

Abbreviations used

BCL10: B-cell CLL/lymphoma 10
 CARD9: Caspase recruitment domain-containing protein 9
 CNS: Central nervous system
 CSF2: GM-CSF gene
 ERK: Extracellular signal-regulated kinase
 FC: French-Canadian
 GAPDH: Glyceraldehyde-3-phosphate dehydrogenase gene
 GUSB: Glucuronidase β gene
 LCL: EBV-induced lymphoblastoid cell line
 MALT1: Mucosa-associated lymphoid tissue lymphoma translocation protein 1
 MUHC: McGill University Health Centre
 NF- κ B: Nuclear factor κ B
 p-ERK: Phosphorylated ERK
 PMA: Phorbol 12-myristate 13-acetate
 RASGRF1: Ras protein-specific guanine nucleotide-releasing factor 1
 sCNSc: Spontaneous central nervous system candidiasis
 SNP: Single nucleotide polymorphism
 WT: Wild-type

These cases provide compelling evidence that autosomal recessive CARD9 deficiency causes human susceptibility to invasive mycoses, including CNS disease. However, clinical characterization of sCNSc has only recently been described,⁶⁻⁸ and a full description of the clinical features will facilitate its recognition and diagnosis. Furthermore, the molecular and cellular bases by which CARD9 defects cause susceptibility to invasive fungal disease remain to be deciphered.

Here we provide comprehensive clinical and radiologic characterization of sCNSc in a series of French-Canadian (FC) young adults caused by CARD9 deficiency, resulting from the hypomorphic p.Y91H mutation, which arose because of founder effects. We confirm that impaired expression and production of GM-CSF is a consistent cellular phenotype of their PBMCs after fungal stimulation. The molecular basis for this defective GM-CSF response is reduced activation of nuclear factor κ B (NF- κ B), as well as extracellular signal-regulated kinase (ERK) activation, in monocytes. Although the canonical CARD9 signaling pathway involves CARD9 complexing with B-cell CLL/lymphoma 10 (BCL10) and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) to effect downstream signaling, we demonstrate that this interaction is intact with the p.Y91H mutation. Rather, impaired ERK activation results from a decreased interaction between CARD9 with Ras protein-specific guanine nucleotide-releasing factor 1 (RASGRF1). Collectively, the results suggest that the CARD9/RASGRF1/GM-CSF axis is critical for control of *C albicans* and expands our understanding of the immunopathogenesis of CARD9 deficiency.

METHODS**Subjects**

Subjects (n = 4) and their families provided informed consent for the McGill University Health Centre (MUHC) institutional review board-approved research protocol (GEN10-256). Comprehensive medical histories, including review of all available outside records and serial clinical as well as clinical immunologic laboratory testing, were performed at the MUHC. Therapeutic interventions were monitored at the MUHC in conjunction with the physician at the referring center for patients 2 (P2), 3 (P3), and 4 (P4).

Clinical imaging

Imaging of the brain and related structures was performed at various time points during the evaluation by using standard clinical protocols at the time of examination. T1-weighted, T2-weighted, gadolinium enhancement, fluid-attenuated inversion recovery, and diffusion-weighted magnetic resonance imaging were reviewed by an experienced neuroradiologist (JC). Because these scans were reviewed retrospectively, specific scanning protocols were variable.

Cell stimulation experiments

PBMCs and PMNs were isolated, and EBV-induced lymphoblastoid cell lines (LCLs) were established, as previously described.^{6,9} Patients' monocytes were isolated by means of adherence, as described previously.¹⁰ Where cells were stimulated with zymosan (InvivoGen, San Diego, Calif), phorbol 12-myristate 13-acetate (PMA; Sigma, St Louis, Mo), or insulin (Humulin R; Eli Lilly, Indianapolis, Ind). Cytokines were measured from supernatants harvested after stimulation by using the Quansys Q-Plex ELISA Array (Quansys Biosciences, Logan, Utah), according to the manufacturer's instructions.

***C albicans* preparation**

C albicans isolates (from P1; ATCC 14053) were cultured on Sabouraud dextrose agar (Oxoid, Toronto, Ontario, Canada), counted, and diluted in sterile PBS and heat killed at 75°C for 1 hour. Commercial heat-killed *C albicans* was from InvivoGen.

T_H17 cell ex vivo enumeration

T_H17 cell enumeration from whole blood was performed, as previously described.¹¹

PCR and DNA sequencing

The entire *CARD9* gene (NG_021197.1), including 2 kb upstream of the transcription start site, all introns, exons, the 3' region, and predicted regulatory miRNA (miRBase)¹² and the corresponding target (miRDB)¹³ were PCR amplified from genomic DNA by using primers designed to flank the respective regions (primers and sequencing conditions are available on request). Sequencing was performed at the McGill University and Génome Québec Innovation Centre. Sequencing analyses were performed on Sequencher sequence analysis software (Gene Codes, Ann Arbor, Mich).

Haplotype analyses

PCR primers were designed to amplify single tandem repeat markers for 12 markers spanning a 13.8-Mb region on chromosome 9 that includes *CARD9*. PCR products were radiolabeled by adding ³⁵S- α -dATP (PerkinElmer, Waltham, Mass) to the PCR mix with the Qiagen HotStarTaq PCR System (Qiagen, Mississauga, Ontario, Canada; primer sequences and conditions are available on request). Amplified products were separated by means of electrophoresis on a 5% denaturing acrylamide-urea gel and then autoradiographed.

Quantitative real-time PCR

Total RNA was isolated with the RNeasy Kit (Qiagen) and reverse transcribed with the Maxima cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Waltham, Mass). Quantitative real-time PCR was performed by using the Solaris qPCR Gene Expression assay system with probes directed against the GM-CSF gene (*CSF2*; catalog #AX-011166-00-0200), the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*; catalog #AX-004253-00-0200), and the glucuronidase β gene (*GUSB*; catalog #AX-012541-00-0200; Thermo Scientific) on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, Calif). The mRNA input was normalized to expression of the housekeeping genes *GAPDH* or *GUSB*.

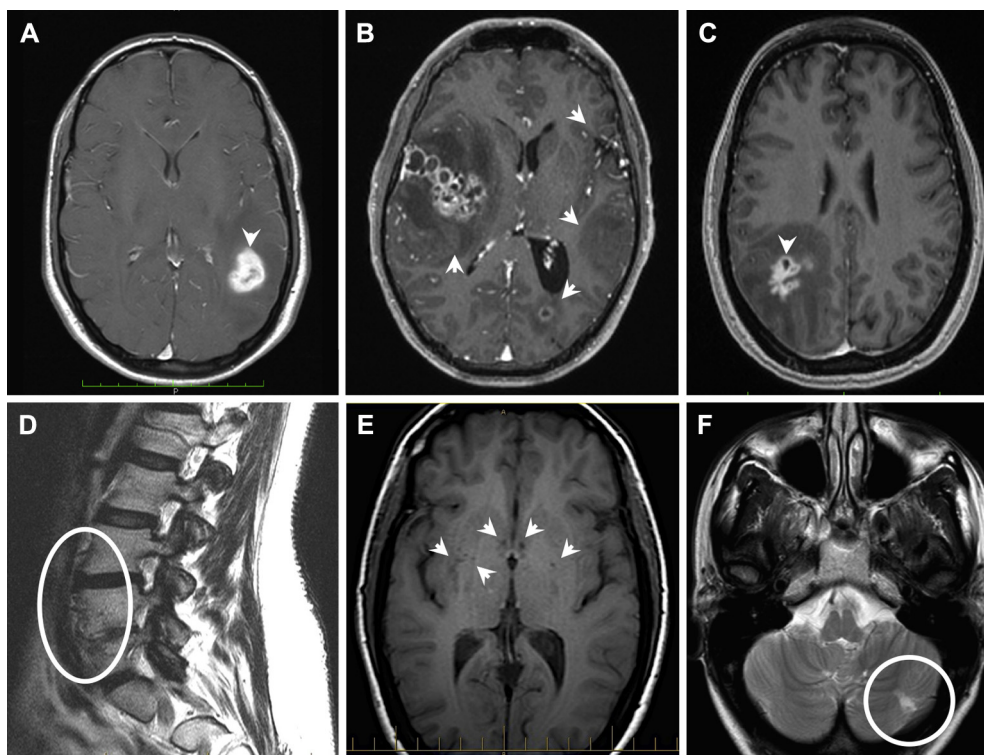


FIG 1. Magnetic resonance imaging of patients with sCNSc. Cerebral images obtained on presentation of P1 (**A**), P2 with diffuse involvement (**arrowheads**, **B**), P3 (**C**) in addition to vertebral magnetic resonance imaging showing osteomyelitis (**circle**, **D**) and P4 with numerous hypodense lesions in the basal ganglia bilaterally (**arrowheads**; **E**) and encephalomalacia in the left cerebellum (**circle**, **F**).

Antibodies

Antibodies against phosphorylated I κ B α , total I κ B α , phosphorylated ERK (p-ERK) 1/2, and total ERK1/2 were from Cell Signalling Technology (Danvers, Mass). Anti-CARD9 was from Abnova (Walnut, Calif). Anti-Flag was from OriGene (Rockville, Md).

Promoter construct studies

The Renilla luciferase reporter construct with the putative human CARD9 promoter region, encoding 1214 bp upstream of the transcription start site, and the Cypridina TK control vector were from Active Motif (Carlsbad, Calif). The wild-type (WT) putative promoter construct was confirmed by means of bidirectional Sanger sequencing and then used to create the c.-529T>C variant by means of site-directed mutagenesis (New England Biolabs, Whitby, Ontario, Canada). Dual-luciferase activity was measured with the Lightswitch dual assay kit (Active Motif).

Co-immunoprecipitation studies

Plasmids for WT green fluorescent protein (GFP)–CARD9, Flag-BCL10, Flag-MALT1, Flag-HRAS, and Flag-RASGRF1 were purchased from OriGene. The WT CARD9 plasmid was used to create the p.Y91H mutant by using a site-directed mutagenesis kit (New England Biolabs). HEK293T cells were transfected with TurboFect (Thermo Scientific), and lysates were incubated with Dynabeads Protein G (Life Technologies, Burlington, Ontario, Canada). Samples were separated on Bis-Tris Polyacrylamide Gels (Life Technologies), transferred onto polyvinylidene difluoride membranes, and immunoblotted. Band densities were measured with ImageJ software (<http://imagej.nih.gov/ij/>, 1997–2014).

