Inherited CARD9 deficiency in otherwise healthy children and adults with Candida species–induced meningoencephalitis, colitis, or both

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Background: Invasive infections of the central nervous system (CNS) or digestive tract caused by commensal fungi of the genus Candida are rare and life-threatening. The known risk factors include acquired and inherited immunodeficiencies, with patients often displaying a history of multiple infections. Cases of meningoencephalitis, colitis, or both caused by Candida species remain unexplained.

Objective: We studied 5 previously healthy children and adults with unexplained invasive disease of the CNS, digestive tract, or both caused by Candida species. The patients were aged 39, 7, 17, 37, and 26 years at the time of infection and were unrelated, but each was born to consanguineous parents of Turkish (2 patients), Iranian, Moroccan, or Pakistani origin.

Meningoencephalitis was reported in 3 patients, meningoencephalitis associated with colitis was reported in a fourth patient, and the fifth patient had colitis only.

Methods: Inherited caspase recruitment domain family, member 9 (CARD9) deficiency was recently reported in...
otherwise healthy patients with other forms of severe disease caused by *Candida*, *Trichophyton*, *Phialophora*, and *Exophiala* species, including meningoencephalitis but not colitis caused by *Candida* and *Exophiala* species. Therefore we sequenced *CARD9* in the 5 patients.

Results: All patients were found to be homozygous for rare and deleterious mutant *CARD9* alleles: R70W and Q289* for the 3 patients with *Candida albicans*–induced meningoencephalitis, R35Q for the patient with meningoencephalitis and colitis caused by *Candida glabrata*, and Q295* for the patient with *Candida albicans*–induced colitis. Regardless of their levels of mutant *CARD9* protein, the patients’ monocyte–derived dendritic cells responded poorly to *CARD9*-dependent fungal agonists (curdlan, heat-killed *C albicans*, *Saccharomyces cerevisiae*, and *Exophiala dermatitidis*).

Conclusion: Invasive infections of the CNS or digestive tract caused by *Candida* species in previously healthy children and even adults might be caused by inherited *CARD9* deficiency. (J Allergy Clin Immunol 2015;136: nn.)

Key words: Inborn error of immunity, primary immunodeficiency, invasive fungal diseases, inherited *CARD9* deficiency, central nervous system, colitis, Candida species, human

*Candida* species are commensal yeasts colonizing the skin and digestive tracts of most healthy subjects. However, they can cause mucocutaneous candidiasis, which when chronic (ie, chronic mucocutaneous candidiasis [CMC]) is commonly observed in patients with broad and profound acquired or inherited T-cell disorders. These patients typically display various other infections. Syndromic CMC, in which CMC is a key element of the description of the clinical entity (eg, autosomal recessive autoimmune polyendocrinopathy type 1), and isolated CMC, which generally affects otherwise healthy subjects (eg, autosomal dominant CMC disease), have both been reported to result from primary immunodeficiencies impairing IL-17 immunity. *Candida* species might also cause severe invasive infections. Candididemia, the most frequent clinical form of invasive candidiasis, is the fourth most frequent cause of bloodstream infection in hospitals and is classically reported in patients with neutropenia, patients with a central catheter receiving broad-spectrum antibiotics and/or parenteral nutrition, or both. In contrast, *Candida* species infection of the central nervous system (CNS) is rare. *Candida* species–induced meningitis is reported principally in preterm neonates (0.59% of neonates weighing <1000 g at birth have *Candida* species–induced meningitis), possibly because of immaturity of the blood-brain barrier. Neurosurgery, abdominal surgery, intravenous catheter use, intravenous drug use, HIV infection, immunosuppressive treatments, and cancer have been associated with the few reported cases of *Candida* species infection of the CNS. *Candida* species infections have also been observed in a few patients with inherited chronic granulomatous disease. Finally, 39 patients with *Candida* species CNS infection and no known underlying risk factors have been reported, although chronic granulomatous disease was not excluded in most of these cases. In these patients the median age at CNS infection was 27 years (1-54 years), 62% were male, and 55% died of infection. Interestingly, *Candida* species–induced meningitis has been reported in 5 patients with inherited caspase recruitment domain family, member 9 (CARD9) deficiency, whereas 2 other patients with CARD9 deficiency had brain abscesses, which were caused by *Exophiala* species in 1 patient and possibly by *Trichophyton* species in the other. *Candida* species causes colitis even less frequently than meningoencephalitis, with only 25 cases reported in the context of neutropenia, cancer, lymphoma, systemic lupus erythematosus, AIDS, corticosteroid treatment, or preterm neonates. Therefore we sequenced *CARD9* in 5 unrelated patients: 4 with *Candida* species infections of the CNS, one of whom had proved *Candida* species–induced colitis, with the final patient having proved *Candida* species–induced colitis solely.

