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Basic

Research Article

Hyphal-associated protein expression is crucial for *Candida albicans*-induced eicosanoid biosynthesis in immune cells

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Candida albicans causes opportunistic infections ranging from mucosal mycoses to life-threatening systemic infections in immunocompromised patients. During *C. albicans* infection, leukotrienes and prostaglandins are formed from arachidonic acid by 5-lipoxygenase (5-LOX) and cyclooxygenases, respectively to amplify inflammatory conditions, but also to initiate macrophage infiltration to achieve tissue homeostasis. Since less is known about the cellular mechanisms triggering such lipid mediator biosynthesis, we investigated the eicosanoid formation in monocyte-derived M1 and M2 macrophages, neutrophils and HEK293 cells transfected with 5-LOX and 5-LOX-activating protein (FLAP) in response to *C. albicans* yeast or hyphae. Leukotriene biosynthesis was exclusively induced by hyphae in neutrophils and macrophages, whereas prostaglandin E_2 was also formed in response to yeast cells by M1 macrophages. Eicosanoid biosynthesis was significantly higher in M1 compared to M2 macrophages. In HEK_5-LOX/FLAP cells only hyphae activated the essential 5-LOX translocation to the nuclear membrane. Using yeast-locked *C. albicans* mutants, we demonstrated that hyphal-associated protein expression is critical in eicosanoid formation. For neutrophils and HEK_5-LOX/FLAP cells, hyphal wall protein 1 was identified as the essential surface protein that stimulates leukotriene biosynthesis. In summary, our data suggest that hyphal-associated proteins of *C. albicans* are central triggers of eicosanoid biosynthesis in human phagocytes.

Keywords: Hwp1 · Host–pathogen interaction · 5-LOX · COX · Macrophages · Leukotriene

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Acute inflammation is an important, life-saving, defense mechanism with the primary goal of eliminating noxious agents, such as pathogens, and restoring tissue homeostasis. Myeloid phagocytes such as macrophages and neutrophils form the first line of myeloid cell defense against pathogens [1, 2]. These cells are equipped with a specific set of effector mechanisms, including the release of pro-inflammatory cytokines, chemokines, and other immune mediators. The latter involves the formation of eicosanoids such as leukotrienes (LTs) and prostaglandins (PGs), which are formed from arachidonic acid (AA, C20:4, ω-6) by 5-lipoxygenase (5-LOX) and cyclooxygenases (COXs), respectively [3]. Cytosolic phospholipase $A_{2\alpha}$ ($cPLA_{2\alpha}$) releases AA from phospholipids upon inflammatory stimulation. Simultaneously, 5-LOX activated by Ca^{2+}-binding and serine phosphorylation by p38-MAPK and ERK1/2-kinase translocates to the nuclear membrane, where it co-localizes with the membrane-bound 5-LOX-activating protein (FLAP), which is required for cellular LT formation [4]. In another branch of the AA cascade, AA is a substrate for COX-1 and COX-2 yielding PGH_2 as an unstable intermediate, which can be further converted by specific synthases to downstream PGs, such as PGE_2 by PGE synthase and thromboxane A_2 by thromboxane synthase [3, 5].

Macrophages are a group of cells with high phenotypic plasticity and functional diversity [6]. The classical two subsets are grouped as M1- and M2-like macrophages. While M1-like macrophages, in vitro polarized by lipopolysaccharides (LPS) and IFN-γ, produce proinflammatory mediators, nitric oxide, and reactive oxygen intermediates, M2-like macrophages are generated in vitro upon exposure to IL-4 and are associated with the resolution phase of inflammation [7]. Differentiation into M1 and M2 subtypes is associated with differential expression of AA cascade enzymes resulting in a phenotypically distinct lipid mediator (LM) profile upon pathogen stimulation, reflecting the central role of macrophage subpopulations in pro- and anti-inflammatory immune responses upon pathogen exposure [8, 9].

Among microbial pathogens, the yeast *Candida albicans* is an increasing cause of infections tightly related to the innovation of immunosuppressive and invasive medical therapies that easily breach the physiological immune barrier and provide a bridge for fungal infections [10, 11]. The commensal *C. albicans* can cause disease, ranging from superficial mucosal infections to severe, systemic candidiasis. The shift from a harmless colonizer to a life-threatening pathogen is a complex process that has been only partially understood [12, 13]. To date, *C. albicans* pathogenicity has been strongly associated with the morphological transition from yeast to hyphae [11, 14]. While both morphological forms are found during host infections, strains unable to form hyphae are less virulent [14, 15]. In addition to the morphological transition itself being considered an important virulence feature, this transition is accompanied by virulence-enhancing processes such as the expression of surface proteins, cell wall remodeling, the secretion of host epithelium-degrading enzymes and proteins, and the expression of the peptide toxin candidalysin [16–19]. As part of the human inflammatory arsenal against invading *C. albicans*, the interplay between yeast or hyphae and human immune cells in terms of LM formation is only rudimentarily understood [20, 21].

In the current report, we investigate the eicosanoid formation in both subpopulations of macrophages, neutrophils, and in 5-LOX/FLAP expressing HEK293 cells that are challenged by *C. albicans*. We further elucidate differences in yeast and hyphae stimulation and unravel the molecular mechanisms that provoke LM formation.

Materials

Acetic acid (UPLC-grade), acetone, acrylamide, agar, ammonium persulfate (APS), glucose, L (+)-glutamine, 2-mercaptoethanol, n-hexane HiPerSolve Chromanorm (HPLC-grade, ≥ 97%), methanol (UPLC-grade), sodium dodecyl sulfate (SDS), potassium chloride (KCl), and tetramethylethylenediamine (TEMED) were purchased from VWR International. Bovine serum albumin (BSA), dextran, glycine (≥99%), hygromycin-B, yeast extract, peptone ex casein, sodium chloride (≥99.5%), trishydroxymethylaminomethane (TRIS), Triton X-100, coverslips and microscope slides were obtained from Carl Roth. Amersham Protan 0.45 μm, Fura-2-AM, ionomycin, Prolong Diamond Antifade mountant DAPI, prestained protein ladder, 16% formaldehyde solution, and methyl formate (≥98%) were from Fisher Scientific. DMEM, fetal calf serum (FCS), glycerophosphate, G418 disulfate salt, leupeptin (LP), phenylmethanesulfonyl fluoride (PMSF), Roswell Park Memorial Institute medium RPMI-1640 (w/o phenolred), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Histopaque-1077, LPS from *Escherichia coli*, penicillin–streptomycin, soy trypsin inhibitor (STI), sodium pyrophosphate ($Na_2P_2O_7$) were purchased from Merck KGaA, and PBS salt (from SERVA Electrophoresis. LM standards and deuterated LM standards were purchased from Cayman (Biomol GmbH). Human granulocyte-macrophage colony-stimulating factor (GM-CSF), human macrophage CSF (M-CSF), IFN-γ, and IL-4 were obtained from Peprotech. The mouse anti-5-LOX monoclonal antibody used for fluorescence assays was a generous gift of Dr. Dieter Steinhilber (Goethe University Frankfurt), the mouse anti-5-LOX antibody from BD Bioscience was applied for Western Blot. The mouse and anti-β-actin antibody, mouse and rabbit anti-GAPDH, rabbit anti-ERK antibody, mouse anti-p-ERK antibody, rabbit anti-COX-1, rabbit anti-COX-2 antibody, rabbit anti-p38 MAPK antibody, and rabbit anti-p-p38 MAPK antibody were from Cell Signaling Technology, Inc. The rabbit anti-FLAP polyclonal antibody was purchased from Abcam. Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 555 goat anti-mouse, and normal goat serum (10%) were obtained from Invitrogen. AA, CaCl, disodium ethylenediaminetetraacetic acid (EDTA), monopotassium phosphate (K_2HPO_4), magnesium sulfate ($MgSO_4$), NP-40, and sodium orthovanadate (Na_3VO_4) were from AppliChem.

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

Strain	Strain/Gene function	Reference
Control strains		
SC5314	Wild type	[24]
Strains with terminated hyphae to yeast transition		
hgc1$^{\Delta/\Delta}$	G1 cyclin-related protein regulating filamentation	[32]
efg1$^{\Delta/\Delta}$ /*cph1*$^{\Delta/\Delta}$	Transcription factors regulating filamentation	[15]
Hyphae-specific genes		
hwp1$^{\Delta/\Delta}$	Adhesin	[16]
hwp1$^{\Delta/\Delta}$ + *HWP1*	Complemented strain	[16]
ece1$^{\Delta/\Delta}$	Polypeptide protein containing the peptide toxin candidalysin	[18]
hyr1$^{\Delta/\Delta}$	Adhesin (hyphae regulated gene)	[34]
als3$^{\Delta/\Delta}$	Adhesin (agglutinin-like sequence 3)	[17, 35]

Blood cell isolation

Experiments with primary human blood cells were approved by the ethical review committee of the University Hospital Jena, Germany (license number: 4968-11/16). Leukocyte concentrates from human peripheral blood of healthy donors were purchased from the Transfusion Medicine, University Hospital Jena. Neutrophils and monocytes were isolated as described previously [22]. Briefly, leukocytes were isolated using dextran sedimentation (2.5% dextran) and subsequently separated by centrifugation on density cell separation medium Histopaque 1077. Neutrophils were isolated from the granulocyte/erythrocyte fraction by hypotonic erythrocyte haemolysis and were washed twice with ice-cold PBS and resuspended in PBS (pH 7.4). In order to obtain monocytes, PBMC were washed twice with ice-cold PBS and resuspended in (PBS, pH 7.4; 1 mM Ca^{2+} and 0.5 mM Mg^{2+}). Cell viability was determined by Trypan Blue on a Beckmann Vi-Cell cell counter. Cells were seeded into cell culture flasks for 1 h at 37°C for monocyte separation. The supernatant was removed and cells were seeded in 1640 (RPMI-1640) supplemented with 10% FCS, 2 mM L-glutamine, and penicillin–streptomycin for subsequent differentiation and polarization.

