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Detection of interferon alpha protein reveals differential levels and cellular sources in disease

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Running title: Ultra-sensitive detection of IFNα in human diseases

Short Caption: We report the direct quantification of interferon alpha protein in monogenic interferonopathies, autoimmunity and infectious disease states, made possible by the combination of digital ELISA and high-affinity autoantibodies isolated from APECED patients, revealing differential levels and cellular sources dependent on underlying pathology.

Abbreviations
AEB: Average enzyme per bead
AGS: Aicardi-Goutières syndrome
CNS: Central nervous system
CSF: Cerebrospinal fluid
JDM: Juvenile dermatomyositis
IFN: Interferon
IQ: Inter quartile (range)
ISG: Interferon stimulated gene
LOD: Limit of detection
mABs: Monoclonal antibodies
MDBK: Mardin-Darby bovine kidney cells
RVCL: Retinal vasculopathy with cerebral leukodystrophy
SLE: Systemic lupus erythematosus
SLEDAI: Systemic lupus erythematosus disease activity index

Type I interferons (IFNs) are essential mediators of anti-viral responses. These cytokines have been implicated in the pathogenesis of autoimmunity, most notably systemic lupus erythematosus (SLE), diabetes mellitus and dermatomyositis, as well as monogenic type I interferonopathies. Despite a fundamental role in health and disease, the direct quantification of type I IFNs has been challenging. Utilizing digital ELISA technology, we recorded attomolar concentrations of IFNα in healthy donors, viral infection and complex and monogenic interferonopathies. IFNα protein correlated well with functional activity and IFN stimulated gene expression. High circulating IFNα levels were associated with increased clinical severity in SLE patients, and a study of the cellular source of IFNα protein indicated disease-specific mechanisms. Measurement of IFNα attomolar concentrations by digital ELISA will enhance our understanding of IFN biology and potentially improve the diagnosis and stratification of pathologies associated with IFN dysregulation.
Introduction

The identification of a soluble factor that protects cells from viral infection was first made by Isaacs and Lindenmann in 1957 (Isaacs and Lindenmann, 1957; Isaacs et al., 1957). We now know that multiple species of type I interferon (IFN) exist, with this heterogeneity arising from the presence of 13 functional α genes and one β gene situated syntenically on human chromosome 9p (Manry et al., 2011). However, despite almost 60 years of active research in this field, the direct measurement of type I IFN protein in biological samples has remained elusive. Type I IFN mRNA is usually present at only trace levels in peripheral blood mononuclear cells (PBMCs) from healthy individuals, and current ELISAs have proven either insensitive or unreliable, leading to the development of proxy assays based on type I IFN signaling (Berger Rentsch and Zimmer, 2011; Hua et al., 2006; Li et al., 2010; Niewold et al., 2009; Seo et al., 2009). Such presumed low levels of circulating IFN protein likely reflect the high biological potency of these cytokines, with most cell types expressing a type I IFN receptor.

Balanced against its beneficial role in anti-viral protection, non-physiological exposure to IFN can have major detrimental effects (Hunt et al., 2014). This point is well illustrated by the complex disorders systemic lupus erythematosus (SLE) and dermatomyositis (DM), and the recently defined group of monogenic autoinflammatory diseases referred to as the type I interferonopathies, where persistent type I IFN-induced signalling is considered causal to pathology (Crow, 2011; Greenberg et al., 2005; Hooks et al., 1979; Rodero and Crow, 2016). However, the mechanistic dissection of these pathologies has been hampered by the inability to directly quantify the disease causing protein. This also represents a major unmet clinical need, since such a test could improve diagnosis and therapeutic monitoring in the context of infection, autoimmunity and type I interferonopathies.

