Two independent killing mechanisms of Candida albicans by human neutrophils: evidence from innate immunity defects

Running title: neutrophil killing mechanisms of Candida albicans

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Key points

- Human neutrophils use two independent mechanisms for the killing of unopsonized and serum-opsonized *Candida albicans*

- Unopsonized *Candida* killing depends on CR3 and CARD9 but not dectin-1 and opsonized *Candida* killing on FcγR’s, PKC and NADPH oxidase activity
Abstract

Invasive fungal infections, accompanied by high rates of mortality, represent an increasing problem in medicine. Neutrophils are the major effector immune cells in fungal killing. Based on studies with neutrophils from patients with defined genetic defects, we provide evidence that human neutrophils use two distinct and independent phagolysosomal mechanisms to kill *Candida albicans*. The first mechanism for the killing of unopsonized *Candida albicans* was found to be dependent on Complement Receptor 3 (CR3), the signaling proteins phosphatidylinositol-3-kinase (PI3K) and CARD9, but was independent of NADPH oxidase activity. The second mechanism for the killing of opsonized *Candida albicans* was strictly dependent on Fc-gamma receptors, protein kinase C (PKC) and Reactive Oxygen Species (ROS) production by the NADPH oxidase system. Each of the two pathways of *Candida* killing required Syk tyrosine kinase activity, but dectin-1 was dispensible for both of them. These data provide an explanation for the variable clinical presentation of fungal infection in patients suffering from different immune defects, including dectin-1 deficiency, CARD9 deficiency or Chronic Granulomatous Disease (CGD).
Introduction

The occurrence of fungal infections such as those with *Candida* and *Aspergillus* spp. has progressively increased over the last decades as a consequence of the extensive use of immunosuppressive therapies\(^1\);\(^2\). Invasive fungal infections, in particular, are associated with mortality rates of 40-50\%\(^3\);\(^4\).

Neutrophils are the most important effector immune cells in fungal killing. It appears that adaptive immunity is primarily responsible for controlling *Candida* infection at the mucosal level by interleukin-17 (IL-17)-producing lymphocytes, whereas cells of the innate immune system are critical for preventing systemic candidiasis\(^5\);\(^6\);\(^8\). The latter is illustrated by the increased prevalence of invasive fungal infections in patients with chemotherapy-induced neutropenia or with neutrophil functional defects, such as genetic deficiencies of the NADPH oxidase as seen in Chronic Granulomatous Disease (CGD) patients\(^9\).

The neutrophil mechanism of fungal killing is poorly understood. First, it is not known which pathogen recognition receptors and their downstream signaling pathways are involved in the killing of *Candida albicans*. Both C-type lectin receptors (CLR), including dectin-1, dectin-2 and mincle, and also integrins, especially complement receptor 3 (CR3, CD11b/CD18, αMβ2), have been demonstrated to recognize the β-glucans and mannans exposed on the *C.albicans* cell wall\(^1\);\(^10\);\(^11\). However, two studies performed with dectin-1 knockout mice contradict each other, with one showing that dectin-1 was required for the control of systemic candidiasis, whereas the other claimed that this receptor was not relevant\(^12\);\(^13\). The differential requirement of dectin-1 appeared to be *Candida* strain-specific\(^14\). In humans, only one family with three dectin-1-deficient
patients has been reported so far, showing clinically mucocutaneous fungal infections, but no invasive fungal infections. Moreover, as we have previously shown for human neutrophils, CR3 and not dectin-1 seems to be the major receptor for β-glucan recognition. Patients with a leukocyte adhesion defect, either LAD-I in which CD11/CD18 integrins are lacking or LAD-III (also known as LAD1/variant) syndrome with a deficiency of kindlin-3 required for leukocyte integrin activation, are genetically predisposed to invasive bacterial and fungal infections.

In mouse models, both dectin-1 and CR3 have been described to trigger spleen-tyrosine kinase (Syk) and phosphatidylinositol-3-kinase (PI3K) to elicit a cytotoxic response and to activate the signaling adaptor protein CARD9. Recently, human patients with CARD9 deficiency have been characterized to suffer, typically, from Candida meningitis, and this was found to be associated with an impaired killing capacity of CARD9-deficient neutrophils towards Candida. Similarly, CGD patients with a defective NADPH oxidase system are also predisposed to invasive candidiasis, which may indicate a strict requirement to activate the NADPH oxidase system for microbial killing.

