Induction of Interferon Regulated Genes in Endothelial Cells:

Differential Regulation by Interferon Subtypes and Pro-Inflammatory Cytokines

Doctoral Thesis by DI Herwig Moll

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Wenn einer, der mit Mühe kaum
gekrochen ist auf einen Baum,
Schon meint, daß er ein Vogel wär,
So irrt sich der. (Wilhelm Busch)

Abstract

Type I interferons (IFNs) are known to exhibit antiviral, antiproliferative and immunoregulatory activity, and are therefore an important tool in the host defense against infection by pathogens. Since the vessel lining endothelial cells (ECs) constitute the link between tissue on the basal side and immune cells in blood on the apical side, they have an essential function in the immunological response to viral or bacterial infections. It is therefore of particular interest to gain more knowledge about IFN signaling and interferon regulated genes in ECs.

In the course of this PhD-thesis, we investigated individual stimuli for their capacity to regulate IFN stimulated genes (ISGs) in primary endothelial cells. We initially observed an unusual IFN-like response of ECs to neutralizing monoclonal antibodies against IFNα or IFNβ. A detailed analysis revealed that the respective antibodies (which were frequently employed by the world wide IFN community) had been contaminated during antibody production. We further explored the contaminating agent and identified a recombinant IFNα subtype, which was not neutralized by the respective antibody, to account for the effects observed.

With respect to the natural diversity of IFNα subtypes, we then characterized the biological activity of 13 different IFNα proteins on human ECs and fibroblasts. We investigated their effect in the regulation of ISGs, namely IFIT1, ISG15, CXCL10, CXCL11 and CCL8. The different IFNα subtypes showed a remarkably consistent potency in ISG mRNA induction, independent of target gene and cellular background. We therefore classified IFNα subtypes in three groups of high, intermediate and low activity. Furthermore, we gained evidence for cell type specific regulation of ISGs, as reflected in different levels of basal and IFNα inducible ISG expression in endothelial cells versus fibroblasts.

In comparison, we investigated ISG regulation in endothelial cells by cytokines other than type I interferon. Our results indicated that treatment of ECs with tumor necrosis factor α (TNFα), lipopolysaccharide (LPS) or interleukin 1β (IL-1β), directly induced transcript levels of IFIT1, ISG15 and CXCL10. Furthermore, we found antiviral effects of pro-inflammatory EC stimulation and assessed a possible contribution of autocrine IFNβ production. We gained evidence that autocrine IFNβ contributed to the sustained induction of ISG

mRNA expression, but did not mediate the antiviral activity of pro-inflammatory cytokines.

Zusammenfassung

Die Typ I Interferone (IFNs) spielen im angeborenen Immunsystem eine wichtige Rolle und ihre antivirale, antiproliferative und immunregulatorische Wirkung ist gut beschrieben. Da Endothelzellen (ECs) das Bindeglied zwischen dem Gewebe auf der basalen Seite und den Immunzellen im Blut auf der apikalen Seite darstellen, haben sie ebenfalls eine zentrale Rolle im Kampf gegen virale oder bakterielle Infektionen. Daher ist es von besonderer Bedeutung, die Wirkungsweise von Interferon bzw. die Regulation von IFN stimulierten Genen (ISGs) in ECs zu erforschen.

Im Rahmen dieser Dissertation haben wir eine Reihe unterschiedlicher Stimuli auf ISG Regulation in Endothelzellen untersucht. Wir haben zunächst die endotheliale Antwort auf Behandlung mit Typ I IFN-neutralisierenden Antikörpern beobachtet, die überraschender Weise einem IFN Signal ähnlich war. Wie sich nach eingehender Analyse herausstellte, wurden die angewendeten Antikörper, die in der weltweiten Interferonforschung weit verbreitet sind, im Zuge der Produktion kontaminiert. Wir konnten die Kontamination als einen rekombinanten IFNα-Subtyp identifizieren, der vom jeweiligen Antikörper nicht neutralisiert werden konnte.

Im nächsten Abschnitt wurde die biologische Vielfalt der IFNα Subtypen untersucht, d.h. deren biologische Effekte in ECs und Fibroblasten verglichen. Es wurden 13 verschiedene IFNα Proteine in Bezug auf die Stimulation von ISGs – insbesondere IFIT1, ISG15, CXCL10, CXCL11 und CCL8 – überprüft. Die untersuchten IFNα Subtypen zeigten Unterschiede in ihrer Stimulationskapazität, die bei allen ISGs in ähnlicher Weise hervortraten und die sowohl in ECs als auch in Fibroblasten beobachtet wurden. Wir konnten die Subtypen aufgrund ihrer unterschiedlichen Aktivität in stark, mittel und schwach stimulierende Kategorien einteilen. Es stellte sich weiters heraus, dass zelltypspezifische Genexpression vorwiegend von einer unterschiedlichen basalen ISG Produktion abhängig war.

In einer weiteren Studie untersuchten wir die ISG Regulation in Endothelzellen durch proinflammatorische Zytokine. Unsere Daten zeigten, dass ISGs direkt durch proinflammatorische Stimuli induziert wurden. Weiters beobachteten wir antivirale Effekte, die durch eine proinflammatorische Aktivierung von ECs ausgelöst wurden. Besonderen Bezug nahmen wir im Rahmen dieser Studie auf

die Produktion und Wirkung von autokrinem IFN. Es zeigte sich, dass autokrines IFNβ zwar die ISG Induktion verstärkte, jedoch nicht maßgeblich zum antiviralen Effekt der pro-inflammatorischen Zytokine beitrug.

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Background

Endothelial cells – structure and function

Endothelial cells (ECs) form the inner lining of blood and lymphatic vessels, thereby constituting a barrier between the blood stream and the underlying tissue. Since endothelial cells cover the entire circulation system – from the heart to the smallest capillaries – the endothelium belongs to the largest "organs" of the human body. The number of ECs in a human adult is estimated to be 1 – 6 x 10^{13} cells, amounting to about 1 kg, and the EC surface would cover 1 – 7 square meters[1].

Vascular endothelial cells in normal, healthy tissue have to fulfill essential functions, among them blood fluidity maintenance, blood flow regulation, control of the exchange of nutrients, fluids and gases between the blood and the tissue, and inhibition of leukocyte attachment[2, 3].

On the apical side, quiescent ECs are non-adhesive to blood cells and adapted to shear stress, whereas on the basal side they express a vast array of adhesion molecules to interact with the basal membrane and surrounding pericytes. Therefore, ECs are characterized by a pronounced polarity.

Depending on the vessel type and tissue, ECs form a tight monolayer or a leaky barrier, such as in the liver or the spleen where blood cells may get in direct touch with the underlying cells. However, in the vessels of most tissues, ECs form a compact layer, with tight junctions governing the interspace between them[4]. Cell-cell interactions at the lateral side of ECs are generally formed by adherens junctions, with VE-cadherin as the major component. Due to this close-meshed net established in a quiescent state, ECs have to mediate a controlled transport through the monolayer[5]. For this purpose, the apical membrane of ECs is rich in caveolae, which can form vesicles and therefore allow for controlled shuttling of macromolecules between blood and tissue[6].

Endothelial cells in inflammation

Activation of ECs in the course of inflammation leads to a change in the phenotype and thus to a functional shift. EC activation is divided in two phases: first, the rapid stimulation or type I activation, which is independent of new protein synthesis. Second, the slower response or type II activation is triggered by the expression of new genes[7]. Main mediators of type II activation are

lipopolysaccharide (LPS), tumor necrosis factor α (TNFα) or interleukin 1β (IL-1β), as reviewed by Pober *et al.*[8]. Ligand binding to the respective receptors on ECs leads to the formation of intracellular signaling complexes, which trigger the cascade of mitogen-activated kinase kinase kinases (MAPKKK), finally activating the transcription factors nuclear factor –κB (NF-κB) and activating protein 1 (AP-1) (as further discussed below). These transcription factors positively regulate the expression of inflammatory genes, like adhesion molecules, cytokines, chemokines and growth factors. Pro-inflammatory stimulation of ECs further leads to the reorganization of actin and tubulin cytoskeletons, thereby inducing a barrier dysfunction allowing for direct contact of plasma compounds and inflamed tissue[9]. In addition, activated ECs are recruiting leukocytes by the combined action of chemokines and leukocyte adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) or E-selectin[3]. Attracted leukocytes initially adhere with weak affinity to EC surface selectins, they decelerate and roll on the luminal membrane. Close contact and further leukocyte activation by chemokines lead to binding of leukocyte integrins to immunoglobulin-domain molecules on the EC surface. Finally, leukocytes transmigrate through gaps between ECs into the underlying tissue in a process called diapedesis (reviewed by Muller[10]).

Endothelial cells in angiogenesis

Another functional change of ECs is observed in angiogenesis. In this process, new capillaries are developed from preexisting vessels[11]. The major steps in angiogenesis comprise the degradation of extracellular matrix proteins by proteolytic digest, EC proliferation and migration, the formation of a new lumen and maturation to a functional vessel. This is not a frequent event in healthy adults, but mainly observed in wound healing and in the female reproductive cycle. However, angiogenesis also plays a major role in disease, e.g. growing tumors are dependent on the formation of new blood vessels to ensure adequate supply with nutrients and oxygen. Thus, the endothelial cell has become a target of interest in cancer therapy, and antiproliferative agents such as interferons are considered to exert antiangiogenic therapeutic effects.

Type I interferons

Interferons (IFNs) were first identified and characterized in 1957 by Isaacs and Lindenmann[12, 13], and have been in the focus of interest since then. Today they are well described as proteins with antiproliferative, antiviral and immunomodulatory activity[14]. Interferons constitute the first line of the innate defense system against infection by pathogens like virus or bacteria. They establish an antiviral state by regulating interferon stimulated genes (ISGs) in target cells and by activating immune effector cells. Initially, the family of IFNs was classified into two groups: type I and type II IFNs. More recently, a third class emerged within the superfamily of IFNs, the type III IFNs. The latter represents a distinct group within this protein family, as the members IFNλ1,2 and 3 (also known as interleukin 29 (IL-29),IL-28A and IL28B, respectively) have a tissue specific impact and mainly mediate host protection in the epithelium[15]. The type II class of interferons is represented by only one member, namely IFNγ, whereas there are several members of the type I IFN family, including IFNα, IFNβ, IFNε, IFNκ and IFNω[16]. All type I genes are clustered on the short arm of chromosome 9. With respect to IFNα, there are 14 non-allelic genes encoding 12 different IFNα proteins. Although a number of studies have addressed this issue[16-19], the physiological relevance and apparent redundancy of IFNα subtypes is still unclear.

Type I IFN mediated signaling

All type I IFNs bind to the same cell surface receptor complex, consisting of the two subunits IFNα receptor 1 (IFNAR1) and IFNAR2[20, 21]. IFNAR2 has higher affinity than IFNAR1 to most type I IFNs. Both receptor chains are in direct interaction with members of the Janus activated kinases (JAKs); IFNAR1 is associated with tyrosine kinase 2 (Tyk2) and IFNAR2 interacts with JAK1. Ligand binding induces dimerization of the receptor chains placing the JAKs in close proximity. Once they have activated each other by cross-phosphorylation, tyrosine residues in the intracellular domain of the two receptor chains become phosphorylated, thereby providing docking sites for the SH2 domain of signal transducers and activators of transcription (STATs). Subsequently, JAKs mediate tyrosine phosphorylation of recruited STAT molecules. Activation of STATs leads to the formation of a STAT1 – STAT2 heterodimer (in type I IFN

signaling), which dissociates from the receptor and joins with a member of the interferon regulatory factor (IRF) – family, IRF9, to form the transcriptional activator complex ISGF3. This transcription factor complex translocates into the nucleus, binds to and activates the interferon stimulated response element (ISRE), a common promoter sequence in ISGs. In ECs, more than 175 genes are upregulated by type I IFNs, among them genes with antiviral and immunoregulatory activity, but also genes with antiproliferative, i.e. antiangiogenic properties[22].

Beside the canonical JAK/STAT signaling pathway, there is growing evidence that IFN signaling involves multiple signaling cascades including the mitogen-activated protein kinase (MAPK)/p38 cascade and the phosphatidylinositol 3-kinase (PI3K) cascade[23, 24]. These signaling pathways have been shown to contribute to both, the early cellular response but also the maintenance of a prolonged cell activation by IFN.

TLR mediated signaling

Toll like receptor (TLR) mediated signaling is essential in the innate immune defense for recognition of pathogen associated molecular patterns. There is an array of different TLRs described, which are located either on the cell surface or in intracellular organelles like endosomes[25, 26]. TLRs are highly specialized in detection of ligands, e.g. TLR3 recognizes intracellular dsRNA whereas ligands for TLR4 are lipopolysaccharide (LPS), viral envelope proteins or endogenous ligands like hyaluronic acid implicated in malignant diseases. However, all TLRs contain intracellular Toll/IL-1 receptor (TIR) domains, thus eliciting overlapping cellular responses after receptor activation via recruitment of adaptor molecules like the myloid differentiation primary response gene 88 (MyD88). Of particular importance is the activation of the NF-κB pathway via IL-1R-associated kinase (IRAK) 4 and 1 as well as TNF-receptor-associated factor 6 (TRAF6)[27]. Binding of TLR ligands also activates MAPKs, including c-Jun N-terminal kinase (JNK), leading to the activation of transcription factor AP-1.

Furthermore, TLR signaling has a deep impact on IRF activity and the expression of type I IFNs[28]. The regulation of type I IFNs occurs mainly at the transcriptional level. The promoter region of IFNβ harbors four regulatory cis-elements, described as positive regulatory domains (PRDs) I, II, III and IV. In

contrast, the promoter of IFNα genes contains only two PRDs, PRD I and PRD III. PRD II and PRD IV provide binding sites for NF-κB and AP-1, respectively, while PRD I and PRD III are the recognition elements for IRFs. Thus, the regulation of IFNα genes is highly dependent on IRFs, whereas IFNβ is also controlled by NF-κB and AP-1.

Until now, nine members of the IRF transcription family have been identified and characterized in the context of various biological processes[29]. These members are characterized by a highly conserved N-terminal DNA binding domain which enables binding to ISRE DNA sequences similar to the PRD I and PRD III sites[30]. Among the known IRFs, IRF1, IRF3, IRF5 and IRF7 have been identified to contribute to type I IFN gene regulation[31].

Regulation of IRFs upon TLR4 activation was shown to be dependent on the adaptor proteins TIR-domain-containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM). Subsequent phosphorylation of IRF3 by TANK binding kinase 1 (TBK1) leads to IRF3 homodimers which associate with the histone acetyl transferases CREB binding protein (CBP) and p300, thus forming a complex termed enhanceosome which binds to the IFNβ promoter.

As reviewed by Taniguchi *et al.*[21], induction of IFNα genes is dependent on both, IRF3 and IRF7. While IRF3 is constitutively present, the expression of IRF7 is mediated by ISGF3. Therefore, in a sequence of action following infection with pathogens, IRF3 phosphorylation leads to the expression of autocrine IFNβ, which activates the JAK/STAT signaling pathway resulting in ISGF3 formation and transcription of IRF7 (among other target genes). Thereafter, pathogen mediated phosphorylation of IRF7 enables the production of IFNαs in this autocrine feedback loop. Hence, IFNβ forms the first line of innate defense against pathogen infection, whereas IFNα contributes to a sustained cellular response.

In addition, a weak constitutive type I IFN signaling has been observed for fibroblasts and immune cells, and was reported to provide low levels of IRF7 within the cells, thereby allowing cells a faster and stronger response in the fight against pathogens.

Interferon stimulated genes

The downstream target genes of TLR and IFN signaling mediate the ascribed biological effects. In large scale gene expression studies, more than 175 genes were identified to be regulated by type I IFNs, among them interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) and ISG15[22].

IFIT1 is a 56 kDa cytoplasmic protein also known as IFI56, ISG56 or p56 due to its molecular weight. IFIT1 was reported to interact with the multisubunit protein complex eukaryotic initiation factor 3 (eIF3), in particular with the subunits eIF3c and eIF3e[32]. Binding of IFIT1 blocks the ability of eIF3 to stabilize the ternary complex of eIF2, GTP, and Met-tRNA$_i$, thus inhibiting initiation of translation[32-34].

Among the best described interferon target genes is ISG15, which was first cloned more than 20 years ago[35]. Sequence analysis revealed that ISG15 is composed of two domains, each of which exhibits approximately 30 % homology to ubiquitin. Similar to ubiquitylation which regulates many aspects of the innate immune response, it was proposed that ISG15 may form protein conjugates and fulfill comparable functions in the response to IFN. For ISG15 activation, multiple E1, E2 and E3 proteins are coordinately induced by type I IFNs, and they catalyze the conjungation of ISG15 to other proteins in a process called ISGylation. To date, at least 158 putative ISG15 target proteins have been identified[36]. Among them are numerous proteins involved in the cellular IFN response, like the signaling molecules JAK1 and STAT1 or antiviral effector proteins such as MxA[37]. However, ISGylation does not promote protein degradation, but has activating functions. For example, ISG15 counteracts the virus mediated degradation of IRF3, thereby increasing IFNβ transcription[38].

Beside the intracellular, antiviral function, ISG15 is also reported to be secreted in high amounts and to act as a cytokine to modulate immune responses. Human lymphocytes, monocytes and other non-immune cells can release ISG15 into culture medium after stimulation with IFNα or –β[39]. ISG15 functions as a chemotactic factor for neutrophils[40] and a stimulus of IFNγ production from CD3$^+$ lymphocytes[41]. Thus, ISG15 has been shown to exert immunoregulatory and antiviral properties.

In summary, the host response to pathogens is mediated by a multitude of IFN downstream effector genes which are regulated at the transcriptional (ISGF3, IRFs) and the protein level (ISGylation).

Aim of the study

The focus of this PhD thesis was the regulation of interferon stimulated genes in endothelial cells, with special emphasis on distinct stimuli of ISG expression, their associated signal transduction and biological effects.

The first study was based on the prior discovery of our group that neutralizing monoclonal antibodies to IFNα or IFNβ were found to trigger rather than inhibit an IFN response in quiescent ECs as characterized by the induction of ISGs. This was of special interest, because IFNs play a crucial role in the pathogenesis of autoimmune diseases such as lupus erythematosus (SLE), type I diabetes or autoimmune thyroid disease[42-44]. Therefore, the application of neutralizing antibodies has been considered for therapeutic approaches in autoimmune diseases[45]. We thus aimed to characterize this phenomenon in more detail and identify the underlying mechanism, to investigate possible detrimental effects to IFN antibody therapy.

However, in the course of the project we discovered contaminations in the antibody preparations applied in our studies. Therefore, we aimed to identify the contaminant and unravel the origin of contamination. Interestingly, most of these antibodies were provided by the leading source for products in the IFN field (founded by one of the pioneers of IFN research). The further examination of this contamination was thought to be of interest, as the findings were expected to be of deep impact for the interferon community supplied with products of this market leader over the last 8 years.

As the contaminant was identified as a particular IFNα subtype, it became of interest to investigate the specificity and redundancy of IFNα subtype signaling in endothelial cells. The study was focused on the regulation of selected ISGs which were known to be interferon targets in ECs and fibroblasts[22]. We thus aimed to analyze and compare the effects of different IFNα subtypes on lymphatic and blood vessel derived ECs versus fibroblasts.

Furthermore, as interferon production and ISG regulation may also occur secondary to a non-IFN stimulus, we extended the analyses to endothelial cell stimulation with pro-inflammatory cytokines. We were particularly interested in autocrine IFN secretion by ECs. Therefore, we investigated ISG expression in the context of inflammatory activated ECs and evaluated the contribution of

autocrine type I interferon production to ISG regulation and to the antiviral response.

Neutralizing Type I Interferon Antibodies trigger an Interferon-Like Response in Endothelial Cells[1]

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EC, endothelial cell; HDMEC, human dermal microvessel endothelial cell; HUVEC, human umbilical vein endothelial cell; IFIT-1, interferon induced protein with tetratricopeptide repeats 1; IFNAR, interferon alpha receptor; IFNGR, interferon gamma receptor; ISG15, interferon stimulated gene 15; ISGF3, interferon stimulated gene factor 3; ISRE, interferon stimulated response element; MEF, mouse embryonic fibroblast; SLE, systemic lupus erythematosus

Keywords: endothelial cell, neutralizing antibody, type I interferon, Fc domain

Abstract

Neutralizing antibodies to type I interferons are of therapeutic significance i.e. are currently evaluated for the treatment of autoimmune diseases with pathogenic IFNα production such as for systemic lupus erythematosus.

Unexpectedly, we observed that several neutralizing antibodies reportedly known to counteract IFNα or -β activity triggered an “IFN-like” response in quiescent primary human endothelial cells leading to activation of the transcription factor ISGF3 and the expression of interferon-responsive genes. Furthermore, these antibodies were found to enhance rather than inhibit type I interferon signals, and the effect was also detectable for distinct other cell types such as PBMCs. The stimulatory capacity of anti-IFNα/β antibodies was mediated by the constitutive autocrine production of sub-threshold IFN levels, involved the type I IFN receptor and was dependent on the Fc antibody domain, as Fab or $F(ab')_2$ fragments potently inhibited IFN activity.

We thus propose that a combined effect of IFN recognition by the antibody paratope and the concomitant engagement of the Fc domain may trigger an interferon signal via the respective type I IFN receptor which accounts for the observed “IFN-like” response to the neutralizing antibodies. With respect to clinical applications, the finding may be of importance for the design of recombinant antibodies versus Fab or $F(ab')_2$ fragments to efficiently counteract IFN activity without undesirable activating effects.

Introduction

The inducible interferon response and the associated anti-virus, anti-tumor and immunomodulatory activities are well characterized hallmarks of the defense system. They are primarily mediated by the rapid activation of the transcription factor ISGF3 (interferon stimulated gene factor 3)[4] which binds to promoter elements termed ISRE (interferon stimulated response element) and induces expression of interferon-response genes such as IFIT-1 (interferon induced protein with tetratricopeptide repeats 1) or ISG15 (interferon stimulated gene 15). In contrast, the low level, constitutive expression of type I interferons (IFNα or -β) has now been recognized to serve distinct functions in cellular signaling and activation[46]: In the absence of any known stimulus, a low basal expression level of type I interferons is maintained which results in a weak signaling event and intracellular tyrosine phosphorylation of IFNAR-1, the type I IFN receptor α-chain. The signal is considered to be “sub-threshold” i.e. does not elicit the signaling cascade that leads to transcriptional activation of interferon-response genes. However, IFNAR-1 is maintained in a “ready state” thereby promoting rapid cellular activation upon stimulation with virus, interferons or other STAT-signaling cytokines. In this context, IFNAR-1 was shown to engage in cross-talk with other receptors such as the type II IFNγ or the IL-6 receptor – thus constituting a common “docking site” for STAT dimerization and an efficient enhancer of cellular activation[47, 48]. Constitutive, low level IFNα/β expression has been reported for mouse embryonic fibroblasts (MEFs), splenocytes, macrophages and bone marrow cells[47, 48], and was further described to promote activation of $CD8^+$ T-cells upon T-cell receptor engagement[49] and to regulate dendritic cell differentiation[50, 51].

Considering the potency of IFNα/β in the immune response, the application of recombinant IFNs has proven an evident and valuable therapeutic tool in the treatment of e.g. viral infections or cancer. On the other hand, type I interferons – in particular IFNα - have been found to play a crucial role in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE), type I diabetes or autoimmune thyroid disease[45, 52]. In this context, the development of a neutralizing antibody directed against multiple IFNα subtypes has been promoted[53], and in April 2006 the first clinical trial has been launched applying a

humanized IFNα blocking mAb to SLE patients. IFN-neutralizing activities are expected to target immune effector cells such as leukocytes, but their impact further extends to other cell types such as the vessel-lining endothelial cells (ECs). In this regard, we have observed and characterized an unexpected “IFN-like” response in ECs upon exposure to neutralizing antibodies directed against type I interferons.

Materials and Methods

Cell culture

Primary ECs were isolated from human foreskin samples by dispase digest, purified via α-CD31 antibody coupled Dynabeads (Invitrogen Corp., Carlsbad, CA) and cultured in fibronectin-containing EGM2-MV growth medium (Cambrex Corp., East Rutherford, NJ) without VEGF supplementation. Purified EC cultures showed ≥ 98 % purity and viability. For separation of lymphatic and blood vessel ECs, α-podoplanin antibody coupled Dynabeads were applied. All isolates were characterized by flow cytometry for EC characteristics i.e. CD31 expression, CD34 as marker of microvessels and for E-selectin induction following stimulation with 100 ng/ml of TNFα for 4 h. Lymphatic ECs were detected by podoplanin expression. To keep the passage number low, experiments were generally repeated with different EC isolates i.e. represent biological replicates with variable inducibility. Representative results of 2-4 comparable experiments are shown (as further specified in the figure legends).
HUVECs (human umbilical vein endothelial cells) were obtained from Cambrex Corp. and grown in fibronectin-containing EGM2 medium without VEGF supplementation (Cambrex Corp., East Rutherford, NJ). 293T cells are derived from human embryonic kidney cells into which the temperature sensitive gene for SV40 T-antigen was inserted. HT-29 was isolated from a human colorectal adenocarcinoma. Both cell lines (293T, HT-29) were supplied by ATCC and cultured in Dulbecco´s modified Eagle medium with 10 % FCS. Peripheral blood mononuclear cells (PBMCs) were isolated from 100 ml EDTA-treated whole blood of a healthy volunteer by standardized density gradient centrifugation using Ficoll-Paque (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK) and were supplied with RPMI1640 medium containing 10 % FCS.

Treatment with neutralizing antibodies and rIFN

Two days prior to stimulation, ECs were seeded in growth medium at $7x10^5$ cells per 30 mm dish (293T and HT-29 cells at $2x10^6$ cells) to yield a confluent cell layer within 24 hours. Culture medium was then exchanged to EGM2-MV containing 5 % FCS but no additional growth factor supplements and cells were

allowed to adopt a quiescent state over the next 24 h. Antibodies were added on day 3 at various concentrations and for the time periods indicated. $7x10^5$ PBMCs in 500 µl RPMI1640 medium were stimulated immediately after isolation without prior culture period.

Recombinant interferon and blocking mAbs targeting either IFNα (clones #2 and #13: MMHA-2 and MMHA-13, respectively) or IFNβ (clones #3 and #12: MMHB-3 and MMHB-12, respectively) as well as the IFNAR chain 2 (MMHAR-2) were all obtained from PBL Biomedical Laboratories (Piscataway, NJ). ELISA assays for detection of human IFNα or -β were also manufactured by PBL and carried out essentially as described[54]. Neutralizing antibodies to IFNγ (NIB42) or the IFNGR α-chain (GIR-208) were supplied by BD Biosciences (San Jose, CA); for mouse IgG_1 isotype control the MOPC-21 clone was applied (Sigma-Aldrich Corp., St. Louis, MO). TNFα was kindly provided by H. R. Alexander (NCI, NIH, Bethesda, MD), whereas LPS was obtained from Sigma-Aldrich Corp.

