Induction of ERK-kinase signalling triggers morphotype-specific killing of Candida albicans filaments by human neutrophils

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Summary

Candida albicans is among the most important fungal pathogens in humans. Morphological plasticity has been linked to its pathogenic potential as filamentous forms are associated with tissue invasion and infection. Here we show that human neutrophils discriminate between yeasts and filaments of C. albicans. Whereas filaments induced targeted motility, resulting in the establishment of close contact between neutrophils and fungal cells, yeast forms were largely ignored during coincubation. In transwell assays, C. albicans filaments induced significantly higher migratory activity in neutrophils than yeasts. Neutrophil motility based on actin rearrangement was essential for killing of C. albicans filaments but not involved in killing of yeast forms. Using inhibitors for MAP-kinase cascades, it was shown that recognition of C. albicans filaments by neutrophils is mediated via the MEK/ERK cascade and independent of JNK or p38 activation. Inhibition of the ERK signalling pathway abolished neutrophil migration induced by C. albicans filaments and selectively impaired the ability to kill this morphotype. These data show that invasive filamentous forms of C. albicans trigger a morphotype-specific activation of neutrophils, which is strongly dependent on neutrophil motility. Therefore, human neutrophils are capable of sensing C. albicans invasion and initiating an appropriate early immune response.

Introduction

Invasive infections caused by Candida albicans remain among the most important fungal infections in immunocompromised patients (Richardson, 2005; Ruhnke, 2006). It is well established that beside a specific T-cell response, neutrophils are crucial in establishing protective immunity against Candida (Romani et al., 1997; Romani, 1999; 2000; Pfaller and Diekema, 2007). Thus, although isolated T-cell defects may predispose to Candida infection, neutropenia is a major risk factor for invasive candidiasis and associated with a more severe cause of disease and a poor outcome. Human polymorphonuclear neutrophils (PMN) are the predominant cell type in peripheral blood and represent a first line of defence in the innate cellular immune system. Activated by inflammatory stimuli, neutrophils migrate to sites of infection and initiate inactivation of invading microorganisms (Segal, 2005). These highly differentiated cells rely on several strategies to eliminate invasive microorganisms including the generation of reactive oxygen intermediates (ROI), phagocytosis and the release of granule enzymes and antimicrobial peptides, the formation of neutrophil extracellular traps (NETs) (McKenzie and Schreiber, 1998; Brinkmann et al., 2004; Quinn and Gauss, 2004; Segal, 2005; Fuchs et al., 2007). Neutrophil invasion to sites of infection is among the fastest immune reactions in the human body. Consequently, neutrophils are among the fastest migrating cells of the human body, migrating at up to 20 μm min⁻¹. Neutrophil migration is usually directed by gradients of both extrinsic and intrinsic chemoattractants like formylated peptides or IL-8. Alternatively, random movement called chemokinesis can occur in the absence of stimulus gradients (Niggli, 2003). In human PMN, chemotactic factors bind to seven-transmembrane-domain receptors and activate heterotrimeric G-proteins, followed by a complex signalling network that can involve several different mitogen-activated protein kinases (MAPK) including p38 MAPK.
the c-Jun N-terminal protein kinase (JNK) or the extracellular regulatory kinase (ERK) (Niggli, 2003). *C. albicans* has been shown to stimulate neutrophil migration and release one or more chemotactic factors (Cutler, 1977; Edens *et al*., 1999; Geiger *et al*., 2004). However, little is known about the effector mechanisms of human neutrophils that contribute to killing of *C. albicans*. Neutrophils can recognize, attach to and damage Candida pseudohyphae (Diamond and Krzesicki, 1978). Recently, it has been shown that neutrophils govern the transcriptome of *C. albicans* in human blood by inducing a carbohydrate and nitrogen starvation response (Fradin *et al*., 2005). Furthermore, neutrophils induce oxidative stress and inhibit filamentation of *C. albicans* (Fradin *et al*., 2005). These results underline the central function of neutrophils in the immune response against invasive Candida infection. NET-formation has been shown to be triggered by *C. albicans* and contribute to extracellular killing of this pathogen (Urban *et al*., 2006). Here we show that neutrophils can discriminate between yeast forms and filaments of *C. albicans*. Filaments, which are formed during invasion, specifically trigger targeted motility by activation of the ERK-kinase cascade. This motility is essential for killing of *C. albicans* filaments. In contrast, killing of yeast forms does not depend on either ERK activation or motility of PMN.

**Results**

*Human neutrophils rapidly engulf filaments but not yeast forms of C. albicans*

To assess the interaction of primary human PMNs with different morphotypes of *C. albicans*, temperature dependent dimorphism was employed to generate either yeast cells or filamentous forms of *C. albicans*. The interaction between PMN and *C. albicans* was monitored microscopically by time-lapse microscopy. Viability and the ability to replicate of both morphotypes were verified before the coincubation. Several independent experiments with neutrophils from different donors showed identical results. Despite numerous events of direct contact resulting from random movement of neutrophils, *C. albicans* yeast cells were not actively attacked by human PMNs via cell–fungus interaction and no phagocytosis or persistent association between PMN and *C. albicans* yeasts was observed (Movie S1). These results are similar to those published previously by Behnsen *et al.* and confirm that under these experimental conditions human PMNs do not establish intimate contact with *C. albicans* yeast cells (Behnsen *et al*., 2007). In contrast to the results obtained with yeast forms, human PMNs thoroughly engaged *C. albicans* filamentous forms derived from the same strain as the yeast cells within one hour of coincubation. Whereas most filamentous structures were too large to be completely phagocytosed, the granulocytes appeared to wrap around fungal elements and remained closely associated with the filaments throughout the observation (Movie S1, Fig. 1). Electron microscopy confirmed a close contact between the intact neutrophil membrane and the fungal cell wall (not shown) similar to interactions described previously by Diamond *et al.* (Diamond and Krzesicki, 1978). Using real-time microscopy, we could show that these neutrophils remain viable throughout the interaction as movement along the fungal structure was clearly observed. Due to the fact that filament preparations of *C. albicans* always contain a small percentage of yeast forms, it could be shown that even in the simultaneous presence of *C. albicans* filaments and yeast cells, human PMNs selectively attacked and associated with the filamentous forms (Movie S1; Fig. 1). This selective activation pattern was however, not restricted to larger filaments (as shown in Fig. 1) but was also observed with smaller germ-tubes (see Fig. 9A).
Candida albicans filaments, but not yeast cells induce targeted motility of human PMN

