UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE MEDICINA

TESIS DOCTORAL

Susceptibilidad mendeliana a las infecciones por micobacterias en el hombre

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Carmen Oleaga Quintas

Directoras

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“Nothing in biology makes sense except in the light of evolution”

— Theodosius Dobzhansky

"Above all, don't fear difficult moments. The best comes from them"

— Rita Levi-Montalcini

“In our obscurity - in all this vastness - there is no hint that help will come from elsewhere to save us from ourselves. It is up to us”

— Carl Sagan

"If you know you are on the right track, if you have this inner knowledge, then nobody can turn you off... no matter what they say"

— Barbara McClintock

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ABBREVIATIONS

Table of abbreviations	
AD	Autosomal dominant
ADA	Adenosine deaminase
AFB	Acid-fast bacilli
AIDS	Acquired immune deficiency syndrome
AK	Adenylate kinase
AML	Acute Myeloid Leukemia
APC	Antigen-presenting cell
AR	Autosomal recessive
BCG	Bacillus Calmette-Guérin
BCG-itis	BCG related local lymphadenitis
BCR	B cell receptor
BWA	Burrows-Wheeler aligner
CADD score	Combined annotation-dependent depletion score
cDC1	Type 1 conventional dendritic cell
cDC2	Type 2 conventional dendritic cell
CDM	Drosophila melanogaster myoblast city
CDS	Coding sequence
CGD	Chronic granulomatous disease
CID	Combined immunodeficiency
CMC	Chronic mucocutaneous candidiasis
CML	Chronic Myeloid Leukemia
CRAC	Calcium release activated channel
DHR	Dihydrorhodamine
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DOCK	Dedicator of cytokinesis
EBV	Epstein-Barr virus
EBV-B cells	Epstein-Barr Virus-Transformed B cells
EDA-ID	Anhidrotic ectodermal dysplasia with immune deficiency
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EM	Environmental mycobacteria
EMSA	Electrophoretic mobility shift assay
EV	Empty plasmid
FBS	Fetal bovine serum
FLT3L	Fms-like Tyrosine Kinase 3 ligand
FOXN1	Forkhead box protein N1

GAF	Gamma-activated factor
GAS	Gamma-activating sequence
GATA2	Guanine-Adenine-Thymine-Adenine 2
gDNA	Genomic DNA
HIES	Hyper-IgE syndrome
HIV	Human immunodeficiency virus
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes Simplex virus
IFN-γR	Interferon-γ receptor
Ig	Immunoglobulin
IGRA	Interferon-γ release assay
IkBα	NF-κB inhibitor α
IKK	Inhibitor of nuclear factor kappa-B kinase
IL	Interleukin
iPSCs	Induced pluripotent stem cells
IRF	Interferon regulatory factor
IRIS	Immune reconstitution inflammatory syndrome
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-α/β sequence response elements
JAK	Janus kinase
LAT	Linker for activation of T cells
LOF	Loss-of-function
MAF	Minor allele frequency
M-CSF	Macrophage colony-stimulating factor
MDDC	Monocyte-derived dendritic cell
MDMs	Monocyte Derived Macrophages
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MSC	Mutation significance cutoff
MSMD	Mendelian susceptibility to mycobacterial disease
MWs	Molecular weights
NADPH	Nicotinamide adenine dinucleotide phosphatase
NF-κ-B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next generation sequencing
NHEJ	Nonhomologous end-joining
NIK	NF-κB-inducing kinase
NK	Natural Killer
NT	Untransfected

NTM	Nontuberculous mycobacteria
OS	Omenn syndrome
PAS	para-aminosalicylic acid
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDCs	Plasmacytoid dendritic cell
PID	Primary immunodeficiency
pLI	Probability of being loss-of-function intolerant
PMA	Phorbol myristate acetate
PNGase-F	N-glycosidase-F
PNP	Purine nucleoside phosphorylase
POLE2	DNA Polymerase Epsilon 2
qPCR	Quantitative real-time PCR
RAG	Recombination activating gene
RLTPR	RGD motif, leucine rich repeats, tropomodulin domain and proline-rich containing
RNA	Ribonucleic acid
RORγ	Retinoic acid-related orphan receptor gamma
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RVIS	Residual Variation Intolerance Score
SCID	Severe combined immunodeficiency
SID	Secondary immunodeficiency
SMARCAL1	Swi/snf-related matrix-associated actin-dependent regulator of chromatin subfamily a-like protein 1
SnIPRE	Selection Inference using Poisson Random Effects
SNPs	Single nucleotide polymorphisms
SPPL2A	Signal peptide peptidase-like 2A
STAT1	Signal transducer and activator of transcription 1
SV40-fibroblasts	Primary human fibroblasts immortalized with SV-40 T antigen
TB	Tuberculosis
TNF	Tumor necrosis factor
TRIM	Tripartite motif
TST	Tuberculin skin test
TYK	Tyrosine kinase
V(D)J	Variable, diversity, joining
WES	Whole-exome sequencing
WT	Wild type
ZAP	Zeta-chain-associated protein kinase

ZF	Zinc-finger
γc	Common gamma chain

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RESUMEN

INTRODUCCIÓN

Desde que Robert Koch descubriera *Mycobacterium tuberculosis* en 1882 hasta hoy, más de 150 especies del género *Mycobacterium* han sido descritas. Algunas micobacterias son virulentas y provocan enfermedades en multitud de individuos. Sin embargo, la mayoría de las micobacterias son poco virulentas y pueden encontrarse en el medio ambiente, por lo que son llamadas micobacterias ambientales. Las enfermedades por micobacterias en el humano pueden estar causadas por la virulencia del microorganismo o por una susceptibilidad a las micobacterias intrínseca del individuo, conocida como inmunodeficiencia. Las inmunodeficiencias pueden ser secundarias (también llamadas adquiridas) o primarias, dependiendo de si la infección por micobacteria es una consecuencia indirecta o directa del defecto. Las inmunodeficiencias que confieren enfermedades por micobacterias son defectos que afectan a la interacción entre células presentadoras de antígeno y células efectoras del sistema inmune. Los individuos afectados por inmunodeficiencias primarias pueden sufrir infecciones de un espectro amplio o reducido. Aquellos con predisposición a un espectro reducido de infecciones pueden tener infecciones por uno o dos microorganismos, entre los que se encuentran las micobacterias. Dentro de esta categoría se encuentra la susceptibilidad mendeliana a micobacterias (MSMD por sus siglas en inglés), donde los individuos afectados están predispuestos a infecciones por micobacterias que pueden estar acompañadas, o no, de infecciones por otros microorganismos.

OBJETIVOS Y METODOLOGÍA

Los pacientes susceptibles a enfermedades por micobacterias, especialmente aquellas de poca virulencia, requieren de un diagnóstico genético y de la caracterización del defecto genético para poder brindarles un tratamiento personalizado y consejo genético. Mediante el uso de secuenciación de última generación y secuenciación Sanger se hallaron mutaciones en genes asociados con infecciones por micobacterias, *IFNGR2* y *GATA2* en concreto, que se consideraron patogénicas y se caracterizaron. Para demostrar su patogenicidad se utilizaron un sistema de sobreexpresión, células inmortalizadas y sangre de los pacientes afectados. Se realizaron ensayos de tipo luciferasa o cambio en la corrida electroforética (EMSA, por sus siglas en inglés) para caracterizar estas mutaciones. Además, se utilizaron herramientas bioinformáticas para predecir *in silico* la consecuencia de las mutaciones encontradas.

RESULTADOS Y DISCUSIÓN

Se hallaron dos nuevas mutaciones en *IFNGR2*, 10 mutaciones conocidas y una nueva mutación en *GATA2*. Para *IFNGR2* se estudiaron tres pacientes con MSMD, dos hermanos procedentes de Turquía y un individuo de la India, mediante secuenciación del exoma completo (WES, por sus siglas en inglés). Se hallaron dos mutaciones en homocigosis en *IFNGR2*, una mutación cambio de sentido afectando al codón de inicio del gen (c.1A>G) en los dos hermanos de Turquía, y otra una deleción de la base inmediatamente posterior al codón de inicio del gen (c.4delC) causando un cambio de lectura del gen en el tercer

individuo. Dada la localización de estas dos mutaciones se esperaba que la respuesta a IFN-γ estuviera totalmente abolida. Sin embargo, se observó por sobreexpresión que ambos mutantes daban lugar a la expresión reducida de la proteína completa de IFN-γR2 y que eran capaces de responder levemente a IFN-γ. En vistas de estos resultados se estudió la respuesta a IFN-γ en células inmortalizadas de los pacientes con la mutación c.1A>G. Los fibroblastos SV40 de estos pacientes mostraron una respuesta débil a IFN-γ, mientras que las células B transformadas con el virus Epstein Barr mostraron una respuesta prácticamente abolida. Para confirmar estos resultados se estudiaron linfocitos T primarios y macrófagos derivados de monocitos de los pacientes con la mutación c.1A>G. Estas células mostraron una respuesta débil a IFN-γ, como se observó en sobrexpresión y en células inmortalizadas. Finalmente, se observó que la proteína IFN-γR2 residual generada por ambos mutantes era producida por la iniciación de la traducción entre el segundo y noveno codón no-AUG dentro del péptido señal. Un péptido señal más corto parece ser suficiente para pasar por las vías de secreción.

Para *GATA2* se reclutaron 14 probandos con infecciones por micobacterias. Cada uno de estos individuos presentaba una mutación germinal en heterocigosis en *GATA2* de entre las 11 halladas en total, una de las cuales nunca había sido reportada. Tres familiares de estos individuos también sufrieron infecciones por micobacterias y eran portadores de una de las mutaciones halladas, llegando a un total de 17 pacientes con deficiencia por GATA2. Las 11 mutaciones fueron estudiadas en un sistema de sobreexpresión, donde se observó que 10 de ellas no producían GATA2 correctamente. Mediante la caracterización de estas mutaciones se observó que siete de las mutaciones eran pérdida de función y cuatro eran hipomórficas. Además, el estudio de mecanismo de dominancia desveló que ninguna mutación actuaba en dominancia negativa y, se demostró que *GATA2* evolucionó mediante selección purificadora.

Esto sugiere que la deficiencia de GATA2 actúa por haploinsuficiencia. Los pacientes sufrieron infecciones por diferentes especies de micobacterias, predominantemente dos micobacterias ambientales (*M. avium* y M. kansasii) y *M. tuberculosis*. La infección por micobacteria fue la primera manifestación clínica en 10 de los pacientes, y la mayoría de los pacientes sufrieron infecciones por otros microorganismos como bacterias piógenas, hongos y virus. En estos pacientes también se observaron problemas hematológicos como monocitopenia, mielodisplasia y leucemia mieloide aguda. Entre los familiares se observó que 16 eran portadores de una mutación en GATA2 pero permanecían completamente asintomáticos, lo que llevó al estudio de la penetrancia de la deficiencia de GATA2, concluyendo que es incompleta. Además, se estudió la penetrancia para las infecciones por micobacterias, que resultó ser similar a la penetrancia de otras manifestaciones clínicas asociadas con la deficiencia de GATA2.

CONCLUSIONES

La caracterización de mutaciones germinales en *IFNGR2* y *GATA2* ha permitido tratar y aconsejar a los pacientes con enfermedades por micobacterias. Las dos mutaciones descritas en *IFNGR2* son la primera muestra de una deficiencia cuantitativa, en vez de cualitativa, en un defecto parcial de IFN-γR2. Su impacto a nivel clínico se encuentra entre los pacientes con una deficiencia completa de IFN-γR2 y aquellos con una deficiencia parcial. Por otro lado, la evaluación de las mutaciones en *GATA2* ha demostrado que la deficiencia autosómica dominante en GATA2 es causada por haploinsuficiencia y su penetrancia clínica es incompleta. Por tanto, se debería considerar una deficiencia en GATA2

en pacientes de cualquier edad que presenten infecciones por micobacterias, con o sin otros signos relacionados con la deficiencia. Además, todos sus familiares deberían ser genotipados. En general, estos resultados demuestran la importancia de caracterizar mutaciones nuevas en genes conocidos. Aunque el gen se conozca, el impacto de la mutación no se puede verificar sin haberla caracterizado.

SUMARY

INTRODUCTION

Since the discovery of *Mycobacterium tuberculosis* in 1882 by Robert Koch, more than 150 species from the genus *Mycobacterium* have been described. A few of these mycobacteria are virulent and cause disease in many individuals. However, most mycobacteria are environmental mycobacteria, weakly virulent and found regularly in the environment. Mycobacterial diseases in human can be caused by the virulence of the microorganism or because the individual has an affection that renders them susceptible to the mycobacterial infection, known as immunodeficiencies. Immunodeficiencies can be secondary (also known as acquired) or primary, depending if the mycobacterial disease is an indirect or direct consequence of the defect. Primary immunodeficiencies underlying mycobacterial disease are defects within the immune system affecting the crosstalk between the antigen presenting cells and the lymphoid effector cells. Individuals with primary immunodeficiencies can suffer from a broad or a narrow spectrum of infections, both including mycobacterial infections. Those predisposed to narrow infections may present with infections by one or two microorganisms, mycobacteria being one of them. Within this latter category Mendelian susceptibility to mycobacterial diseases (MSMD) can be found, which predisposes to mycobacterial infections that may or may not be accompanied by infections caused by other microorganisms.

OBJECTIVES AND METHODOLOGY

Patients susceptible to mycobacterial infections, especially to weakly virulent mycobacteria, require a genetic diagnosis and characterization of the genetic defect in order to provide a customized treatment and counselling for the family. Using next generation sequencing and Sanger sequencing, we have identified mutations in genes associated to mycobacterial infections, *IFNGR2* and *GATA2* respectively, which we hypothesized were causing the disease and that we aimed to characterize. In order to prove this hypothesis, we used an overexpression system, immortalized cells and fresh blood from the patients to perform assays like electrophoretic mobility shift assays (EMSAs) or luciferase reporter assays that allowed us to characterize the mutations. Moreover, we used bioinformatic tools in order to predict *in silico* the consequence of the mutations found.

RESULTS AND DISCUSSION

Two novel mutations in *IFNGR2* and 10 known and one novel mutation in *GATA2* were found to confer predisposition to mycobacterial diseases. For *IFNGR2* three patients with MSMD, two individuals from Turkey and one individual from India, were studied by whole-exome sequencing. A homozygous mutation at the initiation codon (c.1A>G) of *IFNGR2* in the two Turkish patients and another homozygous mutation for a frameshift deletion of one base pair immediately after the initiation codon (c.4delC) in the Indian patient were found. Given the location of these mutations, it was expected to observe an abolished response to IFN-γ. Through overexpression, the mutant alleles were observed to produce low levels of full-length IFN-γR2 and to have an impaired, but not abolished, response to IFN-γ.

In light of this observation, immortalized cell lines from the c.1A>G patients were studied. It was shown that SV40 fibroblasts from these patients responded weakly to IFN-γ, whereas their Epstein Barr virus transformed B cells had an almost abolished response to IFN-γ. In order to confirm the results observed in immortalized cell lines, studies in primary T cells and monocyte derived macrophages from patients with the c.1A>G mutation were performed. Primary cells from the c.1A>G mutation patients showed and impaired but not abolished response to IFN-γ, as seen for the overexpression and immortalized cell lines. Finally, it was observed that the residual protein expression of IFN-γR2 of normal molecular weight and function was due to initiation of translation between the non-AUG second and ninth codons within the signal peptide of the protein. The shorter signal peptide appeared to be sufficient for entering the secretory pathway.

For *GATA2*, 14 index cases with mycobacterial disease were recruited. These patients carried one of a total of 11 different heterozygous *GATA2* mutations, one of which had not been reported before. Three relatives of these index cases also had mycobacterial disease and were carriers for a *GATA2* mutation, adding up to a total of 17 patients. The 11 mutations were studied in an overexpression system, where it was observed that 10 of them affected the correct expression of GATA2. Moreover, the characterizations of these mutations lead to the observation that seven mutations were loss-of-function and four hypomorphic. In addition, no mutation was found to be dominant-negative *in vitro* and, the evaluation of the *GATA2* locus showed that it evolved under purifying selection, suggesting a mechanism of haploinsufficiency for GATA2 deficiency. The mycobacterial infections in these patients were observed to be caused by different species of mycobacteria, predominantly two environmental mycobacteria (*M. avium* and *M. kansasii*) and *M. tuberculosis*. Mycobacterial

infection was the first clinical manifestation in 10 patients and most patients also suffered from other bacterial and/or fungal infections (e.g. *Staphylococcus*, *Clostridium*, *Streptococcus*, *Candida*, and *Aspergillus*). Hematological disorders such as monocytopenia, myelodysplasia or acute myeloid leukemia were also observed in these patients. Sixteen family members were shown to be carriers for a *GATA2* mutation but completely asymptomatic, leading to the evaluation of clinical penetrance, which was found to be incomplete. Moreover, clinical penetrance for mycobacterial disease was found to be similar to other GATA2 deficiency-related manifestations.

CONCLUSIONS

The characterization of mutations in *IFNGR2* and *GATA2* has allowed the treating and counseling of patients suffering from mycobacterial diseases. The two mutations found in *IFNGR2* provide the first evidence of a quantitative, as opposed to qualitative, partial defect of IFN-γR2. Its clinical severity is intermediate between those of known complete and partial IFN-γR2 deficiencies. The evaluation of *GATA2* mutations has shown that autosomal dominant GATA2 deficiency is dominant by haploinsufficiency and displays incomplete penetrance. For this reason, a diagnosis of GATA2 deficiency should be considered in patients of any age with mycobacterial infections, especially, but not exclusively, in the presence of other GATA2 deficiency-related phenotypes. Moreover, all direct relatives should be genotyped for the *GATA2* locus. Overall, these results highlight the importance of characterizing novel mutations in known genes. Even if the gene is known, without proper characterization the impact of the mutation cannot be ascertained.

INTRODUCTION

Mycobacterial diseases are, to date, an important burden to humans and remain a serious and frequent challenge to health systems.[1,2] Infection by *M. tuberculosis*, which causes tuberculosis (TB), is the ninth leading cause of death worldwide and the leading cause from a single infectious agent, ranking above Human Immunodeficiency Virus (HIV).[2] Interestingly, of the estimated 1.7 billion people infected with *M. tuberculosis*, only 5 to 10% will develop TB.[2] TB burden is predominantly seen in low to middle-income countries, most of the current TB cases occurring in South-East Asia (45%), Africa (25%) and Western Pacific (17%).[2] TB cases can be associated to other health factors, such as undernourishment (20% of TB cases), co-infection with HIV (10%), smoking (8%), diabetes (7.5%) or alcoholism (4.5%).[2] Moreover, over the past three decades, environmental mycobacteria (EM), also known as nontuberculous mycobacteria (NTM), have been recognized as important opportunistic pathogens, causing a wide range of diseases, and whose incidence surpasses TB in resource-rich countries.[3–6] Disease caused by EM is worsened by other health risks, as seen for TB.[7] The virulence of the pathogen (including here its drug resistance), as well as the genetic background of the host, will determine the outcome of the infection in the affected individual.[8] The genetic dissection of host predisposition to mycobacterial diseases provides understanding of the physiopathological mechanisms associated with anti-mycobacterial immunity.[8] The study of the immune response to mycobacteria may lead to the discovery of new therapeutic targets and thus overcome the resistance for the existing treatment.

I. History and pathogenicity

1. The discovery of *M. tuberculosis* by Robert Koch

The oldest evidence of mycobacterial diseases dates from a 9000-year-old settlement in the Eastern Mediterranean, where two individuals appeared to have suffered from TB.[9–11] Since then, from old civilizations around the world (such as ancient Egypt, pre-Columbian communities and Indian kingdoms) until now, mycobacterial diseases have always been present.[11–13] However, the discovery of the pathogen responsible for TB is relatively new and was preluded by a series of observations and hypothesis regarding the origin of the disease. In the XVIIIth century, Benjamin Marten hypothesized that consumption (TB at the time) could be caused by "small living creatures" and that these creatures could be passed on by sick individuals to healthy ones.[14] Jean-Antoine Villemin demonstrated in 1865 that TB was transmissible, after observing the high frequency of TB in soldiers in barracks compared to those in the field.[15,16] Thanks to these discoveries, Robert Koch was able to postulate his hypothesis that TB was caused by "foreign parasitic structures" and isolated for the first time the microorganism responsible of TB. To this end, R. Koch developed a staining method that allowed marking the microorganism inside the tissue of individuals with TB. He then isolated the microorganism, which he named *Tuberkelvirus* and was later renamed *Mycobacterium tuberculosis*.[17,18] Once isolated, he inoculated the microorganism into a healthy individual and observed if the same disease was developed, leading to the discovery of the causative agent of TB.[17] The criteria followed to discover the etiology of TB were then transformed into what are known as Koch's Postulates (Figure 1). Both the staining and isolation of *M. tuberculosis* were groundbreaking discoveries that would win R. Koch the Nobel Prize of Medicine in 1905.[11,16]

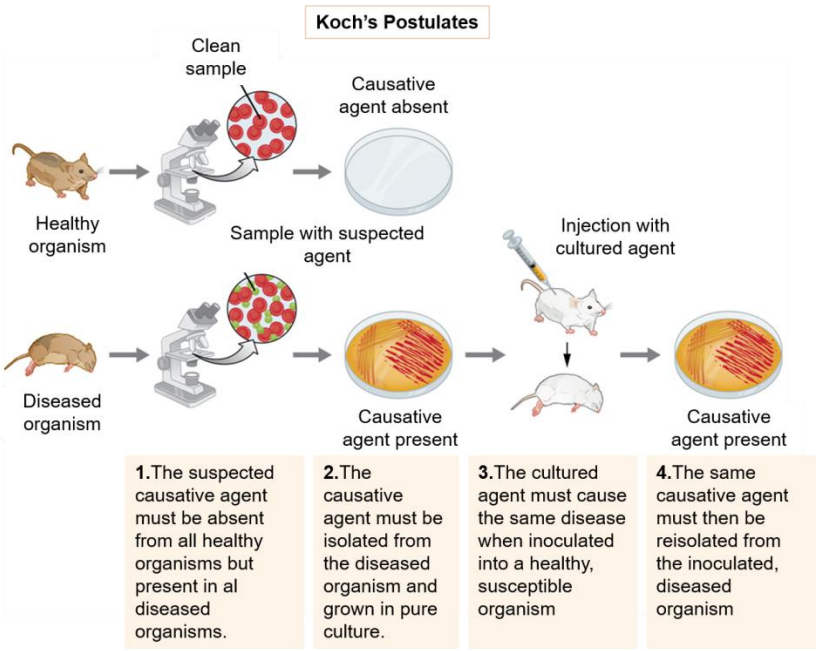

Figure 1 - Koch's postulates, criteria designed to establish a causal relationship between a causative microbe and a disease. Adapted from OpenStax College, Microbiology. OpenStax CNX. Under Creative Commons Attribution 4.0 International License.

2. Mycobacteria and their pathogenicity

To date, there are more than 150 recognized species of the genus *Mycobacterium*, of which only a few have been reported to cause disease in humans.[19] The ground breaking discoveries of R. Koch have allowed the better understanding of the structure, composition and, in the latter years, the pathogenicity of mycobacteria. All mycobacteria are aerobic, non-spore forming, non-motile and rod-shaped. The distinctive feature of the genus, and the reason behind the special staining, is the lipid-rich cell envelope, which has granted them the name of Acid-fast bacilli (AFB) (Figure 2).[20,21] Their genome plays a very important role in the pathogenicity of each species.[22] Whole-genome analyses have revealed key differences between more virulent and less virulent mycobacteria, such as the coding regions and the size of the genome.[22]

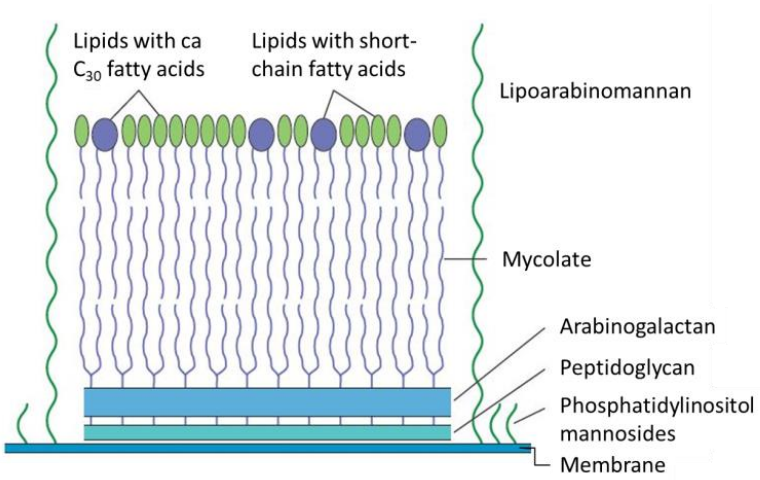

Figure 2 – Mycobacterial cell envelope. (Adapted from Brennan & Draper. AMS; 1994:271-284)[21]

However, the pathogenicity of mycobacterial infections not only depends on the virulence of the mycobacteria but also on the immune response of the host.[22] The main virulence factor of mycobacteria is the capacity to invade, survive and replicate within macrophages. Briefly, the microorganism enters the macrophage by phagocytosis and is surrounded by a membrane-bound vacuole that will give place to a nascent phagosome. The maturation of this phagosome will expose the microorganism to antimicrobial factors such as reactive oxygen species (ROS), hydrolytic activities and acidic pH that will destroy it. Virulent mycobacteria can obstruct the maturation of the phagosome, inhibit or tolerate acidification and can escape into the cytoplasm.[22] At the same time, antigen-presenting cells (monocyte/macrophage lineage, APC) will engulf the mycobacteria and process the antigen in two different ways: the phagosome, that will allow the presentation of these antigens by the major histocompatibility complex (MHC) class II molecules; or the cytoplasm, that will lead to MHC class I molecules presentation. This process will result in secretion of interleukin (IL)-2 and IL-12 leading to clonal proliferation of $CD4^+$ and $CD8^+$ lymphocytes

(α/β T cells). Expanded $CD4^+$ cells and, to a lesser extent, $CD8^+$ cells, natural killer (NK) cells and γδ T cells will release interferon (IFN)-γ that will activate macrophages. This activation will initiate a cascade of events leading to further macrophage activation and tumor necrosis factor (TNF) and IL-12 production, resulting in the search, engulfment and destruction of mycobacteria and affected cells (Figure 3).[22,23]

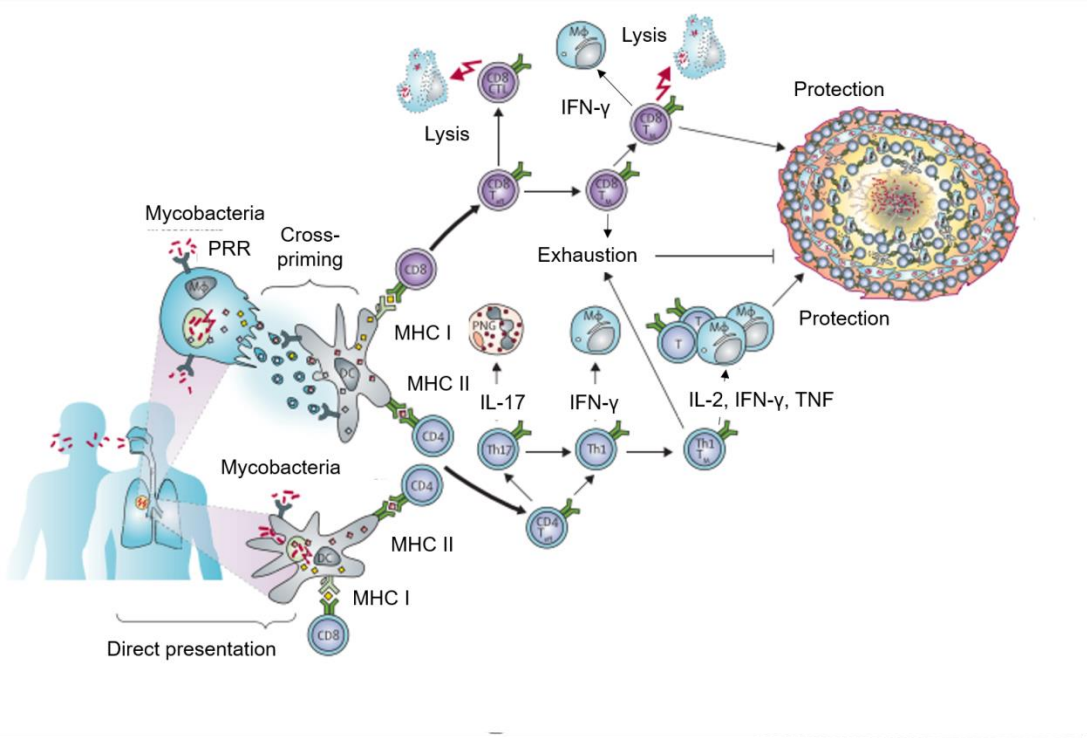

Figure 3 - Overview of the immune response in mycobacterial infection. (Adapted from Kaufmann *et al*. The Lancet; 2010: 2110-2119)[23]

