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A panel of synthetic antibodies that selectively recognize and antagonize members of the interferon alpha family

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Abstract

The 12 distinct subtypes that comprise the interferon alpha (IFNα) family of cytokines possess anti-viral, anti-proliferative and immunomodulatory activities. They are implicated in the etiology and progression of many diseases, and also used as therapeutic agents for viral and oncologic disorders. However, a deeper understanding of their role in disease is limited by a lack of tools to evaluate single subtypes at the protein level. Antibodies that selectively inhibit single IFNα subtypes could enable interrogation of each protein in biological samples and could be used for characterization and treatment of disease. Using phage-displayed synthetic antibody libraries, we have conducted selections against 12 human IFNα subtypes to explore our ability to obtain fine-specificity antibodies that recognize and antagonize the biological signals induced by a single IFNα subtype. For the first time, we have isolated antibodies that specifically recognize individual IFNα subtypes (IFNα2a/b, IFNα6, IFNα8b and IFNα16) with high affinity that antagonize signaling. Our results show that highly specific antibodies capable of distinguishing between closely related cytokines can be isolated from synthetic libraries and can be used to characterize cytokine abundance and function.

Key words: antagonist, antibody engineering, cytokine, interferon alpha interferon signaling, phage display

Introduction

The interferon alpha (IFNα) proteins belong to the class of secreted, alpha helical cytokines produced by leukocytes of both lymphoid and myeloid origins as part of the innate immune response to pathogen challenge (Siegal *et al.*, 1999; Hidmark *et al.*, 2005) Upon binding to receptors, they elicit pleiotropic effects including anti-viral, anti-proliferative and immunomodulatory activities (Tompkins, 1999), which shape innate and adaptive immune responses and counter infection (Tough *et al.*, 1996; Marshall *et al.*, 2011).

The members of the IFNα family, the most numerous of the Type I IFNs, are expressed from 13 functional genes that produce 12 distinct protein products with 78–95% sequence identity (Allen and Diaz, 1994; Diaz *et al.*, 1994; Nyman *et al.*, 1998; Kumaran *et al.*, 2007) (**Figure S2, S3**). The IFNα structure is comprised of 5 α-helices in a bundle that presents two binding sites: a high affinity site for IFNAR2 and a low affinity site for IFNAR1 (Radhakrishnan *et al.*, 1996; Thomas *et al.*, 2011). Type I IFNs exert their biological activity by forming a ternary cell-surface complex through a sequential mechanism whereby the cytokine first binds to IFNAR2 through a high affinity interaction and then subsequently recruits IFNAR1 through a low affinity site (Piehler *et al.*, 2012; Wilmes *et al.*, 2015). This results in intracellular signaling through the JAK/STAT and other pathways, and leads to the expression of a large number of IFN-responsive genes collectively referred to as the IFN signature (de Veer *et al.*, 2001; Baechler *et al.*, 2003).

Although it is known that the various IFNα subtypes exhibit cell- (Hiscott *et al.*, 1984; Nyman *et al.*, 1998; Easlick *et al.*, 2010) and ligand-dependent expression patterns (Hillyer *et al.*, 2012), widely varying potencies (Moll *et al.*, 2011), and potentially divergent activities (Ortaldo *et al.*, 1984; Hu *et al.*, 1993; Langer, 2007), a detailed understanding of their individual roles in the pathology of disease is lacking. Recent studies highlight this void by confirming the role of IFNα in a variety of autoimmune disorders (Burman *et al.*, 1985; Imagawa *et al.*, 1995; Atkinson and Eisenbarth, 2001; Blanco *et al.*, 2001; Nestle *et al.*, 2005; Li *et al.*, 2008; Baccala *et al.*, 2012; Asgari *et al.*, 2013; Ferreira *et al.*, 2014), and suggesting that individual subtypes potentially play a role in the development of human disease (Hirankarn *et al.*, 2008). Unfortunately, investigations of human IFNα subtypes at the protein level have been hampered by the absence of molecular tools capable of distinguishing the subtle molecular differences between subtypes. Clinical studies published to date invariably employ broadly neutralizing antibodies that treat IFNα as if it were a single species rather than a collection of 12 subtypes (Yao *et al.*, 2009; Merrill *et al.*, 2011; Baccala *et al.*, 2012; McBride *et al.*, 2012). Although a variety of antibodies and antibody-based diagnostic tools exist for detecting IFNα proteins, these are either pan-specific or have not been assessed for specificity, and thus yield little insight in to subtype contributions (Seeds and Miller, 2011).

To the best of our knowledge, only one attempt to generate subtype-specific antibodies has been published (Sattayasai, JIR, 1988). This study employed immunogenic peptides to raise antibodies against IFNα1 and IFNα4 and yielded only one antibody specific for IFNα1 and another that exhibited cross-reactivity to both cytokines. Neither however inhibited cytokine activity. These results and the complete commercial absence of inhibitory subtype-specific antibodies to the IFN alphas underscore the challenge that this objective poses.

Despite the challenge, subtype-specific antibodies would aid our ability to quantitatively resolve IFNα subtype activities and may facilitate investigation of their individual biological roles. As an important step towards assembling antibodies that distinguish between the highly similar IFNα subtypes, we used a synthetic library (Persson *et al.*, 2013) to select for antibodies targeting each of the IFNα proteins. For the first time, we provide the sequences of highly selective antibodies that bind a single IFN subtype, and describe the validation of antibodies targeting four subtypes (IFNα2a/b, IFNα6, IFNα8b and IFNα16), three of which showed potent inhibition of IFNα signaling. In summary, we confirm the utility of synthetic antibodies as tools for quantifying and inhibiting individual IFNα subtypes to aid in the characterization of IFNα-mediated disease.

