Review Article

Signal transduction in the type I interferon system and viral countermeasures

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Type I interferons (IFN) including IFNα/β are cytokines of the immune system with critical functions in innate and adaptive immune response. Secreted IFN acts via JAK/STAT signaling pathways to direct a huge gene expression program, including antiviral, apoptotic, survival and immune genes. Only recently, the molecular patterns and their receptors as well as the connected signaling pathways leading to transcriptional activation of IFN genes have been elucidated. Ubiquitous cytosolic RNA helicases like RIG-I which sense intracellular triphosphate RNAs and activate the IFN-controlling transcription factors IRF3 and IRF7 seem to play a major role in antiviral defense and immunity. Recognition of extracellular nucleic acids by a subset of Toll-like receptors in addition contributes to a generalized host IFN response. During co-evolution with the host, viruses have learned to counteract every piece of the IFN network. Learning from viruses how to target the IFN system may lead us to novel strategies for therapeutic intervention.

Keywords: innate immunity / interferon / IRF / TLR / RIG-I

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Introduction

The type I interferon (IFN) system, including a single IFNβ and a dozen IFNα variants, is an indispensable part of the immune system of all vertebrates, probably invented in response to viruses. Initially identified due to their immediate antiviral activity, IFNs are today recognized as multifunctional cytokines with pleiotropic activities in innate host cell defense and in coordinating adaptive immunity. IFN is involved in activating subsets of immune cells like NK, CTL and DC, which helps to eliminate pathogens or tumors, but which may also play a role in the establishment and maintenance of autoimmune disorders. IFN must therefore be under tight control of the host and expressed only in response to an immediate threat, like invading pathogens.

Innate immune recognition of pathogens is based on receptors for conserved, invariant structures, or molecular patterns [1]. Viruses are made in cells and lack an own metabolism. Nucleic acids are therefore the prime candidate patterns for recognition. Indeed, nucleic acids represent the main class of IFN inducers, as was appreciated

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Abbreviations: **BVDV**, bovine viral diarrhea virus; **CARD**, caspase activation and recruitment domain; **CBP**, cAMP-responsive-element-binding protein (CREB)-binding protein; **CSFV**, classical swine fever virus; **EBV**, Epstein-Barr virus; **HAV**, hepatitis A virus; **HCV**, hepatitis C virus; **HSV-1**, herpes simplex virus 1; **IFN**, Interferon, here: type I IFN; **IFNAR**, interferon alpha receptor; **IKKα/-β/-i(ε)**, IκB kinases; **IPS-1**, interferon-beta promoter stimulator 1; **IRAK**, IL-1 receptor associated kinase; **ISG**, interferon stimulated gene; **ISGF3**, IFN-stimulated gene factor 3; **ISRE**, interferon stimulated response element; **IRF**, interferon regulatory factor; **JAK**, Janus kinase; **MDA5**, melanoma differentiation-associated gene 5 product; **MyD88**, myeloid differentiation primary response protein 88; **NF-κB**, nuclear factor kappa-B; **NS/nsp**, non-structural protein; **2′-5′OAS**, 2′-5′oligoadenylate synthetase; **pDC**, plasmacytoid dendritic cell; **PIN**, ; **PKR**, RNA-activated protein kinase; **RIG-I**, retinoic acid inducible gene-I; **RSV**, respiratory syncytial virus; **SARS**, severe acute respiratory syndrome; **SOCS**, suppressor of cytokine signaling; **STAT**, signal transducer and activator of transcription; **TBK1**, TANK-binding kinase 1; **TIR**, Toll/IL-1 receptor (domain); **TRAF**, TNF Receptor-Associated Factor; **TRIF**, Toll/IL-1R domain-containing adaptor inducing IFNβ; **TRAM**, TRIF-related adaptor molecule; **TLR**, Toll-like receptor.

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early. Nevertheless, the first nucleic acid receptors able to trigger IFN induction were identified only during the past few years. These belong to two groups of protein families, the transmembrane Toll-like receptors (TLR), and the cytoplasmic RIG-I-like receptors (RLR). Members of the TLR9 subfamily, TLR3, -7, -8, and -9 sense nucleic acids approaching the cell from outside whereas the cytoplasmic RLRs identify dangerous RNA that has made it into the cytoplasm. Stimulation of these receptors leads to activation of the transcription factor kappa-B (NF-κB) and expression of inflammatory cytokines. In addition, they activate transcription factors of the interferon regulatory factor (IRF) family, IRF3 and IRF7, which control transcription of the early IFNβ and late IFNα genes, respectively. The activation of IRF3 and IRF7 by non-canonical members of the inhibitor of kappa B kinase (IKK) family of Ser/Thr kinases, TANK-binding kinase 1 (TBK1) and IKKi (IKKε), appears to be a central key event for IFN production in all body cells, with a remarkable exception in plasmacytoid dendritic cells (pDC). In a second round of receptor-mediated signal transduction, the secreted IFNs activate giant gene expression programs by the canonical Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling. This causes tremendous pleiotropic effects in all body cells, including a strong positive feedback to nucleic acid sensing.

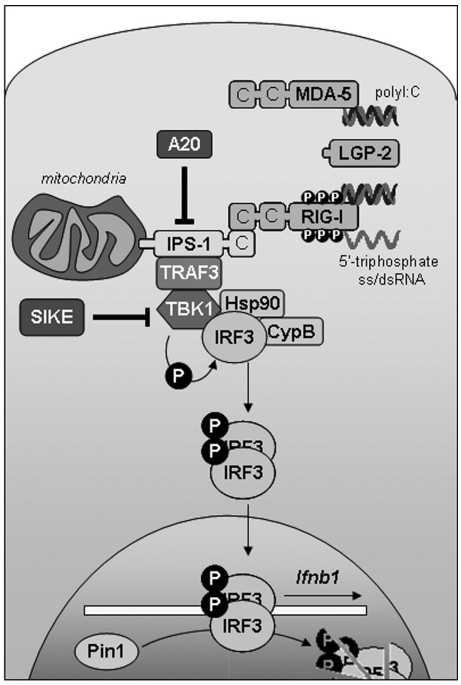

Figure 1. RIG-I mediated induction of type I IFN. The cytoplasmic RNA helicase RIG-I bound to viral triphosphate RNAs is recruited via CARD domain (C) interaction to the adaptor protein IPS-1, localized in the outer mitochondrial membrane. In a complex containing TRAF3 and chaperones Hsp90 and CypB, the kinase TBK1 phosphorylates the transcription factor IRF3 at C-terminal residues. Phospho-IRF3 forms homodimers that translocate to the nucleus and bind to the PRDIII-I enhancer region of the *Ifnb1* gene, driving the mRNA transcription. The nuclear protein Pin1 targets IRF3 to proteasomal degradation. A20 and SIKE are cellular inhibitors of the IFN induction pathway. The helicase LGP-2 that lacks CARD domains probably interferes with RIG-I and MDA-5 function (see text for details).

