Invasive fungal infection and impaired neutrophil killing in human CARD9 deficiency

Running title: CARD9 deficiency in neutrophils

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Category: Phagocytes
Key point

- Human CARD9-deficiency is characterized by a selective neutrophil killing defect, resulting in invasive candidiasis.
Abstract

Caspase recruitment domain-containing protein 9 (CARD9) is an adaptor molecule in the cytosol of myeloid cells, required for induction of T-helper cells producing IL-17 (Th17 cells) and important in anti-fungal immunity. In a patient suffering from *Candida dubliniensis* meningoencephalitis mutations in the *CARD9* gene were found to result in the loss of protein expression. Apart from the reduced numbers of CD4+ Th17 lymphocytes, we identified a lack of monocyte-derived cytokines in response to *Candida* strains. Importantly, CARD9-deficient neutrophils showed a selective *Candida albicans* killing defect with abnormal ultrastructural phagolysosomes and outgrowth of hyphae. The neutrophil killing defect was independent of the generation of reactive oxygen species (ROS) by the NADPH oxidase system. Taken together, this demonstrates that human CARD9 deficiency results in selective defect in the host defense against invasive fungal infection, caused by an impaired phagocyte killing.
Introduction

Opportunistic invasive fungal infections, such as those with *Candida* species, are an increasing problem in medicine, amongst other things as a consequence of chemotherapy-induced leukopenia. The host defense against *Candida* infections in human subjects appears to rely on innate as well as adaptive immune mechanisms.

It has become clear that in particular the responses mediated by the IL17-producing Th17 subset of T lymphocytes play an essential role in the host immunity against *Candida* infections. This is illustrated by a variety of genetic defects associated with the development of Th17 cells in patients and an increased susceptibility to candidiasis. The role of IL-17 in the defense against persistent *Candida* infection has been directly implicated by the identification of IL-17R and IL-17F mutations in pedigrees suffering from chronic mucocutaneous candidiasis (CMC).

On the other hand, the innate complement and phagocyte pathways of host immunity against *Candida* spp in humans have been implied in the clearance of these fungal infections. This is illustrated by the prevalence of invasive *Candida* spp infections in Chronic Granulomatous Disease (CGD), which is caused by defects in the NADPH oxidase enzyme complex needed for microbial killing, and also by the improved survival upon granulocyte transfusion in case of disseminated candidiasis during chemotherapy-related conditions of prolonged neutropenia.

While opsonins such as complement are involved in the recognition of *Candida* by phagocytes, there is evidence that the C-type lectins expressed on the plasma membrane of myeloid cells interact with β-glucan and mannan residues on the surface of *Candida*, and thus play an essential role in recognition as well.

Exactly which relevant effector functions are triggered through recognition of *Candida* by lectins, including Dectin-1, Dectin-2 and other related receptors such as Mincle, is not
known. Experimental evidence from *in vitro* studies and *in vivo* studies in mice suggests that Dectin-1 signals via the tyrosine kinase Syk and via a downstream complex of the cytosolic proteins CARD9, Bcl-10 and MALT1 to the transcription factor NF-kB, which acts as a central regulator in the production of inflammatory cytokines.\(^{11}\) Of interest, a recent report has described several cases of CARD9 deficiency in a human family with a history of *Candida* infections.\(^{14}\) Notably, there was evidence that some of the patients within this family not only displayed CMC, but also suffered from invasive *Candida* infection in the brain. The same study also showed defects in the development of Th17 cells in these patients, linking CARD9 to this pathway of adaptive immunity. However, no studies were performed on myeloid cells, which express CARD9 protein most abundantly.\(^{15}\)

Here we describe a patient with CARD9 deficiency who is suffering from chronic invasive *Candida* infection of the brain, in which we show that – apart from reduced Th17 cell activity – the immune system is more broadly affected. This includes innate defects in inflammatory cytokine production and fungal killing by neutrophils.
Materials and Methods

Cell purification.
The granulocytes and erythrocytes were separated from the mononuclear leukocytes and platelets by centrifugation over isotonic Percoll with a specific density of 1.076 g/ml. PBMCs were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with a 10% FCS, gentamycine and β-mercaptoethanol. Erythrocytes in the pellet were lysed. Granulocytes were washed and resuspended in Hepes-buffered saline solution.16-18