RESULTS

Case reports

We describe 5 patients with proved *Candida* species infections of the CNS, the digestive tract, or both from 5 unrelated consanguineous families originating from Turkey (n = 2), Iran, Morocco, and Pakistan (n = 1 each, Table I).

Kindred A

A 42-year-old woman (P1, A.II.2; Fig 1, A) living in France and born to consanguineous Turkish parents presented at 39 years of age with *Candida albicans*–induced meningitis and brain abscesses. She had recurrent vulvovaginal candidiasis, with episodes occurring about 5 times per year since the age of 36 years. She presented with headache, persistent fever, and vomiting. She then displayed an altered mental state, right-arm paresis, and facial palsy. Brain magnetic resonance imaging (MRI) provided evidence of an infiltrative frontal lesion with a mass effect and contrast enhancement with ventricular dilatation (Fig 2, A). Lumbar puncture revealed the presence of 1100 leukocytes/mm³ (80% lymphocytes and 16% eosinophils) in the cerebrospinal fluid (CSF), together with increased protein levels up to 1.53 g/L and hypoglycorrhachia of 1.5 mmol/L. CSF pressure was high at up to 25 cm H₂O. Three lumbar punctures were performed, and *C albicans* grew from the CSF samples collected. Brain biopsy showed yeasts and numerous pseudohyphae in giant cell granulomas with necrosis (Fig 3, A-D). A culture of the biopsy sample was positive for *C albicans*. Abdominal and thoracic computed tomography (CT) and transesophageal echocardiography provided no evidence

### Abbreviations used

- **CARD9**: Caspase recruitment domain family, member 9
- **CMC**: Chronic mucocutaneous candidiasis
- **CNS**: Central nervous system
- **CSF**: Cerebrospinal fluid
- **CT**: Computed tomography
- **DHR**: Dihydroorotate
- **MDDC**: Monocyte-derived dendritic cell
- **MRI**: Magnetic resonance imaging
- **NF-κB**: Nuclear factor κB
- **NK**: Natural killer
- **PMA**: Phorbol 12-myristate 13-acetate
- **PMN**: Phagocytic-mediated necrosis
- **VSV**: Vesicular stomatitis virus
- **WT**: Wild-type

### METHODS

Details of the materials and methods used in this study are provided in the Methods section in this article’s Online Repository at [www.jacionline.org](http://www.jacionline.org).
TABLE I. Characteristics of the 5 patients with invasive fungal infection and homozygous CARD9 mutations

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age at onset (y)</th>
<th>Age at last follow-up (y)</th>
<th>Sex</th>
<th>Country of origin</th>
<th>Organ involvement</th>
<th>Associated CMC</th>
<th>Fungus</th>
<th>Status</th>
<th>CARD9 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>39</td>
<td>42</td>
<td>F</td>
<td>Turkey</td>
<td>CNS</td>
<td>Yes</td>
<td>C albicans</td>
<td>Alive</td>
<td>R70W/R70W</td>
</tr>
<tr>
<td>P2</td>
<td>7</td>
<td>8</td>
<td>F</td>
<td>Turkey</td>
<td>CNS</td>
<td>Yes</td>
<td>C albicans</td>
<td>Alive</td>
<td>R70W/R70W</td>
</tr>
<tr>
<td>P3</td>
<td>17</td>
<td>28</td>
<td>M</td>
<td>Iran</td>
<td>CNS, sinus, digestive tract</td>
<td>No</td>
<td>C glabrata</td>
<td>Alive</td>
<td>R35Q/R35Q</td>
</tr>
<tr>
<td>P4</td>
<td>37</td>
<td>37</td>
<td>F</td>
<td>Morocco</td>
<td>CNS</td>
<td>Yes</td>
<td>C albicans</td>
<td>Alive</td>
<td>Q289*/?Q289*</td>
</tr>
<tr>
<td>P5</td>
<td>26</td>
<td>34</td>
<td>M</td>
<td>Pakistan</td>
<td>Digestive tract</td>
<td>No</td>
<td>C albicans</td>
<td>Alive</td>
<td>Q295*/?Q295*</td>
</tr>
</tbody>
</table>

F, Female; M, male.

for the dissemination of C albicans infection. Immunologic explorations showed normal CD4+ T, CD8+ T, and natural killer (NK) lymphocyte counts and B-cell lymphopenia at 4% (56 cells/μL). T-lymphocyte proliferations were normal in response to PHA and antigens (tuberculin and candidin). Leukocyte oxidative burst, as assessed by using dihydrorhodamine (DHR) tests, was normal, and IgG, IgA, and IgM levels were also normal. The infection was cured by 2 months of combined intravenous antifungal therapy combining liposomal amphotericin B and 5-flucytosine, which was subsequently replaced with oral fluconazole. A cerebral shunt was performed to treat intracranial hypertension. Two years later, fluconazole treatment is continuing, and the patient is alive without sequel. Neither her parents nor her siblings and children have had any severe infectious disease.

