Cytokine 53 (2011) 52–59

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

# The differential activity of interferon-α subtypes is consistent among distinct target genes and cell types

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ARTICLE INFO

*Article history:*
Received 30 March 2010
Received in revised form 29 July 2010
Accepted 15 September 2010

*Keywords:*
IFN-α
Subtypes
Endothelial cells
Fibroblasts
Interferon stimulated genes

ABSTRACT

IFN-α proteins have been described to originate from 14 individual genes and allelic variants. However, the exceptional diversity of IFN-α and its functional impact are still poorly understood. To characterize the biological activity of IFN-α subtypes in relation to the cellular background, we investigated the effect of IFN-α treatment in primary fibroblasts and endothelial cells of vascular or lymphatic origin. The cellular response was evaluated for 13 distinct IFN-α proteins with respect to transcript regulation of the IFN-stimulated genes (ISGs) IFIT1, ISG15, CXCL10, CXCL11 and CCL8. The IFN-α proteins displayed a remarkably consistent potency in gene induction irrespective of target gene and cellular background which led to the classification of IFN-α subtypes with low (IFN-α1), intermediate (IFN-α2a, -4a, -4b, -5, -16, -21) and high (IFN-α2b, -6, -7, -8, -10, -14) activity. The differential potency of IFN-α classes was confirmed at the ISG protein level and the functional protection of cells against influenza virus infection. Differences in IFN activity were only observed at subsaturating levels of IFN-α proteins and did not affect the time course of ISG regulation.

Cell-type specific responses were apparent for distinct target genes independent of IFN-α subtype and were based on different levels of basal versus inducible gene expression. While fibroblasts presented with a high constitutive level of IFIT1, the expression in endothelial cells was strongly induced by IFN-α. In contrast, CXCL10 and CXCL11 transcript levels were generally higher in endothelial cells despite a pronounced induction by IFN-α in fibroblasts.

In summary, the divergent potency of IFN-α proteins and the cell-type specific regulation of individual IFN target genes may allow for the fine tuning of cellular responses to pathogen defense.



## 1. Introduction

Since interferons (IFNs) were first discovered in 1957 [1] a vast array of family members have been identified. They are divided into three major classes, the type I, II and III IFNs, which are structurally related and share distinct properties. While type II and III IFNs are limited to one (IFN-γ) or three (IFN-λ) members, the type I IFN family includes a multitude of proteins including IFN-α, -β, -ε, -κ and -ω [2]. Among those, IFN-β, -ε, -κ and -ω are derived from single genes, whereas IFN-α comprises a remarkable number of subtypes. A total of 14 human non-allelic IFN-α genes are located on the short arm of chromosome 9, giving rise to 12 distinct human IFN-α proteins: IFN-α1, -2, -4, -5, -6, -7, -8, -10, -14, -16, -17 and -21 [3,4]. Overall, the human IFN-α species show 75–99% amino acid sequence identity; the protein sequence of IFN-α13 is identical to IFN-α1.

All type I IFNs bind to the same cell surface receptor complex termed IFNAR which is composed of the two subunits IFNAR1 and IFNAR2. Ligand binding leads to phosphorylation of the associated tyrosine kinases and to downstream signaling events. IFN-stimulated gene factor 3 (ISGF3) consisting of STAT1, STAT2 and the IFN regulatory factor 9 (IRF-9) is activated and binds to IFN-stimulated response elements (ISREs) to initiate the transcriptional activation of numerous IFN-stimulated genes (ISGs). The resulting proteins mediate the biological responses generally attributed to type I IFN, including anti-viral, anti-proliferative, and immunoregulatory activities [5,6].

In this context, the diversity of type I interferons has been debated for many years. Despite the fact that they share a common receptor, the type I IFN species differ in their potency of anti-viral and anti-proliferative action [7–10]. Distinct receptor affinity and/or different interaction sites with the receptor chains have been proposed to account for the variability in the signals transduced [11,12]. With respect to cell responses triggered by IFN-β versus IFN-α, a distinct amino acid composition conserved among the IFN-α subtypes seems to result in a weaker affinity for IFNAR1 and a generally lower biological activity of the alpha subtypes

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doi:10.1016/j.cyto.2010.09.006

[11]. However, quantitative or qualitative differences in gene regulation by IFN-β versus IFN-α are only detectable at subsaturating concentrations, indicating that responses comparable to IFN-β may be elicited by high levels of IFN-α [13].

Investigations on the differential activity of the distinct IFN-α subtypes have generally been limited to a subset of the IFN-α proteins. A number of studies reported highest and lowest levels of anti-viral protection for IFN-α8 and IFN-α1, respectively [7,14]. However, the potency of subtypes seems to vary with the particular IFN function under investigation [12,15]. Regarding the signaling events triggered by IFN-α subtypes there are indications for differential receptor interaction [12,16] and distinct activation of STAT molecules [9] which may result in divergent ISG regulation and functional impact of the IFN-α family members.

Furthermore, the context of a given cell type influences the gene expression pattern in response to type I IFN [17,18]. For example, Indraccolo et al. reported the selective induction of ISGs (CXCL10, CXCL11 and IFIT1) in endothelial cells (ECs) as opposed to primary fibroblasts after IFN-α/β treatment, indicating that the combination of cell specific factors and the type of interferon determine the ultimate cellular response [19].

