Aspects of interferon alpha signalling in hematopoetic cells

Lennart Carlsson

Department of Molecular Biology
Umeå University
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Department of Molecular Biology
Umeå University, Sweden

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ABSTRACT

The type I interferons (IFN) are a family of cytokines with pleiothropic activities that include inhibition of viral replication, cell proliferation and activation of the immune system. These properties give the IFNs important physiological and pathological roles in infection and cancer and have led to their therapeutic use for many clinical conditions. In humans, the type I IFNs consist of 12 different IFNα subtypes as well as single IFNβ, ω and κ subtypes. They all compete for binding to a common receptor, consisting of two subunits, IFNAR1 and IFNAR2. In almost all cell types proliferation is inhibited by IFNs as a consequence of the antiviral properties. However, previous studies on human peripheral B-lymphocytes have shown increased survival as well as proliferation upon IFN treatment.

We established a purification system for extraction of B-lymphocytes from buffy-coat, utilizing density centrifugation in combination with anti-CD19 magnetic beads. In an attempt to identify the molecular mechanisms of increased survival, the expression and/or activation pattern of different signaling proteins were analysed by Western blot. It was previously reported that phosphatidylinositol 3'-kinase (PI3K) physically interacts with the IFNAR complex, via adaptor proteins. Activated PI3K indirectly activates Akt/PKB, a kinase involved in a pathway leading to both survival and proliferation signals. We were able to show a novel signaling pathway - IFN treatment activated Akt/PKB as well as a downstream effector, one member of the Forkhead family (FOXO1) was inactivated by phosphorylation and as a consequence p27/Kip1 expression was downregulated. Activation of this pathway resulted in increased survival as measured by TUNEL assay, an effect efficiently counteracted by the the synthetic PI3K inhibitor, LY294002.

In additional experiments we investigated the molecular mechanisms of proliferation. Activation of B-cells was ensured by using limiting concentrations of anti-IgM antibodies, mimicing natural activation. Using thymidine incorporation, we discovered that IFN treatment increased the sensitivity to anti-IgM stimulation. As a consequence, more cells proliferated as measured by CFSE staining. However, on its own, IFN was unable to induce proliferation. IFN turned out to be as efficient as IL-2, a classical B-lymphocyte growth factor. In order to distinguish proliferation from increased survival, Rb phoshorylation was analysed by Western blot. Phosphorylation induced by anti-IgM was further enhanced by IFN. As we determined earlier, p27/Kip1 expression was downregulated, releasing the cell cycle block. However, p21/Cip1 expression was upregulated but almost exclusively localised to the cytoplasm, therefore unable to perform the classical growth inhibitory functions. We conclude that type I interferons contribute to increased survival as well as proliferation of human primary B-lymphocytes.

The IFN receptor subunits was studied in a human myeloma cell line (U266), using a variant of which that are totally resistant towards the anti-proliferative properties of IFN. The reason for resistance in clinical situations is seldom elucidated, but is often believed to be due to development of antibodies against interferon. The resistant cells were unable to bind radio-labelled IFN, and through Southern Blot we could determine that the IFNAR1 gene was not functional. Also the IFNAR2 gene was affected, since Northern blot detected an aberrant transcript not present in the wild type cells. Karyotyping showed that the cells had 3-4 copies of chromosome 21, but Southern blot did not detect any cytoplasmic region of IFNAR2. The IFN receptors are close to each other on the genome, and a deletion affecting one receptor gene is likely to affect the other as well. We conclude that the IFN resistance in U266Res cells is due to lack of functional receptor subunits.

LIST OF PUBLICATIONS

This thesis is based on the following articles and manuscripts, which will be referred to by their roman numerals (I-III):

I. Ruuth, K*, **Carlsson, L***, Hallberg, B and Lundgren E
Interferon-alpha promotes survival of human primary B-lymphocytes via phosphatidyl-inositol 3-kinase.
Biochem Biophys Res Commun. 2001 Jun 15;284(3):583-6.

II. **Carlsson, L***, Ruuth, K* and Lundgren E
IFN alpha induced proliferation of human primary B-lymphocytes
(submitted for publication)

III. **Carlsson L**, Dacklin I, Persson H, Golovleva I, Ruuth K and Lundgren E
Characterisation of IFN resistance in a myeloma cell line with an unstable genome
(submitted for publication)

* equal contribution

ABBREVIATIONS

IFN	Interferon
IFNAR	Interferon type I receptor complex
IFNAR1, 2	Interferon type I receptor subunit 1, 2
NIPC	Natural interferon producing cell
ISG	Interferon stimulated gene
ISRE	Interferon stimulated response element
GAS	Gamma activate sequence
GAF	Gamma activated factor
ISGF3	Interferon stimulated gene factor 3
PKR	dsRNA activated protein kinase
PACT	Protein activator of PKR
2-5 AS	2'-5' oligoadenylate synthase
eIF-2α	eukaryotic initiation factor-2a
NF-kB	Nuclear factor-kB
STAT1, 2, 6	Signal transducer and activator of transcription
IRF-1, 2	Interferon regulatory factor
PI3K	Phosphatidylinositol 3'-kinase
FOXO1	Forkhead transcription factor 1
PKB/Akt	Protein Kinase B
GSK3	Glycogen synthase kinase 3
Rb	Retinoblastoma gene product
PP2B	Protein phosphatase 2B
CDK	Cyclin dependent kinase
CPLA2	Cytosolic phospholipase A2
NK-cell	Natural killer cell
PDK1	phophoinositide dependent kinase 1
DAG	Diacylglycerol
PIP3	Phosphatidylinositol (3,4,5) triphosphate
PTEN	Phosphatase and tensin homolog
SHIP	SH2 domain inositol polyphosphate 5-phosphatase
MAPK	Mitogen activated protein kinase
PIAS	Protein inhibitor of activated STAT's
DAG	Diacylglycerol
IP_3	Inositolphosphate
JNK	c-Jun amino terminal kinase
PLCγ	Phospholipase Cγ
PKC	Protein kinase C
IL-15	Interleukin 15

The Interferon Family

Interferon (IFN) was initially discovered almost 50 years ago by researchers investigating mechanisms responsible for viral interference. They observed that infection of chicken cells with heat-inactivated virus caused the cell supernatant to contain an activity that interfered with subsequent live virus infection – hence the name interferon [1]. The biochemical characterisation of this activity was pursued for a long time without success, and it was not until the late 1970's that the first protein sequence was determined [2,3]. The antiviral activity eventually turned out to belong not to a single protein but rather to a large and heterogeneous family containing proteins of two types, denoted type I interferons (IFNα, β and ω) and type II interferon (IFNγ), respectively [4].

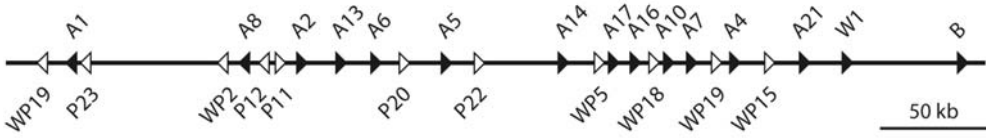


Figure 1: Genomic organisation of human type I IFN genes. Functional genes are indicated as filled arrows with corresponding annotation above the line. Non-functional pseudogenes are indicated as empty arrows with the annotation below the line. Arrowheads indicate transcriptional direction.

