

GM-CSF therapy in human caspase recruitment domain-containing protein 9 deficiency



To the Editor:

Human caspase recruitment domain-containing protein 9 (CARD9) deficiency is associated with spontaneous development of fungal infections that uniquely target the central nervous system (CNS).¹ Recently, we showed that the CNS fungal infection observed in patients with CARD9 deficiency is in part due to a defect in production of CXC neutrophil chemoattractants, leading to a severe reduction in protective neutrophil recruitment to the CNS that was not due to defects in intrinsic neutrophil chemotaxis or neutrophil survival.² This CNS-specific neutropenia observed in our CARD9-deficient patient with homozygous c.170G>A (p.R57H) *CARD9* mutations was replicated in *Card9*^{-/-} mice, which have uncontrolled fungal growth in the brain associated with *Candida* species- and brain-specific impairment in neutrophil recruitment.²

Recent reports by the Vinh group^{3,4} have proposed the use of recombinant GM-CSF as a salvage adjunct immune-based treatment for human CARD9 deficiency based on an impaired fungus-related GM-CSF response observed *in vitro*; indeed, from a cohort of French-Canadian patients that carry the “signature” p.Y91H *CARD9* missense mutation (n = 4), 2 with disease recalcitrant to antifungal therapy alone achieved clinical remission with GM-CSF therapy and had relapse of infection on discontinuation of GM-CSF. The pathway by which CARD9 leads to production of GM-CSF might relate to modular complexing with H-RAS and Ras protein specific guanine nucleotide releasing factor 1 (RASGRF-1) for downstream extracellular signal-regulated kinase (ERK) activation, which were defective in the French-Canadian patient myeloid cells and in a cell line transfected with the p.Y91H *CARD9* mutation.^{3,4}

In the present study we used GM-CSF to treat a patient with distinct homozygous *CARD9* missense mutations (p.R57H)² who had *Candida* species-related meningoencephalitis and did not observe a clinical benefit nor did the patient experience infection relapse on discontinuation of GM-CSF. To examine the reasons for the lack of GM-CSF response in our patient, we investigated the H-RAS–RASGRF-1–ERK pathway in our patient’s myeloid cells and in transfected cells. Furthermore, we evaluated whether exogenous GM-CSF administration promoted protective neutrophil recruitment to the CNS in our CARD9-deficient patient and in *Card9*-deficient mice.

We previously described a CARD9-deficient patient who is homozygous for the p.R57H missense mutation, which results in expression of nonfunctional full-length CARD9 protein by myeloid cells.² Similar to the French-Canadian cohort of CARD9-deficient patients, our patient presented with spontaneous *Candida* species infection of the CNS with associated osteomyelitis,^{2,4} and her PBMCs exhibited reduced GM-CSF production in response to fungal ligands as assessed by means of *in vitro* restimulation experiments.² Briefly, the patient presented at the National Institutes of Health in early December 2013 at age 10 years with malaise, headache, nausea, and vomiting caused by *Candida albicans*-related meningoencephalitis. The infection was initially diagnosed in mid-2012 after symptoms of fever, headache, back pain, and

vomiting, but the patient had multiple interruptions of antifungal therapy since then.² Extensive radiographic abnormalities included pachymeningitis, a brain abscess, cervical spine osteomyelitis and syrinx, and an enlarged fourth ventricle.² The β -D-glucan level in CSF was greater than 500 pg/mL, reflecting marked fungal load in the CNS. She was treated initially with liposomal amphotericin B (3 mg/kg/d), 5-flucytosine (25 mg/kg every 6 hours), and voriconazole (8 mg/kg every 12 hours), with marked improvement in her symptoms. She was transitioned to voriconazole and 5-flucytosine in late December 2013, followed by high-dose fluconazole (12 mg/kg/d) in May 2014.

After the presentation by Gavino et al³ of their initial experience with GM-CSF, because our patient still had significant residual radiographic and CSF microbiological (β -D-glucan) and laboratory (cell count and protein) abnormalities (Fig 1, A–D), we added adjunct GM-CSF and monitored clinical, radiographic, and CSF microbiological (β -D-glucan) and laboratory (cell count and protein) responses. The patient received 200 μ g of GM-CSF (sargramostim) from May 2014 to August 2015. As expected, therapy induced significant peripheral neutrophilia and eosinophilia (Fig 1, A). Although eosinophil counts were increased in the infected CSF (range, 8–84 cells/ μ L), GM-CSF treatment did not alter the patient’s CSF neutropenia (range, 0–3 cells/ μ L; Fig 1, B) and did not decrease CSF protein levels (range, 502–600 mg/dL; Fig 1, C). Strikingly, the patient’s fungal load, as measured based on CSF β -D-glucan levels, did not decrease during GM-CSF therapy but instead further increased (Fig 1, D).

