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Survey

Fine tuning type I interferon responses

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ABSTRACT

Interferon responses are balanced between protection against pathogens and other disease agents versus toxicity and development of chronic diseases. Optimal outcomes are achieved by regulating the nature, strength and duration of Interferon (IFN) production, IFN-receptor interaction and signalling pathways modulated in a manner appropriate for particular target cells. Modification of cell behaviour is mediated by regulation of positive and negative signalling pathways and by proteins encoded by selected groups of IFN-regulated genes. Understanding how these pathways are regulated and how to measure them by biomarkers or gene signatures will enable us to better understand the role of IFN pathways in the pathogenesis of infectious and inflammatory diseases and cancer. This will lead to improved patient stratification and disease treatment.

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1. Introduction

Interferons (IFNs) are a family of cytokines whose name was published in 1957 [1] based on viral interference work that began earlier in the Walter and Eliza Hall Institute in Melbourne [2]. During this period Nagano and Kojima had also described a similar phenomenon [3]. Since then, IFNs have been shown to represent a family of cytokines that exhibit a broad range of properties beyond just antiviral action. Research in Australia in the last 30 years has made significant contributions to the field ranging from cloning one of the human isoforms (rhu IFNα4) [4], structure function studies aimed at identifying the role of conserved amino acids [5], clinical studies in a range of diseases with Ian Mackay from WEHI [6–8], cloning and characterisation of receptors [9,10], generation of IFN receptor deficient mice [11] and negative regulation by SOCS proteins [12–14]. In this review we shall discuss how current knowledge informs us of how IFN signals are regulated or fine-tuned, which, after all is the aim of any therapeutic exploitation of

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these multifunctional cytokines. We will include aspects of our own work consistent with the theme of this volume on cytokine research in Australia.

Interferons are now broadly classified into three major types: Type I IFNs (including α, β, ω, ε), Type II (IFNγ) and Type III (IFNλs, also known as IL28 and 29) [15,16]. They are distinguished by the specificity of their cognate receptors, their primary amino acid sequence homology, genetic locus, and stimulus and cell type of production. This review will focus on the type I IFNs, which have been the focus of our work. In broad terms, type I IFNs have pleiotropic effects on cells – they induce an antiviral state, they can inhibit cell proliferation, modulate cell fate (survival/apoptosis), differentiation and migration. Their effects can be local, at the site of production, or systemic. One major role is the regulation of the development and activity of virtually every effector cell of the innate and adaptive immune responses [17]. Because of the rapid production in response to stimuli, they are considered key effectors of innate immunity that link recruitment and sculpting of the adaptive response [18–20]. The various properties of type I IFNs make them very crucial to host defence and indeed mice that are deficient in IFN responses are susceptible to many diseases including infection (by viruses and some bacteria) and cancers [11,21,22]. However, like many cytokines, the properties of type I IFNs are a double edged sword. Excessive IFN responses are toxic as evident in mice deficient in the negative regulator of IFN signalling, SOCS1 (see below and Linossi et al., 2013). Furthermore, type I IFNs appear to contribute to the pathogenesis of autoimmune diseases such as Systemic Lupus Erythematosus (SLE) [23] and administration of IFNs to patients can result in dose limiting toxicity including side effects of flu-like symptoms, nausea, myalgia, leukopenia and neurological effects [24]. Therefore it is critical to both understanding the type I IFN system and to harnessing its therapeutic potential, that we have an appreciation of how IFN responses are finetuned by a balance of positive and negative regulation of signalling. These issues will be the focus of this review.

2. Description and production of type I IFNs

The type I IFNs are encoded by a family of genes located in a cluster on chromosome 9p in humans and the syntenic region on chromosome 4 in mouse [15]. There are 14 subtypes of IFNα that share 75–100% amino acid identity; a single IFNβ subtype with 30% identity to a consensus IFNα sequence; a single IFNε with 30% amino acid identity to consensus IFNα and IFNβ; IFNω and other subtypes in certain species [15,16]. It is an unusual feature among the cytokine family that 20 or so type I IFNs all signal through the same receptor composed of IFNAR1 and IFNAR2 [25]. It begs the question of why so many subtypes exist. In this regard, it is notable that type I IFNs are rarely produced singly (some features of IFNβ and IFNε may be an exception), never all at once and usually in small groups (of IFNα subtypes +/−IFNβ). In addition to the divergent sequences of the protein coding regions of these (mostly) single exon genes, the regulatory promoter regions of the genes in the type I IFN locus are also divergent in sequence, resulting in different regulatory elements, suggesting different control of spacio-temporal production.