To overcome this limitation we took advantage of a new digital ELISA technology based on counting individual enzyme-labelled immunocomplexes of proteins captured on paramagnetic beads in Single Molecule Arrays (Simoa) (Rissin et al., 2010; Wilson et al., 2015). Combining this technology with unique high affinity antibodies isolated from APS1/APECED mutation patients (Meyer et al., 2016) enabled the direct quantification of IFNα at attomolar (femtogram/ml) concentrations. With this five thousand-fold increase in sensitivity over commercial ELISAs, we could directly measure elevated IFNα protein in patients with adult and juvenile-onset SLE.
(JSLE), juvenile-onset DM (JDM), a group of molecularly distinct type I interferonopathies and acute viral meningitis. IFN protein quantification by Simoa was highly correlated with IFN anti-viral activity and interferon stimulated gene (ISG) expression measured in serum and in the cerebrospinal fluid (CSF) of all patients examined. Finally, differences within disease groups were observed based on serum IFNα protein levels, and between diseases as indicated by the results of our studies on the cellular source of IFNα protein.

These data show that the measurement of IFNα protein by digital ELISA is a sensitive, reliable and biologically relevant method that can be used for the diagnosis, stratification and therapeutic monitoring of pathological states associated with an upregulation of type I IFN signalling. The ability to directly measure IFNα protein levels will facilitate a better understanding of the nature, regulation and impact of the human IFN-induced response.

Results and discussion:

Direct quantification of IFNα in plasma, serum and cerebrospinal fluid

To confirm the specificity of our Simoa assay for human IFNα, and non-cross reactivity for other IFNs, we tested these antibodies against IFNβ, IFNλ1, IFNλ2, IFNω and IFNγ recombinant proteins (Fig. 1 A), and 16 subtypes of IFNα that included 3 commercially available IFNα2 types; namely IFNα2a, IFNα2b, IFNα2c (Fig. 1 B). Our assay did not cross-react with other IFNs and was able to detect all IFNα subtypes, although a lower affinity to IFNα2 was observed (Fig. S1 A). Because its standard curve was representative of all other subtypes, IFNα17 (IFNα17) was chosen as our reference protein. To further define the specificity of these reagents we performed a competition assay in plasma samples from 5 systemic lupus erythematosus (SLE) patients. Addition of anti-IFNα antibody depleted the signal, showing a specific detection of IFNα in the tested samples (Fig. 1 C). Reproducibility was assessed across 3 assays (Fig. 1 D), and the mean of three blanks +3SD was used to calculate the limit of detection (LOD). The mean of all LODs was calculated at 0.23 fg/ml, and this value was multiplied by our standard dilution factor (x 3) to give a measure of 0.69 fg/ml, which was used to replace all undetectable values for presentation purposes. Assay reproducibility was also assessed by the measurement of IFNα protein in 22 plasma samples from 8 SLE, 8 JDM, 3 Aicardi-Goutières syndrome (AGS) and 3 TMEM173-mutated / STING patients, using two independently prepared lots of beads, performed by different users at different times.
Analyzing 10 matched plasma and serum samples from AGS patients revealed a strong correlation (Fig. 1 F, $R_s = 0.958$, $p = 0.0001$), indicating a negligible influence of blood processing on IFNα concentration and the ability to use either sample for retrospective patient screening.