To investigate whether C. albicans-induced migration of human PMNs was morphotype dependent, a transwell assay was used to quantify PMN migration towards yeasts or filaments of C. albicans. For these experiments very small germ-tubes were used to ensure that cell size and weight were comparable between yeast and filamentous forms (Fig. S1). Determination of dry weight revealed that the mass of the filamentous forms was 18% larger than that of the yeast forms. Filaments induced a significantly higher migratory activity in PMN than yeast forms at the same multiplicity of infection (moi = 1) (Fig. 2). Whereas 66 ± 19% of the granulocytes migrated towards C. albicans filaments, only 19 ± 8% were attracted by C. albicans yeasts (P < 0.01) during one hour of coincubation (arithmetic means ± standard deviation). Random motility of PMN without any stimulus resulted in migration of 13 ± 3% of PMN. To exclude that this effect was only due to small difference in cell size between yeasts and filaments, the moi for yeast forms was increased to four, corresponding to 3.3 times the dry weight of the filamentous forms. At this moi, yeast forms induced higher migratory activity (34 ± 5%, P < 0.01 when compared with random motility), confirming that C. albicans yeast cells are able to trigger PMN migration. However, the percentage of migrating PMN was still significantly lower than for C. albicans filaments at an moi of 1 (P < 0.01). To test whether opsonization with human serum affects chemotactic activity of PMN, yeasts and filaments of C. albicans were opsonized in 50% serum of the PMN donor for 30 min before the chemotaxis assay. This resulted in slightly elevated levels of migration (84 ± 27% for filaments and 28 ± 15% for yeasts). However, the significant difference between the two morphotypes was also observed after opsonization. Therefore, C. albicans filaments induced a significantly higher migratory activity in human PMNs than yeast forms. One of the most potent chemotactic factors involved in neutrophil recruitment is IL-8. This cytokine can be produced by a number of cell types and many human cells including epithelial, endothelial and dendritic cells have been shown to produce IL-8 in response to contact with C. albicans. To test whether neutrophils are capable of enhancing their own migratory activity by producing IL-8 in response to Candida, secretion of IL-8 by human PMNs was quantified. Whereas C. albicans filaments induced levels of 581 ± 145 pg ml⁻¹ IL-8, yeast forms of the same strain only induced 190 ± 26 pg ml⁻¹ (P < 0.01, secretion of unstimulated neutrophils: 8.8 ± 2.8 pg ml⁻¹) (Fig. 3). Therefore, IL-8 secretion induced by C. albicans filaments was 3-times higher than that induced by yeast form. In contrast to IL-8, no significant secretion of the regulatory cytokines IL-10 and IL-12 or of the proinflammatory cytokines IL-1 beta, IL-6 and TNF-alpha was observed in response to C. albicans yeast forms or filaments. Thus, C. albicans filamentous forms are a much more potent stimulus for chemotaxis than yeast forms and this effect is further enhanced by autologous IL-8 secretion.
Both filaments and yeast forms of C. albicans are effectively killed by human PMN

Data presented so far suggest significant differences between the response of human neutrophils towards yeast cells and filaments of C. albicans. However, inactivation of invading microorganisms in situ is the main function of neutrophils. Therefore, the capacity of freshly isolated human PMNs to kill both morphotypes of C. albicans was quantified. For that purpose, yeast cells or filaments of C. albicans strain SC5314 were generated by temperature dependent morphogenesis and incubated with freshly isolated human PMNs for different time points at an moi of 2.5. During the incubation period a continuous killing of both yeast cells and filaments was observed by sequential plating and counting of colony-forming units. The peak of fungicidal activity was reached between 150 and 210 min of coincubation with 56 ± 24% of yeast cells and 55 ± 12% of filaments remaining viable after 210 min in comparison to a control without PMN (arithmetic mean ± standard deviation, P = not significant). Differences between yeast forms and filaments could neither be observed with regard to the efficacy of fungal killing nor the kinetics of this reaction. To ensure that plating assays were not affected by different behaviour of yeast cells and filaments, XTT assays were performed to quantify killing of both morphotypes. After 180 min of coincubation, a remaining metabolic activity of 59 ± 12% for yeast forms and 58 ± 27% of filamentous forms was found, confirming the results of the plating assays and showing that killing activity of human PMNs against both morphotypes is equal. Thus, although there are marked differences in the behaviour of human PMNs towards C. albicans yeast cells or filaments, both forms are effectively killed by these cells – leading to the hypothesis that different effector mechanisms are responsible for killing of either morphotype.

