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Cell Type-Specific Signaling in Response to Interferon-γ

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Abstract Type II interferon-γ (IFN-γ) is a pleiotropic cytokine that regulates many different cellular functions. The major signaling pathway activated by IFN-γ involves sequential phosphorylation of the tyrosine residues of the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) proteins, providing the primary mechanism through which gene expression is induced. However, recent work has revealed that the responses are complex, as shown by the activation of kinases in addition to JAKs, differential patterns of activation of STAT1, STAT3, and STAT5 in different cells, and activation of transcription factors other than STATs. This complexity is used to regulate biological functions differentially in a cell type-specific manner, by activating different specific signals and patterns of gene expression.

1 Introduction and Historical Perspective

Interferons (IFNs) are pleiotropic cytokines that were originally discovered because of their antiviral activity. Three major types have been described. The type I IFNs include α, β, ω, τ, δ, κ, and ε. The predominant forms are IFN-α, of which there are 12 separate proteins in humans, and a single IFN-β (Pestka et al. 2004). The recently identified type III IFNs, alternatively named IFN-λ, consist of interleukin-(IL)-28 and IL-29 (reviewed by Ank et al. 2006). Type II IFN, represented only by IFN-γ, is produced by NK cells, $CD4^+$ and $CD8^+$ T cells, in contrast to IFN-β, which is produced by most types of cells. IFN-γ has important immunomodulatory properties in addition to helping to protect cells from viral, bacterial, and parasitic infections (Dupuis et al. 2001; Lu et al. 1998). It is one of the major cytokines responsible for upregulating MHC class I on the surfaces of many cells and for inducing MHC class II on endothelial cells and a variety of leukocytes (Fruh et al. 1999; van den Elsen et al. 1998). In addition, IFN-γ is crucial for activating mononuclear phagocytes in the battle against infectious agents and for stimulating antigen-presenting cells to produce IL-12 and tumor necrosis factor (TNF)-α (Schroder et al. 2004). Furthermore, IFN-γ regulates the humoral immune response by effecting IgG heavy chain switching (Finkelman et al. 1988). Of note, IFN-γ exerts profound antiproliferative effects on a variety of normal and tumor cells (Schroder et al. 2004). It also plays a major role in tumor immunosurveillance, part of a more general process called cancer immunoediting, which is responsible both for eliminating tumors entirely and for sculpting the immunogenic phenotypes of the tumors that eventually do form in immunocompetent hosts (reviewed by Dunn et al. 2004a, 2004b). New, exciting results suggest that IFNs signal in a cell type-specific manner. A recent review summarizes the evidence for type I IFNs (van Boxel-Dezaire et al. 2006), and the current review focuses on IFN-γ-dependent signaling.

Investigation of the signaling pathways activated by type I and II IFNs by genetic and biochemical means led to the discovery of the JAKs and STATs, tyrosine kinases and latent transcription factors that drive the major responses to the IFNs (Darnell et al. 1994; Stark et al. 1998). Further research revealed that JAK1 and JAK2 bind tightly to the two subunits of the IFN-γ receptor, IFNGR1 and IFNGR2, respectively (Sakatsume et al. 1995; Behrmann et al. 2004). Ligand binding induces the assembly and activation of the IFNGR complex (Bach et al. 1996), leading to the cross-phosphorylation and activation of JAK1 and JAK2, and phosphorylation of the cytoplasmic domain of IFNGR1 (Igarashi et al. 1994), providing docking sites for the SH2 domains of STATs. After STAT1 has been phosphorylated on tyrosine 701, it dissociates from the receptor, forming

a homodimer through reciprocal phosphotyrosine-SH2 interactions. STAT1 homodimers translocate to the nucleus and bind to gamma-activated sequence (GAS) elements in the promoters of most IFN-responsive genes (ISGs). Similarly to IFN-γ, type I IFNs can also activate the formation of STAT1 homodimers, but they primarily activate the formation of the trimeric IFN-stimulated gene factor 3 (ISGF3), a complex of activated STAT1, STAT2, and IRF9. The IFN-stimulated regulatory element (ISRE) present in promoters of certain ISGs (Friedman and Stark 1985) binds to ISGF3 in response to type I IFNs (reviewed in Stark et al. 1998). However, some ISREs can also bind to a complex of STAT1 homodimers plus IRF-9 upon IFN-γ stimulation (Bluyssen et al. 1995; Majumber et al. 1998). Interestingly, type I IFNs can activate all seven mammalian STATs in a cell type-specific manner, all of which bind to GAS elements in ISGs (van Boxel-Dezaire et al. 2006). Notably, not only can IFN-γ activate STAT1 homodimers, but STAT1-independent pathways must also exist (Ramana et al. 2002). This aspect is discussed extensively below.

2 IFN-γ Receptor Functions

The IFNGR was initially characterized in the early 1980s by the binding of radiolabeled ligands in a variety of different cell types (reviewed by Farrar et al. 1993). These experiments indicated that most primary cells and cultured cell lines express high-affinity binding sites for IFN-γ. In addition, it was found that human and murine IFN-γ bound to their respective receptors in a strictly species-specific manner and therefore induced biological responses in species-matched cells only (reviewed in Bach et al. 1997). Pestka and colleagues (Jung et al. 1987) demonstrated, using stable murine–human somatic cell hybrids containing all the murine chromosomes but only a few human chromosomes, that all the hybrids containing human chromosome 6 bound to IFN-γ with high affinity. However, only hybrids containing both human chromosomes 6 and 21 demonstrated a biological response to IFN-γ. The human and murine genes for the ligand-binding component of the IFNGR were cloned and are localized on chromosomes 6 and 10, respectively (Aguet et al. 1988; Gray et al. 1989). This chain is IFNGR1, or IFN-γ receptor α chain. The second subunit, designated IFNGR2 or IFN-γ receptor β chain, was subsequently cloned, and the human and murine genes are localized on chromosomes 21 and 16, respectively (Soh et al. 1994; Hemmi et al. 1994).

The activation of STAT3 in response to IFN-γ is more clearly seen in murine than human cells (Costa-Pereira et al. 2005). In rat astrocytes, the GTPase RAC1 associates with IFNGR1 and is activated by JAK1 after IFN-γ stimulation.

Notably, RAC1 deficiency abolishes STAT3 activation and diminishes STAT1 activation in IFN-γ-stimulated rat astrocytes, suggesting that RAC1 may serve as an auxiliary mediator of IFN-γ signaling (Park et al. 2004). If, for instance, RAC1 were to associate only with murine IFNGR1, it could explain perhaps why STAT3 is activated more abundantly by murine IFN-γ. However, to our knowledge, no data are available concerning the possibility that RAC1 might be involved in IFN-γ-dependent signal transduction in human cells. Guanine nucleotide exchange factors such as VAV activate RAC1 by exchanging GDP for GTP. Interestingly, in murine RAW 264.7 macrophages, IFN-γ triggers the prompt, dose-dependent tyrosine phosphorylation of VAV, which is essential for activating lymphocytes. In addition, VAV binds to the SRC-related kinase HCK in murine macrophages, and antisense oligonucleotides specific for murine HCK block IFN-γ-mediated VAV phosphorylation (English et al. 1997). Whether the phosphorylation of VAV is involved in any way in the activation of STATs or other signaling pathways induced by IFN-γ is not known. It is possible that differential involvement of adaptor proteins or expression of cell-surface proteins that modify receptor functions might explain species- or cell type-specific differences in IFN-γ-dependent signaling, as discussed below.

