Taj Ali Khan

**NOVOS DEFEITOS GENÉTICO-MOLECULARES E MECANISMOS
IMUNOPATOLÓGICOS DE PACIENTES BRASILEIROS COM
SUSCETIBILIDADE A INFECÇÕES POR MICOBACTÉRIAS**

Tese apresentada ao Programa de Pós-Graduação em
Imunologia do Instituto de Ciências Biomédicas da
Universidade de São Paulo, para obtenção do Título
de Doutor em Ciências.

São Paulo
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Supervisor: Prof. Dr. Antônio Condino Neto

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"Knowledge was among the first things that ALLAH (GOD) gave to the human beings. The first verse of the Quran revealed to the Prophet (P.B.U.H) started with the word "IQRA" (read). The verse was, in fact, addressed not to the Prophet (P.B.U.H) alone but to the whole of mankind through him"
(TAJ ALI KHAN)

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A Comissão de *Ética em Pesquisas com Seres Humanos* do ICB, na sessão realizada no dia 19.10.2011, **APROVOU** o projeto intitulado: "*Defeitos da imunidade inata associados à infecção por micobactérias*" sob responsabilidade de execução dos autores Prof. Dr. ANTÔNIO CONDINO NETO e o aluno TAJ ALI KAHN.

Cabe os pesquisadores executantes elaborarem e apresentar a este Comitê, relatórios anuais (parciais ou final), de acordo com a resolução 196/06 do Conselho Nacional da Saúde, item IX. 2 letra c.

O primeiro relatório deverá ser encaminhado à Secretaria deste CEP em **19.10.2012.**

Atenciosamente,

Prof. Dr. PAULO M.A. ZANOTTO
Coordenador da Comissão de Ética em
Pesquisas com Seres Humanos - ICB/USP

"I dedicate my thesis: to my loving father, my late mother, my brothers and sisters who always advise me to be conscientious and persevering, who exhort me to have a staunch faith in GOD while pursuing my goals in life. I dedicate my thesis to all the physicians, patients; to my Brazilian mother Sussu, Brazilian brother Otavio Cabral Marques and German sister Lena F. Schimke for being there for me when I was stressed out, for being reassuring and loving whenever I got nostalgic, for encouraging me to keep going despite all the difficulties; last but not the least to my supervisor Professor Antonio Condino Neto for being so supportive, affectionate, patient, trusting and providing me the opportunity to work under his kind supervision. In a nutshell, there is no doubt in my mind that without everyone's continued support and counsel I could not have completed this task. I hope this work will contribute to the public health community"

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ABSTRACT

Taj Ali Khan. Novel molecular genetic defects and immunopathological mechanisms in Brazilian patients with mycobacterial diseases. [Ph. D. thesis (Immunology)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2014.

The occurrence of mycobacterial infections in human subjects suggest impaired anti-mycobacterial immunity and host susceptibility factors remain to be identified. We aimed to characterize novel genetic defects and immunopathological mechanisms in Brazilian patients with susceptibility to mycobacterial diseases. Clinical data of 17 patients were obtained from the medical records. Patients with possible severe combined immunodeficiency (SCID) and CD40L deficiency were screened by lymphocyte count and measurement of immunoglobulin levels followed by CD40L protein expression on CD4⁺ T cell. Peripheral blood mononuclear cells (PBMCs) from the patients and healthy controls were evaluated for the ability to produce IFN- γ and IL-12, to phosphorylate STAT1 (in monocytes) and STAT4 (in lymphocytes), and to express the IFN- γ and IL-12 receptors in order to screen or confirm possible defects in the IL-12/ IFN- γ axis. In addition, defects in toll-like receptor (TLR) signaling were analyzed by measuring the shedding of CD62L on neutrophils, TLR expression, and I κ B α degradation in monocytes. The later was also performed to screen patients with impaired NF- κ B signaling. Defects of NADPH oxidase components were evaluated by dihydrorhodamine (DHR) assay and NADPH proteins expression. Genetic analysis was performed by Sanger sequencing and whole exome-sequencing. Monocytes-derived macrophages (MDMs) were evaluated for their capacity to phagocytose and control intracellular *Mycobacterium tuberculosis* growth. The patients developed different mycobacterial diseases and *M. tuberculosis* was the most frequent species. Different functional abnormalities were found: six patients (P8, P9, P10, P11, P12 and P15) presented impaired ability to produce IFN- γ and four patients (P12, P14, P15 and P16) failed to produce IL-12; defective STAT4 phosphorylation and reduced expression of IL-12 receptors were identified in two patients (P8 and P9). Neutrophils from P7 displayed impaired CD62L shedding following activation with BCG or Pam2CSK4 (TLR2/6 agonist) and neutrophils from P13 showed reduced CD62L shedding in response to BCG, Pam2CSK4 or LPS (TLR4 agonist). In accordance, monocytes from P7 and P13 demonstrated defective I κ B α -degradation in response to the above mentioned agonists and only P7 had defective expression of TLR2. A defect in TLR pathway was identified in another patient (P10) whose lymphocytes displayed reduced expression of TLR10. Failure in oxidative burst was demonstrated by phagocytes from P6 and defective p47^{phox} expression was identified. Genetic analysis revealed mutations in different genes: *RAG1* (P1), *CD40LG* (P2, P3, P4), *NEMO* (P5), *NCF1* (P6), *TLR2* (P7) *IL-12R β 2* (P8), *IL-12R β 1* (P9), *TLR10* (P10), *DKC1*(P11), *SOCS-1*(P12) and *IRAK2* (P13). Finally, MDMs from patients phagocytose normally but were unable to appropriately control intracellular *M. tuberculosis* growth in comparison to MDMs from healthy subjects. We concluded that the Brazilian patients have heterogeneous mutations previously associated with susceptibility to mycobacterial diseases and novel genetic variations were identified suggesting novel PIDs. In addition, the inability of MDMs to control the intracellular growth of *M. tuberculosis* indicates this contributes to patients' susceptibility to mycobacterial infections.

Keywords: Mycobacterial infection. Mutation. Macrophage.

RESUMO

Taj Ali Khan. Novos defeitos genético-moleculares e mecanismos imunopatológicos de pacientes brasileiros com suscetibilidade a infecções por micobactérias. [Tese (Doutorado em Imunologia)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2014.

Pacientes com suscetibilidade a infecções por micobactérias indicam falhas na resposta imune e factores genéticos predisponentes que necessitam ser estudados. Objetivamos identificar novos defeitos genéticos e mecanismos imunopatológicos em pacientes brasileiros com suscetibilidade a infecções por micobactérias. Dados clínicos de 17 indivíduos foram obtidos dos prontuários médicos. Possíveis pacientes com imunodeficiência combinada grave e deficiência de CD40L foram selecionados por imunofenotipagem e valores séricos dos isotipos de imunoglobulina, bem como análise da expressão da proteína CD40L na superfície das células T CD4⁺. Para selecionar ou confirmar possíveis defeitos no eixo IL-12/IFN- γ , células mononucleares do sangue periférico (PBMCs) foram avaliados quanto à capacidade de produzir IL-12 e IFN- γ , fosforilar as moléculas STAT1 (em monócitos) e STAT4 (em linfócitos) e expressar os receptores de IL-12 (IL-12R) e IFN- γ . Além disso, defeitos na sinalização via receptores semelhantes ao toll (TLRs) foram analisados em neutrófilos através da avaliação do “shedding” da molécula CD62L e expressão dos TLRs. Defeitos do complexo NADPH oxidase foram investigados por dihidrorodamine (DHR) e expressão dos componentes da NADPH. A análise genética foi realizada por sequenciamento Sanger e “whole exome sequencing”. Macrófagos derivados de monócitos (MDM) foram avaliados quanto à capacidade de fagocitar e controlar o crescimento intracelular da *Mycobacterium tuberculosis*. Infecções por diferentes espécies de micobactérias foram apresentadas pelos pacientes, sendo *M. tuberculosis* a espécie mais frequentemente identificada. Diferentes disfunções foram encontradas: seis pacientes (P8, P9, P10, P11, P12 and P15) apresentaram reduzida produção de IFN- γ e quatro pacientes (P12, P14, P15 and P16) baixa produção de IL-12; falhas na fosforilação do STAT4 e reduzida expressão do IL-12R foram identificados em dois pacientes (P8 e P9). Os neutrófilos dos pacientes P7 e P13 demonstraram reduzido “shedding” da molécula CD6L após ativação com BCG ou Pam₂CSK₄ (agonista de TLR2/6) e P4 em resposta à BCG, Pam₂CSK₄ ou LPS (agonista de TLR4). Coerentemente, os monócitos do paciente P13 demonstraram reduzida degradação do I κ B α em resposta aos agonistas indicados acima e falhas na expressão do TLR2. Reduzida expressão do TLR10 foi identificado no paciente P10. Os fagócitos do P6 apresentaram defeito na explosão respiratória e na expressão da molécula p47^{phox}. Mutações em diferentes genes foram encontradas: *RAG1* (P1), *CD40LG* (P2, P3, P4), *NEMO* (P5), *NCF1* (P6), *TLR2* (P7), *IL-12R β 2* (P8), *IL-12R β 1* (P9), *TLR10* (P10), *DKC1* (P11), *SOCS-1* (P12) e *IRAK2* (P13). MDMs dos pacientes fagocitaram normalmente *M. tuberculosis*, porém reduzida capacidade em inibir o crescimento da *M. tuberculosis* foi observada. Concluimos que os pacientes estudados possuem defeitos moleculares heterogêneos e que os MDMs desses indivíduos apresentam falhas no controle do crescimento da *M. tuberculosis*. Nossos dados sugerem que esses são fatores subjacentes à susceptibilidade a infecções por micobactérias nesses indivíduos.

Palavras-chave: Infecções por micobactérias. Mutações. Macrófagos.

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LIST OF ABBREVIATIONS

BCG	Bacille Calmette-Guérin
CD	cluster of differentiation
CGD	chronic granulomatous disease
CD40L	CD40 ligand
CD40LG	CD40 ligand gene
CFU	colony forming units
CMV	cytomegalovirus
CYBB	cytochrome b beta
DCs	dendritic cells
DKC1	dyskeratosis congenita 1
DHR	dehydrohodamine
cDNA	complementary <i>deoxyribonucleic acid</i>
DNA	deoxyribonucleic acid
DNAg	genomic <i>deoxyribonucleic acid</i>
DEPC	diethylpyrocarbonate
dNTP	deoxyneucleotide P
DKC	dyskeratosis congenita
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
GATK	genome analysis toolkit
GM-CSF	<i>granulocyte macrophage colony-stimulating factor</i>
H₂O₂	hydrogen peroxide
HIGM	hiper IgM syndrome
IFN-γ	interferon-gamma
IL	interleukin
IKBKG	inhibitor of kappa light polypeptide gene enhancer in B-cells
IRAK2	interleukin-1 receptor-associated kinase 2
IL-12Rβ1	interleukin 12 receptor betta 1
JAK	janus associated kinase
L-selectin	<i>L-selectin</i> or CD62L <i>antigen ligand</i>
LPS	lipopolysaccharide
MDMs	monocytes derived macropahes
MSMD	mendelian susceptibility to mycobacterial disease
MFI	<i>median fluorescence intensity</i>
MyD88	Myeloid differentiation primary response 88
MDMs	monocyte-derived macrophages
NK	natural killer
NCF1	neutrophils cytosolic facto 1
NF-κB	<i>nuclear factor-kappa B</i>
NADPH	nicotinamide adenine dinucleotide phosphate
NEMO	NF-kappa-B essential modulator
NTM	nontuberculosis mycobacteria

PIDs	primary immunodeficiency diseases
PBMCs	Peripheral blood mononuclear cells
PBMCs	Peripheral blood mononuclear cells
PCR	polymerase chain reaction
PBS	phosphate buffered saline
Phox	<i>phagocyte oxidase</i>
PSI-BLAST	position-specific Iterative basic local alignment search tool
PMA	<i>phorbol 12- myristate 13-acetate</i>
RSV	respiratory syncytial virus
RAG1	recombinant activating gene 1
PRR	pattern recognition receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
rhIFN-γ	human recombinant IFN- γ
RT-PCR	<i>reverse transcriptase-polimerase chain reaction</i>
R-10	Meio RPMI 1640 supplemented with 10% serum fetal bovine
RNI s	reactive nitrogen intermediates
SCT	stem cell transplantation
STAT1	signal transducer and activator of transcription-1
STAT4	signal transducer and activator of transcription-4
SOCS-1	suppressor of cytokine signaling 1
SCID	severe combined immunodeficiency
SNP	single nucleotide polymorphism
TNF-α	tumor necrosis factor alpha
TB	tuberculosis
TH-1	T helper 1
TLR	toll-like receptor
UTI	urinary tract infection
XL-EDA-ID	X-linked anhidrotic ectodermal dysplasia with immunodeficiency

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1 INTRODUCTION

The genus *mycobacterium* contains several species, many of which cause human diseases. The *Mycobacterium tuberculosis* and three closely related *mycobacterium* species (*M.bovis*, *M.africanum*, *M.microti*) cause tuberculosis disease, composing what is known as *Mycobacterium tuberculosis complex*. Among these pathogens, *Mycobacterium tuberculosis* is the strict causative agent of *tuberculosis*. Mycobacteria other than those above described which do not make up the *Mycobacterium tuberculosis* complex are called nontuberculosis or atypical mycobacteria. Atypical mycobacteria historically recognized causing mycobacterial disease include *M.avium complex* (*M .avium* and *M. intracellulare*), *M.kansasii*, *M.furtuitum*, *M.chelonae*, *M.abscessus*, *M.gordonae*, *M.szulgae*, *M.marinum*, *M.ulcerans*, and *M.scrofulaceum* (Rastogi et al., 2001)

Tuberculosis (TB) remains a scourge of humanity and a major global health problem, presenting high incidence in Brazil (Cavalcanti et al., 2012). The global prevalence of *M. tuberculosis* infection is about 32%. It is estimated that about one third of the human population carry this pathogen and that the worldwide death toll is over one million per year (Deffert et al., 2014a; Dye et al., 1999) *M. tuberculosis* is spread by small airborne droplets, called droplet nuclei, generated by the coughing, sneezing or talking from a person with pulmonary or laryngeal tuberculosis. These minuscule droplets can remain airborne for minutes to hours after expectoration (Lee et al., 2005). Introduction of *M. tuberculosis* into the lungs leads to infection of the respiratory system (Pulmonary TB); however, the organisms can spread to other organs, such as the lymphatic system, pleura, bones/joints, or meninges, and cause extrapulmonary tuberculosis or disseminated tuberculosis (Brosch et al., 2002).

Bacteria in droplets that bypass the mucociliary system and reach the alveoli are quickly surrounded and engulfed by alveolar macrophages, the most abundant immune effector cells present in alveolar spaces (Korf et al., 2006). The subsequent phagocytosis by macrophages followed by mycobacterial growth control are main events that results in either successful control of the infection followed by latent tuberculosis or progression to active disease called primary progressive tuberculosis.

(Knechel, 2009). One-third of the world's population is infected with *Mycobacterium tuberculosis* and remains asymptomatic. Of the total infected latent individuals, only 5–10% will develop active tuberculosis disease in their lifetime suggesting an underlying genetic background in these subjects (**Figure 1**)

Figure 1 *M. tuberculosis* infection: active or latent TB

Mycobacterium tuberculosis exposure and infection. Exposure to *Mycobacterium tuberculosis* (MTB) can result in different clinical outcomes, which include the absence of any clinical or laboratory evidence of infection, infection without clinically active disease and active disease (O'Garra et al., 2013).

1.1 Immunity to Mycobacteria diseases

The course of mycobacterial infections is dependent on the interactions of the mycobacterium and the immune response of the host. Upon the first encounter with mycobacteria the innate arm of the immune system is rapidly activated and the adaptive arm of the immune system synergistically cooperates to control the mycobacteria growth and spreading (**Figure 2**).

Elimination of *M. tuberculosis* infection mainly depends on the success of the interaction between infected macrophages and T lymphocytes despite the involvement of other cells (O'Garra et al., 2013). Macrophages are important effector cells in immunity against intracellular bacteria. On infection, macrophages (MO) recognize the mycobacteria by Toll like receptor (TLR) engagement (especially TLR1/2, TLR2/6) followed by phagocytosis and control of mycobacteria growth. In addition macrophages and dendritic cells also secrete cytokines such as IL-12 and IL23 to induce IFN- γ production by T and NK cells, which in turn, increases the phagocytosis, phago-lysosomal fusion, oxidative burst and other not fully clear non-oxidative mechanism (Errante et al., 2008). For an efficient T helper 1 (Th1, IFN- γ producer cells) differentiation, co-stimulation (e.g CD40L-CD40, CD28-CD80/CD86 interactions) and NEMO/NF- κ B dependent signaling (Nunes-Alves et al., 2014) are required. On other hand, the negative regulation of IFN- γ production involves different mechanism, including production of Th2 cytokines (IL-4, IL-5, IL-13) (Romani, 2011) and the participation of suppressor of cytokine signaling (SOCS)-1. In accordance, patients with severe pulmonary tuberculosis have been reported to have skewed Th1 to Th2 immune response (Wang et al., 2002) and raised SOCS-1 levels (Masood et al., 2012).

Individuals with defects in the innate or adaptive immunity affecting the above described mechanisms exhibit increased susceptibility to mycobacterial infections (Patel et al., 2008). Therefore, to better understand the immune response to mycobacterial diseases, we describe as follows the primary immunodeficiency disorders (PIDs) in which the occurrence of mycobacterial illustrate the important mechanism of immune protection against Mycobacteria species.

Figure 2 Immunity to mycobacteria

Patients with tuberculosis develop immunological signs of infection; others can eliminate the bacteria during the innate immune phase, without generating T cell memory. T cells are engaged by antigen-presenting cells, and this generates effector and memory T cells (both effector memory T (T_{EM}) and central memory T (T_{CM}) cells). B cells are also activated and *M. tuberculosis* specific antibodies are produced. Infected macrophages and giant foam cells, surrounded by T_{CM} and B cells. An optimal T helper (TH) cell balance is required to control *M. tuberculosis* while limiting immunopathology. This balanced reaction includes pro-inflammatory TH1-type responses (characterized by interferon- γ (IFN γ), tumor necrosis factor (TNF)- α and interleukin-12 (IL-12) production) and TH17-type responses (characterized by IL-17 production). TH2-type responses (associated with IL-4 production) and regulatory T (Treg) cell phenotypes that limit immunopathology (Walzl et al., 2011).

1.2 Mycobacterial infections in primary immunodeficiency diseases

Although previous studies have uncovered some of the genes involved in human predisposition to mycobacterial infections, a comprehensive understanding of genetic susceptibility factors remains elusive. There are major lines of evidence to support a role for host genetics in TB. First, twin studies indicate that TB rates among monozygotic twins

are more than twice the rate of dizygotic twins. Second, several PIDs are associated with susceptibility to mycobacteria (Berrington and Hawn, 2007). PIDs are inherited immune system defects leading to susceptibility to a variety of pathogens. More than 150 types of PIDs with distinct underlying gene defects have been identified so far (Geha et al., 2007). A number of PIDs result in an increase susceptibility to severe mycobacterial disease including those from the Mendelian susceptibility to mycobacterial diseases (MSMD), severe combined immunodeficiency (SCID), defects in the TLRs signaling, CD40L deficiency and chronic granulomatous disease (CGD) (Lee et al., 2011; Norouzi et al., 2012; Uciechowski et al., 2011) which will be described in details below. However, other PIDs in patients who developed mycobacterial diseases remain to be identified.

1.1.1 Well defined PIDs presenting predisposition to mycobacterial infections

Natural human immunity to the mycobacteria relies on the functional IL-12/IFN- γ integrity which is a connection between macrophages and T lymphocyte/NK cells. Following recognition and phagocytosis of mycobacteria by macrophages, macrophages produce IL-12 which in turn, activates in T cells the phosphorylation of the signal transducer and activator of transcription-4 (STAT4). STAT4 translocates to the nucleus where it induces the IFN- γ production which in turn modulates the macrophages activation enhancing the control of mycobacteria growth. Patients with PIDs impairing the IL-12/ IFN- γ circuit and/or the oxidative burst may present increased susceptibility to mycobacterial disease.

1.1.1.1 Severe combined immunodeficiency (SCID)

SCID is a PID caused by mutations in different genes which products are crucial for the development and function of both T and B cells. The most common defects are due to mutations in the genes *IL2RG*, *JAK3*, *IL7RA*, *RAG1*, *RAG2*, and *ADA*. All identified defects are autosomal recessive, except for the *IL2RG* defect that causes X-linked SCID. SCID can be subdivided based on different peripheral lymphocyte immunophenotyping as T-B+NK+ (absent circulation T but B and NK cells present), T-B+NK- (absent T and NK but B cells present), T-B-NK+ (both T and B cells absent but NK cells present), or T-B-NK- (T, B and NK cells are absent), regardless of the functional status of these cells (Al-Herz et al., 2014b).

Children with different SCID forms are vulnerable to almost all classes of microorganisms in the first months of life and are characterized by recurrent severe life

threatening infections (Buckley, 2004; Petricevich et al., 2001) such as chronic diarrhea, interstitial pneumonia and/or therapy-resistant mucocutaneous candidiasis. Infections with opportunistic pathogens, such as *Pneumocystis jiroveci*, *Listeria* sp., *Salmonella typhi*, *Toxoplasma*, *Mycobacterium* sp., *Aspergillus* spp, adenovirus, respiratory syncytial virus (RSV), cytomegalovirus (CMV), herpes simplex virus (HSV) and Epstein - Barr virus (EBV) are often present. Of note, the BCG vaccination causes disseminated infections and mycobacterial infections other than BCG complications may be lethal in SCID patients. Infiltrating and ulcerating lesions at the site of the BCG vaccination and in the regional lymph nodes, and even systemic propagation with papular cutaneous lesions, osteolytic lesions and organ impairment (liver, spleen, lymph-node and lung) may occur (Lee et al., 2011). The lack of adaptive immune response including the absence of IL-12/IFN γ axis, results in defective innate immune development accounting for the susceptibility to mycobacterial disease in SCID patients. Several cases of disseminated mycobacterial infections have been described in SCID patients (Casanova and Abel, 2002; Sadeghi-Shabestari and Rezaei, 2009). Mostly children with SCID present disseminated disease after BCG vaccination but localized BCG infections have also been described (Reichenbach et al., 2001).