Kindred B

P2 is an 8-year-old girl born to a Turkish kindred (P2, kindred B, B.II.1; Fig 1, A) living in Belgium. She has had chronic thrush and onychomycosis since the age of 5 years. At the age of 7 years, she had fever for several weeks, with headache and vomiting. CSF analysis provided evidence of C albicans–induced meningitis but with 920 cells/mm3, 20% of which were eosinophils (fluconazole minimum inhibitory concentration = 0.5 mg/L). Brain MRI revealed the presence of 2 lesions of 11 and 6 mm in diameter, respectively. Medullary MRI revealed several enhancing lesions. C albicans grew from a CNS biopsy specimen. Histologic explorations were carried out as follows: neutrophils; CD4+ T, CD8+ T, and NK lymphocyte blood counts; DHR test results; and IgG, IgA, and IgM plasma levels were normal. IgE levels were high at 1.7 mg/mL. Eosinophil counts; DHR test results; and IgG, IgA, and IgM plasma levels were normal. IgE levels were high at 1.7 mg/mL. Eosinophil counts were also high at up to 1500/mm3. Neither the patient’s parents nor his siblings had any severe infectious disease.

Kindred C

P3 is a 28-year-old man from a consanguineous Iranian kindred (P3, kindred C, C.II.2; Fig 1, A) living in Iran. He had a history of left hemiplegia at the age of 17 years. MRI and CT scans revealed a brain abscess (Fig 2, B). Candida species grew from the surgical biopsy specimen obtained. The patient was subsequently discharged on oral fluconazole treatment. At the age of 20 years, he had fever and right ptosis. Cerebral CT scanning showed soft tissue opacities and calcifications in the sphenoids, ethmoids, and left maxillary and frontal sinuses, with 2 regions of bone erosion in the median wall of the right orbit adjacent to the right orbital apex. There was a 6 × 4-mm region of high-density soft tissue that extended to the frontal sinus. Bone attenuation was observed at the superolateral left orbital rim and the posterolateral wall of the left sphenoid tissue, suggesting fungal sinonasal infection with orbital and intracranial extension. The patient underwent sinus surgery, and the cultures obtained from the surgical specimen were positive for Candida species. The patient was discharged on oral itraconazole treatment. At the age of 22 years, he had bloody diarrhea complicated with anemia. Colonoscopy revealed extensive linear ulcers throughout the colon, a low level of vascular development, low levels of haustration, and many sessile and ulcerative polyps, with a larger number of small polyps and ulcerations in the terminal ileum (Fig 2, C). Two gut biopsies were carried out on the terminal ileum. Histopathologic analysis of the biopsy specimens revealed a diffuse inflammatory lesion characterized by infiltration of macrophages and eosinophils associated with the presence of round yeasts, which was identified by using periodic acid–Schiff and Gomori-Grocott staining and measured up to 4 μm in diameter without pseudohyphae (Fig 3, E and F). Anti–Candida species immunohistochemistry results were positive (Fig 3, G). Collectively, morphologic and immunohistochemical data suggested invasive colonic Candida species infection. Candida glabrata grew from a cultured biopsy specimen. Immunologic explorations were carried out as follows: neutrophils; CD4+ T, CD8+ T, B, and NK lymphocyte blood counts; DHR test results; and IgG, IgA, and IgM plasma levels were normal. IgE levels were high at 1.7 mg/mL. Eosinophil counts were also high at up to 1500/mm3. Neither the patient’s parents nor his siblings had any severe infectious disease.

Kindred D

P4 is a 37-year-old woman from a consanguineous Moroccan kindred (P4, kindred D, D.II.1; Fig 1, A). Three years before presentation, she had thrush, with recurrent episodes occurring about 3 times per year. At the age of 37 years, she suddenly had severe headache, vomiting, and right hemiparesis. Fundus examination provided evidence of papillary edema. Cerebral MRI showed a 30 × 40-mm left temporoparietal lesion (Fig 2, D) with several small peripheral nodules displaying peripheral enhancement after contrast medium injection and a large perilesional edema with a mass effect on the left ventricle. C albicans grew from a CNS biopsy specimen. Histologic examination revealed the presence of a lymphoplasmacytic infiltrate around the vessels, and Gomori-Grocott staining revealed the presence of pseudohyphae. Abdominal and thoracic CT and transesophageal echocardiography provided no evidence for the dissemination of C albicans infection. Immunologic explorations were carried out as follows: neutrophils; CD4+ T,
CD8+ T, B, and NK lymphocyte counts; DHR test results; and IgG, IgA, and IgM plasma levels were normal. Serologic test results for HIV were negative, and the patient did not have diabetes mellitus. The infection was cured after treatment for 15 days with a combination of liposomal amphotericin B and 5-fluorocytosine, followed by fluconazole treatment, which is still underway after 10 months. The patient’s father had a history of recurrent skin dermatophytosis. Neither her mother nor her siblings had any severe infectious disease.