In July 2014, 2 months after GM-CSF initiation, the patient had worsening headaches with progressive enlargement of the fourth ventricle caused by ventricular foramen obstruction, both proximally and distally for which a fourth ventricular shunt was placed (Fig 1, E). In July 2015, the patient underwent a revision of the fourth ventricle shunt secondary to the trapped fourth ventricle. On brain biopsy at that time, fungal elements were noted without a tissue neutrophilic response; instead, we observed abundant eosinophils and granulomatous inflammation (Fig 1, F). We discontinued GM-CSF (after 442 days of therapy) because of a lack of apparent clinical and microbiological responses and because of the concern of potential eosinophil-mediated immunopathology in the CNS given the marked expansion of eosinophils in the CSF and brain tissue during treatment and trapped fourth ventricle. After GM-CSF discontinuation, peripheral white blood cell counts returned to pre-GM-CSF levels (Fig 1, A), with rare eosinophils and no neutrophils observed in the lumbar puncture performed 4 months after cessation of GM-CSF treatment. Moreover, no clinical worsening or ventricle entrapment has been noted during the greater than 2.5 years after GM-CSF discontinuation. The patient currently receives high-dose fluconazole treatment and is clinically stable without symptoms. The size of the fourth ventricle has decreased and slow improvement in the chronic meningeal radiographic abnormalities is seen. Thus in our patient GM-CSF did not show a therapeutic benefit, and no clinical relapse was noted after drug discontinuation.

Because our patient’s response to GM-CSF was apparently different from that seen in the CARD9-deficient French-Canadian patients carrying the p.Y91H mutation, we assessed whether the H-RAS–RASGRF-1–ERK pathway, which was defective in the French-Canadian patients, was also impaired in our patient.⁴ To