Cytoplasmic receptors for RNA

Viral dsRNA, and synthetic dsRNA analogs like poly(I:C), are long known to play a major role both in the induction of IFN [2] and in stimulating antiviral responses [3, 4]. The Ser/Thr protein kinase R (PKR) is activated by dsRNA. In addition to its direct antiviral activity, PKR can activate NF-κB, but not IRF3 or IRF7, and is therefore synergistic in IFNβ induction, but it is not sufficient. The first cytoplasmic receptors able to activate IRF3 and IFN induction have been described only recently. Two related DExD/H box RNA helicases, retinoic acid inducible gene I protein (RIG-I) and the melanoma differentiation-associated gene product (MDA-5) were found essential for IFN production in response to RNA virus infection [5–7] (Fig. 1). RIG-I encodes a protein of 925 amino acids with an N-terminal region containing two caspase activation and recruitment domains (CARD) and a C-terminal region with ATP-dependent RNA helicase activity. MDA-5 has a similar structure, containing two CARD domains and a C-terminal RNA helicase domain. CARD domains may interact with CARDs from other proteins and are platforms for nucleating signaling events. Both the CARD domains and ATPase activity were required for IFN induction in virus-infected cells, suggesting that not only binding to viral RNA but some processing of virus RNA is necessary for downstream signaling. A third member of the RIG-I-like receptor (RLR) family, LGP2, binds dsRNA but lacks a CARD domain. LGP2 may compete with RIG-I or MDA-5 for RNA binding [8, 5] and may exert additional inhibitory activities [9].

Intriguingly, RIG-I and MDA-5 respond to different RNA virus types, although both can bind dsRNA. Whereas RIG-I recognizes a variety of RNA viruses, including negative strand RNA viruses which produce undetectable levels of dsRNA [10] and positive strand RNA viruses, MDA-5-dependent activation of IFN gene expression has been observed so far only in cells infected with a picornavirus, encephalomyocarditis virus (EMCV) [11–13]. Differential activation was also suggested by RNA transfection experiments. RIG-I was activated by *in vitro*-transcribed RNAs, but not by the dsRNA analog poly(I:C), whereas

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MDA-5 did respond to poly(I:C) [11–15]. A study with IFN-inducing 21–27 nt long siRNA-like dsRNAs suggested that blunt dsRNAs are unwound efficiently by the RIG-I helicase activity, whereas those with a 3′-terminal 2nt overhang, as are found in Dicer-products, disturb unwinding and activation [16].

5′ triphosphate RNA is the molecular ligand for RIG-I activation

Besides dsRNA and poly(I:C), RNAs transcribed *in vitro* by the RNA polymerase of bacteriophages T7, T3, and Sp6 were recently found to trigger IFN expression [17]. Like T7 phage, many mammalian RNA viruses initiate viral RNA synthesis in a primer-independent manner, resulting in the presence of a triphosphate moiety at the 5′ end of RNAs. Notably, these include the paramyxoviruses and rhabdoviruses that are recognized by RIG-I, whereas picornaviruses like EMCV which are sensed by MDA-5, produce RNAs that lack a 5′ triphosphate and instead have a protein (Vpg) linked covalently to the 5′ terminus.

The identity of 5′ triphosphate RNA as the first, molecularly defined ligand for RIG-I was confirmed recently [18, 19]. IFN induction in rabies virus-infected cells was found to depend on RIG-I and on the presence of viral triphosphate RNA as a specific RIG-I ligand. RNA from rabies virus-infected cells and from purified rabies virus particles led to induction of IFN after transfection into cells. This activity was completely lost, when the 5′-triphosphate of the RNA was removed or replaced with a 5′-cap structure. Moreover, RIG-I directly bound not only dsRNA but also ssRNA oligonucleotides carrying a 5′-terminal triphosphate. IFN induction in response to triphosphate RNAs was independent of TLR recognition [20] and was abolished in RIG-I knock-out MEFs [18]. As confirmed in independent work, single-strand influenza virus triphosphate RNAs are also recognized by RIG-I [19]. These authors could also show that the viral NS1 protein colocalizes in complexes containing RIG-I and viral triphosphate RNAs, suggesting that NS1 does not only bind to dsRNA but also to viral triphosphate ssRNA, thereby acting as an antagonist of RIG-I. These findings fully support the previous observation that phage-transcribed ssRNA can induce IFN [17] and demonstrate that uncapped 5′-triphosphate RNA present in viruses serves as the molecular signature for the detection of viral infection via RIG-I. Triphosphate-containing RNAs are usually not present or accessible in healthy cells. Although cellular RNA polymerases do initiate transcription with triphosphate nucleotides, these are posttranscriptionally modified or removed in the nucleus. Triphosphate RNA in the cytoplasm of cells is therefore a pattern signature for "non-self" or dangerous [18].