3. Treatment and vaccine

The first available treatment for TB appeared in the XIXth century and involved a change of lifestyle, in which patients were invited to live at high altitudes with fresh air, mild exercise and repose.[24] Since then, different drugs were developed that proved to have a better efficiency when given together, starting what was called "triple therapy" (streptomycin, para-

aminosalicylic acid (PAS) and isoniazid).[24,25] Between 1950 and 1970 new medications were discovered that lead to less secondary effects and that could be used against drug-resistant mycobacteria.[26] Nowadays, treatment against any kind of mycobacteria is evaluated depending on the isolated microorganism, the response to the therapy, tolerance and adherence of the patient. More than 15 drugs have been developed for the treatment of TB, among which the preferred ones are rifampin, ethambutol, isoniazid and macrolides.[24,27,28] Regular treatment lasts six months, having an 85% success rate. If drug resistance is found the treatment can be prolonged from nine to 20 months.[2]

Research for a vaccine against TB was started in 1900 by Albert Calmette and Camille Guérin at the Pasteur Institute in Lille.[29] From 1908 to 1919, they cultivated a virulent strain of *M. bovis* which was subcultured until demonstrated to fail to produce progressive TB in animals. The vaccine was named Bacillus Calmette-Guérin, or BCG, in honor of the two researches that developed it. In 1921, the first administration of the vaccine in humans took place, which was given orally and, by 1931, a special laboratory was set in Paris to manufacture the BCG vaccine.[29] Since this time, several strains of the vaccine have been developed from the original one of 1920, of which five are used in 90% of the cases.[29–31] Since BCG vaccination policies, practices and strains vary depending on the country a BCG World Atlas (http://www.bcgatlas.org) was created to access detailed information about BCG use worldwide.[32] BCG vaccination is recommended in neonates who live in areas that have a high incidence of TB and has been shown to prevent childhood TB meningitis and miliary TB disease, providing also protection against leprosy.[30] Interestingly, the BCG vaccine has been shown to have associated problems in some individuals, where 1 every 2,500 vaccines

cause localized BCG-associated complications (BCG-itis) and 1 every 100,000 vaccines present disseminated complications (BCG-osis).[33]

4. Susceptibility to mycobacteria

Some individuals are prone to disease caused by mycobacteria, which is known as susceptibility to mycobacteria.[28,34–37] It is estimated that around a third of the world's population is infected with *M. tuberculosis* but that only 10% of this group will develop mycobacterial disease.[2,28] EM diseases or disseminated disease caused by BCG vaccine, occur when the host is suffering from an immunodeficiency.[28,38] Immunodeficiencies can be divided in two main groups: primary immunodeficiencies (PIDs), which are due to inherent dysfunction of the immune system and that will be addressed extensively in the next section; and secondary immunodeficiencies (SIDs) or acquired immunodeficiencies, which are a consequence of other causes.[39] Among the causes for SID which lead to mycobacterial infection are: acquired immune deficiency syndrome (AIDS) due to HIV infection, immunosuppression, leukemia and immune reconstitution inflammatory syndrome (IRIS).[28,39]

II. PIDs

PIDs are a group of rare, chronic disorders whose main characteristic are intrinsic defects of the immune system.[40,41] These defects predispose those suffering them to a variety of manifestations, including recurrent infections and unusual adverse reaction to vaccination. As of February 2017, 354 inborn errors of immunity have been reported, caused by mutations in 344 different genes.[41]

1. The genetic approach to understanding PIDs

Four main factors have been claimed to play a role in the development of an infectious disease, explaining the clinical variability existing between patients suffering from the same infection. These factors are: microbial variability (microbiological theory), environmental variability (ecological theory), somatic immunity variability (adaptive and acquired immunity, immunological theory) and germline immunity variability (intrinsic, innate and adaptive immunity, genetic theory) (Figure 4).[42] These four pillars explaining disease development are complementary and overlapping.[42] The genetic theory appeared towards the second half of the XXth century in response to some gaps left by the microbiological theory and the immunological theory (such as vaccination failure in some individuals and interindividual variability in the course of a primary infection), trying to link intrinsic errors of the individual to the observed phenotype.[42,43] This theory was properly validated with the discovery of three PIDs conferring predisposition to a narrow range of diseases (membrane attack complement defects and X-linked lymphoproliferative disease).[42,44,45] From that moment onwards, single-gene mutations underlying susceptibility to different infections (such as herpes simplex encephalitis, pneumococcal disease and mycobacterial disease) were

identified.[44,46,47] It is thanks to these major discoveries that, to this date, mutations described in 344 genes have explained the PIDs presented by several individuals around the world.[41]

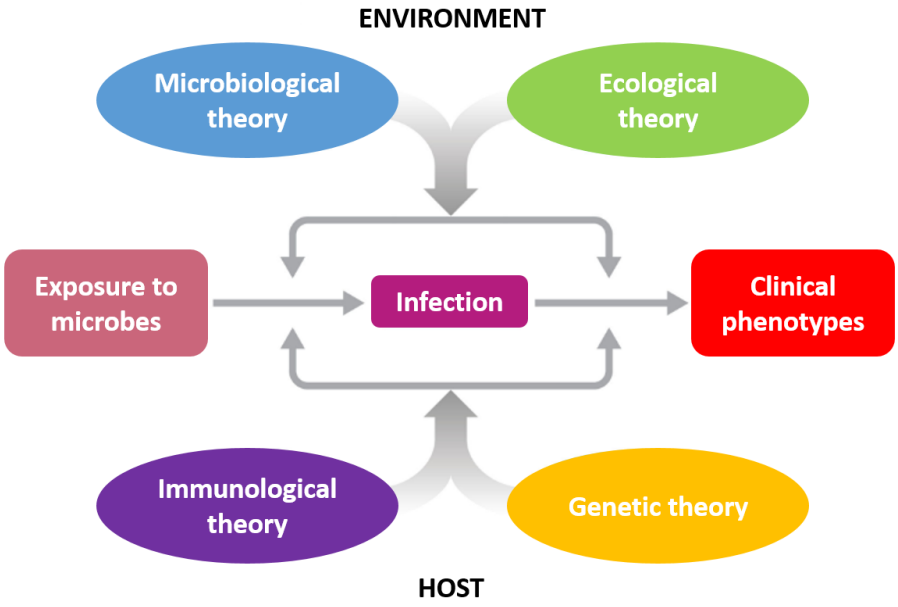

Figure 4 – The four complementary theories of infectious diseases. (Adapted from Casanova and Abel; Annual Review of Genomics and Human Genetics; 2013: 215-243)[42]

2. Mycobacterial diseases in PIDs

Predisposition to mycobacterial diseases in PIDs occurs as the sole infection in otherwise healthy individuals, or it is accompanied by a much broader susceptibility to pathogens due to more profound immune defects.[48,49] To this end, this section has been subdivided into "Broad predisposition to multiple and mycobacterial infections" and "Narrow predisposition to mycobacterial diseases: Mendelian susceptibility to mycobacterial diseases (MSMD)".

a. Broad predisposition to multiple and mycobacterial infections

Classical PIDs are characterized by chronic or recurrent infections caused by a wide range of pathogens (viruses, bacteria, fungi and protozoa), among which mycobacteria are

included. Patients suffering from classical PIDs suffer from life-threatening diseases during early childhood. In this category we can find Severe Combined Immunodeficiency (SCID), Combined Immunodeficiency (CID), Chronic Granulomatous Disease (CGD) and syndromic MSMD, which includes: autosomal dominant (AD) Guanine-adenine-thymine-adenine 2 (GATA2) deficiency, autosomal recessive (AR) Signal Transducer and Activator of Transcription 1 (STAT1) deficiency, AR IRF-8 deficiency, AR Retinoic acid-related orphan receptor gamma/gammaT (RORγ/γT) deficiency, partial AR JAK1 deficiency and AR Tyrosine kinase 2 (TYK2) deficiency. Affected cell types include lymphoid and myeloid lines.

a.1 SCID and CID

SCIDs are immunodeficiencies characterized by the dysfunction of cellular and humoral immunity.[41,50] These patients suffer from disseminated life-threatening infections by poorly virulent agents of viral, bacterial, fungal or parasitic origins in their first months of life.[51–53] Mycobacterial diseases in these patients are mainly caused BCG, being mostly BCG-osis, even though some BCG-itis have been described.[54] Infections by EM and *M. tuberculosis* have been reported but are less common, probably because the patients are not regularly exposed to these mycobacteria.[49,54] The immunological hallmark of these individuals is profound T-cell lymphopenia with or without reduced numbers of circulating B cells, who can also present a small thymus lacking thymocytes, their spleen is deficient in T cell areas and formation of tonsils and lymph nodes are missing.[53] In some cases, numbers of NK cells and myeloid cells can also be affected.[55] So far, X-linked mutations in one gene and AR mutations in 16 genes have been described to cause SCID, the X-linked gene and eight autosomal genes causing profound T cell deficiency with normal B cell count[56–64] and

eight autosomal genes causing profound T and B cell deficiency.[41,65,74,66–73] Of these 17 genes, nine genes have been linked to mycobacterial diseases, four with T cell deficiency but normal B cells (T-B+ deficiency, annex section I. SCID and CID causing genes, Table 1 – SCID causing genes) and five with both T and B cell deficiency (T-B- deficiency, annex section I. SCID and CID causing genes, Table 1 – SCID causing genes). BCG vaccination is contraindicated in SCID patients, given that any of them could develop an adverse effect to the vaccine.[54]

CIDs also affect cellular and humoral immunity but are generally less profound than SCID.[41,49] CIDs can be subdivided into CID or CID with associated syndromic features, which present prominent characteristics not associated to the immune defect (dermatological and neurological defects being the most common), broadening the clinical phenotype of these patients.[75] Patients are susceptible to poorly virulent agents of viral, bacterial, fungal or parasitic origins, suffering from recurrent infections that can be either localized (mostly in the respiratory and gastrointestinal tract) or disseminated.[76] Mycobacterial infections are mostly disseminated and caused mainly by BCG, as the vaccine is administered within the first months of life, followed by EM infections and then *M. tuberculosis* infections.[49,76] These types of immunodeficiencies are characterized by defective development or function of T cells, that maybe accompanied by B cell dysfunction and immunoglobulin defects.[76] Mutations in 102 genes have been reported to cause CID, seven of which are X-linked, 11 have an AD mode of inheritance, three can either be AD or AR and 81 are AR.[41] Only 15 of these genes, of which eight cause syndromic CID, have been linked to mycobacterial diseases (annex section I. SCID and CID causing genes, Table 2 – CID causing genes).[49,77-102] The

severity of the T cell defect in these patients might determine whether the patients will suffer or not from mycobacterial infections.[49]

a.2 CGD

CGD is a PID affecting the nicotinamide adenine dinucleotide phosphatase (NADPH) oxidase complex subunits, responsible of the respiratory burst in phagocytic leukocytes. It can be divided into classic CGD and p40^{phox} deficiency. Classic CGD is the result of mutations in genes encoding for the NAPDH oxidase complex subunits: *CYBB*, encoding for gp91^{phox} and the only X-linked; *CYBA*, encoding for p22^{phox}; *NCF1*, encoding for p47^{phox}; and *NCF2*, encoding for p67^{phox}.[49,103,104] Mutations in *NCF4*, encoding for p40^{phox}, have been described to cause a milder form of CGD (p40^{phox} deficiency) that do not suffer from invasive infections or mycobacterial disease, with the exception of one patient who also carried an AD *STAT1* mutation.[105] Briefly, the catalytic glycoprotein gp91^{phox} will interact with p22^{phox} in the cell membrane to form cytochrome b_{558} which will attract the cytosolic p67^{phox}, p47^{phox} and p40^{phox} which in turn will recruit Rac1/2. This will provoke a conformational change on gp91^{phox} that will enable cytosolic NADPH to give an electron to molecular oxygen in the phagolysosome to form superoxide ions that will be used to generate ROS.[104] Phagocytic cells, i.e. granulocytes, monocytes and macrophages; are the leukocytes affected by this disease.[106] This classic CGD is characterized by life-threatening, recurrent infections (bacterial and fungal) and dysregulated inflammation, which can lead to autoimmunity.[103,104] Sites of infection include the lung, skin, lymph nodes and liver.[103] Mycobacterial diseases, specifically, are usually severe and localized, being relatively common in patients living in endemic countries for TB, where BCG vaccine is compulsory.[49,103,104,106] BCG is the most isolated agent in patients suffering from these type of infections, followed by *M. tuberculosis*.

EM infections have been reported in isolated cases, appearing to be rare when compared to BCG disease and TB, probably because NADPH is not critical in the control of these agents.[49,106] Patients are mainly infected by pyogenic bacteria and fungi, specially *Staphylococci* and *Aspergillus* species.[103,104] The overall estimation of mycobacterial disease in classic CGD patients is at 25%, sometimes being the first observed clinical manifestation.[49,106]

a.3 Syndromic MSMD

Syndromic MSMD is a combination of both mycobacterial disease and other infections associated with a more complex cellular phenotype than pure MSMD,[107] which will be reviewed extensively in the next section. With the exception of Tyrosine kinase 2 (TYK2), the deficiencies listed here have not been linked to purely mycobacterial disease.[107]

a.3.1 AD GATA2 deficiency

AD GATA2 deficiency (OMIM: 614172) was first described in 2011 and is caused by germline heterozygous mutations on the *GATA2* gene.[108–112] GATA2 is a two-zinc finger transcription factor responsible for the development and maintenance of hematopoietic stem cells (HSCs) and various hematopoietic progenitor cells in humans, where it is mainly expressed.[113,114] GATA2 can either bind DNA as a monomer, a dimer or a heteromultimer.[113] It can be modified by phosphorylation, acetylation and sumoylation, being rapidly degraded by ubiquitination.[113] GATA2 deficiency is the cause of a wide spectrum of clinical phenotypes, including monocytopenia with mycobacterial disease (monoMAC syndrome),[109,115] familial myelodysplastic syndrome (MDS),[108,110,116] chronic or acute myeloid leukemia (CML or AML),[110,116] Emberger syndrome (primary lymphedema and

MDS),[111,117,118] and, more rarely, isolated neutropenia,[119] aplastic anemia,[120] and isolated NK cell deficiency.[112,121] *GATA2* mutations are loss-of-function (LOF) or hypomorphic[122] and seem to act by haploinsufficiency.[108,123] GATA2 deficiency predisposes mainly to mycobacterial, viral and fungal infections.[124] Mycobacterial infections are mainly caused by EM, mostly *M. avium* and *M. kansasii*, but some patients have been reported to have suffered from TB.[125,126] Regarding BCG vaccination, information is only available for three patients, of which only one had a disseminated BCG infection.[33] These infections are mostly disseminated, but in some cases can be localized. Infections by viruses occur in approximately two thirds of GATA2 deficient patient, mainly by human papillomavirus.[125] Patients have usually normal numbers of T cells (but they can be diminished in some cases) and show a progressive loss of monocytes, B cells, NK cells and dendritic cells (syndrome known as DCML)[112], and have been shown to have elevated levels of FMS-like tyrosine kinase 3 ligand (FLT3L) in their plasma.[113,122,126,127] The clinical features typically manifest in the second decade of life, but the age of onset varies from early childhood to late adulthood. Some mutation carriers remain asymptomatic throughout their life (the patient has the mutation but no clinical phenotype, presenting incomplete penetrance).[127,128] In order to survive, these patients are advised to undergo hematopoietic stem cell transplantation (HSCT).[113]

a.3.2 AR STAT1 deficiency

AR STAT1 deficiency (OMIM: 613796) is caused by bi-allelic *STAT1* mutations. STAT1, encoded by the gene *STAT1*, is a critical transcription factor belonging to the JAK/STAT signaling pathways that is activated by IFN-mediated signals (IFN-α/β, IFN-γ, and IFN-λ or IL-29) and other cytokines, like IL-27.[124,129] Briefly, binding of the cytokine to

its specific receptor will lead to the activation of the JAKs that are constitutively associated to the receptor. The JAKs will phosphorylate the intracellular domain of the receptors, creating a docking site for STAT1, which will be recruited, phosphorylated in the tyrosine at position 701 (Tyr701) and then released. Once released, phosphorylated STAT1 will either form homodimers with other phosphorylated STAT1 (known as gamma-activated factor, GAF) or heterodimers with STAT2 and interferon regulatory factor 9 (IRF-9) (known as IFN-stimulated gene factor 3, ISGF3). These dimers will then translocate into the nucleus, where they will bind to specific promoters like gamma-activating sequences (GAS) in case of GAF or to IFN-α/β sequence response elements (ISRE) in case of ISGF3. The binding will activate or modulate the transcription of specific target genes, like IL-12 in the case of IFN-γ stimulation or IRF-7 in case of IFN-α/β (Figure 5).[129,130] AR mutations in this gene have been described either as complete (caused by biallelic loss-of-expression and LOF *STAT1* alleles) or partial (caused by biallelic hypomorphic *STAT1* alleles), affecting either the phosphorylation or DNA binding capacity of STAT1. Patients carrying these mutations suffer from severe or mild mycobacterial and viral diseases respectively.[49,129,130] In complete AR STAT1 deficiency IFN-α/β, IFN-γ, IL-27 and IFN-λ signaling are abolished, accounting for the severe susceptibility to both virus and mycobacteria. The main causes of death in AR complete STAT1 deficiency are viral diseases or disseminated infection with BCG, which is why BCG vaccination is contraindicated in these patients.[129,131] Infections with EM have also been reported, particularly with *M. kansasii*.[130] HSCT is recommended in complete AR STAT1 deficiency.[129] Patients with partial AR STAT1 deficiency, as opposed to the complete deficiency, have impaired but not abolished IFN-α/β, IFN-γ, IL-27 and IFN-λ signaling, accounting for the milder clinical phenotype, and get well with treatment.[132–134]

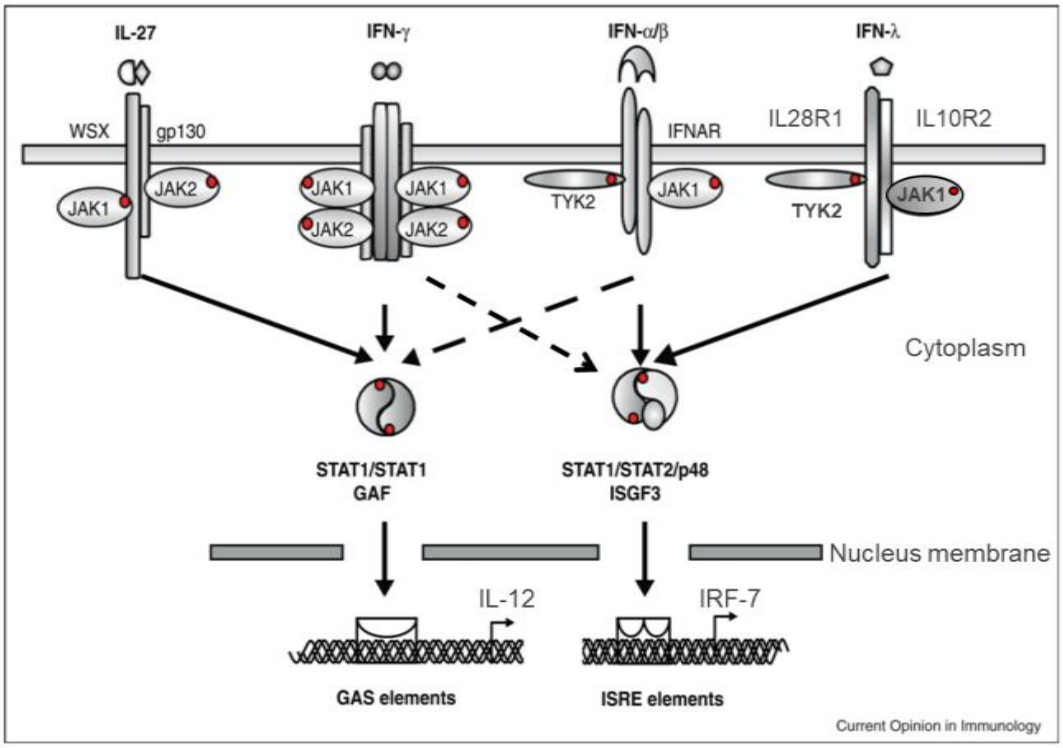

Figure 5 – Representation of IFN-α/β and IFN-γ signaling pathways. Image represents the two different ways STAT1 can be activated to give place to either antimycobacterial or antiviral immunity according to the stimulus. (Adapted from Boisson-Dupuis *et al*. In: *Curr Opin Immunol*. 2012: 364-378)[130]

a.3.3 AR IRF-8 deficiency

AR IRF-8 deficiency (OMIM: 226990) is caused by bi-allelic *IRF8* mutations. IRF-8 plays a critical role in hematopoietic lineage determination, being essential in myeloid cell development, determining granulocyte versus monocyte fate.[135] Moreover, it has been shown to play an important role in the development of plasmacytoid dendritic cells (pDCs) and type 1 conventional dendritic cells (cDC1s), remaining highly expressed in differentiated DCs.[136] Complete AR IRF8 deficiency was first described in 2011, where the patient suffered from BCG-osis and oral candidiasis.[137] Moreover, no monocytes or DCs could be detected in the blood. As a result of this absence, IL-12 production was impaired, rendering T cells anergic and with a very low capacity to produce IFN-γ.[84] Since then, three other patients have been

reported to have AR IRF8 deficiency and EBV infection, but with no associated mycobacterial disease.[138] Interestingly, these last three patients showed reduced but not abolished numbers of monocytes and DCs. However, they did show NK cells deficiency.[139] Differences in these cell numbers may account for the distinctive infectious phenotype of the patient described in 2011, rendering monocytes and dendritic cells key players in the immunity against mycobacteria.

a.3.4 AR TYK2 deficiency

AR TYK2 deficiency (OMIM: 611521) is caused by biallelic *TYK2* mutations. TYK2 is a member of the JAK family, associated to the receptors of IL-12, IFN-α/β, IL-10, IL-6 and IL-23.[49,124,129,140,141] AR recessive TYK2 deficiency was first described in 2006 in a patient that displayed characteristics of hyper-IgE syndrome (HIES): atopic dermatitis, high IgE levels, and recurrent cutaneous staphylococcal infections. This patient also had BCG-itis, gastroenteritis by *Salmonella* an several viral infections (including Herpes Simplex virus, HSV).[142] In 2015, seven other patients with complete AR TYK2 deficiency where described with viral and/or mycobacterial infections but no HIES.[143] Patients were shown to have impaired but not abolished cellular responses to IL-12 and IFN-α/β (explaining the mycobacterial and viral infections), IL-23 (with no mucocutaneous candidiasis), and IL-10 (but with no overt clinical consequences).[143] Between 2016 and 2018 three other patients have been described who did not develop mycobacterial disease.[144,145] To date, 11 AR TYK2 deficient patients have been described, nine of them suffering a complete deficiency and two suffering a partial deficiency. Of these 11 patients, 10 were BCG vaccinated, of whom four showed an adverse reaction to the vaccine. In one of these four patients, BCG-osis was the only disease recorded. Moreover, two other patients suffered exclusively from *M.*

tuberculosis infection (abdominal TB or miliary TB).[143] Overall, three TYK2 deficient patients had a phenotype of pure MSMD, which will be discussed in the next section.

a.3.5 AR RORγ/γT deficiency

AR RORγ, and its isoform RORγT, deficiency (OMIM: 616622) is caused by bi-allelic *RORC* mutations. Both RORγ and RORγT are transcription factors. RORγT, whose expression is restricted to leukocytes, is the most studied of both isoforms, which has been observed to play a role in IL-17A/F and IL-22 producing lymphocytes (including ILC3, γδ T cells, and Th17) cells in mice.[146] Defects in IL-17A/F immunity are linked to chronic mucocutaneous candidiasis (CMC) but not to mycobacterial diseases.[41] However, in 2015, AR RORγ/γT deficiency was described in seven patients suffering from both CMC and mycobacterial disease.[147] All seven patients were BCG vaccinated and developed mycobacterial disease (either by BCG or *M. tuberculosis*).[148] These patients presented T cell lymphopenia, small thymus, and a lack of palpable axillary and cervical lymph nodes. IL17A/F secretion was impaired in T cells from the patients, accounting for CMC. Surprisingly, IFN-γ secretion was found to be impaired in γδ T cells and Th1* cells (cells secreting both IL-17A/F and IFN-γ), accounting for the mycobacterial disease observed in these patients.

a.3.6 Partial AR JAK1 deficiency

Partial AR JAK1 deficiency (OMIM: 147795) is caused by bi-allelic *JAK1* mutations. JAK1 is a tyrosine-kinase involved in the intracellular signalization of many cytokines, including IFN-α/β and IFN-γ.[149] It is associated to the receptors of these cytokines, allowing the activation of transcription factors as described in section a.3.2 AR STAT1 deficiency and

depicted in Figure 5. Partial AR JAK1 deficiency was described for the first time in 2016 in a patient who suffered from systemic atypical mycobacterial disease and who had a history of viral, fungal, and parasitic skin infections.[150] Besides, the patient developed a mild T cell lymphopenia and severe anemia in his mid-teens. The mutant JAK1 was shown to be hypomorphic, accounting for the impaired, but not abolished, response to IFN-α, IFN-γ, IL-27, IL-10, IL-2 and IL-4 in the patient's lymphocytes.[151] Cellular responses to IFN-γ and IFN-α were impaired but not abolished by this mutant allele in in primary fibroblasts and in overexpression system (U4A cells), where phosphorylation of STAT1 and STAT2 was measured. The patient also suffered from urothelial carcinoma. Overall, partial AR JAK1 deficiency causes susceptibility to mycobacteria due to the impairment of IFN-γ signaling and to other infections due to the impairment of response to other cytokines including IFN-α.[151]

b. Narrow predisposition to mycobacterial diseases: MSMD

MSMD is a rare group of PIDs which predisposes otherwise healthy individuals to infection by weakly virulent mycobacteria and, in few cases, to *Salmonella* and *Candida* infections.[35,152] Onset of the disease is usually in childhood, but it can also be observed in adolescence and adulthood. Clinical manifestations observed in these patients range from localized to disseminated, and are mostly due to EM and BCG, but some cases of infection by *M. tuberculosis* have also been described.[35,152] Prevalence is 1 every 50,000 individuals and no overt immunological and hematological abnormalities are observed.[35,153] The first description of MSMD was probably in 1951, in a child with disseminated disease caused by BCG who remained otherwise healthy.[154] However, it was not until 1996 that the first genetic etiology for MSMD was described, when a homozygous mutation in *IFNGR1* was shown to

be responsible for a fatal BCG disseminated disease in an otherwise healthy patient.[46] Since then, mutations in 11 genes conferring MSMD (*IL12B*, *IL12RB1*, *ISG15*, *TYK2*, *IRF8*, *SPPL2A*, *CYBB*, *IFNGR1*, *IFNGR2*, *STAT1*, *NEMO*) have been found, whose allelic heterogeneity is responsible for 21 different genetic disorders.[35,49,143,155] The products of these genes (Figure 6) either control the production (IL-12p40, IL-12Rβ1, TYK2, NEMO, ISG15, IRF-8 and SPPL2a) and/or the response to IFN-γ (IFN-γR1, IFN-γR2, STAT1, IRF-8 and gp91^{phox}).[35,143,155] Interestingly, specific modes of inheritance in genes that cause mycobacterial disease with a broader infection phenotype (like NEMO, gp91^{phox}, STAT1 and IRF-8) can also cause pure MSMD.[35,49]