Macrophage differentiation and polarization

Monocytes were cultivated for 6 d (37°C, 5% CO_2) with 20 ng/mL GM-CSF to differentiate them from M1-macrophages or 20 ng/mL (M-CSF) to differentiate them from M2-macrophages. Cells were detached with PBS-EDTA (pH 7.4.; 1 mM EDTA and PBS (pH 7.4), centrifuged, and resuspended to the required density in RPMI-1640 (10% FCS; 2 mM L-glutamine). Subsequently, macrophages were polarized for another 48 h (37°C, 5% CO_2) with 100 ng/mL LPS and 20 ng/mL INF-γ to polarize M1 macrophages or 20 ng/mL IL-4 to polarize M2 macrophages.

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).

HEK_5-LOX/FLAP cell culture

HEK_5-LOX/FLAP cells were cultured as monolayer at 37°C and 5% CO_2 in DMEM supplemented with 10% heat-inactivated FCS with 100 U/mL penicillin and 100 U/mL streptomycin. G418 (400 μg/mL) and (200 μg/mL) hygromycin B were used to select for 5-LOX/FLAP expressing cells [23].

C. albicans culture

C. albicans (ATCC SC5314) [24] and all *C. albicans* mutant strains are listed in Table 1. *C. albicans* glycerol stocks were inoculated on YPD-agar (1% yeast-extract (m/v), 1% peptone (m/v), and 2% dextrose (m/m)). For experiments, single colonies were picked and incubated in a YPD medium at 30°C and 180 rpm overnight. Subsequently, overnight cultures were centrifuged and resuspended with PBS (pH 7.4) twice before adjustment to the required cell density. Yeast cells were transferred to RPMI (without phenol red) for hyphae induction (120 min at 37°C). Hyphae cultures were centrifuged, resuspended, and adjusted to the required cell density using Neubauer improved counting chamber.

Determination of LM formation in macrophages via UPLC-MS/MS

Monocyte-derived macrophages (MDMs) were seeded with RPMI-1640 into six-well plates prior to polarisation described above

(2×10^6 cells/well). The medium was aspirated and MDMs were stimulated with *C. albicans* yeast or hyphae at indicated multiplicity of infections (MOI) in PBS+ (pH 7.4; 1 mM [Ca^{2+}]). To collect LM, the supernatant was removed and mixed with 2 mL of ice-cold methanol containing 10 μL of deuterium labeled internal standard (200 nM d8-5S-hydroxyeicotetraenic acid [HETE], d4-LTB_4, d5-LXA_4, d5-RvD_2, d4-PGE_2, and 10 μM d8-AA; Cayman Chemicals) for means of quantification and sample normalization. Sample purification was performed as previously described [25]. In brief, samples were transferred to –20°C for at least 60 min to ensure protein precipitation and subsequently centrifuged at 1200 × rpm for 10 min, at 4°C. The supernatant was diluted with 8 mL acidified H_2O (0.5 mM HCl), followed by solid phase extraction on Sep-Pak Vac 6cc 500 mg/6 mL C18-columns (Waters). After loading the sample, the column was washed with water and n-hexane, and LMs were eventually eluted with methylformate. Samples were evaporated under nitrogen (TurboVap LV; Biotage) and resuspended in 100 μL 50% methanol for ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) as described [25]. LMs were separated chromatographically applying an ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm; Waters) at 50°C and at a 0.3 mL/min 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 min that was followed by isocratic elution at 98% till 15.5 min. 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). Analysis was performed in the negative ion mode using multiple reaction monitoring 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 min for *d8*-5(S)HETE; *m/z* 339.3 → 197.2 and RT 9.2 min for *d4*-LTB_4 as well as m/z 355.3→193.2 and RT 6.1 min for *d4*-PGE_2. Eicosanoids were analyzed using the following transitions and RTs: et-LTB_4 *m/z* 335.2→195.10 at RT 8.9 min; LTB_4 *m/z* 335.2→195.1 at RT 9.2 min; 20-OH-LTB_4 *m/z* 351.3→195.1 at RT 4.8 min; 5-HETE *m/z* 319.2→115.1 at RT 12.1 min; PGE_2 m/z 351.3→189.1 at RT 6.1 min; PGD_2 m/z 351.3→233.1 at RT 6.3 min; $PGF_{2\alpha}$ m/z 353.3→193.1 at 6.5 min; TXB_2 at m/z 369.3→169.1 at RT 5.8 min. Quantification was performed by applying linear calibration curves with r^2 values of 0.998 or higher (for fatty acids: 0.95 or higher) for each LM as described [25].

Lactate dehydrogenase assay

To assess the cytotoxic effects of *C. albicans* (SC5314) yeast and hyphae on human cells, cell membrane integrity was quantified by determination of activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the supernatants. Macrophages (2×10^6 cells/mL) and HEK_5-LOXFLAP cells (2×10^6 cells/mL) were incubated with *C. albicans* (SC5314) yeast or hyphae at MOI 10, vehicle control, and triton X-100 (0.2%) as full lysis control for 0.5, 1, 2, and 24 h. LDH release from disintegrated cells was measured by CytoxTox96 KIT (PROMEGA GmbH) according to the manufacturer's instructions. The values are presented as a percentage of the full lysis control Triton X-100 (0.2%).

Determination of intracellular Ca^{2+} concentration

To analyze the effect of *C. albicans* (SC5314) yeast or hyphae on $[Ca^{2+}]_i$-levels in macrophages (2×10^6 cells/mL) and HEK_5-LOX/FLAP cells (4×10^6 cells/mL), cells 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 protected from light for 30 min (37°C; 5% CO_2). Cells were washed twice with KREBS-HEPES buffer (1200 rpm, 5 min, room temperature) and finally resuspended 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 10 of *C. albicans* yeast or hyphae, vehicle control (KREBS-HEPES; 1 mM [Ca^{2+}]; 0.1% BSA), or with 2 μM ionomycin as positive control. Fluorescence emission λ_{em} at 510 nm after excitation λex at 340 nm and 380 nm, respectively was measured continuously over 120 min. The ratio of emission maxima was calculated for every time point and normalized to total [Ca^{2+}]-influx under treatment of 1% triton X-100.

Immunofluorescence microscopy

Immunofluorescence microscopy was applied to analyze the subcellular localization of 5-LOX and FLAP under stimulation with *C. albicans* (SC5314) yeast or hyphae. Macrophages (1×10^6 cells/well) and HEK_5-LOX/FLAP cells (1×10^6cells/well) were seeded on coverslips in 12-well plates 2 days prior to the experiment. The supernatant was removed and cells were stimulated in PBS+ (PBS pH 7.4; 1 mM $CaCl_2$) with *C. albicans* at MOI 10 or vehicle control (PBS+) for 60 min (37°C; 5% CO_2). Cells were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 min, followed by permeabilization in acetone on ice for 5 min and Triton X-100 (0.25% in PBS (m/v); pH 7.4) for 10 min. The slides were incubated with primary antibodies mouse monoclonal anti-5-LOX 1:100 and rabbit polyclonal anti-FLAP 1:150) in nonimmune 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 nonimmune goat serum for 30 min 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) coupled with AxioCam MR camera (Carl Zeiss). Displayed Microscopic pictures show a 50 × 50 μm section of macrophages and a 20 × 20 μm section of HEK_5-LOXFLAP cells of the full image.

SDS-PAGE and Western blotting of macrophages

Experiments were stopped and macrophages (2×10^6 cells/mL) were scraped in 80 μL Seamann 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). Cell lysates were transferred on ice followed by centrifugation for 10 min at 15 000 × *g* and 4°C. The supernatant's protein concentration 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 min. SDS-polyacrylamide gels (10%) were used for protein separation and were subsequently blotted to nitrocellulose membrane. To prevent unspecific antibody binding, membranes were blocked in 5% BSA in TBS (1 h, 21°C) prior to incubation with primary antibodies (rabbit monoclonal anti-ERK1/2, 1:1000 (4695S, Cell Signaling); mouse monoclonal anti-phospho-ERK1/2 (Thr202/Tyr204) 1:1000 (9106, Cell Signaling); rabbit monoclonal anti-p38 MAPK, 1:1000 (8690S, Cell Signaling); rabbit polyclonal anti-phospho-p38 MAPK, 1:1000 (Thr180/Tyr182); rabbit polyclonal anti-COX-1, 1:1000 (#4841, Cell Signaling); rabbit monoclonal anti-COX-2, 1:500 (#12282, Cell Signaling)); 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 (926–32210, LI-COR Biosciences), IRDye 800CW Goat anti-Rabbit IgG (H + L), 1:15,000 (926–32211, LI-COR Biosciences), and/or IRDye 680LT Goat anti-Mouse IgG (H + L), 1:40,000 (926–68020, LI-COR Biosciences) for 60 min at room temperature and was stored at 37°C until dryness. Analysis was performed using an Odyssey infrared imager (LI-COR Biosciences).

Statistics

Results are displayed as means + SEM of *n* observations, with *n* representing the number of experiments performed with different donors. Graphs were created and validated with GraphPad Prism 7.04 software (GraphPad Software). A value of $p < 0.05$ was considered statistically significant.

Results

LM biosynthesis provoked by *C. albicans* yeast or hyphae in M1 and M2 macrophages

To further increase our knowledge of *Candida*-induced eicosanoid formation, we investigated the LM formation in monocyte-derived M1 and M2 macrophages and 5-LOX activation in immortalized stably transfected HEK_5-LOX/FLAP cells upon interaction with *C. albicans* yeast and hyphae.