Thus, to address the diversity of IFN-α subtypes in the context of cell-type specific responses in a comprehensive manner, we have evaluated cell activation by 13 different IFN-α proteins for primary endothelial cells and fibroblasts. ISG regulation at the mRNA and protein level as well as anti-viral activity were investigated in human lymphatic endothelial cells (LECs), in blood vessel endothelial cells (BECs) and in primary fibroblasts isolated from the same donor. Based on this detailed analysis we aimed to establish whether a quantitative and/or qualitative difference in gene regulation and functional impact can be attributed to the IFN-α subtypes, in particular for the ISGs previously reported to be differentially induced in endothelial cells and fibroblasts.

## 2. Materials and methods

### 2.1. Culture and stimulation of primary cells

Primary ECs were isolated from human foreskin samples via proteolytic digest, and purified using anti-CD31 antibody coupled magnetic beads (Invitrogen Corp., Carlsbad, CA). Isolates were cultured in microvascular endothelial growth medium EGM2-MV (Lonza, Cologne, Germany) containing 1 μg/ml fibronectin, 5% FCS and human growth factors without the supplementation of vascular endothelial growth factor (VEGF). For further separation of LECs and BECs, anti-podoplanin antibody coupled magnetic beads were applied. Primary fibroblasts were isolated from foreskin samples of the same donor via anti-CD90 antibody coupled beads and cultured in minimal essential medium (Invitrogen) containing 20% FCS and 1 mM sodium pyruvate. All isolates were characterized by flow cytometry for cell-type specific surface markers, i.e. CD31, CD34 and E-selectin expression (following TNF-α treatment) for ECs, podoplanin and CD90 expression for LECs and fibroblasts, respectively. All cultures showed ⩾95% purity and viability. Repetitive experiments were conducted with isolates from different donors, i.e., represent biological replicates with varying inducibility.

Forty-eight hours before IFN treatment all primary cells were seeded in culture dishes supplied with EGM2-MV without VEGF to reach confluence within 24 h. The medium was then changed to EGM2-MV without growth factor supplementation. The following day cells were stimulated with recombinant IFNs diluted in conditioned medium and cells were harvested at the indicated time points for further analysis. All recombinant IFNs applied (PBL InterferonSource, Piscataway, NJ) were produced in *Escherichia coli* and purified by ion exchange, hydrophobic interaction and size exclusion chromatography. Furthermore, all subtypes were tested and certified to be free of endotoxin (<1 EU/μg).

### 2.2. Analysis of ISG mRNA expression

RNA was isolated from IFN-stimulated ECs and fibroblasts with E.Z.N.A. Total RNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA). Subsequently, 250 ng RNA were reverse transcribed with oligo(dT) primers using the DyNAmo cDNA Synthesis Kit (Finnzymes, Espoo, Finland) and the generated cDNA was diluted 1:25 for further analysis. Real-time PCR was performed using either TaqMan probes or SYBR green incorporation with the respective qPCR MasterMix Plus Low ROX (Eurogentec, Seraing, Belgium). Primer and probe sequences are listed in Table 1. Each sample was assayed in triplicate with the 7500 Fast PCR Detection System (Applied Biosystems, Foster City, CA) for 40 cycles of 5 s at 95 °C followed by 1 min at 60 °C. Transcript levels of target genes were calculated using an on-plate standard dilution series and were normalized to the respective mRNA levels of the housekeeping genes β-actin (ACTB) and $\beta_2$-microglobulin (β2m). The value obtained for the untreated sample was generally set to 1 to calculate changes in mRNA expression upon IFN treatment. To compare the amount of target gene transcripts between different cell types, values were normalized to the same level of housekeeping gene expression in LECs, BECs and fibroblasts, and were subsequently expressed in relation to the untreated LEC control sample. Statistical analysis was performed using SPSS 10.0.1 Software (SPSS, Inc., Chicago, IL). Student's *T*-test was applied for comparison of mean values; differences between "potency categories" of IFN-α subtypes were evaluated by Wilcoxon test.

### 2.3. Analysis of ISG protein expression

Immunoblotting was performed to determine IFIT1 protein expression in cytosolic cell extracts. IFN-treated LECs were harvested in lysis buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 1% NP-40 and the Complete Mini Protease Inhibitor Cocktail (Roche, Indianapolis, IN). After adjusting samples to equal protein concentration, proteins were separated on 10% polyacrylamide/SDS gels and subsequently transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) by semi-dry blotting. Blocking with 5% non-fat milk in PBS with 0.05% Tween20 was followed by immunodetection using a polyclonal α-IFIT1 antibody at 1:1000 dilution (Abcam, Cambridge, UK). To test for equal protein loading a monoclonal α-GAPDH antibody was applied at 1:10,000 dilution (Assay Designs, Ann Arbor, MI). Bound antibody was detected by peroxidase-conjugated secondary antibody and the SuperSignal West Femto Detection System (Thermo Fisher Scientific, Rockford, IL).

For assessing CXCL10 secretion, LEC culture supernatants were analyzed by ELISA (Quantikine; R&D Systems, Minneapolis, MN) according to manufacturer's instructions. Absorption at 450 nm was measured with a Wallac Victor$^3$ multilabel counter (Perkin Elmer Life Sciences, Waltham, MA).

