RESEARCH PAPER

Constitutive IFN α/β signaling maintains expression of signaling intermediaries for efficient cytokine responses

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ABSTRACT. Interferons (IFNs) are a family of immunoregulatory cytokines with important roles in anti-viral and anti-tumor responses. Type I and II IFNs bind distinct receptors and are associated with different stages of the immune response. There is however, considerable crosstalk between these two cytokines with enhancement of IFN γ responses following IFN α/β priming and loss of IFN α/β receptor (IFNAR) resulting in diminished IFN γ responses. In this study, we sought to define the mechanism of crosstalk between the type I and II IFNs. Our previous reports demonstrated reduced expression of the canonically activated transcription factor signal transducer and activator of transcription (STAT)1, in cells lacking the IFNAR α chain (IFNAR1). Therefore, we used microarray analysis to determine whether reconstitution of STAT1 in IFNAR1-deficient cells was sufficient to restore IFN γ responses. We identified several biological pathways, including the MHC class I antigen presentation pathway, in which STAT1 reconstitution was able to significantly rescue IFN γ -mediated gene regulation in *Ifnar1*^{-/-} cells. Notably, we also found that in addition to low basal expression of STAT1, cells lacking the IFNAR1 also had aberrant expression of multiple other transcription factors and signaling intermediaries. The studies described herein demonstrate that basal and regulated expression of signaling intermediaries is a mechanism for crosstalk between cytokines including type I and II IFNs.

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INTRODUCTION

The exposure of cells to cytokines, chemokines and other extracellular signals during pathogenic infection, injury and neoplasia allows for potent, and often synergistic, host immune responses.^{1,2} IFNs, a family of immunoregulatory cytokines with important roles in anti-pathogen and anti-tumor responses, can synergise with other stimuli, such as tumor necrosis factor (TNF) α and interleukin (IL) 1α for more potent immune responses.³⁻¹⁰ Upon IFN binding, receptor-associated janus activated kinases (JAKs) are phosphorylated leading to activation of latent signal transducer and activator of transcription (STAT) molecules. Pivotal to IFN signaling is the transcription factor STAT1, the loss of which severely impairs IFN-dependent cellular responses.^{11,12} The canonical, JAK/STAT1-dependent IFN γ signaling pathway has been extensively studied, however more recently alternate signaling pathways triggered by IFN γ such as mitogen activated protein kinase (MAPK) and nuclear factor κ B (NF κ B) pathways have been identified.¹³⁻¹⁶

Although specific IFN family members bind to selective receptors and activate distinct signaling pathways, significant crosstalk between these pathways is evident.^{1-3,6,17,18} Pre-treatment of cells with low levels of type I IFNs (IFN α/β) leads to more potent response to type II IFN (IFN γ) and interestingly loss of the type I IFN receptor (IFNAR) results in diminished type II IFN responses.^{19,20} Although type I IFNs are secreted in abundance in response to viral infection, uninfected cells also constitutively secrete low concentrations of IFN α/β .²¹⁻²⁴ Studies by our laboratory and others, show that cells unable to produce or respond to constitutive IFN α/β have attenuated STAT1 expression. This led to a model of cytokine crosstalk which postulates that priming levels of IFN β regulate STAT1 expression, which is important for mediating IFN γ responses.^{23,25,26} Moreover, we have demonstrated that reconstitution of

STAT1 into *Ifnar1*^{-/-} cells could restore IFN γ -induced STAT1 DNA binding and partially rescued IFN γ -dependent anti-viral responses.²³ In this study we used global gene expression analysis to examine the contribution of STAT1 to the crosstalk between tonic IFN α/β and IFN γ . Our data revealed that STAT1 alone regulates only a small proportion of IFN crosstalk. Moreover, loss of tonic IFN α/β signaling results in aberrant expression of numerous transcription factors and intracellular signaling molecules, thus tonic IFN α/β signaling regulates the expression of multiple signaling intermediaries involved in cytokine signaling.

RESULTS

*STAT1 rescue of IFN γ responsiveness in *Ifnar1*^{-/-} cells is enhanced at earlier time points*

STAT1 is the canonical transcription factor activated in response to IFN γ and we have previously found that its expression is attenuated in *Ifnar1*^{-/-} cells.²³ To determine the extent to which STAT1 reconstitution alone could rescue molecular responses to IFN γ in *Ifnar1*^{-/-} cells, microarray-based gene expression analysis was performed over a 6 hr time course. Prior to microarray analysis STAT1 expression in *wild type*, *Ifnar1*^{-/-}, empty vector-transduced (*Ifnar1*^{-/-}-MSCV) and HA-tagged *STAT1 α* -transduced (*Ifnar1*^{-/-}-HA-STAT1) *Ifnar1*^{-/-} mouse embryonic fibroblasts (MEFs) was confirmed by western blotting (Fig. 1A). Probes in which IFN γ induced a significant ($p < 0.05$) and greater than 1.2-fold increase or decrease in expression were considered to be regulated by IFN γ . Of the 362 probes which had induced expression in *wild type* cells following 1 hr of IFN γ treatment, 109 probes (30.1%) were also induced in *Ifnar1*^{-/-} and/or *Ifnar1*^{-/-}-MSCV cells and *Ifnar1*^{-/-}-HA-STAT1 cells (Fig. 1B).