Statistical analysis

Graphs and statistical analyses were generated with GraphPad Prism software (Version 6.00; GraphPad Software, La Jolla, Calif). Statistical

significance was calculated according to an unpaired 2-tailed Student *t* test, with a *P* value of less than .05 considered significant.

RESULTS

Clinical phenotype and radiology of CARD9 deficiency in FCs

Four patients from 3 unrelated FC families were identified. Patient 1 (P1) has previously been reported. Briefly, he presented with seizures at age 30 years and was found to have a left temporal lobe mass (**Fig 1, A**).⁶ Although initially suspected of malignancy, biopsies were not diagnostic. The mass was resected at age 39 years because of intermittent seizures, from which *C albicans* was isolated with no evidence of malignancy.

Patient 2 (P2) presented at age 38 years with headaches worsening over several weeks and eventual dysphasia and left-sided weakness. Multiple intracranial cystic masses, which were thought to be glioblastoma multiforme, were identified on imaging (**Fig 1, B**). Initial biopsy revealed only reactive astrocytosis. Over the next 2 months, worsening weakness progressing to left hemiparesis and decreasing level of consciousness led to surgical resection, which identified only *C albicans* infection. On the basis of antifungal susceptibility testing, the patient was started on 800 mg/d fluconazole. Despite approximately 3 months of fluconazole, he had ongoing headaches, worsening neurologic deficits, and abnormal cerebrospinal fluid parameters, which improved with adjunctive GM-CSF therapy (see **Fig E1** in this article's Online Repository at www.jacionline.org).

Patient 3 (P3) presented at age 39 years with painless loss of vision in the left eye. The endophthalmitis did not improve on

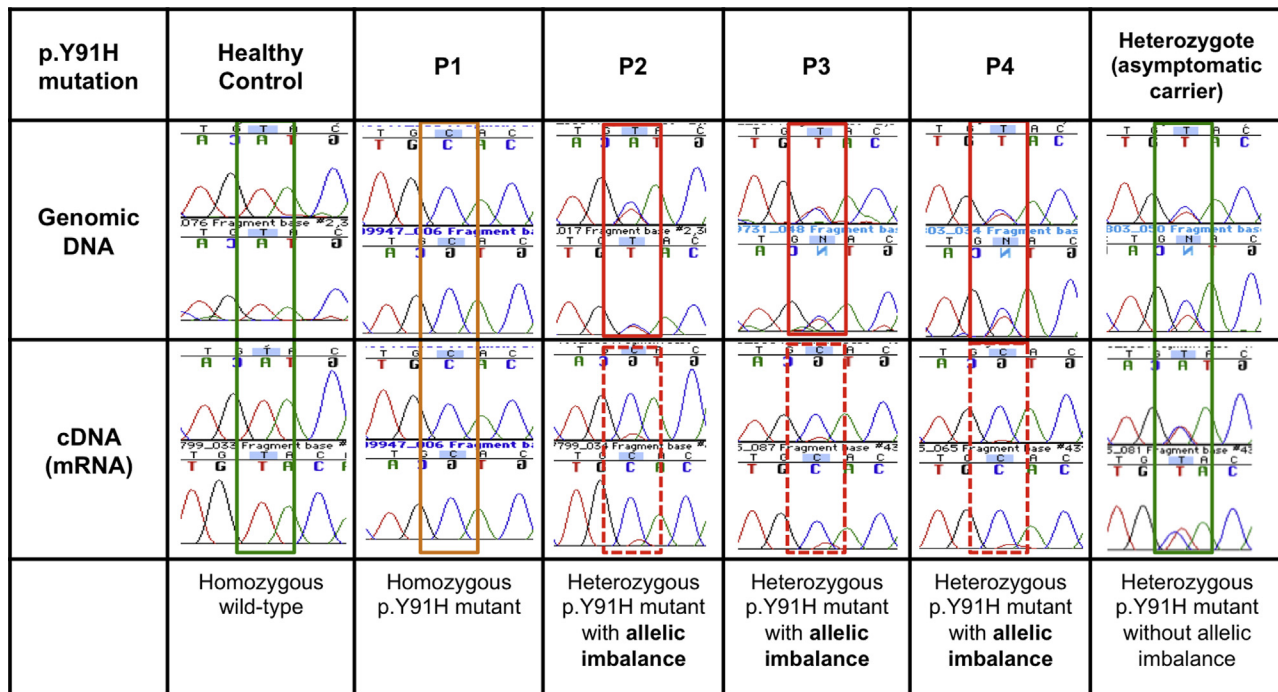


FIG 2. Allelic imbalance of *CARD9*. Electropherograms demonstrate P2, P3, and P4 are germline heterozygous for the p.Y91H allele but express only p.Y91H at the cDNA level, which is consistent with allelic imbalance. P1 is homozygous for the p.Y91H mutant. An asymptomatic heterozygous carrier is shown to demonstrate that *CARD9* is normally biallelically expressed.

empiric antitoxoplasmosis therapy. Vitreous sampling yielded *C albicans*. Imaging revealed lesions in the right parietotemporal area, as well as in the lumbar vertebra (Fig 1, C and D); the patient refused sampling of these lesions. She was started on fluconazole, which eventually resulted in some incomplete improvement in her vision, as well as radiologic resolution of her brain abscesses and vertebral osteomyelitis. She received a diagnosis of bipolar disorder in adolescence (no imaging performed) and had intermittent episodes of tinea versicolor (no cultures available).

Her twin sister, patient 4 (P4), had previous skin lesions diagnosed as tinea versicolor and occasional episodes of white oral plaques presumed to be thrush. P4 had occasional episodes of migraines but no frank neurologic symptoms. However, magnetic resonance imaging screening revealed numerous nonenhancing lesions in the basal ganglia bilaterally (Fig 1, E) and a wedge-shaped area of encephalomalacia in the left cerebellar hemisphere resembling an infarct (Fig 1, F). Because she had no compromising symptoms, she refused both biopsy of these regions and a lumbar puncture. Given these abnormal CNS findings, with her *CARD9* genotype (see below), P4 was considered to be affected. Pedigrees are presented in Fig E2 in this article's Online Repository at www.jacionline.org.

Genetics

***CARD9* deficiency in FCs is associated with p.Y91H and c.-529T>C.** We previously demonstrated that P1 was homozygous for the c.439T>C (p.Y91H) allele and that intrafamilial segregation was consistent with an autosomal recessive trait.⁶ Given the sCNSc phenotype of P2 to P4, we sequenced the *CARD9* locus from genomic DNA and found

them each to be heterozygous for this same c.439T>C (p.Y91H) allele. Because sCNSc caused by *CARD9* deficiency is thought to be a recessive disease, we sequenced the cDNA from PBMCs of P2 to P4, available family members, and unrelated healthy control subjects to search for a second mutation. Single nucleotide polymorphism (SNP) analysis revealed that the *CARD9* locus is normally biallelically expressed. In contrast, P2, P3, and P4 abnormally demonstrated allelic imbalance, expressing only the mutant c.439T>C (p.Y91H) allele (Fig 2). This was similarly seen in their PMNs and LCLs (data not shown). Sequencing showed that unaffected family members of P2 and P3/P4 expressed either the c.439T>C allele in heterozygosity with the WT allele or had allelic imbalance in the absence of the c.439T>C allele (Fig 2). Thus P2, P3, and P4 harbor the c.439T>C mutation on one allele but also do not express the other *CARD9* allele, rendering them the transcriptional equivalent to P1 at the *CARD9* locus. Failure to express the other *CARD9* allele, resulting in allelic imbalance, invoked the presence of a putative second mutation.

To identify this second mutation, we sequenced the entire *CARD9* locus and surrounding regions, including all exons, introns, 2 kb upstream of the transcription start site, the 5' and 3' untranslated regions, the poly-A tail region, miRNA-3621 (predicted to regulate *CARD9* [miRDB]), and the predicted target sequence. The only variant common to P2 to P4 that was not a known SNP (dbSNP build 141) was identified to be 529 bp upstream of the ATG initiation codon (c.-529T>C; see Table E1 in this article's Online Repository at www.jacionline.org for all identified SNPs). Pedigree analysis revealed that healthy family members carrying only the c.-529T>C variant also demonstrated allelic imbalance at the *CARD9* locus. Comparison of germline and expressed *CARD9* SNPs in our pedigrees confirmed that the

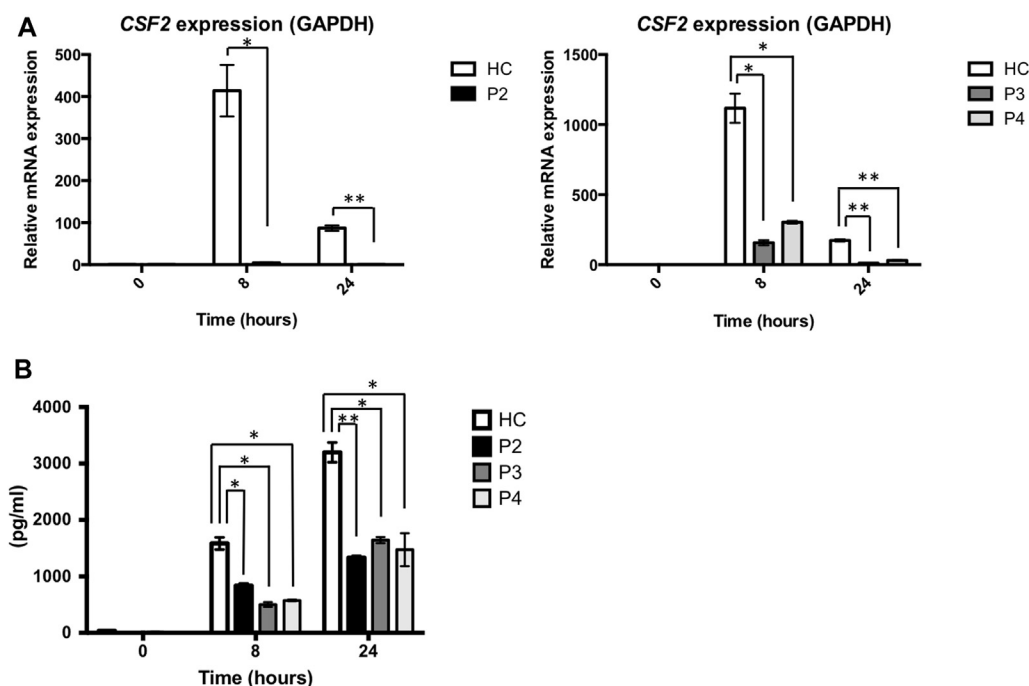


FIG 3. Impaired GM-CSF response after fungal stimulation in *CARD9*-deficient PBMCs. Zymosan-stimulated PBMCs of patients with the p.Y91H mutation have blunted *CSF2* expression relative to the housekeeping gene *GAPDH* (A) and GM-CSF production in supernatants (B). HC, Healthy control subject. * $P < .05$ and ** $P < .01$.

c.-529T>C variant was on the allele distinct from that harboring the p.Y91H mutation. However, there was no significant difference in expression between the c.-529T>C variant and the WT control in a luciferase reporter vector driven by the putative promoter region of *CARD9* (see Fig E3 in this article's Online Repository at www.jacionline.org), suggesting that the c.-529T>C variant is likely in linkage disequilibrium with the causal mutation rather than being the direct cause of allelic imbalance.

