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UNRAVELLING THE GENETIC CAUSE OF LIFE-THREATENING INFECTIONS IN CHILDREN

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LIST OF ABBREVIATIONS

AD:	Autosomal dominant
ADA2:	Adenosine deaminase 2
APC:	Antigen presenting cell
AR:	Autosomal recessive
Arp2/3:	Actin-related protein 2/3 complex
BTK:	Bruton tyrosine kinase
CDC42:	Cell division cycle 42
CGD :	Chronic granulomatous disease
CLR:	C-type lectin receptor
CMC:	Chronic mucocutaneous candidiasis
CMV:	Cytomegalovirus
CNV:	Copy number variation
CVID:	Common variable immunodeficiency
DC:	Dendritic cell
DOCK8:	Dedicator of cytokinesis 8
F-actin:	Filamentous actin
GAS:	IFN-γ activated site
GOF:	Gain of function
HLH:	Hemophagocytic lymphohistiocytosis
HSCT:	Hematopoietic stem cell transplantation
HSV-1:	Herpes simplex virus 1
IAV:	Influenza A virus
IEI:	Inborn error of immunity
IFN:	Interferon
Ig:	Immunoglobulin
IFNAR:	Type I interferon receptor
IFNGR:	Type II interferon (IFN-γ) receptor
ILC:	Innate lymphoid cell
IPD:	Invasive pneumococcal disease
IRF:	Interferon regulatory factor
ISG:	Interferon stimulated gene
ISGF3:	Interferon stimulated gene factor 3
ISRE:	Interferon stimulated response element
JAK:	Janus-activated kinase
LOF:	Loss of function
LPS:	Lipopolysaccharide
MAPK:	Mitogen-activated protein kinase
MAVS:	Mitochondrial antiviral signaling protein
MDA5:	Melanoma differentiation-associated protein 5

MEFV:	Mediterranean fever
MSMD:	Monogenic susceptibility to mycobacterial disease
NGS:	Next generation sequencing
NK:	Natural killer
NLR:	Nod-like receptor
OAS:	2′,5′-oligoadenylate synthetase
PBMC:	Peripheral blood mononuclear cell
PCR:	Polymerase chain reaction
pDC:	Plasmacytoid dendritic cell
PI3K:	Phosphatidyl-inositol 3-kinase
PID:	Primary immunodeficiency
RAP1:	Ras-related protein 1
RLR:	Retinoic-acid-inducible protein 1 (RIG-I)-like receptor
SNV:	Single nucleotide variants
STAT:	Signal transducer and activator of transcription
STING:	Stimulator of IFN genes
TLR:	Toll-like receptor
TRIF:	TIR-domain-containing adaptor molecule 1
TY2:	Tyrosine kinase 2
USP-18:	Ubiquitin specific peptidase 18
WASP:	Wiskott-Aldrich syndrome protein
WES:	Whole exome sequencing
WGS:	Whole genome sequencing
XL:	X-linked

INTRODUCTION

INTRODUCTION

Parts of introduction adapted from:
Giorgia Bucciol, Leen Moens, Barbara Bosch, Xavier Bossuyt, Jean-Laurent Casanova, Anne Puel and Isabelle Meyts. Lessons learned from the study of human inborn errors of innate immunity. J Allergy Clin Immunol. 2019 Feb;143(2):507-527.
Giorgia Bucciol, Erika Van Nieuwenhove, Leen Moens, Yuval Itan and Isabelle Meyts. Whole exome sequencing in inborn errors of immunity: use the power but mind the limits. Curr Opin Allergy Clin Immunol. 2017 Dec;17(6):421-430.
Stuart G Tangye, Giorgia Bucciol, Jose Casas-Martin, Bethany Pillay, Cindy S Ma, Leen Moens, Isabelle Meyts. Human inborn errors of the actin cytoskeleton affecting immunity: way beyond WAS and WIP. Immunol Cell Biol. 2019 Apr;97(4):389-402.

1. General overview of human inborn errors of immunity

Primary immunodeficiency diseases (PIDs) are a heterogeneous group of genetically determined diseases involving one or more components of the immune system. The principal hallmarks of these disorders are susceptibility to infections, autoimmunity and higher risk of cancer (1). Although there are no precise data on the prevalence of these disorders, it is estimated that 1:1500 to 1:2500 people suffer from a PID. Selective IgA deficiency may even be as common as 1:500, although only 5% of patients are clinically symptomatic (2). Most PIDs present in childhood and an inverse correlation between severity of the disease and age of onset can generally be seen, although better diagnostic tools and increased awareness have led in recent years to a rise in the number of adults diagnosed with a PID (2–5). The great advances of the last decades in human genetics have permitted an acceleration in the discovery of new genes associated with known or new phenotypical PIDs, with the greatest leap determined by the implementation of next-generation sequencing (NGS) in 2009. Since then, more than 200 new genes have been discovered to underlie a primary immunodeficiency, doubling the total number of diseases so far described (6). An attempt at comprehensive genotypic and phenotypic classifications is made by the International Union of Immunological Societies (IUIS) with biannual publications (1,6). The most recent from 2019 reveal a shift from the concept of "primary immunodeficiencies" as fundamental defects in the immune response to infection towards the broader concept of "inborn errors of immunity" (IEIs) as a heterogeneous group of different phenotypes including infection, autoinflammation, autoimmunity, allergy and malignancy (1,6). The genotypic classification groups IEIs in 10

tables according to the immunological mechanism most fundamentally affected by the genetic defect, from cellular to humoral to innate immunity, even if many diseases show in fact overlapping features (6). The phenotypic classification, instead, is designed to be of assistance in clinical practice, containing a tree-based algorithm to aid in the diagnostic process. Based on the immunological and clinical phenotype, IEIs are thus divided in severe combined immunodeficiencies, combined immunodeficiencies, antibody deficiencies, immune dysregulation diseases, phagocyte defects, defects of intrinsic and innate immunity, autoinflammatory disorders, complement deficiencies, bone marrow failure, and phenocopies of other diseases. The more numerous are antibody deficiencies, representing 45% of all IEIs, followed by combined immunodeficiencies (19%, of which 13% with syndromic features), phagocyte defects (5%), autoinflammatory diseases (5%), complement disorders (5%), diseases of immune dysregulation (3%) and disorders of intrinsic and innate immunity (1%) (Fig. 1, (7)). Further down the decision tree, they are categorized based on presentation or other clinical features. These include characteristic infections, ectodermal anomalies, failure to thrive, autoimmune or autoinflammatory manifestations, lymphoproliferation, combinations of syndromic features, allergy, eczema, angioedema, and bone marrow abnormalities. Starting from this pattern recognition, the clinician is then guided through the diagnostic steps necessary to identify the causative defect. In reality, however, this almost invariably demands an NGS approach.

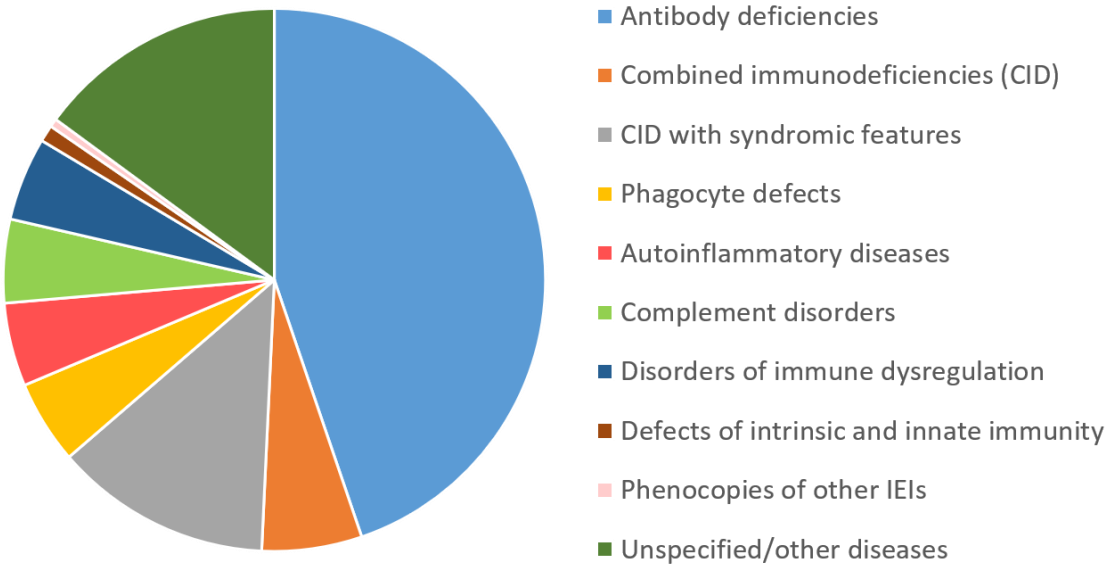

Fig. 1. The relative frequency of IEIs by major type of defect.

Isolated severe infections as inborn errors of immunity: the genetic theory of infectious diseases

Infectious diseases remained the main cause of death in humans until the middle of the 19th century, with half of the children dying of "fever" before the age of 15 years. The major burden was caused by primary infections, targeting children and young adults. The great work of Pasteur and Koch in the second part of 1800 led to the discovery of microbes and to the germ theory of disease, which revolutionized the approach to the ill. The consequent adoption of hygiene and aseptic practices together with the discovery of antibiotics and vaccines had a great epidemiological impact and led to a dramatic drop in human mortality curves, increasing life expectancy at birth from 20 to 80 years (8).

However, the germ theory does not explain why the clinical course of infection is so variable among individuals, spanning from inapparent infection to a critical or lethal disease for almost every known pathogen. This inter-individual heterogeneity in the context of primary infections in young children cannot be simply ascribed to individual acquired immunity or microbial variation, both of which require a long time to arise. It points instead to a genetic, albeit often not completely penetrant, origin of human susceptibility to specific microbes (8–10). A large Danish epidemiological adoptee study showed indeed a strong correlation between early death by infection in the children and their biological parents, but not in their adoptive parents, thus suggesting that predisposition to severe infectious diseases is genetically determined (11).

The search for genetic, and specifically monogenic, causes of susceptibility to infection has therefore been at the forefront of scientific advances in the field of IEIs in the last decades. Aided by the progress in genetic sequencing, the study of subjects affected by unusual, recurrent or severe infections shed light on many different immune mechanisms and defects relevant to human immunity against microbes. In turn, this led to a great change in our perception of what constitutes an immunodeficiency.

Non-conventional PIDs: a paradigm shift

The study of IEIs was indeed long dominated by the concept of "conventional" primary immunodeficiencies, in which rare Mendelian traits confer susceptibility to infection with a multitude of pathogens, in a completely penetrant manner and with a measurable effect on

immune cells number or function. The prototype of such PIDs is X-linked (XL) agammaglobulinemia, which is caused by deleterious mutations in the Bruton tyrosine kinase gene (*BTK*). BTK is expressed in hematopoietic cells, and defects of this protein render the host susceptible, for life, to recurrent life-threatening infections with various microbes and result in a detectable immunological phenotype (little or absent immunoglobulin (Ig) production and few or absent B lymphocytes).

In the 1910s, Nicolle described asymptomatic infections and documented the natural variability in host susceptibility to infection. In line with these observations, "monogenic infections" were described from the 1940s onward, and their molecular basis from the 1970s onward. Increased and selective susceptibility to infections with one or a narrow range of pathogens can be referred to as "non-conventional" PIDs and include many inborn errors of innate immunity (8). An example is the susceptibility to invasive Neisseria infections in patients with late complement pathway defects (12–14). Over the last two decades, susceptibility to a narrow range of microbes, such as monogenic susceptibility to invasive pneumococcal disease (IPD), mycobacterial disease (MSMD), Herpes simplex virus 1 (HSV-1) encephalitis and fungal disease, particularly chronic mucocutaneous candidiasis (CMC), have been ascribed to defects in single genes, often but not always encoding components of innate immunity (14,15).

However, dichotomous classifications making distinctions between adaptive and innate immunity, not to mention cell-intrinsic immunity, represent an oversimplification and prove difficult to apply to inborn errors of immunity. In fact, inborn errors of innate immunity may result in the absence or impaired production of cytokines essential for the development, survival, proliferation or function of B or T cells, and can lead to allergy and autoimmunity, indications of adaptive immune dysregulation. Indeed, as we learn more about these defects and more cases are reported, their phenotypes appear to be broader than initially described.

A second paradigmatic shift in the field of IEI is the recognition of autoinflammatory disorders as innate disorders of immune dysregulation, in juxtaposition with adaptive immunity-driven autoimmunity. Previously classified as rheumatological diseases, they are now accepted as immune disorders and their impact on immune and hematopoietic functions demonstrated by numerous studies (16,17). Moreover, autoinflammatory manifestations are being discovered as part of the phenotype of both well-known and novel IEIs, such as combined

immunodeficiencies, common variable immunodeficiency (CVID), chronic granulomatous disease (CGD), signal transducer and activator of transcription (*STAT1*) gain of function (GOF) mutations, actin cytoskeleton defects, and many others.

2. Understanding the pathophysiology of non-conventional PIDs

In the changing landscape of what is considered a PID, a tremendous effort has been devoted to discovering novel genetic etiologies of seemingly fortuitous presentations, such as life-threatening infections in children without a detectable immunological phenotype, and to understanding the underlying mechanisms of these and other so-called non-conventional PIDs. Starting from this framework, our line of research focuses on patients, encountered in daily practice, with unexplained phenotypes that could indicate an immune defect, such as severe course of infection, autoinflammation, or syndromic features with immunodeficiency. By following a stepwise approach based on immunological screening followed by genetic testing and functional analysis of potential mutations, we can gain understanding of the mechanisms of immune dysfunctions while making the most efficient use of our resources. In this setting, targeted gene sequencing is of limited use, and an unbiased approach with next generation techniques is often required. This is due to the phenotypical variability, overlapping manifestations, and genetic heterogeneity of IEIs, not to mention the great number of etiologies still unknown, reflected by the constant flux of newly reported defects in recent years.

The work reported in this thesis stems directly from this approach, always starting from the patients with a diagnostic question in mind and coming back to them with insight into their disease and, hopefully, the possibility of offering targeted treatment. The application of this diagnostic approach to both children referred to the immunodeficiency clinic and children admitted to the intensive care unit for life-threatening infections has been fruitful. The identification of monogenic defects affecting immunity enables us to offer specific care, therapy, and counseling to the patients. Even in the absence of a curative or targeted treatment, the knowledge of the underlying defect allows us to make predictions on the course of disease and to choose the best preventive strategy, for example antimicrobial therapy in case of a high risk of recurrent invasive infections. This is especially important for inborn errors of innate immune pathways without readily detectable immunological defects. The severity and

spectrum of these diseases can range from a single episode of infection in childhood to multiple life-threatening complications protracting into adulthood and into the next generation, which is why an etiological diagnosis is of the utmost importance in the follow-up of these patients.

Since most of these diseases are extremely rare, to the point that for some defects only one or a few patients are described, the extent of their clinical features is largely unknown. For this reason, an extensive genetic diagnostic program is of great assistance in expanding our knowledge of these diseases and in increasing our awareness of the possible multi-faceted manifestations characteristic of a specific IEI. In particular, the idea that a life-threatening course of infection with common germs in apparently healthy children could be the first or only sign of an immune defect as opposed to just bad luck, is still insufficiently credited by general pediatricians and intensive care practitioners. On the other hand, the pathophysiology of complex or syndromic presentations is often ill understood, and insight in the basic mechanisms of disease can potentially be a game changer in the care of these difficult cases.

Finally, the recent tremendous advances in therapeutic options give us the means of treating some IEIs in a targeted, personalized way, allowing us to i) correct the defective pathway and potentially reverse the symptoms of the disease; ii) treat diseases that are not curable by hematopoietic stem cell transplantation (HSCT) or gene therapy; iii) target all affected cells and not only blood cells, as with HSCT, thus acting also on extra-hematopoietic disease manifestations; iv) avoid the toxicity of HSCT.

3. From next generation sequencing to functional validation

Next generation sequencing (NGS) is a high-throughput sequencing method in which thousands or millions of small DNA fragments are sequenced in parallel (18). Starting from the patient's genomic DNA, a sequencing library is prepared by random fragmentation of the DNA into short strands that are then fused with adaptors. These fragments are amplified and sequenced in parallel multiple times, usually at least 20 times for any nucleotide, to attain sufficient read depth. The obtained sequences are then mapped to a reference human genome and the differences between the patient's DNA and the reference are "called" as variants (Fig. 2). A read depth of at least 20 is considered essential to discriminate sequencing errors from true variants. These variants must be then analyzed in the setting of the patient's clinical and

cellular phenotype and pedigree. This step is warranted in order to disregard irrelevant variants and instead detect mutations in known disease-causing genes or in potentially novel candidate genes (19).

NGS can be used to sequence the entire genome (whole genome sequencing, WGS), a targeted panel such as the whole exome (WES), or a more limited set of genes of interest. The exome is defined as all the exons for about 20,000 protein-coding genes and for microRNA, small nucleolar RNA and large intergenic noncoding RNA genes, constituting about 1% of the human genome (20). In the construction of the DNA library, WES and panel sequencing require enrichment in exons through hybridization with target-specific probes and polymerase chain reaction (PCR) amplification of the selected regions (21). This additional step generates bias at different levels: since the enrichment process is not perfectly homogeneous and there is an a priori reference bias with the choice of probes, some exons are underrepresented or missing in the library; moreover, the PCR amplification step can introduce errors in the sequences of nucleotides because of polymerase errors, guanine-cytosine related errors, stochastic errors and template switch errors (19,22).

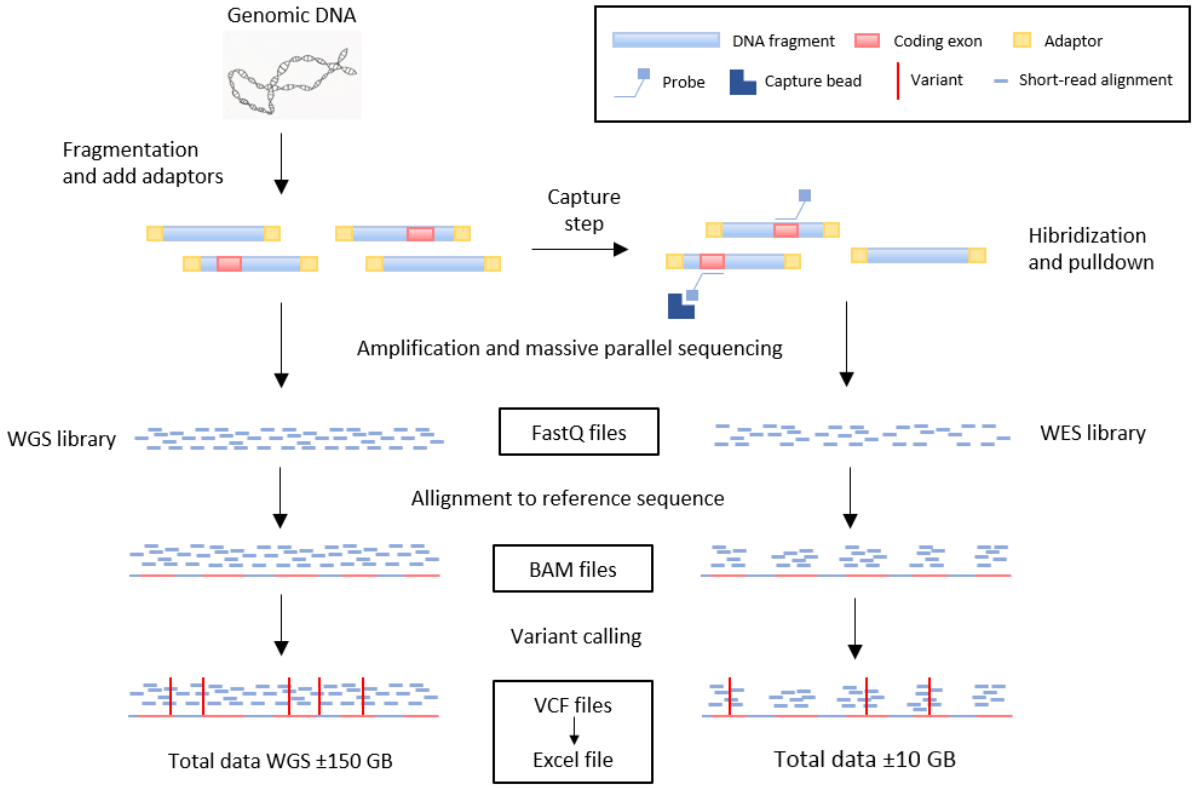

Figure 2. The process of whole genome (WGS) and whole exome sequencing (WES). The files that are finally derived from the analysis are of three types: FASTQ files, including all the raw data; BAM files, for the alignment to the reference sequence; and VCF files, subsequently convertible to Excel files, for the variant calling. Adapted from (23).

Accordingly, WGS was shown to be more powerful than WES for detecting exome variants (single nucleotide variants (SNVs) as well as small insertions/deletions (indels) and copy number variations (CNVs)). WGS provides a much more uniform distribution of sequencing-quality parameters and a more homogeneous coverage of the genome (24). Moreover, thanks to the current shift in the clinical setting from fixed gene panels and WES towards WGS, various deep intronic variants were shown to be disease-causing, broadening the field of diagnostics in IEI even more (19,25).

Next generation sequencing for inborn errors of immunity

Inborn errors of immunity are likely to be monogenic and display genetic pleiotropy (different clinical and immunological phenotypes originate from mutations of the same gene) and genetic heterogeneity (mutations in different genes give rise to the same phenotype) (26,27). For these reasons, the unbiased approach allowed by NGS techniques has accelerated the unraveling of these disorders at the genetic level. Incomplete penetrance is also a common feature of inborn errors of immunity. This can originate from oligogenic/polygenic models of disease, which are sometimes difficult to characterize. Epigenetic or environmental factors (e.g. previous encounter with a pathogen) may also play a role (28). In this complex context, only in selected cases does Sanger sequencing of single genes lead to a definitive diagnosis. Moreover, many novel genetic etiologies are yet to be unraveled and many new phenotypes are likely to be ascribed to known genotypes (29).

WES generates on average 20,000 high quality coding variants. In order to minimize the presence of common variants and maximize the inclusion of probable disease-causing mutations, a gene-level and a variant-level filtering approach are applied (Fig. 3) (30). The first crucial step is the generation of a genetic hypothesis that takes into consideration the potential mode of inheritance, the severity of the phenotype, the penetrance, etc. The presence of multiple affected siblings in a family with healthy ancestors is suggestive of autosomal recessive (AR) inheritance, so homozygous (especially in the case of consanguineous unions) or compound heterozygous variants should be prioritized. The inclusion of multiple affected and unaffected siblings is extremely useful to discern the potential disease-causing variant. Nevertheless, the possibility of autosomal dominant (AD), X-linked (XL) recessive inheritance or de novo mutations should not be neglected in consanguineous unions. Trio analysis, in

which the index patient and parents are sequenced simultaneously, is particularly efficient to pinpoint disease-causing de novo mutations; it is also very informative in the detection of homozygous and compound heterozygous mutations.

Severe phenotypes in single patients should be investigated in particular for de novo, homozygous or compound heterozygous variants, although in the case of incomplete penetrance this approach could exclude the causal mutation. Finally, AD inheritance implies that a heterozygous mutation is disease-causing. In accordance with the suspected inheritance, a hypothesis must be formed on the detected variants.

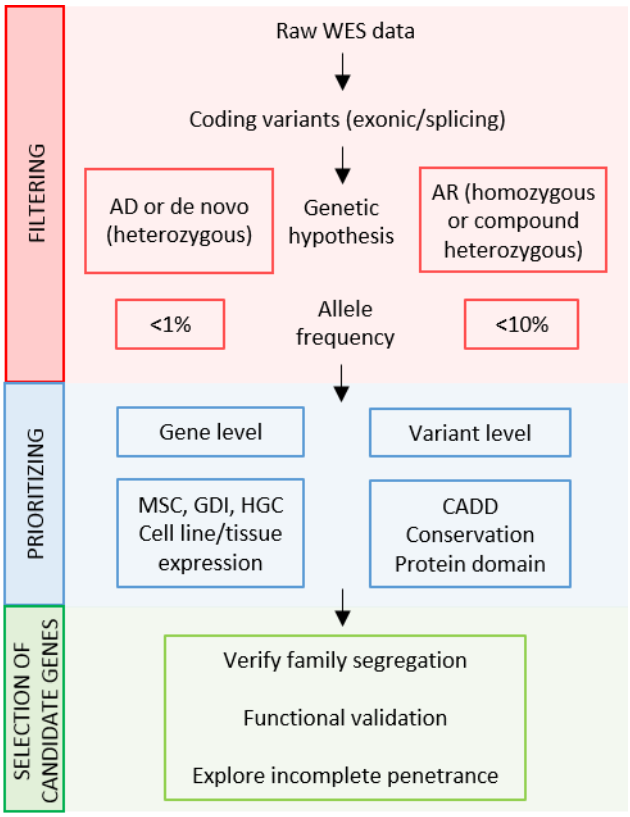

Figure 3. Flow chart depicting the general filtering method to screen whole exome data for disease-causing mutations. The process is divided into a "hard" filtering to eliminate all non-interesting variants, a second step where the variants are prioritized through a gene level approach and a variant level approach, and finally the selection of candidate variants based on the genetic hypothesis with family segregation, demonstrating a functional effect and, if necessary, exploring the possibility of incomplete penetrance. Adapted from (23).

AD: autosomal dominant; AR: autosomal recessive; CADD: combined annotation-dependent depletion; GDI: gene damage index; HGC: human gene connectome; MSC: mutation significance cutoff; WES: whole exome sequencing.

Heterozygous dominant variants are generally considered GOF mutations, where the encoded protein acquires a new function or expression pattern. However, dominant loss of function (LOF) variants can also occur through haploinsufficiency, where a reduction of gene function by 50% is sufficient to result in disease, or by a dominant negative effect, where the mutant gene product acts antagonistically to the wild-type protein function (27). This can occur for instance if the mutant protein hampers multimer formation. Disease-causing compound heterozygous or homozygous variants are presumed loss-of-function mutations, with few exceptions (31,32).

Functional validation of novel variants

The following criteria represent a general guideline to consider a gene to harbor potential disease-causing variants, even if they are not always valid and several counter-examples can be found: 1) it encodes a protein involved in a pathway related to the phenotype; 2) it is expressed in the cells and tissues of interest; 3) it is intolerant to mutations in the general population, indicating that mutations in that gene have a higher probability of being deleterious. There are databases reporting the expression of human genes across human cell lines, cell types, tissues and organs (33–35). It is reasonable to hypothesize that the gene of interest is expressed in the affected cell or tissue, although mutations in ubiquitously expressed genes can result in a phenotype that is highly tissue specific (36).

At the variant level, the allele frequency, based on different public databases in accordance with the ethnicity of the studied kindred, is the main filter to substantially reduce the number of variants. The hypothesized inheritance model dictates the frequency cutoff that should be applied. In general, heterozygous variants with a frequency in the general population of more than 1% are considered polymorphisms, with at most a weak modulating effect on disease. In the context of monogenic disorders, for AD transmission a frequency cutoff of less than 1% is thus applied. For AR disorders, instead, a higher frequency cutoff is recommended to minimize false negatives: to obtain a frequency cutoff of 1% for the homozygous state, we need to combine two parental alleles with a frequency of 10% respectively to respect Hardy-Weinberg equilibrium. Exceptions can derive from population genetics phenomena, such as founder effect, resulting in a high carrier frequency (such as in ADA2 deficiency (37)), or evolutionary adaptation (for example MEFV mutations that provide a selective advantage

against *Yersinia pestis* infection (38)). These cutoffs can be further reduced based on knowledge on the disease prevalence and on the predicted clinical penetrance. In the research setting, more strict cutoffs are used to screen for novel disease-causing variants, which usually have a very low allele frequency. Coding variants that are synonymous, i.e. variants that do not cause a change in the amino acid at that position, and non-coding variants that don't affect splicing, are usually excluded. This step significantly reduces the data load to be analyzed, since in most cases synonymous variants are silent or do not cause a change to the protein. Rarely, synonymous variants alter disease susceptibility or cause disease, for example by impairing splicing, or by affecting mRNA stability or protein folding (39). Various algorithms that attempt to predict the functional effect and assess the conservation/intolerance of a variant are publicly available and are used as an *in silico* prediction model of its impact.

At any rate, no prediction tool has the power to prove the pathogenicity of a variant. This is why, upon discovery of a potential new disease-causing gene or variant, it is fundamental to experimentally ascertain the causal connection between the variant and the clinical and immunological phenotype, to prevent ascribing to a false-positive result a disease-causing effect (27). Especially for single-patient genetic studies, solid evidence to show that the genetic variant is responsible for the destruction, impairment of function or alteration of expression or function of the protein product is necessary (40). First, the effect of the variant on RNA or protein expression must be investigated. In the case of unaltered protein expression, altered function can lead to disease: functional studies are therefore a crucial step. For instance, in the study of mutations in genes affecting the type I interferon pathway, the in vitro assay of viral load in cell lines relevant to the phenotype can be pursued. In case of a de novo mutation, different cell types should be tested to exclude a somatic mutation. Finally, for loss-of-function mutations the cellular phenotype should be rescued by transduction with the wild-type allele, while for gain-of-function mutations rescue should be obtained by knockdown/knockout or correction of the mutant allele (40). The functional confirmation of causality represents the most difficult and time-consuming part in the characterization of new disease-associated genes or mutations, but remains essential for the scientific validation of the findings and hence also for the diagnostic value of a reported variant. Reports of novel "mutations", even if in

genes known to cause inborn errors of immunity, should be interpreted with caution if the validation step is missing.

4. Exploring "old" pathways with new insights

It is beyond the scope of this introduction to discuss all pathways relevant to IEIs in detail. I will therefore focus on the pathways and principles relevant to this work.

Innate immunity is a germline-encoded system that enables eukaryotes to defend themselves against infection (41,42). It consists of three main components: 1) anatomical barriers, such as the physical barrier of intact skin and the chemical barrier of low gastric pH; 2) soluble proteins secreted onto mucosal surfaces or into the bloodstream, such as complement factors; 3) a cellular compartment composed of both hematopoietic and non-hematopoietic cells. Antigen presenting cells (APC), such as dendritic cells (DCs), phagocytes, such as neutrophils, natural killer (NK) cells and other innate lymphoid cells (ILCs), together with non-hematopoietic cells, such as fibroblasts, keratinocytes, epithelial cells, and neurons, harbor pathways that allow an adequate response to infection. Pathogens are first detected via innate immune sensors or receptors. Several classes of microbial receptors have been described: Toll-like receptors (TLRs), retinoic-acid-inducible protein 1 (RIG-I)-like receptors (RLRs), Nod-like receptors (NLR), C-type lectin receptors (CLRs), and DNA sensors (43–45). These receptors recognize microbial structures, such as lipoproteins, lipopolysaccharide (LPS), flagellin, peptidoglycan or microbial nucleic acids. Recognition activates downstream signaling cascades, leading to a rapid (occurring within hours) but short-lived immune response. This early immune response enables the host to survive until an adaptive response is generated, but its excessive activation can trigger auto-inflammation, which is defined as damage caused by dysregulated innate immune cells to host tissues (46,47). Cell-intrinsic immunity is a branch of innate immunity in which a set of cellular antimicrobial defenses protects individual hematopoietic and non-hematopoietic cells against microbes, especially viruses, through autocrine pathways and intracellular mechanisms, such as the production of viral restriction factors (48,49). A failure of this cell-intrinsic immunity can be the result of several monogenic defects of immunity, mostly involved in anti-viral defense.

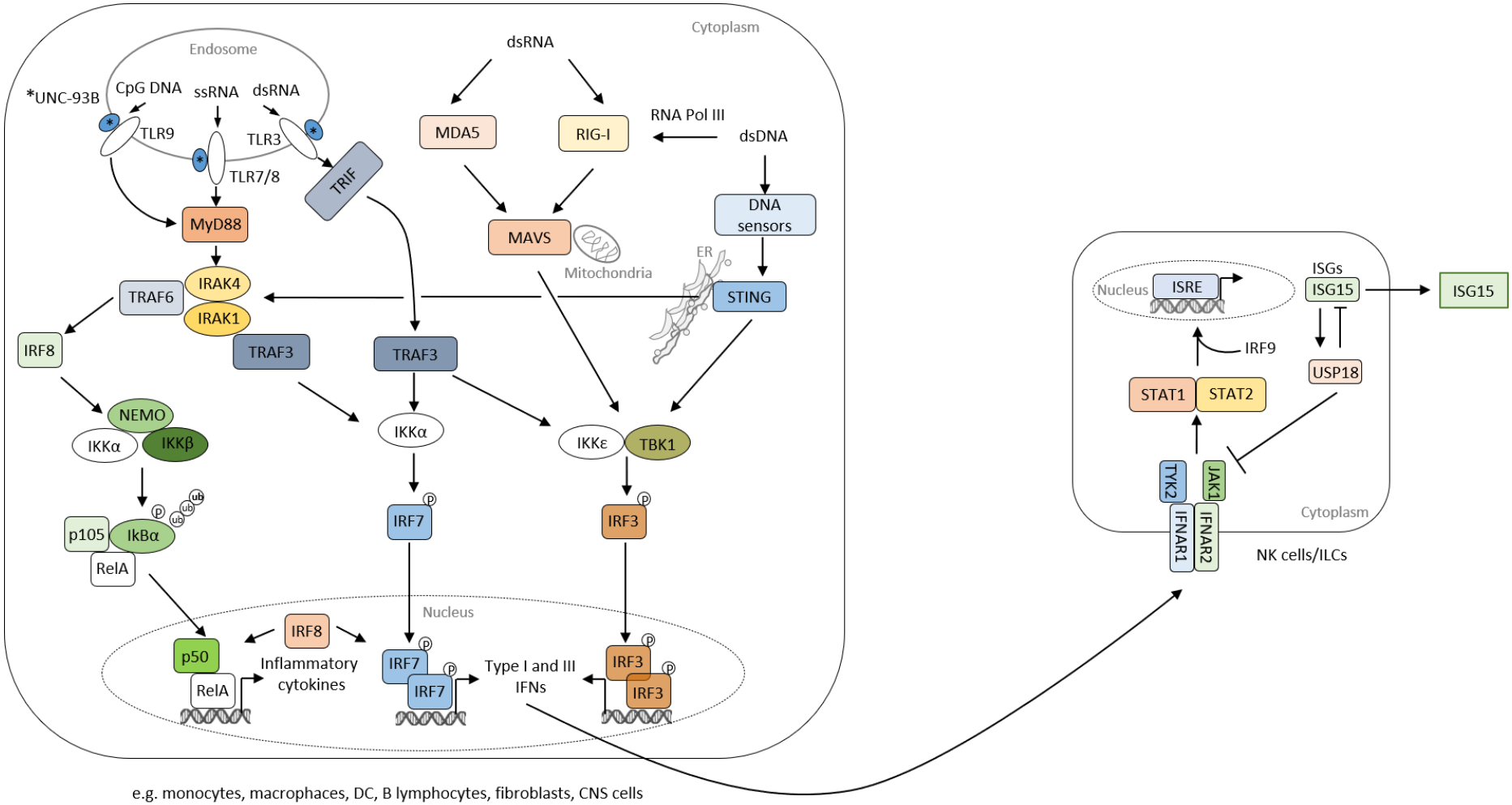

Figure 4: Recognition of viral nucleic acids by endosomal TLRs, cytosolic RLRs and DNA sensors. UNC-93B protein guides TLR7/8 and TLR9 trafficking from the ER to the endosome, where they recognize viral ssRNA and CpG DNA, respectively. These TLRs recruit MyD88, IRAK4 and IRAK1, which activate TRAF3 and TRAF6. TRAF6 mediates the activation of NF-κB, leading to the induction of inflammatory cytokine genes, whereas TRAF3 activates IKKα, which catalyzes the phosphorylation of IRF7 and induces type I IFN genes (classic MyD88-dependent pathway). After recognizing viral dsRNA, UNC-93B-dependent endosomal TLR3 recruits TRIF for the activation of TBK1 and IKKε, followed by the activation of IRF3 and upregulation of IFN production (alternative TRIF-dependent pathway). IRF8 facilitates the activation of NF-κB (shown here in the forms of its subunits p105, p50 and RelA) and IRF7. MDA5 and RIG-I recruit mitochondrial MAVS adapter molecules after the recognition of cytoplasmic viral dsRNA. Various cytoplasmic dsDNA sensors signal via the ER-located adapter molecule STING. The activation of these RNA and DNA signaling pathways results in the production of inflammatory cytokines via NF-κB activation, and IFN production via TBK1-IKKε-IRF activation. Secreted type I IFNs interact with their surface-expressed receptor IFNAR1/2, leading to the recruitment and activation of TYK2 and JAK1, which in turn phosphorylate and activate STAT1 and STAT2. STAT1/STAT2/IRF9 form complexes that interact with ISRE promoters for the induction of ISGs expression. Intracellular ISG15 is type I IFN-inducible and is involved in the USP18-dependent regulation of type I IFN and the prevention of type I IFN-dependent auto-inflammation. For all proteins shadowed in color mutations have been described. Adapted from (50).