Our recent work has revealed that the IFN-γ-induced activation of both STAT1 and STAT3 depends totally on the phosphorylation of tyrosine 419 in the cytoplasmic domain of murine IFNGR1 (Qing and Stark 2004). In contrast, when the corresponding tyrosine 440 in the cytoplasmic domain of human IFNGR1 is mutated, STAT1 is no longer phosphorylated on serine 727, but low levels of STAT1 phosphorylation of tyrosine 701 and STAT3 activation are still detectable (Costa-Pereira et al. 2005). Notably, these IFNGR1 mutant human cells are still able to induce IRF-1, MHC class I, and CIITA after IFN-γ stimulation, although at reduced levels. Because Costa-Pereira et al. (2005) used human IFNGR1$^{-/-}$ diploid fibroblasts to express human IFNGR1 mutants, whereas IFNGR1$^{-/-}$ mouse embryonic fibroblasts (MEFs) were used to express murine IFNGR1 mutants (Qing and Stark 2004), the different outcomes might be explained by differences between species, cell type-specific differences, or both. However, when mutant human Y440F IFNGR1 was expressed in murine fibroblasts, the upregulation of MHC class I, induction of IFN regulatory factor 1 (IRF1) mRNA and stimulation of nitric oxide production were totally abolished (Farrar et al. 1992), suggesting that some of the differences noted above might indeed be species-specific.

Sustained exposure to IFN-γ is harmful (Starr et al. 1998; Naka et al. 1998; Marine et al. 1999) and, therefore, it is of the utmost importance to have a variety of mechanisms in place that tightly regulate the activation of STAT1 by IFN-γ (reviewed by Wormald and Hilton 2004). Many studies show that suppressor of cytokine signaling (SOCS)-1 is the most potent inhibitor of STAT1-mediated

signaling in response to IFN-γ (reviewed by Alexander and Hilton 2004). SOCS-1 inhibits the kinase activity of JAK2 by binding directly to the active site loop domain in vitro (Yasukawa et al. 1999). In addition, SOCS-1 can polyubiquinate VAV and JAK2, which then become targets of proteasome-mediated degradation (Kile et al. 2002). By employing mutants of IFNGR1, we investigated the roles of the other tyrosines in the cytoplasmic domain of mouse IFNGR1, namely Y285, Y370, and Y441. Stronger activation of STAT1 and enhanced antiviral activity was observed only with IFNGR1 variants mutated in Y441 (Qing et al. 2005). In addition, constitutive overexpression of SOCS-1 inhibited IFN-γ-dependent signaling only in cells expressing mutants that retained Y441 and Y419. Interestingly, mutation of Y441 blocked the ability of SOCS-1 to bind to IFNGR1 in response to IFN-γ. Based on these results, we proposed that the phosphorylation of Y441 creates a docking site for SOCS-1, which then binds to JAK2 to partially inhibit JAK2 phosphorylation. Furthermore, the binding of SOCS-1 to Y441 also blocks the access of STAT1 to Y419, and this effect may be the principal mechanism of inhibition of IFN-γ-dependent signaling (Qing et al. 2005). In addition to SOCS-1, other negative regulators of IFN-γ-dependent signaling are important. SHP-2, an SH2 domain-containing protein tyrosine phosphatase that is constitutively associated with IFNGR1, inhibits STAT1 activation, probably by affecting JAK1, but without affecting the phosphorylation of IFNGR1 (You et al. 1999). Protein inhibitor of activated STAT1 (PIAS-1) acts by blocking the binding of phosphorylated STAT1 dimers to DNA and thus inhibits STAT1-mediated gene activation in response to IFN-γ (Liu et al. 1998). Finally, the nuclear phosphatase TC45 dephosphorylates STAT1 in the nucleus (ten Hoeve et al. 2002).

Other important sites in the cytoplasmic domain of IFNGR1 are leucine 270 and isoleucine 271, which play a critical role in directing receptor trafficking after ligand binding (Greenlund et al. 1994), and proline 267, which plays a dominant role in constitutive JAK1 association and thus is crucial for IFN-γ-dependent signal transduction (Kaplan et al. 1996). The cytoplasmic domain of IFNGR2 is much shorter than that of IFNGR1 (66 amino acids versus mouse or human IFNGR1, 200 and 221 amino acids, respectively). Within this domain, two closely spaced sequences, at 262–267 and 270–274, block IFNGR2 function when mutated to alanine residues (Bach et al. 1996). Co-precipitation studies showed that these sequences are necessary for the specific and constitutive association of the IFNGR2 chain with JAK2. The IFNGR1 promoter contains a GC-rich region with no TATA box, like promoters for uninducible housekeeping genes (Bach et al. 1997), suggesting that IFNGR1 expression is not regulated by external stimuli, which has been largely confirmed experimentally. However, the critical region −128 to −109 of the IFNGR1 promoter appears to possess a phorbol ester-responsive element. Binding of the transcrip-

tion factor SP1 to this element causes the upregulation of IFNGR1 expression, explaining why phorbol ester causes upregulation of IFNGR1 in differentiating monocytes (Sakamoto and Tanaguchi 2001). In contrast, the transcription of IFNGR2 seems to be tightly regulated.

Differential expression of IFNGR2 is another cause of cell type-specific responses. Potential binding sites in the mouse IFNGR2 promoter were found for SP1, AP-2, NF1, EGR, and NF-kB (Ebensperger et al. 1996). Whereas IFNGR1 is highly expressed on membranes of T, B, and myeloid cells, IFNGR2 seems to be expressed highly only on myeloid cells, moderately on B cells, and poorly on T cells (Bernabei et al. 2001). In contrast to Th2 cells, Th1 cells, which are characterized by high IFN-γ production, are unresponsive to IFN-γ because they do not express IFNGR2 (Pernis et al. 1995). Unresponsiveness is due to IFN-γ-dependent downregulation of IFNGR2 and is not directly linked to T cell differentiation: human peripheral blood T cells and murine Th2 cells also downregulate IFNGR2 upon exposure to IFN-γ (Bach et al. 1995). Notably, ligand-dependent IFNGR2 downregulation did not occur in certain fibroblast cell lines, suggesting that IFN-γ regulates the expression of IFNGR2 and concomitant sensitivity only in certain cell types. Other causes of selective IFNGR2 downregulation on T cells are stimulation with insulin-like growth factor (IGF)-1 (Bernabei et al. 2003) and uptake of iron by the transferrin receptor (Regis et al. 2005). Interestingly, primary T cells downregulate IFNGR2 but still express IFNGR1 after ligation of the T cell receptor (TCR) and IL-2 stimulation and are unresponsive to IFN-γ (Sakatsume and Finbloom 1996). Expression of IFNGR2 and thus sensitivity to IFN-γ can be restored by secondary TCR ligation or by treatment with phorbol ester, showing that the expression of IFNGR2 can also be positively regulated. Therefore, as T cells progress from primary TCR activation through IL-2-dependent proliferation, followed by secondary TCR stimulation, their responsiveness to IFN-γ varies, and this may affect their ability to participate in an ongoing immune response (Sakatsume and Finbloom 1996). Other factors that upregulate IFNGR2 on T cells are serum deprivation (Bernabei et al. 2001), exposure to nitric oxide (Allione et al. 1999), or low extracellular pH (Bosticardo et al. 2001). It is not clear why T cells are particularly sensitive to regulation of IFNGR2 expression in contrast to other cell types.

