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Paracoccidioidomycosis Associated With a Heterozygous *STAT4* Mutation and Impaired IFN-γ Immunity

Lena F. Schimke,[1,2] James Hibbard,[3] Ruben Martinez-Barricarte,[4] Taj Ali Khan,[1] Ricardo de Souza Cavalcante,[5] Edgar Borges de Oliveira Junior,[1] Tabata Takahashi França,[1] Asif Iqbal,[6] Guilherme Yamamoto,[7] Christina Arslanian,[1] Claudia Feriotti,[1] Tania Alves Costa,[1] Jacinta Bustamante,[4,8,9,10] Stéphanie Boisson-Dupuis,[4,8,9] Jean-Laurent Casanova,[4,8,9,11,12] José Alexandre Marzagao Barbuto,[1] Mayana Zatz,[7] Rinaldo Poncio Mendes,[5] Vera Lucia Garcia Calich,[1] Hans D. Ochs,[3] Troy R. Torgerson,[3] Otávio Cabral-Marques,[1,2,a] and Antonio Condino-Neto[1,13,a]

[1]Department of Immunology, University of Sao Paulo, Brazil; [2]Department of Rheumatology and Clinical Immunology, University of Lübeck, Germany; [3]Department of Pediatrics, University of Washington School of Medicine and Seattle Children's Research Institute; [4]St Giles Laboratory of Human Genetics of Infectious Diseases, the Rockefeller University, New York; [5]Tropical Diseases Area, Botucatu Medical School, São Paulo State University, Brazil; [6]Laboratory of Biochemistry and Biophysics, Butantan Institute, Sao Paulo, Brazil; [7]Human Genome and Stem Cell Research Center, University of Sao Paulo, Brazil; [8] Imagine Institute, Paris Descartes University; [9]Laboratory of Human Genetics of Infectious Diseases, [10]Center for the Study of Primary Immunodeficiencies, and [11]Pediatric Hematology-Immunology Unit, Necker Hospital for Sick Children, Paris, France; [12]Howard Hughes Medical Institute, the Rockefeller University, New York; and [13]Institute of Tropical Medicine, University of Sao Paulo, Brazil

Background. Mutations in genes affecting interferon-γ (IFN-γ) immunity have contributed to understand the role of IFN-γ in protection against intracellular pathogens. However, inborn errors in *STAT4,* which controls interleukin-12 (IL-12) responses, have not yet been reported. Our objective was to determine the genetic defect in a family with a history of paracoccidioidomycosis.

Methods. Genetic analysis was performed by whole-exome sequencing and Sanger sequencing. STAT4 phosphorylation (pSTAT4) and translocation to the nucleus, IFN-γ release by patient lymphocytes, and microbicidal activity of patient monocytes/macrophages were assessed. The effect on STAT4 function was evaluated by site-directed mutagenesis using a lymphoblastoid B cell line (B-LCL) and U3A cells.

Results. A heterozygous missense mutation, c.1952 A>T (p.E651V) in *STAT4* was identified in the index patient and her father. Patient's and father's lymphocytes showed reduced pSTAT4, nuclear translocation, and impaired IFN-γ production. Mutant B-LCL and U3A cells also displayed reduced pSTAT4. Patient´s and father´s peripheral blood mononuclear cells and macrophages demonstrated impaired fungicidal activity compared with those from healthy controls that improved in the presence of recombinant human IFN-γ, but not rhIL-12.

Conclusion. Our data suggest autosomal dominant STAT4 deficiency as a novel inborn error of IL-12–dependent IFN-γ immunity associated with susceptibility to paracoccidioidomycosis.

Keywords. STAT4 deficiency; primary immunodeficiency; IL-12/IFN-γ axis; paracoccidioidomycosis.

Interleukin-12 (IL-12)–dependent interferon-γ (IFN-γ)–regulated immunity plays an important role in the protection of humans against infections caused by intracellular bacteria, fungi, and protozoa [1]. Among others, the activation of antigen-presenting cells such as monocytes/macrophages and dendritic cells following pathogen recognition results in the secretion of IL-12, which induces naive $CD4^+$ T lymphocyte differentiation into IFN-γ–producing T helper 1 (Th1) cells. This process involves cell signaling molecules that lead to the activation of the signal transducer and activator of transcription 4 (STAT4) [2, 3]. In turn, IFN-γ activates microbicidal mechanisms in macrophages to eliminate the engulfed pathogen [4]. In this context, mutations in 6 genes (*ISG15*, *IFNGR1, IFNGR2, STAT1, IL12B, IL12RB1, TYK2*) encoding proteins that participate directly in the IL-12/IFN-γ axis [1] and in a number of different genes that indirectly affect proteins involved in IFN-γ–dependent immunity (eg, *IRF8, NEMO, CYBB, CD40L*) [1, 5–7] have been described in humans susceptible to opportunistic infections caused by intracellular bacteria, fungi, and protozoa. Primary immunodeficiencies (PIDs) represent a unique opportunity to study and understand human immune system responses in natura [6]. Whereas classic PIDs, such as severe combined immunodeficiency, confer predisposition to a broad spectrum of infectious diseases [6], recent reports have described nonclassic PIDs resulting in susceptibility to a narrow spectrum of pathogens. This is, for instance, the case in patients with inborn errors of IFN-γ immunity [1, 8], which leads to selective predisposition to weakly virulent mycobacteria and in some single cases [1] to other opportunistic infections caused by different fungi such as candidiasis [9], histoplasmosis [10], coccidioidomycosis [11,

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[a]These authors contributed equally.

Correspondence: L. F. Schimke, MD, Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, 1730 Lineu Prestes Avenue, São Paulo, SP, ZIP 05508-000, Brazil (lenaschimke@hotmail.com).

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12], and paracoccidioidomycosis (PCM) [13]. Notably, although the latter is an endemic Latin American disease [14], it has been associated with only 2 well-defined PIDs: CD40 ligand (CD40L) deficiency [5, 15], a pivotal regulatory molecule of the IL-12/IFN-γ axis [16], and IL-12 receptor β1 (IL12Rβ1) deficiency [13]. PCM is caused by the fungus *Paracoccidioides brasiliensis* and is characterized by granulomatous lesions manifesting in 2 clinical forms: the acute (juvenile) form presenting with disseminated fungal lesions [17], and the chronic (adult) form generally caused by reactivation of a latent endogenous focus consisting most commonly as involvement of lungs and mucous membranes of the upper airways and digestive tract [18]. Noteworthy, less than 1% of the individuals detected by serological testing as infected with *P brasiliensis* develop the clinical disease [13]. Therefore, it is possible that a subset of infected patients have underlying genetic factors leading to clinical manifestation of PCM. As a first approach to tackle this issue, we studied a multiplex family with a history of acute PCM disease.

MATERIALS AND METHODS

Family History

The index case is a 30-year-old Brazilian woman, born to nonconsanguineous parents (Figure 1A). She received routine Bacillus Calmette-Guérin vaccination at infancy, but developed no adverse reaction. At 20 years of age, she presented with a 5-month history of epigastric pain associated with nausea, vomiting, and a 6-kg weight loss. Physical examination revealed bilateral lymph node enlargements in the submandibular and inguinal chains. Histopathological examination of duodenal micronodules and fibrinous gastrointestinal lesions revealed typical intracellular *P brasiliensis* yeast forms. Specific serum antibody to PCM antigen, determined by immunodiffusion, was highly positive at a 1:256 dilution. The patient was treated with trimethoprim–sulfamethoxazole for 3 years with improvement of the clinical manifestations and up to the time of writing, she had no further symptoms. Laboratory studies conducted during treatment of acute PCM revealed normal serum immunoglobulin levels and blood cell counts. Moreover, no alteration in lymphocyte subsets was observed (Table 1).

Family history revealed that a paternal uncle died in early infancy due to infection of unknown etiology, and that the patient's father, currently 76 years of age, had PCM disease when he was 20 years old. He had a history of weight loss and papular and ulcerative skin lesions in the face and on the scalp causing hair loss, which are symptoms typical for cutaneous manifestation of PCM. He was referred to a tertiary clinic for treatment; however, no further histological studies or laboratorial characterization are available from that time. The study was approved by the local ethical review board according to the Brazilian Ministry of Health (Resolution 96/196) and the Helsinki Convention, and written informed consents were obtained from the patient, her parents, and healthy controls.