Genomic sequencing has resulted in the identification of 13 functional IFNα genes in humans, as well as single copies of the more distantly related IFNβ and IFNω genes. All of them are intronless and located close to each other on the short arm of chromosome 9 [5]. In addition, a number of non-functional pseudogenes are located within the same region (Fig. 1). The IFNα proteins constitute a homologous group with 12 distinct members (subtypes), as α1 and α13 are identical. These monomeric proteins all contain 166 amino acids (except alpha2 – 165 aa) with 79-95% homology on the amino acid level [6]. IFNβ has the same number of amino acids but display only 30% homology to a consensus IFNα sequence [7,8]. IFNω is slightly larger (172 aa), displaying about 60% homology to a consensus IFNα sequence [9,10].

Surprisingly, some additional members of the interferon family were recently discovered. The gene encoding IFNκ is located adjacent to the other members of the interferon type I family. The corresponding protein (180 aa) is approximately 30% homologous to a consensus IFNα sequence and competes for binding to the same receptor complex [11]. Also IFNε, the latest member in the family, has been localised to the same chromosomal region. The protein consists of 187 aa and display highest homology to IFNα. However, the receptor has not yet been identified, but may be the same as for the other type I IFNs [12].

Despite the heterogeneity within the family, all type I IFNs compete for binding to the same receptor complex (IFNAR), although the binding affinity differs between subtypes [13]. The specific activity of individual subtypes has been compared in a number of reports [14,15] and most of them have found extensive variations. However, different specific activities have often been reported for the same subtype when tested in different laboratories, resulting in no clear consensus regarding the potency of individual subtypes.

The fact that all IFNα genes are intronless and share a high degree of homology suggests that they are all derived from a common ancestral gene by successive duplication. However, the biological significance of this multiplicity of proteins is not yet established, as all type I IFNs seem to exhibit functional redundancy.

Type II IFN (IFNγ) is different from type I IFNs in many aspects. It is a dimeric glycoprotein with subunits consisting of 146 amino acids, without any obvious sequence homology to type I interferons. Human IFNγ is encoded by a single gene containing 4 exons, located on chromosome 12 [16]. Contradictory to type I IFN, expression and secretion of type II IFN is not the result of infection itself, but rather a result of recognition of infected cells by activated T and NK cells, which are the principal producers [17]. Although IFNγ possesses an antiviral activity, it functions primarily as an immune modulator responsible for pathogen clearance, rather than an antiviral agent [18,19]. As the mode of action is different from type I IFNs, non-redundant functions are displayed in many cases [20]. However, significant functional similarities exist as well, as both types of IFN induce a largely overlapping set of genes [21].

Biological Activities of Interferons

Cytokines act as intercellular messengers, creating a regulatory network modulating many different cellular functions. They often act locally, in an autocrine or paracrine fashion and due to their high potency, their release and activity is tightly controlled. As an important part of this intricate network, type I IFNs display pleiotropic physiological effects on many cell types, both normal and transformed.

Antiviral activity

Type I IFNs act primarily as antiviral agents, essential for the early innate immune response against viruses and microorganisms. Therefore, almost all nucleated cells in the body has the capacity to produce IFN, resulting in induction of an antiviral state in infected as well as neighbouring uninfected cells [22]. The IFN system is rapidly induced upon virus infection and function both to decrease virus load and to modulate signals necessary for activation of the adaptive immune response. In addition to viruses, IFN can also be produced upon infection with many other pathogens, such as bacteria and parasites. The mode of action is complex and not yet fully characterised, although mice deficient in different components of the interferon system has shown the importance and efficiency of the system – mutant mice were extremely sensitive to infection using various viruses and pathogens compared to control mice [20,23]. During evolution, different pathogens have developed a number of mechanisms in order to successfully replicate inside mammalian cells. As a consequence, various countermeasures (Fig. 2) have evolved in order to inhibit these pathogens [24,25].

The most studied protein involved in the establishment of an antiviral state is the dsRNA activated protein kinase (PKR), with multiple functions in control of transcription and translation. Viral infection as well as IFN induced signalling results in increased expression of this homodimeric Ser/Thr kinase, which becomes activated through auto-phosphorylation upon binding to dsRNA. The active kinase subsequently phosphorylates several targets, most importantly the translation initiation factor eIF-2α. This event leads to

formation of an inactive translation initiation complex, resulting in inhibition of protein synthesis [26,27]. The activity of several transcription factors, such as NF-kB, STAT1, IRF-1 and p53 are also regulated by PKR [28]. Active NF-kB is essential for transcriptional induction of many genes, including IFNβ (but not IFNα). However, although PKR has a major role in regulating virus infection, it is not sufficient to mediate a full antiviral response [29,30].

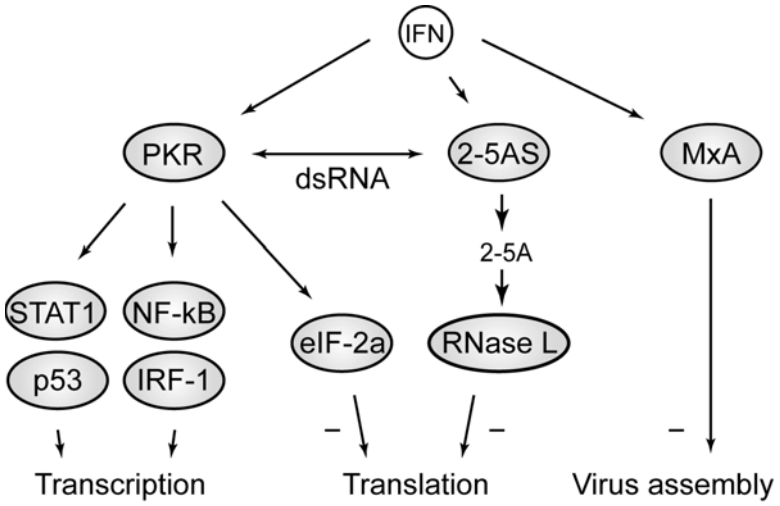


Figure 2: Schematic representation of important IFN-induced antiviral pathways. Both PKR and 2-5AS are activated by dsRNA, while Mx proteins interact with and inhibit assembly of viral components.

Other important proteins that are induced upon IFN stimulation are the family of 2´-5´A oligoadenylate synthetases (2-5 AS) and RNase L. Interaction between 2-5 AS and dsRNA result in conversion of ATP into short, unusually linked polyA stretches that function as activators of RNase L. Subsequent homo-dimerization leads to activation of Rnase L and extensive cleavage of different RNA species, resulting in inhibition of translation [31].

Also the MxA protein, which is a large GTPase, has been shown to be important in suppression of virus replication. MxA interferes with viral assembly through inhibition of intracellular transport of key components [32].

The use of microarrays has shown that IFN regulate the expression of many (>200) other proteins as well, many of which are very poorly characterized [21,33,34]. It is likely that a number of these will prove to be involved in regulating virus infection. At least one study has also pointed in that direction, as mice deficient in all the IFN-induced pathways mentioned above could still resist infections to a certain degree [35].

Antiproliferative activity

Viral replication is inhibited as a result of the establishment of an antiviral state, and as a consequence cellular proliferation is affected. IFN has been shown to elicit an antiproliferative effect on many cell types, both normal and transformed. However, contradictory to the general situation, IFN-induced growth stimulation has been reported as well [36].

The anti-proliferative effect differs significantly, ranging from extremely sensitive to totally resistant [37]. Although IFN–induced cell cycle arrest has been studied extensively in transformed cell-lines, the exact mechanism is still not elucidated. All phases of the cell cycle are susceptible, and different cell types are targeted at different stages. However, a common theme is down-regulation of cyclin dependent kinase (CDK) activity as a result of increased expression of cyclin dependent kinase inhibitors [38], [39].

