The *Candida albicans* factor *MNN9* modulates cytokine production in distinct epithelial cell types

Dissertation

To Fulfill the
Requirements for the Degree of
„doctor rerum naturalium“ (Dr. rer. nat.)

FRIEDRICH-SCHILLER-
UNIVERSITÄT
JENA

Submitted to the Council of the Faculty of
Biological Sciences
of the Friedrich Schiller University Jena

by Diploma Biologist

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28th of August 2020, Jena

This thesis was prepared at the “Leibniz-Institute for Natural Product Research and Infection Biology – Hans Knöll Institute” in the Department of Microbial Pathogenicity Mechanisms under the supervision of Prof. Bernhard Hube and in the Research Group Microbial Immunology under the supervision of Prof. Ilse D. Jacobsen.

This work was financed by the University Hospital Jena and the HKI.

Table of contents

1 Summary i

2 Zusammenfassung iii

3 Introduction 1

3.1 The human pathogen *Candida albicans* 1

3.1.1 Clinical relevance 1

3.1.2 Mucosal infections 1

3.1.3 Morphological plasticity 2

3.1.4 Stages of pathogenesis 2

3.2 The cell wall of *Candida albicans* 3

3.2.1 Composition and genes involved in cell wall synthesis 3

3.2.2 The *Candida albicans* mannosyltransferase Mnn9 6

3.3 Interaction of *Candida albicans* with epithelial cells 7

3.3.1 Different recognition by myeloid and epithelial cells 7

3.3.2 Discrimination between commensal and pathogenic *Candida albicans* 9

3.3.3 Role of the inflammasome in the host response 10

4 Aims of this study 12

5 Material and methods 13

5.1 Material 13

5.1.1 Strains 13

5.1.2 Cell lines 14

5.1.3 Plasmids and oligonucleotides 14

5.1.4 Media, buffers, kits, enzymes, and chemicals 16

5.1.5 Antibodies 21

5.2 Methods 23

5.2.1 *Escherichia coli* storage and cultivation 23

5.2.2 *Candida albicans* storage, cultivation, and growth conditions 23

5.2.3 Cell lines cultivation 24

5.2.4 Isolation of genomic DNA from *Candida albicans* 24

Table of contents

5.2.5 DNA amplification via polymerase chain reaction (PCR) 24
5.2.6 Generation of *C. albicans MNN9* mutant and revertant strains 25
5.2.7 Southern Blotting 27
5.2.8 Epithelial cell damage via LDH release 27
5.2.9 Cytokine determination 27
5.2.10 Large-scale screening of a *C. albicans* mutant collection 28
5.2.11 Stimulation of epithelial PRRs with agonists 28
5.2.12 PRR expression in epithelial cells by flow cytometry 28
5.2.13 Yeast PAMP surface exposure by flow cytometry 29
5.2.14 Hyphal PAMP surface exposure by fluorescence microscopy 29
5.2.15 Adhesion, invasion, and hyphal length 29
5.2.16 Protein isolation and determination of protein concentration 30
5.2.17 Target expression analysis via western blotting 31
5.2.18 Quantitative target expression via Smart Protein Layer (SPL) western blotting 31
5.2.19 Inflammasome activation, blocking of the IL-1 receptor, and blocking of the NLRP3 inflammasome 33
5.2.20 Murine systemic candidiasis model 33
5.2.21 Ethic statement 33
5.2.22 Statistical analysis 34
6 Results 35
6.1 Epithelial damage and cytokine release in response to *C. albicans* infection 35
6.1.1 C. albicans causes less damage to oral epithelial cells in comparison to hepatic and renal epithelial cells 35
6.1.2 The cytokine response towards *C. albicans* infection differs between epithelial cells 36
6.2 Pattern recognition receptors (PRRs) expressed by different epithelial cells 38
6.2.1 Hepatic, renal, and oral epithelial cells express a similar repertoire of PRRs . 38
6.2.2 Epithelial cells differ in the responsiveness to PRR ligands 40
6.3 Fungal factors modulating the epithelial cytokine response 41

6.3.1 Identification of *C. albicans* factors that influence epithelial cytokine secretion 42

6.3.2 A defect in the mannosyltransferase II partly encoded by *MNN9* modulates the epithelial-specific cytokine response 43

6.4 Influence of cell wall mannosylation on the epithelial immune response 44

6.4.1 The degree of mannosylation affects epithelial damage and cytokine response 45

6.4.2 Induction of the epithelial inflammasome-associated IL-1 secretion 46

6.4.3 Strain-dependent adhesion and invasion properties do not correlate with the induced cytokine response 49

6.4.4 Normal yeast cell growth of glycosylation mutant strains 51

6.4.5 Mannosylation mutant strains expose more β-1,3-glucan and chitin on their surface 52

6.5 Analysis of signaling pathways involved in the epithelial immune response 55

6.5.1 Activation of *C. albicans*-induced mitogen-activated protein kinase (MAPK) signaling 56

6.5.2 Activation of *C. albicans*-induced NF-κB signaling 59

6.6 *MNN9* is required for full virulence *in vivo* 60

7 Discussion 63

7.1 Mucosal and parenchymal epithelial cells produce cytokines that could contribute to the innate immune response 63

7.2 Colonized epithelial cells express inflammasomes that enable early recognition of pathogen-induced damage by the secretion of IL-1β 64

7.2.1 Aggregating *C. albicans* strains could elevate the host response induced by PAMPs and DAMPs 65

7.3 Contribution of epithelial PRRs to the immune response 66

7.4 Activation of signaling cascades that induce cytokines is epithelial-specific 68

7.5 Influence of the glycosylation status on the epithelial immune response 70

7.5.1 Deletion of *MNN9* uncouples the correlation in damage and cytokine release 70

7.5.2 Exposure of PAMPs has no exclusive role in the activation of an epithelial response 71

Table of contents

7.5.3 Fungal chitin might have an influence on the epithelial immune response ... 71

7.5.4 Glycosylation mutant strains mimic the cell wall of clinically relevant isolates 72

7.5.5 Impaired adhesion does not necessarily alter the epithelial immune response 73

7.5.6 Candidalysin, the “missing link” in morphogenesis and cell damage?..... 73

7.6 Early recognition of the β-glucan unmasked *MNN9* mutant could induce neutrophil recruitment in the kidney that reduces virulence in murine disseminated candidiasis.... 73

7.7 Concluding remarks and outlook 75

8 Supplement I

9 List of abbreviations..... IX

10 References XI

11 Appendix..... XXIII

11.1 Curriculum vitae XXIII

11.2 Publication..... XXIII

11.3 Talks and poster presentations..... XXIII

11.4 Additional activities XXIV

11.5 Supervision XXV

11.6 Affiliations XXV

11.7 Grants and awards..... XXV

11.8 Acknowledgements XXVI

11.9 Selbstständigkeitserklärung..... XXVII

1 Summary

The opportunistic fungal pathogen *Candida albicans* colonizes mucosal surfaces of the majority of healthy human individuals. However, the fungus can cause superficial infections in otherwise healthy people and could develop life-threatening systemic candidiasis in immunocompromised patients. Mucosal epithelial cells can discriminate the shift from the commensal to the pathogenic lifestyle of *C. albicans* by the activation of a biphasic MAPK mediated by PRRs. Previous studies mainly focused on the mucosal cytokine response from oral and vaginal epithelial cells that can be affected by superficial infections. This study aimed to compare the cytokine release from oral mucosal epithelial cells with parenchymal hepatic and renal epithelial cells. While all three tested cell lines released IL-6 and IL-8 in response to *C. albicans*-induced cell damage, secretion of other inflammatory cytokines was cell line-specific. The PRR-mediated damage-dependent cytokine secretion suggested an epithelial-specific expression of PRRs. However, all cell lines expressed a similar pattern of TLR2, TLR4, dectin-1, dectin-3, and MR that were not inducible by *C. albicans* infection or stimulation with cytokines. Different responsiveness of PRR stimulation with ligands and the epithelial-specific cytokine release further suggested an epithelial-specific induction of signaling cascades downstream of PRRs. Indeed, MKP1 phosphorylation displayed cell-specific kinetics that were independent of early host cell damage. The epithelial immune response can be modulated by *C. albicans* factors that was observed during a large-scale host response screening. This study focused on Mnn9 that is crucial for the *N*-mannosylation of the cell wall and therefore has an influence on host recognition. A deletion in the *MNN9* gene induced stronger inflammatory cytokine releases by hepatic and oral epithelial cells without altering the damage potential. Using a set of mannosylation mutant strains, the influence of the glycosylation status of *C. albicans* cell wall on epithelial damage and cytokine induction was further analyzed. Compared to the *MNN9* mutant a similar phenotype in the epithelial immune response was observed for the *MNN2* sextuple mutant. To activate the epithelial immune response adhesion to host cells is considered as a prerequisite step. However, the *MNN9* mutant adhered weaker on hepatic cells and was less invasive in all tested cell lines, suggesting a more complex interaction of different fungal factors with epithelial cells. The *MNN9* mutant induced an elevated oral IL-1α and IL-1β release that was partly dependent on the IL-1 receptor but independent of the NRLP3 inflammasome. Notably, the release of inflammasome-associated cytokines was only observed in oral but not renal or hepatic epithelial cells. Differences observed in the epithelial cytokine response by different glycosylation statuses of the cells suggested an altered PAMP exposure. However, all tested strains displayed a similar and strong surface exposure of β-1,3-glucan and chitin. While the process of the epithelial receptor-mediated recognition of *C. albicans* is still unknown, previous findings in this study suggested differences in the activation of MAPK signaling causing elevated cytokine releases induced by the *MNN9* mutant. Interestingly, while the *MNN9* mutant induced a delayed activation of oral MKP1 as well as of ERK1/2 and JNK, consistent phosphorylation indicates stronger and prolonged activation of all three MAPK pathways during infection that could explain the increased cytokine release. A mouse model of disseminated candidiasis revealed that *MNN9* is required for full virulence, demonstrating the importance of an

intact mannosylation layer of the *C. albicans* cell wall. Taken together, this study could show that the epithelial immune activation in response to *C. albicans* differs not only between mucosal and parenchymal cells, it is also cell type-specific. Moreover, the cytokine secretion can be modulated by alterations in the fungal cell wall in the absence of Mnn9.

2 Zusammenfassung

Der opportunistische Pilzpathogen *Candida albicans* besiedelt die Schleimhäute bei der Mehrzahl der gesunden Individuen. Der Pilz kann jedoch Oberflächeninfektionen im ansonsten gesunden Menschen auslösen, sowie lebensbedrohliche, systemische Candidosen in immungeschwächten Patienten entwickeln. Mukosale Epithelzellen sind in der Lage, eine Veränderung vom kommensalen hin zum pathogenen Lebenszyklus von *C. albicans* mit Hilfe der Mustererkennungsrezeptoren (PRR) zu unterscheiden und ein zweistufiges Signalkaskadesystem (MAPK) zu aktivieren. Vorhergehende Studien fokusierten sich hauptsächlich auf die mukosale Zytokinantwort von oralen und vaginalen Epithelzellen, die durch Oberflächeninfektionen betroffen sein können. Diese Studie zielte darauf ab, die Zytokinsekretion von oralen, mukosalen Epithelzellen mit der von parenchymalen, hepatischen und renalen Epithelzellen zu vergleichen. Während alle drei getesteten Zelllinien IL-6 und IL-8 als Reaktion auf die von *C. albicans*-induzierte Schädigung freisetzen, wurden andere inflammatorische Zytokine zelltypspezifisch sekretiert. Die PRR-vermittelte und schädigungsabhängige Zytokinsekretion suggerierte eine epithelspezifische Expression von PRRs. Jedoch exprimierten alle Zelllinien ähnlich viel TLR2, TLR4, Dectin-1, Dectin-3 und MR, welche durch Infektion mit *C. albicans* oder Stimulierung mit Zytokinen nicht stärker induziert werden konnten. Die unterschiedliche Stimulierbarkeit von PRRs mittels Liganden und die epithelspezifische Zytokinfreisetzung legten nahe, dass es zu einer unterschiedlichen Aktivierung von Signalkaskaden kommt. Tatsächlich erfolgt eine MKP1-Phosphorylierung zeit- und zelltypspezifisch, ist jedoch unabhängig von einer frühen Zellschädigung. Die Immunantwort von Epithelzellen kann durch *C. albicans*-Faktoren reguliert werden, welche im Rahmen eines Screenings der Wirtsimmunantwort analysiert wurde. Diese Studie fokussierte sich auf den Faktor Mnn9, welcher einen entscheidenden Einfluss auf die *N*-Mannosylierung der Zellwand hat und deshalb auch direkt die Erkennung durch den Wirt beeinflusst. Ein Defekt im *MNN9*-Gen führte zu einer stärkeren Freisetzung inflammatorischer Zytokine durch hepatische und orale Epithelzellen, ohne das Schädigungspotential im Vergleich zum Wildtyp zu verändern. Durch die Verwendung einer Kollektion von verschiedenen Mannosylierungsmutanten wurde der Einfluss des Glykosilierungsstatus der *C. albicans*-Zellwand auf die Schädigung und Zytokinfreisetzung von Epithelzellen weitergehend untersucht. Im Vergleich zur *MNN9*-Mutante wurde ein ähnlicher Phänotyp in der Immunantwort von Epithelzellen bei der *MNN2*-Sechsfachmutante beobachtet. Um eine Immunantwort von Epithelzellen zu induzieren, wird angenommen, dass die Adhäsion an Wirtszellen eine Grundvoraussetzung darstellt. Die *MNN9*-Mutante jedoch adhärierte schwächer an hepatischen Zellen und war weniger invasiv in allen drei getesteten Zelllinien gewachsen, was mutmaßlich durch ein komplexes Zusammenspiel aus verschiedenen Pilzfaktoren mit Epithelzellen hervorgerufen wird. Außerdem induzierte die *MNN9*-Mutante eine erhöhte IL-1α- und IL-1β-Sekretion von oralen Epithelzellen, die teilweise vom IL-1-Rezeptor abhängig war, jedoch unabhängig vom NLRP3-Inflammasom. Auffallend war, dass Inflammasom-assoziierte Zytokine nur von oralen, und nicht von renalen und hepatischen Epithelzellen freigesetzt wurden. Die durch einen veränderten Glykosylierungsstatus der Zellen hervorgerufenen Unterschiede in der Zytokinsekretion führten zu der Annahme, dass die Zellwand

von *C. albicans* ein verändertes Pathogen-assoziiertes, molekulares Muster (PAMPs) exponiert. Alle getesteten Stämme zeigten jedoch eine ähnliche sowie stark erhöhte Exponierung von β-1,3-Glukan und Chitin. Zwar ist der Prozess der Rezeptor-basierten Erkennung von *C. albicans* weiterhin unbekannt, die vorangegangenen Ergebnisse dieser Studie deuten jedoch auf eine unterschiedliche Aktivierung des MAPK-Signalweges, das wiederum zur erhöhten Zytokinfreisetzung durch die *MNN9*-Mutante führte. Interessanterweise stellte sich dabei heraus, dass es zu einer verzögerten Aktivierung von MKP1, ERK1/2 und JNK in oralen Epithelzellen durch die *MNN9*-Mutante kommt. Eine kontinuierlich ansteigende und über einen längeren Zeitraum anhaltende Phosphorylierung aller drei Signalkaskaden könnte jedoch die zuvor beobachtete, erhöhte Zytokinfreisetzung erklären. Weiterhin stellte sich heraus, dass im systemischen Candidose-Model der Maus, die *MNN9*-Mutante eine stark reduzierte Virulenz aufweist. Dadurch wird deutlich, dass eine intakte Mannosylierung der Zellwand wichtig für die Pathogenität ist. Zusammengefasst kann gesagt werden, diese Arbeit konnte zeigen, dass die Immunantwort von Epithelzellen in Bezug auf *C. albicans*-Infektionen nicht nur davon abhängt, ob die Epithelzellen aus der Schleimhaut oder dem Parenchym innerer Organe stammen, sondern zelltypspezifisch ist. Die Zytokinsekretion kann zudem durch die Veränderung der pilzlichen Zellwand, hergerufen durch das Fehlen von Mnn9, moduliert werden.

3 Introduction

3.1 The human pathogen *Candida albicans*

Fungi are ubiquitously distributed and represent approximately 7% of all species on earth [1]. Some of them substantially contribute to life-threatening human infections that cause high mortality rates including the genera *Cryptococcus*, *Aspergillus*, *Pneumocystis*, and *Candida* [2]. From more than 150 known *Candida* species, 15 of them are opportunistic human pathogens with *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* frequently isolated [3]. In contrast to other fungal pathogens, some *Candida* species and in particular *C. albicans* and *C. glabrata* are commensals of the human microbiota colonizing mucosal surfaces [4].

3.1.1 Clinical relevance

C. albicans is a harmless member of the endogenous human mycobiota of about 30-70% of healthy individuals and colonizes mucosae of the urogenital- and gastrointestinal tract and oropharynx [4]. The lifestyle of *C. albicans* can shift from commensal to pathogenic affecting immunocompromised but also immunocompetent individuals and occurs in neonatal intensive care [2,5]. Life-threatening systemic candidiasis can develop through the breakdown of gastrointestinal barriers, in patients with immunosuppression, or is associated with the use of medical devices such as central venous catheters [6]. *Candida spp*. are the fourth most common cause of nosocomial bloodstream infections with the highest incidence of *C. albicans* [3,7,8]. Candidaemia-caused crude mortality ranges from >20% observed in German ICU's [7] up to 49% at ICU's in the United States of America [9,10]. Early fungal-specific diagnosis is limited, resulting in delayed or inappropriate antifungal therapy. Besides that, antifungal drugs that directly target fungal virulence factors such as *C. albicans* dimorphism are not available [11]. Currently, classes of polyenes, azoles, and echinocandins are applied in antifungal prophylaxis. These antifungals, however, are often fungistatic that gives the fungus time to develop resistance [12]. Azoles (e.g. fluconazole, voriconazole) and polyenes (e.g. amphotericin B) target ergosterol formation that is a major component of the fungal cell membrane, while echinocandins (e.g. caspofungin) target the cell wall by inhibiting the β-1,3-glucan synthase [13]. Even though the prevalence of resistant strains to fluconazole increases, this antifungal is most commonly administered to patients suffering from bloodstream infections [10]. Nevertheless, resistance to the newest, clinical approved antifungal class of echinocandins has also been reported in recent years [14].

3.1.2 Mucosal infections

While systemic infections caused by *C. albicans* display very high mortality rates, much higher morbidity rates are caused by superficial infections affecting the oral cavity and vaginal tract that usually do not lead to death [2,15].

Thrush or oropharyngeal candidiasis (OPC) affects worldwide 10 million people annually. Particularly common risk factors for OPC are immunosuppression, e.g. by HIV, but also cancer, transplantations, antibiosis, and the uses of dentures [16]. OPC is characterized by the invasion of epithelial cells from the oral mucosa that induces host cell damage by the secreted peptide-toxin Candidalysin [17].

The most prevalent form of *Candida* infection is vulvovaginal candidiasis (VVC) affecting 50-75% of immunocompetent women at least once in their lifetime [18]. One-tenth of them further develop recurrent or chronic VVC suffering at least four episodes annually [18]. Yeast cells of *C. albicans* are often isolated from healthy women indicating that this morphology is tolerated by the host. In contrast to OPC, *C. albicans* induced inflammatory cytokines that recruit neutrophils are detrimental to vaginal immunopathology. This immune cell recruitment during VVC induces an overwhelming immune response that worsens inflammation without reducing fungal burden [19].

Due to its high prevalence, both superficial fungal infections have been studied in different *in vitro* models such as monolayer cell cultures (oral epithelial cells, vaginal epithelial cells) or reconstituted human epithelium (oral RHE and vaginal RHVE [20,21]) and mouse models of OPC [22] and VVC [23]. To investigate *C. albicans* virulence in systemic infections the mouse intravenous challenge model is most commonly used.

3.1.3 Morphological plasticity

C. albicans is a polymorphic fungus that can grow as unicellular yeast, pseudohyphae or true hyphae [24]. It can switch between these morphotypes that enables colonization of the host (yeast) and invasion (filaments). This morphological plasticity is a critical step in *C. albicans* pathogenicity but not in non-filamenting *C. glabrata* and *C. parapsilosis*. Filamentation in *C. albicans* is mediated by the core response of eight gene products including the cell wall-associated proteins Als3, Ece1, Hwp1, and Rbt1 [25] and involves transcription factors such as Rfg1 [26]. The thigmotropic orientation of growing filaments further facilitates *C. albicans* to invade and damage host cells [27]. Different cell morphologies have a divergent influence on the host immune response. While *C. albicans* yeast cells induce a strong cytokine response of human PBMCs, pseudohyphae and hyphae induce a weaker innate immune response [28]. In contrast, *C. albicans* hyphae induce a stronger TNF-α response in macrophages [29], and induce more cytokines in oral and vaginal epithelial cells [30,31] than yeast cells. The shift of different environmental signals are considered to initiate or affect filamentation, e.g. the presence of *N*-acetylglucosamine, or serum, neutral pH, CO2, temperature, the presence of quorum sensing molecules such as farnesol, and nutrients availability [32,33]. Following hyphal initiation, hyphal maintenance is also important in virulence mediated by the *C. albicans* factor Eed1 [34].

3.1.4 Stages of pathogenesis

In order to cause fungal disease, three stages of pathogenesis can be discriminated in the shift from a commensal to a pathogenic lifestyle of *C. albicans* [35]: adhesion, invasion, and host cell damage. Some factors that are involved in these stages have functional redundancy that cannot distinctly be separated. Besides this, *C. albicans* is continuously interacting with the host also in the commensal phase, and therefore adhesion only is not an intrinsic characteristic of pathogenesis. The majority of human mucosae are comprised of epithelial cells that coat outside surfaces of organs or the human body [36]. Therefore, these epithelial cells stay in direct contact with the outlining microbial environment and represent a critical role in the first host response.

Adhesion is mediated through fungal cell wall moieties that interact with host cell receptors to allow a covalent attachment [37]. Fungal Als3 (agglutinin-like sequence 3) and Ssa1 interact with surface expressed E-Cadherin and EGFR/HER2 (epidermal growth factor receptor/human epidermal growth factor 2) expressed by oral cells [38]. Other fungal adhesion proteins include Eap1 (enhanced adhesions to polystyrene), Iff4, and Hwp1 (hyphal wall protein 1) that interact with epithelial cells but the epithelial-mediated recognition is unknown. The initial contact to host cells is likely mediated by yeast cells, while a morphological switch from yeast to hyphae induces the expression of more adhesins that confers stronger adhesion [39]. Following adhesion, a strong induction of hypha formation occurs in less than one hour *in vitro* and is accompanied with the expression of hypha-associated proteins that are involved in further adhesion, invasion, and host cell damage that ultimately result in immune activation [40]. Opposing roles in epithelial adhesion has been observed for different members of the Als family of cell surface adhesins: while disruption of *ALS2* [41] and ALS3 [42] reduces epithelial adhesion, deletion of *ALS5*, *ALS6*, or *ALS7* leads to increased adhesion [43].

Invasion into host cells is hypha-dependent and can occur by host-driven induced endocytosis that does not require viable hyphae, or fungal-driven active penetration [44]. Receptor-mediated induced endocytosis is a critical step in OPC and requires the interaction of fungal Als3 and Ssa1 with host E-cadherin [45] and the receptors EGFR/HER2 [38] that causes remodeling of the actin cytoskeleton, and the downstream AhR (aryl hydrocarbon receptor) [46]. This invasion route, however, occurs in oral [47] but not in intestinal epithelial cells [48,49]. The more prevalent invasion strategy in distinct cell types is believed to be active penetration [47] that relies on fungal turgor pressure and secreted proteinases from the Sap family [50–52]. Disruption of *SAP* genes has been demonstrated to impair both fungal invasion and adhesion properties with a reduced adhesion caused by a deletion of *SAP1*, *SAP2*, *SAP3*, and *SAP10*, and increased adhesion caused by a deletion of *SAP9* [53,54]. Compared to induced endocytosis, active penetration occurs at later time points during infection *in vitro*. However, oral mucosa is comprised of an outermost layer with terminally differentiated cells and an underlying layer with proliferating epithelial cells that require both processes to facilitate fungal invasion and to establish infection [37].

Damage of host cells is caused by fungal hyphae during deep invasion and is accompanied by the secretion of hydrolases [55]. Recently, fungal-induced epithelial damage was directly linked with the secretion of Candidalysin, an Ece1-derived peptide toxin [17,56] that intercalates into membranes causing lesions. Translocation from mucosal surfaces into the bloodstream can cause life-threatening fungal disease, however, this does not necessarily induce host cell damage [49]. Damage of oral epithelial cells induced by *C. albicans* can occur via necrosis or apoptosis [57,58]. Translocation through the intestinal barrier is induced by necrosis only [49].

3.2 The cell wall of *Candida albicans*

3.2.1 Composition and genes involved in cell wall synthesis

The fungal cell wall equally provides rigidity to convey the cell shape and flexibility to enable cell wall remodeling during growth, morphogenesis, and pathogenesis. 90% of the *C. albicans* cell wall consists

of the three major polysaccharides mannan, β-glucan, and chitin (Fig. 1). Compositions of cell wall polysaccharides vary between strains and conditions, however, about 60% are β-glucans, 35-40%

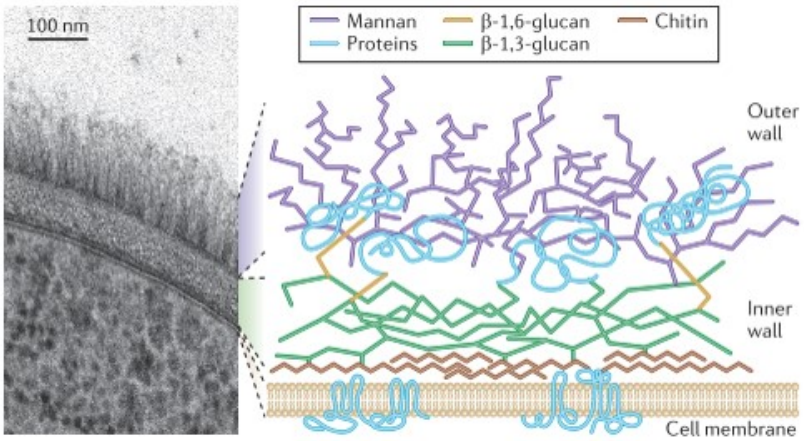

Fig. 1. *Candida albicans* cell wall structure.
The *C. albicans* cell wall consists of an inner layer of β-glucan and chitin, and an outer layer made of *N*- and *O*-linked mannans that are linked with proteins. Modified from Gow *et al*., 2012 [82].

mannoproteins, and 1-2% chitin [59]. β-1,3-glucan is covalently linked to chitin within the inner layer and to mannoproteins of the fibrillary outer cell wall layer via β-1,6-glucan. These mannoproteins are connected via glycosylphosphatidylinositol (GPI)-anchors to β-glucans or to the cell membrane.

Cell wall proteins are post-translationally modified through the addition of *N*- and *O*-linked mannans [59] that are (besides β-glucan and chitin) important pathogen-associated molecular patterns (PAMPs) recognized via host pattern recognition receptors (PRRs). Linear *O*-linked glycosylation consists of α-1,2-mannose residues and requires gene activity of *MNT1*, *MNT2*, members of the *PMT* family, and indirectly *PMR1* [60]. *O*-linked glycosylation is required for mucosal adhesion and virulence in murine disseminated candidiasis [61–63].

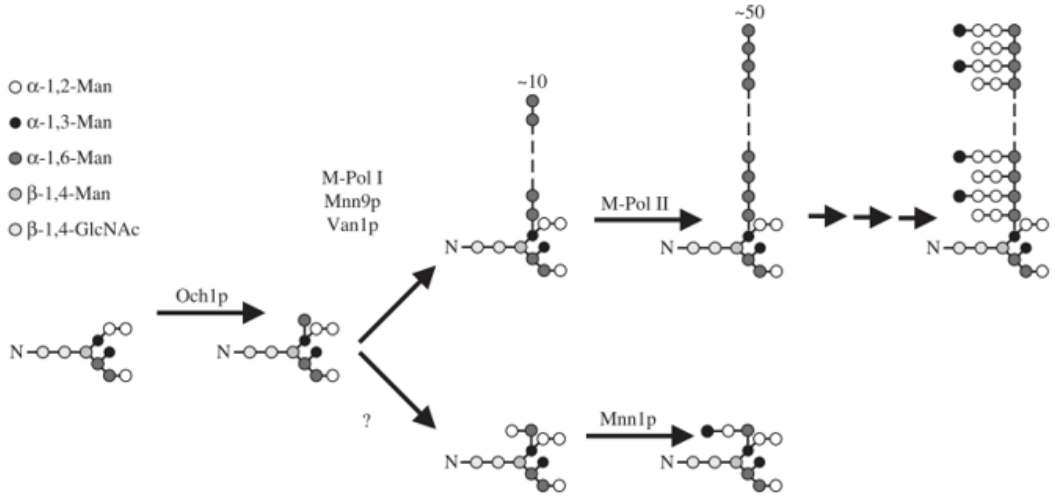

Fig. 2. Involvement of polymerase I and II in the *N*-mannan backbone biosynthesis.
The mannosyltransferase Och1 catalyzes addition of the first α-1,6-mannosyl residue to the *N*-mannan core. Further elongation of the backbone is performed by the polymerase I and II complex. Decoration of the backbone with different sidechains mainly involves the activity of members from the Mnn1 and Mnn2 family. Modified from Striebeck *et al*., 2013 [69].

Complex branched *N*-mannosylation is important in the fungal-epithelial interaction inducing a host response and is required for full virulence [60]. The *N*-glycosylation structure can be divided into the *N*-mannan core that branches into the backbone and sidechains. Synthesis of *N*-linked glycans has been studied in the nonpathogenic *Saccharomyces cerevisiae* [64], and many related genes found in *C. albicans* are orthologues with an equal activity. The process of mannosylation starts with the formation of the *N*-mannan core during protein synthesis in the lumen of the endoplasmic reticulum [65,66]. The core consists of a dolichol pyrophosphate anchored oligosaccharide $Glc_3Man_9GlcNAc_2$ that is transferred onto asparagine residues within the polypeptide [67]. After processing by glycosidases, the mature core $Man_8GlcNAc_2$ is formed that is essential for the outer chain of branched *N*-mannan. Further attachments of mannose are then performed by mannosyltransferases in the Golgi apparatus. The *N*-mannan backbone synthesis starts with the first α-1,6-mannosyl residue added to the core by the mannosyltransferase Och1 [68] (Fig. 2). Then, the polymerase I complex composed of the mannosyltransferases Mnn9 and Van1 form the first part of the α-1,6-mannose backbone of about 10 α-1,6-mannosyl residues [69,70]. This backbone is further extended by the mannan polymerase II complex composed of Mnn9 and Anp1 [71]. Recently, Mnn10 was identified as another important subunit of the mannan polymerase for the extension of the α-1,6-mannose backbone [72]. The backbone is decorated by sidechains of α-1,2-mannose and involves gene activity of *MNN5* [73,74] and members of the *MNN2* family (*MNN2*, *MNN21*, *MNN22*, *MNN23*, *MNN24*, *MNN26*). Sequential deletion of *MNN2* genes results in altered cell wall composition, enhanced sensitivity against cell wall stressors, and attenuation in virulence, with the most severe phenotype when all members are deleted in a *MNN2* sextuple mutant [75]. The addition of α-1,2-mannosyl sidechains can also be performed by Mnt5, showing a functional redundancy in the highly branched *N*-mannosylation [76].

Outer chains are further capped with either β-1,2-mannose by Bmt1 and Bmt3 [77], or α-1,3-mannose units by members of the *MNN1* gene family (*MNN1*, *MNN12*, *MNN13*, *MNN14*, *MNN15*, *MNN16*). While the gene products of the *MNN1* family are considered to display functional redundancy, deletion of *MNN14* results in avirulence in murine disseminated candidiasis and is associated with only subtle defects in mannosylation [78].

Further branching of the α-1,2-mannose sidechains occurs by adding phosphomannan through a phosphodiester bond via Mnn4 and Mnn6 [79] and is required for macrophage phagocytosis [80]. Glycosylation elaborated by mannosyltransferases in the Golgi apparatus requires manganese ions as an essential cofactor [74]. The P-type ATPase Pmr1 is involved in calcium and manganese ion transport into the Golgi apparatus maintaining manganese homeostasis. Therefore, disruption of *PMR1* impairs the general activity of mannosyltransferases influencing *N*- and *O*-mannosylation that leads to attenuated virulence in mice [81]. Experimentally determined *N*- and *O*-glycosylation structures [75] show that a *pmr1*Δ/Δ strain displays less severe defects compared to an *och1*Δ/Δ strain. However, the structure of both mutants is most impaired followed by *mnn9*Δ/Δ, *mnn2*Δ/Δ sextuple, single *mnn2*Δ/Δ, and *mnn1*Δ/Δ.

Underneath the mannoprotein layer, the inner skeletal cell layer consists of β-glucans and chitin that conveys the cell shape [82]. β-1,3-glucan is the structural backbone of the cell wall matrix and is

synthesized by β-1,3-glucan synthase complex consisting of the catalytic subunit Fks1, Gsl1, and the regulatory subunit Rho1 [83]. It is an important antifungal target by inhibiting the β-1,3-glucan synthesis with the antifungal drug echinocandin [13]. Exposure of β-1,3-glucan at yeast bud scars induces a strong inflammatory cytokine response in immune cells and triggers *C. albicans* phagocytosis that is recognized by dectin-1 and TLR2 [84]. Unmasking of the glucan layer by heat inactivation, antifungal drugs, during the progression of infection, or defects in the mannan layer by gene deletions further increases the immunostimulatory effect [85]. Masking the immune activating β-1,3-glucan from the immune system has also been observed by other fungi, e.g. *Histoplasma capsulatum* [86].

Cell wall chitin counts for about 2% of the total cell wall composition in yeast [87], while hyphae contain five times more chitin [88]. Chitin is a polymer of β-1,4-GlcNAc that is synthesized by the chitin synthases Chs1, Chs2, Chs3, Chs8 [89]. However, only Chs1 is essential for *C. albicans* viability [90]. While Chs3 is responsible for about 85% of yeast and hyphal cell wall chitin, Chs2 is hypha-specific [91]. Similar to β-glucan, chitin is only exposed at bud scars [84] and unmasking can be induced by heat and echinocandins but also during neutrophil attack [92]. The immune recognition of chitin is so far not resolved [93], but chitin of specific molecular sizes can induce a cytokine response in macrophages that involves NOD2, TLR9, and MR [94,95].