Type I interferon responses

Host antiviral defense (Fig. 4) is based on the recognition of viral nucleic acid intermediates by membrane-bound TLRs or, in the cytoplasm, by intracellular viral sensors for DNA or RNA, such as RNA-sensing RIG-I, DNA sensors, and RLRs like melanoma differentiation-associated protein 5 (MDA5). Recognition of the microbial ligand by these receptors induces the production of antiviral mediators, such as type I and III interferon (IFN), via IFN regulatory factor (IRF) 3 and IRF7, through unique adaptors [TIR-domain-containing adaptor molecule 1 (TRIF), mitochondrial antiviral signaling protein (MAVS) and stimulator of IFN genes (STING)] (44,51). Type I and III IFNs then activate innate cells such as NK and ILCs to eliminate virus-infected cells. In more detail, type I IFNs (IFN-α, IFN-β, IFN-ε, and IFN-ω in humans) bind the type I IFN receptor (IFNAR), a heterodimer composed by the low affinity IFNAR1 subunit and the high affinity IFNAR2 subunit. The intracellular domain of the receptor then activates tyrosine kinase 2 (TYK2) and Janus-activated kinase 1 (JAK1), which initiate multiple pathways, including the induction of IFN stimulated genes (ISGs) by signal transducers and activators of transcription (STATs), activation of Ras-related protein 1 (RAP1) via CRKL phosphorylation, of the mitogen-activated protein kinase (MAPK) p38 pathway, and of the phosphatidyl-inositol 3-kinase (PI3K) signaling pathway. Upon phosphorylation, various STAT molecules form hetero- and homodimers. In particular, type I IFN signaling produces STAT1-STAT2 heterodimers, which combine with IRF9 to form the ISG factor 3 (ISGF3), and STAT1-STAT1 homodimers. ISGF3 translocates to the nucleus and binds to IFN-stimulated response elements (ISRE) in the promoter regions of ISGs, while STAT1 homodimers bind to IFN-γ-activated site (GAS) elements. STAT1 homodimers mediate also the main activation pathway downstream of the type II IFN (IFN-γ) receptor (IFNGR), a heterodimer formed by IFNGR1 and IFNGR2. Similar to IFNAR, its intracellular domain associates to the kinases JAK1 and JAK2, which phosphorylate STAT1 and initiate the IFN response as described above. There are numerous ISGs activated by IFN stimulation. They encode pattern recognition receptors that detect viral particles, transcription factors that increase IFN production, and proteins with direct antiviral activity, such as ISG15, an ubiquitin homologue that prevents virus-mediated degradation of IRF3, MXA, a GTPase with a strong antiviral activity both against cytoplasmic and nuclear viruses, and the 2′,5′-oligoadenylate

synthetase (OAS) proteins, that mediates viral RNA degradation (52). Type I and II IFN pathways are summarized in figure 5.

The importance of the strict regulation of these pathways has been shown by the study of type I interferonopathies, a clinically heterogenic group of hereditary diseases with a constitutive activation of this pathway (17), while their relevance and relative redundancy in anti-viral defense has been once again brought to light by several inborn errors of immunity. In parallel, several defect of the IFN-γ/IL-12 pathway underlie Mendelian susceptibility to mycobacterial disease (MSMD) (53).

The key role of type I IFN in host antiviral defense is indeed highlighted by reports on human AR IFNAR2 and STAT2 deficiencies, displaying a similar phenotype and incomplete penetrance (54–57). These patients typically present with complicated vaccine-strain measles after immunization with the live vaccine but can also suffer from life-threatening infections with other viruses. Homozygous mutations in both IFN-γ receptor 2 (*IFNGR2*) and *IFNAR1* were found in a child who suffered from *Streptococcus viridans* sepsis, severe Cytomegalovirus (CMV) infection and mycobacteriosis at the age of 2 months, and went on to develop hemophagocytic lymphohistiocytosis (HLH) (58). Of note, he later received attenuated measles, mumps and rubella virus vaccines without any adverse effect. The mutation in *IFNGR2* is LOF and considered responsible for the mycobacterial susceptibility, while the mutation in *IFNAR1* was predicted to be deleterious and the cause of the CMV infection (58). In an otherwise healthy child suffering from life-threatening acute respiratory distress syndrome during primary Influenza A virus (IAV) infection at the age of 2.5 years, compound heterozygous null mutations of IRF7 were identified (59). By the time at which this case was reported, the child had not suffered from other severe infections, implying that human IRF7 is largely redundant in host defense against most common viruses (59). AR IRF9 deficiency was recently characterized in a child who presented with complications after measles-mumps-rubella vaccine, recurrent viral infections and severe IAV infection requiring intensive care treatment, a clinical picture overlapping with both IRF7 and STAT2 deficiency (60).

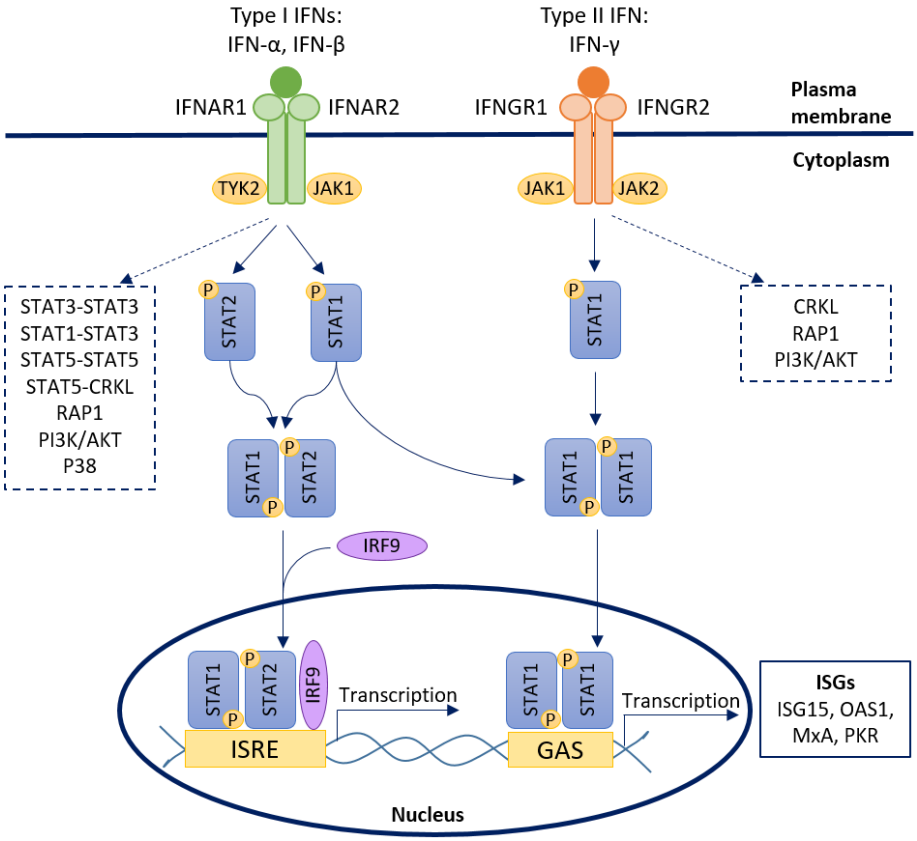

Figure 5. Activation of the JAK-STAT pathways by type I and type II interferons. Upon binding to their respective receptors, both composed by heterodimers, type I and type II interferons (IFN) activate the receptor-associated kinases TYK2, JAK1, and JAK2. They phosphorylate STAT molecules, allowing them to form homo- or heterodimers. STAT1-STAT2 heterodimers combine with IRF9 to form a complex called ISG factor 3, which translocates to the nucleus and binds to IFN-stimulated regulatory elements on the promoters of IFN stimulated genes (ISGs). STAT1-STAT1 homodimers bind instead to IFN-γ-activated site (GAS) elements on ISGs promoters.

CDC42 and the actin cytoskeleton

In eukaryotic cells, actin cytoskeleton formation and dynamics are essential for multiple cellular processes, such as mitosis, endocytosis, vesicular trafficking, cell migration and cytokinesis. Cytoskeleton mechanics rely on a multitude of highly conserved actin-binding proteins, which regulate nucleation, elongation, severing, branching, depolymerization and cross-linking of filamentous actin (F-actin) (Fig. 6). Wiskott–Aldrich syndrome protein (WASP) is one of the nucleation promoting factors that promote branching of F-actin via the actin-related protein 2/3 complex (Arp2/3) (61,62). Cytoplasmic WASP is in an auto-inhibited conformation that is activated following phosphorylation by cell division cycle 42 (CDC42), a member of the Rho family of GTPases. Prior to activating WASP, CDC42 needs to be loaded

with guanosine triphosphate by dedicator of cytokinesis 8 (DOCK8), a guanine nucleotide exchange factor (63).

Mutations in genes encoding the effector and regulatory proteins involved in the cytoskeleton have been detected in human IEIs (6). The prototype actin cytoskeleton disease is Wiskott-Aldrich syndrome, characterized by thrombocytopenia, eczema, autoimmunity, malignancy and susceptibility to infections. It is worth noting that immunodeficiency and vascular malformations are common features shared by other cytokine skeleton defects, such as DOCK8 deficiency and *CDC42* mutations. In particular, different *CDC42* defects cause either a constellation of developmental phenotypes with variable immunodeficiency, or a severe immune dysregulation syndrome with neonatal-onset autoinflammation (64–67).

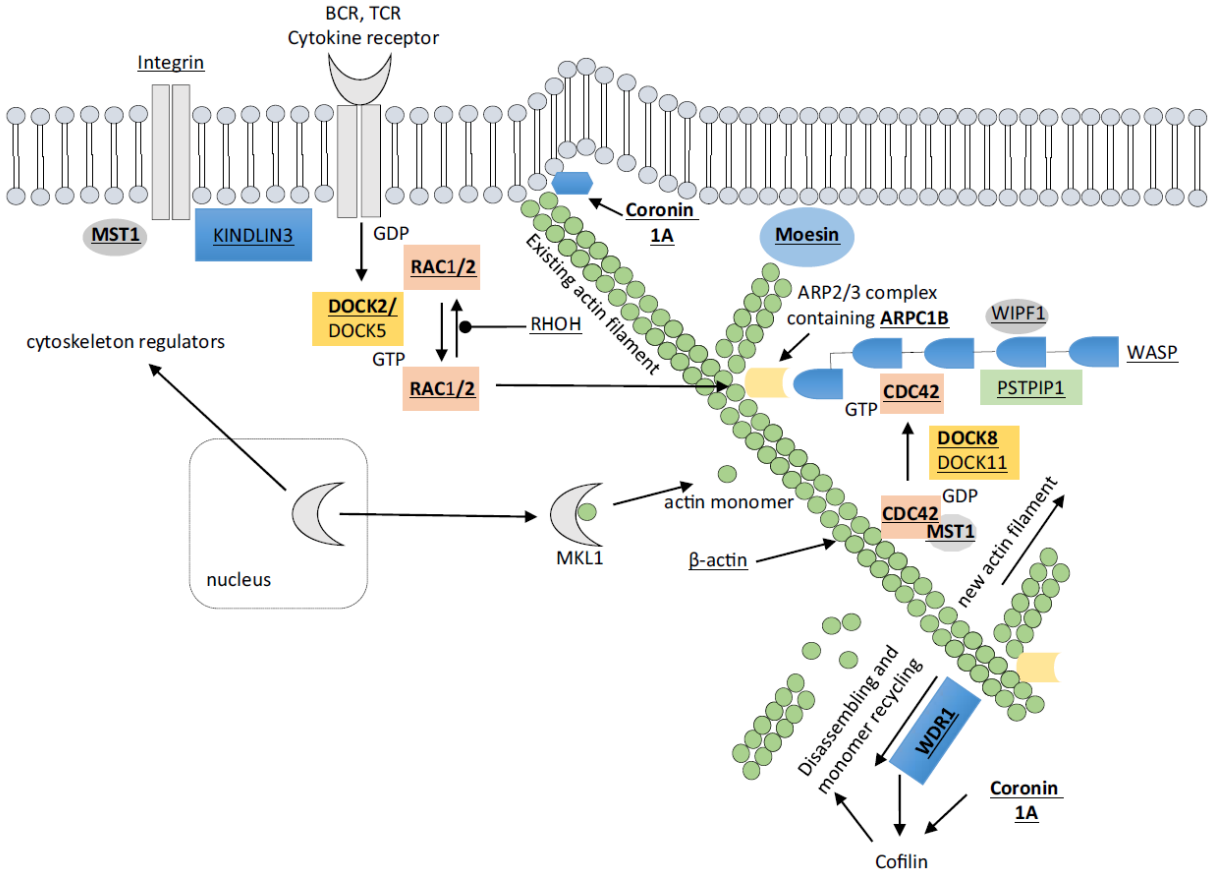

Figure 6. Actin cytoskeleton dynamics. Activated DOCK proteins convert inactive GDP-bound Rho family proteins (RAC, CDC42) to active GTP-bound forms. They, in turn, activate the actin nucleator Arp2/3 complex via WASP. WDR1 promotes the cofilin-dependent actin filament turnover. RHOH is a negative regulator of Rac GTPase-mediated signaling in hematopoietic cells. PSTPIP1 is a member of the leukocyte uropod that regulates endocytosis and cell migration. MKL1 regulates the pool of actin monomers available for filament assembly and gene expression of other actin regulators. Moesin and Coronin 1A protein are involved in linking assembled actin filaments to the plasma membrane. Coronin 1A and WDR1 augment F-actin severing via cofilin. Integrins and downstream signaling molecules (MST1, KINDLN3) are needed for the regulation of F-actin assembly and dynamics in response to the environment. Genes causing IEIs are underlined. Adapted from (68).

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OBJECTIVES

OBJECTIVES

The primary objective of this thesis is the identification of monogenic immune defects in children, especially those causing isolated life-threatening infections, and their functional characterization. A secondary objective is the expansion of the clinical and immunological phenotype of rare errors of intrinsic immunity. The foundation of this research approach rests on unbiased NGS-based genetic testing followed by in-depth functional studies, clinical description, and immune phenotyping.

In chapter 1, we uncover the molecular mechanism of a novel defect affecting the type I interferon receptor 1 (IFNAR1) and causing susceptibility to overwhelming infection with live vaccines. The children of two unrelated families suffered from severe disseminated disease caused by vaccine-strain viruses, namely measles after measles-mumps-rubella inoculation and yellow fever vaccine-associated viscerotropic disease after yellow fever vaccination. After identification by WES of candidate variants in the IFNAR1 gene, we performed a complete assessment of their consequences in vitro (at the gene, protein and cell level) to establish their pathogenicity.
This chapter clearly illustrates the potential of an unbiased approach with NGS in the discovery of rare inborn errors of intrinsic immunity with no obvious immunological phenotype and an apparently unremarkable presentation.

Chapter 2 and chapter 3 focus on better understanding the genetics, pathophysiological mechanisms, clinical presentation, and immunological phenotype of patients with rare IEIs.
In chapter 2, we examine a cohort of patients with STAT2 deficiency. Discovered in 2014, STAT2 deficiency causes severe disseminated vaccine-strain measles after inoculation and other fatal viral illnesses. The expertise in the IFN-STAT pathway acquired during the previous project was applied to the collection and study of newly identified patients with STAT2 loss-of-function mutations. Extensive in vitro analyses add new molecular insight to the previously described variants in STAT2, and a more complete picture of the clinical and immunological manifestations of the disease is provided.
Finally, in chapter 3 we apply WES to diagnose a patient with a complex syndrome manifesting with dysmorphisms, developmental delay, thrombocytopenia, recurrent lung

infections and systemic inflammation. We discover a *de novo* mutation in *CDC42*, encoding a Rho GTPase essential for the activation of WASP and actin cytoskeleton dynamics, and we expand the already heterogeneous clinical and immunological phenotype of this syndrome.

CHAPTER 1

Inherited IFNAR1 deficiency in otherwise healthy patients with adverse reaction to measles and yellow fever live vaccines

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Abstract

Vaccination against measles, mumps, and rubella (MMR) and yellow fever (YF) with live attenuated viruses can rarely cause life-threatening disease. Severe illness by MMR vaccines can be caused by inborn errors of type I and/or III interferon (IFN) immunity (mutations in *IFNAR2*, *STAT1*, *STAT2*). Adverse reactions to the YF vaccine have remained unexplained. We report two otherwise healthy patients, a 9-year-old boy in Iran with severe measles vaccine disease at 1 year and a 14-year-old girl in Brazil with viscerotropic disease caused by the YF vaccine at 12 years. The younger sister of the Iranian patient died following MMR vaccination. We find a homozygous *IFNAR1* mutation in the Iranian patient and compound heterozygous *IFNAR1* mutations in the Brazilian patient. The three mutant alleles are loss-of-expression and loss-of-function. Patient-derived fibroblasts fail to activate the JAK-STAT pathway and to induce IFN-stimulated genes (ISGs) after treatment with IFN-α2b or -β. Moreover, we show that patients' fibroblasts are highly susceptible to viruses, including the YF and measles virus vaccine strains, in the absence or presence of exogenous type I IFN. Finally, we rescue the fibroblasts' phenotypes with wild-type *IFNAR1.* We thereby demonstrate that autosomal recessive, complete IFNAR1 deficiency can result in life-threatening complications of vaccination with live attenuated measles and YF viruses in previously healthy children and adolescents.

Introduction

There are currently eleven live attenuated viral (LAV) vaccines in use worldwide. All have been shown or suspected to cause life-threatening disease in rare individuals. So far, severe disease by only three LAV vaccines has been explained by inborn errors of immunity, in at least some patients. Live (oral) poliovirus (OPV) vaccine can cause vaccine-associated paralytic poliomyelitis in patients with agammaglobulinemia due to a variety of inborn errors of T or B cells (1). Oral rotavirus vaccine can cause life-threatening dehydration in patients with severe combined immunodeficiencies (2). The measles vaccine strain in the measles, mumps, and rubella (MMR) vaccine can cause disseminated infections in patients with inborn errors of IFNAR2 (3), STAT1 (4), or STAT2 (5,6), which control cellular responses to various IFNs (type I only in the case of IFNAR2, type I and type III IFNs for STAT2, and all three types for STAT1). Vaccine-strain mumps infections are extremely rare, with only one life-threatening case reported in a patient with RAG1 deficiency and a lack of T and B cell adaptive immunity (7). Patients with inborn errors of IRF7 (8), which controls the amplification of type I and III IFNs, or IRF9 (9), which controls the cellular responses to these IFNs, have been reported to suffer from an ill-defined adverse reaction to MMR vaccination. Most cases of severe MMR vaccine disease remain unexplained. None of the life-threatening adverse reactions to live vaccines for rubella virus, yellow fever virus (YFV), influenza virus, varicella zoster virus, vaccinia virus, or hepatitis A virus have been explained genetically. We studied two otherwise healthy patients who developed disseminated, life-threatening disease following LAV vaccination: a 9-year-old Iranian boy following MMR vaccination at 1 year, whose younger sister died at 1 year following MMR vaccination, and a 14-year-old Brazilian girl following YF vaccination at 12 years.

Results

Two unrelated patients with adverse reactions to measles and YF vaccines

We studied two unrelated patients whose pedigrees are shown in Figure 1A. Full case reports are available in the supplemental materials. Briefly, patient 1 (P1) was born in Iran to consanguineous parents and presented at one year of age with disseminated vaccine-strain measles (Fig. 1A). About ten days after inoculation with the MMR vaccine, he presented with a generalized exanthema, fever and neurological symptoms consistent with encephalitis. A

computed tomography (CT) of the brain showed mild cerebral edema and analysis of cerebrospinal fluid (CSF) demonstrated leukocytosis (350 lymphocytes/μL, 2 neutrophils/μL). PCR for measles was positive both in blood and CSF. He had no notable past medical history. P1 is now 9 years old, in good health and has not manifested any other invasive infections. P1 had a younger sibling who died four weeks after routine MMR vaccination at age 1 year but for whom material was not available for genetic analysis. His mother electively terminated a third pregnancy following prenatal genetic identification of the same homozygous variant in *IFNAR1.* Patient 2 (P2) is a 14-year-old Brazilian girl born to non-consanguineous parents (Fig. 1A). Otherwise healthy until age 12, she presented with fever, hypotension, vomiting, and lethargy 7 days following YF vaccination. Her condition deteriorated, she developed renal and hepatic dysfunction and required intubation and mechanical ventilation. A CT scan of the chest demonstrated pleural effusions and atelectasis. YFV RNA was detected in the patient's blood by PCR. Sequencing confirmed that this was the vaccine-strain virus (Figure S1) (10). A diagnosis of yellow fever vaccine-associated viscerotropic disease (YEL-AVD) was made. She does not belong to any known risk groups for YEL-AVD (11). One year after recovery, she remains healthy. Notably, P2 had received two MMR vaccinations at 12 and 16 months of age, and other live vaccines (oral poliovirus and bacillus Calmette-Guérin), without incident. She developed neutralizing antibodies to YFV in levels associated with seroprotection and seropositivity to measles, rubella, diphtheria, and tetanus.

Bi-allelic private nonsense or splicing *IFNAR1* variations in the patients

We performed whole exome sequencing (WES) analysis on both patients. The percentage of homozygosity was 2.9% and 0.25% in P1 and P2, respectively, consistent with the former being born to consanguineous parents. Principal component analysis (PCA) confirmed the ethnicities of the two families (12). We then analyzed copy number variations (CNVs) and single nucleotide variations (SNVs). We filtered out noncoding and synonymous SNVs, other than those at exonic and intronic essential splice sites. We additionally filtered out common variants (minor allele frequency [MAF] >0.01), variants predicted to be benign (combined annotation dependent depletion [CADD] score below the mutation significance cutoff [MSC]), and variants occurring in very frequently damaged genes (those with a gene damage index [GDI] >13.84) (13–15). Finally, we tested a model of autosomal recessive (AR) inheritance and

focused our attention on genes with homozygous or compound heterozygous lesions.

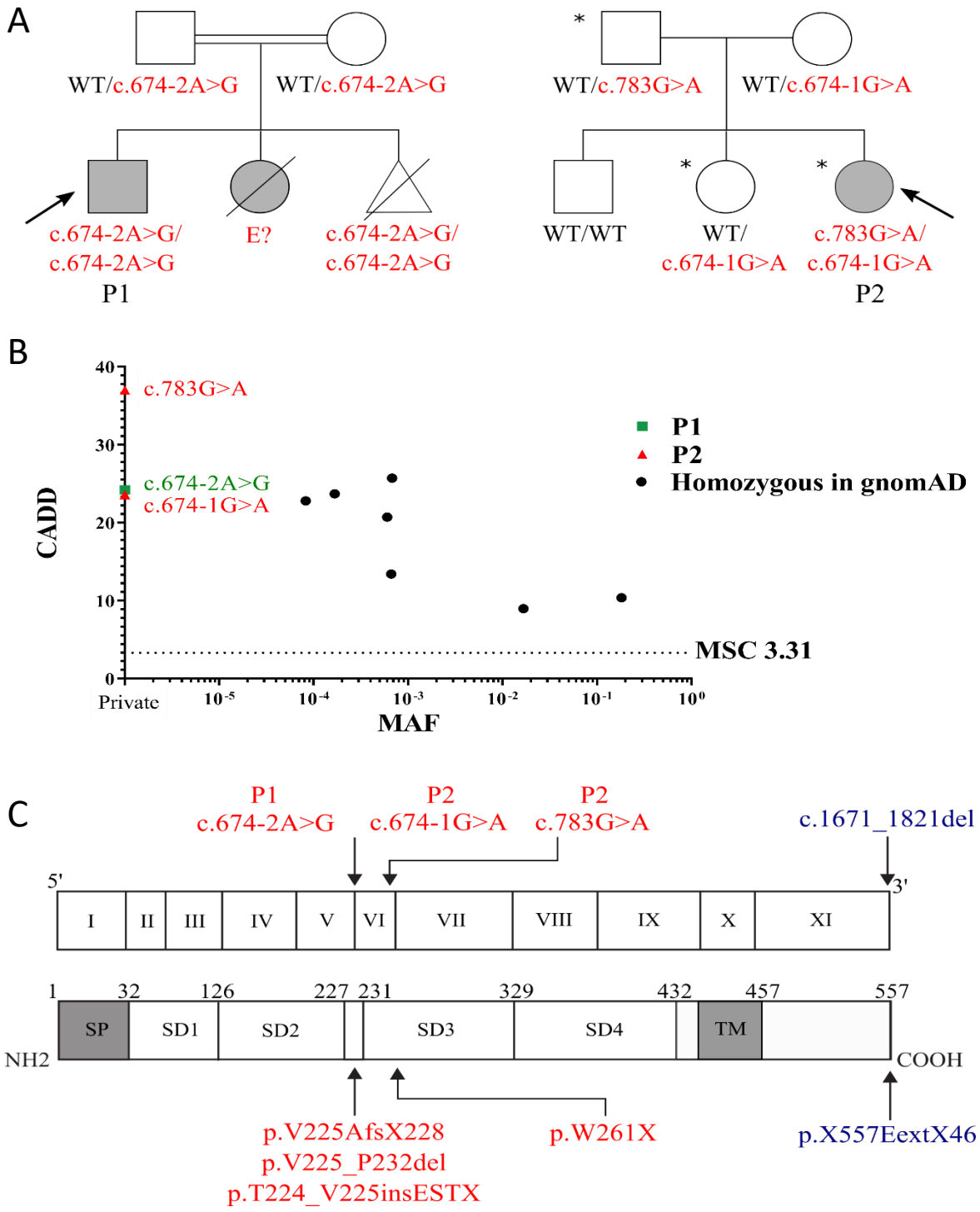

Figure 1. Three private *IFNAR1* variants are present in two kindreds associated with life-threatening LAV vaccine disease. A) Pedigrees of the IFNAR1-deficient families. Asterisks demarcate those individuals who received vaccination against YFV. B) Population genetics of homozygous coding missense and predicted loss-of-function *IFNAR1* mutations taken from GnomAD and in-house cohorts. The patients' variants are private and shown in red and green, while seven variants detected in GnomAD are shown in black. MAF, mean allele frequency; MSC, mutation significance cutoff. C) Schematic illustration of the *IFNAR1* gene with 11 coding exons (I-XI) and of the IFNAR1 protein with its four fibronectin type III subdomains (SD1–4). SP, signal peptide; TM, transmembrane domain. The previously reported *IFNAR1* variant is indicated in blue, while those of P1 and P2 are indicated in red.

In P1, we found a private homozygous mutation in *IFNAR1*, c.674-2A>G, among 48 variants in 39 genes (Table S1). In P2, 11 variants in 6 genes passed filtering criteria (Table S1), including two variants in *IFNAR1*, c.783G>A (p.W261X) and c.674-1G>A (Fig. 1C). None of these three *IFNAR1* variants are found in online databases including 1000 Genomes, Bravo, and gnomAD (Genome Aggregation Database) – a database that encompasses the ExAC (exome aggregation consortium) database (Fig. 1B). The *IFNAR1* gene, together with *IFNAR2*, encodes the heterodimeric receptor for the type I IFNs (16,17). The three *IFNAR1* mutations are predicted to be deleterious by either disrupting the same essential splice site, (c.674-2A>G and c.674-1G>A, in P1 and P2, respectively), or by creating a premature stop codon (p.W261X in P2). The three mutations were confirmed by Sanger sequencing of the patients and their relatives, which also verified that the mutations present in P2 were compound heterozygous (Fig. 1A and S2A). We did not have genomic DNA from P1's sister who died in her first year of life following MMR vaccination. A total of only 7 non-synonymous or splicing *IFNAR1* variants were found in homozygosity in gnomAD, all of which were missense (Fig. 1B). Collectively, these findings suggested that the two patients had a novel and rare inborn error of immunity, AR IFNAR1 deficiency.

The three mutant *IFNAR1* alleles encode truncated proteins

Because the c.674-1G>A and c.674-2A>G mutations occur at an essential splice acceptor site of exon 6, we hypothesized that they would lead to an aberrantly spliced transcript (Fig. 1C). We therefore amplified the *IFNAR1* cDNA from SV40 large T antigen-transformed fibroblasts (SV40-F cells) derived from both patients and a healthy control (Fig. 2A, S2B and S2C) and performed Sanger sequencing on individual cDNA clones. All *IFNAR1* transcripts present in healthy control cells contained normal splicing at exon 5-6 and 6-7 junctions, whereas three alternative splice products were observed in P1 and P2. In both patients, about 60% of transcripts lacked exon 6, which induces a frameshift and a premature stop at amino acid (aa) 228 (p.V225AfsX228); 30-40% of transcripts lacked the first 24 base pairs of exon 6 in P1 and P2, which results in the deletion of aa 225 to 232 (p.V225_P232del); a trace amount of transcripts contained a 56 bp segment retained from intron 5, which introduces a premature stop after three amino acids (p.T224_V225insESTX). For simplicity, we will refer to these mutations as V225fs, V225_P232del, and T224ins. A small percentage of transcripts from P2's

cells, about 10%, were found to contain normal splicing at exon 5-6 and 6-7 junctions, yet the nonsense mutation c.783G>A, p.W261X (hereafter W261X) was present in all such transcripts. Western blot analysis of HEK293T cells overexpressing the 4 patient-derived transcripts and WT *IFNAR1* transcripts showed that all 4 proteins are expressed but with a molecular weight (MW) lower than the WT protein (Fig. 2B). These findings suggested that any proteins encoded by the three mutant *IFNAR1* alleles are not functional.

The patients' cells do not express IFNAR1 and do not respond to type I IFNs

qRT-PCR on patients' cells showed a strong reduction of *IFNAR1* mRNA when compared with healthy controls, suggesting nonsense-mediated decay (Fig. S2D). We tested if any IFNAR1 protein was expressed in patients' cells by western blotting and flow cytometry (Fig. 2C, 2D, 2E, and S3A). We observed a complete lack of IFNAR1 expression in patients' SV40-F and B-lymphoblastoid cell lines (B-LCLs) when compared with healthy controls and patients with other defects in the type I IFN signaling pathway, such as IRF7 -/-, STAT1 -/-, STAT2 -/-, and IRF9 -/- patients (Fig. 2C, 2D, 2E, and S3A). As previously reported, we detected severely decreased expression of IFNAR1 in TYK2-deficient cells (18). IFNAR2 surface expression was normal in both patients' cells (Fig. S3B). In summary, IFNAR1 expression was undetectable in both fibroblastic and lymphoid cell lines derived from both patients. We cannot exclude the remote but finite possibility that residual levels of IFNAR1 are expressed on the surface of other cells. We next tested type I IFN signaling in patients' fibroblasts. Phosphorylation of STAT1 and STAT2 was abolished in patients' SV40-F cells in response to IFN-α2b (Fig. 2F). Moreover, patients' SV40-F cells did not substantially upregulate expression of IFN stimulated genes (ISGs) *MX1* and *IFIT1* in response to IFN-α2b and IFN-β, whereas their *CXCL9* induction in response to IFN-γ was intact and elevated to a level above that of healthy controls, in contrast to previous reports suggesting that the absence of type I IFN signaling impairs type II IFN signaling (Fig. 2G) (19). This result indicates that the patients have major defects in type I IFN signaling with impairment of *MX1* and *IFIT1* induction similar to other deficiencies in this pathway, including STAT1, STAT2, IRF9, and IFNAR2. Altogether, these data showed that the patients' fibroblasts had IFNAR1 deficiency, with a complete lack of responses to IFN-α2b and -β. We did not exclude the possibility that these or other cells can respond to some of the 15 other type I IFNs, at least for some ISGs.

