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A novel renal epithelial cell in vitro assay to assess *Candida albicans* virulence

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Candida albicans, an opportunistic fungal pathogen, can cause severe systemic infections in susceptible patient groups. Systemic candidiasis is mainly studied in the mouse intravenous challenge model, where progressive infection correlates with increased early renal chemokine levels.

To develop a new in vitro assay to assess *C. albicans* virulence, which reflects the events occurring in the murine infection model, renal M-1 cortical collecting duct epithelial cells were evaluated as the early producers of cytokines in response to *C. albicans*. We show that renal epithelial cells respond only to live *C. albicans* cells capable of forming hyphae, producing chemokines KC and MIP-2, with levels correlating with epithelial cell damage. By assaying epithelial cell responses to strains of known virulence in the murine intravenous challenge model we demonstrate that renal epithelial cells can discriminate between virulent and attenuated strains. This simple, novel assay is a useful initial screen for altered virulence of *C. albicans* mutants or clinical isolates in vitro and provides an alternative to the mouse systemic infection model.

Introduction

Candida albicans and other *Candida* species, such as *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. dubliniensis*, and *C. krusei,* are common commensal fungal species found in the gastrointestinal and reproductive tracts of healthy individuals.[1] However, *Candida* species can also cause mucosal (superficial) and systemic infections.[2] Systemic candidiasis is one of the most important nosocomial infections in Europe and the US and is associated with high mortality (40%) rates among hospitalized patients, particularly those in intensive care units (ICU), people undergoing major surgery and in immunocompromised individuals.[2-6]

Mucosal and systemic candidiasis is mainly studied in animal models.[7-10] However, there are limitations to these models; *C. albicans* is not a natural colonizer of small mammals[10] and there are ethical and cost implications.[11,12] These disadvantages, as well as Society's wish to reduce the numbers of animals used in research, have encouraged scientists to explore in vitro models to refine, reduce, or replace (3Rs) animals in research.[13]

For mucosal infections, in vitro models include mucosal explants,[14] monolayer cell cultures,[15-17] multiple layer cell cultures, and reconstituted human epithelium.[18-20] A good correlation has been found between immune responses measured in in vitro models and fungal virulence assayed in animal models.[20-23]

Although numerous models have been developed to investigate superficial candidiasis,[24-27] in vitro models to study systemic infection are currently limited to interactions with immune cells or with blood vessel endothelial cells.[28-33] Systemic infection is primarily studied in model hosts, such as invertebrate mini-hosts,[34-39] the chick chorioallantoic membrane model,[40,41] and small mammals.[42-46] However, the mouse intravenous (IV) challenge model remains the most commonly used model to investigate *C. albicans* virulence.[47-50] During infection the bloodstream and the majority of organs are cleared of the pathogen, but fungal burdens increase in the kidneys and brain, accompanied by increasing levels of renal cytokines and chemokines.[51,52] Increased renal cytokine levels correlate with lesion severity and eventual infection outcome,[49,52] with high levels of pro-inflammatory cytokines eventually causing sepsis and death of infected animals.

The escape of fungi from the bloodstream during systemic candidiasis has been modeled in vitro using endothelial cells.[28-30,53] Endothelial cell damage and cytokine production was induced only by live, germinated fungal cells,[53] and those *C. albicans* strains unable to damage endothelial cells were found to be less virulent in the mouse model of systemic candidiasis.[29] However, as it is early cytokine and chemokine levels in the kidneys which correlate with *C. albicans* murine systemic infection outcome,[52] we hypothesized that the host innate immune response is initiated in the kidneys. Epithelial cells, including renal cortical epithelial cells, are known to be capable of initiating an innate immune response through proinflammatory cytokine production, e.g., IL-8, IL-6, and IL-1.[22,54-61] We, therefore, chose to evaluate murine renal epithelial cells as the basis for the development of a new assay to allow in vitro assessment of *C. albicans* virulence.

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Results

Candida albicans–renal cells interactions

Based upon previous work,[52] it was hypothesized that the interaction between fungal and renal epithelial cells initiates the innate immune response in the kidneys during systemic *C. albicans* infection. To examine how *C. albicans* physically interacts with a renal epithelial monolayer, murine M-1 cortical collection epithelial cells were co-incubated with *C. albicans* SC5314, a virulent strain in the mouse systemic infection model, at a co-incubation ratio of 1:1 *C. albicans*:M-1 cells. Within 3 h hyphae were evident (**Fig. 1A**) and had penetrated the epithelial monolayer (**Fig. 1D**). By 6 h some elongated hyphae were observed (**Fig. 1B**), with further evidence of penetration into the epithelial monolayer (**Fig. 1E**), while masses of hyphae were seen at 24 h post-infection (**Fig. 1C**).

In order to further characterize the response of murine renal epithelial cells to *C. albicans*, chemokines and cytokines produced in response to *C. albicans* SC5314 were measured from 6 to 96 h post-infection. The majority of cytokines assayed (IL-6, TNF-α, IFN-γ, IL-12, IL-17, IL-10, and IL-1β)[51,52,62] were undetectable over 96 h (data not shown), whereas KC and MIP-2 (equivalent to human IL-8) chemokine levels increased (**Fig. 2A and B**). Both KC and MIP-2 levels were significantly higher than uninfected controls at 6 h, increasing further by 8 h. However, later in infection control KC and MIP-2 levels also increased but remained significantly lower than co-incubations at 24 h, and even at 48 h in the case of MIP-2 (**Fig. 2B**). By 48 h control KC levels were similar to the 1:1 co-incubation (**Fig. 2A**) and by 96 h control MIP-2 had increased to levels similar to the 1:1 co-incubation (**Fig. 2B**).

To assay whether *C. albicans* damaged renal cells during their interaction lactate dehydrogenase (LDH) release was measured (**Fig. 2C**). LDH levels reflected chemokine production by epithelial cells, where significantly higher damage occurred in the co-incubations compared with uninfected epithelial cells at 6 h post-infection. Incubation of 10 times more *C. albicans* cells with renal cells resulted in enhanced epithelial cell damage at earlier time points, although there was little difference later in infection (**Fig. 2C**).

