Interferons at age 50: past, current and future impact on biomedicine

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Abstract | The family of interferon (IFN) proteins has now more than reached the potential envisioned by early discovering virologists: IFNs are not only antivirals with a spectrum of clinical effectiveness against both RNA and DNA viruses, but are also the prototypic biological response modifiers for oncology, and show effectiveness in suppressing manifestations of multiple sclerosis. Studies of IFNs have resulted in fundamental insights into cellular signalling mechanisms, gene transcription and innate and acquired immunity. Further elucidation of the multitude of IFN-induced genes, as well as drug development strategies targeting IFN production via the activation of the Toll-like receptors (TLRs), will almost certainly lead to newer and more efficacious therapeutics. Our goal is to offer a molecular and clinical perspective that will enable IFNs or their TLR agonist inducers to reach their full clinical potential.

The discovery and molecular understanding of the cellular mechanisms and clinical use of interferons (IFNs) have been a major advance in biomedicine over the past 50 years. This family of secreted autocrine and paracrine proteins stimulates intracellular and intercellular networks that regulate resistance to viral infections, enhance innate and acquired immune responses, and modulate normal and tumour cell survival and death. After their discovery in 1957 (Ref. 1), it was soon appreciated that IFNs were critically important to the health of animals and humans and that the IFN system had potential as therapies for infections for both RNA and DNA viruses (TIMELINE).

However, advances in molecular biology a decade later and into the 1970s were required before the promise could be realized. The 1980s saw their introduction into the clinic as the first pharmaceutical products of the budding biotechnology industry, and, importantly, as a demonstration of the effectiveness of IFNs not only for viral diseases and cancer but also for multiple sclerosis (MS). The 1990s were marked by an expansion in their clinical applications with regulatory approvals worldwide and a further understanding of molecular events influencing biological actions. Current studies have led to new insights into IFNs as a fundamental component of the innate immune system. Additionally, studies have revealed how IFN production is induced through Toll-like receptors (TLRs), the actions of IFN-stimulated genes (ISGs), the identification of viral mechanisms that resist actions of this potent protein, and how mutation and suppression of gene products of the IFN system in and by malignant cells may affect the initiation and progression of cancer.

After binding to high-affinity receptors, IFNs initiate a signalling cascade through signalling proteins that can also be activated by other cytokines, which were first identified through studies of IFNs. Cellular actions are mediated through specific ISGs, which underlie the antiviral effects, as well as immunoregulatory and antitumour effects. Future drugs that could act as molecular activators for ISGs, many of which exist in a latent state or as agonists for TLRs, might be expected to have potent antiviral, antitumour and/or immunomodulatory effects.

From more than 100,000 published papers, we offer a perspective with a focus on human IFNs to stimulate the future investigation of important questions. Although IFNs function as an integrated system, conceptually it helps to consider their production, which is mediated through TLR activation, and their action, which is mediated through JAK/STAT (Janus kinase/Signal Transducers and Activators of Transcription) and other signalling pathways. The production of IFNs is important for understanding the role of IFNs in innate immunity, while their effects relate to dissecting underlying and future mechanisms of action and application. Highlighting initial pivotal discoveries and more recent findings of conceptual importance, we review how IFNs...
are induced, the cellular actions of IFNs and ISGs, human therapeutic applications, and summarize important
questions for biomedicine and drug and clinical development
initiatives.

How synthesis is induced
Production of IFNs, both in vitro and in vivo, is transient
and requires stimulation by viruses, microbial products
or chemical inducers. In the course of the discovery
of IFNs, either live or heat-inactivated influenza
viruses were initially identified as inducers1. Subsequently other
microbial products, including those of bacteria, proto-
zoa, and RNA and DNA viruses, were also recognized
to induce IFN. It was also shown that microbial nucleic
acids, lipids, polysaccharides or proteins trigger induction
of IFNs through activation of TLRs (FIG. 1). An early
pivotal discovery identified double-stranded (ds) RNAs,
both natural and synthetic, as potent inducers1, leading
to the simplistic paradigm that viruses induce IFNs by
producing dsRNA; in reality, it is only one of the viral
gene products responsible for induction. Nonetheless,
dissection of cellular responses that lead to induction
were spearheaded by the analysis of dsRNA-mediated
signalling pathways.

dsRNA is recognized by TLR3, which is present
mostly in endosomal membranes4, and also by two
cytoplasmic RNA helicases, retinoic acid-inducible
gene I (RIG-I) and melanoma differentiation associated
protein 5 (MDA5)5 (FIG. 2). The cytoplasmic proteins
can also recognize single-stranded (ss) RNAs but only if they
have 5′-triphosphates. Mice in which the TLR3 (REF 6),
the RIG-I or the MDA5 (REF 8) gene has been disrupted
are more susceptible to virus infection; however the relative
importance of the three proteins for inhibitory
activity varies for the immune defence against different
viruses. Surprisingly, the presence of TLR3 can enhance
pathogenesis in mice infected with influenza A virus4
or West Nile virus10. An adapter protein for TLR3
signalling is TLR adapter molecule 1 (TRIF), whereas
the mitochondrial protein IFN-β-promoter stimulator 1
(IPS1; also known as VISA) is an adapter for RIG-I and
MDA5; both TRIF and IPS1 recruit inhibitor of nuclear
factor-κB (NFκB) kinase (IKK) and TANK-binding
kinase (TBK1), the common activator kinases11. Other
nucleic acids such as ssRNA, acting through TLR7 and
TLR8, and bacterial oligodeoxynucleotides, acting
through TLR9, are also potent inducers12. Bacterial
lipopolysaccharides induce IFNs through TLR4 and also
recruit TRIF; viral glycoproteins bind and activate dif-
f erent TLRs. An additional cytoplasmic receptor exists
for recognizing viral DNA13.

IFN genes, which are normally transcriptionally
silent, are induced by the binding of TLR-activated
transcription factors to their promoters. Transcriptional
induction of IFN-β has been a model experimental
system for defining interactions of transcription factors as
an enhanceosome multiprotein complex with DNA14.
The most important transcription factors for induction
are proteins of the IFN regulatory factor (IRF), specifi-
cally IRF3 and IRF7, and NFκB families15,16. IRFs
are activated by the kinases TBK1 or IKKe; activated IRFs
then dimerize and translocate to the nucleus17. The
IKK protein kinase complex phosphorylates 1KB and
releases it from NFκB; NFκB is then further activated
by phosphorylation by other kinases18.