Founder effects of p.Y91H and c.-529T>C. All affected patients in our cohort are FCs. The c.439T>C (p.Y91H) and c.-529T>C alleles are not seen in any public SNP database, nor have they been reported in other (non-FC) cases of *CARD9* deficiency. These variants in apparently unrelated pedigrees might be either due to sites for independent recurrent mutations (ie, mutational hotspots) or reflect that the families share a common ancestry (ie, founder effect). Because the FC population of Quebec is known for other genetic disorders attributed to common founders, we hypothesized that the *CARD9* variants in our cohort represent founder effects. This is supported by the absence of genetic elements around our *CARD9* variants typically associated with mutational hotspots (eg, direct repeats, [quasi]palindromes, polypurine/polypyrimidine runs, and consensus motifs). Further circumstantial evidence for a founder effect is the identification of 6 other published reports of FCs with sCNSc¹⁴⁻¹⁷; despite our best efforts, no samples from these cases (from 1945–1994) are available for genotyping. However, genotyping of 156 subjects defined as FCs from an unrelated study on hereditary cancers revealed 1 additional carrier of c.439T>C and a different carrier of c.-529T>C, providing additional

evidence for a possible founder effect. Thus in our cohort we have 7 subjects with the c.-529T>C variant and 12 subjects with the c.439T>C mutation (3 of these with both).

To confirm the founder-effect hypothesis, we performed haplotype analysis with polymorphic intragenic and extragenic short tandem repeat markers, as done previously.¹⁸ Haplotypes were reconstructed from genotype information by using the known relationships between different family members and assuming the smallest possible number of recombination events between chromosomes in any given parent individual. Haplotypes observed in each family are summarized in Fig E4 in this article's Online Repository at www.jacionline.org. The presence of conserved regions (808 kb for the c.439T>C mutation in exon 3 and 2.2 Mb for the c.-529T>C mutation in the putative promoter region) show that each mutation resides on a chromosome derived from an ancestor common to all carriers (ie, founder effect). Because the size of the conserved region is inversely proportional to the time elapsed since the appearance of the mutation (regions surrounding older mutations underwent a larger number of recombination events that introduce new alleles on the haplotype over time), we can conclude that the c.439T>C mutation in exon 3 is older than the c.-529T>C variant in the putative promoter region. Based on these observed allele frequencies in control subjects (1/312 chromosomes each) in presumed Hardy-Weinberg equilibrium, with an estimated FC population in Quebec of 6.5 million, there would be an estimated 67 subjects homozygous for c.439T>C (p.Y91H), 67 subjects homozygous for the c.-529T>C variant, and 134 subjects who were compound heterozygotes. Thus approximately 268 FC subjects in Quebec would be genetically predisposed to this phenotype because of these 2 alleles.

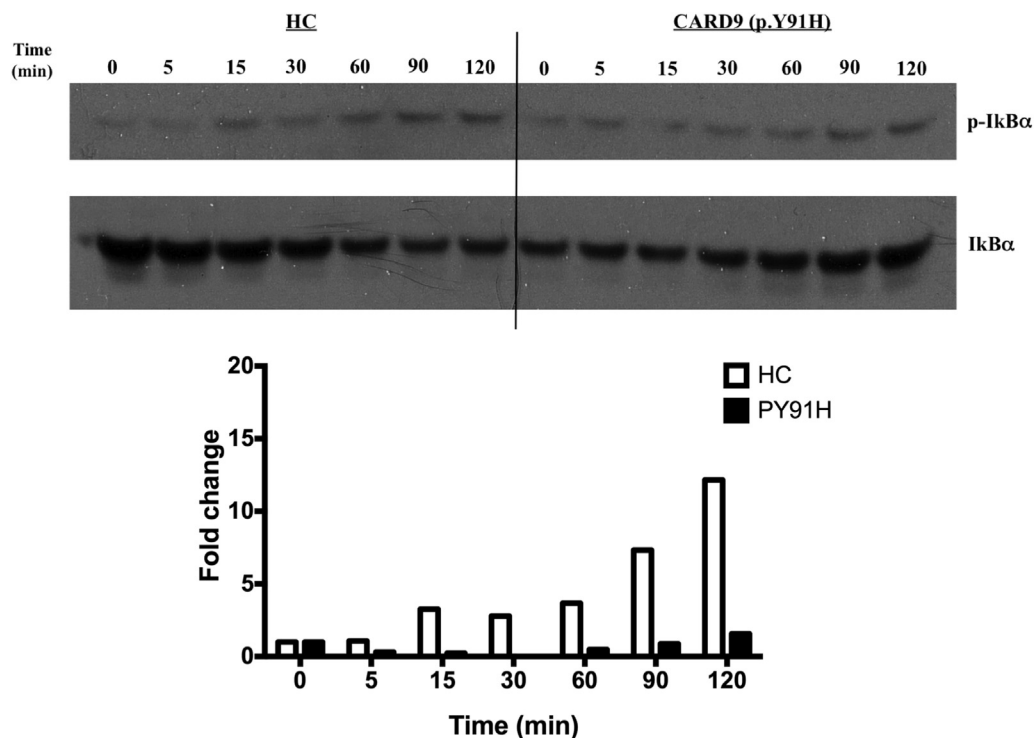


FIG 4. Impaired NF- κ B activation in CARD9-deficient cells. Lymphoblastoid cells from CARD9-deficient patients demonstrate impaired NF- κ B activation after zymosan stimulation, as assessed by using densitometric analysis of p-I κ B α relative to total I κ B α . A single experiment representative of 3 independent experiments performed is shown. HC, Healthy control subjects.

Functional studies

Impaired GM-CSF responses after fungal stimulation is a consistent feature of CARD9 deficiency in FCs. We have previously shown that PBMCs from P1 demonstrated profoundly blunted induction of *CSF2* expression and corresponding GM-CSF cytokine production in response to zymosan (a fungal agonist).⁶ To determine whether the impaired GM-CSF response was a consistent characteristic of CARD9 deficiency in our series, we evaluated PBMCs from P2 to P4. P2, P3, and P4 all demonstrated similarly blunted *CSF2* induction and GM-CSF production after stimulation with zymosan (Fig 3), a commercial heat-killed *C albicans* preparation, the heat-killed *C albicans* strain isolated from P1, and another heat-killed clinical strain of *C albicans* (ATCC 14053, see Fig E5 in this article's Online Repository at www.jacionline.org). Production of other proinflammatory (eg, IL-1 β , IL-2, IL-6, IL-8, IL-12, IFN- γ , and TNF- α) and anti-inflammatory (eg, IL-4 and IL-10) cytokines was not statistically significantly different from corresponding responses of healthy control subjects (data not shown). Thus impaired expression and production of GM-CSF in response to fungal stimulation is a consistent cellular phenotype of CARD9 deficiency in our cohort.

Given our previous successful use of rhGM-CSF adjunctive therapy in P1 and the dire situation of P2 (ie, wheelchair bound, ongoing headaches, and fluctuating mental status, with abnormal cerebrospinal fluid parameters despite appropriate antifungal therapy), adjunctive therapy with rhGM-CSF was initiated. As with P1, serial lumbar punctures demonstrated normalization of cerebrospinal fluid, which occurred only after introduction of rhGM-CSF, along with progressive radiologic improvement (see

Fig E1). P3 and P4 did not undergo adjunctive therapy with rhGM-CSF.

The p.Y91H mutation does not impair CARD9 interactions with BCL10 or MALT1. It has been previously shown that CARD9 forms a complex with BCL10 and MALT1 to mediate NF- κ B activation in response to zymosan.¹⁹ To further elucidate the functional consequences of the c.439T>C (p.Y91H) mutation, we examined its effect on the NF- κ B signaling cascade. Fungal stimulation of patient-derived LCLs revealed impaired NF- κ B activation relative to that seen in healthy control subjects (Fig 4), as has been recently reported.⁸ To determine the molecular basis for this, we evaluated the effect of p.Y91H on the ability of CARD9 to complex with BCL10, MALT1, or both by means of cotransfection of HEK293T cells, followed by co-immunoprecipitation studies. Comparing WT with p.Y91H CARD9, no difference in their interaction with either BCL10 (see Fig E6 in this article's Online Repository at www.jacionline.org) or MALT1 (data not shown) was observed. Thus impaired complex formation between CARD9, BCL10, or MALT1 does not account for the CARD9 deficiency in our cohort.

