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Article

High levels of short-chain fatty acids secreted by *Candida albicans* hyphae induce neutrophil chemotaxis via free fatty acid receptor 2

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Abstract

Candida albicans belongs to our commensal mucosal flora and in immune-competent individuals in the absence of epithelial damage, this fungus is well tolerated and controlled by our immune defense. However, *C. albicans* is an opportunistic microorganism that can cause different forms of infections, ranging from superficial to life-threatening systemic infections. *C. albicans* is polymorphic and switches between different phenotypes (e.g. from yeast form to hyphal form). *C. albicans* hyphae are invasive and can grow into tissues to eventually reach circulation. During fungal infections, neutrophils in particular play a critical role for the defense, but how neutrophils are directed toward the invasive forms of fungi is less well understood. We set out to investigate possible neutrophil chemoattractants released by *C. albicans* into culture supernatants. We found that cell-free culture supernatants from the hyphal form of *C. albicans* induced both neutrophil chemotaxis and concomitant intracellular calcium transients. Size separation and hydrophobic sorting of supernatants indicated small hydrophilic factors as responsible for the activity. Further analysis showed that the culture supernatants contained high levels of short-chain fatty acids with higher levels from hyphae as compared to yeast. Short-chain fatty acids are known neutrophil chemoattractants acting via the neutrophil free fatty acid receptor 2. In line with this, the calcium signaling in neutrophils induced by hyphae culture supernatants was blocked by a free fatty acid receptor 2 antagonist and potently increased in the presence of a positive allosteric modulator. Our data imply that short-chain fatty acids may act as a recruitment signal whereby neutrophils can detect *C. albicans* hyphae.

Keywords: GPR43, acetate, granulocyte, infection, inflammation

1. Introduction

Neutrophils are our main defense against pathogens invading our tissues. For this purpose, they need to leave the circulation and migrate to the site of infection following a trail of a variety of chemotactic factors. These chemotactic factors can be released from tissue-resident cells (e.g. interleukin 8 and leukotriene B4)[1] or be activated on bacterial or fungal surfaces (e.g. the complement cleavage product C5a).[2,3] The chemotactic receptors expressed by neutrophils belong to the group of G-protein coupled receptors (GPCRs), and stimulation of such receptors directs cell migration toward increasing concentrations of chemoattractants. The ligation of chemotactic GPCRs triggers characteristic intracellular calcium transients, and assessment of such calcium transients can be used as a proxy for measurement of chemotaxis *in vitro*.[4,5]

Chemoattractants can also be released directly from microorganisms, and such end-point chemoattractants are likely key for guiding neutrophils all the way to the microbial intruders.[6] For bacteria, one main group of end-point chemoattractant released are the formylated peptides, short peptide remnants and unique hallmarks of prokaryotic protein synthesis.[7] Formylated peptides are recognized by the high-affinity GPCR formyl peptide receptor (FPR).[8] Fungi, which are eukaryotic microbes, do not use formylated methionine for protein synthesis, and fungal chemoattractants are very poorly described. Still, several previous studies have indicated that *Candida albicans*, the most common opportunistic human fungal pathogen, directly attracts neutrophils. For instance, Losse et al.[9] showed that the expression of pH-regulated antigen 1 (Pra1p) had a significant impact on neutrophil migration *in vitro*; overexpression of Pra1p resulted in increased chemotaxis, whereas yeast lacking *PRA1* had decreased ability to attract neutrophils. Whether Pra1p, which indeed is secreted, may act as a chemoattractant directly has, however, not been described. β-Glucans, components of the fungi cell wall, have also been shown to affect neutrophil chemotaxis. Harler et al.[10] demonstrated that β-glucan bound to fibronectin directed and enhanced neutrophil migration toward formylated Met-Leu-Phe (fMLF) *in vitro*, while Sato et al.[11] showed a direct effect of β-glucans in a study where the β-1,6-long glucosyl side chain–branched β-glucan, isolated from *C. albicans*, induced concentration-dependent neutrophil chemotaxis *in vitro*.

Short-chain fatty acids (SCFAs; e.g. acetate, propionate, and butyrate) are commonly found in the intestines, mainly produced

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by local commensals during their fermentation of partly indigestible fibers from our diet.[12] The SCFAs have been coupled to a healthy gut flora with maintenance of intestine homeostasis and a decreased risk of developing various diseases (e.g. colorectal cancer,[13] inflammatory bowel disease,[14,15] and type 2 diabetes[16]). SCFAs have been shown to be chemotactic for neutrophils, but while most chemotactic factors attract cells at extremely low concentrations, SCFAs need considerably higher concentrations to induce chemotaxis.[17,18] SCFAs are ligands to the free fatty acid receptor 2 (FFAR2, previously called GPR43),[19] a GPCR expressed by human neutrophils.[17,20] We have previously demonstrated that the anaerobic bacteria *Porphyromonas gingivalis* and *Fusobacterium nucleatum* release high levels of SCFAs during culture *in vitro*. In these studies, microbial supernatants containing SCFAs induced chemotaxis and calcium transients in neutrophils via FFAR2 signaling.[21,22]