Polymorphonuclear neutrophil motility based on actin rearrangement is necessary for killing of C. albicans filaments

In real-time microscopy analyses a morphotype-specific increase in neutrophil motility leading to at least partial engulfment of the fungi, was observed when these cells were coincubated with C. albicans filaments. To address the role of neutrophil motility, cytochalasin D, a potent inhibitor of actin polymerization was added to the experiments. At a concentration of 10 µg ml⁻¹ cytochalasin D efficiently prevented the establishment of close contact between C. albicans filaments and PMN. However, PMN remained fully viable and their integrity was not disturbed throughout the course of the experiment as indicated by residual non-productive motility observed in time-lapse microscopy and proven by live dead staining and FACS analyses of the neutrophils (data not shown). In addition, cytochalasin D at this concentration did not negatively affect growth or viability of C. albicans yeast forms or filaments. PMN-induced killing and growth inhibition of C. albicans filaments were assessed in independent assays. Inhibition of C. albicans growth in the presence of human neutrophils was determined microscopically using FITC labelled filamentous forms. Whereas a considerable inhibition of C. albicans growth was conferred by human neutrophils alone when compared with control experiments (~80%, P < 0.01), this effect was completely abolished in the presence of cytochalasin D (Fig. 4). In plating experiments, 10 µg ml⁻¹ cytochalasin D was able to impair the fungicidal activity of PMN against C. albicans filaments (Fig. 5). Even 1 µg ml⁻¹ cytochalasin D was sufficient to achieve a significant effect (P < 0.01) on killing of C. albicans filaments by PMN. In contrast, at these concentrations no effect of cytochalasin D on killing of C. albicans yeast cells could be observed (Fig. 5), indicating that motility of PMN is not essential for killing of this morphotype. No dosage effect of cytochalasin D could be observed at concentrations leading to paralysis but not cell death of PMN (Fig. 5). When higher concentrations were used, PMN became apoptotic and their killing efficacy towards both morphotypes of C. albicans was severely diminished (data not shown). These results fit well with the observation of neutrophil behaviour towards either morphotype in the time-lapse microscopy experiments. Taken together, these data indicate that inhibition of neutrophil motility by interfering with reorganization of the actin cytoskeleton has a profound effect on the activity of neutrophils against C. albicans filaments but not yeast forms. To test, whether other components of the cytoskeleton were also involved in PMN anti-Candida activity, experiments were performed with colchicine (10 µg ml⁻¹), a toxin inhibiting polymerization of microtubules. In contrast to cytochalasin D, this substance did not interfere with targeted motility of neutrophils. As expected, no effect of colchicine on growth inhibition of C. albicans filaments by human PMNs could be observed (data not shown).

Recognition and killing of C. albicans filaments by PMN is mediated by the MEK/ERK MAP-kinase cascade

Different cascades have been shown to mediate granulocyte motility and targeted migration. To determine the signalling pathways involved in recognition of C. albicans filaments by human neutrophils, chemotaxis assays were performed in the presence of inhibitors of several regulatory cascades. In control experiments, addition of the solvent DMSO in concentrations identical to those used for diluting the inhibitors did not impair neutrophil migration (data not shown) and all inhibitors except the ERK 1/2...
inhibitor did not affect growth, viability or filamentation of *C. albicans*. The ERK 1/2 inhibitor 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione-HCl did not affect viability of *C. albicans* but severely reduced filamentation under inducing conditions. Although induction of the filamentous morphotype was performed before the experiments (without the inhibitor) this could potentially indicate an influence of this specific inhibitor on *C. albicans*, which might modulate the outcome of experiments. Neither inhibition of the p38 MAP-kinase cascade by SB202190 (final concentration 2 μM) nor inhibition of the JNK1/2 kinases by SP600125 (2 μM) impaired neutrophil migration induced by *C. albicans* filaments (Fig. 6). In contrast U0126 (2 μM), an inhibitor of MEK1/2 activation, markedly impaired migration of PMN towards *C. albicans* filaments, suggesting a central role of the ERK kinase cascade (Fig. 6). To confirm this, inhibitors of tyrosine kinases (genistin, 10 μM), and ERK1/2 were used in identical experiments. Both inhibitors showed an almost complete reduction of a specific PMN migration induced by *C. albicans* filaments (Fig. 6). Therefore, activation of the ERK cascade, but not of JNK or p38 MAPK signalling, is crucial for neutrophil migration induced by *C. albicans* filaments. To directly monitor ERK activation in human PMNs after contact with both morphotypes of *C. albicans* Western blot analyses were performed with antibodies specific for phosphorylated ERK 1/2 kinases. Fifteen min after contact of human PMNs with *C. albicans* filaments, a strong phosphorylation of ERK was detected in neutrophils (Fig. 7). In contrast, only very low or no ERK phosphorylation could be found after contact of human PMNs with *C. albicans* yeast forms. In control experiments, no cross-reactivity of the antibodies used for detection of ERK or the actin control with *C. albicans* proteins could be detected (data not shown). As a temperature shift (25°C to 37°C) was used to induce filamentation, the differential activation of ERK could have been dependent on ambient temperature rather than the morphotype. To exclude this, a pH-shift (pH 4 to pH 7) was used to induce filamentation without altering the temperature. However, identical results were obtained, indicating that ERK phosphorylation is indeed triggered by filaments and not solely dependent on temperature or pH dependent effects. To test, whether inhibition of ERK signalling also interfered with the fungicidal activity of human neutrophils against *C. albicans* filaments, killing assays were performed in the presence of the specific MEK1/2 inhibitor U0126 (2 μM). Addition of U0126 resulted in a significantly reduced fungicidal activity of human PMNs against *C. albicans* filaments (Fig. 8). In contrast, this inhibitor showed no effect on killing of *C. albicans* yeast forms by human PMNs (Fig. 8). Therefore, PMN motility induced by *C. albicans* filaments via activation of the ERK signalling cascade is essential for killing of this morphotype, whereas neither activation of the ERK cascade nor motility play a role in killing of *C. albicans* yeast forms. 

