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“A novel multigenic immunodeficiency affecting interferon mediated immunity”

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A Thesis Submitted to the Faculty of

The Harvard Medical School

in Partial Fulfillment of the Requirements

for the Degree of Master of Medical Sciences in Immunology

Harvard University

Boston, Massachusetts.

“A novel multigenic immunodeficiency affecting interferon mediated immunity”**ABSTRACT**

Primary immunodeficiencies are classically considered monogenic disorders characterized by host susceptibility to specific infectious pathogens. Patients with genetic defects in the IL-12/interferon gamma (IFN- γ) axis have susceptibility to weakly virulent mycobacteria, *Mycobacteria tuberculosis*, and *Salmonella*, but are resistant to the majority of other infectious pathogens. Host susceptibility to disseminated cytomegalovirus (CMV) infections typically results from genetic defects in T cell development or function. We studied a patient with disseminated *M. tuberculosis* and cytomegalovirus viremia. Whole exome sequencing identified two novel homozygous mutations affecting two distinct IFN pathways: the signal transducing chain of the IFN- γ receptor (*IFNGR2*) and the signaling chain of the IFN- α receptor (*IFNAR1*). The frameshift deletion in *IFNGR2* resulted in a truncated protein with significantly decreased protein expression and absent downstream signaling, as demonstrated by a lack of STAT1 phosphorylation and HLA-DR upregulation in response to IFN- γ stimulation. Human defects in *IFNAR1* have not been previously described. The patient's *IFNAR1* mutation replaced the protein's native stop codon with 46 novel C-terminal amino acids (*IFNAR1*^{*557Gluext*46}). The *IFNAR1*^{*557Gluext*46} mutant was expressed in patient fibroblasts at a level comparable to that of wild-type *IFNAR1* in control fibroblasts. However, the *IFNAR1*^{*557Gluext*46} mutant led to impaired STAT1/STAT2 phosphorylation and decreased nuclear translocation of STAT1 in response to IFN- α stimulation. Furthermore, the patient had impaired expression of the IFN- α stimulated genes, *IFIT1*, *IFIT2*, and *IRF7*, which are critical for host immunity to CMV. Patient and control fibroblasts were equally susceptible to CMV entry; however, cells expressing

IFNAR1^{*557Gluext*46} presented impaired expression of the ISG *IFIT2*, *IFN-β* and *IFI16* after infection with CMV. Moreover, pretreatment with IFN-α did not prevent CMV replication in patient fibroblasts compared to cells expressing the wild type IFNAR1. The patient's susceptibility to multiple types of infectious pathogens thus resulted from dual defects in Type I and Type II IFN signaling. These findings highlight the utility of whole exome sequencing in the discovery of multigenic primary immunodeficiencies.

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1. CHAPTER 1: BACKGROUND

1.1. INTRODUCTION

Innate immunity constitutes the first line of host defense against infectious organisms¹. The identification of monogenic defects in innate immunity has highlighted its crucial role in the normal immune response². The innate immune response is initiated after sensing pathogen using a limited number of germline encoded pathogen recognition receptors (PRRs) that recognize pathogen-associated molecular patterns shared amongst several classes of infectious agents³. During viral infections, the innate immune system recognizes viral proteins and nucleic acids using different PRRs, which include Toll-like receptors (TLRs), RIG-I-like receptors and DNA sensors like IFI16 and AIM2⁴. The activation of PRRs leads to signal transduction pathways that activate NF- κ B, the transcription factor ATF-2/c-Jun and interferon regulatory factors (IRF) 1, 3 and 7⁵, culminating in expression of genes important for host immunity, including those encoding for type I interferons.

Interferons (IFN), originally discovered by Isaacs and Lindenmann in 1957⁶, are proteins secreted by the host's cells upon infection. There are three families of IFNs: type I (IFN- α , IFN- β and IFN- ω), type II (IFN- γ), and type III (IFN- λ 1, IFN- λ 2 and IFN- λ 3). Each type of IFN has its specific receptor (IFNAR, IFNGR, IFNLR) and distinctive signaling pathway. Additionally, crosstalk exists among the IFN signaling pathways. Although the type I IFN receptor (IFNAR) signals primarily through heterodimers of STAT1 and STAT2 transcription factors, IFNAR also signals through STAT1 homodimers, which is a signaling pathway classically associated with the IFN- γ receptor (IFNGR)⁷. The crosstalk between the IFNR signaling pathways can also be initiated by the IFNGR, which can alternatively signal via STAT1 and STAT2 heterodimers⁸. The most widely studied function of the IFNs is their role in the protective response against viral infections by inducing the cellular antiviral response. Stimulation of the IFNAR and to some

extent the IFNGR, allow the initiation of the antiviral state in several cell types. Cross talk between the IFNAR and IFNGR signaling pathways allows IFN- γ to induce the expression of a limited number interferon stimulated genes (ISG); however, type I IFNs are the most potent inducers of the antiviral response and stimulate the expression of a bigger number of ISG⁹.

The genes coding for type I IFNs are clustered in one locus on the human chromosome 9 and include 13 genes coding IFN- α , 1 gene coding for IFN- β and 1 gene coding IFN- ω ¹⁰. Type I IFNs constitute a major component of the innate immune response against viral infections and are rapidly induced after sensing of viral proteins or nucleic acids by the PRRs. Type I IFNs produced during early phases of infection stimulate the IFNAR in both an autocrine and a paracrine fashion, activating the transcription of ISGs important for establishing antiviral defense and amplifying type I IFN production via a positive feedback mechanism¹¹.

The IFNAR is a complex consisting of two receptor subunits, IFNAR1 and IFNAR2¹², the receptor associated kinases Tyk2 and Jak1, and the IFNAR2 associated signaling protein STAT2¹³ (Schematic Figure 1). IFNAR1 is a transmembrane protein of 557 aminoacids coded by the *IFNAR1* gene, has low affinity for type I IFNs and acts as the signaling chain of the IFNAR¹⁴. IFNAR2 is a transmembrane protein with three described isoforms (IFNAR2c, IFNAR2b, sIFNAR2a)¹⁵, all of them encoded in a single *IFNAR2* gene in chromosome 21 and generated by means of exon skipping, alternative splicing and differential usage of polyadenylation sites. The functional isoform of the IFNAR2 protein, IFNAR2c, has high affinity for type I IFNs and acts as the ligand binding chain of the IFNAR, while IFNAR2b and sIFNAR2a have a modulatory function on the response to type I IFNs¹⁴.

Intracellular signaling downstream of the IFNAR is initiated upon binding of the type I IFNs, resulting in activation of the receptor associated kinases Jak1 and Tyk2 (Schematic Figure 1). These kinases phosphorylate the intracellular portion of IFNAR1, thereby creating a docking site for STAT2. STAT2 translocates from the IFNAR2 chain to the IFNAR1 chain and is

subsequently phosphorylated by Tyk2 and Jak1, which enables STAT1 binding (Schematic Figure 1). STAT1 is then activated by phosphorylation and causes the release of the phosphorylated STAT1/STAT2 heterodimer (pSTAT1/pSTAT2) from IFNAR1. pSTAT1/pSTAT2 binds to IRF-9 in the cytoplasm, forming a trimer known as IFN stimulated gene factor 3 (ISGF3), which then translocates to the nucleus and binds to the IFN stimulated response element (ISRE) within the promoter of ISGs to activate the transcription of hundreds of genes (Schematic Figure 1)^{13, 16}. The importance of the response to type I IFNs in controlling viral infections has been recently highlighted by the description of human mutations in the genes encoding IFNAR2 and STAT2. These patients exhibit complete abrogation of the response to type I IFNs and developed fatal infections with vaccine strain measles after inoculation with the live attenuated measles, mumps and rubella vaccine (MMR)^{17, 18}. These patients were not susceptible to other viruses, including cytomegalovirus (CMV), Epstein-Barr virus (EBV), varicella zoster virus (VZV), and multiple respiratory viruses, suggesting that the type I IFNs have a contribution to host immunity against specific viral pathogens.

IFN- γ is the sole member of the type II IFN family. IFN- γ is structurally unrelated to the type I IFN family and binds to its own receptor (IFNGR)¹⁰. In contrast to the type I IFNs, produced by most cell types, IFN- γ is exclusively produced by immune cells: CD4⁺ T cells, CD8⁺ T cells, NK cells, NKT cells, monocytes, macrophages and dendritic cells⁸. During the initial phases of infection, pathogen recognition by monocytes and macrophages induces the production of chemokines and IL-12. Recruited NK cells produce IFN- γ in response to IL-12 at site of infection and provide the initial source of IFN- γ during the innate phase of the immune response¹⁹, while T cells are the most important source of IFN- γ during the adaptive phase.

The IFNGR is a four-chain complex, composed of two ligand binding chains (IFNGR1) and two signal transducing chains (IFNGR2) (Schematic Figure 2). The IFNGR2 chain, while critical for signaling downstream of the IFNGR, does not bind directly to IFN- γ and is not

capable of initiating signaling in absence of IFNGR1^{20, 21}. The abundance of each chain varies within different cell types and with the activation state of the cell, but overall, the cell's responsiveness to IFN- γ is determined primarily by the surface expression of IFNGR2. Like the chains of the IFNAR, IFNGR1 and IFNGR2 lack intrinsic enzymatic activity and depend on the association with the tyrosine kinases Jak1 and Jak2 to initiate downstream signaling events⁸ (Schematic Figure 2).

Signaling downstream of the IFNGR begins after IFN- γ binds to the IFNGR1 chain (Schematic Figure 2). Subsequent auto-phosphorylation of Jak1 and trans-phosphorylation of Jak2 enables phosphorylation of critical cytoplasmic residues (YDHPK at amino acids 440-444) on both IFNGR1 chains, which serve as docking sites for STAT1⁸. After binding to IFNGR1, the recruited STAT1 proteins undergo phosphorylation at Tyr⁷⁰¹ and dissociate from the receptor as a pSTAT1 homodimer known as IFN- γ activated factor (GAF). The GAF translocates to the nucleus and binds to IFN- γ activation sites (GAS) within the promoter of IFN- γ regulated genes to activate or suppress transcription^{8, 22}. In addition to signaling through the canonical GAF pathway, the IFNGR can signal to a lesser extent via pSTAT1:pSTAT1:IRF-9 heterotrimers and ISGF3 complexes to induce the expression of ISGs⁸ (Schematic Figure 2).