Materials and Methods

Expression, purification and characterization of IFN alpha cytokines

The IFNα subtype proteins were purified as described (Kuruganti *et al.*, 2014). Briefly, constructs encoding all IFNα subtypes used were obtained from DNA 2.0, cloned into pPAL7 and transformed into BL21 (DE3) cells for expression by autoinduction at 20°C (Studier, 2005). IFNα proteins that were not soluble when expressed by autoinduction were induced using isopropyl β-D-1-thiogalactopyranoside (IPTG). IFNα subtypes expressed in inclusion bodies were refolded prior to purification. IFNα protein was purified using eXact resin followed by ion-exchange and size exclusion chromatography (SEC).

The molecular weights of purified IFNα proteins were determined by MALDI-TOF mass spectrometry and compared with expected molecular weights. SEC of the IFNα subtypes was performed as a purification and diagnostic step to confirm proper folding of the molecules. All 12 IFNαs (plus the IFNα2b variant) exhibited SEC profiles consistent with properly folded monomeric proteins. Additionally, the bioactivity of each IFNα subtype was evaluated using an HL116 reporter cell line as described below. Dose response curves were generated for each IFNα subtype from at least six independent measurements, to derive half-maximal effective concentration (EC_{50}) values. The specific activity of each subtype was also determined using the World Health Organization NIH standard for IFNα2a. The bioactivity of IFNα1, IFNα8 and IFNα14 produced from subtilisin protease domain fusion proteins was also compared against commercial protein preparations. IFNα1 and IFNα8 exhibited EC_{50} values and specific activities equivalent to the commercial preparations, while IFNα14 exhibited 5-fold higher specific activity compared to commercial IFNα14. Specific activity was also used to estimate the reproducibility of the protein expression and purification protocols. For these studies, four IFN subtypes (IFNα1a, IFNα2a, IFNα4ab and IFNα14) were expressed and purified three separate times resulting in three distinct preparations of each. Specific activity measurements for these IFNα preparations were essentially identical, suggesting the protein expression and purification protocols reproducibly generate biologically active IFNα proteins.

Selections and characterization of Fab-phage

Phage from Library F were cycled through rounds of binding selections with IFNα subtype antigen coated on 96-well Maxisorp Immunoplates (Nunc) as described (Sidhu and Fellouse, 2006). After three or four rounds of selection, phage were produced from 48 individual clones grown in a 96-well format and the culture supernatants were used in phage ELISAs to detect specific-binding clones (Persson *et al.*, 2013). To assess specificity, Fab-phage were assayed for binding to each of the 12 IFNα subtypes and bovine serum albumin (BSA, Sigma-Aldrich) as a negative control. Clones of interest were subjected to DNA sequence analysis. A more detailed explanation of efforts taken to obtain subtype-specific clones is outlined in the results section.

Surface plasmon resonance

Fab proteins were expressed and purified as described (Studier, 2005). The binding kinetics of serial dilutions of Fab proteins were evaluated with IFNα immobilized by amine coupling on a GLC sensor chip using a Biorad ProteOn XPR36 instrument as described (Persson *et al.*, 2013). Data were analyzed using ProteOn software Version 3.1.0.6 and fit independently using a 1:1 Langmuir binding model from which estimates of K_D were obtained from the ratio of the k_a and k_d values.

To investigate Fab-IFNα-binding relative to the receptor-binding sites, Fc-tagged IFNAR2 was produced as described (Deshpande *et al.*, 2013), and immobilized as above. Sensor responses arising from IFNα binding to IFNAR2 were assessed and compared to responses from an equivalent amount of IFNα pre-incubated with a 2-fold excess of the cognate Fab or Fab alone as control.

IFNα-responsive cell-based luciferase assay

HL116 cells were derived from HT1080 cells (a human fibrosarcoma cell line) by stable transfection of a plasmid carrying the luciferase reporter gene under the control of the IFN-inducible 6–16

promoter (Uzé *et al.*, 1994). HL116 cells, grown in the DMEM-glutamax complete media (DMEM, 1X HAT, 10% FBS) at 37°C in a 5% CO_2 environment were plated in white opaque plates (Costar) at 4×10^5 cells/ml (100 µl/well) and incubated overnight. IFNα-induced luciferase activity was measured after 5 h stimulation of HL116 cells at 37°C with IFNα in the presence or absence of Fab, or Fab alone control, as described (Uzé *et al.*, 1994). Following incubation for 10 min at 37°C, luciferase reagent was added (50 µl/well; SteadyGlo, Promega). Luminescence was measured in a Biotek Synergy2 plate reader with a 5 s integration time and dose-response curves were fit with PRISM (Graphpad Inc) using the sigmoidal dose response equation.

Inhibition profiles were obtained using a pan-specific IFNAR1/2-FCkh heterodimer shown previously to block IFN signals and reproduce the affinity of the cell-surface receptor for IFNα (K_i = 12.5–18.5 pM)(Deshpande *et al.*, 2013). As a potent antagonist that neutralizes all IFNα subtypes, the IFNAR1/2-FCkh heterodimer was used to establish a baseline luciferase signal in the absence of any stimulation by IFNα, which was used to normalize luciferase signals that vary amongst IFNα subtypes. The Fab specificity/neutralization assay was conducted as described (Kuruganti *et al.*, 2014) by measuring IFN-mediated luciferase signals at the EC_{50} concentration of the subtype (S1), and comparing to signals obtained at the same IFNα concentration following incubation with either 10 nM IFNAR1/2-FCKh (S2) (Kalie *et al.*, 2007; Deshpande *et al.*, 2013) or 100 nM anti-IFNα Fab (S3). Normalized inhibition by each Fab was calculated as follows: (S3-S2)/(S1-S2).

