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*J Immunol* published online 30 July 2012
http://www.jimmunol.org/content/early/2012/07/29/jimmunol.1200185
Differential Interaction of the Two Related Fungal Species *Candida albicans* and *Candida dubliniensis* with Human Neutrophils

Eliška Svobodová,* Peter Staib,† Josephine Losse,* Florian Hennicke,† Dagmar Barz,‡ and Mihály Józsi*

*Candida albicans*, the most common facultative human pathogenic fungus is of major medical importance, whereas the closely related species *Candida dubliniensis* is less virulent and rarely causes life-threatening, systemic infections. Little is known, however, about the reasons for this difference in pathogenicity, and especially on the interactions of *C. dubliniensis* with the human immune system. Because innate immunity and, in particular, neutrophil granulocytes play a major role in host antifungal defense, we studied the responses of human neutrophils to clinical isolates of both *C. albicans* and *C. dubliniensis*. *C. dubliniensis* was found to support neutrophil migration and fungal cell uptake to a greater extent in comparison with *C. albicans*, whereas inducing less neutrophil damage and extracellular trap formation. The production of antimicrobial reactive oxygen species, myeloperoxidase, and lactoferrin, as well as the inflammatory chemokine IL-8 by neutrophils was increased when stimulated with *C. dubliniensis* as compared with *C. albicans*. However, most of the analyzed macrophage-derived inflammatory and regulatory cytokines and chemokines, such as IL-1α, IL-1β, IL-1ra, TNF-α, IL-10, G-CSF, and GM-CSF, were less induced by *C. dubliniensis*. Similarly, the amounts of the antifungal immunity-related IL-17A produced by PBMCs was significantly lower when challenged with *C. dubliniensis* than with *C. albicans*. These data indicate that *C. dubliniensis* triggers stronger early neutrophil responses than *C. albicans*, thus providing insight into the differential virulence of these two closely related fungal species, and suggest that this is, in part, due to their differential capacity to form hyphae. *The Journal of Immunology*, 2012, 189: 000–000.

Despite their similarity, *C. dubliniensis* appears to be less virulent in animal and mucosal infection models (4–7) according to its infection rate and the severity of disease. *C. dubliniensis* strains are more rapidly cleared from the site of infection and are less able to cause disseminated infections. The phylogenetic properties of *C. dubliniensis* have been well described in the literature (2, 8). A DNA hybridization array (9) and lately comparison of both genomes (10) uncovered particular differences between *C. dubliniensis* and *C. albicans* in regard to putative virulence genes, including those encoding hyphae-associated factors such as secreted proteases or invasins. The ability of *C. albicans* to reversibly switch between yeast and filamentous growth forms has widely been documented as an important virulence attribute of *C. albicans*, facilitating host invasion and colonization. *C. dubliniensis* also produces true hyphae, however, with a far lesser efficiency than *C. albicans*, which putatively contributes to its comparatively poor virulence (6, 11).

The control of fungal infection in the human host critically depends on cells of the innate immunity. Neutrophil granulocytes are major phagocytic cells that are recruited early to the infection site, where they activate a large repertoire of antimicrobial mechanisms including phagocytosis, the generation of reactive oxygen species (ROS), the release of granule enzymes and antimicrobial peptides, and the formation of neutrophil extracellular traps (NETs) (12–14). Attracted monocytes possess less microbicidal capacity but are important in alerting cells of the innate and adaptive immune system, and give rise to macrophages, important phagocytic and APCs. These innate immune cells recognize fungal pathogens via three major receptor families (i.e., the TLRs, the lectin receptors, and the NOD-like receptors), leading to activation of signaling pathways that result in tightly regulated and complementary immune responses (15, 16).
To date, little is known about the Candida species-specific responses of human innate immune cells. Because C. dubliniensis has a reduced ability to cause severe (i.e., systemic) infections, which are usually brought in context with C. albicans, one would expect a better clearance of the former by host immune cells. A previous report found no difference in the phagocytosis, oxidative burst, or killing induced by these two fungal species in whole blood (17). Nevertheless, it has been shown that even though both Candida species release a chemotactic factor for neutrophils, the chemotaxis toward C. dubliniensis is enhanced compared with C. albicans (18). Studies using epithelial cell infection models demonstrated that C. dubliniensis causes minimal invasion (5, 19) and cytokine production (20, 21) compared with C. albicans, and that this effect is attributed to the ability of the latter to switch from yeast to hyphal morphology under cell culture conditions (21).

It is important to understand their interaction with the host immune system in detail to decipher species-specific differences in the pathogenicity of these two closely related Candida species. Therefore, we compared the activation of human neutrophil granulocytes upon coinoculation with C. albicans and C. dubliniensis in vitro, using a set of previously characterized clinical isolates. We found that C. dubliniensis strains enhanced neutrophil migration and phagocytosis, as well as IL-8, ROS, and lactoferrin release, but caused less extracellular trap formation and neutrophil damage, in comparison with C. albicans strains. In addition, a reduced ability of C. dubliniensis to stimulate the production of IL-17 by mononuclear cells, a cytokine involved in neutrophil recruitment and activation, was observed. These results demonstrating a differential interaction of the two Candida species with cells of the human innate immune system may, in part, explain the differences in their virulence.