Next Generation and Sanger Sequencing

Genomic DNA was extracted from ethylenediaminetetraacetic acid (EDTA) blood using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to manufacturer's instructions. Whole exome sequencing (WES) was performed as previously described [19]. Briefly, variant calls were made with GATK Unified Genotyper. All calls with a read coverage ≤10 × and a Phred-scaled single nucleotide polymorphism (SNP) quality of ≤ 30 were removed from consideration [20]. Potential impact of nonsynonymous variations was evaluated using common conservation status and prediction programs (SIFT, MutationTaster, CADD Score). The identified variations were confirmed by Sanger sequencing using specific primers (available upon request), annotated according to the Ensembl gene annotation system [21], and checked for family segregation according to the phenotype.

STAT4 Phosphorylation in Lymphocytes

Tyrosine phosphorylation of STAT4 was analyzed in T lymphocytes from peripheral blood mononuclear cells (PBMCs) as previously described [22]. In brief, preactivated PBMCs (8 µg/mL phytohemagglutinin [PHA] for 3 days) were stimulated with 10 ng/mL recombinant human IL-12 (rhIL-12) (Peprotech, Rocky Hill, NJ) for 30 minutes and stained with anti-CD3 antibody. The cells were fixed, permeabilized, and stained with anti-pY693 STAT4 antibody (BD Biosciences) for analysis by flow cytometry. In parallel, a second aliquot of cells was transferred to 13-mm round glass cover slips and visualized by a Zeiss LSM 780 Multiphoton microscope with a 1.30 oil DIC M27 objective (Carl Zeiss, Jena, Germany) after staining the nuclei with Hoechst 33342.

Site-Directed Mutagenesis Studies

To limit the number of blood draws and collections due to institutional ethical rules, we extended the analysis of the defects observed in T cells to site-directed mutagenesis studies. We used a lymphoblastoid B cell line (B-LCL), and the plasmid pLZRS-IRES-ΔCD271, containing STAT4-wildtype (WT), STAT4-E651V, and STAT4-Y693W/S721T that were transfected into retroviral-producing Phoenix A cells and retrovirus collected when all of the cells were positive for CD271, as previously described [23]. We choose Y693W/S721T as a mutant control because the phosphorylation of tyrosine 693 and serine 721 are essential for STAT4 function [3, 24]. B-LCL cells were transduced with the retrovirus described above and stimulated with several concentrations of IFN-α (10 to 100 000 U/mL). Because B-LCL cells express only the IL12Rβ1 subunit of the IL12R (ie, a nonfunctional IL12R) and U3A cells (used in a second cell model described below) lack the IL-12 receptor, IFN-α, which is a stronger inducer of STAT4 phosphorylation [25], was used instead of IL-12. After stimulation, phosphorylation of STAT4 and STAT1 (used as control to show that the results obtained are not

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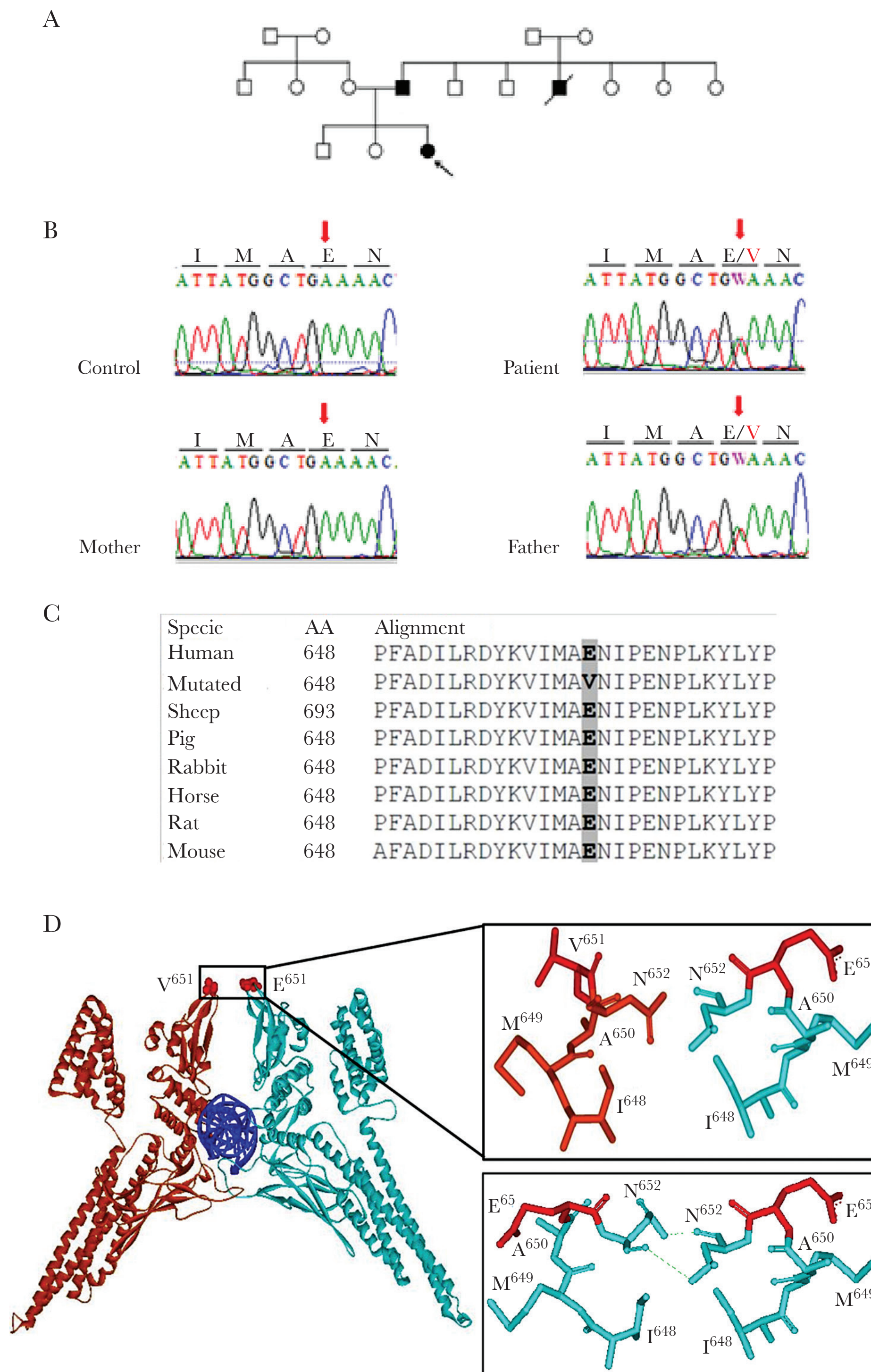

Figure 1. Heterozygous variation in *STAT4* in two family members with PCM disease. *A*, Family pedigree. Filled symbols represent individuals with history of severe infectious disease. Paracoccidioidomycosis was diagnosed in the index case (indicated with a black arrow) and her father. *B*, Chromatograms of genomic DNA bearing the wild-type *STAT4* sequence from a healthy control and patient's mother, and the g.119147A>T (c.1952 A>T) *STAT4* variant identified in the index patient and her father. *C*, Multiple sequence alignment of STAT4 indicating the conserved amino acid E651 (highlighted). *D*, Crystal structure of STAT4 showing a mutated–wild-type dimer with affected amino acid change E651V in the SH2 domain, which might disturb specific hydrogen bonding (enlarged wild-type–mutant dimer in upper quadrant versus wild-type–wild-type dimer with intact hydrogen bonding in lower quadrant). Method of in silico analysis are described in Supplementary Material.

due to differences in overall IFN-α response) was evaluated by flow cytometry using antibodies against pSTAT4 and pSTAT1 (BD Biosciences) and by western blot using rabbit antihuman STAT4 (Cell Signaling Technology, Danvers, MA). We also assessed if the mutations indicated above affect STAT4 protein expression in HEK293T cells. For this, HEK293T cells were

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Table 1. Lymphocyte Subsets

	Patient Sample[b]		Control Sample		Normal Range[a]
Cell population	%	Cells/μL	%	Cells/μL	%
lymphocytes					
T Lymphocytes	74	7278	53	5395	43–76
CD4	39	3811	30	3080	23–48
CD8	20	1964	20	2005	14–33
B Lymphocytes	10	1027	9	810	4.3–23.1
$CD4^+$ T lymphocytes					
CD45RA	24	1746	41	2183	21–57
CD45RO	32	2348	26	1411	38–60
$CD8^+$ T lymphocytes					
CD45RA	16	1183	24	1288	45–77
CD45RO	23	1697	15	786	16–49
$CD19^+$ B lymphocytes					
$CD27^+$	46	473	55	458	4.6–49.1
NK cells					
$CD56^+CD16^+$	10	977	21-	1948	5.0–27.0

[a]Mayo Medical Laboratories, Unit Code 88800.

