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Multifaceted Activities of Type I Interferon Are Revealed by a Receptor Antagonist

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Abstract

Type I interferons (IFNs), including various IFN- α isoforms and IFN- β , are a family of homologous, multifunctional cytokines. IFNs activate different cellular responses by binding to a common receptor that consists of two subunits, IFNAR1 and IFNAR2. In addition to stimulating antiviral responses, they also inhibit cell proliferation and modulate other immune responses. We characterized various IFNs, including a mutant IFN- α 2 (IFN-1ant) that bound tightly to IFNAR2 but had markedly reduced binding to IFNAR1. Whereas IFN-1ant stimulated antiviral activity in a range of cell lines, it failed to elicit immunomodulatory and antiproliferative activities. The antiviral activities of the various IFNs tested depended on a set of IFN-sensitive genes (the “robust” genes) that were controlled by canonical IFN response elements and responded at low concentrations of IFNs. Conversely, these elements were not found in the promoters of genes required for the antiproliferative responses of IFNs (the “tunable” genes). The extent of expression of tunable genes was cell type-specific and correlated with the magnitude of the antiproliferative effects of the various IFNs. Although IFN-1ant induced the expression of robust genes similarly in five different cell lines, its antiviral activity was virus- and cell type-specific. Our findings suggest that IFN-1ant may be a therapeutic candidate for the treatment of specific viral infections without inducing the immunomodulatory and antiproliferative functions of wild-type IFN.

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INTRODUCTION

Type I interferons (IFNs) are a family of cytokines that are characterized by their antiviral, antiproliferative, and immunomodulatory activities (1, 2). The type I IFNs act on and can be produced by nearly every nucleated cell (3). In humans, there are 16 type I IFNs, including many IFN- α isoforms and a single IFN- β , all of which act by binding to the same receptor complex, which consists of two subunits, IFNAR1 and IFNAR2 (4). Upon formation of the ternary complex, the IFN signal is transduced through receptor-associated Janus kinases (JAKs), which activate members of the signal transducer and activator of transcription (STAT) family of proteins. Subsequently, STAT1 and STAT2 proteins translocate to the nucleus, where together with the transcription factor IRF9 (interferon-regulatory factor 9), they form the interferon-stimulated gene factor 3 (ISGF3) transcription complex, which induces the expression of interferon-stimulated genes (ISGs) (5). In addition to members of the canonical JAK-STAT pathway, IFNs also signal through other, less well-defined factors (3). We previously showed that even low amounts of weak-binding IFNs induce the transcription of some genes, whereas the activation of other genes requires a high concentration of high-affinity IFN and a high concentration of receptors on the cell surface (6). We refer to this first group as “robust” genes, with many of them related to antiviral activities, whereas the second group of genes, whose products have immunomodulatory and antiproliferative functions, are termed “tunable” genes.

Type I IFNs share a similar spectrum of activities, but they vary substantially in their potency against different viruses, their antiproliferative activity, and their ability to activate cells of the immune system (7, 8). Studies of these overlapping yet differential cellular responses have suggested that the dynamics of ligand interaction with the receptor subunits and the stability of the ternary complex play a key role in regulating cellular response patterns (9–12). We previously showed that increasing the binding affinity of IFN- α 2 to either IFNAR1 or IFNAR2 enhances its antiproliferative activity (6, 11, 13). Accordingly, an IFN- α 2 variant that combines the His⁵⁷→Tyr (H57Y), Glu⁵⁸→Asn (E58N), Gln⁶¹→Ser (Q61S) mutations (termed YNS) and has its C-terminal tail substituted with that of IFN- α 8 (YNS- α 8tail) was previously constructed. This mutant binds to IFNAR1 and IFNAR2 with 50- and 15-fold higher affinities, respectively, than those of wild-type IFN- α 2. This results in a ~200-fold increase in its antiproliferative activity compared to that of IFN- α 2 (6). On the other side of the spectrum, we identified an IFN- α 2 mutant, R120E- α 8tail (IFN-1ant), which has markedly reduced binding to IFNAR1, but enhanced binding to IFNAR2 (14). This mutant does not confer any antiproliferative activity and antagonizes the activities of other type I IFNs. Decreasing binding affinity to one of the receptors is a known strategy to design antagonists, because it prevents the formation of a functional signaling complex (15).

Here, we showed that at high concentrations of IFN-1ant, a partial IFN signal was induced that activated the expression of only robust genes, whereas it suppressed the antiproliferative response stimulated by IFN- α and IFN- β proteins. We next characterized the robust and tunable patterns of IFN activities by focusing on the biological responses to IFN-1ant in a number of cell lines. Studying several cell lines and viruses showed that the antiviral activity of IFN-1ant was both virus and cell-type-specific, ranging from no antiviral response to full protection. Hence, IFN-1ant is an IFN- α mutant with differential antiviral activity.

Examination of IFN-1ant-induced gene expression suggested gene(s)-specific protection against viruses. Finally, analysis of gene induction profiles implied that different transcriptional programs mediate the robust versus tunable responses of type I IFNs.

RESULTS

IFN-1ant has increased binding to IFNAR2, whereas its binding to IFNAR1 is undetectable

The structure of the type I interferon ternary complex marking the locations of the mutations used in this study is shown in Fig. 1A (16). Whereas the YNS triple mutation increases the binding of this variant to IFNAR1 compared to that of wild-type IFN- α 2 (9, 11), the R120E mutation abolishes it (14). On the opposite side of the molecule, the unstructured C-terminal tail has a role in modulating its affinity for IFNAR2 (13). IFN-1ant was generated by mutating both sides of IFN- α 2, thereby altering its binding characteristics to both IFNAR1 and IFNAR2. With surface plasmon resonance (SPR) measurements, we compared the binding affinity of IFN-1ant to those of the wild-type IFN- α 2 and the two super-agonists: YNS and YNS- α 8tail. Whereas both YNS and YNS- α 8tail bound with higher affinity than did IFN- α 2 to IFNAR1, IFN-1ant showed no detectable binding (Fig. 1B). Both IFN-1ant and YNS- α 8tail exhibited higher binding affinities for IFNAR2 (Fig. 1C) because of the C-terminal tail of IFN- α 8 (13).

IFN-1ant antagonizes the antiproliferative activity of other type I IFNs

The biological activity of IFNs is related to their binding affinity for the receptor. Here, we examined the biological activity of IFN-1ant on five cell lines: WISH, OVCAR3, T47D, A549, and Huh7.5 cells. These experiments revealed that OVCAR3 cells were highly sensitive to the antiproliferative effects of IFN- α 2 or YNS; WISH, A549, and Huh7.5 cells exhibited intermediate sensitivity; whereas T47D cells were completely resistant to the antiproliferative activity of these IFNs (Fig. 2A). IFN-1ant did not induce an antiproliferative effect in any of the cell lines, even at a high concentration (1 μ M) (Fig. 2A). Notably, this was true even for OVCAR3 cells, which were highly sensitive to the IFN-induced antiproliferative effect (Fig. 2, A and B and Table 1).

We previously showed that concentrations of type I IFN that have an antiproliferative effect also result in a continued decrease in the amount of IFNAR on the cell surface (12, 16). Consistent with its lack of antiproliferative activity, IFN-1ant did not cause a reduction in the cell-surface abundance of IFNAR, even at 50 nM (fig. S1).

Because IFN-1ant binds tightly to IFNAR2, but has minimal binding to IFNAR1, it has the ability to antagonize ternary complex formation by other type I IFNs (14). Indeed, when used at 2 and 20 nM, IFN- α 2 had no antiproliferative effect on WISH cells in the presence of 200 nM IFN-1ant (Fig. 2C). Similarly, 200 nM IFN-1ant fully antagonized the antiproliferative activity of 0.2 nM of the high-affinity IFNs, IFN- β and YNS. At higher, non-physiological, concentrations of IFN- β and YNS, the antagonistic effect of IFN-1ant was incomplete (Fig. 2C). A reciprocal experiment in which the amount of IFN-1ant was varied demonstrated that increasing its concentration enhanced its antagonistic effect,

consistent with the simple mass action equilibrium of competitive binding of the ligands (fig. S2).

The antiviral activity of IFN-1ant is virus- and cell-type-specific

In a previous study (14), the antiviral activity of IFN-1ant was found to be low compared to that of wild-type IFN- α 2; however, this conclusion was based solely on the calculated 50% effective concentration (EC_{50}) values for inhibition of viral replication. Here, we evaluated the EC_{50} values of the various IFNs in terms of both viral inhibition and cell survival. After infection of WISH cells with vesicular stomatitis virus (VSV), IFN-1ant protected about 60% of the cells from virus-induced death, which was similar to the protection conferred by 1 pM IFN- α 2 (Fig. 3A). Moreover, when VSV-infected WISH cells were treated with 200 nM IFN-1ant in combination with 100 pM IFN- α 2, IFN- β , or YNS, only the IFN- α 2-treated cells exhibited a reduction in cell survival compared to cells not treated also with IFN-1ant (Fig. 3B). Increasing the concentration of IFN- α 2 to 1 nM almost completely restored its antiviral activity (fig. S3). Hence, in contrast to its inhibitory effect on the antiproliferative response (Fig. 2C), the ability of IFN-1ant to inhibit antiviral activity was partial.