Although the specific antiviral function of many ISGs have yet to be studied, the IFN-induced protein with tetratricopeptide repeats (IFIT) family of ISGs has been well characterized. The human IFIT family is composed of four genes: *IFIT1*, *IFIT2*, *IFIT3* and *IFIT5*, none of which are constitutively expressed²³. The presence of ISRE sequences in the promoter region of the IFIT genes enables rapid and robust expression in response to type I IFNs produced during the early stages of viral infections²⁴. All IFIT proteins are cytoplasmic and contain tetratricopeptide motifs that facilitate binding to host and viral proteins, thereby enabling them to block viral replication²⁵. IFIT1 and IFIT2 block the initiation of translation by binding to the eukaryotic initiator factor eIF3 and decrease viral replication by decreasing cellular translation itself²⁶.

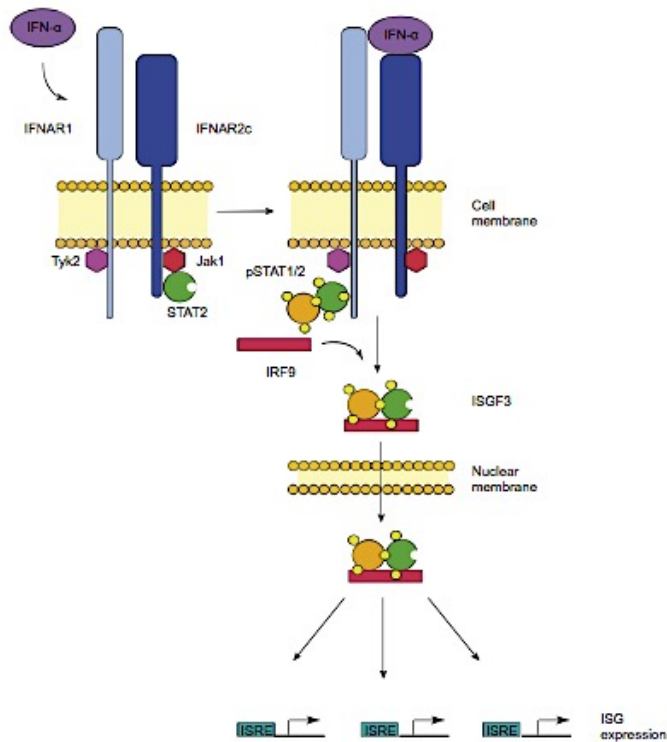
Besides decreasing cellular translation, IFIT1 blocks viral replication more specifically by interacting with viral proteins. For example, IFIT1 binds and sequesters the human papilloma virus helicase E1 in the cytoplasm and effectively reduces the viral replication process²⁷. The IFIT family of proteins also blocks viral replication by interacting with viral nucleic acids. During infection with negative stranded RNA viruses, IFIT1 recognizes and binds to the virus uncapped 5' triphosphate RNA and provides a scaffold for IFIT2 and IFIT3 to form a complex that sequesters viral RNA and decreases the pool of actively replicating viral nucleic acids²⁸. Due to its important role in the antiviral response and their upregulation upon IFNAR signaling, expression of the IFIT genes is commonly used to evaluate the cellular response to exogenous type I IFNs²⁹ or viral infections³⁰.

The description of Mendelian susceptibility to mycobacterial diseases (MSMD), a group of monogenic PID affecting the IL-12/IFN- γ axis, has demonstrated the importance of type II IFNs in host immunity. Mutations underlying MSMD have been found in six genes (*IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1* and *NEMO*) and result in thirteen disorders that differ in their inheritance pattern, levels of protein expression and overall functionality of the IL-12/IFN- γ axis^{31, 32}. MSMD patients can be further classified based on the cellular response to IFN- γ stimulation, as having complete (no response) or partial (residual response) forms of the disease³³. Patients with MSMD are predisposed to infections with weakly virulent mycobacteria, such as *Mycobacterium bovis* in the Bacillus Calmette-Guérin (BCG) vaccine, as well as *Mycobacterium tuberculosis* and *Salmonella*³³. Patients with defects in the IL-12/IFN- γ axis are usually resistant to infections caused by most other infectious pathogens. However, individual case reports have identified patients with additional concomitant infections, including listeriosis³⁴, klebsiellosis³⁵, nocardiosis^{36, 37}, histoplasmosis³⁸, paracoccidioidomycosis³⁹, VZV and CMV⁴⁰. It is not known if these atypical infections occurred because of an additional role of the IL-12/IFN- γ axis in host defense against these organisms, or from patient-specific genetic

variations affecting other mechanisms of host immunity. We here present a patient who presented with disseminated mycobacterial, streptococcal and cytomegalovirus infections resulting in secondary hemophagocytic lymphohistiocytosis. Using whole exome sequencing (WES) we have identified two novel pathogenic mutations that affect the *IFNGR2* and the *IFNAR1* genes in this patient. To our knowledge, this is the first description of an immunodeficiency affecting the response to both type I IFNs and IFN- γ , as well as the first description of a pathogenic mutation affecting the *IFNAR1* gene in humans. Our studies highlight the utility of WES in the discovery of multigenic primary immunodeficiencies.

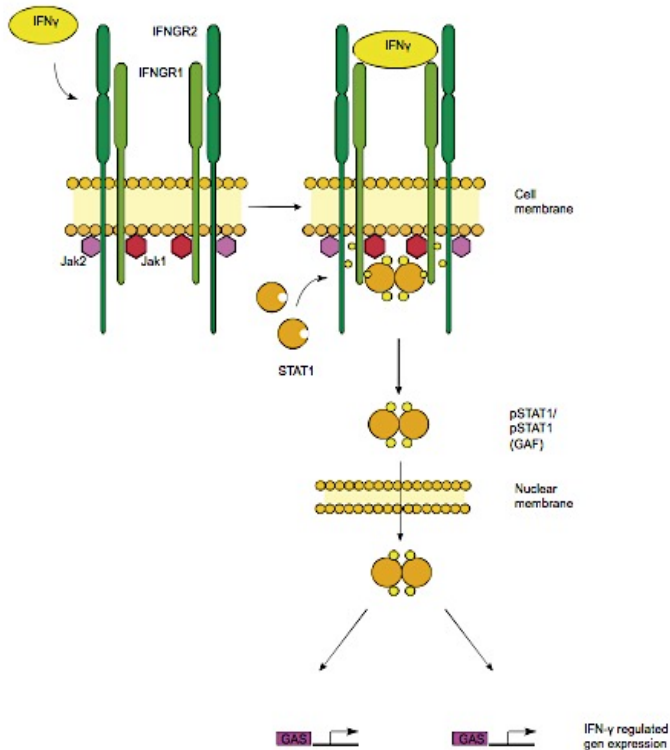
1.2. SCHEMATIC FIGURES

Schematic Figure 1. Model of the IFNAR signaling pathway.



The IFNAR is composed of two chains, IFNAR1 and IFNAR2c. Upon IFN α binding to the receptor, the receptor pre-associated kinases Tyk2 and Jak1 phosphorylate the intracellular domains of the receptor creating a docking site for STAT2 at the IFNAR1 chain. STAT2 is phosphorylated and recruits STAT1, STAT1 is activated by phosphorylation and the pSTAT1/pSTAT2 dimer is released to the cytoplasm where binds with IRF9 to form the IFN stimulated gene factor 3 (ISGF3) complex. ISGF3 translocates to the nucleus and binds to the IFN stimulated response elements (ISRE) sequences in the promoter region of ISG and activates gene expression.

Schematic Figure 2. Model of the IFNGR signaling pathway.



The IFNGR is composed of two chains, IFNGR1 and IFNGR2. Upon binding of IFN γ to the IFNGR1 chain, the kinases Jak1 and Jak2 activate each other by cross-phosphorylation. The kinases then phosphorylate the intracellular domain of the IFNGR1 chain creating a docking site for STAT1. STAT1 binds to Tyr457 of each IFNGR1 and is phosphorylated by the Jak kinases. The pSTAT1 proteins form a dimer known as gamma activating factor (GAF) and are released from the receptor. The GAF then translocates to the nucleus and binds to IFN γ activation sites (GAS) in the promoter region of IFN γ regulated genes to induce gene expression.

2. CHAPTER 2: METHODS AND DATA

2.1. INTRODUCTION

We studied a two months old boy presenting with simultaneous disseminated *Mycobacterium tuberculosis*, CMV and *Streptococcus viridans* infection causing secondary HLH. Due to the simultaneous isolation of multiple infectious agents and the atypical development of secondary HLH, this patient was thought to have an underlying primary immunodeficiency. To perform genetic studies in this patient, blood samples were obtained and prepared for DNA isolation and WES analysis. To functionally prove the pathogenic nature of the mutations found by WES, a skin biopsy was obtained to establish a fibroblast cell line. Immunoblotting, flow cytometry and qt-PCR techniques were used to evaluate the cellular response to IFN stimulation. To further test the cell's anti-viral response, mutant and control skin derived fibroblasts were subjected to infection with CMV.

2.2. MATERIALS AND METHODS

Study participants

The proband and his parents were enrolled in this study. After informed consent, peripheral blood was obtained from the study participants; a skin biopsy was obtained from the proband to generate a fibroblast cell line. All studies performed on blood or skin derived fibroblasts from the study participants were approved by Boston Children's Hospital Institutional Review Board (Protocol 04-09-113R).

Genetic analysis

Genomic DNA was isolated from peripheral blood samples of the proband using the Gentra Puregene Blood Kit (Qiagen) as per kit protocol. WES was performed by Illumina HiSeq-2000 (Illumina Inc., San Diego, CA) on the proband. The paired-end Illumina libraries were captured in solution according to the Agilent SureSelect protocol. Average coverage of the exome was 150x. The Burrows-Wheeler Aligner (BWA) was used to map reads to the human reference genome assembly GRCh37⁴¹. Variants were called using the Genome Analysis Toolkit (GATK)⁴², Sam Tools^{43, 44} and Picard tools (<http://picard.sourceforge.net>)

Sanger sequencing

Sanger sequencing was used to validate the mutations in the *IFNGR2* and *IFNAR1* genes identified on the proband by WES and to identify the carrier status of the parents. Two pairs of gene specific nested primers were used to cover the mutation site in each gene: *IFNGR2* (F1: 5'-GTGAGAAGAGTGCTGAACTG-3'; F2: 5'-GTGCGTAGGAAGATCATTCT-3'; R1: 5'-ACCATTAAGATGTCTGCGTG-3'; R2: 5'-AGAGATTGTACCATGACACT-3'), *IFNAR1* (F1: 5'-TAGTATTTCTCTGAACAGCCAT-3'; F2: 5'-TCAACTTCTGAGGAACAAATCG-3'; R1: 5'-TGACTCATCTCATCCAATGC-3'; R2: 5'-GACCTATGATCTGAAGATGT-3').

cDNA sequencing

RNA from skin derived fibroblasts was extracted using the RNeasy Mini Kit (Qiagen) and was reverse-transcribed with the OneStep RT-PCR kit (Qiagen) as per kit protocol. cDNA was sequenced with nested *IFNGR2*^{C266fs} (F1: 5'-CTTTGACATCGCTGATACCTC-3'; F2: 5'-CTCCAGAGTGTAAGTGTGTTTACA-3'; R1: 5'-AAGCGTTTGGAGAACATCTTCT-3'; R2: 5'-

GAACATCTTCTTGCTCCTTTT-3') or IFNAR1^{*557next*46} specific primers (F1: 5'-TTCTCTGAACAGCCATTGAAGAA-3'; F2: 5'-GCTACAGTAGAAGAACTAAT-3'; R1: 5'-TTAGGACCTATGATCTGAAGATGT-3'; R2: 5'-TCTGACTCATCTCATCCAATGC-3').

