/2/3 were analyzed by western blot. (D) Dose response profiles of Tyk2 and Stat1/2/3 phosphorylation after IFN $\alpha$ 2 or IFN $\beta$  treatment of Daudi and Jurkat cells. Cells were treated with the indicated doses (from 2.5 to 500 pM) of IFN $\alpha$ 2 or IFN $\beta$  for 30 min. Lysates (40  $\mu$ g) were analyzed as described in (A). Data are representative of at least 3 different experiments.

*Figure 2. IFN $\alpha$ 2 and IFN $\beta$ -induced phosphorylation profiles in WISH cells.*

(A) Kinetics of Tyk2 and Stat1/2/3 phosphorylation in WISH cells treated with 500 pM of IFN $\alpha$ 2 or IFN $\beta$ , for the indicated time. (B) Dose response profiles of Tyk2 and Stat1/2/3 phosphorylation in WISH cells treated for 15 min with IFN $\alpha$ 2 or IFN $\beta$ . Lysates (40  $\mu$ g) were analyzed by western blot. Data are representative of at least 3 different experiments.

*Figure 3. Levels of IFNAR1 and IFNAR2 and IFN $\alpha$ 2-induced phosphorylation profiles in WISH-derived clones.*

(A) Surface IFNAR1 and IFNAR2 levels in stable WISH clones measured by flow cytometry. Three stable clones were derived from WISH parental cells, one depleted of endogenous IFNAR1 (CPO2), one over-expressing IFNAR1 (R16) and one expressing IFNAR1 as in parental cells (R23). (B) Total IFNAR1 content and IFN $\alpha$ -induced Jak/Stat phosphorylation in CPO2, R23 and R16 clones. Cells were incubated with 500 pM IFN $\alpha$ 2 for the indicated time. Lysates (40  $\mu$ g) were analyzed by western blot. Representatives of at least 3 different experiments are shown. The lower band visible in the R16 samples represents a 95 kDa incompletely processed IFNAR1 species which accumulates when the protein is overexpressed [15].

*Figure 4. Dose response profiles of IFN $\alpha$ 2-induced Jak/Stat phosphorylation in WISH-derived clones.*

(A) CPO2 and R23 cells were treated with increasing doses of IFN $\alpha$ 2 for 15 min. Note the maximal dose (2500 pM) added on CPO2 cells. (B) R23 and R16 cells were treated with IFN $\alpha$ 2 (from 1 to 100 pM) for 15 min. Total lysates (40  $\mu$ g) were resolved by SDS/PAGE and levels of phosphorylated Tyk2, Jak1 and Stat1/2/3 were analyzed by western blot. The arrow points to a band, identified as phospho-Tyk2, which cross-reacts with anti-phospho-Jak1 Abs.

*Figure 5. Ligand-induced post-translational modifications of IFNAR1 and IFNAR2.*

Daudi cells were treated with 500 pM IFN $\alpha$ 2 or IFN $\beta$  for the indicated times. Total lysates (5 mg) were used to immunoprecipitate endogenous IFNAR1 and IFNAR2. Immunoprecipitates were resolved by SDS/PAGE and the extent of ubiquitination of the receptors was analyzed using anti-ubiquitin Abs (top panels). Note the shift in migration of IFNAR1 due to induced ubiquitination. The left membrane was stripped and reprobed with anti-phosphotyrosine 4G10 mAb, P-Ser535-IFNAR1-specific Abs and anti-IFNAR1 mAb, as indicated. The right membrane was stripped and reprobed with anti-phosphotyrosine 4G10 mAb and anti-IFNAR2 mAb, as indicated. Migration of the 116 kDa marker is indicated on the left. Data are representative of at least 3 different experiments.

*Figure 6. Basal decay of surface IFNAR1 and IFNAR2.*

Surface stability of IFNAR1 and IFNAR2 in unstimulated cells. Decay of IFNAR1 (empty squares) and IFNAR2 (filled squares) from the surface of Daudi, Jurkat, 293T and HeLa cells treated with cycloheximide (CHX) was measured by flow cytometry. The results are plotted as percentages of the mean fluorescence at time 0. Mean values of 4 different experiments are presented.

*Figure 7. Ligand-induced down-regulation of IFNAR1 and IFNAR2.*

(A) IFNAR1 (left panels) and IFNAR2 (right panels) decay from the cell surface were measured by flow cytometry in cells treated with CHX and 500 pM IFN $\alpha$ 2 (empty circles) or IFN $\beta$  (filled circles) for the indicated time. Data are plotted as the percentage of mean fluorescence at time 0. Mean values of at least 3 different experiments are shown. In the insets the difference between mean fluorescence values measured on untreated and IFN-

treated cells is plotted. (B) Daudi, Jurkat, 293T and HeLa cells were treated with 500 pM IFN $\alpha$ 2 or IFN $\beta$  for the indicated time. Total lysates (40  $\mu$ g) were resolved by SDS/PAGE and levels of IFNAR1 and IFNAR2 were analyzed by western blot using corresponding antibodies.

*Figure 8. Ligand-induced down-regulation of IFNAR2 in 293T cells expressing different levels of IFNAR1.*

293T cells were transiently cotransfected with EGFP and either an IFNAR1-silencing vector or an IFNAR1-expression vector. An aliquot of the transfected cells was monitored for EGFP and IFNAR1 levels. In parallel, cells were treated with 200 pM IFN $\alpha$ 2 or IFN $\beta$  for 2 h, or left untreated, and levels of IFNAR2 were measured in chosen EGFP-positive subpopulations. IFNAR1 relative level (horizontal axis) represents the amount of surface IFNAR1 in transfected cells relative to endogenous IFNAR1 (marked as 1) in untreated cells. The extent of IFNAR2 down-regulation (vertical axis) is expressed as the difference in the Geo Mean of IFNAR2 specific fluorescence between untreated cells and cells treated with IFN $\alpha$ 2 (empty circles) or IFN $\beta$  (filled circles).

