# Elucidating the function of Ece1 peptides during *Candida albicans*-macrophage interactions

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# List of Abbreviations

| Abbreviation | Explanation |
|---|---|
| % | percent |
| × g | times gravity acceleration |
| µL | microlitre |
| Abs | optical densitiy |
| *ACT1* | gene encoding actin 1 |
| AIDS | acquired immune-deficiency syndrome |
| *ALS* | agglutinin-like sequence |
| Amp | ampicillin |
| AMP | antimicrobial peptide |
| AP-1 | activator protein 1 |
| APC | antigen-presenting cell |
| arg | arginine |
| *ARG4* | gene encoding the argininosuccinate lyase |
| ASC | apoptosis-associated speck-like protein containing a CARD |
| ATP | adenosine triphosphate |
| AUC | area under the curve |
| bp | base pair(s) |
| C- | carboxy- |
| CaL | candidalysin |
| cAMP | cyclic adenosine monophosphate |
| *CAMP* | gene encoding the LL37 precursor |
| CARD | caspase recruitment domain-containing protein |
| CCL | C-C motif ligand |
| cDNA | complementary DNA |
| cf. | *confer*, from latin, "compare" |
| CLR | C-type lectin receptor |
| $CO_2$ | carbon dioxide |
| CR | complement receptor |
| cRNA | complementary RNA |
| CXCL | C-X-C motif ligand |
| *CYBA* | gene encoding the cytochrome b-245, alpha polypeptide |
| *CYBB* | gene encoding the cytochrome b-245, beta polypeptide |
| DAMP | danger-associated molecular pattern |
| DC | dendritic cell |
| DC-SIGN | dendritic cell-specific ICAM-3-grabbing nonintegrin |
| *DCK1* | gene encoding a guanine nucleotide exchange factor |
| *DEFB* | gene encoding a β-defensin |
| dept. | department |
| DIG | digoxigenin |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide phosphate |
| *DUOX* | gene encoding the dual oxidase |
| *ECE1* | gene encoding the *Extent of Cell Elongation 1* |
| *EFB1* | gene encoding the *Elongation Factor-1 beta* |
| EDTA | ethylenediaminetetraacetic acid |
| EGFR | epidermal growth factor receptor |
| ELISA | enzyme-linked immunosorbent assay |
| ER | endoplasmic reticulum |
| ERK | extracellular signal-regulated kinase |

| | |
|---|---|
| EtBr | ethidium bromide |
| FBS | fetal bovine serum |
| FC | fold change |
| FcγR | Fcγ receptor |
| gDNA | genomic DNA |
| GPI | glycosylphosphatidylinositol |
| *GPX3* | gene encoding the glutathione peroxidase 3 (plasma) |
| Gro | growth-regulated oncogene |
| h | hour |
| *HAC1* | gene encoding the transcriptional activator *HAC1* |
| HER2 | human epidermal growth factor receptor 2 |
| *HGT2* | gene encoding the high-affinity glucose transporter gene 2 |
| his | histidine |
| *HIS1* | gene encoding the ATP phosphoribosyl transferase |
| hMDM | human monocyte-derived macrophage |
| *HWP* | hyphal wall protein |
| *IFF/HYR* | IPF family F/hyphally up-regulated protein |
| IFN | interferon |
| *IHD1* | gene encoding the induced during hyphae development protein 1 |
| IL | interleukin |
| iNOS | inducible nitric oxide synthase |
| JNK | c-Jun N-terminal kinase |
| *KAR2* | gene encoding the endoplasmic reticulum chaperone BiP |
| Kex | kexin protease |
| LB | lysogeny broth |
| LC-MS/MS | liquid chromatography tandem mass spectrometry |
| LDH | lactate dehydrogenase |
| LiAc | lithium acetate |
| $log_2$ | binary logarithm |
| LPS | lipopolysaccharide |
| LysM | lysin motif |
| MAMP | microbe-associated molecular pattern |
| MAPK | mitogen-activated protein kinase |
| MBL | mannose-binding lectin |
| mBMDM | murine bone marrow-derived macrophage |
| MCP | monocyte chemoattractant protein |
| M-CSF | macrophage colony-stimulating factor |
| $MgCl_2$ | magnesium chloride |
| MHC | major histocompatibility complex |
| min | minute |
| MIP | macrophage-inducible protein |
| mL | millilitre |
| MMLV | Moloney Murine Leukemia Virus |
| MOI | multiplicity of infection |
| MPM | Microbial Pathogenicity Mechanisms |
| MR | mannose receptor |
| mRNA | messenger RNA |
| N- | amino- |
| NADPH | nicotinamide adenine dinucleotide phosphate, reduced form |
| NCEP | Non-Candidalysin Ece1 Peptide |
| *NCF* | gene encoding a neutrophil cytosolic factor |
| NET | neutrophil extracellular trap |
| NF-kB | nuclear factor “kappa-light-chain-enhancer” of activated B-cells |
| NLR | nucleotide-binding oligomerisation domain-like receptor |
| NLRC4 | NLR family CARD domain-containing protein 4 |

| | |
|---|---|
| NLRP3 | NLR family, pyrin domain containing 3 |
| *NOS* | gene encoding a nitric oxide synthase |
| *NOX* | gene encoding an NADPH oxidase |
| o/n | over night |
| PAMP | pathogen-associated molecular pattern |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| pH | potential of hydrogen |
| PRR | pattern recognition receptor |
| PSM | peptide spectrum match |
| r | recombinant |
| *RBT1* | gene encoding the repressed by *TUP1* protein 1 |
| RIN | RNA integrity number |
| RNA | ribonucleic acid |
| RNAse A | ribonuclease A |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| rpm | rounds per minute |
| RPMI | Roswell Park Memorial Institute |
| rSAP | recombinant shrimp alkaline phosphatase |
| s | second |
| Sap | secreted aspartyl protease |
| SD | standard deviation |
| SD medium | synthetic defined medium |
| Sod | superoxide dismutase |
| SP | signal peptide |
| SPE | solid-phase extraction |
| TAE | tris-acetate-ethylenediaminetetraacetic acid |
| *Taq* | *Thermus aquaticus* |
| TBE | tris-borat-ethylenediaminetetraacetic acid |
| TE | tris-ethylenediaminetetraacetic acid |
| TF | transcription factor |
| Th1 | type 1 T helper cell |
| Th17 | type 17 T helper cell |
| Th2 | type 2 T helper cell |
| TLR | toll-like receptor |
| Treg | regulatory T-cell |
| *TXNRD1* | gene encoding the thioredoxin reductase 1 |
| UPR | unfolded protein response |
| *URA3* | gene encoding the orotidine-5'-phosphate decarboxylase |
| ura | uridine |
| UV | ultra-violet |
| v/v | volume per volume |
| v-ATPase | vacuolar ATPase |
| *vs.* | *versus* |
| w/v | weight per volume |
| Wt | wild type |
| *XDH* | gene encoding the xanthine dehydrogenase |
| YPD | yeast peptone dextrose |

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## Summary

The opportunistic, polymorphic fungus *Candida albicans* is a frequent colonizer of human mucosal surfaces on which it usually resides as a harmless commensal. However, under certain circumstances, *C. albicans* is able to overgrow the microbiota, damage host barriers and cause disease. One main virulence trait of the fungus is its ability to transition between a yeast and a hyphal morphology. The hypha-associated gene *ECE1* is highly expressed during filamentation and invasion of epithelial cells. The gene encodes a polypeptide consisting of eight individual peptides separated by Kex2 protease cleavage sites. Of these, the third peptide is a cytolytic toxin, also referred to as candidalysin.

In epithelial cells, which represent the first line of mechanical defence against invading microorganisms, the toxin induces membrane damage and activates a danger response pathway, contributing to the recruitment of innate immune cells like macrophages. Macrophages phagocytose and usually kill *C. albicans* cells upon contact. However, a proportion of *C. albicans* cells can survive and produce hyphae within these immune cells. Prolonged filamentation of the fungus leads to immune cell rupture, providing an escape route for *C. albicans* from this hostile environment. The fungus can further hijack pyroptosis, a pro-inflammatory, NLRP3 inflammasome-dependent host cell death pathway as an escape route. The NLRP3 inflammasome is essential for the anti-*Candida* defence in mice and its activation results in secretion of the pro-inflammatory cytokines IL-1β and IL-18 as well as in pyroptotic host cell lysis. Hypha formation is a necessary but not sufficient trigger to induce NLRP3 inflammasome activation, underlining the importance of hypha-associated factors during this process. This thesis demonstrates that the hypha-associated toxin candidalysin provides the activating stimulus for the NLRP3 inflammasome, as the toxin alone was unable to induce inflammasome activation without a prior priming step and toxin-dependent IL-1β secretion was abrogated in murine phagocytes lacking distinct NLRP3 inflammasome components. This activation is facilitated by toxin-induced potassium efflux from the macrophage, as no IL-1β release was detectable upon treatment of macrophages with high extracellular potassium or a potassium channel inhibitor. However, candidalysin does not trigger pyroptotic host cell death in macrophages, as it still induced damage in NLRP3

inflammasome knock-out phagocytes. In contrast, the lytic properties of the toxin rather induce necrosis, likely by triggering direct host cell lysis.

Besides candidalysin, the *ECE1* gene encodes seven further Non-Candidalysin Ece1 Peptides (NCEPs, Ece1-I – Ece1-VIII). The secretion of nearly all predicted peptides (or fragments thereof) alongside with the toxin by *C. albicans* hyphae indicates yet unknown, putative effector-like functions of these NCEPs. This thesis demonstrates that mutants lacking single or combined NCEP-encoding sequences often exhibit defects in hypha formation and diminished *ECE1* gene expression. Furthermore, many of these mutant strains experienced ER stress and showed an induction of the unfolded protein response when grown under hypha-inducing conditions, presumably due to improper folding and processing of the modified Ece1 proprotein. This might in turn negatively affect hyphal morphogenesis and thus, in a circuit, the expression of the hypha-associated gene *ECE1* and Ece1 peptide secretion. LC-MS/MS analyses of hyphal supernatants revealed that almost all NCEP-knock-out mutants exhibited an altered Ece1 peptide secretion pattern. Importantly, mutants lacking the Ece1-II, -IV, or -VII-encoding sequence exhibited a reduced secretion of candidalysin into the hyphal supernatant, indicating that the respective peptides might be involved in processes required for candidalysin secretion. To gain first insights regarding putative effector-like functions, macrophages were challenged with synthetic versions of the most abundant NCEP fragments Ece1-Va, -VIa, and -VIIa alone and in combination with candidalysin. First results indicate distinct roles of mainly Ece1-Va and Ece1-VIIa during the infection process. The tested fragments alone did not damage macrophages. However, Ece1-VIIa, in combination with Ece1-VIIb, reduced the candidalysin-induced macrophage damage. Transcriptional profiling and immune mediator secretion assays revealed that candidalysin treatment alone results in substantial changes in the macrophage transcriptional response and the secretion of immune mediators. This was mostly unchanged by an additional co-incubation with Ece1-Va, indicating that the main macrophage response is induced by the peptide toxin itself. In addition, macrophage co-incubation with Ece1-Va alone resulted in the up-regulation of genes involved in metal stress without triggering strong inflammatory responses, whereas Ece1-VIIa alone seemed to the modulate the macrophage oxidative stress responses and triggered the release of mainly chemotactic immune mediators.

Taken together, this thesis shows a dual function of candidalysin in macrophages. On the one hand, it acts as a classical virulence factor by facilitating immune evasion *via* membrane destruction, whereas on the other hand, the toxin induces host-protective, pro-inflammatory signalling, thus acting as an avirulence factor causing clearance of *C. albicans*.

First data generated in this thesis also indicate that NCEPs are important for Ece1 folding, processing and/or secretion of the processed Ece1 peptides including candidalysin, as genetic modifications of the *ECE1* gene often resulted in ER stress, UPR induction, deficiencies in hyphal morphogenesis, and diminished *ECE1* expression. Furthermore, some NCEPs also possess effector-like functions by modulating the macrophage transcriptional response and immune mediator secretion. These results provide the first data about potential biological functions of NCEPs and their role during *C. albicans*-host cell interactions.

## Zusammenfassung

Der opportunistische, polymorphe Pilz *Candida albicans* ist ein häufiger Besiedler menschlicher Schleimhautoberflächen, auf denen er normalerweise als harmloser Kommensale vorkommt. Unter bestimmten Umständen ist *C. albicans* jedoch in der Lage die Mikrobiota zu überwachsen, Wirtsbarrieren zu schädigen und Infektionen zu verursachen. Ein wesentliches Virulenzmerkmal des Pilzes ist seine Fähigkeit zwischen einer Hefe- und einer Hypheform zu wechseln. Das hyphen-assoziierte Gen *ECE1* wird während der Filamentierung und Invasion von Epithelzellen stark exprimiert. Es kodiert für ein Polypeptid das aus acht einzelnen Peptiden besteht, die durch Kex2-Protease Spaltstellen getrennt sind. Das dritte Peptid ist ein zytolytisches Toxin und wird auch als Candidalysin bezeichnet.

In Epithelzellen, die die erste mechanische Verteidigungslinie gegen eindringende Mikroorganismen darstellen, induziert das Toxin eine Membranschädigung und aktiviert einen Gefahrenabwehrweg, der zur Rekrutierung von Immunzellen wie Makrophagen beiträgt. Normalerweise phagozytieren und töten Makrophagen *C. albicans* Zellen bei Kontakt ab. Ein Teil der *C. albicans* Zellen kann jedoch in diesen Immunzellen überleben und Hyphen bilden. Die Filamentierung des Pilzes führt zur Zerstörung der Immunzellen und stellt einen Fluchtweg für *C. albicans* aus dieser feindlichen Umgebung dar. Weiterhin kann der Pilz den pro-inflammatorischen, NLRP3-Inflammasom-abhängigen Wirtszelltod Pyroptose als Ausweg nutzen. Das NLRP3-Inflammasom ist essentiell für die anti-*Candida* Immunantwort in Mäusen und die Aktivierung resultiert in der Sekretion der pro-inflammatorischen Zytokine IL-1β und IL-18 sowie der pyroptotischen Wirtszelllyse. Die Hyphenbildung ist ein notwendiger, aber nicht ausreichender Auslöser für die NLRP3-Inflammasom-Aktivierung, was die Bedeutung hyphen-assoziierter Faktoren während dieses Prozesses unterstreicht. Diese Arbeit zeigt, dass das hyphen-assoziierte Toxin Candidalysin den aktivierenden Stimulus für das NLRP3-Inflammasom liefert, da das Toxin allein nicht dazu in der Lage war die Inflammasom-Aktivierung ohne einen vorherigen Priming-Schritt zu induzieren. Weiterhin trat die toxin-abhängige IL-1β-Sekretion in murinen Phagozyten, denen bestimmte NLRP3-Inflammasomkomponenten fehlen, nicht auf. Die Inflammasom-Aktivierung wird durch einen toxin-induzierten Kaliumefflux aus den Makrophagen

vermittelt, da keine IL-1β-Freisetzung nach Behandlung der Makrophagen mit hohen Konzentrationen an extrazellulärem Kalium oder einem Kaliumkanal-Inhibitor nachweisbar war. Allerdings induziert Candidalysin in Makrophagen keinen pyroptotischen Wirtszelltod, da es in NLRP3-Inflammasom-defizienten Phagozyten immer noch Membranschäden hervorrief. Im Gegensatz dazu induzieren die lytischen Eigenschaften des Toxins eher einen nekrotischen Zelltod, der wahrscheinlich durch einer direkte Wirtszelllyse vermittelt wird.

Neben Candidalysin kodiert das *ECE1* Gen für sieben weitere, Non-Candidalysin Ece1 Peptide (NCEPs, Ece1-I - Ece1-VIII). Die Sekretion fast aller vorhergesagten NCEPs (oder deren Fragmente) zusammen mit dem Toxin durch *C. albicans* Hyphen weist auf noch unbekannte, putative effektor-ähnliche Funktionen dieser Peptide hin. Diese Arbeit zeigt, dass Mutanten, denen einzelne oder mehrere NCEP-kodierende Sequenzen fehlen, häufig Defekte in der Hyphenbildung und eine verminderte *ECE1* Genexpression aufweisen. Darüber hinaus zeigten viele dieser Stämme Anzeichen von ER-Stress sowie eine Induktion der ungefalteten Protein-Antwort, wenn sie unter hyphen-induzierenden Bedingungen wuchsen. Vermutlich wird dies durch eine fehlerhafte Faltung und Prozessierung des modifizierten Ece1-Proteins verursacht. Dies könnte sich wiederum negativ auf die Hyphenmorphogenese und damit in einem Kreislauf auf die Expression des hyphen-assoziierten Gens *ECE1* und die Ece1 Peptidsekretion auswirken. LC MS/MS Analysen von Hyphenüberständen zeigten, dass fast alle NCEP-Knock-out-Mutanten ein verändertes Ece1-Peptidsekretionsmuster aufwiesen. Mutanten, denen die für Ece1-II, -IV oder -VII kodierende Sequenz fehlte, zeigten eine reduzierte Sekretion von Candidalysin in den Hyphenüberstand. Dies deutet darauf hin, dass die entsprechenden Peptide an Prozessen beteiligt sein könnten, die für die Candidalysin-Sekretion erforderlich sind. Um erste Erkenntnisse über mutmaßliche, effektor-ähnliche Funktionen zu gewinnen, wurden Makrophagen mit synthetischen Versionen der am häufigsten vorkommenden NCEP-Fragmente Ece1-Va, -VIa und -VIIa allein und in Kombination mit Candidalysin inkubiert. Erste Ergebnisse weisen auf unterschiedliche Rollen von hauptsächlich Ece1-Va und Ece1-VIIa während des Infektionsprozesses hin. Die getesteten Fragmente allein schädigten die Makrophagenmembran nicht. Ece1-VIIa reduzierte jedoch in Kombination mit Ece1-VIIb die Candidalysin-induzierte Makrophagenschädigung. Transkriptionsprofile und Immunmediator-Sekretionsassays

zeigten, dass eine Behandlung mit Candidalysin allein zu erheblichen Veränderungen in der transkriptionellen Antwort der Makrophagen und der Sekretion von Immunmediatoren führt. Dies blieb durch eine zusätzliche Koinkubation mit Ece1-Va weitgehend unverändert, was darauf hindeutet, dass die Hauptantwort der Makrophagen durch das Peptidtoxin selbst induziert wird. Darüber hinaus führte die Koinkubation von Makrophagen mit Ece1-Va allein zu einer Hochregulierung von Genen die am Metallstress beteiligt sind, ohne eine starke pro-inflammatorische Reaktion auszulösen. Eine Koinkubation mit Ece1-VIIa schien die oxidative Stressantwort in Makrophagen zu modulieren und induzierte die Freisetzung von vor allem chemotaktisch wirksamen Immunmediatoren.

Zusammenfassend zeigt diese Arbeit eine Doppelfunktion von Candidalysin in Makrophagen. Einerseits agiert es als klassischer Virulenzfaktor, indem es die Immunevasion durch Wirtsmembranschädigung erleichtert, andererseits induziert das Toxin eine pro-inflammatorische Immunantwort, die protektiv für den Wirt ist und stellt somit einen Avirulenzfaktor dar, der die Beseitigung von *C. albicans* bewirkt.

Erste Daten, die in dieser Arbeit generiert wurden, deuten auch darauf hin, dass NCEPs wichtig für die Ece1-Faltung, -Prozessierung und/oder Sekretion der prozessierten Ece1-Peptide einschließlich Candidalysin sind, da genetische Modifikationen des *ECE1* Gens häufig zu ER-Stress, UPR-Induktion, Defiziten in der Hyphenmorphogenese und verminderter *ECE1* Expression führten. Darüber hinaus besitzen einige NCEPs auch effektor-ähnliche Funktionen, indem sie die Transkriptionsantwort von Makrophagen und die Sekretion von Immunmediatoren modulieren. Diese Ergebnisse liefern die ersten Daten über potenzielle biologische Funktionen von NCEPs und ihre Rolle während der Interaktion von *C. albicans* und Wirtszellen.

# 1 Introduction

## 1.1 The human pathogenic fungus *Candida albicans*

From all described 3 to 5 million fungi inhabiting the world, only a minority of a few hundred species has been associated with human infections (O'Brien *et al.* 2005, Blackwell 2011, Köhler *et al.* 2017). Still this low number of species infects more than 1 billion people annually, which makes fungal infections clinically highly relevant (Brown *et al.* 2012, Köhler *et al.* 2017).

The polymorphic, diploid fungus *Candida albicans* usually resides as a harmless commensal and part of the normal human microbial flora on mucosal surfaces such as the oral cavity, the gastrointestinal and the genitourinary tract. Healthy adults are commonly colonised by *C. albicans* without any symptoms, with rates up to 70 % (Soll *et al.* 1991, Kleinegger *et al.* 1996, Huffnagle and Noverr 2013). However, under predisposing conditions such as systemic or local immunosuppression, or prolonged antibiotic treatment, the fungus is able to overgrow and cause disease (Ellepola and Samaranayake 2001, Xu *et al.* 2008, Delaloye and Calandra 2014).

Fungal overgrowth can lead to superficial infections that are very common but non-life-threatening (Hay 2018). For example, 75 % of all women are prone to a vulvovaginal infection at least once in their lifetime, which is frequently associated with preceding antibiotic treatment (Sobel *et al.* 1998, Shukla and Sobel 2019). Furthermore, the vast majority of patients suffering from the acquired immunodeficiency syndrome (AIDS) will experience oropharyngeal candidiasis at some stage of their disease progression (de Repentigny *et al.* 2004).

Invasive *C. albicans* infections (invasive candidiasis) are less frequent and only occur when the fungus breaches the host barrier and is able to gain access to and disseminate throughout the bloodstream. In these cases, typically sterile organs like kidney, liver, heart, spleen, or brain are colonised by *C. albican*s (Fernandez *et al.* 2000, Kullberg and Arendrup 2015). These systemic infections are usually acquired nosocomially (in the hospital) and associated with a prolonged intensive care unit residence, cardiac and gastrointestinal surgeries, sustained broad-spectrum antibiotic treatment, central venous catheters, and immunosuppression (Das

*et al.* 2011, Wisplinghoff *et al.* 2014). *Candida* bloodstream infections (candidemia) and especially *Candida*-induced sepsis are accompanied by a crude mortality rate of up to 75 % depending on the given circumstances of infection (Brown *et al.* 2012, Duggan *et al.* 2015, Kaur and Chakrabarti 2017, Pappas *et al.* 2018).

Amongst all diagnosed nosocomial bloodstream infections, *Candida* species rank the fourth most common cause in the USA, with *C. albicans* being the most prevalent, followed by *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. lusitaniae* and *C. guilliermondii*. The respective incidence of infection with non-*albicans Candida* species is depending on the geographic region, age, and underlying antifungal drug treatment (Wisplinghoff *et al.* 2004, Arendrup 2010, Lamoth *et al.* 2018, Pfaller *et al.* 2019). In the recent years, the species *C. auris* has emerged and is responsible for numerous outbreaks in hospitals worldwide causing candidemia with crude mortality rates up to 66 % (Spivak and Hanson 2018, de Jong and Hagen 2019).

## 1.2 Virulence factors of *Candida albicans*

During infection under predisposing conditions in a susceptible host, *C. albicans* expresses several virulence factors. These virulence factors contribute to adhesion to, invasion into, and damage of host tissue as well as to avoidance, evasion, or counteraction of the host's defensive system. The broad repertoire of *C. albicans*' virulence determinants includes, amongst others, the morphological ability to transition from yeast to hypha, phenotypic switching, the expression of adhesins and invasins, as well as the secretion of hydrolytic enzymes and the cytolytic toxin candidalysin (Mayer *et al.* 2013, Moyes *et al.* 2016). Intriguingly, a single factor is not sufficient for an infection; rather a combination of several factors for specific infection stages is required to drive pathogenicity. Furthermore, not all factors are required simultaneously (Mayer *et al.* 2013).

### 1.2.1 Morphology

*Candida albicans* is a polymorphic fungus which is able to grow in a yeast, a pseudohyphal, and a true hyphal form depending on the growth conditions (Sudbery *et al.* 2004). Apart from these structures, it can also form chlamydospores, which rather resemble a resting, non-

proliferating stage. However, whether chlamydospores show a prolonged viability and a distinct function during infection is still unclear (Staib and Morschhauser 2007, Bottcher *et al.* 2016). Furthermore, *C. albicans* is able to undergo phenotypic switching between white, grey, and opaque cells with distinct phenotypes as well as different mating and virulence potentials depending on the respective niche infected (Pomes *et al.* 1985, Slutsky *et al.* 1985, Kvaal *et al.* 1997, Kvaal *et al.* 1999, Lachke *et al.* 2003, Tao *et al.* 2014).

The yeast-to-hypha transition is a major virulence trait of *C. albicans*. The ovoid yeast form is predominantly found at pH<6 and high cell densities (>$10^7$cell/mL) (Odds 1988, Villa *et al.* 2020). In contrast, an elevated temperature of 37 °C, the presence of serum, 5 % $CO_2$, a pH>7, lower cell densities (<$10^7$cell/mL), surface contact and the availability of certain amino acids favours filamentation of the fungus, all of the latter resembling conditions *C. albicans* faces during infection of the human body (Odds 1988, Sudbery *et al.* 2004, Brand *et al.* 2007, Sudbery 2011, Garbe and Vylkova 2019, Villa *et al.* 2020). The induction of hyphal growth is controlled *via* a complex regulatory network that involves numerous transcription factors (TFs) like, amongst others, Efg1, Cph1, Cph2, and Rim101, that act downstream of different signalling pathway pathways like the cyclic AMP (cAMP) or the mitogen-activated protein kinase (MAPK) pathway (Kornitzer 2019, Villa *et al.* 2020). Interestingly, during the complex process of hypha induction, *C. albicans* expresses a minimal core set of eight co-regulated genes, namely *ALS3, DCK1*, *ECE1*, *HGT2*, *HWP1*, *IHD1*, *RBT1*, and orf19.2457, that are consistently up-regulated upon different hypha-inducing conditions at different time points (Martin *et al.* 2013). Furthermore, filamentation results in the expression of hypha-dependent virulence factors like adhesins, invasins, secreted hydrolases, superoxide dismutase (Sod)5, Ece1/candidalysin, and others (Martchenko *et al.* 2004, Mayer *et al.* 2013, Moyes *et al.* 2016), which collectively contribute to the infection process and will be discussed in more detail in the following paragraphs.

Both morphological forms appear during systemic infections. However, the hyphal form of *C. albicans* is more often implicated with invasion of the fungus into the tissue and damage, whereas the yeast form seems to be essential for dissemination throughout the bloodstream (Wilson and Hube 2010, Zhu and Filler 2010, Martin *et al.* 2011, Jacobsen *et al.* 2012).

Therefore, both morphologies are important during the infection process and consequently, mutants locked in either morphological form are attenuated in virulence (Lo *et al.* 1997, Braun *et al.* 2000, Braun *et al.* 2001, Murad *et al.* 2001, Gow *et al.* 2002, Saville *et al.* 2003).

### 1.2.2 Adhesion, invasion, and damage

As a first step during the transition of *C. albicans* from the commensal to the pathogenic state, the fungus needs to overcome host barriers. Therefore, it initially needs to adhere to epithelial cells, which are representing the first line of mechanical defence against invading microorganisms (Richardson *et al.* 2018a). Contact with a biotic surface induces filamentation of the fungus and the formed hyphae grow directionally due to $Ca^{2+}$-guided thigmotropism (Watts *et al.* 1998, Brand *et al.* 2007). To ensure adhesion to the tissue, *C. albicans* expresses several adhesins of three main groups comprised of the agglutinin-like sequence (encoded by the *ALS* genes), the IPF family F/ hyphally up-regulated protein (encoded by the *IFF*/*HYR* genes*)*, and the hyphal wall protein (*HWP)* gene family (de Groot *et al.* 2013). Especially the hypha-specific proteins Als3 and Hwp1 are essential for fungal adherence to host cells (Staab *et al.* 1999, Zhao *et al.* 2004, Martin *et al.* 2013). However, adhesion is not restricted to the hyphal growth form, but also occurs in the yeast stage due to changes in the surface hydrophobicity and thus the change in outermost protein content (Hazen 1989, Hazen *et al.* 1990, Ener and Douglas 1992).

Following adhesion, the fungus is able to breach the barrier and invade the host cells. Invasion into the tissue is a prerequisite for subsequently occurring host cell damage and is mediated by fungal invasins and distinct invasive mechanisms (Richardson *et al.* 2018a). Up to date, three routes of *C. albicans*-epithelial cell invasion have been reported: (I) active penetration by mechanical force of the growing hypha (major route, approximately 70 % within the first 3 h of infection for e.g. epithelial cells), which is supported by proteolytic degradation of host barrier proteins, (II) invasion between epithelial cells, also referred to as paracellular invasion, and (III) the passive process of induced endocytosis (Wächtler *et al.* 2012).

Interestingly, epithelial cells discriminate between the commensal (yeast) and the pathogenic (hyphal) state of *C. albicans via* a differential, biphasic activation of MAPK-p38/c-Fos

signalling (Moyes *et al.* 2010, Naglik *et al.* 2014). A low fungal burden and mainly yeast cell occurrence does not elicit an epithelial danger response, whereas increased fungal burden and hypha formation leads to exceedance of a certain threshold of the proto-oncogene c-Fos (Naglik *et al.* 2014). Together with c-Jun, c-Fos forms the heterodimeric activator protein (AP)1, which subsequently activates epithelial cells, mediating immune activation and fungal clearance (Naglik *et al.* 2014). When fungal burdens cannot be reduced below the threshold level, damage of host cells predominates. Fungus-induced host cell damage is a consequence of hypha formation and thus mechanical forces, the secretion of hydrolytic enzymes like secreted aspartyl proteases (Saps), lipases, and phospholipases, as well as the secretion of the peptide toxin candidalysin, which will be discussed in detail below (Naglik *et al.* 2003, Schaller *et al.* 2005, Naglik *et al.* 2014, Moyes *et al.* 2016).

## 1.3 The interaction of *Candida albicans* with the immune system

*Candida albicans* not only encounters epithelial and endothelial cells upon infection, but also immune cells. The human immune system is divided into the innate and the adaptive immunity. The former represents the first line of defence during infection, whereas the latter mediates pathogen clearance at later stages of infection and is responsible for the generation of an immunological memory (Murphy and Weaver 2016).

Monocytes, macrophages, neutrophils, and dendritic cells (DCs) are cells of the innate immune response. They express a variety of pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs) on the pathogen surface. The presentation of pathogen-derived antigens on the surface of an antigen-presenting cell (APC) is necessary for the shift from the innate to the adaptive immune response (Murphy and Weaver 2016). The adaptive immunity is mediated by T- and B-cells, which derive from leukocyte progenitor cells. Upon random recognition of a presented antigen, these lymphocytes proliferate and differentiate into antigen-specific effector cells, which combat the infection by elimination of the pathogen (Murphy and Weaver 2016).

Different virulence factors and strategies allow *C. albicans* to avoid the recognition by the host's immune system, modulate the immunogenic outcome, and to mediate escape from immune cells (Seider *et al.* 2010), which will be discussed in detail in paragraph 1.3.3.

### 1.3.1 Recognition of *Candida albicans*

As a first step during interaction of the fungus with cells of the innate immunity, fungal immunogenic surface attributes are recognised by host PRRs, which leads to immune cell activation, the production of immunological effectors like cytokines, and ultimately fungal clearance (Naglik 2014).

The fungal cell wall, as the first point of contact between fungus and immune cells, is comprised of two distinct layers that differ structurally and chemically from one another. The inner, skeletal layer is composed of chitin and β-(1,3)-glucans, the latter being covalently linked to β-(1,6)-glucans. The outer layer appears to be more fibrillar and contains highly glycosylated proteins with mannose-residues (mannans), which are often attached to β-(1,3)-glucan *via* a glycosylphosphatidylinositol (GPI)-remnant bound to a β-1,6-glucan linker (Klis *et al.* 2001).

*Candida albicans*' β-glucan has been shown to be a major immunostimulatory component of the fungal cell wall recognised by the C-type lectin receptor (CLR) dectin-1 and toll-like receptor (TLR)2 on the host side (Brown and Gordon 2001, Gantner *et al.* 2003, Gow *et al.* 2017). The complement receptor (CR)3 has been implicated with the detection of less frequently exposed β-(1,6)-glucan (Rubin-Bejerano *et al.* 2007).

Besides glucans, mannans have been shown to exhibit immunostimulatory properties, providing another target for fungal recognition by the host (Hall and Gow 2013). The mannose receptor (MR), a CLR, has been reported to sense N-linked mannans of the fungal cell wall (Cambi *et al.* 2008). Furthermore, the CLRs dectin-2, dectin-3, mincle, and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), as well as galectin-3 and the soluble mannan binding lectin (MBL) have been implicated with mannan detection (Cambi *et al.* 2003, Jouault *et al.* 2006, Lillegard *et al.* 2006, McGreal *et al.* 2006, Cambi *et al.* 2008, Saijo *et al.* 2010, Zhu *et al.* 2013). Due to a short cytoplasmic tail, dectin-2, -3 and mincle need to engage the Fcγ receptor (FcγR) to induce the respective signalling pathways (Sato *et al.* 2006, Graham and Brown 2009, Zhu *et al.* 2013, Haider *et al.* 2019). Outermost phospholipomannans and O-linked mannosyl residues are bound by host TLR2/TLR6 hetero- and TLR4 homodimers (Tada *et al.* 2002, Jouault *et al.* 2003, Netea *et al.*

2006, Netea *et al.* 2008), whereas fungal deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) is detected intracellularly by TLR9 and TLR7, respectively (van de Veerdonk *et al.* 2008, Biondo *et al.* 2012). Toll-like receptor 6 has been reported to modulate the balance between Th1 and Th2 cytokines during disseminated candidiasis (Netea *et al.* 2008). An overview of the main immunostimulatory fungal moieties and the respective recognising host PRRs is given in Figure 1.

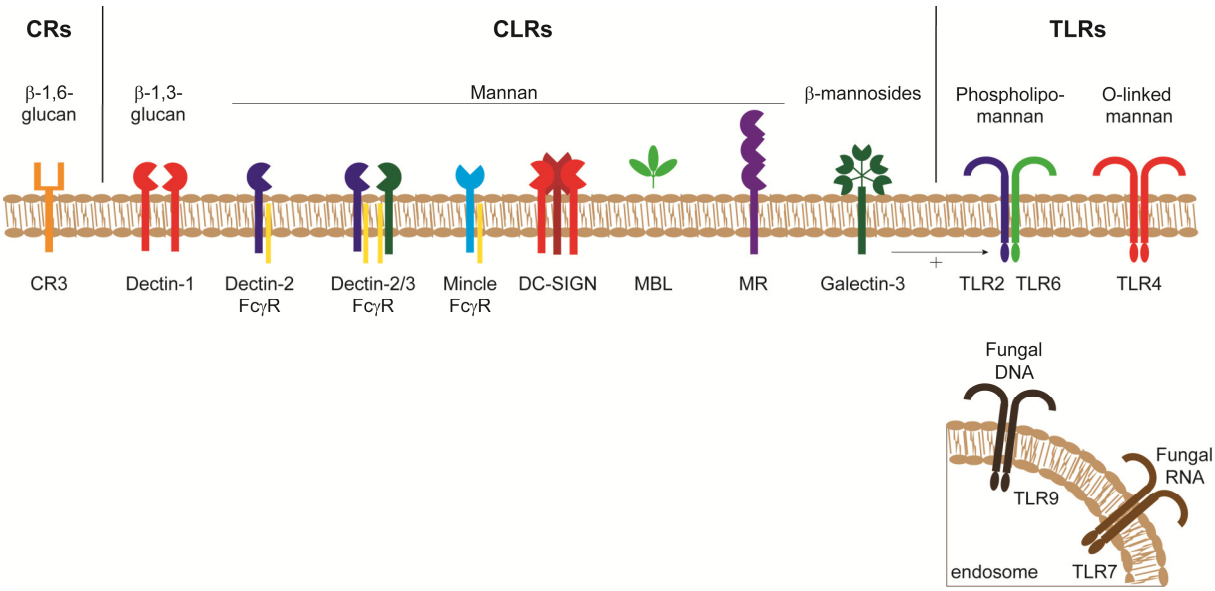


**Figure 1: Recognition of *C. albicans* by innate immune cells.**

The *C. albicans* cell wall moieties glucan and mannan are sensed by different host PRRs which are expressed on the surface of innate immune cells. Intracellularly, TLR7 and TLR9 sense fungal RNA and DNA, respectively.

The innermost chitin layer of the *C. albicans* cell wall is mainly important for providing the required rigidity and resistance to physical stress (Free 2013). However, also chitin has been implicated with immunomodulatory functions. For a long time it has been referred to as an "orphan" microbial-associated molecular pattern (MAMP) as no dedicated receptor had been found. Up to date, several studies provided evidence that chitin is clearly involved in the response to fungal pathogens depending on the size of chitin polymers, immune cell types, and the experimental settings. One study reported chitin-recognition in a dectin-1-dependent manner (Mora-Montes *et al.* 2011), whereas others have shown MR-mediated signalling and TLR2-dependency (Wagener *et al.* 2014, Fuchs *et al.* 2018). In contrast to other PAMPs, chitin potentially mediates an immunomodulatory instead of an immunostimulatory response (Mora-Montes *et al.* 2011, Gow and Hube 2012, Wagener *et al.* 2014).

### 1.3.2 Phagocytosis and killing of *Candida albicans*

Following the recognition of *C. albicans*, professional phagocytic cells readily ingest and kill the fungus. Neutrophils are the most important immune cells mediating *C. albicans* clearance during infections, and neutropenia is a major risk factor for systemic candidiasis in the human as well as in the murine system (Uzun *et al.* 2001, Pasqualotto *et al.* 2006, Koh *et al.* 2008, Drummond *et al.* 2015, Desai and Lionakis 2018, Kato *et al.* 2019, Swidergall *et al.* 2019). These immune cells phagocytose non-opsonised *C. albicans* cells upon recognition *via* TLRs and CLRs, whereas opsonised fungi are recognised *via* CR3 and the FcγR (Miramon *et al.* 2013, Naglik 2014). Once taken up, the fungus is killed by nitrosative, oxidative, and non-oxidative mechanisms. Nitrosative killing is limited to intracellular compartments due to the cytosolic or perinuclear localisation of the inducible nitric oxide synthase (iNOS) (Stuehr 1999, Kolodziejska *et al.* 2005). Oxidative stress through generation of reactive oxygen species (ROS) occurs both intra- and extracellularly and is mediated by the NADPH oxidase, which is located in the plasma and the phagosomal membrane (Segal *et al.* 2012). The phagocytic pathway in neutrophils does not involve a decrease in pH but is mediated by the fusion of the phagosome with cytoplasmic granules. These granules contain, amongst other, antimicrobial peptides (AMPs) like lactoferrin and defensins, as well as the enzymes lysozyme and elastase, all contributing to non-oxidative killing of phagocytosed microbes (Lee *et al.* 2003, Amulic *et al.* 2012). Neutrophils have additionally been shown to migrate preferentially towards germinating or filamentous forms of *C. albicans* and to inhibit filamentation of the fungus upon phagocytosis, thus counteracting a main virulence trait of the fungus (Rubin-Bejerano *et al.* 2003, Fradin *et al.* 2005, Wozniok *et al.* 2008). Furthermore, in particular long hyphal cells, which are too large for phagocytosis, can be trapped and killed extracellularly by neutrophil extracellular traps (NETs). These NETs are structures consisting of chromatin fibres, released granular proteins, and AMPs like calprotectin (Urban *et al.* 2006, Urban *et al.* 2009).

In addition to neutrophils, macrophages play a critical role in the defence against *C. albicans* infections (Austermeier *et al.* 2020). Tissue-resident macrophages located in naïve tissue contribute to tissue homeostasis and represent the first line of innate immune defence against

invading *C. albicans* (Xu and Shinohara 2017). Moreover, monocytes migrate to the site of infection upon stimulation by cytokines and differentiate into macrophages. Several studies have shown that macrophages significantly contribute to fungal clearance, orchestration of the immune response, and neutrophil recruitment in *Candida*-infected tissues such as the brain, the liver, the spleen, the peritoneum, the kidney, and the gastrointestinal tract (Qian *et al.* 1994, Lionakis *et al.* 2011, Lionakis *et al.* 2013, Leonardi *et al.* 2018, Drummond *et al.* 2019, Sun *et al.* 2019). Macrophages rapidly phagocytose the fungus upon recognition of opsonised fungal cells *via* CR3 and FcγR, whereas non-opsonised fungi are recognised *via* TLRs and CLRs (Vazquez-Torres and Balish 1997, Miramon *et al.* 2013, Naglik 2014). In contrast to neutrophils, the phagosome matures through subsequent fusion and fission events, which results in a decreased pH (Pitt *et al.* 1992, Levin *et al.* 2016, Pauwels *et al.* 2017). This pH decrease is mediated *via* rapid recruitment of v-ATPase to the phagosomal membrane and results in the activation of phagosomal, acid-dependent proteases like cathepsins, which contribute to a non-oxidative degradation of phagocytosed microbes (Yates *et al.* 2005, Haas 2007, Kinchen and Ravichandran 2008). In addition, the phagosome is a place of limited nutrient availability (Haas 2007). Similar to neutrophils, macrophages also generate oxidative and nitrosative stress to eliminate phagocytosed *C. albicans* cells (Sasada and Johnston 1980, Vazquez-Torres and Balish 1997). In terms of nitrosative stress, macrophages largely rely on the production of candidacidal peroxynitrite (Vazquez-Torres *et al.* 1996).

As an additional arm of the innate immunity, intracellular nucleotide-binding oligomerisation domain-like receptors (NLRs) are activated upon recognition of *C. albicans* and initiate a pro-inflammatory immune response, which mediates anti-fungal host responses (Joly *et al.* 2009). Especially the NOD-, LRR- and pyrin domain-containing protein (NLRP)3 inflammasome is essential during *C. albicans* infections (Gross *et al.* 2009, Hise *et al.* 2009), which will be discussed in more detail in paragraph 1.3.3. Furthermore, also the NLR family CARD domain-containing protein (NLRC)4 and NLRP10 inflammasomes play important roles during mucosal infection and systemic candidiasis in mice, respectively (Tomalka *et al.* 2011, Joly *et al.* 2012).

Macrophages infected with *C. albicans* typically express and release large amounts of pro-inflammatory cytokines like interleukin (IL)1α, IL-1β, tumor necrosis factor (TNF)α and the

pleiotropic cytokine IL-6, all contributing to recruitment of neutrophils into the infected tissue (Castro *et al.* 1996, Torosantucci *et al.* 2000, Kim *et al.* 2005, Seider *et al.* 2011). Furthermore, *C. albicans*-infected macrophages induce the expression and secretion of chemokines that are associated with the activation and recruitment of phagocytic cells, like macrophage inflammatory protein (MIP)1α, MIP-1β, MIP-3α, MIP-4, growth-regulated oncogene (Gro)α, Gro-γ, monocyte chemoattractant protein (MCP)1 and IL-8 (Castro *et al.* 1996, Torosantucci *et al.* 2000, Kim *et al.* 2005, Seider *et al.* 2011, Bachelerie *et al.* 2014). Thus, macrophage-mediated pro-inflammatory signalling crucially contributes to antifungal effects (Heung 2020).

Antigen-presenting, monocyte-derived DCs play a crucial role in bridging the innate and the adaptive immune response by presenting processed antigens to T- and B-lymphocytes in the draining lymph nodes (Banchereau *et al.* 2000, Savina and Amigorena 2007). To do so, phagosomal maturation and pathogen destruction occurs slower than in neutrophils and macrophages, putatively to preserve as much antigen information as possible. Thus, DCs are not considered as conventional immune effector cells dedicated to kill the pathogen, but they still importantly contribute to controlling an infection (Savina and Amigorena 2007). Figure 2 summarises the main killing mechanisms used by innate immune cells to degrade *C. albicans* cells, as well as the role of DCs in bridging innate and adaptive immunity.

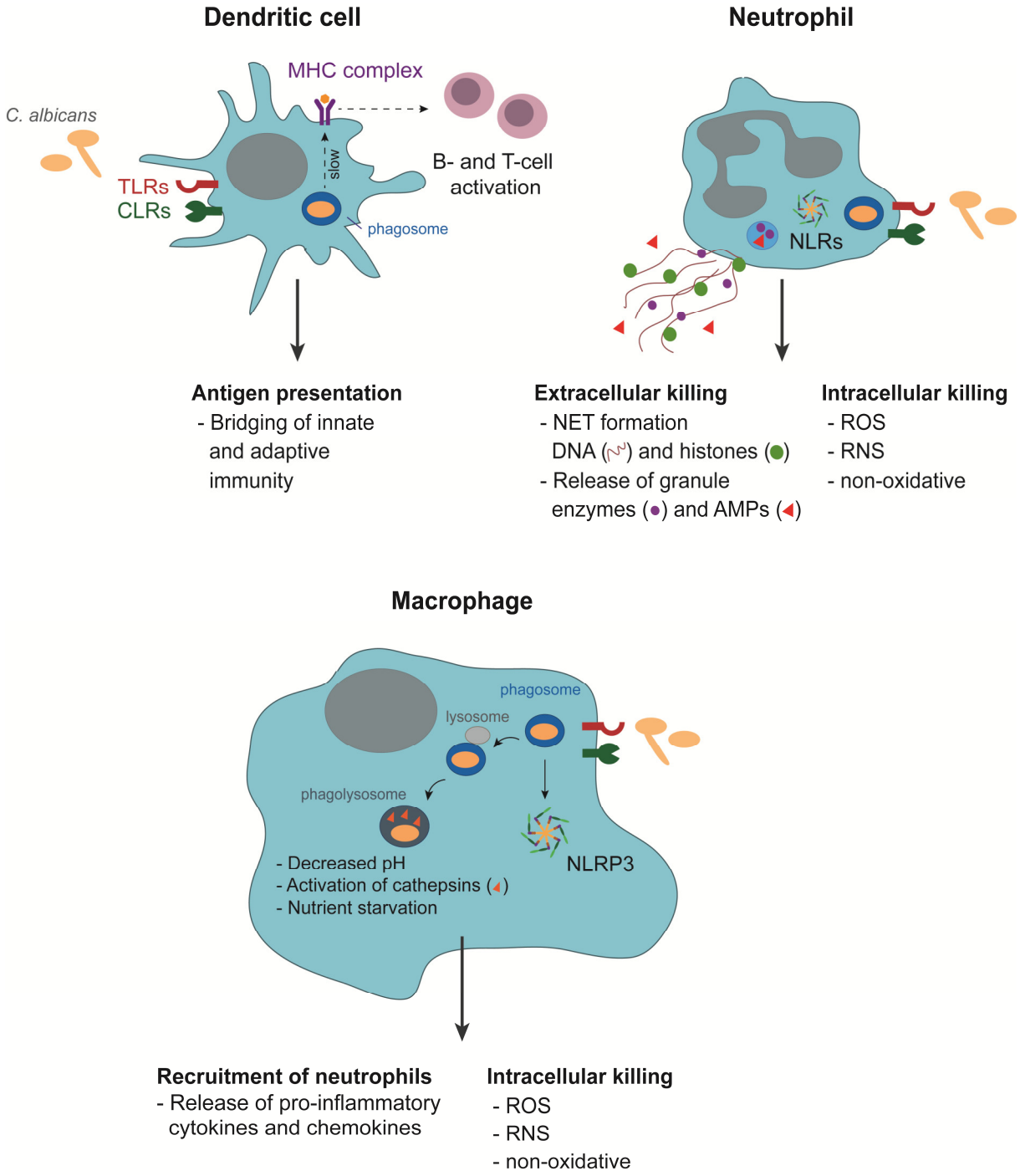


**Figure 2: Innate immune response to *C. albicans*.**

*Candida albicans* is recognised by innate immune cells *via* different PRRs (TLRs, CLRs, NLRs). Upon recognition, the fungus is killed intra- or extracellularly using a combination of various mechanisms like the generation of ROS or RNS, AMP and enzyme release, NET formation, cathepsin activation, and nutrient starvation. Macrophages further release immune mediators contributing to neutrophil recruitment. Slow processing in DCs leads to presentation of fungal antigens *via* MHC complexes, which bridges the innate with the adaptive immunity. Not shown is phagocytosis of opsonised *C. albicans via* CR3 and FcγR. Figure is modified from (Naglik *et al.* 2017).

### 1.3.3 *Candida albicans* strategies to evade the immune response

To counteract the killing mechanisms and stresses induced by immune cells, *C. albicans* up-regulates stress response pathways and interferes with an initiated immune response. Furthermore, the fungus is able to evade immune cell uptake by preventing recognition and phagocytosis by shielding its outermost β-glucan layer with mannoproteins at early stages of infection, whereas late upon infection β-glucan is unmasked in yeast and hyphal cells allowing immune recognition by innate immune cells (Wheeler *et al.* 2008, Gow *et al.* 2017). During budding of yeast cells, β-glucans and chitin are exposed at the bud scar (Gantner *et al.* 2005). Moreover, it has been shown that the fungus actively masks its β-glucan components upon triggers like hypoxia or lactate exposure (Ballou *et al.* 2016, Pradhan *et al.* 2018).

Once taken up, engulfed cells are confronted with the killing abilities of professional phagocytes. Still, a proportion of internalised fungi is able to survive and counteract the engaged detrimental pathways. To detoxify ROS, *C. albicans* produces the surface-bound superoxide dismutases (Sod)4 and 5 (Lorenz *et al.* 2004, Fradin *et al.* 2005, Frohner *et al.* 2009, Dantas Ada *et al.* 2015) as well as a glutathione reductase Grx2 and thioredoxin Trx1 (Miramon *et al.* 2012). Additionally, the fungus suppresses ROS generation by the immune cells, thereby inhibiting oxidative and nitrosative stress (Wellington *et al.* 2009). To cope with nutrient starvation inside the macrophage phagosome, phagocytosed *C. albicans* cells rapidly reprogram their metabolic pathways towards the utilisation of alternative carbon sources like the glyoxylate cycle or β-oxidation of fatty acids, as well as the usage of amino acid transporters and permeases. In contrast, genes involved in protein biosynthesis are downregulated (Lorenz *et al.* 2004, Munoz *et al.* 2019, Laurian *et al.* 2020). *Candida albicans* can additionally modulate the phagosomal pH. Phagosomes containing living *C. albicans* cells change their pH from acidic to neutral, which is putatively a fungus-driven process mediated by production of neutralising metabolites and/or by phagosomal damage and thus proton leakage due to hyphal growth (Vylkova and Lorenz 2014, Westman *et al.* 2018). This hyphal growth not only leads to neutralisation of the phagosomal pH, but also enables the fungus to pierce membranes and escape from the immune cell. Escape of *C. albicans* due to prolonged hypha formation and ultimately piercing of the phagocytic membrane is a long-known

phenomenon (McKenzie *et al.* 2010). Apart from that, glucose consumption by growing hyphae has been reported to trigger host cell death (Tucey *et al.* 2018, Tucey *et al.* 2020).

In the past years, an additional mechanism of *C. albicans*-induced macrophage damage, pyroptosis, which is relying on inflammasome activation, has been identified (Uwamahoro *et al.* 2014, Wellington *et al.* 2014). Inflammasomes are multimeric cytosolic protein complexes that induce a caspase-dependent production of mature, pro-inflammatory IL-1β and IL-18 upon infection. *Candida albicans* induces the NLRP3 inflammasome in innate immune cells like macrophages, DCs and neutrophils (Gross *et al.* 2009, Joly *et al.* 2009, Ganesan *et al.* 2014, Tucey *et al.* 2016, Niemiec *et al.* 2017). This inflammasome has been shown to crucially contribute to host defence against fungal infection (Hise *et al.* 2009, van de Veerdonk *et al.* 2015). It consists of Nlrp3, the adapter protein apoptosis-associated speck-like protein containing a CARD (ASC) and the inactive pro-form of caspase 1. Activation of the NLRP3 inflammasome requires two steps: Initially, the inflammasome is primed upon sensing microbial PAMPs like LPS from bacterial or β-glucan from fungal pathogens by host PRRs like TLR4 or dectin-1, respectively (Kelley *et al.* 2019). This leads to NF-κb-mediated *NLRP3*, *IL1B* and *IL18* transcription, assembly of the primed NLRP3 inflammasome and the production of pro-IL-1β and pro-IL-18. Subsequently, the primed inflammasome is activated by a variety of stimuli such as ion fluxes, ROS, mitochondrial or lysosomal damage, resulting in cleavage of pro-caspase 1 into its mature form. Upon that, caspase 1 cleaves pro-IL-1β and pro-IL-18 into the mature, secreted forms (Kelley *et al.* 2019). Inflammasome activation not solely results in secretion of pro-inflammatory cytokines, but can also lead to induction of pyroptosis, a pro-inflammatory, programmed cell death pathway mediated *via* NLRP3-dependent caspase 1 activation and subsequent cleavage of gasdermin D into its active form, the latter representing the executor protein of pyroptotic cytolysis (Kelley *et al.* 2019). Recent studies have shown that macrophages undergo a pyroptotic host cell death, specifically within early stages (within the first 8 h) of infection with *C. albicans* and when the fungal burden is low (Uwamahoro *et al.* 2014, Wellington *et al.* 2014). Hypha formation is a necessary but not sufficient trigger for inflammasome activation and hyphal factors or activities seem to be important to mediate this process (Joly *et al.* 2009, Wellington *et al.* 2012). Importantly, this

induced immune cell death can provide an escape route for the fungus early upon infection in addition to escaping the hostile environment *via* hypha formation at later stages of infection (≥8 h) (McKenzie *et al.* 2010, Uwamahoro *et al.* 2014). Nevertheless, fungal factors triggering this process remained largely unknown (Krysan *et al.* 2014).

In contrast to NLRP3 inflammasome activation, no beneficial function of NLRC4 or NLRP10 inflammasome activation for *C. albicans* during infection has been proposed so far.

Taken together, these adaptations and strategies allow *C. albicans* to survive and proliferate in and even escape from the hostile environment inside macrophages. Figure 3 depicts the interaction of *C. albicans* with macrophages and summarises fungal strategies to overcome the detrimental immune cell actions to ultimately mediate fungal escape.

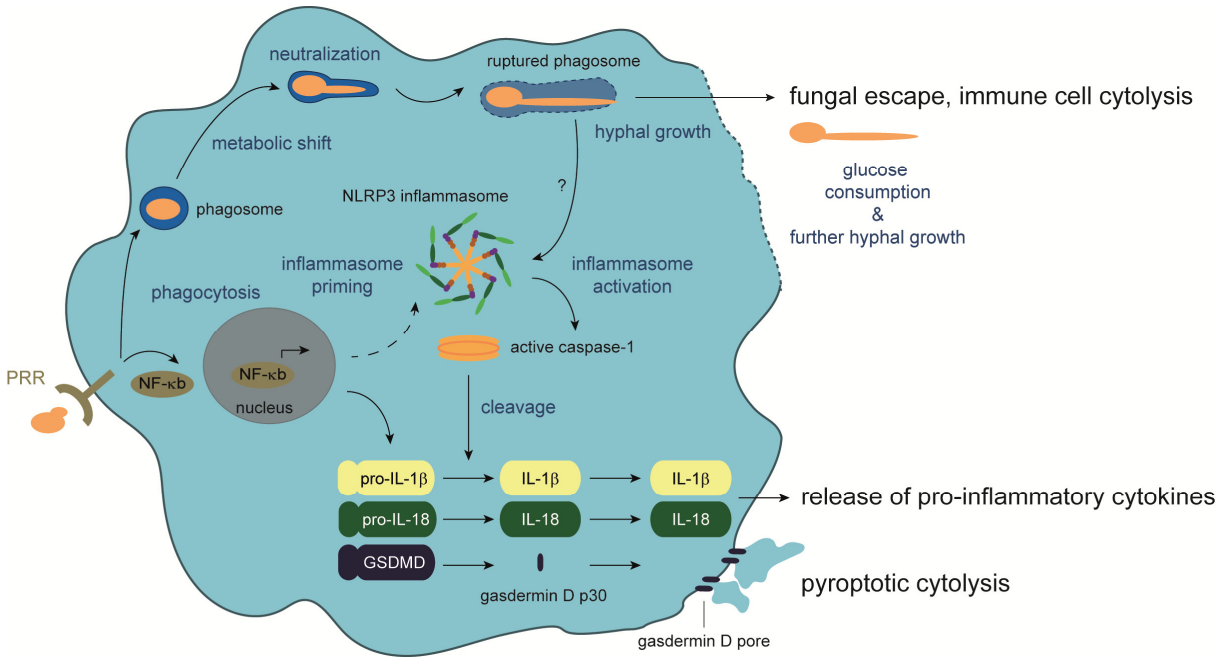


**Figure 3: The *C. albicans*-macrophage interaction.**

*Candida albicans* PAMPs are recognised by host PRRs, which leads to phagocytosis of the fungus and, amongst others, NF-κb signalling. Contact with the phagosomal content leads to reprogramming of metabolic pathways to cope with nutrient starvation. Modulation of the phagosomal pH results in a neutralised phagosome, which enables fungal escape by hyphal growth. Glucose consumption by growing hyphae similarly mediates immune cell death. Activation of the inflammasome and the induction of pyroptosis results in the release of pro-inflammatory cytokines, immune cell lysis, and fungal escape. Figure is modified from (König *et al.* 2020a).

## 1.4 The hypha-specific *Candida albicans* gene *ECE1*

As hypha formation has been implicated with the ability of *C. albicans* to escape from immune cells, hypha-specific genes are of great interest when elucidating the fungus-phagocyte interplay. The gene *Extent of Cell Elongation* (*ECE*)1 has already been described in 1993 as highly expressed during filamentation of *C. albicans* despite being dispensable for the process itself (Birse *et al.* 1993). In 2013, the *ECE1* gene has been, amongst seven other genes, reported to be part of *C. albicans*' core filamentation response (Martin *et al.* 2013). Still, the function of *ECE1* remained unknown. The gene is expressed within minutes after germ tube formation and the expression increases up to 10,000-fold as compared to the yeast morphology (Birse *et al.* 1993, Moyes *et al.* 2016). The encoded protein Ece1 is comprised of eight peptides separated from one another by repetitive lysine-arginine residues (KR sites), representing cleavage sites for the Golgi-located fungal subtilisin-like endopeptidase Kex2 (Figure 4) (Bader *et al.* 2008, Moyes *et al.* 2016, Richardson *et al.* 2018b). After cleavage, the deriving peptides are further processed by the fungal exopeptidase Kex1, which removes the terminal arginine (Moyes *et al.* 2016, Richardson *et al.* 2018b).

### 1.4.1 The cytolytic toxin candidalysin

Of all eight Ece1 peptides, only the third has been shown to adopt an α-helical structure, which allows the peptide to intercalate into host cell membranes (Moyes *et al.* 2016). It is able to damage several human epithelial cell types *in vitro* and to induce hemolysis in erythrocytes (Moyes *et al.* 2016). Furthermore, the toxin rapidly induces heterogeneous and transient membrane lesions in artificial membranes, indicating a carpet-like insertion into the membrane (Moyes *et al.* 2016). Due to these features, the peptide has been termed candidalysin, representing the first cytolytic peptide toxin described in a human pathogenic fungus (Moyes *et al.* 2016).

As stated above, candidalysin is released from Ece1 upon sequential processing by Kex2 and 1 (Moyes *et al.* 2016). The resulting peptide toxin consists of 31 amino acids (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK) and exhibits an amphiphatic nature, as it contains an N-terminal hydrophobic and a C-terminal hydrophilic region (Moyes *et al.* 2016).

At least the cleavage sites before and after the candidalysin-encoding sequence are indispensable for the maturation of candidalysin (Figure 4) (Richardson *et al.* 2018b). Thus, the correct processing of the peptide toxin is critical for fungal virulence (Richardson *et al.* 2018b).

Several studies have shown that candidalysin crucially contributes to fungal infection of different host niches. In 2016, candidalysin has been described as critical during mucosal candidiasis, as the absence of the toxin lead to an abolished damage potential of the fungus against oral epithelial cells (Moyes *et al.* 2016). Therefore, candidalysin can be seen as the long-sought missing link between *C. albicans* hypha formation and host cell damage (Moyes *et al.* 2016, Wilson *et al.* 2016). Moreover, the toxin drives the induction of innate type 17 responses in oral epithelial cells (Verma *et al.* 2017).

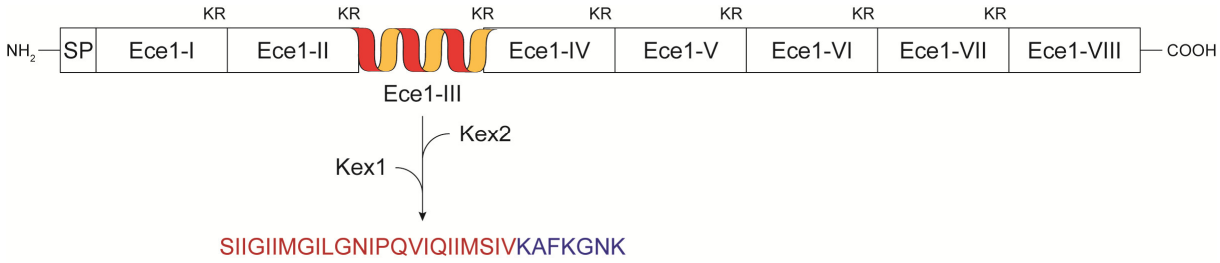


**Figure 4: Structure of Ece1 and maturation of candidalysin.**

Candidalysin derives from the third of eight peptides within the preproprotein Ece1. It is released after sequential processing by the two Golgi-located fungal proteases Kex2 and 1, exhibits an amphiphatic nature and adopts an α-helical structure. SP - signal peptide, red - hydrophobic part, blue - hydrophilic part, yellow/red: α-helical structure adopted by Ece1-III. Ece1-I, -II and -IV to -VIII: Non-Candidalysin Ece1 peptides (NCEPs).

As candidalysin shares features with other cytolytic toxins and the membrane-perturbing action of bacterial pore forming toxins is well known to play an essential role during confrontation with macrophages by inducing inflammatory responses, inflammasome activation and inflammatory host cell death (Abrami *et al.* 1998, Keyel *et al.* 2011, Gonzalez-Juarbe *et al.* 2015, Greaney *et al.* 2015, Cavaillon 2018), it is tempting to speculate that candidalysin similarly triggers inflammasome activation.

### 1.4.2 Non-Candidalysin Ece1 peptides

Apart from candidalysin, *C. albicans* simultaneously expresses seven other peptides Non-Candidalysin Ece1 Peptides (NCEPs, Figure 4) that derive from the same Ece1 precursor protein as discussed above. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of hyphal supernatants revealed the secretion of candidalysin and NCEPs, mainly Ece1-V, -VI, and -VII fragments, upon cleavage of Ece1 by Kex2 and 1 proteases (Moyes *et al.* 2016, Richardson *et al.* 2018b). Table 1 exemplarily shows the LC-MS/MS data for a wild type (Wt) culture grown under hypha-inducing conditions.

**Table 1: LC-MS/MS analysis of a *C. albicans* Wt culture**

Ece1 peptide name and amino acid sequence after Kex2 digest are depicted in black. Retrieved peptide fragments from LC-MS/MS analysis of a Wt culture grown under hypha-inducing conditions and alternative peptide fragment name are given in grey. Peptide Spectrum Match (PSM) values are the mean of 3 replicates and show the relative incidence of peptide/peptide fragment retrieval. Table is adapted from supplementary data published by (Richardson *et al.* 2018b).

| Peptide | Peptide amino acid sequence/fragments retrieved by LC-MS/MS | PSM value |
|---|---|---|
| Ece1-I | MKFSKIACATVFALSSQAAIIHHAPEFNMKR | |
| | AIIHHAPEFNM | 3 |
| Ece1-II | DVAPAAPAAPADQAPTVPAPQEFNTAITKR | |
| | DVAPAAPAAPADQAPTVPAPQEFNTAIT | 8 |
| Ece1-III | SIIGIIMGILGNIPQVIQIIMSIVKAFKGNKR | |
| Candidalysin | SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK | 508 |
| Ece1-IV | EDIDSVVAGIIADMPFVVRAVDTAMTSVASTKR | |
| | EDIDSVVAGIIADMPFVV | 1 |
| Ece1-V | DGANDDVANAVVRLPEIVARVATGVQQSIENAKR | |
| Ece1-Va | DGANDDVANAVVRLPEIVA | 165 |
| Ece1-Vb | VATGVQQSIENAK | 4 |
| Ece1-VI | DGVPDVGLNLVANAPRLISNVFDGVSETVQQAKR | |
| Ece1-VIa | DGVPDVGLNLVANAPR | 24 |
| Ece1-VII | DGLEDFLDELLQRLPQLITRSAESALKDSQPVKR | |
| Ece1-VIIa | DGLEDFLDELLQRLPQLIT | 164 |
| Ece1-VIIb | SAESALKDSQPV | 181 |
| Ece1-VIII | DAGSVALSNLIKKSIETVGIENAAQIVSERDISSLIEEYFGKA | |
| Ece1-VIIIa | DAGSVALSNLIK | 5 |
| Ece1-VIIIb | SIETVGIENAAQIVSER | 3 |
| Ece1-VIIIc | DISSLIEEYFGKA | 8 |

The repetitive Kex2 protease cleavage sites in the Ece1 sequence and the secretion of these NCEPs into *C. albicans* hyphal supernatants (Moyes *et al.* 2016, Richardson *et al.* 2018b) point

towards a specific function of these peptides. However, no NCEP function has been reported yet. Inspired by observations made in bacterial and plant pathogenic fungi (next paragraph) or obvious questions regarding the self-protection of *C. albicans* against candidalysin, a number of possible function is feasible. For example, it is possible that the NCEPs are directly relevant for fungal biology. They might be important for proper Ece1 folding (e.g. chaperone-like), processing, and/or delivery. Furthermore, a function during hypha formation *per se* or an involvement in internal signalling cascades is possible. In addition, NCEPs might protect the fungus against candidalysin self-toxicity in an antitoxin-like manner. Apart from fungal biology, NCEPs can putatively also modulate the candidalysin function in terms of counteraction or support of membrane insertion. As commensal factors, also a function in an antimicrobial peptide (AMP)-like manner by targeting competing microbes, during nutrient acquisition by binding and/or shuttling, or an involvement in microbial communication is conceivable. Lastly, NCEPs might function as effector peptides on or in host cells. It is possible, that NCEPs induce signalling cascades or modulate the host metabolism, e.g. by binding to host receptors on the cell surface or upon translocation into the host cytoplasm. Putatively, NCEP translocation into the host cells is facilitated by candidalysin-mediated membrane destabilisation or pore formation.

## 1.5 Effector peptides and proteins

To modulate the host response upon infection, microbes can produce effector peptides or proteins that aid the pathogen during the infection process. Effectors have been implicated for example with immune evasion, modulation of the host response and exploitation of host-derived micro- and macronutrients and have been mainly described in bacterial and fungal plant pathogens (Galan 2009, Stergiopoulos and de Wit 2009, König *et al.* 2021).

Bacterial pathogens possess a broad variety of highly specific secreted effector proteins manipulating their host to create suitable niches for survival and proliferation. For example, gram-negative bacteria use type I-VI secretion systems to inject virulence proteins directly into the host cytoplasm. In case of gram-positive bacteria, further secretion apparatus systems like the injectisome or the type VII secretion system are used (Green and Mecsas 2016). The variety of host pathways targeted by these effectors is broad. Depending on the respective

lifestyle, cell death induction is inhibited or induced, signalling cascades are remodelled, and many more (Galan 2007, Mattoo *et al.* 2007, Reddick and Alto 2014).

Plant pathogenic fungi are of great economical concern, as infection of cultured plants can have a devastating impact on the crop yield and further cause intoxication in humans when inhaled or eaten (Doehlemann *et al.* 2017, Köhler *et al.* 2017). Commonly used effector proteins of plant pathogenic fungi are LysM effectors, which contain chitin-binding motifs, thereby manipulating the chitin-triggered immunity in host plants (Kombrink and Thomma 2013). Another strategy is the induction or prevention of plant cell death, depending on the pathogenic lifestyle of the fungus. During their biotrophic phase, (oomycete-like) fungi like *Phytophthora* spp., *Ustilago maydis* or *Botrytis cinerea* secrete diverse effectors suppressing cell death induction in the plant tissue (Dou *et al.* 2008, Bos *et al.* 2010, Kelley *et al.* 2010, Gilroy *et al.* 2011, Rabe *et al.* 2013, Weiberg *et al.* 2013). On the other hand, during necrotrophic growth, fungal pathogens like *Colletotrichum orbiculare, Sclerotinia sclerotiorum or Magnaporthe oryzae* have been shown to secrete cell death-inducing effector proteins (Yoshino *et al.* 2012, Lyu *et al.* 2016, Wang *et al.* 2016).

Regarding *C. albicans*, no "classical" effector peptides or proteins, meaning secreted fungal factors which modulate the biological activity of distinct host targets (Lo Presti *et al.* 2015), have been described so far. Thus, the characterisation of putative *C. albicans* effectors like the above introduced NCEPs remains to be conducted.

# 2 Aims of the study

Whilst usually residing as a harmless commensal on mucosal surfaces, *C. albicans* can also cause disease under predisposing conditions reaching from relatively harmless superficial infections to life-threatening systemic candidiasis.

The hypha-specific peptide toxin candidalysin has recently been identified as a crucial contributor to epithelial damage induction during infection and the missing link between *C. albicans* filamentation and damage (Moyes *et al.* 2016, Wilson *et al.* 2016). However, it further induces a danger response in oral epithelial cells, which leads to recruitment of immune cells and results in a host-protective response. The functions of candidalysin during the *C. albicans*-epithelial interaction and its involvement in the induction of mucosal immunity have been summarised in **manuscript I**.

Hyphae are not only formed during the interaction of *C. albicans* with epithelial surfaces, but also during the interaction with immune cells (McKenzie *et al.* 2010), the latter crucially contributing to fungal clearance upon translocation of the fungus through epithelial and endothelial barriers (Netea *et al.* 2015). Recent studies have further shown that the fungal pathogen *C. albicans* hijacks the programmed, pro-inflammatory, NLRP3 inflammasome-dependent cell death pathway pyroptosis to escape from macrophages, thereby evading the immune response (Uwamahoro *et al.* 2014, Wellington *et al.* 2014). Hypha formation has long been known to trigger the activation of the NLRP3 inflammasome in response to *C. albicans* infection, suggesting that hyphal factors play a role in this process (Joly *et al.* 2009). However, the exact mechanism or the role of hypha-associated factors remained largely unknown.