To evade the IFN system, viruses have evolved many
mechanisms to block IFN synthesis and actions — acting
at almost every step of the signalling pathway18,19. For
example, a hepatitis C virus (HCV)–encoded protease
can cleave IPS1 off the mitochondrial membrane and
block RIG-I/MDA5-mediated signalling20. Hepatitis B
virus (HBV) ORF-C and terminal proteins can also block
induction. Influenza virus, Ebola virus, papilloma viruses
and the human herpes Kaposi’s sarcoma–associated virus
(KSHV) encode proteins that interfere with IRF activa-
tion or induction and actions of IFNs21. For example,
KSHV can downregulate one of the receptor chains for
IFN-γ, and the NS1 protein of influenza viruses prevents

Timeline | Major milestones and discoveries in 50 years of interferon research

| Year | Event |
|------|-------|
| 1957 | Virus-induced IFN |
| 1964 | Non-viral IFN inducers: dDNA |
| 1967 | IFNα: a family |
| 1969 | IFNγ (immune IFN) |
| 1970 | Human antitumour effects |
| 1973 | Clinical trials with impure IFNs |
| 1975 | IFN has antiviral effect in HBV-infected humans |
| 1976 | Purification, cloning, sequencing of IFN-α1, IFN-α2, IFN-β |
| 1980 | IFN-γ cloned |
| 1981 | ISG promoters |
| 1982 | IFN antiangiogenic effects |
| 1983 | Clinical MS effects |
| 1984 | IFN gene promoter |
| 1985 | Recombinant IFN cancer clinical trials |
| 1986 | IFN-α2 FDA approval for hairy cell leukaemia |
| 1987 | IFN-α4 clinical trials |

The molecular understanding and clinical use of interferons (IFNs) has been a major advance in biomedicine over the past half century. Discovery of IFNs evolved from studies of viral interference beginning in 1955 with evidence for inhibition by one virus with replication of a second. 1980 marked a particularly ‘big year’, which saw the purification, cloning and sequencing of IFN-α1, IFN-α2 and IFN-β. FDA, Food and Drug Administration; HBV, hepatitis B virus; HCV, hepatitis C virus; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; JAK, Janus kinase; MS, multiple sclerosis; STAT, signal transducer and activator of transcription.
CpG oligonucleotides

Bacterial DNA oligodeoxynucleotide sequences that include a cytosine–guanosine sequence and certain flanking nucleotides that have been found to induce innate immune responses through interaction with Toll-like receptor 9.

DMXAA

5, 6-dimethylxanthenone-4-acetic acid. An experimental antiviral drug currently in clinical trials for lung and prostate cancer. It is classified as a vascular disruption agent, causing apoptosis (death) of vessel endothelial cells and the release of vasoactive molecules, which inhibit the formation of new tumour blood vessels.

Antiviral unit

An antiviral unit is the concentration of an interferon required to inhibit virus replication in vitro by 50%; an international WHO standard provides a reference base for each major interferon type.

establishment of an antiviral state through the interaction with RIG-I22,23. Conversely, patients with defects in the production of type I IFNs, due to mutation of the UNCG3B gene, are highly susceptible to herpes simplex virus 1 (HSV-1) encephalitis24.

Development of small-molecule activators of induction are only beginning; however, delineation of the responsible signalling pathway has identified many target proteins. CpG oligonucleotides are activators of TLR9 (REF 25); the quinolinamine imiquimod26 and its analogues activate TLR7 (REFS 25, 26); and DMXAA induces IFN synthesis through a TLR-independent pathway27. Development of chemical modulators that selectively activate IFN synthesis or block the synthesis of inflammatory cytokines could have a broad therapeutic potential28,29.

IFN proteins and their receptors

The realization that IFNs constitute a protein family arose initially from the definition of antigenic differences between human fibroblast (IFN-β) and leukocyte IFNs (IFN-α)30. Although protein purification studies suggested the potential multigene nature of IFN-α, this was only firmly established by the cloning of IFN-α1, IFN-α2 and IFN-β31–33, which, when accomplished, helped solidify the financial future of the nascent biotechnology industry. The number of functional genes identified that encode type I IFNs has grown subsequently: 17 non-allelic genes have now been described in humans. All lack introns and cluster on chromosome 9 (IG.3). Of the type I IFNs, there are 13 IFN-αs (plus an additional synthetic consensus sequence and also additional minor allelic variants), whereas there is only one type of IFN-β, IFN-ω, IFN-κ or IFN-κ (FIG. 3). Among mammals, the number of type I IFN genes is variable; some have unique types (for example, IFN-δ occurs only in pigs and IFN-τ only in ruminants) and others are devoid of a particular type (for example, IFN-ω in mice). Consistent with the universal biological definition of IFNs (that is, proteins inducing relatively species-specific antiviral effects), all type I IFNs, which are mostly non-glycosylated proteins of 165–200-plus amino acids, share homologies that range from 30–85% within a species. Essentially all have relatively high specific potencies (1 × 107 to 1 × 109 antiviral units per mg protein).

Although type I IFNs have qualitative and quantitative differences in their antiviral and other actions34,35, the reason for origin and maintenance through evolution of these related proteins is unknown. All mammalian species have retained, however, at least one IFN-α and one IFN-β36. In humans, expression of IFN-κ and IFN-ε seems tissue specific36,37, but all cells are able to produce other IFNs. In monocyte-derived dendritic cells, in which viral infection induces expression of the 15 IFN-α/IFN-β/IFN-ω subtypes, stimulation of TLR3 or TLR4 induces mostly IFN-β and IFN-α1, which emphasizes the differences in the promoter sequences for the IFN-αs, IFN-ω, and IFN-β genes that govern the response to different inducers38.

