

## Human IFNAR2 deficiency: lessons for antiviral immunity

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**Abstract** Type I interferon (IFN- $\alpha/\beta$ ) is a fundamental antiviral defense mechanism. Mouse models have been pivotal to understanding the role of IFN- $\alpha/\beta$  in immunity, although validation of these findings in humans has been limited. We investigated a previously healthy child with fatal encephalitis following inoculation of the live-attenuated measles, mumps and rubella (MMR) vaccine. By targeted resequencing we identified a homozygous mutation in the high-affinity interferon- $\alpha/\beta$  receptor (*IFNAR2*) in the proband, as well as a newborn sibling, that rendered cells unresponsive to IFN- $\alpha/\beta$ . Reconstitution of the proband's cells with wild-type *IFNAR2* restored IFN- $\alpha/\beta$  responsiveness and control of IFN-attenuated viruses. Despite the severe outcome of systemic live-vaccine challenge, the proband had previously shown no evidence of heightened susceptibility to respiratory viral pathogens. The phenotype of *IFNAR2* deficiency, together with similar findings in *STAT2* deficient patients, supports an essential but narrow role for IFN- $\alpha/\beta$  in human antiviral immunity.

**Summary:** Human *IFNAR2* deficiency causes fatal susceptibility to live viral vaccines, revealing a vital but narrow nonredundant role for IFN- $\alpha/\beta$  in viral immunity.

**Introduction:** Understanding the concerted antiviral immune response is an important goal for immunologists, vaccinologists and virologists. In the current paradigm, viral sensing leads to the induction of an antiviral program that is powerfully amplified and propagated by innate interferons (IFNs  $\alpha/\beta$  and  $\lambda$ ). These soluble cytokines induce an antiviral, antiproliferative state by signaling at specific cell-surface receptors, inducing the expression of hundreds of interferon-stimulated genes (ISGs) to inhibit viral replication (1). Furthermore, IFNs contribute to the recruitment and activation of both innate and adaptive immune effectors (2, 3). Whilst responses to IFN- $\lambda$  are limited by receptor expression to the mucosal epithelium, all nucleated cells respond to IFN- $\alpha/\beta$ . This potent systemic antiviral defense exerts ongoing selective pressure, as reflected by the fact that most pathogenic viruses have evolved mechanisms of IFN evasion (4).

Mouse knockout models have been pivotal to our understanding of IFN- $\alpha/\beta$  biology (5-8). Mice lacking the ubiquitously expressed receptor for IFN- $\alpha/\beta$  (Ifnar), or downstream signaling components (tyrosine kinase 2 [Tyk2], signal transduction and activator of transcription-1 [Stat1] or Stat2) manifest complex defects in viral resistance and immune homeostasis (9). As a result, IFN- $\alpha/\beta$  has been used in humans to treat diverse pathological states including chronic viral infection, multiple sclerosis and cancer (10). However, evidence for the nonredundant role of IFN- $\alpha/\beta$  in human antiviral immunity is scant. No human defects in IFNAR are reported; currently recognised primary immunodeficiencies (PIDs) that affect downstream signaling do so either nonspecifically (TYK2, STAT1) (11, 12), due to their involvement in other critical signaling pathways, or incompletely (STAT2) (13). Nonetheless, human STAT2 deficiency results in a narrow but severe phenotype of innate antiviral immunodeficiency, particularly following systemic challenge with live-viral vaccines such as measles, mumps and rubella (MMR) (13).