**Materials and Methods**

**Candida strains**

The C. albicans and C. dubliniensis wild-type strains that were used are listed in Table 1. All strains were routinely maintained on yeast peptone dextrose agar (20 g peptone, 10 g yeast extract, 20 g glucose, 15 g agar per liter). For experiments, cells were grown in yeast peptone dextrose medium at 30°C with shaking for 16 h and washed twice in Dulbecco's PBS (DPBS; Lonza, Wuppertal, Germany) before use. In case of C. albicans, hypha formation was induced in RPMI 1640 medium containing 1-glutamine and 25 mM HEPEs (Invitrogen, Karlsruhe, Germany) at 37°C for 1 h. For C. dubliniensis, hypha formation was induced in RMPI 1640 medium containing 10% FCS (PAA Laboratories, Co1be, Germany) at 37°C for 1 h (11). The hyphal morphology for both species was monitored microscopically to ensure a similar hyphal percentage and length.

C. dubliniensis reporter strain construction

To visualize Candida cells during interaction with neutrophils, we also used GFP-labeled fungal cells. For this purpose, C. albicans and C. dubliniensis strains that express the GFP gene from the constitutively active C. albicans and C. dubliniensis ADH1 promoter, respectively. The C. albicans reporter strain SCADH1G4A carrying a P_{CaADH1}-GFP fusion in one of the ADH1 alleles of the wild-type strain SC5314 had been described previously (26). Analogously, the C. dubliniensis strain CaADH1GFP1A, which harbors a P_{CaADH1}-GFP fusion in one of the CaADH1 alleles of the wild-type strain W284, was constructed as follows. An ApaI-XhoI fragment with CaADH1 upstream sequences was amplified by PCR with the primers CaADH1-1 (5'-TATATAGGGCCCCCGGGTATAGG-3') and CaADH1-2 (5'-TATATACTCGAGGTTTTTGTATTGTAGAACATTTACGTTAGTTG-3'). A Psol-Sall fragment containing CaADH1 downstream sequences was obtained by PCR with the primers CaADH1-1 (5'-GGGCTGTTAGGCGCTGTCAGGTAATGTCGC-3') and CaADH1-4 (5'-GGCGCTGTTAGGCGCGCGGTCG-3') and CaADH1-4 (5'-GGGCGCTGTTAGGCGCGCGGTCG-3'). The resulting construct was amplified from C. dubliniensis strain CAH111 and was used as a template for the PCR. The CaADH1 upstream and downstream fragments were successively substituted for CaADH1 upstream and downstream fragments in plasmid pADH1E1 (27) via the introduced restriction sites, respectively, to result in pCADH1E12. A SalI-BglII GFP fragment from pNIM1 (28) was cloned in the XhoI-BglII digested plasmid pCADH1E12, resulting in pCADH1GFP1. Flanked by CaADH1 upstream and downstream sequences, pCADH1GFP1 contains a cassette with the GFP gene under control of P_{CaADH1}, followed by T_{CAGT} termination sequences, and the SAT1 marker for transformant selection after transformation. Transformation by electroporation of C. dubliniensis W284 by use of the linear ApaI-SacII DNA cassette from pCADH1GFP1, transformant selection on neomycin (Werner Bioagents, Jena, Germany), and Southern analysis of genomic DNA with ECL-labeled (GE Healthcare, Freiburg, Germany) upstream and downstream CaADH1 probes for proving correct insertion of the GFP construct in the CaADH1 locus of the resulting transformant CaADH1GFP1A were performed by standard procedures as described previously (29).

**Human cell culture and stimulation**

Human neutrophil granulocytes were isolated from peripheral blood of healthy individuals as previously described (30). All blood donors gave informed consent. If not otherwise stated, neutrophils were cultured in serum-free X-VIVO 15 medium (Lonza) at a concentration of 2.5 x 10^6 cells/ml and cultivated at 37°C in a humidified atmosphere containing 5% CO2. If not stated otherwise, neutrophils were incubated with Candida cells at a multiplicity of infection (MOI) of 1. Culture supernatants were collected either after 1.5 h for ROS, myeloperoxidase (MPO), and lactoferrin measurement or after 20 h of coinoculation for IL-8 measurement. All stimulations for each donor were performed in duplicates.

PBMCs were isolated by Ficoll-Hypaque (GE Healthcare) density gradient separation, and erythrocytes were lysed using a hypotonic salt solution. PBMCs were washed with DPBS and cultured in X-VIVO 15 medium containing 10% pooled normal human serum at a concentration of 5 x 10^6 cells/ml. For stimulation experiments, PBMCs were incubated in medium alone or in medium containing 1 x 10^6 live Candida cells/ml, and supernatants were collected after 7 d for IL-8 measurement.

Monocytes were obtained from PBMCs by positive selection with CD14 magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany), following the manufacturer’s instructions. In brief, PBMCs were resuspended in MACS buffer (DPBS with 0.5% BSA and 2 mM EDTA) containing CD14 microbeads. After incubation for 30 min at 4°C, cells were washed and resuspended in MACS buffer and loaded onto MiniMACS LS Separation Columns (Miltenyi Biotech). After washing out of unlabeled cells with MACS buffer, monocytes were eluted and the purity of CD14+ monocytes was determined by FACS.

Monocyte-derived macrophages (MDMs) were obtained by culturing isolated monocytes for 7 d in X-VIVO 15 medium supplemented with 10% heat-inactivated FCS, 2 mM ultraglutamine (Lonza), and 50 µg/ml gentamicin sulfate (Lonza), and by addition of 500 IU/ml GM-CSF (Immuno-nutools, Friesoythe, Germany) every 48 h. MDMs were resuspended at 2 x 10^6 cells/ml in serum-free X-VIVO 15 medium and cultivated at 37°C in a humidified atmosphere containing 5% CO2. Cells were infected with Candida at a MOI of 1 or 4, depending on experiment. Culture supernatants were collected 20 h later, and cytokine profiles were determined by cytokine array and/or ELISA.