Type I IFNs belong to the helical cytokine family with secondary structures of a five-α-helix bundle held in position by two disulphide bonds39. They act through a cell-surface receptor composed of two ubiquitously expressed transmembrane proteins, IFN (α, β and ω) receptor 1 (IFNAR1) and IFNAR2 (the genes for which are clustered on chromosome 21), and are associated with two cytoplasmic tyrosine kinases, TYK2 and JAK1 (REF 39) (FIG. 3). Formation of the IFN–receptor complex involves one side of the IFN protein interacting with IFNAR2 in a region forming the hinge between the two fibronectin type III (FnIII) domains (FIG. 3); binding affinity is in the nanomolar range39. IFNAR1 binds IFNs with an affinity 1,000-fold weaker than that of IFNAR2, with a binding site located opposite to the IFNAR2 binding site. Binding studies are consistent with the ternary complex between IFNAR1, IFN and IFNAR2 having a 1:1:1 stoichiometry, and a similar if not identical architecture for all type I IFNs. Ternary complex assembly is a two-step process; the ligand binds first to one IFNAR and then recruits the second with no identified interaction between the two IFNARs39.

As affinities for IFNAR2 are generally much higher than for IFNAR1, a binding pathway in which IFNs bind first to IFNAR2 and then IFNAR1 should have a higher probability. However, with IFN-ω1 having a low affinity for IFNAR2, the relevance of the reverse-binding pathway, which could lead to differing cellular effects, has been confirmed40. If differences in the structures of the IFN–receptor complexes cannot account for the differential activities of type I IFNs, then a body of argument — which includes studies on the activities of engineered IFNs — suggests that differential affinities for IFNARs and thus, ternary complex stability, govern differential biological activities39,41–45. The cell-surface concentration of IFNARs and their lateral organization into microdomains could also be important cellular parameters that shape responsiveness to individual IFNs46. Similar or other changes in receptor organization may also account for the increased susceptibility to HBV infection that occurs with polymorphisms in class II cytokine receptor genes47.
IFN-γ is a single glycosylated protein of 140 amino acids that is designated as a type II IFN because of its distant amino-acid sequence homology with type I IFNs, and its production by natural killer (NK) or activated T cells. Like type I IFNs, it binds to two class II cytokine receptor proteins; when ligand bound it forms a complex of two of each of the receptor proteins linked to an antiparallel homodimer of IFN-γR1 (Fig. 3). IFN-γ receptor 1 (IFNGR1) maps to chromosome 6 and has a JAK1 binding domain and a STAT1 docking site. IFNGR2 contains a JAK2 binding domain and maps to chromosome 21q22.1 in a cluster that also contains IFNAR1, IFNAR2 and interleukin 10 receptor 2 (IL10R2; also known as IL10RB)10. Although IFNGR1 is constitutively present on all cells, IFNGR2 is tightly regulated and less widely expressed. Promoter polymorphisms and/or mutations within both chains have been associated with increased susceptibility to malaria and mycobacteria — in a few patients these defects have been reconstituted by marrow transplantation51. The type III IFN family with three subtypes of IFN-λ, which are co-produced with IFN-β, activate the same main signalling pathway as type I IFNs but have evolved a completely different receptor structure48 (Fig. 3).

How cells respond to IFNs
Initial studies identified several genes that were induced by type I IFNs and analysis of their promoters identified conserved DNA elements53–55. Proteins bound to these elements after treatment with type I IFNs were purified and identified as STAT1, STAT2 and IRF9 (REFS 59–62). To identify other components of IFN-dependent signalling cascades, the promoter element of the 6–16 gene was used to drive IFN-dependent expression of guanine phosphoribosyl transferase; cells that did not respond to IFNs were then selected with 6-thioguanine. Following chemical mutagenesis, several mutant cell lines were obtained, each lacking a protein essential for signalling. For example, mutant U1A was shown by complementation to lack the tyrosine kinase TYK2 (REFS 60,62). Subsequently seven STAT and four JAK family members were identified; these transcription factors and tyrosine kinases have been shown to be essential for responses not only to IFNs but also to other cytokines and growth factors as well63.

Minimum requirements for a response to type I IFNs are the heterodimeric IFN receptor; the tyrosine kinases TYK2 and JAK1, which reciprocally transphosphorylate the receptor chains when activated; STAT1 and STAT2, which are phosphorylated in response to signalling; and the unphosphorylated IRF9 (Fig. 3). Transcription in response to IFN-dependent signalling is initiated by high-affinity binding to specific palindromic promoter sequences of the trimeric complex of STAT1, STAT2 and IRF9. The response to IFN-γ requires only the two receptor proteins, the kinases JAK1 and JAK2, and STAT1. STAT1 and STAT3 bind competitively to the same phosphotyrosine residue of IFNGR1, with the binding of STAT1 greatly favoured49. Although initial work was carried out primarily in human fibroblasts, recent studies have identified additional complexity that allows individual cell types to respond by activating different STATs in response to the same IFN (reviewed in REFs 64,65). Analysis of defects in the IFN system has identified germline mutations in humans that result in deficiencies of STAT1 or TYK2, with enhanced susceptibility to infection by viruses55–58. Although the mouse has been a useful model, the human defects are not always identical to the effects that result from the targeted deletions of these genes in mice41.

Upon activation of receptors, the JAKs undergo autophosphorylation and transphosphorylation to increase their activity, and then phosphorylate the IFN receptors and finally STATs. However, the kinase activities of the JAKs are not sufficient to explain all nuances of signalling. Tissue-specific differences in activating additional protein kinases probably contribute to the differential responses of various cells to a single type of IFN. In at least some cell types, the p85 subunit of phosphatidylinositol 3-kinase (PI3K) is associated with IFNAR1. The activation of p85 by IFN leads to AKT phosphorylation and expression of the chemokine (C-X-C motif) ligand 11 (CXCL11) gene, encoding an important chemokine67. Type I IFNs also activate p38, and inactivation of p38 blocks induction by IFN-β of CXCL11 and TNFSF10 (encoding tumour...
necrosis factor-related apoptosis-inducing ligand, APO2L/TRAIL) and of CXCL10 (encoding the chemokine IP-10; also known as IFN-γ-induced peptide, 10 kDa) in primary leukocytes.