Thus, one major aim of this thesis was to characterise the role of candidalysin during the interaction of *C. albicans* with primary human and murine mononuclear phagocytes (**manuscript II**). The involvement of the toxin in inducing macrophage damage and the respective time course of this damage induction was surveyed measuring the release of cytoplasmic lactate dehydrogenase (LDH) or propidium iodide staining. Moreover, the ability to activate programmed or non-programmed cell death pathways like apoptosis, pyroptosis, necroptosis, or necrosis was examined using specific inhibitors, western blotting and staining

protocols. Furthermore, the potential of candidalysin to elicit a specific, inflammasome-dependent immune response in primary mononuclear phagocytes was monitored. The activation of the inflammasome was assessed by measuring IL-1β release from macrophages infected with different *C. albicans* mutant strains or co-incubated with synthetic candidalysin. To unravel the mechanism of inflammasome activation, inhibitors of known inflammasome-activating triggers were applied before measuring the release of mature IL-1β. Apart from that, immune cells derived from specific knock-out mice and inhibitors were used to characterise the respective inflammasome type responsible for the candidalysin-mediated pro-inflammatory cytokine release.

To summarise and discuss results regarding the role of candidalysin during the interaction of immune cells with *C. albicans* (presented in **manuscript II**) and its function during *C. albicans*-epithelial interaction (partially summarised in **manuscript I**), **manuscripts III** and **IV** were prepared focusing on the dual function of candidalysin; on the one hand as a crucial damage factor and on the other hand as an inducer of pro-inflammatory, host-protective responses. **Manuscript III** additionally puts complex *in vivo* studies into context with *in vitro* data.

Despite the fact, that candidalysin has been shown to be a key hypha-specific factor involved in many infection-related processes, the *ECE1* gene encodes seven further peptides with highly conserved cleavage sites. It was thus hypothesised that these NCEPs function as effector peptides, putatively supporting candidalysin function, processing, and/or secretion or even exhibiting own, distinct functions during infection.

Effector molecules are a common theme in bacteria and plant pathogenic fungi. To gain a first overview of infection and immune evasion strategies as well as host modulation and exploitation during infection with different human and plant pathogenic fungi, **manuscript V** was prepared, covering exemplary fungal effector molecules and strategies that are involved in the above-mentioned processes.

To further dissect the role of NCEPs (presented as **additional results** of this thesis in chapter 4), NCEP-knock-out mutants were constructed, verified and screened for *ECE1* expression and Ece1 peptide secretion. Furthermore, fungal properties like hypha formation,

the induction of an unfolded protein response, and the damage potential against primary human macrophages were monitored. In addition to the generated NCEP-knock-out mutants, synthetic NCEP peptides were used to elucidate the function of these putative effector peptides in more detail. To investigate the overall response of primary human macrophages to treatment with NCEPs and/or candidalysin, transcriptional profiling was performed using whole human genome microarrays. To elucidate potential NCEP effector functions during an interaction with macrophages, the cytokine profile of primary human macrophages upon co-incubation with synthetic peptides was monitored using multiplex and standard ELISAs.

# 3 Manuscripts

## 3.1 Manuscript I – Naglik *et al.*, *Current Opinion in Microbiology* 2017

### *Candida albicans*-epithelial interactions and induction of mucosal innate immunity

Julian R. Naglik, **Annika König**, Bernhard Hube, Sarah L. Gaffen.

*Current Opinion in Microbiology* 2017 Dec; 40:104-112. doi: 10.1016/j.mib.2017.10.030. Review.

Summary:

This review summarises the interaction of *C. albicans* with the epithelium during infection. As a first step during infection, the fungus adheres to epithelial cells and activates them *via* candidalysin secreted by invading hyphae. This activation results in a danger response characterised by the release of cytokines, chemokines, and AMPs. These in turn lead to the recruitment of immune cells like neutrophils, macrophages and innate type 17 cells. Neutrophils and macrophages contribute to fungal clearance by intra- and extracellular killing mechanisms. Innate type 17 cells mediate the further release of cytokines, chemokines and AMPs, thus promoting fungal clearance as well as strengthening of the epithelial barrier.

Own contribution:

Annika König conducted literature research, wrote the part "Innate immunity at mucosal surfaces: neutrophils and macrophages" and created the figure.

Estimated authors' contributions:

| | |
|---|---|
| Julian R. Naglik | 30 % |
| **Annika König** | **25 %** |
| Bernhard Hube | 20 % |
| Sarah L. Gaffen | 25 % |

Prof. Dr. Bernhard Hube

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# *Candida albicans*-epithelial interactions and induction of mucosal innate immunity

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## Abstract

*Candida albicans* is a human fungal pathogen that causes millions of mucosal and life-threatening infections annually. *C. albicans* initially interacts with epithelial cells, resulting in fungal recognition and the formation of hyphae. Hypha formation is critical for host cell damage and immune activation, which are both driven by the secretion of Candidalysin, a recently discovered peptide toxin. Epithelial activation leads to the production of inflammatory mediators that recruit innate immune cells including neutrophils, macrophages and innate Type 17 cells, which together work with epithelial cells to clear the fungal infection. This review will focus on the recent discoveries that have advanced our understanding of *C. albicans*-epithelial interactions and the induction of mucosal innate immunity.

### Keywords

Candida; Candidalysin; pathogenicity; virulence; hyphae; epithelium; mucosal; adhesion; invasion; damage; innate immunity; neutrophil; macrophage; dendritic cell; Type 17 immunity; IL-17

## Introduction

*Candida albicans* is normally a harmless commensal organism within the normal microbiota in approximately half the world's population. In the commensal phase, *C. albicans* most

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likely resides in the mucus layer of mucosal surfaces. However, occasionally and under certain circumstances, *C. albicans* may encounter host cells directly, which may result in the attachment of the fungus to epithelial cells. Depending on the strain of *C. albicans* and the physiological and immune status of the host, this interaction event can lead to superficial overgrowth and epithelial invasion, followed by disease and immune activation. *C. albicans* is the most prevalent *Candida* species causing infections in humans and is the causative agent of oral and vaginal candidiasis (e.g. thrush), giving rise to severe morbidity in millions of individuals worldwide. Given that potentially fatal systemic infections can arise from breaches of the mucosal barrier (predominantly from the gut) it is of paramount importance to understand how *C. albicans* interacts with cells of the innate immune system and how this fungus is restricted to the mucosal surface in health. Critical to this is an understanding of how epithelial cells are able to discriminate between harmless (commensal) and dangerous (pathogenic) *C. albicans* cells, which determines whether a mutually beneficial commensal relationship or immune activation takes place.

## *C. albicans* interaction with epithelial cells: adhesion and invasion

Epithelial cells at mucosal surfaces are the first point of contact with *C. albicans* and constitute the first line of defence. Although fungal pathogenicity depends on the type of mucosal tissue, there are common virulence mechanisms and principles. *C. albicans* adhesion to epithelial cells is mediated through the interaction of fungal cell wall moieties and surface proteins with host receptors (Table 1). *C. albicans* yeast cells are recognized by oral epithelial cells (in the TR146 cell line) and induce three signalling pathways within 15 min; the nuclear factor-kappaB (NF-κB) pathway, the phosphatidylinositol-4,5-bisphosphate 3-kinase (Pi3K), and all three mitogen-activated protein kinase (MAPK) pathways (p38, JNK (c-Jun N-terminal kinase) and ERK1/2 (extracellular signal-regulated protein kinase)). This results in the activation of the p65/p50 transcription factor *via* NF-κB, the c-Jun transcription factor *via* JNK and ERK1/2, and AKT (protein kinase B) and mTor (mammalian target of rapamycin) *via* Pi3K signaling [1,2]. Initial binding may constitute recognition of fungal cell wall mannans and β-glucans but this does not fully activate epithelial cells as proinflammatory cytokines were not induced [2]. Lack of activation by *C. albicans* cell wall polysaccharides was also found in skin keratinocytes [3], suggesting that fungal polysaccharides play a limited role in inducing epithelial/keratinocyte immune responses. While many other yeast-associated secreted/cell-surface proteins (e.g. Sap1-3/9/10, Als1/3/4/9, Mp65, Phr1, Iff4, Sun41, Pra1, Eap1, Utr2 and Ecm33), cell wall processing proteins (e.g. Big1, Mnt1/2, Mnn9), and protein trafficking/vesicle transport proteins (e.g. Vps11) are thought to promote epithelial adhesion, this is likely to be *via* indirect mechanisms given that these proteins possess complex, multi-factorial functions that contribute to cell wall integrity and hypha formation [4–7].

Adhesion of *C. albicans* to an epithelial cell is a strong inducer of hypha formation. The formation of hyphae occurs within 30 – 60 min and this is accompanied by the expression of hypha-associated proteins, which are known to possess critical roles in adhesion, invasion, damage induction and immune activation/evasion. The two key hyphal proteins that promote epithelial adhesion are Hwp1 (hyphal wall protein 1) [8] and Als3 (agglutinin-like sequence 3) [9,10]. Hwp1 is highly expressed in human oral infections [11] and acts as a substrate for

epithelial transglutaminases, enabling strong covalent links with other epithelial proteins [12]. Als3 is both an adhesin and an invasin, and together with Ssa1 (heat shock protein) promotes the endocytosis of *C. albicans* into epithelial cells *via* E-cadherin [13–15] and the EGFR/Her2 (epidermal growth factor receptor/human epidermal growth factor 2) complex [16]. Endocytosis is an entirely host driven process and does not require viable hyphae [17]. Other pathways that promote *C. albicans* endocytosis include the PDGF BB (platelet-derived growth factor BB) and NEDD9 (neural precursor-cell-expressed developmentally downregulated protein 9) pathways, which both require hypha formation and Als3 expression [18•]. However, despite possessing adhesion/invasin activities, Als3 does not directly induce epithelial cell damage or cytokine production [19]. The AhR (aryl hydrocarbon receptor) also contributes to the endocytosis of *C. albicans via* Src family kinase phosphorylation of EGFR, but AhR is not involved in epithelial damage or cytokine induction by *C. albicans* and it is unknown how AhR is activated [20•]. Currently, the level of redundancy between these different pathways (E-cadherin, EGFR/Her2, AhR, PDGF BB and NEDD9) and how they communicate to promote C. *albicans* endocytosis is unclear. It is important to note that induced endocytosis is not the only invasion route of *C. albicans*. Indeed, active penetration, which does not require host activities, seems to be the dominant invasion route depending on the type of epithelial cell [21].

## Epithelial damage and immune activation by Candidalysin

While *C. albicans* adhesion and invasion leads to fungal recognition and signal pathway activation, surprisingly this does not translate into epithelial damage or innate immune activation [2,17]. Recently, it was discovered that *C. albicans* hyphae induce both epithelial damage and innate immunity through the secretion of a cytolytic peptide toxin called Candidalysin, which is encoded by the hypha-associated *ECE1* gene [22••]. Candidalysin is an amphipathic peptide that adopts an α-helical structure and is the first peptide toxin to be identified in any human fungal pathogen. In oral epithelial cells, Candidalysin induces calcium ion influx and lactate dehydrogenase (LDH) release, which are characteristics of cell damage and membrane destabilization (Figure 1). Notably, *C. albicans* mutants where the entire *ECE1* gene or the Candidalysin-encoding region has been deleted, have full invasive potential *in vitro* but are incapable of inducing tissue damage or cytokine release, and are highly attenuated in a murine model of oropharyngeal candidiasis and a zebrafish swimbladder mucosal model [22••].

Candidalysin induces epithelial immunity predominantly *via* MAPK signalling, specifically (i) the p38 pathway, resulting in the activation of the AP-1 transcription factor c-Fos, and (ii) the ERK1/2 pathway, resulting in the activation of MKP1 (MAPK phosphatase 1) that regulates immune responses [22••]. Together, these pathways lead to the production of pro-inflammatory cytokines including IL-1α/β, IL-6, GM-CSF and G-CSF. Importantly, p38/c-Fos and MKP1 is also activated in human vaginal epithelial cells [23] and by other hypha-forming *Candida* species [24]. Therefore, these signalling pathways may enable different mucosal tissues to detect fungal hyphae, thereby potentially identifying when certain *Candida* species have become pathogenic. Notably, epithelial activation by Candidalysin is not mediated *via* C-type lectin receptors (CLRs) or Toll-like receptors (TLRs) [2], suggesting that epithelial cells utilise different sensing mechanisms than myeloid cells;

whereby myeloid cells respond to *C. albicans* cell wall moieties (β-glucan and mannans) (see below) and epithelial cells respond to damage-inducing *C. albicans* through p38/c-Fos/MKP1 by detecting Candidalysin activity [25–27]. Similar p38 activation has been observed in murine intestinal epithelial cells with bacterial pathogens (*Citrobacter rodentium*) [28] and in *C. elegans* (nematode worm) with *C. albicans* [29], indicating that p38 signalling may be a common epithelial mechanism for the detection of pathogenic microbes.

## Innate immunity at mucosal surfaces: neutrophils and macrophages

*C. albicans*, predominantly through Candidalysin activity, induces proinflammatory cytokines, chemokines and antimicrobial peptides (e.g. IL-1α, IL-1β, IL-8, G-CSF, GM-CSF, β-defensin 3, CCL20 and S100A8/9) from epithelial cells that are required for immune cell recruitment [2,22••,30]. The key myeloid cells that are initially recruited to the site of infection include neutrophils and macrophages (Figure 1). These immune cells recognize *C. albicans* cell wall mannans and DNA *via* TLR2, 4 and 9, and fungal β-glucan *via* CLRs including Dectin-1/−2, DC-SIGN or Mincle [31–33]. Activation of TLRs and CLRs leads to the induction of NF-κB, MAPK and Syk signaling and the production of pro-inflammatory cytokines and further downstream immune effector functions. Nod-like receptors (NLRs) can also be activated by danger signals or internalized fungal compounds and this leads to inflammasome activation and the secretion of IL-1β and IL-18, which help protect against superficial and disseminated *C. albicans* infection [34].

Upon *C. albicans* infection, neutrophils are rapidly recruited to the site of entry. Even without physical contact to invading hyphae, neutrophils respond to epithelial derived chemokines and growth factors and release TNFα, which in turn triggers a protective effect in epithelial cells *via* the upregulation of TLR4 [35]. Neutrophils also inhibit hyphal formation without direct contact [36,37]. Neutrophils can also be recruited by responding directly to *C. albicans*-derived factors such as the secreted aspartic proteases (Saps) [38•]. Furthermore, neutrophils phagocytose (e.g. *via* CLRs) and kill *C. albicans* yeast cells and short hyphae intracellularly predominantly via oxidative burst mechanisms. *C. albicans* hyphae that are too large to be phagocytosed are either growth-inhibited or killed extracellularly through the formation of neutrophil extracellular traps (NETs or NETosis), *via* the release of granule enzymes and through secretion of antimicrobial peptides such as calprotectin [39,40,41•]. Indeed, the zinc binding properties of calprotectin inhibits *C. albicans* growth during NET formation [40]. *C. albicans* hyphae trigger NETosis more effectively and rapidly than yeast cells, but both morphologies can induce NETs *via* autophagy and oxidative mechanisms [42•]. Reactive oxygen species [43], fibronectin [44] and Dectin-1 signaling [45] have also been implicated in NET formation. However, the role of Dectin-1 is controversial as other studies indicate that NET release by β-glucan is mediated via complement receptor 3 (CD11b/CD18) and not Dectin-1 [44].

Macrophages are also recruited to the site of infection and ingest non-opsonized *C. albicans* after recognition by TLRs and CLRs [47]. While macrophages phagocytose and kill *C. albicans* intracellularly in the phagolysosome through oxidative and nitrosative mechanisms, their activity and efficiency of killing is lower than that of neutrophils. Thus, *C. albicans* is readily able to survive within and escape from macrophages *in vitro* [48] and macrophages

play a more minor role *in vivo* during murine disseminated infections [32,34]. Macrophages also recognize *C. albicans via* intracellular NLRs, which activates the NLRP3 inflammasome, leading to production of pro-inflammatory IL-1β and IL-18 as well as pyroptotic host cell death [49,50]. Although immune cell death was originally thought to be hypha-dependent, hypha-independent triggers of pyroptosis have also been described [51,52•]. Therefore, filamentation alone may not be sufficient to trigger NLRP3 inflammasome-mediated pyroptosis [52•,53]. Intracellular hypha formation is driven by active alkalinization of the phagosome [54] and causes macrophage cell death by at least two different mechanisms: pyroptosis and physical piercing of the macrophage membrane [49,50,54]. Notably, NLRP3 inflammasome activation is not necessarily coupled with pyroptosis and the fungal trigger that activates the inflammasome still remains unknown. Finally, while inflammasome activation can lead to IL-1β and IL-18 production, IL-1β has been implicated with Th17 responses whereas IL-18 appears to promote Th1 activity [55].

## Innate immunity at mucosal surfaces: innate Type 17 cells

A key insight into requirements for host defense against mucosal candidiasis came from the recognition that mice lacking the IL-17 receptor or its key downstream signaling adaptor Act1 are highly susceptible to oropharyngeal candidiasis (OPC) [30,56,57]. Even more strikingly, when humans were subsequently identified with loss-of-function mutations in the same genes, their dominant disease susceptibility was chronic mucocutaneous candidiasis (CMC) [58,59,60••]. IL-17 is the eponymous cytokine of the Th17 lineage, and a common misconception is that this cytokine functions mainly in the adaptive immune response. However, a variety of innate cells of lymphoid origin produce IL-17, including γδ-T, natural killer T (NKT), innate lymphoid cell type 3 (ILC3) and TCRβ+ 'natural' Th17 cells (nTh17) [61]. In the context of OPC, IL-17 is produced mainly by γδ-T and nTh17 cells, and mice lacking a TCR (e.g., Rag1−/− or IL-7Rα−/− mice) are highly susceptible to infection [62]. Although ILC3s have also been reported in this context [63], Rag1−/− mice have ILC3 cells but still show the same high susceptibility to OPC as IL-17R-deficient mice [62]. The role of neutrophils in producing IL-17 is controversial, but data in the murine OPC model argues against neutrophils as a source of this cytokine [64].

Surprisingly, activation of innate Type 17 cells appears to be quite distinct from activation of adaptive Type 17 immunity. A Dectin-1-Syk-CARD9 pathway was shown to be important for activating immunity to systemic candidiasis [65]. Consistently, CARD9 is essential for the adaptive Th17 recall response in oral candidiasis. However, this adaptor was largely dispensable for induction of the acute innate IL-17 response [66], a finding that was also recently verified for Dectin-1 and TLR2 (A Verma *et al.*, unpublished). This new study finds that Candidalysin production by *C. albicans* hyphae is the triggering factor for innate IL-17 production in the murine model of OPC (Figure 1). Mice infected with *ECE1*-deficient strains show only minimal induction of IL-17 or activation of nTh17 cell production of this cytokine. Additionally, IL-1R signaling is required for activation of the innate Type 17 response, with contributions from both hematopoietic and non-hematopoietic compartments (A Verma *et al.*, unpublished). A vital role for IL-1 in defense against OPC was shown previously in studies of the inflammasome in mouse OPC [67] and was recently verified in contributing to neutrophil activation in this setting [68]. Collectively, these data indicate that

the early, innate response to *C. albicans* in the oral mucosa depends on sensing of tissue damage through Candidalysin, and not simply the presence of β-glucan components revealed upon fungal filamentation.

Although the IL-17 receptor is expressed ubiquitously, we found that the essential responder cell in the context of oral candidiasis is the superficial oral epithelial cell [69•]. Mice with a conditional deletion of the IL-17 receptor in Keratin 13+ cells (including oral and buccal epithelial cells, but not skin, gut or other tissues) show a similar fungal susceptibility as mice with a full knockout of this receptor. Moreover, gene pathway signatures induced in the oral mucosa during acute infection were highly conserved with genes induced by *C. albicans* and IL-17 in human oral keratinocytes [69].

The anti-fungal functions of IL-17 are multi-fold. First, IL-17 is a potent activator of the neutrophil response, which it triggers by inducing expression of neutrophil-recruiting chemokines and cytokines such as G-CSF, CXCL1/2 and 5 in oral tissue [30]. It should be noted that the extent to which IL-17 drives neutrophil signals may be variable [30,64,70]. Second, IL-17 potently induces anti-microbial peptides (AMPs), particularly β-defensins-1 and −3. Mice lacking these defensins show markedly increased susceptibility to OPC [30,69•,71]. IL-17 may also act on salivary gland cells, contributing to the production of antifungal AMPs such as histatins [72,73]. The combined action of IL-17 signaling promotes effective, non-redundant host defense to mucosal candidiasis.

As noted above, several human kindreds were identified with inherited mutations in the IL-17 receptor signaling pathway that cause CMC [58–60]. Additionally, other gene defects that predispose to CMC are associated with defective IL-17 production or function, including mutations in *STAT3* (Hyper-IgE Syndrome, HIES), *STAT1* and *AIRE* (APECED) [74]. In the latter case, neutralizing antibodies against Type 17 cytokines are found in affected patients, raising the possibility that disease is associated with reduced IL-17 signaling. Of course, in all these cases, IL-17 is likely produced by both innate cells and conventional (adaptive) Th17 cells. In this regard, HIV patients with low CD4 T cells counts are highly prone to OPC, and this has been particularly associated with Th17 loss [75]. Finally, in 2016 the first biologic drugs (Secukinumab, Ixekizumab) targeting IL-17 (specifically, IL-17A and the IL-17A/F heterodimer) directly came to the market to treat psoriasis, a strongly IL-17-driven autoimmune disease [76]. Surprisingly, the incidence of OPC is quite low, in the range of 4–8% of patients [77]. This may simply mean that blockade is incomplete, due either to dose effects or access of anti-IL-17 antibodies to the oral mucosal tissue. Alternatively, these biologics spare IL-17F, which has been shown to cooperate with IL-17A in promoting resistance to OPC [63,78]. Cumulatively, these findings all support a central role for IL-17 receptor signal transduction in mucosal host defense, at both innate and adaptive levels.

## Conclusion

The epithelial cell plays a fundamental role in the host response to *C. albicans* (Figure 1). Both *C. albicans* yeast and hyphae are recognized, but only hyphae are able to invade epithelial cells by induced endocytosis and/or active penetration, causing activation of

epithelial cells. Endocytosis of *C. albicans* is mediated *via* multiple epithelial receptors and the fungal invasin Als3, but epithelial cells are predominantly activated by the hypha-associated peptide toxin Candidalysin. Candidalysin damages epithelial membranes and activates danger response pathways mediated *via* p38/cFos and ERK/MKP1, which results in immune activation and the secretion of cytokines and chemokines. These effector molecules recruit innate immune cells such as neutrophils, macrophages and innate Type 17 cells. Neutrophils (and macrophages) directly kill or restrict the fungus through phagocytosis mechanisms and/or NET formation, and innate Type 17 cells secrete IL-17 and other inflammatory effectors to further recruit neutrophils and promote mucosal barrier function. These innate immune responses work in conjunction with epithelial cells to control the fungal infection. It is clear that *C. albicans* hypha formation is critically important for both fungal pathogenicity and the host response. Additional advances into these epithelial-hyphal interaction events will no doubt provide valuable insights into our understanding of *C. albicans* infections in the future.

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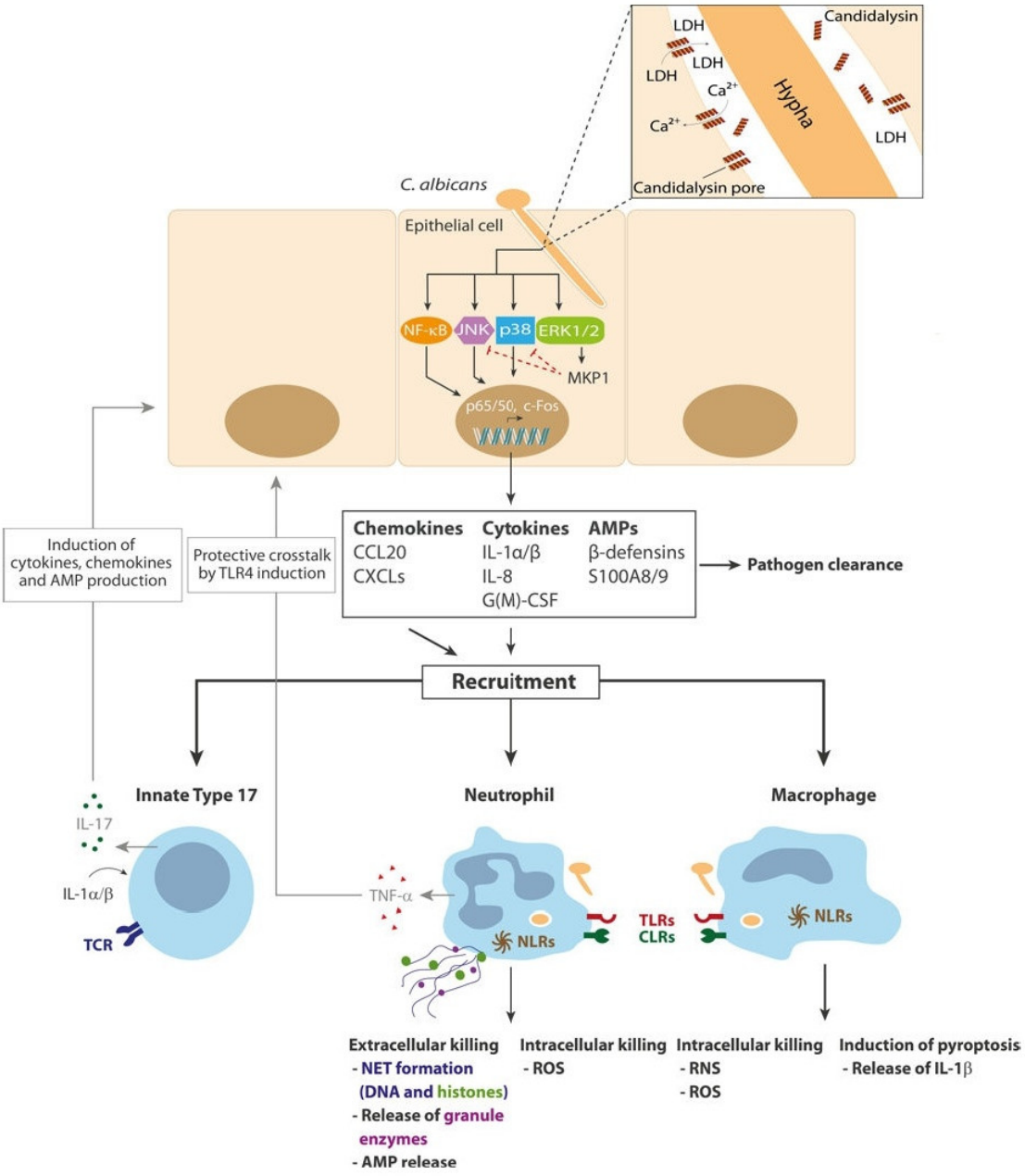


**Figure 1.**

Innate immunity against C. albicans at the oral mucosa. C. albicans hypha formation induces the formation of an invasion pocket and the production of Candidalysin (helical peptide; top right panel). At high concentrations (>15 μM) Candidalysin forms pores that result in membrane damage (LDH release), calcium influx, and the activation of the epithelial cell, predominantly via the MAPK signalling pathways and the transcription factor c-Fos. MKP1 activation (via ERK1/2) contributes to the regulation of the epithelial immune response. Epithelial activation leads to chemokine, cytokine and antimicrobial peptide (AMP) release

and the subsequent recruitment of innate immune cells, including macrophages, neutrophils and TCRβ+ type 17 cells. Macrophages and neutrophils recognise and phagocytose the fungus through traditional pattern recognition receptors such as TLRs and CLRs. This results in death of the fungus via oxidative or nitrosative (ROS/RNS) killing or the induction of pyroptosis in macrophages (and the release of IL-1β) or NET formation in neutrophils. Neutrophils also release TNFα, which induces the upregulation of TLR4 in epithelial cells. IL-1α/β released by epithelial cells, macrophages and potentially other cell sources activate TCRβ+ type 17 cells, which in turn release IL-17 that subsequently induces the release of additional chemokines, cytokines and antimicrobial peptides (AMP) from epithelial cells, further promoting fungal clearance and barrier function.

**Table 1**

*C. albicans* genes involved during interactions with epithelial cells

| Fungal component/gene | Epithelial function or target receptors | Reference |
|---|---|---|
| **Structural polysaccharides** | | |
| β-glucan | Induces epithelial signalling. Recognised by Epha2. | [2]. M Swidergall *et al* (abstract) |
| Mannans | Induces epithelial signalling. Receptors not identified. | [2] |
| Chitin | Induces epithelial signalling. Receptors not identified. | [2] |
| **Adhesins** | | |
| *HWP1* | Adhesion to epithelial cells via transglutaminase activity. Specific host receptors unknown. | [8] |
| *ALS1-9* | Adhesin family. Structural studies indicate this family has multiple epithelial targets. | [14,79–82] |
| *INT1* | Interaction with epithelial integrins. | [83] |
| **Toxins** | | |
| *ECE1* | Parent protein of Candidalysin. Induces c-Fos and MKP1 signalling. Receptor activation indicated but not identified. | [22••] |
| **Endocytosis** | | |
| *ALS3* | Activation of or interaction with E-cadherin, EGFR/Her2, AhR, NEDD9 and PDGF BB | [13,15,16,18•,20•] |
| *SSA1* | HSP70 family member. Activation of or interaction with EGFR/Her2 | [15] |
| **Active Penetration/hydrolysis** | | |
| *SAP1-8* | Secreted aspartic proteases – digestion of epithelial tissues. Sap5 degrades E-cadherin | [84,85] |
| *PLB1* | Phospholipase B1 – digestion of epithelial tissues | [86] |
| *LIP1-10* | Lipase family – digestion of epithelial tissues | [87] |

## 3.2 Manuscript II – Kasper, König, Koenig *et al.*, *Nature Communications* 2018

### The fungal peptide toxin Candidalysin activates the NLRP3 inflammasome and causes cytolysis in mononuclear phagocytes

Lydia Kasper*, **Annika König***, Paul-Albert Koenig*, Mark S. Gresnigt, Johannes Westman, Rebecca A. Drummond, Michail S. Lionakis, Olaf Groß, Jürgen Ruland, Julian R. Naglik, Bernhard Hube

*These authors contributed equally to this work

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Summary:

The fungal peptide toxin candidalysin is the first described peptide toxin in a human pathogenic fungus so far. This publication shows that candidalysin is a major inducer of NLRP3 inflammasome-mediated IL-1β secretion from murine and human mononuclear phagocytes upon *C. albicans* infection. However, the NLRP3-dependent cell death pathway pyroptosis seems to occur independently of candidalysin, suggesting that an activation of the NLRP3 inflammasome is not necessarily coupled to pyroptosis. The toxin candidalysin rather directly causes cytolysis in a necrosis-like manner.

Own contribution:

Annika König conducted parts of the hMDM (Western Blot, ELISAs, PI staining, LDH measurement, phagocytosis and escape assays, hyphal length measurements) and mBMDM (Western Blot, ELISA) experiments, analysed the respective experimental data, performed statistical tests, wrote large parts of the manuscript and created the figures.

Estimated authors' contributions:

| | |
|---|---|
| Lydia Kasper | 23 % |
| **Annika König** | **23 %** |
| Paul-Albert Koenig | 23 % |
| Mark S. Gresnigt | 8 % |
| Johannes Westman | 6 % |

Prof. Dr. Bernhard Hube

| | |
|---|---|
| Rebecca A. Drummond | 5 % |
| Michail S. Lionakis | 1 % |
| Olaf Groß | 1 % |
| Jürgen Ruland | 1 % |
| Julian R. Naglik | 1 % |
| Bernhard Hube | 8 % |

Prof. Dr. Bernhard Hube

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# The fungal peptide toxin Candidalysin activates the NLRP3 inflammasome and causes cytolysis in mononuclear phagocytes

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Clearance of invading microbes requires phagocytes of the innate immune system. However, successful pathogens have evolved sophisticated strategies to evade immune killing. The opportunistic human fungal pathogen *Candida albicans* is efficiently phagocytosed by macrophages, but causes inflammasome activation, host cytolysis, and escapes after hypha formation. Previous studies suggest that macrophage lysis by *C. albicans* results from early inflammasome-dependent cell death (pyroptosis), late damage due to glucose depletion and membrane piercing by growing hyphae. Here we show that Candidalysin, a cytolytic peptide toxin encoded by the hypha-associated gene *ECE1*, is both a central trigger for NLRP3 inflammasome-dependent caspase-1 activation via potassium efflux and a key driver of inflammasome-independent cytolysis of macrophages and dendritic cells upon infection with *C. albicans*. This suggests that Candidalysin-induced cell damage is a third mechanism of *C. albicans*-mediated mononuclear phagocyte cell death in addition to damage caused by pyroptosis and the growth of glucose-consuming hyphae.

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*Candida albicans* is an opportunistic human pathogenic fungus that causes severe morbidity and mortality in millions of individuals worldwide, with approximately 200,000 deaths attributed to invasive systemic infections each year[1,2]. The ability to undergo a yeast-to-hypha transition is considered one of the main virulence attributes of *C. albicans*[3] and is accompanied by the expression of infection-associated genes that facilitate adhesion, invasion, nutrient acquisition, host cell damage, biofilm formation, and immune evasion. Consequently, mutant strains locked in the yeast morphology have an attenuated virulence potential to cause systemic infection[3,4]. *C. albicans* filamentation impacts on fungal recognition by phagocytes (macrophages and dendritic cells (DCs)) of the host innate immune system, activation of pro-inflammatory signalling for host defence, and also on fungal survival and immune escape[5–13].

After recognition of fungal pathogen-associated molecular patterns (PAMPs; e.g., cell wall β-glucan) by phagocyte pattern recognition receptors (PRRs), including Dectin-1[14], *C. albicans* cells are efficiently phagocytosed by macrophages. Once phagocytosed and contained within a phagosome, *C. albicans* can still form hyphae, which leads to stretching of phagocyte membranes and host cell killing, thereby facilitating *C. albicans'* survival and outgrowth[15]. This piercing of host cell membranes by physical forces was thought to be the major pathway of *C. albicans* immune escape and fungus-induced macrophage damage[9]. However, recent discoveries have led to a paradigm shift in our understanding of *C. albicans*-phagocyte interactions[16]. Murine-based studies demonstrated that phagocytosed *C. albicans* induces pyroptosis during early interaction with macrophages, while later events leading to cell damage are mechanistically distinct from pyroptosis, depend on hypha formation[12,17] and are associated with glucose consumption by growing hyphae[18]. Pyroptosis is characterized as an inflammasome-mediated, caspase-1-dependent cell death pathway resulting in IL-1β secretion through pores in the cell membrane, subsequent cell swelling with membrane rupture and, ultimately, cell death[16,19]. Collectively, these data suggest that macrophage killing by *C. albicans* is a two-stage process, with early pyroptosis-mediated inflammatory damage, followed by physical damage by hyphal piercing[16] and competition for glucose[18].

*C. albicans*-induced pyroptosis is dependent on NLRP3 (NACHT, LRR, and PYD domains-containing protein 3) inflammasome signalling, a major pro-inflammatory pathway that can integrate multiple cellular stress signals, including those from fungal, bacterial, and viral pathogens or sterile insults[8,20–22]. In general, NLRP3 inflammasome activation requires two sequential events, a priming and an activation step[23–25]. The priming signal (signal 1) is provided by microbial ligands such as fungal β-glucans or bacterial lipopolysaccharide (LPS), leading to the NF-κB-dependent *IL1B* (pro-IL-1β) and *NLRP3* transcription. A subsequent triggering signal (signal 2) activates the inflammasome resulting in the assembly of a multiprotein complex consisting of the sensor protein NLRP3, the adapter protein ASC (apoptosis-associated speck-like protein containing a C-terminal CARD) and the pro-form of the inflammatory protease caspase-1[24–26]. This NLRP3 inflammasome complex serves as a platform for pro-caspase-1 activation and thereby facilitates the processing of its substrates, including pro-IL-1β, for the release of mature bioactive IL-1β[16,21]. Signal 2 can be provided by multiple stimuli, such as extracellular ATP, particulate matter, or viral RNA, but also bacterial pore-forming toxins (PFTs) that activate NLRP3 through still poorly defined mechanisms[25,27,28]. *C. albicans* hypha formation is known to promote, although not being essential for, inflammasome activation and pyroptosis[7,8,10–13,29]. However, the fungal molecular effectors providing signal 2 are unknown. Furthermore, hypha formation is essential for fungal escape[30] and is required for macrophage lysis by mechanisms distinct from those causing pyroptotic cell death[12].

We recently identified the cytolytic peptide toxin Candidalysin as the missing link between *C. albicans* hypha formation and host cell damage[31,32]. Candidalysin is encoded by *ECE1*, one of the core filamentation genes expressed under most hyphae inducing conditions[33], and is therefore exclusively released by *C. albicans* hyphae, but not yeast cells. *ECE1* codes for a polyprotein consisting of eight distinct peptides. After proteolytic processing[34], these peptides, including Candidalysin, are secreted into the extracellular space. Candidalysin is able to directly damage epithelial membranes via membrane intercalation, permeabilisation, and pore formation, causing the release of cytoplasmic constituents[31].

Given the functional similarities to bacterial PFTs[27,28], in this study we dissect the role of Candidalysin in the phagocyte inflammatory and damage response to *C. albicans* hyphae using a combination of human and murine macrophages and murine DCs. We identify the fungal toxin Candidalysin as a trigger of NLRP3 inflammasome activation and a critical factor required for inflammasome-independent cytolysis.

## Results

**Candidalysin is required for IL-1β release in vivo**. During systemic candidaemia, *C. albicans* disseminates to vital organs. Organ-specific fungal morphologies and innate immune responses determine if and how *C. albicans* is cleared in different organs[35]. Given that *C. albicans* hypha formation[7,8] and bacterial toxins[28] can activate the inflammasome, we hypothesized that the recently discovered hypha-associated cytolytic toxin, Candidalysin[31], can cause IL-1β production, as a key marker of inflammasome activation. Therefore, we investigated the potential of a *C. albicans* mutant lacking Candidalysin to induce IL-1β production as compared to wild-type (Wt) cells during systemic infection. *C. albicans* Wt cells infecting kidneys grow predominantly in the hyphal form[35] and high levels of IL-1β were observed (Fig. 1a). In contrast, *ece1*Δ/Δ mutant cells deficient for Candidalysin[31] showed significantly lower levels of IL-1β responses in the kidney (Fig. 1a). In the spleen, an organ where predominantly yeast cells are observed[35], no significant differences in IL-1β levels were observed between Wt and *ece1*Δ/Δ infected mice (Fig. 1b). The observation that Candidalysin-deficient *ece1*Δ/Δ mutants induce significantly lower IL-1β levels

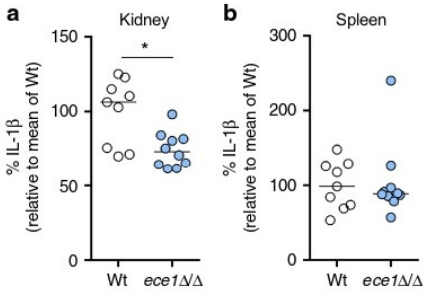


**Fig. 1** Kidney and spleen IL-1β levels during systemic candidemia. **a**, **b** IL-1β levels measured in **a** kidney and **b** spleen homogenates that were obtained at 1 day post infection from C57/Bl6 mice infected intravenously with *C. albicans* Wt or the *ece1*Δ/Δ mutant strain. Values are represented as scatterplot and the median of two independent experiments. The mean of the Wt control group was set at 100% to determine the percentage reduction in IL-1β levels in the mice infected with the *ece1*Δ/Δ mutant. The means of experimental groups were compared for statistical significance using the Mann–Whitney *U* test. *$p \leq 0.05$

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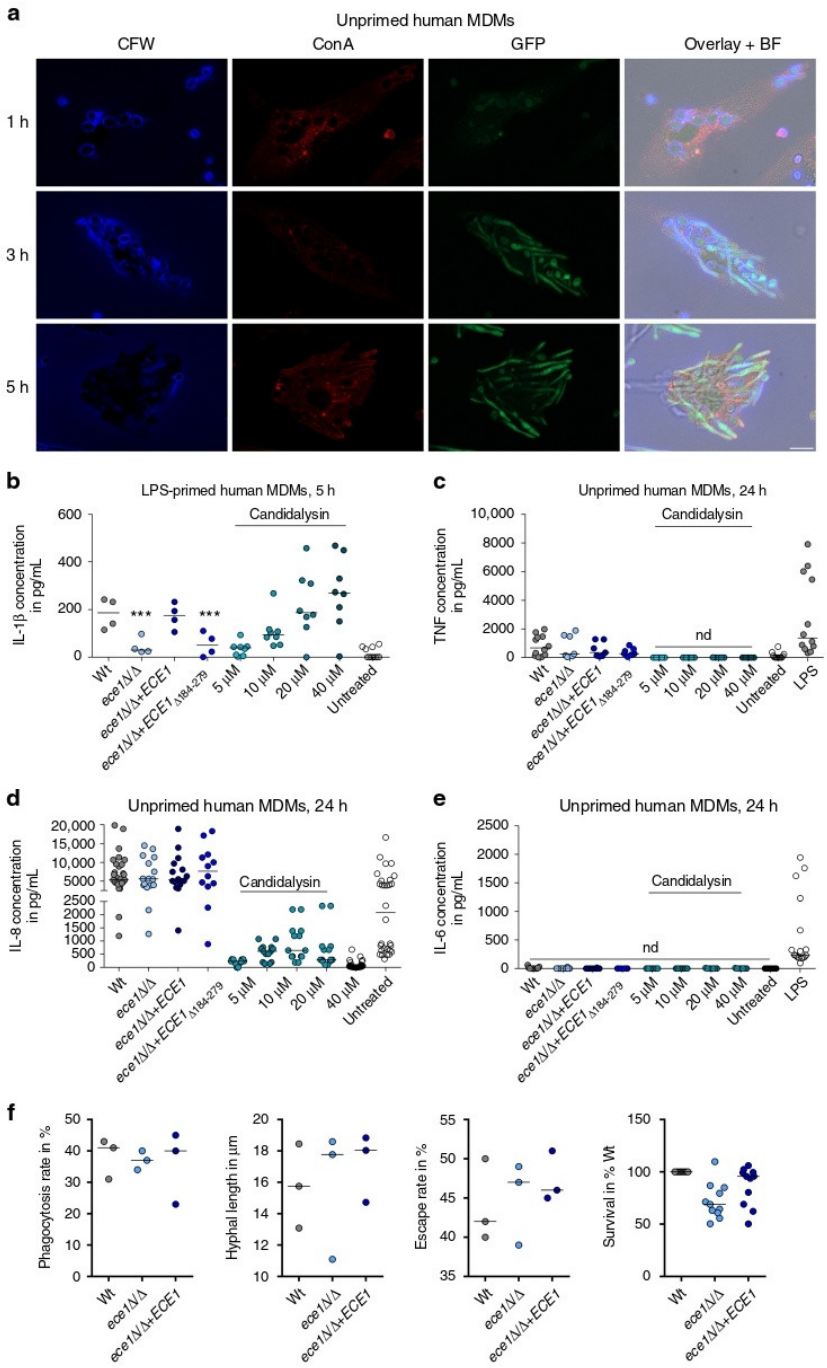


in an organ where predominantly hyphae are observed highlights an important role for Candidalysin in IL-1β induction.

**Candidalysin induces IL-1β release by human macrophages**. To test whether Candidalysin is a major driver of inflammasome activation in macrophages, we first investigated *ECE1* (coding for Candidalysin) expression using a *C. albicans* reporter strain expressing GFP under the control of the *ECE1* promoter after phagocytosis by primary human monocyte-derived macrophages (hMDMs) (Fig. 2a). Phagocytosed yeast cells produced hyphae within 3 h and hyphal cells showed a clear GFP fluorescence signal after 3 and 5 h, but not at initial stages (1 h) before hyphal formation was induced. Therefore, *ECE1* is strongly induced in *C. albicans* hyphae after phagocytosis by macrophages.

To study the influence of Candidalysin on inflammasome activation, we measured IL-1β secretion by LPS-primed primary

**Fig. 2** Candidalysin induces IL-1β release by human macrophages. **a** Fluorescence imaging of hMDMs infected with *C. albicans* cells expressing GFP under the control of the *ECE1* promoter. At indicated time points, samples were stained with ConA (non-phagocytosed fungal cells or extracellular hyphae) and Calcofluor White (CFW, phagocytosed and non-phagocytosed fungal cells). Single fluorescence channel images and a composite image of CFW, ConA, GFP, and the bright field (BF) image of one representative experiment out of three are shown. Scale bar 10 µm. **b** IL-1β release measured by ELISA in culture supernatants of LPS-primed hMDMs infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 10) or co-incubated with synthetic Candidalysin for 5 h. **c** TNF, **d** IL-8, and **e** IL-6 release measured by ELISA in culture supernatants of unprimed hMDMs infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 6) or co-incubated with synthetic Candidalysin for 24 h. **f** Phagocytosis rate (1 h *p.i.*), hyphal length of intracellular hyphae (3 h *p.i.*), the rate of hyphae piercing the macrophage membrane (10 h *p.i.*), and and the survival rate of *C. albicans* (3 h *p.i.*, cfus) is shown for human MDMs exposed to *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strain (*ece1*Δ/Δ) (MOI 1). Values are represented as scatterplot and the median of at least three different donors in at least two independent experiments. For statistical analysis, a one-way ANOVA with Dunnett's multiple comparison test was used. ***$p \leq 0.001$, nd not detectable. Significance compared to Wt

hMDMs after infection with Wt *C. albicans* and mutants lacking the entire *ECE1* gene (*ece1*Δ/Δ) or only the Candidalysin-encoding sequence (*ece1*Δ/Δ + $ECE1_{\Delta 184-279}$). Both mutant strains triggered significantly less IL-1β secretion from hMDMs compared to the Wt or to an *ECE1* re-integrant strain (*ece1*Δ/Δ + *ECE1*) (Fig. 2b). LPS-primed hMDMs stimulated with synthetic Candidalysin also secreted IL-1β in a dose-dependent manner (Fig. 2b). In contrast, secretion of inflammasome-independent cytokines IL-6, IL-8, and TNF from non-primed hMDMs was unaltered when stimulated with the Wt, *ece1*Δ/Δ or *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$ strains. Synthetic Candidalysin induced only low levels of IL-8 and no IL-6, or TNF (Fig. 2c–e). Thus, Candidalysin-deficient *C. albicans* strains exhibit specific defects in IL-1β induction, although they are fully capable of inducing inflammasome-independent pro-inflammatory cytokines in hMDMs.

To understand why the *ece1*Δ/Δ and *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$ mutant strains stimulated much less IL-1β secretion as compared to the Wt, we quantified the influence of *ECE1* deletion on the phagocytosis rate, hyphal length inside macrophages, the rate of hyphal outgrowth from macrophages, and fungal survival after phagocytosis. Deletion of *ECE1* did not influence any of these parameters and no significant differences to the Wt control were observed (Fig. 2f). Therefore, the decreased inflammasome activation in the absence of *ECE1* was not due to reduced uptake of fungal cells or hyphal defects.

An EL4.NOB-1 cell-derived IL-1 bioassay[36] verified that the IL-1β released in the supernatant of human MDMs stimulated by Wt *C. albicans* and the synthetic Candidalysin peptide is indeed bioactive (Fig. 3a). Western blot analyses revealed mature IL-1β in supernatants of LPS-primed phagocytes upon stimulation with *C. albicans* or synthetic Candidalysin (Fig. 3b). Of note, IL-1β secretion was absent in unprimed macrophages stimulated only with Candidalysin (Figs. 3b and 5a, see below). Thus, the priming step (signal 1) is indispensable for Candidalysin-mediated IL-1β production, indicating that Candidalysin selectively provides signal 2 for inflammasome activation. In addition to LPS, a PAMP-derived from gram-negative bacteria, β-glucan-containing molecules, such as Zymosan and Curdlan, were also sufficient as a priming signal for significant IL-1β production (Fig. 3c), which is consistent with the findings of Gross et al.[21]. Thus, while dispensable for the priming step of inflammasome induction (signal 1), Candidalysin is a potent trigger of inflammasome activation (signal 2) upon priming with bacterial or fungal PAMPs.

We conclude that Candidalysin is a major activator of the inflammasome and IL-1β secretion in primed hMDMs.

**Candidalysin induces IL-1β release by bone-marrow-derived macrophage (mBMDMs) and bone-marrow-derived dendritic cells (mBMDCs)**. To test for the specificity of inflammasome activation in phagocytic cells of different origin, we extended our analysis to primary murine mBMDMs and murine mBMDCs. In contrast to hMDMs, the *ece1*Δ/Δ and *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$ mutants induced Wt-like IL-1β secretion in mBMDMs (Fig. 4a–c), and a moderate, but non-significant reduction in IL-1β induction in mBMDCs (Fig. 4d). However, extracellularly administered synthetic Candidalysin induced a robust, dose-dependent IL-1β response in both mBMDMs and mBMDCs (Fig. 4a–d), whereas secretion of inflammasome-independent TNF in mBMDCs was not affected (Fig. 4e). Therefore, similar to human phagocytes, Candidalysin is able to induce IL-1β secretion from mBMDMs and mBMDCs.

Candidalysin thus acts as a potent inflammasome inducer in both human and murine phagocytes. While Candidalysin alone is sufficient for optimal inflammasome activation in human and murine macrophages and murine DCs, other fungal factors exhibit redundancy in stimulating IL-1β through inflammasome activation in murine phagocytes.

**Candidalysin-activates the NLRP3 inflammasome**. Secretion of IL-1β upon inflammasome activation requires proteolytic processing by caspase-1[26,37]. To investigate whether Candidalysin-triggered processing of pro-IL-1β into mature IL-1β is mediated by caspase-1, we inhibited caspase-1 with the irreversible inhibitors Z-YVAD-FMK or Ac-YVAD-cmk.

Caspase-1 inhibition reduced IL-1β secretion in both *C. albicans*-infected human and murine mononuclear cells after exposure to Candidalysin (Fig. 5a). Yet, both inhibitors did not globally reduce cytokine secretion, because IL-8 or TNF levels were mainly unaltered by Z-YVAD-FMK or Ac-YVAD-cmk treatment (Fig. 5b). Thus, Candidalysin-induced IL-1β secretion is dependent on caspase-1 proteolytic activity. In line with these findings, we observed caspase-1 activation in Candidalysin-treated hMDMs using the fluorescent probe FAM-YVAD-FMK (Fig. 5c). Using a Caspase-GLO assay we detected caspase-1 activity in Wt *C. albicans* stimulated mBMDCs, but significantly reduced caspase-1 activity in mBMDCs exposed to the *ece1*Δ/Δ and *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$ mutants (Fig. 5d). By western blotting, we observed cleaved caspase-1 in culture supernatants of Candidalysin-treated hMDMs as well as mBMDMs and mBMDCs (Fig. 5e). A direct comparison between unprimed and LPS-primed phagocytes showed that the initial priming step is indispensable for Candidalysin-mediated inflammasome activation not only in human macrophages (see above), but also in murine mononuclear phagocytes (Figs. 5a–e and 4c, see above).

Inflammasomes are large protein complexes that include NLR proteins, the adapter protein ASC and pro-caspase-1. Besides NLRP3, which has been demonstrated to be crucial for *C. albicans*-induced inflammasome activation[21,22], several other NLRs, including NLRC4 and NLRP1, trigger the formation of inflammasomes. By using a genetic approach to test whether the

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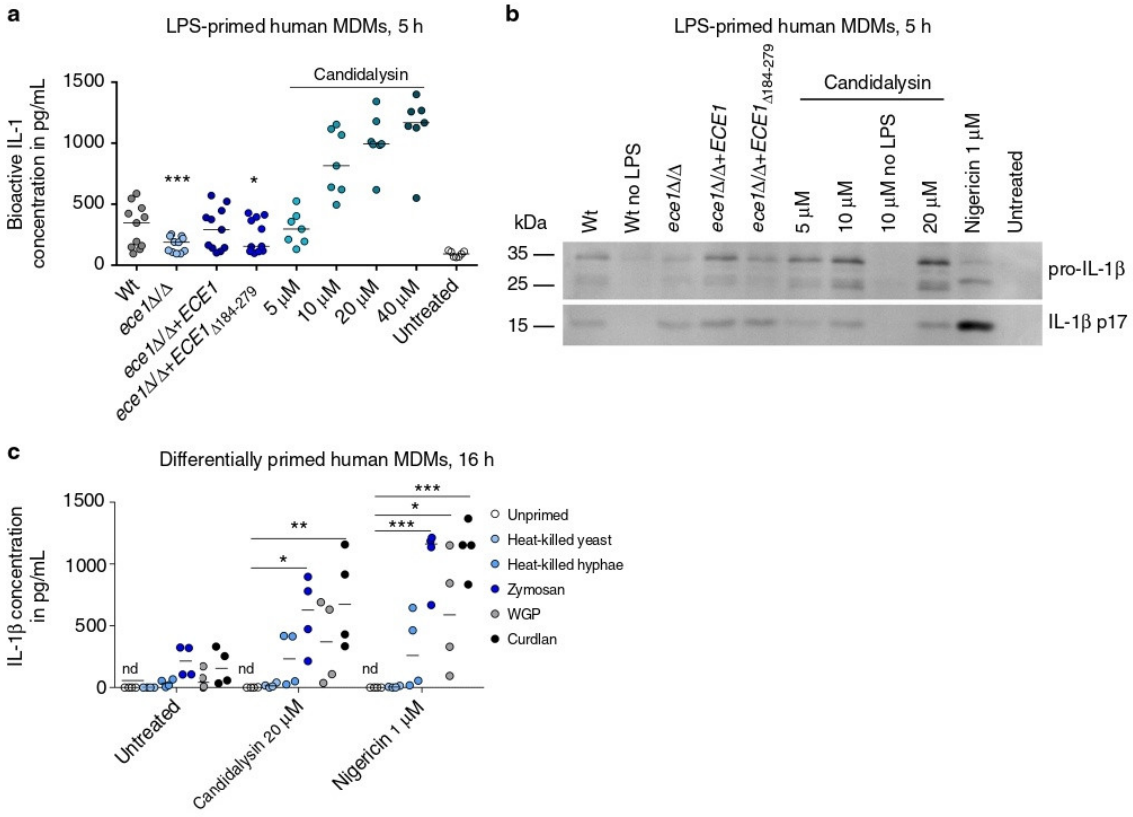


**Fig. 3** Candidalysin-dependent release of bioactive, mature IL-1β by primed hMDMs. **a** Levels of bioactive IL-1 measured in culture supernatants of LPS-primed hMDMs that were infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 10) or co-incubated with synthetic Candidalysin. Bioactive IL-1 was quantified by stimulation of EL4.NOB-1 cells culture supernatants and correlation of the secreted murine IL-2 to a concentration range of recombinant human IL-1β. **b** The presence of processed IL-1β (p17) detected by western blotting in the supernatant of LPS-primed or unprimed (no LPS) hMDMs that were infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 10) or co-incubated with synthetic Candidalysin for 5 h. A representative image of three independent experiments or donors is shown. **c** IL-1β levels were determined by ELISA in culture supernatants of human MDMs that were primed for 16 h with heat-killed *C. albicans* yeasts or hyphae, Zymosan (*Saccharomyces cerevisiae* cell wall), WGP (whole glucan particles; *S. cerevisiae* β-glucan) or Curdlan (β-1,3 glucan) followed by treatment with synthetic Candidalysin or Nigericin for 5 h. Values are represented as scatterplot and the median of at least three different donors in at least two independent experiments. For statistical analysis, a one-way ANOVA with Dunnett's multiple comparison test was used. $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$, nd not detectable. Significance compared to Wt (**a**) or to unprimed cells (**c**)

NLRP3 inflammasome is activated by Candidalysin, we stimulated LPS-primed mBMDCs from *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$ or *Casp1*$^{-/-}$ mice[21]. IL-1β secretion was dependent on NLRP3, ASC and caspase-1 respectively (Fig. 5f). Secretion of the inflammasome-independent cytokine TNF was indistinguishable among all tested genotypes (Fig. 5g). These data demonstrate that caspase-1 is fundamentally required for Candidalysin-induced IL-1β secretion via classical NLRP3 inflammasome activation.

**Actin-mediated events and filamentation induce inflammation.** Candidalysin is secreted by *C. albicans* hyphae[31]. Since phagocytes can be exposed to hyphae either pre-phagocytosis or post-phagocytosis, immune cells may be exposed to Candidalysin intracellularly or extracellularly. Therefore, we asked whether internalization of Candidalysin is required for inflammasome activation. hMDMs pre-treated with Cytochalasin D, a well-characterized inhibitor of phagocytosis that impairs actin filament assembly, showed significantly decreased Candidalysin-dependent IL-1β, but not IL-8 secretion (Fig. 6a). In contrast, IL-1β secretion induced by the potassium ionophore Nigericin was unaffected (Fig. 6a). This suggests that cytoskeletal movement and/or peptide internalization are required for inflammasome activation by Candidalysin and that the mechanism of inflammasome activation by Candidalysin and Nigericin differs.

Candidalysin is necessary for optimal inflammasome activation by *C. albicans* in human macrophages and murine phagocytes (see above). However, deletion of *ECE1* did not completely abrogate IL-1β secretion, indicating that other fungal factors or hypha formation per se (e.g., via physical forces) may be crucial for inflammasome activation[7,8,10–13]. In agreement with this, the *C. albicans* strain *efg1*Δ/Δ/*cph1*Δ/Δ, which is defective in hyphal formation and the expression of hypha-associated factors[4] induced even lower IL-1β secretion by hMDMs than the *ece1*Δ/Δ mutant (Fig. 6b). However, the *hgc1*Δ/Δ mutant which is defective in hyphal induction, but still can express Candidalysin to some extent[38], induced similar IL-1β levels as the *ece1*Δ/Δ mutant that can form hyphae, but cannot produce Candidalysin (Fig. 6b). Nonetheless, supplementation of synthetic Candidalysin to *efg1*Δ/Δ/*cph1*Δ/Δ, *hgc1*Δ/Δ, or *ece1*Δ/Δ *C. albicans* cells

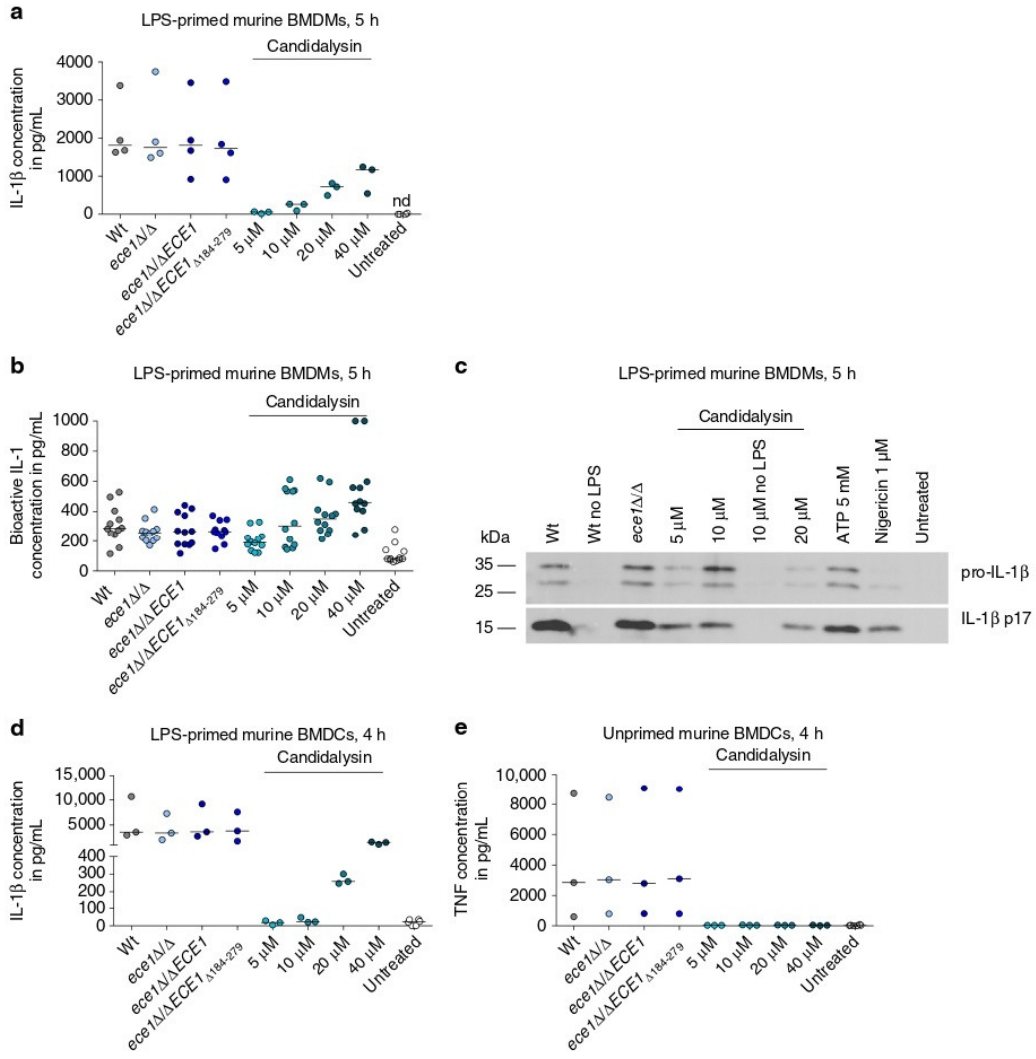


**Fig. 4** Candidalysin induces IL-1β release in murine mononuclear cells. **a** IL-1β release measured by ELISA in culture supernatants LPS-primed mBMDMs infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 6) or co-incubated with synthetic Candidalysin for 5 h. **b** Levels of bioactive IL-1 measured in culture supernatants of LPS-primed mBMDMs that were infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 6) or co-incubated with synthetic Candidalysin. Bioactive IL-1 was quantified by stimulation of EL4.NOB-1 cells culture supernatants and correlation of the secreted murine IL-2 to a concentration range of recombinant human IL-1β. **c** The presence of processed IL-1β (p17) detected by western blotting in the supernatant of LPS-primed or unprimed (no LPS) mBMDMs that were infected with *C. albicans* Wt re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 10) or co-incubated with synthetic Candidalysin for 5 h. A representative image of three independent experiments or donors is shown. **d** IL-1β and **e** TNF levels measured by ELISA in culture supernatants of LPS-primed or unprimed mBMDCs respectively, that were infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 5) or co-incubated with synthetic Candidalysin for 5 h (mBMDMs) or 4 h (mBMDCs). Secreted IL-1β (**a**, **d**) and TNF (**e**) were determined by ELISA. Values are represented as scatterplots and the median of at least three different replicates ($n \geq 3$). nd not detectable

restored IL-1β secretion to Wt levels (Fig. 6b). This demonstrates that Candidalysin is necessary for inflammasome activation and can compensate for the lack of other inflammasome-stimulating attributes of *C. albicans*. Interestingly, this compensatory mechanism requires fungal viability, as the rescue effect was not observed with heat-killed *C. albicans* cells as compared to untreated LPS-primed hMDMs.

**Candidalysin activates the inflammasome via $K^+$ efflux**. Several mechanisms, such as lysosomal destabilization followed by the release of lysosomal cathepsins, production of reactive oxygen species (ROS), or the permeation of cell membranes leading to ion fluxes are discussed as upstream activators of the NLRP3 inflammasome during fungal infection[39]. To elucidate how Candidalysin triggers inflammasome activation, we first inhibited

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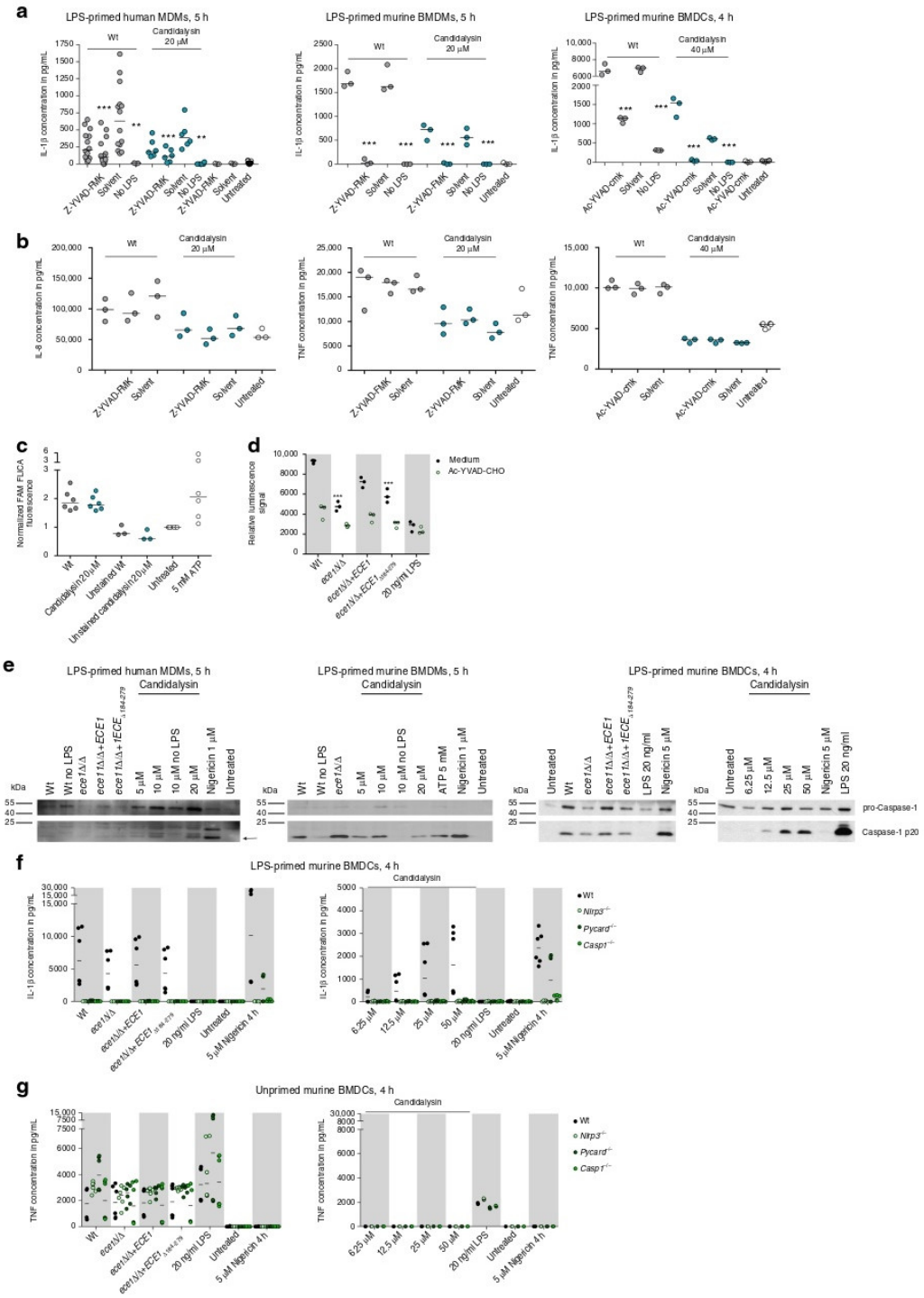


potassium efflux, a common mechanism of inflammasome activation by bacterial toxins and *C. albicans*[21,40]. Inhibition of potassium efflux was achieved by increasing the extracellular potassium concentration or by blocking ATP-dependent potassium channels with glibenclamide. Similar to the potassium ionophore Nigericin, Candidalysin-dependent IL-1β secretion by

**Fig. 5** Candidalysin activates the NLRP3 inflammasome. **a** IL-1β and **b** IL8 (hMDMs) or TNF (mBMDMs, mBMDCs) release measured by ELISA in culture supernatants of LPS-primed or unprimed (no LPS) hMDMs, mBMDMs, or mBMDCs that were infected with *C. albicans* Wt (MOI 10, 6, or 5 respectively) or co-incubated with synthetic Candidalysin for 5 h (hMDMs, mBMDMs) or 4 h (mBMDCs). The caspase-1-inhibitor Z-YVAD-FMK (88.9 μM, hMDMs and mBMDMs) or Ac-YVAD-cmk (20 μM, mBMDCs) or the inhibitor solute control DMSO was added 1 h prior to infection. **c** Caspase-1 activation measured by fluorescence intensity after staining with FAM-YVAD-FMK FLICA™ in LPS-primed hMDMs that were infected with Wt *C. albicans* (MOI 10), co-incubated with synthetic Candidalysin for 5 h, or treated with ATP for 30 min. **d** Caspase-1 activity measured by luminescence intensity (Caspase1-Glo inflammasome assay) in cell culture supernatants of LPS-primed mBMDCs that were infected for 5 h with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 5). **e** Cleavage of caspase-1 into the active p20 form (arrow) assessed by western blotting in LPS-primed or unprimed (no LPS) hMDMs, mBMDMs, or mBMDCs that were infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 10 mBMDMs, hMDMs or 5 mBMDCs) or co-incubated with synthetic Candidalysin or Nigericin for 5 h (mBMDMs, hMDMs) or 4 h (mBMDCs). Representative images of three independent experiments or donors are shown. **f** IL-1β and **g** TNF levels measured by ELISA in culture supernatants of f LPS-primed or **g** unprimed Wt, *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$ or Casp1$^{-/-}$ mBMDCs that were infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 5) or co-incubated with synthetic Candidalysin or Nigericin for 4 h. Values are presented as scatterplots and the median of at least three different donors or replicates ($n \geq 3$). For KO mBMDCs, all technical replicates are shown of the experiments that were performed in duplicates. For statistical analysis (**a**–**c**), a one-way ANOVA with Dunnett's multiple comparison test was used. ***$p \leq 0.001$, **$p \leq 0.01$, nd not detectable

human MDMs was inhibited by blocking potassium efflux, while IL-8 secretion was not affected (Fig. 6c). In murine BMDMs and BMDCs potassium efflux was similarly important for Candidalysin-dependent IL-1β secretion, but not for TNF secretion (Fig. 6d, e).

Next, we investigated the impact of ROS on Candidalysin-triggered inflammasome activation by inhibiting the NADPH-oxidase-dependent ROS system with (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (PDTC). This inhibitor exhibited no effect on Candidalysin-induced IL-1β secretion by hMDMs (Fig. 6f). Consistently, the deletion of *ECE1* or the Candidalysin-encoding sequence alone did not reduce *C. albicans*-induced ROS production in hMDMs, suggesting that Candidalysin does not contribute to fungal ROS induction. Accordingly, ROS levels induced in hMDMs by synthetic Candidalysin are low compared to ROS levels induced by *C. albicans* cells (Fig. 6g, h).