In the present study we demonstrate that human neutrophils use two distinct and independent mechanisms to kill C. albicans, governed by the presence of opsonins on the yeast. The first mechanism, for the phagolysosomal killing of unopsonized C. albicans, depends on CR3 recognition and signaling via Syk, PI3K and CARD9, but is completely independent of NADPH oxidase activity. The second mechanism, for phagolysosomal killing of serum-opsonized C. albicans, is strictly dependent on the Fc-gamma receptors, protein kinase C (PKC) and Reactive Oxygen Species (ROS) production by the NADPH oxidase system, in which the tyrosine kinase Syk does have a role but PI3K does not.
Further evidence for these distinct routes of fungal killing was obtained with cells from patients with well characterized neutrophil dysfunctions. Our study clearly demonstrates the presence of two independent pathways for fungal killing that may contribute to the identification of novel therapeutic targets and provide an explanation for the clinical phenotype observed in dectin-1 deficiency, CARD9 deficiency and CGD.
**Materials and Methods**

**Killing of microorganisms**

Short-term-microbial activity of granulocytes was determined as previously described 24. In brief, PMN(5.0x10^6 cells/ml) were cultured overnight with the *C. albicans*-GFP, at a yeast:neutrophil ratio of 4:1. The appearance of the hyphenated form of *C. albicans* was assessed with a digital fluorescence microscope (Evos, Westburg, Belgium). To determine the role of dectin-1 and CR3 in the *C. albicans* killing, neutrophils were treated with a monoclonal antibody against human dectin-1(clone 259931, R&D systems), CD11b(clone 44A,ATCC, Rockville,MD,USA), CD18(clone IB4, ATCC), FcγRI (clone 10.1, BD Pharmingen, San Diego, CA, USA), FcγRII (clone AT10, AbD Serotec, Oxford, UK) or FcγRIII (clone 3G8, BD-Pharmingen). The inhibitors of Syk signaling were R406 (Selleckchem, Houston, TX, USA) and Bay 61-3606 (Sigma-Aldrich, Diegum, Belgium). The PI3K-inhibitors were Wortmannin and LY294002 (Sigma). The PKC-inhibitor was Go6983 (Calbiochem, Darmstadt, Germany).

**Statistics**

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 by the Mann-Whitney test. The results are presented as the mean ± SEM. Data were considered significant when *p*<0.05.
Study approval

The study was performed according to national 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 the Academic Medical Centre in Amsterdam according to the Declaration of Helsinki principles (version Seoul 2008).
Results

Unopsonized versus serum-opsonized Candida killing by human neutrophils

The pathogenicity of fungi is determined by their ability to germinate, invade tissues and spread via the bloodstream\(^{25}\). Although neutrophils are essential for antifungal host defense, the mechanism(s) for the killing of *Candida* have remained poorly defined, which led us to study *Candida* conidia killing and containment of hyphal growth by human neutrophils in more detail. We noticed that the capacity of human neutrophils from healthy controls to inhibit germination of *C.albicans* into clusters of hyphae in an overnight assay as well as to kill *Candida* conidia (2 hours), was strongly enhanced by opsonization with human serum (Figure 1A-B and Figure S1A). The observed increase in *Candida* hyphae coincided with an increase in viability of *Candida* conidia after overnight incubation (Figure S1B). This enhancing effect of opsonization was completely dependent on IgG antibodies, as demonstrated as follows. First, we did not detect any significant reduction upon complement inactivation in the serum by prior heat-treatment at 56°C for 30 minutes (Figure 1B and Figure S1). Secondly, IgG in immunoglobulin preparations for intravenous use (IVIG) in patients resulted in similar killing enhancement as observed with intact whole serum (Figure 1B and Figure S1). Thirdly, the serum from an untreated X-linked agammaglobulinemia (XLA) patient who was completely lacking antibodies because of an inherited B-cell deficiency, did not enhance killing upon opsonization (Figure 1B). And – finally –monoclonal antibodies (mAbs) against Fcγ receptors completely blocked the enhancement, supporting the notion that the serum opsonization of *C.albicans* depends on the IgG directed against the candida cell wall (Figure 1B). Neutrophils treated with cytochalasin B to prevent actin...
polymerization for phagocytosis and particle uptake, were impaired in inhibition of unopsonized and opsonized *C. albicans* germination, which indicates that this process under these test conditions occurs through intracellular phagolysosomal killing (**Figure S1C**).

