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A Homozygous *CARD9* Mutation in a Family with Susceptibility to Fungal Infections

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ABSTRACT

BACKGROUND

Chronic mucocutaneous candidiasis may be manifested as a primary immunodeficiency characterized by persistent or recurrent infections of the mucosa or the skin with candida species. Most cases are sporadic, but both autosomal dominant inheritance and autosomal recessive inheritance have been described.

METHODS

We performed genetic studies in 36 members of a large, consanguineous five-generation family, in which 4 members had recurrent fungal infections and an additional 3 members died during adolescence, 2 after invasive infection of the brain with candida species. All 36 family members were enrolled in the study, and 22 had blood samples taken for DNA analysis. Homozygosity mapping was used to locate the mutated gene. In the 4 affected family members (patients) and the 18 unaffected members we sequenced *CARD9*, the gene encoding the caspase recruitment domain-containing protein 9, carried out T-cell phenotyping, and performed functional studies, with the use of either leukocytes from the patients or a reconstituted murine model of the genetic defect.

RESULTS

We found linkage (lod score, 3.6) to a genomic interval on chromosome 9q, including *CARD9*. All four patients had a homozygous point mutation in *CARD9*, resulting in a premature termination codon (Q295X). Healthy family members had wild-type expression of the *CARD9* protein; the four patients lacked wild-type expression, which was associated with low numbers of Th17 cells (helper T cells producing interleukin-17). Functional studies based on genetic reconstitution of myeloid cells from *Card9*^{-/-} mice showed that the Q295X mutation impairs innate signaling from the antifungal pattern-recognition receptor dectin-1.

CONCLUSIONS

An autosomal recessive form of susceptibility to chronic mucocutaneous candidiasis is associated with homozygous mutations in *CARD9*.

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CHRONIC MUCOCUTANEOUS CANDIDIASIS is characterized by impaired clearance of fungal infections and results in colonization and infections of the mucosa or skin, predominantly with *Candida albicans*.^{1,2} A variety of clinical conditions, such as infection with the human immunodeficiency virus or the use of corticosteroids, favor the development of chronic mucocutaneous candidiasis, but the disease may also be a primary immunodeficiency arising from unknown genetic defects.^{1,3} In chronic mucocutaneous candidiasis, the most common infections are due to *C. albicans*; however, patients may also have an increased susceptibility to dermatophytes.^{1,3} Severe complications rarely develop in patients with chronic mucocutaneous candidiasis, although reports on invasive infections with candida species — *Cryptococcus neoformans* or *Histoplasma capsulatum* — have been published.⁴⁻⁶

Research conducted in the eight decades since the first report on primary chronic mucocutaneous candidiasis appeared⁷ has shown that it is a heterogeneous syndrome that may be accompanied by endocrine and inflammatory disorders, including hypothyroidism and adrenocortical failure.² Most cases of chronic mucocutaneous candidiasis are sporadic, but multiplex families with dominant⁸⁻¹² and recessive^{13,14} inheritance have been described.

Recurrent and severe candidiasis can have a defining role in primary immunodeficiencies. The autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) syndrome is caused by biallelic mutations in *AIRE*, the autoimmune regulator gene. Heterozygous mutations in the signal transducer and activator of transcription 3 gene (*STAT3*) cause the hyper-IgE syndrome, another multisystem disorder in which candidiasis is a common clinical feature.¹⁷⁻²⁰ Genetic linkage of an autosomal dominant candidiasis–thyroiditis syndrome to chromosome 2 has been reported,¹² and candidiasis associated with a low expression of intercellular adhesion molecule 1 (*ICAM-1*) has been traced to chromosome 11,²¹ but in both cases the causative genes remain unknown.