*Figure 9. IFN $\alpha$ 2-induced recycling of IFNAR2 in HeLa cells.*

(A) IFN-induced antiproliferative effect in HeLa cells. Cells were either left untreated or treated with the indicated concentrations of IFN $\alpha$ 2 (open circles) or IFN $\beta$  (closed circles) for 72 hr. Cell density was monitored by crystal violet staining as described in Experimental procedures. The mean optical density values of triplicate datasets are presented. (B) HeLa cells were either left untreated or treated with 25  $\mu$ M monensin for 30 min in the presence of transferrin (50  $\mu$ g/ml) and the level of TrR at the cell surface was measured by flow cytometry (left panel). Data are plotted as percentages of the mean fluorescence in control condition. The mean value of 4 different experiments is presented. In the right panel, the basal decay of IFNAR2 from the cell surface was monitored in the presence (open squares) or absence (filled squares) of monensin for the indicated times using flow cytometry. The results are plotted as percentages of the mean fluorescence at time 0. The mean values of 3 different experiments are presented. (C) IFN $\alpha$ 2-induced IFNAR2 recycling. HeLa cells were treated with 500 pM IFN $\alpha$ 2 (left panels) or IFN $\beta$  (right panels) in the presence (open circles) or absence (closed circles) of monensin for the indicated times and the surface levels of IFNAR1

and IFNAR2 were analyzed by flow cytometry. The results are plotted as percentages of the mean fluorescence at time 0. The mean values of 3 different experiments are presented.

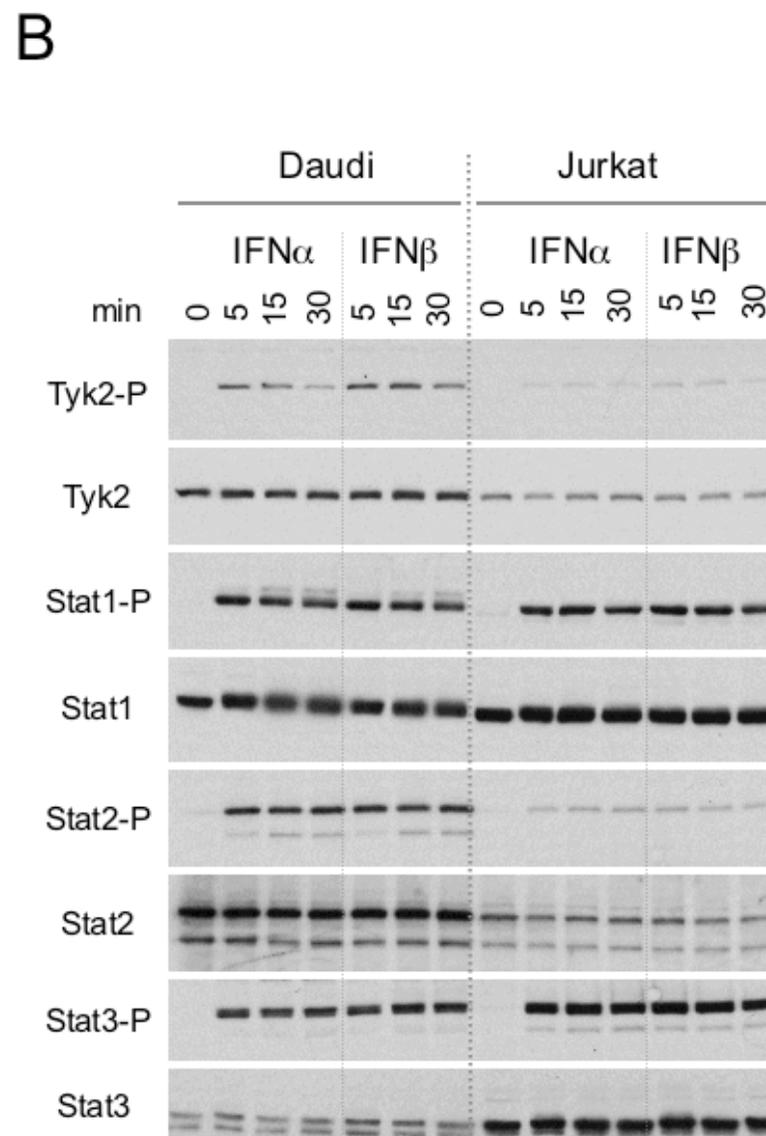
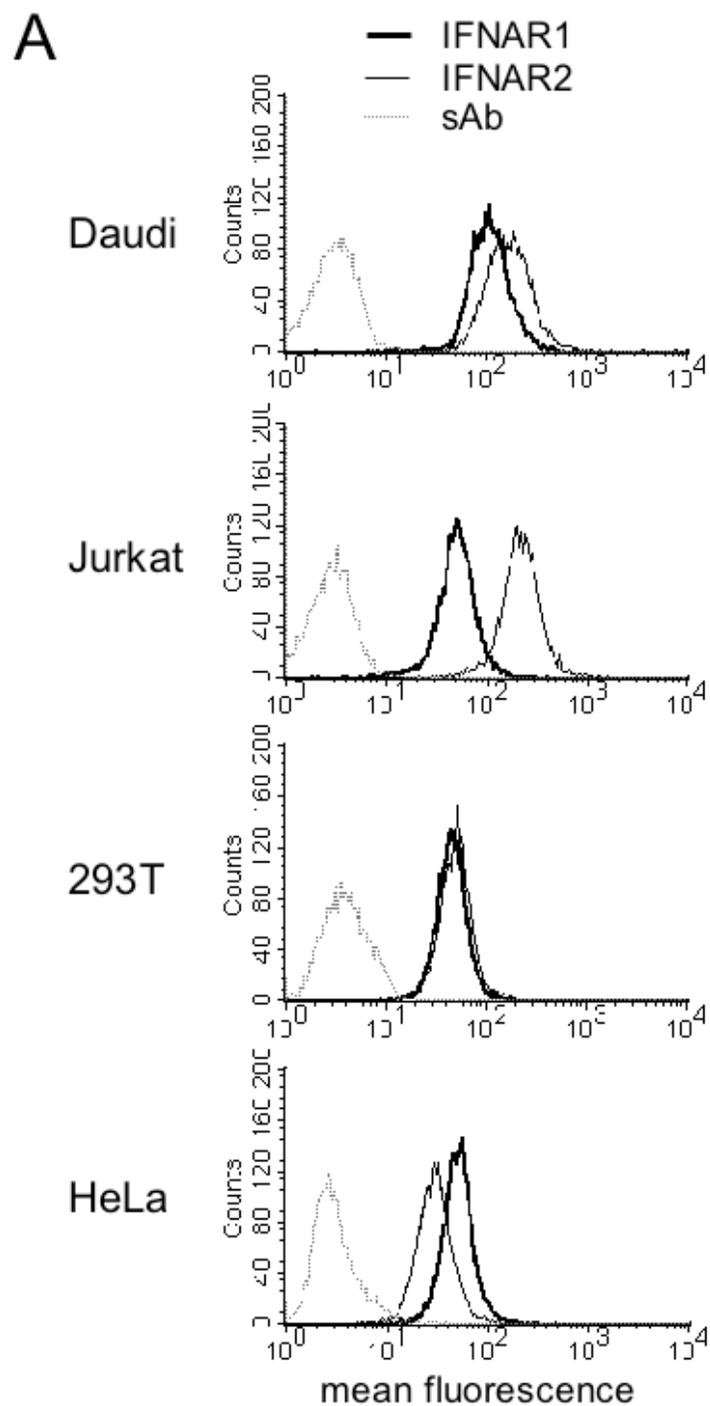