**No role for dectin-1 (CLEC7A) in Candida killing by human neutrophils**

With the understanding that serum opsonization was dependent on IgG, we subsequently focused on the surface molecules on human neutrophils involved in the uptake and killing of unopsonized *C. albicans* conidia. The β-glucans form a major constituent of the *C. albicans* cell wall. Dectin-1-deficient mice have been reported to suffer from *Candida* infections in experimental models\(^{13}\). Dectin-1 on mice macrophages was shown to signal towards CARD9 to mediate NF-κB translocation and cytokine production, which is generally believed to contribute to survival from severe *Candida* infection\(^{20}\).

Since little is known about the role of dectin-1 in the neutrophil cytotoxic response, we first treated neutrophils of healthy controls with a blocking mAb against dectin-1. This mAb effectively blocked the binding of *C. albicans* to dectin-1-transfected human embryonic kidney (HEK) cells (**Figure S2**).

Upon treatment with this blocking dectin-1 antibody human neutrophils still effectively inhibited the germination of both unopsonized and opsonized *C. albicans* conidia (**Figure 2**). Neutrophils treated with the antibody against dectin-1 also exhibited normal phagocytosis and killing of both the unopsonized and opsonized *C. albicans* conidia (**Figure S3A and B**).
To confirm these findings we subsequently tested the candidacidal neutrophil activity of previously identified patients with a homozygous stop codon Tyr238X in the CLEC7A gene, leading to a partial deletion of the carbohydrate recognition domain of the dectin-1 receptor (Table S1, patients A and B)\(^1\). HEK cells expressing a dectin-1 receptor with this rare mutation did not bind *C. albicans*, in contrast to cells expressing wild-type human dectin-1 (Figure S2). Remarkably, the dectin-1-deficient neutrophils from these patients were completely normal in the inhibition of both unopsonized and opsonized *C. albicans* germination (Figure 2). The killing of unopsonized and opsonized *C. albicans* conidia by the dectin-1-deficient neutrophils was also normal (Figure S3A). In addition, dectin-1 signaling was dispensable for the patients’ neutrophil capacity to generate hydrogen peroxide in response to zymosan or (un)opsonized *C. albicans* (Figure S4). Also the related CLRs dectin-2 and mincle are unlikely to be involved in neutrophil reactivity to *C. albicans*, since HEK cells transfected with either dectin-2 or mincle did not show *Candida* binding (Figure S2). Taken together, these findings demonstrate that human neutrophils are capable of phagocytizing and killing (un)opsonized *C. albicans* through a dectin-1-independent pathway.

**The lectin-binding role of CR3 in the killing of *C. albicans***

We next examined the role of the integrin CR3 (CD11b/CD18, αMβ2) in the uptake and killing of *C. albicans*. CR3 is highly expressed on neutrophils and has been reported to recognize β-glucans and mannans of the *Calbicans* cell wall\(^1\). Neutrophils of healthy controls were treated with a blocking mAb against the common β2 integrin subunit (clone IB4) and one against the αM chain (clone 44A) of the integrin CR3.
These cells showed a clear defect in the inhibition of unopsonized *C. albicans* germination after overnight incubation (Figure 3A). This killing defect induced by CR3 blocking was prevented by prior opsonization of *C. albicans* with normal human serum (Figure 3A). Treatment of neutrophils with blocking mAbs against CR3 and against the FcγRs resulted in an impaired killing of unopsonized and serum-opsonized *C. albicans* (Figure 3A). Neutrophils incubated with blocking mAbs against CR3 displayed a slight reduction in early phagocytosis of *C. albicans* conidia (Figure S3D).