**Neutrophil migration assays**

Migration assays were performed as described previously (30). Neutrophils were labeled with calcein AM (Sigma-Aldrich, Taufkirchen, Germany), allowed to migrate toward Candida cells through a 3-µm-pore polycarbonate membrane of a transwell system (Corning Life Sciences, Amsterdam, The Netherlands) for 60 min and were quantified in the lower well.

**Neutrophil phagocytosis assay**

Neutrophils (5 x 10^5) in X-VIVO 15 medium were added to coverslips, precoated with 0.01% poly-l-lysine, in 24-well plates and allowed to attach for 40 min. In some experiments, neutrophils were pretrained with 15 µg/ml Cytochalasin D (Sigma-Aldrich) for 30 min at 37°C before phagocytosis. Candida cells constitutively expressing GFP were added at a MOI of 2 and sedimened by centrifugation at 1400 rpm for 3 min. After 40 min of coincubation at 37°C and 5% CO2, plates were cooled on ice and nonphagocyotised Candida cells were stained with 10 µg/ml calcofluor white (CWF; fluorochrome brightener 28; Sigma-Aldrich) for 20 min at room temperature. Cells were fixed with 4% paraformaldehyde (Carl Roth, Karlsruhe, Germany) for 15 min at 20°C, coverslips were mounted on glass slides, and phagocytosis was analyzed using a 63 x immersion oil objective at room temperature with an LSM 710 confocal laser-scanning mi-
croscope running ZEN software (Carl Zeiss, Jena, Germany). At least four of 3 × 3 microscopic fields (200 × 200 μm) were analyzed for the colocalization of GFP and CFW staining using an argon laser with a wavelength of 488 nm and corresponding filters. The percentage of phagocytosed cells was calculated from the GFP signal that does not colocalize with the CFW signal.

**Release of NETs**

Neutrophils were attached to coverslip as described earlier for the phagocytosis assay. Afterward, 1 × 10⁶ GFP-expressing yeast cells or 100 nM PMA as a positive control were added. Fungal cells were sedimented by centrifugation at 1400 rpm for 3 min. The coverslips were then incubated for 3 h at 37°C in a humidified atmosphere containing 5% CO₂. Extracellular DNA was stained with 20 μg/ml propidium iodoide (Sigma-Aldrich) for 20 min at 37°C. After washing gently with DPBS, the coverslips were placed on mounting solution on a glass slide, and NETs were analyzed using an LSM 710 confocal laser-scanning microscope (Carl Zeiss). For quantification of NETs, neutrophils were coincubated with Candida cells in white 96-well plates. Released DNA was stained with 5 μg/ml SYTOX Orange (Invitrogen), and the fluorescence was measured in a fluorescence reader (Tecan, Crailsheim, Germany) with emission and absorption filters of 540 and 575 nm, respectively.

**Neutrophil granulocytes were seeded in a flat-bottom, 96-well plate at a concentration of 5 × 10⁶ cells/ml. After addition of Candida cells at a MOI of 1, the plate was incubated for 20 h at 37°C and 5% CO₂. For control, neutrophils and Candida cells were incubated in medium only. The plate was equilibrated to 20°C, supernatants were collected in a white 96-well plate, and the lactate dehydrogenase (LDH) activity was measured using a fluorescence reader (Tecan, Crailsheim, Germany) with excitation and emission filters of 485 and 538 nm. MPO and lactoferrin were detected using a sandwich ELISA. In brief, 1 × 10⁶ GFP-expressing yeast cells or 100 nM PMA as a positive control were added. Fungal cells were sedimented by centrifugation at 1400 rpm for 3 min. The coverslips were then incubated for 3 h at 37°C in a humidified atmosphere containing 5% CO₂. Extracellular DNA was stained with 20 μg/ml propidium iodoide (Sigma-Aldrich) for 20 min at 37°C. After washing gently with DPBS, the coverslips were placed on mounting solution on a glass slide, and NETs were analyzed using an LSM 710 confocal laser-scanning microscope (Carl Zeiss). For quantification of NETs, neutrophils were coincubated with Candida cells in white 96-well plates. Released DNA was stained with 5 μg/ml SYTOX Orange (Invitrogen), and the fluorescence was measured in a fluorescence reader (Tecan, Crailsheim, Germany) with emission and absorption filters of 540 and 575 nm, respectively.

**Neutrophil damage assay**

Neutrophil granulocytes were seeded in a flat-bottom, 96-well plate at a concentration of 5 × 10⁶ cells/ml. After addition of Candida cells at a MOI of 1, the plate was incubated for 20 h at 37°C and 5% CO₂. For control, neutrophils and Candida cells were incubated in medium only. The plate was equilibrated to 20°C, supernatants were collected in a white 96-well plate, and the lactate dehydrogenase (LDH) activity was measured using a fluorescence reader (Tecan, Crailsheim, Germany) with excitation and emission filters of 485 and 538 nm. MPO and lactoferrin were detected using a sandwich ELISA. In brief, 1 × 10⁶ GFP-expressing yeast cells or 100 nM PMA as a positive control were added. Fungal cells were sedimented by centrifugation at 1400 rpm for 3 min. The coverslips were then incubated for 3 h at 37°C in a humidified atmosphere containing 5% CO₂. Extracellular DNA was stained with 20 μg/ml propidium iodoide (Sigma-Aldrich) for 20 min at 37°C. After washing gently with DPBS, the coverslips were placed on mounting solution on a glass slide, and NETs were analyzed using an LSM 710 confocal laser-scanning microscope (Carl Zeiss). For quantification of NETs, neutrophils were coincubated with Candida cells in white 96-well plates. Released DNA was stained with 5 μg/ml SYTOX Orange (Invitrogen), and the fluorescence was measured in a fluorescence reader (Tecan, Crailsheim, Germany) with emission and absorption filters of 540 and 575 nm, respectively.