**Fig. 4.** Inhibition of targeted motility by cytochalasin D prevents the inhibitory effect of human PMNs on growth of *C. albicans* filaments.

A. Growth of filamentous forms was quantified by incubating FITC labelled filaments at 37°C for 1 h. At the end of experiments FITC labelled fluorescent parts of the filaments, and newly grown, non-fluorescent parts were measured using the Zeiss Axiovision software and the proportion of new growth during the experiment was calculated. Nuclei of neutrophils are counterstained with DAPI (blue fluorescence).

B. Cytochalasin D completely restored growth of *C. albicans* filaments in the presence of neutrophils. Bars represent arithmetic means from three independent experiments (each including data from 100 randomly chosen filaments) ± standard deviation.
Filamentation defective mutants fail to induce ERK-dependent activation of neutrophils

Morphogenesis of C. albicans is dependent on the activation of signalling cascades and central transcription regulators such as Efg1 or Cph1 (Lo et al., 1997; Stoldt et al., 1997; Kumamoto and Vinces, 2005). Consequently, a Δefg1/cph1 mutant has been described to exhibit severe defects in morphogenesis, rendering it unable to form filaments under most conditions. In addition, this mutant displays a severe defect in virulence (Lo et al., 1997). Furthermore, a C. albicans mutant lacking Ras1, a factor of the cAMP-signalling pathway upstream of Efg1, has been shown to lack filamentation under many inducing conditions including pH and temperature shift, whereas only minor growth defects were observed in the yeast form (Feng et al., 1999; Leberer et al., 2001). Both mutants were grown under either yeast or filament inducing conditions and used for stimulation of human neutrophils. Microscopic examination of coincubation experiments revealed that neither the Δras1 nor the Δph1/efg1 deletion mutant were able to trigger human neutrophils to establish direct contact and engulf the fungi. In contrast, wild-type filaments were engulfed within 1 h of coincubation as observed before in real-time microscopy (Fig. 9). Western blot analysis of ERK phosphorylation in PMN stimulated with the C. albicans Δras1 or the Δcph1/efg1 mutant showed that both mutants were unable to induce ERK-phosphorylation in human PMN, regardless whether they were raised in yeast or filament inducing conditions (Fig. 10). Therefore, mutants defective in central regulators of filamentation have lost their capacity to initiate morphotype-specific activation of human PMN.

Candida albicans Δtup1 does not induce neutrophil activation despite its filamentous morphotype

Tup1 is a major repressor of filament formation in C. albicans (Braun and Johnson, 1997). Consequently, C. albicans Δtup1 mutants have been described to constitutively display a filamentous phenotype. To test whether C. albicans Δtup1 cells were able to activate human PMNs we performed coincubation experiments. Interestingly, although the Δtup1 mutant grew as filaments, no establishment of direct contact and engulfment by human PMNs was observed (Fig. 9). This observation was identical for C. albicans Δtup1 cells grown under yeast or under filament inducing conditions, indicating that the Δtup1 hyphae lack essential factor(s) for activation of human PMN. Western blots were performed with total protein extracts from human PMNs stimulated with C. albicans Δtup1 cells to test whether ERK phosphorylation could be observed. However, independent of the culture conditions, C. albicans Δtup1 did not induce ERK phosphorylation in human PMN.

Discussion

Candida albicans is the predominant cause of invasive candidiasis. A key virulence factor of this fungus is its ability to switch between yeast and filamentous forms and thereby adapt to different environmental conditions inside the human host. This morphological transition is quite
Fig. 6. Induction of targeted motility in human neutrophils by C. albicans filaments depends on activation of the ERK signalling cascade but is independent of p38 MAPK and JNK signalling. C. a. = C. albicans, SB20219 = p38 MAPK inhibitor, SP600125 = JNK inhibitor, ERK-inhibitor = 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione-HCl (ERK1/2 inhibitor), U0126 = MEK 1/2 inhibitor, genistin = tyrosine kinase inhibitor (all inhibitors are listed in detail in the methods section). Chemotaxis experiments were performed in a transwell system for 1 h at 37°C. n.s., not significant (P > 0.05) compared with C. albicans filaments, **P < 0.01 compared with C. albicans filaments.

Fig. 7. Coincubation of human PMNs with C. albicans filaments results in ERK phosphorylation. Human PMNs were coincubated with medium only (lane 1), C. albicans yeast cells (lane 2) or C. albicans filaments (lane 3) generated by a shift in either temperature (A) or pH (B) for 15 min and 25 min. Subsequently cells were lysed and phosphorylated ERK was detected in a Western blot. Detection of actin provides a loading control. A strong phosphorylation of ERK is induced by C. albicans filaments but not by yeast cells.

