UNRAVELLING NOVEL GENETIC AND FUNCTIONAL MECHANISMS OF PRIMARY IMMUNODEFICIENCIES

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UNRAVELLING NOVEL GENETIC AND FUNCTIONAL MECHANISMS OF PRIMARY IMMUNODEFICIENCIES

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"Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows tracings of her workings apart from the beaten paths; nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of nature, by careful investigation of cases of rarer forms of disease"

William Harvey, English physician (1578-1657)

PREFACE

Immunology has fascinated me from a very early age. As a pediatrician, I have been struck by the importance of genetics in young developing individuals. It soon became clear to me that serious infections did not solely depend on the microorganism, but on the way the hosts themselves responded to them. Classic, serious infections, such as herpetic encephalitis or invasive meningococcal disease, could be due to genetic defects. Then further down the line, poly-autoimmunity, auto-inflammation and hyper-inflammation. Understanding the interception between genetics and immunology had become a must.

I had the privilege of growing up with the generalization of the molecular biology techniques, leading to the identification of hundreds of new congenital immune defects (Fig.1).

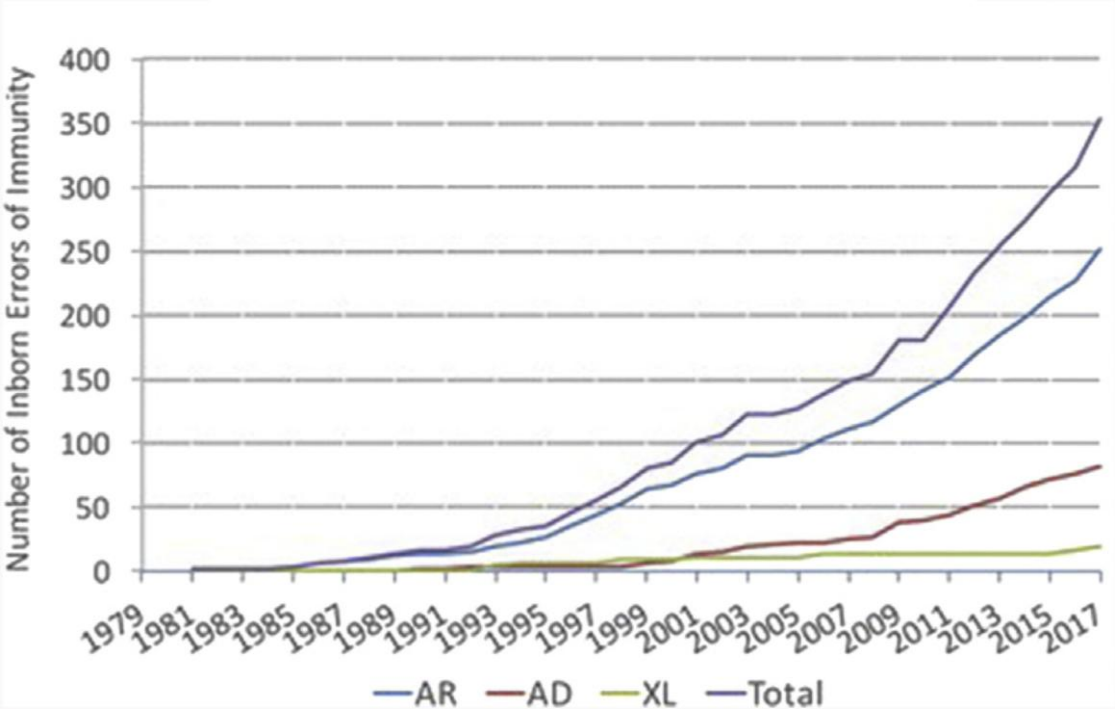

Figure 1: Evolution of Primary Immunodeficiencies in the last 40 years. AR- autosomal recessive, AD- Autosomal dominant, XL- X-linked. Adapted from Heimall et al, 2019[1]

I was able to follow this growth, witnessing several facts as follows:

- The discovery of new genetic defects that caused primary immunodeficiencies (PIDs) has broadened the knowledge about the immune system, especially in terms of new components and new interactions.

- The clarification of genetic defects associated with atypical PID has made it possible for us to have a better understanding of genes, diseases and particularly the impact that a certain genetic alteration has at protein level, and the relevance that the same alteration has on the functioning of the immune system.

- This new knowledge has had a real impact on the survival and quality of life of these patients. Understanding the genetic defects and pathophysiology behind these rare diseases, such as PIDs, has made it possible to use new treatments, anticipate complications and improve care.

This thesis has emerged out of a necessity and an obligation to be part of this explosion of knowledge. A personal necessity, based on the assumption that knowledge will improve clinical practice. An obligation, because having the privilege of being able to accompany patients with rare and complex diseases, I have been forced to understand and, in some cases, decipher their diseases, responding to the doubts not only of patients and their parents, but also of science itself.

According to article 4º of the Diário da República, 2nd series Nº111, June 9th, 2015 (regulation nº 320/2015) and according to article 19º of the Diário da República, 2nd series, nº 153, August 7th, 2015 (Regulation nº 519/2015), some of the results presented in this thesis were published in international scientific journals with peer reviews:

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  (Under submission to the American Journal of Human Genetics)

"Although the way ahead (form immunology) is full of pitfalls and difficulties, this is indeed an exhilarating prospect. There is no danger of a shortage of forthcoming excitment in the subject. Yet, al always, the highlights of tomorrow are the unpredictabilities of today."

Cesar Milstein (Nobel lecture, 1984)

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RESUMO

As imunodeficiências primárias (IDP) constituem um grupo heterogéneo de doenças congénitas que podem afetar o desenvolvimento e/ou a função de qualquer componente do sistema imunitário. Isto pode conduzir à alteração de qualquer uma das funções do sistema imune e portanto os doentes com IDP podem apresentar infeções recorrentes ou susceptibilidade especifica a determinado micro-organismo , mas também podem apresentar auto-imunidade, linfoproliferação, híper-inflamação ou até neoplasias. A recente generalização da disponibilidade das técnicas de sequenciação de nova geração permitu não só a identificação de genes responsáveis por IDP previamente descritas, mas também a descoberta de novas IDP. É sabido que a grande maioria das IDP são defeitos monogénicos que podem apresentar expressão e penetrância incompletas. O reconhecimento que mutações no mesmo gene podem conduzir a diferentes fenótipos, mas também que mutações em múltiplos genes diferentes podem conduzir ao mesmo fenótipo, tornou mandatória a utilização de técnicas de sequenciação de nova geração para elucidar os quadros clínicos destes doentes ou grupos de doentes. Ao compreendermos o impacto destas experiências da Natureza no sistema imunitário, foi possível não só expandir o conhecimento, mas também mudar condutas terapêuticas e o aconselhamento das famílias.

Esta mudança de paradigma conduziu a uma necessidade continua de redefinir os sinais de alarme para a suspeição de IDP. Além da clássica susceptibilidade infeciosa, as manifestações auto-imunes de início precoce, a poli-autoimunidade, a hiper-inflamação ou a infalamação recorrente (sistémica ou específica de órgão), a alergia exuberante (especialmente associada a outras manifestações), e até algumas neoplasias (como a leucemia juvenil mieolomonocítica) podem ser manifestações reveladoras de IDP.

Esta tese teve como objectivo revelar novos mecanismos genéticos e/ou funcionais de IDPs. Para isso foram investigados doentes seguidos na Unidade de Imunodeficiências Primárias do Hospital Dona Estefânia, Centro Hospitalar de Lisboa

Central, que tivessem uma IDP com sintomas incomuns e fatais ou uma IDP com sintomas atípicos que não permitisse estabelecer um diagnóstico genético definitivo.

A investigação destes doentes permitiu adicionar novos fenótipos a IDP previamente conhecidas: hiperplasia nodular regenerativa num doente com imunodeficiência combinada grave causada por uma nova mutação hipomórfica no gene IL2RG; síndrome hemofagocítico secundário a infeções por BCG e *Citomegalovirus* num doente com deficiência do receptor do IFNγ; doença inflamatória intestinal e um fenótipo assemelhando-se ao sindrome trico-entero-hepático causado por uma mutação hipomórfica do gene TTC7A; cutis laxa num doente com síndrome de APLAID causado por uma nova mutação do gene PLCG2.

A investigação de um conjunto de doentes com doença inflamatória intestinal de início infantil (IO-IBD) permitiu a identificação de uma causa monogénica para a doença em 54% dos doentes e em muitos desses casos, isto conduziu a alterações terapêuticas. Pudemos descrever uma nova causa de IO-IBD, imunodeficiência e surdez neurossensorial, causada por mutações em STXBP3, que não tinha sido previamente associada a doença humana.

Adicionalmente, num doente com um fenótipo IPEX-like, identificamos uma mutação homozigótica do gene ZC3H12A, codificando uma proteína Regnase-1 disfuncional condicionando uma regulação pós-transcricional aberrante de IL-6.

Finalmente, numa doente com fasceites graves recorrentes, identificamos uma variante de significado incerto no gene IRG1 e provamos que essa variante conduz a uma alteração do metabolismo de ácido itacónico nos macrófagos, causando incapacidade de detectar adequadamente a resolução da infeção e conduzindo a hiperinflamação.

ABSTRACT

Primary immunodeficiencies (PIDs) constitute a heterogeneous group of congenital diseases that affect the development and/or the function of any component of the immune system. As any function of the immune system can be affected, patients with PIDs can present with recurrent infections or a higher specific susceptibility to certain microorganisms, but also with autoimmunity, lymphoproliferation, hyper-inflammation or even cancer. With the massive generalization of the next-generation sequencing (NGS) techniques, the discovery of new PIDs and novel genetic causes for previously known PIDs has been boosted. It is known that the vast majority of PIDs are monogenic, with variable expressivity and often with incomplete penetrance. The recognition that mutations in the same gene can present with several clinical phenotypes, but also that mutations in multiple different genes can manifest with the same phenotype, has made mandatory the use of NGS to elucidate the clinical picture of individual patients or patient groups. By understanding the impact of such nature's experiments on the immune system, it has been possible to expand knowledge, change therapeutic strategies and family counselling.

This has led to a continuous change of the warning signs that can reveal PIDs. Adding to the infectious susceptibility, early onset autoimmune manifestations, poly-autoimmunity, hyper-inflammation, or recurrent inflammation (systemic or organ-specific), exuberant allergy (especially if associated with other manifestations), and even some neoplasms (such as juvenile myelomonocyte leukaemia), could be indications to investigate the presence of a PID.

This thesis aimed to reveal new genetic and/or functional mechanisms of PIDs. Patients enrolled in this thesis were followed at the Primary Immunodeficiencies Unit of Hospital Dona Estefânia, Centro Hospitalar de Lisboa Central, having either a known PID that presented fatal and uncommon symptoms or an undiagnosed PID presenting atypical clinical symptoms.

The investigation of these patients added new phenotypes to previously described PIDs: nodular regenerative hyperplasia in a patient with leaky X-linked

severe combined immunodeficiency caused by a novel hypomorphic mutation in the IL2RG gene; hemophagocytic lymphohistiocytosis secondary to BCG and *Cytomegalovirus* infections in a patient with IFNγ receptor deficiency; inflammatory bowel disease (IBD) and a phenotype resembling tricho-entero-hepatic syndrome caused by a hypomorphic TTC7A mutation; cutis laxa in an APLAID patient caused by a novel mutation in the PLCG2 gene.

The investigation of an Infantile-onset IBD cohort of patients allowed the identification of a monogenic cause for the disease in 54% of the patients and in many cases, this led to changes in the therapeutic approach. We were able to identify a novel cause of IO-IBD, immunodeficiency and sensoryneural immunodeficiency, caused by STXBP3 mutations, not previously associated with human disease.

Moreover, in a patient presenting an IPEX-like phenotype we have identified a novel PID caused by homozygous mutations in the ZC3H12A gene, leading to a dysfunctional regnase-1 protein that caused aberrant post-transcriptional regulation of IL-6 production. Finally, in a patient with severe recurrent fasciitis we identified a variant of unknown significance in the IRG1 gene and aimed to prove that the IRG1 variant lead to altered itaconate metabolism in macrophages, thus resulting in inappropriate sensing of the resolution of the infection and hyperinflammation.

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ACRONYMS

ADA- Adenosine deaminase

AIRE- Autoimmune Regulator

ALPS- Autoimmune lymphoproliferative syndrome

APECED- Autoimmune Poliendocrinopathy, Candidiasis, Ectodermal Distrophy

APLAID- Autoinflammation and PLCγ2-associated antibody deficiency and immundysregulation

ARE- Adenine/Uridine Rich elements

CAD- Cis-acotinic acid decarboxylase

CGD- Chronic granulomatous disease

ChrD- Crohn's Disease

CD- Cluster of differentiation

CNS- Central Nervous System

CTLA4- Citotoxic T lymphocyte antigen-4

CVID- Common Variable Immunodeficiency

DIRA- Deficiency of the IL1 receptor antagonist

DITRA- Deficiency of the IL36 receptor antagonist

EBV- *Epstein-Barr virus*

ELISA- Enzyme-linked immunosorbent assay

fHLH- Familial hemophagocytic lymphohistiocytosis

Gb- *Granulibacter bethesdensis*

GM-CSF- Granulocyte-macrophage colony stimulating factor

GvHD- Graft versus host disease

GWAS- Genome-wide association Studies

HDE-CHULC- Hospital Dona Estefânia, Centro Hospitalar Universitário de Lisboa Central

HDFa- Primary human dermal fibroblasts

HPV- *Human papilloma virus*

HSCT - Haematopoietic stem cell transplantation

HSV- *Herpes Simplex virus*

IBD- Inflammatory Bowel Disease

IFNγ- Interferon γ

IKB- Inhibitor of Nuclear Factor Kappa B

IL- Interleukin

IO-IBD- Infantile Onset Inflammatory Bowel Disease

IPEX- Immune dysregulation, poliendocrinopathy, X-linked)

IRG1- Immune-responive gene protein 1

IRT- Immunoglobulin replacement therapy

IVIG- Intravenous Immunoglobulin

LPS- Lipopolysaccharide

MALT- Mucosa-associated lymphoid tissue

mTOR- Mammalian target of rapamycin

MHC- Major histocompatibility complex

Mb- Mycobacterium bovis

MIA- Multiple Intestinal Atresias

MIA-CID- Multiple Intestinal Atresia associated with combined immunodeficiency

NADPH- Nicotinamide adenine dinucleotide phosphate

NEMO- NFKB essential modulator

NFkB- Nuclear factor kappa light chain enhacer of activated B cells

NGS- Next-generation sequencing

NK- Natural Killer

NOD2- Nucleotide-binding oligomerization domain-containing protein

PAMPs- Pathogen-associated molecular patterns

PRR- Pattern recognition receptors

PBMCs- Peripheral blood mononuclear cells

PBS- Phosphate buffer saline

PCR- Polymerase Chain Reaction

PI3K- Phosphoinositide 3 kinase

PI3KCD- Phosphoinositide 3 kinase catalytic subunit Delta

PID- Primary Immunodeficiency

PIDs- Primary Immunodeficiencies

PTEN- Phosphatase and Tensin Homologue

qPCR- Quantitative PCR

RAG- Recombination Activating Gene

ROS- Reactive oxygen species

SCID- Severe Combined Immunodeficiency

SDH- Succinate dehidrogenase

SLAM- Signalling lymphocytic activation molecule

STAT- Signal transducer and activator of transcription

TCR- T-cell receptor

Th- T helper

TLR- Toll-like receptors

TNFα- Tumor necrosis factor alpha

Treg- T regulatory

TPP- Tripeptidyl peptidase

TTC7A- Tetratricopeptide repeat domain 7A

UC- Ulcerative colitis

VEO-IBD- Very early-onset inflammatory bowel disease

VUS- Variant of unknown significance

WES- Whole exome sequencing

XLP- X-linked lymphoproliferative syndrome

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CHAPTER I

General introduction and state of the art

1. IMMUNE SYSTEM

The immune system is classically divided into innate and adaptive. The response of innate immunity is fast, nonspecific and does not generate immunological memory, unlike adaptive immunity, which takes days to mount a response, but is specific and generates immunological memory.[8] Despite these functional concepts, the great difference between them is the type of receptor used for pathogen recognition: innate immune recognition is mediated by pattern recognition receptors (PRR), coded in the genome, with general specificity for essential structures of microorganisms. On the other hand, adaptive immune recognition is mediated by antigen receptors, generated by somatically recombined germ gene segments, allowing the generation of a highly diverse repertoire, capable of an ultra-specific recognition of antigens.[9]

Moreover, it is well known that exposure to pathogens not only generates immune memory through adaptive immunity, but also improves the response of innate immune components, which evokes the concept of trained immune memory in the innate immune system.[10-12] Despite this academic division, all components of the immune system interact with each other in a complex functional network.[13]

Innate Immunity

Throughout the evolution of nature, an effective and sophisticated immune system has depended on the ability to preserve the components that demonstrated the best results.[14] Innate immunity corresponds to what nature has selected as being crucial over millions of years of evolution, which clearly demonstrates its vital importance.[15] Its main functions are not only in the early recognition and detection of microorganisms, but also in the initiation and regulation of inflammation,[16] as well as in the maintenance of a homeostatic response,[17,18] and the activation and instruction of adaptive immunity.[13,16,18] In short, over and above the physical and enzymatic barriers,[11-13] innate immunity is mostly composed of circulating PRR (such as defensins, catelecidins, colectins, lectins and pentraxins)[13,16,19-27] and PRR-containing cells, such as granulocytes, macrophages and dendritic cells, but also mast cells, eosinophils and Natural Killer (NK) cells. Through a limited number of

PRRs, these cells recognize ultra-conserved structures of pathogens, called pathogen-associated molecular patterns (PAMPs).[28-30]

PAMPs are characterized by being invariant among microorganisms of the same class, as they are crucial for their survival, thus limiting the evolutionary ability of the microorganism to evade the immune system. As they are products of pathways that are unique to microorganisms, this also allows proper discrimination between self and non-self)[9,28-30] Some examples of PAMPs and respective PRR can be found in table 1.

PAMP	TYPE	PRR
Lipopolysaccharide	Gram-negative bacteria	TLR4
Flagellin	Flagellated bacteria	TLR5
Peptidoglican	Gram positive Bacteria	TLR2
Porins	*Neisseria*	TLR2
Triacyl Lipopeptides	Bacteria and Mycobacteria	TLR1/2
Diacyl Lipopetides	*Mycoplasma*	TLR6/2
ssRNA	RNA Virus	TLR7/8
dsRNA	Virus	TLR3
ssRNA	RNA Virus	TLR7/8
DNA	Virus	TLR9
Hemozoin	Plasmodium	TLR9
Mannan	Candida albicans	TLR4

Table 1: Examples of PAMPs and respective PRR

Interestingly, PRRs don't have the ability to distinguish between pathogenic and symbiotic microorganisms (as PAMPs are not restricted to pathogenic bacteria). Considering that humans are colonized by trillions of symbiotic bacteria, it is very

important that the innate immune response maintains a strightly-regulated homeostasis.[17,31] Any disruption of these interactions can lead to hyper-inflammatory conditions.

Regarding cell-associated PRR, they can be divided into PRR associated with phagocytosis (such as dectin-1),[32] but also intracellular (such as Toll-like receptors, endosomal TLR 3,7,8,9,10, NOD-like receptors and RIG-I-like receptors)[29,30,33-35] and surface receptors (transmembrane TLR 1,2,4,5 and 6).[9,29,33,36] Following the recognition of the respective PAMPS, different signalling pathways are activated and promote different cellular functions, namely the production of pro-inflammatory cytokines, which not only cause inflammation, but also regulate and activate the adaptive immune system.[33,36]

Adaptive immunity

The main characteristic of adaptive immunity is the use of specific antigen receptors in T and B cells, which brings an immune response in two main phases: antigen presentation and effector response.

The first one consists of the presentation of the antigen that will be recognized by the specific T and B cells. This phase usually occurs in the specialized lymphoid tissue environment.[37]

The effector response, comprises either the movement of the activated T cells to the specific area of action, or the secretion of antibodies by the activated B cells (plasma cells) to the blood and tissues.[38]

T and B lymphocytes develop from progenitors in the bone marrow. While B precursors remain in the bone marrow during their development, T cells migrate to the thymus at an early stage of their development, where they are called thymocytes. The production of specific antigen receptors occurs at an early stage in the development of both cells, prior to exposure to the antigens as a result of a random rearrangement and splicing of multiple DNA segments that code for the antigen-binding areas of the receptors.[13,39].[40] This mechanism, known as V(D)J

recombination is absolutely critical for the T and B cell development, allowing a diversity of over 10^8 T-cell receptors and 10^{10} antibody specificities. In brief, during lymphocyte development, the exons that encode the antigen binding domains are assembled by sequential chromosomal breakage and rejoining of the V (variable), D (diversity) and J (joining) gene. This process is mediated by a V(D)J recombinase, consisting of two lymphoid specific proteins, coded by recombination activating gene 1 and 2 (RAG1/RAG2).[41] This process also implies the presence of several cytokines. In fact, it the discovery of the IL2RG gene, in which mutations lead to X-linked Severe Combined Immunodeficiency (XL-SCID), improved the understanding that different cytokines are important for the correct development of different lymphoid lineages. IL2RG encodes a protein called γ-chain, which is part of multiple cytokine receptors, thus causing abnormal development of different lymphocyte subsets.[42]

The T and B lymphocytes that leave the thymus and bone marrow, respectively, are naïve (they did not find their specific antigen), and will go on to form the lymphoid population of secondary lymphoid organs in the spleen, tonsils, lymph nodes and mucosa-associated lymphoid tissue. This microenvironment in the secondary lymphoid organs is conducive to the maintenance of lymphocytes and their contact with antigens through antigen-presenting cells.[43]

During the thymus period, the T-cells learn the self-nonself discrimination. In the cortex, the double-positive lymphocytes (CD4+CD8+) that do not get a rescue signal through T-cell receptor (TCR) are programmed to undergo death by neglect.[44] in fact, a limited percentage of these cells bind major histocompatibility complex (MHC) ligands with the correct avidity, obtaining a signal that induces the maturation of double positive lymphocytes into CD4+CD8- or CD8+CD4- single positive T cells, in a process called positive selection.[44,45] Single positive T cells are then directed to the thymic medulla by the recognition of various chemokines. There, the single positive T cells that over-react to self-antigens are eliminated by a process called negative selection, which is mediated by medullar thymic epithelial cells and medullary dendritic cells. These cells broadly express tissue-specific antigens (proteins restricted to pancreas, stomach, eye, muscle, etc), in a process regulated by the transcription factor autoimmune regulator (AIRE). Thus, only non-self reactive

thymocytes will overcome negative selection and leave the thymus as mature naive T-cells. However, along this recognition processes n the thymus, some of the thymocites that recognize self-MHC (Major histocompatibility complex) peptide complexes with high affinity start expressing FOXP3, and survive negative selection, maturing as regulatory T cells (Treg), that will migrate to the periphery and act as suppressive autoreactive T cells.[46,47]

Antigens that are produced endogenously within the cell (usually tumour and viral proteins, besides self antigens) are complexed with MHC class I molecules and presented to CD8+ T cells. Exogenous antigens are processed via a different pathway after being endocytosed, and are presented in MHC class II molecules to CD4+ T cells..[13,48]

CD4+ T cells only recognize antigens presented by MHC class II and CD8+ T cells only recognize antigen presented by MHC class I. Therefore, the MHC molecule that is used for antigen presentation will determine the type of effector response.[13,48]

The activation of T cells is then dependent on proper antigen presentation. When a CD4+ T cell finds a complementary peptide-MHC class II complex in an antigen presenting cell (APC), i.e. macrophages, dendritic cells (DC) and B lymphocytes, it further requires strong co-stimulatory signals (namely through the interaction between CD28 expressed on T cells and CD80 and CD86 expressed on APC) to avoid the state of anergy, which is a very important mechanism of peripheral tolerance. The inducible T cell costimulator (ICOS) is a CD28 homologue that is inducibly expressed on activated T cell and augments T cell activation. On the other hand, after activation, the expression of cytotoxic T lymphocyte antigen-4 (CTLA-4) is increased in T cells. CTLA-4 has a higher affinity for CD80 and CD86 than CD28, leading to loss of costimulation and cessation of proliferation and cytokine production, thus controliing excessive T cell activation.[49,50]

The functional phenotype of the activated T cell depends on the condition under which the T cell is activated and stimulated. The route of antigen entrance (skin, gut, lungs, etc.), its form (living micro-organisms, inert molecules, etc.), or the

amount of antigen present, are all important factors.[51] The T cell subsets are then defined by the set of transcription factors that they express to maintain their differentiated state, as well as the production of certain citokines,[52] with which they mediate their effector responses. It is now understood that the fate of the T cell is less static than previously thought, depending on the extra-cellular environment and the transcription factors (such as T-bet and STAT4 for Th1, FOXP3 or STAT5b for Treg and GATA3 or STAT6 for Th2) (Fig.2).[53]

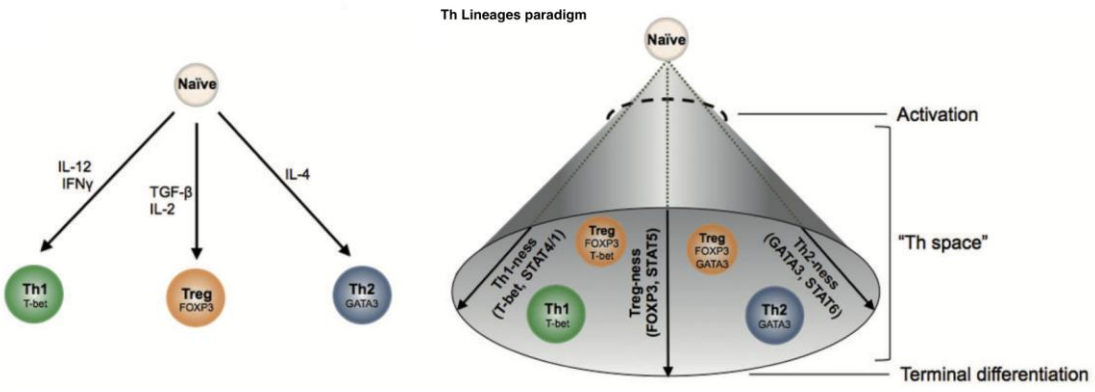

Figure 2: Th lineages paradigm. Adapted from Evan CM et al, 2013.[53]

Several distinct subsets can arise upon CD4+ T cell activation.

- T helper (Th) 1 cells are the subset involved in cell mediated inflammation and delayed-type hypersensitivity reactions. They lead to the production of opsonizing antibodies, induce cellular cytotoxicity and macrophage activation. These cells mostly produce interleukin (IL) 2 and interferon γ (IFNγ) and their response is critical for the control of intracellular pathogens (virus, intracellular bacteria), but may also be related to certain autoimmune diseases.[52,54]

- Th2 mostly produce IL-4 and IL-5 that respectively promote the production of IgE and stimulate eosinophil development. Th2 responses are very important for the anti-helminthic responses and are involved in the pathogenesis of atopic diseases.[52,54,55]

- Th17 and its major cytokine IL17 have a role in chronic inflammatory responses in chronic infection, allergy and autoimmunity. They are also very important for the antifungal defense.[56]

- The thymic regulatory T cells (Treg) have been discussed earlier in this chapter. However, inducible (or peripheral) Treg cells differentiate from naive T cells and are critical for the establishment of peripheral tolerance, especially at interfaces, such as the mucosal surfaces of the gut and lung, where the immune system comes into contact with the exterior and is chronically exposed to foreign antigens.[57]

- Follicular helper T cells are prominent in the germinal centres of secondary lymphoid tissues and are crucial to the production of high affinity antibodies.[58]

- Other T cell subsets have distinct functions, such as the induction of mucosal tolerance (Th3), antitumor immunity (Th9) or antibacterial (Th22), being the latter also implicated in pathologic inflammatory responses in the skin, related to atopic dermatitis and psoriasis.[52,54]

Dynamic changes in gene expression mediate the antigen-induced B-cell activation and differentiation in secondary lymphoid tissues. There, clonal expansion and class switch recombination at the IgH locus, somatic hypermutation of VH genes and affinity maturation for a unique antigenic epitope occurs.

The differentiation of naïve B cells is driven by antigenic stimuli but also by non-antigenic signals (such as complement deposition on the surface of microbes that bind to complement receptors on B cells). B cells can respond to thymus-independent and dependent antigens.[59] Thymus independent antigens possess highly repetitive molecular structures that are capable of cross-linking specific B-cell receptors and directly activate the B cell to secrete IgM antibody. Some examples are the polysaccharide capsules of *Streptococcus pneumoniae* or *Haemophillus influenzae*. Importantly, as polysaccharides cannot be loaded into MHC II, these bacteria have the ability to evade T cell recognition but still elicit specific antibody responses mediated by immature (transitional) B cells.[60] Until the age of two, this mechanism of defence is very immature, thus rendering children very susceptible to invasive infection by encapsulated organisms. Thymus dependent antigens are proteins that can be loaded into MHC class II molecules and presented to the TCR of CD4+ T cells, driving their differentiation into a subset that is specialized to promote antibody production by B cells.[61] The interaction between CD40 on the B cell surface

and CD40 ligand on the T cell induces isotype switching from the originally produced IgM.[62] This allows high-affinity, class-switched antibodies to be produced, which occur mainly in the germinal centres of the lymph nodes.[63] Despite being, unquestionably, the major function of B-cells, antibody production is not the only function of these cells. They also present antigens to T cells, participate in CD4+ T cell stimulation and regulate the immune responses via cytokine production. Over recent years, regulatory B cells emerged not only as IL-10 producing cells that inhibit the pro-inflammatory responses, but also as regulators of the maturation and function of DC.[64,65]

Naïve CD8+ T cells circulate in the periphery and when they recognize a known peptide presented by an APC harbouring a MHC class I molecule, they become activated and differentiate as effector cells capable of exerting their cytotoxicity function and kill an infected cell presenting the same antigen. The cytotoxic mechanisms are mostly performed through two complex pathways: granule exocytosis and expression of the Fas ligand, thus initiating programmed cell death in the target cell.[66]

2. PRIMARY IMMUNODEFICIENCIES

PIDs are a heterogeneous group of diseases that affect the development or function of any component of the immune system.[67-70]

The history of the PIDs is extremely rich, beginning classically in the 1950s. The description of agammaglobulinemia by Colonel Bruton in 1952 in a child with recurrent infections and absence of gamma fraction in protein electrophoresis is considered to be the first historical report of PID,[71,72] although a few months earlier, Bruton and Janeway had presented five cases of this pathology at the congress of the Society of Paediatric Research.[73] Curiously, the first report of Wiskott-Aldrich syndrome was described in Germany in 1937,[74] but its association with recurrent infections was only referred to in 1954,[75] and its relationship to an immune deficiency only 14 years later.[76] The first reference to "alymphocytosis" was made by Glanzmann in 1950, and it was only in 1953[77] that the first publication of two children with alymphocytosis and absence of lymphoid tissue appeared.[78] However, in 1958, it was still considered to be a kind of Swiss type agammaglobulinemia [79,80]. It was successfully demonstrated in 1962 that these patients did not reject skin grafts or presented late hypersensitivity, and had altered thymic development.[81] The term SCID was only defined as late as 1975.[82] A few years earlier, in 1965, DiGeorge demonstrated the importance of the thymus in the development of T lymphocytes, by describing a different group of children with combined immunodeficiency associated with other anomalies, such as peculiar facies, hypoparatiroidism and heart disease.[81,83] The strange discovery of hypergammaglobulinemia in children with severe and recurrent infections was made by Janeway in 1954,[84] leading to the description of phagocyte defects for the first time in 1959.[85] These have been brilliantly characterized by Landing in 1965.[86] In 1967 two different studies showed that these patients had a defect in the bactericidal capacity of their serum.[87,88] Interestingly, back in 1922 Schulz had already described the existence of the "agranulocytic angina" probably the first account of a PID.[89]

The first PID classification was published in the New England Journal of Medicine in 1970, consisting of 14 immunodeficiencies.[90] The 1980s and 1990s were

marked by the identification of dozens of new congenital defects of immunity, but also by the discovery of the first genes responsible for these diseases, allowing for the understanding of the pathophysiological mechanisms of many of them, and even the first gene therapy trials.[83,91-96]

The classification published in 2007 already included more than 150 PID and more than 120 described genes.[96] The Human Genome Project, that ended in 2003,[97] was instrumental in pushing the development of high-throughput technologies for preparing, mapping and sequencing DNA.[98] Since the first massively parallel pyrosequencing platform was commercialized in 2005,[99] the classical methods used to discover genes responsible for diseases (classic linkage studies and homozygosity-mapping),[100] have been replaced by the use of new generation sequencing (NGS) techniques.[101,102] Since then, the discovery of new PIDs and novel genetic causes for previously known PIDs has been boosted.[103-105] It is known that the vast majority of PIDs are monogenic, with variable expressivity and often with incomplete penetrance.[106] The recognition that mutations in the same gene can present with several clinical phenotypes, but also that mutations in multiple different genes can manifest with the same phenotype, has made it mandatory to use NGS to elucidate the clinical picture of individual patients or patient groups.[107-109] By understanding the impact of such nature's experiments on the immune system, it has been possible to expand knowledge, change therapeutic strategies and family counseling,[110-112] but it has also raised a multitude of very important ethical issues.[113,114]

With the evolution of knowledge, the signs classically defined as PID revealers are now a relic of the past.[115-117] It is not only the susceptibility to infections, whether serious, recurrent or caused by unusual agents, that is indicative of PID.[118,119] Early onset autoimmune manifestations, poly-autoimmunity,[120-124], hyper-inflammation, [125-127] or recurrent inflammation (systemic or organ-specific),[125,128-131] exuberant allergy (especially if associated with other manifestations),[132-136] and even some neoplasms (such as juvenile myelomonocyte leukaemia),[137-139] could be indications for suspicion of PID. In this way, the warning signs for PID have been redefined, with an emphasis on the different affectation of the various systems.[140-142]

3. CLASSIFICATION OF PIDs

In parallel with the evolution of knowledge about congenital immune defects, the classical classification of PIDs into combined immunodeficiencies, humoral, well-defined PIDs, immunoregulatory diseases, phagocyte defects, innate immune defects, auto-inflammatory and complement defects,[67,143-145] has been adapted, reflecting the greater complexity and intersection of different types of immune defects.[146] In the most recent classification more than 350 PIDs are described and of those, 344 with an identified genetic defect.[147]

This chapter summarises the different PID groups, focusing more closely on those that will be most relevant to the results of this thesis.

3.1. IMMUNODEFICIENCIES AFFECTING CELLULAR AND HUMORAL IMMUNITY

3.1.1. SEVERE COMBINED IMMUNODEFICIENCY (SCID)

SCIDs are a group of genetic disorders that result in the absence or dysfunction of T and B lymphocytes, with an estimated incidence of 1:58.000 births.[148] It is considered to be a paediatric emergency, since patients are highly susceptible to every type of infection and usually succumb to it before the age of 12 months, if they are not diagnosed and treated in a timely and appropriate manner.[149] The typical symptoms are recurrent, severe or opportunistic infections, often in children with poor weight progression and chronic diarrhoea. Some patients have a variant, with generalised erythroderma, hepatosplenomegaly and lymphadenopathy caused by the development of self-reactive lymphocytes (Omenn syndrome). In some cases, the maternal engraftment (which occurs due to the absence of T lymphocytes from the foetus) causes a very similar clinical picture.[150-152]

SCIDs are usually classified according to the main pathways that are affected by the molecular defect. Thus, there may be cases where there is a T deficiency with a normal number of B-lymphocytes (SCID T-B+) or an absence of T and B-lymphocytes (SCID T-B-). These groups are further subdivided according to the presence or absence of NK cells (NK+ or NK-).[148-152]

There are cases in which the genetic defect causes a less severe phenotype, with the presence of some hypofunctional lymphocytes, leading to an atypical condition that is less severe and has greater chances of survival. The vast majority of these cases, called leaky-SCID, are associated with RAG or Artemis defects, essential proteins for the V(D)J rearrangement of B and T cell receptors.[153,154] There are very few reports of leaky-SCID patients caused by mutations in the IL2RG, many of them caused by somatic reversion.[155-161]

3.1.2. LESS SEVERE COMBINED IMMUNODEFICIENCIES

This group includes immunodeficiencies that affect cellular and humoral immunity, but whose impairment is less severe than SCID. It is a heterogeneous group of diseases in which recurrent infections, particularly by less common agents, are the most frequent characteristics.[162]. In all of them there is cellular dysfunction, which can be quantitative, either of CD8 (as in class I MHC defect),[163] or of CD4 (in the class II MHC defect).[164] However, this dysfunction can only be qualitative (as in the case of Hyper-IgM syndromes caused by CD40 (member of the tumour necrosis factor superfamily, expressed constitutively on B lymphocytes) or CD40 ligand (expressed on activated CD4+ cells that interact with CD40).[165,166]

3.2. COMBINED IMMUNODEFICIENCIES WITH ASSOCIATED OR SYNDROMIC FEATURES

This is one of the groups that best reflects the complexity of PIDs, including diseases with very heterogeneous phenotypes, caused by changes in different components of the immune response. Among many others, here we can find classic PIDs such as Wiskott-Aldrich syndrome,[167], immuno-osseous dysplasia,[168,169] thymic defects (such as DiGeorge syndrome, CHARGE syndrome or FOXN1 deficiency),[162,170,171] and Hyper-IgE Syndromes.[172-175] This group also includes multiple intestinal atresias with combined immunodeficiency (MIA-CID), in which the affected patients are carriers of biallelic mutations of the TTC7A gene and classically present multiple intestinal atresia (MIA) and a phenotype of combined immunodeficiency or even SCID.[176] In recent years, the spectrum associated with this

disease has been widely broadened, allowing diagnosis of patients with atypical clinical characteristics, namely very early onset inflammatory bowel disease (VEO-IBD), with or without intestinal atresia.[177]

3.3. IMMUNODEFICIENCIES PREDOMINANTLY OF ANTIBODIES

This is the most prevalent group, corresponding to about half of all PIDs. In most of these diseases, infectious susceptibility predominates, namely to capsulated agents. However, there may also be non-infectious complications such as inflammatory bowel disease, systemic granulomatous disease or autoimmunity. This includes more complex diseases such as agammaglobulinemia (absence of B cells and pan-hypogammaglobulinemia),[178] common variable immunodeficiency (CVID, where there is a deficit of IgG and another immunoglobulin isotype associated with absent response to vaccines),[179-181] or hyper-IgM syndromes (where there is a defect of IgG and IgA with normal or elevated IgM),[165,166] but also less complex and more frequent defects such as IgA defect and specific antibody production defects.[179,181,182]

3.4. DISEASES OF IMMUNE DYSREGULATION

This group of PIDs reflects the defects that lead to a disturbance in the regulation of the immune response. This includes genetic defects that lead to:

- Hyper-inflammation, such as IL10 pathway defects that cause VEO-IBD, but also familial hemophagocytic lymphohistiocytosis (fHLH), in which cytotoxicity defects cause a cytokine storm that leads to multi-organ failure and death if not properly diagnosed and treated.[183-189]
- Loss of central tolerance, due to mutations in the AIRE gene causing APECED syndrome (Autoimmune Poliendocrinopathy Candidiasis Ectodermal Dystrophy). Patients have a classic triad of chronic mucocutaneous candidiasis, adrenal insufficiency and hypoparatiroidism, but often have other autoimmune manifestations of various organs and systems (urticaria,

autoimmune hepatitis, thyroiditis, anaemia, thrombocytopenia and enteropathy, among others).[124]

- Loss of peripheral tolerance, in which patients may present numerous manifestations of immune dysregulation, particularly eczema, polyautoimmunity and lymphoproliferation. They may be caused by apoptosis defects (autoimmune lymphoproliferative syndrome, ALPS)[123,190,191] and of Treg (Immune dysregulation, Polyendocrinopathy, X-linked (IPEX) syndrome, caused by mutations in FOXP3)[120,192]. Nevertheless , in recent years many patients with IPEX-like phenotype without mutations in the FOXP3 gene have been identified, leading to the discovery of other genes responsible for compromising peripheral tolerance and leading to autoimmunity and infectious susceptibility (defects in CD25, CTLA4 and LRBA genes or gain function from STAT3).[121,122,193-198]
- Susceptibility to *Epstein-Barr Virus* (EBV), a very heterogeneous group of diseases where patients share the common characteristic of having a particular susceptibility to severe EBV infection.[199-203]

3.5. CONGENITAL DEFECTS OF PHAGOCYTE NUMBERS OR FUNCTION

This group contains diseases that have the dysfunction of granulocytes in common. As a consequence of this dysfunction, patients present severe and recurrent infections, most frequently of the skin, soft tissues and deep abscesses caused mostly by *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

In isolated quantitative defects (congenital neutropenias), most cases involve a maturative blockade of granulocyte differentiation in the promyelocyte-myelocyte phase and, in addition to infections, patients have an increased risk of acute myeloid leukaemia and myelodysplasia.[204-208] Congenital neutropenia is often associated with other extra-hematopoietic manifestations or appears in the context of a syndrome, which makes it possible to predict the underlying genetic defect.[209-222]

There are several types of functional defects, which add healing defects and periodontitis, among other symptoms to the infectious susceptibility.[223-225]

Chronic Granulomatous Disease (CGD) is one of the most well-studied PIDs, in which there is an oxidative burst defect by alteration of the nicotinamide complex adenine dinucleotide phosphate (NADPH) oxidase, responsible for the transfer of an electron from NADPH to molecular oxygen, generating a superoxide ion, precursor of H2O2 and other reactive oxygen species (ROS), essential for the microbicide activity of phagocytes.[226] It is a rare disease that occurs in about 1:250000 live births,[227] and can be caused by mutations in any of the components of NADPH oxidase.[228] The X-linked disease, caused by mutations in the CYBB gene encoding gp91^{phox} is responsible for about 70% of the cases, but mutations in p22phox, p47phox, p67phox are responsible for the autosomal recessive forms of the disease.[229,230] Rare cases of p40phox and Rac2 deficiency have also been described.[231,232] The degree of impairment of the oxidative function is directly related to the severity of the clinical presentation, and patients with CYBB gene mutation have almost absent oxidative capacity and therefore a worse prognosis than those with autosomal recessive disease and residual oxidative function.[233] Despite having inflammatory complications (particularly of the gastrointestinal tract),[234,235] the predominant manifestations of CGD patients are recurrent, often invasive and life-threatening, infections caused by bacteria and fungi. Pneumonia is the most frequent infectious manifestation, caused primarily by *Staphylococcus aureus, Nocardia spp, Burkholderia spp, Serratia spp mycobacteria* and *fungi*, particularly *Aspergillus spp*.[227,230,236-238] Skin infections, another common manifestation, are usually caused by *Staphylococcus aureus*.[227,230,236-238] Lymphadenitis and lymph node abscesses are usually located in the cervical region and are caused by *Staphylococcus aureus, Burkholderia spp* and, rarely by *Granulibacter bethesdensis (Gb)*.[227,230,236-238] Around 1/3 of the patients present hepatic abscess, caused by *Staphylococcus aureus* and *Burkholderia spp*. These abscesses are dense, septated, caseous, often with pseudo-capsules, classically requiring multiple drains.[227,230,236-240] Septicaemia is less frequent and is usually caused by *Salmonella spp* and other Gram negatives.[227,230,236-240] Central nervous system infection is relatively uncommon and is usually caused by *fungi* and, in rare cases, by *Salmonella.*[227,230,236-240]

3.6. DEFECTS IN INTRINSIC AND INNATE IMMUNITY

This is a group of heterogeneous diseases which, due to the deregulation of a certain component or pathway of the innate immunity, have a marked infectious susceptibility. Some of them only have infectious susceptibility to a particular agent, such as the *human papilloma virus* (HPV),[241] *Influenza virus*,[242] *Rhinovirus* [243] or *herpes simplex virus* (HSV).[244-246] In other cases, there is a particular, but not exclusive, susceptibility to some pathogens, such as mendelian susceptibility to *Mycobacterium tuberculosis*.[247-249] Finally, there are defects of the innate immunity that render the host broadly susceptible to viral infections,[250,251] fungal infections,[252-256] bacterial infections,[257-260] or even viral and mycobacterial infections.[261]

3.7. AUTOINFLAMMATORY DISORDERS

This area has been one of the most developed in recent years, with the genetic elucidation and pathophysiology of a large part of these diseases, so there are now many different groups with very heterogeneous clinical manifestations. Although there are several possible classifications, the one used by most experts is based on the underlying mechanism:[130,262,263]

- Interferonopathies: they are caused by multiple genetic defects of different components of the intra-cellular surveillance, leading to the disruption of cellular homeostasis, thus causing neurologic impairment,[264,265] and/or early-onset systemic vasculitis.[266,267]
- Proteasome-related: these are caused by mutations in genes that regulate proteasome activity causing cellular stress leading to cytokine dysregulation and systemic manifestations such as fever, nodular skin rash, myositis, panniculitis-induced lipodistrophy and basal ganglion calcification.[268-271]
- Inflammasomopathies: these are many different diseases that are caused by hyper-function of the NLRP3, NLRC4, NLRP1 and pyrin inflammasomes. Each one of these lead to substantially different phenotypes that share some common manifestations, such as recurrent fever and skin involvement.[269,272-281]

- Non-inflammasome related diseases: In this group there are diseases with pathophysiological mechanisms that are not grouped in any of the previous ones. They are very distinct, and may for example cause pustular psoriasis, (deficiency of the IL1 receptor antagonist, DIRA, and deficiency of the IL36 receptor antagonist, DITRA),[128,129,282], early onset sarcoidosis (Blau Syndrome)[283] or stroke (ADA2 deficiency).[284,285]

3.8. COMPLEMENT DEFICIENCIES

Patients may present hereditary angioedema (C1 inhibitor defect), immunocomplex deposition diseases and infections by capsulated bacteria (C1q, C1r, C1s, C2, C3 and C4 defects), invasive meningococcal disease (C5-C9, properdin and factor D) or atypical haemolytic uremic syndrome (C3 gain of function, , factor B and D and loss of function of factor H and factor I).[271,286-288]

3.9. PHENOCOPIES OF INBORN ERRORS OF IMMUNITY

Finally, this group includes diseases with a similar presentation to PIDs, but which are not caused by germline mutations. They can be caused by somatic mutations, such as those caused by RAS-associated leukoproliferative syndrome,[137,289] late onset cryopyrinopathies or hyper-eosinophilic syndrome.[290,291] It should be noted that they may also be caused by the presence of anti-cytokine autoantibodies.[292-296]

4. NEW CONCEPTS

The above classification shows that the paradigm regarding PIDs has changed in recent years with the generalisation of NGS techniques. After many years in which the discovery of new genes was based on a bottom-up strategy, starting from the clinical phenotype (educated guesses based on known molecular pathways), the top-down strategy was generalized, in which the exome or genome of a patient or group of patients is sequenced and the discovery of the responsible gene leads to the elucidation or clarification of the function of the codified protein in the immune system.[105-111] Using this new approach, new phenotypes have been identified and many diseases that were not classically associated with immune defects have become part of the possible clinical presentations of congenital immune defects. For example, in recent years, some hyper-inflammatory diseases have been associated with defects in inflammation control. One of these diseases is the very early onset inflammatory bowel disease (VEO-IBD). There are other examples of this, such as poly-autoimmunity or early-onset autoimmunity. Of these diseases, very early onset diabetes mellitus (before the age of 2), especially when associated with other autoimmune manifestations, has been associated with various monogenic defects of immunity.[194,297] Classically associated with autoimmune lymphoproliferative syndrome (ALPS), paediatric-onset Evans syndrome has been, in recent years, found to be the first manifestation of numerous PIDs, namely the haploinsufficiency of CTLA4, deficiency of LRBA or TPP2 or even the gain-of-function of STAT3.[194,298-301]

Curiously, the interest in post-transcriptional regulation of cytokines is not recent and it allows us to recognize the importance of the fine control of cytokine production through these mechanisms.[302] However, to date, there has been no monogenic defect in any of these components that was associated to PID.

On the other hand, one area that has been expanding enormously in recent years is immunometabolism. [303] The importance of different metabolic approaches in controlling and even triggering the immune response is being understood more clearly,[304] since the intersection between these two areas has allowed the clarification of many of these mechanisms. One of these examples, which is

becoming increasingly more enlightening, is the preponderant role of the mammalian target of rapamycin (mTOR) pathway in the immune response. [305,306] The discovery of the rapamycin molecule in 1975 made it possible to identify mTOR as its target in 1991.[307,308] In the decades that followed, this led to the description of the PI3K-AKT-mTOR pathway, with multiple functions in the different cell types, such as the control and regulation of its survival, proliferation, metabolism and growth.[309,310] Initially, germinal and somatic mutations associated with overgrowth syndromes were described.[311-313] In recent years, PIDs caused by mutations leading to mTOR hyperactivation, such as the gain in function of PI3KCD or the loss of function of the regulatory protein PTEN were identified.[314-316] This pathway, highly focused on the function of the mTOR complex, allows immune system cells to dynamically switch from a quiescent, catabolic and autophagic state, using beta-oxidation as an energy source, to an activated, anabolic state, using glycolysis as an energy source. This allows the adequate expansion, contraction and homeostasis of the immune response.[317,318] In the recently described PI3KCD or PTEN mutations lead to gain of function and consequent hyperactivation of mTOR, disrupting this balance and causing autoimmunity, as well as a greater infectious susceptibility and greater incidence of lymphoid neoplasms.[314-317,319] In the coming years, this growing interest on congenital defects of immunometabolism will receive a very important part of the attention of the researchers in this fascinating field of hereditary defects of immunity.

4.1. INFANTILE ONSET INFLAMMATORY BOWEL DISEASE (IO-IBD) AND PIDs

INFLAMMATORY BOWEL DISEASE (IBD)/PEDIATRIC IBD

Inflammatory bowel disease (IBD) comprises two chronic intestinal diseases: Crohn's disease (CrhD) and ulcerative colitis (UC). These complex diseases are characterised by chronic and recurrent inflammation of the intestine. Classically, CrhD discontinuously affects any part of the gastrointestinal tract, from the mouth to the anus, and usually has transmural inflammation. On the other hand, UC mainly involves the colon, in a continuous way, and the inflammation is restricted to the mucosa.[320-322]

They have a high prevalence (58/100,000) and incidence (2.5-11.4/100,000), and there has been a progressive increase in the number of cases, both in adult and paediatric age (Fig.3). [2,323-327]

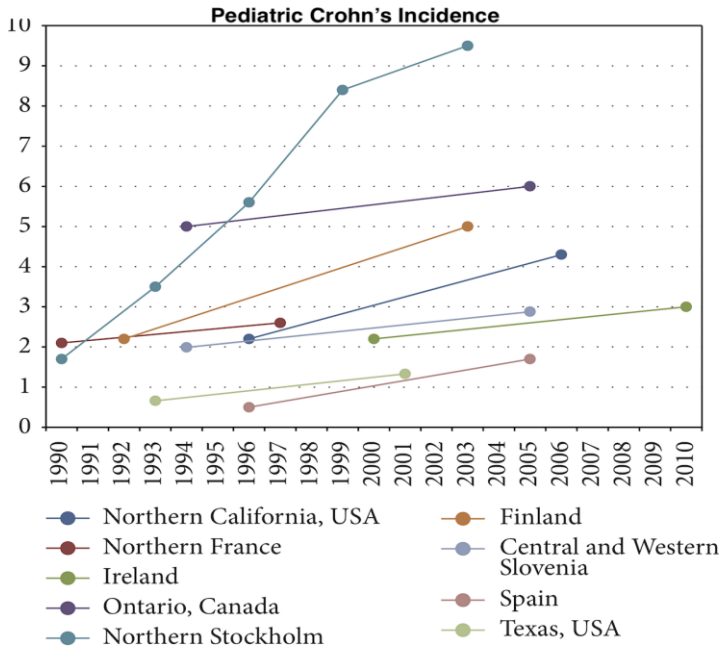

Figure 3: Incidence of IBD in children. Adapted from Muller KE et al, 2014.[2]

About 25% of the cases are diagnosed in the paediatric age and of these, 20% start before the age of 10 (early-onset IBD), 3% before the age of 6 (Very-early onset

IBD, VEO-IBD) and about 1.5% before the age of 2 (Infantile-onset IBD, IO-IBD).[323,325,328] Interestingly, it has been recently recognized that their incidence in industrialized countries has begun to plateau, whereas emerging economies continue to have an increasing incidence.[327] Despite the fact that some studies stress that, contrary to the generality of IBD, the incidence of VEO-IBD has remained relatively stable throughout the years,[328] the majority of the work indicates that incidence has increased in all age groups.

Additionally, it is interesting to note that immigrants rapidly acquire the risk profile of their new country, stressing the importance of both environment and epigenetics. On the other hand, IO-IBD has been associated with positive family history and consanguinity, supporting the concept that earlier onset of disease is strongly related with genetic inheritance.[329]

Etiopathogenesis

The precise aetiology of IBD remains unclear, but many factors have been shown to contribute. In this review, we will focus on those factors that are most relevant to VEO-IBD: the microbiome and genetic predisposition.

a) MICROBIOME

The role of the microbiome in the etiopathogenesis of IBD has been under evaluation for several years. Given the sterile nature of the intra-uterine environment, until birth, the foetus is devoid of microbial colonization. During birth, the newborn is exposed to the mother's microbiota and the process of colonization of the mucous membranes (digestive, respiratory, urogenital) with which we coexist throughout our lives begins.[330 331-334]

The infant's microbiota is very oscillative and variable, stabilizing from the age of 3. At this age, it is very much alike the adult microbiota. The host immune system develops and establishes a symbiotic relationship throughout this period under the influence of the microbiota. [332,333]

Interactions between the microbiota and the host are complex and dynamic, and are particularly important in the intestinal tract, where the greatest diversity and

abundance of microorganisms resides. (typically 10^{11}–10^{12} microbes/ml of luminal content).[330-333]

The interaction that is established between the intestinal microbiota and the human host has a mutual benefit: the microbiota establishes a stable ecosystem, benefiting from the warm, nutrient-rich environment of the gut. The host receives protection from pathogens, stimulation of angiogenesis, promotion of vitamin production and expansion of its ability to obtain nutrients from food.[330,332,333]

However, the host-microbiota relationship, namely at the intestine, is not always normal, symbiotic and mutualist. Alteration of the intestinal microbiota composition (dysbiosis), favouring the growth of certain species in detriment of others, may lead to an inability to maintain the fine immunological regulation of inflammation, leading to hyperinflammation, translated as IBD. Several studies have shown that differences in microbiome are found in patients with IBD, with a relative decrease of Firmicutes and Bacteroidetes and a relative increase of Proteobacteria and Actinobacteria. As the establishment of a stable microbiome occurs in the first three years of life, this would be an important window of vulnerability that may be related to the development of VEO-IBD.[125,332,335,336]

Multiple factors, such as travelling, smoking or diet, have been reported to be at the origin of this altered host-microbiota relationship.[125,335,337] However, these factors are of little relevance in young children. On the other hand, the increasing consumption of antibiotics at a critical time when the microbiome is being established has raised questions about their importance in the increasing incidence of VEO-IBD. Several studies have shown that the consumption of antibiotics is related to an increase in IBD in children.[125,338,339] However, two recent studies further paved the way, proving that exposure to antibiotics during pregnancy or labour, are linked to an increased incidence of VEO IBD in both human and animal models.[340,341] It is interesting to note that the association of antibiotic exposure during pregnancy or early childhood, with the consequent impact on the microbiome, has also been associated with allergic and autoimmune diseases.[342,343]

Finally, the efficacy of using non-absorbable antibiotics to induce remission in some patients confirms the influence of the microbiome in some cases of VEO-IBD.[341,344,345]

b) GENETICS, EPITELIAL BARRIER FUNCTION AND THE IMUNE SYSTEM

In 1996 a first susceptibility loci for IBD was identified in chromosome 16. It was later characterized as being related to variants in NOD2, conditioning a deregulation of the interaction between the microbiota and inflammation via NF-κB.[346-348] After the sequencing of the human genome and the generalization of NGS techniques, numerous susceptibility loci were identified for IBD using Genome-Wide Association Studies (GWAS).[349-352] While mechanisms implicating these susceptibility loci are not entirely clear, they have increased our knowledge of the different ways of controlling intestinal inflammation, namely the importance of the epithelial barrier integrity and of a finely controlled innate response (namely through the NF-κB route and the maintenance of intra-cellular homeostasis).[3,352] However, it seems clear that IBD starting in childhood has very different particularities from adult IBD, namely a greater family burden, severity, extent and refractority to treatment.[3,353,354] In addition, it is considered that the earlier the age of onset, the lower the influence of the environment and the greater the genetic load. Thus, VEO-IBD is a subgroup of IBD in which the genetic component will have a much greater importance, and it is considered that in this group the monogenic causes will have a much greater preponderance (Fig.4).