C. albicans is a yeast found in the human commensal microflora and colonizes, for example, the oral cavity and the gastrointestinal tract.[23,24] Normally, our innate immune cells effectively protect against fungal infections, but *C. albicans* is an opportunist, and local use of anti-inflammatory drugs, which disturbs the immune cells, or treatment with antibiotics that affects the commensal microflora can cause local infections. Such infections are often easily treated with antifungal therapy. Moreover, local disturbances in our immune defense (e.g. due to radiation therapy as part of a treatment of head and neck cancer) can result in painful oral infections, often by *C. albicans* and other *Candida* species.[25] Also, severe or even lethal invasive infections in the tissues or circulation can occur due to systemic immune deficiencies (e.g. neutropenia or if the neutrophil killing mechanisms are defective).[26,27] Thus, neutrophils are critical for fungal defense, and recruitment of these cells is imperative for avoiding severe *Candida* infections.[28] *C. albicans* is polymorphic (i.e. able to undergo phenotypical transitions between growth forms). The organism can reversibly change between yeast form, pseudohyphal growth, and hyphal growth. The ability to change between growth forms is thought to be the cause for variation of virulence.[29,30] Hyphae are elongated and branching filaments that are able to cover large areas, to penetrate cells and invade into the bloodstream. Hyphae express a number of virulence factors like adhesins and tissue-degrading enzymes and are thereby considered a more virulent phenotype compared to yeast,[31–33] even if also the yeast form can contribute to infection.[30,34] Neutrophils have been shown to be more reactive to the hyphal form of *C. albicans* compared to the yeast form with regards to targeted migration,[35] formation of leukotrienes,[36] and neutrophil extracellular traps.[37] Peters et al.[38] showed that the innate immune defense is weaker against *C. albicans* strains defective for hyphal formation. Furthermore, factors secreted from *C. albicans* hyphae modulate neutrophil responses, for example, Pra1,[9] secreted aspartic proteases (Sap),[39] and the cytolytic peptide toxin candidalysin.[40–42] It seems clear that neutrophils can distinguish between yeast and hyphae phenotype and that the latter trigger a more pronounced inflammatory response.

In this study, we identify SCFAs, ligands for the neutrophil receptor FFAR2, as major fungal chemoattractants released by *C. albicans*, particularly during hyphal growth. These findings shed new light on the molecular cues involved in recognition and recruitment of neutrophils to *C. albicans* infections.

2. Materials and methods

2.1 Isolation of human neutrophils from buffy coats

Neutrophils were isolated from freshly drawn blood from healthy volunteers or from one-day-old buffy coats obtained at the Sahlgrenska hospital blood central, according to Boyum.[43] In short, erythrocytes were removed by dextran sedimentation and the white blood cells were thereafter separated by a density gradient (Ficoll-Paque) in a centrifugation step. After collection of the neutrophil pellet, remaining erythrocytes were lysed with distilled water, and the neutrophils were thereafter washed twice in Krebs-Ringer phosphate buffer (KRG) containing glucose (1 mM) and Mg^{2+} (1.5 mM). The neutrophil cell pellet was suspended in KRG with Ca^{2+} (1 mM) and kept on ice until use.

In some experiments, leukocytes were used (instead of neutrophils) by only subjecting the buffy coats to erythrocyte removal and washing (as above) before use.

Ethics: The study was approved by the regional ethical board of Gothenburg, Sweden, and in accordance to Declaration of Helsinki.

For chemotaxis experiments, fresh peripheral blood was drawn from healthy individuals after informed consent. For other experiments, buffy coats were obtained from the Sahlgrenska hospital blood bank after deidentification, and therefore no informed consent is needed according to Swedish law (Swedish legislation section code 4§ 3p SFS 2003:460).

2.2 Preparation of supernatants from yeast and hyphal cultures

C. albicans (SC5314, a clinical isolate from a patient with disseminated candidiasis[44]) was cultured on Sabouraud dextrose agar plates for 24 h at 37 °C, and thereafter, 4 to 5 colonies were suspended in RPMI (without phenol red, 5 mL) and further cultured in a water bath at 30 °C for 24 h (softly shaken). The fungal cells were counted by flow cytometry (Accuri C6; BD) and thereafter diluted to 1×10^6/mL in RPMI and further cultured at 30 °C (water bath) for 4 h for growth in yeast form. To induce hyphal growth, the cultured yeast cells were washed twice in phosphate-buffered saline (PBS) (500 × *g*, 5 min, room temperature), suspended in RPMI to 1×10^6/mL, and incubated for 6 h at 37 °C on a rocking board. The cultures of yeast and hyphae were visualized by Giemsa and May Grünwald staining (both from Sigma-Aldrich) on glass slides, using a microscope (Olympus BX41; Olympus).

Supernatants from yeast and hyphal cultures were prepared by centrifugation at 21,000 × *g* for 20 min followed by sterile filtration (0.2 µm; Corning), and the supernatants were stored at −80 °C. Unless indicated, both yeast and hyphal supernatants were centrifuged through an Amicon ultra 0.5 centrifugal filter unit (3 kDa MWCO; Merck Millipore) at 21,000 × *g* for 20 min, 4 °C to obtain low molecular weight molecules.

The protein content in the supernatants was measured using a BCA protein assay kit (Pierce) according to the manufacturer's instructions.

2.3 Reverse-phase chromatography of fungal supernatants

The fungal supernatants were sorted in hydrophobic (eluate) and hydrophilic (flowthrough) fractions using C18 solid-phase extraction columns (Strata C18-E; Phenomenex). In short, the C18 columns were activated with 40% acetonitrile in 0.05% formic acid and equilibrated with 0.05% formic acid. The fungal supernatants were passed over the column, and the unbound flowthrough was collected in Eppendorf tubes. Retained molecules were eluted by 40% acetonitrile in 0.05% formic acid, dried by vacuum centrifuge (Concentrator Plus; Eppendorf) at 45 °C for 3 h, and redissolved in 1% DMSO (Sigma-Aldrich) in KRG with Ca^{2+}.

2.4 Analysis of *C. albicans* supernatants with liquid chromatography/tandem mass spectrometry

Liquid chromatography (LC) and tandem mass spectrometric analysis (MS/MS) using an ExionLC UHPLC system coupled to a 6500+ QTRAP (AB Sciex LLC) was performed on size-separated (<3 kDa) supernatants as described in Dahlstrand Rudin et al.[22] to quantitatively determine the contents of SCFAs in the fungal supernatants.