The antiproliferative effects of IFN are successfully used in clinical medicine to treat a number of malignancies, for example hairy cell leukaemia, chronic myeloid leukaemia (CML), multiple myeloma (MM) and non-Hodgkin's lymphoma [40].

Immunomodulatory activity

Although type II IFN is the major immuno-modulatory interferon, type I IFN also modulate the adaptive immune system through direct and indirect effects (reviewed in [41]).

A major function for type I IFN is to enhance the cytotoxicity of NK cells by upregulation of the perforin expression levels [42] [43]. IFN also

stimulates proliferation of NK cells indirectly via induction of IL-15 secretion from macrophages/monocytes [44] [45]. IFN-induced IL-15 can also stimulate proliferation of memory T cells [46], while the survival of activated T-cells appears to be affected directly by IFN [47]. The immunomodulatory activity of type I IFN also includes upregulation of HLA class I expression, thereby promoting a cytotoxic (CD8+) T cell response. Also B-cell survival and proliferation is affected directly by IFN [48,49]. The immunomodulatory effects of IFN are utlized in the treatment of multiple sclerosis (MS).

Spontaneous IFN production in the absence of viral infection, although at very low levels, has been reported both *in vitro* and *in vivo* (reviewed in [50]). The proposed function would be to prepare the adaptive immune system for future infections [51].

Cytotoxic activities

In cells not infected with virus, IFN can protect against or promote apoptosis, depending on cell type. In virus-infected cells [52], as well as in some transformed cells, IFN has been shown to efficiently induce apoptosis, although the mechanisms are generally poorly investigated. dsRNA has been shown to be a potent inducer and there is strong evidence that PKR is a critical mediator of this response [52-57]. The mechanism is not fully characterized, as downstream targets remain to be identified. PKR has also been associated with apoptosis due to other stimuli. Protein activator of PKR (PACT), an activator of PKR which acts independently of dsRNA or viral infection has been implicated in cellular stress responses [58].

Contradictory to the general observation described above, there are also situations where IFN has been shown to promote survival, especially in hematopoetic cells of normal or transformed origin [47-49,59-61].

Transcriptional regulation of IFN genes

Upon viral infection the presence of the hostile intruder is detected inside the cell. The mechanism by which this is achieved is not yet completely elucidated as viruses differ significantly by the means that they carry genetic information (RNA or DNA). It is assumed that the inducer is intracellular dsRNA, provided by the viral genome itself or as a result of viral replication or transcription [62]. Although almost all cells have the capacity to produce IFN, the major amount *in vivo* is produced by a small subset of precursor dendritic cells, known as natural interferon producing cells (NIPC).

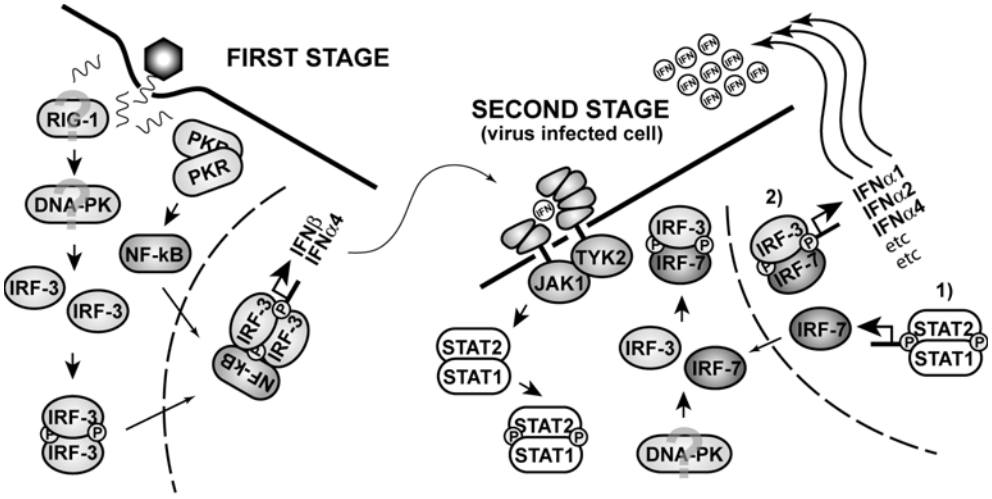


Figure 3: Virus induced pathways resulting in expression/secretion of IFN. Induction of IFN expression takes place according to a two-stage model described in the text. Proteins marked with (?) has been implicated in this pathway but has not yet been confirmed.

The mechanisms responsible for IFN transcription are not completely understood but a positive feedback mechanism has been shown to be important [63,64] (Fig. 3). Upon viral infection, the latent interferon regulatory factor 3 (IRF-3) becomes phosphorylated on several positions. The kinase(s) involved are not completely defined, although several candidates have been presented [65-67]. The phosporylation triggers activation and subsequent homo-dimerization of IRF-3. Nuclear translocation and binding to specific

sequences in the promoters of the IFNα4 and IFNβ gene triggers transcription and subsequent secretion. The second phase of the interferon response is elicited upon binding of secreted IFN to the IFNAR complex.

JAK/STAT signal transduction triggers expression of IRF-7, another member of the IRF family. If IRF-7 is expressed in a virally infected cell, IRF-7 is phosphorylated. Subsequent hetero-dimerization with IRF-3 produces a promiscuous transcription factor able to support trancription from most if not all IFN genes. This two-step activation mechanism could be speculated to be important in order to release as little IFN as possible. It is a cytokine with powerful effects on neighbouring cells, therefore the regulation is of outmost importance [68] [69] [70] [71,72].

As mentioned briefly above, spontaneous IFN production in the absence of viral infection has been reported *in vitro* as well as *in vivo*, creating a positive feedback loop where expression of IRF-7 is dependent on IFN induced signalling. IRF-7 has a short half-life and must be continuously produced [73], demanding continuos expression of IFN in order to retain a functional loop. If IRF-7 expression exceeds the threshold level, the feedback loop will be efficient due to the constant production of IFN. Conversely, if IFN-dependent IRF-7 expression falls below the threshold level, the feedback loop operates inefficiently. However, the exact mechanism of spontaneous IFN production is not known, but it is speculated that the promotors are intrinsically leaky, resulting in low levels of IFN production.

The IFN receptor

As secreted intercellular messengers, IFNs bind to and transduce signals through specific cell surface receptors. The genes coding for the IFNAR subunits are located very close to each other on chromosome 21. (Fig 4) [74].

Figure 4: Genomic structure of the human interferon type I receptor locus. IFNAR2 and IFNAR1 are located very close to each other chromosome 21, clustered together with two other cytokine receptor subunits.

Molecular cloning and expression studies have so far indicated a functional IFN type I receptor consisting of two subunits, both of which are members of the class II cytokine receptor family [75]. IFNAR1 contains an extracellular part consisting of two distinct domains (D200), each of which is further divided into two sub-domains (SD100), with structural similarity to the constant region of immunoglubulins [13,76-79](Fig 5). Three different variants of IFNAR2 can be produced through alternative splicing. All have an identical extracellular part consisting of a single D200 domain, divided into two SD100 sub-domains in a similar fashion as IFNAR1 [74]. One splice variant is soluble (IFNAR2a) and was initially purified from human urine [80]. Soluble receptors are relatively common among cytokines and can be speculated to function as negative regulators, absorbing excessive ligand. The membrane-associated variants (IFNAR2b and IFNAR2c) have different cytoplasmic domains [81], but only IFNAR2c participates in signal transduction.