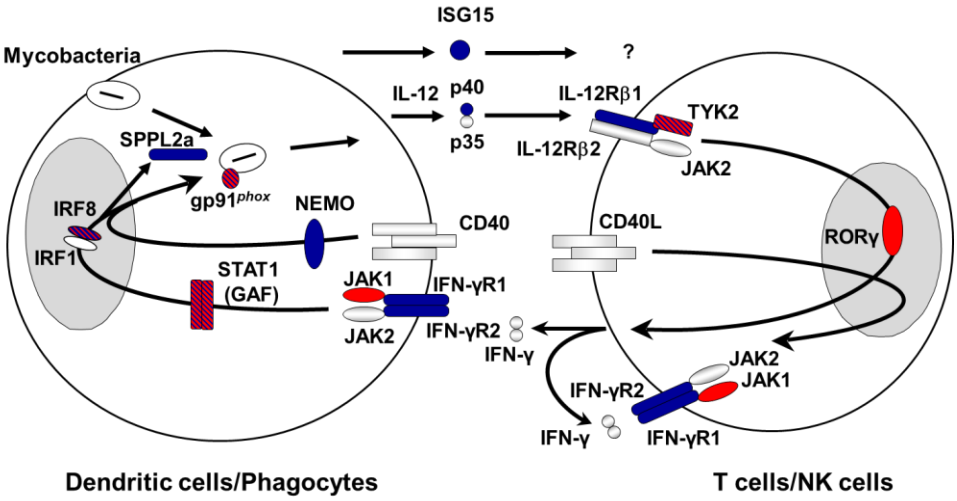

Figure 6 – MSMD causing genes and their cellular implication in the production or response to IFN-γ. Protein deficiencies that cause MSMD are depicted in blue, those responsible only for mycobacterial disease with a broader infection phenotype are depicted in red, and those described for both MSMD or MSMD combined with other infections are depicted in red and blue. (Adapted from Rosain *et al*. Immunology and Cell Biology; 2018; in press)[107]

b.1 Defects in IFN-γ production

b.1.1 AR IL-12p40 and IL-12Rβ1 deficiencies

IL-12, also known as IL-12p70, is a cytokine composed of the subunits IL-12p40 and IL-12p35.[124] IL-12 signals through IL-12 receptor (IL-12R), which is formed by the subunits IL-12Rβ1 and IL-12Rβ2 and are associated to TYK2 and JAK2 respectively. IL-12 signaling will result in IFN-γ production and secretion.[124] IL-12Rβ1 is encoded by *IL12RB1* and IL-12p40 is encoded by *IL12B*. Recessive mutations in both of these genes (either homozygous or compound heterozygous) give place to a complete deficiency of their encoded proteins that can be with or without protein expression. To date, 220 patients with AR complete IL-12Rβ1 deficiency have been described (OMIM: 614891).[129] NK cells and T cells of IL-12Rβ1 patients have a lack of response to both IL-12 and IL-23 (IL-23 receptor is formed by the heterodimer of IL-12Rβ1 and IL-23R)[19] that lead to low IFN-γ production. These patients are prone to infections by BCG, EM or *M. tuberculosis*, and half of them also present nontyphoidal salmonellosis.[156] Some of the patients also show mild forms of CMC, probably due to the impairment of IL-23 signaling. On the other hand, AR complete IL-12p40 deficiency (OMIM: 614890) has been reported in 50 patients who show a low production of IFN-γ. Infections in these patients are usually caused by BCG after vaccination, but infections caused by EM and *M. tuberculosis* have also been described. Clinical phenotype is thought to be mild, but it has been shown that it can range from asymptomatic to death at early stages.[35,157] Overall, prognosis for these patients is good since recurrence of disease is rare. A combination of antibiotics and IFN-γ injection should suffice as treatment.[129]

b.1.2 AD IRF-8 deficiency

In contrast to AR IRF-8 deficiency (see section a.3.3 AR IRF-8 deficiency), AD IRF-8 deficiency (OMIM: 614893) is caused by heterozygous mutations in *IRF8*, of which one has been shown to have a negative effect on the wild type (WT) protein (dominant negative).[139,158] These mutations give place to an expressed hypomorphic IRF-8 protein that causes the diminishment of conventional dendritic cells in charge of producing IL-12 and an impaired production of IFN-γ by mycobacterium-specific memory Th1* cells.[155] Patients with AD IRF-8 deficiency suffer from BCG disease, without any other infectious disease, unlike those who suffer from AR deficiency. Prognosis for these patients is good and antibiotics are the only recommended treatment.[124,129]

b.1.3 AR SPPL2a deficiency

In 2018 one new genetic etiology of MSMD has been described. Homozygous mutations in signal peptide peptidase-like 2A (*SPPL2A*) gene, encoding for SPPL2a, were described in three patients suffering from BCG disease after vaccination.[155] SPPL2a is a protease in charge of degrading the amino-terminal fragment of the human leukocyte antigen (HLA) invariant chain (CD74) of antigen-presenting cells. If this fragment is not degraded it accumulates in the cells, having a toxic effect on them. SPPL2a deficient patients have been shown to have a selective depletion of IL-12 and IL-23-producing $CD1c^+$ conventional type 2 dendritic cells (cDC2s) and their circulating progenitors. Moreover, Th1* cells have been shown to have an impaired IFN-γ production after stimulation with mycobacterial antigens. These results have been corroborated in Sppl2a deficient mice, which were susceptible to mycobacterial diseases. Overall, SPPL2a deficiency causes MSMD through a

quantitative defect of IL-12 and IL-23-producing cDC2s and through an impaired production of IFN-γ by mycobacterium-specific memory Th1* cells. These patients have been found to be similar to AD IRF-8 deficient patients, and their prognosis seem to be the same.[155]

b.1.4 AR ISG15 deficiency

Interferon-stimulated gene 15 (ISG15), encoded by *ISG15*, is a ubiquitin-like protein that attaches to substrates in a process called ISGylation, which is similar to ubiquitination.[159] ISG15 can be secreted by neutrophils, monocytes and lymphocytes and induces potently IFN-γ production in lymphocytes when synergized with IL-12. Since its discovery in 2012, complete AR ISG15 deficiency (OMIM: 616126) has been described in six patients with loss of expression and function mutations.[160,161] The common clinical feature of these patients was calcification of the basal ganglia in the brain. Of these six patients, three developed mycobacterial disease due to BCG vaccination. T and NK cells of the patients displayed an impairment, but not abolishment, of IFN-γ production after stimulation with BCG and IL-12.[160] Addition of recombinant ISG15 rescued the IFN-γ production in both T and NK cells of the patients. Interestingly, calcification in the brain can be explained by and enhanced IFN-α/β activity, which is caused by the destabilization of USP18, a negative regulator of this cytokine.[162]

b.1.5 X-linked recessive NEMO deficiency

NEMO or *IKBKG* encodes for inhibitor of nuclear factor kappa-B kinase subunit γ (IKKγ) or nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) essential modulator (NEMO). This gene plays a role in NF-κB signaling.[124]. Regarding its function, NF-κB is a transcription factor conformed by either homo or hetero-dimers of five different

proteins in the Rel family (p50, p52, RelA, c-Rel and RelB). At the steady state, NF-κB is inhibited by IkBα, IkBβ and IkBε. When the cell is stimulated, IKKs complex (IKKα, IKKβ and the regulatory subunit IKKγ) will phosphorylate the IkBs allowing NF-κB's translocation to the nucleus and the transcription of several immune system receptors and receptors of ectodermal and bone cells.[124] X-linked recessive NEMO deficiency is linked to a syndromic CID called anhidrotic ectodermal dysplasia with immune deficiency (EDA-ID, OMIM: 300291).[101,102] However, 11 patients suffering from X-linked recessive NEMO deficiency have been reported with purely mycobacterial diseases (OMIM: 300636). These patients carry very specific mutations (p.E315A, p.R319Q, c.1-16G>C and c.1-16+1G>T), responsible for their narrow phenotype.[107] These mutations are located in the region of *NEMO* that encodes for the leucine zipper domain that forms a salt bridge, which is fundamental in CD40-triggered IL-12 production through c-Rel.[35] Cellular phenotypes of the patients present low IFN-γ and IL-12 production after T cell specific stimulation.[163] Patients with this specific NEMO deficiency suffered from purely mycobacterial infections by EM, BCG and TB.[35,129] The prognosis of the patients is variable but they can, however, benefit from antibiotic treatment and IFN-γ injection.[35,49]

b.2 Defects in IFN-γ response

b.2.1 IFN-γ receptor deficiencies

IFN-γ receptor (IFN-γR) is a heterodimer composed of two subunits, IFN-γR1 and IFN-γR2, encoded respectively by *IFNGR1* and *IFNGR2*. IFN-γR1 is the ligand binding chain of the receptor and is associated to JAK1, whereas IFN-γR2 is the signal transducing chain and is associated to JAK2.[149] IFN-γ stimulation will result in the dimerization of these

two receptors, which will activate JAK1 and JAK2 that will then phosphorylate STAT1, initiating the transcription of target genes (Figure 5).[164] Three types of deficiencies for these two molecules have been described: AR complete deficiency (for IFN-γR1 OMIM: 209950; for IFN-γR2 OMIM: 614889), caused by bi-allelic null mutations (with or without protein expression) resulting in abolished IFN-γ response; AR partial deficiency, caused by bi-allelic hypomorphic mutations which lead to impaired response to IFN-γ; and AD partial deficiency (for IFN-γR1 OMIM: 615978), caused by mono-allelic mutations impairing IFN-γ signaling by either negative dominance (IFN-γR1) or haploinsufficiency (IFN-γR2).[129] Mutations in *IFNGR1* have been reported in more than 100 individuals, whereas mutations in *IFNGR2* have only been reported in 24.[35,129,165] AR complete deficient patients suffer from severe life-threatening mycobacterial infections, mostly EM and BCG, in early childhood and present high levels of IFN-γ in plasma.[35,129] Viral infections have been reported in few cases.[129] Prognosis for these patients is very poor and HSCT is recommended once the mycobacterial disease has been controlled.[129] AR partial deficient patients, as opposed to complete deficiency, present a less severe clinical phenotype with EM and BCG diseases mostly, with other bacterial diseases being reported in some cases. High IFN-γ levels in plasma may also be detected in these patients. Prognosis is relatively good in most cases, with antibiotic treatment and recombinant IFN-γ injections.[129] Interestingly, one AR complete *IFNGR2* mutation (p.T168N) and AR partial IFN-γR2 deficiency have been shown to be caused by mutations that lead to high levels of N-glycosylation of the protein and lead to retention in the endoplasmic reticulum (ER).[166] AD partial deficiency has been mostly described for IFN-γR1 since, to date, AD partial IFN-γR2 deficiency has only been reported in one patient.[167,168] AD partial deficiency predisposes to EM, TB and BCG infections, mostly affecting the bones

(osteomyelitis). Prognosis is relatively good, with antibiotic treatment and recombinant IFN-γ injection.[129] Of note, AD partial IFN-γR2 deficiency has been shown to have incomplete penetrance, since only one heterozygous individual has developed mycobacterial disease.[129,167]

b.2.2 AD STAT1 deficiency

In contrast with AR STAT1 deficiency (reviewed in section a.3.2 AR STAT1 deficiency), LOF AD STAT1 deficiency (OMIM: 614892) has been purely linked to mycobacterial diseases.[129] Mutations causing this deficiency have been shown to reduce the cellular response to IFN-γ but not to IFN-α, explaining the pure mycobacterial disease, and can either affect the phosphorylation, DNA binding capacity of STAT1 or both.[129] Mycobacterial infections in these patients can be either local or disseminated and be caused by EM, BCG or TB. Osteomyelitis in these patients is frequent, similar to what is observed in AD partial IFN-γR1 patients.[129] Interestingly, some mutation carriers have remained asymptomatic, presenting therefore incomplete penetrance. Prognosis and treatment are similar to those for AD partial IFN-γR1 patients.[129]

b.2.3 X-linked recessive gp91^{phox} deficiency

CGD causing gp91^{phox} deficiency has been reviewed in section a.2 . Two specific mutations in *CYBB* (p.Q231P and p.T178P) have been shown to predispose patients who carry them to only mycobacterial diseases (BCG-osis, BCG-itis or TB) (OMIM: 300645).[35] Interestingly, it was observed that ROS production was only abolished in monocyte-derived macrophages (MDMs) and Epstein-Barr Virus-Transformed B (EBV-B) cells, when activated with BCG and IFN-γ. Moreover, mutated gp91^{phox} expression in these cells was

shown to be impaired. In contrast to CGD, where all phagocytic cells are affected, neutrophils, monocytes and monocyte-derived dendritic cells (MDDCs) showed normal ROS production after stimulation in these patients. These results highlighted the importance ROS production in macrophage against mycobacterial infection, whereas ROS production by granulocytes and monocytes is more important against fungi and pyogenic bacteria.[35]

III. Study strategy

The genetic diagnosis of patients with mycobacterial diseases can follow two strategies: immunological approach or genomic approach.

The immunological approach consists on clinical suspicion, standardized functional tests and Sanger sequencing of a candidate gene (a known gene to cause mycobacterial disease). Clinical suspicion is based on the distinctive clinical phenotype that is displayed by patients who are mutated in certain genes. The clinical suspicion is strengthened by the functional standardized tests. These tests include the evaluation of the IFN-γ axis, which is the gold procedure for MSMD diagnosis. This assay was developed by Feinberg *et al*, [153,169] and consists in the measurement of different cytokines (IL-12p40, IL-12p70 and IFN-γ) by enzyme linked immunosorbent assay (ELISA) after whole blood or peripheral blood mononuclear cells (PBMCs) activation. This activation is performed with live BCG with or without recombinant IL-12p70 or recombinant IFN-γ for 48 hours. Levels of the produced cytokines after these stimulations can point towards an IFN-γ production or response defect. Further studies can be performed, like measurement of IFN-γ levels in patients' plasma (useful to determine AR IFN-γR deficiencies);[153,170] evaluation by flow cytometry of receptor expression (like IL-12Rβ1 or IFN-γR1) to determine whether the proteins are being expressed or not;[153] and cytometric evaluation of downstream signaling (such as STAT1 for IFN-γ and STAT4 for IL-12) that allows to detect defects of phosphorylation.[153] The results obtained in these functional tests may point towards a candidate gene that can be Sanger sequenced in the search of a reported or novel mutation.

The genomic approach consists on the use of next generation sequencing (NGS) to identify the gene responsible for the disease.[171] This method is used for those patients that, despite their clear clinical phenotype, are not mutated in any of the known genes to cause mycobacterial disease. The working hypothesis is that there are novel mutations in undescribed genes that may affect, or not, the IFN-γ axis and that are mycobacterial disease causing.

JUSTIFICATION, HYPOTHESIS AND OBJECTIVES

Since the discovery of the first genetic etiology of MSMD in 1996, 501 individuals with mycobacterial disease have been genetically diagnosed in the Laboratory of Human Genetics of Infectious Diseases. However, the genetic etiology of approximately 500 other patients suffering from mycobacterial diseases is still unknown. Mutations in known MSMD causing genes require characterization in order to discern the severity of the genetic defect encountered. The genetic diagnosis and functional characterization of the mutation of the candidate gene are key in providing a proper treatment and genetic counseling for the family, as well as identifying a good donor in case HSCT is required. This thesis is focused on the characterization of novel mutations in *IFNGR2* and known and novel mutations in *GATA2* in patients suffering from mycobacterial diseases. We hypothesize that these mutations impair the function of the IFN-γR2 and GATA2 proteins respectively, and are responsible for the mycobacterial disease in these patients.

Specific objectives were set with the aim of proving this hypothesis:

1. To characterize the novel *IFNGR2* mutations by overexpression and in patients' cells in order to determine whether they confer a complete or partial IFN-γR2 deficiency.
2. To characterize in detail at the molecular, cellular, immunological, and clinical levels patients with *GATA2* mutations and mycobacterial disease; a study that is lacking in the current literature.

MATERIALS AND METHODS

Patients and kindreds

Patients were referred to the Laboratory of Human Genetics of Infectious Diseases in France due to mycobacterial infectious and hematological abnormalities. The parents and/or patients, as requested and approved by the institutional review boards (IRB) of the various institutions involved, signed an informed consent form. The experiments described here were performed in France, in accordance with local regulations, and with the approval of the IRB of Necker Hospital for Sick Children, France. Particularly for the patients diagnosed with GATA2 deficiency, the physicians in charge completed a detailed questionnaire including date of birth, date of first clinical manifestations, BCG vaccination, infectious diseases (in particular mycobacteria), hematological disorders, HSCT, living status and, if dead, cause of death. The data were collected from 2011 to 2018 and sent to the laboratory. Our analysis focused mainly on mycobacterial infectious disease in GATA2 patients. We did not consider clinical signs for other infectious diseases for the recruitment of these patients.

Mycobacterial infection diagnosis

Mycobacterial infectious disease was diagnosed based on clinical and radiologic features, staining for acid-fast bacilli (AFB), molecular PCR and/or microbiological culture results when available. Recurrence was defined as a subsequent episode of mycobacterial disease with the same microbe after a period free from clinical disease and treatment. Diagnosis of EM has been categorized in lung disease, according to the criteria of the American Thoracic Society (ATS)/Infectious Disease Society of America (IDSA), and extra-pulmonary disease, referred as skin and soft tissue, lymph node, bone and joint, disseminated

or catheter-related infections.[172,173] Tuberculosis was diagnosed according to the criteria proposed by S. Graham *et al*.[174] Diagnosis of confirmed tuberculosis was defined with the presence of at least one sign or symptom of tuberculosis with positive culture for *M. tuberculosis.* Probable infection by tuberculosis was defined by if the patient had one sign or symptom suggestive of tuberculosis, a chest radiography consistent with intrathoracic tuberculosis and at least one of the following: i) positive clinical response to anti-tuberculosis treatment, ii) documented exposure or close contact with a patient with tuberculosis, or iii) immunological evidence of *M. tuberculosis* by a positive tuberculin skin test (TST) or interferon-γ release assay (IGRA). Possible tuberculosis was defined by, in addition to signs or symptoms suggestive of tuberculosis infection, if the patient had: i) chest radiography not consistent with pulmonary tuberculosis but he has at least a positive clinical response to anti-tuberculosis treatment; or documented exposure or close contact with a patient with tuberculosis, or immunological evidence of *M. tuberculosis* by TST or IGRA.

Extraction of DNA and WES

Genomic DNA (gDNA) was extracted from whole blood from healthy controls, the patients and family members, using the iPrep PureLink gDNA Blood kit and the iPrep instruments from Thermo Fisher Scientific. For one patient genomic DNA was extracted from plasma with the QIAmp DNA Blood Mini Kit (Qiagen, 51104). WES was performed with 3 µg of DNA. Exome capture was performed with the SureSelect Human All Exon 72 Mb kit (Agilent Technologies, Santa Clara, CA, USA). Paired-end sequencing was performed on a HiSeq 2000 (Illumina, San Diego, CA, USA), generating 100-base reads.

The sequences were aligned with the GRCh37 reference built of the human genome, using the Burrows-Wheeler aligner (BWA).

Polymerase chain reaction (PCR) and sequencing

IFNGR2 and *GATA2* were sequenced using designed primers flanking each of their exons and part of their intronic borders. Additionally, intron 5 of *GATA2* was also sequenced in search of intronic regulating variants. PCRs were carried out using FastStart™ Taq (Roche) and a GeneAmp PCR system 9700 (Applied Biosystems). PCR products were then analyzed by electrophoresis in 1% agarose gels, purified by centrifugation through Sephadex G-50 Superfine resin (GE Healthcare) and sequenced with the Big Dye Terminator cycle sequencing kit (Applied Biosystems). Sequencing products were purified by centrifugation through Sephadex G-50 Superfine resin, and sequences were analyzed with an ABI Prism 3700 apparatus (Applied Biosystems). The sequences were aligned to the genomic sequence of *IFNGR2* (NM_005534.3) or *GATA2* (NM_032638.4) with Genalys, version 2.0 β software.

Cell culture and stimulation

EBV-B cells, primary human fibroblasts immortalized with SV-40 T antigen (SV-40 fibroblasts) and human embryonic kidney (HEK)293-T cells were cultured in Roswell Park Memorial Institute medium (RPMI, Gibco) or Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco), referred as complete medium.[175,176] EBV-B cells and SV40-fibroblasts were stimulated with the indicated doses of IFN-γ (Imukin, Boehringer Ingelheim) and IFN-α2b (IntronA, Schering Plough) for 20 minutes.

Expression plasmids and cell transfection

WT *IFNGR2* allele was inserted into the V5-topo-pcDNA3.1 (Invitrogen) according to the manufacturer's instructions and an insert-less V5-topo-pcDNA3.1 plasmid was kept as an empty plasmid (EV). *GATA2*-WT isoform 1 (RC208554) and *GATA2*-WT isoform 2 (RC227514) in a PCMV6 backbone were bought from Origene, as well as the insert-less backbone. Plasmids were transformed into TOP 10 competent cells (Invitrogen). Multiplied plasmid extraction was performed using HiSpeed Plasmid Maxi Kit (Qiagen). Mutations in all the plasmids were generated by site-directed mutagenesis (PfuUltra II Fusion HS DNA polymerase or QuickChange II XL Site-Directed Mutagenesis Kit, both from Agilent) according to the manufacturer's instructions, followed by DpnI digestion (New England Biolabs). HEK293-T cells were transfected with one of the various *IFNGR2* V5-tagged pcDNA3.1 plasmids or *GATA2* PCMV6 plasmids using either X-treme GENE9 transfection reagent (Sigma Aldrich) or Lipofectamine transfection kit (LTX and PLUS reagent, Invitrogen), according to the manufacturer's instructions. IFN-γR2 deficient fibroblasts[177] and IFN-γR2 patient's fibroblasts were also transfected with *IFNGR2* V5-tagged pcDNA3.1 plasmids. Cells were plated at $0.6x10^6$ in six well plates and grown in 2 ml of complete DMEM and incubated at 37˚C in 5% CO_2. After 24 hours, transfection was carried out. After 48 hours, the cells were collected and proteins were extracted.

Cell lysis, immunoblotting and immunoprecipitation

Total proteins were solubilized in extraction buffer (25 mM Tris-HCl, pH 7.6, 150 mM $NaCl_2$, 1% NP-40, 1 mM EDTA, 1× proteinase inhibitor cocktail mix, 1 mM PMSF, and 1 mM Na_3VO_4). Their concentration was then measured by the Bradford method and they

were incubated with Laemmli buffer 1x and NuPage (Invitrogen) as a reducing agent. Samples were then loaded into SDS-PAGE, 10% polyacrylamide gels (Bio-Rad) and then transferred into low fluorescence or normal PVDF membranes or nitrocellulose membranes (Bio-Rad) with the Trans-Blot Turbo Transfer System (Bio-Rad) or iBlot system (Invitrogen). Immunoblotting was performed using antibodies against: anti-IFN-γRβ (sc-377291, Santa Cruz Biotechnology, Inc.), anti-V5 tag (Thermo Fisher Scientific), anti-GATA2 antibody (sc-267, Santa Cruz Biotechnology, Inc.), anti-DDK antibody (TA5001, Origene) GAPDH (14C10, Cell Signaling Technology), pY701 STAT1 (612133, BD Biosciences), STAT1 (610116, BD Biosciences) or α-tubulin (sc-23948, Santa Cruz Biotechnology, Inc.). We detected bound antibody with ECL western blotting detection reagents (Bio-Rad) on Chemidoc or with fluorescence detector LI-COR Clx machine.

V5 tagged *IFNGR2* transfected HEK293-T cells were lysed and immunoprecipitated using anti-V5 tag antibody and agarose A/G beads (Santa Cruz Biotechnology, Inc.) after a background-clearing step with an isotype control and agarose A/G beads (Santa Cruz Biotechnology, Inc.). Immunoprecipitated proteins were subsequently resolved on SDS-PAGE. Blots were either probed with anti-IFN-γRβ or the SDS gel was incubated with Coomasie.

Electrophoretic mobility shift assay (EMSA) and luciferase reporter assays

SV40 fibroblasts or EBV-B cells were stimulated for 20 min with IFN-γ or IFN-α at the indicated doses. A two-step protein extraction was performed to separate the cytoplasmic and nuclear content of the cells; cells were first mixed with a membrane lysis buffer (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.05 % NP40, 25 mM NaF

supplemented with 1 mM PMSF, 1 mM DTT, 10 µg/ml leupeptin, 10 µg/ml aprotinin) and incubated for 30 min on ice. The lysate was centrifuged and the supernatant was discarded. The remaining pellet, nuclear fraction, was mixed with nuclear lysis buffer (20 mM Hepes pH 7.9, 0.4 M NaCl, 1,mM EDTA, 1, mM EGTA, 25% glycerol supplemented with 1 mM PMSF, 1 mM DTT, 10 µg/ml leupeptin, 10 µg/ml aprotinin) and incubated for 30 min on ice. Five to 10 µg of nuclear extract was incubated with ^{32}P-labeled (α-dATP) or IRDye 700 GAS probe, corresponding to the Fc-γR1 promoter, and subjected the mixture to electrophoresis in a polyacrylamide gel.

Luciferase reporter assays were performed in HEK293-T cells. $3x10^6$ cells were plated in 96 wells culture plates in 100 µl of complete DMEM and incubated at 37˚C and 5% CO_2. Lipofectamine kit was used for the transfection with various plasmids: EV, *GATA2*-WT and mutated isoform 1. After 24 hours, the supernatant was discarded and luciferase activity was measured with the Dual-Luciferase Reporter Assay kit (Promega) following the manufacturer's instructions. Other plasmids used in the experiment were *PML* (RC220236, Origene), *GATA2* Luciferase reporter plasmid (LR0030, Panomics) and Renilla pRL-SV40-d238 created by Ho and Strauss.[178] Samples were read using a Luminometer (Victor X4 model 2030, PerkinElmer).

Flow cytometry

Briefly, 5 x 10^5 SV-40 fibroblasts were incubated with 10 and 10^3 IU/ml of IFN-γ for 48 h. Cells were washed with staining buffer (1X Phosphate-buffered saline or PBS, 2 mM ethylenediaminetetraacetic acid or EDTA, and 2% FBS) and HLA-DR levels on the cell surface were then evaluated by flow cytometry with an anti-human HLA-DR FITC antibody

(556643, BD Biosciences). STAT1 phosphorylation was assessed by activating EBV-B cells, previously stained with Aqua Dead Cell Stain kit (Thermo Fisher Scientific), with 10^5 IU/ml of IFN-γ or IFN-α for 30 min, washing them in cold PBS 1X, following nuclear permeabilization by BD phosflow buffers. Cells were then washed and incubated for 1 h at 4ºC with either an antibody against phosphorylated STAT1 (612597, BD Biosciences) or with the corresponding isotype antibody (565357, BD Biosciences). Cells were washed three times, samples were acquired on a Gallios (Beckman Coulter), using FlowJo as the analysis software.

Whole blood assay of the IFN-γ circuit and ELISA

Heparin-treated blood samples from healthy controls, patients and parents were stimulated *in vitro* with BCG alone or with BCG plus IFN-γ or IL-12 (R&D), as previously described.[153,169] Supernatants were collected after 48 h of stimulation and ELISA were performed with specific antibodies directed against IFN-γ, IL-12p70, or IL-12p40, using the human Quantikine HS kits for IL-12p70 and IL-12p40 (R&D Systems) and the human Pelipair IFN-γ (Sanquin), according to the manufacturer's instructions. ELISA was also performed on plasma from GATA2 patients, their family members (when available) and healthy donors for human FLT3L (R&D Systems) and human macrophage colony-stimulating factor (M-CSF) (R&D Systems) following the manufacturer's instructions.

Differentiation of MDMs and $CD4^+$ T cells

$CD14^+$ monocytes from PBMCs were isolated using a positive selection kit from Miltenyi Biotec. Briefly, 0.5 million monocytes were incubated in 6-well plates with 50 ng/ml M-CSF (216-MC, R&D systems) and 50 ng/ml IL-4 (204-IL, R&D systems), to induce

MDMs differentiation during 14 days.[179] $CD4^+$ naive T cells were obtained using human naive $CD4^+$ T cell isolation kit II (Miltenyi Biotec). The cells were stimulated with 10^5 IU/ml of IFN-γ or IFN-α.

PBMCs and ribonucleic acid (RNA) analysis

PBMCs were obtained from heparin-treated whole blood from patients and healthy controls by centrifugal separation through a Ficoll gradient and washing with RPMI. Different PBMCs populations ($CD3^+$, $CD56^+$, $CD19^+$, $CD14^+$, dendritic cells, myeloid dendritic cells and plasmacytoid dendritic cells) were obtained from buffy coat from healthy donors through Ficoll gradient and then separated by positive or negative selection with MACS Miltenyi beads and columns following the manufacturer's instructions. Isolated peripheral blood $CD34^+$ cells were bought from STEMCELL technologies (# 70040). Total RNA was extracted from different peripheral myeloid and lymphoid cells, from frozen vials of PBMCs from patients and healthy donors using the RNeasy Micro Kit (Qiagen). Retrotranscription was carried out with SuperScript II Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was performed in the ABI Prism 7500 (Applied Biosystems) using *SYBR* Green master mix (Biorad) with specific primers for *GATA2*. *GAPDH* expression was used as an internal control. Data were analyzed using the comparative cycle threshold (ΔCt) method.

Statistical analysis

Statistical analysis was performed for the luciferase assay experiments using GraphPad Prism version 5.03. After checking whether the data were normally distributed, an unpaired t-test (Wilcoxon matched-pairs signed rank test) was used for statistical analysis, as

at all times two sets were compared: WT versus mutants, combinations of WT and EV versus combinations of WT and mutant, or WT versus combination of WT and mutant. Data were considered significant when $p<0.01$.

Penetrance calculation

We estimated the penetrance as a function of age for all type of GATA2-related disease and according to disease type (mycobacterial infection and other disease) in GATA2-deficient first degree relatives of index cases using the non-parametric cumulative incidence function taking into account competing risks, as implemented in the R package "cmprsk". Confidence intervals (CIs) were computed using log-log transformation to ensure that the lower limits of the 95% CIs were positive.

Selective pressure measures

Three different methods to analyze selective pressure were used to study the purifying selection of GATA2.[180–182] Further information can be found in Annex section: II. Selective pressure measures.

RESULTS

Chapter I – IFN-γR2 deficiency

Description of three MSMD patients

We investigated three MSMD patients from two unrelated kindreds (Figure 7A). P1 and P2 were born to consanguineous parents in Turkey and P3 was born to consanguineous parents in India.