Cell-culture and stimulation conditions

Fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum, 50,000IU penicillin, 50,000µg streptomycin, 10µM HEPES, and 2mM Glutamine. PBMCs were isolated using Ficoll solution then were cultured in RPMI medium supplemented with 10% fetal bovine serum, 50,000IU penicillin, 50,000µg streptomycin, 10µM HEPES, and 2mM Glutamine overnight. Cells were stimulated with IFN-α or IFN-γ (R&D systems) 1000IU/mL for the described time points.

Fibroblasts were seeded at a density of 1×10^5 cells/well in 6 well plates and 48 hours later were stimulated with IFN-α or IFN-γ. For immunoblotting experiments, cells were stimulated for 15, 30 or 60 minutes with the corresponding IFN. For subcellular fractionation and gene expression experiments, cells were stimulated for 8, 16 or 24h with IFN-α.

Immunoblotting

Cultured skin fibroblasts were homogenized in PBS that contains 30mM Tris-HCl pH 7.5, 120mM NaCl, 2mM KCl, 1% Triton X-100 and 2mM EDTA supplemented with protease and phosphatase inhibitors (Complete and PhoStop, Roche). Proteins were separated by electrophoresis on 4-15% precast polyacrylamide gels (Bio-Rad) and were transferred to 0.45µm nitrocellulose membrane (Bio-Rad). Membranes were blocked in a 1x solution Tris-Buffered Saline/Tween 20 (TBST) with 5% (w/v) nonfat dry milk for one hour at room temperature and then incubated overnight at 4°C with purified rabbit polyclonal antibody against

pSTAT2-Tyr690 (Cell signaling, 4441), pSTAT1-Tyr701 (Cell signaling, 9167) diluted 1:1000 or 1:2000, respectively, in 1x TBST with 5% BSA. Immunoblot detection of IFNAR1 was done with a purified rabbit polyclonal antibody (Bethyl, A304-289A) diluted 1:800 in 1x TBST with 5% milk. As a loading control, membranes were re-probed with a purified mouse monoclonal antibody against β -actin (Abcam, ab3280) diluted 1:20000 in 1x TBST with 5% milk or a purified polyclonal rabbit antibody against STAT1 (Cell signaling, 14995) or STAT2 (Cell signaling, 4594) diluted 1:1000 in 1x TBST with 5% BSA. Antigen-antibody complexes were visualized with anti-rabbit or anti-mouse secondary antibodies respectively (GE Healthcare). Quantification of the signal intensities of pSTAT1, pSTAT2, STAT1, STAT2, was done using the ImageJ analyzer software (1.48v).

Subcellular fractionation

To assess pSTAT1 nuclear translocation after 8 or 24h of stimulation with IFN- α , cells were subjected to subcellular fractionation using a Nuclear Fractionation Kit (Active motif) as per kit protocol. pSTAT1 nuclear translocation was determined by immunoblotting and quantified as previously described. As loading controls for each subcellular fraction, membranes were probed with a polyclonal antibody against Rab5 (Cell signaling, 2143) diluted 1:400 in 1X TBST with 5% milk or PARP (Cell signaling, 9532) diluted 1:200 In 1X TBST with 5% milk for the cytoplasmic and nuclear fraction, respectively.

Gene expression analysis

After 8, 16 or 24h of stimulation with IFN- α (1000IU/ml), RNA was extracted from skin derived fibroblasts using the RNeasy Mini Kit (Qiagen) and was reverse-transcribed with the iScript cDNA synthesis kit (BioRad). To measure gene expression after IFN- α stimulation,

expression of the ISGs *IFIT1* (Hs00356631_g1), *IFIT2* (Hs01922738_s1) and *IRF7* (Hs01014809_g1) and the housekeeping gene *GUSB* (Hs00939627_m1 Life Technologies, USA) was measured. Results were analyzed using the $2^{-\Delta\Delta CT}$ method.

For gene expression analysis following viral infections, total RNA was harvested from mock-, HSV-1 d109 or HCMV-infected cells using the RNeasy kit (Quiagen) and DNase treated (DNA-free, Ambion) according to the manufacturer's instructions. RNA (500 ng) was reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies), and cDNA was analyzed by real-time PCR (Step One Plus, Applied Biosystems) using Power SYBR green mix. Samples were normalized to cellular 18S RNA levels. For PCR using DNA, viral DNA copy numbers were normalized to a cellular GAPDH pseudogene. The primers used in this study are as follows: Human IFN β : (Fp: 5'-AAACTCATGAGCAGTCTGCA-3'; Rp: 5'-AGGAGATCTTCAGTTTCGGAGG-3'), Human IFIT2 (Fp: 5'-ACGGTATGCTTGGAACGATTG-3'; Rp: 5'-AACCCAGAGTGTGGCTGATG-3'), 18S RNA (Fp: 5'-GCATTCGTATTGCGCCGCTA-3'; Rp: 5'-AGCTGCCCGGCGGGT-3'), GAPDH (Fp: 5'-CAGGCGCCCAATACGACCAAATC-3'; Rp: 5'-TTCGACAGTCAGCCGCATCTTCTT-3'), Human IFI16 (Fp: 5'-ACTGAGTACAACAAAGCCATTTGA-3'; Rp: 5'-TTGTGACATTGTCCTGTCCCCAC-3'), IE1 (Fp: 5'-CAAGTGACCGAGGATTGCAA-3'; Rp: 5'-CACCATGTCCACTCGAACCTT-3'), CMVexon4 (Fp: 5'-GATAGTCGCGGGTACAGGGGACTCT-3'; Rp: 5'-GGTCACTAGTGACGCTTGTATGATGACCATCTACGG-3')

Fluorescence-activated cell sorting (FACS) analysis

Standard flow cytometric methods were used for staining of cell-surface proteins. Anti-human mAbs to the following molecules with the appropriate isotype-matched controls were used for staining: CD14 (Biolegend, 325606), HLA-DR (Biolegend, 307617) and IFNGR2 (R&D systems, FAB773). IFNGR2 studies were done on PBMCs. For HLA-DR upregulation studies,

fibroblasts were cultured at a density of 1×10^5 cells/well in 6 well plates and stimulated with IFN- γ 1000IU/mL for 4 days before staining. For STAT1 phosphorylation studies, fibroblasts were cultured at a density of 1×10^5 cells/well in 6 well plates and stimulated with IFN- α 1000IU/mL for 1, 3, 5, 15, 30, 45 or 60 minutes. For intracellular staining experiments, BD Phosflow Lyse/Fix Buffer and Phosflow Perm Buffer III (BD biosciences) were used as per kit protocol, an anti-human mAb to pSTAT1 (Cell signal, 9174) with the appropriate isotype-matched control was used for staining.

All flow cytometry data was collected with an LSRFortessa (BD Biosciences, San Jose, Calif) cell analyzer and analyzed with FlowJo software (Tree Star, Ashland, Ore).

Viral infection

The HCMV laboratory strain AD169 was obtained from the NIH AIDS Reagent Program, and was propagated in human foreskin fibroblasts (HFF) as described⁴⁵. The HSV-1 replication-defective virus *d109* lacking expression of immediate-early genes was grown and titrated as previously described⁴⁶. For infections, viruses were diluted in PBS containing 1% BCS and 0.1% glucose and incubated with cells for 1.5 h on a 37°C shaker. Cells were infected at a multiplicity of infection (MOI) of 1. After viral adsorption, the cells were washed with PBS and overlaid with DMEM supplemented with 10% FBS. For viral entry studies, cells were infected at a MOI of 10 and DNA was isolated immediately after viral adsorption.

Immunofluorescence

Fibroblasts grown on glass coverslips were mock- or HCMV-infected and fixed with 2% formaldehyde at the indicated times post infection. Coverslips were permeabilized with 0.5% NP-40 for 10 min and blocked in DMEM containing 5% normal goat serum for 1 h at room temperature. The coverslips were incubated with mouse anti-IE antibody (1:500, Millipore)

followed by a secondary anti-mouse Alexa-488 antibody (1:1000; Jackson ImmunoResearch). Nuclei were counterstained with DAPI. Cells were imaged using an Axioplan 2 microscope (Zeiss) at 40x magnification.

Statistical analysis

All data is presented as mean \pm S.E.M, and compared using the unpaired t-test or two-way ANOVA. Statistical analysis was performed using GraphPad Prism software (version 6.0).

2.3. RESULTS

Clinical presentation and immunologic phenotyping

The proband is the son of consanguineous parents of Saudi Arabian ancestry. There was no family history of PID. Healthy until two months of age, when he presented with a two-week history of poor feeding, lethargy and fever. His blood culture was positive for *Streptococcus viridans*. He developed splenomegaly, lymphadenopathy, thrombocytopenia, anemia, and elevated ferritin levels consistent with secondary HLH (Supplementary Table 1). Histologic evaluation of his bone marrow revealed hemophagocytosis. Cultures of his bone marrow and lymph nodes were positive for *Mycobacterium tuberculosis*. At this time, he was also found to have CMV viremia (4,000,000 copies/mL). Immunological evaluation was normal (Table 1) and HIV PCR was negative. Sanger sequencing of genes associated with familial HLH (*PRF1*, *UNC13D*, *STX11*, *STXBP2*) was normal. He was treated with dexamethasone and cyclosporine for HLH and gancyclovir for CMV viremia. His mycobacterial infection was treated with isoniazid, rifampin, clarithromycin, ciprofloxacin and amikacin. The patient has been healthy on prophylactic anti-tuberculosis medications and has received attenuated viral vaccines without development of vaccine-associated diseases.