An adapter for RIG-like-receptors

A direct and common downstream adapter to the CARD domains of active RIG-I and of MDA-5 required for activation of IFN and of NF-κB-controlled cytokines has recently been identified and was dubbed IFNβ promoter stimulator 1 (IPS-1) [21], mitochondrial antiviral signaling protein (MAVS) [22], virus-induced signaling adaptor (VISA) [23], and CARD adapter inducing IFNβ (Cardif) [24]. Intriguingly, IPS-1 is a transmembrane protein located to the outer mitochondrial membrane (Fig. 1). Its function in relaying signals to IFN induction is destroyed by targeting the protein to the plasma or ER membranes, suggesting that the mitochondrial localization is of functional relevance [22, 25]. IPS-1 contains a single N-terminal CARD domain, and a C-terminal region which interacts with TRAF6, FADD and RIP1 which are involved in NF-κB signaling [21, 23]. Details of how IPS-1-mediated downstream activation of the IRF kinases TBK1 and IKKi can occur, await clarification. However, TRAF3 is required [26] which is also involved in relaying signals of the TIR adapter TRIF to activation of IFN (see below), suggesting an important node function of this protein in IFN induction [27-29]. A negative regulator of the RIG-I-IPS-1-TBK1 activation cascade is A20, an NF-κB-inducible ubiquitin-editing protein [30, 31] whose C-terminal zinc-finger motifs are required for IRF3 and NF-κB inhibition.

Cytosolic receptors for DNA

Whereas cytoplasmic recognition of dsRNA has been appreciated for years, the existence of intracellular systems sensing DNA and responding with IFN production was corroborated only recently. TLR-independent induction of IFN was observed in response to transfected B-DNA [32], or to DNA escaped from apoptotic degradation [33] and required IRF3 [34]. Previously, TLR- and NOD-independent IFN induction by intracellular bacteria like *Listeria monocytogenes* has been observed [35]. The analysis of IPS-1$^{-/-}$ mice finally revealed that IPS-1 is not required for IFN induction in response to transfected DNA, infection with *Listeria,* or the dsDNA vaccinia virus [14, 15]. These observations strongly suggest a so far not appreciated distinct IFN inducing pathway which may have evolved to appropriately respond to DNA virus.

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Transcriptional activation of IFN genes: Activation of IRFs by phosphorylation

Transcription of the IFNα/β genes is primarily controlled by proteins of the IFN regulatory factor (IRF) family, in particular the latent IRF3, and the IFN-inducible IRF7 (for a detailed review see [36]). In most body cells, activation of IRF3 triggers expression of a small subset of IFN genes, in particular IFNβ. Transcription is promoted by an enhanceosome containing in addition the activated transcription factors, NF-κB and AP-1 (ATF-2-c-Jun)[37]. The secreted early IFN stimulates the synthesis of IRF7, which controls transcription of many additional members of the IFNα gene family.

Activation of the transcription factors IRF3 and IRF7 involves phosphorylation [38] by Ser/Thr kinases of the IKK family. Two non-canonical IKKs, TBK1 and IKKi which is expressed in hematopoietic cells after IFN stimulation, phosphorylate C-terminal Ser clusters of IRF3 and IRF7 [39, 40]. This is followed by IRF dimerization, import into the nucleus, and formation of the enhanceosome to initiate IFN mRNA synthesis. The major role in the initial activation of IRF3 is attributed to the ubiquitous and constitutively expressed kinase TBK1. Transcriptional activation of IRF3-controlled genes, including IFNβ, is abrogated in TBK1 deficient mice in response to Sendai virus infection [41].

In cells not activated by TLR or RLR ligands, TBK1 and IKKi are bound to a protein called suppressor of IKKε (SIKE) that prevents their association with IRF3 [42]. In contrast, a member of the peptidyl-prolyl isomerase proteins, cyclophilin B (CypB), and its interaction with latent IRF3, was found required for phosphorylation and dimerization of IRF3 and IFNβ production. [43]. Another molecular chaperone, Hsp90, is part of the signaling complex leading to IFN induction (Fig. 1). Only upon viral infection, IRF3 dissociated from an IRF3-Hsp90-TBK1 complex to drive transcription of IFN mRNA. It seems that Hsp90 stabilizes TBK1 preventing its proteasome mediated degradation [44]. Stability of IRF and TBK1 seems to play an important role for IFN expression from a regulatory point of view. The half-life of the IFN-induced IRF7 controlling expression of the IFNα genes, is only 0.5–1 hour [45]. A nuclear peptidyl-prolyl isomerase, PIN1, appears to be involved in limiting IFN transcription. Via a WW domain, PIN1 binds to phosphorylated IRFs and causes their proteasomal degradation [46]. PIN1 knock-out mice show delayed IRF3 turnover and increased IFN production in stimulated cells. A positive feedback on IRF stability by IFN signaling was suggested by the observation that IRF3 is stabilized by conjugation with ISG15 [47].

Toll-like receptor pathways inducing IFN

TLRs are single-span transmembrane proteins located at the cell surface or endosomal membranes of cells and act as sensors for microbial patterns. Upon ligation of extracellular ligands, TLR signals are transmitted via adapters that associate with the intracellular Toll-Interleukin receptor (TIR) domains of TLRs (Fig. 2). This leads to activation of NF-κB and expression of inflammatory cytokines, a general feature of TLR signaling [48]. Of the twelve mammalian TLRs identified, five TLRs can in addition activate IRFs and IFN induction, and four of these recognize nucleic acids. TLR3 binds dsRNA and poly(I:C) [49], TLR7 and 8 recognize ssRNA [50], and TLR9 binds hypomethylated CpG DNA [51]. These TLRs are located in endosomal membranes [52]. A remarkable exception is the cell surface TLR4, as it can mediate IFN induction in response to a non-nucleic acid ligand, namely bacterial lipopolysaccharide (LPS). Activation of TLR4 to induce IFN involves a co-receptor, CD14, and binding of a complex of bacterial LPS with the MD-2 protein (for review see [53]). It must be noted that, in contrast to the ubiquitous RIG-I, the expression of TLRs is cell-type dependent. While TLR3 and TLR4 are expressed on many cell types including epithelial cells, fibroblasts and monocytes, TLR7, 8 and 9 are primarily expressed on myeloid DC (mDCs) and pDC [54]. pDC are known as the major producers of IFNα in humans. In contrast to other cells, pDC express high levels of IRF7 which otherwise is induced only after IFN JAK/STAT signaling by "early" IFNs, in particular IFNβ [55–57].