Figure 1 (A and B)

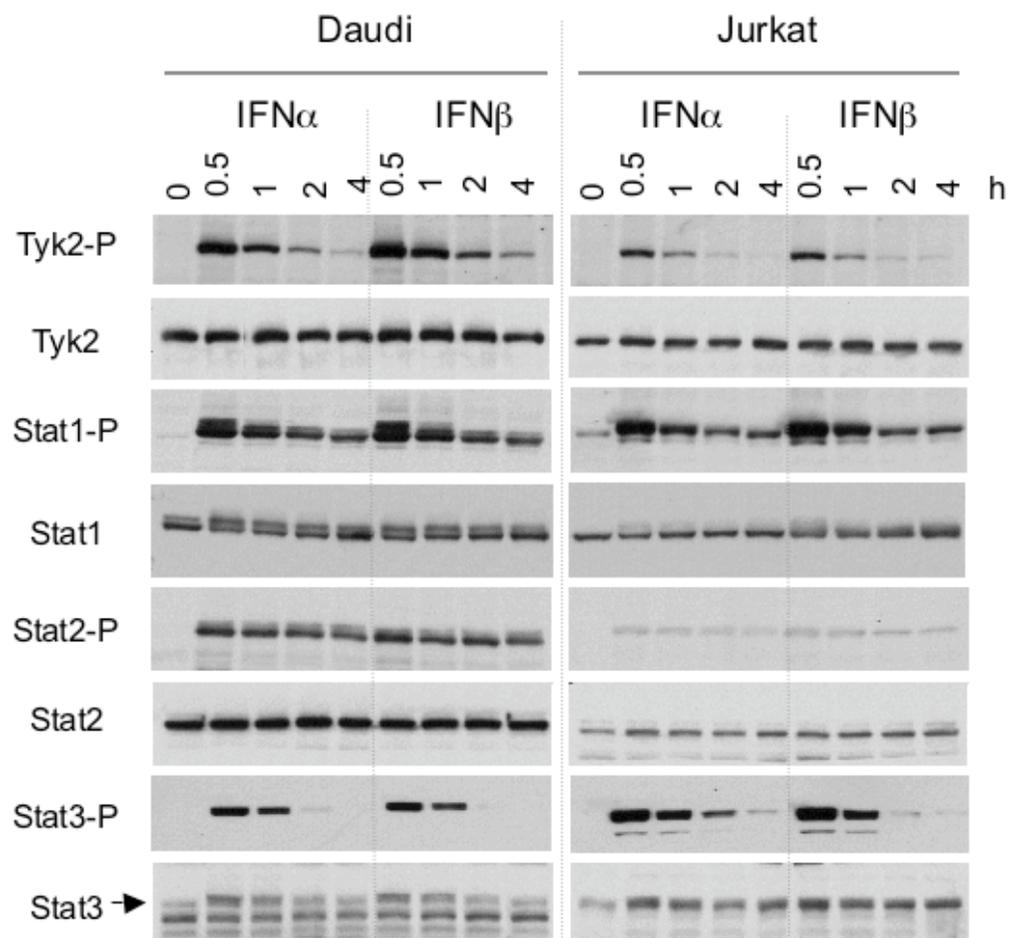


Figure 1 C

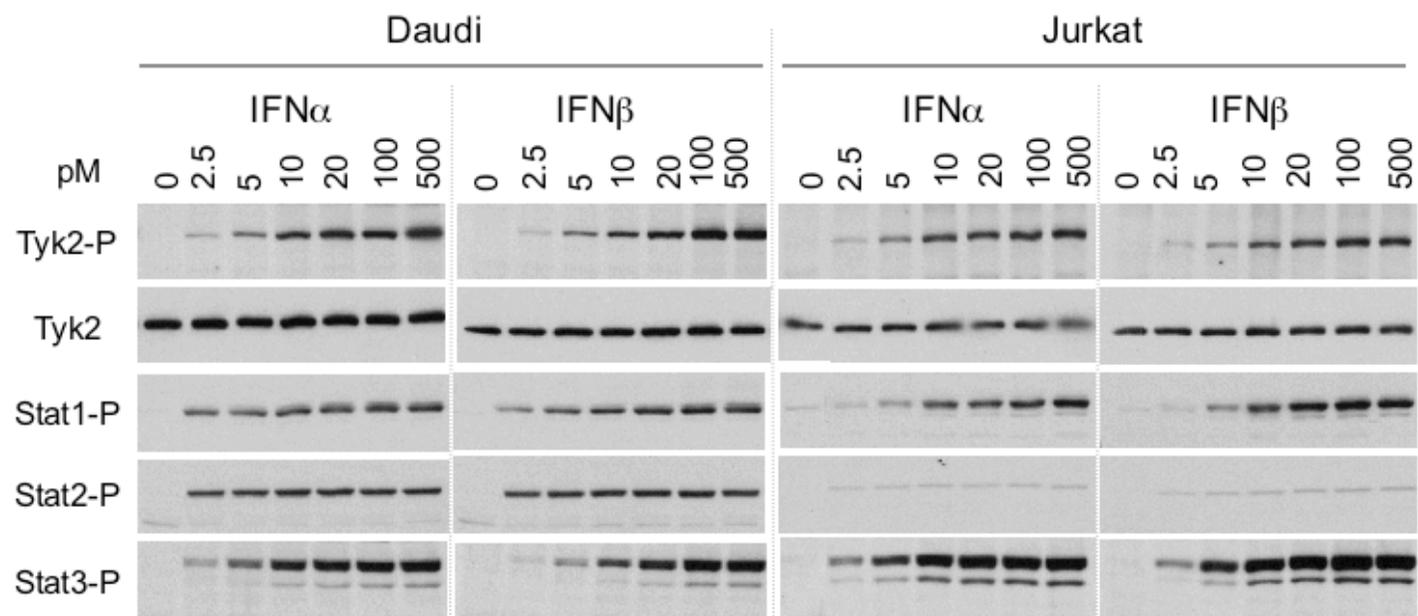