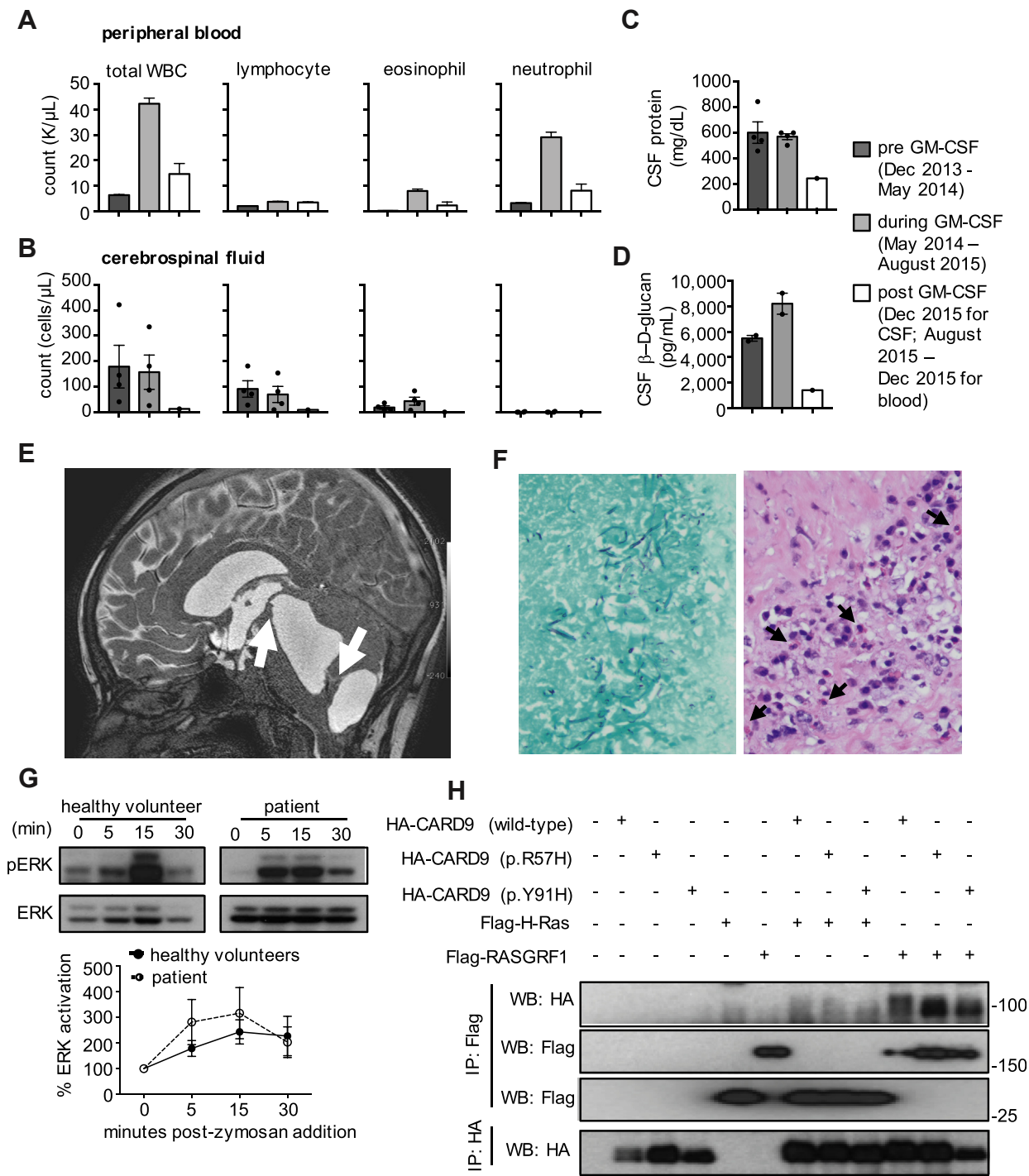


FIG 1. Patient response to GM-CSF treatment and effects of the c.170G>A (p.R57H) *CARD9* mutation on the H-RAS–RASGRF-1–ERK axis. **A** and **B**, Total WBC, lymphocyte, eosinophil, and neutrophil counts in the peripheral blood (Fig 1, A) and cerebrospinal fluid (CSF; Fig 1, B) for our *CARD9*-deficient patient before, during, and after GM-CSF therapy. **C** and **D**, Protein (Fig 1, C) and β -D-glucan (Fig 1, D) concentrations in CSF before, during, and after GM-CSF therapy. **E**, Magnetic resonance imaging scan of the patient in July 2014 (during GM-CSF treatment). Arrows denote proximal and distal fourth ventricular foraminal obstruction. **F**, Brain biopsy specimen from our *CARD9*-deficient patient in July 2015 (during GM-CSF treatment) showing fungal invasion (GMS stain, left), lymphoplasmacytic infiltration, the presence of eosinophils (black arrows, right image), and the absence of neutrophils (hematoxylin and eosin stain, right). **G**, ERK phosphorylation in CD14⁺ monocytes stimulated with depleted zymosan (62.5 μ g/mL) for 5, 15, and 30 minutes. Western blots shown are representative of 4 independent experiments; pooled data are shown in the graph. **H**, HEK293T cells were transfected with hemagglutinin (HA)-tagged WT, p.R57H *CARD9*, or p.Y91H *CARD9* plasmids with or without Flag-tagged H-Ras or RASGRF-1. Cell lysates were immunoprecipitated (IP) with anti-Flag antibody or anti-hemagglutinin antibody, followed by immunoblotting (Western blotting [WB]) against the indicated antibodies (HA and Flag). Images are representative of 6 independent experiments.