Fig. 8. Activation of the ERK signalling cascade is necessary for killing of C. albicans filaments but not yeast forms. After 210 min of coincubation, addition of MEK 1/2 inhibitor U0126 at a concentration of 2 μM completely prevented killing of C. albicans filaments but did not influence killing of C. albicans yeasts. In control experiments, U0126 at this concentration did not affect PMN vitality or C. albicans growth or viability. Inhibitors and their concentrations used in the experiments can be found in Experimental procedures.
Although both, yeast and filamentous forms are found in infectious lesions caused by *C. albicans*, the morphological switch to filamentous forms, including germ-tubes, pseudohyphae and hyphae, has been associated with invasiveness (Kumamoto and Vinces, 2005). Several studies suggested that continuous elongation of filaments may contribute to host cell damage and lead to tissue destruction and invasion (Farrell *et al.*, 1983; Zink *et al.*, 1996; Hausauer *et al.*, 2005; Kurzai *et al.*, 2005). In addition, several proteins contributing to the pathogenesis of *Candida* infection have been shown to be predominantly expressed in filamentous forms. These include secreted aspartic proteases Sap4–6, adhesins like Hwp1 and members of the Als family such as the invasin Als3 (Hube *et al.*, 1994; Hoyer *et al.*, 1998; Staab and Sundstrom, 1998; Staab *et al.*, 1999; Staib *et al.*, 2000; Naglik *et al.*, 2003; Phan *et al.*, 2007). Expression of many of these

**Fig. 9.** Microscopic images of *C. albicans–PMN* interaction after 1 h of coincubation. All fungal strains were grown under hyphal inducing conditions.
A. *C. albicans* SC5314 wild type.
B. *C. albicans* Δ*cpfl/efg1*.
C. *C. albicans* Δ*ras1*.
D. *C. albicans* Δ*tup1*.
Left: overview; right: higher magnification. Under filament inducing conditions, the wild-type strain and the constitutively filamentous Δ*tup1* mutant form filaments whereas the mutants are arrested in the yeast form. Only the wild-type strain (A) triggers neutrophils to establish direct contact and engulf the fungi.

**Fig. 10.** Western Blot detection of phosphorylated ERK1/2 in human neutrophils after stimulation with *C. albicans*. 1, *C. albicans* Δ*tup1* grown under yeast form inducing conditions (pH 4); 2, *C. albicans* Δ*tup1* grown under filament inducing conditions (pH 7); 3, *C. albicans* Δ*ras1* (pH 4); 4, *C. albicans* Δ*ras1* (pH 7); 5, *C. albicans* Δ*cpfl/efg1* (pH 4); 6, *C. albicans* Δ*cpfl/efg1* (pH 7); 7, *C. albicans* SC5314 (pH 7). Only filaments of the wild-type strain are able to induce strong ERK phosphorylation in human neutrophils. Identical results were obtained with yeasts and filaments induced by temperature shift.
Morphotype-specific activation of neutrophils by C. albicans

A significant proportion of the innate immune system is controlled by the mitogen-activated protein (MAP)- or cAMP-signalling pathways and deletion of the key transcriptional factors of these pathways, Efg1 and/or Cph1, prevents filament formation under many environmental conditions and results in severely reduced virulence of C. albicans in several infection models (Lo et al., 1997; Stoldt et al., 1997; Kumamoto and Vinces, 2005). Similar observations were made for mutations locking C. albicans in the filamentous form, indicating that morphological plasticity rather than just the filamentous phenotype is important for virulence (Liu, 2002). Based on these observations it has been speculated for some time that the immune system might be capable of differentiating between C. albicans yeast forms and filamentous forms. Dendritic cells, which are known to exert main regulatory functions, were the most promising candidates for differential reception of C. albicans morphotypes (Pulendran et al., 2001). These cells provide a link between early innate responses and the adaptive immune system and can induce activation as well as suppression of proinflammatory stimuli (Banchereau et al., 2000). It could be shown that murine dendritic cells are uniquely capable of sensing different morphotypes of C. albicans and respond by specifically activating distinct arms of adaptive cellular immunity. Phagocytosis of yeasts triggered interleukin-12 production and priming of protective T helper type 1 cells in murine dendritic cells whereas contact with filamentous forms inhibited interleukin-12 secretion and Th1 priming and instead induced IL-4 production (d’Ostiani et al., 2000). In contrast, in differential activation of human dendritic cells depended on the engagement of different receptors rather than only the morphotype of C. albicans (Romani et al., 2004). In addition, it been shown that human dendritic cells react differently to C. albicans yeast forms and filaments with regard to the release of proinflammatory cytokines (Kurzai et al., 2005).