In contrast to induced gene expression, only 5 of the 440 probes which showed significant repression in response 1 hr of IFN γ treatment in *wild type* cells, were also repressed in *wild type*, *Ifnar1*^{-/-} and/or *Ifnar1*^{-/-}-MSCV cells and *Ifnar1*^{-/-}-HA-STAT1 cells (Fig. 1B). Similar patterns of gene expression were seen at the later time points with 18.2% and 40.3% of probes induced and 3.2% and 2.5% of probes repressed in *wild type*, *Ifnar1*^{-/-} and/or *Ifnar1*^{-/-}-MSCV cells and *Ifnar1*^{-/-}-HA-STAT1 cells in response to 3 and 6 hrs of IFN γ treatment respectively (Fig. 1C-D).

To determine the genes for which loss of IFNAR1 expression attenuated IFN γ responsiveness, we compared the *wild type* and *Ifnar1*^{-/-} microarray data sets and considered probes which lacked a significant, >1.2-fold change in expression or had >1.2-fold attenuation of gene regulation compared to *wild type* cells as having lost IFN γ responsiveness. In response to 1, 3 and 6 hrs of IFN γ treatment, 85.6%, 93.6% and 75.3% of probes had attenuated gene induction in *Ifnar1*^{-/-} cells compared to *wild type* cells respectively (Fig. 1B-D). For IFN γ -dependent gene repression 99.3%, 99.8% and 98.1% of probes had attenuated responses following 1, 3 and 6 hrs of IFN γ treatment (Fig. 1B-D). These data indicate that the vast majority of IFN γ regulated genes are affected by the absence of IFNAR1.

STAT1 α reconstitution of *Ifnar1*^{-/-} cells was considered to have rescued IFN γ responses if the following criteria were met: (i) The gene had lost IFN γ responsiveness in *Ifnar1*^{-/-} and *Ifnar1*^{-/-}-MSCV cells compared to *wild type* cells (ii) The gene had *wild type*-equivalent responsiveness to IFN γ in *Ifnar1*^{-/-}-HA-STAT1 cells (i.e. a significant and >1.2-fold change in expression relative to untreated and <1.2-fold attenuation of gene regulation in *Ifnar1*^{-/-}-HA-STAT1 cells compared to *wild type* cells). Of the 310 probes in which IFN γ -induced gene expression was attenuated in *Ifnar1*^{-/-} cells following 1 hr of IFN γ treatment, 43 (13.8%) probes had rescued IFN γ responsiveness in *Ifnar1*^{-/-}-HA-STAT1 cells (Fig. 1E). In response to 3 hrs and 6 hrs of IFN γ treatment, induction of gene expression of 62 (8.4%) and 38 (7.4%) probes with attenuated responses in *Ifnar1*^{-/-} cells was rescued in *Ifnar1*^{-/-}-HA-STAT1 cells respectively (Fig. 1F-G). In contrast to the effects on IFN γ -dependent gene induction, Stat1 α reconstitution of *Ifnar1*^{-/-} cells only rescued gene repression of 0.7%, 1.0% and 0.4% of probes following 1, 3 and 6 hrs of IFN γ treatment respectively. These data indicated that the effect of STAT1 reconstitution on IFN γ -dependent gene regulation was greatest during early phase gene induction.