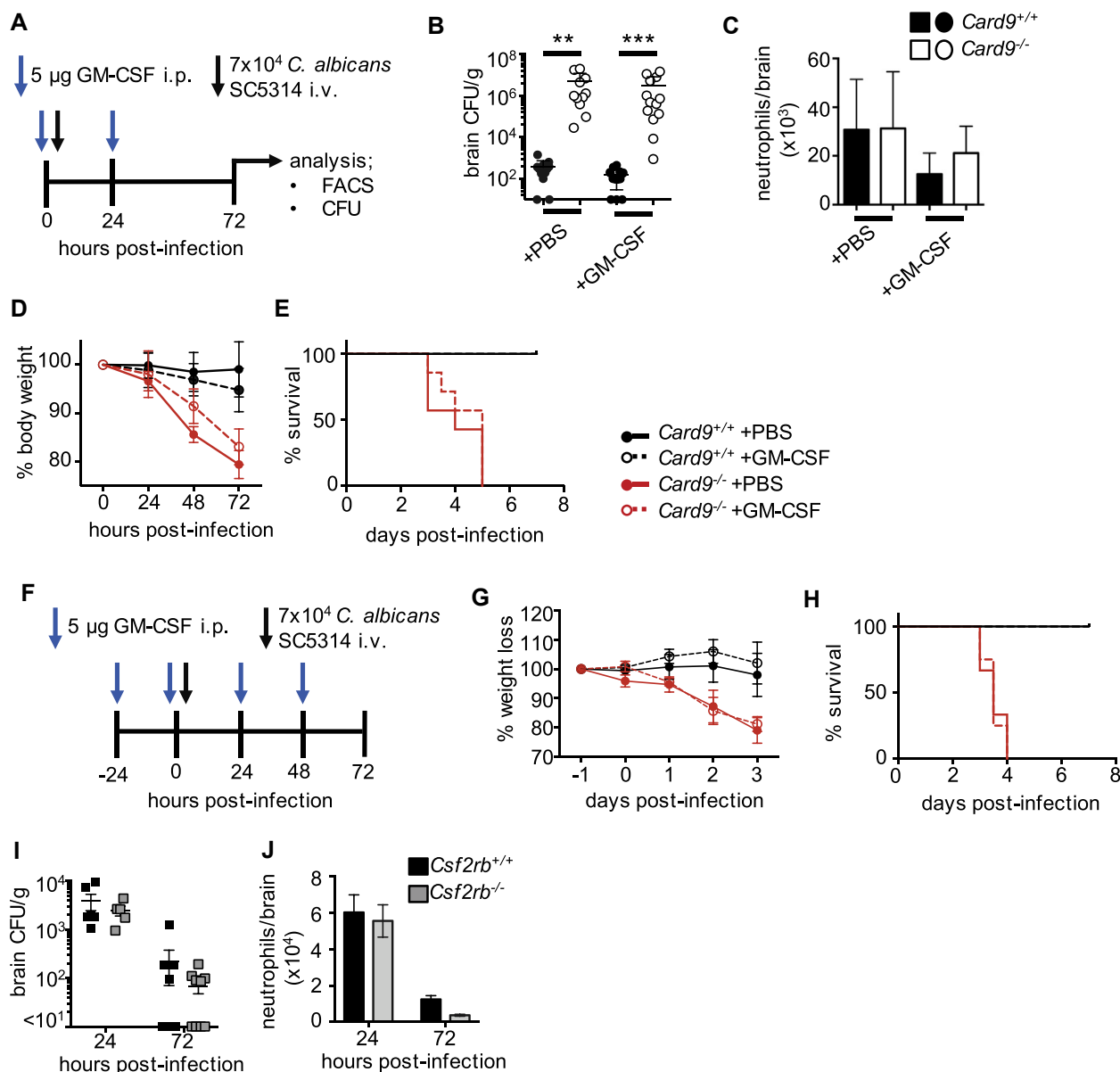


FIG 2. Exogenous GM-CSF treatment or ablation of GM-CSF signaling has no effect on the outcome of acute *C. albicans* CNS infections in mice. **A**, Schematic showing GM-CSF treatment regimen. **B** and **C**, WT (solid circles, black lines) and *Card9*^{-/-} (open circles, red lines) mice were treated with PBS (solid lines) or 5 µg of GM-CSF (broken lines) and analyzed for brain fungal burden (Fig 2, **B**) and neutrophil recruitment (Fig 2, **C**) to the brain at 72 hours after infection (n = 5-6 per group). **P < .01 and ***P < .005, 2-tailed Student t test. **D** and **E**, Weight loss (Fig 2, **D**) and survival (Fig 2, **E**) of WT and *Card9*^{-/-} mice treated with either PBS (WT, n = 6; *Card9*^{-/-}, n = 7; ***P < .001, log-rank test) or GM-CSF (WT, n = 6; *Card9*^{-/-}, n = 7; ***P < .001, log-rank test) by using the regimen shown in Fig 2, **A**. Data were pooled from 2 independent experiments. **F**, Schematic showing an extended GM-CSF treatment regimen; animals were treated 24 hours before infection and then every 24 hours thereafter. **G** and **H**, Weight loss (Fig 2, **G**) and survival (Fig 2, **H**) of WT and *Card9*^{-/-} mice treated with either PBS (WT, n = 4; *Card9*^{-/-}, n = 3; *P < .01, log-rank test) or GM-CSF (WT, n = 4; *Card9*^{-/-}, n = 4; **P < .005, log-rank test) by using the regimen shown in Fig 2, **F**. Data are from 1 experiment. **I** and **J**, Fungal burdens (Fig 2, **I**) and neutrophil recruitment (Fig 2, **J**) to the brain in infected (dose, 1.3 × 10⁵) *Csf2rb*^{-/-} mice (n = 6-8 per group). Data were pooled from 2 independent experiments.

examine this, we isolated CD14⁺ monocytes from our patient and healthy volunteers and stimulated them *ex vivo* with depleted zymosan to activate the cells specifically through the C-type lectin receptor Dectin-1. In contrast to the French-Canadian

patients, our patient's monocytes induced ERK phosphorylation normally (Fig 1, **G**).

We next assessed the interaction of CARD9 with RASGRF-1 and H-Ras using an immunoprecipitation approach, directly

comparing cells transfected with wild-type (WT) *CARD9* or *CARD9* carrying the p.R57H mutation (our patient) or the p.Y91H mutation (French-Canadian cohort). We found a modest decrease in the interaction of p.Y91H *CARD9* with RASGRF-1 that was in line with previous findings⁴; instead, the interaction of p.R57H *CARD9* with RASGRF-1 was intact (Fig 1, H). We found minimal interactions of *CARD9* with H-Ras, and this appeared similar in patients with both mutations (Fig 1, H). Therefore despite both the p.Y91H and p.R57H mutations resulting in reduced GM-CSF responses on fungal stimulation *ex vivo*,^{2,3} our data show that the p.R57H *CARD9* missense mutation does not affect the H-RAS–RASGRF-1–ERK pathway in monocytes and cannot be rescued by adjunctive GM-CSF therapy. Hence future work will be required to examine the H-RAS–RASGRF-1–ERK-independent pathway or pathways that might account for impaired GM-CSF production on fungal stimulation in *CARD9*-deficient myeloid cells from different mutations. Additional studies will also be required to investigate the H-RAS–RASGRF-1–ERK response and GM-CSF clinical effect in *CARD9*-deficient patients with different mutations.

To further examine the potential therapeutic role of GM-CSF against fungal disease in the setting of *CARD9* deficiency in a more controlled setting, we used *Card9*^{−/−} mice infected systemically with *C albicans*. Although *Card9*^{−/−} mice have the first exon of *Card9* deleted⁵ and do not carry the p.Y91H or p.R57H mutations, we have shown previously that these animals phenocopy the susceptibility to CNS candidiasis, as observed in *CARD9*-deficient patients, and mimic many aspects of the pathology observed in patients.²

First, we measured GM-CSF concentrations in naive and infected organs in WT and *Card9*^{−/−} mice. We were unable to detect GM-CSF protein or transcript in the brain for either mouse strain, and thus GM-CSF was not induced by acute fungal infection in this tissue (data not shown). We found that GM-CSF concentrations in the kidney, another target organ of *C albicans*, did not increase significantly with infection, although we did note a reduction in GM-CSF in *Card9*^{−/−} mice at 72 hours after infection (see Fig E1 in this article's Online Repository at www.jacionline.org).

