



A hot spot on interferon α/β receptor subunit 1 (IFNAR1) underpins its interaction with interferon- β and dictates signaling

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The interaction of IFN- β with its receptor IFNAR1 (interferon α/β receptor subunit 1) is vital for host-protective anti-viral and anti-proliferative responses, but signaling via this interaction can be detrimental if dysregulated. Whereas it is established that IFNAR1 is an essential component of the IFNAR signaling complex, the key residues underpinning the IFN- β -IFNAR1 interaction are unknown. Guided by the crystal structure of the IFN- β -IFNAR1 complex, we used truncation variants and site-directed mutagenesis to investigate domains and residues enabling complexation of IFN- β to IFNAR1. We have identified an interface on IFNAR1-subdomain-3 that is differentially utilized by IFN- β and IFN- α for signal transduction. We used surface plasmon resonance and cell-based assays to investigate this important IFN- β binding interface that is centered on IFNAR1 residues Tyr²⁴⁰ and Tyr²⁷⁴ binding the C and N termini of the B and C helices of IFN- β , respectively. Using IFNAR1 and IFN- β variants, we show that this interface contributes significantly to the affinity of IFN- β for IFNAR1, its ability to activate STAT1, the expression of interferon stimulated genes, and ultimately to the anti-viral and anti-proliferative properties of IFN- β . These results identify a key interface created by IFNAR1 residues Tyr²⁴⁰ and Tyr²⁷⁴ interacting with IFN- β residues Phe⁶³, Leu⁶⁴, Glu⁷⁷, Thr⁷⁸, Val⁸¹, and Arg⁸² that underlie IFN- β -IFNAR1-mediated signaling and biological processes.

The type I IFNs, including 14 IFN- α and lone IFN- β , IFN- ϵ , and IFN- ω , have critical roles in response to viral and bacterial infections and cancers (1, 2). They are also applied clinically for the treatment of hepatitis virus B and C (1), cancers including melanoma (3), and multiple sclerosis (4). Although they show clinical efficacy, their use is restricted by dose-limiting toxicities, including leukopenia, nausea, fatigue, neurological disorders (3), and localized cutaneous effects (5). All type I IFNs engage their cognate receptors, IFNAR1 and IFNAR2, to activate the canonical JAK-STAT signaling pathway, but ligand engagement can also activate alternative signaling pathways (6). Despite sharing these receptor components, there are IFN subtype-specific elements to signaling; compared with IFN- α , IFN- β has specific roles in osteoclastogenesis (7), control of chronic viral infection (8), the potent induction of apoptotic pathways required for control of tumor cell growth, and the development of B cells and myelopoiesis (9).

Structural insight into the IFN-IFNAR⁵ interactions has been gleaned from the crystal structures of a human IFN- α 2 variant and human IFN- ω in complex with both IFNAR1 and IFNAR2 (10). Furthermore, specific insight into the mode of IFN- β -mediated activation of IFNAR1 was obtained from the crystal structure of the murine IFN- β -IFNAR1 complex (11). Comparison of these structures and evidence from the literature (12) suggests that the minimal ligand binding domains for human and mouse IFNAR1 are similar and sit broadly across the three membrane distal SDs (SD1–3) of the receptor with limited involvement of the membrane proximal subdomain, SD4 (Fig. 1A). It has also been shown that key residues discriminate between ligands and that there is potential for ligand-specific interaction interfaces (10, 11).

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This article contains supplemental Figs. S1 and S2.

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⁵ The abbreviations used are: IFNAR, IFN- α/β receptor; SD, subdomain; ECD, extracellular domain; ISG, interferon stimulated gene; SPR, surface plasmon resonance; MEF, mouse embryonic fibroblasts; ISRE, interferon stimulated response element; ANOVA, analysis of variance; CI, cell index; OBB Odyssey blocking buffer; m-, mouse.

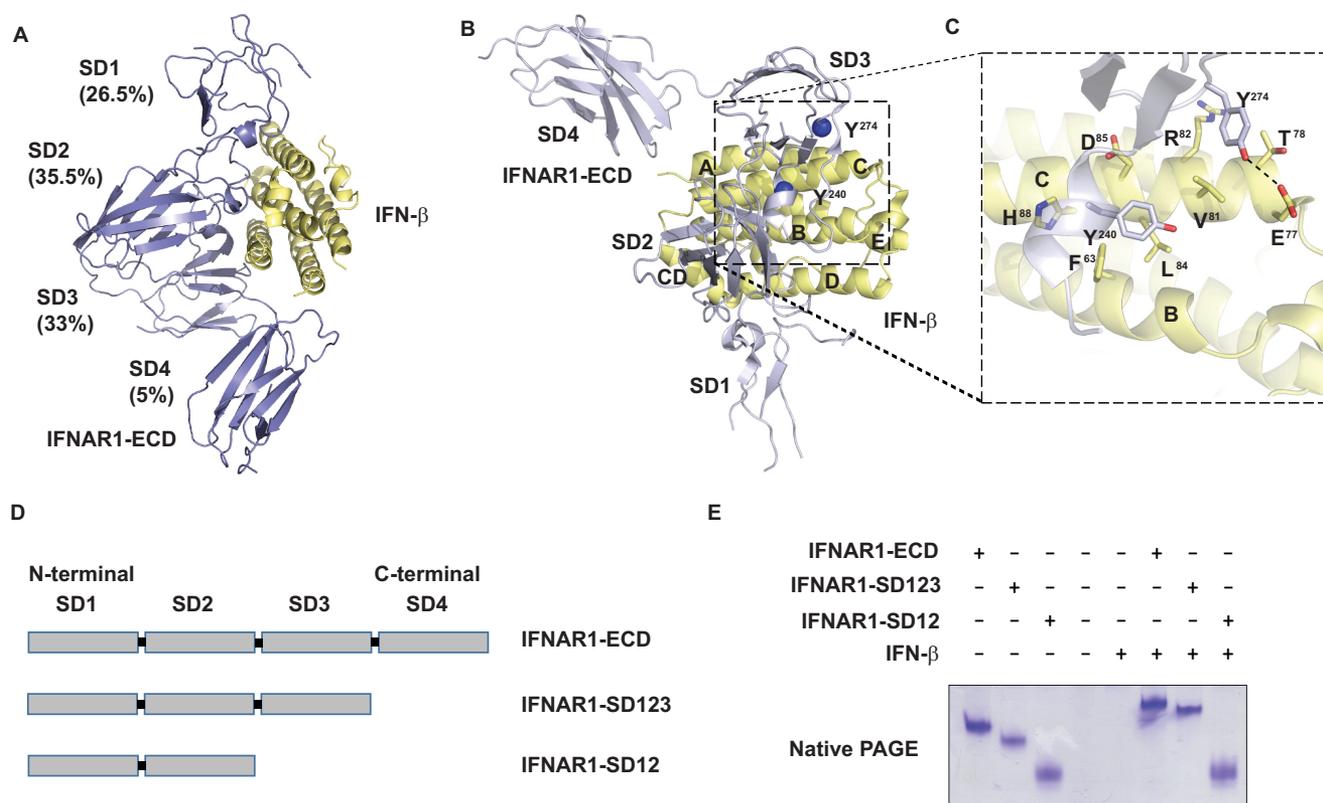


Figure 1. Contributions of IFNAR1 SD1–4 to IFN- β binding. *A*, crystal structure of IFNAR1 (blue) in complex with IFN- β (yellow). Shown are relative percentage contributions of each domain of IFNAR1 to the overall IFN- β binding interface (crystal structure of the IFN- β -IFNAR1 complex from de Weerd *et al.* (11); PDB code 3WCY). *B*, the helices of IFN- β (A–E) and the IFNAR1 subdomains (SD1–4) are indicated. The positions of Tyr²⁴⁰ and Tyr²⁷⁴ are indicated with dark blue spheres. *C*, close-up view of the binding of Tyr²⁴⁰ and Tyr²⁷⁴ (blue sticks) to residues on the B and C helices of IFN- β (yellow sticks). *D*, diagrammatic representation of IFNAR1-ECD truncation variants generated in this study. *E*, native PAGE (10% v/v) analysis of IFNAR1-ECD, IFNAR1-SD123, and IFNAR1-SD12 alone and with IFN- β . These interactions were carried out in triplicate.

However, experimental validation of these predictions is lacking.