3.2.2 The *Candida albicans* mannosyltransferase Mnn9

Cell wall mannosylation is critical in host recognition and immune activation of myeloid and epithelial cells. The *C. albicans* gene *MNN9* (*ORF19.7383*) is an ortholog of *S. cerevisiae MNN9* that is 1107 bp long (Candida Genome Database, *C. albicans* Assembly 22). The gene product encodes for a subunit of the mannosyltransferase complex in the Golgi apparatus that elongates the α-1,6-mannose backbone of the *N*-mannosyl residue. Its role in glycosylation and modification of secreted proteins was first described in *S. cerevisiae* [96] and later in *C. albicans* [97]. *MNN9* is expressed in yeast and hyphae with highest rates during filament-inducing conditions (thesis of Carlos Cívicos Villa, 2014; https://gredos.usal.es/jspui/handle/10366/125512). Deletion of *MNN9* results in aggregates of slightly larger yeast cells and, depending on the study, results in formation of pseudohyphae [97] or normal hyphae [98] under filament-inducing conditions. In accordance with other mannosylation mutant strains, the cell wall composition of a *MNN9* deletion strain displays reduced mannan but elevated β-glucan levels [97,99]. As a consequence, the cell wall integrity is affected, and the mutant shows increased sensitivity to Congo Red [99], fluconazole, amphotericin B, and itraconazole (thesis of Carlos Cívicos Villa, 2014). A deletion of *MNN9* further impairs biofilm formation at later time points [100] and decreases the adhesion capability on oral epithelial cells [98]. Fungal factors that are commonly expressed in *C. albicans* to enable adhesion, invasion, and damage of host cells (e.g. members of the Als and Sap family, Ssa1, Ece1) show no obvious differences in expression compared to the wild type [17,97,100]. Impaired *N*-glycosylation caused by deletion of *MNN9* enables this strain to damage oral epithelial cells and activates MKP1 and c-Fos which results in the release of the inflammatory cytokines G-CSF, GM-CSF, IL-1a, and IL-6 [98]. In contrast to many other mannosylation mutant strains, the pathogenicity of a *MNN9* mutant strain has not yet been analyzed in murine models of disseminated candidiasis.

3.3 Interaction of *Candida albicans* with epithelial cells

In order to prevent invasion of pathogenic microorganisms, the human body has evolved a set of immune responses that include physical barriers and the activation of an innate and adaptive immune system. Epithelial cells at mucosal surfaces are constantly exposed to the external environment and represent physical barriers that are not only passive in function but rather constitute the first line of defense [36]. Moreover, epithelial cells are also part of the parenchyma of internal organs, such as kidney and liver. In the past years, epithelial cells are considered as active sensors in the innate immunity that differ from the myeloid immune response mechanisms to recognize, signal, and secret immune effectors in response to fungal infection.

3.3.1 Different recognition by myeloid and epithelial cells

The innate immune response of invading *C. albicans* has been extensively studied and is predominantly mediated by neutrophils, monocytes, macrophages, and dendritic cells that requires recognition of conserved microbial signatures [101]. Recognition of these so-called PAMPs is accomplished by the major classes of PRRs including TLRs (toll-like receptors), CLRs (C-type lectin receptors), and NLRs (nucleotide-binding oligomerization domain (NOD)-like receptors) [101,102] (Fig. 3). TLRs are transmembrane receptors that recognize bacterial, viral, and fungal PAMPs via an

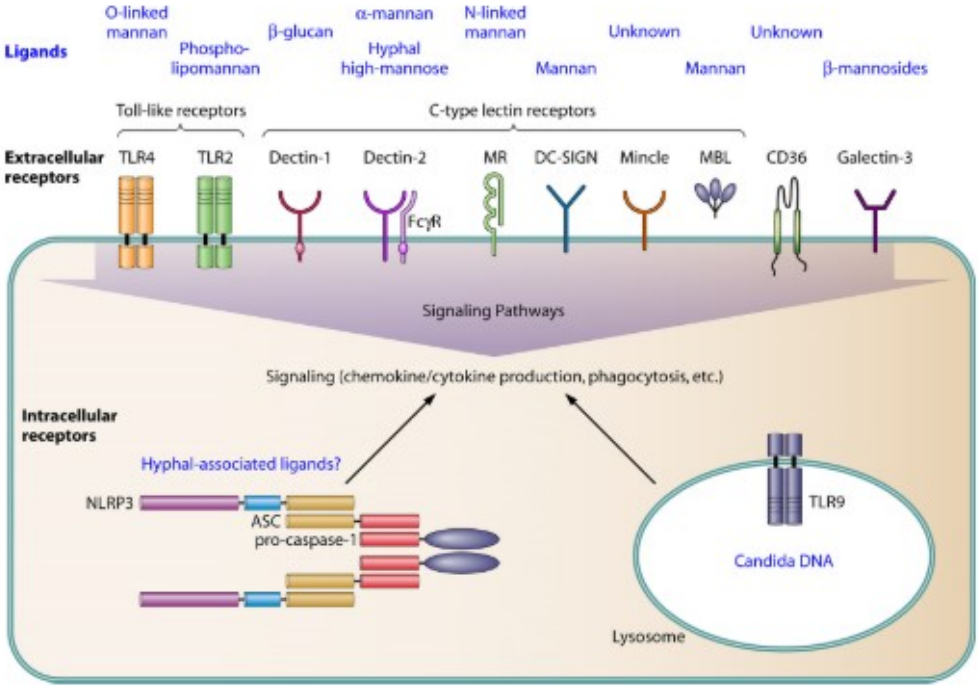

Fig. 3. Pattern recognition receptors that are involved in the recognition of *C. albicans* pathogen-associated molecular patterns.
Toll-like receptors and C-type lectin receptors extracellularly recognize *C. albicans* cell wall components and induce downstream signaling resulting in an immune response. Modified from Cheng and Joosten., 2012 [236].

extracellular LRR domain (leucine-rich repeat), and signal into the cytoplasm via a TIR domain (Toll/interleukin-1 receptor) [103]. CLRs recognize PAMPs via an extracellular CRD (carbohydrate-recognition domain) or CTLD (C-type lectin-like domain) and signal through the cytoplasmic ITAM/ITIM (immunoreceptor tyrosine-based activation/inhibition motif) [104]. NLRs recognize both

pathogen-associated pattern and host-derived molecules. Together with ASC (apoptosis-related speck-like protein containing a CARD) and procaspase-1 they form the inflammasome that activates caspase-1 resulting in the cleavage of pro-IL-1β and pro-IL-18 into their mature and active form [105]. Another class that recognize *C. albicans* involves galectin-3 [106].

PRRs from different classes can recognize *C. albicans* cell wall polysaccharides such as mannans, β-glucan, and chitin. Immune sensing of *C. albicans* by monocytes and macrophages is mediated by the recognition of *N*- and *O*-linked mannans and β-glucan that requires CLRs and TLRs involving MR (mannose receptor), TLR4 and the dectin-1/TLR2 complex [102,107]. Apart from MR, dectin-2 has also been described to recognize *N*-linked mannan [108], while TLR4 only recognizes *O*-linked mannan [107]. β-glucan is thought to be masked by the fibrillary mannan layer, and its exposure is restricted to bud scars or occurs during cell wall rearrangement in the process of hyphae formation and infection [84,85]. However, recent findings doubt the static view of the cell wall and suggest at least partial surface exposure of β-glucan [109]. Recognition of β-glucan involves the complement receptor CR3, TLR2, TLR6, and the CLR dectin-1 [107,110]. Because of the predominant exposure of β-1,3-glucan at bud scars dectin-1 is considered to recognize only *C. albicans* yeast but not hyphal cells [84]. Recently, the non-classical receptor for β-glucan EphA2 (ephrin type-A receptor 2) was discovered on oral epithelial cells that sense the presence of both yeast and hyphae [111]. Likewise β-glucan, chitin lines the inner skeletal layer of *C. albicans* cell wall and is rarely exposed to surfaces [84], but unmasking can occur during neutrophil attack [92]. Macrophages are able to recognize chitin and induce an inflammatory cytokine response depending on the size of the molecule [94]. Recently, TLR2 has been identified to directly bind fungal chitin in a size-dependent manner [237]. Previous studies also suggested involvement of NOD2, TLR9, and MR in chitin recognition [95]. Furthermore, the intracellularly expressed TLR9 senses microbial DNA [112].

Many epithelial cells also constitutively express TLRs, CLRs, and NLRs with some contrary results depending on the study. In contrast to myeloid cells, type and composition of PRRs vary between primary cells and cell lines, mucosal localization, and species. The majority of TLRs are expressed by many epithelial cells such as the oral epithelium [113], intestinal, lung, and hepatic cells [106]. The role of epithelial TLRs in the epithelial activation has been questioned [114], however, stimulation of TLRs with microbial ligands induces antimicrobial peptides and cytokines indicating a functional role in the epithelial immune response [106]. Even though oral cells express only low levels of TLR4, it is required for an epithelial activation once neutrophils are recruited, resulting in immunological crosstalk between the epithelium and immune cells to protect from *C. albicans* infection [113]. It, therefore, appears that some PRRs are not required for the initial recognition but mediate antifungal protection in the presence of neutrophils [106].

In contrast to TLRs, only a few reports have described the expression of epithelial CLRs including the expression of dectin-1 in bronchial and intestinal epithelial cells [115,116]. Non-PRRs may also be involved in recognition of *C. albicans* such as EGFR and HER2 that can recognize hyphae [38].

3.3.2 Discrimination between commensal and pathogenic *Candida albicans*

The identification and discrimination of endogenous, opportunistic colonizers from pathogenic microbes is a fundamental ability of mucosal epithelial cells [106] (Fig. 4). Recognition of microbial PAMPs leads to PRR signaling and downstream activation of MAPK (mitogen-activated protein kinase) and NF-kB (nuclear factor kappaB) pathways [30,31]. Once activated, MAPK signaling results in the release of chemokines, inflammatory cytokines, and antimicrobial peptides that clear infection or further activate the innate and adaptive immunity [106]. Therefore, the activation of MAPKs is a tightly

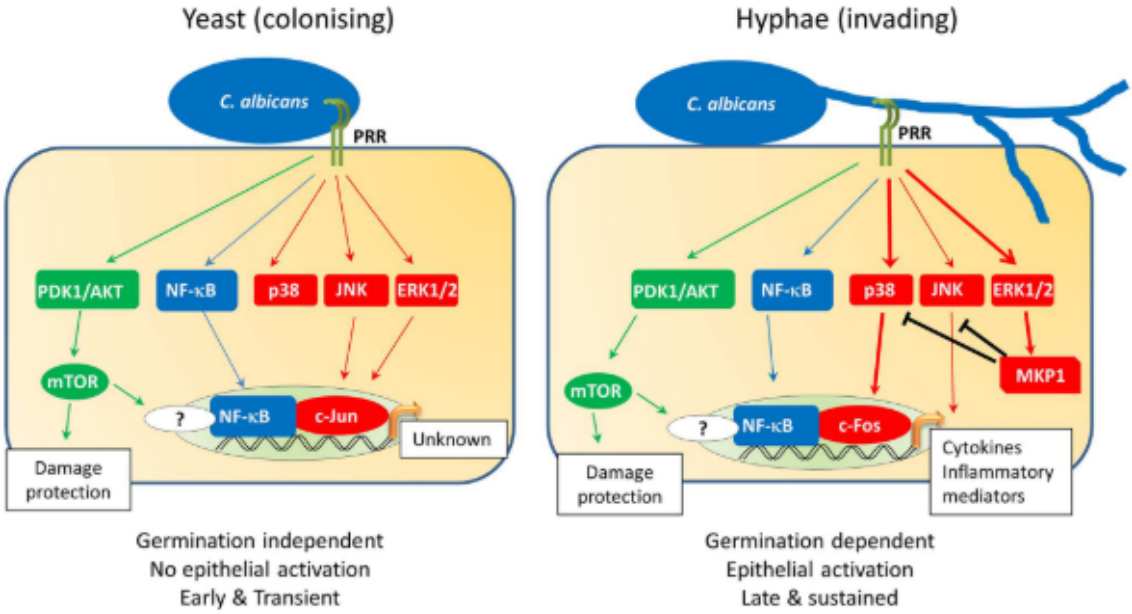

Fig. 4. Mucosal epithelial cells discriminate between colonizing yeast cells and invading hyphae. Colonizing yeast cells activate transient signaling that does not result in an epithelial cytokine secretion (left site). When *C. albicans* filaments, reaches high levels of fungal burdens, and invades epithelial cells, a second MAPK signaling pathway including c-Fos and MKP1 is activated that induces an inflammatory cytokine response. Modified from Naglik *et al.*, 2014 [182].

regulated process to enable a fine-tuned immune response to prevent overreaction [117]. Colonizing yeast cells of *C. albicans* are recognized by conventional PRRs that induce a weak and transient activation of NF-kB, Pi3K (phosphatidylinositol-4,5-bisphosphate 3-kinase), and the MAPK pathways p38, JNK (c-Jun *N*-terminal kinase), and ERK1/2 (extracellular signal-regulated protein kinase), independent of fungal viability [30]. Induction of these pathways occurs within 15 min in oral epithelial cells and induces further activation of the transcription factor c-Jun via JNK and ERK1/2 pathways. The activation of c-Jun, however, represents a non-inflammatory epithelial response without cytokine secretion [30]. During mucosal infection, hyphal formation and epithelial invasion, as well as an increasing fungal burden, lead to sustained activation of NF-κB and PI3K signaling pathways and further increases MAPK signaling by the activation of the transcription factor c-Fos via p38 [30,31,118,119]. Fungal invasion also activates the MAPK phosphatase MKP1 that is induced and stabilized by ERK1/2. Mucosal activation of c-Fos and MKP1 regulation depend on hypha formation and correlate with cell damage and the induction of epithelial inflammatory cytokines [30,118]. Strains that are not able to produce or maintain hyphal formation do not induce an epithelial cytokine response. In addition to

the triggered danger response, infection of oral mucosa also induces damage protection via PI3K/mTOR signaling pathway [119].

The MAPK p38/c-Fos signaling mechanism is considered as a common mucosal epithelial danger response activated during dangerous levels of invasive hyphae [30,99]. However, the induced cytokine response is site-specific, and the threshold of hyphal burden differs between oral and vaginal cells. While oral epithelial cells secrete IL-1α, IL-1β, G-CSF, GM-CSF, IL-6, and IL-8 in response to *C. albicans* infection [30], secretion of G-CSF and IL-6 is almost absent by vaginal epithelial cells [31]. These cytokines stimulate neutrophil generation and activation from granulocyte-macrophage progenitors in the bone marrow [120]. Neutrophils are the principal effector cells to clear *C. albicans* infection by phagocytosis [121]. They also interact with epithelial cells to provide mucosal protection. Phagocytosed *C. albicans* cells are killed intra- or extracellularly by reactive oxygen or nitrogen species (ROS and RNS), and by neutrophil extracellular traps (NETs) released during neutrophil cell death [122,123]. This protective role of neutrophils in oral candidiasis is detrimental in vaginal candidiasis as overt inflammation exacerbates disease [124]. Besides the epithelial cytokine response, antimicrobial peptides such as defensins (β-defensin-1/2/3), cathelicidins (LL-37), and histatins (histatin-5) are released from oral epithelial cells to limit fungal growth [125]. The interaction of epithelial cells with immune cells has been demonstrated for the PMN-dependent secretion of epithelial antimicrobial peptides that is important in the clearance of *C. albicans* in oral mucosal infection [113,126]. In addition, the mucosal epithelial immune response also induces protective adaptive immunity by recruiting CCR6-expressing dendritic cells that phagocyte *C. albicans* and present antigens to T cells [127]. IL-17A/F secreted by Th17 cells acts on mucosal epithelial cells to support barrier function and to induce epithelial antimicrobial peptides such as β-defensin-3 [128,129], but neutrophil mobilization is independent of the IL-17 pathway [130].

3.3.3 Role of the inflammasome in the host response

Inflammasomes are intracellular multiprotein complexes composed of a sensor PRR of the NLR family, in some cases an ASC adaptor protein (apoptosis-related speck-like protein containing a CARD), and the cysteine protease procaspase-1 [105]. Sensor proteins are NLRP1, NLRP3, NLRC4, NLRP6, and AIM (absent in melanoma 2) that can recognize both pathogen-associated patterns and host-derived molecules. *C. albicans*, however, is only recognized by the inflammasomes NRLP3 and NLRC4 [131,132]. The canonical NLRP3 inflammasome is expressed in myeloid cells and nonkeratinizing epithelial cells from the oral cavity and is by far the best-characterized inflammasome [133,134]. Formation of the inflammasome activates procaspase-1 into caspase-1 that is responsible for the cleavage of pro-IL-1β and pro-IL-18 into their mature secreted form. To activate the NLRP3 inflammasome, priming and activation signals are required [135–137]. In the initial step, activation of the transcription factor NF-κB by upstream PRRs such as TLRs, the IL-1 receptor IL-1R1, and TNFR leads to the transcription and translation of pro-IL-1β and NLRP3. In a second step, a diverse group of agonists such as cytokines, pore-forming toxins, ATP, and crystalline molecules lead to the activation of NLRP3 [138–140], the assembly of the NLRP3 inflammasome, and consequently the activation of caspase-1. In macrophages, *C. albicans* filaments activate caspase-1 through the NLRP3 inflammasome that

induces IL-1β secretion [131,136] or a cytokine-dependent programmed cell death called pyroptosis [141]. IL-1β is mainly produced by macrophages and is important in the acute phase response during infection by recruiting neutrophils and monocytes, and induces additional cytokines [142]. The presence of host molecules released by injured cells causing sterile inflammation, can also activate the inflammasome that leads to the production of pro-inflammatory IL-1β [143]. Both cytokines from the IL-1 family, IL-1β and IL-1α, the latter which is released during cell death [138], bind to the IL-1 receptor and induce their own production and secretion via a positive feedback loop. IL-1R signaling regulates the neutrophil response and plays a critical role in the defense of OPC [144].

4 Aims of this study

Disseminated candidiasis in mice revealed that the kidney is the principal target organ accompanied by a low number of resident immune cells and delayed neutrophil recruitment [145,146]. However, inflammatory cytokines appear early during infection and correlate with the immunopathology in the kidney [147]. In contrast, early accumulation of neutrophils in the liver controls *C. albicans* fungal burden. This organ-specific control of *C. albicans* assumes an contribution of epithelial cells to the progression of disease.

Previous studies about the epithelial cytokine response focused on mucosal sites that can be affected by superficial *C. albicans* infection resulting in OPC or VVC. Mucosal epithelial cells can discriminate between yeast and hyphal forms of *C. albicans* that requires PRR signaling and downstream activation of the MAPK pathway. This activation is mediated by MKP1 and c-Fos that depends on *C. albicans* hyphal formation and host cell damage [30,31,118].

During systemic infection almost all organs can be affected by *C. albicans*. Thus, the fungus also interacts with epithelial cells that are part of the parenchyma of internal organs, e.g. from kidney, spleen, and liver. While parenchymal epithelial cells are usually sterile, mucosal epithelial cells are in direct contact with the invading pathogen. This suggests that both types of epithelial cells differently activate an innate immune response. Therefore, this study aimed to analyze and to compare the cytokine induction from mucosal and parenchymal epithelial cells.

C. albicans can bypass the host immune system by metabolic adaptation or through masking of cell wall components. This suggests that fungal factors might modulate the epithelial immune response. Therefore, in this study a collection of *C. albicans* mutant strains was analyzed for their ability to influence the epithelial cytokine release without affecting host cell damage. As a result, a *C. albicans* strain with a defect in the *N*-mannan biosynthesis encoded by *MNN9* modulated the epithelial cytokine response and was further analyzed *in vitro* and in a mice model of disseminated candidiasis.

5 Material and methods

5.1 Material

5.1.1 Strains

All *C. albicans* and *E. coli* strains used are listed in Table 1 and Table 2.

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

Strain	Parental strain	Genotype	Source, reference
SC5314	-	wild type	Gillum *et al*., 1984
NGY152	CAI-4	*RPS1*/*rps1*::*URA3*	Sandini *et al*., 2007
***mnn9*Δ/-**	SC5314	*MNN9*/*mnn9*::FRT	This study
***mnn9*Δ/Δ**	SC5314	*mnn9*::FRT/*mnn9*::FRT	This study
+MNN9	SC5314	*mnn9*::FRT/*mnn9*::FRT, *ADH1*/*adh1*::MNN9-SAT1	This study
***och1*Δ/Δ**	CAI-4	*och1*::*hisG*/*och1*::*hisG*, *RPS1*/*rps1*::Clp10	Bates *et al*., 2006
+OCH1	CAI-4	*och1*::*hisG*/*och1*::*hisG*, *RPS1*/*rps1*::Clp10-OCH1	Bates *et al*., 2006
***pmr1*Δ/Δ**	CAI-4	*pmr1*::*hisG*/*pmr1*::*hisG*, *RPS1*/*rps1*::Clp10	Bates *et al*., 2005
+PMR1	CAI-4	*pmr1*::*hisG*/*pmr1*::*hisG*, *RPS1*/*rps1*::Clp10-PMR1	Bates *et al*., 2005
***MNN2* sextuple mutant**	CAI-4	*mnn21*::dpl200/*mnn21*::dpl200, *mnn24*::dpl200/*mnn24*::dpl200, *mnn26*::dpl200/*mnn26*::dpl200, *mnn22*::dpl200/*mnn22*::dpl200, *mnn2*::dpl200/*mnn2*::dpl200, *mnn23*::dpl200/*mnn23*::dpl200, *ura3*::imm434/*ura3*::imm434, *RPS1*/*rps1*::Clp10	Hall *et al*., 2013

Table 2. *E. coli* strains used in this study

Strain	Parental strain	Genotype	Source, reference
DH5α	-	F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK-, mK+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1	Thermo Fisher Scientific

Stellar™	-	F-, *endA1, sup*E44, *thi*-1, *rec*A1, *rel*A1, *gyr*A96, *pho*A, Φ80d *lac*ZΔ M15, Δ *(lac*ZYA - *arg*F*)* U169, Δ *(*mrr - *hsd*RMS - *mcr*BC*)*, Δ*mcr*A, λ-	Clontech
ECO_pSFS2A	DH5α	pSFS2A	This study
ECO_pMNN9-SAT1-Flipper	Stellar™	pMNN9-SAT1-Flipper	This study
ECO_pSK-ADH1-CaGFP	DH5α	pSK-ADH1-CaGFP	This study
ECO_pSK-ADH1-MNN9	DH5α	pSK-ADH1-MNN9	This study

5.1.2 Cell lines

Human epithelial cell lines used in this study are listed in Table 3.

Table 3. Cell lines used in this study

Cell line	Origination	Cultivation medium	Source
A498	human kidney carcinoma, proximal tubule	MEM, 10% FBS	DSMZ, ACC 55
Caco2	human colon carcinoma	DMEM with 4.5 g/L Glc, L-Glutamine, 10% FBS, 1% of 100x NEAA	DSMZ, ACC 169
HepaRG	human hepatic progenitor cells, immortalized	Williams' Medium E with GlutaMAX , HepaRG™ Thaw, Plate & General Purpose Medium Supplement	Gibco (life technologies)
TR146	human buccal cell carcinoma	DMEM with 4.5 g/L Glc, L-Glutamine, 10% FBS	Episkin

5.1.3 Plasmids and oligonucleotides

Plasmids and oligonucleotides (primers) that were used to generate and verify the *MNN9* mutant and revertant strains are listed in [Table 4] and [Table 5].

Table 4. Plasmids used in this study

Plasmid	Comment	Source, reference
pSFS2A	SAT1 flipper cassette	Reuß *et al*., 2004
pMNN9-SAT1-Flipper	*MNN9* deletion cassette	This study
pSK-pADH1-CaGFP	integrates into *ADH1* locus	Polke *et al*., 2017
pSK-pADH1-MNN9	*MNN9* complementation cassette	This study

Table 5. Used primers in this study

Primer	Sequence (5' → 3') with the respective restriction site	Comment	Source, reference
SAT1 mm9-up-for	GTTTATGGGGCCCGTAGCTGTTCTTCGACATTG	restriction site for ApaI	This study
SAT1 mnn9-up-rev	AGGTACTCGAGCCTATAGTGAACCATGATTGCTG	restriction site for XhoI	This study
SAT1 mnn9-down-for	CCATTCCGCGGAGTAAATCAAGTGGGGGACAAC	restriction site for SacII	This study
Sat1 mnn9-down-rev	AGTAGGAGCTCTTCTTTGGGTACGGTTCAACAC	restriction site for SacI	This study
mnn9up	AGTGAGAGAGTGGCAGAAAG	MNN9 verification forward	This study
mnn9down	CTCTGGTTGTCCCCCACTTG	MNN9 verification reverse	This study
probe-mnn9-for	GACTGCTAAACTGATCTCCCATTG	probe for Southern blotting	This study
probe-mnn9-rev	CATTTCTATTTGTAGCTGTTGGTGG	probe for Southern blotting	This study
SAT1-mnn9-comp-for	GTTTATGCTCGAGGTAGCTGTTCTTCGACATTG	restriction site for XhoI	This study
SAT1-mnn9-comp-rev	AGTAGCACGTGCCCACTTGATTTACTCATTAT	restriction site for PmlI	This study
ADHp-fwd	GTCATGTGCACGGACAAGC	integration in *ADH1* locus forward	Polke *et al*., 2017

mnn9down2	CCCACTTGATTTACTCATTAT	integration in *ADH1* locus reverse	This study
probe-mnn9-adh1-for	CATGGAAATTAAACTGGGTGTTGTG	probe for Southern blotting	This study
probe-mnn9-adh1-rev	CAGAATGCGATCGTAGCACTATTTC	probe for Southern blotting	This study

5.1.4 Media, buffers, kits, enzymes, and chemicals

Media, buffers, commercial kits, enzymes, and chemicals used in this study are listed in [Table 6], [Table 7], [Table 8], [Table 9], and [Table 10].

Table 6. Media used in this study

Medium	Composition	Application
Dulbecco's Modified Eagle Medium (DMEM) with high glucose	composed by Gibco (life technologies)	cultivation of TR146 and Caco2
HepaRG Thaw, Plate & General Purpose Medium Supplement	composed by Gibco (life technologies)	cultivation of HepaRG
LB (agar)	1% tryptone, 0.5% yeast extract, 1% NaCl, (2% agar)	cultivation of E. coli
Minimum Essential Medium (MEM) with L-glutamine	composed by Gibco (life technologies)	cultivation of A498
NTC YPD agar	YPD agar, 200 µg/ml Nourseothricin	mutant generation
RPMI 1640	composed by Gibco (life technologies)	cultivation of C. albicans
William's Medium E (WME) with GlutaMAX	composed by Gibco (life technologies)	cultivation of HepaRG
YPD (agar)	2% peptone, 1% yeast extract, 2% D-glucose, (2% agar)	cultivation of C. albicans
YPM	2% peptone, 1% yeast extract, 2% maltose	mutant generation/ marker recycling

Table 7. Buffers used in this study

Buffer/Solution	Composition	Application
10x Tris-EDTA buffer, pH 7.5	100 mM Tris-HCl, 10 mM EDTA	mutant generation/ transformation
20x SSC, pH 7.0	3 M NaCl, 0.3 M sodium citrate	Southern blotting
5x maleic acid buffer, pH 7.5	0.5 M maleic acid, 0.75 M NaCl	Southern blotting
5x sample loading buffer	1.5 M Tris-HCl pH 6.8, 50% glycerol, 25% β-mercaptoethanol, 10% w/v SDS, 0.2% Bromphenol Blue	western blotting
alkaline phosphatase buffer, pH 9.5	0.1 M Tris-HCl, 0.1 M NaCl	Southern blotting
blocking solution	1% w/v blocking reagent (Roche), 0.1 M maleic acid, 0.15 M NaCl pH 7.5	Southern blotting
blotting buffer	200 mM glycine, 25 mM Tris	western blotting
cell detachment solution	0.001% trypsin, 10 mM EDTA, PBS	flow cytometry
collection gel buffer, pH 6.8	0.5 M Tris	western blotting
denaturing solution	0.5 M NaOH, 1.5 M NaCl	Southern blotting
depurination solution	250 mM HCl	Southern blotting
DNA lysis buffer	1 M sorbitol, 100 mM trisodium citrate pH 5.8, 50 mM EDTA pH 8, 0.06% lyticase, 2.5% β-mercaptoethanol	DNA isolation
DNA proteinase buffer	10 mM Tris-HCl pH 7.5, 50 mM EDTA pH 7.5, 0.5% SDS, 0.1% proteinase K 1000x stock	DNA isolation
FACS buffer	1x PBS, 3% FBS, 0.1% sodium azide	flow cytometry
Foxp3/Transcription Factor Staining Buffer Set	composed by eBioscience	flow cytometry
Harju buffer	20% Triton X-100, 10% SDS, 5 M NaCl, 1M Tris-HCl pH 8, 0.5 M EDTA	DNA isolation
high stringency wash buffer	0.1x SSC, 0.1% SDS	Southern blotting
LiAc transformation solution	100 mM lithium acetate, 10 mM Tris-HCl, 1 mM EDTA,	mutant generation/ transformation

lithium acetate buffer	1 M lithium acetate	mutant generation/ transformation
low stringency wash buffer	2x SSC, 0.1% SDS	Southern blotting
migration buffer	200 mM glycine, 25 mM Tris, 3.5 mM SDS	western blotting
neutralization solution	1 M Tris-HCl pH 7.5, 1.5 M NaCl	Southern blotting
Odyssey Blocking Buffer TBS-based	composed by LI-COR	western blotting
PBS, pH 7.4	140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4	diverse
PEG/LiAc solution	80% PEG (50%), 10% lithium acetate (1 M), 10% Tris-EDTA (10x)	mutant generation/ transformation
PEG3650 solution	50% PEG3650	mutant generation/ transformation
RIPA lysis buffer, modified	50 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25 % sodium deoxycholate, 0.1% SDS, 1% NP-40	protein isolation
separation gel buffer, pH 8.8	2 M Tris	western blotting
TBST, pH 7.6	10 mM Tris, 100 mM NaCl, 0.1% Tween 20	western blotting
Tris-HCl, pH 9.0 staining buffer	0.1 M	adhesion assay
washing buffer	1x maleic acid buffer, 0.3% Tween 20 (v/v)	Southern blotting

Table 8. Kits used in this study

Kit	Company	Application
ArC Amine Reactive Compensation Bead Kit	Invitrogen	flow cytometry
Cytotoxicity Detection Kit (LDH)	Roche	damage assay
Foxp3/Transcription Factor Staining Buffer Set	eBioscience	flow cytometry
Human CCL2 (MCP-1) ELISA Ready-SET-Go!	eBioscience	cytokine measurement
Human CCL4 (MIP-1β) ELISA Ready-SET-Go!	eBioscience	cytokine measurement
Human GM-CSF ELISA Ready-SET-Go! (2nd Generation)	eBioscience	cytokine measurement

Human IL-10 ELISA Ready-SET-Go!	eBioscience	cytokine measurement
Human IL-17E ELISA Kit	OmniKine	cytokine measurement
Human IL-1α ELISA MAX Deluxe	BioLegend	cytokine measurement
Human IL-1β ELISA Ready-SET-Go! (2nd Generation)	eBioscience	cytokine measurement
Human IL-6 ELISA Ready-SET-Go!	eBioscience	cytokine measurement
Human IL-8 ELISA Ready-SET-Go! (2nd Generation)	eBioscience	cytokine measurement
Human TNF-α ELISA Ready-SET-Go!	eBioscience	cytokine measurement
Human TSLP ELISA Ready-SET-Go!	eBioscience	cytokine measurement
innuPrep DOUBLEpure kit	Analytik Jena	PCR purification
LEGEND MAX Human IL-33 ELISA	BioLegend	cytokine measurement
PCR DIG Probe Synthesis Kit	Roche	Southern blotting/ probe labeling
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	protein concentration
SPL Blue Kit	NHDyeAgnostics	quantitative western blotting (SPL)
SPL Kit Red-IR	NHDyeAgnostics	quantitative western blotting (SPL)

Table 9. Enzymes used in this study

Enzyme	Company	Application
ApaI	New England Biolabs	mutant generation
EcoRV	New England Biolabs	mutant generation
KpnI	New England Biolabs	mutant generation
LongAmp Taq DNA polymerase	New England Biolabs	mutant generation
PciI	New England Biolabs	mutant generation
Phusion HF DNA polymerase	New England Biolabs	mutant generation
PmlI	New England Biolabs	mutant generation
SacI	New England Biolabs	mutant generation
SacII	New England Biolabs	mutant generation
T4 DNA ligase	New England Biolabs	mutant generation
Taq DNA polymerase	New England Biolabs	mutant generation
XhoI	New England Biolabs	mutant generation

Table 10. Chemicals, antibiotics, and ligands used in this study

Chemicals	Company	Application
1 kb DNA Ladder	New England Biolabs	agarose gel electrophoresis
6x Loading Dye	New England Biolabs	agarose gel electrophoresis
adenosine 5'-triphosphate (ATP)	Sigma	inflammasome induction
albumin bovine fraction V (BSA)	Serva	staining, western blotting
ammonium persulfate (APS)	Serva	western blotting/ SDS-PAGE
anakinra (Kineret)	Sobi	IL-1R1 blocking
CDP Star	Roche	Southern blotting
chitin from shrimp shells	Sigma-Aldrich	PRR stimulation
collagen I from rat tail	Gibco (life technologies)	coating
concanavalin A from *Canavalia ensiformes*	Sigma	coating/ fluorescence microscopy
DIG Easy Hyb	Roche	Southern blotting
EDTA	Sigma-Aldrich	protein isolation, western blotting
ethanol 100%	Roth	DNA isolation
Fluorescent Brightener 28 (calcofluor white, CFW)	Sigma	adhesion assay
furfurman from *Malassezia furfur* cell wall	InvivoGen	PRR stimulation
glybenclamide	InvivoGen	inflammasome blocking
IL-1β	Invitrogen	ligand
Immuno Blue reagent	NHDyeAgnostics	quantitative western blotting (SPL)
***iso*-propanol (2-propanol)**	Roth	DNA isolation
lipopolysaccharides from *E. coli* 055:B5 (LPS)	Sigma	inflammasome induction
L-Lactate Dehydrogenase	Roche	LDH standard/ damage assay
LPS-B5 ultrapure LPS from *E. coli* 055:B5	InvivoGen	PRR stimulation
lyticase from *Arthrobacter luteus*	Sigma-Aldrich	DNA isolation
mannan from *Saccharomyces cerevisiae*	Sigma-Aldrich	PRR stimulation
***β*- mercaptoethanol**	Roth	western blotting
Molecular Weight Marker II	Roche	Southern blotting
N,N,N',N'-tetramethylethylen-diamine (TEMED)	Serva	western blotting/ SDS-PAGE
Nourseothricin	HKI	mutant generation

OneComp eBeads	eBioscience	flow cytometry
Pam2CSK4	InvivoGen	PRR stimulation
phenol/chloroform/isoamylalcohol	Roth	DNA isolation
phorbol 12-myristate 13-acetate (PMA)	Sigma	PRR stimulation
PhosSTOP EASYpack	Roche	protein isolation
poly-*D*-lysin	Chemicon	coating
ProLong Gold Antifading Reagent	Invitrogen	fluorescence microscopy
Protease Inhibitor Mix 100x	Jena Bioscience	protein isolation
proteinase K	Biozym	DNA isolation
Roti-Histofix 4%	Roth	adhesion, invasion/ fixation
sheared Salmon Sperm DNA	Invitrogen	mutant generation
sodium azide	Roth	flow cytometry, western blotting
TNF-α	Invitrogen	ligand
trehalose-6,6-dibehenate (TDB)	InvivoGen	PRR stimulation
Triton-X 100	Sigma	invasion assay
Trypsin-Versene	Lonza	cell detachment
Tween 20	Serva	invasion assay, western blotting
Western Lightning Plus-ECL	PerkinElmer	western blotting
zymosan A from *Saccharomyces cerevisiae*	Sigma	PRR stimulation

5.1.5 Antibodies

Antibodies used in this study are stated in [Table 11].