To determine the potential diagnostic capability of our assay, we examined collections of plasma and serum established during the study of Mendelian type I interferonopathies (27 patients in total) - attributable to mutations in $TREX1$ ($n = 4$), $RNASEH2A$ ($n = 1$), $RNASEH2B$ ($n = 6$), $RNASEH2C$ ($n = 2$), $SAMHD1$ ($n = 2$), $ADAR1$ ($n = 2$), $IFIH1$ ($n = 3$) and $TMEM173$ gain-of-function ($n = 7$) (STING). We also analysed samples from patients with JDM ($n = 43$), and SLE ($n = 72$) (both juvenile and adult forms; $n = 6$ and 66 respectively). We compared these data with samples from 20 healthy control subjects, and patients with specific autosomal dominant mutations in the 3’ end of $TREX1$ causing retinal vasculopathy and cerebral leucodystrophy (RVCL) which has not previously been associated with increased type I IFN signalling ($n = 30$). Clinical characteristics of these patient and control groups are described in Tables S1 – S6. A one-way ANOVA test (Kruskal-Wallis), and Dunn’s post-test controlling for multiple comparison testing, revealed significantly elevated IFNα in the monogenic interferonopathy ($p < 0.0001$), JDM ($p < 0.0001$) and SLE ($p < 0.0001$) groups as compared to healthy controls, confirming the previously recognised associations with these clinical phenotypes (Fig. 2 A). In contrast, RVCL patients showed no difference in comparison to healthy controls (median 1.6 fg/ml; Inter Quartile range (IQR) 0.95 - 4.6 fg/ml) (Fig. 2 A). Multiple group testing also demonstrated that patients with a molecularly determined interferonopathy had significantly higher IFNα (median 310 fg/ml; IQR 71 - 2223 fg/ml) in comparison to SLE (median 20 fg/ml; IQR 0.69 - 234 fg/ml) ($p < 0.001$), but not to JDM (median 56 fg/ml; IQR 14 - 120 fg/ml) patients (Fig. 2 A).

As expected given the fundamental role of IFN in antiviral protection, testing of CSF from patients with central nervous system (CNS) infection (acute meningitis, $n = 9$; acute encephalitis, $n = 9$; acute meningo-encephalitis, $n = 1$) revealed very high levels of IFNα protein (Fig. 2 B: median 4174.2 fg/ml; IQR 2437.4 - 11173 fg/ml). In contrast, all but one patient with RVCL ($n = 12$) demonstrated an IFNα protein level of less than 10 fg/ml ($p < 0.001$, Mann-Whitney test as compared to infection samples).

$IFN\alpha$ concentration correlates with functional antiviral activity
To investigate the functional relevance of such low protein levels, we compared IFNα protein measurements with IFN activity as assessed by a cytopathic protection assay (Lebon et al., 1988; Lebon et al., 1979; Palmer et al., 2007). Specifically, IFN activity is determined by dilution of patient material incubated with Mardin-Darby bovine kidney cells. Cells are challenged the next day with vesicular stomatitis virus, and examined 18 hours post infection to measure the viral cytopathic effect. Cell protection against infection reveals the biological activity of type I IFN, which is compared with serial dilutions of an IFN standard. Comparing these assays on serum samples (Fig. 3 A: 25 JDM, 2 JSLE and 11 monogenic interferonopathy patients – either AGS or STING, Table S7) demonstrated a positive correlation ($R_s = 0.83$ $p < 0.0001$). Considering the potential contribution of type I IFNs to CNS disorders, we performed a similar comparison on the CSF samples presented in Fig. 2 B. This analysis revealed an even stronger positive correlation between IFN activity and concentration ($R_s = 0.924$, $p < 0.001$). To note, statistical analysis was performed on samples with either detectable IFN activity ($\geq 2$ IU/ml) or IFN protein ($> 0.69$ fg/ml) (Fig. 3 B). Interestingly, 10 serum and 1 CSF samples had IFNα concentration $> 10$ fg/ml with no detectable anti-viral activity, reflecting either the increased sensitivity of the Simoa assay or the presence of post-translational (or other) modifications that may alter functional potency.

**Comparison of IFNα concentration with ISG expression**

We have previously described a screening tool for the identification of monogenic type I interferonopathies based on qPCR assessment of six ISGs expressed by leukocytes (Rice et al., 2013; Rice et al., 2016). In this test, the median fold change of the ISGs when compared to the median of healthy controls is used to create an IFN score. Scores two standard deviations above the median of 29 controls are designated as positive (score of $> 2.466$). We compared Simoa IFNα measurements with the ISG score of samples from 31 SLE (Fig. 3 C), 24 JDM (Fig. 3 D) and 29 monogenic interferonopathy (Fig. 3 E) (Table S8) patients, excluding patients who had negative results for both tests (10 SLE and 1 JDM patients). This analysis revealed a positive correlation between the two parameters in all three groups (SLE, $R_s = 0.787$, $p < 0.0001$ ($n = 31$); JDM, $R_s = 0.665$, $p = 0.0004$ ($n = 24$); interferonopathies, $R_s = 0.635$, $p = 0.0002$ ($n = 29$)). As type I interferonopathies are associated with a broad range of phenotypes at least partially determined by genotype, we grouped patients according to identified mutations (colour coded in Fig. 3 E), but this did not obviously affect the correlation (data not shown). To note, two
outliers in the SLE cohort showed high ISG expression with no detectable IFNα and vice versa, although the explanation for such phenotypes is not clear.