**Results:** We evaluated a 13-month old infant who developed severe, prolonged and ultimately fatal encephalitis, as a complication of MMR vaccination (Fig. 1A and supplementary case report). There was clear evidence of sustained replication of vaccine viruses, as well as human herpes virus 6 (HHV6), in systemic and brain samples (Table S1), despite an appropriate serological response to MMR (Table S2). Given the clinical similarities to STAT2-deficiency, in a patient with normal adaptive immune parameters (Table S3), we suspected a PID involving the innate antiviral response. We therefore examined the ability of patient dermal fibroblasts to control the replication of attenuated viruses deleted for specific IFN- $\alpha/\beta$  antagonists (14, 15), and observed that, unlike control cells, they supported the formation of large plaques (Fig. 1B). Patient cells were also unable to develop an antiviral state in response to exogenous IFN- $\alpha$ , showing unhindered wild-type viral replication as compared with significant inhibition in controls (Fig. 1C). In contrast, IFN- $\gamma$  treatment was capable of inducing a partial antiviral state in patient and control cells (Fig. S1). These findings implied an IFN- $\alpha/\beta$  signaling defect. Indeed, IFN- $\alpha$  failed to induce the expression of classical antiviral gene products in patient cells (Fig. 1D). To determine the degree to which IFN- $\alpha/\beta$  signaling was impaired, we measured the global transcriptional response of fibroblasts to IFN- $\alpha$ , IFN- $\beta$  and IFN- $\gamma$  by whole genome microarray. There were no differences in basal transcriptional profile between the patient and three independent controls, however we observed a striking failure of transcriptional responses to IFN- $\alpha/\beta$  in the patient (Fig. 1E). Of 230 and 374 probes differentially expressed in response to IFN- $\alpha$  or IFN- $\beta$  respectively (224 overlapping), none were induced in the patient (datasets S1 and S2). In contrast, IFN- $\gamma$  responses were preserved (Fig. 1E, dataset S3).

We sought to localize this profound defect by examining successive phosphorylation steps within the IFN- $\alpha/\beta$  signaling pathway. IFNAR is a heterodimer composed of low affinity (IFNAR1) and high affinity (IFNAR2) subunits, which are respectively associated with the kinases TYK2 and JAK1 (16). In normal cells, IFNAR ligation by IFN- $\alpha/\beta$  results in reciprocal transphosphorylation of JAK1 and TYK2. These phosphorylate STAT1 and STAT2, which associate with IRF9 to form Interferon-Stimulated Gene Factor 3 (ISGF3) and thereby effect gene transcription (1) (Fig. 2A). We observed complete failure of tyrosine phosphorylation of JAK1, TYK2, or STAT 1/2 in patient cells exposed to IFN- $\alpha$  (Fig. 2B) or IFN- $\beta$  (Fig. S2). By contrast, phosphorylation of JAK1 and STAT1 following IFN- $\gamma$  treatment was normal (Fig. 2C). These differential effects on JAK1/STAT1 phosphorylation localized the signaling defect proximally, to IFNAR itself (Fig. 2A).

To identify the putative genomic variant, we first sequenced *IFNAR1* and *IFNAR2* in complementary DNA. There were no variants in *IFNAR1* and corresponding protein expression was normal (Fig. 3C). In contrast, we identified a single rare variant in *IFNAR2*, namely a single-base deletion in exon 5 (c.A311del; Ref. ENST00000342136, Fig. 3A). This variant proved to be homozygous in genomic DNA of the proband as well as a newborn sibling, and heterozygous in both parents, but was absent from a healthy control sequenced in parallel and from public databases of genomic variation such as dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) and Ensembl release 78 (<http://www.ensembl.org>). A311 is highly conserved in bovine to rodent to ape orthologues of *IFNAR2* and is present in all *IFNAR2* transcript variants. The c.A311del variant introduces a frameshift substitution of glycine in place of glutamic acid at position 104, generating a downstream premature stop codon (pE104fs110X). IFNAR2 has three protein isoforms, generated by exon skipping, alternative splicing, and differential use of polyadenylation sites (16) (Fig. 3B), although only one (IFNAR2c) is functional (17). The c.A311del variant is predicted to truncate all isoforms at the first N-terminal fibronectin III domain (Fig. 3B). In keeping with a loss of full length protein, we could not detect IFNAR2 by immunoblot with a C-terminal antibody (Fig. 3C and Fig. S3) and in this respect the patient cells resembled the IFNAR2-deficient sarcoma cell line U5A (18) (Fig. S4).