P1 (Kindred A, II.1, Figure 7A) was born in 2009 from a Turkish consanguineous family. He was BCG vaccinated at 2 months of age. At 9 months of age, he developed a local lymphadenitis (BCG-itis) which was apparently resolved by antibiotics but he had a relapse at 18 months of age. At the age of 5 years, he developed multiple cervical lymphadenopathies, deep neck abscesses, cervical bone lesions affecting movement of the neck. *Salmonella* serology was positive but it was not found in blood culture. At the age of 7 years, he was admitted to the hospital for osteomyelitis in cervix, thorax, lumbar spine and sacral bones. *M. bovis*-BCG was characterized as the responsible microorganism. Subsets of T, B and NK cells were normal, as well dihydrorhodamine (DHR) assay on neutrophils for respiratory burst evaluation after phorbol myristate acetate (PMA) activation. Diagnosis of MSMD was suspected. He received multiple antibiotics and subcutaneous IFN-γ as treatment and he proved clinical improvement. He is currently 9 years-old and healthy.

P2 (Kindred A, II.2, Figure 7A) was born in 2012 and she is the sister of P1. She received BCG vaccination at 2 months of age and developed suppurative lymphadenitis related to the vaccine that drained spontaneously. She was treated with multiple antibiotics and subcutaneous IFN-γ. No other infectious diseases have been detected in this patient.

Laboratory investigations showed a normal immunophenotyping of T, B and NK cells according to the age; respiratory burst was evaluated by DHR assay, being normal on neutrophils after PMA activation. IgG, IgA and IgM serum levels were normal for the age of the patient. She is 6 years-old and she is well, without any treatment.

P3 (Kindred B, II.4, Figure 7A) was born in 2011 from Indian non consanguineous parents. He received BCG vaccine at 10 days of life. At 3 months he developed swelling of the left axillary lymph node which was drained. Histopathology showed reactive follicular hyperplasia with no granulomas, but AFB smear was positive. Treatment history at that point was not available. At 11 months P3 showed inguinal and axillary lymphadenitis and hepatosplenomegaly. Chest X-ray showed persistent right middle lobe patch. Gastric AFB was positive; mycobacterial culture was negative. Fine needle aspiration cytology from inguinal nodes revealed ill-defined granulomas with AFB. Mycobacterial culture from lymph node yielded EM, which was identified as *M. chelonei*. Started treatment with Rifampicin, Ethambutol, Clarithromycin and Aminoglycoside. IFN-γ could not be procured and parents were unaffordable for stem cell transplantation. He died at 5 years old of age with persistent disseminated EM disease. He had one elder brother who died of possible tuberculosis. His sister is clinically well.

Identification of novel *IFNGR2* mutations

WES was carried out on the patients' gDNA. We searched for rare variations (minor allele frequency, MAF < 0.01) in known MSMD-causing genes. In kindred A (P1 and P2), we found a homozygous nucleotide substitution at position c.1A>G of *IFNGR2* (Figure 7B), which leads to the replacement of the first methionine-encoding codon by a valine-encoding

codon. In kindred B (P3), we identified a homozygous nucleotide deletion, c.4delC (Figure 7B), which is predicted in silico to cause a premature stop codon 22 amino acids after the first methionine (M1). Both parents of the patients were heterozygous for the corresponding mutations (Figure 7B). We mined public databases (GnomAD, ExAC and BRAVO) and found that mutation c.1A>G was reported in heterozygosity in one European individual in GnomAD (out of 14,324 individuals, of which 7,150 individuals were European), whereas mutation c.4delC was not reported. Both the M1 residue (c.1ATG) and the arginine that follows (R2, c.4CGA) are highly conserved in the species for which *IFNGR2* was sequenced (Figure 8A). These mutations had a high combined annotation dependent depletion (CADD) score,[183] higher than the mutation significance cutoff (MSC) of *IFNGR2*[184] and similar to other IFN-γR2 reported patients (Figure 8B). All mutations were confirmed by Sanger sequencing. No DNA sample was available from the siblings of P3. These mutations thus probably underlie AR MSMD by IFN-γR2 deficiency, whether complete or partial.

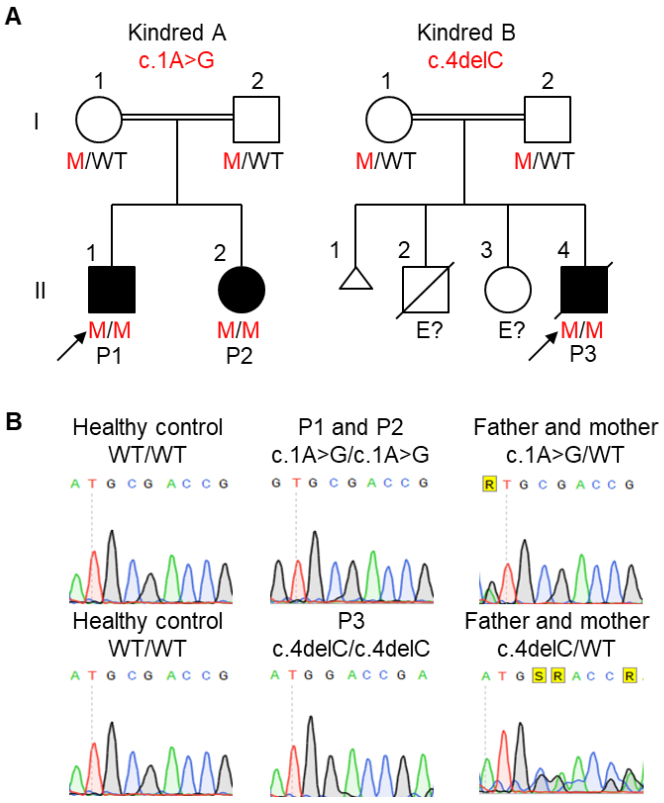

Figure 7 - Identification of 3 new patients with IFN-γR2 deficiency and MSMD. **(A)** Familial segregation of the c.1A>G and c.4delC mutations (mutations marked in red). Each kindred is designated by a capital letter (A-B), each generation by a roman numeral (I-II), and each individual by an Arabic number. Healthy individuals are shown in white. Solid black shapes indicate patients with MSMD. The probands are indicated by arrows. E? stands for not genotyped. **(B)** Electropherogram showing the ATG-GTG mutation in P1 and P2, and the CGA-GA mutation in P3. (Adapted from Oleaga-Quintas *et al*. Human Molecular Genetics; 2018; in press)[185]

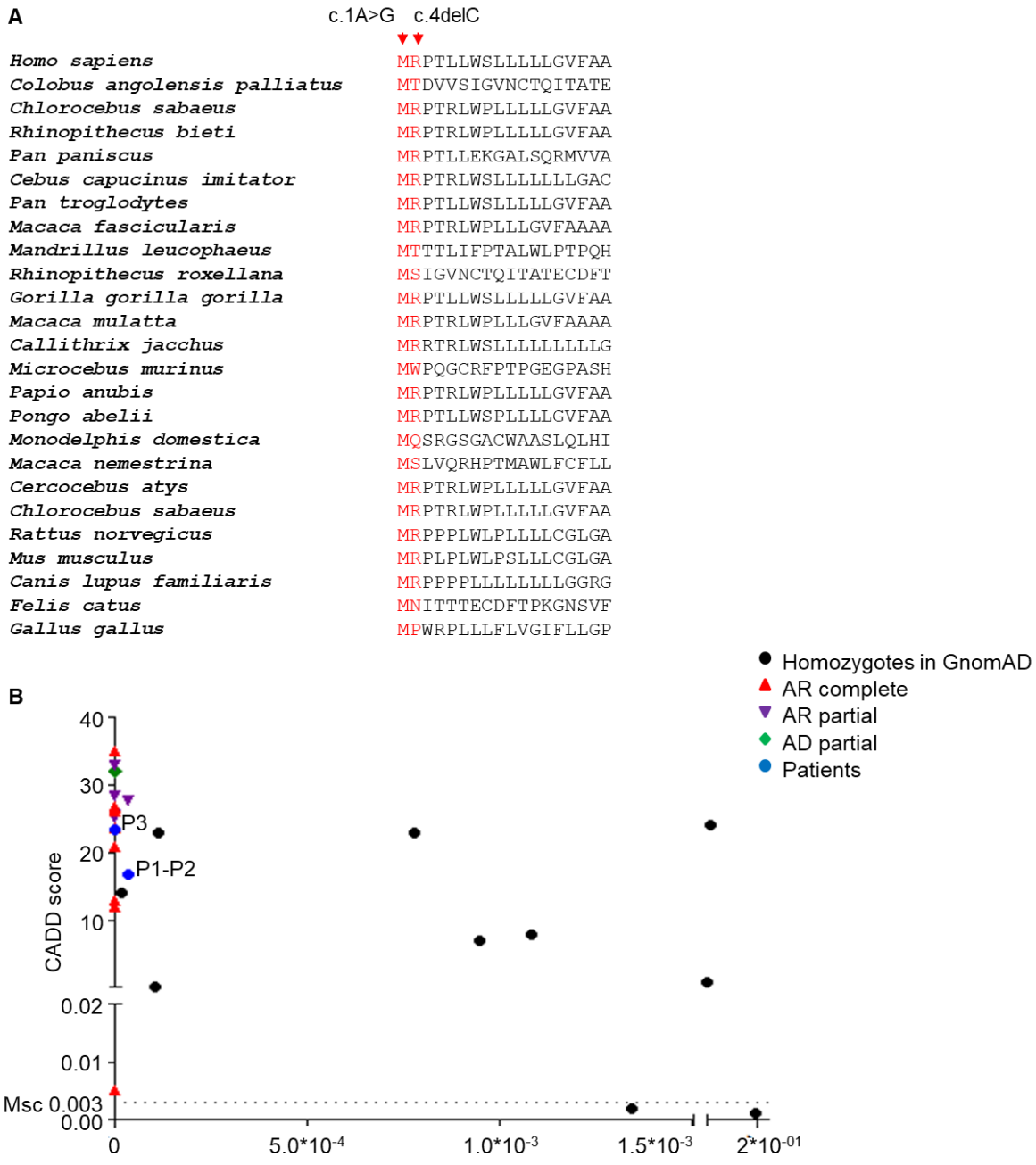

Figure 8 - Consequences of the aminoacid alteration. **(A)** First 18 aminoacids of IFN-γR2 in different species showing conservation of the first methionine and the second arginine, indicated by arrows and in red. **(B)** CADD score of the published *IFNGR2* mutations, the reported homozygous variations in public databases and of the mutations c.1A>G and c.4delC. (Adapted from Oleaga-Quintas *et al*. Human Molecular Genetics; 2018; in press)[185]

Expression evaluation of mutated IFN-γR2 proteins

We first characterized the c.1A>G and c.4delC alleles *in vitro* by overexpressing the corresponding cDNAs in HEK293-T cells or IFN-γR2-deficient SV40-fibroblasts[177] (homozygous for the c.278delAG mutation). We either left cells untransfected (NT) or transiently transfected cells with WT, c.1A>G, and c.4delC *IFNGR2* cDNAs, or with EV, and analyzed the corresponding products by Western blotting.[177] *IFNGR2* c.278delAG, which is a reported loss-of-expression and LOF mutation, was included as a negative control.[177] As expected, the WT-*IFNGR2* plasmid encoded a single full-length 70-kDa protein; the negative control and EV showed no protein expression (Figure 9). Interestingly, both mutants c.1A>G and c.4delC also showed a single full-length 70-kDa protein, albeit of lower amounts than WT (Figure 9A). IFN-γR2 belongs to the type I transmembrane proteins and its glycosylation is an important post-translational modification.[186] Glycosylation ensures correct folding of the protein and proper ligand binding to it, being also responsible for the range of molecular weight (MW) of this and other membrane proteins.[187] The lysates from transient transfections of WT, c.1A>G, c.4delC, and c.278delAG were treated with peptide N-glycosidase-F (PNGase-F) to remove N-glycans.[188] The same expression pattern was observed, with c.1A>G and c.4delC displaying products of equal MW (approximately 45kDa) but lower amounts than WT (Figure 9B). These findings suggested that although one mutation affects the first methionine and another creates an upstream frameshift, there seems to be proper translation of the encoded mRNAs, ensuring the expression of a seemingly full-length protein. These findings also confirmed that the two mutations were pathogenic and the patients had AR IFN-γR2 deficiency, whether complete or partial.

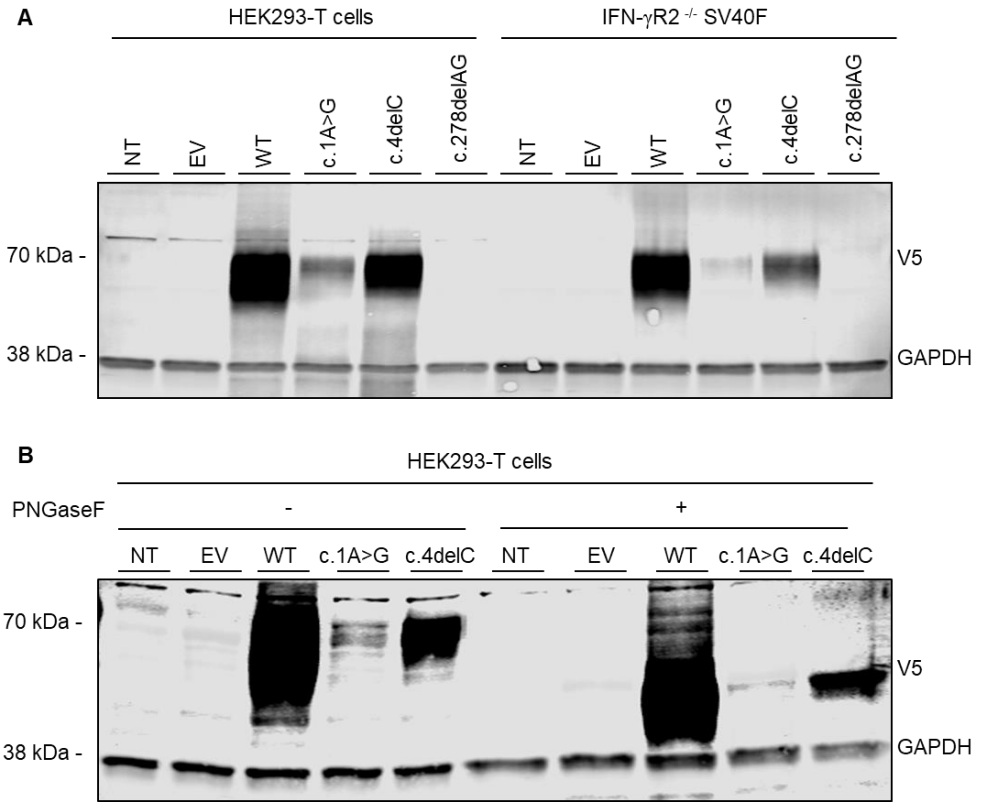

Figure 9 - IFN-γR2 protein expression. **(A)** HEK 293-T cells or IFN-γR2 deficient SV40-fibroblasts (IFN-γR2$^{-/-}$ SV40F) were left NT or were transiently transfected with EV, WT, c.1A>G, c.4delC and c.278delAG *IFNGR2* plasmids. Total lysis and western blot were performed with anti-V5 antibody and used GAPDH as a loading control. **(B)** We performed total lysis and left untreated (-) or treated (+) these lysates with an inhibitor of glycosylation, PNGaseF, revealing with anti-V5 antiboy and used GAPDH as a loading control. (Adapted from Oleaga-Quintas *et al*. Human Molecular Genetics; 2018; in press)[185]

Defective IFN-γ response in overexpressed *IFNGR2* mutants

We then assessed the functional consequences of c.1A>G and c.4delC mutations. We transiently transfected EV, WT, c.1A>G, c.4delC, and c.278delAG in IFN-γR2-deficient SV40-fibroblasts. We then either left the cells unstimulated (NS) or stimulated them with IFN-γ (10^5IU/ml) for 20 minutes. Nuclear proteins were extracted and GAF-DNA binding activity was assessed by EMSA with a GAS probe. As expected, cells transfected with the WT-*IFNGR2* plasmid displayed high binding activity after stimulation, whereas cells transfected with EV or the negative control did not show any activity (Figure 10A).

Interestingly, cells transfected with c.1A>G or c.4delC cDNA showed impaired, but not abolished, responses to IFN-γ (Figure 10A). These findings correlate with the protein expression levels, showing that both c.1A>G and c.4delC *IFNGR2* mutants encode IFN-γR2 proteins with reduced but not abolished expression and function. In order to compare the observed activity with previously reported forms of partial AR deficiency, we tested the five corresponding hypomorphic mutations (G227R, G141R, S124F, R114C and c.958insT)[166,189–191]. After stimulation, the mean level of binding followed a gradient: WT > G227R > c.1A>G > G141R> c.958insT> S124F> R114C> c.4delC> c.278delAG (Figure 10B), where overexpressed c.1A>G showed one of the strongest bindings and c.4delC showed the lowest binding, with G227R and c.1A>G being the least deleterious of all tested alleles in overexpression. We then transfected SV40-fibroblasts from P1 (cells from P3 were not available) with WT *IFNGR2* and their response to IFN-γ was evaluated by EMSA. GAF binding activity was restored in the transfected cells, when compared with EV-transfected and un-transfected cells (Figure 10C). These results strongly suggested that the c.1A>G mutation of *IFNGR2* is responsible for the poor response to IFN-γ observed in the overexpression system. Collectively, these data indicated that the patients from the two kindreds had a severe form of AR partial IFN-γR2 deficiency.

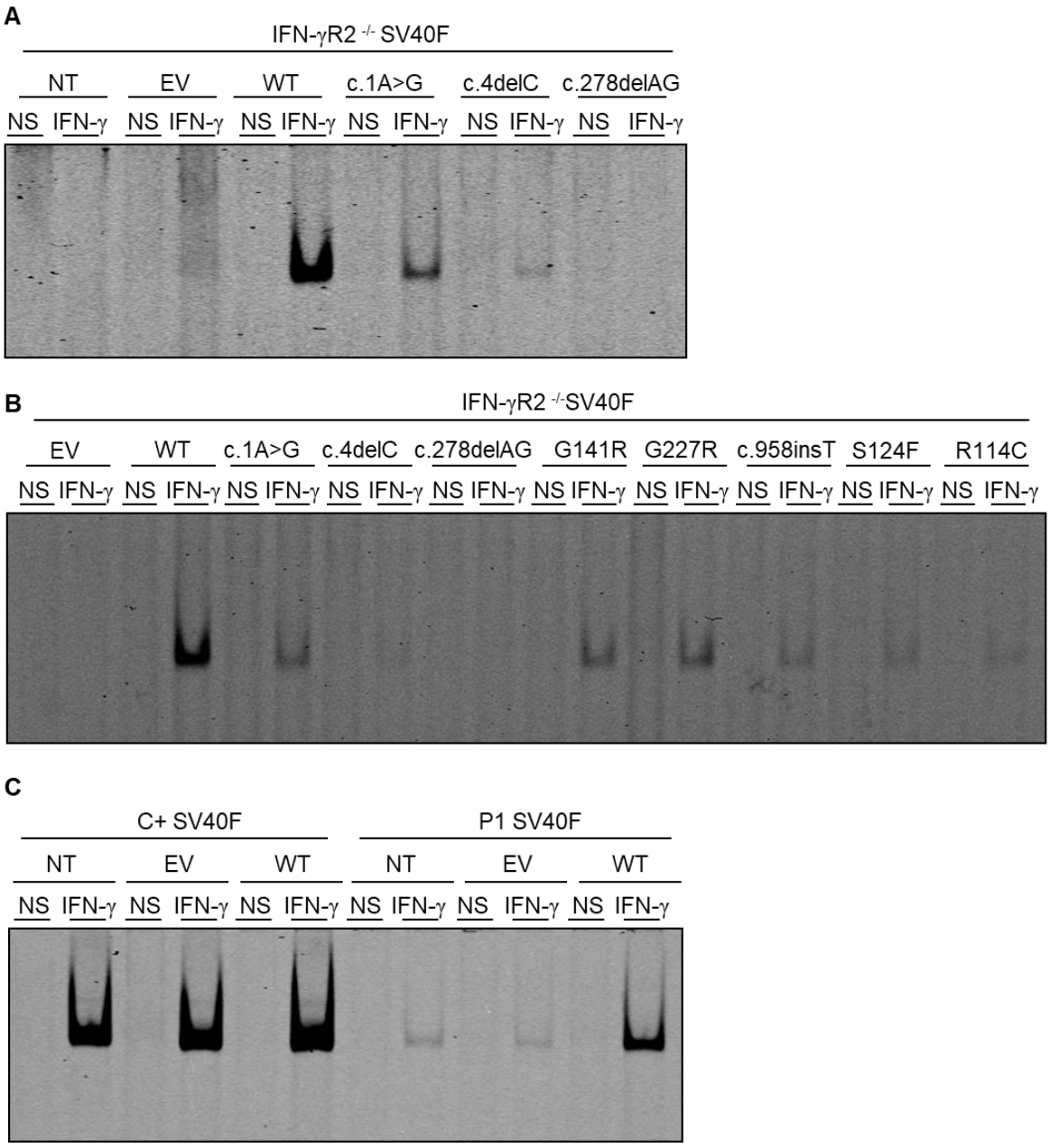

Figure 10 - Impaired STAT1-DNA-binding activity in response to IFN-γ stimulation *in vitro* and complementation of the IFN-γ response with WT *IFNGR2*. **(A)** IFN-γR2$^{-/-}$ SV40-fibroblats were left NT or were transiently transfected with EV, WT, c.1A>G, c.4delC and c.278delAG *IFNGR2* plasmids. **(B)** IFN-γR2$^{-/-}$ SV40-fibroblasts (SV40F) were transiently transfected with EV, WT, c.1A>G, c.4delC, c.278delAG, G141R, G227R, 958insT, S124F and R114C (these last five are reported AR partial *IFNGR2* mutations) *IFNGR2* plasmids. **(C)** SV40-fibroblasts from a healthy control and P1 were left NT or were transiently transfected with EV and WT *IFNGR2* plasmid. **(A)**, **(B)** and **(C)** were either left NS or stimulated with 10^5 IU/ml of IFN-γ (IFN-γ). DNA-binding activity was then analyzed by EMSA with a GAS probe in LI-COR, A700. (Adapted from Oleaga-Quintas *et al*. Human Molecular Genetics; 2018; in press)[185]

STAT1 phosphorylation upon IFN-γ stimulation in SV40 fibroblasts

Interestingly, a mutation of the first ATG of *IFNGR1* has previously been described in a patient with MSMD, where the first methionine is replaced by a lysine (p.M1K).[192] In homozygous EBV-B cells for this *IFNGR1* mutation, the response to IFN-γ is impaired but not abolished, whereas it is completely abolished in the corresponding SV40-fibroblasts. We set out to compare the early response to IFN-γ and IFN-α (as a control of stimulation) of SV40-fibroblasts from a healthy control, P1, P2 (cells from P3 being not available), a patient with AR complete IFN-γR2 deficiency (homozygous for the c.278delAG mutation, hereafter referred to as negative control), a patient with AR complete IFN-γR1 deficiency (compound heterozygous for the c.104_107dupTTAC and c.200+1G>A mutations), three patients with AR partial IFN-γR2 deficiency[166,189] (corresponding to S124F, G141R and R114C), and the patient with AR partial IFN-γR1 deficiency due to the p.M1K mutation (hereafter referred to as M1K). We assessed the ability of these cells to phosphorylate tyrosine 701 of STAT1 (p-Y701-STAT1) after IFNs stimulation by western blotting. The healthy control showed p-STAT1 after stimulation with IFN-γ, whereas the negative control, the AR complete IFN-γR1 deficient cells and the M1K-IFN-γR1 deficient patient did not show any response (Figure 11A). Cells from the three patients with AR partial IFN-γR2 deficiency had impaired, but not abolished, response to IFN-γ. Both P1 and P2 showed an equally and profoundly diminished response (Figure 11B), not abolished but lower than that of the three other patients with AR partial deficiency. The response was however detectable, unlike in cells from the patient mutated in the initiation codon of *IFNGR1*. The phosphorylation after IFN-α was found to be comparable in all cell lines (Figure 11). The levels of GAPDH were also similar across cell lines, attesting that the differences of STAT1 phosphorylation were not

caused by differences in protein loading. The IFN-γ signaling pathway is shown to be impaired but not abolished in the SV40-fibroblasts of P1 and P2, unlike in the M1K-IFN-γR1 SV40-fibroblasts, which show a complete abolition of IFN-γ signaling in terms of phosphorylation of STAT1.

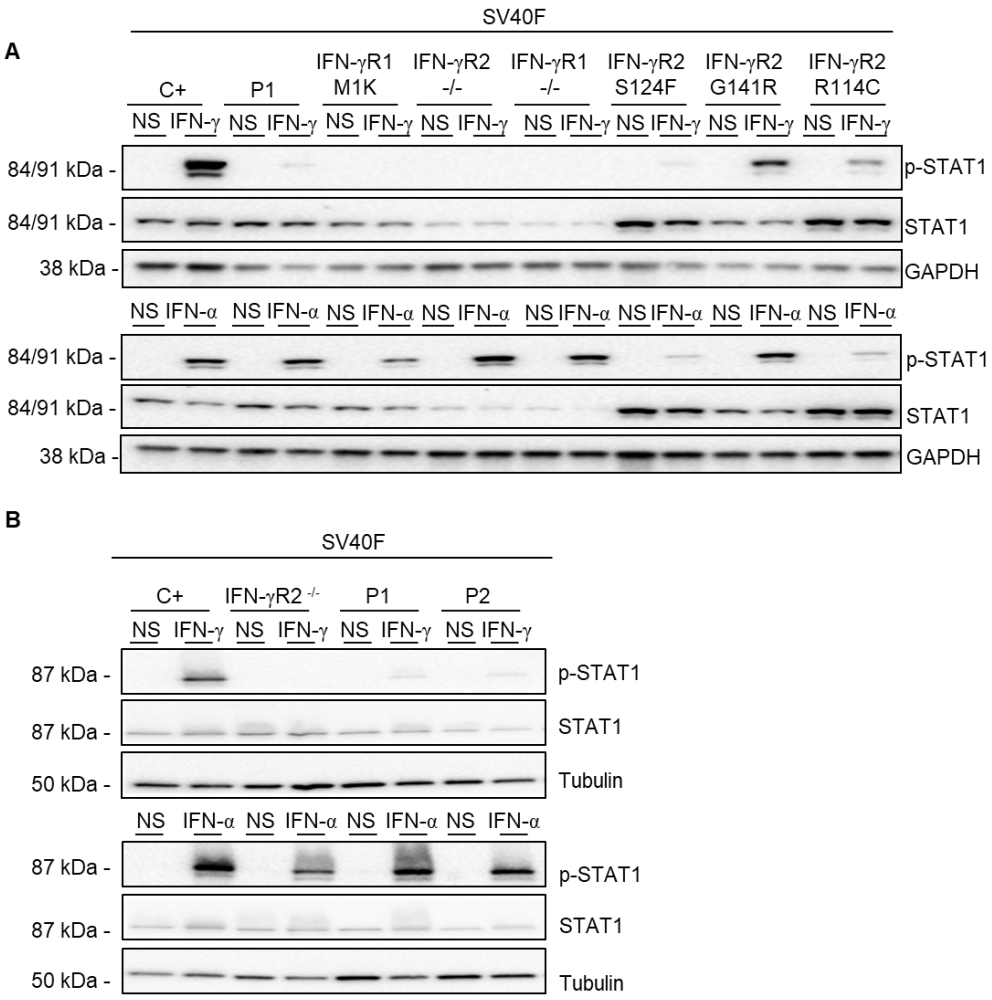

Figure 11 - Impaired phosphorylation of STAT1 in P1's and P2's SV40-fibroblasts after IFN-γ stimulation. **(A)** SV40-fibroblasts from a healthy control (C+), an IFN-γR2 deficient patient (IFN-γR2 $^{-/-}$), an IFN-γR1 deficient patient (IFN-γR1 $^{-/-}$), P1, an IFN-γR1 patient mutated on the first methionine (IFN-γR1-M1K) and three recessive partial IFN-γR2 patients (S124F, G141R and R114C). **(B)** SV40-fibroblasts from a healthy control (C+), an IFN-γR2 deficient patient (IFN-γR2$^{-/-}$), P1 and P2. **(A)** and **(B)** cells were left NS or were stimulated with 10^5 IU/ml of IFN-γ (IFN-γ) or IFN-α (IFN-α) for 20 minutes. Phosphorylation of STAT1 in Tyr-701(p-STAT1), total levels of STAT1 and levels of GAPDH (as loading control) were measured by western blot using specific antibodies. Upper panel: IFN-γ stimulation, lower panel: IFN-α stimulation. (Adapted from Oleaga-Quintas *et al*. Human Molecular Genetics; 2018; in press)[185]

DNA binding and late response to IFN-γ stimulation in SV40 fibroblasts

We next studied the ability of IFN-γ to stimulate the formation of GAS-binding nuclear complexes by EMSA. We performed the same activation as for the western blot in SV40-fibroblasts. The healthy control showed binding activity after stimulation with IFN-γ, whereas the negative control and the M1K patient did not show any binding (Figure 12A). Interestingly, P1 and P2 showed an equally and profoundly diminished, but detectable, binding activity after IFN-γ stimulation. The binding after IFN-α was found to be similar in all cell lines (Figure 12A), confirming the results seen by western blot. We then analyzed later events in the IFN-γ pathway, and measured the induction of HLA-DR after stimulation of two concentrations of IFN-γ stimulation (10 or 10^3 UI/ml) for 48 hours. The healthy control's cells showed an increase of HLA-DR with the low concentration of IFN-γ, and a higher increase with the higher dose, whereas the negative control and M1K-IFN-γR1 cells showed no expression for either concentration (Figure 12B). S124F and R114C cells did not induce HLA-DR for both concentrations of IFN-γ (Figure 12B). In G141R cells, low concentrations of IFN-γ did not induce HLA-DR expression, whereas higher concentrations did, albeit to a lower extent than in control cells (Figure 12B). The results obtained for these patients with AR partial IFN-γR2 deficiency are consistent with previous reports[166]. P1 and P2 cells displayed a slight increase in HLA-DR expression in the presence of low concentrations of IFN-γ, higher than the other three AR partial IFN-γR2 patients tested, and a stronger increase in response to higher concentrations, much lower than that of the healthy control but similar to the G141R patient and higher than the S124F and R114C patients (Figure 12B). Thus, both early and late events in the IFN-γ signaling pathway are impaired

but not abolished in the SV40-fibroblasts of P1 and P2, unlike in the M1K-IFN-γR1 SV40-fibroblasts, which show a complete abolition of IFN-γ signaling.