[b]Performed after completion of paracoccidioidomycosis treatment.

transfected with 10 μg of pCMV6 plasmid (OriGene, Beijing, China) containing WT, E651V, or Y693W/S721T *STAT4* and total STAT4 expression was assessed by western blot (rabbit anti-STAT4, Cell Signaling Technology). Furthermore, expression of *STAT4* mRNA in transduced B-LCL and HEK293T cells was analyzed by RT-qPCR using Hs01028017_m1 probe (Applied Biosystems, Foster City, CA).

In a second cell model STAT4 nonexpressing U3A cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 1 × GlutaGro medium to 80% confluence and transfected with WT, mutant (E651V) STAT4, or both (WT and E651V STAT4) plasmids with directly fused green fluorescent protein (GFP) at the C-terminus. In brief, cells were suspended in 100 μL of E2 buffer containing 7.5 μg of STAT4 plasmids. Each 100 μL cell mixture was electroporated for two 26-ms pulses at a voltage setting of 1225 using Invitrogen's Neon Transfection System in 100 μL neon tips. Transfected cells were incubated for 36 hours to allow for maximum STAT4 expression. Transfected cells were mobilized, suspended in 50 μL of media, and stimulated with different concentrations of IFN-α (0 to 5000 U/mL). After 20 minutes in the presence of IFN-α, the cells were fixed in 4% paraformaldehyde, stained with anti-pSTAT4 (p.Y693) antibody (BD Bioscience) and analyzed using an LSR II flow cytometer.

Evaluation of IFN-γ Production

Intracellular IFN-γ production by patient's and father's lymphocytes was assessed as previously described [16]. Moreover, extracellular IFN-γ release was evaluated in the supernatants of PHA-preactivated PBMCs (0.2×10^6 in 200 μL Roswell Park Memorial Institute medium [RPMI]) stimulated or not with IL-12 (10 ng/mL) for 48 hours at 37°C. The IFN-γ concentration was analyzed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Becton Dickinson, Mountain View, CA).

Fungicidal Activity

The ability of PBMCs and monocyte-derived macrophages (MDMs, generated as previously described [26]) alone or cocultured with autologous lymphocytes (MDM/lymphocyte ratio of 1:10) to kill *P brasiliensis* (Pb18) was determined by counting colony forming units (CFU), as previously reported [26, 27]. Briefly, PBMCs or MDMs alone or cocultured with lymphocytes were challenged with *P brasiliensis* (ratio of 1:20 fungus/PBMCs and 1:2 fungus/MDMs) for 48 hours at 37°C in a humidified 5% CO_2 atmosphere. Moreover, when indicated, the cultures were simultaneously activated or not with rhIL-12 (10 ng/mL) or rhIFN-γ (20 ng/mL). CFU were counted on day 6 and *P brasiliensis* survival determined in relation to *P brasiliensis* growth on plates from untreated PBMCs from healthy control subjects.

Statistical Analysis

Data were analyzed using GraphPad Prism 5 statistical software (GraphPad Software, San Diego, CA). Results are expressed as mean with standard error of the mean of 3 individual experiments, if not indicated differently. Significance difference was determined using Mann-Whitney test when $P \leq .05$.

RESULTS

Heterozygous p.E651V Mutation in STAT4 of Subjects With PCM

Considering the complexity and number of genes involved in the immune response against pathogens and the history of nonclassical PID represented by members of the family studied here, we carried out WES of the index case in order to identify candidate genes predisposing to PCM. WES revealed a total of 229 relevant variants (eg, nonsense, insertion, deletion, frameshift, etc), which were not found in 1000 Genome Project (www.genome.gov), dbSNP (www.ncbi.nlm.nih.gov/SNP/), Exome Aggregation Consortium (http://exac.broadinstitute.org), Exome Variant Server (http://evs.gs.washington.edu/EVS/), or Genome Aggregation Database (http://gnomad.broadinstitute.org/about). Among these 229 genes, we selected variations in 4 genes possibly associated with antifungal immune responses: *JAK3, IL13RA, TRAIP,* and *STAT4.* However, only the variant g.119147A>T (c.1952 A>T), which causes a single amino acid change altering glutamic acid (Glu/E) to valine (Val/V) at position 651 (p.E651V) in STAT4, was also present in the patient's father and not present in the healthy mother as confirmed by Sanger sequencing (Figure 1B) and in accordance with an assumed autosomal dominant inheritance. The variant c.1952 A>T (p.E651V) was not present in the exome data of 609 healthy Brazilian controls (in-house database).

Conservation analysis demonstrated that the p.E651V mutation, located within the STAT4 SH2 binding domain, affects a residue that is conserved among different species (Figure 1C).

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Mutation Taster and SIFT Score analysis indicated this alteration as deleterious and the CADD Score for this variation is reported to be 24.2, which is way above the mutation significance cutoff (MSC) for this gene of 3.3 [28]. Gene damage index (GDI) prediction revealed a score of 36.38 (GDI Phred score of 1.09081) for STAT4, which correlates to a medium damage prediction for this gene in the general population [29]. Additional in silico investigation using a STAT4 3D structural construct showed that the p.E651V mutation may affect STAT4 phosphorylation and binding to the cytoplasmic domain of the IL-12R as well as STAT4 dimerization due to disturbed SH2 interaction through hydrogen bonding between mutant and WT monomers (Figure 1D).

Defective STAT4 Phosphorylation in Patient's Cells and in Cells Expressing the Mutant Allele

Successful STAT4 signaling requires phosphorylation of STAT4 (pSTAT4) on tyrosine 693 and seronine S721, which triggers STAT4 dimerization and translocation to the nucleus where it induces transcription of *IFNG* [3]. IL-12 activation induced normal STAT4 phosphorylation in the patient's mother and healthy control $CD3^+$ T cells, while $CD3^+$ T cells from the patient and her father had reduced phosphorylation of STAT4 (Figure 2A). Confocal microscopy analysis confirmed the impaired IL-12–induced STAT4 phosphorylation, and revealed markedly reduced pSTAT4 translocation to the nucleus in $CD3^+$ T cells from the patient and her father (Figure 2B) compared with cells from healthy controls. Furthermore, the patient´s PHA-preactivated $CD3^+$ T cells showed normal IL-12Rβ1 and IL-12Rβ2 expression in comparison to lymphocytes from healthy controls, suggesting that impaired pSTAT4 was not due to the absence of IL-12R (Supplementary Figure 1).

In agreement with the primary cell studies, B-LCL cells transduced with the STAT4-E651V mutant displayed reduced pSTAT4 in response to IFN-α in a dose-dependent manner when compared with B-LCL cells transduced with WT STAT4 (Figure 3A). Normal IFN-α–induced pSTAT1 in transduced B-LCL cells confirmed the specificity of STAT4 defects (Figure 3B). In parallel to normal expression of total STAT4, reduced IFN-α–induced pSTAT4 was also observed in B-LCL cells transduced with the mutant constructs, as shown by western blot analysis (Figure 3C), while IFN-α–induced pSTAT1 protein expression was normal in the same set of cells (Figure 3D). Normal protein expression of total STAT4-p.E651V in HEK293T cells (Figure 3E) and normal mRNA levels in both HEK293T and B-LCL cells when compared to WT STAT4 (Figure 3F), suggest that the E651V mutation does not affect STAT4 transcriptional activity.