In order to have a full understanding of the regulation of type I IFN signalling, it is important to consider where and when IFNs are produced. Classically, like other cytokines involved in acute responses, type I IFNs are thought to be only expressed after a stimulus, the usual stimulus being infection [18]. Thus there has been considerable recent excitement about the discovery of the growing families of pattern recognition receptors as factors that sense pathogens and drive the production of type I IFNs, as well as other cytokines and chemokines that are the effectors of the innate inflammatory response [19,26]. IFNs are induced by cell surface and endosomal TLR signalling pathways: IFNβ specifically via TLR4 stimulation by lipopolysaccharide (LPS); mixtures of IFNα subtypes and IFNβ by others and usually not by TLR2, which acts in consort with TLRs 1 or 6 [18,19] (although an unusual myeloid cell type in the GI tract has been reported to produce IFNβ via an atypical TLR2 pathway [27]). Cytosolic Rig I – Like Helicases (RLHs) drive IFN production after activation by viral nucleic acids via signalling pathways activated by recruitment of these receptor complexes to the mitochondria associated membranes. Other nucleic acid sensors (Aim2, DD/HX proteins, etc.) have been located in the cytosol and nucleus [28,29]. These families of pattern recognition receptors (PRRs) appear to be located at a variety of subcellular locations such that they will sense different parts of a pathogen (cell surface LPS or peptidoglycan or single or double stranded nucleic acids: RNA or DNA) that are exposed to the host cell during the course of infection. PRR recognition initiates a series of signal transduction pathways, the important one for type I IFN production appears to be the activation of one or more of the Interferon Regulatory Factor (IRF) family of transcription factors. IRF 1, 3, 5 and 7 appear to be particularly important in the induction of IFNα gene expression, whereas IRF3 is important in the induction of IFNβ gene expression together with NFκB (which is not involved in IFNα gene expression) [30].

It is generally considered that most cells in the body can produce type I IFNs, but haemopoietic cells are the predominant source and of these, plasmacytoid dendritic cells (pDC) can produce very high levels. The latter observation may be due to the high levels of constitutive IRF7 expression compared to other cells in which the expression of this transcription factor (TF) is usually low and must be primed by an initial burst of IFNβ before optimal levels of other type I IFNs are produced [31]. This two-stage mechanism of type I IFN production has been demonstrated in cultured fibroblasts (and GM mice) but the extent it occurs in various cell types has not been ascertained. Indeed in a recent study, we unexpectedly demonstrated that breast cancer cells expressed IRF7, which drove type I IFN production, which, in turn, activated immune surveillance mechanisms that suppressed metastasis [32].

Breast cancer is not the only situation in which type I IFNs are produced in the absence of infection. While the type I IFN system has likely evolved to respond to pathogens, either their cell wall components or nucleic acids that are 'exposed' during the course of infection, the same sensing systems can detect DNA from dying cells [33], aberrant nucleic acid metabolism (degradation), which is the underlying cause of Aicardi Goutiere's Syndrome [34,35] and antibody complexes in autoimmunity – all leading to type I IFN production and contributing to pathology [36].

IFNβ is induced by CSF1 during macrophage differentiation where it has an inhibitory effect on the cell [37]. Similarly, IFNβ is induced by RANKL during osteoclast generation, again inhibiting proliferation of these cells [38]. Accordingly, Ifnar1−/− mice have been reported to be slightly osteoporotic because of excessive numbers of osteoclasts. In both the previous situations, IFNβ is produced via a pathway that activates the TF, AP1, which binds to cognate sites in the promoter. This is a distinct mechanism from the IRF3/NFκB pathway that activates pathogen driven IFNβ production. Another situation where type I IFNs are produced in the absence of pathogen stimulation is the recent observation that lactobacillus, a component of commensal flora, stimulated very high production of IFNβ [39]. This implies that a "physiological" production of conventional type I IFN may play a role in the "resting" mucosal organs in the maintenance of homeostasis and perhaps in priming innate protection. We recently described an even more specialised mucosal function of a type I IFN in the constitutive production of IFNε in the female reproductive tract. Surprisingly the IFNε promoter contains no binding elements for

IRFs and is therefore not induced by pathogens [40]. Instead it is constitutively expressed, hormone regulated (stimulated by oestrogen and suppressed by progesterone), and its expression levels fluctuate during the oestrus cycle and are switched off during pregnancy at the time of embryo implantation.

In general terms, what we believe this means is that the rules for regulating IFN production are adapted to suit the location and time. Accordingly, depending on the characteristic local influences (e.g. hormones, exposure to environment, microflora, etc.), the production and action of the type I IFN system appears to be adapted to ensure protection is provided but adequately controlled by site-specific mechanisms to ensure toxicity does not ensue.

Therefore, the first level where the type I IFN system is "finetuned" is when and where it is produced. It important to realise that this is not only in pathological, but also "physiological" circumstances. It is interesting to note that viruses often target critical parts of the host immune response to evade it, survive and establish productive or chronic infection. Thus several viruses target the production of type I IFNs e.g. NS1 and NS3/4 proteases that act at the level of IRF activation – turning down the finetuning to suit their survival [41,42].