Another mechanism of NLRP3 inflammasome activation involves lysosomal destabilization and lysosomal content release to the cytosol. Proteases such as cathepsins, which require lysosomal acidification to become catalytically active, have been suggested to mediate this effect[39]. Blocking lysosomal acidification with the vacuolar $H^+$ ATPase inhibitor Bafilomycin A1 did not reduce Candidalysin-induced IL-1β secretion of hMDMs (Fig. 7a), suggesting that phagosomal destabilization is also not involved in Candidalysin-dependent inflammasome activation. Similarly, co-localization of Wt, *ece1*Δ/Δ, or *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$ cells with the late endo(lyso)somal marker LAMP1, the late maturation markers Phosphatidylinositol 4-phosphate (PI(4)P) and Rab7[41], as well as with the acidic organelle dye LysoTracker, indicated that phagosome maturation is not affected by Ece1 (Fig. 7a–g). Lastly, administration of synthetic Candidalysin did not lead to a loss of acidification of mature phagosomes loaded with heat-killed *C. albicans* cells as monitored by LysoTracker staining (Fig. 7d). Consistent with the fact that most activators engaging the lysosomal pathway are particles like alum or uric acid crystals, our data indicate that lysosomal mechanisms are not involved in inflammasome activation by Candidalysin. Together, we conclude that induced potassium efflux operates as a main trigger of Candidalysin-induced NLRP3 inflammasome activation comparable to the role of potassium efflux in NLRP3 activation by bacterial PFTs[40].

**Candidalysin is required for damage of hMDMs and mBMDCs**. Previous studies indicate that *C. albicans* causes macrophage damage by two different mechanisms: programmed caspase-1-dependent and inflammation-associated cell death (pyroptosis) within the first hours of infection, .followed by physical cell membrane rupture due to sustained hypha formation[9,12,17] and glucose consumption[18] at later time points. As Candidalysin is essential for fungal-induced epithelial cell damage[31], but also activates caspase-1 (see above), we tested whether Candidalysin contributes to *C. albicans*-induced cell damage of mononuclear phagocytes at different time points of infection. By measuring the release of cytoplasmic LDH into the supernatants as a read-out for host cell damage we demonstrate that externally administered synthetic Candidalysin dose-dependently induces cell lysis of human and murine macrophages and murine DCs already at early time-points (Fig. 8a–d). Using human macrophages infected with *C. albicans* for 24 h, we demonstrate that loss of the *ECE1* gene is associated with a loss of the full damage potential of *C. albicans* (Fig. 8a). This coincided with a reduction of metabolic activity of hMDMs (Fig. 8b). LDH levels released from *C. albicans*-infected hMDMs at 5 h were similar to those from an uninfected control. While early *C. albicans*-induced mBMDM damage measured by LDH release did not indicate an *ECE1*-dependency or Candidalysin-dependency (Fig. 8c), damage to mBMDCs induced by *C. albicans* was again partly *ECE1*- and Candidalysin-dependent (Fig. 8d).

To study the damage kinetics of primary hMDMs in more detail, we used propidium iodide (PI) staining to monitor dead immune cells as described in ref. [12]. Similarly, we observed that damage of hMDMs by *C. albicans* Wt (Fig. 8e) occurs in a characteristic biphasic pattern[12]. The first 10–12 h are characterized by a slow increase in host cell damage, whereas in the second phase between 12–24 h damage occurs more rapidly. When *ECE1* or only the Candidalysin-encoding sequence was deleted, the damage potential of *C. albicans* was reduced in both phases in hMDMs (Fig. 8e) highlighting a significant contribution of Candidalysin to *C. albicans*-induced cell damage in human macrophages. Incubation of primary hMDMs with synthetic Candidalysin showed direct, dose-dependent cytotoxicity, as damage (PI-positive cells) occurred rapidly and was saturated within 6 h (Fig. 8f).

In summary, Candidalysin is sufficient to cause rapid damage to both human and murine mononuclear cells and is a major contributor to fungal-mediated damage of hMDMs and mBMDCs.

**Candidalysin-induced cell death is caspase-1-independent**. We observed both Candidalysin-dependent inflammasome activation and early damage of phagocytes. We, therefore, asked whether the inflammatory response and host cell damage in response to Candidalysin are connected and whether cell damage is associated with pyroptosis.

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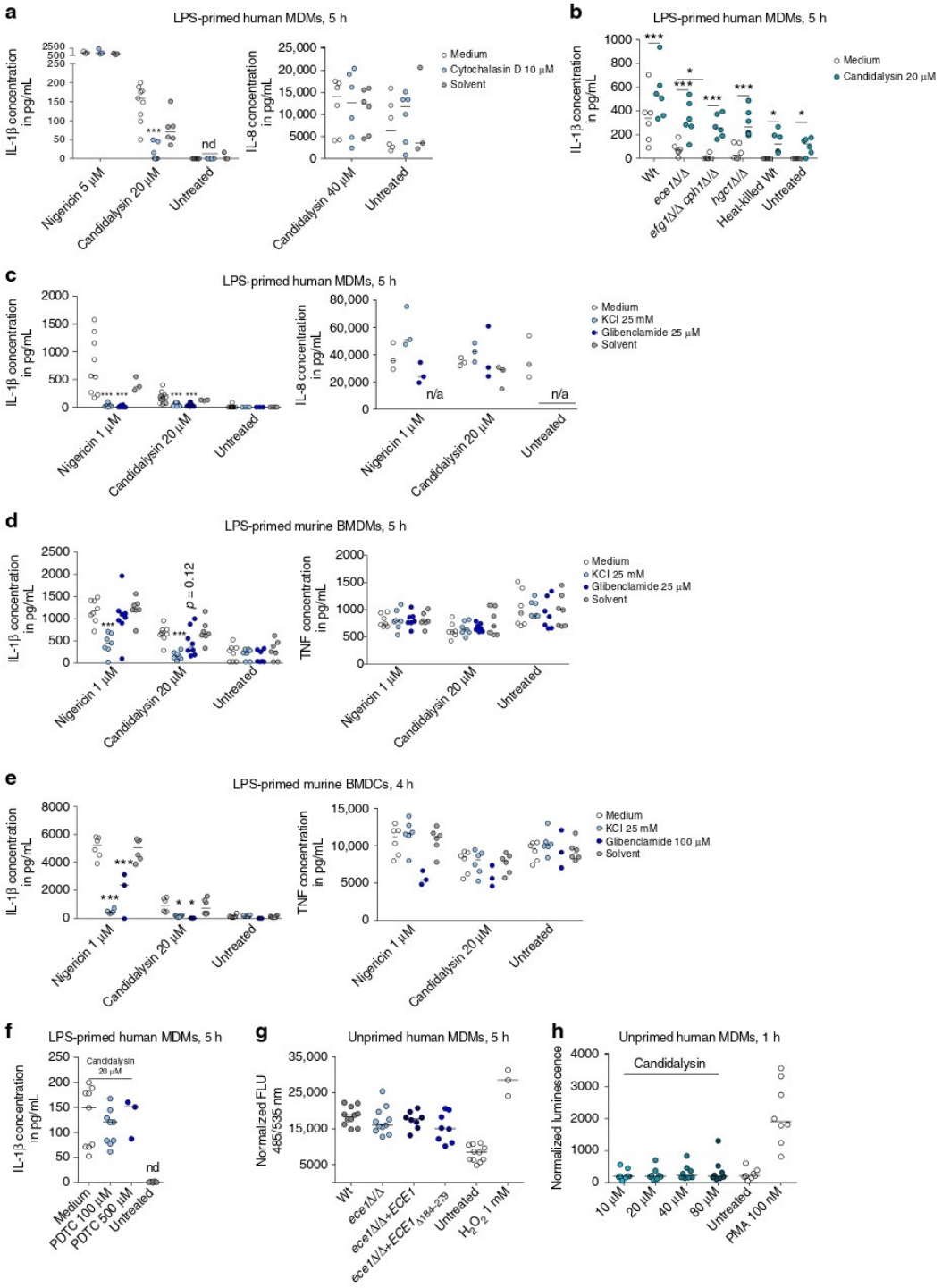


First, to exclude other forms of programmed cell death, we determined whether Candidalysin can induce apoptosis or necroptosis in primary hMDMs. *C. albicans* is able to trigger apoptosis[42] and many bacterial PFTs can induce a programmed form of necrosis, necroptosis[43–45]. Annexin V staining suggested minimal exposure of cell surface phosphatidylserine in Candidalysin-treated hMDMs and hMDMs infected with Wt or *ece1*Δ/Δ *C. albicans* strains (Fig. 9a). Since Annexin V does not exclusively stain apoptotic but also necroptotic cells, we assayed for the activation of the apoptotic caspases 3 and 7. Both caspases

**Fig. 6** Potassium-dependent and actin-dependent inflammasome activation. **a** IL-1β and IL-8 levels measured by ELISA in culture supernatants of LPS-primed hMDMs treated with synthetic Candidalysin or Nigericin for 5 h. Selected samples were pre-treated with the actin cytoskeleton inhibitor Cytochalasin D or the inhibitor solute control DMSO 1 h prior to administration of Candidalysin. **b** IL-1β release measured by ELISA in culture supernatants of LPS-primed hMDMs that were infected with *C. albicans* Wt, *ece1*Δ/Δ, *efg1*Δ/Δ/*cph1*Δ/Δ, *hgc1*Δ/Δ mutant strains, or heat-killed Wt (MOI 10) in presence or absence of synthetic Candidalysin for 5 h. **c**–**f** IL-1β and **c** IL-8 or **d**, **e** TNF levels measured by ELISA in culture supernatants of LPS-primed **c**, **f** hMDMs **d** mBMDMs, and **e** mBMDCs. Phagocytes were treated with synthetic Candidalysin or Nigericin for 5 or 4 h (BMDCs). Selected samples were pre-treated with the following inhibitors 1 h prior to administration of Candidalysin: **c**–**e** the potassium channel inhibitor glibenclamide or inhibitor solute control DMSO, KCl was added after LPS priming, **f** (2R,4 R)-4-aminopyrrolidine-2,4-dicarboxylate (PDTC). **g** Intracellular ROS production in hMDMs pre-loaded with 20 µM H2DCF-DA for 30 min and infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 10) or treated with $H_2O_2$ (positive control) for 5 h. Fluorescence (Ex 485/Em 535) measured immediately after infection was subtracted from fluorescence (Ex 485/Em 535) measured after 5 h. **h** Total ROS production in hMDMs subjected to synthetic Candidalysin or PMA (positive control) was monitored by Luminol-enhanced chemiluminescence. Relative luminescence units (RLU) were recorded for 60 min and the difference between maximum and minimum luminescence values was calculated. Data are represented as scatterplot and median of at least three different donors ($n \geq 3$) or independent experiments. For statistical analysis, a one-way ANOVA with Dunnett's multiple comparison test was used. For analysis of the different *C. albicans* mutants, a two-way ANOVA with Sidak's multiple comparison test was applied. ***$p \leq 0.001$, *$p \leq 0.05$, n/a not applicable, nd not detectable

were weakly activated upon co-incubation with Candidalysin and no differences were observed when comparing hMDMs stimulated with Wt or *ece1*Δ/Δ *C. albicans* cells (Fig. 9b). Inhibition of necroptosis with the RIP1-kinase inhibitor Necrostatin-1 also did not diminish macrophage damage (Fig. 9c). Thus, Candidalysin does not appear to trigger apoptosis or necroptosis in human macrophages .

As pyroptosis is characterized by inflammasome activation and subsequent caspase-1-dependent IL-1β secretion[19,46], we measured early macrophage damage in human and murine mononuclear cells after inflammasome priming and the addition of the caspase-1 inhibitor Z-YVAD-FMK. While Caspase-1 inhibition reduced Candidalysin-dependent IL-1β secretion (Fig. 5a, see above), inhibitor treatment had no effect on Candidalysin-induced host cell lysis in hMDMs or mBMDMs, and damage was independent of LPS priming (Fig. 10a, b), though LPS priming was required for cell death of mBMDCs (Fig. 10c). Although previous reports demonstrated that pyroptosis contributes to *C. albicans*-mediated damage of mBMDMs[12,17], LDH levels released by *C. albicans*-infected mBMDMs and mBMDCs were slightly but non-significantly reduced after caspase-1-inhibition (Fig. 10b, c). In line with this, blocking inflammasome activation by inhibiting the host actin cytoskeleton or potassium efflux reduced Candidalysin-induced inflammasome activation (IL-1β release), but not Candidalysin-induced cell damage (Figs. 6a–e and 9d, e, see above).

Damage by Candidalysin is, therefore, mainly independent of inflammasome activation. To exclude that there are differences in the dynamics of *C. albicans* and Candidalysin-induced cell death and to verify our analysis using a different caspase-1 inhibitor, MDMs were LPS-primed and exposed to the caspase-1 inhibitor VX-765. Caspase-1 inhibition did not significantly influence the dynamics of *C. albicans* (Fig. 10d) or Candidalysin (Fig. 10e) induced cell death, although it was effective in reducing inflammasome-dependent IL-1β secretion (Fig. 10f).

Finally, we applied a genetic approach to show that Candidalysin-mediated damage is not pyroptotic. We exposed mBMDCs deficient in the inflammasome components NLRP3, ASC, or caspase-1 to synthetic Candidalysin. Similar to the other immune cell types tested, Candidalysin-induced damage in mBMDCs was independent of LPS-priming, caspase-1, ASC, or NLRP3 (Fig. 10g). As expected, cell lysis induced by live Wt, but also the *ece1*Δ/Δ mutant, *C. albicans* cells was at least partially dependent on the inflammasome, as the overall damage was reduced in *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$, or *Casp1*$^{-/-}$ as compared to Wt mBMDCs (Fig. 10g). Thus, *C. albicans* lacking Candidalysin can still induce inflammasome-dependent cell death (pyroptosis). Importantly, the reduction in damage caused by the *ece1*Δ/Δ or *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$ mutant compared to the *C. albicans* Wt was still present in DCs lacking Nlrp3, ASC, or caspase-1.

In summary, these data indicate that *C. albicans*-induced pyroptosis in mononuclear phagocytes is independent of Candidalysin. Moreover, while Candidalysin induces the NLRP3 inflammasome and caspase-1 activation, Candidalysin-induced host cell lysis is independent of the inflammasome and caspase-1.

## Discussion

Phagocytes of the host's innate immune system, such as macrophages and DCs, are pivotally important for efficient clearance of *C. albicans* infections and initiation of inflammatory responses[47]. The cytolytic peptide toxin Candidalysin has recently been identified as a critical virulence factor that intercalates into host membranes and damages epithelial cells during mucosal *C. albicans* infections[31]. Furthermore, Candidalysin drives protective innate type 17 cell responses during oral candidiasis[48], immunopathology during vaginal infections[49], and mediates translocation through intestinal barriers[38].

In this study, using human and mouse mononuclear phagocytes, we show that Candidalysin activates the NLRP3 inflammasome (signal 2 agent), resulting in the secretion of mature IL-1β in a caspase-1-dependent manner. Intriguingly, however, Candidalysin-induced cytolysis is independent of the inflammasome and pyroptosis. Our work identifies Candidalysin as the first fungal toxin with such dual action on phagocytes of the innate immune system.

Inflammasome activation is a two-step process, requiring an initial priming step and a second, inflammasome-activating step[21,23,24]. Our data show that Candidalysin selectively provides a stimulus for the second, inflammasome-activation step, as the toxin alone was not able to induce inflammasome activation without priming by LPS or β-glucan-containing molecules like Zymosan or Curdlan, similar to other NLRP3-inflammasome activators, such as Nigericin or ATP. Multiple stimuli for inflammasome activation, such as mitochondrial damage, ROS production, endo-lysosomal damage, and potassium efflux have been identified[50]. Potassium efflux, in particular, seems to be a central trigger for inflammasome activation for many bacterial PFTs, but also for *C. albicans*[21,40]. We demonstrate that Candidalysin triggers inflammasome activation via potassium efflux in human macrophages, as well as murine BMDMs and BMDCs, suggesting that Candidalysin functions similarly to bacterial PFTs, most likely by inducing membrane permeabilisation and a subsequent drop in cytosolic potassium levels[40,51].

While ROS have previously been implicated in *C. albicans*-dependent inflammasome activation in mBMDCs[21], ROS inhibition with PDTC had no effect on IL-1β secretion in primary

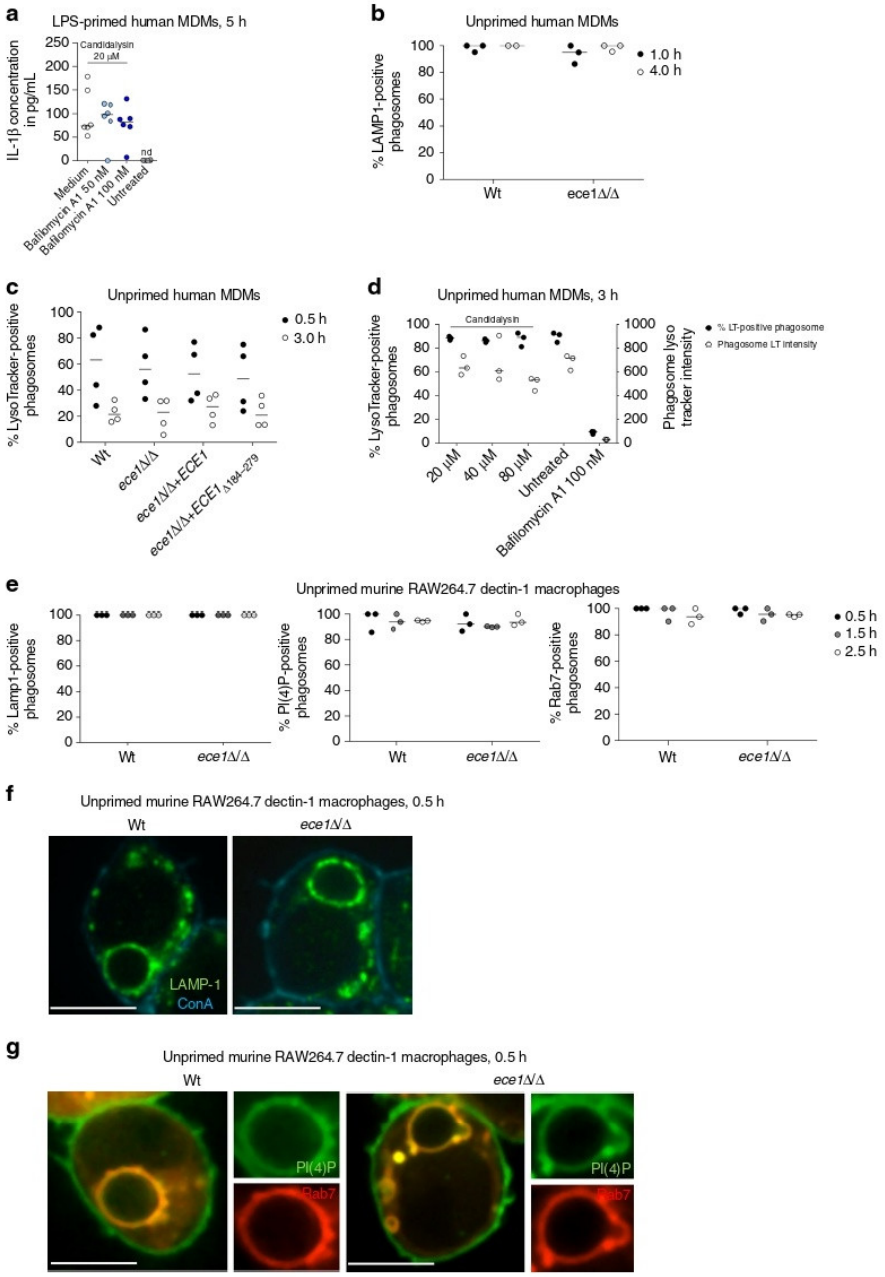


**Fig. 7** Ece1-independent phagosome maturation. **a** IL-1β levels measured by ELISA in culture supernatants of LPS-primed hMDMs. Cells were treated with synthetic Candidalysin for 5 h. Selected samples were pre-treated with the vacuolar H+ ATPase inhibitor Bafilomycin A1 1 h prior to administration of synthetic Candidalysin. **b**, **c** Human MDMs were infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 5) and co-localization of *C. albicans*-containing phagosomes with **b** the phagosomal marker LAMP1 or **c** the lysosomal acidification marker LysoTracker was quantified at indicated time points. **d** Human MDMs pre-stained with LysoTracker were infected with heat-killed *C. albicans* Wt (MOI 5) for 3 h in presence or absence of Bafilomycin A1 (phagosomal acidification inhibitor) or synthetic Candidalysin. *C. albicans*-containing phagosomes were quantified for the percentage of LysoTracker-positive phagosomes and LysoTracker intensity. **e** Murine RAW264.7 Dectin-1 macrophages were infected with *C. albicans* Wt or *ece1*Δ/Δ mutant yeasts (MOI 2) and co-localization of *C. albicans*-containing phagosomes with the phago(lyso)somal markers Lamp1, PI(4)P, and Rab7 was quantified at indicated time points. **f**, **g** Murine RAW264.7 Dectin-1 macrophages were infected with *C. albicans* Wt or *ece1*Δ/Δ mutant strain as described in **e**. Representative image of Lamp1 (**f**) or PI(4)P and Rab7 (**g**) acquisition 30 min after phagocytosis. ConA staining of non-phagocytosed *C. albicans*. Scale bar 8 μm. Values are represented as scatterplot with median of three independent donors or experiments ($n \geq 3$)

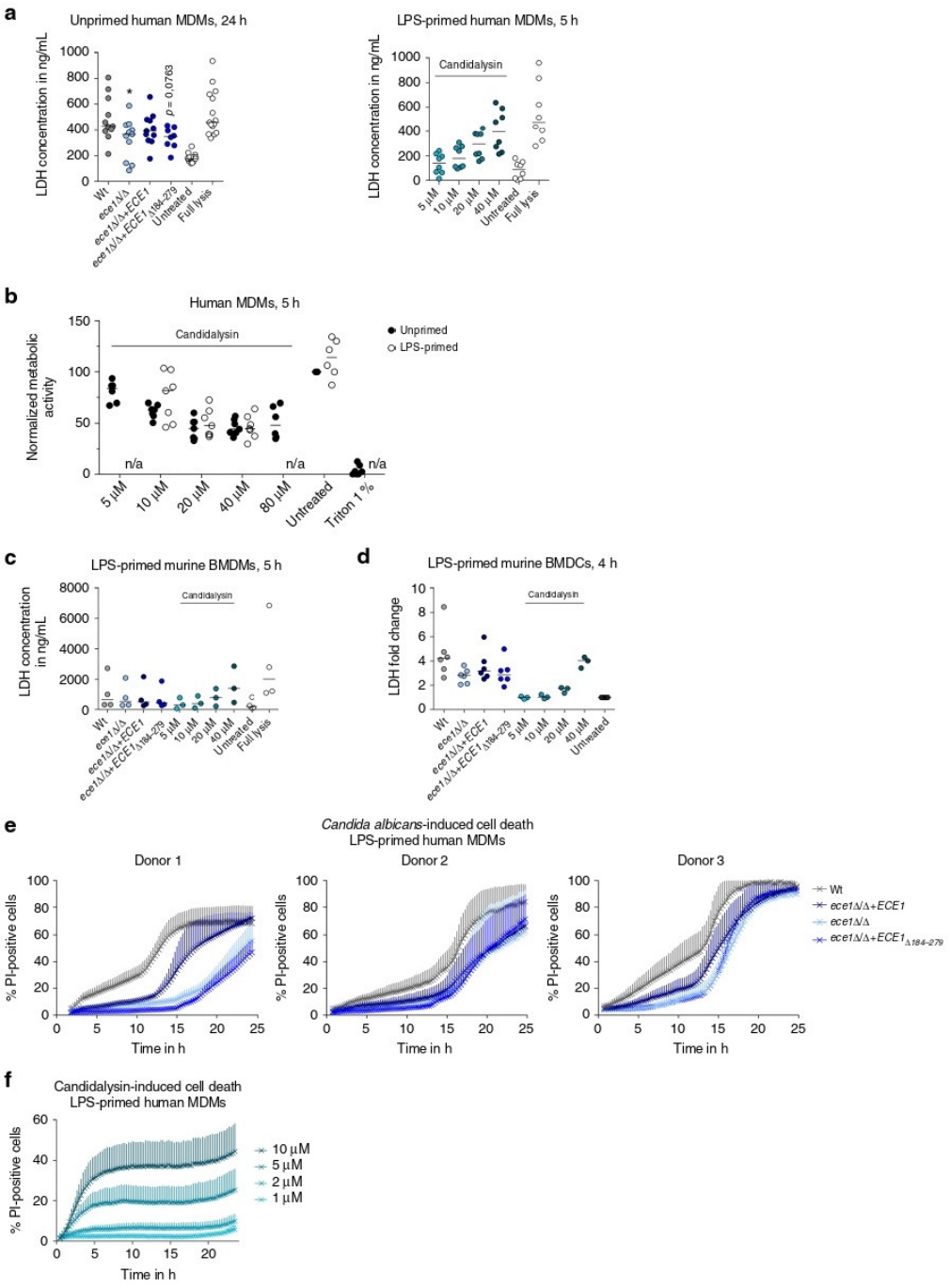


**Fig. 8** Candidalysin-dependent damage of hMDMs. **a**, **c**, **d** Macrophage lysis was quantified by measuring LDH release in **a** unprimed hMDMs or LPS-primed **c** mBMDMs or **d** mBMDCs that were infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 6) for 5 or 24 h and in LPS-primed **a** hMDMs, **c** mBMDMs, or **d** mBMDCs that were incubated with synthetic Candidalysin for 5 h. **b** Metabolic activity of LPS-primed or unprimed hMDMs treated with synthetic Candidalysin for 5 h was measured using XTT. 1% Triton X-100 was added as a positive control. **e**, **f** Macrophage damage over time was assessed by quantifying propidium iodide (PI)-positive cells in LPS-primed hMDMs infected with **e** *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$) (MOI 6) or **f** incubated with synthetic Candidalysin. **a**–**d** Values are represented as scatterplot with median of at least three different donors ($n \geq 3$). For statistical analysis, a one-way ANOVA with Dunnett's multiple comparison test was used. $^*p \leq 0.05$, significance compared to Wt infection. **e** The results of three different donors are displayed separately due to strong donor variability. Data are shown as mean + SD of two independent positions in two wells. **f** Data are shown as mean + SD of six independent donors

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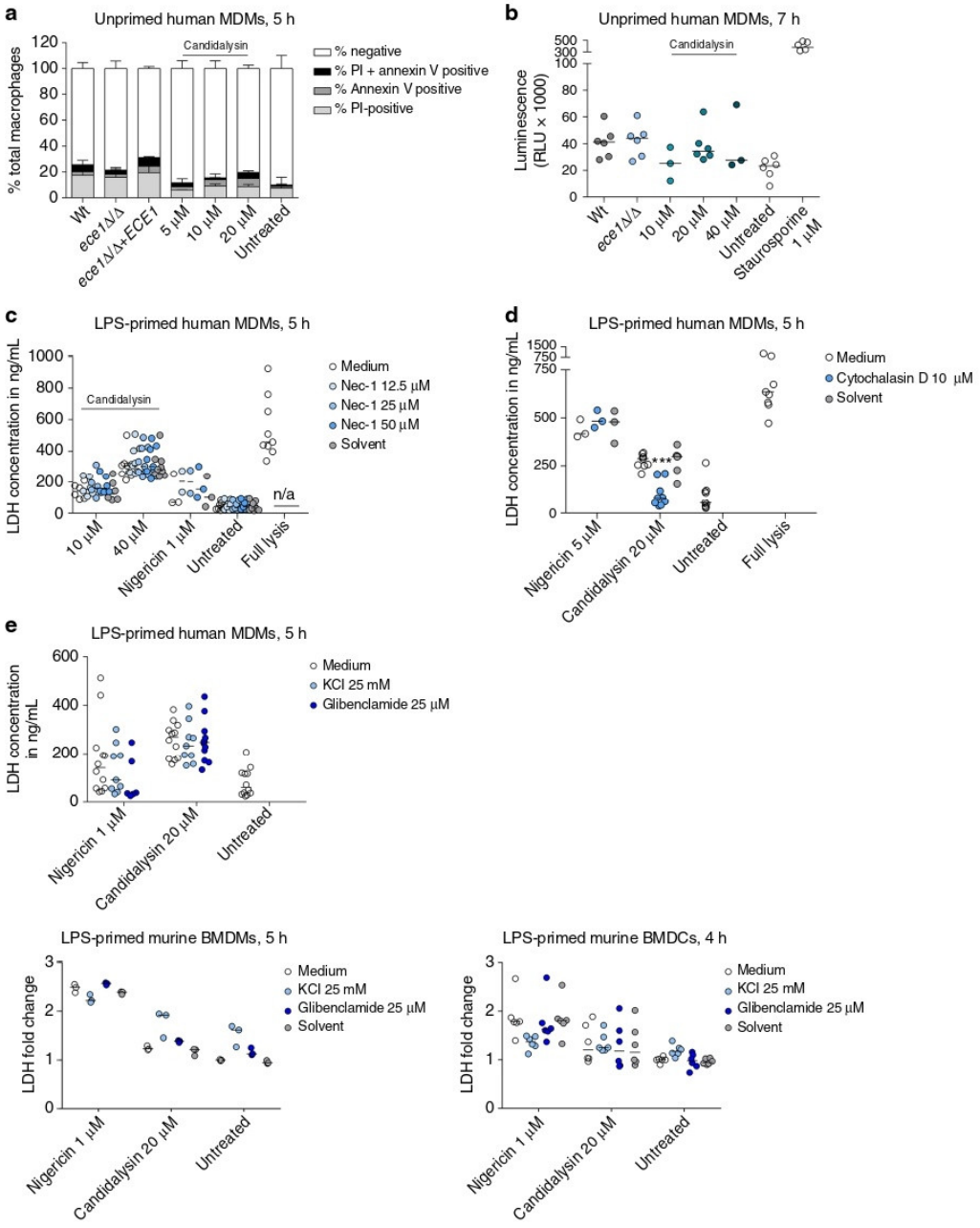


human macrophages. Phagosomal destabilization may also activate the inflammasome, a process thought to involve the release of lysosomal enzymes including cathepsins[52]. However, we found no evidence for phagosomal destabilization and resulting inflammasome activation, which we had hypothesized as a potential result of the intra-phagosomal onset of hypha transformation and lytic activities of Candidalysin produced.

Of note, Candidalysin-dependent inflammasome activation and cellular damage were strongly inhibited by the F-actin polymerisation inhibitor Cytochalasin D. To our knowledge, this is the first description of a pathogen-derived PFT whose inflammasome activation properties depend on the host cell actin cytoskeleton. In contrast, the ability of bacterial PFTs like Nigericin to activate Nlrp3 is not affected by cytoskeleton inhibitors (this study)[53]. These data suggest that inflammasome activation by Candidalysin may depend on toxin internalization[40,52] or actin-mediated pore-assembly at the cell surface[54].

Our experiments with synthetic Candidalysin peptide isolate the Candidalysin-induced effects from other fungal factors and show a clear role for Candidalysin in inflammasome activation and induction of cell damage in human MDMs, murine BMDMs, and BMDCs. Although analysis of *C. albicans* mutants lacking the

**Fig. 9** Neither apoptosis nor necroptosis is triggered by Candidalysin. **a** Phosphatidylserine exposure and cell viability of hMDMs infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strain (*ece1*Δ/Δ) (MOI 10) or treated with synthetic Candidalysin for 5 h were quantified by staining with FITC-Annexin V and PI, respectively. The number of single-stained or double-stained macrophages was evaluated by manual counting of at least 200 macrophages. **b** Caspase 3/7 activity was assessed by measuring luminescence of hMDMs 7 h post infection with *C. albicans* Wt or *ece1*Δ/Δ mutant strain (MOI 10) or co-incubation with Candidalysin. Staurosporine served as a positive control. Shown are relative luminescence values (RLU) after background subtraction. **c**, **d** LPS-primed hMDMs were treated with synthetic Candidalysin or Nigericin for 5 h. Selected samples were pre-treated with **c** the necroptosis inhibitor Necrostatin-1 (Nec-1) or **d** the actin cytoskeleton inhibitor Cytochalasin D or inhibitor solute control DMSO 1 h prior to administration of synthetic Candidalysin or Nigericin. Macrophage lysis was quantified by measuring LDH release. **e** LPS-primed hMDMs, mBMDMs or mBMDCs were treated with synthetic Candidalysin or Nigericin for 4–5 h. Selected samples were pre-treated with the potassium channel inhibitor glibenclamide or inhibitor solute control DMSO 1 h prior to administration of synthetic Candidalysin or Nigericin. KCl was added after LPS priming. Macrophage lysis was quantified by measuring LDH release. **a** Data are shown as mean + SD of two different donors. **b**–**e** Values are represented as scatterplot with median of three independent donors or experiments ($n \geq 3$). For statistical analysis, a one-way ANOVA with Dunnett's multiple comparison test was used. ***$p \leq 0.001$, significance compared to Candidalysin treatment

Ece1- (Candidalysin-) encoding sequence, demonstrated that Candidalysin drives both *C. albicans*-induced inflammasome activation and cellular damage in human macrophages, deletion mutant phenotypes were less prominent or absent in murine BMDMs and BMDCs. This suggests that Candidalysin seems to be more important for human cells as compared to murine cells, but could also be interpreted by the fact that several fungal factors exhibit redundancy in stimulating IL-1β and inducing cell death, particularly in murine phagocytes. Similarly, distinct inflammatory response patterns of murine and human macrophages have been observed when challenged with *Aspergillus fumigatus*[55].

One of such redundant triggers for both, inflammasome activation and damage is likely *C. albicans* filmentation[6,9,56] (this study). Besides, fungal aspartic proteases are known inflammasome inducers[29], and fungal cell wall architecture, ergosterol biosynthesis and phosphatidylinositol-4-kinase signalling play a role in macrophage cytolysis[10–12,17,57].

Importantly, when applying an in vivo systemic candidiasis model, we observed reduced IL-1β levels in mice infected with the *ece1*Δ/Δ mutant as compared to mice infected with wild-type cells. Of note, these differences were only observed in the kidney, an organ where the fungal morphology is dominated by hyphae[35], whereas no differences in the IL-1β release were observed in the spleen, where infecting *C. albicans* cells are predominantly in the yeast morphology. These data highlight that the strictly hyphal associated *ECE1* gene and thus Candidalysin is essential for full IL-1β release during systemic murine infections with *C. albicans*.

The concept that phagocytosed *C. albicans* cells trigger macrophage damage exclusively by mechanical means through sustained filamentation, macrophage membrane stretching and, eventually, host cell lysis, leading to fungal escape[9] has been challenged by a number of recent studies, suggesting a more complex picture of *C. albicans*-macrophage interactions. In murine macrophages, *C. albicans* infection triggers pyroptosis, a regulated inflammatory form of cell death, by activating the NLRP3 inflammasome[12,17]. Pyroptosis is characterized by host cell damage mediated by caspase-1, subsequent pore formation, cell swelling, and eventually membrane rupture[19,46]. Pyroptosis-mediated macrophage damage may thus be an escape route for *C. albicans* within the first six to eight hours of infection before sustained hypha formation results in host cell damage[12,16,17]. However, our data indicate that Candidalysin-induced macrophage lysis is independent of pyroptosis and inflammasome activation, as neither caspase-1 inhibition nor inhibition of potassium efflux nor genetic ablation of caspase-1, Nlrp3, or ASC led to a significant reduction in toxin-induced phagocyte lysis at early time points. In addition, LDH release by phagocytes exposed to the *ece1*Δ/Δ mutant was reduced in inflammasome knockout as compared to wild-type phagocytes. This indicates that pyroptosis still plays a major role in *C. albicans*-induced cell death by Candidalysin-deficient cells. The bi-phasic cell death dynamics with live *C. albicans* similar to the study of Uwamahoro et al.[12] supports the view that pyroptosis plays a role in the *C. albicans* cell induced cell death. The fact that the predominantly pyroptotic first wave of death[12] is clearly reduced in the *ece1*Δ/Δ mutant, may suggest a minor role for Candidalysin in pyroptosis or that Candidalysin contributes to non-pyroptotic processes in this phase. However, our genetic approach with murine cells lacking key components of the NLRP3 inflammasome clearly demonstrates that Candidalysin induced cell death is predominantly pyroptosis-independent. We can, however, not exclude that Candidalysin, in the setting of live *C. albicans* cells, may facilitate the induction of pyroptosis by other fungal molecules. We also found no evidence for Candidalysin triggering other regulated cell death pathways such as apoptosis or necroptosis. Thus, Candidalysin seems to cause cell death differently from (regulated cell death-inducing) bacterial PFT's such as *Bacillus anthracis* lethal toxin, *Serratia marcescens* hemolysin ShlA, *Clostridium perfringens* β-toxin, or *Staphylococcus aureus* α-hemolysin, while sharing the ability to activate the inflammasome[27,28,43–45,58].

While most known NLRP3 activators including bacterial PFTs kill myeloid cells in an NLRP3 and ASC-dependent manner, there is precedence for NLRP3 activators killing these cells independent of the inflammasome. Three prominent examples are insoluble activators like monosodium urate crystals (MSU) or alum crystals[59], membrane damage by mixed-lineage kinase domain-like protein (MLKL) during necroptosis[60], and cytoplasmic LPS activating Gasdermin D-dependent pyroptosis through caspase-4/11[61]. Similar to Candidalysin, these activators all engage Nlrp3 via $K^+$ efflux, suggesting that membrane perturbations that lead to inflammasome-independent cell death can in parallel activate Nlrp3 through the $K^+$ efflux-mediated mechanism. Furthermore, besides inducing regulated host cell death, Cullen et al.[51] have suggested that signal 2-inducing PFTs, such as streptolysin or listeriolysin, can lead to non-selective permeabilisation of plasma membranes and subsequent necrotic host cell death.

The evidence we have collected so far point to a direct interaction of Candidalysin with host cell membranes as the main cause for toxin-induced necrotic damage.

This study demonstrates that Candidalysin has the ability to damage mononuclear phagocytes and to activate the inflammasome and that these two observations are putatively independent events. Inflammasome activation results in the production of the pro-inflammatory cytokine IL-1β, which, when secreted, induces the recruitment of other immune cells to the site of infection[62,63]. Indeed, the NLRP3 inflammasome has been implicated with an anti-*Candida* response[21,22] and has been shown to induce a protective antifungal Th1/Th17 response[64]. Toxin-dependent inflammasome activation may thus be a disadvantage for the

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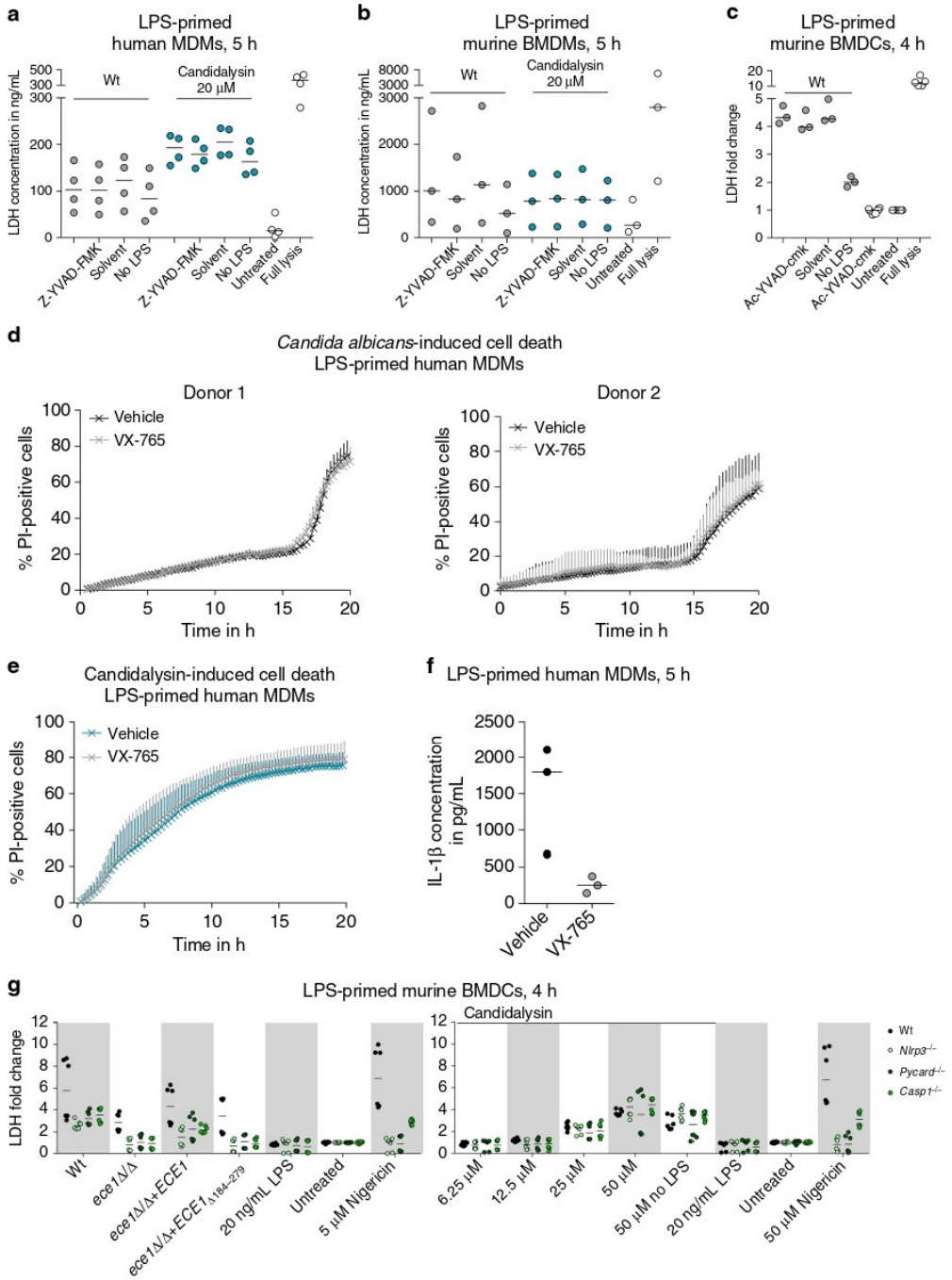


fungus. In contrast, phagocyte damage may be a benefit for the fungus, by supporting immune evasion and escape from macrophage killing by host cell lysis[16].

In light of the literature, we propose the following model for the role of Candidalysin in *C. albicans*-macrophage/DC interaction: The recognition of *C. albicans* PAMPs and/or bacterial ligands of commensal microbes by immune cell PRRs leads to fungal phagocytosis and inflammasome priming. Phagocytosed fungal cells form hyphae, leading to rapid production of hypha-associated factors such as Candidalysin and other inflammasome-inducing fungal factors. Candidalysin intercalates into host membranes, causing direct plasma membrane permeabilisation leading to ion fluxes that cause a drop in cytosolic potassium. This triggers NLRP3 inflammasome activation and caspase-1-dependent IL-1β processing. Further membrane destabilization ultimately leads to lytic host cell death, thereby contributing to

**Fig. 10** Candidalysin-induced damage is mainly caspase-1-independent. **a**–**c** Cell damage was quantified by measuring LDH release in LPS-primed or unprimed (no LPS) **a** hMDMs, **b** mBMDMs, or **c** mBMDCs that were infected with *C. albicans* Wt (MOI 6 or 5) for 5 or 4 h respectively or synthetic Candidalysin. The caspase-1-inhibitor Z-YVAD-FMK (88.9 μM, **a**, **b**), Ac-YVAD-cmk (20 μM, **c**) or the inhibitor solute control DMSO was added 1 h prior to infection. **d**, **e** Macrophage damage over time was assessed by quantifying PI-positive cells in LPS-primed hMDMs that were infected with **d** *C. albicans* Wt or **e** incubated with synthetic Candidalysin in the presence or absence of the caspase-1 inhibitor VX-765. **f** Nigericin (5 μm)-induced IL-1β release in LPS-primed hMDMs the presence or absence of the caspase-1 inhibitor VX-765. **g** Cell damage was quantified by measuring LDH release in LPS-primed or unprimed (no LPS) Wt, *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$ or *Casp1*$^{-/-}$ mBMDCs that were infected with *C. albicans* Wt, re-integrant (*ece1*Δ/Δ + *ECE1*) or mutant strains (*ece1*Δ/Δ, *ece1*Δ/Δ + *ECE1*$_{\Delta 184-279}$) (MOI 5) or incubated with synthetic Candidalysin for 4 h. **a**–**c**, **g** Values are represented as scatterplot with median of three independent experiments or donors ($n \geq 3$). **d** The results of two different donors are displayed separately due to strong donor variability. Data are shown as mean + SD of four independent positions in at least 2 wells. **e** Data are shown as mean + SD of six independent donors

the release of mature IL-1β. Concomitantly, caspase-1 activation results in early pyroptotic damage of host cells—a multifactorial process induced in the first hours of infection, which depends on hyphal formation and certain cell wall components[10–12,17,57,65,66], but not Candidalysin. In later phases of infection, mechanical destruction of phagocytes is initiated by hyphae that are formed inside macrophages and pierce the host cell membrane[9]. Hyphal membrane piercing and outgrowth is independent of Candidalysin as mutants lacking Candidalysin have the full potential to escape from host cells.

Our data presented here, collectively with previously published studies on Candidalysin, clearly point towards dual roles of Candidalysin in *C. albicans* pathogenesis, with different outcome depending on the type of infection. First, Candidalysin suits the description of a classical virulence factor[67] that damages host cells. Second, the current study demonstrates that Candidalysin is an immunomodulatory molecule. Such molecules which are sensed by the host immune system to initiate a protective response have been designated as avirulence factors[68,69]. The outcome of the two effects, damage potential vs. protective immune response, dictates the outcome of the infection. During oral infections, epithelial cells recognize Candidalysin via the danger response pathway (via p38 and c-Fos)[31], causing cytokine release and recruitment of phagocytes, in particular neutrophils. This neutrophil recruitment is crucial for pathogenicity, but with oppositional outcome in different tissues (and depending on the immune status of the host). During oral infections, the attraction of neutrophils is protective in immunocompetent mice[48], while neutrophil recruitment during vaginal infections is associated with collateral damage and immunopathology[34]. We believe that similar processes occur in *C. albicans*-infected organs during systemic infections; with macrophages being key players responsible for neutrophil attraction. This concept is, for example, in agreement with the observation that organ-specific fungal morphology and neutrophil attraction correlates with pathogenesis[35].

## Methods

**Ethics statement**. Blood was obtained from healthy human volunteers with written informed consent. The blood donation protocol and use of blood for this study were approved by the Jena institutional ethics committee (Ethik-Kommission des Universitätsklinikums Jena, Permission No 2207–01/08). Animal experiments were performed in compliance with the German animal protection law or approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases, USA.

***C. albicans* strains and growth conditions**. *C. albicans* strains included the wild-type (Wt) strain SC5314[70], a derivate of SC5314, a parental strain of the mutant strains used (BWP17-CIp30)[71], an *ECE1* deletion strain (*ece1*Δ/Δ), an *ECE1*-complemented strain (*ece1*Δ/Δ + *ECE1*), a strain lacking only the Candidalysin-encoding region in Ece1 (*ece1*Δ/Δ + *ECE1*$_{\Delta 184-279}$), a *ECE1*-GFP reporter strain (SC5314 + p*ECE1-GFP*; *ECE1* promoter-GFP)[31], and the hypha deficient mutants *efg1*Δ/Δ/*cph1*Δ/Δ[30] and *hgc1*Δ/Δ[72]. Cells were routinely grown overnight in YPD shaking cultures (1% yeast extract, 2% peptone, 2% glucose) at 30 °C and 180 rpm. Prior to infection experiments, cultures were washed with PBS, counted and adjusted to the desired concentration. *C. albicans* hyphae were prepared by inoculating PBS-washed yeast cells into RPMI 1640 (Thermo Fisher Scientific) at $6.66 \times 10^6$ cells/mL and incubating for 2 h at 37 °C, 180 rpm. For preparation of heat-killed cells, 500 μL yeast or hyphal cultures were incubated at 70 °C for 10 min.

**Preparation of hMDMs**. Human peripheral blood mononuclear cells (hPBMC) were isolated by Histopaque-1077 (Sigma-Aldrich) density centrifugation from buffy coats donated by healthy volunteers. CD14 positive monocytes were selected by magnetic automated cell sorting (autoMACs; MiltenyiBiotec). To differentiate monocytes into human MDMS (hMDMs), $1.7 \times 10^7$ cells were seeded into 175 $cm^2$ cell culture flasks in RPMI 1640 media with 2 mM L-glutamine (Thermo Fisher Scientific) containing 10% heat-inactivated fetal bovine serum (FBS; Bio&SELL) (RPMI + FBS) and 50 ng/mL recombinant human M-CSF (ImmunoTools) and incubated for seven days at 37 °C and 5% $CO_2$. Adherent hMDMs were detached with 50 mM EDTA in PBS, seeded in 6, 24 or 96-well plates to a final concentration of $1 \times 10^6$, $1–2 \times 10^5$ or $4 \times 10^4$ hMDMs/well, respectively in RPMI + FBS and incubated overnight. Macrophage infection experiments were performed in serum-free RPMI medium.

For the differential staining of macrophage phagocytosis and hypha formation after phagocytosis, hMDMs were differentiated by using an adherence method. Briefly, hPBMCs isolated by Histopaque-1077 density centrifugation (see above) were seeded into 100 mm Petri dishes ($4 \times 10^7$ cells/dish) in RPMI 1640 media with 2 mM L-glutamine without FBS and incubated at 37 °C and 5% $CO_2$ for 1–2 h. Following, non-adherent cells were removed by washing twice with PBS. Adherent cells were then differentiated for seven days in RPMI + FBS medium with 50 ng/mL M-CSF as described above.

**Preparation of murine macrophages and DCs**. Murine bone-marrow-derived macrophages (mBMDMs) were generated by culturing bone marrow cells isolated from the femur and tibia of 9 to 19 week old healthy female C57BL/6J mice. For differentiation, $5 \times 10^6$ cells were seeded into a 175 $cm^2$ cell culture flask in RPMI + FBS containing 1% Penicillin/Streptomycin (PAA Laboratories) and 40 ng/mL recombinant murine M-CSF (ImmunoTools) and incubated for seven days at 37 °C and 5% $CO_2$. Adherent cells were detached by scraping in RPMI + FBS, seeded in 6 or 24-well plates to a final number of $1.5 \times 10^6$ or $5 \times 10^5$ mBMDMs/well and incubated overnight.

Murine bone-marrow-derived dendritic cells (mBMDCs) were generated by culturing bone marrow cells from 6 to 20 week old C57BL/6J Wt or *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$ or *Casp1*$^{-/-}$[73-75] mice for seven days in mBMDC medium (GlutaMAX-supplemented RPMI + FBS containing 1% Penicillin/Streptomycin (Gibco), 50 μM β-mercaptoethanol (Gibco) and 20 ng/mL recombinant murine GM-CSF (ImmunoTools)). On day 7, the mBMDC culture was harvested. Adherent cells were detached with 5 mM EDTA in PBS, mBMDCs were seeded in 96-well plates in mBMDC medium to a final number of $1 \times 10^5$ mBMDCs/well. Macrophage and DC infection experiments were carried out in serum-free medium.

**Cultivation and transfection of RAW264.7-Dectin-1 cells**. The RAW264.7-Dectin-1-LPETG-3 × HA macrophage cell line (RAW Dectin-1)[76] was grown in RPMI 1640 (Wisent Bioproducts) supplemented with 10% heat-inactivated FBS at 37 °C and 5% $CO_2$ and tested negative for mycoplasma contamination. For transient transfections with plasmids GFP-2xP4M-SidM and iRFP-FRB-Rab7[77], 80% confluent monolayers of RAW264.7 Dectin-1 were collected by scraping and plated onto 1.8 cm glass coverslips at a density of $5 \times 10^4$ cells/coverslip. Macrophages were allowed to recover for 18 h prior to transfection with FuGENE HD (Promega) according to the manufacturer's instructions. Briefly, 1 μg of plasmid DNA and 3 μL of FugeneHD were mixed in 100 μL serum-free RPMI and incubated for 15 min at room temperature. This mix was then distributed equally into four wells of a 12-well plate (Corning Inc.) containing the RAW264.7 Dectin-1 in 1 mL RPMI + FBS. Cells were imaged 18–24 h after transfection.

**Synthetic peptides**. Candidalysin peptide[31] was synthesized commercially (Proteogenix or Caslo). The peptide was dissolved in water and added to phagocytes in concentrations ranging from 1 to 80 μM.

**Infection of hMDMs, mBMDMs, and mBMDCs**. For simultaneous measurement of phagocyte damage and cytokine release 5 h *post infection* (*p.i.*), $2 \times 10^5$ hMDMs or $5 \times 10^5$ mBMDMs/well were seeded into 24-well plates. Murine BMDCs were seeded into 96-well plates to a density of $1 \times 10^5$ mBMDCs/well. For cytokine release or phagocyte damage measurements 24 h *p.i.*, $4 \times 10^4$ hMDMs or $1 \times 10^5$ mBMDCs/well were seeded into 96-well plates. If necessary, phagocytes were primed prior to infection for 2 h (hMDMs, mBMDMs) or 3–4 h (mBMDCs) with 50 ng/mL LPS (Sigma Aldrich). Alternatively, heat-killed yeasts or hyphae (multiplicity of infection (MOI) 10), 100 µg/mL Zymosan (Sigma Aldrich), 100 µg/mL Curdlan (Invivogen) or 100 µg/mL whole glucan particles (WGP dispersible; Invivogen) were used as priming agents. For inhibitor studies, the following compounds were added 1 h prior to infection: the caspase-1-inhibitor Z-YVAD-FMK (88.9 µM; Merck) or Ac-YVAD-cmk (20 µM, Invivogen), the caspase-1-inhibitor VX-765 (50 µg/mL, Invivogen) or vehicle control, the actin cytoskeleton inhibitor Cytochalasin D (10 µM; Sigma Aldrich), the V-ATPase inhibitor Bafilomycin A1 (50–500 nM; Sigma Aldrich), the ROS inhibitor 4-Aminopyrrolidine-2,4-dicarboxylate (PDTC) (100, 500 µM; Enzo Life Sciences), the potassium channel inhibitor glibenclamide (25 µM; Sigma Aldrich) or the RIP1-kinase inhibitor Necrostatin-1 (12.5–50 µM; Biomol). Human MDMs and mBMDMs were infected in 300 µL (24-well plate) or 100 µL (96-well plate) with *C. albicans* at MOI 1 (24 h infection mBMDMs), MOI 6 (24 h infection hMDMs), MOI 6 (5 h infection mBMDMs) or MOI 10 (5 h infection hMDMs) or co-incubated with synthetic Candidalysin. Murine BMDCs were infected in 300 µL in 96-well plates with *C. albicans* at MOI 5 for 4 h or co-incubated with synthetic Candidalysin. Nigericin (1, 5 µM for 4–5 h; Sigma Aldrich), LPS (1 µg/ml; Sigma Aldrich) or ATP (5 mM for 30 min; Invivogen) were used as positive controls. After incubation at 37 °C, 5% $CO_2$ for 4, 5 or 24 h, plates were centrifuged at $250 \times g$ for 10 min and supernatants were harvested.

**IL-1 bioassay**. The murine cell line EL4.NOB-1, which was kindly provided by Prof. L. Joosten (Radboudumc, Nijmegen, The Netherlands), has a high level of surface IL-1 receptor expression, which can recognize both human and murine IL-1. The cell line tested negative for mycoplasma contamination. Constitutively the cells produce practically undetectable IL-2 levels, but in response to bioactive IL-1, the cells produce high concentrations of IL-2. Furthermore, these cells are unresponsive to other cytokines like tumour necrosis factor (TNF), colony stimulating factors (CSFS), IL-3, IL-5, IL-6, and IFN-γ[36]. EL4.NOB-1 cells were seeded in 96 well flat-bottom plates at a final density of $1 \times 10^6$ cells/mL and grown in RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS at 37 °C and 5% $CO_2$ and were stimulated for 24 h using culture supernatants of hMDMs or mBMDMs stimulated in presence or absence of various concentrations of Candidalysin or *C. albicans* Wt, *ece1*Δ/Δ, *ece1*Δ/Δ + *ECE1* or *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$ (as described above). As a control EL4.NOB-1 cells were stimulated with recombinant human IL-1β (R&D systems) in concentrations ranging from 1000 pg/mL to 7.8 pg/mL. After 24 h of incubation at 37 °C, 5% $CO_2$ the culture supernatants were collected and IL-2 production was measured by ELISA (eBioscience), and bioactive IL-1 levels were calculated based on the response to recombinant IL-1β.

**In vivo infections and IL-1β quantification**. Eight week old female C57BL/6 mice (Taconic) were maintained in individually ventilated cages under specific pathogen-free conditions at the 14BS facility at the National Institutes of Health (Bethesda, MD, USA). With 10 mice per group (two independent experiments with five mice each) a power was estimated of 80% ($\beta = 0.80$) with a type I error below 5% ($\alpha = 0.05$) for a variance of 15%. Animals were randomly infected intravenously with $2 \times 10^5$ yeast cells of the indicated fungal strains and humanely euthanized 24 h later for analysis of tissue IL-1β levels. Groups infected with Wt and *ece1*Δ/Δ were unblended for researchers. Kidneys and spleens were aseptically removed and homogenized in PBS supplemented with protease inhibitor cocktail (Roche) and 0.05% Tween 20. Homogenized organs were centrifuged twice to remove debris and resulting supernatants snap-frozen on dry ice and stored at −80 °C prior to analysis. IL-1β concentration in the tissue homogenates was determined by ELISA (R&D Systems), following the manufacturers' instructions.

**LDH-based cell damage assay and cytokine quantification**. Lysis of macrophages and DCs was assayed by measuring the concentration of the cytoplasmic enzyme lactate dehydrogenase (LDH) in cell culture supernatants using the non-radioactive Cytotoxicity Detection or CytoTox-ONE™ Kit (Roche, Promega). Cytokines were quantified in cell culture supernatants by Enzyme-linked Immunosorbent Assay according to the manufacturer's instructions (Ready-SET-Go! ELISA; Thermo Fisher Scientific).

**Quantification of macrophage damage by time-lapse imaging**. For analysis of macrophage cell death kinetics, a method adapted from Uwamahoro et al.[12] was used. Briefly, $6 \times 10^4$ cells/well (hMDMs) were seeded in µ-Slide 8-well chambered coverslips (ibidi) and primed for 2 h with 50 ng/mL LPS prior to infection. Macrophages were then infected with *C. albicans* (MOI 6) or co-incubated with synthetic Candidalysin. Non-phagocytosed yeasts were removed by washing after 1 h. Propidium iodide (PI; 3.33 µg/ml; Sigma Aldrich) was added to stain non-viable immune cells, chamber slides were transferred to the inverted Zeiss AXIO Observer.Z1 microscope. At least two independent fields/well were imaged every 15 min at 10× magnification for a maximal time span of 24 h using a bright field channel and a DsRed filter. Red channel images were processed using the Fiji software (ImageJ[78]). After conversion to binary images, the number of PI-positive cells was determined using the Particle Analyzer tool. The total number of macrophages was determined manually by counting PI-negative macrophages in an overlay picture of the last time point and adding the Fiji-calculated number of PI-positive macrophages for the same time point.

**XTT assay**. To determine the metabolic activity of Candidalysin-treated macrophages, $4 \times 10^4$ hMDMs/well were co-incubated with synthetic Candidalysin in triplicates in a 96-well plate for 5 h at 37 °C and 5% $CO_2$ in 200 µL RPMI 1640 medium without phenol red (ThermoFisher Scientific). Subsequently, 50 µL of pre-warmed 1 mg/mL XTT and 100 µg/mL coenzyme Q0 (Sigma Aldrich) diluted in RPMI were added and samples were incubated for 2 h at 37 °C. The absorbance at 450 nm was measured with a Tecan Infinite microplate reader, with reference readings at 570 and 690 nm.

**Phagocytosis assay and staining of phagosomes**. $1 \times 10^5$ hMDMs were allowed to adhere to coverslips in a 24-well plate overnight. Acidification of the phagosomes was assessed by adding the acidotropic dye LysoTracker Red DND-99 (Thermo Fisher Scientific; diluted 1:10,000 in RPMI) 1 h prior to infection and during co-incubation with fungal cells. Where indicated, macrophages were pre-treated with 100 nM Bafilomycin A1 (Sigma Aldrich) 1 h before infection. Macrophages were infected with *C. albicans* (MOI 1 to 5) or treated with synthetic Candidalysin. For synchronization of phagocytosis, plates were incubated on ice for 20 min after infection. Unbound yeast cells were removed by washing with RPMI, and phagocytosis was initiated by incubating at 37 °C and 5% $CO_2$. Cells were fixed with 4% paraformaldehyde at the indicated time points. Non-internalized *C. albicans* cells were stained with Alexa Fluor 647-conjugated Concanavalin A (ConA; Thermo Fisher Scientific) for 45 min. For staining of non-internalized and internalized fungal parts, macrophages were permeabilised with 0.5% Triton X-100 in PBS and stained with Calcofluor White (Sigma-Aldrich). For immunofluorescence staining of LAMP1, samples were blocked with 5% BSA in PBS after fixation, followed by incubation with a mouse anti-LAMP1 antibody (sc-20011; 1:100; Santa Cruz Biotechnology) for 2 h and with an Alexa Fluor 555-conjugated anti-mouse IgG antibody (A-21424, 1:500; Thermo Fisher Scientific) for 1 h. Coverslips were mounted and fluorescence images were recorded using the Zeiss AXIO Observer.Z1 (Carl Zeiss Microscopy). Phagocytosis and outgrowth rates of intracellular hyphae were calculated by manually counting a minimum of 50 yeast cells/sample. Hyphal length of internalized *C. albicans* cells was measured for 20 cells/sample. The percentage of LAMP1 or LysoTracker-positive phagosomes was evaluated by counting at least 20 or 50 yeast-containing phagosomes/sample, respectively. For evaluation of LysoTracker fluorescence intensities of heat-killed cell-containing phagosomes, the profile option of the Zeiss software ZEN was used. Line-profiles were placed across at least 10 *Candida* cells/sample and intensity peaks of DsRed channel images were recorded.

For analysing phagosomal maturation in murine macrophages, RAW264.7 Dectin-1 macrophages were infected with *C. albicans* Wt or *ece1*Δ/Δ mutant (MOI 2). Yeast cells were centrifuged onto macrophages at $300 \times g$ for 1 min and phagocytosis was allowed for 20 min at 37 °C and 5% $CO_2$, before non-adherent cells were removed. Remaining non-phagocytosed yeasts were outside-labelled with Alexa Fluor 647-conjugated ConA for 10 min. All outside-labelled yeast cells were excluded from the experiment. At given time points, macrophages were fixed in ice-cold 100% methanol for 5 min at −20 °C, followed by extensive washing in PBS. Phagolysosomes were detected using rat anti-Lamp1 hybridoma (1D4B, 1:20, Developmental Studies Hybridoma Bank), and visualized using a donkey anti-rat Alexa Fluor 488-coupled secondary antibody (712-545-150, 1:1000, Jackson ImmunoResearch). For quantification of Phosphatidylinositol 4-phosphate (PI(4)P) and Rab7 acquisition, RAW264.7 Dectin-1 macrophages were transiently co-transfected with GFP-2xP4M (PI(4)P binding domain) and RFP-Rab7. At given time points, micrographs were acquired using a spinning-disk confocal microscope (Quorum Technologies), and at least 16 Lamp1-, PI(4)P- and Rab7-positive phagosomes were quantified using Volocity 6.3 (Perkin Elmer Inc.), counting at least 16 phagosomes/sample.

**Survival assay**. $4 \times 10^4$ hMDMs were seeded in 96-well plates in RPMI + FBS containing 100 U/mL IFN-γ (Immunotools), infected with *C. albicans* (MOI 1) and incubated at 37 °C and 5% $CO_2$. The assay was performed in triplicates. The survival of yeast cells internalized by macrophages was assessed after 3 h by removing non-hMDM-associated fungal cells by washing with RPMI, subsequent lysis of hMDMs with 20 µL 0.5% Triton X-100 per well and plating lysates on YPD plates to determine fungal burdens (colony forming units (cfus)). Lysate cfus were normalized to cfu numbers of the respective inoculum.

**Caspase-1 activation assay**. Caspase-1 activation in hMDMs was assayed using the FAM FLICA™ caspase-1 Kit (Bio-Rad). Briefly, $1 \times 10^5$ hMDMs/well were seeded into 24-well plates containing glass coverslips and incubated overnight at

37 °C and 5% $CO_2$. Prior to infection, macrophages were primed for 2 h with 50 ng/mL LPS. Cells were then infected with *C. albicans* (MOI 10) or subjected to synthetic Candidalysin. Non-phagocytosed fungal cells were removed after 1 h by washing. After 4 h, FAM-YVAD-FMK FLICA™ reagent was added to a final concentration of 1× and macrophages were incubated for an additional 1 h. Subsequently, nuclei were stained with Hoechst, samples were fixed according to the manufacturer's instructions and fluorescence images were recorded by a Leica DM5500B microscope, using appropriate filters for the detection of FAM FLICA (green channel) and Hoechst (DAPI channel) signals. Fluorescence intensity of FAM FLICA in macrophages was quantified using the quantification tool for region of interest (ROI) of the Leica LAS AF microscope software. ROIs were placed around macrophages and mean grey values were recorded. Background values (ROI placed in region without macrophages) were subtracted.

Caspase-1 activity in mBMDCs was determined using the CaspaseGlo 1 Inflammasome assay (Promega). $1 \times 10^5$ mBMDCs/well were seeded into 96-well plates and primed with LPS for 3 h or left untreated for 3 h before infection with *C. albicans* (MOI 5) for 5 h. For the CaspaseGlo 1 inflammasome assay, supernatant were transferred to white 96-well plates and mixed with the supplemented substrate mix in the presence or absence of the Caspase-1 inhibitor Ac-YVAD-CHO, according to the manufacturer's manual. The plotted values were detected 60 min after mixing samples and the supplemented substrate mixes. Blank values of medium without cells were subtracted from sample values.

**Annexin V-based cell death assay**. Phosphatidylserine exposure on hMDMs was quantified using FITC-Annexin V (Biolegend), according to the manufacturer's instructions. Briefly, $1 \times 10^5$ hMDMs/well were seeded into 24-well plates containing glass coverslips and incubated overnight at 37 °C and 5% $CO_2$. Cells were infected with *C. albicans* (MOI 10) or treated with synthetic Candidalysin and incubated for 3 or 7 h. Staurosporine (1 µM; Sigma Aldrich) was used as a positive control. Cells were incubated with 5 µL FITC-Annexin V and 5 µg/mL PI (Sigma Aldrich) in 200 µL annexin binding buffer for 15 min, mounted with DAPI and imaged immediately using the Zeiss AXIO Observer.Z1 (Carl Zeiss Microscopy). Images were evaluated manually for Annexin V- and PI-positive cells by counting at least 200 macrophages.

**Caspase-3/7 activation assay**. Caspase-3/7 activity in hMDMs was determined using the Caspase-Glo 3/7 Assay (Promega). $4 \times 10^4$ hMDMs/well were seeded into white clear-bottom 96-well plates and incubated overnight at 37 °C and 5% $CO_2$. Macrophages were infected with *C. albicans* (MOI 10) or treated with synthetic Candidalysin and incubated for 7 h. Staurosporine (1 µM; Sigma Aldrich) was used as a positive control. Caspase-Glo substrate was added and cells were incubated at room temperature for 60 min. Luminescence was recorded using a Tecan Infinite microplate reader.

**Luminol-based ROS detection**. Total ROS production by hMDMs was quantified by chemiluminescence. Briefly, $4 \times 10^4$ hMDMs/well were seeded into white clear-bottom 96-well plates and incubated overnight at 37 °C and 5% $CO_2$. All cells and reagents were prepared in RPMI 1640 without phenol red. Cells were subjected to synthetic Candidalysin or 100 nM PMA as a positive control. All samples were prepared in triplicates. Fifty microliters of a mixture containing 200 mM luminol and 16 U horseradish peroxidase were added immediately prior to quantification. Luminescence was measured every 3 min over a 60 min incubation period at 37 °C using a Tecan Infinite microplate reader. For each sample, minimum and maximum luminescence values were determined and the difference was calculated (MAX–MIN).

**Intracellular ROS measurement ($H_2$DCF-DA)**. $4 \times 10^4$ hMDMs/well were seeded into black clear-bottom 96-well plates and incubated overnight at 37 °C and 5% $CO_2$. Immediately before infection, cells were loaded with 20 µM $H_2$DCF-DA (Sigma Aldrich) in pre-warmed PBS for 30 min at 37 °C, 5% $CO_2$ and washed with pre-warmed PBS. Macrophages were then infected with *C. albicans* (MOI 10) or treated with $H_2O_2$ (1 mM) or PMA (1 µM). Fluorescence (Ex 485/Em 535) was recorded immediately after infection and after 5 h incubation at 37 °C and 5% $CO_2$ using a Tecan Infinite microplate reader and fluorescence increase over time was calculated Ex 485/Em 535 (5 h–0 h). Candidalysin treatment led to an unspecific fluorescence signal and was therefore excluded from analysis. All samples were prepared at least in duplicates.

**Western blot analysis**. LPS-primed (50 ng/ml, 2 h or 4 h) and unprimed (no LPS) hMDMs, mBMDMs or mBMDCs were seeded at $1.5 \times 10^6$ hMDMs or mBMDMs/well in 6-well (hMDMs, mBMDMs) or $1 \times 10^5$ mBMDCs/well in 96-well plates. Cells were infected with *C. albicans* (MOI 6 or 5), treated with synthetic Candidalysin, or 1 µM (hMDMs, mBMDMs)/5 µM (mBMDCs) Nigericin as a positive control. Supernatants were collected 5 h *p.i.*

For hMDMs and mBMDMs, supernatant proteins were precipitated with chloroform/methanol (1:4). The resulting protein pellets were resuspended in 1 × Laemmli buffer (31.25 mM Tris-HCl pH 6.8, 12.5% glycerol, 1% SDS, 0.005% Bromophenol blue). For SDS-PAGE, 10 µL of supernatant sample (heat-denatured, with β-mercaptoethanol) were separated and transferred to a PVDF membrane. Membranes were blocked with 5% bovine serum albumin (SERVA) in TBS-T (50 mM Tris, 0.15 M NaCl, 0.05% Tween 20, pH 7.6) and incubated with primary antibodies specific for IL-1β (AF-201-NA for human samples or AF-401-NA for murine samples; 1:800, R&D Systems) or caspase-1 (AG-20B-0048 for human samples or AG-20B-0042 for murine samples, 1:500, Adipogen) in TBS-T overnight at 4 °C. After washing three times with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated anti-goat (HAF109, 1:2000, R&D Systems) or anti-mouse (1:2000, HAF007, R&D Systems) antibodies in TBS-T followed by three washing steps. Immunoreactivity was detected by enhanced chemiluminescence (ECL Plus western blotting Substrate; Thermo Fisher Scientific, Inc.). For mBMDCs, 15 µL of supernatant triplicate samples were pooled and boiled in 1× Laemmli buffer containing β-mercaptoethanol. Fifteen microliters of the pooled supernatant sample were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked and washed as described above and incubated with primary antibody specific for caspase-1 (AG-20B-0042, 1:1000, Adipogen). The membrane was washed as described above and incubated with a horseradish peroxidase-conjugated anti-mouse antibody (#7076, 1:2000, Cell Signaling). Ponceau or Coomassie staining of membrane or gel was used to ensure equal loading of supernatant samples to the gel. Full-size scans of western blots are provided in Supplementary Fig. 1.