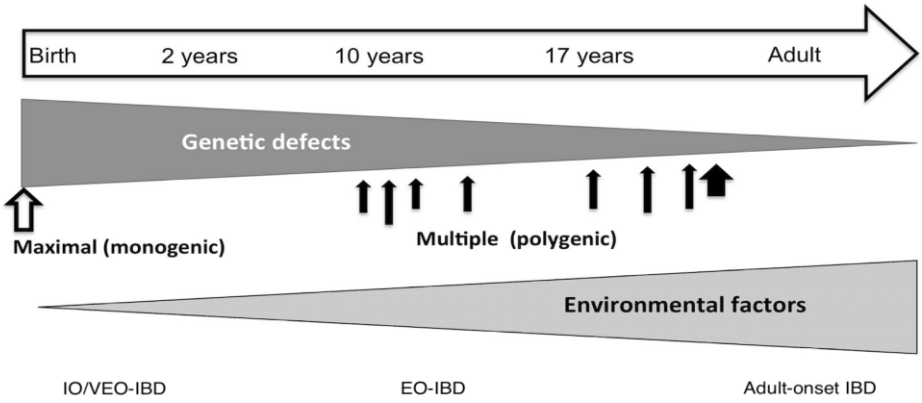

Figure 4: Importance of the different predisposing factors for the development of IBD according to age. Adapted from Okou et al, 2014.[3]

Thus, in recent years there have been numerous advances in the characterization of genes that predispose to VEO-IBD, and from more than 60 genes identified, the vast majority codifies crucial proteins of the immune system or proteins that are important for the maintenance of the intestinal barrier intactness (Fig.5).[4,125,355,356]

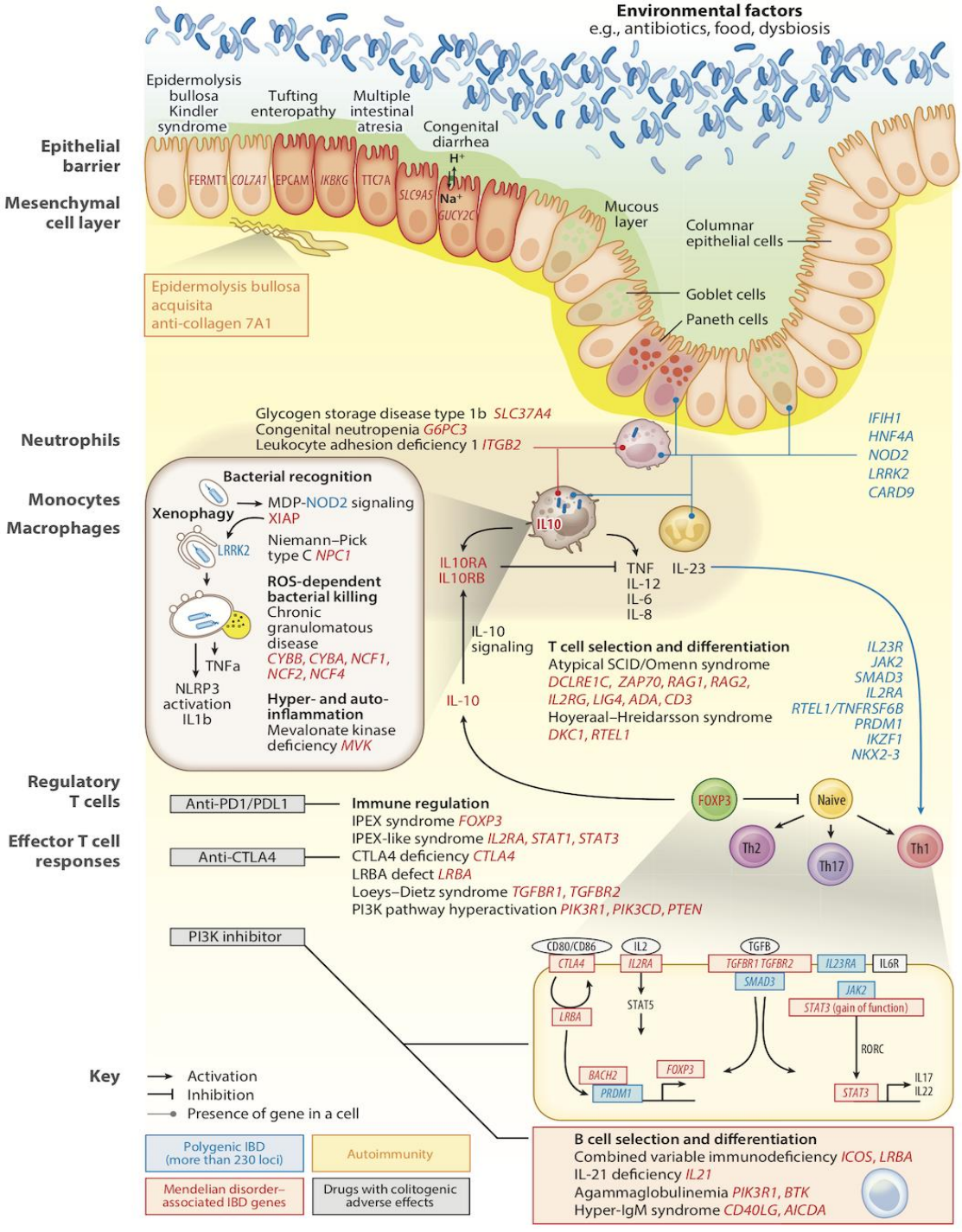

Figure 5: Schematic representation of genes and proteins involved in the genesis of VEOIBD. Adapted from Uhlig HH et al, 2018.[4]

b1) Genetic defects affecting the integrity of the intestinal barrier/epithelial response.

As mentioned previously, intestinal homeostasis depends on complex interactions between the microbiome, the intestinal epithelium and the host immune system.[357] Since only a single layer of epithelial cells serves as a barrier between the microbiota present in the intestinal lumen and the immune cells of the lamina propria, it is not surprising that defects in these layers may lead to intestinal inflammation. Thus, COL7A1 mutations (epidermolysis bullosa dystrophica) were described as being associated with VEO-IBD. [358] Interestingly, patients with autoantibodies against collagen VII (epidermolysis bullosa acquisita) also have IBD, reflecting the importance of collagen VII in maintaining the integrity of the intestinal epithelial barrier.[359]

Other examples of monogenic defects that affect the intestinal barrier function and originate VEO-IBD are Kindler syndrome, where mutations in the gene FERMT1 lead to a dysfunctional Kindlin-1 protein that is critical to an adequate barrier function of the epidermis[360] and also ADAM17 deficiency, where loss-of-function mutations in this gene (which encodes a disintegrin and metalloproteinase 17) lead to a very low rate of epithelial production.[361]

Finally, a paradigmatic case is the IkBkG defect (ectodermic dysplasia and X-linked immunodeficiency), which encodes the NF-kB essential modulator (NEMO) protein. In this well-known immunodeficiency, enterocolitis is one of the most common symptoms.[362] However, haematopoietic stem cell transplantation (HSCT) does not ameliorate IBD despite correcting the immune defect, suggesting that it is the epithelial deficiency of NEMO, and not the hematopoietic cells deficiency that leads to intestinal inflammation.[363]

b2) Congenital defects of immunity

We can divide this high number of genes associated with VEO-IBD into two large groups:

- Genes in which the VEO-IBD is not universal, oftenly not the main symptom and follows other clinical symptoms (such as CGD, G6PC3 defect, IPEX syndrome, among others). In these genes the intestinal phenotype is not entirely monogenic, depending on other genetic susceptibilities and environmental factors, as is well illustrated in CGD where only 30% of patients present IBD.
- Genes in which the VEO-IBD is the main symptom and, quite often, the only symptom of the disease (as in the IL-10 signalling defects). In such cases, the genetic defect confers complete penetration and there is a clear genotype-phenotype relationship.

This knowledge is important in the choice of the etiological investigation of VEO-IBD. In any case, monogenic defects can be grouped into the following subtypes,[355] already briefly described in chapter I.

- i) Neutropenia and phagocytic defects
- ii) Hyper and/or self-inflammation
- iii) Selection and activation defects of T and B cells
- iv) Immune dysregulation

i) Neutropenia and phagocytic function defects

Neutrophils are components of the innate intestinal barrier, responding constantly to translocation or bacterial invasion.[364] The accumulation of these cells during inflammation leads to the characteristic histology of IBD: crypt abscesses in ulcerative colitis and granulomas in Crohn's disease.[321,322] However, congenital defects that lead to granulocyte hypofunction, whether quantitative or qualitative, have been associated with IBD, VEO-IBD or even IO-IBD. It is interesting to note that diseases that present altered oxidative function (CGD),[365,366] as well as diseases with altered neutrophil migration (leukocyte adhesion deficiency),[225] and even diseases that lead to an increase in neutrophil apoptosis in the context of cellular stress (G6PC3 or Glycogenosis type IB),[367,368] are associated with VEO-IBD.

ii) Hyper and/or auto-inflammation

Given the need for fine regulation of intestinal microbiota-immunity interaction, it is not surprising that several syndromes with hyper or autoinflammation are associated with VEO-IBD. Of these, mevalonic aciduria/hyper-IgD syndrome stands out, a condition where the defect of an enzyme in the pathway of the cholesterol synthesis, mevalonate kinase (which phosphorylates mevalonate into geranylgeranyl pyrophosphate), leads to a decrease in geranylgeranylated proteins and subsequent hyper-production of IL1. There are several reports of VEO-IBD or even IO-IBD, with excellent response to IL1 blockade.[280,281,369]

X-linked lymphoproliferative syndrome (XLP) type 2, caused by XIAP deficiency, is one of the main monogenic causes of isolated, often fistulizing, VEO-IBD and is related to dysfunctional NF-kB signalling mediated by NOD2.[200,370-373]

iiia) B cell Defects

Despite having a relatively high incidence in immune disorders classically associated with humoral deficiencies, it is believed that intestinal symptoms in such patients are not due to the absence/reduction of B lymphocytes or to antibody deficits. Nowadays, it is well known that most humoral defects with IBD/VEOIBD are not due to intrinsic B defects but to multiple other immune defects.[198,356,374-377] An illustrative example of this is agammaglobulinemia, in which the occurrence of VEO-IBD is not due to a deficit in antibody production, but instead the cause of intestinal inflammation is the absence of IL10-producing B cells, affecting the regulatory T cell compartment.[378,379]

iiib) Mainly T cell defects

Cellular defects are classically associated with VEO-IBD, being the prototype condition SCID[380] and the Wiskott-Aldrich Syndrome, in which inflammatory bowel disease is relatively frequent and can be corrected with HSCT.[381] The MIA-CID syndrome (described in detail in chapter IV.I) is a complex disease in which the deficiency of the TTC7A protein causes cytoskeletal alteration and deficient cellular

adhesion and polarity. In complete defects there are multiple intestinal atresias, but in hypomorphic defects patients can present with isolated VEO-IBD.[176,177,382,383]

iv) Immune Dysregulation

Within this complex group of immune dysregulation defects, the presence of VEO-IBD is relatively common. However, we would highlight two groups of patients: patients with IPEX, where colitis is usually early and resistant to treatment[192] and IL10 signalling defects, which were the first monogenic cause of VEO-IBD to be identified. IL10 is one of the most potent cytokines with anti-inflammatory and immunosuppressive properties and is crucial for intestinal homeostasis. In both groups, the presentation is almost always of isolated IO-IBD (70% of patients with onset in the first month of life), with severe peri-anal disease, and fistulizing processes, intractable except with HSCT. Interestingly, the incidence of B cell lymphomas in these patients is surprisingly high, highlighting the role of the IL10/IL10R axis in the lymphoma genesis.[384-387]

CHAPTER II

Objectives

The general objectives of this thesis were to clarify the genetic and functional mechanisms of primary immunodeficiencies, particularly in patients with abnormal infectious susceptibility, but also in those with autoimmunity, autoinflammation or early, multiple or severe hyperinflammation.

Here are the specific objectives in more detail, as follows:

1- Clarification of PIDs of patients with atypical clinical symptoms, making it difficult to predict the affected gene.
    a. To describe in detail the immunological and clinical presentation of these patients.
    b. To identify the genetic defect using next generation sequencing techniques.
    c. To test hypotheses about the immune mechanisms that have influenced this presentation.
2- Extensive clinical and immunological study of patients suffering from PIDs with fatal and uncommon symptoms, and the elaboration of explanatory hypotheses for these cases.
3- Identification of new PIDs:
    a. To identify new genetic defects that cause primary immunodeficiency using new generation sequencing techniques.
    b. To prove the pathogenicity of identified variants and describe the immunological and clinical consequences associated in detail.

CHAPTER III

Material and methods

STUDY POPULATION

Between January 2012 and December 2017, at the Hospital Dona Estefânia, Centro Hospitalar Universitário de Lisboa Central (HDE-CHULC), we have prospectively studied all children that could be categorized in one of the following groups:

A. Clinical manifestations and/or laboratory tests that made it possible to diagnose a specific PID, but who showed severe and unusual symptoms.

B. Clinical manifestations and/or laboratory tests compatible with an immune deficiency, but whose phenotype did not provide an accurate clinical diagnosis.

Eighteen children (1 month to 18 years of age) were identified and included in this study. The laboratory techniques that have been used to achieve the objectives of the thesis shall be briefly described. In order to achieve the specific objectives of each of the subchapters of the scientific output, there will be a detailed description of the technical characteristics that were carried out in each work project.

GENERAL STUDY PROCEDURES

Detailed clinical data were collected from all patients following verbal and written informed consent, and images of exams and photos of the patients were also recorded in cases where the clinical phenotype justified it. Immunological characterization of all patients was carried out, guided by the phenotype and the suspected diagnosis. We present the methods that were applied to all patients. In each subchapter of the Scientific Outputs, we specify in each scientific article the methodologies used to achieve each specific objective of the thesis.

Flow Cytometry

For the evaluation of lymphocyte subsets, peripheral blood was analysed by Flow Cytometry in a 4-color BD FACS Calibur (BD, San Jose, California, USA), using the BD IMK kit with Trucount tubes (BD Biosciences) according to the manufacturer's

instructions. An additional panel of monoclonal antibodies (including CD45RA, CD45RO, CD62L, and HLA-DR) was also performed for further characterization of T-cell differentiation, using a lyse-wash protocol. Finally, the IOTest® Beta Mark kit (Immunotech SAS, a Beckman Coulter Company, Marseille, France) was also used for the characterization of the TCR Vβ repertoire.

Flow cytometry data analysis was performed using Multiset and CellQuestPro software (BD Biosciences).

Proliferation assays

Proliferative capacity was performed with a thymidine incorporation assay. In brief, peripheral blood mononuclear cells (PBMCs) were incubated with mitogens (PHA and PMA+ionomycin) for 3 to 4 days, and with antigens (PPD, C. albicans antigen and Tetanus toxoid) for 7 to 8 days, at 37ºC, in a 5% CO_2 atmosphere. The cells were labelled with tritiated thymidine (3H-thymidine) (Perkin Elmer, Boston, MA, USA) in the last 18h of incubation. Cells were then harvested and transferred to a Filtermat A filter (Perkin Elmer), and a Meltilex cintillation sheet (Perkin Elmer) was applied after the Filtermat was dried. The radioactivity in DNA recovered from the cells was read in a MicroBeta counter (Perkin Elmer). Results were presented as stimulation indexes, obtained from the ratios of cpm from stimulated and unstimulated cells (incubated in parallel).

Antibody response evaluation

Immunoglobulins were quantified in serum. Immunoturbidimetry was used to quantify IgG, IgA and IgM. As for specific responses, antibodies for diphtheria, tetanus, and pertussis were evaluated by Enzyme-linked immunosorbent assay (ELISA).

Whole exome sequencing and bioinformatic analysis

In group B (patients whose clinical manifestations and/or laboratory tests were compatible with an immune deficiency, but whose phenotype did not provide an accurate clinical diagnosis), Whole Exome Sequencing (WES) was performed in an

attempt to identify the gene presumably affected and responsible for the phenotype.

DNA samples were isolated from blood or PBMCs. For exome target enrichment Agilent SureSelect 50 Mb kit was used. Sequencing was done using Illumina HiSeq 2000 with 94 bp paired-end reads. FASTQ files were aligned to the hg19 reference sequence using Novoalign version 2.07.19, including hard and soft clipping, quality calibration and adapter trimming. Duplicate reads were excluded using the PICARD tool MarkDuplicates. Calling was performed using SAMtools v0.18 and single sample calling. The resulting calls were annotated with the software ANNOVAR. Candidate variants were filtered based on function: loss-of-function, non-synonymous or potential splicing altering variants (defined as being with 5 bp of the actual splice site) and frequency. Library preparation, exome capture and sequencing were done according to the manufacturers' instructions.

ETHICS

According to the alínea e) of the Article 19 from Diário da República, 2. series, N.o 153, 7th August 2015 (Regulation n.o 519/2015), this PhD research project was approved by the Ethics Committee of the Centro Hospitalar de Lisboa Central (process nº 131/2014) and by the Ethics Committee of NOVA Medical School. Moreover, the administration board of Dona Estefania Hospital approved this protocol.

Families and patients were not paid for their participation in this project. They have signed an informed and voluntary consent, ensuring anonymity and ensuring follow-up in case of non-acceptance of participation in the project. In case of detecting changes that were considered relevant to the patient's clinical condition, they were communicated to the parents, and this premise was made clear in the informed consent. As mentioned above, images of exams and photos of the patients were also recorded in cases where the clinical phenotype justified it, following verbal and written informed consent.

The inherent costs of the laboratory assessment in the context of clinical integration were supported and approved by the Hospital Administration, since they

were PIDs under study, whose clarification may have implications for the therapeutic approach of patients.

CHAPTER IV

Scientific Outputs

IV.I. ATYPICAL PRIMARY IMMUNODEFICIENCIES

NOVEL IL2RG MUTATION CAUSES LEAKY $T^{LOW}B^{+}NK^{+}$ SCID WITH NODULAR REGENERATIVE HYPERPLASIA AND NORMAL IL-15 STAT5 PHOSPHORYLATION

CLINICAL AND LABORATORY OBSERVATIONS

Novel *IL2RG* Mutation Causes Leaky $T^{LOW}B^{+}NK^{+}$ SCID With Nodular Regenerative Hyperplasia and Normal IL-15 STAT5 Phosphorylation

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Summary: X-linked severe combined immunodeficiency disease (SCID) is caused by mutations in the interleukin (IL)-2 receptor γ (*IL2RG*) gene and patients usually present with a $T^{-}B^{+}NK^{-}$ SCID phenotype. Nevertheless, a minority of these patients present with a $T^{-}B^{+}NK^{+}$ phenotype, similar to the IL-7R-deficient patients. We report a patient with a novel missense p.Glu297Gly mutation in the *IL2RG* gene presenting with a leaky $T^{low}B^{+}NK^{+}$ SCID with delayed onset, moderate susceptibility to infections, and nodular regenerative hyperplasia. He presents with preserved STAT5 tyrosine phosphorylation in response to IL-15 stimulation but not in response to IL-2 and IL-7, resulting in the NK^{+} phenotype.

Key Words: hypomorphic SCID, γc, IL2RG, STAT5 phosphorylation, nodular regenerative hyperplasia

(*J Pediatr Hematol Oncol* 2018;00:000–000)

Severe combined immunodeficiency diseases (SCIDs) are a group of genetic disorders that result in the absence or dysfunction of T and B lymphocytes. Without adequate diagnosis and treatment, patients usually succumb to infection during the first 2 years of life.[1]

Classically considered as having an incidence of 1 in 75,000 to 100,000 births, its real incidence is becoming clearer with the introduction of newborn screening and is estimated to be around 1:58,000 births.[2]

X-linked SCID is the most common form of SCID and is caused by mutations in the interleukin (IL)-2 receptor γ (*IL2RG*) gene (OMIM 208380), which encodes the common cytokine receptor γ chain (γc). This protein is a subunit of several receptors that recognize cytokines IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. It is essential for the signal transduction, by interaction with Janus Kinase 3 (JAK3) and subsequent phosphorylation and activation of the signal transducer and activator of transcription (STAT) proteins that regulate the induction of gene transcription.[3,4]

The pivotal role of signaling of each of these cytokines has been clearly showed: IL2 signaling is crucial for the maintenance of peripheral tolerance, IL-4 and IL-21 signaling defects are responsible for the intrinsic B-cell deficiency, IL-7 signaling defect causes abnormal/absent T-cell development, and abnormal signaling of IL-15 leads to the natural killer (NK) deficiency. The combination of these cytokine signaling defects explain the $T^{-}B^{+}NK^{-}$ SCID phenotype observed in the majority of patients harboring mutations in the *IL2RG* gene.[3–5]

There are few reports of leaky-X-SCID patients, presenting with a less severe phenotype, prolonged survival, and atypical manifestations (including 2 adult patients previously diagnosed as having common variable immunodeficiency) (Table SI, Supplemental Digital Content 1, http://links.lww.com/JPHO/A255).[3,6–10]

These leaky-XSCIDs can be caused by hypomorphic regulatory mutations or reversion mutations in the *IL2RG* gene.[7–10]

Interestingly, a minority of X-SCID patients present with a $T^{-}B^{+}NK^{+}$ phenotype, similar to the IL-7R-deficient patients.[11]

This is in accordance with the preserved signaling by the IL-15R, as it has been shown before that signal transduction by the IL-7R is crutial for T-cell development but is dispensable for NK cell development, whereas adequate signaling via the IL-15R is essential for NK cell but not for T-cell development.[3,4,12]

Here, we report a patient with a novel missense p.Glu297Gly mutation in the *IL2RG* gene presenting with a leaky $T^{low}B^{+}NK^{+}$ SCID with delayed onset of disease,

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J.F.N.: designed the research study and wrote the paper. C.M. and L.M.B.: performed immunologic analysis. A.I.C. and C.N.: medical doctor of the patient and designed the research study. M.F.: performed histopathologic analysis and illustrations. V.P., J.C., S.B., and S.N.: performed genetical analysis. D.M.: performed HSCT and analyzed the data. K.G.: performed the research and analyzed the data.

The authors declare no conflict of interest.

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moderate susceptibility to infections, inflammatory bowel disease, and nodular regenerative hyperplasia (NRH). He had no signs of a reversion mutation and had reduced but not absent expression of γc leading to a preserved STAT5 tyrosine phosphorylation (STAT5-pTyr) in response to IL-15 stimulation but not in response to IL-2 and IL-7 stimulations.

MATERIALS AND METHODS

Flow Cytometry

For the evaluation of lymphocyte subsets, peripheral blood was analyzed by flow cytometry in a 4-color BD FACS Calibur (BD, San Jose, CA), using the BD IMK kit with Trucount tubes (BD Biosciences) according to the manufacturer's instructions. An additional panel of monoclonal antibodies (including CD45RA, CD45RO, CD62L, and HLA-DR) was also performed for further characterization of T-cell differentiation, using a lyse-wash protocol. Finally, the IOTest Beta Mark kit (Immunotech SAS, a Beckman Coulter Company, Marseille, France) was also used for the characterization of the TCR Vβ repertoire.

For the liver biopsy, the sample was mechanically disaggregated in a Medicon chamber (BD Biosciences), recovered, and resuspended in phosphate buffered saline. The cell suspension was incubated with the BD IMK kit (BD Biosciences) in regular flow cytometry tubes, using a lyse-wash protocol.

Flow cytometry data analysis was performed using Multiset and CellQuest Pro software (BD Biosciences).

Proliferation Assays

Proliferative capacity was performed with a thymidine incorporation assay. In brief, peripheral blood mononuclear cells were incubated with mitogens: PHA (6.25 μg/mL) and PMA+ionomycin (10^{-7} M+10^{-5} M) for 3 to 4 days, and with antigens: PPD (5 μg/mL), *Candida albicans* antigen (50 μg/mL), and tetanus toxoid (230 ng/mL) for 7 to 8 days, at 37°C, in a 5% CO_2 atmosphere. The cells were labeled with tritiated thymidine (3H-thymidine) (Perkin Elmer, Boston, MA) in the last 18 hours of incubation,. Cells were then harvested and transferred to a Filtermat A filter (Perkin Elmer), and a Meltilex cintillation sheet (Perkin Elmer) was applied after the Filtermat was dried. The radioactivity in the DNA recovered from the cells was read in a MicroBeta counter (Perkin Elmer). Results were presented as stimulation indexes, obtained from the ratios of counts per minute from stimulated and unstimulated cells (incubated in parallel).

Antibody Response Evaluation

Immunoglobulins were quantified in serum. Immunonephelometry (BN II, Siemens Healthcare, Germany) was used to quantify IgG, IgA, and IgM. As for specific responses, antibodies for diphtheria, tetanus (Binding site, UK), and Pertussis (EuroImmune, Germany) were evaluated by enzyme-linked imumnosorbent assay.

Exome Sequencing and Bioinformatics Analysis

We isolated DNA samples from blood or peripheral blood mononuclear cells. Library preparation, exome capture, and sequencing have been carried out according to the manufacturers' instructions. For exome target enrichment Agilent SureSelect 50 Mb kit was used. Sequencing was done using Illumina HiSeq. 2000 with 94 bp paired-end reads. FASTQ files were aligned to the hg19 reference sequence using Novoalign version 2.07.19, including hard and soft clipping, quality calibration, and adapter trimming. A total of 82.8 million reads were generated and analyzed. Duplicate reads were excluded using the PICARD tool MarkDuplicates. Calling was performed using SAMtools version 0.18 and single sample calling. The resulting calls were annotated with the software ANNOVAR. Candidate variants were filtered based on function: loss-of-function, nonsynonymous, or potential splicing altering variants (defined as being with 5 bp of the actual splice site) and frequency.

Histopathologic Examination

A wedge liver biopsy was performed, 2 cm in length, stained using hematoxylin eosin, Masson trichrome, Red sirius, Perls, reticulin, and CK7 immunohistochemistry.

IL2RG Expression and STAT5 Phosphorylation Assay in Patient Blood

The 3 μL of anti–human IL2RG (BD Biosciences) or matched isotype control (BD Biosciences) was added to 100 μL of blood, incubated for 10 minutes at room temperature. Red blood cells were lysed with FACs Lsye (BD Biosciences) for 10 minutes and then washed with Cell Wash (BD Biosciences) before being fixed (FACsFix, BD Bioscience). In total, 10,000 lymphocytes were acquired (FACs Calibur, BD Biosciences) and analyzed (CellQuest Pro, BD Biosciences) For STAT5 phosphorylation assay, cells were left unstimulated or 1×10^4 IU/mL IL-2 (Chiron) or 50 ng/mL IL-7 or IL-15 (R&D systems) were added to 100 μL of whole blood and placed at 37°C for 10 minutes. Two milliliters of prewarmed FACs Lyse/Fix (BD Biosciences) were then added to the blood, mixed, and placed at 37°C for a 10 minutes. The cells were pelletted and washed once with (Invitrogen) containing 1% fetal calf serum (STAT wash). The cells were resupended in cold Perm Buffer III (BD Biosciences) and placed at 4°C for 30 minutes. The cells were then washed once with STAT wash before 5 μL of antibodies (STAT5-ptyr and CD4 PerCP [BD Biosciences]) were added, and the cells were incubated at room temperature for 30 minutes in the dark, washed with STAT wash, and fixed (FACsFix, BD Biosciences). In total, 10,000 lymphocyte events were acquired (FACs Calibur, BD Biosciences) and analyzed using CellQuest Pro software (BD Biosciences).

RESULTS—CASE REPORT

A 3-year-old boy was admitted for pneumococcal pneumonia with empyema, requiring intensive care. His parents were not consanguineous and he had 2 maternal uncles that died due to overwhelming infection before the age of 12 months. He also had chronic diarrhea since the age of 2 years. His immunologic workup revealed moderate $CD3^+$ lymphopenia (538 cells/μL), very reduced naive $CD4^+$ counts but normal numbers of B and NK cells. The Vbeta repertoire was diverse and polyclonal and there was no maternal engraftment. His proliferative responses were maintained after stimulation with mitogens (anti-CD3, PHA, and PMA), but responses to recall antigens (PPD, candida, and TT) were severely depressed. He had normal IgG, borderline IgA, and borderline-low IgM. He mounted a moderate response to diphteria, but not to tetanus, 4 weeks after vaccination with DTPa (diphteria, tetanus, acellular pertussis), but 3 months after vaccination the titere became nonprotective (Table 1).

He was diagnosed with $T^{low}B^+NK^+$ leaky-SCID but his parents refused hematopoietic stem-cell transplantation (HSCT). Subcutaneous immunoglobulin replacement, as well as cotrimoxazole prophylaxis were promptly initiated. From the age of 3 to 8 years, he had *Cryptosporidium parvum* infection for which he

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TABLE 1. Immunologic Assessment of the Patient

T-Cell Function	3 y Old	4 y Old	5 y Old	6 y Old	7 y Old	9 y Old
Absolute lymphocyte count ($\times 10^9$/L)	1120	1043	1175	1270	1400	375
$CD3^+$ (cells/μL) (n [%])	538 (44)	684 (54)	641 (54)	720 (55)	869 (66)	287 (79)
$CD4^+$ (cells/μL) (n [%])	257 (21)	313 (24)	262 (23)	279 (21)	296 (23)	139 (39)
$CD4^+CD45RA^+$ (%)	30	28	20	19	26	9
$CD8^+$ (cells/μL) (n [%])	171 (14)	230 (1)	230 (20)	264 (20)	360 (27)	105 (30)
$CD8^+CD45RA^+$ (%)	68	70	50	58	59	43
$CD16^+/56^+$ (cells/μL) (n [%])	171 (14)	147 (12)	334 (27)	337 (26)	318 (24)	48 (13)
Proliferative responses to PHA (CPM $\times 10^3$) (stimulation Index; normal > 50)	25,621 ± 1762 (133)	12,083 ± 2982 (182)	ND	ND	17.366 ± 1.045 (10.7)	4.195 ± 1.138 (3.8)
Proliferative responses to PMA+I (CPM $\times 10^3$) (stimulation Index, normal > 50)	21,665 ± 2320 (75)	35,372 ± 3071 (531)	ND	ND	6.677 ± 0.864 (12.9)	0.773 ± 0.432 (1.9)
Proliferative responses to anti-CD3 (CPM $\times 10^3$) (stimulation Index, normal > 50)	ND	9743 ± 610 (146)	ND	ND	ND	1.235 ± 1.233 (2.2)
Proliferative responses to PPD+I (CPM $\times 10^3$) (stimulation Index, normal > 10)	177 ± 40 (1)	599 ± 340 (7)	323 ± 220 (3.5)	ND	0.325 ± 0.048 (0.6)	2.716 ± 0.828 (1.7)
Proliferative responses to *Candida albicans* (CPM $\times 10^3$) (stimulation Index, normal > 10)	149 ± 26 (0.8)	98 ± 28 (1.2)	ND	ND	0.249 ± 0.088 (0.4)	1.368 ± 0.505 (0.9)
Proliferative responses to TT (CPM $\times 10^3$) (stimulation Index, normal > 10)	ND	0.324 ± 0.209 (0.6)	ND	ND	0.272 ± 0.109 (0.5)	2.713 ± 1.056 (1.7)

B-Cell Function	3 y Old	3.5 y Old	4 y Old	5 y Old	6 y Old	7 y Old	9 y Old
$CD19^+$ (cells/μL) (n [%])	403 (33)	408 (32)	212 (16)	200 (16)	212 (16)	108 (8)	17 (5)
$CD19^+CD27^-IgD^+IgM^+$ (%)	ND	ND	95	ND	ND	ND	89
$CD19^+CD27^+$ (%)	2	ND	3	ND	ND	ND	10
$CD19^+CD27^+IgD^-$ (switch) (%)	1	ND	1	ND	ND	ND	2.5
$CD19^+CD38^+$IgM high (transitional)	ND	ND	16	ND	ND	ND	22
IgG (g/L)	10.2	12.2	12.4	10.6	11.8	12.6	11.5
IgM (g/L)	0.46	0.70	ND	0.6	0.4	0.3	0.3
IgA (g/L)	0.92	1.0	ND	1.1	0.9	1.2	1.2
Antidiphteria	0.27	1.25	0.05	ND	ND	ND	ND
Antitetanus	0.12	0.05	0.05	ND	ND	ND	ND

†Diphteria, tetanus, acellular pertussis vaccination.
†Subcutaneous immunoglobulin substitution.
CPM indicates counts per minute; ND, not determined.

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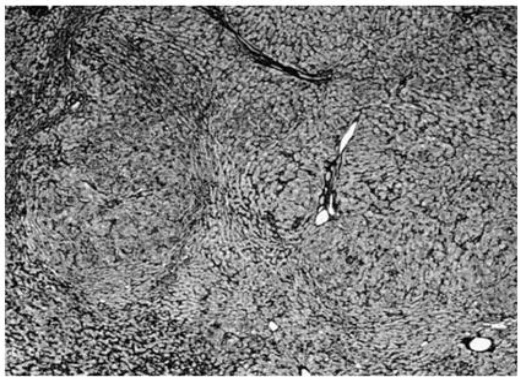

FIGURE 1. Hepatic histology reticulin silver impregnation highlighting subtle distortion of liver architecture with diffuse nodules of hyperplastic hepatocytes and regions of internodular hepatocyte atrophy, at low power (original magnification, ×25).

received paromomycin and nitazoxanide; Epstein-Barr Virus viremia complicated by severe colitis and toxic megacolon; mild varicela and 2 episodes of zoster; at the age of 8, he had *Microsporum canis* tinea corporis for which he received a 3-week regimen of itraconazole and then received prophylatic itraconazole. At the age of 9 years, he presented splenomegaly which led to the diagnosis of severe portal hypertension caused by NRH. The liver biopsy revealed that the normal architecture was mildly distorted by widespread atrophy and numerous monoacinar regenerative nodules without fibrosis (Fig. 1). There was a lymphocytic infiltrate, composed of 60% of $CD3^+$, of which 25% $CD4^+$, 50% $CD8^+$, and 25% $CD4CD8^-$, and 40% of NK cells. No pathogens were identified.

Simultaneously, he presented with intestinal bleeding and the biopsies were suggestive of inflammatory bowel disease. He had positive norovirus PCR in the stools and colonic biopsies. He received oral IvIG, as well as low-dose steroids and infliximab. He has recently received HSCT from a matched unrelated donor following a reduced-intensity conditioning protocol.

No mutation was found in the targeted analysis of the *IL7Rα* and *RAG1/2* genes of the patient. However, whole exome sequencing identified a novel hemyzigous missense p.Glu297Gly mutation in the *IL2RG* gene (ENST00000374202: exon 7: c.890 A > G) (Fig. 2). Sanger sequencing confirmed the finding and

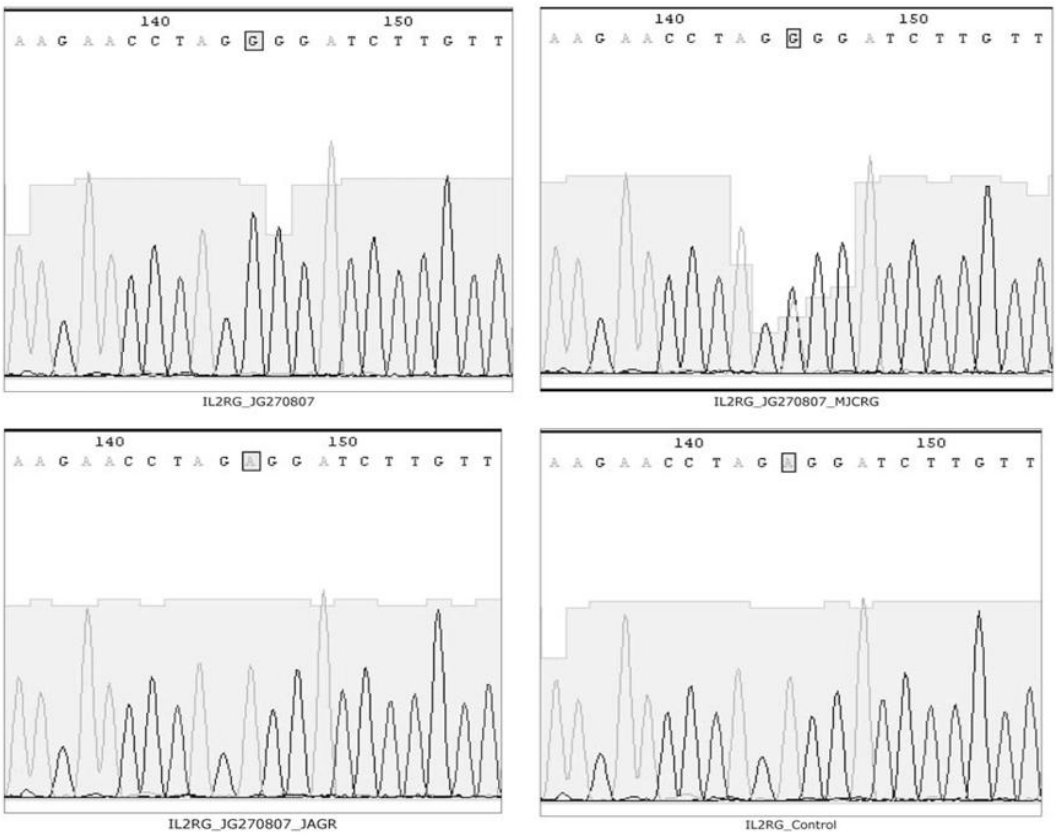

FIGURE 2. p.Glu297Gly mutation in the *IL2RG* gene.

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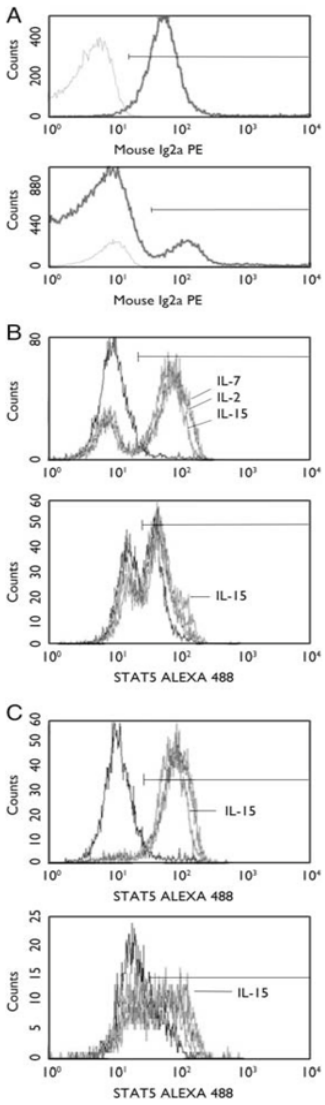

FIGURE 3. A, Reduced γc expression, shown by control (top) patient (bottom) gray is isotype control and black is gamma chain. Control has 96% expression and patient has 15% expression. B, Stat5 tyrosine phosphorylation analyzed on total lymphocytes (control top panel and patient bottom panel). Lymphocytes were defined by FSC versus SSC. Cells were unstimulated (black line) or stimulated with IL-2 (medium gray), IL-7 (dark gray), or IL-15 (lightest gray). C, Stat5 tyrosine phosphorylation analyzed on $CD4^+$ lymphocytes (control top panel and patient bottom panel). Lymphocytes were gated by FSC versus SSC followed by CD4 versus SSC to define $CD4^+$ lymphocytes. Cells were unstimulated (black line) or stimulated with IL-2 (medium gray), IL-7 (dark gray), or IL-15 (light gray). FSC indicates forward scatter; SSC, side scatter.

showed that his mother was heterozygous for the same mutation, whereas his father had a wild-type allele. The p.Glu297Gly mutation was never detected previously, for example, it is absent from single nucleotide polymorphism database and > 120,000 subjects in the gnomAD database. Bioinformatics analysis predicted that the mutation affected a highly conserved amino-acid residue. The p. Glu297Gly mutation was predicted to be probably pathogenic (SIFT—deleterious; PolyPhen2—possibly damaging; Mutation Taster—neutral). His mother was heterozygous for the same mutation.

He did not have signs of reversion mutation as assessed by next-generation sequencing in DNA isolated from whole blood. FACS analysis showed significantly reduced γc expression on patient's cells (Fig. 3A). To understand his clinical and immunologic phenotype, we studied STAT5 phosphorylation in lymphocytes after stimulation with various cytokines.

We found reduced STAT5-pTyr in the patient after stimulation with IL-2 and IL-7. However, he had very high basal level of STAT5-pTyr and a largely preserved level of STAT5-pTyr after IL-15 stimulation (Figs. 3B, C).

DISCUSSION

The hypomorphic presentation of the disease, as well as the parental refusal of HSCT at the moment of the diagnosis, allowed longer than usual observation of the natural course of this disease caused by a novel mutation in the *IL2RG* gene. The patient progressively had more severe and frequent infections that were mostly caused by viruses. In parallel with this, his immune function worsened over time, with progessive decline of the number of T cells, as well as loss of the proliferative responses to mitogens which were preserved in the first years after the diagnosis.

To our knowledge, this is the first X-SCID patient that presented NRH, a rare liver condition characterized by the presence of widespread small regenerative nodules in the absence of fibrosis. It is thought to result from intrahepatic vasculopathy leading to alterations in microvascular perfusion, hepatocyte injury, and regeneration which contribute to the formation of nodules that compress the portal and central veins and thus potentially causing portal hypertension.[13] NRH has been well described in common variable immunodeficiency patients and is thought to result from the invasion of the hepatic parenchyma by $CD8^+$ cells, leading to hepatocyte loss, followed by regeneration which causes vascular abnormalities and portal hypertension.[14] It is plausible that in our patient the imune dysregulation caused by T-cell dysfunction led to a similar mechanism, specially given the lymphocytic infiltrate ($CD8^+$ and $CD56^+$) that was detected in the patient.

The p.Glu297Gly mutation may have altered stability of γc leading to its reduced level in the patient's cells (Fig. 3A). This defect should ultimately lead to suboptimal signaling by IL-7R pathway and aberrant T-cell development, which is in agreement with the naive $CD4^+$ lymphopenia, as well as the progressive loss of $CD3^+$ cells and proliferative responses to mitogens, observed in the patient.

However, the residual γc expression on patient's lymphocytes probably allows normal signaling via IL-15R, as suggested by the normal NK cell count and largely preserved STAT5 phosphorylation after stimulation with IL-15. As shown previously by Smyth and colleagues, in Epstein-Barr Virus-transformed B-cell lines derived from X-SCID patients, the amount of γc required for the correct signaling of various signaling pathways is different. Interestingly, as γc expression becomes limiting, the IL-15R signal transduction is preferentially preserved relatively to

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the IL7R, in terms of STAT5 phosphorylation, thus allowing NK cell development but not T-cell development.[11,15] Our findings corroborate this mechanism.

In summary, our report adds NRH to the clinical manifestations of atypical X-SCID and shows that a novel hypomorphic IL2RG p.Glu297Gly (and probably other hypomorphic mutations) can lead to a progressive loss of T-cell function but preserved NK cells, resulting in a $T^{low}B^{+}NK^{+}$ leaky-SCID.

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HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS IN 2 PATIENTS WITH UNDERLYING IFNγ RECEPTOR DEFICIENCY

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Hemophagocytic lymphohistiocytosis in 2 patients with underlying IFN-γ receptor deficiency

To the Editor:

Hemophagocytic lymphohistiocytosis (HLH) is a severe hyperinflammatory disorder, caused by mutations in genes required for lymphocyte cytotoxicity or may be secondary to infections, malignancy, and autoimmunity.[1,2] Animal models have indicated a central role for IFN-γ produced by $CD8^+$ T cells in the pathophysiology of HLH,[3] and a possible clinical efficacy of anti–IFN-γ antibody therapy for the control of HLH.[4]

Here, we detail 2 unrelated cases of fatal IFN-γ receptor (IFN-γR) deficiency with mycobacterial infections who were initially diagnosed with HLH. Patient A, a 2-month-old girl, and patient B, a 4-year-old girl (see Case Reports and Fig E1, *A* and *B*, in this article's Online Repository at www.jacionline.org), fulfilled, respectively, 7 of 8 and 5 of 6 HLH diagnostic criteria[1] (see Table E1 in this article's Online Repository at www.jacionline.org).

For information on methods, see this article's Online Repository at www.jacionline.org.

In patient A, cytotoxic lymphocyte function was evaluated twice with the consistent finding of pathologically low natural killer (NK)-cell cytotoxic function, even after IL-2 stimulation (see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org). This reflected low NK cell numbers (Fig 1, *A*), possibly caused by antithymocyte globulin treatment. NK-cell cytotoxic activity was not evaluated in patient B because of the very low frequency of peripheral blood NK cells, already present at admission (Fig 1, *B*). In both patients, additional characterization of NK-cell and T-cell function did not reveal defects consistent with primary HLH caused by an impairment in lymphocyte cytotoxicity (Fig 1, *C-F*; also see Fig E2, *C* and *D*). Accordingly, no mutations were found in known HLH-associated genes.

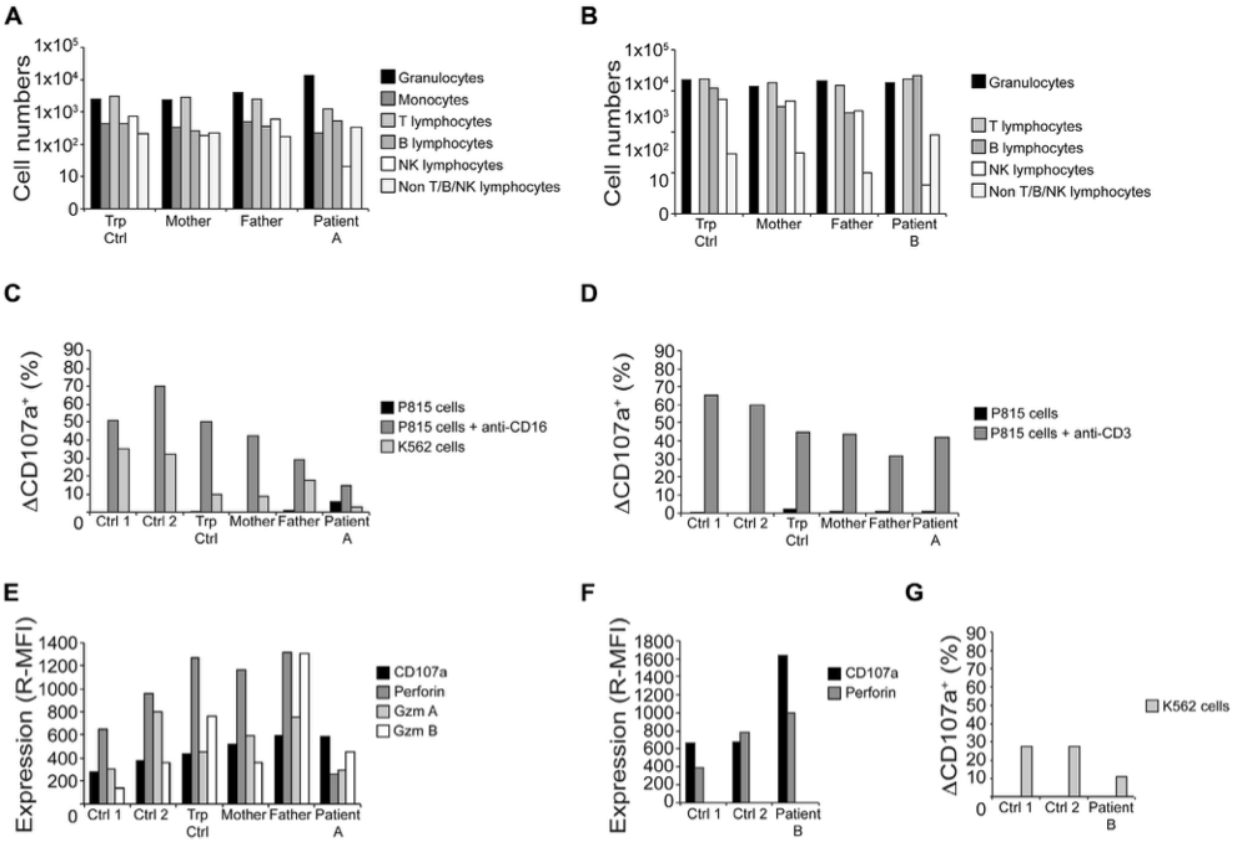

FIG 1. Immunologic analyses. Both patients (**A** and **B**) displayed a low number of NK cells. Neutrophil counts in patient B were lower at initial diagnosis of HLH compared with this later measurement. Flow-cytometric analyses of patient A showed abnormal NK-cell degranulation toward K562 **(C)**, normal T-cell degranulation **(D)**, and normal granule content **(E)**. Granule content **(F)** and NK-cell degranulation toward K562 **(G)** were normal in patient B. *Ctrl*, Control; *R-MFI*, relative median fluorescence intensity; *Trp*, transport.

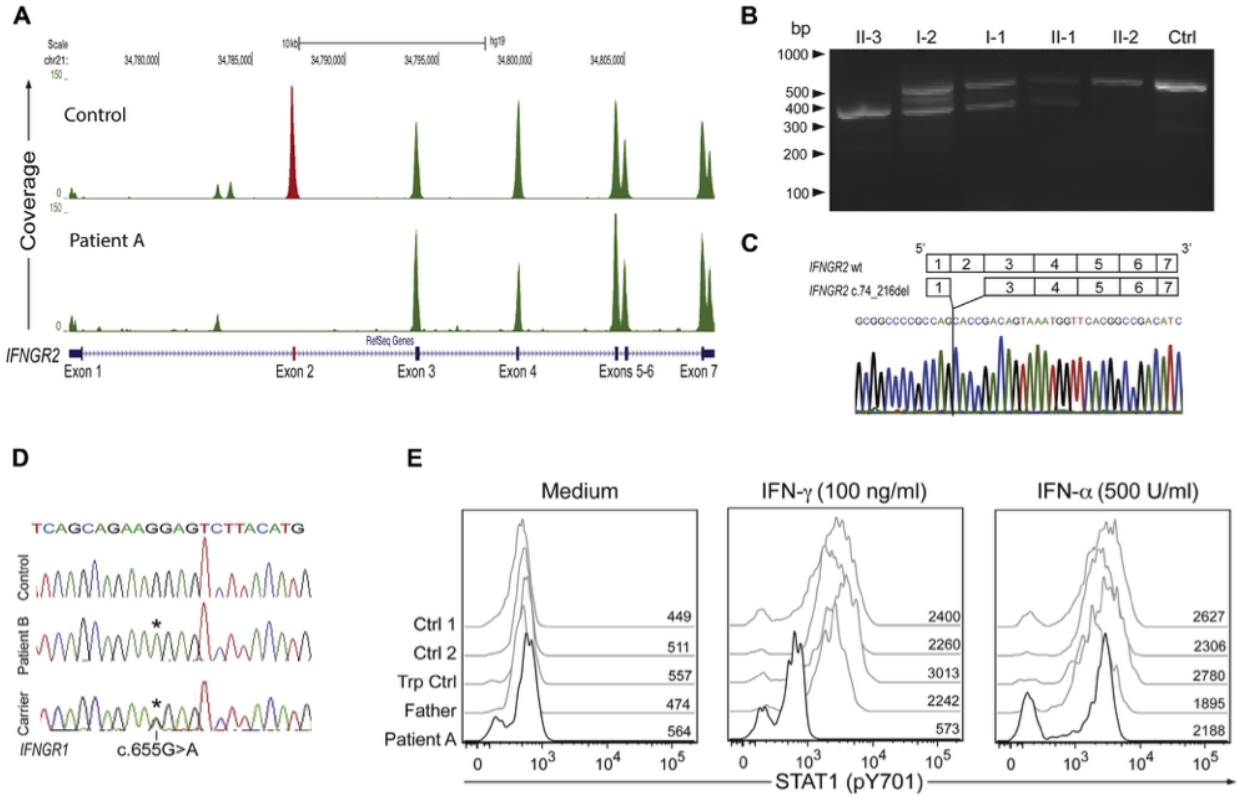

FIG 2. Coverage analysis in patient A suggests an exonic deletion of exon 2 of *IFNGR2* **(A)**, validated by RT-PCR of *IFNGR2* **(B)** and cDNA sequencing **(C)**, which showed lack of exon 2. **D,** Sanger electropherograms of G219R *IFNGR1* missense mutation in patient B. **E,** STAT1 phosphorylation on IFN-γ stimulation is defective in patient A, but not in the controls. *Ctrl,* Control; *STAT1,* signal transducer and activator of transcription 1; *Trp,* transport.

After the diagnosis of HLH, the patients were found positive for cytomegalovirus and *Mycobacterium bovis* infections, and for EBV and *Mycobacterium tuberculosis* (MTB) infections, respectively. Moreover, in patient B, plasma IFN-γ levels were constitutively elevated, without increasing further on stimulation with mitogen or MTB antigen (see Fig E3, *A* and *B* in this article's Online Repository at www.jacionline.org). In light of a disseminated mycobacterial infection, a differential diagnosis of Mendelian susceptibility to mycobacterial disease (MSMD) was considered, although fulminant HLH in the context of defective IFN-γ signaling was deemed unlikely.