Next, we determined the ability of the IFNs to protect T47D and OVCAR3 cells from VSV and encephalomyocarditis virus (EMCV). IFN-1ant did not protect T47D cells from VSV infection, while the other IFNs tested provided only partial protection (Fig. 3C). Moreover, YNS led to a substantially lower fraction of surviving cells compared to that in response to IFN- α 2, which was in contrast to the 17-fold lower EC_{50} value of YNS (Table 2). The inability of YNS to fully protect T47D cells from VSV was not due to its antiproliferative activity, as T47D cells were resistant to this effect (Fig. 2A). Infection of T47D cells with EMCV did not provide any information, because T47D cells are resistant to this virus even in the absence of IFN (fig. S4).

Although OVCAR3 cells were sensitive to the antiproliferative effects of IFNs, relatively high IFN concentrations were required to protect them from VSV infection (Fig. 3D and Table 1). As a consequence, in the VSV antiviral assay, we observed both the antiviral and antiproliferative effects of IFNs, which resulted in a failure to obtain complete survival of infected OVCAR3 cells upon treatment with IFN- α 2 or YNS (Fig. 3D). IFN-1ant, which did not elicit any antiproliferative effect (Fig. 2B), partially protected OVCAR3 cells from VSV. OVCAR3 cells were fully protected from EMCV infection by IFN-1ant, IFN- α 2, and YNS because of the lower EC_{50} values of these IFNs in protecting against EMCV versus VSV (Fig. 3, D and E and Table 2). That IFN-1ant fully protected OVCAR3 cells from EMCV, but only partially protected them from VSV suggests that different genes are required to defend cells from these two viruses, and that these genes also differ from the genes involved in the antiproliferative activities of IFNs.

We further evaluated the cell line-specific antiviral response by infecting four different cell lines with a fluorescent reporter yellow fever virus (YFV). This assay has the benefit of directly measuring the relative numbers of infected cells, as determined by flow cytometry, independently of the antiproliferative activity of IFN. In addition to type I IFNs, we also tested type III interferon (IFNL3), which signals through interleukin-10 receptor beta (IL-10RB) and IFNLR1 (17, 18). Type III IFNs are thought to have activities similar to

those of weak type I IFNs (19). Examining the antiproliferative activity of IFNL3 on A549, Huh7.5, OVCAR3, and T47D cells revealed that, with the exception of Huh7.5 cells, IFNL3 had very little or no effect on cell proliferation (Fig. 2A). Whereas IFN- α 2 fully protected all four cell lines from YFV infection, IFN-1ant conferred only partial protection from infection at any concentration, except in the case of Huh7.5 cells, for which high concentrations of IFN-1ant were effective (Fig. 3, F and G). The ability of IFNL3 to induce cell survival from YFV infection was cell line dependent, ranging from 40% survival to full rescue. Treating A549 cells with a combination of IFN-1ant and IFNL3 resulted in a near complete inhibition of YFV replication (fig. S5), indicating an additive effect of these IFNs (which signal through different receptors). Similarly, combining IFN- α 2 at sub-saturating concentrations with IFNL1 elicits increased antiviral activity against hepatitis C virus (HCV) than do the two IFNs separately (20). Finally, because IFN is used to treat chronic hepatitis B virus (HBV) and HCV infections, we examined the efficiency of the IFNs against a fluorescent reporter HCV. We found that both IFN-1ant and IFNL3 had partial antiviral activity against HCV in Huh7.5 cells, whereas IFN- α 2 conferred full protection (Fig. 3H). In summary, examining the antiviral dose-response curves of the different IFNs on these cell lines (Fig. 3, D and G) revealed that the maximal antiviral activity of IFN-1ant was equivalent to that of ~1 pM IFN- α 2, with EC₅₀ values being 300- to 3000-fold higher than those of IFN- α 2 (Table 2).

IFN-1ant induces STAT phosphorylation to a small extent

IFN-1ant stimulated the phosphorylation of STAT1 and STAT2 in WISH cells, albeit to a low extent (Fig. 4A). Expanding our analysis to other cell lines showed that the amount of phosphorylated STAT (pSTAT) in response to 200 nM IFN-1ant was similar to that generated by 1 pM IFN- α 2 (Fig. 4, B to D), whereas the abundances of pSTAT1 and pSTAT2 in response to 2 nM IFNL3 were intermediate (Fig. 4D). These data are consistent with the biological activity of IFNL3 and its EC₅₀ values in comparison to those of the other IFNs. Because pSTAT amounts in T47D, WISH, and OVCAR3 cells were similar in response to IFNs, this suggests that the differential activation of antiproliferative and antiviral responses is not related to the extent of STAT activation.

IFN-1ant activates robust genes and inhibits induction of tunable genes

Phosphorylated STATs translocate to the nucleus and drive target gene expression (21, 22). To examine the ability of IFN-1ant to activate gene expression, we treated WISH cells with IFN-1ant at concentrations ranging from 1 to 500 nM for 6 and 24 hours. Through quantitative polymerase chain reaction (qPCR) assays, we measured the gene induction patterns of several robust (*MX1* and *IFI6*) and tunable (*CXCL11*, *IL8* and *IRF1*) genes (Fig. 5A). Consistent with its lack of an antiproliferative activity, IFN-1ant induced little or no expression of tunable genes at either time point. In contrast, IFN-1ant induced high expression of both *MX1* and *IFI6*. Moreover, the extent of IFN-1ant-induced gene expression peaked at 10 nM, suggesting saturation of IFNAR2 on WISH cells, which is consistent with the binding affinity of IFN-1ant for this receptor. These results are in contrast to the substantial increase in transcript abundance observed when IFN- α 2 concentrations were increased from 1 pM to 10 or 100 pM (fig. S6). To verify that IFN-1ant-induced gene activation was not solely dependent on its binding to IFNAR2, we

treated IFNAR1 knockout cells (23) with high concentrations of IFN-1ant, YNS, and YNS- α 8tail. We did not detect induction of ISGs, regardless of the IFN tested (fig. S7), which suggests that gene induction by IFN-1ant is a result of ternary complex formation. We next compared the gene expression profiles of the same ISGs in cells treated with 200 nM IFN-1ant or 2 nM YNS for 6 and 24 hours (Fig. 5B). The abundances of all of the examined transcripts were greater in response to YNS; however, whereas the difference between the two IFNs in their ability to induce expression of robust genes was relatively small, the expression of tunable genes was induced only by YNS.

IFN-1ant is an antagonist of the antiproliferative, but not antiviral, activities of IFNs (Figs. 2C and 3B). Noting that IFN functions are mediated by ISGs, we next determined the antagonistic effect of IFN-1ant on YNS-induced gene expression. As predicted, gene expression profiles correlated with the biological effect of IFN-1ant (Fig. 5C). Whereas combining IFN-1ant with YNS had almost no effect on the induction of robust gene expression, a substantial reduction in the expression of tunable genes was observed when 200 nM IFN-1ant was combined with 0.2 nM YNS (Fig. 5C). This effect was even more pronounced when the cells were treated for 24 hours, because of the delayed induction of these genes (Fig. 5C).

Different IFNs induce distinct gene expression profiles

To identify the molecular basis behind the seemingly contradictory effects of different IFNs on different cell lines and viruses, we assessed gene expression profiles with the BioMark real-time PCR system, which enables high-throughput analysis of 96 cDNAs with 96 different primer pairs in a single run, with a similar quality to that of standard qPCR (fig. S8). Primers were chosen for ISGs that are involved in the antiviral, antiproliferative, and immune-modulating activities of IFN to obtain a broad perspective on gene expression profiles. Cells were treated with 200 nM IFN-1ant, 1 pM IFN- α 2, 1 nM YNS, 1 nM IFN- β , or 2 nM IFNL3 for 8 and 24 hours. We showed earlier that these IFN concentrations (except for 1 pM IFN- α 2) conferred maximal biological activity (Figs. 2 and 3). Gene expression was analyzed by several bioinformatics methods. First, gene expression profiles were evaluated by principal component analysis (PCA) and hierarchical clustering analysis (Fig. 6, A and B). Note that in the PCA analysis, the x-axis accounts for most of the variability (59 and 68% for 8 and 24 hours, respectively), whereas the y-axis accounts for only 14 and 12.5% of the variability at these times. Whereas most qPCR analyses are focused on fold-differences in mRNA (based on C_T values), we also explored C_T values, which represent absolute mRNA amounts. PCA of C_T values for the various conditions (cell lines and IFNs) revealed that YNS and IFN- β clustered closely together (Fig. 6, A to C), which was also true for IFN-1ant and 1 pM IFN- α 2 (Fig. 6, A, B and D). Both pairs of IFNs were clearly separable by PCA. In addition, although the gene expression profile of IFNL3-treated cells was similar to that of IFN-1ant-treated cells at the 8-hour time point, IFNL3 led to a more distinct gene expression profile at 24 hours (Fig. 6, A, B and E). This likely is a result of the inhibitory mechanisms that affect IFN-1ant-dependent, but not IFNL3-dependent, gene expression (24, 25). Furthermore, clustering the C_T data (Fig. 6B), which represents absolute mRNA amounts, revealed that the gene expression profiles for a specific

cell line clustered together, and are separated from the other cell lines. This indicates cell line-specific differences in basal gene expression.