**Measurement of cytokine production**

The production of IL-8 by human neutrophils was measured from supernatants of cocultures taken after 20 h using a commercial sandwich ELISA kit (ImmuNoTools). IL-1β and IL-17 were quantified from culture supernatants of MDMs or PBMCs using commercial sandwich ELISA kits from ImmunoTools and eBioscience (Frankfurt, Germany), respectively. The amounts of other inflammation-related cytokines were measured after 24 h from the supernatants of MDMs or PBMCs using commercial sandwich ELISA kits at a MOI of 1 in X-VIVO 15 medium using a Proteome Profiler Human Cytokine Array Kit (Panel A; R&D Systems, Wiesbaden, Germany).

**Statistical analysis**

Statistical analysis of the results was carried out using an unpaired two-tailed Student t test. A p value ≤ 0.05 was considered significant.

### Results

**Human neutrophils migrate at a higher rate toward C. dubliniensis**

Because both *C. albicans* and *C. dubliniensis* were shown to release chemotactic factor(s) for neutrophils (18), we studied the migratory activity of neutrophils toward different strains of both *Candida* species (Table I) in a transwell assay. All studied strains of *C. dubliniensis* significantly enhanced the number of neutrophils that migrated through the porous membrane of the transwell system compared with the strains of *C. albicans* (Fig. 1). We did not observe significant differences in the growth rate between the studied strains in suspension cultures. During the 1-h incubation time, the fungal cells did not multiply in the wells as monitored by microscopic analysis, and the *C. albicans* cells only started to germinate. Thus, the observed difference in supporting neutrophil migration was not due to difference in fungal mass among the fungal species.

**C. dubliniensis is rapidly phagocytosed by human neutrophils**

To assess the phagocytosis of both *Candida* species, we coincubated blood-derived human neutrophils with GFP-labeled yeast cells (*C. albicans* strain SCADH1G4A and *C. dubliniensis* strain CdADH1GFP1A; for details, see Materials and Methods) at a MOI of 2. After 40 min, nonphagocytosed fungal cells were stained with CFW and the percentage of engulfed cells was determined microscopically. Under these conditions, ~70% of the *C. dubliniensis* cells were internalized compared with only ~20% of *C. albicans* cells (Fig. 2). As a negative control, neutrophils were pretreated with Cytochalasin D before phagocytosis. Under this condition, almost no cells were internalized after the specified time point (data not shown).

**Reduced ability of C. dubliniensis to induce NETs**

To analyze whether *C. dubliniensis* induces the production of extracellular traps by human neutrophils, similar to *C. albicans* (14), we coincubated GFP-expressing yeast cells of both *Candida* species at a MOI of 1 in X-VIVO 15 medium containing 10 mM dihydrorhodamine (Sigma-Aldrich) for 15 min at 37°C followed by lysis of neutrophils with DPBS supplemented with 1% Triton X-100. The fluorescence signal of the oxidized dihydrorhodamine was measured in a fluorescence reader (Tecan, Crailsheim, Germany) with excitation and emission filters of 485 and 538 nm. MPO and lactoferrin were detected using a sandwich ELISA. In brief, 1 × 10⁶ GFP-expressing yeast cells or 100 nM PMA as a positive control were added. Fungal cells were sedimented by centrifugation at 1400 rpm for 3 min. The coverslips were then incubated for 3 h at 37°C in a humidified atmosphere containing 5% CO₂. Extracellular DNA was stained with 20 μg/ml propidium iodoide (Sigma-Aldrich) for 20 min at 37°C. After washing gently with DPBS, the coverslips were placed on mounting solution on a glass slide, and NETs were analyzed using an LSM 710 confocal laser-scanning microscope (Carl Zeiss). For quantification of NETs, neutrophils were coincubated with Candida cells in white 96-well plates. Released DNA was stained with 5 μg/ml SYTOX Orange (Invitrogen), and the fluorescence was measured in a fluorescence reader (Tecan, Crailsheim, Germany) with emission and absorption filters of 540 and 575 nm, respectively.

### Table I. *C. albicans* and *C. dubliniensis* clinical isolates used in this study

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ATCC, American Type Culture Collection; DSM, German collection of microorganisms.
Neutrophil responses to *C. albicans* and *C. dubliniensis*

The ability of *C. dubliniensis* to damage neutrophils is reduced

One of the pathogenic attributes of *C. albicans* is the production and elongation of hyphae, through which the pathogen is able to damage and escape from host phagocytes (31). The ability of *C. dubliniensis* to switch from the yeast to the hyphal form is reduced, especially in a nutrient-rich environment (11), which can be found in the infection site in the human body. To test whether *C. dubliniensis* can cause damage to human neutrophils to the same extent as *C. albicans*, we determined the amounts of LDH released upon damage of the plasma membrane of the host cells, in culture supernatants after 20 h of coincubation of *C. albicans* cells (strains SC5314, ATCC38696, and ATCC44808) and *C. dubliniensis* cells (strains Wu272, Wu284, and H1) with neutrophils. As a positive control, neutrophils were lysed, and as a negative control, LDH release was measured from neutrophils incubated in medium only. Even though *C. dubliniensis* was rapidly phagocytosed, the yeast cells were not able to switch into hyphae as efficiently as *C. albicans*, as observed microscopically (Supplemental Fig. 3), and caused only slight damage (~20%) to neutrophils compared with the negative control representing dead neutrophils after 20 h (~16%). In contrast, all *C. albicans* strains caused more damage (30–50% after 20 h) to human neutrophils (Fig. 5). Similarly, *C. dubliniensis* strains caused less neutrophil damage compared with *C. albicans* strains when the fungal cells were added after 1-h hypha induction (data not shown).