Based upon the greatest significant differences for all parameters between uninfected and infected cells (**Fig. 2**), and in attempts to reflect localized fungal:epithelial cell ratios in the kidney, an 8 h time point and a 1:1 co-incubation was chosen as the basis for an in vitro assay, with KC, MIP-2, and LDH levels assayed.

Renal cells respond only to live *C. albicans* capable of switching morphology

Previous studies with peripheral blood mononuclear cells (PBMC) have shown that heat-killed (HK) *C. albicans* yeast cells stimulate higher cytokine production than live cells.[32,33,62] Endothelial cells have been shown to respond only to live *C. albicans* cells[53] and oral epithelial cells produce significantly lower levels of cytokines in response to killed *C. albicans* cells.[58] To investigate whether renal epithelial cell respond to killed cells, *C. albicans* yeast cells were killed either by heat-killing, by chemical killing, or by exposure to UV.[63,64] Dead cells were then used in the assay and the responses compared with those induced by live cells. Using two different *C. albicans* strains known to be virulent in the mouse model, it was shown that only live cells induce significant KC and MIP-2 levels (**Fig. 3A and B**). Similar results were found when hyphal cells were assayed, with only live cells capable of inducing chemokine responses (**Fig. 3A and B**).

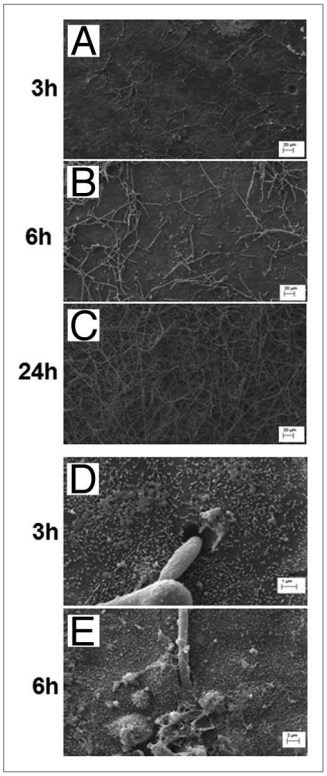

Figure 1. Scanning electron microscopy (SEM) images of *C. albicans*-renal epithelial cell co-incubation. M-1 renal epithelial cells and *C. albicans* SC5314 at 1:1 (**A–C**) *C. albicans*:renal cell ratio were co-incubated and visualized by SEM at 3 h (**A**), 6 h (**B**), and 24 h (**C**). Hyphal cell adherence to and penetration into renal cell monolayer is evident at 3 h and 6 h (**D and E**). Scale bars represent 20 μm (**A–C**), 1 μm (**D**), and 2 μm (**E**).

In our assay yeast cells rapidly form hyphae during co-incubation with renal epithelial cells. To investigate the importance of hyphal formation in the induction of innate immune responses by renal cells, morphological mutants were tested in our assay. The *hgc1*Δ and *egf1*Δ/*cph1*Δ (**Table 1**) null mutants, which are unable to form true hyphae, stimulated significantly lower KC and MIP-2 production (**Fig. 4A and B**), and caused less damage to the epithelial cells (**Fig. 4C**). Controls for these mutants, i.e., DAY185 and CAF2, did not differ in either chemokine production or epithelial cell damage relative to SC5314 (data not shown).

Renal epithelial cells discriminate between virulent and attenuated *C. albicans* isolates and mutants

To explore whether renal epithelial cells can differentiate between attenuated and virulent *C. albicans* strains, the assay was performed using *C. albicans* clinical isolates of known virulence in the mouse intravenous challenge model of systemic candidiasis