Kindred E

A 34-year-old man (P5, kindred E, E.II.3; Fig 1, A) from a consanguineous Pakistani kindred had been living in the United Kingdom for the last 8 years. While he was living in Pakistan, he was given a diagnosis of cervical lymphadenitis ascribed to tuberculosis, although not microbiologically proved, and had a full course of antituberculous chemotherapy. Several months after completing his antituberculous treatment, he noticed bloody diarrhea, lost weight, and became anemic. On initial assessment at the age of 26 years, the patient was found to have oral and esophageal candidiasis and florid right-sided colitis with multiple pseudopolyps. Biopsy specimens showed multiple fungal organisms. Chest radiography and abdominal CT scans showed no sign suggestive of tuberculosis, intra-abdominal lymphadenopathy, or hepatosplenomegaly, and colonic biopsy results were consistently negative for tuberculosis. Initially, a diagnosis of histoplasmosis was retained. Therefore the patient was treated with fluconazole for 2 years, followed by itraconazole. His symptoms improved. However, colonoscopy showed persistent infection, and symptoms recurred when the treatment was stopped. The patient was HIV seronegative, with normal lymphocyte subsets. At the age of 29 years, he was found to be anemic, with iron deficiency. Immunoglobulin levels (IgG, IgA,
and IgM) were within normal ranges, but IgE levels increased at 4979 IU/mL. After a diagnosis of possible histoplasmosis, the patient was treated with a short induction course of liposomal amphotericin B, followed by posaconazole. His symptoms improved, but after a colonoscopy performed in September 2009, obvious histologic signs of ongoing infection were observed on colonic samples, and *C. albicans*, resistant to triazoles but susceptible to amphotericin B and caspofungin, grew from cultured samples. The tissue samples had positive test results for *Candida* species by using PCR. Results of histoplasmosis complement fixation, precipitin, and immunodiffusion antibody tests all remained negative, and results of the serum Histoplasma Antigen EIA performed in Indianapolis by the laboratory of Dr Joe Wheat were also negative, as was the test for cryptococcal serum antigen. This patient had no history of other recurrent infections, and there was no family history of susceptibility to any particular infection.

**Identification of homozygous CARD9 mutations**

We investigated the above 5 patients from unrelated consanguineous kindreds. They displayed CNS candidiasis and *Candida* species–induced colitis but no detectable immunodeficiency in the first round of routine immunologic tests (HIV serology, neutrophil counts, and T-cell, B-cell, and NK cell lymphocyte counts). We sequenced CARD9 exons and found homozygous CARD9 mutations in all 5 patients. P1 and P2 had a homozygous CARD9 missense mutation, c.208C>T in exon 3, replacing the arginine residue in position 70 with a tryptophan (R70W) within the CARD domain of the CARD9 protein (Fig 1, B). P1 has 4 healthy children, all of whom were heterozygous for the mutation. The parents of P2 are heterozygous for the mutation. P3 had a homozygous CARD9 missense mutation, c.104G>A in exon 2, replacing the arginine residue in position 35 with a glutamine (R35Q), within the CARD domain of the CARD9 protein (Fig 1, B). His healthy brother is heterozygous for the mutation (wild-type [WT]/R35Q). P4 and P5 had homozygous c.865C>T and c.883C>T mutations in exon 6, resulting in premature termination codons at positions 289 (Q289*) and 295 (Q295*), respectively, in the region encoding the coiled-coil domain of CARD9 (Fig 1, B). Genotypes were not available for the other members of the family.

FIG 2. Clinical, pathologic, and radiologic features of patients. A and B, Brain abscess of P1 (Fig 2, A) and brain CT scan of P3 (Fig 2, B). C, a–d, Colonoscopy results for P3. D, Brain MRI of P4.
The segregation of the 4 mutations in the 5 kindreds was consistent with autosomal recessive CARD9 deficiency with complete clinical penetrance. None of the mutations reported here was found in any of the various public databases checked (HGMD, Ensembl, and 1000 Genomes or our in-house whole-exome sequencing database [>2000 exomes]). We also sequenced 1052 control subjects from the Human Genome Diversity Cell Line Panel and 30, 90, and 83 Iranian, Turkish, and Algerian healthy control subjects, respectively, in whom we found none of the 4 variants described here. These data ruled out the possibility of R35Q, R70W, Q289*, or Q295*, being irrelevant polymorphisms. The 2 missense mutations were predicted to be probably damaging by using PolyPhen 2 (with the highest possible score of 1) and damaging by using SIFT (scores of 0 for R35Q and 0.02 for R70W). In addition, the Q289* and the Q295* mutations have already been reported and shown to be rare and deleterious CARD9 alleles. Collectively, these data strongly suggest that all 5 patients tested are homozygous for rare and deleterious mutant CARD9 alleles.