### 2.4. Analysis of influenza virus infection and anti-viral IFN activity

Influenza A virus (PR8) was propagated in Vero cells in serum-free AIMV medium (Invitrogen) containing trypsin (5 μg/ml). LECs were pretreated with IFN (100 pg/ml) for 16 h. Cells were then exposed to the virus for 30 min in serum-free OPTI-PRO medium (Invitrogen) containing 5 μg/ml trypsin. The MOI (multiplicity of infection) was set to 1. Subsequently, viral supernatant was removed and cells were supplied with fresh OPTI-PRO medium.

For the detection of viral protein expression, LECs were harvested 7 h after influenza A infection, permeabilized with

**Table 1**
Sequences of primers and probes applied in real-time PCR analysis.

| Gene | Primer/probe | Sequence |
|---|---|---|
| β2m | Forward primer | 5′-CGCTCCGTGGCCTTAGC-3′ |
| | Reverse primer | 5′-AATCTTTGGAGTACGCTGGATAGC-3′ |
| | Probe (Yakima Yellow/BHQ-1) | 5′-TGCTCGCGCTACTCTCTCTTTCTGGC-3′ |
| ACTB | Forward primer | 5′-CCTGGCACCCAGCACAAT-3′ |
| | Reverse primer | 5′-GCCGATCCACACGGAGTACT-3′ |
| | Probe (6-FAM/ BHQ-1) | 5′-ATCAAGATCATTGCTCCTCCTGAGCGC-3′ |
| IFIT1 | Forward primer | 5′-GATCTCAGAGGAGCCTGGCTAA-3′ |
| | Reverse primer | 5′-TGATCATCACCATTTGTACTCATGG-3′ |
| | Probe (Yakima Yellow/BHQ-1) | 5′-CAAAACCCTGCAGAACGGCTGCC-3′ |
| ISG15 | Forward primer | 5′-GAGAGGCAGCGAACTCATCT-3′ |
| | Reverse primer | 5′-AGGGACACCTGGAATTCGTT-3′ |
| | Probe (6-FAM/ BHQ-1) | 5′-TGCCAGTACAGGAGCTTGTG-3′ |
| CXCL10 | Forward primer | 5′-CGATTCTGATTTGCTGCCTTAT-3′ |
| | Reverse primer | 5′-GGCTTGCAGGAATAATTTCAAGT-3′ |
| CXCL11 | Forward primer | 5′-CTTGGCTGTGATATTGTGTGC-3′ |
| | Reverse primer | 5′-GGGTACATTATGGAGGCTTTC-3′ |
| CCL8 | Forward primer | 5′-AATGTCCCAAGGAAGCTGTG-3′ |
| | Reverse primer | 5′-GGGAGGTTGGGGAAAATAAA-3′ |

IntraPrep reagent (Beckman Coulter, Inc., Fullerton, CA) and further stained with a combination of two fluorescence-labeled monoclonal antibodies detecting viral matrix protein as well as nucleoprotein (Dako, Glostrup, Denmark). ECs were analyzed for viral protein expression with an FC500 flow cytometer (Beckman Coulter).

The amount of virus produced by endothelial cells was evaluated 24 h after initial infection, i.e. the virus released into the serum-free supernatant was harvested and subjected to a standard plaque assay to determine the viral titer as previously described [20]. Results are given in plaque-forming units per ml (pfu/ml) of virus supernatant.

## 3. Results

### 3.1. Induction of ISG mRNA by IFN-α subtypes

Since ISG regulation by type I IFN occurs primarily at the transcript level, we first investigated ISG mRNA expression in response to 13 different IFN-α proteins: IFN-α1, -2a, -2b, -4a, -4b, -5, -6, -7, -8, -10, -14, -16, and -21 (with IFN-α2a and -2b, as well as IFN-α4a and -4b representing allelic variants of the same gene). Primary human LECs, BECs and fibroblasts isolated from the same donor samples were treated with 100 pg/ml of recombinant IFN for 4 h. Transcript levels of ISGs were determined by quantitative RT-PCR and mean values of three independent experiments are shown in Fig. 1 for five selected genes involved in anti-viral and immunoregulatory IFN functions (IFIT1, ISG15, CXCL10, CXCL11, CCL8). Data were calculated as fold induction of ISG mRNA by IFN-stimulation in relation to the respective untreated control (Fig. 1A–E). Furthermore, we aimed to compare the actual transcript levels present in LECs, BECs and fibroblasts. Since expression of the housekeeping genes $\beta_2$-microglobulin and β-actin did not vary substantially between the three cell types (data not shown), the ISG values were normalized to a constant level of housekeeping gene transcripts (Fig. 1G–K).