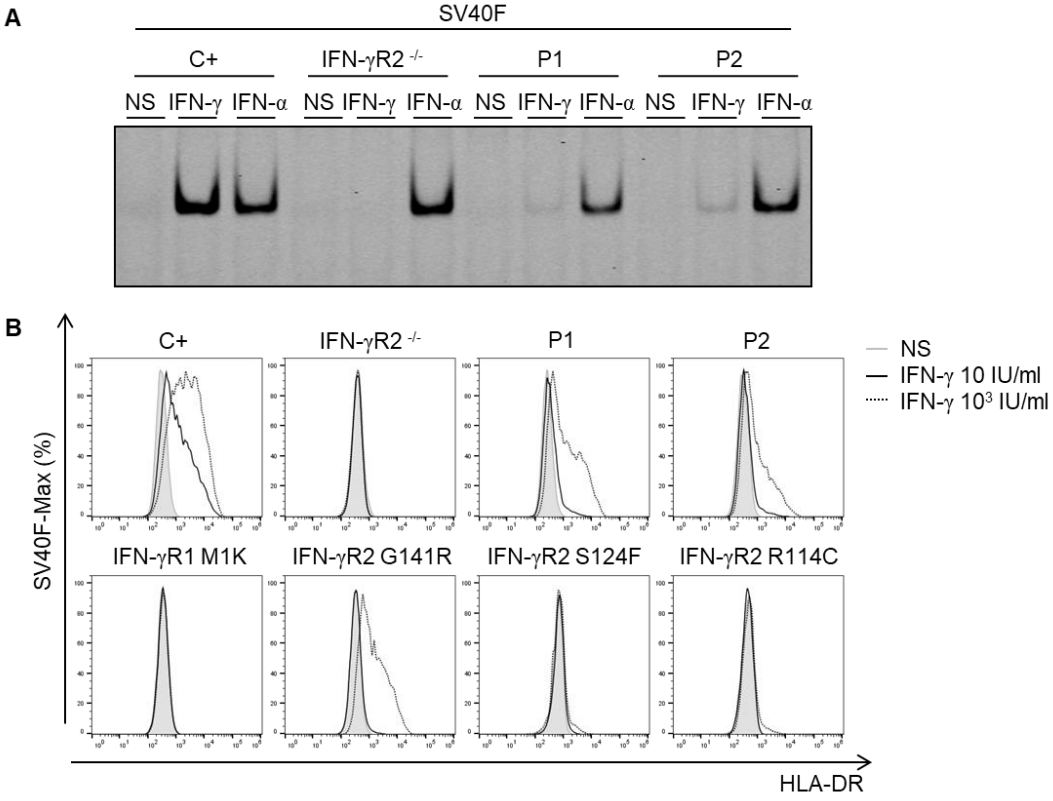

Figure 12 - Impaired response of P1's SV40-fibroblasts to IFN-γ stimulation. **(A)** SV40-fibroblasts from a healthy control (C+), an IFN-γR2 deficient patient (IFN-γR2 $^{-/-}$), P1 and P2 were left NS or were stimulated with 10^5 IU/ml of IFN-γ (IFN-γ) or IFN-α (IFN-α) for 20 minutes and GAS probe-binding nuclear proteins were measured by fluorescent EMSA with LI-COR, A700. **(B)** SV40-fibroblasts from a healthy control (C+), an IFN-γR2 deficient patient (IFN-γR2 $^{-/-}$), P1, P2 the IFN-γR1 patient mutated on the first methionine (IFN-γR1-M1K) and three recessive partial IFN-γR2 patients (S124F, G141R and R114C) were stimulated with the indicated doses of IFN-γ for 48 hours. HLA-DR induction was determined by flow cytometry. Overlapping histograms are represented relative to mode. Gray area: no stimulation (NS), continuous black line: 10 IU/ml IFN-γ, discontinuous black line: 10^3 IU/ml IFN-γ. The results shown are representative of 3 independent experiments. (Adapted from Oleaga-Quintas *et al*. Human Molecular Genetics; 2018; in press)[185]

STAT1 phosphorylation upon IFN-γ stimulation in EBV-B cells

We then characterized EBV-B cells from P1 (P2 and P3 EBV-B cells were not available). We analyzed the early response to IFN-γ and IFN-α of a healthy control, P1, the AR complete IFN-γR2 deficient patient (homozygous for c.278delAG), an AR complete

IFN-γR1 deficient patient (homozygous for the c.202-1G>T mutation), S124F, G141R, R114C, cells from a patient with AD partial IFN-γR2 deficiency[167] (heterozygous for the c.186delC mutation), and M1K-IFN-γR1 deficiency. We first assessed the ability of these cells to phosphorylate STAT1 after IFNs stimulation, as measured by western blotting. The healthy control showed p-STAT1 after stimulation with IFN-γ, whereas the negative control, the AR complete IFN-γR1 and the M1K patient did not show any response (Figure 13). P1 showed a severely impaired, but not abolished, response to IFN-γ (Figure 13). The three patients with IFN-γR2 partial deficiency previously reported, and the AD c.186delC patient, had an impaired, but not abolished, response to IFN-γ (Figure 13). The phosphorylation of STAT1 in response to IFN-α was found to be comparable in all cell lines (Figure 13). The levels of GAPDH were also similar across cell lines, attesting that the differences of STAT1 phosphorylation were not caused by differences in protein loading. The IFN-γ signaling pathway is severely impaired in terms of phosphorylation of STAT1 by western blot in the EBV-B cells of P1.

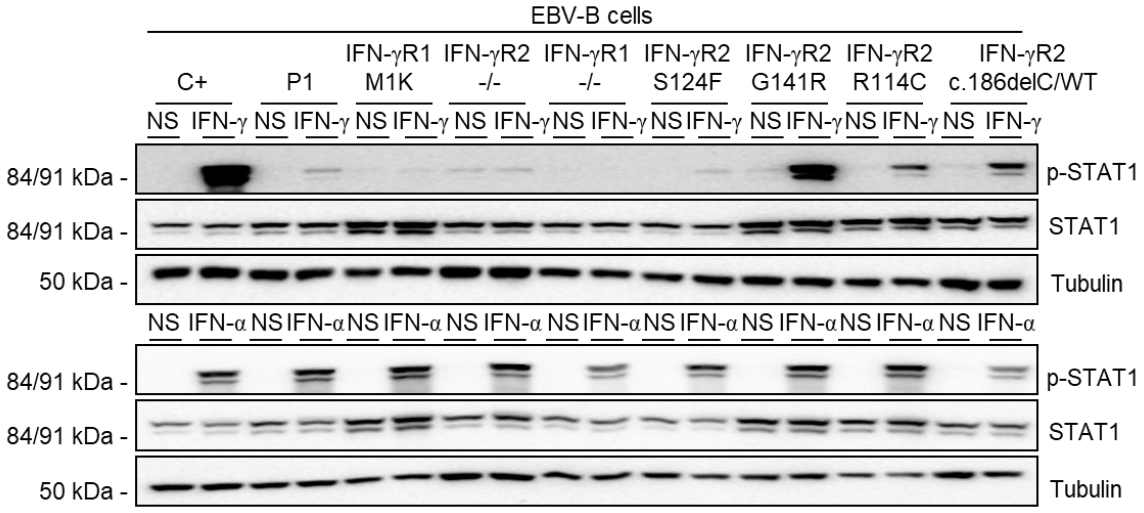

Figure 13 – Impaired phosphorylation of STAT1 in P1's EBV-B cells after IFN-γ stimulation. EBV-B cells from a healthy control (C+), an IFN-γR2 deficient patient (IFN-γR2 $^{-/-}$), an IFN-γR1 deficient patient (IFN-γR1 $^{-/-}$), P1, an IFN-γR1 patient mutated on the first methionine (IFN-γR1-M1K), three recessive partial IFN-γR2

patients (S124F, G141R and R114C) and a dominant partial IFN-γR2 patient (c.186delC) were left NS or were stimulated with 10^5 IU/ml of IFN-γ (IFN-γ) or IFN-α (IFN-α) for 20 minutes. Phosphorylation of STAT1 in Tyr-701 (p-STAT1), total levels of STAT1 and levels of GAPDH (as loading control) were measured by western blot using specific antibodies. Upper panel: IFN-γ stimulation, lower panel: IFN-α stimulation. (Adapted from Oleaga-Quintas *et al*. Human Molecular Genetics; 2018; in press)[185]

Abolished DNA-binding activity upon IFN-γ stimulation in P1's EBV-Bs

We investigated the ability of STAT1 homodimers to bind to the GAS probe by EMSA in EBV-B cells. We performed the same activation as for the western blot in cells from a healthy control, the negative control, the AR complete IFN-γR1 patient, P1, IFN-γR2 (S124F, G141R, R114C, c.186delC), and IFN-γR1 (M1K) deficient cells. As expected, the healthy control showed binding activity after stimulation with IFN-γ, unlike the negative control and the AR complete IFN-γR1 patient (Figure 14A). Consistent with what has been published, G141R, S124F, R114C, and c.186delC showed impaired but not abolished binding to GAS after IFN-γ stimulation (Figure 14A). As for P1 and the M1K IFN-γR1patient, no GAS binding was detected (Figure 14B). The binding after IFN-α was found to be more or less similar in all cell lines (Figure 14). This result supports the hypothesis that *IFNGR2* c.1A>G mimics *IFNGR1* M1K in EBV-B cells. However, the defect in P1's EBV-B cells is more pronounced than that in P1's SV40-fibroblasts, whereas it is the opposite for the M1K IFN-γR1 patient. The impact of the mutations of the first methionine of the two chains of the IFN-γ receptor seems to depend on the mutated chain and the cell type tested.

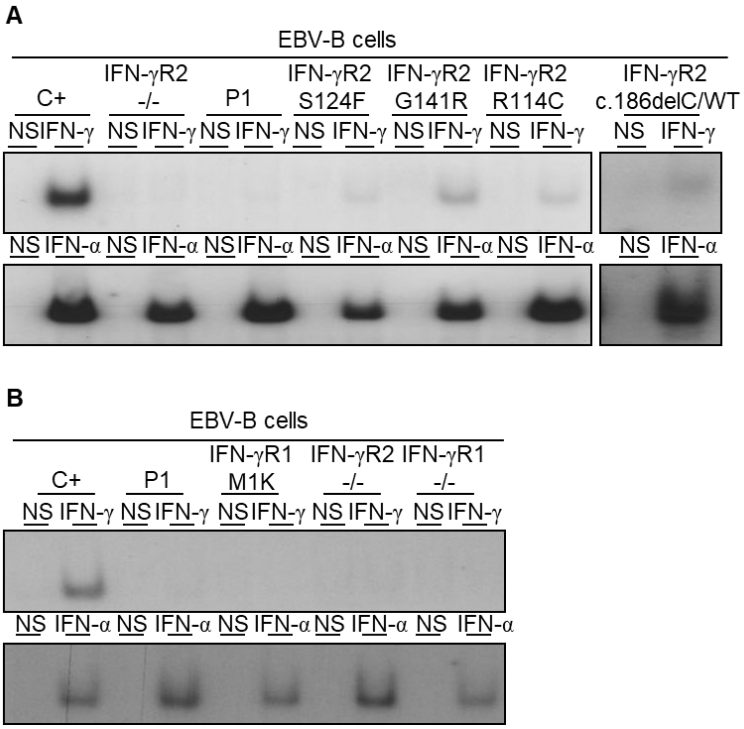

Figure 14 - Impaired GAS binding of P1's EBV-B cells after IFN-γ stimulation **(A)** EBV-B cells from a healthy control, a negative control, P1, three recessive partial patients and an autosomal dominant patient. **(B)** EBV-B cells from a healthy control, P1, the IFN-γR1 M1K patient, a negative control for IFN-γR2 (IFN-γR2 $^{-/-}$) and a negative control for IFN-γR1 (IFN-γR1 $^{-/-}$). **(A)** and **(B)** cells were left non stimulated (NS) or were stimulated with 10^5 IU/ml of IFN-γ (IFN-γ) or IFN-α (IFN-α) for 20 minutes and GAS probe-binding nuclear proteins were measured by radioactive EMSA, p32 dATP. Upper panel: IFN-γ stimulation, lower panel: IFN-α stimulation. (Adapted from Oleaga-Quintas *et al*. Human Molecular Genetics; 2018; in press)[185]

Impairment of IFN-γ signaling in fresh leukocyte subset

IFN-γR2-deficient patients show high levels of IFN-γ in their plasma and low or abolished production of IL-12p40 and IL-12p70 in their whole blood after BCG and IFN-γ stimulation.[35] We measured IFN-γ levels in the plasma of P1, P2, and P3 by ELISA, and compared them with healthy controls and patients with complete or partial IFN-γR2 deficiency. The healthy controls and the patient with AD partial IFN-γR2 deficiency showed no detectable IFN-γ in their plasma, whereas P1, P2, and P3 showed high IFN-γ levels in

their plasma, which were similar to the levels observed in patients with AR partial IFN-γR2 deficiency, whether complete or partial (Figure 15).

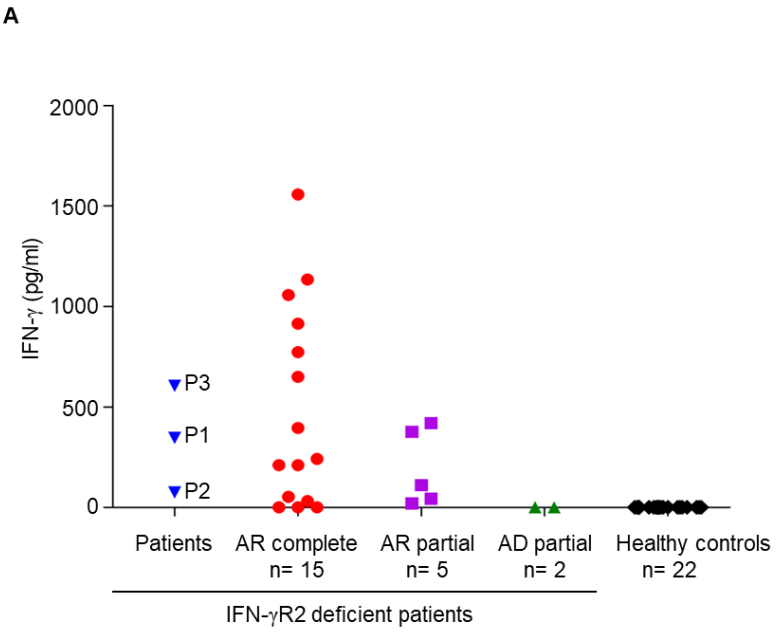

Figure 15 - IFN-γ levels in plasma from P1, P2, P3, AR complete IFN-γR2 deficient patients (n=16), AR partial IFN-γR2 deficient patients (n=5), AD partial patient (n=2) and healthy controls (n=22). (Adapted from Oleaga-Quintas *et al*. Human Molecular Genetics; 2018; in press)[185]

We also assessed the IFN-γ, IL-12p40 and IL-12p70 production of whole blood from healthy controls, P1, and P2 (P3 was not available) after 48 hours stimulation with live BCG, with or without IFN-γ or IL-12, as previously described,[169] as compared with patients with complete or partial IFN-γR2 deficiency. Healthy controls, IFN-γR2-deficient patients, P1, and P2 showed similar secretion of IFN-γ after BCG stimulation and a higher production after BCG and IL-12 (data not shown). All individuals tested showed production of IL-12p40 after BCG stimulation, but no IL-12p70 production (Figure 16B). When stimulating with both BCG and IFN-γ, the healthy controls showed a higher increase in IL-12p40 production compared with BCG alone (Figure 16A), as well as an induction of IL-12p70 production

(Figure 16B). In contrast, patients with AR complete IFN-γR2 deficiency did not show any response, in terms of the production of IL-12p40 in response to BCG plus IFN-γ, compared with BCG alone, and that of IL-12p70 in response to either stimulation (Figure 16). Patients with AR partial IFN-γR2 deficiency showed an increase in IL-12p40 levels, upon stimulation with BCG plus IFN-γ, yet lower than controls (Figure 16A). Patients with an AR partial defect showed no IL-12p70 production, whereas those with an AD partial defect showed a normal production of IL-12p70 (Figure 16B). P1 and P2 also showed an increase of IL-12p40 in response to BCG plus IFN-γ, and no IL-12p70 production, like known patients with an AR partial IFN-γR2 deficiency (Figure 16). These results showed that the IFN-γR2 deficiency in P1 and P2 is more severe than that in previously reported patients with AR IFN-γR2 partial deficiency but less so than in patients with AR complete IFN-γR2 deficiency.

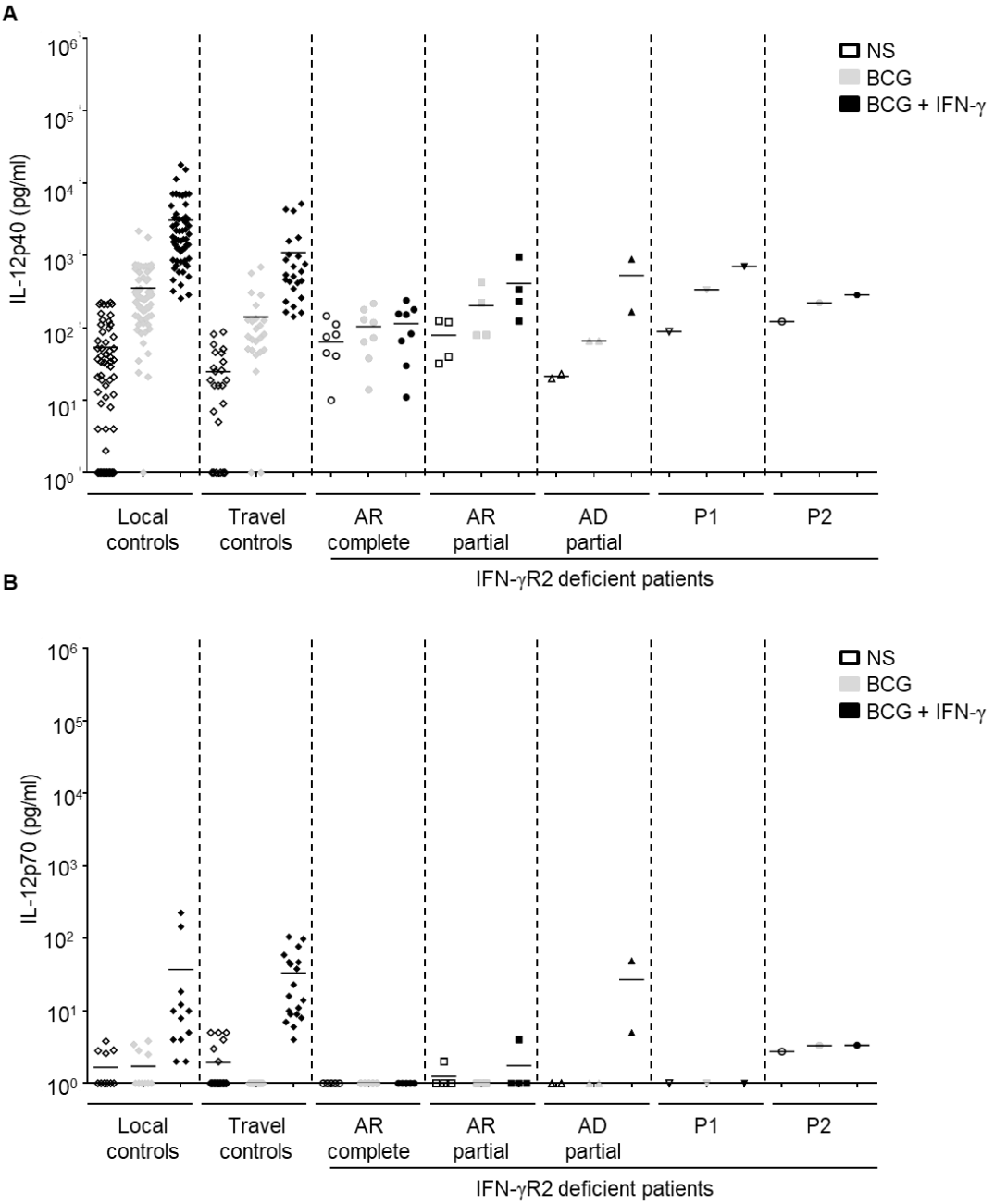

Figure 16 - Impaired response to IFN-γ in terms of the production of IL-12 in the whole- blood supernatant from the patients. **(A)** Production of IL-12p40 and **(B)** IL-12p70 in whole blood cells from local controls, travel controls, AR complete IFN-γR2 deficient patients, AR partial IFN-γR2 deficient patients, AD partial patient, P1 and P2 either NS or stimulated for 48 hours with live BCG alone or live BCG plus IFN-γ, as assessed by ELISA. Each symbol represents a value from an independent test and horizontal bars represent means. (Adapted from Oleaga-Quintas *et al*. Human Molecular Genetics; 2018; in press)[185]

Study of individual leukocyte subsets

We then investigated the consequences of the c.1A>G mutation in fresh leukocyte subsets from two healthy controls, P1, P2, and an AR complete IFN-γR2 deficient patient (c.278delAG mutation). It has been previously reported that p-STAT1 levels in AD partial IFN-γR2 patient's naive $CD4^+$ T cells and memory interleukin (IL)-4 producing T cells responded poorly to IFN-γ, whereas MDMs did not.[167] We tested naïve $CD4^+$ T cells and MDMs generated by stimulation of monocytes *in vitro* with M-CSF plus IL-4 (monocytes from the AR complete IFN-γR2-deficient patient were not available). The different cell subsets were stimulated with IFN-γ and IFN-α, as previously done. In naïve $CD4^+$ T cells a slight phosphorylation of STAT1 was observed in P1 and P2 after IFN-γ stimulation, whereas in the healthy controls the levels of p-STAT1 were high and in the IFN-γR2-deficient patient phosphorylation was not detected (Figure 17A). MDMs from P1 and P2 showed a slight phosphorylation after IFN-γ stimulation, much lower than the healthy controls but not abolished (Figure 17B). The levels of p-STAT1 after IFN-α stimulation were comparable between all cell types (T lymphocytes and MDMs) of the patients and the controls (Figure 17). Total STAT1 levels were similar among all points and GAPDH was used as a loading control (Figure 17). These results correlate with published data[167], and the c.1A>G mutation seems to affect more strongly the cell types that normally express low levels of IFN-γR2 at their surface. They also support the hypothesis that the c.1A>G mutation confers partial activity of IFN-γR2, lower than that of the known hypomorphic mutations underlying AR partial IFN-γR2 deficiency.

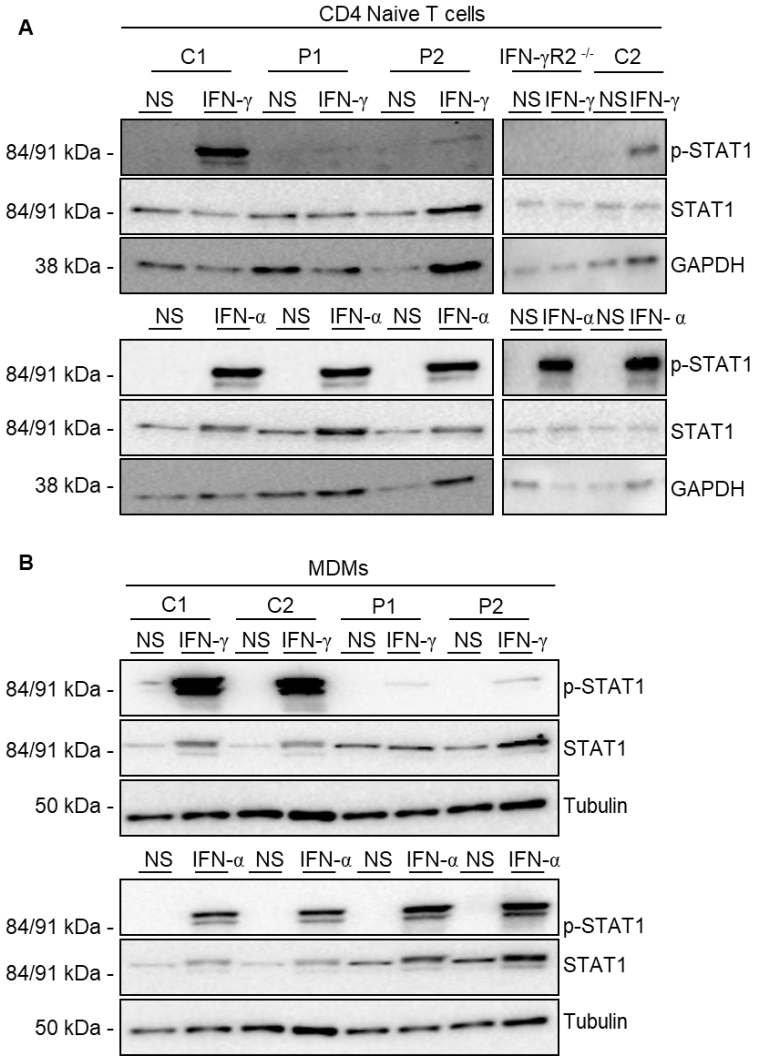

Figure 17 - Functional study of T cells and MDMs. **(A)** $CD4^+$ naïve T cells from two healthy controls (C1 and C2), an IFN-γR2 deficient patient (IFN-$\gamma R2^{-/-}$), P1 and P2. **(B)** $CD14^+$ cells from a healthy control (C1), P1 and P2 were isolated and differentiated to MDMs. **(A)** and **(B)** cells were isolated and left NS or were stimulated with 10^5 IU/ml of IFN-γ (IFN-γ) or IFN-α (IFN-α) for 20 minutes. Phosphorylation of STAT1 in Tyr-701(p-STAT1), total levels of STAT1 and levels of GAPDH or tubulin (as loading control) were measured by western blot using specific antibodies. Upper panel: IFN-γ stimulation, lower panel: IFN-α stimulation. (Adapted from Oleaga-Quintas *et al*. Human Molecular Genetics; 2018; in press)[185]

Lack of re-initiation of translation at distal AUG

Several studies have shown that the most common start of translation in mammalian mRNA is at an AUG codon in a favorable context for translation initiation, which is -3 A or

G and + 4 G, collectively defining a Kozak consensus sequence.[193–195] These two mutations, c.1A>G and c.4delC, underlie a novel form of AR partial IFN-γR2 deficiency, where the expressed receptor is poorly abundant but normal. It is a quantitative, as opposed to the qualitative defect previously reported for mutations p.G227R, p.G141R, p.R114C, p.S124F and c.958insT. We thus attempted to identify the amino-acid responsible for the initiation of the residual IFN-γR2 expression. We searched in the *IFNGR2* sequence an AUG codon downstream of c.1ATG that could match these characteristics through the Prediction of Translation Initiation ATG Web site (http://atgpr.dbcls.jp).[196] The next AUG predicted to re-initiate translation (and the next AUG in the sequence) is located in position c.238, aminoacid position 80. Usage of this initiation codon would lose the leader sequence and part of the extracellular domain. We, nevertheless, constructed several plasmids designed as "Start" [c.1delATG (Start2), c.1delATGC (Start2 + c.4delC), c.238AT>GC (M80A), c.1_237del (Start80) and Start2 + M80A] to define the start of translation after position c.4, and test if there would be IFN-γR2 protein expression by deleting the first 237 base pairs (first 79 aminoacids) or changing the methionine in this position by an alanine (Figure 18A). All the mutants except Start80 resulted in a product of normal MW but weaker intensity than WT *IFNGR2*, although it was stronger than that of c.1A>G (Figure 18B). In the case of Start80, no product was detected, similar to what is observed with c.278delAG point serving as a negative control (Figure 18B). As expected, these data ruled out a re-initiation of translation by the Kozak codon c.238.

