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RESEARCH ARTICLE

Candida albicans-induced leukotriene biosynthesis in neutrophils is restricted to the hyphal morphology

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Abstract

Neutrophils are the most abundant leukocytes in circulation playing a key role in acute inflammation during microbial infections. Phagocytosis, one of the crucial defence mechanisms of neutrophils against pathogens, is amplified by chemotactic leukotriene (LT)B_4, which is biosynthesized *via* 5-lipoxygenase (5-LOX). However, extensive liberation of LTB_4 can be destructive by over-intensifying the inflammatory process. While enzymatic biosynthesis of LTB_4 is well characterized, less is known about molecular mechanisms that activate 5-LOX and lead to LTB_4 formation during host–pathogen interactions. Here, we investigated the ability of the common opportunistic fungal pathogen *Candida albicans* to induce LTB_4 formation in neutrophils, and elucidated pathogen-mediated drivers and cellular processes that activate this pathway. We revealed that *C. albicans*-induced LTB_4 biosynthesis requires both the morphological transition from yeast cells to hyphae and the expression of hyphae-associated genes, as exclusively viable hyphae or yeast-locked mutant cells expressing hyphae-associated genes stimulated 5-LOX by $[Ca^{2+}]_i$ mobilization and p38 MAPK activation. LTB_4 biosynthesis was orchestrated by synergistic activation of dectin-1 and Toll-like receptor 2, and corresponding signaling *via* SYK and MYD88, respectively. Conclusively, we report hyphae-specific induction of LTB_4 biosynthesis in human neutrophils.

This highlights an expanding role of neutrophils during inflammatory processes in the response to *C. albicans* infections.

KEYWORDS

5-lipoxygenase, fungi, immune response, inflammation, lipid mediator, oxylipins

1 | INTRODUCTION

Inflammation is a protective response to microbial infection, aiming to eliminate pathogens and restore tissue integrity and homeostasis. Alongside with resident tissue macrophages,[1] neutrophils form the first line of myeloid cells that mediate defence against pathogens.[2,3] Combating microbial infections, human neutrophils possess a distinct armoury of mechanisms including, phagocytosis, generation of reactive oxygen species, degranulation, and secretion of several pro-inflammatory mediators.[3] Among the latter, oxygenated lipid mediators (LM) derived from arachidonic acid (AA) are considered pivotal regulators of inflammation.[3] Above all, leukotriene (LT)B$_4$ is biosynthesized from AA by 5-lipoxygenase (5-LOX), which is a non-heme iron-containing dioxygenase and abundantly expressed in neutrophils.[4] Under inflammatory conditions, stimulated cytosolic phospholipase A$_2$ (cPLA$_2$) translocates to the nuclear membrane and releases AA from membrane-bound phospholipids. 5-LOX is activated by Ca^{2+}-binding at the N-terminal C2-like domain and serine phosphorylations through p38-MAPK and ERK1/2, and equally translocates from the cytosol to the nuclear membrane, where it co-localizes and assembles with the membrane-bound 5-LOX–activating protein (FLAP).[5] This 5-LOX/FLAP complex formation together with the AA release provides optimal conditions for the generation of LTB$_4$. LTs act in a paracrine manner and evoke several immunoregulatory functions including chemotaxis,[6-8] induction of phagocytosis,[9,10] enhancement of neutrophil swarm amplification,[11,12] and thereby promoting antimicrobial defence.[13-15]

While the physiological functions and the enzymatic biosynthesis of LTs are well characterized, less is known about microbial molecules and cellular mechanisms that drive LT formation in neutrophils in response to pathogens. Early studies suggested complement factors, bacterial peptides, endotoxins, and cell wall components to stimulate LT biosynthesis in immune cells, but elucidations of cellular mechanisms are incomplete.[16,17] Recently, we showed that amphipathic peptide toxins, called phenol-soluble modulins (PSMs), released by the bacterium *Staphylococcus aureus* induce LT-biosynthesis in neutrophils by stimulating the formyl-peptide receptor 2 (FPR2/ALX).[18] Besides bacterial stimulation, previous studies of others suggested that pathogenic fungi can also induce LM-biosynthesis. For example, *Candida albicans* can stimulate eicosanoid formation in murine macrophages through cPLA$_2$ activation *via* activation of the pattern-recognition receptors (PRRs) dectin-1 and dectin-2 by fungal cell wall components β-glucan and mannan, respectively.[19-21] Additionally, an involvement of toll-like-receptors (TLRs) was discussed during *C. albicans*-mediated cPLA$_2$ activation.[20]

The polymorphic yeast *C. albicans* is both a human commensal and a prevalent opportunistic pathogen that very frequently causes superficial mucosal infections[22] and, in severe cases, life-threatening invasive candidiasis associated with high mortality rates.[23-26] As *C. albicans* is an asymptomatic colonizer in healthy human individuals, the majority of infections originate endogenously, and are closely linked to predisposing factors that range from antibiotic treatments to immunosuppressive therapies and invasive medical technology.[27,28] The morphological shift from yeast to hyphae, which are capable of invading host tissues, is a crucial process during infections. The associated increase in pathogenicity by the transition from yeast to hyphae is a conserved mechanism accompanied by the induction of various virulence-associated processes including remodeling of the fungal cell wall and expression of effector proteins and peptides.[29]

In this study, we investigated the induction of LT formation by *C. albicans* in neutrophils. We specifically focused on how the yeast and hyphae morphologies differentially activate neutrophils for LT formation, and elucidated the receptors and underlying pathways leading to the formation of these pro-inflammatory LM.

2 | MATERIAL AND METHODS

2.1 | Materials

Acetic acid (UPLC-grade), acetone, acrylamide, agar, ammonium persulfate, BAPTA-AM, glucose, L (+)-glutamine, 2-mercaptoethanol, *n*-hexane (HPLC-grade, ≥97%), methanol (UPLC-grade), sodium dodecyl sulfate (SDS), potassium chloride (KCl), and tetramethylethylenediamine (TEMED) were purchased from VWR International (Darmstadt, Germany). Bovine serum albumin (BSA), dextran, glycine (≥99%), yeast extract, peptone ex casein, sodium chloride (≥99.5%), trishydroxymethylaminomethane (TRIS), triton X-100, coverslips and microscope slides were obtained

from Carl Roth (Karlsruhe, Germany). Amersham Protan 0.45 μm, Fura-2 AM, ionomycin, Prolong Diamond Antifade mountant with DAPI, pre-stained protein ladder, 16% formaldehyde solution and methyl formate (≥98%) were from Fisher Scientific (Schwerte, Germany). Glycerophosphate, leupeptin (LP), phenylmethylsulfonyl fluoride (PMSF), RPMI 1640 (w/o phenol red), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Histopaque-1077, lipopolysaccharide (LPS) from *Escherichia coli*, zymosan A from *Saccharomyces cerevisiae*, soybean trypsin inhibitor (STI), sodium pyrophosphate ($Na_4P_2O_7$) and UO126 were purchased from Merck KGaA (Darmstadt, Germany), and phosphate buffered saline (PBS) were bought from SERVA Electrophoresis (Heidelberg, Germany). A23187, skepinone-L and LM standards as well as deuterium labelled LM standards and MK886 were purchased from Cayman (Biomol GmbH, Hamburg, Germany). The mouse anti-5-LOX monoclonal antibody was a kind gift of Dr D. Steinhilber (Goethe University Frankfurt, Germany). The mouse anti-β-actin antibody, rabbit anti-GAPDH antibody, rabbit anti-ERK antibody, mouse anti-phospho-ERK antibody, rabbit anti-p38 antibody and rabbit anti-phospho-p38 antibody were ordered from Cell Signaling Technology, Inc (Danvers, MA, USA). The rabbit anti-FLAP polyclonal antibody was purchased from Abcam (Cambridge, MA, USA). Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 555 goat anti-mouse and normal goat serum (10%) were obtained from Invitrogen (Carlsbad, CA, USA) and R406 was from Invivogen (Toulouse, France). Acrylamide, calcium chloride ($CaCl_2$), disodium ethylenediaminetetraacetic acid (EDTA), monopotassium phosphate (K_2HPO_4), magnesium sulfate ($MgSO_4$), NP-40 and sodium orthovanadate (Na_3VO_4) were from AppliChem (Darmstadt, Germany).

