Candida albicans adhesin Als3p is dispensable for virulence in the mouse model of disseminated candidiasis

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The presence of specific proteins, including Ece1p, Hwp1p and Als3p, distinguishes the *Candida albicans* hyphal cell wall from that of yeast-form cells. These proteins are thought to be important for the ability of *C. albicans* cells to adhere to living and non-living surfaces and for the cell-to-cell adhesion necessary for biofilm formation, and also to be pivotal in mediating *C. albicans* interactions with endothelial cells. Using an *in vitro* flow adhesion assay, we previously observed that yeast cells bind in greater numbers to human microvascular endothelial cells than do hyphal or pseudohyphal cells. This is consistent with previous observations that, in a murine model of disseminated candidiasis, cells locked in the yeast form can efficiently escape the bloodstream and invade host tissues. To more precisely explore the role of Als3p in adhesion and virulence, we deleted both copies of *ALS3* in a wild-type *C. albicans* strain. In agreement with previous studies, our *als3Δ* null strain formed hyphae normally but was defective in biofilm formation. Whilst *ALS3* was not expressed in our null strain, hypha-specific genes such as *ECE1* and *HWP1* were still induced appropriately. Both the yeast form and the hyphal form of the *als3Δ* strain adhered to microvascular endothelial cells to the same extent as a wild-type strain under conditions of flow, indicating that Als3p is not a significant mediator of the initial interaction between fungal cells and the endothelium. Finally, in a murine model of haematogenously disseminated candidiasis the mutant *als3Δ* remained as virulent as the wild-type parent strain.

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INTRODUCTION

The ability of the opportunistic fungal pathogen *Candida albicans* to interact with host cells is an important component of systemic infections, which have mortality rates ranging from 30 to 50 % (Viudes *et al.*, 2002; Wey *et al.*, 1988). The use of implanted medical devices provides a substrate upon which *C. albicans* can form biofilms, complex structures which consist of a mixture of yeast, pseudohyphae and hyphae embedded in an extracellular matrix (Ramage *et al.*, 2005). Fungal cell wall proteins are believed to be important for the ability of *C. albicans* cells

to adhere to living and non-living surfaces and for the cell-to-cell adhesion necessary for biofilm formation, and also to be pivotal in mediating *C. albicans* interactions with endothelial cells. It had been thought that filamentation was necessary to penetrate the vascular endothelium in order to enter tissues, but in a murine model of systemic candidiasis, *C. albicans* cells can rapidly escape the bloodstream after infection, with fewer than 10 % of introduced cells recoverable from blood 10 min after injection (MacCallum & Odds, 2005). Further, although strains that are locked in the yeast form are avirulent in the murine model of systemic candidiasis (Lo *et al.*, 1997; Saville *et al.*, 2003; Stoldt *et al.*, 1997), it has been demonstrated that yeast cells can effectively escape the bloodstream and penetrate organs (Bendel *et al.*, 2003; Chen *et al.*, 2006; Saville *et al.*, 2003; Spellberg *et al.*, 2003). The importance of yeast morphology for dissemination is further supported by our recent

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Abbreviations: ALS, agglutinin-like sequence; GMS, Grocott–Gomori methenamine-silver; H&E, haematoxylin and eosin.

observation that yeast-form cells adhere better than hyphal cells to endothelial cells under conditions of flow (Grubb *et al.*, 2009). Although cells locked in the filamentous state also display reduced virulence (Braun & Johnson, 1997; Braun *et al.*, 2000; Murad *et al.*, 2001), the ability to form hyphae is clearly important for *C. albicans* to cause pathology after dissemination: cells locked in the yeast form remain avirulent until they are permitted to form hyphae, after which point, mice succumb to the infection (Saville *et al.*, 2003). To better understand the capacity of *C. albicans* to cause disease, previous studies have examined not only the regulation of hypha formation, but also the importance of specific cell wall proteins in *C. albicans* virulence.