We undertook genetic studies of a large, consanguineous Iranian family with multiple cases of chronic mucocutaneous candidiasis to determine whether a mutated gene was associated with this form of nonsyndromic candidiasis. Recent work

has shown that innate antifungal immunity in mice is controlled by a signaling pathway that does not involve toll-like receptors. Mice lacking either the C-type lectin receptor dectin-1 (encoded by the *Clec7a* gene) or the intracellular adapter molecule *Card9*, which is essential for dectin-1 signaling, have impaired antifungal immunity.^{22,23}

We identified a homozygous mutation in *CARD9* that results in a loss-of-function mutation due to a premature stop codon in the coding sequence. Experiments in the murine *Card9*^{-/-} model showed that only wild-type *CARD9* — not the mutated human *CARD9* gene found in patients — could restore cytokine production in response to the triggering of dectin-1, a pattern-recognition receptor for fungal cell-wall antigens.

METHODS

STUDY PARTICIPANTS

We enrolled 36 members of a large, consanguineous Iranian family in the study. Blood samples were obtained for DNA analysis, and the participants were classified as likely to be affected or likely to be unaffected according to the results of physical examination and microbiologic culture. Laboratory personnel were unaware of the classification of the samples. The participants provided written informed consent on forms approved by local ethics committees. DNA samples from 50 healthy Iranian donors and 180 healthy white donors of other nationalities were used as controls.

GENOTYPING AND ANALYSIS

DNA samples from five family members deemed likely to be affected and eight deemed likely to be unaffected were genotyped with use of the Affymetrix 250k *NspI* single-nucleotide-polymorphism (SNP) mapping array, as described previously.²⁴ Genotypes of the SNP arrays were assigned with the use of the Bayesian Robust Linear Modeling and Mahalanobis (BRLMM) distance method, which was implemented as described in the Genotyping Console software and introduced as an improvement over the RLMM method.²⁵

To further evaluate one region on chromosome 9 that was suggestive of linkage, four microsatellite markers were genotyped on 13 available samples, as described previously.²⁶ Single-marker lod scores were computed with Fastlink²⁷⁻²⁹; multipoint lod scores were computed with Su-

perlink.^{30,31} (For details on the use of the BRLMM method, Fastlink, and Superlink, see the Supplementary Appendix, available with the full text of this article at NEJM.org.)

MOLECULAR GENETICS AND T-CELL PHENOTYPING

DNA from the study participants was isolated, and coding regions of *CARD9* (Ensembl number, ENSG00000187796) were amplified and sequenced. Total RNA was isolated and transcribed into complementary DNA (cDNA). SYBR green-based real-time polymerase-chain-reaction quantification of *CARD9* was performed with the use of standard curves. Screening for the Q295X mutation with the use of restriction-enzyme digestion was carried out in heterozygous and homozygous healthy family members as well as in healthy controls.

T-cell phenotyping, regulatory T-cell staining, and detection of Th17 cells (helper T cells producing interleukin-17) were performed in accordance with protocols published previously.³²⁻³⁶ Extracts of peripheral-blood mononuclear cells from patients with a deficiency of the *CARD9* protein were stained with a polyclonal goat anti-*CARD9* antibody. (Details of these procedures are available in the Supplementary Appendix.)

RETROVIRAL TRANSDUCTION OF HUMAN *CARD9* VARIANTS

CARD9 cDNA was generated from human peripheral-blood mononuclear cells and cloned into a retroviral expression vector (based on murine stem-cell virus) expressing green fluorescent protein.³⁷ The Q295X mutation was introduced with the use of site-directed mutagenesis. Retroviruses were generated by transfecting the Phoenix ecotropic-packaging cell line and were used to infect bone marrow cells as described previously.³⁷ Bone marrow cells were differentiated into macrophages and stimulated with either curdlan, a selective dectin-1 agonist, or ultrapure lipopolysaccharide. Concentrations of tumor necrosis factor α (TNF- α) in the supernatants were analyzed with the use of an enzyme-linked immunosorbent assay.^{22,23,38}

RESULTS

PATIENTS' MEDICAL HISTORY

The pedigree (Fig. 1A) for the consanguineous Iranian family shows that multiple members were

affected by chronic mucocutaneous candidiasis through a presumably autosomal recessive mode of inheritance. Recurrent fungal infections were diagnosed clinically in eight family members, three of whom died in early adolescence — two with proven and one with presumed invasive candida infection of the brain. None of the eight infected patients had unusual bacterial or viral infections, suggesting that the host defense against these pathogens was normal.