FIGURE 1 (see next page). Reconstitution of STAT1 in *Ifnar1*^{-/-} MEFs partially rescues IFN γ responses. (A) Protein lysates from *wild type*, *Ifnar1*^{-/-}, *Ifnar1*^{-/-}-MSCV and *Ifnar1*^{-/-}-HA-STAT1 129/C3H MEFs were separated by SDS-PAGE and probed with antibodies specific for STAT1 and actin as a loading control. (B-G) *wild type*, *Ifnar1*^{-/-}, *Ifnar1*^{-/-}-MSCV and *Ifnar1*^{-/-}-HA-STAT1 129/C3H MEFs were treated with 100 U/ml IFN γ for 1, 3 and 6 hrs and mRNA expression was assessed by Affymetrix microarray (n = 3). (B-D) Venn diagram of the number of genes that are up-regulated ($\uparrow$ green) or down-regulated ($\downarrow$ red) relative to untreated (p < 0.05, >1.2-fold) following (B) 1 hr, (C) 3 hrs or (D) 6 hrs of IFN γ treatment and whether they are specific or common to each cell type. (E-G) Left panel. Heat maps of probes with attenuated IFN γ -dependent gene induction in *Ifnar1*^{-/-} MEFs relative to *wild type* MEFs (p > 0.05, <1.2-fold change relative to untreated or >1.2-fold attenuation in change compared to *wild type*). Right panel. Heat maps of probes with attenuated (as defined above) IFN γ -dependent gene induction in *Ifnar1*^{-/-} MEFs and *Ifnar1*^{-/-}-MSCV MEFs relative to *wild type* MEFs and rescued (p < 0.05 and >1.2-fold change relative to untreated and <1.2-fold attenuation in change compared to *wild type*) in *Ifnar1*^{-/-}-HA-STAT1 MEFs. The 10 attenuated (left panel) and rescued (right panel) probes with the greatest fold change in *wild type* MEFs following (E) 1 hr, (F) 3 hrs and (G) 6 hrs of IFN γ treatment are displayed. Heatmaps were generated using Java Treeview. Total number of probes with induced ($\uparrow$) and repressed ($\downarrow$) expression in *wild type* MEFs that had attenuated (left panel) or rescued (right panel) expression are listed below heatmaps.

FIGURE 1. (For caption, see previous page.)

STAT1 rescues IFN γ -mediated responses to a select range of biological pathways in IFNAR1 cells

Ontological analysis of the microarray data was performed to identify the biological pathways rescued following IFN γ treatment of *Ifnar1*^{-/-}HA-STAT1 cells. The 2106 genes

with attenuated regulation by IFN γ in *Ifnar1*^{-/-} MEFs over the 6 hr time course of IFN γ treatment were analyzed using MetaCorZe's Genego. Genes with aberrant expression following IFN γ treatment in *Ifnar1*^{-/-} MEFs were involved in a diverse range of biological pathways, with the most significantly affected including developmental,

immunological response, reproduction and apoptosis pathways (Fig 2A). Surprisingly, only one pathway directly associated with STAT signaling was found among the 10 most significantly affected biological pathways. Subsequently, genes with attenuated regulation by IFN γ in *Ifnar1*^{-/-} MEFs were compared to the genes in which IFN γ responses were significantly rescued by reconstitution of STAT1 using MetaCore's Genego comparative analysis for pathway maps. This analysis revealed, unsurprisingly, that among the 10 pathways most significantly affected by both the loss of IFNAR1 and reconstitution of STAT1 cells were the IFN α/β signaling pathway and multiple JAK/STAT signaling pathways (Fig. 2A). Of the 10 most significantly affected pathways, 7 were associated with immune responses with the most significantly affected pathway being the MHC class I antigen presentation pathway (Fig. 2A-B). These data suggested that despite genes involved in a diverse array of biological responses pathways being aberrantly regulated by IFN γ in the absence of IFNAR1, the pathways in which STAT1 reconstitution of IFNAR1 deficient cells rescued IFN γ -mediated gene regulation were enriched for immune response pathways.

STAT1-reconstitution of *Ifnar1*^{-/-} cells rescues IFN γ -inducible protein expression of genes involved in the MHC class I antigen presentation pathway

Several components of the MHC class I-dependent antigen presentation pathway were significantly rescued by STAT1 reconstitution of *Ifnar1*^{-/-} cells as determined by microarray analysis (Fig. 2C). We had previously found

that STAT1 reconstitution of *Ifnar1*^{-/-} cells rescued IFN γ -dependent induction of RNA expression for a key molecule in antigen presentation, β 2 microglobulin (B2M).²³ Therefore the MHC class I antigen presentation pathway represented an ideal candidate to validate the attenuation of IFN γ -dependent biological responses in IFNAR1 deficient cells and its rescue by STAT1 reconstitution.