**Increased release of antimicrobial substances and IL-8 by neutrophils stimulated with *C. dubliniensis* versus *C. albicans***

Antimicrobial compounds and cytokines produced by the innate immune cells are important factors that affect the outcome of fungal infections. We therefore analyzed the production and/or release of ROS, MPO, lactoferrin, and the proinflammatory cytokine IL-8 by human neutrophils coincubated with *C. albicans* or *C. dubliniensis*. Supernatants from neutrophils incubated in medium only or with PMA (potent neutrophil stimulator) were used as a negative and positive control, respectively. All analyzed strains of *C. dubliniensis* caused an increased release of ROS, MPO, lactoferrin, and IL-8 when compared with the strains of *C. albicans* (Table II).

To analyze in more detail whether the morphology of the *Candida* cells per se differentially influences this phenotype, hypha formation was induced for 1 h at 37°C before addition to the neutrophils (Supplemental Fig. 1). *C. dubliniensis* strains again caused less NET formation compared with *C. albicans* strains (data not shown). Notably, after several hours of coincubation with neutrophils, most *C. dubliniensis* cells did not maintain the hyphal morphology (Supplemental Fig. 2), as also reported by Moyes et al. (21).

**Phagocytosis of *C. albicans* and *C. dubliniensis* by human neutrophils.** Blood-derived neutrophils were coincubated with GFP-expressing *C. albicans* and *C. dubliniensis* cells for 40 min at a MOI of 2. Extracellular fungal cells were stained with CFW and analyzed at ×630 magnification in a laser-scanning microscope. (A) Pictures represent overlay images of green (internalized fungal cells) and blue (nonphagocytosed fungal cells) fluorescence channels with bright field. Scale bar, 10 μm. (B) The percentage of internalized fungal cells was quantified microscopically. Results shown are percentage of phagocytosed cells + SD from at least five experiments with different blood donors. ***p < 0.001, Student *t* test.
Candida products acting on neutrophils. For this purpose, we compared the neutrophil response when coincubated with Candida cells directly and when separated by a porous membrane to allow exchange of medium and products. Whereas C. albicans caused only a slightly elevated lactoferrin production upon direct contact compared with separated cells, the induction of lactoferrin production by C. dubliniensis was largely dependent on the Candida–neutrophil contact (Fig. 7).

C. dubliniensis stimulates less production of fungal immunity-related cytokines

Because macrophages are important innate immunity cells that recruit additional cell types to the site of infection, we measured chemokines and cytokines from MDMs after coincubation with both Candida species for 24 h at a MOI of 1 using a commercially available inflammation array. C. dubliniensis and C. albicans induced several inflammatory molecules under these experimental conditions in comparison with MDMs incubated without fungal cells (Fig. 8). Whereas some molecules were detected in similar amounts for both C. dubliniensis and C. albicans, such as C5/C5a, growth-regulated oncogene-α (also known as CXC1), RANTES (CCL5), and MIP-1α (CCL3), a markedly reduced induction of G-CSF, GM-CSF, IL-1α, IL-1β, IL-1ra, IL-10, IL-16, macrophage migration inhibitory factor, serine protease inhibitor 1, TNF-α, and soluble triggering receptor expressed on myeloid cells was detected when MDMs were exposed to C. dubliniensis. In contrast, the chemoattractant IFN-γ-induced protein 10 (also known as CXCL10) was more strongly induced by the two tested C. dubliniensis strains (Wu¨272 and Wu¨284) compared with C. albicans SC5314 (Fig. 8).

Because the production of IL-17, an important cytokine in host antifungal defense and a potent activator of neutrophils (32), is also mediated through the action of the proinflammatory macrophage-derived IL-1β, we further analyzed the IL-1β production. MDMs were coincubated with yeast cells of C. albicans strains (SC5314 and ATCC38696) and C. dubliniensis strains (Wu¨272 and Wu¨284) for 20 h at a MOI of 4, and IL-1β was measured from the culture supernatants by ELISA. Both strains of C. albicans triggered significantly higher IL-1β production than did the C. dubliniensis strains (Fig. 9), which remained in yeast form, compared with the hypha-forming C. albicans (Supplemental Fig. 3).

In addition, we have compared the amounts of IL-17A produced by PBMCs upon stimulation with C. albicans and C. dubliniensis. PBMCs at a concentration of 5 × 10⁴/ml were coincubated with 1 × 10⁶/ml yeast cells of C. albicans strains (SC5314, ATCC38696, ATCC44808, and WO-1) and C. dubliniensis strains (H1, Wu¨272, Wu¨284, and VK9603-29) for 7 d, and IL-17A was measured from the supernatants by ELISA. C. albicans stimulated a higher IL-17A production in PBMCs compared with C. dubliniensis (Fig. 10). This assay was also performed with fungal cells stimulated for 1 h at 37˚C to produce hyphae. Again, C. albicans cells caused more IL-17A release than C. dubliniensis cells, and the levels of IL-17A were increased for both fungal species compared with the yeast cells (Fig. 10).