Table 1. Immune profile of the patient

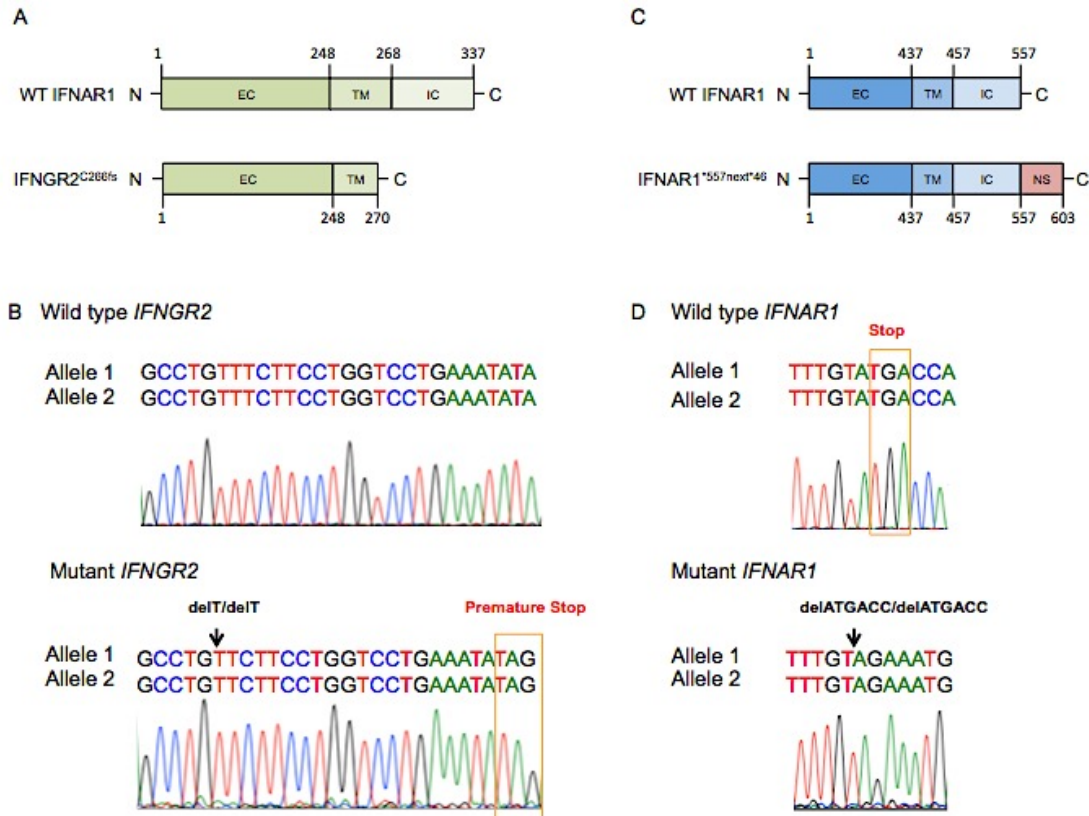
Lymphocyte subsets, cells/mL (normal range for age) ⁴⁷	
CD3 ⁺	5,250 (2,500 – 5,500)
CD3 ⁺ CD4 ⁺	1,180 (1,600-4,000)
CD3 ⁺ CD8 ⁺	3,610 (560 – 1,700)
CD4 ⁺ /CD8 ⁺	0.32
CD19 ⁺	2,390 (300 – 2,000)
CD19 ⁺ HLA-DR ⁺	100%
CD16 ⁺ /CD56 ⁺	1,100 (170 – 1100)
Immunoglobulins, mg/dL (normal range) ⁴⁸	
IgG	670 (206 – 601)
IgA	40 (2.8 – 47)
IgM	90 (17-105)
Lymphocyte proliferation (% of normal control)	
Phytohaemagglutinin	76
Concavalin A	36
Pokeweed	143

Identification of homozygous mutations in *IFNGR2* and *IFNAR1*

Due to the consanguinity of the proband's parents, we anticipated an autosomal recessive cause of immunodeficiency. WES of the proband revealed 37 homozygous variants (Supplementary Table 2), which were not found in multiple public databases or our own in-house database of exomes.

A homozygous one base pair deletion in *IFNGR2* (*IFNGR2*^{C266fs}, NM_005534:c.798delT:p.C266fs) was considered a plausible causative variant due to the known contribution of IFN- γ in host defense against mycobacteria. *IFNGR2*^{C266fs} is predicted to cause a frameshift followed by premature truncation of the protein after residue 270, which resides in the intracellular domain of *IFNGR2* (Figure 1A). Additionally, a homozygous mutation in the *IFNAR1* gene (*IFNAR1*^{*557Gluext*46}, NM_000629:c.1671_1821del: p.*557Gluext*46) was also identified in this patient. The *IFNAR1*^{*557Gluext*46} mutation is predicted to disrupt the protein's stop codon, leading to the addition of 46 novel aminoacids to the protein's C terminus (Figure 1C). Sanger sequencing of genomic DNA confirmed that both mutations were present in the homozygous state in the proband and in the heterozygous state in both parents (Figure 1 and Supplementary Figure 1).

Figure 1. Predicted effect in protein structure and Sanger sequencing of the *IFNGR2* and *IFNAR1* mutations.



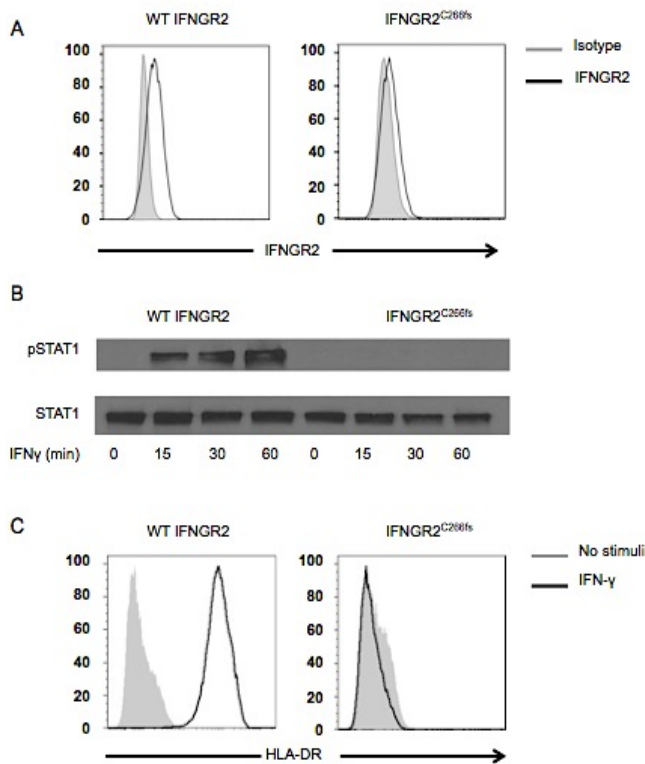
A, Schematic of wild type (WT) and mutant (*IFNGR2*^{C266fs}) *IFNGR2*. The *IFNGR2*^{C266fs} protein is truncated at residue 270. B, Sanger sequencing demonstrating the *IFNGR2* mutation (798delT) in the homozygous state in the patient. Sequencing of a control subject is included. C, Schematic of wild type (WT) and mutant (*IFNAR1*^{*557next*46}) *IFNAR1* protein. The *IFNAR1*^{*557next*46} protein has 46 extra aminoacids at the protein C terminus. D, Sanger sequencing demonstrating the *IFNAR1* mutation (1671_1821del) in the homozygous state in the patient. Sequencing of a control subject is included. EC = extracellular domain, TM = transmembrane domain, IC = intracellular domain, NS = novel sequence.

Impaired response to IFN- γ in fibroblasts expressing IFNGR2^{C266fs}

The intracellular domain of IFNGR2 contains the binding site for the kinase Jak2. The association of IFNGR2 and Jak2 is critical for intracellular signaling downstream of the receptor after IFN- γ binding to the IFNGR^{20, 21}. The premature stop codon in IFNGR2^{C266fs} would result in a mutant protein lacking the binding site for Jak2. This was confirmed by Sanger sequencing of cDNA generated by RT-PCR of mRNA from the patient's fibroblasts (Supplementary Figure 2A). We next evaluated the effect of IFNGR2^{C266fs} on protein expression. IFNGR2 surface expression was evaluated by flow cytometric analysis using an antibody specific to the protein's N-terminus. Monocytes from the proband exhibited severely decreased expression of the mutant IFNGR2^{C266fs} protein compared to expression of the native protein on control monocytes (Figure 2A). We aimed to confirm the effect of the *IFNGR2*^{C266fs} mutation at the protein level by immunoblotting, however, analysis of the IFNGR2 expression on fibroblasts was not possible using commercially available antibodies directed against the protein N terminus.

Stimulation of the IFNGR complex with IFN- γ leads to phosphorylation of STAT2 and upregulation of the MHC class II protein HLA-DR on fibroblasts. Patient fibroblasts had complete abrogation of STAT1 phosphorylation and no upregulation of HLA-DR after IFN- γ stimulation (Figure 2B and 2C). This data demonstrates that the 67 C-terminal residues in IFNGR2 are essential for receptor signaling.

Figure 2. Analysis of the expression and functionality of the mutant IFNGR2^{C266fs} protein.