Real-time RT-PCR

Total RNA was isolated from cell cultures with Qiagen RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), 500 ng RNA were reverse transcribed with oligo(dT) primers using the Superscript III First Strand Synthesis System (Invitrogen Corp., Carlsbad, CA) and the generated cDNA was diluted 1:25 prior to PCR analysis. Real-time PCR was performed with SYBR Green PCR Core Reagents (Applied Biosystems, Foster City, CA) and the following primer sets for IFIT-1 (900 nM forward primer 5'-GCA GAA CGG CTG CCT AAT TT-3', 900 nM reverse primer 5'-TCA GGC ATT TCA TCG TCA TC-3'), ISG-15 (300 nM forward primer 5'-GAG AGG CAG CGA ACT CAT CT-3', 300 nM reverse primer 5'-AGC TCT GAC ACC GAC ATG G-3'), IFNβ (50 nM forward primer 5'-AGC ACT GGC TGG AAT GAG AC-3', 300 nM reverse primer 5'-TCC TTG GCC TTC AGG TAA TG-3'), IFNα1 (300 nM forward primer 5'-GCC TCG CCC TTT GCT TTA CT-3', 300 nM reverse primer 5'-CTG TGG GTC TCA GGG AGA TCA-3', PrimerBank ID 13128950a1), IFNα2 (300 nM forward primer 5'-GCT TGG GAT GAG ACC CTC CTA-3', 300 nM reverse primer 5'-CCC ACC CCC TGT ATC ACA C-3', PrimerBank ID 11067751a1), BCoR (300 nM forward primer 5'-AGA CGA CAT GCT CTC AGC AA-3', 50 nM reverse primer 5'-GAT

CCT ATG GGC CGT GCT 3'), housekeeping genes β_2-microglobulin (50 nM forward primer 5'-GAT GAG TAT GCC TGC CGT GTG-3', 50 nM reverse primer 5'-CAA TCC AAA TGC GGC ATC T-3') and β-actin (900 nM forward primer 5'-CTG GAA CGG TGA AGG TGA CA-3', 300 nM reverse primer 5'-AAG GGA CTT CCT GTA ACA ATG CA-3'). With the exception of IFNs which do not contain introns, all primer sets span at least one exon/intron gene boundary. Primer sets for IFNα1 and IFNα2 were retrieved from the PrimerBank[55]. Each sample was assayed in triplicate with the GeneAmp 5700 Sequence Detection System (Applied Biosystems) for 45 cycles of 15 sec at 95 ºC followed by 1 min at 60 ºC and a final dissociation protocol to screen for false amplification products. mRNA levels for IFIT-1, ISG15, BCoR or IFNs were deduced from the on-plate dilution series of a standard cDNA and were normalized to housekeeping gene values as previously described[56]. Real-time PCR data are given as mean and standard deviation of triplicate samples. The value obtained for the untreated control sample was generally set to 1 and changes in mRNA expression upon stimulation are given in relation to the untreated control.

Immunoblotting

Endothelial whole cell extracts were generated essentially as described[57, 58] i.e. in lysis buffer containing 1 % NP-40, 0.5 % deoxycholic acid and the Complete Mini-Protease Inhibitor Cocktail (Roche, Indianapolis, IN). 30 µg of total protein were separated on PAGE mini gels and subjected to wet blotting. Immunodetection was performed with polyclonal rabbit antiserum against IFIT-1 (generous gift by Ganes Sen, Cleveland, OH) at a 1:2000 dilution or ISG-15 (Rockland Immunochemicals, Inc., Gilbertsville, PA) at a 1:200 dilution.

EC transfection with siRNA

Three distinct Stealth siRNA duplex oligonucleotides for IFNβ gene silencing (HSS10523-2, -3, -4) as well as the respective negative control siRNAs with matched GC-content were obtained from Invitrogen (Carlsbad, CA). $2x10^6$ proliferating ECs were harvested in 400 µl RPMI1640 with 10 % FCS and siRNA was added. Cells were then subjected to electroporation at 200 V, 960 µF

essentially as described[59]. Stimulation of ECs with anti-IFN mAbs or rIFN was performed 24 to 48 h post transfection. A mix of the three IFNβ siRNAs (f.c. 1 µM each) proved to be more effective than single application of the IFNβ siRNA variants (data not shown).

ISGF3 Reporter Gene Assay

Electroporation of $2x10^6$ ECs at 200 V, 960 µF was performed with a total of 30 µg DNA. A combination of 29 µg pISRE-Luc plasmid with 5 ISRE sites directing expression of the luciferase reporter gene (Stratagene, La Jolla, CA) and 1 µg of the constitutive β-galactosidase expression plasmid pCMVβ for normalization (Clontech, Mountain View, CA) was applied. Cells were then seeded in 30 mm wells at $1x10^6$ to yield a confluent cell layer within 24 hours. Stimulation with anti-IFNα mAb #2 (6 µg/ml) or rIFNα (100 pg/ml) was carried out for 4 and 24 hours. Cells were then harvested in 40 µl lysis buffer and samples (à 10 µl) were assayed in triplicate for luciferase as well as β-galactosidase activity applying the Tropix Dual-Light System according to manufacturer's instructions (Applied Biosystems, Foster City, CA) for chemiluminescent detection with a Wallac Victor^3 multilabel counter (Perkin Elmer Life Sciences, Boston, MA). Luciferase activity as measured in relative light units (RLU) was normalized to the corresponding β-galactosidase value.

Generation of Fab and F(ab')$_2$ fragments

The anti-IFNα mAb #2 or mIgG$_1$ isotype control antibody were subjected to ficin digest at 37 ^oC in the presence of 10 mM cysteine for Fab versus 1 mM cysteine for F(ab')$_2$ fragment generation according to the instructions of the "ImmunoPure IgG$_1$ Fab and F(ab')$_2$ Preparation Kit" (Pierce Biotechnology, Rockford, IL). For control, an antibody fraction was treated comparably in ImmunoPure Digestion Buffer but without addition of ficin protease. The resulting antibody fragments were evaluated under reducing as well as non-reducing conditions by Western blotting with ImmunoPure peroxidase-conjugated goat anti-mouse IgG (H+L) antiserum (Pierce) and digest efficiency was found to be ≥ 90 %. Control human

Fab/kappa fragments were obtained from Bethyl Laboratories, Inc. (Montgomery, TX).

Results

Primary endothelial cells as isolated from human dermal microvessels (HDMECs) were subjected to standard *in vitro* culture. When cells were exposed to increasing doses of neutralizing antibodies directed against IFNα or -β (in the absence of any exogenously added type I interferon) an "interferon-like" response was observed (Fig. 1): The dose-dependent induction of IFN-responsive genes such as IFIT-1 and ISG15 was detected at the mRNA level by real-time RT-PCR as well as at the protein level by immunoblotting. The effect was verified for four different, commercially available blocking mAbs targeting either IFNα (clones #2, 13) or IFNβ (clones #3, 12) as illustrated in figures 1 and 4. Furthermore, the stimulatory capacity of anti-IFNα and -β mAbs was additive (Fig. 2 A) and IFIT-1 mRNA induction was observed at all time-points (2, 4, 6, 8 h) investigated (data not shown). All mAbs tested were of the mouse IgG_1 isotype with κ light chains. An appropriate isotype control did not induce IFIT-1 or ISG15 expression in HDMECs.

A first indication on the potential mechanism underlying the extraordinary "intrinsic" ability of the type I interferon antibodies to induce IFN-responsive genes came from the observation that IFIT-1 induction by anti-IFNα or -β mAbs was abolished in the presence of IFNAR-blocking antibodies (Fig. 2 B). Thus, the type I interferon receptor seemed to be involved even in the absence of exogenously added IFN. Furthermore, antibody treatment resulted in the activation of the ISGF3 transcription factor as monitored by reporter gene assay (Fig. 2 C) indicating an interferon-like signal resulting in the transcriptional regulation of IFIT-1 and ISG15. Exposure of endothelial cells to the IFN neutralizing antibodies was well tolerated and did not result in any apparent changes in morphology (Fig. 2 D) or signs of apoptosis (data not shown) over prolonged time periods. Concomitant pro-inflammatory activation of ECs by LPS or TNFα led to a partial reduction but could not prevent the endothelial "interferon-like" response to the antibodies (Fig. 3 A).

The tested cell isolates mostly consisted of a mixture of vascular and lymphatic ECs originating from human skin microvessels. When analyzed in separate cultures, comparable responses were elicited in both cell populations (Fig. 3 B). In addition, endothelial cultures derived from larger vessels (human umbilical vein endothelial cells, HUVECs) were highly responsive to the type I interferon

antibodies. When investigating other human cell types, we found that the phenomenon was not restricted to ECs but could also be observed in freshly isolated PBMCs when treated with the respective IFN blocking mAbs (Fig. 3 C). In contrast, the effect was essentially absent in antibody-treated 293T or HT-29 cell cultures representing a human embryonic kidney and colon carcinoma cell line, respectively.

Since all the antibodies tested on HDMECs were established neutralizing monoclonals, we proceeded to test their blocking abilities in EC combination treatment with rIFN and mAb (Fig. 4). Four hours of incubation with rIFNβ (10 pg/ml) induced IFIT-1 mRNA levels by about 40-fold. Interestingly, addition of blocking mAbs (at 12 µg/ml) targeting IFNβ resulted in a further increase of IFIT-1 expression by 2 to 10-fold depending on the antibody applied (clone #12 and #3, respectively). A similar phenomenon was observed when combining rIFNα with anti-IFNα blocking mAbs. In contrast, a neutralizing antibody directed against the type I interferon receptor IFNAR completely abrogated the endothelial response to rIFNα. Furthermore, when IFIT-1 expression was triggered by recombinant type II interferon (10000 U/ml rIFNγ), the induction was efficiently blocked by the addition of neutralizing antibody targeting either IFNγ or the corresponding type II receptor.

As the ability of the type I interferon antibodies to induce IFN-responsive genes was not dependent on but could be enhanced by exogenously added IFN, we hypothesized that endothelial cells might constitutively express low levels of autocrine type I interferon contributing to the effects observed. To test our hypothesis, silencing of IFNβ gene expression was achieved by transiently transfecting HDMEC cultures with double-stranded siRNAs. When HDMECs were challenged with IFNβ siRNA or non-specific (control) siRNA, no induction of IFIT-1 was observed i.e. there was no endothelial response to the uptake of chemically modified dsRNA oligonucleotides (Fig. 5 A). 24 hours post-transfection, ECs were exposed to anti-IFNα or -β mAbs at 12 µg/ml. Induction of IFIT-1 mRNA was markedly inhibited by IFNβ gene silencing as opposed to control siRNA treatment (Fig. 5 A): IFIT-1 expression levels in response to type I interferon antibodies were reduced to about 40 %. The IFN silencing efficiency of these samples was evaluated by real-time RT-PCR analysis and equaled the effect seen for IFIT-1. However, not only IFNβ mRNA levels but also IFNα1 and

IFNα2 transcripts were decreased by about 60 % in the presence of IFNβ siRNA (Fig. 5 D). In contrast, the detectable mRNA expression of non-related EC genes such as the transcriptional regulator BCoR or the housekeeping gene β-actin were not affected by IFNβ versus control siRNA (Fig. 5 E). To reverse the effect of IFNβ gene silencing, ECs were pretreated with "sub-threshold" concentrations of IFNβ at 1 pg/ml (corresponding to 0.1 U/ml) for 2 h and were then challenged with anti-IFNβ blocking mAb (Fig. 5 B). As expected, rIFNβ at 1 pg/ml was below the signal threshold i.e. was insufficient *per se* to induce IFIT-1 expression. However, the low-dose pretreatment restored EC responsiveness to anti-IFNβ neutralizing mAb to 84 %. Comparably, EC activation by high-dose (100 pg/ml) IFNα was inhibited to 38 % due to IFNβ gene silencing, whereas high-dose IFNβ could partially overcome the block and achieve expression levels of 76 % (Fig. 5 C).

Having established that endogenously expressed type I interferon mediates the unexpected EC responsiveness to IFN-neutralizing mAbs, we then questioned whether antibody binding to Fcγ receptors on the cell surface might contribute to the effects observed. Despite the fact that all our *in vitro* experiments were conducted in the presence of 5 % FCS in culture medium thus supplying an excess of bovine IgG, binding of the mouse monoclonal anti-IFN antibodies to human endothelial Fcγ receptors could not be excluded. We therefore added increasing concentrations of $mIgG_1$ isotype antibody to our reactions (Fig. 6 B): A dose-dependent decline in EC responsiveness i.e. in IFIT-1 mRNA induction by interferon neutralizing mAbs was observed. This prompted us to further investigate the requirement for the Fc domain i.e. we generated Fab as well as $F(ab')_2$ fragments from anti-IFNα blocking mAb #2 by ficin digest. When comparing the Fab and $F(ab')_2$ fragments to the intact antibody (subjected to a mock treatment without addition of ficin protease, for control) the intact molecule retained its dose-dependent stimulatory capacity, whereas the corresponding Fab or $F(ab')_2$ fragments could not induce IFIT-1 mRNA expression in HDMECs (Fig. 6 A). However, the generated fragments exhibited strong neutralizing capacity for recombinant IFNα which was not observed when Fab or $F(ab')_2$ fragments of the control $mIgG_1$ isotype antibody were applied (Fig. 6 C): IFIT-1 mRNA induction in response to EC treatment with 10 pg/ml of rIFNα was

entirely abolished by 1 µg/ml of Fab or $F(ab')_2$ fragments from anti-IFNα blocking mAb #2 which documents the actual interferon neutralizing potency attributed to the orginal antibody by the manufacturer. In comparison, the fragments where less potent in competing i.e. inhibiting the activity of the intact Ig molecule: Increasing the amount of Fab fragment while maintaining the concentration of intact anti-IFNα mAb #2 at 1 µg/ml led to a 64 % drop in IFIT-1 transcript levels at a ratio of 2:1. In contrast, a comparable amount of unrelated control Fab fragment led to a non-specific quenching of IFIT-1 induction in the range of 25 % (Fig. 6 B).

Discussion

In the work presented, we have investigated the unexpected induction of IFN-regulated genes IFIT-1 and ISG15 in primary endothelial cells which were exposed to antibodies known to neutralize type I interferon. Despite the absence of exogenously added IFN, an “IFN-like” signal was observed involving the type I interferon receptor and leading to the activation of the transcription factor ISGF3. The potency of eliciting this response varied among the antibodies applied – apparently relating to differences in binding affinities rather than the specificity for IFNα or -β. Depending on the clone, antibody concentrations in the range of 1-10 µg/ml were sufficient to induce IFIT-1 mRNA levels comparably achieved by EC stimulation with 10 pg/ml of rIFNβ (40-fold). It is of interest to note that plasma levels of neutralizing antibodies reached in clinical applications are well within or even higher than the concentration range tested in our *in vitro* setting[60, 61].

Since all antibodies applied in our analyses were reported to neutralize interferon bioactivity - as confirmed by the manufacturer in cytopathic effect inhibition assays and as subsequently verified for the Fab and $F(ab')_2$ fragments in our experiments - we investigated the combined application of rIFN and mAb on HDMECs. The full-length type I IFN blocking antibodies were found to enhance rather than inhibit the endothelial response to IFNα or IFNβ. In contrast, neutralizing antibodies targeting IFNγ or the interferon receptors IFNAR and IFNGR potently repressed IFIT-1 induction by recombinant interferon. We have thus provided evidence that the ability of neutralizing antibodies to elicit an “interferon-like” response or to further enhance the effects of type I interferon, is only observed for mAbs targeting IFNα or -β in this cellular context.

Since the response was inhibited by concomitant administration of IFNAR blocking antibodies even in the absence of exogenously added rIFN, we hypothesized that endothelial cells might constitutively express low levels of type I interferons – as previously reported for other cell types[47, 48] – which might contribute to the effects observed. When testing EC culture supernatants, interferon was not detectable at ELISA sensitivities of 4 U/ml (IFNα) or 10 U/ml (IFNβ). However, considering that basal “sub-threshold” IFN concentrations were reported to range around 0.1 U/ml[47, 48] ELISA sensitivity may have been

limiting (data not shown). We therefore proceeded to block the potential constitutive production of type I interferon by siRNA application. The approach was limited to IFNβ, since silencing of IFNα genes is difficult to accomplish due to the variety of IFNα subtypes that may be expressed. Furthermore, basal expression of IFNα in fibroblasts was shown to be dependent on the constitutive IFNβ production – arguing for a predominant role of IFNβ in the low level, basal expression of type I interferons[62]. Comparably, we observed that the application of IFNβ siRNA led to the concurrent down-regulation of IFNβ and IFNα subtypes generally expressed in endothelial cells[63]. Silencing of IFNβ expression in HDMECs was found to greatly reduce the "interferon-like" response to IFN blocking mAbs: IFIT-1 expression levels were reduced to about 40 % irrespective of the antibody specificity to IFNα or -β, thus reflecting the impact of IFNβ siRNA on the overall type I interferon expression. We therefore propose that ECs maintain a basal level of IFN expression and IFNAR phosphorylation which allows for their "interferon-like" response to IFN-blocking mAbs. Since the constitutive, weak IFN signal is also known to be a prerequisite for the efficient cellular response to a high-level IFN challenge in e.g. MEFs[48], we conducted a control experiment with 100 pg/ml of rIFNα or -β. IFIT-1 induction by high-dose interferon was similarly impaired by IFNβ silencing. These results further support our argument for an essential, basal IFNβ expression in primary endothelial cells which promotes their capacity for efficient and rapid cellular activation.

The further investigations focused on the potential involvement of Fc domains in the endothelial activation by IFN-neutralizing antibodies. As the mAbs tested were of the mIgG$_1$ isotype, a cross-reaction with human Fc gamma receptors seemed feasible. When comparing the full-length antibody with Fab or F(ab')$_2$ fragments of an IFNα blocking mAb, the fragments did not elicit an "interferon-like" response in endothelial cells thus pointing to the importance of the antibody Fc domain. Furthermore, the fragments potently inhibited IFIT-1 induction by recombinant interferon i.e. they exhibited the expected IFN neutralizing capacity and they could compete for the activity of the corresponding full-length antibody. The latter was, however, not as effective as the inhibition of rIFN. This might potentially relate to a better accessibility of intact antibody to autocrine interferon if the antibody was membrane-associated i.e. bound to Fc receptors. The

observation that the EC response to intact anti-IFN mAb was also reduced in the presence of a $mIgG_1$ isotype antibody (containing an Fc-portion), further suggested involvement of Fc receptors. Endothelial cells are known to express Fcγ receptors with an apparent heterogeneity depending on the vessel type. Various isoforms of FcγRII as well as FcγRI and FcRn have been detected on ECs with CD32 being the most prominent on HDMECs[64, 65]. Yet, we could not demonstrate CD32 expression on our endothelial isolates nor block the effect of the anti-IFN antibodies by concomitant treatment with a neutralizing antibody directed against CD32 (data not shown). However, these results do not exclude the potential involvement of an endothelial Fc receptor other than CD32.

Interestingly, Fc receptors have been localized to EC caveolar membrane sections which sets them in close proximity to IFNAR molecules[48, 66]. With respect to their interrelation, two settings may be envisioned. The mere local proximity of IFNAR and Fc receptors might serve to sequester autocrine interferon at the cell surface: IFN-neutralizing antibodies on FcRs might thus increase the local type I IFN concentration beyond the signal threshold provided that IFN bound to the blocking mAbs can be released i.e. passed onto IFNAR. Alternatively, a direct receptor interaction between FcR and IFNAR could occur, initiated by the anti-IFNα/β antibodies and resulting in IFNAR activation beyond the basal “ready state”. Both players, IFNAR and Fcγ receptors have been reported to engage in diverse receptor cross-talk[47, 48, 67]. Thus, whether Fc receptors are indeed involved in the “IFN-like” response to the neutralizing mAbs or whether the antibody Fc domain mediates engagement of another, as yet unidentified cellular factor is of prime interest for further investigations.

The activating potential of neutralizing IFN antibodies was observed for all types of endothelial cells tested and was not abolished by concomitant pro-inflammatory activation of ECs. In addition, the fact that IFNα/β neutralizing antibodies did not only trigger an “IFN-like” response on quiescent cells but could also enhance the effect of a high-dose interferon challenge, emphasizes the potentially adverse systemic implications. The induction level of interferon-responsive genes varied to some extent with primary endothelial cell isolates. When other cell types were investigated, a heterogeneous response was observed which may relate to Fc repertoire or differences in the potency of the signaling cascade. Interestingly, the “IFN-like” response to monoclonal

neutralizing antibodies directed against human type I interferon was also recorded for human PBMCs. While this observation extends the potential clinical impact these antibody effects might have, it seems intriguing why these effects have not been noted previously by other groups in comparable experiments on leukocytes. In preliminary investigations we have gathered an indication pointing to the importance of the antibody type: While all the antibodies presented herein were monoclonals of the $mIgG_1$ isotype, we did not find an IFN-like response to rabbit polyclonal anti-IFN antiserum in our experimental setting (data not shown). Thus, the nature and isotype of the antibody may be a crucial determinant.

The clinical application of recombinant monoclonal antibodies targeted at IFNα is at the current therapeutic focus of autoimmune diseases. In light of the recent launch of the first clinical trial testing an IFNα neutralizing mAb for the treatment of SLE, a possibly pleiotropic antibody effect would seem of particular concern. Our results would suggest that enhanced neutralizing efficiency might be achievable by testing Fab or $F(ab')_2$ fragments versus full-length immunoglobulins in systemic settings.

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Figures

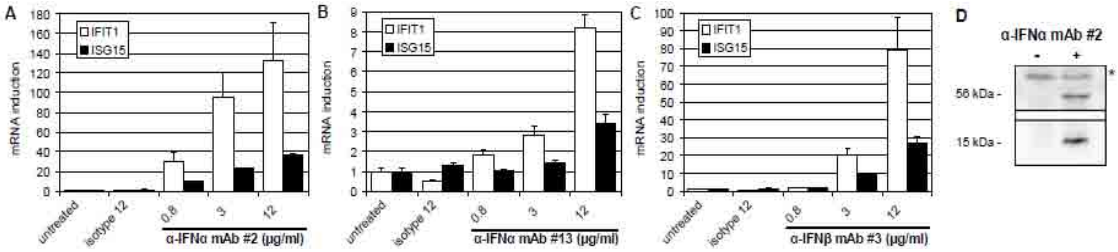

FIGURE 1. Dose-dependent induction of interferon-responsive genes by neutralizing antibodies to type I IFN. Endothelial cell cultures were treated for 4 h with 0.8, 3 or 12 µg/ml of anti-IFNα mAb clone #2 (A, n=7), clone #13 (B, n=2) or anti-IFNβ mAb clone #3 (C, n=3). The non-specific $mIgG_1$ isotype control was applied at 12 µg/ml. Real-time RT-PCR analysis of endothelial RNA was performed to evaluate mRNA levels of IFIT-1 and ISG15. Each sample was assayed in triplicate and the number of comparable experiments performed is given for each figure (n). IFIT-1 induction by antibody treatment was statistically significant ($p \leq 0.03$) for all mAbs and concentrations. Comparably, p-values ≤0.01 were recorded for ISG15 induction with the exception of anti-IFNα mAb clone #13 at the lowest concentration (p=n.s.). Protein expression (D, n=2) of IFIT-1 and ISG15 was investigated by immunoblotting of whole cell extracts following 5 h of stimulation with anti-IFNα mAb clone #2 at 12 µg/ml (*non-specific, cross-reactive protein).

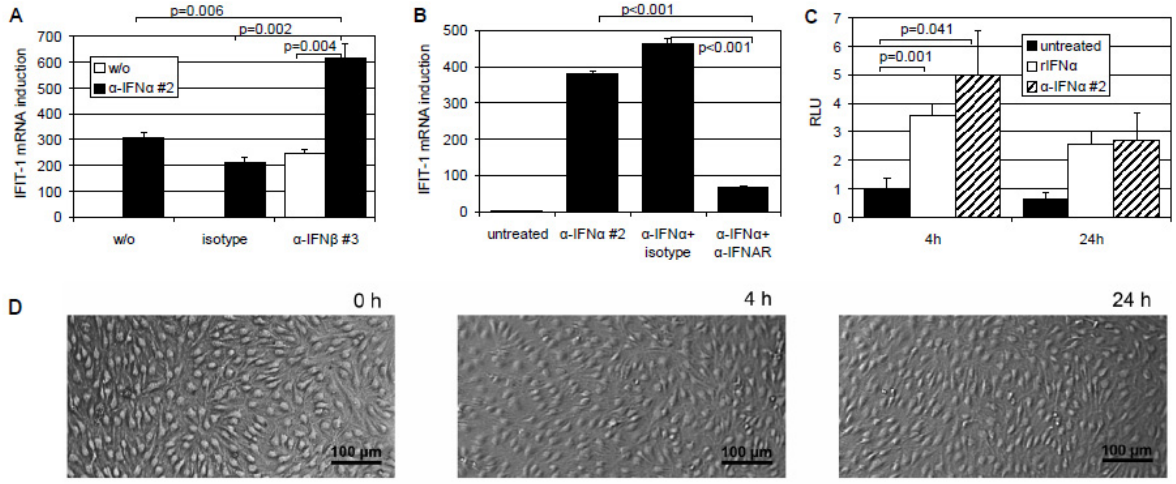

FIGURE 2. Characterization of the mechanisms involved in the induction of interferon-responsive genes by neutralizing antibodies to type I IFN. (A, n=3) HDMECs were challenged for 4 h by single or combined treatment with 3 µg/ml of anti-IFNα mAb clone #2 and anti-IFNβ mAb clone #3 (or $mIgG_1$ isotype control). Endothelial RNA and corresponding cDNA were then analyzed for IFIT-1 mRNA expression by real-time PCR which demonstrated an additive effect of anti-IFNα and -β antibodies. (B, n=3) IFIT-1 induction by 3 µg/ml of anti-IFNα mAb clone #2 was blocked by the addition of anti-IFNAR antibody at 1 µg/ml versus $mIgG_{2a}$ isotype control. (C, n=2) Activation of the interferon-responsive transcription factor ISGF3 was evaluated by reporter gene assay measuring luciferase activity in relative light units (RLU) after EC stimulation for 4 h or 24 h with 6 µg/ml of anti-IFNα mAb clone #2 or rIFNα (100 pg/ml) for comparison. (D) Endothelial cultures after 0, 4 or 24 h of stimulation with 6 µg/ml of anti-IFNα mAb clone #2 were investigated by phase contrast microscopy to document that no apparent changes in EC morphology were triggered by antibody treatment.

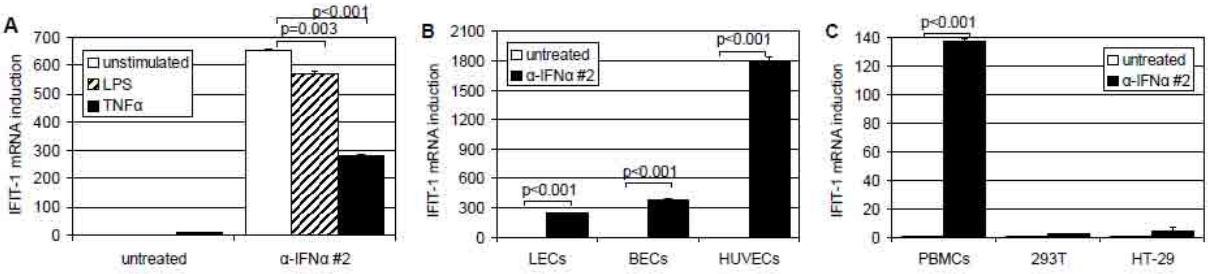

FIGURE 3. "Interferon-like" response to neutralizing antibodies by different EC variants or other cell types. (A, n=2) HDMECs stimulated with pro-inflammatory mediators such as LPS at 1 µg/ml or TNFα at 100ng/ml were concomitantly treated with 12 µg/ml of anti-IFNα mAb clone #2 for 4 h. (B, n=2) EC populations isolated from blood (BECs) or lymphatic (LECs) skin vessels or from human umbilical veins (HUVECs) were exposed to 12 µg/ml of anti-IFNα mAb clone #2 or mIgG$_1$ isotype control for 4 hours. (C, n=2) Comparably, freshly isolated PBMCs (peripheral blood mononuclear cells) or in vitro cultures of 293T and HT-29 cells were exposed to 12 µg/ml of anti-IFNα mAb clone #2 for 4 hours. Total RNA and corresponding cDNA were analyzed for IFIT-1 mRNA expression by real-time PCR.