An important function of the activation of protein serine kinases such as p38 and protein kinase C (PKC) in response to IFN-dependent signalling is phosphorylation, directly or indirectly, of transcription factors. Serine 727 of STAT1 is phosphorylated in response to IFN-γ by the kinase cascade P38K—AKT—PKC—MKK4—p38 (MKK4, mitogen-activated protein kinase kinase 4; also known as MAP2K4), with some variation in the activation of different PKC or MKK proteins in different cells. IFN-dependent activation of PI3K, extracellular response kinases (ERKs) and p38 stimulates the phosphorylation of NFκB (but not IκB), AP-1 and possibly PU.1, respectively. These activated transcription factors may then either drive gene expression independently of activated STATs or cooperate with activated STATs on certain promoters (Fig. 4). Conversely, transcription initiated by phosphorylated STATs does not proceed indefinitely; homeostasis and balance result from the actions of phosphatases such as SHP1 and SHP2 and a family of ISGs, the suppressor of cytokine signalling (SOCS) proteins. SOCS inhibit receptor signalling both by directly inhibiting JAKs and by targeting the receptor complex for proteasomal degradation.

Prior exposure to other cytokines conditions how a cell will respond and, conversely, IFNs condition responses to other cytokines. An excellent example of such an effect is prior exposure of human macrophages to IFN-γ, which changes the response to IL10 from activation of STAT3 to activation of STAT1. Although, as reductionist scientists, we tend to study the responses of cells in culture to treatment with IFNs alone, the situation in vivo is obviously much more complex.

Figure 2 | Different interferon (IFN) signalling pathways activated by dsRNA and viruses. Extracellular double-stranded (ds) RNA or intracellular dsRNA produced during viral replication can activate different signalling pathways triggered by either membrane-bound Toll-like receptor 3 (TLR3) or cytoplasmic retinoic acid-inducible gene I (RIG-I; also known as DDX58) or melanoma differentiation associated protein 5 (MDA5; also known as IFI16). In TLR3 recognizes dsRNA in the lumen of the endosome, which causes phosphorylation of specific tyrosine residues in TLR3 by an unidentified protein tyrosine kinase (PTK). TLR3 dimerizes, binds to CD14 and activates the signalling complex assembled by TLR adaptor molecule 1 (TRIF). Two major pathways bifurcate from TRIF. One, composed of tumour necrosis factor (TNF) receptor-associated factor 3 (TRAF3) and TANK-binding kinase (TBK1/IKKE), leads to TRIF. The other branch acts through TRAF6 and transforming growth factor-β-activated kinase 1 (TAK1; also known as MAP3K7) leading to the activation of nuclear factor-kB (NFκB), JUN and activating transcription factor 2 (ATF2) transcription factors. The activated transcription factors translocate from the cytoplasm to the nucleus, bind to the cognate sites in the promoters of the target genes and singly or in combinations induce their transcription. The cytoplasmic RNA helicases RIG-I and MDA5 recognize dsRNA or 5′ triphosphorylated single-stranded (ss) RNA and use the mitochondrial membrane-bound protein IFN-β-promoter stimulator 1 (IPS1; also known as VISA) as the specific adaptor. IPS1 functions like TRIF and activates the same transcription factors leading to the induction of similar genes. In addition, they cause apoptosis by activating caspases 8 and 10 through the interaction of FADD with IPS1. Solid arrows denote steps that have been fully delineated, stippled arrows show steps that contain as yet unknown intermediaries. AIP3, atrophin-1 interacting protein 3; CS, c-Jun NH2-terminal kinase; DME, different interferon signaling pathways activated by dsRNA and viruses; JUN, c-jun; MDA5, melanoma differentiation associated protein 5; NK, natural killer; NLR, nucleotide-binding oligomerization domain containing 1; p38, mitogen-activated protein kinase 1; RIG-I, retinoic acid-inducible gene I; RIG-I/MDA5 IPS1, different interferon signalling pathways activated by dsRNA and viruses.
ISGs: molecular mechanisms of antiviral action

ISGs are a diverse group of more than 300 genes (which and how many are a function of cell-type signalling variations as discussed above) that mediate the biological and therapeutic effects of IFN stimulation99,100 (TABLE 1). Studies of their mode of action have resulted in fundamental discoveries concerning translational control, regulation of RNA stability and editing, and protein transport and turnover11. Furthermore, proteins that are induced upon IFN stimulation, especially those that can be activated or inhibited in vivo, are targets for high-throughput screening for identification of new modulators of the IFN system.

Examples for this are 2′,5′-oligoadenylate synthetases (OASs) and ribonuclease L (RNASEL), which inhibit a broad range of RNA viruses81. Viral dsRNA can directly activate one of several human OAS proteins to produce a unique 2′-to-5′ linked oligoadenylate of 3–6 bases (2–5A) from ATP82. The only well-established function of 2–5A is activation of the ubiquitous, latent enzyme, RNASEL83. 2–5A binding to RNASEL induces monomeric, inactive RNASEL to dimerize into a potent endoribonuclease that cleaves single-stranded regions of RNA on the 3′ side of UpUp and UpAp dinucleotides84–86. The OAS–RNASEL pathway can inhibit the replication of encephalomyocarditis virus, Coxsackie virus B4, West Nile virus, some retroviruses and HCV87. Furthermore, degradation of cellular mRNA and rRNA by RNASEL damages the host cell machinery that is required for viral replication and can result in apoptosis, contributing to both antiviral and antitumour actions84–89.

RNASEL also cleaves self-RNA into small degradation products that activate the recognition receptors, RIG-I and MDA5, to induce IFN-β, similar to that of non-self viral RNA90, thus perpetuating and amplifying the production of IFN-β. A high-throughput screen has resulted in the identification of small molecules that can activate RNASEL and produce broad-spectrum antiviral effects91.

The dsRNA-activated protein kinase (PKR) and OAS were the first enzymes identified that uniquely respond to IFNs82,83. PKR is a serine/threonine kinase that mediates translational and transcriptional control in response to dsRNA and other signals82–86. In addition, the cellular protein PACT (also known as PRKRA) activates PKR in the absence of dsRNA87. PKR mediates translational control by phosphorylating the protein synthesis initiation factor EIF2α, resulting in an inactive complex between EIF2–GDP and the recycling factor, EIF2B. These events produce global inhibition of protein synthesis that blocks further viral replication and full amplification of the viral-induced cellular stress response. Many viruses, however, evade PKR through a range of strategies such as binding and sequestering dsRNA, thus depriving PKR of its activator or inhibition of its kinase activity88.