The cell-type specific response to IFN-α was apparent with the individual target genes rather than the IFN-α subtypes applied; differences were more distinct between endothelial cells and fibroblasts than between lymphatic and blood vessel derived ECs. The basal expression level varied significantly for three of the investigated IFN target genes (Fig. 1L); IFIT1 mRNA was most abundant in fibroblasts whereas highest constitutive expression of CXCL10 and CXCL11 was found in BECs. Interestingly, the selected target genes also showed distinct inducibility by IFN-α (irrespective of subtype) in the three cell types investigated (Fig. 1F). IFIT1 mRNA was highly induced in LECs and in BECs upon IFN-α treatment, whereas fold induction was substantially lower in fibroblasts (Fig. 1A and F). However, when comparing the transcript levels present after IFN-stimulation (Fig. 1G) we found that IFIT1 mRNA was equally abundant in fibroblasts and ECs due to the high level of basal IFIT1 expression in human skin fibroblasts (Fig. 1L). In contrast, fibroblasts showed pronounced induction of CXCL10 and CXCL11 by IFN-α (Fig. 1C, D and F) but the cellular transcript levels remained low in comparison to endothelial cells (Fig. 1I and J) which presented with a higher level of basal expression (Fig. 1L). ISG15 mRNA concentration was comparable in all three cell types following IFN-stimulation (Fig. 1H) with LECs showing low baseline expression (Fig. 1L) but the highest induction values (Fig. 1B and F). CCL8 induction (Fig. 1E and F) and IFN-induced transcript levels (Fig. 1K) were distinctly higher in fibroblasts than ECs.

With respect to the induction profile of these five genes in response to the various IFN-α subtypes, a recurring pattern of stimulation was observed, i.e., individual IFN-α proteins showed consistently high, intermediate or low activity irrespective of target gene or cellular background. Therefore, we compared the relative potency of IFN-α subtypes in ISG induction in relation to IFN-α1 (values set to 1), which generally showed the lowest activity. The data collected for the five ISG transcripts were assembled in a box plot illustration (Fig. 2A) and demonstrated a reproducible potency of IFN-α subtypes consistent among cell types. Hence, we further combined the values obtained for the three different cell types (Fig. 2B) to yield an induction profile for the investigated IFN-α proteins. Interestingly, three categories of biological activity emerged, i.e., groups of high, medium and low ISG induction. IFN-α2b, -6, -7, -8, -10 and -14 were the most potent inducers of ISG expression with a 4–5-fold higher median level than elicited by IFN-α1. The subtypes IFN-α2a, -4a, -4b, -5, -16 and -21 displayed an intermediate capacity to induce ISGs (2–3-fold higher) as compared to IFN-α1 which consistently gave the lowest induction values (set to 1). To further substantiate this classification, a comparison of ISG induction values was performed by Wilcoxon test and revealed that IFN-α subtypes of the same cluster were significantly different from all members of the other two "potency categories" ($p < 0.05$ following Bonferroni-Holm correction).

### 3.2. Induction of ISG protein by IFN-α subtypes

To investigate whether the distinct potency of IFN-α subtypes to induce ISG transcripts was also reflected in the amount of generated protein, a member of each cluster was chosen for subsequent experiments: IFN-α1 with low activity, IFN-α4b with intermediate and IFN-α2b with high activity. Since IFN-α subtypes displayed a consistent induction pattern in all cell types, the investigations were limited to LECs. After stimulation with recombinant IFNs, cells were analyzed for mRNA (at 4 or 8 h) as well as protein expression (at 4, 8 or 24 h).

IFIT1 protein detectable in cytosolic extracts after 8 h of IFN-stimulation closely correlated with IFIT1 transcript levels (Fig. 3). While IFN-α2b treatment led to a strong induction of IFIT1 protein, the expression was substantially lower for IFN-α4b stimulation. IFN-α1 showed particularly weak induction of IFIT1 protein.

Furthermore, CXCL10 protein levels were evaluated in LEC supernatant following IFN-stimulation. Protein expression (after 4 h) was in accordance with transcript levels and reflected the ascribed potency of IFN-α subtypes: while IFN-α1 led to minor CXCL10 secretion, IFN-α4b showed moderate and IFN-α2b high