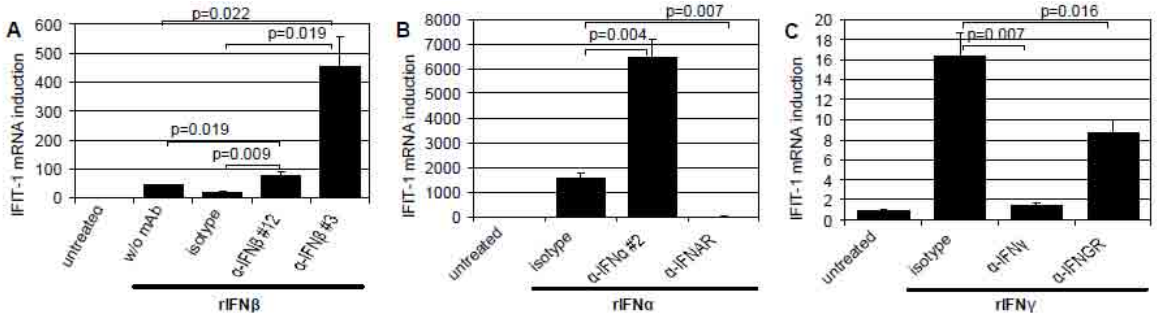

FIGURE 4. Enhanced response to rIFNα or -β in the presence of neutralizing antibodies to type I IFN. (A, n=3) HDMECs were stimulated with rIFNβ (10 pg/ml) for 4 h in the absence or presence of anti-IFNβ mAb clone #3 or #12, or $mIgG_1$ isotype control at 12 µg/ml each. (B, n=2) EC treatment for 6 h with rIFNα (10 pg/ml) was combined with 12 µg/ml of $mIgG_1$ isotype control, anti-IFNα mAb clone #2 or anti-IFNAR antibody. (C, n=3) Comparably, ECs were exposed for 6 h to 10000 U/ml of rIFNγ and 12 µg/ml of $mIgG_1$ isotype control, anti-IFNγ or anti-IFNGR antibody. Endothelial RNA was analyzed for IFIT-1 mRNA expression by real-time RT-PCR.

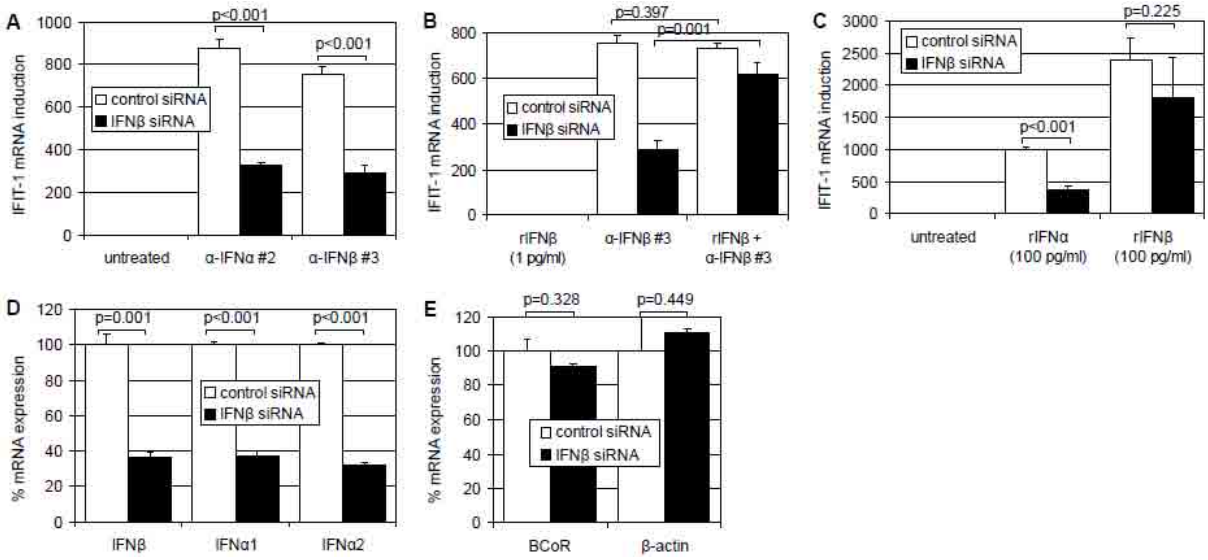

FIGURE 5. IFNβ gene silencing inhibits the "IFN-like" response of ECs to IFN-neutralizing mAbs. Following EC transfection with IFNβ siRNA or non-specific control siRNA, cells were challenged with 12 µg/ml of anti-IFNα mAb clone #2 or anti-IFNβ mAb clone #3 for 2 h (A, n=3). To reverse the effect of IFNβ gene silencing, EC cultures were pretreated with "sub-threshold" concentrations of rIFNβ (1 pg/ml) for 2 h with or without subsequent addition of 12 µg/ml of IFNβ mAb clone #3 for another 2 h (B, n=2). Treatment of siRNA transfected ECs with high-dose (100 pg/ml) rIFNα or -β for 4 h (C, n=4) was followed by RNA isolation and real-time RT-PCR detection of IFIT-1 expression. (D, n=3) Silencing efficiencies for IFNβ as well as IFNα1 and IFNα2 were determined by real-time RT-PCR and are shown for EC samples stimulated for 2 h with 12 µg/ml anti-IFNβ mAb clone #3. Comparably, the effect of IFNβ siRNA versus non-specific control siRNA was tested on transcript levels of non-related EC genes such as BCoR and β-actin for the same samples (E, n=3).

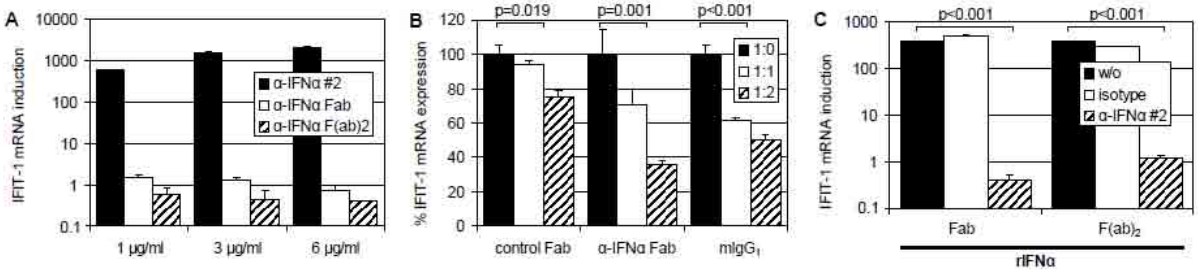

FIGURE 6. Antibody domains involved in IFIT-1 induction by IFN-neutralizing antibodies. (A, n=4) HDMECs were exposed for 4 h to 1, 3 or 6 µg/ml of intact (control-treated) anti-IFNα mAb clone #2 or a generated Fab or F(ab')$_2$ fragment of clone #2. Incubation for 4 h was followed by real-time RT-PCR analysis of endothelial RNA for IFIT-1 mRNA expression. In comparison to the full-length antibody, the fragments were incapable of eliciting IFIT-1 mRNA expression ($p \leq 0.029$ for all concentrations of intact mAb versus fragments applied). (B, n=4) The full-length anti-IFNα mAb clone #2 (1 µg/ml) was mixed at a ratio of 1:1 or 1:2 with the corresponding generated Fab fragment, a non-specific pool of unrelated Fab fragments or with an intact mIgG$_1$ isotype control. (C, n=3) HDMEC stimulation with recombinant IFNα at 10 pg/ml for 4 h was challenged with concomitant administration of 1 µg/ml Fab or F(ab')$_2$ fragments generated from anti-IFNα mAb clone #2 or from a mIgG$_1$ isotype antibody.

Contamination with Recombinant Interferons Accounts for the Unexpected Cell Activation by Commonly Applied Interferon Blocking Antibodies[1]

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EC, endothelial cell; IFIT-1, interferon induced protein with tetratricopeptide repeats 1; IFN, interferon; IFNAR, type I interferon receptor; IP, immunoprecipitation; ISG15, interferon stimulated gene 15; ISGF3, interferon stimulated gene factor 3; MOI, multiplicity of infection; pfu, plaque-forming units

Running Title: Type I IFN Contamination of Blocking Antibodies

Keywords: antibodies, cytokines, cell activation, endothelial cells, human

Abstract

The cellular response to interferons is an essential part of immune reactions and is triggered by pathogen-associated signals. We recently observed an unusual “IFN-like” response in human endothelial cells (ECs) upon exposure to neutralizing antibodies directed against human IFNα or -β. The signal seemed to involve the antibody Fc domain, the autocrine low-level production of IFNs and the type-I interferon receptor. A model was proposed of antibody binding to surface Fc-receptors with sequestration of autocrine IFN and subsequent release to nearby interferon receptors which would result in the observed “IFN-like” signal.

We now present evidence that the “IFN-like” activity is indeed a distinct factor, separable from the antibody moiety. As the signal signature argued against contamination with mouse-derived microbial products, the activity was further characterized and identified as human rIFNα subtype not recognized by the respective blocking antibody. Sequential production of recombinant interferons and neutralizing antibodies was established as the source of contamination which had evaded standard techniques for antibody purification and required an additional step of hydrophobic interaction chromatography for substance removal. The occurrence of IFN contaminations in commercially available anti-IFN antibody preparations was found to be frequent and to reach rIFN concentrations commonly applied in *in vitro* experiments. Since most of the tested antibodies are among the standard tools that have been employed in IFN research over the past years, the presented analyses may be beneficial to the IFN research community in re-evaluating experiments conducted with these antibodies.

Introduction

Human type I interferons comprise a family of at least 13 IFNα subtypes, the IFNβ and the IFNω proteins which bind to the common IFNAR (interferon alpha receptor) consisting of two polypeptide chains[68, 69]. The signal is transduced by a JAK-STAT pathway resulting in the activation of the transcription factor ISGF3 (interferon stimulated gene factor 3) and the downstream induction of target genes that mediate the IFN-associated immune response[70, 71]. The intra- and intercellular functions of interferon regulated genes such as IFIT-1 (interferon induced protein with tetratricopeptide repeats 1) or ISG15 (interferon stimulated gene 15) have been investigated in detail to elucidate the mechanisms of IFN action[32, 72].

The interferon response is also initiated by pattern recognition receptors detecting pathogen associated molecular patterns of viral or bacterial components[73]. Among those, the Toll-like receptors have been extensively characterized and described to trigger the autocrine production of type I interferons resulting in a feedback IFN response[74]. Apart from the associated activation of ISGF3, TLR ligands like LPS are known to engage additional transcription factors such as AP-1 or NF-κB in the pro-inflammatory activation of target cells[75].

We have recently described a novel and unusual “IFN-like” response initiated by neutralizing antibodies to type I interferons in primary endothelial cells (ECs). In the absence of exogenously added recombinant interferon, the exposure of ECs to increasing concentrations of IFN blocking mAbs resulted in the dose-dependent activation of ISGF3 and induction of the interferon response genes IFIT-1 and ISG15 at the mRNA and protein level [76]. The effect was observed for 4 different monoclonal antibodies directed against IFNα or -β and was additive when antibodies were combined or given in conjunction with recombinant interferons. Furthermore, the “IFN-like” response to these antibodies was not restricted to endothelial cells but was also seen for peripheral blood mononuclear cells, and seemed to be absent from transformed cell lines. With respect to the possible mechanism of EC activation by IFN blocking mAbs, we found that it involved the type I interferon receptor, i.e., was inhibited in the presence of neutralizing antibodies to IFNAR. Silencing of the endogenous IFNβ production in ECs resulted in the concomitant down-regulation of IFNα genes

and greatly reduced the cellular response to IFN blocking mAbs. When the antibodies were processed with ficin protease for the generation of Fab or $F(ab´)_2$ fragments, their stimulatory capacity was lost and their neutralizing activity towards type I interferon prevailed[76].

Based on these results, we concluded that an intrinsic feature of the IFN blocking mAbs was responsible for the observed “IFN-like” activation of ECs. It seemed to involve the antibody Fc-domain and to depend on endothelial IFN production and IFNAR function. A model was proposed with respect to the spatial proximity of IFNAR and Fc receptors in caveolae which might promote an autocrine IFN signal. A complex formed by the antibody and Fc receptor would capture (enrich) type I interferon at the cell surface and possibly release the ligand to the nearby IFN receptor resulting in an interferon signal.

We now present evidence that refutes this hypothesis showing that the “IFN-like” activity associated with IFN blocking mAbs is indeed a discrete component which can be separated from the antibody moiety. We have identified distinct subtypes of human recombinant IFNα as the likely source of contamination. These IFNα subtypes are not neutralized by the respective antibody in contrast to the majority of other IFN subtypes. Furthermore, we have obtained evidence that a considerable number of commercially available IFN antibody preparations contain detectable and substantial levels of contaminating interferons that are likely to affect the outcome of *in vitro* experiments involving neutralization of type I interferon.

Materials and Methods

Endothelial cell isolation, characterization and culture

Primary ECs were isolated from human foreskin samples by dispase digest, purified via α-CD31 antibody coupled Dynabeads (Invitrogen) and cultured in fibronectin-containing EGM2-MV growth medium (Cambrex) without VEGF supplementation. Purified EC cultures showed ≥ 98% purity and viability as characterized by immunostaining with fluorescence-labeled antibodies (Serotec) and flow cytometry for EC markers CD31, CD34, CD54 and CD62E (E-selectin) following stimulation with 100 ng/ml of TNF or 1 µg/ml of LPS for 4 h. To keep the passage number low, experiments were generally repeated with different EC isolates, i.e., represent biological replicates with variable inducibility. Representative results of 2-3 comparable experiments are shown (as further specified in the figure legends).

Treatment with neutralizing antibodies and rIFN

Two days prior to stimulation, ECs were seeded in growth medium at $1.25x10^5$ cells per 15 mm dish (24 well plate) to yield a confluent cell layer within 24 hours. Culture medium was then exchanged for EGM2-MV containing 5% FBS but no additional growth factor supplements and cells were allowed to adopt a quiescent state over the next 24 h. Antibodies and recombinant IFN were added on day 3 at the indicated concentrations and cells were generally stimulated for 4 h.

Recombinant interferons, sheep polyclonal human IFNα antiserum and blocking mAbs targeting either human IFNα (clones no. 2, no. 9, and no. 13: MMHA-2, MMHA-9, and MMHA-13, respectively) or IFNβ (clone no. 3: MMHB-3) were all from PBL Biomedical Laboratories. ELISA assays for detection of human IFNα were also manufactured by PBL and carried out essentially as specified. For mouse IgG1 isotype control the MOPC-21 clone was applied (Sigma-Aldrich). Further reagents obtained from Sigma-Aldrich include LPS and polymyxin B; TNFα was kindly provided by H. R. Alexander (NCI, NIH, Bethesda, MD).

Real-time RT-PCR

Endothelial RNA was isolated with E.Z.N.A. Total RNA Kit (Omega Bio-tek), 500 ng of RNA were reverse transcribed with oligo(dT) primers using the Superscript III First Strand Synthesis System (Invitrogen) and the generated cDNA was diluted 1:25 prior to PCR analysis. Real-time PCR was performed with the qPCR MasterMix Plus Low ROX (Eurogentec) and the following probe (200 nM) and primer (450 nM) sets for IFIT-1 (forward primer 5'-GAT CTC AGA GGA GCC TGG CTA A-3', reverse primer 5'-TGA TCA TCA CCA TTT GTA CTC ATG G-3', YakimaYellow/BHQ-1 labeled probe 5'-CAA AAC CCT GCA GAA CGG CTG CC-3'), ISG-15 (forward primer 5'-GAG AGG CAG CGA ACT CAT CT-3', reverse primer 5'-AGG GAC ACC TGG AAT TCG TT-3', 6-FAM/BHQ-1 labeled probe 5'-TGC CAG TAC AGG AGC TTG TG-3'), housekeeping genes β_2-microglobulin (forward primer 5'-CGC TCC GTG GCC TTA GC-3', reverse primer 5'-AAT CTT TGG AGT ACG CTG GAT AGC-3', YakimaYellow/BHQ-1 labeled probe 5'-TGC TCG CGC TAC TCT CTC TTT CTG GC-3') and β-actin (forward primer 5'-CCT GGC ACC CAG CAC AAT-3', reverse primer 5'-GCC GAT CCA CAC GGA GTA CT-3', 6-FAM/BHQ-1 labeled probe 5'-ATC AAG ATC ATT GCT CCT CCT GAG CGC-3'). Simultaneous detection (multiplexing) of IFIT-1 and ISG15 as well as of β_2-microglobulin and β-actin PCR products was performed. Each sample was assayed in triplicate with the 7500 Fast PCR Detection System (Applied Biosystems) for 45 cycles of 3 sec at 95ºC followed by 30 sec at 60ºC. mRNA levels for IFIT-1 and ISG15 were deduced from the on-plate dilution series of a standard cDNA and were normalized to the respective housekeeping gene values. Real-time PCR data are given as mean and standard deviation of triplicate samples. The value obtained for the untreated control sample was set to 1 and changes in mRNA expression upon stimulation are given in relation to the untreated control. While data are generally shown for IFIT-1 transcripts, all experiments have been evaluated for IFIT-1 and ISG15 regulation yielding comparable results.

Protein digest with ficin

The two-step purified anti-IFNα mAb no. 2 (20 µg) was compared to a combination of compounds designed to reproduce the contaminated antibody preparation: rIFNα_{14} (375 pg) was supplied with 20 µg of non-contaminated,

three-step purified anti-IFNα mAb no. 2 and 4 µg of BSA. Digest with ficin protease or control treatment in the presence of 1 mM cysteine was performed for 24 h at 37°C as specified for $F(ab')_2$ fragment generation according to the instructions of the "ImmunoPure IgG1 Fab and $F(ab')_2$ Preparation Kit" (Pierce). Following centrifugation at 1000 x g for 5 min, the supernatant was applied in appropriate dilution to EC cultures.

EC infection with influenza virus

Influenza A virus (PR8) was propagated in Vero cells in serum-free AIMV medium (Invitrogen Life Technologies) containing trypsin (5 µg/ml) as established in our laboratories[77]. Endothelial cells were pre-treated with rIFNα (100 pg/ml) or anti-IFNα mAb clone no. 2 (6 µg/ml) for 14 h prior to influenza infection. Cells were then exposed for 30 min to virus in serum-free OPTI-PRO medium (Invitrogen Life Technologies) at a multiplicity of infection (MOI) equaling 1. Viral supernatant was removed, and cells were supplied with serum-free medium (for virus harvest) or with regular EC growth medium (for all other analyses) in further culture.

Detection of viral protein expression

ECs were harvested by trypsin treatment 7 h post-infection with influenza A virus. Cells were permeabilized with IntraPrep reagent (Beckman Coulter) and further stained with the IMAGEN reagent A (Dako), a combination of two fluorescence-labeled monoclonal antibodies detecting the viral matrix protein as well as nucleoprotein. Following fixation in 0.5% formaldehyde, ECs were analyzed for viral protein expression with an FC500 flow cytometer (Beckman Coulter).

Cell viability assay

24 hours post-infection, a tetrazolium compound (EZ4U assay; Biomedica) was added to the culture medium in 1:10 dilution. ECs were then cultured for another 2.5 hours to allow for metabolic conversion of the substrate before spectrophotometric analysis of the generated formazan derivative at 450 nm.

Plaque formation assay

The amount of virus produced by endothelial cells was evaluated 48 hours after initial infection, i.e., the virus released into the serum-free supernatant was harvested and activated by addition of trypsin (5 µg/ml). A standard plaque assay (agar overlay) was applied to determine viral titers in serial dilution of virus suspensions on Vero cells as previously described[77]. Results are given in plaque-forming units per ml (pfu/ml) of virus supernatant.

Immunoprecipitation and immunoblotting

Anti-IFNα mAb clone no. 2 (6 µg) was diluted in 1 ml of EGM2-MV medium (without supplementation of growth factors). Three steps of immunoprecipitation (IP) were performed by addition of 20 µl protein A/G PLUS agarose (Santa Cruz), incubation at 4^{o}C for 1 h and centrifugation for 5 min at 2000 x g and 4^{o}C. At each step, 200 µl of supernatant as well as the IP pellet were set aside for subsequent analysis. The remaining supernatant was again supplied with 20 µl protein A/G PLUS agarose for the next IP cycle. For EC stimulation, 180 µl of IP supernatant were directly applied to endothelial cultures for 4 hours, prior to RNA isolation and quantitative RT-PCR analysis. For Western blot analysis, 10 µl of IP supernatant or original antibody solution (equaling 60 ng) were separated on PAGE mini gels and subjected to semi-dry blotting. IP pellets were treated with 20 µl of SDS/PAGE loading buffer (for 5 min at 95^{o}C) to retrieve bound proteins; 10 µl thereof were also analyzed by Western blotting with ImmunoPure peroxidase-conjugated goat anti-mouse IgG (H&L chain) antiserum (Pierce).

NF-κB Reporter Gene Assay

Electroporation of 2x10^{6} ECs at 200 V, 960 µF was performed with a total of 30 µg DNA. A combination of 29 µg reporter plasmid with 3 NF-κB binding sites directing expression of the luciferase reporter gene[57] and 1 µg of the constitutive β-galactosidase expression plasmid pCMVβ for normalization (Clontech) was applied. Cells were then seeded in 30 mm wells at 1x10^{6} to yield a confluent cell layer within 24 hours. Stimulation with anti-IFNα mAb no. 2 (6 µg/ml), TNFα (100 ng/ml) or rIFNα (100 pg/ml) was carried out for 4 and 8 hours. Cells were then harvested in 50 µl phosphate buffer and exposed to 3 cycles of freeze-thaw

lysis. Samples (supernatant à 10 µl) were assayed in triplicate for luciferase as well as β-galactosidase activity applying the Tropix Dual-Light System according to manufacturer's instructions (Applied Biosystems) for chemiluminescent detection with a Wallac Victor^3 multilabel counter (Perkin Elmer Life Sciences). Luciferase activity as measured in relative light units was normalized to the corresponding β-galactosidase value and expressed as fold induction compared to the non-stimulated control.

Cytopathic effect (CPE) inhibition assay

Antiviral assays were conducted on A549, human lung carcinoma (ATCC CCL-185), and L929, murine fibroblast (ATCC CCL-1) cells. For the A549 assay, 10000 cells in 0.1 ml of DMEM with 10% FBS were plated in each well of a 96 well tissue culture plate and incubated at 37 ^{o}C/5% CO_2 for 4 hours. Serial dilutions of the test samples (interferon or antibody preparations) were prepared in media and 0.1 ml was transferred to the plate containing the cells. Additional wells containing no interferon were included. After incubation for 18 hours a dilution of murine encephalomyocarditis virus (EMCV, ATCC VR-129B) which had been empirically determined to yield 100% cytopathic effect in 40 hours was added in 0.05 ml of media. Wells which received virus but no IFN served as the viral killing control, wells receiving 0.05 ml of media but no virus or IFN served as the cell control. The assay was allowed to develop for 40-48 hours in the incubator; the remaining cells were stained with crystal violet. After air drying the plates were read visually and then the crystal violet was solubilized with 0.1 ml of 70% methanol; the absorbance at 570 nm was determined in a Molecular Devices VMax microplate reader. CPE protection was determined relative to the cell control (100%) and virus control (0%) and the EC50 was determined in units/ml relative to a laboratory standard of human IFNα_{2a} (PBL# 11100) calibrated to the international standard for human IFNα (BEI Resources Gxa01-901-535).

The L929 assay was performed in a similar manner with the following exceptions. The media used was MEM with 10% FBS, 30000 cells were plated in 0.1 ml, the virus was added and the assay allowed to resolve for 24 hours and the standard used to calibrate the samples was murine IFNα A (PBL #

12100) calibrated versus the international standard for murine IFNα (BEI Resources Ga02-901-511).

Antibody purification

All antibodies were purified from mouse ascites fluid. Two procedures were used. A 2-step procedure consisting of ammonium sulphate precipitation and ion exchange chromatography was applied. The three step procedure used for later preparations involved the first two steps followed by hydrophobic charge induction chromatography. Frozen ascites fluid was thawed and filtered through 2 layers of cheese cloth. The ascites fluid was diluted with 2 volumes of dPBS and ammonium sulfate precipitation was done at 1.85 M final concentration, incubation on ice for 1 hour and centrifugation. The precipitate was resuspended in dPBS and the precipitation step was repeated. The final pellet was resuspended in 50 mM Tris pH 8.3, 40 mM NaCl and dialyzed extensively versus this buffer. The dialysate was loaded onto a 100 ml packed volume DEAE-ToyoPearl (Tosoh Bioscience), washed with 5 column volumes of buffer and eluted with a 40 mM to 500 mM gradient of NaCl over 20 column volumes. Samples containing monoclonal antibody were identified by NuPAGE Bis-Tris electrophoresis, and were then dialyzed versus storage buffer and frozen.

The third step used on later batches was MEP-Hypercel (Pall Corp.). Eluted mAb from the DEAE column was adjusted to 0.5 M NaCl, was loaded onto a 25 ml MEP-Hypercel column and washed with 5 column volumes of the same buffer. Elution was performed step-wise with 5 column volumes each of dPBS, 50 mM citrate-phosphate buffer pH 6.0, 5.5, 5.0, 4.5, 4 and 2.5. Fractions containing mAb were determined by gel electrophoresis followed by direct binding ELISA. Briefly, Costar Hi Bind Strip wells (Corning) were coated with 1 µg/ml IFN and blocked with 1% BSA/PBS. Serial dilutions of the mAb in 0.1% BSA/PBS/0.05% Tween were applied. The plates were washed twice with PBS/0.05% Tween and bound antibody was detected by incubation of a 1:10000 dilution of donkey anti-mouse IgG-HRP conjugate (Jackson Immuno) followed by 3 washes and detection with TMB substrate (Neogen). Color evolution was stopped by acidification and absorbance read at 450 nm in a VMax plate reader. Active fractions were pooled and frozen for long term storage.

Results

In the pursuit to further characterize the “IFN-like” activity of neutralizing antibodies their functional equivalence to type I interferons in terms of anti-viral activity was evaluated. ECs were subjected to infection with influenza wildtype virus with or without prior activation by rIFNα or anti-IFNα blocking mAb (Table I). An antibody dose of 6 µg/ml was comparable to 100 pg/ml of rIFNα in partially protecting endothelial cells from viral replication as assayed by intracellular virus protein expression, virus release and EC viability. The anti-viral activity was confirmed for anti-IFNα mAb clone no. 2 by the manufacturer PBL (Pestka Biomedical Laboratories) in a standardized CPE inhibition assay (compare Fig. 2*A* and Table III).

While previous experiments had pointed to a direct effect of the IFN-blocking mAb involving the Fc-domain[76] the first indication that the “IFN-like” activity was separable from the antibody moiety was gained when the antibody was further purified. Three sequential rounds of antibody binding to protein A/G were required to remove > 90% of immunoglobulin (Fig. 1*A*). However, the remaining fraction (IP supernatant) largely retained its capacity to stimulate the expression of IFN-regulated genes such as IFIT-1 (Fig. 1*B*).