2.5 Measurements of intracellular calcium transients in leukocytes

Suspensions of leukocytes, or isolated neutrophils, at 6×10^6/mL were labeled with the fluorescent calcium indicators Fluo-3 (4 µg/mL; Invitrogen) and Fura Red (10 µg/mL; Invitrogen) suspended in KRG with Ca^{2+} and 1% fetal calf serum (FCS) for 30 min at 37 °C. The cells were thereafter washed twice and resuspended in KRG with Ca^{2+} and 1% FCS. When all leukocytes were used, cells were labeled with an APC-conjugated anti-CD45 antibody (1:500; Abcam) to facilitate the gating of distinct leukocyte subsets. For each sample, labeled cells (50 µL) were mixed with KRG with Ca^{2+} and incubated at 37 °C for 5 min with or without the FFAR2 antagonist GLPG0974 (1 µM; Tocris) or the allosteric modulator Compound 58 (Cmp58, 1 µM; Tocris). The assay was performed by flow cytometry (Accuri C6, BD) with fMLF (10 nM), *C. albicans* supernatant (1:10 final dilution), or buffer being added with a gel-loading tip after a 20-s running time (the pipette tips were quickly filled and emptied a few times in the cell sample to ensure adequate mixing) during measurements (10 measurements per second) of fluorescence intensities of Fluo-3 (ex 488 nm, em 525 nm) and Fura Red (ex 488 nm, em 660 nm). When a Ca^{2+}-inducing stimulus is added, the Fluo-3 signal increases while the Fura Red signal decreases.[45] From the mean fluorescence in each channel (excluding unlabeled events), intracellular Ca^{2+} fluctuations were plotted as the Fluo-3/Fura Red ratio over time using FlowJo software (V10; TreeStar) and GraphPad Prism (version 9.2.0; GraphPad Software). Assays typically ran over 3 min and encompassed roughly 45,000 gated events, meaning that the mean fluorescence in each time point was based on approximately 25 events per time point. When whole leukocyte samples were used, distinct leukocyte populations were identified based on CD45 expression and side scatter and analyzed separately.

2.6 Evaluation of neutrophil transmigration

Neutrophils (60,000 cells/sample, 2×10^6/mL [30 µL, sample volume]) in KRG with Ca^{2+} (1 mM) with 0.3% bovine serum albumin (BSA) were added on top of a chemotaxis membrane (with a pore size of 3 µm; ChemoTX Disposable Chemotaxis System; Neuroprobe Inc) and allowed to migrate over the membrane toward fungal supernatants (diluted 1:1, 1:3, 1:5, or 1:10), fMLF (10 nM), or buffer (30 µl) for 90 min at 37 °C in 5% CO_2. After incubation, the membrane was removed and the migrated cells were lysed with 2% cetyltrimethylammonium bromide in PBS with 2% BSA. The lysed cells were quantified by myeloperoxidase activity measurements with peroxidase reagent (OPD; Sigma-Aldrich) in phosphate citrate buffer. Absorbance at 450 nm was measured in a plate reader (CLARIOstar; BMG Labtech), and the results were calculated as percent migration (of 60,000 cells).

2.7 Statistical analyses

Statistical analyses were performed with parametric or nonparametric tests using GraphPad Prism (version 9.2.0) as described in the figure legends. A *P*-value below 0.05 was considered statistically significant.

3. Results

3.1 *C. albicans* hyphae release neutrophil chemoattractants

In this study, we evaluated whether *C. albicans* culture supernatants contain chemotactic factors for neutrophils. *C. albicans* is polymorphic, and since the neutrophils are reportedly more responsive toward the hyphal form than toward the yeast form,[35,36] we first cultured *C. albicans* in temperatures beneficial for the formation of the yeast (30 °C) and hyphae phenotype (37 °C), respectively.[46] A microscopic examination determined that the cultures of the two *C. albicans* growth forms were successful (Fig. 1A). We were unable to properly enumerate hyphal cells, but a BCA protein assay performed on supernatants from four individual cultures showed that supernatants from yeast and hyphal cultures contained similar concentrations of proteins (Fig. 1B).

Chemotactic factors are often small soluble molecules, and the supernatants were therefore sorted based on size using a centrifugal filter column, and the fraction <3 kDa was used to stimulate neutrophils in the subsequent experiments. Neutrophil transmigration involves activation of GPCRs and typically results in an intracellular Ca^{2+} transient as the Ca^{2+} stores are emptied. For example, the chemoattractant fMLF induced a Ca^{2+} transient in neutrophils via the GPCR FPR1 (Fig. 2A, inset). The supernatants from hyphal cultures induced distinct intracellular Ca^{2+} transients, while the supernatants from yeast cultures did not affect the neutrophils in this assay (Fig. 2A and B). Next, the supernatants were assayed for their chemotaxis-inducing capacity. Even though the absolute levels of chemotaxis were somewhat lower compared to that induced by the potent positive control fMLF, undiluted supernatants from hyphal cultures (1:1) induced significant neutrophil chemotaxis in vitro. However, also the yeast supernatant showed a similar trend of low but dose-dependent chemotaxis, even though no statistical significance was reached (Fig. 2C).

3.2 Hydrophilic molecules in the supernatants from hyphal cultures trigger neutrophil Ca^{2+} signaling

As supernatants from yeast cultures showed low effect on neutrophil chemotaxis and did not trigger intracellular Ca^{2+} release, we continued with supernatants from hyphal cultures. To further evaluate the content of chemoattractants in the hyphae supernatant, a < 3-kDa filtered supernatant was passed over a C18 solid-phase extraction column to separate hydrophobic and hydrophilic molecules. The fraction that did not bind to the column (flowthrough with hydrophilic molecules) and the fraction eluted from the column using organic solvent (eluate with hydrophobic molecules) were collected separately. Of the two fractions, neutrophils responded with intracellular calcium transients to the flowthrough, while the eluate did not affect the cells in this assay (Fig. 3A and B). This indicates that the chemoattractants released by *C. albicans* are small (<3 kDa), hydrophilic molecules.