Furthermore, U3A cells that received homozygous (E651V/E651V) or heterozygous (WT/E651V) constructs showed reduced pSTAT4 in response to IFN-α in a dose-dependent manner when compared with those receiving WT STAT4 (Figure 4A). Notably, heterozygous constructs impaired the IFN-α–induced pSTAT4 stronger than the homozygous (Figure 4A and B). Although these data require further investigation, our finding is in agreement with a previous report showing that, because the assembly of nonphosphorylated STAT4 dimers occurs before receptor-driven pSTAT4, STAT4 mutant homodimers have more affinity to form intramolecular rearrangement compared to mutant heterodimers. Therefore, STAT4 heterozygous mutations cause a stronger impairment in pSTAT4 than homozygous [30]. Taken together, our data suggest a dominant negative effect of the E651V mutant allele that underlies an autosomal dominant phenotype in heterozygous cells.

Lymphocytes Carrying the p.E651V Mutation Produce Significantly Less IFN-γ Following IL-12 Stimulation

Because pSTAT4 was defective in T cells from the patient and her father, we evaluated IL-12–induced IFN-γ production by preactivated PBMCs. We observed reduced intracellular (Figure 5A) and secreted (Figure 5B) IFN-γ levels when studying $CD4^+$ T cells from the patient and her father after activation with rhIL-12 in comparison to healthy controls. In contrast, compared with healthy control cells, patient´s and father´s $CD3^+$ T cells produced normal levels of intracellular IFN-γ when stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin. Notably, although both individuals with the p.E651V *STAT4* mutation present lower levels of IFN-γ positivity compared to controls, IFN-γ–expressing cells were slightly more frequent in the patient when compared to her father, while the IFN-γ secretion by father´s PBMCs was higher than in the index patient. We considered this slightly differences as experimental artifacts.

Impaired P brasiliensis Fungicidal Activity by PBMCs and MDMs From STAT4-Deficient Individuals

We asked whether PBMCs and MDMs from *STAT4*-deficient individuals cocultured with autologous lymphocytes are able to kill *P brasiliensis*. Both PBMCs (Figure 6A) and MDMs/T cell coculture (Figure 6B) from the patient and her father displayed reduced fungicidal activity when challenged with *P brasiliensis* compared to healthy controls. In agreement with the defects in STAT4 phosphorylation and nuclear translocation, exogenous rhIL-12 failed to increase their impaired fungicidal activity against *P brasiliensis* in comparison to PBMCs or MDMs from healthy controls (Figure 6A and B). Furthermore, we observed that MDMs alone also present impaired fungicidal activity (Figure 6C), thus confirming that macrophages require IFN-γ to develop normally [31]. In this latter experiment, exogenous IFN-γ enhanced fungicidal activity of macrophages from the control and the STAT4-deficient individuals, albeit fungicidal activity was still lower by MDMs from the latter.

DISCUSSION

Here, we report a family with a daughter and father who developed symptomatic PCM and identified a heterozygous

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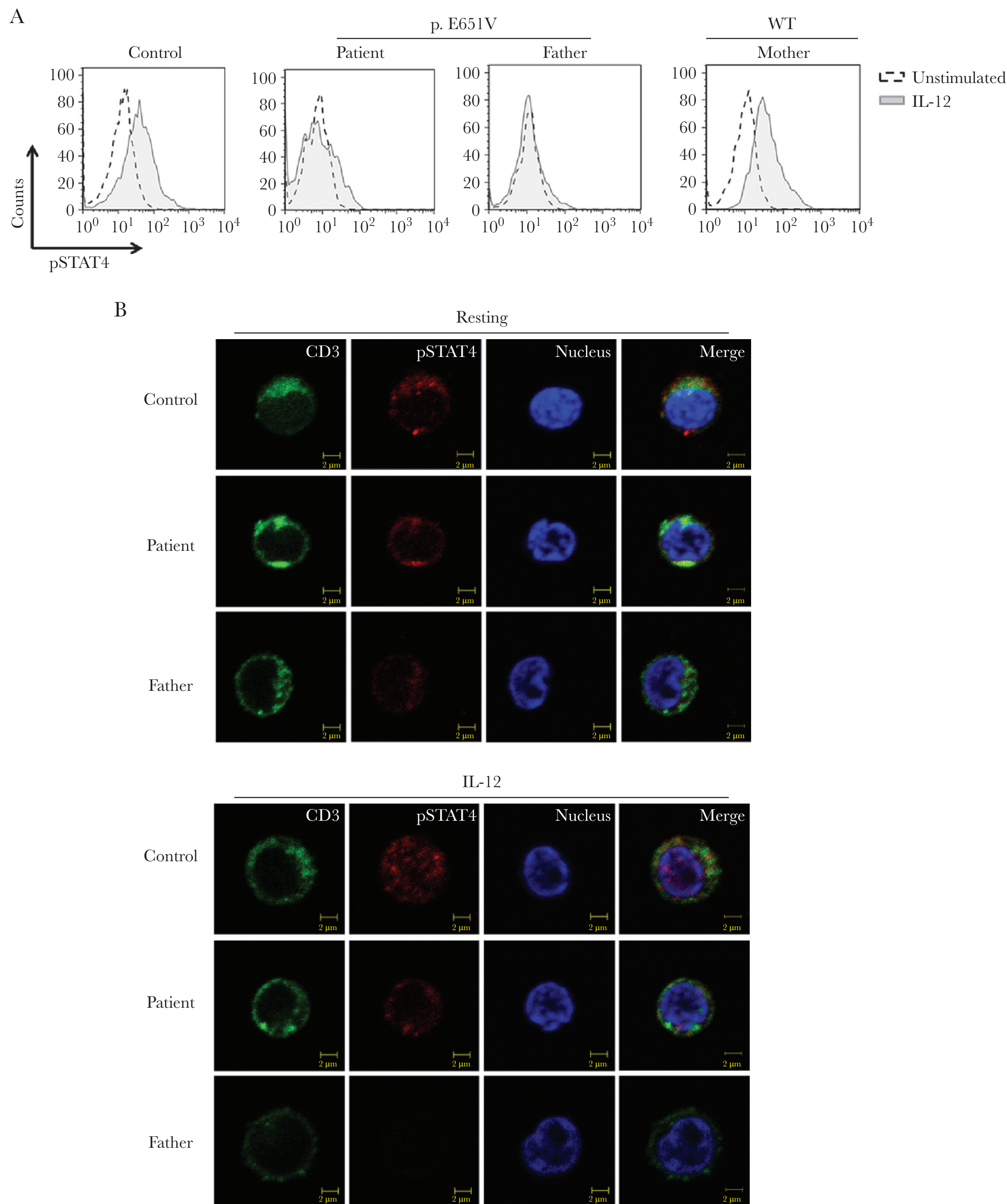

Figure 2. Defective phosphorylation and nuclear translocation of STAT4. *A*, CD3+ T lymphocytes, preactivated with phytohemagglutinin (PHA) for 3 days to induce interleukin-12R (IL-12R) expression on the cell surface, from the index patient, her parents, and a healthy control were analyzed for phosphorylation of STAT4 by flow cytometry after stimulation with rhIL-12. Histograms showing phosphorylated STAT4 levels in nonstimulated (dashed lines) and stimulated (filled grey area) cell samples representing 1 of 3 independent experiments. *B*, Confocal microscopy of PHA-preactivated lymphocytes revealing phosphorylated STAT4 levels (second row from left) in the cytoplasm and pSTAT4 translocation to the nucleus after stimulation with rhIL-12. The panel shows 1 representative cell of various cells analyzed in 2 independent experiments.