Figure 1 D

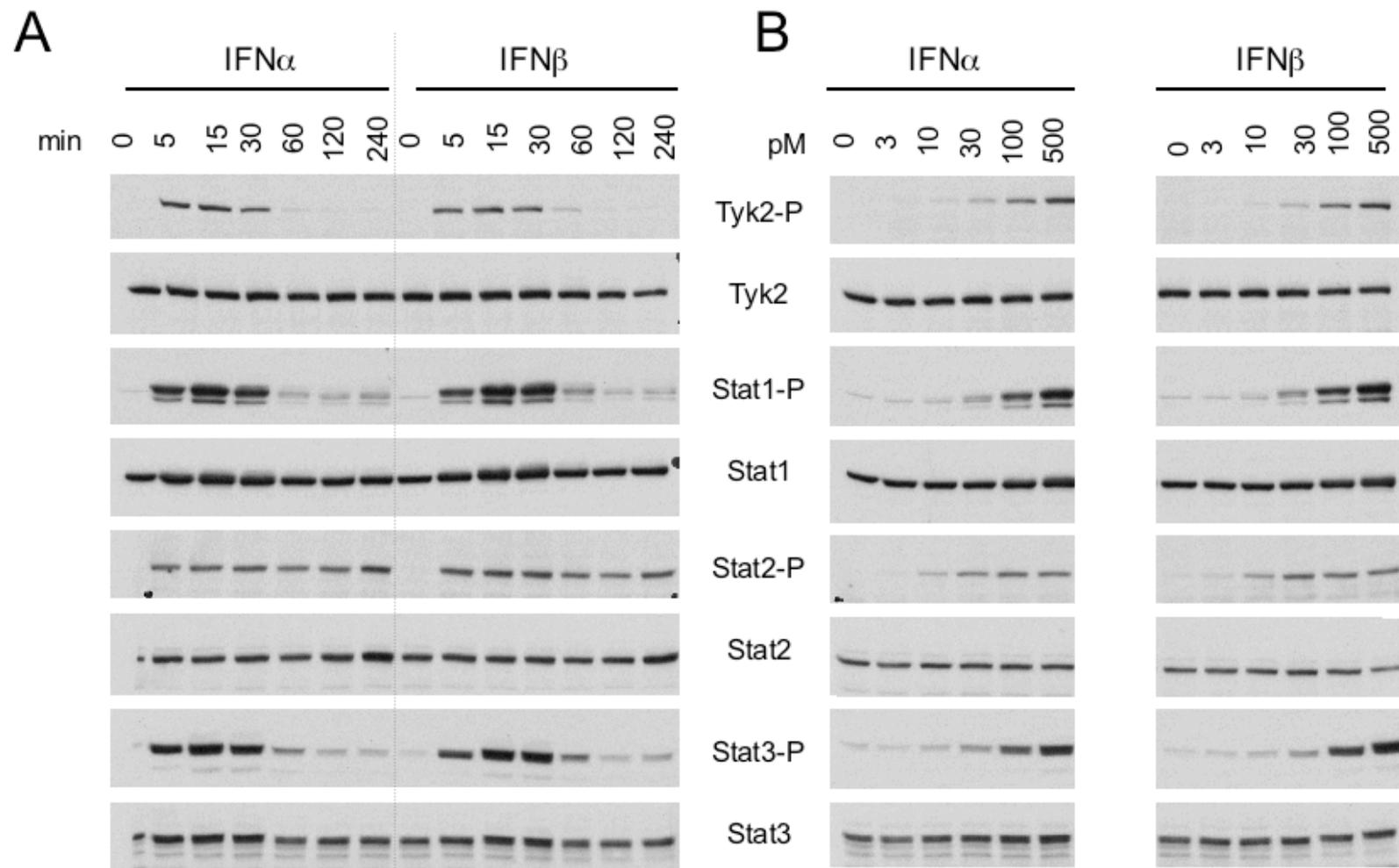


Figure 2