SCFAs are small hydrophilic molecules shown to be released by other microorganisms.[12,14,21,22] Therefore, using LS-MS, we analyzed the content of SCFAs in the fungal supernatants (<3 kDa). Supernatants from both hyphal and yeast cultures contained multiple SCFAs at µM levels (Table 1), and especially acetate (acetic acid) was particularly abundant (millimolar concentrations). The acetate concentration was approximately 4 times higher in

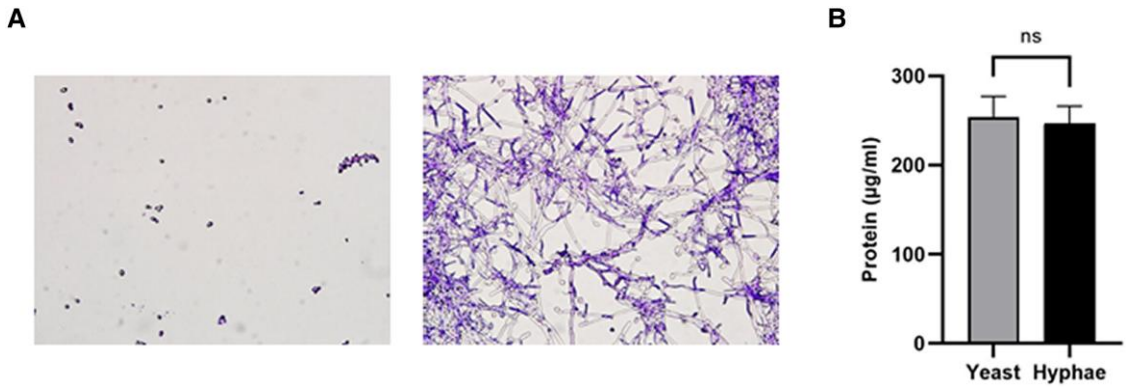

Fig. 1. Culture supernatants from yeast and hyphae of *C. albicans* contain similar protein levels. *C. albicans* was incubated under conditions optimal for culture of yeast and hyphae phenotype at 30 °C or 37 °C, respectively. Phenotypes were confirmed by Giemsa and May Grünwald staining and depicted after ocular examination (40×) (A). The protein content in the cell-free supernatants were analyzed by a BCA assay in a total of 4 supernatants of each culture. A Mann–Whitney test was used for statistical evaluation; ns, not significant (B).

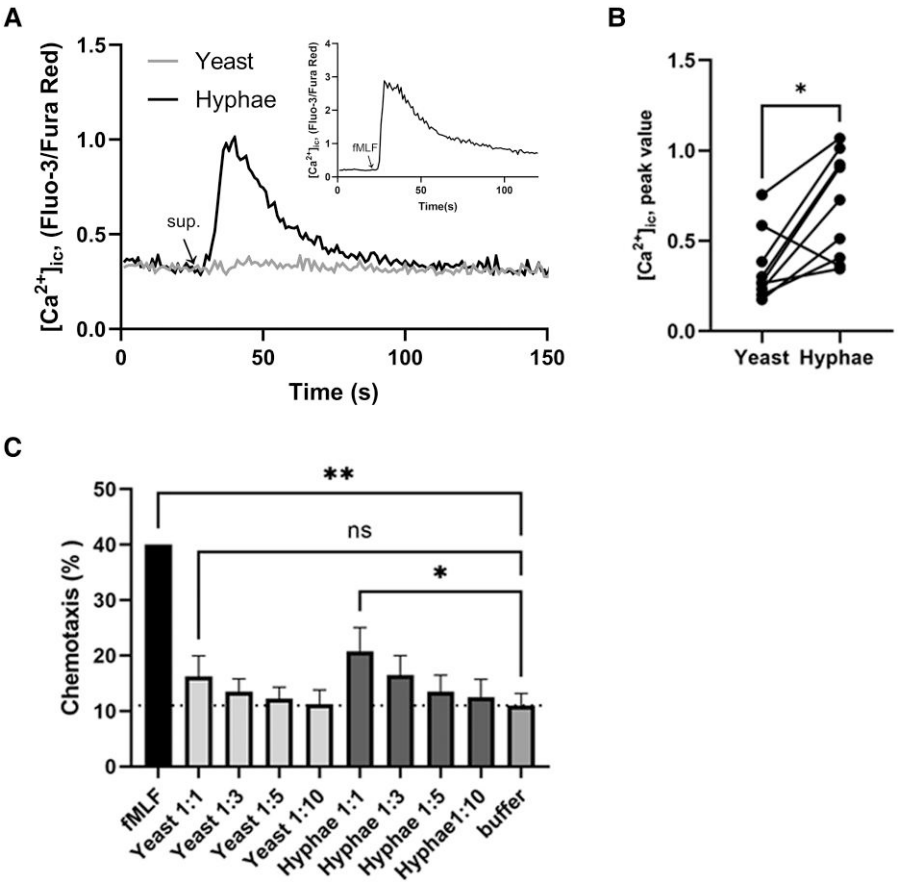

Fig. 2. Supernatants from cultures of *C. albicans* hyphae are chemotactic for neutrophils. Supernatants from hyphae and yeast cultures (1:10 dilution, arrow) were added to neutrophils during measurement of intracellular Ca^{2+} levels (ratio of mean Fluo-3/Fura Red fluorescence over time) shown in one representative curve (A; inset shows a typical response to fMLF) and peak values from neutrophils of 9 individual buffy coats (B). Wilcoxon matched-pair signed rank test was used for statistical evaluation. Supernatants from yeast or hyphae cultures in the indicated dilutions, with fMLF (10 nM, positive control) and buffer (negative control) used in a chemotaxis assay. Chemotaxis was evaluated as the mean of triplicates and calculated as percentage of 100% neutrophils (cell solution of 60,000 cells); statistical evaluation: Friedman's test followed by Dunn's multiple comparison test, $n = 4$ (C). $*P < 0.05$, $**P < 0.01$; ns, not significant.

supernatant from hyphal cultures compared to supernatant from yeast cultures. While the levels of propanoic acid, butyric acid, and isobutyric acid were also higher in the supernatant from hyphal cultures, levels of succinic acid and capronic acid were higher in the supernatant from yeast cultures (Table 1). Despite significant levels of SCFAs, the pH in the supernatants was surprisingly slightly alkaline: 8.1 (7.9 to 8.3) for yeast and 8.3 (8.1 to 8.7) for hyphae (mean and range; $n = 5$). This likely reflects that SCFAs are rather weak acids and that multiple other factors are present in the supernatants.