Immunostaining and FACS analysis.
After erythrocyte lysis, expression of surface-bound receptors on granulocytes was assayed in total leukocyte samples by flow cytometry (FACS), with commercially available monoclonal antibodies (MoAbs), mouse anti-human Dectin-2/CLEC6A(Clone: 545943) , Dectin-1/CLEC7A(Clone: 259931) (R&D systems, Minneapolis, USA), indirectly labeled with Alexa488-rabbit-anti-mouse-Ig (Molecular Probes). Samples were analyzed on an LSRII flow cytometer equipped with FACSDiva software (BD). Cells were gated based on their forward and side scatter, and 10,000 gated events were collected per sample.

Western blot analysis.
Analysis of protein expression was performed by Western blot.17 The following antibodies were used for detection: polyclonal rabbit anti-human CARD9 (Protein Tech Group, Chicago, USA), polyclonal mouse anti-human CLEC4E / MINCLE (Abcam, Cambridge, UK), monoclonal mouse anti-human CLEC6A / Dectin-2, CLEC7A / Dectin-1 (R&D system, Minneapolis, USA) and monoclonal mouse anti-human BCL-10 (Clone: 151, Invitrogen, Carlsbad, USA).
Lymphocyte activation in vitro.

PBMCs were resuspended in PBS at a concentration of 5x10^6 cells/mL and labeled with 0.5 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) as described before.19,20 T-cell proliferative capacity was determined by addition of optimal concentrations of MoAbs against CD3 (CLB-CD3/4.E; IgE isotype) plus or minus CD28 (CLB-CD28/15E8; IgG1 isotype), or purified Candida antigen (25 μg/mL).

Cell stimulation and cytokine measurement.

For IL-17 induction peripheral blood mononuclear cells (PBMCs) were cultured at a concentration of 2.5x10^6 T-cells/mL and were stimulated for 72 hours with a combination of anti-CD3 and anti-CD28 monoclonal antibodies (MoAbs).

Where indicated PBMCs were re-stimulated with Phorbol Myristate Acetate (PMA 20ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) 6 hours before supernatant collection - in the absence or presence of Brefeldin A.

Cytokine concentrations of IL-17 in culture supernatant were analyzed with an enzyme-linked immunosorbent assay (ELISA) using anti-human IL-17A capture monoclonal antibody (Ab) (eBioscience, clone eBio64CAP17).

For IL-6 and IL-1β, TNF-α and for IL-8 production, PBMCs (0.25x10^6 monocytes/mL), NB4 or PMN (5.0x10^6 cells/mL) were stimulated overnight with the indicated stimuli. Production of IL-6, IL-1β, TNF-α and IL-8 was measured in the supernatants with a Pelikine ELISA (Sanquin Reagents, Amsterdam, The Netherlands) according to the manufacturer’s protocol.

Killing of microorganisms.

Short-term-microbicidal activity of granulocytes was determined as previously described.21 In brief, Escherichia coli (strain ML-35), Staphylococcus aureus (strain 502A) and Candida
*albicans* (*Candida albicans*) stably transfected with GFP was a generous gift of Dr. Alexander Johnson (Department of Microbiology and Immunology, University of California at San Francisco, CA, USA) were grown under aerobic conditions at 37°C (bacteria) or 30°C (yeast) overnight in Lysogeny Broth (LB). Bacteria and yeasts were then collected by centrifugation, washed twice in PBS and resuspended in HEPES medium. After opsonisation (10% (v/v) pooled serum, 15 min, at 37°C), bacteria were added at a ratio of 5 : 1 neutrophil (4x10⁶ cells/mL) and *Candida* at ratio 4:1. At the desired time points, 50-μL samples were diluted in 2.5 mL of water/NaOH at pH 11.0. At the end of the incubation period, the number of viable microorganisms in each sample was determined by the pour-plate method in LB agar. The CFU were counted after an overnight incubation at 37°C, and the percentage killing was calculated. To assess long-term microbicidal activity of granulocytes, PMN (5.0x10⁶ cells/mL) were cultured overnight with the *C. albicans*-GFP, at a yeast:neutrophil ratio 4:1. The appearance of the hyphenated form of *C. albicans* was assessed with a digital fluorescence microscope (Evos, Westburg, Belgium). This was also determined after addition of G-CSF (optimal concentration, 20 ng/mL), GM-CSF (optimal concentration, 10 ng/mL) and IFN-gamma (optimal concentration, 100 ng/mL) to the overnight culture of neutrophils and *Candida* conidia.