The current study investigates the ligand-receptor subdomains and residues that contribute to the formation of a stable complex between IFN- β and the extracellular domain (ECD) of IFNAR1. Using subdomain truncation variants of IFNAR1-ECD, we initially show that IFNAR1-SD3 is vital to the formation of a stable IFN- β -IFNAR1 complex. We next interrogated the crystal structure of the IFN- β -IFNAR1 complex, focusing on key residues on IFNAR1-SD3 and residues to which they interact on IFN- β . Our data reveal that a key interaction interface exists between two tyrosine residues on IFNAR1-SD3 and a small number of residues on IFN- β helices B and C. Using site-directed mutagenesis, we demonstrate that this interface is used differentially by IFN- β compared with IFN- α 1. Furthermore, we show that this interface significantly influences the affinity of IFN- β for IFNAR1, the IFN- β -mediated internalization of IFNAR1, activation of STAT1, and the induction of interferon stimulated genes (ISGs). Importantly, we also show that this interface influences the magnitude of the biological activities that result from the IFN- β -IFNAR1 interaction.

Results

The structural determination of murine IFNAR1 receptor in complex with IFN- β (PDB code 3WCY) revealed the interaction to be dominated by the three membrane-distal domains of

the receptor with each contributing approximately a third of the binding interface (Fig. 1A). By contrast, the fourth domain of IFNAR1 contributed just 5% to the overall interface (Fig. 1A). We sought to understand the relative importance of IFNAR1 subdomains and individual IFN- β -IFNAR1 residues (Fig. 1, B and C) to the formation of this high affinity complex and the contributions these residues made to the functionality of IFN- β via IFNAR1.

IFNAR1-SD3 is vital for efficient IFN- β binding

To assess the relative importance of SDs of IFNAR1-ECD to IFN- β binding, we generated truncation variants of IFNAR1-ECD by introducing stop codons at the C termini of SD3 (to generate IFNAR1-SD123) and of SD2 (to generate IFNAR1-SD12) (Fig. 1D). Using native PAGE, we compared the ability of these truncated forms and the full-length IFNAR1-ECD to bind IFN- β under native conditions. As we have previously shown, the addition of IFN- β induces an observable shift in mobility of IFNAR1-ECD (11). A similar observable shift in mobility was also seen with the addition of IFN- β to IFNAR1-SD123, indicating that IFN- β bound this truncated form of IFNAR1-ECD under these conditions (Fig. 1E). However, the addition of IFN- β to IFNAR1-SD12 did not alter the mobility of this form of IFNAR1-ECD, indicating that IFN- β did not bind this protein efficiently under these conditions (Fig. 1E). These data suggest that under native PAGE conditions the

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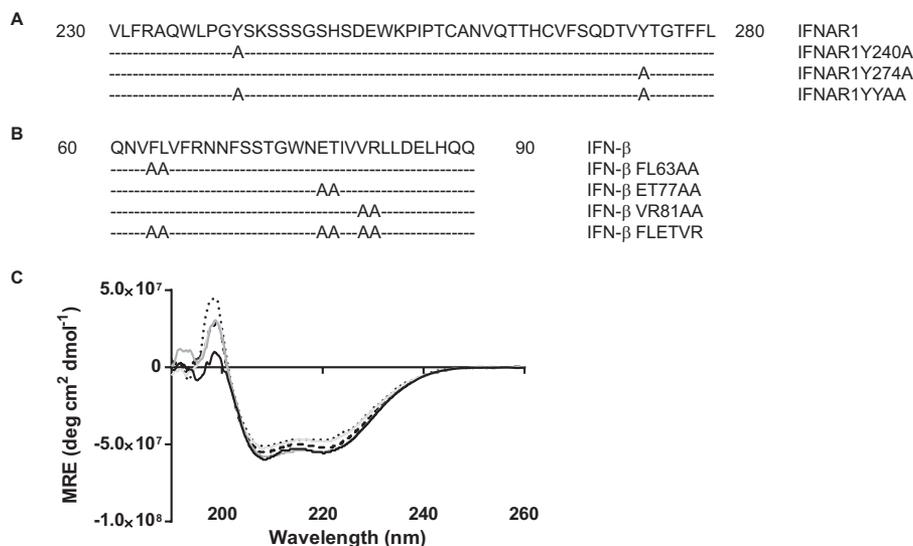


Figure 2. IFNAR1 and IFN- β variants generated and assessed in this study. *A*, residues of IFNAR1 were mutated to alanine residues as indicated. IFNAR1 residues 230–280-only are shown. *B*, residues of IFN- β were mutated to alanine residues as indicated. IFN- β residues 60–90 only are shown. *C*, circular dichroism analysis confirmed the α -helical fold of IFN- β variants: IFN β (black line), IFN- β F63A/L64A (dark gray line), IFN- β E77A/T78A (dotted line), IFN- β V81A/R82A (light gray line) and IFN- β FLETVR (dashed line). MRE, mean residue ellipticity.

presence of SD3 of IFNAR1-ECD was critical for efficient binding of IFN- β .

Two residues, Tyr²⁴⁰ and Tyr²⁷⁴, dominate the interaction interface on IFNAR1-ECD SD3

Examination of the contacts between IFNAR1-ECD and IFN- β in the crystal structure of the IFN- β -IFNAR1 complex revealed that central to the binding of IFNAR1-SD3 were the residues Tyr²⁴⁰ and Tyr²⁷⁴. Using the AREA/MOL program from the CCP4 suite (13), we determined that these residues together contributed 40% of the total binding interface of this subdomain (Fig. 1B). Tyr²⁴⁰, located on the loop between the β 3 and β 4 strands of IFNAR1-SD3, was pivotal to binding the C-terminal of the IFN- β B helix and N-terminal of the IFN- β C helix. Tyr²⁴⁰ sat in a predominantly hydrophobic pocket of IFNAR1-SD3 with principal interactions to IFN- β residues Phe⁶³, Val⁸¹, Leu⁸⁴, and H⁸⁸ (Fig. 1C). IFNAR1 Tyr²⁷⁴, located on the loop between the β 5 and β 6 strands of IFNAR1-SD3, was similarly pivotal to binding the N-terminal of the C helix of IFN- β and sat in a polar pocket characterized by a hydrogen bond to Glu⁷⁷ and by van de Waals interactions with Thr⁷⁸, Val⁸¹, and Arg⁸² (Fig. 1C).

IFNAR1 residues Tyr²⁴⁰ and Tyr²⁷⁴ are important for IFN- β affinity

We expressed recombinant forms of IFNAR1-ECD containing mutations at Tyr²⁴⁰, Tyr²⁷⁴ or both, generating IFNAR1-ECD Y240A, IFNAR1-ECD Y274A, and IFNAR1-ECD Y240A/Y274A (herein referred to as IFNAR1-ECD YYAA), respectively (Fig. 2A). We assessed the binding of IFN- β to these receptor variants using surface plasmon resonance (SPR). Our results show that although alanine substitutions at Tyr²⁴⁰ or Tyr²⁷⁴ showed slight but not statistically significant reductions in IFN- β binding (Table 1), a synergistic effect was observed when these two mutations were combined in IFNAR1-ECD YYAA. The affinity of IFN- β for IFNAR1-ECD YYAA was

significantly reduced \sim 69-fold when compared with IFN- β binding to IFNAR1-ECD (Table 1). These data suggest that individually Tyr²⁴⁰ and Tyr²⁷⁴ make minor contributions to IFN- β binding and affinity for IFNAR1-ECD, but that together they have a synergistic effect, dramatically influencing the interaction.

Mutations introduced onto IFN- β differentially affect IFNAR1 affinity

Because we had demonstrated the importance of IFNAR1 residues Tyr²⁴⁰ and Tyr²⁷⁴ to this interface, we next investigated the importance of IFN- β residues that bind these tyrosine residues to this interface. We generated variants of IFN- β by substituting alanine residues pairwise at either Phe⁶³-Leu⁶⁴, Glu⁷⁷-Thr⁷⁸, or Val⁸¹-Arg⁸², the residues predicted to be the central contacts between IFN- β and IFNAR1 residues Tyr²⁴⁰ and Tyr²⁷⁴ (Fig. 2B). We also generated a multisite variant of IFN- β by substituting alanine residues at the six residues above, generating IFN- β variant F63A/L64A/E77A/T78A/V81A/R82A, herein referred to as IFN- β FLETVR (Fig. 2B). As these residues are predominantly hydrophobic (Phe, Leu, Thr, Val) or ionic (Glu, Arg), their collective mutation may compromise the high affinity binding of the IFN- β -IFNAR1 interaction. Initially, circular dichroism (CD) spectroscopy was used to compare the overall fold of IFN- β and its variants and demonstrated that the single- and multisite alanine substitutions introduced onto IFN- β did not alter the α -helical structure of the proteins (Fig. 2C).