Expression of many of the IFN-induced genes does not confer virus-specific protection

We further analyzed the expression data for WISH, OVCAR3, and T47D cells. As shown earlier, these cell lines exhibited distinct IFN-induced biological activities. We focused on treatments with 200 nM IFN-1ant and 1 nM YNS, and analyzed the expression profiles of two groups of transcripts with the NetWalker analysis tool (26). The highest $-C_T$ values of tunable genes were observed in OVCAR3 cells treated with YNS, with the next highest values found in YNS-treated WISH cells. Conversely, as was the case for all cell lines treated with IFN-1ant, YNS-treated T47D cells exhibited very low $-C_T$ values for the tunable genes (Fig. 7A). These data show a strong correlation between tunable gene induction and antiproliferative responsiveness. On the other hand, robust genes displayed high $-C_T$ values in all cell lines in response to either IFN-1ant or YNS (Fig. 7A). Because there were major differences in the antiviral responses between cell lines and IFNs (Fig. 3, A, and C to E), one cannot pinpoint specific genes from this list as being responsible for the variation in viral defense. The basal mRNA amounts of tunable genes were in general much lower than those of robust genes (Fig. 7A, zero time point). Examining $-C_T$ values (Fig. 7A, right panel) showed that YNS-induced gene expression was generally stronger than that induced by IFN-1ant; however, this does not seem to be the determining factor in antiviral potency between different IFNs and type of viruses.

We next compared the induction of tunable and robust genes by IFN-1ant and YNS (Fig. 7B). Expression of robust genes was activated under all conditions and in all cell lines. Conversely, the expression of tunable genes was activated only by YNS. Moreover, in T47D cells, whose proliferation was not inhibited by IFNs, the extent of tunable gene expression was the lowest, whereas in OVCAR3 cells, the extent of tunable gene expression by YNS was the highest (Fig. 7B). These results clearly show a difference in the transcriptional program between robust and tunable genes.

Differentiating robust from tunable genes by clustering and classification

To formally differentiate between robust and tunable genes, we classified and clustered the IFN-induced gene expression data. This was performed by correlation-based constant shift embedding (CCSE) to extract distances from pairwise similarities between gene expression profiles (fig. S9 and see the Materials and Methods). We used various tunable and robust genes as training sets (Fig. 7A). All of these genes (except *IDO1*) were clustered according to our previous classification (Fig. 7A). *IDO1* is an exception, because its expression in T47D cells was increased by YNS; however, the absolute amount of *IDO1* mRNA in these cells was similar to that in the other cell lines under non-induced conditions. In addition to the genes of the training set, *HRASLS2*, *TNFSF13B*, *CEACAM1*, *IFI16*, *TNFRSF10A*, and *TNFAIP3* were classified as tunable genes (Fig. 8A and table S1). These genes are involved in tumor suppression, apoptosis, and the induction and modulation of immune and inflammatory responses. *SOC31*, *IRF2*, and *USP18* were left uncategorized (Fig. 8A). The expression of *SOC31* and *IRF2* was induced only by a strong IFN signal; however, their expression was also induced with the same amplitude in T47D cells. Analysis of the

expression profile of *USP18* positioned this gene on the border between the two groups. Finally, our mathematical analysis (Fig. 8A) suggests the presence of two distinct clusters within the group of robust genes (with CCSE feature 2 above or below that of *TRIM22*), which requires further examination.

Promoters of robust genes contain canonical transcription factor-binding sites

Differences in transcriptional programs can be linked to differential transcription factor binding in promoter regions. We evaluated the putative binding sites of 445 transcription factors in the promoter sequences of robust, tunable, and housekeeping genes (table S2). For each evaluated transcription factor, we obtained a position weight matrix (PWM) that describes its binding preferences to DNA from the TRANSFAC database. Next, we located the best hits for each PWM across the promoters of each group of genes, and compared the distributions of best-hit scores between the groups by analysis of variance (ANOVA) (see Materials and Methods). We found a number of transcription factors that had a statistically significant difference in binding between the promoters of the robust and tunable genes (Fig. 8B). We found that the binding sites of the three canonical transcription factors IFN-stimulated response element (ISRE), IFN regulatory factor 8 (IRF8), and IRF1 were enriched specifically in the promoters of robust genes, whereas there were no statistically significant differences in their binding to the promoters of tunable and housekeeping groups. In addition, the binding sites of hepatocyte nuclear factor 4 alpha (HNF4A) and Wilms Tumor 1 (WT1) were statistically significantly enriched in the promoters of tunable genes, but not in the promoters of robust or housekeeping genes (Fig. 8B).

To further evaluate mechanistic differences between the activation of robust and tunable genes, we determined the timing of expression for members of each group (Fig. 8B). Whereas expression of robust genes was activated within the first 4 hours of treatment with IFN, the expression of tunable genes was activated after a lag period of ~12 hours. Moreover, the expression of robust genes peaked at 16 hours after treatment (presumably because of negative feedback by SOCS and USP18), whereas the expression of tunable genes was further enhanced even after 36 hours. This would suggest either that tunable genes are not directly activated through the JAK-STAT pathway or that there exists an inhibitor that delays their induction.

To directly assess the importance of ISGF3 in inducing the expression of robust and tunable genes we knocked down STAT2, IRF9, or both with small interfering RNAs (siRNAs). This resulted in a substantial reduction in *MX1* and *IFI6* basal expression as well as in their induction upon IFN treatment (Fig. 8C), indicating the importance of ISGF3 for their expression. Analysis of several tunable genes demonstrated a more complex mechanism of gene activation. The basal expression of *CXCL10* and *CXCL11* was hardly affected by knock down of STAT2 or IRF9, while their induction by YNS was substantially reduced (Fig. 8C). The basal expression of *IL8* increased in cells in which STAT2 and IRF9 were knocked down, implying that an inhibitory mechanism may be involved in regulating its expression. In response to YNS, the expression of *IL8* was only mildly affected by knock down of STAT2 or IRF9. *IRF1* expression was essentially unaffected by knockdown of either STAT2 or IRF9, which is consistent with its activation by STAT1 homodimers (27).

These results imply that at least some tunable genes require ISGF3 for their expression, either directly or indirectly.

To analyze the role of HNF4A and WT1 in IFN-induced gene activation, we performed knockdowns of either or both of them. Whereas knockdown of these transcription factors had almost no effect on the amounts of mRNA for robust genes in response to IFNs (except for having some small effect on *IRF7* expression), knockdown of HNF4A substantially increased their basal expression (Fig. 8D). This suggests that HNF4A may act to repress the expression of robust genes in unstimulated cells. Examining the effect of knockdown of HNF4A on the expression of tunable genes demonstrated that HNF4A activated IFN-dependent *CXCL10* expression (Fig. 8D). On the other hand, WT1 repressed the IFN-induced-expression of *IL6*, *IL8*, and *IL11*. These results suggest that HNF4A and WT1 are indeed involved in the IFN-dependent regulation of expression of tunable and robust genes, as was predicted by the bioinformatics analysis.

DISCUSSION

Type I IFNs are a main component of the host innate immune response against viral infection. They limit early viral replication through multiple direct molecular mechanisms, including inhibition of viral transcription and translation, as well as degradation of viral nucleic acids (28). However, they also activate the adaptive immune response, take part in immunomodulation, and induce antiproliferative activities, such as cell cycle arrest and apoptosis (29–31). Here, we used an IFN mutant (IFN-1ant) that exclusively activates the cellular antiviral response to study the regulation of the different functions of IFNs. IFN-1ant was originally produced as an antagonist of type I IFNs (14), which binds tightly to IFNAR2, but has little or no detectable binding to IFNAR1. Because ternary complex formation is required for signaling [although, non JAK-STAT signaling is suggested to occur in the absence of IFNAR2, but not IFNAR1 (32)], IFN-1ant would not be expected to have the ability to signal. Here, we revealed that despite its undetectable binding affinity for IFNAR1, IFN-1ant elicited a degree of antiviral response at very high concentrations (>1000-fold higher than the EC_{50} of IFN- $\alpha 2$). We found that IFN-1ant activated a signal equivalent to that activated by 1 pM wild-type IFN- $\alpha 2$, a concentration that supports the formation of only few ternary complexes, but was sufficient to induce an antiviral, but not an antiproliferative, response. Because IFN-1ant binds tightly to IFNAR2, this finding also explains how it acts as an antagonist of the antiproliferative activity of IFN by blocking the formation of many more ternary complexes. Furthermore, the IFN-1ant-induced antiviral response was virus- and cell type-specific, ranging from providing no rescue to complete protection from viral infection. This wide range of antiviral activity may result from complex virus-host interactions, together with differences in the cellular composition of antiviral proteins.