Pam3Cys-SKKKK was bought from EMC Microcollections (Tübingen, Germany), RSC-3388 was from Santa Cruz Biotechnology Inc (Dallas, TX, USA) and ST2825 from MedChemExpress (Monmouth Junction, NJ, USA).

2.2 | Neutrophil isolation

Experiments with human neutrophils were approved by the ethical review committee of the University Hospital Jena, Germany. Leukocyte concentrates prepared from peripheral blood of healthy human donors were purchased from the Institute of Transfusion Medicine, University Hospital Jena. All methods were performed in accordance with the relevant guidelines and regulations. Neutrophil isolation was performed as described.[30] Briefly, neutrophils were isolated using dextran sedimentation (2.5% dextran) and subsequent density centrifugation on cell separation medium (Histopaque-1077), followed by hypotonic erythrocyte hemolysis. Neutrophils were washed twice with ice-cold PBS and resuspended in PBS (pH 7.4).

2.3 | *C. albicans* culture

C. albicans (SC5314) and all applied mutant strains are listed in Table 1. *C. albicans* cultures were grown in YPD-medium (1% yeast-extract [m/v]), 1% peptone (m/v) and 2% dextrose (m/m) at 37 or 30°C overnight. Yeast cultures were washed with PBS (pH 7.4) and adjusted to the required cell concentration. For hyphae induction, overnight cultures of *C. albicans* were washed with PBS (pH 7.4) and resuspended in RPMI (without phenol red) for

TABLE 1 *C. albicans* strains used in this study

Strain	Strain/Gene function	References
Control strains		
SC5314	Wild type	31
BWP17 (control for *ece1Δ/Δ, hgc1Δ/Δ*)	Parental strain	32
CAI-4 (control for *efg1/cph1Δ/Δ*)	Parental strain	33
Strains with terminated yeast to hyphae transition		
hgc1Δ/Δ	G1 cyclin-related protein regulating filamentation	34
efg1/cph1Δ/Δ	Transcription factors regulating filamentation	35
Hyphae-specific genes		
ece1Δ/Δ	Polypeptide protein containing the peptide toxin candidalysin	36
Clinical isolates		
C127	Clinical isolate	
C128	Clinical isolate	
C274	Isolated from human blood	ATCC44808

120 minutes at 37°C. Hyphae were washed, resuspended and adjusted to the required cell number using Neubauer improved counting chamber. For specific experiments, *C. albicans* was inactivated by exposure to heat (80°C; 20 minutes) or UV-C radiation (254 nm; 40 minutes).

2.4 | Determination of LM formation in neutrophils via HPLC

Freshly isolated neutrophils (5×10^6 cells/mL) in PBS buffer containing 1 mM $CaCl_2$ and 0.1% glucose (PGC buffer) were preincubated with indicated inhibitors for 10 minutes at 37°C and stimulated with 2.5 µM A23187. The reaction was stopped after 10 minutes at 37°C by ice-cold methanol. 5-LOX products were analyzed by RP-HPLC as described.[37] Briefly, 530 µL acidified PBS and 200 ng of internal standard (PGB_1) were added, followed by solid-phase extraction using C18 RP-columns (100 mg, UCT, Bristol, PA, USA). Analytes were eluted by methanol and samples were analyzed by RP-HPLC using a C-18 Radial-PAK column (Waters, Eschborn, Germany). 5-LOX products include the all-trans isomers of LTB_4 as well as 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) and its corresponding alcohol 5(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HETE).

2.5 | Determination of LM formation in neutrophils via UPLC-MS/MS

Freshly isolated neutrophils (5×10^6 cells/mL) in PGC buffer were co-incubated with *C. albicans* yeast or hyphae at indicated multiplicity of infections (MOI), or stimulated by 2.5 µM A23187, candidalysin or with distinct pathogen-associated molecular patterns (PAMPs) at indicated concentrations and time periods. To determine the influence of inhibitors on LM formation, neutrophils (5×10^6 cells/mL) were preincubated with inhibitors at indicated concentration versus vehicle control for 15 minutes prior respective stimulation. The incubation was terminated with 2 volumes of ice-cold methanol containing 10 µL of deuterium-labelled internal standard (200 nM d8-5S-HETE, d4-LTB_4, d5-LXA_4, d5-RvD2, d4-PGE_2, and 10 µM d8-AA; Cayman Chemicals) by means of quantification and sample normalization. Sample purification was performed as described previously.[38] In brief, samples were transferred to −20°C for at least 60 minutes to ensure protein precipitation and centrifuged at 1200× rpm for 10 minutes, at 4°C. The supernatant was diluted with 8 mL acidified H_2O (0.5 mM HCl), followed by solid-phase extraction (SPE) on Sep-Pak Vac 6 cc 500 mg/6 mL C18-columns (Waters, Milford, MA, USA). After sample loading, the column was washed with water and *n*-hexane, and LMs were eventually eluted with methyl formate. Samples were evaporated under nitrogen (TurboVap LV; Biotage, Uppsala, Sweden) and resuspended in 100 µL 50% methanol for ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) as described.[38] LMs were separated applying an ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm; Waters, Eschborn, Germany) at 50°C and at a 0.3 mL/minutes flow rate using MilliQ water (A) and methanol (B) both acidified with 0.01% acetic acid, starting with 42% of B, increasing up to 86% at 12.5 minutes that was followed by isocratic elution at 98% till 15.5 minutes. Eicosanoids were analyzed with an Acquity UPLC system (Waters) directly interfaced into the electrospray ionization Turbo V Source of a QTrap 5500 Mass Spectrometer (Sciex, Framingham, MA, USA). Analysis was performed in the negative ion mode using multiple reaction monitoring (MRM) with analytes being scheduled in specific retention time (RT) windows. Deuterated internal standards were detected using the following transitions and RTs: m/z 327.3 → 116.1 and RT 12.0 minutes for *d8*-5(S)HETE; m/z 339.3 → 197.2 and RT 9.2 minutes for *d4*-LTB_4. Eicosanoids were analyzed using the following transitions and RTs: et-LTB_4 m/z 335.2 → 195.10 at RT 8.9 minutes; LTB_4 m/z 335.2 → 195.1 at RT 9.2 minutes; 20-OH-LTB_4 m/z 351.3 → 195.1 at RT 4.8 minutes; 5-HETE m/z 319.2 → 115.1 at RT 12.1. Quantification was performed applying linear calibration curves with r^2 values of 0.998 or higher (for fatty acids r^2 values of 0.95 or higher) for each LM as described.[38]

2.6 | Lactate dehydrogenase (LDH) assay

To determine the influence of various *C. albicans* morphologies on neutrophil viability, cell membrane integrity was quantified by the release of the cytoplasmic enzyme LDH. Neutrophils (5×10^6 cells/mL) were incubated with wild type *C. albicans* yeast or hyphae at a MOI 5 and 10, with the vehicle control, and with triton X-100 (0.2%) as full lysis control for 0.5, 1, 2, and 24 hours. LDH release from disintegrated cells was measured by CytoxTox96 KIT (PROMEGA GmbH, Mannheim, Germany) according to the manufacturer's instructions. The values are presented as a percentage of the full lysis control.