Table 11. Antibodies used in this study

Antibody	Host/isotype	Company	Application
anti-*Candida*	rabbit	Acris Antibodies	invasion assay
anti-digoxygenin	anti-DIG-AP Fab fragments	Roche	Southern blotting
anti-human CD206 Brilliant Violet 421	mouse, IgG1	BioLegend	flow cytometry
anti-human CD282 FITC	mouse, IgG2a	BioLegend	flow cytometry
anti-human CD284 PE-Cyanine7	mouse, IgG2a	eBioscience	flow cytometry

anti-human CD368 APC	mouse, IgG2b	BioLegend	flow cytometry
anti-human CD369 PE	mouse, IgG2a	BioLegend	flow cytometry
anti-mouse Alexa Fluor 488	goat	Invitrogen	flow cytometry
anti-mouse HRP	IgG	Cell Signaling Technology	western blotting/ quantitative western blotting (SPL)
anti-mouse PE	goat	BioLegend	fluorescence microscopy
anti-rabbit Alexa Fluor 488	-	Invitrogen	invasion assay
anti-rabbit Alexa Fluor 647	-	Invitrogen	invasion assay
anti-rabbit HRP	IgG	Cell Signaling Technology	western blotting/ quantitative western blotting (SPL)
anti-β-1,3-glucan	mouse, IgG	Biosupplies	flow cytometry/ fluorescence microscopy
c-Fos	monoclonal, rabbit IgG	Cell Signaling Technology	western blotting
concanavalin A 647	-	Invitrogen	flow cytometry/ fluorescence microscopy
FVD eFluor506	-	eBioscience	flow cytometry
human FcR Blocking Reagent	-	Miltenyi Biotec	flow cytometry
IgG1 Brilliant Violet 421	mouse	Biolegend	flow cytometry
IgG2a FITC	mouse	BioLegend	flow cytometry
IgG2a PE	mouse	BioLegend	flow cytometry
IgG2a PE-Cyanine7	mouse	eBioscience	flow cytometry
IgG2b APC	mouse	BioLegend	flow cytometry
JNK	monoclonal, mouse IgG1	Santa Cruz Biotechnology	quantitative western blotting (SPL)
Lectin from *Triticum vulgaris* FITC	-	Sigma	flow cytometry/ fluorescence microscopy

Conjugate (WGA-FITC)			
MKP1	monoclonal, mouse IgG3	Santa Cruz Biotechnology	quantitative western blotting (SPL)
NF-κB p65	monoclonal, mouse IgG2b	Cell Signaling Technology	western blotting
p44/42 MAPK ERK1/2	monoclonal, mouse IgG1	Cell Signaling Technology	quantitative western blotting (SPL)
phospho-DUSP1/MKP1	monoclonal, rabbit IgG	Cell Signaling Technology	quantitative western blotting (SPL)
phospho- NF-κB p65	monoclonal, rabbit IgG	Cell Signaling Technology	quantitative western blotting (SPL)
phospho-p44/42 MAPK ERK1/2 XP	monoclonal, rabbit IgG	Cell Signaling Technology	quantitative western blotting (SPL)
phospho-SAPK/JNK	monoclonal, rabbit IgG	Cell Signaling Technology	quantitative western blotting (SPL)
Smart Infrared Fluorescent anti-rabbit	goat, IgG	NHDyeAgnostics	quantitative western blotting (SPL)
Smart Red Fluorescent anti-mouse	goat, IgG	NHDyeAgnostics	quantitative western blotting (SPL)

5.2 Methods

5.2.1 *Escherichia coli* storage and cultivation

E. coli strains were stored at -80°C in 50% (v/v) glycerol. Prior usage, strains were maintained on LB agar at 37°C and routinely grown in LB broth at 37°C and 180 rpm.

5.2.2 *Candida albicans* storage, cultivation, and growth conditions

All strains were stored at -80°C in 50% (v/v) glycerol. Prior usage, *C. albicans* strains were maintained on YPD agar at 30°C and cultured in YPD broth at 30°C and 180 rpm. Exponential grown cells were used for all experiments and incubated for 3-4 h with a starting inoculum of OD_{600nm} 0.2. Cells were collected by centrifugation at 3200 x g, washed three times in phosphate-buffered saline (PBS pH 7.4) and counted with a hemocytometer. Aggregating mutant strains were physically separated with a 1 ml syringe (Ø 0.5 mm, BD Plastipak) by pipetting for at least 10 times prior counting. The cell number of *C. albicans* strains corresponded with the cell number of seeded host cells for a comparable multiplicity of infection (MOI). Strains were bi-concentrated in the respective cell culture medium without any supplements and ½ volume was added to the wells. For hyphal induction, cells were incubated in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C

and 5% CO_2 for 4 h. Fungal growth was measured in 96-well tissue culture plates in a microplate reader (Tecan Infinite 200 Pro) at 30°C for 24 h. To prepare conditioned cell culture medium for growth analysis, epithelial cells were grown to 80% confluency and the media replaced with fresh medium for 24 h. Then, the supernatant was collected, centrifuged, and filtered through a 0.2 µm membrane.

5.2.3 Cell lines cultivation

Hepatic epithelial cells (HepaRG) were cultivated in William's Medium E (WME) with GlutaMAX and HepaRG Thaw, Plate & General Purpose Medium Supplement. Renal epithelial cells (A498) were cultivated in Minimum Essential Medium (MEM) with L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS). Oral epithelial cells (TR146) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) with high glucose and supplemented with 10% heat-inactivated FBS. All cell line cultivation and infection experiments were performed without antibiotics or antifungal agents in a humidified incubator at 37°C, 5% CO_2 and 95% humidity. Two days prior the experiment, epithelial cells (HepaRG $2x10^5$ cells/ml; A498 and TR146 $1x10^5$ cells/ml) were seeded with a final volume of 200 µl, 1 ml or 2 ml in 96-, 24- or 6-well tissue culture plates (TPP Techno Plastic Products), respectively, to ensure full confluency. At the day of infection, cell culture medium was removed and ½ volume was added to the wells.

5.2.4 Isolation of genomic DNA from *Candida albicans*

Two different methods were used to isolate genomic DNA from *C. albicans*: for quantitative assays, a freeze and thaw DNA isolation protocol was performed [148]. Briefly, 200 µl of Harju buffer was added to a pelleted *C. albicans* culture and incubated for 5 min in -80°C. Afterwards, the frozen sample was transferred into a 95°C water bath for 1 min. The last two steps were repeated twice and the suspension vortexed. Next, 200 µl phenol/chloroform/isoamylalcohol was added, vortexed for 2 min, centrifuged at 20,000 xg for 5 min, and the upper aqueous phase was transferred into 400 µl ice-cold 100% ethanol. After another centrifugation step, the pellet was washed with 70% ethanol, air-dried, and afterwards resuspended in ddH_2O.

Pure and qualitative DNA isolation was used for Southern Blot assays and prepared as the following: 1 ml DNA lysis buffer was added to a pelleted *C. albicans* culture and incubated at 37°C for 45 min. The suspension was centrifuged at 13,000 xg for 5 min and the pellet resuspended in 800 µl DNA proteinase buffer. After incubation at 60°C for 30 min, 800 µl phenol/chloroform/isoamylalcohol was added and mixed by vortexing. The suspension was centrifuged again and the upper hydrophilic phase was transferred into 700 µl isopropanol, mixed, and centrifuged for 5 min. The pellet was washed with 70% ethanol, air-dried, and afterwards resuspended in ddH_2O.

5.2.5 DNA amplification via polymerase chain reaction (PCR)

To amplify genes, polymerase chain reactions (PCRs) were performed in T-Personal Thermocycler (biometra). For sequence verification, Taq DNA polymerase was used. To enable accurate gene amplification, proof-reading DNA polymerases LongAmp and Phusion HF were used. For further applications, PCR products were purified using the innuPrep DOUBLEpure kit or first separated via

agarose gel electrophoresis, cut from the gel, and purified using the above mentioned kit. For analysis, 6x Loading Dye was mixed with the PCR product and separated in an 1% agarose gel with 1 kb DNA ladder as a reference. Content of PCR mixes are listed in Table 12 and conditions are listed in Table 13.

Table 12. Reaction setups for different DNA polymerases

	Taq DNA polymerase	**LongAmp Taq DNA polymerase**	**Phusion HF DNA polymerase**
Reaction buffer (1x)	ThermoPol Reaction Buffer	LongAmp Taq Reaction Buffer	Phusion HF Reaction Buffer
10 mM dNTPs	200 µM	300 µM	200 µM
10 µM primer (forward)	0.2 µM	0.4 µM	0.5 µM
10 µM primer (reverse)	0.2 µM	0.4 µM	0.5 µM
Template DNA	50 ng	50 ng	50 ng
DNA polymerase	0.025 U/µl	0.1 U/µl	0.02 U/µl

Table 13. Thermocycling conditions for different DNA polymerases

		Taq DNA polymerase		**LongAmp Taq DNA polymerase**		**Phusion HF DNA polymerase**	
		Temperature	Time	Temperature	Time	Temperature	Time
Initial denaturation		95°C	30 s	94°C	30 s	98°C	30 s
Denaturation	30x	95°C	30 s	94°C	30 s	98°C	30 s
Annealing		45-68°C	30 s	45-65°C	60 s	45-72°C	30 s
Elongation		68°C	1 min / kbp	65°C	50 s / kbp	72°C	30 s / kbp
Final extension		68°C	5 min	65°C	10 min	72°C	10 min
Hold		8°C	∞	8°C	∞	8°C	∞

5.2.6 Generation of *C. albicans MNN9* mutant and revertant strains

Used plasmids and primers are listed in [Table 4 + Table 5]. All deletion and complementation cassettes were verified by PCR, restriction, and sequencing. All *C. albicans* mutant and revertant strains were verified by PCR, sequencing, and Southern blotting.

To delete *MNN9* (*ORF19.7383*; 1107 bp) the SAT1 flipper method was used in a modified way [149]. The SAT1 flipper cassette in pSFS2A (isolated from ECO_pSFS2A) was used to construct the *MNN9* deletion cassette: an upstream fragment of *MNN9* (position -509 to +26) with the flanking restriction sites ApaI and XhoI was amplified from SC5314 genomic DNA with the primer SAT1 mm9-up-for and

SAT1 mnn9-up-rev. Downstream of *MNN9* (position +1092 to +1606) a SacII and SacI flanking fragment was amplified with SAT1 mnn9-down-for and Sat1 mnn9-down-rev. Both fragments were cloned into pSFS2A resulting in pMNN9-SAT1-Flipper. The original pSFS2A was transformed into *E. coli* DH5α and the recombinant plasmid was transformed into *E. coli* Stellar^{TM} to amplify plasmid DNA. The *MNN9* deletion cassette was excised from pMNN9-SAT1-Flipper with KpnI and SacI, resulting in a 4217 bp linear fragment containing the upstream part of *MNN9*, FRT site, maltose promotor, a flippase, the SAT1 resistance cassette, a second FRT site and the downstream part of *MNN9*. The *MNN9* deletion cassette was introduced by chemical transformation into *C. albicans* SC5314 wild type using the modified lithium method [Walther and Wendland, 2003]. Briefly, 1 µg of *C. albicans* DNA was mixed with 100 µg of single-stranded carrier DNA (sheared Salmon Sperm), 100 µl of a *C. albicans* lithium acetate suspension, and 600 µl of PEG/ lithium acetate solution and incubated for 12-16 h at 30°C under static conditions. To transform the recombinant DNA, heat shock was performed for 15 min at 44°C. Afterwards, the *C. albicans* pellet was resuspended in YPD without antibiotics and incubated at 30°C for 4 h. To select for positive clones, transformants were plated on YPD agar containing 200 µg/ml nourseothricin for 3 days at 30°C. After verification of correct clones via PCR, the SAT1 marker from *C. albicans mnn9*Δ/- was recycled using maltose-containing YPM medium by activating the *Candida* flippase (CaFLP). Then, the second *MNN9* allele was removed in the same way, resulting in FRT sites in the former *MNN9* locus. Gene deletion of *C. albicans mnn9*Δ/Δ was confirmed by the primers mnn9up and mnn9down in PCR, resulting in a 377 bp long fragment. To verify by Southern blotting, the probe was generated with the PCR DIG Probe Synthesis Kit using the primers probe-mnn9-for and probe-mnn9-rev (231 bp). Genomic DNA was digested with KpnI, separated in 1% agarose gel by electrophoresis. Depurinated and transferred DNA on a nylon membrane was hybridized in Dig Easy Hyb with the single stranded DNA probe at 40°C overnight. Using the CDP Star Chemiluminescence System, a homozygous *MNN9* mutant was verified by a single band at 2846 bp, while the wild type resulted in a band at 3995 bp.

To restore the wild type phenotype, one allele of the *MNN9* gene was integrated into the ADH1 locus of *C. albicans* using a modified pSK-pADH1-CaGFP construct [34]. The *MNN9* open reading frame was amplified with flanking XhoI and the blunt cutter PmlI sites using SAT1-mnn9-comp-for and SAT1-mnn9-comp-rev from -509 to +1128. CaGFP was cut out from the prior isolated pSK-pADH1-CaGFP (originated from ECO_pSK-ADH1-CaGFP) with XhoI and the blunt cutter EcoRV and ligated with the *MNN9* amplicon resulting in pSK-pADH1-MNN9. The complementation cassette was amplified in ECO_pSK-ADH1-MNN9, isolated, cut with KpnI and SacI (5032 bp), and transformed into the *MNN9* homozygous mutant resulting in the strain *C. albicans* +*MNN9*. Correct integration into the *ADH1* locus was confirmed by the primers ADHp-fwd (binding genomic DNA) and mnn9down2 (binding in the integrated complementation cassette), resulting in a 2632 bp long fragment. To verify by Southern blotting the primers probe-mnn9-adh1-for and probe-mnn9-adh1-rev were used to generate a 357 bp long probe. Genomic DNA was digested with PciI. The revertant strain was verified by two bands at 6775 bp and 3832 bp, whereas the homozygous mutant resulted in one band at 3832 bp.

5.2.7 Southern Blotting

Southern Blotting was performed in order to verify mutant and revertant strains of *MNN9*. Probes were labeled with digoxygenin using the PCR DIG Probe Synthesis Kit. Probes to verify *mnn9*Δ/Δ were generated by using the primers probe-mnn9-for and probe-mnn9-rev resulting in 231 bp. Probes to verify the *MNN9* revertant strain (*mnn9*Δ/Δ+*MNN9*) were generated by using the primers probe-mnn9-adh1-for and probe-mnn9-adh1-rev resulting in 357 bp. Probe quality was checked via agarose gel electrophoresis. Genomic DNA of *C. albicans* was isolated following section 5.2.4. 12 µg of DNA was digested with KpnI to verify *MNN9* deletion or the integration of *MNN9* into the *mnn9*Δ/Δ strain. Digested genomic DNA was separated in 1% agarose gel at 90 V for 3 h. To estimate band sizes, a DIG-labeled marker was used (Molecular Weight Marker II). Then, the gel was incubated in depurination solution for 20 min, two times in denaturing solution for 10 min, and two times in neutralization solution for 10 min at room temperature. The DNA was transferred onto a nylon membrane (Roche) using a vacuum blotter at 70-100 mbar for 1.5 h and afterwards crosslinked at 120 mJ/cm² for 1 min (Bio-Link, Vilber-Lourmat). The nylon membrane was pre-hybridized with prewarmed DIG Easy Hyb at 40°C for 45 min in a hybridization oven (Biometra). Probes were heated to 99°C for 5 min, cooled on ice and added to the pre-hybridization solution, following incubation overnight at 40°C under rotation. Then, the membrane was washed two times in low stringency buffer for 5 min at room temperature, two times in prewarmed high stringency buffer for 15 min at 68°C, two times in maleic acid buffer with 0.3% Tween 20 for 5 min at room temperature, and once in maleic acid buffer without Tween 20 for 5 min at room temperature. Afterwards, the membrane was blocked for 30 min at room temperature in 1% blocking solution. The anti-digoxygenin antibody was added 1:20,000 to the blocking solution and incubated for 1 h at room temperature. Subsequently, the membrane was washed two times in 1 x maleic acid buffer with 0.3% Tween 20 for 15 min, and two times in 1 x maleic acid buffer without Tween 20 for 15 min. Before detection, the membrane was equilibrated in alkaline phosphatase buffer for 5 min. For detection, CDP Star solution was diluted 1:100 in alkaline phosphatase buffer and the solution evenly distributed on the membrane. After 5 min of incubation, chemoluminescent signals were detected by a Chemoimager with an exposure time of 1-2.5 h. Resulting pictures were inverted and cropped with Adobe Photoshop CS6.

5.2.8 Epithelial cell damage via LDH release

To determine epithelial cell damage after 24 h (standard infection assays) or after 2, 4, and 6 h (in signaling experiments), lactate dehydrogenase (LDH) activity was measured from cell culture supernatant with the Cytotoxicity Detection Kit according to the manufacturer's protocol. L-LDH from rabbit muscle was used to generate the standard curve.

5.2.9 Cytokine determination

Infected samples were centrifuged 24 h post infection and the supernatant was analyzed with commercially available ELISA kits for IL-1α, IL-1β, IL-10, IL-17E, IL-6, IL-8, IL-33, MIP-1β, MCP-1, TNF-α, TSLP, and GM-CSF according to the manufacturer's protocol.

5.2.10 Large-scale screening of a *C. albicans* mutant collection

A collection of 1099 *C. albicans* transposon mutant strains (Knockout sets from Aaron Mitchell, provided by the Fungal Genetics Stock Center) was screened for their ability to damage renal and oral epithelial cells and examined the host cell response by quantification of IL-8. Cell lines were cultivated as described in section 5.2.3. 5 µl of *C. albicans* strains from a glycerol stock were incubated in 96-well tissue culture plates in 95 µl YPD at 30°C and 180 rpm until stationary growth. Then, a dilution of 1:20 in fresh YPD was incubated for 16 h at 30°C and 180 rpm. At the day of infection, cell culture medium was removed from epithelial cells and 95 µl fresh media without any supplement was added. For infection, strains were diluted 1:10 in PBS pH 7.4 and 5 µl was added to the wells. After incubation of 24 h in a humidified incubator at 37°C, 5% CO_2 and 95% humidity, LDH and IL-8 was measured from the supernatant.

5.2.11 Stimulation of epithelial PRRs with agonists

To induce a PRR-specific cytokine response, the following agonists were diluted in the respective culture media without any supplements and incubated for 24 h: 10 ng/ml Pam2CSK4 to stimulate TLR2; 100 µg/ml LPS-B5 ultrapure LPS from *E. coli* 055:B5 to stimulate TLR4; 100 µg/ml zymosan A from *S. cerevisiae* to stimulate dectin-1; 100 µg/ml furfurman from *Malassezia furfur* cell wall to stimulate dectin-2; and 100 µg/ml trehalose-6,6-dibehenate (TDB) to stimulate mincle/dectin-3. Furthermore, 100 µg/ml cell wall extractions of mannan from *S. cerevisiae* and 100 µg/ml chitin from shrimp shell were used to test epithelial activation without PRR-specific stimulation.

5.2.12 PRR expression in epithelial cells by flow cytometry

For flow cytometry experiments, all cell lines were grown in 6-well tissue culture plates. Afterwards all steps were performed on ice, if not otherwise stated. Noninfected epithelial cell samples were washed with PBS and detached with 300 µl 0.001% trypsin and 10 mM EDTA within 15 min. Then, 600 µl cell culture medium with 10% FBS was added to stop enzymatic reaction and keeping cells alive. Infected cell samples containing hyphae were filtered through a 70 µm cell strainer (Corning). Cells were counted in PBS and adjusted to $2x10^5$ cells per staining. Cells were stained for viability with 1:500 FVD eFluor506 according to the manufacturer's protocol and afterwards washed with FACS buffer. To reduce the high autofluorescence background signal that was assessed from all tested epithelial cell lines, samples were initially fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set according to the manufacturer's protocol and afterwards stained for the targets. Therefore, 2.5 µl of the following antibodies were used to stain $1x10^6$ cells for the respective PRR in a multicolor panel: anti-human CD282 FITC (mouse, IgG2a, 1 mg/ml) for TLR2; anti-human CD284 PE-Cyanine7 (mouse, IgG2a, 0.2 mg/ml) for TLR4; anti-human CD369 PE (mouse, IgG2a, 0.2 mg/ml) for dectin-1/CLEC7A; anti-human CD368 APC (mouse, IgG2b, 0.2 mg/ml) for dectin-3/CLEC4D; anti-human CD206 Brilliant Violet 421 (mouse, IgG1, 1 mg/ml) for MR. Isotype controls were used in the same concentration as for the staining antibodies: mouse IgG2a FITC, mouse IgG2a PE-Cyanine7, mouse IgG2a PE, mouse IgG2b APC, mouse IgG1 Brilliant Violet 421. Fc receptors were blocked in the staining procedure using human FcR Blocking Reagent with a final dilution of 1:50. Staining and FcR blocking reagent were diluted in FACS buffer and incubated with the cell samples in 96-well plate

with V-bottom for 15-20 min at 4°C. For compensation, each fluorochrome was mixed with OneComp eBeads. The live/dead stain was compensated using the ArC Amine Reactive Compensation Bead Kit. Samples were analyzed using a FACSVerse flow cytometer and the FACSuite software (BD Biosciences). 10,000 events were measured and cells were gated for live (FSC-A/eF506) and single cells (FSC-A/FSC-H) prior to analysis of the mean fluorescence intensity (MFI) from the geometric mean using FlowJo 10.4.1 (LLJ).

5.2.13 Yeast PAMP surface exposure by flow cytometry

Stationary grown *C. albicans* yeast cells were fixed with Roti-Histofix 4% and adjusted to $5x10^6$ cells/ml in PBS. Heat-inactivated yeast cells (10 min at 70°C) were used as a control for dead cells. To stain for β-1,3-glucan, first cells were blocked with 2% Albumin Bovine Fraction V (BSA) for 10 min at 37°C. Then, the cells were incubated for 1 h with 2.5 µg/ml anti-β-1,3-glucan (mouse, IgG). The second antibody goat anti-mouse Alexa Fluor 488 (1:1000) was incubated for another 1 h. To stain for mannan, yeast cells were incubated with concanavalin A Alexa Fluor 647 (1:1000) for 30 min at 37°C. To stain for chitin, cells were incubated with Lectin from *Triticum vulgaris* FITC Conjugate (WGA-FITC, 1:40) for 1 h at room temperature. Samples were analyzed using a FACSVerse flow cytometer and the FACSuite software (BD Biosciences). 10,000 events from stained and unstained samples were measured and the mean fluorescence intensity (MFI) was analyzed from single cells (FSC-A/FSC-H) using FlowJo 10.4.1 (LLJ).

5.2.14 Hyphal PAMP surface exposure by fluorescence microscopy

Glass cover slips were coated with 0.5 mg/ml concanavalin A from *Canavalia ensiformes* in 24-well tissue culture plates to ensure adhesion of strains with weak adhesion properties. Exponential grown *C. albicans* strains were adjusted to 10^5 cells/ml in RPMI 1640 with 10% FBS and incubated for 4 h under humidified conditions in the culture plate. Non-adhered cells were washed with PBS and the samples fixed with Roti-Histofix 4%. Next, the samples were incubated with 2% Albumin Bovine Fraction V for 10 min at 37°C. For the composite staining the following antibodies were incubate for 1 h at 37°C: 5 µg/ml anti-β-1,3-glucan (mouse, IgG) for β-1,3-glucan, concanavalin A Alexa Fluor 647 (1:1000) for mannan, and Lectin from *Triticum vulgaris* FITC Conjugate (1:200) for chitin. The secondary antibody goat anti-mouse PE (1:50) was incubated for 30 min at 37°C. After washing with PBS, cover slips were mounted on cover slides with ProLong Gold Antifading Reagent and analyzed by fluorescence microscopy (Axio Observer.Z1, Zeiss; Plan-Apochromat 20x/0.8 M27; filter set 38 HE for chitin; filter set 43 HE for β-glucan; filter set 50 for mannan). Picture brightness was improved with whole image adjustments in the histogram using ZEN 2.3 blue edition (Zeiss). The RPe (red), FITC (green), and AF647 (blue) channel were separated with the image export method. Single pictures were cropped to 1/3 of the original size without resize and a bar of 20 µm was added.

5.2.15 Adhesion, invasion, and hyphal length

Experiments to determine adhesion, invasion, and hyphal length were performed in 24-well tissue culture plates on glass cover slides. Because the used cell lines differed in their adherence property to glass slides, different preparatory methods were used before seeding: oral epithelial cells were

seeded on glass cover slides without any additional coating. Hepatic epithelial cells were seeded on glass cover slides coated with 0.5 mg/ml concanavalin A from *Canavalia ensiformes*. Renal epithelial cells adhered to glass slides, but medium replacement and washing steps detached these cells completely. However, glass slides coated with 0.5 mg/ml Concanavalin A, 10 µg/ml Collagen I from rat tail, or 0.1 mg/ml poly-D-lysin did not improve adhesion. Therefore, Clipmax was used that has a cyclic olefin polymer (COP) cover slide (TPP Techno Plastic Products), which improved culture maintenance and stability during washing steps, but the cell morphology was visibly impaired. Clipmax with a polystyrene (PS) cover slide (TPP Techno Plastic Products, commercially not available as of March 2019), did not influence cell morphology compared to normal cell culture tissue flasks (Cellstar TC, PS, 75 cm², Greiner) and was used for all assays with renal cells. All cell lines were cultivated for two days like above mentioned.

For adhesion assays, exponential grown *C. albicans* strains were added with a final concentration of 10^5 cells/well and incubated for 1 h under humidified conditions. Non-adhered *C. albicans* cells were removed by three washing steps with PBS. Then, samples were fixed with Histofix overnight at 4°C. *Candida* cells were stained with 10 µg/ml Fluorescent Brightener 28 (calcofluor white) in 0.1 M Tris-HCl (pH 9.0) for 20 min at room temperature. After washing with water, cover slips were mounted on cover slides with ProLong Gold Antifading Reagent and analyzed by fluorescence microscopy (Axio Observer.Z1, Zeiss). Adhered cells were counted from at least 20 images (EC Plan-Neofluar 10x/0.30 M27; filter set 49) covering 2/3 of the whole slide resulting in at least 100 cells illustrated in cells per high power field. Single cells were counted in cell aggregations which were defined as touching yeast cells.

For invasion assays and hyphal length determination, $0.5x10^5$ cells/well were added for 4 h, washed and fixed as above mentioned. To stain outside *Candida* filaments, samples were incubated with anti-*Candida* primary antibody (rabbit, 1:2000) and anti-rabbit Alexa Fluor 488 secondary antibody (1:5000) each for 1 h. All washing steps were performed with 0.01% Tween 20. Prior inside/total staining of *Candida* filaments, cells were permeabilized with 0.5% triton-X 100 for 5 min. Then, samples were incubated with anti-*Candida* primary antibody and anti-rabbit Alexa Fluor 647 (1:5000) each for 1 h. After washing with water, cover slips were mounted on cover slides with ProLong Gold Antifading Reagent and analyzed by fluorescence microscopy (Axio Observer.Z1, Zeiss). At least 120 filaments were analyzed for invasion (Plan-Apochromat 20x/0.8 M27; filter set 38 HE for outside; filter set 50 for inside/total staining). Hyphal length was measured with the line tool and analyzed in the measurement tab (ZEN 2.3 blue edition, Zeiss).

5.2.16 Protein isolation and determination of protein concentration

Epithelial cells were seeded in 6-well tissue culture plates and grown to confluency for two days. For infection, exponential grown *C. albicans* was adjusted to a tenfold host cell cell number used for seeding (HepaRG $20x10^5$ cells/ml, A498 and TR146 $10x10^5$ cells/ml), following published protocols [30]. Cell culture media were substituted without supplements 16 h prior infection. From mock infected controls the media was also exchanged but not from untreated controls (“resting cells”). Cell samples were lyzed on ice with a modified RIPA lysis buffer containing phosphatase inhibitors and protease

inhibitors. The suspension was incubated on ice for 30 min, centrifuged at 15,000 xg for 15 min and the supernatant was used to determine the protein concentration with the Pierce BCA Protein Assay Kit according to the manufacturer's protocol.

5.2.17 Target expression analysis via western blotting

Expression of c-Fos and NF-κB was determined by standard western blotting. Denaturized 10-15 µg protein (to analyze c-Fos) or 5 µg protein (to analyze NF-κB) in sample loading buffer were separated in 12.5% acrylamide gels via SDS polyacrylamide gel electrophoresis (SDS-PAGE) using the Biometra Eco-Mini system (Analytik Jena) and Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad). Then, proteins were transferred on a PVDF membrane (Amersham Hybond 0.45 PVDF) and blocked afterwards with 5% BSA for 1 h. Primary antibodies were diluted in 5% BSA with 0.02% sodium azide 1:1000 for c-Fos (monoclonal, rabbit IgG), 1:1000 phospho-NF-κB p65 (monoclonal, rabbit IgG), 1:1000 NF-κB p65 (monoclonal, mouse IgG2b) and incubated overnight at 4°C. Washing steps were performed in TBS with 0.1% Tween 20. The secondary antibodies anti-rabbit IgG HRP-linked and anti-mouse IgG HRP-linked were incubated for 1 h at room temperature. Blots were incubated with the chemiluminescent Western Lightning Plus-ECL for 1 min and emitting light was detected for 1-5 min with a ChemoImager (Chemi-Smart 5000, Vilber-Lourmat). Pictures were inverted and cropped for the target with Adobe Photoshop CS6.

5.2.18 Quantitative target expression via Smart Protein Layer (SPL) western blotting

The MAPK signaling targets MKP1, ERK1/2, and JNK were quantified with the Smart Protein Layer technology from NH DyeAgnostics [150]. For MKP1 and JNK, two blots were prepared for the phosphorylated and the total target protein. For ERK1/2, phosphorylated and total target protein was analyzed on the same blot, using mouse and rabbit antibodies to distinguish between both states. 10 to 15 µg protein was used from 2 and 4 h samples and 8 µg protein from 6 h samples. The protein samples were labeled with the SPL Blue Kit or SPL Kit Red-IR according to the manufacturer's protocol with target-specific adjustments: the bi-fluorescent Calibrators A and B (Cal A and B) for experiment-to-experiment comparisons and antibody signal comparisons were not mixed due to different brightness levels. Cal A was diluted with 2 µl of a water-loading buffer mixture instead of Cal B. Cal B was diluted to obtain a similar signal intensity like the targets, which was determined in test blots before. Because mouse and rabbit secondary antibodies were used to detect target signals, Cal B was also used with these two isotypes. Cal B rabbit was diluted 1:5 for ERK1/2, and 1:20 for JNK and MKP1 targets. Cal B mouse was diluted 1:2 for ERK1/2 and MKP1, and 1:4 for JNK targets. Smartalyzer basic S (SMA S, 25 kDa) was used as loading control and added to each protein sample. To label the total protein amount, 2 µl (not 1 µl, to enhance labeling efficiency) Smart Label Reagent working solution was added to the protein samples and mixed with the reaction and loading mix (RL-mix). Denaturized Cal A, Cal B, and protein samples were separated in 12.5% acrylamide gels via SDS polyacrylamide gel electrophoresis (SDS-PAGE) using the Biometra Eco-Mini system (Analytik Jena) and Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad). The running front was cut out from the gel before image acquisition was performed by Octoplus QPLEX (NHDyeAGNOSTICS). Using the SPL Blue Kit, the gel

fluorescence was documented in blue (GDye 100) for gel total protein (GTO), and red (GDye 300) for SMA (gel loading: GLO); or with the SPL Kit Red-IR in red for GTO and infrared (GDye 400) for SMA. Then proteins were transferred on a low-fluorescent PVDF membrane (low-fluorescent PVDF Blotting Kit, NHDyeAGNOSTICS) via sandwich wet transfer blotting in an electrical field. The membrane was blocked for 1 h at room temperature with 5% BSA or Odyssey Blocking Buffer in a TBS-based buffer system. The following primary antibodies were separately incubated in 5% BSA or Odyssey Blocking Buffer (TBS) at 4°C overnight: 1:1000 phospho-DUSP1/MKP1 (monoclonal, rabbit IgG); 1:100 MKP1 (monoclonal, mouse IgG3); 1:1000 phospho-p44/42 MAPK ERK1/2 XP (monoclonal, rabbit IgG); 1:1000 p44/42 MAPK ERK1/2 (monoclonal, mouse IgG1); 1:1000 phospho-SAPK/JNK (monoclonal, rabbit IgG); 1:100 JNK (monoclonal, mouse IgG1). Washing steps were performed in TBS with 0.1% Tween 20. Secondary antibodies Smart Infrared Fluorescent anti-rabbit IgG or Smart Red Fluorescent anti-mouse IgG (both goat) were diluted 1:1000 and incubated for 1 h at room temperature.

Using the SPL Blue Kit, signal intensity from the blot was documented using blue fluorescence channel for blot total protein (BTO), infrared fluorescence for blot target protein 1 (BTA 1, e.g. phosphorylated target, bound through anti-rabbit secondary antibody), or red fluorescence for blot target protein 2 (BTA 2, total target protein on a second blot, bound through anti-mouse secondary antibody). When the total target protein was determined on the same blot (BTA 1 and BTA 2), the (mouse) secondary antibody was incubated for another 1 h at room temperature. Then BTO 2 (blue) was detected a second time, and the blot target protein 2 (BTA 2, total target protein) was detected in red.