We next used SPR to measure the affinity of IFN- β and its variants to IFNAR1-ECD. Comparison of the measured affinities of IFN- β and the IFN- β variants to immobilized IFNAR1-ECD showed that none of the single site variants IFN- β F63A/L64A, IFN- β E77A/T78A, or IFN- β V81A/R82A showed a significant reduction in affinity for IFNAR1 compared with IFN- β (Table 1). By comparison, the multisite variant IFN- β FLETVR showed a significant \sim 165-fold reduction in IFNAR1

Table 1**SPR measurements of IFN- β and mutants binding to IFNAR1 and the mutant receptors as indicated**

Association (k_a), dissociation (k_d), and affinity (K_D) are indicated. Data are represented as the mean \pm S.D. of at least triplicate independent experiments. Significance of comparisons calculated relative to the K_D of the IFNAR1-IFN- β interaction. NS, not significant.

Receptor	IFN	k_a (mean)	k_d (mean)	K_D (mean \pm S.D.)	-Fold increase (K_D) compared to IFN β	Significance (K_D) from IFN β
		$1/Ms$	$1/s$	nM		
IFNAR1-ECD	IFN- β	1.55×10^5	3.77×10^{-4}	$3.34 (\pm 2.23)$	1	
IFNAR1-ECDY240A	IFN- β	4.66×10^5	1.16×10^{-2}	$29.1 (\pm 15.5)$	8.71	NS
IFNAR1-ECDY274A	IFN- β	5.39×10^4	8.58×10^{-4}	$20.6 (\pm 12)$	6.17	NS
IFNAR1-ECDYYAA	IFN- β	4.07×10^5	9.79×10^{-2}	$232 (\pm 67.2)$	69.46	^a
IFNAR1-ECD	IFN- β F63A/L64A	7.40×10^5	7.85×10^{-3}	$16.8 (\pm 5.65)$	3.00	NS
IFNAR1-ECD	IFN- β E77A/T78A	8.87×10^4	8.51×10^{-4}	$12.1 (\pm 5.8)$	3.62	NS
IFNAR1-ECD	IFN- β V81A/R82A	5.27×10^5	1.76×10^{-3}	$7.44 (\pm 3.72)$	2.22	NS
IFNAR1-ECD	IFN- β FLETVR	9.86×10^4	4.65×10^{-2}	$552 (\pm 175)$	165.27	^a

^a $p < 0.0001$ (one-way ANOVA with Dunnett's multiple comparisons testing).

affinity (Table 1). Overall, these results suggest that although the mutations introduced at Phe⁶³-Leu⁶⁴, Glu⁷⁷-Thr⁷⁸ and Val⁸¹-Arg⁸² had insignificant effects on IFNAR1 affinity, the combination of all six residues had the greatest effect on affinity of IFN- β for IFNAR1.

IFNAR1 residues Tyr²⁴⁰ and Tyr²⁷⁴ are important for IFN- β -mediated signaling

To compare the contributions made by residues Tyr²⁴⁰ and Tyr²⁷⁴ to signal transduction by IFN- β , we generated variants of full-length IFNAR1 housing tyrosine-to-alanine mutations at Tyr²⁴⁰ (IFNAR1Y240A), at Tyr²⁷⁴ (IFNAR1Y274A), or at both residues (IFNAR1Y240A/Y274A, herein referred to as IFNAR1YYAA) (Fig. 2A). We used transient transfection of *Ifnar1*^{-/-} mouse embryonic fibroblasts (MEFs) to express full-length IFNAR1 or the variants above on the surface of these cells. We confirmed the presence of equivalent levels of IFNAR1 mRNA in the transfected *Ifnar1*^{-/-} MEFs using quantitative real-time PCR (RT-PCR; supplemental Fig. S1). Our data showed that after treatment with IFN- β , cells transfected with all IFNAR1 variant receptors showed a reduced interferon-stimulated response element (ISRE)-luciferase response compared with cells transfected with IFNAR1 (Fig. 3A; $p < 0.01$). Cells transfected with either IFNAR1Y240A or IFNAR1Y274A showed relatively minor differences in the luciferase response (showing 20 and 28% reductions, respectively, $p < 0.01$), whereas cells transfected with IFNAR1YYAA demonstrated an 85% reduction in luciferase response compared with that measured by IFN- β stimulation through the IFNAR1 receptor (Fig. 3A, $p < 0.001$). To investigate IFN-subtype specificity of the interface on IFNAR1-SD3, we assessed the use of IFNAR1 receptor variants for signaling by IFN- α . Our data show that compared with IFNAR1, transfection of cells with IFNAR1Y240A or IFNAR1YYAA reduced IFN- α -driven ISRE-luciferase responses in these cells by 91 and 100%, respectively (Fig. 3B). By contrast, cells transfected with IFNAR1Y274A showed a 33% reduction (relative to IFNAR1) in the IFN- α -driven ISRE-luciferase response (Fig. 3B). Comparison of the pattern of IFN- β - and IFN- α -induced ISRE-luciferase responses transduced via IFNAR1Y240A was remarkably different between these IFN subtypes, suggesting that the interface on IFNAR1-SD3, incorporating both Tyr²⁴⁰ and Tyr²⁷⁴, is used differentially by IFN- β compared with IFN- α .

IFN- β residues binding IFNAR1 Tyr²⁴⁰ and Tyr²⁷⁴ are important for signaling

Having shown that the IFN- β variant proteins retained their native fold and that some demonstrated reduced affinity for IFNAR1-ECD, we next assessed their ability to signal by driving an ISRE-luciferase reporter in a transient transfection system. We transfected *Ifnar1*^{-/-} MEFs with IFNAR1 and stimulated these cells with 2.5 ng/ml concentrations of either IFN- β , IFN- β F63A/L64A, IFN- β E77A/T78A, IFN- β V81A/R82A, or IFN- β FLETVR. Our results showed that stimulation of cells with the single-site variants, IFN- β F63A/L64A, IFN- β E77A/T78A, and IFN- β V81A/R82A, showed a trend to reduction in the induced luciferase response that was not significantly different from cells stimulated with IFN- β (Fig. 3C). In contrast, cells treated with IFN- β FLETVR induced a consistent and significantly reduced luciferase response (reduced by 45%) compared with cells stimulated with IFN- β (Fig. 3C). Although none of the single-site IFN- β variants showed a significant contribution to the IFN- β -induced ISRE-luciferase response, these data suggest that IFN- β residues Phe⁶³/Leu⁶⁴, Glu⁷⁷/Thr⁷⁸, and Val⁸¹/Arg⁸² cooperate to synergistically support IFN- β -driven signaling via the critical IFNAR1-SD3 interface.

The IFN- β -IFNAR1-SD3 interface controls down-regulation of endogenous IFNAR1

Having shown that the combined substitutions introduced onto IFN- β significantly affected both IFNAR1 binding affinity and signaling, we next measured their effect on the down-regulation of IFNAR1 from the surface of cells (11, 14). We observed that IFN- β significantly reduced surface levels of IFNAR1 in a dose-dependent manner and at all doses investigated (Fig. 4A). In comparison, IFN- β FLETVR did not significantly remove IFNAR1 from the surface of cells, even at doses 30 times higher than IFN- β (at 0.3 ng/ml), which did induce significant IFNAR1 down-regulation (Fig. 4A). Because IFN- β FLETVR showed a lower binding affinity for IFNAR1 than IFN- β , we assessed whether the lack of observable IFNAR1 down-regulation may be due to the short time course of this experiment (1 h) and carried out an experiment over 48 h of continuous IFN- β or IFN- β FLETVR stimulation. Again, IFN- β treatment down-regulated IFNAR1 from the surface of the cells, and maintained reduced levels of surface IFNAR1 until at least 24 h after initiation of treatment; by 48 h of treat-