We first examined the IFN γ -dependent induction of molecules involved in the transport of peptides to the endoplasmic reticulum, transporter associated with antigen processing (Tap)1 and Tap2, in *Ifnar1*^{-/-} cells. In corroboration with the microarray data, we found reduced IFN γ -mediated protein induction of TAP1 expression in *Ifnar1*^{-/-} MEFs compared to *wild type* MEFs following exposure to IFN γ for 6 hrs (Fig. 3A). There was also attenuation of TAP2 induction in *Ifnar1*^{-/-} MEFs in response to 6 hrs of IFN γ treatment and a minor effect of loss of IFNAR1 for cells treated for 24 hrs (Fig. 3B). Reconstitution of STAT1 in *Ifnar1*^{-/-}HA-STAT1 cells rescued IFN γ -mediated induction of TAP1 in response to 6 hrs and TAP2 following 6 and 24 hrs of treatment ((Fig. 3A-B). Similar to the TAP molecules, induction of proteasome subunit, β type (PSMB)9, a catalytic component of the immunoproteasome, was attenuated in *Ifnar1*^{-/-} cells following 6 hrs of treatment (Fig 3A). This attenuated induction of PSMB9 was also evident in *Ifnar1*^{-/-} MSCV cells following 6 hrs of IFN γ treatment and importantly, was rescued to *wild type* equivalent levels in *Ifnar1*^{-/-}HA-STAT1 cells (Fig. 3C). Notably, PSMB9 expression reached *wild type* equivalent levels in all IFNAR1 deficient cell lines by 24 hrs of IFN γ treatment suggesting that, as seen in for multiple genes in the microarray

FIGURE 2 (see next page). Reconstitution of STAT1 α in *Ifnar1*^{-/-} MEFs preferentially rescues immune and inflammatory IFN γ responses. (A-B) Probes from figure 1.E-G listed as attenuated (A-B) or rescued (B) were analyzed using MetaCore's Genego. The 10 most significantly affected pathways are displayed. (A) Pathway enrichment analysis of attenuated genes. EMT = epithelial-to-mesenchymal transition. (B) Comparative pathway enrichment analysis of attenuated and rescued genes. (C) Heatmaps displaying fold change in expression following 1, 3 or 6 hrs IFN γ treatment of genes involved in antigen presentation via MHC class I for *wild type*, *Ifnar1*^{-/-}, *Ifnar1*^{-/-}-MSCV and *Ifnar1*^{-/-}HA-STAT1 MEFs. Heatmaps were generated using Java Treeview.

FIGURE 2. (For caption, see previous page.)

FIGURE 3. STAT1 reconstitution rescues IFN-induced protein expression of MHC class I antigen presentation proteins in *Ifnar1*^{-/-} MEFs. (A-C) *wild type*, *Ifnar1*, *Ifnar1*^{-/-}MSCV and *Ifnar1*^{-/-}HA-STAT1 MEFs were cultured in media alone or treated with 100 U/ml IFN γ for (A) 6 hrs and (B-C) 24 hrs. Protein lysates were separated by SDS-PAGE and probed with antibodies specific for (A) TAP1 (B) TAP2 or (C) PSMB9. Western membranes were re-probed with antibody specific for (A-B) Actin or (C) HSP70 to confirm equivalent loading of proteins. (D-E) *wild type*, *Ifnar1*, *Ifnar1*^{-/-}MSCV and *Ifnar1*^{-/-}HA-STAT1 MEFs were cultured in media alone or were treated with 5 or 10 U/ml IFN γ for 16 hrs. Cells were analyzed by flow cytometry for expression of the MHC class I molecules (D) H2-K^b and (E) H2-D^b. Data represents the IFN γ -induced change in MFI relative to untreated for 11–13 independent experiments and error bars are SEM. Significant changes in MFI relative to untreated (fold increase in MFI) were determined by t-test #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001. Significant differences in fold increase in MFI relative to *wild type* were determined by t-test **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

analysis, IFNAR1 expression was required for early, but not late phases responses.

Finally, we assessed the effects of STAT1 on the ability of IFN γ to regulate cell surface expression of the MHC class I heavy chain on *Ifnar1*^{-/-} cells. Again, in agreement with the microarray analysis, *Ifnar1*^{-/-} and *Ifnar1*^{-/-}MSCV MEFs displayed significantly attenuated IFN γ responses, with minimal changes in H2-K^b and H2D^b expression compared to *wild type* cells (Fig. 3D-E). In

contrast, cell surface expression of H2-K^b and H2-D^b following both 5 and 10 U/ml treatments of IFN γ was, like *wild type* cells, significantly induced on *Ifnar1*^{-/-}HA-STAT1 cells (Fig. 3D-E). Together, these data indicated that STAT1-reconstitution was able to rescue the attenuated IFN γ -dependent regulation of mRNA and protein expression of genes involved in protein processing, peptide transport and cell surface antigen presentation in *Ifnar1*^{-/-} cells.