For further confirmation of these findings in overnight germination we tested neutrophils from patients with a *FERMT3* mutation, causing the leukocyte adhesion defect in LAD-III (Table S1, patients C and D). In LAD-III the hematopoietic protein kindlin3 is absent, resulting in a defect of the integrin CR3 and other leukocyte and platelet integrins to adopt a high-affinity ligand conformation\(^{17,18,26}\). Kindlin3-deficient neutrophils were defective in the inhibition of unopsonized *C. albicans* conidia germination while fully capable to inhibit serum-opsonized *C. albicans* conidia germination in the same overnight killing assay (Figure 3A). Incubation of kindlin3-deficient neutrophils with blocking mAbs against FcγRs resulted in an increase in observed hyphae clusters of serum-opsonized *C. albicans* as compared to untreated kindlin3-deficient neutrophils (Figure 3B). Since the uptake of *Candida* by the neutrophils is an essential aspect of the process of phagolysosomal killing, the immediate responses of the kindlin3-deficient neutrophils were studied and found to be impaired in the phagocytosis and subsequent early killing of unopsonized *C. albicans* conidia, but not in case of IgG-opsonized *C. albicans* conidia (Figure S3C and D).
Thus, the integrin CR3 has an essential role in the recognition, uptake and killing of unopsonized *C. albicans* conidia, which indicates that in human neutrophils not dectin-1, but the integrin CR3 controls the killing of unopsonized *C. albicans*.

**The killing of opsonized and not unopsonized *C. albicans* depends on an intact NADPH oxidase system**

Since the lack of ROS formation by neutrophils upon addition of unopsonized zymosan is used as a diagnostic test for LAD-III\(^1\)\(^8\) and ROS production was also reduced in response to unopsonized *Candida* by kindlin3-deficient neutrophils (Figure S4), we investigated the role of the NADPH oxidase system in the killing mechanisms in greater detail. We studied patients with a complete defect in the NADPH oxidase activity, suffering from Chronic Granulomatous Disease (CGD). The CGD patients (Table S1, patients E-L) were deficient in either the cytosolic NADPH oxidase proteins, *i.e.* p47\(^{phox}\) or p67\(^{phox}\) (N=3 and N=1, respectively) or the membrane-bound subunit (gp91\(^{phox}\); N=4) of the NADPH oxidase system, resulting in a failure to produce ROS\(^2\)\(^7\)-\(^2\)\(^9\). To our surprise, the neutrophils of these patients inhibited the germination of unopsonized *C. albicans* as efficiently as control neutrophils (Figure 4A), but were severely impaired in the killing of serum- or IgG-opsonized *C. albicans*, resulting in massive outgrowth of hyphae in the overnight germination assay (Figure 4A). Incubation of CGD neutrophils with blocking mAbs against Fc\(\gamma\)Rs resulted in normalization of the killing of serum-opsonized *C. albicans* (Figure 4B). CGD neutrophils treated with blocking mAbs against the Fc\(\gamma\)Rs and against CR3 were defective in the killing of both unopsonized and serum-opsonized *C. albicans* (Figure 4B). In the short-term response, the CGD neutrophils showed a
slightly reduced killing capacity of both unopsonized and serum-opsonized *C. albicans* conidia compared to control neutrophils, despite normal phagocytosis (Figure S5A; data not shown). Only the defect in the inhibition of serum-opsonized *C. albicans* germination was strikingly abnormal. Ultimately, the killing of unopsonized *C. albicans* seems to be independent of ROS formation and can therefore not be held responsible for the defect in unopsonized *Candida* killing in case of a failure in CR3 function, as suggested above. These data together point towards distinct and independently activated routes of killing: one being activated by CR3-mediated uptake of unopsonized *C. albicans*, and another being induced by IgG-FcγR-mediated uptake of serum-opsonized *C. albicans*. These routes are activated by the surface receptors involved in recognition and uptake and dictate the subsequent killing mechanism involved. Only in case of the latter route of IgG-mediated *Candida* uptake and not that of unopsonized *Candida* killing, the subsequent ROS formation by the NADPH oxidase activity is essential for phagolysosomal killing.