**Effect of CARD9 mutation on protein level and function**

We investigated the consequences of these mutations for protein levels by carrying out an immunoblot analysis for CARD9 on whole-cell extracts from HEK-293T cells transfected with a pcDNA3.1 V5 (C terminal–tagged) plasmid with no insert or carrying the WT (pcDNA3.1 V5 CARD9 WT) or one of the 4 mutant alleles of CARD9 (pcDNA3.1 V5 CARD9 R35Q, pcDNA3.1 V5 CARD9 R70W, pcDNA3.1 V5 CARD9 Q289*, or pcDNA3.1 V5 CARD9 Q295*). In cells transfected with the CARD9 R35Q allele, CARD9 protein levels and molecular weights were similar to those in cells transfected with the WT allele, whereas cells transfected with the CARD9 R70W allele had reproducibly lower levels of a protein of normal size, and cells transfected with the CARD9 Q289* and Q295* alleles had normal levels of a protein truncated by about 25 kDa, as previously reported (Fig 4, A). Flow cytometric analysis of CARD9 protein levels carried out only on monocyte-derived dendritic cells (MDDCs) from P1 (R70W/R70W) showed this...
protein to be slightly less abundant than in MDDCs from a healthy control subject (63% of MDDCs were CARD9 positive in P1, whereas 87% of MDDCs were CARD9 positive in the control subject, as tested in parallel; Fig 4, B). Collectively, these data are consistent with the previously characterized pathogenic mutations of CARD9.15,49-52,62; nonsense mutations prevented the production of full-length CARD9, whereas missense mutations did not necessarily do so.

We then evaluated the functional consequences of the mutations by studying the production of TNF-α and IL-6 using whole blood cells after 24 or 48 hours of stimulation with zymosan, heat-killed S.cerevisiae, C.albicans, E.deformitidis, S.aureus, VSV, BCG, LPS, and PMA plus ionomycin for P1, P2, and P6; P2’s father; and 7 control subjects. Fig 4, D, TNF-α production after 24 hours of stimulation of MDDCs with curdlan, zymosan, S.cerevisiae, C.albicans, E.deformitidis, S.aureus, LPS, and VSV for P1, P2, and P6; P2’s parents; and 6 healthy control subjects tested in parallel. NS, Not stimulated.

**Fig 4.** Effect of CARD9 mutations on CARD9 protein levels and function. **A** and **B**, Effect of CARD9 mutations on CARD9 protein levels. Fig 4, A, Immunoblot analysis of CARD9 in whole-cell extracts of HEK-293T cells cotransfected with pcDNA3.1 V5 (C-terminally tagged), either empty or carrying the WT or mutant (R35Q, R70W, Q289*, and Q295*) CARD9 alleles, together with a cyan fluorescent protein plasmid as a transfection control. Antibodies against CARD9, V5, cyan fluorescent protein, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; as a loading control) were used. Fig 4, B, Flow cytometric analysis of CARD9 expression in MDDCs from patient P1 and a control subject. **C** and **D**, Effect of CARD9 mutations on CARD9 protein function. Fig 4, C, TNF-α (left) and IL-6 (right) production by whole blood cells after 24 hours of stimulation with zymosan, heat-killed S.cerevisiae, C.albicans, E.deformitidis, S.aureus, VSV, BCG, LPS, and PMA plus ionomycin for P1, P2, and P6; P2’s father; and 7 control subjects. Fig 4, D, TNF-α production after 24 hours of stimulation of MDDCs with curdlan, zymosan, S.cerevisiae, C.albicans, E.deformitidis, S.aureus, LPS, and VSV for P1, P2, and P6; P2’s parents; and 6 healthy control subjects tested in parallel. NS, Not stimulated.
homozygous for the CARD9 R70W allele), and, more strikingly, P5 (Q295*/Q295*) had impaired TNF-α production after 24 hours of stimulation with *S cerevisiae, C albicans*, and *E dermatitidis* in comparison with the 7 healthy control subjects tested in parallel or P2’s father (R70W/WT; Fig 4, C, left). In addition, P1 and P2 had reduced levels of IL-6 production after 24 hours of stimulation with *S cerevisiae* but subnormal to normal levels of IL-6 production in response to *C albicans* and *E dermatitidis*. By contrast, P5 showed a marked impairment of IL-6 production in response to *S cerevisiae, C albicans*, and *E dermatitidis* in comparison with the 7 healthy control subjects tested in parallel or P2’s father (R70W/WT; Fig 4, C, right). Production of both TNF-α and IL-6 in response to zymosan, *S aureus*, VSV, BCG, LPS, and PMA plus ionomycin was comparable in the 3 patients tested (P1, P2, and P5) and in healthy control subjects. Moreover, IL-6 production tested after 48 hours (see Fig E1 in this article’s Online Repository at www.jacionline.org) on whole blood from P1 only was strongly impaired after stimulation with heat-killed *S cerevisiae, C albicans*, and, to a lesser extent, zymosan but was normal after stimulation with LPS, *S aureus*, VSV, BCG, or PMA/ionomycin. Unfortunately, P3 (R35Q/R35Q) and P4 (Q289*/Q289*) could not be tested.