**Association of disease phenotypes with serum IFNα in lupus patients**
To examine whether serum IFNα concentrations were relevant to disease status we focused on the SLE cohort. SLE is a complex and heterogeneous disease, and as such a number of different biomarkers are used to monitor disease severity and progression. For this analysis we divided the SLE patients into 3 groups based on serum IFNα concentrations; < 10 fg/ml (n = 25), 10 - 300 fg/ml (n = 14), > 300 fg/ml (n = 8), (Fig. 4) and in these subgroups examined (A) SLEDAl (systemic lupus erythematosus disease activity index), (B) ESR, (C) CH50 activity, (D) number of autoantibodies, and (E) profile of autoantibodies directed against ribonucleoproteins. An ANOVA with Dunn’s post testing revealed significantly higher α-RNP antibodies (p < 0.0001), ESR (p < 0.01), and SLEDAl (p < 0.01), and lower CH50 activity (p < 0.001) in the patients that had highest serum IFNα. In contrast, anti-ds DNA antibodies and CRP activity showed no differences between the subgroups (Table S9).

**Identification of cellular sources of IFNα**
To explore cellular mechanisms driving disease pathogenesis, we next considered the source of IFNα detected in patients. We sampled blood from 3 STING, 4 AGS, 3 JDM, 3 SLE patients and 4 healthy donors, and isolated CD4+ and CD8+ T cells, B cells, NK cells, CD14+ monocytes and pDCs. A representative flow cytometry panel with the gating strategy is shown in Fig. S2. The percentage of each cell population is presented in Table S10, the purity of sorted cell numbers are detailed in the materials and Table S11, and clinical characteristics of these patients are detailed in Table S12. Cell subsets were isolated, lysed and assessed with our IFNα Simoa assay. To calculate the level of protein present per cell, we divided the concentration of IFNα by the number of cells sorted and normalized for the volume in which the cells were lyzed. This revealed a striking presence of IFNα in the monocytes and pDCs of STING patients, with a mean of 1.03 attogram/cell in pDCs and 1.53 attogram/cell in monocytes (Fig. 5). Notably, no other cell types from the STING patients, nor any cell type analysed from the other patient groups tested, demonstrated levels of IFNα above those in healthy controls. This was despite the high levels of IFNα observed in plasma, suggesting a non-circulating cellular source of IFNα protein in these diseases. This discrepancy in the cellular source of IFNα between monogenic interferonopathy patients highlights a potential cell type specific
mechanism dependent on genes involved in nucleic acid metabolism or sensing.