**Statistical analysis**. Experiments were performed at least in biological triplicates ($n \geq 3$) with at least three different donors (hMDMs) or three independent experiments or mice (mBMDMs, mBMDCs, RAW264.7 Dectin-1), unless stated differently. Experiments performed in *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$ or *Casp1*$^{-/-}$ mBMDCs were performed in biological duplicates. All experiments were performed in an unblinded fashion. All data are reported as the scatterplot with median or for line charts mean + SD. No exclusion of data was performed except for Candidalysin induced TNF release, which was confirmed to be false positive in repeated experiments. Data were analysed using GraphPad Prism 7 (GraphPad Software, Inc. La Jolla, USA) and a one-way ANOVA for inter-group comparisons with a Dunnett's multiple comparison test. For statistical analysis of grouped data, a two-way ANOVA with Sidak's multiple comparison was applied. Statistically significant results are marked with a single asterisk meaning $p \leq 0.05$, double asterisks meaning $p \leq 0.01$ or triple asterisks meaning $p \leq 0.001$, nd—not detectable, n/a—not applicable.

## Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files. All relevant data are available by request from the authors, with the restriction of data that would compromise the confidentiality of blood donors.

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## Author contributions

L.K., A.K., and P.A.K. contributed equally to this work. L.K. and A.K. performed hMDM and mBMDM experiments, analysed the data, wrote the manuscript and prepared the figures. P.A.K. performed mBMDC experiments, analysed the data, and edited the manuscript. M.S.G. performed hMDM and mBMDM experiments, analysed the data, and edited the manuscript. R.D. and M.S.L. performed in vivo experiments, analysed the data, and edited the manuscript. J.W. performed all experiments concerning RAW264.7 Dectin-1 macrophages. O.G. provided bone marrow of knock-out mice and edited the manuscript. J.R. and J.R.N. designed experiments and edited the manuscript. B.H. conceived and designed the study and wrote the manuscript.

## Additional information

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## 3.3 Manuscript III – König *et al.*, *Toxins* 2020

### The Dual Function of the Fungal Toxin Candidalysin during *Candida albicans*-Macrophage Interaction and Virulence

**Annika König**, Bernhard Hube, Lydia Kasper

*Toxins (Basel)* 2020 Aug; 12(8): 469. doi: 10.3390/toxins12080469. Review.

Summary:

This review summarises the role of candidalysin during *C. albicans* infection with a focus on its interaction with macrophages and its implication in NLRP3 inflammasome activation (manuscript II). In several *in vivo* and *in vitro* studies, the toxin has been reported to induce membrane damage and to contribute to translocation and immunopathology, thus representing a classical virulence factor. Still, it can similarly act as an avirulence factor limiting fungal virulence by inducing a danger response in epithelial cells, followed by the recruitment of neutrophils for fungal clearance, and by inducing pro-inflammatory cytokine secretion by macrophages. Thus, candidalysin can be seen as a toxin exhibiting a dual function dependent on the context, time point, and niche of infection, highlighting the complexity of fungus-host interaction.

Own contribution:

Annika König conducted literature research, wrote large parts of the manuscript and created the figures.

Estimated authors' contributions:

| | |
|---|---|
| **Annika König** | **50 %** |
| Bernhard Hube | 5 % |
| Lydia Kasper | 45 % |

Prof. Dr. Bernhard Hube

toxins

MDPI

*Review*

# The Dual Function of the Fungal Toxin Candidalysin during *Candida albicans*—Macrophage Interaction and Virulence

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**Abstract:** The dimorphic fungus *Candida albicans* is both a harmless commensal organism on mucosal surfaces and an opportunistic pathogen. Under certain predisposing conditions, the fungus can overgrow the mucosal microbiome and cause both superficial and life-threatening systemic infections after gaining access to the bloodstream. As the first line of defense of the innate immune response, infecting *C. albicans* cells face macrophages, which mediate the clearance of invading fungi by intracellular killing. However, the fungus has evolved sophisticated strategies to counteract macrophage antimicrobial activities and thus evade immune surveillance. The cytolytic peptide toxin, candidalysin, contributes to this fungal defense machinery by damaging immune cell membranes, providing an escape route from the hostile phagosome environment. Nevertheless, candidalysin also induces NLRP3 inflammasome activation, leading to an increased host-protective pro-inflammatory response in mononuclear phagocytes. Therefore, candidalysin facilitates immune evasion by acting as a classical virulence factor but also contributes to an antifungal immune response, serving as an avirulence factor. In this review, we discuss the role of candidalysin during *C. albicans* infections, focusing on its implications during *C. albicans*-macrophage interactions.

**Keywords:** candidalysin; cytolytic toxin; dual function; inflammasome activation

**Key Contribution:** This manuscript focuses on the fungal peptide toxin candidalysin. It describes how this toxin impacts on the interaction of the human-pathogenic yeast *Candida albicans* with macrophages and discusses how host responses to candidalysin and toxin-induced damage affect fungal virulence.

## 1. Introduction

Of the estimated three to five million fungal species existing worldwide, only a tiny portion (less than 0.01%) cause infections in a human host [1,2]. Still, these few species infect more than one billion people worldwide every year [3,4]. These include primary pathogens that cause disease even in a healthy host, like *Histoplasma capsulatum* or *Paracoccidioides brasiliensis*, and opportunistic fungi like *Aspergillus* and *Candida* spp., which need a susceptible host for disease development [5]. Whereas infections with *Aspergillus* spp. are acquired from the environment, infections with *Candida albicans* mainly originate from endogenous reservoirs like the gut [5–9].

Systemic (invasive) fungal infections pose a serious and often underestimated global health threat due to their crude mortality rate and rising antifungal resistance [4]. The top 10 invasive fungal infections kill approximately 1.5 million people annually, which is more than deaths caused by

tuberculosis or malaria [4]. Many of these infections are typically acquired nosocomially in susceptible hosts, and *Candida* spp. rank as the fourth most common cause, with *C. albicans* accounting for more than half of all *Candida*-induced bloodstream infections [10,11]. Systemic *C. albicans* infections account for more than 400,000 life-threatening infections per year [4].

Both arms of the immune system, innate and adapted immunity, are required for combating *Candida* infections. As significant contributors to innate immunity, macrophages play an important role in immunity against *C. albicans* infections by mediating phagocytosis, clearance of internalized fungi, and recruitment of neutrophils [12–18]. However, most pathogenic fungi, including *C. albicans*, have evolved elegant strategies to counteract killing by phagocytes. For *C. albicans*, these include hypha formation and the production of the hypha-associated cytolytic peptide toxin candidalysin [19]. In this review, we discuss the role of candidalysin during infection, focusing on its role in the interaction of *C. albicans* with macrophages.

## 2. The Pathogen *C. albicans* and Innate Immune Defense by Macrophages

*C. albicans* usually resides as a harmless commensal on mucosal surfaces such as the gastrointestinal and urogenital tract and the oral cavity [20,21]. Under predisposing conditions such as local immunosuppression or antibiotic treatment, the fungus can cause superficial infections that are comparably harmless and relatively easy to treat [22–24]. However, under certain circumstances like systemic immunosuppression, gastrointestinal surgery, central venous catheters, or prolonged stay in intensive care units, the fungus can breach the epithelial barrier, gain access to the bloodstream, and disseminate, causing systemic candidiasis [25,26].

In animal models, neutrophils were identified as key players in controlling disseminated candidiasis, and neutropenia is known to be a major risk factor for systemic candidiasis [27–31]. Besides neutrophils, monocyte-derived immune cells play an essential role in host defense against *C. albicans* infections. Several studies showed that monocytes and monocyte-derived immune cells are indispensable for controlling *C. albicans* infections [13–15]. Tissue-resident macrophages are needed for innate immune defense against *C. albicans* infections [12]. Patrolling monocytes migrate into infected tissues and differentiate into macrophages and dendritic cells, with the latter bridging innate and adaptive immunity against *C. albicans* by presenting fungal antigens to naive T-cells in lymph nodes [32,33]. Monocytes and macrophages contribute directly to fungal clearance by internalization and subsequent intracellular killing, but they also mediate neutrophil recruitment [14,16–18].

## 3. Immune Evasion Mechanisms of *C. albicans*

Macrophages recognize fungal-pathogen-associated molecular patterns (PAMPs) via surface pattern recognition receptors (PRRs) and rapidly phagocytose *C. albicans*. One major *C. albicans* PAMP is its cell wall β-glucan, which is detected via the macrophage PRR dectin-1 [34,35]. Upon phagocytosis of fungal cells, the nascent phagosome matures through fusion steps with lysosomes, ultimately forming the phagolysosome. This cellular compartment represents a hostile environment for the fungus characterized by low pH, few nutrients, and antimicrobial activities (such as oxidative, nitrosative, and proteolytic stress) [36,37].

Despite exposure to the macrophage phagosome's detrimental environment, a fraction of engulfed fungal cells can survive. *C. albicans* counteracts oxidative and nitrosative stress by suppression of ROS generation [38], and production of detoxifying enzymes like superoxide dismutases [39–42]. Internalized fungal cells rapidly reprogram their metabolism to adapt to nutrient starvation inside the phagosome; this includes the up-regulation of genes involved in alternative carbon use (glyoxylate cycle and fatty acid beta oxidation), or encoding oligopeptide transporters and amino acid permeases whereas genes associated with protein biosynthesis are down-regulated [41,43,44]. The data indicate that *C. albicans* cells experience metabolic starvation inside the phagosome.

In addition, *C. albicans* evades the acidic phagosomal environment; *Candida*-containing phagosomes change from acidic to neutral pH over time [45]. This phagosome neutralization is likely mediated

by fungal activities such as the production of neutralizing metabolites [46] or phagosome damage by extending fungal hyphae, which leads to proton leakage [45].

Hypha formation inside macrophages is connected with phagosome damage and the escape of *C. albicans* from these immune cells and immune cell death. *C. albicans* cells engulfed by macrophages rapidly induce hyphal growth, and hyphae are important for many of the above-described immune evasion mechanisms [45–50]. Extending hyphae eventually pierce macrophage membranes, causing macrophage cell death due to physical forces, thus providing an escape route for the fungus from the hostile environment inside the phagocyte [49,51,52]. Recent studies showed that *C. albicans* damages macrophages and escapes by inducing early inflammasome-dependent cell death (pyroptosis) [53,54]. Thus, the induction of macrophage pyroptosis can promote the second pathway of fungal escape in addition to the physical damage caused by extending hyphae [51,55,56]. Escaping hyphae consume glucose in the environment rapidly, which provides a third pathway for *C. albicans*-induced macrophage cell death [57].

In combination, these adaptation mechanisms are thought to promote *C. albicans* survival and even proliferation inside macrophages and ultimately allow escape from these immune cells.

## 4. Candidalysin—A Hypha-Specific Cytolytic Peptide Toxin

The ability to change between its two most important morphologies, yeast and hyphal cells, represents one major virulence trait of *C. albicans* [58]. The hyphal growth program is tightly regulated and induced upon multiple stimuli such as body temperature or contact to host surfaces [59]. During this filamentation process, the fungus expresses virulence factors like the adhesin and invasin Als3, the superoxide dismutase Sod5, or secreted aspartic proteases (Sap4–6) [60–62]. Hyphae contribute to immune evasion of the fungus following phagocytosis by macrophages and allow invasive growth on host epithelia [63–65]. Both processes cause host cell damage, but the damage-mediating fungal factors remained largely unknown. For decades, hydrolases had been thought to be the significant damaging factors of *C. albicans*. In contrast to many bacteria, no pore-forming toxin-like molecules, peptide toxins, or cellular effector proteins were identified in *C. albicans* or any other human pathogenic fungus. As a "toxic surprise" [66], the *C. albicans* toxin candidalysin was recently discovered as the first peptide toxin identified in any human pathogenic fungus [19]. Candidalysin is encoded by the *C. albicans* gene *ECE1*, one of the most highly expressed genes upon hypha formation. The expression of *ECE1* increases within minutes after the induction of filamentous growth up to 10,000-fold [19,67]. *ECE1* is one of the eight core filamentation genes in *C. albicans* induced in response to a wide range of different filamentation stimuli [68], suggesting an important and strictly morphology (hyphal)-associated role during infection. The gene encodes a polypeptide consisting of at least eight peptides separated by lysine-arginine (KR) motifs [69]. The third peptide, candidalysin, is released from the Ece1preproprotein after sequential proteolytic processing by the Golgi-located subtilisin-like protease, Kex2, and the carboxypeptidase, Kex1 [69,70]. The correct processing of the preproprotein is essential for the release of functional candidalysin and, in turn, epithelial damage in vitro and fungal virulence in a model of oropharyngeal candidiasis [70]. This activation mechanism is shared with several bacterial toxins like diphtheria toxin, anthrax toxin protective antigen, or aerolysin, which are similarly activated by proteolytic processing of a precursor protein by subtilisin-like proteases [71–73]. Processed candidalysin is secreted and can be detected in culture supernatants and during growth on epithelial cells [19]. Upon complete processing, the toxin consists of 31 amino acids (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK) and adopts an α-helical structure. It exhibits an amphipathic nature due to containing an N-terminal hydrophobic region and a C-terminal hydrophilic region. The toxin can intercalate into host epithelial membranes through these features, resulting in membrane permeabilization and cell lysis [19].

Genetic analyses underlined the importance of candidalysin for fungal mucosal infection since the deletion of the toxin-encoding sequence from the *ECE1* gene completely abolished *C. albicans*-induced damage to epithelial cells in vitro [19]. The same genetic modification attenuated *C. albicans* virulence in a mouse model of oropharyngeal candidiasis and a zebrafish swim bladder infection model, which

are two in vivo models of mucosal infection [19]. Importantly, candidalysin or *ECE1*-deletion strains show no defects in hypha formation, adhesion, or invasion properties [19,67]. The data suggest that production of candidalysin, rather than hypha formation per se or secreted hydrolases, are the main mediators of the host cell damage caused by *C. albicans* [74].

## 5. Candidalysin in *C. albicans*—Macrophage Interactions

The membrane-perturbing action of bacterial toxins is well known to play an essential role during confrontation with macrophages by inducing inflammatory responses and inflammatory host cell death [73–77].

As the expression of the candidalysin-encoding *ECE1* gene is strongly induced upon *C. albicans* phagocytosis by macrophages [41,43,78], it is likely that mature candidalysin is produced by macrophage-internalized *C. albicans* cells, and toxin-dependent effects occur. *C. albicans* strains lacking the *ECE1* gene or the candidalysin-encoding sequence caused less host cell damage than wild type strains when confronted with primary macrophages over 24 h, pointing to candidalysin-dependent macrophage damage [78]. Hypha formation inside macrophages, and later piercing of macrophage membranes, was unaffected by deletion of the candidalysin-encoding *ECE1* gene; this suggests that the toxin is not needed for physical membrane damage due to hypha extension [53,57,78].

In the first hours of *C. albicans*-macrophage interaction, host cell lysis is mainly exerted via caspase-1-dependent pyroptosis, a regulated cell death pathway depending on the activation of the NOD-like receptor protein 3 (NLRP3) inflammasome [53,54,79,80]. Activation of the NLRP3 inflammasome, resulting in secretion of bioactive IL-1β, is triggered by *C. albicans* in myeloid cells like macrophages, dendritic cells, and neutrophils [81–84], and the NLRP3 inflammasome is an essential component of the host defense against *C. albicans* [55,56].

Canonical NLRP3 inflammasome induction requires a priming and subsequent activating step. Detection of microbial ligands like fungal β-glucans or bacterial LPS by host PRRs like dectin-1 or toll-like receptor (TLR)4 leads to inflammasome priming and to the production of pro-IL-1β and pro-IL-18 [85,86]. The inflammasome is then activated, resulting in caspase-1 cleavage into its active form and processing of pro-IL-1β and pro-IL-18 into the mature, pro-inflammatory, secreted forms. In response to bacterial pathogens, the NLRP3 inflammasome can further be activated non-canonically via direct sensing of intracellular LPS by caspase-4 and caspase-5 in humans and caspase-11 in murine cells [86]. In addition, an alternative activation is possible via TLR4-dependent LPS sensing and caspase-8 activation in human monocytes [87]. In both cases, the final cleavage of pro-IL1β and pro-IL-18 into the mature forms is carried out by caspase-1 [86,87]. Apart from this, serine proteases like elastase, cathepsin G, and proteinase 3 are capable of cleaving pro-IL-1β independently of caspase-1 [88]. In in vitro culture conditions, IL-1β maturation was reported to be dependent on caspase-1. However, using in vivo studies, caspase-1-independent IL-1β processing is the prevailing source of mature IL-1β during the acute phase of infection, which is characterized by strong neutrophil infiltration. The contribution of inflammasome-mediated, caspase-1-dependent pro-IL-1β cleavage increases at later time points, which are rather macrophage/monocyte-dominated [88–92].

*C. albicans* hyphae are a necessary but insufficient trigger of inflammasome activation; this suggests that hypha-associated factors and hyphal activities, which remain largely unknown in detail, contribute to inflammasome activation [56,80,93]. Candidalysin has recently been identified as one major trigger of hypha-dependent NLRP3 inflammasome activation in primary human macrophages and murine dendritic cells [78,94]. A synthetic candidalysin peptide is sufficient to induce secretion of mature IL-1β in human and murine macrophages and murine bone marrow-derived dendritic cells in a strictly caspase-1 and NLRP3-dependent manner [78,94]. Infection of macrophages with candidalysin-deficient *C. albicans* mutants proved that candidalysin crucially contributes to *C. albicans*-dependent IL-1β secretion by murine and human macrophages [78,94,95].

Candidalysin does not provide the inflammasome-priming signal; instead, it is one of the fungal factors triggering the inflammasome-activating step [78]. The activation of the NLRP3 inflammasome

by candidalysin is mediated via potassium efflux, putatively, through toxin-induced membrane perturbances or lesion formation [78]. Potassium efflux is a common inflammasome-activating trigger that is also induced by bacterial toxins [96,97]; this indicates that fungal and bacterial membrane-disturbing toxins can activate similar pro-inflammatory response mechanisms in phagocytes [78,97]. Candidalysin-induced inflammasome activation was not only inhibited by treatment with the potassium channel inhibitor glibenclamide or by the addition of high extracellular potassium [78], but also by the addition of the inflammasome inhibitor MCC950, which directly interacts with the NLRP3 inflammasome by blocking ATP hydrolysis. This suggests that additional mechanisms other than potassium efflux are involved in the candidalysin-mediated inflammasome activation [95].

Despite inducing caspase-1-dependent inflammasome activation, candidalysin does not seem to be a major trigger of caspase-1-dependent pyroptosis in mononuclear cells [78]. Toxin-induced macrophage damage was not reduced in the presence of caspase-1 inhibitors or phagocytes isolated from caspase-1 or NLRP3 knockout mice, and a candidalysin-deficient mutant was still able to induce caspase-1-dependent damage. The data suggest that NLRP3 inflammasome activation is not necessarily coupled to pyroptosis [78]. This is in contrast to many bacterial pore-forming toxins like α-hemolysin from *Staphylococcus aureus* or listeriolysin from *Listeria monocytogenes*, which activate the inflammasome and induce pyroptosis in human and murine monocytic and monocyte-derived cells [97–99].

The data collected suggest that candidalysin triggers a separate pathway of *C. albicans*-induced host cell damage, likely by directly perturbing host cell membranes but independent of hypha-mediated mechanical host cell rupture and pyroptosis. *C. albicans* dependent pyroptosis instead seems to depend on other factors like fungal cell wall components and fungal morphology [79,100]. In addition, secreted aspartic proteases are known as inflammasome inducers [101] and could potentially contribute to this inflammatory cell death.

In summary, the currently available data shed light on candidalysin functions in macrophage membrane damage and inflammasome activation (Figure 1). Many aspects of the toxin action in these immune cells remain to be elucidated. Candidalysin will likely be secreted by growing hyphae inside the phagosome [19], but the subcellular localization inside macrophages and the mechanisms of toxin distribution within the host cell are unknown. As the toxin seems to be mostly dispensable for damage of the phagosomal membrane by growing hyphae [45], it seems that the phagosomal membrane is not the main target of this fungal toxin.

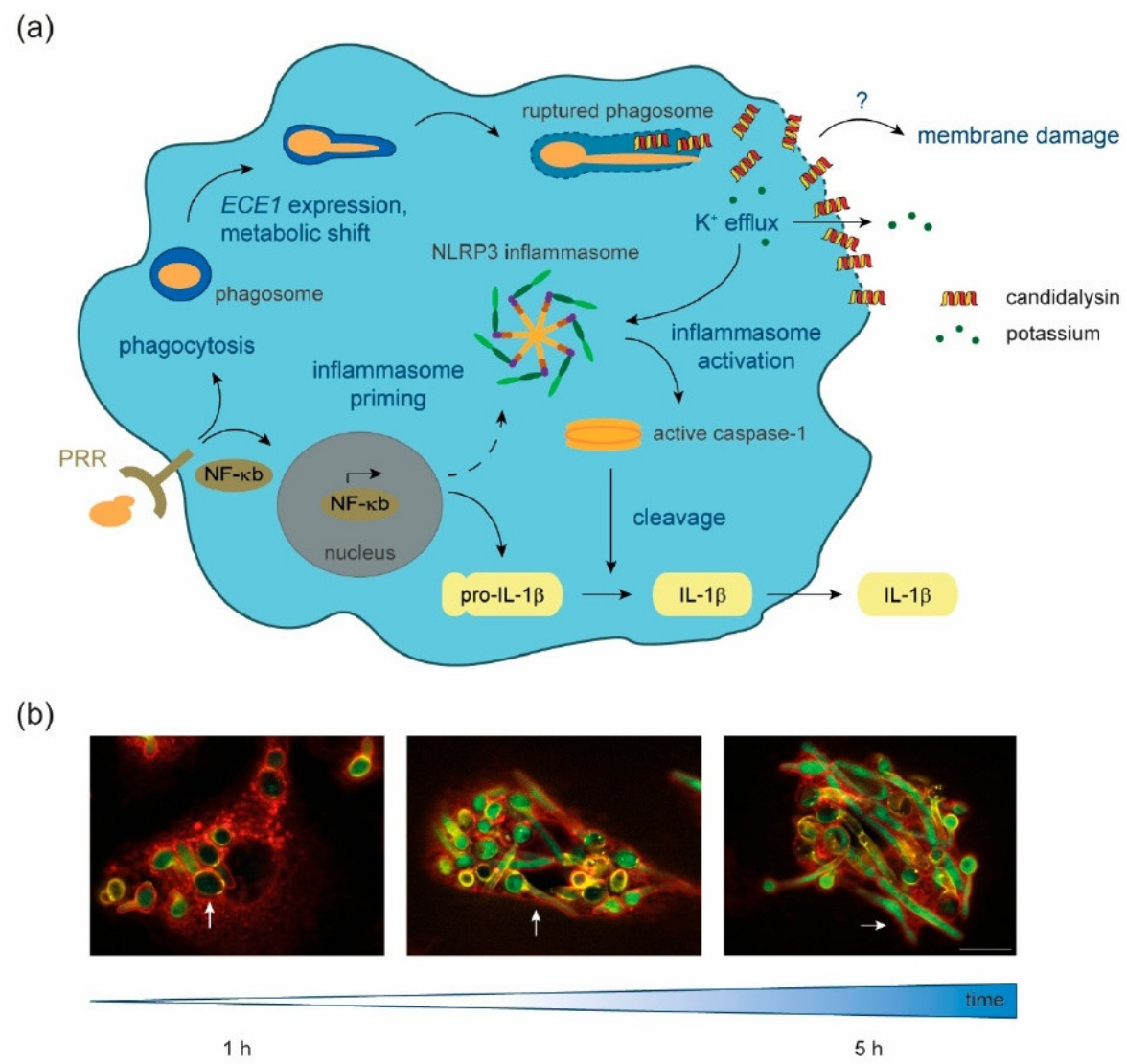


**Figure 1.** Candidalysin–macrophage interaction. (**a**) Upon recognition of fungal-pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRR), *Candida albicans* is phagocytosed and NF-κB signaling is induced. NF-κB signal transduction leads to the formation of the primed NLRP3 inflammasome and the production of pro-IL-1β. Inside the phagosome, *C. albicans* cells undergo a metabolic shift to adapt to nutrient limitation, form hyphae, express *ECE1*, and produce the polypeptide Ece1, which is further processed into candidalysin. The filamentation of the fungus inside the phagosome leads to phagosomal membrane damage and eventually hyphal outgrowth. Our data suggest that candidalysin accumulates in phagocyte membranes, facilitating ion fluxes such as potassium efflux, which in turn activates the primed inflammasome. This activation leads to cleavage of pro-caspase-1 into the enzymatically active form, which processes pro-IL-1β into the mature pro-inflammatory IL-1β, which is then secreted. What remains unknown is the exact mechanism through which candidalysin causes membrane damage. (**b**) Fluorescence microscopy images of *C. albicans* cells, which express GFP under control of the *ECE1* promoter, internalized by primary human monocyte-derived macrophages. Over 1 to 5 h, ingested yeast cells (white arrows) start to filament and induce *ECE1* transcription (green). Green, GFP; red, Concanavalin A lectin staining of host cells; yellow, Calcofluor white fungal cell wall staining. The white scale bar represents 10 μm and applies to all fluorescence microscopy images.

## 6. Dual Function of Candidalysin during Infection

By taking the candidalysin–macrophage interaction as an example, candidalysin can be seen as a microbial factor that exhibits a dual function during interaction with the host. It provides a mechanism for host cell lysis, contributing to escape from these immune cells. Through activation of the NLRP3 inflammasome, it provokes a pro-inflammatory host-protective response that can be beneficial for fungal clearance (Figure 1) [78]. This combination of effects that are both beneficial and detrimental to the host has also been proposed for the action of bacterial pore-forming toxins during the interaction with macrophages [76].

Mouse infection experiments with *C. albicans* mutants lacking *ECE1*, or the candidalysin-encoding sequence only, showed that the candidalysin-dependent induction of IL-1β release transfers from the macrophage in vitro infection model to in vivo models of systemic candidiasis. These experiments showed that candidalysin is required for host IL-1β release in murine kidneys and neutrophil recruitment [30,78]. Similarly, candidalysin-induced IL-1β production by brain microglia can induce antifungal immunity by promoting neutrophil recruitment [18].

The dual function model of candidalysin can also be transferred to the interaction of *C. albicans* with epithelial and endothelial barriers. In a mouse model of oral infections, the toxin showed to be critically important for damage induction and the establishment of infection [19]. Candidalysin-dependent damage has been seen in in vitro models of oral, vaginal, and intestinal epithelial, as well as endothelial models [19,30,65,102]. These data represent the toxin's function as a classical virulence factor [19,103]. However, candidalysin simultaneously activates epithelial PI3K/Akt, NF-κB, p38, JNK, and ERK1/2 MAPK signaling cascades. It thereby elicits a pro-inflammatory response that contributes to the recruitment of immune cells like macrophages, Th17 cells, and neutrophils to the site of infections. It mediates a protective crosstalk via the latter [19,104–107]. This danger response is also activated by candidalysin in endothelial cells and vaginal cells [30,102]. Potentially, this is mostly mediated via the release of alarmins and antimicrobial peptides in epithelial cells [108].

Candidalysin can be seen both as a virulence factor that helps to evade innate immune responses or to breach host barriers, but also as an avirulence factor that can activate host-protective responses in the immunocompetent host and thus limit the pathogen's virulence (Figure 2) [109,110].

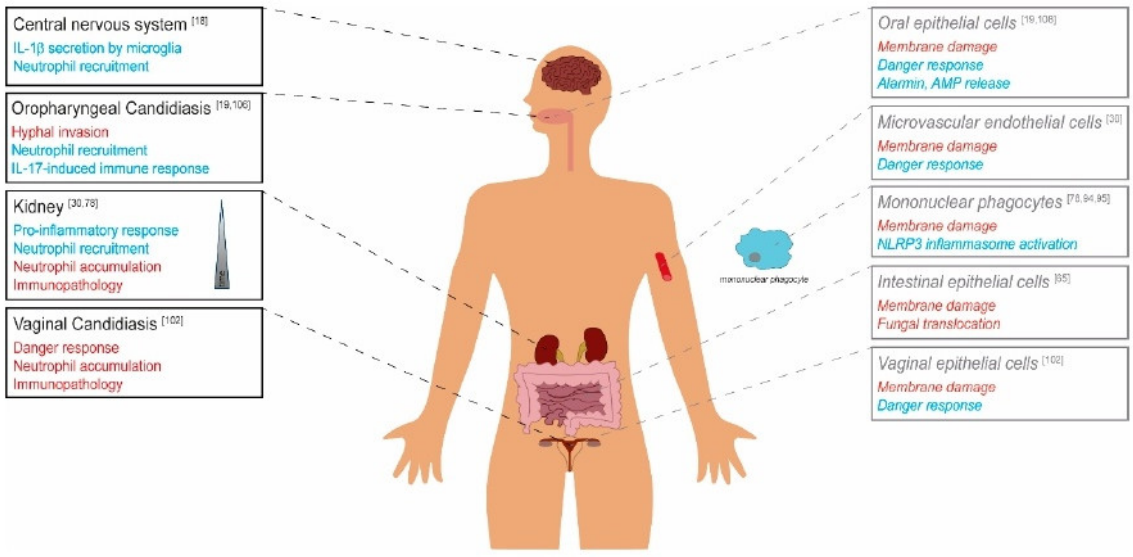


**Figure 2.** The role of candidalysin in *C. albicans* virulence. Candidalysin-dependent effects on the *C. albicans* virulence potential are depicted in red (detrimental for the host, classical virulence factor) and blue (beneficial for the host, avirulence factor) for the respective body site/organ. Black and strong colors show in vivo data derived from murine models. Grey, light colors, and typesetting in italics represent in vitro data from cell culture studies.

Depending on the infection site, candidalysin exhibits differential effects on fungal virulence. The toxin contributes to damage of host cell membranes, which is connected with fungal invasion, translocation through barriers and escape from phagocytes. The activation of host responses like the epithelial danger response pathway or the phagocyte NLRP3 inflammasome, and resulting neutrophil recruitment, in many cases leads to a protective host response. In contrast, massive neutrophil infiltration during vaginal infection and in later stages of systemic infection can cause immunopathology.

Under certain circumstances, the immune system's activation can also promote fungal virulence and worsen the infection outcome. During disseminated candidiasis, for example, neutrophil recruitment stimulated by candidalysin increases mouse mortality during later infection stages, which is likely related to immunopathological effects [30]. Similarly, a strong candidalysin-mediated infiltration of neutrophils is responsible for the typical immunopathology of vaginal *C. albicans* infections [102].

As discussed above, candidalysin expression is associated with filamentation of the fungus [19,67,68]; however, filaments are not the dominating phenotype in all host niches infected by *C. albicans*. For example, hyphae dominate in brain and kidney tissue but seem to be absent in the liver and spleen during disseminated candidiasis in a mouse model of systemic infection [111]. The murine gut is predominantly colonized by yeast cells or a mixture of yeast and hyphae [112,113]. In addition, the inhibition of filamentation by external cues or genetic modification will reduce candidalysin production [114,115]. Thus, candidalysin-induced effects will likely not only depend on niche-specific host responses but also niche-specific levels of filamentation and expression of candidalysin in the respective infection environment.

## 7. Conclusions

Macrophages are, besides neutrophils, crucial for combating disseminated candidiasis. Fungal killing is mediated by a combination of antimicrobial activities within the phagosome. The fungus can counteract these attempts by producing hyphae, which induce pyroptosis, mechanically stretch, and ultimately lyse the phagosomal membrane, thereby inducing immune cell death, further supported by fungal glucose consumption.

The cytolytic peptide toxin candidalysin contributes to macrophage lysis but also mediates the induction of pro-inflammatory cytokine release via the NLRP3 inflammasome. This, as well as its diverse implications during oral, vaginal, and systemic infections, highlights the dual function of this toxin as a classical virulence factor and an avirulence factor during the *C. albicans*-macrophage interaction, mucosal, and systemic infection.

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## 3.4 Manuscript IV – König *et al.*, *derm Praktische Dermatologie* 2020

### Das *Candida albicans* Toxin Candidalysin – das Resultat einer Ko-Evolution von Mensch und Pilz

**Annika König**, Lydia Kasper, Bernhard Hube

*derm Praktische Dermatologie* Ausgabe 04/2020, derm (26) 2020. Review.

Summary:

This short article summarises the findings made in manuscript II and puts these into the context of co-evolution of fungus and host. As the toxin can be seen as a classical virulence factor (e.g. damage induction) as well as an avirulence factor (e.g. induction of pro-inflammatory cytokines), it highlights the continuous co-evolution of *C. albicans* and the human host during which both sides constantly evolve factors and strategies to try to gain the upper hand.

Own contribution:

Annika König wrote large parts of the manuscript and created the figures.

Estimated authors' contributions:

| | |
|---|---|
| **Annika König** | **70 %** |
| Lydia Kasper | 20 % |
| Bernhard Hube | 10 % |

Prof. Dr. Bernhard Hube

# Das *Candida albicans*-Toxin Candidalysin – das Resultat einer Ko-Evolution von Mensch und Pilz

*Annika König, Lydia Kasper, Bernhard Hube*


## Summary

The yeast *Candida albicans* is typically a harmless commensal of the human mucosa including the gastrointestinal tract. However, it is further a main agent of skin and mucosal infections and can cause severe life-threatening infections. Only a few years ago, the fungus-produced peptide toxin Candidalysin has been described as a key virulence factor during mucosal candidiasis. However, the toxin does not exclusively act as a classical virulence factor upon contact with scavenger cells (phagocytes) of the innate immunity. On the one hand it kills macrophages, thereby contributing to survival after phagocytosis and enabling immune evasion of the fungus; on the other hand the toxin mediates the activation of the NLRP3 inflammasome which results in a host-protective pro-inflammatory immune response. Therefore, the toxin seems to be acting as a virulence as well as an avirulence factor, depending on the niche the fungus is occupying. This illustrates, that human and fungus undergo co-evolutionary adaptations whilst interacting, during which each side constantly tries to gain the upper hand.


*Keywords*

*Candida albicans,* toxin, Candidalysin, host cell, dual function, co-evolution.


## Zusammenfassung

Der Hefepilz *Candida albicans* ist ein normaler, meist harmloser Besiedler von Schleimhäuten, gehört aber zu den häufigsten Erregern von Haut- und Schleimhautinfektionen und kann in schweren Fällen lebensbedrohliche Infektionen auslösen. Das von diesem Pilz gebildete Peptidtoxin Candidalysin wurde vor wenigen Jahren als entscheidender Virulenzfaktor bei mukosalen Candidosen identifiziert. Beim Kontakt mit Fresszellen (Phagozyten) der angeborenen Immunität agiert das Toxin jedoch nicht exklusiv als klassischer Virulenzfaktor. Einerseits kann Candidalysin Phagozyten abtöten und es dem Pilz so ermöglichen, die Phagozytose zu überleben (Immunevasion), anderseits resultiert die Toxinbildung aber auch in der Aktivierung des NLRP3-Inflammasoms und führt somit zu einer protektiven, pro-inflammatorischen Antwort. Das Toxin scheint daher, abhängig von der jeweiligen Nische in der sich der Pilz etablieren muss, entweder als Virulenz- oder als Avirulenzfaktor zu agieren. Dies verdeutlicht, dass Mensch und Pilz im Zuge ihrer Interaktion eine Ko-Evolution durchlaufen, während derer jede Seite versucht die Oberhand und Kontrolle zu gewinnen.


*Schlüsselwörter*

*Candida albicans,* Toxin, Candidalysin, Wirtszelle, duale Funktion, Ko-Evolution.

## Die duale Funktion des *Candida albicans*-Toxins Candidalysin

Weltweit existieren zirka 3 bis 5 Millionen Pilzspezies. Eine große wirtschaftliche Bedeutung kommt vor allem den über 8.000 pflanzenpathogenen Pilzen zu, die Ernteeinträge schmälern oder Intoxikationen beim Menschen hervorrufen können. Klinische Relevanz zeigt jedoch nur ein noch kleinerer Bruchteil von 150–400 Spezies, die als humanpathogen gelten. Trotz dieser verhältnismäßig geringen Anzahl potenziell humanpathogener Pilze, infizieren diese jährlich über eine Milliarde Menschen.

Größtenteils manifestieren sich Pilzinfektionen als oberflächliche Infektionen der Haut- oder Schleimhaut (v.a. Dermatophytosen der Hornschichten, aber auch Candidosen der Schleimhaut). Diese Infektionen sind vergleichsweise harmlos und zumeist gut therapierbar. Bei einer gestörten Abwehrlage kommen aber auch zunehmend lebensbedrohliche, invasive systemische Pilzinfektionen vor, die sich durch eine hohe Morbidität und Mortalität auszeichnen. So versterben jährlich mehr Menschen an einer invasiven Pilzinfektion als an Malaria und ungefähr genauso viele wie durch Tuberkulose. In Europa sind systemische Candidosen unter systemischen Pilzerkrankungen, gefolgt von Aspergillosen (vor allem durch *Aspergillus fumigatus* verursacht), am häufigsten. Bei ersteren wird zu einer großen Mehrheit *Candida albicans* aus dem Blut isoliert, gefolgt von (je nach geografischem Standort und Patientengruppe) *C. glabrata, C. parapsilosis, C. tropicalis* und *C. krusei* (2, 10).

In gesunden Menschen ist *C. albicans* ein normaler Besiedler (Kommensale), der größtenteils im Gastrointestinaltrakt, aber auch in der Mundhöhle und der Vagina zu finden ist, ohne jedoch Infektionen hervorzurufen. Im Falle prädisponierender Faktoren wie Immunsuppression (z.B. durch eine HIV-Infektion, nach Transplantationen oder bei Tumortherapien), mechanischer Zerstörung der Wirtsbarrieren (Operationen, Katheter) oder exzessiver Antibiotikatherapie, kann *C. albicans* als opportunistischer Erreger jedoch Infektionen hervorrufen.

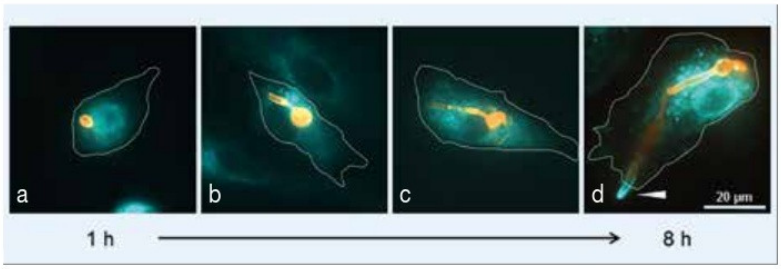


*Abb. 1a–d: Filamentierung des Pilzes in Makrophagen. a) Makrophagen (Umriss als weiße, gestrichelte Linie) phagozytieren C. albicans-Hefen (orange). b) Diese beginnen nach der Aufnahme durch Makrophagen Hyphen zu bilden. c) Über einen Zeitraum von acht Stunden wird die Hyphe immer länger, bis sie d) schließlich die Wirtzellmembran durchstößt (weißer Pfeil, Hyphenspitze wird türkis). Türkis: Färbung von Makrophagen und außenliegenden Pilzzellteilen*

Der Übergang von einem kommensalen Stadium zu einer Infektion beginnt oft mit der Interaktion von *C. albicans* mit Wirts-Epithelien, die eine erste mechanische Barriere darstellen. Der Pilz exprimiert spezielle Adhäsionsfaktoren, welche die Bindung an Wirts-Epithelzellen begünstigen. Im Anschluss an diese Adhäsionsphase, folgt die Invasion des Pilzes in das Gewebe und damit verbunden eine Schädigung der Wirtszellen, welche die Translokation in tiefere Gewebeschichten und den Blutstrom ermöglicht. Während dieser Prozesse muss sich *C. albicans* auch mit Fresszellen (Phagozyten) der Immunabwehr wie zum Beispiel Makrophagen auseinandersetzen. Kommen diese in Kontakt mit dem Pilz, erfolgt eine Erkennung von Oberflächenstrukturen der Pilz-Zellwand und *C. albicans* wird phagozytiert.

Ein wichtiger Virulenzfaktor des Pilzes ist seine Fähigkeit, in unterschiedlichen Morphologien zu wachsen, also vom Hefen- ins Hyphenwachstum überzugehen und umgekehrt. Während des Hyphenwachstums werden wichtige Virulenzfaktoren wie Adhäsine, Invasine und hydrolytische Enzyme (z.B. Proteasen) produziert. Die Hyphenbildung ermöglicht so eine Invasion von Epithelbarrieren. Sie trägt aber auch dazu bei, dass der Pilz nach der Phagozytose durch Makrophagen wieder entkommen kann, indem die Hyphen aus den Phagozyten heraus wachsen. Diese Immunevasion geht mit der Zerstörung der Wirtszellmembran und letztlich dem Zelltod der Immunzelle einher (4) (Abb. 1).

Da die Filamentierung von *C. albicans* als wesentlicher Bestandteil der Pathogenese gilt, sind Gene, die in diesen Prozess involviert sind, von besonderer Bedeutung, um den Infektionsvorgang zu verstehen.

Vorangegangene Studien zeigen, dass das Gen *ECE1* bereits Minuten nach Beginn der Hyphenbildung stark von *C. albicans* exprimiert wird. Gleichzeitig wird dieses Gen aber nicht für die Hyphenbildung oder das Hyphenwachstum selbst benötigt. Tatsächlich war die Funktion dieses Gens für den Pilz und die Infektion bis vor Kurzem unbekannt (1).

Das Gen *ECE1* kodiert für ein Protein, welches von fungalen Proteasen an bestimmten internen Aminosäuremotiven gespalten wird. Es resultieren acht Peptide, von denen eines (Peptid 3) eine α-helikale Struktur annimmt. Dieses Peptid wurde als zentrale Schädigungskomponente gegenüber Epithelzellen charakterisiert und stellt das erste beschriebene Toxin eines humanpathogenen Pilzes dar (8). Aufgrund seiner Membran-pertubierenden und zerstörerischen (lytischen) Eigenschaften wurde das Peptid als Candidalysin bezeichnet. Diese Eigenschaft, die Hyphenassoziation, die hohe und schnelle Genexpression sowie die Tatsache, dass *C. albicans* mittels Hyphenbildung aus Makrophagen entkommen kann, ließen vermuten, dass das Gen *ECE1* beziehungsweise respektive das kodierte Toxin Candidalysin auch bei Interaktionen mit Makrophagen eine wichtige Rolle spielt. In der Tat wird *ECE1* während des Hyphenwachstums von *C. albicans*-Zellen nach der Phagozytose durch Makrophagen stark exprimiert. Es lag daher auf der Hand, den Einfluss von Candidalysin auf die Wirtsschädigung und das Ausbrechen des Pilzes aus Makrophagen näher zu untersuchen.

Für die Interaktion von *C. albicans* mit Makrophagen waren vor Beginn unserer Studie zwei mögliche Wege der Immunzellschädigung bekannt: 1. eine mechanische Zerstörung der Phagozytenmembran durch das Auswachsen von *C. albicans*-Hyphen und 2. der bestimmte, regulierte Zelltodmechanismus der sogenannten Pyroptose, der mit einer Inflammasom-Aktivierung und einer Freisetzung pro-inflammatorischer Zytokine, vor allem Interleukin (IL)-1β, einhergeht.

Inflammasome sind große, zytoplasmatische Proteinkomplexe, die aus einem Sensormolekül, einem Adapterprotein und einer Protease (Caspase-1) bestehen und die essenzielle Funktionen bei mikrobiellen Infektionen haben. Die Inflammasom-Reaktion erfolgt über ein 2-Stufen-Programm. In einem ersten Schritt, dem Priming, wird zunächst über die Aktivierung zellulärer Oberflächenrezeptoren eine Signalkaskade aktiviert, woraufhin unter anderem das Vorläufermolekül von IL-1β (pro-IL-1β) gebildet wird. Ein sich anschließendes Signal bedingt die Aktivierung des geprimten Inflammasoms und führt zur Spaltung von pro-IL-1β durch zum Beispiel Caspase-1 und zur Freisetzung von bioaktivem IL-1β. Die Aktivität von Caspase-1 kann weiterhin einen regulierten pyroptotischen Zelltod durch Wirtszell-Lyse vermitteln.

Die Inflammasomaktivierung durch *C. albicans* ist bereits hinreichend beschrieben, allerdings war bisher weitgehend ungeklärt, welche spezifischen Pilzsignale zum Anschalten dieser

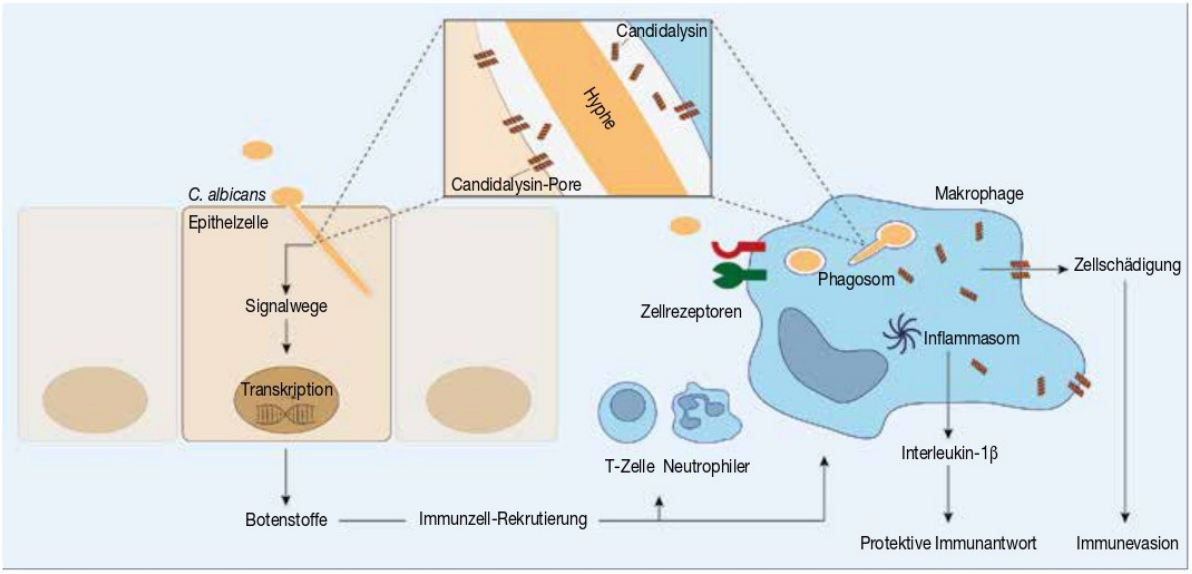


*Abb. 2: Interaktion von C. albicans mit Epithelzellen und Makrophagen. Candida albicans kann durch Hyphenbildung in Epithelzellen einwachsen (Bildung einer Invasionstasche). Dabei kann von den Hyphen freigesetztes Candidalysin die Epithelzellmembran schädigen, gleichzeitig werden aber auch epitheliale Signalwege aktiviert. Daraus resultierend werden Botenstoffe freigesetzt, die die Rekrutierung verschiedener Immunzellen aktivieren, darunter auch Makrophagen. Der Pilz wird von Makrophagen erkannt und phagozytiert. Aufgenommene C. albicans-Zellen bilden daraufhin Hyphen und sezernieren Candidalysin, welches eine Aktivierung des Inflammasoms auslöst. Dadurch werden pro-inflammatorische Zytokine freigesetzt, die wiederum die Rekrutierung weiterer Immunzellen vermitteln (protektive Immunantwort). Ebenfalls schädigt das Toxin jedoch die Zelle. Diese zytotoxischen Eigenschaften können die Fresszelle zerstören, so ein Abtöten des Pilzes verhindern und damit eine Immunevasion ermöglichen*

pro-inflammatorischen Antwort führen (11).

Interessanterweise ist die Sekretion von pro-inflammatorischem IL-1β durch Makrophagen deutlich *ECE1*- beziehungsweise Candidalysin-abhängig. Das Peptid selbst ist dabei nicht am Priming der Immunzellen beteiligt, da eine Zytokinsekretion nach Candidalysinzugabe nur nach vorheriger Stimulation der Phagozyten durch andere immunstimulierende Substanzen wie zum Beispiel bakterielle Lipopolysaccharide (LPS) erfolgt. Candidalysin stellt somit eines der lange gesuchten, *C. albicans*-abhängigen Inflammasom-aktivierenden Signale dar. Weiterhin zeigen unsere Studien, dass das Toxin Candidalysin tatsächlich das NLRP3-Inflammasom aktiviert, welches bereits als wichtig für die Interaktion von *C. albicans* mit Makrophagen beschrieben wurde (3, 5). Aus Studien mit bakteriellen Krankheitserregern sind verschiedene Aktivierungsmechanismen für Inflammasomen, wie reaktive Sauerstoffspezies, lysosomale und mitochondriale Dysbalancen aber auch Ionenfluxe, bekannt. Im Falle von Candidalysin erfolgt die Aktivierung über Freisetzung von Kalium, einem Mechanismus, der auch von vielen Bakterientoxinen genutzt wird (5).

Ein ebenfalls zentraler Schritt der Pyroptose ist die terminale Lyse der Wirtszelle. In Epithelzellen ist die *C. albicans*-vermittelte Schädigung absolut abhängig von *ECE1* und der Präsenz von Candidalysin. Auch in Makrophagen zeigt sich eine Abhängigkeit der Schädigung von *ECE1* und Candidalysin, jedoch nicht in dem gleichen Ausmaß wie bei Epithelzellen. In diesen Immunzellen ist offensichtlich das Zusammenwirken von verschiedenen Faktoren wichtig für die Wirtszell-Lyse. Wir konnten jedoch beobachten, dass im Falle einer Infektion der Makrophagen die Candidalysin-induzierte Immunzell-Lyse unabhängig von Caspase-1 stattfindet und somit nicht einem pyroptotischen Zelltod zuzuordnen ist. Unsere Daten weisen vielmehr darauf hin, dass die Lyse durch eine direkte Interaktion des Toxins mit der Zytoplasmamembran der Makrophagen verursacht wird (5).

Im Hinblick auf das mechanische Auswachsen der Pilz-Hyphen aus den Immunzellen zeigten sich keinerlei Unterschiede zwischen Wildtyppilzzellen und solchen, denen das *ECE1*-Gen fehlt. Auch hat *ECE1* keinen Einfluss auf das Hyphenwachstum von phagozytierten *C. albicans*-Zellen (5).

Somit zeigt sich, dass die Interaktion von *C. albicans* mit Zellen der Immunabwehr noch komplexer ist als bisher angenommen und mindestens drei Möglichkeiten des Pilz-induzierten Zelltods in Betracht gezogen werden müssen: Die beiden bereits etablierten Mechanismen 1. der mechanischen Zerstörung durch Filamentierung des Pilzes (Candidalysin-unabhängig), 2. des pro-inflammatorischen, pyroptotischen, nicht Candidalysin-abhängigen

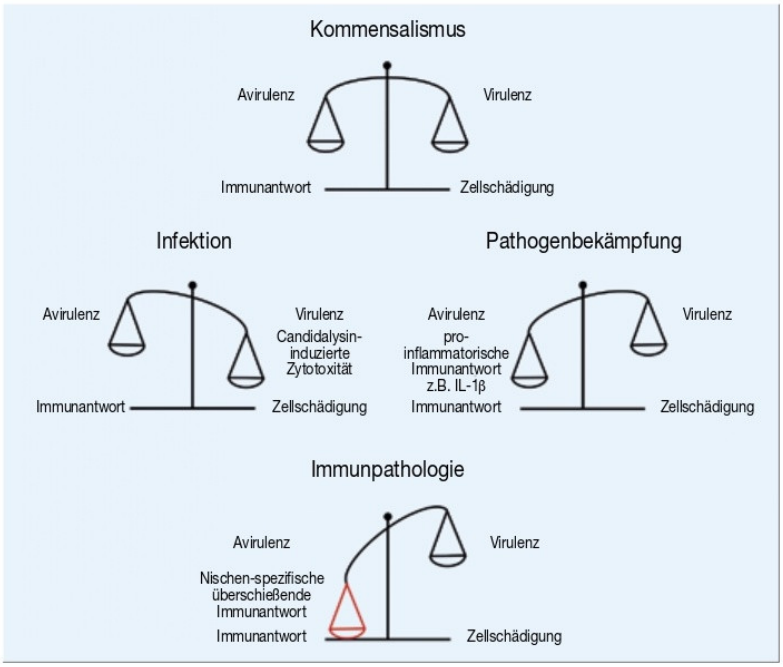


*Abb. 3: Konzept für Gene mit dualer Funktion am Beispiel von Candidalysin. Das Konzept der dualen Funktion von Candidalysin ist gut als Waage darstellbar. Auf der einen Seite steht die Funktion als Avirulenzfaktor, auf der anderen die als Virulenzfaktor. Während der kommensalen Phase wird durch eine geringe Pathogen- und Toxinlast keine, beziehungsweise nur eine sehr mäßige Immunantwort ausgelöst, der Pilz besiedelt die Nische asymptomatisch. Im Falle der Infektion kommt es zu einer durch Proliferation erhöhten Pilz- sowie Toxinlast, es überwiegt die Funktion als Virulenzfaktor, vor allem wenn die Konzentrationen des Toxins sehr hoch sind (in der Invasionstasche, im Phagosom). Die Wirtszelle wird geschädigt, sodass eine Immunevasion des Pilzes möglich ist. Gleichzeitig bedingen erhöhte Candidalysin-Konzentrationen jedoch auch die Aktivierung pro-inflammatorischer Signalwege, die eine Immunzell-Rekrutierung und schlussendlich die Pathogen-Eliminierung vermitteln. Je nach besiedelter Nische kann allerdings auch die eigentlich protektive Immunantwort überschießen und durch massive Rekrutierung von Immunzellen und Dysregulation zur Immunpathologie führen, wie zum Beispiel während vaginaler C. albicans-Infektionen (adaptiert aus 9)*

Zelltods und die neu aufgedeckte 3. Candidalysin-induzierte, direkte Membranschädigung (5–7).

Am Beispiel der *C. albicans*-Makrophagen-Interaktion ergibt sich daher eine interessante neue Sicht auf das bisher fast exklusiv als Zytotoxin beziehungsweise Virulenz-assoziierten Faktor eingeordnete Candidalysin. Offensichtlich ist das Toxin neben seiner Funktion als wichtiger Virulenzfaktor auch ein Avirulenzfaktor des Pilzes. So vermittelt es einerseits die Wirtszellschädigung während der Infektion, wodurch der Pilz Zugang zu Nährstoffen erhält, die Translokation in tiefere Gewebeschichten und – bei Immunzellkontakt – eine Immunevasion für den Pilz. Andererseits induziert das Toxin aber auch eine pro-inflammatorische Immunantwort, die eine schützende Funktion für den Wirt hat (Abb. 2).

Auch bei der Interaktion mit Epithelzellen zeigt sich diese Dualität – Candidalysin vermittelt nicht nur die Epithelzellschädigung und ist wichtig für die Etablierung von oralen Infektionen im Maus-Infektionsmodell, sondern aktiviert auch epitheliale Signalwege, die wiederum zur Stimulierung einer pro-inflammatorischen Immunantwort beitragen (8). Weiterführende Studien zeigen, dass Candidalysin nicht nur wichtig für die Etablierung oraler sondern auch vaginaler und systemischer Infektionen mit Verbreitung des Pilzes über die Blutbahn ist. Hier spielt die Rekrutierung von Neutrophilen sowie eine T-Zell-vermittelte Immunantwort eine Rolle (9) (Abb. 2). Allerdings sind die Konsequenzen der Immunaktivierung gewebeabhängig. Während sich die Stimulierung durch Candidalysin bei oralen Infektionen schützend auswirkt und zur Eliminierung des Pilzes führt, ist die Stimulierung durch Candidalysin bei vaginalen Infektionen ein entscheidender Beitrag zum Krankheitsbild, weil die Rekrutierung von Neutrophilen zu einer Immunpathologie führt, die typisch für vaginale Infektionen ist (9).

Ob die Eigenschaften von Candidalysin als Virulenz- oder als Avirulenzfaktor überwiegen, hängt also vom Immunstatus des Wirts sowie von der Infektionsnische ab. Im immunkompetenten Wirt setzen kolonisierende *C. albicans*-Zellen vermutlich nur wenig Toxin frei – es kommt zu keiner Krankheit. Wenn die Bedingungen jedoch günstig für eine *C. albicans*-Vermehrung und -Infektion sind, wird durch die angeregte Hyphenbildung mehr Toxin freigesetzt und es kommt zur Schädigung des Wirts. Die gleichzeitige Aktivierung des Immunsystems kann dann zu einer Eindämmung der Infektion führen. In bestimmten Infektionsnischen (vaginale Infektionen) oder bei einer fehlregulierten Immunantwort kann Candidalysin aber auch zu Schäden durch eine Überreaktion des Immunsystems führen (Immunpathologie) (9) (Abb. 3).

Weiterhin verdeutlicht diese duale Funktion, dass Mensch und Pilz im Zuge ihrer Interaktion und Ko-Existenz über einen langen Zeitraum eine Ko-Evolution durchlaufen haben und dies weiterhin tun. Beide Interaktionspartner entwickeln konstant Faktoren und Strategien, um sich gegen den jeweils anderen durchzusetzen, während der andere wiederum Gegenmaßnahmen entwickelt und ergreift. So versuchen beide, Mensch und Pilz, das Rennen um die Überhand während der Infektion für sich zu entscheiden.

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## 3.5 Manuscript V – König, Müller *et al.*, *Cellular Microbiology* 2021

### Fungal factors involved in immune evasion, modulation and exploitation during infection

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*These authors contributed equally to this work

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Summary:

Human and plant pathogenic fungi evolved surprisingly similar strategies and features despite infecting hosts from very different kingdoms. This review summarises mechanisms employed by representative human and plant pathogenic fungal species to evade the immune system, modulate specific host targets or pathways, and to access nutrients from the host, with a focus on recent discoveries. Many of these mechanisms involve fungal effector molecules that mediate distinct processes upon infection of the respective host. Furthermore, this manuscript emphasises that a similar evolutionary pressure mediates the evolution of similar factors and strategies even if the host and/or the infected niche differ significantly.

Own contribution:

Annika König designed the review structure, conducted an overall and subsequently detailed literature research, wrote the part on immune evasion, critically read the manuscript and created the figures.

Estimated authors' contributions:

| | |
|---|---|
| **Annika König** | **35 %** |
| Rita Müller | 35 % |
| Selene Mogavero | 20 % |
| Bernhard Hube | 10 % |

Prof. Dr. Bernhard Hube

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MICROREVIEW

WILEY

# Fungal factors involved in host immune evasion, modulation and exploitation during infection

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[Correction added on 26 October 2020, after first online publication: Projekt Deal funding statement has been added.]

## Abstract

Human and plant pathogenic fungi have a major impact on public health and agriculture. Although these fungi infect very diverse hosts and are often highly adapted to specific host niches, they share surprisingly similar mechanisms that mediate immune evasion, modulation of distinct host targets and exploitation of host nutrients, highlighting that successful strategies have evolved independently among diverse fungal pathogens. These attributes are facilitated by an arsenal of fungal factors. However, not a single molecule, but rather the combined effects of several factors enable these pathogens to establish infection. In this review, we discuss the principles of human and plant fungal pathogenicity mechanisms and discuss recent discoveries made in this field.

**KEYWORDS**
effector proteins, fungal virulence factors, host exploitation, host modulation, human pathogenic fungi, immune evasion, plant pathogenic fungi

## 1 | INTRODUCTION

Among the estimated 3–5 million fungal species existing worldwide, relatively few are described as pathogens of plants, animals or humans (Blackwell, 2011). Nevertheless, these fungal species can have dramatic effects in nature, agriculture and human health. For example, many plant pathogenic fungi dramatically reduce crop yields (Doehlemann, Okmen, Zhu, & Sharon, 2017; Kohler, Hube, Puccia, Casadevall, & Perfect, 2017) and only a few hundred human pathogenic fungal species infect more than a billion people every year (Brown, Denning, & Levitz, 2012; Kohler et al., 2017). Although the hosts of these two groups of pathogenic fungi are diverse, the principles of plant and human fungal pathogenicity mechanisms are similar. In this review, we focus on recent studies dealing with factors and properties of selected plant and human pathogenic fungi facilitating infections. We will further focus on the examples of factors produced by fungi from both groups, which facilitate three key pathogenic aspects: immune evasion, host target modulation and exploitation of micro- and macro-nutrients from the host.

Annika König and Rita Müller contributed equally to this study.

## 2 | PROTECTION FIRST OF ALL – FUNGAL FACTORS INVOLVED IN IMMUNE EVASION

Any initial invasive contact of plant or human pathogenic fungi with the host surface will likely induce an immune response detrimental for the fungus. Thus, escaping the host's immune response is a general feature of all successful fungal pathogens. In fact, fungal pathogens have evolved sophisticated strategies to accomplish this, for example, by avoiding recognition and by manipulating host responses.

### 2.1 | Venetian masquerade ball – Shielding of immunostimulatory cell wall moieties

The fungal cell wall is the first immunostimulatory layer coming into direct contact with a host cell. It is comprised of chitin, glucans, polysaccharides (e.g., mannoproteins), waxes and pigments, depending on the pathogen (Gow, Latge, & Munro, 2017). Recognising these cell wall components cannot only alarm the host of a potential attack by a microbial invader, but also induce a targeted immune response towards a fungal pathogen. A well conserved immunostimulatory part of the cell wall of human and plant pathogenic fungi is β-glucan, which is sensed by host pathogen recognition receptors (PRRs), such as dectin-1 on human cells, subsequently initializing an immune response. Shielding is a common fungal strategy to prevent this recognition. Pathogenic *Candida* spp. cover β-glucan with mannoproteins, thus preventing receptor binding (Gow et al., 2017). In the airborne pathogen, *Aspergillus fumigatus*, the surface of hydrophobin RodA has been identified to mask the β-glucan layer (Carrion Sde et al., 2013). *Cryptococcus neoformans* synthesises a polysaccharide capsule to shield its β-glucan in the cell wall (O'Meara & Alspaugh, 2012), whereas *Histoplasma capsulatum* has been shown to shield its β-glucan layer with a coat of α-1,3-linked glucans, rendering it non-immunostimulatory (Rappleye, Eissenberg, & Goldman, 2007). This fungus also secretes Eng1, a β-glucanase, reducing the amount of exposed β-glucan on its surface (Garfoot, Shen, Wuthrich, Klein, & Rappleye, 2016).

Similarly, the plant pathogen, *Colletotrichum graminicola*, selectively down-regulates GLS1, a β-glucan synthase, during biotrophic growth, making hyphae non-immunogenic early on during infection (Oliveira-Garcia & Deising, 2013). Specific proteins that evolved to act exclusively during interactions with the host and thus named "effectors," can play a role in shielding as well, as shown for the root endophyte, *Piriformospora indica*. This fungus secretes FGB1, which binds β-glucan, altering the fungal cell wall composition and suppressing β-glucan-triggered reactive oxygen species (ROS) induction (Wawra et al., 2016).

Chitin is a cell wall component able to activate chitin-triggered immunity (CTI) in the host plant (Gow et al., 2017). Many fungal plant pathogens secrete LysM effector proteins containing chitin-binding LysM motifs, thereby manipulating CTI in host plants. For instance, the causative agent of tomato leaf mould, *Cladosporium fulvum*, secretes the effector protein, Avr4, which binds to fungal chitin, thereby protecting the pathogen against host chitinases and the release of chitin fragments initiating CTI (Stergiopoulos et al., 2010).

Strategies used by human and plant pathogenic fungi to prevent recognition of immunogenic cell wall moieties by the host's immune system are summarised in Figure 1.

### 2.2 | Creating confusion – Fungal activities to evade immune recognition

Immune recognition can also be actively evaded by binding or degrading host factors. For example, the human pathogenic fungus, *Candida*

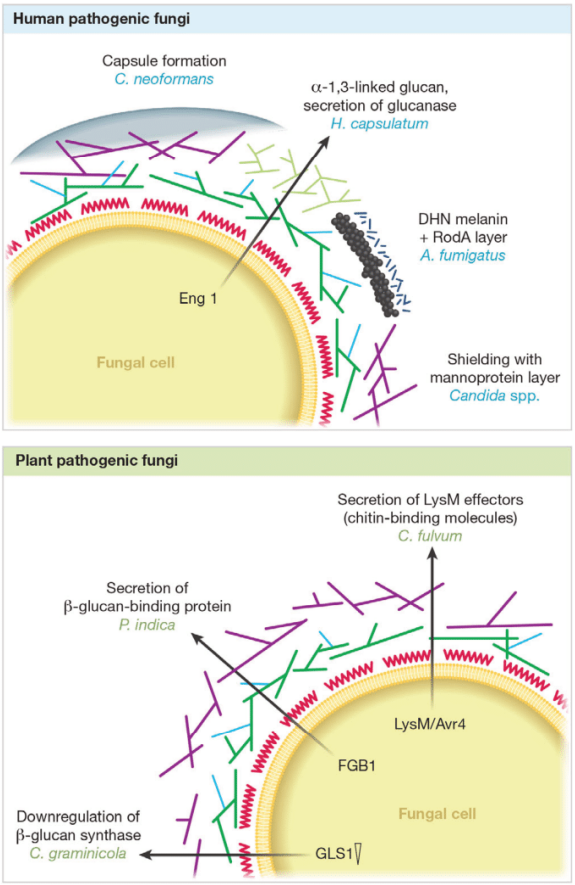


**FIGURE 1** Shielding strategies of human and plant pathogenic fungi. Human and plant pathogenic fungi use elaborate strategies to shield immunogenic cell wall moieties from the host's immune system. Human pathogenic fungi largely rely on shielding of the highly immunogenic β-glucan by capsule formation, a pigment, mannoprotein or hydrophobin layer, and different glucan linkage. The secretion of glucanases also ensures the reduction of surface β-glucan. Plant pathogenic fungi rather actively interfere with the immune recognition by secretion of glucan- and chitin-binding proteins. Furthermore, the down-regulation of β-glucan synthase reduces the amount of the immunogenic molecule in the fungal cell wall. Yellow – plasma membrane, red – chitin, dark green – β-1,3-glucan, turquoise – β-1,6-glucan, light green – α-1,3-glucan, purple – mannan, black – DHN melanin, dark blue – RodA, grey – capsule, scheme is modified from Gow et al. (2017)

*albicans*, secretes several proteins that interfere with the complement system. For example, Gpm1 and Pra1 bind the host complement factor H (FH) and other complement components, thereby impairing complement system activation, opsonization and uptake of the fungus by phagocytes (Luo, Hipler, Munzberg, Skerka, & Zipfel, 2015). *In A. fumigatus*, Aspf2 has been shown to recruit FH (Dasari et al., 2018). Similarly, *C. neoformans* targets the complement receptor 3 (CR3) by secreting App1, an anti-phagocytic effector protein, which binds to CR3 on macrophages preventing the uptake of the fungus, thus facilitating dissemination throughout the bloodstream at early stages of infection (Luberto et al., 2003). Other fungal factors can prevent killing by phagocytes. In *A. fumigatus*, DHN melanin has been shown to interfere with the endocytic pathway following immune cell uptake by suppressing the final acidification step in the phagolysosome (Thywissen et al., 2011). The pigment also inhibits the NADPH oxidase-dependent activation of LC3-associated phagocytosis, which is crucial for host defence against *A. fumigatus* (Akoumianaki et al., 2016).

Plant pathogenic fungi can also produce factors that facilitate evasion from the plant's immune response. *An elegant evasion mechanism achieved by several maize pathogens, including the* corn smut fungus *Ustilago maydis*, relies on the secretion of fungalysins, metalloproteases, which have been described to cleave host chitinases, thereby preventing the release of immunogenic chitin-derived pathogen-associated molecular patterns (PAMPs), which, in turn, would activate CTI in the host plant (Okmen et al., 2018). Furthermore, the *U. maydis* effector, Pit2, inhibits apoplastic host cysteine proteases, which otherwise would induce the plant's defence mechanisms (Mueller, Ziemann, Treitschke, Assmann, & Doehlemann, 2013; van der Linde et al., 2012). Similarly, Avr2, a *C. fulvum* effector, interacts with the plant protease, Rcr3, thereby directly suppressing host immunity (van Esse et al., 2008).

## 2.3 | The counterattack – Antagonising activated immune responses

Although prevention of an immune response can be effective, it may not be enough. When the immune response has already been activated, the fungus can either directly target proteins mediating host defence or inhibit the signalling mechanisms involved. The first has been shown for the *U. maydis* effector, Pep1, which blocks ROS formation by plant POX12, thus suppressing oxidative stress that is usually initiated around invading hyphae (Hemetsberger, Herrberger, Zechmann, Hillmer, & Doehlemann, 2012). Furthermore, the protein, Shy1, has been reported to degrade salicylic acid, an important plant hormone mediating local and systemic resistance against infections (Rabe, Ajami-Rashidi, Doehlemann, Kahmann, & Djamei, 2013). The *Magnaporthe oryzae* AvrPiz-t effector targets rice RING ubiquitin ligase, APIP6, which suppresses the induction of pattern-triggered immunity (PTI) in the host plant (Park et al., 2012). Furthermore, it has been shown to interact with a host potassium channel, thereby interfering with important signalling pathways mediating the plant's immune response to the fungus (Shi et al., 2018). Interestingly, effector proteins, secreted by symbiotic mycorrhizal fungi, can also subvert the plant's defence program. For example, *Glomus intraradices* secretes SP7, which inhibits the transcription of defence-related genes in plant root cells (Kloppholz, Kuhn, & Requena, 2011).

Such modifications of host immune responses by effector proteins, which are secreted and transported into the host cell, are a very common theme for plant pathogenic fungi (Stergiopoulos & de Wit, 2009); however, so far, not a single effector, with related functions, has been found in human pathogenic fungi. Thus, human pathogens often have to live with the consequences of an activated immune response. For example, in many human pathogenic fungi, host-initiated oxidative killing mechanisms are readily detoxified. This is achieved, for instance, in *C. albicans* by the surface-bound superoxide dismutases (Sod) 4 and 5 (Fradin et al., 2005; Frohner, Bourgeois, Yatsyk, Majer, & Kuchler, 2009) or by the glutathione reductase, Grx2, and the thioredoxin, Trx1 (Miramon et al., 2012). Very similar mechanisms are found in *C. neoformans* (Sod1) (Cox et al., 2003), *Candida glabrata* (Sod1, Yap1) (Roetzer et al., 2011) and *H. capsulatum* (Sod3) (Youseff, Holbrook, Smolnycki, & Rappleye, 2012). An alternative strategy is adopted by *A. fumigatus*, where the secondary metabolite, gliotoxin, interferes with ROS production by neutrophils (Tsunawaki, Yoshida, Nishida, Kobayashi, & Shimoyama, 2004).