Using the SPL Kit Red-IR, the signal intensity from the blot was documented using red fluorescence channel for blot total protein (BTO), infrared fluorescence for blot target protein 1 (BTA 1, e.g. phosphorylated target, bound through anti-rabbit secondary antibody), or blue fluorescence for blot target protein 2 (BTA 2, total target protein on a second blot, bound through anti-mouse secondary antibody). When the total target protein was determined on the same blot (BTA 1 and BTA 2), the (mouse) secondary antibody was incubated for another 1 h at room temperature. Then BTO 2 (red) was detected a second time, and the blot target protein 2 (BTA 2, total target protein) was detected in blue.

To increase the signal-to-noise ratio from low-abundance target proteins the HRP-coupled antibodies anti-rabbit IgG HRP-linked or anti-mouse IgG HRP-linked were used as secondary antibodies for incubation instead of the above described fluorescence-conjugated antibodies. Then, blots were analyzed in the blue fluorescent channel to determine BTO. Afterwards the Immuno Blue reagent was added to the blot resulting in a stable blue fluorescent signal.

To avoid overexposed pictures, the fluorescence signal was detected manually just below the saturation threshold. GTO, GLO, BTO1/2 and BTA1/2 pictures were analyzed using LabImage 1D Version 3.4.5 (Kapelan Bio-Imaging) following the SPL-LabImage Software Package manual from NHDyeAGNOSTICS. Lanes and bands on the blot were detected with the automatic lane/band search or manually. For background noise reduction the rolling ball with 45 pixels were applied. SMA and Cal A bands were detected from GLO and transferred on GTO. Then SMA and Cal A bands were detected

from BTO. Cal B and target bands were detected on BTA. A template was adjusted to assign lanes and band names for an automatic normalization with the Project Comparator. For normalization, three steps were (automatically) performed: normalization for equal loading of protein samples based on SMA basic in GLO. Next, the labeling efficiency of the samples was normalized by analyzing the band volume of SMA in GTO. Then, target signals were normalized to total protein in BTO. With this three normalization steps the target protein signals were determined in BTA. SPL normalized target signal intensities were compared to the mock infection control. Because mock infected samples did not differ in their phosphorylation status at all tested time points, the 2 h time point was used as reference for all other time points from infected samples.

5.2.19 Inflammasome activation, blocking of the IL-1 receptor, and blocking of the NLRP3 inflammasome

To induce the inflammasome, epithelial cells were primed with 1 µg/ml LPS for 3 h, washed with PBS, and added 5 mM ATP for 24 h as a second stimulus. Cytokine release of IL-1α and IL-1β was analyzed from the supernatant as described above. 10 µg/ml anakinra (Kineret) was used to block the IL-1 receptor IL-1R1. 25 µg/ml glybenclamide was used as a potassium channel inhibitor to block the maturation of caspase-1 resulting in the blocking of the NLRP3 inflammasome. Both blocking reagents were directly added during infection.

5.2.20 Murine systemic candidiasis model

Female BALB/c mice (eight weeks old, Charles River Germany) were housed without antibiotics. For infection, cryostored *C. albicans* strains were plated on YPD agar, restreaked on plate, and incubated in YPD overnight at 30°C. Cells were washed in ice-cold PBS and afterwards physically separated with a syringe (Ø 0.5 mm, BD Plastipak) by pipetting for at least 10 times prior counting. Mice were infected with $2.5x10^4$ cells/g body weight via lateral tail vein. The health status was monitored twice a day by examination of temperature, weight and behavior. Survival was monitored over 21 days and mice showing severe illness were euthanized. To analyze fungal burden, organs were separated, weighed, homogenized in PBS, plated in serial dilutions on YPD agar, and incubated for two days at 30°C. For histological analysis, cross-section organ slices were fixed, embedded in wax, and stained with periodic acid-Schiff (PAS). Samples were analyzed using Axio Observer.Z1 (Zeiss) in the channel TL Brightfield with a color camera with a 40x magnification (EC Plan-Neofluar 40x/0.75 M27) for single pictures. Whole organ images were generated by taking pictures with the tile method (EC Plan-Neofluar 10x/0.30 M27) and afterwards stitched into one image using the stitch method (ZEN 2.3 blue edition, Zeiss).

5.2.21 Ethic statement

Animal experiments were performed in accordance with the German animal protection law and were approved by the Federal State authority (Thüringer Landesamt für Verbraucherschutz) ethics committee (beratende Komission nach §15 Abs. 1 TierSchG; permit no. 03–023/16). The animals were cared in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

5.2.22 Statistical analysis

All graphs display the mean of biological replicates with standard deviation. Statistical analyses were performed using GraphPad Prism 7.04. Differences between groups were performed using one-way ANOVA with Tukey's multiple comparisons test. Comparisons to one control group were performed using one-way ANOVA with Dunnett's multiple comparisons test.

6 Results

6.1 Epithelial damage and cytokine release in response to *C. albicans* infection

Epithelial cells lining mucosal surfaces represent the first line of defense against invading pathogens. The epithelium not only serves as a physical barrier but also senses host cell injury and induces an innate immune response [36]. Production and secretion of inflammatory cytokines by mucosal epithelial cells activate resident immune cells and further contribute to the recruitment of additional immune cells that could clear infections [151,152]. Previous studies of the epithelial immune response mainly focused on the oral and vaginal epithelium that is commonly colonized by *C. albicans*. These surfaces can be affected by superficial fungal infections that trigger the release of IL-6, IL-8, IL-1α/β, GM-CSF, and TNF-α depending on cell lysis [113,151–153]. Mucosal epithelial cells from the gastro-intestinal tract and lung also respond to *C. albicans* infection [154–156]. However, the fungus also interacts with other types of epithelial cells that form the parenchyma of internal organs, e.g. from the kidney [157]. In systemic candidiasis of mice, the cytokine profile from the kidney does not correlate with the low number of residential immune cells or delayed kinetics of recruited immune cells [145,147,158]. Therefore epithelial cells might contribute to this organ-specific response leading to the immunopathology of *C. albicans* infection.

6.1.1 *C. albicans* causes less damage to oral epithelial cells in comparison to hepatic and renal epithelial cells

Secretion of inflammatory cytokines from oral and vaginal epithelial cells is damage-dependent [20,58,151]. To compare the damaging capabilities of *C. albicans* on different cell lines, $2x10^4$ of oral (TR146) and renal (A498) cells and the double amount of hepatic (HepaRG) cells were seeded in a 96-well tissue culture plate and grown to full confluency within two days. For infection, *C. albicans* wild type SC5314 cells were added with the same cell number of seeded host cells to enable a comparable multiplicity of infection (MOI) by less than one. After 24 h released lactate dehydrogenase (LDH) was determined in the supernatant and unveiled differences between these cell lines (Fig. 5). Nearly all hepatic and renal cells were damaged based on comparison to total cell lysis induced by Triton. In contrast, only about 60% of oral cells were damaged, suggesting epithelial-specific sensitivities to *C. albicans* infection.

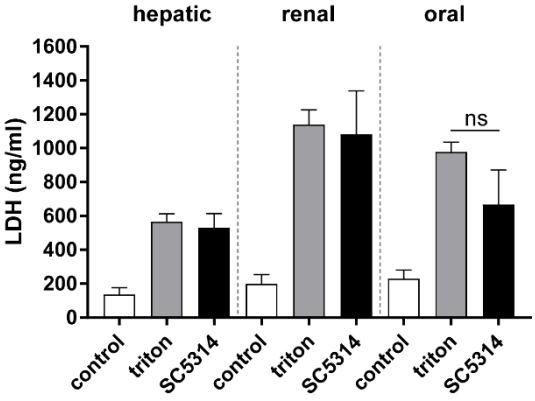

Fig. 5. Epithelial cell damage induced by *C. albicans* SC5314.
Confluent hepatic (HepaRG), renal (A498), and oral (TR146) epithelial cell lines were mock-infected (control) or infected with *C. albicans* SC5314 for 24 h. To induce total cell lysis, triton was added 5 min prior the end of the experiment. Lactate dehydrogenase (LDH) was quantified from supernatants. Data represent mean values +/- SD from at least six independent experiments.

6.1.2 The cytokine response towards *C. albicans* infection differs between epithelial cells

Next, inflammatory cytokines were quantified from the supernatant by enzyme-linked immunosorbent assay (ELISA) and revealed further cell type-specific differences: even though all cell lines secreted interleukin (IL)-6 and IL-8 in response to *C. albicans* infection, hepatic epithelial cells secreted significantly lower amounts of IL-6 and slightly less IL-8 compared to renal and oral epithelial cells (Fig. 6A). Nonetheless, IL-6 and IL-8 secretion by infected hepatic cells was significantly increased compared to mock-infected control (Fig. 6B). TNF-α was also released by all cell lines upon infection with the highest release observed for hepatic cells (Fig. 7A). IL-1α and IL-1β were exclusively secreted by oral epithelial cells (Fig. 7B). While the release of IL-1α is associated with damage, IL-1β release requires active production and secretion mediated by the inflammasome [159]. To test whether an inflammasome-associated cytokine response could be induced in hepatic and renal epithelial cells, LPS was used for initial priming followed by stimulation with ATP [136,137,160], but this did not result in any IL-1 release in hepatic and renal epithelial cell lines (data not shown).

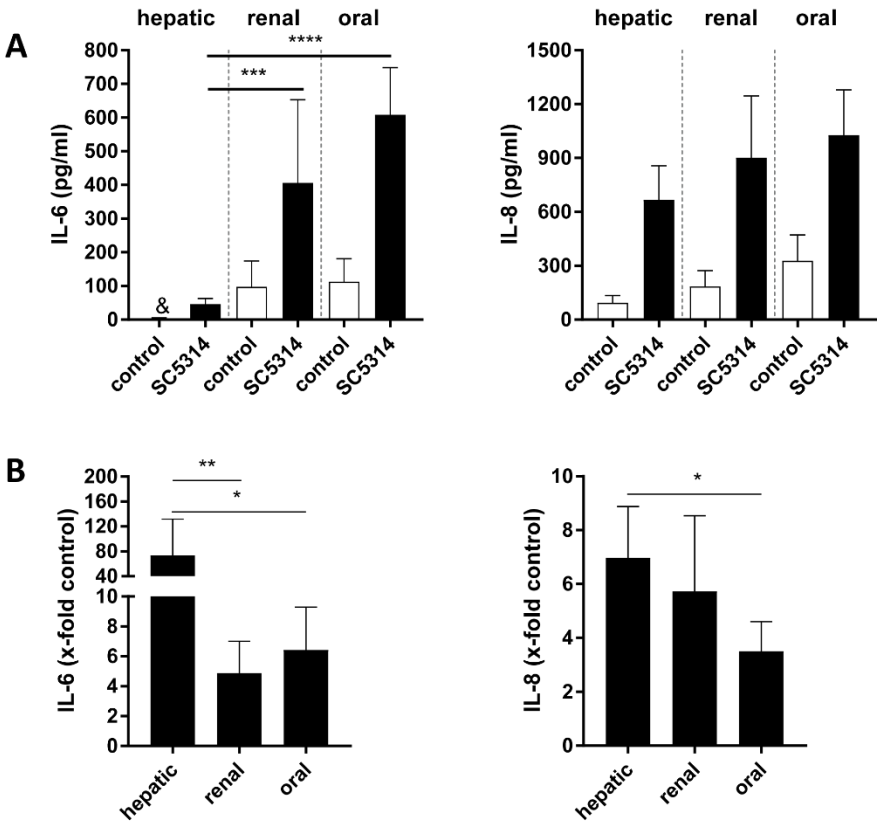

Fig. 6. Cell line-dependent IL-6 and IL-8 release induced by *C. albicans* SC5314.
Confluent hepatic (HepaRG), renal (A498), and oral (TR146) cell cultures were mock-infected (control) or infected with *C. albicans* SC5314 for 24 h. (A) IL-6 and IL-8 were quantified from supernatants by ELISA. (B) Relative increase of IL-6 or IL-8 release compared to the mock-infected control. Data represent mean values +/- SD from at least six independent experiments. &, below detection limit. Statistics: One-way ANOVA with post-hoc Tukey's multiple comparisons test; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.

Furthermore, TSLP was released in small amounts by infected hepatocytes (Fig. 7C), and IL-33 by infected hepatic and oral cells (Fig. 7D). The inflammatory cytokine MCP-1, that generally is involved in the early acute-phase response during systemic infection, was induced by *C. albicans* only in infected renal cells, whereas infection reduced the amount secreted by hepatic and oral cells (Fig. 7E). GM-CSF, MIP-1β, IL-17E (IL-25), and the anti-inflammatory cytokine IL-10 were not detectable in the supernatant of any cell line (data not shown).

Thus, *C. albicans* infection caused damage in epithelial cells from different organs, with susceptibility differing between different cell lines, and induced an innate immune response by a core secretion of the inflammatory cytokines IL-6 and IL-8, and additionally by the secretion of cell line-specific cytokines.

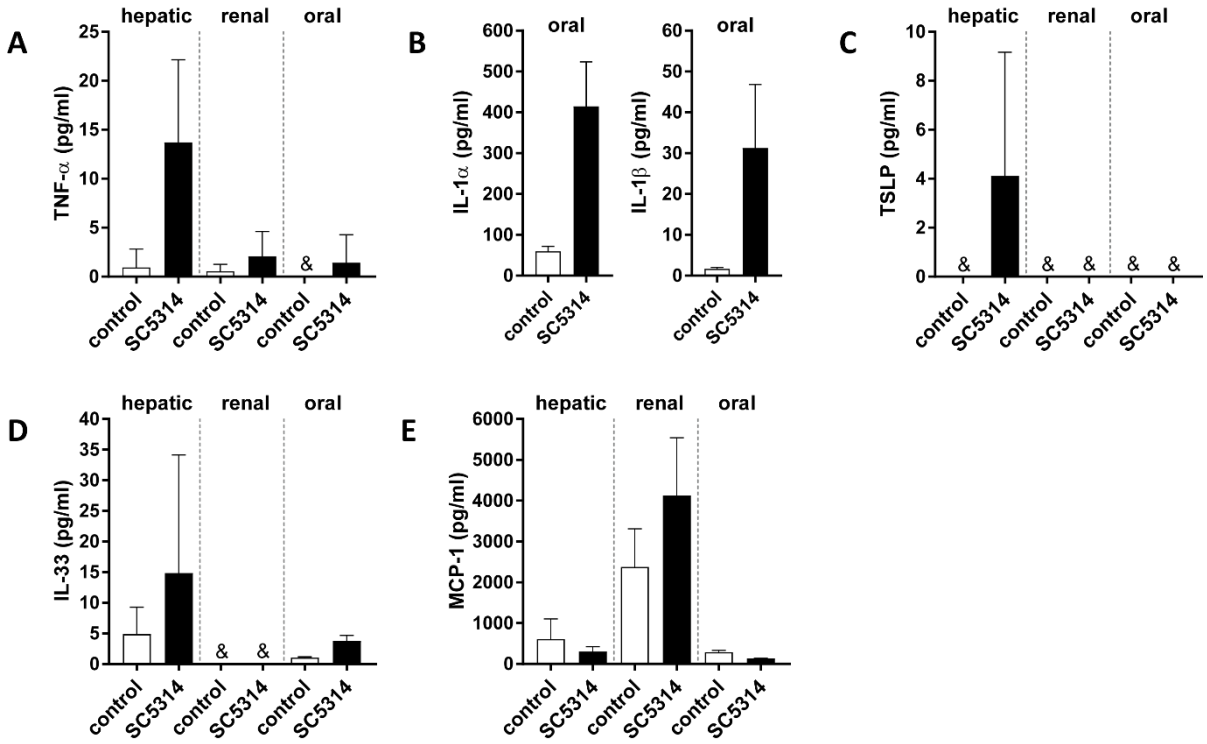

Fig. 7. Epithelial-specific cytokine response induced by *C. albicans* SC5314.
Confluent hepatic (HepaRG), renal (A498), and oral (TR146) cell cultures were mock-infected (control) or infected with *C. albicans* SC5314 for 24 h. TNF-α (A), IL-1α and IL-1β (B), TSLP (C), IL-33 (D), and MCP-1 (E) were quantified from supernatants by ELISA. Data represent mean values +/- SD from at least three independent experiments. &, below detection limit.

6.2 Pattern recognition receptors (PRRs) expressed by different epithelial cells

Pathogen-induced cytokine secretion depends on stimulation of pattern recognition receptors (PRRs) [101,161]. Additionally, induction of cytokine release by oral and vaginal epithelial cells requires cell damage [21,58,151]. The cell lines analyzed in this study were substantially damaged by *C. albicans* infection and therefore insufficient damage could be excluded as an explanation for the differences in cytokine production. This suggested that epithelial-specific expression of PRRs could lead to the differences in cytokine induction in response to *C. albicans*.

6.2.1 Hepatic, renal, and oral epithelial cells express a similar repertoire of PRRs

To investigate the two main classes of PRRs recognizing *C. albicans* cell wall components [101], expression of toll-like receptors (TLRs) and C-type lectin receptors (CLRs) was analyzed by flow cytometry. Preliminary experiments revealed a strong autofluorescence from all analyzed cell lines that could be reduced by permeabilization with saponin resulting in the staining of total PRR expression rather than surface expression. The mean fluorescence intensity (MFI) was analyzed from the geometric mean of all samples. To determine nonspecific binding of the fluorescence antibodies, isotype controls were used, and the resulting MFI were comparable in most cases with the unstained samples (data not shown). However, since not all available isotype controls perfectly matched all specific antibodies, and it is widely accepted to refrain from using isotype controls in quantitative

assays [162], the MFI of stained samples were compared to the unstained one. The relative MFI was increased in all cell lines for TLR2, TLR4, dectin-1, dectin-3, and at low levels for MR (Fig. 8), indicating expression of these PRRs. From all tested PRRs, the MFI for TLR4 showed the strongest intensity in all three cell lines. Compared to renal and oral cells, hepatic cells displayed the highest relative increase

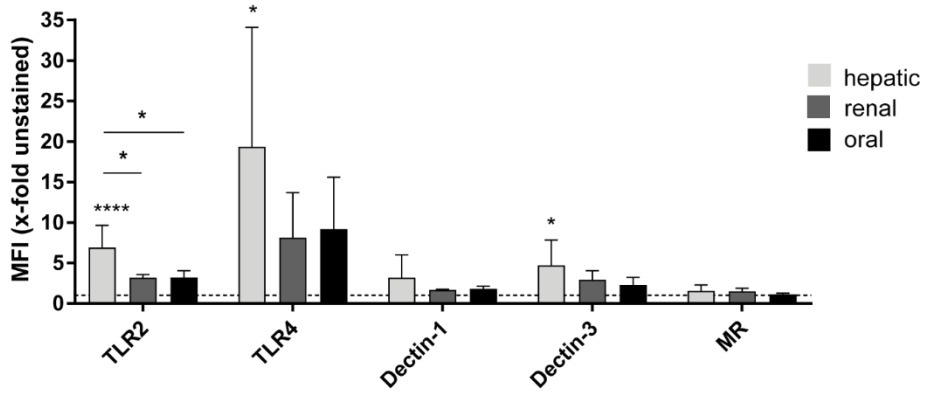

Fig. 8. Similar repertoire of pattern recognition receptors (PRRs) expressed by different epithelial cells. PRR expression from unstimulated hepatic, renal, and oral epithelial cells grown for two days. Cells were removed (0.001 % trypsin + 10 mM EDTA) and stained for dead cells (eF506), and the PRRs TLR2, TLR4, dectin-1, dectin-3, and MR. 10.000 events were gated for live cells (FSC-A/eF506), then for single cells (FSC-A/FSC-H). The mean fluorescence intensity (MFI) was analyzed from the geometric mean and the relative MFI was compared to unstained samples depicted from at least four independent experiments +/- SD. Dotted baseline: MFI=1. Statistics: One-way ANOVA with post-hoc Tukey's multiple comparisons test; *, $P \leq 0.05$; ****, $P \leq 0.0001$.

in the MFI of TLR2, TLR4, dectin-1, dectin-3; suggesting a stronger PRR expression in this cell line.

Some studies have been demonstrated that infection changes PRR gene transcription [163,164]. Therefore, PRR expression was analyzed in cell lines stimulated with both IL-1β and TNF-α, zymosan A, or co-incubated with live *C. albicans* for 7 h. This stimulation, however, did not influence the relative MFI compared to unstimulated controls (Fig. 9).

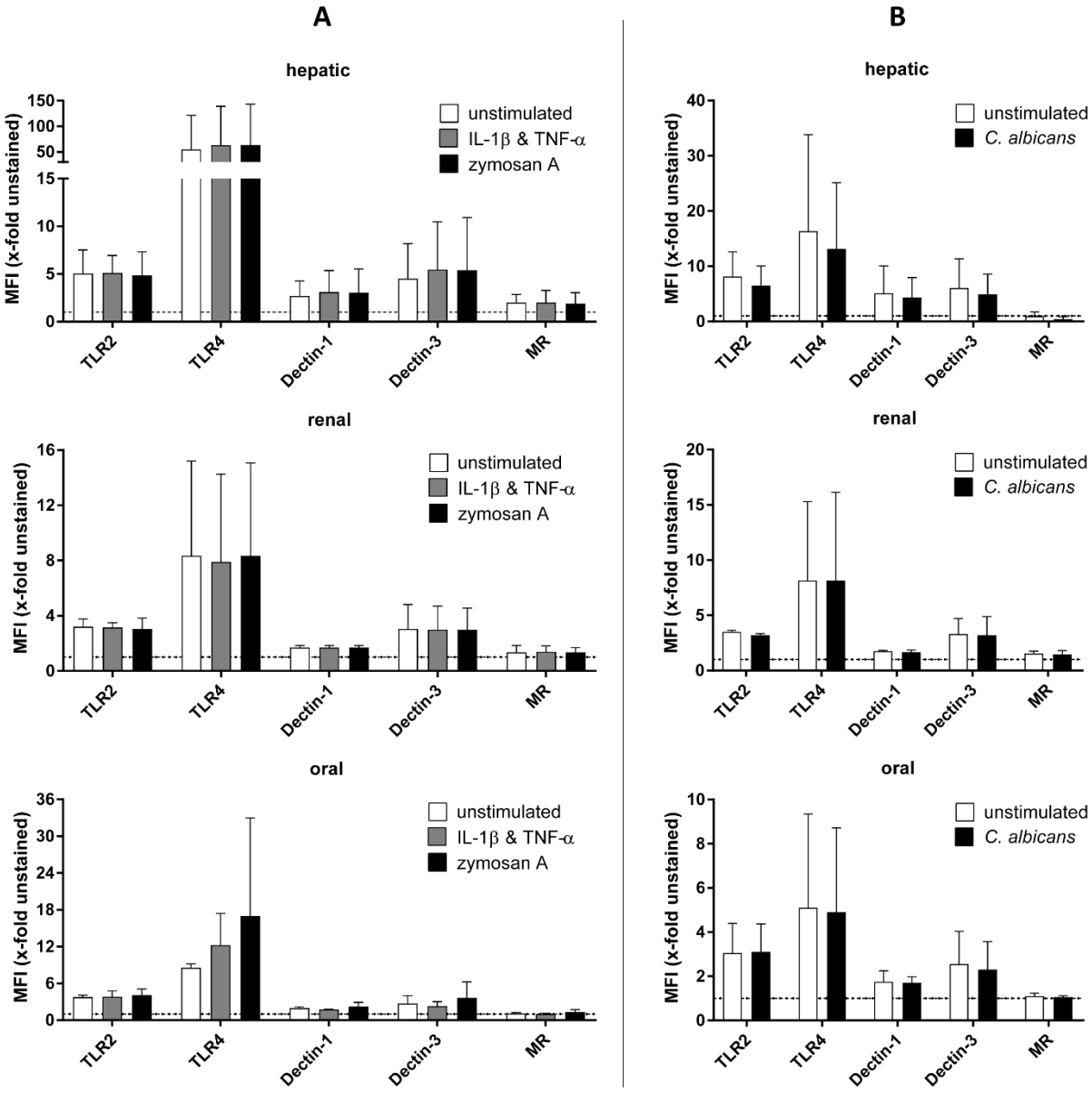

Fig. 9. PRR expression is unaltered after cytokine stimulation or infection with *C. albicans*.
Confluent hepatic, renal, and oral cells were either unstimulated, stimulated with both IL-1β and TNF-α, or zymosan A for 24 h (A) or infected with *C. albicans* for 7 h (B). Cells were removed (0.001 % trypsin + 10 mM EDTA) and stained for dead cells (eF506), and the PRRs TLR2, TLR4, dectin-1, dectin-3, and MR. 10.000 events were gated for live cells (FSC-A/eF506), then for single cells (FSC-A/FSC-H). The mean fluorescence intensity (MFI) was analyzed from the geometric mean and the relative MFI was compared to unstained samples depicted from two independent experiments with +/- SD. Dotted baseline: MFI=1.

6.2.2 Epithelial cells differ in the responsiveness to PRR ligands

Host cells express TLRs and CLRs to recognize microbial pathogens rapidly. While results in this study demonstrate that composition and expression levels of PRRs do not explain the epithelial-specific cytokine response, cells can differ in the responsiveness of PRR-induced signaling cascades. To test this hypothesis, PRRs were stimulated with specific ligands (Pam2CSK4 for TLR2, purified LPS-B5 for TLR4, zymosan A for dectin-1, furfurman for dectin-2, trehalose-6,6-dibehenate (TDB) for dectin-3 stimulation) and IL-8 release was quantified after 24 h of incubation. From all tested cell lines, hepatic epithelial cells were most responsive to all tested PRR ligands and induced a strong IL-8 release after

stimulating TLR2, TLR4, dectin-1, and dectin-2 (Fig. 10) without causing damage (data not shown). Stimulation of PRRs of oral cells did not induce IL-8 secretion compared to the unstimulated control, confirming that the oral cytokine response additionally requires damage-dependent signaling [58]. Renal epithelial cells were solely responsive to TLR2 and dectin-3 stimulation.

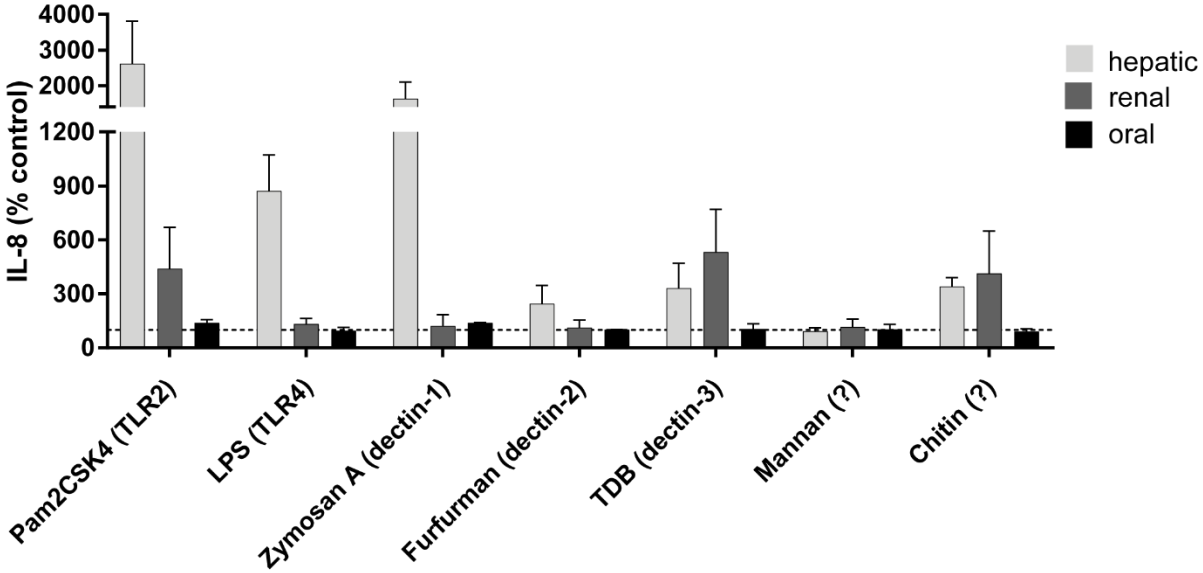

Fig. 10. Responsiveness of TLRs and CLRs differ between epithelial cells.
IL-8 released in the supernatant from hepatic, renal, and oral epithelial cells stimulated with PRR ligands for 24 h. The following PRRs were stimulated: TLR2 (Pam2CSK4), TLR4 (LPS-B5), dectin-1 (zymosan A), dectin-2 (furfurman), and dectin-3 (trehalose-6,6-dibehenate, TDB); mannan and chitin. Data represent mean values +/- SD from at least two independent experiments. Dotted baseline: 100% IL-8 release of the unstimulated control.

It has been shown that myeloid cells, e.g. monocytes and macrophages, respond to fungal cell wall components, specifically mannan [107], β-glucan [165,166], and chitin [94]. In contrast to innate immune cells, cell wall extractions from *S. cerevisiae* mannan or chitin from shrimp shell did not induce IL-8 secretion by oral epithelial cells without damage [151,163]. Likewise, mannan did not induce a response by the other cell lines either. Hepatic and renal cells secreted small amounts of IL-8 when stimulated with chitin.

In summary, while oral epithelial cells require damage to induce a PRR-mediated cytokine response, renal and in particular hepatic cells respond to a variety of ligands by inducing IL-8 secretion. This strong hepatic cytokine secretion could contribute to the overall cytokine response during infection independent from the damage caused by the pathogen.

6.3 Fungal factors modulating the epithelial cytokine response

Disseminated candidiasis in mice results in an early and strong cytokine expression in the kidney that could not originate from the low number of residential immune cells [145,147]. It is, therefore, possible that epithelial cells contribute to the innate immune response by secreting inflammatory cytokines. *C. albicans* can bypass the innate immune system by metabolic adaptation or through masking immunoactive cell wall components [167]. One of the few known fungal factors that directly modulate

the epithelial cytokine response are secreted aspartyl proteinases (SAPs) [21]. Part of this study was to discover new fungal factors that have an immunomodulatory effect on epithelial cells.

6.3.1 Identification of *C. albicans* factors that influence epithelial cytokine secretion

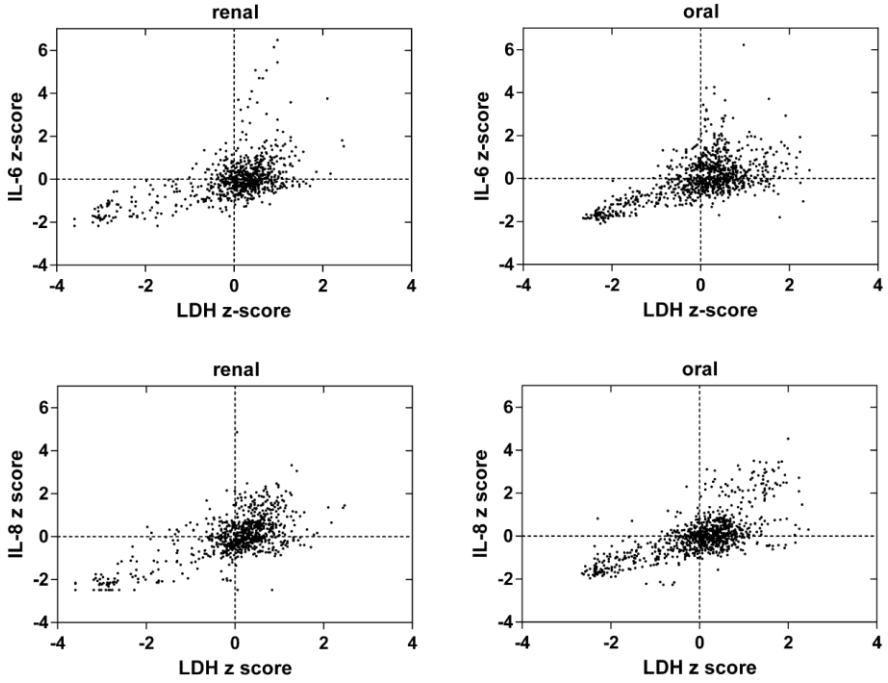

Fig. 11. Damage-dependent IL-6 and IL-8 release of renal and oral epithelial cells.
Confluent renal and oral cell cultures were infected with a collection of 1099 *C. albicans* transposon strains. After 24 h, lactate dehydrogenase (LDH), IL-6, and IL-8 were quantified from supernatants. The z score indicates the distribution of standard deviations from the mean represent by the parental strain and was calculated as $z = (X - \mu) / \sigma$; with X=score, μ=mean, and σ=standard deviation. Data represent one biological replicate.

To screen for fungal factors that can modulate the epithelial cytokine response, a commercially available collection of 1099 *C. albicans* transposon mutant strains [168] was analyzed for their ability to damage renal and oral cells and to induce IL-8 secretion. The capability to induce IL-8 secretion appeared to correlate with host cell damage, and most of the strains showed a similar damage and cytokine response as it was observed for parental strains (Fig. 11, a z score of zero represents the parental strain). While strains with a defect in filamentation (8%) or a growth defect (3%) were excluded from further analysis, as this would reduce damage of host cells, strains that did not correlate in damage and cytokine release, or induced an epithelial-specific immune response, were selected for further analysis. Therefore, the relative cell damage and relative IL-8 secretion were calculated based on the respective parental strain. Mutant strains with a standard deviation of less than 1 in damage were considered to induce unaltered cell damage. For the IL-8 release, strains with a standard deviation of more than 1.5 were considered to induce a stronger or weaker cytokine response. In total, 68 putative candidates were selected for a triplicate repetition that confirmed the phenotype for one-third of these strains. 11 transposon mutants with the strongest alteration in IL-8 induction were selected for further analysis including gene mutations in *CHD1*, *RIC1*, *SUV3*, *RFG1*,

PHO23, *MCM1*, *SCH9*, *SPT20*, *ORF19.764*, *BAS1*, and *MNN9* (Table 14). To further analyze the epithelial-specific immune response; intestinal, hepatic, renal, and, oral epithelial cells were infected with each of the eleven mutant strains. Some strains displayed increased damage to intestinal and oral cells but not hepatic and renal cells (Fig. S1A). A strong increase of IL-8 secretion was observed in oral cells in response to mutant strains such as *suv3*Δ/Δ or *mnn9*Δ/Δ (Fig. S1B). None of the analyzed mutant strains induced a stronger IL-8 release of renal cells compared to the respective parental strain.