1.1.1.2 CD40L deficiency

Mutations in the CD40 ligand gene (*CD40LG*), encoding a surface protein expressed mainly by activated CD4⁺ T cells causes the X-linked hyper-IgM syndrome (X-HIGM) or CD40L deficiency (Fuleihan et al., 1993a; Reichenbach et al., 2001). CD40 is expressed on macrophages, dendritic cells (DC) and B cells (antigen-presenting cells, APC). CD40L deficiency results in impaired defective humoral immune response (Fuleihan et al., 1993b; Allen et al., 1993; DiSanto et al., 1993; Korthauer et al., 1993; Aruffo et al., 1993) and impaired DCs/T cell crosstalk resulting in skewed Th1 (IFN- γ producer cells) to Th2 (IL-4 and IL-5 producer cells) (Cabral-Marques et al., 2012). Therefore, in addition to extracellular and intracellular bacterial infections, CD40L-deficient patients are susceptible to fungal infections, particularly *Pneumocystis jiroveci* pneumonia, and infections with *Candida albicans*, *Cryptococcus* and *Histoplasma* (Nonoyama et al., 1997) and noteworthy the occurrence of mycobacterial infections in these patients have been described (Cabral-Marques et al., 2014; Levy et al., 1997; Wang et al., 2014). The clinical spectrum of mycobacteria infections in CD40L deficiency ranges from moderate BCGitis manifestations to severe central nervous system involvement and disseminated *M. tuberculosis* infection.

1.1.1.3 NF κ B signaling defects

X-linked anhidrotic ectodermal dysplasia with immunodeficiency (XL-EDA-ID) is a rare PID caused by hypomorphic mutations in the gene encoding IKK- γ /NEMO, a protein essential for activation of the ubiquitous transcription factor NF-Kb (Ngadjeua et al., 2013). Patients with NF-kappa-B essential modulator (NEMO) are susceptible to multiple infectious agents, including pyogenic bacteria, viruses and mycobacteria. The occurrence of atypical mycobacterial disease is a relatively common manifestation in defects of NF- κ B signaling (NEMO and I κ Ba defects). The immunodeficiency in patients with XL-EDA-ID is characterized by hypogammaglobulinemia, defective specific antibody production, and impair CD40-triggered induction of IL-12 production by T cells (Dai et al., 2004). NEMO is part of a kinase complex responsible for the I κ B α phosphorylation and consequently I κ B α degradation followed by NF- κ B translation to the nucleus (Doffinger et al., 2001). Given that NF- κ B plays pleiotropic roles in the activation of immune response including regulation of T cell development, toll receptor signaling pathways and activation of IL-12/IFN- γ axis, patients with NEMO and I κ B α deficiencies are prone to a variety of opportunistic infections including those caused by mycobacteria sp. (Lee et al., 2011).

1.1.1.4 Defects in Toll-like receptor pathways

TLRs are phylogenetically conserved mediators which are essential for microbial recognition on macrophages and DC cells. TLRs are one of the four families of pattern recognition receptors (PRR) that were first discovered in 1985; they received their name from their similarity to the protein coded by the Toll gene identified in *Drosophila melanogaster* by Christiane Nusslein Volhard (Hansson and Edfeldt, 2005). To date, 10 members of TLRs have been identified in humans with important function in host defense in early steps of the inflammatory response. TLRs are present in the plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) and endosome (TLR3, TLR7, TLR8, TLR9) of leukocytes (Lim and Staudt, 2013). The cytoplasmic domain of TLR is homologous to the signaling domain of Interleukin-1-receptor (IL-1R) and links to Interleukin-1-receptor-associated-kinases (IRAKs), serine kinases that activates transcription factors like NF- κ B to signal the production of cytokines e.g. TNF- α , IL-1 β , IL-6 and IL-12 (Basu et al., 2012; Schmitz et al.,

2001; van Crevel et al., 2002; Oddo et al., 1998) (**Figure 3**). MyD88, IRAK4 and IRAK2 are critical signaling mediators of the TLRs (Lin et al., 2010).

Figure 3 Immune recognition of *M. tuberculosis* by macrophages

Upon encountering mycobacteria, macrophages are stimulated by mycobacterial-associated molecular patterns, which interact with TLRs on the cell surface of the macrophages. The macrophages are stimulated to produce cytokines (TNF-α, IL-6, IL-1β, IL-12) and to control mycobacteria replication (van Crevel et al., 2002).

TLRs sense microbial products and play an important role in innate immunity. Patients with defective TLR signaling suffer from a broad spectrum of infectious diseases (Puel et al., 2005). So far, TLR2 is the main TLR demonstrated to be critical for the immune response to mycobacterial infections. Mutations in the TLR2 (Bhide et al., 2009; Kang and Chae, 2001) have been shown to confer susceptibility to mycobacteria infections. Mycobacterial lipoarabinomannan (LAM) are recognized by TLR2 which heterodimerizes with TLR1 and TLR6 (van Crevel et al., 2002). In addition, TLR2 cooperates with TLR9 which recognizes *M. tuberculosis* unmethylated CpG (Berrington and Hawn, 2007; Brightbill et al., 1999; Underhill et al., 1999) and with TLR10 which mycobacterial ligand is unknown. However,

mutations in TLR10 can predispose to susceptibility to mycobacterial diseases (Guan et al., 2010; Ma et al., 2007).

1.1.1.5 *Chronic granulomatous diseases (CGD)*

IFN- γ is well known to enhance the oxidative burst of phagocytes which in turn, is essential for the immune response against a variety of microorganisms including mycobacteria. The importance of the oxidative burst is demonstrated by patients with chronic granulomatous disease (CGD) that is caused by genetic abnormalities affecting the phagocyte NADPH oxidase and results in failure of neutrophils and monocytes/macrophages to generate reactive oxygen species (ROS). Consequently these patient phagocytes are unable to kill fungi and intracellular bacteria. The phagocyte NADPH oxidase complex consists of two membrane bound proteins (gp91phox and p22phox) which together form the cytochrome b_{558} and three cytosolic proteins (p47phox, p67phox, and p40phox) (**Figure 4**). Upon cell activation these proteins and the small GTPase Rac assemble at the cell membrane and phagolysosomes to trigger the oxidative burst, essential for microbial killing (Song et al., 2011).

The incidence of CGD is approximately 1 in 250,000 live births (Winkelstein et al., 2000). Among the NADPH oxidase genes, *CYBB* encodes gp91phox and is located on X chromosome and accounts for about 65% of CGD cases. Mutations in the autosomal genes e.g *CYBA* (encoding p22phox), *NCF1* (encoding p47phox), *NCF2* (encoding p67phox), and *NCF4* (encoding p40phox) cause autosomal recessive (AR)-CGD. The most common form of AR-CGD is caused by mutations in *NCF1* (25% of patients) followed by mutations in *NCF2*, *CYBA*, and *NCF4* (Gardiner et al., 2013; Song et al., 2011).

Figure 4 The NADPH oxidase system

The

assembly of NADPH oxidase components, the molecular genetics and the prevalence of CGD subtypes is shown. Adated from (Song et al., 2011)

1.1.1.6 Mendelian susceptibility to Mycobacterial Disease

The idea that life-threatening infectious diseases occurring in otherwise healthy children, during the course of primary infection, may result from single-gene inborn errors of immunity is gaining ground (Alcais et al., 2009; Alcais et al., 2010; Casanova and Abel, 2007). One of the most thoroughly investigated pediatric syndromes is Mendelian susceptibility to mycobacterial disease (MSMD) (OMIM 209950), a rare disorder (affecting about 1 in 100 000 individuals), predisposing individuals to severe clinical disease upon infection with weakly virulent mycobacteria, including the Bacille Calmette-Guérin (BCG). These patients are frequently susceptible to *Salmonella* and *M. tuberculosis*. However, the genetic etiologies of many patients with MSMD remain to be identified (Bogunovic et al., 2013; Casanova and Abel, 2002). Genetic dissection of MSMD has revealed mutations in seven autosomal genes: *IFNGR1*, *IFNGR2*, *STAT1*, *IL-12p40*, *IL-12RB1*, *IL-12RB2*, *IRF8*, *ISG15* (autosomal defects); and two X-linked: NF-kappaB essential modulator (NEMO) gene and *CYBB*. A schematic representation of known genetic etiologies of MSMD and the IL-12/IFN- γ mediated immunity to mycobacteria is illustrated in **Figure 5** and described in **Table 1**.

Figure 5 Inborn errors of IL-12/IFN- γ circuit in patients with MSMD

Proteins
for which
mutations
in the

corresponding genes have been associated with MSMD are shown in red.

Table 1 Genetic defect that cause Mendelian Susceptibility to Mycobacterial Disease

MSMD gene	Year of publication	Mode of inheritance	Reference
<i>IFN-γR2</i>	1996	AR/AD	(Jouanguy et al., 1996)
<i>IFN-γR1</i>	1998	AR	(Dorman and Holland, 1998)
<i>IL-12B</i>	1998	AR	(Altare et al., 1998)
<i>STAT1</i>	2001	AR/AD	(Dupuis et al., 2001)
<i>IL-12Rβ1</i>	2004	AR	(Fieschi et al., 2004)
<i>NEMO</i>	2006	X-linked	(Filipe-Santos et al., 2006)
<i>CYBB</i>	2011	X-linked	(Bustamante et al., 2011)
<i>IRF8</i>	2011	AR	(Hambleton et al., 2011)
<i>ISG15</i>	2012	AD	(Bogunovic et al., 2012)
<i>IL-12R β2</i>	2012	AR	(Kong et al., 2013)

AR,
autos
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AD,
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nant

In some cases depending upon the location and type of mutation, molecular genetic defects in the same gene can lead to autosomal dominant or recessive mode of inheritance. For instance, some mutations leading to partial STAT1 deficiency are autosomal recessive while mutations resulting in complete loss of function (dominant-negative mutations) are autosomal dominant. However, a variety of other genetic defects leading to different STAT1 deficiencies have been previously described (Boisson-Dupuis et al., 2012).

Two X-linked MSMD forms occur due to specific mutations in CYBB. Jacinta et al (Bustamante et al., 2011) reported specific CYBB mutations (p.Q231P and p.T178P) and Filipe-Santos and co-workers reported that mutations (p.E315A and p.R319Q) in the leucine zipper (LZ) domain of the NEMO gene in three unrelated kindreds (Filipe-Santos et al., 2006). The CYBB mutations result in an impaired respiratory burst in monocyte-derived

macrophages but not in monocytes or granulocytes. The macrophage-specific functional consequences of the germline mutation resulted from cell-specific impairment in the assembly of the NADPH oxidase. On the other hand, the NEMO mutant proteins were produced in normal amounts in blood and fibroblastic cells. However, the patients' monocytes presented an intrinsic defect in T cell-dependent IL-12 production, resulting in defective IFN- γ secretion by T cells. IL-12 production was also impaired as the result of a specific defect in NEMO- and NF-kappaB/c-Rel-mediated CD40 signaling after the stimulation of monocytes and dendritic cells by CD40L-expressing T cells and fibroblasts, respectively. However, the CD40-dependent up-regulation of costimulatory molecules of dendritic cells and the proliferation and immunoglobulin class switch of B cells were normal. Moreover, the patients' blood and fibroblastic cells responded to other NF-kappaB activators, such as TNF- α , IL-1 β , and lipopolysaccharide (LPS).

Except the cases described above, patients with NEMO and CYBB deficiencies can develop a wide spectrum of infections caused by different etiological agents besides the increased susceptibility to mycobacterial infections.

2 OBJECTIVES

2.1 General Objectives

To characterize the molecular genetic defects and immunopathological mechanisms underlying the susceptibility to mycobacterial disease in Brazilian patients.

2.2 Specific objectives

- a) To characterize the clinical features of Brazilian patients with mycobacterial diseases;
- b) To screen the patients with mycobacterial diseases for well known PIDs:
 - ✓ SCID: lymphocyte subpopulations immunophenotyping
 - ✓ CD40L deficiency: quantifying the immunoglobulin serum levels and CD40L expression
 - ✓ NF κ B signaling defects: analyzing the I κ B α degradation
 - ✓ TLR defects: evaluating CD62L shedding following TLR agonist stimulation
 - ✓ CGD: measuring the oxidative burst and NADPH expression
 - ✓ Defects of IL-12/IFN- γ axis: measuring the cytokine production and phosphorylation of STAT1 and STAT4
- c) Characterize by Sanger sequencing the molecular genetic defects after the functional screening (objective b)
- d) To identify by whole exome sequencing (WES) novel molecular genetic defects and candidate genes for new PIDs
- e) To analyze the phagocytosis of *M. tuberculosis* by monocytes-derived macrophage (MDM) and their ability to control the *M. tuberculosis* growth.

3 PATIENTS, MATERIALS AND METHODS

3.1 Cohort characteristics

Relevant clinical data of all patients were obtained from the medical records. 18 patients with clinical history of mycobacterial diseases were studied. All patients are born of non-consanguineous marriages except P12 who was born to consanguineous parents (**Figure 6**).

Figure 6 Pedigree of Brazilian patients with mycobacterial diseases

Signed consent forms were obtained from all patients or their parents, and blood samples were collected under institutional guidelines. The study was approved by the Ethics Committee at the Institute of Biomedical Sciences, University of São Paulo, according to the Helsinki Convention and the Brazilian Department of Health. The minimum and maximum age of patients at the time of disease onset were 2 months and 20 years, respectively. The minimum and maximum age at the clinical diagnosis of PID were 2 months and 36 years, respectively. The minimum and maximum age at the time of molecular diagnosis were one year and 36 years, respectively (**Table 2**).

Table 2 Age of first manifestation, clinical and genetic diagnosis of patients with susceptibility to mycobacterial disease

Pt	Gender	Date of birth	First symptoms (years)	Clinical diagnosis of PIDs (years)	Molecular diagnosis (years)
P1	Male	2006	1	6	7
P2	Male	2007	<1	<1	6
P3	Male	2006	<1	1	7
P4	Male	1993	<1	1	15
P5	Male	2012	<1	<1	1
P6	Female	1997	<1	1	16
P7	Male	2009	3	3	4
P8	Female	1979	36	36	37
P9	Male	2010	2	2	3
P10	Female	2012	<2	<1	1
P11	Male	2003	8	8	10
P12	Female	1991	3	4	22
P13	Female	2001	<1	11	12
P14	Male	20000	4	7	NI
P15	Female	2012	<1	<1	NI
P16	Male	2002	10	10	NI
P17	Female	2014	<1	<1	NI
P18	Female	1993	<1	2	NI

NI, not identified

Mycobacterial infections were caused by different mycobacterium species. The predominant species was *M. tuberculosis* followed by *M. bovis* (BCG) (Table 3). Clinical manifestations of some patients are shown in Figure 7.

Table 3 Infections and etiologic agents of patients with mycobacterial disease

Pt	BCGitis	clinical manifestation - <i>Mycobacterium sp</i>	Other infections - etiologic agents**
P1	+	disseminated TB - <i>M. avium complex</i>	pneumonia - <i>H. influenza</i> , diarrhoea-Rotavirus , bronchitis, juvenile xanthogranulomatous, hear loss, anemia
P2	-	disseminated TB - <i>M. tuberculosis</i>	pneumonia - <i>P. jirovecii</i> , otitis, pneumonias, sinusitis, stomatitis
P3	-	disseminated TB - <i>M. tuberculosis</i>	pneumonia, respiratory failure
P4	-	disseminated TB - <i>M. tuberculosis</i>	paracoccidioidomycosis - <i>P. brazilienses</i> , pneumonia - <i>P. jirovecii</i> -, otitis
P5	+	-	Pneumonia - <i>K.pneumoniae</i> , pneumonia- <i>S.aureus</i> ,
P6	-	pulmonary TB - <i>M. tuberculosis</i>	mucomycosis - <i>C.albican</i> , pneumonia, fungal endocarditis - <i>C.albican</i>
P7	-	pulmonary TB - <i>M. tuberculosis</i>	leishmania- <i>L.Visceral</i> , pneumonia- <i>K.multi</i> Resistance, persistent fever hepatosplenomagly, adenopathy
P8	-	pulmonary TB - <i>M. tuberculosis</i>	cryptococcal infection - <i>Cryptococcus gatti</i> , recurrent fever, chest pain, pneumonia
P9	-	pulmonary TB - <i>M. tuberculosis</i>	hypertonia, lymphadenopathy, tonsillitis
P10	+	-	hyperplasia, fever, lymphadenopathy
P11	-	<i>M.tuberculosis</i> - disseminated TB	Otitis, pneumonias, tonsillitis, meningitis, sepsis, diarrhea
P12	-	pulmonary TB - <i>M. tuberculosis</i>	Leishmania - <i>L.visceral</i> , actinomycosis, tonsils, recurrent pneumonia
P13	-	vertebral column TB - <i>M. tuberculosis</i>	urinary tract infection - <i>E.coli</i> , osteomyelitis - <i>S.aureus</i> , pus
P14	-	pulmonary TB - <i>M. tuberculosis</i>	pneumonia, tonsillitis
P15	-	meningitis TB - <i>M. tuberculosis</i>	-
P16	-	pulmonary TB - <i>M. tuberculosis</i>	pneumonia, tonsillitis
P17	+	-	pneumonia
P18	-	cutaneous mycobacteriosis - <i>M. avium complex</i>	chickenpox - varicella zoster virus, pneumonia, otitis

Pt, patient, TB,tuberculosis ; -, absent; +, present

Figure 7 Clinical pictures of Brazilian patients with mycobacterial disease

(A) Images of BCGtis, (B) cutaneous mycobacteriosis caused by MAC, (C) Vertebral TB caused by *M.tuberculosis*, (D) calcaneus osteomyelitis caused by MAC. (E) On the top CT scan of lung shows hilar lymphadenopathy on the right and left side and on the bottom caseous granuloma with multi nucleated langhans cells and multiple histiocytes with acid fast bacilli consistent with *M.tuberculosis* and further confirmation of *MTB* by Ziehl neelsen stain.

The leukocytes percentage of all patients was in normal range (**Table 4**). However, one patient (P14) presented low absolute number of T and B lymphocytes populations. Immunoglobulin levels were in normal range except in 3 patients (P2, P3 and P4) with clinical symptoms of CD40L-deficiency and one (P5) with clinical diagnosis of EDA-ID (**Table 4**).

Table 4 Laboratorial features of patients with susceptibility to mycobacterial disease

Pt	Lymphocytes ^a %			Age (years)	Serum Igs Level ^b (mg/dL)		
	CD3+	CD4+	CD8+		IgG	IgA	IgM
1	19	17	7	6	900	50	180
2	42	35	21	8	210	6	90
3	44	33	15	6	341	11	335
4	43	31	18	21	97	6	464
5	41	29	17	2	147	15	51
6	40	33	18	17	1200	134	92
7	44	29	18	5	892	124	100
8	45	24	17	36	860	100	100
9	42	28	18	4	1051	80	91
10	40	29	17	2	560	102	90
11	42	35	21	11	934	110	76
12	47	21	15	23	1000	102	80
13	46	28	19	13	944	102	108
14	43	17	18	6	900	50	180
15	41	22	21	2	800	80	90
16	39	19	16	10	1023	90	60
17	39	19	16	<2	500	30	60
18	46	21	18	21	600	61	50

3.2 Strategies for functional screening in Brazilian patients with Susceptibility to Mycobacterial Disease and to identify the novel molecular genetic defects

The summary of experiments performed in this study is shown in a flow chart (**Figure 8**) which details are described in the following sections. Patients who were HIV negative and developed mycobacterial infections caused by different mycobacterium species with and without comorbidities were selected for this study. The data were obtained from cellular analysis and functional assays using whole blood, PBMCs and MDMs from the patients and unrelated healthy controls.

Figure 8. Diagnostic testing flow chart to identify functional and molecular genetic defects in patients with mycobacterial diseases.

Immunophenotyping and expression of CD40L

3.2.1 Isolation of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) of patients and healthy control were separated by Ficoll-Paque density gradient centrifugation as before described with slightly modification (de Almeida et al., 2011). Briefly, 10 ml of heparinized blood from patients and healthy donors were mixed with 10 ml phosphate buffered saline (PBS) and layered over 12.5 ml of Ficoll-paque (1.077 ± 0.001 g/ml, GE Healthcare) in a 50 ml conical tube (Corning, NY, Mexico). Density gradient centrifugation was performed at 900xg for 30 min, after centrifugation the interface layer containing PBMCs was collected in another 50 ml conical tube (Corning, NY, Mexico), washed two times with phosphate buffered saline (PBS) and the cells were used for experiments.

3.2.2 Cell staining and flow cytometry analysis

T cells ($CD3^+CD4^+$, $CD3^+CD8^+$) and B ($CD19^+CD40^+$) cell counts were determined by flow cytometry and CD40L protein expression was assessed on the surface of activated $CD3^+CD4^+$ T cells using monoclonal antibodies as previously described (Seyama et al., 1998). Activation of cells was confirmed by staining $CD3^+CD4^+$ T cells with specific antibodies to CD69 (Becton Dickinson, BD). The tubes were stored for 30 minutes at 4 ° C protected from light for binding of the antibodies to the receptors. The incubated cell suspension with the antibody was washed twice by adding 1 mL PBS and the tubes were centrifuged at 900 xg at 25°C for 5 minutes. The cells were resuspended and fixed in 300 μ L of PBS containing PBS plus 1% paraformaldehyde.