In patient A, Sanger sequencing of *IFNGR1* and *IFNGR2* was followed by whole exome sequencing. Variant calling did not identify mutations able to explain the phenotype. Only a homozygous missense variant in *FANCA* (p.H1417D), described in patients with Fanconi anemia, was found. Instead, analysis of copy number variation revealed a possible homozygous deletion of exon 2 of *IFNGR2* (Fig 2, *A*). Of note, exon 2 was not amplified by PCR. On evaluation of *IFNGR2* transcripts, the patient expressed only a truncated *IFNGR2* transcript lacking exon 2 (c.74_216del, p.Asp25Alafs*38; Fig 2, *B* and *C*). In patient B, sequencing of *IFNGR1* and *IFNGR2* revealed a homozygous *IFNGR1* c.655G>A (p.G219R) missense variant, affecting a conserved residue in the second domain of the extracellular region of IFN-γR1. The parents and the healthy sister were heterozygous carriers (Fig 2, *D*). The G219R variant was predicted damaging and was not found in the 1000 Genomes database and in 150 healthy Chinese individuals. An in-frame deletion affecting residue 218 of IFN-γR1 was described in a patient with MSMD carrying compound heterozygous *IFNGR1* mutations (the other mutation being p.V61Q), resulting in normal IFN-γR1 surface expression but severely impaired IFN-γ binding.[5] The G219R mutation may have a similar effect. With mycobacterial infections and mutations in *IFNGR1* or *IFNGR2*, the patients fulfilled a diagnosis of MSMD in addition to HLH. A molecular defect in either chain of the IFN-γR heterodimer impairs signaling and causes increased susceptibility to severe *Mycobacterium* species infections.[6] After IFN-γ stimulation, no signal transducer and activator of transcription 1 phosphorylation was observed by flow cytometry in monocytes from patient A (Fig 2, *E*), establishing a diagnosis of complete IFN-γR deficiency. In patient B, it is not clear whether the IFN-γR1 G219R mutation causes complete or partial IFN-γR1 deficiency because such data were not available. Nonetheless, the high levels of IFN-γ found in patient B may suggest a complete deficiency. Both the identified genetic aberrations were novel. Importantly, this represents the first report of HLH in patients with genetic aberrations impairing IFN-γ signaling. Because IFN-γ is considered a cardinal cytokine in the development of HLH, a link between HLH and defects in IFN-γ signaling is surprising.

Mouse models indicate a crucial role of IFN-γ for the development of primary HLH.[3] Fatal HLH-like disease in *Prf1* knockout mice infected with lymphocytic choriomeningitis virus can be treated by antibody-mediated IFN-γ neutralization.[3] Intriguingly, antibody-mediated TNF neutralization ameliorated organ damage in *Prf1* knockout mice challenged with murine cytomegalovirus.[7] Moreover, in wild-type mice, high levels of IL-4 or repeated Toll-like receptor 9 (TLR9) stimulation can induce the development of an HLH-like or macrophage activation syndrome–like disease.[8,9] On TLR9 stimulation, IFN-γ–knockout mice developed macrophage activation syndrome–like disease with a severity comparable to that of wild-type mice, suggesting that immune pathology may arise independent of IFN-γ.[9] This model may provide an explanation for HLH in our patients. DNA from *M tuberculosis* is a potent stimulus for TLR9-dependent responses.[10] We speculate that genetic susceptibility to severe and sustained mycobacterial infection may predispose to strong TLR stimulation, resulting in HLH. The low NK cell numbers in our patient may have also enhanced the risk of developing HLH.

Interestingly, hemophagocytosis was not found in both cases. Hemophagocytosis is often viewed as a late symptom and is not required for a diagnosis of HLH.[1] In mice, infusion of IFN-γ can induce hemophagocytosis by macrophages.[11] Notably, such macrophage-mediated macropinocytosis depends on IFN-γR signaling in macrophages.[11] Therefore, our patients may further support a role for IFN-γR signaling in driving hemophagocytosis, but not other pathologic features of the HLH syndrome.

Based on experiments in animal models, a clinical efficacy of anti–IFN-γ antibody treatment in patients with primary HLH has been proposed.[3,4] Clinical trials are ongoing, notably with active infections, including mycobacterial, representing exclusion criteria. Our findings suggest that other cytokines, for example, TNF, may be important drivers of immune pathogenesis. In patients infected by multiple pathogens, blocking IFN-γ may accommodate other infections, leading to TLR stimulation and HLH. Therefore, at least in settings of HLH associated with primary defects in immune function other than lymphocyte cytotoxicity, immune suppression besides anti–IFN-γ antibody should be considered. It is noteworthy that antithymocyte globulin controlled HLH in both patients.

In conclusion, we report 2 cases of HLH in children with novel IFN-γR mutations. Importantly, our results highlight the significance of IFN-γ–independent mechanisms in the immune pathology of HLH, provide new views on pathogenic mechanisms of human hyperinflammatory syndromes, and expand the spectrum of genetic conditions conferring susceptibility to HLH. Clinically, because different mechanisms seem to be involved in HLH pathogenesis, novel therapies, beside anti–IFN-γ therapy, should be investigated.

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CASE REPORTS

Patient A, a 2-month-old girl, born to consanguineous Portuguese parents (Fig E1, *A*), was initially admitted for impetiginized atopic eczema treated with flucloxacilin, which triggered an episode resembling a drug reaction with eosinophilia and systemic symptoms syndrome. Subsequently, she developed worsening fever, hepatosplenomegaly with cholestatic hepatitis, hyperferritinemia, hypofibrinogenemia, as well as thrombocytopenia, anemia, and elevated sCD25 level (Table E1). Hemophagocytosis was not evident in bone marrow, liver, or skin. The patient fulfilled 7 of 8 HLH diagnostic criteria,[E1] establishing a diagnosis of HLH (Table E1). HLH treatment was initiated with dexamethasone, antithymocyte globulin, and cyclosporine, resulting in partial control of disease. Subsequently, the patient was found positive for cytomegalovirus (>10,000,000 viral copies/mL) and for *Mycobacterium bovis* in bone marrow and hepatic culture. Despite treatment for the infections, the clinical conditions worsened and the patient developed acute respiratory distress syndrome with fatal outcome at the age of 4.5 months.

Patient B, a 4-year-old girl, was born in Italy to apparently nonconsanguineous Chinese parents (Fig E1, *B*). The patient was admitted for persistent fever, leucocytosis, and a mediastinal mass. She subsequently developed hepatosplenomegaly, anemia, neutropenia, thrombocytopenia, hypertriglyceridemia, and hyperferritinemia. Bone marrow aspirate, performed twice, did not show hemophagocytosis. However, the patient fulfilled 5 of 6 HLH diagnostic criteria examined (Table E1). The patient was positive for EBV infection (180,000 viral copies/mL), while tuberculin skin and Quantiferon-TB test results were, respectively, negative and indeterminate. Interestingly, plasma IFN-γ levels were constitutively high, without further increase in response to mitogen or TB-antigen stimulation (Fig E3, *A* and *B*). Dexamethasone, anti-CD20, and cyclophosphamide and gancyclovir were administrated, leading to partial remission of HLH and infection control. Two weeks later, the patient reactivated (with ferritin up to 395,644 ng/mL) and developed life-threatening conditions. Rabbit antithymocyte globulin was administered with sudden improvement, albeit persistent fever and the onset of ocular and neurologic symptoms. At this point, disseminated infection by *M tuberculosis* was diagnosed by PCR and cultures of blood, cerebral spinal fluid, and eye swab. A 5-drug antitubercular therapy was started with transient control followed by death from multiorgan failure.

METHODS

The studies were approved by the Regional Ethical Review Board in Stockholm, Sweden, and by the Regional Committee in Florence, Italy.

Genetic analyses

Genomic DNA was isolated from peripheral blood according to a standard procedure. Informed consent was obtained from the patients and their relatives. Genes responsible for familial HLH, *IFNGR1*, and *IFNGR2* were analyzed by direct sequencing. For patient A, whole exome sequencing was performed on a HiSeq2000 Illumina machine. Agilent SureSelect v5+UTRs kit was used for target enrichment. The sequencing reads were aligned to the human genome build37 (*hg19*) using BWA/0.7.4.[E2] Unified Genotyper GATK (v.2.5)[E3] and Annovar software (version 2013Jun21)[E4] were used for variant calling and annotation, respectively. Copy number variations were analyzed with the R package ExomeDepth.[E5] RNA was isolated from blood or fibroblasts of patient A and her family members and used for cDNA synthesis and evaluation of *IFNGR2* transcripts according to standard procedures. Primers are available on request. One hundred and fifty healthy controls of Chinese ethnicity were analyzed for the missense variant in *IFNGR1* identified in patient B. Primers are available on request.

Immunologic analyses

Absolute lymphocyte counts were performed according to standard procedures (Trucount, BD Biosciences, San Jose, Calif). PBMCs were isolated by density gradient centrifugation (Lymphoprep, Axis-Shield, Dundee, United Kingdom) and maintained in complete medium (RPMI-1640 supplemented with 2 mM L-glutamine and 10% FBS; all Hyclone, South Logan, Utah). Intracellular expression of perforin was evaluated by using flow cytometry in both patients as previously described.[E6] NK-cell and cytotoxic T lymphocytes degranulation was assessed by using flow cytometry as previously described.[E7] NK-cell cytotoxicity against K562 target cells was evaluated in patient A with a standard 4-hour ^{51}Cr-release assay using PBMCs, as previously described.[E8] To evaluate signal transducer and activator of transcription 1 signaling, PBMCs from patient A were thawed and stained, after overnight incubation, with fixable dead cell stain (Invitrogen, Carlsbad, Calif). PBMCs (1×10^6) were resuspended and either left untreated or stimulated with 500 U/mL IFN-α (PBL Interferon Source, Logan, Utah) or 100 ng/mL IFN-γ (Peprotech, Rocky Hill, NJ) for 30 minutes. The cells were fixed, washed in PBS, and permeabilized for 30 minutes at −20°C (Perm Buffer III, BD Biosciences). The cells were thereafter stained with anti-CD14 and anti–signal transducer and activator of transcription 1 pY701 antibodies (both BD Biosciences). The cells were washed and analyzed by using flow cytometry (LSR Fortessa, BD Biosciences). Dead cells were excluded and monocytes identified by forward scatter and side scatter characteristics and CD14 staining. All analyses were performed using FlowJo software (v9.7.5; Tree Star, Ashland, Ore). IFN-γ release assay was performed using the Quantiferon-TB Gold test (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, whole blood was incubated for 16 hours at 37°C with mitogen (positive control), TB antigen, or nothing (negative control), respectively. Plasma IFN-γ levels were then quantified using an ELISA plate.

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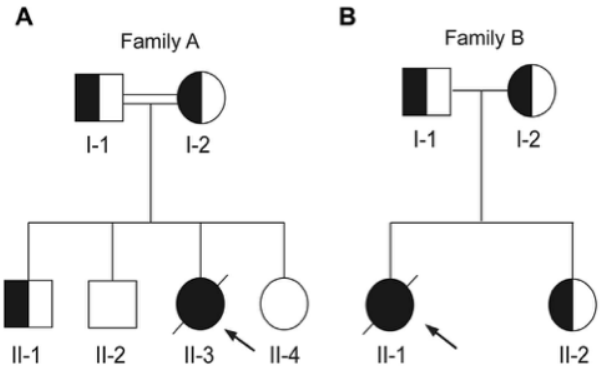

FIG E1. Family pedigree of patient A (II-3) **(A)** and patient B (II-1) **(B)**.

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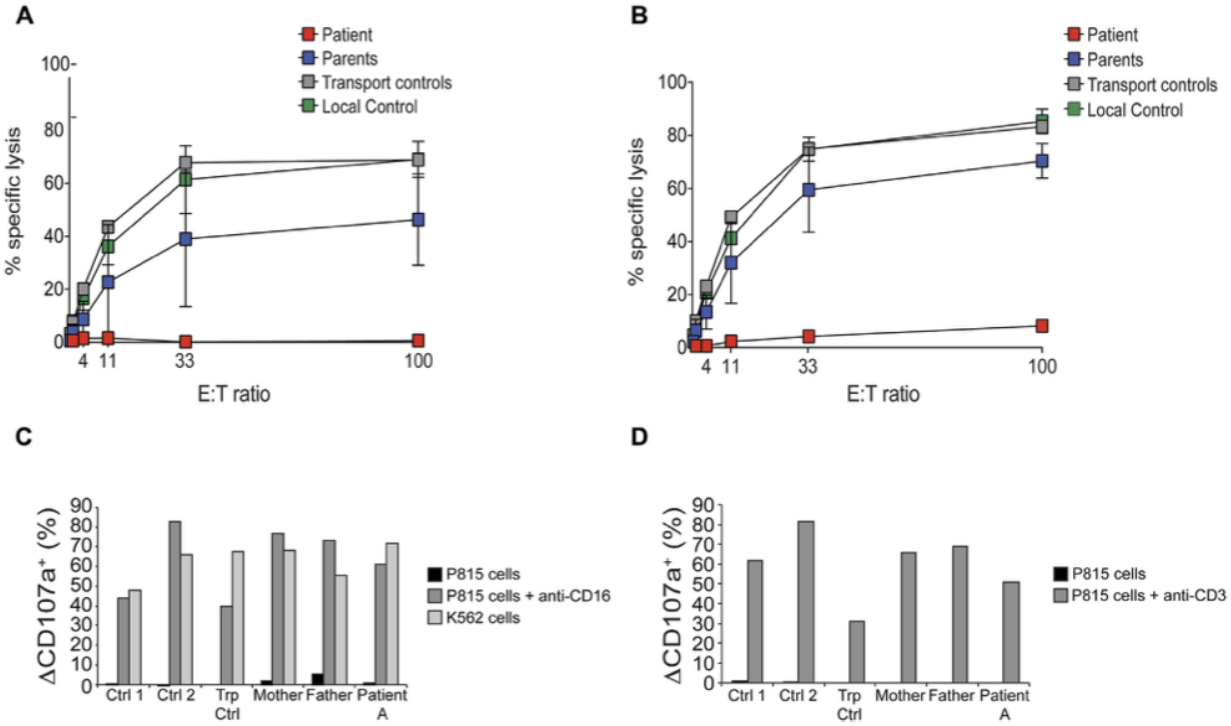

FIG E2. NK-cell cytotoxic activity measured in patient A in resting NK cells **(A)** and after stimulation with IL-2 **(B)**. Flow-cytometry analyses of NK-cell **(C)** and T-cell degranulation **(D)** after stimulation with IL-2 in PBMCs from patient A. *Ctrl*, Control; *Trp*, transport.

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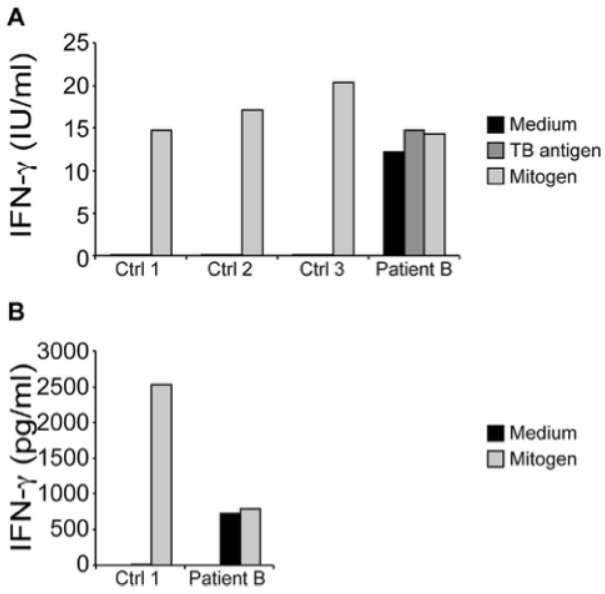

FIG E3. IFN-γ release by patient B and control PBMCs. IFN-γ release was evaluated with 2 different assays that quantified IFN-γ by active units **(A)** or concentration **(B)**. *Ctrl*, Control.

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TABLE E1. Clinical and laboratory findings of the patients at disease onset

Finding	A	B
Ethnical origin	Portuguese	Chinese
Familial disease	No	No
Parental consanguinity	Yes	No
Sex	Female	Female
Age at diagnosis of HLH	2 mo	4 y
Fever	Yes	Yes
Splenomegaly	Yes	Yes
Hepatomegaly	Yes	Yes
Hb (g/L)	67	77
Neutrophils (109/L)	2.5	6.7
Platelets (109/L)	10	21
Triglycerides (mmol/L)	15.2	7.4
Fibrinogen (g/L)	0.9	2.7
Hemophagocytosis	No	No
Ferritin (ng/mL)	5,434	36,292
sCD25 (U/mL)	>200,000	nd
NK-cell activity*	Defective	nd
NK-cell degranulation	Normal	Normal
Neurologic manifestations†	None	None
Pathologic CSF	nd	nd
Treatment of active disease	Dexa, CsA, ATG	Dexa, VP16, ATG
Remission at 2 mo	Yes	No
Age at HSCT	Not done	Not done
Outcome	Deceased	Deceased

ATG, Antithymocyte globulin; *CsA*, cyclosporine A; *Dexa*, dexamethasone; *HSCT*, hematopoietic stem cell transplantation; *nd*, no data; *VP16*, etoposide.
*Defective: 10 lytic units or less.
†Reported at some point during the course of the disease.

MISSENSE MUTATION OF TTC7A MIMICKING TRICHO-HEPATO-ENTERIC (SD/THE) SYNDROME IN A PATIENT WITH VERY-EARLY ONSET INFLAMMATORY BOWEL DISEASE

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Missense mutation of TTC7A mimicking tricho-hepato-enteric (SD/THE) syndrome in a patient with very-early onset inflammatory bowel disease

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ABSTRACT

Tricho-hepato-enteric syndrome (SD/THE) and **Multiple intestinal atresia with combined immune deficiency** (MIA-CID) are autosomal recessive disorders that present immunological and gastrointestinal features.

There are two different phenotypes of patients with TTC7A mutations: the severe form, caused by null mutations and leading to the classical MIA-CID; and the mild form, caused by missense mutations and leading to predominant features of VEO-IBD, less severe immunological involvement and hair abnormalities.

We expand the knowledge about TTC7A deficiency, describing a patient with the mild phenotype of TTC7A deficiency but presenting overlapping features of SD/THE and MIA-CID: intestinal atresia and inflammatory bowel disease evocative of MIA-CID, but also dental abnormalities, huge forehead, liver abnormalities, autoimmune thyroiditis and hypogammaglobulinemia, evocative of SD/THE.

1. Introduction

Tricho-hepato-enteric syndrome (SD/THE) is a rare autosomal recessive disorder that is classically associated with facial dysmorphism (wide forehead, hypertelorism, broad nasal root); abnormal hair (described as wooly, breakable, brittle, unbrushable and associated with trichorrhexis nodosa); chronic diarrhea and immune deficiency (mostly humoral deficiency) (Hartley et al., 2010; Fabre et al., 2011). Moreover, about half of the patients were prematurely born, present hepatic disease including cirrhosis, siderosis, hepatomegaly and have skin abnormalities like café au lait spots or xerosis (Monies et al., 2015; Fabre et al., 2013a). This syndrome is caused by mutations in the TTC37 or SKIVL2 genes, which encode for sub-units of the Ski complex, a co-factor of the cytoplasmatic exosomal RNA degradation pathway (Fabre et al., 2012, 2013b). Over the last few years, milder phenotypes have been described and even patients with isolated immune defects have been reported (Oz-Levi et al., 2015; Rider et al., 2015).

Multiple intestinal atresia with combined immune deficiency (MIA-CID) is another rare autosomal recessive disorder. It is classically associated with intestinal atresias that can occur throughout the gastrointestinal tract, particularly involving the small bowel. Short bowel syndrome due to intestinal resection and requirement of total parenteral nutrition are common features of this syndrome (Notarangelo, 2014; Chen et al., 2013). Associated combined immunodeficiency is also commonly present, allegedly caused by deficient thymopoiesis and lymphocyte differentiation, proliferation, and survival. Therefore, recurrent and opportunistic infections are also present (Notarangelo, 2014; Lemoine et al., 2014). This disease is caused by mutations in the TTC7A gene, involved in the regulation of the RhoA signaling pathway, crucial to normal ROCK function (allowing adequate cytoskeleton organization and epithelial cell polarity) (Notarangelo, 2014; Samuels et al., 2013; Fernandez et al., 2014). Recently, some patients with MIA-CID having only mild intestinal defects and even patients without structural defects presenting only with very-early-onset inflammatory bowel disease (VEO-IBD) have been reported (Woutsas et al., 2015; Avitzur et al., 2014).

We present the clinical report of a patient with overlapping features of both syndromes that was initially diagnosed as SD/THE. Whole

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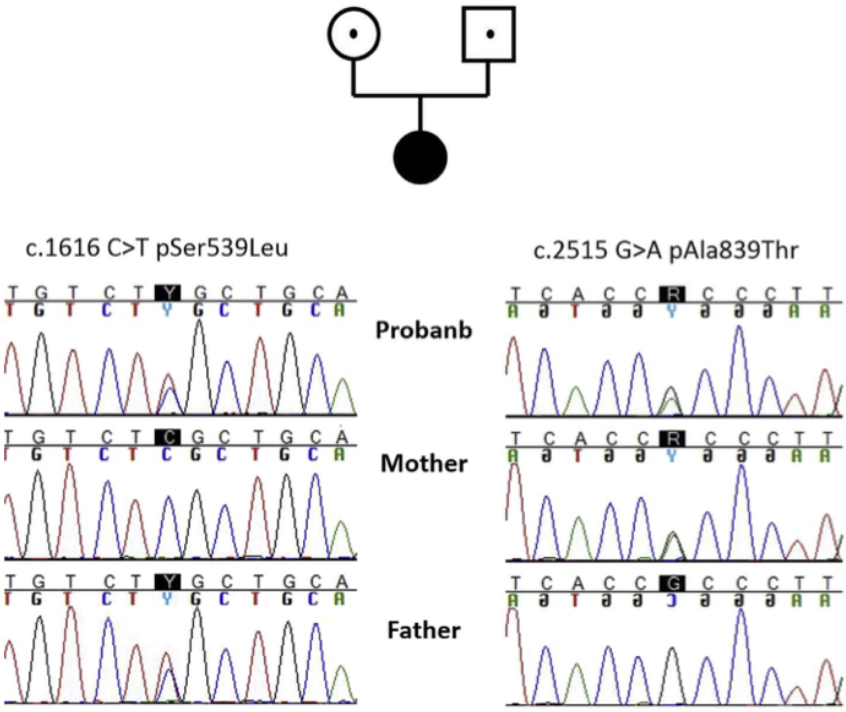

Fig. 1. Famliy predigrees showing mutation segregation. TTC7A transcript sequencing: left, variant c.1616 C > T transmitted by the father and right, variant c.2515 G > A transmitted by the mother. The variant is highlighted in black.

Exome Sequencing (WES) allowed the diagnosis of TTC7A deficiency.

1.1. Clinical report

A caucasian female infant was born at 33 weeks of gestation to unrelated parents. Her weight (2015 g), height (41 cm) and head circumference (30 cm) were on the 50th percentile. In the first weeks of her life, she presented feeding intolerance and at the age of two months, a type 1 ileal atresia was diagnosed. An ileostomy was performed and she was kept on both parenteral and enteral nutrition. At the age of 5 months she developed bloody diarrhea despite the presence of an ileostomy. The colonic histology revealed inflammatory pseudo-polyps, caliciform cells depletion, glandular distortion/atrophy, leading to the diagnosis of VEO-IBD. Her physical examination was notable for the presence of a prominent forehead, hypertelorism and enamel dysplasia (Fig. 2). She didn't have any hair/dermatological or cardiac abnormalities. (Relevant clinical and laboratory features are summarized in Table 1). Her colitis worsened after the re-establishment of the continuity of the intestinal tract. At the age of 20 months she started to present self-limited but recurrent elevation of AST and ALT. The hepatic biopsy revealed the presence of mild fibrosis and interface hepatitis. There were no autoantibodies related to autoimmune hepatitis present in the serum.

Fig. 2. Phenotype of the patient: wide forehead and hypertelorism; enamel dysplasia.

She also presented unexplained episodes of leukocytosis (70-80% neutrophils) and thrombocytosis, not related to infections. The immunological evaluation showed normal lymphocyte subpopulations but severe and symptomatic hypogammaglobulinemia (recurrent respiratory infections), as well as absent serological response to vaccines. She also presented autoimmune thyroiditis.

She started subcutaneous immunoglobulin replacement at the age of 2 leading to a dramatic decrease of the number of infections. Moreover, the episodes of intermittent leukocytosis disappeared and she presented an improvement of the colonic inflammation, allowing adequate enteral nutrition.

Nevertheless, from the age of 3 to the age of 5, she has presented 3 episodes of simultaneous elevation of AST/ALT and bloody diarrhea, which responded to low-dose prednisolone (1 mg/kg/day) and azathioprine. At the moment, at the age of 5 years-old, she has been asymptomatic for the last 12 months. She is growing on the 50th percentile of weight, height and head circumference. Her neurodevelopment is perfectly normal.

The association of enteropathy, facial dysmorphy, dental abnormalities (enamel dysplasia), hepatic abnormalities and mild immunological involvement (isolated hypogammaglobulinemia) was evocative of SD/THE, despite the absence of hair abnormalities and the presence of a type 1 intestinal atresia (that had never been reported in SD/THE). In fact, type 1 atresia is not related to an interruption of the continuity of the intestinal tract, but is instead the presence of a membrane in the inner intestine.

However the direct sequencing of the TTC37 and SKIV2L genes revealed no mutation. A whole exome analysis was performed and showed a compound heterozygosity of TTC7A, with two missense

Table 1
Clinical and laboratory features of the patient.

Sex	Female
Current Age	3 years-old
Gestational Age	33 weeks
Dysmorphology	
IUGR	No
Wide forehead	Yes
Hypertelorism	Yes
Skin alterations	No
Enamel Dysplasia	Yes
Hair (namely trichorrhexis nodosa)	No
Gastrointestinal/Nutrition	
Intestinal atresia	Yes (Jejunal Type 1 atresia)
Multiple Intestinal Atresia	No
IBD	VEO-IBD
Hepatitis	Yes
Parenteral Nutrition (PN)	2-10 months

Hematology/Immunology	Value	Reference value
Leukocytes (x10^3/mm^3) (maximum value without identifiable cause))	35	4–12
Platelets (cells/μL) (maximum value without identifiable cause)	950 000	150–500.000
Absolute lymphocyte count (x10^9/L)	3600	2500–5500
CD3 + (cells/μL)	2860	1400–3700
CD4 + (cells/μL)	2140	700–2200
CD4 + CD45RA + (%)	85%	53-86%
CD8 + (cells/μL)	547	490–1300
CD8 + CD45RA + (%)	80%	42-82%
CD16 + /56 + (cells/μL)	234	130–720
CD19 + (cells/μL)	464	190–1400
CD19 + CD27-IgD + IgM + (%)	90%	69-90%
CD19 + CD27 + (%)	8%	7-24%
CD19 + CD27 + IgD- (switch)	4%	2,7-12,5%
CD19 + CD38 + IgMhigh (transitional)	18%	
IgG (g/L)	3,63	5,3-10,7
IgM (g/L)	0	0,46-1,9
IgA (g/L)	0	0-1,5
Anti-Diphteria	0	Protective > 0,5
Anti-Tetanus	0	Protective > 0,5
Free T4 (ng/dL)	0,75	0,89-1,35
TSH (uUI/mL)	16,6	(0,7-4,1)
Anti-TPO Antibodies (UI/mL)	9,9	< 5,5

mutations in exon 14 and 21, which were confirmed in Sanger sequencing. In fact, the patient had VEO-IBD and an intestinal atresia, which were both previously linked to TTC7A deficiency. To our knowledge, this is the first report of a patient with TTC7A deficiency who presents simultaneously an intestinal atresia and VEO-IBD and also has hepatic involvement and the phenotypic features previously described.

1.2. Matherial and methods

Written consent for genetic and exome analysis were obtained from the patient and her parents. The Biobank of the Department of Genetics of La Timone Hospital proceeded with the DNA extraction and ensured the long-term storage of samples. Exome sequencing was performed after exome enrichment using *Agilent XT Capture Exome V5* in *Illumina HiSeq (2000)/2500* platform *(Aros Applied Biotechnology* A/S, Denmark). The variants were first filtered with the in-house "VarAFT" system (http://varaft.eu) focusing on the 29 genes linked to congenital diarrheal syndrome to date (Table 1 SI. (Canani et al., 2015; Posovszky, 2016).

Using frequency below 0.01 on Exac Browser (http://exac.broadinstitute.org/) only one candidate gene remained: TTC7A. The patient has compound heterozygous mutations in the TTC7A gene (NG_034143.1):

- NM_001288951.1: c.2587G > A; p (Ala863Thr), inherited from the mother
- NM_001288951.1:c.1616C > T; p (Ser539Leu). Inherited from the father

Both were rare with respectively three and two alleles in Gnomad for c.2587G > A and c.1616C > T, with no description of homozygosity. Both variants were predicted to be pathogenic using bioinformatics tools (Polyphen: probably damaging; SIFT: deleterious; Mutation Taster: disease causing; UMD predictor: pathogenic). The variants were deposed in clinvar (SUB3050809). The TTC7A variants were confirmed by Sanger sequencing on a 3500XL Genetic Analyzer® (ThermoFisher, Carlsbad, CA) and parental DNA samples were tested to confirm inheritance. (Fig. 1). A second analysis without gene filtering did not find any other relevant candidate gene.

2. Discussion

We present the case of a patient with clinical features evocative of SD/THE but presenting missense mutations (probably hypomorphic) in TTC7A. We considered SD/THE to be the most probable diagnosis due to the presence of VEO-IBD, large forehead and the absence of combined immunodeficiency, despite the presence of a type 1 ileal atresia.

Indeed, there is significant clinical overlap of both syndromes (Table 2). This patient presents some features that had never been described before in patients with TTC7A mutations: hepatic involvement, dental anomalies, facial dysmorphism and mild immunological involvement). Additionally, even the coexistence of intestinal atresia and VEO-IBD in the same patient with TTC7A deficiency had never been described.

MIA-CID linked to TTC7A deficiency was only characterized in 2013 and in the following years a more precise characterization of the syndrome has been described. To date, there are 52 patients described worldwide and the syndrome seems to have two different phenotypes:

- Severe type, with multiple intestinal atresia and combined immunodeficiency, leading to premature death.
- Mild type, with predominant features of VEO-IBD, less severe immunological involvement (some with auto-immune features), and hair abnormalities.

Interestingly enough, in a very recent past, some patients with TTC7A deficiency were described to present some dermatological features, namely ichthyosis. This characteristic was expected to be present, since the animal model KO to TTC7A presents severe dermatological features (Leclerc-Mercier et al., 2016).

There seems to be some genotype/phenotype correlation with less severe phenotype linked to missense (probably hypomorphic) mutations, whereas the most severe phenotype is related to null mutations.

We believe that, despite the overlap that these two different conditions may present, the presence of intestinal atresia seems to be linked with TTC7A mutations, whereas woolly hair and trichorhexis nodosa

Table 2
Comparison of phenotypic feature of SD/THE, MIA-CID/VEOIBD and patients (constant: > 90% of the cases reporded, frequent: 50-70% of the cases, rare < 50% of the cases.

	SD/THE	Patient	MIA_CID/VEOIBD
Intestinal atresia	Non recorded	Yes unique	Frequent
Intractable diarrhea	Constant	Yes	Frequent
IBD like feature	Rare	Yes	Rare
Facial dysmorphy	Constant	Yes	Not reported
Hair abnormalities	Constant	No	Rare
Immune deficiency	Constant	Yes	Constant
Liver disease	Frequent	Yes	Rare
IUGR	Constant	No	Not reported
Skin abnormalities	Frequent	No	Rare

are tipically linked to SD/THE. However, there are less than 60 patients with both diseases reported. Thus, a clearer vision of these syndromes will most certainly arise as more patients are described.

Finally, this case illustrates once again the importance of broad gene analysis (either targeted on a gene panel or whole exome sequencing) for the clarification of rare diseases with overlapping phenotypes.

Conflict of interest statement

The authors declare that they don't have any conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmg.2017.11.014.

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NOVEL *PLCG2* MUTATION IN PATIENT WITH APLAID, CUTIS LAXA AND SENSORINEURAL DEAFNESS

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CASE REPORT
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Novel *PLCG2* Mutation in a Patient With APLAID and Cutis Laxa

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Background: The auto-inflammation and phospholipase Cγ2 (PLCγ2)-associated antibody deficiency and immune dysregulation (APLAID) syndrome is a rare primary immunodeficiency caused by a gain-of-function mutation S707Y in the *PLCG2* gene previously described in two patients from one family. The APLAID patients presented with early-onset blistering skin lesions, posterior uveitis, inflammatory bowel disease (IBD) and recurrent sinopulmonary infections caused by a humoral defect, but lacked circulating autoantibodies and had no cold-induced urticaria, contrary to the patients with the related PLAID syndrome.

Case: We describe a new APLAID patient who presented with vesiculopustular rash in the 1st weeks of life, followed by IBD, posterior uveitis, recurrent chest infections, interstitial pneumonitis, and also had sensorineural deafness and cutis laxa. Her disease has been refractory to most treatments, including IL1 blockers and a trial with ruxolitinib has been attempted.

Results: In this patient, we found a unique *de novo* heterozygous missense L848P mutation in the *PLCG2* gene, predicted to affect the PLCγ2 structure. Similarly to S707Y, the L848P mutation led to the increased basal and EGF-stimulated PLCγ2 activity *in vitro*. Whole blood assays showed reduced production of IFN-γ and IL-17 in response to polyclonal T-cell stimulation and reduced production of IL-10 and IL-1β after LPS stimulation. Reduced IL-1β levels and the lack of clinical response to treatment with IL-1 blockers argue against NLRP3 inflammasome hyperactivation being the main mechanism mediating the APLAID pathogenesis.

Conclusion: Our findings indicate that L848P is novel a gain-of-function mutation that leads to PLCγ2 activation and suggest cutis laxa as a possible clinical manifestations of the APLAID syndrome.

Keywords: APLAID, PLCγ2, cutis laxa, sensorineural deafness, IL-10, IL-1b, auto-inflammatory syndromes

INTRODUCTION

The strict control of inflammation is one of the most important functions of the immune system (1). While the majority of the autoimmune inflammatory diseases are mediated by effectors of the adaptive immune system, auto-inflammatory syndromes are caused by an aberrant activation of the innate immune cells (1–4). These disorders are characterized by systemic inflammation and diverse organ-specific manifestations, such as arthritis, aseptic meningitis, deafness, or urticaria (4, 5). More than 20 years ago, mutations in the *MEFV* and *TNFRSF1A* genes were shown to cause familial Mediterranean fever and Tumor necrosis factor receptor-associated periodic syndrome (TRAPS) (6). Since then, inherited deficiencies in the components of the innate immune system that control inflammation have been identified, e.g., cryopyrin-associated periodic syndrome (CAPS) and Auto-inflammation with infantile enterocolitis (AIFEC) caused by gain-of-function mutations in NLRP3 and NLRC4 that lead to the release of pro-inflammatory cytokines (such as IL-1β) (7–9). These findings expanded the knowledge about mechanisms mediating auto-inflammation and provided scientific basis for the targeted treatment of such disorders (4–7).

Phosphatidylinositol and its derivatives are essential regulators of the human immune cell functions. Phospholipase C (PLC) is a family of enzymes that catalyze hydrolysis of phosphatidylinositol 4,5-bisphosphate, producing second messenger molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 induces release of calcium from endoplasmic reticulum to the cytoplasm, while DAG can activate cell signaling proteins, e.g., protein kinase C. PLCγ is structurally distinct from other PLC enzymes and has two isoforms. PLCγ1 is ubiquitously expressed and acts downstream of growth factor receptors, while PLCγ2 is found mainly in hematopoietic cells, where it is activated by tyrosine kinases recruited to immune cell receptors, e.g., BCR and Fc receptors or, as subsequently shown, also by Rac GTPase (10–15).

Recently, 27 patients presenting with cold-induced urticaria, recurrent sinopulmonary infections, antibody deficiency, and autoimmunity, were found to have heterozygous in-frame deletions affecting the *PLCG2* gene, resulting in constitutive activation of PLCγ2. This syndrome was designated the PLCγ2-associated antibody deficiency and immune dysregulation (PLAID) (10). Thereafter, the auto-inflammation and PLCγ2-associated antibody deficiency and immune dysregulation (APLAID) syndrome was described in two patients from another family (11). APLAID patients presented with early-onset blistering skin lesions, eye inflammation with ocular hypertension, inflammatory bowel disease (IBD), arthralgia, and recurrent sinopulmonary infections caused by a humoral defect, but lacked circulating autoantibodies and had no cold-induced urticaria. This distinctive phenotype was caused by a missense mutation S707Y in the *PLCG2* gene that disrupted an auto-inhibitory cSH2 domain, causing increased production of intracellular IP_3 and calcium release resulting in hyperactivation of the PLCγ2 signaling pathway (11). Contrary to the PLAID mutants, this mutation does not lead to constitutive enzymatic activity, requiring upstream signaling events for its activation.

Here, we report a new patient with APLAID caused by a unique activating mutation in PLCγ2 that presented with novel manifestations, including cutis laxa and sensorineural deafness.

MATERIALS AND METHODS

Ethics

All materials were obtained with informed consent in accordance with the Declaration of Helsinki and with approval from the ethics committees in Portugal (131/2014) and UK (15/WS/0019). Written informed consent for publication of this case report was obtained from the parents of the patient.

Flow Cytometry

For the evaluation of lymphocyte subsets, peripheral blood was analyzed by Flow Cytometry in a 4-color BD FACS Calibur (BD, San Jose, California, USA), using the BD IMK kit with Trucount tubes (BD Biosciences) according to the manufacturer's instructions. An additional panel of monoclonal antibodies (including CD45RA, CD45RO, CD62L, and HLA-DR) was also performed for further characterization of T-cell differentiation, using a lyse-wash protocol. Finally, the IOTest® Beta Mark kit (Immunotech SAS, a Beckman Coulter Company, Marseille, France) was also used for the characterization of the TCR Vβ repertoire.

Flow cytometry data analysis was performed using Multiset and CellQuestPro software (BD Biosciences).

Proliferation Assays

Proliferative capacity was performed with a thymidine incorporation assay. In brief, peripheral blood mononuclear cells (PBMCs) were incubated with mitogens (PHA, final concentration and PMA+ionomycin) for 3–4 days, and with antigens (PPD, C. albicans antigen and Tetanus toxoid) for 7–8 days, at 37°C, in a 5% CO_2 atmosphere. The cells were labeled with tritiated thymidine (3H-thymidine) (Perkin Elmer, Boston, MA, USA) in the last 18 h of incubation. Cells were then harvested and transferred to a Filtermat A filter (Perkin Elmer), and a Meltilex scintillation sheet (Perkin Elmer) was applied after the Filtermat was dried. The radioactivity in DNA recovered from the cells was read in a MicroBeta counter (Perkin Elmer). Results were presented as stimulation indexes, obtained from the ratios of cpm from stimulated and unstimulated cells (incubated in parallel).

Antibody Response Evaluation

Immunoglobulins were quantified in serum. Immunoturbidimetria was used to quantify IgG, IgA, and IgM. As for specific responses, antibodies for Diphtheria, Tetanus, and Pertussis were evaluated by ELISA.

Whole-Blood Cytokine Production Assays

Whole blood was diluted 1:5 in RPMI into 96-well F plates (Corning) and activated using the following stimulants Phytohemagglutinin (PHA; 10 μg/ml; Sigma-Aldrich) PMA/IONO, LPS (1 μg/ml, List Biochemicals), Pam2CSK4 (Invivogen, 1 micg/ml), Pam3CSK4 (Invivogen,

1 micg/ml), Flagellin (Invivogen, 1 micg/ml), CL-097 (Invivogen, × micg/ml). Supernatants were taken at 24 h. Cytokines were measured using multiplexed particle based bead array (IFNγ, TNFα, IL-17,IL-1b, IL-10, IL-6, R+D Systems Fluorokinemap) on a Luminex analyser (Bio-Plex, Bio-Rad, UK). Data were statistically analyzed by the two-tailed Mann-Whitney test using Prism 6 (GraphPad Software).

Exome Sequencing and Bioinformatics Analysis

We isolated DNA samples from blood or peripheral blood mononuclear cells (PBMCs). Library preparation, exome capture, and sequencing have been done according to the manufacturers' instructions. For exome target enrichment Agilent SureSelect 50 Mb kit was used. Sequencing was done using Illumina HiSeq 2000 with 94 bp paired-end reads. FASTQ files were aligned to the hg19 reference sequence using Novoalign version 2.07.19, including hard and soft clipping, quality calibration and adapter trimming. Duplicate reads were excluded using the PICARD tool MarkDuplicates. Calling was performed using SAMtools v0.18 and single sample calling. The resulting calls were annotated with the software ANNOVAR. Candidate variants were filtered based on function: loss-of-function, non-synonymous or potential splicing altering variants (defined as being with 5 bp of the actual splice site) and frequency.

Cell Culture and Transfections

Plasmids for the expression of full-length human pTRIEX-PLCg2 constructs in mammalian cells have been described previously (11).

QuikChange PCR mutagenesis (Stratagene) was used to introduce a point mutation in the PLCg2 WT variant. All mutants were fully sequenced to verify the fidelity of PCR. COS7cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2

In Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and 2.5 mM glutamine. Prior to transfection cells were seeded into 6-well plates at a density of 2.5×10^5 cells/well and grown for 16 h in 2 ml/well of the same medium. For transfection, 1.0 ug of PLCg2 DNA was mixed with 1 µl of PlusReagent™ and 7 µl of Lipofectamine™ (Invitrogen) and added to the cells in 0.8 ml of DMEM without serum. The cells were incubated for 3.5 h at 37°C, 5% CO2 before the transfection mixture was removed and replaced with DMEM-containing serum.

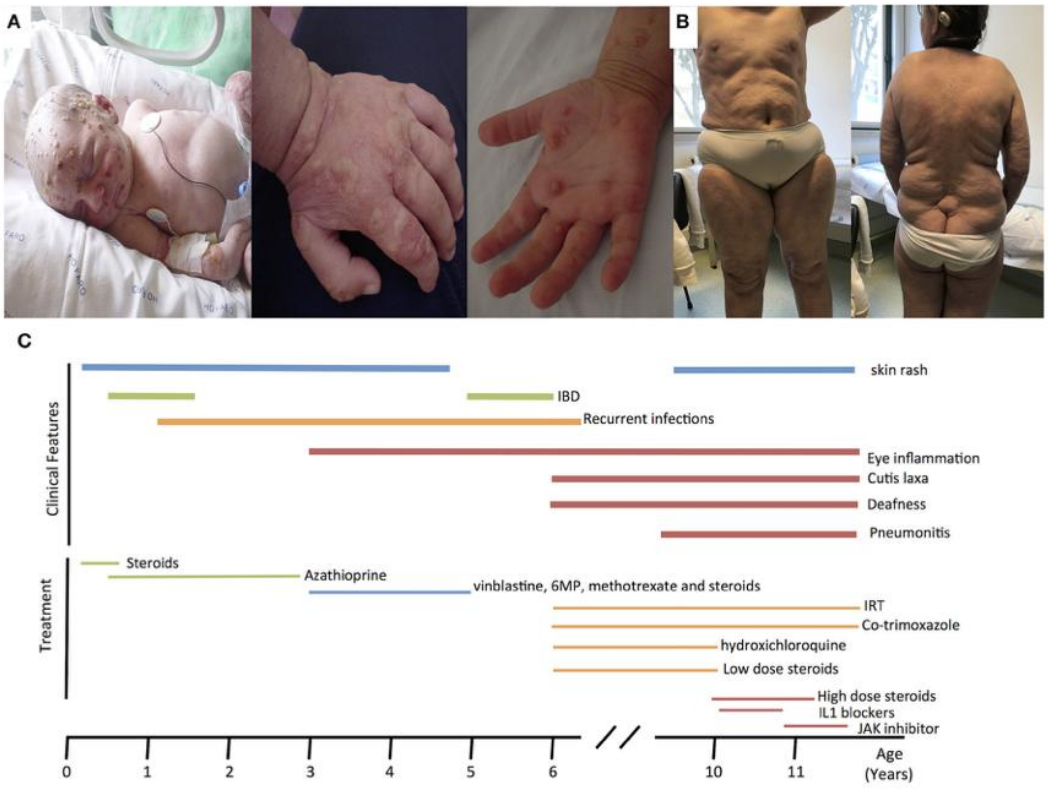

FIGURE 1 | (A) Neonatal pustulosis. **(B)** Cutis laxa. **(C)** Timeline for the clinical manifestations and treatments of the patient. IRT, Immunoglobulin replacement therapy.

TABLE 1 | Immunological assessment of the patient.

Immunology	6 Years-old	10 Years-old	Reference value
Absolute lymphocyte count ($\times 10^9$/L)	3,600	3,350	2,500–5,500
CD3+(cells/μL)	1,685	1,250	1,200–3,000
CD4+(cells/μL)	985	634	650–1,500
CD4+CD45RA+ (%)	65%	59%	53–86%
CD8+(cells/μL)	640	529	370–1,100
CD8+CD45RA+ (%)	83%	79%	42–82%
CD16+/56+ (cells/μL)	100	143	100–480
CD19+(cells/μL)	**↓ 55**	**↓ 143**	270–860
CD19+CD27-IgD+IgM+ (%)	**↑ 90%**	**↑95%**	47–70%
CD19+CD27+ (%)	**↓ 6%**	**↓ 3%**	7–24%
CD19+CD27+IgD- (switch)	**↓ 0.5%**	**↓ 0**	2.7–12.5%
CD19+CD38+IgM^{high} (transitional)	**↑ 50%**	**↑64%**	
IgG (g/L)	**3,563**	**10.5 (on IRT)**	5.3–10.7
IgM (g/L)	**0**	**0**	0.46–1.9
IgA (g/L)	**0**	**0**	0–1.5
Anti-diphteria	**0**	**2.4 (on IRT)**	Protective >0.5
Anti-tetanus	**0**	**1.5 (on IRT)**	Protective >0.5
Proliferative responses to PHA (CPM × 10^3) (stimulation index)	25,621 ± 1,762 133	Not performed	Control 20,076 ± 3,179 45.8
Proliferative responses to PMA+I (CPM × 10^3) (stimulation index)	21,665 + 2,320 75	Not performed	Control 13,069 ± 1,645 47.7

Analysis of Inositol Phosphate Formation in Intact COS7 Cells

Inositol Phosphate formation was assessed as described in Everett et al. (13). Briefly, 24 h after transfection, the cells were washed twice with inositol-free DMEM without serum and incubated for 24 h in 1.5 ml of the same medium supplemented with 0.25% fatty acid free bovine serum albumin (Sigma) and 1.5 uCi/ml myo-[$^{2-3}$H]inositol (MP Biomedicals). After a further 24 h, the cells were incubated in 1.2 ml of inositol-free DMEM without serum containing 20 mM LiCl with or without stimulation with 100 ng/ml EGF (Calbiochem). The cells were lysed by addition of 1.2 ml of 4.5% perchloric acid. After incubating the samples on ice for 30 min, they were centrifuged for 20 min at 3700 × g. Supernatants and pellets were separated. The supernatants were neutralized by addition of 3 ml of 0.5 M potassium hydroxide/9 mM sodium tetraborate and centrifuged for a further 20 min at 3700 × g. Supernatants were loaded onto AG1-X8 200–400 columns (Bio-Rad) that had been converted to the formate form by addition of 2 M ammonium formate/0.1 M formic acid and equilibrated with water. The columns were washed three times with 5 ml of 60 mM ammonium formate/5 mM sodium tetraborate, and inositol phosphates were eluted with 5 ml of 1.2 M ammonium formate/ 0.1 M formic acid. Five milliliter Ultima-Flo scintillation fluid (PerkinElmer Life Sciences) was added to the eluates and the radioactivity quantified by liquid scintillation counting. The values represent total inositol phosphates. The pellets from the first centrifugation were resuspended in 100 μl of water and 375 μl of chloroform/methanol/HCl (200:100:15) was added. The samples were vortexed, and an additional 125 μl of chloroform and 125 μl of 0.1 M HCl were added.

After further vortexing, the samples were centrifuged at 700 × g for 10 min. Ten microliter of the lower phase were placed in a scintillation vial with 3 ml of Ultima-Flo scintillation fluid and the radioactivity quantified by liquid scintillation counting. The obtained values correspond to radioactivity in inositol lipids. PLC activity is expressed as

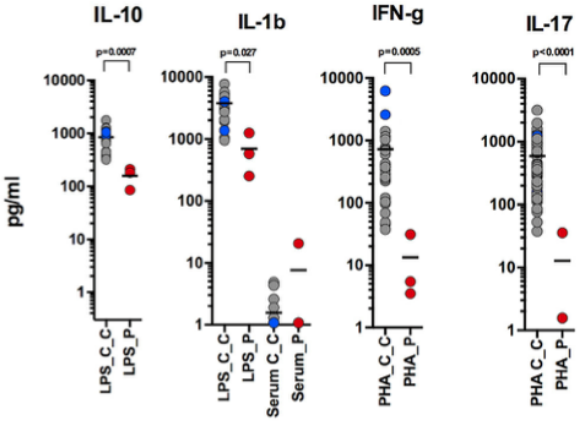

FIGURE 2 | Reduced production of IL-10 and IL-1β after LPS stimulation; reduced production of IFN-γ and IL-17 in response to polyclonal T-cell stimulation (performed at 9, 10, and 11 years-old).

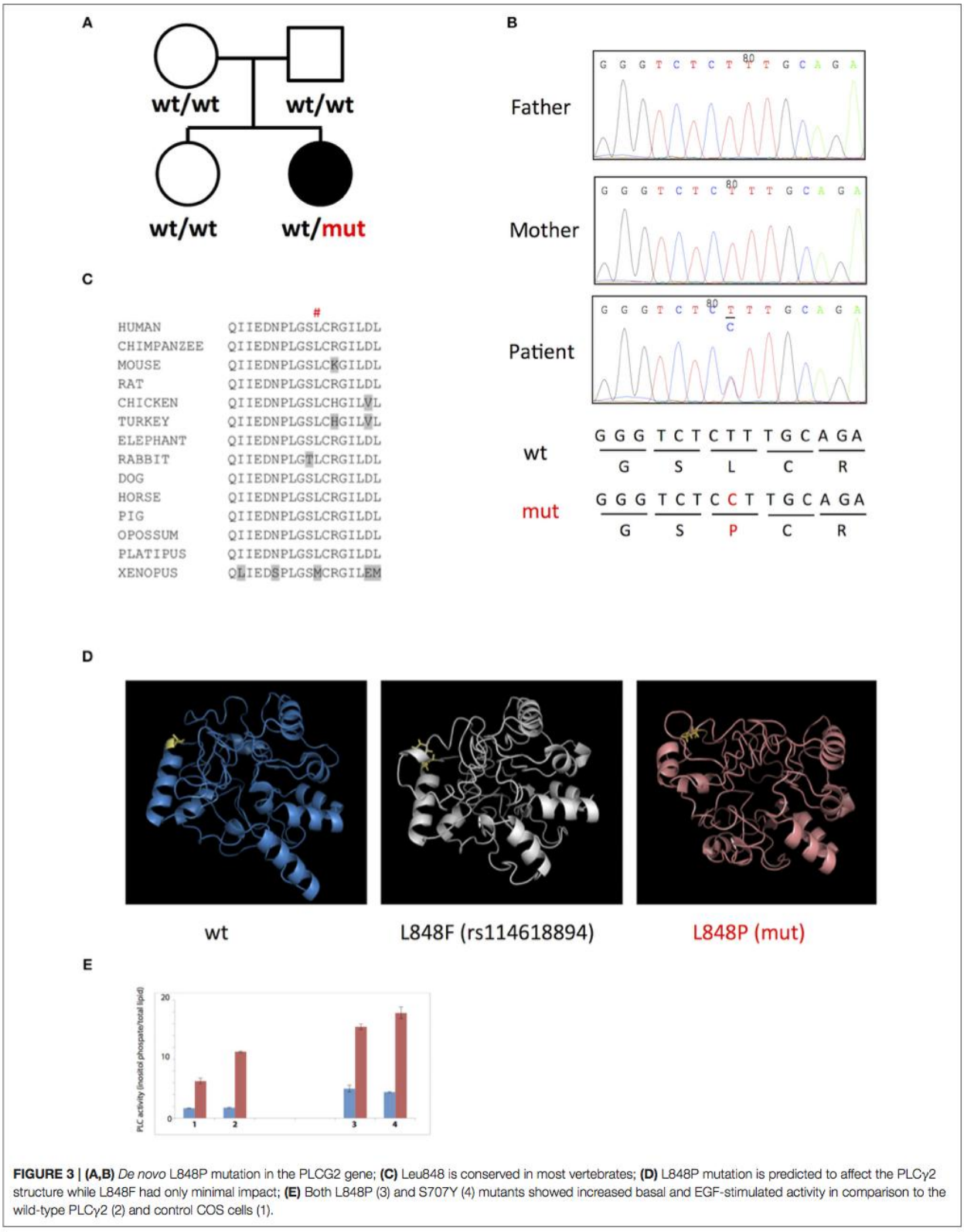

FIGURE 3 | (A,B) *De novo* L848P mutation in the PLCG2 gene; **(C)** Leu848 is conserved in most vertebrates; **(D)** L848P mutation is predicted to affect the PLCγ2 structure while L848F had only minimal impact; **(E)** Both L848P (3) and S707Y (4) mutants showed increased basal and EGF-stimulated activity in comparison to the wild-type PLCγ2 (2) and control COS cells (1).

TABLE 2 | Clinical and immunological manifestations of PLAID patients, previously published APLAID patients and this patient.

	PLAID ($n = 27$)	APLAID S707Y ($n = 2$)	APLAID L848P ($n = 1$)
Genetics	heterozygous in-frame deletions PLCG2 gene	Missense mutation	Missense mutation
Mutation effect	Gain-of-function	Gain-of-function	Gain-of-function
PLCγ2 activation status	Constitutive activation	Requires upstream signaling for activation	Requires upstream signaling for activation
Affected PLCγ2 domain	cSH2	cSH2	spPH
CLINICAL MANIFESTATIONS			
Cold urticária	✓	✗	✗
Allergic disease	✓	✗	✗
Autoimmunity	✓	✗	✗
Recurrent chest infection	✓	✓	✓
Cutaneous granulomas	✓	✓	✓
Inflammatory bowel disease	✗	✓	✓
Posterior uveitis	✗	✓	✓
Interstitial pneumonitis	✗	✓	✓
Cutis laxa	✗	✗	✓
Sensorineural deafness	✗	✗	✓
IMMUNOLOGY			
T cells	Normal	Normal	Normal
Class-switched memory B cells	↓	↓	↓
NK cells	↓	Normal	Normal
IgG	↓	Normal	↓
IgA	↓	↓	↓
IgM	↓	↓	↓
Circulating auto antibodies	✓	✗	✗

the total inositol phosphates formed relative to the amount of [^{3}H]myo-inositol in the phospholipid pool. Because the differences in steady state labeling of inositol lipids are small (within 20%), this normalized PLC activity corresponds closely to PLC values expressed as total inositol phosphates; however, the error bars between the duplicates are generally smaller.

