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Expanding the Clinical and Genetic Spectrum of Human CD40L Deficiency: The Occurrence of Paracoccidioidomycosis and Other Unusual Infections in Brazilian Patients

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Abstract CD40 ligand (CD40L) deficiency or X-linked hyper-IgM syndrome (X-HIGM) is a well-described primary immunodeficiency in which *Pneumocystis jiroveci* pneumonia is a common clinical feature. We have identified an unusual high incidence of fungal infections and other not yet described infections in a cohort of 11 X-HIGM patients from nine unrelated Brazilian families. Among these, we describe the first case of paracoccidioidomycosis (PCM) in X-HIGM. The molecular genetic analysis of CD40L was performed by gene sequencing and evaluation of CD40L protein expression. Nine of these 11 patients (82%) had fungal infections. These included fungal species common to CD40L deficiency (*P. jiroveci* and *Candida albicans*) as well as *Paracoccidioides brasiliensis*. One patient presented with PCM at age 11 years and is now doing well at 18 years of age. Additionally, one patient presented with a

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simultaneous infection with *Klebsiella* and *Acinetobacter*, and one with condyloma caused by human papilloma virus. Molecular analysis revealed four previously described CD40L mutations, two novel missense mutations (c.433 T>G and c.476 G>C) resulting in the absence of CD40L protein expression by activated $CD4^+$ cells and one novel insertion (c.484_485insAA) within the TNFH domain leading to a frame shift and premature stop codon. These observations demonstrated that the susceptibility to fungal infections in X-HIGM extends beyond those typically associated with X-HIGM (*P. jiroveci* and *C. albicans*) and that these patients need to be monitored for those pathogens.

Keywords *Paracoccidioides brasiliensis* · CD40 ligand · primary immunodeficiency · X-linked hyper-IgM syndrome

Introduction

Congenital susceptibility to fungal infections is most frequently observed in primary immunodeficiency disorders associated with deficiency or dysfunction of T cells or phagocytes (APECED, STAT1, IL-17R, and STAT3 deficiencies) [1–4]. Mutations in CD40 ligand (CD40L; CD154, MIM #300386), a surface protein expressed by activated $CD4^+$ T cell deficiency, is a well-defined T cell defect that causes the X-linked hyper-IgM syndrome (X-HIGM) [5–9]. In addition to extracellular and intracellular bacterial infections, patients with X-HIGM are susceptible to fungal infections, particularly *Pneumocystis jiroveci* pneumonia, and infections with *Candida albicans*, *Cryptococcus* and *Histoplasma* [10–12]. Interestingly, the majority of these infections occur within the first 5 years of life and are much less common thereafter [11–13].

In humans, the effective immune response against fungi is dependent on both innate and adaptive responses. Appropriate activation of phagocytic cells and particularly the production of reactive oxygen intermediate by nicotinamide adenine dinucleotide phosphate oxidase are important for the control of fungal infections [14]. The activation of this system is coordinated by the interleukin (IL)-12/interferon-γ (IFN-γ) axis, which is triggered by CD40/CD40L interaction [15] and mediated by Th1 cells. On the other hand, the humoral immune response orchestrated by antibodies plays an essential role in the defense against extracellular bacteria as illustrated by the increased susceptibility to these infections by patients with predominantly antibody deficiencies [16]. In this context, the CD40–CD40L interaction plays an important role by facilitating the expression of activation-induced cytidine deaminase and other proteins involved in the class switch recombination and somatic hypermutation of immunoglobulin (Ig) variable region genes [17].

Here we report the clinical features and molecular defects of a cohort of X-HIGM patients identified in Brazil, with a high incidence of fungal infections (82%) compared to the incidence described in Europe and North America (34% to 57%) [11, 13]. The spectrum of fungal infections in our patients includes species that have never been described in X-HIGM such as paracoccidioidomycosis (PCM). In addition, we observed a patient who developed sepsis by being simultaneously infected with *Klebsiella pneumonia* and *Acinetobacter* sp. and a case of condyloma caused by human papilloma virus (HPV) and extended the number of known CD40L mutations by four.