As for RIG-I-mediated IFN induction, stimulation of TLR3 and TLR4 leads to the "classical" phosphorylation and activation of IRF3 and IRF7 by TBK1 and IKKi. This critically involves the TIR adapter TRIF [58] also known as TICAM1 [59], and is independent of IPS-1. TRIF binds to the TLR3 TIR directly, and to the TLR4 TIR via another TIR adaptor, TRAM [60, 61]. TRIF associates with TBK1 and IRF3. Similar to RIG-I/IPS-1-signaling, TRAF3 is required, suggesting an important role of this molecule at the merge of different upstream pathways [27, 28].

Notably, a completely different mode of IFN activation is observed for TLR7-9 (Fig. 2). This pathway is based on other adapters, other kinases and results in activation of exclusively IRF7. It plays a major role in pDC which express latent IRF7 and which therefore can produce the entire IFNα repertoire immediately upon stimulation of TLR7 and TLR9. This pathway is dependent on the TLR adapter MyD88 which otherwise is engaged in NF-κB activation [62, 63]. IRF7 activation requires a complex including MyD88, IRF7, and TRAF6 [63]. Three kinases, IRAK4, IRAK1, and IKKα, appear to be crucially involved.

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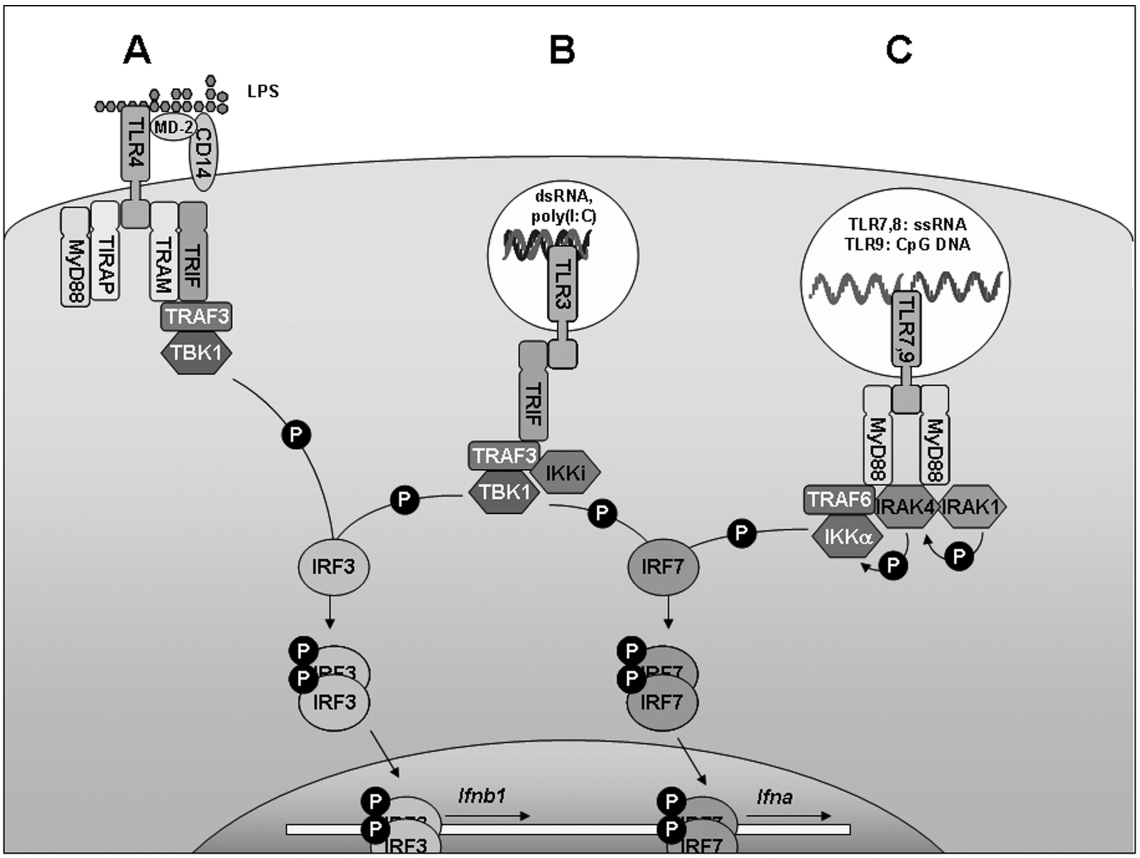

Figure 2. Interferon induction by Toll-like receptors. (A) TLR4 is the only Toll-like receptor that leads to IFN production in response to a non-nucleic acid ligand, namely the bacterial membrane component lipopolysaccharide (LPS). LPS binding to TLR4 and the co-receptors CD14 and MD-2 leads to signal transmission via the TIR adaptors TRAM and TRIF. Recruitment of TBK1, IRF3 and TRAF3 results in IRF3 phosphorylation by TBK1. (B) TLR3 is expressed in many cell types and located in endosomes. TLR3 recognizes dsRNA, released from disrupted viral particles or virus-infected cells. Signaling involves the TIR adaptor TRIF. TBK1 and IKKi phosphorylate IRF3 and 7 leading to production of IFN. (C) The endosomal TLR7, -8 and -9 are expressed predominantly in specialized hematopoetic cells like pDC. IFN induction depends on the adaptor protein MyD88 and the ubiquitin ligase TRAF6. In a complex containing IRAK1, IRAK4 and IKKα, IRF7, but not IRF3, is phosphorylated. Homodimers of phospho-IRF7 are imported into the nucleus and switch on transcription of IFNα mRNAs.

Although in the absence of both IRAK1 and IRAK4 MyD88-dependent IRF7 activation is severely impaired [64, 65] it is suggested that IKKα is the kinase responsible for the final phosphorylation of IRF7 [66].