3
Activation of Kinases Other than JAKs

JAK-STAT signaling alone is not sufficient to explain all the biological effects of IFN-γ and several other kinase pathways have emerged as critical additional components of IFN-γ-induced signal transduction. First of all, the phos-

phorylation of STAT1 on Y701 is not enough to induce the full expression of IFN-γ-induced genes: additional phosphorylation of serine 727 is necessary (Wen et al. 1995; Kovarik et al. 2001; Varinou et al. 2003). Serine phosphorylation of STAT1 facilitates the association of chromatin-bound STAT1 with the co-activator CBP and the subsequent recruitment of histone acetylases, important for chromatin remodeling (Varinou et al. 2003). Inhibition of p38 mitogen-activated protein kinase (P38MAPK) led to defective serine phosphorylation of STAT1 in fetal brain astrocytes after stimulation with IFN-γ (Lee et al. 2003). In addition, the function of serine-phosphorylated STAT1 was dependent on P38MAPK activation after stimulation of human epithelial cells or mouse fibroblasts with type I or type II IFN (Goh et al. 1999). MKK6 turned out to be the upstream activator of P38MAPK in these cells (Goh et al. 1999). Of note, dsRNA-activated protein kinase (PKR)-deficient MEFs show defective phosphorylation of S727 upon IFN-γ stimulation, suggesting that PKR might function upstream of P38MAPK in these cells (Ramana et al. 2000b). In several different cell types activation of phosphatidylinositol 3-kinase (PI3K) and subsequently AKT by IFN-γ are needed to phosphorylate S727 (Nguyen et al. 2001). A member of the protein kinase C (PKC) family, PKC-δ, is rapidly activated in human promyelocytic cells downstream of PI3K and associates with STAT1, which then is phosphorylated on S727 (Deb et al. 2003). Notably, the activation of PKC-δ and serine phosphorylation of STAT1 are crucial for induction of pro-apoptotic genes and mitochondria-dependent apoptosis (DeVries et al. 2004). Other PKC family members might also be involved in cell type-specific responses to IFN-γ. An IFN-γ-induced PI3K/PKC-ε/MAPK signaling pathway is involved in S727 phosphorylation in mesangial cells (Choudhury 2004). In contrast, in human embryonic kidney cells, PCK-ε seems to be involved in the tyrosine phosphorylation of STAT1 (Ivaska et al. 2003), but this might occur through the activation of SRC-family kinases instead of MAPKs, as discussed below. In T cells, IFN-γ activates a PI3K/mTOR/PKC-θ/MKK4 signaling pathway, which does not affect the tyrosine phosphorylation of STAT1. However, since the transcription of GAS-containing genes is enhanced by this pathway, it is likely that this enhancement is also a result of increased phosphorylation of S727 (Srivastava et al. 2004). Although which MAPK is activated downstream of PI3K and PKC activation was not investigated in any of the above-mentioned studies, it is possible that a serine-threonine kinase such as P38 (Goh et al. 1999) or perhaps c-Jun kinase (JNK; Zhao et al. 2005) is directly responsible for serine phosphorylation of STAT1. The IFN-γ-stimulated signaling pathway that is emerging from all of these data is: PI3K→AKT→PKC(-δ, -ε, or -θ)→MKK(4 or 6)→P38MAPK→ serine phosphorylated STAT1. The cell type-specific variation of this proposed pathway seems to be the activation of different PKC and MKK family members.

The adaptor protein that couples the activated IFNGR to PI3K activation is presently unknown, but the CRK/CBL adaptor protein complex has been proposed to play this role (Platanias 2005).

Different cells are likely to employ alternative strategies to phosphorylate STAT1 on S727. IFN-γ has been shown to elicit a calcium ion flux in thyroid cells, microglia, neutrophils, T cells, monocytes, and fibroblast-like cells, suggesting that an increase of free calcium ions is involved in IFN-γ-dependent signaling in several cell types (Aas et al. 1999; Koide et al. 1988; Kung et al. 1995; Buntinx et al. 2002; Franciosi et al. 2002; Nair et al. 2002). In response to IFN-γ, human fibrosarcoma cells and MEFs activate calcium/ calmodulin-dependent kinase (CAMK) II, which can interact directly with STAT1 and induce the phosphorylation of S727 in vitro (Nair et al. 2002). In keratinocytes, an increase in free calcium ions leads to activation of the annexin II/PYK2/ MEKK4/ MKK6/P38 MAPK/ATF2 signaling pathway upon IFN-γ stimulation (Halfter et al. 2005). It is likely that P38 MAPK activation through this pathway also increases the serine phosphorylation of STAT1 and the consequent enhancement of ISG-transcription, although the authors did not investigate this point. Because the serine-threonine kinase CAMKII and the calcium-regulated tyrosine kinase PYK2 are both sensitive to an increase in calcium ions, it is possible that these two pathways are intertwined, particularly since it has also been described that transcription factors such as CREB, ATF, and C-EBP-β are substrates of CAMKII (White et al. 1998; Cruzalegui et al. 2000). Indeed, IFN-γ also activates CREB and C-EBP-β in addition to ATF2 (see Sects. 6.1 and 6.2). However, more research is needed to comprehend the individual, and possibly overlapping signaling pathways that lead to the activation of these transcription factors and subsequent cell type-specific transcription. It is possible that the activation of PYK2 in certain cell types leads to downstream enhancement of the serine phosphorylation of STAT1 in addition to the activation of additional transcription factors. Interestingly, PYK2 activation by IFN-γ leads to the activation of another MAPK, ERK2, eventually leading to the serine phosphorylation of STAT1 and maximal transcriptional activation in MEFs (Takaoka et al. 1999). The adaptor protein GRB2 complexed with SOS might couple the activation of PYK2 to ERK activation in response to IFN-γ (Blaukat et al. 1999). However, the coupling of PYK2 with another adaptor protein such as CRK leads to activation of JNK (Blaukat et al. 1999), suggesting that IFN-γ-activated PYK2 might be involved in the activation of multiple downstream signaling pathways by coupling to different adaptor proteins.

Although, as described above, IFN-γ-induced PKC activation leads to MAPK activation, PKC also seems to be involved in activating SRC-family tyrosine kinases. In human alveolar epithelial cells, IFN-γ activates PLC-γ2 via an upstream tyrosine kinase to induce the activation of PKC-α and c-SRC or LYN,

resulting in the activation of STAT1 and expression of ICAM-1, and thus the initiation of monocyte adhesion (Chang et al. 2002). SRC family kinases are required for IFN-γ to activate STAT3 (but not STAT1) by tyrosine phosphorylation, whereas JAK1 and JAK2 are required to activate both STAT1 and STAT3 in MEFs (Qing and Stark 2004). FYN could be involved in STAT3 activation, because this SRC-family member associates through its SH2 domain with activated JAK2 upon IFN-γ stimulation (Uddin et al. 1997). Interestingly, the tyrosine kinase PYK2 amplifies c-SRC-dependent STAT3 activation in response to epidermal growth factor (Shi et al. 2004), and it is possible that it does the same in response to IFN-γ, because PYK2 becomes phosphorylated upon stimulation of MEFs by IFN-γ (Takaoka et al. 1999).

In addition to affecting the activation of STATs and other transcription factors, the activation of kinases other than JAKs seems to be involved in activating other signaling pathways. For instance, the activation of mTOR downstream of PI3K leads to selective regulation of the translation of IFN-γ-induced mRNAs, but not transcription, by activating p70S6K and phosphorylating the S6 ribosomal proteins, and by phosphorylating the repressor of mRNA translation EIF4E-binding protein 1 (4EBP1), which deactivates 4EBP1, leading to its dissociation from EIF4E and the subsequent initiation of translation (Platanias 2005). In addition, treatment with IFN-γ leads to the tyrosine phosphorylation of CBL, followed by the sequential activation of C3G and RAP1, resulting in subsequent growth inhibitory effects in promyelocytic cells (Alsayed 2000). Furthermore, studies performed with MEFs that lack both the α and β subunits of IKK revealed that a subset of IFN-γ-induced genes is dependent on IKK activation (Sizemore et al. 2004). The IKK complex is best known as a regulator of NF-κB-dependent signaling and its effect on IFN-γ-dependent signaling is currently being studied in our laboratory. Finally, IFN-γ induces a MEKK1/MEK1/ERK/ C/EBP-β signaling pathway to induce the transcription of GATE-dependent ISGs (see also Sect. 6.2 and Roy et al. 2002). In summary, it is well accepted that, in addition to the JAKs, several different kinases are activated in response to IFN-γ, and one can safely predict that the cell type-specific expression of these kinases and their substrates will help to determine cell type-specific responses.