Upon binding to their cell-surface receptor, type I IFNs activate the expression of more than 1000 genes involved in various activities (21, 22). IFN-induced genes can be divided into at least two groups: (i) genes that are highly sensitive and require only picomolar concentrations of IFNs for their expression (so-called robust genes), and (ii) genes that require 100-fold higher concentrations of IFN for their expression (so-called tunable genes).

Analysis of gene array results showed that genes whose products mediate IFN-dependent antiviral activity belong to the first group, whereas the products of genes from the second group are involved in cell proliferation, chemokine activity, inflammation, and other biological processes (9, 12, 16, 33–36). IFN-1ant is unique among IFN variants in that it only activates the expression of robust genes, consistent with its inability to elicit antiproliferative activity in all of the five cell lines that we examined.

Although IFN-1ant and YNS induced similar profiles of robust gene expression in all cell lines, these gene activation patterns were not consistent with their degrees of antiviral activity, particularly in T47D cells, which were not protected from VSV by IFN-1ant and were only partially protected by YNS. This finding may relate to the activation of virus-specific antiviral genes that were not examined in this study. Indeed, two studies in mice demonstrated that classical robust genes (*MX1*, *OAS*, and *EIF2AK2*) are either insufficient or nonessential to protect from viral infection (37, 38). A study of the role of ISGs in viral defense revealed that different genes stimulated by IFNs were required to target different viruses (39). Results from another study also suggest that the mRNA amounts of robust genes in different cell lines do not simply correlate with the inhibition of HIV-1 infection (40). Therefore, although we examined the expression of about 100 genes, we may have missed specific genes that are responsible for the antiviral response of a particular cell line to a specific virus. An unbiased approach, such as the use of microarrays or RNA-sequencing in combination with knockdown of suspected targets may be useful in identifying the regulatory mechanisms responsible for the cell line-specific and virus-specific functions of IFNs. Because IFN-1ant elicited only antiviral activity, this approach might also uncover the mechanisms responsible for differential IFN activity with respect to antiviral versus antiproliferative signaling pathways.

IFN-1ant and IFNL3 mediated similar biological activities and gene expression profiles, at least at 8 hours after treatment. Both IFNs mainly activated expression of the robust group of genes, but did not induce expression of the tunable group of genes in all of the cell lines examined, or at least did so to a very low extent. At later times, negative feedback by USP18 inhibits signaling by type I, but not type III, IFNs (24, 25), and, at least under the experimental conditions used here, the expression of IFNL3-induced genes was not inhibited. It may be clinically relevant that combining IFN-1ant and IFNL3 resulted in an additive antiviral effect while blocking the tunable type I IFN response that may relate to some of its side effects (41). Another clinical advantage of combining these two IFNs comes from the independent activities of the type I and type III receptor complexes. In vitro and in vivo experiments demonstrated that type I or type III antiviral systems are functional in cells lacking receptors from the opposite receptor complex (42–44).

Activating a strong IFN signal (with YNS, IFN- β , or a high concentration of IFN- α 2) resulted in maximal gene expression, including the expression of tunable genes. Nevertheless, YNS did not enhance the expression of tunable genes in T47D cells. This is consistent with the lack of an antiproliferative response in these cells. The observation that not all tumor cells, irrespective of their origin, are susceptible to the apoptotic effects of IFNs (45, 46), together with our gene expression data and promoter analysis, suggest that unique transcriptional signatures are present in IFN-sensitive cells, and that these signatures

are distinct from those in IFN-resistant cells. Because STAT phosphorylation and the expression of robust genes did not vary between the cell lines that we tested, the analysis of downstream signaling components, which are not well defined, should distinguish between responsive and nonresponsive cells. This was also suggested in a study of renal cancer cells that are resistant to the antiproliferative activity, but not the antiviral activity, of IFN- α or to IFN-induced gene activation (47).

Bioinformatics analysis of the extent of gene expression in response to IFN-1ant, IFN- α 2, IFN- β , YNS, and IFNL3 in a number of cell lines provided insights into the relationship between gene induction, signaling, and biological outcome. Three methods of clustering revealed two basic sets of IFN-sensitive genes: robust and tunable. Members of the first set are directly related to the antiviral response according to Gene Ontology (GO) biological process terms in DAVID, and their expression is driven by canonical, IFN-induced transcription factors, such as ISGF3, IRF8, and IRF1. Moreover, the basal mRNA amounts for these genes were already high and were further increased by minute quantities of IFN- α 2, IFN- β , or YNS. Conversely, basal mRNA amounts of tunable genes were low, and much higher concentrations of IFN were required to increase their abundance.

According to DAVID, the activities of tunable genes mainly cluster to the cytokine, chemokine, inflammatory, and immune responses, as well as the regulation of cell proliferation, cell death, and locomotion functions. Moreover, the promoters of tunable genes are not enriched for binding sites for ISRE, IRF8, or IRF1. This does not mean that ISGF3 is unimportant for the expression of tunable genes, because knockdown of STAT2 or IRF9 substantially reduced their expression. Nevertheless, activation of tunable gene expression by IFN may be a secondary response, as was suggested by the delayed onset of their activation and by the absence of canonical ISRE-binding sites in their promoters, or it may be delayed by specific transcriptional inhibitors. Our bioinformatics analysis also showed enrichment for the binding of HNF4A and WT1 to putative sequences within the promoters of tunable genes. Knockdown of these two transcription factors differentially affected basal and IFN-induced transcription of robust and tunable genes. Whereas knockdown of HNF4A resulted in a substantial increase in the basal expression of robust genes, transcriptional activation of some of the tunable genes in response to IFN was reduced.

At the early stages of IFN signaling, the rates of receptor endocytosis and degradation or recycling rather than the kinetics of ligand dissociation determine the decomposition of the IFN signaling complex (3). This could explain why similar activities can be observed for all IFNs except for IFN- α 1, which has the weakest binding affinity and biological activity of all of the IFN α forms (8, 30). IFN- α 2 mutants with lower binding affinity, as well as IFN- α 1, have dissociation kinetics that are more rapid than the kinetics of receptor endocytosis and, for this reason, show reduced activity compared with that of wild-type IFN- α 2. We did not observe any endocytosis of IFNARs in response to IFN-1ant. Furthermore, the low binding affinity and the high EC₅₀ values of IFN-1ant are reminiscent of the properties of IFN- α 1, but are more extreme. IFN- α 1 is reported to be among the major subtypes of type I IFN that are produced upon viral infection (48–50). That the IFN- α isoform with the lowest activity

is one of the highest produced in response to a pathogen may enable a careful adjustment of cellular activation during host defense.

Because of its potent antiviral activity, IFN-based therapies have been developed for chronic infections with HBV and HCV, as well as for HIV infections; however, a poorly understood phenomenon has been the persistence of virus despite the induction of antiviral immune responses by type I IFNs (29). Teijaro *et al.* and Wilson *et al.* (51, 52) addressed this question and found that IFNs can also suppress the immune system in ways that promote viral persistence. The authors showed that blocking IFN signaling led to an initial increase in the amount of lymphocytic choriomeningitis virus (LCMV) in mice, but to a substantial reduction in viral load by two months after infection and to an improved immune response. The conclusion from these studies is that although the early antiviral effects of IFNs are critical, the potential immunoregulatory roles of IFNs later during chronic infection could explain the paradoxical clinical observations with IFN-based treatments. This conclusion should, however be taken with caution. In a very recent study on SIV-infected rhesus macaques treated with IFN-1ant we showed that inhibition of interferon activity during early infection resulted in a more severe disease over time (53). IFN-1ant may also have therapeutic applications in the treatment of systematic lupus erythematosus (SLE), a disease that appears to be driven by constant IFN signaling (54, 55). Moreover, combining IFN-1ant with IFN- α 2 or IFN- β may provide useful alternatives to conventional IFN-based therapy, because this combination promotes full antiviral potency while reducing the occurrence of side effects driven by the tunable genes.

Here, we demonstrated that IFN-1ant does not have antiproliferative activity or induce the expression of inflammation-associated genes, but that it elicits antiviral effects over a wide range of concentrations. Moreover, we used IFN-1ant as a tool to differentiate between the robust and tunable responses of type I IFNs, and we suggest that these relate to differences in gene regulation programs induced by different IFNs at different concentrations and in various cell types. Future studies should focus on how this relates to the varied activities of IFNs at the organismal level to assess how to optimize the use of IFN-1ant as a drug for the multiplicity of conditions for which it is applied.

MATERIALS AND METHODS

Cell lines and viruses

WISH is a human amniotic epithelial cell line. The OVCAR3 ovarian cancer cell line and the T47D breast cancer cell line are part of the NCI60 panel of human tumor cells. A549 is an adenocarcinomic human alveolar basal epithelial cell line. Huh7.5 cells derived from the human hepatoma cell line Huh7 and are highly permissive to HCV infection. YFV-Venus and HCV-YPet were described previously (56–58).