We next assessed whether exogenous GM-CSF treatment in WT and *Card9*^{−/−} mice could improve outcomes after systemic *C albicans* challenge (Fig 2, A) using a therapeutic scheme previously shown to reverse immunologic defects mapping to GM-CSF production in *C albicans*-infected mice.⁶ GM-CSF treatment did not reduce fungal burden in the brain or any other organ analyzed (Fig 2, B, and see Fig E2 in this article's Online Repository at www.jacionline.org) nor did it increase neutrophil recruitment into the *Card9*^{−/−} brain at day 3 after infection, a time at which WT mice have controlled fungal invasion, whereas *Card9*^{−/−} mice exhibit inexorable fungal brain growth without neutrophil recruitment (Fig 2, C).² In line with this, we did not observe any reductions in the rapid weight loss (Fig 2, D) and increased mortality (Fig 2, E) observed in infected *Card9*^{−/−} mice treated with GM-CSF by using either the aforementioned regimen (Fig 2, A) or an enhanced alternative scheme that delivered more GM-CSF (Fig 2, F–H). We confirmed that the administered GM-CSF was biologically active by phosphorylated signal transducer and activator of transcription 5 (pSTAT5) staining in stimulated granulocytes (see Fig E3 in this article's Online Repository at www.jacionline.org).

Given these results, we next examined the direct role of GM-CSF signaling during CNS candidiasis. We infected *Csf2rb*^{−/−} mice, which are deficient in the GM-CSF receptor and thus unable to respond to GM-CSF. Notably, we found that these animals did not phenocopy the CNS susceptibility of *Card9*^{−/−} mice because they recruited normal numbers of neutrophils and controlled fungal proliferation in the brain similar to WT (Fig 2, I and J). Therefore these data demonstrate the dispensable role of GM-CSF in control of acute CNS *C albicans* infections in mice.⁷ In agreement with this, patients with neutralizing autoantibodies against GM-CSF and those with germline mutations in the α subunit of the GM-CSF receptor (*CSF2RA*) have not been reported thus far to have spontaneous systemic *Candida* species infection in the CNS or elsewhere.^{8,9}

In summary, our results show that human *CARD9* deficiency caused by homozygous c.170G>A (p.R57H) *CARD9* mutations does not impair the H-RAS–RASGRF-1–ERK pathway and is not responsive to adjunct GM-CSF therapy, despite impaired GM-CSF production by the patients' myeloid cells on *in vitro* fungal stimulation.² The contrasting responses of the French-Canadian patients and our *CARD9*-deficient patient to GM-CSF could be associated with other *CARD9*-independent genetic or molecular defects; variations in the onset, chronicity, and severity of fungal infection; or fungal strain virulence. These are important factors for future study. Indeed, human *CARD9* deficiency is a highly heterogeneous disease associated with a wide array of clinical phenotypes, making patient care challenging.¹ Future work should aim to further characterize the immunologic defects associated with human *CARD9* deficiency caused by different mutations, which might lead to immune-based GM-CSF-dependent or other therapeutic strategies and immunologic readouts to predict clinical responses for patients with *CARD9* deficiency.

When physicians take care of *CARD9*-deficient patients with *Candida* species-related meningoencephalitis, it is important to (1) aggressively institute intensive antifungal treatment early to avoid infection-related structural brain abnormalities that require shunt placement and (2) to use adjunct GM-CSF with the understanding that different patients can exhibit differential responses to GM-CSF treatment, including awareness for the potential of promoting eosinophil-mediated CNS immunopathology, particularly in patients with high-burden CNS fungal infection and/or infection-related structural brain abnormalities (hydrocephalus and trapped ventricles) at treatment onset.

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Intralymphatic immunotherapy with 2 concomitant allergens, birch and grass: A randomized, double-blind, placebo-controlled trial



To the Editor:

Allergen immunotherapy improves symptoms and prevents disease progression in patients with allergic rhinitis. However, enthusiasm for this therapy is dampened by the risk of allergic side effects, as well as the long treatment duration, namely 3 to 5 years of repeated subcutaneous injections or daily sublingual tablet administration.¹ Hence intralymphatic administration of the vaccine has been suggested as an alternative therapeutic option with less side effects and a much shorter treatment duration.² In intralymphatic immunotherapy (ILIT) a low dose of allergen is injected in a groin lymph node every fourth week, in total 3 injections, 8 weeks. To date, a small number of

size-limited trials have been conducted. Most,^{3,4} but not all,⁵ trials have supported the concept of ILIT.

A majority of patients with allergic rhinitis are polysensitized.¹ This is the first randomized, double-blind, placebo-controlled study that investigates ILIT with more than 1 allergen at the same time. This is also the first trial to investigate the mechanisms behind tolerance development through direct evaluation of tolerance-inducing cells derived from lymph nodes.

The study was conducted during 2012-2015 at 2 separate Ear, Nose, and Throat Departments in Sweden. Sixty patients between 18 and 55 years old with moderate-to-severe allergic rhinitis to both birch and grass pollen were included. The study was approved by the local ethics committee, registered with ClinicalTrials.gov ID 2009-016815-39, and conducted according to good clinical practice guidelines. All participants provided written informed consent.