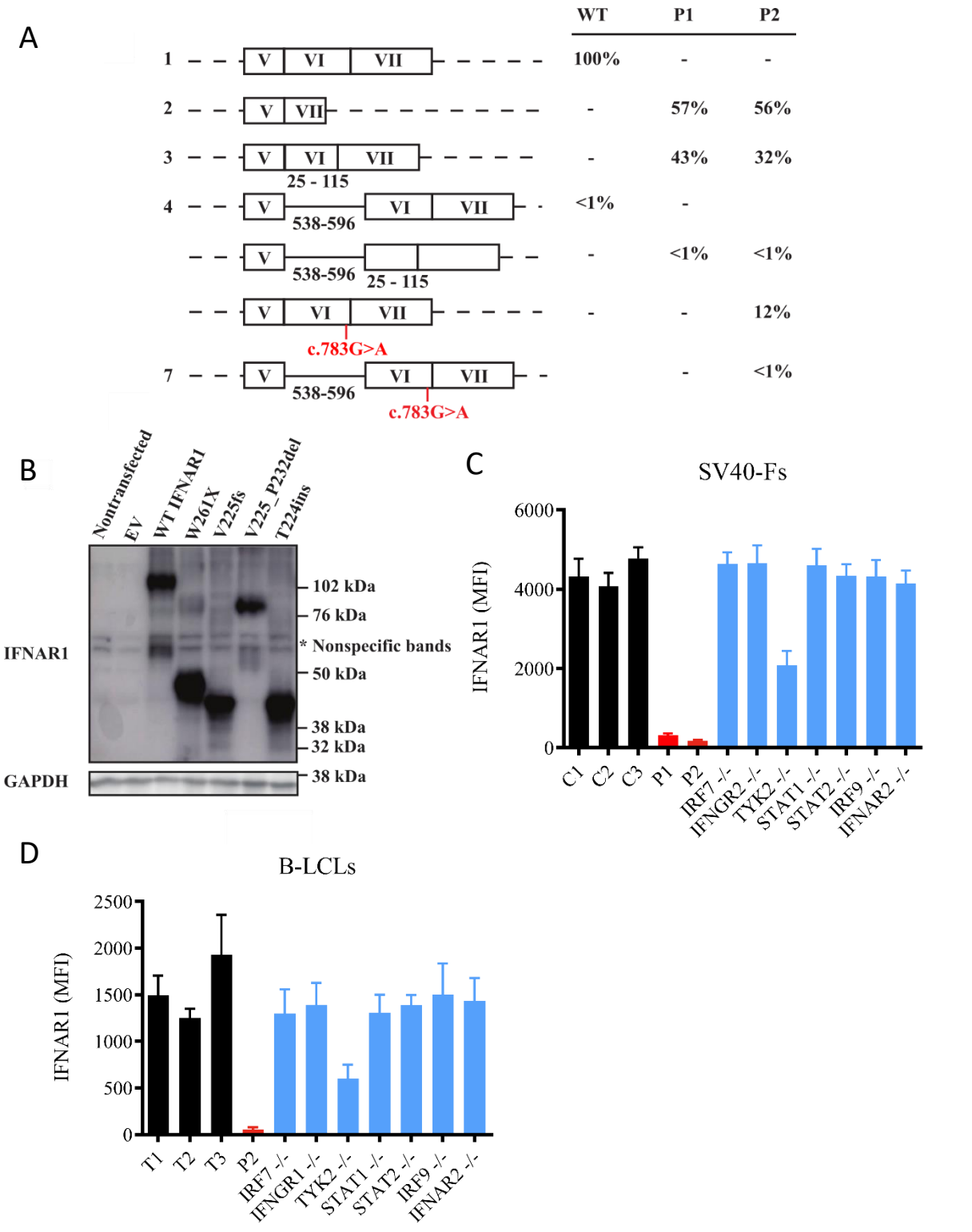

Figure 2. Impact of IFNAR1 variants on type I IFN signaling. A) cDNA sequencing results demonstrate aberrant splicing of *IFNAR1* mRNA from patients' SV40-F cells. At least 150 transcripts were sequenced for each individual. The black numbers under the schematic representations indicate the positions of the included elements relative to their start sites. B) Western blot of IFNAR1 in HEK293T cells transiently transfected with IFNAR1 constructs. C, D) Mean fluorescence intensity (MFI) of IFNAR1 staining on SV40-F (D) and B-LCL (F) cells from three healthy controls (C1, C2, and C3 or T1, T2, and T3), P1, P2, IRF7−/−, IFNGR2−/−, IFNGR1−/−, TYK2−/−, STAT1−/−, STAT2−/−, IFNAR2-/- and IRF9−/− patients as assessed by flow cytometry.

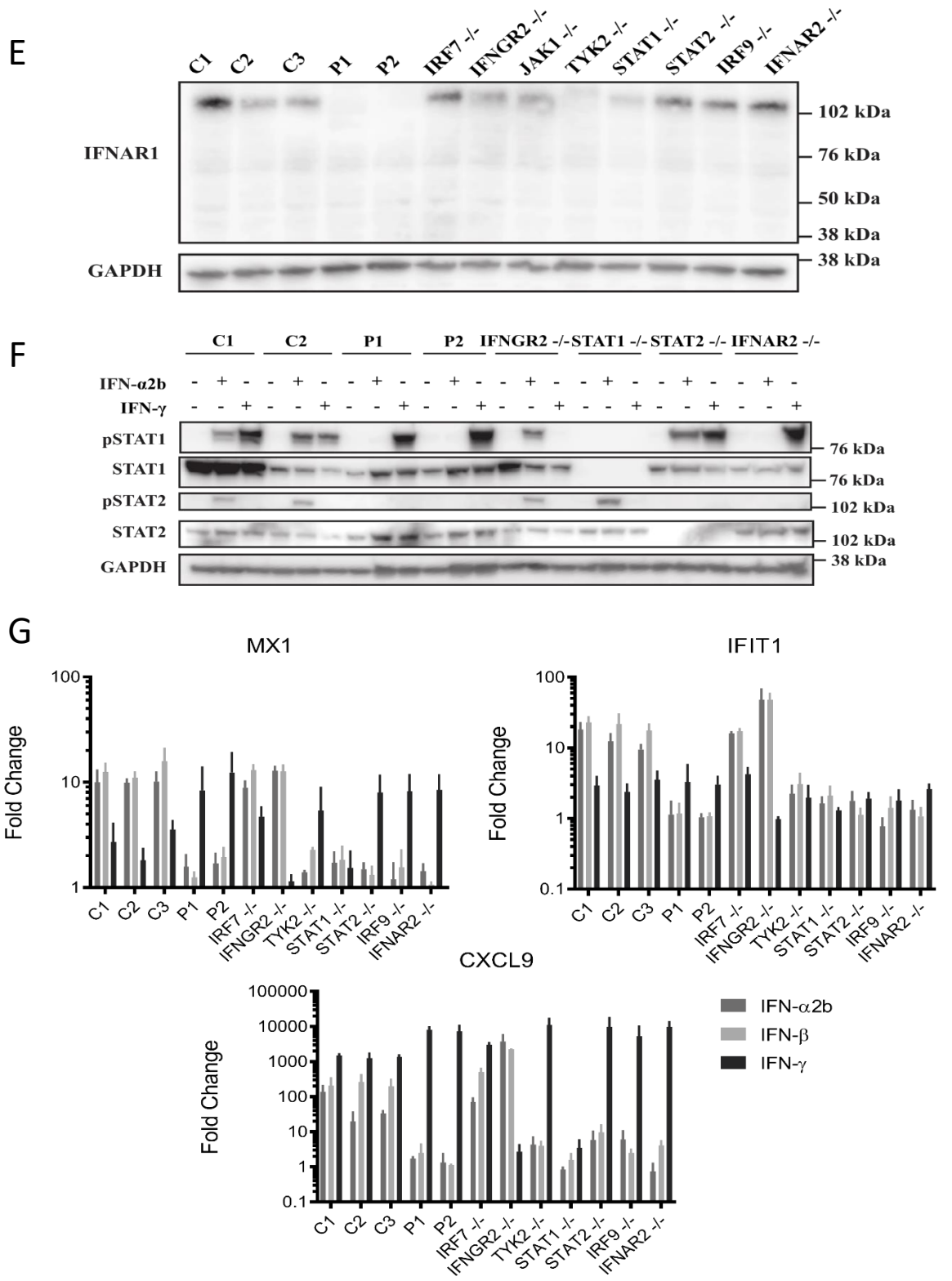

Figure 2 - continued. E) Western blot of endogenous IFNAR1 in SV40-F cells from three healthy controls (C1, C2, and C3), P1, P2, IRF7−/−, IFNGR2−/−, TYK2−/−, STAT1−/−, STAT2−/−, IRF9−/−, and IFNAR2−/− patients. F) Western blot analysis of phosphorylated STAT1 (pSTAT1) and pSTAT2 levels in SV40-F cells stimulated with 1,000 U/ml of IFN-α2b or IFN-γ for 20 min. Cells were from two healthy controls (C1, C2), P1, P2, IFNGR2−/−, STAT1−/−, STAT2−/−, and IFNAR2−/− patients. G) Transcription levels of MX1, IFIT1, and CXCL9 assessed by qRT-PCR on SV40-F cells treated with 1,000 U/ml of either IFN-α2b, -β, or -γ for 2 h. Cells were from three healthy controls (C1, C2, and C3), P1, P2, IRF7−/−, IFNGR2−/−, TYK2−/−, STAT1−/−, STAT2−/−, IRF9−/−, and IFNAR2−/− patients.

The patients' fibroblast-intrinsic type I IFN immunity to viruses is impaired

To test if patients' cells were able to control viral infections in the presence of type I IFNs, we pretreated SV40-F cells from both patients with IFN-α2b and infected them with VSV, a virus commonly used in testing type I IFN responses (Fig. 3A). When pre-treated with IFN-α2b, healthy control cells were able to control VSV replication 24 hours after infection. In contrast, viral replication could not be suppressed in patients' cells treated with IFN-α2b, a finding also seen in IFNAR2, STAT1, STAT2, and IRF9-deficient patient cells. We observed similar results with YFV-17D, a vaccine strain highly similar to that responsible for P2's infection (Fig. 3B). Interestingly, IRF7-deficient cells were able to control YFV-17D replication to the same degree as healthy controls, in the presence or absence of exogenous IFN-β (Fig. 3B). To re-assess if the patients' cellular phenotypes were caused by the lack of normal IFNAR1, we transduced patients' primary fibroblasts with wild-type (WT) *IFNAR1*, *IFNAR2*, or an empty vector (EV). Only transduction with *IFNAR1* was able to rescue the defect in IFNAR1 expression levels (Fig. S3C and S3E) and patients' responses to type I IFN as assessed by induction of *MX1* and *IFIT1* following IFN-α2b or -β stimulation (Fig. 3C). Importantly, transduction with WT *IFNAR1* rescued viral titers, thus rescuing, at least partially, the phenotype of increased VSV, measles virus, and YFV susceptibility in P1 and P2's fibroblasts (Fig. 3D, 3E, S3F, and S3H). Then, to assess the extent to which the cellular phenotype replicated the patients' infectious phenotype, we also infected patients' cells with influenza A virus (IAV, A/CA/07/2009/H1N1 strain) and herpes simplex virus-1 (HSV-1), two viruses they had been exposed to without developing severe disease (Fig. S4A-S4F). Again, the patients' SV40-F cells supported higher levels of viral replication than healthy controls' for both HSV-1 (Fig. S4A-S4D) and for IAV (Fig. S4E and S4F). We also assessed the ability of patients' SV40-F cells to control viral replication of YFV-Asibi and Zika viruses (Fig. S4G-S4J). Consistent with other viruses, both patients displayed an inability to control viral replication even following pretreatment with exogenous IFN-β when compared to cells from a healthy control (Fig. S4G-S4J). Collectively, these data indicate that IFNAR1 deficiency results in a broad defect in fibroblast-intrinsic immunity. Finally, in the course of viral infection, we quantified the production of IFN-β, known as the fibroblastic IFN because it is the predominant type I IFN produced by these cells. We observed that elevated amounts of IFN-β were produced in IFNAR1-deficient cells upon infection by YFV-17D, when compared with healthy control cells, indicating that the observed

antiviral defects arose from abolished type I IFN signaling, and not from impaired IFN-β production (Fig. S3G). These findings also confirmed that IFN-β production, unlike that of other type I IFNs, does not require IFNAR1- and IRF7-dependent amplification (20). Altogether, these data showed that cell-intrinsic type I IFN immunity to VSV, YF vaccine-strain virus, IAV, HSV-1, and measles vaccine-strain virus was abolished in the patients' cells. These findings strongly suggest that AR IFNAR1 deficiency underlies measles vaccine disease in P1 (and by inference in his deceased sister) and YEL-AVD in P2 by disruption of cell-intrinsic type I IFN immunity to viruses.

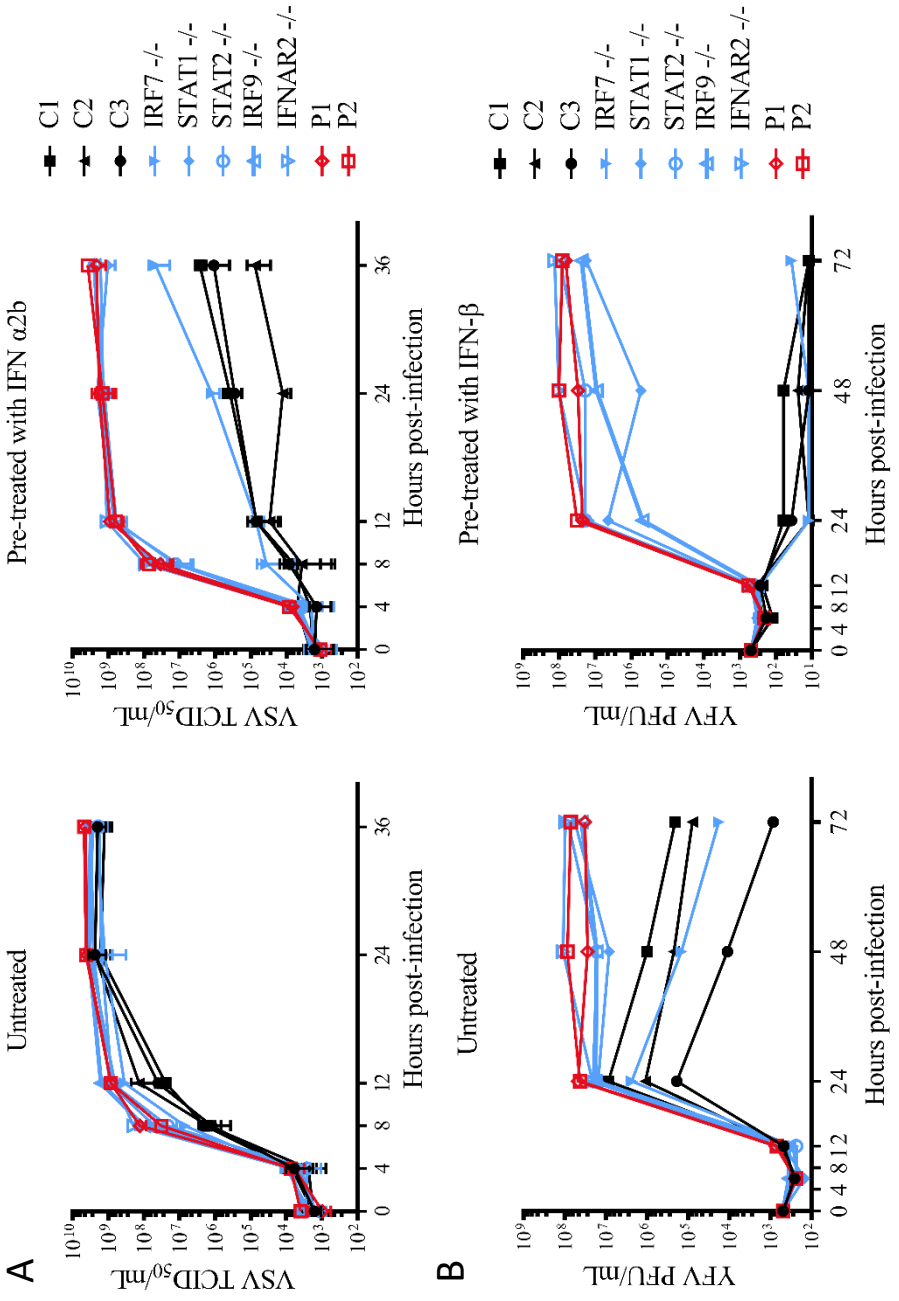

Figure 3. IFNAR1 is required for type I IFN–mediated cell intrinsic immunity to viral infections. A) VSV titers in SV40-F cells unstimulated (left) or pretreated (right) with 1,000 U/ml IFN-α2b for 16 h, followed by VSV infection at an MOI of 3. B) YFV titers in SV40-F cells unstimulated (left) or pretreated (right) with 1,000 U/ml IFN-β for 16 h, followed by infection with YFV-17D at MOI = 0.05. Mean and range (n = 2) are shown. Cells from three healthy controls (C1, C2, and C3), P1, P2, IRF7−/−, STAT1−/−, STAT2−/−, IRF9−/−, and IFNAR2−/− patients were included in (A, B).

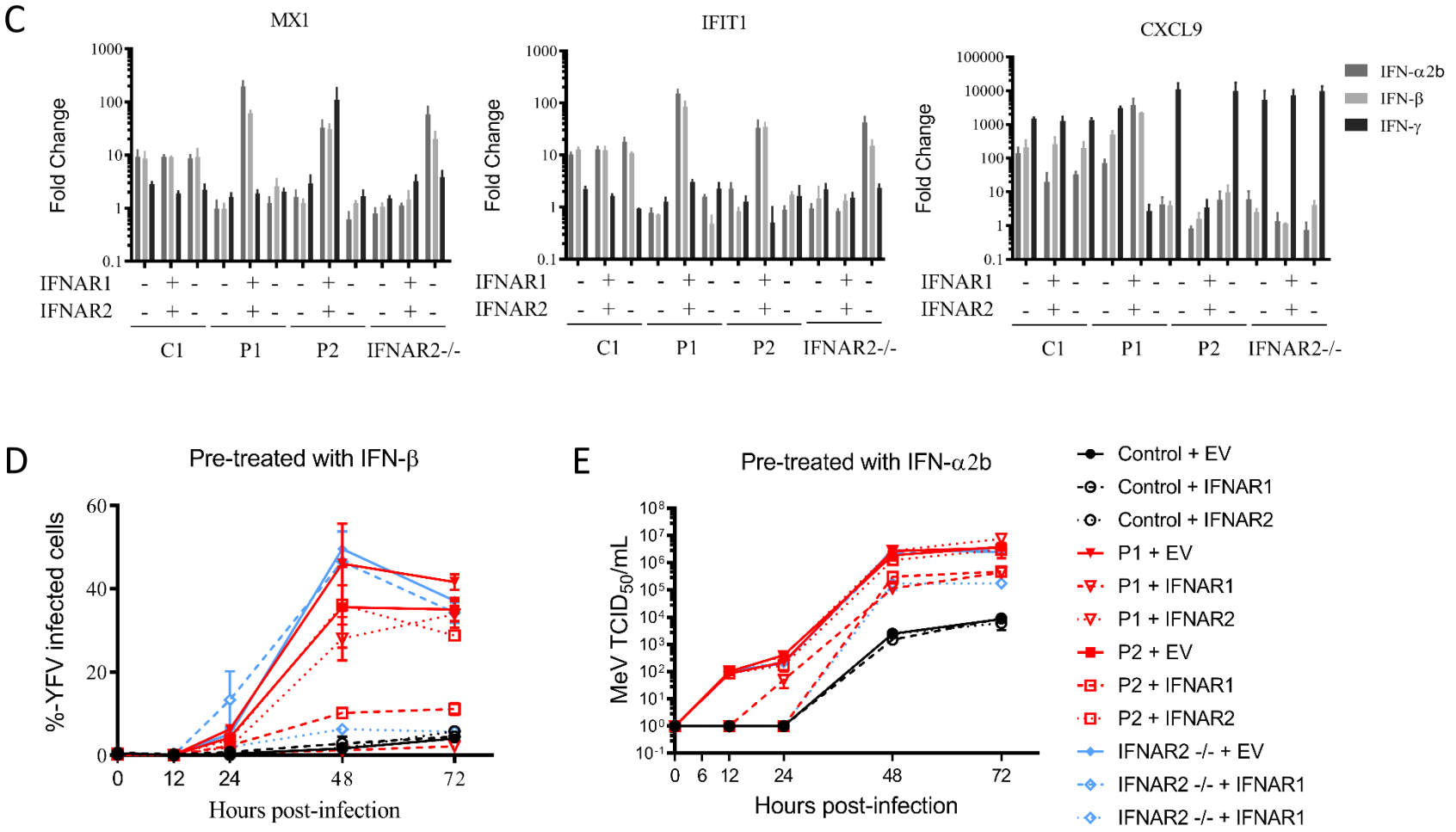

Figure 3 – continued. C) Transcription levels of *MX1*, *IFIT1*, and *CXCL9* assessed by qRT-PCR on SV40-F cells treated with 1,000 U/ml of IFN-α2b, -β, or -γ for 2 h. D) SV40-F cells were infected with YFV-17D at an MOI of 0.05, and the percentage of cells positive for YFV viral proteins was assessed by flow cytometry. Cells were stimulated with 1,000 U/ml IFN-β for 16 h before infection. E) SV40-F cells were infected with MeV at an MOI of 0.1, and viral titer was assessed at the given time points. Cells were stimulated with 1,000 U/ml IFN-α2b for 16 h before infection. Cells were from a healthy control, P1, P2, and an IFNAR2−/− patient that were transduced with WT *IFNAR1*, WT *IFNAR2*, or EV in (C, D, E). TCID, tissue culture infectious dose.

Discussion

We herein describe AR IFNAR1 deficiency in two unrelated families. We demonstrate that this monogenic error of immunity can underlie life-threatening, isolated measles vaccine disease and YEL-AVD in previously healthy children and adolescents. Patients with complete forms of AR IFNAR2, STAT1, and STAT2 deficiencies have also presented with life-threatening complications following MMR vaccination (Table 1) (3–6,21), whereas IRF7- or IRF9-deficient patients are apparently less susceptible to measles vaccine infection (8,9). Interestingly, patients deficient in JAK1 or TYK2, whose cells show impaired but not abolished response to type I IFNs, have not been reported to experience disease following MMR vaccination (22–25). Collectively, these data differ from experimental work done in mice, where STAT1 appears dispensable for defense against both WT and attenuated measles viruses (26,27). They suggest that a set of genes jointly regulated by human IFNAR1, IFNAR2, STAT1, and STAT2, and perhaps by IRF7 and IRF9, are critical for control of vaccine-strain measles virus (26,27). Furthermore, the fact that IFNAR1 and IFNAR2 deficiencies both result in measles virus vaccine related-disease, while deficiency in IL-10RB, a subunit of the type III IFN receptor, has not been reported to produce this infectious phenotype, suggest that type I IFN signaling is more critical for defense against measles vaccine-strain virus than type III IFN signaling (28–31). Our data also establish IFNAR1 deficiency as the first genetic etiology for YEL-AVD – and as its first explanation (11). Despite the development of the YF vaccine in the 1930's, YFV remains a global health threat partially due to deficient rates of vaccination. As the eponymous flavivirus, the study of YFV and its vaccine is of interest to studies of other flaviviruses. Chimeric YF viruses in which prM-E sequences are introduced into the YF vaccine virus backbone are the basis of ongoing vaccine trials against dengue and Japanese encephalitis viruses (32) with controversial results in clinical trials (33,34). Although still considered a safe and effective vaccine, the uncertainty regarding the cause(s) of YEL-AVD have encouraged attempts to develop an inactivated alternative vaccine (35–37). The difficulties encountered while attempting to develop safer vaccines related to YFV-17D for the past decade underline that a better understanding of host defenses against the virus is necessary. Recent studies have demonstrated that the YFV-17D virus promotes the induction of critical type I IFN mediators such as STAT1 and IRF7 in humans (38–40).

Table 1. Patients suffering from MMR vaccine-related disease.

Gene mutated	Age (mo) at first MMR vaccination	Age (yr) at last known follow-up	Clinical manifestations
STAT2 P1 (5)	18	5	P1: 6 d following MMR vaccination, the patient developed fever, rash, conjunctivitis, and lymphadenopathy, followed by hepatitis and pneumonitis requiring supplemental oxygen. Patient recovered from MMR infection.
STAT2 P2, P3 (21)	12	3	P2: 1 wk after MMR vaccination, the patient developed a febrile illness. Readmitted 1 mo later with opsoclonus-myoclonus and lymphocytes in the CSF, readmitted again at 2.5 yr with impaired renal and liver function, opsoclonus-myoclonus, and seizures. Patient recovered from MMR infection.
	13	2.5	P3: 1 wk after MMR vaccination, the patient developed a febrile illness, anemia, and lymphopenia. CSF was positive for mumps virus by PCR. Readmitted at 15 mo due to persistent fever and malaise, eventually developed septic shock and metabolic acidosis. Patient recovered from MMR infection.
STAT2 P4, P5 (6)	24	7 (deceased)	P4: 1 wk after MMR vaccination, the patient developed a morbilliform rash, tonsillitis, conjunctivitis, lymphadenopathy, hepatosplenomegaly, and arthritis. Patient recovered from MMR infection. Patient experienced frequent severe viral infections during the first 3 yr and died at 7 yr of fulminant viral infection.
	18	11	P5: 1 wk after MMR vaccination, the patient developed disseminated measles, complicated by hepatitis and pneumonitis. Patient recovered from MMR infection.
IFNAR2 (3)	13	1.4 (deceased)	6 d after MMR vaccination, the patient developed a marked injection-site reaction and generalized morbilliform skin rash, recurrent maculopapular rash, fever, and intractable seizures. Patient died on day 81 after vaccination.
IRF7 (8)	12	9	1 wk after MMR vaccination, the patient developed a rash suggestive of measles. Patient recovered from MMR infection. Patient also suffered from a life-threatening influenza infection at 3 yr of age.
IRF9 (9)	16	5	2 wk after MMR vaccination, the patient developed an idiopathic biliary perforation. Patient recovered from MMR infection. Patient also suffered a life-threatening influenza infection at 2 yr of age, parainfluenza virus at age 4, and recurrent fevers without a causative pathogen identified throughout her childhood.
STAT1 (4)	13	2	1 wk after MMR vaccination, patient developed fever, irritability, rash, diarrhea, encephalitis, a hemophagocytic lymphohistiocytosis-like syndrome, and was positive for human herpesvirus 6. Patient was readmitted 7 d later with refusal to walk, unsteady gait, and inflammatory cells in the CSF. Patient recovered from MMR infection.
IFNAR1 P1 (this report)	12	9	10 d after MMR vaccination, patient developed a generalized exanthema, fever, and encephalitis. Patient recovered from MMR infection.

Significantly, YFV-17D viremia was typically detectable in healthy vaccine recipients 5-7 days following vaccination and no deficiency of adaptive immunity has been found in individuals with YEL-AVD, suggesting that the disease is most likely the result of a failure of innate or intrinsic immunity (41–43). Consistent with this suggestion, recent work in mice has shown that intact type I IFN signaling is critical for control of YFV-17D (44). Moreover, deficiency in this pathway can be exacerbated by defects in type III IFN signaling (45). Our data provide proof-of-principle that single gene inborn errors of innate immunity, in a broad sense, and specifically of type I IFN cell-intrinsic immunity, can underlie life-threatening adverse reaction to the YF vaccine.

Strikingly, these two patients harboring bi-allelic *IFNAR1* variants, have lived until 9 and 14 years of age, without other clinically significant viral infections. A patient with combined partial IFNAR1 and complete IFNGR2 deficiencies recently reported (46) suffered from cytomegalovirus (CMV) infection, in addition to BCG disease. In contrast, P1 and P2 were IgG^{+} and IgM^{-} for CMV infection, and CMV DNA was not detected in their plasma by PCR, confirming that they had been exposed to CMV. The other patient's combined deficiency in IFNAR1 (partial) and IFNGR2 (complete) may have contributed to CMV disease, consistent with its occurrence in a few patients with isolated, complete IFNGR1 or IFNGR2 deficiency (47–50). Three hypotheses could explain the robust anti-viral defense in the two patients with AR complete IFNAR1 deficiency. A first hypothesis is that the *IFNAR1* splice allele is hypomorphic, i.e. leaky, in some cell types that were not tested. An alternative hypothesis is that IFNAR1 is redundant for some type I IFNs, in some cells that have a complete deficiency and were not tested, at least for some ISGs, as previously shown for IFNAR2 (51). A third hypothesis is that IFNAR1 and all type I IFNs are redundant for immunity against most viruses, at least in certain conditions of infection. It is probably relevant that patients with IFNAR2, STAT2, or IRF9 deficiency, which impair responses to type I IFNs, and type III IFNs for STAT2 and IRF9, are apparently also normally resistant to a broad range of viruses, including CMV, and have survived until 5-11 years of age (3,5,6,9,21), whereas all patients with complete STAT1 deficiency, which impairs responses to type I, type II, and type III IFNs, died by 2 years of various viral infections (4,52–54), unless they received both hematopoietic stem cell transplantation and IgG substitution (4). Future studies are required to understand

the mechanisms of protective immunity to viruses and to develop safer vaccines against viral diseases in patients with inborn errors of type I and/or type III IFNs.

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Supplementary Material

Materials and methods

Study oversight

The study was approved by the institutional review boards of The Rockefeller University, INSERM, University Hospitals Leuven, and the National Institute of Infectious Diseases, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. Written informed consent was obtained from all patients or their guardians.

Case Reports

Patient 1 (P1) was born in Iran to consanguineous parents. He was evaluated at the age of one year for disseminated vaccine-strain measles. He had previously received Hepatitis B virus (HBV), Bacillus Calmette-Guérin (BCG) and influenza vaccinations without any adverse effect. Ten days after inoculation with the MMR vaccine, he presented with a generalized exanthema, fever and neurological symptoms consistent with encephalitis. Laboratory findings only showed moderate lymphocytosis with 7800 total leukocytes/μL, 29% neutrophils, 45% lymphocytes, 10% monocytes, 4% eosinophils, and mild hyponatremia. A computed tomography (CT) of the brain showed mild cerebral edema and analysis of CSF demonstrated leukocytosis (350 lymphocytes/μL, 2 neutrophils/μL). PCR for measles was positive both in blood and CSF, with negative bacterial cultures. Antibodies against measles and mumps were at the lower limit of the normal range, isohemagglutinin titers were normal and antibody responses to pneumococcal and HBV-antigens were also normal. Additionally, P1 was IgG^{+} and IgM^{-} for CMV, and CMV DNA was not detectable in his plasma. Lymphocyte subpopulations (T, B and NK cells) were in the normal range. His past medical history was characterized by frequent viral upper respiratory tract infections with a hospital admission for bronchiolitis, but was otherwise not significant. His growth and development were normal. His younger sister also developed meningoencephalitis one week after receiving MMR vaccination, and died of complications four weeks later. P1 is now 9 years old, in good health and has not manifested any other invasive infection (Fig. 1A).

Patient 2 (P2) is a 14 year-old Brazilian girl born to non-consanguineous parents. Otherwise healthy until age 12, she presented with fever, hypotension, vomiting, and lethargy 7 days

following yellow fever vaccination. Laboratory findings after admission demonstrated leukocytosis with total leukocytes at 28,200/μL, and thrombocytopenia with a platelet count of 21,000/μL. Consistent with renal and hepatic dysfunction, the patient was icteric on exam with an international normalized ratio (INR) of 2.0 and a partial thromboplastin time (PTT) of 66.4. She demonstrated ALT and AST levels of 187 IU/L and 623 IU/L, respectively, and had a creatinine of 2.71 mg/dL. Her condition rapidly deteriorated with bradycardia and diminished consciousness with a Glasgow Coma Score of 8, necessitating admission to the ICU, intubation, and mechanical ventilation. A CT of the chest demonstrated pleural effusions and atelectasis (Fig. 1B). Neutralizing antibodies against YFV were detectable (by plaque reduction neutralization test, PRNT) at a dilution titer of 1:640, as were antibodies against herpes 1 and 2 viruses, while antibodies against HAV, HBV, and HCV, as well as dengue virus and leptospirosis were negative. P2's serum was similarly IgG^+ and IgM^- against CMV antigens, with CMV DNA not detectable in her plasma by PCR. YFV was detected in the patient's blood by PCR analysis, and sequencing confirmed that this was the vaccine-strain virus, not wild-type YFV (Fig. S1). A diagnosis of yellow fever vaccine-associated viscerotropic disease (YEL-AVD) was made. She was treated with an aggressive course of IV fluids, plasma and platelet transfusions, vasopressors, hydrocortisone, and was prophylactically given IV broad spectrum antibiotics. Her hypotension and thrombocytopenia resolved over the next week and, two weeks after her admission, she was discharged from the ICU and made a full recovery. One year after this episode, she remains healthy. Her circulating Ig levels and leukocyte subsets are normal. Notably, the patient received two MMR vaccines at 12 and 16 months of age, and other live vaccines (oral poliovirus and bacillus Calmette-Guérin), without incident (Fig. 1A).

WES

Exome capture was performed with the SureSelect Human All Exon 50 Mb kit (Agilent Technologies). Paired-end sequencing was performed on a HiSeq 2000 (Illumina) generating 100-base reads. We aligned the sequences with the GRCh38 reference build of the human genome using the BWA aligner (55). Downstream processing and variant calling were performed with the Genome Analysis Toolkit, SAMtools, and Picard (56,57). Substitution and InDel calls were made with GATK Unified Genotyper. All variants were annotated using an annotation software system that was developed in-house (13,58,59).

Genetics

WES analysis of each patient revealed a total of 16,797 variants in P1 and 50,634 variants in P2. We filtered out all variations found in 1000 genomes database, the Genome Aggregation Database (GnomAD), our own database of 4,892 exomes for infectious diseases at a frequency of >1%, leaving 48 and 11 non-synonymous coding variants in P1 and P2's exomes, respectively: 30 and 6 were homozygous and 18 and 5 were heterozygous. The heterozygous variants inherited from one parent were not considered in a model of complete penetrance. Excluding those in *IFNAR1*, none of the variants affected genes related to immunity. The nonsense c.783G>A mutation had a CADD score of 37, and the two splicing mutations, c.674-1G>A and c.674-2A>G, had CADD scores of 23.6 and 24, respectively. A genomic measure of individual homozygosity was plotted for P1 and P2, two European individuals from consanguineous families, and 37 individuals from non-consanguineous families from our in-house WES database. Homozygosity was computed as the proportion of the autosomal genome belonging to runs of homozygosity (ROHs). The ROHs were defined as ranging at least 1 Mb of length and containing at least 100 SNPs, and were estimated using the homozyg option of the PLINK software (60). The centromeres were excluded because they are long genomic stretches devoid of SNPs and their inclusion might inflate estimates of homozygosity if both flanking SNPs are homozygous. The length of the autosomal genome was fixed at 2,673,768 kbs as previously described (61). We estimated the selective pressure acting on IFNAR1 to be 2.762 (indicative of positive selection), by estimating the neutrality index (NI) (62) at the population level: (PN/PS)/(DN/DS), where PN and PS are the number of non-synonymous and synonymous alleles, respectively, at population level (1000 Genomes Project) and DN and DS are the number of non-synonymous and synonymous fixed sites, respectively, for the coding sequence of *IFNAR1*.

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient (Lymphoprep, Proteogenix) from the blood of patients and healthy donors. SV40-immortalized dermal fibroblasts and Vero cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. B-LCLs were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum. Primary fibroblasts were grown in DMEM/F-12 (1:1) containing L-glutamin and Hepes

supplemented with 10% fetal calf serum, amphotericin B (0.5 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml).