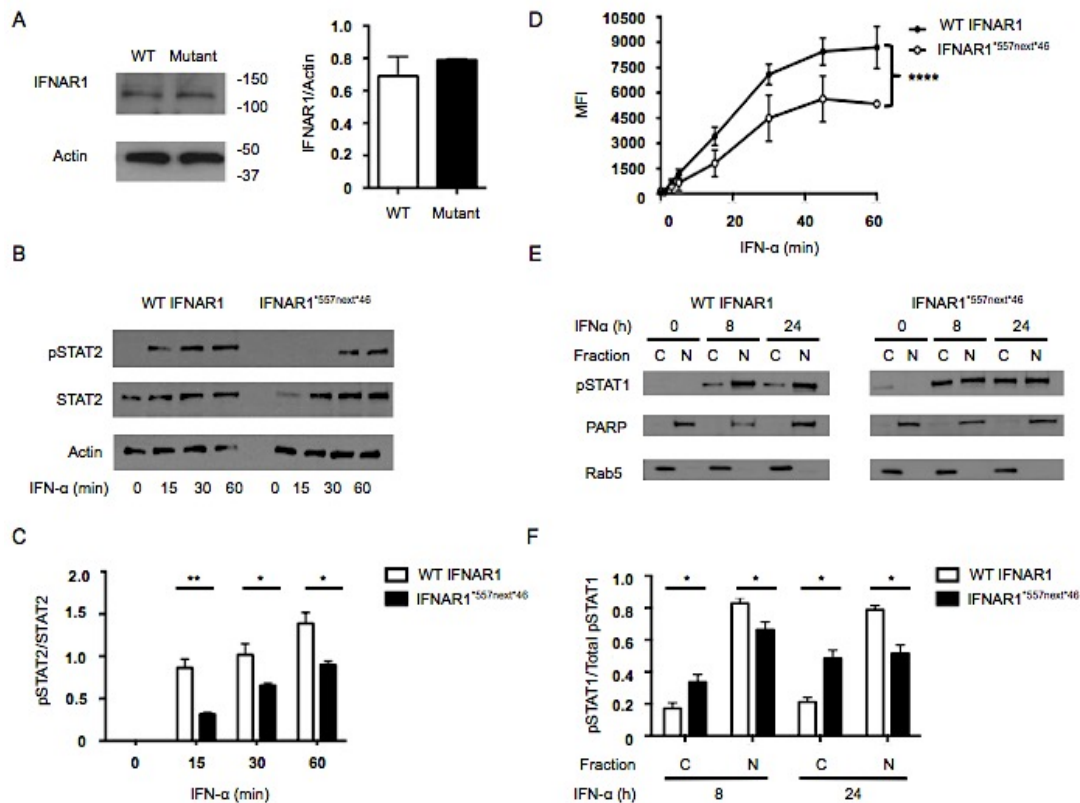


A, Flow cytometric analysis of the wild type and mutant IFNGR2 protein expression at the surface of CD14⁺ cells from a healthy control (WT IFNGR2) and the proband (IFNGR2^{C266fs}). B, Immunoblot analysis of STAT1 phosphorylation after IFN- γ stimulation in lysates of skin derived fibroblasts from the proband and healthy controls. C, Flow cytometric analysis of the HLA-DR expression at the cell surface of skin derived fibroblasts from the proband and healthy control after IFN- γ stimulation.

***IFNAR1*^{*557Gluext*46} preserves protein expression and adds a novel 46 amino acid sequence to the C-terminus of IFNAR1**

The proband's history of disseminated CMV is not typical for a defect in IFNGR, since CMV infections have been previously reported in only three patients with mutations affecting the IL-12/IFN- γ axis. In addition to the mutation in *IFNGR2*, the proband we present also had a novel mutation in *IFNAR1*. *IFNAR1* functions as the signaling chain of the IFNAR and is required for responsiveness to type I IFNs. Due to the known importance of the type I IFNs in controlling viral infections, we investigated the effect of the patient's *IFNAR1*^{*557Gluext*46} on *IFNAR1* expression and signaling. The *IFNAR1*^{*557Gluext*46} mutation is predicted to disrupt the native stop codon and add 46 novel C-terminal amino acids with a molecular weight of an additional 5 kilo Daltons, while otherwise preserving the normal *IFNAR1* protein sequence. Immunoblotting of fibroblast lysates from the patient using an antibody targeting aminoacids 450-500 of *IFNAR1* C-terminus, revealed expression of *IFNAR1*^{*557Gluext*46} protein at a level comparable with that of WT *IFNAR1* in control fibroblasts (Figure 3A). Since the increase of 5 kilo Daltons in the molecular weight of the mutant *IFNAR1* was not distinguishable on immunoblotting, the sequence of the novel 46 C-terminal amino acids was confirmed by Sanger sequencing cDNA generated from mRNA isolated from the patient's fibroblasts (Supplementary Figure 2B).

Figure 3. Analysis of the expression and functionality of the mutant IFNAR1^{*557next*46} protein.



A, Immunoblot analysis of IFNAR1 in lysates of skin derived fibroblasts from the proband (Mutant) and healthy controls (WT). B, Immunoblot analysis of STAT1 phosphorylation after IFN α stimulation in lysates of skin derived fibroblasts from the proband (IFNAR1^{*557next*46}) and healthy controls (WT IFNAR1). C, Quantitative analysis of STAT2 phosphorylation. * P < 0.05; ** P < 0.01 by unpaired t test. D, Flow cytometric analysis of STAT1 phosphorylation after IFN α stimulation in skin derived fibroblasts from the proband and healthy controls. The means and SEs of three independent experiments are shown. **** P < 0.0001 by two way ANOVA. E, Immunoblot analysis of pSTAT1 nuclear translocation after IFN α stimulation of skin derived fibroblasts from the proband and healthy controls. After IFN α stimulation, fibroblasts were fractionated into cytoplasmic (C) and nuclear (N) fractions.

Figure 3 (Continued) F, Quantitative analysis of pSTAT1 nuclear translocation. pSTAT1 density in each fraction was normalized to the corresponding loading control and added to obtain total pSTAT1. pSTAT1/Total pSTAT1 represents pSTAT1 distribution between the C and N fractions. Columns and bars represent means and SEMs and are representative of three experiments. * P <0.05 by unpaired t test.

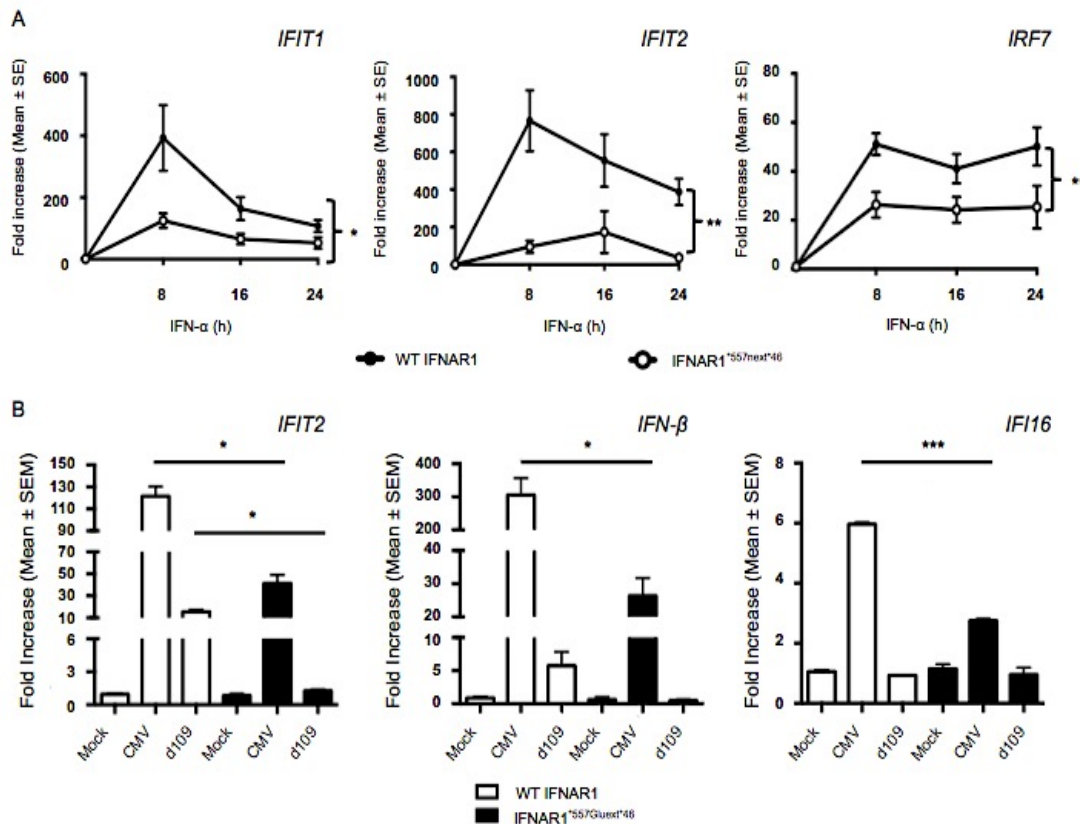
Defective intracellular signaling downstream of *IFNAR1*^{*557Gluext*46}

Binding of IFN- α to the IFNAR complex leads to STAT2 activation and subsequent STAT1 phosphorylation. Assembly of the pSTAT1/pSTAT2 heterodimer triggers a cascade of intracellular signaling events that ultimately induce the ISGs necessary for controlling viral replication⁷ and spread⁴⁹. We first investigated the patient's cellular response to IFN- α by quantifying STAT2 and STAT1 phosphorylation downstream of IFN- α stimulation in fibroblasts. Although the mutant *IFNAR1*^{*557Gluext*46} protein preserves all necessary docking sites required for downstream intracellular signaling events, STAT2 phosphorylation, as quantified by immunoblotting, was significantly decreased after IFN- α stimulation of the patient's fibroblasts (Figure 3B-C). Flow cytometric analysis of STAT1 phosphorylation was also decreased after IFN- α stimulation of the patient's fibroblasts (Figure 3D), as would be expected in the setting of impaired STAT2 phosphorylation. After phosphorylation of receptor associated STAT1 and STAT2, the pSTAT1/pSTAT2 heterodimer is released to the cytoplasm and associates with IRF-9 to form ISGF3 complexes that translocate to the nucleus. Since after IFN- α stimulation, pSTAT1 exists in the nuclear compartment mostly as part of the ISGF3 complex, we assessed the subcellular distribution of pSTAT1 after IFN- α stimulation in patient and control fibroblasts. Compared to controls, an increased proportion of pSTAT1 was retained in the cytoplasm of patient fibroblasts 8 and 24 hours after IFN- α stimulation (Figures 3E-F), suggesting that the presence of the additional 46 amino acids at the C terminus in *IFNAR1*^{*557Gluext*46} stabilizes the interaction between the receptor and the pSTAT1/pSTAT2 dimer. Collectively, these data indicate that the novel 46 amino acids in the mutant *IFNAR1*^{*557Gluext*46} receptor result in impaired phosphorylation of STAT1 and STAT2 as well as defective nuclear translocation of pSTAT1/pSTAT2 heterodimers.