Flow cytometry studies were performed on a BD FACSCanto II Cytometer. Lymphocytes were gated according to size (forward scatter, FSC) and complexity (side scatter, SSC) and the results were analyzed using the FlowJo software (Treestar, Inc, Ashland, Ore).

3.3 Analysis of IL-12/IFN- γ axis

3.3.1 Production of IL-12 and IFN- γ by PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood after Ficoll-Hypaque fractionation and analyzed for the production of IL-12 and IFN γ as previously described (Feinberg et al., 2004). Briefly, 2×10^5 PBMCs were activated at 37 °C in 5% CO₂, in 96-well round-bottom plates (Greiner Bio-One, Frickenhausen, Germany) in a final volume of 200 μ L of RPMI 1640, supplemented with 10% FCS. The production of IL-12 and IFN- γ were measured after 24 hours and 48 of incubation with IFN- γ (200 IU/ml, Imukin[®], Boehringer Ingelheim) or/and live BCG (1 PBMC/10 BCG), and with IL-12 (5 ng/ml, R&D Systems[®]) or/and live BCG (1 PBMC/10 BCG), respectively. BCG was cultured as previously described (Petricevich et al., 2001). The production of IL-12 and IFN- γ in the supernatants was analyzed by ELISA according to the manufacturer's instructions (Becton Dickinson).

3.3.2 Analysis of activation of IFN γ R/STAT1 and IL-12R/STAT4 pathways

In order to detect possible defects in the intracellular pathways of IFNGR and IL-12R, the phosphorylation state of STAT1 and STAT4 were analyzed in monocytes and lymphocytes respectively, as previously described with some modifications (Fleisher et al., 1999; Uzel et al., 2001). For the STAT1 assay, cells were stimulated with IFN- γ for 30 min and then cells were fixed and permeabilized with Cytotfix/Cytoperm Plus kit (Becton Dickinson) according to the manufacturer's instructions. After washing with 100 μ L Cell Staining Buffer twice at 2500 rpm for 5 minutes, the cells were stained with anti pY701 STAT-1 antibody (BD) for 30 min at 4 °C, protected from light. The cells were washed with 100 μ L Cell Staining Buffer twice at 2500 rpm for 5 minutes, resuspended in 300 μ L of PBS and analyzed by flow cytometry. For IFN γ R1 expression on the surface of monocytes, the PBMCs were stained with PE-conjugated anti-IFN γ R1 (eBiosciences Inc. San Diego, CA) at 4 °C for 30 min in the dark. After incubation, cells were washed twice with PBS and the cells were fixed in PBS plus 1% paraformaldehyde and analyzed by flow cytometry.

For the STAT4 assay, PBMCs obtained were stimulated for 5 days in the presence of phytohemagglutinin (PHA, 8 µg/ml, Life technologies, NY, USA) in order to induce the expression of IL-12R on the CD4⁺ T cells. 1×10^6 PBMCs per 150 µL of RPMI were incubated for 2 h at 37 °C to rest, followed by stimulation with IL-12 (10 ng/ml) (Peprotech) for 30 min. The lymphocytes were fixed and permeabilized with Cytofix/Cytoperm Plus kit (Becton Dickinson) according to the manufacturer's instructions., followed by staining with APC-conjugated pY693-STAT4-alexa 647 antibody (BD Biosciences) and was evaluated by flow cytometry on a BD FACSCanto II Cytometer and the data obtained were analyzed by using FlowJo software (Treestar, Inc, Ashland, Ore). For analysis of surface expression of IL-12Rβ1 and IL-12Rβ2 on the CD4⁺ T cells, lymphocytes were stained with PE-conjugated anti-IL-12Rβ1 (BD Biosciences) and APC-conjugated anti-IL-12Rβ2 (BD Biosciences) respectively at 4°C for 30 minutes. The cells were washed with PBS and fixed with PBS plus 1% paraformaldehyde, and the surface expressions of proteins were analyzed by flow cytometry.

3.4 Investigation of TLR and NFκB signaling defects

As CD62L molecule is present on granulocytes and this molecule shed or cleavage when TLRs have normal downstream signaling after stimulations. Therefore, we assessed the cleavage of the membrane-bound L-selectin (CD62L) on neutrophils and monocytes by flow cytometry for detection of possible inborn defect in Toll-like receptor signaling as previously described (von Bernuth et al., 2006). Briefly, Briefly, 100 µL of whole blood were incubated for 1 hour without stimulation or with PMA (400 ng/mL), live BCG (1 cell/10 BCG), synthetic diacylated lipopeptide (Pam₂CSK₄, agonist for TLR2/6, 100 ng/mL Invivogen, San Diego, CA), synthetic triacylated lipopeptide (Pam₃CSK₄, agonist for TLR1/2, 1 µg/mL, Invivogen, San Diego, CA), Zymosan, agonist for TLR2 and Dectin-1 (1 cell: 8 particles, InvivoGen, San Diego, CA 92121 - USA), LPS, agonist for TLR4 (100 ng/mL Sigma, St Louis, MO), TNF-α (20 ng/mL, R&D systems) at 37°C and 5% CO₂. After erythrocytes lyses, the cells were stained with anti-human CD62L antibody (BD Biosciences), and analyzed by flow cytometry. The results obtained were expressed as median fluorescence intensity (MFI).

3.4.1 *Flow cytometric analysis of surface expression of TLRs*

We determined the expression of specific surface molecules functioning know as receptors (TLR2, TLR4 and TLR10) on neutrophils, monocytes and lymphocytes in relation to possible anomalies in these signaling pathways. For this, the phagocytic cells were incubated with APC- conjugated anti-TLR2 (eBiosciences Inc. San Diego, CA), FITC-conjugated antiTLR4 (eBiosciences Inc. San Diego, CA) and Anti-human TLR10 (PE, eBiosciences Inc. San Diego, CA) at 4°C for 30 min in the dark. After incubation cells were washed twice with PBS and the cells were fixed in PBS plus 1% paraformaldehyde. The protein surface expression was analyzed by flow cytometry on a BD FACSCanto II Cytometer and the data obtained were analyzed by using FlowJo software (Treestar, Inc, Ashland, Ore).

3.4.2 *Evaluation of NFκB activation by measuring the IκBα degradation*

NFκB is a transcription factor regulating the expression of a number of genes involved in innate immunity and inflammatory responses. In resting cells NFκB dimers are associated with inhibitory IκB proteins such as IκBα. Upon activation by a variety of pathogens, cytokines and TLR agonists, IκBα is phosphorylated and degraded leading to translocation of NFκB dimers to the nucleus and regulation of target gene expression (DiDonato et al., 1996; Sun et al., 1996).

In order to evaluate the degradation of IκBα, the PMN cells of the patients under study and normal controls were left untreated or stimulated with phorbol myristate acetate (PMA, 300 ng/ml, Sigma Laboratories, St. Louis, MO, USA) , live BCG (1 cell/10 BCG), synthetic diacylated lipopeptide (Pam₂CSK₄, agonist for TLR2/6, 100 ng/mL Invivogen, San Diego, CA), synthetic triacylated lipopeptide (Pam₃CSK₄, agonist for TLR1/2, 1 µg/mL, Invivogen, San Diego, CA), LPS, agonist for TLR4 (100 ng/mL Sigma, St Louis, MO), TNF-α (20 ng/mL , R&D systems, Minneapolis, MN) incubated for 20 minutes at 37°C. To determine the degradation of IκBα cells were fixed and permeabilized (Cytofix/PermBufferII, BD Biosciences) and stained with PE Mouse anti-IκBα antibody (BD Biosciences). After 20 minutes of incubation cells were re-suspended in cell staining buffer and degradation of IκBα

was analyzed by flow cytometry. NF κ B activation was assessed in response to TLR agonists regarding to defects in TLR signaling

3.5 Analysis of oxidative burst

3.5.1 Dihydrorhodamine-123 (DHR) assay

Oxidative burst in neutrophils and monocytes was assessed as previously described (Hasui et al., 1989). Peripheral leukocytes were incubated for 5 min at 37 °C with dihydrorhodamine 123 (Sigma-Aldrich) after activation for 1 hour with phorbol myristate acetate (PMA, 300 ng/ml, Sigma Laboratories, St. Louis, MO, USA) , live BCG (1 cell/10 BCG), synthetic diacylated lipopeptide (Pam₂CSK₄, agonist for TLR2/6, 100 ng/mL Invivogen, San Diego, CA) and LPS, agonist for TLR4 (100 ng/mL Sigma, St Louis, MO), TNF- α (20 ng/mL , R&D systems).

3.5.2 Flow cytometric analysis of NADPH oxidase complex

Expression of NADPH oxidase complex was detected by flow cytometry as previously described (Koker et al., 2007; Wada et al., 2013). For membrane bound NADPH oxidase proteins, peripheral blood monocytes (PBMs) were incubated with anti-gp91phox, anti-p22phox monoclonal antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, CA) and after washed twice with PBS, the cells were fixed with PBS plus 1% paraformaldehyde and analyzed by flow cytometry. For cytoplasmic proteins, prior the cells were fixed and permeabilized with Cytofix/Cytoperm Plus kit (Becton Dickinson) according to the manufacturer's instructions and then stained with anti-p40phox, anti-p47phox, and anti-p67phox monoclonal antibodies (Bioss inc, Woburn, MA) at 4 °C for 30 min. After washing tow time with PBS, the cells were analyzed by flow cytometry on a BD FACSCanto II Cytometer and the data were analyzed by using FlowJo software.

3.6 Characterization of molecular genetic defects

3.6.1 Genetic analysis by Sanger sequencing

3.6.1.1 Isolation of gDNA

Genomic DNA was obtained from peripheral blood leukocytes using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The samples were stored at 4°C overnight and then stored at -20°C.

3.6.1.2 Polymerase chain reaction (PCR)

Candidate genes were amplified by using standard PCR and sequencing procedures. For the PCR reaction a solution containing Milli-Q water, 10x buffer, deoxynucleotide triphosphate, MgCl (250mM), Taq polymerase, 1 µg of cDNA (or gDNA) and the according forward and reverse primers was prepared. The PCR was run on an Eppendorf® Mastercycler Gradient thermocycler using the following program: heating the mixture to 94°C, followed by 30 cycles of 60°C for annealing of the primers and 72°C for initiation of duplication by the polymerase. The obtained PCR products were analyzed on a 1.5% agarose gel.

To eliminate unincorporated nucleotides or remnants of primers, the PCR products were purified prior to sequencing. For this procedure, we used the kit GFX PCR DNA and Gel Band Purification (GE Healthcare) according to the manufacturer's instructions. The purified DNA was quantified again by NanoDrop and then stored at -20°C until sequencing.

3.6.1.3 Sequencing reactions

PCR products were sequenced using the BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA) on an ABI PRISM 3700 sequencer (Applied Biosystems) in the center of studies of the human genome at the University of São Paulo. For the analysis of the sequenced data, we used the sequencing software: BioEdit and compared the sequences to the gene sequences published on GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). The primers used for PCR and sequencing of candidates genes are given in **Table 5**, **Table 6**, **Table 7**, and **Table 8**

Table 5 Primers used for Sanger sequencing and PCR of *CD40LG*

Amplification regions	Primer	Sequencing (5'-3')	T _m
Promotor	foward	GATCTCTGACTTGGGCAATT	54 ⁰
	reverse	CAAACATTATGCCACAAGAC	
Exon 1	foward	CCAGAAGATACCATTTC	52 ⁰
	reverse	AAGGCTTGTGGTTCATCT	
Exon 2	foward	AGCTGTATTCTCCTTCCGAATGAC	52 ⁰
	reverse	CAGTTTCCCGATCTAGCA	
Exon 3	foward	AGCCCCTGTCAAAATGAC	52 ⁰
	reverse	CCTGATGCAACAACACTG	
Exon 4	foward	ATGAGACCGATGGAAACAGC	60 ⁰
	reverse	CAGATTGCATTCCACCTCCT	
Exon 5	foward	CAAGTCTCTGAAATATGCTCAA	60 ⁰
	reverse	CCATAAGGAGGATCCTAGGGTTA	

Table 6 Primers used for Sanger sequencing and PCR of *IL-12RB1* gene

Amplification regions	Primer	Sequencing (5'-3')	T _m
Exon 4	foward	GGCTGAGGATTCGGAATAGG	60 ⁰
	reverse	ACTGAAGCCCAGGAAGGAGT	
Exon 5	foward	TGGGATGTGATGCACAAAGT	59 ⁰
	reverse	TTGGTAAGGGGACCATGAAA	
Exon 6	foward	TAGCTGGGACCTGCAAGTGT	60 ⁰
	reverse	ACCACCTCAGGGGCTAGAGT	
Exon 7-8	foward	TAAGCCATGATTGCACCACT	59 ⁰
	reverse	CCCAGCCCCATTTTATTTTA	
Exon 9	foward	TGGGGATGCAAATTGTGTTA	60 ⁰
	reverse	AGTCATGTGCCTCTCCTGCT	
Exon 10	foward	GGCCTTTGTCCAAGTCTGTC	60 ⁰
	reverse	ACCCCTAAATCAGGCACTCC	
Exon 12	foward	GGGTGTTGCATAGGGAAGAG	59 ⁰
	reverse	CACTGTGCCAGCCTCTATT	
Exone 13	foward	TGGCCTCAAATGACTTACCC	59 ⁰
	reverse	TTGATAACCAAGGCCAGAG	
Exone 14	foward	CCTGGCCTCTGAGGAGTAAA	60 ⁰
	reverse	AGGGAGCCATAGAGGGAGTG	

Table 7 Primers used for sequencing and PCR of *TLR2* gene

Amplification regions	Primer	Sequencing (5'-3')	T _m
Exon 1	foward	CCCATTCATTTCGTCCATTC	60 ⁰
	reverse	TTCCCACTCTCAGGATTTGC	
Exon 1	foward	CCTGGGCAGTCTTGAACATT	59 ⁰
	reverse	GAGCAAAGTCTCTCCGGTTTTT	
Exon 1	foward	TTGAAAAATTTCAGCCTGTGAGG	60 ⁰
	reverse	TCAGCAGGAACAGAGCACAGC	
Exon 1	foward	GGCCAGCAAATTACCTGTGT	59 ⁰
	reverse	GCAAGTCCTCAAATGACGGTA	

Table 8 Primers used for Sanger sequencing and PCR of candidate genes

Gene	Fwd primer sequencing	Rev primer sequencing	T _m *
<i>SOCS-1</i>	5'CTTAGCGTGAAGATGGCCTC 3'	5'ACACGGCATCCCAGTTAATG 3'	59 ⁰ C
<i>RAG1</i>	5'CCACCTTGGGACTCAGTTCT 3'	5'GCTCTTCTCTTGCTGACG 3'	59 ⁰ C
<i>DKC1</i>	5'GGAAGAGCCAGGACTGAAGA 3'	5'AGCAAGCCTCATCTTTGGAG 3'	61 ⁰ C
<i>IRAK2</i>	5'CATTGTCTGGAGGGAGTGTG 3'	5'ATTACAAAGGAGCCGTGGGA3'	58 ⁰ C
<i>TL10</i>	5'CAGAGGGTGATGCTCCAGAG 3'	5'GCCCCACTCAAACCTAGGAT 3'	60 ⁰ C
<i>IL-12Rβ2</i>	5'CATGAGGTCCAGGCACAGAG 3'	5'GGGCCTGCAGGAGATAAGAG3'	
<i>NCF1</i>	5'AAGATGGTCCTCACCCCAAT3'	5'CCACACTCACATGGAACCTCG3'	60 ⁰ C

3.6.2 Isolation of RNA

Total RNA was extracted from mononuclear cells which have been isolated with Ficoll- Hypaque centrifugation from peripheral blood, using the TRIzol Reagent kit (Life technologies). Briefly, 5×10^7 cells were washed one time with RPMI medium in RNase - free eppendorf tubes, and resuspended in 1ml of Trizol. After homogenization, the solution was incubated for 5 minutes at 30°C and 200µl of chloroform were added. After mixing vigorously for 15 seconds and incubation for 3 minutes at 30°C, the sample were centrifuged for 15 minutes at 12,000 g and 4°C. Then, the aqueous phase (top) was carefully transferred to a new RNase -free eppendorf tube and 500µl isopropanol were added. After incubation for 10 minutes at 30°C and centrifugation for 10 minutes at 12000 g at 4°C, the RNA was precipitated in the bottom of the tube. The RNA was washed one time with 1 ml 75% ethanol,

air dried and resuspended in 15-30µl RNase-free water. The gDNA and RNA were quantified by measuring the absorbance with a spectrophotometer (NanoDrop).

3.6.2.1 Synthesis of cDNA

Total RNA was subjected to reverse transcription to obtain cDNA. Briefly, 2 µg of total RNA were mixed with 2µl oligo dT primer and DEPC water to complete the total volume to 12µl and samples were incubated at 70°C for 3 minutes. A solution containing 4 µl of 5X buffer, 2µl dNTP (2.5 mM), 1µl DTT, and 1µl RNase inhibitor (Superscript III ®, Invitrogen, USA) was added and the mixture incubated for 5 minutes at 4°C. Then followed an incubated of 60 minutes at 42 ° C and 10 minutes at 92°C to inactivate the reverse transcriptase enzyme. The resulting cDNA was frozen at -20°C for subsequent use in PCR reactions and sequencing. Primer for PCR and sequencing are given in **Table 9**.

Table 9 Primers used for Sanger sequencing and PCR of cDNA of STAT4

Primer	Forward sequencing	Reverse sequencing	T _m
Primer 1	5'TGGTGTTTTGATCCTGGCTA3'	5'AAGCATTTTCCTGCAGTGTCA3'	59 ⁰ C
Primer 2	5'GACAAGAATAGTGCCATGGTGA3'	5'TGGCTTTGACATTAGTTCCACA3'	59 ⁰ C
Primer 3	5'TCAGGTAAAGGTTAAGGCATCA3'	5'TTGCTAACAAAGCCCATGAC3'	61 ⁰ C
Primer 4	5'TCCATATGCTGGCAGAGAAG3'	5'GCTTCAGATGTCAAACATTTC3'	58 ⁰ C

3.6.3 Whole exome sequencing

Human exome deep sequencing was performed at St. Giles Laboratory of Human Genetics of Infectious Disease, The Rockefeller University, US. We extracted genomic DNA from peripheral blood, and an adaptorligated library was prepared with the TruSeq DNA Sample Preparation Kit (Illumina). Exome capture was performed with the SureSelect Human All Exon kit (Agilent). Paired-end sequencing was carried out on an Illumina HiSeq 2000 generating 100 bp reads. For sequence alignment, variant calling and annotation, the sequences were aligned with the human genome reference sequence (GRCh37 build), with the Burrows–Wheeler Aligner. Downstream processing was carried out with the Genome analysis toolkit (GATK) and SAMtools. Variant calls were made with a GATK Unified Genotyper. (Bolze et al., 2010; Soltesz et al., 2013). All calls with a read coverage two times or less and a

Phred-scaled quality of 20 or less were excluded. All variants were annotated with an in-house annotation software system.

3.7 Mutation prediction

Missense variations found were checked for previously described single nucleotide polymorphism (SNP) in the Ensembl (<http://www.ensembl.org/>) and dbSNP Short Genetic Variation databases (<http://www.ncbi.nlm.nih.gov/projects/SNP/>). In addition, *in silico* analysis for missense variations were evaluated using various tools including SIFT (<http://sift.jcvi.org/>), PolyPhen2 (<http://genetics.bwh.harvard.edu/pph2/>), MutationTaster <http://www.mutationtaster.org/> and PolyPhen2 <http://genetics.bwh.harvard.edu/pph2/> scores.

3.8 3D protein structure modeling

3D analysis were performed by analyzing each amino acid substitution on the structure and function. For this the sequences were blasted using PSI-BLAST (<http://blast.ncbi.nlm.nih.gov>) against PDB (<http://www.rcsb.org>) to obtain related three dimensional coordinates (template). The command model multiple of program MODELLER 9v7 was used to construct comparative models of the proteins. The Output of MODELLER contains three dimensional models for the target sequences (wild type and mutant) including all non-hydrogen main-chain and side-chain atoms was performed as previously described (Sali and Blundell, 1993). The evaluations of the comparative models were conducted using PROCHECK and ProSa programs. Furthermore, the quality of the mutant models were also compared by superposition of the Ca traces. We have constructed multiple models for each wild type and mutant and the best models were selected using the mentioned evaluation procedures.

3.9 Generation of monocyte-derived macrophages

Human MDMs were obtained as described by Esquivel-Solís *et al*; (Esquivel-Solis *et al.*, 2009). Briefly, PBMCs were isolated from heparinized blood after Ficoll-Hypaque sedimentation and adherent monocytes were cultured at 37°C in a humidified 5% CO₂ atmosphere for 7 days in the presence of 5 ng/ml of macrophage colony stimulating factor (M-CSF, Peprotech).

3.9.1 *Evaluation of phagocytosis and M. tuberculosis growth control by MDMs*

A virulent *M. tuberculosis* H37Rv strain (ATCC 25618) was kindly provided by Dr. Philip Suffys from Fundação Oswaldo Cruz (FIOCRUZ, Brazil) and cultured as previously described (Wong et al., 2007). 4×10^4 MDMs were seeded in 96-well plates (Corning) in complete RPMI medium (Gibco; 1 mM sodium pyruvate, 2 mM glutamine and 3% FCS) and kept overnight at 37°C in a 5% CO₂ atmosphere. MDMs were infected with H37Rv at 1:2 ratio (MDM: bacilli) for 3 h (day 0) and the cells washed to remove extracellular mycobacteria. On day 0 and after days 3 and 6, the MDMs were lysed with 0.1% saponin for 10 min and the homogenates were diluted and plated in Middlebrook 7H10 medium supplemented with 10% OADC (Difco; acid/albumin/dextrose/catalase). The colonies were determined after 21 days incubation at 37°C. Bacterial uptake (phagocytosis) data were obtained from CFU counts on day 0. Bacterial growth index was determined by CFU number ratio on days 3 and 6 per CFU number relative to day 0. For some experiments, once differentiated, macrophages were stimulated by treating cells for 22 h in serum free RPMI-1640 medium containing human recombinant IFN- γ (500 IU/ml, Imukin[®], Boehringer Ingelheim) as previously described (Esquivel-Solis et al., 2009). Macrophages were certified as uninfected prior to our experiments.