Among the various branches of the AA cascade, AA can be converted by the 5-LOX/FLAP pathway to 5-H(p)ETE, which is then further metabolized to LTA_4 the unstable intermediate and substrate for LTA_4 hydrolase, to form the pro-inflammatory and chemoattractant LTB_4. In cells, LTB_4 is degraded to 20-OH-LTB_4 (Fig. 1A). COX-1/2 metabolizes AA to PGH_2, which is further converted to PGs and thromboxane depending on the tissue (Fig. 1A). M1 and M2 macrophages are obtained in vitro following differentiation of monocytes and subsequent polarization with LPS/IFNγ and IL-4, respectively, resulting in differential expression of AA cascade enzymes and consequently in different LM profiles. Western blot revealed protein expression of enzymes involved in AA metabolism in both M1 and M2 macrophages (Fig. S1). 5-LOX and low levels of COX-1 are expressed in both subpopulations, whereas FLAP expression was strongly reduced in M2 macrophages, attenuating the 5-LOX pathway. Conversely, COX-2 was strongly expressed in LPS-polarized M1 macrophages.

M1 and M2 macrophages were stimulated with *C. albicans* yeast and hyphae for 60 min and phenotype-specific eicosanoid formation was analyzed via UPLC-MS/MS (Fig. 1B and F; Figs. S2 and S3). LTB_4 products (sum of LTB_4, t-LTB_4, and 20-OH-LTB_4) and PGE_2 were chosen as representatives for the activation of the 5-LOX and COX biosynthetic pathways, respectively. *C. albicans* yeast failed to activate the 5-LOX pathway in M1 and M2 macrophages. However, a small but significant increase in PGE_2 formation was observed in yeast-stimulated macrophages. In contrast, total eicosanoid biosynthesis was significantly increased in M1 macrophages upon stimulation with *C. albicans* hyphae, as reflected by the formation of LTB_4 products and PGE_2. Low levels of PGE_2 comparable to yeast-activated macrophages were detected in M2 macrophages upon stimulation with *C. albicans* hyphae, with total LM formation in M2 macrophages reaching less than a quarter of the LM levels detected in M1 macrophages. Note that IL-4 polarization of M2 macrophages strongly induces 15-LOX-1 expression [26], which shifts the LM biosynthesis towards 15-LOX-derived LM, a phenomenon seen in plenty of reports [8]. Nevertheless, unexpectedly neither yeast nor hyphae of *C. albicans* stimulate 15-LOX product formation.

Cellular LT formation is dependent on the nuclear co-localization of 5-LOX and its helper protein FLAP [4]. By monitoring the subcellular distribution of these proteins, we found that exclusively in hyphae-challenged M1 macrophages 5-LOX translocated and co-localized with FLAP at the nuclear membrane (Fig. 1C and G). Interestingly, in M2 macrophages and under stimulation with both morphologies 5-LOX remained disseminated within the nucleus (Fig. 1G), explaining the lack of LT formation. Both $cPLA_2$ and 5-LOX are stimulated by $[Ca^{2+}]_i$ mobilization [27, 28]. Therefore, changes of intracellular $[Ca^{2+}]_i$ levels were continuously monitored during the 60 min incubation period stimulated by *C. albicans* yeast or hyphae in both macrophage phenotypes (Fig. 1D and H). Although the increase in $[Ca^{2+}]_i$-mobilization under fungal stimulation was not significant, *C. albicans* hyphae induced a slightly stronger increase in intracellular $[Ca^{2+}]$ compared with the yeast cells. To exclude cytotoxic effects and macrophage membrane disintegration as the underlying cause of eicosanoid formation, potential cell damage of *C. albicans* yeast or hyphae-stimulated macrophages was monitored

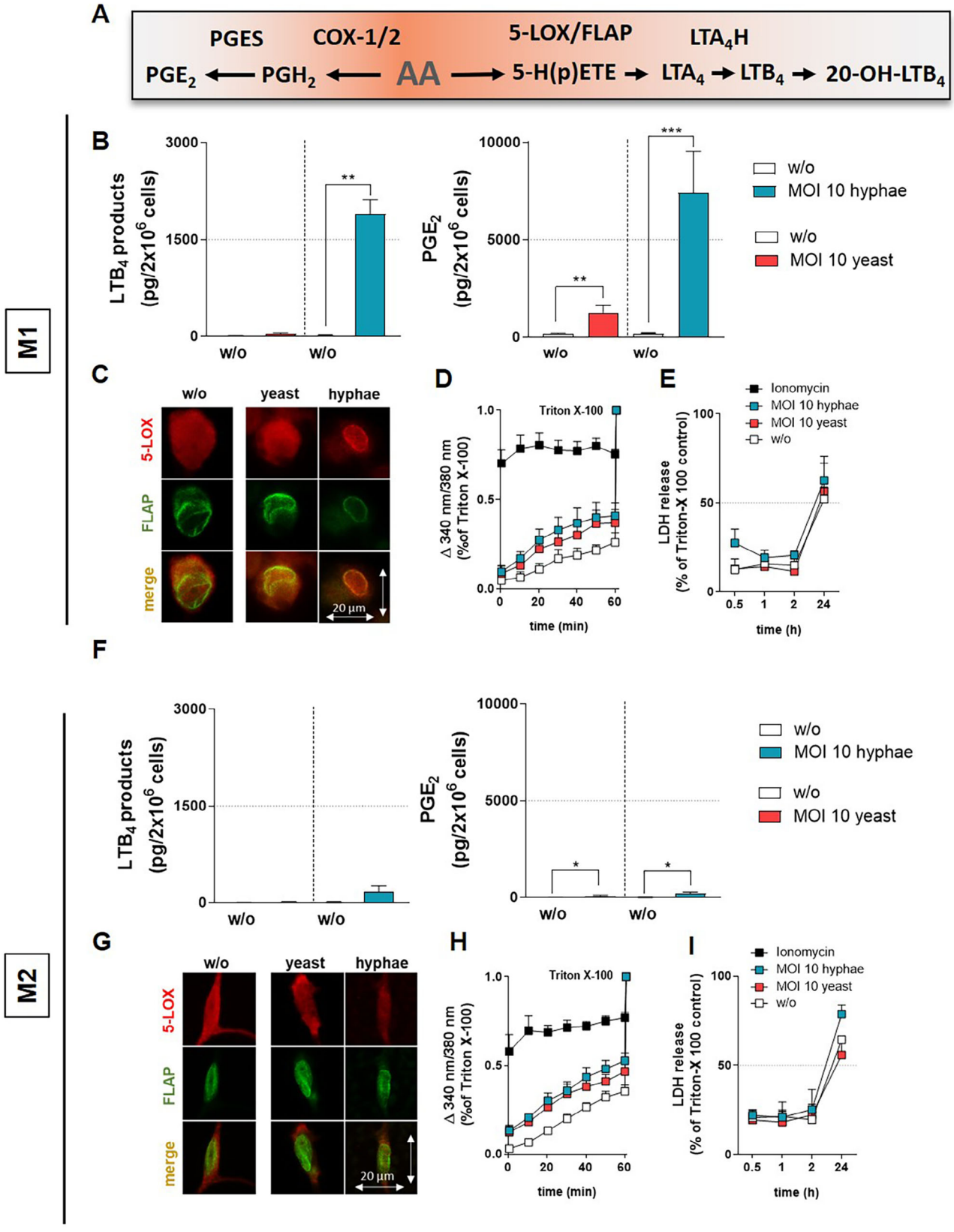

Figure 1. *C. albicans* morphology determines eicosanoid formation in M1 and M2 macrophages (A) Illustration of two branches of the AA cascade. (B, F) LTB_4 and PGE_2 formation in 2 Mio. human M1 (B) and M2 (F) macrophages upon stimulation with vehicle control (PBS; pH 7.4, 1mM $[Ca^{2+}]$) or MOI 10 of *C. albicans* (SC5314) yeast (red) and hyphae (blue), *n* = 4-5. (C, G) Subcellular localization of 5-LOX and FLAP in M1 (C) and M2 (G) macrophages

by LDH activity in supernatants. No significant LDH release was observed during the first 2 h of fungi–macrophage interaction but strongly increased after 24 h in both M1 and M2 macrophages (Figure 1E and I). In conclusion, LT formation was solely induced by *C. albicans* hyphae in M1 macrophages, and PGE_2 biosynthesis was stimulated in M1 and M2 macrophages by yeast and hyphae, but to a much higher extent in hyphae-stimulated M1 macrophages.

C. albicans hyphae provoke MAPKs activation in M1 and M2 macrophages

Stimulation of mitogen-activated protein kinases (MAPKs) is globally involved in the activation of $cPLA_2$, 5-LOX, and ultimately in the expression of COX-2 [29, 30]. While 5-LOX can be activated through phosphorylation at Ser 271 and Ser 663 via p38-MAPK-dependent MAKAPK-2 and ERK1/2, respectively, $cPLA_2$ activity is induced by phosphorylation at Ser505 *via* ERK1/2 [31]. Western blot analysis confirmed p38-MAPK and ERK1/2 phosphorylation in response to *C. albicans* hyphae in both macrophage phenotypes (Fig. 2A and B). The progression of phosphorylation differed between the MAPKs but was phenotype-independent. While the level of p38-MAPK phosphorylation remained constant over 180 min, ERK1/2 phosphorylation peaked after 45 min of stimulation and decreased continuously over the next 135 min.