Methods

Subjects Between 2006 and 2011, we identified 11 patients from nine unrelated families with CD40L deficiency in our clinic. The criteria for diagnosing CD40L deficiency complied with the International Union of Immunological Societies Expert Committee on Primary Immunodeficiencies [16], based on characteristic clinical criteria and confirmed by the lack of CD40L expression by activated T cells and the presence of mutation in the CD40L gene. Informed consent was obtained from all patients or their parents, and blood was 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.

Cell Culture and Activation Peripheral blood mononuclear cells were isolated from heparinized blood after Ficoll-Hypaque sedimentation and cultured (1×10^6 cells/well) for 5 h at 37°C in 5% CO_2 in flat-bottomed microtiter plates in a final volume of 200 μl of RPMI 1640 (Gibco Laboratories), supplemented with 10% fetal calf serum, 1% glutamine, and antibiotics, with or without phorbol myristate acetate (15 ng/ml, Sigma Laboratories, St. Louis, MO, USA) and calcium ionophore (ionomycin, 300 ng/ml, Sigma Laboratories).

Lymphocyte Counts and CD40L Expression T ($CD3^+CD4^+$, $CD3^+CD8^+$) and B ($CD19^+CD40^+$) cell counts were determined by flow cytometry analysis using specific monoclonal antibodies. CD40L expression was assessed on the surface of resting and activated $CD3^+CD4^+$ T cells using the monoclonal antibody TRAP1 (Mouse IgG1) as previously described [18]. Activation of cells was confirmed by staining $CD3^+CD4^+$ T cells with specific antibodies to CD69 (Becton Dickinson, BD) (data not shown). Prior to flow cytometry, samples were washed and fixed in 1% paraformaldehyde. The results obtained were analyzed using FlowJo software (Treestar Inc., Ashlan, OR, USA).

Sequence Analysis of CD40L Gene Genomic DNA (gDNA) was isolated from EDTA blood using the Wizard® Genomic DNA Purification kit (Promega). All five exons of the CD40L gene, including the exon–intron boundaries, were individually amplified by polymerase chain reaction (PCR), as previously described [18]. Primers are available upon request. 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). The observed sequences were compared with the CD40L mutation registry (http://bioinf.uta.fi/CD40Lbase/), and structural analysis of missense mutations was performed based on the crystal structure of the protein (PDB 1ALY). CD40L complementary DNA (cDNA) was also sequenced as previously described [18]. To provide evidence that the two novel missense mutations identified are not single-nucleotide polymorphisms (SNPs), we evaluated both mutations using MutationTaster, a bioinformatic method to evaluate disease-causing potential of sequence alterations [19] and sequenced gDNA from 100 healthy Brazilian controls.

Results

Patient Baseline Characteristics

We enrolled 11 male patients ranging in age from 1 to 23 years in whom a diagnosis of X-HIGM syndrome was suspected. All patients came from nonconsanguineous Brazilian families (Fig. 1). Median age at time of disease onset was 6 months (ranging from 1 to 7 months). The median age at the time of X-HIGM diagnosis was 2 years (ranging from 5 months to 6 years).

High Incidence of Fungal Infections and the Occurrence of Paracoccidioidomycosis

Nine of 11 (82%) patients in this cohort presented with fungal infections, many of which were being unusual or severe (Table I). Six patients had *P. jiroveci* pneumonia, an infection that typically occurs in young (under the age of 5 years) CD40L-deficient patients [11]; three patients had infections caused by *C. albicans* which were either of an unusual type or particularly severe: P5 was diagnosed with severe candida esophagitis, P6 with oral and perianal candidiasis, and P9 with persistent cutaneous candidiasis on the scalp (Fig. 2a). It is important to note that at the time of the candidiasis, P5 and P6 but not P9 were treated prophylactically with trimethoprim–sulfamethoxazole to prevent *P. jiroveci* pneumonia, but no other antibiotics were used. None presented with neutropenia at the time the *Candida* diagnosis was established.