Intracellular recognition of virus RNA is crucial for antiviral defense

Although TLR-mediated activation may contribute considerably to systemic IFN levels, the recent characterization of knock-out mice suggests an outstanding importance of cytoplasmic recognition of viruses for antiviral host defense. Intriguingly, the absence of TLRs or of TLR adaptors did not have severe effects on the susceptibility of mice to RNA virus infection, in spite of a reduction of systemic IFN levels in some cases (for review see [67]). This indicated that intracellular recognition of viruses is not only required but is sufficient for mounting a protective antiviral inflammatory. Indeed, the lack of RIG-I or MDA-5 correlated with increased susceptibility to viruses recognized by these receptors [11]. In IPS-1 knock-out mice, IRF3 and NF-κB responses to viruses and poly (I:C) was severely impaired. IPS-$1^{-/-}$ mice were more susceptible to EMCV, which is sensed by MDA-5. In addition, IPS-$1^{-/-}$ mice and even heterozygous IPS-$1^{+/-}$ mice were highly susceptible to infection with vesicular stomatitis virus (sensed by RIG-I) in spite of high IFNα levels probably produced by pDC, which are equipped with the TLR-dependent MyD88/IKKα/IRF7 pathway [14, 15]. These observations emphasize the importance of cytosolic recognition of viruses. They suggest that the mere production of sys-

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temic endogenous IFN, as well as therapeutic application of exogenous IFN, does not guarantee the critical triggers for initiating an appropriate and sufficient innate antiviral response. Further work towards integrating the expression profiles of IRF, STAT, NF-κB, AP-1, and MAPK target genes may lead to identification of the relevant components beyond IFN [68].

IFN JAK/STAT signaling

All type I IFN family members have a common single and ubiquitous receptor, the IFNα receptor (IFNAR) which mediates the pleiotropic effects of IFN. Nevertheless, different IFNs may cause somewhat differential responses in a cell, and the biological response of different cell types to IFN may vary considerably (for comprehensive reviews see [69–71]. The major pathway of IFNAR signaling involves the activation of STAT family members by Janus kinases [72, 73] (Fig. 3). The two chains of IFNAR1 and IFNAR2c (a splice variant) are associated with the Janus kinases TYK2 and JAK1, respectively. Binding of IFN results in the tyrosine phosphorylation of the Janus kinases and of the recruited STAT1 and STAT2. STAT1 and STAT2 form heterodimers through SH2-phosphotyrosine interactions which associate with p48 (IRF9). This complex, known as IFN-stimulated gene factor 3 (ISGF3), binds to characteristic DNA sequences known as IFN-stimulated response elements (ISRE) in the promoters of more than hundred genes. STAT1 homodimers which are minor products of IFNAR signaling, but abundant in IFNγ signaling, activate a partially overlapping set of genes specified by gamma-activated sequences (GAS). IFNAR signaling can lead to activation of other STATs as well, resulting in a variety of STAT homo- and heterodimers. In addition, not only Janus kinases, but other kinases can contribute to STAT activation. Moreover, transcription factors other than STATs may participate in the expression of ISGs. The relative abundance of STATs, kinases, and other transcription factors may contribute to the observed flexibility of biological responses to IFN [69, 70].

Negative regulation of the JAK-STAT pathways occurs primarily at two levels, the activation of STATs, and transcription of ISG by activated STATs. Members of the suppressor of cytokine signaling proteins (SOCS), SOCS1 and SOCS3, can inhibit JAK activity by binding through their SH2 domains to JAKs and to the receptor, respectively [74]. Members of the protein inhibitor of activated STAT (PIAS) family act as small ubiquitin-like modifier (SUMO) E3 ligases that target phosphorylated STAT in the nucleus and interfere with their transcriptional activity [75].

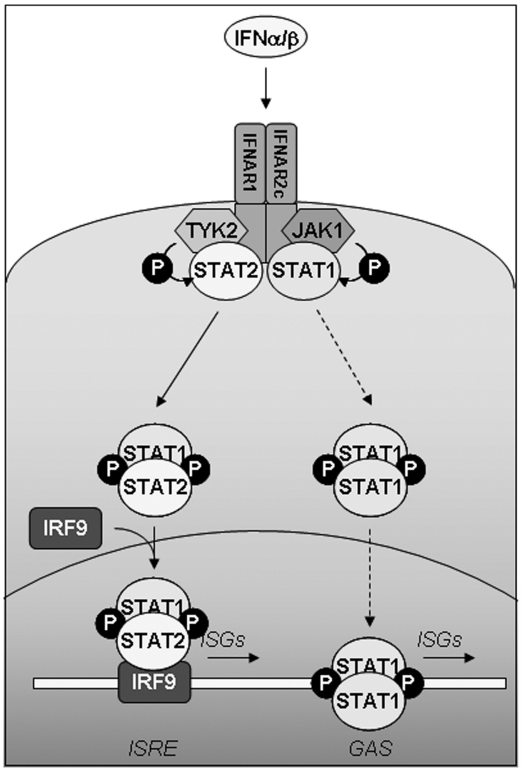

Figure 3. Type I IFN signaling. The receptor for IFNβ and IFNα subtypes is the ubiquitous IFNα receptor (IFNAR). IFN binding and receptor dimerization leads to cross-activation of receptor-associated Janus kinases, JAK1 and Tyk2, which phosphorylate the transcription factors STAT1 and STAT2 at specific tyrosine residues. Tyrosine phosphorylation allows STAT interaction via their SH2 domains. STAT1/2 dimers form a complex with IRF9, called ISGF3, and drive the transcription of interferon stimulated genes (ISGs) containing *ISRE* sequence in the promoter region. STAT1 homodimers (which are more efficiently formed after IFNγ signaling) promote transcription of ISGs controlled by the GAS (gamma activated sequence) motif.

Antiviral activities of IFN

An important consequence of paracrine IFN signaling is the establishment of an antiviral state in the surrounding of virus-infected cells [76–78]. If not able to limit virus replication in previously infected cells in which viral antagonists are active, IFN response should at least impede further spread of viruses. Among the hundreds of ISGs induced are several coding for potent antiviral proteins. Antiviral mechanisms well established in cell culture and animal models comprise the PKR system [79], the 2′-5′OAS/RNaseL system [80], and the Mx protein family [81]. Additional proteins with potential antiviral activities are ISG20, promyelocytic leukaemia protein (PML), guanylate-binding protein 1 (GBP-1), P56, and the RNA-specific adenosine deaminase 1 (ADAR1) (for review

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see [78]. An IFN-induced protein family with rather specific activity is the apolipoprotein B editing catalytic polypeptide 3 (APOBEC3) family of cytidine deaminases, which incorporate into vif-deficient HIV-1 virions [82] and which can block HIV-infection in resting T4 cells [83].