3.10 *Statistic analysis*

Statistical significance was assessed by using the nonparametric Mann-Whitney test. The statistical analyses were performed by using the GraphPad PRISM 4.03 software (GraphPad Software, San Diego, Calif) and differences with a P value of less than .05 were considered significant.

4 RESULTS

4.1 Patients with mycobacterial infections and signs of well known PIDs

Following the flow chart shown in **Figure 8**, the heterogenous genetic background identified in our cohort will be described in the next section. The criteria to conclude each genetic identification complies with the International Union of Immunological Societies Expert Committee on Primary Immunodeficiencies (Al-Herz et al., 2014b)

4.1.1 “Leaky” SCID is characterized in P1

Due to absent or reduced number of lymphocytes and consequently absence of impaired adaptive immune response (including impaired IFN- γ production) patients with SCID are highly susceptible to mycobacterial diseases (Geha et al., 2007; Rezaei et al., 2006). Because the immunophenotyping of P1 revealed low T and B cells (**Table 4**) P1 was investigated for a possible defect in *RAG1* which is the most common autosomal form of SCID (Tasher and Dalal, 2012). Genetic sequencing revealed three different novel deletions (c.351delT, c.343_350delGGGAATT, c.304_311delATCCATCA) (**Figure 9A**). A single nucleotide polymorphism (SNP) (c.302C>A) was also identified in *RAG1* of P1. *In silico* prediction indicates that these genetic alterations are pathogenic. In accordance, computational 3D structure analysis indicates that the three deletions disrupt the RAG1 protein structure (**Figure 9B**). Considering the molecular genetic defect identified in P1 and the reduced T and B cells associated with his clinical history, our data suggest that patient P1 has the typically mild SCID called “leaky” SCID.

Figure 9 Genetic and molecular analysis of RAG1 of P1

A)

B)

(A) Gene sequencing revealed compound heterozygous mutations (c.351delT, c.343_350delGGGAATT, c.304_311delATTCCATCA, c.302C>A) in *RAG1* of P1. (B) *In silico* analysis of RAG1 protein predicts truncated RAG1 protein caused by compound heterozygous mutations (c.351delT, c.343_350delGGGAATT, c.304_311delATTCCATCA, c.302C>A).

4.1.2 Three patients (P2, P3 and P4) were identified with CD40L deficiency

Increasing number of reports have shown that CD40L-deficient patients are susceptible to mycobacterial diseases (Cabral-Marques et al., 2014; Levy et al., 1997; Wang et al., 2014). Patients P2, P3 and P4 (all male subjects) had normal T and B cell counts, imbalanced immunoglobulin levels (suggesting defective class switch recombination, CSR), and family history of X-linked PID. Because CD40L deficiency is the most common form of hyper-IgM syndrome accounting for 70% of the cases (Levy et al., 1997), we analysed the expression of CD40L protein on the surface of CD3⁺CD4⁺ T cells from the three patients (P2, P3 and P4). Reduced CD40L expression was found (**Figure 10A**). Genetic analysis revealed three heterogeneous mutations: c.G520A (p.W140X), c.633_634insAGCC (p.L193fx201), and c.345_402del (p.345_402del) in the *CD40LG* of P2, P3 and P4, respectively (**Figure 10B**) (Cabral-Marques et al., 2014). In agreement, computational 3D structure (*in silico* analysis) of CD40L demonstrated that these three molecular modifications disrupt the CD40L protein structure (**Figure 10C**).

Figure 10 Molecular and genetic analysis of *CD40LG* from P2, P3 and P4

B)

C)

D)

(A) Abolished expression of CD40L on the surface of activated CD3⁺ CD4⁺ T cells from CD40L-deficient patients (P2, P3 and P4) compared to the healthy control. (B) Genetic analysis of *CD40LG* revealed three different mutations: c.G520A (p.W140X); c.633_634insAGCC (p.L193fsX201) and p.345_402del (exon 3 skipping) in P2, P3 and P4, respectively. (C) *In silico* analysis suggesting truncated CD40L protein expression in P2, P3 and P4 compared to the control.

4.1.3 X-linked EDA-ID caused by NEMO deficiency in P5

The transcription factor NF- κ B, in resting cells, is present in the cytoplasm in association with inhibitory κ B proteins (I κ B, e.g I κ B- α). Upon stimulation I κ B- α is phosphorylated on specific serine residues S32 and S36 by the IKK complex consisting of IKK- α , IKK- β and IKK- γ (NEMO), followed by I κ B- α polyubiquitination and subsequent degradation by the 26S proteasome (Chen et al., 1999; DiDonato et al., 1996; Sun et al., 1996). Mutation in different genes can lead to EDA-ID. However, mutations in *NEMO* cause the X-linked form of the disease (Doffinger et al., 2001). Therefore, patient P5 with positive family history of X-linked PID, who showed the classical X-linked EDA phenotype characterized by absence of sweat glands, sparse scalp hair, rare conical teeth (**Figure 11A**) and impaired CSR (**Table 4**) was investigated for NEMO deficiency.

Lymphocytes from P5 showed failure in I κ B α degradation in response to PMA + PHA and TNF- α (**Figure 11A**). *NEMO* was sequenced and a potential novel missense hypomorphic mutation (c.1238A>C/p.P413H) localized in exon 10 was identified in a hotspot mutations area previously described (Kawai et al., 2012) (**Figure 11B**). MutationTaster analysis indicated that the p.P413H substitution is not a SNP.

It is important to note that despite NEMO is located on the X- chromosome, P5 DNA chromatogram shows a heterozygous change due to the presence of a NEMO pseudogene which spans from exon 3 to 10 (Aradhya et al., 2001a; Aradhya et al., 2001b; Smahi et al., 2000). For this reason further evaluation of NEMO cDNA is required to avoid invalid conclusions based upon genomic DNA alone. However, the c.1238A>C mutation associated with the defective I κ B α degradation suggests that this finding underlys the X-linked EDA-ID phenotype of P5.

Multiple sequence alignment of NEMO demonstrated that the mutation p.P413H is localized in a conserved domain among different species of NEMO zinc finger domain probably impairing NF κ B activation. 3D protein structure modeling shows that this mutation disrupts NEMO binding to ubiquitin resulting in impairment of I κ B α degradation (**Figure 11D**).

Figure 11 Molecular and genetic characterization of NEMO deficiency in P5

(A) P5 showing sign of EDA. Defective I κ B α degradation in response to PMA/PHA and TNF- α in lymphocytes from P5. (B) gDNA sequencing identified a novel hemizygous mutation (c.1238A>C/p.P413H) in NEMO gene. (C) The mutated amino acid is conserved across the species. (D) *In silico* analysis of NEMO predicts that the p.P413H mutation is localized in the zinc finger domain. This fact impairs NF κ B activation by disrupting the NEMO binding to ubiquitin and consequently abolished the I κ B α degradation.

4.1.4 The occurrence of *M. tuberculosis* infection in CGD in P6 caused by p47phox deficiency

CGD patients frequently have an increased susceptibility to mycobacterial diseases (Bustamante et al., 2007), however, only those with mutations in the genes encoding gp91phox, p22phox, and p67phox proteins have been described with mycobacterial infections (de Oliveira-Junior et al., 2011; Deffert et al., 2014b; Koker et al., 2013; Lee et al., 2008; Ying et al.). Retrospective analysis of P6 clinical history showed that she developed pneumonia at 8 months of age and remained without clinical manifestations until 6 years of age, when she presented with persistent high fever and was diagnosed simultaneously with pneumonia, pleural empyema, granulomatous pleuritis, osteomyelitis of the rib, and septicemia with poor response to different antibiotic regimens. A lung biopsy showed pulmonary mycosis due to *Aspergillus sp.* and zygomycetes. The latter species could not be identified. One year later, the patient presented with other severe infections such as meningitis, endocarditis, and pulmonary mycosis with positive culture for *Candida albicans*.

Laboratory results revealed normal leukocyte counts and normal serum IgA, IgM, and IgG levels. At 12 years of age, the patient developed persistent fever, dry cough, and weight loss. CT scan and chest radiograph suggested severe TB and *Mycobacterium tuberculosis* was identified by lung biopsy. The patient was treated for three years with rifampicin, isoniazid, and pyrazinamide.

Based on P6 clinical history, defects in NADPH oxidase components were suspected. Neutrophils and monocytes from P6 presented a reduced oxidative burst in response to PMA and BCG (**Figure 12A-C**) compared to phagocytes from healthy controls, strengthening our hypothesis. The expression of p47phox was abolished while the other NADPH subunits were expressed normally in patient's monocytes (**Figure 12D**). Sequencing of *NCF1* gene identified the common deletion g.3307_3308delGT frequently reported in patients with autosomal CGD. This is a frameshift homozygous GT deletion (c.75–76delGT; p.Val25X51) located in the beginning of exon 2 (**Figure 12E**). In the control chromatogram, the co-amplification of both functional gene and two pseudogenes (containing the GT deletion) was evident because the sequences diverge after the initial GT. In contrast, due to the homozygous GT deletion also in functional *NCF1* gene from patient P6, sequences of the gene and pseudogene overlap each other as previously described (Noack et al., 2001; Prando-Andrade et al., 2004). The 3D structure modeling analysis showed a truncated protein (**Figure 12F**).

Figure 12 Molecular and genetic analysis of P6

A)

B)

C)

D)

E)

F)

(A) Neutrophils and (B) monocytes from P6 showed a reduced oxidative burst after stimulation with PMA or BCG using DHR assay. (C) DHR MFI of neutrophils and monocytes. (D) Histograms showing the expression of NADPH components. (E) Chromatogram showing the homozygous deletion (g.3307_3308delGT) identified in gDNA of P6. (F) Comparative 3D structure of our index case demonstrating a truncated protein. MFI, Mean fluorescence intensity.

Following the other patients of our cohort who presented with normal lymphocyte counts and immunoglobulin levels were investigated for oxidative burst defects by DHR assay. Neutrophils and monocytes from patients P7 to P18 showed similar oxidative burst in response to PMA in comparison to healthy controls ruling out the CGD diagnosis. On the other hand the patients neutrophils and monocytes displayed a significantly difference in the oxidative burst response in response to BCG stimulation (**Figure 13A-B**). This data suggest that other mechanisms regulating the BCG immune response and NADPH oxidase activity can be affected in some patients of our cohort.

Figure 13 Analysis of phagocyte oxidative burst of patients with susceptibility to mycobacterial infections

(A) H_2O_2 production by neutrophils (B) monocytes from patients and healthy controls following PMA and BCG activation. Significant difference is denoted by asterisk ($P \leq 0.05$, Mann-Whitney test). MFI, Mean fluorescence intensity.

4.1.5 Patients P7 and P8 showed abnormal CD62L shedding

Among the TLRs, mutations and single nucleotide polymorphism (SNPs) in human TLR2 (most frequently) and in TLR10 genes have been associated with increased susceptibility to mycobacterial infections (Bhide et al., 2009; Merx et al., 2007; Thuong et al., 2007). Therefore, we evaluated patients TLR signaling by measuring the shedding of membrane-bound L-selectin (CD62L) which is a well recognized procedure to detect defects in TLRs activation (Hayashi et al., 2003; von Bernuth et al., 2006). After neutrophil TLRs bind their ligands, signaling pathways are activated triggering the shedding of CD62L. We performed

the analysis of the CD62L shedding following different stimulation: PMA (receptor independent stimulus), Pam₂CSK₄ (TLR2/TLR6 agonist), LPS (TLR4 agonist), TNF- α (as TLR independent agonist but a NF- κ B activator) and BCG. No statistic difference was observed between patients and healthy controls (**Figure 14**). However, two patients displayed reduced shedding: P7 in response to Pam₂CSK₄ and BCG and P10 in response to Pam₂CSK₄, LPS and BCG, suggesting different defects affecting the TLR signaling and BCG recognition.

Figure 14 Evaluation of TLR signaling by the shedding CD62L on the surface of neutrophils from patients with susceptibility to mycobacterial diseases

(A) CD62L shedding on the surface of neutrophils after activation without or with PMA, BCG, Pam2CSK4, LPS and TNF- α . P7 showed reduced CD62L shedding in response to specific TLR2 agonists (BCG and Pam2CSK4) while P10 showed impaired CD62L shedding in response to TLR2 agonists (BCG and Pam2CSK4) and TLR4 agonist (LPS). MFI, Mean fluorescence intensity.

4.1.5.1 Characterization of TLR2 deficiency in P7

All TLR signaling pathways culminate in activation of the transcription factor NF- κ B which has pleiotropic effects in the regulation of the innate immune response (Kawai and Akira, 2007). Therefore, to further characterize what is the signaling defect leading to the defective CD62L shedding by P7 neutrophils in response to Pam₂CSK₄ we first evaluated the NF- κ B activation by the I κ B α degradation in response to the same stimuli used in the CD62L shedding assay. Because of the limitation in the amount of blood that can be drawn safely from the P10 who had only 1 year of age to identify his genetic defect gDNA of P10 was directly sequenced by WES as described in the next section.

In agreement with CD62L shedding, P7 monocytes presented defective I κ B α degradation in response to TLR2 agonists (Pam2CSK4) and BCG while normal degradation to LPS (**Figure 15**).

Figure 15 Defective I κ B α degradation in monocytes from P7

I κ B- α degradation in monocytes from P7. Reduced I κ B- α was observed in monocytes from P7 when stimulated with TLR2 agonists (Pam2CKS4 and BCG) while normal I κ B- α degradation to LPS and TNF- α .

To further characterized a possible defect in the TLR2 signaling we measured the IFN- γ and IL-12 production as shown in **Figure 16**. Accordingly to the CD62L shedding and I κ B α , PBMCs from P7 produced low levels of IFN- γ when activated with Pam2CSK4 + IL-12 and BCG + IL-12. In addition, the IL-12 production in response to Pam₂CSK₄ + IFN- γ and BCG + IFN- γ . On the other hand, the production of IL-12 and IFN- γ in response to LPS + IFN- γ , LPS + IL-12 and PMA + Ionomycin was normal (**Figure 16A-D**).

Figure 16 IL-12 and IFN- γ by PBMCs from P7

Cytokine production by PBMCs from P1. (A, B) PBMCs from P1 displayed reduced IL-12 and IFN- γ production after stimulation with TLR2 agonist (Pam₂CSK₄) and (C, D) BCG + IFN- γ and BCG + IL-12 while normal amount of IL-12 and IFN- γ following activations with LPS + IFN- γ or LPS + IL-12 or PMA + Iononmycin were observed. The cytokine quantification was performed by ELISA.

A possible defect in IL-12/IFN- γ intracellular signaling, was analyzed by measuring the phoshorylation of STAT1 and STAT4 in monocytes and lymphocytes from P7. Following IFN- γ and IL-12 stimulation P1 cells displayed normal STAT1 and STAT4 phoshorylation (**17A and 17B**) ruling out an intrinsic defect in the intracellular IL-12/IFN- γ signaling.

Figure 17 Cells from P7 displayed normal activation of IL-12/IFN- γ axis

(A) Monocytes and (B) lymphocytes from P7 demonstrated normal STAT1 and STAT4 phosphorylation after activation with rhIFN- γ and rhIL-12 respectively.

Taking together our data suggested that P7 has a TLR2 deficiency. Therefore we performed the molecular genetic analysis of TLR2 of P7. Initially the surface expression of TLR2 on neutrophils from P7 showed partial reduced expression of TLR2 in comparison to neutrophils from healthy controls (**Figure A-B**). Sequencing of P7 *TLR2* demonstrated a heterozygous genetic variation (c.1736G>A/p.R579H) in the exon 1. This genetic variation was not found in 100 healthy Brazilian healthy control subjects. In addition, multiple sequence alignment revealed that this base pair substitution affects conserved amino acids among different species (**19A-B**). Additionally, *in silico* analysis predicts contradictory of the p.R579H variation in the protein structure: while MutationTaster predicts p.R579H variation as SNP, polyphen prediction indicates that p.R579H variation is pathogenic. In its turn, 3D structure modeling shows that the p.R579H variation disrupts the physio-chemical properties of TLR2 (**Figure 19C**) due to substitution from large size and basic amino acid (R) to a medium size and polar (H) amino acid.

Figure 18 Reduced surface expression of TLR2 on neutrophils from P7

Flow cytometry analysis showing (A) histograms and (B) graphics of partial reduced expression of TLR2 by neutrophils from P7 in comparison to healthy controls. MFI, Mean fluorescence intensity.

Figure 19 Molecular genetic analysis of P7

A) Genetic analysis confirmed the heterozygous mutation c.1736G>A/p.R579H in TLR2 gene of P7. (B) conservative analysis showing that this amino acid is conserved among different species. (C) 3D structure modeling analysis demonstrated that the mutation p.R579H may disrupt the physio-chemical properties of TLR2.

4.1.6 Investigation of patients without sign of classical PIDs

During our investigation we have molecular genetically characterized seven patients (P1-P7) who developed mycobacterial infections with mutations in five well known PIDs disease-causing genes (*RAG1*, *CD40LG*, *NEMO*, *NCF1*, *TLR2*) by using well established methods in the literature. However, still 11 patients (P8-P18) without evident sign of well-known (classical) PID remained without a molecular genetic characterization. Because patients with susceptibility to mycobacterial diseases have a high level of genetic heterogeneity (Al-Muhsen and Casanova, 2008; Doffinger et al., 2000), we decided to evaluate the IFN- γ /IL-12 axis and following perform the WES analysis of P8-P18 from those who showed defective IFN- γ /IL-12 axis. Despite PBMCs from the patients group produced reduced amounts of IFN- γ and IL-12, no statistic difference was found in comparison to PBMCs from healthy controls. However, comparing the production of IFN- γ and IL-12 by PBMCs from each patient with PBMCs from the healthy control group, six patients (P8, P9, P10, P11, P12 and P15) produced low level of IFN- γ while four patients (P12, P14, P15 and P16) produced reduced amounts of IL-12p40 in response to the indicated stimuli (**Figure 20A, 20B**).

Figure 20 Analysis of IL-12/IFN- γ axis of Brazilian patients with susceptibility to mycobacterial disease

Investigation of IL-12/ IFN- γ axis (A) PBMCs from patients with mycobacterial infections were activated by IL-12, BCG or BCG + IL-12 and the production of IFN- γ analyzed. (B) For the IL-12 release the PBMCs were stimulated by IFN- γ , BCG or BCG + IFN- γ .

4.1.6.1 Whole exome sequencing in association with Sanger sequencing revealed candidate genes underlying susceptibility to mycobacterial diseases

We sequenced gDNA by WES from the patients whose cells showed impaired IFN- γ /IL-12 production (P8 to P12). In addition, gDNA from P10 was at the first month of life during the present study and had limitation in the amount of blood that could be drawn safely from him. WES sequencing identified genetic variations in different genes: *IL-12R β 2* (P8), *TLR10* (P10), *DKC1* (P11), *SOCS-1* (P12) and *IRAK2* (P13). The genetic variations in these genes were then confirmed by Sanger sequencing as shown in the following sections. P9, whose PBMCs showed low IFN- γ production was directly evaluated for defects in IL-12R signaling because his blood samples were ship to our lab in a time frame unable to analyze his gDNA by WES. For didactic reasons considering that both patients have defects in the IL-12 receptors we show P9 data following P8 data. Unfortunately, blood shipment of patients P14, P15, P16, P16, 17 and P18 was not available in a reasonable time frame to be included in the molecular genetic analysis performed in this thesis. However, the search for their molecular defect is under investigation.

It is important to mention that the following genetic variations will require sequencing of gDNA from healthy controls to exclude that they are present in Brazilian population as a SNP and deep further mutagenesis and immunopatologic studies to assure their mutational characteristics. Therefore we call them as genetic variations instead of mutations.

4.1.6.2 IL-12R β 2

Sanger sequencing confirmed the heterozygous missense variation c.1187C>T/p.A396V in *IL-12R β 2* of P8 (**Figure 21A**). Sequencing of P8's mother and father gDNA presented wild type *IL-12R β 2* sequence (data not shown) suggesting that the c.1187C>T variation is a *de novo* mutation. *In silico* analysis predicted contradictory results for this genetic variation: MutationTaster and polyphen analysis predict that this variation is non-pathogenic; We performed additional SIFT prediction (<http://sift.jcvi.org>) which indicates the p.A396V variation is pathogenic. Ensemble and the dbNSP report the p.A396V variation as a SNP found in 0.023 of the human population, however the clinical relevance of this variation is not available. Conservation analysis of the IL-12R β 2 protein from different species showed that the novel base pair substitution (c.1187C>T) affects a conserved amino acids within the IL-12R β 2 extracellular domain (**Figure 21B**). In addition, 3D structure

modeling analysis of IL-12Rβ2 predicts that the p.A396V variation produces a truncated protein affecting the secondary structure of IL-12Rβ2 and may affect the downstream signaling of IL-12Rβ2 (**Figure 21C**)

Despite the contradictory data obtained from the bioinformatic analysis, we analyzed the STAT4 phosphorylation in lymphocytes from P8 after recombinant human IL-12 (rhIL-12) activation. P8 lymphocytes demonstrated impaired phosphorylation of STAT4 following rhIL-12 stimulation in comparison to lymphocytes from healthy controls (**Figure 22A**)

We asked if the expression of IL-12Rβ2 by PHA-treated (to induce the IL-12R expression) T lymphocytes could be affected. Flow cytometric analysis demonstrated that the T lymphocytes from P8 have partial reduced expression of IL-12Rβ2 while IL-12Rβ1 was expressed normally (**Figure 22B**).