At the age of 6 months, patient P10, born and living in Sao Paulo city, was diagnosed with X-HIGM while presenting with *P. jiroveci* pneumonia, recurrent otitis media, and sinusitis. At the age of 11 years while on intravenous immunoglobulin (IVIG) treatment, he presented with mild prolonged fever and cough. Chest CT scans showed mediastinal lymphadenopathy and a lymph node biopsy confirmed tuberculoid granulomatous inflammation as a consequence of *Paracoccidioides brasiliensis* within multinuclear cells (Fig. 2b). In addition, bone marrow biopsy revealed hypoplasia and tuberculoid granuloma, a clinical feature compatible with the acute form of PCM. It is important to note that P10 did not

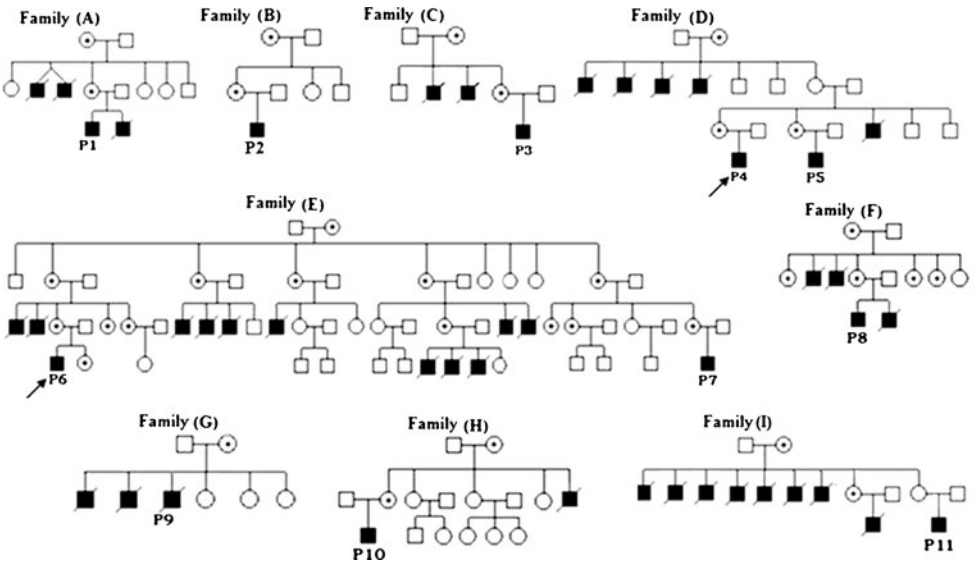

Fig. 1 Patients' pedigrees. *Black squares* represent males with recurrent, severe infections. *Black squares with slashes* indicate deceased individuals. All female carriers, represented by *circles with dots*, were clinically and immunologically healthy

Table I Infections observed in Brazilian patients with X-HIGM

Pt	Birth year	Infections and pathogens			
		Fungi	Bacteria	Other etiologic agents	Other infections associated with unidentified pathogens
1[a]	2008	Pneumonia—*P. jiroveci*			Pneumonia
2[a]	1987	Pneumonia—*P. jiroveci*	Urinary tract infection—*E. coli*		Otitis, tonsillitis
3[a]	2007	Pneumonia—*P. jiroveci*		Diarrhea—*C. parvum*	Pneumonia
4[a]	2007				Pharyngitis, laryngitis, otitis, pneumonia
5[a]	2003	*Candida* esophagitis			
6[a]	2005	Oral and perianal candidiasis		Condyloma—HPV, herpes simplex	Otitis, sinusitis, pneumonia
7[a]	1997	Pneumonia—*P. jiroveci*			Otitis, sinusitis, pneumonia
8[a]	2006	Pneumonia—*P. jiroveci*			sepsis, pneumonia
9[b]	2002	Cutaneous candidiasis on the scalp	Sepsis—*Klebsiella pneumonia*, sepsis—*Actinobacter* sp., otitis—*P. aeruginosa*		Pneumonia
10[a]	1993	PCM—*P. brasiliensis*, pneumonia—*P. jiroveci*			Otitis media, sinusitis, pneumonia
11[a]	2009				Pulmonary granuloma pneumonia

PCM paracoccidioidomycosis, *Pt* patient, *UP* unidentified pathogen, *HPV* human papillomavirus

[a] Alive

[b] Dead

present with neutropenia 6 months before or during PCM. He was treated with an 8-month course of itraconazol and recovered.