IFN secreted from infected cells bind with high affinity to the IFNAR complex. In the human system, IFNAR2 has been shown to be the major ligand binding subunit, while IFNAR1 makes only a minor contribution in this respect [13], [79]. The receptor subunits do not display any intrinsic affinity for

each other, all interactions are ligand induced. The cytoplasmic regions do not contain any active domains of any kind; signal transduction is totally dependent on accessory proteins presented below.

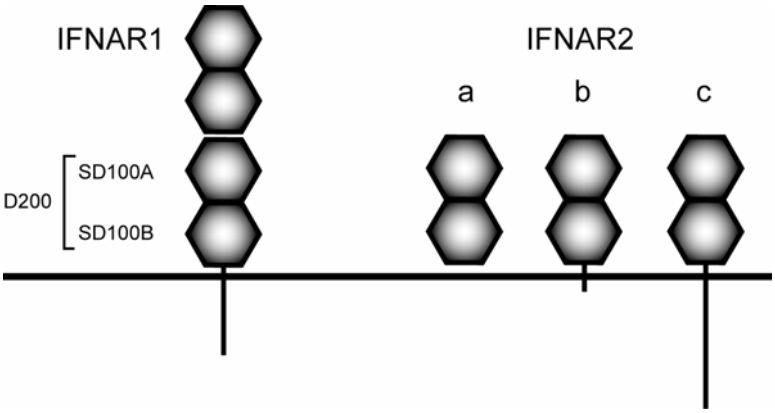


Figure 5: Schematic structures of type I interferon receptors. IFNAR1 is shown on the left, and the three splice variants of IFNAR2 are on the right. IFNAR2a is soluble, IFNAR2b is non-functional (unable to signal) and IFNAR2c is fully functional.

JAK/STAT Signal Transduction

The first attempts to dissect the signalling pathway utilized by IFNs, included the cloning of genes induced upon IFN treatment [82,83]. Upon analysis of the promoter regions of different interferon stimulated genes (ISGs), the first sequence to be identified was the interferon stimulated response element (ISRE) (Fig. 6) [84]. This sequence was found to be both necessary and sufficient for type I IFN inducibility, and transcriptional responses were both rapid and resistant to inhibition of protein synthesis, indicating that the necessary factor was already present in the cell. Consequently, a factor that bound to the ISRE was identified, and denoted IFN stimulated gene factor 3 (ISGF3) [85]. However, ISRE containing reporter constructs responded to both type I and type II IFNs.

A similar approach was used in order to identify a sequence and factor responsible for IFN type II inducibility. This resulted in the identification of a sequence denoted gamma activation site (GAS) [86], and subsequently the gamma activated factor (GAF) [87], which binds to GAS sequences. Unexpectedly, type I IFNs were reported to induce the activity of a factor identical to GAF. Thus, both type I and type II IFNs utilize GAS sequences for transcriptional induction. Also the reverse situation is possible, as type II IFN has been shown to induce genes containing ISRE sequences but lacking GAS sequences [88].

Purification of ISGF3 resulted in the characterisation of three different proteins. Cloning of the corresponding genes identified the first members of a novel family of transcription factors, the signal transducers and activators of transcription (STATs) [89]. One of the characterised proteins turned out to represent splice variants of the same gene, STAT1. These splice variants can be used interchangeably in the formation of ISGF3. STAT2, a related but distinct gene product that is unique to IFN type I signalling was also identified. In addition, a member of the interferon regulatory factor (IRF) family, p48/IRF-9 was demonstrated to be part of the ISGF3 complex [84]. It functions as an affinity switch towards ISRE sequences. The GAF factor has been

demonstrated to consist of homo or heterodimers of STAT1 and STAT2 [90], although the mechanism of homo-dimerisation remains obscure.

In parallel with the biochemical dissection of the signalling pathway, a genetic approach [91] was used in order to identify all required components in the interferon induced pathway. Together, these studies revealed an important general signal transduction pathway, the JAK/STAT pathway, that is used in cytokine and growth factor signalling [92].

The biological activities of IFNs are initiated upon ligand binding to the components of the type I IFN receptor, IFNAR1 and IFNAR2. Since the receptor subunits do not display any intrinsic affinity for each other, the cytoplasmic domains of the receptor subunits are believed to come in close proximity of each other only upon ligand binding. Constitutively attached to the cytoplasmic domain of the receptor subunits are members of the Janus kinase family of tyrosine kinases. Tyk2 is constitutively associated with IFNAR1 [93] and Jak1 is constitutively associated with IFNAR2c [80]. In addition, STAT1 and STAT2 have been shown to be pre-associated with IFNAR2 [94].

Upon ligand interaction Jak1 and Tyk2 activate each other [95]. It is believed that they phosphorylate the receptor subunits, providing docking sites for a number of proteins, most importantly members of the signal transducers and activators of transcription (STAT) family. All STAT proteins contain a SH2 domain with affinity for phosphorylated tyrosines [96]. Upon phoshorylation of the receptor subunits, they become phoshorylated by the JAK kinases. The phosphorylated STATs heterodimerize, dissociate from the receptor complex and through an unknown mechanism translocate to the nucleus where they function as transcription factors (Fig 6). Upon binding of the STAT1:2 heterodimer to p48/IRF-9, the transcription factor ISGF3 is formed [97]. p48/IRF-9 act as a specificity switch, changing the affinity for the promoter sequence from GAS to ISRE. The signalling cascade results in an altered transcription profile of a large number of genes, providing the basis for the pleiotropic effects induced by IFN.

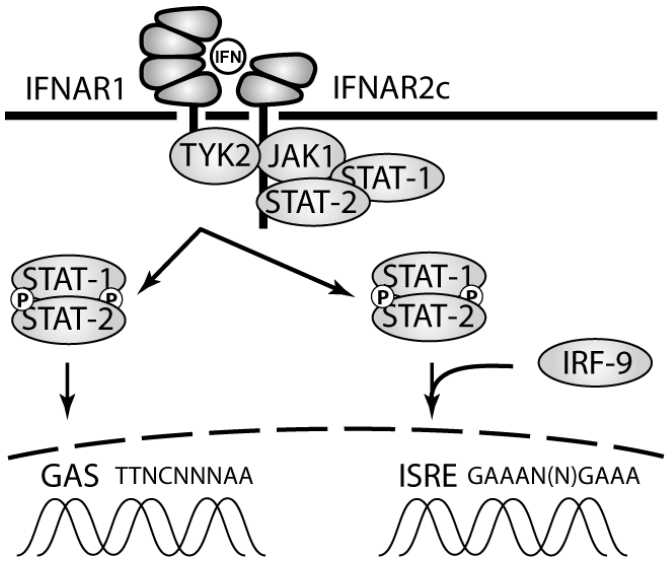


Figure 6: Simplified schematic outline of the JAK/STAT signalling pathway. Upon activation of IFNAR, different STAT complexes can be formed

IFN-induced transcription is dependent upon STAT1 phosphorylation on S727 [98] by p38 MAPK kinase [99]. PKR has also been reported to play a role [100], [101]. STAT1 then aquire the ability to interact with members of the basal transcriptional machinery, for example CBP/p300 family members (reviewed in [102]). However, although STAT2 does not contain any MAPK consensus sequence, and is not known to be serine phosphorylated in response to IFN, it also binds CBP/p300 and facilitates interaction with the basal transcription machinery [103].