To determine the PMN killing of *C. albicans* hyphae, conidia (OD: 0.2, 625 nm) were cultured in LB for 16 hours at 37°C for formation of a hyphae monolayer, 100-120 μm long and confluence. Neutrophils were added for 2 hours in different effector target (E:T) ratios, lysed in water (pH=11), washed in PBS and incubated for 3 hours with yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide). The hydrogenases of the functional hyphae can cleave MTT to its purple derivative MTT-formazan, which can be dissolved in isopropanol and quantified by spectrophotometry. The percentage of viable *C. albicans* hyphae was then determined.
**EM analysis of phagolysosomes.**

Purified neutrophils were fixed in 2%(w/v) paraformaldehyde with 0.2%(w/v) glutaraldehyde and then processed for ultrathin cryosectioning. 19 50-nm-thick cryosections were cut at −120°C with diamond knives (diatome) in a cryo-ultramicrotome (Leica, Vienna, Austria) and transferred onto carbon/formvar-coated copper grids. The grids were placed on 2%(w/v) gelatin plates at 37°C, embedded in methylcellulose with 0.6% uranyl acetate and examined with a CM10 electron microscope (Philips, The Netherlands).

**Statistics.**

Graphs were drawn and statistical analysis was performed with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Data were evaluated by paired, two-tailed student’s *t*-test and two-way ANOVA with post hoc Bonferroni test. The results are presented as the mean ± SEM, as indicated. Significance is mentioned when *p*<0.05.

**Study approval.**

The study is performed according to Dutch rules and regulations with respect to the use of human materials from healthy, anonymized volunteers with written informed consent and all experiments were approved by the Medical Ethical Committee of Sanquin Research and the Academic Medical Centre in Amsterdam which acts according to the Declaration of Helsinki principles (version Seoul 2008).

**Supplemental methods.**

Detailed methodology of the supplemental figures is described in the Supplemental Materials.
Results

Case report

We describe an Asian 13-year-old girl who was adopted as an infant. When she first presented at the age of 7 years, she was diagnosed with Candida dubliniensis meningoencephalitis without any obvious underlying risk factors for fungal meningitis. Fever, headaches, behavioral changes and seizures were the predominant clinical findings. CSF analysis revealed an eosinophilic pleocytosis with increased protein level and decreased CSF glucose. Appropriate antibiotics were initiated adding antifungics (amphotericine B, followed by fluconazole) which resulted in clinical recovery and normalization of the CSF values. Initial magnetic resonance imaging (MRI) showed mainly a deep infarction of the left striatum, meningeal enhancement and mild ventricular dilatation. After 6 months of treatment the discontinuation resulted in a clinical relapse. Repeated cultures CSF later disclosed Candida dubliniensis. Clinical signs and CSF pleocytosis resolved within several weeks of restarting combined antifungal therapy (fluconazole, 5-flucytocin). This patient is studied in more detail (Emmanuel Scalais, in preparation).

CARD9 deficiency and mutation analysis

Surface staining for Dectin-1 and -2 on neutrophils and monocytes, and Western blotting for Dectin-1 and -2, Mincle and Bcl-10 revealed normal levels of these proteins in the lysates of the patient’s neutrophils and monocytes (Fig. 1 A and B; data not shown). However, the CARD9 protein appeared absent (Fig. 1 B), and this was confirmed with various anti-CARD9 antibodies directed toward different epitopes of the protein. Sequence analysis revealed that the girl was compound heterozygote for two previously undescribed mutations c.214G>A and c.1118G>C in CARD9 (Fig. S1 A), resulting in the amino acid substitutions p.Gly72Ser and p.Arg373Pro, which both comprise highly conserved residues in
the CARD9 protein in vertebrates (Fig. S1 B). These mutations were not found among 100 healthy donors (not shown). Regarding the medical family history, the adoption agency was able to contact both parents. Although they refused collection of DNA for mutation analysis, clinical data indicated that both parents were over forty years of age and healthy to date.