The index patient (Patient 2B2) was a 19-year-old man who had had oral candidiasis (thrush) since the age of 3 years. Candida infection was confirmed with the use of microbiologic testing, and prophylaxis with ketoconazole was ongoing. He was otherwise healthy.

Patient 2B1, a sibling of Patient 2B2, had had intermittent thrush since early childhood. Seizures developed suddenly, with loss of consciousness, when he was 18 years old. Hydrocephalus developed, and he died of candida meningitis at the age of 19 years.

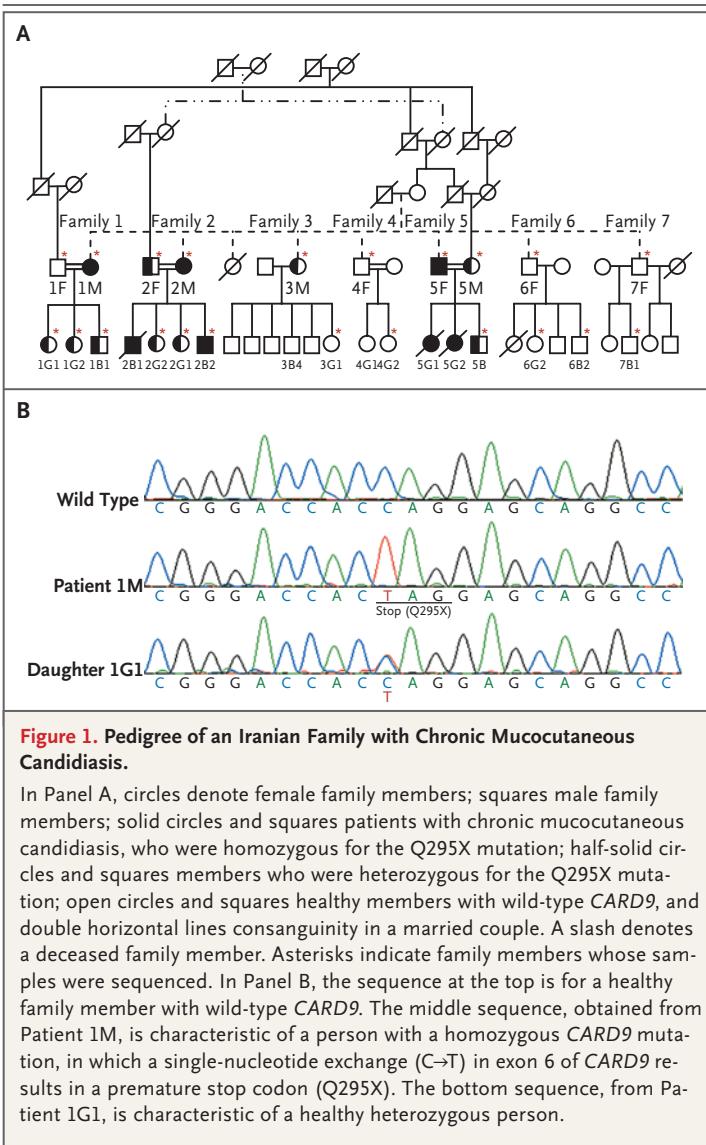
Patient 2M, the mother of Patients 2B1 and 2B2, was 50 years old at the time of our study and had had vaginal candidiasis since the age of 42 years. In addition to being infected with *C. albicans*, she had a 5-year history of dermatophytosis of her hands and neck. She also had intermittent aphthous lesions, type 2 diabetes mellitus, and nephrolithiasis.

Patient 1M, the sister of Patient 2M, had had oral and vaginal candidiasis since early childhood. She also had tinea corporis on her chest and neck. She was otherwise healthy.

Patient 5F, the affected brother of Patients 1M and 2M, had had dermatophytosis since childhood, with little improvement in response to local treatment. Both of his daughters were given a post-mortem diagnosis of invasive candida infection.

The older daughter (Patient 5G1) had a ventricular septal defect during infancy and had a geographic tongue, which is suggestive of chronic candidiasis. A unilateral paresthesia developed when she was 13 years old, and she died from what was vaguely defined as a brain tumor, with severe skull destruction, at the age of 15 years.