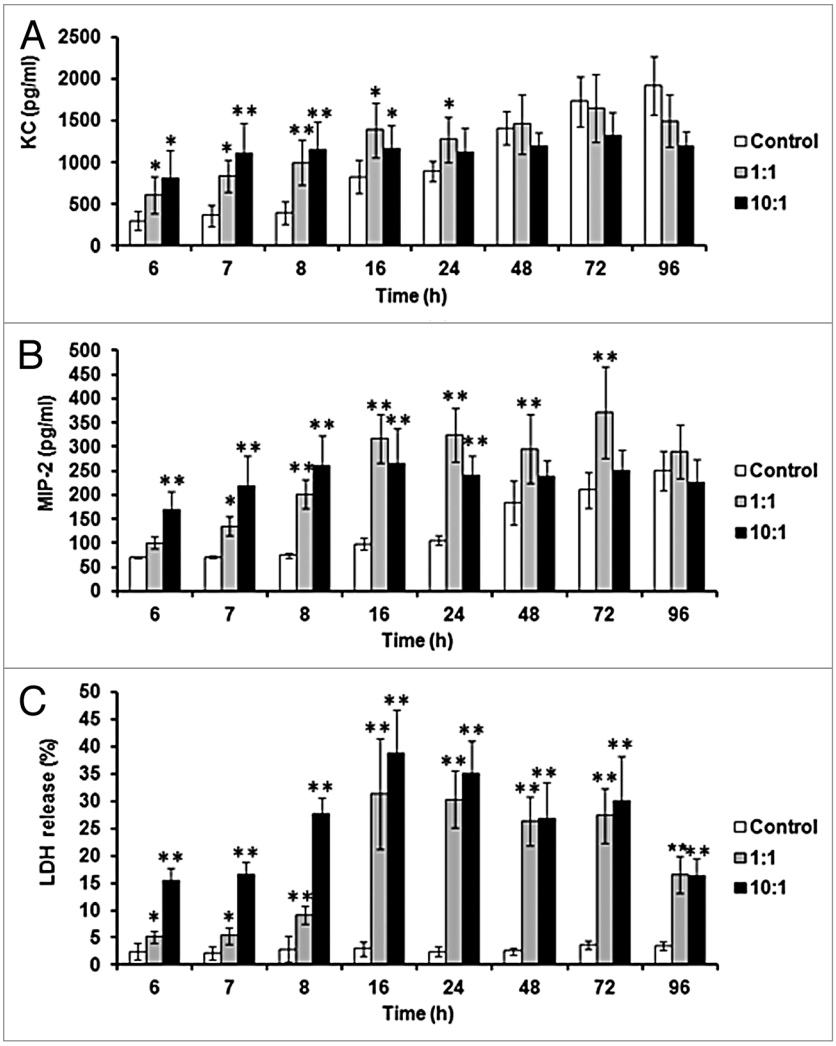

Figure 2. Renal epithelial chemokine production and damage increases during incubation with *C. albicans*. KC (**A**) and MIP-2 (**B**) levels were measured over 96 h of co-incubation. Chemokine levels and LDH release (**C**) were measured for control, 1:1 (*C. albicans*:renal cells) and 10:1 co-incubations. Results represent the means ± standard deviation of three separate experiments using triplicate samples. Significant differences relative to uninfected control were determined by ANOVA and the Tukey post-hoc test (*$P \leq 0.05$, **$P < 0.001$).

(**Table 1**).[52] Only virulent isolates induced significant levels of KC, with the exception of isolate AM2003-020, relative to uninfected controls (**Fig. 5A**). MIP-2 production showed a similar pattern but significant increases in chemokine levels were also found for two attenuated isolates, AM2003/0100 and AM2003-0069 (**Fig. 5B**). Again, LDH release was highest for virulent strains, with little damage seen when known attenuated strains were tested (**Fig. 5C**).

C. albicans null mutant strains known to be affected in virulence (**Table 1**)[65-68] were also tested in the model, and were compared with the control strain CAI4 + CIp10[68] (**Fig. 6**). *pmr1*Δ + CIp10, an attenuated strain in the mouse systemic model,[65] showed reduced KC levels (**Fig. 6A**), MIP-2 levels (**Fig. 6B**), and epithelial cell damage (**Fig. 6C**) when compared with the control. This defect was restored in the reintegrant strain. However, a strain of intermediate virulence, *mnt1/mnt2* + CIp10 mutant,[67] stimulated similar levels of KC, MIP-2, and LDH release as the parental and reintegrant strains (**Fig. 6**).

Further clinical isolates of known virulence were tested in blinded assays during evaluation of the assay to prevent unconscious bias. Virulence of the isolates in the mouse model was revealed after the assays had been completed. Similar to previous assays with clinical isolates (**Fig. 5**), virulent *C. albicans* isolates stimulated high KC and MIP-2 production, with the exception of a single strain, AM2003/0191, while intermediate and attenuated isolates induced similar chemokine levels (**Fig. 7A and B**) and LDH release (**Fig. 7C**) as uninfected controls. Clinical isolate AM2003/0191 formed a mix of yeast and hyphae in co-culture, rather than true hyphae as seen for other virulent strains. The majority of attenuated strains (**Figs. 5 and 7**), with the exception of 78/028 and AM2003/0074, formed long pseudohyphae in co-culture. However, the correlation is not complete as strains b30708/5 and AM2003/0100 formed true hyphae in co-culture, but did not cause significant damage to the epithelial cells or stimulate high levels of chemokines (data not shown).

Chemokine levels and epithelial cell damage

Generally, in our assay, trends for KC, MIP-2, and LDH levels were similar, with low levels for attenuated strains and higher levels for virulent strains. Correlation analyses of damage and chemokine production suggests that the two are related, with $R^2 = 0.778$ for KC and LDH and $R^2 = 0.6918$ for MIP-2 and LDH (**Fig. 8**). In order to establish whether chemokine levels merely reflect release of intracellular chemokine stores when epithelial cells are damaged chemokine levels from lysed epithelial cells after 8 h incubation were measured. Both KC and MIP-2 levels released from lysed cells were similar to those found for uninfected, intact M-1 epithelial cells, while chemokine production was significantly higher for epithelial cells co-incubated with *C. albicans* (**Fig. 9A**). Active production of both KC and MIP-2 was further confirmed by analysis of KC and MIP-2 gene expression during co-incubation with *C. albicans*, with KC levels increased from 1 h post-infection and MIP-2 significantly increased from 4 h post-infection (**Fig. 9B**). This suggests that chemokine production is an active process in response to damage caused by fungal cells.

Discussion

Animal models are most commonly used to investigate pathogenesis, infection progression and virulence of *C. albicans*.[42-45,47-52,65-71] However, these experiments require the use of many animals, particularly mice. The aim of the present study

was to develop a new in vitro model to assay *C. albicans* virulence, allowing a reduction in the number of mice used in systemic candidiasis studies, thus addressing the 3Rs.[13]

Our results clearly demonstrate that renal epithelial cells are capable of initiating an immune response to fungal pathogens, similar to other epithelial cell types.[18,54,58,60,72] Interactions between renal epithelial cells and fungal cells, using a cell culture monolayer, showed that *C. albicans* formed hyphae and penetrated into M-1 epithelial cells at 3 h post-infection. *C. albicans* had previously been shown to adhere to the HEK293 human kidney cell line, but chemokine and cytokine responses were not investigated.[73] Physical interaction between *C. albicans* and endothelial or oral and vaginal epithelial cells has previously been demonstrated to be required for initiation of a host response, with a switch to hyphal growth required.[29,53,58,74] This ability to switch between yeast and hyphal forms is also required for full virulence of *C. albicans* in the mouse intravenous challenge model.[34,75,76] However, results for endothelial and epithelial cells differ significantly from in vitro assays with human peripheral blood mononuclear cells (PBMCs), where the highest cytokine levels induced are in response to heat-killed, yeast form *C. albicans*.[33,77,78]