Impaired ISG upregulation downstream of *IFNAR1*^{*557Gluext*46}

Nuclear translocation of the STAT1/2 heterodimer is followed by the expression of genes important for the anti-viral response. Some ISGs like *IFIT1* and *IFIT2* are important for blocking viral replication by decreasing cellular translation and interacting directly with viral proteins, while other ISGs like *IRF7* are important for amplifying type I IFNs production or pathogen recognition like IFI16. Since the proband presented with disseminated CMV, we evaluated the expression of *IFIT1*, *IFIT2*, and *IRF7* after IFN- α stimulation of patient and control fibroblasts. Induction of *IFIT1*, *IFIT2*, and *IRF7* was significantly decreased in the patient's fibroblasts compared to control cells (Figure 4A). Notably, the expression of *IFIT1* and *IFIT2* is exclusively controlled by type I IFNs²⁴ and fibroblasts do not secrete IFN- γ . Therefore, these data demonstrate that the mutant *IFNAR1*^{*557Gluext*46} receptor significantly impairs the induction of genes critical for control of viral expansion and that the patient's *IFNGR2*^{C266fs} mutation does not contribute to the diminished expression of *IFIT1*, *IFIT2*, and *IRF7* after IFN- α stimulation.

Figure 4. IFNAR1^{*557next*46} results in decreased expression of ISG after IFN α stimulation and viral infection.



A, Skin derived fibroblasts from the proband (IFNAR1^{*557next*46}) or healthy controls (WT IFNAR1) were stimulated with IFN α and ISG expression was evaluated by qt-PCR. The means and SEs of three independent experiments are shown. * P < 0.05; ** P < 0.01 by two way ANOVA. B, Skin derived fibroblasts from the proband (IFNAR1^{*557next*46}) or healthy controls (WT IFNAR1) were infected with CMV or HSV-1 d109 for 4 hours after which ISG expression was evaluated by qt-PCR. * P < 0.05; *** P < 0.001

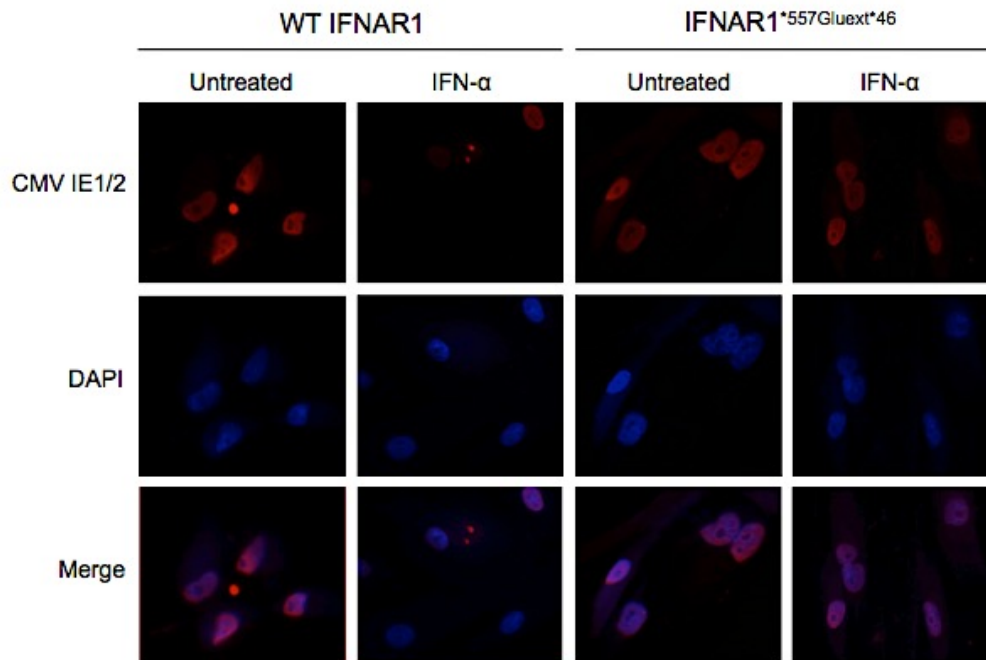
Impaired upregulation of ISGs after CMV infection and defective anti-viral state in fibroblasts expressing *IFNAR1*^{*557Gluext*46} (*)

The expression of ISGs is important for establishing the cellular anti-viral response and blocking the viral replication and protein expression. We have shown that upregulation of ISGs is diminished in cells expressing *IFNAR1*^{*557Gluext*46} after stimulation with IFN- α . To prove the biological significance of these findings, we next evaluated the upregulation of ISGs after infection with CMV or the Herpes simplex virus-1 (HSV-1) d109, a potent inducer of the type I IFN response. Induction of the ISGs *IFIT2*, *IFN- β* and *IFI16* was significantly decreased in fibroblasts expressing *IFNAR1*^{*557Gluext*46} compared to control cells after infection (Figure 4B). The decreased upregulation of ISGs observed after infection with CMV or HSV-1 d109 is not explained by different expression of PRRs important for sensing infection with herpes viruses or differences in viral entry, as shown by similar baseline expression of the DNA sensor *IFI16* and quantification of the CMV *IE1* gene after viral adsorption (Supplementary Figure 3A and B).

We next evaluated the effectiveness of the anti-viral state induced by type I IFNs to block CMV replication in patient and control fibroblasts. Pre-treatment with IFN- α completely abrogated the expression of the CMV derived proteins IE1 and IE2, a maker of viral replication, in control fibroblasts while it had no effect in fibroblasts expressing *IFNAR1*^{*557Gluext*46} (Figure 5). This data demonstrate that the dampened response to type I IFNs as a result of *IFNAR1*^{*557Gluext*46} is insufficient to control CMV replication *in vitro* and contributes to the patient increased susceptibility to the virus.

(*) This data was kindly generated by Dr. David M. Knipe and Maggie Angelova, PhD.

Figure 5. Proband derived fibroblasts are unable to control CMV replication after IFN- α pre-treatment.



Proband (IFNAR1^{*557next*46}) and healthy control (WT IFNAR1) derived fibroblasts were stimulated with IFN- α for 12 hours prior to infection with CMV. Expression of the CMV IE1 and IE2 proteins was evaluated by immunofluorescence 16 hours post infection.

2.4. DISCUSSION

We report a novel multigenic cause of primary immunodeficiency affecting the cellular response to IFNs. Using WES we identified two candidate homozygous mutations affecting the *IFNGR2* and the *IFNAR1* genes. The *IFNGR2*^{C266fs} mutation causes the expression of a truncated protein that lacks the intracellular domains necessary for signalling downstream of the receptor and abrogates the cellular response to IFN- γ . The *IFNAR1*^{*557Gluext*46} mutation adds 46 novel amino acids to the C-terminus of IFNAR1. The presence of these extra 46 amino acids decreases phosphorylation of STAT1 and STAT2 and nuclear translocation of pSTAT1/pSTAT2 dimers, significantly decreasing the expression of ISGs after stimulation with IFN- α and infection with CMV and the effectiveness of the anti-viral state in blocking viral replication. These results confirm the pathogenic nature of both candidate mutations and their role on the proband's susceptibility to infection with *Mycobacterium tuberculosis* and CMV.

Supplementary Table 1. HLH diagnostic criteria in the patient.

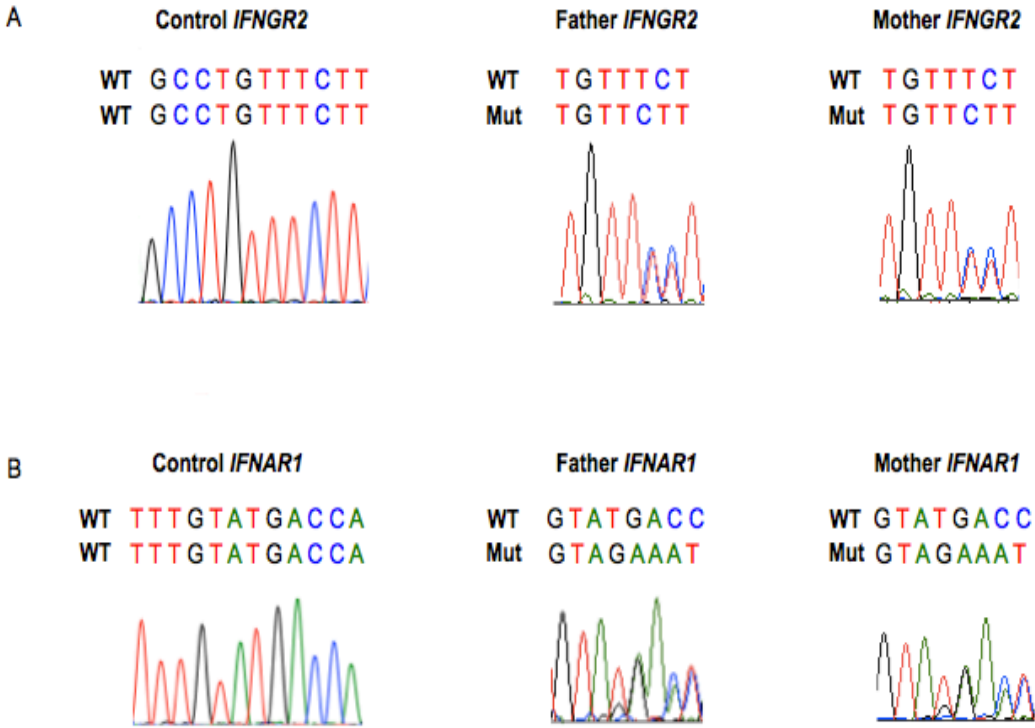
Diagnostic criteria for HLH (2004)⁵⁰	Findings in Patient
Fever	Present
Splenomegaly	Present
Cytopenias	
Hemoglobin < 90 g/L	9.6g/L
Platelets < 100 x 10 ⁹ /L	21.5 x 10 ⁹ /L
Neutrophils < 1 x 10 ⁹ /L	9.66 x 10 ⁹ /L
Hypertriglyceridemia and/or hypofibrinogenemia	
Triglycerides ≥ 3.0 mmol/L	7.5mmol/L
Fibrinogen ≤ 1.5 g/L	1.5 g/L
Hemophagocytosis in bone marrow, spleen or lymph nodes	Present
Low or absent NK cell activity	Not analyzed
Ferritin ≥ 500 µg/L	4,000 µg/L
Soluble CD25 ≥ 2,400 U/mL	6,500 U/mL
Pathogenic mutations in genes causing HLH ⁱ	Not found

ⁱ No mutations in the *PRF1*, *UNC13D*, *STX11* or *STXBP2* genes.

Supplementary Table 2. Homozygous variants found in the patient using WES.