By observing the interaction between neutrophils and different C. albicans morphotypes in real-time microscopy, it was demonstrated that C. albicans filaments but not yeast cells induce targeted motility of human neutrophils. This motility led to the establishment of a close contact between PMN and C. albicans filaments resulting in an engulfment process. Neutrophils remained viable throughout this interaction and were observed moving along fungal filaments. Even in mixed coincubations, where neutrophils were simultaneously stimulated with yeast forms and filamentous forms of C. albicans, these cells selectively attacked the filamentous forms. Consequently, migration activity of PMN induced by filaments is much higher than that induced by yeast forms, which could in part be explained by the fact that filamentous forms trigger a significantly higher release of the chemotactic factor IL-8. Behnsen et al. recently described that neutrophil reactions towards fungal pathogens are controlled by the dimensionality of the environment (Behnsen et al., 2007). Whereas Aspergillus fumigatus conidia were efficiently collected in a dragging process in a two-dimensional setting resembling the experimental approaches used in this study, C. albicans yeasts were found to be largely ignored in the same setting (Behnsen et al., 2007). Here we show that by simple alteration of the morphotype from yeast to filament, neutrophils can easily react to the same pathogen and are vigorously activated, resulting in targeted motility and IL-8 secretion. Thus, whereas environmental dimensionality is an important modulator of neutrophil activation it is dominated by a capacity of these cells to specifically sense the invasive form of C. albicans. The reason for differential induction of motility in PMN by C. albicans yeast forms and filaments can be ascribed to a strong activation of neutrophil chemotaxis by filamentous forms of C. albicans. C. albicans has been known to release chemotactic factors and previously, cell wall components and peptides of this fungus were suggested to act as chemoattractants (Cutler, 1977; Edens et al., 1999; Sato et al., 2006). Here we show that yeast cells and filaments of identical C. albicans strains possess a differential ability to induce migration in neutrophils. A similar effect has already been observed for phenotypic switching of C. albicans between the white and opaque form, which does also affect the chemotactic potential (Geiger et al., 2004). We demonstrated that activation of ERK plays a central role in initiating targeted motility towards C. albicans filaments, whereas p38 MAPK and JNK signalling pathways are not involved in this process. Inhibition of the ERK cascade at several levels of regulation, including inhibition of ERK1/2 as well as inhibition of upstream MAP kinase kinase MEK1/2 and general inhibition of tyrosine kinases all result in significantly impaired neutrophil motility in response to C. albicans filaments. Other studies confirmed that ERK signalling is triggered by C. albicans and can contribute to neutrophil and macrophage activation (Hii et al., 1999; Ibata-Ombetta et al., 2001; Zhong et al., 2003). However, published data with regard to the participation of ERK signalling in killing of C. albicans are contradictory (Hii et al., 1999; Zhong et al., 2003). This might well be due to the fact that only killing of C. albicans filaments but not yeast forms requires ERK activation. By interfering with ERK signalling or actin reorganization it was shown that targeted motility mediated by ERK activation is indeed a necessary prerequisite for killing of C. albicans filaments. Agents, such as cytochalasin D, preventing actin polymerization have been shown to suppress polarity and migration of PMN and treatment of human PMNs with cytochalasin D results in reduced mean cell velocity and phagocytic activity. These effects were also observed in this study. However, at the concentrations used in this study, cytochalasin D

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did not affect neutrophil viability by inducing apoptosis or necrosis. In contrast to the killing of *C. albicans* filaments, ERK signalling and actin based motility are dispensable for killing of *C. albicans* yeast forms. Previous observations demonstrated that the ability of PMN to prevent morphotype switching and formation of filaments by *C. albicans* is also dependent on direct contact (Fradin et al., 2005). Therefore, contact dependent antifungal effector mechanisms dominate the protective activation pattern of human neutrophils in the interaction with invasive *C. albicans*. However, it should be noted that, despite the lack of ERK activation and targeted motility towards *C. albicans* yeasts, neutrophils are well capable of killing this morphotype with the same efficiency as filamentous forms. Therefore, these data do not imply immune evasion of the yeast cells but rather suggest a rapid and specific additional activation towards filaments and a different killing mechanism.

The results presented above suggest the existence of a molecular activator of human neutrophils on the surface of *C. albicans* filaments, which is absent or only weakly accessible on the surface of *C. albicans* yeasts and able to induce ERK phosphorylation. Indeed, Tang et al. have shown that *C. albicans* can release a factor that triggers ERK but not p38 MAPK or JNK phosphorylation when cultured under experimental conditions, which in our hands strongly induce filamentation (Tang et al., 2004). Recently, some studies suggested a central role of beta-glucan detection, which might potentially involve complement receptor 3 (CR3) or dectin 1, in the activation of human neutrophils (Lavigne et al., 2006; Kennedy et al., 2007). Interestingly, Lavigne et al. were able to show that *C. albicans* filaments strongly expose beta-1,3-glucan at their surface, whereas yeast cell beta-1,3-glucan exposition was restricted to the locus of budding. In contrast to these findings, however, another study suggested that only yeast form budding scars contain sufficient amounts of accessible beta-glucan, whereas this cell wall component might be masked during filamentous growth due to diminished cell division and separation (Gantner et al., 2005). Therefore, the role of glucan in neutrophil activation and the exact nature of the stimulus for ERK signalling remain to be determined. Hints towards the compounds of *C. albicans* filaments relevant for activation of human PMNs may come from a detailed analysis of mutants. As expected, mutants defective in filament formation, namely *C. albicans Δras1* and *C. albicans Δcph1/efg1*, were found to fail in triggering ERK dependent neutrophil activation. This finding further underlines that the described activation program is morphotype-specific and not dependent on ambient growth conditions. Most notably, however, contact with *C. albicans Δtup1* did also not initiate ERK phosphorylation and fungal engulfment, although this mutant displays a constitutive filamentous phenotype. Data from the literature indicate that filaments induced by deletion of *tup1* do not exactly resemble wild-type filaments but rather represent a specific entity (Braun and Johnson, 1997). Obviously the stimulus for PMN activation is not present (or not sufficiently exposed) in Δtup1 filaments compared with wild-type filaments. A thorough comparison of the cell wall composition and protein expression pattern between wild-type and Δtup1 filaments could therefore provide important insight into the factors relevant for interaction of *C. albicans* with human neutrophils.