2.7 | Determination of intracellular Ca^{2+} concentration

To analyze the effect of *C. albicans* yeast or hyphae on $[Ca^{2+}]_i$ in neutrophils, cells (10^7 neutrophils/mL) were resuspended in modified KREBS-HEPES buffer (20 mM

HEPES buffer, pH 7.4 containing 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.4 mM KH_2PO_4, and 5.5 mM glucose) and stained with 2 µM Fura-2-AM in the dark for 45 minutes (37°C; 5% CO_2). Cells were washed twice with KREBS-HEPES buffer (1200 rpm, 5 minutes, room temperature) and adjusted to a concentration of 5×10^6 cells/mL in KREBS-HEPES supplemented with 1 mM $CaCl_2$ and 0.1% BSA. The cell suspension was aliquoted into a black 96-well-plate (100 µL/well) and incubated with indicated MOI of *C. albicans* yeast or hyphae, vehicle control (2% DMSO, KREBS-HEPES), or with 2 µM ionomycin as the positive control. Fluorescence emission λ_{em} at 510 nm after excitation λ_{ex} at 340 and 380 nm, respectively was measured continuously over 120 minutes. Ratio of emission maxima was calculated for every time point and normalized to total Ca^{2+} influx under treatment of 1% triton X-100.

2.8 | Immunofluorescence microscopy (IF)

Immunofluorescence microscopy was applied to analyze subcellular localization of 5-LOX and FLAP under stimulation with *C. albicans* yeast or hyphae. Neutrophils (2×10^6) were seeded in PBS +/+ (PBS, pH 7.4; 1 mM Ca^{2+} and 0.5 mM Mg^{2+}) on coverslips and incubated for 30 minutes (37°C, 5% CO_2) to promote cell adherence. Cells were stimulated in PGC buffer with *C. albicans* at the indicated MOI, with 2.5 µM A23187 as positive control versus vehicle control (PGC) for 60 minutes (37°C; 5% CO_2). Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 20 minutes, followed by permeabilization in acetone on ice for 5 minutes and triton X-100 (0.25% in PBS (v/v); pH 7.4) for 10 minutes. The slides were incubated with primary antibodies (mouse monoclonal anti-5-LOX 1:100 and rabbit polyclonal anti-FLAP 1:150) in non-immune goat serum overnight. Samples were incubated with secondary antibodies (Alexa Fluor 488 goat anti-rabbit (1:1000) and Alexa Fluor 555 goat anti-mouse (1:1000) fluorophore-labelled) in non-immune goat serum for 30 minutes at 25°C in the dark and slides were mounted with DAPI-containing ProLong Diamond Antifade Mounting medium on glass slides. Samples were visualized using Zeiss Axiovert 200 M microscope and a Plan-APOCHROMAT 40×/1.3 Oil DIC (UV)Vis–IR 0.17/∞ objective (Carl Zeiss, Jena, Germany) coupled with AxioCam MR camera (Carl Zeiss). Displayed microscopic pictures show a 10×10 µm section of the full image, representative of three independent experiments.

2.9 | SDS-page and western blotting

For generation of lysates, incubations were stopped by transferring samples, corresponding 10^7 cells/mL, on ice for 5 minutes followed by centrifugation for 5 minutes at 500 g and 4°C. Cell pellets were lysed in 100 µL Saemann lysis buffer (Tris plus NaCl [pH 7.4]); NP-40 1%; Na_3VO_4 1 mM; NaF 10 mM; $Na_4P_2O_7$ 5 mM; β-glycerolphosphate 25 mM; EDTA 5 mM; leupeptin 1 mg/mL; STI 6 mg/mL; PMSF 100 mM) and kept on ice for 20 minutes. Lysates were centrifuged at 4°C and 15 000 g for 10 minutes and the protein concentration of the supernatant was determined using the Bio-Rad Protein Assay according to the manufacturer's instructions. Laemmli Buffer (TRIS-HCl 0.2 M, SDS 8%, glycerol 40%, EDTA 50 mM, bromophenol blue 0.08% and 8% mercaptoethanol) was added and samples were boiled at 96°C for 5 minutes. SDS-polyacrylamide gels (10%) were used for protein separation and were subsequently blotted to nitrocellulose membranes. To prevent unspecific antibody binding, membranes were blocked in 5% BSA in TBS (4°C, overnight) prior to incubation with primary antibodies (rabbit monoclonal anti-ERK1/2, 1:1000 (4695S, Cell Signalling); mouse monoclonal anti-phospho-ERK1/2 (Thr202/Tyr204) 1:1000 (9106, Cell Signalling); rabbit monoclonal anti-p38 MAPK, 1:1000 (8690S, Cell Signalling); rabbit polyclonal anti-phospho-p38 MAPK (Thr180/Tyr182), 1:1000 (9211S, Cell Signalling) in TBS with 5% BSA overnight at 4°C. Immunoreactive bands were stained with IRDye 800CW Goat anti-Mouse IgG (H + L), 1:10 000 (926-32 210, LI-COR Biosciences, Lincoln, NE), IRDye 800CW Goat anti-Rabbit IgG (H + L), 1:15 000 (926-32 211, LI-COR Biosciences) and/or IRDye 680LT Goat anti-Mouse IgG (H + L), 1:40 000 (926-68 020, LI-COR Biosciences) for 60 minutes at room temperature and was stored at 37°C until dryness. Analysis was performed using Odyssey infrared imager (LI-COR Biosciences).

2.10 | Statistics

Results are presented as means + SEM of n observations, where n represents the number of experiments performed with different donors. Graphs were created and validated with GraphPad Prism 7.04 software (GraphPad Software, La Jolla, CA, USA). A value of $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | *C. albicans* hyphae but not yeast cells stimulate LT biosynthesis in human neutrophils

The potential of different *C. albicans* morphologies to induce LT formation was investigated by coincubation

of neutrophils with yeast or hyphae at different MOI, and 5-LOX products formation was monitored over a period of 60 minutes. The metabolites 5-HETE, LTB_4 and its trans-isomer t-LTB_4 together with 20-OH-LTB_4, the rapidly formed metabolite of LTB_4, were considered as 5-LOX products (Figure 1A). Apart from Figure 1, the sum of LTB_4 and 20-OH-LTB_4 are presented as LTB_4 products. Remarkably, only hyphae were able to evoke 5-LOX activation and LM biosynthesis, whereas yeast cells failed to induce LT formation (Figure 1B). While 5-HETE formation peaks around 30 minutes upon stimulation, other 5-LOX products such as LTB_4 and 20-OH-LTB_4 reached their maximum at 45 and 60 minutes, respectively