Table 14. Possible *C. albicans* factors that modulate the epithelial-specific cytokine response

Open Reading Frame (ORF)	gene	(putative) function
orf19.2823	*RFG1*	transcriptional repressor
orf19.7383	*MNN9*	protein mannosylation
orf19.6036	*RIC1*	unknown
orf19.829	*SCH9*	protein kinase
orf19.4519	*SUV3*	RNA helicase
orf19.3809	*BAS1*	transcription factor, purine biosynthesis
orf19.422	*SPT20*	transcriptional co-activator
orf19.1759	*PHO23*	unknown
orf19.7025	*MCM1*	transcription factor
orf19.3035	*CHD1*	unknown
orf19.764	-	unknown

6.3.2 A defect in the mannosyltransferase II partly encoded by *MNN9* modulates the epithelial-specific cytokine response

The large-scale host response screening revealed 11 possible fungal factors that could modulate an epithelial cytokine response. To verify the phenotype of these transposon mutants, homozygous gene deletion mutants were generated with the *SAT1* flipper method in the marker-free genetic background of the *C. albicans* wild type SC5314 [149]. Successfully created homozygous gene deletion strains of *RIC1*, *SUV3*, and *MNN9* were used for infection of hepatic, renal, and oral epithelial cells (data not shown). From all tested candidates, deletion of *MNN9* showed the strongest phenotype: while hepatic and oral cell damage was similar compared to the parental strain (Fig. 12A), *mnn9*Δ/Δ induced significantly more IL-6 and IL-8 secretion by hepatic cells and elevated levels of IL-6 and significantly more IL-8 by oral cells (Fig. 12B+C). In renal cells, however, a reduced damage also resulted in a decreased cytokine response. The elevated cytokine response was reversed in the heterozygous complementation strain +*MNN9*.

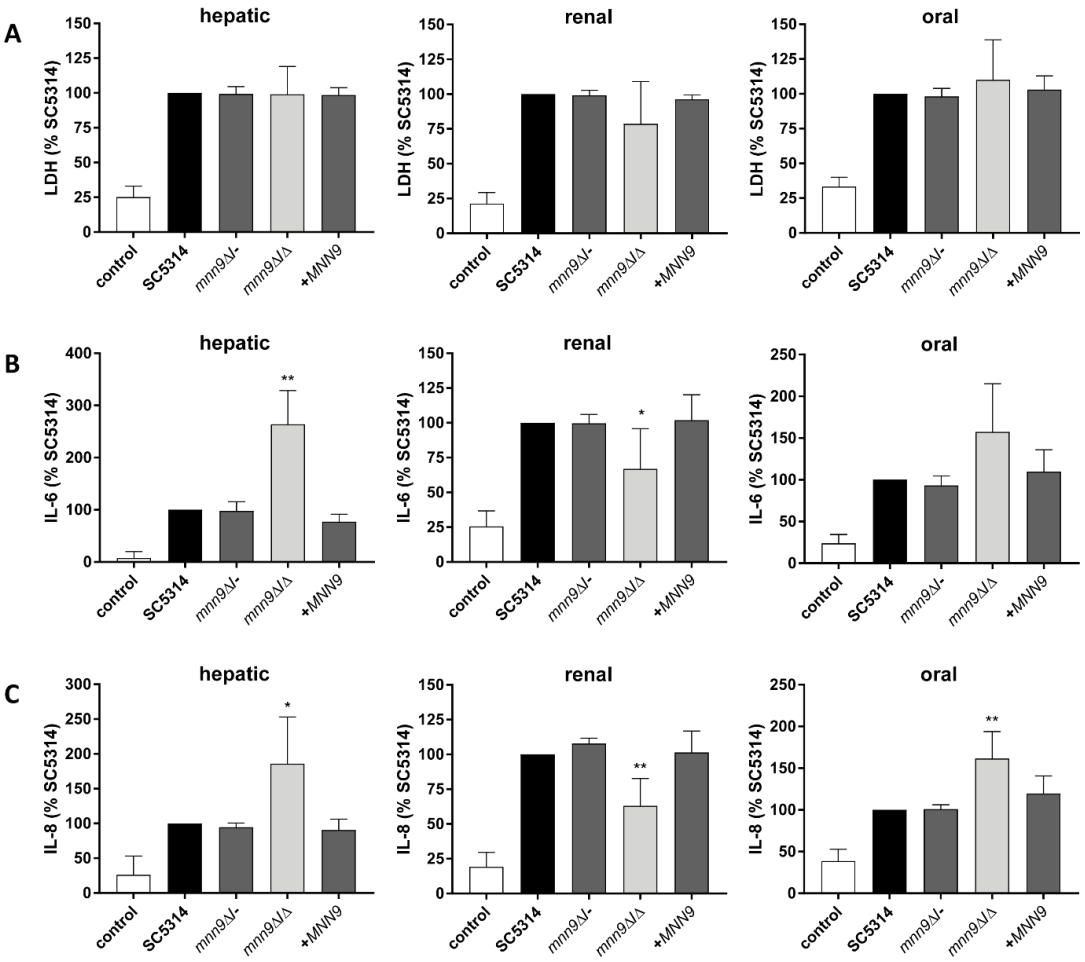

Fig. 12. Deletion of *C. albicans MNN9* modulates the epithelial-specific cytokine response.
Confluent hepatic, renal, and oral epithelial cell lines infected with *C. albicans mnn9*Δ/Δ for 24 h under standard culture conditions. Cell culture supernatant was assessed for lactate dehydrogenase (LDH, A) measuring epithelial cell damage, or the amount of IL-6 (B) and IL-8 by ELISA (C). Data represent mean values +/- SD from at least four independent experiments (three for the heterozygous mutant). Statistics: One-way ANOVA with post-hoc Dunnett's multiple comparisons test; *, P ≤ 0.05; **, P ≤ 0.01.

6.4 Influence of cell wall mannosylation on the epithelial immune response

Deletion of *MNN9* caused an increased hepatic and oral epithelial cytokine response while the damage potential did not differ. *MNN9* encodes for a subunit of the mannan polymerase complex II and is involved in the *N*-linked outer-chain mannan and protein glycosylation by adding α-1,6-mannose to the *N*-mannan backbone [169]. As a consequence, *MNN9* activity influences the elongation of the α-1,6-mannose backbone that is essential for further additions of α-1,2-mannose sidechains and phosphomannan capping [69]. During infection, fungal cell wall components are crucial for the stimulation of host cells by their interaction with PRRs [35]. Mannosylation has a significant impact on the accessibility of cell wall components and thus subsequent host cell responses [163,170,171]. To gain further insight into the role of *N*-mannosylation on epithelial cell type-specific immune responses, *C. albicans* mutants were selected that display gradually different degrees of the mannosylation status. This included an *OCH1* mutant with an absent α-1,6-mannose backbone [68]; a *PMR1* mutant with

shorter *N*- and *O*-mannosylation chains [172]; and a strain lacking all α-1,2-mannose side chains due to the deletion of the whole *MNN2* gene family (the mmn2Δsextuple mutant [75]).

6.4.1 The degree of mannosylation affects epithelial damage and cytokine response

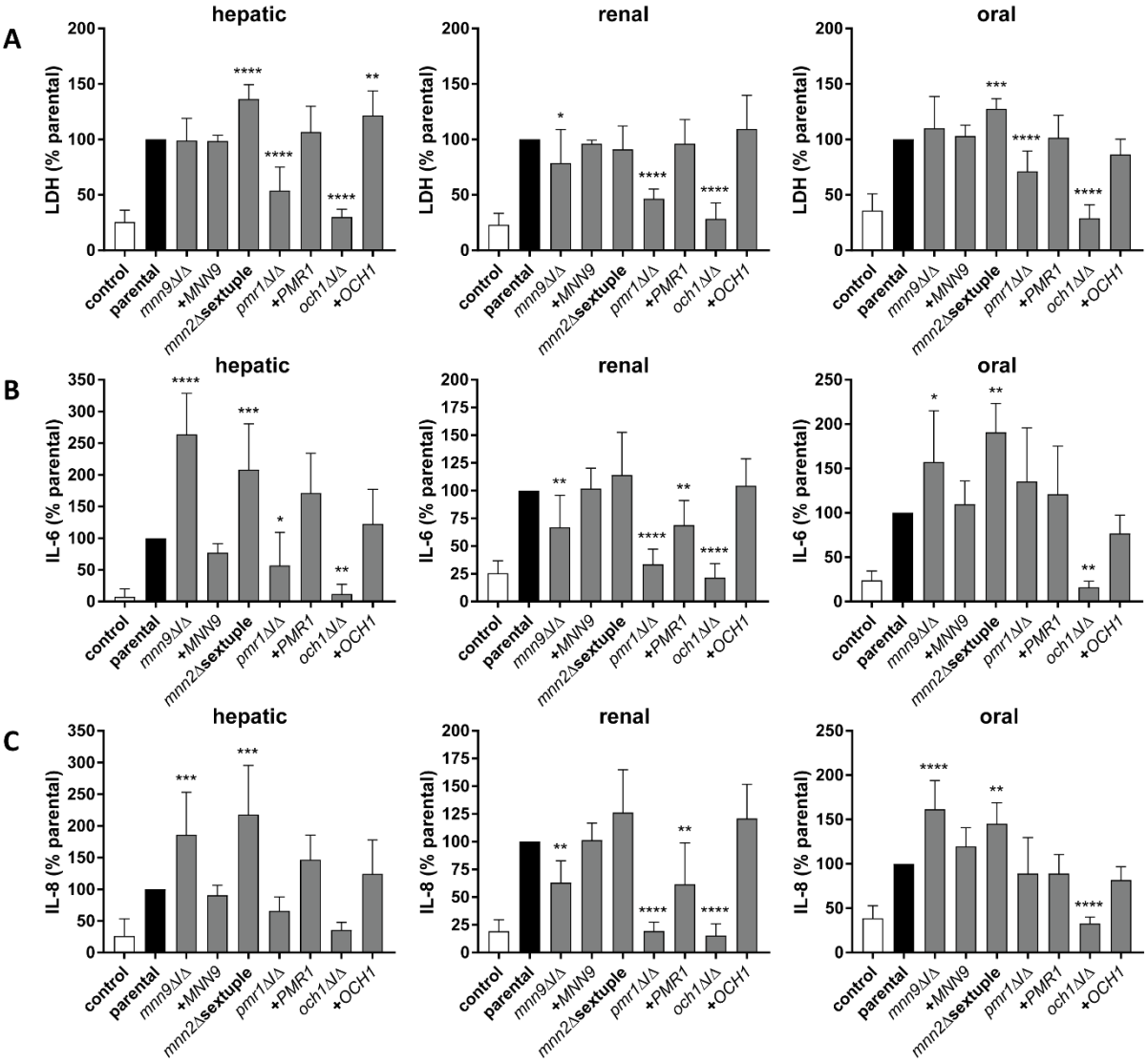

Fig. 13. The cell wall mannosylation status affects epithelial cell damage and cytokine secretion. Confluent hepatic, renal, and oral epithelial cell lines infected with different *C. albicans* mannosylation mutants for 24 h under standard culture conditions. Cell culture supernatant was assessed for lactate dehydrogenase (LDH, A) measuring epithelial cell damage, or the amount of IL-6 (B) or IL-8 by ELISA (C). Data represent mean values +/- SD from at least three independent experiments. Statistics: One-way ANOVA with post-hoc Dunnett's multiple comparisons test; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.

Oral epithelial cells differentially respond to *N*- and *O*-mannosylation defects of the *C. albicans* cell wall that particularly depends on fungal pathogenicity rather than the glycosylation status [98]. To test the influence of an altered mannosylation status on mucosal and parenchymal epithelial cells, the supernatant from infected hepatic, renal, and oral epithelial cells was analyzed for released LDH, IL-6, and IL-8; and compared the results with the respective parental strain. A deletion in *OCH1* abolished hyphal formation and did neither induce host cell damage nor any cytokine response by

the tested epithelial cells (Fig. 13). Deletion of *PMR1* decreased the damage observed in hepatic and renal cells and induced less IL-6 and IL-8 secretion, particularly in renal cells. Oral cell damage was also reduced by the *pmr1*Δ/Δ; however, secreted levels of IL-6 and IL-8 were comparable with the parental strain. The *MNN2* sextuple mutant displayed an increase in cytokine response by hepatic and oral cells similar to the *mnn9*Δ/Δ. However, while hepatic and oral damage induced by the *mnn9*Δ/Δ was comparable to the parental strain, the *MNN2* sextuple mutant caused more damage to these cell lines. Another cell type-specific difference was observed for renal epithelial cells: the *mnn9*Δ/Δ caused slightly less renal cell damage and consequently induced less cytokine release. However, damage induced by the *MNN2* sextuple mutant was comparable to the parental strain, but the cytokine response was moderately increased. Other strains from the transposon mutant collection that have an unaltered α-1,6-mannose *N*-mannan backbone, but subtle alterations in side chains of α-1,2-mannose or α-1,3-mannose (mutation in either *MNN14* or *MNN22* [75,78]) did not alter the epithelial cytokine response (Fig. S2).

6.4.2 Induction of the epithelial inflammasome-associated IL-1 secretion

In this study, it could be shown that only oral epithelial cells but not hepatic or renal epithelial cells, secreted IL-1 in response to *C. albicans* infection. While IL-1α is usually released upon cell damage, IL-1β is actively secreted depending on caspase-1 [159]. Activation of myeloid caspase-1 requires TLR2 and dectin-1 signaling as a first stimulus and formation of the NLRP3 inflammasome as a second stimulus. Both, IL-1α and IL-1β bind to the same IL-1 receptor (IL-1R1), induce their own production and secretion via a positive feedback loop and can stimulate IL-8 and other inflammatory cytokines [105,131,173].

6.4.2.1 The release of oral IL-1 is damage-dependent and correlates with IL-6 and IL-8

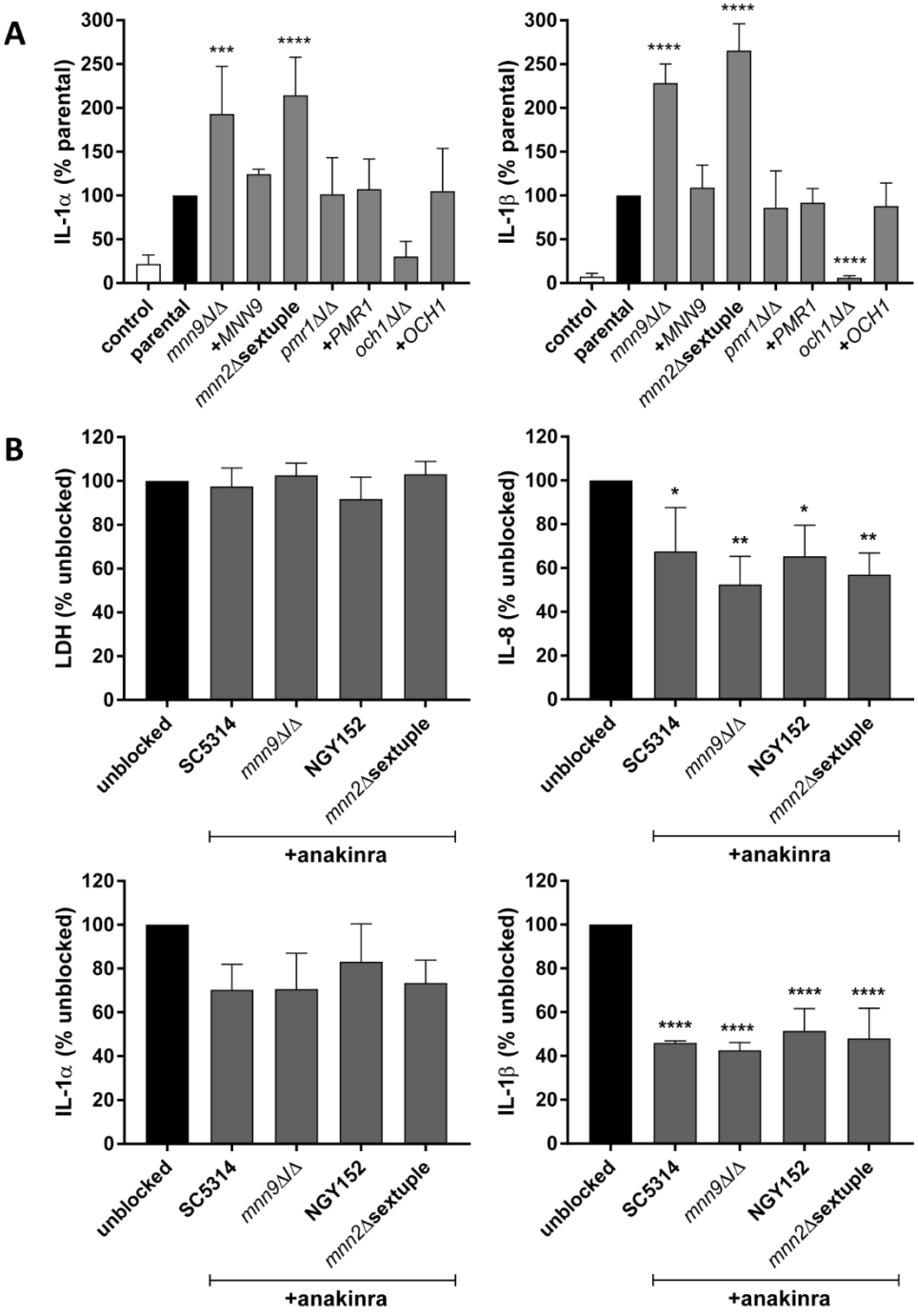

Fig. 14. Blocking of the IL-1 receptor (IL-1R1) in oral epithelial cells decreases the release of inflammatory cytokines independent from the mannosylation status.
Oral cells were infected with different mannosylation mutants and corresponding parental strains for 24 h. (A) Cell culture supernatants from at least four (two for *och1*Δ/Δ and +*OCH1*) independent experiments were used to determine the release of IL-1α and IL-1β. The values obtained for the parental strains were set to 100%. (B) Effect of IL-1R1 blocking with 10 µg/ml anakinra on damage determined by LDH release into supernatants and the relative secretion of IL-8, IL- 1α, and IL-1β. The values obtained for unblocked cells were set to 100%. Data are depicted as mean +/- SD from at least three independent experiments. Statistics: One-way ANOVA with post-hoc Dunnett's multiple comparisons test; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.

Elevated IL-6 and IL-8 release triggered by the *mnn9*Δ/Δ and *MNN2* sextuple mutant strains raised the question whether the release of IL-1 is also affected by the glycosylation status. Like IL-6 and IL-8 secretion, the release of oral IL-1α has been shown to be dependent on *C. albicans*-mediated damage [174]. This could also be shown for the damage-deficient *och1*Δ/Δ, which did not induce IL-1 secretion (Fig. 14A, for damage see Fig. 13A). Although the damage capacity of the *pmr1*Δ/Δ was reduced (Fig. 13A), secreted amounts of IL-1α and IL-1β were similar to the parental strain (Fig. 14A) and corresponded with secreted IL-6 and IL-8 levels (Fig. 13B+C). While the *MNN2* sextuple mutant caused enhanced damage and induced a twofold IL-1 secretion, the *mnn9*Δ/Δ strain induced a similar IL-1 release compared to the *MNN2* sextuple mutant but damaged oral cells to the same extent as the parental strain.

6.4.2.2 Defects in mannosylation do not affect IL-1R1-dependent IL-1 release

Binding of IL-1 to the IL-1R1 induces the production and secretion of inflammatory cytokines [105,175]. Therefore, the increased amount of IL-1 release by oral epithelial cells in response to infection with the *mnn9*Δ/Δ and *MNN2* sextuple mutants could potentiate the cytokine response, as it was observed for IL-8, via binding of IL-1R1 (Fig. 13C). To examine the role of IL-1R1 signaling in the overall cytokine response, the receptor was blocked during infection with the antagonist Anakinra (also known as Kineret), and the release of LDH, IL-1α, IL-1β, and IL-8 was determined from the supernatant. Compared to the infected and unblocked sample, the addition of Anakinra reduced the relative IL-8 secretion independent from the glycosylation status (Fig. 14B). Likewise, IL-1α was reduced, even though blocking the IL-1R1 did not influence cell damage. Secretion of IL-1β was reduced by half when oral cells were blocked with Anakinra, showing that IL-1β production partially depends on IL-1 receptor signaling.

6.4.2.3 Oral IL-1 secretion is independent of the NLRP3 inflammasome

Processing and secretion of IL-1β by caspase-1 requires induction of the inflammasome [139]. The canonical NLRP3 inflammasome is by far the best characterized inflammasome that is also expressed in many epithelial cells [134]. To test whether the oral cytokine response is dependent on the NLRP3 inflammasome, the potassium channel inhibitor glybenclamide was used to inhibit maturation of caspase-1. Inhibition did not reduce IL-1β secretion after infection with any of the tested strains and did also not affect IL-8 and IL-1α secretion (Fig. 15). Thus, the NLRP3-independent release of oral IL-1β suggests the involvement of other inflammasomes, e.g. the NLRC4 inflammasome.

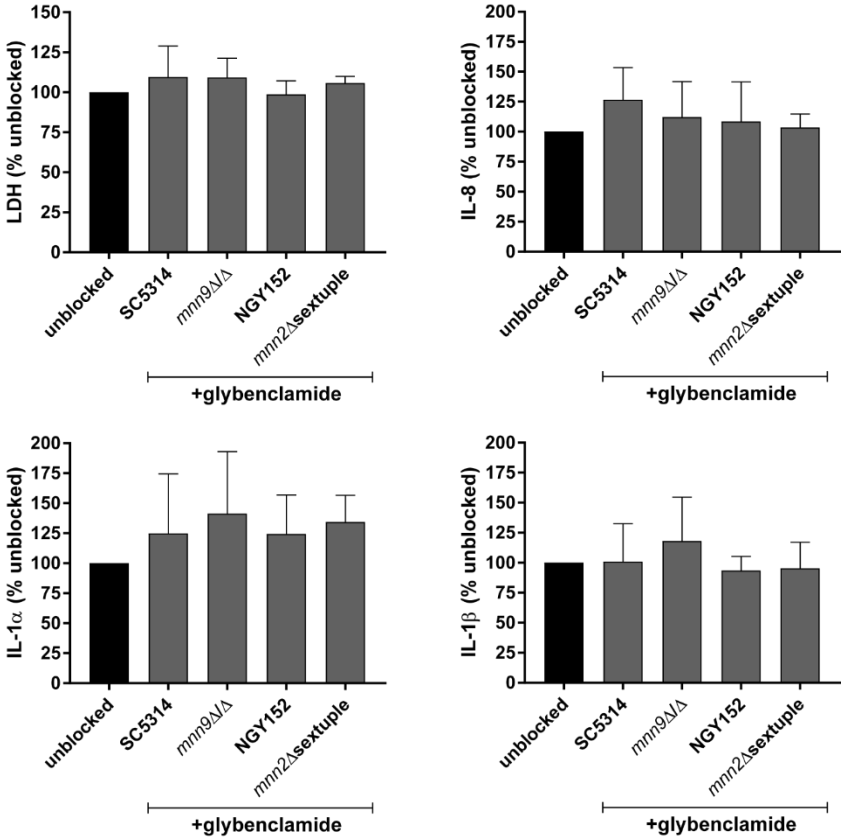

Fig. 15. Inhibition of caspase-1 maturation does not influence the inflammatory cytokine release.
The maturation of caspase-1 was inhibited with 25 µg/ml glybenclamide during *C. albicans* infection and the cell culture supernatant from three independent experiments was assessed for LDH, or IL-8, IL-1α, and IL-1β by ELISA (B). Data represent mean values +/- SD.

6.4.3 Strain-dependent adhesion and invasion properties do not correlate with the induced cytokine response

Adhesion to host cells is a prerequisite for *C. albicans* to colonize mucosal surfaces and is also required for PRR-mediated signaling to induce an epithelial immune response [176]. Therefore, increased adhesion by the *mnn9*Δ/Δ and *MNN2* sextuple mutant might contribute to the higher cytokine response observed by oral and hepatic cells. To test this hypothesis, epithelial cells were seeded on glass cover slides and infected with *C. albicans* strains for 1 h and subsequently stained to enable counting of fungal cells. While adhesion to oral cells was unaltered of the *mnn9*Δ/Δ and *MNN2* sextuple mutant, both strains adhered less to hepatic cells than the parental strain (Fig. 16A). It thus appears unlikely that the enhanced hepatic cytokine production can be attributed to *C. albicans* adhesion properties. The *MNN2* sextuple mutant adhered better on renal epithelial cells, which might contribute to the observed inflammatory cytokine release in comparison to *mnn9*Δ/Δ. Yeast cells of both mannosylation mutant strains clustered in large cell aggregates on the cell layer of all cell lines (Fig. 16B). This cell aggregation has been described before and could be explained by cell wall separation defects or a change in the overall net charge of the cells [68,97,171,172,177].

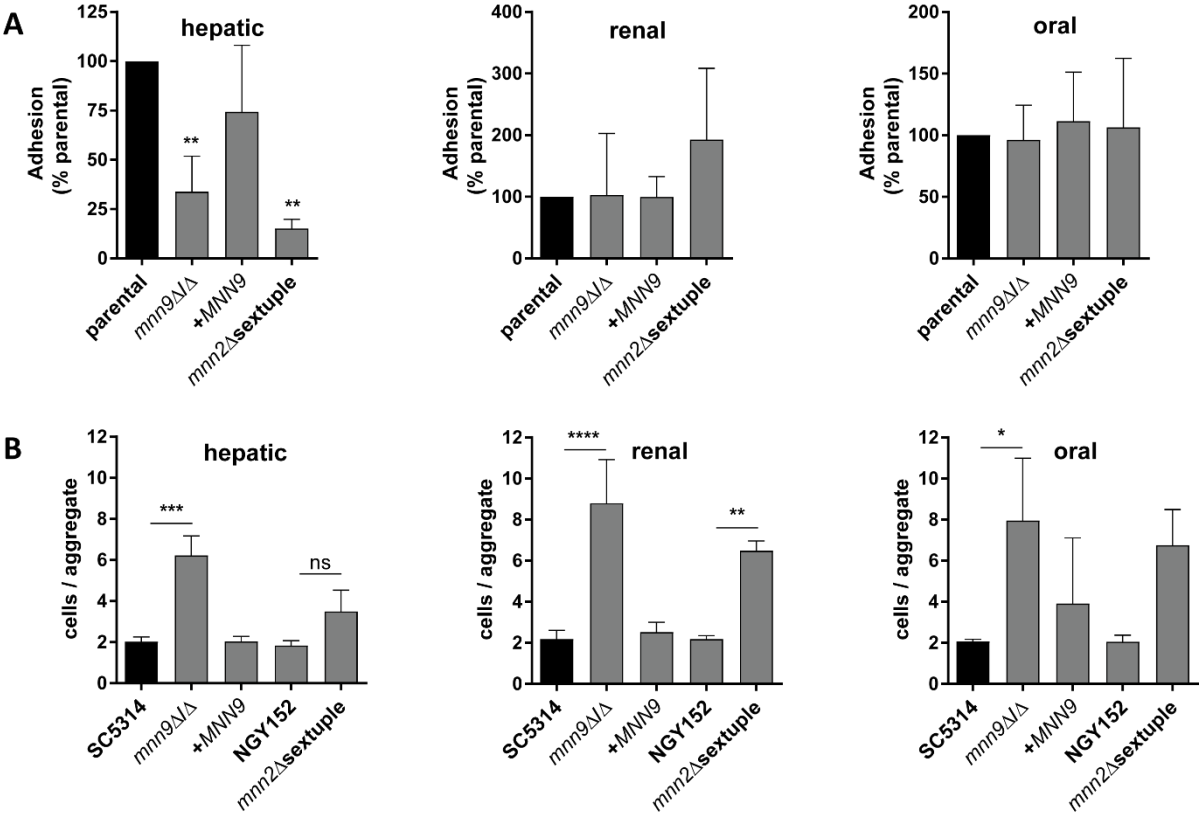

Fig. 16. Strain-specific adhesion on hepatic, renal and oral epithelial cells.
To determine adhesion, *C. albicans* strains were co-incubated for 1 h with confluent epithelial cells and afterwards stained for counting fungal cells. (A) Relative adhesion of *C. albicans mnn9*Δ/Δ, +*MNN9*, and *MNN2* sextuple mutant compared to the parental strain. Statistics: One-way ANOVA with post-hoc Dunnett's multiple comparisons test; **, P ≤ 0.01. (B) Number of yeast cells forming one aggregate, defined as touching yeast cells. Statistics: One-way ANOVA with post-hoc Tukey's multiple comparisons test; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001; ns = not significant. (A+B) Data represent mean values +/- SD from at least three independent experiments. SC5314 is the parental strain from the *MNN9* mutant and +*MNN9*; NGY152 is the parental strain from the *MNN2* sextuple mutant.

Hyphal growth is considered to be crucial for fungal invasion and critical for virulence [118]. The ability to invade epithelial cells was analyzed after 4 h of infection by differential fluorescent staining that discriminates non-invasive from invasive *C. albicans* hyphae. The staining revealed that the *mnn9*Δ/Δ and *MNN2* sextuple mutant grew less invasively into hepatic, renal, and oral cells (Fig. 17A). Hyphal formation has been previously demonstrated for the *mnn9*Δ/Δ and *MNN2* sextuple mutant under hypha-inducing conditions [75,97]. However, after 4 h of infection of epithelial cells, both strains developed shorter hyphae than the wild type (Fig. 17B). In contrast, hyphal length was not impaired of the *MNN2* sextuple mutant when incubated without epithelial cells on glass cover slides (Fig. S3).

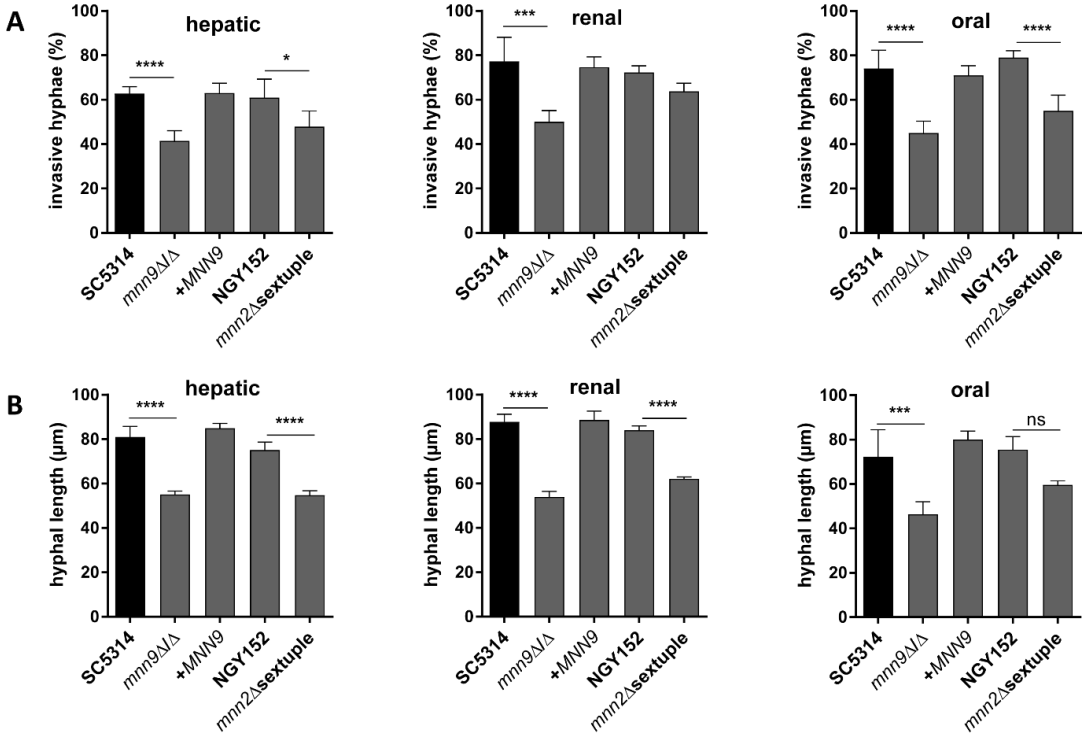

Fig. 17. Strain-specific invasion in hepatic, renal and oral epithelial cells.
C. albicans strains were co-incubated for 4 h on confluent epithelial cells. To analyze invasion properties, outside hyphal structures, invaded hyphae, and total hyphae were discriminated by differential fluorescent staining and the relative amount of invasion calculated (A); or the total hyphae length was measured (B). SC5314 is the parental strain from the *MNN9* mutant and +*MNN9*; NGY152 is the parental strain from the *MNN2* sextuple mutant. (A+B) Data represent mean values +/- SD from at least three independent experiments. Statistics: One-way ANOVA with post-hoc Tukey's multiple comparisons test; *, P ≤ 0.05; ***, P ≤ 0.001; ****, P ≤ 0.0001; ns = not significant.

6.4.4 Normal yeast cell growth of glycosylation mutant strains

The *mnn9*Δ/Δ and *MNN2* sextuple mutant induced a stronger hepatic and oral cytokine response that could not be explained by alterations in adhesion, invasion, or hyphal length. To test whether any of the observed phenotype is influenced by alterations in propagation, mutant strains and the respective wild types were analyzed in growth at 30°C in 96-well tissue culture plates. Yeast growth of both strains was not fundamental impaired in standard YPD medium at 30°C (Fig. 18). Since all infection experiments were performed in the respective cell culture medium, *C. albicans* yeast growth was analyzed in pure (data not shown) or conditioned DMEM (incubated with oral cells), conditioned MEM (incubated with renal cells), and conditioned WME (incubated with hepatic cells) without supplements at 30°C. While the *MNN2* sextuple mutant displayed a slight growth delay in conditioned DMEM, *mnn9*Δ/Δ grew slightly slower in conditioned WME (Fig. 18). Both mutant strains showed no alterations in growth when incubated with conditioned MEM. Thus, even though the *mnn9*Δ/Δ and *MNN2* sextuple mutant displayed aggregating phenotypes and a reduced hyphal growth on epithelial cells, yeast cell growth was not impaired.