To prove definitively that the homozygous c.A311del *IFNAR2* variant was responsible for the functional defect in IFN signaling, we transduced fibroblasts from the proband with wild-type *IFNAR2* (IFNAR2c) (Fig. 4A). IFNAR2c complementation restored responsiveness to IFN- $\alpha$  as measured by STAT1 tyrosine phosphorylation (Fig. 4B), ISG induction (Fig. 4C) and reduction in WT viral protein expression (Fig. 4E; see also Fig. 1C for control responses). Importantly, it also reinstated the ability to control the replication of IFN-sensitive viruses (Fig. 4D).

**Discussion:** This previously unreported PID, IFNAR2 deficiency, directly informs our understanding of the role of IFN- $\alpha/\beta$  in human antiviral immunity. Despite a profound defect in IFN- $\alpha/\beta$  signaling, conferring serious viral susceptibility *in vitro* and *in vivo*, there was no evidence of clinically relevant vulnerability to commonly encountered viruses prior to the MMR-related illness in the proband. The precise contribution of each attenuated vaccine virus to the final illness is difficult to quantify but both mumps and rubella were detected in brain biopsy more than 7 weeks after vaccination. HHV6 was also detected, but HHV6 latency is ubiquitous

by the first years of life, and its role as a pathogen or commensal in the CNS is not fully resolved (19). Whether HHV6 was directly involved in neuropathology, or acting as a bystander - as has been reported with other neurotropic viral illness (20) - is unclear. However, susceptibility to neurotropic herpesviruses is a recognized feature of human defects in innate viral sensing (21, 22), possibly reflecting the tissue-specific importance of IFN- $\alpha/\beta$  in CNS antiviral immunity (23). Intriguingly, the control of systemic cytomegalovirus (CMV) replication in the IFNAR2 proband indicated the presence of additional effective means of herpesvirus surveillance. This reflects the situation in STAT2-deficient patients, where individuals controlled Epstein-Barr virus (EBV) and varicella zoster virus (VZV), as well as various respiratory viruses (13), despite severe illness arising from live-vaccine challenge. These data, which contrast starkly with the mouse models (5, 8), imply unappreciated redundancy in IFN- $\alpha/\beta$ -mediated antiviral defense. The discordance between immunological and clinical infection phenotypes also recalls other PIDs affecting innate immune recognition and/or signaling, such as deficiencies of IRAK4 (24) and MyD88 (25). These generate narrow bacterial susceptibility profiles in humans (26), as a consequence of signaling network redundancy and/or complementation by adaptive immunity. Indeed, the capacity of IFN- $\gamma$  to generate an antiviral state in IFNAR2 deficient cells suggests a potentially important therapeutic avenue for patients with IFN- $\alpha/\beta$  signaling defects. The apparent absence of a functional T cell defect in patients with IFNAR2 or STAT2-deficiency implies that the murine paradigm, in which aspects of T cell activation and maintenance are attributed to IFN- $\alpha/\beta$  signaling (9), does not translate into a clinically relevant phenotype in humans. Similarly, other important homeostatic abnormalities reported in *Ifnar*<sup>-/-</sup> mice, such as impaired myelopoiesis or defects in thymic T cell development (16), are not a feature of human deficiencies of IFNAR2 or STAT2, presumably as a consequence of divergent species evolution (27).