(Figure 1B). Based on this finding, we chose an incubation period of 60 minutes for further experiments to reflect an informative LT biosynthesis and present a sum of LTB_4 and 20-OH-LTB_4 as LTB_4 products as. In neutrophils, LT generation requires translocation of the cytosolic 5-LOX to the nuclear membrane to form the essential biosynthetic complex with the scaffold protein FLAP. Therefore, immunofluorescence microscopy was performed to monitor subcellular localization of 5-LOX and FLAP upon *C. albicans* stimulation. After exposure of neutrophils to yeast cells, FLAP was located at the nuclear membrane with 5-LOX remaining dispersed in the cell comparable to the unstimulated vehicle control (Figure 1C). However,
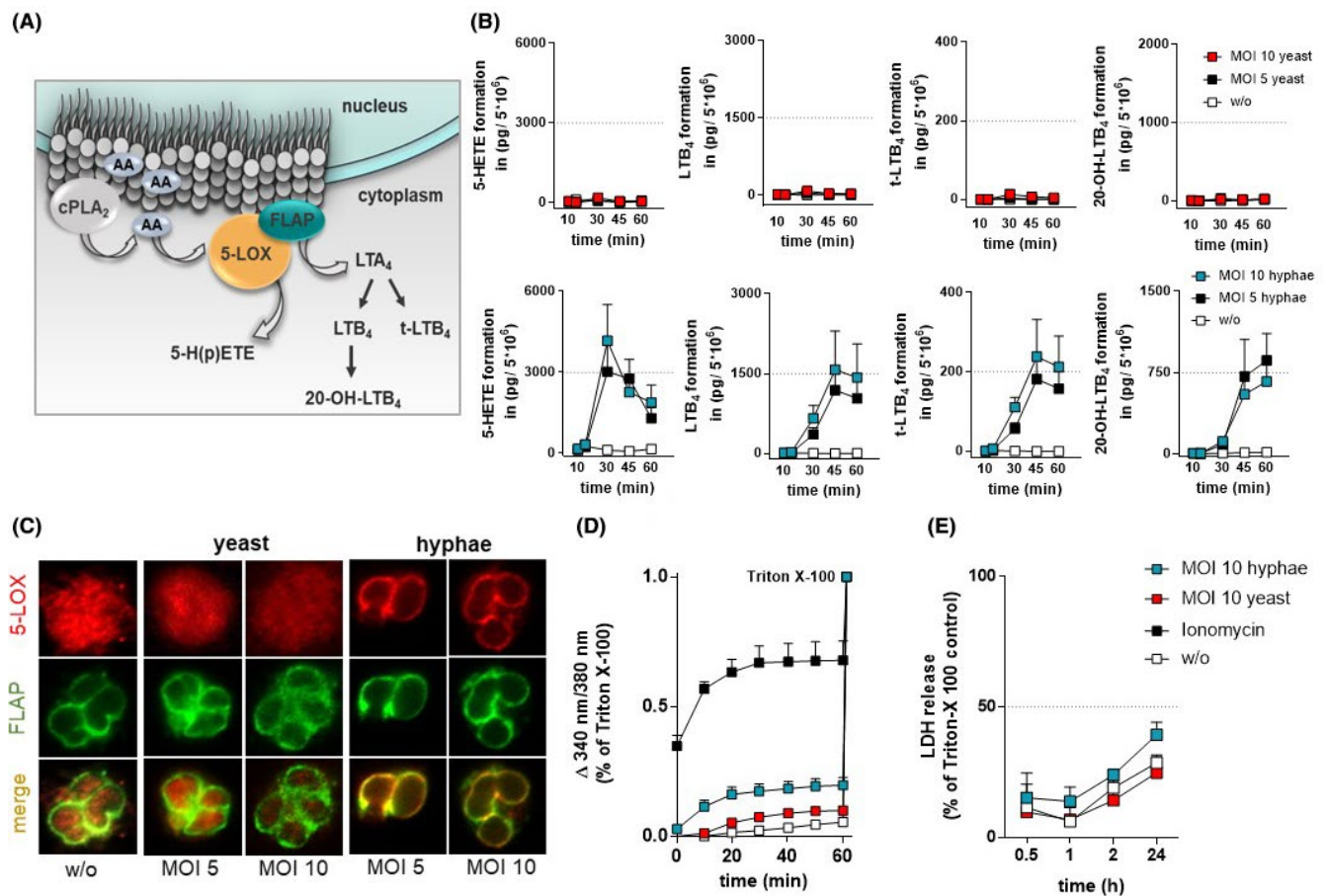
FIGURE 1 Yeast and hyphae of *C. albicans* (SC5314) differentially impact 5-LOX activation, LM biosynthesis and host cell viability. A, Schematic overview of cellular LT biosynthesis. B, Human neutrophils (5×10^6 cells/mL) were exposed to *C. albicans* (SC5314) yeast or hyphae at indicated MOI for 60 minutes. Formed LM were extracted via SPE and analyzed by UPLC-MS/MS. 5-HETE, LTB_4 and its trans-isomers, as well as 20-OH-LTB_4 are representative for 5-LOX activation. Data are presented as mean + SEM (pg/5×10^6 cells) from n = 5-7 independent experiments. C, Subcellular localization of 5-LOX and FLAP in human neutrophils upon stimulation with indicated MOI of *C. albicans* yeast or hyphae for 60 minutes was monitored by indirect IF microscopy. Cells were stained with antibodies against 5-LOX (Alexa Flour 555, red) and FLAP (Alexa Flour 488, green). A representative of 3 independent experiments was selected. D, Detection of $[Ca^{2+}]_i$ measured by continuous fluorescence reading. Fura-2/AM-labelled neutrophils (5×10^6 cells/mL) were incubated with 2 μM ionomycin, *C. albicans* hyphae or yeast or vehicle control. Data are shown as mean + SEM from n = 9-15 independent experiments. E, Cell membrane integrity of human neutrophils (5×10^6 cells/mL) treated with *C. albicans* yeast or hyphae or vehicle control (PBS; pH 7.4) was determined by measurement of LDH release and normalization to 0.2% Triton X-100 as lysis control over 24 hours. Data are shown as percentage of triton X-100 control, mean + SEM; n = 3 donors

hyphae elicited 5-LOX translocation to the nuclear membrane and promoted co-localization with FLAP (Figure 1C), causing optimal conditions for LT formation and 5-LOX activation. 5-LOX as well as cPLA$_2$ are activated by increased levels of intracellular calcium. Therefore, we determined the $[Ca^{2+}]_i$-level in neutrophils. Surprisingly, both yeast and hyphae induced intracellular $[Ca^{2+}]$ mobilization within the first 10 minutes of stimulation compared to the vehicle control. However, neutrophil responses evoked by hyphae resulted in a stronger $[Ca^{2+}]_i$ increase compared to yeast cells (Figure 1D), which let us conclude that the moderate $[Ca^{2+}]$ influx caused by yeast is insufficient for 5-LOX activation.

To exclude that the observed effects originated from *C. albicans*-mediated cytotoxic effects, extracellular LDH levels were quantified over 24 hours to assess neutrophil membrane integrity and cell vitality. When incubated with hyphae at a MOI of 10, neutrophils showed a minor increase in LDH release compared to cells exposed to yeast cells or vehicle control, with over 50% of the cells remaining intact after 24 hours of incubation (Figure 1E). Notably, within the first hour of stimulation, in which the LM biosynthesis occurred, there was no cytolysis detectable.

3.2 | Hyphae induced LT biosynthesis is Ca^{2+}-dependent

The binding of intracellular calcium is relevant for both cPLA$_2$ and 5-LOX activation and thus crucial for LT formation.[39,40] Since hyphae induced potent Ca^{2+}-mobilization (Figure 1D) its role for LT biosynthesis was explored by preincubation with the $[Ca^{2+}]$-chelating agents BAPTA-AM or EDTA. Both chelators inhibited 5-LOX product formation (Figure 2A,B), indicating the demand of abundant extracellular and intracellular Ca^{2+}-mobilization for cPLA2 respective 5-LOX activation and LT biosynthesis.

3.3 | *C. albicans* hyphae activate LT biosynthesis in neutrophils via p38 MAPK and ERK1/2