These examples show that plant as well as human pathogens have evolved strategies and effectors to evade an activated immune response, for example, by detoxifying oxidative stress or through inhibition of important signalling cascades. However, secreted effector proteins that are transported into the host cell are still to be described for human pathogenic fungi.

## 2.4 | The final strike – Interference with host cell death pathways

The ultimate outcome of a fungal–host cell interaction during infection may be host cell death. This can be caused by regulated processes or by the consequence of overwhelming host cell damage and host cell death, in general, can be beneficial or detrimental for either the fungal pathogen or the host.

For example, when the induction of defence mechanisms is not sufficient to limit the infection, the host can activate cell death pathways, thus sacrificing infected host cells to limit pathogen spreading and mediate fungal clearance. However, pathogens have evolved strategies and factors to interfere with this process for their own purpose. Depending on the type of pathogenic lifestyle, host cell death can either be a disadvantage or an advantage for the fungus. Some fungi (e.g., necrotrophic plant pathogenic fungi) actively induce cell death of the respective host to gain access to nutrients or to promote spreading within the host, whereas others inhibit the induction of host cell death in order to evade the immune system or due to the requirement of a living host (e.g., facultative intracellular pathogens, biotrophic plant pathogens). Furthermore, the interference with host cell death pathways can be divided into active modulation of regulated cell death pathways and indirect induction of killing due to toxin secretion or massive fungal proliferation, which overwhelm the host and, in turn, cause host cytolysis.

In the biotrophic fungus-like oomycete *Phytophthora infestans*, the effector protein, Avr3a, has been shown to suppress programmed cell death (PCD) of the plant host cell (Bos et al., 2010), while SNE1 inhibits necrotic cell death and interferes with other cell death inductor pathways (Kelley et al., 2010). Similarly, *Phytophthora sojae* Avr1b suppresses programmed plant cell death induction (Dou et al., 2008).

Necrotrophic plant pathogenic fungi, which feed on dead plant tissue, use a variety of effector proteins to induce plant cell death. This not only facilitates nutrient acquisition, but also limits detrimental ROS release by the plant's defence mechanisms. *Sclerotinia sclerotiorum*, *the causative agent of white mould in many plants*, *secretes the small protein*, SsSSVP1, which relocates a protein of the mitochondrial cytochrome b-$c_1$ complex in the host plant, thereby triggering plant cell death (Lyu et al., 2016). Many necrotrophic fungi have been shown to elicit necrosis by activating the host plant's PCD machinery. Exemplarily, Ecp2 from *C. fulvum* and its homologues in *Mycosphaerella fijiensis* induce necrosis in a susceptible host (Stergiopoulos et al., 2010). Furthermore, secreted toxins contribute to host cell death. Fumonisin B1, a toxin of *Fusarium moniliforme* is capable of inducing apoptosis-like host cell death by interfering with the jasmonate/ethylene (JA/ET) and salicylate (SA) signalling pathways (Asai et al., 2000). Victorin, a toxin produced by the oat pathogen, *Cochliobolus victoriae*, similarly triggers an apoptosis-like cell death in susceptible plants but via mitochondrial malfunction (Curtis & Wolpert, 2004). Furthermore, the toxin binds TRXh5, which is sensed by LOV1, a protein which subsequently induces PCD (Wolpert & Lorang, 2016). Another inductive strategy is used by the AT toxin of *Alternaria alternata*, which has been shown to induce PCD in tobacco, involving caspase-like proteases and ROS accumulation in the plant tissue (Yakimova, Yordanova, Slavov, Kapchina-Toteva, & Woltering, 2009).

Interfering with host cell death pathways is not a feature limited to plant pathogenic fungi. For example, immune evasion can be accomplished by molecules from human fungal pathogens inducing apoptosis in host cells. DHN melanin of *A. fumigatus* suppresses the induction of apoptosis in macrophages via PI3K/Akt signalling pathways (Isaac et al., 2015) and thus inhibits the exposure of pathogen-derived antigens to dendritic cells, which bridge innate and adaptive immunity (Albert, 2004). Furthermore, the programmed, pro-inflammatory host cell death pyroptosis can be activated by fungi upon infection of macrophages. A well-studied example is given by *C. albicans*. Different factors and mechanisms have been discussed as triggers for pyroptosis, such as cell wall composition and the formation of hyphae (Joly et al., 2009; O'Meara et al., 2018; Uwamahoro et al., 2014; Wellington, Koselny, Sutterwala, & Krysan, 2014). In addition, secreted aspartyl proteases (Saps) have been shown to activate the NLRP3 inflammasome and could thus potentially induce pyroptosis upon *C. albicans* infection (Pietrella et al., 2013). Apart from these, Ahr1 and Stp2, transcriptional regulators of amino acid transport, have been reported to be involved in inflammasome activation and pyroptosis by preventing phagosomal acidification (Vylkova & Lorenz, 2017). Clearly, the induction of pyroptosis in innate immune cells is triggered by a combination of several fungal factors. The induction of this type of cell death can provide an escape route for the fungus from the hostile environment inside immune cells; however, the release of pro-inflammatory cytokines associated with pyroptosis causes attraction of neutrophils and thus contributes to pathogen clearance.

Fungal pathogens can also proliferate within immune cells for longer periods without engaging host cell death programs, but finally causing cell death due to overwhelming host cell damage. For example, the facultative intracellular pathogen, *C. neoformans*, resides and replicates in acidic phagolysosomes, which are maintained at an optimal pH by the buffering capacity of the fungal capsule, thus ensuring an immunological silent replicative niche for the pathogen (De Leon-Rodriguez, Fu, Corbali, Cordero, & Casadevall, 2018). Also residing and replicating, this time in non-acidified phagolysosomes, is *C. glabrata*, which has also been shown to manipulate macrophage cytokine profiles towards a less pro-inflammatory pattern. A constant proliferation of the fungus inside the phagocyte ultimately leads to host cell lysis and pathogen release, 2–3 days after infection (Seider et al., 2011). Recent observations propose that fungal biotin homeostasis plays a role in *C. glabrata*'s potential to persist and evade the host immune system (Sprenger et al., 2020). In contrast, *C. albicans* readily forms hyphae upon phagocytosis, which leads to mechanical damage of the phagocyte membrane starting already hours after infection (McKenzie et al., 2010).

However, host cell death is not always the ultimate outcome once a pathogenic fungus has entered a host cell. During the later stages of infection, *C. neoformans* favours phagocytosis and uses macrophages as a Trojan horse to cross the blood–brain barrier after replicating within immune cells (Santiago-Tirado, Onken, Cooper, Klein, & Doering, 2017). Furthermore, some pathogens are capable of escaping from the phagolysosome by inducing their own expulsion. The above-mentioned *C. neoformans* uses non-lytic expulsion (vomocytosis) as a side escape route (around 10–27% in the first 10 hr of infection depending on the host cell type), a process that requires actin, involves urease activity as well as autophagy mechanisms, and can still occur several hours after phagocytosis (Fu et al., 2018; Johnston & May, 2010; Ma, Croudace, Lammas, & May, 2006; Nicola et al., 2012). This non-lytic expulsion has additionally been described to occur as a rare event in *C. albicans*, *C. krusei* and *C. parapsilosis*-infected immune cells, however, the factors mediating this process are still under investigation (Bain et al., 2012; Garcia-Rodas, Gonzalez-Camacho, Rodriguez-Tudela, Cuenca-Estrella, & Zaragoza, 2011; Toth et al., 2014).

Apart from this non-lytic process, the induction of direct host cell lysis via toxin production can mediate fungal escape from immune cells. This has recently been reported for the *C. albicans* peptide toxin candidalysin, which has been shown to directly damage mononuclear phagocyte membranes, thus facilitating fungal escape without activating PCD pathways (Kasper et al., 2018). As discussed above, both human and plant fungal pathogens have evolved similar strategies to prevent recognition by the host's immune system, to circumvent the host's defence mechanisms, and to manipulate the immune response as depicted in Figure 2.

However, there are also clear differences between the two groups of fungal pathogens. For example, human and plant pathogenic fungi share mechanisms of interference with the host's cell death machinery, but one striking difference between pathogens of different hosts is evident. Plant pathogenic fungi mainly interfere with host cell death pathways to facilitate their respective lifestyle (biotrophic or

necrotrophic) and thus nutrient acquisition, whereas human pathogenic fungi usually have to cope with an activated immune response and rather use this interference to mediate evasion of immune recognition, escape from immune cells and spreading inside the host. Nevertheless, all described mechanisms of plant and human pathogens aim at the same: fungal survival.

## 3 | GAINING ADVANTAGES – FUNGAL FACTORS INVOLVED IN MODULATION OF HOST TARGETS DURING INFECTION

Immune evasion is a key aspect of all pathogenic fungi. Further pathogenicity mechanisms shared by plant and human pathogenic fungi include invasion strategies and, once the host has been invaded, the production of fungal factors that actively modulate host cells to their advantage. An overview of these general infection strategies of human and plant pathogenic fungi, in addition to immune evasion, is given in Figure 3 and examples are discussed below.

### 3.1 | Gaining a foothold – Modulation of fungal uptake

Invasion into the host can be an active, fungus-driven process, or a passive, host-driven one and can be seen as a prerequisite to optimise the manipulation of the host by effectors. In both active and passive cases, specific fungal moieties are required. Plant pathogens are known to actively penetrate plants as they have to overcome the plant's cell wall. Many plant pathogens, therefore, build defined structures, such as appressoria and haustoria, during invasion, extensively reviewed in a study by Lo Presti et al. (2015).

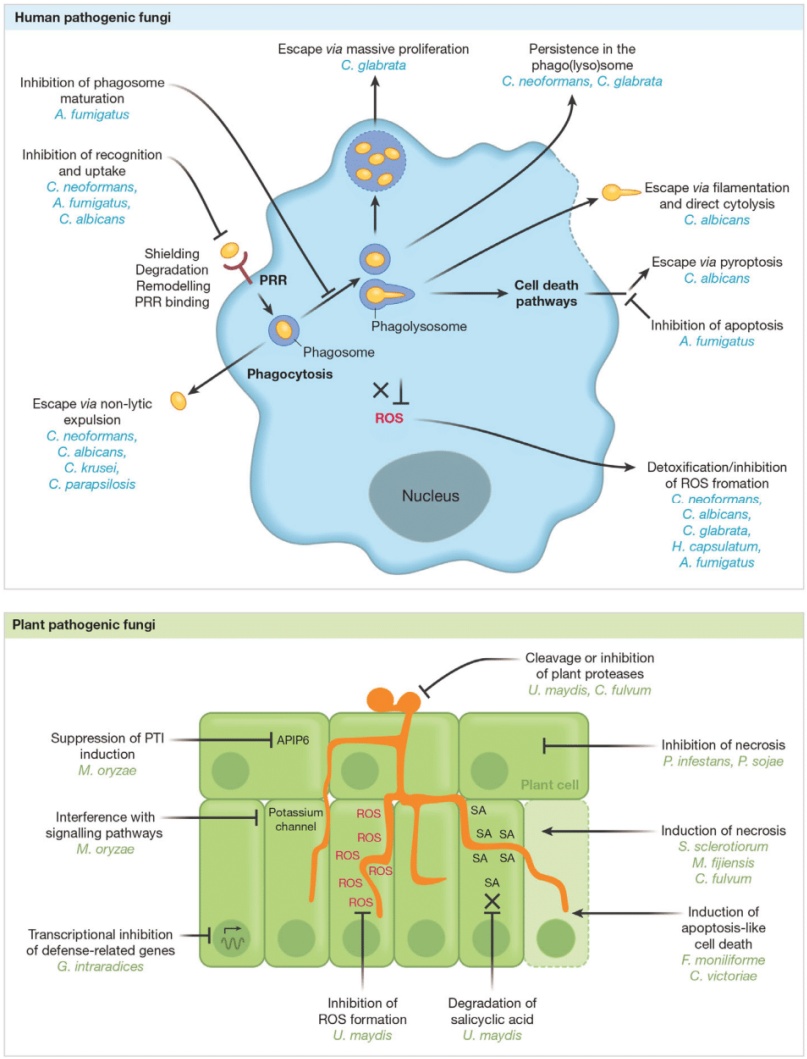


**FIGURE 2** Immune evasion strategies of human and plant pathogenic fungi. In addition to shielding (Figure 1), both human and plant pathogenic fungi need activities to evade immune recognition and/or antagonise activated immune responses. Human pathogenic fungi are usually confronted with cells of the innate immunity, such as macrophages and neutrophils. Plant pathogenic fungi have to deal with the effects of an activated immune response of the plant cell and have evolved strategies to interfere with it by either suppressing it (biotrophic fungi or hemibiotrophs in the initial biotrophic stage) or by inducing the activation of cell death pathways in case of necrotrophic fungi

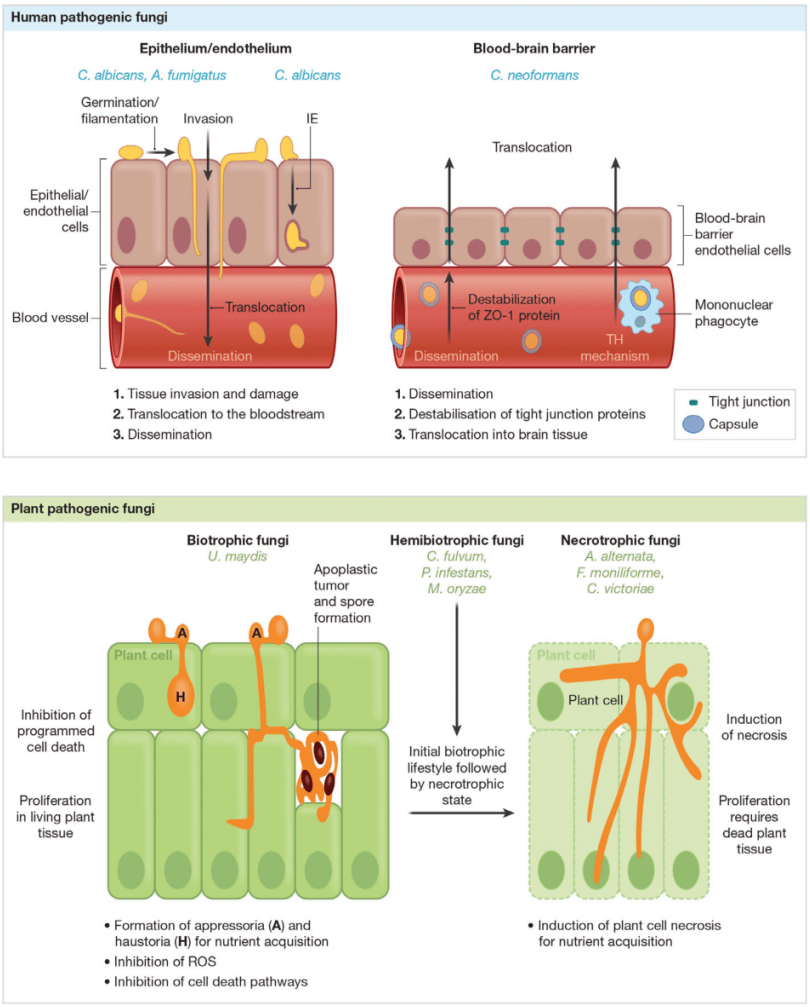


**FIGURE 3** Infection strategies of human and plant pathogenic fungi. Plant and human fungal pathogens share general infection strategies. Human pathogenic fungi like *C. albicans* or *A. fumigatus* adhere in the yeast/conidial form to epithelial or endothelial cells. Upon germination, hyphae actively penetrate the tissue and mediate fungal translocation, thus facilitating dissemination throughout the blood stream. *C. albicans* can further invade host cells by induced endocytosis (IE), a host-driven process. Disseminating *C. neoformans* cells destabilise tight junction proteins in blood–brain barrier endothelial cells, or use macrophages as a Trojan horse (TH), thereby promoting transcellular translocation into the brain parenchyma. Upon contact with immune cells, immune evasion strategies (Figures 1 and 2) come into play. Plant pathogenic fungi, which need to breach the cell wall of plant cells, form appressoria (A), structures dedicated to generate the pressure for invasion. Biotrophic fungi (e.g., *U. maydis*) usually form haustoria (H) for nutrient acquisition, secrete effectors to inhibit cell death induction and proliferate in living plant tissue. Hemibiotrophic fungi (e.g., *C. fulvum*) have an initial biotrophic stage, followed by a necrotrophic lifestyle. Necrotrophic fungi like *C. victoriae* secrete effectors dedicated to induce cell death pathways like necrosis for nutrient acquisition and spreading in dead plant tissue. Details are discussed in the text

Human fungal pathogens, which only need to breach a cell membrane during infection, can actively penetrate host cells but also, to a minor extent, induce their own endocytosis via invasins (Wachtler et al., 2012).

Active penetration is a common theme of plant and human pathogenic fungi and requires hyphal morphologies. The physical forces of growing hypha per se are sufficient to penetrate many host tissues. Human pathogenic yeasts thus have to apply other invasion strategies or, as in the case of *C. albicans*, switch from a yeast to a hypha morphology during invasion (Brand, 2012; Mendgen, Hahn, & Deising, 1996). In fact, *C. albicans* is a rare example of a human pathogenic fungus, which is able to invade host cells using both mechanisms, active penetration and induced endocytosis (Wachtler et al., 2012). Als3, a protein highly expressed during hypha formation of this fungus, binds N-cadherins on endothelial cells and E-cadherins on oral epithelia, thereby triggering endocytosis pathways of the host cell (Liu & Filler, 2011; Martin et al., 2013).

A pathogenic attribute of *C. neoformans* is its urease, which has been described to be relevant for invasion into brain tissue during disseminated infections, putatively by interfering with ZO-1 protein stability thus potentially enabling paracellular invasion through the endothelial barrier (Singh et al., 2013).

One example of a plant pathogen that evolved a strategy to manipulate the host plant's physiology after invasion is *S. sclerotiorum*. This fungus produces oxalate, which mediates a permanent opening of the host plant's stomata by increasing the amount of osmotic relevant molecules and preventing stomata closure by blocking the action of the plant hormone, abscisic acid (Guimaraes & Stotz, 2004).

## 3.2 | The inside job – Host manipulation after uptake

Once inside a host cell, a pathogenic fungus might need to protect its new position, to further invade and access more nutrients (see below),

or to reprogram host metabolism to its own benefit. Plant pathogens use effectors for this purpose, whereas, up to date, no such effector molecule of a human fungal pathogen has been described. In the following paragraph, we will, therefore, discuss the examples of specific host cell manipulation by distinct effectors of plant pathogens.

In *U. maydis*, Tin2, a secreted, virulence-associated protein effector, interacts with ZmTTK1, a plant protease. This leads to the modulation of the biosynthesis of lignin by enhancing the activity of the anthocyanin biosynthesis, thus reducing the precursors for lignin. As a result, lignification of the plant cell wall is reduced, which is beneficial for massive proliferation of the fungus (Tanaka et al., 2014). Furthermore, modulation of host metabolism has been reported for this fungus, which interferes with the plant's salicylic acid pathway by secreting the chorismate mutase, Cmu1. This enzyme interacts with ZmCm2 in the cytosol of the plant cell, leading to a reduced accumulation of salicylic acid and to a changed metabolic status of the host cells, beneficial for the pathogen (Djamei et al., 2011). For biotrophic plant pathogenic fungi, the structural integrity of the host is important to obtain functional haustoria. The rust fungus, *Uromyces fabae*, effector, RTP1p, translocates into the host cell to stabilise and protect the haustorium from degradation by forming amyloid-like fibrillar structures (Kemen, Kemen, Ehlers, Voegele, & Mendgen, 2013). In addition to the examples discussed in the context of immune evasion, these examples highlight that fungal pathogens are able to manipulate their respective host to their advantage, facilitating the progression of infection.

### 3.3 | Open offence – Fungal toxins

A special case of fungal pathogenicity factors are toxins, which contribute to the virulence arsenal of pathogens during infection in many ways. Some toxins were already discussed above in the context of cell death pathways. In contrast to human pathogenic fungi, the diversity of toxins in plant pathogenic fungi is immense and only a few can be covered here. Toxin production by plant pathogenic fungi is a long-known phenomenon comprising of, for example, secondary metabolites, peptides and proteins. Counting as a secondary metabolite, the already described victorin of *C. victoriae* targets the mitochondrial glycine decarboxylase complex of the host plant and thereby inhibits biosynthesis of the proteinogenic amino acid serine (Curtis & Wolpert, 2004). Other strategies are used by the *Cochliobolus carbonum* HC-toxin, which inhibits the histone deacetylases and, therefore, directly interferes with the plant's gene expression, or the phytotoxic tentoxin from *Alternaria* spp., which induces an energy breakdown in certain chloroplasts due to ATP hydrolysis (Horbach, Navarro-Quesada, Knogge, & Deising, 2011). In terms of low molecular weight peptides, *Rhynchosporium secalis* secretes necrosis-inducing peptides, which probably modulate plasma membrane $H^+$-ATPase function. As examples of toxic proteins, *Alternaria brassicicola* secretes AB and AP toxins, and further proteinaceous toxins have been described in the necrotrophs, *Pyrenophora tritici-repentis* (PtrToxA, ToxB) and *Stagonospora nodorum* (Sn1-4Tox) (Horbach et al., 2011). Details about these and several other toxins of plant pathogenic fungi directly targeting specific host cell factors or proteins have been extensively reviewed by Horbach and colleagues in 2011 (Horbach et al., 2011).

Surprisingly, toxins do not seem to be common in human pathogenic fungi. The first and only cytolytic peptide toxin of a human pathogenic fungus described so far is produced by *C. albicans* and has been termed candidalysin (see also above). This toxin has been shown to be crucial for *C. albicans*-induced host cell damage on oral epithelia and during mucosal infections (Moyes et al., 2016; Naglik, Gaffen, & Hube, 2019). However, the damage caused by candidalysin is also key in activating a protective immune response via the danger response pathways (Moyes et al., 2016). Therefore, this toxin has virulence as well as avirulence functions (König, Hube, & Kasper, 2020). It is thus clear that toxins play an important role during the interaction of pathogenic fungi with the respective host, not only by inducing PCD pathways like apoptosis or pyroptosis (see above), but also by directly damaging the host cell membrane or cell wall resulting in cytolysis, by interfering with important pathways within the host cell and by inducing a protective host response. Therefore, toxins crucially contribute to processes like immune evasion or immune induction, host cell damage and thus also nutrient acquisition in both human and plant pathogenic fungi. This particular aspect of gaining nutrients from the host is discussed in the following paragraph.

## 4 | FEASTING ON HOST'S EXPENSES – HOST EXPLOITATION FOR NUTRIENT ACQUISITION

During the infection process, pathogens usually face nutrient limitation. The ability of pathogenic fungi to cope with nutrient-restricted conditions is thus one of their key virulence attributes. Among others, metals, sugar and nitrogen are essential nutrients during infection. Metals, for example, are co-factors for several eukaryotic proteins. The host sequesters those resources from pathogens in a process termed “nutritional immunity.” However, fungi developed strategies to access those nutrients from their hosts (Gerwien, Skrahina, Kasper, Hube, & Brunke, 2018).

### 4.1 | Pick-pocketing – Fungi collect metals from the host environment

Pathogenic fungi have several ways to scavenge metals from the host environment, including the production of surface proteins that bind metal-rich host proteins, transporters, reductive up-take systems, metal-binding proteins and siderophores.

Although metal acquisition is essential for both plant and human fungal pathogens, this aspect of fungal pathogenicity has been more frequently investigated in human pathogenic fungi. *C. albicans* possesses a whole repertoire of iron scavenging systems, comprising proteins and hemophores for heme extraction (e.g., Rbt5, Pga7 and

Csa2), ferric reductases (e.g., Cfl1 and Cfl95) and the ability to capture xenosiderophores (e.g., ferricrocin), extensively reviewed in studies by Fourie, Kuloyo, Mochochoko, Albertyn, and Pohl (2018) and Gerwien et al. (2018). The already mentioned invasin Als3, required for induced endocytosis, is also crucial for acquiring iron from ferritin, which is the main iron storage protein in oral epithelial cells (Almeida et al., 2008).

*Aspergillus fumigatus* and *A. nidulans* are able to exploit iron via their siderophores, triacetylfusarinine C and ferricrocin. In addition, it has been shown that *A. fumigatus* is able to utilise iron from the human serum protein, transferrin (Almeida, Wilson, & Hube, 2009; Eisendle, Oberegger, Zadra, & Haas, 2003; Haas et al., 2003; Hissen, Chow, Pinto, & Moore, 2004). Furthermore, *H. capsulatum* has been reported to produce siderophores comprising dimerum acid, acetyl dimerum, coprogen B, methyl coprogen B and fusarinine (Brechting & Rappleye, 2019; Howard, Rafie, Tiwari, & Faull, 2000).

Siderophore production and xenosiderophore exploitation are also described for plant pathogenic fungi. *U. maydis*, for example, produces ferrichrome and ferrichrome A (Budde & Leong, 1989), whereas the citrus pathogen, *Geotrichum candidum*, has been described to utilise iron bound by the xenosiderophore, ferrioxamine B (Mor, Pasternak, & Barash, 1988).

Another important micronutrient that needs to be taken up from the environment is zinc. *C. albicans* secretes the zincophore, Pra1, a protein that binds zinc from host cells. The metal is then brought inside the fungus by Zrt1, a co-expressed plasma membrane zinc transporter. The system is dependent on hyphal development in alkaline pH (Citiulo et al., 2012). *A. fumigatus* expresses a similar system comprising Aspf2 and ZrfC for the exploitation of zinc (Amich, Vicentefranqueira, Leal, & Calera, 2010), and so does *H. capsulatum*, in which the expression of Zrt2 has been reported to be crucial for zinc homeostasis (Dade et al., 2016).

The exploitation of copper requires transporters as well. *H. capsulatum* requires Ctr3 for growth under low copper concentrations, like in copper-depleted macrophages (Shen, Beucler, Ray, & Rappleye, 2018). *C. neoformans* can acquire copper through the metallothionein, Cmt, and the copper transporters, Ctr1 and Ctr4, similar to *C. albicans*, which expresses a protein homologous to Ctr1 (Ding et al., 2013; Marvin, Williams, & Cashmore, 2003).

Taken together, the sequestration of metals from the host by pathogenic fungi using (xeno) siderophores is a key process during infection shared by human and plant pathogenic fungi, again underlining that successful strategies and factors can evolve independently under a similar evolutionary pressure.

### 4.2 | Looting the host – Fungi exploit macro-nutrients

Further nutrients required for growth are carbon, nitrogen and phosphate. Plant as well as human pathogenic fungi developed transporter systems, which ensure phosphate, sugar and amino acid import.

In both *C. albicans* and *C. neoformans*, the phosphate-responsive transcription factor, Pho4, regulates the expression of phosphate transporters, such as Pho84, Pho840 and Pho89, thereby enabling phosphate acquisition (Ikeh et al., 2016; Lev et al., 2017).

During infection, fungi also crave for sugar as a carbon source. In *C. neoformans*, glucose is taken up by the high-affinity hexose transporter, Hxs1, which is also critical for virulence (T. B. Liu et al., 2013). Furthermore, transporters have been described in haustoria, which allow biotrophic fungal pathogens to take up nutrients from their host. For example, the hexose transporter of HXT1p from *U. fabae* is specific for D-glucose and D-fructose (Voegele, Struck, Hahn, & Mendgen, 2001). The hemibiotrophic plant pathogen, *C. graminicola*, has been reported to possess five hexose transporters (CgHXT1-5) with varying affinity and expression time points, depending on the phase of infection (Lingner, Munch, Deising, & Sauer, 2011). Not only monosaccharides, but also disaccharides can be transported from the plant into the fungus. For example, *U. maydis* expresses the sucrose transporter, Str1, during infection, which is important for fungal virulence (Wahl, Wippel, Goos, Kamper, & Sauer, 2010).

Nitrogen is taken up by plant pathogenic fungi mostly as amino acids. *U. fabae* expresses Aat1 for histidine uptake and Aat3 has been described to mediate mainly leucine, methionine and cysteine uptake (Struck, Ernst, & Hahn, 2002; Struck, Mueller, Martin, & Lohaus, 2004). *Fusarium oxysporum*, on the other hand, produces Gap1, a general amino acid permease (Divon, Rothan-Denoyes, Davydov, A, & Fluhr, 2005). For *C. albicans*, it has been reported that the deletion of the amino acid transporter, Csh3, influences the amino acid uptake and virulence in a mouse model (Martinez & Ljungdahl, 2004), and transcription factors, Stp1 and 2, are regulators for amino acid and peptide utilisation (Miramon, Pountain, van Hoof, & Lorenz, 2020). In *C. neoformans*, only the permeases, Aap4 and Aap5, are relevant for virulence (Martho et al., 2016).

These examples show that general principles for micro- and macro-nutrient acquisition, such as siderophores, and especially transporters for sugars and amino acids, are commonly shared between pathogenic fungi independent of the host, however, adapted to their own specific niche.

## 5 | CONCLUSION

The tight relationship between fungal pathogens and their host, may it be human or plant, requires that such pathogens carry a whole arsenal of factors needed for survival. From immune evasion to modulation of specific targets of the host (not associated with the immune system) and host exploitation, diverse peptides, proteins and secondary metabolites enable fungi to infect their hosts in very similar ways. Independently of the respective host, plant and human fungi usually do not rely on only one factor but on several ones, all collectively contributing to the virulence of the pathogen to counteract the complex defence strategies of their host. In summary, although plants and humans are very different hosts, fungi infecting them share surprisingly similar pathogenicity mechanisms. This suggests that several of the attributes discussed in this review are essential for all established pathogenic fungi and that successful strategies have evolved independently.

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## CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

## DATA AVAILABILITY STATEMENT

Data sharing not applicable to this Micro-Review as no datasets were generated or analysed during the current study.

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# 4 Additional results – Dissecting the role of Non-Candidalysin Ece1 Peptides for fungal biology and during infection

As the *ECE1* gene encodes seven further peptides besides candidalysin (NCEPs), it was hypothesised that these peptides exhibit distinct functions like supporting candidalysin processing and secretion, mediating candidalysin-dependent effects and/or exhibiting other, candidalysin-independent, effector peptide-like functions in the host.

## 4.1 The generation of *Candida albicans* NCEP-knock-out strains

To elucidate the function of the respective NCEPs, knock-out strains were generated lacking single peptides encoded within the *ECE1* gene or peptide combinations. As Ece1-I additionally encodes the signal peptide responsible for guiding the Ece1 protein to the ER, no mutant lacking this sequence was generated to avoid effects due to impaired protein trafficking. Furthermore, the Δ*P7* mutant strain was already generated and verified in a Master's thesis conducted in the MPM department (König 2015). Regarding the knock-out of peptide sequence combinations, sequences of the Ece1 central (Δ*P4+5*) and the C-terminal peptides (Δ*P6-8*) were deleted to gain information about the importance of the central and C-terminal Ece1 regions. To investigate the effect of an Ece1 polyprotein containing more than one candidalysin peptide, a mutant was generated harbouring three candidalysin-encoding sequences (TripleP3). In this mutant, the Ece1-V and Ece1-VII-encoding sequences were exchanged against candidalysin-encoding gene fragments. For easement of reading, abbreviations for the mutant strains are used in the following. The strain name, the respective abbreviation along with the *ECE1* genotype and the encoded peptide(s) deleted are summarised in Table 2. All sequences for the respective *ECE1* sequence in comparison to the Wt *ECE1* sequence are shown in Table 3.

The following chapter describes the construction and verification of mutant strains lacking specific NCEP sequences or combinations thereof. The complete methodological strategy to generate these mutants is described in paragraph 7.1.3.

**Table 2: NCEP-knock-out strains generated in this study.**

Detailed information about the generated NCEP-knock-out strains, their strain name and abbreviation, the deleted *ECE1* region, and the respective peptide(s) missing due to the genetic modification. Strains Δ*P3* and Δ*P7* have been generated previously. Information on these strains is given along with the others as the strains were used in further analyses.

| Strain name | Abbreviation | Deleted *ECE1* region | Encoded peptide(s) missing |
|---|---|---|---|
| *ece1*Δ/Δ+*ECE1*$_{\Delta P2}$ | Δ*P2* | *ECE1*$_{\Delta 94\text{-}183}$ | Ece1-II |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P3}$ | Δ*P3* | *ECE1*$_{\Delta 184\text{-}279}$ | Ece1-III |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P4}$ | Δ*P4* | *ECE1*$_{\Delta 280\text{-}378}$ | Ece1-IV |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P5}$ | Δ*P5* | *ECE1*$_{\Delta 379\text{-}480}$ | Ece1-V |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P6}$ | Δ*P6* | *ECE1*$_{\Delta 481\text{-}582}$ | Ece1-VI |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P7}$ | Δ*P7* | *ECE1*$_{\Delta 583\text{-}684}$ | Ece1-VII |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P8}$ | Δ*P8* | *ECE1*$_{\Delta 685\text{-}813}$ | Ece1-VIII |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P4+5}$ | Δ*P4+5* | *ECE1*$_{\Delta 280\text{-}480}$ | Ece1-IV and Ece1-V |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P6\text{-}8}$ | Δ*P6-8* | *ECE1*$_{\Delta 481\text{-}813}$ | Ece1-VI, Ece1-VII, and Ece1-VIII |
| *ece1*Δ/Δ+*ECE1*$_{\text{TripleP3}}$ | TripleP3 | *ECE1*$_{379\text{-}480::\ 184\text{-}279,\ 583\text{-}684::184\text{-}279}$ | Ece1-V and Ece1-VII<br>Instead, candidalysin is present 3× |

### 4.1.1 Generation of *Candida albicans* plasmids harbouring NCEP-knock-out *ECE1* sequences

Commercially ordered pUC57 plasmids (Biomatik, Ontario (CAN)) containing modified *ECE1* gene sequences that lack certain NCEP sequences (Table 3) flanked by cleavage sites for the enzymes *Blp*I and *BsmB*I were transformed into *Escherichia coli*. Upon isolation, pUC57 donor plasmid and *C. albicans* vector plasmid pCIp10+*ECE1* were double-digested with *Blp*I and *BsmB*I yielding an insert (*ECE1* fragment from the pUC57 plasmid) and a vector backbone (pCIp10), which were subsequently purified by gel elution. Upon ligation, the pCIp10+*ECE1*Δ$_{Px}$ plasmids were subcloned into *E. coli* and transformants harbouring the correct pCIp10+*ECE1*Δ$_{Px}$ plasmid were verified *via* polymerase chain reaction (PCR) using *ECE1* promotor- and terminator-binding primers (ECE1Promfwd, ECE1Termrev, Table 10). For all modifications in the *ECE1* gene, at least three positive clones were obtained. Positive clones were cultivated, the plasmid was amplified, and plasmid deoxyribonucleic acid (DNA) was extracted from an overnight (o/n) culture.

**Table 3: *ECE1* sequence of Wt and NCEP-knock-out strains.**

Wild type or NCEP-knock-out strains with their respective *ECE1* sequence, peptide sequences are separated from one another by an underscore.

| Strain | *ECE1* sequence |
|---|---|
| Wt | MKFSKIACATVFALSSQAAIIHHAPEFNMKR_DVAPAAPAAPADQAPTVPAPQEFNTAITKR_SIIGIIM GILGNIPQVIQIIMSIVKAFKGNKR_EDIDSVVAGIIADMPFVVRAVDTAMTSVASTKR_DGANDDVAN AVVRLPEIVARVATGVQQSIENAKR_DGVPDVGLNLVANAPRLISNVFDGVSETVQQAKR_DGLEDFL DELLQRLPQLITRSAESALKDSQPVKR_DAGSVALSNLIKKSIETVGIENAAQIVSERDISSLIEEYFGKA |
| Δ*P2* | MKFSKIACATVFALSSQAAIIHHAPEFNMKR_SIIGIIMGILGNIPQVIQIIMSIVKAFKGNKR_EDIDSVVA GIIADMPFVVRAVDTAMTSVASTKR_DGANDDVANAVVRLPEIVARVATGVQQSIENAKR_DGVPDV GLNLVANAPRLISNVFDGVSETVQQAKR_DGLEDFLDELLQRLPQLITRSAESALKDSQPVKR_DAGS VALSNLIKKSIETVGIENAAQIVSERDISSLIEEYFGKA |
| Δ*P3* | MKFSKIACATVFALSSQAAIIHHAPEFNMKR_DVAPAAPAAPADQAPTVPAPQEFNTAITKR_EDIDS VVAGIIADMPFVVRAVDTAMTSVASTKR_DGANDDVANAVVRLPEIVARVATGVQQSIENAKR_DGV PDVGLNLVANAPRLISNVFDGVSETVQQAKR_DGLEDFLDELLQRLPQLITRSAESALKDSQPVKR_D AGSVALSNLIKKSIETVGIENAAQIVSERDISSLIEEYFGKA |
| Δ*P4* | MKFSKIACATVFALSSQAAIIHHAPEFNMKR_DVAPAAPAAPADQAPTVPAPQEFNTAITKR_SIIGIIM GILGNIPQVIQIIMSIVKAFKGNKR_DGANDDVANAVVRLPEIVARVATGVQQSIENAKR_DGVPDVGL NLVANAPRLISNVFDGVSETVQQAKR_DGLEDFLDELLQRLPQLITRSAESALKDSQPVKR_DAGSVA LSNLIKKSIETVGIENAAQIVSERDISSLIEEYFGKA |
| Δ*P5* | MKFSKIACATVFALSSQAAIIHHAPEFNMKR_DVAPAAPAAPADQAPTVPAPQEFNTAITKR_SIIGIIM GILGNIPQVIQIIMSIVKAFKGNKR_EDIDSVVAGIIADMPFVVRAVDTAMTSVASTKR_DGVPDVGLNL VANAPRLISNVFDGVSETVQQAKR_DGLEDFLDELLQRLPQLITRSAESALKDSQPVKR_DAGSVALS NLIKKSIETVGIENAAQIVSERDISSLIEEYFGKA |
| Δ*P6* | MKFSKIACATVFALSSQAAIIHHAPEFNMKR_DVAPAAPAAPADQAPTVPAPQEFNTAITKR_SIIGIIM GILGNIPQVIQIIMSIVKAFKGNKR_EDIDSVVAGIIADMPFVVRAVDTAMTSVASTKR_DGANDDVAN AVVRLPEIVARVATGVQQSIENAKR_DGLEDFLDELLQRLPQLITRSAESALKDSQPVKR_DAGSVALS NLIKKSIETVGIENAAQIVSERDISSLIEEYFGKA |
| Δ*P7* | MKFSKIACATVFALSSQAAIIHHAPEFNMKR_DVAPAAPAAPADQAPTVPAPQEFNTAITKR_SIIGIIM GILGNIPQVIQIIMSIVKAFKGNKR_EDIDSVVAGIIADMPFVVRAVDTAMTSVASTKR_DGANDDVAN AVVRLPEIVARVATGVQQSIENAKR_DGVPDVGLNLVANAPRLISNVFDGVSETVQQAKR_DAGSVA LSNLIKKSIETVGIENAAQIVSERDISSLIEEYFGKA |
| Δ*P8* | MKFSKIACATVFALSSQAAIIHHAPEFNMKR_DVAPAAPAAPADQAPTVPAPQEFNTAITKR_SIIGIIM GILGNIPQVIQIIMSIVKAFKGNKR_EDIDSVVAGIIADMPFVVRAVDTAMTSVASTKR_DGANDDVAN AVVRLPEIVARVATGVQQSIENAKR_DGVPDVGLNLVANAPRLISNVFDGVSETVQQAKR_DGLEDFL DELLQRLPQLITRSAESALKDSQPVKR |
| Δ*P4+5* | MKFSKIACATVFALSSQAAIIHHAPEFNMKR_DVAPAAPAAPADQAPTVPAPQEFNTAITKR_SIIGIIM GILGNIPQVIQIIMSIVKAFKGNKR_DGVPDVGLNLVANAPRLISNVFDGVSETVQQAKR_DGLEDFLD ELLQRLPQLITRSAESALKDSQPVKR_DAGSVALSNLIKKSIETVGIENAAQIVSERDISSLIEEYFGKA |
| Δ*P6-8* | MKFSKIACATVFALSSQAAIIHHAPEFNMKR_DVAPAAPAAPADQAPTVPAPQEFNTAITKR_SIIGIIM GILGNIPQVIQIIMSIVKAFKGNKR_EDIDSVVAGIIADMPFVVRAVDTAMTSVASTKR_DGANDDVAN AVVRLPEIVARVATGVQQSIENAKR |
| Triple P3 | MKFSKIACATVFALSSQAAIIHHAPEFNMKR_DVAPAAPAAPADQAPTVPAPQEFNTAITKR_SIIGIIM GILGNIPQVIQIIMSIVKAFKGNKR_EDIDSVVAGIIADMPFVVRAVDTAMTSVASTKR_SIIGIIMGILGNI PQVIQIIMSIVKAFKGNKR_DGVPDVGLNLVANAPRLISNVFDGVSETVQQAKR_SIIGIIMGILGNIPQV IQIIMSIVKAFKGNKR_DAGSVALSNLIKKSIETVGIENAAQIVSERDISSLIEEYFGKA |

### 4.1.2 Generation of *Candida albicans* NCEP-knock-out strains

For the generation of NCEP-knock-out mutants, the uridine auxotrophic *C. albicans ece1*Δ/Δ strain (*ece1*Δ/Δ ura$^-$, M2042, Table 8) was transformed with the respective pCIp10+*ECE1*Δ$_{Px}$ plasmids. The integration of the knock-out construct occurred in the *C. albicans RPS1* locus *via* homologous recombination and simultaneously restored the uridine auxotrophy of the *C. albicans* mutant strain (Figure 5).

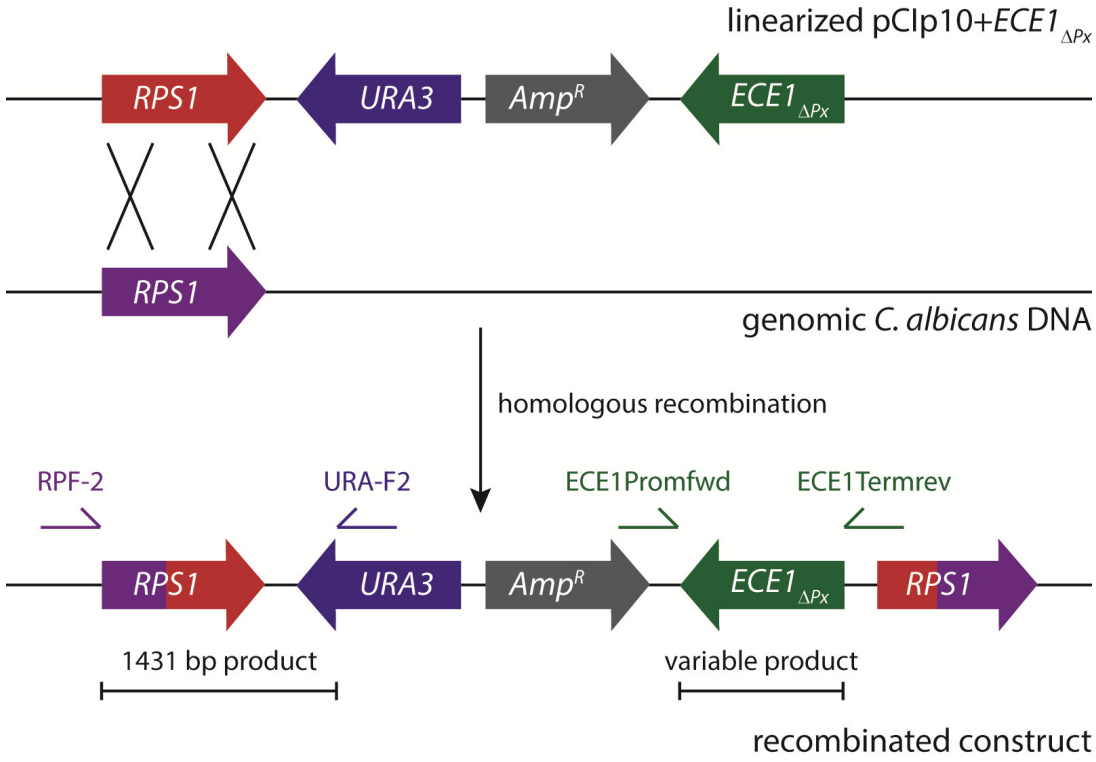


**Figure 5: Homologous recombination of knock-out construct into genomic *C. albicans* DNA.**

Using homologous recombination (black crosses), the construct harbouring the mutated *ECE1* gene, the *URA3* gene as well as an Amp resistance cassette (AmpR) was integrated into the native *RPS1* locus in the *C. albicans* genomic DNA. Verification of correct integration and NCEP sequence deletion was possible via PCR using different primer pairs (violet, blue, and green half-headed arrows).

*Candida albicans* transformants were verified for correct insert integration into the *RPS1* locus by amplification of the *URA3-RPS1* region (Figure 6) *via* PCR using the primer pair URA-F2 and RPF-2, binding in the *URA3* gene and the *RPS1* locus, respectively. Only in case of correct integration, a PCR product of 1431 bp was expected (Figure 5). As the Wt did not harbour the *URA3* gene within the *PRS1* locus, no product was expected. As shown in Figure 6, all mutants were verified in terms of correct insert integration.

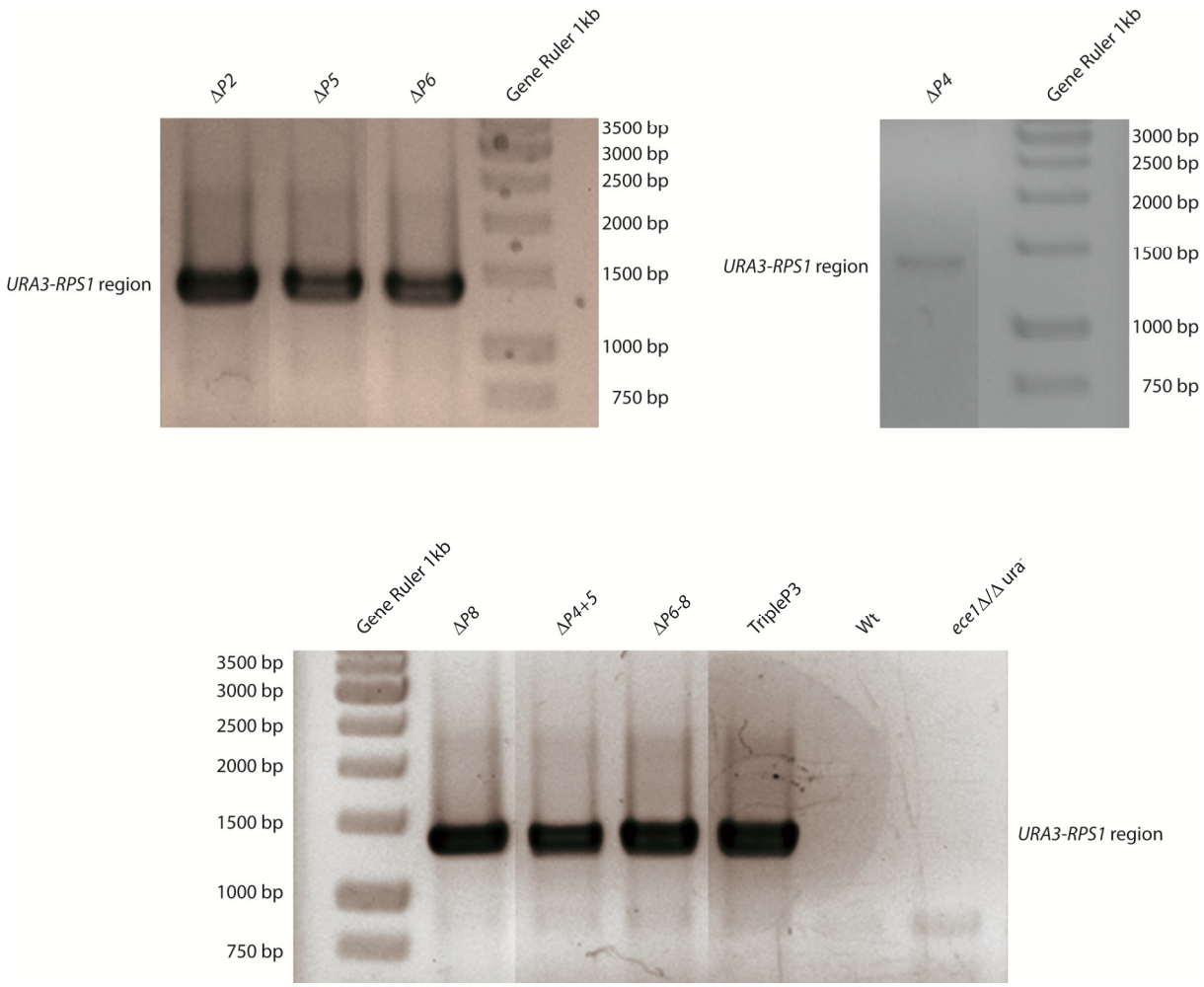


**Figure 6: Verification of correct construct integration in *C. albicans* NCEP-knock-out strains.**

Verification of correct construct integration into the *C. albicans RPS1* locus using the primer pair URA-F2 and RPF-2. A band at 1431 bp shows a correct integration. Separate pictures have been cropped to this selection from one continuous gel. As a marker, the GeneRuler™ 1 kb Ladder (NEB Biolabs GmbH, Frankfurt am Main) was used.

Subsequently, the correct deletion of the respective NCEP-encoding sequence within the *ECE1* insert (Figure 7) was verified by PCR using the primer pair ECE1Promfwd and ECE1Termrev. In case of a correct NCEP-encoding sequence deletion within the *ECE1* gene, band sizes as given in Table 4 were expected. As both native *ECE1* alleles had been replaced in the background strain *ece1*Δ/Δ ura$^-$ by a *HIS1* or an *ARG4* cassette, also an amplification of these cassettes with ECE1Promfwd and ECE1Termrev was possible yielding products of 1641 bp (*HIS1*) and/or 2279 bp (*ARG4)*. All generated mutants were verified for correct deletion of the respective NCEP-encoding sequence within the *ECE1* insert (Figure 7).

**Table 4: PCR products expected upon correct NCEP deletion within the *ECE1* gene.**

Strain and respective expected band size upon PCR amplification using the primer pair ECE1Promfwd and ECE1Termrev.

| Strain | Expected band size in bp |
|---|---|
| Wt | 1080 |
| Δ*P2* | 990 |
| Δ*P4* | 981 |
| Δ*P5* | 978 |
| Δ*P6* | 978 |
| Δ*P8* | 951 |
| Δ*P4+5* | 879 |
| Δ*P6-8* | 747 |
| TripleP3 | 1068 |

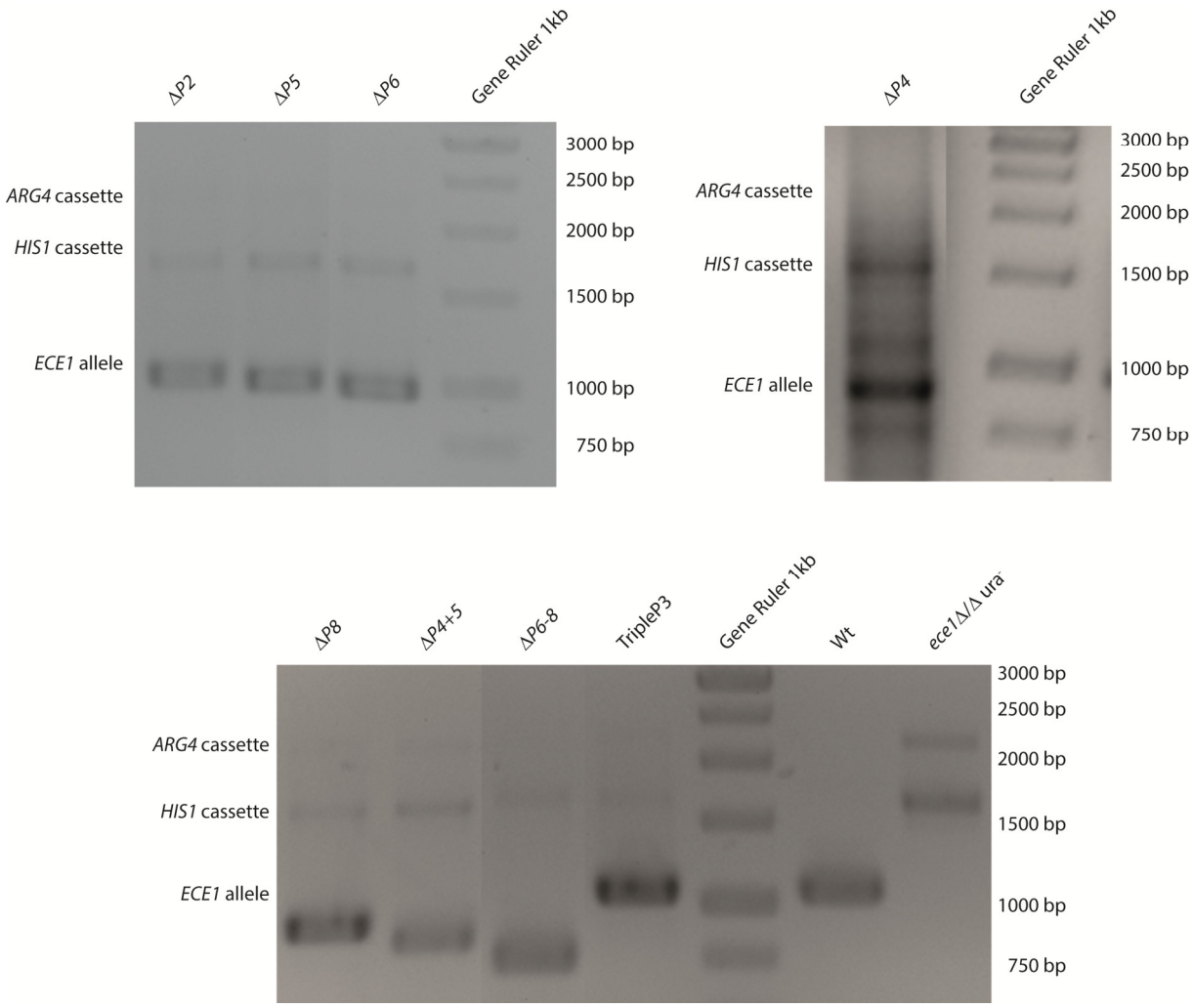


**Figure 7: Verification of *ECE1* modification in *C. albicans* NCEP-knock-out strains.**

Verification of peptide deletion in the *ECE1* gene using the primer pair ECE1Promfwd and ECE1Termrev. The shift in band size compared to the Wt band (1080 bp) shows the correct deletion of the peptide-encoding sequence in the mutant (Table 4). Separate pictures have been cropped to this

selection from one continuous gel. As a marker, the GeneRuler™ 1 kb Ladder (NEB Biolabs GmbH, Frankfurt am Main) was used.

### 4.1.3 Southern Blot verification

The genotype verification of NCEP-knock-out mutant strains using Southern Blot was part of the supervised Master's thesis of Lina Dally (Dally 2018). All results regarding the genotypic verification can be found in this thesis. Figure 8 schematically illustrates the strategy of genotypic verification *via* Southern Blot.

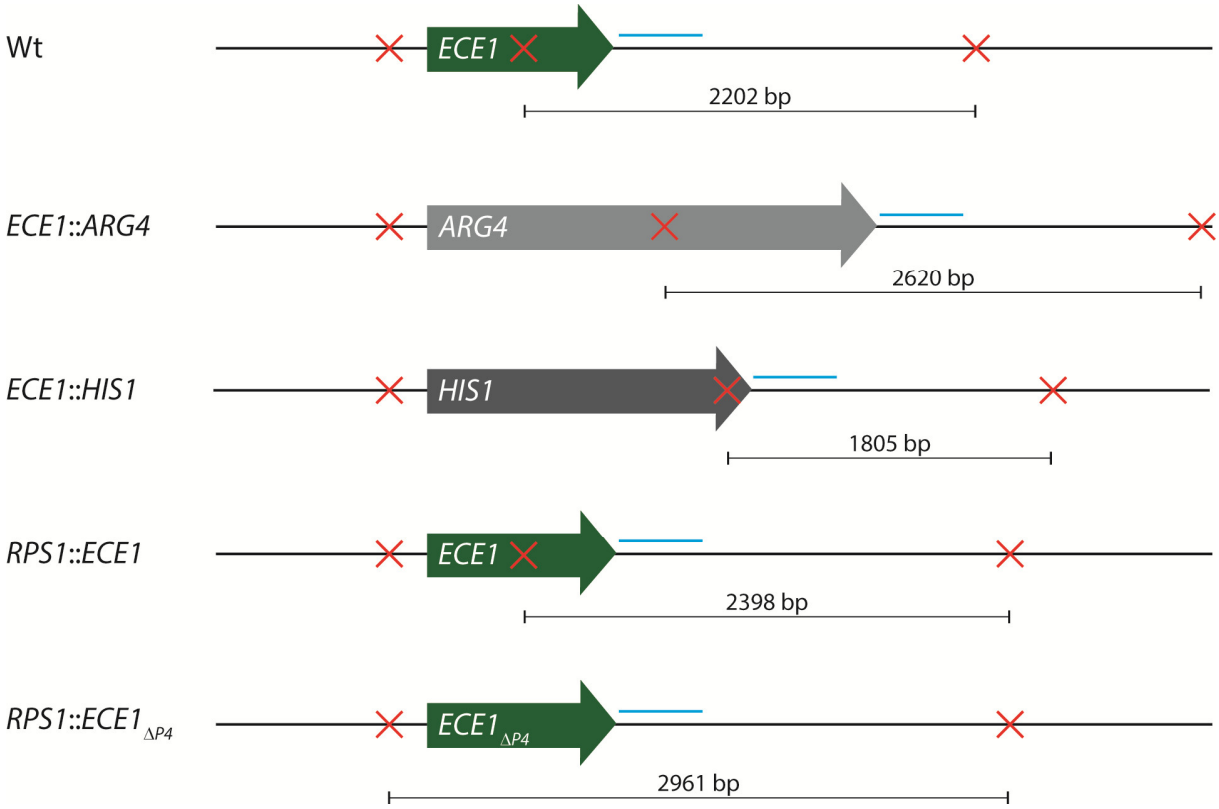


**Figure 8: Schematic illustration of Southern Blot verification of Δ*P4* mutant.**

Scheme of genetic verification of mutants harbouring deletions in the *ECE1* gene (green). Different restriction sites of *Msl*I (red crosses) in the genes lead to differently sized products using a probe binding in the *ECE1* terminator region (blue). *ARG4* and *HIS1* alleles (light and dark grey) in the native *ECE1* loci are detected similarly. Figure is adapted from (Dally 2018).

Except for the mutants Δ*P3* and Δ*P5*, a probe binding in the *ECE1* terminator region was used. For mutants lacking the Ece1-III or Ece1-V-encoding sequence, a promoter probe was applied. All mutants, except the mutant lacking the Ece1-II-encoding sequence (Δ*P2*), showed the expected band sizes in the Southern Blot and were therefore verified in terms of their genotype. Regarding the Δ*P2* mutant, the results obtained from the Southern Blot are questionable and require further evaluation, as already described and suggested in (Dally

2018). Table 5 exemplarily states the expected band sizes for the Southern Blot verification of the Δ*P4* mutant and Figure 9 exemplarily illustrates the Southern Blot result of this mutant.

**Table 5: Expected band sizes for Southern Blot verification of the Δ*P4* mutant.**

Alleles or cassettes in the respective integration locus and the expected band sizes upon *Msl*I digest.

| Allele/cassette [in locus] | Expected product band size in bp |
|---|---|
| Wt *ECE1* allele | 2202 |
| *HIS1* cassette [*ECE1*] | 1805 |
| *ARG4* cassette [*ECE1*] | 2620 |
| *ECE1* allele [*RPS1*] | 2398 |
| $ECE1_{\Delta P4}$ allele [*RPS1*] | 2961 |

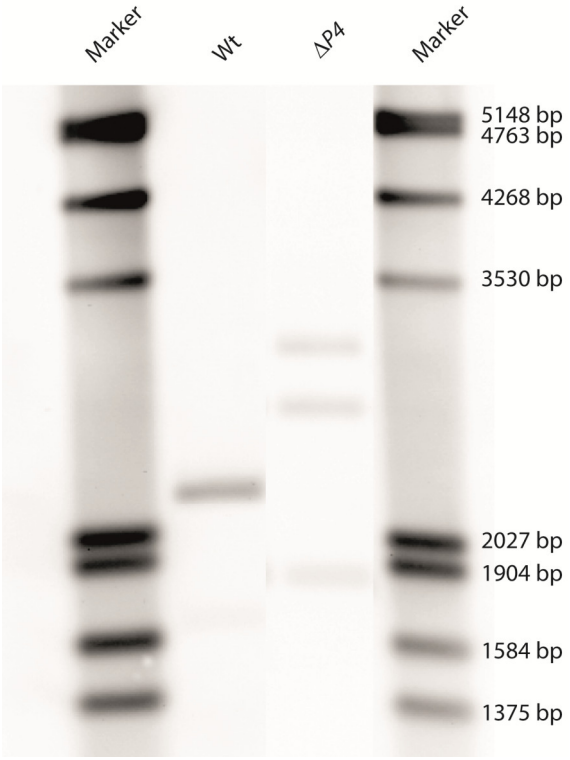


**Figure 9: Southern Blot of Δ*P4* mutant and Wt.**

Band pattern is depicted for the Wt and the Δ*P4* mutant. Genomic DNA was digested with *Msl*I, expected band sizes are given in Table 5. Figure is adapted from (Dally 2018) and cropped from one continuous gel. As a marker, the DIG-labelled DNA Molecular Weight Maker III (Roche, Grenzach-Whylen) was used.

## 4.2 Yeast growth and filamentation of NCEP-knock-out strains

To analyse whether NCEPs possess functions within the fungus, e.g. during growth in the yeast or hyphal morphology, the growth of both morphological states was monitored in the generated NCEP-knock-out mutants over a period of 24-48 h.

To evaluate whether genetic changes in the *ECE1* gene impair the yeast growth of the fungus, growth curves were recorded by measurement of the absorbance at 600 nm ($Abs_{600}$). All mutants grew like the Wt under yeast growth conditions (Figure 10).

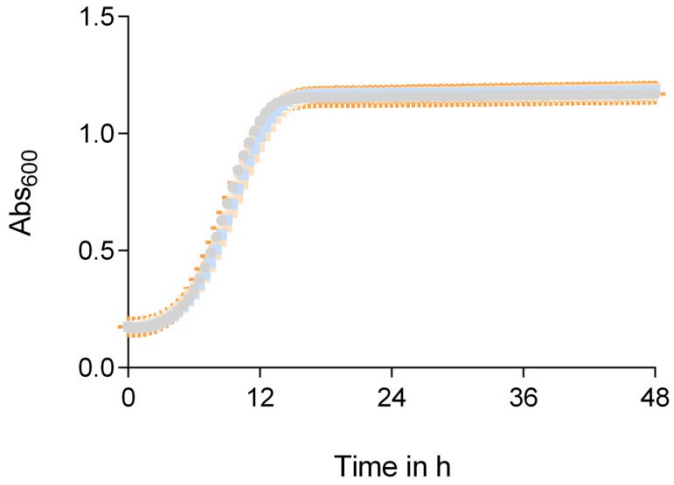


**Figure 10: Growth curve of *C. albicans* NCEP-knock-out mutants.**

Growth curve of NCEP-knock-out mutants (Δ*P2*, Δ*P3*, Δ*P4*, Δ*P5*, Δ*P6*, Δ*P7*, Δ*P8*, Δ*P4+5*, ΔP6-*8*, TripleP3; orange), Wt (grey), and *ece1*Δ/Δ mutant (light blue) over a time period of 48 h at 30 °C and continuous shaking at 180 rpm. Growth was measured at $Abs_{600}$ in YPD medium.

To elucidate whether NCEP deletions result in altered filamentation properties of the fungus, the hyphal length as well as the microcolony diameter was determined microscopically for all strains at 6 and 24 h after hypha induction, respectively. Furthermore, to ensure that defects in hyphal length are not due to defects in the filamentation initiation, the percentage of hypha formation was determined after 6 and 24 h.

After 6 h of hypha induction, all mutants except Δ*P3*, Δ*P4* and Δ*P7* exhibited a significantly reduced hyphal length as depicted in Figure 11A. After 24 h of hypha induction, most of the mutants that initially had shorter hyphae still exhibited a reduced microcolony diameter after 24 h (Δ*P2*, Δ*P5,* Δ*P6*, Δ*P4+5*, and Δ*P6-8*). However, these microcolony diameters were mostly

no longer significantly smaller as compared to the Wt. Only in case of the TripleP3 mutant microcolonies formed after 24 h were significantly reduced in diameter (Figure 11B).

Regarding the initiation of filamentation, the mutants lacking the Ece1-V-encoding sequence in combination with other changes in the *ECE1* gene (Δ*P4+5* and TripleP3) exhibit dramatic deficiencies in initiation of hyphal growth, as shown as the percentage of hypha formation after 6 h of induction (Figure 12A). Similar to the hyphal length, the deficiency in hypha induction of the Δ*P4+5* mutant observed after 6 h was abrogated after 24 h. However, the TripleP3 mutant still displayed a slight defect (Figure 12B).

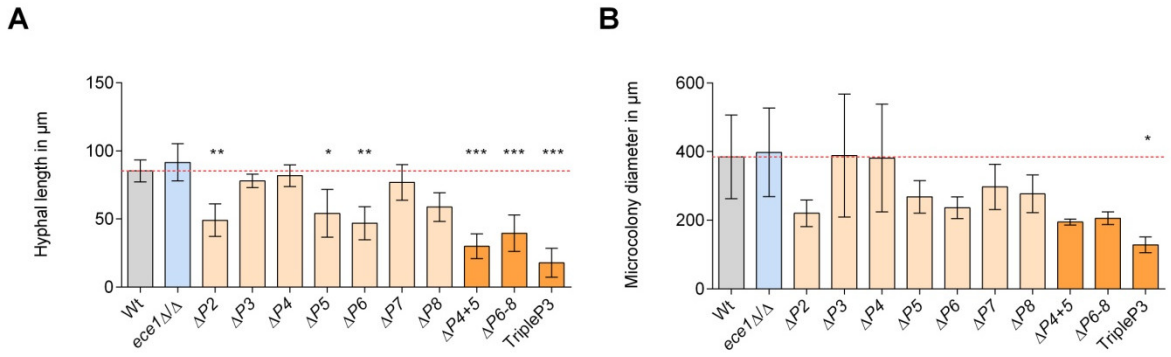


**Figure 11: Hyphal length after 6 h and microcolony diameter after 24 h of *C. albicans* NCEP-knock-out strains.**

Mean hyphal length (A) or microcolony diameter (B) in µm of generated NCEP-knock-out strains compared to the Wt and the *ece1*Δ/Δ mutant after 6 and 24 h of hypha induction, respectively. * p<0.05, ** p<0.01, *** p<0.001, significance compared to the Wt, red line indicates the Wt level, n=3, values are given as mean ± standard deviation (SD).

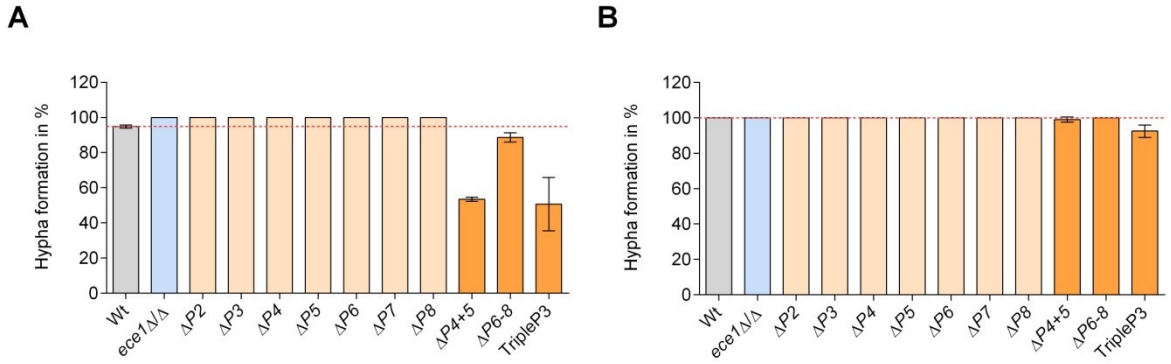


**Figure 12: Percentage of hypha formation of *C. albicans* NCEP-knock-out mutants after 6 and 24 h.**

Percentage of hypha formation (number of hyphal cells per 100 cells) of generated NCEP-knock-out strains compared to the Wt and the *ece1*Δ/Δ mutant after 6 (A) and 24 h (B) of hypha induction. Red line indicates the Wt level, n=2, values are given as mean ± SD.

## 4.3 *ECE1* expression in NCEP-knock-out mutants

As the induction of hypha formation is correlated with the expression of the hypha-associated gene *ECE1* (Birse *et al.* 1993), and many NCEP-knock-out mutants exhibited filamentation defects, all mutants were screened for *ECE1* expression after 3 h of hypha induction.

When normalised to the Wt yeast culture, all mutants except Δ*P2* and Δ*P3* exhibited deficiencies in the *ECE1* expression (less than 75 % of Wt *ECE1* expression in hyphal samples, Figure 13). Especially the mutants Δ*P5*, Δ*P4+5*, and TripleP3 showed a completely abolished *ECE1* expression (Figure 13). Thus, the majority of modifications within the *ECE1* gene led to an impaired gene expression.

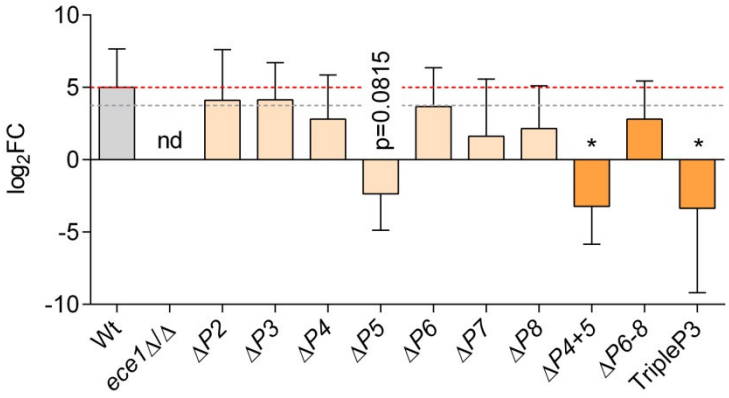


**Figure 13: $log_2$FC of *ECE1* expression in Wt, *ece1*Δ/Δ, and NCEP-knock-out strains under hypha-inducing conditions.**

$Log_2$ fold change (FC) of *ECE1* expression of hyphal samples compared to the Wt yeast sample. Gene expression was normalised to the expression of the housekeeping gene actin (*ACT1*). Red line indicates Wt level of *ECE1* $log_2$FC, grey line indicates 75 % of Wt level, ** $p<0.01$, significance compared to the Wt, nd – not detectable, n=3, values are given as mean + SD.