STAT activation cell-based assay

IFNα-induced STAT1 phosphorylation in Daudi cells was evaluated as described (Takeuchi *et al.*, 2003). Daudi cells were cultured in serum-free RPMI media (Gibco) for 24 h at 37°C in a 5% CO_2 environment. Aliquots of 10^5 cells were stimulated for 15 min with IFNα that had been pre-incubated with or without Fab. Cells were collected by centrifugation at 2000 *g* and lysed on ice with 200 µl of 50 mM Tris buffer (pH 8.0), 150 mM NaCl, 1.25% Triton X-100, supplemented with 1:100 volume of protease/phosphatase inhibitor solution (Cell Signaling). Cell lysates were subjected to SDS-PAGE and western blotting with a 1:1000 dilution of anti-pSTAT1 (Tyr701) primary antibody (58D6, Cell signaling) in PT buffer. Blots were developed with a 1:2500 dilution of anti-rabbit HRP antibody (SC-2030, Santa Cruz Biotech) and a 1:1 mixture of chemiluminescent reagent (Biorad), and were imaged for 0.5–10 s on a Biorad Imager. Load control blots were prepared and imaged using the same lysates, except that an anti-STAT1 antibody (9172, Cell Signaling) was used in place of the anti-pSTAT1 antibody.

Results

Isolation and characterization of phage-displayed anti-IFNα antibodies

For binding selections against each of the 12 IFNα subtypes, we used library F, a phage-displayed library of synthetic antigen-binding fragments (Fabs) that has been described previously (Persson *et al.*, 2013), and has yielded tight and specific Fabs for many antigens (Hornsby *et al.*, 2015; Huang *et al.*, 2015; Kuruganti *et al.*, 2016; Na *et al.*, 2016). Library F was built using a highly stable human framework by incorporating optimized diversity within the three heavy chain complementarity determining regions (CDR-H1, -H2, and –H3) and the third light chain CDR (CDR-L3). Positions in CDR-H1 and -H2 were diversified in a limited manner with solvent accessible residues restricted to an equimolar distribution of tyrosine and serine. In contrast, CDR-H3 and CDR-L3, which are usually the most important CDRs for antigen recognition, were more extensively diversified by allowing for substantial length variation and by using a tailored mixture of nine amino acids.

Following three rounds of selection for binding to each IFNα protein, 48 individual clones were isolated and evaluated for specificity by phage ELISAs that assessed binding to the entire panel of IFNα subtypes. These assays revealed some clones that bound with high specificity to only their cognate antigen, but most clones exhibited weak binding to additional IFNs as well. The most specific clones for each antigen were subjected to DNA sequencing and the amino acid sequences of the diversified CDRs were decoded (Fig. 1).

For seven IFNα subtypes (IFNα1a, IFNα2a/b, IFNα5, IFNα6, IFNα7, IFNα14 and IFNα16), this analysis revealed highly specific Fabs that only exhibited high binding signals for their cognate antigen (Fig. 1). For two other subtypes (IFNα8b and IFNα21b), we obtained Fabs with high binding signals for their cognate antigens but also low-level binding to a number of other subtypes. In the case of IFNα4ab, the most specific Fab we obtained also bound to IFNα7a. Finally, we were unsuccessful in obtaining selective Fabs for IFNα10 and IFNα17. For IFNα10, the most selective Fab also bound strongly to IFNα14ab and IFNα7a, and for IFNα17, the most selective Fab also bound to half of the other subtypes. Taken together, these results show that direct-binding selections using the naïve library F can yield specific, or highly selective, Fabs for most of the closely related IFNα subtypes without the need for negative selections or affinity maturation.

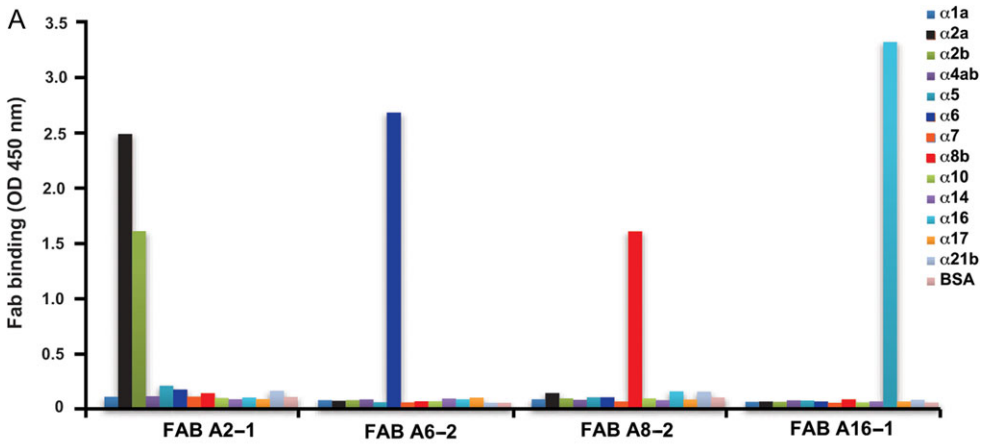
Characterization of specificity and affinity with purified Fab proteins

For four of the antigens (IFNα2a, IFNα6, IFNα8b and IFNα16), we purified Fab proteins that exhibited high specificity for their cognate antigens in phage ELISAs. ELISAs with the purified Fab proteins against the entire panel of IFNα subtypes confirmed specific binding, as three of the four Fabs exhibited a strong signal for binding only to their cognate antigen. The only exception was the Fab selected for binding to IFNα2a, which bound to both IFNα2a and IFNα2b, but this was expected since the sequences of these two variants differ at only one position (Fig. 2A).