Discussion

C. albicans is the most virulent species of the genus Candida, and causes not only superficial but also life-threatening systemic infections. C. albicans and C. dubliniensis are very closely related and share numerous phenotypes; intriguingly, however, systemic
Neutrophils play a major role in innate antifungal defense. However, the understanding of neutrophil–fungal species interaction is, in part, associated with the different capacity of the two species. Therefore, our data suggest that this differential neutrophil interaction is, in part, associated with the different capacity of the two Candida species to form hyphae.

Previous studies demonstrated that C. albicans releases a chemoattractant that can act through the formyl peptide receptor on neutrophils and induce migration (18, 33). Using the single-cell chemotaxis assay, Geiger et al. (18) showed that also C. dubliniensis releases chemokinetic and chemoattractant factors for human neutrophils, and that the conditioned media of C. albicans resulted in a lower chemokinetic index than did the conditioned supernatant of C. dubliniensis. In agreement with this, we detected a higher migration of human neutrophils toward all tested isolates of C. dubliniensis compared with isolates of C. albicans (Fig. 1). The chemoattractant of C. albicans has been identified as a small peptide (18, 33), but there is evidence for the involvement of other factors in the neutrophil migration toward C. albicans, such as soluble β-glucans (34). Although C. albicans hyphae induce a higher neutrophil motility than the yeast form (35), in our assays, C. dubliniensis caused a higher migration rate of neutrophils than C. albicans (Fig. 1) despite its incapability of forming true hyphae under the tested cell culture conditions, suggesting that the chemoattractant produced by C. dubliniensis is not a hypha-specific product.

Neutrophils also internalized C. dubliniensis cells more efficiently than cells of C. albicans (Fig. 2). In a previous study, Peleroche-Llassahuanga et al. (17) found no significant difference in the phagocytosis between different isolates of both fungal species. However, in this study, we unraveled that C. dubliniensis species specifically induces increased migration, uptake, and antimicrobial effects, but less formation of NETs by neutrophils as compared with C. albicans, thereby providing new insights into the differential virulence of the two species. Furthermore, our data suggest that this differential neutrophil interaction is, in part, associated with the different capacity of the two Candida species to form hyphae.

Table II. Increased release of ROS, MPO, lactoferrin, and IL-8 from neutrophils stimulated with C. dubliniensis compared with C. albicans

<table>
<thead>
<tr>
<th>Strain</th>
<th>Donors Tested</th>
<th>% RFI</th>
<th>t Test</th>
<th>% Conc.</th>
<th>t Test</th>
<th>% Conc.</th>
<th>t Test</th>
<th>% Conc.</th>
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<td>C. albicans</td>
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<tr>
<td>ATCC44808</td>
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<td>92 ± 11</td>
<td>** 69 ± 9</td>
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<td>WO-1</td>
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<td>66 ± 22</td>
<td>80 ± 39</td>
<td>108 ± 76</td>
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<td>89 ± 5</td>
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<td>100 ± 6</td>
<td>86 ± 3</td>
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<tr>
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<td>87 ± 11</td>
<td>98 ± 6</td>
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<tr>
<td>C. dubliniensis</td>
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<td>H1</td>
<td>8</td>
<td>143 ± 24</td>
<td>** 192 ± 26</td>
<td>** 355 ± 146</td>
<td>** 196 ± 25</td>
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<td>H12</td>
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<td>** 188 ± 18</td>
<td>** 377 ± 168</td>
<td>** 195 ± 35</td>
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<td>** 194 ± 32</td>
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<td>** 209 ± 20</td>
<td>** 387 ± 195</td>
<td>** 176 ± 30</td>
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<tr>
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<td>** 133 ± 18</td>
<td>* 220 ± 47</td>
<td>134 ± 4</td>
<td>*</td>
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<td>Wu367</td>
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<td>135 ± 5</td>
<td>* 140 ± 11</td>
<td>* 273 ± 38</td>
<td>** 151 ± 14</td>
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<tr>
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<td>147 ± 2</td>
<td>** 137 ± 3</td>
<td>* 249 ± 30</td>
<td>** 123 ± 4</td>
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<td>VK9603-29</td>
<td>3</td>
<td>129 ± 9</td>
<td>* 144 ± 20</td>
<td>* 291 ± 106</td>
<td>121 ± 2</td>
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<tr>
<td>VK9603-34</td>
<td>8</td>
<td>138 ± 22</td>
<td>169 ± 17</td>
<td>** 309 ± 128</td>
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<td>123 ± 9</td>
<td>137 ± 5</td>
<td>* 230 ± 36</td>
<td>129 ± 8</td>
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<td>VK9603-63</td>
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<td>115 ± 7</td>
<td>123 ± 4</td>
<td>178 ± 21</td>
<td>116 ± 7</td>
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Neutrophils were coincubated with live yeast cells of the indicated strains at a MOI of 1, or left untreated as a negative control. ROS were measured intracellularly by detecting the fluorescence of ROS-converted fluorescent dye. MPO, lactoferrin, and IL-8 were measured from coculture supernatants by ELISA. Results shown are percentages of relative mean fluorescence intensities (RFI; for ROS) or concentrations (Conc.; for MPO, lactoferrin, IL-8) ± SD from at least three experiments with different blood donors. Data were normalized to the value of C. albicans strain SC5314 (set to 100%) after the subtraction of the negative control.