The classical members of the STAT family involved in interferon type I signaling is STAT1 and STAT2, but recently other members of the family has been implicated as well. STAT3 has been reported to function as an adaptor for PI3K, as well as being a transcriptionally active partner for STAT1. Also

STAT6 has been reported to form complexes with STAT2 in B-lymphocytes, although no functional data has been assigned to this complex [104].

Several other proteins have been identified that may also be required for IFN signaling. For example, cytosolic phospholipase A2 (CPLA2) has been shown to be phoshorylated and activated upon IFN treatment, and inhibitors of CPLA2 can block induction of ISRE containing genes. This implies that CPLA2 has a role in the transactivation of interferon responsive genes [105] and reviewed in [106]. The scenario is further complicated by the fact that IFN induced proteins, for example members of the interferon regulatory factors (IRF), can also bind to ISRE sequences and modulate transcription. IRF-1 is an IFN induced, positive regulator of transcription (i.e. HLA class I), involved in expression of a number of genes [107] [108].

In contrast to the extended knowledge about the induction of signal transduction, little is known about the mechanisms of negative regulation. IFN induced proteins play a major role as protein synthesis inhibitors prolong the transcription of IFN induced genes [83], [109]. Suppressors of cytokine signalling (SOCS) family members are expressed upon treatment with many cytokines, including IFN [110] and inhibits signal transduction either through interaction with phosphorylated Janus kinases or phosphorylated receptor subunits [111], [112], [113], reviewed in [114]. The protein inhibitors of activated stats (PIAS) proteins are constitutively expressed and have been shown to interact with phosphorylated STATs, thereby inhibiting their DNA binding activity [115]. SHP-1, a tyrosine phosphatase which associates with IFNAR-1 upon IFN treatment has been shown to decrease JAK1 and STAT1 phosphorylation, implying a role in signal attenuation [116]. Also IRF family members, including IRF-2 [117] are known to bind ISRE sequences and negatively regulate transcription. IRF-2 is an inhibitor of transcription that is expressed by IRF-1 in an autoregulatory negative feedback loop.

PI3K/Akt Signal Transduction

It has been demonstrated that the regulatory subunit (p85) of phosphatidylinositol 3' kinase (PI3K) associates to the IFNAR complex via IRS1/2 [118,119] or STAT3 [120]. PI3K is a very important lipid kinase as it phosphorylates PIP_2 to form PIP_3 [121], a second messenger with the intrinsic capacity to attract proteins containing a plextrin homology (PH) domain to the cell surface. PI3K is counteracted by the phosphateses PTEN (3' P removal) and SHIP (5' P removal).

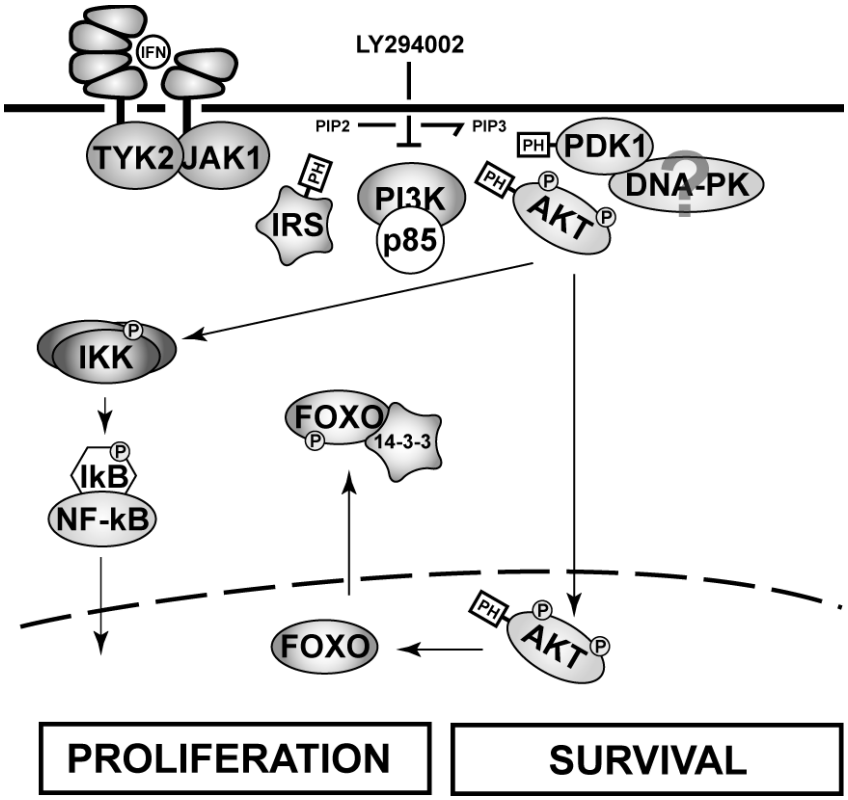


Figure 8: Simplified schematic outline of the IFN-induced PI3K/AKT signalling pathway. LY294002 is a synthetic inhibitor of PI3K.

Many proteins contain a PH domain, but one protein of particular interest is the Ser/Thr kinase protein kinase B (AKT) that has been shown to be an important mediator of both survival and proliferative signals [122], [123]. AKT is activated by phosphorylation on two residues (Thr308 and Ser473). PDK1 is responsible for the Thr308 phoshorylation, while the kinase responsible for Ser473 phosporylation is still a controversial issue [124]. However, a recent paper suggests DNA-dependent protein kinase (DNA-PK) to be the long-sought answer [125].

Once phosphorylated, PKB/Akt translocates mainly to the nucleus, where several downstream targets are affected. A major target for PKB/Akt are members of the Forkhead family of transcription factors [126], which upon phosphorylation become inhibited and redistributed to the cytoplasm where they are retained partly as a result of an interaction with the adaptor protein 14-3-3. The Forkhead family has been shown to be responsible for the transcriptional control of the cell cycle inhibitor, p27 [127].

Also NF-kB has been reported to be activated through PKB/Akt, via the IKK complex leading to phosphorylation and subsequent degradation of IkB.

Cell Cycle Control

IFN is generally associated with inhibition of proliferation, but recent studies have shown that the picture is somewhat more complicated [49]. The cell cycle is driven by the sequential expression and activity of different cyclin/CDK complexes (Fig. 9). As quiescent cells is about to enter the cell cycle in response to mitogenic signals, cyclin D is expressed. Cyclin D assemble with the catalytic partner CDK4/6 in the cytoplasm (reviewed in [128]) and after nuclear translocation, cyclin-CDK4/6 complex is activated by the cyclin activating kinase (CAK).

Mitogenic signals regulate the turnover of cyclin D though modulation of glycogen synthase kinase 3β (GSK-3β) activity. Inhibitory phosphorylation of GSK-3β by activated Akt/PKB leads to decreased phosphorylation of cyclin D. This results in decreased nuclear export and a subsequent increase in cyclin D half-life.

A major checkpoint in G1/S transition is the phosphorylation of Rb, resulting in inactivation. Phosphorylation of Rb is initiated by activated cyclin D-CDK4/6 [129], releasing E2F transcription factor family members. E2F induced expression of cyclin E in late G1 results in a positive feedback loop, where cyclin E/CDK2 contributes to the irreversibility of the G1/S transition through additional phosphorylation of Rb [130].