We then assessed TNF-α production by MDDCs from P1, P2, and P5; P2’s father and mother; and 6 healthy control subjects stimulated for 24 hours with curdlan and the same agonists as used in the whole blood assay (Fig 4, D). The patients displayed a strong impairment of TNF-α production in response to stimulation with all fungal ligands used (curdlan, heat-killed *S cerevisiae, C albicans, E dermatitidis*, and, to a lesser extent, zymosan), whereas it was within the range of healthy control subjects after stimulation with *S aureus*, LPS, and VSV.

In addition, 293 HEK cells transfected with the R35Q and the R70W CARD9 alleles displayed impaired nuclear factor κB (NF-κB) transcriptional activity, as shown by NF-κB–luciferase reporter assays, comparing these cells with cells transfected with the WT CARD9 allele at the basal level and after stimulation with curdlan, *S cerevisiae*, or *C albicans* (Fig 5). Surprisingly, under these conditions, the 2 nonsense mutations (Q289* and Q295*) were associated with constitutive NF-κB–luciferase activity. These results can be accounted for by the production in this overexpression system of normal amounts of truncated CARD9-truncated proteins able to interact through their intact coiled-coil domain with the downstream partner BCL10.

Finally, we evaluated the proportion of ex vivo IL-17A–producing T cells using flow cytometry because low proportions of IL-17 T cells have been reported in some, 15,49,50,62 but not all, 51,52 CARD9-deficient patients. Under these conditions, no differences were observed between the patients tested (P1, P2, and P5), P2’s parents (CARD9 R70W/WT), and the 10 healthy control subjects tested in parallel (Fig 6, A). Moreover, IL-17A production by whole blood cells after 24 hours of stimulation with PMA and ionomycin, as measured by using ELISA, was similar for the 3 patients tested, P2’s father, and 7 healthy control subjects tested in parallel (Fig 6, B). Therefore we conclude that the homozygous R35Q and R70W mutations led to the production of normal or small amounts of loss-of-function CARD9 proteins, whereas the Q289* and Q295* mutations led to the absence of a normal CARD9 protein. This resulted in the impairment of proinflammatory cytokine production by CARD9-deficient whole blood cells and particularly by MDDCs specifically in response to various fungal ligands, as well as an impairment of NF-κB transcriptional activity in transfected HEK cells, whereas IL-17 T-cell production was normal.