In *C. albicans*, the agglutinin-like sequence (ALS) genes encode a family of cell wall proteins involved in adhesion (Hoyer *et al.*, 2008). The cell surface glycoprotein Als3p is a major component of the hyphal but not the yeast cell wall (Argimón *et al.*, 2007; Coleman *et al.*, 2009; Zhao *et al.*, 2004). Strains lacking Als3p can still form hyphae (Nobile *et al.*, 2006; Zhao *et al.*, 2004), but biofilm formation *in vitro* is impaired (Nobile *et al.*, 2006; Zhao *et al.*, 2006). In the absence of Als3p, hyphal cells grow in parallel orientation rather than in the tangled arrangement observed with wild-type cells (Zhao *et al.*, 2006). This results in a weaker structure and reduced biofilm mass. Conversely, although overexpression of *ALS3* does not restore the ability of the *efg1Δ* mutant strain to form hyphae, it does lead to an increase in biofilm mass (Zhao *et al.*, 2006). Biofilms formed *in vivo* exist in polymicrobial environments and Als3p appears to be important for these interactions as well. Without Als3p, *C. albicans* hyphae are impaired in their ability to adhere to the human oral cavity bacterium *Streptococcus gordonii* and form a biofilm (Silverman *et al.*, 2010). Although Als3p is clearly important, it is not the sole determinant of biofilm formation. An *als3Δ* mutant is able to form an extensive biofilm *in vitro* under hypoxic conditions (Stichternoth & Ernst, 2009) and *in vivo* using a rat venous catheter model (Nobile *et al.*, 2006), whilst overexpression of *HWP1*, but not *ALS3*, effectively restores the ability of a *bcr1Δ* null strain to form biofilms on murine mucosal surfaces (Dwivedi *et al.*, 2011).

Previous studies have demonstrated that a lack of Als3p impairs the ability of *C. albicans* to adhere to human buccal epithelial cell (BEC) monolayers, and that incubation with specific anti-Als3p monoclonal antibodies blocks *C. albicans* adhesion to human BEC or human umbilical vascular endothelial cell (HUVEC) monolayers (Coleman *et al.*, 2009). Without Als3p, *C. albicans* has reduced ability to damage TR146 oral squamous cell carcinoma cells cultured *in vitro* either as a reconstituted human epithelium model (Zhao *et al.*, 2004) or as monolayers (Almeida *et al.*, 2008), and is endocytosed less efficiently by HUVEC or oral epithelial cells (Phan *et al.*, 2007). This endocytosis has been shown to be mediated by *C. albicans* Als3p interactions with E-cadherin on oral epithelial cells and N-cadherin on endothelial cells. These observations

have led to the supposition that Als3p might play an important role during disseminated infections, although no study using the haematogenously disseminated murine model to test this hypothesis has yet been published. During a disseminated *C. albicans* infection, fungal cells must form the initial attachments to host tissues not under static conditions, but subject to the stresses of fluid moving through the blood vessels. Our recent data show that, in contrast to the situation under static conditions, yeast-form cells adhere better than hyphae under conditions of flow (Grubb *et al.*, 2009). We therefore sought to test the ability of an *als3Δ* null strain to adhere to endothelial cells under these conditions and, since it has not previously been reported, to test the capacity for virulence of the mutant strain in the widely used murine model of haematogenously disseminated candidiasis.

METHODS

Strains and media. The yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Strains were routinely maintained as -80°C frozen stocks and grown on yeast extract-peptone-dextrose (YPD). For filamentation assays in liquid medium, strains were grown overnight at 28°C , washed in sterile PBS, diluted 1:20 into fresh medium such as RPMI 1640 supplemented with L-glutamine and buffered with MOPS (Angus Buffers and Chemicals), and incubated with shaking at 37°C . Biofilm formation was determined as described by Nobile & Mitchell (2005). Briefly, cells from an overnight culture were diluted to OD_{600} 0.5 in 2 ml Spider medium and added to a well of a 12-well plate containing a $15\text{ mm} \times 15\text{ mm}$ square cut from a silicone sheet (Bentec Medical) and pretreated overnight in fetal bovine serum (FBS) (Lonza). The plate was incubated at 37°C with gentle shaking for 90 min and then the squares were washed with sterile PBS to remove non-adherent cells. Incubation was continued with fresh Spider medium for 48 h and the wells were photographed. For the flow adhesion assay, *C. albicans* was grown as described previously (Grubb *et al.*, 2009). Briefly, cultures were grown overnight at 25°C in YPD broth. For yeast-form growth, cells were diluted 1:10 in fresh YPD and grown with shaking at 25°C for 3 h, while, to induce hypha formation, cells were diluted 1:10 in fresh YPD supplemented with 5% FBS and grown with shaking at 37°C for 1 h. Microscopic examination of the cells confirmed the morphology and that they remained separated with no evidence of significant clumping. After culture, *C. albicans* cells were washed once in HEPES-buffered Hank's balanced salt solution (HBSS) and resuspended at 2×10^6 c.f.u. ml^{-1} in HBSS (Sigma). All plasmid manipulations were performed with *Escherichia coli* strain DH5 α with selection on Luria-Bertani plates containing 100 μg ampicillin ml^{-1} when necessary.