We next used surface plasmon resonance (SPR) to measure the binding kinetics of the Fab proteins. SPR experiments were performed with all four antigens, and each Fab exhibited-binding signals against its cognate antigen (**Figure S1**). Analysis of the binding curves revealed affinities in the low to sub-nanomolar range (Fig. 2B), and thus confirmed that the Fabs recognized their cognate antigens with high affinity. All four Fabs exhibited slow off-rates but differed significantly in their on-rates. Fab A8-2 exhibited extremely tight affinity for IFNα8b (K_D = 0.29 nM) due to its fast on-rate. Fabs A6-2 and A16-1 exhibited somewhat lower but still tight affinities for IFNα6 (K_D = 6.5 nM) or IFNα16 (K_D = 3.1 nM), respectively, and exhibited somewhat slower on-rates than Fab A8-2. Fab A2-1 exhibited the slowest on-rate for its cognate antigen IFNα2a, and consequently, it exhibited the lowest but still respectable affinity (K_D = 23 nM). Taken together, these results show that the Fabs not only exhibit high specificities, but also exhibit high affinities comparable to those of optimized natural antibodies.

Antigen	Fab ID	CDRL3 (107 108 109 110 111 112 113 114 115 116)	CDRH1 (30 35 36 37 38 39)	CDRH2 (55 56 57 58 59 62 63 64 65 66)	CDRH3 (107 108 109 110 111 111.1 111.2 111.3 111.4 111.5 112.5 112.4 112.3 112.2 112.1 112 113 114 115)	α1a	α2a	α2b	α4ab	α5	α6	α7a	α8b	α10	α14	α16	α17	α21b
IFNα1a	A1-1	S Y - - - - - Y L I	L S S Y S I	Y I S S Y Y S S T Y	G W - - - - - - - - - - - - - - - D L	45.4	1.0	0.9	1.4	0.9	0.9	0.9	1.0	0.9	0.9	0.9	1.3	1.1
	A1-2	S Y - - - - - Y L I	L S S Y S I	Y I S S Y Y S S T Y	G W - - - - - - - - - - - - - - - A L	58.9	1.0	1.0	1.4	1.1	1.0	1.1	1.1	1.0	1.2	1.1	1.1	1.1
	A1-3	S Y - - - - - Y L I	F S S Y S I	Y I S S Y Y S S T Y	G W - - - - - - - - - - - - - - - A L	62.2	1.1	1.2	3.0	1.2	1.2	1.2	1.1	1.1	1.0	1.0	1.0	1.0
IFNα2a	A2-1	Y Y - - - - - W L I	I S S Y Y I	Y I S S S S S Y T S	V H G Y - - - - - - - - - - - S V G M	1.2	27.3	17.6	1.3	2.3	1.9	1.2	1.5	1.1	0.9	1.1	1.0	1.8
IFNα4ab	A4-1	Y W G V - - P A L I	I Y Y S S I	Y I S S Y S S Y T Y	T V R G S K K P - - - - Y F S G W A M	1.1	1.1	1.0	24.2	1.6	1.8	28.4	2.1	23.5	12.7	1.6	17.0	1.1
	A4-2	A F W F - - Y H L I	I Y S Y Y I	Y I Y P Y Y G Y T Y	S - - - - - - - - - - - - - - - A L	1.1	1.1	1.0	15.1	1.8	1.2	10.8	1.3	0.8	1.1	1.6	0.8	1.1
IFNα5	A5-1	S A F Y - - A F P I	I Y Y Y Y M	S I S S Y S G Y T Y	S W V - - - - - - - - - - - - - W G M	2.6	2.5	2.3	2.4	33.9	3.2	8.8	4.1	24.2	3.3	3.5	5.0	9.8
	A5-2	P F P W - - V S L I	I S Y Y S M	S I Y P S Y S Y T S	H A W Y S - - - - - - - - - G Y S F A L	1.0	1.1	1.0	1.0	38.0	1.3	3.2	25.4	3.5	1.0	12.3	2.4	2.6
	A5-3	S W - - - - - Y P F	F S S S S I	S I S S S Y G Y T Y	G Y Y W Y - - - - - - - - - A G W G A M	0.9	0.9	4.4	1.2	46.0	2.5	1.3	5.2	1.7	0.7	2.0	2.0	1.6
	A5-4	V Y Y G - - Y Y P I	F S S S S I	S I S S S Y G Y T Y	T V R G S K K P - - - - Y F S G W A M	0.8	1.0	1.4	1.3	20.3	1.2	1.0	1.0	1.0	0.9	0.8	0.9	0.9
IFNα6	A6-1	S G - - - - - H L I	F S S S S I	S I S S S Y G Y T Y	G A Y S - - - - - - - - - - H P G W A M	1.1	1.1	1.1	1.9	1.8	56.1	1.3	1.0	1.0	1.1	1.1	1.2	1.2
	A6-2	S V - - - - - Y L I	I S S S S M	S I S S Y Y S Y T Y	A F Y S - - - - - - - - - - W P G W G I	1.3	1.5	1.4	1.5	2.8	35.3	1.7	1.5	1.3	1.2	1.7	1.2	1.2