Hyphae-triggered eicosanoid formation is dependent on hyphal-associated protein expression

Based on the observation that *C. albicans* yeast cells failed to stimulate sufficient eicosanoid formation in M1 and M2 macrophages, we questioned whether hyphal-associated virulence factors activate the 5-LOX and COX pathways. Utilizing yeast-locked *C. albicans* mutant strains is an elegant approach to assess the importance of morphological and transcriptomic adaptations during the transition from yeast to hyphae. The yeast-locked mutant *C. albicans efg1*Δ/Δ/*cph1*Δ/Δ is unable to filament and express hyphal-associated genes [15]. *C. albicans hgc1*Δ/Δ mutant shows defective hyphal formation but still expresses hyphal-associated proteins, including hyphal wall protein 1 (Hwp1), Ece1, Hyr1, and Als3 [32]. Consequently, these strains differ in their protein-expression profile. All *C. albicans* strains were treated under hyphae-inducing conditions. In M1 macrophages 5-LOX and COX activity was significantly reduced upon stimulation with *C. albicans efg1*Δ/Δ/*cph1*Δ/Δ compared with *C. albicans* wild-type, as reflected by a significant decrease in LTB_4 and PGE_2 formation (Fig. 3, Fig. S4). Similarly, LTB_4 and PGE_2 formation in M2 macrophages was attenuated upon stimulation with *C. albicans efg1*Δ/Δ/*cph1*Δ/Δ compared with wild-type, but as mentioned above (Fig. 1B and F) starting from a less abundant level of LM formation than in M1 macrophages. However, co-incubation of macrophages with the *hgc1*Δ/Δ *C. albicans* mutant still resulted in eicosanoid formation comparable to that of the wildtype strain (Fig. 3, Fig. S4). Since the *hgc1*Δ/Δ mutant cannot form true hyphae, yet is still able to express hyphal-associated proteins, this suggests a strong involvement of the latter in hyphae-induced LM biosynthesis.

C. albicans hyphae stimulate 5-LOX activation in HEK_5-LOX/FLAP cells

During disseminated candidiasis, the fungus infects vital tissues during the late stages of infection. Drastic immune activation by the infected tissue cells not only drives protective immunity, but particularly, in the case of the kidney, leads to uncontrolled neutrophil influx [33]. Here, we used HEK293 cells stably expressing 5-LOX and FLAP (HEK_5-LOX/FLAP) [23] to exclude innate immune-specific recognition pathways as underlying mechanisms for fungal-induced eicosanoid formation. These experiments allowed us to extend the validity of our results obtained in professional innate immune cells and prove them as universal pathogenicity mechanisms that may contribute to continuous inflammation. We monitored 5-LOX activation in HEK_5-LOX/FLAP cells by determining their cellular distribution using immunofluorescence microscopy. Similar to previous observations in macrophages, *C. albicans* hyphae strongly induced 5-LOX activation, visible in translocation, after 60 min of co-culture, whereas *C. albicans* yeast failed in this respect (Fig. 4A). Intracellular Ca^{2+} mobilization as 5-LOX activator was monitored for 60 min in HEK_5-LOX/FLAP cells stimulated with both *C. albicans* morphologies. Surprisingly, in contrast to macrophages (Fig. 1D) and neutrophils [21], both yeast and hyphal cells of *C. albicans* failed to stimulate a detectable Ca^{2+}-influx (Fig. 4B), suggesting the requirement of more precise activation factors for 5-LOX translocation. To exclude fungal cytotoxicity and to ensure membrane integrity during incubation, LDH activity in supernatants was examined. In contrast to macrophages and neutrophils [21], HEK_5-LOX/FLAP cells showed slightly but not significantly increased LDH levels during the first 2 h of incubation with *C.*

upon stimulation with MOI 10 of *C. albicans* yeast or hyphae for 60 min was monitored by IF microscopy. Cells were stained with antibodies against 5-LOX (Alexa Flour 555, red) and FLAP (Alexa Fluor 488, green). A representative of four independent experiments is shown. (D, H) Determination of $[Ca^{2+}]_i$ mobilization through continuous fluorescence measurement. FURA-2AM-labeled M1 (D) and M2 (H) macrophages were incubated with 2 μM ionomycin, *C. albicans* hyphae, yeast, or vehicle control (PBS; pH 7.4, 1 mM $[Ca^{2+}]$). Data are shown as mean + SEM from $n = 3$ independent experiments. E/I) cell membrane integrity of M1 (E) and M2 (I) macrophages under treatment with *C. albicans* yeast or hyphae, vehicle control (PBS; pH 7.4, 1mM $[Ca^{2+}]$) was determined by analysis of LDH release and normalization to 0.2% triton X-100 as full lysis control over 24 h. Data are shown as percentage of triton X-100 control, mean + SEM, $n = 3$ donors. Statistical analysis in (B, F) was performed on logarithmically transformed data using paired Student's *t*-test; stimulus vs. control; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$.

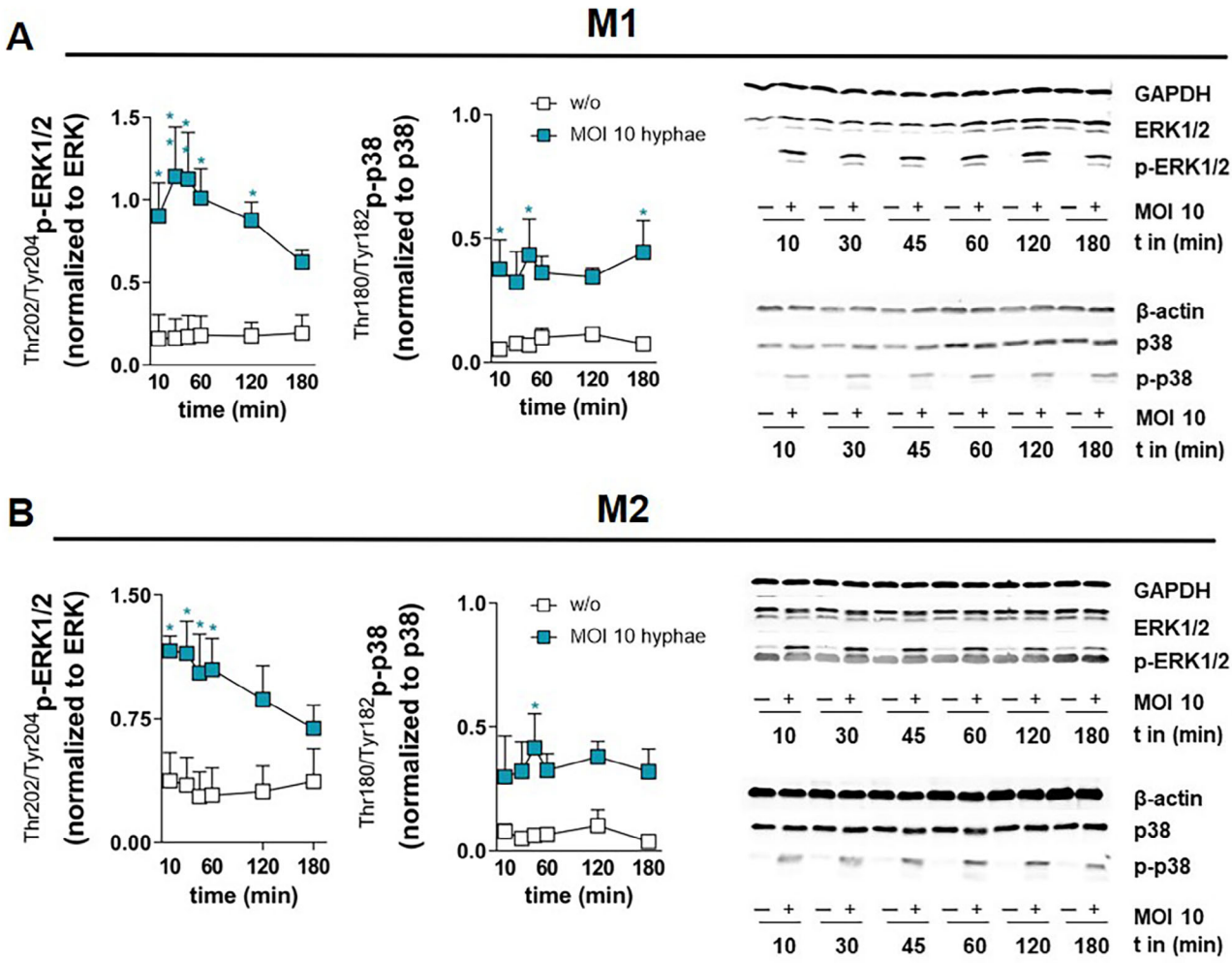

Figure 2. Activation of p38-MAPK and ERK1/2 in M1 and M2 macrophages by *C. albicans* hyphae. Total protein expression and phosphoprotein expression of p38 MAPK and ERK-1/2 were analyzed by Western blot and densitometric analysis in (A) M1 macrophages and (B) M2 macrophages (2×10^6 cells/mL). Host cells were incubated with *C. albicans* (SC5314) hyphae (MOI 10) or vehicle control (PBS; pH 7.4; 1 mM Ca^{2+}) for the indicated times, $n = 3$-4. Results are given as mean + SEM and statistical analysis was performed using two-way ANOVA with Sidak's multiple comparison post hoc test, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.005$, stimulus vs. control. Original blots are included in Supporting Information.

albicans yeast and hyphae, leading to a significant LDH release after 24 h of treatment (Fig. 4C). This observation suggests an expected higher susceptibility of HEK_5-LOX/FLAP cells compared with immunocompetent cells, but also rules out cytotoxic effects as trigger for 5-LOX activation within the first 2 h of interaction.

Deletion of HWP1 in *C. albicans* results in loss of 5-LOX activation in HEK cells and neutrophils, but not in macrophages

Based on our findings in macrophages (Fig. 3) and neutrophils [21], we investigated the influence of the hyphae-locked mutant strains *efg1*Δ/Δ/*cph1*Δ/Δ and *hgc1*Δ/Δ on 5-LOX activation in HEK_5-LOX/FLAP cells. Both yeast-locked strains *efg1*Δ/Δ/*cph1*Δ/Δ and *hgc1*Δ/Δ were unable to stimulate 5-LOX translocation and co-localization with FLAP (Fig. 5A) suggesting that hyphal formation is a prerequisite for 5-LOX translocation in the engineered HEK cell system.