amino acid change (p.E651V) in STAT4 that causes a dominant negative effect on STAT4 phosphorylation, thus impairing IL-12–induced IFN-γ immunity. Notably, while classic PIDs are characterized by recurrent and severe infections, which often result in increased clinical complications with aging, the patients with nonclassic PIDs show susceptibility to a narrow spectrum

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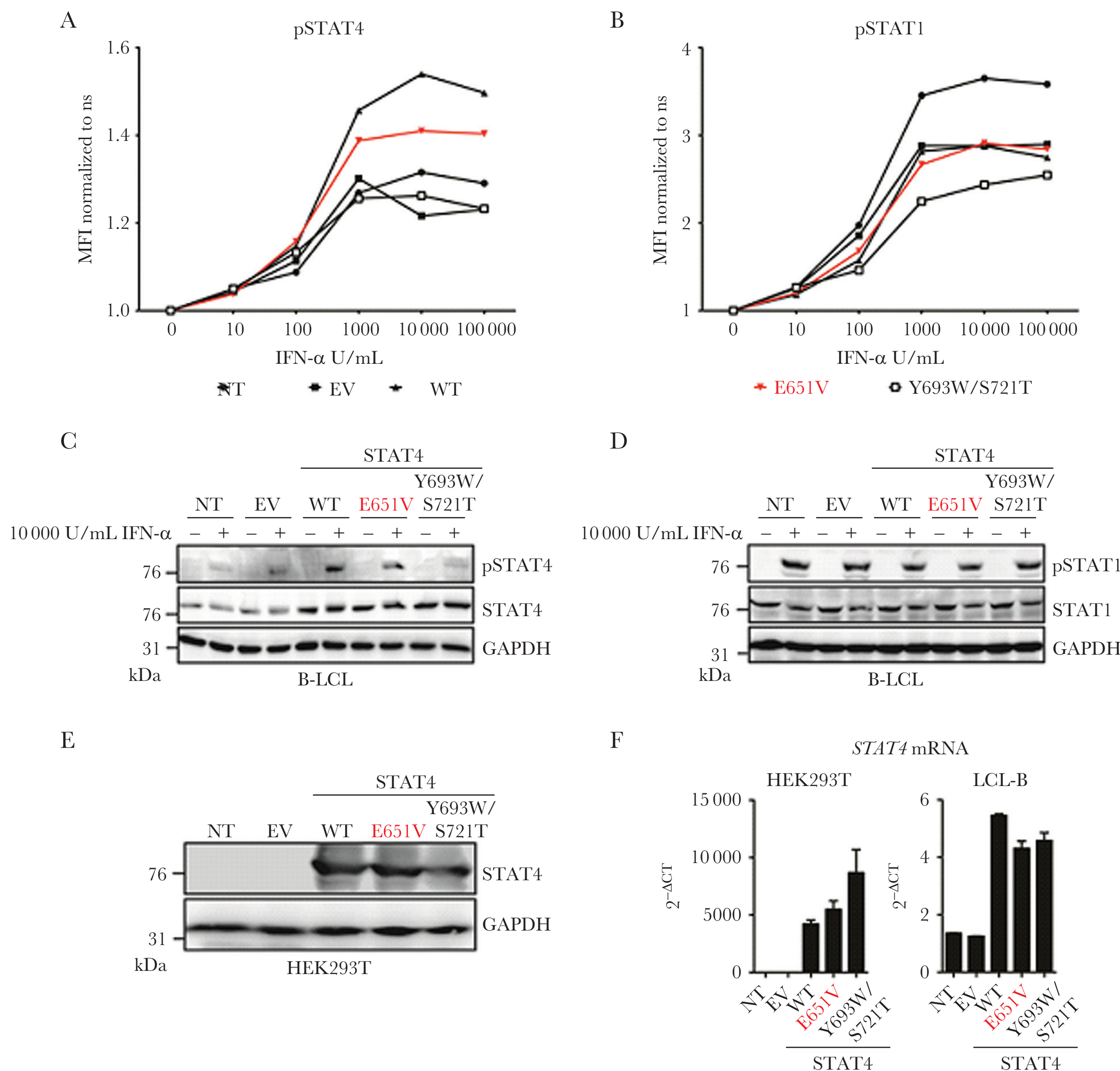

Figure 3. Defective STAT4 phosphorylation in B-LCL and HEK293 T cells. *A*, Phosphorylation of STAT4 was assessed by flow cytometry by stimulating B-LCL cells with a gradient of interferon (IFN)-α and measuring the mean fluorescence intensity (MFI) that was normalized by the values of the nonstimulated (ns). *B*, Phosphorylation of STAT1 showing that the results in (*A*) are not due to differences in overall IFN-α response. *C*, B-LCL cells were stimulated with 100 000 U/mL of IFN-α and phosphorylation of STAT4 assessed by western blot. *D*, The same cell lysates from (*C*) were analyzed in a separate gel and phosphorylation of STAT1 assessed showing equal response to IFN-α in all conditions. *E*, Overexpression of WT, E651V, or Y693W/S721T STAT4 in HEK293T cells showing that none of the variants impair the expression of the protein. *F*, Real-time quantitative polymerase chain reaction (RT-qPCR) data for *STAT4* in transfected HEK293T from (*E*) and in B-LCL nontransduced (NT), transduced with a retrovirus generated with an empty vector (EV), or with WT, E651V, and Y693W/S721T conditions showing similar mRNA expression levels. N = 2 for all experiments. Abbreviations: NT, nontransfected; EV, empty vector; WT, wild type; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

of etiologic agents, and often clinical improvement with age or may even develop only a single episode of an infection with good clinical outcome [32, 33]. For instance, patients with mutations in *TLR3, TBK1, TRAF3, TRIF, IRF3*, or *UNC93B1* are susceptible to herpes simplex encephalitis [8], and genetic defects in proteins of the IFN-γ pathway result in Mendelian susceptibility to mycobacterial disease [1]. In the latter category, infections are mainly caused by atypical mycobacteria, but a considerable proportion of patients may also suffer from infections caused by *Salmonella spp.* [1] and sporadic cases of severe infections caused by other intracellular bacteria, fungi, and parasites, such as *Listeria monocytogenis* [34], *Klebsiella* [35], *Leishmania* [36], *Toxoplasma gondii* [37], *Histoplasma capsulatum* [10], *Coccidioides* [11, 12], and *P brasiliensis* [13] have also been reported. Particularly, PIDs such as IL-12p40, IL-12Rβ1, IL-1 receptor-associated 4 (IRAK-4), UNC93B1, and Toll-like receptor 3 (TLR3) deficiencies have been reported to predispose patients to a single episode of infections with generally

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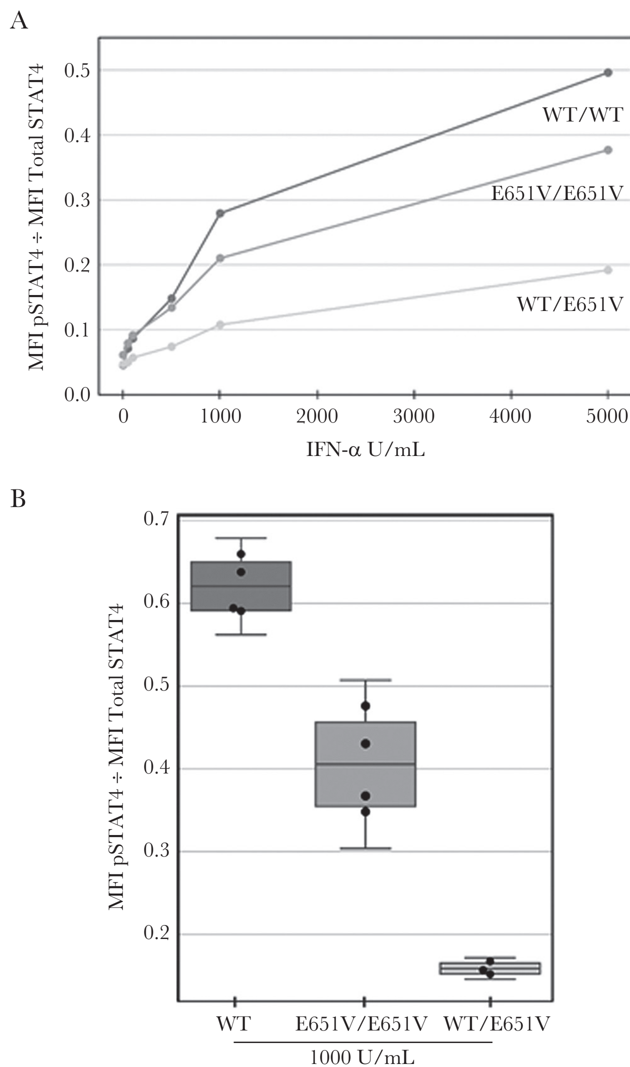