**T cell function and Th17 cells**

The presence of AIDS-related invasive *Candida* infections suggests a role for CD4⁺ T cells in such fungal infections. We tested T cell proliferation in response to *Candida* antigen as well as polyclonal CD3/CD28 stimulation and detected robust T cell responses - as was also true for tetanus toxoid, chickenpox and herpes simplex virus antigen (not shown).

Of the various CD4⁺ T lymphocyte subsets, Th17 cells are activated by various interleukins and characteristically release IL-17. This Th17 cell is thought to be a major cell type upon switching from innate to adaptive immunity. In our patient, cytokine profiles from T cell cultures activated by CD3/CD28 showed clear induction of various cytokines in the supernatant of these cells (not shown) but reduced levels of Th17-derived IL-17 (136 ± 28 and 770 ± 91 pg/mL, patient and controls, respectively; p<0.001), consistent with the data of Glocker et al.

**Monocyte and neutrophil function in CARD9 deficiency**

Since CARD9 is primarily expressed in myeloid cells, these cells were explored further. We first investigated the release of inflammatory cytokine release by PBMCs and neutrophils in response to activation by various (un)opsonized pathogens in several independent experiments. This showed the virtual absence in *Candida*-induced monocytic IL-6 and IL-1β release in the patient PBMCs, while the response to bacteria (Fig. 2 A and B) and various TLR ligands (not shown) was not or only partly affected. To obtain direct evidence
that CARD9 was causative for the defect we performed lentiviral reconstitution with wild-type CARD9 of hematopoietic stem cells from bone marrow cultured for 14 days in the presence of M-CSF. The cultured cells were successfully transduced with CARD9 (80-90% positive for the reporter nerve growth factor receptor (NGFR)). Partial reconstitution coincided with recovery of bone marrow (BM)-derived monocytic cytokines (as shown for IL-6 upon overnight activation with unopsonized *Candida albicans*) (Fig. 2 C).

We next measured neutrophil-derived IL-8 production in overnight cultures. While there is considerable interexperimental variation in IL-8 release, the response of the patient neutrophils to *Candida albicans* was consistently decreased (Fig. 2 D), although not as strongly as the monocyte-derived cytokines. Support for defective cytokine release by CARD9-deficient neutrophils was obtained from experiments with myeloid NB4 cells in which CARD9 was efficiently knocked down (Fig. S3 A and B).

In the patient neutrophils, we observed no defects in the immediate induction of NADPH oxidase activity by the various stimuli tested – either primed with platelet-activating factor (PAF) or TLR ligands for subsequent fMLP-induced NADPH oxidase activity, or directly activated by fMLP or the phorbol ester PMA. The same was true for induction of the respiratory burst by the yeast-derived particle zymosan, *C. albicans* particles or aggregated IgG (Fig. 5 A and B). These findings indicate that the patient cells had a completely normal capacity to generate reactive oxygen species (ROS) and normal recognition and signalling for these immediate responses. Adhesion and chemotaxis upon activation by TLR ligands or chemoattractants such as complement fragment C5a, PAF or the chemokine IL-8, were all normal (not shown).

When neutrophil killing capacity was studied, the immediate killing response, within 1-2 hours, by CARD9-deficient cells was significantly impaired with unopsonized *Candida albicans* conidia and normal with serum-opsonized conidia (Fig. 3 A). In contrast, the killing
of opsonized Gram-positive *Staphylococcus aureus* or opsonized Gram-negative *Escherichia coli* was not different from control neutrophil killing (Fig. S4 A). We investigated the cytotoxic response to *Candida albicans*. Even though the patient was infected with the philogenetically closely related dubliensis strain, it has been described that neutrophils have a similar cytotoxic response towards *Candida albicans* than to *Candida dubliensis* in terms of phagocytosis, oxidative burst and killing. 