In addition to Ca^{2+} binding, 5-LOX is activated by phosphorylation at serine 271 and serine 663 *via* MAPKAPK-2/3 and ERK1/2, respectively.[41,42] To decipher the intracellular signalling cascade activated by *C. albicans* hyphae, neutrophils were pre-incubated with inhibitors interfering with different target sides along the 5-LOX activation pathway prior to stimulation. Skepinone-L selectively inhibits p38 MAPK[43] the upstream kinase of MAPKAPK-2/3, UO126 blocks ERK1/2 activation,[44] RSC-3388 directly targets cPLA$_2$[45] and MK886 abrogates 5-LOX product formation *via* FLAP inhibition.[46] All tested inhibitors showed a significant reduction of LTB$_4$, and 20-OH-LTB$_4$ formation compared to vehicle control (0.1% DMSO) (Figure 3A). While inhibition of cPLA$_2$ and FLAP directly affected the enzymatic biosynthesis of LT, the impressive impact of p38 MAPK and ERK1/2 inhibitors implicated a strong involvement of kinase-mediated signalling in 5-LOX activation and LT biosynthesis evoked by *C. albicans* hyphae. Interestingly, when neutrophils were activated by the non-pathophysiological stimulus Ca^{2+}-ionophore A23187, total 5-LOX product formation was less influenced by kinase inhibitors as LTB$_4$ formation was only attenuated to 47% and 93% by UO126 and Skepinone-L, respectively (Figure 3B), as reported before.[42,47] Western Blot analysis confirmed p38 MAPK and ERK1/2 activation in response to *C. albicans* hyphae and to A23187. However, marked differences were observed in
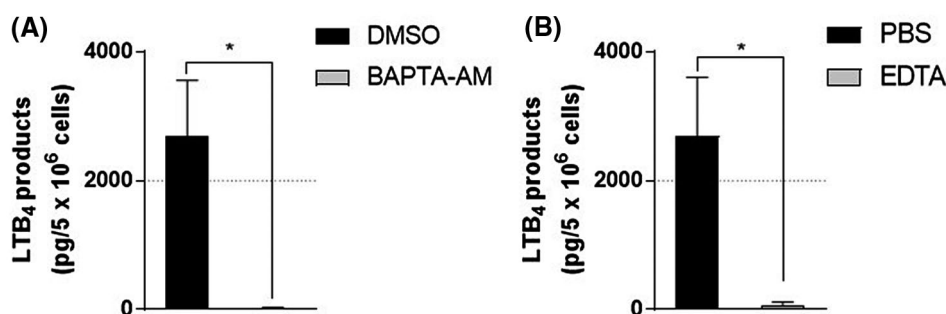
FIGURE 2 Role of $[Ca^{2+}]$ for *C. albicans*-induced LM biosynthesis in human neutrophils. Neutrophils (5×10^6 cells/mL) were preincubated (A) with 15 μM BAPTA-AM versus vehicle control (0.1% DMSO) or (B) with 1 mM EDTA versus vehicle control (PGC; PBS pH 7.4; 0.1% glucose, 1 mM CaCl$_2$) for 15 minutes at 37°C prior to stimulation with MOI 10 hyphae 60 minutes. LM were extracted *via* SPE and analyzed by UPLC-MS/MS. Data was obtained from n = 5-6 donors. Bars represent mean + SEM, with individual replicates shown. Statistical evaluation was performed using Student's paired *t* test; *$P < .05$ inhibitor versus vehicle control
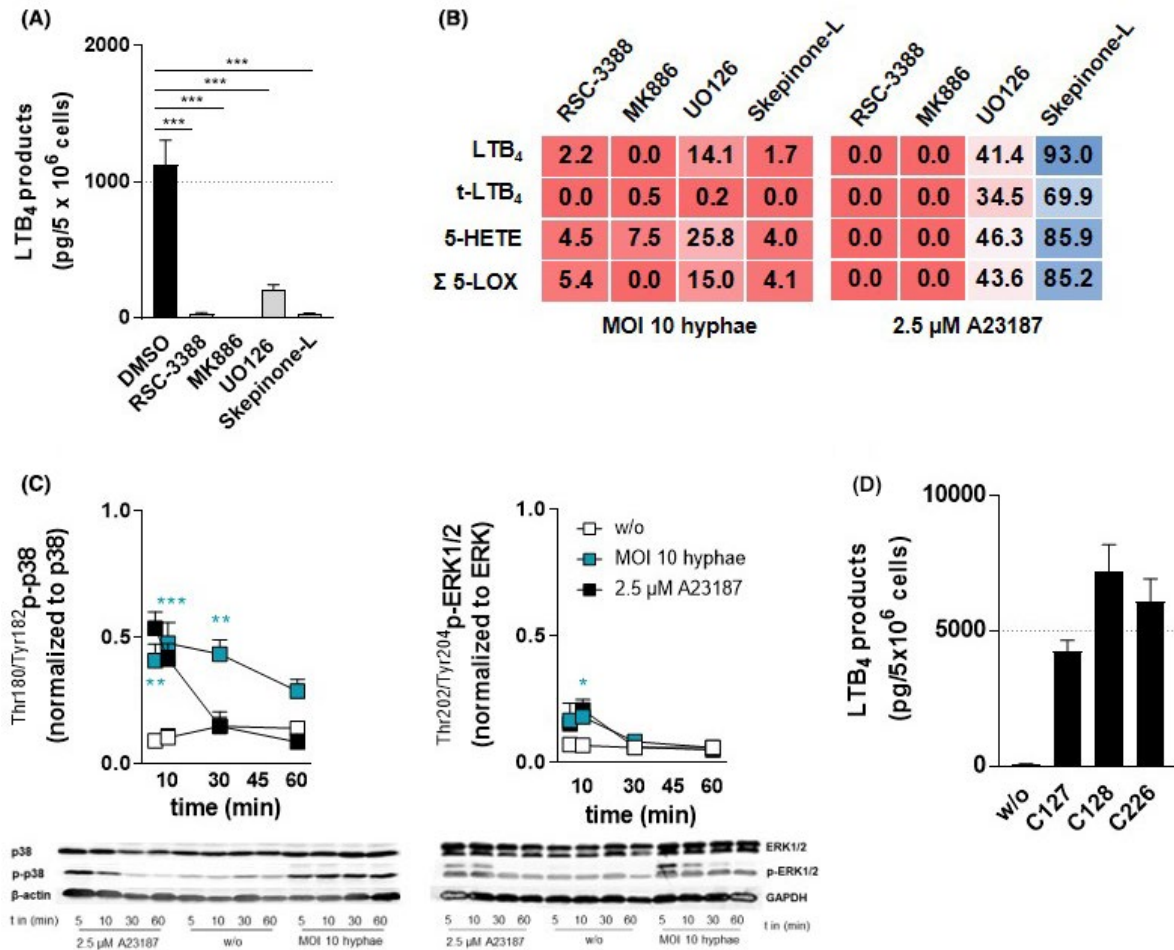
FIGURE 3 Activation of p38 MAPK and ERK-1/2 during incubation with *C. albicans* hyphae. Neutrophils (5×10^6 cells/mL) were preincubated with 10 μM RSC-3388, 0.3 μM MK886, 1 μM UO126, 1 μM skepinone-L and vehicle control (0.1% DMSO) for 15 minutes and (A) subsequently stimulated with *C. albicans* hyphae MOI 10 for 60 minutes or (B) with 2.5 μM A23187 for 10 minutes at 37°C. Formed LM were analyzed *via* HPLC or UPLC-MS-MS. Data are displayed as percentage of stimulated control and were obtained from n = 3 donors. C, Phosphorylation of p38 MAPK and ERK-1/2 was analyzed by immunoblotting. Neutrophils (10^7 cells/mL) were incubated with MOI 10 *C. albicans* hyphae, 2.5 μM A23187 or vehicle control (PGC; PBS pH 7.4; 0.1% glucose, 1 mM $CaCl_2$) for the indicated time periods, n = 4-5. D, Neutrophils (5×10^6 cells/mL) were exposed to hyphae of clinical isolates for 60 minutes. Formed LM were extracted *via* SPE and analyzed by UPLC-MS/MS. Results are given as mean + SEM and statistical analysis in (A) was performed using two-way ANOVA with Dunnett's post-hoc test and in (C) was performed applying two-way ANOVA with Bonferroni post-hoc test; *$P < .05$, **$P < .01$ and ***$P < .005$; stimulus/inhibitor versus vehicle controls

p38 MAPK stimulation. While A23187 induced kinase activation declined rapidly below the vehicle control within few minutes, hyphal stimulated p38-MAPK phosphorylation peaked after 10 minutes followed by only a modest decline over 60 minutes (Figure 3C, left panel). This is in line with the detection of LT biosynthesis over a period of 60 minutes (Figure 1B) and suggests LM formation mediated by p38-MAPK. ERK1/2 phosphorylation was equally stimulated by A23187 and hyphae within the first 10 minutes of incubation. This activation decreased to the phosphorylation state of the vehicle control already after 30 minutes, which may reflect a minor impact of ERK1/2 on LT biosynthesis (Figure 3C, right panel).