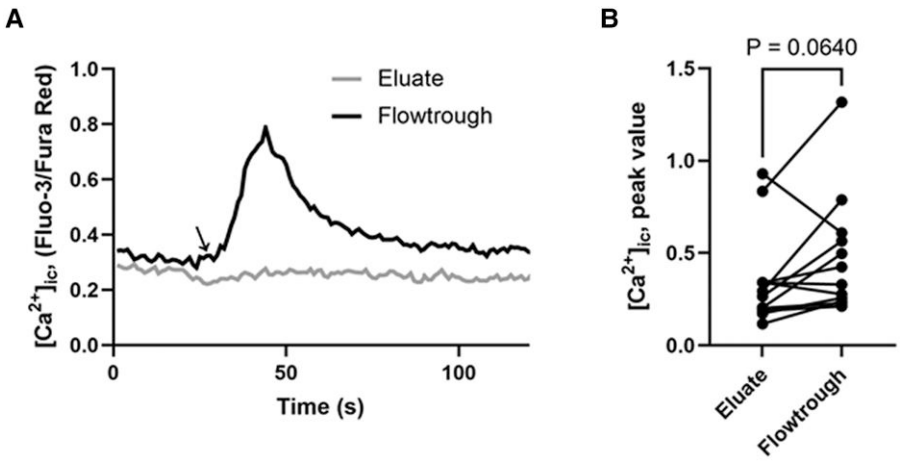

Fig. 3. Neutrophils respond with intracellular Ca^{2+} signaling to hydrophilic molecules in the supernatant from hyphal cultures. Culture supernatants from hyphae of *C. albicans* were separated on C18 columns in hydrophobic (eluate) and hydrophilic (flowthrough) fractions. The eluate and flowthrough fractions (1:10 dilution, arrow) were added to neutrophils during measurement of intracellular Ca^{2+} transients (ratio of mean Fluo-3/Fura Red fluorescence over time). Results are shown as 1 representative curve (A) and peak values from neutrophils of 12 individual buffy coats (B). Wilcoxon matched-pair signed rank test was used for statistical evaluation.

Table 1. Content of SCFAs in yeast and hyphae supernatant of *Candida albicans*.

SCFAs	Chemical structure	Hyphae supernatant, µM	Yeast supernatant, µM
		SCFAs (µM)	
Acetic acid	$C_2H_4O_2$	3,952.9	1,086.5
Propanoic acid	$C_3H_6O_2$	12.5	5.9
Butyric acid	$C_4H_8O_2$	14.1	9.6
Isobutyric acid	$C_4H_8O_2$	10.9	6.9
Succinic acid	$C_4H_6O_4$	12.3	53.6
Valeric acid	$C_5H_{10}O_2$	ND	ND
Isovaleric acid	$C_5H_{10}O_2$	ND	ND
Caproic acid	$C_6H_{12}O_2$	0.5	1.1

Abbreviation: ND = non detectable.

3.3 SCFAs released by hyphae activate neutrophils via FFAR2

The SCFAs are recognized by FFAR2, a receptor shown to be expressed and functional on neutrophils. Monocytes have also been shown to express FFAR2, at least at the mRNA level,[47] but Björkman et al.[20] reported that monocytes are nonresponsive to FFAR2 agonists. To test which leukocyte populations, other than neutrophils, responded to the hyphae supernatant, Fluo-3/Fura Red–labeled leukocytes were stained with a CD45 antibody to evaluate calcium transients in specific leukocyte populations (Fig. 4). In contrast to neutrophils, monocytes did not respond to the supernatant from *C. albicans* hyphae (Fig. 4, left). Both monocytes and neutrophils were highly responsive to fMLF (Fig. 4, right). Lymphocytes did not respond to any of the stimuli (Fig. 4). Interestingly, this indicates that the chemoattractants released by *C. albicans* hyphae are neutrophil specific.

To test whether FFAR2 was directly involved in mediating neutrophil responses to supernatants from hyphal cultures, we pretreated neutrophils with the specific FFAR2 antagonist GLPG0974 before stimulation. The FFAR2 antagonist blocked the hyphae supernatant-induced Ca^{2+} signal completely (Fig. 5A and B). Importantly, the antagonist at the concentration used did not affect the Ca^{2+} signal induced by other GPCRs such as the FPR1 agonist fMLF (Supplementary Fig. 1A).

Signaling via FFAR2 in neutrophils has previously been shown to be positively modified by the allosteric modulator Cmp 58; Cmp 58 does not activate receptor signaling per se but increases the neutrophil response to the FFAR2 agonist acetate.[48,49] The presence of Cmp 58 potently increased the intracellular Ca^{2+} signaling in neutrophils after stimulation with supernatants from hyphal cultures (Fig. 5A and B) while an fMLF-triggered Ca^{2+} signal was not affected (Supplementary Fig. 1B). When the concentration of hyphae supernatant was reduced to a nonactivating level (diluted 1:100), Cmp 58–treated cells still responded well to stimulation (Fig. 5C and D).

For comparison, we prepared a mix of different SCFAs, corresponding to the concentration and content in the hyphae supernatants of *C. albicans* (Table 1), and used this acid mix to stimulate neutrophils. Quite similar to the hyphal supernatants, the acid mix induced clear FFAR2-dependent Ca^{2+} transients in neutrophils (Supplementary Fig. 2).

Taken together, these data demonstrate that SCFAs and FFAR2 are the critical players responsible for neutrophil chemotaxis triggered by the size-filtered (<3 kD) *C. albicans* supernatants from hyphal cultures. This is, to our knowledge, the first study to identify fungal-derived neutrophil chemoattractants with identification of the responsible receptor.

3.4 SCFA are main chemoattractants released during hyphal growth

The above experiments were conducted using only the low molecular weight fractions (<3 kDa) of *C. albicans* supernatants. To explore the relative importance of SCFA and FFAR2 for the sensing of released *C. albicans* factors in a broader sense, we tested the effects of the FFAR2 modulators when stimulating cells with complete, not size-fractionated, supernatants. Responses induced by full supernatants from hyphal cultures were significantly increased by Cmp 58 and significantly decreased by the FFAR2 antagonist (Fig. 6). The FFAR2 antagonist did not completely block the responses induced by complete supernatants, indicating the existence of larger (>3 kD) chemoattractants that employ receptors distinct from FFAR2. However, these data suggest that the main chemoattractants released during hyphal growth are indeed SCFAs.