Constitutive IFN α/β signaling regulates the expression of multiple signaling molecules

Having found that STAT1 reconstitution of IFNAR1-deficient cells only rescued a proportion (~15%) of IFN γ regulated genes and that these genes were enriched for involvement in immune and inflammatory responses, we investigated whether constitutive IFN α/β signaling regulated the expression of other transcription factors to modulate IFN crosstalk. We first examined expression of other members of the JAK/STAT signaling pathway known to be regulated by and involved in IFN signaling. In corroboration with our previous qRT-PCR findings, there was attenuated RNA expression of *Stat1* and *Stat2*, but not *Socs3*, in unstimulated *Ifnar1*^{-/-} cells, as determined by microarray and qRT-PCR analysis (*Stat1* and *Socs3*) (Supp. Fig. 1A, Supp. Table 1–2).²³ Similarly, we validated our previously identified decrease in basal STAT3 protein expression using primary C57BL/6 *Ifnar1*^{-/-} MEFs and confirmed that the attenuated STAT3 protein expression evident in 129/C3H MEFs lacking IFNAR1 was related to a significant decrease in RNA expression (Supp. Fig. 1A–B).²³ Given that both IFN γ and IL-6 can activate STAT3 and that, IL-6 mediated STAT3 DNA binding is attenuated in the absence of IFNAR1,²⁷ we assessed STAT3 activation following IFN γ or IL-6 treatment of *Ifnar1*^{-/-} cells. In contrast to previous findings,²⁷ we found reduced STAT3 phosphorylation following IL-6 stimulation of *Ifnar1*^{-/-} cells (Fig. 4A). Similarly, IFN γ -mediated STAT3 activation was also attenuated in IFNAR1-deficient cells (Fig. 4A). These data indicate that constitutive IFN α/β signaling regulates expression of multiple STAT transcription factors and that this may influence crosstalk with cytokines other than IFNs.

To determine the effect that loss of constitutive IFN α/β signaling has on the global transcriptional profile of resting cells, we assessed transcription factor expression in unstimulated *wild type* and *Ifnar1*^{-/-} MEFs by microarray analysis. 1264 genes had ≥ 1.2 -fold ($p \leq 0.05$) differential basal expression in *Ifnar1*^{-/-} MEFs compared to *wild type* MEFs (Supp. Table. 1).

Of the 1264 genes with differences in basal expression between *wild type* and *Ifnar1*^{-/-} cells, 106 were known transcription factors (Supp. Table. 2). Furthermore, qRT-PCR analysis of intracellular signaling molecules known to crosstalk with the type I IFN signaling pathway confirmed the reduced expression of genes involved in TLR and NF- κ B signaling, *Myd88*, *Nfkb1a* and *Nfkb1e* (Fig. 4B) in *Ifnar1*^{-/-} cells (Fig. 4.b and Supp. Table. 2).

We also assessed RNA and protein expression of several transcription factors known to regulate IFN γ responses that were not identified by the microarray analysis as having attenuated expression in *Ifnar1*^{-/-} MEFs. We found significantly reduced RNA expression of the transcription factors *c-Jun* and *Irf1* by qRT-PCR analysis (Fig. 4B). As shown in Figure. 4C, *Ifnar1*^{-/-} MEFs also expressed less *c-Jun* protein than *wild type* MEFs and thus the decrease in *c-Jun* mRNA expression observed correlated with a similar decrease in total protein expression. Similarly, although we found no reduction in extracellular signal-regulated kinase (ERK)1/2 expression by microarray analysis, basal expression of ERK1, but not ERK2 was reduced in *Ifnar1*^{-/-} MEFs (Fig. 4D). Overall, these data revealed that the absence of IFNAR1 results in reduced aberrant expression of multiple transcription factors which are involved in a range of cytokine and inflammatory signaling responses.

DISCUSSION

The canonical signal transduction pathways activated by cytokines to modulate cellular responses and gene expression have been extensively investigated. These pathways are comprised of signaling intermediaries such as kinases, G proteins, adaptor proteins and transcription factors that get turned ‘on’ and ‘off’ in the presence of a stimulus. In contrast, the physiological scenario of concomitant exposure to several cytokines and the consequences and mechanisms regulating these interactions are relatively undefined.²⁸ Cytokine crosstalk can occur at the level of the receptor, by regulation of the expression of receptor components or the

FIGURE 4. Aberrant expression and activation of signaling intermediaries in unstimulated *Ifnar1*^{-/-} MEFs. (A) *wild type* and *Ifnar1*^{-/-} MEFs were cultured in media alone or treated with 100 U/ml IFN γ or 100 ng/ml IL-6 and 100ng/ml IL-6R for 30mins. Protein lysates were separated by SDS-PAGE and probed with an antibody specific for pSTAT3. Western membrane was re-probed with an antibodies specific for Actin to confirm equivalent loading of proteins. (B) RNA was extracted from untreated *wild type* and *Ifnar1*^{-/-} 129/C3H MEFs and mRNA expression of *c-Jun*, *Irf1*, *Myd88*, *Nfkb1a* and *Nfkb1e* was determined by qRT-PCR. Expression of each gene was normalized to *L32* and untreated *wild type* MEFs. Data represents the mean of 4–6 independent experiments and error bars are SEM. Significance determined by t-test **p* < 0.05, ***p* < 0.01, ****p* < 0.001. (C-D) Protein lysates from untreated *wild type* and *Ifnar1*^{-/-} MEFs were separated by SDS-PAGE and probed with antibodies specific for (C) c-JUN, and (D) ERK1/2. Western membranes were re-probed with antibodies specific for HSP70 to confirm equivalent loading of proteins.