The elucidation of pathways driving LT formation in neutrophils by hyphae were predominately performed using the *C. albicans* wild type strain SC5314. To validate the specificity of hyphae-induced LT-biosynthesis in neutrophils, different clinical isolates were evaluated for their ability to activate 5-LOX and stimulate formation of chemotactic LTB_4. Hyphae from all isolates showed inducible effects comparable to SC5314 (Figure 3D).

Summarizing, 5-LOX activation and LT biosynthesis in neutrophils induced by *C. albicans* is restricted to the hyphal morphology of this dimorphic fungal pathogen, depends on Ca^{2+} mobilization and is mediated by p38 MAPK signaling.

3.4 | Viable hyphae are required to induce LT biosynthesis

Encouraged by these findings, we aimed at elucidating hyphae-related stimulating factors and mechanisms that cause the pro-inflammatory response in terms of 5-LOX activation and LT biosynthesis. To evaluate the role of intact fungal cell walls and the ability of *C. albicans* to actively participating molecules, neutrophils were exposed to viable and inactivated hyphae and yeast. Although heat-inactivated yeast cells showed a tendency to stimulate LT formation, this was significantly lower compared to the levels induced by viable hyphae. In contrast, heat-inactivated hyphae completely lost their ability to induce LT biosynthesis (Figure 4A). This suggests a need for viable hyphae to induce 5-LOX activation. Since it is well known, that heat inactivation of *C. albicans* results

in remodeling of the cell wall associated with enhanced β-glucan exposure,[48] hyphae were also killed by UV-C radiation. Affirmative, comparable to heat inactivation, UV-killed hyphae were incapable of inducing LT formation in neutrophils (Figure 4A). Conclusively, LT biosynthesis in neutrophils requires features of viable hyphae and recognition of molecular patterns on the fungal cell wall is insufficient to activate 5-LOX and LT biosynthesis.

3.5 | Hyphae induce LT biosynthesis via SYK and MYD88-dependent pathways

Heat-inactivated yeast cells with β-glucan exposure on the cell surface did not activate robust LT biosynthesis, yet viable hyphae that unmask β-glucan at the hyphal tip strongly induce LT biosynthesis. Previous studies
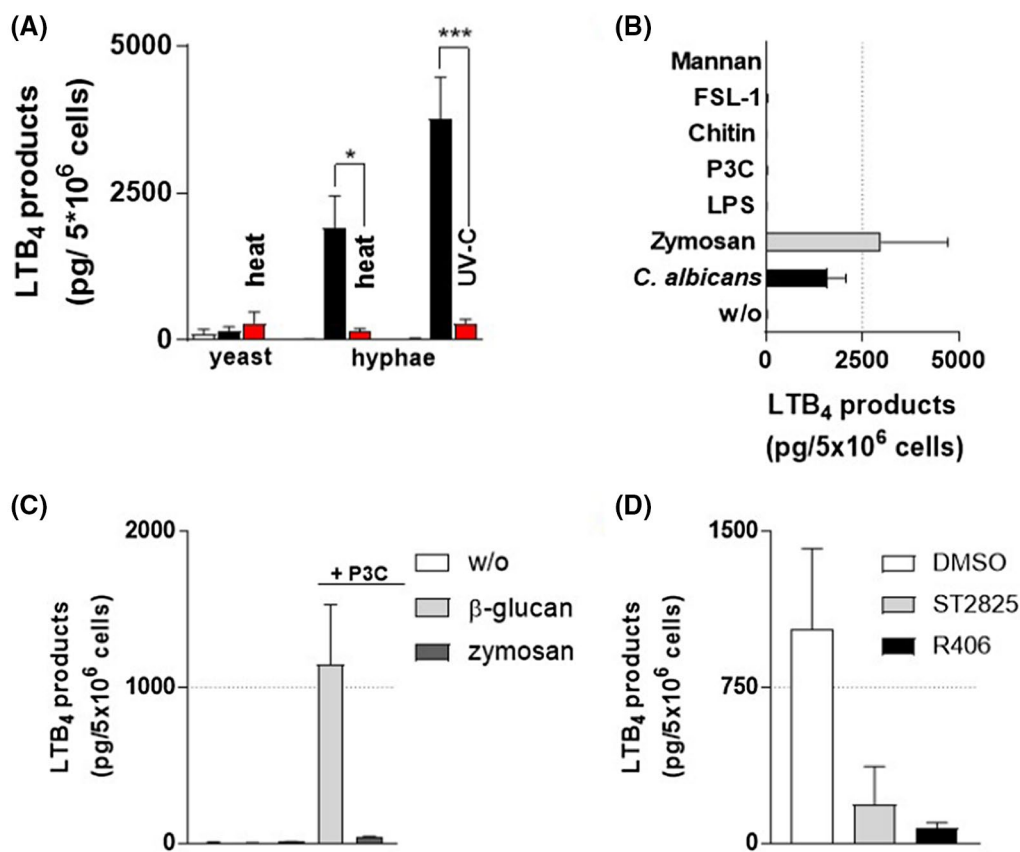
FIGURE 4 *C. albicans*-induced 5-LOX activation is mediated *via* PRRs. A, Neutrophils (5×10^6 cells/mL) were stimulated for 60 minutes with a MOI 5 of *C. albicans* yeast and hyphae versus heat-inactivated (80°C; 20 minutes) and ultraviolet-inactivated (254 nm; 40 minutes) *C. albicans* yeast and hyphae and vehicle control (PGC; PBS pH 7.4; 0.1% glucose, 1 mM CaCl$_2$). B, Neutrophils (5×10^6 cells/mL) were stimulated MOI 10 *C. albicans* (SC5314) hyphae, 100 μg/mL zymosan, 10 ng/mL LPS, 10 μg/mL P3Cys, 100 μg/mL chitin or vehicle control (PGC; pH 7.4; 0.1% glucose; 1 mM CaCl$_2$) for 60 minutes; n = 4 and in (C) with 100 μg/mL zymosan, 100 μg/mL β-glucan ± 10 μg/mL P3Cys or vehicle control (PGC; pH 7.4; 0.1% glucose; 1 mM Ca^{2+}) for 60 minutes; n = 3. D, Neutrophils (5×10^6 cells/mL) were preincubated with 10 μM ST2825, 1 μM R406 and vehicle control (0.1% DMSO) and stimulated by MOI 10 *C. albicans* (SC5314) hyphae; n = 3. LM were isolated *via* SPE and analyzed using UPLC-MS-MS. Values are presented as mean + SEM, statistical analysis was performed using paired Student's *t* test; *$P < .05$, **$P < .01$ and ***$P < .005$; stimulus versus vehicle control

demonstrated strong synergistic effects between dectin-1 and TLR 2 and 4 signaling regarding cytokine release upon *C. albicans* recognition.[49] Therefore, several pattern recognition receptor (PRR) ligands were examined alone and in combination for their ability to induce LT formation in neutrophils. Fungal ligands such as mannan that mainly targets the C-type lectin dectin-2,[50] chitin that modulates dectin-1, and Fc-γ-receptor-signalling,[51] but also the TLR agonist palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH (P3Cys) activate TLR1/2 heterodimers,[52] LPS activates TLR4[53] and the lipoprotein Pam2CGDPKHPKSF (FSL-1) activating TLR2/6 heterodimers[54] induced no LT biosynthesis (Figure 4B). Interestingly, only zymosan, a yeast cell wall preparation containing various protein-carbohydrate complexes that stimulates dectin-1 and TLR2, induced LT formation predominantly reflected by LTB$_4$ biosynthesis (Figure 4B). This observation suggested a requirement of complex stimulation for LT biosynthesis rather than induction by a single PRR ligand. Concurrent stimulation of dectin-1 together with TLR2 by a combination of β-glucan and P3Cys, respectively, stimulated LT formation that surpassed activation by β-glucan alone (Figure 4C). Still, the amounts of 5-LOX products yielded by viable hyphae stimulation could not be reached, suggesting an additional inducing factor.