Protein expression and purification

Recombinant IFNs and IFNARs were expressed and purified as detailed previously (11, 59, 60).

In vitro binding assays

The binding affinities of wild-type and mutant forms of IFN- α 2 for IFNAR1-EC or IFNAR2-EC were measured by SPR with the ProteOn XPR36, as previously described (6, 13, 59).

Antiviral and antiproliferative assays

Assays of the antiproliferative effects of the IFNs were performed as described previously (61). Cell numbers were monitored after 72 or 96 hours of IFN treatment by staining the cells with crystal violet or by measuring cellular ATP content with the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's protocol. Antiviral activity against VSV and EMCV was assessed by determining the extent of inhibition of the cytopathic effects of the viruses (39, 61, 62). In general, interferon was added at serial dilutions to cells grown on flat-bottomed 96-well plates. Four hours later, the virus was added to all wells. Incubation times were determined after calibration, and were 17, 23, and 25 hours for VSV in WISH, OVCAR3, and T47D cells, respectively, and 25 hours for EMCV in OVCAR3 cells. Cell density was measured by crystal violet staining. The overlap between those concentrations of IFN that promoted an antiproliferative effect and those that promoted antiviral activity was marginal (fig. S10), except for OVCAR3 cells treated with YNS (in reality, it was even smaller because antiviral activity was measured after ~24 hours, a time point at which antiproliferative effects were limited). To further limit this problem we also used the fluorescent reporter viruses YFV-Venus and HCV-YPet. In these experiments, the fluorescent proteins served as markers for viral protein production. For the YFV or HCV antiviral assays, cells were grown in 24-well plates. Six hours after the addition of IFN, the virus was added for 1 hour, removed, and then medium-containing IFN was added back to cells. After 24 hours (YFV) or 48 hours (HCV), cells were harvested with Accumax (Millipore) and fixed with 2% paraformaldehyde before being analyzed by flow cytometry. For normalization, virus infected, untreated cells were taken as one for measuring virus infection efficiency. EC₅₀ values for antiproliferative and antiviral assays were calculated as previously described (61).

Flow cytometric analysis of cell-surface receptors

Relative cell-surface amounts of IFNAR1 and IFNAR2 were assessed by flow cytometry using the AA3 and 117.7 antibodies as previously described (9, 63).

Analysis of pSTAT1 and pSTAT2

STAT phosphorylation was determined by Western blotting as described previously (6), with the following antibodies: polyclonal anti-pSTAT1 (Tyr⁷⁰¹) (Santa Cruz Biotechnology, Inc.), polyclonal anti-pSTAT2 (Tyr⁶⁸⁹) (Millipore), polyclonal anti-STAT1 (Santa Cruz Biotechnology, Inc.), polyclonal anti-STAT2 (Delta Biolabs), and monoclonal α -tubulin (Sigma). Quantitative analysis of band intensities on Western blots was performed with ImageQuant software (GE healthcare).

Quantitative PCR analysis

The extent of expression of human IFN-stimulated genes was measured as described previously (6). High-throughput qPCR was performed with BioMark 48×48 and 96×96 Dynamic Arrays (Fluidigm Corporation) according to the manufacturer's protocol. cDNAs (50 ng/μl) were pre-amplified with all the primers together (see table S3) and analyzed by the BioMark real-time PCR instrument. Initial data analysis was performed with the Fluidigm real-time PCR analysis software.

siRNAs

WISH cells were transfected for 48 hours with the following human siRNAs: HNF4A or WT1 siGENOME SMARTpool siRNAs, ON-TARGETplus STAT2 or IRF9 siRNAs, ON-TARGETplus Non-Targeting siRNA #2 (control siRNA), or a combination of these siRNAs (Dharmacon). Transfection was performed with INTERFERin (Polyplus-transfection) according to the manufacturer's recommendations.

Data analysis

Principal component analysis (PCA) was performed with Partek Genomics Suite 6.6 software. Hierarchical clustering was performed in Partek with Pearson dissimilarity and complete linkage. To test whether different transcription factors were more likely to regulate the expression of robust genes rather than that of tunable genes, PWMs for 445 transcription factors from the TransFac database (version 11.4) were retrieved. Next, we extracted the promoter sequences, which were defined as the region from 2000 base pairs (bp) upstream to 500 bp downstream of the transcription start site (TSS). For each transcription factor–promoter pair, we searched for the highest matching score, which was defined as the log-ratio sum between the PWM and the background nucleotide distribution. For example, for a PWM of length N and a sequence $S = S_1..S_N$:

$$score(S) = \sum_{i=1}^N \log(p_i(S_i) / b(S_i)) \quad (\text{Eq. 1})$$

where $P_i(S_i)$ is the probability to see nucleotide S_i at position i of the PWM, and $b(S_i)$ is the probability to see nucleotide S_i given the nucleotide distribution in the promoter. Finally, for each transcription factor, we computed the mean and standard error of the best score across the promoters of robust and tunable genes (as well as for the housekeeping genes, see table S2), and computed a P value for the statistical significance of the difference by one-way ANOVA. Further analysis for classification and clustering of the IFN-induced gene expression data consisted of three parts. First, we performed binary classification to generalize the existing manual set of binary labels (robust and tunable) for the genes. Classification was performed with a linear support vector machine trained on the constant shift embedding in two dimensions of the genes, in which the Pearson correlation of the pairwise distance between all features (all time points for all conditions) has been employed a dissimilarity metric (64, 65). Next, we proceeded with unsupervised analysis, which was performed based on the same constant shift embedding in 2D based on correlation dissimilarity. The analysis consisted of clustering with Gaussian mixture models. The Bayesian information criteria, a principle for model order selection, selected three

components (65, 66). Finally, we used a system for the analysis of logical queries to identify genes on the basis of given queries. For each, we calculated the empirical cumulative density functions $P_{\text{gene}}(X)$ over all combinations of time points and conditions. Queries were combined according to the rules of probability calculus to yield joint distributions on the basis of an assumption of independence. In the query system, atomic literals for fold changes were combined into conjunctive expressions by multiplying their estimated cumulative distributions (67). The complete list of genes used for computational data analysis is found in table S1. Functional categories enriched in the different gene clusters were identified with the functional annotation and clustering tool of the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v 6.7 (68, 69).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES AND NOTES

- Haller O, Kochs G, Weber F. The interferon response circuit: induction and suppression by pathogenic viruses. *Virology*. 2006; 344:119–130. [PubMed: 16364743]
- Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat. Rev. Immunol.* 2005; 5:375–386. [PubMed: 15864272]
- Piehler J, Thomas C, Garcia KC, Schreiber G. Structural and dynamic determinants of type I interferon receptor assembly and their functional interpretation. *Immunol. Rev.* 2012; 250:317–334. [PubMed: 23046138]
- Brierley MM, Fish EN. Review: IFN- α /beta receptor interactions to biologic outcomes: understanding the circuitry. *J. Interferon Cytokine Res. Off. J. Int. Soc. Interferon Cytokine Res.* 2002; 22:835–845.
- Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu. Rev. Biochem.* 1998; 67:227–264. [PubMed: 9759489]
- Levin D, Harari D, Schreiber G. Stochastic receptor expression determines cell fate upon interferon treatment. *Mol. Cell. Biol.* 2011; 31:3252–3266. [PubMed: 21690295]
- Uzé G, Schreiber G, Piehler J, Pellegrini S. The receptor of the type I interferon family. *Curr. Top. Microbiol. Immunol.* 2007; 316:71–95. [PubMed: 17969444]
- Lavoie TB, Kalie E, Crisafulli-Cabatu S, Abramovich R, DiGioia G, Moolchan K, Pestka S, Schreiber G. Binding and activity of all human alpha interferon subtypes. *Cytokine*. 2011; 56:282–289. [PubMed: 21856167]
- Jaitin DA, Roisman LC, Jaks E, Gavutis M, Piehler J, Van der Heyden J, Uze G, Schreiber G. Inquiring into the differential action of interferons (IFNs): an IFN- α 2 mutant with enhanced affinity to IFNAR1 is functionally similar to IFN- β . *Mol. Cell. Biol.* 2006; 26:1888–1897. [PubMed: 16479007]