**DISCUSSION**

Four of the 5 patients described here displayed *Candida* species infections of the CNS, one of which was associated with *Candida* species–induced colitis, whereas the fifth patient had *Candida* species–induced colitis solely. Our findings demonstrate that CARD9 deficiency is a genetic cause of rare forms of invasive candidiasis and that CARD9 plays an essential role against *Candida* species infection in the brain and colon. This is concordant with the recently reported role of Card9 in mouse antifungal immunity of the gut. 53 Indeed, Card9-deficient mice were shown to display particularly strong fungal colonization of the digestive tract with fewer than normal numbers of colonic IL-17–producing T cells and innate lymphoid cells, strongly suggesting a critical role for CARD9 in gut IL-17 immune responses and in fungal control. The 5 unrelated patients described here are homozygous for 4 different *CARD9* mutations, 2 of which have never before been described. In addition to the Q295* homozygous nonsense mutation previously reported in a large multiplex consanguineous Iranian family, 49 a homozygous missense (R101C) mutation found in a consanguineous Moroccan family, 51 a homozygous nonsense (Q289*) mutation found in 5 Algerian and 2 Tunisian kindreds 15 and an Egyptian patient, 54 compound heterozygous missense mutations (G72S and R373P) reported in a child of Korean origin, 55 a homozygous missense mutation (Y91H) reported in a French-Canadian patient, 56 a homozygous missense mutation (R181W) found in a patient of Angolan origin, 57 a homozygous in-frame deletion (E323del) in an Iranian patient, 58 compound heterozygous nonsense mutations (L64fs*59 and Q158*) reported in a Chinese patient, 62 and a homozygous frameshift mutation (D274fs*40) reported in 3 unrelated Chinese patients. 62 We identified 2 new homozygous *CARD9* missense mutations, R35Q and R70W, in an Iranian kindred and 2 unrelated Turkish kindreds. We also report a patient from Morocco with the previously described Q289* *CARD9* allele 15,64 and a patient from Pakistan with the previously described Q295* mutation. 49 In all kindreds studied, none of the heterozygous subjects were symptomatic, whereas all homozygotes were symptomatic, which is consistent with an autosomal recessive mode of inheritance with complete clinical penetrance (albeit often late in adulthood, up to 39 years). In total, 38 patients from 23 families in 9 countries have been identified with CARD9 deficiency caused by 13 different alleles, mostly with invasive fungal infections: deep dermatophytosis in 17 patients, 15 superficial or extensive dermatophytosis in 4 patients, 49,64 CMC in 15 patients (present report), 15,56 CNS infection with *Candida* species in 9 patients (present report), 15,51 *Candida* species–induced colitis in 2 patients (present report), *Exophiala* species–induced CNS and liver disease in 1 patient, lung and bone disease in another patient, 52 and *Phialophora verrucosa*–induced subcutaneous disease in 4 patients. 62 These mutations are all loss of function, with a partial or complete defect, as suggested based on whole blood TNF-α or IL-6 production on stimulation, with fungal agonists being more impaired in P5 (Q295*) than in P1 or P2 (R70W). However, this difference was no longer observed when using MDDCs, suggesting some cell-specific compensatory mechanisms. None of these patients had other fungal, parasitic, bacterial, or viral
infections, whereas CARD9-deficient mice are susceptible not only to Candida species but also to Mycobacterium tuberculosis and Listeria monocytogenes.65-67 Intriguingly, individual CARD9-deficient patients seem to be prone to a single type of invasive fungal disease. 68 Patients with invasive dermatophytic disease do not have invasive candidiasis and vice versa. Moreover, none of these patients were reported to have Aspergillus species infections, which is concordant with a CARD9-independent neutrophil killing of Aspergillus species.50

In any case our report shows that Candida species infection of the CNS is a major clinical phenotype in CARD9-deficient patients. Patients with Candida species infections of the CNS should be explored for possible CARD9 deficiency, even if they are previously healthy adults. The same principle applies to rare patients with unexplained histologically documented colitis caused by Candida species. Patients with Candida species-related meningoencephalitis, colitis, or both should be investigated for possible CARD9 deficiency, even if they are previously healthy adults. The same principle applies to rare patients with unexplained histologically documented colitis caused by Candida species. Patients with Candida species-related meningoencephalitis, colitis, or both should be investigated for possible CARD9 deficiency, even if they are previously healthy adults. The same principle applies to rare patients with unexplained histologically documented colitis caused by Candida species.

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Clinical implications: CARD9 deficiency might predispose to meningoencephalitis, colitis, or both caused by Candida species. Therefore CARD9 deficiency should be investigated in patients with Candida species–related meningoencephalitis, colitis, or both without any known risk factors for fungal infection.

REFERENCES


METHODS

Patients

We recruited 5 patients with a history of proven meningococcalitis, colitis, or both caused by Candida species (Table 1) and no known underlying associated condition. Diagnosis was based on the revised European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group Consensus Group (EORTC/MSG) criteria. This study was conducted in accordance with the Helsinki Declaration. All patients and their relatives provided written informed consent for participation in the study.

Molecular genetics

Genomic DNA was isolated from whole blood cells. CARD9 was amplified with specific primers (PCR amplification conditions and primer sequences are available on request). PCR products were sequenced with the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif) and analyzed on an ABI Prism 3700 apparatus (Applied Biosystems). E3

Control subjects

We sequenced exons 2, 3, and 6 of the CARD9 gene for all 105 healthy unrelated control subjects from the Human Genome Diversity Cell Line Panel originating from 52 different ethnic groups initially sampled for population genetics studies, and 30 subjects from Iran, 90 from Turkey, and a total of 83 from Algeria.