## 4.4 Ece1 secretion in NCEP-knock-out mutants

The expression of *ECE1* is a prerequisite for the subsequent peptide secretion by *C. albicans* hyphae. As NCEPs might potentially be involved in the candidalysin processing or secretion process, the effect of peptide deletion on the secretion pattern was investigated. Mutants were grown under hypha-inducing conditions and Ece1 secretion into the culture supernatant was analysed using LC-MS/MS. The results are summarised in Table 6 and show the relative abundance of peptide retrieval in PSMs.

Regarding the hyphal supernatant of a Wt culture, it is evident that candidalysin (Ece1-III lacking the terminal arginine residue) was by far the most abundant peptide detected by LC-MS/MS analysis. As previously reported (Richardson *et al.* 2018b), the peptides Ece1-V, -VI, -VII and -VIII were not found as full length versions in the supernatant but as processed fragments (a-c). Ece1-Va, Ece1-VIIa, and Ece1-VIIb (Table 1) were frequently retrieved from the hyphal supernatants of a Wt. All other fragments were only detectable in very low PSMs. Ece1-I, Ece1-II, and Ece1-IV were found in their respective full length version in the hyphal supernatants, but only to a very low extent.

**Table 6 LC-MS/MS data of Wt, *ece1*Δ/Δ, and NCEP-knock-out strains.**

PSM values of retrieved peptides and peptide fragments from a hyphal culture of a Wt, an *ece1*Δ/Δ mutant or NCEP-knock-out strains analysed by LC-MS/MS. Values below an arbitrary threshold of 20 are considered as background (grey). n=3 for Wt and the TripleP3 mutant, for all other strains n=2. Fragments of a peptide (Table 1) are given subsequently separated by slashes.

| Strain | Ece1 peptide | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | VII | VIII |
| | | | candidalysin | | a/b | a/b | a/b | a/b/c |
| Wt | 3 | 5 | 862 | 1 | 162/5 | 33/0 | 219/154 | 3/3/7 |
| *ece1*Δ/Δ | 0 | 0 | 0 | 0 | 0/0 | 0/0 | 0/0 | 0/0/0 |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P2}$ | 3 | 0 | 118 | 0 | 28/0 | 6/0 | 117/31 | 0/0/0 |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P3}$ | 1 | 2 | 0 | 4 | 99/4 | 71/0 | 35/113 | 3/0/11 |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P4}$ | 0 | 9 | 125 | 0 | 48/2 | 24/0 | 97/50 | 0/0/0 |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P5}$ | 0 | 7 | 73 | 1 | 0/0 | 28/0 | 130/30 | 0/0/3 |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P6}$ | 0 | 11 | 159 | 4 | 78/0 | 0/0 | 172/52 | 0/0/0 |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P7}$ | 0 | 1 | 27 | 3 | 32/1 | 13/0 | 0/0 | 0/0/0 |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P8}$ | 0 | 3 | 355 | 3 | 153/3 | 13/0 | 301/20 | 0/0/0 |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P4+5}$ | 0 | 0 | 36 | 0 | 0/0 | 0/0 | 58/5 | 0/0/0 |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P6-8}$ | 0 | 0 | 31 | 8 | 18/2 | 0/0 | 1/0 | 0/0/0 |
| *ece1*Δ/Δ+*ECE1*$_{\text{TripleP3}}$ | 0 | 0 | 14 | 0 | 0/0 | 0/0 | 0/0 | 0/0/0 |

Interestingly, Ece1-II was more often detected as a dipeptide of Ece1-II and -III with the amino acid sequence DVAPAAPAAPADQAPTVPAPQEFNTAITKRSIIGIIMGILGNI-PQVIQIIMSIVKAFKGNK and a PSM average of 41 in a Wt culture than the cleaved form (not shown in Table 6, cf. Additional data CD). As an internal control, the respective peptide or fragments thereof were not found in the hyphal supernatants of the associated knock-out strain.

Notably, the absence of candidalysin secretion in the Δ*P3* mutant occurred simultaneously with a drastic reduction of Ece1-VIIa release despite an otherwise Wt-like secretion of other NCEPs. The concomitant absence or reduced secretion of candidalysin and Ece1-VIIa was further observed in the mutants Δ*P2*, Δ*P4*, Δ*P5*, and Δ*P4+5*. However, this reduction was often also accompanied by a lower Ece1-Va secretion (Δ*P2*, Δ*P4*, Δ*P7*, and Δ*P6-8*). Mutants lacking the Ece1-VII-encoding sequence (Δ*P7*, Δ*P6-8*, TripleP3) were unable or nearly unable to secrete any peptide into the hyphal supernatant. In case of the Δ*P8* mutant, all peptides were secreted in a Wt-like manner except the neighbouring peptide fragment Ece1-VIIb. Regarding the example of Δ*P3* or Δ*P8*, it is evident, that deletions of certain peptides do not necessarily result in an overall diminished secretion of Ece1 in general. These deletions rather caused a more specific reduction of certain peptides fragments (Ece1-VIIa and Ece1-VIIb, respectively). The deletion of the Ece1-VII-encoding sequence, as well as the double deletion of *P4* and *P5*, was accompanied by an overall reduction of peptide secretion, whereas in the mutants Δ*P2*, Δ*P4*, Δ*P5*, and Δ*P6* intermediate secretion patterns were observed.

## 4.5 Damage potential of NCEP-knock-out mutants against host cells

Candidalysin has been shown to be critical for the damage potential of *C. albicans* against oral epithelial cells (Moyes *et al.* 2016). However, our study (manuscript II) revealed that at least in mononuclear phagocytes, the damaging potential of the fungus is not exclusively dependent on the toxin (Kasper *et al.* 2018), but also strongly depends on hypha formation. Since differences in hypha formation and candidalysin secretion in several NCEP-knock-out strains were observed, the influence of single peptide deletions within the *ECE1* gene on the fungal damaging potential was evaluated. Thus, primary human monocyte-derived macrophages (hMDMs) were infected with NCEP-knock-out mutant strains and the damaging potential was analysed by measurement of cytoplasmic LDH release.

Except for the mutants Δ*P6,* Δ*P7*, Δ*P8*, and Δ*P6-8*, which showed no decreased damage potential against hMDMs, all other mutants exhibited a more or less strong defect in causing host cell damage (Figure 14). The strongest defect on the damaging potential against hMDMs was observed in the mutant lacking candidalysin (Δ*P3*) and the one lacking the complete *ECE1* gene (*ece1*Δ/Δ). However, also the deletion of the Ece1-II-, -IV-, -V-

and -IV+V-encoding sequences in the *ECE1* gene resulted in a significantly diminished damaging potential against hMDMs. This was additionally true for the TripleP3 mutant, in which the Ece1-V- and -VII-encoding sequences had been substituted with candidalysin-encoding sequences.

Changes in the N-terminal parts of *ECE1* from the Ece1-I- to the Ece1-V-encoding sequence seem to have the strongest effect on the damaging potential of the fungus towards hMDMs. However, this effect seems to be not necessarily connected to the potential of the fungus to produce Wt-like hyphae or express *ECE1* and secrete the respective peptides in a Wt-like manner (Figure 11, Figure 13, Figure 14, Table 6).

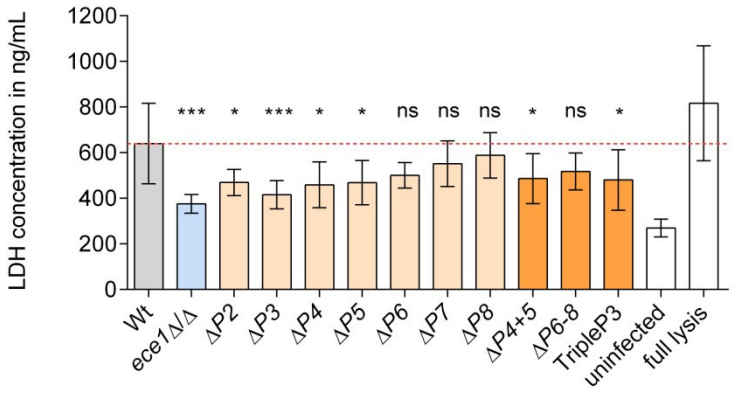


**Figure 14: Damage of hMDMs by Wt, *ece1*Δ/Δ, and NCEP-knock-out strains after 24 h.**

Damage of primary hMDMs upon infection with Wt, *ece1*Δ/Δ mutant, and the NCEP-knock-out mutants for 24 h was measured as LDH release from hMDMs. Red line indicates Wt-like damage level, full lysis represents 100 % host cell lysis by Triton-X-100. ns - not significant, * $p<0.05$, *** $p<0.001$, significance compared to the Wt, $n\geq4$, values are given as the mean ± SD.

Summarising, deletions in the *ECE1* gene affect the filamentation ability of the fungus, *ECE1* expression, and secretion of Ece1 peptides into the hyphal supernatant. These processes are often, but not necessarily correlated. Thus, the present data do not allow drawing a clear conclusion on specific NCEP functions so far. Furthermore, the damaging potential of NCEP-knock-out strains against hMDMs can so far not be predicted on the basis of hypha formation, *ECE1* expression, and Ece1 secretion.

## 4.6 Analysis of the unfolded protein response in NCEP-knock-out mutants

The data presented so far evidently show that genetic manipulation within the *ECE1* gene results in more or less dramatic changes in the *ECE1* expression, Ece1 secretion, hyphal

length, and the damage potential of the mutant strains against hMDMs. Amino acid changes in the peptide sequence or even the deletion of complete peptide sequences might lead to incorrect protein folding, processing, and secretion, as indicated by the LC-MS/MS data presented in paragraph 4.4 and published in 2018 by Richardson *et al.* (Richardson *et al.* 2018b). This potentially leads to endoplasmic reticulum (ER) stress and subsequently an induction of the unfolded protein response (UPR) (Gardner *et al.* 2013, Richardson *et al.* 2018b), which in turn might negatively affect filamentation. Indeed, a treatment with tunicamycin, an ER-stress inducer (Guillemette *et al.* 2011), resulted in abnormal hypha formation of the Wt, comparable to the phenotype of generated NCEP-knock-out mutants (unpublished data from Deniz Yildirim, HKI Jena, MPM department).

To measure ER stress in the generated NCEP-knock-out mutants, the induction of the UPR was monitored by analysing messenger RNA (mRNA) splicing of the transcriptional activator *HAC1* and gene expression of the UPR-responsive gene *KAR2*, an ER-resident molecular chaperone (Okamura *et al.* 2000, Pincus *et al.* 2010).

### 4.6.1 The transcription factor *HAC1* is activated in some NCEP-knock-out mutant strains

*HAC1* mRNA is spliced upon induction of the UPR during ER stress (Sidrauski and Walter 1997), resulting in a potent transcriptional activator, which induces gene expression of UPR-responsive genes like *KAR2* (Travers *et al.* 2000). To test whether NCEP mutants experience ER stress, first the splicing of *HAC1* mRNA was monitored by PCR amplification from cDNA using the primer pair HAC1SP-F and HAC1SP-R. An amplification of full length *HAC1* cDNA yielded a product of 109 bp, whereas cDNA of the spliced *HAC1* mRNA was 90 bp in size. Occurring splice products were quantified using ImageJ.

When Wt *C. albicans* cells were grown under treatment with the ER stress-inducing agent tunicamycin, splicing occurred in the yeast as well as in the hyphal sample (Figure 15). In the untreated Wt and the *ece1*Δ/Δ mutant, no splicing was detectable when grown under hypha-inducing conditions, indicating the absence of ER stress (Figure 15). Figure 15 further shows that in the mutants Δ*P5*, Δ*P6*, Δ*P8*, Δ*P4+5*, Δ*P6-8*, and especially in the TripleP3 mutant splicing occurred under hypha-inducing conditions, indicating the presence of ER stress in

these mutants upon hypha induction. Regarding the TripleP3 mutant, *HAC1* was spliced to a greater extent than under tunicamycin treatment of the Wt. In all other strains no splicing was detectable. Furthermore, none of the NCEP-knock-out strains tested exhibited a spliced product when grown in the yeast morphology, indicating that no ER stress occurs in the mutants under these conditions. This was additionally true for the untreated Wt and the *ece1*Δ/Δ mutant strain.

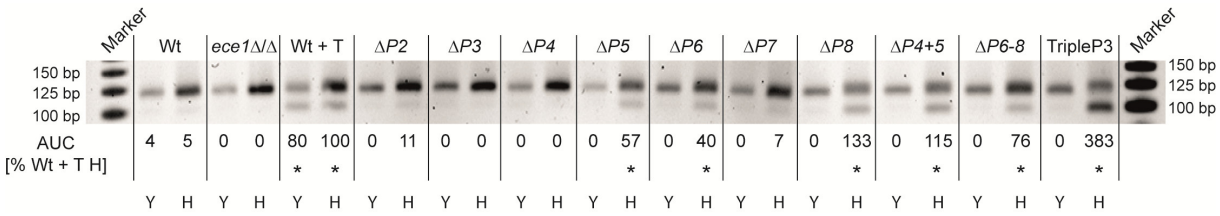


**Figure 15: *HAC1* mRNA splicing and quantification in Wt, *ece1*Δ/Δ, and NCEP-knock-out strains.**

Amplification of full length *HAC1* cDNA (109 bp) or spliced *HAC1* cDNA (90 bp) by PCR using the primer pair HAC1SP-F and HAC1SP-R. Splice products exhibiting an area under the curve (AUC) of more than 25 % of the Wt + T hyphal sample are indicated with an *. Separation was conducted in a 4 %, high resolution agarose gel. Picture is cropped from one double row gel. Wt + T - 2 µg/mL tunicamycin treatment, Y - yeast sample, H - hyphal sample, n=3. The 25 bp DNA Step ladder (Promega GmbH, Walldorf) was used as a marker. One representative replicate is shown, pictures of all three replicates are provided on the CD added to this thesis.

### 4.6.2 *KAR2* is up-regulated in several NCEP-knock-out mutant strains

The gene encoding the molecular chaperone Kar2 is UPR-responsive and up-regulated under ER stress (Okamura *et al.* 2000, Pincus *et al.* 2010). As many NCEP-knock-out mutants showed an activation of the transcriptional activator *HAC1* by mRNA splicing, the expression of *KAR2* was measured in the Wt, a tunicamycin-treated Wt, the *ece1*Δ/Δ mutant and the NCEP-knock-out mutants after 3 h of hypha induction. Under tunicamycin-induced ER stress in the Wt, *KAR2* was up-regulated (Figure 16). In contrast, the untreated Wt and the mutants *ece1*Δ/Δ, Δ*P2,* Δ*P3*, and Δ*P7* did not show an up-regulation of *KAR2* expression, but rather expression levels similar to or slightly lower than the Wt sample (Figure 16). All other mutants tested exhibited different degrees of a slight up-regulation of *KAR2* gene expression (Figure 16).

Comparing the *KAR2* expression data with the data obtained from the *HAC1* mRNA splicing, it is evident that in all mutants that showed splicing of *HAC1* mRNA, *KAR2* expression was

up-regulated, indicating that these mutants experience ER stress. Furthermore, it is clear that the molecular chaperone *KAR2* is involved in the stress response in the majority of the generated NCEP-knock-out mutants.

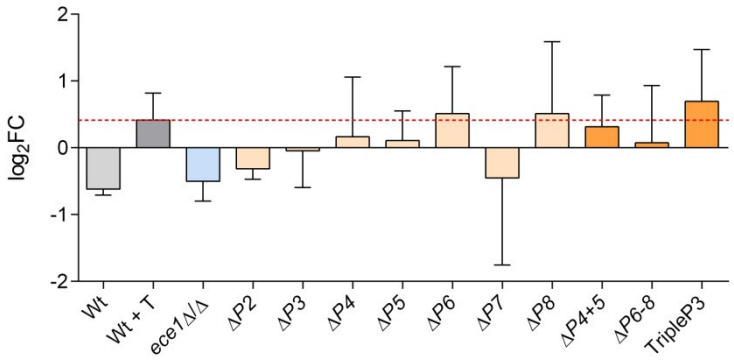


**Figure 16: $log_2$FC of *KAR2* expression in Wt, *ece1*Δ/Δ, and NCEP-knock-out strains.**

*KAR2* expression normalised to the Wt yeast sample. Gene expression was measured after 3 h of hypha induction and normalised to the expression of the housekeeping gene *ACT1*. Wt + T – 2 µg/mL tunicamycin treatment. Red line indicates gene expression level in the tunicamycin-treated Wt. n=3, values are given as the mean + SD.

## 4.7 Summary of NCEP-knock-out mutant characterisation

All data collected for the characterisation of NCEP-knock-out mutants in paragraphs 4.2 to 4.6 are summarised in a heat map (Figure 17). In most of the cases, the expression of *ECE1* was correlated with hypha formation, whereas the secretion of candidalysin was impaired in all strains with a modified *ECE1* sequence. Especially modifications in the C-terminal region (*P5-P8*) and deletions of peptide combinations resulted in the induction of the UPR (*HAC1* mRNA splicing and up-regulation of *KAR2* expression), which in turn probably accounts for the filamentation defects of these mutants and thus an impaired *ECE1* gene expression and candidalysin secretion. These strains exhibited no or only a moderately diminished damaging potential against hMDMs. In contrast, deletions in the N-terminal regions of *ECE1* (*P1–P4*) did not result in a UPR induction and rather exhibited no deficiencies in hypha formation. However, these strains mostly showed an impaired candidalysin and Ece1-VIIa secretion, as well as defects in the damaging potential against hMDMs. Combinatory peptide deletions or modifications mostly exhibited a stronger effect than single peptide deletions and, in line with that, the TripleP3 mutant showed the most severe defects and exhibited deficiencies in all processes tested except during yeast growth (during which *ECE1* is not expressed).

Concluding, several aspects like UPR induction, hypha formation, *ECE1* expression, and Ece1 secretion are influenced by deletions or modifications within the *ECE1* gene. This supports the hypothesis that NCEPs are required for Ece1 folding and processing. Importantly, defects in hypha formation or candidalysin secretion do not necessarily directly translate to a diminished damaging potential against hMDMs, highlighting that neither candidalysin secretion nor hypha formation alone is sufficient for damage induction in these host cells.

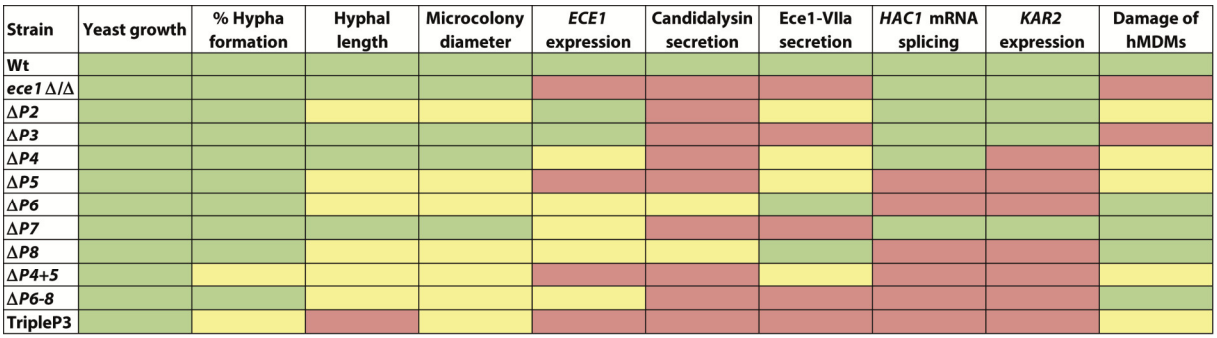


**Figure 17: Heat map of all data collected for NCEP-knock-out mutant generation.**

Heat map shows the result of all read-outs used to characterise the generated NCEP-knock-out mutants. Yeast growth (time course over 48 h), % of hypha formation and hyphal length (6 h), microcolony diameter (24 h), *ECE1* and *KAR2* expression (3 h), candidalysin and Ece1-VIIa secretion (18 h), as well as *HAC1* mRNA splicing (3 h) and the damaging potential against hMDMs (24 h). Green - ≥ 75 % of Wt level, yellow – 25<x<75 % of Wt level, red - ≤ 25 % of Wt level. For *HAC1* mRNA splicing and *KAR2* expression green colour indicates no splice product or no up-regulation of gene expression, whereas red indicates *HAC1* mRNA splicing or up-regulation of *KAR2* expression, respectively.

## 4.8 The effect of synthetic Ece1 peptides on macrophages

To evaluate potential NCEP functions during the interaction with host cells, the response of hMDMs to treatment with synthetic peptides was monitored. For this, synthetic full length versions of the peptides and peptide fragments (Peptide Protein Research Ltd., Hampshire (UK)) detected in the supernatant of a hyphal Wt culture (Table 1) were screened.

As synthetic candidalysin alone strongly induces macrophages damage (Kasper *et al.* 2018) and NCEPs are co-expressed and -secreted with the toxin (Richardson *et al.* 2018b), the damaging potential of the full length peptides and NCEP fragments against macrophages was monitored by measuring host LDH release. To evaluate whether full length NCEPs or NCEP fragments can subvert or enhance the candidalysin effect, the damaging potential of the toxin was additionally analysed in combination with NCEPs or NCEP fragments in equimolar

amounts. Furthermore, NCEPs have been hypothesised to act as effector peptides on or in the host cells. Thus, the macrophage transcriptional response upon co-incubation with NCEP fragments as well as the induction of cyto- and chemokines release from these host cells was evaluated using microarrays or enzyme-linked immunosorbent assays (ELISAs), respectively.

### 4.8.1 Damage of macrophages induced by synthetic Ece1 peptides

To test the damaging potential of synthetic NCEPs towards hMDMs, full length synthetic peptides with the sequence predicted upon sequential Kex2 and Kex1 digest (Table 1 in black) or peptide fragments which occurred in the supernatant of Wt hyphal cultures (Table 1 in grey) were co-incubated with these immune cells.

Figure 18 illustrates the damaging potential of synthetic candidalysin and full length NCEPs measured by the peptide capability to induce the release of LDH from macrophages into the supernatant. It is evident that only candidalysin exhibited a damaging potential against hMDMs, whereas all other full length NCEPs did not induce any cytolysis of these immune cells (Figure 18A). When hMDMs were challenged with candidalysin in combination with full length synthetic NCEPs in equimolar amounts, no striking effect for most NCEPs in terms of damage enhancement or reduction was observed (Figure 18B). Only upon addition of full length Ece1-VII to candidalysin, the damage of hMDMs was significantly reduced as compared to the candidalysin only-induced macrophage damage (Figure 18B).

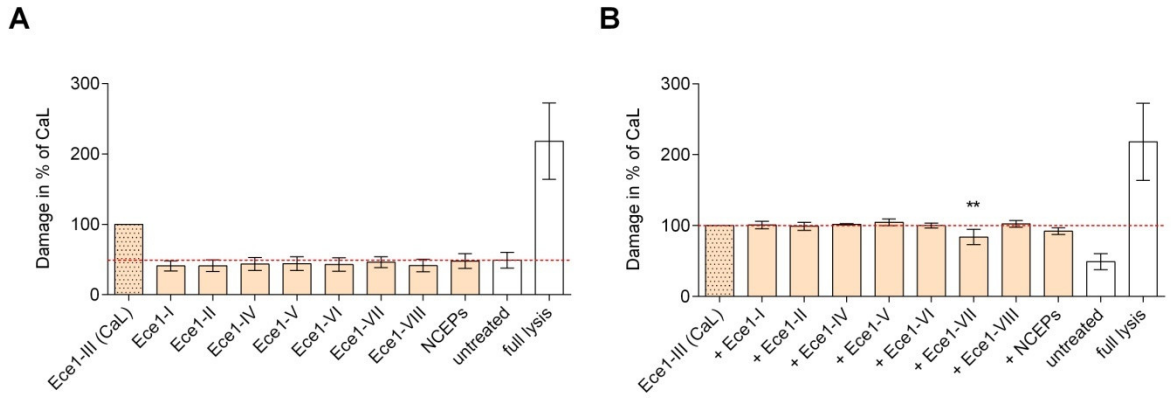


**Figure 18: Damage of hMDMs by candidalysin and/or full length NCEPs.**

Damage of primary hMDMs after 24 h (in % of candidalysin induced damage) was measured as LDH release from the host cells. (A) Damage upon co-incubation with 5 µM candidalysin or the respective NCEPs in full length. (B) Damage upon co-incubation with candidalysin + full length NCEPs, 5 µM each. Full lysis represents 100 % lysis by Triton-X-100. Red line indicates untreated control damage

level (A) or candidalysin-induced damage level (B). CaL - candidalysin, ** p≤0.01, significance compared to CaL treatment, n=4, values are given as the mean ± SD.

To further evaluate whether the Ece1-V, -VI, -VII and -VIII fragments found in the LC-MS/MS analysis (Table 1) modulate the candidalysin-induced damage of host cells, hMDMs were co-incubated with a mix of synthetic candidalysin and/or NCEP fragments in equimolar amounts, and screened for the damaging effect by measuring LDH release.

As depicted in Figure 19A, none of the synthetic NCEP fragments tested exhibited any damaging potential against hMDMs upon co-incubation for 24 h. Similar to the full length NCEPs (Figure 18B), most fragments did not enhance or reduce the candidalysin-induced damage of hMDMs (Figure 19B). However, in case of a co-incubation of hMDMs with candidalysin and the Ece1-VII fragments a and b in equimolar amounts, a significant damage reduction was observable as seen with full length Ece1-VII (Figure 18B, Figure 19B).

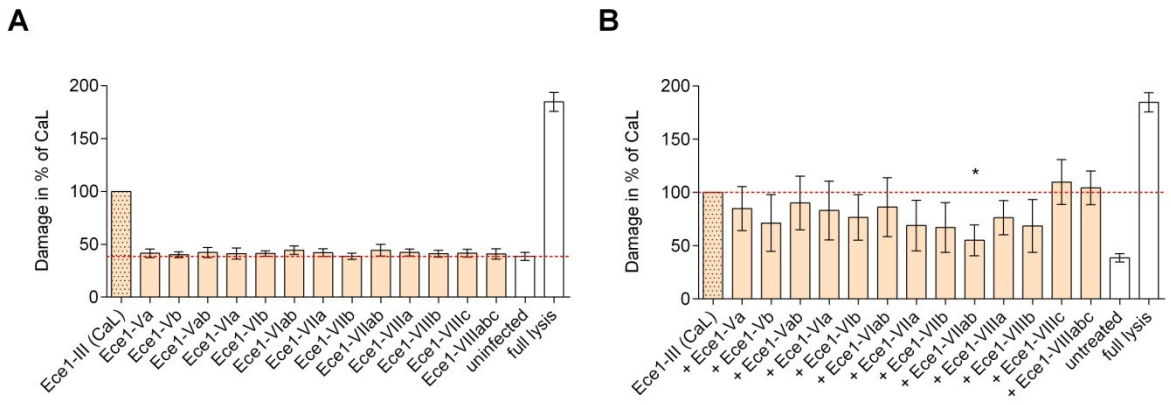


**Figure 19: Damage of hMDMs by candidalysin and/or NCEP fragments.**

Damage of primary hMDMs (in % of candidalysin-induced damage) after 24 h measured as LDH release from the host cells. (A) Damage upon co-incubation with 5 µM candidalysin or Ece1-V, -VI, -VII or -VIII peptide fragments. (B) Damage upon co-incubation with candidalysin + NCEP fragments, 5 µM each. Full lysis represents 100 % lysis by Triton-X-100. Red line indicates damage of control damage level (A) or candidalysin-induced damage level (B). CaL - candidalysin, ab - co-incubation with peptide fragments a and b, abc - co-incubation with peptide fragments a, b, and c. * p≤0.05, significance compared to CaL treatment, n=4, values are given as the mean ± SD.

### 4.8.2 Transcriptional profiling of Ece1 peptide-treated macrophages using microarrays

Apart from the hypothesis that NCEPs can aid or subvert the candidalysin action, they are also thought to be putative effector peptides. In case of an effector-like activity, it was

hypothesised that a co-incubation of macrophages with NCEPs would induce a specific response in these immune cells. To elucidate that hypothesis, transcriptional profiling of hMDMs was performed using microarrays to evaluate the influence of co-incubation with NCEPs on macrophage gene expression.

Since Ece1-Va, -VIa and -VIIa were the most abundant peptide fragments detected by LC-MS/MS (Table 1), these peptides were used for the initial screening. Furthermore, the peptide toxin candidalysin was included. To test whether candidalysin mediates the translocation of putative effector NCEPs into the host cytoplasm, a combination of candidalysin and Ece1-Va was included in the analysis. All synthetic peptides were applied at non-damaging concentrations of 5 µM, which was confirmed *via* LDH measurement and microscopic evaluation prior to further experiments (data not shown).

To remove the strong donor-dependency, which is characteristic for results obtained from hMDM experiments, the mean of all biological replicates was calculated by averaging the results of treatment *vs.* no treatment per donor and time point (paragraph 7.1.4.4). Subsequently, a paired t-test was used to determine the statistical significance of the observed regulation. Results obtained from microarray experiments were subjected to a filtering process for differentially expressed genes (DEGs) fulfilling the requirements of $\log_2 FC \geq 1$ or $\leq -1$ and a p-value $\leq 0.05$ of treatment *vs*. no treatment. Subsequently, an enrichment analysis using ShinyGo (Ge *et al.* 2020) followed by a reduction of redundant pathways with REVIGO (0.7 similarity) (Supek *et al.* 2011) was conducted. Apart from the data presented in the following, lists showing the complete results per treatment as well as the lists of genes enriched in the respective pathways can be found on the CD added to this thesis (Additional Data).

The number of DEGs for each treatment is shown in Table 7 and Figure 20. A treatment of macrophages with candidalysin or candidalysin in combination with Ece1-Va resulted in the highest number of DEGs, whereas a treatment with the NCEP fragments alone reached lower DEG numbers (Table 7). The Venn diagrams depicted in Figure 20 further indicate a rather peptide-specific response of macrophages upon co-incubation with candidalysin and/or NCEP fragments for both time points, as only very few DEGs were found to be overlapping upon different peptide treatments.

To identify functions enriched among DEGs, these genes were subjected to an enrichment analysis and a reduction of redundant pathways as described above. Besides this, the gene expression of selected DEGs involved in the detected pathways is displayed in heat maps for all peptide treatment conditions.

**Table 7: Number of DEGs upon co-incubation with candidalysin and/or NCEP fragments**

Number of DEGs ($log_2FC \geq 1$ or $\leq -1$, $p \leq 0.05$) upon treatment with 5 µM candidalysin and/or 5 µM Ece1-Va, Ece1-VIa, or Ece1-VIIa at different time points.

| Time point | Treatment | DEGs |
|---|---|---|
| 6 h | Candidalysin | 326 |
| | Candidalysin + Ece1-Va | 325 |
| | Ece1-Va | 174 |
| | Ece1-VIa | 103 |
| | Ece1-VIIa | 193 |
| 24 h | Candidalysin | 830 |
| | Candidalysin + Ece1-Va | 951 |
| | Ece1-Va | 273 |
| | Ece1-VIa | 196 |
| | Ece1-VIIa | 306 |

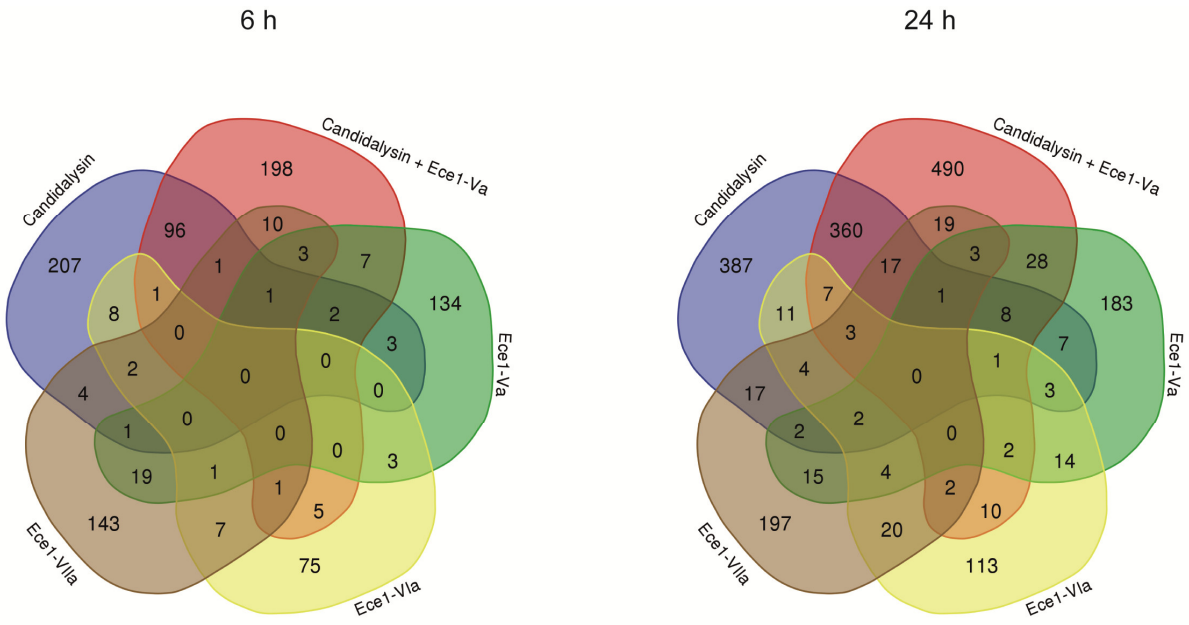


**Figure 20: Venn diagrams of macrophage DEGs upon Ece1 peptide treatment.**

Venn diagrams illustrate DEGs ($log_2FC \geq 1$ or $\leq -1$, $p \leq 0.05$) upon co-incubation with candidalysin and/or NCEP fragments after 6 and 24 h. Diagrams were prepared online using the Venn web tool on the website http://bioinformatics.psb.ugent.be/webtools/Venn/ and show treatment-specific DEGs as well as genes that are differentially expressed upon different treatments (overlapping regions).

Figure 21 and Figure 24 depict pathways enriched amongst genes differentially regulated in hMDMs upon candidalysin treatment and expression of selected genes involved in these pathways, respectively. Especially genes involved in pathways mediating a heat or stress response, such as the heat shock protein genes *HSPA1A*, *HSPA1B*, *HSPA6*, *HSPE1*, *HSPH1*, *DNAJA1*, *DNAJA4*, *DNAJB1*, *DNAJB6*, the superoxide dismutase *SOD2* gene as well as kinase genes like *IRAK*2 and *TAOK*2 were up-regulated upon 6 h of co-incubation with candidalysin (Figure 21, Figure 24). Furthermore, genes involved in phosphorylation and protein modification were up-regulated such as dual specific phosphate genes (*DUSP1*, *DUSP4*, *DUS5P*, *DUSP14*), the histone deacetylase gene *HDAC4* or genes encoding zinc-finger proteins (*DBF4*, *SNAI2, BCL11A*) (Figure 21, Figure 24). Apart from these, also genes involved in the response to a cytokine stimulus (Figure 21) like the chemo- and cytokine genes *CXCL2*, *CCL18*, and *TNF*, the receptor gene *CCR7*, genes encoding the TFs *STAT4* and *CEBPB* as well as genes encoding suppressors of cytokine secretion (*SOCS1*, *SOCS3*) were up-regulated (Figure 24). In contrast to these up-regulations, genes involved in the regulation of type-I IFN production (*TLR3*, *TLR8*, *RNF125*, *HERC5*) and IL-1β production or secretion were down-regulated (*TLR8*, *NOD2*, *AIM2*, *P2RX7) (*Figure 21, Figure 25). However, *IL1B* gene expression itself was up-regulated after 6 h of co-incubation (Figure 24).

Upon 24 h of co-incubation of hMDMs with candidalysin, genes involved in pathways connected to a heat or stress response such as genes encoding TFs and zinc finger proteins like *CEBPB*, *NFKB1A*, *NFKB2*, *ZC3H12A*, *SNAI1*, *BCL11A* were up-regulated, partially overlapping with the response after 6 h. Furthermore, an up-regulation of genes was found in the enriched pathway of the immune response including many cyto- and chemokine genes (*IL1A*, *IL1B*, *IL8*, *IL32*, *IL36B*, *CCL2*, *CCL3*, *CCL5*, *CCL8*, *CCL23*, *CXCL10*, *TNF*), genes encoding cyto- or chemokine receptors (*CCR7*, *IL1RN*, *IL7R*, *IRAK2*, *IRAK3*), and the CLR genes *CLEC4D* (dectin-3) and *CLEC4E* (mincle). Moreover, also the expression of several matrixmetalloprotease-encoding genes was induced. Amongst the down-regulated genes, no enrichment in a certain pathway was detected after 24 h of co-incubation.

When hMDMs were co-incubated with a mix of candidalysin and Ece1-Va in equimolar amounts, overall the same pathways and gene expression profiles were detected as during

incubation with candidalysin only (enriched pathways Figure 21 and Figure 22, gene expression Figure 24-Figure 26).

For the 6 h time point, the strongest up-regulation was seen for genes encoding heat shock proteins like *HSPA1A*, *HSPA1B*, *HSPA6*, *HSPE1*, *HSPH1*, *DNAJA1*, *DNAJA4* and *DNAJB1* (Figure 24), involved in pathways mediating a heat and stress response (Figure 22), as shown upon candidalysin treatment only (Figure 21, Figure 24).

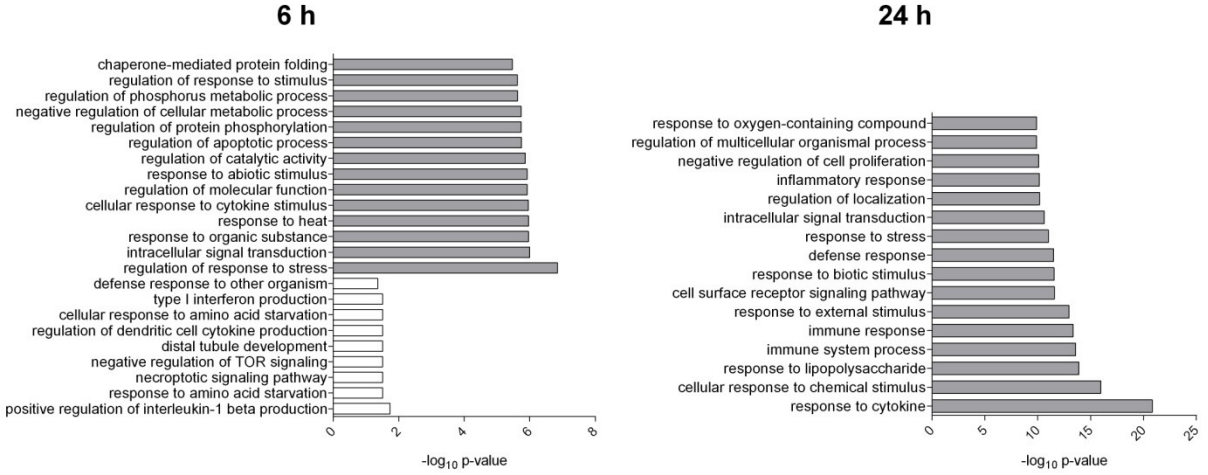


**Figure 21: Transcriptional profiling of hMDMs co-incubated with candidalysin.**

Results obtained for transcriptional profiling upon co-incubation of hMDMs with 5 µM candidalysin. Analysis of pathways enriched among DEGs *via* ShinyGo. Genes were filtered by a $\log_2$FC of ≥ 1 or ≤ -1 and a p-value ≤ 0.05 of treated *vs.* untreated prior to the pathway analysis. Reduction of redundant pathways was conducted with REVIGO and an allowed similarity of 0.7 (medium), grey - pathways enriched among up-regulated genes, white - pathways enriched among down-regulated genes. n=3.

Similar to the results obtained from candidalysin-only treatment, the 24 h time point was dominated by up-regulation of genes involved in pathways like cytokine response and cytokine-mediated signalling (*IL1A*, *IL1B*, *IL6*, *IL8*, *IL32*, *IL36B*, *CCL2*, *CCL3*, *CCL5*, *CCL8*, *CCL15*, *CCL17*, *CCL18*, *CCL23*, *CXCL2*, *CXCL1*, *CXCL5*, *CXCL10*, *TNF*, *IL1RA*, *IL2RA*, *IL7R*, *CCR7*, *IRAK2*, *IRAK3*, *JAK3*). Furthermore, the matrixmetalloprotease genes *MMP9*, *MMP12*, *MMP14* and *MMP19* were up-regulated and occurred in some enrichment pathways upon candidalysin + Ece1-Va treatment. Importantly, the MMPs 12, 14, and 19 were up-regulated to a greater extent when candidalysin was administered in combination with Ece1-Va. Also pathways like "neutrophil chemotaxis" and "granulocyte migration" appeared in the enrichment list for Ece1-Va treatment (Figure 21). Genes involved in this pathway were

mostly the above mentioned cyto- and chemokines, as well as additionally up-regulated genes like *VEGFA* and *S100A8*, which were similarly regulated in candidalysin-only and candidalysin + Ece1-Va-treated samples (Figure 24). Importantly, these pathways also occurred in the candidalysin-only treated sample analysis, but were not recorded as they did not belong to the 30 most significant terms detected. Concerning down-regulated genes upon 24 h treatment with candidalysin + Ece1-Va, it seems that mainly organic acid metabolism was repressed, including many enzyme genes like, amongst others, *ACACB*, *ALDH5A1*, *CPT1A*, *DAO*, *HADH*, *PFKM*, and *PPAT*. Furthermore, genes involved in the pathways "cold-induced thermogenesis", a mechanism of heat-generation by increasing metabolism, such as *FFAR4, HADH*, *IGF1R*, and *UCP2* and were down-regulated (Figure 25). Again, these genes were similarly regulated in the samples treated with candidalysin only, however, no enrichment was found, as the down-regulation was not significant for some of these genes. Thus, hMDMs seem to down-regulate metabolism of important compounds like e.g. fatty acids in response to sensing candidalysin, putatively a strategy of the host to limit nutrient availability for the fungus upon infection.

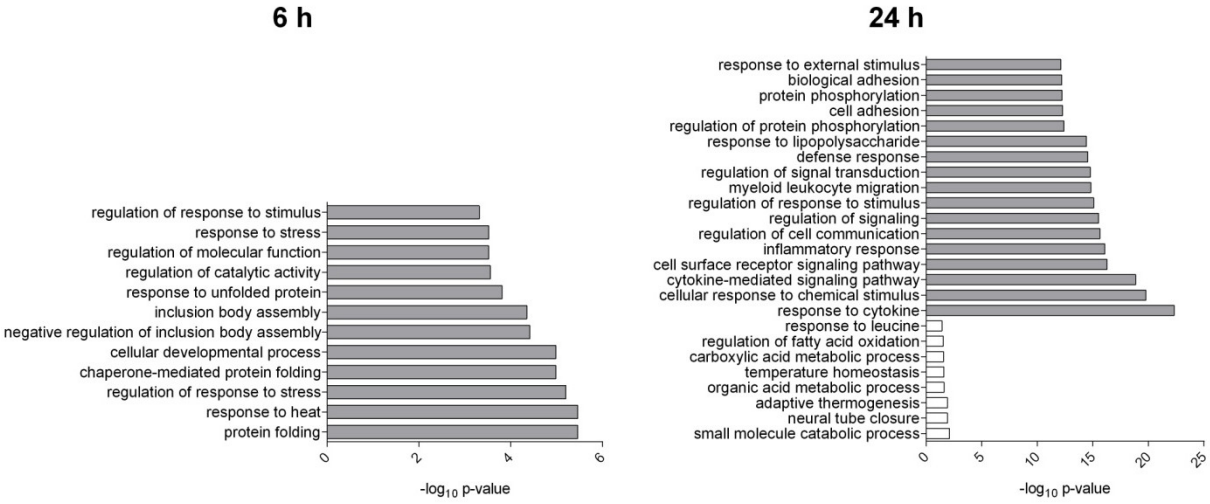


**Figure 22: Transcriptional profiling of hMDMs co-incubated with candidalysin and Ece1-Va.**

Results obtained for transcriptional profiling upon co-incubation of hMDMs with candidalysin and Ece1-Va, 5 µM each. Analysis of pathways enriched among DEGs *via* ShinyGo. Genes were filtered by a $\log_2$FC of ≥ 1 or ≤ -1 and a p-value ≤ 0.05 treated *vs*. untreated prior to the pathway analysis. Reduction of redundant pathways was conducted with REVIGO and an allowed similarity of 0.7 (medium), grey - pathways enriched among up-regulated genes, white - pathways enriched among down-regulated genes. n=3.

Notably, the co-incubation of hMDMs with Ece1-Va alone for 6 h led to the up-regulation of genes encoding metallothioneins (*MT1E*, *MT1G* and *MT1H*), which are involved in pathways related to metal stress and detoxification of divalent cations (Figure 23, Figure 26). In contrast, candidalysin-treated hMDMs down-regulated these genes at that time point and Ece1-VIa as well as Ece1-VIIa did not induce a transcriptional change (Figure 26). However, after 24 h of candidalysin and candidalysin + Ece1-Va treatment, these genes were up-regulated.

Concerning down-regulated genes, an enrichment of genes involved in the pathway of blood circulation was found upon 6 h of co-incubation with Ece1-Va alone, including genes involved in sodium and calcium transport such as *SCN5A* and *ATP2B3* as well as the gene encoding the vasopressin receptor *AVPR1B*, and the TF genes *FOXN4* and *GATA4*. This down-regulation was characteristic for the treatment of hMDMs with Ece1-Va, as only Ece1-VIIa also slightly down-regulated these genes but to a minor extent (Figure 26). Upon 24 h of co-incubation, no significant enrichment was found amongst up- or down-regulated genes (Figure 23, Figure 26).

The analysis of the Ece1-VIa effect exhibited only a slight up-regulation of genes involved in muscle hypertrophy (*BMP10*, *IL6ST*, and *PDE9A*), glia cell migration (*RBFOX2*, *SOCS7*) and aminoglycoside metabolism (*AKR1B10*, *AKR1C4*) upon 24 h of co-incubation (Figure 26). However, these genes were similarly regulated in all samples (Figure 26). Apart from that, no gene enrichment in any pathway was found (Figure 23, Figure 26).

Regarding the co-incubation of hMDMs with Ece1-VIIa, genes involved in glutathione metabolism (*CHAC1*, *GGT6*, *GSR*), in the response pathway to inactivity (*SCN5A*, *UTRN*,) and in the purine ribonucleoside metabolism (*SULT2B1*, *SULT4A1*) were down-regulated (Figure 23, Figure 26). Of note, all of these genes also showed a trend of down-regulation under candidalysin treatment. Furthermore, also treatment with Ece1-Va and Ece1-VIa led to a down-regulation of genes involved in purine ribonucleoside metabolism (Figure 26). Nevertheless, the regulation induced by Ece1-VIIa treatment was stronger than the one induced by treatment with the other peptides for the majority of these genes (Figure 26).

Summarising, the peptide toxin candidalysin seems to exert the strongest effect on gene expression in macrophages. Moreover, the response of these immune cells to the toxin was

not dramatically changed by an additional co-incubation with Ece1-Va, further indicating that the main response is driven by the peptide toxin. Interestingly, the reaction of macrophages to the NCEP fragments Ece1-Va, -VIa, and -VIIa seemed to be rather peptide-specific, than characterised by a common response, but still comparably small to the response towards candidalysin.

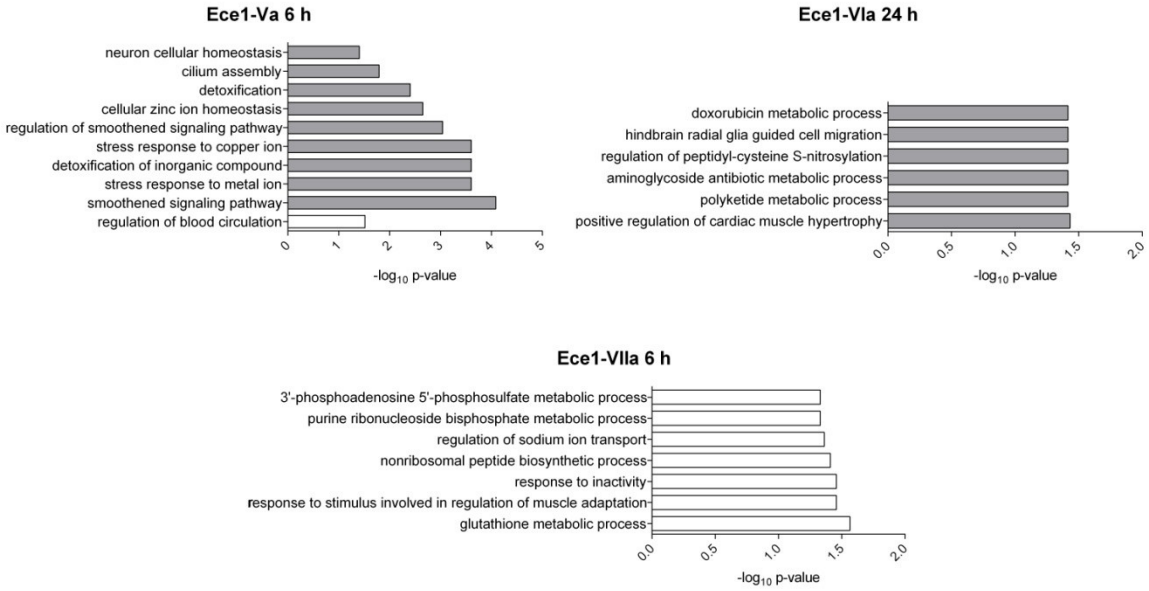


**Figure 23: Transcriptional profiling of hMDMs co-incubated with Ece1-Va, Ece1-VIa or Ece1-VIIa.**

Results obtained for transcriptional profiling upon co-incubation of hMDMs with 5 µM Ece1-Va, Ece-VIa, or Ece1-VIIa. Analysis of pathways enriched among DEGs *via* ShinyGo. Genes were filtered by a $\log_2$FC of ≥ 1 or ≤ -1 and a p-value ≤ 0.05 treated *vs.* untreated prior to the pathway analysis. Reduction of redundant pathways was conducted with REVIGO and an allowed similarity of 0.7 (medium), grey - pathways enriched among up-regulated genes, white - pathways enriched among down-regulated genes. n=3.

| Pathway | Gene | Treatment/ time point | | | | | | | | | | Gene name |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CaL | | CaL + Ece1-Va | | Ece1-Va | | Ece1-Vla | | Ece1-Vlla | | |
| | | 6 h | 24 h | 6 h | 24 h | 6 h | 24 h | 6 h | 24 h | 6 h | 24 h | |
| cyto-/chemokine response | *IL1A* | | | | | | | | | | | interleukin 1, alpha |
| | *IL1B* | | | | | | | | | | | interleukin 1, beta |
| | *IL6* | | | | | | | | | | | interleukin 6 |
| | *IL7* | | | | | | | | | | | interleukin 7 |
| | *IL10* | | | | | | | | | | | interleukin 10 |
| | *IL12A* | | | | | | | | | | | interleukin 12 |
| | *IL15* | | | | | | | | | | | interleukin 15 |
| | *IL18* | | | | | | | | | | | interleukin 18 |
| | *IL23* | | | | | | | | | | | interleukin 23 |
| | *IL27* | | | | | | | | | | | interleukin 27 |
| | *IL32* | | | | | | | | | | | interleukin 32 |
| | *IL36A* | | | | | | | | | | | interleukin 36, alpha |
| | *IL38B* | | | | | | | | | | | interleukin 36, beta |
| | *TNF* | | | | | | | | | | | tumor necrosis factor |
| | *TGFB* | | | | | | | | | | | transforming growth factor beta |
| | *CXCL1* | | | | | | | | | | | chemokine (C-X-C motif) ligand 1 |
| | *CXCL2* | | | | | | | | | | | chemokine (C-X-C motif) ligand 2 |
| | *CXCL3* | | | | | | | | | | | chemokine (C-X-C motif) ligand 3 |
| | *CXCL5* | | | | | | | | | | | chemokine (C-X-C motif) ligand 5 |
| | *CXCL8* | | | | | | | | | | | chemokine (C-X-C motif) ligand 8 |
| | *CXCL9* | | | | | | | | | | | chemokine (C-X-C motif) ligand 9 |
| | *CXCL10* | | | | | | | | | | | chemokine (C-X-C motif) ligand 10 |
| | *CXCL12* | | | | | | | | | | | chemokine (C-X-C motif) ligand 12 |
| | *CXCL13* | | | | | | | | | | | chemokine (C-X-C motif) ligand 13 |
| | *CCL1* | | | | | | | | | | | chemokine (C-C motif) ligand 1 |
| | *CCL2* | | | | | | | | | | | chemokine (C-C motif) ligand 2 |
| | *CCL3* | | | | | | | | | | | chemokine (C-C motif) ligand 3 |
| | *CCL5* | | | | | | | | | | | chemokine (C-C motif) ligand 5 |
| | *CCL8* | | | | | | | | | | | chemokine (C-C motif) ligand 8 |
| | *CCL11* | | | | | | | | | | | chemokine (C-C motif) ligand 11 |
| | *CCL15* | | | | | | | | | | | chemokine (C-C motif) ligand 15 |
| | *CCL17* | | | | | | | | | | | chemokine (C-C motif) ligand 17 |
| | *CCL18* | | | | | | | | | | | chemokine (C-C motif) ligand 18 |
| | *CCL20* | | | | | | | | | | | chemokine (C-C motif) ligand 20 |
| | *CCL22* | | | | | | | | | | | chemokine (C-C motif) ligand 22 |
| | *CCL23* | | | | | | | | | | | chemokine (C-C motif) ligand 23 |
| | *CCL24* | | | | | | | | | | | chemokine (C-C motif) ligand 24 |
| | *FGF2* | | | | | | | | | | | fibroblast growth factor 2 |
| | *CCR7* | | | | | | | | | | | chemokine (C-C motif) receptor 7 |
| | *IL1RA* | | | | | | | | | | | interleukin 1 receptor antagonist |
| | *IL2RG* | | | | | | | | | | | interleukin 2 receptor, gamma |
| | *IL2RA* | | | | | | | | | | | interleukin 2 receptor, antagonist |
| | *IL7R* | | | | | | | | | | | interleukin 7 receptor |
| | *IRAK1* | | | | | | | | | | | interleukin-1 receptor-associated kinase 1 |
| | *IRAK2* | | | | | | | | | | | interleukin-1 receptor-associated kinase 2 |
| | *IRAK3* | | | | | | | | | | | interleukin-1 receptor-associated kinase 3 |
| | *TAOK2* | | | | | | | | | | | TAO kinase 2 |
| | *JAK3* | | | | | | | | | | | Janus kinase 3 |
| | *SOCS1* | | | | | | | | | | | suppressor of cytokine signaling 1 |
| | *SOCS3* | | | | | | | | | | | suppressor of cytokine signaling 3 |
| | *VEGFA* | | | | | | | | | | | vascular endothelial growth factor A |
| | *S100A8* | | | | | | | | | | | S100 calcium binding protein A8 |
| heat shock/ stress response | *HSPA1A* | | | | | | | | | | | heat shock 70kDa protein 1A |
| | *HSPA1B* | | | | | | | | | | | heat shock 70kDa protein 1B |
| | *HSPA6* | | | | | | | | | | | heat shock 70kDa protein 6 (HSP70B') |
| | *HSPE1* | | | | | | | | | | | heat shock 10kDa protein 1 |
| | *HSPH1* | | | | | | | | | | | heat shock 105kDa/110kDa protein 1 |
| | *DNAJA1* | | | | | | | | | | | DnaJ (Hsp40) homolog, subfamily A, member 1 |
| | *DNAJA4* | | | | | | | | | | | DnaJ (Hsp40) homolog, subfamily A, member 4 |
| | *DNAJB1* | | | | | | | | | | | DnaJ (Hsp40) homolog, subfamily B, member 1 |
| phosphate metabolism | *DUSP1* | | | | | | | | | | | dual specificity phosphatase 1 |
| | *DUSP4* | | | | | | | | | | | dual specificity phosphatase 4 |
| | *DUSP5* | | | | | | | | | | | dual specificity phosphatase 5 |
| | *DUSP14* | | | | | | | | | | | dual specificity phosphatase 14 |
| detoxification | *SOD2* | | | | | | | | | | | superoxide dismutase 2, mitochondrial |
| CLRs | *CLEC4D* | | | | | | | | | | | C-type lectin domain family 4, member D |
| | *CLEC4E* | | | | | | | | | | | C-type lectin domain family 4, member E |
| zinc finger proteins, transcription factors, histone deacetylase | *BCL11A* | | | | | | | | | | | B-cell CLL/lymphoma 11A (zinc finger protein) |
| | *SNAI1* | | | | | | | | | | | snail family zinc finger 1 |
| | *SNAI2* | | | | | | | | | | | snail family zinc finger 2 |
| | *ZC3H12A* | | | | | | | | | | | zinc finger CCCH-type containing 12A |
| | *CEBPB* | | | | | | | | | | | CCAAT/enhancer binding protein (C/EBP), beta |
| | *NFKBIA* | | | | | | | | | | | NF-κ light polypeptide gene enhancer in B-cells inhibitor, α |
| | *NFKB2* | | | | | | | | | | | NF-κ light polypeptide gene enhancer in B-cells 2 (p49/p100) |
| | *STAT4* | | | | | | | | | | | signal transducer and activator of transcription 4 |
| | *HDAC4* | | | | | | | | | | | histone deacetylase 4 |
| matrixmetallo-proteases | *MMP1* | | | | | | | | | | | matrix metallopeptidase 1 (interstitial collagenase) |
| | *MMP9* | | | | | | | | | | | matrix metallopeptidase 9 (gelatinase B) |
| | *MMP12* | | | | | | | | | | | matrix metallopeptidase 12 (macrophage elastase) |
| | *MMP14* | | | | | | | | | | | matrix metallopeptidase 14 (membrane-inserted) |
| | *MMP19* | | | | | | | | | | | matrix metallopeptidase 19 |

**Figure 24: Gene expression of selected genes involved in pathways modulated mainly by candidalysin treatment I.**

$Log_2FC$ of exemplary genes involved in the pathways stated in Figure 21 and Figure 22. Red - up-regulation, blue - down-regulation. Stronger colour indicates stronger regulation. n=3.

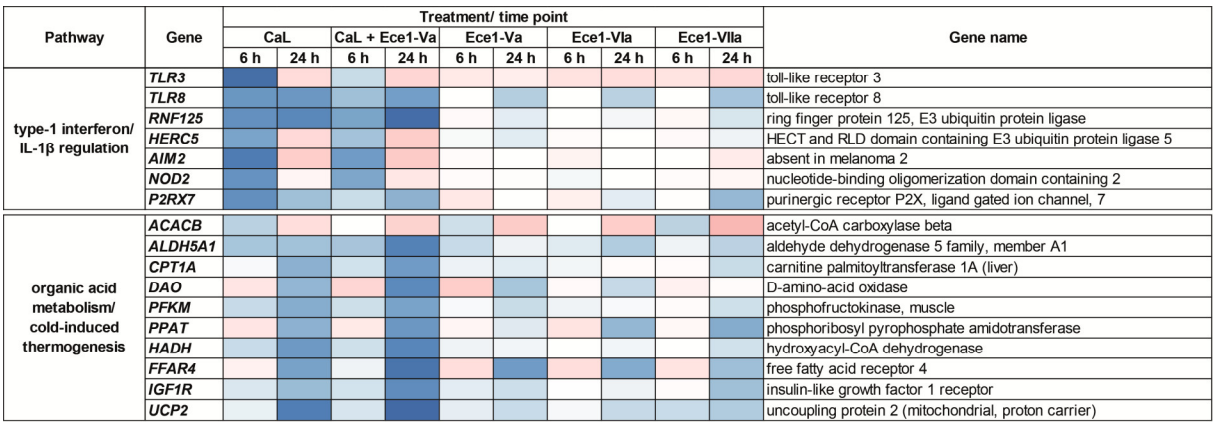


**Figure 25: Gene expression of selected genes involved in pathways modulated mainly by candidalysin treatment II.**

$Log_2FC$ of exemplary genes involved in the pathways stated in Figure 21 and Figure 22. Red - up-regulation, blue – down-regulation. Stronger colour indicates stronger regulation. n=3.

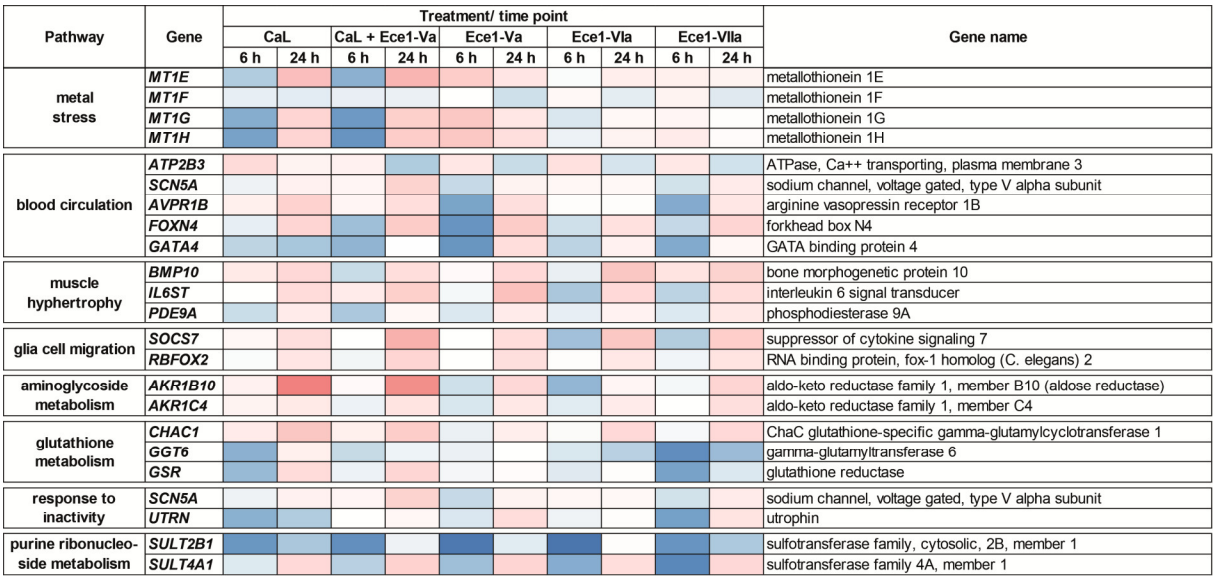


**Figure 26: Gene expression of selected genes involved in pathways modulated mainly by Ece1-Va, Ece1-VIa or Ece1-VIIa treatment.**

$Log_2FC$ of exemplary genes involved in the pathways stated in Figure 23. Red - up-regulation, blue - down-regulation. Stronger colour indicates stronger regulation. n=3.

### 4.8.3 Cytokine and chemokine response of macrophages induced by synthetic Ece1 peptides

Apart from the transcriptional response of hMDMs to a treatment with NCEPs and/or candidalysin, especially the immune reaction in terms of cyto- and chemokine secretion is of great importance to analyse potential effector functions of the peptides. Many cyto- and chemokines have intracellular stores or are activated on a post-transcriptional level, which makes analyses on the protein level important for conclusion of a putative NCEP effector

function. To screen for secretion of multiple cyto- and chemokines, multiplex and single ELISAs were used to determine the amount of 37 different, well known immune response mediators secreted by macrophages (Table 24) (Bachelerie *et al.* 2014, Palomino and Marti 2015). Furthermore, the expression of many of the respective genes was regulated upon treatment with candidalysin and/or NCEP fragments as presented in paragraph 4.8.2. The secretion of immune mediators was measured after 6 and 24 h of co-incubation with NCEPs. Candidalysin co-incubation or infection with the *C. albicans* Wt, an *ece1*Δ/Δ mutant strain or a strain lacking only the candidalysin-encoding sequence (Δ*P3*) was used as a control, as these have already been described to induce the release of different immune mediators from mononuclear phagocytes (Castro *et al.* 1996, Torosantucci *et al.* 2000, Kim *et al.* 2005, Seider *et al.* 2011, Kasper *et al.* 2018, Rogiers *et al.* 2019). To evaluate a putative effector function of NCEPs, the most abundant fragments secreted into the hyphal supernatant by Wt *C. albicans* (Ece1-Va, Ece1-VIa, Ece1-VIIa, and Ece1-VIIb, Table 1) were analysed for their potential to induce the release of the above-mentioned immune mediators. As it was hypothesised that candidalysin might be involved in the translocation of these putative effectors into the host cell, the NCEP fragments were further tested in a combination with the toxin in equimolar amounts.

The chemokines MIG (CXCL9), IP-10 (CXCL10), RANTES (CCL5), MCP-2 (CCL8), and the cytokine IL-12p70 were induced upon hMDM infection with *C. albicans* for 24 h. This secretion was independent of *ECE1* or the candidalysin-encoding sequence, and in most cases secretion was not induced upon co-incubation with synthetic NCEP fragments and/or candidalysin (Figure 27). Regarding IP-10, RANTES and MCP-2, a minor induction upon 24 h of co-incubation with candidalysin, Ece1-VIa (only IP-10), Ece1-VIIa (only MCP-2), or candidalysin in combination with NCEP fragments was observed (Figure 27). However, chemokine levels induced upon peptide co-incubation were approximately 10 times lower than levels elicited upon *C. albicans* infection.

Figure 28 depicts that several pro-inflammatory cytokines were triggered by a co-incubation of hMDMs with synthetic candidalysin or an infection with *C. albicans*. The peptide toxin potently induced the release of the danger-associated molecular pattern (DAMP) IL-1α, as

well as of the inflammasome-related pro-inflammatory cytokines IL-1β and IL-18. In agreement with this, the *ece1*Δ/Δ mutant and the mutant lacking the candidalysin-encoding sequence induced less secretion of these cytokines than the *C. albicans* Wt. Apart from that, also a co-incubation with Ece1-VIIa resulted in an induction of IL-1α and IL-18 release.

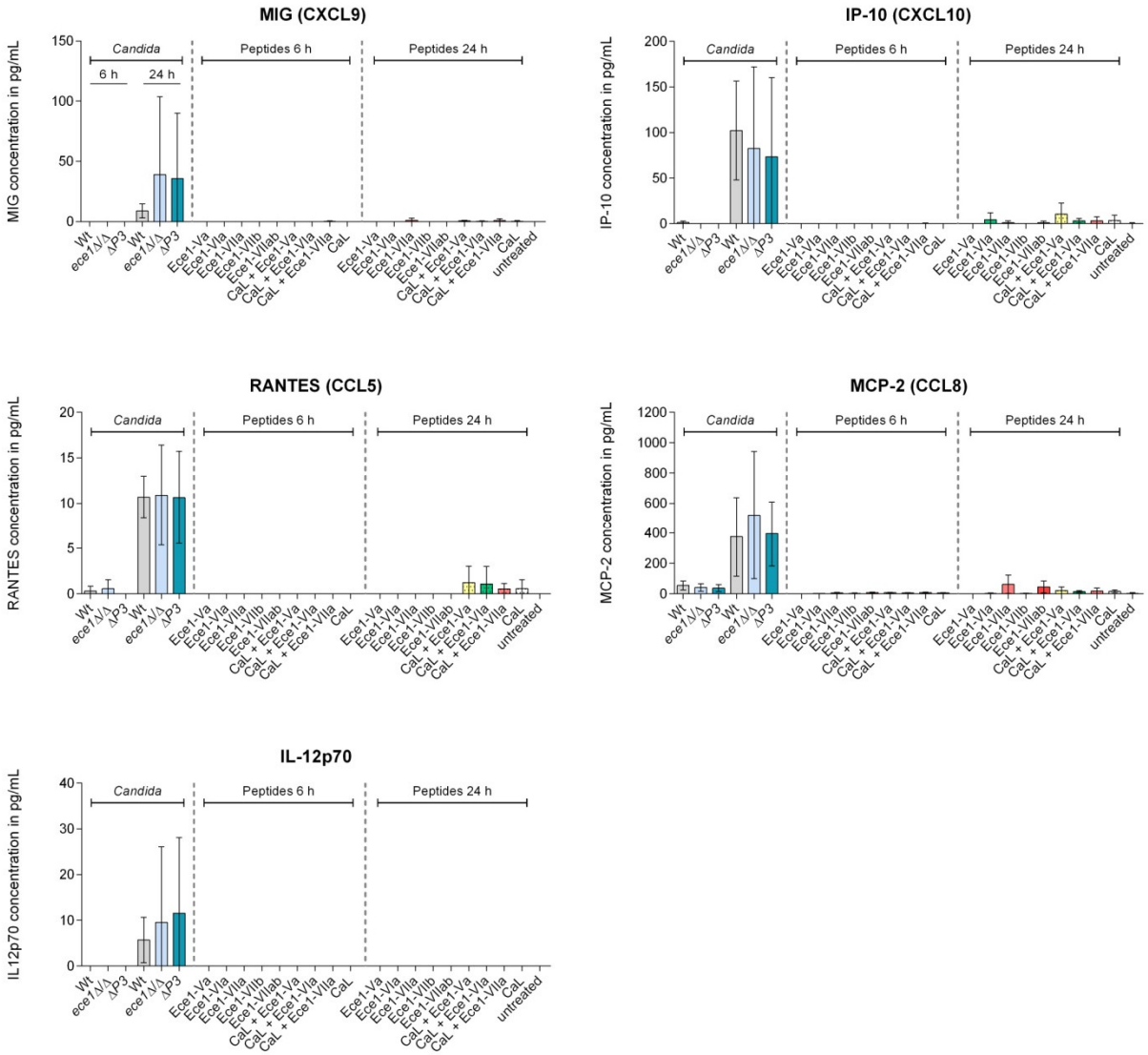


**Figure 27: *C. albicans* induces *ECE1*-independent chemokine secretion.**

Primary hMDMs were co-incubated with 5 µM candidalysin or NCEP fragments or infected with *C. albicans* multiplicity of infection (MOI) 5 for 6 or 24 h. All cyto- and chemokines were measured in a multiplex ELISA. CaL - candidalysin, ab - co-incubation with peptide fragments a and b, n=3.