In conclusion, we report here that the ultrasensitive detection of IFNα protein in human material can provide novel insights into disease causing pathways. The transformational increase in sensitivity over conventional methods that we present here derives from the combination of the Simoa digital ELISA, and the extremely high affinity of the human monoclonal antibodies (mAbs) isolated from APS1/APECED mutation patients (Meyer et al., 2016). In this way, we were able to identify circulating pDCs and monocytes as a constitutive source of IFNα protein in STING mutation patients. Although pDCs are recognised as the major type I IFN producing leukocyte (Siegal et al., 1999), they were not apparently implicated in AGS, JDM or SLE patients in our study, possibly suggesting tissue sequestration of these cell types or an additional cellular source of IFNα in these conditions. However, the number of pDCs isolated was low, in particular from SLE patients, so that these results will require additional confirmatory experiments. The current lack of a test to measure type I IFN protein in routine medical practice represents a major unmet clinical need. The potential of direct measurement of IFN protein per se as a disease biomarker is obvious, and will be immediately relevant in SLE where anti-IFNα and anti-IFNAR therapies are currently being tested (Lauwerys et al., 2014). Indeed, our data identified lupus patients with low or high IFNα concentration, which differentiated patients based on a number of disease relevant phenotypes, and as such could be used for future patient stratification, as well as for on-treatment monitoring. Such changes might also be of clinical relevance in other situations, as evidenced by the risk of developing IFN-driven pathology in the context of TNFa blockade (Conrad et al., 2015). Furthermore, our ability to detect and quantify IFNα in different sample types from virally infected patients suggests possible clinical utility in the assessment of disease severity. For these reasons, we consider that digitalised ELISA technology can play a central role in future management of many different disease states.

Methods

Subjects and samples

Historical serum, plasma and CSF samples were collected from patients demonstrating the presence of biallelic mutations in TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1 and ADAR1, as well as recognised dominant disease-causing mutations in TREX1, and from patients with dominant mutations in IFIH1 and TMEM173 (STING). Samples from patients with systemic lupus
erythematous (SLE) and dermatomyositis (DM) demonstrated clinical criteria conforming to American College of Rheumatology and Bohan and Peter criteria respectively. Historical samples for IFN activity studies were collected across the period 1985 – 2015. All samples were collected with informed consent. The study was approved by the Leeds (East) Research Ethics Committee (reference number 10/H1307/132), and by the Comité de Protection des Personnes (ID-RCB / EUDRACT: 2014-A01017-40). In Figure 2, only the first sample collected from each individual patient was plotted (Table S1-S5). For the correlation analysis in Fig. 3, serial samples from the same patient were used (Tables S7 and S8). SLE disease activity was assessed by an experienced rheumatology nurse specializing in SLE, who interviewed each patient and had access to all medical data and blood results using the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI). SLE Patients also had blood tests at the relevant clinic visits including that for SLE disease activity (C3, C4, and anti–double stranded DNA) and for routine inflammatory markers (CRP and erythrocyte sedimentation rate (ESR)), which were performed in a fully accredited, NHS laboratory (http://www.edinburghlabmed.co.uk).

Simoa assay development
The Simoa IFNα assay was developed using a Quanterix Homebrew Simoa assay according to the manufacturer’s instructions, and utilizing two autoantibodies specific for IFNα isolated and cloned from 2 APS1/APECED patients recently described (Meyer et al., 2016 and patent application WO2013/098419). The 8H1 antibody clone was used as a capture antibody after coating on paramagnetic beads (0.3mg/ml), and the 12H5 was biotinylated (biotin/Ab ratio = 30/1) and used as the detector. EC50 binding of the mAbs was determined by ELISA as previously described (Meyer et al., 2016), and observed to be 4.02 ng/ml and 4.4 ng/ml for 8H1 and 12H5 respectively, for IFNα5. The IC50 was determined using an ISRE-Luciferase reporter assay (described in Meyer et al., 2016), with results presented for both antibodies against all IFNα subtypes in Fig. S1_A. For the Simoa assay, recombinant IFNα17/αI (PBL Assay Science) was used as a standard curve after cross-reactivity testing (Fig. 1_B). The limit of detection (LOD) were calculated by the mean value of all blank runs + 3SDs and was 0.23 fg/ml. Non-specificity was demonstrated against IFNβ, IFNλ1, IFNλ2, IFNω, and IFNγ (Peprotech & PBL Assay Science), and cross reactivity was tested against IFNα1, IFNα1 (Val114), IFNα2b, IFNα4a, IFNα4b, IFNα5, IFNα6, IFNα7, IFNα8, IFNα10, IFNα14, IFNα16, IFNα17, IFNα21 (all PBL Assay Science), IFNα2a (Peprotech) and IFNα2c (eBioscience). Additional specificity of the assay was demonstrated in a competition assay where SLE patient plasma samples (n = 5)
were 1/3 diluted with PBS and pre-incubated with the IFNα capture antibody (50 μg/ml) for 30 minutes at room temperature prior to Simoa analysis. For Simoa measurements, biological samples were diluted from 1/3 to 1/30 depending on the amount of material available and to avoid saturation. Samples with signal below the LOD were normalized to 0.69 fg/ml (LOD*3, the minimal dilution factor) for presentation and analysis purposes.