One of the most relevant hyphal surface proteins is the Hwp1, which facilitates attachment to the target cell and is required for cell-to-cell adhesion [16, 34]. *C. albicans hwp1*Δ/Δ and its revertant *hwp1*Δ/Δ+*HWP1* were used to evaluate the involvement of hyphal adhesins in 5-LOX activation in HEK_5-LOX/FLAP cells. The *HWP1*-deficient strain *hwp1*Δ/Δ failed to induce 5-LOX translocation, whereas *C. albicans hwp1*Δ/Δ+*HWP1* stimulated 5-LOX translocation comparable to the wild-type (Fig. 5B). Thus, *C. albicans* appears to require the mature hyphal morphology with full Hwp1 expression at the fungal surface to trigger 5-LOX activation in stably transfected HEK_5-LOX/FLAP cells. Motivated by this finding, we stimulated macrophages and neutrophils with *C. albicans hwp1*Δ/Δ and its revertant. Surprisingly, whereas in macrophages the absence of the Hwp1 did not disrupt a proper

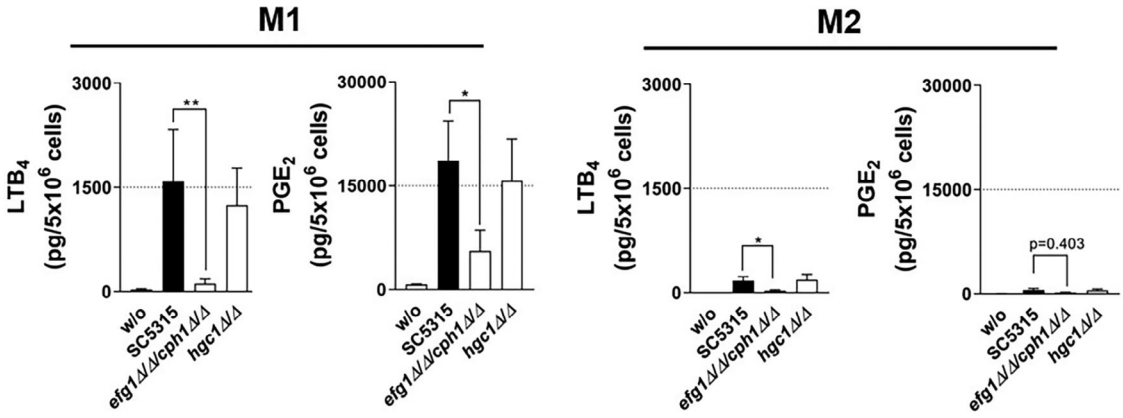

Figure 3. Hyphal-associated protein expression triggers LM formation in macrophages. M1 and M2 macrophages (2×10^6 cells/mL) were stimulated (A) with MOI 10 hyphae of *C. albicans* wildtype (SC5314) or yeast-locked mutant strains *efg1*Δ/Δ/*cph1*Δ/Δ and *hgc1*Δ/Δ as well as vehicle control (PBS; pH 7.4; 1 mM [Ca^{2+}]), $n = 7$ LM were isolated via SPE and analyzed using UPLC-MS/MS. Values are presented as mean + SEM. Data were log-transformed for two-way ANOVA with Dunnett's post hoc test; *C. albicans* mutant strain vs. *C. albicans* wildtype (SC5314). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

eicosanoid biosynthesis (Fig. 5C), in neutrophils LTB_4 formation was significantly reduced by *hwp1*Δ/Δ and was restored by the revertant *hwp1*Δ/Δ+*HWP1* (Fig. 5D), which confirms Hwp1as essential surface protein to facilitate LT biosynthesis. Another virulent hyphae-associated protein is the cytolytic peptide candidalysin, which is expressed and released during filamentous growth. However, the deletion mutant extent of cell elongation 1 protein (*ece1*Δ/Δ), incapable of expressing candidalysin, showed comparable virulence regarding eicosanoid formation in M1 macrophages as the wild-type strain (Fig. 5E). Comparable findings were reported before in neutrophils [21]. These observations let us conclude that 5-LOX activation and accompanied LT formation are clearly associated with hyphae-specific protein expression but differ between different immunocompetent cells. Motivated by the finding that hyphae-specific protein expression plays a crucial role in hyphae-induced eicosanoid formation, we evaluated other surface proteins that are exclusively expressed in hyphae. Our focus turned toward the hyphae wall protein Hyr1 [35] and the surface protein Als3, belonging to the agglutinin-like sequence (Als) family [17, 36]. However, the deletion mutants *hyr1*Δ/Δ and *als3*Δ/Δ showed comparable potential to activate eicosanoid biosynthesis as the wild-type strain (Fig. 5E).

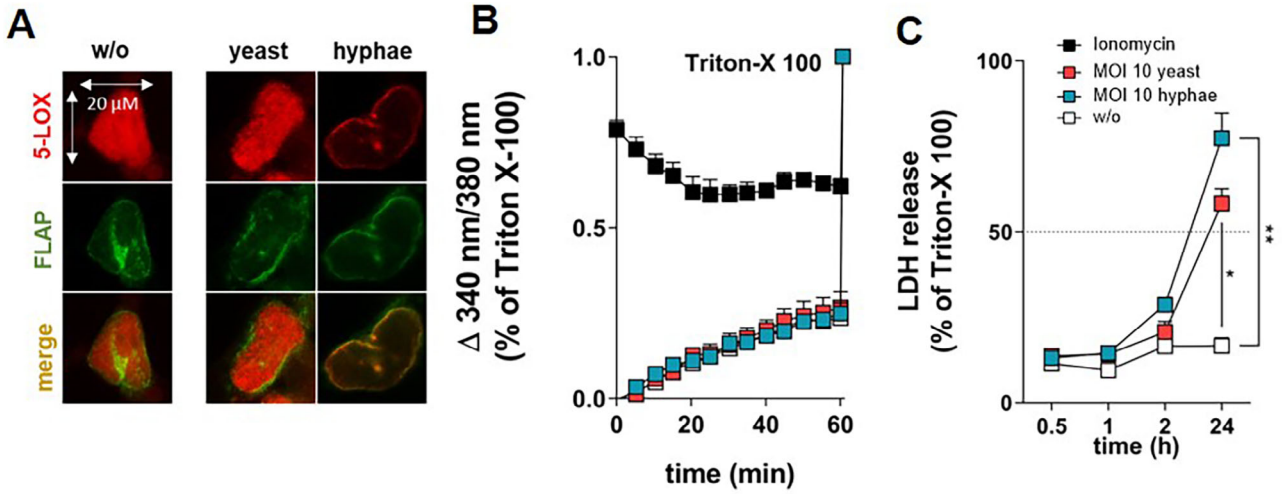

Figure 4. *C. albicans* triggers 5-LOX activation in HEK_5-LOX/FLAP. Subcellular localization of 5-LOX and FLAP was detected by immunofluorescence microscopy in HEK293 cells stably expressing human 5-LOX and FLAP (A) HEK_5-LOX/FLAP cells were incubated with MOI 10 of *C. albicans* (SC5314) yeast or hyphae for 60 min. Cells were stained with antibodies against 5-LOX (Alexa Flour 555, red) and FLAP (Alexa Flour 488, green). A representative of $n = 4$ independent experiments is shown. (B) Determination of Ca^{2+} mobilization through continuous fluorescence measurement. FURA-2AM-labeled HEK_5-LOX/FLAP cells were incubated with 2 µM ionomycin, *C. albicans* (SC5314) hyphae, yeast, or vehicle control (PBS; pH 7.4, 1 mM Ca^{2+}). Data are shown as mean + SEM, $n = 3$. Membrane integrity of HEK_5-LOX/FLAP cells under treatment with *C. albicans* (SC5314) yeast or hyphae, vehicle control (PBS; pH 7.4, 1 mM Ca^{2+}) was determined by analysis of LDH release and normalization to 0.2 % triton X-100 as full lysis control over 24 h. Data are shown as a percentage of triton X-100 control, mean + SEM, $n = 3$ experiments. Statistical analysis in (C) was performed two-way ANOVA with Tukey's post hoc test; stimulus vs. control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