3. Type I IFN signalling is initiated at the receptors

All type I IFNs initiate signalling via interaction with the type I IFN components, IFNAR1 and IFNAR2 [15,16,25]. These were cloned in the 1990s as were their murine homologues [9,43–45], which reinforced the view that the murine IFN system is similar in complexity of ligands and specificity of receptors and signalling components and thus represented a good system in which to model human effects. Nineteen years ago, we and others generated mice with null mutations in the Ifnar1 component of the receptor [11,21]. These mice demonstrated the critical importance of this system in protection from viral infection and in transducing signals from all type I IFNs including the recently described IFNε (indeed this was the result that really defines this cytokine as a type I IFN) [40]. Subsequently there have been over 1,400 papers published using these mice, demonstrating a role for IFN in protection against viral infections, some bacterial infections, cancer and autoimmune diseases. Furthermore, studies with these mice have demonstrated that IFNs contribute to diseases including SOCS1 suppressed multi-organ inflammation and lethality [13], septic shock, bacterial infections (e.g. Chlamydia) [40,46], SLE type autoimmune diseases [47,48], and neurotoxicity associated with nucleic acid-sensing PRRs inducing pathogenic IFN production [33–36]. These mice have also contributed to our understanding of in vivo and cellular type I IFN signal transduction mechanisms including the existence of priming [49].

The IFNAR1 chain of the receptor that was the target for generating the aforementioned knockout mice is considered the "signal transducing chain" for all type I IFNs, whereas the IFNAR2 chain is the high affinity binding chain. However, there have been suggestions that interaction with IFNAR1 could be significant: (1) this receptor was initially cloned by virtue of its ability to bind a particular IFNα subtype and IFNβ [43]; and (2) interactions with IFNAR1, in the context of a ternary complex, have been correlated with potency of activity [50]. Even before the receptors were cloned, several studies at Monash and other laboratories, investigated important residues in the ligands that influenced binding. These demonstrated the importance of cysteine residues that "pinned" the 3-dimensional structure of IFNs together[5]. These studies identified conserved hydrophilic residues such as Arg33 and Tyr123 were critical for activity, whereas other conserved residues were not [51]. These data were consistent with the observations that differences in amino acid sequences of different type I IFNs (even different IFNα subtypes) accounted for differences in the affinity of interaction with cell surface receptors and the potency of the IFN biological effect [52]. While it is well documented that different type I IFN subtypes have different biological potencies, there are no convincing, quantitative experiments to determine that any IFN subtype has a particular activity that others do not. Based on current data, this could only occur if differences in affinity of receptor engagement can result in a different signal.

Experiments mutating residues on the ligand and on cloned receptors were extended over the years to generate an impression of what the binding interfaces on this alpha-helical cytokine were with each receptor component [52,53]. Our understanding of the fine structure of the interaction of type I IFNs with the extracellular domains of IFNAR1 and IFNAR2 were elucidated in 2012 when an X-ray crystallography characterization of the ternary complex was published by the Garcia and Schreiber groups [50]. This had implications for how different IFN subtypes might interact with the two receptor components and transduce signals, and critical interacting residues on all three proteins were predicted.

What remains to be determined is whether any cellular differences in the absolute or relative levels of Ifnar1 and Ifnar2 either permanently or at a particular stage of development or response occur, as has been reported for an IFNγ receptor subunit, IFNGR2, in particular T cell subsets [54].

4. Signal transduction pathways activated via type I IFN receptors.

The ternary complex formed by a type I IFN ligand engaging with the extracellular domains of IFNAR1 and IFNAR2 also bring into proximity the intracellular domains of these receptors and their associated signalling adaptors, thus enabling interactions that initiate the signalling cascade. The best characterised signalling proteins that are pre-associated with the receptor chains are Tyk2 (with IFNAR1) and JAK1 (with IFNAR2) [15,55,56]. These are members of the Janus kinase (Just Another Kinase) family that were discovered by Andrew Wilks working at the Melbourne branch of the Ludwig Institute, and have proven to be important in signalling by many cytokines [57,58]. Once activated by receptor dimerisation/multimerisation, JAK kinases phosphorylate each other and tyrosine residues on the receptors, which in turn act as docking sites for Signal Transducers and Activators of Transcription (STAT) proteins, which dock onto phosphotyrosine residues via their SH-2 domains [59]. In addition to the importance of their kinase activation of STATs, JAKs have other activities [60]. For example, the Tyk2 interaction with IFNAR1 stabilises its expression at the plasma membrane [61].