Antibody preparations of several of these clones were available with differing purification schemes. The initial studies were performed with preparations subjected to a two-stage purification based on ammonium sulfate precipitation and ion exchange chromatography. Later preparations utilized a three-stage process involving an additional purification step by hydrophobic interaction chromatography (HIC), including more rigorous cleaning of the equipment between purification procedures. When anti-IFNα mAb clone no. 2 and anti-IFNβ mAb clone no. 3 were purified from ascites produced in Balb/C or SCID mice, the “IFN-like” activity was only observed for antibody preparations by the two-step method (Fig. 2*A* and 2*B*). In contrast, the additional HIC procedure abolished the capacity of anti-IFN mAb preparations to protect from viral infection or to elicit expression of IFN-responsive genes. Furthermore, three-step purified antibodies displayed a potent IFN-neutralizing activity which could not be confirmed for the two-step preparations (Fig. 2*C*).

Having established that the described “IFN-like” activity was attributable to a discrete contaminant of the applied anti-IFN antibody preparations, the source

and identity of the activity was further investigated. Contamination with microbial products stimulating a toll-like receptor (TLR) pathway and an associated autocrine feedback-loop of type I interferon signals was a possible trigger which would result in the concomitant pro-inflammatory activation of endothelial cells[58, 78, 79]. However, when comparing the TLR4 agonist LPS with contaminated anti-IFNα blocking mAb and rIFNα, only LPS was found to induce the expression of the pro-inflammatory adhesion molecule E-selectin (CD62E) which was potently blocked by the LPS-antagonist polymyxin B (Fig. 3*A*). With respect to the activation of interferon-regulated genes, LPS treatment of ECs resulted in a moderate induction of ISG15 transcript levels and did not regulate IFIT-1 mRNA expression which was in stark contrast to the pronounced induction of these genes by rIFNα or the contaminated antibody (Fig. 3*B*). Again, the LPS effect was selectively blocked in the presence of endotoxin antagonist, whereas polymyxin B had no impact on the other two stimulants. With NF-κB and ISGF3 being the key transcription factors activated by TLR pathways, a potential TLR signal was further investigated by NF-κB reporter assay. While the contaminated anti-IFNα blocking mAb was previously shown to activate ISGF3[76], an effect on NF-κB activity could not be observed (Fig. 3*C*) which was a further argument against the involvement of a TLR agonist.

Considering the original observation of an “IFN-like” signal elicited by the antibody preparations, a possible contamination with type I interferon (exceeding the neutralizing capacity or specificity of the respective antibody) was then questioned. In support of this hypothesis we found a similar time and dose response of ECs stimulated with rIFNα or two-step purified anti-IFNα mAb clone no. 2 (Fig. 4*A* and 4*B*). Importantly, a sheep polyclonal antiserum directed against human IFNα potently blocked the induction of IFN-responsive genes by the contaminated anti-IFNα mAb clone no. 2 or anti-IFNβ mAb clone no. 3 thus pointing towards contamination with an IFNα subtype (Fig. 4*C*). Sheep antiserum retrieved from the same animal prior to immunization did not interfere, whereas a similar inhibitory effect was observed with a rabbit polyclonal anti-IFNα antiserum (data not shown). It is of interest to note that the three-step purified monoclonal antibodies (either separately or in combination) did not alter IFIT-1 induction by the contaminated anti-IFNα mAb clone no. 2 (Fig. 4*D*).

Since the polyclonal antisera directed against human IFNα exhibited weaker but detectable neutralizing capacity towards mouse IFNα (data not shown), the initial characterization of the contaminating IFN subtype was aimed at the distinction between human and mouse IFNα(Fig. 5*A*). When testing two-step purified anti-IFNα mAb clone no. 2 or no. 9 in CPE inhibition assays on human (A549) versus mouse (L929) target cells, the contaminant in either antibody preparation showed a more potent (about 1000-fold higher) effect on human cells. Thus, with human IFNα being the most likely contaminant we proceeded to investigate which subtype(s) might be involved. Considering that the three-step purified anti-IFNα mAb clone no. 2 could not reduce the IFN activity of the contaminated antibody preparation, the various subtypes of human IFNα were compared with respect to neutralization by (non-contaminated) mAb no. 2. IFNα subtypes 8, 14, and 16 were not blocked by anti-IFNα mAb clone no. 2 whereas all other subtypes showed partial or complete inhibition (Fig. 5*B*). Comparably, the sheep polyclonal anti-human IFNα antiserum potently blocked all tested IFNα subtypes. Among the three likely candidates, rIFNα$_8$ and rIFNα$_{14}$ had been produced by PBL shortly prior to the preparation of anti-IFNα mAb clone no. 2 which may have been the source of contamination. To further substantiate this notion and discriminate between the two IFNα subtypes, the detection of rIFNα$_8$ and rIFNα$_{14}$ by two different ELISA subtypes was compared (Table II). In contrast to standard rIFNα$_{2a}$ (100%) the detection level of rIFNα$_{14}$ was poor (0.2 and 4%) in either assay, whereas rIFNα$_8$ was only recognized by one ELISA subtype (10%). When the contaminant of anti-IFNα mAb no. 2 was evaluated, the detection pattern resembled the profile established for rIFNα$_{14}$, i.e., the interferon was detectable by both ELISA subtypes but at different sensitivities. It should be mentioned that the contaminant was separated from mAb no. 2 by protein G column prior to ELISA testing to avoid interference by the antibody. Furthermore, when the antibody was added to rIFNα in spike recovery experiments, the ELISA detection of rIFNα$_8$ and rIFNα$_{14}$ was not affected (data not shown). Finally, to reconcile the conclusion of human rIFNα$_{14}$ being a discrete contaminant in the two-step purified preparation of anti-IFNα mAb no. 2 with our original finding that the “IFN-like” activity is destroyed by ficin digest in F(ab´)$_2$ generation[76], we further confirmed that rIFNα$_{14}$ is a target for the protease and its activity is lost upon treatment with ficin (Fig. 5*C*).

Furthermore, our original findings on “IFN-like” activities associated with interferon neutralizing antibodies were not confined to the anti-IFNα mAb clone no. 2 but were shown for four different monoclonal antibodies[76]. Therefore, the selection of antibodies supplied by PBL as well as anti-IFN antibodies obtained from other companies were tested for potential contamination with type I interferons. Table III lists a variety of antibodies found to contain detectable levels of anti-viral activity and protein recognized by IFNα ELISA illustrating the prevalence and significance of comparable contaminations in commercially available antibody preparations. Specific activities calculated for the respective IFN contaminants (in units of anti-viral activity per mg detectable IFN protein) are unusually high which is likely to be the result of the diminished capacity of the IFN ELISA to detect select IFNα subtypes.

Discussion

The preparation of antibodies from ascites is prone to contamination with mouse-derived peptides and DNA as well as pathogen-associated factors such as viral and bacterial components[80]. Appropriate methods have been developed for antibody purification and process monitoring[81, 82], and have similarly been applied to the investigated anti-IFN blocking mAbs. Nevertheless, the antibody preparations were found to contain a soluble contaminant triggering an “IFN-like” activation of target endothelial cells which pointed to a possible residue of ascitic components initiating an immune response. Since the majority of pathogen-associated signals leading to the IFN pathway are mediated by the TLR family, we screened for hallmarks of TLR activity. However, we did not observe the induction of the transcription factor NF-κB nor the pro-inflammatory activation of ECs, strongly arguing against TLR involvement.

First indications towards a potential contamination with type I interferon were gained from the “signal signature” on IFN-responsive genes: The time course and dose-dependency of gene regulation closely resembled the direct activation of endothelial cells by rIFNα, while rIFNβ showed a distinct and more prolonged activation of target genes (data not shown). Further proof for the involvement of IFNα came from competition studies with sheep or rabbit polyclonal antibodies showing that the contaminant was neutralized by the anti-IFNα antiserum. The co-purification of mouse interferon upon monoclonal antibody isolation from ascites was a potential source of contamination which was addressed by comparing the IFN effect in CPE inhibition assays on mouse versus human target cells. There was a significantly higher impact on the human target cells. In addition, the subsequently applied ELISA tests for the detection and quantitation of IFNα protein did not recognize murine interferon isoforms (data not shown), again arguing for the presence of human rather than mouse IFNα.

Thus, the previously observed activation of ISGF3 and the regulation of IFN target genes such as IFIT-1 and ISG15 were consistent with our finding that human IFNα was the most likely source of contamination and the origin of the “IFN-like” activity associated with the anti-IFN antibody preparations. Furthermore, the reported signal dependency on the autocrine production of IFNβ by endothelial cells was comparable to the impact of IFNβ silencing on EC stimulation with recombinant interferons[76] and was explained by a reduced

activation state of IFNAR in the absence of low-level endogenous IFNβ expression. However, the question remained as to why the contaminating human IFNα was not neutralized by the respective anti-IFNα blocking mAb. When investigating the neutralizing capacity towards various rIFNα subtypes, the monoclonal antibody failed to inhibit individual family members while the polyclonal antiserum potently repressed all IFNα subtypes. Protein detection by IFNα ELISA further substantiated the conclusion that a distinct IFNα subtype ($IFN\alpha_{14}$) not neutralized by the monoclonal (anti-IFNα mAb no. 2) was present in the antibody preparation. Since the interferon activity associated with anti-IFN blocking mAbs had previously been detected for 4 different commercially available antibodies directed against IFNα or -β[76], a comparable contamination with type I interferon subtypes not recognized by the respective antibody seemed likely. This notion was supported by our finding that sheep polyclonal anti-IFNα antiserum also potently repressed the interferon activity in anti-IFNβ mAb no. 3 preparations.

The source of contamination could be traced to the sequential production of recombinant type I interferons and anti-IFN blocking antibodies with common equipment. Despite the considerable time window of several months between productions, despite the standard procedure for equipment cleansing and the sensitivity of interferons to inactivation by temperature and pH changes[83], the contamination of antibody preparations with functional type I IFN was substantial. Furthermore, the regular two-step purification procedure involving antibody precipitation and ion exchange chromatography was insufficient to remove the contaminating agent, i.e., separation of IFN and antibody required an additional step of hydrophobic interaction chromatography. Also, attempts to purify the monoclonal antibody by binding to protein A/G were inefficient and resulted in partial co-segregation of the contaminant unless repeatedly performed. This complication had led to the previous misinterpretation regarding the involvement of antibody domains in the observed “IFN-like” signal: Prior to antibody treatment with ficin protease for the generation of Fab or $F(ab')_2$ fragments, the immunoglobulin was subjected to a single round of purification by protein A column[76]. A considerable amount of rIFN must have remained associated with the antibody as reflected in the activation of endothelial cells by “purified” material. As now demonstrated, proteolytic processing by ficin was not

selective for the generation of immunoglobulin fragments but also led to the inactivation of rIFNα. Hence, rather than the separation of the antibody Fc domain, the proteolytic destruction of the contaminating interferon accounted for the loss of “IFN-like” activity in the antibody preparation. It is of interest to note that the presence of antibody seemed to have a stabilizing effect on the contaminant. In control experiments exposing $rIFN\alpha_{14}$ to the required buffer systems without the addition of ficin protease, interferon activity was rapidly lost in the absence of antibody or high-dose albumin (data not shown). We therefore propose that antibody binding to interferon (without neutralizing effect) or unspecific protein interactions as reported for rIFNα and albumin[84] may promote the persistence of IFN contaminations in antibody preparations.

The importance of our observation and characterization of functional type I IFN contaminants in neutralizing antibody preparations is demonstrated by the frequent occurrence of detectable IFN activity in a substantial number of tested antibodies. Apart from various mouse monoclonals against human IFNα and IFNβ (MMHA-2, MMHA-3, MMHA-9, MMHA-13, MMHB-3, MMHB-12) also rat anti-mouse antibodies directed against IFNα (RMMA-1) or IFNγ (RMMG-1) presented with considerable amounts of recombinant type I interferon. While most of these antibodies were produced by PBL, additional examples for contaminated antibodies by an alternative supplier were found. Two anti-pig IFN mAbs (K9, F17) similarly showed contamination with rIFNα. The range of detectable interferon activity or protein varied considerably (by a factor of 1000) which may reflect a difference in the extent of contamination and/or the sensitivity of detection systems towards the contaminating IFN subtypes. The highest levels of anti-viral activity as recorded for the anti-IFNα mAb no. 2 equalled a concentration of 800 U/ml of human rIFNα when applying the antibody at a common dilution of 50 µg/ml. The amount of contaminating rIFNα is well within the concentration range preferred for *in vitro* experiments. For example, stimulation of target cells with 1000 U/ml of biological or recombinant $IFN\alpha_{2a}$ in the presence of 50 µg/ml of contaminated blocking mAb no. 2 would be expected to result in the complete neutralization of the α_{2a} subtype, while exposing the cells to 800 U/ml of $rIFN\alpha_{14}$. The net inhibitory effect on the target cells would be minor leading to the false interpretation of results, especially for experimental set-ups where the involvement and concentration of type I

interferon is the unknown parameter under investigation. In contrast, for experiments based on a lower ratio of antibody to neutralized IFNα, the impact of the contaminating rIFNα_{14} might be negligible. Thus, the information given in this report may be of help in interpreting previously conducted experiments with the listed antibodies. With respect to PBL products, all antibody preparations have been carefully evaluated, and contaminated mAbs were found to date back to the last 1-8 years. More stringent purification and equipment cleaning procedures as well as routine testing for contaminating activity have been put in place at PBL in part due to these experiments. It is also easy to envision that reagent providers who prepare multiple cytokines and monoclonal antibodies could face similar issues.

Figures

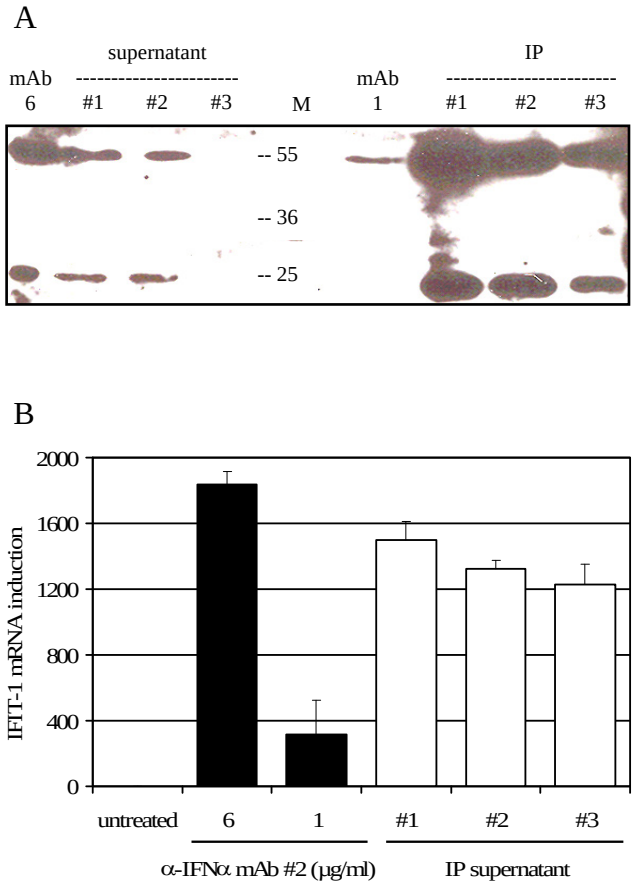

FIGURE 1. The “IFN-like” activity of neutralizing IFN-antibody preparations is separable from the antibody moiety. The anti-IFNα mAb clone no. 2 (6 µg in 1 ml EC medium) was subjected two three rounds of immunoprecipitation using protein A/G PLUS agarose. Aliquots of the supernatant as well as the bead pellet were retrieved after each step of one experiment. *A*, Western blot analysis of the supernatants and precipitated (IP) proteins was performed with goat anti-mouse IgG antiserum to detect the heavy (55 kDa) and light (25 kDa) chains of the monoclonal antibody. For comparison, preparations of anti-IFNα mAb clone no. 2 (6 µg/ml and 1 µg/ml) were loaded. The majority of antibody was precipitated within the first IP step; after the third cycle the antibody was no longer detectable in the supernatant. *B*, The IP supernatant was also applied to ECs cultures in comparison to the original anti-IFNα mAb clone no. 2 (at 6 or 1 µg/ml). Endothelial RNA was isolated 4 h thereafter for quantitative RT-PCR analysis of IFIT-1 expression. Despite progressive removal of the antibody the IP supernatant samples essentially retained their “IFN-like” activity.

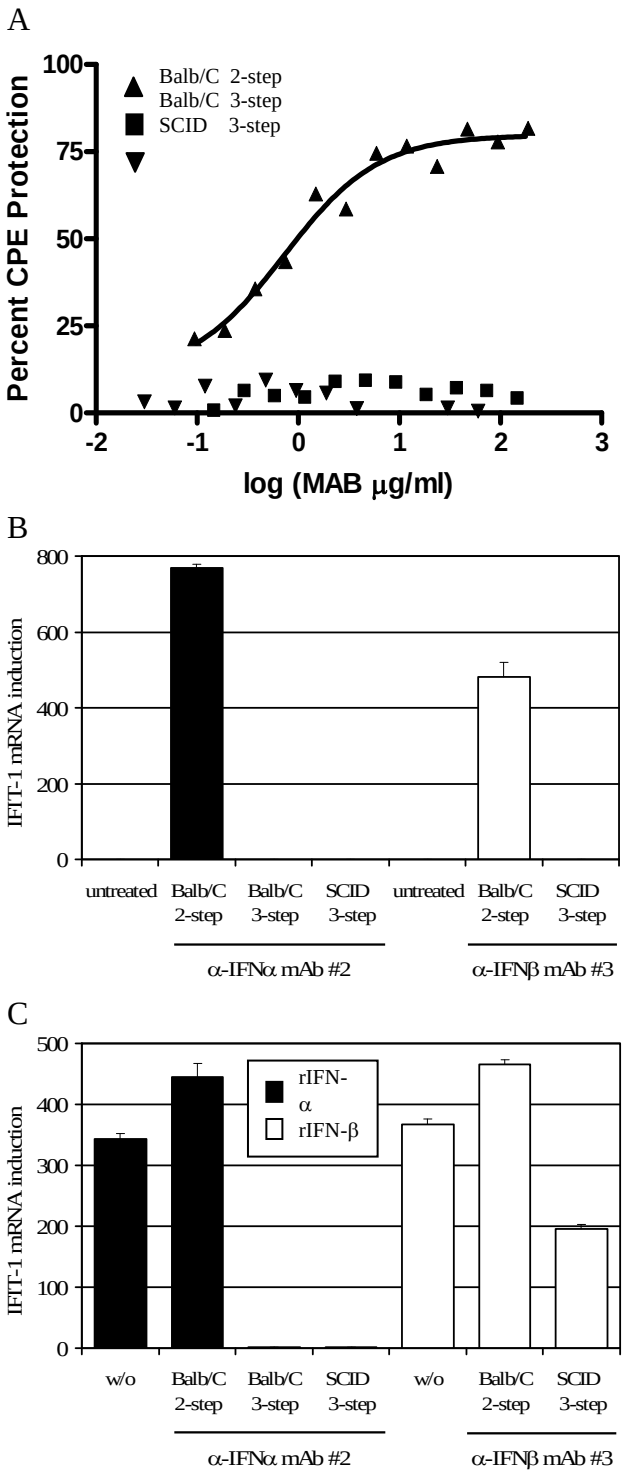

FIGURE 2. The “IFN-like” activity in antibody preparations is dependent on the mode of antibody purification. The neutralizing anti-IFNα mAb clone no. 2 and anti-IFNβ mAb clone no. 3 were isolated from Balb/C or SCID mouse ascites by a 2-step or 3-step procedure. Both methods involved ammonium sulfate precipitation and DEAE chromatography; additional purification by MEP-hypercel chromatography was included in the 3-step protocol. *A*, When the antibody preparations were compared in a standard A549/EMC virus cytopathic effect (CPE) inhibition assay, the 2-step purified anti-IFNα mAb clone no. 2 showed anti-viral activity equivalent to 1.25E+05 U/ml of IFNα activity, while 3-step purified antibody preparations had no detectable activity. *B*, Comparably,

only two-step purified anti-IFNα mAb clone no. 2 and anti-IFNβ mAb clone no. 3 (6 µg/ml) elicited a strong induction of the IFN-responsive gene IFIT-1 in endothelial cells, as assayed by quantitative RT-PCR. *C*, In contrast, when ECs were stimulated with rIFNα_{2a} or rIFNβ (100 pg/ml) for 4 h in the absence or presence of the various antibody preparations (6 µg/ml), only the 3-step purified blocking mAbs showed detectable neutralizing activity. All EC stimulation experiments were performed twice; differences between 2-step and 3-step antibody preparations were significant ($p \leq 0.05$).

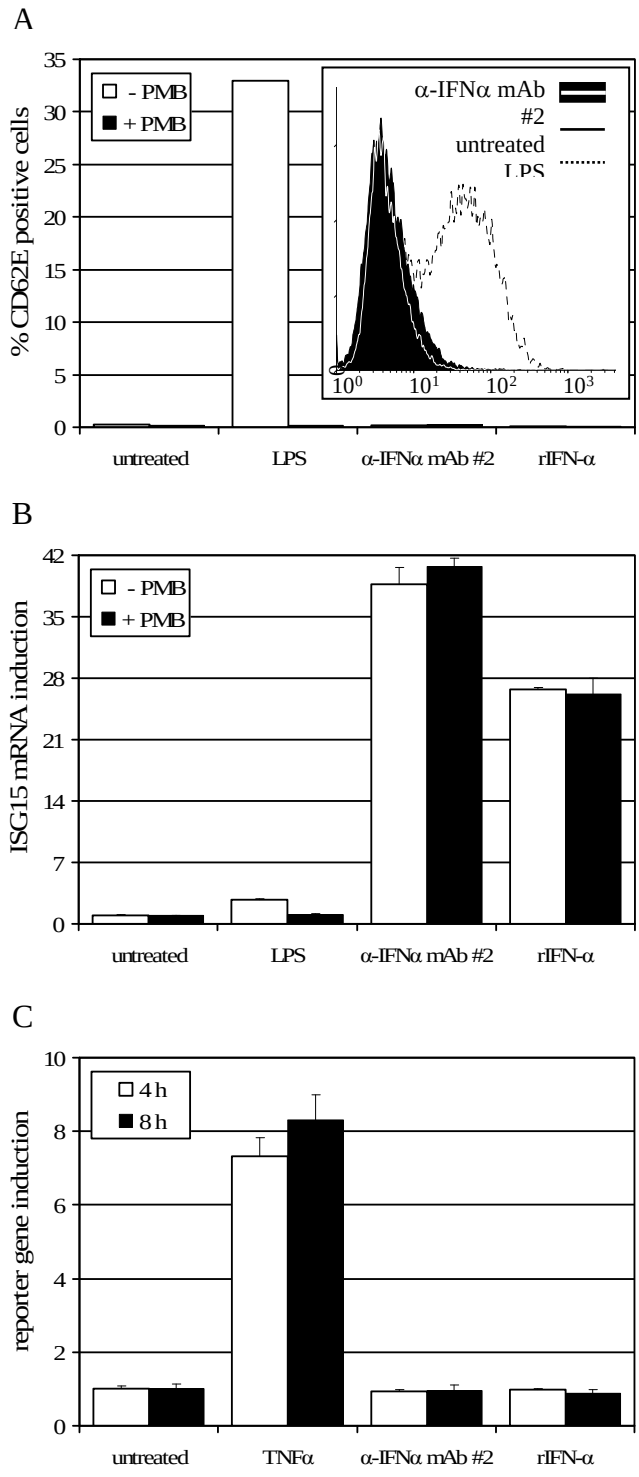

FIGURE 3. The contaminant in neutralizing IFN-antibody preparations has no pro-inflammatory activity. ECs were left untreated or were exposed to polymyxin B (PMB) at 50 µg/ml for 30 min prior to stimulation with LPS (1 µg/ml), anti-IFNα mAb clone no. 2 (6 µg/ml) or rIFNα$_{2a}$ (100 pg/ml) for 4 h. *A*, Cells were subsequently harvested for surface staining of E-selectin (CD62E) and flow cytometric analyis. *B*, Alternatively, EC RNA was analyzed by quantitative RT-PCR for ISG15 mRNA induction. *C*, To test for activation of NF-κB, ECs were transfected with an NF-κB reporter plasmid and subsequently stimulated with anti-IFNα mAb no. 2 (6 µg/ml), TNFα (100 ng/ml) or rIFNα$_{2a}$ (100 pg/ml) for 4 and 8 hours. Reporter gene expression is given in fold

induction compared to non-stimulated control cells. All experiments were performed twice. Pro-inflammatory activity could only be attributed to TNF-α and LPS controls; the latter was blocked by the endotoxin antagonist polymyxin B.

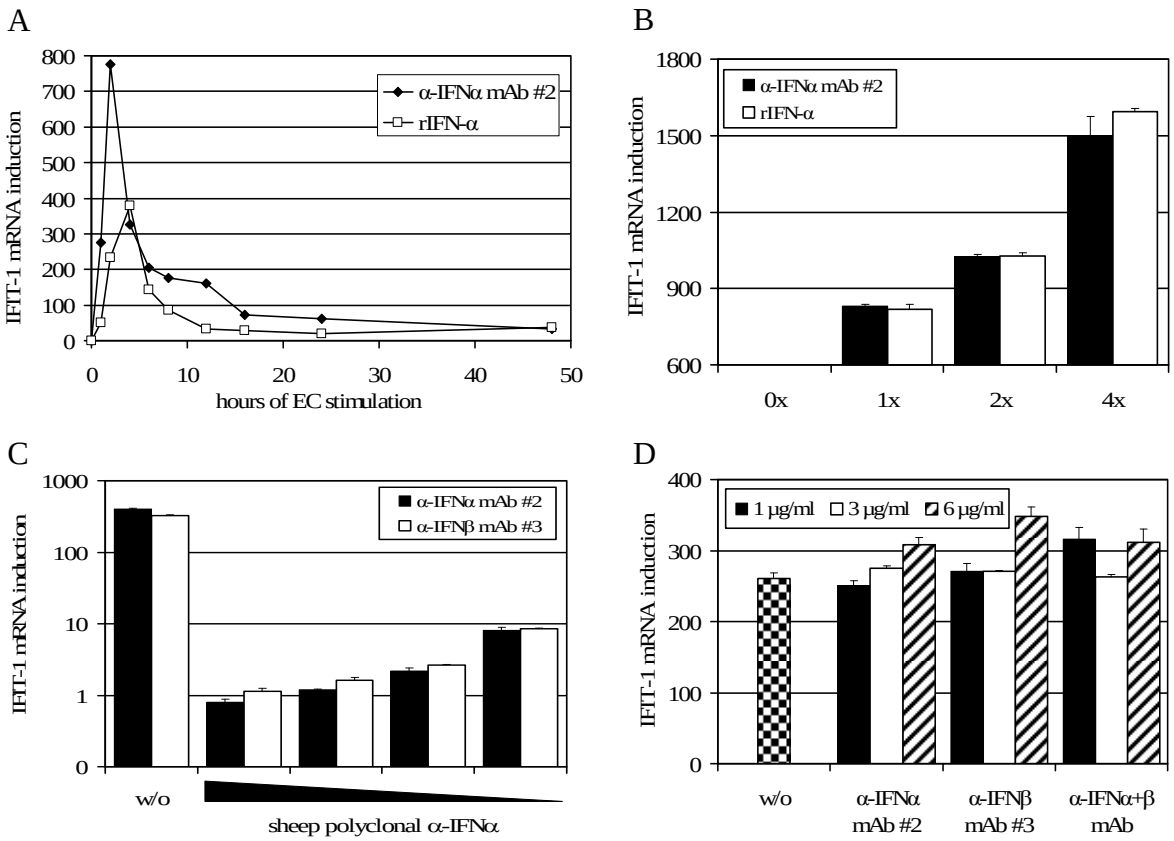

FIGURE 4. The contaminant in neutralizing IFN-antibody preparations shows a time- and dose-dependent signal reminiscent of type I IFN and can be inhibited by polyclonal anti-IFNα antiserum. *A*, ECs were exposed to anti-IFNα mAb no. 2 (6 µg/ml) or rIFNα$_{2a}$ (10 pg/ml) which was supplemented with 6 µg/ml mIgG1 to account for the presence of antibody during stimulation. Total RNA was analyzed by quantitative RT-PCR for IFIT-1 mRNA induction after 1, 2, 4, 6, 8, 12, 16, 24, and 48 hours of stimulation. Two comparable experiments were performed. *B*, For dose-response studies, ECs were treated for 4 h with anti-IFNα mAb no. 2 (6 µg/ml) or rIFNα$_{2a}$ (100 pg/ml) as well as with 2-fold and 4-fold higher concentrations of either substance. IFIT-1 mRNA regulation was evaluated in two separate experiments. *C*, When ECs were stimulated with anti-IFNα mAb clone no. 2 or anti-IFNβ mAb clone no. 3 (6 µg/ml) in the presence of increasing concentrations of neutralizing sheep polyclonal anti-human IFNα antiserum (dilutions of 1:6400, 3200, 1600, 800) the "IFN-like" activity in mAb preparations was entirely blocked ($p \leq 0.05$ at all concentrations in two separate experiments). A comparable amount of sheep antiserum obtained prior to immunization did not have any inhibitory activity (data not shown). D, In contrast, the contaminant of the two-step purified anti-IFNα mAb no. 2 preparation (3 µg/ml) could not be neutralized by three-step purified anti-IFNα mAb clone no. 2 and/or anti-IFNβ mAb clone no. 3 (at 1, 3, and 6 µg/ml), as monitored by IFIT-1 mRNA expression after 4 h of EC treatment (one experiment).