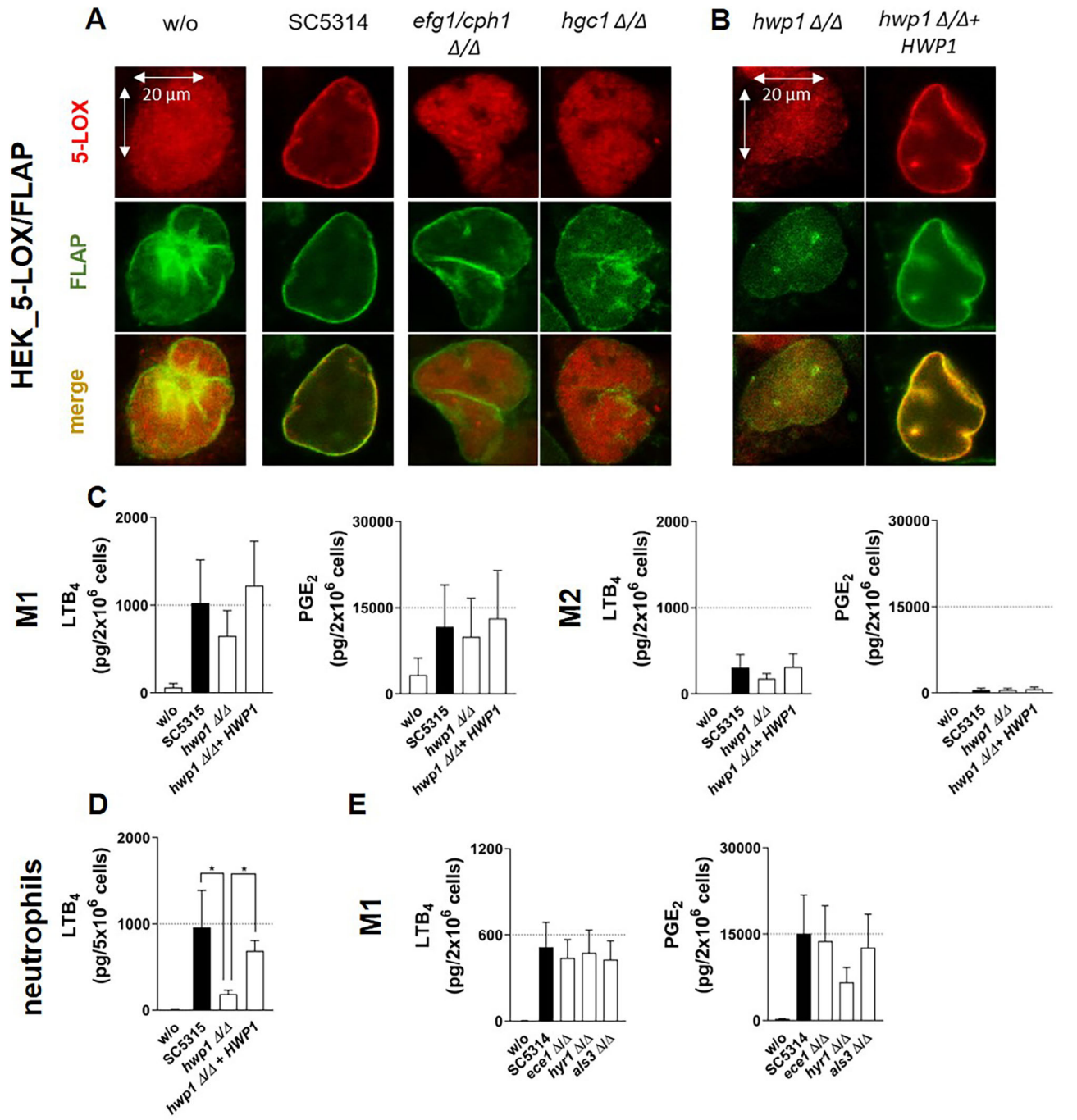

Figure 5. Deletion of *HWP1* in *C. albicans* results in loss of 5-LOX activation in HEK cells and neutrophils, but not in macrophages. (A/B) Subcellular localization of 5-LOX and FLAP was detected by immunofluorescence microscopy in HEK293 cells stably expressing human 5-LOX and FLAP HEK_5-LOX/FLAP cells were incubated with MOI 10 hyphae of (A) *C. albicans* wildtype (SC5314) yeast-locked mutant strains *C. albicans efg1*Δ/Δ /*cph1*Δ/Δ and *hgc1*Δ/Δ and in (B) with *C. albicans hwp1*Δ/Δ knock-out strain and its *hwp1*Δ/Δ+*HWP1* revertant-strain, or vehicle control (PBS; pH 7.4; 1 mM [Ca^{2+}]) for 60 min. Cells were stained with antibodies against 5-LOX (Alexa Flour 555, red) and FLAP (Alexa Flour 488, green). A representative of $n = 4$ independent experiments is shown. (C) M1 and M2 macrophages ($2 \times 1\ 10^6$ cells/mL) and (D) human neutrophils (5×10^6 cells/mL) were stimulated with MOI 10 hyphae of *C. albicans* wildtype (SC5314), *hwp1*Δ/Δ, its *hwp1*Δ/Δ+*HWP1* and vehicle control (PBS; pH 7.4; 1 mM Ca^{2+}), (E) M1 macrophages were stimulated with MOI 10 hyphae of *C. albicans* wildtype (SC5314), *ece1*Δ/Δ, *hyr1*Δ/Δ, *als3*Δ/Δ and vehicle control (PBS; pH 7.4; 1 mM Ca^{2+}). $n = 4$–7. LM was isolated by SPE and analyzed using UPLC-MS/MS. Values are presented as mean + SEM. Data were log-transformed for two-way ANOVA with Dunnett's post hoc test; *C. albicans* mutant strain vs. *C. albicans* wildtype (SC5314). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

Discussion

In the current study, we investigated *C. albicans*-induced eicosanoid formation in macrophage phenotypes and HEK cells while unraveling the underlying inducing mechanisms. We demonstrated that hyphae in particular, but not yeast cells, induce LT and PG formation predominantly in M1 macrophages and that the expression of hyphal-associated proteins plays a crucial role in this process. In HEK cells overexpressing 5-LOX and FLAP and in neutrophils, 5-LOX activation and LT formation were also exclusively induced by hyphae and strongly associated with the expression of the surface protein Hwp1.

A successful human immune response to an invading pathogen requires unambiguous recognition of the micro-organism, followed by transmission and amplification of the proinflammatory signal to ensure the involvement of other mediators and cells of the immune system. Pathogens have developed sophisticated strategies to invade the host while evading the immune system. Among them, the fungus *C. albicans* undergoes a characteristic morphogenetic transition from yeast to hyphae during infection. This morphological change is not only associated with increased virulence [18, 37] but is also an essential survival strategy of the fungus to adapt to the changing environment of the host and complicate immunological clearance [38, 39]. Hyphal formation *per se* is acknowledged as pathogenicity mechanism, but equally important for pathogenicity is the expression of hyphal-associated genes such as *ECE1* and *HWP1*. While the extent of cell elongation protein 1 (Ece1) is the precursor of the amphiphilic peptide toxin candidalysin [18], Hwp1 is associated with adhesion and biofilm formation [16, 40]. Hwp1 is reported to serve as a substrate for mammalian transglutaminases, covalently linking *C. albicans* hyphae to host cells [16]. Moreover, *HWP1*-deficient *C. albicans* strains showed attenuated virulence properties [16, 41]. We have shown that the formation of lipid mediators and the activation of 5-LOX in human macrophages and HEK_5-LOX/FLAP cells after stimulation by *C. albicans* is closely associated with the successful filamentation of the fungus. Using yeast-locked (*efg1*Δ/Δ/*cph1*Δ/Δ) and hyphal-impaired (*hgc*Δ/Δ) mutant strains of *C. albicans*, we confirmed this key finding and linked the activation to hyphal-associated proteins. This phenomenon has already been shown recently by us in neutrophils [21] and suggests that the host specifically mounts a distinct immune response to host–hyphal interactions in terms of the release of pro-inflammatory LM. In this report, we were able to extend our knowledge by identifying Hwp1 as the crucial surface protein that stimulates LT formation in neutrophils and HEK_5-LOX/FLAP cells. Most interestingly, while 5-LOX activation in neutrophils and HEK_5-LOX/FLAP cells is dependent on Hwp1, the expression of other hyphal-associated proteins appears to be involved in macrophages. Neither other adhesins, such as Hyr1 and Als3, nor the hyphal-specific toxin candidalysin seem to play a role in the stimulation of eicosanoid formation in macrophages. Such cell-type-specific stimulation of LM biosynthesis has been shown before for *Staphylococcus aureus* where LM biosynthesis in neutrophils is induced by phenol-soluble modulins, but in macrophages, it is stimulated by the pore-forming toxin alpha-hemolysin [9, 42]. Thus, it seems that different immune cells are equipped with distinct activation mechanisms stimulating LM biosynthesis. It should be noted that synergistic activation may also be required for eicosanoid formation in macrophages. A similar observation has been made during oropharyngeal infection by *C. albicans*, where both Hyr1 and Als3 are required for stimulation of c-Met and EGFR to develop full virulence and epithelial cell invasion [43]. Future studies will have to show which hyphal-associated proteins or what kind of interplay between different actors stimulate LT or PG biosynthesis in macrophages.

During host invasion, *C. albicans* hyphae can breach mucosal and epithelial surfaces. Epithelial cells recognize the fungal pathogen and initiate damage response pathways [37]. These signalling cascades lead to the release of chemokines, cytokines, and antimicrobial peptides that activate the immune system and promote the recruitment of neutrophils and macrophages [37, 44]. Hereby, macrophages are key players in fungal immune recognition. Tissue-resident macrophages (TRMs) were proven to function as sentinel cells, crucial during the early stages of *C. albicans* dissemination into organs [1, 45, 46]. Previous studies have highlighted the relevance of TRM populations in various organs, such as the spleen and liver for *C. albicans* eradication as well as attenuation of fungal dissemination and filamentation [47, 48]. Shortly after *C. albicans* tissue invasion, the immune cell population substantially increases, mainly originating from neutrophils and MDM migrating toward the side of inflammation [33, 49]. Both TRMs and MDMs possess a distinct amatory of defense mechanisms against fungal pathogens, such as phagocytic clearance and subsequent secretion of cytokines or oxygenated lipids to amplify the proinflammatory signal. Eicosanoids, namely LTs and PGs tailor such an innate immune response. They are synthesized in myeloid cells in a cell-type distinct manner and mainly act locally through G-protein coupled receptors [50, 51]. Hereby, LTB_4 is a strong proinflammatory chemoattractant, induces ROS formation, and stimulates the release of chemokines from neutrophils and macrophages [8, 52, 53]. PGE_2, abundant in inflammatory environments, enhances inflammation by causing local vasodilation and attracting leukocytes. It also has immunosuppressive effects, such as stimulating the formation of IL-10. [54–57]. Thus, we identified the cellular mechanism of how *C. albicans* hyphae accelerate inflammation by pushing biosynthesis of inflammatory lipid mediators. In addition, early reports indicated that PGE_2 enhances serum-induced germination and hyphae formation, whereas COX inhibition reduces biofilm formation and yeast-to-hyphae transition [58–60]. It is therefore conceivable that small amounts of PGE_2, already produced by yeast cells, then trigger the formation of hyphae and lead to dramatically increased inflammatory conditions through increased biosynthesis of LT and PG. However, the role of LM in *C. albicans* infection remains controversial in the scientific literature. While Peters *et al*. [61] could show that during co-infection with *C.*