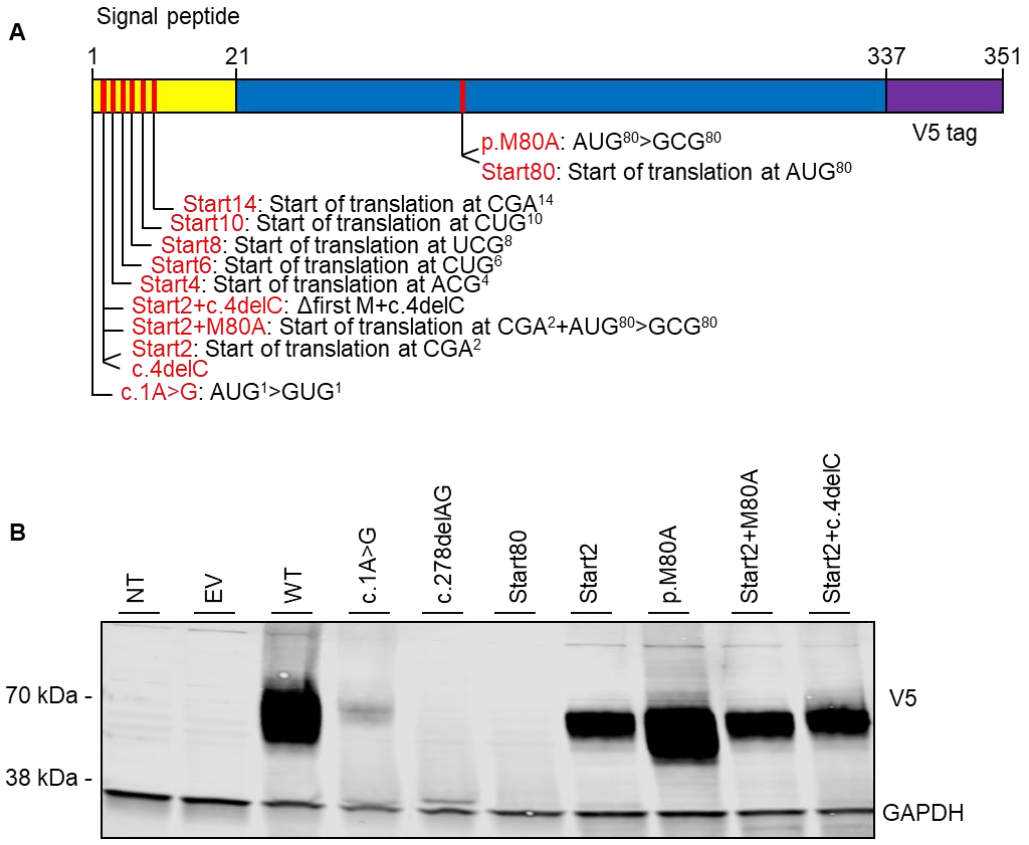

Figure 18 - Study of potential initiation codons. **(A)** Schematic representation of the *IFNGR2* gene cloned in a pcDNA3.1-V5 plasmid. The positions of various translation start codon candidates in the signal peptide and mutations are indicated. **(B)** HEK 293-T cells were either left NT or were transiently transfected with EV, WT *IFNGR2*, c.1A>G, c.4delC, c.278delAG, Start80, Start2, M80A, Start2 combined with M80A or Start2 combined with c.4delC. After 48 hours we performed total lysis and revealed by western blot with anti-V5 antibody and used GAPDH as a loading control. (Adapted from Oleaga-Quintas *et al*. Human Molecular Genetics; 2018; in press)[185]

Re-initiation of translation at a proximal non-canonical codon

Instead, these data suggested that the two mutants c.1A>G and c.4delC were hypomorphic due to initiation of translation at a non-AUG codon, most likely within the leader sequence (signal peptide) of IFN-γR2, and even possibly, in the case of c.1A>G, by the first codon itself (GUG).[197] Indeed, translation can be carried out through non-AUG codons such as CUG, GUG and ACG.[198–200]Analyzing the *IFNGR2* sequence, we found that

there was one ACG codon at position c.10, two consecutive CTG codons starting at positions c.13 and five consecutive CTG codons starting at position c.25. Using a signal peptide predictor (http://www.predisi.de), PrediSi[201], we deleted *in silico* aminoacids of IFN-γR2 from M1 until no signal peptide was predicted, which happened at L10 (c.28CTG). We then created plasmids starting at different non-AUG codons until the predicted loss of the signal peptide (Start 2, 4, 6, 8, 10 and 14) (Figure 18A). IFN-γR2 was expressed at the expected MW (70 kDa) for the mutations encompassing the start at aminoacids 2, 4, 6 and 8 at lower levels than the WT (Figure 19). The Start2 mutation produced similar proteins levels to the c.4delC mutation, whereas Start4 and Start6 produced similar protein levels to the c.1A>G mutation, with Start8 being lower than both of the mutations reported in this study (Figure 19). Both Start10 and Start14 did not show any detectable protein expression, correlating with the predictions made *in silico* (Figure 19). This result suggested that there are several codons involved in the translation of *IFNGR2*, all within the signal sequence, and that c.1ATG codon is the strongest site for the start of translation. This hypothesis was confirmed by immunoprecipitation and protein sequencing of the overexpressed IFN-γR2 WT, c.1A>G and c.4delC. A full-length protein from position 22 (position after the leader sequence is cut) to 337 (last aminoacid of IFN-γR2) was detected for the three lysates tested. Altogether, these results show that the two novel mutations herein described encode, at lower levels than the WT, a full-length mature IFN-γR2 due to initiation of translation by non-AUG codons located between codons two and nine within the 21-long signal peptide.

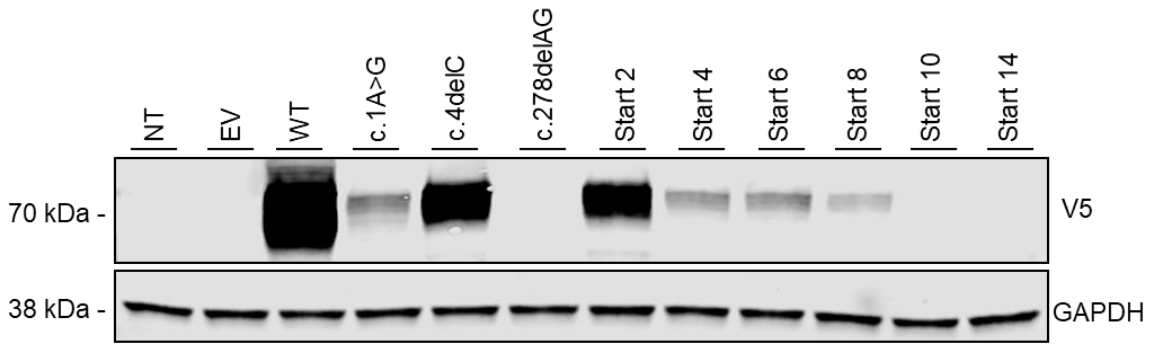

Figure 19 – Identification of reinitiation of translation points. HEK 293-T cells were either left NT or were transiently transfected with EV, WT, c.1A>G, c.4delC, c.278delAG, Start2, Start4, Start6, Start8, Start10 and Start14 *IFNGR2* plasmids. After 48 hours total lysis was performed and revealed by western blot with anti-V5 antibody and used GAPDH as a loading control. (Adapted from Oleaga-Quintas *et al*. Human Molecular Genetics; 2018; in press)[185]

Chapter II – GATA2 deficiency

Demographics and diagnosis of GATA2 deficiency in 14 unrelated kindreds

We studied 14 index cases and three symptomatic relatives from 13 non-consanguineous families and one consanguineous family with mycobacterial diseases and hematological anomalies, adding up to a total of 17 patients (Figure 20) who were referred to the laboratory of Human Genetics of Infectious Diseases between 2011 and 2018. Patients are from Brazil (kindred A and B), Colombia (C), France (D and E), Germany (F), Mexico (G), Portugal (H), Spain (I, J and K), Tunisia (L), Turkey (M), and USA (N). Kindred D and E were reported in a previous study.[126] Using Sanger sequencing, we determined the sequences of seven coding exons and flanking intronic regions of *GATA2* in 10 of the 14 patients (P2-P5, P9, P11-P13, P15 and P17). WES was performed in 4 of the 14 patients (P1, P7 P8 and P16). We identified five missense mutations [c.1163T>C (p.M388T) in kindred A and J, c.1193G>A (p.R398Q) in kindred D, c. 1192C>T (p.R398W) in kindred B and F, c.1187G>A (p.R396Q) in kindred G and c.1186C>T (p.R396W) in kindred M], one nonsense mutation [c.988C>T (p.R330*) in kindred I], one insertion [c.1035_1036insTCTGGCC (predicted p.G346Sfs*40) in kindred K], two duplications [c.599dup (predicted p.S201*) in kindreds H and L and c.1099dup (predicted p.D367Gfs*17) in kindred N] and two deletions [c.915_916del (predicted p.W306Afs*77) in kindred C and c.1023del (predicted p.A342Pfs*45) in kindred E], all in heterozygosity (Figure 20). Ten mutations have previously been reported in other patients,[119,121,123,126,127,202–204] whereas one is novel (c.1035_1036insTCTGGCC). This novel variant was not found in public databases (ExAC, GnomAD and BRAVO database) or in our own WES database of around 4,500 exomes of

patients with a variety of infectious diseases. We analyzed the familial segregation of the mutant alleles in the 14 kindreds. Three family members with clinical features suggestive of GATA2 deficiency (Mycobacterial disease and cutaneous warts for P6; pulmonary tuberculosis for P14; mycobacterial disease and MDS for P10) were found to be heterozygous for *GATA2* mutations. Seventeen individuals were WT. Surprisingly, 16 individuals were carriers of a *GATA2* mutation, but not clinically affected (Kindreds A, B, D, J, L, M and N; Figure 20). Biological material was not available for other 18 individuals (Figure 20). Collectively, these data suggested that 33 individuals (17 patients and 16 carriers) had AD GATA2 deficiency

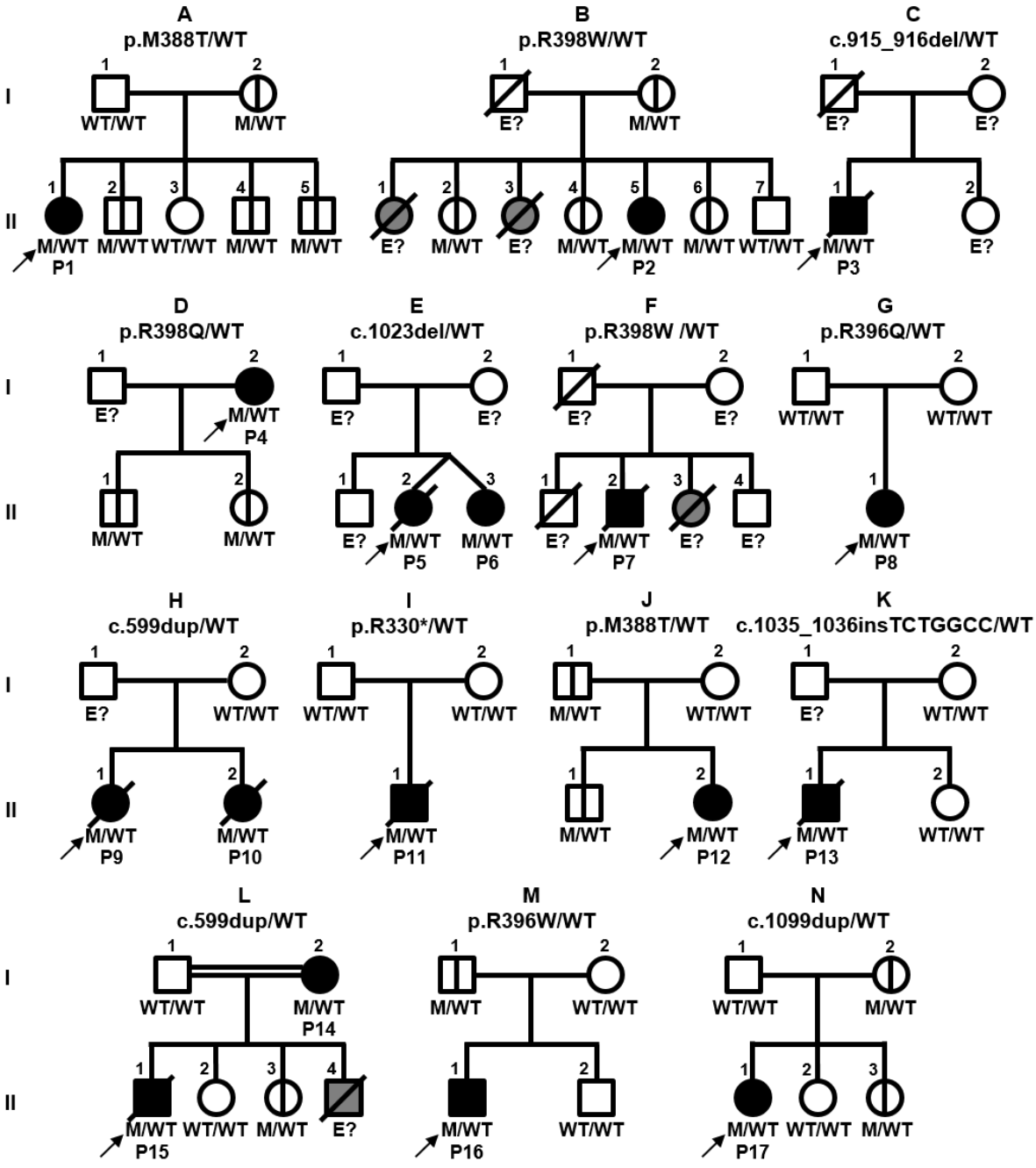

Figure 20 - Schematic representation of the 14 kindreds with germline *GATA2* mutations. Each kindred is designated by a capital letter (A-N), each generation by a roman numeral (I-II), and each individual by an Arabic number. The proband is indicated by arrows. Solid black shapes indicate GATA2 deficient patients and clinical disease. Solid grey shapes indicate individuals with GATA2-related clinical features (lymphedema or AML) but not genotyped. Individuals crossed with a black vertical line represent asymptomatic GATA2 deficiency. Individuals crossed with a diagonal black line represent deceased members. Healthy individuals are shown in white.

Expression of the mutant *GATA2* alleles in HEK293-T cells

Human *GATA2* encodes two isoforms: Isoform 1 with 480 aminoacids (aa, 50.5 kDa) and isoform 2 with 466 aa (49.1 kDa).[113] We evaluated the expression of the GATA2 protein by transiently transfecting HEK293-T cells with constructs encoding C-terminally DDK-tagged WT or mutant *GATA2* cDNAs. Similar levels of *GATA2* mRNA were detected in HEK293-T cells with WT or mutant alleles (data not shown). The WT GATA2 protein was detected by western blot at the expected MW (50 kDa). The missense mutants (p.M388T, p.R396Q, p.R396W, p.R398Q and p.R398W) were expressed at the expected MW (50 kDa), like the WT. The protein expression levels for all missense mutations were normal for both isoforms, as detected with both GATA2- and DDK-specific antibodies (Figure 21). The c. 1099dup, c.599dup, c.1035_1036insTCTGGCC, c.915_916del, c.1023del and p.R330* mutants produced a truncated protein for isoform 1, as detected with an antibody against GATA2 (Figure 21). Protein levels were lower than the WT for the c.599dup, c.1035_1036insTCTGGCC and c.915_916del for isoform 1, whereas c.1099dup, p.R330* and c.1023del produced protein levels similar to the WT. c.1035_1036insTCTGGCC and c.1023del are part of the intron for isoform 2, therefore not being present in the isoform 2 constructs. The c. 1099dup, c.915_916del and p.R330* produced a truncated protein for isoform 2, as detected with the antibody against GATA2, while the mutant c.599dup did not show detectable protein expression for this isoform (Figure 21).

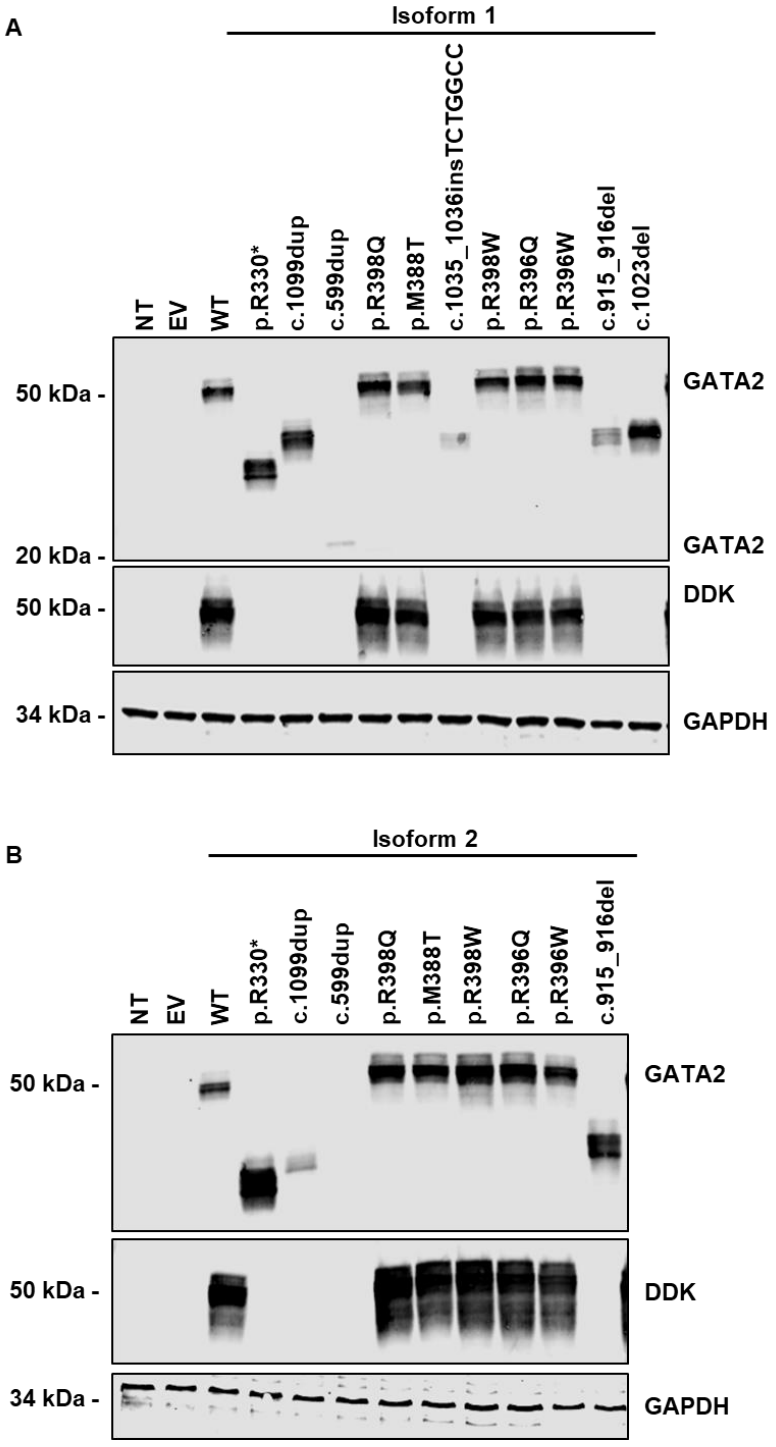

Figure 21 - Protein expression of *GATA2* alleles in overexpression system. HEK293-T cells either left NT or were transfected with an EV, a construct encoding *GATA2* WT-DDK-tagged or mutated alleles corresponding to isoform 1 **(A)** or isoform 2 **(B)** of GATA2 (p.R330*, c.1099dup, c.599dup, p.R398Q, p.M388T, c.1035_1036insTCTGGCC, p.R398W, p.R396Q, p.R396W, c.915_916del and c.1023del) for 48 hours. GATA2 protein expression was evaluated by western blotting with an anti-GATA2 antibody and an anti-DDK tag. GAPDH was included as loading control.

Functional activity of the mutant *GATA2* alleles in HEK293-T cells

We then assessed the functional activity of the GATA2 mutants by luciferase assay in HEK293-T cells transfected with the different cDNAs. Only isoform 1 of GATA2 has transcriptional activity. The promyelocytic leukemia protein (*PML*) gene is a member of the tripartite motif (TRIM) family that has been reported to potentiate the transactivational activity of GATA2.[205] Thus, we evaluated the impact of the different mutant alleles in comparison with *GATA2*-WT, with or without *PML*. HEK293-T cells transfected with *GATA2*-WT showed a functional activity that was enhanced when the cells were co-transfected with *PML* (Figure 22). However, the function of the different *GATA2* alleles was severely impaired or abolished in both conditions depending on the allele tested, being statistically significantly different from the WT (Figure 22). The missense alleles (p.M388T, p.R396Q, p.R396W, and p.R398Q) showed an impaired but not completely abolished luciferase activity compared to the WT, suggesting that these alleles are functionally hypomorphic, whereas one missense (p.R398W), the nonsense (p.R330*) and frameshift alleles (c.599dup, c.915_916del, c.1023del, c.1035_1036insTCTGGCC and c.1099dup) are complete LOF, at least in these conditions of over-expression (Figure 22).

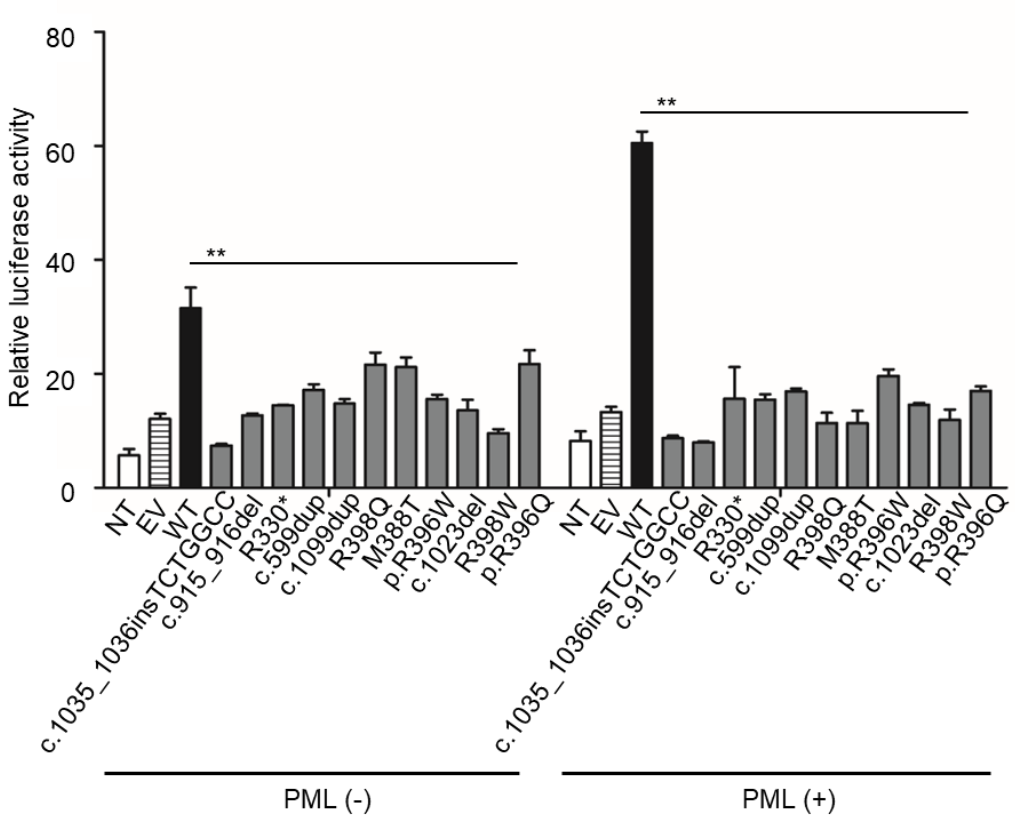

Figure 22 - Functional characterization of *GATA2* alleles. Luciferase activity of GATA2 measured in HEK293-T cells. GATA2-dependent transactivation potential was evaluated with *GATA2* luciferase reporter plasmid. Cells were transiently transfected with (+) or without (-) *PML*, EV and different *GATA2*-plasmids (WT, p.R330X, p.M388T, p.R396W, p.R396Q, p.R398Q, p.R398W, c.599dup, c.915_916del, c.1023del, c.1035_1036insTCTGGCC and c.1099dup).

Study of the mechanism of dominance in HEK293T cells

Afterwards, we studied the mechanism underlying dominance (haploinsufficiency or dominance negative) in heterozygous cells. We performed luciferase assays in transient co-transfections with *PML* and constant levels of WT, changing the amount of mutant plasmid and compensating with EV to reach the same final concentration (Figure 23). The luciferase activity for the different amounts of mutant plasmid was constant and increased compared to the EV, being not statistically significant when compared to the corresponding WT with EV

point (Figure 23). This result strengthens the hypothesis that GATA2 deficiency is caused by haploinsufficiency, since the luciferase activity is predicted to decrease in the presence of higher amounts of mutant plasmid in a dominance negative model.

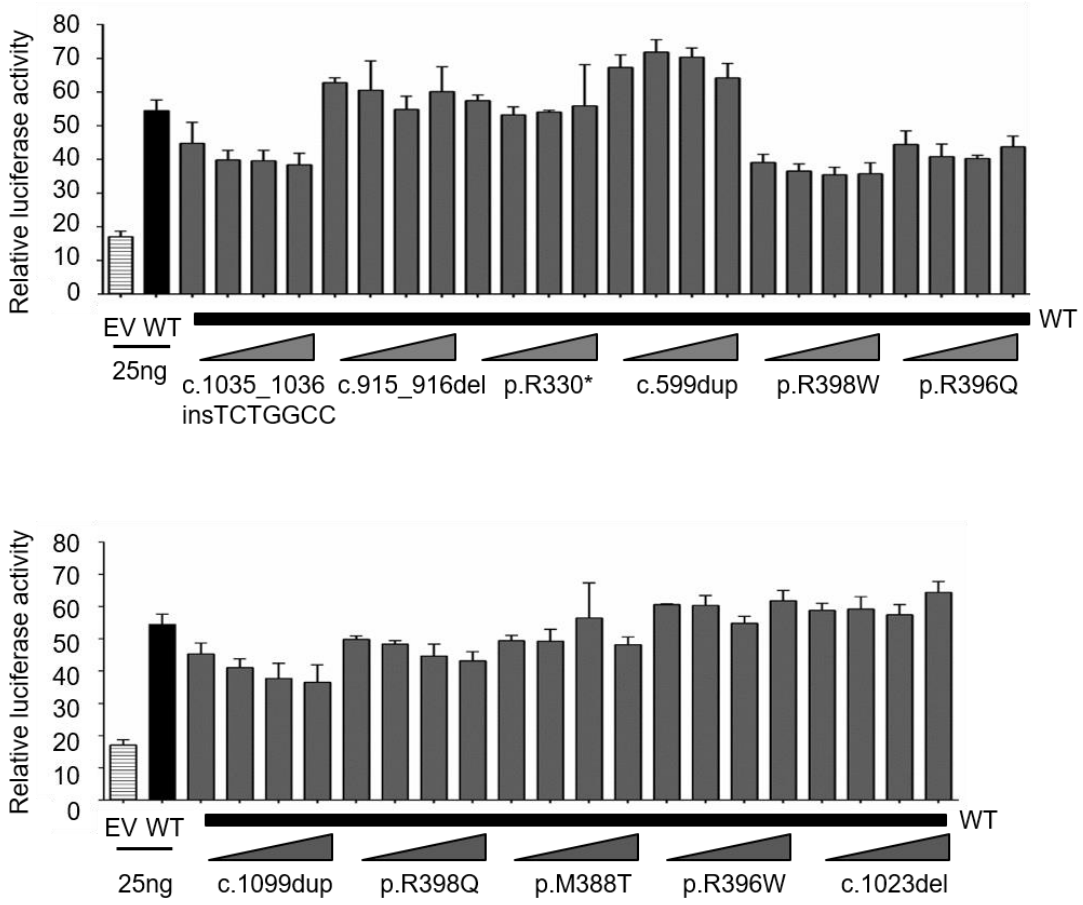

Figure 23 – Dominance test for *GATA2* mutant alleles. Transient co-transfection of cells with *PML* and constant amount *GATA2*-WT plasmid (25 ng) with decreasing amounts of mutant plasmids (p.R330X, p.M388T, p.R396W, p.R396Q, p.R398Q, p.R398W, c.599dup, c.915_916del, c.1023del, c.1035_1036insTCTGGCC and c.1099dup), different amounts of EV were used to keep the final plasmid concentration constant. Error bars indicate standard error of the mean. NS stands for not significant in the statistical analysis.