To confirm the involvement of the dectin-1 receptor and TLR2 in LT biosynthesis in neutrophils, the influence of spleen tyrosine kinase (SYK) and myeloid differentiation primary response 88 (MYD88) mediating the receptor response was determined. Pharmacological inhibition of either of these signaling proteins with R406 (SYK inhibitor) or ST2825 (MYD88 inhibitor) significantly abolished LT formation (Figure 4D). Collectively, these data suggest an involvement of dectin-1 and TLR2 in *C. albicans* stimulated LT formation and provide evidence for crosstalk between both receptors and pathways.

3.6 | Hyphae-associated protein expression mediates LT biosynthesis in neutrophils

Given that 5-LOX activation is strongly induced by living hyphae, while yeast cells and typical PAMPs such as β-glucan alone are insufficient to induce LT biosynthesis, we also considered virulence factors expressed during hyphae formation to be potential inducers. First, human neutrophils were challenged with the *C. albicans* yeast-locked mutant strains *efg1Δ/Δ/cph1Δ/Δ*[35] and *hgc1Δ/Δ*,[34] grown under hyphae-inducing conditions, (Figure 5A) to investigate the importance of the yeast-to-hyphae transition and the expression of hyphal associated genes. While both mutant strains have defects to form true hyphae[34]

only *efg1Δ/Δ/cph1Δ/Δ* failed to induce 5-LOX-product formation, which was still induced by *hgc1Δ/Δ* (Figure 5A). Interestingly, both mutants induce calcium mobilization (Figure 5B), one of the required conditions for 5-LOX activation and translocation to the nuclear membrane. However, while *hgc1Δ/Δ* induces a calcium influx in a same manner as the respective wildtype strain, *efg1Δ/Δ/cph1Δ/Δ* cause a delay in calcium mobilization. Especially within the first 20 minutes of stimulation, *efg1Δ/Δ/cph1Δ/Δ* showed a significant reduced Ca^{2+} influx compared to *hgc1Δ/Δ* and wildtype strains. Since the *hgc1Δ/Δ* mutant has reduced potential to form hyphae, but is still able to express hyphae-associated genes,[34] we questioned whether the cytolytic peptide candidalysin, which is expressed and released during filamentous growth, may be critical for LTB$_4$ formation.[36] Synthetic candidalysin failed to stimulate LT biosynthesis in human neutrophils even at concentrations up to 50 μM (Figure 5C) and deletion mutants lacking the *ece1Δ/Δ* gene showed comparable inducing activity as the wild type strain (Figure 5D).

4 | DISCUSSION

In this report, we investigated the ability of *C. albicans* to induce LT formation in neutrophils, thereby contributing to pro-inflammatory immune responses during infections. We demonstrated that exclusively viable hyphae induced LTB$_4$ biosynthesis and conditional 5-LOX activation. Using yeast-locked and hyphal-impaired mutants we pinpointed the effect towards changes associated with early filamentation. In neutrophils, hyphae-induced LT biosynthesis is mediated by synergistic signalling of dectin-1 and TLR2 *via* kinases SYK and MYD88. Although the hyphae-specific toxin candidalysin did not contribute to chemotactic LTB$_4$ formation, hyphae-associated gene expression has a decisive role within this process.

While others already described the immunoregulatory role of LTB$_4$ during fungal infections,[11,55] this study contributes to understanding the pathophysiologic molecules and cellular mechanisms that drive LT formation in response to pathogens.

Early studies focused on lipopolysaccharide (LPS) that activates TLR4 signalling, formylpeptides binding on formyl peptide receptor-1, and zymosan that activates TLR2 and dectin-1 receptors to stimulate LTB$_4$ formation in neutrophils.[56,57] However, convincing reports were missing that irrevocably disclose cellular pathways and involved receptors. The observation that microbial effector proteins can drive LT formation argues for highly complex activation processes and an involvement of several cellular mediators and receptors distinct from known PAMP-PRR interactions. Recently, we demonstrated that *S aureus*
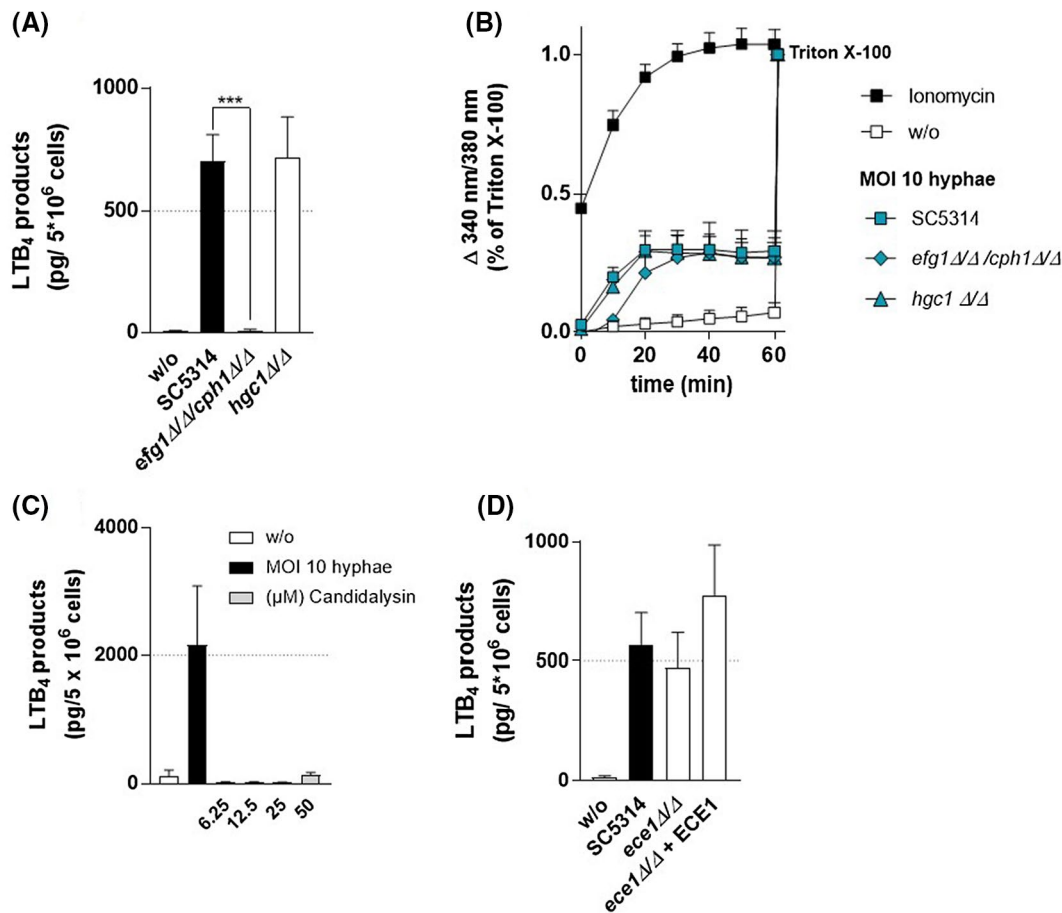
FIGURE 5 *C. albicans*-induced LT formation is dependent on hyphae-specific genes. Neutrophils (5×10^6 cells/mL) were stimulated (A) with MOI 10 hyphae of *C. albicans* wildtype (SC5314) or indicated yeast-locked mutant strains; n = 6; (B) with 2 μM ionomycin, MOI 10 *C. albicans* wildtype (SC5314) hyphae, indicated yeast-locked mutant strains or vehicle control. Detection of $[Ca^{2+}]_i$ was monitored by continuous fluorescence reading of Fura-2/AM-labelled neutrophils. Data are shown as mean + SEM from n = 3 independent experiments; (C) with MOI 10 *C. albicans* (SC5314) hyphae, the indicated concentrations of synthetic candidalysin or vehicle control (PGC; pH 7.4; 0.1% glucose; 1 mM $CaCl_2$); (D) with MOI 10 hyphae of *C. albicans* wildtype (SC5314) *C. albicans* ece1Δ/Δ or *C. albicans* ece1Δ/Δ + ECE1; n = 3. Values in (A) are presented as mean + SEM (pg/5×10^6 cells), statistical analysis was performed in using two-way ANOVA with Dunnett's post-hoc test *$P < .05$, **$P < .01$ and ***$P < .005$, wildtype versus mutant strains

derived PSMs stimulate the FPR2/ALX receptor resulting in LT formation in neutrophils.[18] This effect turned out to be highly neutrophil-specific, as PSMs did not activate 5-LOX in macrophages, while α-hemolysin, a pore-forming toxin, stimulated LM formation in macrophages but not so in neutrophils.[58]