Blunted GM-CSF responses result from impaired ERK activation caused by diminished CARD9/RASGRF1 interaction.. Regulation of GM-CSF expression involves the ERK signaling cascade.^{20,21} To further elucidate the mechanisms by which the c.439T>C (p.Y91H) mutation in CARD9 results in blunted GM-CSF responses to fungi, we evaluated ERK activation by measuring the kinetics of phosphorylated ERK normalized to total ERK after fungal stimulation. Monocytes isolated from our CARD9-deficient patients demonstrated significantly impaired ERK activation in

response to zymosan relative to that seen in healthy control subjects (Fig 5, A). To confirm that the impaired ERK activation was responsible for the impaired CSF2/GM-CSF response, we evaluated the effect of the pharmacologic ERK activators PMA and insulin on zymosan-stimulated monocytes. In monocytes from healthy control subjects, stimulation with zymosan, PMA, or insulin alone resulted in ERK activation (Fig 5, B), although only stimulation with zymosan or PMA induced CSF2 expression (Fig 5, C); these responses were diminished in CARD9-deficient subjects. PMA enhanced zymosan-induced ERK activation (Fig 5, B), as well as CSF2 expression (Fig 5, C) and GM-CSF production (data not shown), in patient-derived monocytes, although it did not normalize them relative to those in healthy control subjects (Fig 5, C). Insulin alone did not induce CSF2 expression in either patients or control subjects, nor did it enhance CSF2 expression in combination with zymosan (Fig 5, C). These results suggest that the defect in ERK-associated GM-CSF responses observed in our CARD9-deficient patients is specific to inflammatory agonists (eg, zymosan and PMA) but that ERK activation alone is not sufficient to regulate GM-CSF responses. Overall, these findings confirm that the impaired ERK activation after fungal stimulation in CARD9-deficient monocytes contributes to the blunted GM-CSF responses.

Ras guanine nucleotide releasing factor 1 (RASGRF1) is a known major regulator of ERK activation,²² and it has been recently shown that CARD9 induces ERK activation in response to *C albicans* by interacting with HRAS and RASGRF1.²³ To interrogate the integrity of this axis, we coexpressed WT or mutant CARD9 with HRAS or RASGRF1 in HEK293T cells and assessed protein interaction by means of co-immunoprecipitation. We found that the p.Y91H mutation in CARD9 decreased its association with RASGRF1 compared with WT (Fig 6, A and B). This impaired interaction was associated with reduced ERK activation (Fig 6, C), establishing the molecular basis by which p.Y91H is deleterious (Fig 6, A and C). Taken together, these findings strongly suggest that CARD9 deficiency caused by the p.Y91H mutation results from decreased complex formation with RASGRF1, impairing ERK activation and resulting in reduced CSF2 expression and GM-CSF production.

DISCUSSION

Although CARD9 deficiency clearly predisposes to invasive fungal disease, the clinical spectrum of disease appears to be variable, which might hinder diagnosis. This series in FCs demonstrates an adult age of onset of clinically significant disease (median, 38 years), with initial radiologic findings misinterpreted as brain malignancies but from which only infection with *C albicans* was identified. A great-uncle of P1 had died in his 30s of a “brain tumor” that in retrospect was likely sCNSc. In P3 her sentinel manifestation was endophthalmitis, and she was found to have probable candidal vertebral osteomyelitis, neither of which has been previously reported with CARD9 deficiency. Her twin, P4, was asymptomatic at the time of evaluation, but neuroimaging revealed abnormalities suggestive of infarcts, which could be compatible with a previous sCNSc episode or episodes that appeared to have resolved spontaneously. If so, it suggests that there is variable penetrance of the sCNSc phenotype in patients with CARD9 deficiency, as seen by the absence of sCNSc in the sequenced homozygous null family members by Glocker et al.¹ Alternatively, this might reflect the natural waxing

and waning history of the disease, as described by Drewniak et al² and Gavino et al.⁶ Thus our FC cohort demonstrates that CARD9 deficiency can have adult-onset manifestation, variable expressivity, and variable penetrance.

The mechanisms by which CARD9 deficiency causes susceptibility to fungal infections are only beginning to be deciphered. The CARD9-deficient patients in our cohort distinctively express the hypomorphic p.Y91H mutation, which might explain the later onset of disease. Because the majority of previous CARD9 mutations are amorphic and few are hypomorphic, our cohort provides an opportunity to dissect the processes by which CARD9 mediates susceptibility to fungal diseases. Our findings demonstrate that PBMCs from our CARD9-deficient cohort consistently demonstrate severely impaired induction of CSF2 expression and subsequent GM-CSF production after fungal stimulation. The importance of GM-CSF in human responses to *Candida* species is supported by 2 recent studies showing that it is distinctly and robustly induced in response to *C albicans* compared with other microbial stimulants.^{24,25} GM-CSF exerts a variety of effects, including stimulating growth, mobilization and survival of myeloid cells, enhancing responsiveness to chemotaxis, and increasing phagocytosis, activation of macrophages, and proliferation of microglia.²⁶ Perhaps important for CARD9 deficiency, murine models have demonstrated the importance of GM-CSF for the recruitment of inflammatory macrophages to the CNS.²⁷ Together, these results suggest that GM-CSF is a distinct component of the PBMC response to *C albicans*, and by corollary, the severe impairment of such a response in our cohort is both abnormal and pathophysiologic.

In addition to our previous report,⁶ other groups characterizing CARD9 deficiency have not reported on GM-CSF responses. Initial cases identified a profoundly reduced T_H17 response in their CARD9-null subjects.^{1,2} Although numerous other primary immunodeficiencies with impaired or abolished T_H17 responses are marked by chronic mucocutaneous candidiasis,²⁸ the salient absence of invasive candidiasis (particularly sCNSc) in patients with these disorders suggests that a blunted T_H17 response is not in itself sufficient to account for invasive candidiasis. In fact, T_H17 responses were demonstrated intact in our patients and in other reports of CARD9-deficient patients with either phaeophomycosis caused by *Exophiala* species⁵ or invasive candidiasis (eg, sCNSc and colitis).⁸ Collectively, these cases clearly indicate that a reduced T_H17 response is neither required nor sufficient to permit invasive mycoses, at least by the spectrum of fungal pathogens identified to date. Rather, additional or alternative pathways mediate invasion, as suggested by differential CARD9 signaling in the mouse.²⁹ The production of other proinflammatory cytokines from subject-derived cells, particularly IL-1 β , IL-6, and TNF- α , has also been reported to be impaired.¹⁻³ Again, the absence of sCNSc, deep dermatophytosis, or phaeophomycosis in patients with abolished IL-6 responses (eg, autosomal dominant hyper-IgE syndrome or autoantibodies to IL-6) suggests that this observation might be an epiphenomenon or a redundant process rather than critical for disease. Interestingly, Lantermier et al⁵ have shown that such a blunted response from whole blood was not recapitulated in monocyte-derived dendritic cells, suggesting a cell-specific compensatory mechanism. Our identification of impaired GM-CSF responses appears to be a consistent cellular phenotype in our FC series and requires evaluation in others. Because GM-CSF is thought to contribute to the generation of

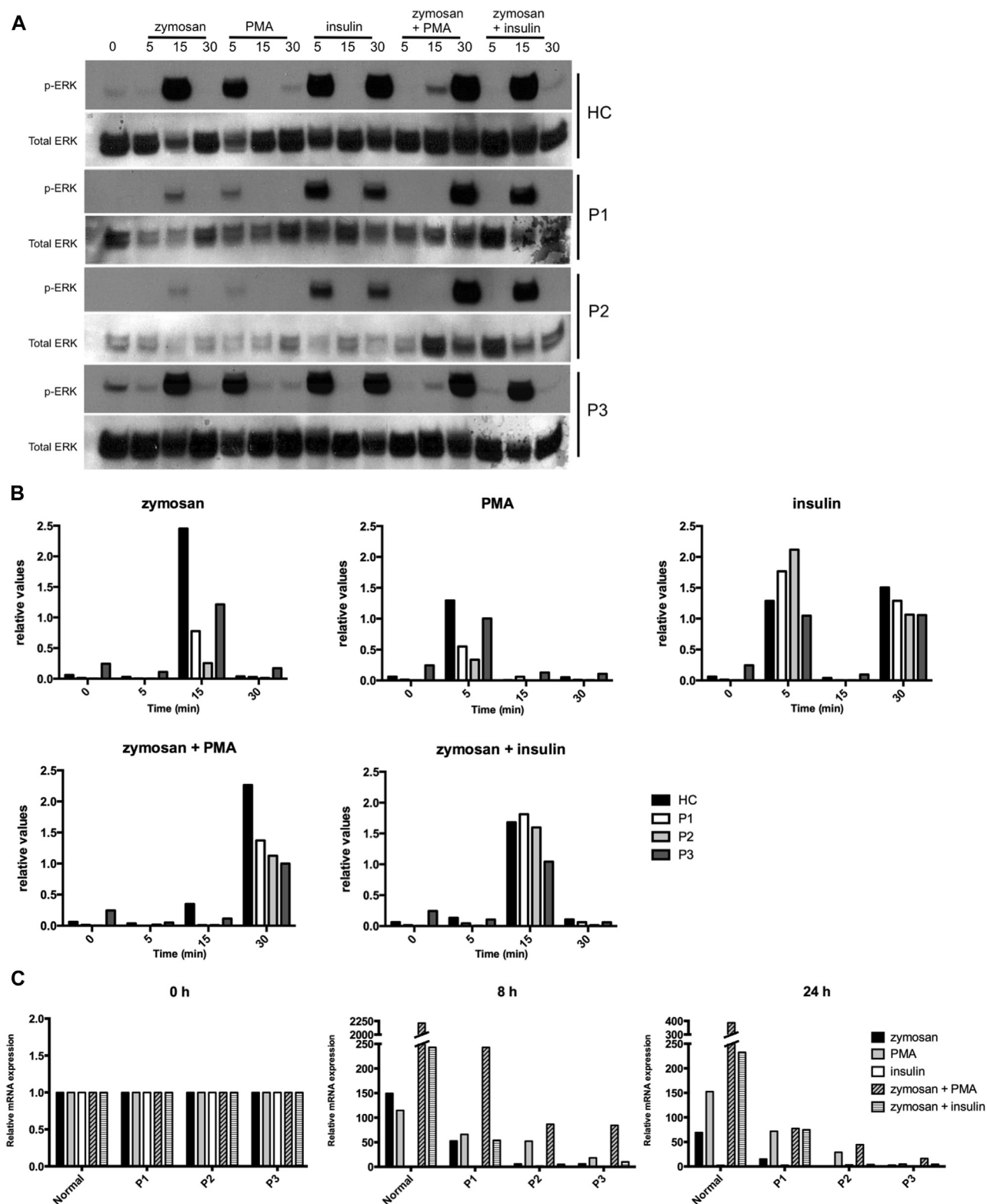


FIG 5. Increased fungus-induced ERK activation in monocytes by PMA enhances GM-CSF responses. Monocytes from subjects were stimulated with zymosan (62.5 μ g/mL), PMA (20 ng/mL), insulin (5 IU/mL), zymosan and PMA, or zymosan and insulin. **A** and **B**, At the indicated time points, cell lysates were harvested for immunoblot analysis of p-ERK and total ERK (Fig 5, A) with densitometric analysis of p-ERK relative to total ERK, as summarized in Fig 5, B. **C**, Stimulated monocytes from subjects were analyzed for *CSF2* expression relative to the housekeeping gene *GAPDH* at indicated time points. HC, Healthy control subjects.