**Selective role of CARD9 in the killing of *C. albicans***

We have recently reported on the inability of neutrophils from a patient with a CARD9-deficiency to restrict the germination of *Candida* conidia, which may contribute to the associated invasive candidiasis observed in these patients (Table S1, patient M). In the current study we confirmed this defect in a second, unrelated patient with CARD9-deficiency suffering from *Candida* meningitis (Table S1, patient N). The killing defect was overcome by opsonization of the *C. albicans* conidia with human serum or IgG (Figure 5). As demonstrated, the short-term killing of unopsonized *C. albicans* conidia by
the CARD9-deficient neutrophils was already reduced (Figure S5B). The inability of CARD9-deficient neutrophils to control germination of unopsonized C.albicans was not due to a defect in phagocytosis, as we already had excluded previously\textsuperscript{23}. Collectively, these data demonstrate that CARD9 is selectively required for the cytotoxic response of neutrophils against unopsonized C.albicans but not against opsonized C.albicans. Under both conditions, ROS generation was normal (Figure S4).

Thus, the recognition of either the unopsonized or the opsonized C.albicans cell wall activates two distinct killing mechanisms by human neutrophils, i.e. a CARD9- versus an NADPH oxidase-dependent pathway, respectively.

**Signal transduction pathways in the killing of C.albicans**

To confirm the notion of two distinct routes of killing that depend on the way how Candida is recognized and phagocytized, i.e. either via CR3 or the FcγRs, we tested well-characterized compounds that are known to inhibit some key enzymes in major upstream signal transduction pathways.

Both the β\textsubscript{2} integrin CR3 and the FcγRs have been described to signal through the tyrosine kinase Syk to elicit a cytotoxic response\textsuperscript{19,21}. To determine the role of Syk signaling in the killing of C.albicans we used two different pharmacological inhibitors of this kinase, i.e. Bay61-3606(2.5 µM) and R406(2.5 µM). Neutrophils from healthy controls incubated with Bay or R406 were impaired in the inhibition of both the unopsonized as well as the opsonized C.albicans germination as compared to untreated neutrophils (Figure 6). We investigated whether these effects were already explained by a defective early response. Indeed, treatment of neutrophils with Bay or R406 also
decreased the short-term killing of unopsonized and opsonized \textit{C.albicans} conidia when compared to untreated neutrophils (\textbf{Figure S6A}). Whereas R406 or Bay reduced the phagocytosis of unopsonized \textit{C.albicans} for 50\%, the same human neutrophils normally phagocytized serum-opsonized \textit{C.albicans} (\textbf{Figure S6B}).

To explore the signal transduction events downstream of Syk signaling, we studied the effect of PI3K inhibition by Wortmannin (100nM) and LY294002 (10µM). Neutrophils incubated with Wortmannin or LY294002 were found to be only inhibited in their capacity to prevent germination of unopsonized \textit{C.albicans} and not that of opsonized \textit{C.albicans} (\textbf{Figure 6}). PI3K inhibition by Wortmannin or LY294002 resulted in a decreased rate of phagocytosis and short-term killing of unopsonized \textit{C.albicans}, but a normal response to opsonized \textit{C.albicans} was found (\textbf{Figure S6B}). Whereas Syk was involved in the killing of unopsonized and opsonized \textit{C.albicans}, PI3K signaling seemed to be restricted to the killing of unopsonized \textit{C.albicans} in the CARD9 pathway, but not in the FcγR-mediated route to kill serum-opsonized \textit{C.albicans} by toxic ROS generation (\textbf{Figure 7}). To determine how the FcγRs activated the NADPH oxidase system we investigated protein kinase C (PKC) by the inhibitor Go6983 (20µM). Neutrophils treated with the PKC inhibitor were impaired in the generation of ROS by the NADPH oxidase system but showed normal levels of \textit{Candida} phagocytosis and proteolytic activity (\textbf{Figure S6}). The Go6983 treated neutrophils were not able to inhibit the germination of opsonized \textit{C.albicans} and normally inhibited the germination of unopsonized \textit{C.albicans} (\textbf{Figure 6}).
Discussion

The last two decades have witnessed a significant increase in the number of sepsis patients caused by fungal infections, due to the common use of immunosuppressive treatments or high-dosed chemotherapy-induced neutropenia. Neutrophils are the most numerous and potent effector cells in fungal killing, although the mechanism of killing is rather poorly understood. The aim of the current study was to investigate which surface receptors and signaling pathways are involved in the neutrophil cytotoxic response to *C. albicans*. We identified two distinct and independent pathways for the killing of either unopsonized or IgG-opsonized *C. albicans*. The first pathway for the phagolysosomal killing of unopsonized *C. albicans* was dependent on CR3, Syk, PI3K and CARD9 signaling, but independent of NADPH oxidase activity. The second pathway involved FcγRs, Syk and ROS formation by the NADPH oxidase system and proved essential for the killing of serum-opsonized *C. albicans*.