	A6-3	S V - - - - - Y L I	I S S Y Y M	S I S P Y Y G Y T Y	H S Y - - - - - - - - - - - - - G A M	0.9	0.9	0.9	1.0	2.1	41.9	1.6	1.7	0.9	1.1	1.1	1.2	1.9
	A6-4	G A A Y - - W F P I	I S S S Y M	Y I Y P S Y S Y T Y	A A W W G W Y - - - - - S G V W G G F	1.1	1.3	1.8	1.9	1.3	43.5	1.4	1.1	1.1	1.2	2.3	4.7	2.2
	A6-5	S Y - - - - - F P I	L S Y S S I	Y I S P Y S G Y T S	G Y H W S - - - - - - - - - W A Y W G M	1.2	1.1	0.8	0.8	1.2	42.6	0.8	1.3	1.3	1.9	1.6	1.5	1.8
	A6-6	G A - - - - - V L I	F S S S S I	S I S S S Y G Y T Y	G S Y G W - - - - - - - - - P G W A M	1.2	1.6	1.6	1.4	1.9	54.7	1.1	1.0	1.0	1.3	1.6	2.1	1.1
IFNα7a	A7-1	Y W Y - - - - A L I	L Y Y Y S M	S I Y P Y Y G Y T Y	G Y Y - - - - - - - - - - - - - A M	0.9	0.9	1.0	9.2	8.4	1.4	34.8	0.9	5.6	3.9	3.0	16.3	3.6
	A7-2	Y W Y - - - - A L I	L Y Y Y S M	S I Y P Y Y G Y T Y	G F Y - - - - - - - - - - - - - A N	1.2	1.2	1.2	8.7	3.3	1.3	58.8	1.3	13.9	7.5	2.1	51.5	2.8
	A7-3	G V V - - - - S L I	I Y Y S S M	S I Y S Y Y S Y T S	S P A Y H W G Y P Y A Y Y F Y W G G L	1.9	1.7	1.4	3.2	4.6	4.7	24.7	1.3	1.9	1.3	1.8	1.2	1.9
IFNα8b	A8-1	Y V T W - S Y Y L I	I Y S Y Y I	S I S S Y Y G S T Y	T V R G S K K P - - - - Y F S G W A M	1.2	1.8	2.0	1.2	16.0	5.7	1.4	51.3	1.8	1.8	6.3	1.4	1.5
	A8-2	S V Y Y - H G H L F	L S Y Y S M	Y I Y P S S G Y T Y	G W S - - - - - - - - - - - - - A M	1.2	1.5	6.4	5.8	1.9	2.1	1.6	46.9	6.1	4.1	1.9	2.8	1.4
	A8-3	H Y S Y - W W G P I	I Y S S S I	S I Y S Y Y G Y T Y	Y S - - - - - - - - - - - - - - G M	1.9	3.0	4.0	5.3	2.4	4.9	9.4	53.4	11.7	11.0	47.6	5.3	3.6
IFNα10	A10-1	Y G W - - - S A L I	L S Y S Y I	S I S P S S G Y T S	S W Y Y W S F Y - - - Y Y Y V G G A F	1.1	1.3	1.1	2.7	14.5	7.6	11.0	1.0	54.7	2.1	1.1	2.3	10.6
	A10-2	A A H V - - G Y P I	I S Y S Y I	S I S P Y Y S S T S	S Y S W S G Y Y W - - Y Y G V Y G A L	16.2	12.8	15.5	9.2	19.9	13.3	13.5	1.9	42.5	12.8	15.0	8.1	10.8
	A10-3	W Y A V - - G A L I	L Y Y S S M	Y I S S Y S S Y T S	T V R G S K K P - - - - Y F S G W A M	1.0	1.1	1.1	5.3	12.9	3.1	10.9	1.5	50.9	4.0	1.4	1.3	8.2
	A10-4	W Y V H - H G V L I	I Y S S S M	Y I Y P Y Y S Y T Y	T V R G S K K P - - - - Y F S G W A M	7.4	2.7	2.4	6.6	23.2	3.7	6.8	14.4	29.5	4.4	3.6	1.8	4.4
	A10-5	S Y A Y - Y S S P F	I S Y Y S I	S I S S S Y G Y T Y	T V R G S K K P - - - - Y F S G W A M	0.9	1.1	1.1	16.5	1.6	1.8	22.8	1.3	17.8	1.0	1.8	5.5	1.1
IFNα14	A14-1	G Y W - - - - F P I	F S S S S I	Y I Y P Y Y G Y T Y	T V R G S K K P - - - - Y F S G W A M	1.1	1.1	1.1	1.7	1.2	1.5	1.3	1.2	1.2	19.5	1.1	1.0	1.1
	A14-2	Y S - - - - - H L F	I Y S S Y I	S I S S Y Y G Y T S	S W S S Y A Y - - - - - F Y V G F A M	1.1	1.8	1.1	1.1	1.5	2.7	1.5	1.6	13.3	17.8	1.4	1.8	1.2
IFNα16	A16-1	W A W Y - P H S P I	F S S S S I	S I S S Y Y G S T Y	G A - - - - - - - - - - - - - - - M N	2.1	1.3	2.0	1.5	1.3	1.6	2.2	2.9	1.3	1.2	46.3	1.3	1.1
	A16-2	G P G Y - A H A L F	I S Y Y Y M	S I S S S Y G Y T Y	T V R G S K K P - - - - Y F S G W A M	5.4	4.4	6.4	3.8	8.1	6.6	6.2	10.8	5.3	4.1	28.2	4.7	6.2
IFNα17	A17-1	Y Y A Y - - Y S L I	I S Y Y Y M	S I Y P S S G Y T Y	Y W S S - - - - - - - - - - - F Y G M	1.2	32.1	32.5	36.6	2.9	1.5	36.6	1.2	36.6	2.1	34.6	36.6	1.5
IFNα21b	A21-1	S G S - - - V S P F	I Y S S S M	Y I S S Y Y G S T S	T V R G S K K P - - - - Y F S G W A M	0.8	1.0	1.6	6.2	0.8	0.8	27.0	0.7	30.8	26.8	1.2	3.5	37.3
	A21-2	G A W W - Y G Y L I	I S Y Y Y M	S I Y S S Y G Y T Y	S Y W - - - - - - - - - - - - - V A L	1.0	5.0	4.8	4.6	7.4	1.9	4.4	4.6	3.0	6.4	1.0	4.5	30.7