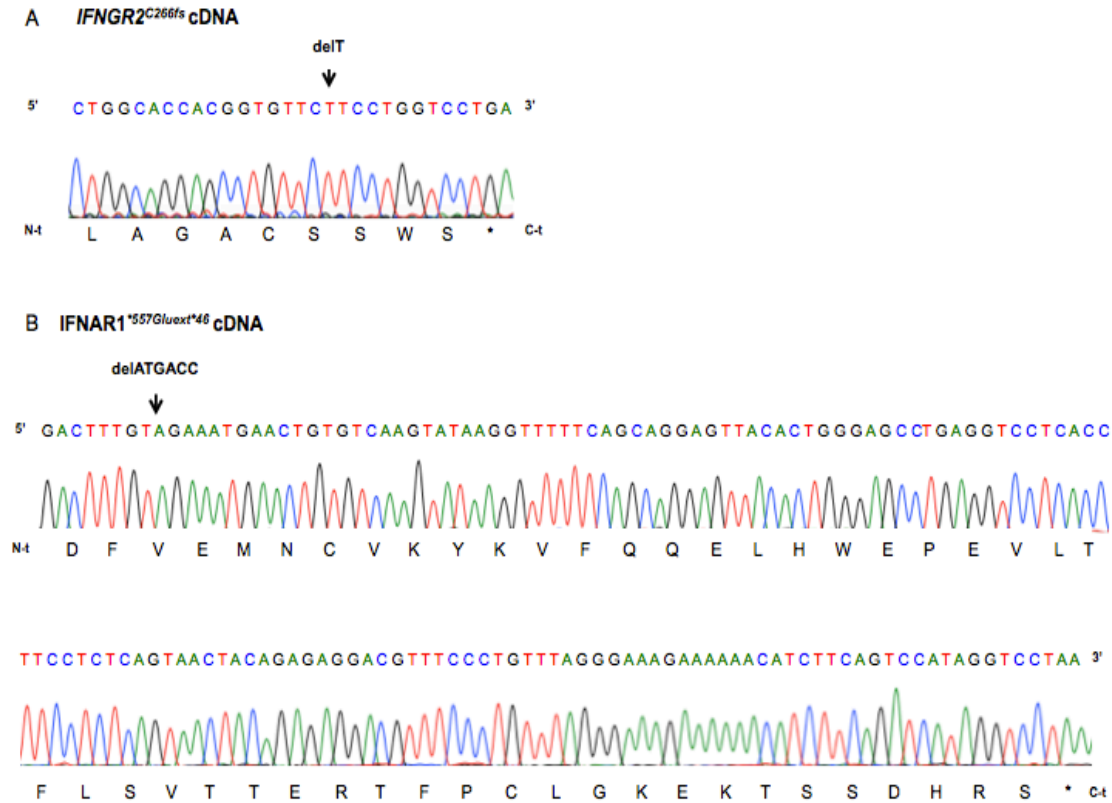
Gene	Chromosome	Mutation start	Mutation end	Reference base	Altered base(s)
TCTEX1D1	1	67242069	67242069	-	C
DDAH1	1	85930490	85930490	A	G
FAM171B	2	187559029	187559029	-	CAG
SLC38A3	3	50251833	50251833	-	G
MUC4	3	195518112	195518112	-	GTCTCCTGCGTAACA
ZNF595	4	85995	85995	-	A
DOPEY1	6	83845388	83845388	G	T
LFNG	7	2552882	2552882	GATG	-
IQCE	7	2598818	2598818	G	A
AOAH	7	36552790	36552790	-	T
UPK3B	7	76144774	76144774	A	T
UPK3B	7	76144775	76144775	T	C
AGAP3	7	150783922	150783922	T	G
RHOBTB2	8	22864881	22864881	G	A
RASSF7	11	562408	562408	T	C
C11orf40	11	4592708	4592708	-	AG
SLC6A12	12	310979	310979	C	T
WNK1	12	974308	974308	-	C
AKAP3	12	4736727	4736727	T	G
ANKLE2	12	133338366	133338366	C	T
MLNR	13	49796269	49796269	TC	-
FLVCR2	14	76045601	76045601	A	G
PKD1L3	16	71981414	71981414	-	TTTGG
OR7C2	19	15052983	15052983	-	ATC
LTBP4	19	41123093	41123093	-	G
GCFC1	21	34143903	34143903	T	G
IFNAR1	21	34727852	34727852	ATGACC	-
IFNGR2	21	34805097	34805097	T	-
CARD10	22	37904680	37904680	G	A
BAIAP2L2	22	38485609	38485609	C	T
DMD	X	31893387	31893387	T	C
ITIH6	X	54777534	54777534	C	T
ITIH6	X	54785283	54785283	A	T
SLC16A2	X	73641251	73641251	A	T
TCEAL6	X	101395784	101395784	C	G
SERPINA7	X	105279192	105279192	AA	-
RBMXL3	X	114424364	114424364	-	C

Supplementary Figure 1. Sanger sequencing confirmation of the carrier state in the proband's parents for the IFNGR2 and IFNAR1 mutations.



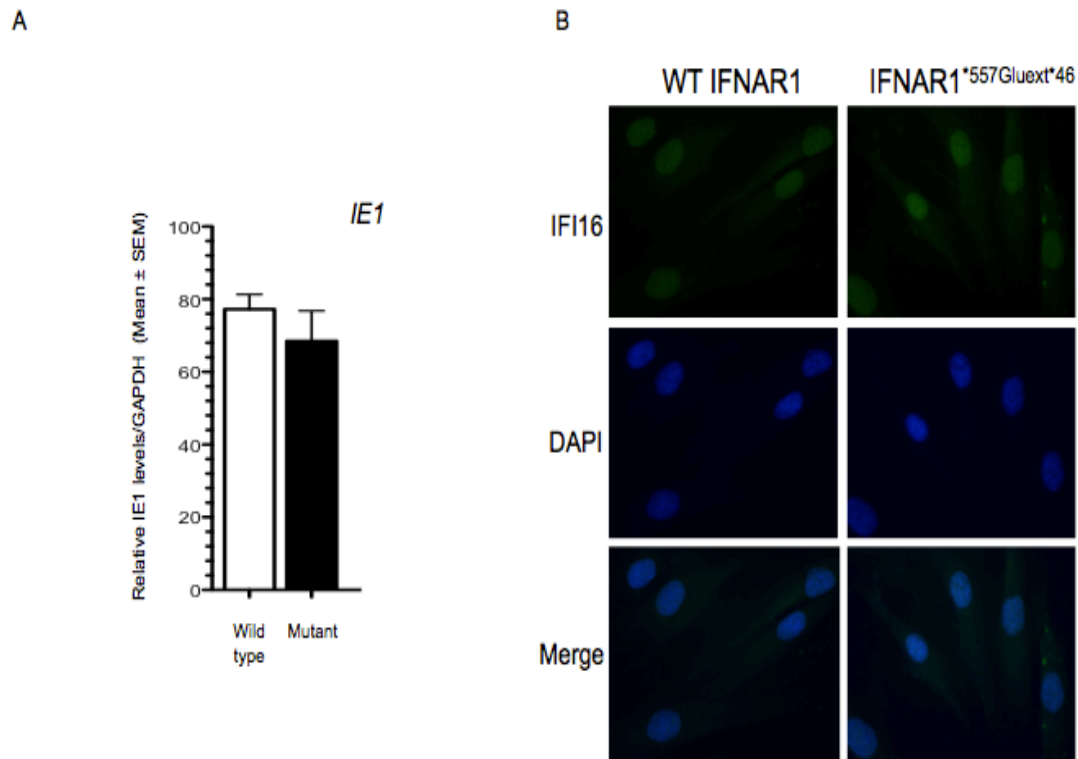
A, Sanger sequencing demonstrating the heterozygous state in the proband's parents for the *IFNGR2* mutation (798delT). B, Sanger sequencing demonstrating the heterozygous state in the proband's parents for the *IFNAR1* mutation (1671_1821del). Sequencing of a control subject is included. WT = wild type, Mut = mutant.

Supplementary Figure 2. cDNA Sanger sequencing confirmation of the predicted modifications to the protein structure as a result of the IFNGR2 and IFNAR1 mutations.



A, cDNA Sanger sequencing confirming the premature truncation at residue 270 of the *IFNGR2*^{C266fs}. B, cDNA Sanger sequencing confirming the addition of a novel amino acid sequence to the *IFNAR1*^{*557Gluext*46} protein C terminus.

Supplementary Figure 3. IFNAR1^{*557Gluext*46} expression does not affect CMV entry or baseline levels of IFI16 expression.



A, CMV entry was evaluated by qt-PCR quantification of IE1 after an adsorption time of 2 hours.

B, Immunofluorescence evaluation of baseline expression levels of IFI16 in proband (IFNAR1^{*557Gluext*46}) and healthy control (WT IFNAR1) derived fibroblasts.

3. CHAPTER 3: DISCUSSION AND PERSPECTIVES

3.1. DISCUSSION

We here report a patient with dual novel defects in the IFN pathway: a nonsense mutation in IFNGR2 and a mutation in *IFNAR1* that adds 46 amino acids to the protein's C-terminus. IFNGR2^{C266fs} was expressed at a significantly lower level compared to wild-type IFNGR2 in control cells and the patient's fibroblasts has no STAT1 phosphorylation or HLA-DR upregulation in response to IFN- γ , which is consistent with his susceptibility to disseminated *Mycobacterium tuberculosis*. However, the patient's CMV viremia is not a common feature of MSMD. Although a small subgroup of ISGs can be induced in response to both type I IFNs and IFN- γ , type I IFNs activate transcription of a wider set of genes important for host immunity against viruses⁹. We found that the patient's IFNAR1 mutation resulted in impaired intracellular signaling downstream of IFN- α stimulation and defective expression of ISGs crucial for controlling CMV⁵¹.

Mutations that affect the *IFNGR2* gene are a rare cause of MSMD and account for only 4% of the cases reported to date⁵². The expression of IFNGR2 is essential for the intracellular signaling events in response to IFN- γ . IFNGR2 lacks intrinsic enzymatic activity and its signaling function depends on the association with the kinase Jak2, which binds to two sequences between aminoacids 284-295 within the receptor's intracellular domain⁵³. We have shown that the IFNGR2^{C266fs} mutant protein is truncated at residue 270, thus eliminating both Jak2 binding sequences. Loss of the 67 C-terminal amino acids of IFNGR2 results in impaired protein expression and a lack of response to IFN- γ , demonstrating that the patient has a novel genetic cause of autosomal recessive MSMD.