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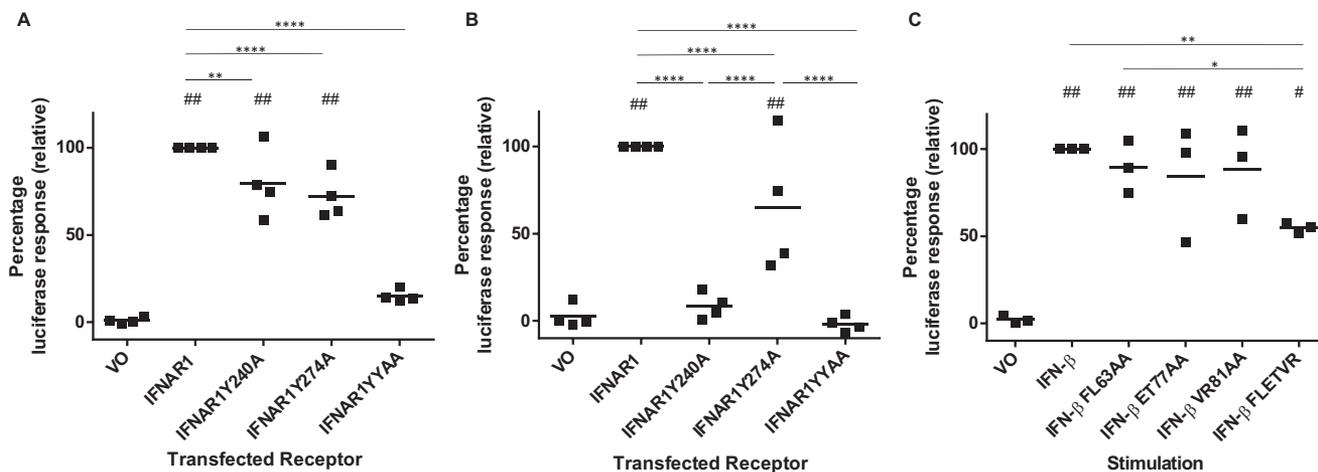


Figure 3. IFN specificity and signaling via IFNAR1-ECD SD3 residues. *A* and *B*, measurement of luciferase activity in cells transfected with vector only (VO), IFNAR1, or the IFNAR1 variant receptors IFNAR1Y240A, IFNAR1Y274A, IFNAR1YYAA after stimulation with 2.5 ng/ml of either IFN-β (*A*) or mIFN-α1 (*B*) for 4 h. *C*, measurement of luciferase activity in cells transfected with IFNAR1 after stimulation with 2.5 ng/ml of IFN-β or variants, IFN-β F63A/L64A, IFN-β E77A/T78A, IFN-β V81A/R82A, and IFN-β FLETVR. Data are expressed as the mean of at least triplicate independent experiments, all performed with technical triplicates. Significance of response calculated relative to cells transfected with either IFNAR1 constructs (*A* and *B*) or treated with IFN-β (*C*). *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$ (two-way ANOVA with Tukey's multiple comparisons testing). Significance of response calculated relative to cells transfected with empty vector only. #, $p < 0.05$; ##, $p < 0.01$ (two-way ANOVA with Tukey's multiple comparisons testing).

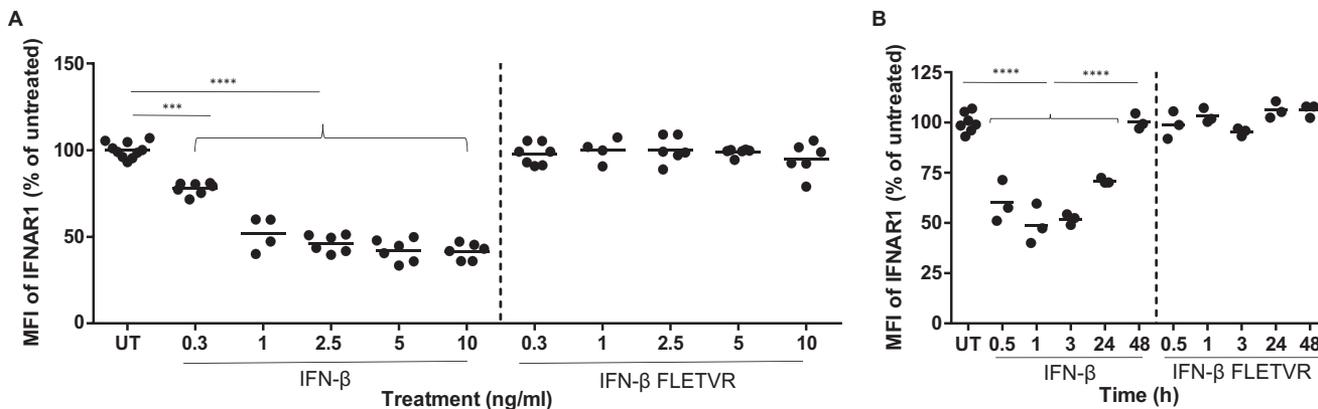


Figure 4. Abundance of surface levels of IFNAR1 on L929 cells treated with either IFN-β or the IFN-β FLETVR variant (indicated) as measured by flow cytometry. *A*, cells were treated with increasing doses of protein (0.3, 1.0, 2.5, 5.0, and 10 ng/ml) as indicated for 1 h before harvesting and staining. *B*, cells were treated with 1 ng/ml concentrations of the proteins indicated and harvested after 0.5, 1, 3, 24, or 48 h of incubation before staining. Data were expressed as the mean of at least triplicate independent experiments, all performed in technical triplicate. Significance of response was calculated relative to untreated cells. ***, $p < 0.001$; ****, $p < 0.0001$ (1-way ANOVA with Dunnett's multiple comparisons testing). Vertical dashed lines on the X-axes indicate the transition between IFN-β and IFN-β FLETVR treatments. *MFI*, mean fluorescence intensity.

ment the levels of IFNAR1 on the surface of the cells had returned to levels measurable on untreated cells (Fig. 4*B*). In comparison, cells treated with IFN-β FLETVR did not show any significant reduction in IFNAR1 surface levels throughout the 48-h time-course (Fig. 4*B*). These data suggest that the residues of IFN-β mutated to generate the IFN-β FLETVR variant are crucial for the IFN-β-driven down-regulation of endogenous IFNAR1 from the cell surface.

The IFN-β-IFNAR1-SD3 interface governs STAT1 activation and gene induction

Because our results had shown that the IFN-β FLETVR variant had a reduced ability to activate the STAT-responsive ISRE reporter, we next determined whether IFN-β FLETVR could activate STAT1 via the endogenous IFNAR1 receptor on mouse cells. Our results showed that stimulation of cells with IFN-β induced rapid phosphorylation of STAT1 Tyr⁷⁰¹ within 30 min of treatment, with no discernible difference between the

low (1 ng/ml) and high (5 ng/ml) doses applied (Fig. 5, *A* and *B*); a significant reduction in IFN-β-induced STAT1 phosphorylation was evident after 120 min. After stimulation with IFN-β FLETVR, we observed reduced STAT1 phosphorylation after 30 min of treatment compared with cells treated with IFN-β at both doses (1 and 5 ng/ml); STAT1 phosphorylation was barely detectable after 120 min (Fig. 5, *A* and *B*). Overall, our results show that although IFN-β FLETVR could induce some STAT1 phosphorylation, levels were significantly reduced compared with those measured in cells treated with IFN-β (Fig. 5, *A* and *B*).