Figure 4. Defective STAT4 phosphorylation in U3A cells. *A*, U3A cells transfected with wild-type (WT)-STAT4, WT/E651V STAT4, and E651V/E651V STAT4 mutant were stimulated with different concentrations of interferon (IFN)-**α** for 20 minutes and phosphorylated STAT4 (pSTAT4) measured by flow cytometry. Data are shown as mean fluorescence intensity (MFI) of pSTAT4 normalized to the MFI of total amount of STAT4 (GFP-STAT4) in the cell. One of 3 independent experiments is shown. *B*, Box blots showing mean and standard deviation of the ratio of MFI pSTAT4 normalized to MFI of total STAT4 from similarly transfected U3A cells activated with 1000 U/mL IFN-**α**. N = 4 for WT-WT, and E651V/E651V; n = 3 for WT/E651 construct.

a favorable outcome and without relapse in most cases [38]. In accordance, the index patient and her father developed only a single, although severe, episode of PCM disease with no further infections with age and both are well without administration of prophylactic treatment. In the case of patients with inborn errors of the IL-12 signaling pathways that secondarily affect IFN-production, alternative mechanism could justify the improvement of clinical manifestations with aging because other in vivo inducers of IFN-γ or other effector cytokines that can compensate for the absence of IFN-γ, such as IFN-α, which also signals through STAT1 and activates downstream effector genes similar to IFN-γ [39, 40].

So far, a specific genetic diagnosis was assigned to only 2 patients who had developed PCM disease: one with defective IL12Rβ1 [13] and another with CD40L deficiency [5], which are both essential molecules for IFN-γ production. Several genetic defects involving IFN-γ–mediated immunity have been described [1]. The family described here displays autosomal dominant STAT4 deficiency associated with susceptibility to PCM disease. Another unique heterozygous mutation (c.1337C>T, p.T446I) in *STAT4* was recently reported by Aavikko et al to be associated with susceptibility to classic Kaposi sarcoma caused by human herpesvirus 8 [41]. Interestingly, one affected heterozygous family member also developed mycobacterial disease, but no investigation was performed in order to characterize the impact of p.T446I mutation in STAT4 function. Both, our data and those reported by Aavikko et al resemble the clinical phenotypes observed in Stat4 and IL-12p40 knockout mice, which are highly susceptible to intracellular pathogens [25] including *P brasiliensis* [42].

Here we provide evidences that the heterozygous p.E651V mutation compromises STAT4 function. However, the contribution of STAT4 deficiency to the pathogenesis of PCM disease will only be provided by further evidence such as additional families with STAT4 deficiency who develop PCM. Notably, in South America the availability of laboratories performing genetic analysis of patients with unusual infectious diseases is still limited and molecular genetic defects in patients with PCM disease are rarely identified [15]. Interestingly, the importance of the IL-12/IFN-γ circuit for the protection against mycobacteria diseases is well known [1], and tuberculosis has frequently been observed in coinfection with PCM [43–46], suggesting that these two infectious diseases may have common immunopathological mechanisms. Thus, additional investigations exploring possible molecular genetic defects underlying PCM disease in Latin American countries may lead to new therapeutic opportunities for patients with PCM.

The interaction between T cells and monocytes/macrophages mediated by the IL-12/IFN-γ axis plays a pivotal role in host defense against intracellular bacteria, fungi, and parasites by activating microbicidal activity of monocytes/macrophages [4], which are key players during *P brasiliensis* infection. While IFN-γ enhances macrophage microbicidal activity [47–49], its effects are not limited to mature phagocytes [50] as confirmed by defects of microbicidal mechanisms of macrophages from patients with inherited IFN-γR1 or IFN-γR2 deficiencies [31]. Accordingly, we could show that, PBMCs and MDMs alone or cocultured with lymphocytes from our index patient and from her father had decreased fungicidal activity against *P brasiliensis* when compared to healthy controls. As expected from defective STAT4 phosphorylation and reduced IFN-γ production, only IFN-γ but not rhIL-12 was able to increase the patient's and her father's PBMCs and MDMs fungicidal activity, while

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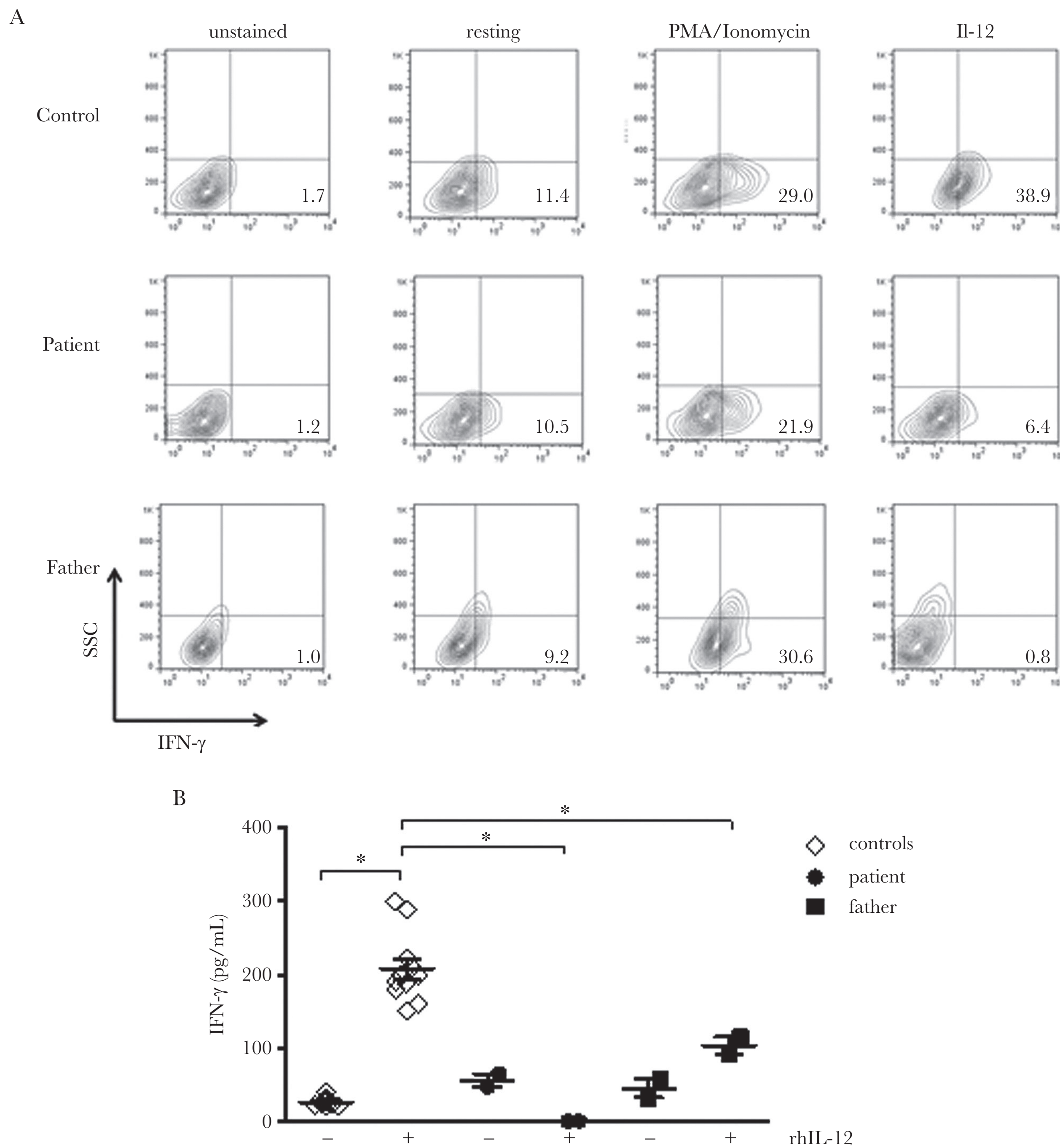