**Interferon activity assay**
Type I IFN activity was measured by determining the cytopathic reduction i.e. protection of Madin-Darby bovine kidney cells against cell death following infection with vesicular stomatitis virus, afforded by patient CSF / serum. A reference of human IFNα, standardized against the National Institutes of Health reference Ga 023-902-530, was included with each titration. IFNα activity in normal healthy serum is < 2 IU/ml.

**Assessment of interferon stimulated gene expression in blood cells**
Blood was collected into PAXgene tubes (PreAnalytix) and total RNA was extracted using a PAXgene (PreAnalytix) RNA isolation kit. RNA concentration was assessed using a spectrophotometer (FLUOstar Omega, Labtech). Quantitative reverse transcription polymerase chain reaction (qPCR) analysis was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems), and cDNA derived from 40ng total RNA. Using TaqMan probes for IFI27 (Hs01086370_m1), IFI44L (Hs00199115_m1), IFIT1 (Hs00356631_g1), ISG15 (Hs00192713_m1), RSAD2 (Hs01057264_m1), and SIGLEC1 (Hs00988063_m1), the relative abundance of each target transcript was normalised to the expression level of HPRT1 (Hs03929096_g1) and 18S (Hs999999001_s1), and assessed with the Applied Biosystems StepOne Software v2.1 and DataAssist Software v.3.01. For each of the six probes, individual (patient and control) data were expressed relative to a single calibrator (control C25). The median fold change of the six ISGs, when compared to the median of the combined 29 healthy controls, was used to create an interferon score for each patient. RQ is equal to $2^{-\Delta\Delta Ct}$ i.e. the normalized fold change relative to a control. When a patient was assayed on more than one occasion, the data for repeat measurements were combined to calculate a mean value (using Applied Biosystems DataAssist software v.3.01).

**Cell sorting and lysis for Simoa analysis**
PBMCs were isolated from blood using lymphocyte separation medium. Just after
isolation, PBMCs were labeled with CD3 Krome Orange, CD8 pCp-Cy5, CD11c PE, CD19 PE-Cy7, CD56 FITC, CD14 APC-Alexa Fluor 750 and HLA-DR Pacific Blue. PBMC subsets were isolated using a BD FACS Aria II according to the gating strategy presented in Figure S1. Purity of the cell sorting was verified for 9 individual donors, and was high for all populations (mean +/- SD for CD4: 97% +/- 3.2, CD8: 97.2% +/- 1.4, B cells: 97.5% +/- 2.3, NK: 97.9% +/- 1.6, monocytes: 97.7% +/- 2.6, pDCs: 96.9% +/- 2.3). After sorting, cells were pelleted and lysed in 50µl of RIPA buffer containing 1x of Halt Protease inhibitor Cocktail. Details of cell numbers and IFNα concentration are indicated in Table S11. Clinical characteristics of these patients are described in Table S12. Cell populations were not sorted from virally infected patients because of ethical restrictions on obtaining sufficient blood volumes.

Statistical analysis
GraphPad Prism was used for statistical analysis. ANOVA tests (Kruskal-Wallis) with Dunn’s post testing for multiple comparisons were utilized to test for differences between patient groups, with median and IQ ranges reported. Correlations between the different assays were calculated using Spearman test.

Supplemental Information
Supplemental Figures S1 shows affinity measurements of the antibodies used in the Simoa assay, S2 the gating strategy for cell sorting, and supplemental Tables S1-7 list clinical characteristics of the patient cohorts.