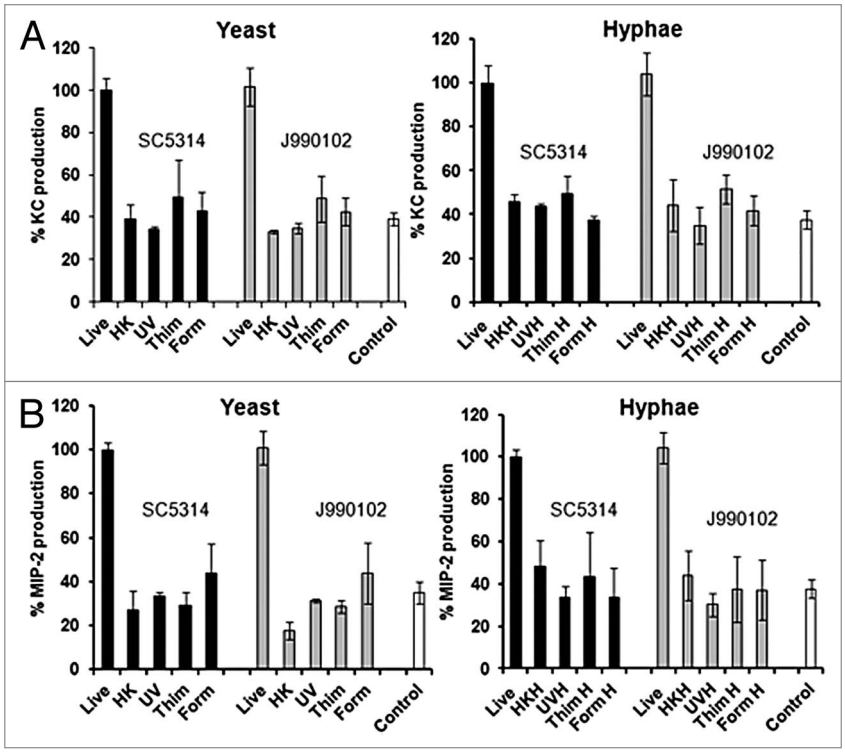

Figure 3. Renal epithelial cells respond only to live *C. albicans* cells. KC (**A**) and MIP-2 (**B**) measured at 8 h for a 1:1 (*C. albicans*-epithelial cell) co-incubation with live or killed *C. albicans* virulent strains, SC5314 or J990102. Live, heat killed (HK), UV killed (UV), formaldehyde (Form), and thimerosal (Thim) killed yeast and hyphal cells (**A and B**) were used. All values are expressed relative to SC5314 and results represent the means ± SEM of three separate experiments using triplicate samples. Significant differences relative to uninfected control were determined by ANOVA and the Tukey post-hoc test (*$P < 0.05$, **$P < 0.001$).

In our in vitro model early, active production of chemokines KC and MIP-2, important for the neutrophil recruitment,[79] by renal epithelial cells was demonstrated in response to virulent *C. albicans* strains. These results reflect early events in the mouse model of systemic infection, where KC levels increase within the first 12 h of infection in the kidneys of mice infected with virulent isolates[52] and virulent strains generally induce higher early levels of renal KC than attenuated strains.[43,52] Other cytokines, e.g., IL-6, TNF-α, and IL-1β, also begin to increase in the kidneys after this time,[52] but were undetectable in our in vitro assay, suggesting that these cytokines may be produced by infiltrating immune cells. However, it is also possible that the cell line used may be defective in pro-inflammatory cytokine production. This is in contrast to oral and vaginal epithelial cells which have been demonstrated to secrete IL-8, IL-1β, IL-6, and TNF-α in response to *C. albicans*.[74,80]

In general only *C. albicans* strains which are virulent in the mouse systemic model induced significant KC and MIP-2 production and caused damage to the renal epithelial cells. Damage to endothelial cells has been shown previously to induce production of immune modulators, and hence initiate an immune response.[81] However not every virulent isolate behaved in the same way in our assay. Virulent isolates AM2003/0191 (clade 2) and AM2003-020 (clade 4)[82] did not elicit significantly increased KC and MIP-2 production by epithelial cells, and two attenuated isolates, AM2003/0069 (clade 2) and AM2003/0100 (clade 2),[82] stimulated significantly higher MIP-2 levels than uninfected controls. Although classed as a virulent isolate in the mouse model, AM2003-020 induces lower levels of KC in the kidneys of infected mice, relative to other virulent isolates,[52] showing agreement with results obtained here. An imperfect correlation between murine virulence and endothelial cell damage has been observed previously, where some strains capable of causing endothelial cell damage were attenuated in a mouse infection model.[29] Adaptation of the fungus in vivo may also play a role, as strain-specific differences in adaptation to the host environment, which could not be reproduced in vitro, were recently demonstrated.[83] These alterations in in vivo adaptation could underlie results found for a minority of *C. albicans* strains where renal epithelial assay results did not correlate with virulence in the mouse model. However, one of the limitations of our model is that phagocytosis and blood clearance of fungi is not accounted for, which would affect the numbers of fungal cells reaching the kidneys in the mouse model, and thus infection outcome. Hence, mutants affected in susceptibility to neutrophils, e.g., the *sod5* mutant, may not be detected as attenuated, even though this mutant is attenuated in the mouse.[84,85]

Our new model using renal epithelial cells is a useful, simple, and rapid assay of *C. albicans* virulence, which can be used as a screen prior to proceeding to animal infection models, thus reducing the numbers of mice used in virulence assays. In the