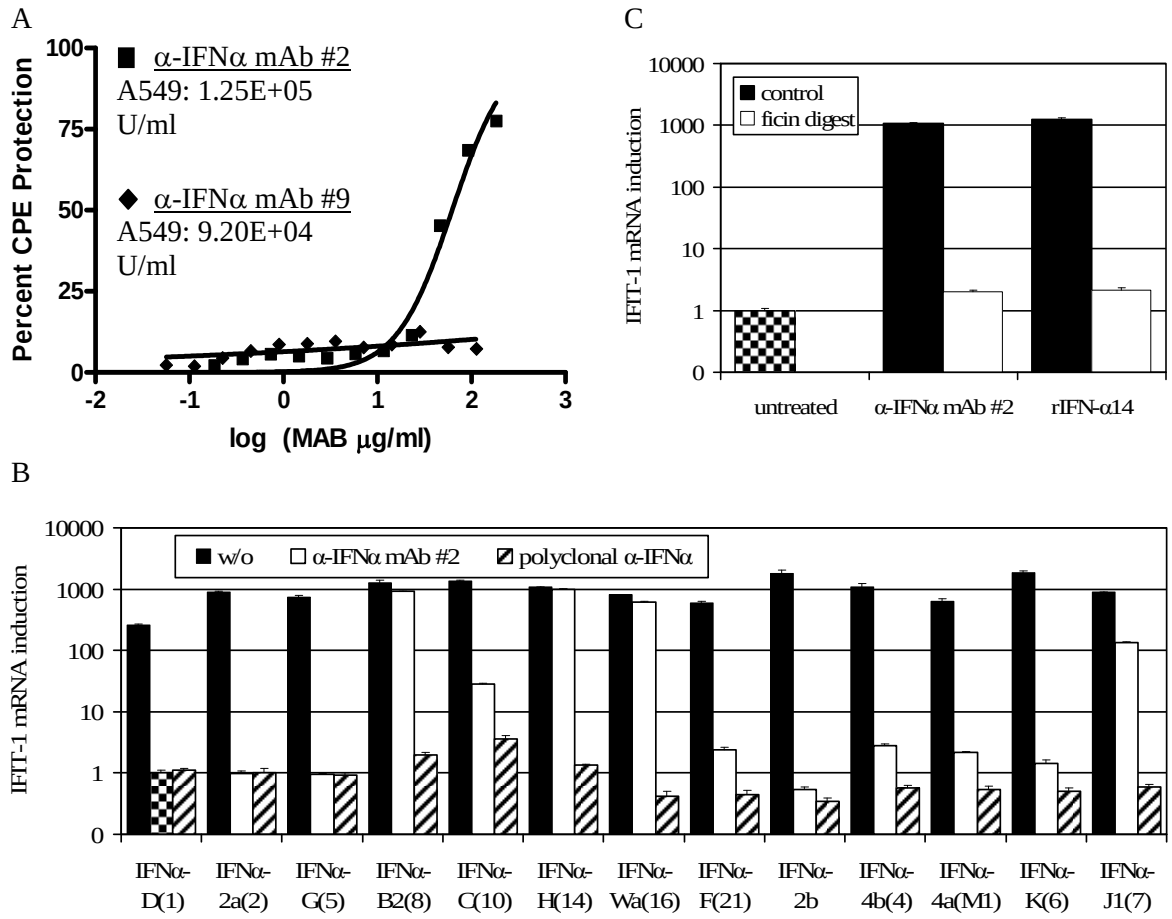

FIGURE 5. The "IFN-like" activity in neutralizing antibody preparations is likely to be due to contamination with particular human IFNα subtypes. *A*, When two antibody preparations (anti-IFNα mAb no. 2 and anti-IFNα mAb no. 9) were tested in a standard human (A549/EMCV) versus mouse cell CPE inhibition assay (L929/EMCV), the anti-viral activity was clearly more pronounced towards the human target, thus pointing to contamination with human rather than mouse IFNα. The equivalent to protection by recombinant IFNα is given in U/ml; the graphic illustration depicts the mouse cell assay. *B*, In a single set of experiments, ECs were stimulated for 4 h with rIFNα (100 pg/ml) of all known subtypes, in the absence or presence of three-step purified anti-IFNα mAb no. 2 (3 µg/ml) or sheep polyclonal anti-human IFNα antiserum at 1:800 dilution. While the polyclonal antiserum potently inhibited all tested IFNα subtypes, the monoclonal did not neutralize rIFNα subtypes 8, 14 and 16. *C*, Digest with ficin protease (versus control treatment in the respective buffer system without addition of protease) was applied to the contaminated, 2-step purified anti-IFNα mAb no. 2. In comparison, rIFNα$_{14}$ was combined with the appropriate amount of non-contaminated, 3-step purified mAb as well as BSA (reflecting the ratio of components in the contaminated antibody preparation). Following ficin or control treatment, the anti-IFNα mAb no. 2 (diluted to 12 µg/ml) and rIFNα$_{14}$ (at 100 pg/ml) were applied to EC cultures. Total RNA was analyzed after 4 h of EC stimulation for IFIT-1 mRNA expression by quantitative RT-PCR. The experiment was performed twice; the differences in IFIT-1 induction by control versus ficin treated compounds were found to be statistically significant ($p \leq 0.05$).

Tables

Table I. *Anti-viral activity by anti-IFNα neutralizing antibody*[a]

Treatment	Viral Protein Expression (%)	Virus Production (pfu/ml)	EC Viability (%)
untreated	0	0	100
virus	50	31425	38
rIFNα + virus	29	7850	62
α-IFNα + virus	27	5475	70

[a] ECs were exposed to infection with influenza A (PR8 wt) virus at the MOI of 1, with or without prior incubation with rIFNα (100 pg/ml) or anti-IFNα mAb clone no. 2 (6 µg/ml). The experiment was performed twice; results given are based on one set of analyses. The percentage of cells positive for intracellular expression of the viral matrix and nucleoprotein was determined 7 h post-infection by flow cytometry. EC viability was assayed 24 hours after exposure to virus. The replication of virus in endothelial cells was evaluated after 48 hours by determining the amount of virus released into the supernatant via a plaque formation assay.

Table II. *Detection of rIFNα subtypes by ELISA*[a]

Sample	Input (pg/ml)	ELISA #41100 human IFNα (pg/ml)	Detection (%)	ELISA #41105 human IFNα (pg/ml)	Detection (%)
$rIFN\alpha_{2a}$	10 000	10 000	100.0	9 887	99
$rIFN\alpha_{8}$	10 000	0	0.0	1 035	10
$rIFN\alpha_{14}$	10 000	20	0.2	385	4
α-IFNα mAb #2	unknown	730		3 083	

[a]

The sensitivity of commercially available ELISA variants (#41100 and #41105) towards different subtypes of rIFNα was evaluated in comparison to the unknown contaminant in the preparation of α-IFNα mAb #2. To avoid interference by the antibody, the contaminant was separated from the monoclonal by protein G column. The flow-through was applied to the ELISA tests and represents a 10-fold dilution of the starting material. The ratio of IFNα levels recorded for the contaminant by the two ELISA variants resembles the "detection signature" of human $rIFN\alpha_{14}$.

Table III. *Level of detectable contamination with rIFNα in various antibody preparations*[a]

Antibody	Antiviral activity (U/ml)	IFNα detected by ELISA #41105 (ng/ml)	Specific activity (U/mg)	Total protein in mAb preparation (mg/ml)	Contamination with rIFNα (%)
α-hIFNα mAb #2	125000	36.7	3.4E+09	7.5	4.9E-04
α-hIFNα mAb #9	92000	50.0	1.8E+09	4.5	1.1E-03
α-hIFNα mAb #13	750	< 0.1	> 6E+09	4.2	< 3.0E-06
α-hIFNβ mAb #3	1282	4.0	3.2E+08	3.6	1.1E-04
α-pigIFNα mAb K9	1200	3.0	4.0E+08	4.4	6.8E-05
α-pigIFNα mAb F17	140	0.1	1.1E+09	4.4	< 3.0E-06

[a] The antibody preparations were tested in the human A549/EMCV CPE inhibition assay and antiviral activity was expressed in equivalents of recombinant human IFNαU/ml). Concentrations of rIFNα (ng/ml) were measured by ELISA #41105 and applied to calculate the specific IFN activity in U/mg. To determine the percentage of contamination, the detected IFNα level was set in relation to the total amount of protein in antibody preparations.

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

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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 endothelial cells and fibroblasts. 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, intermediate and high 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.

Cell type specific regulation was apparent for distinct target genes (IFIT1, CXCL10, CXCL11) independent of IFN-α subtype as reflected in different levels of basal and inducible expression in endothelial cells versus 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.

Keywords: IFN-α, subtypes, endothelial cells, fibroblasts, interferon stimulated genes

1. Introduction

Since interferons (IFNs) were first discovered in 1957[12] 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[14]. 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[16, 85]. 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 antiviral, antiproliferative, and immunoregulatory activities[23, 86].

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 antiviral and antiproliferative action[19, 87-89]. Distinct receptor affinity and/or different interaction sites with the receptor chains have been proposed to account for the variability in the signals transduced[90, 91]. 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[90]. 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[92].

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[19, 93]. However, the potency of subtypes seems to vary with the particular IFN function under investigation[18, 91]. Regarding the signaling events triggered by IFN-α subtypes there are indications for differential receptor interaction[91, 94] and distinct activation of STAT molecules[88] 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[95, 96]. 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[22].

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 hours. 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 E.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 sec 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 β_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) and was based on non-parametric tests (Spearman, Mann Whitney U, and Wilcoxon test). The Bonferroni-Holm procedure was applied to correct for errors of multiplicity.

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 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 1:10000 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 antiviral 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 hours. 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 hours after influenza A infection, permeabilized with 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 hours 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[77]. 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 hours. Transcript levels of ISGs were determined by quantitative RT-PCR and mean values of 3 independent experiments are shown in figure 1 for five selected genes involved in antiviral and immunoregulatory IFN functions. To discriminate between fold induction in response to IFN stimulation (Fig. 1A) and the relative transcript levels present in the three cell types (Fig. 1B) the ISG values were evaluated in relation to the expression levels of two housekeeping genes in LECs, BECs and fibroblasts.

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. IFIT1 mRNA was highly induced in LECs and in BECs upon IFN-α treatment, whereas fold induction was substantially lower in fibroblasts. However, when comparing the transcript levels present in these three cell types following IFN stimulation, we found that IFIT1 mRNA was equally abundant in fibroblasts and ECs due to a high level of basal IFIT1 expression in human skin fibroblasts. In contrast, CXCL10 and CXCL11 showed low transcript levels in fibroblasts as compared to ECs but were highly inducible by IFN-α. ISG15 was regulated in a comparable manner in all three cell types, while endothelial cultures showed lower inducibility as well as lower transcript levels of CCL8 than fibroblasts.

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 Mann Whitney U 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 hours 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 hours) 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 stimulatory capacity. CXCL10 protein was stable in EC supernatant for 24 hours.

3.3. Antiviral 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 antiviral 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 hours 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 hours; virus released into the supernatant was detected at 24 hours post infection. The antiviral activity of the investigated subtypes correlated with their capacity to regulate ISG mRNA and protein (table 2). IFN-α1 exhibited the weakest potency to protect LECs against influenza infection and propagation whereas IFN-α4b and IFN-α2b showed intermediate and strong antiviral activity, respectively.

3.4. Variation in 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 hours. The maximum level of transcript expression was comparable between subtypes but was achieved at distinct ligand concentrations (Fig. 4). 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. 5). 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 hours. IFIT1 transcript levels exhibited a sharp peak at 4 hours post stimulation and were close to baseline after 8 hours. An identical time course was observed for the three IFN-α subtypes applied. ISG15 mRNA peaked at 4 to 8 hours; 24 hours 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[14, 97]. 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[98, 99]. Interestingly, IFN-α1 has repeatedly been reported to be among the major subtypes produced[98, 100, 101].

When we analyzed the potency of 13 different IFN-α proteins in ISG regulation and antiviral protection we found a reproducible pattern of activity in LECs, BECs and fibroblasts, i.e. the subtypes 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, -5, -16, -21) and weak activity (IFN-α1). The differential activity of IFN-α subtypes was confirmed at the protein and functional (antiviral 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 or anti-proliferative activity were repeatedly attributed to IFN-α8 and IFN-α1, respectively[19, 93, 102]. 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[103], 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[91]. These differences may result in divergent signaling events elicited by IFN-α proteins with respect to STAT activation[88]. 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 antiviral protection by IFNs[104, 105] and ISG15 is reported to have immunoregulatory effects[106]. 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[22]. 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[17], 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.

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Figures

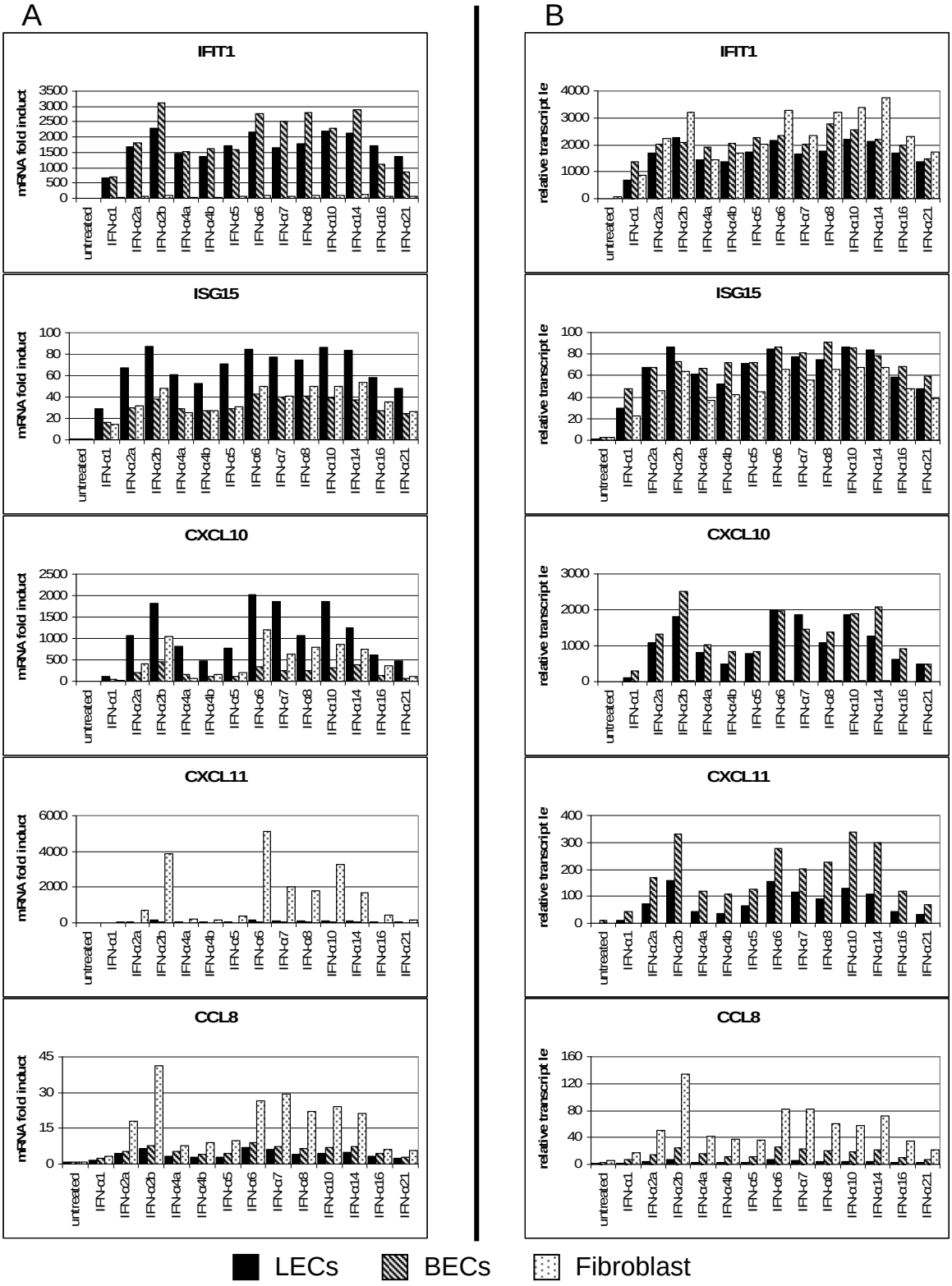

FIGURE1. ISG mRNA induction and transcript levels upon IFN-α stimulation of LECs, BECs and fibroblasts. Primary cells were stimulated with 100 pg/ml of each IFN-α subtype. Total RNA was analyzed by quantitative RT-PCR after 4 hours of stimulation. The fold induction of ISG mRNA was calculated in relation to the respective untreated control sample of each cell type (A). To compare the transcript levels between cell types, ISG values were

normalized to the same level of housekeeping gene expression and expressed in relation to the untreated LEC sample (B). 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.

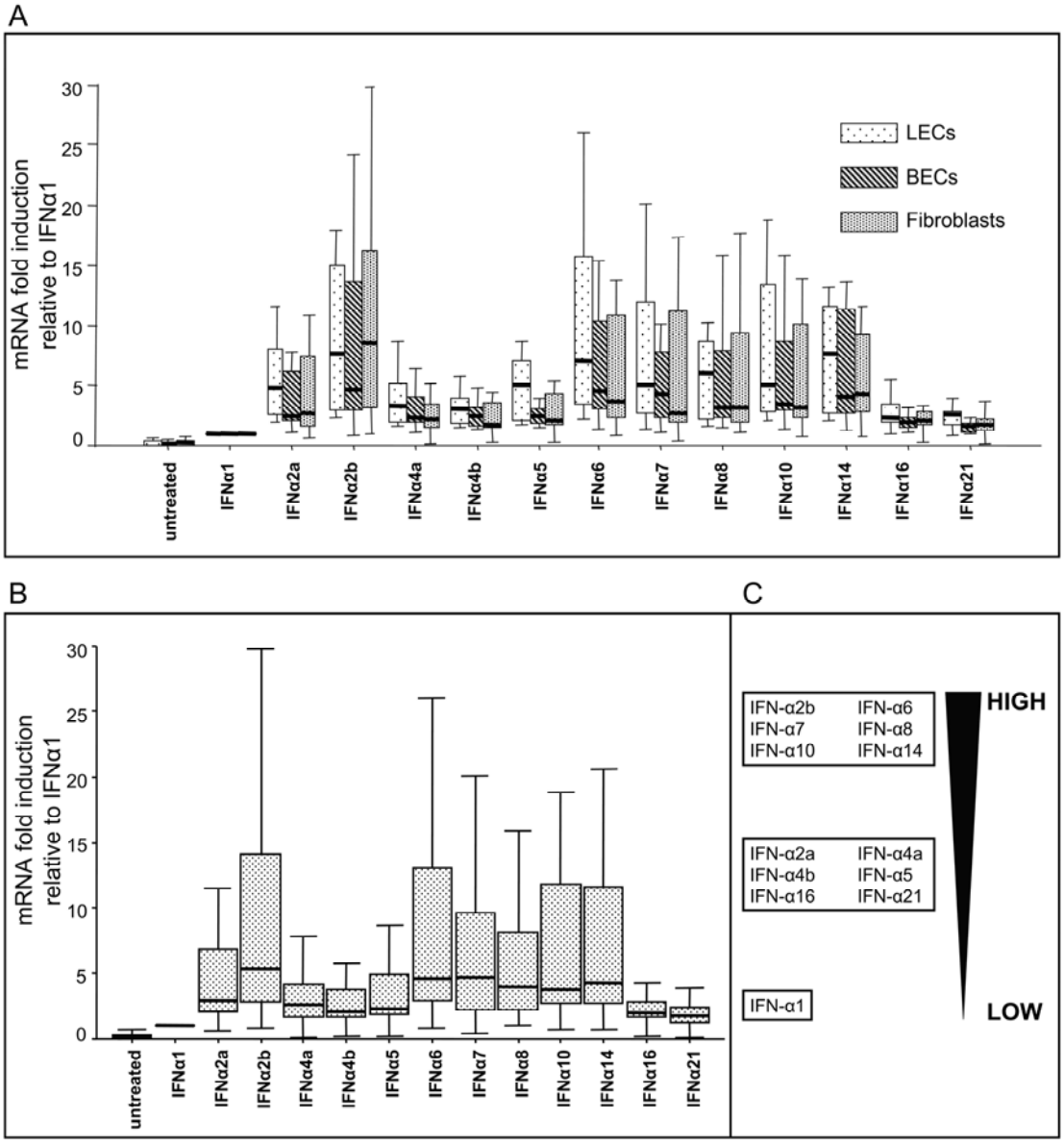

FIGURE 2. Relative potency of IFN-α subtypes in ISG stimulation. Induction values for IFIT1, ISG15, CXCL10, CXCL11 and CCL8 were calculated in relation to IFN-α1 levels set to 1. Data distribution is illustrated by box plot and is given separately for LECs, BECs and fibroblasts (A) or for the combined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

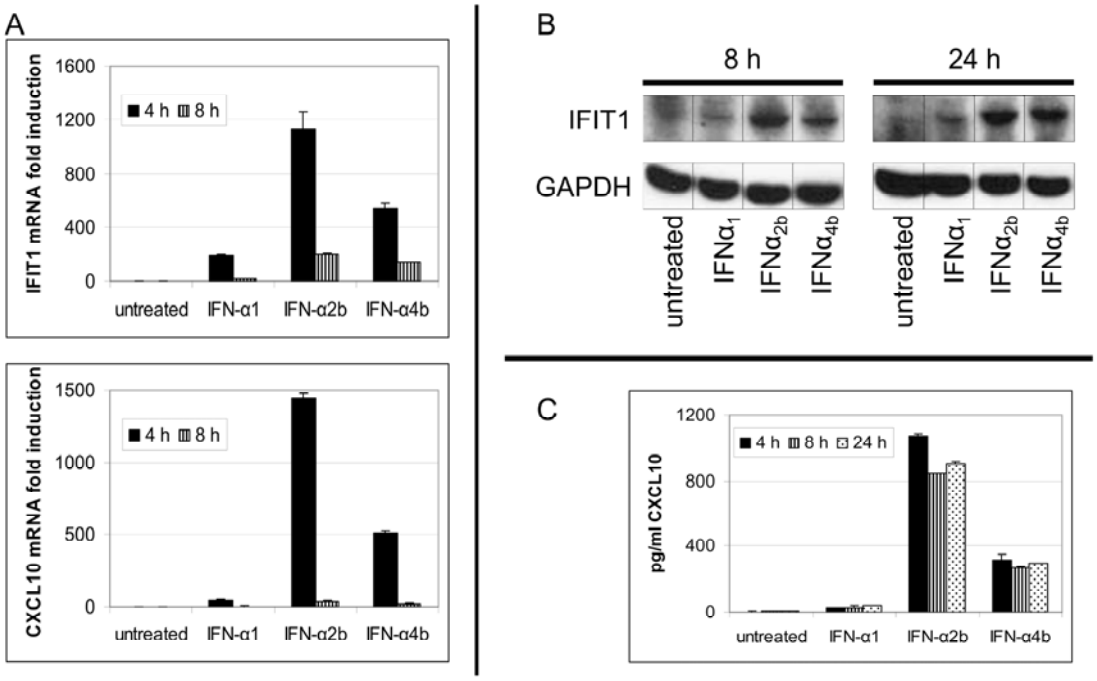

FIGURE 3. Comparison of ISG transcript and protein levels after IFN-α stimulation. LECs were treated with 100 pg/ml of IFN-α1, -2b, or -4b. At the indicated time points, cells and culture supernatant were harvested. Cell samples were subjected to concomitant mRNA and protein isolation. (A) IFIT1 and 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.

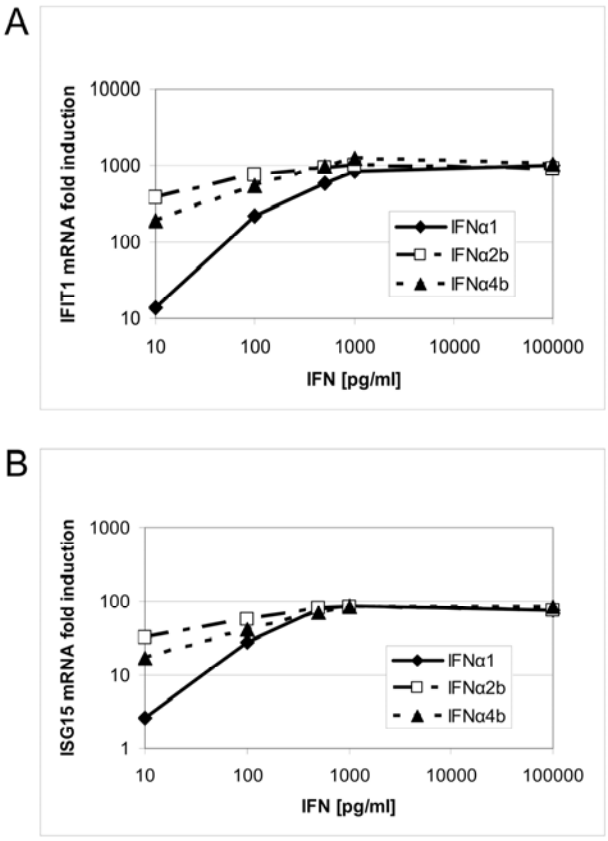

FIGURE 4. Dose dependence of ISG regulation by IFN-α subtypes. LECs were treated with increasing concentrations of IFN-α1, -2b, or 4b for 4 hours. Transcript levels of IFIT1 (A) and ISG15 (B) were analyzed by real-time RT-PCR. Data is given as mean and standard deviation of triplicate samples.