We excluded an imminent role of C-lectins, in particular dectin-1 (CLEC7A). Dectin-1 recognizes β-glucans of the *C. albicans* cell wall and has been found essential in mouse models of *Candida* infection. In the present study, it was demonstrated that in human neutrophils dectin-1 is completely dispensable for the cytotoxic response to *C. albicans*. As previously reported in β-glucan binding and the production of cytokines in response to *C. albicans*, we could confirm that dectin-1-deficient monocytes were indeed impaired in the production of IL-6 in response to *C. albicans* (Figure S7C). Impaired cytokine production by macrophages or dendritic cells may result in reduced numbers of *Candida*-specific Th17 cells, associated with mucocutaneous candidiasis, which is consistent with the observed clinical susceptibility to superficial *C. albicans* infections in dectin-1-
deficient individuals. Instead, human neutrophils use the lectin-binding integrin CR3 for *Candida* uptake and killing, in line with our previous observation that CR3 and not dectin-1 is the major receptor for β-glucan recognition by human neutrophils. In contrast to our findings, Li et al showed in a mouse model that dectin-1 is required to activate CR3 for the cytotoxic response to *C. albicans*. Also LAD-III patients with an activation defect of the integrins are predisposed to invasive fungal infections.

Indeed, kindlin3-deficient neutrophils (as well as control neutrophils treated with blocking antibodies against CR3) were impaired in the capacity to inhibit germination of unopsonized *C. albicans* resulting in massive outgrowth of hyphae, whereas kindlin3-deficient neutrophils showed a normal inhibition of serum-opsonized *C. albicans* germination. Since the latter was IgG-mediated, treatment of these kindlin3-deficient neutrophils with antibodies against FcγRs resulted in an impaired inhibition of *C. albicans* germination. Although deposition of complement fragment C3b has previously been indicated to facilitate phagocytosis of serum-opsonized *Candida* by CR3, our study supports the major role of FcγRs in the phagocytosis of serum-coated pathogens such as *Candida*. Whether the FcγRs inhibit the signaling through CR3 in case of serum-opsonized *Candida* or merely represent a stronger signal for killing, cannot be deduced from our data.

In fact, upon investigation of the role of signaling pathways in the cytotoxic response to *C. albicans*, we show that for human neutrophils both the integrin CR3- and the FcγR-mediated killing of unopsonized and opsonized *C. albicans*, respectively, are Syk dependent. Studies with mice have shown that the integrin CR3 activates Syk to elicit anti-microbial responses such as ROS generation and the release of proteases in the
phagolysosome\textsuperscript{33}. Syk is also involved in mice in FcγR-mediated phagocytosis of IgG-coated erythrocytes\textsuperscript{34}. Others have shown that β-glucan activates a CR3-Syk-PI3K pathway in neutrophils and thereby enhances tumor cell killing\textsuperscript{21}. In line with Honda et al. we found PI3K involved in superoxide production in response to PAF/fMLP and unopsonized \textit{C.albicans} but not in response to serum-opsonized \textit{C.albicans} (data not shown)\textsuperscript{35}. Our results confirm the role of PI3K in neutrophils to be also critical in killing of unopsonized \textit{C.albicans}. Clearly, this CR3-Syk-PI3K pathway acts through the adaptor protein CARD9. In the present study we extend our previous findings on CARD9 in the cytotoxic response to \textit{C.albicans}\textsuperscript{23}. In two unrelated CARD9-deficient patients suffering from \textit{Candida} meningitis, the neutrophils were impaired in the killing of unopsonized but not of serum-opsonized \textit{C.albicans}. Wu et al. demonstrated in mice that CARD9 is involved in bacteria-elicited production of ROS by bone marrow-derived macrophages and in \textit{in-vivo} host response\textsuperscript{36}. In contrast to these murine results the CARD9-deficient neutrophils from two different patients showed a normal NADPH oxidase activity in response to various stimuli including \textit{S.aureus}. This could be explained by the fact that Wu et al. used a different cell type and another Gram-positive bacterium \textit{L.monocytogenes} or this simply illustrates the difference between CARD9-deficient mice and men. To date, four reports have been published including 26 CARD9-deficient patients suffering from severe invasive fungal infections but none of them had severe bacterial infections\textsuperscript{22,23,37,38}. Moreover, we have previously shown that the neutrophil killing of \textit{S.aureus} by the patient cells was completely normal\textsuperscript{23}. These results suggest that non-oxidative killing mechanisms are important in the CARD9-mediated killing of unopsonized \textit{C.albicans}. It has been demonstrated in mice that a double
knockout of elastase and cathepsin G results in susceptibility to invasive candidiasis.\textsuperscript{39} Future research needs to determine how exactly CARD9 regulates the non-oxidative killing of \textit{C. albicans}.