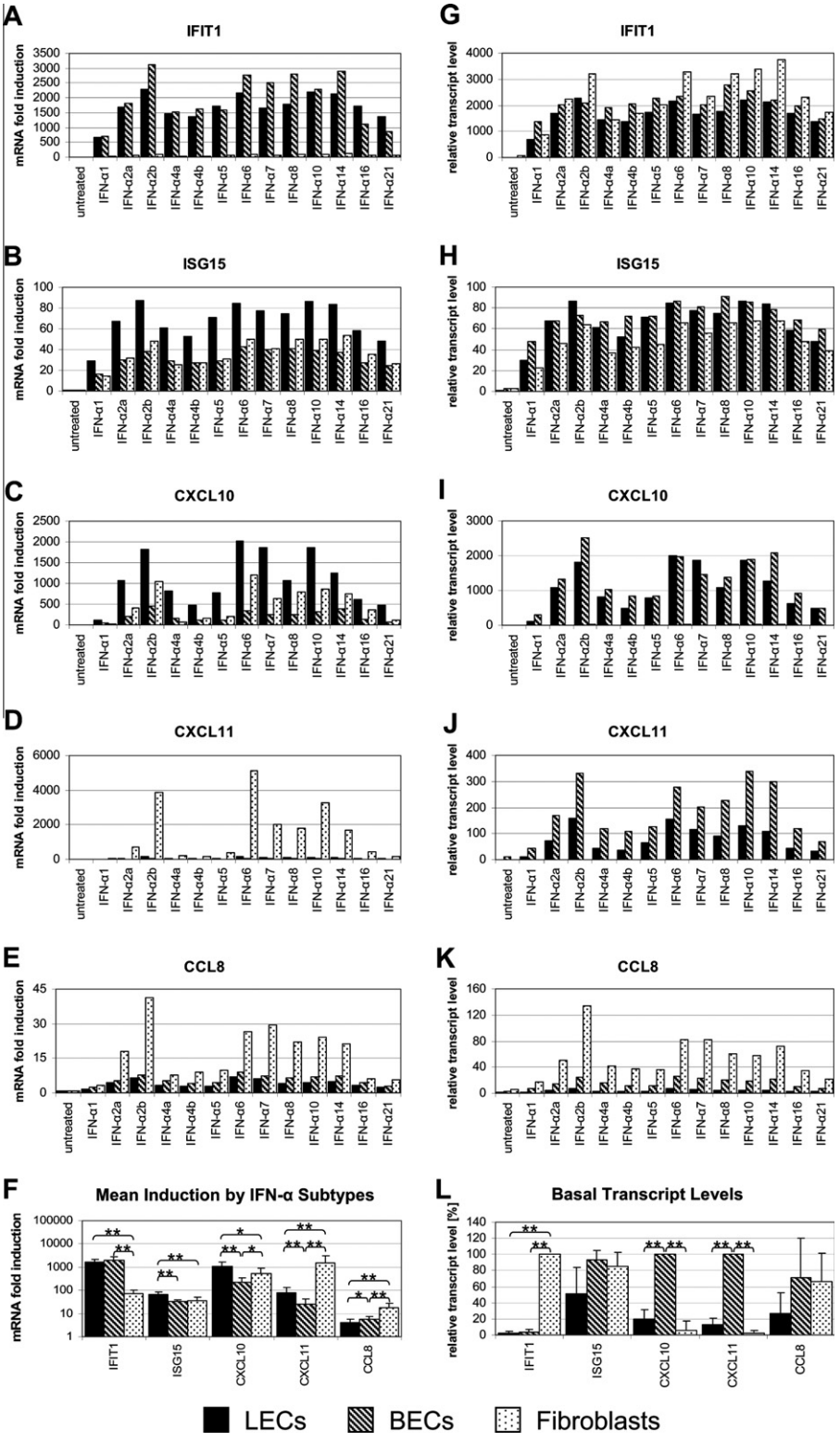


**Fig. 1.** ISG mRNA induction and transcript levels upon IFN-α stimulation of LECs, BECs and fibroblasts. Primary cells were stimulated with 100 pg/ml of each IFN-α subtype. Total RNA was analyzed by quantitative RT-PCR after 4 h of stimulation. The fold induction of IFIT1 (A), ISG15 (B), CXCL10 (C), CXCL11 (D), and CCL8 (E) mRNA was calculated in relation to the respective untreated control sample of each cell type. Three independent experiments were performed, the mean values of all experiments are given. Due to high differences in the overall induction values achieved in the three assays (biological variation) the standard deviations are not shown. (F) The mean induction value in response to all IFN-α subtypes was determined for the five target genes in the three distinct cell types. Mean and SD are given; significant differences (based on Student's *T*-test) in ISG induction between cell types are indicated by one ($p < 0.05$) or two ($p < 0.01$) asterisks. (G–L) Since housekeeping gene expression (i.e. transcript level in relation to total RNA) did not vary substantially between LECs, BECs and fibroblasts (data not shown), ISG values were further normalized to a constant level of housekeeping gene transcripts to be able to compare ISG mRNA expression between cell types (''relative transcript level''). IFIT1 (G), ISG15 (H), CXCL10 (I), CXCL11 (J), and CCL8 (K) values were then expressed in relation to the untreated LEC sample (set to 1). (L) To illustrate the variance in basal ISG expression between cell types, ISG transcript levels of untreated cells were expressed in percent of the highest recorded value. Mean and SD of the three experiments are shown. Differences in basal ISG transcript levels between cell types were evaluated by Student's *T*-test; significance levels of $p < 0.05$ and $p < 0.01$ are indicated by one and two asterisks, respectively.