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, Student t test.
ability of whether species-specific differences in the expression or availability of fungi by neutrophils involves complement receptor 3, FcRs, and dectin-1 (36–38). In our serum-free assays, the participation of a complement-dependent uptake or an Ab-mediated uptake through FcRs could be excluded. Dectin-1 is a major receptor for Candida (39, 40), recognizing β-glucan components of the fungal cell wall and inducing phagocytosis, defense processes, and cytokine production by neutrophils, but its important role in yeast recognition is contradictory (41). Further studies are needed to analyze whether species-specific differences in the expression or availability of β-glucan might explain a more efficient uptake of C. dubliniensis compared with C. albicans.

The release of NETs is thought to be an immune defense strategy that prevents the distribution of pathogens, trapping them into a milieu of highly concentrated antimicrobial substances (42–44). NET formation is appreciated as a killing mechanism against different morphotypes of fungi (14, 45), because neutrophils that are unable to produce NETs lack the ability to inhibit fungal infection efficiently (43, 46). However, the ability of NETs to directly kill pathogens such as C. albicans is not unequivocally established (47). Even though NETosis requires autophyagy and the activation of the respiratory burst machinery involving MPO and the production of ROS (46, 48), our results show that despite the higher MPO and ROS production by neutrophils stimulated with C. dubliniensis, these neutrophils do not undergo NETosis so efficiently as they do when in contact with C. albicans (Figs. 3, 4). Moreover, Yost et al. (49) demonstrated that ROS are necessary but not sufficient for the induction of NETs, but the exact mechanism of NETosis and all factors involved are yet unknown. One might speculate that because C. dubliniensis is more efficiently phagocytosed than C. albicans, the formation of NETs would be dispensable; in contrast, C. albicans is also able to escape from the neutrophils by producing hyphae, and the large hyphae of C. albicans can be prevented from spreading when trapped in the NETs (14).

Candida species can cause cell and tissue damage during interaction with the host. Although C. dubliniensis is able to damage host cells (6), our results show that isolates of C. dubliniensis do not cause as severe damage to human neutrophils in vitro as C. albicans strains (Fig. 5). These data are in line with earlier reports showing that the ability of C. dubliniensis to disseminate and to cause cell damage in vitro and in vivo is reduced (4–7). This reduction in host cell damage is likely associated, in part, with the reduced hyphal formation of C. dubliniensis, because the hyphal morphology serves the penetration of the host cells through mechanical force of the hyphal tip (50). We have observed that under the used cell culture conditions, C. dubliniensis strains were not able to efficiently form hyphae and even after induction of hypha formation, this morphology was not maintained over time (Supplemental Figs. 2, 3). Thus, both the ability to switch between the two morphological forms and to maintain hyphal growth seems to be pivotal for Candida cells to adapt to different host niches and survive in the host (51). In addition, the hyphal morphology is associated with the production of several virulence factors, such as proteases and adhesins, which contribute to the virulence in the host (51). Thus, the observed differences between C. dubliniensis hyphae compared with C. albicans hyphae in inflicting damage to neutrophils could be, in part, also due to differences in some of the virulence factors between these fungal strains.

Upon the encounter with the fungus, neutrophils use a set of killing mechanisms and can further activate and recruit other immune cells by producing cytokines. Neutrophils produce microbicidal amounts of ROS such as superoxide, hydrogen peroxide, and hypochlorous acid, which damage fungi (13, 52). ROS production is catalyzed by the NADPH system and subsequently by MPO, a component of the azurophilic granules. In addition to the oxidative mechanisms, neutrophils can fight bacteria and fungi through a set of antimicrobial substances such as lactoferrin, defensins, and cathelicidins. We found that in comparison with isolates of C. albicans, the studied C. dubliniensis isolates stimulated increased levels of ROS, MPO, and lactoferrin by human...
neutrophils (Table II). These data point to an early and more pronounced activation of neutrophil antifungal activity by *C. dubliniensis*. These observations are in agreement with the study of Vilela et al. (7) where a relatively strong inflammatory reaction of mice infected with *C. dubliniensis* was seen compared with those infected with *C. albicans*. Furthermore, our results indicate that the strong stimulation of lactoferrin production in neutrophils by *C. dubliniensis* requires the fungus–host cell contact (Fig. 7). This suggests that either *C. dubliniensis* more strongly activates neutrophils, for example, because of surface-expressed molecular patterns engaged with host cell receptors, or this species lacks certain factor(s) that counteract or suppress the reaction of the host cells.

We have also investigated the release of fungal immunity-related cytokines upon encounter with both *Candida* species. IL-8 is released by neutrophils and several other cell types in response to fungal or bacterial infection and triggers the mobilization of inflammatory cells into the site of infection (52). Similar to the antimicrobial substances, the amount of IL-8 released from neutrophils was also significantly higher when cocultivated with *C. dubliniensis* than with *C. albicans*. In addition, several differences in the response of macrophages to the two related fungal species were observed. Most molecules detected with the used array were less induced by *C. dubliniensis*, such as G-CSF, GM-CSF, IL-1α, IL-1β, IL-1ra, IL-10, IL-16, serine protease inhibitor-1, TNF-α, and soluble triggering receptor expressed on myeloid cell, but IFN-γ–induced protein 10 was enhanced, indicating important differences in the chemokine and cytokine pattern induced by the two *Candida* species. Because the production of several of these cytokines and chemokines was shown to be induced by dectin-1– and/or dectin-2–mediated signaling (53, 54), the differential cytokine and chemokine induction by *C. albicans* and *C. dubliniensis* may indicate their differential recognition via these host cell pattern recognition receptors. Similarly, it has been shown that the more invasive *C. albicans* induces considerably higher production of IL-1α, IL-8 (20), G-CSF, GM-CSF, and IL-6 (21) in reconstituted human epithelium than *C. dubliniensis*.