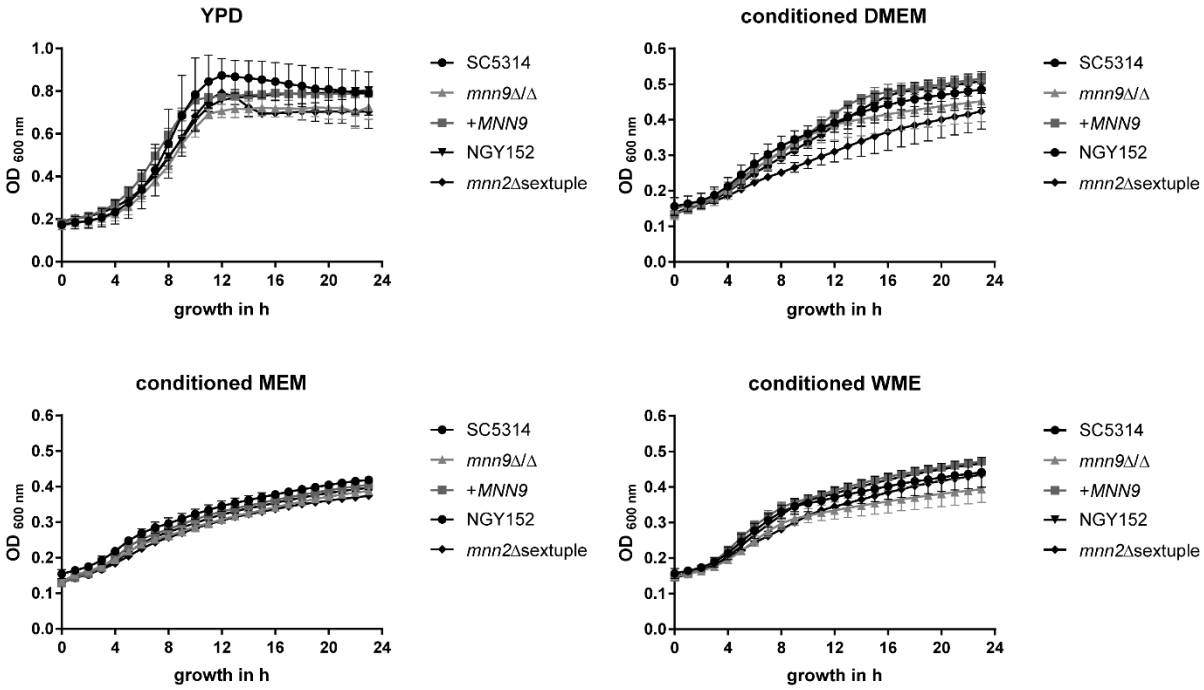

Fig. 18. Deletion of *MNN9* or *MNN2* does not impair growth in different media.
C. albicans strains were grown for 24 h in YPD, conditioned DMEM, MEM, or WME in 96-well tissue culture plates. The optical density was measured by an Tecan ELISA reader at 600 nm for at least two independent experiments.

6.4.5 Mannosylation mutant strains expose more β-1,3-glucan and chitin on their surface

The *C. albicans* cell wall consists of 35-40% mannoproteins, 60% β-glucans, and 1-2% chitin [59]. This ratio can change by deletion of genes involved in mannosylation that elevates glucan and chitin levels due to compensatory regulation [178]. However, changes in cell wall composition also affect the expression of epitopes that are in direct contact to host cells. Thus, an altered pathogen-associated molecular pattern (PAMP) exposure might contribute to differential recognition resulting in the observed changes in the host cells' cytokine response.

Therefore, exposure of the cell wall PAMPs mannan, β-1,3-glucan, and chitin was analyzed from yeast cells by flow cytometry (Fig. S4). The relative MFI compared to the parental strain revealed a decreased mannan exposure on the *MNN2* sextuple and *pmr1*Δ/Δ but not on *mnn9*Δ/Δ or *och1*Δ/Δ (Fig. 19). In contrast, β-1,3-glucan exposure was increased in all glycosylation mutants. As a positive control for β-1,3-glucan exposure, heat-inactivated yeast cells of SC5314 were used that expose more β-1,3-glucan evoked by structural distortion of the cell wall than intact wild type cells [178]. The inner skeletal layer consists of chitin and is usually exposed only at bud scars [84]. However, all mannosylation mutants showed a strong increase in chitin exposure. Although heat-inactivated SC5314 cells used as a positive control for exposed chitin displayed slightly more chitin exposure compared to intact wild type cells, this control exposed less chitin compared to the tested mannosylation mutant strains. This suggests a compensatory *de novo* chitin synthesis in these glycosylation mutant.

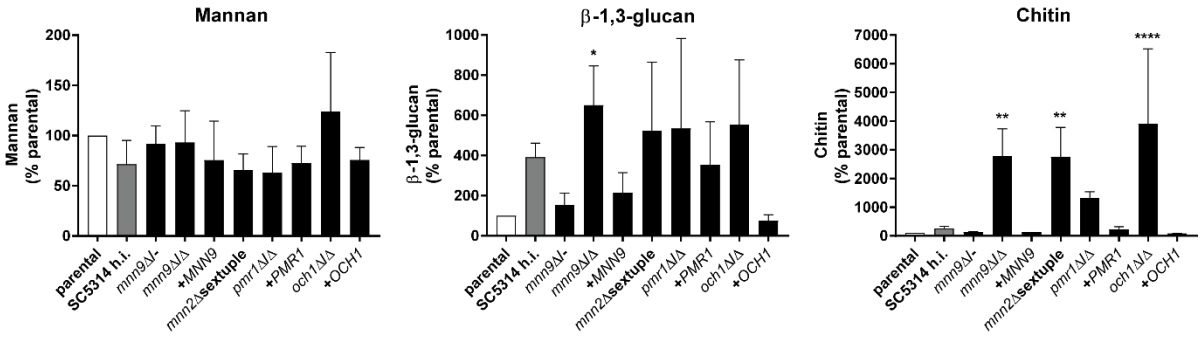

Fig. 19. Exposure of cell wall mannan, β-1,3-glucan, and chitin on *C. albicans* glycosylation mutant strains.

Exposure of mannan, β-1,3-glucan, and chitin from different glycosylation mutants measured by the mean fluorescence intensity (MFI) relative to their respective parental strains. Data is depicted as mean +/- SD from at least three independent experiments. h.i., heat-inactivated. Statistics: One-way ANOVA with post-hoc Dunnett's multiple comparisons test; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.

C. albicans filamentous growth is associated with cell wall remodeling that alters PAMP exposure [35]. As a consequence, different PAMP exposure on filaments from glycosylation mutants might contribute to an altered recognition and thus cytokine response. To test this hypothesis, *C. albicans* strains were incubated for 4 h under filamenting conditions (RPMI 1640 with 10% FBS, 37°C, 5% CO_2) and differentially stained for exposed mannan, β-1,3-glucan, and chitin. As flow cytometry is not suitable to analyze *C. albicans* hyphae, fluorescence microscopy was used to analyze PAMP exposure. As already observed for yeast cells analyzed by flow cytometry, yeast cells from the SC5314 wild type and *MNN9* revertant strain exposed less β-1,3-glucan (red) and chitin (green) compared to the *mnn9*Δ/Δ and *MNN2* sextuple mutant (Fig. 20). Both mutant strains displayed different cell wall component exposure patterns also on filaments: while β-1,3-glucan exposure was observed along all filaments of *mnn9*Δ/Δ and *MNN2* sextuple mutant, the staining appeared only on hyphal tips, secondary and branching hyphae of the wild type and revertant strain. Wild type and revertant strain exposed chitin at bud scars and the first hyphal septa. On filaments of the *mnn9*Δ/Δ, chitin septa were not specifically visible between hyphal cells; instead, a pattern of laterally dense chitin rings was visible.

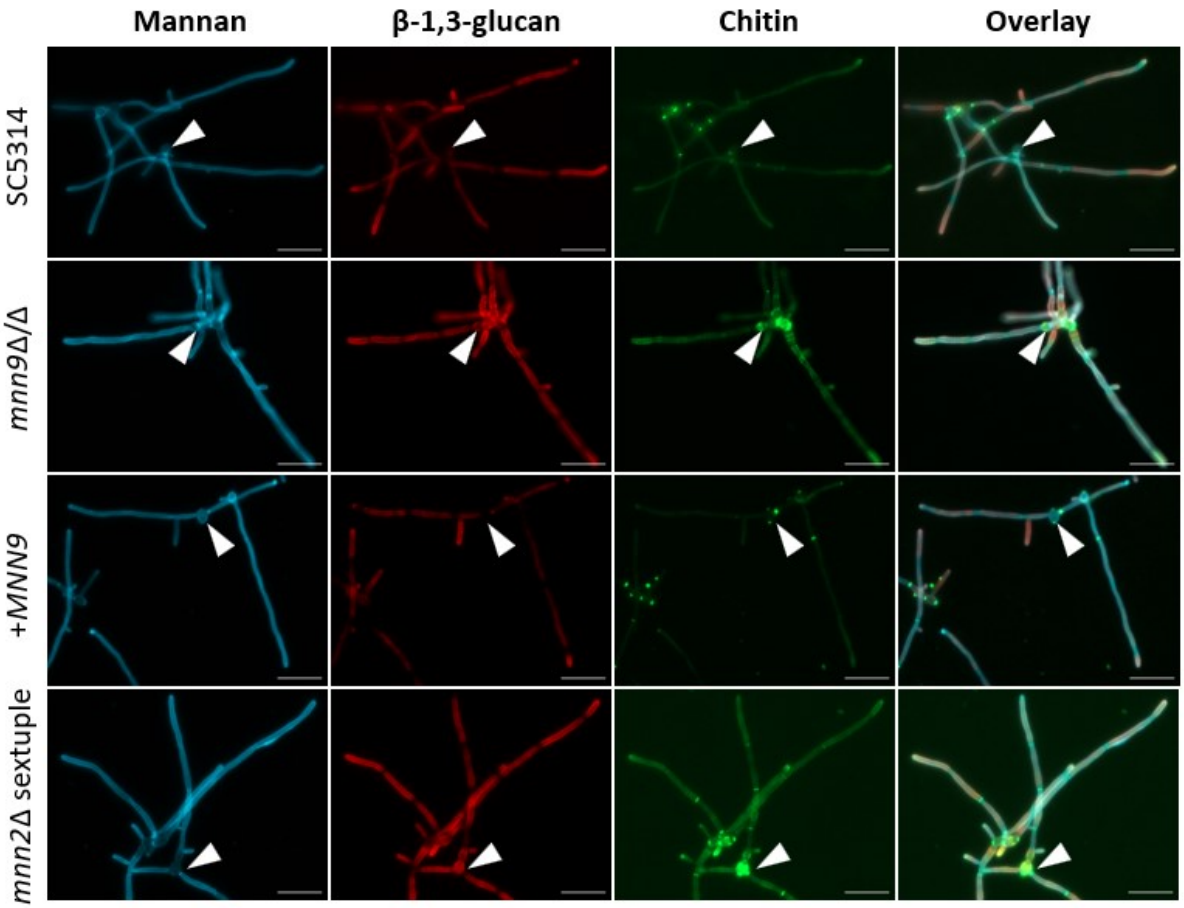

Fig. 20. Exposure of hyphal cell wall PAMPs on *C. albicans* SC5314, *mnn9*Δ/Δ, *+MNN9*, and *MNN2* sextuple mutant determined by fluorescence microscopy.
C. albicans hyphae were induced by 4 h incubation in RPMI 1640 with 10% FBS, 37°C, 5 % CO_2. Representative images of at least two independent experiments. The white arrow indicates yeast cells. Grey bar: 20 µm.

In contrast, the *MNN2* sextuple mutant exposed chitin on all hyphal septa. Only the *mnn9*Δ/Δ strain did not expose chitin at hyphal septa (Fig. 21), suggesting a defect in hyphal separation. However, total staining of chitin with the diffusible molecule calcofluor white displayed normal separated hyphal cells (Fig. S5).

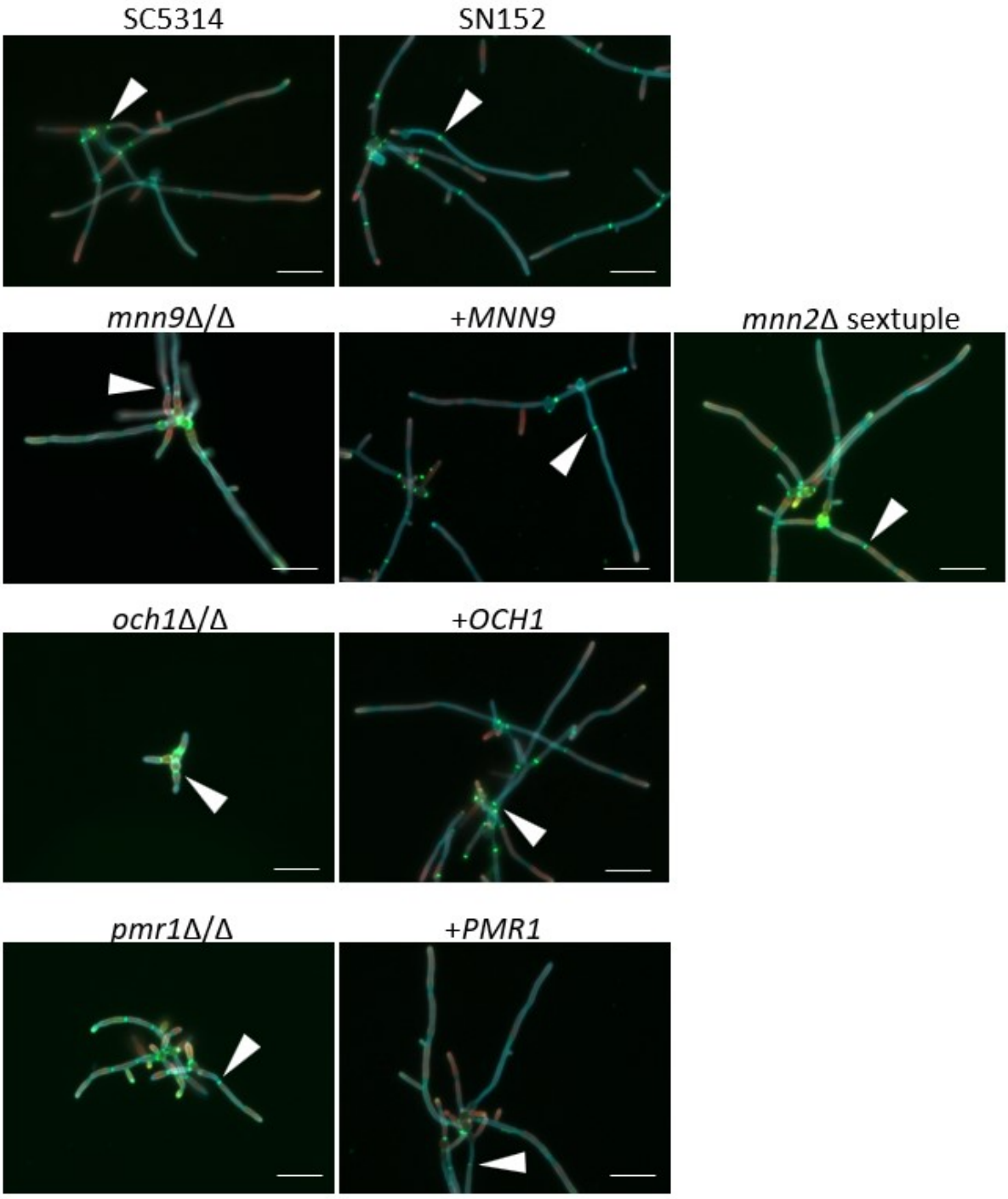

Fig. 21. Glycosylation mutants differ in their filamentous chitin exposure.
C. albicans strains were grown for 4 h under hyphae-inducing conditions and stained for exposed mannan (blue), β-1,3-glucan (red), and chitin (green), displayed as an overlay. Representative images of at least two independent experiments. Arrows indicate the first chitin separation ring. Note: The *mnn9* mutant displays irregular patterns of chitin rings. Bar = 20 µm.

6.5 Analysis of signaling pathways involved in the epithelial immune response

C. albicans infected epithelial cells responded with a distinct secretion pattern of inflammatory cytokines. Even though the process of receptor-mediated recognition of *C. albicans* is still unknown [37], different epithelial cells express a similar repertoire of PRRs but respond differently to ligands. This

suggests a cell type-specific induction of signaling cascades downstream of PRRs. Previous studies identified the activation of mitogen-activated protein kinase (MAPK) signaling including MKP1 and c-Fos in response to *C. albicans*-mediated cell damage [151]. However, while the activation of these pathways is considered to be crucial for cytokine release by oral and vaginal cells [151,152], epithelial cells from different origins might differentially activate signaling pathways.

6.5.1 Activation of *C. albicans*-induced mitogen-activated protein kinase (MAPK) signaling

MKP1 phosphorylation in oral and vaginal cells has been demonstrated to appear earliest by 2 h upon *C. albicans* infection [151,152]. This activation was induced by a dose-dependent fungal burden and required a tenfold higher MOI in vaginal cells. To enable MKP1 activation in hepatic, renal, and oral cell lines, the increased MOI was also used in this study.

6.5.1.1 Damage-dependent activation of MKP1 phosphorylation is cell line-specific

MKP1 phosphorylation was analyzed after 2 h, 4 h, and 6 h of *C. albicans* infection by quantitative western blotting using the fluorescence-based Smart Protein Layer (SPL) method [150]. This method allowed normalization to the total protein amount instead of a single housekeeping protein. Mock-infected cell lines did not display changes in MKP phosphorylation during incubation determined by standard western blotting (data not shown), or in case of the oral cell line by SPL (Fig. 22A).

Infection of hepatic cells with *C. albicans* SC5314 decreased SPL normalized intensities of both MKP1 and p-MKP1 (Fig. 22B). In contrast, p-MKP1 increased by 4 h post-infection in renal and oral cells, while intensities for MKP1 remained stable in these cell lines. The ratio of p-MKP1/MKP1 compared to the mock-infected control revealed cell type-specific kinetics: while p-MKP1 immediately decreased after infection of hepatic cells, it increased by 4 h in renal and oral cells and slightly decreased afterwards in oral cells (Fig. 22C).

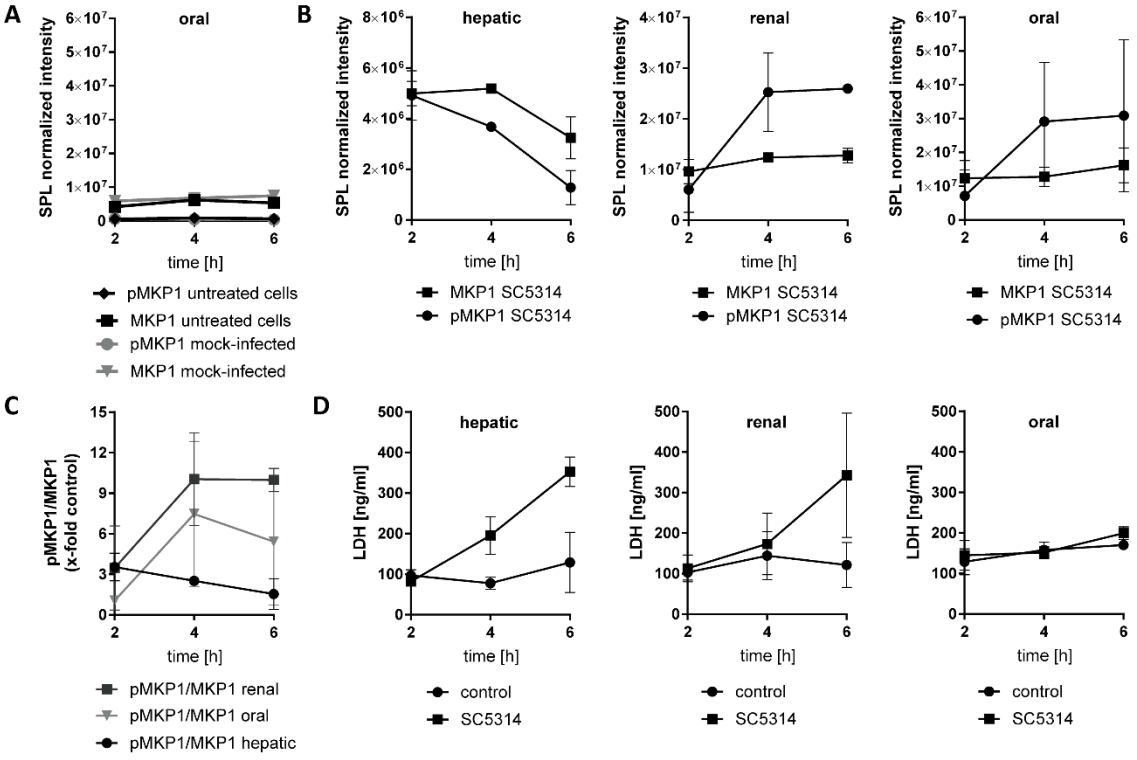

Fig. 22. Cell type-specific activation of MKP1 during *C. albicans* infection.
(A) Oral cells were either untreated or mock-infected for 2, 4, and 6 h and p-MKP1 and MKP1 analyzed by quantitative western blotting using the Smart Protein Layer technology (SPL, from NHDyeAGNOSTICS). (B) Confluent hepatic, renal, and oral cells were either mock-infected or infected with *C. albicans* for 2, 4, and 6 h and analyzed by quantitative western blotting . Relative amount of MKP1 and p-MKP1 normalized to the whole protein amount over time. (C) p-MKP1/MKP1 ratio after infection with *C. albicans* over time. (D) Lactate dehydrogenase (LDH) released into the supernatant during the infection period. Data from A displays one experiment analyzed by SPL and was verified in two independent experiments by standard western blotting (not shown). Data from B+C+D represent mean values +/- SD from at least two independent experiments.

Cytokine release by oral epithelial cells is induced by the activation of signaling cascades that requires sensing by PRRs and cell lysis [20,58,151]. Released LDH, however, revealed cell line-specific differences in the early induction of cell damage: LDH was detected as early as 4 h after infection of hepatic cells but was very low or absent of renal and oral cells (Fig. 22D). Cell damage further increased of hepatic and renal cells by 6 h, but oral damage was almost absent. It thus appears unlikely that the reduced MKP1 phosphorylation in hepatic cells is due to the absence of damage.

6.5.1.2 Activation of c-Fos is independent of *C. albicans* infection

Activation of MKP1 and c-Fos might not be directly linked but are crucial for a hypha-associated induction of the cytokine response in oral and vaginal cells [151,152]. In the discrimination of commensal and pathogenic lifestyle of *C. albicans*, activation of c-Fos results in the production of cytokines [151]. Induction of c-Fos was analyzed by standard western blotting and revealed *C. albicans*-independent activation by 2 h (Fig. 23). Mock-infected hepatic and renal cells already induced c-Fos and the signal intensity did not increase in response to infection. Oral cells did not respond to mock or *C. albicans* infection after 2 h (Fig. 23) or after 4 h (Fig. S.7).

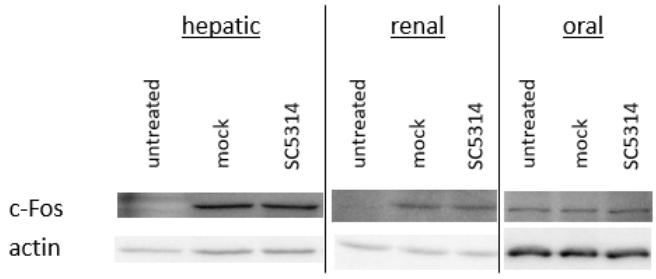

Fig. 23. *C. albicans*-independent induction of c-Fos.
c-Fos expression in untreated, mock-infected, and with *C. albicans* SC5314 infected epithelial cells after 2 h. Representative western blots from at least two independent replicates.

6.5.1.3 A defect in *MNN9* induces a delayed but strong MAPK signaling in oral epithelial cells

The *mnn9*Δ/Δ strain induced an elevated oral cytokine response and caused normal cell damage. The strong exposure of β-1,3-glucan and chitin on both yeast and hyphae suggests differences in the activation of MAPK signaling. Since activation of MKP1 is crucial in the regulation of cytokine induction [151], *C. albicans*-induced phosphorylation of MKP1 might alter the response to infection with *mnn9*Δ/Δ. Activated MKP1 is stabilized by ERK1/2 [179], and in case of increased or prolonged activation of ERK1/2, phosphorylation of MKP1 might also increase. Furthermore, ERK1/2 is supposed to regulate the cytokine response mediated by p38 and JNK signaling. JNK is dephosphorylated by MKP1 in order to control the epithelial immune response and it has been demonstrated that the activation of both proteins displayed opposing kinetics [151].

To examine the different activation of MKP1, ERK1/2, and JNK signaling pathways in oral cells, phosphorylation levels were quantified by SPL western blot. MKP1 phosphorylation was delayed when infected with *mnn9*Δ/Δ but reached wild type levels by 6 h (Fig. 24A). Early induction of phosphorylated ERK1/2 by *mnn9*Δ/Δ appeared similar compared to wild type infection, but further increased in *mnn9*Δ/Δ infected samples that support the hypothesis of a stronger and prolonged MKP1 activation by ERK1/2 (Fig. 24B). The *mnn9*Δ/Δ strain did not induce JNK signaling at early time points, but phosphorylation increased during infection with similar kinetics of wild type-infected cells (Fig. 24C). This increase in JNK phosphorylation, however, is in contrast to the previously demonstrated opposing phosphorylation of MKP1 and JNK [151].

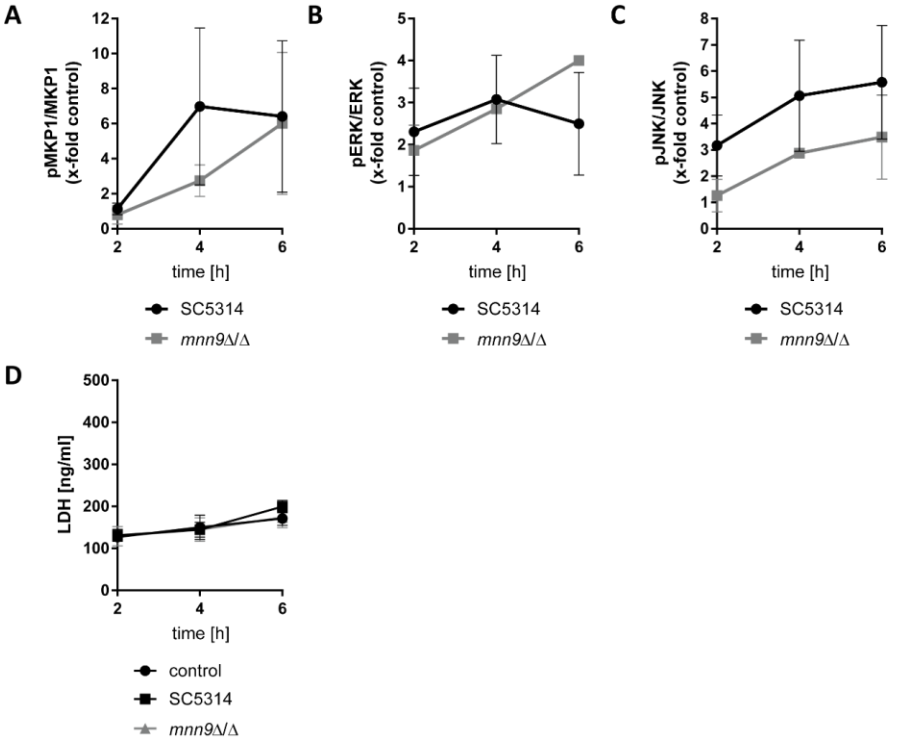

Fig. 24. Delayed but increased phosphorylation of ERK1/2, MKP1 and JNK after infection with the *MNN9* mutant.
MKP1, ERK1/2, and JNK phosphorylation in oral cells was quantified similar to Fig. 22 by using the Smart Protein Layer technology (SPL, from NHDyeAGNOSTICS). Relative p-MKP1/MKP1 (A), p-ERK/ERK (B), and p-JNK/JNK (C) ratios after infection with *C. albicans* SC5314 or *MNN9* mutant normalized to mock-infected controls. (D) Lactate dehydrogenase (LDH) released in the supernatant of oral cells after incubation with SC5314 or *MNN9* mutant. Data represent mean values +/- SD from at least two independent experiments.

Early host cell damage induced by *mnn9*Δ/Δ could contribute to a progressive activation of MAPK signaling pathways and an increased oral cytokine release. However, damage was not detectable after 6 h of infection with the *mnn9*Δ/Δ strain, suggesting MAPK activation before damage (Fig. 24D).

6.5.2 Activation of *C. albicans*-induced NF-κB signaling

The activation of NF-κB is independent of *C. albicans* morphology and only partially involved in the epithelial cytokine response [151]. However, it has been shown that blocking of the NF-κB pathway significantly reduces the amount of secreted cytokines, even though it does not reduce MKP1 and c-Fos activation [151]. The authors suggest that cytokine production might be partly independent of the MAPK-induced activation of c-Fos. Furthermore, the *C. albicans* cell wall moieties mannan, β-glucan, and chitin activate NF-κB [151], which might also contribute to the elevated cytokine response induced by the *mnn9*Δ/Δ. However, phosphorylated NF-κB analyzed by standard western blotting was not induced by *C. albicans* infection by 2 h (Fig. 25), or at later time points (data not shown). Likewise the induction of c-Fos, phosphorylated NF-κB was already observed in mock-infected samples.

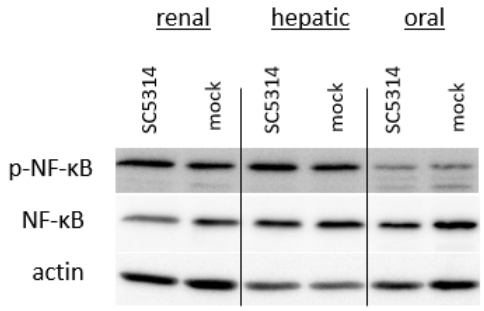

Fig. 25. *C. albicans*-independent activation of NF-κB.
Expression and activation of NF-κB in mock-infected and with *C. albicans* SC5314 infected epithelial cells after 2 h. Representative western blots from at least two independent replicates.

6.6 *MNN9* is required for full virulence *in vivo*

Alterations in the mannosylation status of the cell wall have been shown to often reduce virulence in disseminated candidiasis in mice. Structural defects in mannosylation caused by minor alteration in the α-1,2-mannose side-chains in a *MNN14* mutant strain [78]; the loss of *N*-mannan sidechains in a *MNN2* sextuple mutant [75]; partly loss of *O*- and *N*-mannan in a *PMR1* mutant strain [172]; a shorter α-1,6-mannose backbone in a *MNN10* mutant strain [72]; or the lack of the whole *N*-mannosyl residue in the *och1*Δ/Δ [68,107] improved mice survival upon infection with these strains. So far, the influence of alterations in the mannosylation status caused by the *mnn9*Δ/Δ mutant was not tested *in vivo* before. The structural similarity of the *mnn9*Δ/Δ mutant with the *mnn10*Δ/Δ mutant suggested a similar virulence in systemic candidiasis in mice.

To test the virulence of *C. albicans mnn9*Δ/Δ *in vivo*, BALB/c mice were intravenously infected with $5x10^5$ cells of the SC5314 wild type, *mnn9*Δ/Δ, or revertant strain. Infection with the wild type resulted in a median survival of 4 days (Fig. 26A). In contrast, infection with the *mnn9*Δ/Δ strain resulted in 100% survival of the group after 21 days post infection. The revertant strain containing a single *MNN9* copy caused a median survival of 6.5 days.

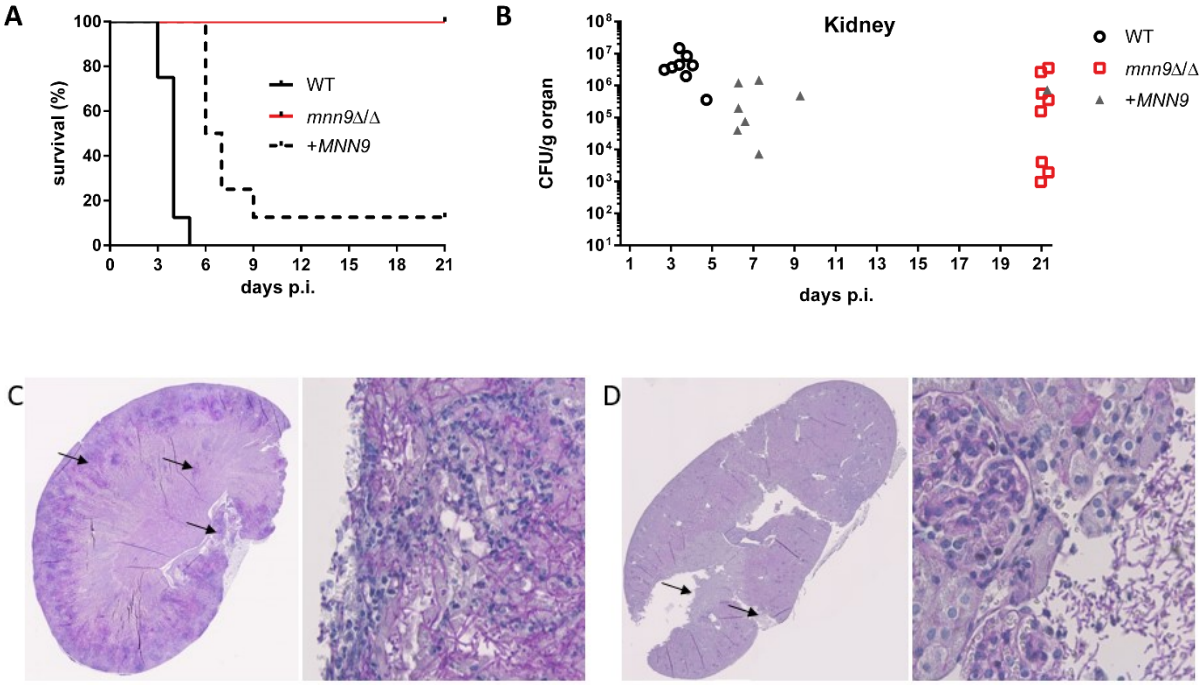

Fig. 26. *MNN9* is required for full virulence of *C. albicans* in disseminated candidiasis in mice.
BALB/c mice were infected intravenously with 5x10^5 *C. albicans* strains and survival was assessed for 21 days (A). Depicted is one representative from two independent experiments with 8 mice per group. Median survival infected with the WT: 4 days; with the mutant: 21 days; with the revertant: 6.5 days. Statistics: log-rank (Mantel-Cox) WT to mutant ****; WT to revertant ****; mutant to revertant: ***. Fungal burden in the kidney at the day by death determined as colony forming units per gram organ (B). Colonized kidney section after 4 days of infection with the WT (C) or after 21 days with the *mnn9*Δ/Δ (D). Arrows indicate *C. albicans* filaments. Pictures are representative PAS staining from kidney sections. For whole organ sections tiles where arranged using Zeiss Zen 2.

Organ fungal burden was analyzed by colony forming unit (CFU) plating from all mice and revealed a high fungal burden in the kidney (Fig. 26B), spleen, liver, and brain (Fig. S6) of mice infected with the wild type strain. The fungal load in the kidney was reduced when infected with the revertant strain, suggesting a partial clearing of the fungus, but this was not sufficient for mice to survive the infection. Mice that were infected with *mnn9*Δ/Δ and euthanized 21 days post infection showed less fungal burden in the kidney, but the infection was not fully cleared. Instead, histological periodic acid-Schiff staining (PAS) showed that in these kidneys granulomas filled with filamentous *mnn9*Δ/Δ were formed (Fig. 26D and Fig. 27). In contrast, wild type filaments were located in the cortex and renal papilla accompanied by a strong inflammatory immune cell infiltration (Fig. 26C).