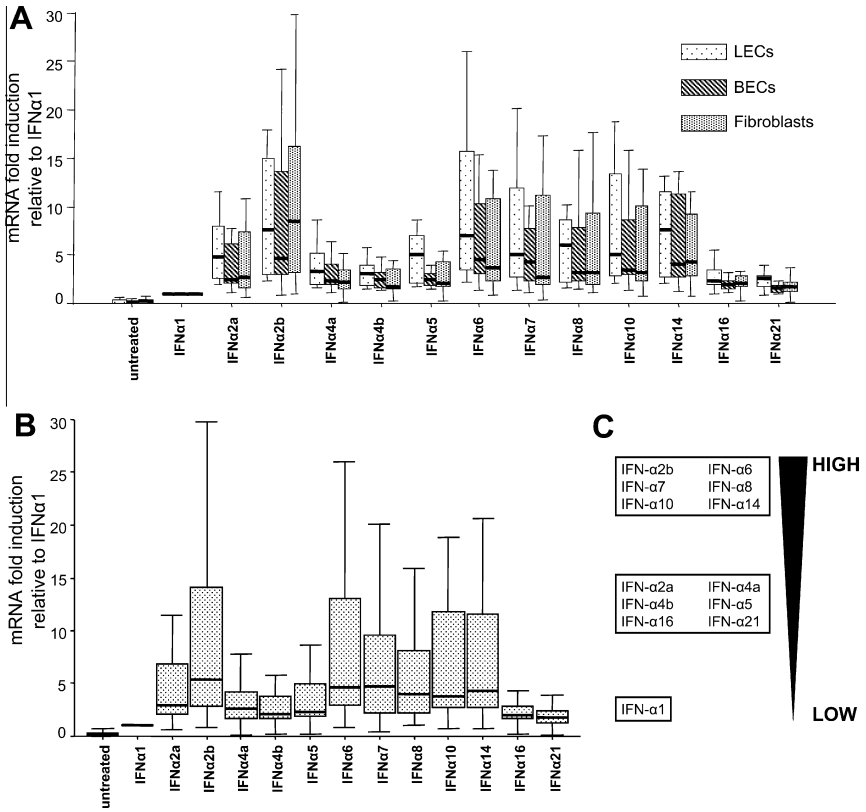


**Fig. 2.** Relative potency of IFN-α subtypes in ISG stimulation. Induction values for IFIT1, ISG15, CXCL10, CXCL11 and CCL8 were calculated in relation to IFN-α1 levels set to 1. Data distribution is illustrated by box plot and is given separately for LECs, BECs and fibroblasts (A) or for the combined set of data (B). The deduced classes of high, medium and low ISG inducers are summarized in (C).

stimulatory capacity. CXCL10 protein was stable in EC supernatant for 24 h.

### 3.3. Anti-viral activity of IFN-α subtypes

The distinct potency of IFN-α subtypes was further evaluated with respect to biological function. Since the investigated ISGs are functionally related to anti-viral defense and immunoregulation, we tested the capacity of IFN-α subtypes to prevent virus replication. IFN-α1, -2b and -4b were applied in representation of the established "potency categories". After 16 h of pretreatment with IFN, LECs were infected with influenza A wildtype virus. The expression of viral matrix and nucleoprotein was determined in LECs after 7 h; virus released into the supernatant was detected at 24 h post infection. The anti-viral activity of the investigated subtypes correlated with their capacity to regulate ISG mRNA and protein (Fig. 4). IFN-α1 exhibited the weakest potency to protect LECs against influenza infection and propagation whereas IFN-α4b and IFN-α2b showed intermediate and strong anti-viral activity, respectively.

### 3.4. Comparison of time course and dose response to IFN-α subtypes

To establish whether the differences in potency among IFN-α subtypes could be leveled by increasing the concentration of available ligand, a dose response experiment was conducted for the three representatives of IFN-α "potency categories". LECs were treated with increasing concentrations of IFN-α1, -2b and -4b; mRNA levels of IFIT1 and ISG15 were determined after 4 h. The maximum level of transcript expression was comparable between subtypes but was achieved at distinct ligand concentrations (Fig. 5). The dose required for half-maximal ISG induction was 40 pg/ml for IFN-α2b as compared to 200 pg/ml and 400 pg/ml for IFN-α4b and IFN-α1, respectively.

With respect to potential differences in signal transduction in response to IFN-α subtypes, the kinetics of ISG mRNA induction was also investigated (Fig. 6). LECs were exposed to comparable biological activities of IFN-α proteins as established before, i.e., were stimulated with 400 pg/ml IFN-α1, 200 pg/ml IFN-α4b and 40 pg/ml IFN-α2b for 2 to 24 h. IFIT1 transcript levels exhibited a sharp peak at 4 h post stimulation and were close to baseline after 8 h. An identical time course was observed for the three IFN-α subtypes applied. ISG15 mRNA peaked at 4 to 8 h; 24 h post stimulation ISG15 transcript levels were still elevated. The kinetics of ISG15 mRNA induction was comparable between IFN-α subtypes.

## 4. Discussion

The exceptional diversity of type I interferons and their functional distinction is an intriguing and largely unanswered question in the field of interferon research. To date 18 human type I IFN genes have been described, with 14 genes representing the IFN-α family [2,21]. The IFN proteins produced in response to a pathogen challenge vary with the particular stimulus and the affected cell type. Plasmacytoid dendritic cells and peripheral blood mononuclear cells are the major sources of IFN-α production, and have been shown to secrete all subtypes of IFN-α [22,23]. Interestingly, IFN-α1 has repeatedly been reported to be among the major subtypes produced [22,24,25].

When we analyzed the potency of 13 different IFN-α proteins in ISG regulation and anti-viral protection we found a reproducible pattern of activity in LECs, BECs and fibroblasts, i.e. the subtypes