YFV Plaque Reduction Neutralization Test

Serum samples were used to quantify the levels of neutralizing antibodies specific to the 17DD-YF virus employing the micro plaque reduction neutralization test (micro-PRNT50). The assays were performed at Laboratório de Tecnologia Virológica, Bio-Manguinhos (LATEV, Laboratory of Virological Technology of Instituto Oswaldo Cruz [Fiocruz-RJ], Brazil). First, a viral suspension was prepared with YF 213/77 #002/16, diluted previously with the objective to obtain approximately 30 lysis plaques per well. Before neutralization, the serum sample was inactivated at 56°C for 30 min and submitted to serial 2-fold dilutions (1:5 to 1:640), and afterwards about thirty plaque-forming units (pfu) of yellow fever virus suspension was added to the samples in 96 well tissue culture plates. After addition of virus, the plates were incubated for 1 hour at 37°C in an incubator with 5% CO_2. A 50-μl cell suspension with 1,600,000 cells/ml were inoculated in all 96 wells of the plate and incubated at 37°C for 3 h under 5% CO_2 for adsorption/sedimentation. The medium was then discarded and carboxymethylcellulose (2.5%) added and the cells were incubated for 6 days at 37°C with 5% CO_2. After incubation, the plates were fixed in formaldehyde (5%) for at least 1 hour at room temperature and stained with 2% crystal violet for 30 minutes at room temperature. Both steps were followed by cleansing with running water. The arithmetic mean of all viral plaques obtained without serum were estimated. From the mean, the 50% endpoint of the number of plaques was calculated. Afterwards, using the dilution with plaque numbers immediately above and below the endpoint, the serum dilution that would result in the 50% endpoint was estimated by linear regression. The results were expressed in reciprocals of dilution.

Measurement of antibodies against diphtheria, tetanus, rubella and measles

An enzyme-linked immunosorbent assay (ELISA) standardized at the Immunological Technology Laboratory of Bio-Manguinhos (LATIM, Fiocruz-RJ) was used for antibody measurement. Standard diphtheria and tetanus curves were prepared using in house sera titrated against National Institute for Biological Standard Controls in England (NIBISC) International References. The diphtheria and tetanus antigen for coating ELISA plates were obtained from NIBISC. Anti-diphtheria and anti-tetanus ELISA titers were considered to be protective if they were equal to or higher than the cut-off of 0.1 IU/ml. Serum titers were

calculated by four parameter logistic curve using SoftMax Pro, version 5.2 (Molecular Devices, USA) (63).

The ELISA tests for rubella and measles were performed at Fiocruz-RJ utilizing the kits Anti-Measles Virus ELISA (IgG) and Anti-Rubella Virus ELISA (IgG) (EUROIMMUN, Germany) according to manufacturer instructions. Anti-measles and anti-rubella ELISA titers were considered to be positive if they were equal to or higher than the cut-off of 275 IU/ml and 11 IU/ml respectively.

Plasmids

The cDNA of *IFNAR1* was cloned into pGEMT cloning vector (Promega, Madison, WI, USA). Site-directed mutagenesis was performed to obtain the indicated mutant constructs. All *IFNAR1* constructs were then subcloned into pCAGGS for overexpression studies. For lentiviral vector production, lentiviral vector transfer plasmids pCH_EF1a_IFNaR1_IRES_Bsd and pCH_EF1a_IFNaR2_IRES_Bsd were generated by cloning the codon optimized coding sequence for human *IFNAR1* isoform 1 (NM_000629 or NP_000620) and human *IFNAR2* into the multiple cloning site of the HIV-based lentiviral vector transfer plasmid pCH_EF1a_MCS_IRES_Bsd. Both cDNAs were ordered as a gBlock (IDT Haasrode, Belgium). Sequences were cloned in frame with the IRES-Bsd sequence. All constructs were resequenced to ensure no adventitious mutations were generated during the cloning.

Production of IFNAR1 and IFNAR2 lentiviral vectors

HIV-based viral vectors were produced by the Leuven Viral Vector Core as previously described (Ibrahimi et al. Hum Gene Therapy, 2009), by triple transient transfection of 293T cells with a Vesicular Stomatitis Virus Glycoprotein G envelope encoding plasmid, and a packaging plasmid together with the respective transfer plasmids using polyethylenimine (Polysciences, Amsterdam, The Netherlands), resulting in LV_IFNAR1, LV_IFNAR2 and LV_control, respectively. After collecting the supernatant, the medium was filtered using a 0.45-μm filter (Corning Inc, Seneffe, Belgium) and concentrated using a Vivaspin 50,000 MW column (Vivascience, Bornem, Belgium). The vector containing concentrate was aliquoted and stored at -80°C.

Generation of stably reconstituted cell lines

HIV-based vectors were used to transduce primary fibroblasts, in a serial dilution series. Cells were cultured and subjected to blasticidin selection (5 μg/ml). IFNAR1 expression was corroborated by qPCR, WB, and cell surface staining.

Western blotting

Fibroblasts, with or without pretreatment with IFN-α2b (Schering, Kenilworth, NJ, USA) or IFN-γ (Imukin, Boehringer Ingelheim) for the specified times, were lysed in NP40 lysis buffer (280mM NaCl, 50mM Tris pH 8, 0.2mM EDTA, 2mM EGTA, 10% glycerol, 0.5% NP40) supplemented with 1mM DTT, PhosSTOP (Roche, Mannheim, Germany) and Complete protease inhibitor cocktail (Roche, Mannheim, Germany). 40 ug protein lysate per lane was resolved by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane, which was probed with unconjugated primary antibodies and HRP-conjugated secondary antibodies. An anti-GAPDH (Santa Cruz) antibody was used as a loading control. Endogenous IFNAR1 was probed with an antibody recognizing the amino-terminus at a dilution of 1:1000 (64G12, generously provided by Sandra Pellegrini), while a polyclonal anti-IFNAR1 was used to detect overexpressed protein (AP8550c, ABGENT, San Diego, CA, USA). SuperSignal West Femto Chemiluminescent substrate (Thermo Fisher Scientific) was used to visualize HRP activity, and this signal was detected by an Amersham Imager 600 (GE Life Sciences).

Flow Cytometry

For measuring surface expression on patients' cells, B-LCL and SV40-F cells (5×10^5 cells per well) were plated in 96-well plates and surface-stained with either purified mouse anti-IFNAR1 AA3 (provided by Sandra Pellegrini) or PE mouse anti-IFNAR2 (PBL Assay Science, Piscataway, NJ, USA) antibodies. Cells stained with AA3 were then washed once with PBS and incubated with a biotinylated rat anti-mouse secondary (Thermo Fisher Scientific) for 30 minutes before being washed once with PBS and incubated for 30 minutes with a PE-conjugated streptavidin (Thermo Fisher Scientific). The cells were then washed twice with PBS and analyzed by flow cytometry. Data were acquired on an LSRII flow cytometer (BD), and the results were analyzed with FlowJo (Tree Star). For YFV infections, transduced fibroblasts were submitted to IFN-β pretreatment and YF17D infection as described below. Cells were detached after incubation for 10 minutes at room temperature in Accumax (STEMCELL, cat. 07921). Cells were then fixed in 4% paraformaldehyde for 20 minutes at room temperature

prior to permeabilization with BD perm buffer (BD Biosciences, cat. 554723). BD perm buffer was used for antibody incubations and washes. Cells were incubated with primary antibodies for 1 hour on ice, washed, incubated with secondary antibodies for 30 minutes at room temperature, washed, and resuspended in PBS with 1% FBS prior to analysis using a BD LSRII flow cytometer. The primary antibody was a mouse anti-YFV (Santa Cruz Biotechnology cat. sc-58083, RRID:AB_630447) diluted 1:250, while the secondary antibody was a goat anti-mouse Alexa Fluor 647 (Invitrogen cat. A-21203) diluted 1:1000.

Quantitative RT-PCR

SV40-F cells stimulated with 1,000 IU/ml IFN-α2b, IFN-β, or IFN-γ for 2 or 8h, or primary fibroblasts treated for 6h, were lysed with RNA lysis buffer, treated with DNase, and RNA was purified according to the manufacturer's protocol (Zymo Research, Irvine, CA). Reverse transcriptase PCR was performed using random hexamers and the Superscript III reverse strand synthesis kit according to the manufacturer's recommendations (Thermo Fisher Scientific, Springfield Township, NJ). Quantitative real-time PCR (qRT-PCR) was performed with Applied Biosystems Taqman assays using the β-glucuronidase (GUS) housekeeping gene for normalization for SV40-F cells, or B-actin for primary fibroblasts. Results are expressed using the $\Delta\Delta$Ct method, as described by the manufacturer.

Virus assays

VSV infections

SV40-F cells were infected with vesicular stomatitis virus (VSV) at a multiplicity of infection (MOI) of 3. The inoculum was absorbed onto the cells for 30 minutes at 25°C, washed twice with PBS and cultured in DMEM with 10% FBS at 37°C. Virus samples were collected at the indicated time points. Viral titers were determined by endpoint dilution on Vero cells using the Reed and Muench calculation. Where indicated, cells were pretreated with IFN-α2b for 16h prior to virus infection. Primary fibroblasts were infected with Indiana stock VSV (MOI=9.3), with or without pretreatment with 10,000 U/ml IFN-α2b for 24 hours prior to infection. The inoculum was left on the cells, and cell survival was monitored with Resazurin assay kit (Abcam, catalog number ab112119) at 1, 2, and 3 days post-infection.

IAV and HSV-1 infections

SV40-fibroblasts were infected with influenza virus (A/California/4/2009) as previously described (9). Briefly a viral inoculum was absorbed onto the cells (MOI=0.5) for 30 minutes at

25°C. Cells were then washed twice with Hank's balanced salt solution and cultured at 37°C in the presence of 0.1μg/ml TPCK-trypsin (Sigma-Aldrich, St. Louis, MO, USA). Virus samples were collected at the indicated times after infection and influenza titers were determined by plaque assay on MDCK cells. For HSV1-GFP infection, SV40-transformed fibroblasts were plated in a 96-well dish and were infected with HSV1-GFP (MOI=0.01) in DMEM supplemented with 2% FCS. GFP fluorescence was then assessed at 0, 8, 24, 48, and 72 hours post infection. HSV1-GFP was a gift from Dr. Desai (64). For HSV-1 (KOS strain, ATCC), SV40-Fs were seeded at a density of 10^5 cells per well in a 24 well plate and infected (MOI=0.01) in DMEM + 2% FCS. Supernatants were collected at the given time points post-infection and $TCID_{50}$ values were calculated following the method of Reed and Muench, after inoculation of Vero cells in 96-well plates.

YFV infections

Preparation of virus stocks.

YF17D viral stocks were derived from pACNR-2015FLYF-17Da plasmid, a derivative, to be fully described elsewhere, of the previously described pANCR-FLYF-Dx containing the full-length infectious YF17D genome under an SP6 promoter (65). In pANCR-2015FLYF-17Da, the linearization site for preparation of RNA was changed to AfIII, an XhoI site within the YF coding region was restored, and adventitious mutations that had occurred during bacterial propagation of pANCR-FLYF-17Dx in the lab were corrected. Asibi viral stocks were derived from an analogous full length Asibi cDNA infectious clone designated pACNR-2015FLYF-Asibi, to be fully described elsewhere. *In vitro*-generated RNA transcripts were electroporated in Huh7.5 cells (66). Virus was harvested 24 h after transfection and titers of $6x10^5$ PFU/ml (17D) and $6x10^3$ FFU/ml (Asibi) were determined by plaque or focus forming assay on C3 fibroblasts. Single-use aliquots were stored frozen at –80°C until use.

Infection of cells.

Prior to infection, patient-derived fibroblasts were pretreated with or without 1000 U/ml IFN-β (PBL Assay Science) for 16 hours. 17D (or Asibi) was then inoculated to approximately 50,000 (or 20,000) fibroblasts in 24-well (or 48-well plates, respectively). For 17D infections, media was discarded and cells were inoculated with 250 μl of 17D diluted in Opti-MEM (MOI=0.05) for 2 h at 37°C. Asibi infections were similar to 17D, except cells were inoculated with 100 μl of Asibi diluted in Opti-MEM (MOI=0.03). Cells were then washed twice with culture media

and incubated at 37°C with 0.5 ml of fresh culture media. At the indicated time points post-infection, media containing the virus produced by the fibroblasts were harvested and titered on Huh7.5 cells by plaque-forming assay.

Plaque assay.

Harvested media was serially diluted 1:10 in Opti-MEM. Approximately 0.5 million Huh7.5 cells were inoculated with 400 µl of diluted virus in 6-well plates, for 2 hours at 37°C. After removal of the inoculum, a 3 ml of overlay of 1.2% Avicel in DMEM supplemented with 1x Pen/Strep and 2% FCS was added and cells were incubated for 3 days (17D) or 4 days (Asibi) at 37°C. After formaldehyde fixation, the cells were stained with crystal violet and the plaques were enumerated.

IFN-ß measurement

The concentration of IFN-β in the supernatant of infected cells was measured by ELISA using the VeriKine-HS™ Human IFN-β Serum ELISA Kit (PBL Assay Science), according to the manufacturer's instructions, using 50 µL of media.

ZIKV infections

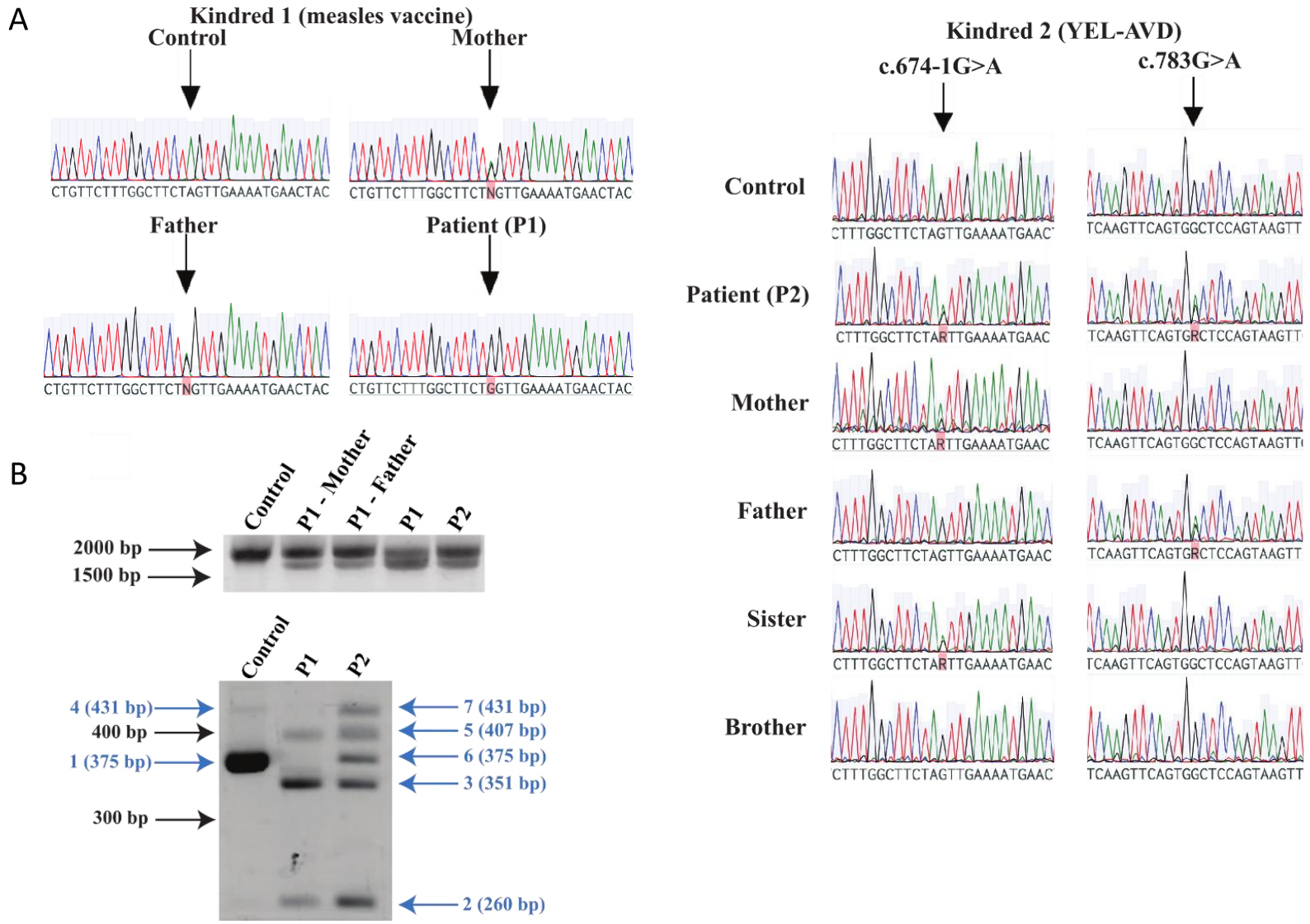
Prior to the infection, the ZIKV stock titer of $1x10^6$ FFU/ml was determined by focus forming assay on C1 fibroblasts. IFN-β pre-treatment, virus infection (ZIKV MOI=0.05), and quantification of virus production were conducted on patient-derived fibroblasts as described above for Asibi, except Vero cells were inoculated with 300 µl of virus for the plaque-forming assays.

MeV infections

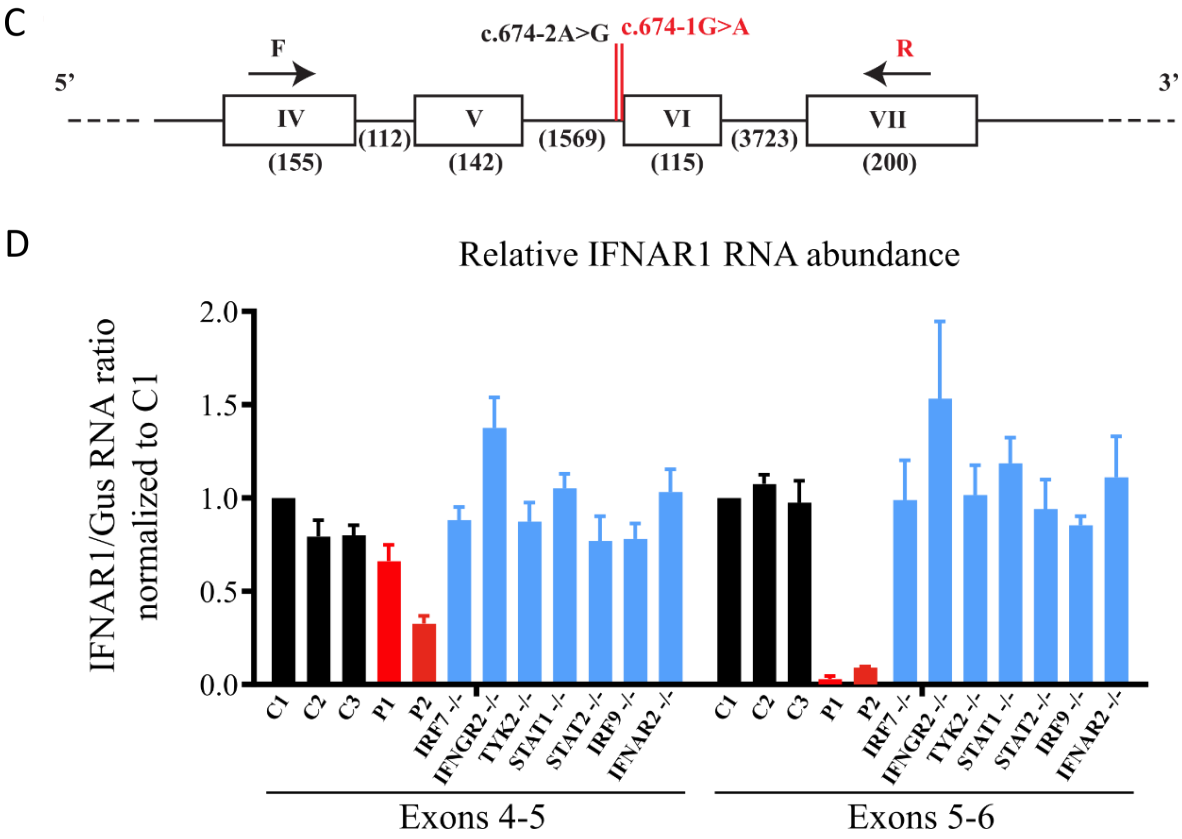
Approximately 50,000 patient-derived SV40-F cells were seeded in 48-well plates and pretreated or not with 1,000 U/ml IFN-α2b (PBL Assay Science) for 16 hours. Cells were incubated for one hour at 37°C with measles virus diluted in DMEM with 10% FCS (MOI=0.1). After virus inoculation, cells were washed twice with PBS and incubated at 37°C with 0.2 ml of fresh culture media. At the indicated time points post-infection, media containing the virus produced by the fibroblasts was harvested and $TCID_{50}$ values were calculated following the method of Reed and Muench after inoculation of Vero cells in 96-well plates.

Supplementary figures and tables

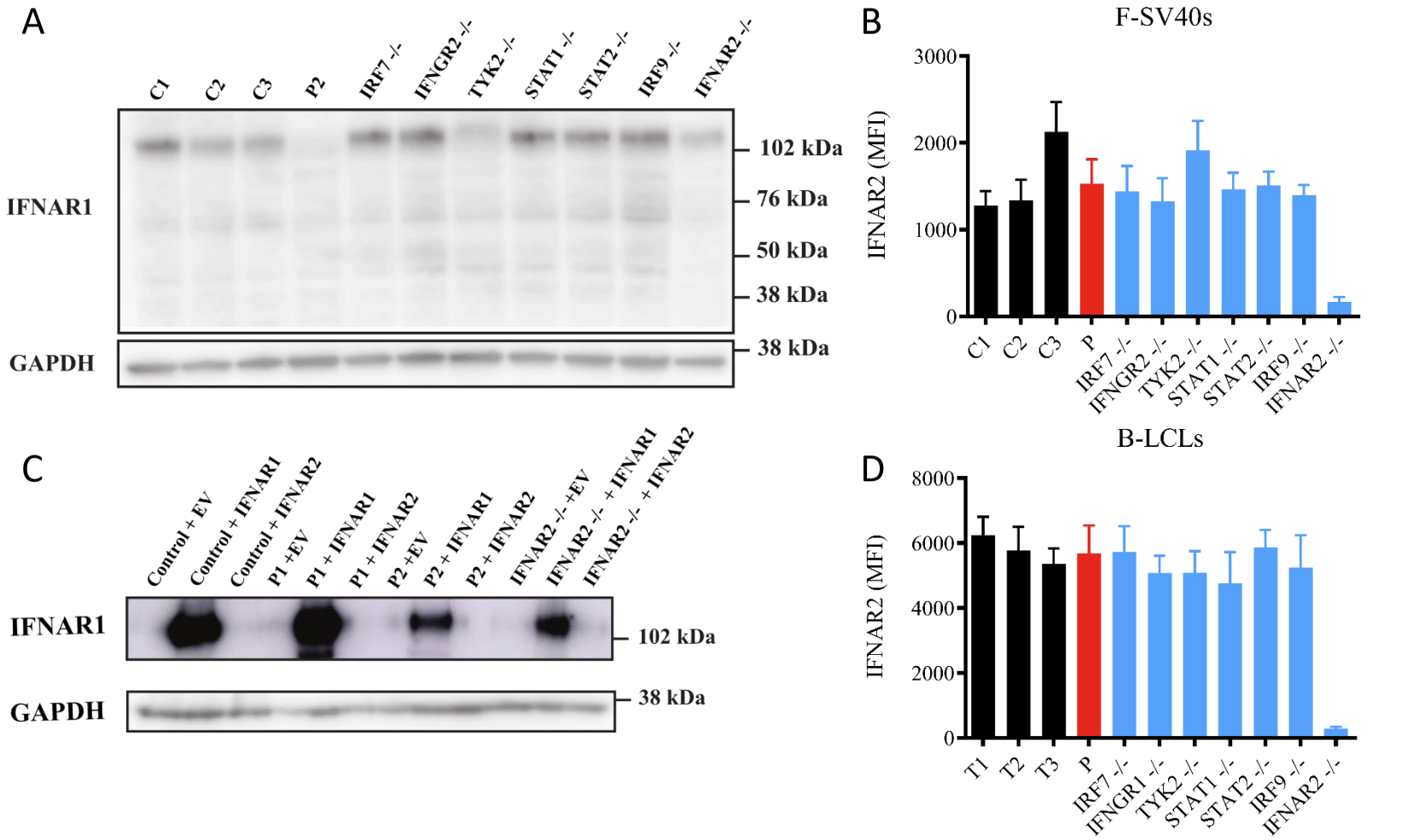
Supplementary figure S1. Evidence of YFV virus infection in P2. Sanger sequencing alignments demonstrating an identical insertion in the YFV genome recovered from P2 during her infection. The consensus WT YFV sequence is shown in the top row, while two YFV-17D strains (102_84 and YF993FV013Z) sequenced are directly below that, followed by the sequence from the YFV isolated from the patient (RNA_2882). Environmental YFV isolates are shown in the bottom four rows.



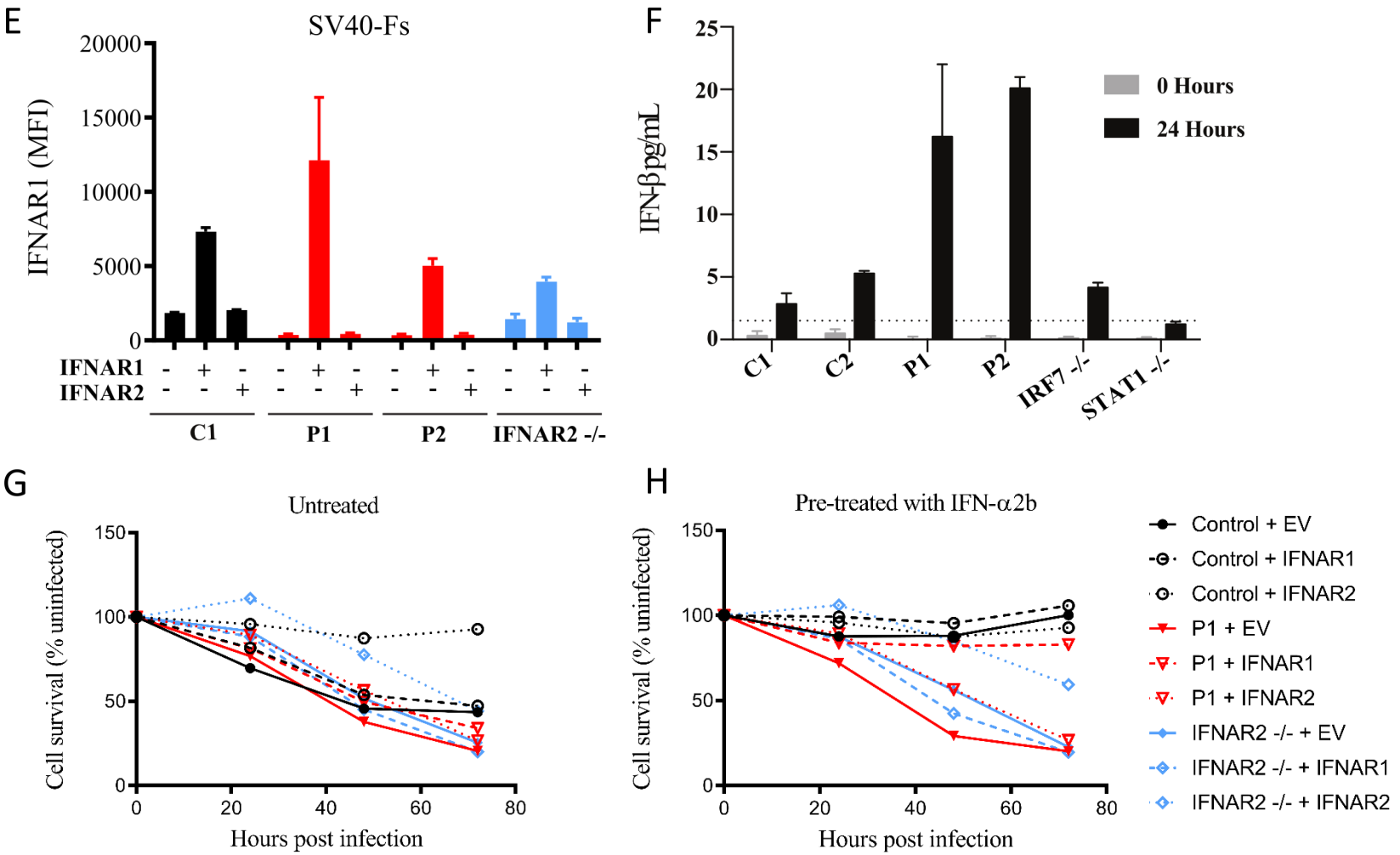
Supplementary figure S2. Genetic analysis of IFNAR1-deficient patients. A) Sanger sequencing chromatograms demonstrating c.674-2A>G, c.674-1G>A, and c.783G>A mutations in patientDNA (black arrows). B) Agarose gel electrophoresis of IFNAR1 cDNA products generated from patient mRNA transcripts. The upper image shows subtle changes in the size of the full-length transcript (expected length of 1,674 base pairs), while the bottom image demonstrates that additional alternatively spliced mRNAs can be detected at low abundances when only exons IV through VII are amplified, as depicted schematically in C. Numbers in blue correspond to the structure of the transcripts present in a given band, as diagrammed in Fig. 2A, with nucleotide lengths in parentheses.



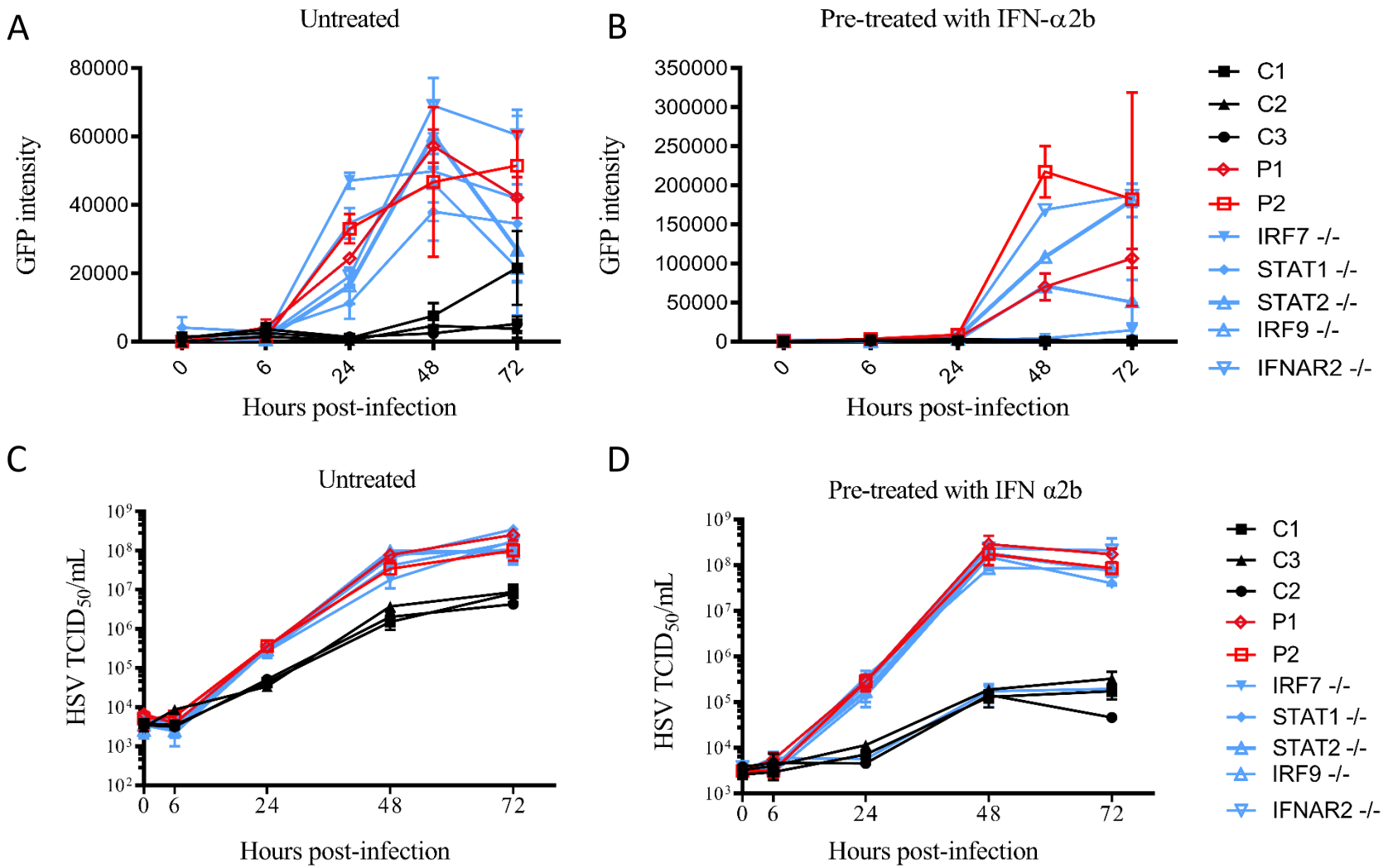
Supplementary figure S2 - continued. C) Diagram of the IFNAR1 gene in the region containing exon 6. Exons are depicted with boxes, while introns are depicted with lines. mRNA transcripts from this region were amplified by PCR and inserted into the pGEM-T Easy system. Mutations present in patient cells are indicated with vertical red lines. Primers used in cDNA sequencing are demonstrated (forward, F-59-ATAGCTTAGTTATCTGGAAAAACTCTTCAGGTG-39; reverse, R-59-GGGTAGTTTTGACATTTTCACAGTCAGG-39), and the nucleotide lengths of the features are shown in parentheses. D) qRT-PCR measuring IFNAR1 mRNA levels in SV40-F cells from P1, P2, three healthy controls (C1, C2, and C3), IRF7−/−, IFNGR2−/−, TYK2−/−, STAT1−/−, STAT2−/−, IRF9−/−, and IFNAR2−/− patients. IFNAR1/GUS mRNA ratios are shown after normalization to C1.