Case Report

Here, we report an 11 years-old girl, second daughter of healthy non-consanguineous parents of Portuguese origin. She presented with very severe vesiculo-pustular rash in the 1st week of life (**Figure 1A**). Biopsy showed dermis infiltration by CD68+CD163+S100–CD1a–histiocytes and a presumptive diagnosis of juvenile xanthogranuloma was made. At the age of 2 months, she started bloody diarrhea and early-onset IBD was diagnosed based on the endoscopic and histologic findings. The bowel disease improved after treatment with prednisolone and maintenance with azathioprine. Nevertheless, she continued to have recurrent episodes of blistering skin rash and presented recurrent chest infections. At the age of 3 years she started to have recurrent eye inflammation that was associated with ocular hypertension. Systemic xanthogranuloma was suspected and she was treated with vinblastine, 6-mercaptopurine, methotrexate, and steroids. Her symptoms did not resolve and she had more frequent infections, including acute otitis media, recurrent chronic sinusitis, recurrent pneumonia, and on one occasion, pulmonary *Aspergillus* infection that responded to itraconazole. Cutis laxa was evident since she was 6 years-old (**Figure 1B**) and progressive sensorineural deafness was diagnosed at the age of 7, requiring a hearing-assistive device. Immunological analysis at that age revealed low IgM and IgA, absent responses to protein antigens and low B cells, with almost absent class-switched memory B cells. T-cell proliferation responses were preserved. No auto-antibodies were found (**Table 1**). Whole blood stimulation assays showed a strongly reduced production of IFN-γ and IL-17 in response to polyclonal T-cell stimulation and reduced production of IL-10 and IL-1β after LPS stimulation (**Figure 2**). However, there was a significant innate induction of IL-6 and TNFα in response to various TLR agonists including LPS, PAM 2/3, Flagellin, and CL-097 (**Supplementary Figure 1** and **Supplementary Table 1**). She started prophylactic co-trimoxazole, immunoglobulin replacement, as well as low dose steroids (5 mg every other day) and hydroxychloroquine (**Figure 1C**), which led to substantial improvement. Nevertheless, since the age of 10 years she started to have several relapses of eye inflammation, skin rash, and pulmonary inflammation (interstitial pneumonitis) that improved only partially with high-dose steroids. Anakinra (IL1-receptor antagonist) and canakinumab (IL1β monoclonal antibody) were tried without any effect. In view of the excellent results of the use of JAK inhibitors in auto-inflammatory diseases (16), and given that PLCγ2 requires upstream signaling events for its activation, we have very recently decided to treat the

patient with ruxolitinib as a bridge to hematopoietic stem cell transplantation.

To uncover genetic basis of the disease, we used whole exome sequencing and identified a heterozygous missense L848P mutation in the *PLCG2* gene. Sanger sequencing showed that this mutation appeared *de novo* in the patient, while being absent from both parents and a healthy sibling (**Figures 3A,B**). The mutation affected amino acid residue Leu848 in the split pleckstrin homology (spPH) domain of the PLCγ2 protein that is conserved in most vertebrates (**Figure 3C**). The L848P mutation is unique to this patient: it was never found in humans, e.g., it is absent from more than 120,000 subjects in the gnomAD database (12). Interestingly, DNA polymorphism rs114618894 that has ∼2% frequency in Africans affects the same amino acid residue leading to the L848F substitution. Bioinformatics analysis predicted that the patient's mutation L848P was damaging (e.g., SIFT = 0.99, MutationTaster = 0.99), while L848F was neutral. We used I-Tasser (17) to model the PLCγ2 protein fragment between amino acids 770 and 1044 containing the L848P and L848F substitutions and found that L848P is predicted to affect the PLCγ2 structure, while L848F had only minimal impact (**Figure 3D**). The spPH domain contributes to auto-inhibition and also binds Rac. A mutation in this domain has been shown to cause gain of function leading to severe auto-inflammatory disease in mice (13). Furthermore, an activating spPH mutation L845F, which is close to residue L848, has been described in cancer resistance to Ibrutinib, the drug acting on Btk upstream of PLCγ2 (14). In order to test the functional effect of the newly found L848P mutation, we cloned PLCγ2 and introduced by site directed mutagenesis L848P or the previously reported APLAID mutation S707Y. We transfected these constructs into COS-7 cells and measured IP production. In this standard cellular assay, both L848P and S707Y mutants showed increased basal and EGF-stimulated activity in comparison to wild-type PLCγ2 (**Figure 3E**). Taken together, our data show that L848P is a *de novo* gain-of-function mutation that is very likely to have caused the disease in our patient.

DISCUSSION

Here, we describe a novel patient with the APLAID syndrome caused by a new missense PLCγ2-activating mutation. This is only the third reported APLAID patient. The clinical and immunological manifestations of our patient resembled the phenotype of the other two APLAID patients described in a single, previous study (11) with the additional presentation of sensorineural deafness and cutis laxa (**Figure 1C**, **Table 2**). Interestingly, cutis laxa was also present in two recently identified Spanish patients with APLAID (18) which suggests that it may be frequent among APLAID patients. The *Plcg2*^{Ali5} mice, with a gain-of-function missense mutation in the PLCG2 gene, present inflammatory dermatitis (19). This, and the fact that PLCγ2 is expressed primarily in hematopoietic cells, makes us speculate that this new manifestation is probably driven by persistent skin inflammation.

On the other hand, despite the fact that congenital and perinatal causes of sensorineural deafness (infection, malformation, hypoxia) were absent, the patient had received ototoxic medication (such as vinblastine), so it is difficult to define causality.

Recently, Chae et al. (20) found that PBMCs of APLAID patients secreted IL-1β after LPS stimulation, while PBMCs of controls did not, and suggested that systemic inflammation in APLAID is driven by increased activation of the NLRP3 inflammasome (20). Despite this, the two APLAID patients did not respond to IL1 blockers (21). In contrast, we report that levels of IL-1β in whole blood of our patient were decreased after LPS stimulation (**Figure 2**). The inefficacy of IL1 blockers in our patient also argues against the IL-1-driven mechanism of her disease.

Interestingly, we noticed that levels of IL-10 were substantially less in whole blood of our patient than in controls. IL-10 is an immunomodulatory cytokine that is well known to balance immune response in the gut and impaired signaling in the IL-10 pathway has been associated with IBD (22). Given that all APLAID patients have IBD, it is tempting to speculate that impaired IL-10 production contributes to the immune dysregulation and is responsible for gut inflammation in these patients.

In summary, our report shows that a novel heterozygous missense gain-of-function L848P mutation the *PLCG2* gene causes APLAID in a patient who additionally presented cutis laxa which appears to extend the APLAID phenotype.

AUTHOR CONTRIBUTIONS

JN designed the study and wrote the paper. RD designed, performed, and analyzed the cytokine assays. CM, DK, and LB performed immunological analysis. GB-M performed and analyzed the cytokine assays. OP, VP, and JC performed genetic analysis. MM and MK performed the functional validation of the PLCG2 mutation. AC and CN medical doctor of the patient + designed the study. SN designed the study and analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02863/full#supplementary-material

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IV.II. ATYPICAL AND FATAL MANIFESTATIONS OF PRIMARY IMMUNODEFICIENCIES

FATAL CENTRAL NERVOUS SYSTEM LYMPHOCYTIC VASCULITIS AFTER TREATMENT FOR BURKITT LYMPHOMA IN A PATIENT WITH A *SH2D1A* MUTATION

PATHOGENESIS AND HOST RESPONSE

Fatal Central Nervous System Lymphocytic Vasculitis after Treatment for Burkitt Lymphoma in a Patient with a *SH2D1A* Mutation

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Abstract: Very rarely, patients with X-linked lymphoproliferative syndrome type 1 present central nervous system vasculitis. We report a patient carrying a *SH2D1A* mutation that, after treatment for lymphoma developed fatal central nervous system vasculitis. He lacked signs of ongoing Epstein–Barr virus infection. We propose that impaired T cell homeostasis caused by SAP deficiency facilitates aberrant $CD8^+$ T cell activation against vascular antigens promoting clinical manifestations.

Key Words: XLP1, SAP, CNS vasculitis, lymphoma, EBV, SH2D1A

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Abstract: Very rarely, patients with X-linked lymphoproliferative syndrome type 1 present central nervous system vasculitis. We report a patient carrying a *SH2D1A* mutation that, after treatment for lymphoma developed fatal central nervous system vasculitis. He lacked signs of ongoing Epstein–Barr virus infection. We propose that impaired T cell homeostasis caused by SAP deficiency facilitates aberrant $CD8^+$ T cell activation against vascular antigens promoting clinical manifestations.

X-linked lymphoproliferative syndrome type 1 (XLP1) is a rare primary immunodeficiency with an estimated incidence of 2 males/1.000.000 newborns.[1] XLP1 is caused by hemizygous mutations in *SH2D1A*, located at Xq25, that encodes the signaling lymphocytic activation molecule (SLAM)–associated protein (SAP).[2] SAP is a cytoplasmic protein expressed in NK and T cells. Upon recruitment to SLAM family receptors (e.g., 2B4), SAP promotes activating signaling. SAP deficiency renders SLAM receptors on lymphocytes inhibitory, resulting in impaired NK and $CD8^+$ T cell cytotoxic responses toward SLAM family receptor-expressing hematopoietic target cells.[2,3] Notably, $CD8^+$ T cell cytotoxic responses in XLP1 are mostly preserved when antigens are presented by tissue cells as well as dendritic cells, but compromised in response to many hematopoietic cells, including B cells. Accordingly, patients are particularly susceptible to Epstein–Barr virus (EPV), which establishes latency in B cells.[4] During EBV infection, infected B cells upregulate expression of CD48 that binds 2B4 expressed on T and NK cells. In XLP1 patients, impaired killing of EBV-infected cells may culminate in fulminant mononucleosis with excessive release of cytokines. Without treatment, a majority of the patients ultimately die.[2,5]

Another frequent presentation of XLP1 is hypo or dysgammaglobulinemia, secondary to defective interactions between B cells and follicular helper T cells.[1,3] One fifth of XLP1 patients present with lymphoma, while a minority of them present other manifestations such as aplastic anemia or vasculitis.[1]

We report an XLP1 patient who developed fatal central nervous system (CNS) vasculitis in the absence of active EBV-infection, 6 months after completing treatment for Burkitt lymphoma.

MATERIALS AND METHODS

Flow Cytometry

For the evaluation of lymphocyte subsets, peripheral blood was analyzed by Flow Cytometry in a 4-color BD FACS Calibur (BD, San Jose, Calif.), using the BD IMK kit with Trucount tubes (BD Biosciences) according to the manufacturer's instructions. An additional panel of monoclonal antibodies (including CD45RA, CD45RO, CD62L, and HLA-DR) was also performed for further characterization of T-cell differentiation, using a lyse-wash protocol. Finally, the IOTest Beta Mark kit (Immunotech SAS, a Beckman Coulter Company, Marseille, France) was also used for the characterization of the TCR Vβ repertoire.

Flow cytometry data analysis was performed using Multiset and CellQuestPro software (BD Biosciences).

Antibody Response Evaluation

Immunoglobulins were quantified in serum. Immunoturbidimetria was used to quantify IgG, IgA and IgM. As for specific responses, antibodies for Diphtheria, Tetanus, and Pertussis were evaluated by enzyme-linked immunosorbent assay.

SAP Expression

Rested peripheral blood mononuclear cells (PBMCs) from the patient and controls were surface labeled for CD3 (OKT3), CD8 (SK1), CD19 (HIB19) and CD56 (NCAM16.2) before fixed in 4% paraformaldehyde and intracellularly stained for SAP (1C9, Abnova). Cells were acquired on an LSRFortssa (BD Bioscience) and analyzed with Flowjo 9.9.

Western Blot

Freshly isolated PBMCs were lysed in RIPA buffer (Santa Cruz Biotechnology) before protein content was quantified using a standard Bradford assay (ThermoFisher). Equal concentrations were run on a 12% Bis-Tris gel (Invitrogen) in the presence of 10 mM DTT. Anti-SAP (1C9) from Abnova and rat anti-SAP (1D12) from Cell Signaling Technology were used for immunoblotting. Mouse anti-actin (C4) from MP Biomedicals was used as the loading control.

Polymerase Chain Reaction

One million PBMCs from a healthy control and the patient were harvested for generating cDNA (M-MLV, Invitrogen) following

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A written informed consent from the patient's mother has been obtained.

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manufacturer's protocol. Taq polymerase (Invitrogen) was used for the polymerase chain reaction (PCR) reaction. Primers synthesized: β2-microglobulin forward 5' AGTATGCCTGCCGTGTGAACC 3'; β2-microglobulin reverse 5' TGCGGCATCTTCAAACCTCCA 3'; *SH2D1A* exon 1/2 5' CCTATGTGTGCTGTATCACGG 3'; *SH2D1A* exon 3 5' TGGCTTCTGAAATGCTGAAATG 3'.

RESULTS

A previously healthy 14-year-old boy presented with abdominal pain and loss of weight and was diagnosed with sporadic ileocaecal Burkitt lymphoma (Fig. 1A and fig., Supplemental Digital Content 1, http://links.lww.com/INF/D232). No evidence of ongoing EBV infection was found, as PCR for EBV in the blood was negative and serology provided evidence of past exposure (VCA-IgM negative, VCA-IgG positive, negative EBNA). His brother had died of fulminant mononucleosis at 2 years of age, prompting further investigation. The patient manifested hypogammaglobulinemia, reduced class-switched memory B-cells, and lack of NKT cells (Table, Supplemental Digital Content 2, http://links.lww.com/INF/D233). Moreover, SAP expression was absent, as measured by Western blot and flow cytometry in PBMCs (Fig. 1B, C).

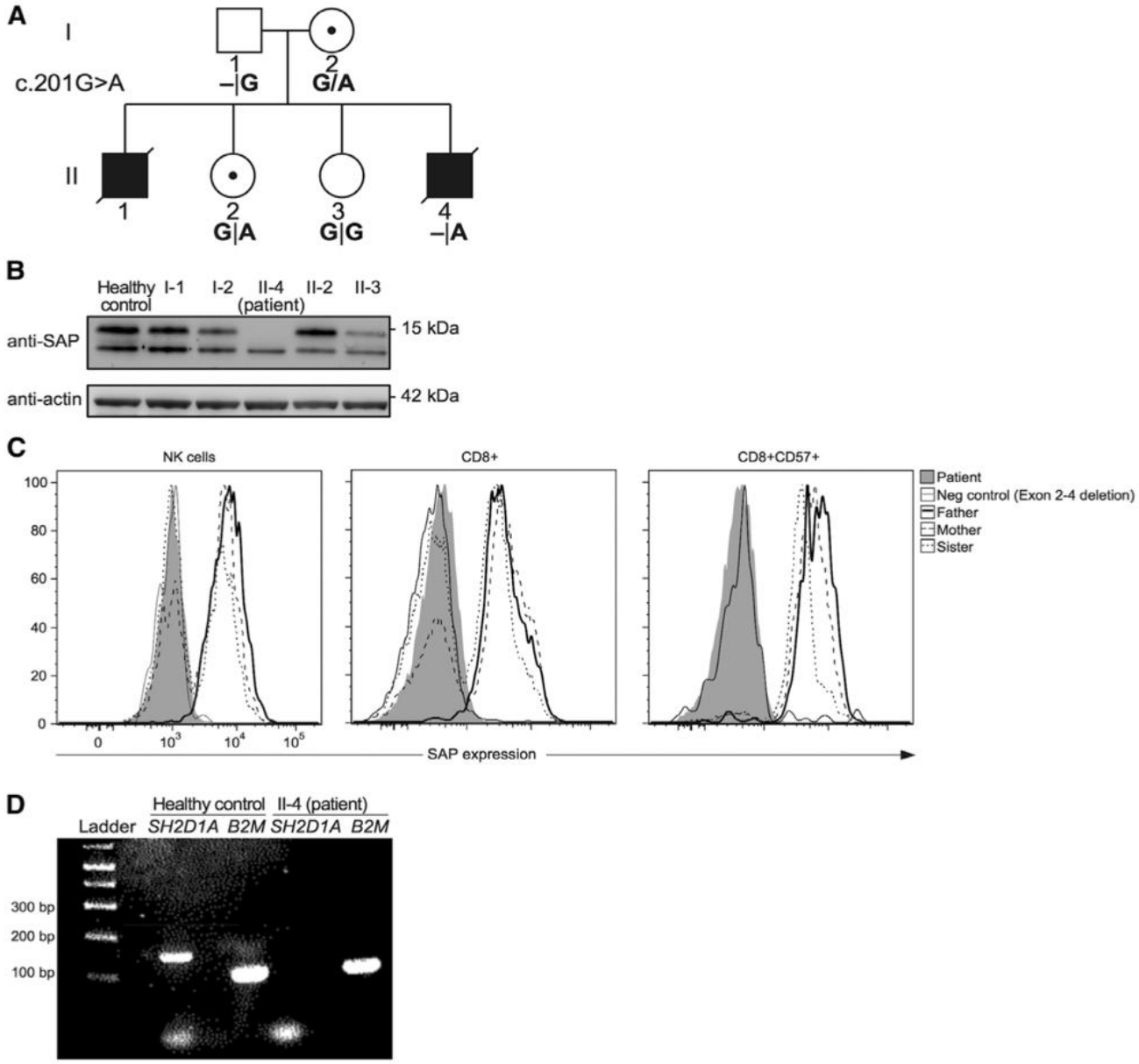

FIGURE 1. Pedigree and molecular diagnostics. A: Pedigree of the family including *SH2D1A* genotype. B: Analysis of SAP expression in PBMC lysates by Western blot in family members. C: Flow cytometric evaluation of SAP expression in NK and T cell subsets in controls and family members, as indicated. D: Sanger sequencing traces covering the patient *SH2D1A* mutation. E: PCR on PBMC-derived cDNA from a healthy control as well as the index patient using primers for *SH2D1A* and *B2M*, as indicated.

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Congruently, a hemizygous *SH2D1A* c.201G>A (p.E67E) synonymous mutation in exon 2 was identified (Fig. 1D), disrupting splicing of exon 2 as evaluated by reverse transcription–PCR (Fig. 1E). The lack of exon 2 in *SH2D1A* mRNA results in a SAP protein lacking the crucial SH2 domain, as previously described by Debeljak et al.[6]

The patient was treated according to the mature B cell lymphoma 96 (LMB 96) protocol and achieved complete remission. The patient was proposed for hematopoietic stem cell transplantation (HSCT), but 6 months after completing the lymphoma treatment, he presented with a short-term memory deficit. Magnetic resonance imaging revealed multiple infra and supratentorial edematous and hemorrhagic lesions with restricted diffusion (Fig., Supplemental Digital Content 3, http://links.lww.com/INF/D234).

Extensive investigation failed to identify any infectious agent. PCR on multiple samples (blood, stools, respiratory secretions, cerebrospinal fluid [CSF], brain tissue) were negative for EBV, CMV, adenovirus, HHV6-8, enterovirus, HTLV, LCMV, HSV, VZV, parvovirus, JC and BK virus, arbovirus, bacterial 16s DNA, *Rickettsia, Bartonella, Coxiella and Leptospira*. Cultures from blood, stools, CSF, and brain biopsy were also negative. Examination of the Vβ T cell repertoire in peripheral blood did not detect any clonal expansions (data not shown). A cerebral stereotactic biopsy revealed signs of vasculitis, with perivascular invasion by $CD8^+$ T lymphocytes, without B-cells (Fig., Supplemental Digital Content 4, http://links.lww.com/INF/D235). Epstein-Barr encoding region staining for EBV in this biopsy was negative.

The patient received broad-spectrum antibiotics and acyclovir, as well as an aggressive treatment with intravenous immunoglobulin, high-dose steroids, rituximab and intrathecal methotrexate and dexamethasone. Nonetheless, his mental and motor condition worsened. One month after the first symptoms, he was unable to walk, talk or recognize his parents. He received cyclophosphamide and alemtuzumab but continued to deteriorate. Finally, the patient became comatose. There was a notion of neurologic irreversibility, so the patient did not receive HSCT. He ultimately died 4 months after the first neurologic symptoms.

DISCUSSION

We report an XLP1 patient who presented Burkitt lymphoma followed by CNS vasculitis at 14 years of age. His brother, who died of fulminant infectious mononucleosis at 2 years of age, likely carried the same mutation. Specific *SH2D1A* mutations generally present with a spectrum of manifestations without predictable genotype-phenotype correlations.[5]

Malignant lymphomas are a well known manifestation of XLP1, being the initial manifestation of the disease in 14% of the patients.[1] Most of the cases arise in extranodal sites, with Burkitt lymphoma of the ileocecal region being the most common presentation.[1,5,7] Remarkably, 20% of these patients are allegedly seronegative for EBV.[2] A lack of correlation between EBV infection and lymphoma can be explained by the incapacity of the SAP-deficient $CD8^+$ cells to respond to B-cells.[2] Recent studies have determined that 3% of male patients with B-cell non Hodgkin lymphoma harbour a *SH2D1A* mutation.[7]

Central nervous system vasculitis is a very rare manifestation of XLP1. To our knowledge, there are only 8 previous patients with XLP1 manifesting central nervous system vasculitis (Table, Supplemental Digital Content 5, http://links.lww.com/INF/D236).[8–10] In the first descriptions, it was proposed that the vasculitis was a complication of EBV infection of the cerebral vessel walls (as a result of defective viral clearance) or by aberrant activation of cytotoxic $CD8^+$ T cells by EBV-infected B cells. However, in subsequent reports, most of the cases had no evidence of ongoing EBV infection. The first 2 case reports to propose that CNS vasculitis could occur independent of active EBV infection did not perform PCR analysis of the CSF or brain tissue. In 2009, Talaat et al[10] reported 2 patients with XLP1 that developed CNS vasculitis in the absence of ongoing EBV infection, substantiated by negative PCR in the affected tissues. We found EBV PCR repeatedly negative in the blood, CSF or brain tissue, as was Epstein-Barr encoding region in the brain tissue. Thus, our results substantiate the notion that severe manifestations of XLP1, including CNS vasculitis, may occur irrespective of EBV status.

Our report strengthens the idea that CNS vasculitis may occur irrespective of the EBV status of the recipient. The mechanisms that drive this fatal outcome in XLP1 patients remain obscure. Notably, reactivation-induced cell death is impaired in SAP-deficiency.[2,5] Thus, we speculate that SAP-deficiency renders autoreactive T cells directed against vascular antigens resistant to apoptosis, potentially contributing to this rare clinical manifestation of XLP1.[4,9]

Notably, our patient is the fourth (4/9) reported to develop CNS vasculitis after completing chemotherapy for Burkitt lymphoma. Opportunistic infections could be responsible for promoting aberrant immune responses, that is, CNS vasculitis. However, the fact that the abnormal cytotoxic responses in XLP1 are mostly related to antigens presented by B cells and that most pathogens are not restricted to these cells argues against this hypothesis. Moreover, we performed an extensive microbiologic investigation that was negative, which may rather suggest an aberrant response to endogenous ligands.

In summary, our report confirms that CNS vasculitis is a fatal manifestation of XLP1 that may occur in the absence of active EBV infection. Following diagnosis, HSCT should therefore be performed in a timely manner to avoid potentially fatal complications of the disease.

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METHYLOTROPH INFECTIONS AND CHRONIC GRANULOMATOUS DISEASE

SYNOPSIS

Methylotroph Infections and Chronic Granulomatous Disease

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Chronic granulomatous disease (CGD) is a primary immunodeficiency caused by a defect in production of phagocyte-derived reactive oxygen species, which leads to recurrent infections with a characteristic group of pathogens not previously known to include methylotrophs. Methylotrophs are versatile environmental bacteria that can use single-carbon organic compounds as their sole source of energy; they rarely cause disease in immunocompetent persons. We have identified 12 infections with methylotrophs (5 reported here, 7 previously reported) in patients with CGD. Methylotrophs identified were *Granulibacter bethesdensis* (9 cases), *Acidomonas methanolica* (2 cases), and *Methylobacterium lusitanum* (1 case). Two patients in Europe died; the other 10, from North and Central America, recovered after prolonged courses of antimicrobial drug therapy and, for some, surgery. Methylotrophs are emerging as disease-causing organisms in patients with CGD. For all patients, sequencing of the 16S rRNA gene was required for correct diagnosis. Geographic origin of the methylotroph strain may affect clinical management and prognosis.

Chronic granulomatous disease (CGD) is a primary immunodeficiency characterized by recurrent infections of the lung, skin, lymph nodes, and liver, as well as granulomatous inflammation affecting those organs and hollow viscera. The immunodeficiency results from deficiencies in any 1 of the 5 subunits forming the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase 2 (Nox2)–based complex, which leads to impaired production of reactive oxygen species in phagocytes. Defects in the Nox2 (gp-91^{phox}) enzymatic subunit (*CYBB* [cytochrome b-245, β polypeptide]) are inherited in an X-linked manner, whereas defects in subunits p47^{phox} (*NCF1* [neutrophil cytosolic factor 1]), p22^{phox} (*CYBA* [cytochrome b-245, α polypeptide]), p67^{phox} (*NCF2* [neutrophil cytosolic factor 2]), and p40^{phox} (*NCF4* [neutrophil cytosolic factor 4]) are inherited in an autosomal recessive manner (*1*,*2*).

CGD infections are often caused by a characteristic group of pathogens, including *Staphylococcus aureus*, *Serratia marcescens*, *Burkholderia cepacia* complex, *Nocardia* spp., and *Aspergillus* spp. (*1*). However, new pathogens are emerging, and some reportedly are found almost exclusively in patients with CGD. Methylotrophs are bacteria that can use single-carbon organic compounds as their sole source of energy, the widespread availability of which makes these organisms versatile environmental inhabitants. However, they rarely cause disease in immunocompetent persons (*3*).

We previously reported 7 methylotroph infections in patients with CGD and here describe 5 more (Table). From these 12 infections, we have isolated the methylotrophs *Granulibacter bethesdensis*, *Acidomonas methanolica*, and *Methylobacterium lusitanum*. These infections were difficult to diagnose and required prolonged courses of antimicrobial drugs and sometimes surgery for complete resolution.

Patient 1

In 2008, a 1-year-old girl from Mexico who had p67^{phox}-deficient CGD was examined for fever, weight loss, and enlarged cervical lymph nodes; she had been receiving ceftriaxone, clindamycin, itraconazole, and interferon-γ for treatment of CGD. CGD was diagnosed when she was 6 months of age, at which time she had *Penicillium* sp. pneumonia and an abnormal dihydrorhodamine oxidation assay result. At 3 weeks of age, she had had a methicillin-sensitive *S. aureus* labial abscess, followed at 8 months of age by 3 episodes of pneumonia and an *S. marcescens* buttock abscess.

A computed tomographic (CT) scan performed at the time of admission showed cervical, mediastinal, and mesenteric lymphadenopathy and a right middle lung lobe infiltrate and hepatosplenomegaly. Excisional cervical lymph node and lung biopsy samples were processed for bacterial,

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nocardial, fungal, and mycobacterial cultures and staining. Direct Gram staining revealed moderate mononuclear cells but no organisms. After 11 days of incubation on chocolate agar at 37°C, the lung biopsy culture grew 1 pink colony. Species level identification conducted by full 16S rRNA gene sequencing (≈1,500 bp) showed a 99.8% match to the *M. lusitanum* type strain (*7*). Etest (bioMérieux Diagnostics, Marcy l'Etoile, France) showed the following MICs (in μg/mL): amikacin (MIC = 4), cefepime (MIC = 8), ceftriaxone (MIC = 2), ciprofloxacin (MIC = 16), piperacillin-tazobactam (MIC = 2), imipenem (MIC = 2), meropenem (MIC ≥32), trimethoprim/sulfamethoxazole (MIC >32), and aztreonam (MIC >256). Culture of the cervical lymph node biopsy sample grew *S. marcescens*.

After 7 months of treatment with ceftriaxone, clindamycin, and itraconazole, the patient completely recovered from

Table. Summary of methylotroph infections in patients with chronic granulomatous disease*

Patient no., reference	Patient age, y/sex	CGD genetics†	Clinical findings	Microbiological findings	Treatment‡	Outcome
1 (this study)	1/F	Autosomal recessive (*NCF2* c.304C>T; p.R102X)	Pneumonia, cervical lymphadenitis	*Methylobacterium lusitanum* (lung), *Serratia marcescens* (lymph node)	CRO/CLI/ITZ, HSCT	Recovered
2 (this study)	19/M	X-linked (*CYBB* intragenic deletion)	Necrotizing cervical lymphadenitis	*Granulibacter bethesdensis; Staphylococcus epidermidis*	VAN/CRO, CFD/DOX/RIF	Recovered
3 (this study)	16/M	X-linked (*CYBB* 13-exon deletion)	Meningitis	*G. bethesdensis*	MEM/CIP/AMK/DOX/TEC/VCZ, MEM/CIP/AMK/DOX/CSP/ LAMB/LZD/RIF/INH/CLR	Died
4 (this study)	9/M	X-linked (*CYBB* point mutation; exon 10)	Cervical abscess, lymphadenitis	*Acidomonas methanolica*	TZP/VAN/LAMB/CIP, MEM/VAN/LAMB/CIP, CIP/VCZ/TMPSMX/ IFN-γ, HSCT	Recovered
5 (this study)	36/M	X-linked (*CYBB* c.1139 G>A; p.W380X)	Multifocal lymphadenitis	*G. bethesdensis; S. epidermidis*	VAN/CRO, CRO/DOX/TMPSMX/ITZ	Still receiving treatment as of 2015
6 (*4*)	10/M	X-linked (*CYBB* p.Arg226X)	Necrotizing cervical lymphadenitis	*A. methanolica*	TMPSMX/CRO/DOX, TMPSMX/RFB/GEN	Recovered
7 (*5*)	10/M	X-linked	Bacteremia	*G. bethesdensis*	TMPSMX/CAZ/MTZ/LZD/VCZ	Died
8 (*6*)	39/M	X-linked	Necrotizing cervical, mediastinal, axillary lymphadenitis	*G. bethesdensis*	MEM/DOX, CRO/DOX	
9 (*6*)	36/M	X-linked	Multifocal necrotizing lymphadenitis, splenic lesions, ascites	*G. bethesdensis*	MEM/TMPSMX/ITZ, MEM/TMPSMX/VCZ/TOB, CRO/TMPSMX/IFN-γ, CPD/DOX/TMPSMX/IFN-γ, splenectomy/TGC	Recovered
10 (*6*)	13/M	X-linked	Necrotizing thoracic lymphadenitis	*G. bethesdensis, S. epidermidis, Candida glabrata, Streptococcus mitis group*	MEM/VCZ, CRO/TOB/DOX/VCZ, CRO, DOX, CRO, CFD	Recovered
11 (*6*)	17/M	X-linked	Necrotizing cervical and mediastinal lymphadenitis	*G. bethesdensis*	LVX, DOX, lymph node excision	Recovered
12 (*6*)	37/M	X-linked	Necrotizing supraclavicular lymphadenitis, splenic and liver lesions	*G. bethesdensis*	CRO/GEN/VAN, CRO/DOX/TMPSMX, CFD/DOX/TMPSMX, DOX/TMPSMX	Recovered

*AMK, amikacin; CAZ, ceftazidime; CFD, cefdinir; CGD, chronic granulomatous disease; CLI, clindamycin; CLR, clarithromycin; CIP, ciprofloxacin; CPD, cefpodoxime; CRO, ceftriaxone; CSP, caspofungin; *CYBB*, cytochrome b-245, β polypeptide; DOX, doxycycline; GEN, gentamicin; HSCT, hematopoietic stem cell transplant; IFN-γ, interferon- γ; INH, isoniazid; ITZ, itraconazole; LAMB, liposomal amphotericin B; LVX, levofloxacin; LZD, linezolid; MEM, meropenem; MTZ, metronidazole; *NCF2*, neutrophil cytosolic factor 2; RFB, rifabutin; RIF, rifampin; TEC, teicoplanin; TGC, tigecycline; TMPSMX, trimethoprim/sulfamethoxazole; TOB, tobramycin; VAN, vancomycin; VRZ, voriconazole.
†Information in parentheses indicates, when known, the mutation that led to CGD.
‡Slashes separate drugs in the same regimen; commas separate regimens.

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the pneumonia and cervical lymphadenitis. She subsequently underwent successful hematopoietic stem cell transplant.

Patient 2

In 2011, a 19-year-old white man from Ohio, USA, who had X-linked CGD was examined for right neck swelling and tenderness (2 weeks' duration), a yellow ulcerated lesion on the right side of the hard palate, and an enlarged right tonsil with copious exudate. He had been receiving prophylactic trimethoprim-sulfamethoxazole and posaconazole. Erythrocyte sedimentation rate (ESR) was 28 mm/h, and C-reactive protein (CRP) concentration was 82 mg/L.

CGD had been diagnosed at birth on the basis of a positive family history. The patient had had hydrocephalus, catheter-associated fungal meningitis, and *Aspergillus fumigatus* pneumonia. When he was 15 years of age, CGD proctitis developed. Fourteen months before hospital admission, he had undergone right neck dissection for *Rothia aeria* infection, which was successfully treated with β-lactams (*8*).

CT images showed new bulky lymphadenopathy in the right neck, involving all nodal planes, and increased thickening and asymmetry of the right oropharynx with hypoattenuation of the right palatine tonsils. Culture of the right tonsillar exudate and empirical treatment with meropenem were not helpful. Antimicrobial therapy was switched to ceftriaxone and high-dose penicillin for empirical coverage of *G. bethesdensis* and *Actinomyces* spp. Right neck dissection with tonsillectomy yielded *Staphylococcus epidermidis*, and full 16S rRNA gene sequencing (≈1,500 bp) of 1 colony of a gram-negative bacillus showed a 99.8% match to the *G. bethesdensis* type strain. Nine weeks of vancomycin and ceftriaxone followed by 8 weeks of cefdinir, doxycycline, and rifampin led to complete resolution of the lymphadenitis.

Patient 3

In 2012, a 16-year-old boy from Portugal who had X-linked CGD was examined for fever, cervical lymphadenopathy, and elevated inflammatory markers. CT images showed a deep cervical abscess, from which nothing grew on culture but which completely resolved after 5 weeks of intravenous ceftriaxone, doxycycline, and ciprofloxacin and 6 weeks of oral amoxicillin/clavulanate, ciprofloxacin, and doxycycline, along with prophylactic itraconazole and interferon-γ.

Immediately after completion of that course of antimicrobial drugs, pneumonia with pleural effusions developed. Results of all cultures (blood, lymph node, bronchoalveolar lavage, and pleural fluid) were negative, but full 16S rRNA gene sequencing (≈1,500 bp) of pleural fluid showed a >99% match to *Cupriavidus* spp. The patient received meropenem, ciprofloxacin, amikacin, doxycycline, teicoplanin, and voriconazole. Two weeks later, fever with splenomegaly, pancytopenia, low fibrinogen levels, and elevated ferritin and soluble CD25 levels were noted. Interferon-γ prophylaxis was discontinued and the patient was administered dexamethasone and intravenous immunoglobulin, after which the presumed exuberant inflammatory response quickly resolved. CT images of the neck and lung were unremarkable, as were positron emission tomography images.

A month later, the boy was examined for fever, cough, and altered mental status; he required intubation and transfer to the intensive care unit. Magnetic resonance imaging revealed bilateral pneumonia and multiple intraparenchymal brain abscesses. Cerebrospinal fluid (CSF) was unremarkable. A lung biopsy sample, collected while the patient was receiving meropenem, ciprofloxacin, amikacin, doxycycline, teicoplanin, and voriconazole, was sterile. Voriconazole was switched to caspofungin and liposomal amphotericin B, and teicoplanin was switched to linezolid. The patient eventually recovered and was transferred out of the intensive care unit.

One month later, fever with focal neurologic deficits developed. CSF examination confirmed persistent pleocytosis with low glucose and elevated protein levels, and CT images indicated leptomeningitis. A 4-day culture of CSF on chocolate agar showed brownish colonies 1–2 mm in diameter. Full 16S rRNA gene sequencing (≈1,500 bp) performed on the isolate from CSF showed a 99.7% match to the *G. bethesdensis* type strain. Etest showed the following MICs (in μg/mL): tobramycin (MIC = 12), ceftriaxone (MIC >32), doxycycline (MIC = 24), and trimethoprim/sulfamethoxazole (MIC = 0.25). Isoniazid, clarithromycin, and rifampin had already been added to the patient's treatment regimen. Despite the above interventions, the patient died of obstructive hydrocephalus and multiorgan failure.

Patient 4

In 2013, a 9-year-old multiracial boy from Iowa, USA, who had X-linked CGD was examined for a 1-day history of fever, fatigue, decreased appetite, headache, and neck pain. He had been receiving oral trimethoprim/sulfamethoxazole, voriconazole and interferon-γ for CGD prophylaxis. When he was 1 month of age, he had had disseminated *Candida lusitaniae* infection with retropharyngeal, parapharyngeal, hepatic, and splenic abscesses. At 6 months of age, he was hospitalized for a progressively enlarging left posterior neck mass. A lymph node biopsy sample showed necrotizing granulomata and rare yeast forms suggestive of *Histoplasma*, but no specific organism was identified. He also had recurrent otitis media, tonsillitis, and aphthous stomatitis. Two months before the visit reported here, he had had *Aspergillus versicolor* pneumonia complicated by granulomatous appendicitis.

At the time of this hospital admission, he had 2 enlarged right anterior cervical nodes, which were soft, mobile, and not tender. ESR was 41 mm/h, and CRP concentration was 76 mg/L. CT images showed a 23 × 9 × 18-mm abscess adjacent to the right sternocleidomastoid muscle, extensive left supraclavicular lymphadenopathy, and left-sided pneumonia and pleural effusion.

Excisional biopsy of the right cervical lymph nodes yielded pus but no organisms. After 3 days of culture on chocolate agar, ≈20 tan colonies of an aerobic gram-negative bacillus were seen. The organism was oxidase-positive, catalase-positive, and indole-negative. After 6 days, abundant growth of a morphologically identical organism was seen on potato dextrose agar without antimicrobial agent and on Mycosel agar with chloramphenicol and cycloheximide but not on brain heart infusion agar with chloramphenicol and gentamicin (all media from Remel, Lenexa, KS, USA). Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (Biotyper system version 3.1; Bruker Daltonics Inc., Billerica, MA, USA) from directly smeared colonies with and without formic acid overlay (*9*) yielded no identification, and growth was insufficient for biochemical identification or susceptibility testing. Species-level identification conducted by 16S rRNA gene sequencing (ABI MicroSeq 500 kit; Thermo Fisher Scientific, Grand Island, NY, USA, and the IDNS SmartGene system, version 3.6.10; SmartGene Inc., Raleigh, NC, USA) was interpreted as *A. methanolica* (100% identity >388 bp with type strain CGDAM1) (*7*). No other pathogens were grown or amplified from any specimen.

The patient initially received piperacillin/tazobactam and vancomycin; liposomal amphotericin B was administered in view of his recent *A. versicolor* pneumonia. After 4 days, the piperacillin/tazobactam was switched to meropenem and ciprofloxacin was added. Levels of inflammatory markers eventually returned to reference values, and the lymphadenopathy improved after 5 weeks of intravenous meropenem and intravenous and oral ciprofloxacin. The patient was discharged with ciprofloxacin, voriconazole for *A. versicolor* pneumonia, prophylactic trimethoprim/sulfamethoxazole, and interferon-γ. He subsequently underwent successful transplant of matched unrelated hematopoietic stem cells.

Patient 5

In 2014, a 36-year-old white man from Georgia, USA, who had X-linked CGD was hospitalized with cervical and abdominal lymphadenopathy; he had 1-year history of fever, malaise, and weight loss. X-linked CGD had been diagnosed when the patient was 6 months of age, when he had had hepatosplenomegaly, cervical lymphadenitis, and recurrent *S. aureus* infections. *Aspergillus* spp. pneumonia developed when he was 2 years of age, a liver abscess required incision and drainage when he was 4 years of age, and multiple abdominal abscesses required surgery when he was 6 years of age. Prophylactic trimethoprim/sulfamethoxazole for CGD had been effective until the hospitalization reported here.

A year before admission, the patient had experienced sudden onset of fever, cough, and shortness of breath. Right-sided pleural effusion was treated with antimicrobial drugs and thoracentesis. Cough and dyspnea improved, but fever, fatigue, malaise, myalgia, and weight loss of >15 kg were refractory to hydroxychloroquine and interferon-γ. At 2 months before admission, he had had cervical and abdominal lymphadenopathy with ascites. Peritoneal fluid examination, esophagogastroduodenoscopy, and colonoscopy were not informative. Excisional biopsy of a right posterior cervical lymph node, tuberculin skin testing, and QuantiFERON–TB Gold (QIAGEN, Valencia, CA, USA) testing produced negative results. Ciprofloxacin and metronidazole did not abate symptoms and fever.

At admission, the patient had right supraclavicular and left axillary lymphadenopathy and hepatosplenomegaly. ESR was 88 mm/h, and CRP concentration was 131 mg/L. CT images showed lung scarring, splenomegaly, pericardial effusion, and multifocal adenopathy of the left axilla, mediastinum, celiac, periaortic, retroperitoneal, and mesenteric regions. Culture of excised axillary lymph node grew *S. epidermidis,* and *G. bethesdensis* (100% match to the *G. bethesdensis* type strain by full 16S rRNA gene sequencing, ≈1,500 bp). Etest of *G. bethesdensis* isolate showed the following MICs in μg/mL: tobramycin (MIC = 4), ceftriaxone (MIC = 32), doxycycline (MIC = 8), trimethoprim/sulfamethoxazole (MIC = 2), and tigecycline (MIC = 16).

The patient was empirically administered vancomycin and ceftriaxone and was discharged with ceftriaxone, doxycycline, prophylactic trimethoprim/sulfamethoxazole, and itraconazole. His medication was eventually switched to cefdinir along with CGD prophylaxis. After 15 months, inflammatory markers and left neck and supraclavicular lymphadenopathy had improved but had not yet normalized.

Discussion

We have identified a total of 12 infections caused by 3 methylotroph bacteria in patients with CGD: 2 *A. methanolica*, 1 *M. lusitanum*, and 9 *G. bethesdensis* infections. Infections caused by *A. methanolica* and *G. bethesdensis* have been reported only for patients with CGD, whereas *M. lusitanum* in a patient without CGD undergoing chemotherapy for leukemia has been reported (*10*). These observations suggest that Nox2-based complex activity (superoxide production) is critical for protection against methylotroph infections. Consistent with this hypothesis, previous studies have demonstrated that *G. bethesdensis* persists in Nox2-based complex-deficient myeloid cells

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and is largely resistant to oxygen-independent microbicidal activity (*11,12*).

Methylotroph infections in CGD patients typically result in elevated inflammatory markers and lymphadenopathy, which may progress to necrotizing lymphadenitis with or without abscess formation. The clinical course may be protracted because of infection persistence, antimicrobial drug resistance, and relapse. Culturing these bacteria is difficult, requiring atypical media and prolonged incubation. These infections have not been fatal for patients in North and Central America, but *G. bethesdensis* infections in patients from Portugal and Spain (*5*) have caused fatal meningitis and bacteremia, respectively. The different clinical courses and outcomes for these patients compared with those in North and Central America suggest that the *G. bethesdensis* strain from Europe may be more virulent; animal studies are needed to explore this possibility. Furthermore, the strains isolated in Spain and Portugal showed only a 99.7% match with the 16S rRNA sequence of the type strain from North America, whereas most strains from North and Central America showed a 100% match with the type strain. Moreover, the strain from Europe displayed more in vitro resistance to antimicrobial agents and was resistant to colistin, most β-lactams, and quinolones (*5*). Although the value and accuracy of in vitro susceptibility testing for *G. bethesdensis* are unknown and may lack predictive value, the clinical and laboratory differences between *G. bethesdensis* strains from the United States and Europe may have substantial implications for therapy.

The facultative methylotrophs we report were difficult to culture; their correct identification required non–culture-based techniques, such as 16S rRNA gene sequencing, and a high index of suspicion. Application of 16S rRNA sequencing and molecular probes to target tissues may identify previously unrecognized bacteria, which may accompany and possibly facilitate methylotroph infections.

G. bethesdensis is a gram-negative, aerobic, oxidase-negative, and catalase positive bacillus. Culture is facilitated by specimen centrifugation and plating on buffered charcoal yeast extract agar or solid mycobacterial media; incubation takes up to 2 weeks (*6*). *A. methanolica* is a gram-negative, aerobic, acid-tolerant, catalase-positive, urease-positive, oxidase-positive, non–spore-forming, and nonmotile rod-shaped bacterium (*13*). It forms tan colonies in <5 days on chocolate agar and grows well on potato dextrose agar. *M. lusitanum* is a vacuolated gram-negative aerobic rod that is positive for indophenol oxidase, catalase, and urease and produces a pink pigment. It also reduces nitrate and assimilates malate (*10*). Methanol dehydrogenase serology is currently under investigation as a potential supportive diagnostic or prognostic tool for tracking methylotroph infections, particularly those caused by *G. bethesdensis* (*14*).

Management of methylotroph infections is often prolonged and may require combination antimicrobial drug therapy and surgery. Drug susceptibilities are difficult to determine and interpret. In vitro, *G. bethesdensis* is typically resistant to most penicillins, cephalosporins, carbapenems, and quinolones, but it is sometimes susceptible in vitro to ceftriaxone, aminoglycosides, doxycycline, and trimethoprim/sulfamethoxazole; combinations of these drugs have helped achieve initial resolution (*6*). Although antimicrobial drug susceptibilities for *A. methanolica* are not well defined, infections seemed to respond to combinations including meropenem, ciprofloxacin, gentamicin, doxycycline, and trimethoprim/sulfamethoxazole. *M. lusitanum* seems to be susceptible to aminoglycosides, cephalosporins, ciprofloxacin, piperacillin/tazobactam, and imipenem but not to meropenem, trimethoprim/sulfamethoxazole, or aztreonam. The role of surgery in treating methylotroph infections has not been defined, but its successful use in several cases is noteworthy.

In conclusion, methylotrophs are environmental organisms that can cause necrotizing infections in patients with CGD. Infectious prodromes and clinical courses may be prolonged. Diagnosis requires a high index of suspicion so that appropriate culture conditions and culture-independent techniques can be established for diagnosis. The difficulty of growing methylotrophs from infected lesions gives pause for the use of the label "sterile inflammation" with regard to CGD patients. Methylotrophs should be aggressively sought as the cause of chronic necrotizing infections in patients with CGD.

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FATAL MENINGITIS IN A PATIENT WITH X-LINKED CHRONIC GRANULOMATOUS DISEASE CAUSED BY A VIRULENT *GRANULIBACTER BETHESDENSIS* STRAIN

Fatal Meningitis in Patient with X-Linked Chronic Granulomatous Disease Caused by Virulent *Granulibacter bethesdensis*

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Granulibacter bethesdensis is a pathogen reported to cause recurrent lymphadenitis exclusively in persons with chronic granulomatous disease. We report a case of fatal meningitis caused by a highly virulent *G. bethesdensis* strain in an adolescent in Europe who had chronic granulomatous disease.

Chronic granulomatous disease (CGD) is a primary immunodeficiency characterized by a deficient nicotinamide adenine dinucleotide phosphate oxidative burst that impairs phagocyte superoxide formation and killing of certain pathogens. Mutations can occur in any of the 5 subunits of nicotinamide adenine dinucleotide phosphate oxidase. Most cases are inherited as X-linked defects (gp-91^{phox}), but they also can occur in an autosomal recessive manner (*1*). Increased susceptibility develops to recurrent infections of the skin, lymph nodes, lungs, and other organs (*2*), mostly caused by bacteria and fungi, including *Staphylococcus aureus*, *Serratia marcescens*, *Burkholderia cepacia*, *Salmonella* spp., *Nocardia* spp., and *Aspergillus* spp. (*2*). Emerging organisms, such as *Granulibacter bethesdensis* and other methylotrophs, occur almost exclusively in CGD patients (*3*,*4*).

G. bethesdensis was first described in 2006, when it was isolated in a CGD patient with lymphadenitis (*4*). It is a gram-negative, aerobic, oxidase-negative, catalase-positive, nonmotile coccobacillus to rod-shape bacterium belonging to the *Acetobacteraceae* family (*5*,*6*). *G. bethesdensis* was the first of these *Acetobacteraceae* family bacteria with proven pathogenicity in humans, causing invasive disease in CGD patients and mice (*4*). It has been mostly linked to indolent nonfatal lymphadenitis and deep neck infections in patients in North America. The infection can recur over several years by reactivation of the same strain or reinfection with different strains (*3*,*7*–*9*). The first fatal infection was reported in a 10-year-old boy from Spain, who died of fulminant sepsis (*10*). In vitro, *G. bethesdensis* shows extensive resistance to various antimicrobial drugs, although its slow growth makes susceptibility testing difficult. Ceftriaxone, aminoglycosides, doxycycline, and trimethoprim/sulfamethoxazole showed activity in vitro (*7*).

We report a case of *G. bethesdensis* meningitis in a patient with X-linked CGD. We also report animal data comparing this *G. bethesdensis* strain with the strain recovered from recurrent lymphadenitis in a US CGD patient.

The Study

The patient was a 16-year-old boy whose X-linked CGD (CYBB exon 13 deletion) was diagnosed when he was 2 years old. His disease had been well-controlled with cotrimoxazole, itraconazole, and interferon-γ. In September 2014, he was hospitalized with a deep cervical abscess (Figure 1, panel A) and received a 5-week course of intravenous ciprofloxacin, doxycycline, and ceftriaxone that resulted in complete clinical and radiologic resolution, followed by 6 weeks of oral amoxicillin/clavulanic acid, doxycycline, and ciprofloxacin along with his usual prophylaxis. No pathogen was identified despite blood cultures, bronchoalveolar lavage, and lymph node biopsy cultures and broad-range bacterial PCR.

After this regimen was completed, the boy was readmitted for 8 weeks with pneumonia with pleural effusion (Figure 1, panel B). Full 16S rRNA gene sequencing (≈1,500 bp) identified *Cupriavidus* spp. in pleural fluid. He received meropenem, amikacin, ciprofloxacin, teicoplanin, doxycycline, and voriconazole, and his condition improved. However, 2 weeks later, fever returned, along with splenomegaly, hemodynamic instability, pancytopenia,

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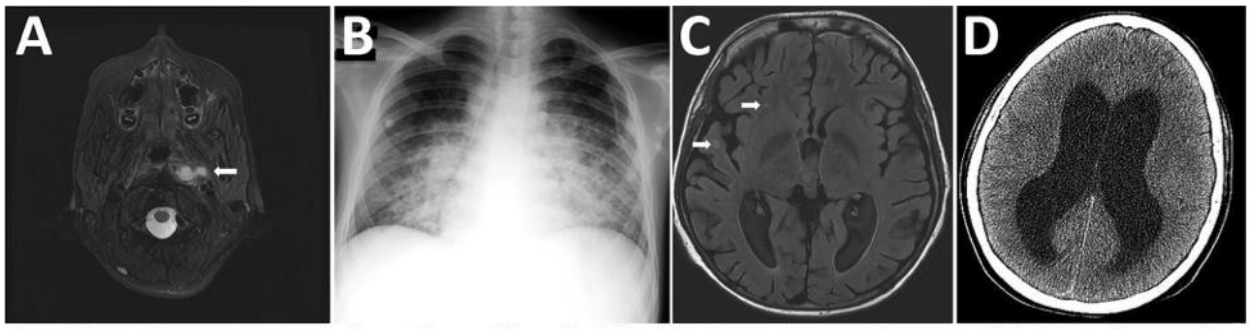

Figure 1. Most relevant imaging results from a 16-year-old boy with X-linked chronic granulomatous disease who died of meningitis caused by a virulent *Granulibacter bethesdensis* strain. A) Computed tomography image showing deep cervical abscess (arrow), July 2012. B) Radiograph showing pneumonia with pleural effusion, October 2012. C) Cranial magnetic resonance image showing multiple intraparenchymal brain abscesses (arrows), December 2012. D The patient died of obstructive hydrocephalus (shown) and multiorgan failure, April 2013.

hypofibrinogenemia, hyperferritinemia, and elevated soluble CD25. He received intravenous immunoglobulin and dexamethasone for this inflammatory condition and fully recovered. Neck and lung computed tomography images and positron emission tomography performed 1 month later showed no signs of active infection.

Nevertheless, a few days later, the patient sought care for altered mental status, hallucinations, aggressiveness, and respiratory instability requiring admission to the pediatric intensive care unit. He had extensive bilateral pneumonia and multiple intraparenchymal brain abscesses (Figure 1, panel C). Meropenem, ciprofloxacin, amikacin, doxycycline,

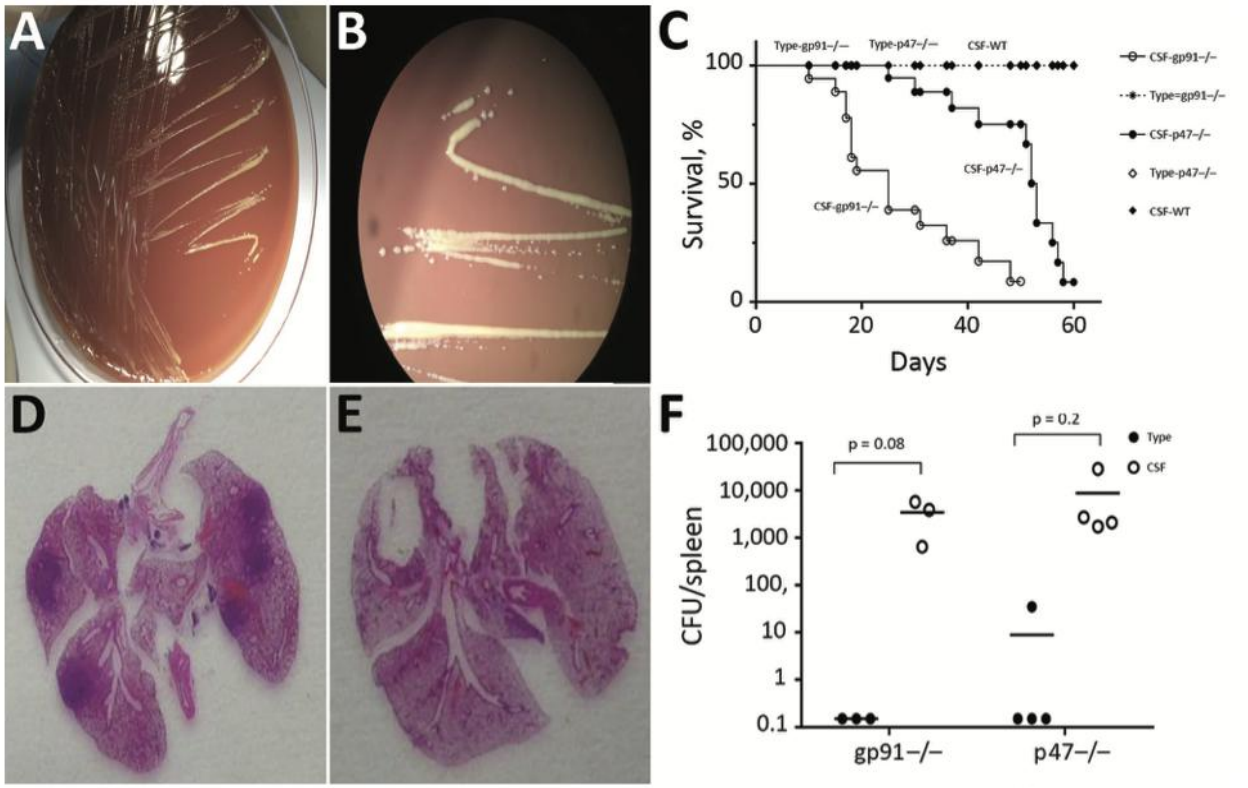

Figure 2. *Granulibacter bethesdensis* colonies and pathology results after inoculation on mouse models. A, B) CSF on chocolate agar for 4 days showed slowly growing brown-yellow, shiny colonies 2–4 mm in diameter. Full 16S RNA sequencing (≈1,500 bp) led to identification of *G. bethesdensis* (with 99.7% match). C) Survival of Gp91/p47 KO mice after inoculation of different *G. bethesdensis* strains. D) Pathology images of gp91KO mouse lung after CSF strain infection. E) Pathology images of gp91KO mouse lungs after type strain infection. F)Quantification of *G. bethesdensis* strains in spleens of Gp91/p47 KO mice after inoculation. CSF, cerebrospinal fluid; type, National Institutes of Health type strain; WT, wild type.