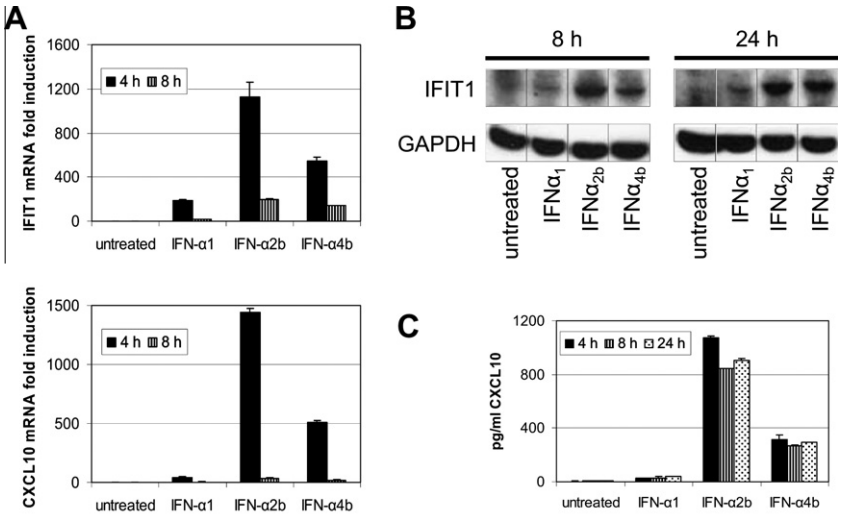


**Fig. 3.** Comparison of ISG transcript and protein levels after IFN-α stimulation. LECs were treated with 100 pg/ml of IFN-α1, -2b, or -4b. At the indicated time points, cells and culture supernatant were harvested. Cell samples were subjected to concomitant mRNA and protein isolation. (A) IFIT1 and CXCL10 mRNA levels were analyzed by real-time RT-PCR. Data is given as mean and standard deviation of triplicate samples. (B) For analysis of IFIT1 protein expression, cytosolic extracts were subjected to SDS–PAGE and immunoblotting with anti-IFIT1 antiserum. For loading control, membranes were re-probed with anti-GAPDH antibody. (C) Culture supernatant was analyzed for CXCL10 protein by ELISA.

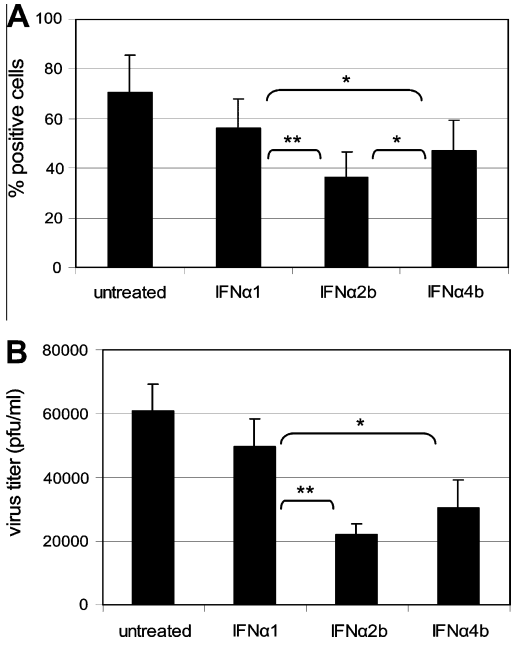


**Fig. 4.** Antiviral activity of IFN-α subtypes. LECs were exposed to IFN-α for 16 h or were left untreated before infection with influenza A (PR8 wt) virus at an MOI equaling 1. (A) Cells positive for intracellular expression of viral matrix and nucleoprotein were determined 7 h post infection by flow cytometry ($n = 6$). (B) The amount of virus released into the supernatant 24 h after virus infection was determined by a standard plaque formation assay ($n = 4$). Data presented are the mean and SD of all experiments performed. Differences in anti-viral activity of IFN-α subtypes were evaluated by Student's *T*-test; significance levels of $p < 0.05$ and $p < 0.01$ are indicated by one and two asterisks, respectively.

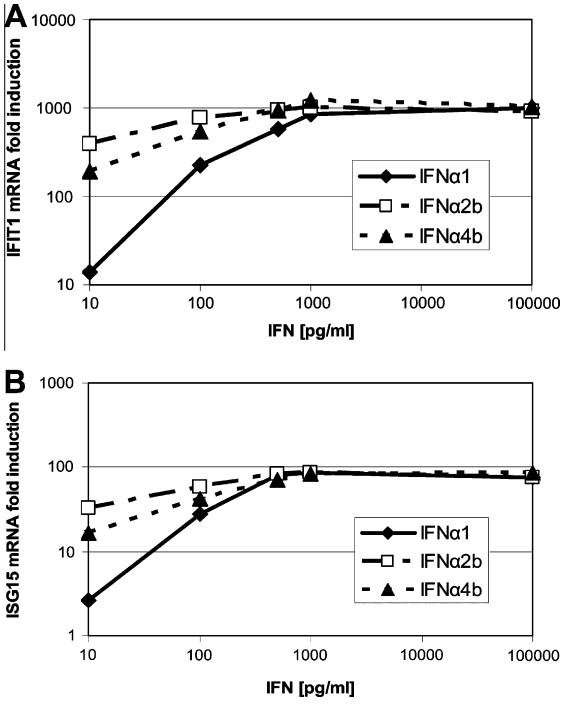


**Fig. 5.** Dose dependence of ISG regulation by IFN-α subtypes. LECs were treated with increasing concentrations of IFN-α1, -2b, or -4b for 4 h. Transcript levels of IFIT1 (A) and ISG15 (B) were analyzed by real-time RT-PCR. Data is given as mean and standard deviation of triplicate samples.

displayed a distinct potency which was consistent in all three cell types and for the genes investigated. To exclude the possibility of variation due to incorrect protein concentration we confirmed the protein content of recombinant IFN preparations by protein gels (data not shown); minor deviations from the calculated concentration did not correlate with the stimulatory potency of IFN-α proteins.