Another ISG family that influences translation is the strongly induced p56-related proteins (IFIT gene products). Two of these, p56 and p54, inhibit protein synthesis by blocking the action of the translation initiation factor EIF3 (REF 99). p56 and p54 bind to different subunits

Both STAT1 and STAT3 have activities in addition to their roles as cytokine-activated transcription factors. STAT1 is activated in response to both type I and type II IFNs and STAT3 is activated in response to gp130 cytokines such as IL6. As STAT1 and STAT3 drive essentially opposite biological responses, large signal-dependent changes in their concentrations will affect their relative activation by a further signal. Indeed, an increase in the ratio of STAT1–STAT3 after IFN-α/β treatment of patients with melanoma correlated with survival89. Another consequence of cytokine-dependent increases in STAT expression is that unphosphorylated STAT1 and STAT3 have important functions that are quite distinct from those of the phosphorylated proteins89–92. For example, unphosphorylated STAT3 activates a subset of kB-dependent genes by forming a complex with NFkB93. Thus the role of STATs in signalling after receptor binding has expanded from kinase-activated transcription factors to proteins that, even in the absence of ligand activation, activate transcription and participate in cell-type specificity, resulting in diverse patterns of ISG induction in different cell types in response to a single IFN.

Figure 3 | Receptor activation or ligand–receptor complex assembled by type I, type II or type III interferons. Type I interferons (IFNs) (α, β, ω, κ, ε, δ, ψ, τ, φ) interact with IFN-α receptor 1 (IFNAR1) and IFNAR2; type II IFN-γ with IFN-γ receptor 1 (IFNGR1) and IFNGR2; and type III IFN-λs with IFN-λ receptor 1 (IFNLR1); also known as IL28RA) and interleukin 10 receptor 2 (IL10R2; also known as IL10RB). Type II IFN-γ is an antiparallel homodimer exhibiting a two-fold axis of symmetry. It binds two IFNγR1 receptor chains, assembling a complex that is stabilized by two IFNγR2 chains. These receptors are associated with two kinases from the JAK family: JAK1 and TYK2 for type I and II IFNs; JAK1 and JAK2 for type II IFN. All IFN receptor chains belong to the class 2 helical cytokine receptor family, which is defined by the structure of the extracellular domains of their members: approximately 200 amino acids structured in two subdomains of 100 amino acids (fibronectin type III modules), themselves structured by seven β-strands arranged in a β-sandwich. The 200-amino-acids domain usually contain the ligand binding site. IFNAR2, IFNLR1, IL10R2, IFNGR1 and IFNGR2 are classical representatives of this family, while IFNAR1 is atypical as its extracellular domain is duplicated. GAS, IFN-γ-activated site; IRF9, IFN regulatory factor 9; ISGF3, IFN-stimulated gene factor 3, refers to the STAT1–STAT2–IRF9 complex; ISRE, IFN-stimulated response element; P, phosphate; STAT1/2, signal transducers and activators of transcription 1/2.
IFN activates the Janus kinases (JAKs) that have been primed for the purpose, either by prior treatment with IFNs or through the binding of gp130-linked cytokines. JAKs activate transcription factors of two main types: STATs and IRFs. STATs are activated by phosphorylation, which causes them to dimerize and bind to the IFN-stimulated response elements (ISREs) of the cytokine gene. ISREs are known as GAS. IRFs are activated by phosphorylation, which causes them to dimerize and bind to the IFN regulatory elements (IREs) of the cytokine gene. IREs are also known as GAS. STAT and IRF activation can lead to the expression of a variety of genes, depending on the type of cell and the nature of the stimulus. Additionally, STATs and IRFs can work together to activate genes, leading to the expression of a more complex set of proteins. The ISGs that are expressed in response to IFNs can have a variety of functions, including the inhibition of viral replication, the induction of apoptosis, and the modulation of immune responses. The ISGs are expressed in response to the activation of the JAK-STAT and IRF pathways that occurs in many types of cancer, suggesting an additional role in tumorigenesis.

**Figure 4 | Complexity of the signalling response.** Different types of cells respond differentially to a single type of interferon (IFN) by varying the activation of specific signal transducers and activators of transcription (STATs), additional transcription factors (TFs) and kinases in addition to the Janus kinases (JAKs). Priming of cells by pre-treatment with another cytokine modulates the response further by increasing the amounts of negative regulators and by modulating other processes. Most genes require STATs, with or without additional TFs, and several genes respond only to activated TFs and not to STATs. The STATs bind to IFN-γ-activated site (GAS) elements or, together with IFN regulatory factor (IRF) proteins, to IFN-stimulated response elements (ISREs), and the TFs bind to specific binding elements (TFBE). CIS, cytokine inducible SH2-containing protein; PTP, protein tyrosine phosphatase; SOCS1, suppressor of cytokine signalling 1.
probable or confirmed antiviral activities include the guanylate-binding protein 1 (GBP1); a 3′,5′-exonuclease encoded by ISG20; the promyelocytic leukemia protein (PML); adenosine deaminase (ADAR1); the endoplasmic reticulum-associated protein Viperin (cig5) that can inhibit human cytomegalovirus; inducible nitric oxide synthase (iNOS); and the nucleoporins Nup98 and Nup96 (REFS 121–128).

Finally, many IFN-pathway signalling proteins are themselves ISGs, thus providing an autocrine loop that amplifies IFN responses; examples are IRF7, RIG-I, MDA5 and STAT1. As ISGs with high levels of transcriptional induction are still poorly characterized functionally, some could prove to be critical mediators of antiviral and other actions. Because all biological effects of IFNs are mediated through the action of ISGs (TABLE 1), further research into understanding the functions of the protein products of these may lead to more efficacious antiviral and other therapeutics.