Fig. 1 Sequences and specificities of anti-IFNα Fab-phage. The sequences of the CDRs diversified in Library F are shown for the most specific Fabs selected for binding to each IFNα subtype. Residues are numbered according to IMGT standards,[65] and dashes indicate gaps in the alignment. The specificity ELISA ratios are the phage ELISA signals for the IFNα subtypes divided by the signals for BSA, and are colored as follows: 10.0 < blue, 5.0 < yellow < 10.0 and 5.0 > white.



B

Fab ID	Antigen	k_a x 10^4 M^{-1} s^{-1}	k_d x 10^{-4} s^{-1}	K_D (nM)
A2–1	IFNα2a	3.0 ± 0.1	7.1 ± 0.1	23 ± 4
A6–2	IFNα6	58 ± 4	37.2 ± 0.7	6.5 ± 0.1
A8–2	IFNα8b	193 ± 6	6.0 ± 0.1	0.3 ± 0.1
A16–1	IFNα16	10.6 ± 0.1	3.3 ± 0.1	3.1 ± 0.2

Fig. 2 Specificity and binding kinetics of IFNα subtype-specific Fabs. (**A**) Fab specificity for IFNα subtypes by ELISA. (**B**) The kinetic constants and associated errors for Fabs binding to IFNα determined by SPR.

Effects of Fabs on IFNα signaling

We used a luciferase-based IFN reporter assay in HL116 cells to assess the effects of Fabs on the signaling activities of their targets (Uzé *et al.*, 1994). This assay showed that Fabs targeting IFNα2a, α6 or α8b were able to completely inhibit signaling at high concentration (1 μM) and exhibited potent IC_{50} values of 10 nM, 45 nM or 130 nM, respectively (Fig. 3A). In contrast, despite its high affinity (Fig. 2B), the anti-IFNα16 Fab showed only a partial inhibitory effect on IFNα16 at 1 μM and did not inhibit significantly at lower concentrations (Fig. 3A).

To corroborate these results with an alternate method for assessing IFN signaling, each IFNα was incubated with its cognate Fab and the mixture was used to stimulate Daudi cells as described (Grimley *et al.*, 1998). Stimulation induced by IFNα was visualized by western blotting of cell lysates to detect phosphorylation of Tyr701 on STAT1, a well-characterized marker of IFN receptor activation (Grimley *et al.*, 1998; Forster, 2012). For Fab A2-1, pre-incubation of a fixed concentration of Fab with varying concentrations of IFNα2a resulted in a marked decrease in levels of phosphorylated STAT1 but did not affect overall levels of STAT1 (Fig. 3B). For Fabs A6-2 and A8-2, a similar assay was used except that a fixed concentration of IFNα6 or α8b, respectively, was incubated with varying concentrations of Fab, and again, levels of phosphorylated STAT1 were reduced but overall levels of STAT1 remained constant (Fig. 3C, D). Furthermore, the Fabs exhibited specificity in this assay, as Fab A6-2 did not inhibit the activity of IFNα8b or α2a (Fig. 3C) and Fab A8-1 did not inhibit the activity of IFNα6 or α2a (Fig. 3D). Taken together, these results show that in a cellular context Fabs A2-1, A6-2 and A8-2 are potent and specific antagonists of IFNα2a, α6 or α8b, respectively, whereas Fab A16-1 binds to IFNα16 with high affinity but does not inhibit activity significantly.

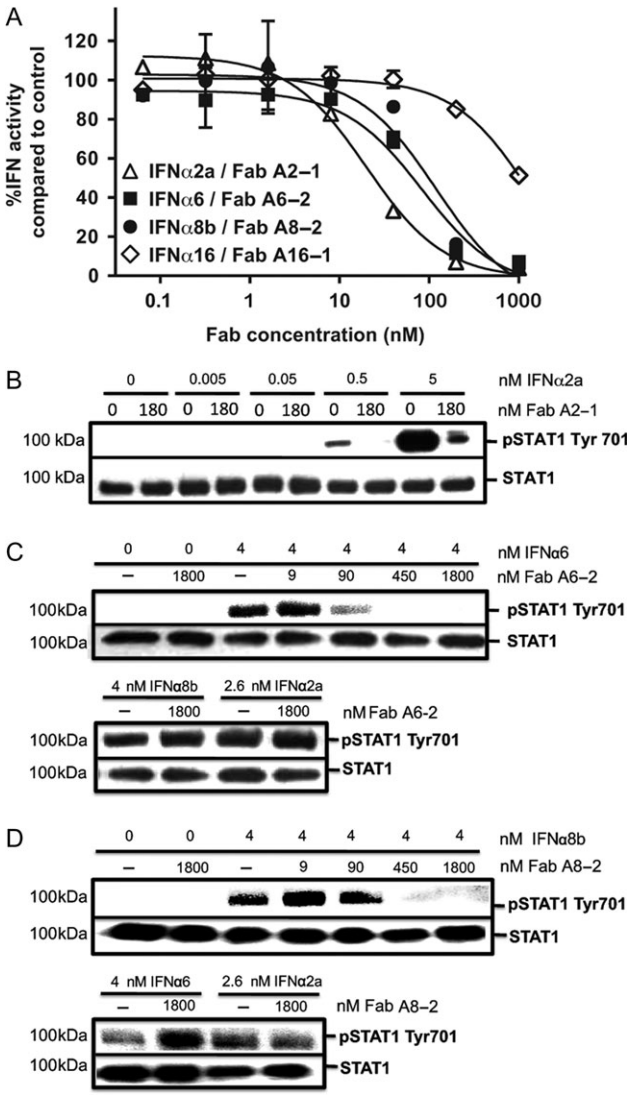


Fig. 3 Effects of Fabs on IFNα signaling. (**A**) Dose response curves for Fabs inhibiting signaling by cognate IFNα subtypes in HL116 cells as measured by luciferase reporter assay. Error bars are shown as the standard error of triplicate measurements The following IC_{50} values were determined: A2-1 (10 nM), A6-2 (45 nM) and A8-2 (130 nM). (**B–D**) Western blots of cell lysates of Daudi cells stimulated with the indicated concentration of IFNα pre-incubated with (B) Fab A2-1, (C) Fab A6-2 or (D) Fab A8-2. Blots were developed with an antibody that recognized phosphor-Tyr701 on STAT1 to detect IFNα-induced phosphorylation of STAT1 or an anti-STAT1 antibody as load control.