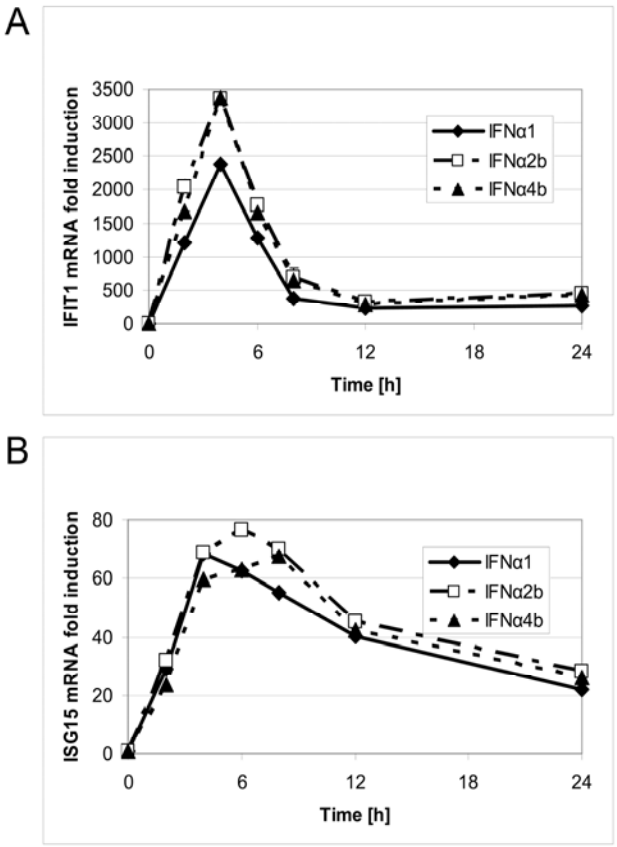

FIGURE 5. Kinetics of ISG induction in response to IFN-α subtypes. LECs were exposed to 400 pg/ml of IFN-α1, 200 pg/ml of IFN-α4b, or 40 pg/ml of IFN-α2b for 2, 4, 6, 8, 12 and 24 hours. Induction of IFIT1 (A) and ISG15 (B) mRNA was analyzed by real-time RT-PCR. Data is given as mean and standard deviation of triplicate samples.

Tables

Table I: *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´

Table II: *Antiviral activity of IFN-α subtypes*

Treatment [a]	Viral Protein Expression [%]	Virus Production [pfu/ml]
untreated	0	0
virus	88	66500
IFN-α1 + virus	66	56000
IFN-α2b + virus	44	23500
IFN-α4b + virus	57	35500

[a] LECs were infected with influenza A (PR8 wt) virus at an MOI equaling 1, with or without IFN-α pretreatment for 16 hours. Cells positive for intracellular expression of viral matrix and nucleoprotein were determined 7 hours post infection by flow cytometry. The amount of virus released into the supernatant 24 hours after virus infection was determined by a standard plaque formation assay. Two comparable experiments were performed; the results of one assay are presented.

Endothelial cells activated by pro-inflammatory stimuli establish an antiviral state independent of autocrine interferon beta secretion

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Abstract

Pro-inflammatory stimulation with lipopolysaccharide (LPS), tumor necrosis factor alpha (TNFα) or interleukin 1 beta (IL-1β) has been described to trigger the activation of interferon (IFN) regulatory factors (IRFs) and the autocrine expression of IFNβ in distinct cell types. This results in a synergistic effect on cellular and immune responses like enhanced resistance to bacterial or viral infections and may also be critical in chronic inflammatory diseases. The mechanism of interaction between pro-inflammatory activation and IFN signaling seems to be cell type specific and complex and is therefore poorly understood.

Here we have investigated the antiviral effects of pro-inflammatory stimulation of human microvascular endothelial cells (HMECs) against infection with influenza A PR8 wildtype virus, and have evaluated a possible contribution or dependence on autocrine IFNβ production.

Activation with TNFα, LPS or IL-1β led to a protective effect against virus infection, as detected by reduced expression of viral matrix- and nucleoprotein in ECs and reduced virus replication. Since cellular protection might be mediated by the direct regulation of interferon stimulated genes (ISGs) upon inflammatory activation of ECs, we evaluated the mRNA levels of IFIT1, ISG15 and IP-10. In comparison to recombinant IFNβ, EC stimulation with TNFα, LPS or IL-1β led to a substantial but delayed induction of IP-10 transcripts. Interestingly, IFIT1 was only induced by IFNβ and TNFα, whereas LPS and IL-1β did not elicit IFIT1 mRNA expression. Applying a neutralizing antiserum against IFNβ, the early ISG induction by TNFα remained unchanged, while autocrine IFNβ seemed to contribute to the sustained ISG mRNA expression at later time points. However, TNFα induced antiviral activity was not affected by blocking extracellular IFNβ.

Thus, we give evidence that TNFα, LPS and IL-1β mediate an antiviral program distinct from type I IFNs. In contrast to previous reports on non-endothelial cell types, autocrine IFNβ production does not enhance protection against virus infection in activated HMECs.

Keywords: endothelial cells; pro-inflammatory activation; antiviral effects; autocrine IFNβ; ISG regulation

Introduction

The endothelium is one of the key players in inflammation due to its barrier function between blood and tissue where infections occur[107]. Thus, immune cells in blood have to cross the endothelial barrier in inflammatory reactions to fulfill their protective functions at the site of infection. Endothelial cells (ECs) play an active role in the diapedesis process by expression of adhesion molecules, cytokines, chemokines and growth factors. Upon pro-inflammatory activation ECs mediate chemoattraction, rolling, adhesion and transmigration of immune cells towards the respective pathogen[8].

Stimulation of ECs with lipopolysaccharide (LPS), a membrane component of gram negative bacteria, with tumor necrosis factor α (TNFα) or interleukin-1β (IL-1β) secreted by leukocytes, induces an activated EC state[108]. Hence, these stimuli are applied in *in vitro* studies of inflammatory processes, and their mode of action is well described[109-112]. LPS is recognized by endothelial cells in complex with the serum derived LPS binding protein and the soluble receptor CD14[113], leading to the activation of toll like receptor 4 (TLR4)[114]. TLRs and the IL-1 receptor exhibit high sequence homology of the cytoplasmic domain which is therefore known as Toll/IL-1R (TIR) domain[115]. Ligand binding to either TLR4 or IL-1R leads to receptor dimerization and recruitment of adaptor molecules, among them the myeloid differentiation primary-response protein 88 (MyD88), IL-1R-associated kinases (IRAKs), and the TNF-receptor-associated factor 6 (TRAF6)[111, 112, 116]. Via phosphorylation and activation of IRAK1, subsequent modification of TRAF6 and the release of an adaptor protein complex into the cytoplasm, the major effects of TLR/IL-1R signaling are mediated, resulting in the activation of kinases IKK and JNK and the downstream transcription factors NF-κB and AP-1[117, 118].

In comparison, TNFα is a homotrimer with pleiotropic function, which binds to the respective receptors TNFR1 and TNFR2[119]. Ligand binding induces trimerization of the receptor complex and binding of TNF receptor associated death domain (TRADD), receptor interacting protein (RIP), TNF receptor associated factor 2 (TRAF2) and FAS associated death domain (FADD), thereby enabling the recruitment of the IKK and JNK to the receptor complex[120]. Thus, TNFα signaling results in the activation of NF-κB and AP-1 by a pathway distinct from LPS and IL-1β.

More recently, the cross-talk between inflammatory activation and interferon pathways has been addressed. In this context, it was shown that cell exposure to LPS induced so-called interferon stimulated genes (ISGs) like IFIT1 or CXCL10 in a MyD88 independent manner[121]. Moreover, LPS, TNFα and IL-1β signaling was found to result in the transcriptional induction, phosphorylation and translocation of interferon regulatory factors (IRFs), which bind to positive regulator domains in the promoter sequence of IFNβ[121-124], thereby promoting the production and secretion of autocrine IFNβ in distinct cell types.

IFNβ belongs to the type I interferons which exhibit immunomodulatory, antiproliferative and antiviral actions[86]. The production of interferon is generally induced upon pathogen recognition by TLRs resulting in the activation of IRF3 and IRF7. IFNβ is under the immediate transcriptional control by IRF3. The subsequent expression and autocrine signaling of IFNβ induces over 100 ISGs with effector function, including antiviral genes like IFIT1, ISG15, Mx1 or OAS1[22]. Moreover, IRF7 induction is a downstream event of IFNβ signaling and is required for IFNα gene transcription. In summary, the first line of IFN mediated innate immunity is dependent on IFNβ, whereas subsequent actions are enhanced by an IFNα/IFNβ feedback loop[125].

All type I IFNs bind to the same cell surface receptor complex, consisting of the two subunits IFNα receptor 1 (IFNAR1) and IFNAR2[20, 21]. The activation of the JAK-STAT pathway leads to the formation of a STAT1 – STAT2 heterodimer which associates with IRF9 to form the transcriptional activator complex ISGF3. This transcription factor complex translocates into the nucleus, binds to and activates the interferon stimulated response element (ISRE), a common sequence in ISG and IFN promoters. Among the ISG effector proteins, IFIT1 is central in the antiviral defense. It is reported to interact with the multisubunit complex eukaryotic initiation factor 3 (eIF3), thereby inhibiting the initiation of protein translation[32-34]. In support, ISG15 counteracts the virus mediated degradation of IRF3 and contributes to the activation of immune cells in pathogen defense[38, 41]. Other interferon regulated proteins such as the chemokine CXCL10 enhance the host reponse by attracting leukocytes to the site of infection.

In comparison, the antiviral effects of pro-inflammatory stimuli were first described more than 30 years ago and were suggested to involve autocrine IFN

signals[126-128]. Interestingly, these studies were mostly performed in monocytes or fibroblasts, cell types which are known to be major producers of IFNβ. However, the underlying mechanism of cellular protection against virus infection were not entirely resolved which is why we set out to further investigate the antiviral effects of pro-inflammatory stimuli and their dependence on autocrine IFNβ production. We chose human microvascular endothelial cells (HMECs) as a cellular model for our *in vitro* study because of their central role in inflammation.

Materials and Methods

Cell culture and stimulation of primary cells

Primary cultures were prepared from human foreskin samples as described previously[76]. In brief, HMECs were isolated by proteolytic digest and subsequently purified via anti-CD31 coupled magnetic beads (Invitrogen Corp., Carlsbad, CA). HMECs were cultured in EGM2-MV (Cambrex Corp., East Rutherford, NJ) containing fibronectin (1 µg/ml) and without supplementation of VEGF. Fibroblasts were isolated from the same foreskin samples via CD90 antibody (Dianova, Hamburg, Germany) coupled beads and propagated in MEM (Invitrogen) containing 20% FCS and 1 mM sodium pyruvate. PBMCs were isolated from EDTA treated blood of a healthy donor by gradient centrifugation using Ficoll-Paque (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK).

48 hours before stimulation, HMECs and fibroblasts were seeded in culture dishes supplied with EGM2-MV without VEGF to reach confluence within 24 hours. Thereafter, medium was exchanged for EGM2-MV without the supplementation of growth factors to allow cells to become quiescent for the next 24 hours. Then, cells were stimulated for the indicated time periods with 100 pg/ml rIFNβ (PBL – InterferonSource, Piscataway, NJ), 1 µg/ml LPS (Sigma-Aldrich, Corp., St. Louis, MO), 100 ng/ml TNFα (kindly provided by H.R. Alexander, NCI, NIH, Bethesda, MD) or 100 pg/ml IL-1β (Sigma-Aldrich). PBMCs were propagated in EGM2-MV without supplementation of growth factors and stimulated immediately after isolation.

Neutralizing sheep anti-IFNβ serum and the matching control, i.e., serum of the same animal before immunization, were supplied by PBL – InterferonSource and were applied at 1:800 to cell cultures.

Analysis of influenza virus infection and propagation

To determine the antiviral effects of cytokines, ECs were pretreated with the respective stimulus over night (15-18 hours). Thereafter, cells were thoroughly washed and then exposed to influenza A PR8 wt virus at a multiplicity of infection (MOI) equal to 1, in serum-free OPTI-PRO medium (Invitrogen) containing 5 µg/ml trypsin. Subsequently, cells were again washed and supplied with fresh OPTI-PRO medium containing trypsin.

For detection of viral uptake and protein expression, ECs were harvested 7 hours after virus infection, fixed and permeabilized using IntraPrep reagent (Beckman Coulter Inc., Fullerton, CA). Thereafter, cells were stained with a mixture of fluorescence labeled antibodies directed against viral matrix- as well as nucleoproteins (Dako, Glostrup, Denmark), and were analyzed with an FC500 flow cytometer (Beckman Coulter).

Virus production was determined by a standard plaque formation assay as described previously[77]. In brief, EC supernatant was retrieved 24 hours after influenza infection. Subsequently, Vero cells were exposed to a dilution series of EC supernatant for 30 minutes, washed and covered with OPTI-PRO and trypsin containing agar. After a two day incubation period the emerging plaques were counted and the viral titer was calculated based on the dilution factor. Results are given in plaque forming units per ml (pfu/ml).

Real-time RT-PCR

For analysis of ISG mRNA expression, cells were harvested and RNA was isolated with E.Z.N.A Total RNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA) according to manufacturer's instructions. Subsequently, 250 ng of RNA were reverse transcribed with oligo(dT) primers using the DyNAmo cDNA Synthesis Kit (Finnzymes, Espoo, Finland). Prior to PCR analysis, the generated cDNA was diluted 1:25. Real-time PCR was performed with TaqMan probes or SYBR green incorporation (for CXCL10 analysis). The respective master mix was obtained from Eurogentec (Seraing, Belgium). Primer (450 nm) and probe (200 nm) sets for the investigated transcripts are given in table 1.

Each sample was assayed in triplicates with the 7500 Fast PCR Detection System (Applied Biosystems, Foster City, CA) for 40 cycles of 5 sec at 95°C and 1 min at 60°C. Transcript expression levels were calculated using an on-plate standard dilution series and were normalized to the respective housekeeping gene values of β2-microglobulin (β2m) and β-actin (ACTB). The value for the untreated control sample was generally set to 1, and PCR data are given as fold induction of gene transcripts (mean and standard deviation of triplicate samples).

Immunoblotting

Cells were incubated for 15 min in cytosolic lysis buffer containing 10 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM DTT and 0.4% NP40 and the Complete Mini Protease Inhibitor Cocktail (Roche, Indianapolis, IN). After centrifugation, the supernatant (cytosolic extract) was retrieved, and the pellet was further processed to nuclear extracts. Therefore, the pellet was incubated for 2 hours with nuclear lysis buffer containing 20 mM HEPES, 10 mM NaCl, 100 µM EDTA, 1 mM DTT, 10% glycerol and the Complete Mini Protease Inhibitor Cocktail. Subsequently, protein content was measured by the Bradford method and extracts were adjusted to equal protein concentration. After SDS-PAGE, samples were transferred to a polyvinylidine fluoride membrane (Millipore, Billerica, MA) by semi-dry blotting. Blocking with 5% BSA in PBS containing 0.05% Tween20 was followed by immunodetection using polyclonal antibodies against pSTAT1-Y701 (Abcam, Cambridge, UK). To test for equal protein loading of nuclear extracts an anti-laminB1 antibody (Abcam) was applied. Secondary antibodies were peroxidase coupled and detected with SuperSignal West Femto Detection System (Thermo Fisher Scientific, Rockford, IL).

Enzyme-linked immunosorbent assay

For assessing IFNβ protein secretion, EC supernatants were analyzed with a high sensitivity human IFNβ ELISA (PBL – InterferonSource). Absorption at 450 nm was measured with a Wallac Victor^3 microtiter plate reader (Perkin Elmer Life Science, Waltham, MA) and IFNβ concentrations were calculated based on a standard dilution series.

ISGF3-ISRE Reporter Gene Assay

For the ISGF3-ISRE reporter gene assay, HMECs were transfected with a total of 20 µg DNA, consisting of 19 µg pISRE-Luc plasmid carrying three ISRE sites directing expression of the luciferase reporter gene (SABioscience, Frederick, MD) and 1 µg of a β-galactosidase expression plasmid with a constitutive CMV promoter (for normalization). Transfection was performed by electroporation at 200 V and 960 µF and HMECs were seeded into 30 mm wells to reach confluence within 24 hours. Cells were then exposed to the various stimuli of

interest. 6 or 18 hours after stimulation, cells were harvested in 35 µl lysis buffer (Applied Biosystems). Samples à 10 µl were assayed in duplicates for luciferase as well as β-galactosidase activity using the Tropix Dual-Light System (Applied Biosystems). Chemiluminescent detection was performed with a Wallac Victor^3 microtiter plate reader. Luciferase activity was measured in relative light units and normalized to the respective β-galactosidase values.

Results

Pro-inflammatory HMEC activation protects cells against influenza A infection

While antiviral protection caused by pro-inflammatory stimuli has previously been described for distinct cell types[127, 129, 130], we have now investigated the impact of TNFα, LPS and IL-1β on influenza A infection of primary, microvascular endothelial cells. After 16 hours of pretreatment with pro-inflammatory stimuli, cells were exposed to influenza A wild type virus. The expression of viral matrix- and nucleoprotein was determined in HMECs after 7 hours; the amount of newly synthesized virus released into the supernatant was measured at 24 hours post initial infection. Inflammatory activation of HMECs triggered antiviral effects comparable to stimulation with IFNβ, but with slightly weaker potency. While pretreatment with 100 ng/ml TNFα or 1 µg/ml LPS reduced viral protein expression by about 25–35% as compared to untreated and therefore unprotected cells, application of 100 pg/ml IL-1β elicited only a weak protective effect of 10-15%. Since the absolute values of viral infection varied strongly with the experiment and the HMEC isolate, data of one representative experiment are shown in table 2.

It should be mentioned that the concentrations of TNFα, LPS or IL-1β applied in EC treatment were saturating and shown to induce similar levels of E-selectin and ICAM-1 protein expression as measured by flow cytometry to confirm HMEC activation (data not shown).

ISG mRNA is induced upon pro-inflammatory EC stimulation

IFN mediated cellular protection against virus infection is dependent on the regulation of downstream ISGs to express proteins with antiviral activity; therefore, we further evaluated mRNA expression levels of IFIT1, ISG15 and CXCL10 upon pro-inflammatory activation of HMECs. In comparison to IFNβ, treatment with TNFα, LPS or IL-1β led to a delayed induction of CXCL10 transcripts as measured by qRT-PCR (figure 1). Regarding the regulation of ISG15, the pro-inflammatory EC treatment resulted in a weak increase of target gene mRNA. In contrast, IFNβ stimulation triggered a 10-fold higher ISG15 mRNA induction. The expression of IFIT1 transcripts after TNF-α treatment was comparable to the kinetics observed for CXCL10 mRNA, leading to a delayed but considerably lower induction of IFIT1 mRNA by TNF-α as compared to

IFNβ. Intriguingly, HMEC treatment with LPS or IL-1β resulted in a decrease of IFIT1 mRNA levels. The observed IFIT1 downregulation was specific for HMECs, since LPS treatment of primary fibroblasts or PBMCs caused a pronounced increase of IFIT1 transcripts measured at 8 and 24 hours after stimulation (figure 2).

HMECs activated by pro-inflammatory stimuli show ISGF3 activity

To explore the dependence of antiviral protection and ISG regulation by pro-inflammatory stimuli on the activation of the canonical type I IFN – JAK – STAT signaling pathway, we first examined ISGF3 activity in endothelial cells. Exposure of HMECs to TNFα, LPS or IL-1β resulted in the activation of the ISGF3 transcription factor as monitored by reporter assay, using a firefly luciferase reporter gene under the transcriptional control of multiple ISRE sites. However, LPS and IL-1β led only to weak ISGF3 activation which was hardly detectable at 6 hours after stimulation (figure 3a). To further confirm the contribution of the type I IFN signaling pathway, we investigated the phosphorylation and translocation of STAT1 by analysis of nuclear extracts with an antibody specific for STAT1 phosphorylated at Tyr701. While EC treatment with IFNβ led to the expected redistribution of phosphorylated STAT1 to the nucleus within a time period of 4 hours, TNFα did not trigger detectable STAT1 phosphorylation (figure 3b).

IFNβ release contributes to ISG induction at later time points

To further investigate the role of autocrine IFNβ production in ISG regulation, we applied neutralizing antiserum against IFNβ during EC activation with pro-inflammatory stimuli. The antiserum was confirmed to be very potent in neutralizing high amounts of recombinant IFNβ in cell stimulation assays (data not shown). Comparing the time course of IFIT1 and ISG15 regulation after stimulation with TNFα in the presence of IFNβ neutralizing antiserum or control antiserum retrieved from the same animal prior to immunization, we observed no substantial difference up to 8 hours post stimulation (figure 4). In contrast, at later time points of TNFα stimulation the application of IFNβ antiserum diminished ISG mRNA levels, indicating that an IFNβ feedback loop contributed to sustaining elevated ISG mRNA levels. Nevertheless, when we applied

cycloheximide in combination with TNFα, we could not confirm the dependence of ISG mRNA induction at later time points on the de novo protein synthesis of autocrine IFNβ (data not shown). Furthermore, we analyzed HMEC supernatant for IFNβ content after 4 or 8 hours of cell treatment with TNFα or LPS. We did not detect secreted IFNβ despite applying a high sensitivity ELISA with a detection limit of 2.5 pg/ml IFNβ.

IFNβ release does not contribute to ISGF3 activation by pro-inflammatory stimuli and is not required for cellular protection against influenza virus

Pro-inflammatory stimulation of HMECs led to the activation of the transcription factor ISGF3 as monitored by reporter assay (figure 3a). We next examined the dependence of ISGF3 activation on autocrine, secreted IFNβ. For this purpose, we applied neutralizing IFNβ antiserum to block the activity of released, extracellular IFNβ during EC stimulation with TNFα, LPS or IL-1β. Intriguingly, the application of neutralizing IFNβ antiserum did not reduce ISRE reporter activity after pro-inflammatory EC stimulation, even though the antiserum potently blocked the effect of recombinant IFNβ supplied at 500 pg/ml (figure 5a).

We further investigated the influence of autocrine IFNβ production on the antiviral effects of pro-inflammatory stimuli. Thus, we activated HMECs by 16 hours of pretreatment with TNFα. Neutralizing anti-IFNβ serum was applied during cytokine pretreatment and / or immediately after infection with influenza PR8 wt virus (MOI=1). Seven hours post infection the expression of viral matrix- and nucleoprotein was measured in HMECs by immunostaining and flow cytometry. In conclusion, blocking extracellular IFNβ in the supernatant of HMECs with TNFα treatment did not promote a higher viral infection rate (figure 5b), indicating that autocrine IFNβ secretion did not contribute to the antiviral activity of TNFα in HMECs.

Discussion

The innate immune response constitutes the first line of defense against foreign pathogens like bacteria or virus, and type I IFN production is a central event in establishing the cellular antiviral response. Several reports have demonstrated the induction of autocrine IFNβ after pro-inflammatory stimulation with ligands like LPS, TNFα or IL-1β, via the activation of NF-κB, AP-1 and IRFs[121-124]. These transcription factors bind to positive regulatory cis-elements in the promoter region of IFNβ, leading to gene expression and subsequent secretion of IFNβ[131]. However, it remains unclear whether the antiviral state established by pro-inflammatory cell activation is exclusively mediated by the induction of autocrine IFNβ, or if there is an IFN-independent mechanism eliciting cellular protection against viral infection.

Since the antiviral effects of pro-inflammatory stimuli were reported to vary with cell type and virus, we first confirmed for our experimental setup the potency of TNFα, LPS and IL-1β to protect HMECs against influenza A wt virus. Pro-inflammatory stimulation led to a reduction of viral protein expression in HMECs as well as of virus propagation by 10–30%, depending on the stimulus applied (table 2). IFNβ or $IFN\alpha_{2a}$ (data not shown) generally exhibited a higher antiviral activity of 50-60%.

We then explored the regulation of ISGs by pro-inflammatory stimuli. While CXCL10 mRNA was highly induced by all stimuli applied, ISG15 and IFIT1 transcripts were only moderately elevated after TNFα stimulation as compared to IFNβ treatment. Moreover, LPS or IL-1β did not induce IFIT1 mRNA and showed limited stimulatory capacity for ISG15. ISG15 and IFIT1 are coding genes for proteins with crucial antiviral functions[132, 133]. This is of particular interest, because the antiviral effects elicited by the different pro-inflammatory stimuli were in a comparable range despite their differences in ISG activation. Furthermore, the distinct pattern of ISG regulation by pro-inflammatory stimuli argued against the involvement of IFNβ feedback production, because an IFNβ feedback signal would be expected to induce substantial levels of all ISGs investigated. Interestingly, when we compared cell types such as primary fibroblasts and peripheral blood mononuclear cells which are known to be major producers of IFNβ, the cells responded to LPS stimulation with a robust induction of IFIT1 mRNA, which might point to an IFN feedback loop.

Preliminary results after applying neutralizing IFNβ antiserum support the involvement of a feedback loop in these cell types.

There is little information available on the basal expression level of IFNβ in ECs. Multiple groups performed gene expression studies with microarrays after pro-inflammatory activation of different EC types but did not provide evidence for IFNβ induction after stimulation with TNFα, LPS or IL-1β[134-136]. Accordingly, we did not detect IFNβ protein in the supernatant of activated HMECs using a high sensitivity ELISA. However, after influenza A infection ECs showed a rapid and robust IFNβ induction, as we measured up to 100 pg/ml IFNβ in the supernatant of HMECs at 7 hours post infection (data not shown).

Due to the possibility that undetectable yet present levels of IFNβ could synergistically act with inflammatory stimuli[137], we applied a highly potent antiserum to block the possible effects of subthreshold levels of extracellular IFNβ. We found that neutralizing IFNβ antiserum did not inhibit the pro-inflammatory ISG induction which further argues for a direct action of pro-inflammatory stimuli in ISG regulation. This effect might be mediated by NF-κB and AP-1 binding sites beside ISRE sequences in the promoter regions of ISGs[28]. Furthermore, anti-IFNβ antiserum did not reduce the antiviral effect of pro-inflammatory EC stimulation. However, to which extent the moderate ISG upregulation in response to pro-inflammatory cell activation contributes to establish the antiviral state is still not entirely resolved.

Intriguingly, TNFα activates the ISGF3 transcription factor to a similar extent like IFNβ, independent of autocrine expression and secretion of IFNβ (figure 3 and 5). However, implication of the canonical JAK-STAT signaling pathway could not be confirmed, since phosphorylation of STAT1 was not detected after HMEC exposure to TNFα. Although Cheon *et al.* described the impact of unphosphorylated STATs on ISG regulation[138], we could not convincingly demonstrate an accumulation of unphosphorylated STATs in the nucleus (data not shown). The induction of ISRE containing promoters by pro-inflammatory stimuli might also be mediated by IRF action. There are several reviews highlighting the function of IRF3 both in the regulation of IFNβ expression as well as in mediating antiviral effects[139, 140]. However, in our experimental setting we did not obtain evidence for activation and nuclear translocation of IRF3 as analyzed by immunoblotting of nuclear extracts or confocal microscopy of

HMECs (data not shown). Furthermore, it was reported that pretreatment with TNF-α or IL-1β of an IFN-insensitive STAT-1$^{-/-}$ U3A human epithelial-like fibrosarcoma cell line had similar protective effects against RSV infection as for the respective wt cells[141], concluding that the protective effect is a downstream event of NF-κB rather than IFN-JAK/STAT signaling.

Since the IFNβ antiserum was applied to cell supernatants to neutralize secreted IFNβ, the contribution of an intracellular IFNβ feedback mechanism could not be ruled out. According to the literature, intracellular IFNβ is sufficient to trigger the JAK/STAT signaling cascade[142, 143] and would not be affected by extracellularly applied neutralizing serum. An appropriate tool to investigate this possibility would be the application of siRNAs for silencing endogenous IFNβ. Unfortunately, transfection with siRNAs may lead to the activation of innate immunity[144]. Even small changes in this hypersensitive system would obscure results and interpretations.