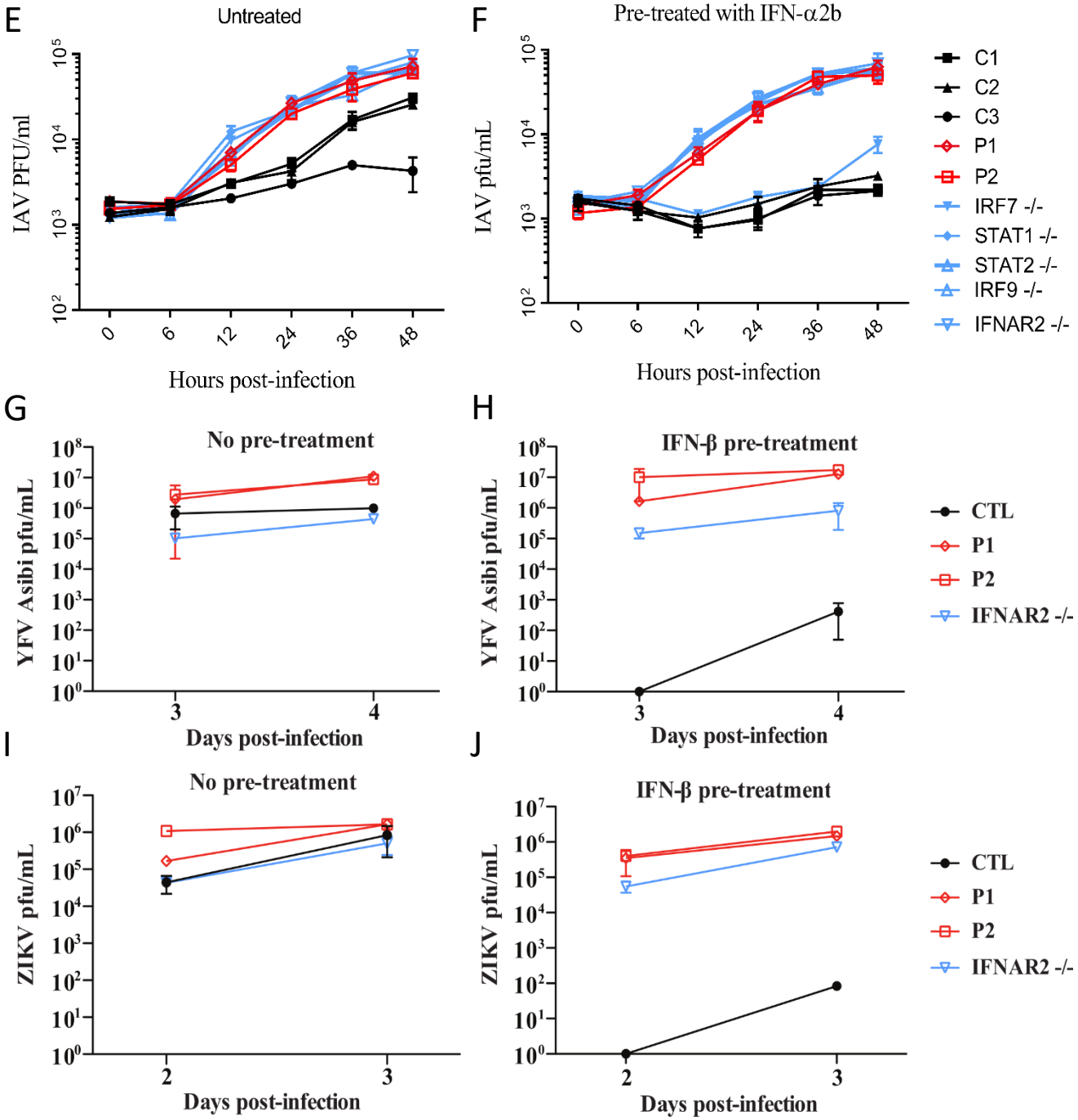
Supplementary figure S3. Disrupted function of the type I IFN signaling pathway in patient cells. A) Western blot of endogenous IFNAR1 in B-LCL cells from three healthy controls (C1, C2, and C3), P2, IRF7−/−, IFNGR1−/−, TYK2−/−, STAT1−/−, STAT2−/−, IRF9−/−, and IFNAR2−/− patients. B and D) IFNAR2 mean fluorescence intensities (MFI) from patient SV40-F (B) and B-LCL (D) cells stained with an anti-IFNAR2 fluorescent antibody. Cells were from three healthy controls (C1, C2, and C3 or T1, T2, and T3), P1, P2, IRF7−/−, IFNGR2−/−, IFNGR1−/−, TYK2−/−, STAT1−/−, STAT2−/−, IRF9−/−, and IFNAR2−/− patients and were assessed by flow cytometry. C) Western blot of IFNAR1 in SV40-F cells from a healthy control, P1, P2, and an IFNAR2−/− patient transduced with WT IFNAR1, WT IFNAR2, or EV.



Supplementary figure S3 - continued. E) MFIs of SV40-Fs from a healthy control, P1, P2, and an IFNAR2−/− patient and transduced with WT IFNAR1, WT IFNAR2, or EV and stained with an anti-IFNAR1 antibody. F) IFN-β levels detected in the supernatant of SV40-F cells either 0 or 24 h after infection with YFV-17D. Cells from two healthy controls (C1 and C2), P1, P2, an IRF7−/− patient, and a STAT1−/− patient were included. The limit of quantitation is indicated by a dotted line. G and H) Survival rates following VSV infection of primary fibroblasts either untreated (G) or pretreated (H) with 1,000 U/ml IFN-α2b for 24 h before infection. Cells were from a healthy control, P1, and an IFNAR2-deficient patient that were either transduced with EV, WT IFNAR1 (IFNAR1), or WT IFNAR2 (IFNAR2).



Supplementary figure S4. IFNAR1 is essential for type I IFN–mediated intrinsic immunity to multiple viruses. A-B) HSV-1-GFP replication levels, as assessed by GFP intensity, in SV40-F cells unstimulated (A) or pretreated (B) with 1,000 U/ml IFN-α2b followed by infection with HSV-1-GFP (MOI = 0.01). C-D) HSV-1 titers in SV40-F cells unstimulated (C) or pretreated (D) with 1,000 U/ml IFN-α2b for 16 h, followed by infection with HSV-1 (MOI = 0.01).



Supplementary figure S4 - continued. E-F) IAV titers in SV40-F cells unstimulated (E) or pretreated (F) with 1,000 U/ml IFN-α2b for 16 h, followed by infection with (A/H1N1/CA/2009) IAV at MOI = 1. Cells from three healthy controls (C1, C2, and C3), P1, P2, IRF7−/−, STAT1−/−, STAT2−/−, IRF9−/−, and IFNAR2−/− patients were included in (A-F). G-H) SV40-F cells stimulated for 16 h with 1,000 U/ml IFN-β before infection (H) or left untreated (G) were infected with 100 µL of YFV Asibi, and viral titer was assessed by plaque assay on Huh7.5 cells at the given time points. I-J) SV40-F cells stimulated for 16 h with 1,000 U/ml IFN-β before infection (J) or left untreated (I) were infected with ZIKV (MOI = 0.05), and viral titer was assessed by plaque assay on Vero cells at the given time points. SV40-Fs from a healthy control, P1, P2, and an IFNAR2−/− patient were included in (G-J). Different time points represent independent infections. TCID, tissue culture infectious dose.

Table S1. Rare homozygous or possible compound heterozygous variants found by exome sequencing of P1 and P2

P1	P2
URI1 (c.840_842delTGA) **CNTF (c.T101A, c.T494G)** SLC45A1 (c.130_131insCGGGAGATG) HTR2B (c.T848C) MTMR9 (c.T1153A) PSG11 (c.G44C) MEOX2 (c.228_230delTGG)b CLN8 (c.A806T) CNDP1 (c.43_44insTGC) MPRIP (c.534_539delCAGCAG) TMEM247 (c.382_383insAGCGGCAGCACGAGGTGG TGATGGAGCAGCTGCAGCGGG) FADS6 (c.17_18insGATGGAACCTACGGAGCCCATGGAACCTA CGGAGCCCATGGAACCTACGGAGCC) KIAA2018 (c.4410_4418delTGCTGCTGC) **ZNF407 (c.A515T, c.C4145T)** SMPD1 (:c.108_113delGTCGGC)b TBL1Y (c.G274A) AOX1 (c.G173A) BSN (c.G11015A) MAP3K4 (c.3566_3568delCTG)b ATG3 (c.920dupT) KIRREL2 (c.32_34delTCC) CNTD2 (c.T524A) ACSL3 (c.G1043A) MAFA (c.621_623delTGG) TMPRSS13 (c.248_262del GGGCTGGAGATGCCT) DYTN (c.C679T) THAP11 (c.367_369delCAG) CXorf23 (c.1727_1728delTA) TMEM54 (c.G136A) NLRP10 (c.T752C) SESTD1 (c.A1310G) APEX1 (c.953_954delTG)b DLC1 (c.G161T)b PLBD1 (c.74_76delGCA) ANKRD62 (c.G1197C) MAML3 (c.1506delG)b RBM23 (c.1077_1078insGCC) HOXD9 (c.794_795insGCA)b **DCP1B (c.782_783insGCA, c.C1532T)** IFNAR1 (c.674-2A>G)[b]	KCNN3 (c.G1092C) **KIAA1875-WDR97 (c.3123+1G>T, c.4750G>A)** MADCAM1(c.736_737insAGGAG) **IFNAR1 (c.674-1G>A, c.G783A)[a,b]** **PIWIL3 (c.A2629C, c.A1996G)**

Unbolded genes are homozygous, while bolded genes may be compound heterozygous or may occur on the same allele. The consequences of the variations on coding sequences are given in parenthesis following each gene. a) Gene containing a stop–gain mutation. b) Gene that is predicted to be related to primary immunodeficiencies via a connectome analysis (67).

CHAPTER 2

Clinical, immunological and molecular study of a cohort of patients with STAT2 deficiency

Bucciol G, Moens L, Delafontaine S, Duncan CJA, Hernandez N, Cale CM, Lachaux A, Bousfiha A, Al Saud BK, Hambleton S, Béziat V, Zhang Q, Casanova JL, Meyts I.

Abstract

Autosomal recessive deficiency of signal transducer and activator of transcription 2 (STAT2) causes a specific defect of innate immunity with susceptibility to viral infections and a characteristic, although not fully penetrant, presentation with disseminated viral disease and encephalitis after inoculation with live measles-mumps-rubella (MMR) vaccine. We report the clinical, immunological and genetic characteristics of sixteen patients from seven unrelated kindreds with STAT2 deficiency. Eight different mutations were identified in the seven kindreds, six of them homozygous and two combined in compound heterozygous form. Five are nonsense mutations, two are splice mutations, and one is a small deletion resulting in frameshift and premature stop. The mutations resulted in variable loss of STAT2 expression and phosphorylation, increased in vitro susceptibility to live vaccine-strain measles and diminished induction of interferon stimulated genes. The clinical phenotype is characterized by viral infections and disseminated disease after MMR vaccination in 7/8 of the patients who received the vaccine, but diarrhea, failure to thrive, bacterial infections, and cytopenia are also reported. The patients are not susceptible to fungal or mycobacterial disease, even when exposed to vaccination with Bacillus Calmette-Guérin. Three patients (19%) succumbed to overwhelming infection in early life (range 2 months-7 years). The penetrance of viral infections is incomplete, and once patients reach adulthood they seem to be protected from severe infections. In all, human STAT2 deficiency leads to a narrow clinical phenotype in stark contrast with the central role of STAT2 type I IFN pathway in anti-viral host defense.

Introduction

Human type I and III interferons (IFNs) constitute a first line of defense against viral infections (1,2). Type I IFNs (IFN-α/β) are secreted by most cell types, with each of the 17 individual IFNs having a specific pattern of expression. In particular, IFN-α are mainly secreted by leukocytes

and IFN-β is produced in abundance by fibroblasts (3). The secretion of type III IFNs (IFN-λ) is instead restricted to epithelial cells of barrier organs and dendritic cells (2,4). In turn, human type II IFN (IFN-γ) is mostly secreted by lymphocytes and has a weak intrinsic anti-viral activity, being instead an important macrophage-activating factor (3). The type I IFN receptor (IFNAR) is a ubiquitously expressed heterodimer composed of a low and a high affinity subunit, called IFNAR1 and IFNAR2 respectively. The type III IFN receptor (IFNLR) is also a heterodimer, found mostly on epithelial cells and on a subset of immune cells (5). Upon binding their receptor, type I and III IFNs trigger a signaling cascade starting with the phosphorylation of tyrosine kinase 2 (TYK2) and janus kinase 1 (JAK1). In turn, they phosphorylate and activate signal transducer and activator (STAT) molecules, that translocate to the nucleus as homo- and heterodimers and initiate transcription. The main signaling pathway downstream of IFNAR and IFNLR involves STAT1 and STAT2 (6). Upon activation, they combine with IFN regulatory factor 9 (IRF9) to form an IFN-stimulated gene factor 3 (ISGF3) complex. ISGF3 then translocates to the nucleus and binds IFN-stimulated response element (ISRE) promoters to induce the transcription of IFN-stimulated genes (ISGs), reinforcing the antiviral response. Both receptors can also induce the formation of STAT1 homodimers, which can drive the induction of ISGs by binding IFN-γ activated sites (GAS) in their promoters.

The first inborn error of human anti-viral IFNs was discovered in 2003 with autosomal recessive (AR) STAT1 deficiency, with up to 23 patients reported by now (7, personal communication). There are two forms of AR STAT1 deficiency, complete and partial, predisposing to mycobacterial disease because of impaired type II IFN signaling and to viral infections because of impaired type I and III IFN signaling. The consequences of impaired IL-27 signaling are unknown. The group of Hambleton et al. described a homozygous splice defect in STAT2 underlying susceptibility to viral infections, with a typical, albeit not fully penetrant, presentation with disseminated viral disease and encephalitis after inoculation with live measles-mumps-rubella (MMR) vaccine (8). Other reports of STAT2-deficient patients followed (9,10). More recently, patients with inherited deficiency of IFNAR2 (11), IRF9 (12), and IFNAR1 (13) have been reported. A striking feature of these five IEIs is that they seem to be associated with different viral infections and clinical outcomes. This might result from the small number of subjects, with three IFNAR1, one IFNAR2, 23 STAT1, nine STAT2 and a single

IRF9-deficient patient reported so far. Additionally, some defects may be complete and others partial, as neatly illustrated by STAT1 disorders. The ethnicity and viral environment may also play an important role. We therefore analyzed the genetic, immunological and clinical characteristics of 16 patients from seven kindreds with autosomal recessive (AR) STAT2 deficiency. Three kindreds (P1-6, P7-8, P9-10) have previously been studied (8–10).

Patients, materials and methods

Patients were recruited from previous reports by contacting physicians and via an international call for inclusion, as well as from the Human Genetics of Infectious Diseases Laboratory Cohort of patients with severe viral infections. All patients or their legal guardians consented to the study. Ethical approval for the study was obtained from the Ethical Research Committee of Leuven University Hospitals (primary center, protocol number S60905). The data were collected via a standardized case record form.

Study of the STAT2 variants

Plasmids containing the mutations under investigation were created by 5' site directed mutagenesis (New England Biolabs) of a pCMV vector containing wild type (WT) *STAT2* cDNA (NM_005419.3) as described by the supplier. WT *STAT2* cDNA or the *STAT2* variants under investigation were further cloned into a lentiviral pTRIP-SFFV-GFP-2A vector (Addgene) with In-Fusion HD cloning kit (Takara Bio). Transient transfection of HEK293T cells to produce lentiviral particles was obtained with XtremeGENE 9 (Roche) in optimized combination of pCMV-VSV-G (Addgene), psPAX2 (Addgene), pHXB2 (NIH-AIDS Reagent Program) and the desired pTRIP_STAT2 plasmid. After collecting the supernatant, the medium was filtered using a 0.45 μm filter and supplemented with protamine sulfate (8ng/mL) for further spinoculation for 2 h at 1200 g at 25°C of primary fibroblasts of a previously described STAT2 deficient patient (150.000 cells/well in 6-well plates) (10). Green fluorescent protein (GFP) expression was monitored via flow cytometry and GFP-expressing fibroblasts were sorted (FACSAria III flow cytometer, BD Biosciences) and cultured for further experiments.

Expression and phosphorylation of STAT1 (anti-STAT1 p84/p91, M-22, sc-592, Santa Cruz Biotechnology, and anti-pSTAT1 pY701, 612232, BD Biosciences) and STAT2 (anti-STAT2 B-3, sc-514193, Santa Cruz Biotechnology, and anti-pSTAT2 Y689, AF2890, R&D Systems) were

evaluated after stimulation with IFN-α2a (10^4 IU/mL, Life Technologies) or IFN-γ ($5*10^3$ IU/mL, R&D systems) via immunoblotting as previously described (10). Furthermore, GFP-sorted fibroblasts were exposed to vaccine strain measles virus (MOI 0,015) with or without IFN-α or IFN-γ pretreatment. Syncytia formation (Floid microscope) on day 3 and IFN-β production (ELISA, R&D systems) on day 5 were evaluated. STAT2-deficient primary fibroblasts transduced with empty vector expressing GFP resulted in increased GFP expression (commonly observed in the hosting laboratory), which precluded syncytia evaluation in this condition.

To study IFN stimulated gene (ISG) induction, total RNA was extracted with TRIZOL reagent (Lift Technologies) from primary fibroblasts left unstimulated or stimulated with IFN-α2a (10^4 IU/mL) or IFN-γ ($5*10^3$ IU/mL) for 6 hours. mRNA was then reverse transcribed directly with Superscript Vilo cDNA synthesis kit (Life Technologies). *MxA* (Hs00895608), *ISG15* (Hs00192713), *OAS1* (Hs00973635) and *ACTB* (Hs1060665, housekeeping gene) transcript levels were identified via Taqman gene expression assays (Applied Biosystems).

Results

Mutations and kindreds

Each of the seven unrelated kindreds carried private mutations in STAT2, four already reported and five novel mutations (8–10). In total we report seven single nucleotide substitutions, five in homozygous and two in compound heterozygous form, and one homozygous small deletion. Of the single nucleotide substitutions, five are nonsense mutations and two are splice mutations. The mutations span all domains of STAT2 (Fig. 1, supplementary Table S1).

Six patients in two generations of kindred I carry the homozygous splice mutation c.381+5G>C, which was shown to result in loss of expression and loss of function (8). The homozygous nonsense mutation c.1836C>A (p.C612X) is present in both children of kindred II. The compound heterozygous c.1528C>T (p.R510X)/c.1576G>A nonsense/splice mutations is carried by two out of four children of kindred III. Two out of five children of kindred IV are homozygous for c.1883_1884del (p.V628fs14X), resulting in a frameshift and premature stop. The two oldest out of five children of kindred V are homozygous for the nonsense mutation c.988C>T (p.R330X), and the youngest out of four children of kindred VI is homozygous for

c.820C>T (p.Q274X). In both these kindreds a sibling died without the possibility of performing genetic analysis. One subject in kindred VII is homozygous for the nonsense mutation c.1999C>T (p.R667X), currently under study by another group. Kindreds are summarized in Fig. 2 and Table 1.

Interestingly, an additional patient with a *STAT2* variant and a clinical phenotype compatible with STAT2 deficiency was originally included in the study. This 10-year-old boy born from consanguineous parents suffered from aseptic meningitis after both doses of MMR vaccination, he had chronic diarrhea and arthritis, and he experienced severe viral infections with Influenza, herpes simplex, varicella-zoster, and hepatitis A, and recurrent otitis and pneumonia with development of bronchiectasis. He carried a homozygous missense c.G1114A (p.G372S) mutation in *STAT2* which however did not result in aberrant expression or phosphorylation of STAT2, nor in a splice variation, nor in the reduction of ISGs expression upon stimulation of the type I IFN pathway (supplementary Fig. 1-3). We have therefore concluded that his phenotype could not be ascribed to STAT2 deficiency and did not further include him in this study. He carried indeed other variants of unknown significance in genes relevant for the immune response, such as DOCK8, that need to be further analyzed.

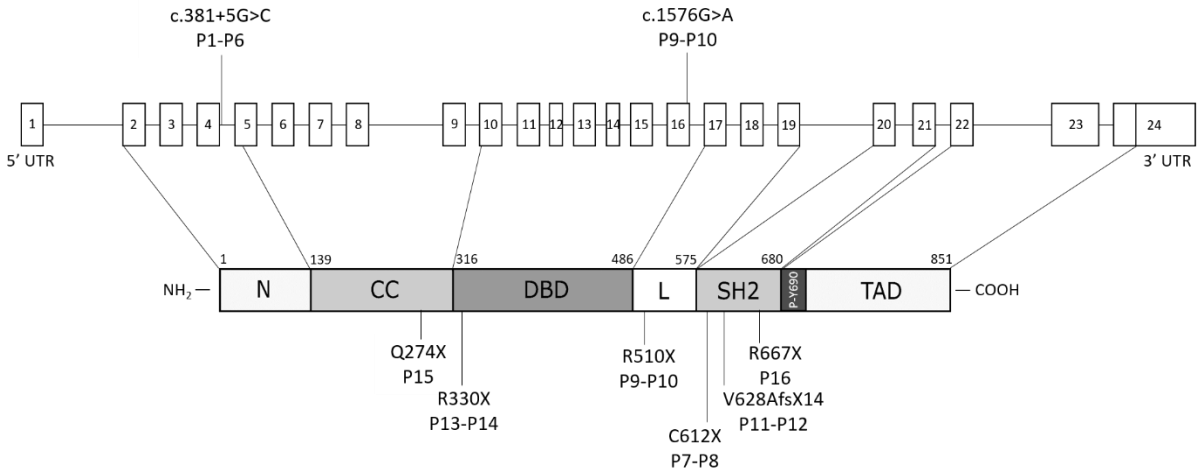


Figure 1. Schematic representation of the STAT2 gene and protein. Mutations and corresponding patients are indicated. Above: numbered boxes indicate exons. Below: numbers above the protein schematics indicate amino acid positions. Protein domains: N: N-terminus, CC: coiled-coil, DBD: DNA-binding, L: linker, SH2: Src homology 2, TAD: transcriptional activation domain.

Previously described cellular phenotypes

The molecular and cellular effect of c.381+5G>C and of c.1528C>T/c.1576G>A have been studied by Hambleton et al. and Moens et al., respectively (8,10). The mutations determined a complete loss of expression of STAT2, with absent STAT2 phosphorylation and absent induction of transcription of downstream ISGs upon IFN-α stimulation. Moreover, fibroblasts from STAT2-deficient patients were more susceptible to infection with a multitude of viruses in vitro even after treatment with IFN-α, which conferred instead protection to fibroblasts from healthy controls (8,10). These results demonstrated a deficient type I IFN response in patients with complete STAT2 deficiency, at least in the conditions and cell type studied. Additionally, c.1836C>A was studied by Shahni et al., who found that the patients' fibroblasts and muscle cells harbored a mitochondrial fission defect with extremely elongated mitochondria (9). The same mitochondrial defect was also confirmed in one patient from the first kindred reported by Hambleton et al. (c.381+5G>C/c.381+5G>C) (9). The mechanism appeared to be a lack of STAT2-mediated activation of the main mitochondrial fission factor dynamin related protein 1 (DRP1), and it also resulted in defective IFN-α-induced apoptosis (9).

Clinical features

Demographics

Sixteen patients from seven kindreds originating from seven countries were included in the study. Two kindreds were from the United Kingdom (kindred I, P1-6, of unknown ethnicity, and kindred II, P7-8, of Albanian origin), one kindred (kindred III, P9-10) from Belgium, one kindred from France (kindred IV, P11-12, of Algerian origin), one kindred from Morocco (kindred V, P13-14), one kindred from Saudi-Arabia (kindred VI, P15), and one kindred from USA (kindred VII, P16, of unknown ethnicity). All described cases are familial and four kindreds are reported to be consanguineous (kindred I, V, VI and VII). The median age at the time of the study was nine years (range: 3-36 years). The median age at the time of onset of symptoms was nine months (range: 1-24 months) and male-to-female ratio was 1:3. Patients' characteristics are summarized in Table 1.

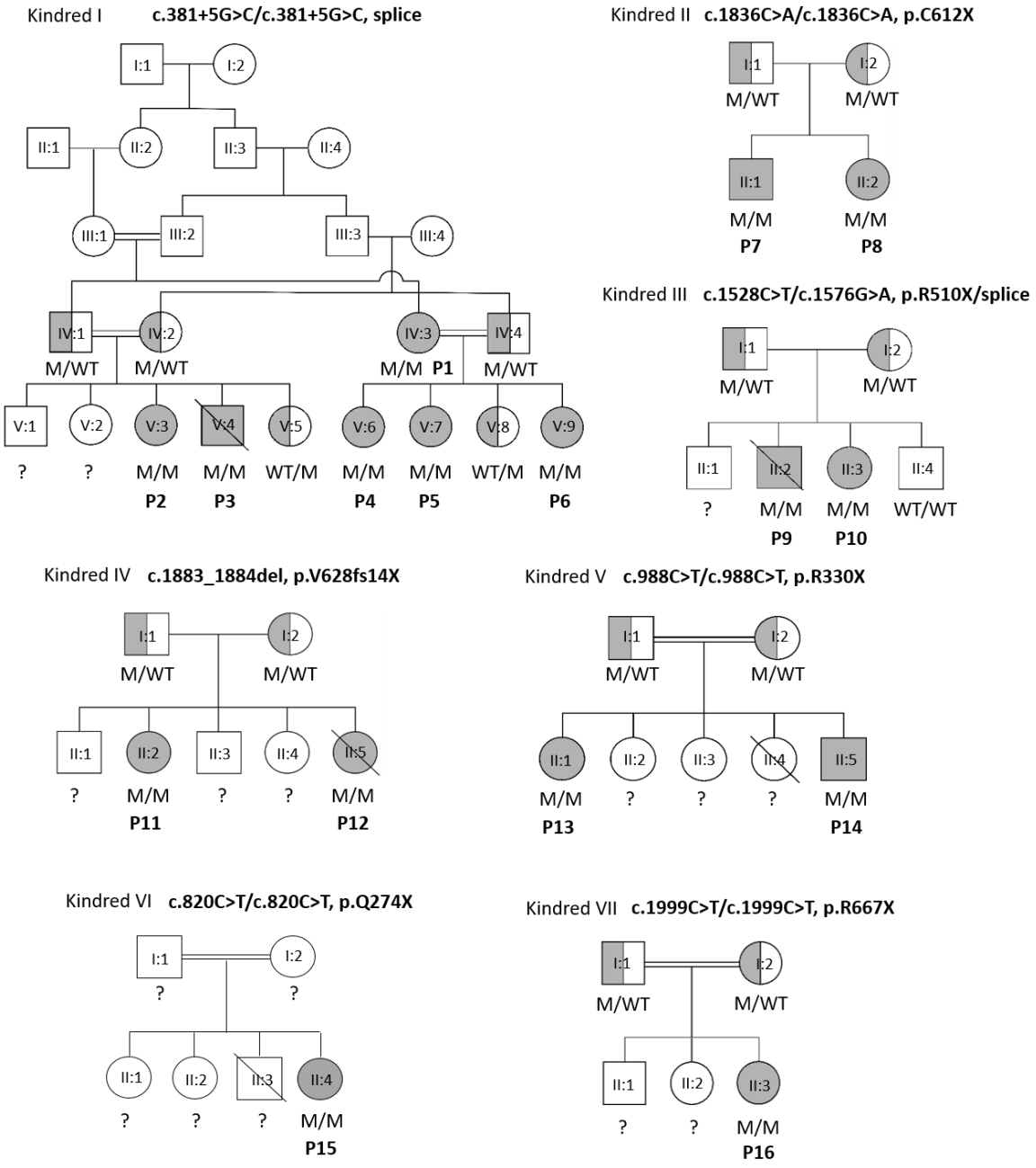


Figure 2. Pedigrees of the STAT2 deficient families. Patients are indicated by full shapes and P. M indicates presence of the variant (specified above the family tree), WT: wild type, ?: not tested/unknown.

Disseminated vaccine-strain measles

Eight out of 16 patients received the live attenuated measles-mumps-rubella (MMR) vaccine, while for five patients this information is not available. Seven MMR-vaccinated subjects subsequently developed disseminated disease, in two cases with neurological involvement (encephalitis/meningoencephalitis or meningismus – P7, P11), in six cases with a multisystem involvement or sepsis-like presentation (P2, P7, P8, P9, P10, P11), and in one case with severe

post-vaccination rash and fever with subsequent hearing loss (P4). In three patients (P7, P10, P11) the clinical picture was reported as consistent with Kawasaki-like disease.

In kindred I, P2 presented aged 18 months with disseminated measles and pneumonia after MMR vaccination and P4 suffered from prolonged fever and rash after MMR vaccination also aged 18 months, which was complicated by a profound acquired sensorineural hearing loss. Information regarding the vaccinal status or the childhood medical history of P1 is not available, while P3, P5 and P6 did not receive the MMR vaccination. In kindred II, P7 had a prolonged post-vaccinal disease at the age of 12 months, starting with a febrile syndrome with systemic inflammation one week after MMR vaccination, when an atypical Kawasaki disease was suspected. This was followed by a first episode of aseptic encephalitis and opsoclonus-myoclonus a month later, which resolved. At the age of 2.5 years he developed fever with diarrhea, hyperinflammation, impaired renal and liver function, metabolic acidosis, hyperammonemia, thrombocytopenia and meningoencephalitis with seizures and opsoclonus-myoclonus, which resulted in significant neurological impairment. His sister P8 developed a febrile illness with anemia, lymphopenia and coagulopathy one week after MMR vaccination at the age of 13 months. Mumps virus was found in her CSF, and mumps, measles and rubella viruses were detected on a throat swab. A month later she developed septic shock requiring intensive care, and no etiologic agents could be identified by culture or PCR for herpes simplex virus (HSV), Varicella-Zoster virus (VZV) and enterovirus. Her recovery was slow and characterized by prolonged marked lymphopenia. In kindred III, P9 developed a Kawasaki-like illness with multi-organ involvement one week after MMR vaccination at the age of 24 months, characterized by a morbilliform rash, systemic inflammation, tonsillitis, conjunctivitis, lymphadenopathy, hepatosplenomegaly, arthritis, hepatitis and pancytopenia. His sister P10 also developed disseminated measles after MMR immunization at the age of 18 months, complicated by hepatitis, pneumonia and coagulopathy. In kindred IV, P11 presented with a febrile syndrome and rash one week after MMR vaccination, which worsened to a picture of Kawasaki-like disease and respiratory distress, requiring intensive care. She developed transient meningismus, but viral PCR were negative in plasma and CSF. A throat swab resulted positive for Rubella and Mumps. Information on the vaccinal status of her sister P12 is not available. P16 received the MMR inoculation without any adverse effect but developed severe disseminated disease after VZV vaccination.

Other infectious phenotypes

Three out of 16 patients presented with bacterial infections, mostly otitis and pneumonia, but the majority of patients suffered from recurrent viral infections, often showing an unusually severe or prolonged course (Table 1). The viruses determined by PCR included Influenza (4 patients), enteroviruses (4 patients), varicella (4 patients, of which 1 vaccine-strain VZV), Epstein-Barr virus (EBV, 2 patients), rotavirus (2 patients), adenovirus (2 patients), respiratory syncytial virus (RSV, 1 patient), coronavirus (1 patient), human herpesvirus 6 (HHV-6, 1 patient), and herpes simplex 1 (HSV-1, 1 patient).

P2 suffered from severe HSV-1 gingivo-stomatitis, requiring hospitalization, and episodes of Influenza and EBV infection without a severe course. P4, P5 and P6 in kindred I, P9 in kindred III and P13 and P14 in kindred V suffered from recurrent respiratory tract infections in infancy. Additionally, P5 experienced Influenza and Varicella infections without complications, P13 had recurrent bronchopneumonia, diarrhea and urinary tract infections, and P14 suffered from recurrent diarrhea. An additional child in kindred V died in infancy from primary HSV-1 infection. P10 suffered from otitis, one episode of pneumonia and episodes of impetigo. She was admitted for severe varicella at the age of 6 months and for Influenza at 7 months of age. At the age of 2.5 years she developed severe primary EBV infection with splenomegaly and lymphadenopathy, experiencing prolonged viremia in the blood and CSF until the age of 5 years. Then she developed Coxsackie B meningitis, and moreover suffered from multiple warts and molluscum contagiosum over several years. P11 had two episodes of bacterial otitis between one and two years and suffered from enteroviral meningitis at the age of two months. Her sister P12 was hospitalized for RSV bronchiolitis at 1 year and experienced an enteroviral infection. No patient showed fungal or mycobacterial susceptibility. Five patients received Bacillus Calmette–Guérin (BCG) vaccination at birth or soon after, without complications.

Additional clinical features and outcome

One out of 16 patients (P12) manifested failure to thrive and diarrhea, and two experienced chronic or intermittent diarrhea (P13 and P14 from kindred V).

P9 and P10 from kindred III and P8 showed intermittent infection-related anemia, lymphopenia, neutropenia and thrombocytopenia. P5 suffers from urticaria, asthma and has a short index and ring finger. P11 has asthma and manifested oral ulcers. P6 experienced an

Table 1. Summary of genetic and clinical characteristics of the patients.

Kindred patient	Genetic defect in STAT2	Year of birth	Gender	Age at onset	Bacterial infections	Viral infections	Fungal infections	Myco-bacterial infections	Warts/ molluscum	Failure to thrive	Diarrhea
I P1	c.381+5G>C	1982	F	NA	No	NA (VZV and CMV positive serology)	No	No	No	No	No
I P2	c.381+5G>C	2006	F	2 y	No	Influenza pneumonia, severe HSV-1 stomatitis, EBV positive serology	No	No	No	No	No
I P3	c.381+5G>C	2007	M	2 m	No	N/A	No	No	No	No	No
I P4	c.381+5G>C	2006	F	1 y	No	Several URTI, Varicella	No	No	No	No	No
I P5	c.381+5G>C	2008	F	NA	No	Several URTI, Influenza, Varicella	No	No	No	No	No
I P6	c.381+5G>C	2014	F	NA	No	Several URTI	No	No	No	No	No
II P7	c.1836C>A	2009	M	1 y	No	N/A	No	No	No	No	No
II P8	c.1836C>A	2010	F	1 y	No	No	No	No	No	No	No
III P9	c.1528C>T c.1576G>A	1995	M	3 m	Otitis Pneumonia	Recurrent severe viral infections, RSV, adenovirus and enterovirus	No	No	No	No	No
III P10	c.1528C>T c.1576G>A	2004	F	6 m	Otitis Pneumonia	Recurrent viral infections, severe VZV, severe EBV, enterovirus meningitis	No	No	Yes	No	No

IV P11	c.1883_1884 del	2002	F	1 y	Otitis	Enterovirus meningitis	No	No	No	No	No
IV P12	c.1883_1884 del	2012	F	9 m	No	Rotavirus, Influenza, RSV and enterovirus	No	No	No	Yes	Yes
V P13	c.988C>T	1998	F	3 m	Pneumonia, urinary tract infections	N/A	N/A	N/A	N/A	N/A	Yes
V P14	c.988C>T	2015	M	5 m	Pneumonia	N/A	N/A	N/A	N/A	N/A	Yes
VI P15	c.820C>T	N/A	F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
VII P16	c.1999C>T	2017	F	9 m	No	Influenza, coronavirus, VZV, HHV6	No	No	No	No	No

Abbreviations: CMV: cytomegalovirus; EBV: Epstein-Barr virus; F: female; HHV6: human herpesvirus 6; HSV-1: Herpes simplex 1; m: months; M: male; N/A: not available; RSV: respiratory syncytial virus; URTI: upper respiratory tract infections; VZV: Varicella-Zoster; y: years.

Table 1 - continued.