Her sister (Patient 5G2) had recurrent, severe, refractory thrush starting in early childhood. At 15 years of age, a severe headache and fevers developed, followed by diplopia. A brain tumor was suspected, but candida meningoencephalitis was



identified during surgery. She died 6 months after the onset of symptoms.

None of the patients with invasive fungal infections had a condition or took any medication that predisposed them to infection. The three deceased patients could not be enrolled in the study because of the lack of available tissue samples.

Patient 1B1, a child of Patient 1M and a cousin of the index patient (Patient 2B2), had had one mild episode of candida infection in adulthood. In our genetic analysis, we considered Patient 1B1 to be affected. We found that the episode of infection in Patient 1B1 was the result of a phenocopy, which is consistent with the fact that it was clinically much milder than the infections in all the other

affected family members (see the Supplementary Appendix for the definition of phenocopy).

CELL COUNTS AND T-CELL PHENOTYPING

In Patients 1M, 2M, and 5F, complete blood counts were within the normal range, as were total counts of CD3+ T cells, CD4+ T cells, CD8+ T cells, memory T cells, follicular helper T cells, effector memory T cells, regulatory T cells, B cells, and natural killer cells (for results see Table S1 in the Supplementary Appendix). Basal levels of serum immunoglobulin were also normal. In Patient 2B2, the results of a delayed-type hypersensitivity skin test were negative for tuberculin but positive for candida.

AIRE was shown to be of wild-type sequence in Patient 2B2. The absence of a specific immunologic disorder led us to use a positional cloning approach to identify the patients' underlying genetic defect.

GENETIC LINKAGE ANALYSIS

Analysis of the SNP genotypes showed a region of perfect segregation on chromosome 9 (137.5 to 138.8 Mbp in human genome build 36), provided that Patient 1B1's episode of candida infection was the result of a phenocopy. This finding was confirmed by genotyping four microsatellite markers, yielding a peak multipoint lod score of 3.6 (see the Supplementary Appendix).

There were 121 genes in the maximal linkage interval defined by the microsatellite markers D9S2157 (135.0 Mbp) and D9S1838 (139.8 Mbp). Among these 121 genes, 41 are located in the perfectly segregating 1.3-Mbp subinterval suggested by the SNP data. After exploring the literature, we identified *CARD9* — which is among the 41 ideally located genes (see Table S2 in the Supplementary Appendix) — as a functional candidate because *Card9*^{-/-} mice are susceptible to fungal infections (for details, see the Supplementary Appendix).^{22,39}

HOMOZYGOUS MUTATIONS IN *CARD9*

We sequenced *CARD9* in the four affected patients and 18 other relatives and identified in all affected persons a single homozygous point mutation from C to T in exon 6 at codon 295, resulting in a premature termination codon (Q295X). Patient 1B1 and his healthy relatives had either a heterozygous Q295X mutation or wild-type alleles only (Fig. 1B).

To assess the frequency of this previously un-

known genetic abnormality in *CARD9* and to exclude the possibility of a genetic variation, we examined the affected site in 50 unrelated healthy Iranians and 180 unrelated healthy white subjects by means of a sequencing assay or a restriction-enzyme assay. None of the 230 controls had the Q295X mutation in *CARD9*.

CARD9 mRNA LEVELS AND PROTEIN EXPRESSION

Among *CARD9* wild-type cells, average levels of *CARD9* mRNA were highest in monocytes, followed by granulocytes, B cells and T cells, and the colon-cell line HT-29. The peripheral-blood mononuclear cells from our patients still had substantial levels of mutated *CARD9* mRNA, thereby escaping nonsense-mediated RNA decay (see the Supplementary Appendix).

To examine the effect of the *CARD9* Q295X mutation at the protein level, we assessed the expression of *CARD9* in peripheral-blood mononuclear cells from the patients, using Western blotting. As compared with unrelated healthy controls and homozygous or heterozygous healthy family members, patients with the homozygous Q295X mutation completely lacked expression of the wild-type *CARD9* protein (Fig. 2), indicating the detrimental consequences of the mutation. However, expression of the truncated *CARD9* (amino acid positions 1 through 294) protein cannot be ruled out, since the polyclonal antibody used is directed against the C-terminal part of *CARD9*.