Patients were randomized 1:1 to either a combination of ALK Alutard 5-grasses and ALK Alutard Birch (ALK-Abelló, Hørsholm, Denmark) or double placebo. For each allergen, 0.1 mL of 10,000 SQ-U (1000 SQ-U) was injected with ultrasonographic guidance in the inguinal lymph nodes (1 allergen in each side). Three injections were administered at 4-week intervals. Baseline characteristics and demographics were the same for both groups. A more detailed account of the methods and results can be found in the Methods and Results sections in this article's Online Repository at www.jacionline.org, including Table E1 for baseline characteristics and Fig E1 for a diagram of the patient flow.

The treatment was generally well tolerated, with some mild-to-moderate reactions⁶ in the active group. One patient discontinued participation after an episode of reactivated genital herpes. No other severe reactions were recorded. See Fig 1, A; Table I; and Fig E2 in this article's Online Repository at www.jacionline.org for more details.

According to the study design, changes in the local nasal response to timothy allergen was the primary treatment outcome. A nasal provocation test (NPT) was performed before and after treatment with 0.1 mL of ALK Aquagen SQ Timothy (10,000 SQ-U/mL) in each nostril. Nasal and ocular symptoms were scored as 0 to 3 points during the first 30 minutes. In the active group the total symptom score was significantly reduced by 28% at 6 to 9 months after treatment, from a mean score of 127.3 to 86.6 (SD, 62.3; 95% CI, 8.1-62.1; $P < .05$; $n = 23$). The corresponding reduction in the placebo group was 12%, from a mean score of 115.7 to 102.3 (SD, 59.5; 95% CI, -10.7 to 37.4; $P = .89$; $n = 26$; Fig 1, C), and did not reach statistical significance. Consequently, active ILIT showed a 16% relative reduction over placebo. Evaluated separately, each of the nasal symptoms of runny, blocked, and itchy nose was also reduced in the active group. The sum of the nasal symptoms was reduced by 25% at 6 to 9 months after active treatment (Fig 1, D). No such changes were seen in the placebo group.

For logistical reasons, birch NPTs were not included in the study design. Daily symptoms and medication diaries are presently considered the most effective way of evaluating the clinical efficacy of allergic rhinitis treatments. However, surrogate markers, such as NPT responses, are well acknowledged for early proof-of-concept or dose-finding studies, such as the present study.⁷ See Table E2 in this article's Online Repository at www.jacionline.org for details on complete NPT results.

METHODS

Study approval

The CARD9-deficient patient and healthy volunteers were enrolled in protocols approved by the National Institute of Allergy and Infectious Diseases' and National Cancer Institute's Institutional Board Review committees and provided written informed consent for participation in the study. This study was conducted in accordance with the Helsinki Declaration.

Monocyte isolation and zymosan stimulations

Peripheral blood was collected from the CARD9-deficient patient and healthy volunteers, and CD14⁺ monocytes were negatively selected from the PBMC fraction by using magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were plated into 96-well round-bottom plates (1×10^6 cells/well) in RPMI without serum and starved for 3 hours at 37°C. At indicated time points (see figure legends), depleted zymosan (Invitrogen, Carlsbad, Calif) was added to the monocytes at a final concentration of 62.5 µg/mL. After stimulation, cells were moved to ice-cold Eppendorf tubes (3×10^6 cells per experimental condition), spun down, and lysed on ice in RIPA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 140 mmol/L NaCl) supplemented with protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail; Thermo Fisher Scientific, Waltham, Mass). Cell lysates were sonicated for 30 seconds and centrifuged at 13,000 rpm for 10 minutes at 4°C, and each supernatant was mixed with equal amounts of 2× SDS sample buffer. Samples were stored at −20°C until further analysis.

Histopathology

Brain biopsy samples from our CARD9-deficient patient were removed at indicated time points relative to GM-CSF therapy and fixed in 10% formalin for 24 hours before embedding in paraffin wax. Tissue sections were stained with periodic acid–Schiff and hematoxylin and eosin.

CSF β-D-glucan measurements

CSF samples obtained by means of lumbar puncture from the patient before, during, and after GM-CSF therapy were used to measure β-D-glucan levels. Many of the values were greater than the limit of detection (>500 pg/mL), and therefore CSF samples were diluted 1:20 and analyzed (MiraVista Diagnostics, Indianapolis, Ind).

DNA constructs and site-directed mutagenesis

Plasmids for WT green fluorescent protein (*GFP*)–hemagglutinin (*HA*)–CARD9 and the 2 mutant variants (p.Y91H and p.R57H) were produced by VectorBuilder. Flag–H-RAS and Flag–RASGRF1 plasmids were from OriGene (Rockville, Md), as previously reported.^{E1} These plasmids were used for transfection (see below).

Coimmunoprecipitation studies

HEK293T cells were cultured as monolayers in DMEM medium supplemented with 10% FBS. All cultures were maintained at 37°C in a 5% CO₂ atmosphere and 100% humidity. When used for experiments, cells were plated on poly-L-lysine (Fisher Scientific) and transfected when they reached 80% confluence. HEK293T cells were transfected with 2 µg of the aforementioned constructs (single or in combination) by using TurboFect transfection reagent (Thermo Scientific). Cells were harvested 24 hours after transfection and lysed on ice in RIPA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 140 mmol/L NaCl) supplemented with protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail; Thermo Scientific). Cell lysates were centrifuged at 13,000 rpm for 10 minutes at 4°C, and each supernatant was incubated with anti-Flag or anti-HA antibodies (D6W5B or C29F4; Cell Signaling Technology, Danvers, Mass)–conjugated Dynabeads with protein A (Thermo Scientific) and protein G

(Thermo Scientific) for 2 hours at 4°C under continuous agitation. After washing with ice-cold RIPA buffer, beads and bound proteins were collected according to the manufacturer's instructions. The beads were resuspended in 50 µL of 2× SDS sample buffer and stored at −20°C until further analysis.