4
Differential Activation of STATs in Different Cell Types

Type I IFNs are unique for their ability to activate all seven known mammalian STATs. Cell-type specific activation of various STATs by type I IFNs has been reviewed recently (van Boxel-Dezaire et al. 2006). IFN-γ mediates its important

antiproliferative effects through the activation of STAT1 and the downstream induction of IRF-1, FAS/CD95, and proteolytic cleavage of caspases 2, 3, and 7 (Bromberg et al. 1996; Porta et al. 2005; Sironi et al. 2004). Although it was first thought that STAT1 was the sole mediator of responses to IFN-γ, more recent work has shown that biologically significant STAT1-independent pathways must be active in addition (Ramana et al. 2000a, 2002). For instance, STAT1-deficient mice are more resistant to murine cytomegalovirus (MCMV) and Sindbis virus than are IFNGR, IFNAR double knockout mice (Gil et al. 2001). In addition, IFN-γ suppresses the cell growth induced by growth factors and cytokines, but enhances the proliferation of STAT1-deficient bone marrow macrophages and MEFs (Gil et al. 2001; Ramana et al. 2000b). Stimulation of cell growth could be explained partly by the fact that only in STAT1-deficient cells are the immediate-early genes *c-myc* and *c-jun* induced (Ramana et al. 2000b). Of note, our more recent data indicate that the activation of STAT3 is increased and prolonged in STAT1-deficient MEFs, and this effect is probably responsible for activating most or all of the IFN-γ-induced ISGs in these cells (Qing and Stark 2004; Ramana et al. 2005). Y419 of murine IFNGR1 is needed to activate both STAT1 and STAT3 in wild-type MEFs upon IFN-γ stimulation, revealing that STAT1 and STAT3 compete for the same binding site (Qing and Stark 2004). A previous study showed that the SH domain of STAT1 has a much higher affinity for tyrosine 419 of IFNGR1 than does the SH domain of STAT3 (Greenlund et al. 1995), explaining why STAT1 is preferentially activated in response to IFN-γ. Of note, when STAT1 levels are low or when STAT1 is absent, STAT3 becomes more highly activated in MEFs (Qing and Stark 2004). In contrast, bone marrow macrophages from STAT1-deficient mice activate even less STAT3 than do the cells from wild-type mice (Gil et al. 2001), suggesting that different cell types have different intrinsic capacities to activate STAT3 in response to IFN-γ.

Interestingly, in addition to STAT1, STAT3 becomes abundantly activated in IFN-γ-activated rat astrocytes. In these cells, STAT3 activation is totally dependent on RAC1 expression (Park et al. 2004), which suggests that species and or cell type-specific activation of STAT3 by IFN-γ might depend on differential adaptor protein expression. Murine adipocytes activate both STAT1 and STAT3 in response to IFN-γ (Stephens et al. 1998). Notably, eosinophils and (mature) monocytes isolated from peripheral human blood activate only STAT1, whereas human neutrophils activate both STAT1 and STAT3 in response to IFN-γ (Caldenhoven et al. 1999). Furthermore, IFN-γ delays the apoptosis of human neutrophils by activating STAT3 and subsequently inducing cellular inhibitor of apoptosis 2 (cIAP2) mRNA and protein expression

(Sakamoto et al. 2005). Notably, the activation of STAT3 and ERK1/2 by IFN-γ is crucial for antagonizing signaling by TGF-β in human renal tubular epithelial cells (Giannopoulou et al. 2006). Interestingly, retinas from mouse embryos and neonates activate STAT3 but not STAT1 after stimulation by IFN-γ (Zhang et al. 2005). Immature mouse macrophages show reduced binding of STAT1 to GAS elements, despite normal activation of STAT1 by IFN-γ, and high constitutive expression of PIAS-1 in immature macrophages could explain these findings (Coccia et al. 2002). In contrast, human neonatal monocytes and monocyte-derived macrophages do not activate STAT1 in response to IFN-γ, despite normal expression levels of IFNGR and STAT1 (Marodi et al. 2001). The authors suggest deficient IFNGR signaling as an explanation, but activation of other STATs was not investigated. Notably, another study showed that neonatal macrophages respond selectively to IFN-γ, in that CIITA mRNA was not induced but other mRNAs were, such as IRF-1 (Lee et al. 2001). Since the induction of CIITA mRNA by IFN-γ depends on activated STAT1, USF-1, and IRF-1 (Muhlethaler-Mottet et al. 1998; O'Keefe et al. 2001), whereas IRF-1 mRNA can be induced by activated STAT1, STAT3, or STAT5 (Heim et al. 1999), these data collectively suggest that neonatal monocytes activate STAT3 or STAT5 instead of STAT1 in response to IFN-γ.

Interestingly, IFN-γ activates STAT5b in human promyelocytic cells, but not in human epithelial cells, whereas IFN-α activates STAT5a in both cell types (Meinke et al. 1996). IFN-γ, which promotes differentiation but not proliferation of human promyelocytic cells, activates STAT5b only in immature monocytes; monocytic differentiation leads to a strong decrease in IFN-γ-mediated activation of STAT5b but not of STAT1 (Meinke et al. 1996). Of note, STAT5 activation by IFN-γ requires Y419 or Y440 of mouse or human IFNGR1, respectively, and the binding of SOCS-3 to this tyrosine residue inhibits STAT5 activation by IFNGR (Woldman 2001). The biological significance of STAT5 activation by IFN-γ is also demonstrated by the fact that activation of STAT5b is crucial to induce IGF-I production in primary human dermal fibroblasts (Hwa et al. 2004). Interestingly, IFN-γ preferentially activates STAT5b in these cells, but in the absence of STAT5b IFN-γ will activate STAT5a, which does not lead to IGF-1 induction. In summary, it is becoming clear that STAT3 and STAT5 are also activated in response to IFN-γ, often in a cell type-specific or maturation stage-specific manner. However, much more research is needed to understand how and why different cell subtypes activate different STATs and also to comprehend fully how the activation of each specific STAT in each specific cell type leads to the induction of specific genes, thus contributing to specific biological responses.

5 Priming

5.1 How Prior Exposure to Other Cytokines Affects Responses to IFN-γ

Exciting new data have emerged indicating that, especially during inflammation, pre-exposure to certain cytokines profoundly changes how cells respond subsequently to IFN-γ. One obvious mechanism through which priming can effect IFN-γ-dependent signaling is by enhancing the expression or activity of specific transcription factors. Notably, priming of human macrophages with low doses of IFN-γ for 2 days leads to increased STAT1 activation when the macrophages are subsequently restimulated with a slightly higher dose of IFN-γ for 10 min (Hu et al. 2002). This phenomenon could be explained by increased STAT1 levels due to IFN-γ priming (Hu et al. 2002), in line with previous results showing that activated STAT1 and IRF-1 drive the expression of STAT1 mRNA and STAT1 protein (Pine et al. 1994; Harada et al. 1994; Wong et al. 2002). These finding are particularly significant, because ligand-induced feedback inhibition and desensitization occur normally, as demonstrated by the priming effects of type I IFN on subsequent stimulation by type I IFN (Sakamoto et al. 2004). Of note, IFN-γ-primed macrophages also show a changed pattern of IFN-γ-induced gene activation. Expression of the STAT1-dependent genes *CCR2*, *IP10*, and *IRF1* are increased, but expression of the STAT3-dependent genes *EGR2*, *BCL2A1*, *IL1B*, *IL6*, *MMP1*, *RANTES*, *VCAM1*, and *FAS*, implicated in tissue destruction during inflammation, is attenuated (Hu et al. 2002, 2005). In this manner, priming by IFN-γ may deliver a homeostatic signal by attenuating the IFN-γ-dependent induction of certain tissue-destructive genes (Hu et al. 2005). One way that activated STAT3-dependent gene expression is attenuated is by downregulation of STAT3 function by increased STAT1 levels, as predicted by our finding that STAT1 and STAT3 compete for binding to Y419 of IFNGR1 (Qing and Stark 2004). For that matter, since the activation of STAT5 by IFN-γ also depends on Y419 (Woldman 2001), priming by IFN-γ could also lead to diminished STAT5 activation upon subsequent stimulation by IFN-γ due to increased competition by STAT1.