Astonishingly little is known about LM biosynthesis during fungal infections. Leslie and co-workers reported that *C. albicans* triggers eicosanoid formation in murine macrophages *via* dectin-1-mediated $cPLA_2$ activation and AA release.[20,21,59] However, activation mechanisms and involved mediators remained unclear on a molecular level. Since the morphological transition of *C. albicans* between yeast and hyphal cells is essential for pathogenicity of this fungus,[35,60] we questioned whether induction of LT biosynthesis in neutrophils depends on the morphology. In fact, we found that LT biosynthesis was exclusively

induced by hyphae. All cellular processes crucial for 5-LOX activation such as Ca^{2+} mobilization, 5-LOX translocation to the nuclear membrane, and co-localization with FLAP, as well as p38 MAPK activation[4] were initiated by *C. albicans* hyphae resulting in LT biosynthesis, while yeast cells were incapable to stimulate the 5-LOX pathway. These findings are in line with previous reports that demonstrated an increase in neutrophil activation and motility evoked specifically by hyphae.[61] Interestingly, in that study *C. albicans* recognition was mediated *via* the MAPK/ERK-1/2 pathway but was independent of p38 MAPK activation. Here, we confirm a role for ERK-1/2, yet also show that p38 MAPK signalling contributes to LT-biosynthesis in neutrophils. Since cPLA2 activation is mediated by p38 MAPK phosphorylation[62] and *C. albicans*-induced AA release was attenuated by a p38 MAPK inhibitor,[20] our findings would support this outcome. Of note, activation of LT

synthesis by the commonly used Ca^{2+}-ionophore A23187 was independent on p38 MAPK activation and only modestly mediated by ERK-1/2, suggesting a pathogen-specific signalling pathway for LT formation.

We further aimed to elucidate hyphae-specific factors, host receptors and activated downstream pathways that lead to LT formation in response to *C. albicans*. Filamentation of *C. albicans* is a complex process characterized not only by the morphological switch from ovoid yeast to elongated hyphae, but also by cell wall remodeling and expression of hyphae-associated genes.[63-65] These mechanisms orchestrate the expression and exposure of fungal cell wall components, virulence and avirulence factors that result in differential immune recognition of yeast and hyphae followed by distinct immune responses. The *C. albicans* cell wall structure has been investigated intensively and its highly flexible architecture changes during the morphological shift from yeast to hyphae. These composition changes, lead to variations in the accessibility of PAMPs, and modified activation of PRRs.[66] In comparison to yeast cells, early formed *C. albicans* filaments (germ tubes) differ in their exposure of immunogenic cell wall components on their surface, and mutant strains with unmasked β-glucan cell surface structures provoke stronger cytokine response.[48,67] Apart from β-glucan, several *C. albicans* cell wall structures were described to be involved in immune recognition as the fungal cell wall is the primary site of confrontation between the host innate immune system and the pathogen. Human neutrophils express multiple PRRs such as the C-type lectin receptor (CLRs) dectin-1 and complement receptor 3 (CR3) recognizing β-glucan, TLR2 binding phospholipomannans, and TLR4 sensing mannoproteins of the fungal cell wall.[68-70] Activation of these PRRs is known to initiate pro-inflammatory host responses, characterized by the release of cytokines and chemokines.[61,66,68,71] Based on observations in *C. albicans*-challenged murine macrophages,[20] we investigated cell wall components such as β-glucan, chitin, mannan, and the TLR2 agonist P3Cys. Interestingly, single stimulation did not induce LT formation, but zymosan a yeast cell wall preparation that acts on TLR2 and dectin-1[72] stimulated LT biosynthesis in neutrophils, suggesting a requirement of synergistic activation on both PRRs. This finding could be substantiated by β-glucan (dectin-1)/P3Cys (TLR2) co-stimulation, which induced LT-formation. Furthermore, it also strengthens the role of previously described dectin-1/TLR2 synergism observed in monocytes and macrophages.[49] The involvement of these PRRs was confirmed by pharmacologic inhibition of the downstream effector proteins SYK and MYD88, which indeed attenuated LTB_4 formation.

Nevertheless, LT levels induced by incubation with viable hyphae could not be obtained by combinations of PAMPs, which let us hypothesize that an additional factor promoted LT formation. This was supported by UVC-inactivated hyphae, which have an intact cell wall morphology, but are not replicating or producing any effectors. Moreover, the yeast-locked strain *efg1/cph1Δ/Δ* that is incapable to form hyphae and does not express any hyphae-associated proteins[35] failed to activate 5-LOX, whereas the *hgc1Δ/Δ* mutant that has reduced potential to form hyphae and still expresses hyphae-associated proteins[34] induced LTB_4 formation. Note, while *efg1/cph1Δ/Δ* did not induce 5-LOX product formation, it stimulated a Ca^{2+} influx, which is one of the 5-LOX activators. However, compared to the wildtype and *hgc1Δ/Δ* strain, the calcium mobilization was time delayed. This observation allows us to discuss two scenarios. Either the Ca^{2+} influx of the first 20 minutes of stimulation was not sufficient to orchestrate AA release and conversion by 5-LOX, or additional activators are required for full 5-LOX activity and LM biosynthesis. Surprisingly, the cytolytic toxin candidalysin[36] failed to stimulate 5-LOX activity and a candidalysin deficient strain *ece1Δ/Δ* and *ece1Δ/Δ + ECE1* induced comparable amounts of LTs as the wild-type strain. At least in neutrophils under our experimental settings, the potent virulence factor candidalysin has no impact on LT formation. However, it should be noted that we detected 5-LOX activity already after 30 minutes in neutrophils upon coincubation with hyphae, a time point where no cytolytic processes were observed. It seems reasonable that LT biosynthesis is induced at an early stage of infection, rather than in a process of pathogen invasion during cytolysis. Future experiments need to disclose whether other hyphae-associated as surface proteins (eg the adhesins Hwp1 and Als3)[73,74] or secretion of hydrolytic enzymes (eg the secreted aspartic protease Sap4-6)[75,76] have an impact on LT biosynthesis in neutrophils.

In summary, we found that exclusively hyphae of *C. albicans* induce LTB_4 formation in neutrophils. This process occurs at an initial stage of infection and is mediated by dectin-1 and TLR2 signaling on the host side and is promoted by the expression of the hyphae-specific proteins. Our findings contribute to the understanding of immune-cell driven host–fungal interactions at early stages of infection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

J. Fischer performed the experiments. J. Fischer, M.S. Gresnigt, and U. Garscha designed the study and performed data analysis. All authors contributed to the discussion and manuscript preparation.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The research was conducted in accordance with the Declaration of Helsinki. Experiments with human neutrophils were approved by the ethical review committee of the University Hospital Jena, Germany.

CONSENT FOR PUBLICATION

All authors declare their participation in the study and development of this manuscript. All authors have read the final version of the manuscript and give consent of the publication in Cellular and Molecular Life Sciences.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article or are available on request from the corresponding author (UG).

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