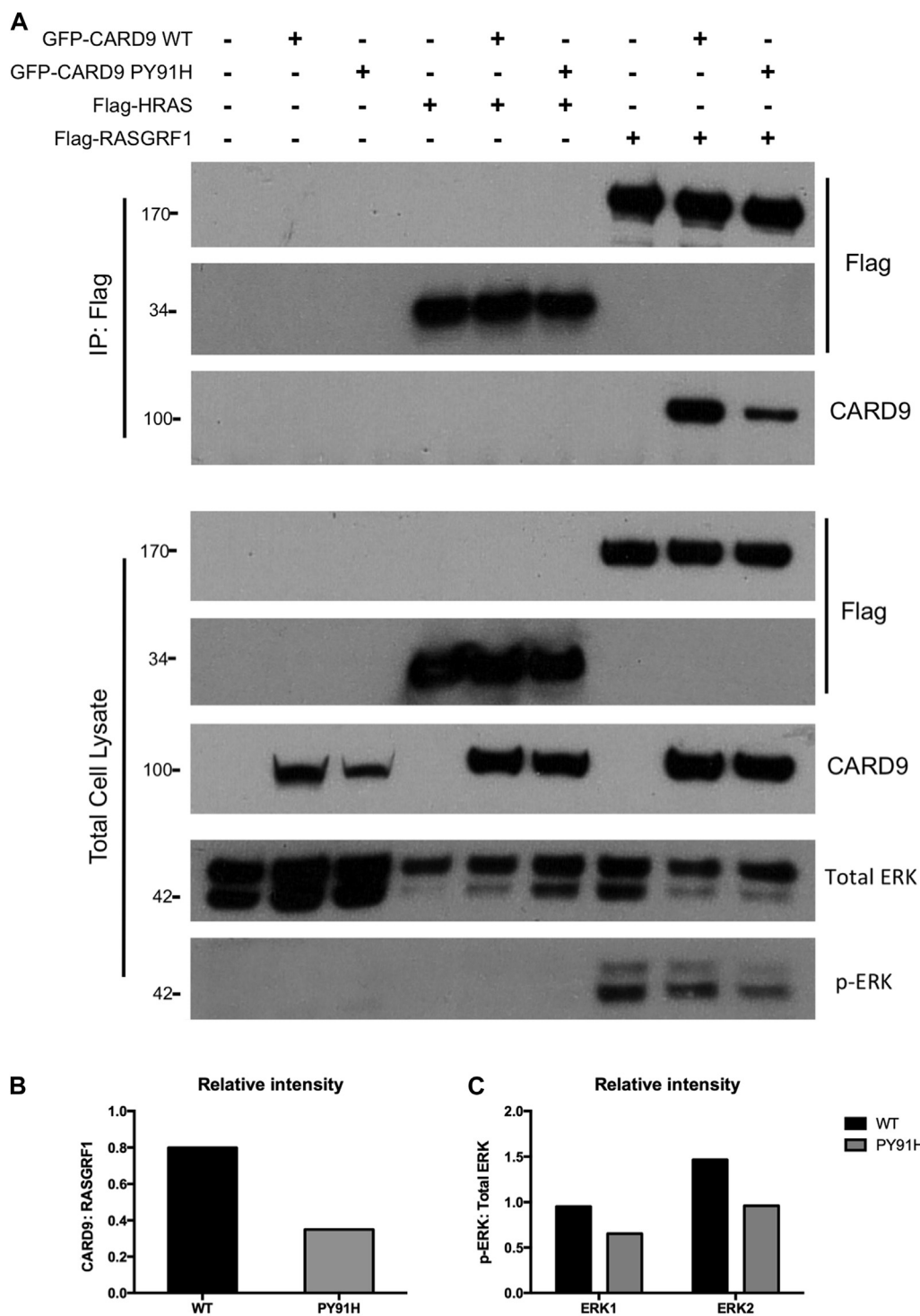


FIG 6. The p.Y91H mutation impairs interaction of CARD9 with RASGRF1. **A**, HEK293T cells were transfected with Flag-tagged HRAS or RASGRF1 and green fluorescent protein-tagged WT or p.Y91H CARD9. Cell lysates were immunoprecipitated (IP) with anti-Flag antibody. Cell lysates and immunoprecipitates were analyzed by means of immunoblotting with antibodies as indicated. **B**, Immunoblots of immunoprecipitates were analyzed by using densitometry. Densitometry of Flag-tagged RASGRF1 relative to CARD9 shows decreased binding in p.Y91H compared with WT. **C**, Densitometry of p-ERK1/2 relative to total ERK1/2 in total cell lysates shows decreased ERK activation in HEK293T cells expressing RASGRF1 and p.Y91H compared with WT.

T_H17 cells and production of proinflammatory cytokines by monocytic cells,²⁷ the impaired GM-CSF responses we demonstrate might ultimately explain these previously reported findings.

An expanding number of *CARD9* mutations are being reported (ie, L64fs*59,⁴ R70W,^{5,8} G72S,² R101C,³ Q158X,⁴ D274fs*60,⁴ Q289X,³ Q295X,¹ E323del,⁵ and R373P²). The variants identified in our FC cohort, c.439T>C (p.Y91H) and c.-529T>C, are distinct: the p.Y91H mutation does not impair gene expression or production of full-length protein, although it diminishes cellular GM-CSF responses, indicating that it is hypomorphic.⁶ Conversely, the c.-529T>C variant, which lies upstream of the gene and thus in a putative promoter region, segregates in our respective pedigrees with *CARD9* loss of allelic expression, resulting in expression of only the contra-allele. This allelic imbalance is abnormal and, when in compound heterozygosity with the c.439T>C (p.Y91H) allele, results in *CARD9* deficiency in P2 to P4. However, this c.-529T nucleotide is not highly conserved, and our dual-luciferase reporter studies demonstrate that this transition to cytosine is not sufficient to significantly impair expression; thus this private variant is likely in linkage disequilibrium with the causal mutation³⁰ or perhaps is more prone to DNA modifications that regulate expression. Further work is in progress to address this.

Nonetheless, our findings suggest that allelic imbalance of *CARD9* expression in compound heterozygosity with another mutant allele can contribute to autosomal recessive *CARD9* deficiency, illustrating the range of potential underlying mechanisms of this disease. Given that the promoter region of *CARD9* has not been well studied and transcription factors regulating its expression (other than *SALL4*³⁰) are limited to *in silico* analysis without validation, our findings should prompt investigations into the mechanisms regulating *CARD9* expression.

The identification of 2 shared unique variants among seemingly unrelated FC families suggested that *CARD9* deficiency might have been established in Quebec because of founder effects, as is seen with select inborn errors of metabolism. The identification of 6 other sCNSc cases in the literature occurring explicitly in FCs¹⁴⁻¹⁷ and our identification of heterozygous carriers of each variant in a repository of unselected FCs further support this hypothesis, which was confirmed by means of haplotype analysis. Genealogic retracing of each of our pedigrees revealed origins from isolated areas in northern coastal France. The FC population arose from approximately 6500 colonials originating from France during the 17th and 18th centuries, with distant consanguinity occurring through the history of Quebec primarily because of geographic isolation.³¹ Thus based on our calculated allele frequencies, *CARD9* deficiency might be relatively more frequent in FCs; the prevalence could actually be higher if there are additional deleterious alleles in this population. The identification of these mutations in descendants from France might also explain previous reports of sCNSc in French persons.³²⁻³⁴

To understand the molecular basis of *CARD9* deficiency, we evaluated the “canonical” pathway, whereby *CARD9* complexes with BCL10 and MALT1, and found no difference in these interactions. These findings suggest that *CARD9* deficiency does not confer susceptibility to invasive fungal disease through this pathway, which is consistent with the absence of invasive fungal disease in families with deficiency of BCL10³⁵ or MALT1.³⁶⁻³⁸

Although “candidal pneumonia” was reported in one subject with MALT1 deficiency,³⁶ the diagnosis was based on the isolation of *C albicans*, along with *Pseudomonas* species and *Streptococcus pneumoniae*, from bronchoalveolar lavage fluid. In the absence of histopathology, this finding is insufficient to establish a diagnosis of candidal pneumonia.³⁹ Collectively, the data suggest that the *CARD9*/BCL10/MALT1 hub might be redundant for invasive fungal disease and that alternative pathways might be central.

Because impaired GM-CSF responses were a consistent feature of *CARD9* deficiency, we dissected the upstream components of this phenotype. We showed that ERK activation in response to fungal stimulation was consistently decreased in *CARD9*-deficient monocytes. To confirm that this process was indeed responsible for the impaired GM-CSF responses seen, we demonstrated that activation of ERK specifically within the context of an inflammatory signaling cascade (ie, PMA) augments fungus-induced GM-CSF responses in *CARD9*-deficient monocytes. This finding establishes a role for monocytes in the pathogenesis of *CARD9* deficiency, adding to the previous data of others showing impaired neutrophilic responses *in vitro* in the absence of serum.^{2,7,40,41} It further couples *CARD9* signaling to ERK activation. The molecular basis for the decreased ERK activation appears to be caused by the inability of *CARD9* to properly complex with RASGRF1 because of the pY91H mutation. These findings provide clinical relevance to the *CARD9*/RASGRF1 complex recently identified²³ and establish this axis as a candidate pathway for other inborn causes of spontaneous invasive fungal diseases. It is interesting to note that RASGRF1 is preferentially, although not exclusively, expressed in the CNS,^{22,42} which might account for the distinct predilection of this organ in *CARD9* deficiency.

In summary, our findings reveal that *CARD9* deficiency in FCs results from at least 2 variants that were established through founder effects. The resulting sCNSc can have adult-onset manifestation of disease, and the natural history might be one of variable expressivity and variable penetrance. Taken together, our findings support a model whereby fungal stimulation results in a *CARD9*/RASGRF1 complex, leading to activation of ERK and NF- κ B and inducing expression of GM-CSF. This axis appears to critically mediate susceptibility to at least sCNSc. It might also be relevant for the other observed invasive fungal diseases.