### Innate and adaptive immunity

In addition to direct inhibition of viral replication by ISGs, a second level of IFN action augments adaptive and acquired immune responses. Early warning of pathogen presence is delivered by tissue-associated and circulating dendritic cells, one type of which, the plasmacytoid dendritic cell, is the circulating type I IFN-producing cell. In addition to TLR activation on cells at the sites of pathogen invasion or replication, this response culminates with dendritic cell-mediated presentation to CD4+ T cells of pathogen-derived peptide fragments that are bound to surface major histocompatibility complex (MHC) class II molecules. MHC class II proteins are selectively upregulated by IFN-γ, whereas type I IFNs fail to do so owing to the STAT2-dependent induction of SOCS1 (REF. 130). Infected cells that display peptide fragments associated with MHC class I molecules on the surface are recognized and subsequently eliminated by CD8+ T cells, thereby clearing the virus. Either type I IFNs or IFN-γ can mark dendritic cells for destruction by IFNγR1- and IFNγR2-deficient mice (REF. 131).

IFNs also promote accumulation of leukocytes at sites of pathogen invasion; specifically, IFNs (along with cytokines such as TNFα and IL1β), strongly promote the expression of vascular adhesion molecules including intracellular adhesion molecule 1 (ICAM1). Furthermore, IFNs induce the production of chemotactic
cytokines (chemokines), which participate in leukocyte recruitment. As examples, three closely related chemokines involved in accumulation of activated T cells and macrophages are the ISGs CXCL9 (also known as MIG, monokine induced by IFN-γ); CXCL10 (also known as IP-10, IFN-γ 10 kD inducible protein); and CXCL11 (also known as I-TAC, interferon-inducible T-cell α-chemoattractant)136-139. True to their names, these chemokines are not expressed in the absence of IFN signalling.

In the development of an adaptive immune response, IFN-γ is produced by an early warning NK cell, or by activated T cells140. IFN-γ governs expression of class II transactivator (CIITA), a master regulator of transcription of the MHC class II molecules themselves, as well as the associated invariant chain, which helps stabilize MHC class II heterodimers. HLA-DM catalyses the displacement of the invariant chain from the MHC class II peptide binding site as the mature MHC class II-peptide conjugate is finalized for insertion into the plasma membrane141-144. Finally, of substantial importance in the host response to the virus is the IFN-mediated activation of cytotoxic effector function among cells of innate and adaptive immunity including NK cells, dendritic cells, macrophages and T cells. Indeed, the property of stimulating macrophages contributed substantially to the recognition of IFN-γ as a biologically important lymphokine and as a ‘different’ IFN156.

**Human therapeutic applications**

Based on preclinical studies of broad spectrum inhibition of virus replication, IFNs were initially investigated as antivirals with activity against RNA and DNA viruses. Clinical effectiveness for both has now been established. But development of relatively specific, low molecular mass antivirals has largely supplanted broad application except for HBV and HCV chronic infections. The first US Food and Drug Administration (FDA) approval for IFN-α2, however, was not for virus infection but for cancer, which was driven by interest created by publicity resulting from its effectiveness in American Cancer Society trials. Subsequently, placebo-controlled randomized trials established the effectiveness of IFN-β for relapsing, remitting MS — an apparent paradox in terms of the mechanistic understanding of IFN actions, as IFNs, as discussed above, are generally immune augmenting rather than immunosuppressive. Presently, a number of drugs are being or have been designed to target different components of the IFN system for different therapeutic indications (FIG. 5).

** Viruses.** The recognition that HBV often caused a chronic infection leading to cirrhosis and hepatocellular carcinoma suggested that infected patients might benefit from IFNs151. Initial clinical trials of impure IFN-α2 suggested benefit but studies with impure IFN-β were less promising153. These low-dose studies were followed, however, by higher doses of recombinant IFNs, when they became available, which then confirmed beneficial effects154. HBV chronic infection evolves with hepatitis e antigen (HBeAg)-positive quiescent viruses escaping inhibition during conversion of an immunotolerant to an immunoactive phase, with enhanced immune elimination of infected hepatocytes155,156. In many, this immune response causes suppression of viral replication and HBeAg loss. A quiescent phase or ‘healthy carriage’ may ensue but disease reactivation is common (HBeAg-negative disease157). In HBeAg-positive early HBV infection, IFNs have not been particularly effective. However, in the immunoactive chronic phase, HBV is sensitive to IFN-α2 and the ongoing immune response is augmented, leading to quiescent HBeAg-negative disease in up to 40% of patients. IFN-α2 (Roferon-A, Hoffmann-LaRoche; Intron-A, Schering–Plough), now usually in the form of a long-acting pegylated version, has been widely used to treat HBeAg-positive HBV infections158. IFN-α2 has also been used in the HBeAg-negative disease that develops when viral mutations permit viral reactivation following HBeAg loss. IFNs reduce viraemia (usually by over 90%) and induce host responses, but drug withdrawal often leads to disease recurrence; however, a proportion of patients (approximately 10–15%) have a prolonged period of viral suppression159. Therapy for chronic HBV infection illustrates the two complementary activities of IFNs: in HBeAg-positive disease IFN increases an immune response, whereas in HBeAg-negative disease IFNs act as direct antivirals.

In the late 1980s ‘non-A, non-B hepatitis’ or ‘post-transfusion hepatitis’ was effectively treated with IFNs160. Subsequent studies identified the causative agent as HCV161. Initial clinical studies of IFN-α2 resulted in sustained and curative virological responses in up to 20% of patients162. Response rates to monotherapy with IFN-α2 for chronic HCV infection were transformed in the mid-1990s by combined use with the weak antiviral ribavirin — over 40% of patients responded163. These results mimic studies for HSV keratitis in which therapy with a combination of IFNs and a weak antiviral agent (acyclovir) were synergistic164. Therapy for chronic HCV infection has now evolved and current regimes commonly use a long-acting pegylated IFN-α2 plus ribavirin with cure of up to 60% of patients. Possibly as a result of selection against RNASEL cleavage sites in its genome, or inhibition of PKR, effectiveness is much less for genotype 1 than for genotypes 2 and 3 of HCV162,164,166.