To assess the specificity of Fab-mediated inhibition, we examined the ability of each Fab to neutralize its cognate antigen in a mixture of three IFNα subtypes. A mixture of IFNα2a, α6 and α8b was used to stimulate a luciferase-based reporter in HL116 cells, either with or without pre-incubation with Fab A2-1, A6-2 or A8-2 (Fig. 4A). In each case, activity was reduced to a level that was the same as the activity of a mixture lacking the cognate antigen (compare black bar to red and blue bars). Furthermore, none of the Fabs had any effect on the activity of mixtures that lacked their cognate antigen (compare red bar to white bar). To further test specificity, we assessed the effect of each Fab on the activity of each of the twelve IFNα subtypes, and as expected, each Fab inhibited only its cognate antigen (Fig. 4B).

Assessment of the effects of Fabs on the interaction between IFNα and IFNAR2

The potent and specific antagonism of IFNα signaling by Fabs A2-1, A6-2 and A8-2 raised the possibility that these Fabs may block binding of their cognate antigens to either IFNAR2 or IFNAR1. Due to the low affinity of IFNAR1 for IFNα, we could not detect this interaction with purified proteins, but the high affinity interaction between IFNAR2 and IFNα was readily detected by SPR, which enabled us to set up an assay to assess inhibition of this interaction by Fabs (Fig. 5). Pre-incubation of IFNα2a, α6 or α8b with Fab A2-1, A6-2 or A8-2, respectively, did not inhibit binding of each cytokine to immobilized IFNAR2, but rather, resulted in an increase in the response units that was indicative of greater mass binding and is consistent with the IFNα-Fab complex binding to IFNAR2. These results show that none of the Fabs block the high affinity site for IFNAR2. Thus, we speculate that these Fabs likely act as antagonists by blocking the low affinity site and preventing recruitment of IFNAR1 to the IFNα-IFNAR2 binary complex.

Discussion

Few studies have investigated the bioactivities of the various IFNα subtypes in a comprehensive manner (Lavoie *et al.*, 2011), and it has been posited that the clinical utility of IFNα has been restricted by a lack of understanding of the differences between the subtypes (Gibbert *et al.*, 2013). Although recombinant cytokines and

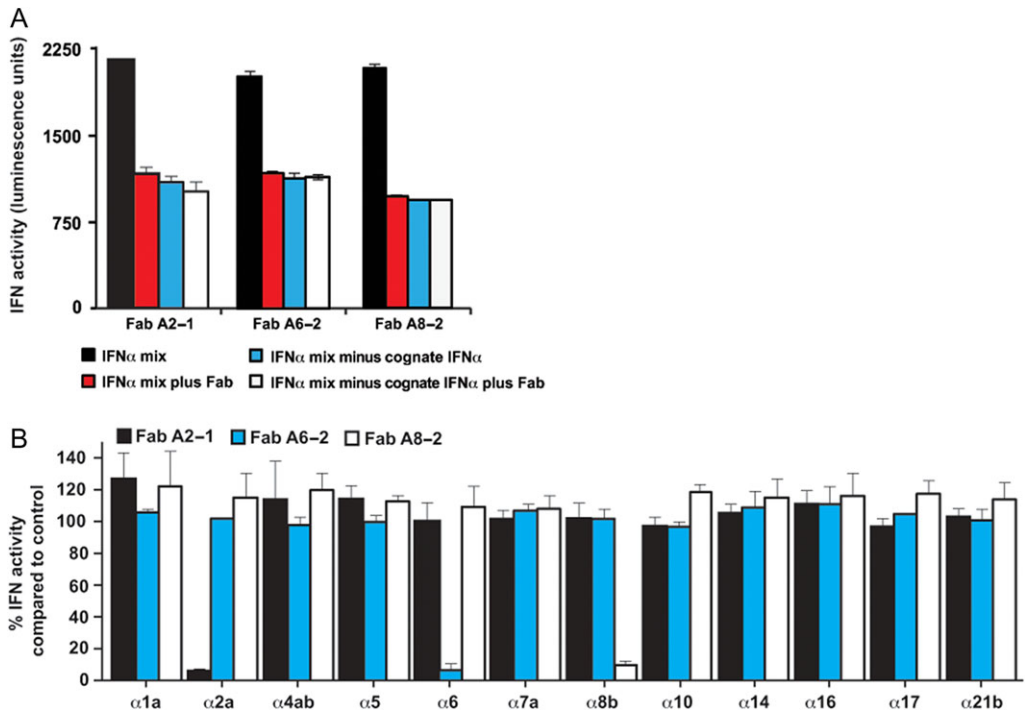


Fig. 4 Specificity of Fabs for inhibition of signaling by IFNα subtypes. (**A**) Effects of Fabs on the activity of IFNα mixtures assessed by luciferase assay in HL116 cells. Each Fab (100 nM) was incubated with a mixture of different concentrations of IFNα2a, IFNα6 and IFNα8b as follows: A2-1 (6 pM, 1 pM, 0.5 pM), A6-2 (1 pM, 4 pM, 1 pM) and A8-2 (4 pM, 0.5 pM, 2 pM). The following mixtures were used to stimulate HL116 cells: IFNα mix (black bars), IFNα mix with indicated Fab (red bars), IFNα mix missing cognate IFNα subtype (blue bars) and IFNα mix missing cognate IFNα subtype with indicated Fab (white bars). Error bars are shown as the standard error of triplicate measurements. (**B**) Effects of Fab A2-1 (black bars), A6-2 (blue bars) or A8-2 (white bars) on the activity of each of the indicated IFNα subtypes (*x*-axis) measured by luciferase assay in HL116 cells. The % IFN activity (*y*-axis) was normalized with the signal in the presence of the pan-IFNα inhibitor IFNAR1/2-FCKh, as described in Materials and Methods section.