formation of receptor complexes.^{19,27,29-31} Crosstalk can also occur at the level of gene promoters as a result of multiple promoter binding sites or chromatin priming.^{32,33} In this study, we examined the impact that the regulation of the basal levels of intracellular signaling molecules has on cytokine responses.

We had previous established that STAT1 reconstitution of *Ifnar1*^{-/-} cells rescued IFN γ -dependent STAT1 activation and DNA binding and partially rescued IFN γ -mediated protection from the cytopathic effects of ECMV.²³ Using microarray analysis to ascertain the effects of STAT1 reconstitution of IFNAR1-deficient cells

on a genome wide scale we found that although STAT1 is the canonical transcription factor activated by IFN γ , only up to 15.4% and 3.2% of IFN γ -dependent gene induction and repression were rescued by STAT1 respectively. Moreover, the ability of STAT1 to rescue IFN γ responses diminished over time. The relatively minor effect of STAT1 expression of IFN γ mediated gene repression was unsurprising however, as gene repression by IFN γ is purported to be mediated via STAT1 independent pathways³⁴ such as CIITA mediated histone deacetylation.³⁵⁻³⁷ Also, as STAT1 is induced by IFN γ signaling,³⁸⁻⁴⁰ it is plausible that at

later time points *de novo* STAT1 production in *Ifnar1*^{-/-} cells in response to IFN γ treatment was able to rescue the IFN γ responsiveness of genes at later time points.

Treatment of primary human fibroblasts with both IFN β and IFN γ synergistically induces expression of genes involved in apoptosis and inflammatory responses.⁴¹ Similarly, the biological response pathways which showed attenuated IFN γ responses in *Ifnar1*^{-/-} cells and for which STAT1 reconstitution significantly rescued IFN γ responsiveness were both enriched for immune responses, apoptosis and alternate cytokine signaling pathways. As expected, our microarray and protein analysis indicated that IFN γ -dependent regulation of the JAK/STAT signaling pathway was significantly affected by the loss of tonic IFN α/β however, interestingly, the MHC class I antigen presentation pathway was the most sensitive to loss of IFNAR1 and was also significantly rescued by STAT1 reconstitution. Expression of a number of genes involved in MHC class I antigen presentation, including *Tap1*, *Psmb9* and MHC class I heavy chain, are known to be mediated by type I and type II IFNs in a STAT1-dependent manner.^{12,42-45} It was however surprising, given that this regulation is co-operatively mediated by other factors including IRF1, IRF2 and the ISGF3 complex for which basal expression was also reduced (Fig. 4 and Supp. Table 2), that STAT1 alone was able to rescue the IFN γ -dependent regulation of these genes.

Although reconstitution of STAT1 in *Ifnar1*^{-/-} cells had a major effect on several biological pathways, ~85% of IFN γ -mediated gene regulation was not rescued. Mediation of cytokine crosstalk by the regulating the expression of signaling intermediaries has also been suggested for STAT4,^{46,47} I κ B,⁴⁸ IRF9⁴⁹ and IRF2.⁵⁰ Our studies, which found that expression of over 100 transcription factors and numerous other signaling intermediaries, including *Myd88*, IKK γ and MAPK kinases, were decreased in cells that lacked tonic IFN α/β , suggests that rather than being regulated by a single transcription factor or signaling complex, cytokine crosstalk likely involves regulation of the expression of a multitude of signaling molecules.

This level of regulation may enhance specific biological responses when particular sets of transcription factors are involved. For example, although expression of STAT1 had a profound effect on IFN γ dependent regulation of genes involved in antigen presentation, other IFN γ -dependent responses, including regulation of genes involved in development and TLR-dependent immune responses, were not rescued by STAT1 reconstitution of *Ifnar1*^{-/-} MEFs. As RNA expression of the signaling intermediary *Myd88*, a fundamental component of the TLR signaling pathway,^{51,52} was, like STAT1, attenuated in cells lacking constitutive IFN β signaling, it is plausible that tonic IFN α/β augments TLR responsiveness by regulating the expression of *Myd88*. Moreover, as expression or activation of transcription factors involved in signaling via other cytokines known to engage in crosstalk with IFN α/β such as IL-6, IL-1 and TNF α ^{6,27,28,53-55} were also revealed by our studies to be attenuated in IFNAR1-deficient cells, it suggest that tonic IFN α/β also regulates responsiveness to these cytokines by this mechanism.