In addition to HBV and HCV infections, other chronic viral infections have been effectively treated. Both systemic and topical IFN-α and IFN-β have reduced virus titres and decreased clinical manifestations of herpes zoster, HSV and cytomegalovirus infections165-169. Almost simultaneous introduction of acyclovir and its analogues, however, which proved to have greater clinical efficacy and reduced side effects, ended clinical development of IFNs for these indications. Papilloma virus infections of skin, larynx and genitals were found to respond with regression of warts upon either intralesional or systemic administration of IFN-α and IFN-β170-175. When compared with placebo, useful therapeutic effects resulted for patients with extensive or refractory disease, but permanent eradication was infrequent. These studies did, however, establish a basis for the use of the TLR7 IFN-inducing agonist imiquimod topically for genital warts with decreases in HPV DNA and with complete
Multiple sclerosis (MS). In about 85% of patients with MS, an inflammatory demyelinating disorder of the central nervous system, disease begins with approximately annual episodes of transient neurological dysfunction (relapsing–remitting MS or RR-MS). Initial studies of IFNs in the 1970s followed tissue-culture studies suggesting that cells from MS patients secreted less IFN-like activity following viral induction than did controls. These findings, combined with a notion that a slow or chronic viral infection might be causative, resulted in the evaluation of using an intrathecal, impure IFN-β as therapy that identified a reduction in relapses,189; however, subsequent clinical trials were either inconclusive (IFN-α2 or detrimental (IFN-γ)190–192. But in 1993, recombinant IFN-β, given subcutaneously in a randomized placebo-controlled trial for RR-MS reduced relapses by about a third and resulted in marked reductions in subclinical disease, as assessed by magnetic resonance imaging (MRI)193,194. This report ushered in the modern age of MS therapeutics: by showing that the natural history of MS could be modified; by documenting that IFN-β was clinically beneficial; and by the demonstration of MRI lesions as a useful surrogate of clinical effectiveness, now widely used in MS drug development. It is now common clinical practice to initiate IFN-β (Betaseron/Betaserom, Bayer Schering/Chiron; Avonex, Biogen Idec; Rebif, Merck Serono (or medications of comparable efficacy)) at the time of diagnosis.195–197. Attacks decrease by about 30%, numbers of new and active MRI lesions (which reflect inflammation) are often reduced as soon as 1-month after initiation, and long-term clinical benefits are now considered plausible.198. Although it has unequivocally represented a breakthrough, improvements on IFN-β are needed as it is only partially effective and is expensive for the life-long, non-curative use.195–197.

Pathogenesis of MS remains unknown but evidence implicates genetic–environmental interactions with critical timing of exposures to initiating factors. Epidemiological studies highlight Epstein–Barr virus and low plasma levels of vitamin D, and genetic studies implicate several polymorphic variants of immune-response genes.198–200. The most obvious clinical outcome from IFN-β is a reduction in MRI lesions200–204, and protein products of ISGs probably mediate these effects.

As one example, an IFN-β ISG product, CD69, forms an inhibitory association with a sphingosine 1-phosphate receptor (SIPR). The consequence in vivo is suppressed lymphocyte exit from lymph nodes and restriction in numbers of circulating lymphocytes available to cross the blood–brain barrier.205 Reduction in expression of matrix metalloproteinase 9 (MMP9) in activated lymphocytes and increased soluble vascular cell adhesion molecule (sVCAM) levels in plasma have also been identified and assigned putative roles in the beneficial effects of IFN-β for patients with MS.206,207. Expression-array studies and candidate gene evaluations have been applied, without
success, in attempts to identify molecular biomarkers of the therapeutic effect of IFN-β in MS208. Development of validated outcome measures for treatment success (and failure) will aid in this process209.

Cancer. Based on the reduction in disease morbidity210,211, initial regulatory approvals for the marketing of IFN-α2 for a chronic B (hairy) cell leukaemia occurred within 5 years from clinical introduction as a result of close collaboration between academic institutions, government and industry. In hairy cell leukaemia and chronic myelogenous leukemia (CML), IFN-α2 decreased marrow infiltration with malignant cells and normalized peripheral haematological parameters210,212–214. In CML, in addition to reductions in leukaemic cell mass, a decrease resulted in cells with the abnormal, activated BCR–ABL kinase212–214. Over 90% of patients with CML with complete cytogenetic response were in remission at 10 years213. However, the survival advantage for IFN-α2, when compared with chemotherapy for CML, has now been exceeded by the even greater effectiveness of the targeted inhibitor of the activated BCR–ABL kinase, such as imatinib (Gleevec; Novartis) and now other newer tyrosine kinase inhibitors.

In addition to hairy cell leukaemia and CML, therapeutic effectiveness of IFN-α2 in causing at least partial disease regression has been identified in more than a dozen other malignancies including myeloma, lymphomas, melanoma, renal cell and bladder carcinoma, and Kaposi’s sarcoma215. For example, in lymphomas of various histologies and of both B-cell and T-cell phenotypes, IFN-α2 has been effective in inducing tumour regressions in almost half of the patients involved in the study, and even in patients previously treated with chemotherapy215,216. Prolonged disease-free and overall survival in intermediate prognosis lymphomas has resulted from IFN-α2 in combination with chemotherapy, even given for limited periods, in randomized multicenter trials215,216. International Phase III trials have been conducted with survival impact confirmed in metastatic renal carcinoma, but like in CML, the orally active, targeted tyrosine kinase inhibitors have changed the natural history of renal carcinoma, extending survival in metastatic disease more than the injectable IFN-α2.

Cure of metastatic malignancies can result when micrometastases are eliminated in patients at highest risk for recurrence after surgical removal of a primary tumour. Effectiveness as a surgical adjuvant for murine tumours provided the rationale leading to pioneering clinical studies that suggested benefit of impure IFN-α when given after surgery for osteosarcoma217,218. This surgical adjuvant approach was the basis for evaluation of IFN-α2 in patients at high risk for recurrence of melanoma. Initial beneficial effects of significant prolongation of disease-free survival have now largely been validated by combined analyses of multi-institutional trials, by subsequent studies that have included evaluation of pegylated IFN-α2 and by meta-analyses219–221. Like other potent physiological mediators such as glucocorticoids, IFNs have toxicities when administered with pharmacological intent222,223. These have been dose related and particularly difficult at the high dose used for melanoma. With the initial dose, malaise, fever and chills, which last for a few hours, dominate but tachyphylaxis occurs with subsequent injections. Fatigue and anorexia, the aetiology of which is not understood, are often dose-limiting with chronic administration for cancer or MS; at higher doses weight loss occurs and may be significant (>10%). Reversible elevation of hepatic transaminases may occur, as may haematological effects, most markedly granulocytopenia.