Extracellular Bacterial Infections

One patient (P9) presented with local pyoderma and oropharyngitis beginning at 3 months of age followed by recurrent otitis and mastoiditis. One of the pathogens causing otitis was *Pseudomonas aeruginosa*. At the age of 3 years, he developed severe mastoiditis followed by sepsis caused by *K. pneumonia and Acinetobacter* sp., leading to the diagnosis of X-HIGM syndrome. Under treatment with cefepime and vancomycin, he fully recovered and IVIG therapy was started. P9 died at 8 years of age due to complications resulting from progressive multifocal leukoencephalopathy.

Other Isolated Etiologic Agents

Other etiologic agents causing infections were isolated in P3 and P6 while on adequate IVIG prophylaxis. The protozoan *Cryptosporidium parvum*, a relatively common pathogen observed in CD40L-deficient patients [11], caused chronic

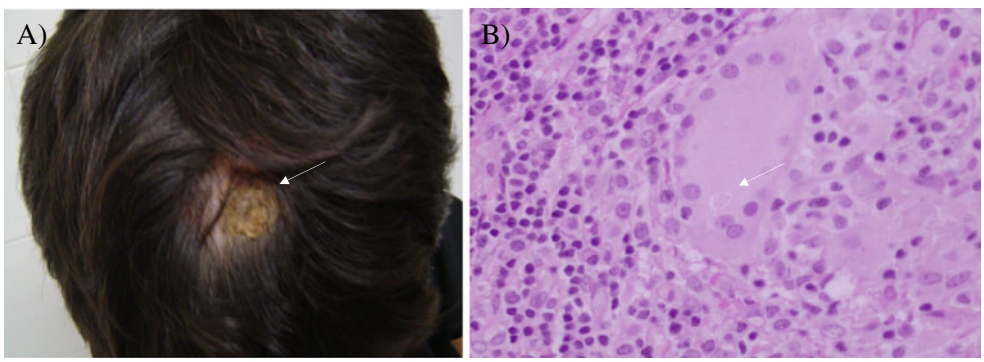

Fig. 2 Paracoccidioidomycosis and cutaneous candidiasis in CD40L-deficient patients. **a** Picture of persistent candidiasis on the scalp of P9. **b** Histopathologic characteristics of a lymph node biopsy of patient P10. Hematoxylin–eosin-stained specimen showing the tuberculoid granulomatous inflammation with *Paracoccidioides brasiliensis* within a multinucleated cell

diarrhea in P3 at 4 years of age. P6 presented with a herpes simplex virus infection, which has been previously reported in X-HIGM patients [13]. In addition, he had perianal condyloma caused by HPV.

Laboratory and Immunologic Findings

Patient lymphocyte counts ($CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+CD40^+$) were similar to healthy controls (Table II). Neutropenia was identified in six patients (P2, P3, P4, P5, P6, and P10). In P3, P4, and P11, neutropenia was associated with recurrent oral or esophageal ulcers but not with other clinical features. Neutropenia was effectively treated with recombinant human granulocyte colony-stimulating factor in all patients except P4. Despite presenting persistent neutropenia, P4 did not have fungal infections.

All patients had low serum levels of IgG and IgA (Table II). Serum IgM was elevated in patients P1, P2, P7, P9, P10, and P11, but was normal in patients P3, P4, and P6 and reduced in patients P5 and P8.

Molecular Genetic Analysis of CD40L

Expression of the CD40L protein on the surface of activated $CD3^+CD4^+$ T cells was impaired in all patients except for P3, who had an expression level similar to the healthy control (Fig. 4). Seyama et al. [18] used a CD40–Ig fusion protein to demonstrate that the c.496 C>A mutation identified in P3 affects binding of CD40 to CD40L on activated T cells.

Sequence analysis of the CD40L gene revealed three novel mutations. These are two missense mutations, one in exon 4 (c.433 T>G, p.V126G, P1), the other in exon 5 (c.476 G>C, p.W140C, P2), and an insertion of 2 base pairs (c.551_552insAA, p.R165X190) within the tumor necrosis factor homologous (TNFH) domain found in one patient (P11) (Table III, Fig. 3b). MutationTaster analysis indicated no SNPs for these altered regions and neither of these two mutations was found in 100 healthy Brazilian control subjects. Furthermore, comparative analysis of the CD40L protein from different species showed that the two novel base pair substitutions affect conserved amino acids within the CD40L TNFH domain (Fig. 3a). These amino acid residues are essential for the CD40L structure, thereby abolishing expression of the CD40L protein on the surface of activated $CD3^+CD4^+$ T cells (Fig. 3a).