In contrast to IFN-γ, stimulation by IL-6 leads to increased STAT3 levels (Narimatsu et al. 2001; Yang et al. 2005). Based on our findings, we predict that priming by IL-6 would result in enhanced STAT3 activation by IFN-γ. In accordance, priming of human neuroblastoma cells for 5 h with cytokines from the GP130 family (such as CNTF or IL-6) leads to the activation of STAT3 in addition to STAT1 after stimulation by IFN-γ, whereas unprimed nerve cells activate only STAT1 in response to IFN-γ (Kaur et al. 2003). However, priming

by CNTF or IL-6 for 5 h left STAT3 levels unchanged. Expression of mutant GP130 revealed that a functional STAT binding site and a functional SHP-2 binding domain are both needed for subsequent IFN-γ-induced STAT3 activation. In addition, treatment with kinase or protein synthesis inhibitors during the priming phase revealed that MAPK and PKC activation, in addition to new protein synthesis, were crucial for STAT3 activation by IFN-γ in nerve cells (Kaur et al. 2003). The authors proposed that cross-talk between GP130 cytokines and IFN-γ involves the induction of an as yet unknown gene that requires STAT, PKC, and MAPK activation, and that the product of this gene is necessary to promote the interaction of STAT3 with the IFNGR. More studies are needed to determine the mechanism of these phenomena in nerve cells, and also to investigate whether increased STAT3 levels in response to IL-6 lead to increased STAT3 activation by IFN-γ in other cell types.

Similarly to IFN-γ, stimulation with IFN-β leads to a subsequent increase in STAT1 levels through activation of STAT1 homodimers (Pine et al. 1994; Harada et al. 1994). However, priming by IFN-β decreases the transcriptional activation of genes normally induced by IFN-γ in human fetal astrocytes and mouse macrophages (Gao et al. 2000; Hua et al. 2002), indicating that increased levels of STAT1 do not automatically lead to increased STAT1-dependent transcriptional activation by IFN-γ. The transcriptional defect caused by IFN-β priming could be related to decreased STAT1 activation or decreased binding of activated STAT1 to GAS-containing ISGs after IFN-γ stimulation (Revel et al. 1995; Gao et al. 2000; Hua et al. 2002). Furthermore, the decrease in STAT1 phosphorylation concerns only the transactivating form of STAT1 (STAT1α), whereas phosphorylation of STAT1β, which lacks the transactivation domain and thus is potentially a negative regulator, is even slightly increased in macrophages (Gao et al. 2000). Notably, priming macrophages with IFN-β results in an increase in the kinetics of STAT1α dephosphorylation, suggesting that priming causes these changes by increasing protein tyrosine phosphatase activity (Gao et al. 2000). These results are in line with data showing that the desensitizing effect of priming by IFN-β on subsequent IFN-β stimulation of fibroblasts is due to increased activity of the tyrosine phosphatase TC-PTP (Sakamoto et al. 2004). However, decreased activity of a tyrosine kinase due to IFN-β priming cannot be excluded. Indeed, prolonged incubation with LPS also leads, via the intermediate production of IFN-β, to diminished IFN-γ-induced STAT1 activation (Crespo et al. 2002; Dalpke et al. 2003). In these studies, the IFN-β-stimulated induction of CIS, SOCS-1, and SOCS-3 could be related to decreased STAT1 activation by inhibited tyrosine kinase activity of the JAKs. In summary, priming can positively or negatively change the quantity and quality of signal transduction by IFN-γ, and several possible mechanisms have been described. Therefore, the nature of the IFN-γ response can be altered

or reprogrammed. This flexibility is certain to play an important role during infection and inflammation, since in both situations cells are exposed repeatedly to bursts of cytokines. It remains to be established whether IFN-γ-induced signaling can be altered similarly in various blood cell subsets after priming, especially with IFN-β, IL-6, or IFN-γ. More work is also needed to determine exactly how long the effects of priming last.

5.2 Receptor Cross-talk

In mouse cells, IFN-γ and IL-6 cannot signal properly in the absence of type I IFN receptors, indicating constitutive cross-talk between these cytokine receptors. The low levels of IFN-β always present in tissues cause IFNAR1 to interact with IFNGR2 and GP130 in caveolar domains of plasma membranes (Taniguchi and Takaoka 2001). The associated IFNAR1 may provide an additional docking site that promotes the dimerization of STATs and concomitant increases in DNA binding and transcription in response to IFN-γ or IL-6, or transphosphorylation of the receptors may lead to enhanced STAT activation (reviewed by Taniguchi and Takaoka 2001; Ivashkiv et al. 2003). Of note, all of the data cited above were generated with mouse cells, and it is not known whether the same receptor cross-talk also takes place in human cells, or whether it is restricted to certain cell types. Tanaguchi and Takaoka (2001) suggest in their "revving-up model" that low-dose priming with type I IFN is necessary and results in normal IL-6 and IFN-γ signaling. In contrast, as discussed above, higher doses of type I IFNs abolishes STAT1 activation, and perhaps also STAT3 and STAT5 activation by IFN-γ. It is also not known whether priming by IL-6 via GP130 influences IFN-γ-dependent signaling via the proposed connection to IFNAR1. Alternatively, the lipid raft-STAT signaling hypothesis suggests that membrane complexes that contain STATs may be sites where the combinatorial effects of different cytokines and different activation pathways are integrated (Sehgal et al. 2002).

We discuss above that stimulation by LPS through TLR4 negatively influences IFN-γ-dependent signaling via the intermediate production of IFN-β and via priming by IFN-β. Nevertheless, IFN-γ signaling can be enhanced by the simultaneous stimulation of defined TLR agonists or whole-bacterial lysates and IFN-γ (Dalpke et al. 2003). Short-term co-stimulation results in the amplification of IFN-γ-dependent signaling attributable to P38MAPK-dependent serine phosphorylation of STAT1 in macrophages (Dalpke et al. 2003). The data suggest that cross-talk between IFNGR and TLRs can also positively influence IFN-γ-dependent signaling. Similarly, IFN-γ and LPS synergistically induce

the expression of pro-inflammatory factors such as IL-1, IL-6, IL-12, NO, and TNF-α (Zhao et al. 2006). Comparable synergism is observed between IFN-γ and peptidoglycan (a TLR ligand) or poly(I:C) (a TLR3 ligand) in inducing the IL-12 promoter. Notably, IRF-8-deficient macrophages do not show enhancement of LPS-induced ERK and JNK phosphorylation by IFN-γ, and the synergistic production of pro-inflammatory factors by LPS and IFN-γ is also lost. Zhao et al. (2006) suggest that the observed interaction between IRF-8 and TRAF6 modulates TLR signaling and may contribute to the cross-talk between the IFN-γ and TLR signaling pathways.

In addition to the known synergism between simultaneous TNF-α and IFN-γ signaling, leading to macrophage activation, there seems to be cross-talk between TNF-α- and IFN-γ-induced signaling. Normally, activated TNF-α receptor 1 (TNFR1) recruits TRADD, which in turn triggers two opposing pathways, leading to either caspase activation and induction of apoptosis or to NF-κB activation and induction of anti-apoptosis genes. In human epithelial cells, the association of STAT1 with TRADD and FADD increases after TNF-α binds to TNFR1 (Wang et al. 2000). STAT1-deficient cells show an apparent increase in the formation of TNF-α-induced TRADD–Rip and TRADD–TRAF2 complexes, leading to enhanced NF-κB activation (Wang et al. 2000). These data indicate that STAT1 acts as a negative regulator of signaling through TNFR1. Interestingly, the binding of IFN-γ to IFNGR causes nuclear translocation of STAT1, which might inhibit STAT1 from being recruited to the TNFR1, providing an explanation for why TNF-α-dependent activation of NF-κB is stronger in the presence of IFN-γ signaling (Wesemann and Benveniste 2003). Notably, stimulation by IFN-γ causes the formation of a TRADD-STAT1α complex in the nucleus. Because IFN-γ-mediated STAT1 activation is prolonged in macrophages in which the expression of TRADD is knocked down, TRADD is identified as a negative regulator of STAT1 activation by IFN-γ (Wesemann et al. 2004).