Overall we have demonstrated that, in addition to maintenance of STAT1 expression, constitutive IFN α/β -signaling mediated by IFNAR1 regulates the expression of multiple transcription factors and signaling intermediaries. Moreover, based on the sub-set of biological responses pathways affected by STAT1 expression, we suggest that, the regulation of signaling molecules involved in specific biological pathways or activated by specific cytokines may be a mechanism whereby synergistic biological responses by multiple cytokines are fine-tuned.

MATERIALS AND METHODS

Mice

C57BL/6 J *wild-type* mice were purchased from the Walter and Eliza Hall Institute of Medical Research or bred in house at the Peter MacCallum Cancer Center. C57BL/6 IFNAR1-deficient (*Ifnar1*^{-/-})⁵⁶ mice were bred in house

at the Peter MacCallum Cancer Center. All experiments were performed in accordance with the animal ethics guidelines ascribed by the National Health and Medical Research Council of Australia. All experiments were approved by the Peter MacCallum Cancer Center Animal Ethics Committee.

Reagents and cell culture

Generation of *wild type* and *Ifnar1*^{-/-} 129/C3H MEFs and retroviral transduction of *Ifnar1*^{-/-} 129/C3H MEFs was described previously.²³ Briefly, *Ifnar1*^{-/-} 129/C3H MEFs were transduced with viral supernatants using murine stem cell virus (MSCV) encoding GFP alone or cDNA encoding HA-tagged STAT1 (generous gift from Thomas Decker). Primary MEFs were derived from day 13.5–14.5 *wild type* or *Ifnar1*^{-/-} C57BL/6 embryos and used during early passages (passage 2–6). For all culture conditions, cells were maintained at 37°C in Dulbecco's Modified Eagle Medium (DMEM) (JRH Biosciences) supplemented with 10% foetal bovine serum (FBS) (ThermoTrace), 2 mM L-glutamine (JRH Biosciences), 50 µM 2-mercaptoethanol (Sigma Chemical Co.) and penicillin/streptomycin (Invitrogen Life Technologies Corp.). Recombinant cytokines and receptors utilised in these experiments were IFNγ (Roche, 11276905910), IL-6 (Peprotec, 200-06) and IL-6 receptor (IL-6R) (R & D systems Inc., 227-SR).

Microarray analysis

RNA was extracted from *wild type*, *Ifnar1*^{-/-}, *Ifnar1*^{-/-}-MSCV and *Ifnar1*^{-/-}-HA-STAT1 transduced 129/C3H MEFs using Trizol (Invitrogen) according to the manufacturer's instructions. RNA quality was assessed by bioanalyzer using RNA Nano Chip (Agilent technologies) as per the manufacturer's instructions. RNA samples were considered degraded if RNA integrity number (RIN) was below 8.

All reagents (except 100% and 80%v/v Ethanol) were purchased from Affymetrix (Millennium Science) as part of the One-Cycle Target Labeling and Control Reagents (P/N 900493)

package and GeneChip® Hybridization control kit (P/N 900454). cDNA preparation, sample hybridization and scanning of the oligonucleotide microarray GeneChip Arrays (Affymetrix) was performed as per manufacturer's instructions. Briefly 1 µg RNA was reverse transcribed using Superscript II and T-7 Oligo (dT). Second strand cDNA synthesis, mediated by RNase H was followed by an *in vitro* transcription (IVT) reaction with T7 RNA polymerase, biotin-11-CTP and biotin-16-UTP to generate biotin labeled cRNA. Purified cRNA was fragmented by mild alkaline treatment, then hybridized to the GeneChip® Mouse Genome 430 2.0 Array Mouse chips (probe arrays) (Affymetrix) and washed using the GeneChip® Fluidics Station 450. Array chips were then stained with Streptavidin Phycoethrin (SAPE) (Molecular Probes) Biotinylated antibody (Vector Labs). Cartridges were loaded into autoloader of the GeneChip® Scanner 3000 7 G laser scanner.

The data (.dat) file, was automatically converted to a cell intensity (.cel) file and the quality of the individual arrays was assessed using GCOS. Microarray CEL files (Affymetrix probe identities and intensities) were converted to annotation files using Affy software (Affy).⁵⁷ All microarray data was assessed for degradation (degradation plots, Affy) normalized by Robust multi-array (RMA) normalization (Affy), p-values determined by Students t-test using Linear Models for Microarray Analysis (LIMMA) and multiple test corrections for each sample set comparison was performed using Benjamini-Hochberg multiple test correction method.⁵⁸ Significance cut offs of $p \leq 0.05$ and fold change ≥ 1.2 for each comparison were implemented.