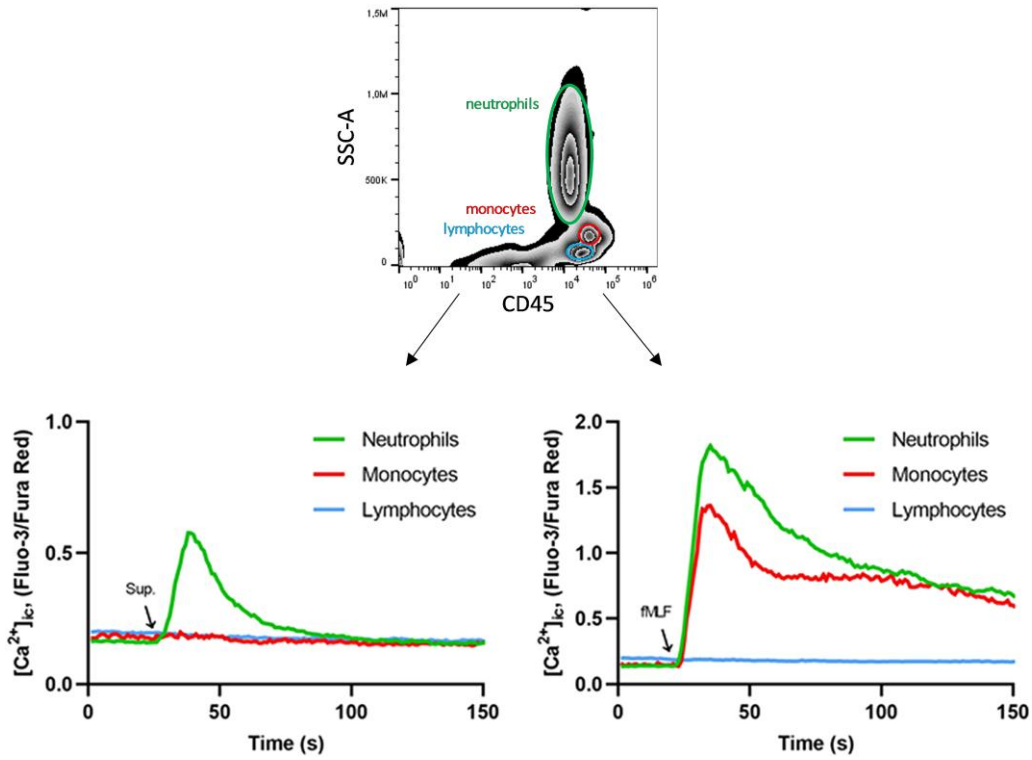

Fig. 4. Monocytes and lymphocytes do not respond with intracellular Ca^{2+} signaling to supernatant from hyphal cultures. Neutrophils (green), monocytes (red), and lymphocytes (blue) were identified based on CD45 staining and granularity (side scatter), and intracellular Ca^{2+} transients (ratio of mean Fluo-3/Fura Red fluorescence over time) were assessed in the different cell populations. Supernatant from hyphae (1:10 dilution) or fMLF (10 nM) was added to the cells during measurement. Representative curves from 1 experiment out of 17 (supernatant from hyphae) and 9 (fMLF) are shown.

4. Discussion

Neutrophils are central players in our innate defense to invading microbes, including pathogenic fungi. Individuals with neutropenia or dysfunctional neutrophils are highly susceptible to severe fungal infections.[26–28] Whereas the cues used by neutrophils to correctly find their way to invading bacteria have been studied intensively, for fungi, the situation is much less clear. To recognize and seek out microbes in the tissues, neutrophils rely on a complex network of different chemoattractants forming gradients. Endogenous molecules (such as the chemokine IL-8) released by resident cells in the affected tissue are central for getting the cells to the general area of infection, and bacteria-derived molecules ascertain that the cells reach all the way to contact the microbial cells.[6] One such molecule is C5a, the generation of which occurs on the surface of microbial cells (bacteria as well as fungi) given that complement factors are available and that activation of the complement cascade takes place. Other central end-point chemoattractants are the formylated peptides that constitute a unique hallmark of prokaryotic protein synthesis.[7] Specific chemoattractant receptors on neutrophils, the FPRs, recognize formylated peptides with high affinity and endow the cells a means to migrate toward higher concentrations of formylated peptides (i.e. closer to the bacteria). Formylated peptides are not produced by eukaryotic microbes, such as fungi, but other microbe-derived chemoattractants (and receptors) likely exist to lead neutrophils all the way to fungal cells.

In this study, we found that *C. albicans* releases neutrophil chemoattractants during in vitro growth and identified these factors as SCFAs. We found all neutrophil-activating potential in the hydrophilic fraction (containing the SCFAs) of the supernatants, but this does not necessarily mean that the hydrophobic fraction is completely devoid of neutrophil chemoattractants. The elution of the hydrophobic fraction from the column is rather harsh and may inactivate chemotactic factors in this fraction. However, the fact that most of the effect triggered by supernatants *not* subjected to hydrophobic-hydrophilic sorting was clearly mediated by FFA2R (Fig. 5) indicates that hydrophilic SCFAs are key components for the chemotactic effects seen.