Importantly, when *Candida* killing after 18-24 hours incubation was evaluated, with CARD9-deficient neutrophils and opsonized *Candida albicans* conidia a clear outgrowth of hyphae was observed which was even more striking with unopsonized *Candida albicans* (Fig. 3 B and C; Fig. S5, movie 1 to 4). In contrast to CARD9-dependent killing, such candida outgrowth was not observed after overnight incubation of neutrophils from CGD patients with unopsonized *Candida albicans* (Fig. 3 B and C). These data demonstrate that a non-oxidative CARD9-dependent and non-redundant mechanism in neutrophils prevents outgrowth of unopsonized *Candida*. There is a definite role of reactive oxygen species in *Candida* killing, but predominantly with the opsonized *Candida albicans* conidia. Under these conditions, CGD neutrophils fail to prevent conidia from hyphenation. Of note, CARD9-deficient neutrophils appeared to kill preformed unopsonized *Candida albicans* hyphae with similar efficiency as control neutrophils (Fig. 3 D), indicating that the process of hyphenation itself is insufficiently controlled by CARD9-deficient neutrophils while uptake is intact (Fig. S4 B). Furthermore, measuring the oxidation of dihydrorodamine (DHR) following the phagocytosis of labeled *Candida* conidia by neutrophils revealed that patient and control cells showed identical responses (Fig. 5 B). The normal DHR response means that in the complex process of uptake degranulation and NADPH oxidase activity gets normally activated upon phagolysosome formation. Only after degranulation of the MPO from azurophilic granules together with the NAPDH-oxidase derived ROS generation a DHR signal will be generated.
Finally, addition of activating factors G-CSF, GM-CSF or interferon-gamma (IFN-\(\gamma\)), which are known to enhance neutrophil function and survival\(^{27}\), were without any effect on outgrowth of *Candida* hyphae (not shown).

Because of the normal ROS generation in the patient’s neutrophils upon activation by yeast particles, the possibility that the lysosomal killing was dysfunctional was further investigated by ultrastructural analysis. Although neither the release of the content of azurophilic or specific granules nor the NADPH oxidase activity in these CARD9-deficient neutrophils were found to be different from control neutrophils (Fig. 5 A and S4 C), the phagolysosomes in the patient cells following unopsonized *Candida albicans*, but not *Staphylococcus aureus*, uptake were abnormal, as clearly demonstrated by electron microscopy (Fig. 4 A and B). Taking all these data together, a hitherto unidentified, non-redundant and relatively selective killing mechanism in neutrophils for fungal organisms is implicated, which depends on the normal function of CARD9.
Discussion

*Staphylococcus aureus* and *Candida* species are the second and third leading causes of bloodstream infections in hospitalized patients.\(^\text{28}\) These organisms jointly cause at least 150,000 clinical bloodstream infections resulting in very large health-care expenditures and ~40,000 deaths per year in the US and Europe each.\(^\text{29-31}\) Identification of immune mechanisms of protective adaptive immunity against these organisms is critical to lay the groundwork for development of active preventive and therapeutic strategies against these microorganisms.

The induction of cell-mediated immunity to *C. albicans* is one of the main tasks of cells of the innate immune system, and *in vitro* evidence suggests that integrin \(\alpha_M\beta_2\) (CR3, Mac-1, CD11b/CD18) is the principal leukocyte receptor involved in recognition of the fungus.\(^\text{32}\)