Table. Bacterial culture from blood and brain samples of GP91KO mouse infected with *Granulibacter bethesdensis* after 4 weeks*

Mouse	Strain	Sample	
		Blood	Brain
gp91$^{-/-}$, n = 10	Type, USA	0	0
gp91$^{-/-}$, n = 10	**CSF, Portugal**	**5**	**2**
p47$^{-/-}$, n = 5	Type	0	0
p47$^{-/-}$, n = 5	CSF	0	0
WT, n = 9	CSF, Portugal	0	ND

*Bold indicates the results of the inoculation of CSF strain in the gp91 KO mouse. CSF, cerebrospinal fluid; ND, not done; WT, wild type.

teicoplanin, and voriconazole were started; results of cerebrospinal fluid (CSF) and lung biopsy samples were unremarkable. Teicoplanin was switched to linezolid and voriconazole to caspofungin and liposomal amphotericin B because of toxicity concerns. Four weeks later, he was discharged from the intensive care unit. One month later, fever, vomiting, and focal neurologic deficits developed. CSF showed pleocytosis and hypoglycorrhachia with elevated protein levels. Cerebral imaging confirmed leptomeningitis. Isoniazid, clarithromycin, and rifampin were added to his treatment regimen, but his neurologic status continued to deteriorate. Obstructive hydrocephalus (Figure 1, panel D) and multiorgan failure developed, and he died 3 months later.

CSF cultures yielded yellow-brown, shiny, small colonies (2–4 mm) on chocolate agar after 4 days incubation. (Figure 2, panels A, B). Full 16S RNA sequencing (≈1,500 bp) showed 99.7% match to the type strain of *G. bethesdensis* CGDNIH1T (ATCC BAA-1260T, DSM 17861T) from North America and 100% match to a previously reported *G. bethesdensis* strain from Spain. Nonstandardized susceptibility test using Etest, performed in Mueller Hinton agar supplemented with 5% sheep blood agar with an overnight air incubation at 37°C, showed resistance to doxycycline (MIC 24 mg/L) and ceftriaxone (MIC >32 mg/L).

We used mouse models of CGD to determine whether differences existed in immune response, pathogenicity, or severity of disease between the European (CSF strain) and the US (type strain) strains. We intraperitoneally infected gp91$^{phox-/-}$ mice with 10^7 CFU of *G. bethesdensis* type strain and p47$^{phox-/-}$ mice intraperitoneally with 10^7 CFU of CSF strain and monitored moribundity during infection. We euthanized gp91$^{phox-/-}$ mice 4 weeks and p47$^{phox-/-}$mice 8 weeeks after infection and collected brain, spleen, lung, lymph nodes, and blood for culture, bacterial enumeration, and histopathologic examination. Plasma cytokines were assayed.

Although we observed differences between gp91$^{phox-/-}$ and p47 $^{pho-/-}$ mice, both CGD mice models showed high rates of death when infected with CSF strain. No deaths occurred in mice infected with type strain, nor did CSF strain cause disease in wild-type mice (Figure 2, panel C). CSF strain–infected mice showed more severe pathologic organ changes than did type strain–infected mice (Figure 2, panels D, E; Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/25/5/18-1505-App1.pdf). We performed quantitative cultures to assess bacterial load in the spleens of inoculated mice. CSF strain CFUs were 100–1,000 times higher than those of type strain 4 and 8 weeks after infection. (Table; Figure 2, panel F). In addition, infection with CSF strain yielded 100–1,000 times higher CFUs in spleens of CGD mice than in wild-type mice 4 and 8 weeks after infection. (Appendix Figure 2). CSF strain showed faster growth on solid and in liquid media and a higher optimal growth temperature (37°C) than previously described North America lymph node isolates (Appendix Figure 3). CGD mice infected with CSF strain showed higher plasma interleukin-1β, tumor necrosis factor–α, and interleukin-6 than those infected with type strain 4 and 8 weeks after infection, which correlated with differences in tissue bacterial load. Cytokine levels did not increase in wild-type mice infected with CSF strain (Appendix Figure 4).

Conclusions

G. bethesdensis is an emerging pathogen shown to cause infection exclusively in CGD patients and has a spectrum of disease severity ranging from chronic and recurrent infections to fulminant sepsis, central nervous system infection, and death (*3*,*7*,*10*). Until recently, all reported North America cases were nonfatal chronic infections; 1 case from Europe (Spain) was fatal. Recently, Mayer et al. reported a X-linked CGD patient in the United States who died of fulminant infection with an organism with 100% identity to 500 bp of *G. bethesdensis* 16S (*11*). Unfortunately, that *G. bethesdensis* isolate was not available for analysis and comparison with other *G. bethesdensis* strains. The previous strain from Europe was highly resistant to antimicrobial agents, including colistin, most β-lactams, and quinolones (*10*).

We found that a CSF *G. bethesdensis* strain, showing an identical 16S sequence to a previously described fulminant strain from Europe, was more virulent and lethal in a mouse model than the *G. bethesdensis* US type strain and more virulent in gp91$^{phox-/-}$ than in p47 $^{phox-/-}$ mice. A fatal case of *G. bethesdensis* infection in the United States suggests that heterogeneity might exist among North America *G. bethesdensis* strains. Bacterial genome sequencing may identify discrete virulence factors. *G. bethesdensis* must be included as a cause of fatal disseminated infection in CGD.

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About the Author

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IV.III. NOVEL PIDS

A. REGNASE 1 DEFICIENCY

Introduction

The fine control of the immune response is crucial to prevent manifestations of autoimmunity and hyperinflammation. APECED[124] and IPEX[120,192] syndromes are, respectively, the prototype of the loss of central and peripheral regulation, and patients show symptoms of poly-autoimmunity, often early and life threatening. In recent years, the discovery of genetic defects of various components involved in immune tolerance has allowed us to expand our knowledge of this complex regulation. Moreover, one of the most important functions of the immune system is to mount a potent inflammatory response to protect the host against pathogens and to repair tissue damage. Nevertheless, prolonged and excessive inflammation can lead to deleterious consequences. Therefore, the regulation of inflammation is very critical and under strict regulation. The precise regulation of the cytokine production is essential for a tailor-made immune response for each new challenge.

Many stimuli that trigger the inflammatory response simultaneously trigger a programme that will resolve inflammation. Both transcriptional and post-transcriptional mechanisms can control the beginning and resolution of inflammation.[388-391] Although transcriptional activation is required for the synthesis of the encoding inflammatory effector proteins, it does not determine the level of protein expression. Instead, the post-transcriptional regulation will precisely determine the levels of protein that will be expressed.[392] Focusing on the post-transcriptional mechanisms, the regulation of mRNAs is one of the critical steps at which gene expression is controlled by proteins through multiple processes, including mRNA splicing, modification, polyadenylation, translation and mRNA degradation.[302,393-395] Among these, the control of mRNA stability, by induction of the mRNA decay mediated by Adenine/Uridine-rich elements (ARE) are among the best characterized. Many of the pro-inflammatory transcripts (such as GM-CSF, TNF, IL-2, IL-6 or IL-8) have an ARE in their 3'UTR, whose function is to stabilize or destabilize mRNA.[396] The ARE provide binding sites for transacting RNA binding proteins, which ensure proper regulation of its function.[392,396,397] Many of these ARE-

binding proteins, that are responsible for the strict regulation of their function, have been identified.[392] Some of them, like the zinc finger proteins TTP or BRF1, bind to ARE through their characteristic C3H zinc finger domains. This binding promotes the rapid degradation of transcriptes of the cytokines (such as TNFα or IL-1) by targeting them to the cellular RNA degradation machinery.[398] Mice that are deficient in TTP develop severe inflammatory diseases resulting from excessive TNFα and IL-1β production.[399,400] Another of these proteins is endoribonuclease Regnase-1, originally identified as MCPIP1, encoded by gene Zc3h12a.[401,402] This cytoplasmic protein is expressed primarily in the immune cells of both the adaptive and innate systems.[5] In macrophages, where its function has been best described, the expression of Regnase-1 mRNA is induced rapidly in a MyD88-dependent manner after LPS stimulation, and its levels then gradually decrease with time.[402,403] Upon transcription, Regnase-1 binds to the ribosome, localizes in the endoplasmatic reticulum and/or cytoplasm and degrades translationally active mRNA of different cytokines (IL-6 and IL12 are the most studied cytokines to be regulated by Regnase-1) via its endonucleolytic activity.[5] Its expression is tightly and dynamically controlled in both transcriptional and post-transcriptional levels. When there is engagement of a ligand to the TLR of the macrophage, Regnase-1 is phosphorylated and undergoes proteasomal degradation by IkB kinases or through cleavage by the paracaspase MALT-1. Recently, its regulation through a super-enhancer around the ZC3H12A gene was reported.[404] When Regnase-1 is degraded, this highly controlled mechanism allows for IL-6 mRNA to escape from degradation and it accumulates during the inflammatory responses. Once Regnase-1 is resynthesized via transcriptional activation, it promotes the degradation of cytokine mRNAs, ensuring the resolution of inflammation and homeostasis maintenance (Fig.6).[404-408]

The importance of Regnase-1 in vivo has been documented by the experiments performed in Regnase-1$^{-/-}$ mice. These mice were born at a Mendelian ratio but most of them died within 12 weeks after birth, following severe lymphoproliferation and auto-immunity.[402,409]

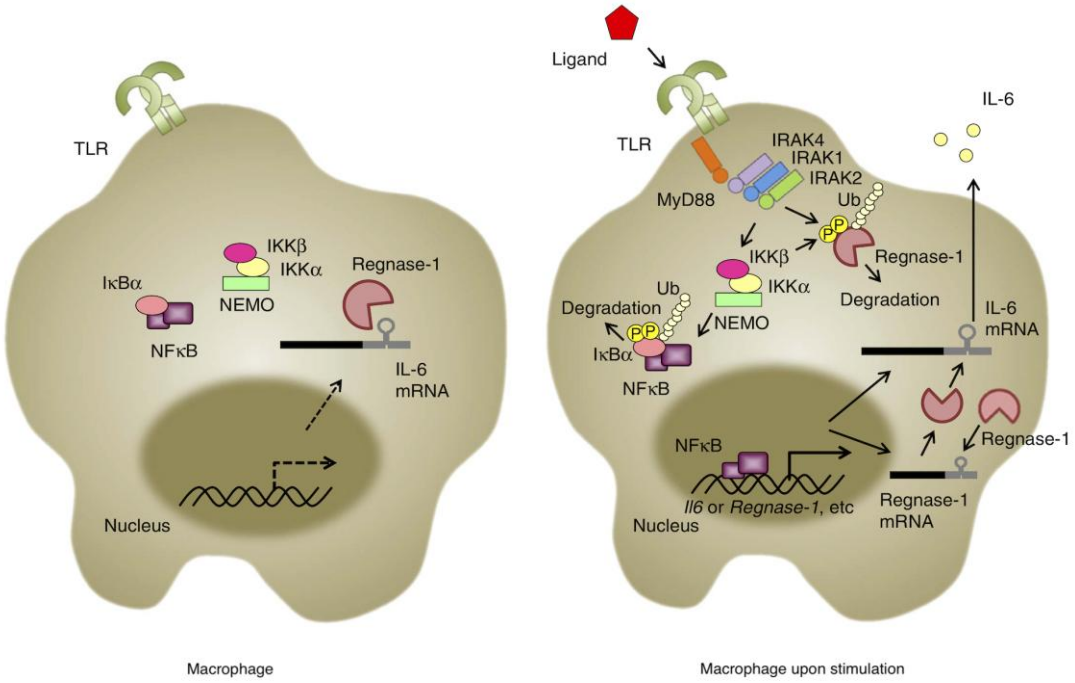


Figure 6: Proposed model of Regnase-1 function. Adapted from Uehata T et al, 2013.[5]

Case Report

The patient was the third son of a consanguineous couple (parents are first cousins), but his family history was unremarkable: his parents and older brothers (19 and 16 years-old) were healthy. At the age of 14 days he presented with a severe, non-bloody diarrhoea, severe dehydration and metabolic acidosis. He was hospitalized but no aetiology was identified despite extensive investigation. At the age of 4 weeks, he presented with Evans syndrome and splenomegaly. There was no response to IVIG, and steroids were started (prednisolone 2 mg/Kg/day) with prompt normalization of the haematological parameters, splenomegaly and diarrhoea. In the following months his clinical condition was stable but many attempts to stop steroids failed due to relapses of diarrhoea and autoimmune haemolytic anaemia and thrombocytopenia. Rituximab failed to improve his condition. The immunological investigation was inconclusive. At the age of 4 years-old he had elevation of the liver enzymes, and the following auto- antibodies were present in his peripheral blood: anti-nuclear, anti-kidney liver microsome and anti-smooth muscle. Azathioprine was started with complete normalization of the hepatic enzymes, allowing gradual

weaning steroids to < 0,5 mg/Kg/day. No hepatic biopsy was performed. During this period, he also presented recurrent otitis and chest infections (leading to bronchiectasis, Fig.7) and chronic sinusitis. Find more detailed laboratory results in annex 1.

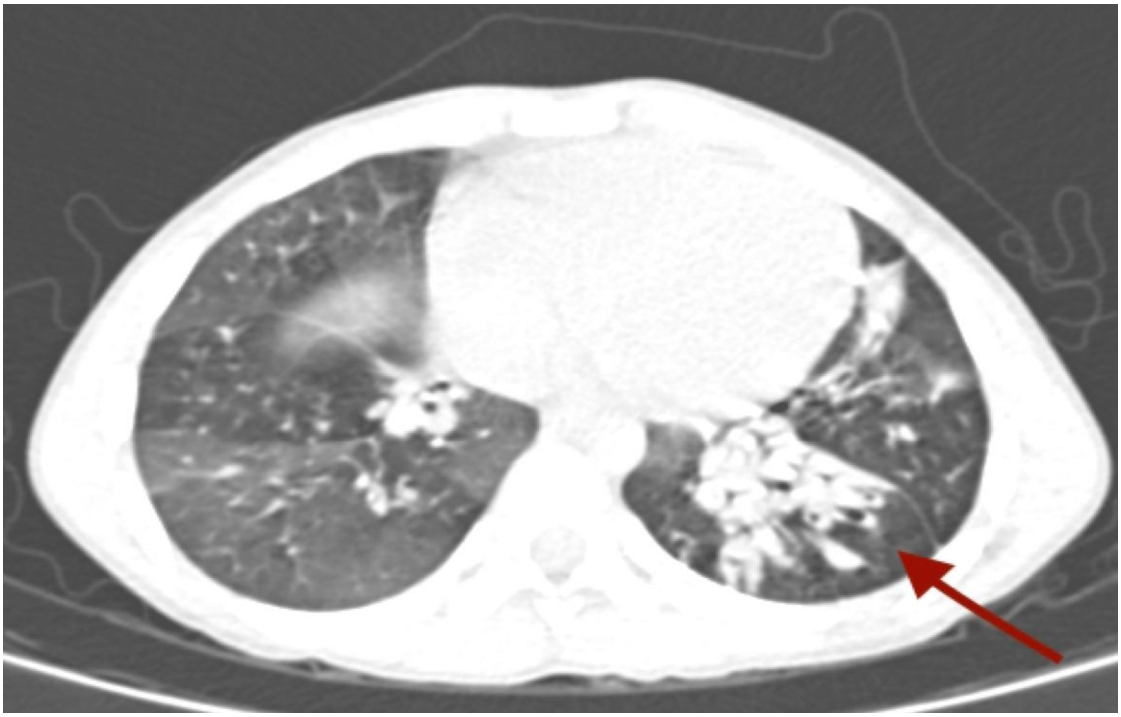


Figure 7: Bronchiectasis in the Regnase-1 deficient patient

He was then sent to our PID Unit at the age of 5. At that time, he had very high IgM and IgG, absent IgA, no response to vaccines and complement consumption. Hyper-IgM, IPEX, IPEX-like and ALPS related genes were sequenced and were normal.

At the age of 7 years-old, following an intimate contact with varicella, he had varicella despite adequate post-exposure prophylaxis. Six months later he had uncomplicated zoster (involving 2 dermatomes) and at the age of 8 he had another zoster reactivation and zoster meningitis (Fig.8).

Figure 8: Zoster in the Regnase-1 deficient patient. (photos authorized by parents after proper informed consent)

After a severe relapse of autoimmune haemolytic anaemia and splenomegaly, azathioprine was stopped and mycophenolate mofetil was started at the age of 8 and half years-old, allowing minimal dose of daily steroids. He had no relapses in the subsequent 2 years, but since then he had severe autoimmune haemolytic anaemia (with intra-medullary immune destruction) and (presumably auto-immune) myelofibrosis, as well as splenomegaly and lymphadenopathies, which were refractory to multiple therapeutic trials (azathioprine, mycophenolate, rituximab, sirolimus) and became steroid-dependant. WES revealed a homozygous mutation in the ZC3H12A gene. At that point, a trial with tocilizumab was also attempted, but again ineffective. At the age of 13, he received HSCT from a matched-related sibling donor (his brother, who did not carry ZC3H12A variants), after conditioning with alemtuzumab, treosulfan, fludarabine and thiotepa. (Fig.9)

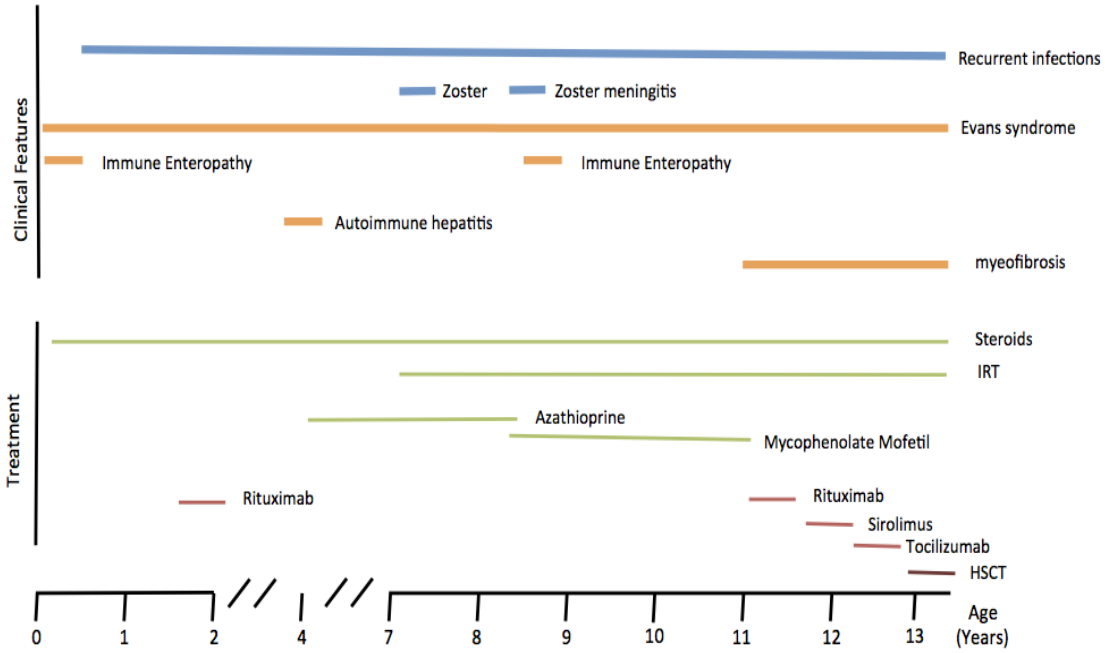


Figure 9: Clinical features and treatment of the patient (IRT=Immunoglobulin replacement therapy)

The clinical manifestations of this PID were corrected with HSCT: the autoimmune cytopenias were corrected, the myelofibrosis and splenomegaly resolved. Despite that, he developed severe skin GvHD, requiring multiple immunosupression and extracorporeal photopheresis. He eventually succumbed to an infection 15 months post-HSCT.

According to this evolution, the diagnostic hypothesis of Regnase-1 deficiency was postulated.

SPECIFIC METHODS

These experiments have been performed in collaboration with Ilie Hashim, Davide Eletto and Danijela Markovic from Sergey Nejentsev's laboratory, Cambridge University, United Kingdom.

Cell isolation and sorting

Peripheral blood mononuclear cell (PBMC) isolation from fresh blood

The patient's PBMC were isolated from whole blood. 10 ml of blood was received in an EDTA vacutainer. The blood sample was then taken to a designated

containment level 2^+ laboratory where the sample was diluted 1:2 in sterile Dulbecco's 1x phosphate buffer saline (PBS). 20 ml of the sample mixture was then layered onto 10 ml Ficoll-Paque PLUS (GE Healthcare Bio-Sciences) followed by centrifugation using Sorvall™ Legend™ X1 centrifuge (Thermo Scientific) at 2000 rpm for 23 minutes without the brake. At this point, the plasma layer which would be used to make wash buffer for cell separation in the top layer was taken off and stored at -20°C. Most of the PBMCs were retained in the buffy coat layer between the interface of the serum layer and the Ficoll-Paque layer and it was removed carefully using sterile Pasteur pipettes. It was then washed twice in sterile PBS, resuspended in PBS, and enumerated by haemocytometer counting

Lymphoblastoid cell line preparation (LCL)

Separation of PBMCs was done as mentioned above. PBMCs were seeded in a sterile 24-well-plate at a density of 1.5-2 $x10^6$ cells/ml in Roswell Park Memorial Institute (RPMI) 1640 containing 10% foetal bovine serum (FBS) (Labtech), 1x Penicillin-Streptomycin solution (PAA laboratory GmbH). EBV crude stock at 1:1 ratio was added and placed in an incubator maintained at 37°C with 5% CO_2. After 6 hours, medium containing viral supernatant was aspirated without disturbing the cells and fresh complete RPMI 1640 was added. After 3-4 weeks of incubation rosette morphology of cells ascertained the transformed phenotype of PBMCs. Cells were mixed thoroughly to break up clumps before splitting to ensure multiclonal population.

Cell culture

Tissue culture media and cell lines

HEK293T, HeLa, HeLa FITR, primary human dermal fibroblasts (HDFa) and patient's primary skin fibroblast were cultured under the same conditions of 5% CO_2 at 37°C in 75 cm^2 flasks. These entire cell lines are grown in Dulbecco's modified eagle media (DMEM) (Lonza BioWhittaker™) supplemented with 10% foetal bovine serum (FBS) (Labtech), 1x Penicillin-Streptomycin solution (PAA laboratory GmbH). Epstein-Barr Virus (EBV) transformed Lymphoblast Cell Line

(LCL) was maintained in 25 cm2 culture flasks in RPMI-1640 (Life Technologies) supplemented with 10% FBS and 1x Penicillin-Streptomycin solution (PAA laboratory GmbH).

Protein analysis

Protein extraction: The cells were washed in PBS before cold RIPA lysis buffer (Sigma) containing 1% protease inhibitor cocktails (Roche) was added. The cells were left on ice for 15 minutes. The cell lysate was then centrifuged for 10 minutes at 13000 rpm (4°C). The supernatant was collected and an equal volume of 2x Laemmli sample buffer (BioRad) containing β-mercaptoethanol (Sigma) was added before storage a in a -20°C freezer.

Protein separation: Protein samples were diluted to 1:1 in 2x Laemmli sample buffers (BioRad) containing β-mercaptoethanol (Sigma) to a total volume of between 15µl-60µl and then boiled at 95°C for 5 minutes. Precision Plus Protein™ Dual Colour Standards (BioRad) markers were used to determine molecular weight values of detected bands. Samples were run on 10% Tris-HCL gels at 200V for 1 hour and 15 minutes in 1% running buffer.

Immunoblotting: A transfer sandwich was created consisting of sponges, filter papers, gel and nitrocellulose (Thermo Scientific) membrane. The transfer sandwich was relocated to the transfer apparatus, which was placed on ice to maintain a temperature of 4°C. 1% transfer buffer was added to the transfer apparatus and was made sure to cover the transfer sandwich. The electrodes were placed on top of the sandwich with the nitrocellulose membrane placed between the gel and a positive electrode. Gels were blotted onto PVDF membrane at 200mA for 2 hours and 30 minutes. The same process was repeated if more than one gels need to be transferred. Membrane was blocked using 5% BSA in 0.1% PBS/Tween-20 (PBS-T) for an hour. Primary antibody was diluted to 1:1000 in 5% BSA and incubated overnight in 4°C on a shaker followed by washing in PBS-T for three times in 5 minutes interval. The membrane was probed with a secondary antibody at 1:5000

dilutions in 5% BSA for an hour. Similar washing steps were repeated.

Proteins were detected using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare) following manufacturer's protocol with subsequent exposure of membrane to Fuji Medical X-ray film (Fujifilm) and then developed using Compact X4 (XOgraph). Exposure times were optimized for minimum background and clear detection of the protein of interest (5 seconds to 1 minute).

Reverse transcriptase PCR

Complementary DNA (cDNA) was made from RNA that was extracted from cell line. 500ηg of total RNA was added. Two micrograms of oligo-(dT)20 (New England Biolabs) was added to 5µg of total RNA, and 1µg of 10 mM of dNTP mix (New England Biolabs) and was incubated at 65°C for 5 minutes. Then 5X First Strand Buffer (New England Biolabs), 0.1M DTT (Life Technologies), 40 units of RNaseOut (Life Technologies) and 200 units of SuperScript™II (Life Technologies) were added. The samples were chilled for 5 minutes and incubated at 42°C for 1 hour, then 95°C for 3 minutes. A sample that had no Reverse Transcriptase added was also set up as a second control to test for genomic contamination. The cDNA was then stored at -20°C.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is the method of choice for selectively amplifying minute amounts of target DNA, quickly and accurately. First the double stranded DNA was separated by raising the temperature to 95°C. The temperature then dropped rapidly to 55°C - 70°C for 30 seconds. The temperature then rose to 72°C for 30 to 60 seconds. The cycle then repeats for 34 times this step amplified the DNA in a multiplicative manner.

A master mix containing final concentrations of 1x PCR buffer, 200uM of each dNTP, 1uM sense primer, 1uM antisense primer, 1-unit Taq DNA Polymerase, and ultra-pure water to a final total volume of 20 ul was added to approximately 50 ng of DNA template. Reactions were carried out either in 0.2 ml thin-walled tubes

(Alpha Laboratories Limited), or PCR microplate Thermo-Fast 96 well (Thermo Scientific) that was sealed with Adhesive PCR Film Seal (Thermo Scientific). All PCR reactions were carried out in Veriti® 96-Well Thermal Cycler using the block settings.

Correct PCR products were confirmed by loading 4µl of the PCR reaction onto a 2% 1 x TAE agarose gel which contained 50 ng of ethidium bromide. DNA was mixed with 6x DNA loading dye before being loaded on the gel. The gel was electrophoresed in 1x TAE buffer. Five microliters of HyperLadder II (Bioline) was run beside the PCR product to check that the product was the expected size. Gels were then photographed under ultraviolet light to visualise DNA.

Quantitative Polymerase Chain Reaction (qPCR)

Quantitative Polymerase Chain Reaction (qPCR) is a widely used method to quantitate DNA or RNA. For quantitative analysis of mRNA expression, comparative real-time PCR was performed with the use of the SYBR Green PCR Master Mix (Applied Biosystems).

Culture of the induced pluripotent stem (iPS) cells and differentiation of macrophages

Culture of the iPS cells

Mitomycin C inactivated Murine Embryonic Fibroblast (MEF) feeder cells were plated onto 0.1% gelatinised (Sigma, G1393) 6 well tissue culture treated dishes (Corning, 353046) in Advanced DMEM F12 (Gibco, 12634 010) supplemented with 10% heat inactivated Foetal Bovine Serum (Labtech), 2 mM L Glutamine, 50 IU/ml Penicillin, 50 IU/ml Streptomycin, 50 µM β 2 mercaptoethanol (Sigma, M3148) and used for a maximum of ten days.

hiPSC were grown on MEF cells in Advanced DMEM F12 (Gibco, 12634 010) supplemented with 20% Knock Out Serum Replacement (Gibco, 10828 010), 2mM L Glutamine, 50 IU/ml Penicillin, 50 IU/ml Streptomycin, 50µM β 2 mercaptoethanol (Sigma, M3148) and the growth factor 4ng/ml rhFGF b (Millipore, GF003), which was

added to the medium prior to use. Daily medium changes of 3 ml per well were performed. Cells were passaged using a solution containing 1:1 Collagenase IV/Dispase 2 (Gibco, 17104 019 and 17105 041 respectively).

Differentiation of iPS cells to macrophages

Differentiation of human iPS cells to macrophages was performed in three stages: formation of Embryoid Bodies (EBs), plating of the EBs to produce myeloid progenitors ('monocytes') and terminal differentiation of the myeloid progenitors to mature macrophages.

Human iPS cells colonies were detached from the MEF feeder cells using the Collagenase / Dispase solution mentioned previously and transferred to 9 cm low adherence bacteriological dishes (Sterilin, 101VR20) in 20ml of Advanced DMEM F12 (Gibco, 12634-010) supplemented with 20% Knock Out Serum Replacement (Gibco, 10828-010), 2 mM L-Glutamine, 50 IU/ml Penicillin, 50 IU/ml Streptomycin and 50 µM β 2 mercaptoethanol (Sigma, M3148) to form EBs. After three days the mature EBs were transferred to 0.1% gelatinised (Sigma, G1393) 10cm tissue culture treated dishes (Corning, 430167) in 12ml of 'X Vivo 5.0%' medium comprised of X Vivo 15 (Lonza, BE02 060Q) with 5.0% heat inactivated Fetal Bovine Serum (Labtech), 2mM L Glutamine and 50µM β 2 mercaptoethanol (Sigma, M3148) supplemented with 50ng/ml human M CSF (Miltenyi Biotec, 130 096 493) and 25 ng/ml human IL 3 (R&D, 203 IL 010/CF). After three/four days the media was topped up with 7 ml of 'X Vivo 5.0%' medium. The EBs then attach to the surface to form myeloid progenitor cells.

Media changes and top ups after the first week use 'X Vivo 0.0%' medium comprised of X Vivo 15 (Lonza, BE02 060Q) with 2 mM L Glutamine and 50µM β 2 mercaptoethanol (Sigma, M3148) supplemented with 50ng/ml human M CSF (Miltenyi Biotec, 130 096 493) and 25 ng/ml human IL 3 (R&D, 203 IL 010/CF). From week two alternatively a full media change was performed at three/four days using 12 ml of 'X Vivo 0.0%' and a media top up of 7 ml after another three/four days. At the end of the third week a 'myeloid progenitor harvest' was performed. Floating myeloid progenitor cells were aspirated along with the medium and passed through

a 40-uM cell strainer (Falcon, 352340) counted and plated onto tissue culture treated dishes in the 'Macrophage Differentiation' medium consisting of RPMI 1640 (Gibco, 21875 034) with 10% heat inactivated Fetal Bovine Serum (Labtech), 50 IU/ml Penicillin, 50 IU/ml Streptomycin supplemented with 100 ng/ml human M-CSF (Miltenyi Biotec, 130 096 493). 50% medium changes were performed using 'Macrophage Differentiation' medium every two/three days; mature macrophages were used for the assay on days seven to ten. Myeloid progenitor harvesting was performed on a weekly basis until their formation ceased.

RESULTS

Identification of the genetic cause of this disease

As the patient's parents were healthy and were first cousins, we hypothesized that the disease was caused by a homozygous Mendelian mutation. In order to identify the causative mutation, WES was performed at Professor Sergey Nejentsev's laboratory (University of Cambridge). Bioinformatics was done by Sergey Nejentsev (University of Cambridge) and Vincent Plagnol (UCL Genomics Institute). The patient's exome had 21,287 single nucleotide variants and small insertions/deletions. Homozygosity mapping (regions with at least 20 consecutive homozygous single nucleotide polymorphisms) revealed 34 potential regions ranging from 93 Kb to 24 Mb. Known polymorphisms were excluded and the remaining 158 rare variants were studied. Of these, 45 were synonymous (thus, unlikely to be causative). Of the remaining 113 rare variants only two variants were homozygous: an in-frame 6-nucleotide insertion and a frameshift 11-nucleotide deletion in exon 6 of the *ZC3H12A* gene.

The *ZC3H12A* gene is located on chromosome 1 and this frameshift deletion was the only homozygous loss-of-function variant in the patient. Sanger sequencing confirmed that the patient had a homozygous frameshift deletion (Fig.10), while his parents and one brother were heterozygous and the second brother was homozygous for the wild type allele (Fig.11). Given that both parents and brothers are healthy, the deletion co-segregated with the disease in the family.

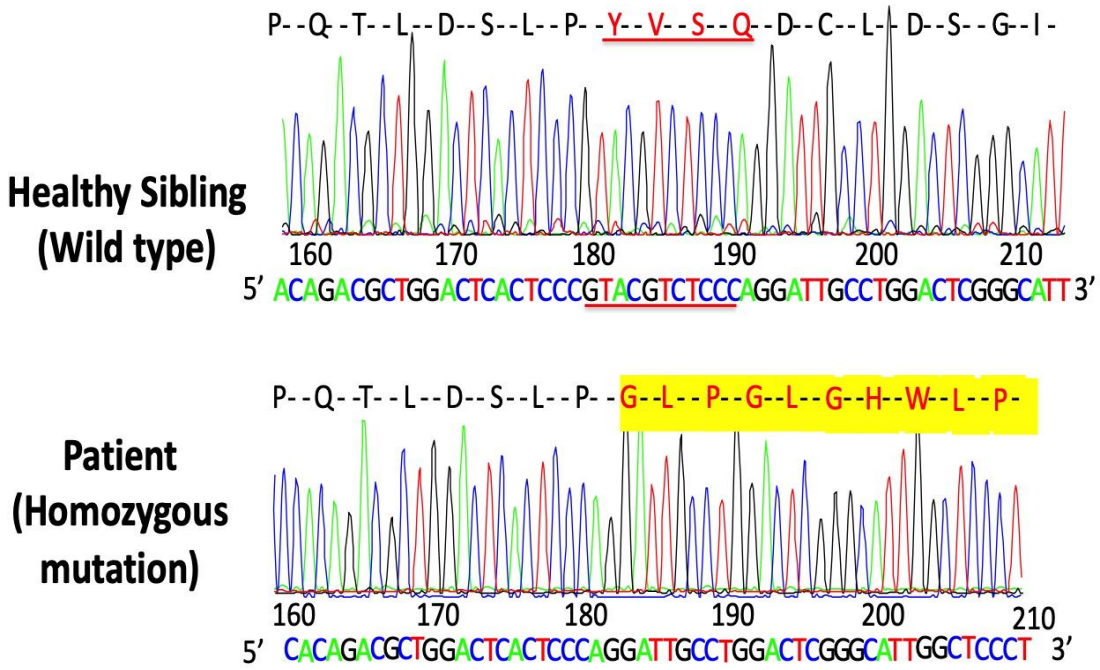


Figure 10: Sanger sequencing confirms homozygous deletion in the *ZC3H12A* gene in the patient. 11-nucleotide deletion is underlined. Yellow shows amino acid sequence that is translated in the patient using a different reading frame. Image generated by Dr Ilie Hashim

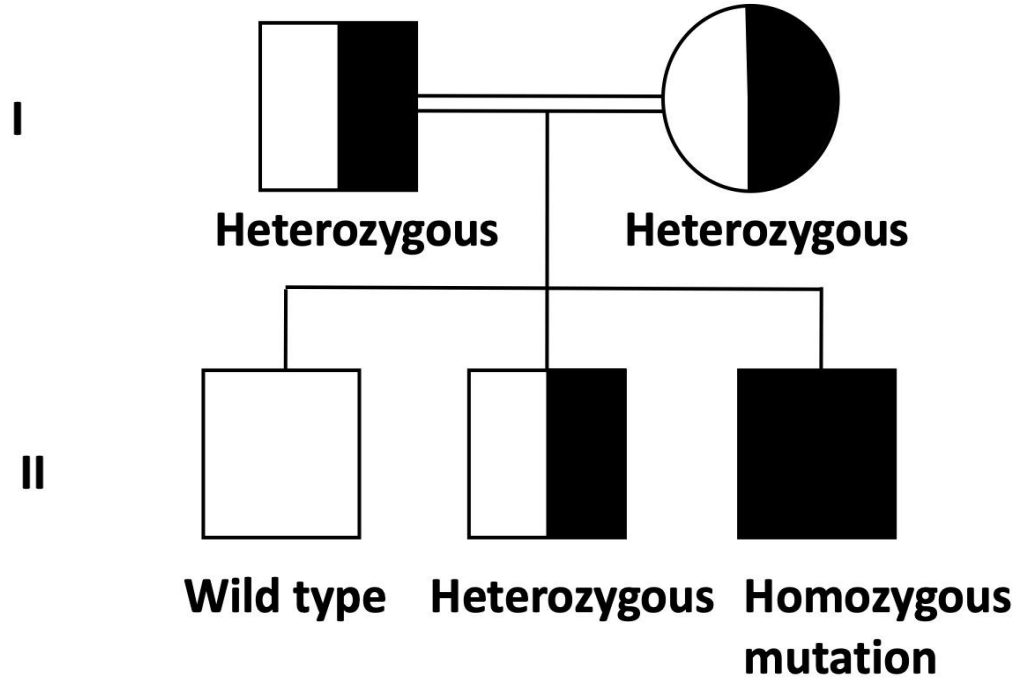


Figure 11: Family tree showing segregation of the mutation in the *ZC3H12A* gene. Open square, unaffected; filled square, affected; partially filled circle and squares, unaffected. Both parents and one of the siblings are carriers of Regnase-1 mutation.

Interestingly, no homozygous loss-of-function variants in the *ZC3H12A* gene were ever reported (they are absent from the ExAC database that contains exome data from approximately 60,000 subjects), suggesting that such mutations are likely to be pathogenic. Taken together, our data strongly suggested that the frameshift deletion in the *ZC3H12A* gene causes biallelic loss-of-function in this gene and is likely to be the causative mutation in this patient.

Regnase-1 is a 599 amino-acid protein and the mutant Regnase-1 protein found in the patient is predicted to have an insertion of two amino-acids following residue 413 and a part translated using a new reading frame following residue 429 of the wild-type protein sequence with a premature stop codon and a truncated 457 amino-acid protein (Fig.12).

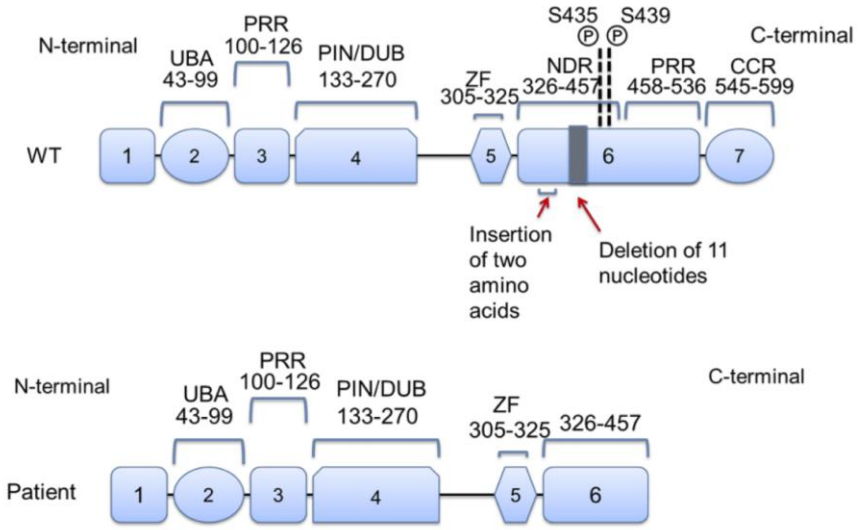


Figure 12: Schematic presentation of the wild type and mutant Regnase-1 protein encoded by the in the *ZC3H12A* gene**.** Domains of the Regnase-1 protein. PRR, Proline-rich region (100–126 and 458–536); PIN, PilT N-terminus domain (133–270); DUB, deubiquitinating enzyme domain (133–270); ZF, zinc-finger motif (305–325); CCR, C-terminal conserved region (545–599). The mutations resulting in premature stop codon, which leads to truncated Regnase-1 protein.
Image generated by Dr Ilie Hashim

Analysis of the mutant Regnase-1 protein

The structure and the length of the Regnase-1 protein are predicted to be affected by the frameshift deletion harbored by the patient. Western Blot of the protein lysates of the patient and control cells have shown that the patient's cells did not express the full Regnase-1 protein. Using the mouse monoclonal MAB7875 from R&D, endogenous Regnase-1 was detected in the control line of the EBV-transformed B cells as well as in the control primary fibroblasts, but no protein band of the expected molecular weight was detected in the patient-derived EBV-transformed B cells or in primary fibroblasts, as it was expected from our previous genetic analysis (Fig.13).

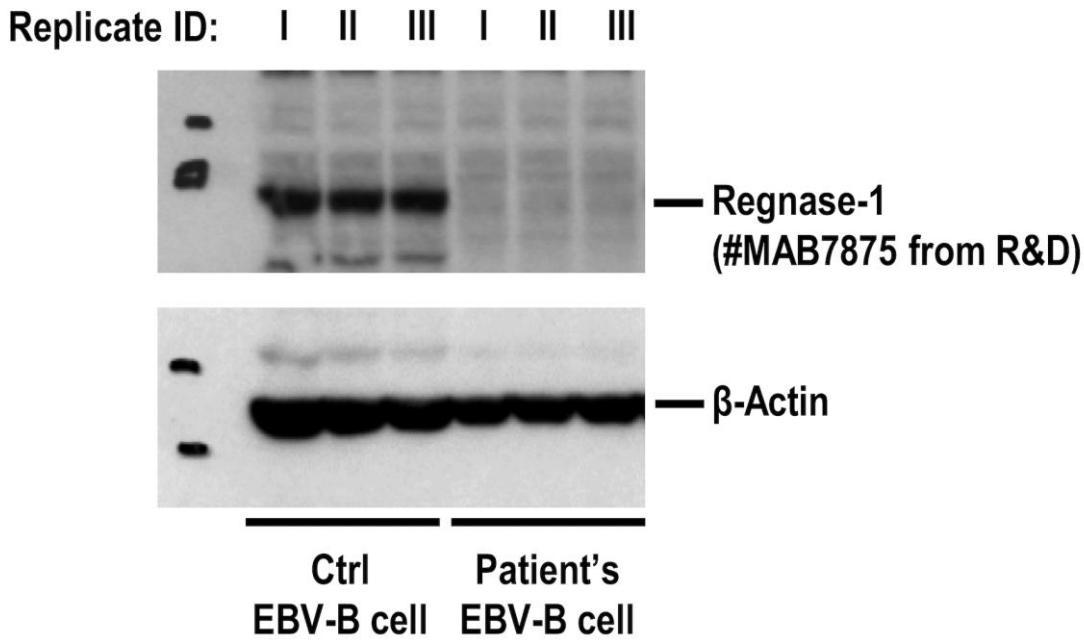


Figure 13: Regnase-1 protein is not detected in patient's cells

To confirm this, immunofluorescent staining was used followed by confocal microscopy to study the patient's and control fibroblasts, using the MCPIP1-P12 antibody sc-136750 from Santa-Cruz. Accordingly, confocal microscopy results have shown the complete absence of Regnase-1 in the patient's cells (Fig.14).

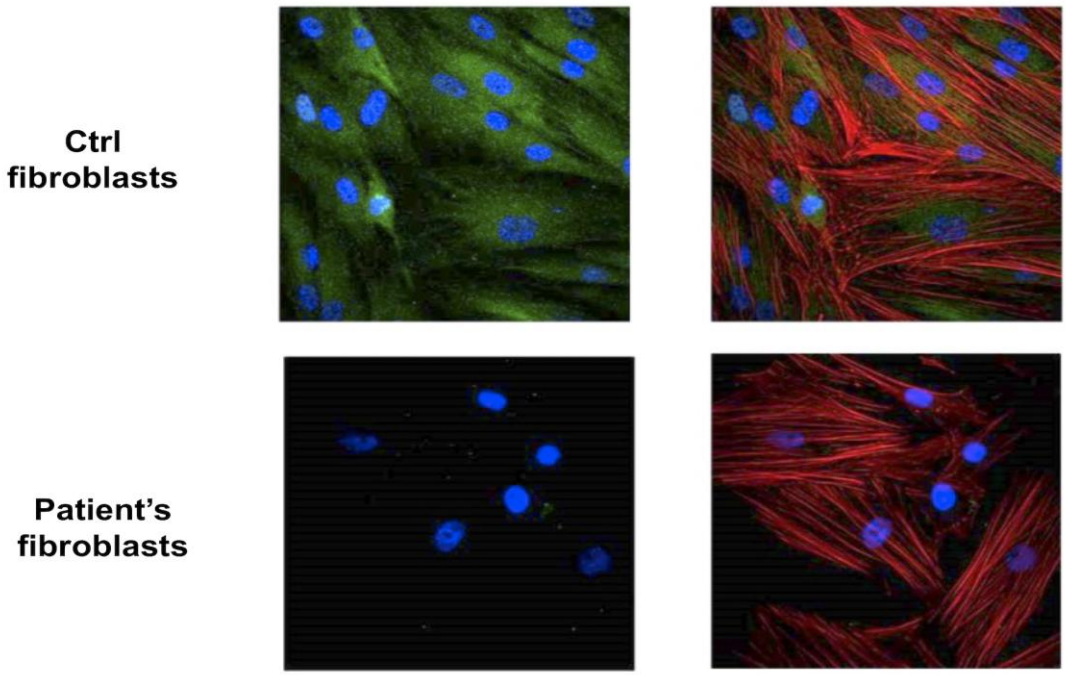


Figure 14: Confocal microscopy of the patient's and control primary fibroblasts. Regnase-1 (#MCPIP1-P12 from Santa-Cruz), actin (red) and DNA in the nucleus (DAPI, blue) are shown

In control fibroblasts the protein was found in the cytoplasm but not in the nucleus, which is in acordance to most of the previous reports on subcellular localisation of Regnase-1. Antibody ab118746 from AbCam that was generated against a peptide corresponding to a region that is predicted to be intact in the patient's cells (between amino acids 320 and 369) was used. Some fluorescent signal in the cytoplasm of the patient's fibroblasts was detected, which was much weaker than the signal observed in control fibroblasts (Fig.15), seeming to suggest that the truncated Regnase-1 protein may be expressed in the patient's fibroblasts, but its amount is strongly reduced.

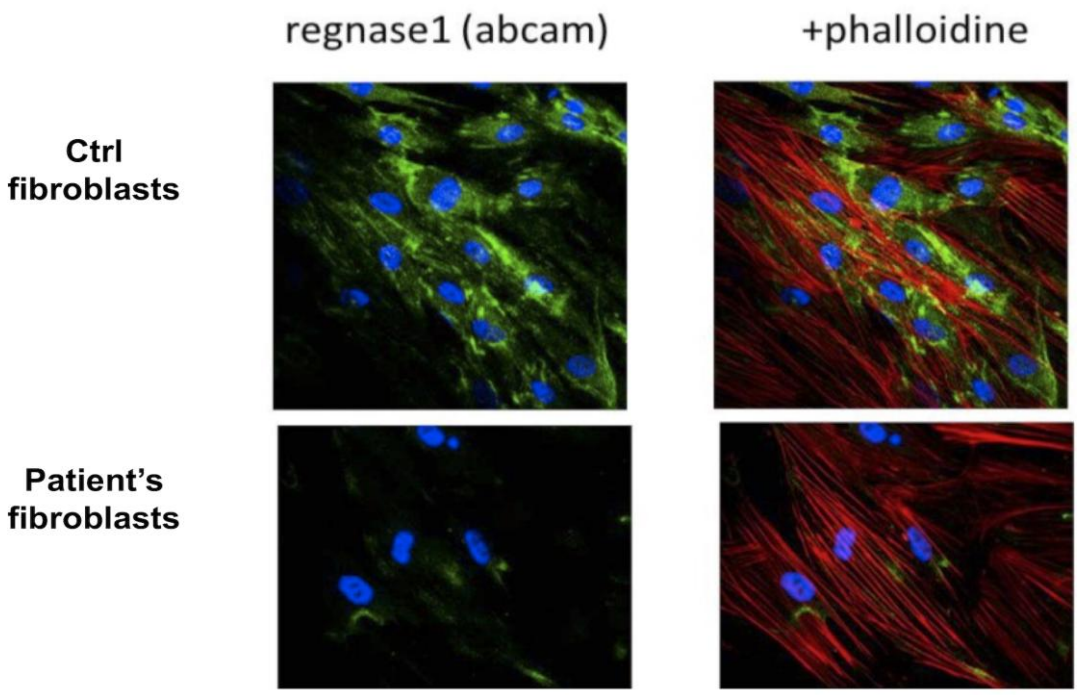


Figure 15: Confocal microscopy of the patient's and control primary fibroblasts. Regnase-1 (#ab118746 from AbCam, green), actin (red) and DNA in the nucleus (DAPI, blue) are shown.

These experiments confirmed the absence of the full-length protein in the patient's fibroblasts and suggested that there was a minimal expression of mutant truncated Regnase-1 protein.

Functional analysis of the Regnase-1 deficient cells

In order to understand how Regnase-1 deficiency leads to the disease in the patient with homozygous mutation in the *ZC3H12A* gene, an analysis of the intra-cellular pathway that involves Regnase-1 was performed.

As discussed in the introduction of this chapter, one the best characterized functions of Regnase-1 is the post-transcriptional regulation of mRNA for the IL-6 cytokine.[390] Previously it was shown that Regnase-1 knockout mice had increased production of IL-6.[402] In agreement with the animal model, whole blood assays done in our patient have shown either increased or well-preserved IL-6 production after various stimulations, despite aggressive immunosuppression, which was evident in the analysis of other cytokine responses.

We decided to analyse the IL-6 mRNA. For this, an EBV transformed B cell line derived from the patient's PBMCs was compared to an EBV transformed B cell line derived from a healthy subject. Using qPCR, it was found that cells of the patient had approximately 9-fold higher level of the IL-6 mRNA than cells of the healthy control (Fig.16).

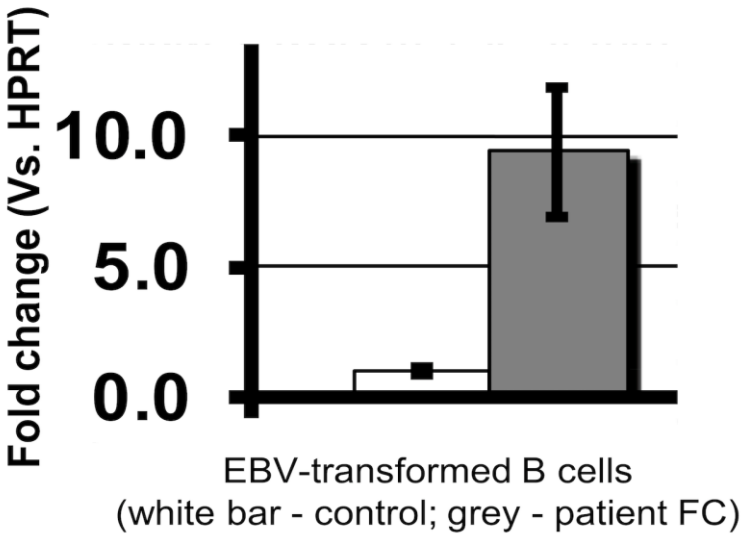


Figure 16: qPCR analysis of the IL-6 mRNA in the EBV transformed B cell lines of the patient FC and healthy control. Results were normalised on the level of expression of the housekeeping gene HPRT. Three technical replicates were done. Averages are shown on the graph.

Afterwards, the patient's primary fibroblasts were compared with fibroblasts of the healthy control subject. Again, a markedly increased level of the IL-6 mRNA in the patient was found, which was approximately 100-fold higher than the level of IL-6 mRNA in fibroblasts of the control. Fibroblasts were stimulated with 10 ng/ml of cytokine IL-1β and analysed levels of the IL-6 mRNA at various time points after stimulation, including 3 hours, 6 hours and 24 hours (Fig.17).

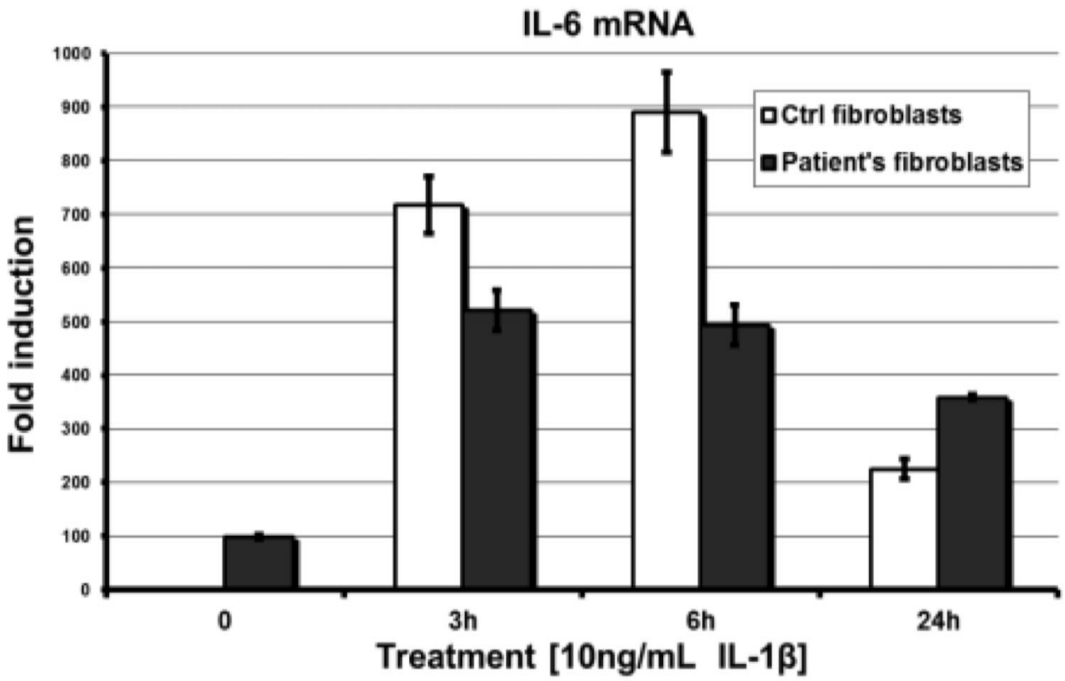


Figure 17: qPCR analysis of the IL-6 mRNA in dermal fibroblasts of the patient FC and healthy control. Results were normalised on the level of expression of the housekeeping gene HPRT. Three technical replicates were done. Averages are shown on the graph.