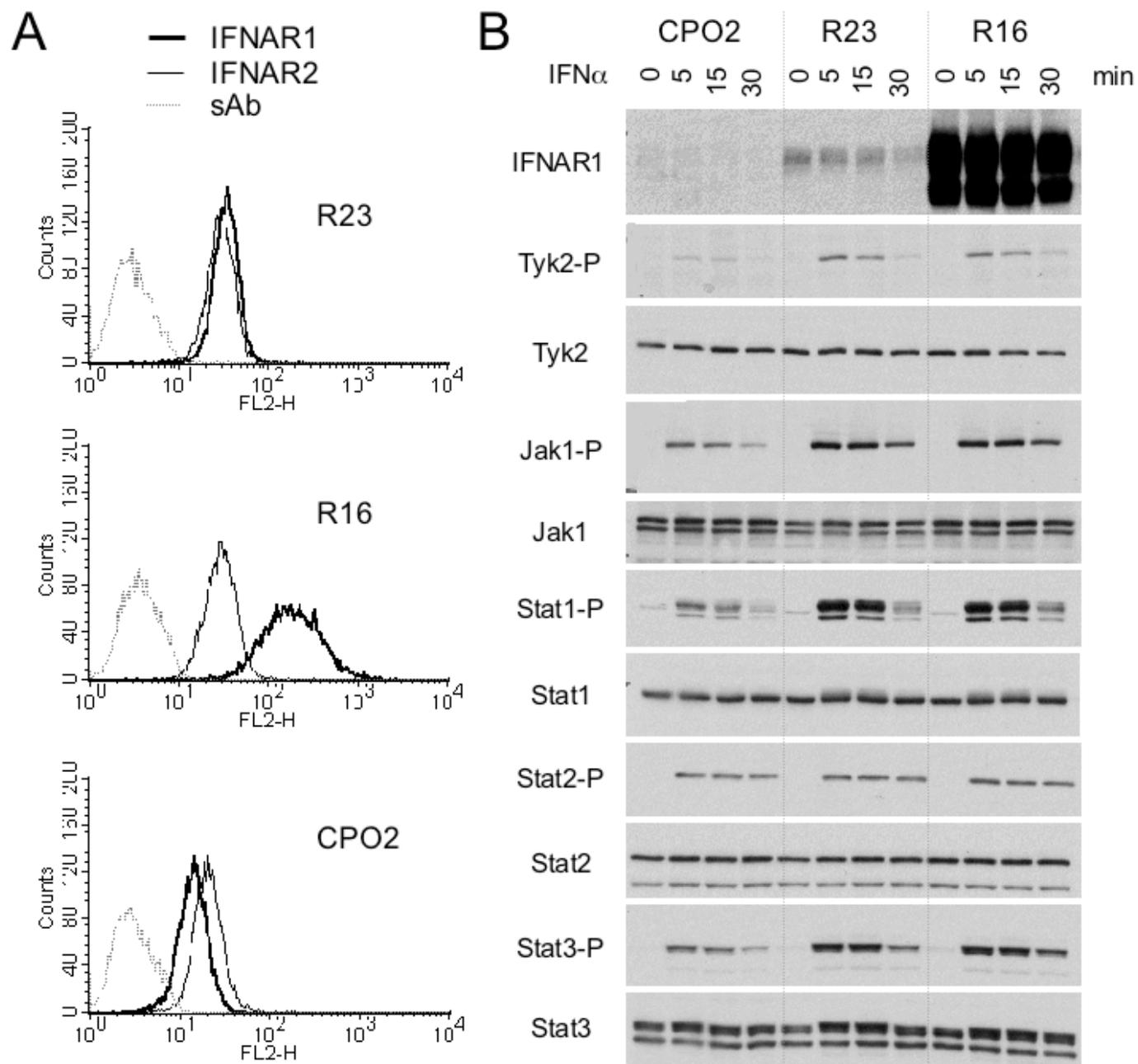


Figure 3

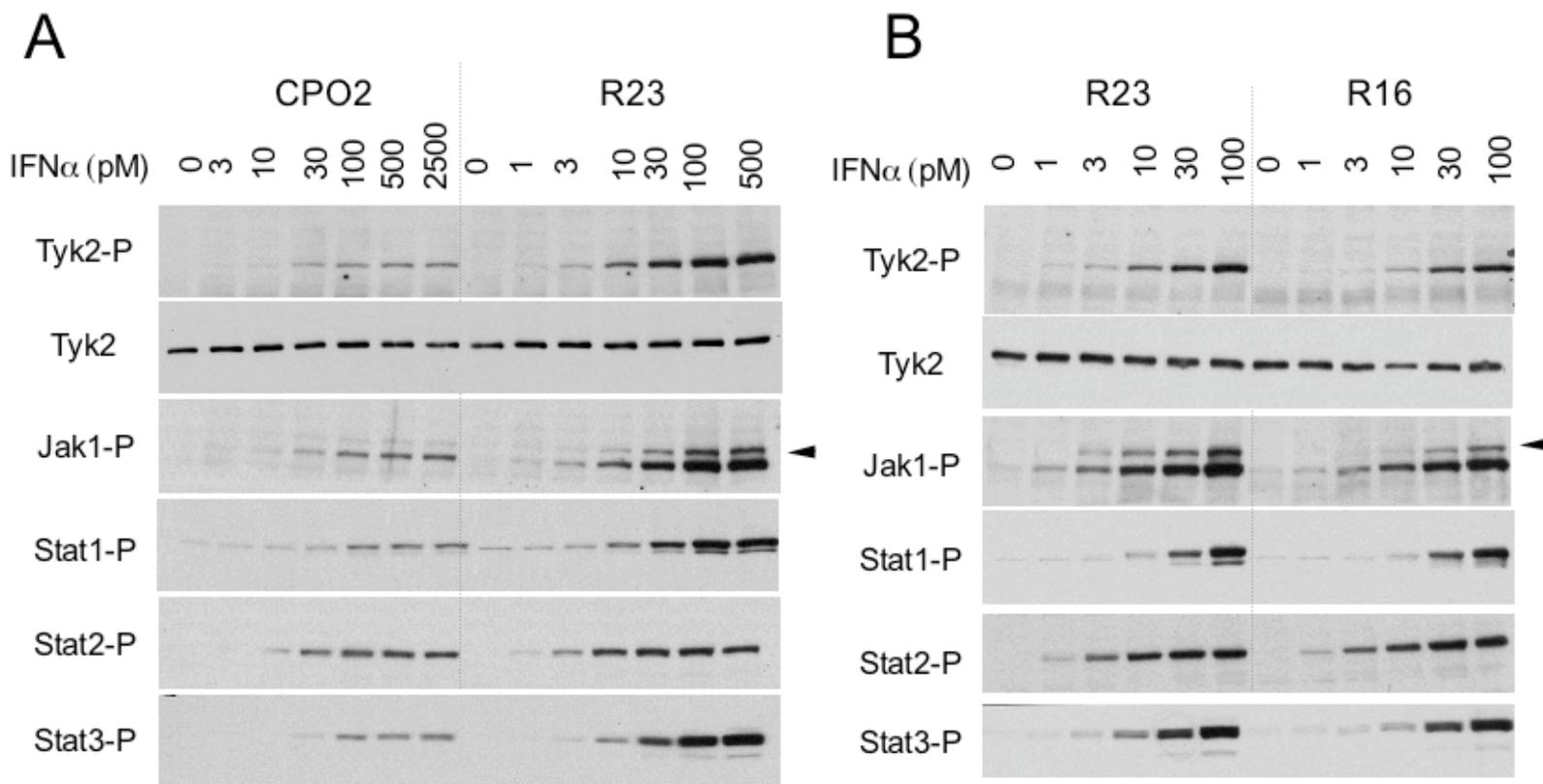


Figure 4