Finally, there is evidence of cross-talk between the IFNGR and the B cell antigen receptor (BCR) or T cell antigen receptor (TCR). Cross-linking of the BCR or TCR enhances the serine phosphorylation of STAT1 and consequent increased induction of STAT1 target genes (Zu et al. 2005). Enhancement of STAT1 serine phosphorylation by cross-linking of BCR involves the activation of P38MAPK and CaMKII, indicating that signaling pathways other than those stimulated by cytokines can also modulate signaling by IFN-γ (Xu et al. 2005). Interestingly, T cell activation 4 h in advance of IFN-γ stimulation leads to the loss of STAT1 phosphorylation, most likely due to the induction of SOCS1 (van de Wiele et al. 2004). However, loss of sensitivity to IFN-γ is still apparent after 3 days, and a mechanism other than inhibition of STAT1 activation by SOCS1 seems to be the reason.

6
New Signaling Pathways

6.1
Activation of Transcription Factors Other than STATs

In addition to differential STAT activation in various cell types (discussed in Sect. 4), differential activation of transcription factors other than STATs is likely to be another strategy used by various cell types to generate specific responses to IFN-γ. Notably, Oncostatin M activates STAT1, but does not activate the STAT1-dependent genes TAP1 and IRF-1, as does IFN-γ, suggesting that signals in addition to STAT1 activation must emanate from the IFNGR (Mahboubi et al. 2002). Interestingly, the constitutive transcription factor SP1 collaborates with STAT1 in inducing certain IFN-γ-stimulated genes. Examples are the *ICAM-1* and *MCP-1* genes in human epithelial and astrocytoma cells (Look et al. 1995; Zhou et al. 1998). Activated STAT1 and SP1 bind, respectively, to GAS elements and GC-rich sequences (GC boxes) to induce transcription (Zhou et al. 1998). Interestingly, the transcription factor AP-1 is critical for the constitutive and IFN-γ-stimulated expression of IFI16 (Clarke et al. 2003). It is likely that AP-1 collaborates with STAT1 in the IFN-γ-dependent induction of IFI16, because two GAS-like elements have been identified in the promoter of this gene (Trapani et al. 1994). Constitutively expressed transcription factors can also negatively regulate IFN-γ-dependent transcription. The promoter of MCP-1 contains an IFN-responsive-inhibitory sequence (IRIS) that consists of a 13-bp CT-rich sequence adjacent to the GAS site (Valente et al. 1998). In gel-shift assays, STAT1 and an as-yet-unknown constitutive transcription factor present in nuclear extracts from IFN-γ-stimulated osteoblastic cells bind to a probe containing the GAS/IRIS.

Transcription factors of the IRF family also collaborate with STAT1 for the induction of certain IFN-γ-dependent genes. IRF-1 is expressed weakly in most cells, but after IFN-γ stimulation IRF-1 is induced quickly by the binding of activated STAT1α to the GAS element in the promoter (Pine et al. 1994). IRF-1 is subsequently involved in the induction of many IFN-γ-stimulated genes through binding to ISREs (Dror et al. 2007). Of note, the following genes are expressed because of the cooperative action of IRF-1 and activated STAT1: *STAT1* (Pine et al. 1994; Wong et al. 2002), *CIITA* (Muhlethaler et al. 1998), *GP91PHOX* (Kumatori et al. 2002), indoleamine 2,3-dioxygenase (*IDO*) (Chon et al. 1996), *vcam1*, *icam1*, *mig*, *ena78*, *itac*, and *ip10* (Jaruga et al. 2004). IFN-γ activates the transcriptional activity of IRF-1 by activating PKC-α (at least in mouse macrophages), which results in post-translational modifications of IRF-1 such as tyrosine phosphorylation, but perhaps also acetylation (Sharf

et al. 1997; Giroux et al. 2003). Additional transcription factors can assist activated STAT1 and IRF-1 in inducing some of these genes in response to IFN-γ. For instance, the induction of the transactivator CIITA also requires the cooperation of USF-1, a constitutively and ubiquitously expressed transcription factor (Muhlethaler et al. 1998). CIITA is subsequently involved in inducing MHC Class II by IFN-γ (reviewed by van den Elsen 2004). As mentioned above, the expression of ICAM-1 needs the cooperative action of the constitutive transcription factor SP1. Furthermore, the induction of IDO requires the activation of PKR and NF-κB and MIG/CXCL9 induction requires NF-κB activation in addition to activated STAT1 and IRF-1 after stimulation by IFN-γ (Du et al. 2000; Hiroi et al. 2003).

All the transcription factors mentioned above are not expressed in a cell type-specific manner, and thus are unlikely to be responsible for cell type-specific gene induction by IFN-γ. GP91(PHOX), an essential component of NADPH oxidase, which is crucial for generating superoxide anions that kill ingested microorganisms, is an example of a gene that is expressed only in differentiated cells of the myeloid linage (Royer-Pokora et al. 1986). Study of *GP91PHOX* transcription might provide clues about the way IFN-γ induces cell type-specific genes. Interestingly, in addition to the activation of STAT1 and IRF-1 (Kumatori et al. 2002), serine/threonine phosphorylation of PU.1 by PKC-α- or PKC-βI is needed for *GP91PHOX* expression in monocytes (Mazzi et al. 2004). Notably, PU.1, a member of the ETS family, is selectively expressed in neutrophils, monocytes, and B cells (Suzuki et al. 1998), probably explaining why *GP91PHOX* is stimulated by IFN-γ only in myeloid cells. In addition, in myeloid cell lines, PU.1 interacts with IRF-1, IRF-8, and CBP to form the complex hemopoietic-associated factor-1 (HAF-1), which plays an important role in activating the *GP91PHOX* promoter in response to IFN-γ in myelomonocytic cells (Eklund et al. 1995). IRF-8 is also expressed selectively in myeloid cells and functions primarily in macrophages and some types of dendritic cells (reviewed by Kanno et al. 2005). Therefore, the myeloid-specific activation of PU.1 and IRF-8 can explain why GP91(PHOX) is selectively induced in monocytes/macrophages by IFN-γ. Interestingly, IRF-8 expression also directs the differentiation of myeloid progenitor cells into mature macrophages (Tamura et al. 2000) and drives the development of plasmacytoid dendritic cells (Tsujimura et al. 2003). Based on all of these results, we propose that combinatorial interactions between cell type-specific and developmentally restricted transcription factors and ubiquitous factors account for cell type-specific gene expression in response to IFN-γ.

PU.1 also cooperates with STAT1 in inducing FcγRI upon stimulation by IFN-γ of myeloid cells. Interestingly, PU.1 is required for both basal and IFN-γ-induced promoter activity, whereas activated STAT1 is needed only for

IFN-γ-induced activation of *FCGRI* (Aittomaki et al. 2002). Furthermore, the STAT1-mediated activation of the *FCGRI* promoter critically requires CBP/p300 (Aittomaki et al. 2002), and this requirement is likely to be important for the expression of many IFN-γ-dependent genes (Zhang et al. 1996). In human monocytic cells, additional requirements for FcγRI induction by IFN-γ are PKR-dependent serine phosphorylation of STAT1 and activation of the NF-κB pathway (Karehed et al. 2007). Another transcription factor that collaborates with STAT1 and CBP/p300 is GATA-1, which is involved in the IFN-γ-mediated induction of HLA-E. Notably, a cell type-restricted enhancer has been identified in the promoter of HLA-E, which binds to GATA-1 upon stimulation with IFN-γ (Barrett et al. 2004). Activation of the annexin II/PYK2/MEKK4/MKK6/P38 MAPK pathway by IFN-γ in keratinocytes is needed to activate the transcription factor ATF2 (Halfter et al. 2005). The authors propose that ATF2 activation by IFN-γ might induce the expression of COX2, but this has not been proven yet. ATF2 will certainly not induce this expression by itself, because others have shown that COX2 induction in response to IFN-γ depends on binding of IRF-1 to ISRE elements in the *cox2* promoter (Blanco et al. 2000). Finally, the transcription factor CREB becomes activated in murine macrophages through the activation of cAMP and PKA following stimulation by IFN-γ (Liu et al. 2004). It is not yet clear which IFN-γ-stimulated genes depend on transcriptional activation by CREB or ATF2, or whether these transcription factors operate totally independently of STAT1. We speculate, based on the papers discussed above, that transcription factors in addition to STATs, such as PU.1, IRF-8 and perhaps CREB, and additional yet-to-be-defined factors are necessary for cell type-specific gene induction in macrophages in response to IFN-γ. These unique macrophage-related signaling pathways must also play a role in the IFN-γ-dependent induction of genes involved in the novel cell type-specific antiviral activation of these cells, which does not involve the induction of IFN-α/β, TNF-α, PKR, RNASEL, or MX1 (Presti et al. 2001).