10. Jaks E, Gavutis M, Uzé G, Martal J, Piehler J. Differential receptor subunit affinities of type I interferons govern differential signal activation. *J. Mol. Biol.* 2007; 366:525–539. [PubMed: 17174979]
11. Kalie E, Jaitin DA, Abramovich R, Schreiber G. An interferon alpha2 mutant optimized by phage display for IFNAR1 binding confers specifically enhanced antitumor activities. *J. Biol. Chem.* 2007; 282:11602–11611. [PubMed: 17310065]
12. Kalie E, Jaitin DA, Podoplelova Y, Piehler J, Schreiber G. The stability of the ternary interferon-receptor complex rather than the affinity to the individual subunits dictates differential biological activities. *J. Biol. Chem.* 2008; 283:32925–32936. [PubMed: 18801736]
13. Slutzki M, Jaitin DA, Yehezkel TB, Schreiber G. Variations in the unstructured C-terminal tail of interferons contribute to differential receptor binding and biological activity. *J. Mol. Biol.* 2006; 360:1019–1030. [PubMed: 16815442]
14. Pan M, Kalie E, Scaglione BJ, Raveche ES, Schreiber G, Langer JA. Mutation of the IFNAR-1 receptor binding site of human IFN-alpha2 generates type I IFN competitive antagonists. *Biochemistry (Mosc.)*. 2008; 47:12018–12027.
15. Schreiber G, Walter MR. Cytokine-receptor interactions as drug targets. *Curr. Opin. Chem. Biol.* 2010; 14:511–519. [PubMed: 20619718]
16. Thomas C, Moraga I, Levin D, Krutzik PO, Podoplelova Y, Trejo A, Lee C, Yarden G, Vleck SE, Glenn JS, Nolan GP, Piehler J, Schreiber G, Garcia KC. Structural Linkage between Ligand Discrimination and Receptor Activation by Type I Interferons. *Cell*. 2011; 146:621–632. [PubMed: 21854986]
17. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, Donnelly RP. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat. Immunol.* 2003; 4:69–77. [PubMed: 12483210]
18. Donnelly RP, Sheikh F, Kotenko SV, Dickensheets H. The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10R2) chain. *J. Leukoc. Biol.* 2004; 76:314–321. [PubMed: 15123776]
19. Meager A, Visvalingam K, Dilger P, Bryan D, Wadhwa M. Biological activity of interleukins-28 and -29: comparison with type I interferons. *Cytokine*. 2005; 31:109–118. [PubMed: 15899585]
20. Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, MacDonald MR, Rice CM. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology*. 2006; 131:1887–1898. [PubMed: 17087946]
21. Der SD, Zhou A, Williams BR, Silverman RH. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc. Natl. Acad. Sci. U. S. A.* 1998; 95:15623–15628. [PubMed: 9861020]
22. De Veer MJ, Holko M, Frevel M, Walker E, Der S, Paranjape JM, Silverman RH, Williams BR. Functional classification of interferon-stimulated genes identified using microarrays. *J. Leukoc. Biol.* 2001; 69:912–920. [PubMed: 11404376]
23. Naka K, Takemoto K, Abe K, Dansako H, Ikeda M, Shimotohno K, Kato N. Interferon resistance of hepatitis C virus replicon-harboring cells is caused by functional disruption of type I interferon receptors. *J. Gen. Virol.* 2005; 86:2787–2792. [PubMed: 16186233]
24. Makowska Z, Duong FHT, Trincucci G, Tough DF, Heim MH. Interferon- β and interferon- λ signaling is not affected by interferon-induced refractoriness to interferon- α in vivo. *Hepatology*. Baltim. Md. 2011; 53:1154–1163.
25. François-Newton V, Magno de Freitas Almeida G, Payelle-Brogard B, Monneron D, Pichard-Garcia L, Piehler J, Pellegrini S, Uzé G. USP18-based negative feedback control is induced by type I and type III interferons and specifically inactivates interferon α response. *PloS One*. 2011; 6:e22200. [PubMed: 21779393]
26. Komurov K, Dursun S, Erdin S, Ram PT. NetWalker: a contextual network analysis tool for functional genomics. *BMC Genomics*. 2012; 13:282. [PubMed: 22732065]
27. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. *Nat. Rev. Immunol.* 2014; 14:36–49. [PubMed: 24362405]
28. Bowie AG, Unterholzner L. Viral evasion and subversion of pattern-recognition receptor signalling. *Nat. Rev. Immunol.* 2008; 8:911–922. [PubMed: 18989317]

29. Odorizzi PM, Wherry EJ. Immunology. An interferon paradox. *Science*. 2013; 340:155–156. [PubMed: 23580520]
30. Moll HP, Maier T, Zommer A, Lavoie T, Brostjan C. The differential activity of interferon- α subtypes is consistent among distinct target genes and cell types. *Cytokine*. 2011; 53:52–59. [PubMed: 20943413]
31. Berry CM, Hertzog PJ, Mangan NE. Interferons as biomarkers and effectors: lessons learned from animal models. *Biomark. Med.* 2012; 6:159–176. [PubMed: 22448790]
32. De Weerd NA, Vivian JP, Nguyen TK, Mangan NE, Gould JA, Braniff S-J, Zaker-Tabrizi L, Fung KY, Forster SC, Beddoe T, Reid HH, Rossjohn J, Hertzog PJ. Structural basis of a unique interferon- β signaling axis mediated via the receptor IFNAR1. *Nat. Immunol.* 2013; 14:901–907. [PubMed: 23872679]
33. Leaman DW, Chawla-Sarkar M, Jacobs B, Vyas K, Sun Y, Ozdemir A, Yi T, Williams BR, Borden EC. Novel growth and death related interferon-stimulated genes (ISGs) in melanoma: greater potency of IFN-beta compared with IFN-alpha2. *J. Interferon Cytokine Res. Off. J. Int. Soc. Interferon Cytokine Res.* 2003; 23:745–756.
34. Samarajiwa SA, Forster S, Auchettl K, Hertzog PJ. INTERFEROME: the database of interferon regulated genes. *Nucleic Acids Res.* 2009; 37:D852–857. [PubMed: 18996892]
35. Coelho LFL, Magno de Freitas Almeida G, Mennechet FJD, Blangy A, Uzé G. Interferon-alpha and -beta differentially regulate osteoclastogenesis: role of differential induction of chemokine CXCL11 expression. *Proc. Natl. Acad. Sci. U. S. A.* 2005; 102:11917–11922. [PubMed: 16081539]
36. Schoggins JW, Rice CM. Interferon-stimulated genes and their antiviral effector functions. *Curr. Opin. Virol.* 2011; 1:519–525. [PubMed: 22328912]
37. Bowick GC, Airo AM, Bente DA. Expression of interferon-induced antiviral genes is delayed in a STAT1 knockout mouse model of Crimean-Congo hemorrhagic fever. *Virol. J.* 2012; 9:122. [PubMed: 22713837]
38. Zhou A, Paranjape JM, Der SD, Williams BR, Silverman RH. Interferon action in triply deficient mice reveals the existence of alternative antiviral pathways. *Virology*. 1999; 258:435–440. [PubMed: 10366581]
39. Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, Rice CM. A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature*. 2011; 472:481–485. [PubMed: 21478870]
40. Kane M, Yadav SS, Bitzegeio J, Kutluay SB, Zang T, Wilson SJ, Schoggins JW, Rice CM, Yamashita M, Hatzioannou T, Bieniasz PD. MX2 is an interferon-induced inhibitor of HIV-1 infection. *Nature*. 2013; 502:563–566. [PubMed: 24121441]
41. Fuchs SY. Hope and fear for interferon: the receptor-centric outlook on the future of interferon therapy. *J. Interferon Cytokine Res. Off. J. Int. Soc. Interferon Cytokine Res.* 2013; 33:211–225.
42. Ank N, Iversen MB, Bartholdy C, Staeheli P, Hartmann R, Jensen UB, Dagnaes-Hansen F, Thomsen AR, Chen Z, Haugen H, Klucher K, Paludan SR. An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. *J. Immunol. Baltim. Md 1950*. 2008; 180:2474–2485.
43. Zhou Z, Hamming OJ, Ank N, Paludan SR, Nielsen AL, Hartmann R. Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. *J. Virol.* 2007; 81:7749–7758. [PubMed: 17507495]
44. Mordstein M, Kochs G, Dumoutier L, Renauld J-C, Paludan SR, Klucher K, Staeheli P. Interferon-lambda contributes to innate immunity of mice against influenza A virus but not against hepatotropic viruses. *PLoS Pathog.* 2008; 4:e1000151. [PubMed: 18787692]
45. Rodríguez-Villanueva J, McDonnell TJ. Induction of apoptotic cell death in non-melanoma skin cancer by interferon-alpha. *Int. J. Cancer J. Int. Cancer.* 1995; 61:110–114.
46. Sgorbissa A, Tomasella A, Potu H, Manini I, Brancolini C. Type I IFNs signaling and apoptosis resistance in glioblastoma cells. *Apoptosis Int. J. Program. Cell. Death.* 2011; 16:1229–1244.