Although T cells are important in anti-fungal defense, T cell reactivity seems to be particularly important for epithelial defense against molds. Among the various subsets of IL-17-producing cells, memory CD4\(^+\) Th17 cells have received most attention regarding their participation in antifungal immune reactivity.\(^\text{2}\) Apart from the release of G-CSF and chemokines to activate neutrophils for microbial clearance, IL-17 also directly induces epithelial cells to produce microbial peptides against, for instance, *Staphylococcus aureus* and *Candida* species.\(^\text{33}\) Recently, Glockler et al. studied a family with homozygous CARD9 mutation and confirmed the defective Th17 response.\(^\text{14}\) In addition, we demonstrate that in human PBMCs the production of IL-6 and IL-1\(\beta\) in response to *Candida albicans* is dependent on CARD9. Hereby, we were able to confirm these findings, as described in the *card9*-knockout mice by LeibundGut-Landmann et al.\(^\text{34}\) The decrease in *Staphylococcus*-triggered IL-1\(\beta\) in monocytes may suggest a role for CARD9 in this pathway. Indeed there is evidence that CARD9 signals facilitate the TLR and Nod2 pathways to activate MAPK and tyrosine kinase pathways to activate NF-\(\kappa\)B for cytokine production.\(^\text{35}\)
We now show that the neutrophils in CARD9 deficiency have a selective defect in *Candida* killing. While monocytes and/or DCs may fail to induce Th17 responses in CARD9-deficiency, because they do not produce pivotal cytokines such as IL-1β for Th17 differentiation, the killing defect in neutrophils may directly contribute to the invasive nature of the fungal manifestations in CARD9 deficiency and thus contrasts with the Th17-associated non-invasive, more localized yeast infections in CMC.

The exact mechanism by which CARD9-defective neutrophils fail to eliminate *Candida* molds remains to be identified. Some important observations about a potential working mechanism have been obtained. First, the NADPH oxidase activity and, second, the degranulation of azurophilic and specific granules in the patient’s neutrophils are normal upon activation (Fig. 5 and S4 C). This was further supported by the normal oxidative response over time when DHR-labeled *Candida* conidia were taken up by CARD9-deficient neutrophils. In turn, CGD neutrophils which are unable to produce superoxide for oxidative killing were unaffected in their killing activity under conditions where massive *Candida* conidia outgrowth and hyphenation was observed in case of CARD9 deficiency. The CARD9-mediated killing mechanism differs from the opsonin-mediated killing pathway, which seems to be completely dependent on fully functional NAPDH oxidase activity as illustrated by the results with neutrophils from CGD patients. These findings are clearly in contrast with a study on BM-derived macrophages from *card9*-knockout mice, showing less reactive oxygen species in response to *Candida albicans.*

Moreover, the CR3 expression and integrin activity were considered to be normal in all adhesion and chemotaxis assays, as well as in the uptake of zymosan or *Candida* particles. The *ex vivo* immediate, early killing response of unopsonized *Candida* conidia was impaired, but it could be restored upon opsonization with human serum. This indicates that yeast β-glucan pattern recognition receptors, such as Dectin-1 and/or CR3, depend on CARD9 to
activate a selective (non-oxidative) cytotoxic mechanism. This notion is supported by the normal killing of hyphae, which are known to shield their β-glucans, and are instead recognized by mannan-specific receptors, such as TLR-2, TLR-4, CR3 and Dectin-2. Still, the ultimate Candida evasion from the patient’s cells in a prolonged, late killing assay indicates that the rapid and complete elimination of Candida is inadequate and results in escape of viable yeast after uptake, resulting in the outgrowth of hyphae. Early after activation of uptake and the intracellular killing reaction, these phagolysosomes tend to swell because the osmotic pressure increases during the breakdown of microbial proteins. The key machinery required for the export of such osmotically active phagolysosomal breakdown products is unknown, but a role for CARD9 in this process may explain the ultrastructural findings. Alternative explanations may be linked to the disregulation of autophagy, since CARD9 has been found to associate with Rubicon, a protein known to inhibit autophagosome maturation. Autophagosomes have been morphologically defined by a quintessential feature: i.e. the presence of ‘double membrane vesicles’ as most critical hallmark of this cellular process. We assessed this phenomenon by analyzing electron microscopy (EM) pictures taken at 2 hours, 4 hours and overnight co-incubation of neutrophils and Candida strains. In none of the EM pictures, either in the patient or in control neutrophils the characteristic autophagy signature of double membranes was observed (data not shown). In the absence of CARD9, uninhibited Rubicon activity would lead to increased autophagy which seems apparently not to be the case in the patient’s neutrophils.