Taken together, our data indicate that human neutrophils are differentially activated by distinct morphotypes of *C. albicans*. Only the filamentous form, which is associated with tissue invasion and the expression of virulence-associated factors, activates ERK-signalling in neutrophils, which in turn leads to targeted motility and establishment of intimate contact between neutrophils and *C. albicans* filaments. Motility is mediated by reorganization of the actin cytoskeleton and essential for killing of *C. albicans* filaments. In contrast, *C. albicans* yeast forms neither induce ERK-mediated migration nor is their killing dependent on neutrophil motility. Thus, unlike previously thought, neutrophils are well capable of sensing danger and do not only display uniform reaction patterns. This also sheds some light on the opportunistic nature of candidosis and the fact that neutropenia poses a risk for the development of invasive Candida infection.

### Experimental procedures

#### Isolation of human neutrophils and FACS analyses

Human PMNs were isolated from peripheral blood of healthy volunteers by density gradient centrifugation using Polymorphrep (Nycomed, Oslo, Norway) (Radsak et al., 2000). Remaining red blood cells were lysed with ACK buffer (Sigma) for 5 min. Purity of isolated PMN was analysed by FACS using anti-CD66b antibody (Becton Dickinson, Heidelberg, Germany) and found to be >95%. For assessment of neutrophil viability a standard propidium iodide staining protocol was used. FACS analyses were performed on a FACSCalibur (Becton Dickinson).

#### Fungi

*Candida albicans* strain SC5314 was used for all experiments. Yeasts were stored in a 35% glycerol stock collection, plated on Sabouraud dextrose agar and incubated at 28°C for 24 h before use. Different morphotypes were induced by a temperature shift. Filaments were induced in RPMI 1640 medium supplemented with 5% fetal calf serum at 37°C for 2 h or until the desired length of filaments for real-time microscopy and microscopic monitoring of growth inhibition. Filament formation was monitored and confirmed microscopically. Very short filaments were used in the experiments to avoid clumping and ensure that mass differences between yeast cells and filaments were kept to a minimum. Clumping was further minimized by homogenizing samples...
through a syringe. Yeast cells were inoculated into RPMI 1640 medium supplemented with 5% fetal calf serum and kept at 25°C before the experiments. Alternatively, a pH shift was used to control morphogenesis. *C. albicans* was inoculated into the Sabouraud Agar into either M199 medium at pH 4 or M199 medium at pH 7, both at a temperature of 30°C for 3 h. In the acidic environment, exclusively yeast cells were found, whereas neutral pH triggered filamentation. For dry weight determination of fungi, 1⁰⁶ yeast forms or filaments were suspended in medium and filtered through a membrane (pore size 0.2 μm) with a vacuum pump. Loaded membranes were dried overnight at 56°C and the weight of loaded membranes was compared with that of the unloaded membrane before the filtration. The *C. albicans Δcph1/Δefg1* mutant (Δcph1::hisG/Δcph1::hisG, Δefg1::hisG/Δefg1::hisG URA3-hisG) (Feng et al., 1997) and the Δras1 mutant (Δras1::hisG/Δras1::hisG) (Feng et al., 1999) were provided by G. Fink, the Δtup1 mutant (Δtup1::hisG/Δtup1::hisG) was constructed by B. Braun and A. Johnson and provided by A. Brown (Braun and Johnson, 1997).

### Cytokine measurements

Supernatants of infection experiments were collected at indicated time points and immediately frozen at −80°C until cytokine quantification. Cytokine concentrations in the supernatants were determined with the Luminox technology (Biosource/Invitrogen), as described previously (Kurzai et al., 2005). Supernatants were diluted 1:5 in sterile phosphate-buffered saline (PBS) and 50 μl of the dilution were used in the assay according to the manufacturer’s instructions. Data were evaluated with the MasterPlex QT software (MiraiBio, Alameda, USA). Alternatively, standard ELISA assays were used to determine TNF-alpha and IL12-p40 (CytoSets, Biosource/Invitrogen).

### Killing and growth inhibition assays

For plate based killing assays, 8 × 10⁴ PMN and 2 × 10⁵ fungal cells (moi = 2.5) were incubated in 40 μl of RPMI 1640 + 5% for 0–3 h at 37°C. PMN were lysed by addition of 2 ml of ice-cold distilled water (4°C). Three serial dilutions (10⁻¹, 10⁻², 10⁻³) of the samples were plated on Sabouraud agar and incubated for 24 h at 37°C. Growth inhibition assays were performed using viable FITC labelled fungi. A total of 1 × 10⁵ fungal cells in 1 ml of RPMI 1640 + 5% FCS were stained with 0.1 mg fluorescein 5(6)-isothiocyanate (FITC, Sigma) for 15 min at 37°C, followed by two washing steps with RPMI 1640 + 5% FCS. After coincubation with PMN for 1 h, fungi were centrifuged on glass slides, fixed with 3.7% formaldehyde for 15 min and washed with PBS. Slides were mounted in Fluoprep (Biormerieux) for immune fluorescence microscopy. Growth of the fungi during the experiment (as indicated by non-fluorescent parts of the fungi) was quantified in relation [%] to fungal size before incubation (indicated by fluorescent parts of a fungal cell) using Zeiss Axiosvision software and measuring technology. One hundred randomly chosen fungal cells were measured for each experimental setting and parameter.

### Real-time wide-field microscopy

A total of 2.5 × 10⁶ FITC coloured viable fungi were mixed with 5 × 10⁵ PMN and filled directly into a slide chamber. After closing the chamber with a wax-vaseline mixture, the slide was examined with wide-field microscopy using an Olympus BX61 with 60 × water objective lenses and 10 × oculars. For images, every 30 s a photo was taken automatically, at 37°C over 1–2 h with an Olympus U-CMAD3 camera.