We next investigated whether IFN-β FLETVR could induce the expression of ISGs in mouse cells. IFN-β induced the expression of all ISGs investigated (*Ccl2*, *Cxcl10*, *Ccl7*, *Ifit1*, *Irf1*, *Bst2*, *Irf7*, *Stat1*, and *Oas2*) with different dose-dependencies (Fig. 6). In comparison, the magnitude of ISG induction was significantly reduced upon stimulation with IFN-β FLETVR (Fig. 6). We observed that IFN-β FLETVR induced

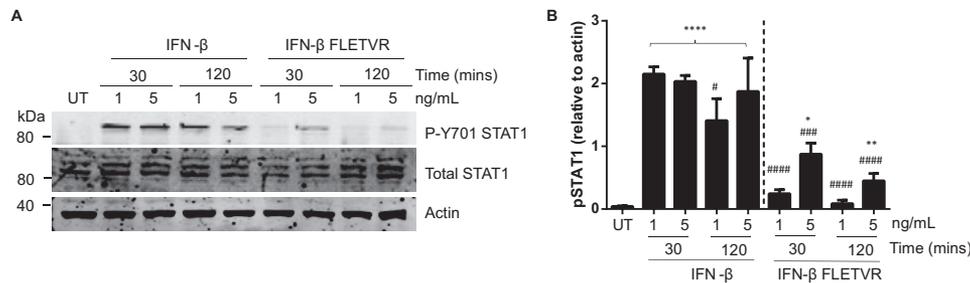


Figure 5. IFN-β FLETVR variant induces reduced STAT1 phosphorylation compared with IFN-β. A, L929 cells were treated with either 1 ng/ml or 5 ng/ml IFN-β or the IFN-β FLETVR variant for either 30 or 120 min. STAT1 phosphorylated at Tyr⁷⁰¹, total STAT1, and actin were detected in whole cell lysates. This result is representative of triplicate independent experiments. B, densitometry of Western blots; data from the triplicate independent experiments are represented as intensity of phospho-STAT1 relative to intensity of actin. Data are expressed as the mean ± S.D. of triplicate independent experiments. Significance relative to untreated samples. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$ (two-way ANOVA with Tukey's multiple comparisons testing); significance relative to treatment with 1 ng/ml IFN-β for 30 min. #, $p < 0.05$; ###, $p < 0.001$; ####, $p < 0.0001$ (2-way ANOVA with Tukey's multiple comparisons testing).

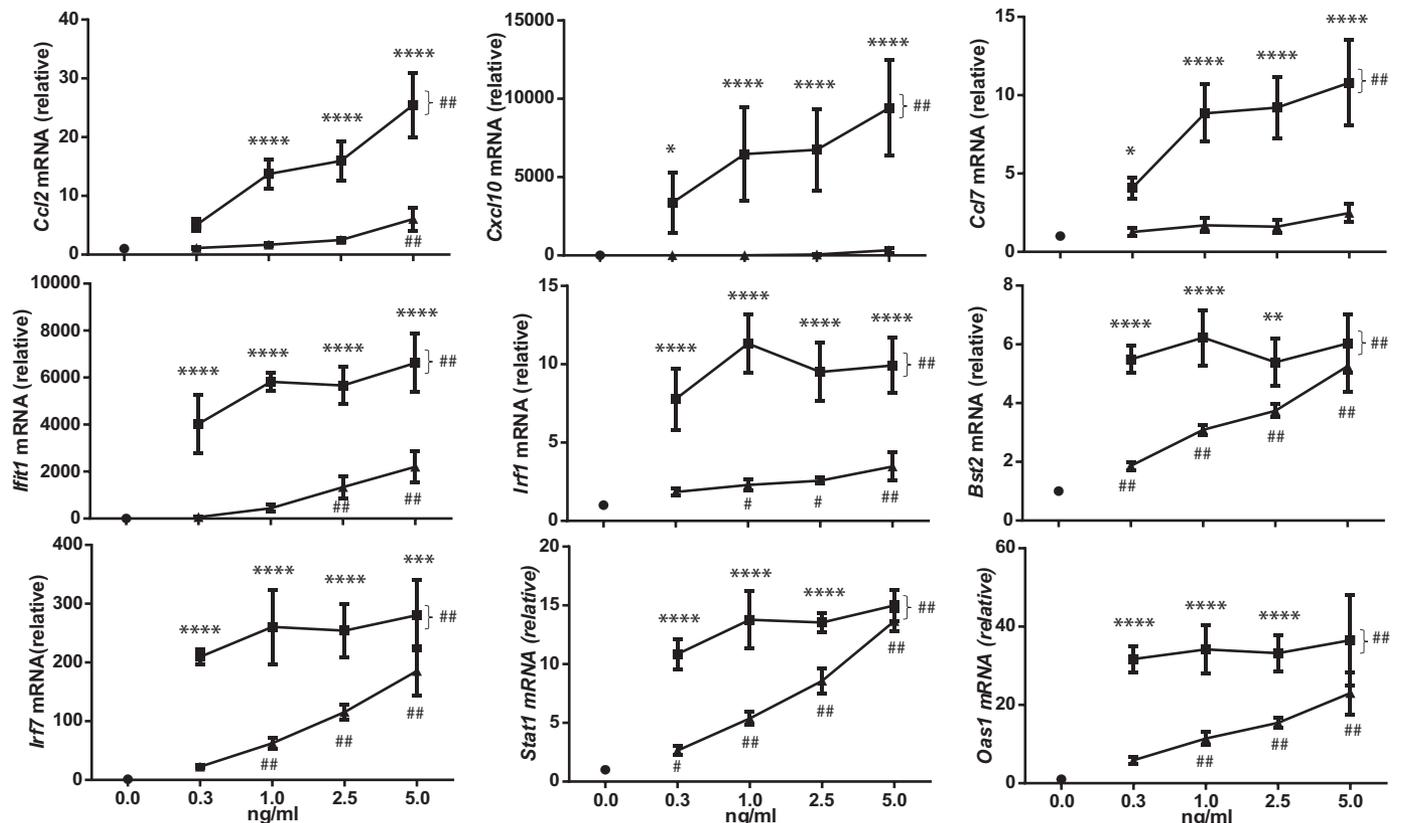


Figure 6. Quantitative RT-PCR analysis of the response of L929 cells to treatment with IFN-β (■) or IFN-β FLETVR (▲) for 3 h. The amplified target from each sample is relative to the levels of 18S in the same sample. All data is normalized to mRNA levels detected in untreated cells (●) and expressed as the mean ± S.D. of at least three independent experiments performed in technical triplicate. Significance indicated above the data points compares treatment between IFN-β and IFN-β FLETVR at the same protein concentration (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ (two-way ANOVA with Sidak's multiple comparisons testing)). All IFN-β-treated samples (as demonstrated by the bracket at the right-hand side of each graph) show -fold induction significantly greater than the untreated samples (##, $p < 0.01$ or less; two-way ANOVA with Dunnett's multiple comparisons testing). Significant difference in -fold induction between IFN-β FLETVR-treated and untreated samples is indicated (##, $p < 0.01$; #, $p < 0.05$; two-way ANOVA with Dunnett's multiple comparisons testing).

some ISGs (*Ccl2*, *Ccl7*, and *Cxcl10*) at levels not significantly different from those measured in untreated cells, suggesting that efficient induction of these genes is reliant on a high affinity IFN-β-IFNAR1 interaction (Fig. 6). Interestingly, for another subset of genes, *Ifit1*, *Irf1*, *Bst2*, *Irf7*, *Stat1*, and *Oas2*, we observed induction by IFN-β FLETVR in a dose-dependent manner but significantly less than that observed with IFN-β (Fig. 6). Taken together, these data suggest that the residues mutated to generate IFN-β FLETVR are important

for efficient IFN-β-driven STAT1 phosphorylation and gene induction.

The IFN-β-IFNAR1-SD3 interface regulates ligand-dependent biological activities

We next investigated the effect that these mutations and the resultant altered downstream signaling events had on the biological activities elicited by the IFN-β FLETVR variant. We compared the anti-viral and anti-proliferative activities of

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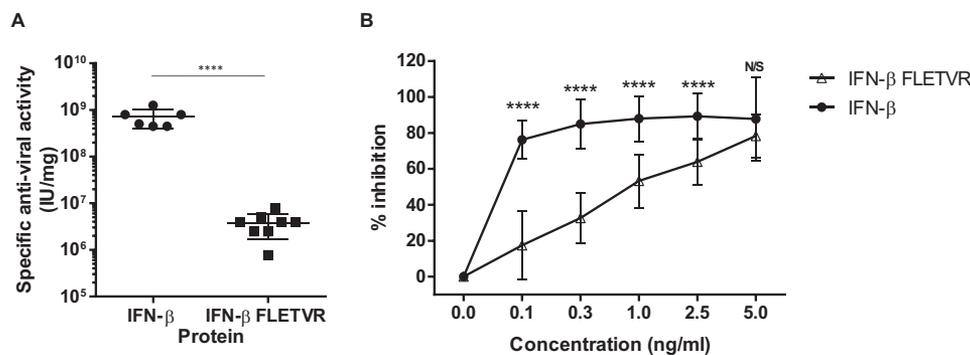


Figure 7. Comparison of the biological responses of IFN- β and the IFN- β FLETVR variant on L929 cells. *A*, the specific anti-viral activities (IU mg⁻¹) of IFN- β and IFN- β FLETVR are shown. Data shown are individual data points and mean \pm S.D. of independent experiments. ****, $p < 0.0001$ (Student's *t* test). *B*, comparison of the anti-proliferative activity of IFN- β and IFN- β FLETVR variant. Cell proliferation was monitored over 72 h in the presence of the indicated doses of either IFN- β or IFN- β FLETVR. Data shown are the 72-h time point and are expressed as the mean \pm S.D. of triplicate independent experiments, performed in technical quadruplicate, analyzed using two-way ANOVA with Sidak's multiple comparisons test. ****, $p < 0.0001$.