Figure 5. Impaired secretion of interferon-**γ** (IFN-**γ**) by patient PBMCs in response to interleukin-12 (IL-12). *A*, Contour plots of phytohemagglutinin (PHA)-preactivated PBMCs from patient and father showing normal IFN-**γ** production in response to phorbol 12-myristate 13-acetate (PMA) plus ionomycin, whereas there was reduced intracellular IFN-**γ** concentration in response to rhIL-12 when compared to a healthy control. The cells were stimulated with PMA plus ionomycin or rhIL-12 for 12 hours. Contour plots represent 1 of 2 individual experiments. *B*, Impaired IFN-**γ** release by PHA-preactivated PBMCs from the index patient and her father when compared to 11 healthy controls. Cells were stimulated with rhIL-12 for 48 hours. Standard deviation of 2 experiments performed with PBMCs from the patient and her father is shown.

the fungicidal activity of healthy control cells could be increased significantly by both reagents.

In conclusion, our data suggest that autosomal dominant STAT4 deficiency represents a novel defect of IL-12–dependent IFN-γ immunity and that it is associated in this kindred with susceptibility to PCM disease. This raises the possibility that patients with symptomatic PCM and other related fungal infections suffer from this or related inborn errors. Therefore, searching for defects in the IL-12/IFN-γ axis, including mutations in STAT4, should be considered for these patients. The identification of additional patients with alterations in the *STAT4* gene are needed to define the clinical spectrum of STAT4 deficiency

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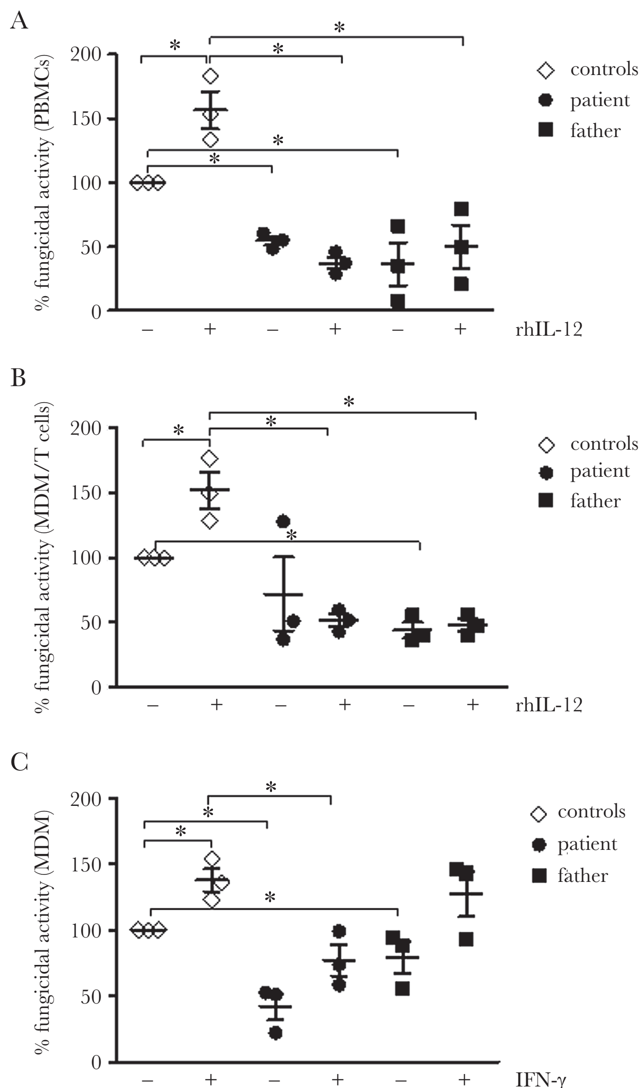

Figure 6. The reduced fungicidal activity against *Paracoccidioides brasiliensis* by peripheral blood mononuclear cells (PBMCs) and monocyte-derived macrophages (MDMs) from the patient and the father is not improved by recombinant human interleukin-12 (rhIL-12). The fungicidal activity was assessed by counting colony forming units (CFU) from recovering internalized fungi. The fungicidal activity was determined in relation to the CFU number on culture plates from healthy control subjects studied on the same day, which was considered as 100%. Each dot represents the mean of triplicates from independent experiments. Standard deviation of the 3 independent experiments performed is shown: (*A*) PBMCs, (*B*) cocultures of MDMs with lymphocytes, and (*C*) MDMs alone were challenged with *P brasiliensis* for 48 hours in the presence or absence of rhIL-12 or interferon-γ (IFN-γ).

and to clarify the immunopathological mechanisms associated with this PID.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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References

1. Bustamante J, Boisson-Dupuis S, Abel L, Casanova JL. Mendelian susceptibility to mycobacterial disease: genetic, immunological, and clinical features of inborn errors of IFN-γ immunity. Semin Immunol **2014**; 26:454–70.
2. Athie-Morales V, Smits HH, Cantrell DA, Hilkens CM. Sustained IL-12 signaling is required for Th1 development. J Immunol **2004**; 172:61–9.
3. Jacobson NG, Szabo SJ, Weber-Nordt RM, et al. Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. J Exp Med **1995**; 181:1755–62.
4. Ramirez-Alejo N, Santos-Argumedo L. Innate defects of the IL-12/IFN-γ axis in susceptibility to infections by mycobacteria and salmonella. J Interferon Cytokine Res **2014**; 34:307–17.
5. Cabral-Marques O, Schimke LF, Pereira PV, et al. Expanding the clinical and genetic spectrum of human CD40L deficiency: the occurrence of paracoccidioidomycosis and other unusual infections in Brazilian patients. J Clin Immunol **2012**; 32:212–20.
6. Picard C, Al-Herz W, Bousfiha A, et al. Primary immunodeficiency diseases: an update on the classification from the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency 2015. J Clin Immunol **2015**; 35:696–726.
7. Cabral-Marques O, Arslanian C, Ramos RN, et al. Dendritic cells from X-linked hyper-IgM patients present impaired responses to Candida albicans and *Paracoccidioides brasiliensis*. J Allergy Clin Immunol **2012**; 129:778–86.
8. Bustamante J, Boisson-Dupuis S, Jouanguy E, et al. Novel primary immunodeficiencies revealed by the investigation of paediatric infectious diseases. Curr Opin Immunol **2008**; 20:39–48.
9. Ouederni M, Sanal O, Ikinciogullari A, et al. Clinical features of *Candidiasis* in patients with inherited interleukin 12 receptor β1 deficiency. Clin Infect Dis **2014**; 58:204–13.
10. Zerbe CS, Holland SM. Disseminated histoplasmosis in persons with interferon-gamma receptor 1 deficiency. Clin Infect Dis **2005**; 41:e38–41.