Figure 21 Molecular and genetic analysis of P8

C)

(A) Heterozygous genetic variation c.1187C>T/p.A396V in *IL-12R β 2* of P8. (B) Conservative analysis of IL-12R β 2. (C) 3D structure modeling analysis showing that the mutation p.A396V may affect IL12R downstream signaling.

Figure 22 Impaired IL-12R/STAT4 signaling by CD4⁺ T cells associated with IL-12 β 2 deficiency

A)

B)

(A) T cells from P8 had shown significantly reduced phosphorylation following rhIL-12 activation; (B) and low expression of IL-12R β 2 on T cells from P8. IL-12R β 1 expression is demonstrated.

Taking together our data suggest that the variation c.1187C>T/p.A396V may affect the IL-12R β 2 function. However, considering the contradictory database reporting this genetic variation as a SNP without clinical data description, we reinforce that future mutagenesis studies will be required to determine if this variation indeed affects the IL-12R β 2 function. The reduced IFN- γ production by P8 PBMCs in response to IL-12 plus BCG and the defective STAT4 phosphorylation reinforce that the p.A396V is pathogenic.

4.1.6.3 IL-12R β 1

Following IL-12 stimulation PHA-treated T lymphocytes from another patient (P9) displayed partially impaired STAT4 phosphorylation in comparison to T lymphocytes from healthy control. Analysis of IL-12R expression showed slightly reduced IL-12R β 1 expression on T lymphocytes from P9 while the IL-12R β 2 expression was normal in comparison to T lymphocytes from healthy (**Figure 23A-B**). We then sequenced *IL-12R β 1* gene from P9 and identified the novel heterozygous genetic variation c.1430T>A/p.V447D in the exon 12 of IL-

12R β 1 gene. Mother and father gDNAs are wild type (data not shown). Mutationtaster predicts this genetic variation as SNP while polyphen predicts possible damaging. Conservation analysis of the IL-12R β 1 protein from different species showed that the novel base pair substitution c.1430T>A affects a conserved amino acids within the IL-12R β 1 extracellular domain. 3D structure modeling analysis predict that the c.1430T>A variation generates a truncated protein (**Figure 24A-C**) with impaired IL-12R β 1 signaling. These data are in accordance with the observed low IFN- γ production by P9 PBMCs in response to IL-12 plus BCG stimulation.

Figure 23 Molecular and genetic analysis of P9

(A) CD4⁺ T cells from P9 had shown partially reduced phosphorylation following rhIL-12 activation; (B) Partial reduced surface expression of IL-12R β 1 and normal expression of IL-12R β 2 on T cells from P9.

Figure 24 Molecular and genetic analysis of P9

(A)Molecular analysis of IL-12Rβ1 gene revealed the novel heterozygous missense genetic variation c.1429T>A/p.V447D (B) The mutated amino acid is partially conserved among species (C) 3D structure modeling analysis predicts that p.V447D disrupt the IL-12Rβ1 protein

1.2.1.1 TLR10

TLR10 as TLR1 and TLR6 mediates the immune responses to a variety of microbial components in cooperation with TLR2. However the TLR10 mycobacterial ligand remains unknown (Guan et al., 2010). However, genetic variation in TLR10 can predispose to susceptibility to mycobacterial diseases (Ma et al., 2007). The heterozygous genetic variation c.175C>T/p.L59F identified in TLR10 gene of P10 could be validated by Sanger sequencing. gDNA from mother and father was not evaluated to analyze their carrier status. MutationTaster and ensembl analysis compared with 3D model (*in silico* analysis) indicated contradictory results: while the firsts indicate this variation as a SNP, the later shows that p.L59F variation results in truncated TLR10. In addition the amino acid p.L59 is conserved among different species (**Figure 25A-C**. The surface expression of TLR10 in lymphocytes from P10 demonstrated low protein levels (**Figure 26A, and 29B**).

Unfortunately, the CD62L shedding and I κ B α degradation in response to a specific TLR10 agonist was not possible to be performed because among the human TLRs, TLR10 is the only family member still without a defined agonist. However, PBMCs from P12 produced reduced amount of IFN- γ following BCG + IL-12 activations as shown in **Figure 20A**, suggesting defective BCG signaling.

Figure 25 Molecular and genetic analysis of P10

B)

p.L59F
↓

Species	AA Alignment
Human	59 PATTTLDLSYN L LFQLQSSDFHSV
Mutated	59 PATTTLDLSYN F LFQLQSSDFHS
Ptroglyodytes	59 PATTTLDLSYN L LFQLQSSDFHS
Mmulatta	59 PATTTLDLSYN L LFQLQSSDFHS
Fcatus	28 PTTTLDLSYN L LSQLQSSDFRS

C)

(A) Heterozygous missense mutation c.175C>T/p.L59F in *TLR10* of P10. (B) Conservation analysis of p.L59 amino acid among different species. (C) *In silico* analysis predicts truncated expression of TLR10

Figure 26 Defective expression of TLR10

(A) Histogram and (B) graphic showing reduced TLR10 expression on lymphocytes from P10 TLR10. MFI, Mean fluorescence intensity.

4.1.6.4 DKC1

Mutations in *DKC1* resulting in X-linked dyskeratosis congenita (DC) is characterized by the failure of skin and haemopoietic system. *DKC1* gene encodes dyskerin which is an important component of the telomerase complex (Dokal, 2000; Knight et al., 2001). Abnormalities in T cell development has been described (Cossu et al., 2002) P11 whose PBMCs produce low amount of IFN- γ (**Figure 20A**), was identified by WES with the previously described hemizygous mutation c.A838C/p.S280R in *DKC1* (Knight et al., 2001) which was confirmed by Sanger sequencing (**Figure 27A**). MutationTaster and polyphen predicted that this mutation is disease causing. Parents' gDNA were sequenced and revealed

the P13’s mother carries the c.A838C mutation. Multiple sequence alignment of dyskerin demonstrated that the amino acid p.S280 is conserved among species. The p.S280R mutation is localized in the NOP10 binding region and the 3D protein structure modeling suggested that p.S280R impairs the bonding between NP10 and DKC1 which is required for maintenance and function of telomerase (Kirwan and Dokal, 2009) (**Figure 27B and 27C**)

Figure 27 Genetic and molecular analysis of DKC1

A)

B)

p.S280

Species	AA Alignment
Huma	280AQWLYDNHKDE S YLRRVVYPLEKL
Mutated	280AQWLYDNHKDE R YLRRVVYPLEK
Ptroglydyte	279AQWLYDNHKDE S YLRRVVYPLEK
Mmulatta	280AQWLYDNHKDE S YLRRVVYPLEK
Fcatus	275NHKDE S YLRRVVYPLEK
Mmusculus	280AQWLYDNHKDE S YLRRVVYPLEK
Ggallus	232AQWQYDNNKDD S YLRRVILP
Trubripes	274AQWQFDHNKDE S YLRRVIFPLEK
Drerio	275AQWQFDHNKDE T YLRRVIFPLEK
Dmelanogaste	278AMWLYENHKDE S MLRRVIKPLEG
Celegan	268AQYLLDTQKDE S YMRHIVRPLEA

(A) Identification of Hemizygous X-Linked mutation A838C/p.S280R in *DKC1* from P11 and his parents (B) Conservation analysis demonstrated that the amino acid p.S280 is conserved among different species. (C) 3D protein structure modeling of *DKC1* predicts that the mutation A838C/p.S280R generates a truncated protein.

4.1.6.5 SOCS-1

SOCS proteins are negative regulators of cytokine-induced Jak/STAT signaling (Kamura et al., 1998; Naka et al., 1997). A novel variation c.493C>T/p.P165S in SOCS-1 was identified by WES and confirmed by Sanger sequencing (**Figure 28A**). This mutation was not found in genomic DNA from mother and father indicating that p.P165S is a *de novo* variation. Multiple sequence alignment (conservation analysis) demonstrated that the p165 amino acid is conserved among species (**Figure 28B**). *In silico* analysis suggests that p.P165S is pathogenic. The 3D protein structure modeling of SOCS-1 indicates that the p.P165S variation is a hypermorphic mutation localized in the SOCSbox domain which is important for SOCS-1/EloinBC interaction. This interaction is necessary for SOCS-1 degradation and consequently activation of IFN- γ signaling. Therefore the p.P165S variation may increase the activity of SOCS-1 and result in the defective IL-12/IFN- γ axis displayed by PBMCs from P12.

Figure 28 Molecular and genetic analysis of SOCS-1

(A) Heterozygous *de novo* mutation c.493C>T/p.P165S in *SOCS-1* gene of P11. (B) Conservation analysis of p.P165 aminoacid among different species. (C) 3D protein structure modeling analysis showing that the substitution of Pro to Ser resulted into extra beta-sheath in the motif of ElonginBC binding site of SOCS-1.

4.1.6.6 IRAK2

IRAK2 is an important molecule for the TLR signaling (Kawagoe et al., 2008; Meylan and Tschopp, 2008). Sanger sequencing confirmed the novel heterozygous genetic variation c.1030G>C/p.D344H found by WES in the *IRAK2* gene of P13. MutationTaster and polyphen predicts that the genetic variation p.D344H is pathogenic. Conservation analysis of IRAK2 confirmed that the mutated amino acid p.D344 is conserved among different species. In addition, the 3D protein structure modeling of IRAK2 predicts that the mutation p.D344H disrupt the ATP binding site may affecting the binding of IRAK2 protein to ATP important for TLR signaling (**Figure 29 A-C**).

Neutrophils and monocytes from P13 displayed reduced CD62L shedding following stimulation with TLR2 (BCG and Pam2CSK4) and TLR4 (LPS) agonists as shown in **Figure 14**. In accordance, monocytes from P13 displayed defective I κ B α degradation when stimulated by BCG and LPS (**Figure 30A**). Further, TNF- α production by the PBMCs from the P9 was evaluated in response to BCG + LPS activation and low level of TNF- α production was observed in comparison to the healthy control. However, TNF- α production was normal in response to PMA + PMA (**Figure 30B**). Taking together, our data suggest that the mutation c.1030G>C in *IRAK2* gene may affects the TLRs signaling.

Figure 29 Molecular and genetic analysis of *IRAK2* gene from P13

B)

p.D344
↓

Species	AA	Alignment
Human	344	HSNVKSSNVLLDQNLTPKLAHPMA
Mutated	344	HSNVKSSNVLLHQNLTPKLAHPM
Mmulatta	344	HSNVKSSNVLLDQNLTPKLAHPM
Fcatus	305	HGNIKSSNVLLDQNFSTSKLAHPM
Mmusculus	342	HSNVKSANVLLDQHLNPKLAHPV
Ggallus	323	HGN IKSSNVLLDENFTP KLG HSG
Dmelanogaster	349	HGD IKPANI LL DQCLQPK
Celegans	324	HGDIKT ANI LL DKHMEPKIGD--
Xtropicalis	338	HGNIKSS N I FL DENSFPKLG HSG

C)

A) Identification of the heterozygous genetic variation c.1030G>C/D344H in IRAK2 gene of P13 (B) conservation analysis of p.D344 in IRAK2. (C) 3D protein structure modeling of wild type and mutated IRAK2.

Figure 30 Cells from P13 demonstrated impaired $\text{I}\kappa\text{B}\alpha$ degradation and $\text{TNF-}\alpha$

(A) Impaired $\text{I}\kappa\text{B}\alpha$ was observed in monocytes from P13 stimulated by BCG and LPS. (B) $\text{TNF-}\alpha$ production by PBMCs from P13 following activation by BCG + LPS.

In order to give an overview of the molecular-genetic defects found in the Brazilian patients with susceptibility to mycobacterial, we summarize all the mutation data obtained in Table 10.

Table 10 Mutations data of Brazilian patients with susceptibility to mycobacterial diseases

Pt	Origin	Type of mutation variation	Mutations	Predicted effect on protein	Reference
P1	RJ	Deletion	(c.351delT, c.343_350delGGGAATT, c.304_311delATCCATCA)	(p.F118fsX139, p.G115fs117, p.F118fsX139)	-
P2	RS	Nonsense	c.475 G > A	p. W140X	(Korthauer et al., 1993)
P3	PI	Insertion	c.633_634insAGCC	p.R165fsX190	(Cabral-Marques et al., 2014)
P4	SP	Deletion	c.345_402del	exon 3 skipping	(Seyama et al., 1998)
P5	SP	Missense	c.1238A>C	p.P413H	-
P6	DF	Deletion	c.75_76delGT	p.Val25X51	(Koker et al., 2013)
P7	SP	Missense	c.1736G>A	p.R579H	(Kormann et al., 2009)
P8	SP	Missense	c.1187C>T	p.A396V	-
P9	SP	Missense	c.1430T>A	p.V447D	-
P10	SP	Missense	c.175C>T	p.L59F	-
P11	RJ	Missense	c.A838C	p.S280R	(Knight et al., 2001)
P12	SP	Missense	c.493C>T	p.P165S	-
P13	SP	Missense	c.1030G>C	p.D344H	-
P14	SP	-	-	-	-
P15	SP	-	-	-	-
P16	SP	-	-	-	-
P17	SP	-	-	-	-
P18	SP	-	-	-	-

4.1.7 Analysis of phagocytosis and mycobacterial growth inside monocytes-derived macrophages

Macrophages are essential for the immune protection against TB by controlling *M. tuberculosis* growth (Deffert et al., 2014a). Finally, we decide to address the macrophage function of the patients from our cohort when challenged with *M. tuberculosis*. MDMs from patients displayed normal capacity to phagocytose *M. tuberculosis* in comparison to healthy controls. However, MDMs from the patients failed to control the intracellular growth of *M. tuberculosis* (**Figure 31A and 31B**).

Figure 31 Phagocytosis and intracellular growth of *M. tuberculosis* in MDMs from patients and healthy controls

A)

B)

(A) Phagocytosis and growth of *M. tuberculosis* in macrophages from patients with susceptibility to mycobacterial infections. The growth index is defined as the number of CFUs at 6 day of culture divided by the number of CFU at 0 day of culture. Significant difference is denoted by asterisk ($P < 0.05$; $n = 15$, Mann-Whitney test).

Considering the activating properties of human recombinant IFN- γ (rhIFN- γ) on phagocytes and its beneficial therapeutic effects for patients with susceptibility to mycobacterial infections (Gao et al., 2011), we pre-treated MDMs from patients and controls with rhIFN- γ (500 U/ml) for 24 hours. Due to limited amount of blood shipment and time frame to conclude this thesis only five patients were analyzed: two patients with IL-12R genetic variations (P8 and P9) and three with CD40L deficiency (P2, P3 and P4) and one patient with DKC1 mutation. MDMs were evaluated for their ability to phagocytose and control intracellular *M. tuberculosis* growth. Despite the patients and controls have

demonstrated increased phagocytose after rhIFN- γ pre-treatment, no statistical significance was observed. However, rhIFN- γ significantly increased the growth control of *M. tuberculosis* by MDMs from control healthy subjects and improved the ability of MDMs from patients to control the growth of *M. tuberculosis*. (**Figure 32A and 32B**).

Figure 32 Phagocytosis and intracellular growth of *M. tuberculosis* in IFN- γ primed MDMs from patients and healthy controls

After differentiation, MDMs were stimulated for 24 h in serum free RPMI-1640 medium containing rhIFN- γ (500 IU/ml). A) Phagocytosis. B) Intracellular growth controls. Significant difference is denoted by asterisk ($P < 0.05$; $n = 5$, Mann-Whitney test).

5 DISCUSSION

This work describes a cohort of 17 Brazilian patients with heterogenous PIDs who developed mycobacterial infections caused by different mycobacterium species. The isolated species were *M. tuberculosis*, *M. bovis* (BCGitis) and *MAC*. Among these infections, pulmonary TB was the most frequent followed by disseminated TB. Besides mycobacterial infections, pneumonia (caused by Mycobact. Or other pathogens?) was the most frequent clinical manifestation. In addition, three patients developed infections not previously described in well-known PIDs. To our knowledge: P1 is the first RAG1-deficient patient who developed juvenile xanthogranulomatous disease; the occurrence of TB in P47phox deficiency (P6) (Manuscript in revision – supplementary data), and *Cryptococcus gattii* infection in IL-12R β 2 deficiency (P8). Sanger sequencing and WES identified a novel mutation in *RAG1*, previously described mutations in *CD40LG*, *NEMO*, *NCF1* and *DKC1* genes, and genetic variations in *TLR2*, *IL-12R β 2*, and *TLR10* which have been previously reported, and novel genetic variations in *IL-12R β 1*, *IRAK2*, and *SOCS-1*. These later requiring deeper investigations to corroborate their mutational status. Five patients (P13, P14, P15, P16 and P17) remain without defined molecular genetic defects due to limited blood sampling or time factor. Finally, MDMs from the patients demonstrated normal capacity to phagocyte *M. tuberculosis*, however, failed to control *M. tuberculosis* growth. Noteworthy, macrophages from CD40L-deficient (P2, P3 and P4), IL-12R-deficient patients (P9: IL-12R β 1; P8: IL-12R β 2) and dyskerin deficient patient (P11), from whom it was possible to obtain enough blood sample to pre-treat the cells and perform MDMs analysis, rhIFN- γ improved the defective mycobacterial growth control.

Mycobacterial diseases remain one of the world's deadliest communicable diseases. In 2013, an estimated 9.0 million people developed TB and 1.5 million died from the disease, 360,000 of whom were HIV-positive (WHO, 2014). Considering our search for genetic defects underlying the susceptibility to mycobacterial diseases only HIV-negative patients were included in our study. As previously described for patients with mycobacterial infections (Smith, 2003) *M. tuberculosis* was the most frequent isolated species which was identified in 13 patients of the 17 patients included in this study. Following *M. tuberculosis*, *M. bovis* (causing BCGitis) was the most frequent species (identified in four patients) and *MAC* in one patient. These data are in accordance with the fact that immunocompromised patients are vulnerable to *M. tuberculosis* and also to adverse complications of BCG vaccine (BCGitis and

BCGosis) and NTM. TB is still epidemic in Brazil and common BCG vaccine is widely practiced and required in Brazil. BCG vaccination aims to prevent early-life infections with *M. tuberculosis*. The BCG vaccination itself is believed to be merely safe for a competent immune system but a potentially lethal infection can be expected in immunocompromised hosts (Rezaei et al., 2006). Future projects need to be encouraged by the Brazilian government to implement newborn screening in Brazil. This will improve the quality of life of Brazilian patients with PID and avoid complications with vaccines based on live attenuated pathogens such as BCG, polio and varicella vaccines.

Here we describe for the first time the occurrence of TB in p47phox deficiency. However, mycobacterial infections are frequently described in CGD caused by mutations in gp91phox, p22phox, and p67phox (de Oliveira-Junior et al., 2011; Deffert et al., 2014b; Koker et al., 2013; Lee et al., 2008; Ying et al.). CGD patients are typically highly susceptible to multiple life-threatening bacterial and fungal infections (Bogunovic et al., 2012; Bustamante et al., 2007; Vinh et al., 2009). So far, 220 cases of CGD patients who developed BCG complications and 72 cases of *M. tuberculosis* infections were described. Approximately 20% of published cases of mycobacterial infection in CGD patients are due to *M. tuberculosis* (Deffert et al., 2014b). Neurocryptococcosis is also described here for the first time in the IL-12R β 2 deficiency, although patients with IL-12R β 1 deficiency have been described with Cryptococcosis (Jirapongsananuruk et al., 2012). Despite not usually described in high frequency, other fungal infections such as candidiasis and histoplasmosis (de Beaucoudrey et al., 2010), Paracoccidioidomycosis (Moraes-Vasconcelos et al., 2005), and Coccidioidomycosis (Vinh et al., 2009) have been reported in MSMD patients.

PIDs are more frequent in human population than previously thought (Bousfiha et al., 2013). Our data suggest that a high number of subjects who developed mycobacterial infection can have a PID and probably the majority of these patients remain without a genetic diagnosis during their entire life with severe impact in their quality of life. The germ theory of diseases indicates that infectious diseases are fundamentally extrinsic and that etiologic agents are mainly responsible for the development of diseases. However, the concept of inborn errors of immunity demonstrates that germline genetic is determinant in individuals and populations.

A number of genetic studies revealed that host genetic makeup is an essential determinant of susceptibility or resistance to infection (Casanova and Abel, 2013). For example individuals lacking DARC expression on erythrocytes are resistant to *Plasmodium vivax*

(Maestre et al., 2010), those lacking functional CCR5 are resistant to R5-tropic strains of HIV (Marmor et al., 2006) and human subjects lacking FUT2 are resistant to noroviruses (Smyth et al., 2011). On the other hand, genetic defects (Mendelian traits) of Bruton's Tyrosine Kinase (BTK) confer susceptibility to extracellular bacteria and virus (Strijbis et al., 2013), defects in TLR3 signaling result in susceptibility to Herpes simplex virus (Guo et al., 2011), defects in *CD40LG* result in susceptibility to a variety of classes of etiologic agents (Cabral-Marques et al., 2012), and MSMD in which specific underlying genetic defects lead to susceptibility to mycobacteria. Considering the nowadays elevated number of HIV-negative individuals and those without behavioral factors which increase the susceptibility to mycobacterial infections (smoking, alcoholism, and other comorbidity factors), a high number of patients who develop mycobacterial infections have underlying genetic defects.