Many studies have demonstrated that IL-17 is a key cytokine in the host defense against *C. albicans* infection (55–58). This cytokine is mainly produced by Th17 cells, which develop in the presence of IL-1β, IL-6, and TGF-β, whereas IL-23 is important for the expansion, maintenance, and function of Th17 cells (32). *C. albicans* induces IL-17 production dependent on macrophage mannose receptor, and this IL-17 production is enhanced by the TLR2/dectin-1 activation pathway (59). Because the hyphal form of *C. albicans* is recognized preferentially by TLR2 on mononuclear cells, in contrast with the yeast cells recognized mainly by TLR4 (60), the morphological plasticity of *Candida* cells might be an important aspect in regulating IL-17 production. Furthermore, IL-1β is produced after the stimulation of the inflammasome (61), which also responds to the morphology shift of *C. albicans* and is crucial in the secretion of IL-17. Indeed, it has been shown that *C. albicans* hyphae specifically induce inflammasome activation in
macrophages in a partly dectin-1–dependent manner, resulting in enhanced IL-1β secretion and induction of IL-17 production (53). In this article, we demonstrate that the production of IL-1β is reduced when MDMs are stimulated with C. dubliniensis in comparison with C. albicans (Fig. 9), supporting the important role of the morphological conversion. Furthermore, the release of IL-17 from PBMCs is also reduced for C. dubliniensis when compared with C. albicans (Fig. 10). These differences are likely due to the fact that C. albicans readily formed hyphae under the used culture conditions, whereas C. dubliniensis did not. IL-17 plays an important role in the recruitment of neutrophils (62). Although C. dubliniensis induced less IL-17A production in our assays, measured after 7 d, it did enhance the migration of neutrophils directly and the production of IL-8, which also supports neutrophil migration. The induction of these immediate and early neutrophil responses, as well as a more efficient phagocytosis, and the limited induction of later responses, including production of inflammatory cytokines and IL-17, suggest that partly because of its restricted capacity to form hyphae, C. dubliniensis poses less danger to the host organism than C. albicans, which is reflected in the attenuated late host response.

Key virulence factors of Candida include the ability to switch between yeast and hypha morphogenesis enabling the fungus in its spreading and invasion of the human host (50, 63), the production of enzymes such as secreted aspartyl proteases and phospholipases (64), and the expression of adhesins (65). Some of these virulence factors, found in C. albicans, however, are lacking or are reduced in C. dubliniensis (9, 10, 66). Aside from C. albicans, C. dubliniensis is the only Candida species that has the capacity to produce true hyphae (3), but with far less efficiency and under fewer conditions. In our experiments, C. dubliniensis did not form filaments during coinoculation with neutrophils in cell culture medium (Supplemental Fig. 3).

We and others previously showed that neutrophils respond more strongly to C. albicans hyphae than to yeast cells (30, 35). In agreement with this, we detected an enhanced production and release of ROS and lactoferrin for neutrophils coinfected with hyphal cells of various C. albicans isolates in comparison with yeast cells (Fig. 6). However, in case of the neutrophil challenge with both morphotypes of C. dubliniensis, the levels of released ROS and lactoferrin remained almost the same. When coinfing neutrophils or PBMCs with fungal cells that were induced for 1 h to form hyphae, increased amounts of ROS, lactoferrin, and IL-17A were detected for both species. Nevertheless, hyphae-induced ROS, lactoferrin, and IL-17A production for isolates of C. dubliniensis did not reach the levels of those induced by C. albicans hyphae. This could be because of the fact that C. dubliniensis does not persist in the state of hyphal form when introduced to the nutrition-rich environment (21), as we also observed during coinoculation with neutrophils (Supplemental Fig. 2).

The lack of the filamentation in C. dubliniensis is supported by expression of the NRG1 repressor and suppression of UME6 induction in a nutrient-dependent manner (11, 67). Thus, filamentation of C. dubliniensis can be increased after deletion of the NRG1 gene, which leads to an improved invasion of human epithelium and survival in macrophage cocultures in vitro (67), or in a nutrient-poor environment (11). The role of hypha production by C. dubliniensis in the interaction with host immune cells could be further studied using such genetically manipulated fungal cells, although the conclusions would be limited because of the multiple effects of NRG1 in addition to influencing filamentous growth.

In conclusion, our data indicate that, in comparison with C. albicans, early responses such as neutrophil migration, phagocytosis, and the release of antimicrobial substances are increased when encountering C. dubliniensis. Late responses, such as NETosis and the release of cytokines by mononuclear cells, including IL-1β and IL-17A involved in antifungal immunity, are reduced, which might indicate a partly dispensable late response because of the early neutrophil activation and fungal clearance. Future studies should identify the fungal and host factors underlying this differential neutrophil response.

Acknowledgments

We thank Joachim Morschhäuser for providing C. albicans and C. dubliniensis clinical isolates and C. albicans strain SCADH14A, as well as plasmids pADH1E1 and pNM1. We also thank Toni Kauffman and Christina Taubert for technical assistance, and the Department of Infection Biology (Hans Knöll Institute, Jena, Germany) for access to their microscope.

Disclosures

The authors have no financial conflicts of interest.

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