Since the predominant neutrophil anti-microbial activity is considered to depend on the formation of ROS by the NADPH oxidase system, we investigated the neutrophils from CGD patients with an inherited defect in ROS production. CGD neutrophils normally inhibited the germination of unopsonized \textit{C. albicans} but were severely impaired in the killing of opsonized \textit{C. albicans}. We also showed that after blocking Fc\textgamma{}Rs the alternative pathway in CGD neutrophils induced the killing of serum-opsonized \textit{C. albicans}. Thus, the killing of opsonized \textit{C. albicans} depends on IgG recognition by Fc\textgamma{}Rs, signaling through PKC and the production of ROS by the NADPH oxidase system.\textsuperscript{40} The finding that CGD neutrophils fail to kill \textit{C. albicans} correspond with the increased prevalence of systemic \textit{Candida} infections described in patients.\textsuperscript{9}

Although suggestions that neutrophil extracellular traps (NETs) of extruded DNA may have a role in the candidicidal activity,\textsuperscript{41} pretreatment of neutrophils with cytochalasin B showed that the inhibition of unopsonized and opsonized \textit{C. albicans} germination occurred through a phagolysosomal killing process.

Taken together, we characterized the presence of two distinct mechanisms used by the neutrophil in the host defense against \textit{C. albicans}. First, the killing of unopsonized \textit{C. albicans} depends on CR3 and the signaling proteins Syk, PI3K and CARD9, but is independent of the NADPH oxidase system. The second mechanism used for the killing of opsonized \textit{C. albicans} depends on IgG recognition by Fc\textgamma{}Rs, signaling through Syk, PKC and activation of the NADPH oxidase system (Figure 7). Apparently, the effects
are binary in the sense that the neutrophil’s choice for one or the other killing mechanism depends on the initial step of *C. albicans* recognition and uptake. There is cross-talk between the two pathways, because binding of IgG-opsonized *C. albicans* to FcγRs on the neutrophils inhibits induction of killing by the CR3-CARD9 pathway, which can be relieved by blocking the FcγRs. In addition, blocking of CR3 and the FcγRs on CGD neutrophils resulted in defective killing of both unopsonized and opsonized *C. albicans*. These findings provide fundamentally new insights into the pathophysiological mechanism underlying immunological defects in the antifungal killing mechanisms of human neutrophils. Further unraveling the relevant molecular signaling pathways will help to identify novel targets for future antifungal therapeutics.
Acknowledgements

R.P.G was supported by the Landsteiner Foundation for Blood Transfusion Research (LSBR 1706) awarded to T.W.K. The authors are very grateful to the patients and their parents for their cooperation. All authors approved the final manuscript revisions.

Authorship

T.W.K. is the principal investigators who conceived and designed the study. R.P.G., J.H., A.T.J.T and M.H. performed the experiments. F.v.d V., M.H. and J.L. assisted in acquisition of the patient samples. T.K.B contributed to the design of the study and D.R. helped with the interpretation and the presentation of the results. R.P.G. devised and performed the analyses and wrote the manuscript together with T.W.K.

Conflict of interest

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


Figure legends

Figure 1. The killing of unopsonized and opsonized *C. albicans* by neutrophils.