SCFAs are small fatty acids with fewer than 6 carbons, and they are frequently found in the intestines as by-products of bacterial fermentation of fiber.[12,50] In the gut, SCFAs are used as nutrients for colonocytes[51] and have been ascribed an abundance of physiological effects[52] coupled to gastrointestinal health and a decreased risk of developing various diseases.[13–15] In addition to health-promoting, physiologic effects, SCFAs also have an important role in generating a proinflammatory milieu. Similar to C5a, SCFAs act as chemoattractants for neutrophils. The chemotactic effect is mediated by the GPCR FFAR2, which is capable of recognizing different SCFAs.[19] In contrast to most chemoattractant–receptor pairs, the interaction between SCFA and FFAR2 requires a comparably higher concentration of ligands.[17] This high threshold concentration needed for SCFAs to affect neutrophil chemotaxis is likely of biological value. For instance, in the gut and in circulation,[53] SCFAs reach levels in the micromolar range during homeostasis; hence, high amounts of SCFAs are present under conditions where neutrophil recruitment is not required or even unwarranted. We have previously shown release of chemotactic concentrations of SCFAs from bacteria associated with periodontal disease, *P. gingivalis* and *F. nucleatum*.[21,22] In deep periodontal

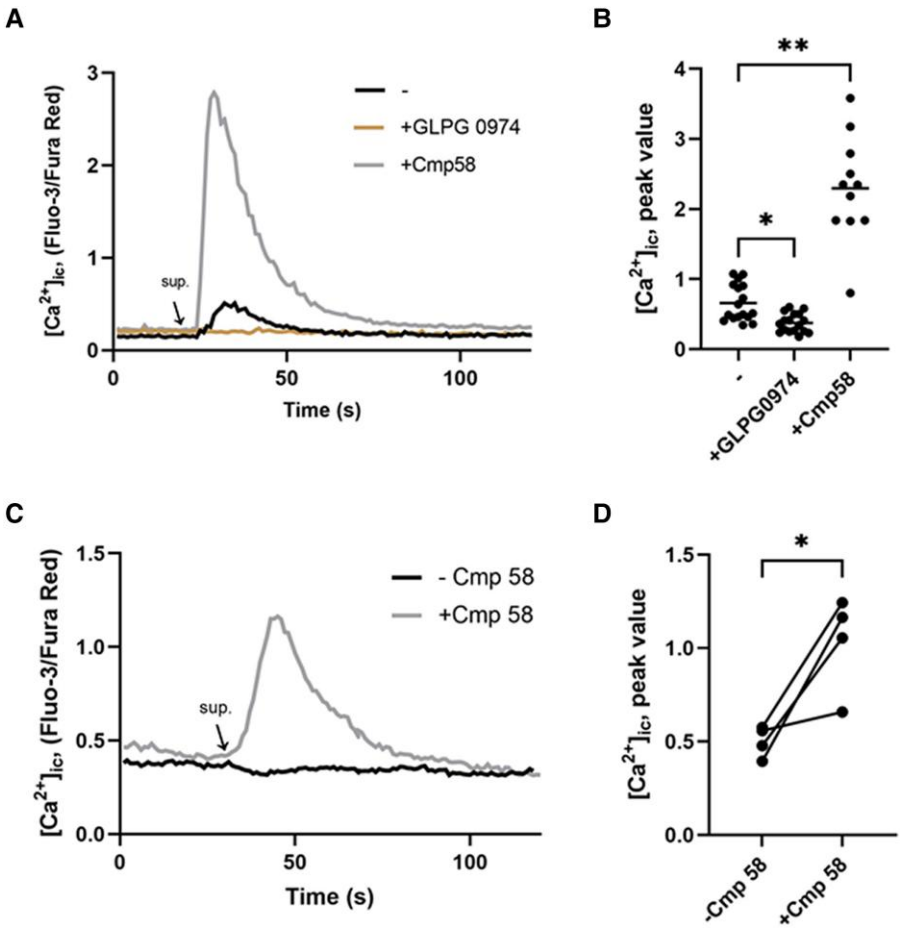

Fig. 5. Supernatant from hyphae culture-induced Ca^{2+} signaling is blocked by a FFAR2 antagonist and potentiated by Cmp 58. Neutrophils were pretreated with buffer, the FFAR2 antagonist GLPG0974 (1 μM), or the positive allosteric modulator Cmp58 (1 μM) for 5 min and intracellular Ca^{2+} levels (ratio of mean Fluo-3/Fura Red fluorescence over time) monitored. Size-filtered culture supernatants (<3 kDa, 1:10 dilution, A and B) from hyphae of *C. albicans* were added during measurement. A representative curve (A) and peak values (mean), calculated from the Fluo-3/Fura Red ratio, $n = 11$ to 17 (B), are shown. A Kruskal–Wallis test, followed by Dunn's multiple comparison test, were used for statistical analysis (B). Neutrophils pretreated with buffer or Cmp 58 (1 μM) were assessed for intracellular Ca^{2+} levels. Culture supernatants from hyphae of *C. albicans* (1:100 dilution) were added during measurement. A representative curve is shown (C) and peak values from neutrophils of 4 individual buffy coats (D). A Mann–Whitney test was used for statistical analysis. $*P < 0.05$, $**P < 0.01$.

pockets, where the bacteria reside, there is a pronounced dominance of neutrophils with only a minor presence of other immune cells.[54] This is in line with the fact that monocytes and lymphocytes lack a functional FFAR2 and do not respond to SCFA stimulation (this study and Björkman et al.[20] and Dahlstrand Rudin et al.[22]). Release of SCFAs by *Candida* metabolism has not been explored intensively, but the production of both acetate and propionate was recently shown by Oliver et al.[55] in a comprehensive analysis of metabolites produced by various *Candida* species. Our findings that SCFA (especially acetate) release is potently increased by the switch from yeast to hyphae and mediates chemotactic activity in neutrophils in vitro may be of importance in vivo. Although our in vitro data should not be directly translated to *Candida* infections in vivo, they are very much in line with the idea that a more pronounced inflammatory response (neutrophil recruitment) would be logical in response to the presence of either large quantities of yeast cells and/or the presence of hyphae. Regarding morphological transition, the switch between yeast and hyphae regulates the expression of virulence factors and seems critical for pathogenicity.[56,57] Thus, a more robust inflammatory response to hyphae as compared to yeast would likely be beneficial.