Kindred patient	Auto immunity	Lympho proliferation	Anemia	Neutro penia	Thrombo cytopenia	Other	MMR vaccine	Complications post MMR	Outcome	Cause of death	Current therapy
I P1	No	No	No	No	No	-	N/A	N/A	Alive	-	None
I P2	No	No	No	No	No	-	Yes	Multi-systemic disease	Alive	-	Acyclovir
I P3	No	No	No	No	No	-	No	-	Deceased at 2 m	Overwhelming viral infection (autopsy)	-
I P4	No	No	No	No	No	-	Yes	Prolonged fever, rash, profound sensorineural hearing loss	Alive	-	Acyclovir
I P5	No	No	No	No	No	Asthma, urticaria	No	-	Alive	-	Acyclovir cetirizine
I P6	No	No	No	No	No	Atypical Kawasaki disease	No	-	Alive	-	Acyclovir
II P7	No	No	No	No	No	-	Yes	Multi-systemic disease, meningoencephalitis	Alive	-	SCIG Levetirac Topiram Lacosanide Omepraz
II P8	No	No	Yes	Yes	Yes	-	Yes	Multi-systemic disease, sepsis-like disease	Alive	-	SCIG
III P9	No	No	Yes	Yes	Yes	-	Yes	Multi-systemic disease Kawasaki-like	Deceased at 7 y	Overwhelming infection	-

III P10	No	No	Yes	Yes	Yes	-	Yes	Multi-systemic disease	Alive	-	None
IV P11	No	No	No	No	No	Oral ulcers, asthma	Yes	Multi-systemic disease with meningismus	Alive	-	Cetirizine Inhaled fluticasone salmeterol
IV P12	No	No	No	No	No	-	Yes	N/A	Deceased at 5 y	Overwhelming infection	-
V P13	N/A	N/A	N/A	N/A	N/A	Bronchiectasis, hypo-IgA	N/A	N/A	Alive	-	N/A
V P14	N/A	N/A	No	No	No	Oral candidiasis	N/A	N/A	Alive	-	N/A
VI P15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Alive	-	N/A
VII P16	No	No	N/A	N/A	N/A	Disseminated vaccine-strain VZV	Yes	No	Alive	-	-

Abbreviations: Ig: immunoglobulin; Levetirac: levetiracetam; m: months; N/A: not available; Omepraz: omeprazole; SCIG: subcutaneous immunoglobulins; Topiram : topiramate; VZV: Varicella-Zoster; y: years.

episode of atypical Kawasaki disease, and also the post-MMR manifestations in P7, P10, P11 resembled Kawasaki disease.

Thirteen patients are alive at the time of the study. Eleven are well while two live with chronic complications: P4 has profound sensorineural hearing loss and P7 survives with significant neurological damage, including spasticity, chorea and cortical visual impairment, following meningoencephalitis with seizures and opsoclonus-myoclonus at the age of 2.5 years. Three patients died (19%). P3, P9 and P12 all succumbed to fulminant infection with an unidentified agent, at the age of two months, seven years and five years respectively. The clinical presentation was of a short febrile illness resembling a viral infection in P3 and P12, and of purpura with catastrophic disseminated intravascular coagulation and multi-organ failure following two days of fever in P9. Etiology could not be determined based on microbiological cultures in any of the deceased, but in P3 a post-mortem examination revealed a picture of enterocolitis, interstitial pneumonia and cerebral cortical edema with neuronal apoptosis, and histopathological changes were consistent with overwhelming viral illness.

Two patients are currently treated with subcutaneous immunoglobulin (Ig) substitution (P7 and P8 from kindred II). Four patients receive prophylactic acyclovir (P2, P4-P6). P5 and P11 use cetirizine, and P11 is also on inhalation therapy for asthma. None of the patients has received hematopoietic stem cell transplantation.

Immunological characteristics

Most patients had normal lymphocyte counts (Table 2) but T and/or B cell lymphopenia was present in three patients (P8, P10, P11). In three out of four tested patients, the percentage of naïve T cells was low (P2, P7, P8). T cell proliferation in response to mitogens was normal in all tested patients, while proliferation in response to antigens was impaired in one out of three (P9). Levels of IgG and IgM were normal or high in all tested patients. Interestingly, four out of eight patients showed low levels of IgA (P2, P8, P11, P13), one of whom suffered from recurrent bronchopneumonia and diarrhea (P13). One out of four tested patients had abnormal antibody production in response to a protein vaccine (tetanus, P12), and both P2 and P8 had a normal pneumococcal polysaccharide vaccine response.

Impact of the mutations on STAT2 expression and STAT2 phosphorylation

STAT2 expression and phosphorylation were examined in STAT2 deficient fibroblasts (obtained from P9, as previously described (10)) reconstituted with lentivirus containing either wild type *STAT2*, c.1836C>A (C612X), c.1528C>T (R510X), c.1883_1884del (V628fs14X), c.988C>T (R330X), or c.820C>T (Q274X) *STAT2*. Immunoblotting for STAT2 demonstrated absence of expression of full length STAT2 protein for all mutants. However, a truncated STAT2 was visible for V628fs14X and for R330X, at least in the lentiviral overexpression system. All mutants resulted in absent STAT2 phosphorylation at Y-690 after stimulation with IFN-α2a. By contrast, STAT1 expression and phosphorylation after stimulation with IFN-γ was normal (Fig. 3).

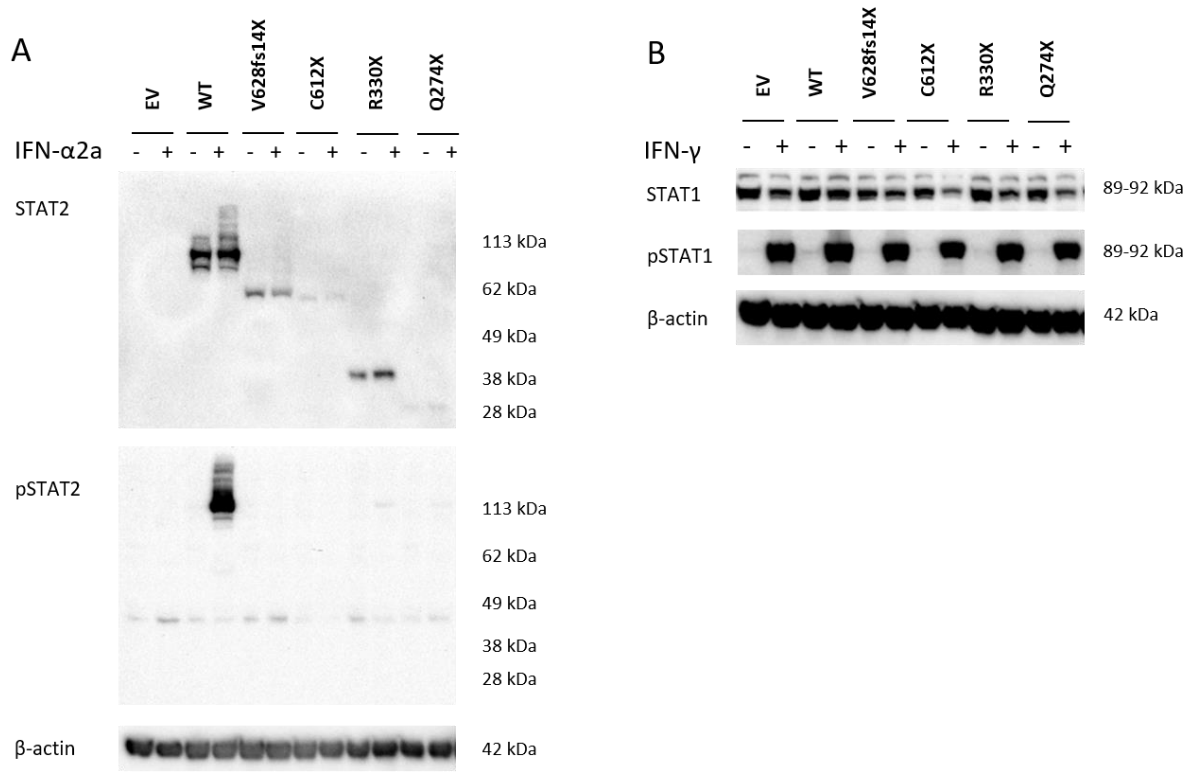


Figure 3. Protein expression and phosphorylation. Primary fibroblasts of a previously described (10) STAT2 deficient patient were lentiviral transduced with a pTRIP_GFP vector containing *STAT2* cDNA or with *STAT2* cDNA harboring the variant under evaluation. GFP-expressing fibroblasts were sorted and cultured for further experiments. STAT1 and STAT2 expression and phosphorylation after pre-treatment with IFN-α2a (A) or IFN-γ (B), respectively, were evaluated via immunoblotting as previously described (10). EV: empty vector; WT: wild type; kDa: kilo Dalton.

Table 2. Immunological characteristics of the patients.

Kindred **Patient**	**I** **P2**	**I** **P3**	**II** **P7**	**II** **P8**	**III** **P9**	**III** **P10**	**IV** **P11**	**IV** **P12**	**V** **P14**
STAT2 variant	c.381+5G>C	c.381+5G>C	C612X	C612X	R510X / c.1576G>A	R510X / c.1576G>A	V628fs14X	V628fs14X	R330X
Age at testing	1 y	1 m	11 m	1 y	3 y	1 y	1 y	1 y	1 y
Total lymphocytes	7270/µL (3600-8900/µL)	4160/µL (3400-7600/µL)	NA	1900/µL (3600-8900/µL)	2300/µL (2300-5400/µL)	1800/µL (3600-8900/µL)	2974/µL (3600-8900/µL)	8350/µL (3600-8900/µL)	12270/µL (3600-8900/µL)
T cells	4781/µL, 65% (2100-6200, 53-75%)	3824/µL, 90% (2500-5500, 53-84%)	85% (49-76%)	650/µL, 34% (2100-6200, 53-75%)	46% (56-75%)	1173/µL, 64% (2100-6200, 53-75%)	1338/µL, 45% (2100-6200, 53-75%)	5770/µL, 69% (2100-6200, 53-75%)	7434, 60% (2100-6200, 53-75%)
CD4+ T cells	3536/µL, 48% (1300-3400, 32-51%)	2937/µL, 70% (1600-4000, 35-64%)	35% (31-56%)	400/µL, 21% (1300-3400, 32-51%)	27% (28-47%)	827/µL, 45% (1300-3400, 32-51%)	476/µL, 16% (1300-3400, 32-51%)	3750/µL, 45% (1300-3400, 32-51%)	3440/µL, 28% (1300-3400, 32-51%)
CD8+ T cells	1207/µL, 16% (620-2000, 14-30%)	1047/µL, 25% (560-1700, 12-28%)	46% (12-24%)	230/µL, 12% (620-2000, 14-30%)	17% (16-30%)	309/µL, 17% (620-2000, 14-30%)	833/µL – 28% (620-2000, 14-30%)	2020/µL, 24% (620-2000, 14-30%)	4125/µL, 33% (620-2000, 14-30%)
Naïve T cells	31% (75-88%)	N/A	55% (76-89%)	65% (75-88%)	N/A	N/A	94% (75-88%)	N/A	N/A
T cell prolif. **Antigens** **Mitogens**	 = =	 N/A N/A	 N/A =	 N/A =	 ↓ =	 = =	 N/A N/A	 N/A N/A	 N/A N/A
NK cells	1134/µL, 15% (180-920, 3-15%)	317/µL, 7% (170-1100, 4-18%)	3% (3-15%)	230/µL, 12% (180-920, 3-15%)	N/A	152/µL, 8% (180-920, 3-15%)	595/µL, 20% (180-920, 3-15%)	N/A	1318/µL, 10% (180-920, 3-15%)

B cells	727/µL, 10% (720-2600, 16-35%)	443/µL, 10% (300-2000, 6-32%)	9% (14-37%)	860/µL, 45% (720-2600, 16-35%)	18% (14-33%)	462/µL, 25% (720-2600, 16-35%)	625/µL,21% (720-2600, 16-35%)	940/µL, 11% (720-2600, 16-35%)	4349/µL, 35% (720-2600, 16-35%)
IgG	7.53 g/L (3.83-10.7)	N/A	9.66 g/L (3.83-10.7)	14.7 g/L* (3.83-10.7)	8.18 g/L (5.39-15)	8.02 g/L (3.83-10.7)	22 g/L (3.83-10.7)	5.32 g/L (3.83-10.7)	8.48 g/L (3.83-10.7)
IgM	0.58 g/L (0.28-1.13)	N/A	0.57 g/L (0.28-1.13)	0.53 g/L (0.28-1.13)	0.67 g/L (0.26-1.06)	3.39 g/L (0.28-1.13)	3.75 g/L (0.28-1.13)	0.99 g/L (0.28-1.13)	1.46 g/L (0.28-1.13)
IgA	0.24 g/L (0.27-1.69)	N/A	0.64 g/L (0.27-1.69)	0.17 g/L (0.27-1.69)	0.45 g/L (0.53-3.36)	1.22 g/L (0.27-1.69)	0.17 g/L (0.27-1.69)	0.58 g/L (0.27-1.69)	1.4 g/L (0.27-1.69)
IgE	235 IU/ml	N/A	N/A	N/A	N/A	30 IU/ml	N/A	N/A	29 IU/ml
Tetanus Ab	4.8 IU/ml	N/A	normal	normal	N/A	N/A	N/A	0.12 IU/ml	N/A
Pneumococcus Ab	528 IU/ml	N/A	N/A	normal	N/A	N/A	N/A	N/A	N/A

*during Ig supplementation. Reference ranges by age are given in brackets. Abbreviations: Ab: antibodies; m: months; N/A: not available; y: years.

Impact of the mutations on *in vitro* vaccine-strain measles susceptibility, IFN-β production and ISG upregulation

Introduction of all *STAT2* mutants in STAT2 deficient primary fibroblasts resulted in a 30% increase in syncytia formation compared to the WT construct in unstimulated conditions (Fig. 4A). Pretreatment with IFN-α2a inhibited syncytia formation in the WT *STAT2*-reconstituted fibroblasts but not in the fibroblasts reconstituted with the mutants. However, there was a decrease by one half in syncytia formation in the R330X and Q274X mutants treated with IFN-α2a compared to the untreated condition (Fig. 4A). IFN-γ pretreatment resulted in some protection from syncytia formation, as described previously (10), in all mutant *STAT2*-reconstituted fibroblasts (Fig. 4A). Finally, we evaluated the secretion of IFN-β, the predominant type I IFN produced by fibroblasts. Elevated amounts of IFN-β were produced in the STAT2 deficient fibroblasts reconstituted with the mutant *STAT2* constructs compared to WT *STAT2* (five- to tenfold increase). This suggests that the increased susceptibility to measles virus in vitro arose from abolished type I IFN signaling, and not from impaired IFN-β production (Fig. 4B). Pretreatment with IFN-α2a resulted in a less pronounced increase in IFN-β compared to the untreated condition, especially evident in the R330X and Q274X mutants, while IFN-β production was almost entirely suppressed after IFN-γ pretreatment (Fig. 4B). It is worth noting that after IFN-α2a stimulation also the empty vector condition produced less IFN-β: this can be caused by a partial activation of ISGs through the formation of STAT1 homodimers, even in the absence of STAT2, and can in part explain the reduced increase of IFN-β production in the cells reconstituted with *STAT2* mutants. Altogether, these data strongly suggest that these newly identified *STAT2* variants are responsible for vaccine-strain measles and viral disease in these patients through the disruption of cell-intrinsic type I IFN immunity to viruses. Finally, we observed severely diminished *ISG15* and *MXA* expression 6h post IFN-α2a stimulation of the STAT2 deficient fibroblasts reconstituted with an empty vector or *STAT2* mutants, while WT *STAT2* could elicit a normal upregulation of transcription (Fig. 5). Interestingly, there was residual *ISG15* and *MXA* induction post IFN-α2a treatment for the R330X and Q274X mutants.

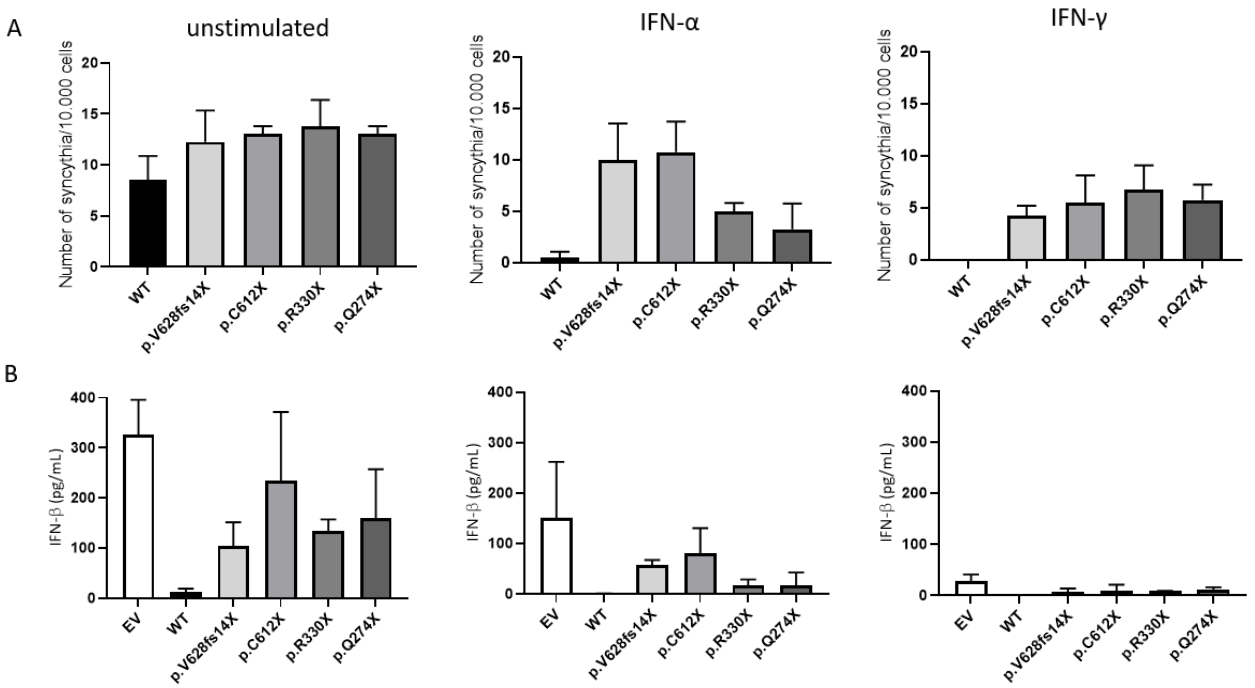


Figure 4. In vitro challenge with measles virus. Fibroblasts transduced with empty vector (EV), wild type *STAT2* (WT) or one of the variants were exposed to vaccine (Edmonston) strain measles virus (MOI 0,015) with or without IFN-α or IFN-γ pretreatment for 24 hours. A) Syncytia formation was evaluated by Floid microscopy on day 3. B) IFN-β production was evaluated by ELISA on day 5.

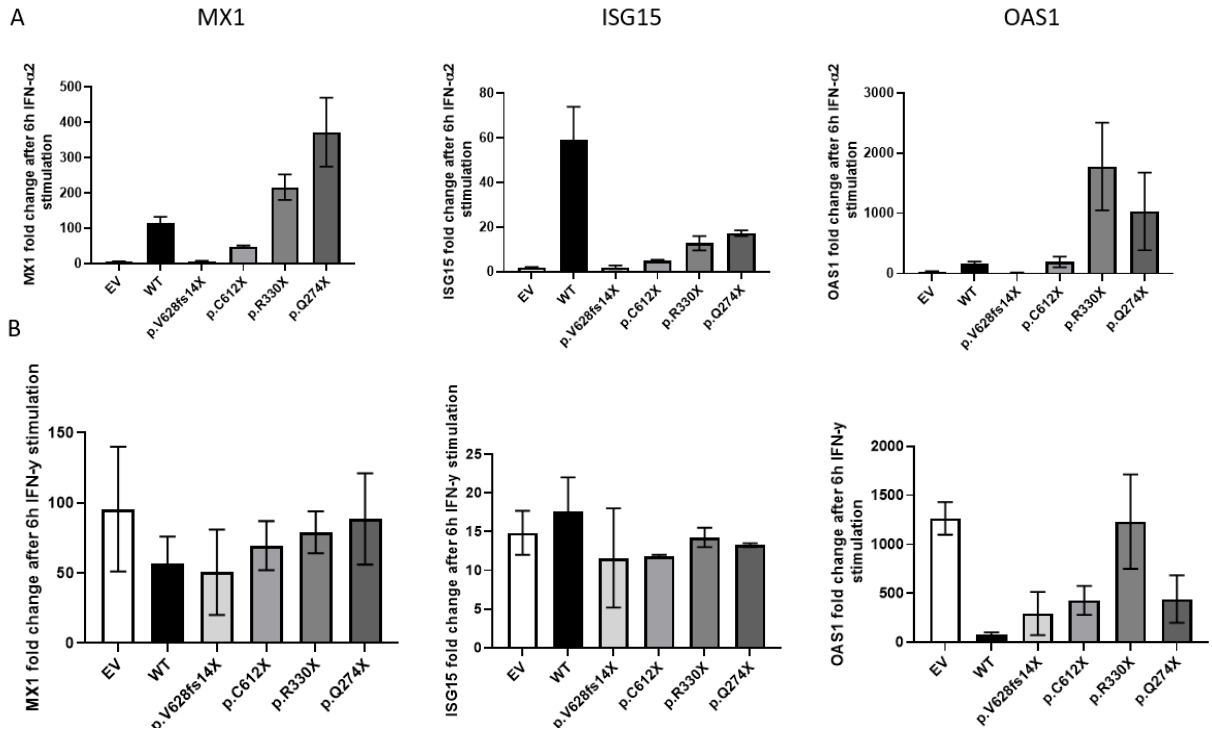


Figure 5. Upregulation of ISGs after type I and II IFN stimulation. Fibroblasts transduced with empty vector (EV), wild type *STAT2* (WT) or one of the variants were pretreated with IFN-α2a (A) or IFN-γ (B) for 6 hours and upregulation of *MX1*, *ISG15* and *OAS1* was evaluated by qPCR. Results normalized to endogenous *ACTB* and fold change of expression calculated with 2-ΔΔCt method.

Discussion

We describe the clinical and immunological phenotype of 16 patients from seven unrelated kindreds carrying eight different mutations in STAT2, a key transcription factor in the type I and III IFN pathway.

The study of the various alleles shows that c.381+5G>C, c.1528C>T/c.1576G>A, C612X and Q274X are complete loss of expression and loss of function, while V628fs14X and R330X result in a truncated STAT2 protein, but with complete loss of STAT2 phosphorylation. Interestingly, R330X and Q274X demonstrate only a partial functional defect when challenged with measles infection in vitro and their ability to upregulate some ISGs upon IFN-α stimulation is in part preserved. Unfortunately, we have very limited clinical data on the two patients carrying these partial defects, so we cannot clinically correlate their phenotype to their genotype.

For the remaining variants, STAT2 deficiency shows incomplete penetrance for several viral infections. Despite a severe impairment of type I IFN responses in vitro, the affected patients display a narrow spectrum of viral susceptibility, the most penetrant manifestation being disseminated disease after MMR vaccine inoculation, even if severe or lethal infections with other or undefined viruses are common. In particular, infection with VZV (in one case vaccine strain), influenza and enterovirus were prevalent in this cohort. Animal studies of the IFNAR1-2/STAT2 pathway defects have also found a significant susceptibility to Zika virus, Adenovirus and other viral infections in STAT2 knockout Syrian hamsters and IFNAR1-2 knockout mice (14–17). Wang et al. have recently described a phosphorylation-independent role of ISGF3 complexes in maintaining a constitutive level of ISGs expression, which represents an early, IFN-independent form of defense against viral infections (18). This allows a very rapid activation of the type I IFN signaling pathway upon viral challenge, since the synthesis of new protein components in response to stimulation with IFN is not required. Lacking this early defense, patients with STAT2 deficiency are therefore more at risk of frequent or uncontrolled viral infections.

Several of the patients in our cohort showed a striking inflammatory response during infections. Four patients were in fact diagnosed with atypical Kawasaki disease, three of them after MMR inoculation. Type I IFNs greatly contribute to the regulation of inflammation in human hosts, with both pro- and anti-inflammatory effects. The anti-inflammatory role of IFN-α/β through the inhibition of inflammasome activity and IL-1 production is well established

and has therapeutic relevance for example in the management of diseases such as multiple sclerosis or familial Mediterranean fever (19). We can speculate that the defect of type I IFN signaling in patients with STAT2 deficiency could in some cases lead to a hyper-inflammatory status, especially when the immune system is challenged by microbial stimuli.

The outcome of patients with STAT2 deficiency is poor. Mortality in this cohort is 19% and the burden of severe infections and their chronic complications is high. Some of the younger siblings in whom MMR vaccination was avoided have up to now led a normal life, mostly experiencing viral infections that were not life-threatening. Despite the substantial mortality, this is in stark contrast with the natural course of complete AR STAT1 deficiency. These patients additionally suffer from severe infections with weakly virulent Mycobacteria and other intracellular bacteria. Their viral phenotype also seems broader and more severe than that of STAT2 deficiency, with around 60% of the patients succumbing to mycobacterial or viral infections in the first years of life if not cured by hematopoietic stem cell transplantation (20,21). Comprehensive analysis of STAT1-deficient cells strongly suggests that the corresponding patients' viral susceptibility is due to impaired IFN-α/β response, whereas their susceptibility to intracellular bacteria is due to defective IFN-γ signaling (22). This conclusion can be drawn from the observation that patients with autosomal dominant STAT1 deficiency, where there is no defect in ISGF3-dependent signaling to type I IFNs, do not experience lethal viral disease. We can thus hypothesize that the milder phenotype of STAT2 deficiency also arises from the conserved type II IFN pathway. This is also suggested by the effect of pre-treatment with IFN-γ in our in vitro measles infection system.

In contrast with AR STAT1 deficiency, STAT2 deficient patient display a cellular and clinical phenotype very similar to the other inborn errors of type I and/or III IFN immunity, including IFNAR2, IFNAR1, IRF7, and IRF9 (11,12,23,24). In all these defects, susceptibility to disseminated disease after MMR vaccination has been described, and other severe viral diseases are reported with incomplete penetrance. In particular, IRF7 and IRF9 deficient patients seem to be particularly susceptible to influenza infection (12,24,25), while a patient with IFNAR1 deficiency who tolerated MMR vaccination without complications later developed post-vaccination visceral yellow fever (23). Of note, also one of the patients with IRF9 deficiency succumbed to enterohemorrhagic fever after yellow fever vaccination (25), and one of the STAT2 deficient patients in our cohort (who incidentally did not suffer from post-

MMR disease) developed instead severe disseminated varicella after VZV vaccination. These data indicate a relevant role of type I IFN in the immune response to live vaccine inoculation, also sustained by studies on the mechanism of attenuation of these viruses. Bankamp et al. have studied the genetic characteristics of nine separate measles vaccine strains currently in use and found that a possible mechanism of attenuation was the loss of IFN resistance, normally mediated by the P, V and C viral proteins (26,27). Moreover, the hemagglutinin protein H of the Edmonston measles vaccine strain has been shown to be responsible for attenuation through strong activation of type I IFN induction in a primate animal model, and the same IFN-inducing effect was traced back to the presence of defecting interfering RNA in another vaccine strain, Hu-191 (28,29). Finally, vaccine-strain yellow fever YFV-17D causes disseminated disease in IFNAR1 deficient mice, but not WT mice (30). Therefore, the absence of a functioning IFN pathway in patients with defects of type I and/or III IFN immunity could explain the extreme virulence shown by these live vaccine viruses. Further studies are required to fully unravel the mechanisms of human anti-viral defense and to develop safer vaccines for these patients.

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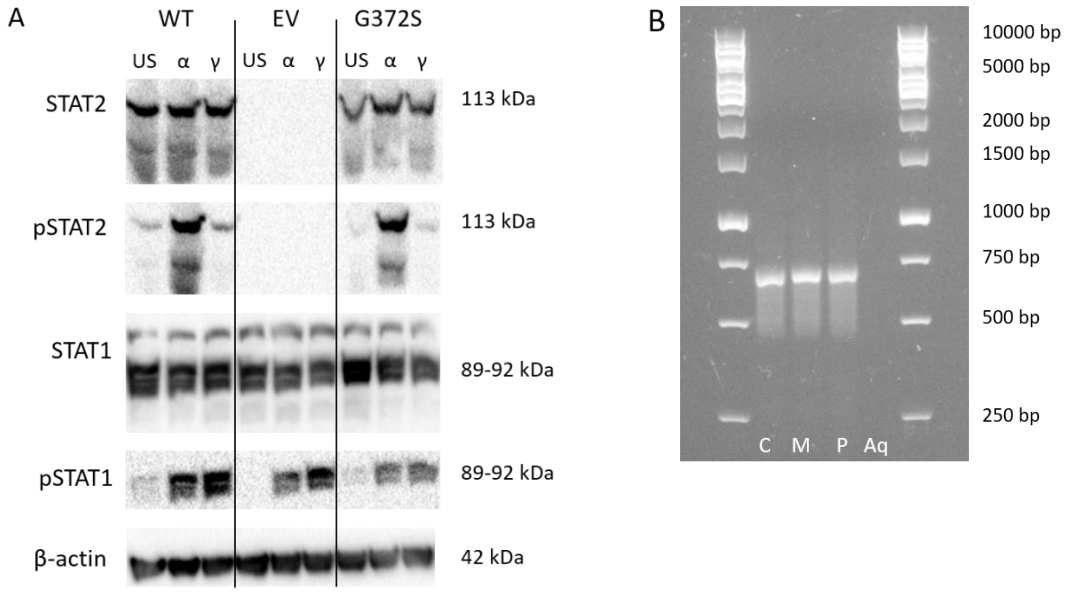
Supplementary material

Table S1. *STAT2* mutations identified with their consequences on *STAT2* gene, transcripts, STAT2 protein and *in silico* prediction of pathogenicity

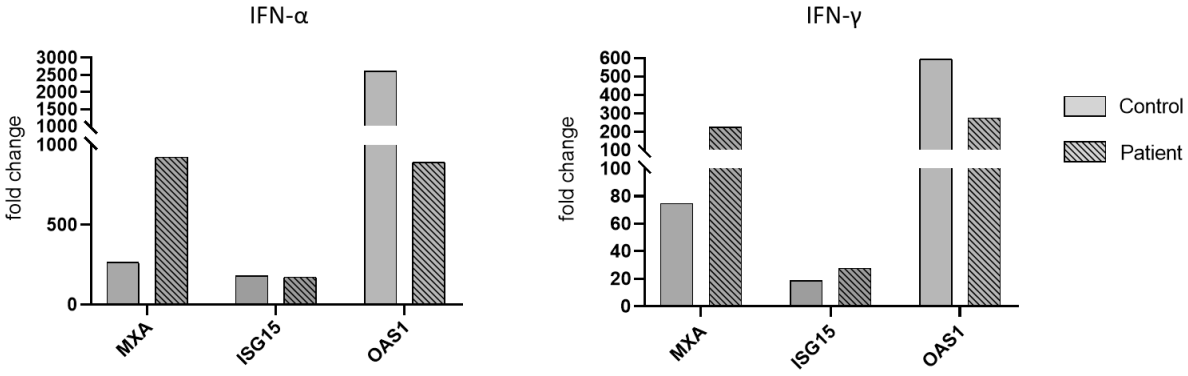
Kindred Patients	Nucleotide change (NM_005419.3)	Deduced amino acid change	Intron/ exon	CADD score *	In silico splicing prediction	Effect on *STAT2* mRNA and protein	STAT2 deficiency	Reference
I P1-6	c.381+5G>C	NA	Intron 4	11.71	Broken WT acceptor splice site	Retaining of intron 4 and 6, skipping of exon 16 and 17, nonsense-mediated decay, complete loss of expression of STAT2	Complete	(1)
II P7-8	c.1836C>A	p.C612X	Exon 20	37	-	cDNA nonsense-mediated decay, complete loss of expression of STAT2	Complete	(2)
III P9-10	c.1528C>T	p.R510X	Exon 17	35	-	cDNA nonsense-mediated decay, complete loss of expression of STAT2	Complete	(3)
	c.1576G>A	NA	Exon 16	33	Broken WT donor splice site			
IV P11-12	c.1883_1884 del	p.V628fs14X	Exon 21	34	-	Truncated STAT2 protein, complete loss of STAT2 phosphorylation	Complete*	Current report
V P13-14	c.988C>T	p.R330X	Exon 10	36	-	Truncated STAT2 protein, complete loss of STAT2 phosphorylation	Partial*	Current report
VI P15	c.820C>T	p.Q274X	Exon 9	34	-	Complete loss of expression of STAT2	Partial*	Current report
VII P16	c.1999C>T	p.R667X	Exon 21	38	-	NA	NA	Current report

MSC CADD score for STAT2 gene is 3.313. *Not tested on patients' material.

Supplementary Figure 1. A) Western blot showing normal expression and phosphorylation of STAT2 and STAT1 for the G372S variant as compared with the wild type (WT) *STAT2* and an empty vector (EV). US: unstimulated, α: stimulated with IFN-α2a, γ: stimulated with IFN-γ, kDa: kiloDalton. B) Normal splicing of STAT2 exons surrounding the G372S variant is shown by agarose gel electrophoresis of PCR products from a healthy control (C), the heterozygous mother of the patient (M), and the homozygous patient (P). Aq: negative control, bp: base pairs.



Supplementary Figure 2. Preserved upregulation of ISGs after type I and II IFN stimulation in the patient carrying the G372S variant compared to a control. Fibroblasts pretreated with IFN-α2a or IFN-γ for 6 hours and upregulation of *MX1*, *ISG15* and *OAS1* were evaluated by qPCR. Results normalized to endogenous *ACTB* and fold change of expression calculated with 2-ΔΔCt method.



CHAPTER 3

Systemic inflammation and myelofibrosis in a patient with Takenouchi-Kosaki syndrome due to *CDC42* Tyr64Cys mutation

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Introduction

Heterozygous mutations in *CDC42*, encoding the small GTP/GDP-binding protein cell division cycle 42 (CDC42) involved in eukaryotic actin cytoskeleton dynamics, cause Takenouchi-Kosaki syndrome, a rare developmental disorder. Hallmarks of Takenouchi-Kosaki syndrome are intellectual and growth delay, dysmorphisms, macrothrombocytopenia, camptodactyly, structural brain abnormalities with sensorineural deafness, hypothyroidism and frequent infections (1). The original patient identified with this condition carried a de novo p.Tyr64Cys (c.191A>G) mutation. Five patients with the Tyr64Cys variant and similar clinical phenotypes have now been described (1-5). Fourteen additional patients with different developmental phenotypes due to autosomal dominant (AD) heterozygous mutations disrupting CDC42 function and downstream signaling were reported by Martinelli et al. (3). Recently, Gernez et al. and Lam et al. described a syndrome of immune dysregulation caused by other *CDC42* mutations in eight additional patients (6,7). These patients presented with neonatal-onset severe autoinflammation with increased serum inflammatory cytokines and chemokines (IL-6, IL-18, IL-18 binding protein (IL-18BP), CXCL9), as well as hepatosplenomegaly and hemophagocytic lymphohistiocytosis (HLH) (6,7). The unrelated patients carried C-terminal *CDC42* mutations (p.Arg186Cys in five; p.Cys188Tyr in two; p.*192Cys*24 in one) (6,7). The exact pathophysiology of inflammation remains elusive, yet Lam et al. did show that Arg186Cys causes aberrant subcellular localization of CDC42 with impaired cell polarity and migration. In contrast, mutations in the switch II domain of CDC42 underlie Takenouchi-Kosaki syndrome, while mutations within or close to the nucleotide binding pocket (NBP) cause growth deficiency, brain malformations and striking facial dysmorphisms, and mutations affecting the association with CDC42/RAC-interacting binding

(CRIB) motifs, especially p.Glu171Lys, underlie a phenotype resembling RASopathies (1-5). The genetic and clinical characteristics of reported patients with *CDC42* mutations are summarized in supplementary table S1, and the protein domains in fig. 1.