Furthermore, after 6 h of co-incubation, an additive effect of Ece1-VIIa and candidalysin was observed for both cytokines (Figure 28, Figure 33). Regarding IL-18, also the synthetic NCEP fragments Ece1-Va and Ece1-VIa induced a slight cytokine secretion and elicited a slight additive effect when co-incubated with candidalysin (Figure 28, Figure 33). The pro-inflammatory cytokine IL-6 was secreted upon treatment with candidalysin or Ece1-VIIa. In

parts, this secretion seems to be candidalysin-dependent during infection, since mutants lacking *ECE1* or the candidalysin-encoding sequence showed defects in cytokine release after 6 h. Similarly, TNF-α secretion was reduced in the Δ*P3* mutant and slightly reduced in an *ece1*Δ/Δ mutant 6 h post infection. However, synthetic candidalysin alone did not induce a strong secretion of this cytokine, indicating a candidalysin-independent induction of TNF-α release by *C. albicans*. The NCEP fragments Ece1-Va and Ece1-VIa did not elicit any TNF-α secretion, whereas Ece1-VIIa triggered a minor secretion of this cytokine, similar to candidalysin. Importantly, of both Ece1-VII fragments (Table 1), only Ece1-VIIa exhibited a cytokine-inducing effect.

Upon secretion of pro-inflammatory cytokines, often also anti-inflammatory cytokines are released as immune-regulatory molecules that modulate the induced pro-inflammatory response (Zhang and An 2007). Indeed, candidalysin and, to a small extent, Ece1-VIIa induced the release of anti-inflammatory IL-10 and IL-1RA (Figure 29). Apart from that, a co-incubation of hMDMs with candidalysin and the NCEP fragments Ece1-Va, -VIa, or -VIIa led to an additive effect of anti-inflammatory cytokine secretion (Figure 29, Figure 33). Infection with *C. albicans* revealed that this induction was not necessarily dependent on candidalysin, as the tested mutant strains showed no clear reduction in cytokine release when compared to the Wt.

Of note, candidalysin was able to strongly induce neutrophil chemoattractive chemokines such as Gro-α (CXCL1), ENA-78 (CXCL5), IL-8 (CXCL8), SDF-1α (CXCL12), and Eotaxin-2 (CCL24) (Figure 30). The eosinophil attractant Eotaxin (CCL11) was only induced to a minor extent (Figure 30). Especially in the case of Eotaxin-2 and ENA-78, a co-incubation with the peptide toxin induced more cytokine release than an infection of hMDMs with *C. albicans* strains. Apart from candidalysin, also Ece1-VIIa induced the above-mentioned chemokines and seemed to have neutrophil chemoattractive properties, even though less potent than candidalysin. As reported for the induction of pro-inflammatory cytokines, the Ece1-VIIb fragment alone was not capable to induce a chemokine response. Similar to the release of IL-1α, IL-18, and IL-6, an additive effect of the NCEP fragments and candidalysin on the

secretion of anti-inflammatory cytokines was observed especially after 6 h of co-incubation (Figure 30, Figure 33).

Apart from inducing neutrophil- and eosinophil-attracting chemokines, candidalysin further stimulated the release of $CD8^+$ T-cell recruiting MIP-1α and MIP-1β (CCL3 and 4, respectively (Honey 2006)). Moreover, the peptide toxin was able to induce MIP-3α (CCL20), a chemokine that mediates recruitment of IL-17 producing type 17 T helper cells (Th17) and regulatory T-cells (Treg).

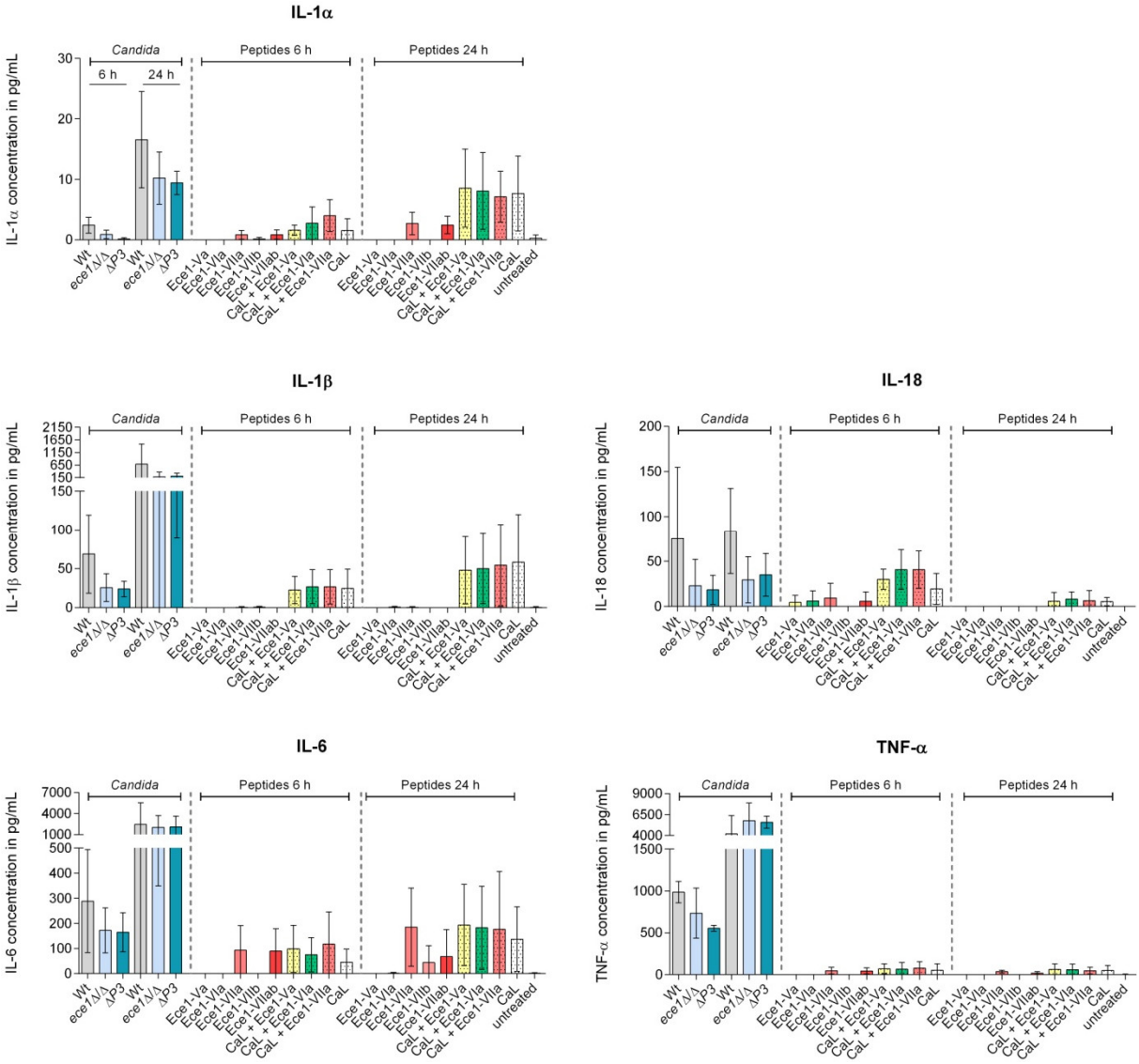


**Figure 28: Candidalysin, Ece1-VIIa, and *C. albicans* induce pro-inflammatory cytokine secretion.**

Primary hMDMs were co-incubated with 5 µM candidalysin or NCEP fragments or infected with *C. albicans* MOI 5 for 6 or 24 h. All cytokines were measured in a multiplex ELISA. CaL - candidalysin, ab - co-incubation with peptide fragments a and b, n=3.

The release of these chemokines was also induced by infection of hMDMs with *C. albicans*. Upon co-incubation with candidalysin, I-309 (CCL1) was induced, which acts chemoattractive for monocytes/macrophages and T-lymphocytes. The monocyte-chemotactic protein (MCP)-1 was secreted in nearly all conditions and seemed to be induced rather unspecifically by infection or treatment with extracellular peptides. Infection with the fungus as well as treatment with candidalysin induced a minor release of IL-7, a cytokine important for the development of B- and T-cells. Figure 31 depicts the release of chemoattractive chemokines, which was mainly induced by *C. albicans* infection and co-incubation with candidalysin.

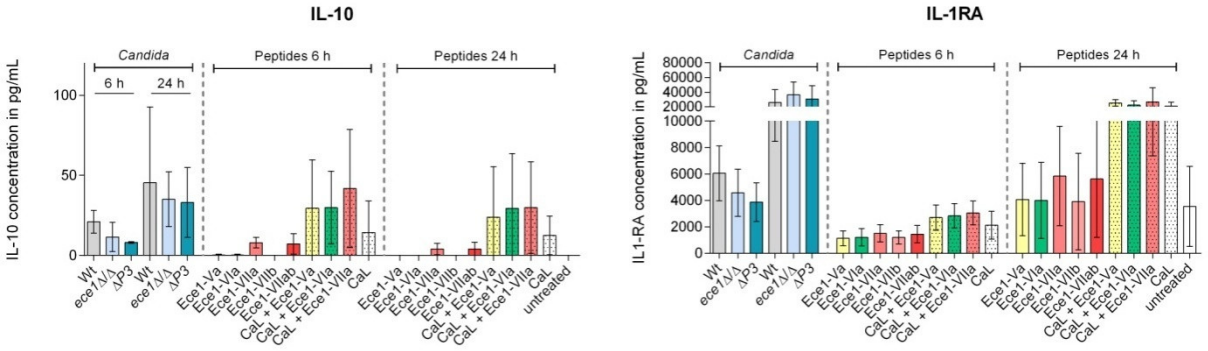


**Figure 29: Candidalysin and *C. albicans* induce anti-inflammatory cytokine secretion.**

Primary hMDMs were co-incubated with 5 µM candidalysin or NCEP fragments or infected with *C. albicans* MOI 5 for 6 or 24 h. Both cytokines were measured in a multiplex ELISA. CaL - candidalysin, ab – co-incubation with peptide fragments a and b, n=3.

Interestingly, a co-incubation with the peptide toxin candidalysin induced the release of matrixmetalloprotease-1 (MMP-1) even stronger than an infection of hMDMs with *C. albicans* (Figure 32). Similarly, MMP-12 release was induced upon candidalysin co-incubation and infection of hMDMs with *C. albicans*. However, this MMP seemed to be additionally activated by the other NCEP fragments tested and to a lesser extent than MMP-1. For the cytokines IL-15, IL-23, IL-27, TGF-β, the chemokines MIP-1δ (CCL15), TARC (CCL17), PARC (CCL18), MDC (CCL22), BLC (CXCL13) as well as for FGF-2 and IL-2RA no secretion or exposure was detected using single or multiplex ELISA (data not shown).

Heat maps of immune mediator secretion in comparison to the Wt- or the candidalysin-induced secretion level were prepared to give a quick overview over all mediators induced in hMDMs upon *C. albicans* infection or Ece1 peptide treatment (Figure 33, Figure 34).

Concluding, of all Ece1 peptides tested on macrophages, mainly the peptide toxin candidalysin induces the release of immune mediators (Figure 33). This peptide induced the secretion of pro-inflammatory cytokines, chemoattractants mediating T-cell, neutrophil,

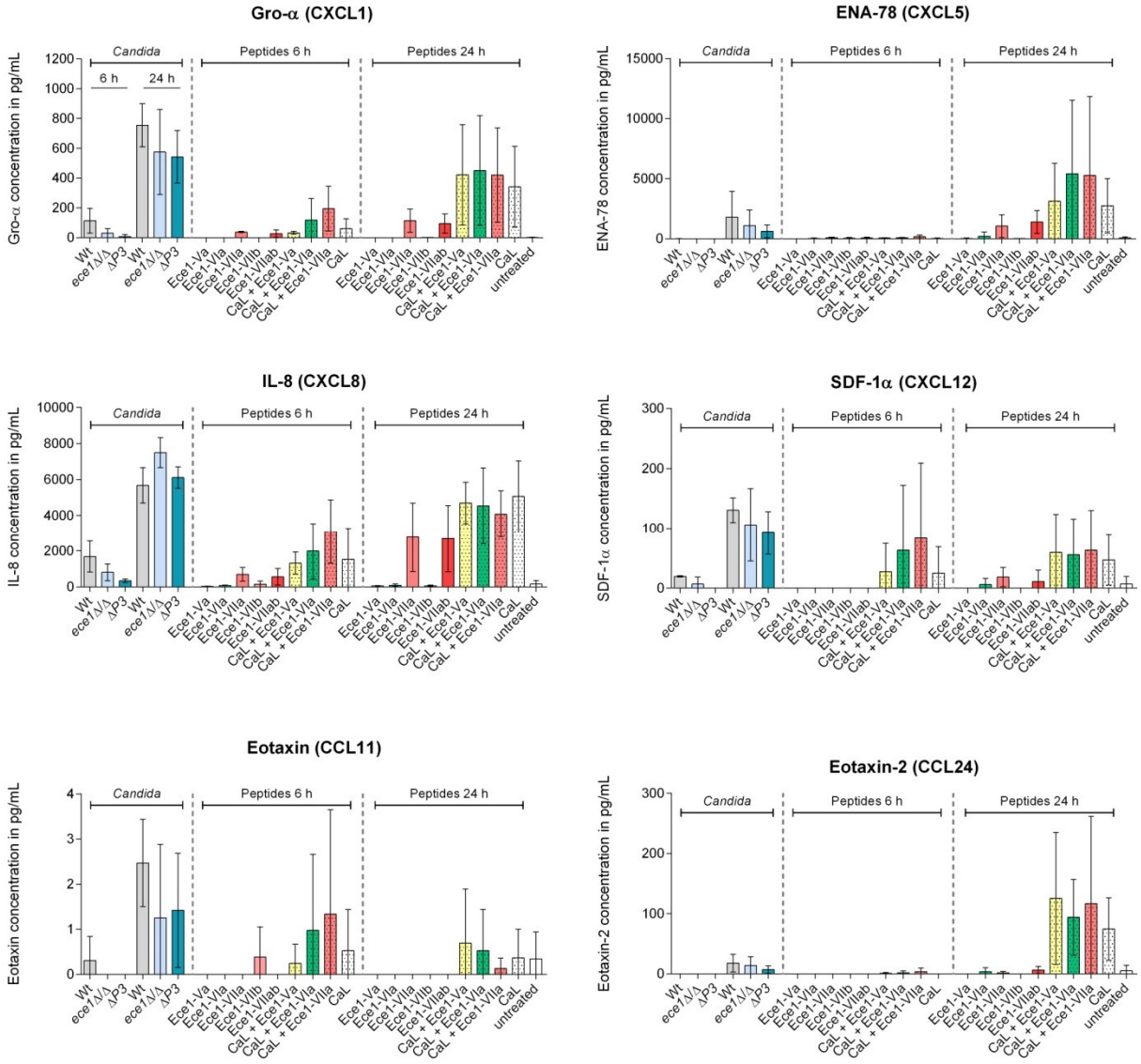


**Figure 30: Candidalysin, Ece1-VIIa, and *C. albicans* induce neutrophil and eosinophil-attracting chemokine secretion.**

Primary hMDMs were co-incubated with 5 µM candidalysin or NCEP fragments or infected with *C. albicans* MOI 5 for 6 or 24 h. All chemokines were measured in a multiplex ELISA. CaL - candidalysin, ab – co-incubation with peptide fragments a and b, n=3.

eosinophil, and monocyte/macrophage recruitment, anti-inflammatory cytokines as well as the release of MMP-1 and -12. Especially in case of the induced pro-inflammatory cytokines at the early time point (6 h), an important function of candidalysin was underlined by diminished immune mediator secretion by *C. albicans* strains lacking the candidalysin-encoding sequence. However, an infection of macrophages with Wt *C. albicans*

cells also elicited the release of many immune mediators in a candidalysin-independent manner, especially after 24 h of infection (Figure 34). Apart from candidalysin, also the a-fragment of Ece1-VII was able to induce mainly neutrophil-attracting chemokines but also some pro- and anti-inflammatory cytokines as well as some T-cell- and monocyte/ macrophage-attracting chemokines (Figure 33). These data clearly demonstrate that NCEPs, especially Ece1-VIIa, are not only by-products of the candidalysin production, but also possess candidalysin-independent functions during the interaction with host cells, further underlining the results obtained from the transcriptional profiling (paragraph 4.8.2).

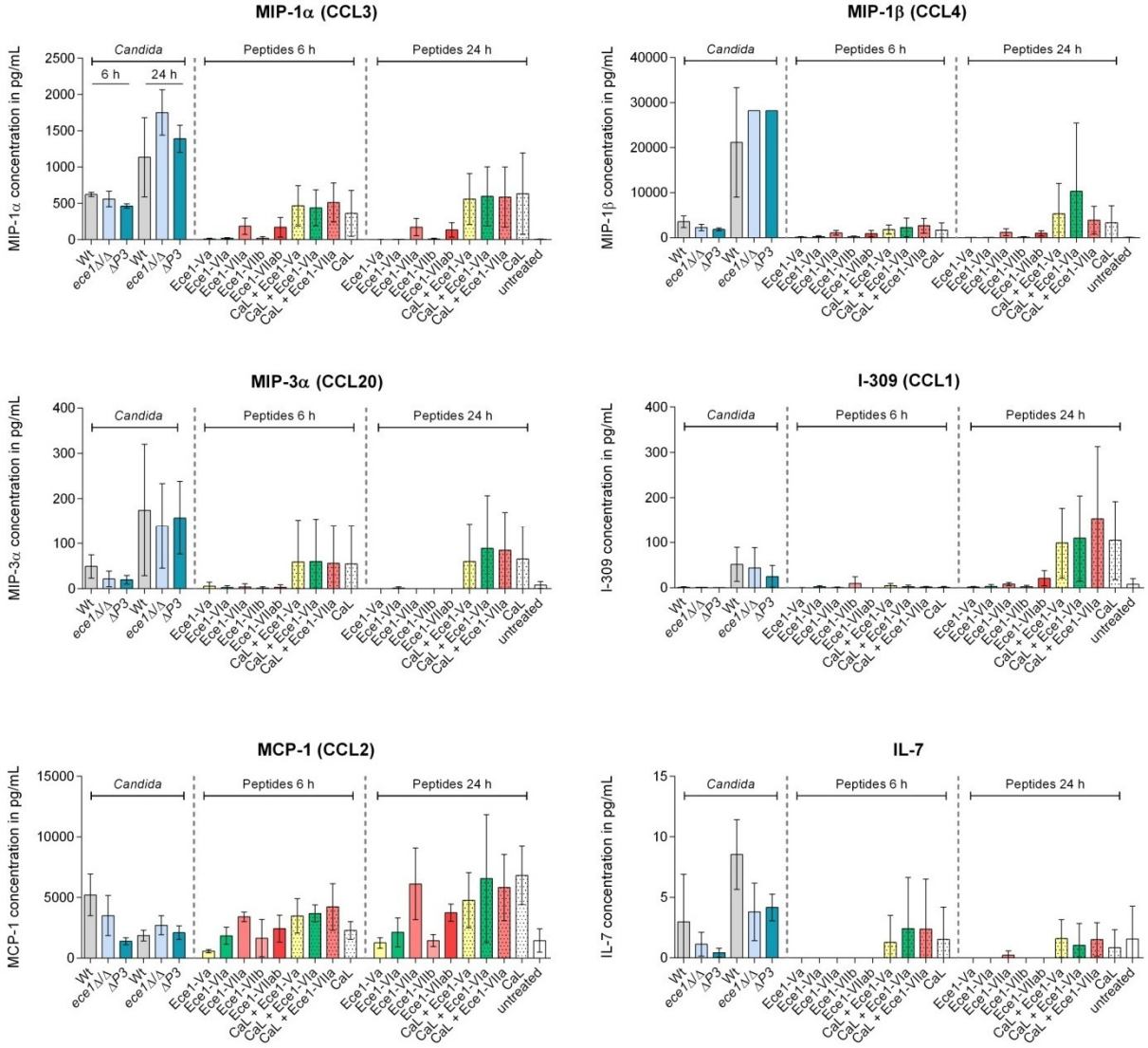


**Figure 31: Candidalysin and *C. albicans* induce T-cell- and monocyte/macrophage-recruiting cyto- and chemokine secretion.**

Primary hMDMs were co-incubated with 5 µM candidalysin or NCEP fragments or infected with *C. albicans* MOI 5 for 6 or 24 h. All mediators were measured in a multiplex ELISA. CaL - candidalysin, ab – co-incubation with peptide fragments a and b, n=3.

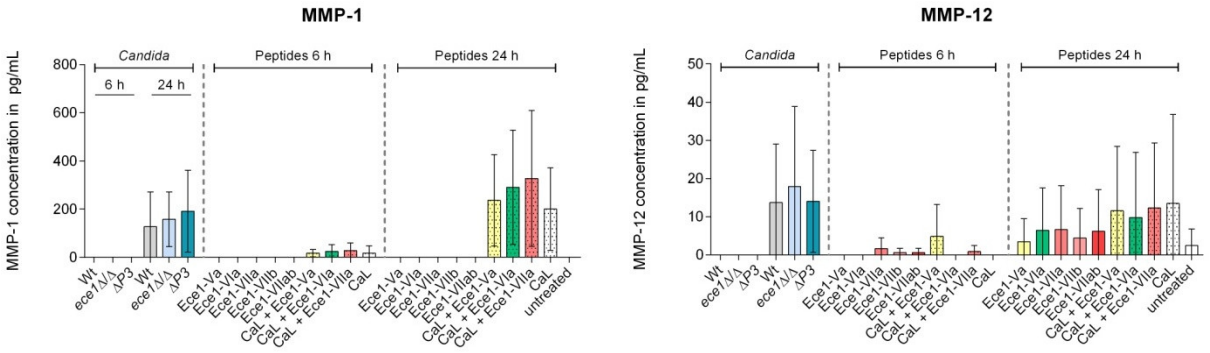


**Figure 32: Induction of MMP-1 and -12 by co-incubation of hMDMs with candidalysin or infection with *C. albicans*.**

Primary hMDMs were co-incubated with 5 µM candidalysin or NCEP fragments or infected with *C. albicans* MOI 5 for 6 or 24 h. Both MMPs were measured in a multiplex ELISA. CaL - candidalysin, ab – co-incubation with peptide fragments a and b, n=3.

| Description | Immune mediator | 6 h | | | | | | | | | 24 h | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CaL | CaL + Ece1- | | | Ece1- | | | | | CaL | CaL + Ece1- | | | Ece1- | | | | |
| | | | Va | VIa | VIIa | Va | VIa | VIIa | VIIb | VIIab | | Va | VIa | VIIa | Va | VIa | VIIa | VIIb | VIIab |
| *C. albicans*-induced mediators | MIG | | | | | | | | | | | | | | | | | | |
| | IP-10 | | | | | | | | | | | | | | | | | | |
| | RANTES | | | | | | | | | | | | | | | | | | |
| | MCP-2 | | | | | | | | | | | | | | | | | | |
| | IL-12p70 | | | | | | | | | | | | | | | | | | |
| Pro-inflammatory cytokines | IL-1α | | | | | | | | | | | | | | | | | | |
| | IL-1β | | | | | | | | | | | | | | | | | | |
| | IL-18 | | | | | | | | | | | | | | | | | | |
| | IL-6 | | | | | | | | | | | | | | | | | | |
| | TNF-α | | | | | | | | | | | | | | | | | | |
| Anti-inflammatory cytokines | IL-10 | | | | | | | | | | | | | | | | | | |
| | IL-1RA | | | | | | | | | | | | | | | | | | |
| Neutrophil/eosinophil attraction | Gro-α | | | | | | | | | | | | | | | | | | |
| | ENA-78 | | | | | | | | | | | | | | | | | | |
| | IL-8 | | | | | | | | | | | | | | | | | | |
| | SDF-1α | | | | | | | | | | | | | | | | | | |
| | Eotaxin | | | | | | | | | | | | | | | | | | |
| | Eotaxin-2 | | | | | | | | | | | | | | | | | | |
| T-cell and monocyte/macrophage recruitment | MIP-1α | | | | | | | | | | | | | | | | | | |
| | MIP-1β | | | | | | | | | | | | | | | | | | |
| | MIP-3α | | | | | | | | | | | | | | | | | | |
| | I-309 | | | | | | | | | | | | | | | | | | |
| | MCP-1 | | | | | | | | | | | | | | | | | | |
| | IL-7 | | | | | | | | | | | | | | | | | | |
| Matrixmetalloproteases | MMP-1 | | | | | | | | | | | | | | | | | | |
| | MMP-12 | | | | | | | | | | | | | | | | | | |

**Figure 33: Heat map of immune mediator release upon Ece1 peptide treatment of hMDMs.**

Release of different immune mediators upon co-incubation of primary hMDMs with different Ece1 peptides (5 µM) for 6 and 24 h. Dark green - ≥ 125 % of CaL secretion level, Green - ≥ 75 % of CaL secretion level, yellow - 25<x<75 % of CaL secretion level, red - ≤ 25 % of CaL secretion level, white - no secretion already in the CaL sample. Percentage of CaL secretion level was calculated individually for each time point. Heat map does neither reflect actual concentration levels nor allow a comparison between *C. albicans* infection and Ece1 peptide treatment due to different normalisations. CaL - candidalysin, ab – co-incubation with peptide fragments a and b.

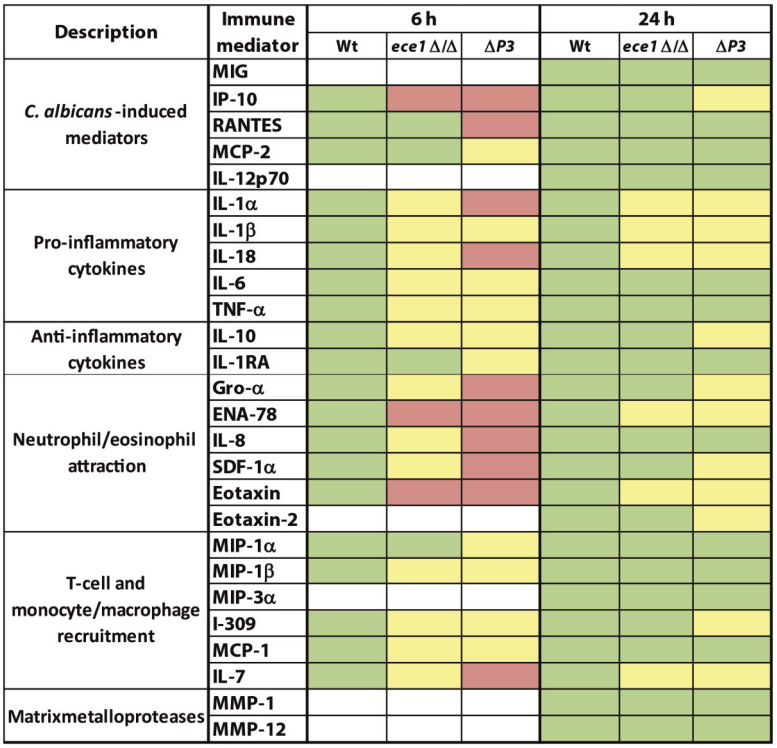


**Figure 34: Heat map of immune mediator release upon *C. albicans* infection of hMDMs.**

Release of different immune mediators upon primary hMDM infection with different *C. albicans* strains (MOI 5) for 6 and 24 h. Green - ≥ 75 % of Wt secretion level, yellow – 25<x<75 % of Wt secretion level, red - ≤ 25 % of Wt secretion level, white - no secretion already in the Wt sample. Percentage of Wt secretion level was calculated individually for each time point. Heat map does neither reflect actual concentration levels nor allow a comparison between *C. albicans* infection and Ece1 peptide treatment due to different normalisations.

# 5 Discussion

*Candida albicans* is a harmless commensal of mucosal surfaces in most healthy individuals, but can also be an opportunistic pathogen causing superficial or even life-threatening diseases when host barriers are breached and the immune system is compromised (Soll *et al.* 1991, Huffnagle and Noverr 2013, Kullberg and Arendrup 2015).

During the commensal state, the fungus resides in different body niches such as the oral cavity, the gastrointestinal, and the genitourinary tract without causing damage of host cells (Soll *et al.* 1991, Moyes *et al.* 2010, Huffnagle and Noverr 2013). Epithelial cells, which represent the first line of mechanical defence against invading microbes, tolerate low fungal burdens during commensalism. However, a high fungal burden and epithelial cell damage induces a danger response pathway *via* which innate immune responses are initiated to clear *C. albicans* infections as reviewed in **manuscript I** (Naglik *et al.* 2017).

These innate immune responses include the recruitment of innate immune cells like macrophages (Naglik 2014, Naglik *et al.* 2017). Once phagocytosed, *C. albicans* activates the NLRP3 inflammasome in mononuclear phagocytes (Gross *et al.* 2009), which mediates the release of pro-inflammatory cytokines. Several studies have shown that this is associated with the induction of the programmed, pro-inflammatory cell death pathway pyroptosis (Uwamahoro *et al.* 2014, Wellington *et al.* 2014, O'Meara *et al.* 2018). **Manuscript II** shows that the fungal peptide toxin candidalysin acts as an important trigger to activate the NLRP3 inflammasome in mononuclear phagocytes and to release mature IL-1β from these immune cells *via* potassium efflux without inducing programmed cell death pathways like pyroptosis, apoptosis, or necroptosis (Kasper *et al.* 2018). In contrast, the toxin rather directly damages host cells in a necrosis-like manner (Kasper *et al.* 2018).

By directly causing host cell damage, candidalysin acts as a classical virulence factor (Moyes *et al.* 2016, Allert *et al.* 2018). However, this toxin-induced host cell damage is sensed by the immune system. As a consequence, candidalysin can mediate host-protective innate immune responses, but can also induce host-detrimental immunopathology and thereby indirect, host-mediated cell damage (Moyes *et al.* 2016, Verma *et al.* 2017, Kasper *et al.* 2018, Richardson *et al.* 2018c, Drummond *et al.* 2019, Ho *et al.* 2019, Rogiers *et al.* 2019, Swidergall *et al.* 2019,

Lowes *et al.* 2020). These dual effects have been discussed in **manuscripts III** and **IV** (König *et al.* 2020a, König *et al.* 2020b).

Apart from the toxin candidalysin, the *ECE1* gene encodes seven other Non-Candidalysin Ece1 peptides (NCEPs), which are secreted into the extracellular space by *C. albicans* hyphae (Richardson *et al.* 2018b). These NCEPs might have diverse functions. They could have an impact on fungal biology or might modulate candidalysin function. Furthermore, NCEPs might act as effector peptides modifying the host response during infection, similar to toxins or effector proteins/peptides secreted by many plant pathogenic fungi, as reviewed in **manuscript V** (König *et al.* 2021). To evaluate the function of NCEPs, *C. albicans* mutants lacking distinct NCEP-encoding sequences or combinations thereof were analysed in terms of fungal biology and their damaging potential against primary human monocyte-derived macrophages (hMDMs) (**chapter 4**). Moreover, the effect of synthetic NCEPs or NCEP fragments on hMDMs was surveyed in terms of macrophage transcriptional and inflammatory response (**chapter 4, additional data on the CD**).

## 5.1 Candidalysin can activate epithelial cell signalling responsible for the induction of innate immune responses

Epithelial cells typically represent the first line of contact or mechanical defence for colonising or invading *C. albicans* cells. Usually, the commensal state is dominated by the yeast form of *C. albicans*, during which many virulence genes are not expressed (Romo and Kumamoto 2020). Yeast cell populations containing no or only low amounts of hyphal cells are tolerated by epithelial cells (Naglik *et al.* 2014). However, upon exceedance of a certain threshold, hypha formation induces a danger response in oral epithelial cells (Naglik *et al.* 2014). This danger response initiates immune cell recruitment and activation, which ultimately leads to fungal clearance during oral infections (Naglik *et al.* 2014).

The ability of *C. albicans* to grow in the hyphal form is a major virulence trait of the fungus. *Candida albicans* can invade epithelial cells by active penetration, representing the dominant route of *C. albicans* tissue invasion, during which the growing hypha mechanically stretches the host cell membrane (Wächtler *et al.* 2012). Depending on the host cell type, invasion is further mediated *via* induced endocytosis, for example in oral epithelial or endothelial cells,

especially at early time points of contact (Sun *et al.* 2010, Wächtler *et al.* 2012). During this process, Als3 and Ssa1 engage the receptors EGFR/HER2 and E- or N-cadherin (Phan *et al.* 2007, Sun *et al.* 2010, Wächtler *et al.* 2012, Zhu *et al.* 2012), resulting in a clathrin-dependent internalisation of the fungus (Moreno-Ruiz *et al.* 2009). Upon active penetration and induced endocytosis, an invasion pocket is formed (Wächtler *et al.* 2012). In this pocket, the fungus-secreted toxin candidalysin can presumably reach concentrations high enough to damage epithelial cells and trigger further signalling *via* calcium-dependent MMP activation, EGFR ligand cleavage and ultimately EGFR activation (Moyes *et al.* 2016, Ho *et al.* 2019). The toxin-dependent epithelial cell damage is the main factor inducing the danger response signalling pathway, which activates c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK)1/2. These in turn induce MAPK signalling resulting in c-Fos activation and ultimately DAMP/alarmin (IL-1α, S100A8, ATP, ROS, RNS) and AMP (human β-defensin 2 and 3, LL37) release (Moyes *et al.* 2016, Naglik *et al.* 2019, Hanaoka and Domae 2020, Ho *et al.* 2020b). DAMPs/alarmins participate in the recruitment of innate immune cells to the site of infection (Naglik *et al.* 2019, Hanaoka and Domae 2020, Ho *et al.* 2020b). Of these, especially the S100 alarmin proteins are important to mediate recruitment of neutrophils and monocytes/macrophages (Yano *et al.* 2010, Yano *et al.* 2014, Xia *et al.* 2018). In contrast, AMPs like defensins directly lyse the fungal cell wall (Jarva *et al.* 2018). Interestingly, genes reported to be involved in the epithelial danger response (*DUSP1*, *JUN*) (Moyes *et al.* 2010, Richardson *et al.* 2018c, Verma *et al.* 2018, Hanaoka and Domae 2020, Ho *et al.* 2020b, Pekmezovic *et al.* 2021) were also up-regulated in hMDMs upon treatment with candidalysin (Figure 24, Additional Data CD). Furthermore, gene expression of *S100A8* and the IL-1α-encoding gene *IL1A* was increased in hMDMs treated with the peptide toxin (Figure 24), indicating that macrophages might also respond to toxin treatment with the induction of a danger response and alarmin release. Regarding ROS and RNS, no up-regulation of genes involved in the generation process (*NOX1*, *NOX3*, *NOX4*, *NOX5*, *XDH*, *CYBA*, *CYBB*, *NCF1*, *NCF2*, *NCF4*, *DUOX1*, and *DUOX2*) was detected upon co-incubation of hMDMs with candidalysin (Additional Data CD). In line with this, the expression of genes encoding subunits of the ATP synthase, an enzyme associated with the generation of mitochondrial ROS, was unaffected by toxin treatment (Additional Data CD).

This suggests that candidalysin is not the *C. albicans*-derived trigger inducing the expression of ROS- or RNS-generating enzymes in these host cells. However, it is still possible, that the toxin activates these enzymes post-transcriptionally, thereby contributing to ROS and RNS production. In case of AMPs, genes encoding β-defensin 2 (*DEFB4A*) and 3 (*DEFB103A*) showed no up-regulation by candidalysin treatment in hMDMs (Additional Data CD). Furthermore, the gene encoding the AMP LL37 (*CAMP*) was down-regulated 1.5-fold after 24 h of candidalysin-hMDM co-incubation (Additional Data CD). Thus, even if candidalysin induces some overlapping responses in epithelial cells and macrophages (e.g. *S100A8* and *IL1A* up-regulation), the main signalling pathways employed by these host cells differ from one another, which makes further research necessary to fully elucidate whether macrophages also induce a specific danger response towards toxin treatment.

## 5.2 Candidalysin activates the NLRP3 inflammasome in mononuclear phagocytes

During infection and upon contact with innate immune cells like macrophages, the fungus is phagocytosed. While a large proportion of *C. albicans* cells can survive phagocytosis under many *in vitro* conditions, it is likely that the majority of phagocytosed *C. albicans* cells are killed by oxidative, nitrosative and non-oxidative mechanisms *in vivo* (Gilbert *et al.* 2014). Furthermore, the induction of pro-inflammatory signalling through activation of the inflammasome is another central pathway to mediate anti-*Candida* activities (Camilli *et al.* 2020). This activation results in cytokine signalling *via* IL-1β and IL-18 and can trigger the programmed, caspase 1-dependent cell death pathway pyroptosis (Broz and Dixit 2016).

*Candida albicans* infections typically activate the NLRP3 inflammasome in myeloid cells like macrophages, neutrophils and DCs (Gross *et al.* 2009, Joly *et al.* 2009, Tucey *et al.* 2016, Niemiec *et al.* 2017, Kasper *et al.* 2018), whereas epithelial cells can further signal *via* the NLRC4 inflammasome upon *Candida* infection (Camilli *et al.* 2020). The NLRP3 inflammasome is a multimeric, cytosolic protein complex comprised of Nlrp3, the adaptor protein ASC, and pro-caspase 1. Its activation requires two steps (Kelley *et al.* 2019). Initially, the inflammasome is primed by NF-κB-mediated production of Nlrp3 and pro-IL-1β, followed by inflammasome assembly. A second, activating step leads to activation of the

inflammasome and subsequently caspase 1, which ultimately cleaves pro-IL-1β and pro-IL-18 into their mature, secreted form (Kelley *et al.* 2019). Filamentation of *C. albicans* is a necessary but not sufficient trigger for inflammasome activation in macrophages, which emphasises the importance of hyphal factors during activation of this process (Joly *et al.* 2009, Wellington *et al.* 2012).

Due to the connection of hypha formation and inflammasome activation, we hypothesised that the fungal peptide toxin candidalysin, which is exclusively highly expressed and secreted during filamentation of the fungus, participates in the process of NLRP3 inflammasome activation in macrophages. Indeed, our study showed for the first time that candidalysin potently triggers the release of mature IL-1β from macrophages in a strictly NLRP3 inflammasome-dependent manner (Kasper *et al.* 2018), which was confirmed by further studies (Rogiers *et al.* 2019, Lowes *et al.* 2020). To elucidate the mechanism of action, we monitored the potential of the toxin to prime and/or activate the NLRP3 inflammasome. Our data evidently show that candidalysin provides the second stimulus to activate the NLRP3 inflammasome in murine and human mononuclear phagocytes (Kasper *et al.* 2018). This is facilitated by toxin-induced potassium efflux from the immune cells, a common trigger also used by bacterial pore-forming toxins to activate the inflammasome (Munoz-Planillo *et al.* 2013, Greaney *et al.* 2015, Kasper *et al.* 2018, Kelley *et al.* 2019). Like candidalysin, some of these toxins (e.g aerolysin, anthrax toxin protective antigen) are activated from a precursor *via* subtilisin-like protease-dependent cleavage (Gordon and Leppla 1994, Gordon *et al.* 1995, Abrami *et al.* 1998, Bader *et al.* 2008, Moyes *et al.* 2016, Richardson *et al.* 2018b). Interestingly, a recent study revealed that also non-potassium-dependent processes contribute to the candidalysin-mediated inflammasome activation, as MCC950, an inhibitor directly interacting with the NLRP3 inflammasome by blocking ATP hydrolysis, inhibited the candidalysin-induced IL-1β release (Lowes *et al.* 2020). Despite the fact that candidalysin is a key trigger of *C. albicans*-induced caspase 1-dependent NLRP3 inflammasome activation and pro-inflammatory IL-1β release, the toxin is not involved in *C. albicans*-triggered caspase 1 and NLRP3-dependent pyroptosis (Uwamahoro *et al.* 2014, Wellington *et al.* 2014, Kasper *et al.* 2018). The latter was proven as caspase 1-inhibition did not reduce the toxin-induced

early damage of macrophages and candidalysin treatment still induced damage in NLRP3 inflammasome knock-out phagocytes (Kasper *et al.* 2018). Nevertheless, a mutant lacking the candidalysin-encoding sequence showed a reduced damaging potential against NLRP3 inflammasome knock-out phagocytes as compared to Wt immune cells. This suggests that NLRP3 inflammasome-associated pyroptosis is induced by live *C. albicans* cells during infection of these immune cells in a toxin-independent manner (Kasper *et al.* 2018). Taken together these data indicate that the processes of inflammasome activation and pyroptosis are not necessarily interconnected but can rather be activated independently (Kasper *et al.* 2018). Importantly, this seems to be a major difference of candidalysin and many bacterial pore-forming toxins (e.g. α-hemolysin or listeriolysin), which usually simultaneously trigger inflammasome-dependent pro-inflammatory signalling and pyroptosis in murine and human monocytic cells (Cervantes *et al.* 2008, Craven *et al.* 2009, Greaney *et al.* 2015). Nevertheless, the human response towards a challenge with membrane-perturbing toxins seems to be conserved in wide ranges (e.g. inflammasome activation *via* $K^+$ efflux, secretion of pro-inflammatory IL-1β) despite different infection strategies employed (Greaney *et al.* 2015, Kasper *et al.* 2018). The cytotoxic effects of candidalysin against human mononuclear phagocytes rather resemble necrotic cell death, as also other programmed cell death pathways like apoptosis and necroptosis were not triggered by the toxin (Kasper *et al.* 2018). This is putatively mediated by insertion of the toxin into the host cell membrane, subsequent membrane destabilisation and lesion formation (unpublished data from Thomas Gutsmann, Research Center Borstel). However, the exact mechanism of this insertion remains unresolved until now and requires further structural characterisation, which could be accomplished by staining with candidalysin-specific antibodies and high-resolution microscopy or nuclear magnetic resonance studies.

Not only hyphae and hypha-associated factors like candidalysin, but also *C. albicans* yeast cells can induce IL-1β release upon macrophage infection and pyroptotic host cell death (Wellington *et al.* 2012, O'Meara *et al.* 2018). These studies demonstrate that also cell surface architecture, ergosterol biosynthesis, and phosphatidylinositol-4-kinase signalling pathways are involved in inflammasome activation and induction of pyroptosis (Uwamahoro *et al.*

2014, Wellington *et al.* 2014, O'Meara *et al.* 2015, O'Meara *et al.* 2016, O'Meara *et al.* 2018). Furthermore, Saps and phagosomal neutralisation have been implicated with inflammasome induction (Pietrella *et al.* 2013, Vylkova and Lorenz 2017, Westman *et al.* 2018). Importantly, first results indicate that NCEPs are not involved in the process of inflammasome activation and IL-1β secretion, suggesting that candidalysin is the only Ece1 peptide mediating this response (paragraph 4.8, (Kasper *et al.* 2018, Rogiers *et al.* 2019)).

In summary, candidalysin is an important trigger of pro-inflammatory signalling and host cell death of macrophages, however, the toxin is not exclusively responsible for these processes and many aspects of the candidalysin-macrophage interaction still require further investigation. For example, it is highly likely that the toxin is secreted by growing *C. albicans* hyphae within the phagosome (Moyes *et al.* 2016). However, damage of the phagosomal membrane induced by *C. albicans* filaments is not dependent on candidalysin (Westman *et al.* 2018). Thus, either this membrane is not the major target of the toxin, or hyphal elongation is simply the overwhelming damaging activity in this compartment. Nevertheless, candidalysin seems to contribute at least to phagosomal leakage resulting in luminal alkalinisation (Westman *et al.* 2018). Studying the subcellular localisation of candidalysin within phagocytes, for example by cell fractionation upon co-incubation or infection and subsequent LC-MS/MS analysis, high-resolution microscopy using specific antibodies, or a co-precipitation of putative binding partners (e.g. membrane proteins, receptors, or NCEPs) using candidalysin-specific antibodies, would shed light on toxin-mediated processes during the *C. albicans*-macrophages interaction.

## 5.3 The dual function of candidalysin

The effects of candidalysin on fungal virulence and host cell response have been extensively studied in the last years, using different *in vitro* and *in vivo* infection models. All studies collectively show that the toxin has a very broad spectrum of action ranging from fungus-beneficial host cell damage or immunopathology to the initiation of host-protective pro-inflammatory signalling.

During oral candidiasis, candidalysin secretion is critical for the induction of epithelial damage (Moyes *et al.* 2016). However, by causing damage, the toxin activates the epithelial danger response pathway during mucosal candidiasis, resulting in the production of pro-inflammatory mediators, which contribute to the recruitment of immune cells (Naglik *et al.* 2017, Ho *et al.* 2019, Hanaoka and Domae 2020, Ho *et al.* 2020b). By eliciting this pro-inflammatory response, it further activates an innate type 17 (Th17) response in an oral candidiasis model, which in turn potently recruits neutrophils, essential players during mucosal *Candida* infections that mediate fungal clearance in the oral mucosa (Romani *et al.* 1996, Huppler *et al.* 2014, Verma *et al.* 2017). In contrast, during vaginal candidiasis, candidalysin induces a massive infiltration of neutrophils resulting in the characteristic immunopathology in this tissue (Yano *et al.* 2012, Richardson *et al.* 2018c). Thus, the recruitment of neutrophils to combat fungal overgrowth can be beneficial or detrimental depending on the site of infection (Verma *et al.* 2017, Richardson *et al.* 2018c).

During infections of the gastrointestinal tract, the influence of candidalysin is less clear. *In vitro* studies have shown that fungus-induced epithelial damage mediates a transcellular translocation across the intestinal barrier in a candidalysin-dependent manner (Allert *et al.* 2018). Nevertheless, it still remains unclear whether this contributes to fungal dissemination *in vivo*.

Additional *in vitro* studies of the interaction of *C. albicans* with macrophages and DCs demonstrate that the toxin damages immune cell membranes, thereby contributing to fungal escape from the hostile environment (Kasper *et al.* 2018). However, it also initiates host-protective pro-inflammatory signalling, probably contributing to fungal clearance (Kasper *et al.* 2018, Rogiers *et al.* 2019).

During systemic infection, *C. albicans* is able to disseminate into virtually every organ of the body, including the central nervous system (CNS) and the kidneys. In CARD9$^{+}$ microglia cells, the most common CNS-resident innate immune cells (Nayak *et al.* 2014), candidalysin activates the IL-1β-CXCL1 pathway, which in turn recruits neutrophils to the site of infection, thus mediating fungal clearance (Drummond *et al.* 2019). In the kidneys, candidalysin similarly provokes neutrophil recruitment and a pro-inflammatory response, which

contributes to controlling the infection at early stages (Kasper *et al.* 2018, Swidergall *et al.* 2019). However, at later stages of disease progression, prolonged candidalysin-dependent neutrophil accumulation rather induces immunopathology in the kidney tissue (Swidergall *et al.* 2019). This is probably the reason for increased mouse mortality, highlighting the diverse contribution of the toxin to fungal virulence during systemic candidiasis (Swidergall *et al.* 2019).

Apart from the above-described functions, candidalysin has been associated with carcinogenesis, IL-17-mediated inflammatory disorders, and fatal outcomes of alcohol-associated hepatitis (Chu *et al.* 2020, Engku Nasrullah Satiman *et al.* 2020, Ho *et al.* 2020a), emphasising the pleiotropic effects of the toxin depending on the circumstances of infections.

Importantly, as candidalysin is a hypha-specific protein, it will not be present in all *C. albicans*-infected tissues, as filaments are not the dominating phenotype in all niches. For example, the hyphal morphology is characteristic for brain and kidney infections, whereas hyphae seem to be absent in the liver and the spleen in *in vivo* mouse models (Lionakis *et al.* 2011). Since candidalysin exhibits these dual tissue- and infection setting-dependent effects, it is obviously not a prime drug target during all types of infections. Accordingly, filamentation inhibitors, which can dampen candidalysin secretion (Romo *et al.* 2019), can in turn be beneficial or detrimental for the infection outcome depending on the toxin-mediated effects in the respective infected tissue.

Concluding, it is obvious that the peptide toxin candidalysin exhibits a dual function (König *et al.* 2020a, König *et al.* 2020b), which is strongly dependent on the infected host niche and the circumstances of infection. On the one hand, it can act as a classical virulence factor damaging membranes or mediating translocation of the fungus, thus being beneficial for the infection process (Moyes *et al.* 2016, Allert *et al.* 2018, Kasper *et al.* 2018, Richardson *et al.* 2018c, König *et al.* 2020a). On the other hand, it as well shows features of an avirulence factor, a factor limiting fungal virulence (Síscar-Lewin *et al.* 2019), by provoking host-protective pro-inflammatory responses or accelerating fungal clearance by neutrophil recruitment (Kasper *et al.* 2018, Drummond *et al.* 2019, Rogiers *et al.* 2019, König *et al.* 2020a).

## 5.4 Modifications in the *ECE1* gene influence filamentation, *ECE1* expression, Ece1 secretion, and the fungal damaging potential

While the role of candidalysin has been extensively studied in the last years, the role of the seven other Ece1 peptides (NCEPs) remained unknown. Therefore, *C. albicans* strains lacking the sequence encoding single peptides or different peptide combinations were generated (Table 2) and analysed regarding their influence on the *C. albicans* filamentation potential, *ECE1* gene expression, Ece1 peptide secretion, and the damaging potential against hMDMs.

### 5.4.1 The impact of *ECE1* modifications on filamentation and induction of the unfolded protein response

In contrast to a complete deletion of *ECE1*, which was not associated with filamentation defects, a lack of certain NCEP-encoding regions was accompanied by defects in hypha formation. Interestingly, the deletion of the Ece1-V-encoding sequence severely changed the morphology of *C. albicans* towards a reduced hyphal length and deficiencies in filamentation induction (Figure 11A, Figure 12A). Furthermore, also a lack of the Ece1-II or -VI-encoding sequence resulted in impaired hyphal elongation without affecting filamentation induction (Figure 11A, Figure 12A). All other mutants produced Wt-like hyphae (Figure 11, Figure 12). Concluding, deletions of the sequences encoding Ece1-II, Ece1-V, and Ece1-VI negatively impact on fungal filamentation, whereas a deletion of the sequence encoding the other NCEPs does not influence the filamentation ability of *C. albicans*.

Importantly, the mutant growth was only affected under hypha-inducing conditions, which are associated with *ECE1* expression, but not in the yeast growth phase, during which the gene is not expected to be expressed (Figure 10-Figure 12). Thus, the observed deficiencies can be accounted to changes in the *ECE1* gene, or to other factors only needed during hyphal morphogenesis.

Since all mutants with filamentation defects recovered with time and produced hyphae to a similar extent as the Wt, it can be concluded that hypha formation in these mutants is solely delayed. Additional investigation by performing a live cell time series of hypha induction would shed further light on the exact timing of hypha induction and the elongation rate.

It is evident that genetic manipulations of the NCEP-encoding regions within the *ECE1* gene have more or less dramatic impact on hypha formation and filamentation induction. During hypha formation, *ECE1* gene expression is massively up-regulated in the *C. albicans* Wt accompanied by a high Ece1 peptide secretion. Genetic modifications of Kex2 KR processing sites, leading to partially unprocessed Ece1 proteins, did not cause any obvious morphological changes (Moyes *et al.* 2016, Richardson *et al.* 2018b). However, the removal of entire NCEP-encoding regions resulted in a delayed filamentation (Figure 11). It was thus hypothesised that these alterations lead to an improper folding and processing of Ece1, putatively resulting in misfolded proteins, imbalances in the protein-folding homeostasis within the ER, and activation of the UPR (Gardner *et al.* 2013). The UPR is an ER stress response, which restores protein homeostasis by up-regulation of genes involved in protein folding and misfolded protein decay alongside with a repression of translation and selective mRNA degradation (Gardner *et al.* 2013).

To analyse the induction of UPR in the generated NCEP-knock-out mutants, splicing of *HAC1* mRNA and *KAR2* gene expression were monitored. The *KAR2* gene encodes a molecular chaperone, which usually binds Ire1, the master sensor of ER stress and prevents its dimerisation. Upon accumulation of misfolded proteins, Kar2 dissociates from Ire1 due to binding of these proteins, thus allowing Ire1 dimerisation. Subsequent Ire1-dependent *HAC1* mRNA splicing and UPR induction was recently demonstrated for *C. albicans* (Sircaik *et al.* 2021). The spliced *HAC1* mRNA acts as a transcriptional activator for UPR-responsive genes involved in restoring ER maintenance and folding capacity like *KAR2* and induces its expression in a regulatory circuit. Thus, UPR-dependent stimulation of Kar2 production restores the ER homeostasis when the stress level is alleviated (Okamura *et al.* 2000, Pincus *et al.* 2010). In case of a UPR induction in the NCEP-knock-out mutants, an engagement of present Kar2 by misfolded Ece1 protein would putatively induce splicing of *HAC1* mRNA, which would in turn presumably lead to an up-regulation of *KAR2* expression.

As expected, neither the Wt nor any of the mutant strains exhibited splicing of *HAC1* mRNA during yeast growth, indicating the absence of ER stress in this non-*ECE1*-expressing morphological state (Figure 15). Furthermore, hypha formation of the Wt did not result in

splicing of *HAC1* mRNA (Figure 15), indicating that folding and processing of Wt-like Ece1 does not induce any ER stress. In line with that, the UPR-responsive gene *KAR2* was down-regulated upon hypha induction in the Wt (Figure 16), further confirming the absence of ER stress. Similarly, the complete *ECE1* knock-out strain (*ece1*Δ/Δ) did not exhibit any *HAC1* mRNA splicing or *KAR2* up-regulation (Figure 15, Figure 16), showing that a complete loss of the Ece1 protein does not result in ER stress upon hypha formation. In concordance with these data, the *ece1*Δ/Δ mutant strain was fully capable of forming proper hyphae (Figure 11). As expected, a treatment of the Wt with tunicamycin, an ER stress inducer which prevents protein N-glycosylation and thus proper ER import and folding (Guillemette *et al.* 2011), induced splicing of *HAC1* mRNA in the yeast as well as in the hyphal samples (Figure 15). Moreover, the expression of *KAR2* was increased under tunicamycin treatment of the Wt, confirming that this gene responds to ER stress (Figure 16). Hypha induction in the mutants Δ*P5*, Δ*P6*, Δ*P8*, Δ*P4+5*, Δ*P6-8*, and TripleP3 resulted in *HAC1* mRNA splicing (Figure 15) and increased *KAR2* gene expression (Figure 16), most prominently in the TripleP3 mutant. Importantly, all these mutants exhibited an impaired hyphal length (Figure 11), suggesting a connection of ER stress, the induction of UPR, and defective hypha formation. Indeed, a treatment of Wt *C. albicans* with tunicamycin also resulted in abnormal hypha formation, comparable to the phenotype of the generated NCEP-knock-out mutants (unpublished data from Deniz Yildirim, HKI Jena, MPM department). In the mutants Δ*P2*, Δ*P3*, Δ*P4*, and Δ*P7*, no or only minor *HAC1* mRNA splicing was observed (Figure 15). Additionally, these mutants did not up-regulate *KAR2* under hypha-inducing conditions (Figure 16, except for Δ*P4*) and filamented Wt-like (Figure 11, except for Δ*P2*), which, together with the splicing data, indicates the absence of ER stress. The different impacts of NCEP-encoding sequence deletions on the induction of ER stress might putatively be also due to a more or less strong influence of the respective sequence deletion on subsequent protein folding and/or accessibility of processing sites. In general, it has to be mentioned that the up-regulation of *KAR2* was not very strong and ranged mainly between a $\log_2$FC of 0 and 1 (Figure 16). It is conceivable, that Kar2 is stronger regulated on the protein than on the expression level, which could be investigated by quantifying protein abundance *via* Western blotting.

Taken together, all data regarding ER stress and UPR induction in the generated mutants indicate that certain changes in the *ECE1* sequence lead to defective Ece1 folding, processing, and delivery *via* the secretory pathway, thus inducing ER stress. This seems to be connected with defects in hypha formation. As the ER is an important source of cell wall components and ergosterol in fungi (Parks and Casey 1995), it is conceivable that ER stress accounts for deficiencies in hypha formation, as components needed during this process cannot be delivered to the hyphal tip in the required amounts. Defects in the *C. albicans* ER-dependent maintenance of protein homeostasis have recently been implicated with defects in hyphal morphogenesis (Sircaik *et al.* 2021). However, to clearly elucidate whether impaired hypha formation is a direct consequence of UPR induction in NCEP-knock-out mutants, it would be interesting to test the gene expression and UPR induction at earlier time points such as 20 or 30 min. At these time points, the hypha-associated gene *ECE1* is known to be already highly expressed (Moyes *et al.* 2016), but probably no or only low amounts of modified Ece1 proteins are present. Furthermore, the induction of the UPR should already be detectable at this time point (Beriault and Werstuck 2013). An absence of UPR induction in these samples would hint towards the UPR being the consequence of problems in hypha formation, whereas a similar pattern as for the 3 h time point would rather suggest that changes in the *ECE1* gene directly lead to ER stress, which than accounts for the reduction or delay of hyphal growth.

### 5.4.2 The impact of *ECE1* modifications on *ECE1* gene expression and Ece1 secretion

In contrast to the Wt, in which *ECE1* gene expression is highly up-regulated under hypha-inducing conditions, mutants lacking the Ece1-V-encoding sequence exhibited a significantly reduced *ECE1* expression (Figure 13). Apart from that, the NCEP-knock-out mutants Δ*P4*, Δ*6*, Δ*P7*, Δ*8*, and Δ*6-8* did not reach 75 % of Wt *ECE1* expression levels (Figure 13), indicating that genetic manipulation within the *ECE1* gene often results in *ECE1* gene expression deficiencies. When combining the *ECE1* expression data and the hyphal length of mutants, it is evident that a Wt-like hyphal length does not necessarily transfer to a Wt-like *ECE1* expression (Δ*P7*) and *vice versa* (Δ*P2*) (cf. Figure 11, Figure 13, Figure 17). However, in most cases, a reduced *ECE1* expression is correlated with impaired hypha

formation and the induction of the UPR. This suggests a feedback mechanism between Ece1 folding, processing, and *ECE1* expression. Imbalances in protein homeostasis and the secretory pathway in turn putatively cause defects in hypha formation and thus, in a circuit, less expression of the hypha-associated gene *ECE1*. Moreover, a cellular sensing of misfolded Ece1 protein and the subsequent UPR induction might result in less production of the "defective" protein in a negative feedback loop to prevent further ER stress. Besides, UPR-independent mechanisms seem to exist which lead to a reduced *ECE1* expression in NCEP mutants with normal filamentation. To gain more insight into possible effects of NCEP deletions within the *ECE1* gene on general gene expression, testing the expression of other hypha-associated genes like *ALS3* or *HWP1* in the NCEP-knock-out strains would be interesting.

While it is proposed that NCEP-knock-out mutants might show reduced levels of hypha-associated genes due to deficiencies in hyphal morphogenesis, *ECE1* mutants lacking the entire gene (*ece1*Δ/Δ) or the candidalysin-encoding sequence only (Δ*P3*) show normal hypha formation (Moyes *et al.* 2016, Kasper *et al.* 2018) and expression of hypha-associated genes like *ALS3* or *HWP1* (unpublished data from Stefanie Allert and Selene Mogavero, HKI Jena, MPM department). These data also suggest that filamentation *per se* and the associated transcriptional pattern is independent of the presence of candidalysin. This further supports the view that the diminished damaging potential of mutants lacking candidalysin is not due to a diminished expression of other virulence-associated genes, but really a consequence of candidalysin absence.

Upon expression of *ECE1*, the gene is transcribed, the preproprotein is guided to the ER by the N-terminal signal peptide and processed in ER and Golgi into the mature peptides by sequential Kex2 and Kex1 digests. These peptides enter the secretory pathway and are released into hyphal supernatants (Moyes *et al.* 2016, Richardson *et al.* 2018b). LC-MS/MS analysis revealed that most of the predicted peptides are present in hyphal supernatants of the *C. albicans* Wt, whilst others were completely absent (Table 1, Table 6) (Moyes *et al.* 2016, Richardson *et al.* 2018b). Furthermore, some only occurred as fragments of predicted peptides (Ece1-V, Ece1-VI, Ece1-VII, and Ece1-VIII), due to cleavage at internal Kex2 protease

recognition sites (Moyes *et al.* 2016, Richardson *et al.* 2018b). No peptides were detectable in a mutant lacking fungal Kex2, suggesting that other fungal proteases are not, or only to a very minor extent, involved in the cleavage process (Richardson *et al.* 2018b). As these peptides and fragments occurred in *Candida*-only samples, the cleavage does further not require host-derived proteases (Richardson *et al.* 2018b). Moreover, the Kex2 cleavage sites separating the peptides from one another are highly conserved amongst clinical *C. albicans* isolates (unpublished observations from Christophe d'Enfert, Institute Pasteur, Paris (FR)), highlighting the putative importance of NCEPs during the infection process.

Interestingly, LC-MS/MS analysis showed that distinct peptides or peptide fragments were not detected in equal amounts as would be expected from the protein structure (Table 1, Table 6). This might be due to different peptide properties like charge or folding that interfere with experimental detection. Moreover, intracellular functions or localisation of certain peptides might retain the respective peptide in the fungal ER, Golgi apparatus, cell membrane, or cell wall, thus preventing the detection in the culture supernatant. LC-MS/MS measurements of equimolar mixtures of synthetic peptides revealed the possibility of detecting all individual peptides. However, the PSMs detected did not reflect the equimolar ratio of the mixture, pointing towards a generally different retrieval of the peptides by this method (unpublished data from Rita Müller, HKI Jena, MPM department). Concluding, the complete absence of peptides in the supernatant must be due to retention of non-detected peptides within the fungus, whereas reasonable differences in the retrieval can be accounted to technical reasons.

As expected, the Δ*P3* mutant did not secrete candidalysin into the hyphal supernatant, whereas most other NCEPs were secreted in a rather Wt-like pattern in this mutant (Table 6). This suggests that a complete loss of the toxin does not significantly influence Ece1 processing and secretion of NCEPs. However, the loss of candidalysin occurred simultaneously with a decreased release of Ece1-VIIa into the supernatant, whereas the release of the Ece1-VIIb fragment was not impaired (Table 6).

In concordance with previously discussed data, all strains lacking the Ece1-V-encoding sequence exhibited a strongly reduced secretion of candidalysin (Table 6), which can likely be

accounted to shorter hyphae due to UPR induction (Figure 11, Figure 15, Figure 16), a significantly reduced *ECE1* expression (Figure 13) and ultimately less Ece1 protein.

When analysing the secretion pattern of other strains tested, it seems that the release of candidalysin is connected to the co-release of Ece1-VIIa, as already described above in case of the Δ*P3* mutant, in which an absence of candidalysin co-occurred with less Ece1-VIIa secretion. Furthermore, whenever Ece1-VIIa secretion was absent or compromised, only low levels of candidalysin were detected in the supernatants (Table 6), suggesting a possible role of Ece1-VIIa in the release of the toxin, for example as a membrane shuttle.
However, the level of the usually highly abundant Ece1-Va was often simultaneously reduced, indicating that a reduced secretion can at least in parts be additionally explained by a general slightly reduced *ECE1* expression (Figure 13) and thus a lower protein level.

Similarly, Ece1-II and -IV seem to be of importance for candidalysin release. Ece1-II is seldom found in the supernatant, if detected, it rather occurs as a dipeptide of Ece1-II and candidalysin. This suggests that the cleavage between Ece1-II and candidalysin occurs as the last of all cleavage steps. Presumably, Ece1-II acts as a guiding protein towards the shuttling peptide Ece1-VIIa and is terminally cleaved from the toxin. According to the fact that Ece1-II is seldom found in the hyphal supernatant, it is probably retained within the trans-Golgi network, fungal cell membrane, or cell wall upon cleavage from candidalysin. This is in concordance with the secretion pattern of the Δ*P2* mutant, in which nearly normal amounts of Ece1-VIIa were present, but only low amounts of mature candidalysin and Ece1-Va were secreted (Table 6), putatively because the release of the toxin is deficient due to missing Ece1-II. Furthermore, unpublished data from our collaboration partner Brian Peters (University of Tennessee (USA)) indicate that changes in the genetic sequence of Ece1-II influence candidalysin secretion, further highlighting the importance of the Ece1-II-encoding sequence for toxin production.
Moreover, also the deletion of Ece1-IV resulted in less secretion of candidalysin and Ece1-Va, whereas Ece1-VIIa was present rather normal amounts (Table 6). As Ece1-IV is usually not found in the supernatant of hyphal cultures, it is presumably intracellularly involved in the Ece1 secretion process. The collected data are in line with previously published results by

Richardson *et al.*, who reported that the candidalysin-adjacent Kex2 protease cleavage sites are of critical importance for candidalysin processing and secretion (Richardson *et al.* 2018b). However, a reduced candidalysin secretion might in parts be explained by shorter hyphae (Δ*P2*, Figure 11) or a reduced *ECE1* expression and thus a reduced Ece1 protein level (Δ*P4*, Figure 13).

Concluding, the peptides Ece1-II, -IV and -VIIa may be important for candidalysin secretion, presumably as a guiding peptide (Ece1-II), during intracellular processes (Ece1-IV), or as a membrane shuttle (Ece1-VIIa). Regarding further investigations, staining with peptide-specific antibodies and high-resolution microscopy could reveal a peptide-peptide or peptide-membrane co-localisation, which would shed further light on the function of NCEPs.

### 5.4.3 The impact of *ECE1* modifications on the *Candida albicans* damaging potential against human monocyte-derived macrophages

As several NCEPs have been shown to be secreted alongside with candidalysin, it is conceivable that NCEPs exhibit an antitoxin-like function or modulate candidalysin function, e.g. by counteracting or facilitating the toxin membrane insertion. As the secretion of the peptide toxin candidalysin has been shown to be a crucial damaging factor of *C. albicans* against different host cells including mononuclear phagocytes (Moyes *et al.* 2016, Wilson *et al.* 2016, Allert *et al.* 2018, Kasper *et al.* 2018), the damaging potential of the generated NCEP-knock-out strains, synthetic NCEP peptides and fragments thereof against hMDMs was monitored.

The damage caused by the strain lacking candidalysin (Δ*P3*) was significantly reduced but not absent in hMDMs (Figure 11, Figure 14, (Kasper *et al.* 2018)). In contrast, the damaging potential against epithelial cells is completely contingent on candidalysin (Moyes *et al.* 2016) (Allert *et al.* 2018). Thus, additional pathways like glucose consumption by growing hyphae and hypha formation *per se* play a role for the damaging potential against mononuclear phagocytes, especially at later time points of infection (McKenzie *et al.* 2010, Tucey *et al.* 2018).

Apart from Δ*P3*, the strains Δ*P2*, Δ*P4*, Δ*P5*, Δ*P4+5*, and TripleP3 exhibited a significantly reduced damaging potential against hMDMs. Notably, all strains lacking the Ece1-V-encoding sequence were affected. In contrast to the candidalysin knock-out strain, these strains also exhibited reduced hyphal length (Figure 11) and partially reduced filamentation induction (Figure 12), which likely explains the reduced damage. Moreover, these strains secreted only very low amounts of candidalysin into the supernatant, exhibited defects in Ece1-VIIa release (Table 6), and showed an activation of the UPR upon hypha induction (Figure 15, Figure 16), suggesting an involvement of Ece1-V-encoding sequence in correct Ece1 protein folding and processing, thereby preventing the induction of ER stress.

In terms of Δ*P2*, the reduced damaging potential is putatively due to a combination of reduced hyphal length (Figure 11) and a reduction of candidalysin secretion due the missing guiding peptide (Table 6). The mutant Δ*P4* formed Wt-like hyphae (Figure 11), but also exhibited a significantly reduced damaging potential against hMDMs (Figure 14). As mentioned, this mutant exhibited a decreased *ECE1* expression (Figure 13) and secreted less candidalysin into the supernatant of hyphal samples as compared to the Wt (Table 6), which might account for the reduced damaging potential.

Interestingly, the mutant lacking the Ece1-VII-encoding sequence formed Wt-like hyphae (Figure 11), did not exhibit a reduction in damaging potential (Figure 14), but secreted only low amounts of candidalysin (Table 6). This mutant therefore shows that candidalysin is not the only damaging factor of *C. albicans* during macrophage infections and that a lack can somehow be compensated, e.g. by Wt-like hyphae. The other way around, the Δ*P6* mutant shows that also a reduced hyphal length (Figure 11) does not necessarily result in a diminished damaging potential (Figure 14), as long as the toxin and Ece1-VIIa are released in reasonable amounts (Table 6).

In terms of synthetic NCEPs, except for Ece1-VII or fragments thereof, no peptide or peptide fragment induced macrophage damage or affected the candidalysin-induced damage of hMDMs (Figure 18, Figure 19). However, when hMDMs were challenged with candidalysin in combination with full length Ece1-VII or Ece1-VIIa and b, the toxin-induced damage was significantly diminished (Figure 18, Figure 19). This suggests that Ece1-VII can modulate the

candidalysin function when both peptide fragments are present. Putatively, a binding, which was hypothesised to be required for shuttling of candidalysin to the supernatant (Ece1-VIIa, paragraph 5.4.2,) diminishes the potential of the toxin to insert into host cell membranes and thus to cause lysis of hMDMs. As the reduction of damage potential was only seen when both Ece1-VII fragments were present, it is possible, that Ece1-VIIb is needed to mediate binding of Ece1-VIIa and candidalysin within the fungus. However, it is rather not involved in the actual release process, as also mutants with normal candidalysin secretion exhibited less Ece1-VIIb in the supernatant (Δ*P8*, Table 6), and mutants with abolished toxin secretion still secreted Ece1-VIIb (Δ*P3*, Table 6).

Concluding, defects in the damaging potential against hMDMs were most prominent when N-terminal parts of *ECE1* (*P2* to *P5*) were absent. However, this correlated with hyphal deficiencies probably due to UPR induction and/or defects in candidalysin secretion. Furthermore, no evidence for a candidalysin-independent function of NCEPs regarding the damaging potential against macrophages was found, as none of the strains exhibited a normal toxin secretion concurrent with reduced host cell damage and none of the synthetic full length peptides or peptide fragments exhibited a damaging potential against hMDMs. Solely Ece1-VII influenced the candidalysin-induced damage potential against macrophages, putatively by preventing or hindering membrane insertion due to binding, which could explain the reduced damage of hMDMs when these immune cells were challenged with the toxin in combination with full length Ece1-VII or Ece1-VIIa and b.

Analyses like immunofluorescence using specific antibodies against the NCEPs and fragments thereof would provide further insight in the intra- and extracellular localisation or co-localisation of several Ece1 peptides, allowing further conclusion on their respective function.