IFN- β and the IFN- β FLETVR variant. Compared with the specific anti-viral activity of IFN- β , IFN- β FLETVR demonstrated an ~ 186 -fold reduction in its ability to protect mouse cells from infection by Semliki forest virus (Fig. 7A), suggesting that the IFNAR1-SD3 interface influences the anti-viral properties of IFN- β .

To assess the effect of IFN- β mutations on the anti-proliferative capacity of the protein, we compared the ability of IFN- β and IFN- β FLETVR to inhibit the proliferation of a mouse cell line. In this assay, IFN- β induced $\sim 80\%$ inhibition in cellular proliferation even at the lowest dose applied (Fig. 7B). In comparison, although treatment of cells with IFN- β FLETVR also showed a dose response in inhibition of cellular proliferation (Fig. 7B), the extent of inhibition was significantly reduced compared with that induced by IFN- β (Fig. 7B). These results suggest that the interface on IFNAR1-SD3 also influences the ability of IFN- β to inhibit cellular proliferation.

Discussion

IFN- β plays important roles in activating innate and adaptive immunity; however, excessive IFN- β signaling has been implicated in the pathogenesis of several diseases. Detrimental roles for IFN- β and/or its receptor IFNAR1 have been described during sepsis (15–17), bacterial infections including *Listeria* and *Mycobacterium* spp. (18), parasitic infections caused by *Trypanosoma* and *Leishmania* spp. (18), chronic viral infection (8, 19), and in the transmission of neuropathic pain (20). It has been hypothesized that a targeted reduction in IFN- β -IFNAR1 signals may be sufficient to protect the host against the lethality of experimental sepsis (21). We previously characterized the importance of IFN- β binding to IFNAR1 in proinflammatory responses, and here we identified a key interaction interface mediated by two residues on IFNAR1, Tyr²⁴⁰ and Tyr²⁷⁴, which interact with particular residues on IFN- β (Phe⁶³, Leu⁶⁴, Glu⁷⁷, Thr⁷⁸, Val⁸¹, and Arg⁸²). We demonstrated that this interface stabilizes the ligand-receptor complex and influences all aspects of IFN- β functionality, suggesting that this interface may be a suitable target for rational drug design to therapeutically modulate IFN- β -mediated signaling.

We and others have shown that the minimal ligand binding region for IFNs on IFNAR1 generally exists on the three membrane distal subdomains of this receptor (12). More specifically

for IFN- β , we have further shown that the interface spanning both Tyr²⁴⁰ and Tyr²⁷⁴ on IFNAR1-SD3 is most vital to IFNAR1 binding and IFN- β function. Because both these residues made multiple interactions with residues on IFN- β , not just via their hydroxyl groups, we chose to replace both residues with alanine to generate the most unambiguous results. Of these residues, Tyr²⁴⁰ is well conserved across species (supplemental Fig. S2A); our data clearly demonstrate a greater reliance on this residue for efficient IFN- α -mediated compared with IFN- β -mediated signal transduction, an observation that is supported in the literature (10). The second tyrosine residue we identified in the interface on IFNAR1-SD3, Tyr²⁷⁴, is not conserved across species (supplemental Fig. S2A); indeed, the residue to which it aligns in human IFNAR1 (Gln²⁷²) was not identified as important in the IFN- ω -IFNAR1 interface (10). Our data, however, suggest that IFN- β and IFN- α , both, partially utilize this residue on mouse IFNAR1 for an efficient ISRE-dependent response. That IFN- β seems to utilize this residue in synergy with Tyr²⁴⁰ for efficient signal transduction suggests that the interface spanning these two residues may be a site of species or IFN subtype specificity.

From the ligand perspective, residues we identified as important in the mouse IFN- β -IFNAR1-SD3 interface are variably conserved across species and/or IFN subtype (supplemental Fig. S2B). Phe⁶³, Glu⁷⁷, and Thr⁷⁸ are highly conserved across IFN subtype and species; although the homolog to Phe⁶³ in human IFN- ω (Phe⁶⁷) is important in the IFN- ω -IFNAR1 interface, the residues to which Glu⁷⁷ and Thr⁷⁸ align (Met⁸¹ and Thr⁸², respectively) were not identified as important to the IFN- ω -IFNAR1 interaction, supporting the potential involvement of this site in the ligand discrimination mechanism exhibited by IFNAR1 (10). Indeed, Glu⁷⁷, Thr⁷⁸, and Arg⁸² in the IFN- β -IFNAR1-SD3 interface bind exclusively to Tyr²⁷⁴ of IFNAR1 (11), further supporting the unique dependence on this tyrosine residue for IFN- β -mediated signaling. Although the presence of a valine at position 81 (Val⁸¹) seems to be unique to mouse IFN- β , the residue to which Val⁸¹ structurally aligns in human IFN- ω (Asp⁸⁵) has been shown to be involved in the human IFNAR1 interface (10). Disparity in the reduction in signals transduced by IFNAR1 residues *versus* IFN- β residues (Phe⁶³, Leu⁶⁴, Glu⁷⁷, Thr⁷⁸, Val⁸¹, and Arg⁸²) in the IFN-

β -IFNAR1-SD3 interface suggests that other residues on IFN- β may also contribute to signaling.

Other studies have reported that IFNs with a comparatively lower binding affinity for IFNAR1 show reductions in the ability to down-regulate cell-surface IFNAR1 (22, 23), to activate STAT1, and to exert an anti-proliferative response on cells (23). Our findings are consistent with these observations in that targeted abrogation of the high affinity IFN- β -IFNAR1 complex completely abolished down-regulation of endogenous IFNAR1 and activation of these IFN- β -mediated signaling outcomes. From the ligand perspective, our data showed a correlative effect between IFN- β -IFNAR1 binding affinity and the significance of the IFN- β -driven STAT response. These data demonstrate the cumulative effect of IFN- β residues Phe⁶³, Leu⁶⁴, Glu⁷⁷, Thr⁷⁸, Val⁸¹, and Arg⁸² to these biological outcomes of the IFN- β -IFNAR1 interaction. Because we showed that this interface influenced the magnitude of the STAT1-phosphorylation dependent signaling, which has been shown to be vital for protection of cells against viral infection, our findings are also consistent with a role for the identified interface in the anti-viral activity of IFN- β (24). The IFNAR1 and IFNAR2 binding interfaces on the type I IFNs are located on opposing sides of the ligands and seem somewhat independent of each other (10). Therefore, although the residues we targeted in this study were found exclusively within the IFN- β -IFNAR1-SD3 interface, we do not expect the mutations made to IFN- β to have affected its interaction with IFNAR2. However, this remains to be experimentally determined.

Because we had shown that the IFN- β -IFNAR1-SD3 interface influenced the magnitude of STAT1 activation, for investigation of its effect on gene induction, we targeted ISGs that had been reported to be inducible via phospho-STAT1-independent pathways, such as the unphosphorylated STAT1 pathway (24). Analysis of the genes induced by IFN- β in our study revealed that there was one subset of genes (*Ccl2*, *Cxcl10*, *Ccl7*, *Ifit1*, and *Irf1*) reliant on the high affinity IFN- β -IFNAR1 interaction for efficient gene induction, an observation supported by the literature (23, 25–27). Interestingly, the subset of genes previously identified as inducible via an IFN- β -dependent, unphosphorylated STAT1-mediated anti-viral pathway (*Bst2*, *Irf7*, *Stat1*, and *Oas2*) were less affected by the mutations made to the IFN- β -IFNAR1-SD3 interface (24). Our results, therefore, suggest that this pathway may be only partially dependent on the high-affinity IFN- β -IFNAR1-SD3 interface identified. We found that mutations made to this interface impacted not only ISG induction but also the ability of IFN- β to inhibit cellular proliferation, as evident from the reduced dose-response curve of the IFN- β -IFNAR1 variant. Mechanistically, these results may, therefore, point to a role for the identified IFN- β -IFNAR1-SD3 interface in differentially regulating or mediating alternative IFN- β -IFNAR1-driven signaling events or pathways. Our data, demonstrating the functional importance of residues at the IFN- β -IFNAR1-SD3 interface, are in contrast to alanine mutations introduced to the (juxta) transmembrane region which predictably had no effect on IFN binding affinity or signaling (28).