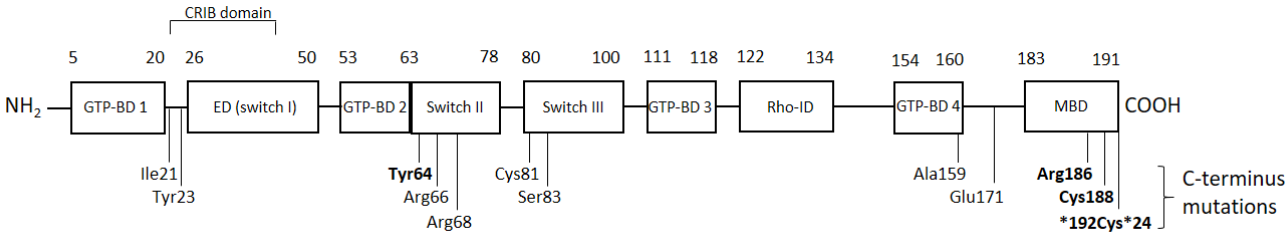

Figure 1. Schematic representation of the CDC42 protein domains with the reported mutations indicated. The mutation carried by our patient (Tyr64) and the mutations in the sequence coding for the C-terminal region recently described by Gernez et al. and Lam et al. (6,7) are highlighted. CRIB: CDC42/RAC-interacting binding domain; GTP-BD: GTP binding domain; ED: effector domain; Rho-ID: Rho insert domain; MBD: membrane binding domain.

Case report

Here, we present a patient with the Tyr64Cys *CDC42* mutation who manifested immune dysregulation, including hepatosplenomegaly, myelofibrosis, systemic inflammation, and pulmonary hemorrhage. The patient was born at term from non-consanguineous Belgian parents. She was small for gestational age (weight -2 SD), microcephalic (head circumference -2.5 SD), hypotonic and mildly dysmorphic. She manifested psychomotor developmental delay, feeding difficulties and growth retardation. A brain MRI scan revealed a Dandy-Walker variant with a posterior fossa arachnoid cyst, requiring cysto-peritoneal drainage at age 18 months. She had microretrognathia, hypertelorism with incomplete eyelid closure, depressed nasal bridge, strabismus, severe astigmatism, arachnodactyly, and sensorineural hearing loss. She developed scoliosis, pes planus, camptodactyly, fingernail exostosis, hepatosplenomegaly with multiple spleen and kidney hyperechogenic lesions (which were not biopsied), liver hemangiomas, and a thoracic-abdominal aortic aneurysm with maximal diameter of 4.5 cm (Fig. 2A). She suffered recurrent lower respiratory tract infections since childhood with diagnosis of bronchiectasis at age 15 years despite antibiotic treatment and physiotherapy. Laboratory evaluations at that time showed profound lymphopenia, especially of B cells, neutropenia, anemia and thrombocytopenia. A bone marrow biopsy at age 16 showed dysmegakaryopoiesis but otherwise normocellular marrow with normal differentiation. She

had hypogammaglobulinemia in the first year of life with IgA and IgM deficiency, but only IgA deficiency persisted later in life. Laboratory results are summarized in table 1.

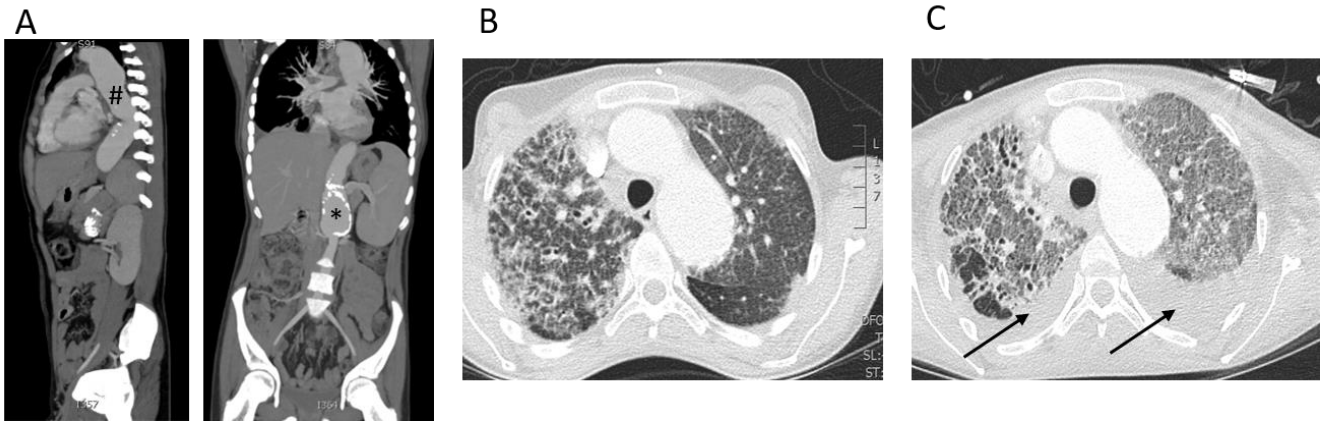

Figure 2. A) Thorax-abdomen CT scan aged 13 years showing prominent aneurysm of the thoracic (#) and abdominal (*) aorta. B-C) Chest CT scans at age 25 (B) and 26 (C) years showing ground glass opacities, bronchiectasis, cyst formation, and crazy paving pattern. In C massive pleural effusion at D+17 of admission can be seen (arrows).

Table 1. Laboratory results and immunological phenotype of the patient.

Test	**Age 3 months**	**Ref. values**	**Age 15 years**	**Ref. values**	**Age 26 years**	**Ref. values**
White blood cells	4000/μL	6000-17.500	2400/μL	4500-13.000	4500/μL	4000-10.000
Neutrophils	800/μL	1000-8500	1400/μL	1800-8000	2500/μL	2500-7800
Monocytes	880/μL	600	200/μL	200-800	200/μL	200-800
Lymphocytes	2000/μL	4000-10.500	700/μL	1100-6500	1000/μL	1200-3600
Red blood cells	3.700.000/μL	3.7-5.3*10^6	4.300.000/μL	4.1-5.1*10^6	4.020.000/μL	3.9-5.6*10^6
Hemoglobin	9.2 g/dL	11-13.5	10.5 g/dL	12-16	10 g/dL	12-16
Platelets	224.000/μL	0.15-0.45*10^6	46.000/μL	0.15-0.45*10^6	41.000/μL	0.15-0.45*10^6
Immunoglobulins						
IgG	2.3 g/L	1.2-8	11.8 g/L	5.76-12.65	15.8 g/L	7.51-15.6
IgG2	0.18 g/L	0.23-1.47	1.44 g/L	1.06-6.1	-	
IgG3	0.3 g/L	0.04-1	0.91 g/L	0.18-1.63	-	
IgM	<0.29 g/L	0.56-3.5	0.48 g/L	0.3-1.59	1.28 g/L	0.46-3.04
IgA	0.05 g/L	0.7-3.1	0.62 g/L	0.81-2.32	0.21 g/L	0.82-4.53
Lymphocyte subpopulations	1640/μL	2300-6500	600/μL	800-3500	858/μL	798-2823
T cells	860/μL	1500-5000	266/μL	400-2100	240/μL	455-1885
CD4+	660/μL	500-1600	300/μL	200-1200	562/μL	219-1124
CD8+	6%	1-9*	-		50%	2-12*
HLA-DR	140/μL	600-3000	25/μL	200-600	13/μL	82-476
B cells	240/μL	100-1300	-		126/μL	66-745
NK cells						
Vβ T cell receptor repertoire	-		Normal		-	

Reference values based on in-house ranges except *Coomans-Bitter et al. J Pediatr. 1997 Mar;130(3):388-93.

At age 25 years, she experienced weight loss (>10%), increased coughing, fever and elevated inflammatory markers. C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) increased to 45 mg/L and 45 mm/h respectively. Fibrinogen was 4 g/L with D-dimers >7500 µg/L (normal <500), but ferritin was normal (highest value 31 µg/L). Approximately 50% of T cells expressed HLA-DR. HLH could not be confirmed. The patient's state did not improve despite broad antifungal and antibiotic treatment but responded to steroids. Bone marrow biopsy showed myeloid hypercellularity, erythroid hypoplasia and fibrosis grade 2/3 according to the WHO classification of myeloid neoplasm (8), suggestive of primary myelofibrosis (Fig. 3). As part of the hematology work-up, an NGS panel (Illumina platform) on peripheral blood was performed to screen for driver variants in *JAK2*, *CALR*, and *MPL*, as well as for non-driver variants in a large set of genes (see list in supplementary material). No somatic or germline variants could be identified. At age 26 years she was admitted with cachexia, dyspnea and hypoxemia (O2 saturation 78%, arterial pO2 34 mmHg). A chest CT scan showed bilateral ground glass opacities, honeycombing and crazy paving pattern with cyst formation (Fig. 2B-C). Bronchus aspirate revealed MRSA and HSV-1 for which she was treated accordingly. Maximal CRP and ESR were 179 mg/L and 50 mm/h, respectively. Ventilatory support was escalated to extracorporeal membrane oxygenation (ECMO) from D+7. Suspecting an inflammatory component, she was started on 80 mg methylprednisolone per day on D+11, after which she could be weaned from ECMO. Upon tapering of steroids, lung infiltrates flared – a pattern seen twice, after which glucocorticoid therapy was maintained from D+41. During this course she had several episodes of pulmonary hemorrhages. CT angiography showed several hypertrophic bronchial arteriae which were embolized. At D+41 after admission Stenotrophomonas maltophilia was cultured from the sputum. Ultimately the patient succumbed to shock associated with Stenotrophomonas maltophilia bacteremia at D+68 after admission.

Whole exome sequencing (WES) was performed on whole blood DNA from the patient and her parents, after informed consent from the parents (research protocol S58466 approved by the ethical committee of the University Hospitals Leuven). WES revealed a de novo heterozygous *CDC42* mutation in the patient, c.191A>G (p.Tyr64Cys), associated with Takenouchi-Kosaki syndrome (supplementary methods and supplementary fig. S1) (1-5).

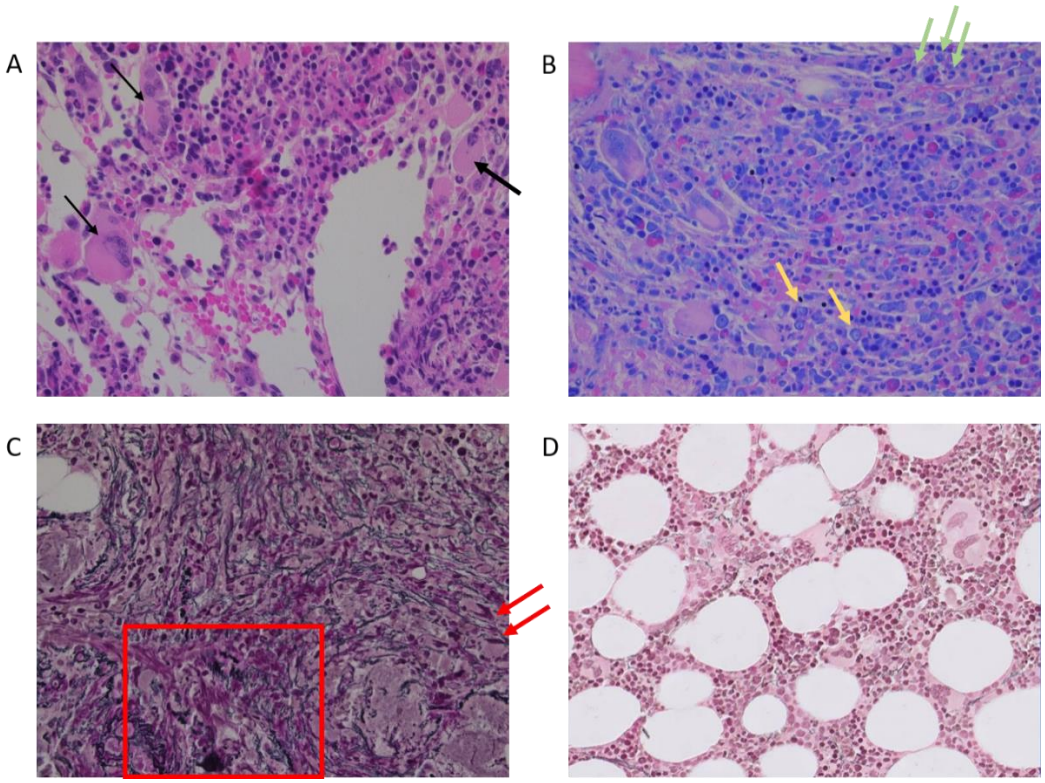

Figure 3. A) Hematoxylin-eosin, B) GIEMSA, C) reticulin staining of the patient's bone marrow. D) Reticulin staining of a control bone marrow. Fibrosis in the patient's bone marrow is identified by the abundance of silver reticulin staining (C) compared to a control (D) and by the presence of crush artifacts (red box), a typical indirect sign of myelofibrosis. Black arrows indicate aberrantly large, hyperlobulated and clustering megakaryocytes, and red arrows aberrantly hyperchromatic megakaryocytes. Green arrows indicate increased presence of polymophonuclear leukocytes and yellow arrows erythroblasts with rod-like nucleoli, seen also in the peripheral leukoerythroblastosis typical of myelofibrosis.

In-depth immune phenotyping at age 25 years showed reduced frequencies of B cells and NK cells with increased proportions of T cells in the patient compared to healthy controls (supplementary methods and fig. 4).

Proportions of total CD4+ and CD8+ T cells were preserved. Differentiation of CD4+ and CD8+ T cells was skewed to effector memory and effector memory expressing CD45RA (TEMRA) subsets respectively, at the expense of naïve cells. No exhaustion signature was present in either CD4+ or CD8+ T cells from the patient (Fig. 4F-G). Normal proportions of NKT and mucosal associated invariant T (MAIT) cells were observed (supplementary fig. S5E). Transitional and memory B cells were decreased and naïve B cells were increased (Fig. 4B-D). Following in vitro activation, CDC42 mutant CD8+ T cells exhibited poor upregulation of the activation markers CD25, CD69 and CD95 when tested after five days. Expression of CD107a was not affected (Fig. 4H). Patient memory CD4+ T cells exhibited normal production of IL-2

as well as cytokines characteristic of T helper (Th)1 (interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α)), Th2 (IL-4, IL-9), Th17 (IL-17A, IL-17F) and T follicular helper (Tfh) (IL-21) cells, although production of IL-13 was reduced (Fig. 4I). Inspired by recent reports (46,47), pro-inflammatory cytokines (IL-6, IL-18, IL-18BPa, CXCL9) were measured in serum samples collected between the age of 25 and 26 years (see supplementary methods). All cytokines tested as well as CRP and ESR were elevated, compared to healthy controls (Fig. 5). Taken together, these data suggest that auto-inflammation is also a feature of Takenouchi-Kosaki syndrome caused by Tyr64Cys in the switch domain of CDC42.

Discussion

The patient manifested the typical traits of Takenouchi-Kosaki syndrome (1-5). The immunophenotype is characterized by lymphopenia, particularly B lymphopenia with excess naïve B cells. The vascular malformations (liver hemangiomas) and the aneurysm are comparable to those described in DOCK8 deficiency, Wiskott-Aldrich syndrome, and/or actin-cytoskeleton disorders in general (9,10). We also show evidence of auto-inflammation in this patient with a *CDC42* mutation outside of the C-terminal domain. Patients with Takenouchi-Kosaki syndrome due to Tyr64Cys have not been reported to suffer from auto-inflammation (1-5), although a single patient with the switch II domain Arg68Gln variant suffered splenomegaly, fever, intermittent skin rash and serositis (3). It is possible that Tyr64Cys is associated with milder auto-inflammation, as reflected by the lower values of inflammatory cytokines in our patient compared to those reported previously with full-blown HLH (6,7). This demonstrates the potential of using serum cytokine pattern analysis in patients with an ill-defined disease course, as it would aid in therapeutic decision making. In hindsight, this patient may have benefited from IL-1 inhibition or anti-IFN-γ antibody emapalumab to control inflammation. On the other hand, given the myelofibrosis and the likely necessity to continue anti-inflammatory treatment lifelong, hematopoietic stem cell transplantation may be a valuable alternative for these patients, as shown in many other recently described severe auto-inflammatory manifestations/primary immunodeficiencies. In general, heterozygous *CDC42* mutations result in a spectrum of immune-deficiency, developmental features and auto-inflammation, much resembling phenotypes caused by mutations in other genes governing the complex actin cytoskeleton dynamics (9).

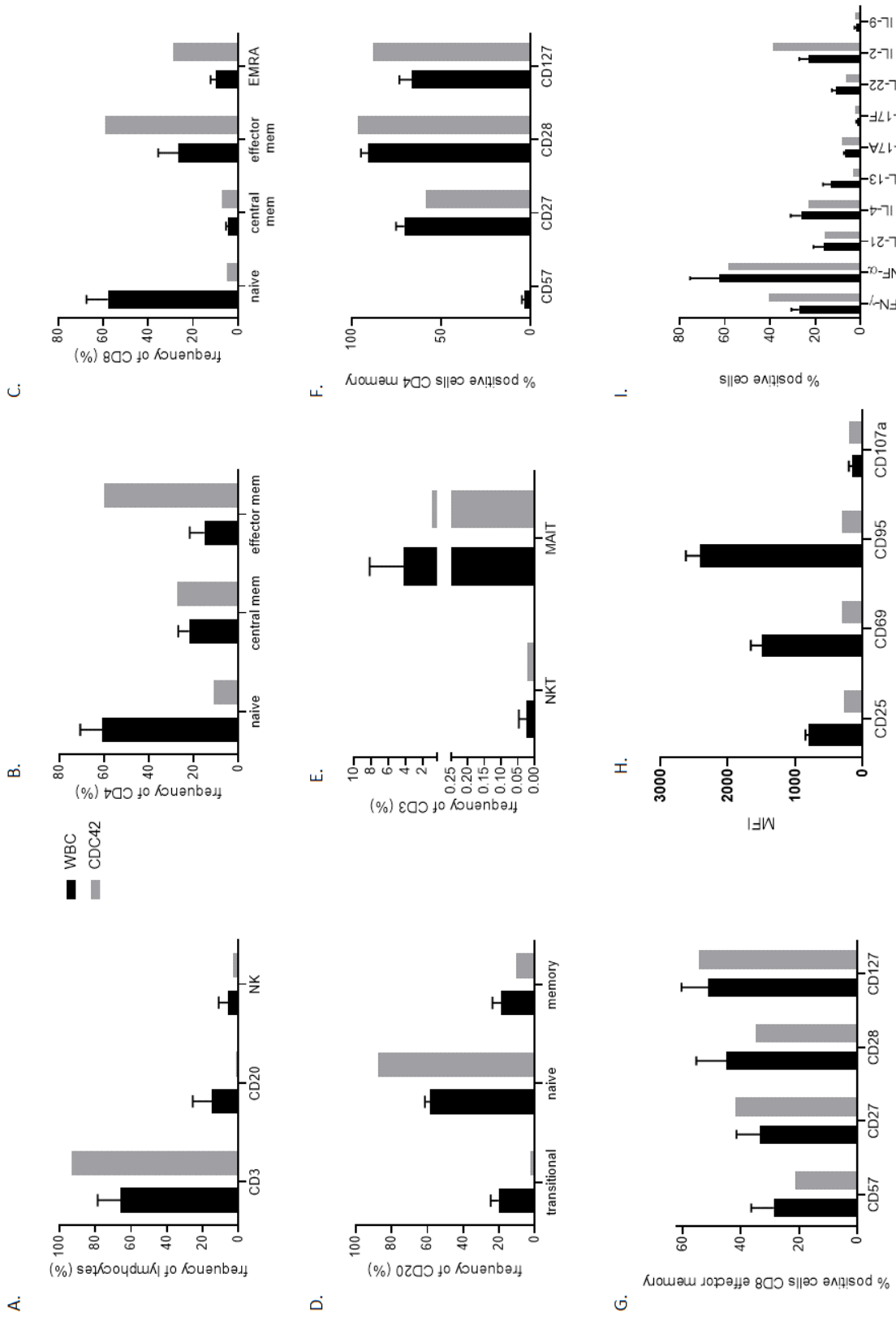

Figure 4. Immunophenotyping performed on PBMCs from three healthy controls (WBC, black bars) and the patient aged 25 years (CDC42, grey bars). For the healthy controls mean and standard deviations are represented. A) T, B and NK cell frequencies. B) CD4+ T cell differentiation. C) CD8+ T cell differentiation. D) B cell differentiation. E) NKT and MAIT frequencies. F-G) Evaluation of the exhaustion signature in CD4+ (F) and CD8+ (G) T cell subsets. H) Activation markers in CD8+ T cells. I) Intracellular production of cytokines in CD4+ T cells: IFN-γ and TNF-α (Th1), IL-4, IL-9 and IL-13 (Th2), IL-17A, IL17F, IL-21 and IL-22 (Th17), IL-2 (Th1 and Th2).

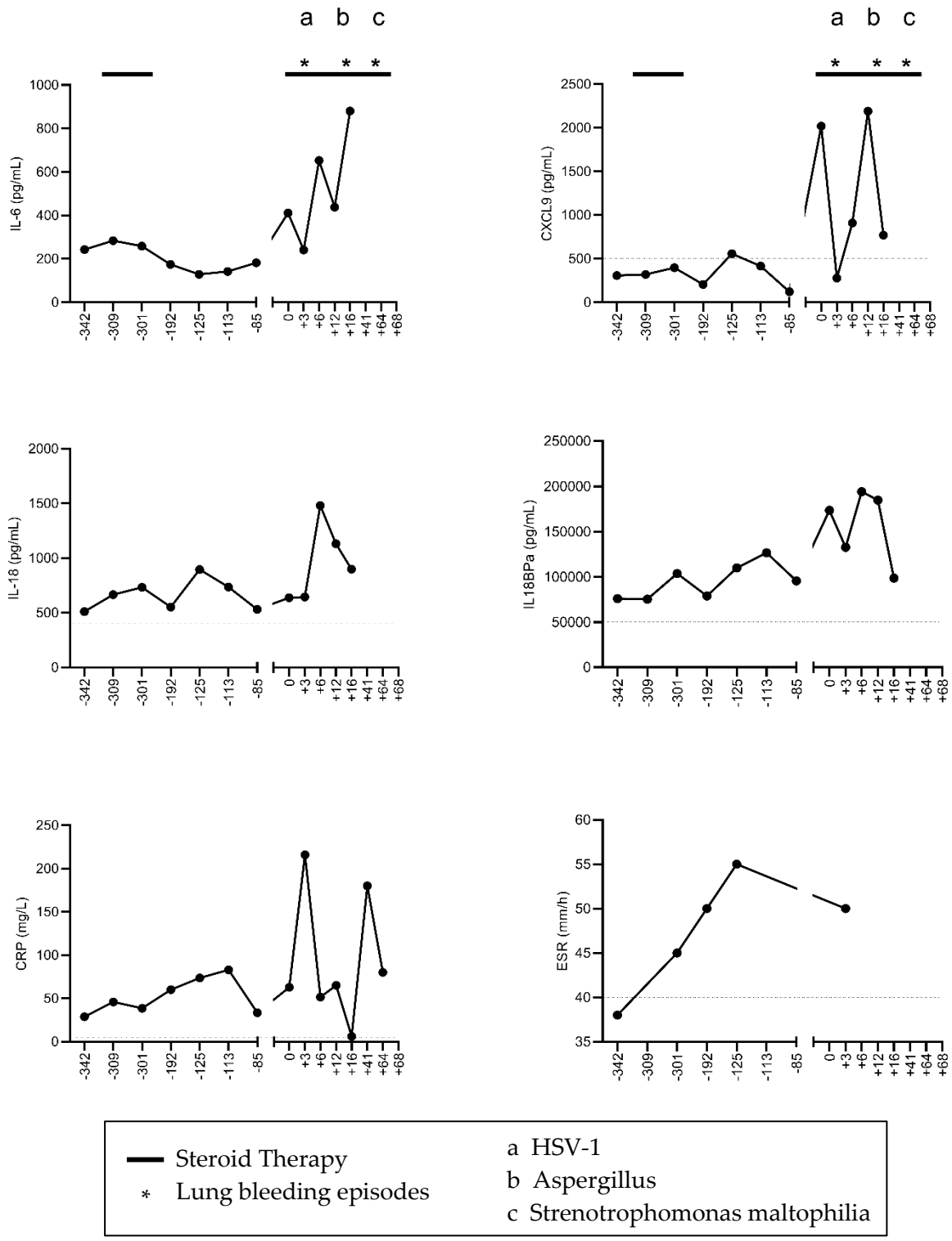

Figure 5. Serum levels of IL-6, CXCL9, IL-18, IL-18BPa, CRP and ESR were measured in the patient. Time points are given in days from the last hospital admission (day 0). Dashed lines represent normal intervals (based on in-house healthy controls for the cytokines measured by ELISA (n=13) and standard ranges for CRP and ESR). Relevant clinical manifestations and treatment are indicated in the legend.

Finally, the patient had grade 2 myelofibrosis with a bone marrow phenotype comparable to a primary form of disease, even though genetic causes of primary myelofibrosis were excluded by gene sequencing and WES. Chronic inflammation is a cause of secondary myelofibrosis, yet this was deemed unlikely given the patient's young age and the absence of amyloid deposits in the bone marrow. Interestingly, downregulation of CDC42 has been identified as a key driver of myeloproliferative disease and erythroid hypoplasia in mice (11). Moreover, CDC42 is significantly downregulated in primary myelofibrosis and its Rho GTPase activity was also shown to play an important role in the development of myelofibrosis by a study that compared differential microRNA and gene expression in the bone marrow of 42 myelofibrosis and 16 healthy individuals (12,13). The Tyr64Cys mutation carried by our patient results in almost completely abolished GTPase activity in response to guanine nucleotide exchange factor (GEF) and a profoundly decreased GTPase activity in response to GTPase activating protein (GAP) (3). This suggests a direct role for the disturbed Rho GTPase activity of the Tyr64Cys mutant in the development of myelofibrosis.

In conclusion, we here demonstrate that auto-inflammation is a feature not only of defects of the C-terminal domain, but also of other *CDC42* mutations, at least of the switch II domain. Moreover, this case expands the clinical phenotype of *CDC42* mutations to include myelofibrosis and aortic aneurysm.

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Supplementary material

Methods

Isolation of peripheral blood mononuclear cells (PBMCs) from the whole blood of patient and controls was performed by Ficoll-Paque density gradient centrifugation (Lymphoprep, Stemcell Technologies).

Serum was separated from the whole blood of patient and controls by centrifugation and frozen at -20°C until analysis.

Genomic DNA was extracted from whole blood (QIAamp DNA Blood Maxi Kit, Qiagen) and sent for whole exome sequencing (Macrogen).

ELISA

IL-6, CXCL9, total IL-18 and IL-18BPA were measured in serum via commercial ELISAs (R&D systems). Serum samples from controls and the patient at different time points were analyzed on the same plate. Serum samples for IL-18BPA measurement were diluted 1:10.

Immune phenotyping

PBMCs were stained with monoclonal Ab (mAb) directed against CD3, CD4, CD8, CD20, CD56, CCR7, CD45RA, CD10, CD27, CD57, CD28 and CD127. The following subsets were determined by flow cytometry: T cells (CD3+), B cells (CD20+), NK cells (CD3-CD56+), CD4+ T cells (CD3+CD4+) and CD8+ T cells (CD3+CD8+); naïve (CD4+CCR7+CD45RA+), central memory (CD4+CCR7+CD45RA-) and effector memory (CD4+CCR7-CD45RA-) CD4+ T cells; naïve (CD8+CCR7+CD45RA+), central memory (CD8+CCR7+CD45RA-), effector memory (CD8+CCR7-CD45RA-) and EMRA (CD8+CCR7-CD45RA+) CD8+ T cells; transitional (CD20+CD10+CD27-), naïve (CD20+CD10-CD27-) and memory (CD20+CD10+CD27+) B cells; MAIT cells (CD3+CD161+Va7.2+), NKT cells (CD3+Vβ11+ Vα24+); CD57, CD27, CD28 and CD127-expressing CD4+ memory T cells and CD57, CD27, CD28 and CD127-expressing CD8+ effector memory T cells.

T cell function analyses

Sorted CD8+ T cells were cultured for 5 days with human T-activation and expansion (TAE) beads (anti-CD2/CD3/CD28, Miltenyi). On day 5, cells were re-stimulated with PMA/ionomycin in the presence of Brefeldin A and stained for surface expression of CD25, CD69, CD95 and CD107a.

Sorted memory CD4+ T cells were cultured for 5 days with human TAE beads. On day 5 cells were re-stimulated with PMA/ionomycin, fixed, permeabilised and intracellularly stained for cytokine expression. Intracellular expression of Th1 cytokines (IFN-γ, TNF-α), Th2 cytokines (IL-4, IL-9 and IL-13), Th17 cytokines (IL-17A, IL-17F and IL-22) and Tfh cytokines (IL-21) was determined by flow cytometry.

Whole exome sequencing

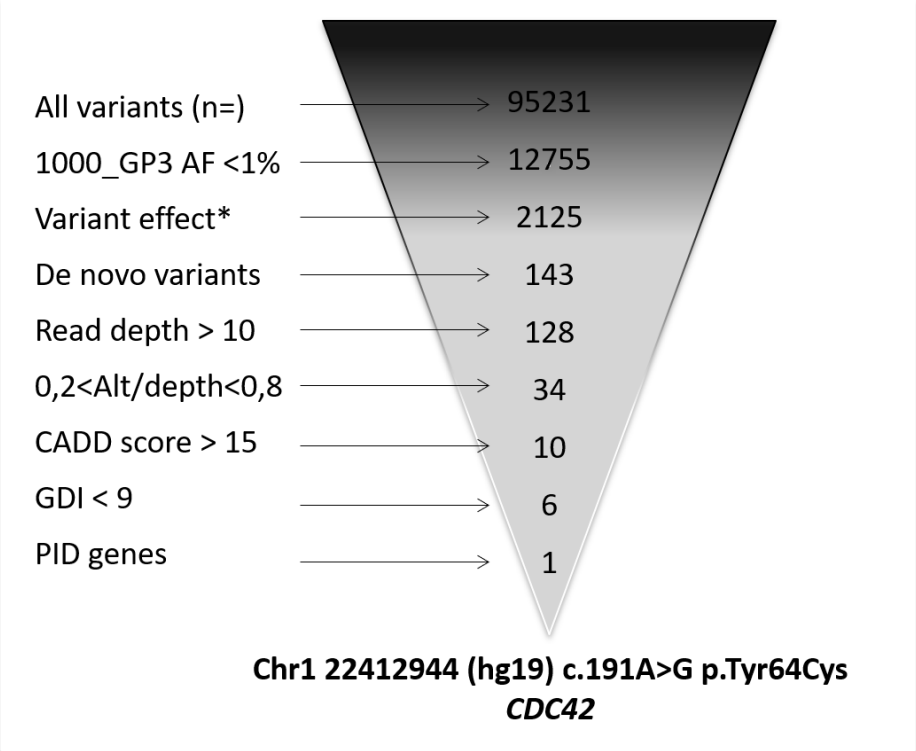
Analysis was performed based on the following filtering strategy (illustrated in supplementary fig. S4): from a total of 95231 variants, we considered 12755 variants with mean allele frequency below 1%; 3' UTR, 5'UTR, downstream gene, intergenic region, intron, non-coding exon, synonymous and upstream gene variants were excluded, leaving us with 2125 variants. Of the 143 de novo variants, 128 had sufficient quality (read depth >10) and 34 of these satisfied the criteria for germline heterozygosity (alternative allele/read depth ratio between 0.2 and 0.8). We prioritized 6 variants based on CADD score and gene damage index, and the mutation in *CDC42* was the only one in a gene related to immunodeficiency and corresponded to the phenotype of our patient.

Primary myelofibrosis screening

A NGS targeted panel (on Illumina Platform) was performed with a coverage of 500x per amplicon for the following genes: ABL1, ASXL1, ATRX, BCOR, BCORL1, BRAF, CALR, CBL, CBLB, CBLC, CDKN2A, CSF3R, CUX1, DNMT3A, ETV6, EZH2, FBXW7, FLT3, GATA1, GATA2, GNAS, IDH1, IDH2, IKZF1, JAK2, KMD6A, KIT, KRAS, MPL, MYD88, NOTCH1, NPM1, NRAS, PDGFRA, PHF6, PTEN, PTPN11, RAD21, RUNX1, SETBP1, SF3B1, SMC1A, SMC3, SRSF2, STAG2, TET2, TP53, U2AF1, WT1 and ZRSR2. No germline or somatic pathogenic mutations were revealed.

Supplementary figure 1. Filtering strategy applied to WES.

1000_GP3: thousand genomes project version 3, AF: allele frequency, Alt: number of reads from the alternative allele, CADD: Combined Annotation Dependent Depletion, GDI: Gene Damage Index, PID: primary immune deficiency (based on Connectome). *3' UTR, 5'UTR, downstream gene, intergenic region, intron, non-coding exon, synonymous and upstream gene variants were excluded.



Supplementary Table S1. Summary of the genetic and clinical characteristics of all reported patients with *CDC42* mutations.