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11. Vinh DC, Masannat F, Dzioba RB, Galgiani JN, Holland SM. Refractory disseminated coccidioidomycosis and mycobacteriosis in interferon-gamma receptor 1 deficiency. Clin Infect Dis **2009**; 49:e62–5.
12. Vinh DC, Schwartz B, Hsu AP, et al. Interleukin-12 receptor β1 deficiency predisposing to disseminated coccidioidomycosis. Clin Infect Dis **2011**; 52:e99–e102.
13. Moraes-Vasconcelos Dd, Grumach AS, Yamaguti A, et al. Paracoccidioides brasiliensis disseminated disease in a patient with inherited deficiency in the beta1 subunit of the interleukin (IL)-12/IL-23 receptor. Clin Infect Dis **2005**; 41:e31–7.
14. Colombo AL, Tobón A, Restrepo A, Queiroz-Telles F, Nucci M. Epidemiology of endemic systemic fungal infections in Latin America. Med Mycol **2011**; 49:785–98.
15. Cabral-Marques O, Klaver S, Schimke LF, et al. First report of the hyper-IgM syndrome registry of the Latin American Society for Immunodeficiencies: novel mutations, unique infections, and outcomes. J Clin Immunol **2014**; 34:146–56.
16. Filipe-Santos O, Bustamante J, Haverkamp MH, et al. X-linked susceptibility to mycobacteria is caused by mutations in NEMO impairing CD40-dependent IL-12 production. J Exp Med **2006**; 203:1745–59.
17. Brummer E, Castaneda E, Restrepo A. Paracoccidioidomycosis: an update. Clin Microbiol Rev **1993**; 6:89–117.
18. Ferreira MS. Paracoccidioidomycosis. Paediatr Respir Rev **2009**; 10:161–5.
19. Byun M, Abhyankar A, Lelarge V, et al. Whole-exome sequencing-based discovery of STIM1 deficiency in a child with fatal classic Kaposi sarcoma. J Exp Med **2010**; 207:2307–12.
20. Kong XF, Vogt G, Itan Y, et al. Haploinsufficiency at the human IFNGR2 locus contributes to mycobacterial disease. Hum Mol Genet **2013**; 22:769–81.
21. Aken BL, Ayling S, Barrell D, et al. The Ensembl gene annotation system. Database (Oxford) **2016**; 2016: baw093.
22. Uzel G, Frucht DM, Fleisher TA, Holland SM. Detection of intracellular phosphorylated STAT-4 by flow cytometry. Clin Immunol **2001**; 100:270–6.
23. Martínez-Barricarte R, de Jong SJ, Markle J, et al. Transduction of *Herpesvirus saimiri*-transformed T cells with exogenous genes of interest. Curr Protoc Immunol **2016**; 115:7.21C.1–7.21C.12.
24. Morinobu A, Gadina M, Strober W, et al. STAT4 serine phosphorylation is critical for IL-12-induced IFN-gamma production but not for cell proliferation. Proc Natl Acad Sci U S A **2002**; 99:12281–6.
25. Kaplan MH. STAT4: a critical regulator of inflammation in vivo. Immunol Res **2005**; 31:231–42.
26. Cabral-Marques O, Ramos RN, Schimke LF, et al. Human CD40 ligand deficiency dysregulates the macrophage transcriptome causing functional defects that are improved by exogenous IFN-γ. J Allergy Clin Immunol **2017**; 139:900–912.e7.
27. Pina A, Saldiva PH, Restrepo LE, Calich VL. Neutrophil role in pulmonary paracoccidioidomycosis depends on the resistance pattern of hosts. J Leukoc Biol **2006**; 79:1202–13.
28. Itan Y, Shang L, Boisson B, et al. The mutation significance cutoff: gene-level thresholds for variant predictions. Nat Methods **2016**; 13:109–10.
29. Itan Y, Shang L, Boisson B, et al. The human gene damage index as a gene-level approach to prioritizing exome variants. Proc Natl Acad Sci U S A **2015**; 112:13615–20.
30. Ota N, Brett TJ, Murphy TL, Fremont DH, Murphy KM. N-domain-dependent nonphosphorylated STAT4 dimers required for cytokine-driven activation. Nat Immunol **2004**; 5:208–15.
31. Conti F, Aragão Filho WC, Prando C, et al. Phagocyte nicotinamide adenine dinucleotide phosphate oxidase activity in patients with inherited IFN-γR1 or IFN-γR2 deficiency. J Allergy Clin Immunol **2015**; 135:1393–5.e1.
32. Casanova J-L. Severe infectious diseases of childhood as monogenic inborn errors of immunity. Proc Natl Acad Sci **2015**; 112: E7128–37.
33. Casanova J-L. Human genetic basis of interindividual variability in the course of infection. Proc Natl Acad Sci **2015**; 112: E7118–27.
34. Roesler J, Kofink B, Wendisch J, et al. Listeria monocytogenes and recurrent mycobacterial infections in a child with complete interferon-gamma-receptor (IFNgammaR1) deficiency: mutational analysis and evaluation of therapeutic options. Exp Hematol **1999**; 27:1368–74.
35. Pedraza S, Lezana JL, Samarina A, et al. Clinical disease caused by Klebsiella in 2 unrelated patients with interleukin 12 receptor beta1 deficiency. Pediatrics **2010**; 126:e971–6.
36. Sanal O, Turkkani G, Gumruk F, et al. A case of interleukin-12 receptor beta-1 deficiency with recurrent leishmaniasis. Pediatr Infect Dis J **2007**; 26:366–8.
37. Sologuren I, Boisson-Dupuis S, Pestano J, et al. Partial recessive IFN-γR1 deficiency: genetic, immunological and clinical features of 14 patients from 11 kindreds. Hum Mol Genet **2011**; 20:1509–23.
38. Casanova JL, Abel L. Primary immunodeficiencies: a field in its infancy. Science **2007**; 317:617–9.
39. Casanova JL, Abel L. Genetic dissection of immunity to mycobacteria: the human model. Annu Rev Immunol **2002**; 20:581–620.
40. Bax HI, Freeman AF, Ding L, et al. Interferon alpha treatment of patients with impaired interferon gamma signaling. J Clin Immunol **2013**; 33:991–1001.
41. Aavikko M, Kaasinen E, Nieminen JK, et al. Whole-genome sequencing identifies STAT4 as a putative susceptibility gene in classic Kaposi sarcoma. J Infect Dis **2015**; 211:1842–51.

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42. Livonesi MC, Souto JT, Campanelli AP, et al. Deficiency of IL-12p40 subunit determines severe paracoccidioidomycosis in mice. Med Mycol **2008**; 46:637–46.
43. Torres-Pereira C, Giovanini AF, Stramandinoli RT, Amenabar JM, Piazzetta CM. Oral paracoccidioidomycosis and pulmonary tuberculosis co-infection: relevance of oral biopsy in establishing the diagnosis and therapeutic approach. Int J Infect Dis **2009**; 13:114–6.
44. Quagliato Júnior R, Grangeia Tde A, Massucio RA, De Capitani EM, Rezende Sde M, Balthazar AB. Association between paracoccidioidomycosis and tuberculosis: reality and misdiagnosis. J Bras Pneumol **2007**; 33:295–300.
45. Guery R, Merat C, Blanchet D, Aznar C, Labbe S, Djossou F. Coinfection with tuberculosis and paracoccidioidomycosis in French Guiana: a common misdiagnosis. Int J Dermatol **2015**; 54:e436–9.
46. Amorim Pellicioli AC, Neves-Silva R, Santos-Silva AR, Vargas PA, Lopes MA. Synchronous oral paracoccidioidomycosis and pulmonary tuberculosis in an immunocompetent patient. Mycopathologia **2015**; 179:459–64.
47. Brummer E, Hanson LH, Restrepo A, Stevens DA. In vivo and in vitro activation of pulmonary macrophages by IFN-gamma for enhanced killing of *Paracoccidioides brasiliensis* or *Blastomyces dermatitidis*. J Immunol **1988**; 140:2786–9.
48. Loures FV, Stroeder H, Almeida I, Calich VL. *Paracoccidioides brasiliensis* lipids modulate macrophage activity via Toll-dependent or independent mechanisms. FEMS Immunol Med Microbiol **2012**; 66:58–70.
49. Parente-Rocha JA, Parente AFA, Baeza LC, et al. Macrophage interaction with paracoccidioides brasiliensis yeast cells modulates fungal metabolism and generates a response to oxidative stress. PLoS One **2015**; 10:e0137619.
50. Ezekowitz RA, Sieff CA, Dinauer MC, Nathan DG, Orkin SH, Newburger PE. Restoration of phagocyte function by interferon-gamma in X-linked chronic granulomatous disease occurs at the level of a progenitor cell. Blood **1990**; 76:2443–8.

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