The cell cycle is a complicated process that requires the coordinated action of many proteins. Therefore, it is not surprising that a number of regulators, cyclin dependent kinase inhibitors, have been identified. They belong to two classes, the INK4 family (p15, p16, p18 and p19) and the Cip/Kip family (p21, p27 and p57). The INK4 family specifically inhibit the activity of CDK4/6, while Cip/Kip family members are broad range inhibitors, affecting the activity of all cyclin-CDK complexes. Initial studies of the Cip/Kip family indicated that they acted only as inhibitors, an activity strongly correlated to nuclear localisation. It has later been shown that although Cip/Kip family members are potent inhibitors of CDK2, they are tolerated by CDK4/6. It has also been shown that assembly of cyclin D-CDK4/6 complexes is significantly enhanced by p21/Cip1 and p27/Kip1

(reviewed in [131]). In addition, as cyclin D/CDK complexes doesn't contain any NLS sequence, nuclear translocation is also enhanced by p21/Cip1 and p27/Kip1.

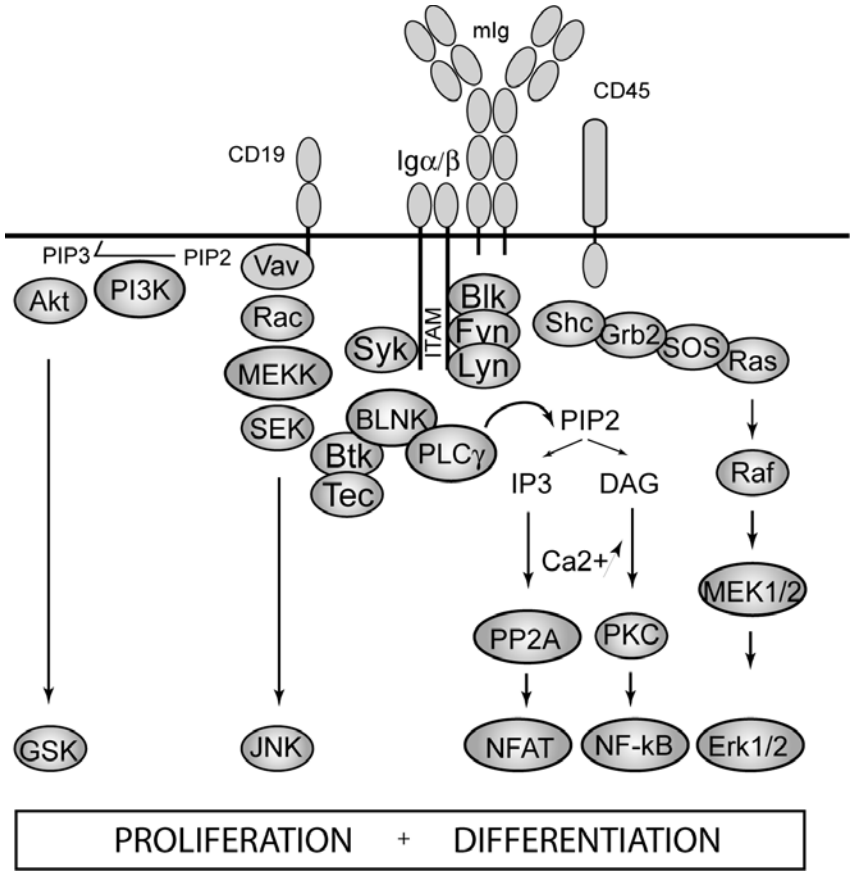
The cyclin dependent kinase inhibitors p21/Cip1 and p27/Kip1 display significant structural homology as well as partial functional redundancy. In lymphocytes, commitment to G0 is maintained through high expression of p27/Kip1 [132-134]. Mitogenic signals decrease p27/Kip1 transcription through the PI3K/Akt/FOXO pathway [135]. Also protein stability is affected, since cyclin E-CDK2 dependent phosphorylation target p27/Kip1 for degradation, thereby releasing the inhibitory constraint [136]. Expression of p21/Cip1 is low in quiescent lymphocytes and increases upon mitogenic stimulation, suggesting cellular functions partially distinct from p27/Kip1. Studies in other cell types have shown that cytoplasmic p21/Cip1 can act as an inhibitor of apoptosis though interaction with ASK1 kinase [137].

Post-translational modifications of p21/Cip1 and p27/Kip1 affecting localisation and cellular function have recently been described. Akt/PKB induced phosphorylation within the NLS sequence of p21/Cip1 and p27/Kip1 has been reported to induce cytoplasmic localisation, at least in some cell types[138]. Proteolytic cleavage within the NLS sequence of p21/Cip1 and p27/Kip1 by caspase 3 has also been reported [139,140]. Interestingly, expression of a cytoplasmic mutant of p21/Cip1 resulted in an apoptosis-resistant phenotype. This suggests a role for p21/Cip1 in protection against apoptosis, maybe through inhibition of cytochrome c release. On the other hand, the function of p27/Kip1 in the apoptosis process remains unclear.

BCR Signalling

Mature peripheral B-lymphocytes circulate in the body, on constant watch for intruders. The mechanisms that maintain a steady-state pool of B-cells remain poorly understood. Signalling through the B-cell antigen receptor (BCR) is required throughout B-cell development and peripheral maturation. Recent findings suggest that lymphocyte survival is a continuous active process and support the role of B-cell receptor engagement in B cell survival.

The BCR constitutes a multi-protein complex made up of a cell-surface immunoglobulin (mIg), associated with invariant accessory proteins (Igα/β) required for initiation of signalling upon ligand interaction (reviewed in [141]).



Cross-linking of surface immunoglobulin molecules activates the receptor-associated Src-family protein tyrosine kinases Blk, Fyn and Lyn. The CD45 phosphatase removes an inhibitory phosphate from these kinases, thus allowing their activation. The receptor-associated kinases phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) in the receptor complex, which bind and activate Syk, another protein kinase (reviewed in [142]). Syk activates B-cell linker protein (BLNK/SLP-65), which help to recruit Tec kinases that in turn phosphorylate and activate phospholipase Cγ (PLCγ). PIP_2 is cleaved by PLCγ into DAG and IP_3, thus initiating two of the three main signalling pathways. IP_3 releases Ca^{2+} from intracellular and extracellular sources and Ca^{2+} dependent enzymes are activated (calcineurin/PP2B) leading to activation of nuclear factor of activated T cells (NFAT). DAG, the other cleavage product of PIP_2, activates PKC leading to activation of NF-kB. Ras, the third main signalling pathway is initiated by Grb2/SOS upon interaction with the adaptor protein Shc. This pathway leads to activation of Erk1/2 (through Raf and MEK1/2) that translocate into the nucleus in order to regulate transcription factor activity.

BCR signalling is modulated by a co-receptor complex of at least three cell-surface molecules, CD19, CD21 and CD81. CD19 is expressed on all B cells from an early stage in their development and contribute to signalling through the BCR. It is phosphorylated by Src-family kinases, which result in recruitment of PI3K and powerful amplification of the BCR signalling cascade.

CD19 signalling also include a MAPK signalling pathway initiated by Vav (through Rac/MEKK/SEK), leading to activation of JNK.

GENERAL AIM:

- to study regulation av type I IFN induced proliferation and survival in experimental systems of importance for tumour treatment.

- to investigate the molecular mechanisms of IFN induced survival in human peripheral B-lymphocytes

- to investigate the molecular mechanisms of IFN induced proliferation in human peripheral B-lymphocytes

- to investigate the molecular mechanisms of IFN resistance in a multiple myeloma cell line.