Receptor-docked STATs are phosphorylated by JAK and other kinases, then dissociate from the receptors, dimerise and translocate to the nucleus via interactions with importins, where they bind to DNA and activate transcription [59]. Type I IFN signalling was initially characterised by the formation of a specific complex called Interferon Stimulated Gene Factor 3 (ISGF3) composed of STAT1, STAT2 and IRF9 (originally called ISGF3γ or p48). STAT1 is certainly critical to type I IFN mediated responses such as antiviral activity and there are many similarities between the phenotype of the Ifnar1−/− and Stat1−/− mice [62]. However, STAT1 is also crucial to type II IFN signalling and that of other cytokines. In addition to activation of the ISGF3 complex, type I IFNs also activate STAT3 and STAT1 to homo- and hetero-dimerise to form complexes that bind to the GAS element (Gamma Activated Sequence because it was first described as a consequence of IFNγ activation). Again these complexes are not specific to IFN signalling but are also activated by IL6 (Mansell and Jenkins, 2013) and other cytokines.

In order to understand the mechanism of IFN signalling in more detail, we and others have undertaken extensive studies to "map" the domains or residues in the cytoplasmic parts of the receptors to which signalling molecules are bound. Firstly, the JAKs are bound to membrane-proximal regions. The summary of several studies demonstrates that STAT1, 2, 3 bind to the Ifnar2 component [14,56,63–65]. Indeed mutation of tyrosine residues on IFNAR1 resulted in no change to STAT reporter activities indicating that this chain played no direct part in recruiting STAT1, 2, or 3 to the receptor complex [14]. This result is at odds with claims that recruitment of IFNAR1 to other receptors such as IFNGR and the IL6 receptor system recruits STAT1 as a mechanism of cytokine cross-talk [66,67]. Since these studies relied on the use of Ifnar1−/− mice, it is more likely that cross talk between these receptor systems occurred by a different mechanism; that is to say, STAT1 levels are low in most cells in Ifnar1−/− mice because of a lack of basal type I IFN priming of many IRGs such as STAT1 (the levels of STAT1 range from 2 to 3 fold below normal levels to 10 fold lower) [49]. Thus JAK/STAT pathway initiation is associated with the IFNAR2 component but requires Tyk2 on IFNAR1.

Other STATs, 4, 5 and 6 are also reportedly activated by type I IFNs, but the receptor chains to which they are associated, or even the upstream signalling pathways that activate them are unknown [59,68,69]. While there is no evidence for IFNAR1 in STAT activation, it may be linked to STAT independent signalling, such as that identified in studies of cells from STAT deficient mice. These studies indicated STAT1-dependent and -independent pathways (for both type I and II IFN signalling). While genes were activated in the absence of STAT1, the nature of the signal transduction pathways were not explored [70]. It should be noted that in addition to factors that transduce activating signals, negative regulation and regulators have also been mapped to the receptor chains (see below).

Supplementing the essential cytokine activated Jak-Stat pathways are alternate signalling pathways that are triggered by IFN receptor engagement to impart optimal transcriptional activation of IRGs and the subsequent biological responses. These include the MAPK (p38 and ERK), NFκB and PI3K/AKT pathways [68,71]. The best characterised of these is the p38 MAP kinase (MAPK) pathway, which is activated coincident with the Jak-Stat pathway [72]. The Erk MAP kinase pathway is also activated by IFN signalling and impacts on ISG mRNA translation via activation of Mnk kinases and eIF4E phosphorylation [73]. Downstream activation of the AKT/mTOR (mammalian target of rapamycin) signalling cascade is also characteristic of IFN receptor engagement but the impact is on mRNA translation of IRGs and production of encoded proteins [74,75]. While the relative contribution of these alternative pathways is likely to be cell and context dependent, many of their functions appear to be essential for IFN-dependent gene transcription, although specific transcript profiles remain to be fully characterised. Indeed a report of type I IFN signalling in T cells indicated a role for T cell receptor signalling molecules [76].

5. IFN Regulated Gene Induction Facilitated by PLZF

The zinc finger and BTB domain containing 16 (*Zbtb16*) gene encoding the protein promyelocytic leukaemia zinc finger (PLZF) is essential for antiviral innate immune responses driven by IFNs [77]. This was initially discovered by studying renal adenocarcinoma cell lines differing in their IFN sensitivity. PLZF stimulated IFN regulated genes that contained putative PLZF binding sites in their promoter regions. Many ISGs including *Oas1g*, *CXCL10*, *Rsad2* (*Viperin*), and *Ifit2* [78] failed to respond to high levels of IFN in *PLZF*−/− mice after viral infection. PLZF also regulates the effector function of NK cells since Granzyme *B* (*GzmB*) induction after IFN activation in splenic isolated NK cells from PLZF deficient mice is impaired. A reduction in IFN-induced CXCL10 expression in NK cell-rich organs is also seen in these mice and likely contributes to their susceptibility to viral infection. The *Zbtb16* gene encoding PLZF is not itself regulated by IFN but instead, PLZF is phosphorylated following IFN signalling. IFN mediates phosphorylation of PLZF at serine residue 76 in vitro via Mitogen-activated protein kinase 8 (JNK) or a kinase in the same pathway. This phosphorylation of PLZF enhances its transcriptional activity and facilitates binding of PLZF to cognates sites in select ISGs. Thus, following IFN stimulation, PLZF acted as a transcriptional activator, a novel finding for this zinc finger protein family member. This activity of PLZF contrasts with its well-described role as a transcriptional repressor [79].