In this study were identified twelve patients who developed mycobacterial infections with heterogenous genetic defects. Mutations in nine different genes previously associated with well known PIDs (*RAG1/SCID*, *CD40LG/CD40L* deficiency, *NEMO/XL-EDA-ID*, *NCF1/CGD*, *TLR2* and *TLR10*/defects in TLR signaling, *IL-12R β 2* and *IL-12R β 1/MSMD*), and possibly novel PIDs due to defects in *IRAK2* and *SOCS-1* (despite requiring further investigations) were characterized. Pathogenicity of the variants was analyzed by allele frequency from public databases (ensembl and dbSNP databases), conservation analysis, *in silico* predictors (MutationTaster, polyphen, 3D structure). However, *in silico* analysis of the mutation in *IL-12R β 2* (mutation: p.A396V) and the mutation in *TLR10* (mutation: p.L59F) showed contradictory results. It is in accordance with previously reports which show that the identification of genetic variations in the clinical investigation of single patients using bioinformatic tools commonly can produce erroneous and contradictory conclusions regarding pathogenicity (Riuro et al., 2014). Therefore, functional assays are required to confirm the pathogenicity of the genetic variations found. The functional tests (IL-12/IFN- γ production, CD62L shedding, STAT4 phosphorylation, I κ B α degradation) which have been performed in the present study suggest that the genetic variations found are indeed pathogenic. However, further mutagenesis studies of the variations found in *TLR2*, *TLR10*, *IL-12R β 1*, *IL-12R β 2*, *IRAK2* and *SOCS-1* will require additional investigation. Moreover, it is important to sequence the DNA from the Brazilian healthy population to confirm that these genetic variations are not SNPs.

The identification of mutations in previously described genes which are associated with well known PIDs was essential for a definitive diagnosis, treatment strategies, prognosis, and

genetic counseling, while the identification of novel genes associated with PIDs will be essential for identification of new genetic etiologies in well-defined PIDs or new PIDs which hampers development of therapeutic or preventive strategies.

Our data are in accordance with the literature which report that patients with SCID (Afshar Paiman et al., 2006; Avila et al., 2010; Schuetz et al., 2008), CD40L deficiency (Levy et al., 1997; Wang et al., 2014; de Oliveira-Junior et al., 2011; Cabral-Marques et al., 2012), CGD (de Oliveira-Junior et al., 2011; Deffert et al., 2014b; Koker et al., 2013; Lee et al., 2008; Ying et al.), XL-EDA-ID (Burns et al., 2014; Dai et al., 2004; Filipe-Santos et al., 2006), defects in TLR signaling (Arji et al., 2014) and MSMD (Senanayake et al., 2014; Aytekin et al., 2011; Boisson-Dupuis et al., 2011) have increased susceptibility to mycobacterial infections. On the other hand it will be necessary to find more subjects with *SOCS-1* and *IRAK2* mutations to well characterize these PIDs. Noteworthy, all the PIDs characterized in our cohort affect the properly triggering of IL-12/IFN- γ axis which is a well characterized part of the immune response required to the protection against infections caused by mycobacteria and other opportunistic pathogens. As well described in the classification of the international union of immunological societies expert committee for primary immunodeficiency (Al-Herz et al., 2014b; Al-Herz et al., 2014a), patients with mutations in pleiotropic genes are susceptible to a broader range of infections beyond those caused by mycobacteria, as is the case of those patients with SCID, XL-EDA-ID, CD40L deficiency, and CGD.

The definition of the genetic diagnosis of the well known PIDs described here (SCID, XL-EDA-ID, CD40L deficiency, CGD) was relatively fast to achieve considering that each of these PIDs have classical clinical manifestations or well established screening methods to identify the underlying genetic defects: SCID - low lymphocyte populations and screening by immunophenotyping; XL-EDA-ID - present clinical characteristics including ectodermal dysplasia and screening by I κ B α degradation; CD40L deficiency - impaired CSR of immunoglobulins and screening by CD40L expression on the surface of CD4⁺ T cells; and CGD patients develop granulomatous infectious and screening by DHR assay. However, the other defects without clear symptoms and many genes involved such as defects in TLR signaling (TLR2 and TLR10, IRAK2) and MSMD (IL-12R β 1, IL-12R β 2, SOCS-1 - a potential MSMD candidate gene), often require support of High-throughput DNA sequencing (WES) as required here. WES has recently advanced and revolutionized the field of genomics,

making it possible for even single research groups to generate large amounts of sequence data very rapidly and at a substantially lower cost (Kircher and Kelso, 2010).

PIDs have been characterized immunologically for the last 60 years and genetically, principally by Sanger DNA sequencing, over the last 30 years. The advent of next-generation sequencing (NGS) in the last decade with the development of whole-exome sequencing in particular, has facilitated the identification of previously unknown genetic lesions. NGS is rapidly generating a stream of candidate variants for an increasing number of genetically undefined PIDs. The use of NGS technology is ushering in a new era, by facilitating the discovery and characterization of new PIDs in patients with infections and other phenotypes, thereby helping to improve diagnostic accuracy (Picard and Fischer, 2014). However, disease gene identification is still a challenge in Brazil and developed countries due to reduced government support despite great efforts of the Grupo Brasileiro de Imunodeficiências (BRAGID, www.imunopediatria.org.br) and the Latin American Society for Immunodeficiencies (LASID, <http://www.lasid.org>). Even though for well known PIDs like CGD or CD40L deficiency the traditional molecular genetic analysis are well established such traditional molecular facilities are only available in a few laboratories in Brazil and other Latin American countries. Molecular genetic diagnosis is limited to a few states/provinces of Brazil, Argentina, Chile and Mexico resulting in difficulties to even determine the actual prevalence and genetic basis of these PIDs in these areas (Cabral-Marques et al., 2014). Therefore, it is urgently required to spread the use of WES to determine the variations in Brazil and Latin America. WES provides coverage of more than 95% of the exons, which contains 85% of disease-causing mutations in Mendelian disorders and many disease-predisposing SNPs throughout the genome (Rabbani et al., 2014). In accordance, the WES identified the genetic variations in the patients without classical signs of well-known PIDs. Of note, WES identified a variant case of *DKC1* mutation with immunodeficiency, however without the classical symptoms of dyskeratosis congenita in P11 and possibly the first cases of *SOCS-1* (12) and *IRAK2* (13) deficiency.

Mutations in *DKC1* gene which is located on Xq28 and encodes dyskerin is responsible for the X-linked recessive form of the disorder. Dyskerin is a component of a small nucleolar ribonucleoprotein (snoRNP) that is highly conserved throughout evolution. The binding of dyskerin to three other proteins (GAR1, NOP10, and NHP2) (**Figure 33**) is important for maintenance of telomerase (Vulliamy et al., 2006).

Figure 33 Interaction of dyskerin with telomerase complex

Dyskerin is an important component of both the H/ACA small nucleolar (snoRNA) ribonucleoprotein (RNP) particles and telomerase complex. (A) Dyskerin, together with GAR1, NHP2, NOP10, and H/ACA snoRNA, comprises snoRNP particles. The H/ACA RNA-protein complex catalyzes pseudouridylation and processing of ribosomal (r) RNA. (B) *TERC* (telomerase RNA component) and TERT (telomerase reverse transcriptase) are the core components of telomerase complex. As an H/ACA snoRNA, *TERC* associates with dyskerin, GAR1, NHP2, and NOP10, which are also present in the telomerase complex (Du, 2008).

To our knowledge the occurrence of TB in P11 is first case of a patient with Dyskeratosis congenita (DKC) and susceptibility to mycobacterial infections. The mechanism by which the p.S280R mutation affects the IFN- γ production needs to be clarified. However, patients with DKC have inherited bone marrow failure syndrome (Knight et al., 2001) and patients with abnormal T cell differentiation has been also reported (Cossu et al., 2002) suggesting the involvement of DKC1 with the T-cell mediated immune response. The severity, age of onset and range of somatic and haematological abnormalities varies widely (Walne et al., 2007). Interestingly, the P11 is currently 11 year old but did not develop the typical clinical picture of DKC. In analogy with some patients with NEMO deficiency who can develop the classical immunodeficiency phenotype without EDA symptoms (Niehues et al., 2004), P11 may be the first patient with a DKC1 mutation who did not develop the DKC signs. However, we can not rule out that P11 will develop DKC symptoms later in life.

Another important data obtained by the WES analysis was the SOCS-1 defect in P12. SOCS-1 deficiency has not been reported in humans; however, *in vitro* studies and mice

models investigation have been conducted. Double SOCS-1 knockout mice died within 3 weeks of birth, with a syndrome characterized by lymphopenia, activation of peripheral T cells, fatty degeneration and necrosis of liver, and macrophages infiltration of major organs (Marine et al., 1999). In other experiments SOCS-1 heterozygous mice exhibit enhanced liver damage, severe liver fibrosis and increased mortality due to cancer compared to the wild type mice (Yoshida et al., 2004).

Physiologically SOCS inhibit phosphorylation of STATs by binding to and inhibiting Jak kinases function (Kamura et al., 1998; Naka et al., 1997). SOCS-1 domains and interacting protein are illustrated in **Figure 34**. SOCS-1 interacts with a variety of proteins including elongin B and elongin C, cullin-5 and RING-box-2 (RBX2), which recruits E2 ubiquitin transferase and function as E3 ubiquitin ligases mediating the degradation of proteins through their N-terminal regions (Yoshimura et al., 2007). E3 ligases carry out the final step in the ubiquitination cascade, catalyzing transfer of ubiquitin from an E2 enzyme to form a covalent bond with a substrate (SOCS-1) and signal for degradation via proteasome (Berndsen and Wolberger, 2014). Moreover, a Socs-1 mutant (L175P, C179F in SOCS box where Elongin BC binds) in mice fails to bind to Elongin BC and was more stable than wild-type Socs-1 suggesting that the Elongin BC complex negatively affects the stability of Socs-1 (Chen et al., 2002) by targeting Socs-1 to the proteasomal degradation pathway. Therefore, the *in silico* analysis prediction of the hypermorphic mutation (c.493C>T/p.P165S) in SOCS-1 of P12 may explain the defective IL-12/IFN- γ axis displayed by PBMCs from P11.

Figure 34 The structure and function of SOCS-1 protein

(A) Structure of SOCS-1 and its three domains (B) SOCS-1 can directly interact with the JAK kinase activation loop but also has affinity for interferon (IFN) receptors Adapted from: (Yoshimura et al., 2007).

The other possible novel PID caused by IRAK2 deficiency was identified in P10. Members of the IRAK family of kinases mediate TLR signaling and among these IRAK1, IRAK2 and IRAK4 are essential for responses to TLR stimulation (Kawagoe et al., 2008). So far IRAK4 deficiency has been described (Picard et al., 2010), however IRAK2 deficiency still not. TLR2, the main TLR involved in mycobacterial immune response, uses IRAK1 and IRAK2 for activation of NF- κ B and MAPK pathways (**Figure 35**) (Kawagoe et al., 2008; Meylan and Tschopp, 2008).

Figure 35 Contributions of IRAK molecules to TLR2 signaling

The TLR2 transduction signaling is demonstrated which culminates with the activation of MAPK kinase and NF-κB. DD, death domain; KD, kinase domain; TIR, Toll-interleukin-1 receptor (Kawagoe et al., 2008; Meylan and Tschopp, 2008).

Human cell lines with knockdown of IRAK2 showed that this molecule participates in NF-κB responses to multiple TLRs and has been proposed to act as a positive regulator of TLR signaling. Macrophages from mice lacking *Irak2* showed impaired cytokine productions (TNF-α, IL-16) in response to all TLRs except TLR3 (Meylan and Tschopp, 2008). In the process of mycobacterial infection control, TNF-α cytokine acts in synergy with IFN-γ, stimulating the production of reactive nitrogen intermediates (RNIs) and reactive oxygen species (ROS), thus mediating the tuberculostatic function of macrophages (Barnes et al., 1993). Together these results reveal the importance of IRAK2 in TLR responses and mycobacterial host defense. In concordance, monocytes from P10 showed impaired CD62L

shedding and NF- κ B activation in response to TLR2 and TLR4 agonist which may account for susceptibility to mycobacterial infection.

Our final aim was to investigate the ability of macrophages from patients with mycobacterial infections to phagocytose and to control intracellular growth of *M. tuberculosis* (O'Brien et al., 1994). Macrophages are the main effector cells involved in the growth control of *M. tuberculosis* and in different body tissues they derive from monocytes. In its turn, monocytes originate from progenitors in the bone marrow and traffic via the bloodstream to peripheral tissues. During both homeostasis and inflammation, circulating monocytes leave the bloodstream and migrate into tissues where, following conditioning by local growth factors, pro-inflammatory cytokines and microbial products, they differentiate into macrophages (Shi and Pamer, 2011).

Macrophages from our patients with susceptibility to mycobacterial diseases showed normal ability to phagocytose *M. tuberculosis*, however failed to control the intracellular growth of *M. tuberculosis* in comparison to those from healthy subjects. It is in accordance with Carranza and collaborators who previously showed that macrophages from TB patients were less capable of controlling *M. tuberculosis* growth (Carranza et al., 2006). The mechanism of impaired growth control of *M. tuberculosis* by our patients MDMS stills needs to be investigated. However, a possible mechanism may be attributed to defective ROS because the patient's monocyte presented reduced oxidative burst in response to *M. bovis* (BCG) this defect may be transferred to macrophages during differentiation from monocytes. On the other hand, considering the heterogenous genetic background of the patients from this study, possibly different mechanisms are involved. The continuation of this research line is required to study increased number of patients with mutations in the same gene in order to study the particularly effect of each genetic defect in the MDM mechanisms of controlling *M. tuberculosis* growth

The optimal *M. tuberculosis* growth control by macrophages requires cytokine activation. *In vitro* models of macrophage activation for the killing of *M. tuberculosis* are rather artificial, and therefore the exact conditions for optimal activation remain unknown (van Crevel et al., 2002). However, it is well established that lymphocyte products, mainly IFN- γ and other proinflammatory cytokines like TNF- α are essential regulators of macrophages activation to control *M. tuberculosis* growth. Pre-activated MDM with rhIFN- γ from our CD40L-deficient patients and from those with defects in the IL-12R improved *M. tuberculosis* growth control.

Unfortunately, blood samples from other patients were not available in a reasonable time frame to analyze if rhIFN- γ improves the *M.tuberculosis* growth control. The improvement of the functional defect of MDM from the CD40L-deficient patients to control *M. tuberculosis* growth after pre-incubation with rhIFN- γ represent an attractive future option to develop clinical trials in order to evaluate the effect of IFN- γ as adjunctive immunotherapy for severe opportunistic infections in CD40L-deficiency, as this cytokine is licensed for use for patients with defects of IL-12/IFN- γ axis and CGD patients. Noteworthy, in a single report despite receiving G-CSF and IVIG treatment, a CD40L-deficient patient from Argentina presented with disseminated mycobacterial infection and poor response to antituberculous therapy. He only showed clinical improvement after adding rhIFN γ as adjuvant therapy (A.G. Seminario and Bezrodnik, 2013). Interesting our ongoing studies with neutrophils from CD40L-deficient patients, who also developed mycobacterial diseases have shown defective fungicidal activity which is improved by pre-treatment with rhIFN- γ (Cabral-Marques et al., 2014, Submitted to Journal of Allergy and Clinical Immunology).

The mechanism by which IFN- γ improves the functional defects of phagocytes occurs both at the level of a progenitor cell and mature cells (Ezekowitz et al., 1987; Ezekowitz et al., 1990). This cytokine enhances the oxidative burst response, but on the other hand, IFN- γ has been shown to contradictorily improve neutrophil microbicidal killing through mechanisms other than enhanced oxidative activity (Woodman et al., 1992). IFN- γ is an important cytokine which plays multifarious roles in different parts of the immune system. It is involved in the process of generating, sustaining and regulating the cells of the innate and adaptive arms of the immune system. Macrophages activated by IFN- γ increased pinocytosis, receptor-mediated phagocytosis and microbial killing ability against mycobacteria.

Taking together the above mentioned data and considering that Brazil is high burden TB country, the treatment of patients with poor responsiveness to TB therapy needs to include IFN- γ as adjuvant therapy. In addition, large randomized controlled trials have been already performed showing that adjuvant therapy using IFN- γ might be beneficial to TB patients (Gao et al., 2011).

In conclusion our data suggest that Brazilian patients with mycobacterial infections have heterogeneous molecular genetic defects. Investigation of more patients with different TB forms in Brazil promise to contribute to expand the clinical and genetic defects associated with the susceptibility to mycobacterial infections which will be an important contribution to the field of infectious diseases worldwide.

6 CONCLUSIONS

- ✓ The most frequent cause of mycobacterial disease was *M. tuberculosis*;
- ✓ Mutations in different genes (*RAG1*, *CD40LG*, *NEMO*, *NCF1*, *TLR2*, *TLR10*, *IL-12R β 1*, *IL-12R β 2*, *SOSC-1* and *IRAK2*) were identified;
- ✓ Monocytes/macrophages from the patients displayed normal phagocytosis, however, showed impaired oxidative burst and *M. tuberculosis* growth control;
- ✓ Possibly SOCS-1 and IRAK2 deficiency are novel PIDs which require additional investigation;
- ✓ rhIFN- γ improved the *M. tuberculosis* growth control by MDMs from patients with IL-12R , CD40L deficiencies and DKC1 Mutation (P11).

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APENDICE A – Manuscript in revision in the Journal of Clinical Immunol about the first occurrence of *M. tuberculosis* infections in p47^{phox} deficiency.

Tuberculosis in autosomal recessive chronic granulomatous disease: mutation in *NCF1* Gene and impaired *Mycobacterium tuberculosis* growth control within monocyte-derived macrophages

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ABSTRACT

Tuberculosis (TB) is a severe and life-threatening infection caused by *Mycobacterium tuberculosis*. Susceptibility to mycobacteria has been reported in a number of primary immunodeficiency diseases (PIDs) including chronic granulomatous disease (CGD). Mycobacterial infections have been reported in CGD patients with mutations in the genes encoding gp91phox, p22phox, and p67phox. Here we report the clinical, molecular, and immunopathological characteristics of the first case of TB in a patient with CGD due to a mutation in p47-phox (*NCF1* gene). The patient displayed normal activation of IL-12/IFN- γ axis, but, impaired oxidative burst and abolished p47phox expression. The sequencing of *NCF1* gene which encodes p47phox revealed the homozygous GT deletion (c.75–76delGT; g.3307_3308delGT) resulting in a premature stop codon (p.Val25X51). Finally, the macrophages from the patient ingest normally *M. tuberculosis*, however, they were unable to control the intracellular growth of the bacilli. This work highlights the essential role of p47phox for controlling the respiratory burst and *M. tuberculosis* growth within human monocyte-derived macrophages.

INTRODUCTION

Tuberculosis remains a major public health problem. It is estimated that about one third of the human population carries the pathogen and that the worldwide death is over one million per year (Deffert et al., 2014b). Susceptibility to infections caused by *Mycobacterium tuberculosis* and other *Mycobacterium* species has been reported in patients with acquired immunodeficiencies (e.g. acquired immunodeficiency syndrome or AIDS) or primary immunodeficiency diseases (PIDs) including defects of the IL-12/IFN- γ axis, severe combined immunodeficiency (SCID), CD40L deficiency and chronic granulomatous disease (CGD) (Reichenbach et al., 2001). The latter is caused by genetic abnormalities affecting the phagocyte NADPH oxidase resulting in the failure of neutrophils and monocytes/macrophages to generate reactive oxygen species and hence an inability to kill fungi and intracellular bacteria. (Song et al.).

The incidence of CGD is approximately 1 in 250,000 live births. (Winkelstein et al., 2000). The phagocyte NADPH oxidase complex consists of two membrane bound proteins (gp91phox and p22phox) which together form the cytochrome b_{558} and three cytosolic proteins (p47phox, p67phox, and p40phox). Upon cell activation these proteins and the small GTPase Rac assemble at the cell membrane and phagolysosomes (El-Benna et al., 2009; Kang et al.) to trigger the oxidative burst, essential for microbial killing. Mutations in the genes encoding components of the NADPH oxidase have been described in CGD. (Kang et al.). Among the NADPH oxidase genes, *CYBB* which encodes gp91phox is located on X chromosome and accounts for about 65% of CGD cases, while mutations in autosomal genes e.g *CYBA* (encoding p22phox), *NCF1* (encoding p47phox), *NCF2* (encoding p67phox), and *NCF4* (encoding p40phox) cause autosomal recessive (AR)-CGD. The most common form of AR-CGD is caused by mutations in *NCF1* (25% of patients) followed by mutations in *NCF2*, *CYBA*, and *NCF4* (Gardiner et al., 2013; Heyworth and Cross, 2002; Matute et al., 2009; Song et al., 2011).

Patients with CGD suffer from recurrent life-threatening bacterial and fungal infections affecting the skin, lungs, liver, spleen, lymph nodes, and bones (Gardiner et al., 2013; Segal et al., 2000; Winkelstein et al., 2000). In North America and Europe, the most common infections in CGD are caused by *Staphylococcus aureus*, *Burkholderia cepacia*, *Salmonella sp.*, *Serratia marcescens*, *Nocardia spp.*, and *Aspergillus spp.* (Holland). On the other hand, in regions such as South America and Asia where TB has high incidence, CGD

patients can additionally develop TB and clinical adverse reactions after *Bacille Calmette–Guerin* (BCG) vaccination (BCGites or BCGosis) (van den Berg et al., 2009).

Mycobacterial infections have been reported in patients with defects of gp91phox, p22phox, and p67phox (de Oliveira-Junior et al., 2011; Deffert et al., 2014b; Koker et al., 2013; Lee et al., 2008; Ying et al.). However, to our knowledge mycobacterial infections caused by *M. tuberculosis* have not been reported in patients with mutations in p47phox (*NCF1* gene). Here we report clinical and molecular features of the first patient with severe TB and the g.3307_3308delGT mutation in *NCF1* gene. In addition, the defective immune response of patient macrophages to *M. tuberculosis* is characterized.