The insertion found in P11 leads to a frame shift mutation with the creation of a premature stop codon resulting in an unstable truncated protein (Fig. 3b). The other four unique mutations identified in the remaining eight patients have been described previously (Table III) and their consequences in CD40L expression are demonstrated in Fig. 4.

Discussion

Common clinical features of CD40L-deficient patients include infections caused by extracellular and intracellular bacterial and fungal pathogens. Pneumonia caused by the commensal fungus *P. jiroveci* is a well-recognized

Table II Laboratorial features in X-HIGM patients

Patient	Neutropenia	Lymphocytes subsets[a] (%)			Serum Ig levels (before IVIG[a])			
		$CD3^+$		$CD19^+CD40^+$	Age (years)	IgG	IgA	IgM
		$CD4^+$	$CD8^+$			(mg/dl)		
1	−	51	36	7	<1	164	21	111
2	+	37	27	5	2	10	9	320
3	+	63	12	13	1	100	6	92
4	+	55	35	7	2	210	6	90
5	+	43	20	7	2	230	24	51.6
6	+	31	23	19	1	90	10	95
7	−	43	12	13	3	270	7	180
8	−	52	23	7	1	44.7	5.2	53
9	−	55	23	19	2	22	5	362
10	+	40	23	6	6	250	5	400
11	−	11	21	31	<1	149	25	107

Persistent neutropenia was observed in P4 and the other patients presented intermittent neutropenia

Ig immunoglobulin, *IVIG* intravenous immunoglobulin therapy

[a] Reference values of IVIG and lymphocyte subsets according to Guerra-Maranhao et al. [52]

Table III Mutations in CD40L

Pt	cDNA mutation	Type of mutation	Affected domain	Affected Exon	Novel or known	References
P1	c. 433 T>G	Missense	TNFH	4	Novel	–
P2	c.476 G>C	Missense	TNFH	5	Novel	–
P3	c.496 C>A	Missense	TNFH	5	Known	[18]
P4	c.475 G>A	Nonsense	TNFH	5	Known	[9]
P5	c.475 G>A	Nonsense	TNFH	5	Known	[9]
P6	c.213_216delATAG	Frame shift deletion	EC	2	Known	[42]
P7	c.213_216delATAG	Frame shift deletion	EC	2	Known	[39]
P8	c.213_216delATAG	Frame shift deletion	EC	2	Known	[39]
P9	c.345_402del	Splice site	EC	3	Known	[18]
P10	c.345_402del	Splice site	EC	3	Known	[18]
P11	c.551_552insAA	Frame shift insertion	TNFH	5	Novel	–

TNFH tumor necrosis factor homology domain, *EC* extracellular

complication in CD40L deficiency with incidence rates ranging from 32% to 42% [11, 13]. However, reports of other fungal infections are rare: *Candida* in two patients [12, 20], *Cryptococcus* in one patient [21], and *Histoplasma* in one patient [10]. While candidiasis and *P. jiroveci* infections usually occur during the first 5 years of life, the patients developing cryptococcosis and histoplasmosis and the Brazilian patient developing *P. brasiliensis* where 12, 19, and 11 years, respectively.

In this study, we identified a high rate of fungal infections (82%) in a cohort of 11 CD40L-deficient patients from Brazil, none of which had neutropenia at the time of presentation. These infections were caused by a broad range of fungal pathogens including *P. jirovecii*, *C. albicans*, and, not previously reported, one patient with *P. brasiliensis* infection. These observations suggest a possible underlying mechanism responsible for the occurrence of fungal infections which remains to be investigated. The reason for the high incidence of fungal infections and the occurrence of PCM described in this cohort of X-HIGM patients agrees with distinct epidemiological features and unique etiologic agents associated with the geographic and socioeconomic conditions in Latin America [22]. For example, in Brazil, the incidence of *Candida* infections is almost ten times higher than that reported in North America and Europe [22]. Accordingly, we identified three X-HIGM patients with severe *Candida* infections. *P. brasiliensis* is a fungus that causes the most important endemic systemic mycosis in South America [23]. Interestingly, both patients with primary immunodeficiency presenting with PCM are Brazilian. One is