The susceptibility to systemic live-attenuated viruses caused by defects of IFN- $\alpha/\beta$  response is striking. Serial passage in nonhuman cells (28) may result in the loss of IFN-evasion genes among other undefined mechanisms of attenuation (29). Such attenuation is rendered inoperative in the IFN- $\alpha/\beta$  deficient host, revealing the viruses' underlying potential for widespread dissemination and pathogenicity (30). This behavior recalls the ability of another live-attenuated vaccine, *Bacille Calmette-Guérin* (BCG), to reveal otherwise cryptic immune defects in Mendelian Susceptibility to Mycobacterial Disease (31). Importantly, the systemic route of vaccine administration evades tissue-specific innate mechanisms that might otherwise complement the lack of IFN- $\alpha/\beta$ , such as mucosal IFN- $\lambda$  (32). As a result, the kinetics and multiplicity of infection likely differ significantly from natural exposure at mucosal surfaces. Our data suggest that pathological dissemination of live-viral vaccines in previously healthy individuals must be assumed to reflect a lesion in the IFN- $\alpha/\beta$  pathway until proven otherwise.

## **Materials and Methods:**

Clinical samples were obtained and testing undertaken following written informed consent from parents in accordance with the Declaration of Helsinki and with local Research Ethics Committee approval. Dermal fibroblasts from patient II.1 and healthy controls were obtained by standard methods and cultured in Dulbecco's Modified Eagle's Medium supplemented by 10% fetal calf serum and 1% penicillin/streptomycin (DMEM-10). Peripheral blood mononuclear cells were isolated by density gradient centrifugation from patient II.2 and healthy controls and

cultured in RPMI medium supplemented by 10% fetal calf serum and 1% penicillin/streptomycin (RPMI-10). The IFNAR2-deficient human sarcoma cell line U5A (18) (a kind gift from Professor Steve Goodbourn) was cultured in DMEM-10. Diagnostic immunology and virology analyses were performed in accredited regional diagnostic laboratories to standard protocols.

### Lentiviral transduction

Lentiviral particles were prepared by AMS Bio (Abingdon, UK). Supplied were target particles encoding the full length open reading frame of human *IFNAR2* transcript variant 1 (NM\_207585) under the control of the constitutive promoter EF1a, with a GFP-Puromycin selection marker under an RSV promoter, as well as null control particles lacking the *IFNAR2* target, but containing the vector backbone. Patient dermal fibroblasts were spinoculated in 6-well plates for 1.5 h at 2000 rpm, with target or null control viral particles, at multiplicity of infection (MOI) 5 in a total volume of 0.5mL DMEM-10 containing hexadimethrine bromide (Polybrene, 8 µg/mL, Sigma). Cells were rested in virus-containing medium for 8 h then incubated in fresh DMEM10 until 48 h, when they were selected in DMEM-10 containing 0.5 µg/mL puromycin (Sigma). Puromycin-containing medium was refreshed every 72 h.

### Viruses and interferons

Viruses, plaque assay methodology and immunofluorescence have been described previously (13). Interferon- $\alpha$  (Intron-A, Schering-Plough, New Jersey, USA), interferon- $\beta$  (Avonex, Biogen Idec, Maidenhead, UK) and interferon- $\gamma$  (Immunikin, Boehringer Ingelheim, Ingelheim am Rhein, Germany) were used at 1000 IU/mL unless otherwise stated.