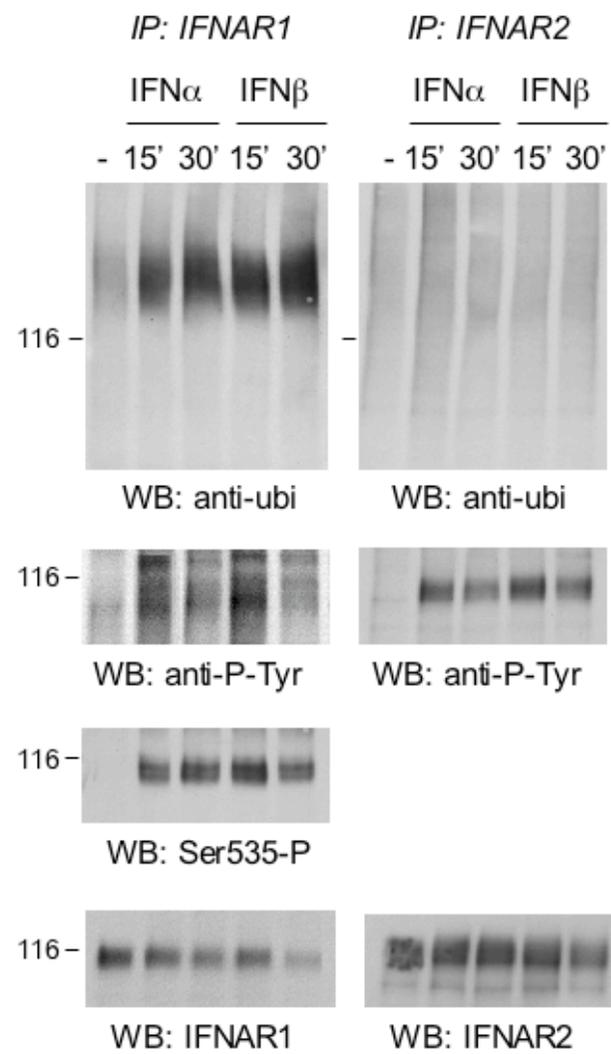


Figure 5

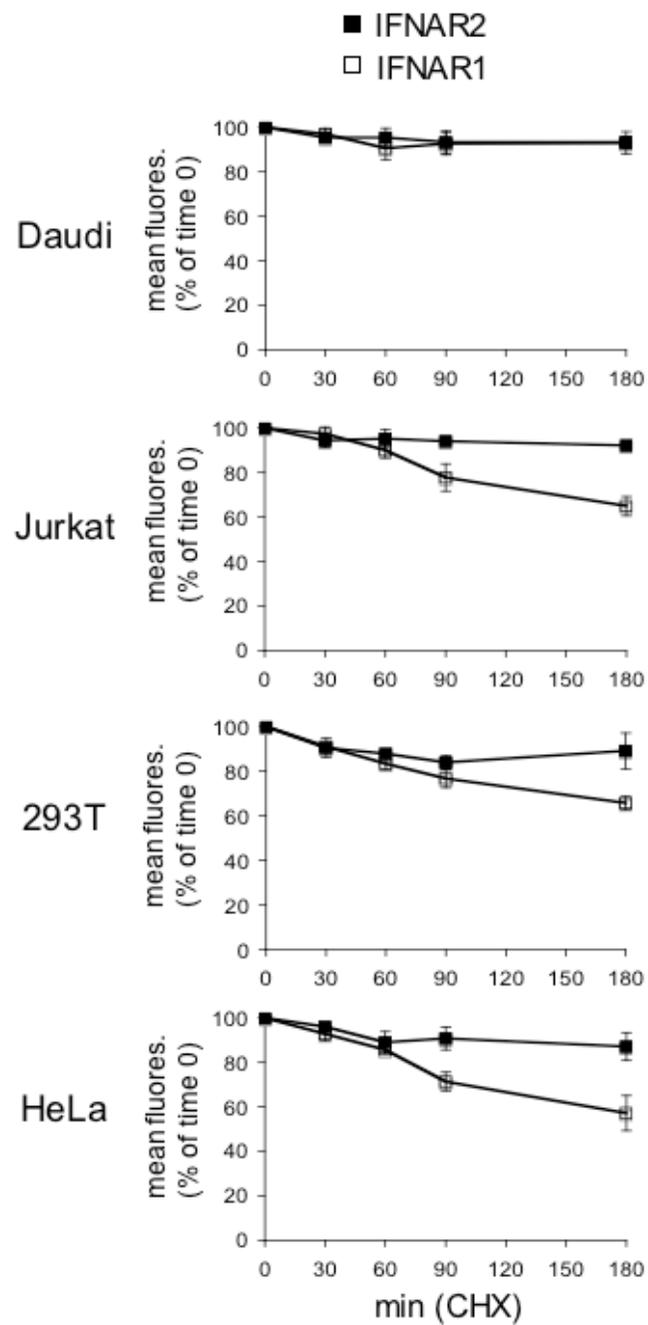


Figure 6