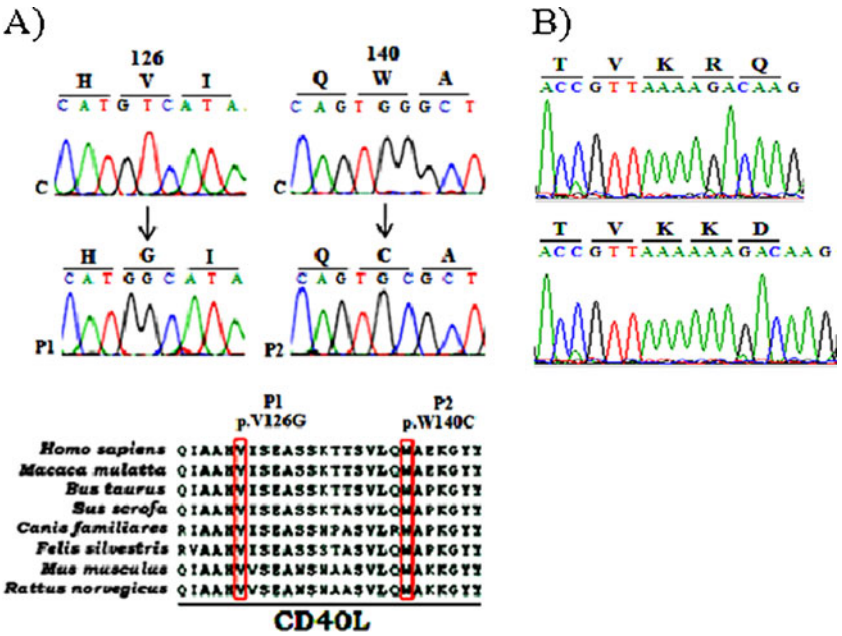

Fig. 3 Novel mutations in CD40L-deficient patients. **a** The missense mutations affect conserved amino acids in the CD40L TNF homology domain (TNFH) of different species and are located at the binding sites with CD40 receptor. **b** The insertion of two adenines (p.R165X190) found in the CD40L gene of P11 results in a frame shift mutation creating a premature stop codon

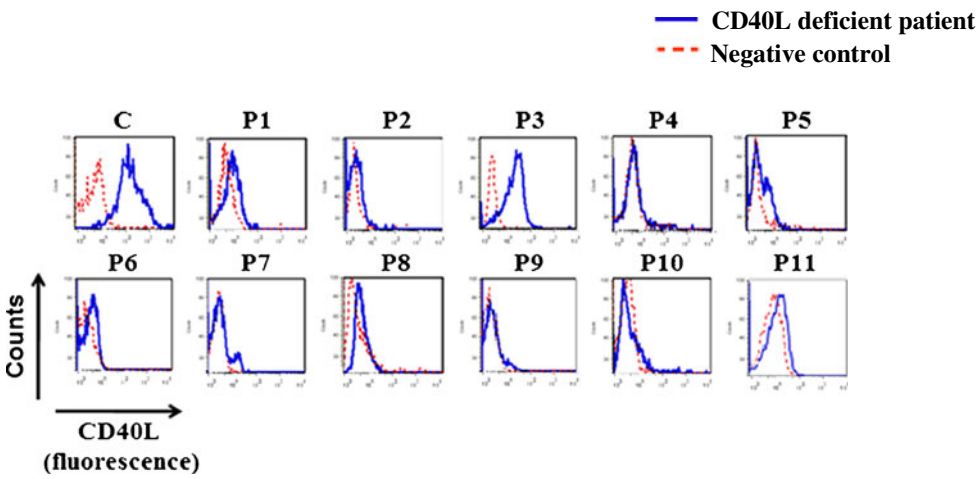

Fig. 4 Defective CD40L protein expression. Flow cytometry histograms showing CD40L expression on the surface of activated $CD3^{+}CD4^{+}$ T cells from patients (P1–P11) demonstrating a lack of protein expression in all but one patient (P3) who expresses a nonfunctional protein as previously demonstrated [18]. Histogram *C* shows data from a healthy control

a patient with a mutation in the beta 1 subunit of the IL-12/IL-23 receptor [24] and the second is the patient (P10) described here. Both patients live in São Paulo, a huge urban area where the incidence of PCM is much lower than in rural regions. The actual incidence and prevalence of PCM is poorly known because reporting this disease is not mandatory in most South American countries, and its distribution is highly variable even in endemic areas [25]. However, some isolated studies reported the PCM average incidence from one to four new cases per 100,000 habitants per year in Brazil [26], whereas in other South American countries, it has been estimated an average incidence of 0.5 cases per 100,000 habitants per year with lower rates being described in Colombia [27, 28].