47. Pfeffer LM, Wang C, Constantinescu SN, Croze E, Blatt LM, Albino AP, Nanus DM. Human renal cancers resistant to IFN's antiproliferative action exhibit sensitivity to IFN's gene-inducing and antiviral actions. *J. Urol.* 1996; 156:1867–1871. [PubMed: 8863634]
48. Nyman TA, Tölö H, Parkkinen J, Kalkkinen N. Identification of nine interferon-alpha subtypes produced by Sendai virus-induced human peripheral blood leucocytes. *Biochem. J.* 1998; 329:295–302. Pt 2. [PubMed: 9425112]
49. Brandt ER, Linnane AW, Devenish RJ. Expression of IFN A genes in subpopulations of peripheral blood cells. *Br. J. Haematol.* 1994; 86:717–725. [PubMed: 7918063]
50. Löseke S, Grage-Griebenow E, Wagner A, Gehlhar K, Bufe A. Differential expression of IFN-alpha subtypes in human PBMC: evaluation of novel real-time PCR assays. *J. Immunol. Methods.* 2003; 276:207–222. [PubMed: 12738374]
51. Teijaro JR, Ng C, Lee AM, Sullivan BM, Sheehan KCF, Welch M, Schreiber RD, de la Torre JC, Oldstone MBA. Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science.* 2013; 340:207–211. [PubMed: 23580529]
52. Wilson EB, Yamada DH, Elsaesser H, Herskovitz J, Deng J, Cheng G, Aronow BJ, Karp CL, Brooks DG. Blockade of chronic type I interferon signaling to control persistent LCMV infection. *Science.* 2013; 340:202–207. [PubMed: 23580528]
53. Sandler NG, Bosinger SE, Estes JD, Zhu RT, Tharp GK, Boritz E, Levin D, Wijeyesinghe S, Makamdop KN, del Prete GQ, Hill BJ, Timmer JK, Reiss E, Darko S, Contijoch E, Todd JP, Silvestri G, Nason M, Norgren RB Jr. Keele BF, Rao S, Langer JA, Lifson JD, Schreiber G, Douek DC. Type I IFN responses in rhesus macaques prevent SIV transmission and slow disease progression. *Nature.* 2014 in press.
54. Crow MK. Interferon pathway activation in systemic lupus erythematosus. *Curr. Rheumatol. Rep.* 2005; 7:463–468. [PubMed: 16303107]
55. Pascual V, Farkas L, Banchereau J. Systemic lupus erythematosus: all roads lead to type I interferons. *Curr. Opin. Immunol.* 2006; 18:676–682. [PubMed: 17011763]
56. Jones CT, Catanese MT, Law LMJ, Khetani SR, Syder AJ, Ploss A, Oh TS, Schoggins JW, MacDonald MR, Bhatia SN, Rice CM. Real-time imaging of hepatitis C virus infection using a fluorescent cell-based reporter system. *Nat. Biotechnol.* 2010; 28:167–171. [PubMed: 20118917]
57. Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset F-L, Bartenschlager R. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc. Natl. Acad. Sci. U. S. A.* 2006; 103:7408–7413. [PubMed: 16651538]
58. Horwitz JA, Dorner M, Friling T, Donovan BM, Vogt A, Loureiro J, Oh T, Rice CM, Ploss A. Expression of heterologous proteins flanked by NS3-4A cleavage sites within the hepatitis C virus polyprotein. *Virology.* 2013; 439:23–33. [PubMed: 23485372]
59. Piehler J, Schreiber G. Biophysical analysis of the interaction of human ifnar2 expressed in *E. coli* with IFNalpha2. *J. Mol. Biol.* 1999; 289:57–67. [PubMed: 10339405]
60. Lamken P, Lata S, Gavutis M, Piehler J. Ligand-induced assembling of the type I interferon receptor on supported lipid bilayers. *J. Mol. Biol.* 2004; 341:303–318. [PubMed: 15312780]
61. Roisman LC, Jaitin DA, Baker DP, Schreiber G. Mutational analysis of the IFNAR1 binding site on IFNalpha2 reveals the architecture of a weak ligand-receptor binding-site. *J. Mol. Biol.* 2005; 353:271–281. [PubMed: 16171819]
62. Rubinstein M, Pestka S. Purification and characterization of human leukocyte interferons by high-performance liquid chromatography. *Methods Enzymol.* 1981; 78:464–472. [PubMed: 6173630]
63. Jaitin DA, Schreiber G. Upregulation of a small subset of genes drives type I interferon-induced antiviral memory. *J. Interferon Cytokine Res. Off. J. Int. Soc. Interferon Cytokine Res.* 2007; 27:653–664.
64. Roth V, Laub J, Kawanabe M, Buhmann JM. Optimal cluster preserving embedding of nonmetric proximity data. *IEEE Trans. Pattern Anal. Mach. Intell.* 2003; 25:1540–1551.
65. Chehreghani MH, Busetto AG, Buhmann JM. Information Theoretic Model Validation for Spectral Clustering. *J. Mach. Learn. Res. - Proc. Track.* 2012; 22:495–503.
66. Schwarz G. Estimating the Dimension of a Model. *Ann. Stat.* 1978; 6:461–464.
67. Jaynes, ET. *Probability Theory: The Logic of Science.* Cambridge University Press; 2003.

68. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 2009; 4:44–57. [PubMed: 19131956]
69. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 2009; 37:1–13. [PubMed: 19033363]

Fig. 1. Characterization of the binding of wild-type IFN- α 2 and its variants to IFNAR1 and IFNAR2

(A) Ribbon representation (based on Protein Data Bank # 3SE3) of the ternary complex of IFNAR1 (R1), IFNAR2 (R2) and the IFN- α 2 mutant YNS drawn with PyMol. Red spheres depict the location of the YNS mutations. Blue spheres depict Arg¹²⁰, which was mutated to Glu in IFN-1ant. The black line represents the C-terminal unstructured tail of IFN. The fourth immunoglobulin domain of IFNAR1 was not resolved in the structure and is depicted as an oval. (B and C) SPR analysis of the binding of IFN-1ant, IFN- α 2, YNS, and YNS- α 8tail to (B) IFNAR1 and (C) IFNAR2, with the receptors immobilized on the surface. The binding signals for IFN- α 2 and IFN-1ant to IFNAR1 were determined by using 100-fold higher concentrations of these IFNs compared to those of YNS and YNS- α 8tail. Data are representative of at least two experiments.

Fig. 2. Analysis of the antiproliferative activities of IFNs

(A) The indicated cells were treated with IFN-1ant, IFN-α2, YNS, or IFNL3 for 72 or 96 hours and the numbers of surviving cells were determined. Data are expressed as the fraction of cells that survived treatment relative to untreated cells. (B) OVCAR3 cells were treated for 72 hours with the indicated concentrations of the indicated IFNs. The fractions of surviving cells were determined by crystal violet staining. (C) WISH cells were treated with the indicated concentrations of IFN-α2, YNS, or IFNβ in the absence or presence of 200 nM IFN-1ant. The fractions of surviving cells were determined by crystal violet staining. Data in all panels are means ± SEM from three experiments. * $P < 0.01$ by two-tailed Student's t test.

Fig. 3. The antiviral activities of IFNs on various viruses in different cell lines

(A) WISH cells were treated for 4 hours with IFN-1ant, IFN-α2, YNS, IFN-β, or left untreated (No IFN), which after they were infected with VSV for 17 hours prior to crystal violet staining for cell survival determination. The optical density of uninfected, untreated stained cells was normalized to 1. (B) WISH cells were treated for 4 hours with 200 nM IFN-1ant (I1a) alone or in combination with 100 pM IFN-α2, YNS, or IFN-β, which after they were infected with VSV and surviving cells counted as described in (A). (C) T47D cells were treated for 4 hours with IFN-1ant, IFN-α2, or YNS which after they were infected with VSV for 25 hours and surviving cells counted as described in (A). (D) OVCAR3 cells were treated with serial dilutions of IFN-1ant, IFN-α2, or YNS for 4 hours which after they were infected with VSV for 23 hours and surviving cells counted as described in (A). (E) OVCAR3 cells were treated for 4 hours with IFN-1ant, IFN-α2, or YNS, which after they were infected with EMCV for 25 hours and surviving cells counted as described in (A). (F) The indicated cell lines were treated for 6 hours with IFN-1ant, IFN-α2, or IFNL3, which after they were infected with the reporter virus YFV and counted as described in the Materials and Methods. Fraction of virus inhibition was calculated by defining the virus infected, untreated sample as zero. (G) As (F) but with serial dilutions of IFN-1ant, IFN-α2, or IFNL3 onto Huh7.5 cells. Here, the fraction of infected cells was determined relative to the virus infected, untreated sample (which is one). (H) As (F) but with IFN-1ant, IFN-α2, or IFNL3 treatments and Huh7.5 cells infected with HCV. Data in all panels are means $\pm$ SEM from three experiments. * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$, by two-tailed Student's t test.

Fig. 4. IFN-1ant stimulates STAT phosphorylation to a low extent
(A) WISH cells were treated for 30 min with the indicated concentrations of IFN-1ant or with 0.2 nM IFN- α 2, and then were analyzed by Western blotting with antibodies against the indicated proteins. (B) T47D, WISH, and OVCAR3 cells were treated for 30 min with 200 nM IFN-1ant, 1 pM IFN- α 2, or 1 nM YNS, and then were analyzed by Western blotting with antibodies against the indicated proteins. (C) T47D, WISH and OVCAR3 cells were treated with either 200 nM IFN-1ant or 1 pM IFN- α 2 for 30 min. Phosphorylation of STAT1 (pSTAT1) and STAT2 (pSTAT2) was monitored by Western blotting, quantified and normalized to total STAT1 and STAT2, respectively. (D) A549 and Huh7.5 cells were treated for 30 min with 200 nM IFN-1ant, 1 pM IFN- α 2, 2 nM IFNL3, or 1 nM YNS, and then were analyzed by Western blotting with antibodies against the indicated proteins. Data in all panels are representative of three experiments.