RESULTS and DISCUSSION:

IFN induces PI3K activation and increased survival (Paper I)

The pleiotropic effects of IFN include immunomodulatory functions, thereby acting as a link between innate and adaptive immunity. The effects of IFN have been studied in many different cell types, both normal and transformed. The large body of available data show that most of the responsive cells react by reduced proliferation, and sometimes even apoptosis. Thus, IFN has been shown to inhibit proliferation of early B- and T-cell progenitors [143] [144] [145]. However, studies on human peripheral B-lymphocytes have shown increased survival as well as proliferation upon IFN treatment [48] [49]. The pathway responsible for this phenomenon is unknown, but a potential pathway could be the PI3K/AKT pathway that has been implicated in survival and proliferation in many different cells, both normal and transformed. In fact, the regulatory subunit of PI3K was recently found to interact with the IFNAR complex [146] [147], but the biological significance was not elucidated. Upon activation, PI3K produces PIP_3, a second messenger that attracts proteins to the inner surface of the cell membrane. Proteins with pleckstrin-homology (PH) domains accumulate at sites of PI3K activation by association to PIP_3. Of particular interest is AKT, a kinase with multiple targets affecting metabolism, survival and proliferation.

We established a robust purification system for extraction of B-lymphocytes from buffy-coat, utilizing density centrifugation in combination with anti-CD19 magnetic beads [148]. Flow cytometry analyses after purification typically displayed approximately 98% mature B-lymphocytes (data not shown).

In an attempt to identify the molecular mechanisms of IFN-induced survival in human peripheral B-lymphocytes, the expression and/or activation pattern of different signaling proteins were investigated. In order to detect activation of Akt, an antibody specific for phosphorylation on S473 was utilized. Using this approach, we were able to demonstrate that both IFNα and IFNβ activated Akt *in vitro* using resting human B-lymphocytes (Fig. 1 and 2). Akt phosphorylation was rapidly induced, reaching a plateau after 2 minutes

and decreasing after 20 minutes (Fig. 2). High concentrations of IFN was used in Fig 1-3, in order to saturate all binding sites simultaneusly, but in the cell culture experiments much lower concentrations (1% compared to Fig. 2 and 3) was routinely used.

FOXO1 (previously known as FKHR) belongs to the forkhead family of transcription factors, found to play critical roles in regulation of apoptosis and proliferation. Activity is inhibited by phosphorylation, which we investigated using an antibody specific for S256 phosphorylation. The kinetics of FOXO1 phosphorylation was slightly delayed compared to AKT phosphorylation, indicating a role downstream of AKT in this pathway. Furthermore, pre-incubation with the PI3K inhibitor LY294002 abrogated Akt as well as FOXO1 phosphorylation, convincingly demonstrating that both Akt and FOXO1 are downstream of and dependent on PI3K in IFN-mediated signaling. The concentration of LY294002 used in our experiments was in the same range (low μM) as previous investigations [148,149]

The cell cycle dependent kinase (CDK) inhibitor p27/Kip1 has previously been found to be regulated by forkhead family members [135]. Thus, phosphorylation-induced inactivation of FOXO1 is expected to result in decreased p27/Kip1 expression. Unexpectedly, IFN treatment did not decrease the high levels of p27/Kip1 expression in resting B-cells (Fig. 4, paper II). However, using anti-IgM activated B-cells, expression of p27/Kip1 was reduced, an effect that was further enhanced in combination with IFN. In addition, p27 expression was restored in a dose-dependent manner by LY294002, strongly suggesting that p27 expression is regulated through the PI3K/Akt/FOXO1 pathway (Fig. 4). The inability of IFN to reduce p27/kip1 expression in resting cells is likely to be due to lack of additional signalling pathways necessary for control of p27/Kip1 expression.

Resting B-lymphocytes in culture have a short life-span due to spontaneous apoptosis, unless the appropriate signals necessary for activation and/or proliferation are provided. Since the PI3K/Akt pathway has been associated with regulation of survival, apoptosis was measured using

independent methods, AnnexinV in combination with Propidium Iodide (PI) and TUNEL staining, respectively. AnnexinV staining of resting B-lymphocytes revealed that IFN treatment by itself dramatically decreased spontaneous apoptosis. (Fig 5). Using TUNEL staining to detect apoptotic cells, survival upon IFN treatment was twice as high after 48h in culture, compared to medium alone (Fig. 6). This response was abrogated in a dose-dependent manner using LY294002, suggesting PI3K as a critical mediator (Fig. 6).

We demonstrate a novel signalling pathway for IFNα in human peripheral B-lymphocytes. Evidence is provided that IFN activate PI3K and the downstream targets AKT and FOXO1. The biological outcome is increased survival, a response that could be efficiently abrogated using the PI3K inhibitor LY294002, thus suggesting PI3K as a critical mediator. As B-lymphocytes are critical mediators of the adaptive immune response, it is tempting to speculate that mature B-lymphocytes are so important in case of an immune-rection, that it is advantageous to enhance their normal function.

IFN act as a co-mitogen in anti-IgM induced proliferation (Paper II) Since a previous investigation has reported that interferon treatment increased proliferation in human peripheral B-lymphocytes [49], and the fact that we previously noticed a dramatic decrease in p27/Kip1 expression upon anti-IgM+IFN treatment prompted us to further investigate this phenomenon.

Thymidine incorporation studies revealed that IFN treatment potentiated the response to anti-IgM induced activation (Fig 1). However, on its own IFN was without effect. The potentiation is likely to be due to the partial use of common signalling pathways. IL-2 is a well-studied co-mitogen for anti-IgM induced proliferation in B-lymphocytes, known to decrease the threshold necessary for activation/stimulation [150]. IFN turned out to be as effective as IL-2, and together they had an additive effect on proliferation (Fig 2). CFSE staining is a sensitive method for measurement of proliferation, which we used

to demonstrate that anti-IgM+IFN treatment stimulated a larger proportion of cells to proliferate one additional cycle compared to anti-IgM treatment (Fig 3).

In order to distinguish proliferation from increased survival, western blots were performed on some key cell cycle regulatory proteins. CDK-induced inactivation of Rb by phosphorylation is an essential event in the cell cycle, releasing E2F transcription factors necessary for progression through G1. Inactivation of Rb is conviniently detected, as the migration in SDS-PAGE is affected by phosphorylation status, requiring only a single antibody for detection of both expression and phosphorylation status. Using this approach, we demonstrated that Rb was phosphorylated in cells treated with anti-IgM, an effect enhanced by anti-IgM+IFN treatment (Fig 4).

In lymphocytes, commitment to G0 is maintained by high expression of p27/Kip1, while p21/Cip1 expression is low (reviewed in [128]). As mentioned earlier, the expression of p27/Kip1 was not affected by IFN treatment in resting cells. After 2 days in culture, the effect is most accentuated when IgM+IFN is combined, most likely a result of stronger activation signals. After 4 days of incubation, anti-IgM and anti-IgM+IFN resulted in a dramatic decrease in p27/Kip1 expression (Fig 4).

Expression of p21/Cip1 was evident at the start of the experiment, probably due to residual signalling from the donor. In B-lymphocytes cultured in medium alone, p21/Cip1 expression went below the limit of detection. It is known that p21/Cip1 expression is upregulated in response to mitogenic signals in B-lymphocytes [151]. Thus, we noticed an increase in p21/Cip1 expression upon anti-IgM and anti-IgM+IFN treatment (Fig 4, Fig 5A and B). However, as determined using both Western blot and immunocytochemistry, expression was detected almost exclusively in the cytoplasm, regardless of treatment (Fig 5A and B). Taken together, these data strongly suggest that the functional role is different from classical anti-proliferation. Further investigations are necessary in order to investigate the mechanism responsible for the cytoplasmic localization.