Clinical implications: *CARD9* deficiency can present with adult-onset subacute/relapsing invasive candidiasis, with variable expressivity (eg, CNS, eye, and bone) and penetrance; it might resemble malignancy. Defective *CARD9*/RASGRF1/ERK signaling impairs GM-CSF responses. Patients could benefit from adjunctive GM-CSF therapy.

REFERENCES

- Glocker EO, Hennigs A, Nabavi M, Schäffer AA, Woellner C, Salzer U, et al. A homozygous *CARD9* mutation in a family with susceptibility to fungal infections. *N Engl J Med* 2009;361:1727-35.
- Drewniak A, Gazendam RP, Tool AT, van Houdt M, Jansen MH, van Hamme JL, et al. Invasive fungal infection and impaired neutrophil killing in human *CARD9* deficiency. *Blood* 2013;121:2385-92.
- Lanternier F, Pathan S, Vincent QB, Liu L, Cypowyj S, Prando C, et al. Deep dermatophytosis and inherited *CARD9* deficiency. *N Engl J Med* 2013;369:1704-14.

4. Wang X, Wang W, Lin Z, Wang X, Li T, Yu J, et al. CARD9 mutations linked to subcutaneous phaeohyphomycosis and TH17 cell deficiencies. *J Allergy Clin Immunol* 2014;133:905, 908.e3.
5. Lantermier F, Barbati E, Meinzer U, Liu L, Pedergrana V, Migaud M, et al. Inherited CARD9 deficiency in 2 unrelated patients with invasive *Exophiala* infection. *J Infect Dis* 2015;211:1241-50.
6. Gavino C, Cotter A, Lichtenstein D, Lejtenyi D, Fortin C, Legault C, et al. CARD9 deficiency and spontaneous central nervous system candidiasis: complete clinical remission with GM-CSF therapy. *Clin Infect Dis* 2014;59:81-4.
7. Herbst M, Gazendam R, Reimnitz D, Sawalle-Belohradsky J, Groll A, Schlegel PG, et al. Chronic *Candida albicans* meningitis in a 4-year-old girl with a homozygous mutation in the CARD9 gene (Q295X). *Pediatr Infect Dis J* 2015;34:999-1002.
8. Lantermier F, Mahdavian SA, Barbati E, Chaussade H, Koumar Y, Levy R, et al. Inherited CARD9 deficiency in otherwise healthy children and adults with *Candida* species-induced meningoencephalitis, colitis, or both. *J Allergy Clin Immunol* 2015;135:1558-68.e2.
9. Vinh DC, Schwartz B, Hsu AP, Miranda DJ, Valdez PA, Fink D, et al. Interleukin-12 receptor beta1 deficiency predisposing to disseminated coccidioidomycosis. *Clin Infect Dis* 2011;52:e99-102.
10. Segal BH, Han W, Bushey JJ, Joo M, Bhatti Z, Feminella J, et al. NADPH oxidase limits innate immune responses in the lungs in mice. *PLoS One* 2010;5:e9631.
11. Renner ED, Rylaarsdam S, Anover-Sombke S, Rack AL, Reichenbach J, Carey JC, et al. Novel signal transducer and activator of transcription 3 (STAT3) mutations, reduced T(H)17 cell numbers, and variably defective STAT3 phosphorylation in hyper-IgE syndrome. *J Allergy Clin Immunol* 2008;122:181-7.
12. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res* 2014;42(Database issue):D68-73.
13. Wong N, Wang X. miRDB: an online resource for microRNA target prediction and functional annotations. *Nucleic Acids Res* 2015;43(Database issue):D146-52.
14. Morris AA, Kalz GG, Lotspeich ES. Ependymitis and meningitis due to *Candida (Monilia) albicans*. *Arch Neurol Psychiatry* 1945;54:361-6.
15. Belisle G, Lachance W, Leblanc C. [Meningitis caused by *Candida albicans*. Report of a case and discussion]. *Union Med Can* 1968;97:710-5.
16. Black JT. Cerebral candidiasis: case report of brain abscess secondary to *Candida albicans*, and review of literature. *J Neurol Neurosurg Psychiatry* 1970;33:864-70.
17. Germain M, Gourdeau M, Hebert J. Case report: familial chronic mucocutaneous candidiasis complicated by deep candida infection. *Am J Med Sci* 1994;307:282-3.
18. Hamel N, Feng BJ, Foretova L, Stoppa-Lyonnet D, Narod SA, Imyanitov E, et al. On the origin and diffusion of BRCA1 c.5266dupC (5382insC) in European populations. *Eur J Hum Genet* 2011;19:300-6.
19. Gross O, Gewies A, Finger K, Schäfer M, Sparwasser T, Peschel C, et al. Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature* 2006;442:651-6.
20. Esnault S, Malter JS. Extracellular signal-regulated kinase mediates granulocyte-macrophage colony-stimulating factor messenger RNA stabilization in tumor necrosis factor- α plus fibronectin-activated peripheral blood eosinophils. *Blood* 2002;99:4048-52.
21. Meja KK, Seldon PM, Nasuhara Y, Ito K, Barnes PJ, Lindsay MA, et al. p38 MAP kinase and MKK-1 co-operate in the generation of GM-CSF from LPS-stimulated human monocytes by an NF- κ B-independent mechanism. *Br J Pharmacol* 2000;131:1143-53.
22. Anborgh PH, Qian X, Papageorge AG, Vass WC, DeClue JE, Lowy DR. Ras-specific exchange factor GRF: oligomerization through its Dbl homology domain and calcium-dependent activation of Raf. *Mol Cell Biol* 1999;19:4611-22.
23. Jia XM, Tang B, Zhu LL, Liu YH, Zhao XQ, Gorjestani S, et al. CARD9 mediates Dectin-1-induced ERK activation by linking Ras-GRF1 to H-Ras for antifungal immunity. *J Exp Med* 2014;211:2307-21.
24. Smeekens SP, Ng A, Kumar V, Johnson MD, Plantinga TS, van Diemen C, et al. Functional genomics identifies type I interferon pathway as central for host defense against *Candida albicans*. *Nat Commun* 2013;4:1342.
25. Duffy D, Rouilly V, Libri V, Hasan M, Beitz B, David M, et al. Functional analysis via standardized whole-blood stimulation systems defines the boundaries of a healthy immune response to complex stimuli. *Immunity* 2014;40:436-50.
26. Francisco-Cruz A, Aguilar-Santelises M, Ramos-Espinosa O, Mata-Espinosa D, Marquina-Castillo B, Barrios-Payan J, et al. Granulocyte-macrophage colony-stimulating factor: not just another haematopoietic growth factor. *Med Oncol* 2014;31:774.
27. McGeachy MJ. GM-CSF: the secret weapon in the T(H)17 arsenal. *Nat Immunol* 2011;12:521-2.
28. Lantermier F, Cypowij S, Picard C, Bustamante J, Lortholary O, Casanova JL, et al. Primary immunodeficiencies underlying fungal infections. *Curr Opin Pediatr* 2013;25:736-47.
29. Goodridge HS, Shimada T, Wolf AJ, Hsu YM, Becker CA, Lin X, et al. Differential use of CARD9 by dectin-1 in macrophages and dendritic cells. *J Immunol* 2009;182:1146-54.
30. Yang J, Chai L, Gao C, Fowles TC, Alipio Z, Dang H, et al. SALL4 is a key regulator of survival and apoptosis in human leukemic cells. *Blood* 2008;112:805-13.
31. Scriver CR. Human genetics: lessons from Quebec populations. *Annu Rev Genomics Hum Genet* 2001;2:69-101.
32. Borha A, Parienti JJ, Emery E, Coskun O, Khouri S, Derlon JM. [*Candida albicans* cerebral granuloma in an immunocompetent patient. A case report]. *Neurochirurgie* 2009;55:57-62.
33. Carra Dalliere C, Thouvenot E, Baptista G, Le Moing V, Charif M. [Meningomyelomalacia in an immunocompetent patient]. *Rev Neurol (Paris)* 2010;166:741-4.
34. Hoarau G, Kerdraon O, Lagree M, Vinchon M, François N, Dubos F, et al. Detection of (1,3)-beta-D-glucans in situ in a *Candida albicans* brain granuloma. *J Infect* 2013;67:622-4.
35. Torres JM, Martinez-Barricarte R, García-Gómez S, Mazariegos MS, Itan Y, Boisson B, et al. Inherited BCL10 deficiency impairs hematopoietic and nonhematopoietic immunity. *J Clin Invest* 2014;124:5239-48.
36. Jabara HH, Ohsumi T, Chou J, Massaad MJ, Benson H, Megarbane A, et al. A homozygous mucosa-associated lymphoid tissue 1 (MALT1) mutation in a family with combined immunodeficiency. *J Allergy Clin Immunol* 2013;132:151-8.
37. McKinnon ML, Rozmus J, Fung SY, Hirschfeld AF, Del Bel KL, Thomas L, et al. Combined immunodeficiency associated with homozygous MALT1 mutations. *J Allergy Clin Immunol* 2014;133:1458-62, e1-7.
38. Punwani D, Wang H, Chan AY, Cowan MJ, Mallott J, Sunderam U, et al. Combined immunodeficiency due to MALT1 mutations, treated by hematopoietic cell transplantation. *J Clin Immunol* 2015;35:135-46.
39. Ruping MJ, Vehreschild JJ, Groll A, Lass-Flörl C, Ostermann H, Ruhnke M, et al. Current issues in the clinical management of invasive aspergillosis—the AGIHO, DMykG, OGMM and PEG web-based survey and expert consensus conference 2009. *Mycoses* 2011;54:e557-68.
40. Liang P, Wang X, Wang R, Wan Z, Han W, Li R. CARD9 deficiencies linked to impaired neutrophil functions against *Phialophora verrucosa*. *Mycopathologia* 2015;179:347-57.
41. Grumach AS, de Queiroz-Telles F, Migaud M, Lantermier F, Filho NR, Palma SM, et al. A homozygous CARD9 mutation in a Brazilian patient with deep dermatophytosis. *J Clin Immunol* 2015;35:486-90.
42. Guerrero C, Rojas JM, Chedid M, Esteban LM, Zimonjic DB, Popescu NC, et al. Expression of alternative forms of Ras exchange factors GRF and SOS1 in different human tissues and cell lines. *Oncogene* 1996;12:1097-107.