Fig. 5. Differential gene expression in WISH cells in response to IFNs

(A) WISH cells were treated with the indicated concentrations of IFN-1ant for 6 (left) or 24 (right) hours and the amounts of the indicated mRNAs were then determined by qPCR. Data are representative of two experiments. (B) WISH cells were treated with 200 nM IFN-1ant or 2 nM YNS for 6 (left) or 24 (right) hours, and the amounts of the indicated mRNAs were determined by qPCR. Data are the relative amounts of mRNAs compared to those in untreated cells, which were set as 1. * $P < 0.05$, ** $P < 0.01$, by two-tailed Student's t test were calculated from three experiments. (C) WISH cells were treated with 200 nM IFN-1ant, 2 nM YNS, or both 0.2 nM YNS and 200 nM IFN-1ant for 6 (left) or 24 (right) hours, and the amounts of the indicated mRNAs were determined as described for (B). YNS versus YNS+IFN-1ant: 6 hours, *IRF1* is statistically significant ($P < 0.05$), 24 hours, *MX1*, *CXCL11*, *IL8* ($P < 0.05$) and *IRF1* ($P < 0.01$) are statistically significant; two-tailed Student's t test from three experiments.

Fig. 6. IFN-dependent gene signatures in different cell lines

(A) Principal component analysis (PCA) of the gene expression profiles (based on C_T values) of the indicated cell lines treated with the indicated IFNs for 8 (left) and 24 (right) hours. The x-axis accounts for most of the variability of the data (see %). (B) Clustering analysis (using $-\Delta C_T$ values) of the gene expression data shown in (A). Individual squares represent the value of a given gene (rows) in a specific sample for the indicated cell lines and IFNs (columns). Genes with a high $-\Delta C_T$ value (high expression) are in red, whereas genes with a low $-\Delta C_T$ value (low expression) are in blue. (C) Comparison of fold change of gene expression of Huh7.5 cells treated with 1 nM YNS (x-axis) or 1 nM IFN- β (y-axis) for 8 hours relative to untreated cells. The abundances of mRNAs were measured with the BioMark real-time PCR system (D) WISH cells were treated with 200 nM IFN-1ant (x-axis) or 1 pM IFN- α 2 (y-axis) for 8 hours and were analyzed as described for (C). (E) Huh7.5 cells were treated with 200 nM IFN-1ant (x-axis) or 2 nM IFNL3 (y-axis) for 8 hours (blue) or 24 hours (red), and were analyzed as described for (C). IFN1a, IFN-1ant. Data are representative of two independent experiments.

Fig. 7. Effects of IFNs on gene expression

(A) Comparison of $-C_T$ values and fold changes in mRNA abundance. T47D, WISH, and OVCAR3 cells were left untreated (0) or were treated with 200 nM IFN-1ant (IFN1a) or 1 nM YNS for 8 or 24 hours. The amounts of the mRNAs of robust and tunable genes were measured with the BioMark real-time PCR system. Values for $-C_T$ and fold-change ($\log_2$) were determined and analyzed with the NetWalker analysis tool (26). Heat map coloring was produced in the range of -14 to 5 (for $-C_T$) or -2 to 10 (for log of the fold-change).

(B) The indicated cells were treated with 200 nM IFN-1ant (left) or 1 nM YNS (right) for 24 hours. The fold-changes in the abundances of mRNAs of the indicated robust or tunable genes were determined, as were the average mRNA abundances of each group of genes listed in (A). mRNA amounts in treated cells were normalized to those in untreated cells. Data are from two independent experiments. All mRNAs for robust and tunable genes were averaged for each of the two treatments (right of the vertical line). $*P < 0.01$, by two-tailed Student's t test.

Fig. 8. Classification of robust and tunable genes and the transcriptional elements in their promoters

(A) Fold-change expression data from Fig. 6B for all of the examined conditions (cell lines, various IFN regimes, duration of treatment) were analyzed by correlation-based constant shift embedding (CCSE) to extract distances from pairwise similarities between gene expression profiles. In the embedding space, machine learning analysis consisted of classification and clustering. Classification was used to generalize existing partial knowledge regarding two classes: robust and tunable genes. Selected genes were marked by name as examples for binary classification. TS, training set. The list of genes used for the analysis is found in table S1. (B) Binding sites for canonical IFN-dependent transcription factors (including ISRE, IRF8, and IRF1) are enriched in the promoter regions of genes from the robust group. PWM, position weight matrix. $*P < 0.01$, $**P < 0.001$, by one-way ANOVA followed by a post hoc Tukey test. Inset: WISH cells were treated with 1 nM YNS for the indicated times, and the amounts of mRNAs of selected robust or tunable genes were determined by qPCR analysis and were calculated and normalized to those in untreated cells. (C) WISH cells were transfected with control siRNA or with STAT2- or IRF9-specific siRNAs individually or in combination. Cells were then left untreated or were treated with 1 nM YNS for 24 hours. The amounts of mRNAs of the indicated robust and tunable genes were determined by qPCR analysis, and are expressed as fold changes relative to those in untreated cells transfected with control siRNA. (D) WISH cells were transfected with control siRNA or with HNF4A- or WT1-specific siRNAs individually or in combination. Cells were then left untreated or were treated with 1 nM YNS for 24 hours. The amounts of

mRNAs of the indicated robust and tunable genes were determined by qPCR analysis and are expressed as fold changes relative to those in untreated cells transfected with control siRNA. Data are from two independent experiments. * $P < 0.01$, ** $P < 0.001$, by one-way ANOVA followed by a post hoc Tukey test.

Table 1

EC₅₀ values for the antiproliferative effects and the fraction of cell-survival of wild-type and mutant forms of IFN-α2 and of IFNL3.

	WISH		OVCAR3		T47D		A549		Huh7.5	
	EC ₅₀ (pM)	Amp	EC ₅₀ (pM)	Amp	EC ₅₀ (pM)	Amp	EC ₅₀ (pM)	Amp	EC ₅₀ (pM)	Amp
IFNα2	1500	0.42	230	0.08	NAP	0.94	439	0.62	390	0.6
YNS	20	0.36	0.8	0.05	NAP	0.96	3	0.56	3	0.46
IFN- lant	NAP	0.99	NAP	0.99	NAP	0.95	NAP	0.99	NAP	0.98
IFNL3	ND	ND	NAP	0.92	NAP	0.95	NAP	0.92	1200	0.78

EC₅₀ values and fractions of cell survival (Amp) for the indicated IFNs were determined from dose-response curve experiments performed with the indicated cell lines, as previously described (61). The experimental error for the EC₅₀ assays was 35%. Therefore, a confidence level of two S.E. would suggest that differences smaller than 2-fold between interferons are not significant. ND, not determined. NAP, no antiproliferative activity.

Table 2

EC₅₀ values for cell-survival or fraction of virus inhibition of wild-type and mutant forms of IFN- α 2 and of IFNL3.

	<u>WISH (VSV)</u>		<u>OVCAR3 (EMCV)</u>		<u>OVCAR3 (YFV)</u>		<u>T47D (VSV)</u>	
	EC ₅₀ (pM)	Amp	EC ₅₀ (pM)	Amp	EC ₅₀ (pM)	Amp	EC ₅₀ (pM)	Amp
No IFN	NAV	0.3	NAV	0.22	NAV	0	NAV	0.36
IFN α 2	0.85	0.97	0.1	0.98	0.53	0.99	18.3	0.74
YNS	0.25	0.96	0.03	1	ND	ND	1.1	0.53
IFN-lant	2000	0.59	98	1	891	0.32	NAV	0.4
IFNL3	ND	ND	ND	ND	152	0.55	ND	ND

	<u>T47D (YFV)</u>		<u>A549 (YFV)</u>		<u>Huh7.5 (YFV)</u>		<u>Huh7.5 (HCV)</u>	
	EC ₅₀ (pM)	Amp	EC ₅₀ (pM)	Amp	EC ₅₀ (pM)	Amp	EC ₅₀ (pM)	Amp
No IFN	NAV	0	NAV	0	NAV	0	NAV	0
IFN α 2	0.46	0.96	0.53	1	0.14	0.98	1.1	0.97
YNS	ND	ND	ND	ND	ND	ND	ND	ND
IFN-lant	1242	0.38	978	0.27	79	0.87	311	0.58
IFNL3	139	0.83	24	0.42	60.5	0.99	50	0.72

EC₅₀ values and fractions of rescue from virus infection (Amp) for the indicated IFNs were determined from dose-response curve experiments performed with the indicated cell lines, as previously described (61). ND, not determined. NAV, no antiviral activity. The experimental error for the EC₅₀ assays was 35%. Therefore, a confidence level of two S.E. would suggest that differences smaller than 2-fold between interferons are not significant.