EFFECT OF *CARD9* Q295X ON SIGNAL TRANSDUCTION

Primary bone marrow cells from *Card9*-deficient mice were retrovirally transduced with human wild-type and mutated (Q295X) *CARD9*, differentiated into macrophages *in vitro*, and analyzed with the use of flow cytometry (see the Supplementary Appendix). We then stimulated the transduced or nontransduced cells with the β -glucan preparation curdlan as a specific and selective agonist for dectin-1 or with the TLR4 ligand lipopolysaccharide and measured TNF- α production to test innate immune cell activation.³⁸ As is consistent with previous data, *Card9*^{-/-} cells showed severe defects in dectin-1-triggered TNF- α production, although they responded normally to stimulation with lipopolysaccharide (Fig. 3A).²² Expression of human full-length *CARD9* corrected the dectin-1 signaling defect in *Card9*^{-/-} cells, indicating that the hu-

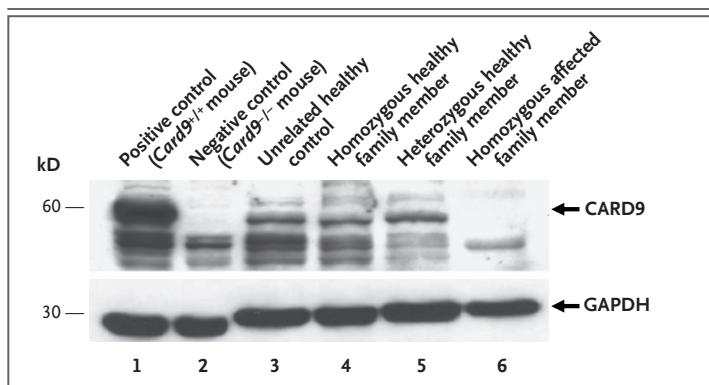


Figure 2. Western Blot Detection of *CARD9* in Peripheral-Blood Mononuclear Cells.

Macrophages from *Card9* wild-type mice (*Card9*^{+/+}) and *Card9* knockout mice (*Card9*^{-/-}) were used as positive and negative controls (lanes 1 and 2), respectively. The blot in lane 4 is from Relative 4F, that in lane 5 from Relative 2G2, and that in lane 6 from Patient 2M. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

man protein can complement the murine mutation. Expression of the human mutant *CARD9* Q295X did not increase TNF- α production, on stimulation with dectin-1, above the level in uninfected cells or those transduced with green fluorescent protein only, showing that *CARD9* Q295X is a loss-of-function mutation (Fig. 3B).

HOMOZYGOUS Q295X MUTATIONS AND TH17 CELLS

Since Th17 cells⁴⁰ are important for antifungal immunity and *Card9*^{-/-} mice have an impairment in Th17 polarization,^{22,38} we compared Th17 cells in our four patients with those in family members with wild-type *CARD9* and nine healthy controls. The mean proportion of Th17 cells in the four affected patients was significantly lower than that in healthy controls (mean, 0.2% of CD4⁺CD45RO⁺ interleukin-17⁺interferon- γ ⁻ cells; *P*=0.004) (Fig. 4). Healthy controls and family members with wild-type *CARD9* had an average of 1.2% Th17 cells *ex vivo*.

DISCUSSION

Pattern-recognition receptors of the innate immune system bind components of microbes and initiate intracellular signal cascades that result in the activation of transcription factors, up-regulation of defense-associated target genes, and release of cytokines. Dectin-1 is a transmembrane pattern-recognition receptor that senses the β -glucan component of fungal cell walls.^{23,41-43} On ligand