### Electron microscopy

Sterile glass plates were placed in a 24-well microtitre plate and fungal cells and human PMNs were seeded. After incubation for 1 h at 37°C plates were centrifuged at 1200 r.p.m. for 10 min. Fixation was performed with 3.7% formaldehyde (in 1 × PBS) for 10 min at room temperature. Plates were washed and incubated with 2% osmium-tetroxide for 1 h. Overnight treatment with 0.5% uranylacetate was followed by washing with PBS. Subsequent steps were performed as described previously (Kurzai et al., 2005). Electron micrographs were taken with a Zeiss EM10 microscope at the Department for Electron Microscopy, University of Würzburg (head: Professor G. Krohne).

### Transwell assay

Migration of PMN was determined by using fluorescently labelled PMN migrating through a membrane of a transwell system (3 μm pore size, polyester, for 24-well plates, Costar) following a protocol described earlier (Dürr et al., 2006). A total of 5 × 10⁶ PMNs in 1 ml of RPMI 1640 + 5% FCS were loaded with 3.3 μM 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM, Invitrogen) for 20 min at room temperature, washed and resuspended in RPMI 1640 + 5% FCS. Fluorescence of neutrophils was checked by fluorescence microscopy. The upper compartment of the transwell system was filled with 100 μl of labelled PMNs and placed into a well containing 600 μl of RPMI 1640 + 5% FCS with or without fungal cells or f-MLP (N-Formyl-Met-Leu-Phe, Sigma) as positive control. After incubation at 37°C for 60 min the inserts were removed and the fluorescence of the wells was read in a fluorescence-reader (485 nm/530 nm, GENios; Tecan, Crailsheim, Germany).

### Inhibitors

The following inhibitors were used in this study at the indicated concentrations: Cytochalasin D (inhibitor of actin polymerization, 10 μg ml⁻¹, Sigma); colchicine (inhibitor of microtubulus polymerization, 10 μg ml⁻¹, Sigma); U0126 (MEK1/2 inhibitor, 2 μM, New England Biolabs); SB202190 (p38 MAPK inhibitor, 2 μM, Sigma); SP600125 (JNK 1/2 kinase inhibitor, 2 μM, Sigma); genistin (tyrosine-kinase inhibitor, 10 μM, Sigma); 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione-HCl (ERK1/2 inhibitor, 25 μM, Calbiochem).

### Western blotting and sample preparation

Polymorphonuclear neutrophils were washed with ice-cold PBS after 20 min of coincubation with yeast cells or filaments of *C. albicans* (moi = 1). Cells were boiled with sodium dodecyl sulfate containing sample buffer for 5 min. Samples were resolved by polyacrylamide gel electrophoresis on SDS –12%
polyacrylamide gels and electrotransferred to nitrocellulose membranes. After blocking in TBS/Tween (25 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) with 5% dry milk (Bio-Rad) or 3% BSA (Albumin Fraction V, AppliChem) for 1 h at room temperature, membranes were incubated with specific primary antibodies (4°C, overnight). Following washing immunoreactivity of actin membranes was detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology). ERK membranes were incubated after washing with the secondary antibody for 1 h at room temperature and following washing immunoreactivity was detected as for actin blots. The following antibodies were used in this study: HRP conjugated anti-actin(1–19) (sc-1616) (Santa Cruz Biotechnology), anti-ERK1 and 2 (pppY185/187) (Biomol), HRP-conjugated secondary antibody (1:5000 in TBS/Tween) (Dianova).

**XTT assays**

XTT assays were prepared as described by Meshulam et al. and Gaviria et al. with modifications (Meshulam et al., 1995; Gaviria et al., 1999). XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide, Sigma) was prepared freshly in 1x PBS at 0.5 mg ml⁻¹ with heating at 55°C for 30 min. Coenzyme Q (2,3-dimethoxy-5-methyl-1,4-benzoquinone, Sigma) was added to a final concentration of 50 μg ml⁻¹ 1 x 10⁶ fungal cells and 1 x 10⁶ human PMNs were suspended in 200 μl of RPMI 1640 + 5% FCS and incubated at 37°C for 2 h. PMNs were lysed by addition of 800 μl of ice-cold distilled water (4°C), followed by centrifugation at 3000 g for 7 min. The supernatant was aspirated and the procedure was repeated once more. Fungal viability was analysed by adding 150 μl of XTT-solution to the pellet for 1 h at 37°C, followed by a centrifugation at 3000 g for 7 min. Absorbance of each sample was determined in 100 μl of the supernatant at 450 nm by use of a microplate spectrophotometer (GENios; Tecan, Greilshiem, Germany). The percentage of fungal damage was calculated with the equation \(1 - \frac{(A_{450fungi – PMNs} - A_{450PMNs})}{A_{450PMNs}} \times 100\).

**Statistical analyses**

Infection experiments were evaluated with Microsoft Excel. Two-tailed Student’s t-test was used to calculate significance values. All experiments were performed at least in triplicate with neutrophils from different donors. A five parameter logistic calculation of the standard curves was used for evaluation of the Luminex data.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Microscopic images of C. albicans yeasts (left) and filamentous forms (right) induced by a temperature shift for the experiments in this study. Very small germ-tubes were used to avoid problems with cell number estimation or extensive clumping. Dry mass determined for the filaments averaged 118% of that determined for the yeast form.

Movie S1. Interaction of human PMNs with C. albicans yeasts and filaments. Every 30 s a picture was taken. For technical details see Experimental procedures. Speed: 10 pictures per second; in the movie 1 s = 5 min. Selected shots from this movie were used in Fig. 1.

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