The intact killing response to bacterial strains by CARD9-deficient neutrophils indicates that a distinct and non-redundant killing mechanism is induced by the yeast species tested and strictly depends on CARD9 in human neutrophils. In addition, we have studied the inhibition of Aspergillus fumigatus germination. CARD9-deficient neutrophils normally inhibited the germination of Aspergillus fumigatus (data not shown). This finding suggests
that the neutrophil killing defect is specific for *Candida* species and this is consistent with the susceptibility to *Candida* infections observed in our patient and those previously reported.\textsuperscript{14}

Our findings show that the defect in *Candida* killing by neutrophils was particularly pronounced in the absence of serum, and this might indeed be relevant in the context of the invasive brain infections observed in our patient and in the patients previously described.\textsuperscript{14}

The central nervous system (CNS) parenchyma is separated from the circulation by the blood-brain-barrier, which limits the access of plasma proteins, including immunoglobulins and complement.\textsuperscript{41} Therefore, in addition to the other potential pathophysiological mechanisms the CNS could be particularly sensitive to *Candida* infections in case of CARD9 deficiency. The lack of inflammatory cytokines and neutrophil chemoattractants produced by monocytes and DCs, and possibly also macrophage-like microglia cells, will exaggerate the defect in fungal clearance. The anti-fungal role of neutrophils is known from clinical experience with intensive cancer treatment.\textsuperscript{9} Our data suggest that hematopoietic stem cell transplantation is the only definite option for curation of CARD9 deficiency.
Acknowledgements

A.D., R.P.G and T.W.K were supported by the Landsteiner Foundation for Blood Transfusion Research (LSBR 1706). The authors are most grateful to the parents of the patient for their collaboration, in particular for their agreeing to investigation studies.

Authorship:

A.D. and R.P.G and T.W.K are the principal investigators, who conceived and designed the study. A.D., R.P.G., A.T.J.T, M.H., M.H.J., J.H., E.M.M.L., and H.J. performed the experiments. E.S. and C.B. assisted in acquisition of the patient samples. D.R. and T.K.B contributed to the design of the study. A.D. and R.P.G. devised and performed the analyses and wrote the first draft of the manuscript with input from all authors supervised by T.W.K. All authors approved the final manuscript revisions.

All authors declare there are no potential conflicts (financial, professional, or personal) that are relevant to the manuscript.
Reference List


Figure legends

Figure 1. CARD9 deficiency in a patient with systemic candidemia
Surface expression of Dectin-1 (left) and Dectin-2 (right) (panel A) in control and patient PMNs and monocytes was measured by flow cytometry. Dotted lines represent controls and solid lines patient cells. Isotype controls are shown in grey.
Comparable amounts of dectins were detected on the surface of patient and control cells.
Western blot detection (panel B) of C-type lectins (Dectin-1, Dectin-2 and Mincle) as well as Bcl-10 and CARD9 in control and patient neutrophils (indicated as C and P, respectively). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Results are representative for three independent experiments.

Figure 2. Cytokine production by CARD9-deficient leukocytes and by the patient’s CD34+ hematopoietic stem cells following CARD9 transduction
Leukocytes from the patient and a control subject were incubated overnight with the indicated stimuli. Culture supernatants were collected and the concentrations of IL-6 and IL-1β by PBMCs (panel A and B) or IL-8 by neutrophils (panel D) were assessed by ELISA. To examine the effect of mutated CARD9 on cytokine production, a human wild-type CARD9 complementary DNA (cDNA) was cloned into a lentiviral expression vector. Following transduction, M-CSF-cultured bone marrow derived monocytic cells of the patient and healthy controls were stimulated with C. albicans or S. aureus, and the production of IL-6 was assessed by ELISA (panel C). Results represent two experiments in triplicate from different bone marrow aspirates of the patient and from healthy controls. Results are means ± SEM of measurement from four to six independent experiments.* p<0.01; ** p<0.001.
Figure 3. Killing of *C. albicans* by CARD9-deficient neutrophils

Killing efficacy of patient and control neutrophils of unopsonized and opsonized *C. albicans* conidia was assessed by standard colony-forming unit assay (panel A). Patient and control neutrophils were incubated with *C. albicans* conidia for two hours and the colonies were counted after overnight incubation of the remaining *Candida* conidia. Results are means ± SEM of at least three different assays. * p<0.05.