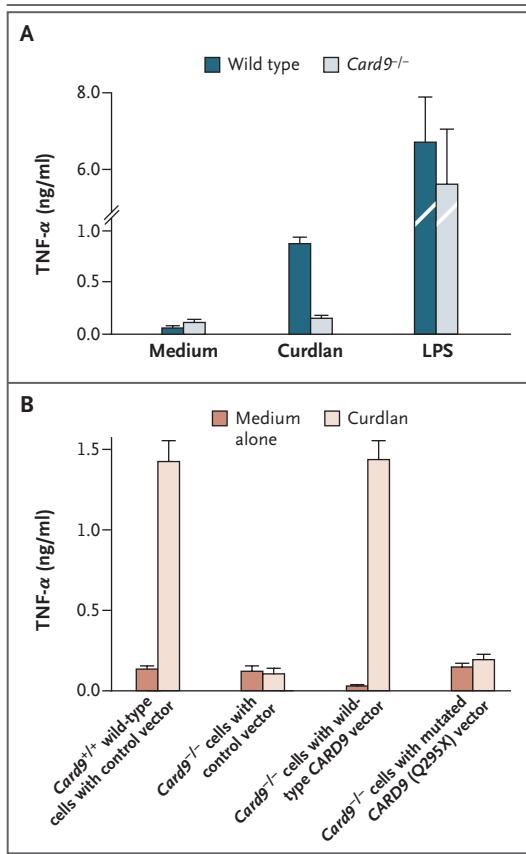


Figure 3. Analysis of the Functional Defect and Genetic Reconstitution of the *Card9* Q295X Mutation.

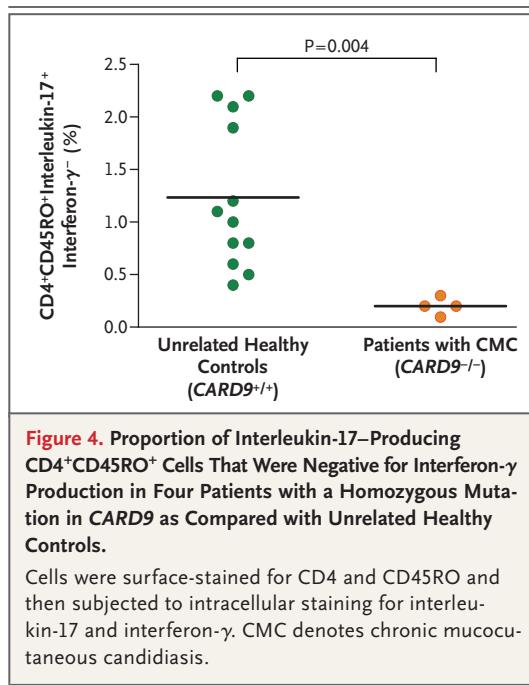
Bone-marrow–derived macrophages from wild-type mice and *Card9*^{-/-} mice were stimulated for 16 hours with both the dectin-1 agonist curdlan (300 μg per milliliter) and the toll-like-receptor agonist lipopolysaccharide (LPS, 100 ng per milliliter) (Panel A). The concentration of secreted tumor necrosis factor (TNF-α) was determined in the cell supernatant with the use of an enzyme-linked immunosorbent assay. To examine the effect of mutated *CARD9* Q295X on signal transduction (Panel B), we cloned human wild-type *CARD9* complementary DNA (cDNA) and mutated *CARD9* Q295X cDNA into a retroviral expression vector. Using these constructs, we retrovirally transduced primary bone marrow cells from *Card9*^{-/-} mice with either wild-type *CARD9* or the mutated *CARD9* Q295X. To establish a control, we transduced bone marrow cells from *Card9*^{+/+} wild-type mice and *Card9*^{-/-} mice with a control vector only. The transduced bone marrow cells were then differentiated into macrophages in vitro. After stimulation of the macrophages with curdlan for 16 hours, the concentration of secreted TNF-α was determined. The macrophages of *Card9*^{-/-} mice transduced with human wild-type *CARD9* secreted as much TNF-α upon stimulation with curdlan as did the control macrophages of wild-type *Card9*^{+/+} mice with the control vector. In contrast with these cells, the macrophages of *Card9*^{-/-} mice transduced with mutated human *CARD9* Q295X or with a control vector only did not respond with increased secretion of TNF-α upon stimulation with curdlan, a finding showing that *CARD9* Q295X is a loss-of-function mutation that cannot correct the dectin-1/*Card9* signaling pathway in the cells of *Card9*^{-/-} mice. T bars indicate standard deviations in three independent experiments.