Table 1. *C. albicans* isolates and strains

Isolate/ mutant	Virulence (mouse model)	Description	References
SC5314	Virulent	Clinical isolate	66
J951361	Virulent	Clinical isolate	52
J990102	Virulent	Clinical isolate	52
AM2003-020	Virulent	Clinical isolate	52
AM2003/0100	Attenuated	Clinical isolate	52
HUN96	Attenuated	Clinical isolate	52
AM2003/0069	Attenuated	Clinical isolate	52
FC28	Attenuated	Clinical isolate	52
RV4688	Attenuated	Clinical isolate	52
AM2003/0191	Virulent	Clinical isolate	52
s20122.073	Virulent	Clinical isolate	52
b30708/5	Attenuated/intermediate	Clinical isolate	52
AM2005/0377	Attenuated	Clinical isolate	52
AM2003/0074	Attenuated	Clinical isolate	52
78/028	Attenuated	Clinical isolate	52
CAI4 + CIp10 (NYG152)	Virulent	Wild-type control strain	68
*pmr1*Δ + CIp10 (NGY355)	Attenuated	*N*- and *O*-glycosylation mutant (*pmr1* null)	65
*pmr1*Δ + *PMR1*–CIp10(NGY356)	Virulent	*PMR1* reintegrant	65
*mnt1/2*Δ + CIp10 (NGY337)	Attenuated/intermediate	*O*-glycosylation mutant (*mnt1 and mnt2* null)	67
*mnt1/2*Δ + *MNT1*–CIp10 (NGY335)	Virulent	*MNT1* reintegrant	67
*hgc1*Δ (WYZ12.1)	Attenuated	G1 cyclin mutant (*hgc1*Δ)	76
*egf1*Δ/*cph1*Δ (HLC54)	Attenuated	Transcriptional regulator and transcription factor double mutant (*egf1*Δ/*cph1*Δ)	75

Clinical isolates and mutant virulence as determined in the mouse intravenous (IV) challenge model.[52,65-68,75,76,82]

future it would be of interest to extend this model to human kidney cell lines to determine whether *C. albicans* interacts in a similar way with human kidney cells.

Materials and Methods

Candida albicans culture and maintenance

C. albicans isolates and strains (**Table 1**) were routinely maintained on YPD agar at 4 °C (1% yeast extract, 2% mycological peptone, 2% glucose, 2% agar) or at −80 °C in glycerol for long-term storage. For co-incubation experiments *C. albicans* cells were grown in YPD at 30 °C 200 rpm overnight. Hyphae were induced by washing YPD overnight cultures three times with PBS, and then inoculating cells into DMEM:Ham's F12 with 2 mM Glutamine (GIBCO) to a final concentration of ~5×10^6 cells/ml. Cultures were incubated for 4 h at 37 °C in 5% CO_2. Cells (~1×10^7 cells/ml) were washed three times with PBS before being used in assays.

Preparation of killed *C. albicans* cells

Killed *C. albicans* cells were prepared either by heat-killing, by chemical killing or by UV killing.[63,64] Heat-killed (HK) cells were obtained by incubating cells (~1×10^8/ml) at 70 °C for 2 h in a water bath. Chemically-killed cells were prepared by treating cells (~1×10^8/ml) overnight with 100 mM thimerosal or 1% formaldehyde, and then washing cells four times in PBS to remove residual chemicals. To UV kill cells, *C. albicans* (~1×10^8/ml) were exposed to 0.100 J/cm^2 UV light 20 times on a UV-crosslinker (CL 508 S, Uvitec, Cambridge) with 254 nm UV emission tubes. All killed cells were stored at 4 °C before used for co-incubation.

For all killed cells, after re-suspension in PBS (~100×10^8 cells/ml), a 10 µl sample was plated onto YPD agar and incubated for 48 h at 30 °C to assay cell viability. The detection limit of the viability assay was 100 cells/ml. Viable cells were not detected in any of the cell suspensions used in these assays.

Renal epithelial cell cultures

The M-1 mouse kidney cortical collecting duct epithelial cell line (CRL-2038, ATCC) was used for co-incubation assays with *C. albicans*. Renal epithelial cells were grown in DMEM:Ham's F12 with 2 mM glutamine (Gibco Life Technologies), 5 µM dexamethasone (Hspira UK Limited), 5% fetal bovine serum (FBS), and maintained in 5% CO_2 at 37 °C. The growth medium was changed every second day. Only passage numbers 35–49 were used for assays.

C. albicans–renal epithelial cell co-incubation assays

For co-incubation of renal epithelial cells with *C. albicans,* 1×10^5 M-1 kidney cells were seeded into each well of a 24-well plate (Greiner Cellstar) and grown for 2 d to reach confluence.

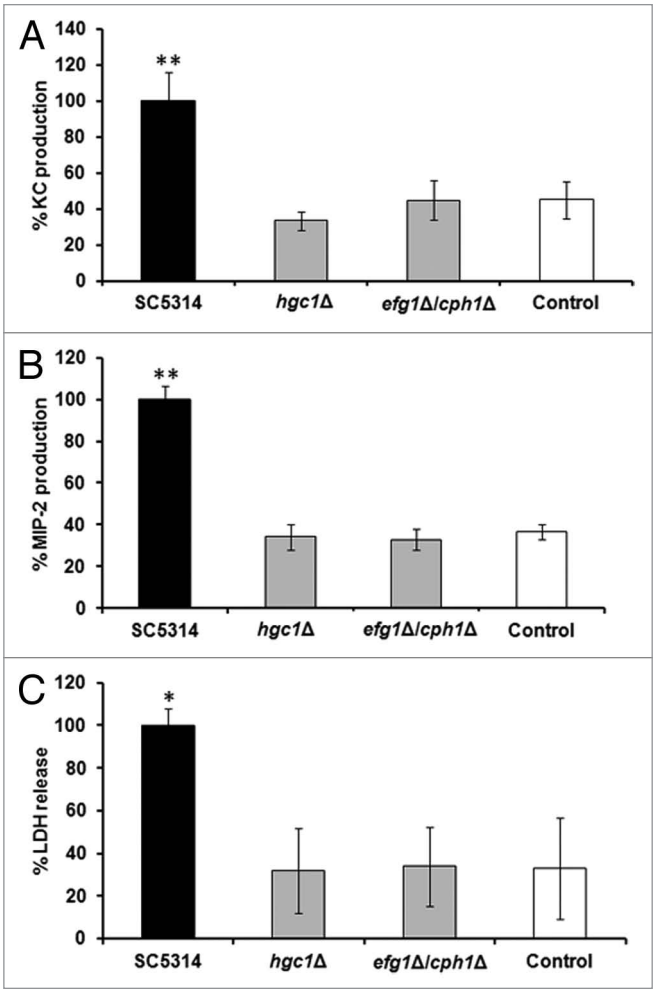

Figure 4. Switching from yeast to hyphal form is required to induce chemokine production and LDH release from renal cells. KC (**A**), MIP-2 (**B**), and LDH (**C**) levels at 8 h post-infection with 1:1 *C. albicans*:renal cells co-incubation with yeast-locked mutants and SC5314. All values are expressed relative to SC5314. Results represent the means ± SD of three separate experiments using triplicate samples. Significant differences relative to uninfected control were determined by ANOVA and the Tukey post-hoc test (*$P < 0.05$, **$P \leq 0.001$).