### Immunoblotting

Confluent monolayers of dermal fibroblasts or  $2 \times 10^6$  PBMC in 24-well plates were washed in phosphate buffered saline (PBS) and lysed on ice in lysis buffer (20 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA) containing 100 mM dithiothreitol (DTT, Sigma-Aldrich, Missouri, USA), 1 x protease inhibitor cocktail (Roche, Basel, Switzerland) with phosphatase inhibitors (10mM sodium fluoride and 1mM sodium orthovanadate, Sigma-Aldrich) and 1 x NuPAGE® Loading Buffer (Life Technologies, Paisley, UK). Lysates were heated to 90°C for 20 min prior to 4-12% Tris-Glycine polyacrylamide gel electrophoresis (Novonex®, Life Technologies, Paisley, UK) in 1 x sodium dodecyl sulfate (SDS) NuPAGE® MOPS Running Buffer (Life Technologies). 5 µL of Prestained Protein Ladder (Thermo Scientific, Life Technologies, Paisley, UK) was used as a size marker. Proteins were transferred to 0.45 µm polyvinyl difluoride (PVDF) membranes (Thermo Scientific Pierce, Life Technologies, Paisley, UK) in NuPAGE® Tris-Glycine Transfer Buffer. Membranes were blocked for 30 min in 5% bovine serum albumin or 5% milk in tris-buffered saline with 0.1% Tween (TBS-T) prior to immunostaining by standard methods. The following anti-human antibodies together with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies were used for immunostaining (all rabbit unless otherwise stated): Sheep IFNAR2 C-terminal (AF7014, R&D Systems, Minnesota, USA); Goat ISG56/IFIT1 (sc-82946, Santa Cruz, Texas, USA); MXA (Mx1/2/3) (sc-34128, Santa-Cruz); IFNAR1 C-terminal (EP899Y, Abcam, Cambridge, UK);

STAT2 (07-140, Millipore, Massachusetts, USA); Phospho-STAT2 (Tyr690, No. 4441); TYK2 (No. 9132); Phospho-TYK2 (Tyr1054/1055, No. 9321); JAK1 (No. 3332); Phospho-JAK1 (Tyr1022/1023, No. 3331); STAT1 (No. 9172); Phospho-STAT1 (Tyr701, Clone D4A7, No. 7649); GAPDH (D16H11, No. 5174), all Cell-Signaling Technologies, Massachusetts, USA. Membranes were washed in TBS-T and developed with Immobilon™ Western Chemiluminescent HRP substrate (Millipore). Visual spectrum and chemiluminescent images were captured on a G:BOX Chemi (Syngene, Hyarana, India) CCD camera using Genesnap software (Syngene). Composite light/chemiluminescent images were generated without manipulation and exported in TIFF format.

### Mutation screening

Complementary DNA was synthesized from purified RNA as previously described (13). *IFNAR1* and *IFNAR2* were PCR-amplified from the cDNA with specific primers designed in Primer3web version 4.0.0 (<http://bioinfo.ut.ee/primer3/>). Primer sequences are available on request. Capillary sequencing was performed according to standard methods. Sequences were aligned with the consensus coding sequence (human genome assembly 38) in nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/blast/>).

### Microarray analysis

Patient dermal fibroblasts in triplicate wells and control fibroblasts from three independent donors in 24-well plates were treated with 1000 IU/mL IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$  or medium alone for 10 h. RNA was isolated by using the ReliaPrep RNA Cell Miniprep System (Promega) according to manufacturers' instructions. Microarrays were performed by ServiceXS Ltd. (Leiden, Netherlands). 200 ng of total RNA were used to synthesize biotinylated cRNA target, which was subject to QC checks, then hybridized (750 ng cRNA per sample) to Illumina HT-12 v4 Expression Bead Chips (Illumina Inc. San Diego, USA). Data from two Illumina HT-12 v4 Expression Bead Chips per sample were background corrected in Illumina Beadstudio with further analysis carried out using the Lumi and Limma Bioconductor packages in R (33, 34). Normalization of background corrected data was applied through variance stabilizing transformation (VST) and robust spline normalization (RSN) in Lumi (35). Testing for differential expression between IFN-treated patient and control cells was done with linear models and empirical Bayesian statistics, utilizing a multi-level experiment approach through Limma. Gene lists for each comparison were generated. Data plots were created using the ggplot2 library in R.

### Statistical analysis

Differential gene expression was considered significant if the Benjamini-Hochberg false discovery rate (FDR) adjusted P value was  $< 0.01$ , and the absolute fold change in expression level was  $\geq 2$  (i.e. 2 fold increase or decrease in expression from baseline).

### **List of supplementary materials:**

## Case Summary S1

Figure S1. Antiviral activity of IFN- $\gamma$ .