Mice infected with the MNN9 mutant displayed a low summarized clinical score that includes estimation of mice behavior, fur, examination of weight, and temperature (Fig. S8). While a clinical score of zero implies a perfect health status, the score observed for mice infected with the MNN9 mutant could hint to a delayed pathogenesis that could also impair survival.

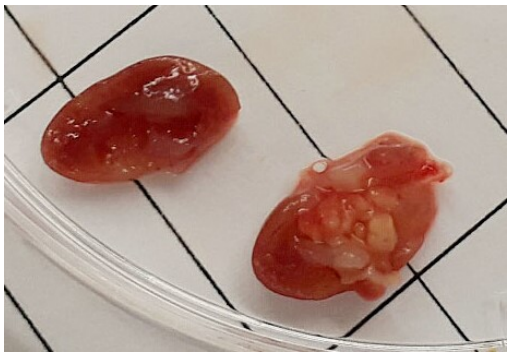

Fig. 27. Kidney granulomas caused by the *MNN9* mutant strain.
Longitudinal section of a healthy kidney (left) and a kidney filled with granulomas from the same mouse after 21 d of infection with *mnn9*Δ/Δ. One rectangle is 25x8 mm in size.

In summary, a defect in the mannosyltransferase II part encoded by *MNN9* resulted in 100% survival of intravenously infected mice but the mutant strain was not fully cleared and mice suffered from relatively high fungal loads in the kidney.

7 Discussion

Innate immunity plays an important role in early *C. albicans* recognition and clearance of infection [180,181]. Epithelial cells lining mucosal surfaces represent not only passive barriers but rather non-professional immune cells that induce an innate immune response by secreting chemokines, cytokines, and antimicrobial peptides [182]. Previous studies focused on the immune response from oral and vaginal epithelial cells, which are representative for mucosal surfaces [30,31,98,183]. They are constantly in contact with pathogens and can be affected by superficial candidiasis. When *C. albicans* reaches the bloodstream, infection of internal organs also leads to the interaction of the fungus with other types of epithelial cells that form the parenchyma of internal organs. These epithelial cells are normally not populated by microbes and do not interact with pathogens under non-infected conditions. How these types of epithelial cells recognize and respond to *C. albicans* is largely unknown.

The aim of this study was to compare the cytokine response of different types of epithelial cells towards *C. albicans* infection. Furthermore, this work identified the glycosylation status of the *C. albicans* cell wall as a factor that modulates the epithelial cytokine response in a cell type-dependent manner.

7.1 Mucosal and parenchymal epithelial cells produce cytokines that could contribute to the innate immune response

All epithelial cell lines investigated in this study secreted IL-6 and IL-8 when challenged with *C. albicans*. This inflammatory cytokine response, however, quantitatively differed between cell types, with more cytokines produced by oral epithelial cells compared to hepatic and renal cells (this study), or vaginal cells [31,184]. The epithelial damage potential also differed between cell lines and revealed that oral cells were more robust to *C. albicans*-induced damage *in vitro*. It is therefore possible that more host cells were intact after oral cell infection which were capable of producing more cytokines under the same conditions compared to renal and hepatic cells. Given that the prevalence of commensal *C. albicans* colonization of oral and vaginal surfaces is very high [2], epithelial cells that form mucosal barriers must have evolutionary evolved mechanisms to adapt to fungal invasion and damage. In contrast, epithelial cells of internal organs are only confronted with microbes, including *C. albicans*, during infection. IL-8 release from epithelial cells *in vitro* was only observed after infection with *C. albicans*, confirming that IL-8 requires *de novo* synthesis [20]. Secreted IL-8 is the most important chemoattractant for neutrophils and macrophages, the principal effector cells to clear *C. albicans* infection [121]. However, it is still unknown, how much epithelial cells contribute to the overall IL-8 production that is necessary to recruit neutrophils for host defense during systemic candidiasis.

IL-6 is considered to be double-edged, operating as pro- and anti-inflammatory cytokine [185]. Infected hepatic cells released low amounts of IL-6, but relatively high amounts compared to the mock-infected control. However, significant amounts of IL-6 is considered to be secreted from neutrophils, macrophages, and monocytes in the hepatic environment [186]. Consistent with the low hepatic IL-6

release compared to other cell lines, it has been shown that IL-6 impairs hepatic detoxification by downregulating drug transporter mRNA expression from the hepatic epithelial cell line HepaRG [187]. Moreover, intestinal epithelial cells did also not secrete IL-6 in response to *C. albicans* infection (unpublished data; and Daig and coworkers (2000) [188]) revealing a tissue-dependent IL-6 secretion.

Bloodstream infections induce a core unit of acute-phase response cytokines including TNF-α, IL-1β, IL-8, and MCP-1 [189] that can cause a cytokine storm resulting in multiorgan failure and lethality [190]. While this cytokine response is usually induced by immune cells, epithelial cells might also contribute to the overwhelming host response. Due to their barrier function against invading pathogens, epithelial cells at mucosal surfaces are most likely stronger involved in the initial immune response than parenchymal epithelial cells that are usually sterile. Interestingly, this study demonstrates that parenchymal epithelial cells not only respond in the secretion of IL-8 in response to *C. albicans* infection but also released cytokines that were absent or have been found at lower levels from oral epithelial cells, e.g. MCP-1, IL-33, TSLP, and TNF-α. MCP-1 was already secreted from mock-infected epithelial cells and the secretion increased after infection only by renal cells. Epithelial MCP-1 secretion is considered to facilitate epithelial-mesenchymal transition [191] and to control bacterial infections [192], but the specific role in fungal immunity is unknown. Steele and Fidel (2002) suggested that constitutively expressed cytokines by epithelial cells could retain homeostasis of the innate immunity [184].

7.2 Colonized epithelial cells express inflammasomes that enable early recognition of pathogen-induced damage by the secretion of IL-1β

Inflammasomes are multicomponent protein complexes that sense inflammation and are involved in wound healing by the of activation caspase-1 to induce IL-1β and IL-18 production, cleavage, and secretion [105]. Production and secretion of IL-1β from macrophages requires first the transcriptional regulation of expression induced by PAMP recognition through TLRs, IL-1R, and TNFR, and next the induction of the inflammasome resulting in caspase-1 activation [139]. The NLRP3 inflammasome consists of the NOD-like receptor NLRP3, and its expression is primed through activated NF-κB [135], the latter which is activated after PRR stimulation and also induces the production of IL-6 and IL-8 [139]. Then, the assembly of the NLRP3 inflammasome is activated by a large variety of signals related to stress and damage, e.g. by TLR ligands and cytokines [138], pore-forming toxins [139,140,193], or NF-κB activators such as UV light and reactive oxygen species [139].

Expression of different inflammasomes has been demonstrated in myeloid compartments and also epithelial cells, e.g. intestinal and oral epithelial cells [133]. The ability to release IL-1β in response to PAMPs, however, differs between tissues and is not solely dependent on inflammasome expression. For example, while several groups observed expression of the NLRP3 inflammasome in human primary bronchial epithelial cells [133,134], stimulation of these cells with *Pseudomonas aeruginosa* PAO1, LPS, and ATP did not induce IL-1β [194].

Mucosal cells from the upper respiratory tract and the intestine are typical ports of microbial entry and therefore evolutionary faced interactions with the harboring microbiome and also pathogens [195].

These so-called lymphoreticular organs show a high IL-1 and a low IL-6 activity [195]. In this study, IL-1β was only secreted by oral epithelial cells in response to *C. albicans* infection. Although few amounts of IL-1β were secreted into the supernatant, its pro-inflammatory effect is already active at low concentrations and therefore might have clinical relevance [196]. However, the main source of IL-1β secretion originates from the myeloid compartment such as monocytes, macrophages, and dendritic cells [196].

Hepatic and renal epithelial cells did not release IL-1β upon *C. albicans* infection or stimulation *in vitro*. This has also been shown for the renal cell line A498 after infection with different *E. coli* strains [197,198], suggesting that parenchymal epithelial cells do not contain inflammasomes to detect the presence of pathogens. Another explanation for the absence of inflammasomes in hepatic epithelial cells could be the negative influence of IL-1 on hepatic detoxification. IL-1β can induce its own expression via an autocrine or paracrine positive feedback loop [199], and additionally can induce the secretion of epithelial IL-6 and IL-8 [173,200,201]. IL-1β and IL-6 have been shown to impair detoxification by downregulating transporter mRNA expression from the hepatic cell line HepaRG [187]. Therefore, hepatic epithelial cells limit uncontrolled inflammation by preventing the release of IL-1β.

Oral epithelial cells contain high constitutive levels of IL-1α which is released during cell death [138] that suggests a correlation between released LDH and IL-1α. Surprisingly, the *MNN9* mutant damaged oral cells like the wild type but induced a stronger IL-1α release presumably by *de novo* synthesis. A similar increase of released IL-1α was also observed when infected with the *MNN2* sextuple mutant, even though the strain damaged oral cells slightly better that could have caused the observed cytokine release.

In macrophages *C. albicans* filaments activate caspase-1 through the NLRP3 inflammasome and induce IL-1β secretion [131,136]. NLRP3 is also expressed in many epithelial cells including oral epithelial cells [134]. This led to the hypothesis that NLRP3 in oral epithelial cells triggers caspase-1 activation and therefore IL-1 production. However, using glybenclamide to block the potassium efflux that inhibits NLRP3 inflammasome activation did not impair any cytokine response. Besides the NLRP3 inflammasome, activation of the NLRC4 inflammasome has been shown to induce IL-1β secretion from murine buccal mucosa [132]. It, therefore, appears that different inflammasomes could activate specific caspases, e.g. caspase-1. However, a noncanonical inflammasome that can activate caspase-11 could also play a role in the oral IL-1 activation [138].

7.2.1 Aggregating *C. albicans* strains could elevate the host response induced by PAMPs and DAMPs

Both the *MNN9* mutant and *MNN2* sextuple mutant strains grew in aggregates and formed an uneven meshwork of filaments causing fungal hotspots on top of epithelial layers. Aggregating yeast cells might cause strong inflammation by influencing the immune response from intact cells in localized areas. Due to presumably high fungal pressure, low amounts of IL-1α might be released early by cell lysis and could impact as damage-associated molecular patterns (DAMPs) on neighboring cells, bind to the IL-1 receptor (IL-1R1) to induce IL-1 and other cytokines as it is known for sterile inflammation

[202]. Dongari-Bagtzoglou and coworkers (2003) demonstrated that *C. albicans* wild type induced 50% less IL-8 of oral epithelial cells when IL-1R1 was neutralized with the IL-1 receptor antagonist (IL-1Ra) [153]. The present study could verify a damage-independent reduction of IL-8, but also IL-1α and IL-1β secretion by oral cells when blocked with the IL-1 receptor antagonist anakinra. However, a tenfold higher concentration of anakinra used in this study did not abolish the inflammatory cytokine release, indicating that secreted IL-6/IL-8 is only partly dependent on IL-1α signaling via IL-1R1 in oral cells. IL-1-dependent induction of inflammatory cytokines seems to differ between epithelial cells, because Kim and coworkers (2013) could abolish IL-1 induced IL-6 secretion of lung epithelium with anakinra in a concentration-depend manner under non-damaging conditions [203]. Notably, the *MNN9* and *MNN2* sextuple mutant induced a stronger IL-1 release. However, IL-1R1 blocking with anakinra dampened IL-1, but also IL-8 secretion to the same extent relative to the other strains. This suggests that the increased IL-1 level induced by these mutants might not be the cause of the observed increase in IL-6 and IL-8 secretion. Expression of IL-1 mRNA has been shown to occur as early as 1 h after *C. albicans* infection of keratinocytes and therefore Egusa and coworkers (2006) proposed an IL-1 signaling receptor [204]. This receptor might recognize *MNN9* and *MNN2* sextuple mutant yeast cells stronger than wild type yeast cells due to aggregation or changes in the glycosylation status.

7.3 Contribution of epithelial PRRs to the immune response

Myeloid PRRs can recognize *C. albicans* cell wall components, such as *N*-linked mannan (by dectin-2 and mannose receptor, MR), *O*-linked mannan (by TLR4), or β-glucan (by TLR2, TLR6, and dectin-1) [101]. Among the repertoire of TLRs, CLRs, NLRs, and RLRs, TLR2 and dectin-1 are by far the best studied PRRs in sensing *C. albicans* [167]. The process of the receptor-mediated recognition of *C. albicans* on epithelial cells is still unknown [37]. However, epithelial cells also express these PRRs, depending on the species, the cellular location in the host, and can differ between primary cells and cell lines. The majority of TLRs are constitutively expressed in the reconstituted human oral epithelium (RHE) [113]. Similarly, human hepatic epithelial cells (HepaRG) also express TLRs (analyzed by flow cytometry [205] and this study). In contrast to this work, one study showed that human renal epithelial cells only express TLR3 and TLR5 but not TLR2 and TLR4 (analyzed by transcriptional profiling of A498 [206]). Some PRRs are known to be expressed intracellularly, e.g. the microbial DNA sensing TLR9 [112]. However, PRRs expressed on cell surfaces can also be expressed intracellularly, which was observed for TLR4 in the lung epithelial cell line A549 [207].

To date, there is limited data on CLR expression and contrary results for dectin-1 have been published showing a crucial role in the *C. albicans* defense [167] or not [208]. One group observed that dectin-1-knockout mice were more susceptible to *C. albicans* systemic infection than wild type mice [167]. However, a sublethal dose of 10^4 CFU did not kill wild type mice, while infection with a lethal dose of 10^5 CFU reduced survival. The other group used a twofold lethal dose and did not see differences in survival of wild type or dectin-1-knockout mice [208]. They concluded that β-glucan on live fungal cells are masked by mannosyl residues and are therefore not accessible for dectin-1. However, the immune response also seems to be dependent on the initial fungal dosage. The contribution of dectin-1 on the epithelial immune response is more obvious, because even though dectin-1 is

expressed in oral RHE [126] and monolayer (this study), renal epithelial cells A489 ([209] and this study), and airway epithelial cells A549 [210], it is not an essential PRR to clear epithelial infections [30,211]. Nevertheless, since stimulation of dectin-1 of hepatic cells induced a strong IL-8 release, dectin-1 particularly seems to has an impact on the epithelial immune response and probably during recognition of C. albicans. For dectin-2 there is only one study showing no expression in oral RHE [126].

In this study, a low basal expression was examined for TLR2, TLR4, dectin-1, and dectin-3 in hepatic, renal, and oral epithelial cells, with a stronger expression rate for all PRRs in hepatic cells. These data was observed from total cell staining that also included intracellularly expressed PRRs. Permeabilization of epithelial cells also circumvented high autofluorescence that was observed in test staining. Given that mucosal and parenchymal epithelial cells showed a similar expression of TLRs and CLRs, this could imply similar recognition and activation of an epithelial cytokine induction in response to *C. albicans*. However, the cell-specific cytokine release observed from oral, hepatic, and renal epithelial cells could conclude that early recognition of *C. albicans* might change PRR expression prior to or during infection to enable a stronger amplification of signal intensities. This study did not observe any change in PRR expression after co-stimulation with both IL-1β and TNF-α for 24 h or infection with *C. albicans* for 7 h. Other studies observed no increase in the transcriptional response of TLRs or dectin-1 during *C. albicans* infection of oral RHE or oral monolayer cells [113,119]. This is in contrast to the work of Wagener and coworkers (2012) where they observed increased mRNA levels of TLR4 after 24 h of incubation of isolated *C. albicans* cell walls with oral epithelial cells [114]. A knockdown of TLR4, however, did not change the cytokine profile during incubation with isolated cell walls. In addition, Bahri and coworkers (2010) described increased transcriptional expression of TLR2, TLR4, and TLR6 in a time-dependent manner in response to *C. parapsilosis* infection of gingival epithelial cells [212]. Although the mRNA expression of TLR2 was also induced in an engineered human oral mucosa model, the transcript for TLR4 was downregulated in response to *C. albicans* after 24 h [164]. However, the comparability of PRR expression in the above mentioned studies is limited due to various experimental setups. For example, the latter used to cultivate *C. albicans* at 37°C that could already induce yeast-to-hypha transition and might change PAMP exposures. Nonetheless, previous findings indicate that the expression of PRRs might change at later time points in oral cells and suggest a quantification of PRR expression at later time points than 7 h. But, due to fast and invasive growth of *C. albicans* on and in the epithelial cell layer, quantification of PRR expression by flow cytometry or quantitative western blotting is technically limited.

No single receptor can solely activate the immune response [213] and changes in the PRR expression was not observed. However, expressed epithelial PRRs might cluster homo- or heterologous into patches to synergistic enhance signal intensities [214]. The collaboration of dectin-1 with TLR2 or TLR4; or dectin-1 with dectin-2; or dectin-2 with dectin-3 was shown in myeloid cells [215,216]. However, in epithelial cells the knowledge about PRR collaboration is limited. The relevance of a specific PRR in *C. albicans* recognition can only be investigated by directly targeting it than analyzing downstream adaptor proteins that are shared within groups of PRRs (such as MyD88 for TLRs and Syk for CLRs [101]). So far, particular blocking of TLR2 or TLR4 by neutralizing antibodies or siRNA knockdown has been

demonstrated not to inhibit c-Fos or MKP1 activation that is needed to induce an epithelial cytokine response [30]. Similarly, blocked TLRs in particular or in combination (e.g. TLR2 and TLR4), or CLRs (and in combination with TLRs), the cytokine response of oral, hepatic, and renal cells was not affected (data not shown). This suggests that a heterologous PRR clustering is not involved in the PRR-mediated cytokine response induced by *C. albicans* and further on raises the question how the receptor-mediated recognition of *C. albicans* results in an epithelial immune response [37].

Although PRR expression remained unaltered and PRR clustering was not observed in monolayer cell cultures, during the interaction of epithelial cells, immune cells, and the pathogen, epithelial PRRs could mediate an innate immune response. Weindl and coworkers (2007) demonstrated a polymorphonuclear leukocyte (PMN)-dependent upregulation of *O*-mannan recognizing TLR4 in oral RHE resulting in antifungal defense [113]. Interestingly, TLR2 that recognizes β-glucan was not involved in this immune response. These results indicate that cell wall *O*-linked mannan might be an important fungal PAMP in the recognition by TLR4 and subsequent epithelial activation. While a relatively strong expression of TLR4 was observed in uninfected oral, renal, and hepatic epithelial cells (this study), infection did not modulate mRNA expression of TLRs in oral RHE after 12 h or 24 h [113] that could also explain why infection did not upregulate TLR expression.

To facilitate dissemination, *C. albicans* must cross endothelial cells lining blood vessels that are also an integral component of the innate immune system. As previously shown for epithelial cells, endothelial cells might also discriminate *Candida* morphology and express various inflammatory cytokines [217]. Even though some studies revealed new signaling pathways that govern the host response in endothelial cells or epithelial cells [218,219], an influence or possible dependency of both cell types in the *C. albicans* immune response is not known.

7.4 Activation of signaling cascades that induce cytokines is epithelial-specific

Oral epithelial cells can discriminate between commensal and pathogenic *C. albicans* via a biphasic MAPK response [30]. Activation of a second-phase MAPK including MKP1 and c-Fos is a crucial event in the hypha-associated cytokine response by oral [30,220] and vaginal [31] mucosa. This activation, however, seems to be dependent on the multiplicity of infection (MOI), which was tenfold higher for vaginal than for oral cells [30,31]. The present study analyzed signaling induction by applying a fungal load that reflects the high MOI of 10 to enable a comparison of the results between all tested cell lines. MKP1 is expressed in the cytoplasm and its primary localization is in the nucleus. Because the subcellular localization of some MKP's have been demonstrated in the nucleus and cytoplasm [221], whole cell lysates including nuclear proteins were examined. Interestingly, while all cell lines secreted IL-8 in response to fungal infection after 24 h, hepatic cells seem to induce this cytokine response independently from MKP1 phosphorylation. Moreover, using higher protein amounts or an even 10 times higher MOI did not increase p-MKP1/MKP1 signal intensities or phosphorylation status in hepatic cells (data not shown). This implies that a *C. albicans*-induced cytokine response depends on cell type-specific signaling also involving other regulators than MKP1.

Activation of oral MKP1 in response to *C. albicans* infection was confirmed in this study. Due to the fact that the *MNN9* mutant induced stronger inflammatory cytokines, this strain could induce an stronger PRR activation that leads to more robust MKP1 phosphorylation resulting in an elevated oral IL-6 and IL-8 release. However, activation of MKP1 did not occur at early time points (within 6 h), since the *MNN9* mutant induced a delayed but constantly increasing MKP1 phosphorylation. Activated MKP1 is stabilized by ERK1/2, and stronger ERK1/2 phosphorylation was observed with the *MNN9* mutant, suggesting a progressive activation of MKP1 at later time points. In turn, *C. albicans* wild type induced highest ERK1/2 phosphorylation level at 4 h that declined afterward, consistent with MKP1 phosphorylation albeit to a lesser extent. Besides consistent MKP1 phosphorylation, the additional JNK activation that was also induced by the *MNN9* mutant might contribute to the increased cytokine release.

Activation of c-Fos could already be induced by mock infection in hepatic and renal cells, and the signal intensity did not increase after infection with *C. albicans*. Oral cells did not at all respond in c-Fos activation towards infection. In contrast, Murciano and coworkers (2011) [98], Moyes and coworkers (2010) [30] and (2011) [31] showed c-Fos induction of infected oral cells. As a negative control, these groups used resting cells that were probably not treated with media exchange after seeding. Such a control is called untreated cells in the present study and did not induce c-Fos in renal and hepatic cells. To further circumvent the activation of MAPK signaling induced by the medium itself, these groups starved the cells prior to infection (oral communication, Marc Swidergall, 2019). The supernatant from mock-infected cells was exchanged prior to infection that already induced c-Fos signal in renal and hepatic cells. This media replacement without supplements was also performed for infected samples 16 h prior to infection following published protocols [98]. It, therefore, appears that c-Fos induction occurs in reaction to stress and not to *C. albicans* itself. Interestingly, a hyperfilamentous *NRG1* mutant with poor adherence capability on vaginal cells displayed only weak activation of c-Fos but was able to induce wild type similar IL-6 levels [31]. The authors suggest further downstream targets of signaling required for cytokine production in different epithelial cells independent from the MAPK-induced c-Fos activation. In addition, they hypothesized that MKP1 and c-Fos might not be directly linked, because their activation is mediated via ERK1/2 and p38, respectively. Consistent with this hypothesis, stronger activation of ERK1/2 in oral cells by the *MNN9* mutant might elevate the activation of MKP1 independent from c-Fos. *Vice versa*, in hepatic cells c-Fos could have a dominant role in the activation of the MAPK signaling resulting in IL-8 and other secreted cytokines in response to the *C. albicans* wild type. On the other hand, early but weak activation of MKP1 that did not increase during infection could explain the low amounts of secreted IL-6.

NF-κB activation was directly analyzed by the phosphorylation of p65 of the p65/p50 NF-κB complex. Canonical activation of the p65/p50 NF-κB complex leads to the ubiquitination and proteasomal degradation of the suppressor IκBα. Free and activated NF-κB enables induction of gene expression that is required for the production of cytokines. The activation of NF-κB is independent of *C. albicans* morphology, and is considered to be a general response to fungi [30,31,118], inflammatory cytokines,

PAMPs, and different stresses [222]. Increased NF-κB activation contributes to inflammation and tissue damage, but in epithelial cells, it also acts on physiological immune homeostasis [223]. It is, therefore, possible that one of these triggers led to the observed activation of phosphorylated p65 in all cell lines when mock or *C. albicans* infected. Moyes and coworkers (2010) observed a reduction of secreted cytokines when they blocked the NF-κB pathway during infection of oral epithelial cells [151]. However, they also concluded that NF-κB is only partially involved in the cytokine response. The NF-κB activation alone did not induce any cytokine secretion by oral, hepatic, or renal epithelial cells and might only moderately contribute to cytokine induction in combination with activated MAPK signaling pathways.

7.5 Influence of the glycosylation status on the epithelial immune response

The influence of the cell wall glycosylation status on the activation of MKP1 and c-Fos, and consequently the epithelial cytokine response, has been demonstrated for a variety of mutant strains with defects in *N*-mannan biosynthesis genes [98]. Even though the recently discovered Candidalysin is the crucial fungal factor for cell lysis and activation of the oral and vaginal epithelial immune response [17,56], cell wall glycosylation [98] and further features [56] might be required to fully activate epithelial cells.

7.5.1 Deletion of *MNN9* uncouples the correlation in damage and cytokine release

Immune activation of epithelial cells that results in cytokine induction is dependent on hyphal burden. This study could show that a mutation in *MNN9* induced stronger cytokine release by hepatic and oral epithelial cells without having an impact on host cell damage. Previous studies demonstrated that the *MNN9* mutant is able to filament, but either develops true hyphae or pseudohyphae [97,98]. Filaments of the *MNN9* mutant stained for exposed chitin displayed no regular pattern for septa compared to the wild type or other mannosylation mutants analyzed in this study. However, when *mnn9*Δ/Δ was stained for total chitin with calcofluor white, regular septation was observed. Based on the characteristics that distinguish hyphal from pseudohyphal cells [224], the *MNN9* mutant displayed no clear phenotype. On the one hand, the mutant did not display consistent parallel-sided walls and showed constrictions at mother-bud neck and many subsequent septal junctions that are characteristics of pseudohyphae. On the other hand, a characteristic for true hyphae was the first septum that appeared within the germ tube instead of the bud. It is possible that these characteristics of hyphal and pseudohyphal morphologies of the *MNN9* mutant might be a result of the defect in mannosylation leading to the upregulation of β-glucan and chitin synthesis.

Any defect in the glycosylation status, e.g. by the deletion in *PMT1*, *MNT1/2*, *PMR1*, or *OCH1*, affecting hyphal growth and resulting in no or weak activation of p-MKP1 and c-Fos signaling, has been shown to induce fewer cytokines from oral epithelial cells [98]. Even though *mnn9*Δ/Δ displayed an altered filamentation as described before, the mutant was capable of activating required signaling of MKP1 and c-Fos [98], which resulted in yet stronger inflammatory cytokine response. This suggests that the glycosylation status indeed is important and exposure of PAMPs influences recognition and epithelial cell signaling.

7.5.2 Exposure of PAMPs has no exclusive role in the activation of an epithelial response

While previous studies usually analyzed the total cell wall composition, the present study focused on PAMP exposure and elucidated differences depending on the glycosylation status. Mannan covers the outer cell wall layer and masks β-1,3-glucan from recognition by dectin-1 in macrophages [167]. Individual gene deletions from the *MNN2* gene family reduce stepwise the α-1,2-mannose side-chain complexity while glucan exposure is increased [75,225]. The glycosylation structure of the *MNN9* mutant displays an even stronger impairment with a shorter α-1,6-mannose backbone and the absence of α-1,2-mannose side-chains [60], which theoretically should facilitate stronger glucan exposure. However, all tested mannosylation mutants exhibited a similar increase in β-glucan exposure on yeast cells, and showed similar patterns on filaments.

Dynamic unmasking of cell wall components is considered to be important during infection [85]. Initially masked β-glucan by mannan becomes subsequently exposed during infection or treatment with caspofungin that allows fungal recognition [85]. In the present study, unmasking was already observed in monocultures, suggesting that this a host-independent process. However, *C. albicans* mannosylation mutants exposed β-glucan along all filaments, whereas wild type filaments were exposed only on hyphal tips, secondary and branching hyphae. This exposure on newly built wild type filaments suggests a delay in the process of β-glucan masking by the mannan layer which was even more impaired in the mannosylation mutant.

7.5.3 Fungal chitin might have an influence on the epithelial immune response

Chitin lines the inner skeletal layer of fungal cell walls and is only exposed at bud scars [84]. While the *C. albicans* yeast cell wall consists of about 2% chitin [87], hyphae contain five times more chitin [88]. Upregulation of chitin synthesis occurs in response to stresses for compensatory regulation [14] and can appear as side effect in *C. albicans* mutants. Increased chitin exposure was observed for all mannosylation mutants by flow cytometry and immunofluorescence microscopy. However, stronger chitin exposure was not just the effect of a thinner mannan cell layer or the loss of cell wall integrity but most likely a compensatory upregulation of chitin synthesis in these glycosylation mutants. This could be proven by a heat inactivated wild type strain that exposed very low chitin levels compared to these mutants.

Unmasking chitin has been shown by chemical perturbation or heat damage [84], and during neutrophil attack [92]. Yeast cells treated with sublethal concentrations of caspofungin induces higher chitin exposure and has been shown to reduce the PRR-mediated cytokine response of PBMCs [226]. Elevated chitin levels, however, protect *C. albicans* from antifungal treatment with caspofungin [227]. Marakalala and coworkers (2013) could also show that elevated chitin levels in β-glucan exposing cells reduced the dependency on dectin-1-mediated controlling of fungal infections *in vivo* [228]. Furthermore, chitin induces cytokines in a chitin particle size-dependent manner in murine macrophages [94] and human PBMCs. In epithelial cells, chitin exposure *per se* was not sufficient to induce an cytokine response. The non-filamenting *OCH1* mutant exposed the highest chitin levels compared to their parental strain but could not induce any cytokine response in any tested cell line. The immunostimulatory effect of

chitin could also be self-triggered by the host, as mammalian chitinase and chitotriosidase lead to chitin degradation into smaller particles that are known to mediate allergic immune responses [89]. Even though so far no chitin-specific receptor is known [93], recognition of chitin involves the activity of NOD2, TLR9, and MR in oral cells [95].

7.5.4 Glycosylation mutant strains mimic the cell wall of clinically relevant isolates

To analyze the influence of cell wall glycosylation on the host immune response, gene deletions involved in mannosylation processes have been used regularly. Interestingly, some of these artificial strains mimic the cell wall from isolates adapted to specific environmental conditions [229]. Phosphomannan is considered for efficient phagocytosis [80] and a defect in the attachment of phosphomannan by *MNN4* mimics the cell wall of strains growing in acidic conditions, e.g. in the female reproductive tract [79,229]. Furthermore, mutations in the *MNN1* or *MNN2* gene family that alters the mannan chain length and complexity could mimic *C. albicans* strains growing in blood and serum [230]. Mannosylation deletion strains are still flexible in PAMP exposure, as it was demonstrated for the *MNN2* sextuple mutant usually exposing high amounts of β-glucan in high glucose medium, while incubation in lactate containing medium restores this phenotype [231]. A strain that exhibits the cell wall of a *MNN9* mutant might not exist under specific conditions, because this defect would occur early in the mannosylation and displays only a rudimentary N-mannan backbone.

Deletion of genes involved in glycosylation can have an impact on the whole cell wall structure, which is not just the removal of a specific epitope [229]. This includes an altered cell wall proteomic structure, which is known for biosynthesis genes with fundamental roles in mannosylation steps, e.g. *OCH1* [68]. One of the well-known cell wall-associated protein families are secreted aspartyl proteinases (Saps) that appear to mediate the epithelial cytokine induction [21,204] and an increased expression might explain the elevated cytokine response induced by the *MNN9* and *MNN2* sextuple mutant. Of the ten known *C. albicans* Saps, Sap9 and Sap10 are incorporated into the cell wall via a glycosylphosphatidylinositol (GPI)-anchor [51]. It can be assumed that Sap9 expression is not reduced in the *MNN9* and *MNN2* sextuple mutant, since the absence of this protease has been demonstrated to increase adhesion to oral cells and attenuates oral cell damage [54]. While data on Sap secretion or incorporation is limited for glycosylation mutant strains, the extracellular matrix proteome of a *MNN9* mutant displayed only minor changes [100]. Bates and coworkers (2013) hypothesized a differed protein surface charge rather than a modified glycosylation status itself for the avirulent *MNN14* mutant [78]. This strain, belonging to the *MNN1* gene family, displays only minor structural defects in the α-1,2-mannose side-chains and no impairments in the α-1,6-mannose backbone. Interestingly, the outcome of altered mannan biosynthesis between *Candida* species can result in contrary virulence, with the most prominent examples of deletion in *ANP1* or *MNN2* resulting in hypervirulence of *C. glabrata* [232] and hypovirulence of *C. albicans* [60]. However, the specific role of cell wall proteins on filamentation, host recognition, and induced immune response needs to be elucidated in more detail. In addition, moonlighting proteins could perform different so far unknown functions, which are not covalently bound to the cell wall, but when located on the cell wall they display distinct functions [233].

7.5.5 Impaired adhesion does not necessarily alter the epithelial immune response

Adhesion to host cells is a prerequisite for *C. albicans* to colonize mucosal surfaces and therefore the pathogen expresses various adhesins, e.g. Als3p, Hwp1p, and Eap1p [40]. A defect in adhesion is also associated with fragile biofilm formation as it was observed for deletion in *MNN9*, *OCH1*, and to a lesser extent in *PMR1* [78,100]. Reduced adhesion capability can decrease the epithelial activation [31], however, it does not necessarily cause a reduced cytokine response [98]. While a defect in *MNN9* or *MNN2* decreased adherence to hepatic cells, these strains induced an elevated cytokine response in hepatic cells. A stronger cytokine response was also observed in oral cells, but adherence to this cell line, as determined in this study, was not impaired in the *MNN9* and *MNN2* sextuple mutant. This is in contrast to observations from Murciano and coworkers (2011) showing reduced adhesion of a *MNN9* mutant to oral epithelial cells determined by CFU counting [98]. A likely explanation for these opposing results could be that glycosylation mutants are known to aggregate [60,68,81,97,177]. In this study, aggregating cells of glycosylation deficient strains were separated with a syringe by physical force, resulting in partially separated cells without impairment in fungal cell viability. A full separation could be achieved by ultrasonic application, but it also decreased viability drastically (unpublished data).

7.5.6 Candidalysin, the "missing link" in morphogenesis and cell damage?

Recently it has been shown that Candidalysin (Ece1), a secreted fungal peptide toxin, is fundamental in damaging epithelial membranes and activating the epithelial immune response [17]. The authors demonstrated that a defect in *ECE1* did not impair filamentation, but abolished epithelial damage and cytokine release. Interestingly, the *MNN9* mutant expresses and secrets more Candidalysin (the functional peptide Ece1-III; analyzed by LC-MS/MS; communicated by Selene Mogavero, HKI Jena). Increasing Candidalysin concentrations elevated the IL-1 release from myeloid cells [140], which might also explain the stronger activation of oral IL-1 release induced by the *MNN9* mutant. An putatively increased secretion of Candidalysin by the *MNN9* mutant, however, did not alter the damage potential of oral epithelial cells. The in this study observed stronger activation of components from the MAPK signaling pathway strengthens the hypothesis of a partly damage-independent cytokine induction by so far unknown factors. Another study supports this theory, where Ece1 expression did not correlate with the degree of LDH release [234]. Moyes and coworkers (2016) suggest that Ece1-III might not solely be secreted into invasion pockets but could also activate surrounding cells without any contact to hyphae [17]. It, therefore, appears possible that Candidalysin is not only *per se* the inducer of epithelial cell damage, but also a main activator of signaling cascades resulting in an epithelial cytokine response.