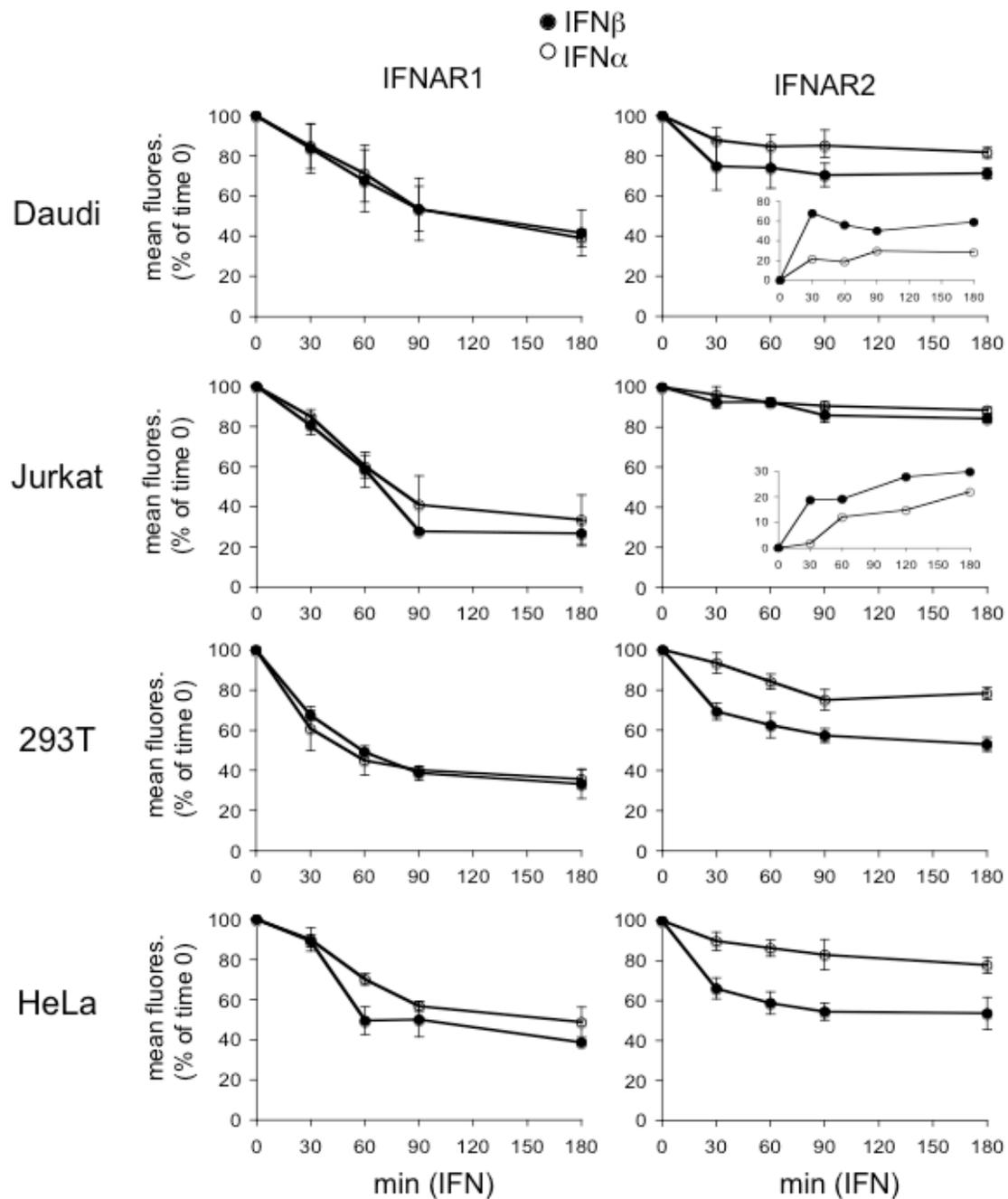


Figure 7A

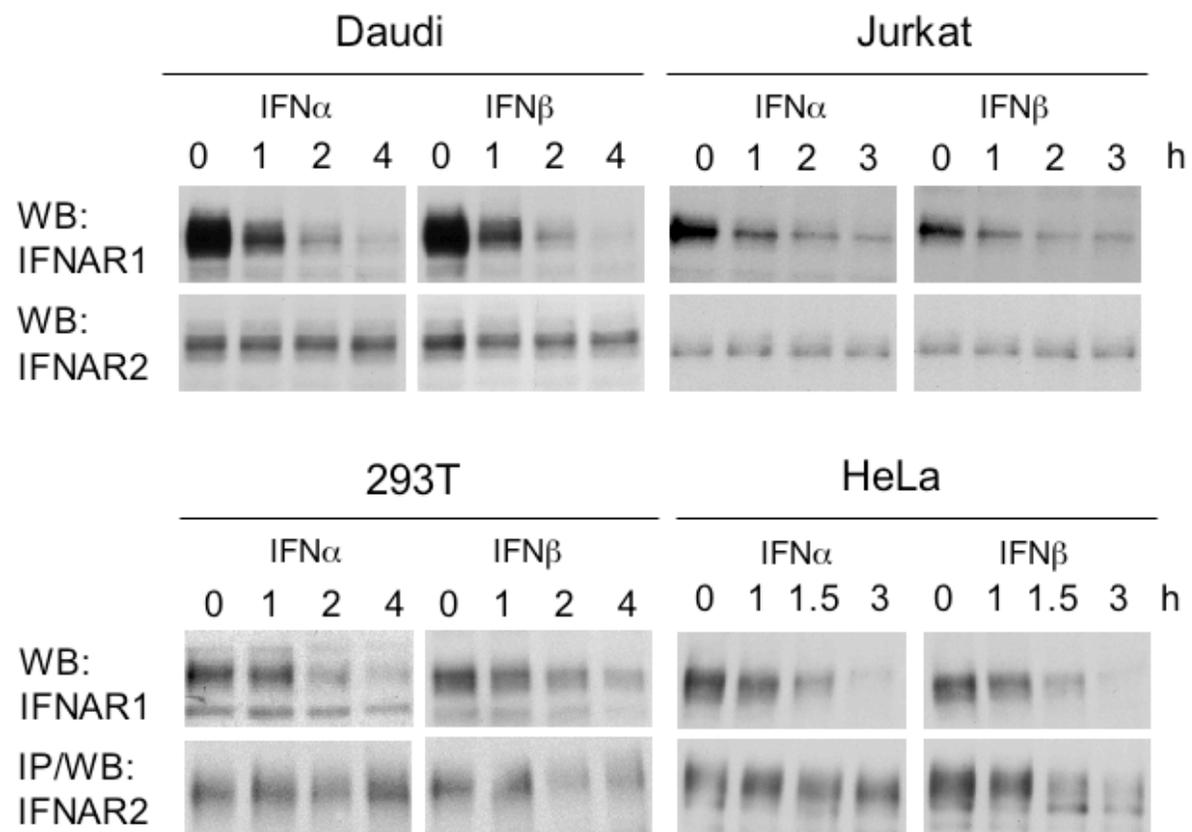


Figure 7B