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collect the SLE cellular samples. Thanks are given to the Clinical Research Unit, Institut Imagine for protocol assistance. AH is cofounder and shareholder, and CH is an employee of ImmunoQure AG, no other financial conflicts are declared.

Author contributions
MPR, JD, GIR and FR performed experiments, analyzed data and wrote the paper. DH led the studies and analysis of the SLE patients. SW, SM & VB performed experiments and analyzed data. MAA, BBM, CB, NB, AB, CB, TAB, ID, MLF, MH, AMM, IM, LM, NP, PQ, GMT, JW, SW, FRL, YR, BN recruited patients. CH and AH provided key reagents and critical review of the manuscript. MA provided critical review and intellectual contribution. YJC & DD designed the study, analyzed data, and wrote the paper.

References


Figure legends

**Figure 1. Specificity, sensitivity, and reproducibility of the Simoa IFNα assay.**
(A) Simoa IFNα assay reactivity with IFNα17, IFNβ, IFNλ1, IFNλ2, IFNω and IFNy recombinant proteins. Lowest concentration is the blank. (B) Simoa IFNα assay cross-reactivity with IFNα1, IFNα1 (Val114), IFNα2a, IFNα2b, IFNα2c, IFNα4a, IFNα4b, IFNα5, IFNα6, IFNα7, IFNα8, IFNα10, IFNα14, IFNα16, IFNα17 and IFNα21. (C) Simoa IFNα competition assay; measurement of IFNα in 5 SLE patient plasma samples following pre-incubation with the human anti-IFNα capture antibody for 30 minutes prior to analysis. (D) Reproducibility testing for each concentration, acquired as duplicates across 3 independent runs. Dashed line represents the LOD, defined by mean blank AEB (Average Enzyme per Bead) + 3SD of all runs. (E) 22 plasma samples (8 SLE, 8 DMJ, 3 AGS and 3 STING) were analysed with two independently prepared lots of beads, by different users and at different times, Spearman correlation is reported. (F) Correlation of IFNα protein measured by Simoa in paired plasma and serum samples from 10 AGS patients, Spearman correlation is reported.

**Figure 2. Quantification of plasma, serum, and CSF IFNα in patient cohorts.** (A) Plasma from healthy controls (n = 20) and patients with RVCL (n = 30), SLE (n = 72), JDM (n = 43) and molecularly defined interferonopathies (n = 27) were assayed by Simoa for IFNα protein. Values were assessed by one-way ANOVA test (Kruskal-Wallis), and Dunn’s multiple comparison testing between groups. (B) CSF samples from acute meningitis (n = 9), acute encephalitis (n = 9), acute meningo-encephalitis (n = 1), and RVCL (n = 12) were assayed by Simoa for IFNα protein. Values were assessed by Mann-Whitney T test., *** p < 0.001, **** p < 0.0001.

**Figure 3. Comparison of IFNα concentration with anti-viral activity and ISG expression.** (A) Correlation of Simoa IFNα protein measurement with IFN activity measured by a cytopathic assay for interferonopathy (n = 10), JDM (n = 26), and JSLE (n = 2) patients. (B) Correlation of Simoa IFNα protein measurement with IFN activity measured by a cytopathic assay for CSF samples from acute viral meningitis (n = 9), acute viral encephalitis (n = 9), acute viral meningo-encephalitis (n = 1), AGS.
Correlation of Simoa IFNα concentration with the ISG score in SLE (C, n = 21), JDM (D, n = 23), and molecularly defined interferonopathy patients (E, n = 29). Spearman correlations were calculated for each patient group, excluding samples where both the ISG score and the IFNα concentration were negative (SLE n = 10, JDM n = 1).