(A) Neutrophils from healthy controls were co-cultured overnight with unopsonized and serum-opsonized *Candida albicans*. White arrows indicate the observed clusters of GFP-positive *C. albicans* hyphae. Scale bar is 50 μm. (B) Neutrophils from healthy controls were co-cultured overnight with unopsonized, serum-opsonized, heat-inactivated(HI)-serum-opsonized, XLA-serum-opsonized, IgG-opsonized *C. albicans*-GFP or after blocking the FcγRs with mAbs (10 μg/ml) and the clusters of hyphae were assessed microscopically. Results are means ± SEM of at least three independent experiments. * P < 0.05.

Figure 2. Dectin-1 is dispensable in the killing of *C. albicans*.

The number of *C. albicans*-GFP hyphae clusters after co-incubation with untreated neutrophils, treated with a blocking antibody against dectin-1 (20 μg/ml) and neutrophils from dectin-1-deficient patients A and B. Results are means ± SEM of at least three independent experiments.

Figure 3. The killing of unopsonized *C. albicans* depends on CR3.

(A) Neutrophils from healthy controls, treated with blocking antibodies against CR3 (10 μg/ml IB4 and 44A) and against FcγRs (10 μg/ml) and from kindlin3-deficient patients C and D were co-cultured overnight with *C. albicans*-GFP and the clusters of hyphae were assessed microscopically. Scale bar is 50 μm. (B) The inhibition of serum-opsonized *C.
Candida albicans germination by untreated and anti-FcγR (10 μg/ml) treated neutrophils from healthy controls and kindlin3-deficient patients. Results are means ± SEM of at least three independent experiments. * P < 0.05.

Figure 4. CGD neutrophils fail to kill serum-opsonized C. albicans.
(A) Neutrophils from healthy controls and CGD patients E to L were co-cultured overnight with C. albicans-GFP, and the clusters of hyphae were assessed microscopically. Scale bar is 50 μm. (B) The inhibition of serum-opsonized C. albicans germination by untreated, anti-FcγR (10 μg/ml) or anti-CR3/FcγR (10 μg/ml) treated neutrophils from healthy controls and CGD patients. Results are means ± SEM of at least three independent experiments. * P < 0.05.

Figure 5. CARD9-deficient neutrophils show a selective C. albicans killing defect.
Neutrophils from healthy controls and CARD9-deficient patients M and N were co-cultured overnight with C. albicans-GFP and assessed microscopically. Scale bar is 50 μm. Results are means ± SEM of at least three independent experiments. * P < 0.05.

Figure 6. Syk, PI3K and PKC in the killing of C. albicans.
Untreated and treated neutrophils with inhibitors of Syk: R406 (2.5 μM) or Bay (2.5 μM), the PI3K inhibitors Wortmannin (100 nM) or LY294002 (10 μM) and the PKC inhibitor Go6983 (20 μM) were co-cultured overnight with C. albicans-GFP and clusters of hyphae were assessed microscopically. Scale bar is 50 μm. Results are means ± SEM of at least three independent experiments. * P<0.05.
Figure 7. Neutrophil-mediated killing of *C. albicans*. Two independent and distinct neutrophil-mediated killing mechanisms of *C. albicans* were characterized. The killing of unopsonized *C. albicans* depends on CR3 recognition, PI3K and CARD9 signaling but is independent of NADPH oxidase activation. The killing of opsonized *C. albicans* involved FcγRs, signaling through PKC and requires ROS formation by the NADPH oxidase system. Syk signaling is essential in both pathways for the killing of unopsonized and opsonized *C. albicans* killing.
Figure 1.

A.

B. Controls of hyphae 20 hours

Clusters of hyphae 20 hours

(N1 x 10^9) neutrophils

unopsonized
serum-opsonized
HI-serum-opsonized
IgG-opsonized
XLA-serum-opsonized
anti-FcγR + serum-opsonized

control
Figure 2.
Figure 3.

A.

Clusters of hyphae (N.10^6 neutrophils) 20 hours

- unopsonized
- serum-opsonized

B.

Clusters of hyphae (N.10^6 neutrophils) 20 hours

- serum-opsonized

* *
Figure 4.

A. Clusters of hyphae (N10^6) (n = 3) in 20 hours
- unopsonized
- serum-opsonized
- IgG-opsonized

B. Clusters of hyphae (N10^6) (n = 3) in 20 hours
- unopsonized
- serum-opsonized
Figure 5.
Figure 6.
Figure 7.
Two independent killing mechanisms of *Candida albicans* by human neutrophils: evidence from innate immunity defects