6.2 STAT-Independent Signals

In Sect. 6.1, we described many examples of collaborative action between STAT1 and other transcription factors needed for the expression of certain genes in response to IFN-γ. Similarly, it is likely that IFN-γ-activated STAT3 or STAT5 cooperates with other transcription factors. However, it is possible that the activation of additional transcription factors leads to the induction of genes without the help of any STAT. It remains to be discovered whether the IFN-γ-dependent, STAT1-independent pathways such as PI3K-dependent monocyte adhesion and induction of concentrative nucleoside transporters

(CNT) in macrophages are truly independent of any STAT (Navarro et al. 2003; Soler et al. 2003). Importantly, a novel type of GAS element with an IRF/ETS binding site has been described that functions independently of STAT1 (Contursi et al. 2000). In a murine macrophage-like cell line, PU.1 and IRF-8 bind to the GAS element present in the promoter of *irf8* (Contursi et al. 2000; Kanno et al. 2005). DNA sequence motif comparison revealed that GAS elements can be divided into two subtypes. One is the classical AAA/TTT palindrome, which contains no IRF/ETS binding motif; examples from this group are PML-GAS and GBP-GAS. The other includes a novel IRF/ETS composite element (Kanno et al. 2005), and examples are GAS elements in the promoters of *irf8*, *irf1*, *CCL2*, *TLR3*, and *cathepsin E preprotein* (Kanno et al. 2005). Notably, all of these genes are important for immune cell functions, particularly in macrophages and dendritic cells.

Other IRF family members can also mediate IFN-γ-stimulated gene induction in a STAT-independent manner. IFN-γ stimulates the expression of the polymeric immunoglobulin receptor (PIGR), which is expressed constitutively on the basolateral surfaces of secretory epithelial cells, where it directs polymeric IgA and pentameric IgM to exocrine secretions (Piskurich et al. 1997). There are three ISRE elements in the promoter of *PIGR*; the two upstream elements are bound to constitutive transcription factors and the third binds to IRF-1 upon IFN-γ stimulation of a human epithelial cell line (Piskurich et al. 1997). Furthermore, constitutive expression of the exonuclease ISG20 depends on the constitutive transcription factors SP1 and USF-1. In contrast, IFN-γ-stimulated expression of ISG20 depends on the binding of IRF-1 to the ISRE element in the promoter, which contains no functional GAS elements (Gongora et al. 2000). These two IFN-γ-activated promoters are driven by the binding of activated IRF-1 to ISRE elements independently of any STAT.

It is becoming clear also that not all ISRE elements are equivalent. Sequence alignment of ISREs has revealed three subtypes thus far (Meraro et al. 2002). The classical ISRE can recruit only IRF dimers, in addition to ISGF3. In contrast, some ISRE subtypes harbor an ETS/IRF binding site named EIRE (ETS/IRF response element; Meraro et al. 2002), different in composition from the ETS/IRF binding element, called EICE, described earlier by Brass et al. (1996). Of note, IRF-8 expression is high in myeloid and B cells, whereas IRF-4 is highly expressed in T and B cells. EICE binds to the immune cell-restricted factor PU.1, which forms a complex with either IRF-4 or IRF-8, and is therefore present in genes whose expression is restricted to immune cells (Kanno et al. 2005). IRF-4 and IRF-8 do not bind effectively to the ISRE element alone, but can do so only when in a complex with PU.1. In contrast, other members of the IRF family (IRF-1, 2, 3, and 7) bind to ISRE elements directly (Honda et al. 2006). Both EICE and EIRE have only one ETS binding site, but EIRE possesses

two IRF binding sites, in contrast to EICE which has only one (Meraro et al. 2002). Because of this difference, promoters that contain EIRE can also become activated after binding to IRF dimers, whereas both types of promoters can become activated by IRF4 or IRF-8/PU.1 heterocomplexes (Meraro et al. 2002). In contrast, IRF heterocomplexes consisting of IRF-8 with either IRF-1 or IRF-2 can bind only to classical ISREs and EIREs and not to EICEs and have been suggested to function as repressors (Bovolenta et al. 1994; Sharf et al. 1995). Interestingly, classical ISRE-containing genes in macrophages, and possibly in other immune cells, can be repressed by IFN-γ through protein–protein interaction between IRF-8 and another ETS family member, TEL, resulting in recruitment of the histone deacetylase HDAC3 (Kuwata et al. 2002). Examples of human genes with EIREs are *ISG15*, *6–16*, *9–27*, *IP10*, *ISG54*, and *CCYBB* (encoding GP91(PHOX)). However, other ways to activate EIRE-containing genes by IFN-γ have also been described. For instance, the human *IP10* and *ISG54* genes possess only an EIRE and no GAS in their promoters and both can bind to STAT1 and IRF-9, probably as a STAT1 homodimer/IRF-9 complex (Majumber et al. 1998; Bluyssen et al. 1995). In addition, the mouse *ip10* promoter can bind to STAT1 and IRF-1 in hepatocytes, but it is not clear what kind of complex is formed (Jaruga et al. 2004). Importantly, the ability of EIRE motifs to recruit not only IRFs but also PU.1/IRF heterocomplexes predicts that some of the genes harboring such elements might be regulated differentially by IFN-γ in immune cells, which constitutively express IRF-4, IRF-8, and PU.1, as has been shown for the *ISG15* gene in macrophages (Meraro et al. 2002) and the *CYBB* gene in myelomonocytic cells, the latter gene showing cooperation with PU.1/IRF-1/IRF-8/CBP (Eklund et al. 1995).

A strong increase in IRF-8 in response to IFN-γ was observed originally in macrophages (Politis et al. 1994), probably because activated PU.1 and IRF-8 binding to the IRF/ETS composite GAS element present in the *irf8* promoter (Contursi et al. 2000; Kanno et al. 2005). Because of the increase in IRF-8 expression, a model was suggested in which IRF-8 and PU.1 play a role in amplifying the expression of genes containing the IRF/ETS composite GAS element by generating a second wave of transcription (Kanno et al. 2005). In addition, it is likely also that the expression of EICE- or EIRE-containing genes is amplified in a macrophage-specific manner through a second wave of transcription. Although it was believed that IRF-8 is only expressed in certain immune cells, it has been shown recently that IFN-γ-stimulated primary colon carcinoma cells also express IRF-8, probably because activated STAT1 binds to the composite GAS element in the *IRF8* promoter (Liu et al. 2003). Similarly, an increase in IRF-1 levels after stimulation by IFN-γ in nonimmune cells is a result of the binding of STAT1 to the IRF/ETS composite GAS element in the *IRF1* promoter. Therefore, since nonimmune cells do

not express PU.1, an increase in IRF-8 may have a quite different effect on classical ISRE or EIRE-containing genes, because complexes formed between IRF-8 and IRF-1 or IRF-2 might function as repressors (Bovolenta et al. 1994; Sharf et al. 1995). However, it cannot be excluded that a concomitant increase in IRF-8 and IRF-1 in immune cells might also lead to the repression of certain genes. However, when IRF-8 levels are not too highly induced in non-immune cells, an increase in IRF-1 homodimers will certainly lead to increased expression of genes containing an ISRE or EIRE element. Moreover, activated STATs may collaborate with IRF-1 to induce the expression of genes that harbor both a classical GAS and an ISRE element. Summarizing these results, we propose that the availability of STATs, IRF, or ETS family members, constitutively present or induced, will determine how a certain cell type responds to IFN-γ, and that the balance among these transcription factors will determine which subtype of ISRE- or GAS-containing genes will be turned on in the first and second phases of transcriptional activation.