Fibroblasts of both patient and control responded to IL-1β stimulation by upregulating transcription of the *IL6* gene and the level of the IL-6 mRNA increased. In control fibroblasts it increased up to a maximum of 900-fold in comparison to the IL-6 mRNA level in the resting fibroblasts, having a peak at the 6-hour time point after IL-1β stimulation and reducing in control fibroblasts at 24-hour time point, although it still remained higher than in the resting cells. Surprisingly, in the patient's fibroblasts the increase of IL-6 mRNA after IL-1β stimulation was not as dramatic, peaking at 6-hour time points to a maximum of 5-fold relative to the level of IL-6 mRNA in the resting patient's fibroblasts. So, at these time points the amount of IL-6 mRNA produced by the control cells was actually larger than in the patient's cells. The patient's fibroblasts also showed decreasing amount of IL-6 mRNA at 24h post stimulation, but not as much as in control, and at this time point it was again larger in the patient's than in control fibroblasts.

These results were confirmed using macrophages derived from the patient's iPS cells and comparing them to macrophages derived from the control iPS cell line (Fig.18).

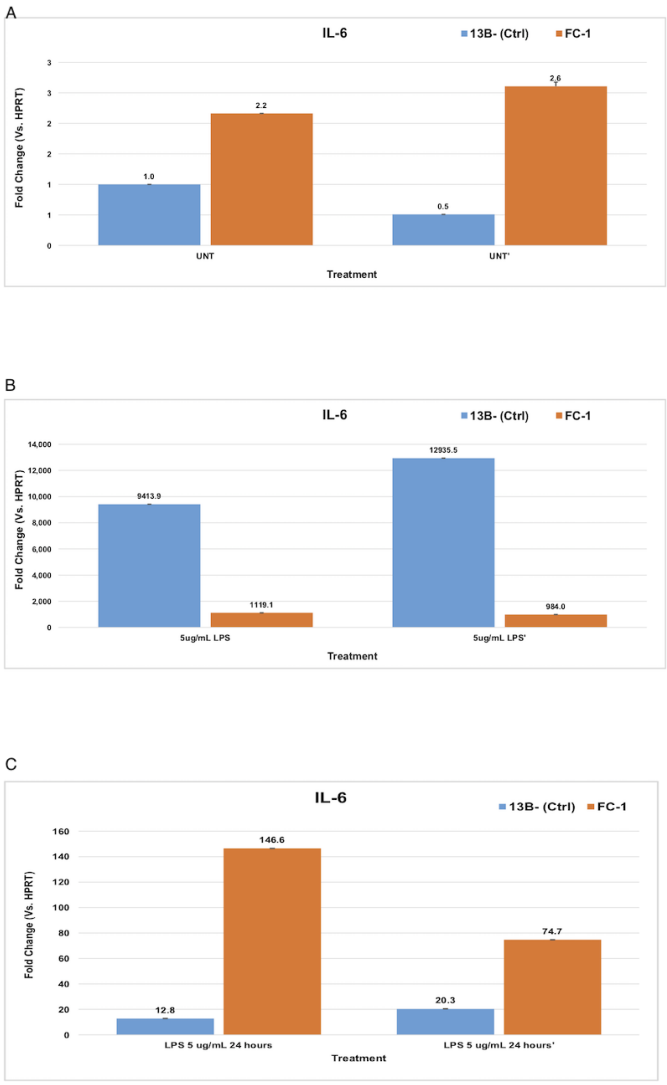


Figure 18: qPCR analysis of the IL-6 mRNA in macrophages derived from iPS cells of the patient FC (clone FC-1) and of the healthy control (clone 13B).

In untreated macrophages of the patient levels of the IL-6 mRNA were 2.2 – 5.2-fold higher than in the control macrophages (Fig.18A). Macrophages were then treated with 5 μg/ml of LPS for 8 hours and 24 hours (Fig.18B and 18C). Both patient's and control macrophages responded to LPS stimulation upregulating the IL-6 mRNA levels. We found higher levels of IL-6 mRNA in control macrophages than in the patient's macrophages at 8 hours after LPS stimulation (Fig.18B). After 24 hours after LPS stimulation, the level of IL-6 mRNA was again higher in the patient's macrophages than in control macrophages.

Overall, these results were surprising and they underlined that the patient's cells did not have persistently elevated amounts of IL-6 mRNA. Instead these cells seemed to have an impaired regulation of IL-6 mRNA, which is very likely related to the impaired function of Regnase-1 (which is the main regulator of the intracellular IL-6 mRNA levels). We would have expected a uniformly increased production of IL-6 mRNA, both in the resting state and after stimulation, if there was a complete Regnase-1 deficiency. These results suggested a more complex mechanism, probably related to residual RNase activity of Regnase-1 towards IL-6 mRNA.

The full length Regnase-1 protein has 599 amino acids, while the patient's mutant Regnase-1 protein is predicted to have a normal amino acid sequence up to position 413, where two amino acids are inserted. Then, up to amino acid position 429 the sequence is again normal. Thereafter, the frameshift mutation leads to a different amino acid sequence and a premature stop codon following amino acid 457. Therefore, if the patient's truncated protein is expressed, it still is predicted to have the PIN domain, which is encoded by amino acids 133 – 270 and is responsible for the RNase activity of Regnase-1. It seems plausible that in the patient's fibroblasts the mutant truncated protein is present and can potentially retain some functions of Regnase-1.

On the other hand, Iwasaki et al have shown that the activity of Regnase-1 is a target of regulation following activation of the NF-κB pathway. In brief, following stimulation of the IL-1 receptor or TLRs, activated IKK complex phosphorylates Regnase-1 and the phosphorylated Regnase-1 then undergoes ubiquitination and degradation.[405] Phosphorylation of Regnase-1 by IKKβ occurs at two serine residues of the DSGXXS motif located at amino acid positions 437 – 442.[405] Interestingly, this motif is absent from the patient's Regnase-1 protein, because it is encoded downstream of the frameshift mutation and this part of the mutant Regnase-1 protein is translated with a different reading frame.

To directly test our hypothesis that the mutant Regnase-1 cannot be degraded in cells after stimulation, we studied HeLa cells that were transfected with construct that expressed either wild type or the truncated mutant protein fused with the FLAG tag. These cells were stimulated with 10 ng/mL of the IL-1β cytokine and

cultured for 30 minutes and 4 hours. To prevent cells from expressing the new Regnase-1 protein, the cells were simultaneously treated with cycloheximide (CHX). At each of the time points the cells were lysed and protein lysates ran on a gel (Fig. 19).

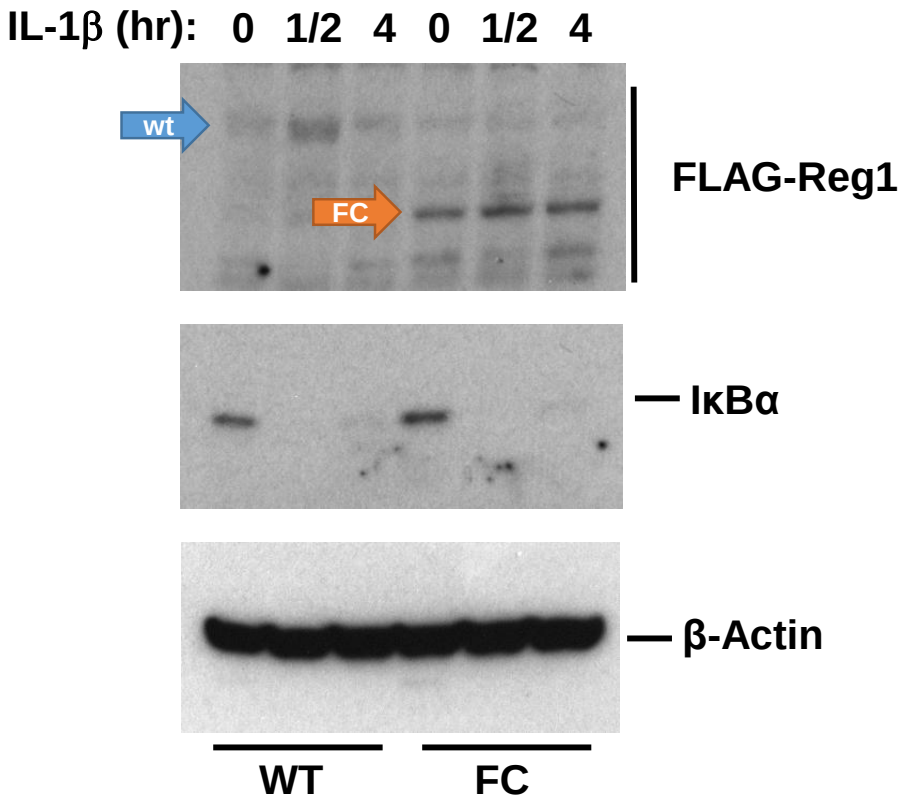


Figure 19: Mutant Regnase-1 protein is refractory to degradation after IL-1β stimulation

In the western blotting the anti-FLAG antibody was used to visualise both the wild type and the mutant Regnase-1 proteins. An antibody against protein IκBα was also used to study its levels in the cells. IκBα is an inhibitory protein that keeps protein subunits of the NF-κB transcription factor in the cytoplasm, thus preventing them from relocating to the nucleus and activating gene transcription. Upon cell stimulation proteins of the IKK complex, e.g. IKKβ, phosphorylate IκBα leading to its degradation and release of the NF-κB transcription factor. As expected, in unstimulated HeLa cellsahigh level of the IκBα protein was found. IκBα is known to be quickly degraded after stimulation. Therefore, it was not yet found in the cells at the 30-minute time point. Cycloheximide prevented it from being expressed again, therefore, we did not find it in the cells at the 4-hour time point either. The wild type

Regnase-1 was clearly detected at the 30-minute time point when it was not yet degraded after stimulation. At 4 hours the amount of the wild type Regnase-1 was much lower because it was degraded and not re-expressed (due of the presence of cycloheximide). Importantly, in cells transfected with the FC mutant construct and expressing the truncated protein, we found stable amounts of Regnase-1. It was completely resistant to degradation after stimulation. This result confirmed that the truncated Regnase-1 protein that lacks the motif used for its phosphorylation is resistant to protein degradation. Therefore, regulation of its intracellular levels and activity after stimulation is impaired.

This leads us to the conclusion that the mutant Regnase-1 protein cannot be phosphorylated after cell stimulation, thus not undergoing ubiquitination and proteasomal degradation.

Discussion

The present clinical case report is a very uncommon presentation that has raised the clinical suspicious of a new cause of IPEX-like syndrome. In order to identify the mutation that caused this disease and to stablish causality, a joint study with Professor Sergey Nejentsev from Cambridge University was conducted.

We have identified for the first time worldwide a novel primary immunodeficiency, caused by a rare homozygous loss-of-function mutation in the ZC3H12A gene. This mutant ZC3H12A gene was predicted to encode a truncated Regnase-1 protein. We demonstrated that the patient's cells lack expression of the full-length Regnase-1 protein, probably corresponding to a truncated protein.

In order to characterize the functional effects of the mutation on the Regnase-1 protein, levels of the IL-6 mRNA were studied in macrophages derived from the patient's iPS cells, fibroblasts and EBV-transformed B cell line. To our surprise, in the patient's cells the kinetics of the IL-6 mRNA levels was different to the one that could be expected in cells that merely lack the Regnase-1 protein. Thus, soon after stimulation the level of IL-6 mRNA in the patient's cells was actually lower than in control cells, which was surprising, given that these cells are deficient for the wild type Regnase-1 protein, a negative regulator of the IL-6 mRNA. At a later point

in time, when the peak of the IL-6 mRNA transcription has passed, the patient's cells again showed a higher level of the IL-6 mRNA. This result suggested a more complex mechanism of the impaired regulation of the IL-6 mRNA by the mutant Regnase-1. It is well known that stimulation of the IL-1 receptor and TLRs leads to the activation of the NF-κB signalling pathway. The NF-κB signalling is known to be important for host protection against infection. For activation, proteins of the NF-κB transcription factor must be released from constitutive interaction with inhibitory IκB proteins (IκBα, IκBβ and IκBε), which sequester NF-κB complexes in the cytoplasm.[410] This occurs through phosphorylation and degradation of IκB proteins by the upstream IκB kinase (IKK) complex, for example following stimulation of cell surface receptors. The IKK complex consists of two catalytically active kinases, IKKα and IKKβ, and the regulatory subunit IKKγ, also known as NEMO (NF-κB essential modulator). In the canonical pathway of NF-κB activation, IκBα undergoes IKKβ-dependent phosphorylation and ubiquitin-mediated degradation, liberating the NF-κB heterodimer, which then translocates to the nucleus.[410] It has been shown that activated IKKβ also phosphorylates Regnase-1, which then leads to the ubiquitination of the Regnase-1 protein and targets it for proteasomal degradation.[405] Through this mechanism IKKβ coordinates an induction of expression of the IL-6 mRNA and at the same time removes Regnase-1 protein, the RNase that degrades IL-6 mRNA, from the cell cytoplasm. This gives time for IL-6 mRNA to be translated and IL-6 cytokine to be secreted. However, transcription of the Regnase-1 mRNA is also upregulated after IL-1 receptor or TLR stimulation and activation of the NF-κB pathway. Therefore, after a while, a new active Regnase-1 protein is expressed again. It cuts the IL-6 mRNA, thus stopping IL-6 secretion and curbing the pro-inflammatory response, which is essential to prevent excessive activation of the target cells, which may lead to autoimmune and inflammatory disorders. Activated IKKβ is known to phosphorylate Regnase-1 protein at two serine residues that are located at amino acid positions 437 – 442 in the DSGXXS motif.[405] However, the patient's truncated Regnase-1 protein lacks this motif. Therefore, regulation of this mutant Regnase-1 protein is impaired. It cannot be phosphorylated after cell stimulation and would not undergo proteasomal degradation, thus remaining in the

cell and exerting its activity on the newly transcriber IL-6 mRNA. Although this activity may be impaired, particularly because some of this mutant Regnase-1 is mislocalized in the cell nucleus, the residual RNase activity might be sufficient to keep IL-6 mRNA levels in the patient reduced in comparison to the levels observed in healthy controls. Hence, this defective mechanism may be particularly damaging for the patient's immune system, as it bears two types of risks. On one hand, the resting cells have increased IL-6 production, which is not properly removed by the mutant Regnase-1. This would lead to over-activation of the immune cells and is likely to be responsible for lymphoproliferation, hyperimmunoglobulinemia, and generation of auto-antibodies. On the other hand, in the case of infection, the immune system is supposed to quickly mount a strong pro-inflammatory response, which is to a large extent orchestrated by the IL-6 cytokine. The patient's cells would be impaired in this capacity, because the mutant Regnase-1 protein would still have some residual activity and would degrade the newly synthesised IL-6 transcript. Thus, patient's immune response in the case of infection may be impaired. This may explain poor responses of the patient's immune system to vaccination and low levels of specific antibodies that may be incapable of preventing infections with common pathogens, such as streptococcus or haemophilus. This results in frequent respiratory infections that already led to bronchiectasis in the patient, which is also a very serious and potentially life-threatening complication.

The nature of the genetic defect and the functional evidence that we produced allowed us to conclude confidently, that the mutation in the Regnase-1 gene is indeed responsible for this patient's disease. This adds to the knowledge about the regulation of the immune response and adds mutations in the ZC3H12A as a potential cause of IPEX-like syndromes.

B. IRG1 DEFICIENCY

Introduction

The role of itaconic acid in biotechonology has been well known for many decades, and is industrially used as a precursor for plastic polymer synthesis, as well as resins, latitices and fibers.[411-413]

It was only in 2011 that the production of itaconic acid was found in mammalian immune cells,[414] and in 2013 the mammalian metabolic pathway for itaconic acid biosynthesis was revealed: it is produced by the decarboxylation of cis-acotinic acid, a tricarboxilic acid cycle (TCA) intermediate, by immune-responsive gene protein 1 (IRG1, also known as *cis*-acotinic acid decarboxylase, CAD), which is encoded by immune-responsive gene 1 (Fig.20).

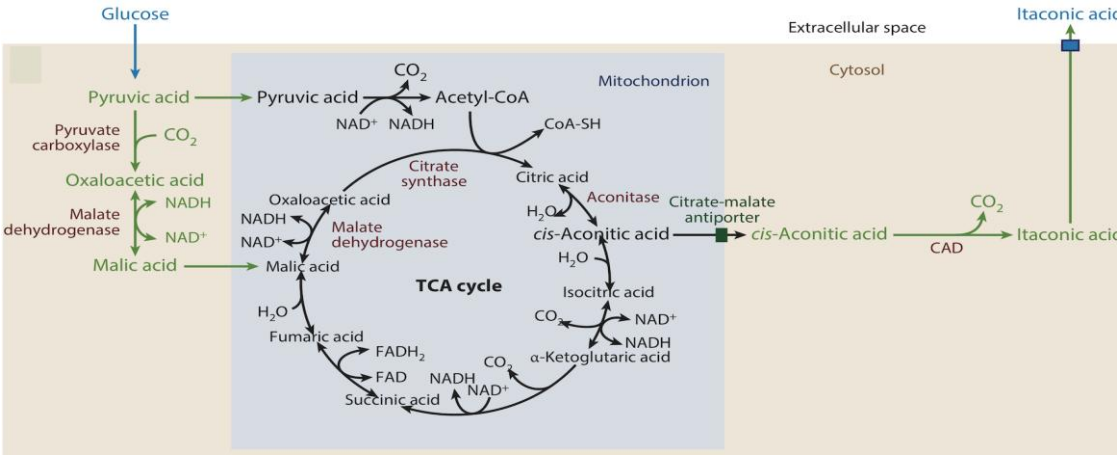


Figure 20: TCA cycle. Adapted from Cordes T et al, 2015.[6]

We will, hereafter, designate the gene as IRG1 and its product as CAD.[6,415] This gene has been known for years to be highly upregulated in mammalian macrophages under proinflammatory conditions.[414,416,417]

Macrophages have the ability to assume different activation states in response to different environmental factors. Resting macrophages (M0) develop pro-inflammatory microbicidal and tumoricidal properties after stimulation with IFNγ and TLR agonists (M1 or classical activation). On the other hand, when M0 are stimulated with IL-4 and IL-13, they will show anti-helminth immune responses and wound healing (M2 or alternative activation).[418-420] Macrophage activation is

accompanied by marked metabolic changes, namely attenuation of oxidative phosphorylation and upregulation of aerobic glycolysis.[421] The M1 metabolic adaptation is marked by transcriptional changes in metabolic genes and a dramatic accumulation of itaconate.[418,421,422]

Itaconate has two main functions:

- Antimicrobial activity. Itaconate is an inhibitor of isocitrate lyase (ICL), the key enzyme of the glyoxylate shunt.[423,424] The activation of this shunt is a survival strategy of invading pathogens, which allows them to utilize acetyl-CoA-generating carbon sources such as fatty acids or cholesterol for biomass production under glucose-limiting condition. Inhibition of ICL renders bacteria vulnerable to the host by blocking their ability to produce energy from fatty acid carbon sources in the absence of glucose, using the glyoxylate shunt.[6,421,425] Some bacteria (such as *Yersinia pestis* or *Pseudomonas aeruginosa*) have developed mechanisms to detoxify itaconate, through its degradation to acetyl-coA and pyruvic acid (Fig.21).[426,427]

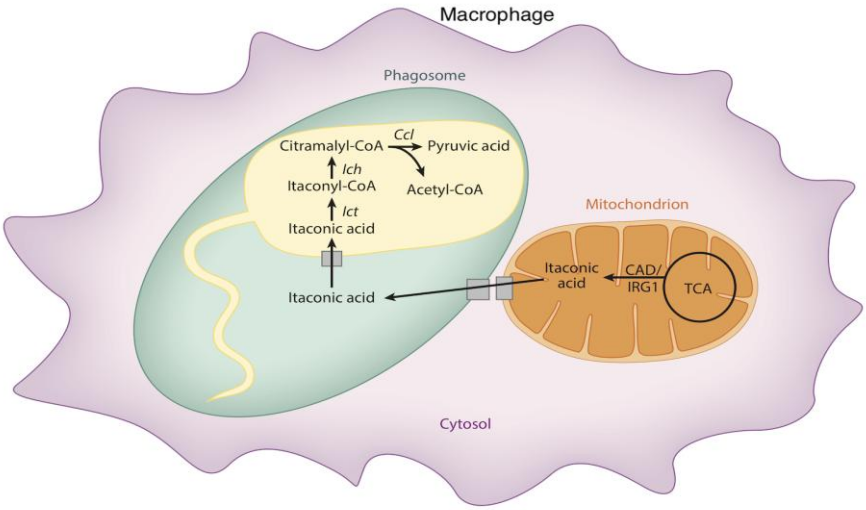


Figure 21: Detoxification of itaconic acid by bacteria. Adapted from Cordes T et al, 2015.[6]

- Modulator of inflammation. Itaconate has been shown to inhibit succinate dehydrogenase (SDH), resulting in succinate accumulation.[425] The oxidation of accumulated succinate promotes reactive oxygen species (ROS), generated from reverse electron transport.[428,429] ROS will directly potentiate inflammasome activation and IL-1 and IL-18 production.[427] Treatment with exogenous dimethyl itaconate (a membrane permeable non-ionic form of itaconate) decreases ROS production in lipopolyssacharide (LPS)-treated macrophages.[421] Interestingly, in an in vivo model of cardiac ischemia reperfusion injury, intravenous infusion of diethyl itaconate during ischemia markedly reduced myocardial infarct size, thus supporting the anti-inflammatory role of itaconate.[421] Moreover, in murine models it has also been shown that following type-I interferon production, IRG1 gene expression is boosted, promoting itaconate production. Itaconate can directly alkylate protein Kelch-like ECH-associated protein 1 (KEAP1, that sequesters Nrf2 in the cytoplasm for proteasomal degradation), enabling Nrf2 to increase the expression of downstream genes with anti-oxidant and anti-inflammatory capacities (Fig.22 and Fig.23).[7,427]

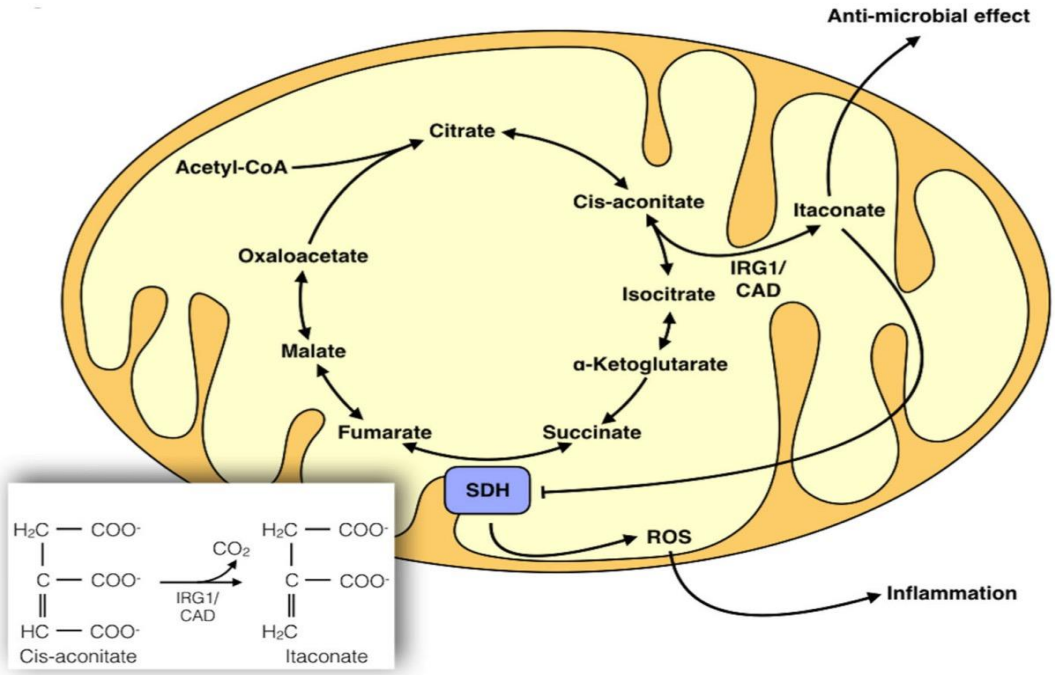


Figure 22: Role of Itaconate in the Immune Response. Adapted from Luan HH et al, 2016.[418]

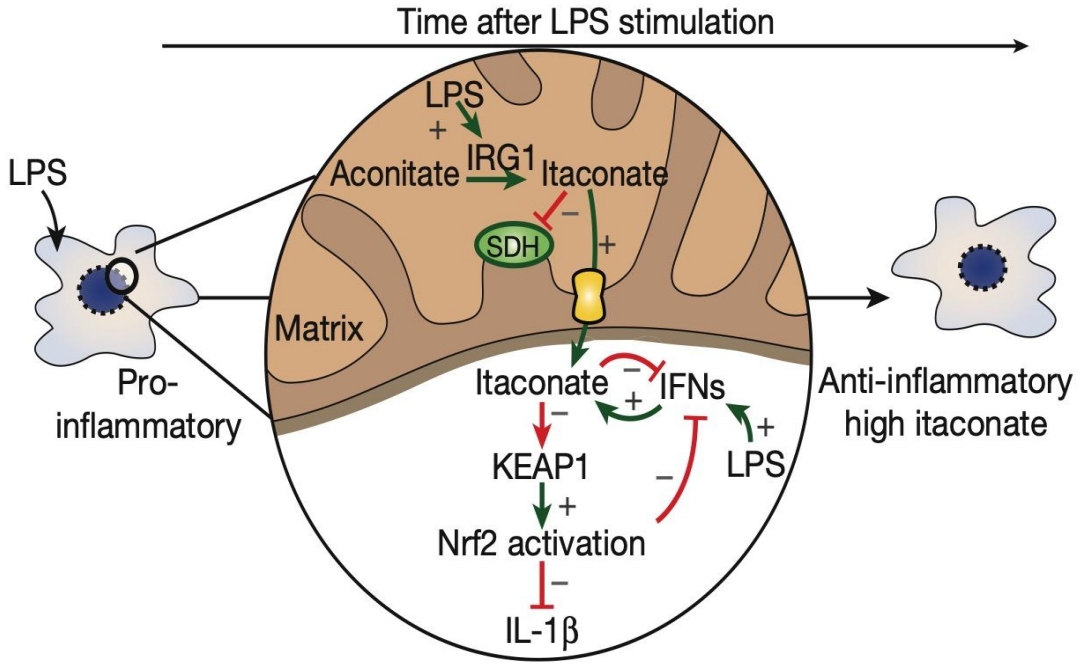


Figure 23: Model functions Itaconate. Adapted from Mills EL et al, 2015.[7]

Very recently, it has been shown that IRG1 knockout mice that are challenged with *Mycobacterium tuberculosis* rapidly succumb to the infection when compared to the wild-type mice. Interestingly, increased mortality was associated with increased infection, but also increased inflammation and pathology, unravelling a crucial role for IRG1 in the host defense against *Mycobacteria*.[430]

Case Report

The patient was the first daughter from a non-consanguineous couple from Cape Verde and the family history was unremarkable. From the age of 2 months until the age of 15 months-old, she had multiple episodes of fever (18 times) and she was hospitalized once because of arthritis of the knee and a second time for elbow arthritis, requiring drainage and surgery. At the age of 15 months she was transferred to Lisbon for extensive myositis and fasciitis of the thigh (Fig.24). She received multiple broad-spectrum antibiotics and required fasciectomy. Multiple bactéria were cultured from the purulent drainage (*Bacteroides fragilis, Klebsiella pneumoniae, Staphylococcus aureus*). Her immunology workup was normal.

Figure 24: A) Myositis and fasciitis of the leg B) gas-producing pathogen C) Brownish purulent drainage; D) Post-fasciectomy E) Fasciectomy scar. (photographs authorized by parents after proper informed consent)

She was discharged after 6 weeks of IV antibiotics, but at the age of 17 months, she was again admitted for myositis of the arm, that resolved after IV antibiotics. At the age of 18 months, she was hospitalized for leg myositis for which she received 3 weeks of IV antibiotics. She was discharged on cotrimoxazole prophylaxis. At the age of 20 months, she had severe myositis and fasciitis of the thigh, requiring multiple surgical drainages, fasciectomy, along with 6 weeks of IV antibiotics. Multiple bacteria were isolated: *Enterobacter cloacae, Escherichia coli, Fusobacter necrophorum, Klebsiella pneumoniae*. From the age of 20 to 26 months,

she didn't have any episode. At the age of 26 months, she presented myositis and fasciitis of the thigh, again requiring fasciectomy and multiple surgical drainages. *Escherichia coli, Fusobacter necrophorum*, *Bacteroides fragilis* and *Streptococus constellatum* were isolated. Empirical IFNγ was added to the 4-week IV antibiotic regimen and she was discharged on IFNγ prophylaxis. She didn't present infectious episodes until the age of 39 months, when she presented myositis and fasciitis of the arm, requiring fasciectomy and IV antibiotics. WES was performed, but there were no variants in the known PID genes. Nevertheless, a rare, heterozygous variant of uncertain significance (VUS) in *IRG1* was identified.

She was discharged on IFNγ prophylaxis and hasn't had any episode of infection in the last 20 months.

Specific methods

In order to identify the causative mutation, WES was performed in the department of laboratory medicine at the National Institute of Health (NIH) and the following experiments have been perfomed in collaboration with Katelyn McCann (NIH/NIAID and UMass Medical School MD/PhD program).

Simultaneous extraction of RNA and intracellular polar metabolites

Primary human monocytes were collected by elutriation. Then, they were plated without differentiation in RPMI (with FBS and pen/strep) and stimulated for indicated time points with LPS (200ng/mL) and/or IFNγ (1000U/mL). RNA and intracellular polar metabolites were collected by simultaneous extraction as published by Sapcariu et al.[431] IRG1 expression was measured by qPCR (TaqMan reagents).

Itaconate levels measured by mass spectrometry

Itaconate was measured by Liquid chromatography-tandem mass spectrometry/Mass spectrometry by Agios Pharmaceuticals. Intracellular metabolite levels were normalized based on total packed cell volume.

Results

Identification of the genetic cause of this disease

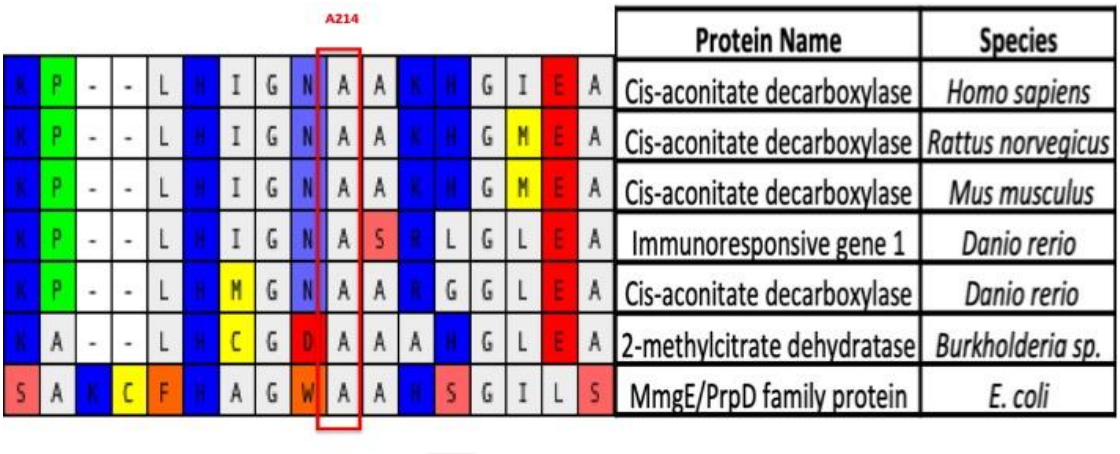
The patient's exome had 199 non-synonymous single nucleotide variants. Known polymorphisms were excluded and the remaining variants were filtered by frequency (<1%) and CADD-phred. This revealed a score a variant of unknown significance (VUS) in IRG1 with a frequency of 0,006% and CADD-phred score of 32. Sanger sequencing confirmed that the patient had a heterozygous missense variant p. A214V in the IRG1 gene, which was not present in the mother. Her father was not available for IRG1 sequencing.

Interestingly, variants in IRG1 are very rare with only 3 heterozygous variants reported (Gnomad Frequency: 3/174,538 alleles = 1.719^{-5}, Table 2)

Population	Allele Count	Allele Number	# of Homozygotes	Allele Frequency
African	3	15,224	0	0.0001971
Ashkenazi Jewish	0	8,400	0	0
Asian	0	16,494	0	0
European	0	87,140	0	0
Latino	0	24,686	0	0
Other	0	22,594	0	0
Total	**3**	**174,538**	**0**	**0.00001719**

Table 2: Reported IRG1 variants.

This residue is very highly conserved across vertebrates, invertebrates and bacteria, indicating a potentially important role for this residue in the structure and/or function of IRG1 (Fig. 25).



Protein Name	Species
Cis-aconitate decarboxylase	*Homo sapiens*
Cis-aconitate decarboxylase	*Rattus norvegicus*
Cis-aconitate decarboxylase	*Mus musculus*
Immunoresponsive gene 1	*Danio rerio*
Cis-aconitate decarboxylase	*Danio rerio*
2-methylcitrate dehydratase	*Burkholderia sp.*
MmgE/PrpD family protein	*E. coli*

Figure 25: Phylogenetical conservation of the IRG1 residue

The bioinformatic analysis of this variant predicted this variant to be disease-causing (Polyphen: probably damaging; SIFT: deleterious). No homozygous loss-of-function variants in the IRG1 gene were ever reported and (they are absent from the ExAC database that contains exome data from approximately 60,000 subjects), suggesting that such mutations are likely to be pathogenic.

IRG1 is a 481 amino acid protein. The crystal structure of mammalian IRG1 has not been published. The *Escherichia coli* protein IDS epimerase (with cis-aconitate decarboxylase activity and about 30% homology with human IRG1 protein sequence), leads us to speculate that, while the enzymatic active site is not known, the patient's variant (A214V) falls within a region with sequence homology to a-helix 11 of IDS epimerase which is thought to be located within the homodimerization domain of the enzyme monomer. Therefore, based on amino acid sequence alignment we predict that the patient's mutation could affect dimerization of IRG1.

Functional assessment of IRG1 in human monocytes

(Data and Figures courtesy of Katelyn McCann (NIH/NIAID and UMass Medical School MD/PhD program)

Since in most of the exhisting data on IRG1 gene, CAD and its product, itaconate, wasn't obtained in human-derived cells, we decided to test it in human monocytes, in order to be able to interpret the patient's data in future exeriments.

As reported in murine experiments, IRG1 mRNA correlates with itaconate in healthy volunteer primary monocytes (Fig.26) and IRG1m RNA and itaconate are almost exclusively expressed/produced in myeloid cells, specifically CD14+ peripheral blood derived monocytes in humans (compared to low gene expression and undetectable itaconate in the CD14- fraction of the PBMC).

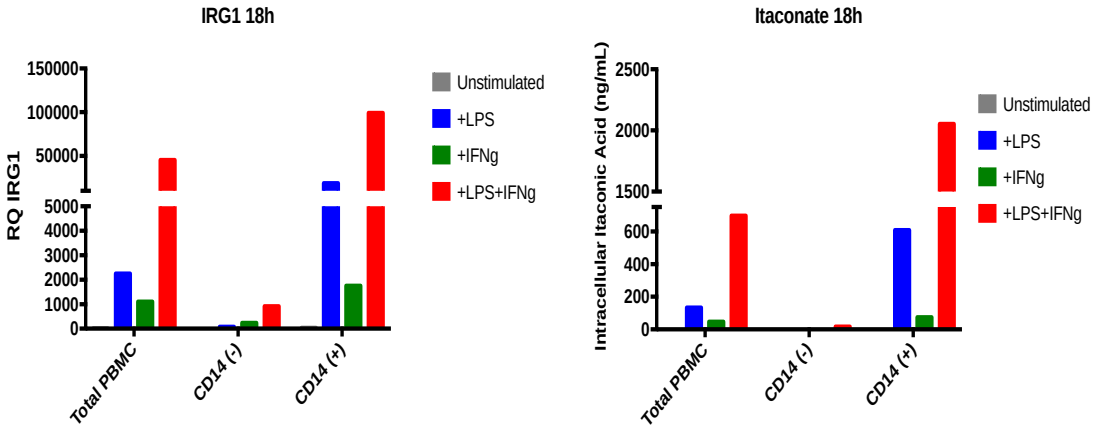


Figure 26: IRG1 mRNA correlates with itaconate in healthy volunteer primary monocytes

The time course of IRG1 and intracellular itaconate Levels in healthy donor monocytes following stimulation with LPS and/or IFNγ is similar to murine models: IRG1 transcript levels peak at 3-6h post stimulation (with LPS and LPS+IFNγ) whereas itaconate metabolite levels peak around 18-24h post stimulation (with LPS and LPS+IFNγ (Fig.27 and Fig.28).

Contrary to what been shown in murine models, LPS+IFNγ but not LPS alone induce high levels of IRG1 mRNA and itaconate levels (Fig.27)

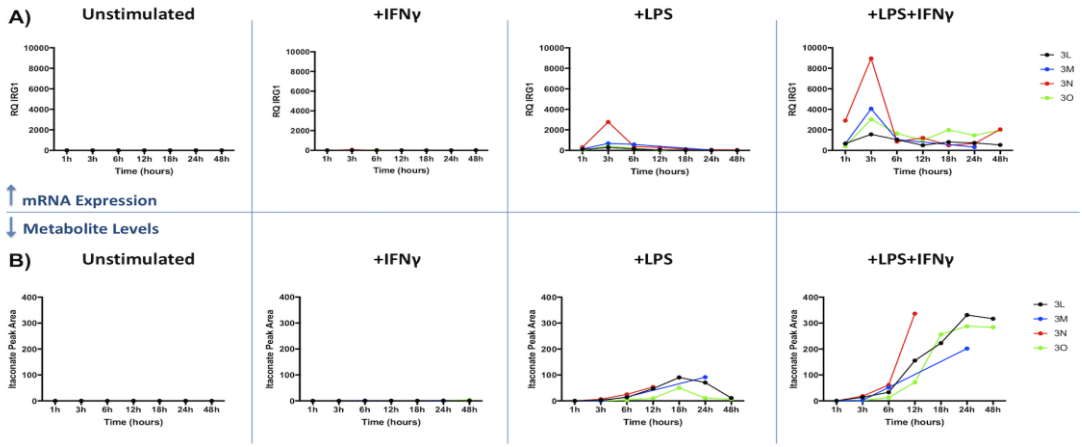


Figure 27: IRG1 transcript levels peak at 3-6h post stimulation (with LPS and LPS+IFNγ) in healthy donor monocytes

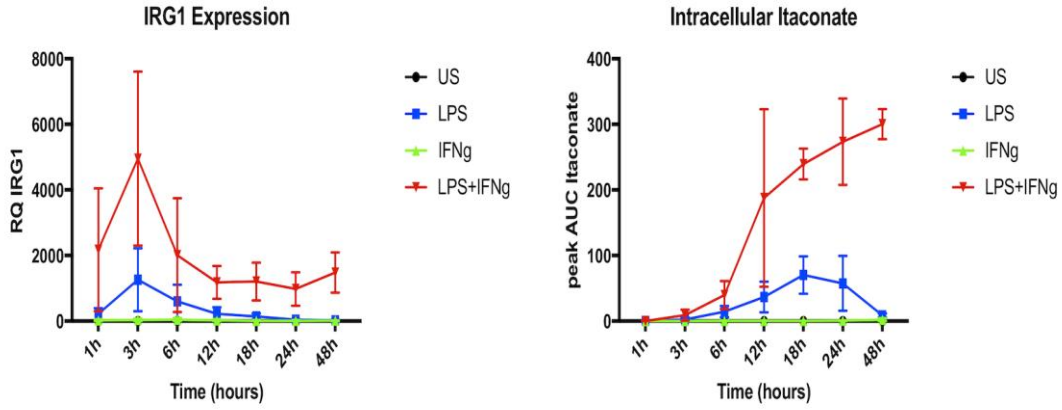


Figure 28: Itaconate metabolite levels peak around 18-24h post stimulation (with LPS and LPS+IFNγ in healthy donor monocytes

Similarly to what has been shown in murine macrophages,[421] intracellular itaconate correlates with succinate accumulation in human monocytes. (Fig.29) However the succinate accumulation was not as dramatic in human monocytes:

- human monocytes = ~1000000 (unstim) to ~4000000 (+stim) ~4x increase
- murine BMDM = ~0.01 (unstim) to ~0.2 (+stim) ~ 20x increase

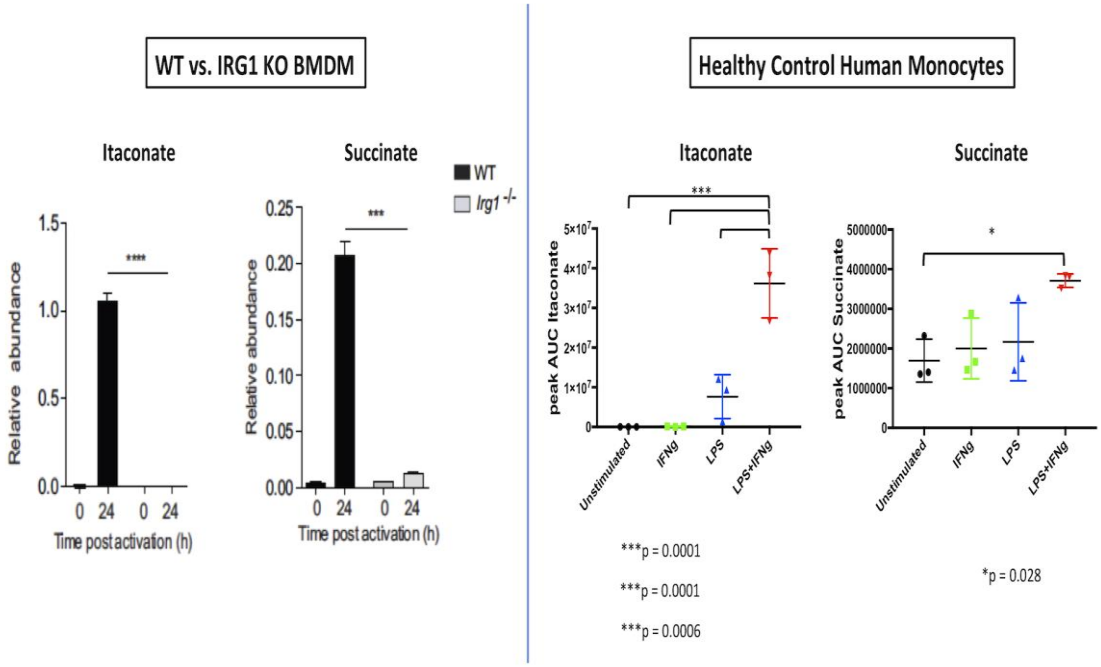


Figure 29: Intracellular itaconate correlates with succinate accumulation in human monocytes

On going studies

Models of total IRG1 KO in human monocyte cell lines are being validated, in order to assess itaconate production and succinate levels, as well as quantify Nfrs/NQO1/anti-oxidant pathway activation, ROS production and bacterial killing. This will allow us to validate whether data that has been published in murine bone marrow derived macrophages is also true in human monocytes in the absence of IRG1.

In order to prove that her mutation in IRG1 causes a functional IRG1 deficiency (either haploinsufficiency or dominant negative effect), the patient's monocytes will be stimulated with LPS+IFN γ for 24 hours (which corresponds to the maximum itaconate levels based on the healthy controls cohort timecourse), and IRG1 mRNA and itaconate levels will be measured. We will compare these to her healthy sibling, parents and controls. This will allow us to prove that the mutation is causing loss of CAD activity in the patient.

If, as expected, we will be able to prove that the patient's variant is indeed functional, we will then try to learn what the sequences of low itaconate in terms of gene expression are by performing RNA sequencing in the patient's cells and then compare them with the IRG1 KO cells.

Discussion

The present clinical case report is a very uncommon presentation that didn't allow us to establish a proper diagnosis, but led to the hypothesis that the underlying immune defect led to the defective intra-cellular killing of bacteria. Thereafter, we established a joint cooperation with Professor Steven Holland of the Division of Intramural Research (DIR) at NIAID/NIH, Bethesda, United States of America, in order to clarify this patient's disease.

We have identified a mutation in IRG1, which probably causes the immunodeficiency in our patient. We have shown that in healthy/normal human monocytes IRG1 mRNA correlates with itaconate levels, and that the time course of IRG1 and intracellular itaconate levels in following stimulation with LPS and/or IFNγ is similar to murine models: IRG1 transcript levels peak at 3-6h post stimulation (with LPS and LPS+IFNγ) whereas itaconate metabolite levels peak around 18-24h post stimulation (with LPS and LPS+IFNγ). Additionaly, we have also shown that in human monocytes, succinate accumulation correlates directly with increasing itaconate, corroborating the murine data of SDH inhibition by itaconate. Interestingly and contrary to what has been shown in murine models, LPS alone does not induce high levels of IRG1 mRNA or itaconate levels, which is achieved by LPS+IFNγ stimulation.

In recent years the functions of IRG1/CAD have become clearer. When macrophage is activated, IRG1 transcript is upregulated and its protein IRG1/CAD catalyzes the production and accumulation of itaconate. Itaconate functions as an anti-inflammatory metabolite through at least two different mechanisms: inhibition of SDH (blocking ROS production) and via alkylation of KEAP1 (allowing overexpression of Nrf2, which will activate downstream genes with anti-oxidant and anti-inflammatory functions). Additionally, it also has antimicrobial effects, mostly by

inhibition of ICL that will block the use of the glyoxylate shunt, an important intracelular survival strategy for many pathogens (Fig.30 and Fig.31).

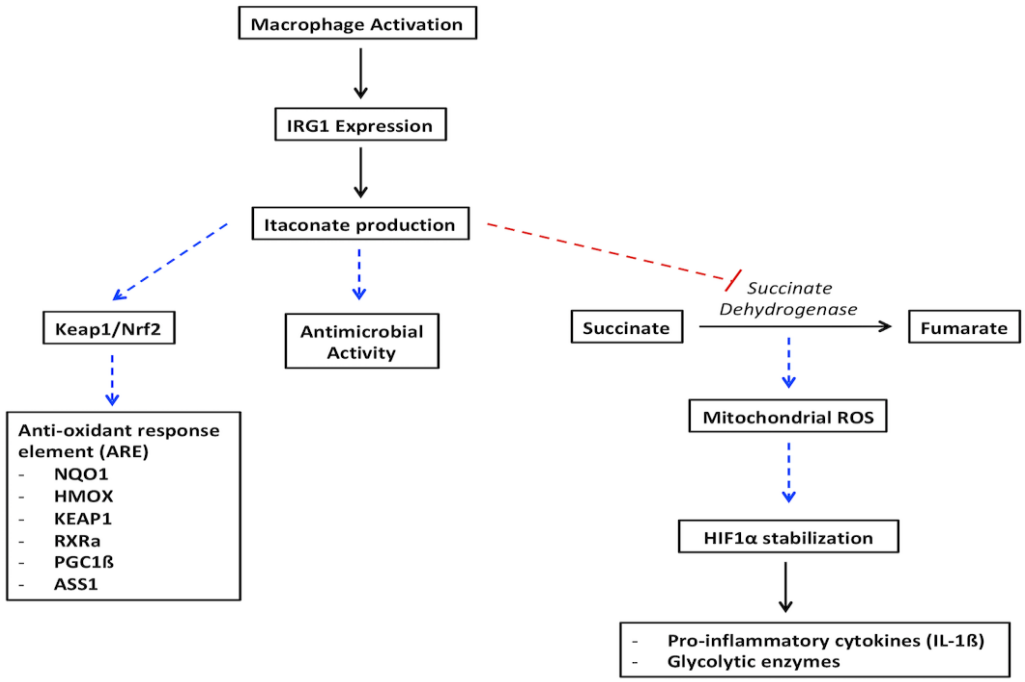


Figure 30: Proposed role of the IRG1-Itaconate Pathway in macrophage activation

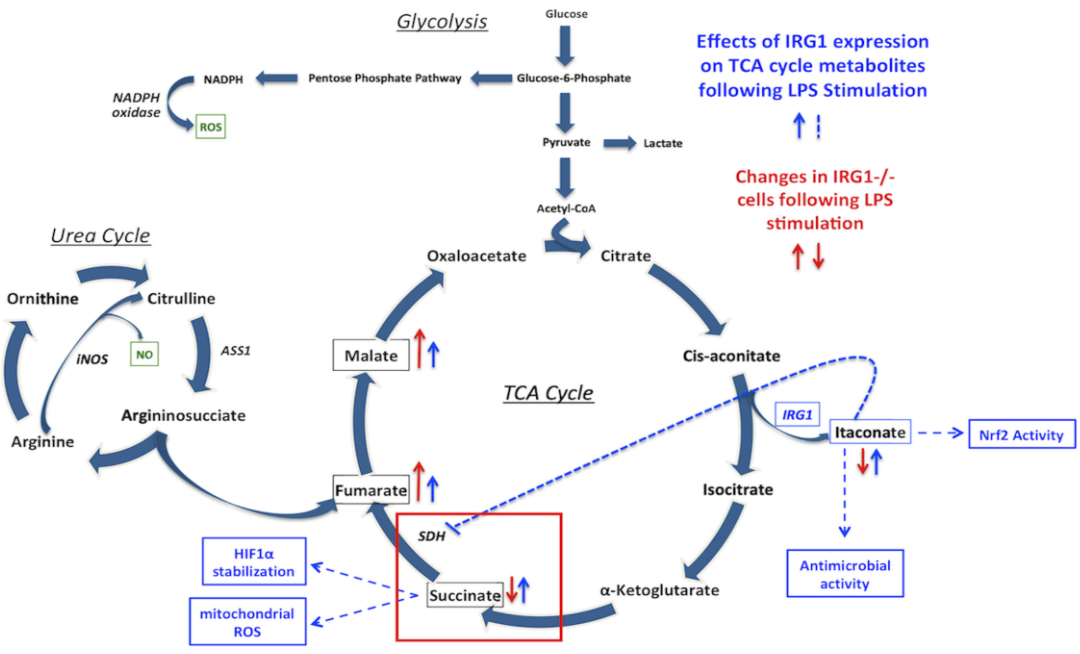


Figure 31: Overview of IRG1 functions

Our patient had severe muscle and soft-tissue infections, caused by multiple microorganisms, requiring multiple surgeries and prolonged courses of IV antibiotics. Surprisingly, she didn't have infections in other locations and most of the episodes happened in the same location as the previous one (either thigh or arm), leading us to speculate that she wasn't able to fully clear the infection, despite a prolonged course of antibiotics and imaging certification of absence of infection (we performed magnetic ressonance imaging before stopping antibiotics after the episodes of fasciitis at the age of 20 and 26 months). Interestingly, after adding prophylactic IFNγ, she only had one episode of infection during the last 3 years.

We speculate that in this patient, the IRG1 variant either causes a dominant-negative effect or leads to haploinsufficiency, leading to dysfunctional CAD activity and reduced itaconate levels. This will render the patient susceptible to infections, probably because her immune system isn't capable of clearing the infection due to reduced itaconate levels, explaining why the infections are restricted to certain localizations. On the other hand, when she is infected she isn't able to control and/or promote the resolution of infection associated inflammation due to the absence of itaconate proper function, leading to the extreme severity of each episode, probably explained by unregulated local inflammation. This would explain the very rapid installation and progression of the disease, leading to recurrent fasciitis, despite rapid diagnosis and beginning of antibiotics. We believe that exogenous IFNγ may have, in the presence of infection (synergistically with TLR stimulation from bacteria), induced higher levels of IRG1 mRNA, resulting in production of near-normal levels of itaconate, which will allow the patient to properly fight infection and avoid hyper-inflammation.

While there is a lot of new data published suggesting mechanisms for the regulation and function of IRG1 and itaconate, the majority of studies only evaluated murine macrophages. Here we have shown that while some of this data holds true in human monocytes/macrophages, there are also diferences that may affect assessment of the function of this pathway and therefore its role in disease pathogenesis. Specifically, we have identified differences in the transcriptional regulation of IRG1 in response to different stimuli.

For this reason, we plan to use the THP-1 (human monocyte) cell line to evaluate the proposed downstream functional effects of itaconate in humans. A method to simultaneously obtain RNA and intracelular metabolites from primary human monocytes has been established so that this patient's variant can be assessed directly in her primary cells. By using this method, we will be able to assess gene expression in the same limited sample of patient primary cells to determine the functional effects of itaconate deficiency in this patient specifically. This method will also help us to eliminate problems associated with studying cell lines or non-human cells as a proxy for human monocytes/macrophages.

C. IO-IBD COHORT

INTRODUCTION

Although the etiology of IBD remains unclear, it is known that several factors influence its appearance. Genetic predisposition and environmental factors have been widely discussed and several loci of genetic susceptibility have been reported recently. In the IO-IBD it is admitted that the genetic contribution, particularly the incidence of monogenic defects, has an even greater relevance.

In recent years, multiple monogenic defects causing VEO-IBD have been revealed, allowing for a better understanding of the pathophysiology of the disease. These genes encode proteins essential for the integrity of the intestinal epithelial barrier and for the fine regulation of the immune system.

It is now understood that distinct changes in the different components of the immune system may be the cause of VEO-IBD, namely the quantitative and qualitative defects of the monophagocytic system, defects in the selection and activation of T and B cells, defects in the regulation of the immune system, and also defects in the control of inflammation.

OBJECTIVES

To understand the immunological and genetic mechanisms of patients with IO-IBD

MATERIAL AND METHODS:

Demographic and clinical variables were obtained from all the patients with IO-IBD followed at HDE-CHULC hospital between January 2012 and December 2017 the most relevant data are summarized in table 3. An immunological investigation was performed in all the patients (oxidative burst, IL10 dosage, lymphocyte subpopulations, immunoglobulin dosage and tetanus and diphtheria vaccine serologies), as previously described in matherial and methods (Capther III)

In an attempt to identify genetic alterations responsible for the phenotype, different approaches were adopted according to 3 groups of patients:

A. IO-IBD patients who presented clinical characteristics compatible with known primary immunodeficiencies (P1-P2). In these patients, Sanger's sequencing of the presumed affected gene was carried out.

B. Patients with IO-IBD without other clinical symptoms (P3-P7). In this group, sequencing of a VEO-IBD related panel was performed (ADAM17, FOXP3, IL15, IRAK1, PRDM1, TGFB1, ATG16L1, FUT2, IL15RA, IRGM, PTEN, TGFB2, CARD9, GATA3, IL17A, LRBA, RNF186, TGFB3, CD40LG, GUCY2C, IL17RA, MYO5B, RORC, TINF2, CDX1, ICOS, IL1RL1, NCF1, RTEL1, TTC7A, CTLA4, IKBKG, IL23A, NCF2, SH2D1A, WAS, CYBA, IKZF2, IL23R, NCF4, STXBP2, WIPF1, CYBB, IL10, IL2RA, NOD2, TERC, WRAP53, DEFB1, IL10RA, IL33, PLCG2, TERT, XIAP, DKC1, IL10RB. In the cases where no genetic cause was identified, WES was performed.

C. Patients with IO-IBD and other clinical symptoms (P8-11), but in which the basic PID could not be predicted, only WES was performed.

In those patients where a new potentially causal variant was identified, studies were carried out to prove the pathogenicity and causality of that variant.

RESULTS

Eleven children with IO-IBD were identified. The main clinical characteristics of these patients, as well as the most relevant genetic studies are shown in table 3.