PCM is an infection acquired by inhalation of airborne propagules produced by fungal mycelium, which then transform into pathogenic yeast [29]. After *P. brasiliensis* invades the host via the respiratory tract, it persists in macrophages causing granuloma formation and disseminates through the reticuloendothelial system [30]. This pathogenesis of persistent *P. brasiliensis* inside macrophages and granuloma formation in lymph nodes and bone marrow was observed in our patient. Two different clinical forms of PCM can be distinguished: an acute, juvenile form and a chronic, adult form [31, 32]. The clinical picture of the acute form reveals a severe disease with reticuloendothelial system organ hypertrophy, bone marrow dysfunction, and septic episodes. The lungs are seldom the primary focus of infection but fungal pathogens can be proven in pulmonary secretions assuming some lung involvement [33]. The disease develops within a few weeks or months and is more frequently reported in children and young adults below 25 years of age [31, 34]. On the other hand, the chronic form results from infection usually acquired during the first two decades of the life staying clinically silent and causing disease in adults, mostly men of 30 to 50 years of age, by reactivation of a latent endogenous focus [32]. The disease progresses slowly with symptoms of cough, expectoration, weight loss, and fever. A characteristic of the chronic form is pulmonary manifestation; however, it can also occur as a multifocal form with fungal spread to more than one organ or tissue such as the oral mucosa, skin, lymph nodes [35–37], central nervous system, and gastrointestinal and ocular manifestations [38–40].

To eradicate PCM, an efficient Th1 immune response is required, characterized by the production of IFN-γ and subsequent activation of phagocytic cells [41]. By enhancing cytochrome *b* expression by phagocytes, IFN-γ is believed to potentiate the oxidative respiratory burst in macrophages and polymorphonuclear leukocytes, a process responsible for many of the microbicidal, tumoricidal, and inflammatory activities of these cells [27, 28]. In accordance, *P. brasiliensis*-pulsed mDCs from CD40L-deficient patients induce a Th2-skewed T cell response characterized by lower IFN-γ and higher IL-4 and IL-5 production compared to healthy subjects [42], an immunological milieu associated with increased susceptibility to fungal infections in humans [43].

The inability to eliminate extracellular bacterial and viral pathogens is also a common clinical feature of patients with X-HIGM and other well-defined humoral immunodeficiencies [11, 44]. However, only one CD40L-deficient patient has been reported with infections caused by *P. aeruginosa* [45], and this is the first report of sepsis caused by *K. pneumonia* and *Acinetobacter* sp. and of condyloma associated with HPV infections in patients with CD40L deficiency. *K. pneumonia* and *Acinetobacter* sp. are two gram-negative bacteria present in the gastrointestinal flora of immunocompetent individuals, causing worldwide nosocomial infections in newborns and in patients with AIDS [46, 47]. Among primary immunodeficient patients, *Klebsiella* sp. infections were only reported in chronic granulomatous disease [48] and X-linked agammaglobulinemia [49]. HPV infections were described in patients with hypogammaglobulinemia, highlighting the role of the antibody in controlling some

viral pathogens. The success of vaccines against HPV in protecting women against HPV infection and cervical cancer [50] underlines the importance of adaptive immunity in the protection against this virus. HPV infections were also observed in patients with autosomal-dominant mutations in the chemokine receptor gene CXCR4 [51], but the underlying mechanism involved in this susceptibility still needs to be clarified.

In conclusion, our data highlight the key role of the CD40/CD40L signaling pathway in mediating immunity against fungal and bacterial infections. It demonstrates that defects in this pathway cause susceptibility to a much broader array of pathogens than those traditionally associated with X-HIGM (i.e., *P. jirovecii*). Therefore, we suggest that patients with CD40L deficiency especially if living in tropical areas should be monitored for these pathogens.

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