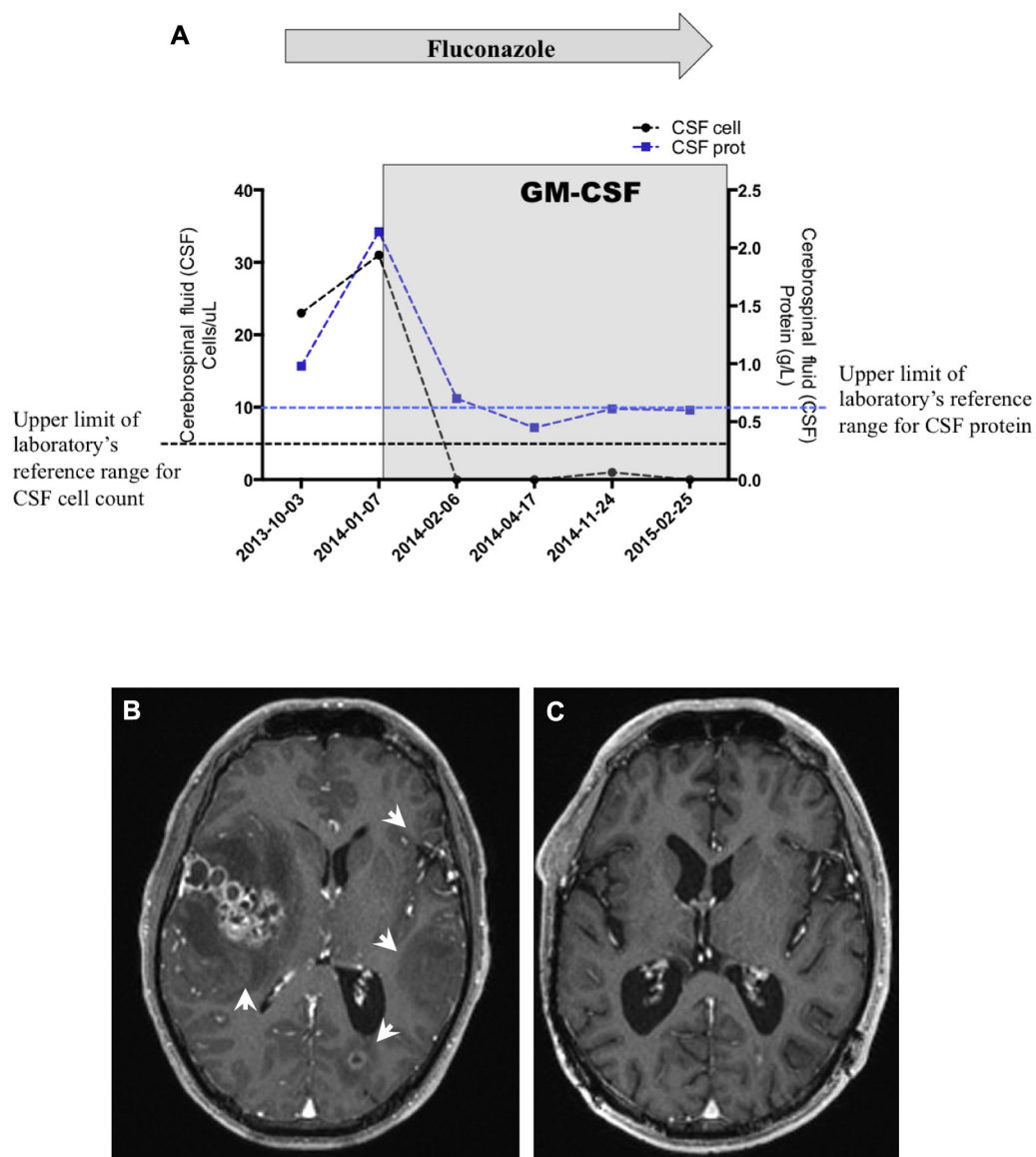


FIG E1. A, Improvement in P2 with adjunctive rhGM-CSF therapy, with serial lumbar punctures demonstrating sustained sterility and normalized parameters of CSF. B and C, Cerebral images obtained on presentation of P2 with diffuse involvement (arrowheads; Fig E1, B) and resolution of lesions on rhGM-CSF therapy (Fig E1, C).

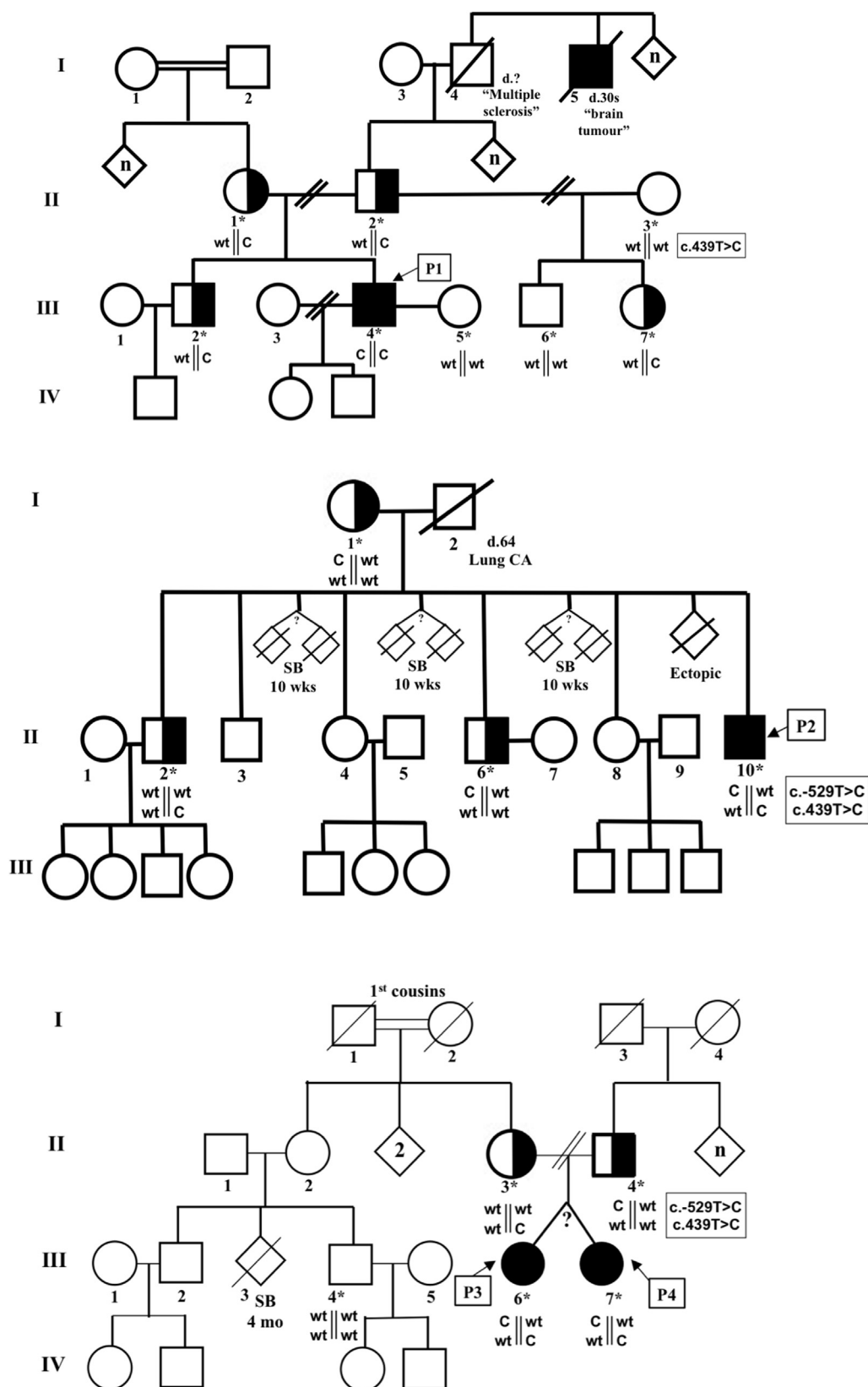


FIG E2. Pedigrees of FC CARD9-deficient patients. Proband are indicated by arrows. Asterisks indicate subjects evaluated and sequenced for CARD9.

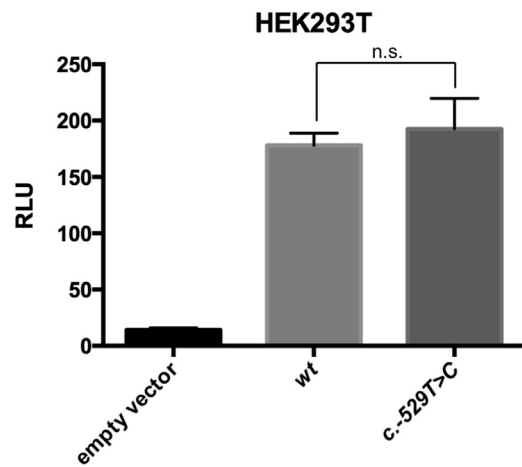


FIG E3. The c.-529T>C variant does not impair *CARD9* expression. HEK293T cells were transfected with a luciferase reporter vector harboring the WT putative promoter region of *CARD9* (ie, 1214 bp upstream of the first exon) or the c.-529T>C variant created by means of site-directed mutagenesis. Luciferase values were normalized to a cotransfection control. *n.s.*, Nonsignificant; *RLU*, relative light units.