7.6 Early recognition of the β-glucan unmasked *MNN9* mutant could induce neutrophil recruitment in the kidney that reduces virulence in murine disseminated candidiasis

The commonly used murine model of disseminated candidiasis resulted in an organ-specific outcome: while spleen and liver controlled *C. albicans* infection by secreting neutrophils, the kidney showed a delay in early neutrophil availability within the first 24 h of infection [145]. However, the chemokine KC

and other cytokines appeared as early as 12 h after infection and further increased within 48 h. Kidney fungal burden consistently increased and as the infection progressed, cytokine-driven accumulation of immune cells ultimately contributed to renal pathology [145]. Moreover, *C. albicans* morphologies also differed between these organs, because hyphae have been not observed in spleen and liver.

A defect in the mannosylation status of *C. albicans* has been demonstrated to generally confer less virulence in murine disseminated candidiasis, regardless how strong the structural impairment was, e.g. by deletion of *OCH1* [68,107], *PMR1* [81], *MNN10* [72], or the *MNN2* sextuple mutant [75]. Likewise, this study demonstrates that the deletion of *MNN9* resulted in complete survival of mice for 21 days. A minor gene-dosage effect was observed for mice infected with the revertant +*MNN9* strain containing one functional *MNN9* allele, resulting in 2.5 days longer median survival compared to the wild type. The fact that the kidney is the target organ in disseminated candidiasis could be confirmed by the highest fungal burden in this organ. Surprisingly, the *MNN9* mutant also displayed a high kidney burden after 21 days that implicates an impaired host immune response. However, kidney burden induced by the *MNN9* mutant was reduced compared to that induced by the wild type between day three and five after infection. This could implicate that the host prevented massive colonization of the *MNN9* mutant or could even partially clear infection. However, limitations are the different time points of fungal burden analysis and lacking data at early time points of infection with the *MNN9* mutant.

50% of mice infected with the *MNN9* mutant displayed one healthy and one enlarged and pale kidney filled with granuloma structures that were not filled with immune cells but rather bulged with fungal filaments. Granuloma formation is expected during chronic infection and suggests a protective mechanism to prevent further fungal proliferation. This unilateral infection of either left or right kidney has been observed for low infectious doses [235]. The infectious dose of $5x10^5$ cells per mice was obtained by single cell counting and verified by CFU plating. However, as a result of the strong aggregating phenotype of the *MNN9* mutant the cell number observed by CFU plating was lower than the single cell counting. Therefore, cell aggregation might have reduced the effective infectious dose calculated by CFU plating that resulted in unilateral kidney infection. On the other hand, CFU plating from organ samples most likely underestimated the number of fungal burden caused by the *MNN9* mutant. A possible solution to calculate total cell numbers for comparison between wild type and mutant strains could have been quantitative PCR to determine the amount of fungal rDNA. It is not completely understood how the fungus systemically translocates into organs, and significantly less is known about translocation of aggregating cells. It might be possible that accumulation of few yeast cells could enter endothelial and epithelial cells, whereas bigger aggregates that are unable to cross these barriers remain in the bloodstream and are cleared subsequently.

Interestingly, kidney fungal burden was also seen by other groups when systemically infected with avirulent or less virulent mannosylation mutant strains: an avirulent *PMR1* mutant [81] with a decreased ability to form normal filaments *in vitro*, or the *MNN2* sextuple mutant [75] forming normal hyphae *in vitro*. Both strains induced high fungal burden in the kidney after 28 days of infection. Surprisingly, an *OCH1* mutant with the most severe structural defects in mannosylation causing a loss in hyphal

formation was not avirulent, but displayed a reduced virulence after 28 days [68,107]. Moreover, wild type-like kidney fungal burden was also observed for the *OCH1* mutant and the authors suggest that this is due to an altered host-fungal interaction driven by the impaired invasive growth by this mutant [68].

Mnn9 and Mnn10 are both part of the mannan polymerase II complex and might share structural similarity, however, the *N*-glycan structure for the *MNN10* mutant has so far not been determined. As well as the *MNN9* mutant, the *MNN10* mutant is reduced in virulence in murine disseminated candidiasis [72]. The authors observed a reduced kidney fungal burden caused by the *MNN10* mutant two and five days after infection, accompanied by increased cytokine levels and early recruited neutrophils and monocytes in the kidney. Consequently, early recruited neutrophils could partially clear the *MNN10* mutant infection, and with decreasing fungal burden, neutrophils and monocytes were also less abundant in the kidney. Since Mnn9 and Mnn10 are both mannosyltransferases involved in the same step of *N*-mannosylation, the *MNN9* mutant might also induce a similar and early neutrophil recruitment that reduces fungal burden.

In conclusion, *C. albicans* cell wall mannosylation might contribute to the modulation of the kidney immune response, whereas unmasking of the cell wall leads to early immune cell recruitment that could result from an elevated epithelial cytokine induction.

7.7 Concluding remarks and outlook

The activation of mucosal epithelial cells is considered to be dependent on *C. albicans*-induced damage. This study demonstrates that epithelial cells from the hepatic and renal parenchyma also require *C. albicans* damage to induce cytokine secretion. Different quantities and qualities of secreted epithelial cytokines suggested site-specific PRR expression and signaling. While PRR expression did not differ between epithelial cells, activation of MKP1 revealed epithelial-specific outcomes that was not dependent on early-induced cell damage. This demonstrates that the epithelial immune response is site-specific and suggests that further signaling components might be involved to induce cytokine releases.

This study further demonstrates that epithelial cells from different sites differently release inflammatory cytokines in response to different glycosylation mutant strains. While the *MNN9* mutant induced a stronger cytokine release by hepatic and oral epithelial cells, renal cells were less activated compared to the parental strain. One remaining question is the impact of epithelial cells on the total host immunity in response to disseminated candidiasis.

Taken together, the results obtained in this thesis demonstrate that commonly known fungal factors leading to adhesion, hyphal growth and maintenance, invasion, Candidalysin secretion and host cell damage, expression of hyphal-associated genes, activation of the epithelial MAPK/c-Fos and MKP1, and masking of immunostimulatory cell wall moieties collaborate in a complex network to confer *C. albicans* virulence but further so far unknown factors are involved to activate epithelial-specific immune responses.

8 Supplement

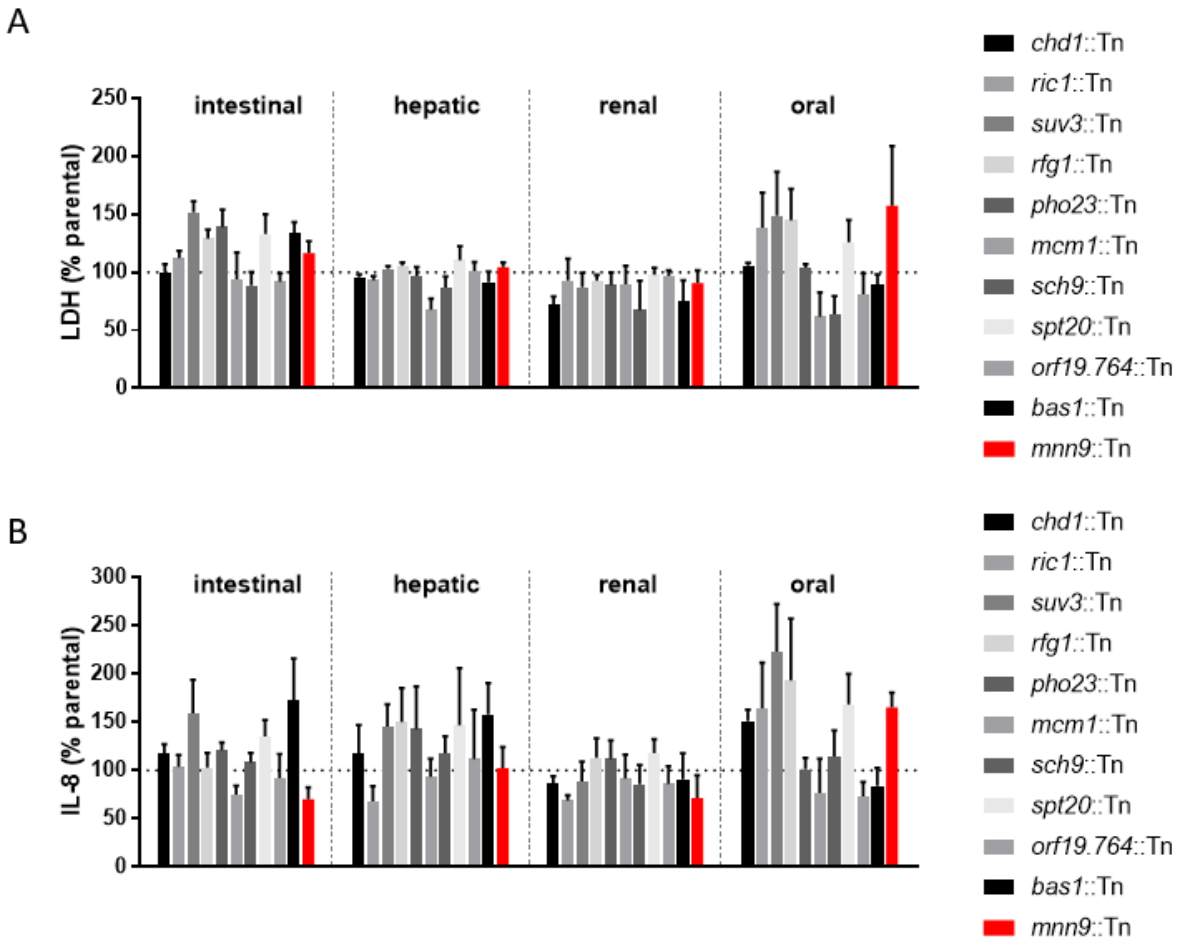

Fig. S1. *C. albicans* mutant strains selected for a putative modulation of the epithelial cytokine response. Confluent intestinal, hepatic, renal, and oral epithelial cell lines were infected with 11 different *C. albicans* transposon mutants for 24 h under standard culture conditions. Cell culture supernatant was assessed for lactate dehydrogenase (LDH, A) measuring epithelial cell damage, or the amount of IL-8 by ELISA (B). Data represent mean values +/- SD from three independent experiments. Dotted baseline: 100% LDH (A) or IL-8 (B) released by the respective parental strain.

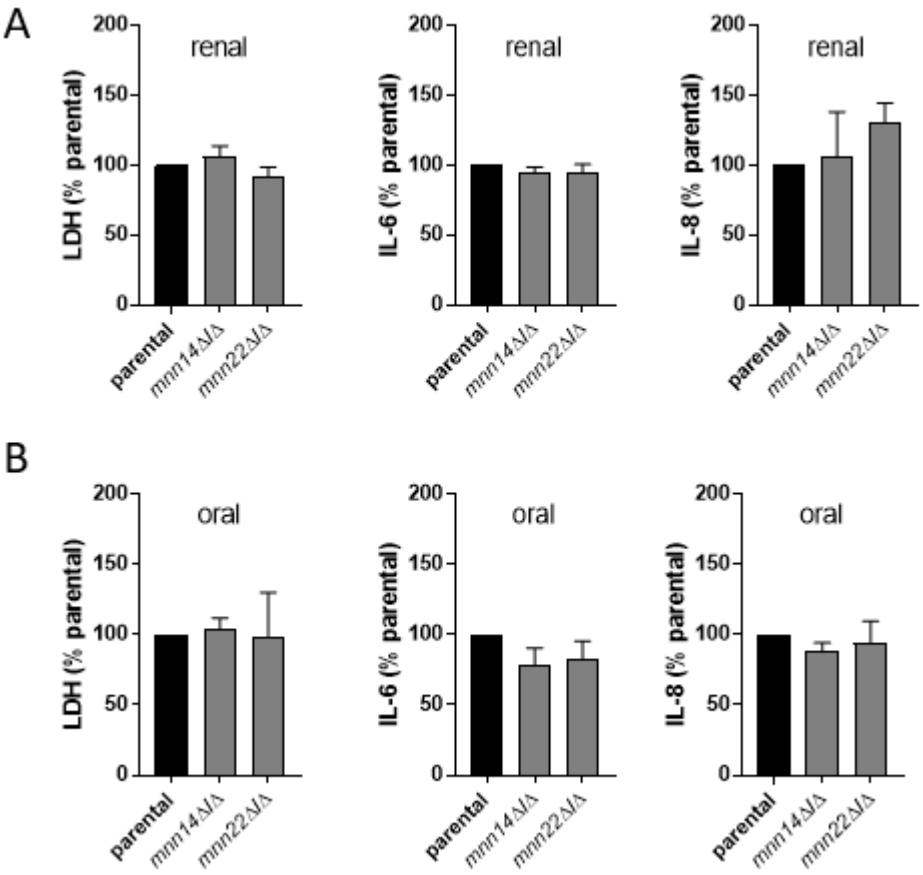

Fig. S2. Subtle defects in *N*-mannosylation by *MNN14* and *MNN22* did not affect epithelial cell damage and cytokine secretion.
Confluent renal (A) and oral (B) epithelial cell lines were infected with *MNN14* or *MNN22* mutants for 24 h under standard culture conditions. Cell culture supernatant was assessed for lactate dehydrogenase (LDH) measuring epithelial cell damage, or the amount of IL-6 and IL-8 by ELISA. Data represent mean values +/- SD from three independent experiments.

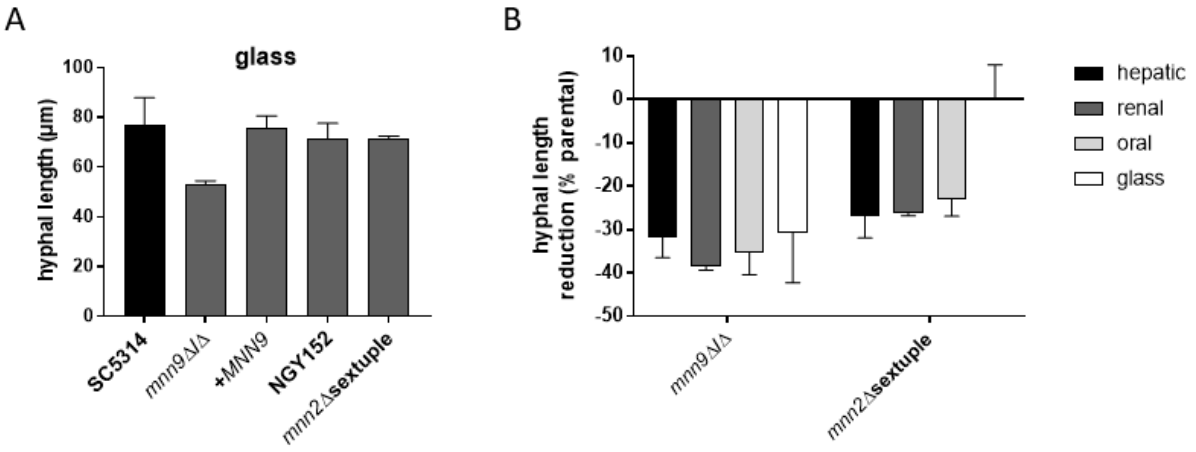

Fig. S3. Epithelial cells influence hyphal growth of the *C. albicans MNN2* sextuple mutant.
Hyphal length of different *C. albicans* strains was measured after incubation on glass (A) or hepatic, renal, and oral cells (B) for 4 h. The hyphal growth was impaired of *mnn9*Δ/Δ on epithelial cells and glass, while hyphal growth of the *MNN2* sextuple mutant was not impaired on glass cover slides without host cells. Data represent mean values +/- SD from two (A) or at least three independent experiments when incubated with epithelial cells.

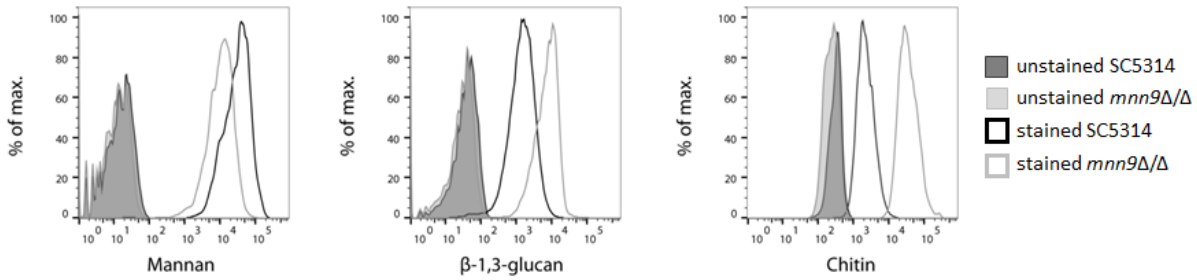

Fig. S4. Exposure of cell wall mannan, β-1,3-glucan, and chitin on *C. albicans* glycosylation mutant strains.
Representative histograms of at least three independent experiments of *C. albicans* SC5314 and *mnn9*Δ/Δ yeast cells stained for mannan, β-1,3-glucan, and chitin exposure; analyzed via flow cytometry.

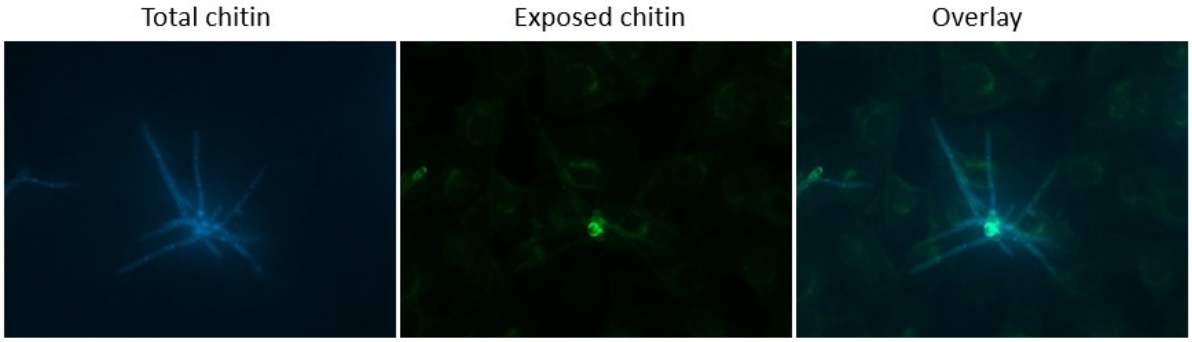

Fig. S5. Chitin staining of the filamentous *mnn9*Δ/Δ reveals normal separated cells.
*C. albicans mnn9*Δ/Δ was co-incubated with oral epithelial cells for 4 h and stained for total chitin (blue) and exposed chitin (green). Total chitin staining was performed once when incubated with oral cells and at least three times without cells (not shown).

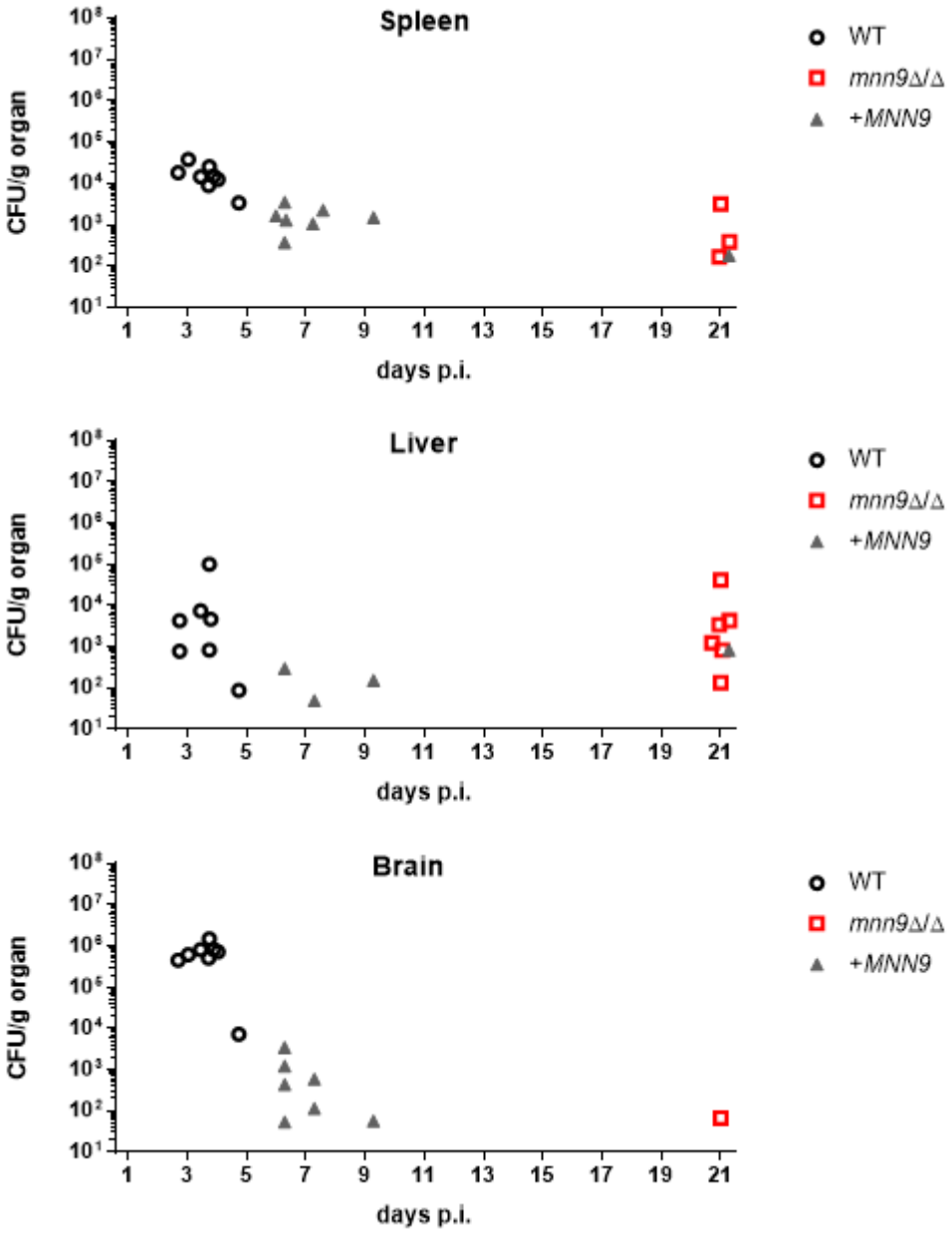

Fig. S6. Fungal burden in spleen, liver, and brain after *C. albicans* systemic infection in mice.
BALB/c mice were infected intravenously with $5x10^5$ *C. albicans* strains and survival was assessed for 21 days. Depicted is one representative from two independent experiments with 8 mice per group. Fungal burden was determined at the day by death as colony forming units per gram organ.

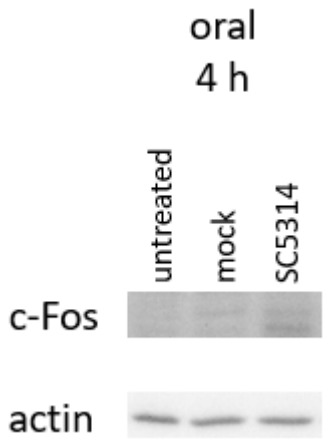

Fig. S7. Analysis of c-Fos activation after 4 h of infection with *C. albicans*.
c-Fos expression in untreated, mock-infected, and with *C. albicans* SC5314 infected epithelial cells after 4 h. Representative western blots from at least two independent replicates.

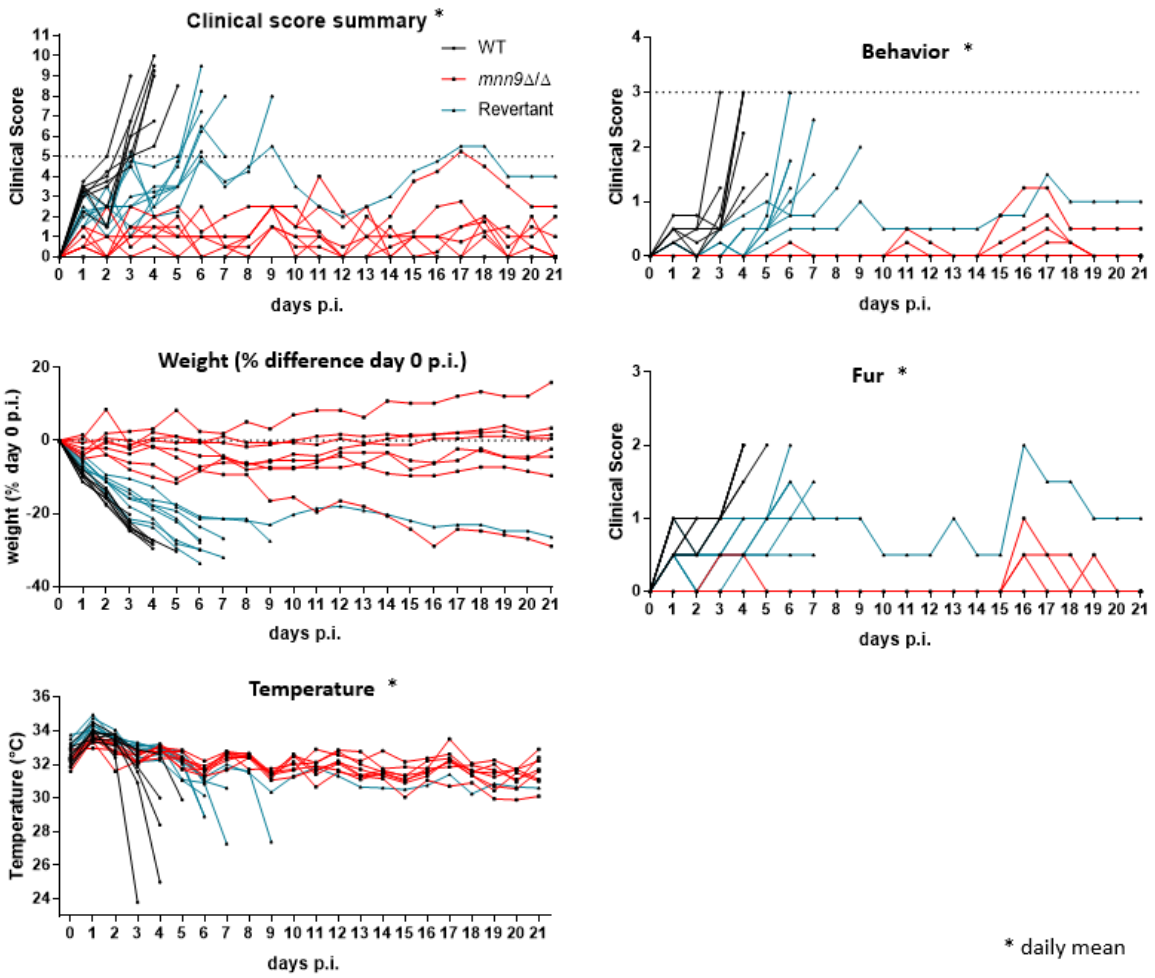

Fig. S8. Clinical score of mice after *C. albicans* systemic infection.
BALB/c mice were infected intravenously with $5x10^5$ *C. albicans* strains of wild type, *mnn9*Δ/Δ or the revertant strain +*MNN9* and survival was assessed for 21 days. To monitor mice health status, weight, temperature, behavior and fur were assessed at least on a daily basis. Changes of weight and temperature were allocated to a specific clinical score, while behavior and fur were directly scored. Mice were euthanized when the clinical score summary reached 5 or more points, or when temperature or weight dropped massively.

9 List of abbreviations

ALS	agglutinin-like sequence protein
ANOVA	analysis of variance
APS	ammonium persulfate
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
C.	Candida
CEACAM	carcinoembryonic antigen-related cell adhesion molecule
CFU	colony forming units
CLR	C-type lectin receptor
CWP	cell wall protein
DAMP	d
ddH2O	bi-distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinases
FBS	fetal bovine serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
HRP	horseradish peroxidase
JNK	c-Jun N-terminal kinases
IL	interleukin
IL1-R1	interleukin-1 receptor-1
LDH	lactate dehydrogenase
LB	Luria Bertani
LPS	lipopolysaccharide
M	molar
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotatic protein 1
MIP-1β	macrophage inflammatory protein-1β
MKP-1	mitogen-activated protein kinase 1
MOI	multiplicity of infection
NLRC4	NLR family CARD domain-containing protein 4
NLRP3	NLR family pyrin domain-containing 3
nm	nanometer

List of abbreviations

OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PRR	pattern recognition receptor
PVDF	polyvinylidenfluorid
ROS	reactive oxygen species
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SAP	secreted aspartyl protease
SD	standard deviation
SDS	sodium dodecyl sulphate
SPL	smart protein layer
TEMED	N,N,N',N'-tetramethylethylendiamine
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
TSLP	thymic stromal lymphopoietin
v/v	volume per volume
w/w	weight per weight
YPD	yeast peptone dextrose

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11 Appendix

11.1 Curriculum vitae

Personal profile

Last name: Pawlik

First Name: Tony

Title: Diploma Biologist

Day of birth: 19-Jun-1985 in Halle

Nationality: Germany

E-Mail: pawlik.tony@t-online.de

Course of studies

04/2013 – 10/2018

PhD student at the Leibniz Institute for Natural Product Research and Infection Biology – Hans Knoell Institute Jena in the Research Group Microbial Immunology and Department of Microbial Pathogenicity Mechanisms. Supervised by Prof. B. Hube and Prof. I.D. Jacobsen. Thesis: "The *Candida albicans* factor *MNN9* modulates cytokine production in distinct epithelial cell types"

10/2006-12/2011

Diploma study of Biology at the Martin Luther University Halle-Wittenberg with microbiology as the main subject. Diploma thesis at the Institute of Biology / Microbiology with the title: "Untersuchungen zum Nitrattransport in *Streptomyces coelicolor*"

1992-2005

Secondary school at the Frieden Gymnasium in Halle, University entrance qualification.

11.2 Publication

Allert S, Förster TM, Svensson CM, Richardson JP, **Pawlik T**, …, Hube B. *Candida albicans*-Induced Epithelial Damage Mediates Translocation through Intestinal Barriers. mBio, 2018.

11.3 Talks and poster presentations

Talks

09/2016

DMyk conference, Essen. Title: The Candida albicans factor MNN9 modulates cytokine production in distinct epithelial cell types

09/2014

DMyk conference, Salzburg. Title: Identification of *Candida albicans* factors that modulate cytokine production in distinct epithelial cell types

02/2014

DGHM Workshop, Göttingen. Title: Molecular characterization of immunomodulation by Candida albicans

Poster

04/2016

ASM Conference on Candida and Candidiasis, Seattle. Title: The *Candida albicans* factor *MNN9* modulates cytokine production in distinct epithelial cell types

09/2015

DGHM, Münster. Title: Identification and characterization of *Candida albicans* factors that modulate cytokine production in distinct epithelial cell types

09/2015

DMyk conference, Jena. Title: Identification and characterization of *Candida albicans* factors that modulate cytokine production in distinct epithelial cell types

09/2015

Weimar Sepsis Update, Weimar. Title: Identification and characterization of *Candida albicans* factors that modulate cytokine production in distinct epithelial cell types

11.4 Additional activities

courses:

07/2018

GMP course, Halle

10/2017

Safeguarding good scientific practice, Jena

09/2016

Effective Presentation – Talks and Posters, Schiller & Mertens, Jena

01/2016

Adobe Photohop & Illustrator Workshop
Scientific Image Processing and Analysis, Jena

03/2015

Gentechnikrecht based on §15 GenTSV in Jena, JenALL

11/2013

Cellular activation by danger signals - Introduction in qPCR and Mass-Spectrometry, Septomics

09/2013

academic writing

07/2013

Time and project management, Oberhof

public relation:

11/2017

Contribution to the Long Night of Science 2017 at the HKI, Microbial Immunology

04/2015

Organization of the Pupil's Science Day at the HKI, Microbial Immunology; Topic: "Volle Hyphe voraus! Wie ein Hefepilz menschliche Barrieren durchbricht"

09/2014

Contribution to the World Sepsis Day, Jena

11/2013

Contribution to the Long Night of Science 2013 at the HKI, Department of Microbial Pathogenicity Mechanisms

11.5 Supervision

07-09/2017

DAAD exchange student from Taiwan

10/2015-03/2017

Master thesis

03/2015-09/2015

Bachelor thesis

11.6 Affiliations

2013-2018

Associate member of the Center for Sepsis Control & Care – Research Training Group (CSCC-RTG)

11.7 Grants and awards

12/2018

medac research award

09/2015

Photo award for 2nd best picture at the DMyk conference 2015 in Jena

DGHM Travel Grant for the attendance of the DGHM 2015 in Münster

01/2014

DGHM Travel Grant for the attendance of the DGHM Workshop 2014 in Göttingen

11.8 Acknowledgements

First of all, I would like to thank Bernhard Hube who gave me the opportunity to start research in Jena. I especially thank Ilse Jacobsen (and of course Bernhard) for supervision during my time as a PhD student. I really enjoyed both state-of-the-art labs, the “cathedral” in A8 but also the cozy Lab House 3.

During my working time, I met many people who deserve acknowledgements. I thank all members of the MPM and MI for the excellent working and post working time. Especially I want to thank “my lab girls” Nicole, Katja, Christine, Melanie, Sarah, Franzi, and many more. In addition, I also wish to thank Pim for proofreading and his laugh I will never forget. By the way, this goes in the same direction to Nicole. You made me smile! I am thankful for the help of my bachelor and master students Julia and Anja, you made a great job!

It is just small thing, but Stefie’s idea to use a syringe to separate aggregating mutants was brilliant and helped me a lot, so thank very much!

I also would like to thank the group of Neil Gow who provided mannosylation strains and Ronny Martin for the plasmid that finally helped me to construct revertant strains.

I appreciate the support of Olaf Kniemeyer and Silke Steinbach who provided and introduced me the really cool machine Octoplus QPLEX that allows quantification of western blots.

Furthermore, I would like to thank Ralf Claus from the University Hospital Jena for his very helpful view from a medical perspective.

Finally, I especially want to thank my parents who supported me every time in every situation. Furthermore, I want to thank Jacki (my cat!), who helped me to relax. And of course, I am thankful to Arthi, our lab dog.

11.9 Selbstständigkeitserklärung

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 aktuell geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena vollumfänglich bekannt.

Personen, die mich bei der Auswahl und Auswertung von Experimenten und des Materials 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, 27.06.2019

Tony Pawlik