Overall, we have characterized and identified an important binding interface between IFN- β and IFNAR1 that is critical for

eliciting the full biological response resulting from IFN- β engagement of IFNAR1, from initial binding to the receptor, to receptor internalization, transcription factor activation, gene induction, and biological processes. Importantly, we demonstrated that by modulating this interface we can distinctly alter the biological effects of IFN- β . Thus, in identifying an IFN- β -specific interface on IFNAR1 and elucidating its importance in modulating IFN- β -mediated responses, we provide further insight into how this cytokine functions and reveal an important target for drug discovery to fine-tune IFN- β -driven responses and perhaps mediate subsequent disease.

Experimental procedures

Cell lines and cell culture

Mouse L929 fibroblast cell line was purchased from the American Type Culture Collection and maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (Gibco), 50 units/ml penicillin, 50 units/ml streptomycin (Gibco) at 37 °C, 5% (v/v) CO₂. MEFs were derived from *Ifnar1*^{-/-} mice as previously published (29) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (Gibco), 50 units/ml penicillin, 50 units/ml streptomycin (Gibco) at 37 °C, 5% (v/v) CO₂. Serum-free adapted insect cell lines Sf9 and High FiveTM (BTI-TN-5B1-4 from *Trichoplusia ni*) were purchased from Life Technologies and maintained in Sf900-II SFM media (Life Technologies) supplemented with 1 μ g/ml gentamicin (Sigma) in a shaking incubator at 27 °C, 120 rpm. For expression cultures, High FiveTM cells were diluted in serum-free Express Five media (Life Technologies) supplemented with 20 mM L-glutamine (Sigma) and 1 μ g/ml gentamicin (Sigma) and incubated at 27 °C, 120 rpm.

Constructs and cloning

The clone of mIFNAR1-ECD was as previously reported (11). Constructs encoding truncation variants of IFNAR1-ECD were generated using this clone and the QuikChange mutagenesis kit (Stratagene) to introduce stop codons at the junctions between IFNAR1-ECD subdomains as directed by specific primer pairs (Table 2). Site-directed mutagenesis was also carried out to introduce alanine mutations at amino acid positions Tyr²⁴⁰ and Tyr²⁷⁴ of this IFNAR1-ECD clone (Table 2). The mIFN- β clone was as previously reported (30). Site-directed mutagenesis was carried out using the QuikChange mutagenesis kit (Stratagene) to introduce pair-wise alanine mutations at amino acid positions Phe⁶³/Leu⁶⁴, Glu⁷⁷/Thr⁷⁸, and Val⁸¹/Arg⁸² of this IFN- β clone as required (Table 2).

Recombinant protein expression, purification, and native PAGE

All recombinant IFNAR1-ECD and IFN- β forms were expressed using a baculoviral expression system and purified as previously published (11, 30). The mIFN- α 1 utilized in this project was expressed by transient transfection in HEK293S cells and purified from culture supernatants as previously described (31). The purity of all protein preparations was checked on

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Table 2

Primers utilized in this study and the purpose for which they were used

Primer name or purpose	Primer sequence 5' to 3' forward	Primer sequence 5' to 3' reverse
Mutagenesis		
Introduce stop codon at Val ²⁰⁵ in IFNAR1	NA	CTTGAGATTTCCTGGTCAAGGCATTTTATTTGC
Introduce stop codon at Pro ³⁰⁹ in IFNAR1	NA	GTTAAGCTTAAGGAGGGAGAATGTGTTT
IFNAR1 Y240A	GTGGCTTCTCGCGCTTCAAAAAGCAG	CTGCTTTTGAAGCGCCAGGAAGCCAC
IFNAR1 Y274A	CTCAAGATACTGTCCGACAGGAACGTTCTTTCTC	GAGAAAGAACGTTCTGTGGCGACAGTATCTTGAG
IFN-β F63A/L64A	GAGTGTCTCCAGAATGTCCGTGTCTTTCAGAAAC AATTTC	GAAATGTCTTGAAGACAGCAGCGACATTTCTGGA GCATCTC
IFN-β E77A/T78A	CTCCAGCACTGGGTGGAATGCGGCTATTGTTGTAC GTCTCCTG	CAGGAGACGTACAACAATAGCCGCATTCCACCCAG TGCTGGAG
IFN-β V81A/R82A	GGAATGAGACTATTGTTGCAGCTCTCCTGGATGAA CTCCAG	GTGGAGTTTCACTCCAGGAGAGCTGCAACAATAGTCT CATTC
E77A/T78A on IFN-β V81A/R82A backbone to generate FLETVR	CTCCAGCACTGGGTGGAATGCGGCTATTGTTGCAG CTCTCCTG	CAGGAGAGCTGCAACAATAGCCGCATTCCACCCAG TGCTGGAG
RT-PCR		
m18S	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG
mOas1a	CCTGCACAGACAGCTCAGAA	AGCCACACATCAGCCTCTCT
mISG15	TGAGAGCAAGCAGCCAGAAG	ACGGACACCAGAAATCGTT
mIRF7	ATCTTGCGCCAAGACAATTC	AGCATTGCTGAGGCTCACTT
mBst2	GGAGTCCCTGGAGAAGAAGG	GGAGTCCCTGGAGAAGAAGG
mCCL2	AGGTGTCCCAAGAAGCTGTA	ATGCTGGACCCATTCCTTCT
mCCL7	AGATCCCAAGAGGAATCTCA	ATAGCCTCCTFCAGCCACTT
mCXCL10	CTGAATCCGGAATCTCCGACC	GAGGCTCTCTGCTGTCCATC
mlft1	TCAAGGCAGGTTCTGAGGA	ACCTGGTCACCATCAGCAT
mIrf1	AGCTGCAAGAGGAACCAGA	CTCACAGAGTTGCCAGCAG
mStat1	TCACATTACATGGGTGGAA	CGGCAGCCATGACTTTGTAG
mlfnar1-Neo	GTGGGCACTGGAGAAACCT	TGACGGATGTATTGCTTTAACTTCT
mlfnar1	GCAGTGTGACCTTTTCAGCA	GAGAATTCACACTTGGTCGTTG

reducing SDS-PAGE before use in experiments. All interactions and native PAGE were carried out as previously published (11) using 8% (v/v) polyacrylamide gels.

CD spectroscopy

CD spectral analyses were measured at room temperature in a Jasco J815 CD spectrophotometer. All scans were run on proteins concentrated to 130 μg/ml in TBS (10 mM Tris, pH 8.0, 150 mM sodium chloride). Triplicate scans were run between 190 and 260 nm. Data were collected and converted to mean residue ellipticity by the equation of Correa and Ramos (32). Data are representative of triplicate experiments.

SPR

All SPR experiments were carried out on a ProteOn XPR36 (Bio-Rad) using an HTG chip for His-tagged proteins and TBS as the running buffer. All ligands (IFNAR1-ECD and variants) were immobilized to the nickel-activated chip via the His tag after dilution to 25 μg/ml in TBS (10 mM Tris, pH 8.0, 150 mM sodium chloride). All analyte samples (IFN-β and variants) were diluted in TBS to various concentrations ranging from 40 nM to 1 μM. All data were referenced according to the manufacturer's instructions (Bio-Rad) and analyzed using the Langmuir binding model. Data were considered for inclusion in the analysis only if the χ^2 value (the measure of error between measured and fitted values) was less than 10% of the R_{\max} as per the manufacturer's instructions (Bio-Rad). k_a (1/MS), k_d (1/s), and K_D (nM) were calculated by the ProteOn Manager software and are represented as the mean from at least triplicate experiments. Significance was determined using one-way ANOVA with Dunnett's multiple comparisons testing.