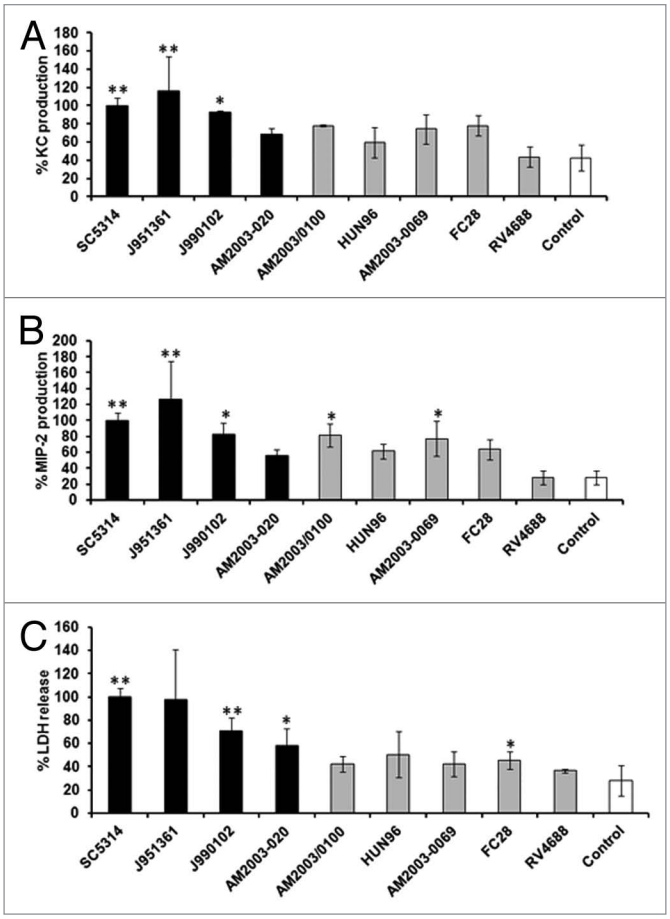

Figure 5. Renal epithelial cells discriminate between virulent and attenuated *C. albicans* isolates. KC (**A**), MIP-2 (**B**), and LDH (**C**) levels at 8 h post-infection for 1:1 *C. albicans*:renal cell co-incubation with different *C. albicans* isolates of known virulence in the mouse model.[82] All values are expressed relative to SC5314. Results represent the means ± SD of three separate experiments using triplicate samples. Significant differences relative to the uninfected control were determined by ANOVA and the Tukey post-hoc test (**$P < 0.001$, *$P < 0.05$). Virulent strains are shown as black columns, attenuated strains as gray and control as white columns.

The growth medium was changed and fungal cells added at a ratio of 1:1 or 10:1 (*C. albicans*:M-1 cells). Plates were incubated for 6–96 h in 5% CO_2 at 37 °C. Culture supernatants were collected at defined time points and stored at –20 °C for cytokine/chemokine measurements or at 4 °C for epithelial cell damage (LDH) assays. To determine intracellular chemokine levels, renal cells were lysed with Triton X-100 (1% final concentration) for 40 min and the supernatant stored at 4 °C. In some experiments *C. albicans* strains were provided to the experimenter in a blinded fashion, e.g., with a code rather than the strain name.

Cytokine and chemokine production

Cytokine production by epithelial cells during *C. albicans* co-incubation was measured by Duoset ELISA development systems or Quantikine colorimetric sandwich ELISAs (R&D Systems) (IL-12, TNF-α, IL-1β, IL-10, IL-17, and IFN-γ, KC, and MIP-2) (R&D Systems), according to the manufacturer's instructions.[17]

RNA extraction and real-time PCR

RNA was extracted from cells using the RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. cDNA was prepared using Superscript II (Invitrogen) as per manufacturer's instructions. The real-time PCR assay was utilized the Roche universal probe library. Primers used were manufactured by Invitrogen: KC forward (5′-ACTCCAACAC AGCACCATGA-3′) and reverse (5′-TGGTCTGCAG GCACTGAC-3′) primers and MIP-2 forward (5′-CCCTGGTTCA GAAAATCATC C-3′) and reverse (5′-CTTCCGTTGA GGGACAGC-3′) primers. PCR was performed using the LC Dual Color Hydrolysis probe assay in a Roche Lightcycler 480. Roche universal probe 49 was used to detect amplification of KC and probe 63 for MIP-2, with the murine GAPDH Taqman assay (Applied Biosystems) included in the same RT-PCR reaction to detect GAPDH as a control, following the manufacturer's instructions. Data was analyzed