albicans and *S. aureus* treatment with non-selective COX-inhibitor indomethacin reduced mortality in mice, Jayaraja *in vivo*. [49] have demonstrated the importance of LTs and PGs for sufficient fungal clearance and survival in mice. Extended studies linking tissue and serum LM concentrations to pathophysiological circumstances are needed to evaluate the role of LM in *Candida* infections. Furthermore, our study showed that eicosanoid formation was significantly reduced in M2 macrophages compared with M1 macrophages, despite comparable activation of MAPK ERK1/2 and p38 MAPK. Both macrophage phenotypes differ in the expression profile of AA metabolizing enzymes resulting in a strongly reduced FLAP and COX-2 expression in M2 cells [25]. It should be further considered that GM-CSF, used as a supplement for M1 macrophage differentiation, is known to upregulate dectin-1 expression [62], a receptor activated by the fungal cell wall component β-glucan [63] and known to be involved in hyphal-induced LT formation in neutrophils [21]. Interestingly, polarization by IL-4 stimulates 15-LOX-1 expression in M2 macrophages [25], but 15-HETE and corresponding metabolites formed from other polyunsaturated fatty acids such as docosahexaenoic acid or eicosapentaenoic acid were not detected. This indicates that 15-LOX-1 in M2 MDM is not stimulated by either yeast or hyphae.

During disseminated candidiasis in mice, the murine kidney is among the primary target organs of *C. albicans*. This susceptibility can be linked to a delayed neutrophil and monocyte influx into kidney tissue compared to other organs [33, 64]. Presupposing myeloid cells and kidney tissue cells differ in their activation toward *C. albicans* yeast and hyphae, we questioned the response of stably transfected HEK_5-LOX/FLAP cells toward both morphologies [37, 44]. Interestingly, we observed a *C. albicans* hyphae-exclusive stimulating effect on 5-LOX. Additionally, the tested HEK cell line showed, comparable to neutrophils, an HWP1-related 5-LOX activation. As HEK-293 cells are no original carriers of 5-LOX and FLAP, it should be stressed, that HEK_5-LOX/FLAP cells represent an artificial approach, originally developed to study 5-LOX and FLAP interaction. However, our data indicate a susceptibility of HEK cells toward *C. albicans* hyphae via Hwp1 with corresponding activation of intracellular signaling cascades.

In conclusion, we report that *C. albicans* filamentation is essential for the activation of eicosanoid biosynthesis in M1 macrophages, neutrophils, and HEK_5-LOX/FLAP. For neutrophils and HEK cells, Hwp1 was identified as an essential hyphae surface protein required for 5-LOX activation and LT formation in the host cells. Whether the activation of the AA machinery contributes to detrimental immunopathology or enhances host-driven clearance of the pathogen is likely niche-dependent and remains to be elucidated. In the future, *in vivo*, infection models will be able to demonstrate the effects of activating eicosanoid biosynthesis on the *C. albicans* infection process in mammals, which could not be presented in this study. Nevertheless, the *in vitro* data highlights a strong specificity of the human immune system toward recognizing *C. albicans* filamentation. Thus, any intervention influencing hyphal growth will impact host–immune responses.

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References

1 **Xu, S. and Shinohara, M. L.**, Tissue-resident macrophages in fungal infections. *Front Immunol*. 2017;**8**: 1798.

2 **Desai, J. V. and Lionakis, M. S.**, The role of neutrophils in host defense against invasive fungal infections. *Curr Clin Microbiol Rep*. 2018. **5**: 181–189.

3 **Funk, C. D.**, Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science*. 2001. **294**: 1871–1875.

4 **Gerstmeier, J., Weinigel, C., Rummler, S., Radmark, O., Werz, O. and Garscha, U.**, Time-resolved in situ assembly of the leukotriene-synthetic 5-lipoxygenase/5-lipoxygenase-activating protein complex in blood leukocytes. *FASEB J*. 2016. **30**: 276–285.

5 **Thulasingam, M. and Haeggstrom, J. Z.**, Integral membrane enzymes in eicosanoid metabolism: structures, mechanisms and inhibitor design. *J Mol Biol*. 2020. **432**: 4999–5022.

6 **Xue, J., Schmidt, S. V., Sander, J., Draffehn, A., Krebs, W., Quester, I., De Nardo, D. et al.**, Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity*. 2014. **40**: 274–288.

7 **Martinez, F. O. and Gordon, S.**, The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep*. 2014. **6**: 13.

8 **Werz, O., Gerstmeier, J., Libreros, S., De la Rosa, X., Werner, M., Norris, P. C., Chiang, N. et al.**, Human macrophages differentially produce specific resolvin or leukotriene signals that depend on bacterial pathogenicity. *Nat Commun*. 2018. **9**: 59.

9 **Jordan, P. M., Gerstmeier, J., Pace, S., Bilancia, R., Rao, Z., Borner, F., Miek, L. et al.**, Werz O. *Staphylococcus aureus*-derived alpha-hemolysin evokes generation of specialized pro-resolving mediators promoting inflammation resolution. *Cell Rep*. 2020. **33**: 108247.

10 **Brown, G. D., Denning, D. W. and Levitz, S. M.**, Tackling human fungal infections. *Science*. 2012. **336**: 647.

11 **Mayer, F. L., Wilson, D. and Hube, B.**, Candida albicans pathogenicity mechanisms. *Virulence*. 2013. **4**: 119–128.

12 **d'Enfert, C., Kaune, A. K., Alaban, L. R., Chakraborty, S., Cole, N., Delavy, M., Kosmala, D. et al.**, The impact of the fungus-host-microbiota interplay upon *Candida albicans* infections: current knowledge and new perspectives. *FEMS Microbiol Rev*. 2021. **45**: fuaa060.

13 **Sprague, J. L., Kasper, L. and Hube, B.**, From intestinal colonization to systemic infections: *Candida albicans* translocation and dissemination. *Gut Microbes*. 2022. **14**: 2154548.

14 **Jacobsen, I. D., Wilson, D., Wachtler, B., Brunke, S., Naglik, J. R. and Hube, B.**, Candida albicans dimorphism as a therapeutic target. *Expert Rev Anti Infect Ther*. 2012. **10**: 85–93.

15 **Lo, H. J., Kohler, J. R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A. and Fink, G. R.**, Nonfilamentous *C. albicans* mutants are avirulent. *Cell*. 1997. **90**: 939–949.

16 **Staab, J. F., Bradway, S. D., Fidel, P. L. and Sundstrom, P.**, Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science*. 1999. **283**: 1535–1538.

17 **Hoyer, L. L., Payne, T. L., Bell, M., Myers, A. M. and Scherer, S.**, Candida albicans ALS3 and insights into the nature of the ALS gene family. *Curr Genet*. 1998. **33**: 451–459.

18 **Moyes, D. L., Wilson, D., Richardson, J. P., Mogavero, S., Tang, S. X., Wernecke, J., Hofs, S. et al.**, Candidalysin is a fungal peptide toxin critical for mucosal infection. *Nature*. 2016. **532**: 64–68.

19 **Felk, A., Kretschmar, M., Albrecht, A., Schaller, M., Beinhauer, S., Nichterlein, T., Sanglard, D. et al.**, Candida albicans hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. *Infect Immun*. 2002. **70**: 3689–3700.

20 **Parti, R. P., Loper, R., Brown, G. D., Gordon, S., Taylor, P. R., Bonventre, J. V., Murphy, R. C. et al.**, Cytosolic phospholipase a2 activation by *Candida albicans* in alveolar macrophages: role of dectin-1. *Am J Respir Cell Mol Biol*. 2010. **42**: 415–423.

21 **Fischer, J., Gresnigt, M. S., Werz, O., Hube, B. and Garscha, U.**, *Candida albicans*-induced leukotriene biosynthesis in neutrophils is restricted to the hyphal morphology. *FASEB J*. 2021. **35**: e21820.

22 **Böyum, A.**, Separation of leucocytes from blood and bone marrow. With special reference to factors which influence and modify sedimentation properties of hematopoietic cells. Oslo, *Universitetsforlaget*. 1968. 108p.

23 **Gerstmeier, J., Weinigel, C., Barz, D., Werz, O. and Garscha, U.**, An experimental cell-based model for studying the cell biology and molecular pharmacology of 5-lipoxygenase-activating protein in leukotriene biosynthesis. *Biochim Biophys Acta*. 2014. **1840**: 2961–2969.

24 **Gillum, A. M., Tsay, E. Y. and Kirsch, D. R.**, Isolation of the Candida albicans gene for orotidine-5'-phosphate decarboxylase by complementation of S. cerevisiae ura3 and E. coli pyrF mutations. *Mol Gen Genet*. 1984. **198**: 179–182.

25 **Werner, M., Jordan, P. M., Romp, E., Czapka, A., Rao, Z., Kretzer, C., Koeberle, A. et al.**, Targeting biosynthetic networks of the proinflammatory and proresolving lipid metabolome. *FASEB J*. 2019. **33**: 6140–6153.

26 **Conrad, D. J., Kuhn, H., Mulkins, M., Highland, E. and Sigal, E.**, Specific inflammatory cytokines regulate the expression of human monocyte 15-lipoxygenase. *Proc Natl Acad Sci U S A.* 1992. **89**: 217–221.

27 **Evans, J. H., Spencer, D. M., Zweifach, A. and Leslie, C. C.**, Intracellular calcium signals regulating cytosolic phospholipase A2 translocation to internal membranes. *J Biol Chem*. 2001. **276**: 30150–30160.