Name	Age (Years)	Age IBD Started (months)	Sex	Other manifestations	Sanger Sequencing	VEO-IBD Panel	WES	Gene identied	Variant
IO-IBD									
P1	Deceased	1	F	Severe perianal disease	IL10RB	NP	NP	IL10RB	DelE3 DupE6
P2	11	8 months	M	Venous collateral circulation Urogenital malformation Neutropenia Pulmonary hypertension	G6PC3	NP	NP	G6PC3	Homozygous c882_903dupl
P3	6	20	F	Severe perianal disease	IL10RA	Yes	NP	IL10RA	R117C R351G
P4	9	18	F	No	NP	Yes	Yes	No	-
P5	5	12	M	No	NP	Yes	Yes	No	-
P6		17	M	No	NP	NP	Yes	No	-
P7	3	15	M	No	NP	NP	Yes	No	-
P8	6	12	M	Uveitis Short stature	NP	NP	Yes	No	-
P9	6	15	F	Hepatopathy Enamel dysplasia Hypogammaglobulinemia Thyroiditis	TTC7A	NP	Yes	TTC7A	S539L A839T
P10	13	3	F	Uveitis Skin rash Pneumonitis	PLCG2	NP	Yes	PLCG2	Homozygous L848P
P11	7	1	M	Deafness Hypogammaglobulinemia Hyponatremia	STXBP3	NP	Yes	STXBP3	Q344S

Table 3: Demographic, clinical and genetic data of the IO-IBD cohort

A. IO-IBD PATIENTS WHO PRESENTED CLINICAL CHARACTERISTICS COMPATIBLE WITH KNOWN PRIMARY IMMUNODEFICIENCIES (P1-P2).

PATIENT 1 (P1): IL10 BETA RECEPTOR DEFECT (IL10RB)

Severe perianal disease started in the neo-natal period with multiple perianal abscesses, and fistulization, which required repeated admission into hospital for surgical treatment and antibiotic therapy (Fig.32) He also had recurrent folliculitis. At 3 months of age, he started mucus, puss and blood excretions, and colonoscopy and histology confirmed the diagnosis of IO-IBD.The genetic study of IL10RA and IL10RB genes revealed the presence of an exon 3 deletion (inherited from the mother) and a duplication of exon 6 (inherited from the father) of the IL10RB gene, conditioning a decrease in STAT3 phosphorylation after IL10 binding, which confirmed the diagnosis of IL10 axis malfunction by a deficiency in IL10 receptor B. The child received an allogeneic HSCT at 18 months from a matched unrelated donor but ultimately died of infectious complications in the post-HSCT period.

Figure 32: Perianal lesions in a IL10RB deficient patient. (photograph authorized by parents after proper informed consent)

PATIENT 2 (P2): TYPE IV CONGENITAL NEUTROPENIA (G6PC3 DEFECT)

An 11-year-old boy, born in Pakistan, son of consanguineous parents in the first degree. He had been living in Portugal since he was 8 years old, and he had presented an IO-IBD that was difficult to control since he was 8 months old, as well as multiple hospitalizations for pneumonia in his country of origin. He also had chronic respiratory failure with pulmonary hypertension, congenital heart disease (tricuspid insufficiency and presence of 3 pulmonary veins). He presented peculiar facies, an enlarged nasal bridge, as well as digital hypocratism, bilateral cryptorchidism and exuberant collateral circulation (Fig.33).

Figure 33: Clinical phenotype of a G6PC3 deficient patient. (photographs authorized by parents after proper informed consent)

Analytically, floating neutropenia and thrombocytopenia, IgM hypogammaglobuminemia, and NK lymphopenia stood out. The myelogram showed no maturative blockages. Given the suspected diagnosis of a G6PC3 defect, Sanger's sequencing of the GGPC3 gene revealed homozygous duplication of the last exon (hom c882_903dup, previously described as associated with this disease), confirming the diagnosis. Initiated therapy with G-CSF, prophylactic antibiotic therapy, with reduction of infectious intercurrences. However, given the difficulty of controlling IBD despite the use of multiple immunosuppressants, the patient received an allogeneic phenotypic allogeneic donor HSCT, with resolution of the intestinal inflammation and resolution of the hematologic phenotype. Currently he is 24 months post-HSCT, without symptoms associated with the underlying disease.

B. PATIENTS (P3-P7) WITH IO-IBD WITHOUT OTHER CLINICAL MANIFESTATIONS

These patients only had IO-IBD in common and presented no other symptoms or signs. No changes were detected in the immunological study of any of these patients.

Patient 3 (P3): Compound heterozygosity of the IL10RA gene

A child of 6 years of age, with IBD onset at 20 months of age, with disease controlled by azathioprine and short cycles of prednisolone. The NGS panel of 56 genes identified a compound heterozygosity of the IL10RA gene (R117C; R351G), previously reported as being associated with the disease.

Patients 4-7 (P4, P5, P6, P7): with no identified etiology

To date, the genetic study has not identified any potentially causal variant. Two of these patients are represented in a multicentre study published in the journal *Inflammatory Bowel Diseases* – annex 2.

C. PATIENTS WITH IO-IBD AND OTHER CLINICAL SYMPTOMS (P8-11), BUT IN WHICH A SPECIFIC DIAGNOSIS COULD NOT BE PREDICTED

Patient 8 (P8): no etiology identified

A 6-year-old child who presented IBD at the age of 12 months. The disease has been refractory to all treatments, including corticotherapy, azathioprine, sirolimus and anti-TNF. Most recently, vedolizumab was initiated: a monoclonal anti-integrin antibody that has recently been shown to be effective in patients with VEO-IBD. The child also has early onset uveitis (15 months) and short stature. To date, the genetic study has not identified a genetic alteration that would justify this clinical presentation.

Patient 9 (P9): HYPOMORPHIC MULTIPLE INTESTINAL ATRESIA ASSOCIATED WITH COMBINED IMMUNODEFICIENCY (MIA-CID)

6-year-old child with MIA-CID. Phenotypic changes were observed that were not previously described in this disease (enamel dysplasia, huge forehead, hepatic changes, hypogammaglobulinemia and autoimmune thyroiditis, reminiscent of Trichoenterohepatic Syndrome). The case is out lined in chapter IV.I. (Atypical PIDs - MISSENSE MUTATION OF TTC7A MIMICKING TRICHO-HEPATO-ENTERIC (SD/THE) SYNDROME IN A PATIENT WITH VERY-EARLY ONSET INFLAMMATORY BOWEL DISEASE).

Patient 10 (P10): APLAID SYNDROME

13-year-old teenager with APLAID. This patient had characteristics that had not been previously reported in APLAID syndrome (sensorineural deafness and lax skin) and a mutation that had not been described previously. The case is represented in Chapter IV.I. (Atypical PIDs -NOVEL *PLCG2* MUTATION IN PATIENT WITH APLAID, CUTIS LAXA AND SENSORINEURAL DEAFNESS)

Patient 11 (P11): NEW GENETIC DEFECT (Syntaxin Binding protein 3, STXBP3)

A 7-year-old child with IO-IBD, refractory to all treatments (including corticosteroids, azathioprine, mycophenolate, anti-TNF), and requiring colectomy at the age of 4 due to intestinal dysplasia. He also suffers from sensorineural deafness. A new STXBP3 deficiency was identified, as will be described in subchapter C.1 (Syntaxin Binding Protein 3 Defect).

DISCUSSION

The explosion of knowledge about IBD in children in recent years has allowed for the identification of multiples genes responsible for VEO-IBD, which has given us an improved understanding of the disease. The multidisciplinary evaluation of children with IO-IBD has enabled us to quickly identify many cases of PID that also present IBD as a clinical symptom, such as the cases of patients with IL10RB defect or G6PC3. In these cases, this recognition has made it possible to change the therapeutic approach, either through the use of different immunosuppression strategies, or through the proposed HSCT at a time when the patients were still not very ill, therefore giving them a greater chance of survival.

In many cases of IO-IBD, it is not possible to identify a genetic defect, and in others, there will be no single gene responsible for the phenotype, but most likely a combination of multiple factors, some genetic and others environmental, which pre-dispose the onset of the disease. Given that this disease is almost always quite complex, requiring repeated surgery and multiple immunosuppression regimens, an exhaustive study, particularly from the genetic point of view, is crucial for the correct assessment of patients as well as the improvement of their prognosis. It should be noted that in many cases of PID, patients are recommended to undergo HSCT without a clearly defined genetic diagnosis. This occurs because the clinical and immunological phenotype is such that it may predict that the problem is of hematopoietic origin. In the particular case of IO-IBD, this should not happen because not all patients will have a monogenic disease and even in those where only one gene is responsible for the phenotype, the problem may be caused by the loss of the intact intestinal barrier and therefore not amenable to be corrected by HSCT. Therefore, the precise identification of the genetic cause must be thoroughly investigated because it may well affect the therapeutic response.

From our research in our group of patients, monogenic disorders for IO-IBD were identified in 6 of the 11 patients studied, including a new PID (STXBP3).

This new concept associated with the paediatric IBD, namely the IO-IBD, in which we do not only aim to assess and control the clinical manifestations, but also

to discover the cause of the disease, has made it possible to improve knowledge about IBDs and to optimize the treatment of patients. Such a multi-disciplinary approach, adding immunologists, geneticists and basic research is fundamental to ensure the best care and outcomes for these complex patients.

C.1 SYNTAXIN BINDING PROTEIN 3 DEFICIENCY

Mutations in *STXBP3* result in Very Early Onset Inflammatory Bowel Disease, Bilateral Sensorineural Hearing Loss and Immune Deficiency

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ABSTRACT

Very early onset inflammatory bowel disease (VEOIBD) is characterized by severe intestinal inflammation affecting infants and children less than 6 years old. Genetic studies of VEOIBD have enabled, in some cases, adoption of life-saving therapeutics, such as bone marrow transplantation. To date, nearly 60 monogenic etiologies of VEOIBD have been identified, many characterized by highly penetrant homozygous recessive or dominant mutations in underlying immune and/or epithelial pathways. Of relevance to this study, damaging homozygous recessive or dominant negative monoallelic mutations in *STXBP2* (Syntaxin binding protein 2) (Munc18-2), a gene known to cause familial-hemophagocytic lymphohistiocytosis (HLH), causes VEOIBD and sensorineural hearing loss. Here, we describe ten patients from five non-consanguineous families presenting with life-threatening infantile-onset diarrhea, consistent with VEOIBD, and eight of these patients with bilateral sensorineural hearing loss. The patients' medical courses were resistant to standard medical therapy. None of the known monogenic etiologies of IBD were identified following next generation sequencing. In each of the 10 patients described, we identified damaging mutations in *STXBP3*, a protein known to regulate intracellular vesicular trafficking and, like STXBP2, is in the syntaxin-binding protein family of molecules, but not previously associated with either VEOIBD or sensorineural hearing loss. Overall, we describe a novel genetic syndrome and identify a role for *STXBP3* in VEOIBD and sensorineural hearing loss.

Inflammatory bowel diseases (IBD) are chronic inflammatory diseases that result in a dysregulated immune response to microbes in the gastrointestinal tract in genetically susceptible individuals[1-4]. Very early onset inflammatory bowel disease (VEOIBD) manifests prior to 6 years of age[5]. VEOIBD is phenotypically distinct from older-onset IBD with some patients exhibiting increased disease progression and severity and poor responsiveness to therapies[5-12]. Given the young age of onset, genetics likely plays a more direct role in VEOBID than in older onset disease. Indeed, next generation sequencing studies focusing on patients with VEOIBD revealed causal monogenic and digenic variants associated with VEOIBD involving primary immunodeficiency genes[6, 7, 13], inflammasomopathies (e.g., *NLR4*, *MEFV*)[14-17], hemophagocytc lymphohistiocytosis (HLH – e.g., *STBP2*, *XIAP*)[18-25] and genes regulating epithelial homeostasis (e.g., *TTC7A, CASP8*)[26, 27]. A growing number of known genetic etiologies underlie VEOIBD, with over 60 identified to date[6, 10, 28]. VEOIBD remains a rare disease entity, though the rate of rise of disease incidence of IBD diagnosed among children under 6 years of age is escalating at an increased rate in some populations (i.e., Canada) as compared to older age groups[11]. While not all cases are as clinically severe and medically refractory as initially believed[11, 29], many are resistant to available medical interventions and remain especially challenging to manage[6, 9, 29].

We describe 10 patients from 5 unrelated and non-consanguineous families with medically-refractory VEOIBD and damaging mutations in *STXBP3*, encoding Syntaxin-Binding Protein 3, a gene not previously associated with VEOIBD. Intriguingly, 8 of these 10 patients also experienced bilateral severe sensorineural hearing loss.

Whole exome sequencing (WES) was performed in 1,517 VEOIBD patients from five centers. We identified 5 patients from 3 families with VEOIBD and bilateral sensorineural hearing loss, with heterozygous mutations in *STXBP3*. A fourth family was identified by GeneMatcher[30] consisting of 3 patients with biallelic mutations in *STXBP3*, VEOIBD, hearing loss, craniofacial defects and hypotonia. A fifth family was later identified, including two siblings with VEOIBD, hypotonia, and biallelic mutations in *STXBP3* (Figure 1 and Table 1); notably, hearing loss was not clinically documented.

All patients presented within months of life with severe diarrhea and in most cases, life-threatening hyponatremia and hypoalbuminemia, often times resulting in repeated admissions to the pediatric intensive care unit. Endoscopy and colonoscopy in most probands revealed erythema and hemorrhagic lesions in the small and large intestine, ileal ulcerations, and colonic pseudopolyps (Figure 2). Histology revealed inflammatory infiltrates, crypt abscesses, villous atrophy, eosinophilia, apoptosis, architectural distortion and crypt destruction (Figure 2). Many patients exhibited recurrent infections with some patients having low NK cell numbers (Table 1). Patients were all resistant to standard medical management of VEOIBD.

An abbreviated summary of each patient is provided below. P1 (Family 1) manifested severe diarrhea within 2 weeks of life and was found to have bilateral severe sensorineural hearing loss for which he received cochlear implants. After failing various immunosuppressants, P1 underwent a diverting ileostomy, without

improvement, and required total parenteral nutrition (TPN). With concern for HLH, the patient underwent hematopoietic stem cell transplant, with resolution of intestinal symptoms 3 years post transplantion. He underwent successful takedown of the diverting ileostomy and repeat endoscopic evaluation demonstrated mucosal healing, confirmed by histology. P2 (Family 2) also presented within weeks of life with bilateral sensorineural hearing loss and bloody diarrhea that was medically refractory to a variety of medications. By 3 years of age, he exhibited several colonic foci of high-grade dysplasia and underwent a total colectomy. Post-operatively he continued to have small intestinal disease. P3 (Family 3) was born deaf and developed marked bloody diarrhea by 6 months of age. He failed various medical interventions and was dependent on TPN. He underwent ileal diversion at 17 months old, with persistent symptoms and finally underwent a colectomy with resolution of symptoms. His sister, P4 (Family 3) developed profound bloody diarrhea by 4 months old and after failing a smaller number of medical interventions underwent colectomy with ileostomy at 8 months of life. Their father, P5 (Family 3), is also deaf since infancy and developed severe bloody diarrhea within the first few months of life and underwent partial colectomy at 18 months of life but required a complete colectomy at 4 years of age. After several surgical revisions, he remains on no medications. P6-P8 (Family 4) have unaffected parents, but each exhibit hypotonia, a blistering skin disorder, multiple joint contractures, craniofacial dysmorphism along with severe bilateral sensorineural hearing loss. Each developed severe secretory diarrhea with electrolyte imbalance and dehydration within 6 weeks of life. They were each admitted multiple times for hypovolemic shock secondary to life threatening diarrhea (one with over 20 admissions). They required TPN and are now in young

adulthood with significant improvement of symptoms. In Family 5, P9-P10 both had severe myopathy, contractures and enteropathy. P9 died from veno-occlusive disease at 8 years of age. P10's myopathy was so striking he required ventilation at birth and manifested diarrhea at 6 weeks old. His disease was initially refractory to a variety of medical interventions but currently is maintained on Sirolimus.

WES revealed *de novo* heterozygous mutations in *STXBP3* in P1 and P2 from Families 1 and 2, respectively. In Family 3, affected siblings (P3, P4) and an affected father (P5) have a heterozygous mutation in *STXBP3*. Each of the mutations in P1-P5 change the last nucleotide of exon 12/intron 12 junction of *STXBP3* (Fig. 1b), predicted to impair the AG/GT exon 12 splice donor (SD) site. Specifically, P1 has a deletion at position 1029, predicted to result in protein product that ends at position K343. P2-P3 each inherited an Adenine instead of a Guanine at position 1029+1 of the 12th intronic segment, predicted to result in retention of an additional 10 amino acids before the protein is prematurely terminated. In each of these patients (P1-P5), a heterozygous mutation, not thought to be independently deleterious[31], was also identified in genes of the cytolytic pathway, or a known VEOIBD gene (See Table 2 for overview of genetic variants). Specifically, P1 inherited a maternally transmitted heterozygous missense mutation in *RAB27A* (c.239C>T, p.R80K, not previously reported). P2 inherited a homozygous variant in *PRF1* (c.272C>T, p.A91V) with a frequency of 0.03 (8444/275566 alleles; 165 homozygous individuals) in gnomAD (http://gnomad.broadinstitute.org/variant/10-72360387-G-A). When present alone, these mutations are usually considered normal polymorphisms, however, they have been described in familial HLH (F-HLH) patients carrying mutations in other cytolytic

pathway genes or as one of the alleles in F-HLH patients with compound heterozygosity in *PRF1*[32]. P3-5 have a heterozygous missense mutation in *LRBA* (c.2170A>G, p.I724V). Although homozygous recessive damaging *LRBA* mutations lead to common variable immunodeficiency with enteropathy[33], p.I724V is reported 94 times in a homozygous fashion in gnomAD, so unlikely to cause VEOIBD alone. In contrast to P1-5 which were associated with heterozygous STXBP3 mutations, P6-P10 have biallelic rare missense mutations in *STXBP3*, specifically, P6-P8 each inherited c.1256C>T or p.T419M (MAF in ExAC of $8.34X10^{-6}$) along with c.205C>T or p.R69C (not reported previously), while P9-P10 inherited c.442T>C or p.Y148H (not previously reported) and c.206G>A or p.R69H (MAF in gnomAD of $4.19X10^{-6}$). P10 also inherited a compound heterozygous mutation in *RTEL1*, and he and his non-affected family members have heterozygous mutations in other genes associated with VEOIBD (*TTC7A, TRIM22, SKIV2L, RTEL1, STAT1)*. See Supplemental Tables

Analysis of the gnomAD database (http://gnomad.broadinstitute.org/gene/ENSG00000116266) revealed 23 heterozygous loss-of-function mutations with high confidence and depth in 138,632 adults (without severe pediatric disease). We suspect that digenic inheritance of a *STXBP3* heterozygous loss-of-function mutation, with an additional pathogenic variant such as in *RAB27a*, *PRF1* or *LRBA,* may be required to explain disease in P1-P5. We suspect that compound heterozygous loss-of-function for *STXBP3* in Families 4-5 are sufficient to cause similar disease with additional phenotypes (Table 1).

To measure the significance of the number of observed putatively causal variants in *STXBP3* or in both *STXBP3* and one of *RAB27A*, *PRF1*, or *LRBA*, we made a null expectation model for these observations, as done previously for single gene defects generated from large population cohorts[34]. Among the 1,517 VEOIBD patients sequenced, we identified the 10 individuals described above with rare (MAF ≤ 0.1%) non-synonymous coding or splice site variants in *STXBP3*. Among these, five affected individuals had rare non-synonymous compound heterozygous variants in *STXBP3*. The number of such variant observations in the VEOIBD cases is significantly higher than in gnomAD ($P<1x10^{-16}$), where the expected frequency is $1.17x10^{-5}$ (or ~1.2/100,000 individuals).

The cases in Families 1, 2, and 3 include 3 probands and 2 additional affected first-degree relatives, each inheriting a rare (MAF ≤ 0.1%) non-synonymous heterozygous variant in *STXBP3* and a heterozygous variant (MAF ≤ 3%) in any one of *RAB27A*, *PRF1*, or *LRBA*. The expected rate of identifying such a combination of variants in gnomAD is $5.64x10^{-4}$ (or ~5.6/10,000 individuals). The number of such variant observations in the VEOIBD cases is significantly higher than in gnomAD ($P=8.67x10^{-3}$). When combined, we find that 10 affected individuals in five families inherited a primary rare variant in *STXBP3* and another variant in *STXBP3*, *RAB27A*, *PRF1*, or *LRBA* to be significantly enriched in VEOIBD cases over gnomAD ($P=5.64x10^{-4}$). Taken together, the occurrence of presumptive *STXBP3* loss-of-function mutations in 5 independent families with life threatening VEOIBD and sensorineural hearing loss strongly supports a causal role for mutations in *STXBP3* in this clinical condition.

To examine whether variants in *STXBP3* are also associated with altered risk in adult IBD, we analyzed variants in the *STXBP3* gene in patients with IBD (UC, CD, or both) in the NIDDK ExomeChip cohort, which is a cohort of European ancestry individuals recruited at IBD centers across North America comprising 12,467 IBD cases and 8,352 non-IBD controls as previously described[35]. None of the 6 *STXBP3* variants genotyped on ExomeChip were observed to be associated with IBD, CD or UC in these cohorts.

We identify *STXBP3* to be associated with VEOIBD, immunodeficiency and sensorineural hearing loss in eight patients from four unrelated families, and VEOIBD in two patients from a fifth family. P1-P5 carry similar heterozygous mutations that likely interfere with splicing and are phenotypically similar, whereas P6-P10 inherited biallelic *STXBP3* missense mutations with additional comorbidities. With the important proviso that P1-P5 carry additional potentially contributory variants in *RAB27A*, *PRF1* and *LRBA,* and possibly in unidentified loci, the common VEOIBD and sensorineural hearing loss phenotypes in these five cases apparently require heterozygous loss-of-function of *STXBP3.* On the other hand, P6-P10 have biallelic missense mutations in STXBP3, sharing mutations in position 69. They exhibit additional clinical features including myopathy and hypotonia, limiting the possibility that these phenotypes reflect co-segregating modifier genes. Intriguingly, P9-P10 did not demonstrate hearing loss. Whereas P1-P5 each retain a WT *STXBP3* allele, none exists in P6-P10. R69 is highly conserved among all members of the Sec/Mun18c protein family, and the crystal structure of STXBP3[36] shows that they reside in critical regions that interact with other membrane trafficking proteins such as SNAREs (STX4

and VAMPs) and DOC2B[37-39]. Y148 is highly conserved among mammals (UCSC Genome Browser[40]). *STXBP3* null mice are embryonically lethal, thus only hypomorphic mutations are likely compatible with life[41, 42].

STXBP3 heterozygous loss-of-function alone may be insufficient to confer VEOIBD and hearing loss, given lack of disease in parents of Families 4-5 and the presence of heterozygous loss-of-function variants in the gnomAD database. We propose two mechanisms whereby *STXBP3* loss-of-function can cause disease. One, exemplified by Families 4-5, involves monogenic biallelic mutations for *STXBP3.* A second, involves digenic inheritance where *STXBP3* heterozygous loss-of-function is compounded with variants in exocytosis pathway genes such as *RAB27A*, *PRF1* or genes involved in VEOIBD such as *LRBA* in P1, P2 and P3-5, respectively.

STXBP3 regulates glucose uptake by controlling the trafficking of the GLUT4 glucose transporter to the plasma membrane[43]. Lack of abnormal glucose homeostasis in our patients might be explained by restrictive nutrient intake and parenteral nutrition. Similarly, *Stxbp3*$^{+/-}$ mice only develop glucose intolerance with a high-fat diet[41].

P1-P8's sensorineural hearing loss suggests STXBP3 may be necessary for normal inner ear hair cell function or vestibulocochlear nerve cells. Notably, a zebrafish insertional mutant in *stxbp3* displays markedly smaller ears and otoliths, pericardial edema and a slightly underdeveloped gut (http://zfin.org/ZDB-FIG-070117-940), paralleling features in these patients.

In conclusion, we identify damaging mutations in *STXBP3* as responsible for VEOIBD and sensorineural hearing loss. Importantly, although mutations in STXBP2 also cause VEOIBD and hearing loss, the role of *STXBP3* must be distinct from and cannot be compensated by *STXBP2*. This work provides a better understanding the genetic etiologies of VEOIBD.

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Clinical Phenotype	**Family 1**	**Family 2**	**Family 3**			**Family 4**			**Family 5**	
	P1	P2	P3	P4	P5 (F3)	P6	P7	P8	P9	P10
Sex	M	M	M	F	M	M	F	F	M	F
Status (Alive/Deceased)	A	A	A	A	A	A	A	A	A	D
Gastrointestinal	+	+	+	+	+	+	+	+	+	+
Surgical intervention	+	+	+	+	+	-	-	-	-	-
Diarrheal episodes	+	+	+	+	+	+	+	+	+	+
Liver disease	-	-	-	-	-	-	-	-	-	+
Intestinal biopsies (SI/C):										
Inflammation	+/+	+/+	+/+	+/+	?/?	-/+	?/?	?/?	?/?	?/?
Apoptosis	+/+	-/+	-/-	-/-	?/?	-/-	?/?	?/?	+/+	?/?
Eosinophilia	+/+	+/+	+/+	+/+	?/?	-/+	?/?	?/?	?/?	?/?
Immunological	+	+	-	-	-	+	+	+	-	-
Recurrent infections	+	+	-	-	-	+	+	+	+	+
HSCT	+	-	-	-	-	-	-	-	-	-
Sensorineural hearing loss	+	+	+	+	+	+	+	+	-	-
Cochlear implants	+	+	-	-	-	-	+	+	-	-
Craniofacial defects:	-	-	-	-	-	+	+	+	-	-
Low set ears	-	-	-	-	-	+	+	-	-	-
Deep set eyes	-	-	-	-	-	+	+	+	-	-
Arched palate	-	-	-	-	-	+	+	+	-	-
Microcephaly	-	-	-	-	-	+	+	+	-	-
Doliocephaly	-	-	-	-	-	-	-	+	-	-
Muscle	-	-	-	-	-	+	+	+	+	+
Hypotonia	-	-	-	-	-	+	+	+	+	+
Limb and body	-	-	-	-	-	+	+	+	-	-
Podiatrial syndactyly	-	-	-	-	-	+	+	+	-	-
General Laboratory										
Bicytopenia	+	-	-	-	-	-	-	-	+	-
NK Cell number	L	L	N	N	-	N	N	N	N	N
Fibrinogen <1.5g	+	-	-	-	-	+	+	+	-	-
Hyponatremia	+	+	+	+	-	-	+	+	+	-
Hypoalbuminemia	+	+	+	+	+	+	+	+	+	+
LDH > 500 U/L	+	+	?	?	+	+	+	+	+	+

Table 1: Summary of clinical case presentation. Bicytopenia: two hematopoietic cell lines are decreased. Abbreviations: M male; F female; A alive; D deceased; SI/C: Small intestine/colon; HSCT hematopoietic stem cell transplant; L low; N normal; LDH, lactate dehydrogenase ? refers to unavailable.

Affected individual	Gene	Genomic location	Allele Frequency	Allele Count	Variant cDNA change	Amino Acid change	Segregation	CADD score	PHRED-scaled score
P1	*STXBP3*	1:109336269	NR	NR	c.1029delG	p.K344fs	*De novo*	5.41205	26
	RAB27A	15:55522599	NR	NR	c.239C>T	p.R80K	Maternal	7.014	33
P2	*STXBP3*	1:109336270	NR[#]	NR[#]	c.1029+1G>A	p.Q344S_ins10*	*De novo*	5.81806	27.2
	PRF1	10:72360387	$2.9X10^{-2}$	8191	c.272C>T	p.A91V	Homozygous	5.40661	26
P3	*STXBP3*	1:109336270	NR[#]	NR[#]	c.1029+1G>A	p.Q344I_ins10*	Paternal	5.81806	27.2
	LRBA	4:151793903	$2.1X10^{-2}$	5728	c.2170A>G	p.I724V	Paternal	1.362	12.59
P4	*STXBP3*	1:109336270	NR[#]	NR[#]	c.1029+1G>A	p.Q344I_ins10*	Paternal	5.81806	27.2
	LRBA	4:151793903	$2.1X10^{-2}$	5728	c.2170A>G	p.I724V	Paternal	1.36232	12.59
P5 (F3)	*STXBP3*	1:109336270	NR[#]	NR[#]	c.1029+1G>A	p.Q344I_ins10*	Unknown	5.81806	27.2
	LRBA	4:151793903	$2.1X10^{-2}$	5728	c.2170A>G	p.I724V	Unknown	1.362	12.59
P6	*STXBP3*	1:109339248	$1.4X10^{-5}$	4	c.1256C>T	p.T419M	Maternal	3.966947	27.7
		1:109299335	NR	NR	c.205C>T	p.R69C	Paternal	4.266642	32
P7	*STXBP3*	1:109339248	$1.4X10^{-5}$	4	c.1256C>T	p.T419M	Maternal	3.966947	27.7
		1:109299335	NR	NR	c.205C>T	p.R69C	Paternal	4.266642	32
P8	*STXBP3*	1:109339248	$1.4X10^{-5}$	4	c.1256C>T	p.T419M	Maternal	3.966947	27.7
		1:109299335	NR	NR	c.205C>T	p.R69C	Paternal	4.266642	32
P9	*STXBP3*	1:109315290	NR	NR	c.442T>C	p.Y148H	Maternal	5.63267	26.6
		1:109299336	$4.1\ X10^{-6}$	1	c.206G>A	p.R69H	Paternal	5.50228	26.2
P10	*STXBP3*	1:109315290	NR	NR	c.442T>C	p.Y148H	Maternal	5.63267	26.6
		1:109299336	$4.1\ X10^{-6}$	1	c.206G>A	p.R69H	Paternal	5.50228	26.2

Table 2: Variants reported in STXBP3 and related pertinent genes (either in vesicular pathway or known VEOIBD gene). MAF- Minor allele Frequency in gnomAD; Allele Count in gnomAD; NR- not reported; [#]- there is a mutation reported a single time in gnomAD at position c. 1029+1 with a different insertion of G>T. fs- Frameshift

Figure 1

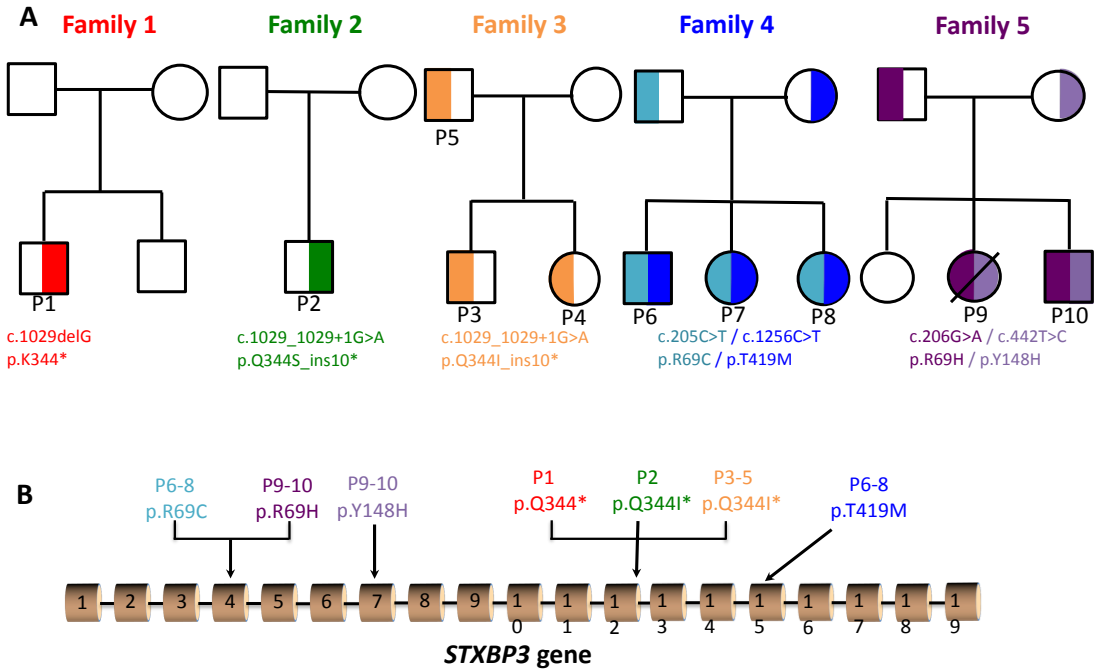

Figure 1: *STXBP3* mutations in 5 families with VEOIBD: (a) Pedigrees of 5 families illustrating 10 patients with VEOIBD and mutations in *STXBP3*. Patient 1 (P1) in Family 1 (red) has a *de novo* heterozygous *STXBP3* mutation c.1029delG, p.Q344*. Patient 2 (P2) in Family 2 (green) has a *de novo* heterozygous *STXBP3* mutation c.1029_1029+1G>A, predicted to result in p.Q344I_ins10*. In Family 3, the two affected siblings (P3, P4) and affected father (P5) carry the same heterozygous mutations in *STXBP3* as does P2 (c.1029_1029+1G>A, predicted to result in p.Q344I_ins10*)(orange). Patients 6, 7, and 8 (P6-8) in Family 4 (blue) have compound heterozygous *STXBP3* mutations c.C205T, p.R69C (light blue) and c.C1256T, p.T419M (royal blue). Patient 9 and 10 of Family 9 (purple) have a compound heterozygous *STXBP3* mutations c.G206A, p.R69H (purple) and c.T442C, p.Y148H (lavender). **(b)** Schematic representation of the *STXBP3* gene showing the position of the identified *STXBP3* mutations of each patient.

Figure 2

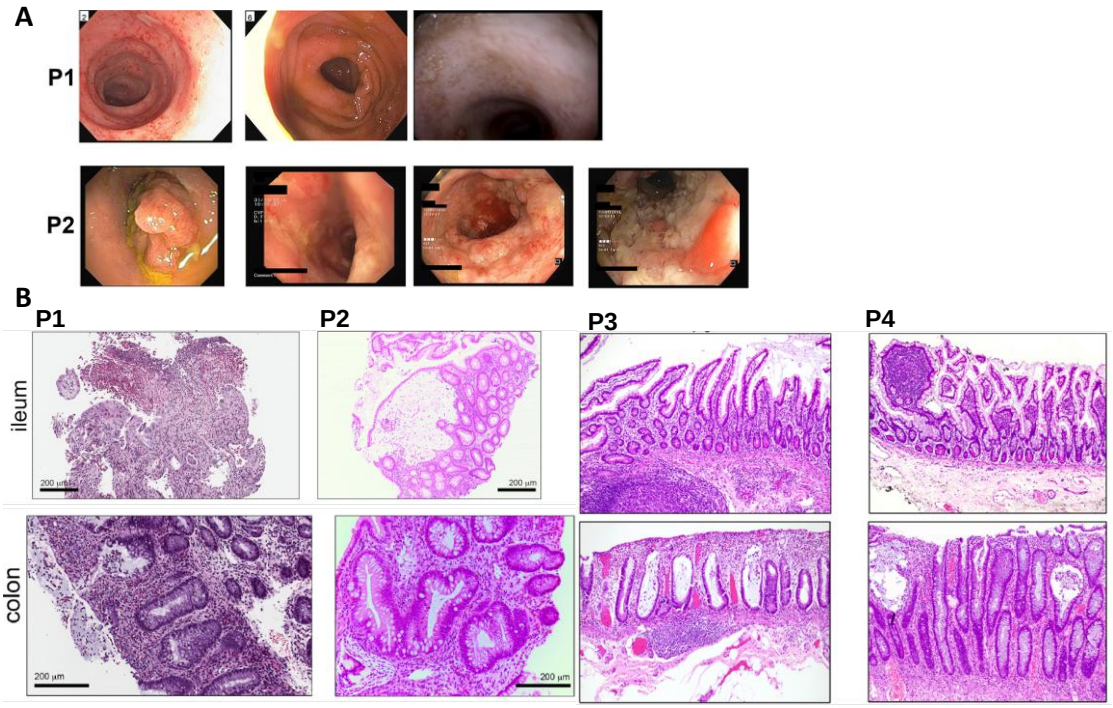

Figure 2: Abnormal colonoscopy and histology of biopsies from ileum and colon of patients with STXBP3 mutations. (a) Colonoscopy images of Patient 1 (P1) and Patient 2 (P2) showing deteriorating lesions of the mucosa with edema, pseudopolyps formations, exudate, crypt abscesses, ulcerative and hemorrhagic lesions. **(b)** Upper Panels: images of endoscopically obtained ileal (upper panel) and colonic (lower panel) biopsies from Patients 1, 2, 3 and (P1-P4) stained with H&E revealing active colitis including neutrophilic crypt abscesses, minimal evidence of crypt architecture distortion with neither significant lamina propria plasmocytosis nor epithelial apoptosis. Mild lamina propria eosinophilia is noted.

CHAPTER V

Discussion

MAIN FINDINGS

The reported number of PIDs has grown steadily in recent decades.[147] This fact was primarily due to the globalisation of laboratory techniques for the study of the immune system, largely because of the pandemic human immunodeficiency virus (HIV) infection.[83] More recently, already in the 21st century, molecular biology techniques became more widespread, making it possible to identify many genes that cause PID. The use of massively parallel sequencing technology (Next-Generation sequencing, NGS) has revolutionised our knowledge of PIDs, allowing us to identify new genes, which have led to the discovery of new mechanisms, subsequently leading to the exploration of new pathways of the immune system.[107-109] This has allowed for the development of new therapies, many of them tailor-made, "designed" for each type of pathology.

This thesis seeks to contribute to this explosion of knowledge through the description and investigation of PIDs with unusual and/or fatal phenotypes, through three different channels: identifying PIDs whose genetic cause has already been described, but presenting previously unreported manifestations (atypical PIDs); clarification of fatal manifestations of known PIDs and the identification of new molecular defects that cause PIDs.

Atypical PIDs

In the 4 studies presented in Chapter IV.I, clinical symptoms and the laboratory investigation of patients did not allow for a definitive diagnosis to be reached. The use of WES has allowed us to make a genetic diagnosis of the patients and, subsequently, the research considered necessary to establish the respective phenotypes has been carried out. Despite the vast difference between each one of these cases, the elucidation of the etiology of the diseases in every one of them has not only contributed to a better knowledge of the pathology in question, but also to a better adequation of the health care provided to patients.

SCID is a medical emergency, and almost all cases are fatal in the first year of life if not properly diagnosed and treated.[149] Hypomorphic SCID cases are rare, but are particularly uncommon in XL-SCID cases (8 cases described in the literature).[152-159] In these, half are due to a widely described phenomenon of somatic reversion, where the most affected strain, in this case the T lymphocytes, revert the mutation by reversing the affected nucleotide.[432] The others are usually due to hypomorphic mutations in the regulatory region. The patient under investigation (had an unusual clinical pattern, exhibiting less susceptibility to infection, but progressive degradation of the T-cell function, which had never been reported before, as well as a liver dysfunction in the form of nodular regenerative hyperplasia. The presence of a novel mutation that had never been seen before and the atypical course of the disease prompted us to further investigate the problem to prove that the mutation was indeed causal. We proved that it was a hypomorphic mutation that led to a diminished, but not abolished expression of the γc protein, causing a loss of phosphorylation of STAT5 post stimulation with IL-2 e IL-7, but not after stimulation with IL-15, justifying the immunological phenotype of SCID $T^-B^+NK^+$.[42,432-434] These findings allowed us to extend our knowledge about the disease, as well as to provide genetic counseling to the family.

As for the hemophagocytic syndrome, or hemophagocytic lymphohistiocytosis (HLH), it is an entity characterized by a toxic immune hyper-activation, which can be triggered by several pathologies and/or genetic defects.[189,435] Classically, primary or familial HLH (fHLH) was defined as resulting from cytotoxicity defects and secondary HLH as being caused by immune hyperactivation due to a wide range of antigenic stimuli.[186,187,436] Although the HLH-2004 criteria were created to identify cases of fHLH, their compliance is not indicative of the presence of a cytolytic defect in all cases.[436,437] However, the correct and timely diagnosis of family HLH (fHLH) is crucial, since the fatality from this disease is more than 90% if it is not properly treated.[187,188,438] The early age of onset of HLH and the presence of consanguinity are among the few characteristics that usually allow the identification of cases of fHLH.[439] The therapy of fHLH cases is based on immunosuppression, inflammation control and subsequent HSCT.[437,440-442] The

immunosuppression chosen varies from one centre to another, but it is based on the intensive chemotherapy (HLH-04 protocol) or on immunotherapy, through the use of drugs that stop the T hyper-response, such as antimocyte serum or alemtuzumab. [442-445] In recent years, more conservative strategies using monoclonal anti-cytokine antibodies have been recommended in order to reduce the toxicity of the therapy[441,443] One of the most advocated strategies is the use of anti-IFNγ monoclonal antibodies, which is considered to be the most important cytokine in the pathophysiology of HLH.[446,447] The patient under investigation was the daughter of consanguineous parents who presented HLH at 2 months of age, triggered by a widespread infection spread by *Mycobacterium bovis* (Mb) and by *Citomegalovirus*. The presence of HLH triggered by two serious infections, namely by Mb, in a small breastfeeding infant born to consanguineous parents, did not limit the diagnostic hypotheses to the genetic causes of fHLH. A complete defect in the receptor 2 of IFNγ was diagnosed. The research carried out has elucidated the pathophysiology of HLH in the presence of signaling deficiency of IFNγ: the presence of PID resulting in a disseminated Mb (intracellular bacteria) infection lead to hyperstimulation of TLR9 and a consequent cytokine storm through an IFNγ independent pathway.[448-451] This study allowed us to expand our knowledge about the pathophysiology of HLH and to understand that even in patients born from consanguineous parents and presenting with very early onset HLH, the existence of fHLH is not certain. This also raises many reservations concerning the application of anti-IFNγ monoclonal antibodies in patients without an established genetic diagnosis, as HLH may originate in other forms, independent of this cytokine.

Rare, autossomal recessive mutations in Tetratricopeptide Repeat Domain 7 (TTC7A) have been linked to a complex and heterogenous syndrome with variable imunodeficiency and intestinal involvement.[177,452-458] TTC7A recruits and targets PI4KIIIα to the plasma membrane,[383,459,460] and then this complex is stabilized by an adaptor protein FAM126 and tethered to the plasma membrane by ERF3B.[383,460] This complex will then allow phosphorylation of phophatidyl inositol (PI) membrane lipids to form PI4-phosphate (PI4P). Normal levels of PIP4 are essential for plasma membrane identity, polarity and cell survival. [382,461] Abnormal TTC7A function will

lead to loss of polarity, increased apoptosis, poor barrier function and dysregulated cytoskeleton.[382,461] TTC7A deficiency was initially linked to multiple intestinal atresia with combined immunodeficiency (MIA-CID),[177,454,456,458,462] and then to VEO-IBD.[177,463] In recent years, very different phenotypes have been described,[452,453,457,464,465] and very recently a genotype/phenotype correlation was proposed.[466] Our patient was the first patient described to present hepatic involvement, dental abnormalities and to present simultaneously intestinal atresia and VEO-IBD.[467] Expanding the clinical features of a complex syndrome facilitates the diagnoses of new patients,[455,463] contributing to timely diagnosis and directed therapy. Interestingly, it is well known that HSCT will rescue the immunologic phenotype but will not resolve the intestinal manifestations, which would require an intestinal transplant.[461,466-468] In recent years, very good results with a RhoA kinase inhibitor (Y27632) were obtained in vitro, raising hope for a directed, tailor-made, therapy.[176,457,463]

Last but not least, PLCγ2 is a protein that is very important for the downstream cellular activation of many diferent pathways, by catalyzing the phosphatidylinositol biphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 will release calcium from the ER to the cytoplasm and Dag will activate different cell signalling proteins.[279,469,470] Autoinflammation and PLCγ2-associated antibody deficiency and immundysregulation (APLAID) is a very rare disease caused by missense mutations in the PLCG2 gene that had only been described in one American family with skin ganullomata, recurrent infections, interstitial pneumonitis and posterior uveitis.[278] These two patients (father and daughter) were both adults and their disease had a mild course except for the severe eye disease. It had been postulated that the hyperinflammation seen in these patients was driven by NLRP3 hyperactivation.[471] Our patient had a novel mutation that led to some new features, namely cutis laxa and sensorineural deafness. Moreover, her clinical course was much more severe, with skin granullomata from birth, systemic granullomatous disease (liver, lungs), eye inflammation and IBD since the first years of life.[472] She was refractory to multiple treatments, namely steroids, azathioprine and IL-1 blockers. Contrary to what has been previously published, we

have proven that her disease was not IL-1 driven, and the inefficacy of IL-1 blockers also supported this idea. This prompted us to start an oral daily JAK inhibitor, ruxolitinib, in view of the need of upstream signalling for PLCγ2 activation in APLAID.[279,469,470] The patient had a dramatic response in terms of normalization of the inflammatory markers and resolution of the eye and skin inflammation. This leads us to suggest that early JAK inhibition could be a good option in the treatment of APLAID patients. Unfortunately, the patient died unexpectedly after a programmed lung biopsy performed for pre-HSCT evaluation of her lung disease.

Fatal PIDs

In the two case studies in the chapter IV.II, the children had a known PID (XLP1 in the first case, CGD in the second) but developed an uncommon fatal complication. In both cases, explanations were sought to explain the severity of the presented phenotype, in order to improve the understanding of the disease and allow that other patients with the same pathology may have a different outcome.

XLP1 is a classically known PID, characterized by a very restricted susceptibility to severe Epstein-Barr virus (EBV) infection.[473] This particularity is due to the fact that the T cytotoxic response is only affected when the antigens are presented by cells with receptors that belong to the SLAM family (*signalling lymphocytic activation mollecule*) like the B lymphocytes, where EBV establishes latency. Most pathogens have antigens that are presented by dendritic cells, that do not have receptors of the SLAM family.[474-476] In recent years, rare cases of patients with clinical symptoms other than fulminant infectious mononucleosis/HLH, such as agammaglobulinemia, lymphoma or vasculitis, have been identified.[138,477-485] It was classically suggested that the EBV infection would trigger these different phenotypes. Our patient presented Burkitt's lymphoma (probably associated with EBV, since EBV DNA was identified in the resected specimen), followed by fatal CNS vasculitis without any evidence of active EBV infection. In addition to our patient, there are only 8 cases of reported vasculitis associated with XLP1 and the absence of EBV infection the absence of EBV infection was proven in only two of them.[477,486-488]

We propose that CNS vasculitis is due to an intrinsic defect of the reactivation-induced cell-death, which leads to the persistence of self-reactive T cells, which would be the basis of this clinical manifestation. The treatment of XLP1 is not consensual and many patients still don't receive HSCT after diagnosis, being treated conservatively. [200]. However, we suggest that the option for HSCT should be taken in all patients with XLP1, based on the fact that the outcome of HSCT has greatly improved in recent years, especially due to high-resolution typing techniques of potential donors, new modalities of conditioning that are far less toxic and the capacity to prepare patients in the pre-HSCT period. Finally, it is also important that the overall survival of the XLP1 patients is higher in the transplanted recipients. The knowledge that HSCT in XLP1 has better better results in patients who did not present HLH, also adds to this proposal.[200] These patients without HLH may develop fatal complications years after the initial diagnosis of XLP1, namely CNS vasculitis. Of the 9 reported cases of CNS vasculitis, only one survived and is in a vegetative state.[489] Altogether, this data prompts us to propose that XLP1 patients presenting manifestions other than HLH are the perfect candidates for HSCT, so this therapeutic option should not be delayed.

To date, Gb infection is exclusive to CGD patients and usually indolent and benign.[490-496] Unlike most of the patients who have been published, our patient had a fatal infection of the CNS by Gb. Our studies revealed that the Gb strain that infected our patient was different from the American strain, and more virulent in murine models than the strain that caused the indolent infection in American patients. Interestingly, our strain had a 100% homology with the only strain causing fatal infection in another European patient. These data should lead to a high level of concern among European patients, as the strain circulating in Europe appears to be more aggressive than in the US. Several authors currently argue that, despite the good overall survival of patients treated with conventional therapy, the HSCT should be considered as a therapeutic alternative to avoid life-threatening infections caused by multidrug resistant pathogens.[497-499]

Novel PIDs

This thesis aimed to discover and describe new genetic causes of PIDs. In order to accomplish this task, and due to the specificity of some of the laboratory work, international partnerships were formed, which greatly contributed to carry out this research, as mentioned in the respective chapters. We were able to describe two new PIDs (Regnase-1 deficiency and STXBP3 deficency) and a third (IRG1 deficiency), which is in the process of being confirmed.

As presented in chapter IV.III, we identified a biallelic mutation of the ZC3H12A gene causing a Regnase-1 deficiency, in a patient presenting an IPEX-like phenotype: an IPEX-like case, in which the patient had infectious susceptibility and immune dysregulation resulting in poly-autoimmunity (immune enteropathy, autoimmune hepatitis, Evans syndrome, immune myelofibrosis) and lymphoproliferation (splenomegaly and generalized lymphadenopathies). It has been shown that this deficiency leads to two distinct effects: on the one hand, it causes a hyper-production of IL-6 by resting cells, by the presence of a protein with decreased RNase activity; on the other hand, it causes a difficulty in the production of IL-6 after stimulation, due to the absence of DSGXXS motif in the protein, preventing its phosphorylation by IKKβ and not permitting its proteosomal degradation. In this respect, the discovery of this new PID has made it possible to broaden the existing view of the fine mechanisms that control the immune response. Post-transcriptional control is a brave new world that can lead us not only to the clarification of many new congenital immunodeficiencies, but also to the prospect of new therapies to regulate the immune response, which can be very important in the treatment of autoimmune diseases. In addition, this knowledge has enabled us to conduct a trial with a guided therapy with a monoclonal antibody against the IL-6 receptor (tocilizumab), which has been found to be ineffective. It is assumed that inefficacy was due to an insufficient dosage to adequately block receptors due to the increase in IL-6 observed in PBMCs and patient fibroblasts, when compared to controls. We did not consider that it was safe to use higher doses on the patient due to the lack of experience in pediatric age. Finally, knowledge of the nature of the disease also made it possible to recommend HSCT as a curative option for the patient's condition.

It was effective in resolving the clinical condition: after the immunological reconstitution, the lymphoproliferation resolved and the patient did not suffer from haemolytic anaemia and immune thrombocytopenia, becoming independent of any transfusions.

In the case described in chapter IV.III of the patient with pyomyositis and recurrent fasciitis, although we could not make a diagnosis of PID in particular, it was possible to hypothesize that the patient had an inability to resolve infections, which could remain latent, in addition to presenting an inflammatory hyper-response that culminated in necrotizing fasciitis. We were able to identify a VUS in the IRG1 gene, which encodes the CAD enzyme and whose product is itaconic acid. Moreover, itaconic acid is critical in preventing hyper-inflammation of macrophages after their activation and has a well-known antibacterial function. These properties of itaconic acid explain the patient's immunodeficiency. As there have only been studies in murine models to demonstrate that these studies were reproducible in human-derived cells, it has been possible to prove that: the production of IRG1 mRNA correlates with itaconate levels; that itaconate levels correlate with succinate accumulation and that unlike murine models, the induction of IRG1 mRNA is induced by joint stimulation with LPS+IFNγ but not LPS or IFNγ alone. The correlation between the clinical and laboratory findings leads us to assume that this is, indeed, the cause of this disease. Still, we aim to prove that the VUS in IRG1 causes a CAD insufficiency in the patient, leading to a decrease in itaconic acid and the consequent decrease in its anti-microbial and anti-inflammatory functions, as explained in the respective chapter. However, it is quite intriguing that infections occur exclusively in the muscle/ subcutaneous tissue and the majority of the times in the same affected localization. We admit that the fact that they only occur in the muscle/ subcutaneous tissue may be due to potential mechanisms of immunological redundancy that allow the IRG1 deficiency to be overcome in other tissues. Although we have not yet had the opportunity to test this technique, there are PIDs where immune redundancy can explain the tropical nature of infectious susceptibility. Although we have not yet had the opportunity to prove this theory, there are PIDs where the redundancy of the immune system explains the restriction of infectious

susceptibility to certain territories. One such example is the herpetic encephalitis due to type-1 IFN production defects, in which it has been proven that TLR3-mediated immunity against the *Herpes Simplex Virus* type 1 (HSV1) is essential for protection against this virus in the central nervous system but not elsewhere.[500,501] This phenomenon is explained by the redundancy of TLR3 for host defences against HSV1 outside the central nervous system.[244] The fact that the infection only occurs in the same localizations (in this case, only in the right thigh and left arm) is difficult to explain, but we speculate that it may be due to the fact that these are the places where an infection was first triggered and subsequently incompletely treated due to the itaconate insufficiency, thus recurring. We also admit that the inoculation of vaccines in those parts of the body may have contributed to the onset of these infections, as the child had received vaccine inoculations in those places a few days before the first episode of pyomyositis. Finally, it is very interesting to note that itaconic acid derivatives such as dimethyl itaconate have a well-established anti-inflammatory effect on animals, namely reducing ischemia-reperfusion injury and ameliorating the skin inflammation in animal suffering from psoriasis.[502] [421] This leaves the future open for using such drugs in patients suffering from these diseases, as well in patients with IRG1 deficiency. Additionally, since the anti-inflammatory properties of itaconate are at least partially due to the activation of Nrf2, it is accepted that drugs that activate these routes may also be good options in overcoming IRG1 deficiency. Dimethyl fumarate is a drug already used for the treatment of multiple sclerosis and psoriasis because of its immunomodulatory and anti-inflammatory effects, due at least in part to the activation of the Nrf2 pathway.[503]

Finally, the research into the group of IO-IBD patients presented in chapter IV.III has proven to be an enormous asset. The evolution of knowledge regarding the IO-IBD has made it possible to identify several monogenic defects that may enable us to carry out targeted therapies, such as HSCT in IL10 or XLP2 pathway defects, monoclonal antibodies in autoinflammatories (Anti-IL1R in cryopyrinopathies, anti-IL18 in NLRC4, for example), abatacept in CTLA4 and LRBA defects, among others.[504] In our cohort, it was possible to identify a monogenic cause in about half of the

patients (6/11). In 5 of these 6 patients our knowledge of the underlying disease enabled us to modify our therapeutic strategy, as previously described. In addition, we were also able to participate in the identification of a new PID in one of the patients due to STXBP3 deficiency.

METHODOLOGICAL CONSIDERATIONS –STRENGTHS AND LIMITATIONS

The main limitation of this thesis is the sample of patients. In addition to being small, it is quite selective: all patients had extreme phenotypes, as can be seen by the number of complications and even mortality. This is due to the fact that our research focused on rare or very severe manifestations of a rare group of diseases. Another limitation is that the investigation of cases has not been standardised and varies from case to case. This was due to the particularities of each patient being investigated, and the nature of the problems was such that it was necessary to adapt the research that was carried out.