IFN-α1 generally exhibited the weakest activity. Thus, ISG induction values were set in relation to IFN-α1 and were combined in statistical analysis of ISG transcript levels. Three categories of inducers emerged with high (IFN-α2b, -6, -7, -8, -10, -14), intermediate (IFN-α2a, -4a, -4b, -5, -16, -21) and weak activity (IFN-α1). The differential activity of IFN-α subtypes was confirmed at the protein and functional (anti-viral protection) level for three representatives of the "potency categories". These results are in line with previous reports that were focused on a limited number of IFN-α subtypes. Highest and lowest levels of anti-viral protection

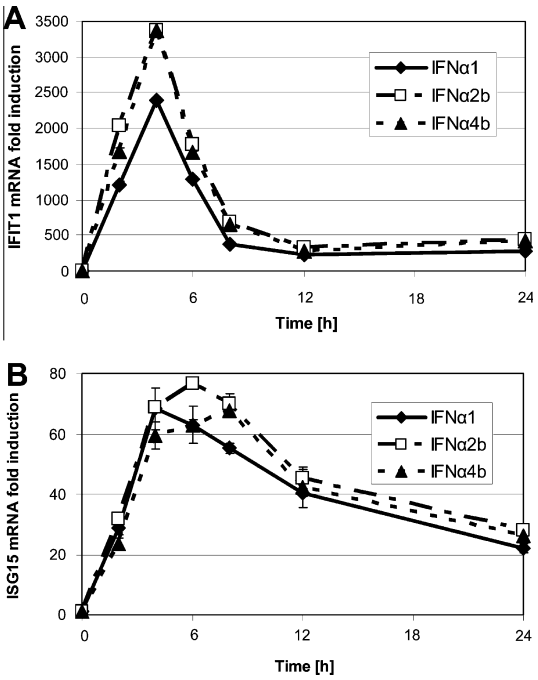


**Fig. 6.** Kinetics of ISG induction in response to IFN-α subtypes. LECs were exposed to 400 pg/ml of IFN-α1, 200 pg/ml of IFN-α4b, or 40 pg/ml of IFN-α2b for 2, 4, 6, 8, 12 and 24 h. Induction of IFIT1 (A) and ISG15 (B) mRNA was analyzed by real-time RT-PCR. Data is given as mean and standard deviation of triplicate samples.

or anti-proliferative activity were repeatedly attributed to IFN-α8 and IFN-α1, respectively [7,14,26]. It is of interest to note that the IFN-α subtype with lowest activity is produced at highest amounts in response to a pathogen challenge which may allow for a careful adjustment of cellular activation during defense.

With respect to the distinct activity of IFN-α subtypes, it has been established that the ligands bind to the IFNAR complex with different affinities. IFN-α1 was described with the lowest affinity to IFNAR2 [27], an observation coinciding with the weakest activity of IFN-α1 in our experiments. Furthermore, IFN-α subtypes have been shown to interact with discrete binding sites on IFNAR2 [12]. These differences may result in divergent signaling events elicited by IFN-α proteins with respect to STAT activation [9]. However, we found that a comparable level and time course of ISG induction is achieved by subtypes of distinct potency (IFN-α1, -2b, and -4b) when ligand concentrations are increased. Thus, qualitative and quantitative differences in IFN signals are only effective at subsaturating levels of IFN-α proteins and may be of minor importance at local sites of high IFN concentration in inflammatory reactions.

While the potency of IFN-α subtypes was strikingly consistent among the three cell types investigated in this study, the selected target genes (IFIT1, ISG15, CXCL10, CXCL11 and CCL8) displayed a high degree of cell-type specific regulation. It should be noted that LECs, BECs and fibroblasts were isolated from the same donor tissue to minimize the influence of biological variation. Regarding the respective function of the selected ISGs, IFIT1 is known to contribute to anti-viral protection by IFNs [28,29] and ISG15 is reported to have anti-viral as well as immunoregulatory effects [30,31]. CCL8, CXCL10 and CXCL11 are chemokines crucially involved in inflammatory processes. The selection of ISGs was based on a previous analysis of genes differentially regulated in endothelial cells and fibroblasts by type I IFN [19]. Indraccolo et al. reported the preferential expression of CXCL10, CXCL11, and IFIT1 in ECs. However, these authors did not discriminate between fold induction and transcript levels. We found that LECs and BECs contained substantially higher levels of CXCL10 and CXCL11 mRNA following stimulation with IFN-α despite a comparable or even higher degree of gene induction observed in fibroblasts. In contrast, fold induction of IFIT1 transcripts was noticeably more pronounced in endothelial cells, but comparable mRNA levels were detectable in all cell types due to the high basal expression of IFIT1 in fibroblasts. Thus, the presence of cell-type specific factors seems to greatly influence the scale of ISG expression with respect to the basal and inducible ISG level. A similar observation has been reported for the differential expression of CXCL10 in dendritic cells versus T-lymphocytes [32], indicating that the cell-type specific regulation of individual IFN target genes may allow for the fine tuning of cellular responses and contributions to pathogen defense.

Thus, with respect to ISG regulation we have shown that IFN-α subtypes elicit differential responses at subsaturating levels with the activity profiles being consistent among cell types. Three categories of high, intermediate and low inducers could be defined. Differences in ISG expression are further determined by cell-type specific factors directing basal and inducible gene regulation.

## Conflict of interest

There is no conflict of interest concerning the authors of this study.

## Acknowledgements

Special thanks to Monika Sachet and Irina Kuznetsova for supplying material and help for the influenza virus experiments. This work was supported by the Austrian Science Fund FWF Grant No. P20074-B13.

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