In conclusion, our results demonstrate that the antiviral effects in TNFα, LPS or IL-1β activated HMECs against influenza A PR8 wt virus are independent of autocrine secretion of IFNβ and do not rely on the function of selected antiviral ISGs like IFIT1 and ISG15.

Conflict of Interest

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

Acknowledgements

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Figures

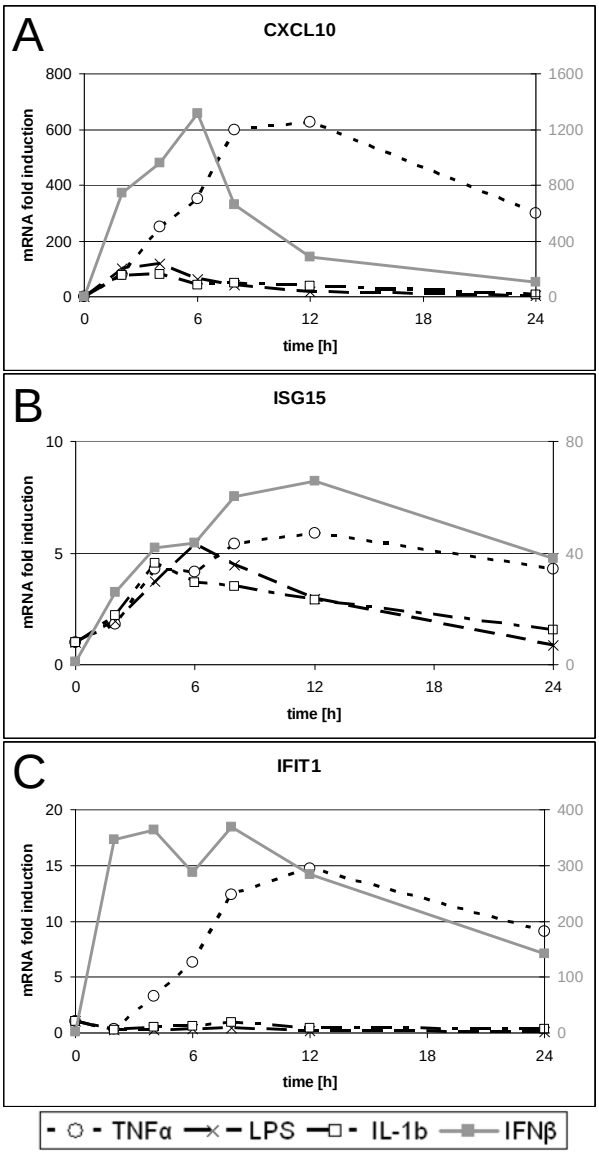

Figure 1: Regulation of ISG transcripts after HMEC stimulation with 100 ng/ml TNFα, 1 µg/ml LPS or 100 pg/ml IL-1β as compared to IFNβ (100 pg/ml) for 2, 4, 6, 8, 12 and 24 hours. CXCL10 (A), ISG15 (B) and IFIT1 (C) mRNA levels were analyzed by qRT-PCR and set in relation to the untreated control sample at time point zero. The data of one representative experiment is given in mean and standard deviation of triplicate samples. ISG induction levels upon IFNβ stimulation are indicated in grey letters of the second ordinate.

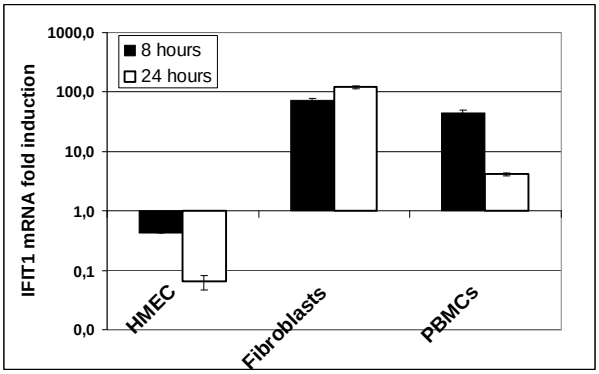

Figure 2: IFIT1 regulation in HMECs compared to primary fibroblasts and PBMCs. Cells were exposed to LPS at 1 µg/ml for 8 and 24 hours and IFIT1 mRNA expression was analyzed by qRT-PCR. For calculation of fold induction the respective untreated control sample was set to 1. The data of one representative experiment is given in mean and standard deviation of triplicate samples.

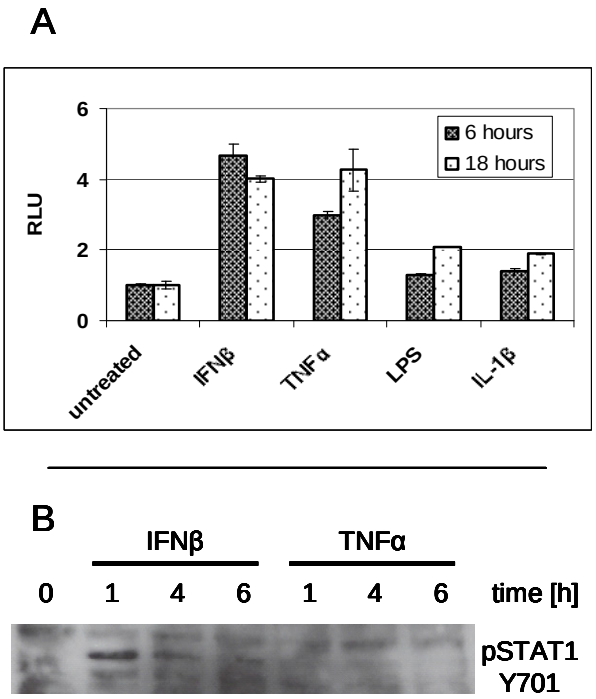

Figure 3: ISGF3 activity in ECs following pro-inflammatory stimulation. (A) Activation of the type I IFN responsive transcription factor ISGF3 was evaluated by reporter gene assay measuring luciferase activity in relative light units (RLU) after stimulation of HMECs for 6 or 18 hours with IFNβ (500 pg/ml), TNFα (100 ng/ml), LPS (1 µg/ml) or IL-1β (100 pg/ml). (B) STAT1 phosphorylation after HMEC exposure to IFNβ or TNFα was examined in nuclear extracts by immunoblotting with a specific antibody against phosphorylated STAT1. Protein concentrations in nuclear extracts were adjusted to compare equal amounts of total protein.

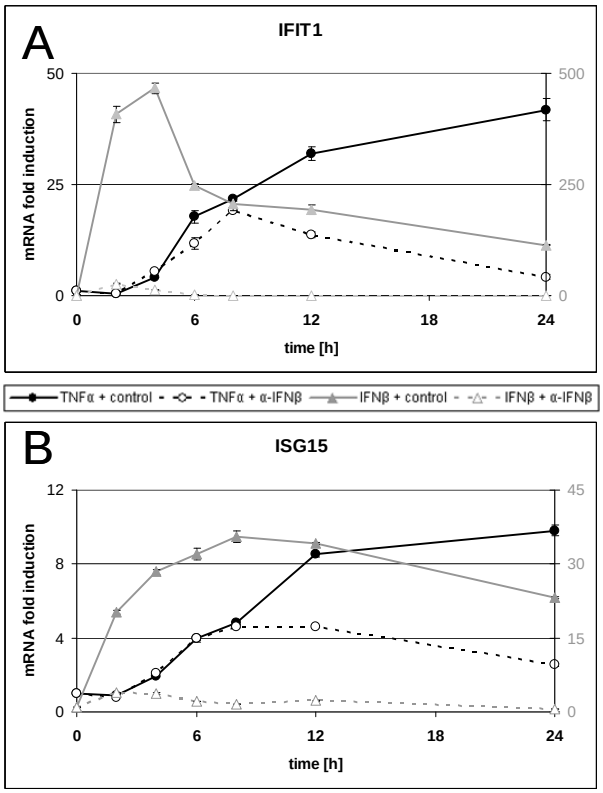

Figure 4: ISG regulation upon HMEC treatment with TNFα (100 ng/ml) or IFNβ (100 pg/ml) in the presence of neutralizing IFNβ antiserum (1:800). For control, sheep serum retrieved prior to immunization was applied. IFIT1 (A) and ISG15 (B) mRNA expression was analyzed by qRT-PCR. Fold induction of transcript levels was calculated in relation to the untreated control sample at time point zero. The data is given in mean and standard deviation of triplicate measurements in one representative out of three conducted experiment.

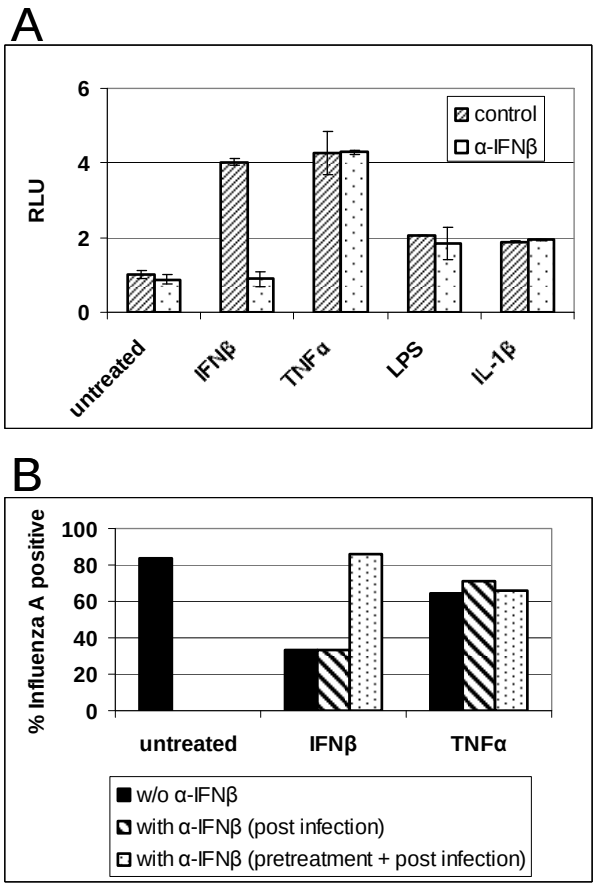

Figure 5: The contribution of autocrine IFNβ to ISGF3 activation and antiviral protection by pro-inflammatory stimuli. (A) Activation of the ISGF3 – ISRE reporter was measured 18 hours after co-incubation of HMECs with anti-IFNβ serum (1:800) and TNFα (100 ng/ml), LPS (1 µg/ml), IL-1β (100 pg/ml) or IFNβ (500 pg/ml). For control, pre-immunization serum was applied. Data of one representative experiment is given. (B) HMECs were stimulated for 16 hours with IFNβ (100 pg/ml) or TNFα (100 ng/ml), with or without application of sheep anti-IFNβ serum. After pretreatment, cells were infected with influenza PR8 wt virus (MOI=1). Subsequently, cells were incubated for 7 hours in the absence or presence of IFNβ antiserum and the expression of viral matrix- and nucleoprotein was measured in HMECs via flow cytometry.

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Tables

Table I: *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´

Table II: *TNFα, LPS and IL-1β have antiviral activity in HMECs*

Treatment [a]	Viral Protein Expression [%]	Virus Production [pfu/ml]
untreated	0	0
virus	83	66 500
IFNβ + virus	33	36 000
TNFα + virus	64	44 500
LPS + virus	66	46 000
IL-1β + virus	72	58 000

[a] HMECs were pretreated with IFNβ, TNFα, LPS or IL-1β for 16 hours as indicated in the table. Subsequently, cells were infected with influenza PR8 wt virus at MOI=1. Infected cells were determined 7 hours post infection by flow cytometric detection of of viral matrix- and nucleoprotein expression. 24 hours after infection the amount of virus released to cell culture supernatant was measured in a standard plaque assay. Four comparable experiments were performed; the results of one assay are presented.

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Appendix

The Journal of Immunology

Neutralizing Type I IFN Antibodies Trigger an IFN-Like Response in Endothelial Cells[1]

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Neutralizing Abs to type I IFNs are of therapeutic significance, i.e., are currently evaluated for the treatment of autoimmune diseases with pathogenic IFN-α production such as for systemic lupus erythematosus. Unexpectedly, we observed that several neutralizing Abs reportedly known to counteract IFN-α or IFN-β activity triggered an "IFN-like" response in quiescent primary human endothelial cells leading to activation of the transcription factor IFN-stimulated gene factor 3 and the expression of IFN-responsive genes. Furthermore, these Abs were found to enhance rather than inhibit type I IFN signals, and the effect was also detectable for distinct other cell types such as PBMCs. The stimulatory capacity of anti-IFN-α/β Abs was mediated by the constitutive autocrine production of "subthreshold" IFN levels, involved the type I IFNR and was dependent on the Fc Ab domain, as Fab or $F(ab')_2$ fragments potently inhibited IFN activity. We thus propose that a combined effect of IFN recognition by the Ab paratope and the concomitant engagement of the Fc domain may trigger an IFN signal via the respective type I IFNR, which accounts for the observed IFN-like response to the neutralizing Abs. With respect to clinical applications, the finding may be of importance for the design of recombinant Abs vs Fab or $F(ab')_2$ fragments to efficiently counteract IFN activity without undesirable activating effects. *The Journal of Immunology,* **2008, 180: 5250–5256.**

The inducible IFN response and the associated antivirus, antitumor, and immunomodulatory activities are well-characterized hallmarks of the defense system. These activities are primarily mediated by the rapid activation of the transcription factor IFN-stimulated gene factor (ISGF)3,[4] which binds to promoter IFN-stimulated response elements (ISREs) and induces expression of IFN response genes such as IFIT-1 (IFN-induced protein with tetratricopeptide repeats 1) or IFN-stimulated gene (ISG)15. In contrast, the low level, constitutive expression of type I IFNs (IFN-α or IFN-β) has now been recognized to serve distinct functions in cellular signaling and activation (1): in the absence of any known stimulus, a low basal expression level of type I IFNs is maintained, which results in a weak signaling event and intracellular tyrosine phosphorylation of the type I IFN receptor α-chain (IFNAR-1). The signal is considered to be "subthreshold," namely does not elicit the signaling cascade that leads to transcriptional activation of IFN response genes. However, IFNAR-1 is maintained in a "ready state" thereby promoting rapid cellular activation upon stimulation with virus, IFNs or other STAT signaling cytokines. In this context, IFNAR-1 was shown to engage in cross-talk with other receptors such as the type II IFN-γ or the IL-6R, thus constituting a common "docking site" for STAT dimerization and an efficient enhancer of cellular activation (2, 3). Constitutive, low level IFN-α/β expression has been reported for mouse embryonic fibroblasts, splenocytes, macrophages, and bone marrow cells (2, 3), and was further described to promote activation of $CD8^+$ T cells upon TCR engagement (4) and to regulate dendritic cell differentiation (5, 6).

Considering the potency of IFN-α/β in the immune response, the application of recombinant IFN (rIFN) has proven an evident and valuable therapeutic tool in the treatment of, e.g., viral infections or cancer. In contrast, type I IFNs, in particular IFN-α, have been found to play a crucial role in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus, type-1 diabetes, or autoimmune thyroid disease (7, 8). In this context, the development of a neutralizing Ab directed against multiple IFN-α subtypes has been promoted (9), and in April 2006 the first clinical trial has been launched applying a humanized IFN-α blocking mAb to systemic lupus erythematosus patients. IFN-neutralizing activities are expected to target immune effector cells such as leukocytes, but their impact further extends to other cell types such as the vessel-lining endothelial cells (ECs). In this regard, we have observed and characterized an unexpected "IFN-like" response in ECs upon exposure to neutralizing Abs directed against type I IFNs.

Materials and Methods

Cell culture

Primary ECs were isolated from human foreskin samples by dispase digest, purified via anti-CD31 Ab-coupled Dynabeads (Invitrogen Life

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[4] Abbreviations used in this paper: ISGF, IFN-stimulated gene factor; EC, endothelial cell; HDMEC, human dermal microvessel EC; IFIT-1, IFN-induced protein with tetratricopeptide repeats 1; IFNAR, IFN-α receptor; IFNGR, IFN-γ receptor; ISG, IFN-stimulated gene; ISRE, IFN-stimulated response element; siRNA, small interfering RNA.

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Technologies) and cultured in fibronectin-containing Microvascular Endothelial Cell Growth Medium EGM2-MV (Cambrex) without vascular endothelial growth factor supplementation. Purified EC cultures showed ≥98% purity and viability. For separation of lymphatic and blood vessel ECs, anti-podoplanin Ab-coupled Dynabeads were applied. All isolates were characterized by flow cytometry for EC characteristics, i.e., CD31 expression, CD34 as marker of microvessels and for E-selectin induction following stimulation with 100 ng/ml TNF-α for 4 h. Lymphatic ECs were detected by podoplanin expression. To keep the passage number low, experiments were generally repeated with different EC isolates, i.e., represent biological replicates with variable inducibility. Representative results of two to four comparable experiments are shown.

HUVECs were obtained from Cambrex and grown in fibronectin-containing EGM2 medium without vascular endothelial growth factor supplementation (Cambrex). 293T cells are derived from human embryonic kidney cells into which the temperature sensitive gene for simian virus SV40 tumor Ag was inserted. HT-29 was isolated from a human colorectal adenocarcinoma. Both cell lines (293T, HT-29) were supplied by American Type Culture Collection and cultured in DMEM with 10% FCS. PBMCs were isolated from 100 ml EDTA-treated whole blood of a healthy volunteer by standardized density gradient centrifugation using Ficoll-Paque (GE Healthcare) and were supplied with RPMI 1640 medium containing 10% FCS.

Treatment with neutralizing Abs and rIFN

Two days before stimulation, ECs were seeded in growth medium at 7×10^5 cells per 30-mm dish (293T and HT-29 cells at 2×10^6 cells) to yield a confluent cell layer within 24 h. Culture medium was then exchanged to EGM2-MV containing 5% FCS but no additional growth factor supplements and cells were allowed to adopt a quiescent state over the next 24 h. Abs were added on day 3 at various concentrations and for the time periods indicated. A total of 7×10^5 PBMCs in 500 μl of RPMI 1640 medium were stimulated immediately after isolation without prior culture period.

The rIFN and blocking mAbs targeting either IFN-α (clones 2 and 13, MMHA-2 and MMHA-13, respectively) or IFN-β (clones 3 and 12, MMHB-3 and MMHB-12, respectively) as well as the IFNAR chain 2 (MMHAR-2) were all obtained from PBL Biomedical Laboratories. ELISA kits for detection of human IFN-α or IFN-β were also manufactured by PBL Biomedical Laboratories and assays were conducted essentially as described (10). Neutralizing Abs to IFN-γ (NIB42) or the IFN-γ receptor (IFNGR) α-chain (GIR-208) were supplied by BD Biosciences. For mouse IgG1 isotype control, the MOPC-21 clone was applied (Sigma-Aldrich). TNF-α was provided by H. R. Alexander (National Cancer Institute, Bethesda, MD), whereas LPS was obtained from Sigma-Aldrich.

Real-time RT-PCR

Total RNA was isolated from cell cultures with RNeasy Mini kit (Qiagen), 500 ng of RNA were reverse transcribed with oligo(dT) primers using the Superscript III First Strand Synthesis System (Invitrogen Life Technologies) and the generated cDNA was diluted 1/25 before PCR analysis. Real-time PCR was performed with SYBR Green PCR Core Reagents (Applied Biosystems) and the following primer sets: IFIT-1 (900 nM forward) 5′-GCA GAA CGG CTG CCT AAT TT-3′, (900 nM reverse) 5′-TCA GGC ATT TCA TCG TCA TC-3′; ISG15 (300 nM forward) 5′-GAG AGG CAG CGA ACT CAT CT-3′, (300 nM reverse) 5′-AGC TCT GAC ACC GAC ATG G-3′; IFN-β (50 nM forward) 5′-AGC ACT GGC TGG AAT GAG AC-3′, (300 nM reverse) 5′-TCC TTG GCC TTC AGG TAA TG-3′; IFN-α1 (300 nM forward) 5′-GCC TCG CCC TTT GCT TTA CT-3′, (300 nM reverse) 5′-CTG TGG GTC TCA GGG AGA TCA-3′ (PrimerBank ID no. 13128950a1); IFN-α2 (300 nM forward) 5′-GCT TGG GAT GAG ACC CTC CTA-3′, (300 nM reverse) 5′-CCC ACC CCC TGT ATC ACA C-3′ (PrimerBank ID no. 11067751a1); BCoR (300 nM forward) 5′-AGA CGA CAT GCT CTC AGC AA-3′, (50 nM reverse) 5′-GAT CCT ATG GGC CGT GCT 3′; housekeeping genes β_2-microglobulin (50 nM forward) 5′-GAT GAG TAT GCC TGC CGT GTG-3′, (50 nM reverse) 5′-CAA TCC AAA TGC GGC ATC T-3′; and β-actin (900 nM forward) 5′-CTG GAA CGG TGA AGG TGA CA-3′, (300 nM reverse) 5′-AAG GGA CTT CCT GTA ACA ATG CA-3′. With the exception of IFNs, which do not contain introns, all primer sets span at least one exon/intron gene boundary. Primer sets for IFN-α1 and IFN-α2 were retrieved from the PrimerBank (11). Each sample was assayed in triplicate with the GeneAmp 5700 Sequence Detection System (Applied Biosystems) for 45 cycles of 15 s at 95°C followed by 1 min at 60°C and a final dissociation protocol to screen for false amplification products. The mRNA levels for IFIT-1, ISG15, BCoR, or IFNs were deduced from the on-plate dilution series of a standard cDNA and were normalized to housekeeping gene values as previously described (12). Real-time PCR data are given as mean and SD of triplicate samples. The value obtained for the untreated control sample was generally set to 1 and changes in mRNA expression upon stimulation are given in relation to the untreated control.

Immunoblotting

Endothelial whole cell extracts were generated essentially as described (13, 14), i.e., in lysis buffer containing 1% Nonidet P-40, 0.5% deoxycholic acid, and the Complete MiniProtease Inhibitor Cocktail (Roche). The 30 μg of total protein were separated on PAGE minigels and subjected to wet blotting. Immunodetection was performed with polyclonal rabbit antiserum against IFIT-1, a gift by G. Sen (Cleveland Clinic, Cleveland, OH) at a 1/2000 dilution or ISG15 (Rockland Immunochemicals) at a 1/200 dilution.

EC transfection with small interfering siRNA

Three distinct Stealth siRNA duplex oligonucleotides for IFN-β gene silencing (HSS10523-2, HSS10523-3, HSS10523-4) as well as the respective negative control siRNAs with matched (guanine-cytosine) GC content were obtained from Invitrogen Life Technologies. A total of 2×10^6 proliferating ECs were harvested in 400 μl of RPMI 1640 with 10% FCS and siRNA was added. Cells were then subjected to electroporation at 200 V, 960 μF essentially as described (15). Stimulation of ECs with anti-IFN mAbs or rIFN was performed 24–48 h posttransfection. A mix of the three IFN-β siRNAs (1 μM each) proved to be more effective than single application of the IFN-β siRNA variants (data not shown).

ISGF3 reporter gene assay

Electroporation of 2×10^6 ECs at 200 V, 960 μF was performed with a total of 30 μg of DNA. A combination of 29 μg of pISRE-Luc plasmid with five ISRE sites directing expression of the luciferase reporter gene (Stratagene) and 1 μg of the constitutive β-galactosidase expression plasmid pCMVβ for normalization (Clontech Laboratories) was applied. Cells were then seeded in 30-mm wells at 1×10^6 to yield a confluent cell layer within 24 h. Stimulation with anti-IFN-α mAb clone no. 2 (6 μg/ml) or rIFN-α (100 pg/ml) was conducted for 4 and 24 h. Cells were then harvested in 40 μl of lysis buffer and samples (10 μl) were assayed in triplicate for luciferase as well as β-galactosidase activity applying the Tropix Dual-Light System according to the manufacturer's instructions (Applied Biosystems) for chemiluminescent detection with a Wallac Victor multilabel counter (PerkinElmer Life Sciences). Luciferase activity as measured in relative light units was normalized to the corresponding β-galactosidase value.

Generation of Fab and F(ab′)$_2$ fragments

The anti-IFN-α mAb clone no. 2 or mouse IgG1 isotype control Ab were subjected to ficin digest at 37°C in the presence of 10 mM cysteine for Fab vs 1 mM cysteine for F(ab′)$_2$ fragment generation according to the instructions of the ImmunoPure IgG1 Fab and F(ab′)$_2$ Preparation kit (Pierce). For control, an Ab fraction was treated comparably in ImmunoPure Digestion buffer but without addition of ficin protease. The resulting Ab fragments were evaluated under reducing as well as nonreducing conditions by Western blotting with ImmunoPure peroxidase-conjugated goat anti-mouse IgG (H+L chain) antiserum (Pierce) and digest efficiency was found to be ≥90%. Control human Fab/κ fragments were obtained from Bethyl Laboratories.

Results

Primary ECs as isolated from human dermal microvessels (HDMECs) were subjected to standard in vitro culture. When cells were exposed to increasing doses of neutralizing Abs directed against IFN-α or IFN-β (in the absence of any exogenously added type I IFN) an "IFN-like" response was observed (Fig. 1): the dose-dependent induction of IFN-responsive genes such as IFIT-1 and ISG15 was detected at the mRNA level by real-time RT-PCR as well as at the protein level by immunoblotting. The effect was verified for four different, commercially available blocking mAbs targeting either IFN-α (clone nos. 2 and 13) or IFN-β (clone nos. 3 and 12) as illustrated in Fig. 1 (also see Fig. 4). Furthermore, the stimulatory capacity of anti-IFN-α and anti-IFN-β mAbs was additive (Fig. 2A) and IFIT-1 mRNA induction was observed at all time points (2, 4, 6, and 8 h) investigated (data not shown). All

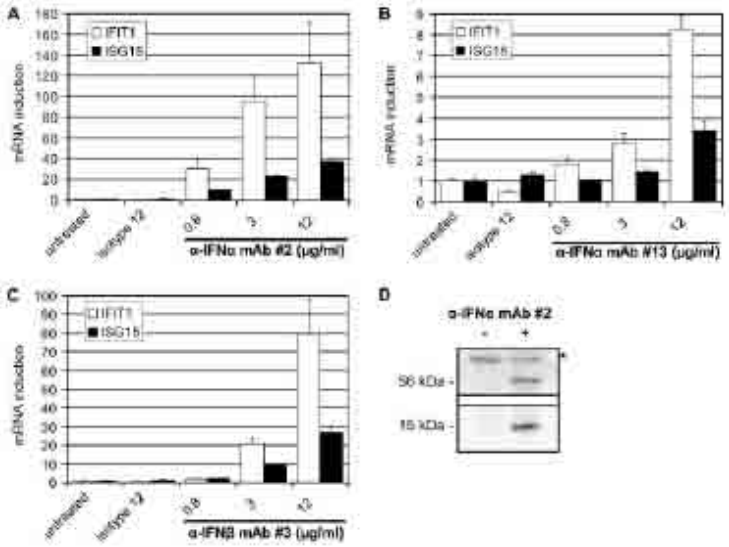

FIGURE 1. Dose-dependent induction of IFN-responsive genes by neutralizing Abs to type I IFN. EC cultures were treated for 4 h with 0.8, 3, or 12 μg/ml anti-IFN-α mAb clone no. 2 ($n = 7$ comparable experiments) (*A*), clone no. 13 ($n = 2$ comparable experiments) (*B*), or anti-IFN-β mAb clone no. 3 ($n = 3$ comparable experiments) (*C*). The nonspecific mouse IgG1 isotype control was applied at 12 μg/ml. Real-time RT-PCR analysis of endothelial RNA was performed to evaluate mRNA levels of IFIT-1 and ISG15. Each sample was assayed in triplicate, and comparable experiments were performed. IFIT-1 induction by Ab treatment was statistically significant ($p \leq 0.03$) for all mAbs and concentrations. Comparably, $p \leq 0.01$ was recorded for ISG15 induction with the exception of anti-IFN-α mAb clone no. 13 at the lowest concentration (p was not significant). *D*, Protein expression of IFIT-1 and ISG15 was investigated by immunoblotting of whole cell extracts following 5 h of stimulation with anti-IFN-α mAb clone no. 2 at 12 μg/ml ($n = 2$ comparable experiments). An asterisk indictes nonspecific, cross-reactive protein.

mAbs tested were of the mouse IgG1 isotype with κ light chain. An appropriate isotype control did not induce IFIT-1 or ISG15 expression in HDMECs.