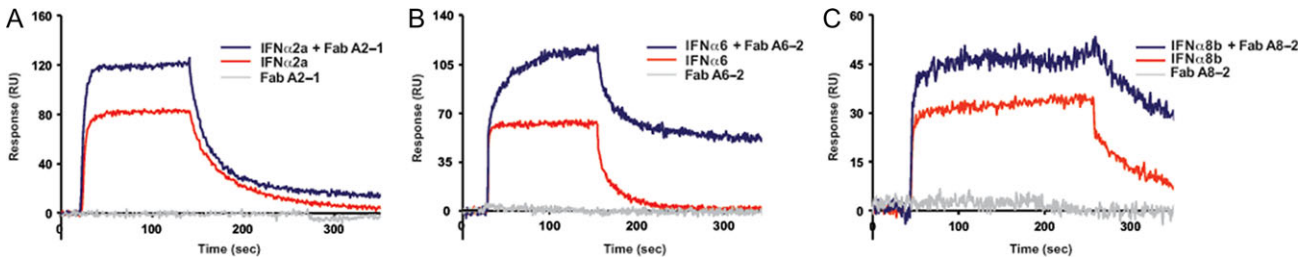


Fig. 5 Effects of Fabs on IFNα binding to IFNAR2. SPR traces are shown for the response of immobilized IFNAR2 to injections of 250 nM IFNα (red), 500 nM Fab (grey) or a mixture of the two (blue). (**A**) IFNα2a and Fab A2-1, (**B**) IFNα6 and Fab A6-2 and (**C**) IFNα8b and Fab A8-2.

engineered variants have been instrumental in revealing key differences in binding and bioactivity (Hu *et al.*, 1993; Blatt *et al.*, 1996; Brideau-Andersen *et al.*, 2007; Kalie *et al.*, 2007), a panel of recombinant antibodies with validated subtype specificities could provide a means of assessing IFNα subtype levels and activities in biological samples. For diseases in which type I IFNs are known to play a role (Hooks *et al.*, 1979; Banchereau and Pascual, 2006; Higgs *et al.*, 2011; Crow, 2014), such as rheumatoid arthritis and systemic lupus erythematosus, these tools could provide insight in to disease pathoetiology and potential therapeutic strategies.

Using a phage-displayed library, we isolated synthetic Fabs against each of the 12 IFNα subtypes. For seven of the subtypes, we achieved absolute specificity and for four others we isolated Fabs that exhibited limited cross-reactivity with other subtypes. Detailed analysis of Fabs targeting four subtypes revealed affinities in the low to sub-nanomolar range, and moreover, Fabs targeting IFNα2a/b, α6 or α8b proved to be potent antagonists of IFNα signaling in cells. Notably, these Fabs did not inhibit the initial high affinity interaction with IFNAR2, but instead, our studies suggest that they inhibit the low affinity interaction with IFNAR1. Regardless of the molecular mechanisms, these Fabs should prove to be valuable tools for probing the functional roles of these cytokines in normal biology and disease.

The inability to isolate specific antibodies to certain subtypes may be due to either a deficiency in the antibody repertoire or to a fundamental limit to discriminating between highly similar subtypes. Though there are likely several reasons why subtype-specific and inhibitory antibodies have not been forthcoming from traditional

hybridoma methods, their absence complicates direct comparison of the use of natural and synthetic libraries for this purpose. However, we note that those subtypes for which we were able to isolate selective antibodies did not possess sequence identities >90% to any other subtype (i.e. IFNα1a, IFNα2a/b, IFNα6, IFNα8b, IFNα16) (Fig. 1 and **S3**), whereas those subtypes that failed to yield absolutely selective Fabs with high affinity possessed >90% sequence identity with one or more other subtypes (i.e. IFNα4ab, IFNα7a, IFNα10, IFNα17, IFNα21b) (**Figure S3**) and exhibited binding to the most similar subtypes (Fig. 1). In future studies, it may be worthwhile to further characterize the antibodies that recognized two or three subtypes and to explore alternate methods for further enhancing specificity.

Although high specificity of antibodies is essential for any diagnostic assay, selective inhibition enables use in cell-based assays that employ functional phenotypic readouts induced by IFNα in biological samples, as described recently (Dall'era *et al.*, 2005; Hua *et al.*, 2006; Niewold *et al.*, 2007). There are a number of phenotypic readouts that could be induced by IFNα activity and antibodies that distinguish between subtypes could reveal aspects of subtype-specific pathoetiology. Indeed, our initial experiments with mixtures of IFNα subtypes show selective inhibition of only the cognate subtype, providing confidence that these antibodies could be used to characterize subtype contributions in biological samples. A broad array of assays have been used to determine IFNα levels, including ELISPOT, cell secretion, *in situ* cytokine expression, bead-capture, flow cytometry-based assays, proximity ligation assays and ELISAs (Bienvenu *et al.*, 1998), and the utility of our antibodies can be explored in these applications. Moreover, it has been recognized that soluble IFNAR2 in serum could interfere with assays that determine IFNα levels (Novick *et al.*, 1992; Mizukoshi *et al.*, 1999), and in this regard, it is notable that we have identified Fabs that can recognize IFNα subtypes in the presence of IFNAR2.

Recent clinical studies have explored the safety and tolerability of anti-IFNα antibody therapies with broad specificity against all (or most) IFNα subtypes in individuals with mild systemic lupus erythematosus (Yao *et al.*, 2009; Merrill *et al.*, 2011; McBride *et al.*, 2012). These studies generally concluded that the therapies were well tolerated and may warrant continued clinical development. However, the ability to alter the IFN signature was limited, and this has resulted in the emerging view that strong inhibition of all IFN subtypes may be necessary to treat the disease. Alternatively, these findings may reflect a limited understanding of the role of IFNα subtypes in disease, which may be addressed by the use of antibodies that recognize and inhibit individual IFNα subtypes or groups of subtypes.

Supplementary Data

Supplementary data are available at *Protein Engineering, Design and Selection* online.

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