6. Negative regulation of IFN signalling

In order to regulate type I IFN signalling and prevent toxicity, there is negative regulation at virtually every step of the signal transduction process: at the level of ligand production, as mentioned above, at the level of receptor, signalling adaptor, signalling enzymes, TFs, co-regulators of transcription, and post-transcriptional and post-translational modifications, as well as inhibitors of the proteins encoded by IFN regulated genes. The effect of these negative regulators can be to limit the strength or duration of signalling.

6.1. Receptor level

The gene encoding IFNAR2, the primary binding chain, can undergo alternative splicing to generate a truncated transmembrane isoform with no apparent signalling capability; this may act as a decoy receptor, thus inhibiting the interaction of ligand with productive signalling receptors [16,25,44,45]. Both human and murine IFNAR2 genes can be alternatively spliced to form a soluble receptor [9,44]. There has been a report that this may also be produced by protease cleavage by ADAM 17 (TACE) [80]. While it is possible that this soluble receptor might inhibit signalling, as is the case in some cytokine systems, this has not yet been demonstrated for the type I IFN. An alternative possibility is that soluble IFNAR2 might engage in trans-signalling (as defined in case of IL6 soluble receptor), whereby the soluble receptor binds ligand and presents it to the signal transducing chain, in this case, IFNAR1 (gp130 in the case of IL6), resulting in the transduction of particular signals[81]. The Ifnar1 component of the receptor also contains a negative regulatory domain that is the target for ubiquitination and degradation [82].

Proteins associated with the intracellular domains of the receptors can also suppress signalling. These include UBP18/43 associated with IFNAR2 [83] and phosphatases that are associated with IFNAR [84,85]. Uncharacterised activities also exist, such as: reports of a negative regulatory domain in the C terminal region of IFNAR1 [86], and a negative impact of the IFNAR2 chain on GAB2 signalling from IFNAR1 [87].

6.2. SOCS proteins

We have recently mapped the binding of the negative regulator SOCS1 to the IFNAR1 component of the type I IFN receptor [14]. The Suppressors Of Cytokine Signalling (SOCS proteins) were discovered concurrently by three groups including the Hilton laboratory at Walter and Elisa Hall Institute in Melbourne (Linossi et al., 2013). There are 7 members of this family that function to inhibit signalling by a range of cytokines and growth factors (Linossi et al., 2013; Mansell and Jenkins, 2013). SOCS proteins are composed of three key domains: KIR, SH2 and SOCS BOX. These domains mediate interaction with their target proteins: they variously bind to phosphor-tyrosine residues on receptors (e.g. IFNGR and g130)

or to JAK kinases and target them for degradation by the proteasome. Of these, SOCS1 and SOCS3 are well characterised to inhibit type I IFN signalling. The in vivo role of these proteins has been defined in gene-targeted mice. SOCS1 mice die pre-weaning due to multi-organ inflammation [12]. This lethality could be rescued by crossing these mice to IFNγ-deficient mice, which is a vivid demonstration of the dangers of unrestricted cytokine signalling [12]. The Socs1−/− lethality at 3 weeks could also be rescued by crossing to Ifnar1−/− mice demonstrating that unrestricted type I IFN signalling was also pro-inflammatory and potentially lethal [13]. Interestingly, mice were not rescued by crossing to Ifnar2−/− mice, which demonstrated that SOCS1 probably associated with the Ifnar1 chain of the receptor (and also that there were potentially phenotypic differences between these two receptor deficient mice). We went on, in collaboration with the WEHI scientists, to demonstrate SOCS1 association with Ifnar1 by co-immunoprecipitation [13]; we then demonstrated that SOCS1 did not associate with phosphotyrosine residues in the receptor (as it did in the IFNγ and IL6 receptor systems), but with the Tyk2 kinase which was associated with the juxta-membrane region of the intracellular domain of Ifnar1 [14].

6.3. Negative Regulation of Transcription

Type I IFN signalling is also negatively regulated at the level of transcription factors by interacting proteins, such as Protein Inhibitors of Activated STATs (PIAS) [88], Akt pathway activation of EMSY [89], TRIM pathways acting via retinoic acid receptor (RAR)α [90], and by several members of the IRF family that can act as negative regulators [91]. Type I IFN signalling also up-regulates expression of Sprouty (Spry) proteins 1, 2, and 4. This is Mnk kinase-dependent and results in suppressive effects on the IFN-activated p38 MAP kinase (MAPK), and consequent suppression of transcription of ISGs [92]. The dual roles of some transcriptional regulators like PLZF are interesting, because while it is a positive regulator of type I IFN signalling, it is at other times, a negative regulator of primary signalling [77–79].