PATIENT, MATERIAL AND METHODS

Case Report: The patient is a female, born in 1997 to non-consanguineous Brazilian parents after an uncomplicated pregnancy. Her mother died of unknown cause and her father is alive and healthy. The patient has two healthy half brothers from the paternal side (Figure 1A). She was vaccinated with BCG without clinical complications. The patient developed pneumonia at 8 months of age and remained without clinical manifestations until 6 years of age, when she presented with persistent high fever and was diagnosed simultaneously with pneumonia, pleural empyema, granulomatous pleuritis, osteomyelitis of the rib, and septicemia with poor response to different antibiotic regimens. A lung biopsy showed pulmonary mycosis due to *Aspergillus sp.* and zygomycetes. The latter species could not be identified. These fungal infections were treated successfully with Amphotericin B.

One year later, the patient presented with other severe infections such as meningitis, endocarditis, and pulmonary mycosis with positive culture for *Candida albicans*. Laboratory results revealed normal leukocyte counts and normal serum IgA, IgM, and IgG levels. At 12 years of age, the patient developed persistent fever, dry cough, and weight loss. CT scan and chest radiograph suggested severe TB and *Mycobacterium tuberculosis* was identified by lung biopsy (Figure 1B). The patient was treated for three years with rifampicin, isoniazid, and pyrazinamide. Currently, the patient is receiving prophylactic cotrimoxazol and itraconazole.

Considering the medical history, the case was investigated for a defect in the IL-12/IFN- γ axis and CGD. The procedures were approved by the Ethics Committee at the Institute of Biomedical Sciences, University of São Paulo, according to the Helsinki

Convention and the Ministry of Health of Brazil. Informed consent was obtained and signed before performing lab exams.

Analysis of IL-12/IFN- γ axis

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood after Ficoll-Hypaque fractionation and analyzed for the production of IL-12 and IFN- γ as previously described (Feinberg et al., 2004). Briefly, 0.2×10^6 PBMCs were activated at 37°C in 5% CO₂, in 96-well round-bottom plates (Greiner Bio-One, Frickenhausen, Germany) in a final volume of 200 μ L of RPMI 1640, supplemented with 10% FCS. The production of IL-12 and IFN- γ were measured after 24 and 48hr incubation with IFN- γ (200 IU/ml, Imukin®, Boehringer Ingelheim) or/and live BCG (1 PBMC/10 BCG), and with IL-12 (5 ng/ml, R&D Systems®) or/and live BCG (1 PBMC/10 BCG), respectively. BCG was cultured as previously described (Petricevich et al., 2001). The production of IL-12 and IFN- γ in the supernatants were analyzed by ELISA according to the manufacturer's instructions (Becton Dickinson).

Dihydrorhodamine-123 (DHR)

In order to determine an intrinsic defect of oxidative burst, the peripheral blood leucocytes (PBL) were incubated for 5 min at 37°C with dihydrorhodamine 123 (DHR, Sigma-Aldrich) after 1 hour activation with phorbol myristate acetate (PMA, 300 ng/ml, Sigma Laboratories, St. Louis, MO, USA) and live BCG (1 cell/10 BCG). The cells were evaluated on a BD FACSCanto II Cytometer and the data obtained were analyzed by using FlowJo software (Treestar, Inc, Ashland, Ore).

Flow cytometric analysis of NADPH oxidase components

Expression of phagocyte NADPH oxidase components was detected by flow cytometry (Koker et al., 2007; Wada et al.). Cells were fixed and permeabilized with Cytofix/Cytoperm Plus kit (Becton Dickinson) according to the manufacturer's instructions. Peripheral blood monocytes (PBMs) were incubated with anti-gp91phox, anti-p22phox (Santa Cruz Biotechnology Inc, Santa Cruz, CA), anti-p40phox, anti-p47phox, and anti-p67phox (Bioss inc, Woburn, MA) at

4 °C for 30 min. After washing, the cells were analyzed by flow cytometry on a BD FACSCanto II Cytometer and the results were analyzed by FlowJo software.

Sequencing of *NCF1* gene

Genomic DNA (gDNA) was isolated from EDTA blood using the Wizard® Genomic DNA Purification kit (Promega). The *NCF1* gene, including the exon–intron boundaries, were amplified by polymerase chain reaction (PCR). PCR products were purified using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare), according to the manufacturer's instructions. PCR products were sequenced on a MegaBACE 1000 sequencer using the DYEnamic ET Dye Terminator kit (Thermo Sequenase™ II DNA Polymerase).

Generation of monocyte-derived macrophages (MDMs)

Human monocyte-derived macrophages (MDMs) were obtained as described by Esquivel-Solís *et al.* (Esquivel-Solis et al., 2009). Briefly, PBMCs were isolated from heparinized blood after Ficoll-Hypaque sedimentation and adherent monocytes were cultured at 37°C in a humidified 5% CO₂ atmosphere for 7 days in the presence of 5 ng/ml of macrophage colony stimulating factor (M-CSF, Peprotech).

Phagocytosis and growth activity of *M. tuberculosis* within MDMs

A virulent *M. tuberculosis* H37Rv strain (ATCC 25618) was kindly provided by Dr. Philip Suffys from Fundação Oswaldo Cruz (FIOCRUZ, Brazil) and cultured as previously described (Wong et al., 2007). 4×10^4 MDMs were seeded in 96-well plates (Corning) in complete RPMI medium (Gibco; 1 mM sodium pyruvate, 2 mM glutamine and 3% FCS) and kept overnight at 37°C in a 5% CO₂ atmosphere. MDMs were infected with H37Rv at 1:1 ratio (MDM:bacilli) for 3 h (day 0) and the cells washed to remove extracellular mycobacteria. On day 0 and after days 3 and 6, the MDMs were lysed with 0.1% saponin for 10 min and the homogenates were diluted and plated in Middlebrook 7H10 medium supplemented with 10% OADC (Difco; acid/albumin/dextrose/catalase). The colonies were determined after 21 days incubation at 37°C. Bacterial uptake (phagocytosis) data were obtained from CFU counts on day 0. Bacterial growth index was determined by CFU number ratio on days 3 and 6 per CFU number relative to day 0.

RESULTS

PBMCs from the index case displayed normal activation of IL-12/IFN- γ axis

Possible defects in IL-12-IFN- γ axis were investigated by analyzing the ability of PBMCs from the index case to produce IL-12 and IFN- γ , which was normal in response to the indicated stimuli (Fig. 2) compared to healthy controls.

Defective oxidative burst suggesting CGD

Neutrophils and monocytes from our index case presented a reduced oxidative burst in response to PMA and BCG (Fig. 3A and 3B) compared to phagocytes from healthy controls, suggesting CGD.

Molecular genetic analysis of NADPH oxidase identified the common GT deletion in *NCF1*

The expression of p47phox was abolished while the other NADPH subunits were expressed normally in our patient's monocytes (Fig 4A). Sequencing of *NCF1* gene identified the common deletion g.3307_3308delGT. This is a frameshift homozygous GT deletion (c.75–76delGT; g.3307_3308delGT; p.Val25X51) located at the beginning of exon 2 (Fig. 4B) (Noack et al., 2001; Prando-Andrade et al., 2004).

Deficient p47phox macrophages present normal phagocytic capacity but fail to control *M. tuberculosis* growth

Macrophages are essential for the immune protection against TB by controlling *M. tuberculosis* growth (Deffert et al., 2014b). Therefore we analyzed the capacity of MDMs from our p47phox deficient patient to phagocyte and control intracellular *M. tuberculosis* growth. The ability of MDMs of the p47phox deficient patient to phagocyte *M. tuberculosis* was similar to healthy controls (Fig 5A). However, MDMs from the patient failed to control the intracellular growth of *M. tuberculosis* (Fig 5B).

DISCUSSION

To our knowledge, this work describes the first patient with TB and CGD with an underlying genetic defect in p47phox. Our patient developed TB at 12 years of age with low response to

anti-tuberculosis treatment and the necessity to be treated longer than usual. Our laboratorial data showed residual oxidative burst and absence of p47phox protein expression due to a homozygous GT deletion (c.75–76delGT; g.3307_3308delGT). Moreover, MDMs from the patient demonstrated normal capacity to phagocyte *M. tuberculosis*, however, failed to control its intracellular growth.

The spectrum of infections in CGD is broader than those with mendelian susceptibility to mycobacterial diseases (MSMD) due to defects of IL-12/IFN- γ axis, that present usual susceptibility mainly to mycobacteria species. On the other hand, patients with CGD are typically highly susceptible to multiple life-threatening bacterial and fungal infections, including mycobacteria (Bogunovic et al., 2012; Bustamante et al., 2007; Vinh et al., 2009). In accordance, the patient presented normal activation of IL-12/IFN- γ axis and showed the CGD clinical phenotype.

Brazil has one of the highest rates of TB infections than other countries where administration of BCG vaccination is a mandatory practice. BCG vaccination can cause clinical complications (BCGitis/BCGosis) in CGD patients. In addition, patients with CGD or defects in the IL-12/IFN- γ axis fail to develop a protective immune response and may develop TB later in life (Ying et al., 2014). Accordingly, despite receiving BCG vaccination without clinical complications, our patient developed TB.

So far, 220 cases of CGD patients who developed BCG complications and 72 cases of occurrence of *M. tuberculosis* infections were described. It is noteworthy that, approximately 20% of published cases of mycobacterial infection in CGD patients are due to *M. tuberculosis* (Deffert et al., 2014b). The gp91phox deficiency accounts for 70% of CGD cases while p47phox mutations occur in 25% of CGD patients which is the most common AR-CGD form. Heterogeneous mutations within the genes encoding the NADPH oxidase components have been described. The unique hot-spot mutation described is the common GT deletion in *NCF1* (Koker et al., 2007) which was also identified in our index case. The reason why the occurrence of *M. tuberculosis* infection has not been described in other patients with mutation in *NCF1* is probably due to a limited number of well-established laboratories performing molecular diagnosis in developing countries where TB is still endemic (Cabral-Marques et al., 2014).

Correlation between molecular defects and severity of CGD is attributed to the type of mutation and its effect on the capacity of the NADPH to trigger ROS production, irrespective

of the affected gene (Kuhns et al., 2010; Roesler et al., 2012). Usually, patients with partial defect of oxidative burst, whose phagocytes are able to produce residual amounts of ROS, show a much milder phenotype, develop infections later in life, and have better prognoses compared with those with complete absence of an oxidative burst response. In accordance, the phagocytes from our patient showed residual ROS production and the first severe infections suggesting CGD occurred after six years of age. In addition, she is currently alive without having received either IFN- γ therapy or bone marrow transplantation.

The relevance of ROS production by macrophages for the immunity against *M. tuberculosis* is highlighted by the discovery of families with peculiar mutations in gp91phox leading to MSMD. These patients lack ROS production by MDMs, but not in neutrophils and show a high susceptibility to mycobacterial infections (Bustamante et al., 2011). Macrophages have been attributed as the main effector cells which controls *M. tuberculosis* growth. (van Crevel et al., 2002). Macrophages surrounded by T cells in healthy subjects form mature granulomas, a process required to control the growth and spreading of *M. tuberculosis* (Saunders and Cooper, 2000). The immunity to mycobacteria has been ascribed to T-cell-mediated immunity, with CD4⁺ T cells playing a crucial role by producing IFN- γ which increases the oxidative burst of phagocytes by modulating NADPH activation (Holland, 2010). Thus, it is clear that the NADPH oxidase plays a major role in microbial killing; however, few studies have investigated *in vitro* function of macrophages from CGD patients (Bustamante et al., 2011; Deffert et al., 2014b; Rahman et al., 2009). Among them, it has been shown that MDMs from CGD patients have reduced capacity to kill *E. coli* and *S. aureus*. However, Fazal *et al.* demonstrated that MDMs from CGD patients are able to control the growth of BCG or *M. avium* (Fazal, 1997). Here we have demonstrated for the first time that MDMs from a CGD patient have normal capacity to phagocyte *M. tuberculosis*, however, failed to control the bacilli growth within MDMs. These data corroborate that a completely normal oxidative burst is required for controlling *M. tuberculosis* growth in humans. Furthermore p47phox knock-out mice have such a similar defective immune response to *M. tuberculosis* (Deffert et al., 2014b).

In conclusion, our data emphasize the relevance of early molecular genetic diagnosis of CGD for the treatment and prevention of TB in patients with mutations in p47phox. Furthermore, our work highlights the important role of the phagocyte NADPH oxidase for the immune response of MDMs against *M. tuberculosis*.

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A)

B)

Figure 1

A)

B)

Figure 2

A)

B)

Figure 3

B)

Figure 4

A)

B)

Figure 5

FIGURE LEGENDS

Figure 1. Family pedigree of our index case and lung biopsy specimen showing *M. tuberculosis*. (A) The black filled circle represents the index case. (B) Ziehl Neelsen stain photomicrograph shows *Mycobacterium tuberculosis* (arrow).

Figure 2. PBMCs from our index case produced normal amounts of IFN- γ and IL-12. (A) PBMCs were activated with IFN- γ , BCG or BCG + IFN- γ to analyze the IL-12 production (B), while for IFN- γ release the cells were stimulated by IL-12, BCG or BCG + IL-12

Figure 3. Impaired phagocytes oxidative burst suggests CGD. (A) Neutrophils and (B) monocytes from our index case showed a reduced oxidative burst after stimulation with PMA or BCG using DHR assay.

Figure 4. Defective p47phox expression and *NCF1* sequencing. (A) Histograms showing defective expression of p47phox in patients monocytes. (B) Sequencing showing the homozygous deletion (g.3307_3308delGT) identified in gDNA of our index case.

Figure 5. Defective growth control of *M. tuberculosis* by MDMs from our index case. The macrophages were cultured with *Mycobacterium tuberculosis* (H37Rv strain) and the bacterial growth index was determined. Patient's MDMs showed normal phagocytosis but failed to control bacilli growth. The results are representative of two independent experiments performed in triplicate

APENDICE B – Manuscript published in the Journal of Clinical Immunol about the First report of the LASID registry in which the occurrence of *M. tuberculosis* infections are described in CD40L-deficient patients.

First Report of the Hyper-IgM Syndrome Registry of the Latin American Society for Immunodeficiencies: Novel Mutations, Unique Infections, and Outcomes

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Abstract Hyper-IgM (HIGM) syndrome is a heterogeneous group of disorders characterized by normal or elevated serum IgM levels associated with absent or decreased IgG, IgA and IgE. Here we summarize data from the

HIGM syndrome Registry of the Latin American Society for Immunodeficiencies (LASID). Of the 58 patients from 51 families reported to the registry with the clinical phenotype of HIGM syndrome, molecular defects were identified in 37

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patients thus far. We retrospectively analyzed the clinical, immunological and molecular data from these 37 patients. CD40 ligand (CD40L) deficiency was found in 35 patients from 25 families and activation-induced cytidine deaminase (AID) deficiency in 2 unrelated patients. Five previously unreported mutations were identified in the *CD40L* gene (*CD40LG*). Respiratory tract infections, mainly pneumonia, were the most frequent clinical manifestation. Previously undescribed fungal and opportunistic infections were observed in CD40L-deficient patients but not in the two patients with AID deficiency. These include the first cases of pneumonia caused by *Mycoplasma pneumoniae*, *Serratia marcescens* or *Aspergillus sp.* and diarrhea caused by *Microsporidium sp.* or *Isospora belli*. Except for four CD40L-deficient patients who died from complications of presumptive central nervous system infections or sepsis, all patients reported in this study are alive. Four CD40L-deficient patients underwent successful bone marrow transplantation. This report characterizes the clinical and genetic spectrum of HIGM syndrome in Latin America and expands the understanding of the genotype and phenotype of this syndrome in tropical areas.

Keywords Latin American Society for Immunodeficiencies · Hyper-IgM syndrome · CD40L deficiency · AID deficiency, infections, mutations

Introduction

Hyper-immunoglobulin M (HIGM) syndrome is a heterogeneous group of primary immunodeficiency disorders characterized by recurrent infections and defects of immunoglobulin class switch recombination (CSR) associated with elevated or normal levels of serum IgM and low levels of serum IgG, IgA, and IgE [1]. HIGM syndrome was first clinically described more than 50 years ago, [2–4] and was molecularly defined in 1993, when 5 groups reported mutations in the *CD40L* gene (*CD40LG*) [5–9], located on the X chromosome. Subsequently, a second X-linked molecular defect caused by mutations in the nuclear factor- κ B (*NF- κ B*) essential modulator gene (*NEMO*) [10, 11], and autosomal recessive HIGM syndromes caused by mutations in *CD40* or by B cell–intrinsic defects of CSR due to mutations in activation-induced cytidine deaminase (*AICDA*) [12] or uracil-DNA glycosylase (*UNG*) [13] have been described. More recently mutations in the post-

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meiotic segregation 2 gene (*PMS2*) [14] have been identified as cause of autosomal recessive HIGM. Among these molecular defects, CD40L and AID deficiencies are the most common forms of HIGM syndrome estimated to represent 75 % and 20 % of cases [15], respectively.

In general, patients with B cell–intrinsic defects are susceptible to extracellular bacterial and rarely viral infections while CD40L- and CD40-deficient patients are in addition susceptible to fungal, opportunistic and intracellular bacterial infections. This difference can be explained by the broader function of CD40L-CD40 interaction in the regulation of B cell [16], dendritic cell (DCs) and T cell immune responses [17, 18].

Following the characterization of the molecular basis of CD40L deficiency 20 years ago, over 200 cases of HIGM syndrome from Europe and North America (http://bioinf.uta.fi/base_root) and approximately 100 cases from a single Center [15], as well as additional cases from the United States [19] have been described. In these geographic regions, the clinical and genetic spectrum of HIGM syndromes are well defined. However, the characterization of these syndromes in Latin America remains to be determined.

Here we describe the genetic and clinical characteristics of CD40L-deficient and AID-deficient patients from Latin America as an ongoing effort of the Latin American Society for Immunodeficiencies (LASID) Registry, aiming to improve diagnosis and treatment of these disorders locally. The LASID Registry contains demographic, genetic, clinical and immunologic information from 58 Latin American patients with HIGM syndrome. We have recently described the occurrence of previously unreported microorganisms causing infections in CD40L deficiency such as *Paracoccidioides brasiliensis*, *Klebsiella* sp. and *Acinetobacter* sp., and human papilloma virus [20]. This report expands the genetic and clinical spectrum of CD40L deficiency globally with the report of five novel mutations in the *CD40LG* gene and previously undescribed infectious agents associated with CD40L deficiency, such as the occurrence of pneumonia caused by *Mycoplasma pneumoniae*, *Serratia marcescens* or *Aspergillus* sp. and diarrhea caused by *Microsporidium* sp. microsporidiosis or *Isospora belli*.

Methods

Data Collection and Construction of Registry

The patients were registered by physicians from different Latin American countries including Argentina, Brazil, Chile, Costa Rica, Mexico, and Peru who care for and followed patients with HIGM syndrome. In June 2011, a 4-page questionnaire asking for detailed information about demographic,

clinical, immunological and molecular characteristics was sent to physicians who are members of LASID and to investigators who perform molecular genetic analysis of patients with HIGM syndrome in Latin America.

Molecular Genetic Diagnosis

Investigators from countries where molecular genetic analyses were feasible started to investigate every male patient with HIGM syndrome for mutations in the *CD40LG* because mutations in this gene lead to the most common form of HIGM syndrome [15, 21]. Analysis of CD40L expression by activated CD4⁺ T cells was performed by flow cytometry using specific monoclonal antibodies (mAbs) (clones available upon request). Because CD40-Ig fusion protein became only recently available in one center in Brazil, the functional assay to evaluate the CD40L-CD40 binding was performed in only a few patients (P12–P17) as previously described [22]. Because AID deficiency is the most common autosomal defect underlying this syndrome, male patients with no abnormality in the molecular genetic analysis of *CD40LG* and female patients were analyzed for mutations in *AID*.

Exons and flanking intron sequences of *CD40LG* and *AID* were amplified by PCR, the PCR products were purified and sequenced (primers and conditions used in each country are available upon request). Mutations are described according to the suggested nomenclature of the Human Genome Variation Society [23, 24].

Diagnostic Criteria

Male patients with the clinical diagnosis of HIGM syndrome were considered to have CD40L deficiency if reduced CD40L protein expression and/or binding of CD40-Ig fusion protein by activated CD4⁺ T cells was identified and a mutation within the *CD40LG* could be ascertained by gene sequencing. On the other hand, patients were considered AID-deficient if they had an autosomal pattern of inheritance and a mutated *AICDA* gene. Patients P1–P11 [20], P20 and P21 [25], and P26–P31 [26] have been reported previously.

Results

Patient Demographic and Baseline Characteristics

The six participating countries reported a total of 58 patients from 51 families with clinical features of HIGM syndrome. The gender ratio was 48 male/10 female. The distribution of patients by countries is shown in Fig. 1. Of these 58 patients, genetic defects were identified in 37. The detailed clinical and laboratory data for these 37 patients were analyzed for this paper.

Fig. 1 Distribution of patients with HIGM among the Latin American countries

In this group of molecularly-defined patients, 35 from 25 non-related families were CD40L-deficient and 2 unrelated (1 male and 1 female) patients were AID-deficient. Seventeen patients (all males with CD40L-deficiency) are from families with a positive history of infections affecting other male members. Patient P36, with AID deficiency is from a consanguineous family. The patients' baseline characteristics including the mean age at the first clinical manifestation, age at diagnosis, serum immunoglobulin levels, etc. are described in Table 1.