**Figure 4. Disease associations of serum IFNα in SLE patients.** Higher serum IFNα levels associate with (A) Higher SLEDAI (systemic lupus erythematosus disease activity index) (p < 0.001), (B) ESR (p < 0.01), (C) lower CH50 activity (p < 0.001) and (D) number of specific auto-antibodies against ribonucleoproteins (anti-Ro, La, Sm, RNP) (p < 0.0001). IFNα <10 fg/ml: n = 25; IFNα = 10 - 300 fg/ml: n = 14; IFNα >300 fg/ml: n = 8. One-way ANOVA (Kruskal-Wallis) p values are reported. (E) Profile of autoantibodies directed against ribonucleoproteins in patients with low, intermediate and high IFN levels. Green = < 25U/ml, yellow = 25 - 50U/ml, orange = 50 - 100 U/ml, red = over 100 U/ml. A positive result is > 25 U/ml. The total number of autoantibodies against ribonucleoproteins (anti-Ro, La, Sm, RNP) is significantly increased in patients with the highest levels of serum IFNα (2-way ANOVA, p < 0.0001). * p < 0.05, **p < 0.01 ***p < 0.001 ****p < 0.0001.

**Figure 5. Identification of circulating IFNα producing cells in STING patients.** IFNα protein levels presented as median attograms per cell in sorted CD4 and CD8 T cells, NK cells, B cells, monocytes and plasmacytoid dendritic cells (pDCs) from STING mutation (red, n = 3), JDM (blue, n = 3), AGS (green, n = 4) and SLE (purple n = 3) patients. The black line on each plot represents the median of 4 control healthy donors.

**Figure S1. IC50 values of mAbs used in Simoa assay for all IFNα subtype**
10,000 HEK 293 MSR cells were seeded in white half-area 96-well plates (Cat. No. 3688, Corning) and reverse-transfected with 50 ng of premixed ISRE-Firefly luciferase reporter and Renilla luciferase constructs (Cat. No. CCS-008L, Qiagen, Hilden, Germany) using Fugene HD according to the manufacturer’s instructions (Promega, Madison, WI, USA). The Renilla luciferase-expressing construct served as an internal normalization control. Cells were incubated overnight in Opti-MEM® I Reduced Serum Medium supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.5% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) at 37°C, 5% CO2 in a humidified atmosphere. Following overnight incubation, cells were stimulated for 24 hours with medium containing mixtures of recombinant
human IFNα with or without anti-IFNα mAbs or control IgG that had been pre-incubated for one hour at 37°C. After 24 hours of stimulation, dual luciferase reporter assays were performed according to the manufacturer’s instructions (Promega).

**Figure S2. Flow cytometry gating strategy.**
Flow cytometry gating strategy for the isolation of CD8+ and CD4+ T cells, B cells, NK cells, monocytes and pDCs.

**Supplemental Tables 1 - 7. Clinical characteristics of patient cohorts and samples tested by IFNα Simoa assay, IFN activity assay and ISG score.**

Table S1. Retinal vasculopathy with cerebral leukodystrophy (RVCL).
Table S2. Juvenile dermatomyositis (JDM).
Table S3. Systemic lupus erythematosus (SLE).
Table S4. Monogenic interferonopathy.
Table S5. Central nervous system inflammation.
Table S6. Controls.
Table S7. Information on data used for Figure 3A.
Table S8. Information on data used for Figure 3C, D and E.
Table S9. Demographic and clinical associations of raised serum IFNα levels in SLE patients.
Table S10. Circulating leukocyte frequencies in STING, AGS, JDM, SLE and controls.
Table S11. Sorted cell numbers and IFN concentrations.
Table S12: Clinical information of patients studied for cell subset IFNα content.
Figure 1
Figure 2

(A) Serum IFNα (fg/mL)

(B) CSF IFNα (fg/mL)

- Healthy (n=20)
- RVCL (n=30)
- SLE (n=72)
- JDM (n=43)
- AGS+STING (n=27)

- RVCL (n=12)
- Meningo-encephalitis (1)
- Encephalitis (n=9)
- Meningitis (n=9)
Figure 3
Figure 4
Figure 5
Figure S1