A first indication on the potential mechanism underlying the extraordinary "intrinsic" ability of the type I IFN Abs to induce IFN-responsive genes came from the observation that IFIT-1 induction by anti-IFN-α or anti-IFN-β mAbs was abolished in the presence of IFNAR blocking Abs (Fig. 2*B*). Thus, the type I IFNR seemed to be involved even in the absence of exogenously added IFN. Furthermore, Ab treatment resulted in the activation of the ISGF3 transcription factor as monitored by reporter gene assay (Fig. 2*C*), indicating an IFN-like signal resulting in the transcriptional regulation of IFIT-1 and ISG15. Exposure of ECs to the IFN-neutralizing Abs was well tolerated and did not result in any apparent changes in morphology (Fig. 2*D*) or signs of apoptosis (data not shown) over prolonged time periods. Concomitant proinflammatory activation of ECs by LPS or TNF-α led to a partial reduction but could not prevent the endothelial IFN-like response to the Abs (Fig. 3*A*).

The tested cell isolates mostly consisted of a mixture of vascular and lymphatic ECs originating from human skin microvessels. When analyzed in separate cultures, comparable responses were elicited in both cell populations (Fig. 3*B*). In addition, endothelial cultures derived from larger vessels (HUVECs) were highly responsive to the type I IFN Abs. When investigating other human cell types, we found that the phenomenon was not restricted to ECs but could also be observed in freshly isolated PBMCs when treated with the respective IFN blocking mAbs (Fig. 3*C*). In contrast, the

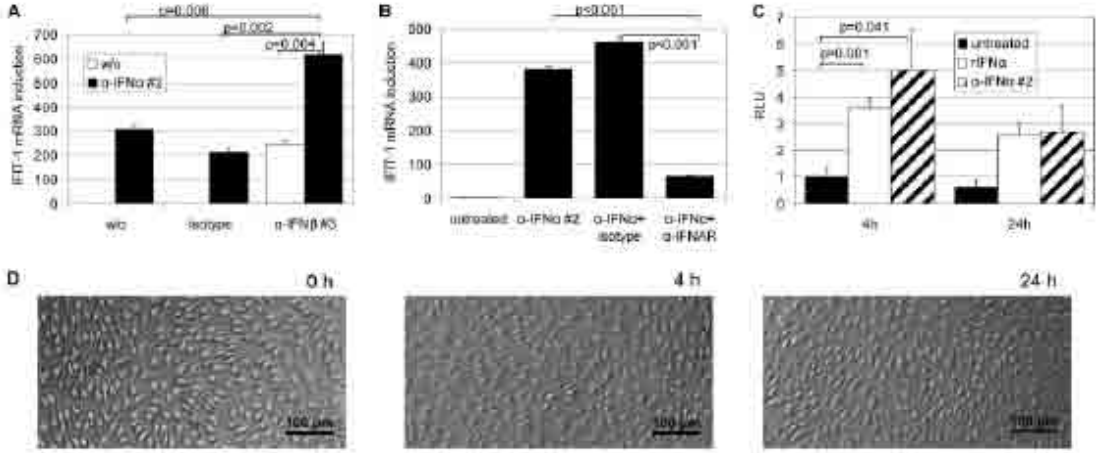

FIGURE 2. Characterization of the mechanisms involved in the induction of IFN response genes by neutralizing Abs to type I IFN. *A*, HDMECs were challenged for 4 h by single or combined treatment with 3 μg/ml anti-IFN-α mAb clone no. 2 and anti-IFN-β mAb clone no. 3 (or mouse IgG1 isotype control) ($n = 3$ comparable experiments). Endothelial RNA and corresponding cDNA were then analyzed for IFIT-1 mRNA expression by real-time PCR, which demonstrated an additive effect of anti-IFN-α and anti-IFN-β Abs. *B*, IFIT-1 induction by 3 μg/ml anti-IFN-α mAb clone no. 2 was blocked by the addition of anti-IFNAR Ab at 1 μg/ml vs mouse IgG2a isotype control ($n = 3$ comparable experiments). *C*, Activation of the IFN responsive transcription factor ISGF3 was evaluated by reporter gene assay measuring luciferase activity in relative light units (RLU) after EC stimulation for 4 or 24 h with 6 μg/ml anti-IFN-α mAb clone no. 2 or 100 pg/ml rIFN-α for comparison ($n = 2$ comparable experiments). *D*, Endothelial cultures after 0, 4, or 24 h of stimulation with 6 μg/ml anti-IFN-α mAb clone no. 2 were investigated by phase contrast microscopy to document that no apparent changes in EC morphology were triggered by Ab treatment.

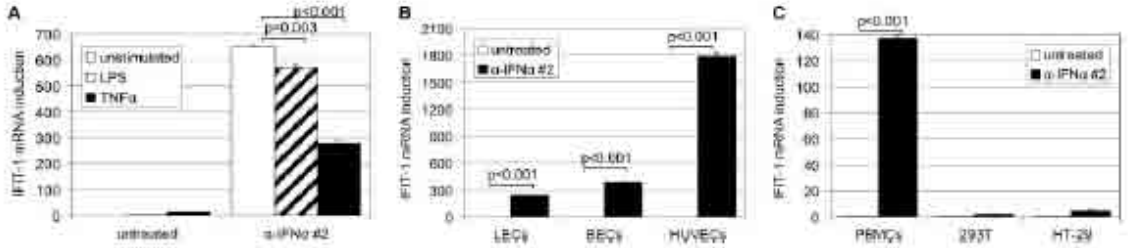

FIGURE 3. IFN-like response to neutralizing Abs by different EC variants or other cell types. *A*, HDMECs stimulated with proinflammatory mediators such as LPS at 1 μg/ml or TNF-α at 100 ng/ml were concomitantly treated with 12 μg/ml anti-IFN-α mAb clone no. 2 for 4 h ($n = 2$ comparable experiments). *B*, EC populations isolated from blood (BECs) or lymphatic (LECs) skin vessels or HUVECs were exposed to 12 μg/ml anti-IFN-α mAb clone no. 2 or mouse IgG1 isotype control for 4 h ($n = 2$ comparable experiments). *C*, Comparably, freshly isolated PBMCs or in vitro cultures of 293T and HT-29 cells were exposed to 12 μg/ml anti-IFN-α mAb clone no. 2 for 4 h ($n = 2$ comparable experiments). Total RNA and corresponding cDNA were analyzed for IFIT-1 mRNA expression by real-time PCR.

effect was essentially absent in Ab-treated 293T or HT-29 cell cultures representing a human embryonic kidney and colon carcinoma cell line, respectively.

Because all the Abs tested on HDMECs were established neutralizing monoclonals, we proceeded to test their blocking abilities in EC combination treatment with rIFN and mAb (Fig. 4). Four hours of incubation with rIFN-β (10 pg/ml) induced IFIT-1 mRNA levels by ~40-fold. Interestingly, addition of blocking mAbs (at 12 μg/ml) targeting IFN-β resulted in a further increase of IFIT-1 expression by 2- to 10-fold depending on the Ab applied (clone nos. 12 and 3, respectively). A similar phenomenon was observed when combining rIFN-α with anti-IFN-α blocking mAbs. In contrast, a neutralizing Ab directed against the type I IFNR, IFNAR, completely abrogated the endothelial response to rIFN-α. Furthermore, when IFIT-1 expression was triggered by type II rIFN (10,000 U/ml, rIFN-γ), the induction was efficiently blocked by the addition of neutralizing Ab targeting either IFN-γ or the corresponding type II receptor.

As the ability of the type I IFN Abs to induce IFN-responsive genes was not dependent on but could be enhanced by exogenously added IFN, we hypothesized that ECs might constitutively express low levels of autocrine type I IFN contributing to the effects observed. To test our hypothesis, silencing of IFN-β gene expression was achieved by transiently transfecting HDMEC cultures with double-stranded siRNAs. When HDMECs were challenged with IFN-β siRNA or nonspecific (control) siRNA, no induction of IFIT-1 was observed, i.e., there was no endothelial response to the uptake of chemically modified dsRNA oligonucleotides (Fig. 5*A*). At 24 h posttransfection, ECs were exposed to anti-IFN-α or anti-IFN-β mAbs at 12 μg/ml. Induction of IFIT-1 mRNA was markedly inhibited by IFN-β gene silencing as opposed to control siRNA treatment (Fig. 5*A*): IFIT-1 expression levels in response to type I IFN Abs were reduced to ~40%. The IFN silencing efficiency of these samples was evaluated by real-time RT-PCR analysis and equaled the effect seen for IFIT-1. However, not only IFN-β mRNA levels but also IFN-α1 and IFN-α2 transcripts were decreased by ~60% in the presence of IFN-β siRNA (Fig. 5*D*). In contrast, the detectable mRNA expression of nonrelated EC genes, such as the transcriptional regulator BCoR or the housekeeping gene β-actin, was not affected by IFN-β vs control siRNA (Fig. 5*E*). To reverse the effect of IFN-β gene silencing, ECs were pretreated with subthreshold concentrations of IFN-β at 1 pg/ml (corresponding to 0.1 U/ml) for 2 h and were then challenged with anti-IFN-β blocking mAb (Fig. 5*B*). As expected, rIFN-β at 1 pg/ml was below the signal threshold, which was insufficient per se to induce IFIT-1 expression. However, the low-dose pretreatment restored EC responsiveness to anti-IFN-β-neutralizing mAb to 84%. Comparably, EC activation by high-dose (100 pg/ml) IFN-α was inhibited to 38% due to IFN-β gene silencing, whereas high-dose IFN-β could partially overcome the block and achieve expression levels of 76% (Fig. 5*C*).

Having established that endogenously expressed type I IFN mediates the unexpected EC responsiveness to IFN-neutralizing mAbs, we then questioned whether Ab binding to FcγRs on the cell surface might contribute to the effects observed. Despite the

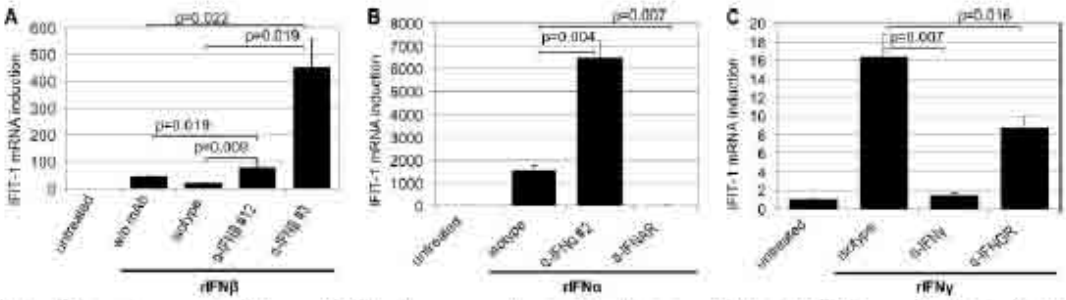

FIGURE 4. Enhanced response to rIFN-α or rIFN-β in the presence of neutralizing Abs to type I IFN. *A*, HDMECs were stimulated with rIFN-β (10 pg/ml) for 4 h in the absence or presence of anti-IFN-β mAb clone no. 3 or clone no. 12, or mouse IgG1 isotype control at 12 μg/ml each ($n = 3$ comparable experiments). *B*, EC treatment for 6 h with rIFN-α (10 pg/ml) was combined with 12 μg/ml mouse IgG1 isotype control, anti-IFN-α mAb clone no. 2 or anti-IFNAR Ab ($n = 2$ comparable experiments). *C*, Comparably, ECs were exposed for 6 h to 10,000 U/ml rIFN-γ and 12 μg/ml mouse IgG1 isotype control, anti-IFN-γ, or anti-IFNGR Ab ($n = 3$ comparable experiments). Endothelial RNA was analyzed for IFIT-1 mRNA expression by real-time RT-PCR.

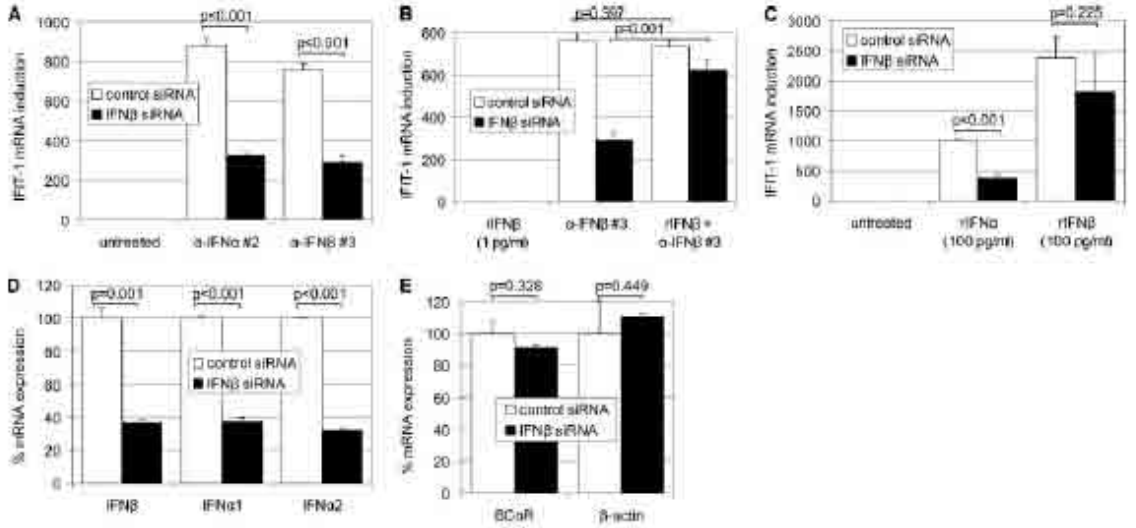

FIGURE 5. IFN-β gene silencing inhibits the IFN-like response of ECs to IFN-neutralizing mAbs. *A*, Following EC transfection with IFN-β siRNA or nonspecific control siRNA, cells were challenged with 12 μg/ml anti-IFN-α mAb clone no. 2 or anti-IFN-β mAb clone no. 3 for 2 h ($n = 3$ comparable experiments). *B*, To reverse the effect of IFN-β gene silencing, EC cultures were pretreated with subthreshold concentrations of rIFN-β (1 pg/ml) for 2 h with or without subsequent addition of 12 μg/ml IFN-β mAb clone no. 3 for another 2 h ($n = 2$). *C*, Treatment of siRNA transfected ECs with high-dose (100 pg/ml) rIFN-α or rIFN-β for 4 h ($n = 4$ comparable experiments) was followed by RNA isolation and real-time RT-PCR detection of IFIT-1 expression. *D*, Silencing efficiencies for IFN-β as well as IFN-α1 and IFN-α2 were determined by real-time RT-PCR and are shown for EC samples stimulated for 2 h with 12 μg/ml anti-IFN-β mAb clone no. 3 ($n = 3$). *E*, Comparably, the effect of IFN-β siRNA vs nonspecific control siRNA was tested on transcript levels of nonrelated EC genes such as BCoR and β-actin for the same samples ($n = 3$ comparable experiments).

fact that all our in vitro experiments were conducted in the presence of 5% FCS in culture medium thus supplying an excess of bovine IgG, binding of the mouse monoclonal anti-IFN Abs to human endothelial FcγRs could not be excluded. We therefore added increasing concentrations of mouse IgG1 isotype Ab to our reactions (Fig. 6*B*). A dose-dependent decline in EC responsiveness, i.e., in IFIT-1 mRNA induction by IFN-neutralizing mAbs, was observed. This response prompted us to further investigate the requirement for the Fc domain, thus we generated Fab as well as $F(ab')_2$ fragments from anti-IFN-α blocking mAb clone no. 2 by ficin digest. When comparing the Fab and $F(ab')_2$ fragments to the intact Ab (subjected to a mock treatment without addition of ficin protease for control) the intact molecule retained its dose-dependent stimulatory capacity, whereas the corresponding Fab or $F(ab')_2$ fragments could not induce IFIT-1 mRNA expression in HDMECs (Fig. 6*A*). However, the generated fragments exhibited strong neutralizing capacity for rIFN-α, which was not observed when Fab or $F(ab')_2$ fragments of the control mouse IgG1 isotype Ab were applied (Fig. 6*C*). IFIT-1 mRNA induction in response to EC treatment with 10 pg/ml rIFN-α was entirely abolished by 1 μg/ml Fab or $F(ab')_2$ fragments from anti-IFN-α blocking mAb clone no. 2, which documents the actual IFN-neutralizing potency attributed to the original Ab by the manufacturer. In comparison, the fragments were less potent in competing, or inhibiting, the activity of the intact Ig molecule. Increasing the amount of Fab fragment while maintaining the concentration of intact anti-IFN-α mAb clone no. 2 at 1 μg/ml led to a 64% drop in IFIT-1 transcript levels at a ratio of 2:1. In contrast, a comparable amount of unrelated control Fab fragment led to a nonspecific quenching of IFIT-1 induction in the range of 25% (Fig. 6*B*).

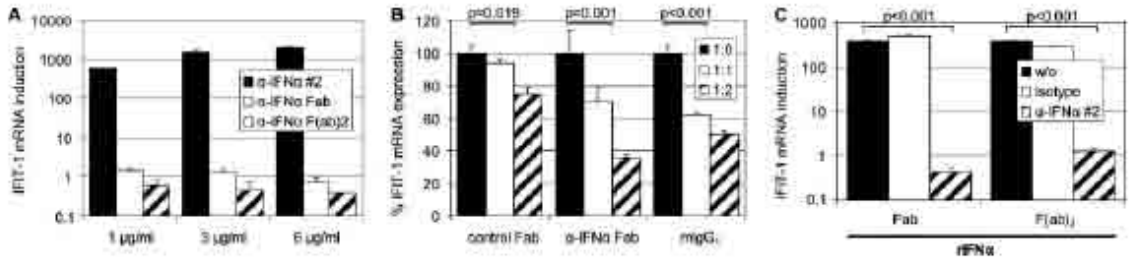

FIGURE 6. Ab domains involved in IFIT-1 induction by IFN-neutralizing Abs. *A*, HDMECs were exposed for 4 h to 1, 3, or 6 μg/ml of intact (control-treated) anti-IFN-α mAb clone no. 2 or a generated Fab or $F(ab')_2$ fragment of clone no. 2. Incubation for 4 h was followed by real-time RT-PCR analysis of endothelial RNA for IFIT-1 mRNA expression ($n = 4$ comparable experiments). In comparison to the full-length Ab, the fragments were incapable of eliciting IFIT-1 mRNA expression, $p \leq 0.029$ for all concentrations of intact mAb vs fragments applied. *B*, The full-length anti-IFN-α mAb clone no. 2 (1 μg/ml) was mixed at a ratio of 1:1 or 1:2 with the corresponding generated Fab fragment, a nonspecific pool of unrelated Fab fragments or with an intact mouse IgG1 isotype control ($n = 4$ comparable experiments). *C*, HDMEC stimulation with rIFN-α at 10 pg/ml for 4 h was challenged with concomitant administration of 1 μg/ml Fab or $F(ab')_2$ fragment generated from anti-IFN-α mAb clone no. 2 or from a mouse IgG1 isotype Ab ($n = 3$ comparable experiments).

Discussion

In the study presented, we have investigated the unexpected induction of IFN-regulated genes IFIT-1 and ISG15 in primary ECs that were exposed to Abs known to neutralize type I IFN. Despite the absence of exogenously added IFN, an "IFN-like" signal was observed involving the type I IFNR and leading to the activation of the transcription factor ISGF3. The potency of eliciting this response varied among the Abs applied, apparently relating to differences in binding affinities rather than the specificity for IFN-α or IFN-β. Depending on the clone, Ab concentrations in the range from 1 to 10 μg/ml were sufficient to induce IFIT-1 mRNA levels comparably achieved by EC stimulation with 10 pg/ml rIFN-β (40-fold). It is of interest to note that plasma levels of neutralizing Abs reached in clinical applications are well within or even higher than the concentration range tested in our in vitro setting (16, 17).

Because all Abs applied in our analyses were reported to neutralize IFN bioactivity, as confirmed by the manufacturer in cytopathic effect inhibition assays and as subsequently verified for the Fab and $F(ab')_2$ fragments in our experiments, we investigated the combined application of rIFN and mAb on HDMECs. The full-length type I IFN blocking Abs were found to enhance rather than inhibit the endothelial response to IFN-α or IFN-β. In contrast, neutralizing Abs targeting IFN-γ or the IFNRs IFNAR and IFNGR potently repressed IFIT-1 induction by rIFN. We have thus provided evidence that the ability of neutralizing Abs to elicit an IFN-like response or to further enhance the effects of type I IFN is only observed for mAbs targeting IFN-α or IFN-β in this cellular context.

Because the response was inhibited by concomitant administration of IFNAR blocking Abs even in the absence of exogenously added rIFN, we hypothesized that ECs might constitutively express low levels of type I IFNs which might contribute to the effects observed. The constitutive low-level expression of type I IFN has previously been reported for other cell types (1, 2). When testing EC culture supernatants, IFN was not detectable at ELISA sensitivities of 4 U/ml (IFN-α) or 10 U/ml (IFN-β). However, considering that basal subthreshold IFN concentrations were reported around 0.1 U/ml (2, 3), ELISA sensitivity may have been limiting (data not shown). We therefore proceeded to block the potential constitutive production of type I IFN by siRNA application. The approach was limited to IFN-β because silencing of IFN-α genes is difficult to accomplish due to the variety of IFN-α subtypes that may be expressed. Furthermore, basal expression of IFN-α in fibroblasts was shown to be dependent on the constitutive IFN-β production, arguing for a predominant role of IFN-β in the low level, basal expression of type I IFNs (18). Comparably, we observed that the application of IFN-β siRNA led to the concurrent down-regulation of IFN-β and IFN-α subtypes generally expressed in ECs (19). Silencing of IFN-β expression in HDMECs was found to greatly reduce the IFN-like response to IFN blocking mAbs: IFIT-1 expression levels were reduced to ~40% irrespective of the Ab specificity to IFN-α or IFN-β, thus reflecting the impact of IFN-β siRNA on the overall type I IFN expression. We therefore propose that ECs maintain a basal level of IFN expression and IFNAR phosphorylation, which allows for their IFN-like response to IFN blocking mAbs. Because the constitutive, weak IFN signal is also known to be a prerequisite for the efficient cellular response to a high-level IFN challenge in, for example, mouse embryonic fibroblasts (3), we conducted a control experiment with 100 pg/ml rIFN-α or rIFN-β. IFIT-1 induction by high-dose IFN was similarly impaired by IFN-β silencing. These results further support our argument for an essential, basal IFN-β expression in primary ECs, which promotes their capacity for efficient and rapid cellular activation.

The further investigations focused on the potential involvement of Fc domains in the endothelial activation by IFN-neutralizing Abs. As the mAbs tested were of the mouse IgG1 isotype, a cross-reaction with human FcγRs seemed feasible. When comparing the full-length Ab with Fab or $F(ab')_2$ fragments of an IFN-α blocking mAb, the fragments did not elicit an IFN-like response in ECs thus pointing to the importance of the Ab Fc domain. Furthermore, the fragments potently inhibited IFIT-1 induction by rIFN, i.e., they exhibited the expected IFN-neutralizing capacity and they could compete for the activity of the corresponding full-length Ab. The latter was, however, not as effective as the inhibition of rIFN. This effect might potentially relate to a better accessibility of intact Ab to autocrine IFN if the Ab was membrane-associated, or bound to Fc receptors. The observation that the EC response to intact anti-IFN mAb was also reduced in the presence of a mouse IgG_1 isotype Ab (containing an Fc portion), further suggested involvement of Fc receptors. ECs are known to express FcγRs with an apparent heterogeneity depending on the vessel type. Various isoforms of FcγRII as well as FcγRI and neonatal Fc receptor have been detected on ECs with CD32 being the most prominent on HDMECs (20, 21). Yet, we could not demonstrate CD32 expression on our endothelial isolates nor block the effect of the anti-IFN Abs by concomitant treatment with a neutralizing Ab directed against CD32 (data not shown). However, these results do not exclude the potential involvement of an endothelial Fc receptor other than CD32.

Interestingly, Fc receptors have been localized to EC caveolar membrane sections, which sets them in close proximity to IFNAR molecules (3, 22). With respect to their interrelation, two settings may be envisioned. The mere local proximity of IFNAR and Fc receptors might serve to sequester autocrine IFN at the cell surface. IFN-neutralizing Abs on Fc receptors might thus increase the local type I IFN concentration beyond the signal threshold provided that IFN bound to the blocking mAbs can be released, i.e., passed onto IFNAR. Alternatively, a direct receptor interaction between Fc receptor and IFNAR could occur, initiated by the anti-IFN-α/β Abs and resulting in IFNAR activation beyond the basal "ready state". Both players, IFNAR and FcγRs, have been reported to engage in diverse receptor cross-talk (2, 3, 23). Thus, whether Fc receptors are indeed involved in the IFN-like response to the neutralizing mAbs or whether the Ab Fc domain mediates engagement of another, as yet unidentified cellular factor, is of prime interest for further investigations.

The activating potential of neutralizing IFN Abs was observed for all types of ECs tested and was not abolished by concomitant proinflammatory activation of ECs. In addition, the fact that IFN-α/β-neutralizing Abs did not only trigger an IFN-like response on quiescent cells but could also enhance the effect of a high-dose IFN challenge, emphasizes the potentially adverse systemic implications. The induction level of IFN response genes varied to some extent with primary EC isolates. When other cell types were investigated, a heterogeneous response was observed that may relate to Fc repertoire or differences in the potency of the signaling cascade. Interestingly, the IFN-like response to monoclonal-neutralizing Abs directed against human type I IFN was also recorded for human PBMCs. Although this observation extends the potential clinical impact these Ab effects might have, it seems intriguing why these effects have not been noted previously by other groups in comparable experiments on leukocytes. In preliminary investigations, we have gathered an indication pointing to the importance of the Ab type. Although all the Abs presented in this study were monoclonals of the mouse IgG1 isotype, we did not find an

IFN-like response to rabbit polyclonal anti-IFN antiserum in our experimental setting (data not shown). Thus, the nature and isotype of the Ab may be a crucial determinant.

The clinical application of recombinant mAbs targeted at IFN-α is at the current therapeutic focus of autoimmune diseases. In light of the recent launch of the first clinical trial testing an IFN-α-neutralizing mAb for the treatment of systemic lupus erythematosus, a possibly pleiotropic Ab effect would seem of particular concern. Our results would suggest that enhanced neutralizing efficiency might be achievable by testing Fab or $F(ab')_2$ fragments vs full-length Igs in systemic settings.

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Disclosures

The authors have no financial conflict of interest.

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