Marker	Distance to CARD9 (bp)	Exon 3 (p.Y91H) mutation					Putative promoter region (c.-529T>C)		
		Fam 1 - Chr 1	Fam 1 - Chr 2	Fam 2	Fam 3	Control B5	Fam 2	Fam 3	Control B4
D9S282	12447510	3	2	3	3	1,5	1	1	2
D9S290	7728903	4	2	1	3	1,4	5	2	2*
D9S1863	5756510	6	3	1	5	3	4	5	4*
D9S164	3000422	7	5	7	6	3,10	6	1	1*
D9S1793	2858119	7	8	7	7	7*	7	8	8*
D9S1826	808031	1	1	1	2	1,2	1	2	1,2
CARD9-MS1	721910	7	7	7	7	7*	8	8	8*
D9S158	157173	4	4	4	4	4	5	5	5*
CARD9-MS5	37825	7	7	7	7	7*	8	8	8*
CARD9		Ex3	Ex3	Ex3	Ex3	Ex3	Int0	Int0	Int0
CARD9-MS6	556325	4	4	1	1	1	5	5	5*
D9S905	618870	1	1	2	2	1,2	1	1	1
D9S1838	1380361	8	8	7	7	7*	12	12	12*

FIG E4. Haplotype analysis demonstrating founder effect. For the p.Y91H mutation in exon 3, the conserved region includes 3 markers in all families (*solid box*) and 6 markers in families 2, 3, and control B5 (*dashed box*). The maximum conserved region for all chromosomes (*solid box*) is 808 kb. For the c.-529T>C variant, the conserved region involves 6 markers (*solid box*). Markers 5' of the short tandem repeat marker CARD9-MS1 suggest one recombination event in ancestors of family 2 up to D9S164, with the ancestral alleles on the other 2 chromosomes in the *dashed box*. These findings support a founder origin for each mutation. For the control subjects, numbers with *asterisks* are alleles consistent with conservation but for which the individual was heterozygous; therefore it is possible that a different allele could be linked because phase is unknown. Both alleles are shown in situations in which both alleles are consistent with conservation or in which neither allele is consistent with conservation.

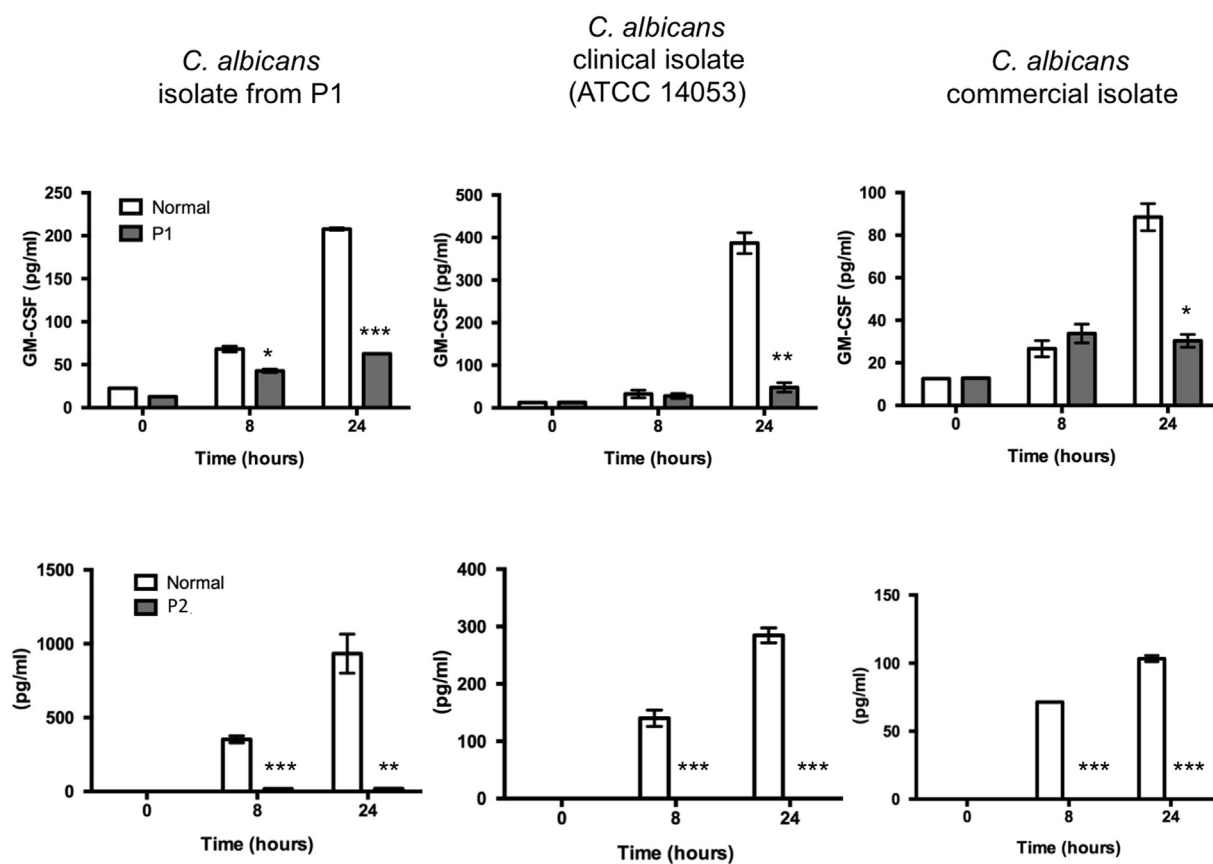


FIG E5. Impaired GM-CSF responses to different *C. albicans* strains. Patient-derived PBMCs were stimulated with a heat-killed *C. albicans* strain isolated from P1, a heat-killed clinical strain of *C. albicans* (ATCC 14053), and a commercial heat-killed *C. albicans* preparation. Supernatants were collected at the indicated time points and analyzed for GM-CSF production. * $P < .05$, ** $P < .01$, and *** $P < .001$.

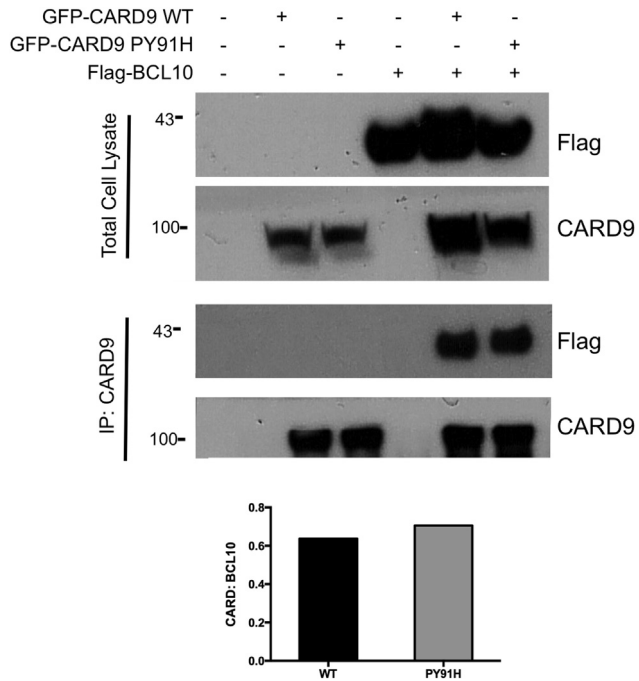


FIG E6. The p.Y91H mutation does not impair CARD9 binding to BCL10. HEK293T cells were transfected, as indicated. Cell lysates were immunoprecipitated (*IP*) with anti-CARD9 antibody and analyzed by means of immunoblotting with the indicated antibodies. Densitometry of BCL10 relative to CARD9 shows no difference in binding between the WT and mutant. A single experiment representative of 3 independent experiments performed is shown.

TABLE E1. Identified SNPs in the *CARD9* locus in our cohort

Chromosome position	Reference allele	Observed	Known SNP?	MAF	Location	P1	P2	Brother of P2 (II-6)	Mother of P2	P3	P4
139270147	A	A/G	rs3088081	G: 39.940% (931/2,331)	5' Promoter	ND	+/-	+/-	+/-	+/-	+/-
139269905	T	C/T	rs3812560	C: 38.761% (1,614/4,164)	5' Promoter	ND	+/-	+/-	+/-	+/-	+/-
139269483	G	A/G	rs3829111	A: 36.231% (942/2,600)	5' Promoter	ND	+/-	+/-	+/-	+/-	+/-
139269336	C	C/T	rs10781500	T: 34.966% (1,456/4,164)	5' Promoter	ND	+/-	+/-	+/-	+/-	+/-
139269196	A	A/G	rs3829110	G: 35.450% (882/2,488)	5' Promoter	ND	+/-	+/-	+/-	+/-	+/-
139268495	T	C/T*	No	NA	5' Promoter	+/-	+/-	+/-	+/-	+/-	+/-
139267533	C	C/T	rs4078099	A: 36.758% (805/2,190)	Intron 1	+/-	+/-	+/-	+/-	+/-	+/-
139266697	T	C/T	rs4075760	C: 36.712% (804/2,190)	Intron 1	+/-	+/-	+/-	+/-	+/-	+/-
139266496	G	G/A	rs4077515	A: 36.635% (3,815.150/10,414)	Exon 2	+/-	+/-	+/-	+/-	+/-	+/-
139266405	C	C/T	rs10781499	A: 36.563% (3,614.620/9,886)	Exon 2	+/-	+/-	+/-	+/-	+/-	+/-
139266221	C	C/T	rs4075078	A: 17.368% (380/2,188)	Intron 2	+/-	+/-	+/-	+/-	+/-	+/-
139265827	T	C/T	No	NA	Exon 3	-/-	+/-	+/+	+/+	+/-	+/-
139263596	A	A/C	rs11145763	C: 36.459% (797/2,186)	Intron 7	ND	+/-	ND	ND	ND	ND
139261933	G	A/G	rs3812555	T: 23.365% (972/4,160)	Intron 8	+/+	+/-	+/-	+/-	+/-	+/-
139261268	C	C/T	No	NA	Exon 10	-/-	+/-	+/+	+/+	+/-	+/-
139261086	T	C/T	rs10781496	G: 35.249% (1,472/4,176)	Intron 10	+/+	+/-	+/-	+/-	+/-	+/-
139259413	C	/A/AGG (3-bp insertion)	rs200873436	A: 32.507% (708/2,178)	Intron 11	+/+	+/-	+/-	+/-	+/-	+/-
139259349	T	C/T	rs4073153	C: 39.323% (860/2,187)	Intron 11	+/+	+/-	+/-	+/-	+/-	+/-
139259344	G	G/C	rs181417685	G: 0.092% (2/2,179)	Intron 11	+/+	+/-	+/-	+/-	+/-	+/-
139259249	T	C/T	rs11145750	G: 37.900% (1,563/4,124)	Intron 11	+/+	+/-	+/-	+/-	+/-	+/-
139258462	T	C/T	rs1135314	C: 25.220% (658/2,609)	e13 (3' UTR)	+/+	+/-	+/-	+/-	+/-	+/-

NA, Not available; ND, not determined; UTR, untranslated region.

*Unique variant, common to P2 to P4, and not a known SNP (dbSNP build 141).