The levels of SCFA, especially acetate, released by *C. albicans* were potently increased during hyphal growth as compared to when it grows in yeast form. Interestingly, this pattern was replicated when assaying FFAR2 activation and chemotaxis; in our in vitro experiments, neutrophils responded with chemotaxis only to supernatants from hyphae but not from yeast. Perhaps the increase in SCFA release during the transition to hyphal growth could help explain why neutrophils are considerably more responsive toward hyphae than toward yeast of *C. albicans*.

It seems clear that the switch from yeast to hyphal growth is associated with more or less profound alterations to cellular metabolism.[58–60] Regarding the production of SCFAs from yeast vs hyphae, details are, to our knowledge, rather scarce but seem to be regulated largely by environmental cues (i.e. growth conditions).[58,59] Further research into what metabolic pathways are altered during yeast to hyphae switching, which could explain the differences we report here, is needed.

The culture supernatants from yeast and hyphae tested in this study are quite complex and likely contain a wide variety of different biomolecules, including proteins and complex carbohydrates. Yeast and hyphae are different phenotypes, grown under different temperatures, and this should affect the release of molecules

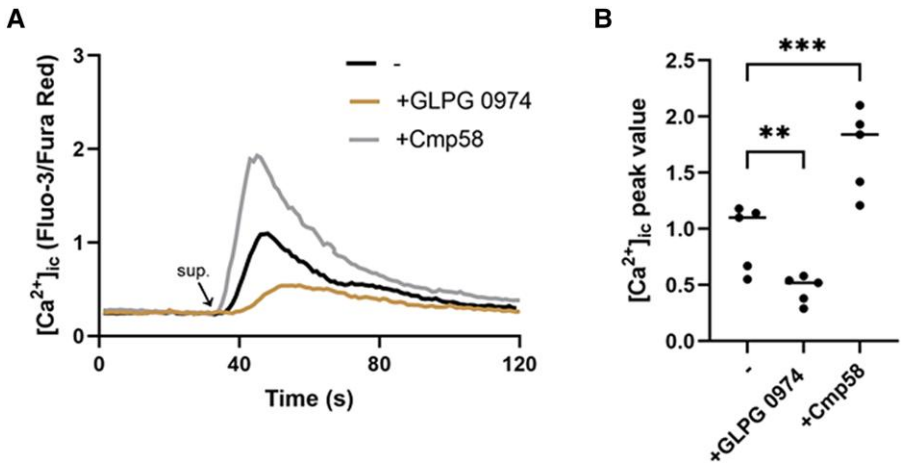

Figure 6. Intracellular Ca^{2+} signaling induced by supernatant from hyphae culture is inhibited by a FFAR2 antagonist and potentiated by Cmp 58. Neutrophils, pretreated with buffer, the FFAR2 antagonist GLPG0974 (1 μM), or Cmp58 (1 μM) for 5 min, were assessed for intracellular Ca^{2+} levels. Complete supernatants (not size filtered, A and B) from hyphae of *C. albicans* were added to the neutrophils during measurement. A representative curve (A) and peak values (mean), calculated from the Fluo-3/Fura Red ratio, from 5 independent experiments (B), are shown. A one-way ANOVA followed by Dunnett's multiple comparison test were used for statistical analysis (B). $**P < 0.01$, $***P < 0.001$.

to the supernatants. It could be argued that the reason for the higher SCFA levels in the hyphae supernatants is only quantitative (i.e. that there are more hyphal cells as compared to yeast cells). It is very hard to normalize our supernatants on the basis of cell amounts (individual hyphae are exceedingly tricky to count), but the fact that our supernatants contained rather similar protein levels makes direct comparisons valid.

Chemoattractants are typically rather small molecules, capable of efficient diffusion, and we employed a 3-kDa cutoff filter to limit the complexity and focus on the small molecules. In the <3-kDa fractions of supernatants from hyphal cultures, practically all chemotactic activity, monitored as intracellular calcium signals, was dependent on the SCFA receptor FFAR2. This would suggest that apart from the SCFAs, *C. albicans* does not release any other small chemotactic molecules during growth. Importantly, whereas C5a is a very potent neutrophil chemoattractant binding the chemotactic receptor C5aR with high affinity, our cultures were grown in the absence of serum and complement factors. Thus, C5a should not be present in our supernatants. When using the complete (not size-filtered) supernatants, FFAR2 was still a major receptor, and the specific FFAR2 antagonist inhibited most of the signal induced without blocking it completely. There is in fact (to our knowledge only) one prior identification of a *Candida*-derived neutrophil chemoattractant, showing that β-glucan from the fungal cell wall is capable of inducing neutrophil chemotaxis.[11] β-Glucan is a rather large molecule, much bigger than 3 kDa, and although it is not known what (chemotactic) neutrophil receptor β-glucan binds to, it is possible that it contributes to the FFAR2-independent signal seen using complete supernatants. In light of the finding that the chemotactic potential of *C. albicans* correlates with Pra1p expression [9] it is possible that this protein (i.e. surface located as well as secreted during hyphal growth)[61] triggers neutrophil chemotaxis in its own right. The >50-kDa Pra1 protein has been demonstrated to interact with the integrin CR3 (i.e. not a chemotactic receptor) on neutrophils,[62] but it is suggested that it also interacts with an additional, possibly chemotactic, receptor(s).[61]

In conclusion, we show that *C. albicans*, in both yeast and hyphae forms, secretes SCFAs during growth, and at least as hyphae, the levels of SCFA (especially acetate) are high enough to induce calcium signals and chemotaxis via FFAR2. Our data suggest that SCFAs are end-point chemoattractants involved in guiding neutrophils all the way to fungal cells growing in tissues. Thus, SCFAs produced by fungi constitute a type of microbe-derived pattern that can be sensed by the innate immune system.

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Author contributions

K.C., J.B., V.V., and A.K. designed the study, and A.K., V.V., H.B., M.S., F.S.K., and A.D.R. performed the experiments. Results were analyzed by A.K., V.V., A.D.R., F.S.K., C.U., J.B., and K.C. A.K. and K.C. wrote the paper with input and critical revision from all the authors.

Supplementary material

Supplementary materials are available at *Journal of Leukocyte Biology* online.

Conflict of interest statement. The authors declare no conflicts of interest.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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