Pt	Age, Sex	*CDC42* mutation	Inheritance	Protein domain	Developmental delay	Brain defects	Growth delay	Dysmorphic	Sensori-neural deafness	Campto dactily
1	22 y, F	Tyr64Cys	Het de novo	Switch II	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
2	26 y, F	Tyr64Cys	Het de novo	Switch II	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
3	14 y, F	Tyr64Cys	Het de novo	Switch II	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
4	16 y, F	Tyr64Cys	Het de novo	Switch II	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
5	4 y, M	Tyr64Cys	Het de novo	Switch II	**Yes**	**Yes**	NA	NA	**Yes**	No
6	†26 y, F	Tyr64Cys	Het de novo	Switch II	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
7	19 y, M	Arg66Gly	Het de novo	Switch II	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	No
8	†8 m	Arg66Gly	Het de novo	Switch II	NA	NA	NA	NA	**Yes**	NA
9	5 y, M	Arg68Gln	Het de novo	Switch II	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	NA
10	6 y, F	Arg68Gln	Het de novo	Switch II	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	No
11	5 y, M	Cys81Phe	Het de novo	Beta4	No	**Yes**	**Yes**	**Yes**	No	No
12	M	Ser83Pro	Het de novo	Beta4	**Yes**	**Yes**	**Yes**	**Yes**	No	NA
13	35 y, M	Ser83Pro	Het de novo	Beta4	**Yes**	**Yes**	No	**Yes**	No	NA
14	8 y, M	Ala159Val	Het de novo	NBP	**Yes**	**Yes**	**Yes**	**Yes**	No	**Yes**

15	M	Ile21Thr	Het de novo	Alpha1	**Yes**	No	NA	**Yes**	Yes	No
16	18 y, M	Tyr23Cys	Het de novo	Alpha1	**Yes**	**Yes**	**Yes**	**Yes**	NA	NA
17	13 y, M	Glu171Lys	Het de novo	CBR	No	No	No	**Yes**	No	No
18	F	Glu171Lys	Het AD	CBR	No	NA	**Yes**	**Yes**	No	NA
19	M	Glu171Lys	Het AD	CBR	No	NA	**Yes**	**Yes**	No	NA
20	F	Glu171Lys	Het AD	CBR	No	NA	**Yes**	**Yes**	No	NA
21	18 m, F	Cys188Tyr	Het de novo	C-terminus	NA	No	**Yes**	**Yes**	No	No
22	22 m, F	Cys188Tyr	Het de novo	C-terminus	NA	No	**Yes**	**Yes**	No	No
23	6 y, F	Arg186Cys	Het de novo	C-terminus	No	No	**Yes**	No	No	No
24	12 y, M	*192Cys*24	Het de novo	C-terminus	**Yes**	No	**Yes**	**Yes**	No	No
25	*6 y, F	Arg186Cys	Het de novo	C-terminus	NA	No	**Yes**	No	No	No
26	†6 m, M	Arg186Cys	Het de novo	C-terminus	NA	No	**Yes**	No	No	No
27	*†18 m, F	Arg186Cys	Het de novo	C-terminus	NA	No	**Yes**	No	No	No
28	†4 m, M	Arg186Cys	Het de novo	C-terminus	NA	No	**Yes**	No	No	No

Abbreviations: CBR: CRIB motif binding region; F: female; GI: gastrointestinal; Het: heterozygous; M: male; m: months; NA: not available; NBP: nucleotide binding pocket; Pt: patient; y: years; †: deceased; *: treated with hematopoietic stem cell transplantation.

Table S1 - continued.

Pt	Hypo-thyroidism	Lymph edema	Cardio-vascular defects	Thrombo cytopenia	Recurrent infections	Lympho penia	Inflamma tion	Other	Ref.
1	No	**Yes**	**Yes**	**Yes**	**Yes**	NA	No	Eczema, inguinal hernia	(1)
2	No	**Yes**	No	**Yes**	No	**Yes**	No	Retinal dysplasia, intestinal lymphangiectasia	(2)
3	**Yes**	No	No	**Yes**	Yes	**Yes**	No	Hypogammaglobulinemia	(4)
4	**Yes**	No	**Yes**	**Yes**	**Yes**	**Yes**	No	Feeding problems, inguinal hernias, scoliosis, multiple allergies, eczema, hypogammaglobulinemia, asthma	(3)
5	**Yes**	No	NA	**Yes**	No	NA	NA	-	(5)
6	No	No	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	Liver hemangiomas, hepatosplenomegaly, spleen and kidney nodules, aortic aneurysm, scoliosis, lung hemorrhages	CR
7	No	No	**Yes**	NA	**Yes**	NA	No	GH deficiency, precocious puberty, hypogammaglobulinemia, vertebral anomalies, chordee, cleft palate, abnormal renal vascular system, annular pancreas, syndactyly	(3)
8	No	No	**Yes**	NA	NA	NA	NA	Deceased from cardiovascular complications	(3)
9	No	NA	**Yes**	**Yes**	NA	NA	**Yes**	Recurrent fever, polyserositis, recurrent skin rash, splenomegaly	(3)
10	No	NA	**Yes**	**Yes**	No	NA	No	Febrile seizures	(3)
11	No	No	No	NA	**Yes**	NA	No	Speech delay and velopharyngeal insufficiency, webbed penis	(3)
12	**Yes**	No	**Yes**	NA	**Yes**	No	No	GH deficiency, anhidrosis, tethered cord and syrinx, asthma, scoliosis, epilepsy, autism	(3)
13	No	**Yes**	No	NA	Yes	NA	No	Seizures	(3)
14	No	No	No	NA	No	NA	No	Hydronephrosis, hypospadias, cerebral palsy, seizures	(3)

15	No	No	No	No	No	**Yes**	No	Tethered cord, unilateral renal agenesis, inguinal hernias, ADHD, pes equinus, scoliosis	(3)
16	NA	NA	NA	No	NA	NA	NA	Epilepsy, autism	(3)
17	No	No	No	No	No	NA	No	Attention deficit, learning difficulties	(3)
18	NA	No	Yes	No	NA	NA	NA	Optic atrophy, pectus deformity	(3)
19	NA	No	No	No	NA	NA	NA	Pectus deformity	(3)
20	NA	No	**Yes**	No	NA	NA	NA	Glaucoma	(3)
21	No	No	No	**Yes**	No	NA	**Yes**	Cytopenias, nodular rashes, arthralgias, hepatosplenomegaly, recurrent fever	(6)
22	No	No	No	**Yes**	**Yes**	NA	**Yes**	Cytopenias, lymphadenopathy, hepatosplenomegaly, skin rash, recurrent fever, cholestasis, HLH	(6)
23	No	No	No	**Yes**	**Yes**	NA	**Yes**	Cytopenias, skin rash, HLH, brain hemorrhage, hepatosplenomegaly, recurrent fever	(6)
24	No	No	No	**Yes**	**Yes**	NA	**Yes**	Cytopenias, brain hemorrhage, hepatosplenomegaly, skin rash, recurrent fever	(6)
25	No	No	No	**Yes**	No	NA	**Yes**	Cytopenias, HLH, skin rash, recurrent fever, myelodysplasia, hepatosplenomegaly, GI bleeding	(7)
26	No	No	No	**Yes**	No	NA	**Yes**	Cytopenias, HLH, hepatosplenomegaly, diarrhea, skin rash, recurrent fever, myelodysplasia	(7)
27	No	No	No	**Yes**	**Yes**	NA	**Yes**	Cytopenias, HLH, skin rash, recurrent fever, hepatosplenomegaly, GI inflammation, myelodysplasia	(7)
28	No	No	No	**Yes**	No	NA	**Yes**	Cytopenias, HLH, skin rash, recurrent fever, cholestasis, ascites, hepatosplenomegaly, GI inflammation, myelodysplasia	(7)

Abbreviations: ADHD: attention deficit and hyperactivity disorder; CR: current report; GH: growth hormone; GI: gastrointestinal; HLH: hemophagocytic lymphohistiocytosis; NA: not available; Pt: patient; Ref: references.

DISCUSSION

DISCUSSION

Parts of discussion adapted from:
Giorgia Bucciol, Isabelle Meyts. Recent advances in primary immunodeficiency: from molecular diagnosis to treatment. F1000Research 2020, peer reviewed and accepted for publication.
Giorgia Bucciol, Leen Moens, Barbara Bosch, Xavier Bossuyt, Jean-Laurent Casanova, Anne Puel and Isabelle Meyts. Lessons learned from the study of human inborn errors of innate immunity. J Allergy Clin Immunol. 2019 Feb;143(2):507-527.
Giorgia Bucciol, Erika Van Nieuwenhove, Leen Moens, Yuval Itan and Isabelle Meyts. Whole exome sequencing in inborn errors of immunity: use the power but mind the limits. Curr Opin Allergy Clin Immunol. 2017 Dec;17(6):421-430.

In the studies here presented, I have applied next generation sequencing (NGS) to the exploration of genetic causes of inborn errors of immunity (IEI). This approach has allowed us to identify IFNAR1 deficiency, a novel IEI, in patients presenting with disseminated vaccine-strain viral disease, and to diagnose Takenouchi-Kosaki syndrome due to a mutation in *CDC42* in a patient presenting with dysmorphisms, developmental delay, thrombocytopenia, immunodeficiency, myelofibrosis and autoinflammation.
Subsequently, I have functionally validated the identified variants by means of molecular and biological analyses and I have explored their relevance for the cellular phenotype expressed by the patients. I then used the same approach when studying a larger cohort of patients with confirmed or suspected STAT2 deficiency.
To summarize, in this thesis I highlight the advantages and limitations of applying NGS to the diagnosis of patients with suspected IEI and I examine the future perspectives allowed by these methods.

Indeed, perhaps more than other medical disciplines, the field of primary immunodeficiency is expanding rapidly thanks to the recent advances in sequencing, gene editing tools, and the introduction of new biological drugs and small molecules that target specific checkpoints relevant to immunity, inflammation, and cancer. We have witnessed first of all a rapid rise in the number of described monogenic inborn errors of immunity, with more than 400 distinct

defects included in the latest International Union of Immunological Societies classification of 2019 (1).

Sequencing in general and NGS techniques in particular are becoming technically more accurate, fast, affordable, and therefore widely available to researchers and physicians. Apart from the obvious result of increasing the sheer number of defined IEI, this rapid expansion has also highlighted the phenotypic heterogeneity and genetic pleiotropy of these disorders. On the other hand, many novel IEI are studied in a single kindred or a small number of kindreds, thus providing little information on the penetrance and phenotype (1). Moreover, their pathophysiology remains often to be unraveled.

The benefit of diagnostic NGS comes at a cost: the necessity of unequivocal functional validation of new variants, albeit in known IEI-related genes. Still too often the pathogenicity of a given variant is based only on the available *in silico* prediction tools, which can be misleading. Indeed, the validation of variants of unknown significance represents one of the biggest hurdles in making WES and NGS technologies the norm in clinical practice. Not only because of the financial cost, but also in terms of the time and competence required first to analyze and study a variant, and afterwards to interpret the results and translate them into clinical treatment and care. Therefore, a tremendous responsibility lies in the hands of geneticists and scientists, and even more of commercial sequencing companies, as the mere reporting of a variant of unknown significance cannot be interpreted as the attribution of pathogenicity to such variant. As an example, in chapter 2 we show that a *STAT2* variant supposed to be pathogenic based on the clinical phenotype of the patient did not in fact lead to aberrant expression or phosphorylation of STAT2 nor to aberrant ISG induction, hence proving not to be the molecular cause of the patient's disease. More than anything, this context calls for collaboration and sharing of data for the benefit of the patients. With limited research resources, the further development of more robust prediction tools/validation models becomes a necessity.

Another current problem in the diagnostics of IEI is the relevance of intronic mutations. With NGS methods routinely available for diagnosis and with a shift from the fixed gene panel and WES towards WGS, we learn more and more that deep intronic variants can be indeed disease-

causing (2,3). This means that with simpler but more accessible sequencing methods many defects can be missed, but also presents the issue of interpreting the relevance of intronic variants. RNA sequencing can be an invaluable tool for the functional analysis of intronic, synonymous and splice variants (4–7). In particular, it can highlight the partial or complete loss of gene expression in the proband compared with controls, which would be missed by traditional sequencing. The increase in diagnostic rate of rare diseases obtained by WGS versus WES can be as high as 6-11% and the use of tissue-specific RNA sequencing can aid the diagnostic process and help clarify the pathophysiology of the disease (6,7). Interestingly, in the case of monogenic rare diseases RNA sequencing as first molecular approach led to a diagnosis in 7-17% of cases (7). No hematological or immunological disorders were among those successfully diagnosed in this cohort, which consisted mostly of patients affected by neuro-muscular disorders. A lower success rate in IEI could also reflect the difficulty of comparing normal controls to patients with significant blood cell abnormalities.

For these reasons, the development of larger genetic facilities dedicated to clinical diagnostics and the training of clinicians in genetics are both becoming very real necessities in the field or rare diseases as IEI. Moreover, the creation of established network of collaborations between the clinical settings and experienced research centers will be necessary, as more and more patients are found to harbor novel variants that cannot be properly assessed without dedicated expertise and resources. Finally, as physician-scientists, we wish to stress the importance of the clinical phenotype as a beacon in the era of so-called unbiased sequencing approaches.

Unraveling the interferon anti-viral immune response: a tale of redundancy

Some of the issues mentioned above are clearly illustrated by the studies presented in this thesis.

Even for IEI where the underlying pathophysiological mechanism is quite clear, such as IFNAR1 and STAT2 deficiency, the bigger picture can remain mysterious. In chapter 1 we identified a novel IEI, IFNAR1 deficiency, as causing life-threatening infections following measles or yellow fever virus vaccination, and we examined its consequences on cell-intrinsic immunity against viruses. In chapter 2 we analyzed the clinical, immunological and genetic characteristics of a large number of STAT2 deficient patients. In comparison with IFNAR1 deficiency, we observed a more severe and varied presentation, although not fully penetrant,

which includes multiple viral susceptibilities (natural and vaccinal) and the risk of overwhelming fatal infections.

Both these type I IFN defects present indeed with a heightened susceptibility to viruses, as expected given the importance of type I IFN in anti-viral responses. The fact that IFNAR1, IFNAR2 and STAT2 deficiencies all result in disseminated vaccine-strain measles while deficiency in IL-10RB, a subunit of the type III IFN (IFN-λ) receptor, has not been reported to produce this infectious phenotype, suggest that type I IFN signaling is more critical for defense against measles vaccine-strain virus than type III IFN signaling (8–11).

Despite this, the narrow clinical and infectious phenotype of these patients is in striking contrast with the central role played by the IFN pathway in human anti-viral defense. This discrepancy can be explained by the supposed redundancy of some innate and cell-intrinsic immune pathways and factors, a hypothesis which seems to find confirmation in the large number of IEI causing infections with one or a narrow range of microbes, sometimes in a single non-hematopoietic cell line, and in the fact that most manifestations cease to exist once the adaptive immune system fully matures (12,13). The innate immune system is believed to be more conserved through the species than the adaptive, nonetheless a great degree of variation has characterized the development of human innate immunity. Patin et al. have recently used flow cytometry and a genome-wide association approach to study 1000 healthy individuals, finding that natural variation in the parameters of innate cells, such as the cell-surface expression of protein markers, is more strongly driven by genetic factors than in adaptive cells (14). These findings hint to the fact that the innate compartment of immunity has evolved over a long period of time to form a tightly intertwined network of defense against microbes, where each component overlaps for most of its functions with other parts. This redundancy can guarantee the best defense in case of failure of one component and explains the narrow spectrum of infections experienced by many patients with an innate immune defect.

The interplay between type I and type II interferon responses

In chapter 2, we show that STAT2 deficient fibroblasts have a partial antiviral response *in vitro* after type I IFN stimulation, probably due to the presence of a partial STAT2 defect and/or the formation of STAT1 homodimers. This mechanism, together with the preserved IFN-γ response of STAT2 and IFNAR1 deficient subjects, suggests that treatment with IFN-γ could

be effective in counterbalancing the lack of type I IFN response during a viral infection in these patients. IFN-γ is approved for the prevention and treatment of bacterial and fungal infections in patients with chronic granulomatous disease (CGD) and proved effective for the treatment of mycobacterial infections, various cancers, lung fibrosis, and osteopetrosis (15,16). Moreover, a study on HIV positive patients showed that prophylactic IFN-γ treatment reduced their risk of opportunistic infections, especially with herpes and cytomegalovirus, thus indicating a possible effect of this IFN on anti-viral defense (17).

Also a combination of IFN-α and IFN-γ treatment could lead to improved viral responses in STAT2 deficient patients, thanks to the immune modulation of IFN-γ and the effect of IFN-α on any residual STAT2 activity. This combination therapy has indeed shown promising results on the viral clearance of patients with hepatitis C who did not respond to IFN-α treatment alone (18). To our knowledge, IFN therapy has not been attempted so far in patients with type I IFN disorders, also because a severe course of infection is usually their first presentation and they are only later diagnosed. However, it could be beneficial in case of subsequent infections and in siblings who receive a genetic diagnosis before showing symptoms.

Nevertheless, these treatments would need to be balanced very carefully as excessive IFN may obviously cause unwanted inflammatory reactions. The need for a fine balance between the activation of IFN responses and their dampening to prevent inflammation is also made clear by the study of these genetic defects. In a previous paper on STAT2 deficiency, we indeed alluded to auto-inflammation as an added feature of the disease and recent evidence now shows that STAT2 plays a role in the USP18-mediated auto-regulation loop (19,20). STAT2 defects could therefore be responsible for both a defect in the IFN anti-viral response and an uncontrolled IFN activation, leading to inflammation (19,21,22).

Immunodeficiency and autoinflammation: expanding the concept of inborn errors of immunity

Heterozygous mutations in *CDC42*, encoding a small Rho GTPase involved in actin cytoskeleton dynamics, were described from 2015 onwards to cause a wide range of different developmental phenotypes with associated thrombocytopenia and immunodeficiency (23–27). Despite attempts to link the genotype with the phenotype, the actual pathophysiology of *CDC42* mutations as well as the impact of the various variant on cell function remains unclear

(26). The picture became even more complicated when two groups described a completely different auto-inflammatory presentation in patients with mutations of the sequence coding for the C-terminal domain (28,29). These patients had none of the previously described manifestations, while presenting with a severe neonatal-onset systemic inflammation and hemophagocytic lymphohistiocytosis (28,29). Given the multitude of functions of CDC42, the underlying mechanisms of the various manifestations of *CDC42* mutations are very difficult to study and the animal model represented by *Caenorhabditis elegans* may be insufficient to fully portrait the human phenotype.

In chapter 3 we reported a patient with both Takenouchi-Kosaki syndrome caused by the common *CDC42* mutation Tyr64Cys, and systemic inflammation with myelofibrosis, further entangling the phenotypic spectrum of the disease. We believe that the presence of myelofibrosis in this patient is neither coincidental nor a nonspecific manifestation of systemic inflammation. Indeed, CDC42 is abnormally expressed in human primary myelofibrosis and its downregulation has been implicated in myeloproliferation and erythroid hypoplasia in mice (30–32). Moreover, another research group has recently identified a second patient with a *CDC42* mutation and pediatric onset myelofibrosis, thus supporting our hypothesis of CDC42 relevance in bone marrow function and systemic inflammation (33,34). Finally, the use of serum cytokine detection to directly measure indicators of systemic inflammation is a valuable tool that could allow the clinician to adopt specific anti-inflammatory treatment even in the absence of a definite genetic diagnosis, which was only obtained post-mortem in our patient.

This case highlights the complex crosstalk between different organs and the central role played by immune components in the homeostasis of multiple systems. It also illustrates the fine tuning required to calibrate the function of most enzymes involved in the immune response. Ultimately, it is another example of the subtle balance between immunodeficiency and excessive inflammation, which can both be present in a single IEI.

Future prospects

The results achieved in this project provide proof that NGS followed by functional studies is an important tool in the discovery of novel IEIs and a substantial aid in the diagnosis of patients with an ill-defined immunodeficiency. As a consequence, a broader project involving

immunological and genetic screening of all children admitted to the intensive care unit with unexplained life-threatening infections is in progress and has already yielded useful results with a direct impact on patient care. The greatest challenge remains the quick characterization of novel variants of unknown significance, which can require a great amount of resources. Within rare defects of intrinsic immunity, this was made even more complex by the discovery of several examples of mutations in one gene which cause a completely different phenotype to the one originally described for that gene, meaning that genotype-phenotype correlations are not to be relied upon. For instance, autosomal dominant defects of TLR3 were found in 2007 to cause susceptibility to herpes simplex encephalitis, and they have now been reported in additional patients who developed acute respiratory distress syndrome during influenza A infection (35,36). Interestingly, both phenotypes show incomplete penetrance, and none of the patients suffered from both infectious diseases despite being exposed to both viruses. Another example is provided by *CDC42* mutations, discussed in this thesis, causing either a developmental phenotype with dysmorphisms, brain defects and immunodeficiency, or neonatal-onset severe inflammation without apparent malformations.

Therefore, the painstaking pursuit of etiological diagnoses for every patient and the identification of the pathophysiological mechanisms at the base of their clinical phenotype remain crucial to advance our understanding of the immune system and its defects. Given the cumbersome validation steps needed for novel variants in known genes, and even more for novel genes, a great deal of innovation is required before molecular diagnosis can become a fast turn-around instrument allowing for decision making in emergency situations. This could be achieved though better *in silico* prediction tools, or, maybe counter-intuitively, thanks to the use of pathway analysis, in which a combination of in depth immunophenotyping and multiplex nanomolecular cytokine measurement could point the physician in the direction of the affected pathway or system. The identification of a specific protein/pathway deficiency or hyperactivation could then allow the initiation of a targeted therapeutic action prior to irreversible organ damage.

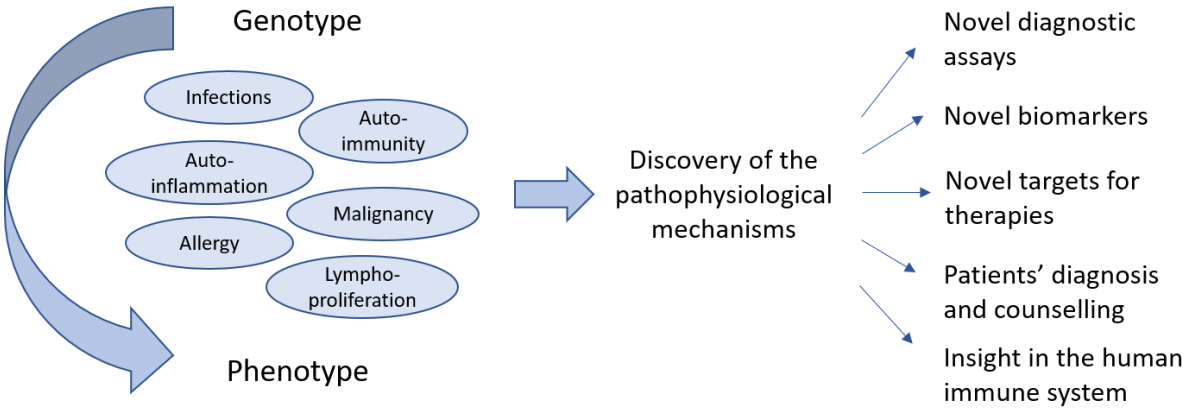
With better knowledge of immune mechanisms and pathology, diseases earlier considered domain of other branches of medicine are now being reclassified as IEIs. This expansion reflects on the one hand the realization of the far-reaching influence of immunity on every organ and system, and on the other the variety of different phenotypes that can arise from the

same immune defect. Examples are the immunological focus in the research on infections of the central nervous system and neuroinflammation, or the recent description of neuromyelitis optica as an additional manifestation of COPA syndrome, an auto-inflammatory disorder characterized by interstitial lung disease, alveolar hemorrhage, kidney disease, and arthritis (21,22,37–39, and Isabelle Meyts personal communication). Indeed, many phenotypes may be due to new PIDs. Considering the great locus and allele heterogeneity demonstrated by IEIs, and taking into account that an *in silico* analysis of candidate PID-causing genes based on the Human Gene Connectome has found 5012 non-redundant novel genes, of which 3110 with biological functions similar to other PID genes, in the top 1% of genes biologically closest to all known PID genes, we can estimate that thousands of diseases are still to be unraveled (40,41). As a direct consequence, a pathway-specific (if not gene/protein-specific) immunotherapy becomes a possibility not only for classical IEIs, but also for many other diseases. For example, anti-IL-1 therapy is useful in *CDC42* mutations with inflammatory features, and the use of JAK inhibitors such as ruxolitinib could be envisaged in patients with COPA syndrome and a high interferon signature.

The expanding knowledge on IEIs has allowed us to gain crucial insights into human immunology that we could not have obtained from any mouse observation. Therefore, by meticulously studying the patients' clinical and immunological phenotypes, by molecular diagnostic steps and intelligent validation, and by being collaborative in our efforts, we can do the best for current and future patients affected by PIDs.

Conclusions

The genetic theory of infectious diseases suggests that many inborn errors of immunity in general and of innate immunity in particular are yet to be characterized by the study of patients with severe infections (12). The discovery and investigation of these and other monogenic disorders give us an incredible insight on the mechanisms of immune responses and allow us to develop new diagnostic and therapeutic approaches. This way, even seminal findings in single patients can improve our understanding of fundamental immunology.



Another valuable method to better understand human immune strategies is the study of the underlying mechanisms of increased resistance to infection with a pathogenic microbe. These findings have a great impact not only on patients with IEIs, but also on thousands of patients with cancer, autoimmune disorders, neurological diseases, and many others, who now benefit from immunotherapies.

Ultimately, these experiments of nature will improve our knowledge and understanding of human immunity and should enable us to provide better treatment to individual patients and entire populations.

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SCIENTIFIC SUMMARY

Scientific summary

Despite the progress of the last century in living conditions and medical care, some previously healthy children are still affected by lethal or potentially lethal infections with pathogens that only cause mild illness in most people. Hypothesizing that monogenic defects of immunity underlie the unusual infectious phenotype in these children, we apply an unbiased diagnostic approach with next generation sequencing to identify possible causes of immunodeficiency and proceed to analyze the identified mutations to characterize their pathophysiological mechanism.

In chapter 1, we study a patient from a consanguineous family who suffered from disseminated vaccine-strain measles and another patient with viscerotropic vaccine-strain yellow fever. Whole exome sequencing (WES) identifies previously not reported biallelic variants in the gene encoding the type I interferon receptor 1 (IFNAR1), involved in anti-viral innate and intracellular responses. A complete functional analysis of the variants is performed and demonstrates their pathogenicity at the gene, protein and cellular level. In particular, we show that patients' fibroblasts are highly susceptible to viruses, including the vaccine-strain measles and yellow fever. Therefore, autosomal recessive complete IFNAR1 deficiency results in life-threatening complications of live attenuated viral vaccinations in previously healthy children, but apparently not other severe infections. This study provides new insight into the specific function and potential redundancy of a pathway of innate immunity and highlights the fact that a severe/unusual course of infection in an otherwise healthy child should always be considered as a potential immunodeficiency.

In chapter 2, we expand our current knowledge of STAT2 deficiency, another autosomal recessive disorder of the type I interferon response. The affected patients present with a characteristic, although not fully penetrant, risk of disseminated disease and encephalitis after inoculation with live measles-mumps-rubella (MMR) vaccine. In contrast with IFNAR1 deficiency, they also seem to be susceptible to other viral illnesses and show a more severe phenotype, with a mortality of 19% in childhood. Analyzing sixteen patients from seven unrelated kindreds with STAT2 deficiency, we find a broader infectious and non-infectious phenotype than previously described, centered on viral susceptibility. Moreover, the functional assessment of the different mutations hints at a possible incomplete form of STAT2 deficiency, where type I interferon responses are partially preserved *in vitro*. As with other

innate immunity defects, once patients reach adulthood they seem to be protected from severe infections.

Finally, in chapter 3 we tackle another inborn error of immunity presenting with a broader phenotype than previously described. A young woman manifesting intellectual and growth delay, dysmorphisms, macrothrombocytopenia, camptodactyly, and structural brain abnormalities, suffered from immunodeficiency, severe lung infections, immune dysregulation and systemic inflammation. WES identified a *de novo* mutation in *CDC42*, causing Takenouchi-Kosaki syndrome. The extended immunophenotyping of the patient showed B cell lymphopenia with increased naïve B cells, decreased naïve T cells, and a global defect of CD8+ T cell activation. Most interestingly, the patient had signs of immune dysregulation and autoinflammation, including myelofibrosis and upregulation of inflammatory cytokines in her blood. These findings expand the phenotypic spectrum of Takenouchi-Kosaki syndrome and link autoinflammation with other *CDC42* mutations than those in the C-terminal domain, recently identified as a cause of neonatal inflammation and hemophagocytic lymphohistiocytosis.

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All the scientific work, experiments and manuscript preparation for this thesis was personally done by me, with the following exceptions:

Introduction and discussion: Isabelle Meyts, Leen Moens, Erika Van Nieuwenhove, José Casas-Martin, Stuart Tangye and Bethany Pillay have written parts of the reviews used in the general introduction and discussion of this thesis.

Chapter 1: all experiments on P2 were coordinated and performed by the St. Giles Laboratory of Human Genetics of Infectious Diseases and the Laboratory of Virology and Infectious Disease, Rockefeller University, and specifically: Nicholas Hernandez, Qian Zhang, Emmanuelle Jouanguy and Laurent Abel analyzed WES data; Nicholas Hernandez and Jeremy Le Pen assessed IFNAR1 expression and performed qPCR experiments; Nicholas Hernandez performed Western Blot analyses, measles infections and VSV infections. For both patients: Nicholas Hernandez performed IAV and HSV infections. Jeremy Le Pen and Andrea Jurado performed YFV-Asibi and ZIKV infections and, along with Dick Zijlmans, YFV-17D infections. Ian Tietjen and Hans-Heinrich Hoffman prepared viral stocks. Tamiris Azamor de Costa Barros performed analysis of anti-tetanus and diphtheria antibodies. Rik Gijsbers assisted with the production of lentiviral vectors. For P1: Mohammad Shahrooei, Afshin Shirkani, Majid Changi-Ashtiani, and Hassan Rokni-Zadeh analyzed WES data; measles experiments were performed by me and Leen Moens with the assistance of Dirk Jochmans, Robert Boudewijns and Ruben Pholien. Nicholas Hernandez, Qian Zhang, Emmanuelle Jouanguy, and Jean-Laurent Casanova prepared the manuscript.

Chapter 2: Leen Moens prepared lentiviral vectors with the assistance of Vivienne Béziat. All other experiments were done by me together with Leen Moens and Selket Delafontaine.

Chapter 3: ELISAs and WES filtering were performed by Leen Moens; immune phenotyping was performed by Bethany Pillay, Cindy Ma and Stuart Tangye; histopathological analysis of the bone marrow was performed by Thomas Tousseyn.

Conflict of interest statement

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For chapter 3, research performed in the Tangye/Ma lab was supported by the National Health and Medical Research Council of Australia, and the Office of Health and Medical Research of the New South Wales Government.

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Education and training

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2017 – 2020	Doctoral Student – Inborn Errors of Immunity, Department of Microbiology, Immunology and Transplantation, KU Leuven (Belgium). Under supervision of prof. Isabelle Meyts, dr. Leen Moens and prof. Xavier Bossuyt.
2016 – 2017	International Scholar – Childhood Immunology, Department of Microbiology and Immunology, KU Leuven (Belgium)
2012 – 2017	Specialization in Pediatrics – Department of Pediatrics, University of Padova (Italy). Full marks and honors.
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2005 – 2011	Medical degree – Faculty of Medicine and Surgery, University of Padova (Italy). Full marks and honors.
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2000 – 2005	High School Diploma – scientific studies, Liceo Scientifico G. Galilei, San Dona' di Piave (Italy). Full marks.

Schools, courses and projects

2020 – First IAPIDS advanced PID school
2018 – Let's talk science summer school, KU Leuven
2018 – UCL Winter School: Advances on primary immunodeficiency
2017 – NGS introduction and variant analysis training, VIB KU Leuven
2017 – Theoretical and practical flow cytometry training, KU Leuven
2016 – UCL Winter School: Advances on primary immunodeficiency
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2013 – Research fellowship in the Primary Immunodeficiency group, Erasmus Medical Center (Rotterdam, The Netherlands), participating in a project studying the immunological mechanisms of PTEN tumor hamartoma syndrome.
2011 – Winner of a grant in the International Exchange Program with Columbia University School of Medicine (New York, USA), where I did internships in Neonatal Intensive Care, Pediatric Emergency Department, Pediatric Intensive Care and Cardiac Intensive Care Unit.

Teaching activity

Co-supervisor for master thesis in Medicine and in Biomedical Laboratory Technology, KU Leuven Faculty of Medicine, and Thomas More University, academic years 2016-2017, 2017-2018.
Teaching assistant for the Primary Immunodeficiency course in Pediatrics, KU Leuven Faculty of Medicine, and UCLL BaNaBa pediatric nursing course, academic years 2017-2018, 2018-2019, 2019-2020.

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Oral presentations at conferences and meetings

2019 – BPIDG meeting, Brussels. G Bucciol, B Pillay, J Casas-Martin, S Delafontaine, M Proesmans, N Lorent, J Coolen, T Tousseyn, X Bossuyt, CS Ma, R Schrijvers, SG Tangye, L Moens, I Meyts. Systemic inflammation and myelofibrosis in a patient with Takenouchi-Kosaki syndrome.

2018 – CIS annual meeting, Toronto. Bucciol G, Arts K, Moens L, Van Ranst M, Brown J, Breuer J, Meyts I. RNA sequencing identifies the cause of chronic infection with lymphoproliferation in a patient with X-linked agammaglobulinemia.

2018 – Symposium celebrating 20 years of BPIDG, Ghent. Bucciol G, Arts K, Moens L, Van Ranst M, Brown J, Breuer J, Meyts I. RNA sequencing identifies the cause of chronic infection with lymphoproliferation in a patient with X-linked agammaglobulinemia.

2016 – PIDs: IPINET-AIEOP working party, Taormina. Bucciol G, Petrone A, Barbato A, Toffolutti T, Basso G, Putti MC. The role of Mycophenolate on autoimmune manifestations of PIDs.

2012 – XXXVII National AIEOP Congress, Bari. Bucciol G, Sanzari MC, Pantano G, Tosato F, Basso G, Putti MC. Lymphocyte subsets and activation markers in children with immunodeficiencies and recurrent infections.

Poster presentations at conferences and meetings

2019 – ESID Focused Meeting, Brussels. G Bucciol, I de la Fuente Garcia, S Delafontaine, J Vandersnickt, L Moens, R Perez de Diego, I Meyts. Life-threatening H1N1 Influenza infection in a patient with leaky SCID associated with compound heterozygous RAG1 mutation. L Moens, G Bucciol, J Casas-Martin, AI Cordeiro, C Neves, C Martins, I Meyts, J Farela Neves. A novel mutation in BLM underlying Bloom syndrome.

2017 – ESID Focused Meeting, Edinburgh. Bucciol G, Willems L, Moens L, Meyts I. Thyroid carcinoma in a child with activated phosphoinositide 3-kinase δ syndrome (APDS): somatic effect of a germline mutation.

2017 – CIS Annual meeting, Seattle. Bucciol G, Boisson-Dupuis S, Bosch B, Meyts I, Moens L. Th17 impairment in a patient with Activated Phosphoinositide-3-Kinase-δ Syndrome (APSD) and Salmonella sepsis.

2016 – 17th ESID Biennial meeting, Barcelona. Bucciol G, Petrone A, Barbato A, Toffolutti T, Basso G, Putti MC. Mycophenolate Mofetil as treatment of autoimmune manifestations of PIDs.

2012 – 15th ESID Biennial Meeting, Florence. Bucciol G, Messina C, Tumino M, Giliani SC, Calore E, Pillon M, Destro R, Gazzola MV, Basso G, Putti MC. Haploidentical TCR$\alpha\beta$ and CD19 depleted hematopoietic stem cell transplantation in a case of SCID. Bucciol G, Sanzari MC, Pantano G, Tosato F, Basso G, Putti MC. Lymphocyte subsets and activation markers in children with immunodeficiencies and recurrent infections.

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