6.4. Post-transcriptional regulation of signalling

The most notable negative regulators of signalling that are being investigated at present are the non-coding RNA, especially micro-RNA (miR) [93]. There are about 800 confirmed genes encoding functional miRs in the human and mouse genome and estimates range up to 10,000s [94]. The genes encode short sequences of RNA that bind Argonaute proteins, which direct the duplexes into the RISC complex where transcripts are degraded. MiR targeting of protein coding sequences thus decreases the half-life of transcripts and the encoded proteins [95]. There is considerable characterisation of the role of miRs in the immune response [96] and cancer [97] but little documentation of their role in regulating innate immune responses and the IFN response in particular, where only a few have been reported [98–100]. The targets include SOCS1 by miR-155 involving auto-regulation of this negative feedback loop [101]; and of Ifnar1 by miR-29a involving thymic involution [102]. There is sufficient data on the regulation of miR transcription to suggest that regulation will be similar to that of protein-coding genes. Therefore the pathways discussed here for regulating the expression of protein-coding IRGs are likely to also transcriptionally regulate miRs, providing another level of networked finetuning.

7. A global view of the type I IFN response

The end result of signalling emanating from an IFN ligand interacting with cognate receptors and initiating various signal transduction pathways is the activation of transcription factors that activate or repress the expression of (sets of) IFN regulated genes (IRGs). It is the proteins encoded by these IRGs that are the effectors of the IFN response. They will elicit protection from viral infection, inhibition of cell proliferation, modulation of immune cell functions, etc. One way to obtain a global view of the IFN response is to perform a microarray analysis of changes in gene expression. The first (using the Affymetrix platform) microarray experiments of an IFN response were performed by the Williams and Silverman laboratories at the Lerner Research Institute, Cleveland Clinic [103,104]. These experiments detected over 1000 genes with altered expression and gene ontology analyses (GO) could identify sets of genes associated with known IFN functions including antiviral activity, cell cycle regulation, survival/apoptosis, immune response, etc. [104]. Ten years later, we decided to collect all of the experiments that had been performed where a cell, mouse or human had been treated with IFN and microarrays performed to measure changes in gene expression (Interferome.org [105]). The tally of IFN regulated genes was then about 2000 (in mouse and human). There was some overlap in genes induced by types I, II and III IFNs, consistent with our knowledge of common signalling pathways [106]. As well, there were a significant number of distinct genes regulated by type I IFNs consistent with our knowledge that these IFNs activate specific pathways.

Having this resource provides a database of the full potential scope of IFN signalling. Analysis of these genes and the pathways leading to their activation is providing a broader view of the IFN response. Analysis of promoter regulatory regions of the IRGs reveals patterns that suggest that sets of genes are co-regulated. We have begun annotating the patterns and pathways and recently have published an updated version of the database with more quantitative analysis of data, thorough annotation of metadata and additional tools such as regulatory regions and tissue expression [107]. This approach has been successfully used to identify subsets of IRGs or pathological significance and the upstream regulatory pathway.

Analysis of transcriptional changes that occur in HIV infected pDC and subsequent Interferome analysis identified that despite inhibition of IFN production, a common feature of viral infection was that a subset of IRGs were induced. Subsequent analysis of the promoters of these genes identified an enrichment of IRF1 sites. These were functionally validated by ChIP experiments. Interestingly, the genes induced by this HIV activated pathway were annotated in GO as anti-apoptotic or pro-survival [108]. Another example of a functional subset of IRGs being activated was our recent study of breast cancer metastases to bone, in which we identified a subset of 540 IRGs that were enriched for IRF7 sites; the gene set and this TF were expressed in the primary tumour cells but were suppressed in metastatic tumour cells [32]. This pathway was demonstrated to activate immune responses to metastasising tumour cells: suppression was necessary to enable metastases to bone, whereas reversal of this process inhibited breast cancer metastases to bone.

One general principle evident from these and other studies is that no situation leads to the induction of all potential IFN regulated genes. Thus, the IFN response is finetuned by the activation of some, but not all possible IFN regulated pathways. It will be important to elucidate why this is if we are to understand and therapeutically manipulate this response. It is the nature, strength, duration, cellular context and activation or repression of these pathways that determine the ultimate response: on the one hand, resolution of infection, repair or healing and return to homeostasis; versus acute disease (e.g. septic shock), chronic disease or aberrant immune response and autoimmunity. We are yet to learn what determines go/no go, strong/weak, short/long signals for each IFN regulated pathway. The selective activation of

pathways likely reflects different IFN subtypes, different cell types containing different levels of signalling factors and potentially expressing different levels of receptor components on the cell surface, as well as the amount and duration of IFN exposure. This different ability of different cells to respond was elegantly illustrated in a recent report of experiments examining a population of cells that responded to a viral stimulus resulting in the production of type I IFNs. It was shown that only a low proportion of cells produced IFN. This was because when the levels of signalling components required for this pathway were measured, most cells were deficient in one or more factors and therefore did not respond. Only cells that contained relatively high levels of all components of the signalling pathway produced IFN [109]. This very important observation is likely to explain why basal IFN is produced as a primer of certain IRGs that are required for an optimal response to a danger signal.