There have been no previous reports of patients with MSMD presenting with HLH. Our proband's secondary HLH occurred in the setting of disseminated infections with multiple organisms: *Mycobacterium tuberculosis*, *Streptococcus viridans*, and CMV. MSMD patients are

considered immunocompetent to pathogens other than *Mycobacteria* and *Salmonella*. A few case reports of patients with MSMD have been reported to have infections with other organisms, but it was not known if the patient's susceptibility to these organisms arose from the genetic defects underlying their MSMD. The unusual clinical presentation of the proband, his history of multi-organism sepsis and the lack of reported cases of HLH secondary to *Mycobacteria* in MSMD patients prompted us to further analyze the potential role of secondary mutations on this patient's phenotype. Given the history of parental consanguinity, an autosomal recessive pattern of inheritance was anticipated for any disease causing mutations. Our unbiased whole exome sequencing approach identified the mutation in *IFNAR1* as the second pathogenic mutation in this patient. The *IFNAR1*^{*557Gluext*46} mutation replaces the protein's native stop codon with 46 novel C-terminal amino acids, while otherwise preserving the amino acid sequence of the wild-type receptor. We have demonstrated that the addition of the 46 extra amino acids in *IFNAR1*^{*557Gluext*46} does not affect protein expression. Despite normal surface expression levels of the mutant receptor, we found that signaling events downstream of *IFNAR1*^{*557Gluext*46} led to decreased phosphorylation of STAT1 and STAT2 and pSTAT1 nuclear translocation after stimulation with IFN- α .

Host immunity against viral infection depends on the expression of ISG downstream of the IFNAR. The role of many ISG in the antiviral response has yet to be studied; however, the IFIT family of genes has been shown to be important for mounting an effective response against viruses by efficiently blocking viral replication^{26, 27, 28}. We have shown that defective signaling downstream of *IFNAR1*^{*557Gluext*46} significantly impairs normal expression of the ISG in response to IFN- α stimulation and viral infection. The DNA sensor IFI16 is an ISG important for the cellular response to CMV and HSV, after sensing viral DNA, IFI16 induces IFN- β expression via the STING/TBK1/IRF-3 pathway⁵⁴ and interestingly, induction of IFI16 expression is important for restriction of CMV replication⁵⁵. We have demonstrated that in spite of similar baseline expression levels of IFI16 in patient and control fibroblasts, cells expressing *IFNAR1*^{*557Gluext*46}

show decreased upregulation of IFI16 after infection with CMV or HSV-1 d109, this defective response might play a role in the proband's susceptibility to CMV facilitating CMV replication. Upregulation of the *IFIT1* and *IFIT2* genes has been reported previously in human fibroblasts in response to CMV infection^{29,30} and the normal response to type I IFNs is crucial for controlling CMV spread and replication *in vitro*⁵¹. The exact mechanism by which the IFIT family members contribute to controlling CMV infections has not yet been definitively proven. It has been proposed that that expression of the IFIT genes blocks CMV replication by nonspecifically decreasing cellular translation or by interacting with CMV specific proteins as has been previously shown for other DNA viruses²⁷. Using an *in vitro* model of viral infection with CMV, we have demonstrated that despite the partial upregulation of ISGs downstream of IFNAR1^{*557Gluext*46}, the dampened cellular response to type I IFNs fails to mount an anti-viral state that effectively prevents viral replication. Additionally, type I IFNs induce the expression of class I HLA genes and stimulate NK and CD8+ T cell mediated cytotoxicity¹⁰, thereby promoting killing of virus infected cells. The defective intracellular signaling events downstream of IFNAR1^{*557Gluext*46} may favor CMV replication by diminishing the induction of ISG that specifically block viral replication and by affecting the normal cytotoxic response against CMV infected cells *in vivo*.

To our knowledge, this is the first report of a patient with a mutation in *IFNAR1*. A kindred with a mutation in the *IFNAR2* gene has been previously reported. Although, in *in vitro* studies have shown the critical role of type I IFNs in the response against CMV, patients lacking *IFNAR2* have been reported to control CMV without developing clinically relevant symptoms¹⁷. The immunocompetence to CMV observed in *IFNAR2* deficient patients may be explained by their normal response to IFN- γ and partial complementation of their defective induction of ISG in response to type I IFNs by IFN- γ mediated formation of ISGF3 complexes. *IFNAR2* and *STAT2* deficient patients^{17,18} have been reported to develop fatal vaccine strain related measles

encephalopathy after receiving the MMR vaccine. However, our proband had no complications after MMR vaccination. The residual signaling and ISG gene expression downstream of IFNAR1^{*557Gluext*46} in our proband may have been sufficient for providing immunity against attenuated, but not wild type, viruses. In contrast, previously reported mutations in IFNAR2 and STAT2 led to complete abrogation of the cellular response to IFN- α . We hypothesize that the high copy number of CMV in the proband may have been due to his dual defect in IFN signaling, specifically, the impaired upregulation of ISGs downstream of IFNAR1^{*557Gluext*46} and the failure of IFNGR signaling to complement this defective response. To assess the contribution of IFNAR1^{*557Gluext*46} in controlling CMV viremia, we are currently performing *in vitro* studies of CMV replication in patient-derived fibroblasts with and without reconstitution with the wild type *IFNAR1* gene.

In addition to CMV viremia, the proband presented with *Streptococcus viridans* bacteremia. The role of type I IFNs in the immune response to virus has been widely studied; however, their role in maintaining host immunity against bacterial pathogens has yet to be clarified^{7, 56}. The effect of the IFN pathways in the response to mycobacteria has been studied using IFNAR^{-/-}, IFNGR^{-/-} or IFNAR^{-/-}IFNGR^{-/-} double knockout mice. Group B *streptococci* (GBS) infection of IFNAR- or IFNGR-deficient mice showed increased lethality and bacterial burden when compared to wild type mice. IFNAR^{-/-}IFNGR^{-/-} double knockout mice died within the first three days post infection, demonstrating significantly more susceptibility to GBS than either of the IFNAR^{-/-} or IFNGR^{-/-} single knockout mice⁵⁷. These data suggest that type I IFNs contribute to host immunity against extracellular bacteria, although the mechanisms are not yet understood. Neither the IFNAR^{-/-} mice nor the patients with IFNAR2 or STAT2 deficiency have been reported to develop mycobacterial infections^{17, 18}, indicating that the immune response to mycobacteria does not depend on type I IFNs. In contrast, the genetic defects associated with MSMD indicate the critical contribution of the type II IFN signaling pathways to immunity against

mycobacteria.

In summary, we have identified a primary immunodeficiency arising from dual defects in the type I and type II IFN pathways in a patient with sepsis due to multiple pathogens. We have shown that the impaired response to type I IFNs downstream of $IFNAR1^{*557Gluext*46}$ fails to establish an effective anti-viral state and promotes CMV infection; furthermore, the patient's defect in *IFNGR2* completely abrogates the response to IFN- γ , which is critical for the immune response against mycobacteria. While PIDs have been classically considered monogenic diseases⁵⁸, our work demonstrates the utility of unbiased next generation sequencing technologies in identifying multigenic causes of immunodeficiency.

3.2. LIMITATIONS

We have shown that the patient's fibroblasts have impaired phosphorylation of STAT1 and STAT2 as well as impaired nuclear translocation of pSTAT2. We hypothesized that the 46 novel C-terminal amino acids of IFNAR1^{*557Gluext*46} sequesters the pSTAT1/pSTAT2 heterodimer and impairs translocation of the heterodimer to the nucleus, thereby preventing subsequent binding of unphosphorylated STAT1 and STAT2 proteins to the receptor. To prove this, we performed co-immunoprecipitation experiments to quantify the amount of pSTAT1 and pSTAT2 bound to IFNAR1 after stimulating patient and control fibroblasts with IFN- α . However, as previously reported by other studies⁵⁹, the weak interaction between the IFNAR and the pSTAT1/pSTAT2 heterodimer is not detectable by co-immunoprecipitation of proteins using primary cells. The use of an over-expression system is an alternative approach to evaluate the interaction between IFNAR1 and the pSTAT1/pSTAT2 dimer. Since the patient's fibroblasts demonstrated residual expression of a mutant IFNGR2 protein as well as levels of IFNAR1^{*557Gluext*46} equivalent to that of wild-type IFNAR1, this experiment requires transfection of a cell line that lacks wild-type forms of the human IFNGR2 and IFNAR1. These experiments are beyond the scope of the current study. The use of paraformaldehyde fixation previous to immunoprecipitation could be an additional approach to evaluate the interaction between IFNAR1^{*557Gluext*46} and the pSTAT1/pSTAT2 dimer.

3.3. FUTURE RESEARCH

The function of type I IFNs expands beyond the establishment of the anti-viral state. Type I IFNs have been reported to possess anti-inflammatory properties and are currently used for the treatment of inflammatory conditions like multiple sclerosis⁶⁰ and familial Mediterranean fever⁶¹. Type I IFNs have been shown to decrease the production of IL-1 β by decreasing the production of proIL-1 β in an IL-10 mediated, STAT3 dependent fashion⁶². Repression of the NLRP3 inflammasome activity has been described in monocytes after treatment with type I IFNs. Repression of the NLRP3 inflammasome has been shown to depend on STAT1 phosphorylation, which suggests it is that the underlying mechanism involves the expression of a yet unidentified ISG⁶². The AIM2 inflammasome is also modulated by type I IFNs via induction of the ISG IFI16, binding of IFI16 to AIM2 prevents the interaction between AIM2 and ASC, hence the blocking AIM2 inflammasome assembly⁶³.

The development of secondary HLH is uncharacteristic of patients with PIDs and has not been described previously in patients with MSMD. In patients with secondary HLH, the uncontrolled infection induces a cytokine storm that causes over activation of the monocyte-macrophage compartment. Human studies on familiar HLH and HLH secondary to systemic onset juvenile idiopathic arthritis (sJIA) have suggested a role for IL-1 signaling in macrophage over-activation^{64, 65}. The administration of Anakinra®, a recombinant IL-1 receptor antagonist, has been reported to be beneficial in the treatment of HLH secondary to sJIA^{66, 67}. We hypothesize that the patient's defect in Type I IFN signaling leads to increased activation of the AIM2 inflammasome in response to infection with CMV, thereby increasing his susceptibility to develop HLH. To test this hypothesis, we are quantifying IL-1 β production after CMV infection of fibroblasts from the patient and controls. These studies will determine if a deficiency in IFNAR1

results not only in a primary immunodeficiency, but also an autoinflammatory state.

4. BIBLIOGRAPHY

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