A probably not less important outcome of IFN signaling is the feedback stimulation of the RNA sensing and IFN induction machinery by inducing critical components like RIG-I, MDA-5, and IRF7. This leads to an amplified capacity to sense intracellular danger signals and allows a more comprehensive set of IFNs to be expressed. In addition, many components of the JAK/STAT pathway itself are among ISGs, including, STAT1, STAT2, and p48 providing a strong positive feedback for IFN signals. The importance of IFNAR signaling is illustrated by experiments with animals lacking the IFNAR or functional STATs. These are highly susceptible to viruses and rapidly succumb even to attenuated viruses [84–86].

Viral IFN antagonists

Most probably, viruses do not go completely unrecognized by the elaborate cellular security system. Exogenous viruses approach the cell as inert complex chemical entities. Although binding of viruses to cell surface receptors may already have inhibitory effects on cell signaling, the time between docking and viral gene expression is critical for detection and triggering of an initial IFN response. As soon as viral gene expression ensuses, the potential of viruses is unchained and viral functions are expressed that can take over control of the cellular signaling pathways. A plethora of viral gene products working as specific IFN antagonists have been described in the past years, and their function characterized. In the following selected examples are provided which illustrate that viral functions can strike every step and piece of the host IFN regulatory network. Those viral proteins that cause a general cell transcription shut down (including IFN genes) are not included.

Virus proteins concealing RNA

Recognition of viral RNA is a key in IFN induction and in activating the antiviral PKR, in case of dsRNA. Any exposure of viral RNA is therefore critical. In this respect, all viral proteins associated with viral RNA may be considered important IFN antagonists by shielding RNA from recognition by host cells. The first viral proteins described as IFN antagonists are dsRNA binding proteins, namely the NS1 protein from influenza A virus, and the E3L of vaccinia virus. NS1 is a multifunctional pleiotropic protein that binds dsRNA, ss triphosphate RNA, RIG-I, and PKR. NS1 also inhibits the 3′end processing of cellular pre-mRNAs, regulates the virus replication cycle, and enhances translation initiation of viral mRNAs [19, 87–89]. More recently, the VP35 protein of Ebola virus, another negative strand RNA virus, was identified as a dsRNA binding protein with multiple functions in IFN escape [90]. Also DNA viruses encode proteins that interfere with cellular dsRNA binding proteins PKR and 2'5'OAS, thereby inhibiting the activation of these key antiviral enzymes, such as the E3L protein from the vaccinia poxvirus [91]. Intriguingly, E3L protein also binds Z-DNA. Whether this relates to a potential protective role in DNA-mediated IFN response, awaits clarification [92]. In any case, large DNA viruses like vaccinia encode a multiplicity of other proteins that interfere with innate and acquired immune response (for a comprehensive review see [93]. A special way of blocking the RNA-triggered induction of IFN is probably enabled by the E^{rns} protein of pestiviruses like BVDV and CSFV. E^{rns} is a structural glycoprotein of the virus and exhibits both RNase and dsRNA binding activity. Both properties of the protein have been found to be involved in blocking IFN induction by extracellular dsRNA by a so far not further specified mechanism [94].

Another strategy to interfere with recognition of viral RNA is targeting the function of the pattern receptors. This is applied by (-)RNA viruses of the *Paramyxovirus* genus, including for example Sendai virus, Simian virus 5, human parainfluenza virus-2, mumps virus and Hendra virus. The V protein of those viruses binds MDA-5, but not to RIG-I, and interfere with IFN induction by transfected RNA [7, 95]. The specificity for MDA-5 is puzzling, since RIG-I, and not MDA-5, appears to be the sensor for paramyxovirus RNAs. The V proteins of most paramyxoviruses are further remarkable as they simultaneously abolish JAK/STAT signaling, mostly by targeting either STAT1 or STAT2 for proteasomal degradation (see below). The typical mode of infection of paramyxoviruses involves fusion at the cell membrane and thereby may further minimize recognition of paramyxoviruses by avoiding encounter with endosomal TLRs. TLR-independent IFN induction in human pDC has been confirmed for respiratory syncytial virus (RSV) [96].

Exciting examples for viral proteins targeting the adaptors of recognition receptors are provided by (+)RNA viruses. The hepatitis C virus (HCV) NS3/4A protease is an essential virus protein required for processing of the immature viral polyprotein, and in addition, is instrumental in preventing IFN induction. HCV NS3/4A cleaves not only the TLR3/4 adapter TRIF [97] but also the RIG-I

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CARD adaptor IPS-1 [24, 25]. Cleavage of TRIF probably disables its association with TRAF3 and/or TBK1. NS3/4A cleavage of IPS-1 removes the transmembrane anchor from IPS-1, and precludes its mitochondrial localization. Treatment of HCV-infected cells with an inhibitor of the NS3 protease (BILN2061) restores IFN induction only partially, suggesting the presence of additional HCV inhibitory activities. The NS3/4A protease of the HCV-related GB virus, which causes generally acute and occasionally chronic hepatitis in small primates, cleaves IPS-1 as well. This observation provides further support for the use of GBV-B infection in small primates as an accurate surrogate model for deciphering virus-host interactions in hepacivirus pathogenesis [98]. Intriguingly, hepatitis A virus (HAV), which belongs to a different virus family, the *Picornaviridae*, has recently been found to follow the same strategy of cleaving IPS-1. Proteolytic inactivation of membrane-bound IPS-1 by HAV requires a protein precursor of the 3C cysteine proteinase (3ABC) that contains a transmembrane domain (SM Lemon, pers. comm.).

The A46R protein of vaccinia is targeting adapter function in a different way. This protein resembles the cytoplasmic TIR domains of the TLR adapter molecules TRIF, TRAM, and MyD88 and competes with TRIF for TLR3-TIR binding, thereby interfering with TRIF-dependent activation of IRF3. In addition MyD88, Mal and TRAM-mediated signaling is affected by A46R [99].