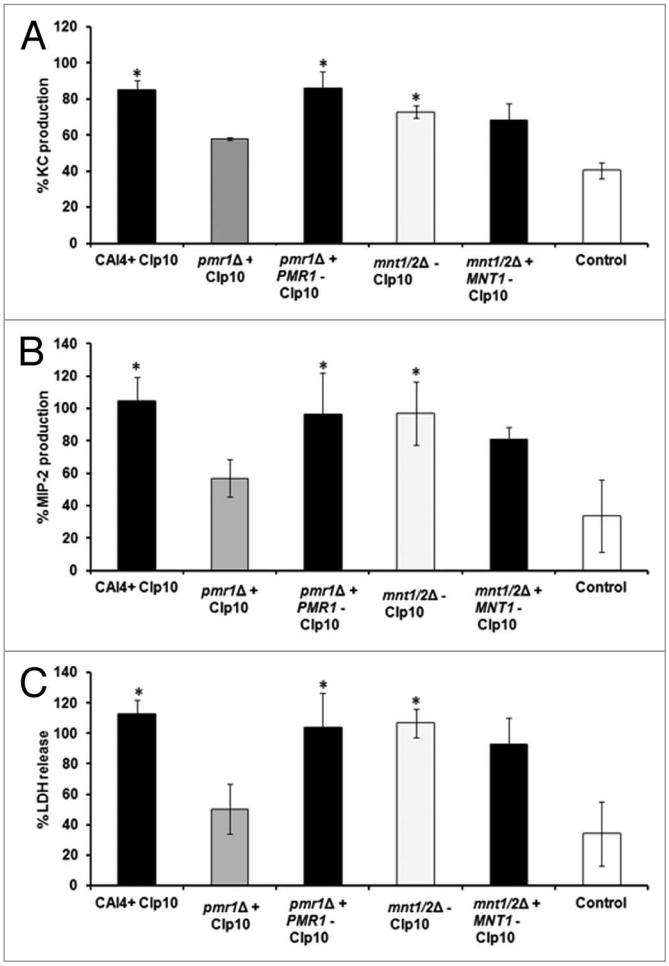

Figure 6. Attenuated *C. albicans* mutant strains fail to induce high levels of chemokine production by renal epithelial cells. KC (**A**), MIP-2 (**B**), and LDH (**C**) levels at 8 h post-infection for a 1:1 *C. albicans:*renal cell co-incubation with *C. albicans* null mutant strains with virulence defects in the mouse intravenous challenge model.[65,67,68] All values are expressed relative to SC5314. Results represent the means ± SD of three separate experiments using triplicate samples. Significant differences relative to uninfected control were determined by ANOVA and the Tukey post-hoc test (**$P < 0.001$, *$P < 0.05$). Virulent strains are shown as black columns, attenuated as gray, intermediate strain as light gray, and control as white columns.

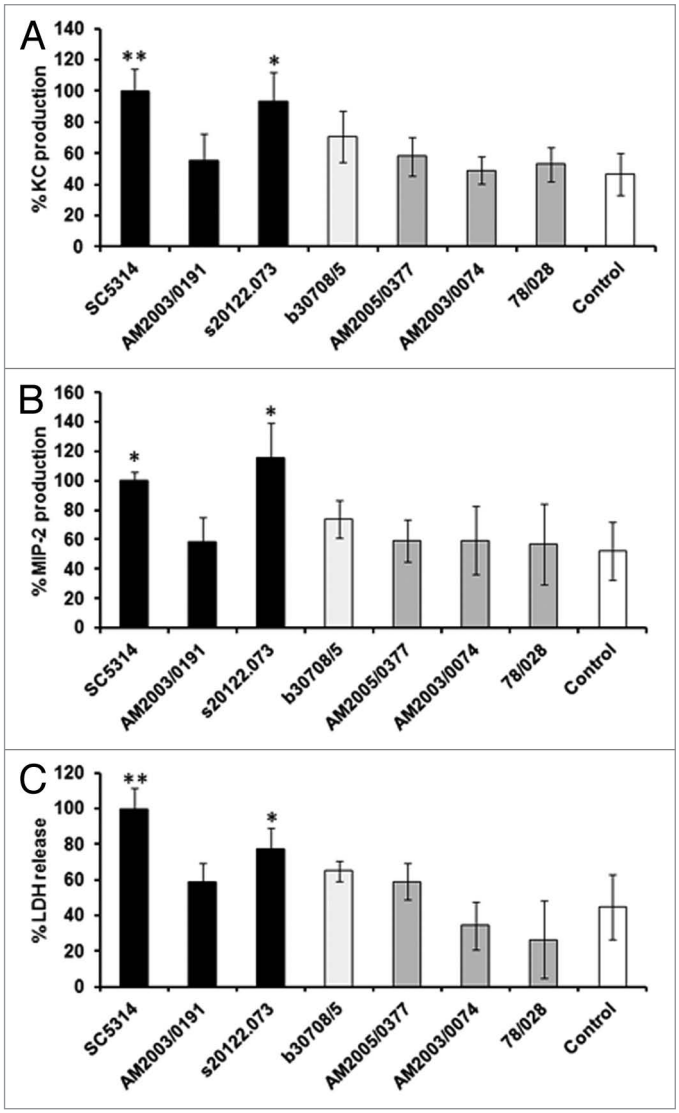

Figure 7. Identification of virulent *C. albicans* strains in a blinded assay. Six *C. albicans* clinical isolates were assayed in a blinded manner, with the experimentalist provided with the mouse virulence data after analysis of the results. KC (**A**), MIP-2 (**B**), and LDH (**C**) levels were determined at 8 h post-infection. All values are expressed relative to SC5314. Results represent the mean ± SD of three separate experiments using triplicate samples. Significant differences relative to the uninfected control were determined by ANOVA and the Tukey post-hoc test (**$P < 0.001$, *$P < 0.05$). Virulent strains (as determined in the mouse model) are shown as black columns, attenuated as gray, intermediate strain as light gray and control as white columns.

with the Lightcycler software by the Absolute Quantification Analysis using the Second Derivative maximum method.

Epithelial cell damage assay

Renal epithelial cell damage was assayed by lactate dehydrogenase (LDH) release into the culture supernatant.[17] At each sampling time, 1 ml of supernatant was collected and LDH release determined using the cytotoxicity detection kit (Roche Applied Science), according to the manufacturer's instructions. LDH levels were determined from an L-Lactate Dehydrogenase (Roche) 8 point standard curve (highest value of 0.125 μg/ml). To determine maximum LDH release with 100% cell death at each time point, Triton X-100 (1% final concentration) was added to the renal cell monolayer and incubated for 40 min to lyse the cells.[17] Total cell lysis was confirmed by light microscopy. LDH release (%) for each time point was calculated relative to the 100% cell death value at the same time point.