8. Fine tuning the type I IFN response by feedback and feed forward mechanisms

We have presented several scenarios that demonstrate that type I IFN may be produced in the absence of an overt pathological stimulus. This included myeloid and osteoclast cell development, gastrointestinal tract microbiota, reproductive tract epithelium and breast cancer and normal epithelial cells. In the latter situation we have not identified an extracellular IFN stimulus, but the IFN regulator, IRF7, is expressed, as are 540 of its potential regulated genes; the impact on the immune response in this case is profound. This "physiological" production is likely to be important in priming the IFN system to ensure the levels of critical signalling factors are maintained at a sufficient level to ensure a robust response. Consistent with this thesis, we and others [110] have demonstrated that gene expression in "resting" cells from Ifnar1 null mice is different from wild type. The expression levels of 100s of (IFN regulated) genes show lower expression, presumably because of the inability of these cells to respond to endogenous IFN production. Among these genes are several, such as STAT1, that are involved in the IFN response. However it must also be remembered that this auto-regulation by IFN of its own response, can be inhibitory as reviewed above for the IFN induced inhibitors, SOCS1 and miRs. Nevertheless, the constitutive IFN does prime positively for its own response because cells with higher levels of signalling components will signal better, while others with low levels will not [109]. Some of the more interesting IRGs are sensors themselves, such as PKR and PRRs (Fig. 1).

8.1. PKR

Protein Kinase R (PKR) is an interferon regulated gene product that is constitutively expressed in all differentiated cells as an inactive protein monomer. It is an effector molecule of the innate immune system that responds to cellular stress, most typically exemplified by viral infection. Viral RNA replication intermediates, or protein activators induced by different stressors, trigger