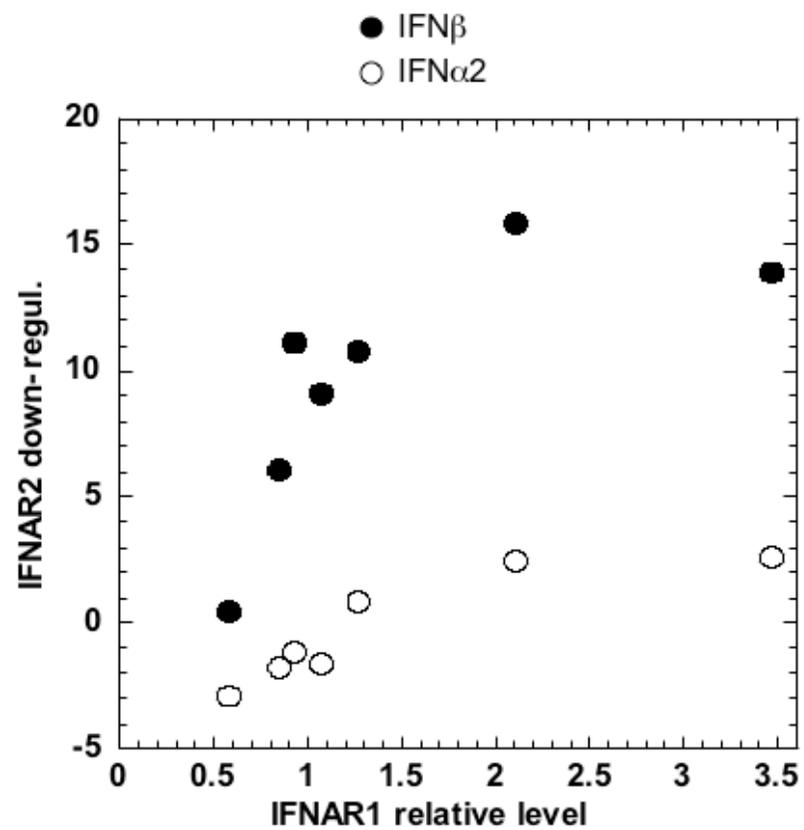


Figure 8

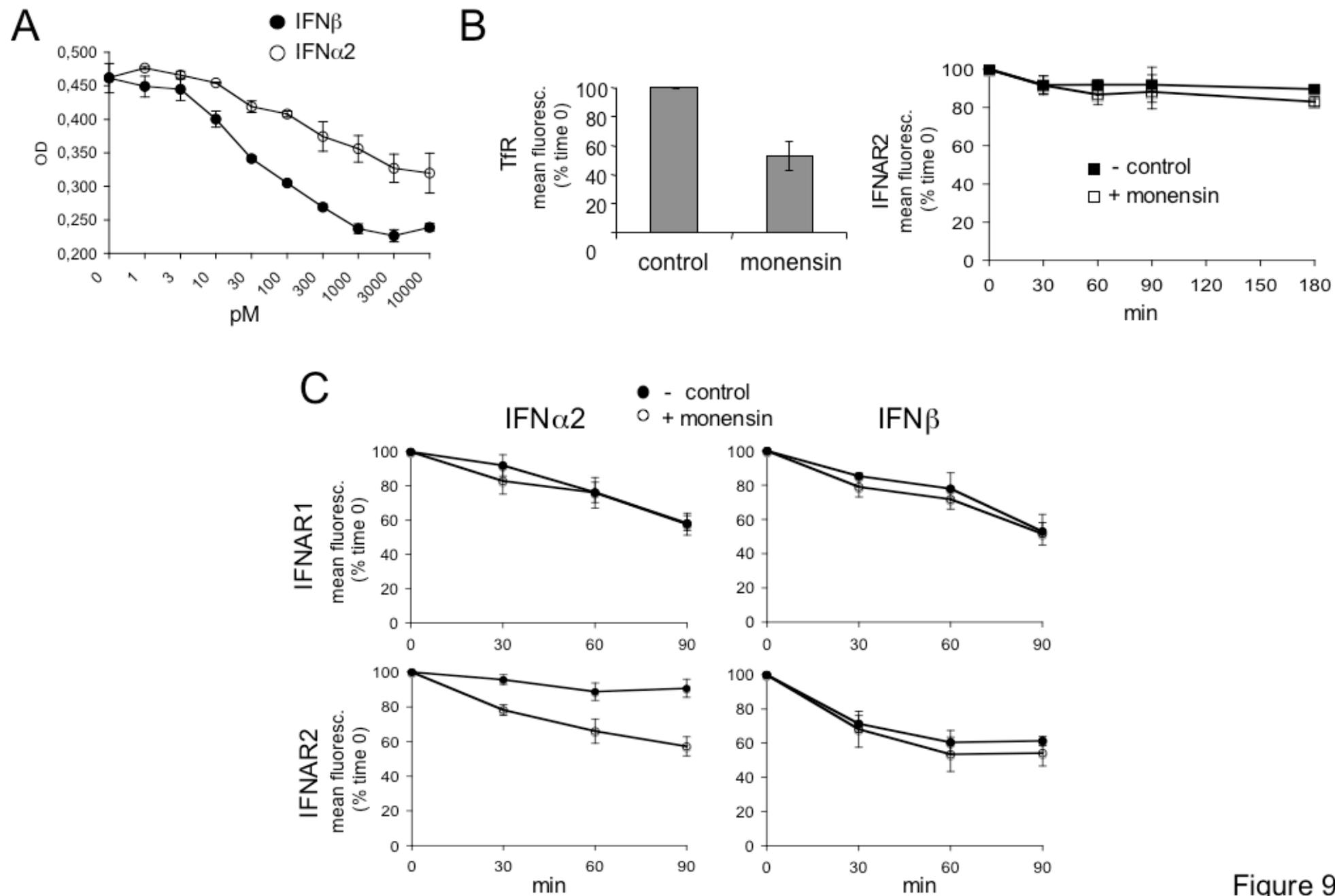


Figure 9