Quantitative real time PCR

RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Alternatively, RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN) as per manufacturer's instructions. cDNA was synthesized from 1 µg RNA using superscript III (Invitrogen) as per the manufacturer's instructions. The abundance of specific genes in the samples

was quantitated using the SYBR Green dye detection method (Applied Biosystems, CA, USA). Primers to murine genes were as follows.

c-Jun ACTCCGAGCTGGCATCCA CCCA CTGTTAACGTGGTTCATG

Irf1 TGGAGATGTTAGCCCGGACACTTT ACGGTGACAGTGCTGGAGTTATGT

L32 TTCCTGGTCCACAACGTCAAG TG TGAGCGATCTCGGCAC

Myd88 AGCAAGGAATGTGACTTCCAG ACCA TGGGAAAGTCCTTCTTCATCGCCT

Nfkbia TGGCCTTCTCAACTTCCAGAA CA TCAGGATCACAGCCAGCTTTTCA

Nfkbie ACATTGATGTACAGGAGGGCA CGA ACAGAGTGGATGAGATGCTGTTGA GG

Socs3 CCTTCAGCTCCAAAAGCGAG GC TCTCCTGCAGCTTGCG

Stat1 CGCGCATGCAACTGGCATATAA CT AAGCTCGAACCCTGTGACATCCT

Stat3 AGTTCCTGGCACCTTGGATTGAG A TTGGACTCTTGACCAATCGGCTA

Threshold cycle numbers (Ct) were measured in the exponential phase for all samples. Relative abundance of sample genes was calculated using the “DDCT method” relative to the *L32* control gene. mRNA abundance was normalized to the untreated samples of each genotype or relative to wild type as indicated.

Flow cytometry

Samples were first incubated in FC block in 2% FBS/PBS for 20 minutes. Antibodies against H2D^b (28-14-8) and H2K^b (AF6-88.5) were obtained from BD Pharmingen. Viability was determined by absence of Fluoro-gold uptake.

SDS-PAGE and protein gel blotting

Cells were washed once in PBS and lysed in whole cell lysis buffer (0.05% Triton X-100, 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM ethylene diamine tetra-acetic acid, 0.1 mM ethylene glycol tetra-acetic acid and 10% glycerol), containing protease and phosphatase inhibitors (1mg/mL aprotinin, 0.5 mg/mL leupeptin, 0.2 mM PMSF, 10 mM NaF, 1 mM Na₃VO₄,

1 mM β glycerophosphate) at 4°C for 10 min. Lysates were then cleared by centrifugation. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were probed with specific antibodies against Actin (Sigma), HSP70 (kind gift from R. Anderson), STAT1 α/β (C terminus) (BD Bioscience), p-STAT3, STAT3, c-JUN, ERK1/2 (Cell Signaling Technology), PSMB9 (Abcam), TAP1 or TAP2 (Santa Cruz Biotechnology). Secondary antibodies were conjugated to horseradish peroxidase and images were visualized by chemiluminescence (ECL, GE Healthcare).

Statistical analysis

Statistical significance was assessed using Microsoft Excel software. For comparisons of means, Student's t-Test (t-test) was used and p-values <0.05 were considered significant. For cluster analysis of microarray probes, k-means organized arrays via Euclidean distance were displayed as logbase2. For cluster analysis of antigen presentation genes, hierarchical organized arrays via Euclidean distance were displayed as logbase2. Cluster analysis was performed using Cluster 3.0⁵⁹ and visualised using Java Tree View.⁶⁰ Pathway enrichment analysis and identification of transcription factors was performed using MetaCore from Thomson Reuters (MetaCore Genego, Thomson Reuters, <https://portal.genego.com/Genego>).

ABBREVIATIONS

B2M	Beta 2 microglobulin
ERK	Extracellular signal-regulated kinase
Hrs	Hours
IFN	Interferon
IFNAR	IFN α/β receptor
IFNAR1	IFN α/β receptor α chain
IL	Interleukin
IRF	Interferon regulatory factor
JAK	Janus activated kinase
MAPK	Mitogen activated protein kinase
MEF	Mouse embryonic fibroblast
MSCV	Murine stem cell virus
NF κ B	Nuclear factor κ B
PSMB	Proteasome subunit, β type

STAT	Signal transducer and activator of transcription
TAP	Transporter associated with antigen processing
TGF	Transforming growth factor
TNF	Tumor necrosis factor
U/ml	Units per ml

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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