Scanning electron microscopy

M-1 cells were grown on BD Falcon cell culture PET track-etched membrane inserts (Becton Dickinson Labware). Inserts had an effective growth area of 0.3 cm^2 and 0.4 μm pore size. Membranes were carefully dissected and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer overnight. Samples were washed in 0.1 M phosphate buffer and then incubated in 1% osmium teroxide for 1 h. Membranes were washed in 0.1 M phosphate

buffer, and then dehydrated through a series of ethanol washes (70%, 80%, 90%, and 100%). Samples were placed in hexamethyldisilazane (HMDS) for 10 min and allowed to dry overnight before attaching to an aluminum stub and coating in gold for viewing.

Statistical analysis

Data were analyzed using IBM SPSS statistics version 20. Significant differences were determined by ANOVA and Tukey Post-hoc test. The correlation factors were calculated using the Spearman non-parametric correlation test, and significant differences in gene expression were determined using the Student *t* test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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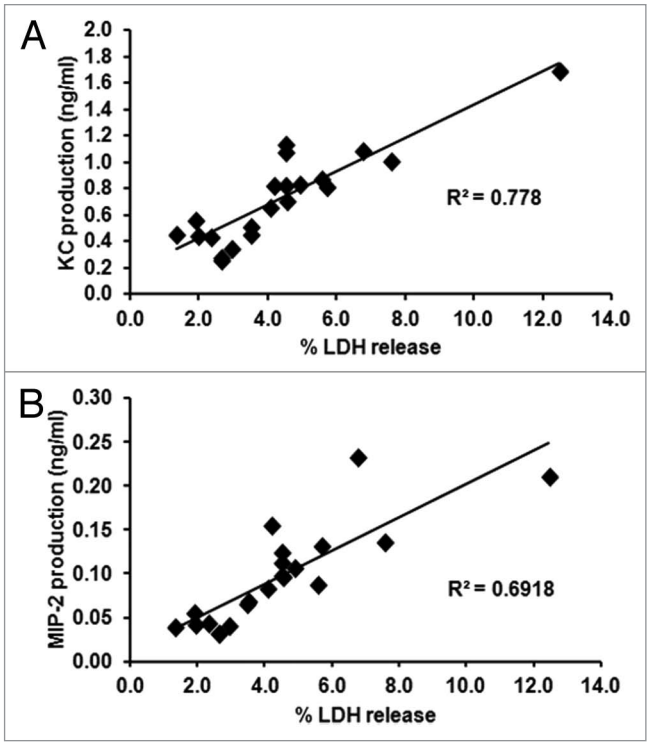

Figure 8. Chemokine production correlates with LDH release from renal epithelial cells. KC (**A**) and MIP-2 (**B**) levels relative to LDH levels for all experiments analyzed. Each data point represents the mean assay values for a *C. albicans* strain or mutant. Correlations were determined by the Spearman correlation test.

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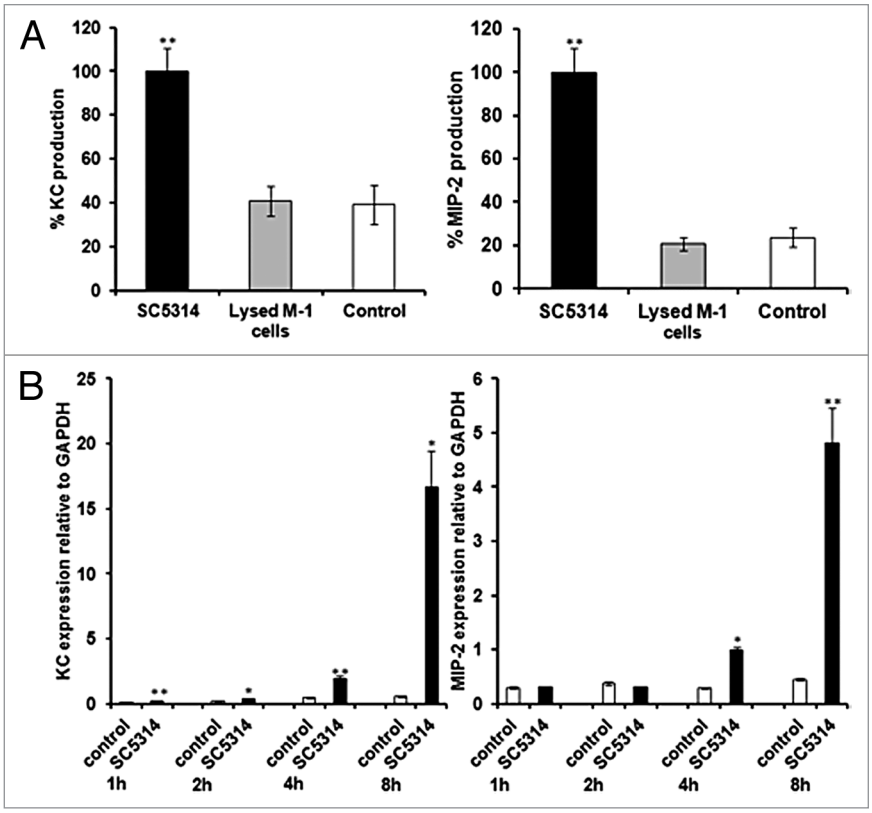

Figure 9. Chemokines are actively produced by renal epithelial cells. (**A**) KC and MIP-2 production by renal cells co-incubated with SC5314 (virulent strain), control uninfected epithelial cells and epithelial cells lysed using Triton-X-100. (**B**) KC and MIP-2 gene expression in renal cells co-incubated with SC5314 (virulent strain) and control cells at 1, 2, 4, and 8 h. Results in (**A**) represent the mean ± SD, and in (**B**) represent the mean ± SEM, of three separate experiments using triplicate samples. Black bars represent the co-incubation, gray bars are lysed epithelial cells and white bars represent uninfected controls. Significant differences relative to the uninfected control were determined by ANOVA and the Tukey post-hoc test (*$P \leq 0.05$, **$P \leq 0.001$) (**A**) or by the Student *t* test (*$P \leq 0.05$, **$P \leq 0.001$) (**B**).

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