Proteins targeting the step of IRF phosphorylation

Obviously, the IRF kinases TBK1 and IKKi as well as the IRFs themselves are major targets for interference with IFN production. The phosphoproteins (P) of several (−)RNA viruses, including Borna disease virus, Ebola virus, and rabies virus, are essential proteins involved in viral RNA synthesis, and interfere with the activation of IRF3 and IRF7 by TBK1, though the mechanisms are mostly poorly understood. For Borna disease virus P, a decoy function was suggested as it was found to be phosphorylated by TBK1 overexpression [100]. Ebola virus P (VP35), in addition to binding dsRNA, interferes with IRF3 activation through TRIF and RIG-I pathways [90]. Rabies virus P prevents phosphorylation of both IRF7 and IRF3, by TBK1 and by IKKi [101] (and unpublished results). Since phosphorylation of the critical IRF3 serine 386 [102] is not possible in the presence of P, IRF3 dimerization, nuclear import and transcriptional activity is precluded. The rabies virus P protein in addition is active in preventing IFN JAK/STAT signaling [103, 104]. This dual function of simultaneously targeting IFN induction and IFN signaling is reminiscent of the situation with paramyxovirus V proteins, but involves distinct mechanisms. For rabies virus (unpublished) and Ebola virus [105] the relevance of P/VP35 IFN antagonistic functions for survival and pathogenesis in the host have been illustrated in animal experiments using engineered viruses. In case of RSV, inhibition of IRF functions requires the coordinate function of two non-essential, non-structural (NS) proteins, NS1 and NS2 [106, 107]. These two proteins mediate also the resistance to exogenous IFN [108, 109]. Finally, (−)RNA viruses may utilize even glycoproteins to fight IFN. Comparison of pathogenic and non-pathogenic hantaviruses revealed a correlation with IFN production which could be linked to mutations in the cytoplasmic tail of the G1 protein. Expression of the G1 cytoplasmic tail of the pathogenic NY-1 strain inhibited RIG-I and TBK1-triggered IFN activation, but failed in inhibition of a phosphomimetic form of IRF3 [110]. Thus, like rabies virus P, G1 appears to target the step of IRF3 phosphorylation by TBK1. Notably, often several viral proteins are employed to counteract IFN induction, as exemplified by the coronavirus causing severe acute respiratory syndrome (SARS). The SARS virus ORF 3b, ORF 6, and N protein all inhibit phosphorylation of IRF3 [111].

Targeting IRF function

IRF3 is the target of a variety of RNA and DNA viruses. The ML protein from Thogoto virus, an influenza-like insect virus, prevents IRF3 dimerization and subsequent CBP interaction, without interfering with the phosphorylation and nuclear import of IRF3 [112]. The Npro protein, a protease of HCV-related pestiviruses like BVDV and CSFV cause a decrease in the cellular IRF3 levels [113, 114]. Both a lack of IFN promoter activation after nuclear translocation of phosphorylated IRF3 dimers and a decrease in cytoplasmic IRF3 levels have been observed in Npro containing cells. The latter effect was attributed to polyubiquitination of IRF3 and proteasomal degradation. Similarly, the NSP1 protein of rotavirus interacts with IRF3 and targets it to proteasomal degradation [115]. Whether the viral proteins leading to IRF degradation mimic or stimulate the functions of the cellular Pin1 is unclear so far. Also IRF7 is a target of virus proteins. An EBV encoded protein, BZLF-1, physically associates with cytoplasmic and nuclear IRF7 and inhibits IRF7 activity after stimulation by dsRNA, as well as the activity of a constitutively active form of IRF7 [116].

Herpes viruses have acquired additional weapons to interfere with the functions of cellular IRFs. Human herpes virus 8 (HHV-8), the causative agent of Kaposi sar-

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coma, encodes several IRF homologues, so-called vIRFs, which are closely related to their mammalian counterparts. vIRFs can act as dominant interfering mutants to block endogenous IRF function or can mimic host IRFs functioning as negative regulators of IFN gene expression, such as IRF2. For example, vIRF1 binds CBP/p300 and IRF3 and thereby prevents the function of the enhanceosome [117]. The vIRF3 (LANA3), in contrast, seems to have a converse effect and stimulates binding of IRF3 and IRF7 to the enhanceosome complex [118] whereas it prevents NF-κB activation by binding of IKKβ [119].

In addition to targeting the classical TBK1/IRF signaling pathways, some viruses are able to shut down IFN induction by MyD88-dependent IRF7 activation in pDC. Infection of human pDCs with clinical isolates of RSV and a measles vaccine strain caused little IFNα expression in contrast to an RSV mutant strain, Long. Moreover, these viruses interfered with IFN induction by potent TLR7 and TLR9-agonists while the mutant strain Long failed in blocking TLR stimulation [120]. The remarkable capacity of RSV and measles virus to shut down activation of human pDC could well contribute to the characteristic Th2 biased immune pathology of measles [121] and RSV.

Interference with IFN function and JAK/STAT signaling

A far-reaching approach to counteract IFN functions is used by the large poxviruses like vaccinia virus. They express soluble IFN-binding proteins ("viroceptors" B8, B18) which compete with the cellular IFNAR for IFN binding [122, 123]. By neutralization of secreted IFN they prevent the establishment of an antiviral state in the non-infected tissue and disable the autocrine IFN feed-back loop.

The function of the IFNAR-associated Janus kinases JAK1 and Tyk2 is targeted by several DNA and RNA viruses. The E6 protein of Human papilloma virus 18 (HPV18) interacts with TYK2 and impairs its function [124] whereas the Large T of polyomaviruses has a preference for JAK1. Also some paramyxoviruses interfere with Janus kinase function, although constitutive targeting of STATs is a more common strategy of the paramyxoviruses. This is due to their non-essential V proteins and/or the essential P proteins. V and P proteins are products of a single gene and share a common N-terminal moiety. Rubulavirus V proteins assemble STAT-specific ubiquitin-ligase complexes from cellular components and target either STAT1 or STAT2 for proteasomal degradation [125, 126]. The V proteins from other paramyxovirus genera do not lead to degradation of STATs, rather prevent phosphorylation of STATs and sequester STAT1 and STAT2 in high molecular mass complexes, as has been shown for Nipah and Hendra viruses. In this case, also P proteins have this activity, though less pronounced. The V protein of measles virus (*Morbillivirus* genus) co-purifies with STAT1, STAT2, STAT3, and IRF9, to recruit STATs to viral inclusion bodies, and was reported to bind to the IFNAR. The Sendai virus P gene encodes another protein (C) targeting STATs [127, 128], and in RSV, the NS2 protein is involved in downregulation of STAT2 [129, 130]. For excellent reviews on paramyxovirus interference with IFN signaling see [131, 132].