In addition, we analyzed several selection parameters, to estimate the evolutionary pressure on the *GATA2* locus (see methods). *GATA2* has a pLI score of 0.97 (Figure 24A) (being 0.9 the threshold where genes are said to be extremely intolerant to LOF variants).

Moreover, *GATA2* was found to be under negative selection when considering the *f* parameter from SnIPRE, which is 0.388 (Figure 24B) (95% confidence interval: 0.223 - 0.675), placing *GATA2* in the 26.4% of the genes under the strongest evolutionary pressure. The RVIS score, with a value of 11.839 (Figure 24C), places *GATA2* in the 11.8% of genes under the strongest negative pressure. These results highly suggest that *GATA2* has evolved under purifying selection, strengthening the hypothesis that GATA2 deficiency results from haplo-insufficiency.[123,206–208]

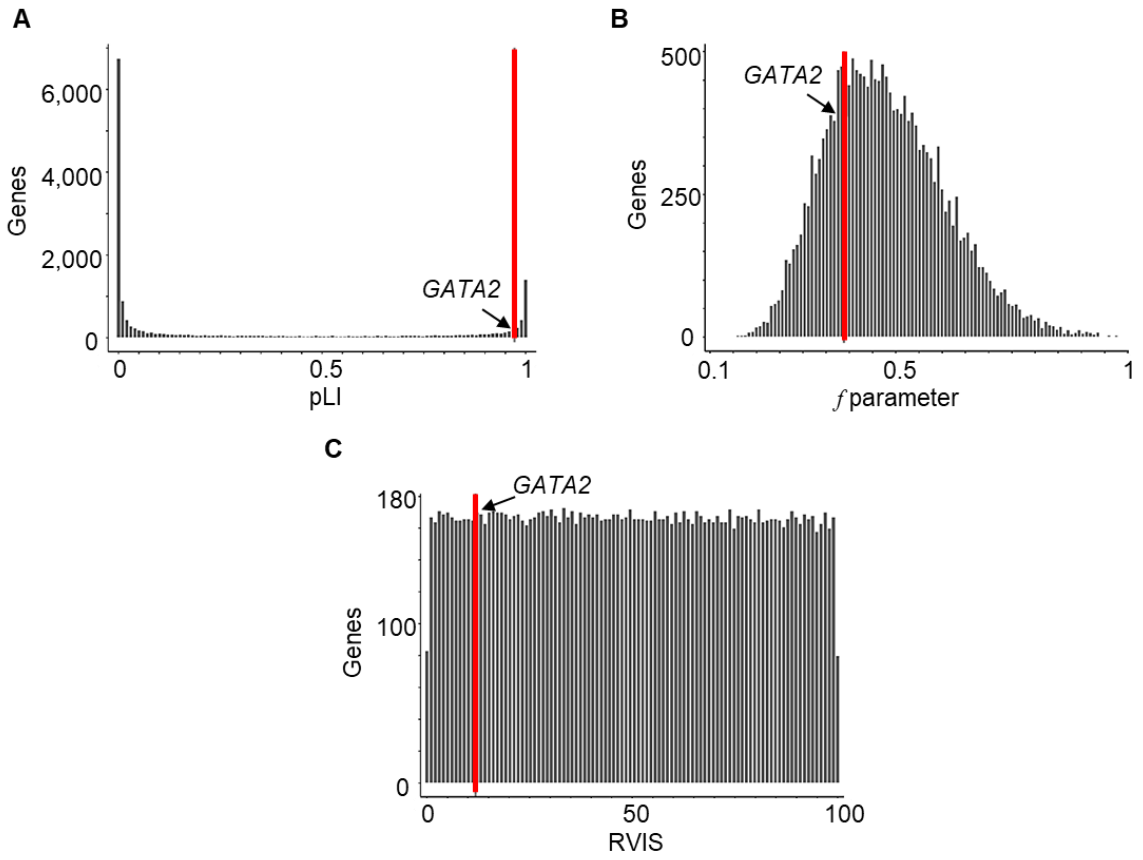

Figure 24 - List of variants and strength of purifying selection on GATA2. Genome-wide distribution of the strength of purifying selection, estimated by the **(A)** pLI score, **(B)** *f* Parameter, and **(C)** RVIS score in bar plots, as described in the methods. The position of GATA2 is indicated by a black arrow and a red vertical bar.

Environmental mycobacterial diseases in GATA2 deficient patients

The mycobacterial diseases in the patients were caused by EM (n=9), *M. tuberculosis* (n=4), EM and *M. tuberculosis* (n=1) and undefined *Mycobacterium* species (n=3) (Figure 25A). Ten patients developed EM disease due to *M. avium* (n=4) and/or *M. kansasii* (n=7). One of these patients (P5) had two different EM infectious diseases caused by *M. avium* and *M. kansasii* and P1 displayed combination of EM disease and tuberculosis. Recurrence of EM disease was observed in P15 who developed an infection by *M. kansasii* affecting lymph nodes at 24 years, and three years later, the patient suffered from a disseminated infection. No other EM was identified in this cohort. Mean age at EM diagnosis was 21 years (range 12 years to 29 years). The diagnosis of EM was made by positive culture (n=9), PCR (n=4), direct detection of acid-fast bacilli (n=2), or clinical suspicion (n=2). Positive culture was documented in two sites (n=4), lung and blood (n=3), or liver and blood (n=1). The sites for EM disease were the lungs (n=7), lymph nodes (n=5), liver (n=3), spleen (n=2), and bone marrow (n=3) (Figure 25B). Up to four organs were affected in infections with EM. In this cohort, EM disease has been not reported to affect soft tissues or the skin. Most episodes involved simultaneous infection of the lung and lymph nodes. Disseminated EM disease was documented in seven patients (P5, P6, P7, P8, P9, P13 and P17). Three patients with pulmonary EM disease had fever and coughing (P2, P6 and P17) and three patients had chest pain, fever and coughing (P5, P7 and P12).

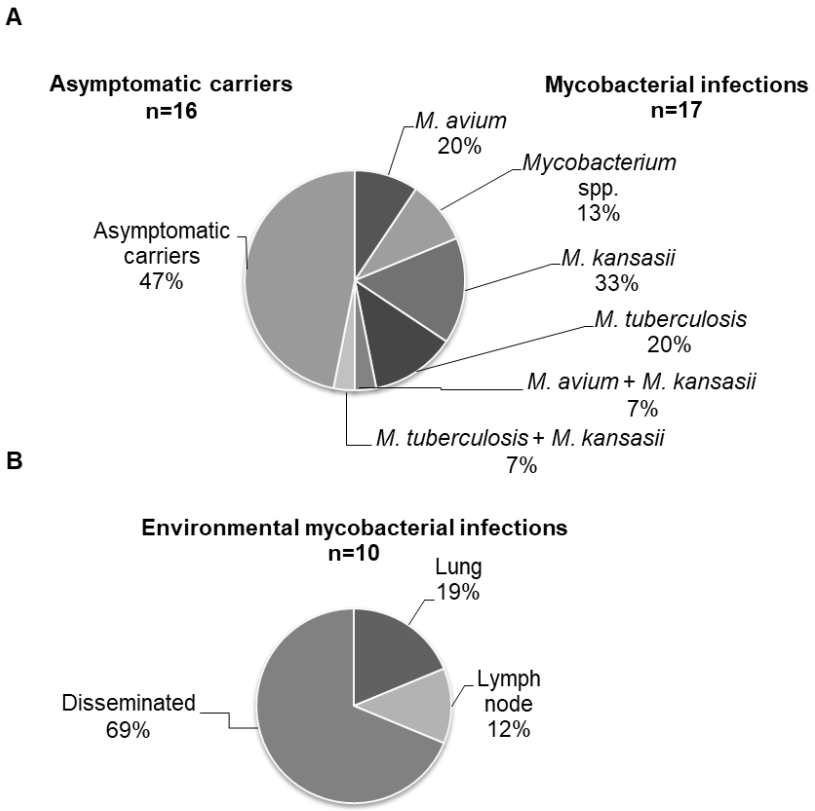

Figure 25 - Mycobacterial infections and distribution in GATA2 deficient patients. **(A)** Spectrum of mycobacterial species infecting GATA2 deficient patients. Patients affected by a type of mycobacterial species are represented by percentages, considering infections by two different species as a type of infection. Asymptomatic carriers represent those individuals carrying the mutation with no clinical phenotype. **(B)** Mycobacterial infection sites in patients with EM infection. Sites of infection are represented by percentages.

Tuberculosis in GATA2-deficient patients

Twelve of the 17 patients studied here received BCG vaccination at birth. However, complications caused by this vaccine were not reported in this cohort. TB has been diagnosed in five patients (P1, P3, P14, P15 and P16). All patients with tuberculosis had been vaccinated with BCG. Mean age at tuberculosis diagnosis was 29 years (range 17 years to 42 years). Tuberculosis was thoracic in four patients (P1, P3, P14 and P15) and disseminated in one patient (P16). The diagnosis of tuberculosis was confirmed in two patients (P1 and P16) by

positive culture of *M. tuberculosis*, whereas probable tuberculosis was diagnosed otherwise in three other patients (P3, P14 and P15). In this cohort, tuberculosis was mostly found in the lungs (n=4) (Figure 26). Recurrence of tuberculosis has been documented in P3 who had two pulmonary episodes (28 years and 32 years) and P16 (29 years and 31 years) who developed two episodes of disseminated tuberculosis affecting the lungs, lymph nodes, spleen and bone marrow. Patients with infections caused by undefined *Mycobacterium* spp. displayed disseminated (P4) or localized (P10 and P11) forms of infectious disease.

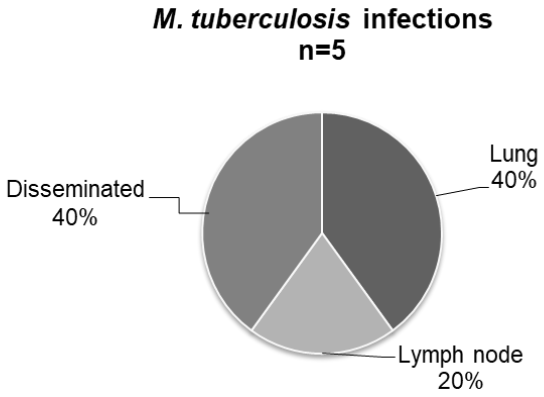

Figure 26 – Distribution of tuberculosis infection. Mycobacterial infection sites in patients with *M. tuberculosis* infection. Sites of infection are represented by percentages.

Other clinical manifestations in GATA2 deficient patients

All patients suffered from non-mycobacterial infections, caused by viruses, bacteria, or fungi. The microorganisms most frequently isolated were: *Streptococcus pneumoniae* (n=2), *Klebsiella pneumoniae* (n=1), *Citrobacter freundii* (n=1), *Clostridium difficile* (n=2), *Haemophilus influenzae* (n=1), *Pseudomona aeruginosa* (n=1), *Streptococcus salivarius* (n=1). Eight patients suffered from bacterial infections other than mycobacterial infections. In addition, five patients displayed mucocutaneous fungal infections caused by *Candida* spp.,

three of them oral infections (P3, P9 and P11), one of them cutaneous infection (P5) and another esophageal infection (P13). Candida infections may have been favored by certain cytopenia (neutropenia in particular, documented in P3 and P13) and medications (such as long antibiotic therapy combined or not with immunosuppressors, P13). One patient had disseminated histoplasmosis at the age of 23 years (P17). P5 and P12 suffered from disseminated aspergillosis, causing the death of P5. One patient suffered from EBV infection (P9). One patient suffered a fulminant hepatitis caused by HSV-2 (P10). Eight patients had recurrent warts caused by human papillomavirus (P1, P2, P3, P5, P6, P10, P14 and P15). P1 required 10 days of hospitalization referred as caused by adverse reactions the yellow fever vaccine. In addition to infectious diseases, some patients had lymphedema (P1, P2, P5, P6, P14 and P115), sarcoidosis (P11 and P16), psoriasis (P5 and P16), monoclonal gammapathy (P11), obesity (P8), thrombosis (P8, P13 and P14), and non-infectious oral ulcers (P4 and P17).

Hematological and immunological anomalies of GATA2-mutated patients

GATA2 mRNA is expressed in total PBMCs and in different subsets of lymphoid (T cells, B cells, NK cells) and myeloid cells (monocytes, total dendritic cells, myeloid dendritic cells, plasmacytoid dendritic cells) and $CD34^+$ cells.[113,209,210] When total PBMCs were analyzed, *GATA2* mRNA was detected in healthy controls (Figure 27). However, when we measured the *GATA2* mRNA levels in different blood cell subsets, we observed that only $CD34^+$ cells expressed detectable *GATA2* mRNA (Figure 27). We then compared the healthy controls to the patients' total PBMCs *GATA2* mRNA levels. We observed that *GATA2* mRNA levels were reduced in the available patients' PBMCs (P4, P11, P13, P16 and P17), when compared

with controls (Figure 27D). These data are consistent with those previously reported in the literature, where *GATA2* mRNA levels were reduced in the PBMCs of a GATA2 deficient patient.[202] In *GATA2* $^{-/+}$ mice, reduced numbers of HSCs have been observed,[202,211] and probably the same situation can occur in *GATA2*-deficient patients.

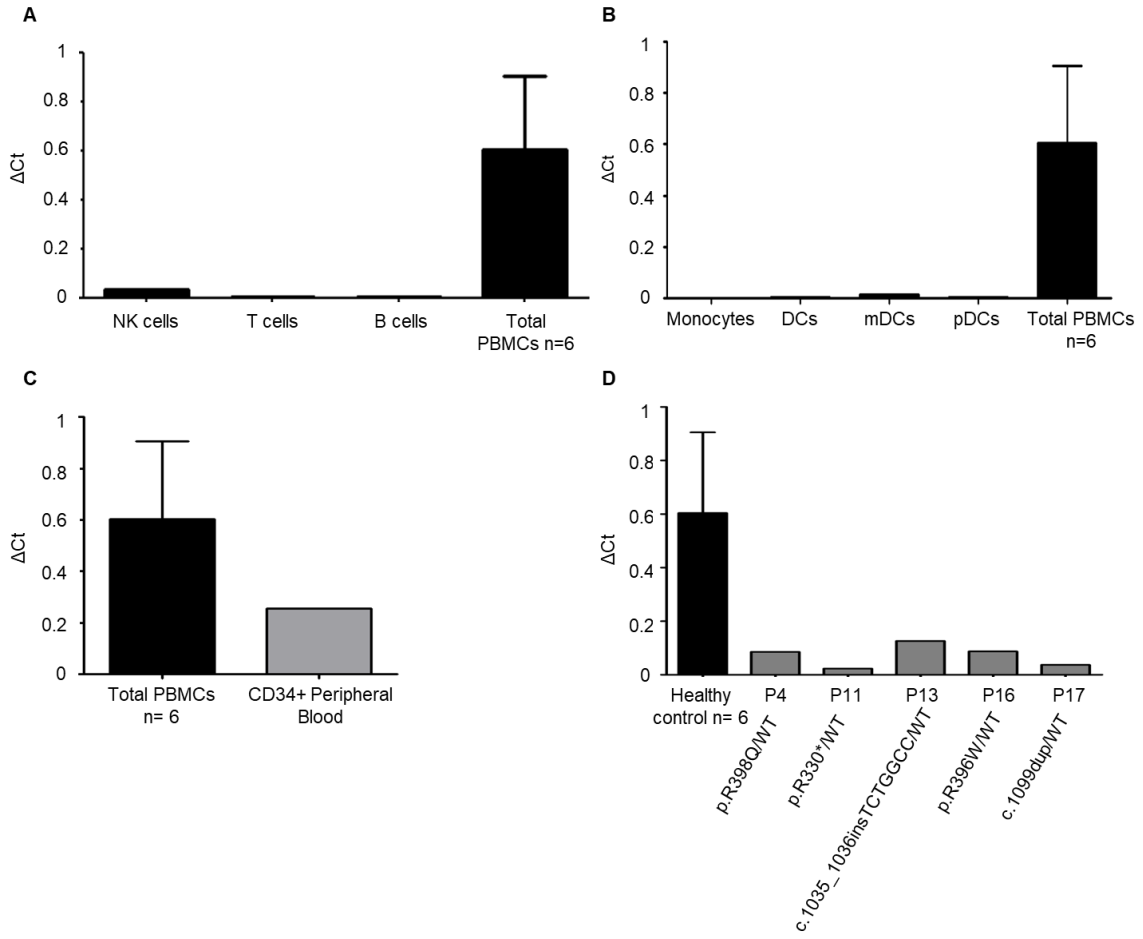

Figure 27 - *GATA2* mRNA levels in peripheral blood subsets. **(A)** *GATA2* qPCR on lymphoid cells (NK, T and B cells) and total peripheral blood mononuclear cells (total PBMCs) from healthy donors (n=6). **(B)** *GATA2* qPCR on myeloid cells (monocytes, dendritic cells [DC], myeloid dendritic cells [mDCs] and plasmacytoid dendritic cells [pDCs]) and on total PMBCs from healthy donors (n=6). **(C)** *GATA2* qPCR on hematopoietic stem cells ($CD34^+$) and total PBMCs from healthy donors (n=6). **(D)** *GATA2* qPCR on total PBMCs from P13, P11, P17, P4 and P16, and total PBMCs from healthy donors (n=6). Error bars indicate the standard error of the mean.

Then, we evaluated the circulating blood counts and we found (when the information was available) that some of the patients presented low neutrophils counts (n=7), low or absent

B cell counts (n=15), low T cell counts (n=11), low or absent NK cells (n=14), and low dendritic cells (n=6). Monocytopenia was the most common feature between the patients, found in 16 out of 17. Bone marrow analysis was performed in twelve patients. Hypoplasia was observed in eight patients (P3, P5, P9, P10, P11, P13, P15 and P17), myelodysplastic syndrome was also observed in seven patients (P4, P5, P9, P10, P11, P13 and P15), acute myeloid leukemia (AML) was observed in P7, and hemophagocytic syndrome secondary to disseminated mycobacterial infection was observed in P13. Cytogenetic analysis was studied in eight patients where six patients have been referred as normal. However, two patients had an aberrant cytogenetic analysis resulting in deletions and aneuploidy in P10; and duplication (15; 1qter->1q12:15p11->15qter) in P11. FLT3L levels on plasma were increased in all the patients tested (n=8) and one carrier, whereas it was undetectable in four carriers (Figure 28A); the plasma was not available for the other patients. Only two patients showed high levels of M-CSF in the plasma; in the others patients (n=7) (Figure 28B), the levels remained low.

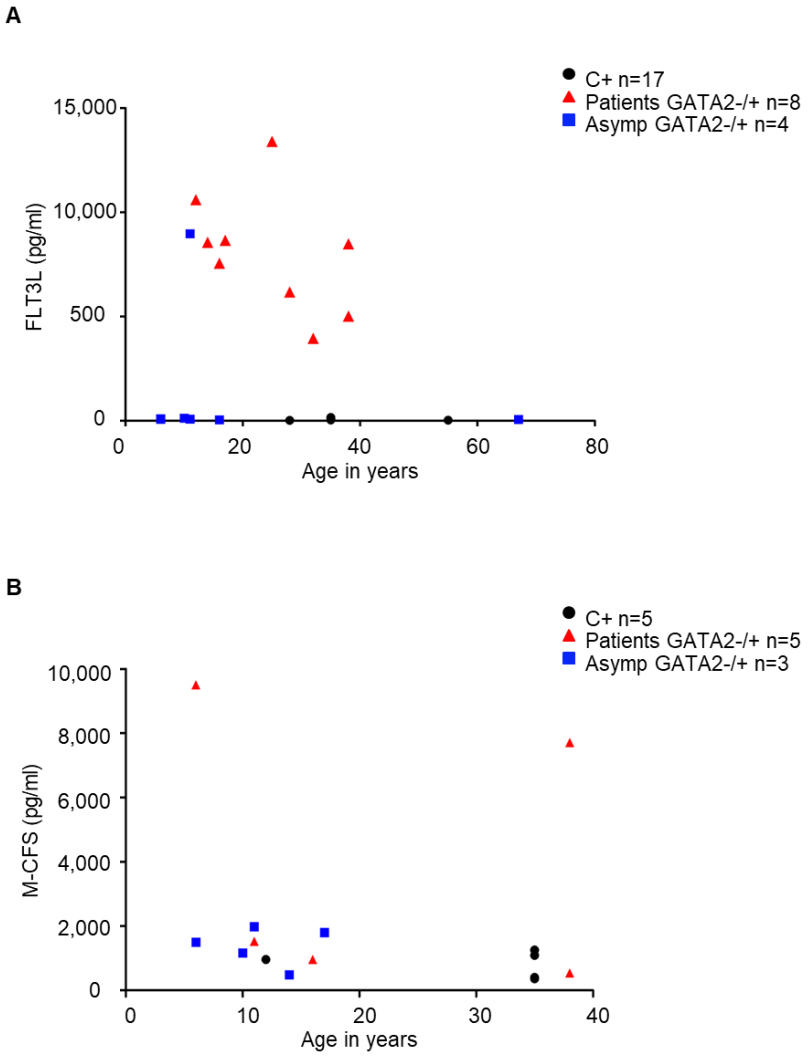

Figure 28 - FLT3L and M-CSF levels in plasma of GATA2-deficient patients. **(A)** FLT3L and **(B)** M-CSF levels in plasma from healthy controls (n=17 and n=5 respectively, black circles), GATA2-deficient patients (n=8 and n=5 respectively, red triangles) and asymptomatic individuals with GATA2 mutations (n=4 and n=3 respectively, blue squares).

Penetrance calculation for GATA2 deficiency

Next, we estimated clinical penetrance as a function of age at the first GATA2 deficiency-related symptom in a total of 18 family members of the index cases who were confirmed to carry a *GATA2* mutation (P14 was not considered due to lack of age information). Of these 18 individuals, only two presented GATA2 deficiency-related

symptoms (P6, at 17 years of age suffered from *M. kansasii* infection; and P10, at 12 years of age suffered from MDS). The other 16 individuals remained healthy at the last follow-up (follow-up range, 6 to 78 years; mean 39.33 years). The estimated clinical penetrance on these 18 individuals reached a plateau of 14% at 20 years (95% confidence interval [CI], 0.02-0.36) (Figure 29A). However, only alive relatives of the index case and one dead relative (P10) were genetically tested for GATA2 deficiency, which can lead to ascertainment bias and underestimation of the penetrance. Thus, we performed a second calculation including four non-genotyped family members who died from diseases very likely to be related to GATA2 deficiency, i.e. AML or lymphedema (Kindred B, II.1 and II.3, who both died from AML at age 36 and 33 years respectively; kindred F, II.3 who died from AML at age 26 years; and kindred L, II.4 who died by lymphedema at birth, considered age 0). Using those 22 individuals, the penetrance was estimated at 15.5% by the age of 25 years (95% CI, 0.034-0.35) and increased to 32.9% by the age of 40 years (95% CI, 0.12-0.55) (Figure 29A). This result suggests that penetrance for GATA2 deficiency increases with age, remaining nonetheless incomplete at the age of 40 years. As mycobacterial infections were the main clinical manifestation in the 14 index cases, we analyzed the penetrance of mycobacterial disease in comparison with the other clinical phenotypes associated with GATA2 deficiency (AML, viral infections or lymphedema) on the 22 individuals afore mentioned. The penetrance for mycobacterial disease was estimated at 10% by the age of 20 years (95% CI, 0.017-0.3) and increased to 22.6% by the age of 40 years (95% CI, 0.06-0.44) (Figure 29B) whereas the penetrance for other clinical phenotypes was estimated at 10% at the age of 20 years (95% CI, 0.15-0.28) and increased to 28% by the age of 40 years (95% CI, 0.095-0.5) (Figure 29B). Overall, we observed no significant differences between the penetrance for

mycobacterial disease or other clinical phenotypes, probably due to the low number of individuals available for this calculation. While the estimation of the clinical penetrance may be biased due to incomplete information regarding family members of the index case, (as illustrated by the difference observed between the two analysis strategy), observing four *GATA2*-mutation carriers who have remained physically healthy at more than 60 years of age (Kindred A, I.2, 78 years; Kindred B, I.2, 63 years; Kindred J, I.1, 68 years; and Kindred M, I.1, 66 years), confirms incomplete penetrance. These results suggest that there might be other elements, such as genetic modifiers or pathogen exposure that play a role in the development of GATA2 deficiency-related disease.[212,213]

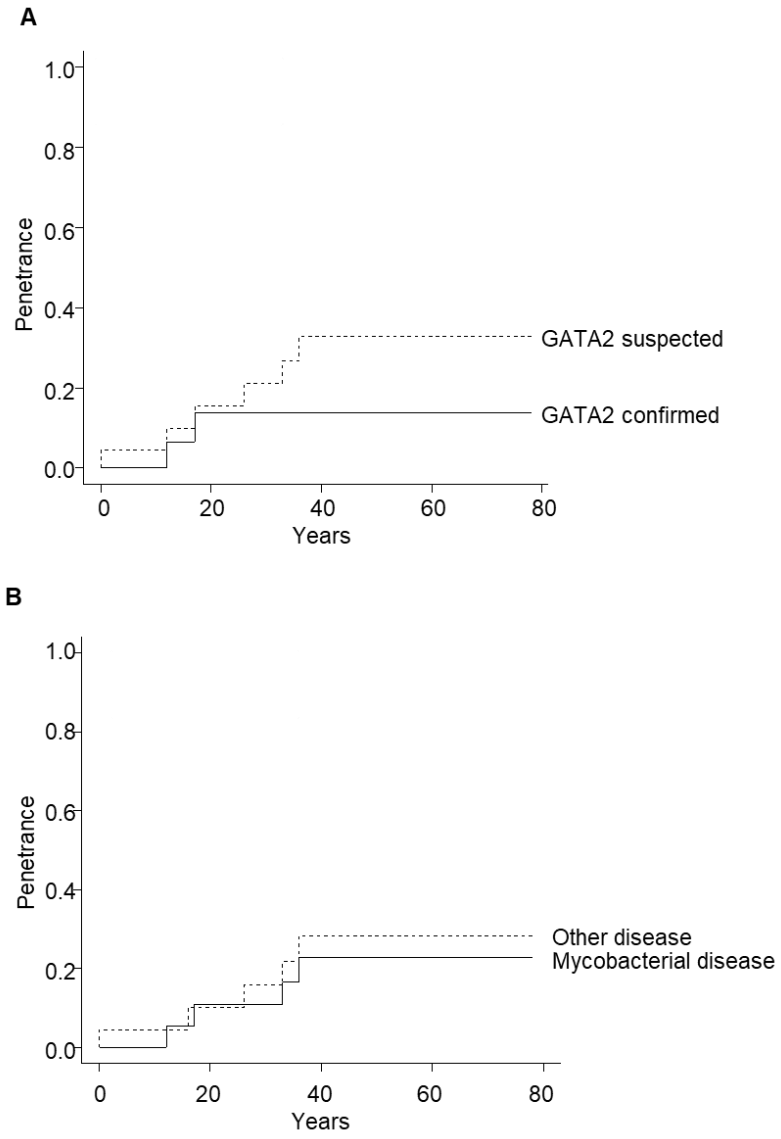

Figure 29 - Penetrance of clinical diseases in GATA2 deficiency. **(A)** Kaplan-Meier curves showing penetrance of *GATA2* confirmed carriers (*GATA2* confirmed, continuous line) and family members with suspected GATA2 deficiency (*GATA2* suspected, discontinuous line) with clinical manifestations at 20 and 40 years. **(B)** Penetrance of mycobacterial infection (Mycobacterial disease, continuous line) and other clinical manifestations of GATA2 deficiency (Other disease, discontinuous line).

Clinical outcome

We focused on the onset of mycobacterial infection and survival of the 17 patients. The age at onset of infection was available for 15 of these patients, being typically in early adulthood. The mean age at onset of the first mycobacterial infection was 23.25 years (range, 12 to 42 years) (Figure 30). Of these onsets, eight were caused by EM (mean age 21 years-range 12 years to 29 years) (Figure 30), four were caused by TB (mean age 29 years-range 17 years to 42 years) (Figure 30) and three were caused by unidentified mycobacteria (mean age 22.6 years-range 12 to 38 years) (Figure 30). Nine of the 17 patients and all asymptomatic individuals are still alive (Figure 20). Their most recent follow-up visit was at a mean age of 35 years (range 25 to 51 years) for the patients and at a mean age of 41.18 years (range 11 to 78 years) for the asymptomatic individuals. Three patients (P4, P12 and P16) underwent HSCT and are alive and well, besides, one patient received liver transplantation (P10). The mortality rate among symptomatic patients was 47% (P3, P5, P7, P9, P10, P11, P13 and P15). The cause of death was infectious diseases for three patients (disseminated infection by *Aspergillus* for P5, disseminated infection by *M. avium* for P9 and disseminated infection by *M. kansasii* for P13), hematological diseases for two patients (myelodysplasia for P3 and AML for P7), and unknown in two patients (P11 and P15). Fulminant hepatitis associated with encephalitis and multiorgan failure caused the death of P10.