CIITA and IRF-9 are examples of transcription factors whose expression is induced by IFN-γ and which therefore play an important role in the second wave of the IFN-γ response. A novel IFN-responsive cis-acting enhancer element, γ-IFN-activated transcriptional element (GATE), distinct from GAS and ISRE, but partly homologous to ISRE, has been identified in the promoter of the *irf9* gene (Weihua et al. 1997). Two transcription factors bind to GATE, GBF1, and GBF2, after they are both synthesized de novo in response to IFN-γ. GBF2 was identified subsequently as C-EBP-β. Its induction by IFN-γ is JAK1- and STAT1-dependent and is probably mediated by the binding of STAT1 to a putative GAS element in the promoter of the *cebpb* gene (Roy et al. 2000). IFN-γ activates C-EBP-β expression by activating the MEKK1/MEK1 or MEK2/ERK1 or ERK2 cascade, resulting in the phosphorylation of C-EBP-β at threonine 294 in the consensus ERK phosphorylation site of the regulatory domain RD2 (Hu et al. 2001). Furthermore, MLK activation by IFN-γ leads to the dephosphorylation of C-EBP-β at serine residue 64 in the transcription activation domain, permitting recruitment of transcriptional co-activators such as p300 (Roy et al. 2005). To date, it is not known whether the MLK-driven dephosphorylation is caused by enhanced phosphatase activity or by inactivation of the kinase that normally phosphorylates serine 64 constitutively (Kalvakolanu et al. 2005). GBF-1 is a novel transcription factor and the gene is located at human chromosome 9q34.13 (Hu et al. 2002). It is not clear which signals from the activated IFNGR induce GBF-1 expression. GBF-1 possesses glutaredoxin-like, PTP-like, RNA Pol II-like, and ribonucleoside diphosphate reductase-like domains, but its precise mechanism of action is not yet known (Hu et al. 2002). The fact that GBF-1 does not bind to monomeric GATE, but does bind to multimeric GATE in a DNA screen, shows that it has very weak DNA binding activity by itself.

However, similarly to IRF-8, which does not bind to DNA alone, GBF-1 possesses strong transactivating activity (Hu et al. 2002). Recent data indicate that C-EBP-β interacts with GBF1 after phosphorylation of C-EBP-β at threonine 294 (Meng et al. 2005). Just as IFN-β induces IRF-9, the induction of IRF-9 by IFN-γ seems to be independent of activated STAT1, but dependent on unphosphorylated STAT1 through an as-yet-unidentified mechanism (Rani et al. 2005). Unphosphorylated STAT1 and STAT3, upregulated in response to IFN-γ or GP130-linked cytokines, respectively, induce sets of genes distinct from those that respond to phosphorylated STAT1 and STAT3 (Chatterjee-Kishore et al. 2000; Yang et al. 2005).

In summary, we describe several IFN-γ-stimulated signaling pathways that involve the activation of different IRF and ETS family members and that function either independently of any STAT or collaborate with STAT1 for the induction of certain genes. These signaling pathways play a very important role in the response of immune cells to IFN-γ. It is not yet known which transcription factors collaborate with activated STAT3 or STAT5 in specific cell types in response to IFN-γ. We expect that many more IFN-γ-activated transcription factors will be discovered that either collaborate with STATs or induce gene expression totally independently of any STAT.

7 Conclusions and Perspective

We propose that different patterns of STAT activation in different cell types contribute to the activation of "STAT only genes" (Fig. 1). In addition to the activation of JAKs, additional kinases such as PI3K, P38, ERK, PYK2, PKC, SRC family members, CaMKII, PKR, MEKK4, and MKK6 become activated. Depending on the cell type, some of these kinases also contribute to the serine phosphorylation of STATs. In addition, they (and other kinases yet to be identified) lead to the activation of transcription factors other than STATs (GATA-1, USF-1, CREB, NF-κB, AP-1, IRF-1, IRF-8, PU.1, and ATF-2). These activated transcription factors either collaborate with STATs on the promoters of "STAT + transcription factor (TF) genes" or function totally independently of any STAT on the promoters of "TF-only genes" (Fig. 1). Furthermore, the differential activation of TFs other than STATs by kinases other than JAKs is an important aspect of cell-type specificity (Fig. 1). Therefore, depending on which STATs and TFs become activated in each cell type, IFN-γ, in addition to its well known antiviral activity, can promote either apoptosis and growth inhibition or survival and proliferation, induce IgG switching in B cells, activate macrophages, and regulate cancer immunoediting. Importantly, priming

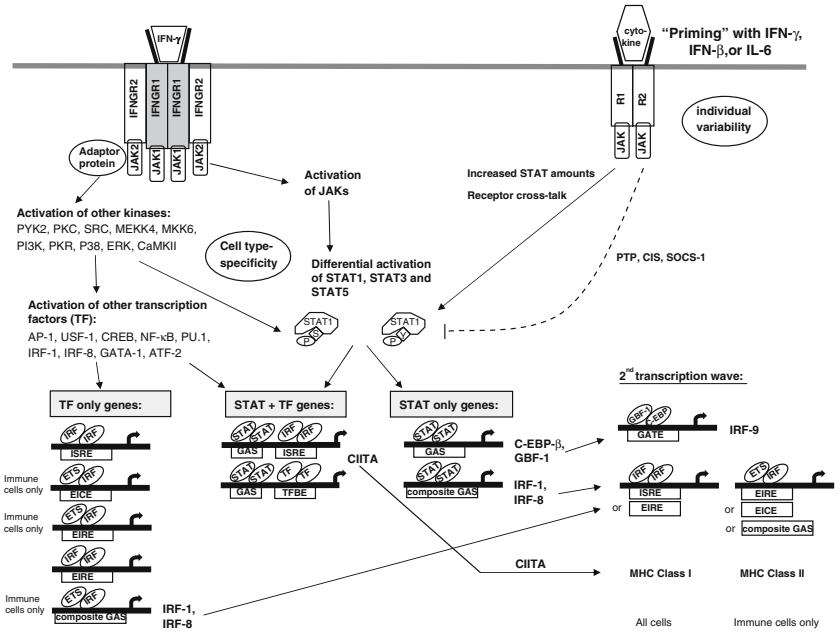

Fig. 1 The complexity of IFN-γ-dependent signaling helps to explain cell type specificity and individual variability in responses. See Sect. 7, "Conclusions and Perspective," for a description of the details

with a cytokine (for example, IFN-γ, IL-6, IFN-β) can either positively (by increasing STAT levels or through receptor cross-talk) or negatively (through PTPs, SOCS-1, and CIS) influence the tyrosine phosphorylation and activation of STATs (Fig. 1). More importantly, priming leads to increased or decreased activation of specific STATs, and thus is likely to have a major impact eventually on which STAT-only genes or STAT + TF genes will be induced (Fig. 1). We propose that priming may well be an important cause of altered IFN-γ-dependent signaling in different individuals (Fig. 1). Notably, the transcription factors IRF-1, IRF-8, CIITA, GBF-1, and C/EPB-β are induced in response to IFN-γ, and play an important role in the secondary transcriptional wave. We propose that the availability of STATs, IRF, or ETS family members, constitutively present or induced, will determine how a certain cell type responds to IFN-γ, and that the balance among these transcription factors will determine which subtype of ISRE- or GAS-containing genes will be turned on in the first and second phases of transcriptional activation.

We hope that the model of Fig. 1 provides a framework for understanding the complex modulation of IFN-γ-dependent signaling, but much is still to be learned. Individual cell types need to be studied in order to understand how and why certain STATs are activated preferentially, and whether different cell types are equally susceptible to priming. Also, the activation of kinases other than JAKs in each individual cell type needs to be linked to the activation of TFs other than STATs, and eventually to the induction of specific genes. More research is also needed to identify cis-acting elements in the promoters of STAT + TF genes and TF-only genes. All of this information is needed to understand fully how different individuals and different cell types react to IFN-γ during infection and inflammation.

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