Like in MS, failure to fully understand the mechanism(s) of antitumour action has slowed further development. Suppression, mutation and polymorphisms of IFNs and their signalling mechanisms in and by malignant cells are emerging as important contributors to cancer development224–226. Mutations in RNASEL have been associated with prostate carcinoma and with presence of the retrovirus XMRV235,273–275. Epigenetic and genetic silencing of IFN-signalling or ISG expression may also influence tumour development216. Reversal of these effects are likely to be the basis for effectiveness of IFNs and/or inducers in murine carcinogen-induced tumours, and may contribute to effectiveness in advanced disease, and provides a rational for developing TLR agonists for...
chemoprevention\textsuperscript{237–239}. Indeed, TLR agonists appear to be effective and are already establishing a role in treatment of malignancy with the proven effectiveness of the TLR7 agonist imiquimod used topically for basal cell carcinomas as an example. Furthermore, relative clinical safety has been established for phosphorothioate oligoribonucleotide agonists for TLR9.

Induction of apoptosis by the ISGs products APO2L/ TRAIL and Fas has been identified in many malignant cell types, as has induction of APO2L/TRAIL on immune effector cell surfaces, thus sensitizing tumour cells to T-cell, NK cell and macrophage-mediated cytotoxicity\textsuperscript{240–243} (TABLE 1). Intranasal administration of IFN-α to basal cell carcinomas increased Fas expression and correlated with regression\textsuperscript{44}. IFN-γ has increased susceptibility to apoptosis by Fas activators and cytotoxic chemotherapies in many cell types including melanoma and colorectal carcinoma\textsuperscript{245,246}. Through interactions with p53 and the inhibitor of apoptosis, XIAP, the ISG product XIAP may allow APO2L/TRAIL to fully activate downstream caspases\textsuperscript{45,248}. In addition, the ISG product IRF1 can suppress another anti-apoptotic protein, Survivin\textsuperscript{249}.

Antitumour activity in vivo may also be mediated by augmented lytic activity of immune effector cells and by enhanced immunogenicity of tumour cells. Both T-cell and NK-cell trafficking, expansion and lytic activity can be promoted by IFNs and ISGs; furthermore, IFN-γ is secreted from these activated cells into the tumour microenvironment\textsuperscript{250–253}. In addition to stimulating immune effector cells, IFNs have critical roles in antigen processing and presentation, as discussed above, both by T cells and dendritic cells\textsuperscript{256}. In addition, IFN-γ can upregulate the tumour-associated antigens, carcinomembryonic antigen and TAG72, both in vitro and in vivo\textsuperscript{257}.

IFNs can also inhibit angiogenesis by altering the stimuli from tumour cells and by directly inhibiting endothelial cells — indeed, they were the first angiogenic inhibitor identified\textsuperscript{258}. Endothelial cells are inhibited in motility\textsuperscript{259} and undergo coagulation necrosis in vitro and inhibition of angiogenesis occurs in vivo within 24 hours of tumour cell inoculation\textsuperscript{250–261}. Suppression of basic fibroblast growth factor (bFGF; also known as FGF2) correlated with reduced vascularization and tumour growth\textsuperscript{262,263}. IFNs also inhibit vascular endothelial growth factor (VEGF) mRNA and protein expression by regulating its promoter\textsuperscript{264}. IL8, a mediator of angiogenesis, was inhibited in vitro and in vivo by IFN-α2b and IFN-β; other angiogenesis inhibitory members of the chemokine family, CXCL9, CXCL10 and CXCL11, are ISGs\textsuperscript{265–267}.

In endothelial cells, the ISG product guanylate binding protein 1, interferon-inducible, 67 kDa (GBP1), functioned as an inflammatory response factor inhibiting endothelial cell proliferation and angiogenesis in part through MMPs\textsuperscript{268}. Clinically, IFN-α2 has proved effective in the treatment of infantile haemangiomas, haemangiolastomas, giant cell tumour of the mandible and Kaposi’s sarcoma\textsuperscript{215,269}. Thus, induction of ISGs that function as angiostatic inhibitors, coupled with secondary downregulation of angiogenic factors, may contribute to antitumour effects\textsuperscript{269}.

**Perspective**

IFNs provide fundamental cellular defence mechanisms against viral infections and cancer and are thus critically important to the health of animals and humans. Because of their clinical effectiveness in limiting virus replication, reducing tumour cell mass, controlling disease symptoms and prolonging survival, IFNs are now licensed worldwide for the treatment of various viral, malignant and immune disorders; market sales approach US$4 billion. As part of the innate immune response, IFNs are not only a principal cytokine that blocks viral replication through the action of specific ISGs, but also (particularly IFN-γ) mediate critical elements of the cellular immune response for recurring bacterial infections in chronic granulomatous disease and for mycobacteria. Because all biological effects of IFNs are mediated through the action of ISGs, understanding the functions of these genes may lead to more efficacious anticancer and antiviral therapeutics. For example, certain IFN-regulated proteins, such as OAS, RNAseL and PKR, exist in either latent inactive or active states, which could be targeted for potent antitumour and/or antiviral effects (FIG. 5).

IFNs have therefore more than reached the effectiveness anticipated by early virologists: they are not only an antiviral with a spectrum of clinical effectiveness against both RNA and DNA viruses, but have been the prototypical biological response modifiers for oncology, and have proved to have effectiveness in suppressing manifestations of MS. The study of IFNs has resulted in fundamental insights into cellular signalling mechanisms and innate and acquired immunity. In addition, their therapeutic use has improved the quality and quantity of life for millions of patients worldwide. However, to fully realize their potential, many questions remain unanswered (BOX 1). As exemplified by recent publications\textsuperscript{267–269}, further investigations will only enable IFNs to have even greater impacts in biomedicine.

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Competing interests statement
The authors declare competing financial interests: see web version for details.

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