Whole blood cell and MDDC stimulation

Whole blood was diluted 1:2 and incubated for 24 or 48 hours with RPMI medium alone, zymosan (5 μg/mL), heat-killed Calicibari (10^7 particles/mL), heat-killed S cerevisiae (10^6 particles/mL), heat-killed E dermatitidis (10^5 particles/mL), LPS (100 ng/mL), heat-killed S aureus (10^3 particles/mL), PMA plus ionomycin (0.2 μg/mL and 2 × 10^{-4} μg/mL, respectively), VSV (10^3/mL) or BCG for P1, P2, and P5, as well as P2’s father and 7 healthy control subjects. Production of IL-6 or TNF-α was assessed by determining the levels of these cytokines in supernatants by means of ELISA, according to the kit manufacturer’s instructions (Sanquin, Amsterdam, The Netherlands). Human PBMCs were isolated from whole blood by using Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia Biotech, Sweden). MDDCs were derived from PBMCs by positive selection of CD14+ cells with CD14 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) by means of differentiation in the presence of GM-CSF (50 ng/mL) and IL-13 (20 ng/mL). After 6 days, 30,000 cells per well were plated in 96-well plates and stimulated for 24 hours with curdlan (25 μg/mL), zymosan (25 μg/mL), heat-killed S cerevisiae (10^3 or 10^6 particles/mL), heat-killed C albicans (10^1 or 10^2 particles/mL), heat-killed E dermatitidis (10^1 particles/mL), heat-killed S aureus (2 × 10^3 particles/mL), and LPS (100 ng/mL) for P1, P2, and P5; P2’s father and mother; and 6 healthy control subjects tested in parallel. TNF-α production was evaluated by means of ELISA, according to the manufacturer’s instructions.

Flow cytometry

Antibody against human CARD9 (5281; Epitomics, Burlingame, Calif), an isotype control antibody, and a secondary goat anti-rabbit–Alexa Fluor 488 antibody (3064-1, Epitomics) were used in accordance with the manufacturers’ protocols.

Cell transfections

Full-length WT CARD9 cDNA was inserted into a pcDNA3.1 expression vector, such that the protein was produced with a V5 tag at the C-terminus. The c.G104A (p.R35Q), c.C208T (p.R70W), c.C865T (p.Q289*), and c.C883T (p.Q295*) mutations were introduced into the cDNA with the QuikChange II XL site-directed mutagenesis kit from Stratagene (La Jolla, Calif, 200522-5), according to the manufacturer’s instructions. Plasmids containing the WT or mutated CARD9 sequences were then amplified and purified with the QIAprep Spin Miniprep Kit from Qiagen (Hilden, Germany; 27106). The resulting plasmids were used to transfect HEK-293T cells in 6 cm-diameter plates, with a calcium phosphate transfection kit (278001; Invitrogen, Carlsbad, Calif) and a cyan fluorescent protein plasmid, according to the manufacturer’s instructions.

Western blotting

Total extracts of HEK-293T cells were prepared 48 hours after transfection. Proteins were separated by means of electrophoresis and transferred to a membrane, which was then probed with anti-V5 (Invitrogen 46-0708), anti–CARD9 H-90 (Sc-99054), anti–cytokeratin fluorescent protein, or anti–glyceraldehyde-3-phosphate dehydrogenase (Sc-25778) antibodies.

NF-kB–luciferase reporter assay

We plated 10^5 HEK-293 cells in Dulbecco modified Eagle medium supplemented with 10% FBS in 96-well plates. These cells were incubated for 6 hours and then transfected in the presence of Lipofectamine LTX, according to the manufacturer’s protocol. The cells were transfected with 6 ng of DECTIN1-, SYK-, and BCL10-expressing pcDNA3 constructs with or without CARD9 WT or CARD9 R35Q, R70W, Q289*, or Q295* constructs and with 100 ng of NF-kB–firefly luciferase vector and 40 ng of pRL-SV40 reporter vector used as an internal control and expressing the Renilla gene under control of the SV40 promotor. Cells were then left unstimulated or stimulated with 25 μg/mL curdlan or 10^3 particles/mL S cerevisiae or C albicans. After 24 hours, cells were lysed and both firefly and Renilla luciferase activities were determined with the Dual-Glo Luciferase Assay System (Promega, Madison, Wis). Results are expressed as means ± SEMs of the ratio of firefly and Renilla luciferase activities adjusted to 1. We used the Student t test to determine the significance of differences. Statistics were calculated with GraphPad Prism version 5 software (GraphPad Software, La Jolla, Calif).

IL-17 production

We evaluated IL-17A production using whole blood ex vivo after 24 hours of stimulation with PMA/ionomycin using ELISA, according to the manufacturer’s recommendations for P1, P2, and P5; P2’s father; and 7 healthy control subjects. We also determined the percentages of CD3+IL-17A+ cells using flow cytometry after 12 hours of stimulation with PMA/ionomycin, as previously described, for P1, P2, and P5, as well as P2’s father and 10 healthy control subjects.

REFERENCES

FIG E1. IL-6 production by whole blood cells after 48 hours of stimulation with zymosan, heat-killed *C. albicans*, *S. cerevisiae*, LPS, heat-killed *S. aureus*, VSV, BCG, and PMA plus ionomycin. NS, Not stimulated.