Our data provide evidence that anti-IgM induced proliferation of human peripheral B-lymphocyte is further increased in combination with IFN. Our evidense is based on thymidine incorporation, CFSE staining and Western blot analysis of Rb phosphorylation. However, IFN alone has no effect on proliferation on its own. Taken together from paper I and II, we conclude that type I interferons contribute to increased survival as well as proliferation of human peripheral B-lymphocytes.

Lack of functional IFNAR1 and IFNAR2 expression in U266Res cells (Paper III) All type I IFNs compete for binding to a common receptor, consisting of two subunits, IFNAR1 and IFNAR2. The reason for resistance in clinical situations is seldom elucidated, but is often believed to be due to development of antibodies against (recombinant) IFN. Genetic instability is a key factor in development and progression of malignant tumours. Multiple myeloma is a B-cell derived tumour, with characteristics of mature plasma cells. Other studies have demonstrated that myeloma cells have marked chromosomal abnormalities, including aneuploidy, deletions and translocations.

We investigated the human myeloma cell line U266, and the variant U266Res totally resistant to the anti-proliferative properties of IFN as a result of culture in increasing amounts of IFN (Fig 1). In an attempt to elucidate the molecular mechanism responsible for the resistance, we performed equilibrium binding studies using radio-labelled recombinant IFNα17. The resistant cell line did not display any ligand binding, in contrast to the wild type cells where the binding was saturable and occured with high affinity (Fig 2).

Using flow cytometry analysis to detect cell surface expression of the receptor components, IFNAR1 was not detected in U266Res cells. IFNAR2 was expressed both in wild type and resistant cells, but the level of expression was reduced in the U266Res cells (Fig 3).

Next, we performed Northern blot analysis in order to investigate transcription of the receptor components. Using an IFNAR1 probe covering a region of the extracellular domain, we were unable to detect the 2.7 kb band corresponding to the full-length IFNAR1 in the resistant cells (Fig 4). Southern blot of IFNAR1 using the same probe was negative for the presence of a band in the U266Res cells (Fig 5), consistent with Northern blot data.

The IFNAR2 Northern blot displayed a peculiar pattern, since the resistant cells displayed strong expression of an abberant transcript, not present in the control cells. However, the 4.5 kb band corresponding to all splice variants of IFNAR2 [74], including IFNAR2c, was also present in the resistant cells, although at reduced levels (Fig 6). The 1.5 kb band present in the control cells has been shown to correspond to a short IFNAR2b transcript.

Immunoblotting was used to determine the expression of JAK/STAT family members. Jak1, Tyk2, STAT1 and STAT2 were analyzed and no differences in expression were detected compared to the wild type cells (data not shown), suggesting the presence of a functional signalling pathway. In order to confirm the functionality of the JAK/STAT pathway, transient transfection reporter assay experiments were performed. The reporter construct contained an IFN-inducible promoter from the 6-16 gene in front of the secreted alkaline phosphatase gene (sAP). Using this approach, we demonstrated that the resistant cells did not respond to IFN, not even transfected with the IFNAR1 cDNA. A response was only detected when exogenous IFNAR1 and IFNAR2 cDNA were transfected in combination, suggesting that the endogenous receptor subunits were non-functional (Fig 7). However, the detected response was constitutive, possibly due to expression and secretion of IFN in the cell culture medium.

The discovery of an abberant IFNAR2 transcript and loss of a functional receptor prompted us to investigate the IFNAR2 gene further. The IFNAR2 gene can be spliced in three different variants, where only the IFNAR2c variant is functional. Southern blot analysis was performed using a probe covering the cytoplasmic domain (exon 9) of IFNAR2c (Fig 8). We were unable to detect a corresponding band in the resistant cells, suggesting that the IFNAR2 gene is non-functional.

Moreover, we used an RT-PCR approach in an attempt to clone and sequence abberant IFNAR2 transcripts from U266Res. From a total of 9 sequenced clones, 8 displayed an aberrant sequence. The sequences were homologous to IFNAR2 from exon 2 through exon 7, but immediately after displayed homology to a intergenic sequence located 5' of IFNGR2, approximately 130 000 bp downstream of exon 7 (Fig 9 and 10). The 9th clone was homologous to IFNAR2b. We also sequenced three clones from the U266 wild type cells, which were all homologous to the membrane bound, but non-functional IFNAR2b, originating from the short 1,5 kb cDNA.

Since both receptor subunits are necessary in order to transduce a signal, resistance is developed as soon as one subunit is missing. However, since they are closely linked on the genomic level, it is not surprising that also the other subunit is affected. However, the fact that the cells retain three or four copies of chromosome 21 is enigmatic. A possible explanation is that at least one copy of the IFNAR2 gene is deleted and that aberrant regulatory mechanisms suppress the expression of non-mutated alleles. Preliminary experiments using FISH technology were negative for deletions using probes covering the proposed region of deletion (Fig 10). Moreover, one out of nine clones displayed an IFNAR2 sequence similar to the three found in the U266 wild type cells. The presence of a 4.4 kb band on Northern blot may predict the expression of IFNAR2c. However, expression of IFNAR2 was demonstrated by FACS analysis, but the data do not differentiate between splice variants. Transient transfection of IFNAR1 did not demonstrate any functional receptor in our sAP reporter assay and Southern blot was negative for exon 9 (=IFNAR2c).

Heterogeneity in the mutations affecting thee IFNAR2 gene must still be present since IFNAR2b was found in U266Res, although at a low frequency. The resistant cell lines might be of value for understanding development of resistance to IFN and other cytokines in clinical settings.

Our studies emphasize the need for additional investigations using primary cells and/or in vivo model systems in order to determine the role for

interferon in normal cells/tissue. Since our studies were conducted, a couple of articles have emerged, addressing this question [152,153].

Also the role of IFN in tumour treatment is important to evaluate. The use of IFN in the treatment of lymphomas has not been particularly successful, maybe due to the mechanisms that has been put forward in this thesis. IFN resistance can be due to several reasons, most commonly due to development of neutralising antibodies. However, also lack of receptor subunits [154,155] or signalling molecules has been described [156-159].

GENERAL SUMMARY:

- We have demonstrated a novel signalling pathway for IFN type I resulting in increased survival in human peripheral B-lymphocytes

- We have demonstrated the IFN type I act as a co-mitogen for anti-IgM induced proliferation in human peripheral B-lymphocytes

- We have characterized the molecular mechanism of IFN type I resistance in a human multiple myeloma cell line.

- In summary, we show the importance of monitoring tumour patients being treated with type I IFN with respect to development of resistance or unusual effects as proliferation or survival.

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The birdwatching community, especially: **Joakim Lessmann** a true expert in bird identification, and a first rank wildlife photographer to be **Jenny Lundqvist** eyes like an eagle, and with the backpack full of nice food (for everyone) **Christer Larsson** always interested in ringing birds and with a burning interest for Stora Fjäderägg **Fredrik Widman** immensely competitive, catching up quickly on the lists**.** Hopefully we can go to Öland next year! Thanks also to my Zeiss Diascope 85*T FL for providing the best opportunity to increase my list(s) and beat my friends!

My parents-in-law **Anita** and **Erik** with family: Many thanks for creating a welcoming (although slightly allergic) atmosphere during our few and short visits!

My parents **Ulf** and **Irma** which understand very little of what I do, but patiently accept the need for long hours and few reunions. My brother and sister **Erik och Maria** and their partners **Kicki and Bosse**, always finding interesting things to do whenever we meet – moose farm visits, for example.

Finally **Christine** my future wife, full of energy and concern – I love you!