In panel B, neutrophils from healthy controls, from the CARD9-deficient patient and from CGD patients (n=9) were co-cultured overnight with *C. albicans*-GFP and assessed microscopically. Massive outgrowth of the hyphenated form of *C. albicans*-GFP was observed, when cocultured overnight with CARD9-deficient cells. Bright-green fluorescence of hyphae indicates viable *Candida*. Such outgrowth was not detected when *C. albicans* was cocultured with control neutrophils. Clusters of hyphae were quantified after overnight incubation of 1*10^5* neutrophils with different numbers of *Candida* conidia (panel C). Data are representative for at least three experiments. The 20x magnification was used and the size of scale bar is 200 μm.

The capacity of patient and control neutrophils to kill *Candida* hyphae was determined by MTT assay (Panel D). Neutrophils in different concentrations were incubated with monolayer of *Candida* hyphae for two hours. The viability of hyphae was assessed by the MTT assay. Results are means ± SEM of at least three different assays.

Figure 4. Phagolysosome formation upon uptake of *C. albicans* by human neutrophils

CARD9-deficient and control neutrophils were co-cultured overnight with unopsonized *C. albicans*. Ultrastructural analysis was performed by Electron Microscopy (EM). EM pictures showed bulging phagolysosome formation upon uptake of *C. albicans* in the patient.
neutrophils in contrast to the control neutrophils, obtained in three independent experiments (Panel A).

In panel B, the ratio of *Candida* conidia diameter to the phagosomal space diameter is significantly reduced in CARD9-deficient neutrophils, taken into consideration that the diameter of the *Candida* conidia was significantly increased in case of CARD9 deficiency compared to the control (1.3 ± 0.2 μm vs. 0.8 ± 0.1 μm, *P*<0.05). Moreover, the phagosomes of CARD9-deficient neutrophils contained more *Candida* conidia than the control neutrophils (1.5 ± 0.1 vs. 1.1 ± 0.1 conidia/phagosome, *P*<0.05). Taken together the phagosomes of the CARD9-deficient patient have a strongly enlarged appearance. Results are means ± SEM of measurement from three independent experiments. ** p<0.01.

Figure 5. NADPH-oxidase activity and phagocytosis of DHR-labeled *C. albicans* in CARD9-deficient neutrophils

To assess the effect of CARD9 deficiency on production of reactive oxygen species, patient and control neutrophils were stimulated with various stimuli: zymosan, serum-treated zymosan, phorbol-12-myristate-13-acetate (PMA), platelet-activating factor (PAF) followed by formyl-Met-Leu-Phe (fMLP), *Candida* particles (ratio with neutrophils, 1:4) and heat-aggregated immunoglobulin (Ig), in the presence of Amplex Red and horseradish peroxidase. Means +/- standard error of the mean of at least three different experiments (Panel A). The phagocytosis of DHR-labeled *C. albicans* by the CARD9-deficient and control neutrophils was determined by flow cytometry (Panel B). Results represent data from two different experiments.
Figure 1

A.

PMNs

Dectin-1

Dectin-2

B.

Bcl-10

Mincle

Dectin1

Dectin2

C

P

CARD9

GAPDH
Figure 3

A. Plot showing the viability of C. albicans unopsonized and opsonized over time in control vs. CARD9 def. patient.

B. Images showing C. albicans unopsonized and opsonized in control, CARD9 def. patient, and CGD patient after 24 hrs.

C. Graph illustrating the counting of hyphae (in 10^3 pm) for C. albicans unopsonized and opsonized in control, CARD9 def. patient, and CGD patient after 24 hrs.

D. Graph depicting the viability of hyphae (%) over Neutrophils (concentration) in control vs. CARD9 def. patient over 2 hrs.
Figure 4

A.

control

CARD9 def. patient

B.

ratio of Candida conidia diameter to phagosome diameter

control

CARD9 def. patient

**
Figure 5

A. 

$H_2O_2$ release (nmol/min.10^9 PMNs)

- unstimulated
- zymosan
- STZ
- PMA
- PAF/fMLP
- $C. albicans$
- IgG

B.

DHR response (MFI)

- control
- CARD9 def. patient

 timelines:
- 0
- 30
- 60
- 90
- 120
- overnight
Invasive fungal infection and impaired neutrophil killing in human CARD9 deficiency