Transient transfections of *Ifnar1*^{-/-} MEFs and luciferase assays

Ifnar1^{-/-} MEFs were used for all transient transfections of IFNAR1 or its variants as previously reported (33). Cells were incubated at 37 °C, 5% (v/v) CO₂ for ~20 h before the addition of any stimuli. To test for comparative expression of introduced *Ifnar1* mRNA, cells were harvested after the 20-h incubation without any stimulation. For luciferase assays, we co-transfected an ISRE-luciferase reporter (as previously published in Ref. 33) as a measure of STAT activation induced by IFN stimulations. All stimulations were carried out with continuous IFN treatment (2.5 ng/ml of culture), with cells harvested for luciferase assays after 4 h incubations at 37 °C, 5% (v/v) CO₂. After incubation, cells were lysed in passive lysis buffer (Promega); luciferase and TK-Renilla activity were assessed as previously described (33). All transfections were carried out in at least biological and technical triplicate for each sample with readings normalized to that of TK-Renilla. Results are presented as luminescence measurable per treatment above those measured in cells transfected with vector alone and then converted to percentage of the luciferase response measured on cells transfected with IFNAR1 and treated with IFN. The significance of responses were calculated using a two-way ANOVA with Tukey's multiple comparisons testing.

Cell lysis, SDS-PAGE, and Western blot

We used L929 cells stimulated with IFN-β or the IFN-β FLETVR variant to compare the ability of these proteins to induce phosphorylation of STAT1 (at Tyr⁷⁰¹). Cells were plated at 6 × 10⁵ cells per well of a 6-well cell culture dish and incubated overnight at 37 °C, 5% (v/v) CO₂. After the end-point of stimulation, medium was aspirated, and cells were rinsed with

PBS and lysed in cell lysis buffer as previously reported (11). Protein concentrations in cell lysates were quantified using Lowry reagents (Bio-Rad) and assayed using a FLUOstar Optima microplate reader (BMG Technologies). 7–15 μg of whole cell lysate was separated on a 10% (v/v) SDS-PAGE (34) and transferred to polyvinylidene difluoride membrane (Immobilon FL, Millipore) using a Mini Trans-Blot apparatus (Bio-Rad). Membranes were blocked in Odyssey blocking buffer (OBB; Millenium Sciences) for 1 h at 22 °C. Membranes were incubated with primary antibodies (anti-phospho-Tyr⁷⁰¹ STAT1 (catalog no. 7649S, clone D4A7; 1:1000, Cell Signaling Technologies), total anti-mouse STAT1 (catalog no. sc-346, clone E-23; 1:200, Santa Cruz Biotechnologies), or anti-actin antibodies (catalog no. A4700, clone AC-40; 1:500, Sigma)) diluted in fresh OBB for 16 h at 4 °C. Binding of secondary antibodies (AlexaFluor 680-conjugated anti-mouse IgG (1:1000, catalog no. A21057, Life Technologies); IR800-conjugated anti-rabbit IgG (1:1000, catalog no. 611-145-002-05, Rockland)) diluted in OBB was carried out for 1 h at 22 °C. Antibody binding was detected using an Odyssey Infra-Red Imager (Li-Cor). Densitometry of the detected bands was quantitated using ImageJ; the levels of detectable phospho-STAT1 were normalized to the level of actin for each sample (triplicate experiments). The blots shown are representative of triplicate independent experiments. The significance of responses were calculated using a two-way ANOVA with Tukey's multiple comparisons testing.

Extraction of RNA and cDNA synthesis for quantitative real-time PCR

To evaluate gene expression by RT-PCR, L929 cells were plated at 6×10^5 cells per well of a 6-well cell culture dish and incubated overnight at 37 °C, 5% (v/v) CO₂. After 3 h of treatment, cells were lysed in RLT buffer (Qiagen) and RNA-purified using the RNeasy column purification kit (Qiagen); all cDNA synthesis was prepared using Superscript III First Strand cDNA kit (Invitrogen) and random hexamers (Invitrogen) following the manufacturer's protocol. Quantitative RT-PCR was performed on an Applied Biosystems 7900HT Fast Real-Time PCR system (ABI) using SYBR reagents (ABI); amplification was directed by the forward and reverse primer pairs indicated (Table 2). All experiments were carried out with biological and technical triplicates (except where stated) with data normalized relative to the expression of 18S and transformed using the $\Delta\Delta C_T$ method (35). Data are presented as -fold induction relative to unstimulated control samples and reported as the mean \pm S.D. of at least triplicate independent experiments. A significant difference in -fold induction between untreated and IFN- β - and IFN- β FLETVR-treated samples was calculated using two-way ANOVA with either Sidak's multiple comparisons testing (to compare mRNA levels measured in IFN- β - and IFN- β FLETVR-treated samples) or Dunnett's multiple comparisons testing (to compare mRNA levels measured in all stimulated cells with that in untreated cells).

Flow cytometry

Flow cytometry was used to measure and compare the effect of stimulation with IFN- β or the IFN- β FLETVR variant

on surface levels of IFNAR1 on L929 cells. The anti-mouse IFNAR1 antibody (catalog no. I-401, Clone Mar1-5A3, Leinco) (as reported in Sheehan *et al.* (36)) and its isotype counterpart (catalog no. I536, Clone HKSP84, Leinco) were biotinylated using EZTM-Link NHS-Biotin following the manufacturer's instructions (Thermo Scientific). For flow cytometry, L929 cells were plated at 2×10^5 cells/well of a 24-well culture plate and incubated at 37 °C, 5% (v/v) CO₂ overnight. After stimulation, cells were harvested from the culture vessel using cell suspension buffer (PBS with 2% (v/v) fetal calf serum) containing 5 mM EDTA and then centrifuged at 1500 rpm for 5 min. Nonspecific antibody interactions were blocked using anti-CD16/CD32 blocking antibody (catalog no. 14-0161-86, clone p3; eBiosciences diluted 1:200) before staining with either biotinylated anti-mouse IFNAR1 or the biotinylated isotype control antibody, both diluted to 10 $\mu\text{g}/\text{ml}$ in cell suspension buffer. Antibody binding was detected using a phycoerythrin-conjugated streptavidin secondary antibody (catalog no. F0040, R&D systems, diluted 1:1000). All cell staining was analyzed on a FACSCanto II (BD Biosciences). Data are given as mean fluorescence intensity of anti-mouse IFNAR1 staining above levels of isotype control antibody staining and are reported as mean of at least triplicate independent biological replicates. Significance of responses was calculated using a one-way ANOVA with Dunnett's multiple comparisons testing.

Anti-viral activity of IFN

Antiviral activities of IFN- β and the IFN- β FLETVR variant were determined by cytopathic effect inhibition assay using mouse L929 cells and Semliki forest virus for infection (37). Activity was measured against a National Institutes of Health reference standard (GU-02-901-511) as published previously (37) and is reported as the concentration of IFN that is required to provide protection to 50% of the exposed cells (ED₅₀). Data are given as specific activity (IU/mg of protein) and are reported as the average from at least triplicate independent experiments. Student's *t* test was applied to the two groups to determine significance.

Measurement of anti-proliferative activity

For assessment of the ability of IFN- β and its variant to inhibit cellular proliferation, 6×10^4 L929 cells were plated per well of a 96-well E-plate (Roche Diagnostics) and monitored using the xCELLigence Real-Time Cell Analyzer SP Instrument (Roche Diagnostics) at 37 °C in 5% (v/v) CO₂. Cell index (CI) measurements were performed in quadruplicate per stimulation, and signal was detected every 30 min. For analysis, the CI index was normalized to time of treatment, and the slope (1/h) was calculated from normalized CI to 72 h post treatment using the Real-Time Cell Analyzer software (Version 1.2, Roche Diagnostics). All treatment analysis was compared with the slope of buffer control-treated cells. Data are expressed as the mean \pm S.D. of triplicate independent experiments, performed in technical quadruplicate, and analyzed using two-way ANOVA with Sidak's multiple comparisons test.

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Author contributions—N. D. W. conceived the idea for the project, conducted most of the experiments, analyzed the data, and wrote the manuscript. A. Y. M. carried out the mutagenesis, prepared the recombinant proteins, and conducted the experiments in anti-viral assays. P. R. P. carried out luciferase assays, gene expression assays, and data analysis. N. M. B. carried out anti-proliferative assays and data analysis and helped prepare the manuscript. S. S. L. carried out Western blots and data analysis. J. P. V. helped conceive the concepts for the project, carried out structure analysis, and helped prepare the manuscript. J. R. contributed to experimental planning and structure analysis and helped prepare the manuscript. P. J. H. contributed to experimental planning and contributed to preparation of the manuscript.

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