Figure S2. IFN- $\beta$  signaling

Figure S3. Failure of ISG induction in patient II.2

Figure S4. IFNAR2 expression in U5A cells

Table S1. Detection of vaccine-strain viruses and HHV6 by PCR or viral culture in patient II.1

Table S2. Antibody responses to MMR in patient II.1

Table S3. Immunological and hematological parameters from patient II.1

Source data (immunoblots)

Dataset S1. IFN- $\alpha$  microarray

Dataset S2. IFN- $\beta$  microarray

Dataset S3. IFN- $\gamma$  microarray

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## Figure Legends

**Figure 1. Failure of IFN- $\alpha$ / $\beta$  antiviral response.** (A) Meningoencephalitis on cortical biopsy. Cortical inflammatory cell nodular infiltrate (left) with marked microglial activation (CD45 staining, middle) and patchy dural inflammation (right). Scale bars 100 $\mu$ m (B) Patient fibroblasts supported the formation of large plaques by parainfluenza 5 and Bunyamwera viruses deleted for IFN- $\alpha$ / $\beta$  antagonists (PIV5 $\Delta$ C, BUN $\Delta$ NSs). Scale bar 1cm (C) Failure of IFN- $\alpha$  to inhibit wild-type (WT) PIV5 replication in patient fibroblasts, revealed by immunofluorescence staining of viral antigen. Scale bars 50 $\mu$ m. (D) Immunoblot showing absence of antiviral protein (MXA and IFIT1/ISG56) induction by IFN- $\alpha$  (representative of n=3 experiments). (E) Absent transcriptional response to IFN- $\alpha$  and IFN- $\beta$  but preserved IFN- $\gamma$  response in patient cells, assessed by microarray; red dots represent significantly differentially expressed probes ( $\geq$  2-fold, adj.  $P < 0.01$ , n=3 replicates). Exact  $P$  values are reported in supplemental datasets S1-3. Patient = patient II.1 (see Figure 3).

**Figure 2. Absence of IFN- $\alpha$ / $\beta$  signaling but preserved IFN- $\gamma$  signaling.** (A) Schematic of IFN- $\alpha$ / $\beta$  and IFN- $\gamma$  signal transduction. (B) Absent tyrosine phosphorylation of TYK2/JAK1/STAT1/STAT2 in response to IFN- $\alpha$ . (C) Normal tyrosine phosphorylation of JAK1/STAT1 in response to IFN- $\gamma$  (C). Data are representative of n=3 experiments. Patient = patient II.1 (see Figure 3).

**Figure 3. Autosomal recessive IFNAR2-deficiency.** (A) Capillary sequencing of *IFNAR2* revealed variant c.A311del was homozygous in patient II.1, resulting in a frameshift mutation p.E104fs110X, heterozygous in both parents, and homozygous in a newborn sibling, II.2. (B) A311del is predicted to truncate all protein isoforms of IFNAR2 at the first N-terminal extracellular domain (ECD). (C) Absent IFNAR2 expression in fibroblasts of the proband by immunoblot with an antibody against C-terminal IFNAR2 (representative of n=3 experiments).

**Figure 4. IFNAR2 complementation restores IFN- $\alpha$ / $\beta$  responses.** (A) Stable expression of WT IFNAR2 or null control by lentiviral transduction in patient fibroblasts restored: (B) STAT1 tyrosine phosphorylation; (C) ISG induction (representative immunoblots of n=3 experiments); (D) Control of IFN-attenuated viruses parainfluenza 5  $\Delta$ C (PIV5 $\Delta$ C) and Edmonton strain measles (MeV) in plaque assays; and (E) overnight IFN- $\alpha$  inhibition of Enders mumps vaccine (MuV), PIV3 and PIV5 by immunofluorescent detection of viral protein. Scale bars 200 $\mu$ m, Patient = patient II.1.