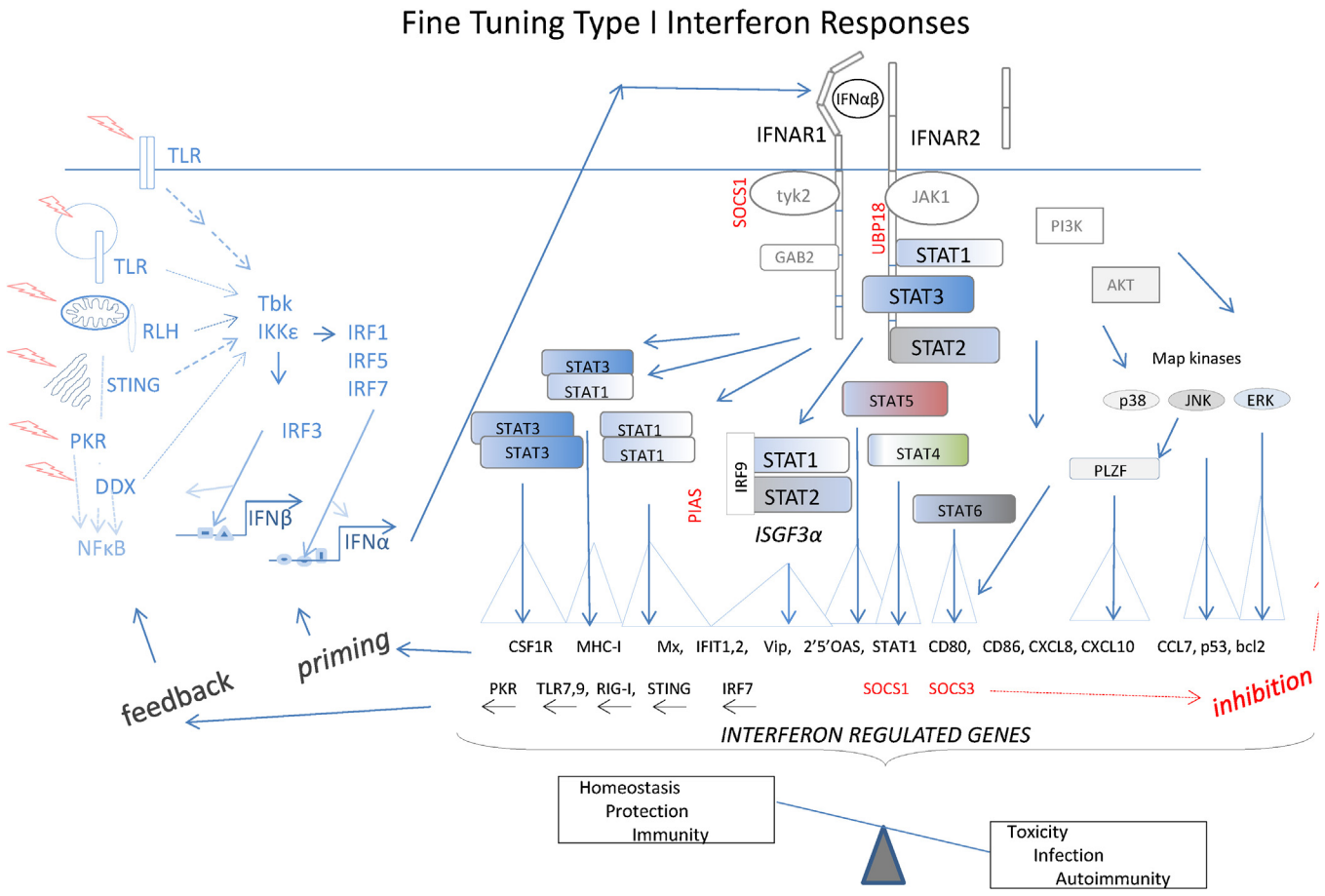

Fig. 1. Fine tuning of type I IFN responses. The figure shows a diagrammatic representation of major factors involved in the production of type I IFNs α and β by upstream pattern recognition receptors such as TLRs, RLHs, STING and DDX which sense pathogens and other stimuli in different subcellular compartments. They activate IF production by phosphorylation of IRF family members by the kinases Tbk or IKKε. IFN acts via its receptors to activate STAT pathways or alternative pathways such as PLZF which in turn contribute to the activation (or repression) of IFN regulated genes (IRGs). Regulation of PRRs constitutes positive feedback loop for its own production (reverse arrows). IFN primes its own signalling by regulating factors such as STAT1. Moreover IFN induces negative regulators (in red) such as SOCS1 and SOCS3, which act with others such as UBP 18/45 and PIAS to dampen the response.

dimerisation and autophosphorylation to generate the active enzyme [111–114]. Activated PKR inhibits protein translation through phosphorylation of eukaryotic initiation factor 2α (eIF2α). PKR has been shown to modulate different cell-signalling pathways including those controlled by the c-Jun Terminal Kinase (JNK) and nuclear factor-κB (NFκB) [115,116]. The precise mechanism by which PKR effects JNK and NFκB has not been established but, in the case of NFκB, appears to be independent of kinase activity [117]. In this case PKR is likely playing a scaffolding role interacting with the adaptor TRAF6 [118]. Recently a role has been proposed for PKR control of the actin cytoskeleton [119]. PKR associates with the key actin regulatory protein Gelsolin (GSN) and regulates its activity. Ablation of PKR affects actin dynamics and GSN-dependent processes that include cell motility, membrane ruffling, endocytosis, and phagocytosis. This identifies a mechanism where the innate immune effector protein PKR controls the actin cytoskeleton by keeping GSN in an inactive conformation. This constitutes a novel means by which the innate immune protein PKR functions in response to pathogens. PKR bolsters innate immunity by limiting pathogen entry into fibroblasts, while increasing macrophage-mediated clearance of infected cells. While the association between PKR and GSN is disrupted by activation of PKR, the active enzyme triggers cell signalling, as described above, and phosphorylates eIF2α limiting viral protein production.

8.2. PRRs

Many PRRs (TLRs, STING, RLHs, Aim2, DNA sensors) are also strongly IFN regulated. Indeed in some cases, this property was an element of their discovery. Some PRR signalling adaptors are IRGs as are some members of the IRF family of TFs, especially IRF7. The constitutive production of type I IFN (or the constitutive activation of certain pathways) thus primes the system responsible for its production to ensure optimal levels are produced in response to a danger signal such as infection. IFN also thus primes the levels of components necessary for its own production.

9. Conclusions and future directions

We have reviewed herein the state-of-the art knowledge on fine tuning type I IFN responses. The balance between a protective response, without harmful effects and a return to homeostasis is offset against the risk of infection (acute and dangerous such as sepsis or chronic infectious disease with sequelae such as cancer in the case of hepatitis B/C), toxicity and autoimmune disease, and is achieved by tightly controlled positive and negative signals. Control occurs at every level from the machinery for stimulating IFN production to its interaction with cognate receptors and transducing signals in the responding cell. We have more yet to learn about the nature and control of IFN signalling, particularly at the global level of induction and suppression of 1000s of genes in signature sets which encode important effector proteins. Much of this new information will come from the application of next generation sequencing technologies and of studies of non-coding RNAs and their role in regulation and as potential biomarkers of disease or responses. Knowledge of fine tuning of the production and response to cytokines such as type I IFNs will be a key to translating this knowledge into clinical application in diagnosing disease, therapeutic use or blocking of their action or tailoring treatments to more responsive patient groups.

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