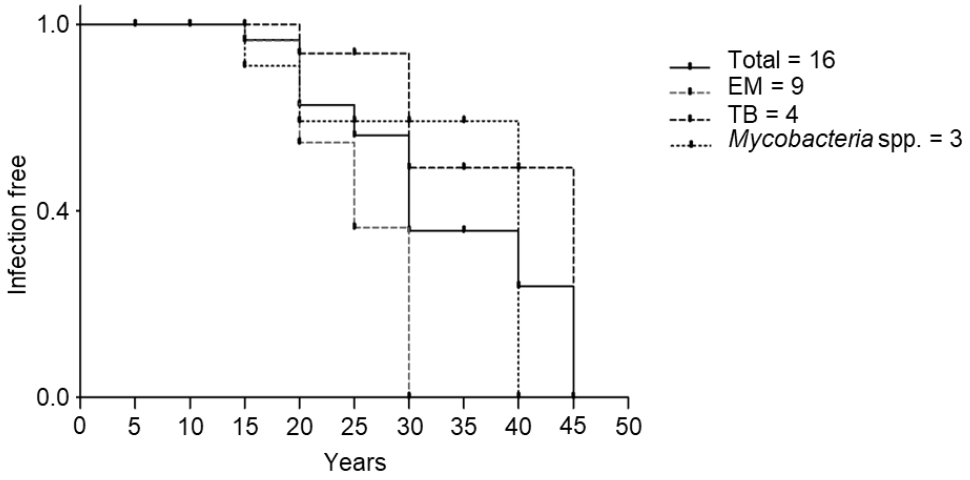

Figure 30 - First onset of mycobacterial infection in GATA2 patients. Kaplan-Meier curve showing percentage of mycobacterial infection free patients in a range of 5 to 45 years. Total number of patients (n=16) is indicated by a continuous black line, patients affected by environmental mycobacteria (EM, n=9) are indicated by a discontinuous grey line, patients affected by *Mycobacterium tuberculosis* (TB, n=4) are indicated by a discontinuous black line and patients affected by unidentified mycobacteria (*Mycobacterium* spp., n=3) are indicated in a black dotted line.

DISCUSSION

The work of this thesis shows the genetic identification of 20 patients of the 500 patients without a genetic diagnosis, of which three suffer from MSMD and 17 are GATA2 deficient. Genetic diagnosis in any germline disease is of utmost importance in order to provide a successful treatment and genetic counseling for the affected family.[8] In this study, we have identified two novel mutations in *IFNGR2* that confer a novel form of AR partial IFN-γR2 deficiency in three patients suffering from mycobacterial diseases. In addition, we have also described a detailed cohort of 17 GATA2 patients with mycobacterial disease as their main clinical phenotype, as well as determined for the first time the incomplete clinical penetrance of this deficiency. Together, these results have helped diagnose and treat patients suffering from mycobacterial diseases.

I. A purely quantitative form of partial recessive IFN-γR2 deficiency

In the first part of this thesis we have reported two novel mutations of *IFNGR2* in three MSMD patients, one being c.1A>G for P1 and P2 in a Turkish family, and the other being c.4delC for P3 in an Indian family. Together, they define a novel form of AR partial IFN-γR2 deficiency that is purely quantitative, with surface expression of diminished but normal receptor chains, whereas previously reported patients expressed diminished levels of receptors on the surface that carried missense mutations. The novelty of this study is the definition of a completely novel type of AR partial IFN-γR2 deficiency, caused by mutations that impair the amount of surface-expressed receptors, but not the nature of these surface receptors. These two IFN-γR2 mutants can be over-expressed, albeit at much lower levels

than WT, and their functional activity was severely impaired, although not abolished. Both SV40-fibroblasts and EBV-B cells from P1 and P2, and by inference from over-expression studies, cells from P3 as well, showed a much more severe impairment of activation of STAT1 and GAF than cells from other forms of AR partial IFN-γR2 deficiency. P1 and P2 fresh leukocyte subsets showed a severe impairment of phosphorylation of STAT1 in naïve $CD4^+$ T cells and in MDMs. The cellular phenotype is therefore more severe than that of the other known form of AR partial IFN-γR2 deficiency, but less severe than that of complete IFN-γR2 deficiency. Therefore, these patients should be treated as AR complete IFN-γR2 deficient patients.

Like other patients with AR complete or partial IFN-γR2 deficiency, these patients had high plasma concentrations of IFN-γ. Their clinical phenotype appears to be intermediate, between that of patients with AR complete deficiency[167,177,221,191,214–220] and patients with AR partial deficiency due to missense mutations.[166,189–191] The three patients presented clinical complications after BCG vaccination. P1 also presented multifocal osteomyelitis caused by BCG at the age of 5 and 7 years, which was treated; he is now healthy at age 9. P2 was treated for the complications after BCG vaccination and is healthy at last follow up at age 6 years. P3 died of *M. chelonei* infection at the age of 5 years. The clinical phenotype of these patients, with the possible exception of P2, is thus globally more severe than that of other patients with AR partial IFN-γR2 deficiency. HSCT is therefore recommended for the two survivors, a treatment generally considered for patients with complete but not partial IFN-γR2 deficiency.[35,220] P1's osteomyelitis adds another patient with a defect in IFN-γ response to show this type of lesion. As it has been previously

suggested, osteomyelitis should be considered a sign to sequence genes involved in the IFN-γ response.[222]

Our study also shows that non-canonical initiation of translation of IFN-γR2 can operate upstream enough in cells lacking the canonical AUG codon upstream the segment encoding the signal peptide. This is physiologically and clinically relevant, as these three patients have a milder phenotype than complete IFN-γR2 deficiency. During translation initiation in eukaryotic cells, the small (40S) subunit of the eukaryotic ribosome binds to the capped 5'-end of the mRNA. It then migrates, stopping at the first AUG codon in a favorable context for translation initiation.[223] If the first ATG is mutated or the context is altered, translation may occur through re-initiation or context-dependent leaky scanning.[175,224–226] In the *IFNGR2* mRNA, the mutation of the first AUG to a GUG, or the loss of a C at position +4, prevents efficient translation initiation at this location. The next AUG codon downstream of the start codon is located 238 base pairs downstream, which would cause the loss of the leader sequence and 58 aminoacids of the extracellular domain. It has been reported, however, that non-AUG codons such as CUG, GUG and ACG, may initiate translation. [192,198–200] The *IFNGR2* gene presents one ACG codon at position c.10, two consecutive CTG codons starting at positions c.13 and five consecutive CTG codons starting at position c.25.

We have shown that codons at position 2, 4, 6 and 8 contribute to the residual translation of IFN-γR2 and, in the case of the mutation c.1A>G, that the GUG codon may contribute as well to this event. The 19 to 12 of 21 residues left in the signal peptide were sufficient for the protein to traffic through the secretory pathway. The residual amounts of IFN-γR2 translated and transported to the cell surface were sufficient to enable diminished

but not abolished cellular responses to IFN-γ. However, the shorter signal peptide was not as efficient as its full-length version, in terms of protein expression on the cell surface. Germline mutations of other signal peptides have previously been reported to underlie human genetic diseases, impairing protein secretion.[227–229] Paradoxically, we found that the total expression of IFN-γR2 proteins encoded by the c.1A>G and c.4delC alleles is inversely correlated with GAS binding activity upon cell stimulation with IFN-γ. Previous studies in prokaryotic cells have shown that protein secretion can be affected by the codon encoding the second aminoacid position of the signal peptide.[230–232] The shorter signal peptide of proteins encoded by c.4delC might thus result in intracellular accumulation of the mutant proteins, thereby accounting for lower levels of receptor expression on the cell surface, when compared with proteins encoded by c.1A>G. Unfortunately, currently available antibodies do not enable a robust detection of IFN-γR2 on the cell surface, preventing us from testing this hypothesis.

Re-initiation of translation downstream the first AUG has previously been reported for other inborn errors of immunity (such as *IFNGR1*[192], *NEMO*[175], *RAG1*[233,234], *FAC*[235] and *NBS1*[236]), as well as in other fields of human genetics.[237,238,247,239–246] Most of these cases are N-terminal frameshift mutations that result in premature stop codons, similar to one of the *IFNGR2* mutations reported here, c.4delC. In these cases, there is an AUG codon downstream the frameshift, where reinitiation of translation has been shown to occur; however, it is not the case for the c.4delC mutation. To our knowledge, in only one case, for *IFNGR1*[192], a missense mutation affecting the first AUG of the gene was reported, like the other *IFNGR2* mutation reported in this study, c.1A>G. The re-initiation of translation in *IFNGR1* was reported to take place at downstream AUGs codons, with additional contributions of non-AUG codons.[192] In contrast, we describe a genetic form of human disease due to re-initiation

of translation by purely non-AUG codons. Translation in non-AUG codons has already been shown to take place in neurodegenerative diseases, cancer and stress response.[248] This is the first example for inborn errors of immunity. This form affects the function of IFN-γR2 differently depending on the cell type. This can be explained by the fact that the efficiency of re-initiation of translation at a codon downstream from the first AUG is poor and may vary from cell type to cell type.[223] The inefficiency of downstream codons of the first AUG can be due to the different amounts of endoplasmic reticulum and post-translational modification procedures across cell types[249], as well as differences in the tissue specific profile of transfer-RNA (t-RNA).[200,250] These factors contribute to the observed re-initiation of translation downstream from the lost canonical AUG, stressing the importance of this compensatory mechanism in human genetics. As reinitiation of translation cannot be predicted *in silico*, mutations that disrupt the canonical AUG should not be considered loss-of-function until experimentally proven to be. [251]

II. GATA2 deficiency and mycobacterial disease: a comprehensive study

Autosomal dominant GATA2 deficiency is a pleiotropic disorder associated with a broad spectrum of phenotypes, including (i) monocytopenia with susceptibility to atypical mycobacteria (monoMAC deficiency),[109,115] (ii) familial MDS,[108,110,116] (iii) CML or AML,[110,116] and (iv) Emberger syndrome.[111,117,118] Rare patients display isolated neutropenia,[119] aplastic anemia,[120] and isolated NK deficiency.[112,121]. The clinical features typically manifest in the second decade of life, but age at onset ranges from early childhood to late adulthood.[113] Only five large cohorts of patients have been characterized at the

immunological, hematological and clinical levels.[113,119,126,127,252] Very few cases of GATA2 deficiency have been studied in detail at the molecular genetic level, and no large series of patients initially diagnosed because of mycobacterial disease (monoMAC deficiency) have been published. The outcome of mycobacterial disease in these patients therefore remains unclear, despite the many published individual case reports for patients with mycobacterial disease.[109,112,211,253–257,115,117,121,123,126–128,203] There has been no description of the clinical, immunological and hematological manifestations associated with mycobacterial disease in these patients, for whom molecular genetic studies are also lacking. For this reason, in the second chapter of this thesis, we have focused on an international series of 17 patients from 14 kindreds in 10 countries with AD GATA2 deficiency and mycobacterial disease. Despite the previous description of various patients with this disorder, information about their ethnicity is often missing. Our patients are from Brazil, Colombia, France, Germany, Mexico, Portugal, Spain, Tunisia, Turkey, and the USA.

We found 11 germline heterozygous mutations in *GATA2* in these 17 patients, one of which was previously unknown, the other 10 having already been reported in other patients. Of the 150 germline *GATA2* alleles reported in the literature, only twelve have been properly characterized *in vitro*, which limits the understanding of the mechanism behind GATA2 deficiency.[108,111,260–262,116,117,122,123,202,211,258,259] We report the biochemical evaluation of 11 *GATA2* alleles *in vitro*, one of which had never been reported before (c.1035_1036insTCTGGCC) and of which only one (p.R330*)[262] had been studied experimentally before. The protein expression was either unaffected in those mutations that were missense or affected by truncation in the case of mutations leading to a premature stop codon when compared to the WT. We have also shown, in a luciferase assay system, the

ability of WT GATA2 to bind and activate its own promoter, which was already reported by Cortes-Lavaud *et al*.[202] The mutant alleles tested were either complete LOF (p.R398W, p.R330*, c.599dup, c.915_916del, c.1023del, c.1035_1036insTCTGGCC and c.1099dup) or hypomorphic (p.M388T, p.R396Q, p.R396W, and p.R398Q) regarding this binding. Differences in terms of function, however, did not correlate with clinical outcome. Patients with hypomorphic alleles did not show striking differences in clinical phenotypes when compared to the patients carrying LOF alleles. When comparing these patients with other patients reported with the same mutations, we found that clinical phenotypes were similar (in terms of infections and hematological manifestations), but not identical.[119,120,127,128] This observation has been previously made by other reports, where clinical outcome differs between individuals carrying the same mutation, even between family members.[127,263]

As for the mechanism of dominance, our results point towards haploinsufficiency being involved. This conclusion is based on (i) the truncating nature of most mutations, (ii) the lack of negative dominance *in vitro*, and (iii) the negative selection operating on *GATA2*. Our results are at odds with a previous report on p.R330*,[262] which was proposed to be dominant negative. The most likely explanation for this discrepancy is that the experimental conditions differed. The use of different promoters, normalization strategies, and transfection reagents, might explain the different results. A previous report has established that depending on the promoter used for luciferase assay, the same allele can be complete LOF or hypomorphic,[260] stressing the different roles that GATA2 has as a transcription factor.

The key clinical presentation in these GATA2-deficient patients is mycobacterial disease, principally marked by the high proportion of EM, in particular *M. kansasii* and *M.*

avium. Interestingly, *M. tuberculosis* is the second most frequent mycobacterium implicated, affecting five patients, probably due to the fact that the affected patients live in countries in which this mycobacterium is endemic. No significant differences in terms of mycobacterial species and geographic or ethnic origin have been observed, when comparing this cohort with other patients reported in the literature.[112,123,126–128,203,211,255,256] Complication of BCG vaccines was not reported in this cohort, consistent with the low susceptibility to BCG documented in other published GATA2-deficient patients.[112,128,203] This probably reflects the age-dependent decline of immunity in these patients, who showed a progressive loss of monocytes, dendritic cells, B cells, NK cells and, in some cases, T cells. Interestingly, one patient had an adverse reaction to the yellow fever vaccine, requiring hospitalization. The loss of peripheral dendritic cells and in particular plasmacytoid dendritic cells could contribute to the adverse effects of this vaccine, as seen in GATA2-deficient patients with severe influenza.[122] Our patients showed no differences with previously reported patients with GATA2 deficiency; who were also prone to develop viral, pyogenic, and fungal infections, as well as malignant hematological disorders and non-hematological disorders, such as lymphedema.[113,126,264]

We observed that clinical penetrance for any GATA2 deficiency-related disease was incomplete at the age of 40 years (32.9%), as 16 of the relatives of the index cases were carriers of the *GATA2* mutations but remained asymptomatic, one of them up to the age of 78 years. Moreover, we observed that the penetrance for mycobacterial infection was incomplete at the age of 40 years (22.6%) and that there were no significant differences between the penetrance for mycobacterial disease or for other clinical phenotypes. Penetrance for GATA2 deficiency in the literature is not properly characterized, since the

high but incomplete penetrance for MDS and AML[113,119,252,260] and low penetrance for lymphedema[111,118,128,212,213,263,265] are assumed based on the number of patients affected, without performing a proper penetrance analysis. In our study we show, by calculating the clinical penetrance of any GATA2 deficiency-associated disease or for mycobacterial disease in particular, excluding the index cases, that GATA2 deficiency penetrance increases with age but remains, however, incomplete by the age of 40 years. Probably, various factors may be involved in this clinical heterogeneity, including gene modifiers in human, environmental exposure and type of microbe. We therefore formerly demonstrate the incomplete penetrance for GATA2 deficiency, which has been assumed throughout the years but had never been properly analyzed.[126,213,263,265]

Altogether, GATA2 deficiency is a complex inborn error of hematopoiesis that requires further study and characterization, given the differences observed between patients and the incomplete penetrance for any GATA2 deficiency-related disease. Search for modifier genes and somatic variations may help discern the broad spectrum within this deficiency. A diagnosis of AD GATA2 deficiency should be considered in young adults with mycobacterial infections, with or without monocytopenia, at any age in life. Moreover, all their direct relatives should be genotyped at the *GATA2* locus.

In summary, we have characterized two mutations in *IFNGR*2 that give place to a non-canonical reinitiation of translation of IFN-γR2. This results in a quantitative partial deficiency of IFN-γR2 which is more severe than the qualitative partial IFN-γR2 deficiencies described to date. Moreover, we have characterized *in vitro* 11 *GATA2* mutations, proving their impairment on GATA2 activity. We have described in detail the clinical features of the

GATA2-mutation carriers and have made a formal calculation of the clinical penetrance, which we observe is incomplete up to 40 years of age.

III. Future perspectives

a. IFN-γR2 deficiency

In the novel form of IFN-γR2 deficiency, it is hypothesized that the differences observed in terms of response to IFN-γ in overexpression for c.1A>G and c.4delC are due to the shorter signal peptide created by c.4delC, causing the possible intracellular accumulation of the latter mutant. In order to prove this, two complementary approaches can be followed: first, comparing the expression of both mutant IFN-γR2 proteins on the cell surface by flow cytometry, and second, to locate both mutant proteins on the cell surface and within the cell by microscopy. The available IFN-γR2 antibodies for flow cytometry do not provide a strong enough signal to detect slight differences between two lowly expressed IFN-γR2 proteins.[166,167,215] Nonetheless, the subcellular localization of both IFN-γR2 mutants can be tested by fluorescent microscopy. By overexpression, both mutants can be marked with an anti-flag antibody and their localization be detected in the cell.[166] With time and the right equipment it is possible to follow this approach.

Moreover, in this project we have observed a more severe form of IFN-γR2 partial deficiency. In previous studies, as well as in this one, it has been observed that each *IFNGR2* hypomorphic mutation responds differently to IFN-γ (in terms of STAT1 phosphorylation) and that this response can vary from one cell type to another.[166,167,191] It could be interesting to study the downstream implications of these differences and rank the mutations from more deleterious to less deleterious. To this end, RNA sequencing could be performed after activation with IFN-γ in available cell lines from IFN-γR2 partial deficient patients. This can

help elucidate the specific effect each mutation has at the signaling level, as well as determine the contribution of each cell type to the response to IFN-γ.

Finally, it is noteworthy that the lack of sufficient biological material from the patient with the c.4delC mutation has led to infer our conclusions about it from an overexpression system. The use of induced pluripotent stem cells (iPSCs) to study MSMD patients has been recently suggested.[266] iPSCs can be generated from different sources and can then be differentiated into several cells of the hematopoietic lineage, such as macrophages.[267,268] Given this, the available material from the IFN-γR2 deficient patient carrying the c.4delC mutation could be used to create iPSCs and study the consequence of the mutation in conditions closer to those *in vivo*.[266]

b. GATA2 deficiency

In the GATA2 cohort, one of the main observations is that half of the individuals carrying a mutation in the *GATA2* remain, to the date of this thesis, clinically healthy or asymptomatic. Previous studies had already observed this trend, but had never calculated the clinical penetrance.[126,212] By calculating the clinical penetrance, we have corroborated that, indeed, GATA2 deficiency shows incomplete penetrance. However, the incomplete clinical penetrance *per se* requires further investigation. It has been suggested that genetic modifiers might play a role in incomplete penetrance for GATA2,[212] but this was not properly investigated. Another study has pointed towards allele specific expression as the main cause for differences in clinical penetrance.[213] Al Seraihi and colleagues[213] investigated differences among *GATA2* mutation carriers of a multiplex kindred. In that study they observed that all symptomatic individuals carried secondary genetic mutations, but that this was not enough

to account for the incomplete penetrance. They then evaluated the endogenous levels of *GATA2*, where they observed that the symptomatic individuals expressed more the mutant allele than the WT, as opposed to the asymptomatic carriers. This lead to an allelic imbalance in WT:mutant *GATA2* ratio, which was shown to be caused by epigenetic mechanisms.[213] Investigating more individuals carrying *GATA2* mutations will strengthen these findings and might, in the future, help find and alternative treatment to HSCT.

Another point worth considering of our study comes from the allele study in overexpression system. For our assays we have used embryonic kidney cells, which are quite different from the cells where GATA2 acts.[113,269] Even though our results in overexpression are robust, it could be worth testing the alleles in myeloid cell lines more closely related to GATA2, such as U937 cells of HEL-60 cells. However, the use of one of these cell lines would require more time and set up, since the allele expression would have to be by viral transduction, as opposed to the transient transfection system used for HEK293-T cells.[108,202]

Finally, we have corroborated that GATA2 is expressed in hematopoietic progenitors but not in fully differentiated leukocytes.[269] It has been observed in mice that GATA2 is most important in the cell fate of common myeloid-restricted progenitors, accounting for the lack of monocytes and dendritic cells in many patients.[270] The evaluation of several hematopoietic progenitors has already been performed for other deficiencies, where the gene's contribution could be assessed.[271] This assay could be performed in available material from GATA2 deficient patients, allowing elucidating GATA2's role in hematopoietic progenitors.

CONCLUSIONS

I. A novel form of IFN-γR2 deficiency

1. Two novel mutations in IFN-γR2, c.1A>G and c.4delC, cause autosomal recessive IFN-γR2 partial deficiency.
2. The mature protein produced by these two mutations is less expressed but its sequence is wild type.
3. The mutations c.1A>G and c.4delC in *IFNGR2* give place to reinitiation of translation in non-AUG codons within the signal peptide of the protein.
4. IFN-γR2 deficiency is these two cases is due to a quantity defect of the protein, unlike the previously reported autosomal recessive IFN-γR2 partial deficiencies, that display a quality defect.

II. A comprehensive study of GATA2 deficiency

1. AD GATA2 deficiency is dominant by haploinsufficiency and displays incomplete penetrance.
2. A diagnosis of AD GATA2 deficiency should be considered in patients of any age with mycobacterial infections, especially, but not exclusively, in the presence of other GATA2 deficiency-related phenotypes, in particular monocytopenia and/or dendritic cell deficiency.
3. All direct relatives of GATA2 deficient patients should be genotyped for the *GATA2* locus.

Taken together, these results highlight the importance of characterizing novel mutations in known genes. Even if the gene is known, without proper characterization the impact of the mutation cannot be ascertained.

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ANNEXES

I. SCID and CID causing genes

Patients with mycobacterial infections have been reported in these PIDs.

Table 1 – SCID causing genes

Disease	Affected gene	Inheritance	Protein function	Reference
T-B+ SCID				
γc deficiency	*IL2RG*	XR	Subunit of six interleukin receptors (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21)	56
JAK3 deficiency	*JAK3*	AR	Signal transducer of the γc	57
IL-7Rα deficiency	*IL7R*	AR	Alpha subunit of IL-7 receptor	58
CD45 deficiency	*PTPRC*	AR	Signaling molecule involved in cell growth and differentiation	59
T-B- SCID				
RAG1 deficiency	*RAG1*	AR	Involved in variable, diversity, joining [V(D)J] recombination for immunoglobulins	65
RAG2 deficiency	*RAG2*	AR	Involved in V(D)J recombination for immunoglobulins	65
DCLRE1C deficiency	*DCLRE1C*	AR	Involved in DNA repair and V(D)J recombination	67
Cernunnos deficiency	*NHEJ1*	AR	Involved in DNA repair	69
ADA deficiency	*ADA*	AR	Catalyzes the irreversible deamination of adenosine and deoxyadenosine in	66, 72-74

			the purine catabolic pathway	

Table 2 – CID causing genes

Disease	Affected gene	Inheritance	Protein function	Reference
CID				
DOCK2 deficiency	*DOCK2*	AR	Responsible for proper chemotaxis	77
CD40 ligand deficiency	*CD40L*	X-linked	Expressed on T cells and responsible of T cell/APC interaction and CD40-dependent IL-12 production	85, 96
ZAP-70 deficiency	*ZAP70*	AR	Interacts with CD3ζ in the T cell receptor	98
MHC class II deficiency group A	*CIITA*	AR	MHC class II transactivator,master regulator of MHC class II gene transcription	99,100
DOCK8 deficiency	*DOCK8*	AR	Involved in actin regulation	101,102
IKBKB deficiency	*IKBKB*	AR	Involved in the NF-κB canonical pathway	78,79
NIK deficiency	*MAP3K14*	AR	NF-κB inducer in the non-canonical pathway	80
CID with associated syndromic features				
Nijmegen breakage syndrome	*NBS1*	AR	Involved in repairing double strand breaks	81,82
POLE2 deficiency	*POLE2*	AR	Involved in DNA repair and replication	83
Winged helix nude FOXN1 deficiency	*FOXN1*	AR	Maintains the balance between	84,86

			growth and differentiation of the epithelial cells of the thymus	
Schimke immuno-osseous dysplasia	*SMARCAL1*	AR	Involved in chromatin remodelation	87,88
Calcium channel ORAI-1 deficiency	*ORAI1*	AR	Essential for lymphocyte activation	89,90
PNP deficiency	*PNP*	AR	Has a key role in purine catabolism essential for maintaining T cell survival	91-93
NEMO deficiency	*IKBKG*	X-linked	Involved in the NF-κB canonical pathway	94,96
EDA-ID due to IKBA mutation	*IKBA*	AD	Gain of function mutation of the inhibitor of NF-κB, IκBα	95,96

II. Selective pressure measures

*S*election *I*nference using *P*oisson *R*andom *E*ffects (SnIPRE)[180] uses a generalized linear mixed model to model genome-wide variability for categories of mutations and estimates two key population genetic parameters for each gene among which *f* quantifies the strength of purifying selection acting on human genes, with 0 corresponding to strong negative selection and 1 to neutral selection. The alignment of the hg19 human genome and the PanTro3 chimp genome from UCSC Genome Browser were used. All differences between the two species were annotated functionally by snpEff 50, using the GRCh37.65 build. All human coding sequences (CDS) >20 base pair (bp) long were retrieved and considered, for each gene, the longest transcript available. All polymorphisms identified in the WES data of phase 1 of the 1,000 Genomes Project were then retrieved. Single nucleotide polymorphisms (SNPs) that were annotated as non-synonymous or synonymous were analyzed, outside of gaps in the human-chimp alignment and polymorphic in at least one human population. Positions that were actually polymorphic in humans or chimps were removed from positions divergent between humans and chimps, using the dbSNP136 chimp database. The number of divergent and polymorphic synonymous and nonsynonymous mutations were obtained, and the proportion of synonymous and nonsynonymous sites, in a total of 18,969 genes. SnIPRE was then used to estimate the *f* parameter.

The Residual Variation Intolerance Score (RVIS)[181] uses allele frequency data to rank genes in terms of whether they have more or less common functional genetic variation relative to the genome wide expectation given the amount of apparently neutral variation the gene has. A RVIS close to 0 has a low number of functional variation, while a gene associated

with an RVIS close to 1 is subject to a high variation rate. The RVIS values calculated using the ExAC dataset were downloaded from the RVIS website.

The probability of being LOF intolerant (pLI)[182] scores the intolerance of each gene to LOF variations. The closer pLI is to 1, the more LOF intolerant the gene appears to be. The authors consider pLI >= 0.9 as an extremely LOF intolerant set of genes. The pLI scores were downloaded from the ExAC website.

III. List of publications

1: **Oleaga-Quintas C**, Deswarte C, Moncada-Vélez M, Metin A, Krishna Rao I, Kanık-Yüksek S, Nieto-Patlán A, Guérin A, Gülhan B, Murthy S, Özkaya-Parlakay A, Abel L, Martínez-Barricarte R, Pérez de Diego R, Boisson-Dupuis S, Kong XF, Casanova, JL, Bustamante J. A purely quantitative form of partial recessive IFN-γR2 deficiency caused by mutations of the initiation or second codon. ***Human Molecular Genetics***. 2018 Aug 23. ddy275.doi: 10.1093/hmg/ddy275. In Press.

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Gallin JI**, Garcés C**, Guedes M**, Hinze CH**, Holland SM**, Hughes SM**, Ibañez P**, Malech HL**, Meyts I**, Moncada-Velez M**, Moriya K**, Neves E**, Oleastro M**, Perez L**, Rattina V**, **Oleaga-Quintas C**, Warner N**, Muise AM**, López JS**, Trindade E**, Vasconcelos J**, Vermeire S**, Wittkowski H**, Worth A**, Abel L**, Dinauer MC**, Arkwright PD**, Roos D, Casanova JL, Kuijpers TW, Bustamante J. Inherited p40phox deficiency differs from classic chronic granulomatous disease. ***Journal of Clinical Investigation***. 2018 Aug 31;128(9):3957-3975. doi: 10.1172/JCI97116. Epub 2018 Aug 6. PubMed PMID: 29969437; PubMed Central PMCID: PMC6118590. *, **Equal contribution

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