Infections and Associated Pathogens

Recurrent severe and unusual infections including those caused by non-opportunistic and opportunistic microorganisms were the hallmark clinical manifestation in the CD40L-deficient cohort. Among them, pneumonia was the most common (28 patients) (Table 1). The most frequent cause of pneumonia (Table 2) was *Pneumocystis jirovecii* (P1-P3, P6, P8, P10, P14, P32 and P34), followed by cytomegalovirus

(P16), *Pseudomonas aeruginosa* (P24), *Streptococcus pneumoniae* (P26), parainfluenza virus type II (P27) and unknown pathogens. In two patients pneumonia was caused by *Aspergillus sp.* (P14, P25) (Fig. 2a and b), and in one each by *Mycoplasma pneumoniae* (P21) and *Serratia marcescens* (P31), which are etiologic agents not previously reported in CD40L-deficient patients. Following pneumonia, upper respiratory tract infections (URTIs) (22 patients) and chronic diarrhea (16 patients) were the most frequent clinical manifestations. In the latter category, *Giardia lamblia* was the most commonly isolated pathogen (P16, P20, P21, P24 and P28) followed by *Cryptosporidium parvum* (P3, P8 and P14) and by the fungus *Microsporidium sp.* (P25) or the protozoa *Isospora belli* (P16 and P17), the latter two being etiological agents not previously described in CD40L deficiency. It is noteworthy that in P25 at age 10 years severe microsporidiosis occurred simultaneously with aspergillosis and in P16 at age 15 years recurrent diarrhea caused by *Isospora belli* led to cachexia with body weight of 22 kg—body mass index (BMI) of 10.4 kg/m² - (Fig. 2c).

Table 1 Patients' baseline, laboratory data and Clinical manifestations

		CD40L-deficient patients (n=35)	AID-deficient patients (n=2)
Baseline characteristics	Mean age at first clinical manifestation (range)	0.7 years (0.1–4)	4.5 years [4, 5]
	Mean age at diagnosis of HIGM syndrome (range)	2.3 years (0.3–13.6)	24.5 years [9, 40]
	Alive	31	2
Laboratory data	Immunoglobulin levels (before IVIG ⁵¹)	IgG (< P3)	2 (100 %)
		IgA (< P3)	2 (100 %)
		IgM (> P97)	2 (100 %)
	Neutropenia	17 (48 %)	–
Clinical manifestations	Pneumonia	28 (80 %)	2 (100 %)
	Upper resp. tract infections	22 (63 %)	2 (100 %)
	Chronic diarrhea	16 (46 %)	2 (100 %)
	Urinary tract infections	3 (9 %)	1 (50 %)
	Sepsis	3 (9 %)	1 (50 %)
	Hepatitis	2 (6 %)	–
	Meningitis/encephalitis	2 (6 %)	–

^a Reference values of immunoglobulin levels are according to Guerra et al. [51]. IVIG intravenous immunoglobulin therapy; P percentile

Table 2 Isolated pathogens from Latin American patients with HIGM syndrome

Patients	Fungi	Extracellular bacteria		Intracellular bacteria	Virus	Protozoa
		Gram-positive	Gram-negative			
CD40L-deficient (n=35)	<i>P. jirovecii</i> (n=9; 26 %)	<i>S. pneumoniae</i> (n=1; 3 %)	<i>P. aeruginosa</i> (n=5; 14 %)	<i>Mycoplasma pneumoniae</i> (n=1; 3 %)	CMV (n=1; 3 %)	<i>G. lamblia</i> (n=5 14 %)
	<i>C. albicans</i> (n=6; 17 %)	<i>S. aureus</i> (n=1; 3 %)	<i>K. pneumoniae</i> (n=3; 9 %)	<i>Mycobacterium tuberculosis</i> (n=3; 9 %)	Hepatitis B virus (n=1; 3 %)	<i>C. parvum</i> (n=3; 9 %)
	<i>Aspergillus sp.</i> (n=2; 6 %)		<i>Actinobacter sp.</i> (n=1; 3 %)		Herpes simplex (n=1; 3 %)	<i>Isospora sp</i> (n=2; 6 %)
	<i>Microsporidium sp.</i> (n=1; 3 %)		<i>S. marcescens</i> (n=1; 3 %)		Molluscum contagiosum (n=1; 3 %)	
					HPV (n=1; 3 %)	
	<i>P. brasiliensis</i> (n=1; 3 %)		<i>E. coli</i> (n=2; 6 %)		Parainfluenza virus type II (n=1; 3 %)	
	<i>H. capsulatum</i> (n=1; 3 %)		<i>E. cloacae</i> (n=1; 3 %)			
	<i>C. neoformans</i> (n=1; 3 %)		<i>B. pertussis</i> (n=1; 3 %)			

CMV cytomegalovirus; HPV human papillomavirus

In addition to pneumonia caused by *P. jirovecii* and *Aspergillus sp.* and diarrhea caused by *Microsporidium sp.*, the CD40L-deficient patients frequently developed other fungal infections such as paracoccidioidomycosis (P10), severe candidiasis which manifested itself in the form of esophagitis

Fig. 2 Pulmonary aspergillosis and *Isospora belli* infection in CD40L-deficiency. **a** Computed tomography (CT) scan of the chest showing *Aspergillus* lung infection in P14. **b** Images showing growth of *Aspergillus niger* from sputum cultures of patient P25. **c** Cachectic state of patient P16 after recurrent diarrhea caused by *Isospora belli*

(P5), candidiasis of the scalp (P9), cellulitis and urinary tract infection (P29), and oral (P7 and P21), genital (P18) and perianal (P6) candidiasis.

Other bacterial infections such as pulmonary (P10 and P19) or disseminated *Mycobacterium tuberculosis* (P25), sepsis caused by *P. aeruginosa* (P15) or caused simultaneously by *K. pneumoniae* and *Actinobacter sp.* (P9), and cellulitis caused by *Klebsiella pneumoniae* or *E. coli* (P29) and urinary tract infection (UTI) caused by *Escherichia coli* (P2) were observed in CD40L-deficient patients. In addition, viral infections caused by human papilloma virus (HPV) (P6), herpes simplex (P6), molluscum contagiosum (P24) and hepatitis B virus (P26) were reported.

The two AID-deficient patients developed recurrent pneumonia, URTIs and chronic diarrhea, while one (P37) reported, in addition, UTI and sepsis. The etiologic agents in these two patients were not identified.

Lymphoid Hyperplasia

Lymphoid hyperplasia was observed in four CD40L-deficient patients characterized by enlarged tonsils (P2 and P10), lymph nodes (P4 and P10), spleen (P4 and P10) and thymus (P13). The latter finding has not been described previously in CD40L-deficient patients and was identified as idiopathic true massive thymic hyperplasia by chest computed tomography when P13 was 7 years old, followed by thymectomy and biopsy (data not shown).

Both AID-deficient patients, P36 and P37 at age 5 and 17 years respectively, presented with lymphoid hyperplasia limited to the tonsils.

Autoimmunity and Inflammatory Disorders

Autoimmunity and inflammatory disorders were infrequent. One CD40L-deficient patient (P27) developed hypothyroidism and two (P13 and P25) had autoimmune hemolytic anemia while

none of the two AID-deficient patients reported inflammatory disorders or clinical autoimmune manifestations.

Molecular Genetic Characterization

Each country in which molecular analysis was feasible investigated every male patient for CD40L deficiency. This approach led to the identification of 35 CD40L-deficient patients. The results of mutation analyses are summarized in Table 3 and Fig. 3. Of those, five novel mutations abolishing CD40L protein expression on activated CD4⁺ T cells were identified: the nonsense mutation c.159C > T affecting codon 35 in exon 1 (p.Q35X), located in the transmembrane domain (TM) in P32; the frameshift insertion c.633_634insAGCC (p.L193fsX201) in exon 5 (P19) and the frameshift deletion c.430_431delAT (p.H125fsX128) in exon 4 (P34) affect the tumor necrosis factor homologous (TNFH) domain. The other two novel mutations were found within the *CD40LG* promoter (a gross deletion, P14) and in the polyadenylation signal sequence (a gross insertion, P15) completely abrogating *CD40LG* transcription or resulting in unstable mRNA. The details of the mutations identified in the *CD40LG* of P14 and P15 will be published elsewhere. The mutations observed in the remaining CD40L-deficient patients (Table 3) have been previously described [9, 15, 20, 22, 25–29].

Two patients (P36 and P37) were found to be homozygous for the previously reported [30] missense mutation (p.C87S) of AICDA that affects exon 3 of the cytidine deaminase domain of the AID protein (Table 3 and Fig. 3).

Treatment and Outcome

All Latin American patients with HIGM syndrome are receiving IVIG replacement therapy resulting in a substantial decrease in the number of infections. The infections, (including 4 deaths) that occurred while receiving IVIG therapy were more frequent in CD40L-deficient patients than in AID-deficient patients. Three CD40L-deficient patients died due to complications resulting from presumptive central nervous system (CNS) infections: P9 died at age 8 years due to progressive multifocal leukoencephalopathy, P20 at 9 years due to progressive panencephalitis, and P32 at 7 years due to encephalopathy. P16, when in a cachectic state, died at age 15 years of sepsis. The survival curve for our cohort of CD40L-deficient patients is shown in Fig. 4.

Sixteen CD40L-deficient patients developed neutropenia (Table 1) which was mostly intermittent and associated with oral, rectal or esophageal ulcers. Neutropenia was effectively treated with granulocyte colony stimulating factor (G-CSF), except P4 who showed only partial response to this treatment. Several CD40L-deficient patients had neutropenia at the time or just before developing fungal infections: P14 and P25 (Aspergillosis), P25 (Microsporidiosis), P3, P5, P29

(candidiasis), P3 and P14 (*P. jiroveci* pneumonia). Twenty seven CD40L-deficient patients are currently receiving antibiotic prophylaxis for *P. jirovecii* or had been on prophylaxis at an earlier age.

Four CD40L-deficient patients underwent successful allogeneic hematopoietic stem cell transplantation (HSCT). Patients P5 and P12 received bone marrow and patients P22 and P23 were given peripheral blood stem cells. Graft-versus-host disease (GVHD) was observed in three patients: P5 developed acute GVHD grade II, P22 cutaneous acute GVHD grade II and P23 cutaneous hyperacute GVHD grade III. In addition, P5 developed systemic CMV infection and autoimmune hemolytic anemia following HSCT and were successfully treated with ganciclovir, methylprednisolone, Rituximab and IVIG. GVHD resolved and all four patients are alive and well at last follow-up (0.5 to 3 years post HSCT) without immunosuppressive therapy.

Discussion

This is the first report of HIGM syndrome across Latin America. This registry study expands the spectrum of microorganisms reported in this disease. Severe and unusual infections caused by microorganisms not previously associated with CD40L deficiency such as *Aspergillus sp.*, *Mycoplasma pneumoniae*, *Serratia marcescens*, *Microsporidium sp.*, and *Isospora belli* were identified in Latin American patients. In addition, infections caused by other not previously described etiological agents such as *Paracoccidioides brasiliensis*, *Klebsiella pneumonia*, *Acinetobacter sp.*, and *human papilloma virus (HPV)* were recently identified in Brazilian patients [20].

The clinical and molecular characterizations of HIGM syndromes in North America and Europe, particularly those caused by CD40L and AID deficiencies [15, 19, 21, 31, 32], have been well established following the discovery of the specific genetic defects underlying the different entities designated as HIGM syndromes [5–9, 12, 13, 33]. In this communication, LASID, which was given the mandate to advance and improve the diagnosis and management of PIDs in Latin America [34, 35], reports the results of its HIGM syndrome registry. In addition to the clinical and immunologic features of the HIGM syndromes described by North American and European investigators [15, 19, 31–33, 36, 37], our report of Latin American patients, which is a genetically heterogeneous group of subjects, expands the spectrum of microorganisms previously reported in HIGM syndromes.

Noteworthy, P24 with gastrointestinal infection caused by microsporidiosis, a clinical complication frequently observed in patients with acquired immunodeficiency syndrome (AIDS) [38], is to our knowledge, the first case of a well-defined primary immunodeficiency disorder suffering from

Table 3 Molecular genetic analysis of CD40L-deficient and AID-deficient patients

Pt	Origin	Type of mutation	cDNA mutation ^a (<i>CD40LG</i>)	Predicted effect on protein	Protein expression by flow cytometry		Ref.
					CD40L mAb	CD40-Ig	
P1	Brazil	Missense	c. 433 T>G	p.V126G	—	N/E	20
P2	Brazil	Missense	c.476 G>C	p.W140C	—	N/E	20
P3	Brazil	Missense	c.496 C>A	p.T147N	+	—	22
P4 ^{c1}	Brazil	Nonsense	c.475 G>A	p. W140X	—	N/E	9
P5 ^{c1}	Brazil	Nonsense	c.475 G>A	p. W140X	—	N/E	9
P6 ^{c2}	Brazil	FS del	c.213_216delATAG	p.I53fsX65	—	N/E	28
P7 ^{c2}	Brazil	FS del	c.213_216delATAG	p.I53fsX65	—	N/E	28
P8	Brazil	FS del	c.213_216delATAG	p.I53fsX65	—	N/E	28
P9 ^c	Brazil	Splice site	c.345_402del	exon 3 skipping	—	N/E	22
P10	Brazil	Splice site	c.345_402del	exon 3 skipping	—	N/E	22
P11	Brazil	FS ins	c.551_552insAA	p.R165fsX190	—	N/E	20
P12 ^{b1}	Brazil	Splice site	c. 213-1G>T	exon 2 skipping	—	—	22
P13 ^{b1}	Brazil	Splice site	c. 213-1G>T	exon 2 skipping	—	—	22
P14	Brazil	Deletion	deletion in promoter ^b	—	—	—	Novel
P15	Brazil	insertion	insertion in poly(A)signal ^b	—	—	—	Novel
P16 ^{b2}	Brazil	Missense	c.817C>T	p.T254M	—	—	22
P17 ^{b2}	Brazil	Missense	c.817C>T	p.T254M	—	—	22
P18	Brazil	Nonsense	c.710 C>A	p.C218X	+	—	22
P19	Brazil	Insertion	c.633_634insAGCC	p.L193fsX201	—	—	Novel
P20 ^{b3 c}	Argentina	FS del	c.500delG	p.M148X153	—	N/E	25
P21 ^{b3}	Argentina	FS del	c.500delG	p.M148X153	—	N/E	25
P22 ^{b4}	Argentina	Missense	c.424 C>A	p.A123E	—	N/E	29
P23 ^{b4}	Argentina	Missense	c.424 C>A	p.A123E	—	N/E	29
P24	Argentina	Nonsense	c.576 C>T	p.Q174X	—	N/E	15
P25	Argentina	Missense	c.813 T>A	p.F253I	—	N/E	25
P26	Mexico	Nonsense	c.714 C>T	p.Q220X	—	N/E	15
P27	Mexico	Splice site	c.465+1 G>A	Exon 4 skipping	—	N/E	15
P28	Mexico	Missense	c.429 C>T	p.H125Y	—	N/E	26
P29 ^{b5}	Mexico	Missense	c.424 C>A	p.A123E	—	N/E	29
P30 ^{b5}	Mexico	Missense	c.424 C>A	p.A123E	—	N/E	29
P31	Mexico	Nonsense	c.750C>T	p.Q232X	—	N/E	27
P32 ^c	Costa Rica	Nonsense	c.159C>T	p.Q35X	—	N/E	Novel
P33	Costa Rica	Nonsense	c.475G>A	p.W140X	—	N/E	9
P34	Costa Rica	FS del	c.430_431delAT	p.H125fsX128	—	N/E	Novel
P35	Chile	Missense	c.473 T>C	p.W140R	—	N/E	28
Pt	Origin		cDNA mutation (<i>AICDA</i>)	Predicted effect on protein	Gene expression by real-time PCR		Ref.
P36	Brazil	Missense	c.260 C>G	p.C87S	D		30
P37	Brazil	Missense	c.260 C>G	p.C87S	D		30

The small letters b and c associated with superscript numbers indicate brothers and cousins, respectively, from the same family. Nucleotide number is based on the sequence data from <http://bioinf.uta.fi/CD40Lbase/to> describe CD40L mutations and from <http://bioinf.uta.fi/AICDAbase/index.php?content=index/IDbases> to describe AID mutations

FS del frame shift deletion; FS ins frame shift insertion; N/E not evaluated

^a The nomenclature for the description of mutations are according to guidelines of Human Genome Variation Society [23, 24]

^b detailed molecular genetic alterations will be published elsewhere

^c patient died

+present; -absent

Fig. 3 Mutations in CD40L and AID. Schematic representation of **a** the *CD40LG* and **b** the *AICDA* coding regions and protein domains. Both genes have 5 exons. The type and position of each mutation observed in Latin American patients are shown. *IC* intracellular tail; *TM* transmembrane domain; *ECU* extracellular unique domain; *TNFH* TNF homology domain; *NLS* nuclear localization signal; *CDD* Cytidine deaminase domain; *NES* nuclear export signal; *UTR* untranslated region

microsporidiosis. On the other hand, infections caused by *Aspergillus sp.*, *Mycoplasma sp.*, *Serratia marcescens*, and *Isospora belli* have been well documented in patients with other PIDs [39–46]. This observations in Latin American patients are important findings which emphasize that patients worldwide with defects in CD40L-CD40 interaction should be monitored for these pathogens.

The high susceptibility to a broad spectrum of pathogens in CD40L deficiency can be explained only in part to be due to neutropenia. CD40L-deficient patients are susceptible to opportunistic infections with high mortality rates even when neutrophil counts are normal and patients are on G-CSF and IVIG treatment [20, 31]. The explanation for this global susceptibility to infections may be the pleiotropic role of CD40L-CD40 interaction in the regulation of immune

responses. In addition to transitory neutropenia, abnormal B cell differentiation and reduced antibody responses, these patients have defective T [18, 47] and dendritic cell (DC) activation [17, 48] which contributes to the immunopathologic mechanisms in CD40L and CD40 deficiencies. Compared with HIGM syndromes caused by B cell-intrinsic defects affecting CSR and somatic hypermutation (eg. AID and UNG deficiencies), patients suffering from CD40L deficiency, with few exceptions [49], manifest the first symptoms of PID at an earlier age, are sicker and more likely to develop severe infections even while on IVIG therapy [31, 32]. This was also the case in our cohort, although only two of our patients had AID deficiency. P36 and P37 did not receive IVIG therapy until the age of 9 and 40 years, respectively, when the diagnosis of AID deficiency was established, while on the other hand, multiple deaths occurred during the first months of life in untreated presumably X-linked HIGM patients reported by families of CD40L-deficient patients included in this study [20].

The clinical manifestations reported by the two Latin American patients with AID deficiency (P36 and P37) are not different from those described by others as recurrent pneumonia, gastrointestinal infections and lymphoid hyperplasia without the occurrence of opportunistic infections [12, 21, 32]. Neither of our two patients, however, developed autoimmune or related inflammatory diseases which have been reported by Quartier et al. in 21 % of their cohort of AID-deficient patients [21] but not by Minegishi et al. [32] and Revy et al. [12]. On the other hand, in accordance with previously studies [19, 31] autoimmune or related inflammatory diseases were observed in approximately 8 % of our CD40L-deficient patients.

Fig. 4 Kaplan-Meier graphs showing the survival rate of CD40L-deficient patients. The graph was made using the GraphPad PRISM 4.03 software (GraphPad Software, San Diego, Calif)

The molecular diagnosis could be established in 37 of the 58 patients reported to the registry with symptoms of HIGM syndrome, 35 with CD40L and two with AID deficiencies. Twenty five different mutations (24 in *CD40LG* and one in *AICDA*) including five novel mutations that abolish CD40L protein expression were identified. Mutation analysis of the most common HIGM syndromes—and a limited number of other PIDs caused by single gene defects—is presently available in only a small number of Latin American countries and in those, is limited to a few states/provinces of Brazil, Argentina, Chile and Mexico, while it is not available in other Latin American countries. For this reason it is still difficult to determine the actual prevalence and genetic basis of HIGM syndromes in Latin America. On the other hand, in the United States, where multiple diagnostic centers are set up to perform sequence analysis for genes associated with HIGM, the estimated minimal incidence of CD40L deficiency is 1/1,035,000 and the incidence of AID deficiency is estimated to be not greater than 2/10,000,000 per year of birth [32]. To overcome this internal problem in Latin America, State and Provincial governments have to be encouraged to support the establishment of new centers dedicated to the study of PID. Established centers have to expand already existing international collaborative studies within Latin America, and with North America and Europe.

Another limitation in Latin American countries is the availability of transplantation centers, which accounts for the small percentage of CD40L-deficient patients who underwent HSCT. This reflects social economic factors because HSCT is one example of high-cost and highly specialized medical care that requires significant infrastructure and a network of specialists from all fields of medicine. HSCT is most frequently performed in U.S and Europe where developed countries have higher gross national incomes and higher governmental health care expenditures. While the total number of performed HSCT is more than 300 per 10 million inhabitants in U.S and Europe, it ranges from zero to 49 per 10 million inhabitants in Latin American countries [50]. However, the economic improvement in some Latin American countries points to the potential for better transplant options over the coming years. For instance, in Brazil, where the highest number of CD40L-deficient patients were diagnosed, the number of HSCT donors available for all diseases demanding this treatment has recently improved (from 12 thousand in 2000 to 3 million in 2013) according to statistical data from the Ministry of Health (<http://www.inca.gov.br>). Associated with these data, the increasing availability of molecular diagnostic centers in Brazil and other Latin American countries will contribute to this improvement.

A phenotype/genotype correlation could not be established in our cohort nor in the previously described cohorts of North American and European patients with HIGM syndromes [12, 19, 21, 31, 32]. Such a correlation is difficult due to the small

number of patients. Additionally, while AID-deficient patients have a relative phenotypic homogeneity [21, 32], this is not the case for CD40L-deficient patients who may develop different clinical phenotypes while carrying the same mutation [20]. More clinical and genetic studies are required to eventually establish a phenotype/genotype correlation.

In conclusion, this first report of the LASID registry highlights the similarities and differences in the clinical and genetic spectrum of HIGM syndromes in Latin America versus other reported cohorts from North America, Europe, and elsewhere and it suggests that patients with HIGM syndrome due to mutations in *CD40LG* are susceptible to a broader species of opportunistic pathogens than previously recognized.

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