The main strengths of this thesis were not only the discovery and description of new PIDs for the first time in the world, but also the increase of the knowledge about other previously known PIDs, allowing proper management of children all over the globe, enhancing their chance of survival and better quality of life. The establishment of multicenter collaborative protocols for the resolution of these complex day-to-day cases has not only allowed for an increase in knowledge about these pathologies, but has also shed light on new defects. This has provided us with a better understanding of the different ways and processes responsible for health and disease. In our view, this is the only way that clinical research on rare diseases can enable new diagnostic methodologies and new therapies to be developed. The expansion of knowledge about the different forms of initiation, control, regulation and finalization of the immune response has made it possible to understand the fundamental role of clinical immunology as a medical discipline that spans across almost all the sub-specialties. At the HDE-CHULC we were able to design an integrated, multi-disciplinary model for monitoring rare, complex pathologies, in which it is assumed that the immune system plays an important role in their genesis.

For example, in this context we have set new models for for the standard-of-care of VEO-IBD and severe eczema, composed of a team that combines the knowledge of immunologists, psychologists, nutritionists, in addition to the clinician responsible for each of the areas (gastroenterology or dermatology, for example). This has allowed proper multi-disciplinary investigation care and treatment of these patients, thus contributing to the best outcome for these patients.

FUTURE PERSPECTIVES

First, in those patients presented in this thesis whose genetic defects have not yet been discovered, we aim to apply new diagnostic methodologies, such as whole genome sequencing, in order to identify the cause of their diseases. This task will be easier if it is possible to establish international multicenter consortia which would allow the identified variants to be brought together in a single database and therefore facilitate the identification of variants shared by patients with identical phenotypes.

Furthermore, we have started the study of new cohorts groups of patients with diverse diseases, such as very early onset type 1 diabetes mellitus, very early onset pustular psoriasis or child-onset Evans syndrome, and we hope that new knowledge will allow the discovery of new targeted therapies that will lead to better prognosis for these children with very rare diseases.

In the short term, we would like to extend our research to other nosological groups, such as patients with serious infections caused by common and frequent agents, such as *papilloma virus, enterovirus, rhinovirus or influenza* virus. As expected, in recent years, some innate immunity defects that cause particular susceptibility to these viruses have been identified. It is clear that a whole new world will have to be explored, given the complexity of the innate immune response to these pathogens.

Although we admit that there will always be a percentage of patients with suspected PID who will be left without a genetic diagnosis, we do think that it is vital

to continue investigating each and every patient with extreme or unusual phenotypes.

"During my residency in Pediatrics I was once told: "Be brave and creative. Dare to defy the paradigm: when you hear hoofbeats, think of horses, not zebras." This was probably the best suggestion I ever received. Zebras are an amazing source of knowledge. Our belief is that knowledge will help them and millions of patients affected with other conditions."

CHAPTER VI

References

Note: Additional references for each study were placed in their respective chapters

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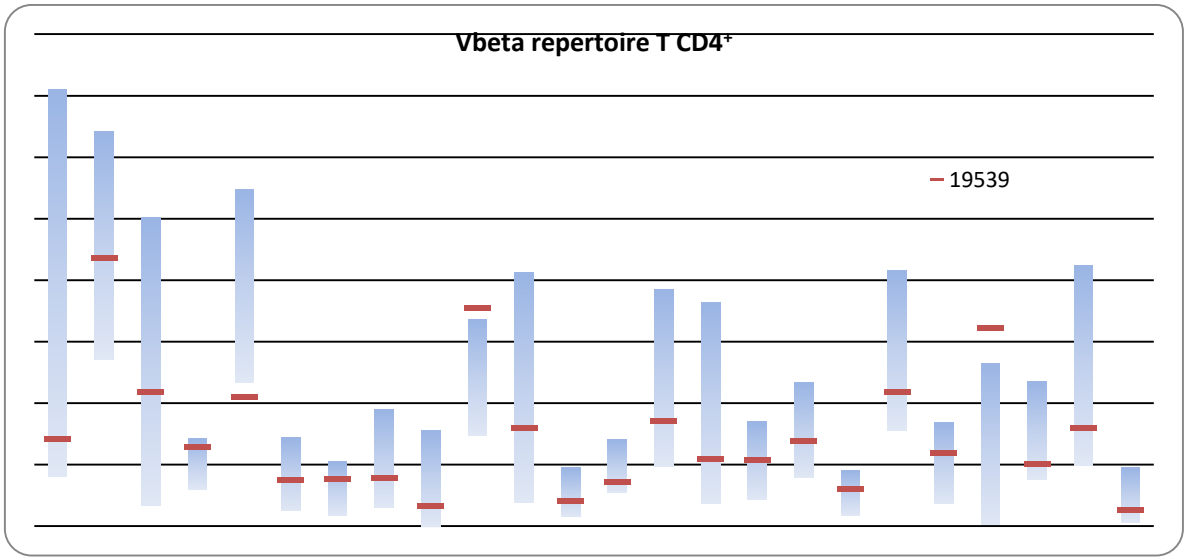
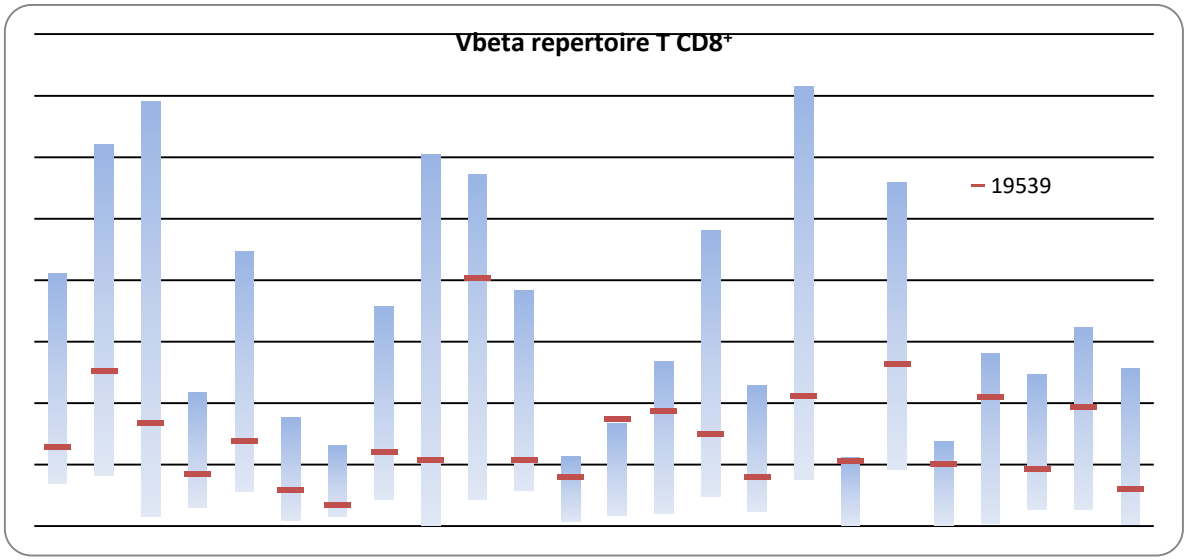
	1 month-old	2 years-old	4 years-old	5 years-old	6	7	8	9	10	11	12	12,5	Age-matched control range
Hemoglobin (g/L)	5	12	10,7	10,9	9,6	13,1	7,4	12,6	11,7	8,9	5,1	7	11,5-13,5
Reticulocyte count (%)	20	3	3,9	1,84	11,7		10				0,3	5	0,5-2,5
Haptoglobin (g/L)	< 0,07	0,2	<0,07	0,35	0								0,34-2
Coombs	IgG+++ C3d+++	IgG+ C3d+	IgG++ C3d+	IgG+ C3d+	IgG+ C3d+	IgG+ C3d+	IgG+++ C3d+++	IgG+ C3d+	C3d+	C3d+	-	-	Negative
Intra-medullary Coombs											++		Negative
Platelets (x10^9/L)	40	250	100	576	480	354	275	181	157	120	60	150	200-450
AST (U/L)	25	30	200	18	20	27	35	27	30	40	274	40	8-35
ALT (U/L)	20	20	171	10	11	17	20	22	20	15	72	27	0-55
Total Bilirrubin (mg/dL)	10,8		0,71	0,9	0,8	0,7	1,2	0,8	0,9	1,4	2,02	1,4	0,3-1,2
Direct Bilirrubin (mg/dL)	0,9		0,3	0,2	0,2	0,2	0,2	0,4	0,2	0,4	0,4	0,2	0-0,5
g-GT (U/L)	40		131			40		25			137	25	<55
Alkaline phosphatase (U/L)	250		444			300	355		280	300	880	320	<500
Calprotectine ug/g	850	<50				<50	600	<50	<50		2800	700	<50
Anti-Gliadine	-	-					-			-			Negative
Anti-transglutaminase	0,08	0,04					-			-			<20
Anti-enterocyte	-	-											Negative
Anti-KLM			+	+	-		-		-	-			Negative
ASMA			+	+	+	-	+	-	-	-			Negative
ANA			1/160	1/160			1/160	1/160		1/160			Negative
Ds-DNA (UI/mL)			12,9	30	15	10	12			0			<20
Anti-thyroglobulin			-			-	-	-		-			

AMA			-				-	-		-			
Anti-TPO			-			-	-	-		-			
Virological studies													
EBV PCR				-		-	-	-	-	-	-	-	
CMV PCR				-		-	-	-	-	-	-	-	
Enterovirus PCR						-			-	-	-	-	
Parvovirus B19 PCR										+	+	+	
Other virus				-		-	-	-	-	-	-	-	
Serum immunoglobulins													
IgA (g/L)	15		0	0	0	0	0	0	0	0	0	0	
IgG (g/L)			32	20,3	23.7	17	10	9.31	7.5	6.5	8,7	8,2	
IgM (g/L)	2,2		2,68	4,58	5,8	5,4	3,44	5,28	5,7	9,8	4,96	0	
IgE (IU/mL)	45				15			9,5	0	0	0	0	
Specific antibodies (IU/mL)													
Anti-Diphteria						0	0,6			0,18			Protective >0,5
Anti-Tetanus						0	3,48			0			Protective > 0,5
Anti-Pneumococcus IgG								19	22				>25

(mg/L)													
Anti-VZV							+		+				
Complement													
C3 (g/L)			1,17	1,34			0,6		0,4		0,75		0,75-1,4
C4 (g/L)			0,15	0,14			0,03		0,12		0,1		0,1-0,34
CH100 (U CH100/mL)			782	800			<392		<392		<392		392-1019
Cell subsets													
Absolute lymphocyte count (cells/ µL))			4500	3200									2500-5500
CD3+ (cells/µL)			2700	2150						3317			1200-3000
CD4+ (cells/µL)			1430	1200						763			650-1500
CD4+CD45RA+ (%)			65	60						26%			53-86%
CD8+ (cells/µL)			1200	800						2146			370-1100
CD8+CD45RA+ (%)			67	62						7%			42-82%

CD16+/56+ (cells/µL)			180	140						132			100-480
FOXP3				9%									
CD3+ CD4- CD8- TCR αβ				3%			4,1						<1%
IL10 (pg/mL)							23						
sFAS-l (pg/mL)							202						40-135
CD19+ (cells/µL)				341			300			480			270-860
CD19+CD27-IgD+IgM+ (%)							84			85			47-70%
CD19+CD27+ (%)							11			8			7-24%
CD19+CD27+IgD- (switch)							3			5			2,7-12,5%
CD19+CD38+IgM^{high} (transitional)							8			0			


Vbeta repertoire T CD4+
19539


Vbeta repertoire T CD8+
19539

Targeted Gene Panel Sequencing for Early-onset Inflammatory Bowel Disease and Chronic Diarrhea

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Background: In contrast to adult-onset inflammatory bowel disease (IBD), where many genetic loci have been shown to be involved in complex disease etiology, early-onset IBD (eoIBD) and associated syndromes can sometimes present as monogenic conditions. As a result, the clinical phenotype and ideal disease management in these patients often differ from those in adult-onset IBD. However, due to high costs and the complexity of data analysis, high-throughput screening for genetic causes has not yet become a standard part of the diagnostic work-up of eoIBD patients.

Methods: We selected 28 genes of interest associated with monogenic IBD and performed targeted panel sequencing in 71 patients diagnosed with eoIBD or early-onset chronic diarrhea to detect causative variants. We compared these results to whole-exome sequencing (WES) data available for 25 of these patients.

Results: Target coverage was significantly higher in the targeted gene panel approach compared with WES, whereas the cost of the panel was considerably lower (approximately 25% of WES). Disease-causing variants affecting protein function were identified in 5 patients (7%), located in genes of the IL10 signaling pathway (3), *WAS* (1), and *DKC1* (1). The functional effects of 8 candidate variants in 5 additional patients (7%) are under further investigation. WES did not identify additional causative mutations in 25 patients.

Conclusions: Targeted gene panel sequencing is a fast and effective screening method for monogenic causes of eoIBD that should be routinely established in national referral centers.

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Key Words: early-onset IBD, infant colitis, chronic diarrhea, next-generation sequencing, genetic screening, immunodeficiency

Inflammatory bowel disease (IBD) refers to a group of chronic inflammatory diseases characterized by abdominal pain, diarrhea and intestinal bleeding, sometimes accompanied by extraintestinal manifestations. In most cases the disease can be classified into 1 of 2 main entities: Crohn's disease (CD) and ulcerative colitis (UC). The age of onset is typically during adolescence or early adulthood, and the incidence is estimated to range from 0.6 to 24.3 per 100,000 per year for UC and 0.3 to 12.7 per 100,000 for CD in Europe.[1]

In 20% to 30% of patients, the disease occurs before the age of 20,[2] and in about one quarter of these pediatric patients, symptoms arise even before the age of 10.[3] It has been observed that patients with such an early onset of disease often show a more severe disease course and a more variable phenotype than adult patients, making a distinction between CD and UC more difficult.[4] Furthermore, these patients frequently show a poor response to treatment, occasionally resulting in life-threatening conditions. To account for the distinct clinical behavior of early-onset and adult-onset IBD, it has been proposed to classify patients with an age of onset during the first 10 years of life as "early-onset," corresponding to subgroup A1a in the Paris modification of the Montreal classification.[5]

Genome-wide association studies (GWAS) have found 231 single-nucleotide polymorphisms (SNPs) within 200 loci to be associated with IBD risk.[6] Cutler et al[7] reported a modestly larger odds ratio for susceptibility variants in *NOD2* in a cohort of pediatric patients compared with those in an adult-onset GWAS.[8] Furthermore, several genes have been identified that seem to be exclusively associated with early-onset IBD. There is, however, significant overlap between the genetic causes underlying early-onset and adult-onset IBD.[9]

In 2009 and 2010, Glocker et al described mutations in the genes encoding interleukin 10 (IL10) and its receptor subunits to be causative in patients from consanguineous parents suffering from very early-onset IBD.[10,11] Complete remission was achieved in these patients following hematopoietic stem-cell therapy (HSCT). Avitzur et al[12] provided evidence for pathogenic effects of biallelic loss-of-function mutations in the tetratricopeptide repeat domain 7 (*TTC7A*) gene in 5 infants with IBD. In several male patients with severe early-onset CD, the disease was traced back to hemizygous deleterious variants or a whole gene deletion of the *XIAP* gene.[13–16] In these cases, IBD proved to be a feature of a Mendelian disorder. The realization that severe cases of IBD can be monogenic disorders enabled new diagnostic and therapeutic strategies directly targeting the molecular defects or HSCT for severe inherited forms of IBD.

Meanwhile, defects in numerous genes have been published to cause eoIBD and IBD-like syndromes like chronic diarrhea in pediatric patients. With an earlier onset, more severe symptoms, or systemic involvement, a monogenic cause becomes more probable. A genetic cause is also more likely with a positive family history. However, monogenic traits may only account for a minority of eoIBD cases. There is growing evidence regarding IBD as a spectrum of genetically and phenotypically diverse diseases, ranging from monogenic traits with complete penetrance to multifactorial diseases also involving the more common single-nucleotide polymorphisms, which merely increase the probability of developing a disease or have a disease-modifying effect.[17]

The increasing number of genes implicated in monogenic conditions with IBD-like symptoms renders the approach of sequencing single genes less useful. Whole genome sequencing and whole-exome sequencing (WES) of individual patients or entire families have successfully identified causative variants in numerous patients. Large-scale sequencing is, however, time-consuming and rather expensive, therefore, genetic screening is not yet performed in all eoIBD cases. Furthermore, Kammermeier et al[18] showed that results from WES can be limited by lack of coverage of certain genomic regions. In addition, WES or WGS may uncover genetic variants associated with other diseases (incidental findings), thus possibly causing ethical conflicts. Targeted

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sequencing of a panel of genes constitutes an alternative, because only genes relevant for a specific phenotype are sequenced. Here, we present a panel of genes sequenced in 71 patients with eoIBD or early-onset chronic diarrhea, and compared results of a subset of 25 patients with the corresponding WES data. To our knowledge, this is so far the largest comparison of gene panel sequencing and WES for IBD.

The aims of this study were to evaluate the applicability of targeted sequencing in routine screening for genetic causes of eoIBD and early-onset chronic diarrhea, compare the quality of variant detection with WES, and finally provide these patients and their physicians with an accurate genetic diagnosis of their condition.

MATERIALS AND METHODS

Study Cohort

Of 71 patients with an unknown genetic background of disease, 24 patients were diagnosed with early-onset CD, 19 with UC, another 19 with indeterminate colitis, and 6 with suspected eoIBD. Three patients suffered from early-onset chronic diarrhea. All patients were diagnosed during the first 10 years of life (Paris classification A1a), while the exact age of onset was unknown for 7 patients. The average age of onset was 3 years. Eight patients were born to consanguineous parents and 4 patients reported affected first-degree relatives. This study was approved by the local ethics committee (No 439/13 from October 18, 2013, University of Freiburg), and written informed consent was obtained from the parents of the patients. Patient characteristics are summarized in Table 1.

Targeted Gene Panel Design

For screening by targeted sequencing, we chose genes where mutations had been reported to be causative for a disease with intestinal inflammation or chronic diarrhea as one of the symptoms. In addition we chose 5 genes for which a strong association with IBD had been shown in genome-wide association studies ("susceptibility genes") to detect novel rare candidates for susceptibility. In total, the panel included 28 genes (Table 2).

Protein coding sequences were selected and a custom panel covering the target regions based on 150 bp reads was designed using the Agilent SureDesign tool. Capture target regions contained flanking sequences of 25 bases at the borders of each exon. The target size was 72.9 kb, covering 99% of the targeted regions.

DNA Library Preparation and Targeted Sequencing

DNA was extracted from peripheral blood leukocytes using QIAGEN cell lysis solution (cat. 8335705) and QIAGEN Protein precipitation solution (cat. 8335767) according to the manufacturers' protocol. Concentration and purity were measured by fluorometric quantification (Qubit; Thermo Fisher Scientific, Waltham, MA).

Enrichment of target regions was performed using Agilent's HaloPlex Target Enrichment System as described in the provider's instructions. In brief, 225 ng gDNA per sample was cleaved and the resulting fragments were hybridized to specifically designed probes and barcoded by indexing primers. The resulting biotinylated circularized target DNA-HaloPlex probe hybrids were captured with streptavidin beads on a magnetic plate and ligated. Target DNA was eluted by adding of NaOH. Target libraries were amplified by PCR and indexed samples were pooled in equimolar amounts. Sequencing was performed on an Illumina MiSeq sequencer.

Whole-Exome Sequencing

For 25 patients, WES data were available in addition to the gene panel data. The exomes were enriched using Illumina's TruSeq enrichment kit according to the manufacturer's protocols. Sequencing of 2 × 100 bp paired-end reads was carried out on an Illumina HiSeq2000.

Data Analysis

Exome sequencing data was mapped against the human reference genome build hg19/GRCh37 using BWA,[19] sorted, converted to bam format and indexed with SAMtools,[20] followed by the removal of PCR duplicates with Picard (http://picard.sourceforge.net). Local realignment around InDels and base quality score recalibration was performed with GATK[21] according to their best practice recommendations followed by variant calling and variant quality score recalibration.

Sequences from gene panel sequencing were analyzed by Agilent's software SureCall version 1.0.4.9. This included removal of adapter sequences and alignment to the human reference genome hg19/GRCh37. GATK was applied for variant calling of all samples together. The multisample VCF was annotated using ANNOVAR.[22] Minor allele frequencies were added from the NHLBI Exome Sequencing Project,[23] the Exome Aggregation Consortium[24] (ExAC), Kaviar,[25] the 1000 genomes project,[26] as well as 145 healthy German in-house controls. For prediction of variant consequences, DANN,[27] CADD[28] and dbscSNV[29] were interrogated in addition to the GERP score[30] as a measure of conservation of the genomic position.

Variant Filtering and Assessment of Pathogenicity

We filtered for nonsynonymous variants, including missense, stop-gain, stop-loss, and splice-site SNVs and small InDels. We used a cutoff of 0.6 for the predictions from dbscSNV, as recommended[29] to additionally detect potential splice-site altering variants further away from the exon-intron boundary (−3 to +8 at the 5' splice site, and −12 to +2 at the 3' splice site). For genes with known recessive or unknown inheritance, and for the susceptibility genes we applied a maximum threshold of 2% for the minor allele frequency in ExAC and filtered accordingly for homozygous, hemizygous, and potentially compound-heterozygous variants. For genes with a known

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TABLE 1. Patient Characteristics of the Cohort and Applied Sequencing

Sample ID	Gender	Ethnicity	Parents Consanguineous	Affected First-Degree Relatives	Age of Onset	Disease Entity	Sequencing
1_01	m	Caucasian	no	no	1	IC	Panel + WES
1_02	f	Caucasian	no	no	1	CD	Panel + WES
1_03	m	Caucasian	no	no	1	CD	Panel + WES
1_04	m	Caucasian	no	no	1	UC	Panel + WES
1_05	m	Caucasian	no	no	1	UC	Panel + WES
1_06	f	Caucasian	no	no	2	CD	Panel + WES
1_07	f	Caucasian	no	no	3	CD	Panel + WES
1_08	m	Caucasian	no	no	3	UC	Panel + WES
1_09	m	Caucasian	no	no	4	CD	Panel + WES
1_10	m	Caucasian	no	no	4	CD	Panel + WES
1_11	f	Caucasian	no	no	4	UC	Panel + WES
1_12	m	Caucasian	no	no	4	CD	Panel + WES
1_13	m	Caucasian	no	no	5	UC	Panel + WES
1_14	f	Caucasian	no	no	5	CD	Panel + WES
1_15	m	Caucasian	no	no	5	UC	Panel + WES
1_16	f	Caucasian	no	no	5	UC	Panel + WES
1_17	m	Caucasian	no	no	5	CD	Panel + WES
1_18	m	Caucasian	no	no	5	UC	Panel + WES
1_19	f	Caucasian	no	no	5	CD	Panel + WES
1_20	m	Caucasian	no	no	7	IC	Panel + WES
1_21	m	Caucasian	no	no	7	CD	Panel + WES
1_22	m	Caucasian	no	no	7	UC	Panel + WES
1_23	f	Caucasian	no	no	7	UC	Panel + WES
1_24	m	Caucasian	no	no	7	CD	Panel + WES
1_25	f	Caucasian	no	no	7	UC	Panel + WES
1_34	f	Pakistani	no	no	2	UC	Panel only
1_35	m	Caucasian	no	no	2	IC	Panel only
1_36	m	Caucasian	no	no	6 mo	IC	Panel only
1_37	m	Arab	no	no	7	CD	Panel only
1_38	f	Arab	yes	no	10 mo	IC	Panel only
1_39	f	Arab	no	no	1 mo	IC	Panel only
1_40	f	Arab	yes	no	1 mo	IC	Panel only
1_41	f	Caucasian	no	no	3 mo	CD	Panel only
1_42	unknown	unknown	unknown	unknown	unknown	suspected IBD	Panel only
1_43	unknown	unknown	unknown	unknown	unknown	suspected IBD	Panel only
2_01	m	Turkish	no	no	4 mo	CD	Panel only
2_02	m	Turkish	yes (first degree)	no	4 mo	CD	Panel only
2_03	m	Caucasian	no	no	1	UC	Panel only
2_04	unknown	Caucasian	no	no	11 mo	UC	Panel only
2_05	m	Caucasian	no	no	1	UC	Panel only
2_06	f	unknown	unknown	unknown	unknown	suspected IBD	Panel only
2_07	unknown	Turkish	no	no	1	IC	Panel only
2_08	unknown	unknown	unknown	unknown	unknown	suspected IBD	Panel only
2_10	m	Caucasian	no	no	6	IC	Panel only
2_11	m	Caucasian	no	no	2	UC	Panel only
2_12	m	Turkish	no	no	2 mo	IC	Panel only

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TABLE 1. (*Continued*)

Sample ID	Gender	Ethnicity	Parents Consanguineous	Affected First-Degree Relatives	Age of Onset	Disease Entity	Sequencing
2_13	m	Caucasian	no	no	2 mo	CD	Panel only
2_14	f	Caucasian	yes (second-degree cousins)	2 siblings	first week	CD	Panel only
2_17	m	Caucasian	no	no	2 mo	IC	Panel only
2_18	m	Caucasian	no	no	2	IC	Panel only
2_19	m	Arab	yes (remote)	sister	5 mo	CD	Panel only
2_20	f	Arab	yes (first degree)	no	1 mo	CD	Panel only
2_21	m	Arab	yes	no	7 mo	chronic diarrhea	Panel only
2_22	m	Arab	no	no	6 mo	chronic diarrhea	Panel only
2_23	m	Caucasian	no	no	4	IC	Panel only
2_24	m	unknown	no	no	5	UC	Panel only
2_25	f	Caucasian	no	no	1 mo	IC	Panel only
2_26	m	Caucasian	no	no	3 mo	IC	Panel only
2_27	m	unknown	no	no	3	UC	Panel only
2_28	f	Turkish	yes	no	first week	IC	Panel only
2_29	m	Caucasian	no	no	3 mo	CD	Panel only
2_30	m	Caucasian	unknown	no	2	CD	Panel only
2_31	f	Caucasian	no	no	6 mo	IC	Panel only
2_33	unknown	unknown	unknown	unknown	unknown	suspected IBD	Panel only
2_34	f	Caucasian/south Asian	no	no	1	UC	Panel only
2_35	f	Arab	no	no	7	chronic diarrhea	Panel only
2_36	m	Caucasian	no	no	4	IC	Panel only
2_37	unknown	Sri Lankan	no	sister	3 mo	CD	Panel only
2_38	m	unknown	unknown	unknown	unknown	suspected IBD	Panel only
2_43	f	Caucasian	no	mother	unknown	CD	Panel only
2_44	m	Caucasian	no	no	8	IC	Panel only

f, female; IC, indeterminate colitis; m, male;.

dominant inheritance we applied a lower frequency cutoff of 0.1%.

Variants filtered out at this stage were treated as not affecting the function of the respective protein and therefore irrelevant in terms of monogenic inheritance. All other variants were further analyzed. We regarded variants with CADD scores above 10 and DANN scores above 0.96 as the most likely candidates for altering the protein structure. Genomic positions with a GERP score above 2.0 were considered to be conserved. All selected variants were classified into the categories (1) affecting function, (2) variants of unknown significance, and (3) probably not affecting function (Fig. 1). The susceptibility genes were treated separately to detect potential novel susceptibility variants, because monogenic inheritance had not been previously described in these genes.

Validation by Sanger Sequencing

All filtered variants were visually confirmed in the respective alignment. Selected variants classified as disease-causing were also validated by Sanger sequencing. Target regions were amplified by a standard PCR protocol, followed by sequencing in both directions. PCR primers as well as PCR conditions are available upon request. Sequences were analyzed using Sequencher version 4.10.1 (http://www.genecodes.com).

RESULTS

Coverage of Targeted Gene Panel

Targeted sequencing resulted in an average overall average target coverage of 598× with 97% covered by at least 10 reads and 95% covered by at least 30×. Table 3 summarizes the metrics of the targeted sequencing panel. One sample (1_41) showed a comparatively low average coverage of 53× (see Table S1, Supplemental Digital Content 1, http://links.lww.com/IBD/B616), however, all genes still achieved sufficient coverage not falling below 17× (see Table S2, Supplemental Digital Content 1, http://links.lww.com/IBD/B616). Apart from this outlier, the remaining 70 samples displayed high average coverage over all genes.

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TABLE 2. Genes Included in the Targeted Gene Panel, Associated Clinical Syndromes and Mode of Inheritance

Gene	Clinical Syndrome	Mode of Inheritance
ADAM17	ADAM17-deficiency	AR
IL2RA	CD25-deficiency	AR
CYBA	Chronic granulomatous disease	AR
CYBB	Chronic granulomatous disease	XR
NCF1	Chronic granulomatous disease	AR
NCF2	Chronic granulomatous disease	AR
NCF4	Chronic granulomatous disease	AR
DKC1	Dyskeratosis congenita	AR/AD
RTEL1	Dyskeratosis congenita	AD
TERC	Dyskeratosis congenita	AR/AD
TERT	Dyskeratosis congenita	AD
TINF2	Dyskeratosis congenita	XR
WRAP53	Dyskeratosis congenita	AR
STXBP2	Familial hemophagocytic lymphohistiocytosis type 5	AR
IL10	IL-10 deficiency	AR
IL10RA	IL-10RA deficiency	AR
IL10RB	IL-10RB deficiency	AR
LRBA	LRBA-deficiency	AR
IKBKG	NEMO-deficiency	XR
TTC7A	TTC7A deficiency	AR
WAS	Wiscott-Aldrich-Syndrome	XR
FOXP3	X-linked immune dysregulation, polyendocrinopathy, enteropathy	XR
XIAP	X-linked lymphoproliferative syndrome 2	XR
ATG16L1	Susceptibility gene	—
IRGM	Susceptibility gene	—
NOD2	Susceptibility gene	—
DEFB1	Susceptibility gene	—
IL23R	Susceptibility gene	—

AR, autosomal recessive; AD, autosomal dominant; XR, X-Linked recessive.

Comparison of Gene Panel Sequencing and WES

For 25 of 71 patients, whole-exome data were additionally available (Table 1). The 2 technologies were compared in terms of target coverage and variant detection to evaluate the performance of gene panel sequencing.

The average coverage of targeted bases in the 25 samples was more than 10-fold higher in panel sequencing (497×) compared with WES (41×). Ninety-five percent of targeted bases were covered by at least 30× in the panel sequencing versus 55% in WES. While only 6% of the target was highly covered by at least 100× in the exomes, more than 87% achieved this in the panel (Fig. 2; see Table S3, Supplemental Digital Content 1, http://links.lww.com/IBD/B616 for a sample-wise comparison). The exome data showed only 75% of reads mapping to or near the target regions, compared with over 93% for the gene panel. This represents a considerable amount of sequence data that is generated during exome sequencing but stays unused due to off-target mapping.

The average pairwise concordance between variants called from exome and gene panel alignments (in the overlapping target regions) of the corresponding sample was 98.8% with an overlap of 37 variants per sample. On average, the gene panel alignments detected 1 variant not found in the respective exome alignment, while the latter showed an average of 2 exclusive variants (see Table S4, Supplemental Digital Content 1, http://links.lww.com/IBD/B616). However, in our case, visual assessment of these variants exclusively revealed false-positive calls, in most cases due to difficult read alignment at the respective positions. Therefore, both methods likely detected all variants present in the target genes, while exome sequencing resulted in slightly more false-positives. However, it has been previously shown that exome data and lower coverage data in general, are more prone to missing variants.[31,32] This is also reflected in the percentage of the target not covered by a single read, which was 4.6% for the exomes, but only 2.6% for the gene panel.

To our knowledge, this is the first direct comparison of targeted gene panel sequencing with WES in a larger cohort (n = 25) of patients diagnosed with eoIBD or similar phenotypes. The high concordance of detected variants demonstrates the reliability of both methods. However, coverage was significantly higher in the targeted sequencing approach, and WES is therefore more likely to miss variants or produce false-positive results due to insufficient coverage, as recently shown.[33]

Targeted sequencing was performed on the Illumina MiSeq sequencer. This system offers a simpler workflow needing fewer structural and human resources and expertise compared with the Illumina HiSeq which was used for WES. Furthermore, material costs for sequencing at the time amounted to 141€ per patient for the gene panel sequencing in contrast with approximately 600€ per patient in WES. Especially in a diagnostic setting, the turnaround time is an important factor, favoring the use of the faster gene panel approach. As samples are pooled for targeted sequencing, exact costs depend on the number of cases and will further decrease with increasing sample numbers.

Detected Variants

In total, 299 variants were detected in targeted panel sequencing of 71 patients. After filtering for rare, functional variants based on the different modes of inheritance as shown in Figure 1, we were left with 19 variants of interest in 9 genes originating from 14 patients (Table 4). Among these, 5 variants were classified as clearly affecting the function of the respective protein. These patients were given a certain genetic diagnosis for their disease. Causative variants were identified in genes of the IL10 pathway (3), *WAS*, and *DKC1*. Of the remaining 14 rare variants, 8 were considered likely to play a role in disease, but

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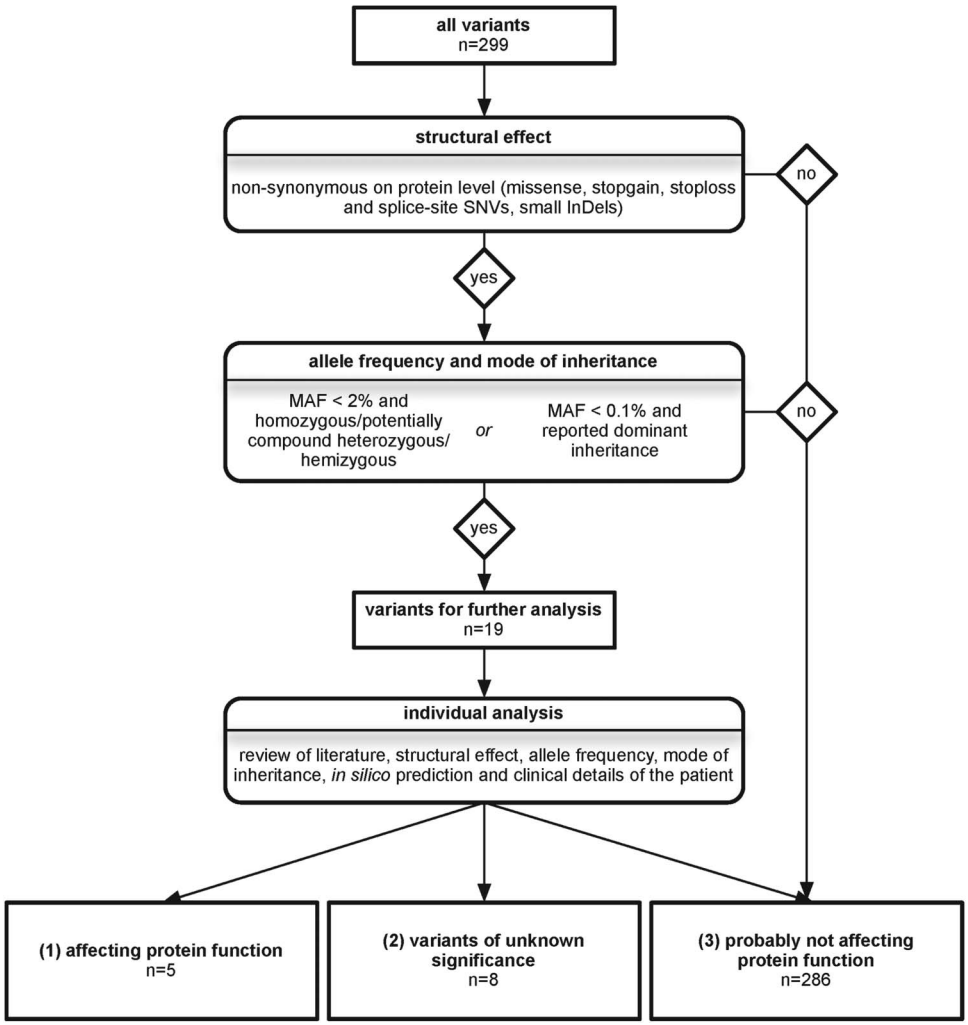

FIGURE 1. Filtering and classification of variants detected in targeted gene panel sequencing. MAF, minor allele frequency; SNV, single nucleotide variant.

their exact functional impact remains uncertain or is currently being analyzed. They were therefore termed variants of unknown significance. The 6 remaining variants were classified as probably not affecting protein function due to being considered too common, not fitting the expected inheritance mode or previous benign classifications.

DISCUSSION

Variants Affecting Protein Function

Interleukin 10 (IL10) is a cytokine expressed by macrophages and lymphocytes that inhibits innate and adaptive immune responses. Mediated by the IL10 receptor, it prevents excessive tissue damage.[34] In the gut, IL10 signaling is essential for intestinal homeostasis,[35] and its abrogation is known to cause severe enteropathy in the first months of life.[10]

We detected disease-causing mutations in 2 genes of the interleukin 10 pathway in 3 patients: A homozygous missense mutation (p.S211F) in *IL10RA*, encoding the alpha subunit of the IL10 receptor, was detected in patient 2_19. This variant was present only once in heterozygous state in the ESP, ExAC, and KAVIAR databases. The locus is highly conserved and the amino acid change is predicted to be damaging. The 2-year-old boy of consanguineous parents had been suffering from a Crohn's-like illness from the age of 5 months, complicated by a failure to thrive, perianal abscesses, and arthralgia. His sister presented with a similar phenotype and died at the age of 8 months. Following our genetic diagnosis, the patient has been treated by HSCT with a matched related graft recently, and is currently in complete remission.

Patient 2_20, a 3-year-old girl of first-degree consanguineous parents, had a novel homozygous nonsense mutation in *IL10RB* (p.C209X). A premature termination of translation at this position will lead to a truncated protein missing the transmembrane and cytoplasmic domains. The patient's clinical history is characterized by a Crohn's-like disease since the first month of

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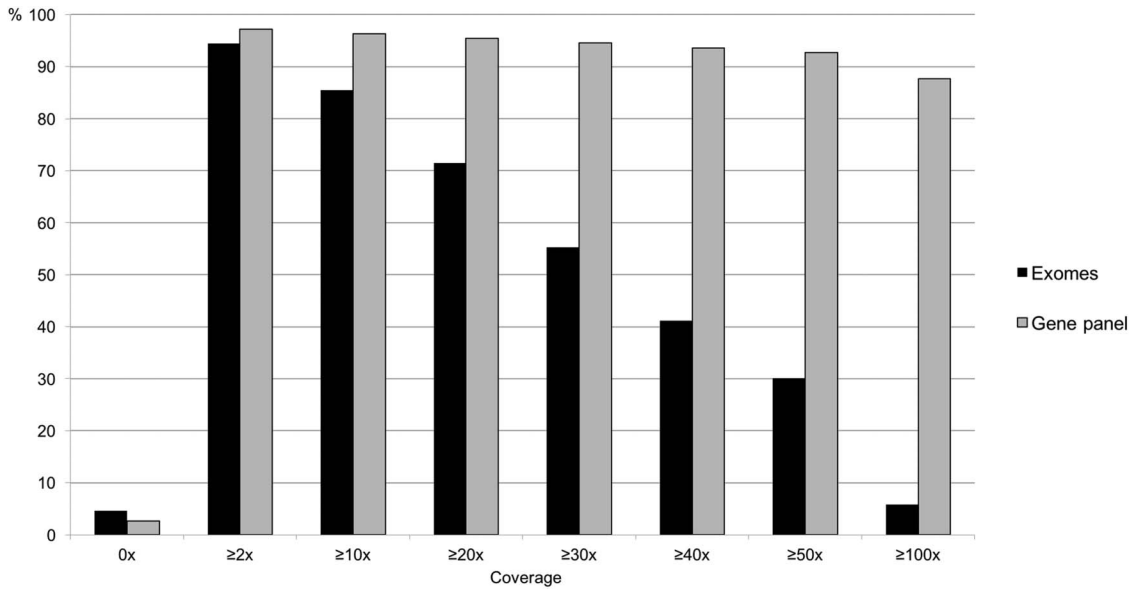

FIGURE 2. Percentage of target region bases covered by the indicated number of reads in gene panel sequencing compared with WES in 25 samples.

life. She suffers from severe failure to thrive, a rectovaginal fistula, mouth ulcers, and gingivitis. Her treatment currently encompasses mesalazine and azathioprine, under which the disease is under control. Currently, no matched donor is available for HSCT in this patient.

In addition, we identified a homozygous variant (c.646 +2T>C) in patient 2_14 in *IL10RB*, encoding for the beta subunit of the IL10 receptor. The novel splice-site mutation is located 2 bases into intron 5 at a conserved genomic position (GERP score 5.35) and is likely to disturb splicing by changing the donor sequence motif, as predicted by an ADA score above 0.99 by dbscSNV. The patient developed colonic Crohn's-like disease in the first month of life, complicated by fistulae, strictures, and failure to thrive. In addition to the intestinal symptoms the patient suffered from joint pain, aphthous stomatitis, folliculitis of the trunk, and human papillomavirus-positive condylomata. She was treated with corticosteroids, azathioprine, and infliximab, and later received adalimumab treatment while being fed an elemental diet. The patient died at the age of 5 years from sepsis with multiorgan failure. She was the child of second-degree consanguineous parents, and 2 of her siblings died in their first year of life from severe enteropathy, suggesting the same genetic cause.

The Wiskott-Aldrich-syndrome protein, encoded by the *WAS* gene, is expressed in hematopoietic cells and necessary for regulation of actin polymerization, and thus, for hematopoietic and immune cell functioning.[36] Abrogation of the protein function by variants in the gene results in microthrombocytopenia, combined immunodeficiency, eczema, and autoimmune symptoms like colitis.[37] Most of the patients are male, as *WAS* is located on the X-chromosome and the defective allele is preferentially inactivated in female carriers. Patient 2_22 harbored a hemizygous splice-site variant (c.777+1G>A), not present in any of the queried databases. It is located at the highly conserved first base in intron 8 and is very likely to disturb normal splicing (ADA score of 1). This variant was previously reported in several patients displaying a classical WAS phenotype.[38,39] The family history of patient 2_22 was positive for WAS and he presented with typical clinical signs including purpura, eczema, and recurrent infections. Laboratory studies showed microthrombocytopenia. As a consequence of the detection of this variant, the patient was therefore diagnosed with WAS and treated by HSCT, receiving a fully matched graft from his brother.

The dyskeratosis congenita 1 gene dyskerin (*DKC1*), is involved in telomere maintenance. Defective variants result in

TABLE 3. Average Coverage Overview of the Gene Panel Consisting of 28 Genes

% Bases on Target	% Bases near Target	% Bases off Target	Average Target Coverage	% 0×	% ≥2×	% ≥10×	% ≥20×	% ≥30×	% ≥40×	% ≥50×	% ≥100×
55.27	37.95	6.78	597.66	2.61	97.30	96.50	95.74	94.96	94.13	93.33	88.90

Near target, 250 bp flanking the target regions; % 0×, percent of target not covered.

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TABLE 4. Filtered Variants, Allele Frequencies and Predictions

Variant Category	Detected in patient(s)	Location	Ref	Alt	State	Gene	Effect	Consequence	ExAC	dbSNP138	DANN	CADD	GERP	ADA Score
1	2_19	chr11:117864808	C	T	homozygous	*IL10RA*	missense	c.C632T, p.S211F	0.00001	rs143645358	1.00	28.80	5.7	
1	2_20	chr21:34655527	T	A	homozygous	*IL10RB*	stopgain	c.T627A, p.C209X			0.99	36.00	0.589	
1	2_14	chr21:34655548	T	C	homozygous	*IL10RB*	splicing	c.646+2 T>C			0.97	22.90	5.35	0.9993
1	2_22	chrX:48546486	G	A	hemizygous	*WAS*	splicing	c.777+1 G>A			0.99	24.90	4.41	1
1	2_23	chrX:154001502	G	A	hemizygous	*DKC1*	missense	c.G1133A, p.R378Q			1.00	33.00	4.62	
2	1_35	chr2:47202157	G	T	heterozygous	*TTC7A*	missense	c.G563T, p.R188L	0.00380		1.00	23.50	5.9	
2	1_35	chr2:47233124	C	G	heterozygous	*TTC7A*	missense	c.C1129G, p.Q377E	0.00940	rs117304542	0.90	7.70	4.03	
2	1_05	chr2:47238530	G	A	heterozygous	*TTC7A*	missense	c.G1348A, p.V450M	0.00160	rs114276698	1.00	25.90	4.47	
2	1_05	chr2:47273468	A	G	heterozygous	*TTC7A*	missense	c.A1817G, p.K606R	0.00180	rs139010200	1.00	25.60	5.51	
2	1_05	chr2:47277182	T	C	heterozygous	*TTC7A*	missense	c.T2014C, p.S672P	0.00180	rs149602485	1.00	27.50	5.01	
2	1_22	chr20:62324600	C	T	heterozygous	*RTEL1*	stopgain	c.C3028T, p.R1010X	0.00009	rs373740199	0.97	35.00	-5.34	
2	2_04	chr20:62326249	G	A	heterozygous	*RTEL1*	missense	c.G3337A, p.D1113N			0.99	17.33	3.8	
2	1_42	chrX:48544230	G	A	hemizygous	*WAS*	pred. splicing	c.463+8 G>A			0.97		4.73	0.9987
3	2_26	chr5:1294352	G	T	heterozygous	*TERT*	missense	c.C649A, p.P217T			0.52	0.01	-1.22	
3	2_07	chr14:24709952	G	T	heterozygous	*TINF2*	missense	c.C734A, p.S245Y	0.00033	rs142777869	0.96	22.70	0.987	
3	2_19	chr20:62293235	G	A	heterozygous	*RTEL1*	missense	c.G334A, p.A112T	0.00023	rs151214675	1.00	28.30	4.84	
3	1_22, 2_04	chr20:62325833	C	A	heterozygous	*RTEL1*	missense	c.C3101A, p.P1034H	0.01457	rs115610405	0.98	16.95	-0.643	
3	2_36	chrX:37663322	G	C	hemizygous	*CYBB*	missense	c.G1090C, p.G364R	0.00341	rs141756032	0.99	23.00	5.77	
3	2_24	chrX:48544502	C	A	hemizygous	*WAS*	missense	c.C538A, p.H180N	0.00133	rs145040665	0.95	14.74	4.32	

Variants are classified into categories: (1) affecting function, (2) candidate variants of unknown significance (VUS), (3) probably not affecting function.
Alt, alternative/variant allele; pred. splicing, splice-site predicted by ADA score; Ref, reference allele.

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shortened telomeres and cause X-linked dyskeratosis congenita or Hoyeraal-Hreidarsson syndrome, a more severe multisystem disorder.[40] As telomeropathies preferably affect tissues with high cell turnover, gastrointestinal manifestations are common.[41] As a consequence of the detection of a hemizygous missense variant (p. R378Q) in *DKC1*, patient 2_23 was diagnosed with Hoyeraal-Hreidarsson syndrome. The detected variant has been previously reported to be disease-causing in a patient with a similar phenotype.[42] Our patient showed delayed psychomotor development since infancy, pancolitis, and an immunodeficiency with hypogammaglobulinemia, low B, and NK cell counts. Given that his severe phenotype includes gastrointestinal and neurological manifestations which cannot be cured by HSCT, his care is palliative. Currently aged 6 years, he receives subcutaneous immunoglobulin replacement and infliximab.

Variants of Unknown Significance

The tetratricopeptide repeat domain-7A (*TTC7A*) gene encodes a protein, expressed in the intestine, thymic epithelial cells, and thymocytes. TTC7A plays an important role in enterocyte and T-cell function. Biallelic loss-of-function variants have been reported to cause a spectrum of diseases characterized by multiple intestinal atresias and severe combined immunodeficiency that manifest in the first months of life.[43,44]

In 2 of our patients, we detected more than 1 heterozygous variant in the *TTC7A* gene after filtering. The 2 variants (p. Q377E, p.R188L) in patient 1_35 were also both present in the patient's unaffected mother and thus reside on the same haplotype. Similarly, the unaffected mother of patient 1_05 also harbored all 3 variants (p.V450M, p.K606R, p.S672P) found in her affected son. Two of these variants have been described as causative together with a deletion on the other allele in a patient suffering from multiple intestinal atresia in the first months of life and a combined immunodeficiency.[44] While it is unlikely that these variants, when inherited from a single healthy parent, are solely responsible for the disease, we hypothesize that they contribute to an overall higher risk for disease development.

The Regulator of Telomere ELongation helicase 1 (RTEL1) is essential for telomere maintenance, and variants in this gene have been reported to cause autosomal recessive or dominant dyskeratosis congenita. In patient 1_22, we detected the heterozygous variant p.R1010X, which leads to a premature stop codon in exon 30 of a total of 35 exons. The resulting truncated protein lacks the C-terminal PCNA (proliferating cell nuclear antigen) Interacting Protein (PIP) motif. The interaction between RTEL1 and PCNA is dependent on this motif, and disruption of this interaction has been shown to result in accelerated cell senescence.[45] This nonsense mutation was not present in the 1000 Genomes Project, but was listed in the ExAC database in 10 individuals in a heterozygous state (MAF of 0.008%). Ballew et al reported 2 brothers diagnosed with autosomal-dominant DC harboring this mutation in a heterozygous state.[46] Their mother proved to be heterozygous for the same variant and showed shortened telomeres despite being healthy. The authors hypothesized genetic anticipation to account for the onset of symptoms in the patients. In addition, Lee et al[47] reported a patient with autosomal-recessive DC who harbored compound-heterozygous mutations in *RTEL1*, one of them being R1010X. Of note, a second heterozygous missense variant in *RTEL1* was detected in patient 1_22 (p. P1034H). The MAF of 1.6% makes a highly penetrant pathogenic effect rather unlikely and, to our knowledge, no associations with telomeropathies have been reported so far for this variant. However, the patient showed constitutional growth retardation, but no bone marrow failure, and developed ulcerative colitis at the age of 7 years. To assess the functional impact of the detected variants we performed telomere length measurement by qPCR which revealed short granulocyte telomeres (data not shown). Thus, we regarded the detected variant p.R1010X in *RTEL1* as likely contributing to the disease in this patient while the role of the common variant p.P1058H remains unclear. As the patient has been in remission for several years, currently at the age of 18, HSCT (which constitutes the only cure for DC) is not an adequate treatment option at the moment. Management concentrates on yearly follow-up including cancer screening by a dermatologist and otolaryngologist to allow early detection of malignancies, which have a higher incidence in these patients.

Patient 2_04 was diagnosed with UC at the age of 11 months with no extraintestinal manifestations. She also possessed the *RTEL1* variant p.P1034H in combination with a second novel missense variant (p. D1113N). The latter is predicted to affect protein function, and is located at a conserved genomic position. However, whether the detected variants affect the protein function so far remains unknown. The patient is currently in follow-up, and further studies may determine the functional effect of the detected variants.

Patient 1_42 harbors a novel hemizygous variant in the *WAS* gene 8bp into intron 4, which is predicted to affect splicing with an ADA score of 0.99. Additionally, this position is highly conserved, which is rare for intronic positions and therefore points to an involvement in splicing. Further testing is needed, but is currently not possible due to loss of contact with the patient.

Susceptibility Variants

In the set of 5 susceptibility genes, we detected 17 variants after filtering (see Table S5, Supplemental Digital Content 1, http://links.lww.com/IBD/B616). As expected, numerous *NOD2* variants (10 missense variants and one frameshift insertion) were among them, most of them previously published to be associated with IBD. However, we detected no homozygous or potentially compound-heterozygous variants in any of the patients that would point to a previously unknown monogenic disease cause in these genes.

Review of Targeted Gene Panels for eoIBD

To date, 2 targeted gene panels have been published for screening of patients with eoIBD. The panel presented by Christodoulou et al[48] included 169 susceptibility genes in order to generate a distinct patient profile containing rare genetic risk

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variants likely to be involved in the individual pathogenesis. Although useful for the understanding of pathogenesis, this approach is unlikely to alter the individual patient management, as there are currently no therapies specific for polygenic risk loci profiles. Xiao et al[49] combined the 3 genes of the IL10 pathway in a single gene panel with 7 risk loci, thereby identifying disease-causing monogenic variants as well as variants with low penetrance. In contrast, Kammermeier et al[18] sequenced 40 genes known to be associated with monogenic diseases potentially causing eoIBD. The panel presented here contains 15 of these genes, plus 8 additional genes associated with monogenic diseases (some of these such as *TTC7A* characterized only recently) and 5 known susceptibility genes. Therefore, this panel may serve to detect disease-causing variants causing Mendelian conditions in a proportion of patients, but also identify rare susceptibility variants in others, thus helping to better understand the disease pathogenesis of polygenic eoIBD. It is evident that the list of genes included needs to be updated regularly due to the frequent identification of new genetic diseases. However, this is easily possible with the Haloplex panel design which was selected.

Sequencing in Clinical Settings

Overall, gene panel sequencing in 71 patients affected by eoIBD or IBD-like symptoms identified disease-causing variants in 5 patients (Table 4), including 3 patients with deficiencies of the IL10 pathway and 1 patient with WAS, dyskeratosis congenita and Hoyeraal-Hreidarsson syndrome, respectively. Of these, 4 patients developed intestinal symptoms during the first year of life and 1 patient at the age of 4 years, although he had shown neurologic abnormalities since his fourth month of life. The parents of 3 of these patients were consanguineous and 2 patients had affected siblings. The exact functional impact of 8 additional candidate variants remains as yet unknown and requires further experiments.

We have shown that gene panel enrichment is a quick and less costly alternative to exome sequencing while generating data of higher quality for genes of interest. Moreover, targeted gene panel sequencing circumvents the ethical problem of detecting and handling incidental findings. These characteristics make the use of gene panels in general, and of the IBD panel presented here in particular, a promising solution for routine screening of early-onset patients with unknown disease cause. However, disease-causing variants that may be present in genes not included in the panel may have been missed. Therefore, the remaining patients for whom no clear candidate genetic variants were identified, may benefit from subsequent exome sequencing. In several of these unsolved cases, however, the genetic basis of the disease may not consist of 1 or few high-impact variants, but rather many variants with small effects, similar to most adult-onset IBD cases. Larger sequencing efforts with more patients are required to detect variants with incomplete penetrance.

Our results also show that the main challenge when working with clinical sequencing data in general is the interpretation of variants of unknown significance.[50] Time-consuming functional experiments are often needed to validate their functional effects. The results of this study, therefore, not only serve a diagnostic purpose, but also facilitate further research. The 8 candidate variants of yet unknown significance will be further analyzed to determine their clinical relevance. In addition, platforms allowing for clinicians and researchers to submit selected variants of unknown significance and find additional patients with identical variants (like the German "VarWatch" project (BMBF project ID01EK1506[51]), or the "MatchMaker Exchange"[52] will further facilitate the evaluation of relevant and benign variants.

Considering the low cost and the simple workflow of the gene panel approach, we believe this method to be well suited for routine use in a diagnostic setting for eoIBD at the current time. As exome and whole genome sequencing becomes cheaper and quicker, these will likely replace gene panels in diagnostics. As yet however, the large data generated with these methods is still a challenge that will need to be addressed in the future before establishing these methods in the routine clinical setting.

Take-Home Messages

1. Targeted gene panel sequencing provides clinicians with higher data quality compared with WES.
2. Targeted gene panel sequencing is a cost-effective method for genetic diagnosis of eoIBD.
3. The IL10 signaling pathway accounts for most gene defects identified in this cohort.
4. Dyskeratosis congenita may be an underlying cause for eoIBD in some patients.
5. The X-linked conditions CGD, XIAP deficiency, and WAS may cause eoIBD, hence a DHR test, NK-cell cytotoxicity assay, and platelet size analysis should be performed in male infants with unclear forms of IBD.

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