## 5.5 The effect of candidalysin and Non-Candidalysin Ece1 Peptide fragments on the macrophage response

As several other peptides (Ece1-II, Ece1-IV) or peptide fragments (Ece1-Va/b, Ece1-VIa/b, Ece1-VIIa/b, and Ece1-VIIIa-c) are secreted alongside with the toxin into the supernatant of hyphal cultures (Table 1, Table 6) (Moyes *et al.* 2016, Richardson *et al.* 2018b), it was

hypothesised that NCEPs might act as effector peptides during the *C. albicans*-host cell interaction. Such effector functions have already been described for many small peptides secreted by different plant pathogenic fungi (Lo Presti *et al.* 2015, König *et al.* 2021). Accordingly, NCEPs might modulate the host cell metabolism by receptor binding on the host cell surface or upon translocation into the host cell cytoplasm, putatively mediated by candidalysin-induced pore formation or membrane destabilisation. Apart from an involvement during the infection process of host cells, NCEPs might also act as commensal factors, e.g. by counteracting the candidalysin-induced effects or by mediating nutrient acquisition for the fungus. To evaluate the potential of the most abundant NCEP fragments Ece1-Va, Ece1-VIa and Ece1-VIIa (Table 1, Table 6) to act as effector peptides during the interaction with human macrophages, macrophage transcriptional profiling and secretion of immune mediators was monitored in comparison to or in combination with candidalysin treatment.

Transcriptional profiling of macrophages co-incubated with synthetic candidalysin and/or Ece1-Va, Ece1-VIa and Ece1-VIIa revealed that a treatment with the toxin resulted in a higher number of DEGs than treatment with the peptide fragments alone (Table 7), suggesting more substantial changes in the host's transcriptome upon contact with candidalysin. Furthermore, toxin treatment resulted in a significant up-regulation of genes involved in the inflammatory and cytokine response (Figure 21). When challenged with candidalysin, macrophages mainly up-regulated a heat shock and stress response as well as pro-inflammatory cyto- and chemokine genes (e.g. *IL1A*, *IL1B*, *IL8, IL36B*). In line with that, candidalysin treatment of the macrophages induced the secretion of pro-inflammatory cytokines (IL-1α, IL-1β, and IL-18) and the chemokine IL-8, especially after 24 h of co-incubation (Figure 28, Figure 30). This is in concordance with previously published work for macrophages (candidalysin-dependent IL-1β secretion, (Kasper *et al.* 2018)) and the additional data presented in this thesis (candidalysin-dependent release of IL-1α, IL-1β, and IL-8, paragraph 4.8.3). At early time points (6 h), a down-regulation of genes involved in the process of IL-1β secretion was observed (Figure 21), putatively representing a negative feedback loop to the strong up-regulation of *IL1B* gene expression itself (Figure 24) and IL-1β secretion (Figure 28) to prevent an overshooting pro-inflammatory response.

Similar to the data obtained from macrophages, the gene expression of *IL36*, *IL1A*, *IL1B*, and *IL8* as well as secretion of IL-1α and IL-18 was likewise observed in oral and vaginal epithelial cells upon candidalysin challenge (Moyes *et al.* 2010, Richardson *et al.* 2018c, Verma *et al.* 2018, Hanaoka and Domae 2020, Ho *et al.* 2020b, Pekmezovic *et al.* 2021), underlining the previously discussed dual function of the toxin depending on the site of infection (paragraph 5.3).

In contrast to the above-mentioned cyto- and chemokines, the release of pro-inflammatory IL-6 and TNF-α was rather induced by *C. albicans* infection *per se* and only partially dependent on candidalysin, confirming previous data (Kasper *et al.* 2018). Still, a candidalysin-dependent up-regulation of gene expression was observed for both cytokines (Figure 24). Simultaneous to the secretion of pro-inflammatory mediators, candidalysin treatment triggered transcriptional up-regulation and the release of the anti-inflammatory cytokines IL-10 and IL-1RA (Figure 24, Figure 29), both involved in dampening the pro-inflammatory signalling as a negative feedback loop (Zhang and An 2007). Apart from that, the toxin induced the secretion of chemokines involved in mediating immune cell migration (Figure 30, Figure 31) (Palomino and Marti 2015).

Furthermore, genes involved in the activation of type-I interferons (IFN) were down-regulated upon challenge of hMDMs with candidalysin (Figure 21, Figure 24). This is in concordance with observations made in *Candida*-infected vaginal cells, which suggest that during early, non-damaging, or commensal stages of infection type-I IFN signalling is up-regulated, whereas upon damage of the host cells, type-I IFN signalling is down-regulated and pro-inflammatory signalling gets activated (Pekmezovic *et al.* 2021). Regarding the hypothesis of NCEPs playing a role during commensalism, at least in terms of type-I IFN signalling, none of the type-I IFN genes up-regulated during early, non-damaging stages of vaginal infections with *C. albicans* (Pekmezovic *et al.* 2021) was similarly regulated upon macrophage co-incubation with the NCEP fragments tested (Additional Data CD). Furthermore, neither was the candidalysin-induced pro-inflammatory cytokine response dampened by a co-incubation with Ece1-Va, Ece1-VIa, or Ece1-VIIa (Figure 24, Figure 28) nor did the tested NCEP fragments induce an anti-inflammatory cytokine response (Figure 24, Figure 29). This suggests that these fragments do probably not act as

commensalism-inducing effectors or counteract the candidalysin-mediated pro-inflammatory response induced in macrophages.

When macrophages were co-incubated with candidalysin and Ece1-Va in combination, the transcriptional response observed was mainly the same as for candidalysin treatment only (Figure 21, Figure 22, Figure 24). Furthermore, the number of DEGs was similar for candidalysin-only and candidalysin + Ece1-Va treatment (Table 7), suggesting that the response of macrophages to the toxin is not dramatically modified by Ece1-Va. This is further supported by the fact that the overlapping transcriptional response of hMDMs towards candidalysin and candidalysin + Ece1-Va treatment was higher (6 h: 96, 24 h: 360 DEGs, Figure 20) as compared to Ece1-Va and candidalysin + Ece1-Va treatment (6 h: 7, 24 h: 28 DEGs, Figure 20).

Importantly, challenging hMDMs with candidalysin alone induced the expression of certain MMP genes (Figure 24). These zinc-dependent enzymes (Nagase and Woessner 1999) have already been reported to be required for the candidalysin-mediated activation of EGFR-related signalling (Ho *et al.* 2019). Apart from being involved in the cleavage of EGFR pro-ligands (Ho *et al.* 2019), MMPs can also potently cleave and thereby post-translationally modify the function of different cytokines (Manicone and McGuire 2008, Young *et al.* 2019). The induction of MMP genes, especially of the macrophage-specific MMP-12, was slightly enhanced during co-incubation of macrophages with candidalysin and Ece1-Va after 24 h as compared to candidalysin treatment alone (Figure 24). An additional effect of Ece1-Va on MMP-12 secretion was already detectable after 6 h when measuring the MMP-12 protein level (Figure 32), whereas at this time point no gene up-regulation occurred (Figure 24). It is known that gene expression is correlated with protein abundance, but only to an extent of around 40 %, as also post-transcriptional and post-translational modifications as well as protein degradation influence the resulting protein levels (Vogel and Marcotte 2012). Thus, the results obtained from the transcriptional profiling can only partially be transferred to the data collected in terms of immune mediator secretion by macrophages and *vice versa*. However, regarding the additive effect of Ece1-Va on the candidalysin-induced up-regulation of *MMP12* expression, this is putatively due to a higher Ece1-Va-dependent zinc availability,

as a treatment with this peptide fragment resulted in an up-regulation of a metal stress response to divalent cations such as zinc, copper, or cadmium (Figure 23, Figure 26, see below). Cleavage of certain cyto- and chemokines by MMPs can result in activation or inactivation depending on the respective cytokine and is further associated with the production of a mediator gradient in proximity to the macrophage resulting in modulation of the immune response (Manicone and McGuire 2008). The co-incubation of macrophages with candidalysin and Ece1-Va further resulted in an additive effect of Ece1-Va on the candidalysin-induced release of certain mediators (Figure 33), however, this was not detectable on the transcriptional level (Figure 24). This additive effect was additionally observed for a co-incubation of macrophages with candidalysin and Ece1-VIa for certain immune mediators (Figure 33). Concluding, candidalysin treatment induces complex transcriptional changes in the macrophages inflammatory response and induces the secretion of many immune mediators, putatively also through assistance by Ece1-Va and Ece1-VIa.

The transcriptional response of macrophages to co-incubation with the synthetic NCEP fragments Ece1-Va, Ece1-VIa and Ece1-VIIa alone was less pronounced than the response to candidalysin (Table 7, Figure 20) and did mainly not result in activation of many specific pathways (Figure 23). However, the analysis of immune mediator secretion from macrophages revealed the release of certain cyto- and chemokines upon treatment with Ece1-VIIa alone, as discussed below.

Most interestingly, a 6 h co-incubation with Ece1-Va resulted in a significant up-regulation of metallothionein genes involved in metal stress (*MT1E*, *MT1F*, *MT1G*, *MT1H*, Figure 23, Figure 26), especially to divalent cations as described above. Furthermore, Ece1-Va treatment alone resulted in up-regulation of the macrophage-specific *MMP12* gene and a slight secretion of the zinc-dependent MMP-12 after 24 h (Figure 24, Figure 32). Apart from that, Ece1-Va-treated macrophages did not release reasonable amounts of other immune mediators (Figure 28-Figure 33). Taken together, it is conceivable, that Ece1-Va somehow enhances the amount of metal ions, e.g. for fungal metal acquisition during nutrient limitation within phagocytes, simultaneously triggering a metal stress response in the affected immune cells due to a higher presence of non-sequestered metal ions.

Only after 24 h of macrophage co-incubation with the NCEP fragment Ece1-VIa, an enrichment of up-regulated genes involved in the processes of muscle hypertrophy, glia cell migration, and aminoglycoside metabolism was detectable (Figure 23, Figure 26). Of these, solely glia cell migration might possibly be linked to a function during infections with *C. albicans*, as glia cells have been shown to crucially contribute to antifungal host defence in the CNS due to a candidalysin-induced IL-1β and CXCL1 neutrophil recruitment (Drummond *et al.* 2019). However, only two (*RBFOX2*, *SOCS7*) out of ten genes involved in this pathway were up-regulated upon Ece1-VIa treatment (Additional Data CD), and these two seem to exhibit further functions, as they are likewise involved in other pathways like cytokine signalling (Additional Data CD). Regarding the release of immune mediators, no reasonable, Ece1-VIa-specific secretion was detectable upon macrophage co-incubation with Ece1-VIa alone (Figure 28-Figure 32).

The exposure of macrophages to Ece1-VIIa resulted in a down-regulation of glutathione metabolism upon 6 h of co-incubation (Figure 23, Figure 26), suggesting that Ece1-VIIa might modulate or dampen the early oxidative stress response induced by macrophages upon *C. albicans* infection in addition to its putative function to shuttle candidalysin to the extracellular space. Changes in the glutathione metabolism could possibly fine-tune the macrophage immune response against *C. albicans* as already described for macrophage infections with the influenza virus (Diotallevi *et al.* 2017). Furthermore, glutathione metabolism has already been implicated with macrophage differentiation and phagocytic activity (Kim *et al.* 2004), which might likewise affect the response towards *C. albicans* during infection. However, none of the other investigated genes involved in the oxidative stress response (*CYBA*, *CYBB*, *GPX3*, *NCF1*, *NCF2*, *NCF4*, *SOD1*, *SOD2*, *TXNRD1*) was down-regulated upon Ece1-VIIa treatment (Additional Data CD). Interestingly, regarding the secretion of immune mediators by macrophages, some were also secreted upon Ece1-VIIa treatment alone (IL-1α, IL-6, IL-8, IL-10, IL-18, IL-1RA, TNF-α, CXCL1, CXCL5, CXCL12, CCL2, CCL3, CCL4; Figure 28-Figure 31), which was partially also seen in the transcriptional response data for Ece1-VIIa treatment (up-regulation of *IL1A*, *IL6*, *IL8*, *CXCL12*, and *CCL2*; Figure 24). In some cases, even an additive effect of candidalysin and Ece1-VIIa was observed (Figure 28-Figure 31, Figure 33). This suggests that this NCEP fragment at least partially

participates in the induction of immune mediator signalling by macrophages (Figure 33), whereas it reduced the candidalysin-induced damage of macrophages when applied in combination with Ece1-VIIa (Figure 19).

Concluding, the response of macrophages is dominated by candidalysin-induced transcriptional changes and cyto-/chemokine release. However, also Ece1-VIIa induces certain immune mediators, which are mainly involved in cell migration, as well as it seems to modulate the early oxidative stress response in macrophages. The NCEP fragment Ece1-Va exhibits a putative function for metal acquisition or accessibility in the analysis of the macrophage transcriptional response, but does mainly not elicit an inflammatory response in macrophages. In terms of Ece1-VIa, no clear conclusion on the function can be drawn from the data presented so far.

Nevertheless, it is clear that NCEPs and fragments thereof possess functions independent from the ones inhabited by candidalysin during the interaction with host cells. To fully elucidate the functions of these and other NCEPs, further research is required, e.g. by analysing the response of different host cells upon challenge with synthetic NCEPs or fragments thereof in terms of host cell damage, transcriptional changes, modulation of host cell-specific signalling pathways, and/or the release of host cell-derived mediators. To ensure that the peptides reach the host cell cytoplasm, where many of the putative functions are predicted to occur, engineered vesicles loaded with synthetic peptides might be used in addition to administering the peptide from the outside (ongoing research in the MPM department, in collaboration with Thomas Gutsmann, Research Center Borstel). Moreover, an analysis of binding partners, for example using pull-down assays, would be of special interest and would shed further light on the relevance of NCEPs during the *C. albicans*-host cell interaction.

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# 7 Appendix

## 7.1 Additional experimental procedures

### 7.1.1 Additional strains and plasmids used or generated in this thesis

#### 7.1.1.1 *Candida albicans* strains

**Table 8: *Candida albicans* strains used in this study.**

Strain name, internal number in parentheses, short name of strain if applicable, genotype and the respective reference.

| Strain name (internal number) | Short name | Genotype | Reference |
|---|---|---|---|
| BWP17+Clp30 Wt strain (M1477) | Wt | *ura3*::λ*imm434*/*ura3*:: λ*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG RPS10*/*rps10*::*Clp30-URA3-HIS1-ARG4* | (Zakikhany *et al.* 2007) |
| *ece1*Δ/Δ ura$^-$ (M2042) | | *ece1*::*HIS1*/*ece1*::*ARG4* | MPM dept, unpublished |
| *ece1*Δ/Δ (M2057) | | *ece1*::*HIS1*/*ece1*::*ARG4 rps1*::*URA3* | (Moyes *et al.* 2016) |
| *ece1*Δ/Δ+*ECE1* (M2059) | | *ece1*::*HIS1*/*ece1*::*ARG4 rps1*::(*URA3 ECE1*) | (Moyes *et al.* 2016) |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P2}$ (M2466) | Δ*P2* | *ece1*::*HIS1*/*ece1*::*ARG4 rps1*::(*URA3 ECE1*$_{\Delta 94\text{-}183}$) | MPM dept., unpublished |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P3}$ (M2174) | Δ*P3* | *ece1*::*HIS1*/*ece1*::*ARG4 rps1*::(*URA3 ECE1*$_{\Delta 184\text{-}279}$) | (Moyes *et al.* 2016) |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P4}$ (M2468) | Δ*P4* | *ece1*::*HIS1*/*ece1*::*ARG4 rps1*::(*URA3 ECE1*$_{\Delta 280\text{-}378}$) | MPM dept., unpublished |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P5}$ (M2470) | Δ*P5* | *ece1*::*HIS1*/*ece1*::*ARG4 rps1*::(*URA3 ECE1*$_{\Delta 379\text{-}480}$) | MPM dept., unpublished |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P6}$ (M2472) | Δ*P6* | *ece1*::*HIS1*/*ece1*::*ARG4 rps1*::(*URA3 ECE1*$_{\Delta 481\text{-}582}$) | MPM dept., unpublished |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P7}$ (M2349) | Δ*P7* | *ece1*::*HIS1*/*ece1*::*ARG4 rps1*::(*URA3 ECE1*$_{\Delta 583\text{-}684}$) | MPM dept., unpublished generated and described in (König 2015) |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P8}$ (M2474) | Δ*P8* | *ece1*::*HIS1*/*ece1*::*ARG4 rps1*::(*URA3 ECE1*$_{\Delta 685\text{-}813}$) | MPM dept., unpublished |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P4+5}$ (M2476) | Δ*P4+5* | *ece1*::*HIS1*/*ece1*::*ARG4 rps1*::(*URA3 ECE1*$_{\Delta 280\text{-}480}$) | MPM dept., unpublished |
| *ece1*Δ/Δ+*ECE1*$_{\Delta P6\text{-}8}$ (M2478) | Δ*P6-8* | *ece1*::*HIS1*/*ece1*::*ARG4 rps1*::(*URA3 ECE1*$_{\Delta 481\text{-}813}$) | MPM dept., unpublished |
| *ece1*Δ/Δ+*ECE1*$_{\text{TripleP3}}$ (M2480) | TripleP3 | *ece1*::*HIS1*/*ece1*::*ARG4 rps1*::(*URA3 ECE1*$_{379\text{-}480::\ 184\text{-}279,\ 583\text{-}684::184\text{-}279}$) | MPM dept., unpublished |

### 7.1.1.2 *Escherichia coli* strain

**Table 9: *Escherichia coli* strain used in this study.**

*Escherichia coli* strain used in this study with strain name, genotype and the respective reference.

| Strain | Genotype | Reference |
|---|---|---|
| *E. coli* DH5α | $F^-$, φ80d l*acZ*Δ M15, Δ(*lacZYA-argF*)U169, deoR, *recA1*, *endA1*, *hsdR17*($r_k^-$, $m_k^+$), *phoA*, *supE44*, $\lambda^-$, *thi-1*, *gyrA96*, *relA1* | Takara Bio Inc., Kusatsu (JPN) (Takara Bio Inc. 2020) |

### 7.1.1.3 Primer sequences

**Table 10: Primers used in this study.**

Primers used in this study with primer name and sequence from 5'-3'.

| Primer name | Sequence 5' – 3' |
|---|---|
| ECE1Promfwd | TGG CTT CTC ATA AAT GAA GGG |
| ECE1Termrev | GTG GTG GTG TTG AAT TGC TT |
| ECE1-F2 | CAC TGG TGT TCA ACA ATC CAT |
| ECE1-R | CTGTCGGTATTGAAAATGCT |
| ECE1KO3fwd | GAA GAT ATT GAT TCT GTT GTT GCT GG |
| ECE1KO6fwd | GAT GGT CTT GAA GAT TTT CTT GAT G |
| ECE1KO6rev | TCT CTT GGC ATT TTC GAT GG |
| URA-F2 | GGA GTT GGA TTA GAT GAT AAA GGT GAT GG |
| RPF-2 | CGC CAA AGA GTT TCC CCT ATT ATC |
| EFB1-F | TCA GAC CAG CTG ATT TAG GTT TG |
| EFB1-R | CAT CTT CTT CAA CAG CAG CTT G |
| ACT1-F | TCA GAC CAG CTG ATT TAG GTT TG |
| ACT1-R | GTG AAC AAT GGA TGG ACC AG |
| KAR2-F | GTG TTG AAG GAT GGT GGT GT |
| KAR2-R | AAC ACT CAA AAC ACC TGC TT |
| HAC1SP-F | CTT CCT CCT CAT CAT CGT TA |
| HAC1SP-R | CAT TTC AGT TGG ACTT TGA |

### 7.1.1.4 Plasmids

**Table 11: Plasmids used in this study.**

Plasmid name and internal P-number given in parentheses, as well as resistance cassette, selection marker, plasmid characteristics, and the respective source or reference. Amp – ampicillin, arg – arginine, his – histidine, ura – uridine.

| Plasmid (internal number) | Resistance cassette | Selection marker | Characteristics | Source/ reference |
|---|---|---|---|---|
| pCIp10 | Amp | ura | Used for integration into the *RPS1* locus and complementation of uridine auxotrophy | (Murad *et al.* 2000) |

| | | | | |
|---|---|---|---|---|
| pClp10+*ECE1* (P250) | Amp | ura | Used for integration into the *RPS1* locus and complementation of uridine auxotrophy, contains the *ECE1* gene | MPM department, unpublished |
| pClp30 | Amp | arg his ura | Used for integration into the *RPS1* locus and complementation of arginine, histidine and uridine auxotrophy | (Dennison *et al.* 2005) |
| pUC57+$ECE1_{\Delta P2}$ (P283) | Amp | | Used for construction of pClp10+$ECE1_{\Delta P2}$ plasmid | Biomatik Corporation, Ontario (CAN) |
| pUC57+$ECE1_{\Delta P4}$ (P284) | Amp | | Used for construction of pClp10+$ECE1_{\Delta P4}$ plasmid | Biomatik Corporation, Ontario (CAN) |
| pUC57+$ECE1_{\Delta P5}$ (P285) | Amp | | Used for construction of pClp10+$ECE1_{\Delta P5}$ plasmid | Biomatik Corporation, Ontario (CAN) |
| pUC57+$ECE1_{\Delta P6}$ (P286) | Amp | | Used for construction of pClp10+$ECE1_{\Delta P6}$ plasmid | Biomatik Corporation, Ontario (CAN) |
| pUC57+$ECE1_{\Delta P8}$ (P287) | Amp | | Used for construction of pClp10+$ECE1_{\Delta P8}$ plasmid | Biomatik Corporation, Ontario (CAN) |
| pUC57+$ECE1_{\Delta P4+5}$ (P288) | Amp | | Used for construction of pClp10+$ECE1_{\Delta P4+5}$ plasmid | Biomatik Corporation, Ontario (CAN) |
| pUC57+$ECE1_{\Delta P6-8}$ (P289) | Amp | | Used for construction of pClp10+$ECE1_{\Delta 6-8}$ plasmid | Biomatik Corporation, Ontario (CAN) |
| pUC57+$ECE1_{\text{TripleP3}}$ (P290) | Amp | | Used for construction of pClp10+$ECE1_{\text{TripleP3}}$ plasmid | Biomatik Corporation, Ontario (CAN) |
| pClp10+$ECE1_{\Delta P2}$ (P296) | Amp | ura | Used for integration of the mutated *ECE1* gene into the RPS1 locus and complementation of uridine auxotrophy | MPM department, unpublished |
| pClp10+$ECE1_{\Delta P4}$ (P303) | Amp | ura | Used for integration of the mutated *ECE1* gene into the RPS1 locus and complementation of uridine auxotrophy | MPM department, unpublished |
| pClp10+$ECE1_{\Delta P5}$ (P297) | Amp | ura | Used for integration of the mutated *ECE1* gene into the RPS1 locus and complementation of uridine auxotrophy | MPM department, unpublished |
| pClp10+$ECE1_{\Delta P6}$ (P298) | Amp | ura | Used for integration of the mutated *ECE1* gene into the RPS1 locus and complementation of uridine auxotrophy | MPM department, unpublished |
| pClp10+$ECE1_{\Delta P8}$ (P299) | Amp | ura | Used for integration of the mutated *ECE1* gene into the RPS1 locus and complementation of uridine auxotrophy | MPM department, unpublished |

| | | | | |
|---|---|---|---|---|
| pClp10+*ECE1*$_{\Delta P4+5}$ (P300) | Amp | ura | Used for integration of the mutated *ECE1* gene into the RPS1 locus and complementation of uridine auxotrophy | MPM department, unpublished |
| pClp10+*ECE1*$_{\Delta P6-8}$ (P301) | Amp | ura | Used for integration of the mutated *ECE1* gene into the RPS1 locus and complementation of uridine auxotrophy | MPM department, unpublished |
| pClp10+*ECE1*$_{TripleP3}$ (P302) | Amp | ura | Used for integration of the mutated *ECE1* gene into the RPS1 locus and complementation of uridine auxotrophy | MPM department, unpublished |

## 7.1.2 Microbiological and molecular biological methods

### 7.1.2.1 Cultivation of *Candida albicans*

Routinely, *C. albicans* strains were cultivated on solid Yeast Peptone Dextrose (YPD) agar plates at 30 °C and kept at 4 °C for short term storage. For experiments, o/n cultures were prepared by inoculating material from a single colony on the YPD plate into liquid YPD and incubating the flask at 30 °C and 180 rpm shaking for 12-16 h. Long term storage at -80 °C was conducted as glycerol stocks, which were prepared from an 50:50 mixture of an stationary *C. albicans* o/n culture and 50 % volume per volume (v/v) glycerol.

### 7.1.2.2 Cultivation of *Escherichia coli*

*Escherichia coli* was routinely cultivated on lysogeny broth (LB) agar plates at 37 °C or in liquid LB at 37 °C and 180 rpm shaking. Short term storage of LB agar plates was performed at 4 °C. The selection of clones harbouring an Amp resistance cassette was performed on LB agar plates containing 50 µg/mL Amp (Sigma-Aldrich Chemie GmbH, Taufkirchen). Long term storage at -80 °C was achieved through preparing glycerol stocks by adding 50 % glycerol (v/v) to an equal amount of an stationary *E. coli* o/n culture supplemented with 50 µg/mL Amp.

### 7.1.2.3 Isolation of plasmid deoxyribonucleic acid

Overnight cultures of selected clones were prepared in 3 mL LB+Amp and grown at 37 °C and 180 rpm shaking. Cultures were harvested by centrifugation for 30 s at 13,000 rpm, the supernatant was discarded and the pellet resuspended in 300 µL P1 buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/mL RNase A). The mixture was vortexed to homogeneity,

300 µL P2 buffer (200 mM NaOH, 1 % SDS) were added prior to inverting the tube to homogenise the suspension. Upon 5 min of incubation at room temperature, 300 µL P3 buffer (3 M potassium acetate pH 5.5) were added and the suspension inverted to homogenise once more. Upon centrifugation for 5 min at 13,000 rpm, the supernatant was transferred into a new tube and 400 µL isopropanol were added before mixing. Upon 10 min of centrifugation at 13,000 rpm, the supernatant was discarded, 500 µL of 70 % ethanol added and the tube centrifuged for 5 min at 13,000 rpm. After discarding the supernatant, the residual pellet was air-dried and finally dissolved in 200 µL of nuclease-free water. Prior to further experiments, the concentration of plasmid DNA was measured as described in paragraph 7.1.2.10.

#### 7.1.2.4 Isolation of genomic *Candida albicans* deoxyribonucleic acid

*Candida albicans* o/n cultures were prepared as stated in paragraph 7.1.2.1. Cells were harvested by 2 min of centrifugation at 10,000 rpm, the supernatant was discarded and the pellet resuspended in 500 µL water. Cells were centrifuged for 30 s at 10,000 rpm, the supernatant was decanted and the tube was briefly vortexed to resuspend the cells in the residual liquid. The suspension was transferred into a screw cap tube containing 300 mg acid-washed glass beads, 200 µL lysis buffer (20 % Triton-X-100, 10 % SDS, 5 M NaCl, 1 M Tris pH 8.0, 0.5 M EDTA) and 200 µL phenol:chloroform:isoamylalkohol 25:24:1 (Carl Roth GmbH + Co. KG, Karlsruhe) were added before cell walls were destroyed by 1 × 10 s 5,000 rpm disruption the cell disruptor and homogenator precellys 24 (Bertin Technologies SAS, Montigny le Bretonneux (FR)). Subsequently, 200 µL Tris-ethylenediaminetetraacetic acid (TE) buffer (10 mM Tris, 1 mM EDTA) was added and the suspension was centrifuged for 5 min at 13,000 rpm. The aqueous layer was transferred into 1 mL 96 %, ice-cold ethanol in a fresh tube and the liquids were mixed by inversion. Upon incubation for 1 h at -20 °C, the mixture was centrifuged for 10 min at 13,000 rpm and 4 °C, the supernatant was discarded and the pellet was resuspended in 400 µL TE buffer. To remove RNA, 3 µL RNAse A (10 mg/mL) were added followed by an incubation of 30 min at 37 °C. In the following, 50 µL 3 M sodium acetate (pH 5.2) were added and the mixture was filled up to 1.5 µL with 96 % ice-cold ethanol, mixed by inversion and incubated for 30 min at -20 °C. Subsequently, the mixture was centrifuged for 10 min at 13,000 rpm and 4 °C. The supernatant was discarded,

the pellet was washed in 500 µL 70 % ethanol and the tubes were centrifuged again as described above. Upon discarding the supernatant, the pellet was air-dried and resuspended in 50 µL nuclease-free water before the DNA concentration was determined as described in paragraph 7.1.2.10.

#### 7.1.2.5 Isolation of *Candida albicans'* ribonucleic acid and quality control

Overnight cultures of *C. albicans* were prepared as stated in paragraph 7.1.2.1 and transferred into round-bottom plastic tubes on the next morning. Cells were harvested by 2 min centrifugation at 3000 × *g*, washed twice in 5 mL phosphate-buffered saline (PBS), centrifuged again as above and the residual pellet was finally resuspended in 1 mL PBS. Upon counting the cells as described in paragraph 7.1.2.1, cell count were adjusted to $1 \times 10^7$ cells/mL in 25 mL clear RPMI-1640 medium (Thermo Fisher Scientific, Waltham (USA)) for hyphal cultures and 1 X $10^8$ cells/mL in 8 mL YPD for yeast cultures. The suspension for the hyphal culture was distributed in 150 $cm^2$ plastic petri dishes and incubated for 3 h at 37 °C and 5 % $CO_2$. Upon incubation, the medium was removed, cells were rinsed with 15 mL ice-cold PBS, hyphae were detached from the surface with a cell scraper and the suspension was transferred into a 15 mL tube. Yeast cultures were incubated in glass flasks for 3 hat 30 °C and 180 rpm shaking. Cultures were transferred into 15 mL tubes, spun for 2 min at 3,000 × *g* and 4 °C and resuspended in 15 mL ice-cold PBS. From this step on, both, yeast and hyphal cultures, were treated equally. Cells were centrifuged for 2 min at 3,000 × *g* and 4 °C before the supernatant was discarded, the pellet was washed with 1 mL ice-cold PBS and cells were pelleted again as described above. The supernatant was removed completely and the pellets were shock-frozen in liquid nitrogen. Until RNA isolation, the pellets were stored at -80 °C. For RNA isolation, all steps were prepared on ice, in an RNAse-free environment ensured by treatment with RNAse*Zap*™ (Thermo Fisher Scientific, Waltham (USA)), filter tips were used for pipetting and diethyl pyrocarbonate (DEPC)-treated water for preparation of buffers. The fungal cell pellet was resuspended in 400 µL AE buffer (50 mM sodium acetate pH 5.3, 10 mM EDTA), 40 µL 10 % sodium dodecyl sulfate (SDS) were added and the mixture was vortexed for 30 s at the highest setting. Upon vortexing, 440 µL phenol:chloroform:isoamylalkohol 25:24:1 (Carl Roth GmbH + Co. KG, Karlsruhe) were added and the mixture was gently mixed by

inversion. Next, samples were subjected to two freeze-thawing steps (5 min at 65 °C in the water bath, 10 min at -80 °C) followed by a 10 min centrifugation step at 14,000 rpm at 4 °C. The aqueous layer including the RNA was carefully transferred into a fresh tube containing 500 µL ice-cold isopropanol with 0.3 M sodium acetate (pH 5.5). The liquids were inverted to homogeneity and fungal RNA was allowed to precipitate for at least 1 h at -20 °C. Upon precipitation, the RNA was pelleted by centrifugation for 12 min at 14,000 rpm and 4 °C. The supernatant was discarded and the pellet was washed with 700 µL ice-cold ethanol. Centrifugation and washing step were repeated once and samples were finally centrifuged for 10 min at 14,000 rpm and 4 °C. The ethanol was completely removed, the pellet was air-dried and subsequently resuspended in DEPC water. To remove residual gDNA, 50 µL DNase solution (42 µL DEPC water, 3 µL Baseline ZERO™ DNAse and 5 µL reaction buffer) were added to the RNA solution and incubated at 37 °C for 45 min in the water bath. Upon gDNA digestion, 350 µL DEPC water, 400 µL isopropanol and 80 µL 3 M sodium acetate (pH 5.5) were added. The liquids were mixed to homogeneity by inversion and the RNA was precipitated o/n at -20 °C. After precipitation, the RNA was pelleted and washed as described above. Upon carefully removing the ethanol, the pellet was air-dried and resuspended in 50 µL DEPC water. The RNA concentration was determined as described in 7.1.2.10.

The quality of isolated RNA from yeast and hyphal samples was determined using the Bioanalyzer 2100 and the RNA 6000 Pico Kit (both Agilent Technologies Inc., Santa Clara (USA)) according to the manufacturer's instructions. The software returned an electropherogram showing a marker peak and two ribosomal peaks in terms of successful preparation. An RNA integrity number (RIN) of at least two, which is calculated from the ribosomal peak ratios, represents high quality RNA which can be used for further experiments.

#### 7.1.2.6 Generation of complementary deoxyribonucleic acid

For the synthesis of complementary DNA (cDNA) from RNA, components as given in Table 12 were used followed by an incubation for 10 min at 70 °C, 2 h at 42 °C and finally 15 min at 70 °C. The generated cDNA was ensured to be free of genomic DNA (gDNA) by performing a PCR as described in paragraph 7.1.2.7.1 using the primer pair EFB1-F and

EFB1-R (Table 10), which amplifies the intron-containing housekeeping gene *Elongation Factor-1 Beta* (*EFB1*). In case of cDNA without gDNA contamination, the product is shorter due to out-splicing of the intron during the maturation process of mRNA, which than serves as a template for cDNA synthesis.

**Table 12: Components used for cDNA synthesis.**

| Component | Volume/amount |
|---|---|
| RNA | 500 ng |
| 5 × First strand buffer | 7 µL |
| DTT | 2 µL |
| dNTPs | 1 µL |
| RNAse Out | 1 µL |
| Reverse Transcriptase Superscript III | 0.5 µL |
| DEPC water | 0.5 µL |
| Oligo dTs | 1 µL |

### 7.1.2.7 Polymerase chain reaction

#### 7.1.2.7.1 Polymerase chain reaction using a *Thermus aquaticus* polymerase

Specific amplification of DNA sequences was achieved by PCR according to the general manufacturer's guidelines. By default, PCR reactions were conducted in a final volume of 25 µL using the components listed in Table 13. A heat-stabile polymerase from *Thermus aquaticus* (*Taq*) was used for amplification of the product. Table 14 shows a typical thermocycler profile used for DNA amplification. For amplification of small fragments (e.g. *HAC1* mRNA splicing), Standard Taq buffer (New England Biolabs GmbH, Frankfurt am Main) was used instead of ThermoPol Buffer.

**Table 13: Standard PCR set-up.**

Polymerase, buffer and dNTPs were purchased from New England Biolabs, Frankfurt am Main. U – units.

| Component | Final concentration |
|---|---|
| ThermoPol buffer with $MgCl_2$ (10 ×) | 1 × |
| dNTPs (10 mM) | 0.2 mM |
| Forward primer (10 µM) | 0.4 µM |
| Reverse primer (10 µM) | 0.4 µM |
| *Taq* polymerase (5 UµL) | 1.25 U |
| DNA template (gDNA/plasmid DNA/cDNA) | 100 ng/10 ng/100 ng |

**Table 14: Standard thermocycler profile used for PCRs.**

| Step | Time | | Temperature in °C |
|---|---|---|---|
| Initial denaturation | 2 min | | 95 |
| Denaturation | 30 s | | 95 |
| Annealing | 30 s | Repeat 29× | 50-68 |
| Elongation | 1 min/kb | | 72 |
| Final elongation | 10 min | | 72 |

#### 7.1.2.7.2 Colony polymerase chain reaction

To screen positive *E. coli* clones for correct genetic manipulation, colony PCR was performed in a final volume of 25 µL and according to Table 13 and a thermocycler profile as given in Table 14. Instead of a dissolved DNA template, the colony of interest was slightly pricked with a pipette tip, which was subsequently placed into the PCR tube containing the PCR master mix without the polymerase. Upon resuspension of colony material in the mixture, the material was incubated for 5 min at 95 °C to lyse the cells. Subsequently, the polymerase was added on ice and the PCR run was performed.

#### 7.1.2.7.3 Quantitative real-time polymerase chain reaction and evaluation

Gene expression was assessed by performing a quantitative real-time PCR (qRT-PCR) using the 2× GoTaq® qPCR Master Mix kit (Promega GmbH, Walldorf) according to the manufacturer's instructions. Primer concentrations of 10 µM were used and annealing temperatures for the different primer pairs are given in Table 15. Template cDNA was used in a 1:10 dilution. Prior to qRT-PCR sample runs, the primer efficiencies of the respective primer pairs used (Table 15) were determined.

Table 16 shows the used cycler program for the qRT-PCR. The genes *ECE1*, *KAR2*, and *ACT1* were analysed, with *ACT1* representing the housekeeping gene and normalisation control for gene expression analysis. Results from the qRT-PCR run were analysed using CFX Manager™ software (Bio-Rad Laboratories Inc., Hercules (USA)). Gene expression was calculated using the ΔΔCT method and additionally taking different primer efficiencies into account as described in (Pfaffl 2004).

Primer efficiency was determined by running the respective qRT-PCR program with the according primer pair for a serial dilution of gDNA in triplicates. Efficiencies were taken from

the generated standard curve (a plot of the mean Cq against the log of the starting quantity) generated by the CFX Manager™ software (Bio-Rad Laboratories Inc., Hercules (USA)).

**Table 15: Primer pairs used for qRT-PCR with respective annealing temperature and efficiency.**

| Primer pair | Annealing temperature in °C | Primer efficiency in % |
|---|---|---|
| ACT1-F + ACT1-R | 59 | 102.9 |
| ECE1-F2 + ECE1-R | 59 | 96.2 |
| ECE1KO3fwd + ECE1KO6rev | 59 | 101.3 |
| ECE1KO6fwd + ECE1-R | 59 | 98.2 |
| KAR2-F + KAR2-R | 56 | 117.3 |

**Table 16: Thermocycler profile used for the qRT-PCR.**

| Step | Temperature in °C | | Time |
|---|---|---|---|
| Initial denaturation | 95 | | 2 min |
| Denaturation | 95 | Repeat 39× | 15 s |
| Annealing | 57/59 | | 20 s |
| Elongation | 72 | | 15 s |
| Final denaturation | 95 | | 15 s |
| Melting curve | 57/59-95 | | 20 s |

### 7.1.2.8 Agarose gel electrophoresis

For separation of DNA templates from PCRs and restriction digests agarose gel electrophoresis was conducted. Routinely, an agarose concentration of 1 % weight per volume (w/v) (Bio&Sell e.K, Feucht) in 1 X Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer (0.4 M Tris acetate pH 8.3, 0.01 M EDTA) was used. For separation of products smaller than 100 bp, a 4 % agarose gel was prepared using a special, high resolution agarose (ROTI®Garose for small fragments, Carl Roth GmbH + Co. KG, Karlsruhe) and the gel was prepared and run in Tris-borate-ethylenediaminetetraacetic acid (TBE) buffer (0.13 M Tris pH 7.6, 45 M boric acid, 2.5 mM EDTA). Depending on the gel size and agarose concentration the electrode potential was set to 60-160 Volt (V). Large gels and gels with a higher agarose concentration were run at higher voltage as compared to smaller or 1 % gels. Before applying the DNA fragments to the gel, the products were supplied with a TriTrack DNA loading dye (Thermo Fisher Scientific Inc., Waltham (USA)). An appropriate marker was applied on each gel. Routinely, the GeneRuler™ 1 kb Ladder (Thermo Fisher Scientific Inc., Waltham (USA)) was

used. For separation of smaller fragments, the 100 bp DNA Ladder (New England Biolabs GmbH, Frankfurt am Main), the HyperLadder™ 25 bp (Meridian Bioscience, Cincinnati (USA)), or the 25bp DNA Step ladder (Promega GmbH, Walldorf) were applied. Upon complete separation, the gel was subjected to ethium bromide (EtBr) staining (0.5 µg/mL) for 15-30 min if not used for Southern Blotting. In the latter case, the gel remained unstained for further analysis. Upon EtBr staining, the DNA bands were visualised with an ultra-violet (UV) transilluminator (E-BOX CX5 TS, VILBER LOURMAT Deutschland GmbH, Eberhardzell). To reduce mutations in the DNA fragments, exposure to UV light was kept as short as possible.

#### 7.1.2.8.1 Quantification of PCR products from an agarose gel electrophoresis picture using ImageJ

To quantify PCR products after agarose gel electrophoresis, the respective gel picture was analysed using ImageJ (Schneider *et al.* 2012) according to a procedure published by the Diamantina Institute of the University of Queensland (AUS) (Diamantina Institute 2017). Briefly, all lanes were surrounded by a rectangular area of identical size and the band intensity was transferred into a histogram for each selection. The respective peaks were selected and the AUCs were determined in arbitrary, numerical values which were exported into a new file. In case of the *HAC1* splice product analysis, all AUCs were normalised to the AUC detected in the Wt + T hyphal sample (Wt treated with 2 µg/mL tunicamycin). Mutants showing splice products that reach more than 25 % of the Wt + T hyphal sample AUC were considered as experiencing ER stress and UPR induction.

### 7.1.2.9 Purification of polymerase chain reaction products

Amplified PCR product were routinely purified using the QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden) according to the manufacturer's instructions. In case of unspecific PCR products or to purify digested vector plasmid or plasmid inserts, gel elution was used for purification of DNA fragments using the QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden) following the manufacturer's protocol. For best separation of fragments, the samples were run in a 2 % agarose gel at low voltage (60 V) for 4-6 h. Prior to the purification procedure, the respective fragments were excised from the gel.

#### 7.1.2.10 Estimation of nucleic acid concentration

As certain procedures require a specific amount of applied DNA or RNA, the concentration of nucleid acids in the respective samples was determined using spectrophometric analysis with the NanoDrop Spectrophotometer ND-1000 (VWR International GmbH, Erlangen). By analysing the absorption peak height at 260 nm ($A_{260}$), which represents the maximum of absorbance of nucleic acids the concentration was determined. Additional analysis of the absorption at 230 and 280 nm ($A_{230}$ and $A_{280}$) indicates contaminations with phenol or proteins, respectively. For DNA samples of good purity, the A260/A280 quotient should range from 1.6-2.0, higher values are usual for RNA samples, whereas a lower quotient typically indicates a contamination with phenol or proteins.

### 7.1.3 Construction of NCEP-knock-out mutants

The respective *ECE1*$_{\Delta Px}$ sequence constructs in the *E. coli* pUC57 plasmids (Table 11) were purchased from Biomatik, Ontario (CAN) and dissolved in nuclease-free water to a final concentration of 500 ng/µL.

#### 7.1.3.1 Restriction digest and ligation

To introduce the *ECE1*$_{\Delta Px}$ sequences into a *C. albicans* plasmid, vector plasmid (pCIp10+*ECE1*) and donor plasmid (pUC57+*ECE1*$_{\Delta Px}$) were digested and subsequently ligated to obtain the pCIp10+*ECE1*$_{\Delta Px}$ construct.

Briefly, plasmids were digested using *Blp*I und *BsmB*I in CutSmart Buffer (all NEB Biolabs GmbH, Frankfurt am Main). Initially, 2 µg plasmid were digested with *Blp*I according to Table 17. Upon 1.5 h incubation at 37 °C, NaCl was added to a final concentration of 100 mM before *BsmB*I was added followed by an additional incubation step of 1.5 h at 55 °C. In case of the vector plasmid, a dephosphorylation was conducted to prevent re-ligation of the digested plasmid. Therefor, 1 µL recombinant Shrimp Alkaline Phosphatase (rSAP) was added and the reaction mixture was incubated for 30 min at 37 °C followed by an enzyme inactivating step of 5 min at 65 °C. To purify the linearised vector and the *ECE1*$_{\Delta Px}$ insert, gel extraction was used as described in paragraph 7.1.2.9. The electrophoresis revealed four bands as shown in

Table 18, of which the desired fragments (1250 bp and 8000 bp) were used for further reactions upon purification.

**Table 17: General plasmid restriction digest set-up.**

Restriction enzyme and respective buffer were purchased from New England Biolabs GmbH, Frankfurt am Main.

| Component | Amount/volume/final concentration |
|---|---|
| Plasmid DNA | 2 µg |
| Restriction enzyme | 1 µL |
| Restriction buffer 10 × | 1 × |

**Table 18: Expected band size of digestion products.**

Products expected upon subsequent restriction digest with *Blp*I and *BsmB*I, and the respective expected band size.

| Digestion Product | Expected band size in bp |
|---|---|
| KO fragment between *Blp*I and *BsmB*I | 1250 |
| Undigested pCIp10+*ECE1* | 4000 |
| Digested plasmid pCIp10+*ECE1* | 8000 |
| Supercoiled and coiled-coil form of pCIp10+*ECE1* | 9400 |

**Table 19: Ligation set-up for pCIp10+*ECE1*$_{\Delta Px}$ generation.**

Ligase and buffer were purchased from Roche. U – unit.

| Component | Amount/final concentration |
|---|---|
| pCIp10+*ECE1* | 50 ng |
| Insert (*ECE1*$\Delta_{Px}$) | 23.5 ng |
| T4 DNA ligase | 400 U |
| T4 DNA Ligase buffer 10 × | 1 × |
| Nuclease-free water | To 20 µl |

Upon purification, vector plasmid and insert were ligated at 16 °C o/n in a ratio of 1:3 vector:insert according to the reaction set-up (Table 19). A vector re-ligation control without insert, and a water control without any DNA were performed. Following the ligation step, the T4 DNA ligase was heat-inactivated for 20 min at 65 °C and the obtained pCIp10+*ECE1*$_{\Delta Px}$ plasmids were cloned into *E. coli*.

### 7.1.3.2 Transformation of *Escherichia coli*

Competent DH5α *E. coli* cells (Takara Bio Inc., Kusatsu (JPN), (Takara Bio Inc. 2020)) were thawed on ice before 5 µL of the ligation mixture was carefully added. The mixture was mixed gently and incubated for 30 min on ice before heat-shocked for 2 min at 42 °C. Immediately, 2 mL of super optimal broth (SOB) medium were added and the mixture was incubated under gentle shaking for 1.5 h at 37 °C. Cells were centrifuged for 5 min at 2,500 rpm and the supernatant was discarded except for one residual drop of medium. In this, cells were resuspended and plated on an LB agar plate containing 50 µg/mL Amp as a selection marker for positive clones. Incubation of plates was conducted o/n at 37 °C.

Upon o/n incubation, clones were re-streaked on fresh LB-Amp plates for further cultivation and the correct genetic manipulation of clones was determined *via* colony PCR (paragraph 7.1.2.7.2) using the primer pair ECE1Promfwd and ECE1Termrev and a subsequent agarose gel electrophoresis.

### 7.1.3.3 Transformation of *Candida albicans*

The background strain *ece1*Δ/Δ ura$^-$ (Table 8) was grown o/n as described in paragraph 7.1.2.1. The o/n cultures were diluted to an $Abs_{600}$ of 0.2 in liquid YPD and grown to an $Abs_{600}$ of 0.6 30 °C and 180 rpm shaking. Subsequently, the cultures were centrifuged for 5 min at 4,000 rpm and washed once with 10 mL of water. Cultures were centrifuged again as previously described, the supernatant was discarded and the pellet resuspended in lithium acetate (LiAc) solution. For the transforming mixture, 100 µg salmon sperm carrier DNA (UltraPure™ Salmon Sperm DNA Solution, Thermo Fisher Scientific, Waltham (USA), previously denatured for 10 min at 98 °C), the prepared *C. albicans*-LiAc, 10 µg of the transforming DNA (pCIp10+$ECE1_{\Delta Px}$ plasmid) and subsequently 600 µL polyethylene glycol (PEG)-LiAc solution were combined and gently mixed to homogeneity. Importantly the transforming pCIp10+*ECE1* plasmid (Table 11) was linearised as described in Table 17 using the restriction enzyme StuI and CutSmart buffer (both New England Biolabs GmbH, Frankfurt am Main) for 2 h at 37 °C prior to usage to maximise the transformation efficiency. The resulting mixture was incubated o/n at 30 °C under gentle shaking followed by a heat shock at 44 °C for 15 min. Upon heat shock, the mixture was incubated 1 min on ice,

centrifuged at 6,000 rpm for 10 min and the pellet was washed once with 900 µL synthetic defined (SD) medium. Subsequently, a further centrifugation step was conducted, the pellet was resuspended in SD medium and the suspension was plated on SD agar plates for selection of positive clones (transformants with restored ura auxotrophy due to insertion of the *URA3* gene upon homologous recombination, Figure 5). The SD agar plates were incubated for 2-4 days at 30 °C to allow growth of positive clones. Positive clones were re-streaked on SD and YPD plates and o/n cultures were prepared as described in paragraph 7.1.2.1 for subsequent isolation of genomic DNA as described in paragraph 7.1.2.4.

#### 7.1.3.4 Southern Blot of selected clones

To verify the genotype of all generated *C. albicans* strains, Southern blotting was conducted for each strain as described in (Dally 2018).

#### 7.1.3.5 Analysis of *Candida albicans*' growth and filamentation ability

To monitor the growth of *C. albicans* in the yeast morphology, cells were adjusted to an $Abs_{600}$ of 0.11 in a total volume of 200 µL in a 96-well plate in YPD. The plate was sealed and incubated at 30 °C in an Infinite® 200 PRO ELISA Reader (Tecan Trading AG, Männedorf (CH)). The $Abs_{600}$ was measured over a time period of 48 h with measurements conducted every 30 min after 30 s of shaking prior to each measurement.

To assess the filamentation ability of different *C. albicans* strains, $5 \times 10^4$ cells/well were inoculated in 1 mL RPMI-1640 medium in 24-well plates and incubated for 6 h. In terms of an incubation time of 24 h, only 200 cells/well were used as an inoculum. Upon incubation, the cells were fixed with ROTI®Histofix (Carl Roth GmbH + Co KG, Karlsruhe) and hyphal length (6 h) or microcolony diameter (24 h) were analysed microscopically using the ZEN2 software (Carl Zeiss Microscopy Deutschland GmbH, Oberkochen).

To determine the percentage of hypha formation, *C. albicans* cells were microscopically counted and analysed regarding an initiation of filamentation (yes or no).

#### 7.1.3.6 Analysis of the Ece1 secretion pattern by tandem mass spectrometry

To monitor the secretion pattern of Ece1 peptides by the generated *ece1*Δ/Δ+$ECE1_{\Delta Px}$ strains, hyphal supernatants were processed by solid-phase extraction (SPE, Daniela Schulz, HKI Jena,

MPM department) according to the previously published protocol (Moyes *et al.* 2016) and analysed using LC-MS/MS as previously published (Moyes *et al.* 2016) in collaboration with Thomas Krüger (HKI Jena, MAM department).

In some cases (Δ*P2,* Δ*P7* and TripleP3), minor hits of genetically deleted peptides were found, even if the PSM values were very low and generally considered to be below the threshold of being taken into account (approximately 20). In all of these cases, an analysis of the direct prior blank sample revealed the same fragments as detected in the mutant samples (cf. Table 6 and Table 20). These false-positive PSMs can therefore be assigned to a peptide carry-over through the LC-MS/MS column instead of incorrect mutant strains. These hits were therefore deleted from Table 6.

**Table 20: Non-corrected table of LC-MS/MS analysis of Δ*P2,* Δ*P7*, and TripleP3 mutant strains.**

Table shows the non-corrected PSM hits for the mutants Δ*P2*, Δ*P7*, and TripleP3. Peptides supposed to be genetically deleted are highlighted in red.

| Strain | Ece1 peptide | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | I | II | III candidalysin | IV | V a/b | VI a/b | VII a/b | VIII a/b/c |
| Δ*P2* | 3 | 1 | 118 | 0 | 28/0 | 6/0 | 117/31 | 0/0/0 |
| Δ*P7* | 0 | 1 | 27 | 3 | 32/1 | 13/0 | 5/2 | 0/0/0 |
| TripleP3 | 0 | 0 | 32 | 0 | 1/0 | 0/0 | 7/0 | 0/0/0 |

## 7.1.4 Cell culturing methods

### 7.1.4.1 Isolation of monocytes from human blood and differentiation into monocyte-derived macrophages

Buffy coats for monocyte isolation were obtained from healthy human volunteers with written informed consent. The blood donation protocol and use of blood for this study were approved by the Jena institutional ethics committee (Ethik-Kommission des Universitätsklinikums Jena, Permission No 2207–01/08). Monocytes were and differentiated into monocyte-derived macrophages as described in (Kasper *et al.* 2018).

### 7.1.4.2 Cultivation of monocyte-derived macrophages

Human MDMs were cultured and detached for experiments as described in (Kasper *et al.* 2018). For damage assays (LDH, paragraph 7.1.4.3) and ELISAs (paragraph 7.1.4.5), $4 \times 10^4$ cells/well were seeded in 96-well plates. For microarray experiments, $1 \times 10^6$ cells/well were seeded in 6-well plates. All assays were performed in serum-free medium.

### 7.1.4.3 Damage assay of monocyte-derived macrophages

To analyse the damaging potential of different *C. albicans* strains or synthetic peptides, the release of the cytoplasmic enzyme LDH was measured upon 24 h of infection or co-incubation. In case of integer cell membranes, the enzyme is retained intracellularly. Upon cell damage, LDH is released into the supernatant and becomes detectable. To obtain a full-lysis control, Triton-X-100 was added to a final concentration of 0.2 %, which resulted in a complete hMDM lysis within 5 min. All samples were diluted 1:10 in RPMI-1640 prior to analysis. To determine the host cell damage, the Cytotoxicity Detection Kit (LDH) from Roche (Grenzach-Whylen) was used according to the manufacturer's instructions, with an internal standard series entrained on each plate to ensure correct concentration determination.

### 7.1.4.4 Microarray experiments of monocyte-derived macrophages

To analyse the transcriptional response of macrophages to a candidalysin and/or NCEP treatment, microarray experiments using the Human Genome CGH Microarray 4x44K (Agilent Technologies, Inc., Santa Clara (USA)). Primary hMDMs were seeded as stated in

paragraph 7.1.4.2. Macrophages were co-incubated for 6 or 24 h with 5 µM of synthetic candidalysin, Ece1-Va, Ece1-VIa, Ece1-VIIa or candidalysin + Ece1-Va (all purchased from Peptide Protein Research Ltd., Hampshire (UK)). Untreated samples were used for each time point for normalisation. For each sample, RNA was isolated and used for subsequent cDNA and cRNA synthesis using reagents purchased from Agilent Technologies, Inc., Santa Clara (USA).

Initially, 1 µg of isolated RNA was added to RNase-free water (total volume of 5.15 µL) and 0.6 µL T7 primer. The mixture was incubated for 10 min at 65 °C in a water bath and subsequently 5 min on ice. Upon that, 4.15 µL of cDNA master mix (Table 21) were added, mixed by pipetting and incubated at 40 °C in a circulating water bath for 2 h. The Moloney Murine Leukemia Virus (MMLV) reverse transcriptase was deactivated by an incubation step at 65 °C. Upon 5 min of incubation on ice, 28.8 µL transcription master mix (Table 22) as well as 1.2 µL Cy5 (samples) or Cy3 (common reference) were added to the sample and incubated for 2 h at 40 °C in a circulating water bath to generate cRNA. Upon cRNA synthesis, cRNA was cleaned for further proceeding. Therefor, 60 µL of RNAse-free water, 350 µL RLT buffer and 250 µL 96 % ethanol were added to the sample and mixed to homogeneity. The whole reaction set-up was transferred to an RNeasy Mini Kit column (Qiagen, Hilden) and centrifuged for 15 s at 8,000 × *g*. The flow-through was discarded and the column was transferred to a new collection tube before adding 500 µL RPE buffer and centrifuging 2 min at 8,000 × *g*. The column was transferred into a new tube and 35 µL RNase-free water were added. The columns were incubated for 1 min at RT and subsequently centrifuged for 1 min at 8,000 × g to elute the cRNA. The concentration of purified cRNA was measured as described in paragraph 7.1.2.10.

To calculate the incorporation rate of dye into the cRNA, the following equation was used:

$$\frac{\text{concentration of dye (Cy3: Cy5) in } \frac{\text{pmol}}{\mu\text{L}}}{\text{concentration of RNA in } \frac{\mu\text{g}}{\mu\text{L}}}$$

Incorporation rates of 6 or more were considered as suitable for further experimental proceeding.

**Table 21: cDNA synthesis master mix per sample.**

| Component | Volume |
|---|---|
| 5 × FS buffer | 2 µL |
| 0.1 M DTT | 1 µL |
| 10 mM dNTPs | 0.5 µL |
| MMLV Reverse Transcriptase | 0.5 µL |
| RNAse inhibitor | 0.25 µL |

**Table 22: cRNA synthesis master mix per sample.**

| Component | Volume |
|---|---|
| 4 × transcription buffer | 10 µL |
| 0.1 M DTT | 3 µL |
| NTP mix | 4 µL |
| RNAse inhibitor | 0.25 µL |
| 50 % PEG | 3.2 µL |
| Inorganic Pyrophosphatase | 0.3 µL |
| T7 RNA Polymerase | 0.4 µL |
| Cy5/Cy3 | 1.2 µL |
| DEPC water | Add to 30 µL |

For sample preparation, components as given in Table 23 were gently mixed, incubated for 30 min at 60 °C in a circulating water bath to fragment the cRNA, and immediately cooled down on ice for 1 min to stop the reaction, 55 µL of 2× HI-RPM hybridisation buffer were added and mixed by pipetting. To remove residual bubbles, the mixture was spun for 1 min at 13,000 rpm.

**Table 23: Sample preparation for cRNA fragmentation.**

Components of fragmentation mix with the respective amount/volume used. The 25 X fragmentation buffer was added last.

| Component | Amount/volume |
|---|---|
| Cy3-labeled cRNA (common reference) | 825 ng |
| Cy5-labeled cRNA (sample) | 825 ng |
| 10 × expression blocking agent | 11 µL |
| 25 × fragmentation buffer | 2.2 µL |
| Nuclease-free water | Add to 55 µL |

Upon centrifugation, 115 µL of each sample were added to the respective array on the slide. The microarray slide was placed upside down on the mask, secured in the holder, and incubated for 17 h at 65 °C. Upon incubation, the arrays were washed in Gene Expression

buffer 1 for 1 min under 400 rpm stirring, 1 min in pre-warmed Gene Expression Wash buffer 2, less than 10 s in pure acetonitrile, and 30 s in pre-warmed Stabilization&Drying solution. Arrays were scanned (Cy5 – 635 nm laser, Cy3 – 532 nm laser) in the GenePix 4000B microarray scanner using the software GenePixPro 7 (both Molecular Devices, LLC., San Jose (USA)). Saved array data files were converted with the Agilent Feature Extraction software. Upon further processing into GeneSpring format, microarrays were analysed using the GeneSpring GX software (Agilent Technologies, Inc., Santa Clara (USA)).

To remove the strong donor-dependency, which is characteristic for results obtained from hMDM experiments, the mean of all biological replicates was calculated by averaging the $log_2$FC of treatment *vs.* no treatment per donor and time point (e.g. mean of $Donor1_{treated\ 6\ h}$ *vs.* $Donor1_{untreated\ 6\ h}$, $Donor2_{treated\ 6\ h}$ *vs.* $Donor2_{untreated\ 6\ h}$, $Donor3_{treated\ 6\ h}$ *vs.* $Donor3_{untreated\ 6\ h}$). Furthermore, a paired t-test was used to determine statistical significance of regulation. Genes regulated more than a $log_2FC \geq 1$ or $\leq -1$ and a p-value of $\leq 0.05$ were considered as significantly up- or down-regulated, respectively. These filtered genes were subjected to an enrichment analysis using ShinyGo (Ge *et al.* 2020) and a subsequent reduction of redundant pathways with REVIGO (0.7 similarity) (Supek *et al.* 2011).

#### 7.1.4.5 Enzyme-linked immunosorbent assay

To detect cytokines, chemokines, the receptor antagonists IL-1RA and IL-2RA, FGF-2, as well as MMP-1 and -12 after 6 or 24 h of infection with *C. albicans* or co-incubation with synthetic Ece1 peptides, ELISAs were used. All ELISA assays were purchased from eBioscience (Thermo Fisher Scientific, Waltham (USA)) and conducted according to the manufacturer's instructions for pre-coated ELISAs and ProcartaPlex multiplex magnetic ELISAs. All samples were diluted 1:10 in RPMI-1640 prior to analysis. The magnetic ProcartaPlex immunoassay plates were run and analysed in the MAGPIX® System (Merck KGaA, Darmstadt) following the manufacturer's instructions. All analytes are shown in Table 24 with their abbreviation, name and method of detection.

**Table 24: Immune response mediators released from hMDMs analysed in this study.**

Immune response mediators released from hMDMs upon co-incubation with candidalysin and/or NCEP fragments or infection with *C. albicans* analysed in this study. Table specifies abbreviation, full name, alternative name (if applicable), and the respective method of detection.

| Abbreviation | Name | Alternative name | Detection method |
|---|---|---|---|
| CCL1 | C-C motif ligand 1 | I-309 | Multiplex ELISA |
| CCL2 | C-C motif ligand 2 | MCP-1 | Multiplex ELISA |
| CCL3 | C-C motif ligand 3 | MIP-1α | Multiplex ELISA |
| CCL4 | C-C motif ligand 4 | MIP-1β | Multiplex ELISA |
| CCL5 | C-C motif ligand 5 | RANTES | Multiplex ELISA |
| CCL8 | C-C motif ligand 8 | MCP-2 | Multiplex ELISA |
| CCL11 | C-C motif ligand 11 | Eotaxin | Multiplex ELISA |
| CCL15 | C-C motif ligand 15 | MIP-1δ | Precoated single ELISA |
| CCL17 | C-C motif ligand 17 | TARC | Multiplex ELISA |
| CCL18 | C-C motif ligand 18 | PARC | Precoated single ELISA |
| CCL20 | C-C motif ligand 20 | MIP-3α | Multiplex ELISA |
| CCL22 | C-C motif ligand 22 | MDC | Multiplex ELISA |
| CCL24 | C-C motif ligand 24 | Eotaxin-2 | Multiplex ELISA |
| TNF-α | Tumor necrosis factor α | | Multiplex ELISA |
| IL-1α | Interleukin-1α | | Multiplex ELISA |
| IL-1β | Interleukin-1β | | Multiplex ELISA |
| IL-6 | Interleukin-6 | | Multiplex ELISA |
| IL-7 | Interleukin-7 | | Multiplex ELISA |
| IL-8 (CXCL8) | Interleukin-8 | | Multiplex ELISA |
| IL-10 | Interleukin-10 | | Multiplex ELISA |
| IL-12p70 | Interleukin-12 | | Multiplex ELISA |
| IL-15 | Interleukin-15 | | Multiplex ELISA |
| IL-18 | Interleukin-18 | | Multiplex ELISA |
| IL-23 | Interleukin-23 | | Multiplex ELISA |
| IL-27 | Interleukin-27 | | Multiplex ELISA |
| TGF-β | Transforming growth factor β | | Precoated single ELISA |
| CXCL1 | C-X-C motif ligand 1 | Gro-α | Multiplex ELISA |
| CXCL5 | C-X-C motif ligand 5 | ENA-78 | Multiplex ELISA |
| CXCL9 | C-X-C motif ligand 9 | MIG | Multiplex ELISA |
| CXCL10 | C-X-C motif ligand 10 | IP-10 | Multiplex ELISA |
| CXCL12 | C-X-C motif ligand 12 | SDF-1α | Multiplex ELISA |
| CXCL13 | C-X-C motif ligand 13 | BLC | Multiplex ELISA |
| IL-1RA | Interleukin-1 receptor antagonist | | Multiplex ELISA |
| IL-2RA | Interleukin-2 receptor antagonist | | Precoated single ELISA |
| FGF-2 | Fibroblast growth factor 2 | | Multiplex ELISA |
| MMP-1 | Matrixmetalloprotease-1 | | Multiplex ELISA |
| MMP-12 | Matrixmetalloprotease-12 | | Multiplex ELISA |

### 7.1.5 Statistical analysis

All results were analysed using GraphPad Prism® 6 (GraphPad Software Inc., La Jolla (USA)).

For all experiments conducted with hMDMs, each individual blood donor was considered as one biological replicate. In terms of experiments performed regarding *C. albicans*-related read-outs (e.g. hyphal length or gene expression), experiments were considered as biological replicates when they were performed on different days and from independent o/n cultures. To ensure technical consistency, at least two technical replicates were analysed per sample, which were averaged prior to statistical analysis. Routinely, data were analysed using one-way ANOVA with Dunnett's multiple comparison correction. In case of microarray experiments, a paired t-test was used for statistical analysis as only two conditions were compared and to remove the strong donor-dependency in hMDM experiments. To specify the statistical analysis, p-values are shown in Table 25.

**Table 25: Significance levels.**

P-values and their respective symbols used to specify statistical significance.

| Symbol | P-value |
|---|---|
| * | ≤ 0.05 |
| ** | ≤ 0.01 |
| *** | ≤ 0.001 |
| ns | Not significant, > 0.05 |
| nd | Not detectable |

## 7.2 Curriculum vitae

### 7.2.1 List of publications

**2021** **König A***, Müller R*, Mogavero S, Hube B

Fungal factors involved in immune evasion, modulation and exploitation during infection, *Cellular Microbiology* 2021 Jan;23(1):e13272. doi: 10.1111/cmi.13272. Review. *these authors contributed equally to this work

**2020** **König A**, Hube B, Kasper L

The Dual Function of the Fungal Toxin Candidalysin during *Candida albicans*-Macrophage Interaction and Virulence, *Toxins (Basel)* 2020 Aug; 12(8): 469. doi: 10.3390/toxins12080469. Review.

**König A**, Kasper L, Hube B

Das *Candida albicans* Toxin Candidalysin – das Resultat einer Ko-Evolution von Mensch und Pilz**,** *derm Praktische Dermatologie* Ausgabe 04/2020, derm (26) 2020. Review.

**2018** Kasper L*, **König A***, Koenig PA*, Gresnigt MS, Westman J, Drummond RA, Lionakis MS, Groß O, Ruland J, Naglik JR, Hube B:

The fungal peptide toxin Candidalysin activates the NLRP3 inflammasome and causes cytolysis in mononuclear phagocytes, *Nature Communications* 2018 Oct 15;9(1):4260. doi: 10.1038/s41467-018-06607-1. * these authors contributed equally to this work

**2017** Naglik JR, **König A**, Gaffen SL, Hube B:

*Candida albicans*-epithelial interactions and induction of mucosal innate immunity, *Current Opinion in Microbiology* 2017 Dec; 40:104-112. doi: 10.1016/j.mib.2017.10.030. Review.

**2015** **Franke A**

"The impact of the *Candida albicans* protein Ece1 on macrophage damage and inflammatory response", Master's thesis, Friedrich Schiller University Jena.

**2013 Franke A**

„Abklärung neuer Aminosäuresubstitutionen in der Thymidinkinase des Herpes Simplex Virus-Typ 2“, Bachelor’s thesis, Friedrich Schiller University Jena.

### 7.2.2 Conference participation

02/2020 2-day workshop “Eukaryotic Pathogens”, Innsbruck (Austria), *Talk*

09/2019 53th Scientific Conference of the German speaking Mycological Society, Mannheim (Germany), *Talk*

02/2018 70th Annual Conference of the German Society of Hygiene and Microbiology, Bochum (Germany), *Poster*

10/2017 JSMC Symposium, Jena (Germany), *Poster*

10/2017 InfectoOptics Conference Life meets Light, Jena (Germany), *Talk and Poster*

04/2017 Conference on Intracellular Niches of Pathogens (SPP 1580 Meeting), Glashütten (Germany), *Poster*

03/2017 Microbiology and Infection 2017 – 5th Joint Conference of the DGHM & VAAM, Würzburg (Germany), *Poster*

03/2017 MiCom 2017 – 6th International Conference on Microbial Communication of Young Scientists, Jena (Germany), *Talk*

12/2016 JSMC Symposium, Jena (Germany), *Poster*

10/2016 IFo-Fun 2016 – Virulence Mechanisms of Phyto- and Human-Pathogenic Fungi, Erlangen (Germany), *Talk and Poster*

05/2016 Cell Death, Inflammation & Immunity Conference, Crete (Greece), *Talk and Poster*

01/2016 2-day workshop “Eukaryotic Pathogens”, Aachen (Germany), *Talk*

### 7.2.3 Awards and travel grants

09/2019 Publication award of the German speaking Mycological Society

Kasper, **König**, Koenig *et al.*, 2018, *Nature Communications*

11/2018 Paper of the Month of the German Society of Hygiene and Microbiology

Kasper, **König**, Koenig *et al.*, 2018, *Nature Communications*

| | |
|---|---|
| 01/2016 | Travel grant from the German Society of Hygiene and Microbiology, Specialist Group Eukaryotic Pathogens to attend their annual status workshop |
| 07/2010 | Best trainee of the year, Award of the German Chemical Society |
| 07/2008 | Best student in the subject chemistry, Award of the German Chemical Society |

### 7.2.4 Additional training and activities

#### Student research assistant

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|---|---|
| 11/2012 – 09/2015 | Student research assistant at University Hospital Jena, Hans Berger Department of Neurology, Group of Fetal Brain Development |

#### Teaching commitment

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|---|---|
| 11/2017 – 04/2019 | Mentoring of a Master Student (practical work + thesis), Lina J. Dally |
| 09/2017 | Assistance with 2-week practical course for biochemistry students: Molecular & Microbial Infection Biology |
| 06/2016 | Assistance with 2-day JSMC/ILRS course: *Ex vivo* and *in vitro* infection models to study host-pathogen interactions |

#### Workshops

| | |
|---|---|
| 09/2019 | Summer School - Imaging technologies for diagnostics in sepsis and infectious diseases |
| 10/2017 | Introduction to the GxPs with focus on GMP and GLP |
| 09/2017 | Scientific writing and publishing |
| 05/2017 | Project management |
| 05/2016 | English Conversation |
| 01/2016 | English Grammar and Pronunciation |

#### Public relation activities

| | |
|---|---|
| 11/2019 | Assistance during the Long Night of Science, HKI, Jena |
| 09/2016 | Assistance of HKI information stand at the Carl-Zeiss-Day, HKI, Jena |
| 04/2016 | Supervision of students during the Forsche-Schüler-Tag, HKI, Jena |
| 02/2016 | Assistance at FEBS course: Realisation of German evening, HKI, Jena |
| 04/2015 | Supervision of students during the Forsche-Schüler-Tag, HKI, Jena |

## 7.3 Statutory Declaration/ Eigenständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbst verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Mir ist die geltende Promotionsordnung der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena bekannt.

Personen, die mich bei den Experimenten, der Datenanalyse und der Verfassung der Manuskripte unterstützt haben, sind als Ko-Autoren auf den entsprechenden Manuskripten verzeichnet. Personen, die mich bei der Verfassung der Dissertation unterstützt haben, sind in der Danksagung der Dissertation vermerkt.

Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen.

Es haben Dritte weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die vorliegende Arbeit wurde in gleicher oder ähnlicher Form noch bei keiner anderen Hochschule als Dissertation eingereicht und auch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung verwendet.

Jena, den _____

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Annika Gerlind Luise König

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