Immunoblot analyses

Equal amounts of proteins (cell signaling experiments) or bead supernatants (coimmunoprecipitation studies) were analyzed by using SDS-PAGE and transferred onto an Immobilon P, polyvinylidene difluoride membrane (EMD Millipore Immobilon, Burlington, Mass). The membranes were incubated with antibodies against the following proteins: phospho-p44/42 mitogen-activated protein kinase (Thr202/Tyr204; 1:2000; Cell Signaling Technology), p44/42 mitogen-activated protein kinase (Erk1/2, 1:2000; Cell Signaling Technology), DYKDDDDK Tag (D6W5B; 1:1000; Cell Signaling Technology), and HA Tag (C29F4; 1:1000; Cell Signaling Technology). Anti-rabbit secondary antibody was from SouthernBiotech (Birmingham, Ala; 1:50,000). The antigens were visualized with the Immobilon Western Chemiluminescent HRP substrate (EMD Millipore Immobilon), according to the manufacturer's instructions, and film was from Agfa (VWR, Radnor, Pa). Western blot quantification was performed by using densitometric analysis with ImageJ analysis software (National Institutes of Health, Bethesda, Md) and Scion Image analysis software (Scion Corporation, Frederick, Md).

Mice

Eight- to 12-week-old female C57BL/6 (WT; Taconic, Albany, NY, or Jackson Laboratory Bar Harbor, Me, as appropriate), *Card9*^{−/−},^{E2} and *Csf2rb*^{−/−} mice were maintained in individually ventilated cages under specific pathogen-free conditions at the National Institutes of Health. Animal studies were performed in accordance with the recommendations in the "Guide for the care and use of laboratory animals" of the National Institutes of Health under the auspices of protocol LCIM14E approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases. Animals were killed by means of cervical dislocation after administration of ketamine/xylene cocktail.

Systemic candidiasis model and GM-CSF treatments

C. albicans strain SC5314 was used for all infections. Yeast was serially passaged 3 times in YPD (yeast extract, Bacto Peptone, and dextrose) broth and grown at 30°C with shaking for 18 to 24 hours at each passage. Yeast cells were washed in PBS, counted, and injected intravenously through the lateral tail vein. For GM-CSF studies, GM-CSF was prepared by diluting cell-culture supernatants of the GM-CSF-expressing X63 cell line (a kind gift from Salome Leibundgutt-Landmann, University of Zurich, Zurich, Switzerland) in sterile PBS. GM-CSF concentrations in supernatants were determined by means of ELISA (R&D Systems, Minneapolis, Minn). Control mice received RPMI media diluted in PBS to the same ratio as the GM-CSF preparation. WT and *Card9*^{−/−} mice were first infected with 7×10^4 yeast cells and then injected with 5 µg of GM-CSF or control PBS intraperitoneally immediately after the infection. Further injections of GM-CSF/control PBS were performed as outlined in the figure legends.

Determination of fungal burdens

At 24 or 72 hours after infection, animals were killed, and organs were weighed, homogenized in PBS, and serially diluted before plating onto YPD agar supplemented with penicillin/streptomycin (Invitrogen). Colonies were counted after incubation at 37°C for 24 to 48 hours.

Determination of GM-CSF concentrations in infected kidneys

Kidneys were isolated from infected or naive animals at the indicated time points and homogenized in sterile PBS supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Homogenized kidneys were

centrifuged to remove cellular debris, and supernatants were snap-frozen on dry ice. GM-CSF concentrations were measured by means of ELISA (R&D Systems), according to the manufacturer's instructions.

Flow cytometry

Leukocytes were isolated from the brain by using previously described methods,^{E3} resuspended in PBS, and stained with Live/Dead fluorescent dye (Invitrogen) for 10 minutes on ice, followed by anti-CD16/32 (eBioscience, San Diego, Calif) for 10 minutes to block Fc receptors. Fluorochrome-labeled antibodies were then added, and cells were incubated on ice for 15 to 30 minutes before acquisition on a BD LSRFortessa (BD Biosciences, Franklin Lakes, NJ). Antibodies used in this study were as follows: CD45 (30-F11), CD11b (M1/70), CD11c (N418), MHC class II (M5/114.15.2), and F480 (BM8; all from eBioscience) and Ly6G (1A8) and Ly6C (AL-21; both from BD Biosciences). Analysis of fluorescence-activated cell sorting samples were performed with FlowJo software (TreeStar, Ashland, Ore).