binding, dectin-1 sends signals through an immunoreceptor tyrosine-based activation motif (ITAM), which becomes phosphorylated by Src family kinases (proto-oncogenic tyrosine kinases), leading to the recruitment and activation of the spleen tyrosine kinase (Syk).^{44,45}

Dectin-1–Syk engages *CARD9*, which together with B-cell leukemia–lymphoma 10 (BCL10) and mucosa-associated lymphoid tissue 1 (MALT1) forms an intracellular signaling complex that in cells recognizing fungi leads to the activation of the transcription factor nuclear factor κB and mitogen-activated protein kinases.^{22,46–48} This signaling pathway is operative in myeloid cells and promotes the production of key cytokines, including interleukin-1β, interleukin-6, and interleukin-23, which are required to control antifungal immune responses.^{24,38,49–52} Apart from dectin-1, the C-type lectins dectin-2 and macrophage-inducible C-type lectin (MINCLE) may also recognize fungi, engage the ITAM adapter FcRγ for Syk activation, and transmit signals through the *CARD9* pathway.^{23,53–55} Thus, *CARD9* plays a central role in antifungal defense by receiving signals from sev-

eral antifungal pattern-recognition receptors and stimulating proinflammatory responses. Since murine *Card9* deficiency results in susceptibility to fungal infections,^{22,39} this signaling pathway seems to be conserved between mice and humans.

Our study shows that a homozygous point mutation in *CARD9*, resulting in a premature termination codon and a loss of function in the adapter protein *CARD9*, is associated with a susceptibility to fungal infections, as evidenced by a chronic mucocutaneous candidiasis phenotype. In the family in our study, two members died from a fungal infection and a third presumably died from a similar cause. Further studies may clarify whether human *CARD9* deficiency accounts only for recurrent mucosal infections or also accounts for an increased susceptibility to severe invasive fungal infections. In this consanguineous family, we cannot exclude the possibility that a second genetic defect may have contributed to a more severe phenotype in the deceased family members.



Unfortunately, we were unable to study viable cells from the family members in vitro because of logistical constraints. However, to understand the function of the human mutated *CARD9* gene, we used an in vivo model with cells from *Card9*^{-/-} mice and showed that the truncated human *CARD9* protein fails to correct the dectin-1 signaling defect. In contrast, the human wild-type *CARD9* protein restores the dectin-1–*Card9* pathway in murine *Card9*^{-/-} macrophages.

In *Card9*^{-/-} mice, stimulation of dendritic cells with the cell-wall component zymosan or whole *C. albicans* cells results in a considerable reduction in the release of cytokines, including interleukin-2, interleukin-6, interleukin-10, and TNF- α , and decreased numbers of Th17 cells, which are implicated in adaptive antifungal immunity.^{22,38} All

CARD9^{-/-} patients had significantly reduced numbers of Th17 cells, further supporting the notion that *CARD9*-mediated signaling contributes to Th17-cell differentiation. Th17 cells and their production of interleukin-17 have been shown to play a pivotal role in mucosal host defense against candidiasis in mice.^{56,57} Moreover, Eyerich et al. reported decreased numbers of Th17 cells in two sporadic cases of chronic mucocutaneous candidiasis,⁵⁸ but the role of these cells in human antifungal immunity remains elusive. If the lack of Th17 cells and their cytokines were critical for the pathogenesis of mucosal candidiasis, one could speculate that in patients with a low total CD4 count, such as in the low-CD4 syndrome, another rare primary immunodeficiency, or in patients with the acquired immunodeficiency syndrome, the lack of CD4 differentiation into Th17 cells is critical for maintaining the mucosal host defense against candida. Patients with the hyper-IgE syndrome, who lack Th17 cells because of heterozygous mutations in *STAT3*, also have recurrent candidiasis.^{18,20} Whether Th17 cells are also implicated in the pathogenesis of candidiasis in APECED is currently being studied. The phenotype of susceptibility to fungal infections in human *CARD9* deficiency serves as another example of a rare primary immunodeficiency that gives insight into the signaling pathways involved in immune regulation.

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APPENDIX

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