28 **Aharony, D. and Stein, R. L.**, Kinetic mechanism of guinea pig neutrophil 5-lipoxygenase. *J Biol Chem*. 1986. **261**: 11512–11519.

29 **Yun, B., Lee, H., Jayaraja, S., Suram, S., Murphy, R. C. and Leslie, C. C.**, Prostaglandins from cytosolic phospholipase A2alpha/cyclooxygenase-1 pathway and mitogen-activated protein kinases regulate gene expression in candida albicans-infected macrophages. *J Biol Chem*. 2016. **291**: 7070–7086.

30 **Tsatsanis, C., Androulidaki, A., Venihaki, M. and Margioris, A. N.**, Signalling networks regulating cyclooxygenase-2. *Int J Biochem Cell Biol*. 2006. **38**: 1654–1661.

31 **Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A. and Davis, R. J.**, Cpla2 is phosphorylated and activated by map kinase. *Cell*. 1993. **72**: 269–278.

32 **Zheng, X., Wang, Y. and Wang, Y.**, Hgc1, a novel hypha-specific G1 cyclin-related protein regulates *Candida albicans* hyphal morphogenesis. *EMBO J*. 2004. **23**: 1845–1856.

33 **Lionakis, M. S., Lim, J. K., Lee, C. C. and Murphy, P. M.**, Organ-specific innate immune responses in a mouse model of invasive candidiasis. *J Innate Immun*. 2011. **3**: 180–199.

34 **Staab, J. F., Datta, K. and Rhee, P.**, Niche-specific requirement for hyphal wall protein 1 in virulence of *Candida albicans*. *PLoS One*. 2013. **8**: e80842.

35 **Bailey, D. A., Feldmann, P. J., Bovey, M., Gow, N. A. and Brown, A. J.**, The *Candida albicans* HYR1 gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. *J Bacteriol*. 1996. **178**: 5353–5360.

36 **Liu, Y. and Filler, S. G.**, Candida albicans Als3, a multifunctional adhesin and invasin. *Eukaryot Cell*. 2011. **10**: 168–173.

37 **Moyes, D. L., Runglall, M., Murciano, C., Shen, C., Nayar, D., Thavaraj, S., Kohli, A. et al.**, A biphasic innate immune MAPK response discriminates between the yeast and hyphal forms of *Candida albicans* in epithelial cells. *Cell Host Microbe*. 2010. **8**: 225–235.

38 **Maxson, M. E., Naj, X., O'Meara, T. R., Plumb, J. D., Cowen, L. E. and Grinstein, S.**, Integrin-based diffusion barrier separates membrane domains enabling the formation of microbiostatic frustrated phagosomes. *Elife*. 2018. **7**: e34798.

39 **Erwig, L. P. and Gow, N. A.**, Interactions of fungal pathogens with phagocytes. *Nat Rev Microbiol*. 2016. **14**: 163–176.

40 **Nobile, C. J., Nett, J. E., Andes, D. R. and Mitchell, A. P.**, Function of *Candida albicans* adhesin Hwp1 in biofilm formation. *Eukaryot Cell*. 2006. **5**: 1604–1610.

41 **Sundstrom, P., Balish, E. and Allen, C. M.**, Essential role of the *Candida albicans* transglutaminase substrate, hyphal wall protein 1, in lethal oroesophageal candidiasis in immunodeficient mice. *J Infect Dis*. 2002. **185**: 521–530.

42 **Romp, E., Arakandy, V., Fischer, J., Wolz, C., Siegmund, A., Loffler, B., Tuchscherr, L. et al.**, Exotoxins from Staphylococcus aureus activate 5-lipoxygenase and induce leukotriene biosynthesis. *Cell Mol Life Sci*. 2020. **77**: 3841–3858.

43 **Phan, Q. T., Solis, N. V., Cravener, M. V., Swidergall, M., Lin, J., Huang, M. Y., Liu, H. et al.**, Candida albicans stimulates formation of a multi-receptor complex that mediates epithelial cell invasion during oropharyngeal infection. *PLoS Pathog*. 2023. **19**: e1011579.

44 **Naglik, J. R., Konig, A., Hube, B. and Gaffen, S. L.**, Candida albicans-epithelial interactions and induction of mucosal innate immunity. *Curr Opin Microbiol*. 2017. **40**: 104–112.

45 **Austermeier, S., Kasper, L., Westman, J. and Gresnigt, M. S.**, I want to break free - macrophage strategies to recognize and kill *Candida albicans*, and fungal counter-strategies to escape. *Curr Opin Microbiol*. 2020. **58**: 15–23.

46 **Kanayama, M., Inoue, M., Danzaki, K., Hammer, G., He, Y. W. and Shinohara, M. L.**, Autophagy enhances NFkappaB activity in specific tissue macrophages by sequestering A20 to boost antifungal immunity. *Nat Commun*. 2015. **6**: 5779.

47 **Qian, Q., Jutila, M. A., Van Rooijen, N. and Cutler, J. E.**, Elimination of mouse splenic macrophages correlates with increased susceptibility to experimental disseminated candidiasis. *J Immunol*. 1994. **152**: 5000–5008.

48 **Sun, D., Sun, P., Li, H., Zhang, M., Liu, G., Strickland, A. B., Chen, Y. et al.**, Fungal dissemination is limited by liver macrophage filtration of the blood. *Nat Commun*. 2019. **10**: 4566.

49 **Jayaraja, S., Dakhama, A., Yun, B., Ghosh, M., Lee, H., Redente, E. F., Uhlson, C. L. et al.**, Cytosolic phospholipase A2 contributes to innate immune defense against *Candida albicans* lung infection. *BMC Immunol*. 2016. **17**: 27.

50 **Narumiya, S., Sugimoto, Y. and Ushikubi, F.**, Prostanoid receptors: structures, properties, and functions. *Physiol Rev*. 1999. **79**: 1193–1226.

51 **Sasaki, F. and Yokomizo, T.**, The leukotriene receptors as therapeutic targets of inflammatory diseases. *Int Immunol*. 2019. **31**: 607–615.

52 **Sumimoto, H., Takeshige, K. and Minakami, S.**, Superoxide production of human polymorphonuclear leukocytes stimulated by leukotriene B4. *Biochim Biophys Acta*. 1984. **803**: 271–277.

53 **Ford-Hutchinson, A. W., Bray, M. A., Doig, M. V., Shipley, M. E. and Smith, M. J.**, Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature*. 1980. **286**: 264–265.

54 **Kalinski, P.**, Regulation of immune responses by prostaglandin E2. *J Immunol*. 2012. **188**: 21–28.

55 **Nakayama, T., Mutsuga, N., Yao, L. and Tosato, G.**, Prostaglandin E2 promotes degranulation-independent release of MCP-1 from mast cells. *J Leukoc Biol*. 2006. **79**: 95–104.

56 **Wang, X. S. and Lau, H. Y.**, Prostaglandin E potentiates the immunologically stimulated histamine release from human peripheral blood-derived mast cells through EP1/EP3 receptors. *Allergy*. 2006. **61**: 503–506.

57 **Strassmann, G., Patil-Koota, V., Finkelman, F., Fong, M. and Kambayashi, T.**, Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2. *J Exp Med*. 1994. **180**: 2365–2370.

58 **Noverr, M. C. and Huffnagle, G. B.**, Regulation of Candida albicans morphogenesis by fatty acid metabolites. *Infect Immun*. 2004. **72**: 6206–6210.

59 **Abdelmegeed, E. and Shaaban, M. I.**, Cyclooxygenase inhibitors reduce biofilm formation and yeast-hypha conversion of fluconazole resistant *Candida albicans*. *J Microbiol*. 2013. **51**: 598–604.

60 **Kalo-Klein, A. and Witkin, S. S.**, Prostaglandin E2 enhances and gamma interferon inhibits germ tube formation in *Candida albicans*. *Infect Immun*. 1990. **58**: 260–262.

61 **Peters, B. M. and Noverr, M. C.**, *Candida albicans-Staphylococcus aureus* polymicrobial peritonitis modulates host innate immunity. *Infect Immun*. 2013. **81**: 2178–2189.

62 **Willment, J. A., Lin, H. H., Reid, D. M., Taylor, P. R., Williams, D. L., Wong, S. Y., Gordon, S. et al.**, Dectin-1 expression and function are enhanced on alternatively activated and GM-CSF-treated macrophages and are negatively regulated by IL-10, dexamethasone, and lipopolysaccharide. *J Immunol*. 2003. **171**: 4569–4573.

63 **Brown, G. D., Herre, J., Williams, D. L., Willment, J. A., Marshall, A. S. and Gordon, S.**, Dectin-1 mediates the biological effects of beta-glucans. *J Exp Med*. 2003. **197**: 1119–1124.

64 **Hebecker, B., Vlaic, S., Conrad, T., Bauer, M., Brunke, S., Kapitan, M., Linde, J. et al.**, Dual-species transcriptional profiling during systemic candidiasis reveals organ-specific host-pathogen interactions. *Sci Rep*. 2016. **6**: 36055.

Abbreviations: **5-LOX**: 5-lipoxygenase · **AA**: arachidonic acid · **BSA**: bovine serum albumin · **COXs**: cyclooxygenases · **cPLA$_{2\alpha}$**: cytosolic phospholipase A$_{2\alpha}$ · **Ece1**: cell elongation protein 1 · **EDTA**: ethylenediaminetetraacetic acid · **FCS**: fetal calf serum · **FLAP**: 5-LOX-activating protein · **GM-CSF**: human granulocyte-macrophage colony-stimulating factor · **HEPES**: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid · **LDH**: lactate dehydrogenase · **LM**: lipid mediator · **LP**: leupeptin · **LPS**: lipopolysaccharides · **LTs**: leukotrienes · **M-CSF**: human macrophage CSF · **PGs**: prostaglandins · **RT**: retention time · **STI**: soy trypsin inhibitor

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