In vitro GM-CSF stimulation and pSTAT5 staining

Isolated bone marrow cells from WT C57BL/6 mice were counted by using Trypan blue stain, and 1×10^6 cells were added to single wells of a round-bottom 96-well plate in 100 μ L of serum-free media (RPMI supplemented with 50 μ mol/L β -mercaptoethanol and penicillin/streptomycin). Cells were serum starved for 2 hours at 37°C and then stimulated with 50 ng/mL GM-CSF (or left unstimulated) for 15 minutes. Cells were then fixed in 4% paraformaldehyde on ice for 20 minutes, washed in PBS (supplemented with 0.5% BSA), and then permeabilized in ice-cold

methanol overnight at -20°C . Cells were washed once in PBS/BSA, followed by a second wash in Triton buffer (PBS supplemented with 0.5% Triton-X and 0.1% BSA), anti-pSTAT5 (clone: pY694; BD Biosciences) was added to cells in Triton buffer. Cells were stained at room temperature and protected from light for 30 minutes. Acquisition and analysis (on forward scatter/side scatter–high granulocytes) were performed, as described above.

Statistics

Statistical analyses were performed with GraphPad Prism 7.0 software (GraphPad Software, La Jolla, Calif). Details of individual tests are included in the figure legends. In general, data were tested for normal distribution by using the Kolmogorov-Smirnov normality test and analyzed accordingly by using the unpaired 2-tailed *t* test or Mann Whitney *U* test. In cases in which multiple data sets were analyzed, 2-way ANOVA was used with the Bonferroni correction. In all cases *P* values of less than .05 were considered significant.

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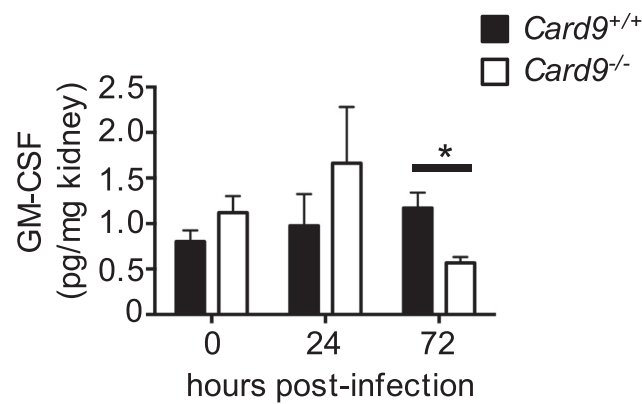


FIG E1. GM-CSF concentrations in kidneys from WT (*solid bars*) and *Card9*^{-/-} (*open bars*) mice. Data were pooled from 2 independent experiments (n = 5-6 per group). **P* < .05, 2-tailed Student *t* test.

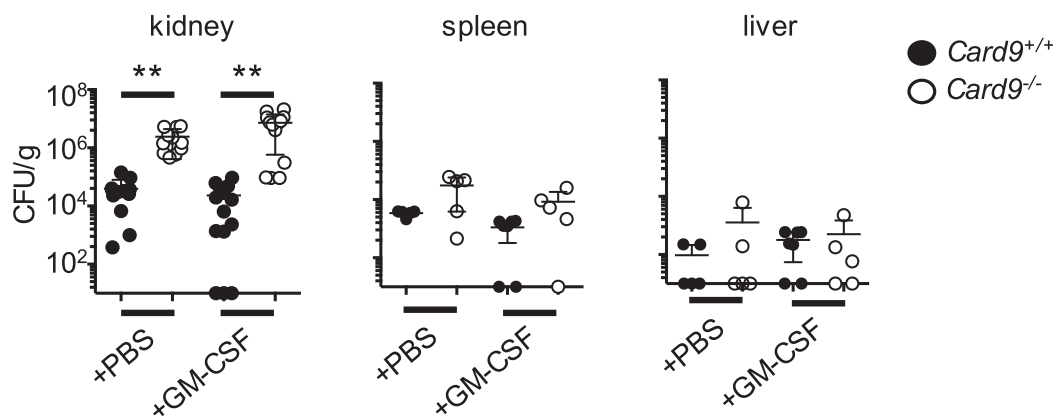


FIG E2. Kidney, spleen, and liver fungal burdens in WT (solid circles) and *Card9*^{-/-} (open circles) mice at day 3 after infection with and without GM-CSF treatment by using the regimen shown in Fig 2, A. Data were pooled from 2 independent experiments (n = 5-7 per group). ***P* < .01, Mann-Whitney *U* test.

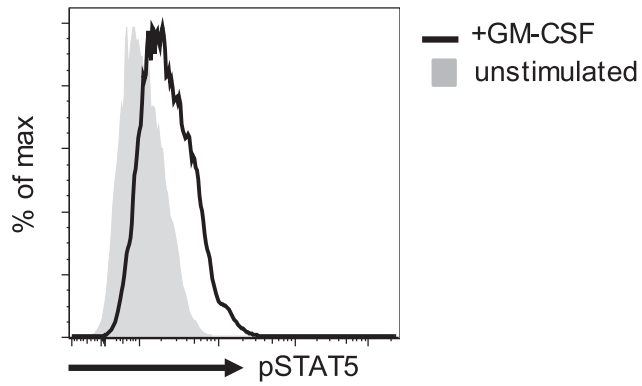


FIG E3. Representative fluorescence-activated cell sorting histogram of pSTAT5 staining of bone marrow granulocytes stimulated with 50 ng/mL GM-CSF compared with unstimulated control cells. The histogram is representative of 2 independent experiments.