In rhabdoviruses like rabies virus which lack a V protein, the essential P protein is responsible for JAK/STAT signal inhibition [103, 104]. Unlike the V proteins of paramyxoviruses, rabies virus P does not target non-activated STATs, but interacts with STAT1 and STAT2 only after tyrosine phosphorylation [104]. The interaction with P retains activated STATs in the cytoplasm, thereby preventing STAT-mediated transcription of ISGs. Such conditional, activation-dependent targeting of STAT1 and STAT2 by rabies virus P to interrupt IFN JAK-STAT signaling is unique among viruses. In view of the multiple tasks of P protein, including engagement in viral RNA synthesis and in IRF inhibition and a limited coding capacity of rhabdoviruses, the observed activity on demand may reflect a specialization to preserve capacity for other duties in non-alerted cells. A less specific mechanism to prevent nuclear import of activated STATs and of other proteins is applied by the Ebola virus VP24, a minor structural protein. VP24 binds importin-α, the main nuclear import factor for STATs. Recruitment of importin-α by overexpressed VP24 led to reduced nuclear accumulation of STATs and ISG transcription [133].

STAT-targeting proteins are known from positive strand RNA viruses or DNA viruses as well. The ORF6 proteins of SARS coronavirus inhibit STAT1 translocation although STAT phosphorylation is not reduced whereas the ORF3b appears to interfere with nuclear transcription factors necessary for the IFN response [111]. The adenovirus E1A protein binds STAT1, and in addition IRF9 (p48) to prevent formation of ISGF3 [134, 135]. The 72-kDa IE1 protein of human cytomegalovirus (HCMV) forms a physical complex with STAT1 and STAT2 in nuclei of infected cells and association of ISGF3 with promoters of IFN-responsive genes in vivo [136]. STAT2 is a specific target for downregulation by the m27 protein of murine CMV (MCMV) [137]. Finally, herpes viruses can also exploit the cellular regulatory pathways of JAK/STAT signaling. HSV-1 induces the expression of SOCS3, to downregulate JAK and STAT phosphorylation [138].

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Apparently, the same strategy can be used by small RNA viruses like HCV [139].

Interference with IFN-induced antiviral proteins

In a recent excellent review a comprehensive description of interference of viruses and viral proteins with IFN-induced antiviral proteins can be found [78]. Of note, virtually all viruses, including RNA viruses, DNA viruses, and retroviruses, have evolved means to interfere with the PKR and 2′-5′OAS/RNAse L system illustrating the importance of these systems in general antiviral defense. Some of the viral proteins competing with PKR for dsRNA are described above as they might also function in preventing recognition by RLR and IFN induction. Rather puzzling are some specific small noncoding viral RNAs like EBV-encoded RNAs (EBERs) or the adenovirus VA RNAs. On the one hand, EBERs are recognized by RIG-I and trigger IFNβ production [140] and on the other hand bind PKR to inhibit its antiviral activity [141, 142]. An intriguing alternative strategy to counteract PKR antiviral activity, just to mention, is applied by HSV-1, whose g34.5 protein "cures" the PKR-mediated shut down by recruiting phosphatase1-alpha to dephosphorylate eIF2 and thereby restore translation [143].

Viral IFN antagonists as factors of tropism and host range

The IFN network as well as the links to other systems, including other innate defense systems, like apoptosis, and the adaptive immune system, is highly variable and it is clear that IFN has different outcome in different cell types, tissues and organs. The tools developed by viruses ideally allow them to grow in the host for some time, at some privileged sites, and without killing the host too early. Whereas some viruses are eliminated after an acute infection, some are controlled partially by the immune system, and persist at low level. The restriction to certain organs may reflect at least in part the incapability of viruses to withstand the innate immunity elsewhere. There is also increasing evidence that the ability of IFN antagonists from viruses to counteract IFN pathways in a certain host species is a critical determinant of its host range. For example, species specific differences have been shown crucial for a couple of paramyxoviruses, including simian virus 5 and Sendai virus [144, 145], RSV [109, 146], measles virus [120], Newcastle disease virus (NDV) [147] and myxoma virus [148]. Indeed, the principle of host-specific IFN antagonism is being utilized for approaches using non-human paramyxoviruses like Newcastle disease virus for oncolytic virotherapy approaches [149]. This is based on the observation that many tumors have defects in the IFN system, particularly in JAK/STAT signaling.

Other members of this virus group do have a broad host spectrum, or are able to cross species barriers. Notably, these include "emerging" and zoonotic viruses, such as rabies- and rabies related viruses, Nipah-, Hendra-, distemper-, and Ebola viruses. In vitro and in vivo many of these viruses are able to enter cells from different species, suggesting that a severe "entry" barrier does not apply. Although successful infection of non-natural hosts, including human, may be a dead end with respect to further virus transmission and spread, the apparent ability to infect a foreign host provides the opportunity to adapt to the new host in different respects. Adaptation to appropriately counteract the new host's IFN system appears therefore to be a key for the emergence of a new human pathogen [150]. The number and degree of molecular changes needed for the viral IFN antagonists to adapt and the intensity of contact may determine the probability of a virus to spill over to human and to convert into a human pathogen.

Outlook

Of the pathogens approaching hosts, viruses are the most intimate ones, because they are produced by host cells. The most singular features of viruses may reside in their nucleic acids. The IFN system appears to have developed primarily in response to nucleic acids. Receptors for external nucleic acids, like TLRs, and for internal nucleic acids, like RIG-I, can activate different defense and emergency conditions, respectively. A major challenge in the future is to exactly define those conditions.

The study of viruses tells us not only how to manipulate different arms of the IFN network but also how to stimulate the IFN system appropriately. Viruses with modified IFN antagonists are promising candidates for attenuated and immunogenic vaccines. Immune stimulatory nucleic acids binding to TLRs already find application as potent immune-modulators, agonists of the recently identified RLRs should provide us with additional tools for distinguishing manipulation of immune responses.

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