Strain construction. Both copies of *ALS3* were sequentially deleted from wild-type strain SC5314 using the SAT1 flipper (Reuss *et al.*, 2004). First, regions flanking the *ALS3* coding sequence (designated LHF and RHFouter and RHFinner, respectively) were PCR-amplified using the primer pairs ALS3_LHF_UPS with ALS3_LHF_DS, ALS3_RHF_outer_UPS with ALS3_RHF_outer_DS and ALS3_RHF_inner_UPS with ALS3_RHF_inner_DS (Table 3). The amplification products were ligated into the *Sma*I site of plasmid pMT3000. The LHF region was liberated from the plasmid by digestion with *Apa*I and *Xho*I at the sites engineered into the primers and ligated between the *Apa*I and *Xho*I sites of the pSFS2 plasmid (Reuss *et al.*, 2004) to form plasmid pALHFS. The inner and outer RHF regions were liberated from the plasmids by digestion with *Sac*II and *Sac*I and

Table 1. Strains used in this study

Strain	Parent	Genotype	Reference or source
SC5314		Wild-type	Gillum <i>et al.</i> (1984)
SC5314-GFP	SC5314	<i>RPS1/RPS1::GFP</i>	Uppuluri <i>et al.</i> (2010)
173	SC5314	<i>ALS3/als3Δ::SAT1-FLIP*</i>	This study
1732	173	<i>ALS3/als3Δ::FRT</i>	This study
17322	173	<i>als3Δ::FRT/als3Δ::SAT1-FLIP</i>	This study
17323	173	<i>als3Δ::FRT/als3Δ::SAT1-FLIP</i>	This study
173221	17322	<i>als3Δ::FRT/als3Δ::FRT</i>	This study
173232	17322	<i>als3Δ::FRT/als3Δ::FRT</i>	This study

**SAT1-FLIP* denotes the *SAT1* flipper cassette.

ligated between the *SacII* and *SacI* sites in pALHFS to form plasmids pASinner and pASouter, respectively. To replace the first copy of *ALS3*, the entire deletion cassette of pASouter was liberated as a *KpnI*–*SacI* fragment and transformed into *C. albicans* strain SC5314 using a modified electroporation transformation method (Köhler *et al.*, 1997). Nourseothricin-resistant transformants were selected on YPD agar plates containing 200 µg nourseothricin ml⁻¹ (Werner Bioagents), as described by Reuss *et al.* (2004). Genomic DNA was prepared from several of the nourseothricin-resistant transformants obtained using the MasterPure Yeast DNA Extraction kit (Epicentre Biotechnologies), digested with *BglII*, transferred to a positively charged Nytran membrane (Whatman) and subjected to Southern blot analysis as described by Church & Gilbert (1984). The LHF PCR product (described above) was labelled with [³²P]dCTP using Ready-To-Go DNA labelling beads (GE Healthcare) and used as a probe. Strains in which the cassette had correctly integrated into one allele of *ALS3* were grown in yeast extract-peptone-maltose to activate the FLP recombinase and remove the deletion cassette. The resulting nourseothricin-sensitive transformants were selected on YPD agar plates containing 20 µg nourseothricin ml⁻¹ (Werner Bioagents), as described by Reuss *et al.* (2004). The transformation and verification process was repeated using the deletion cassette from pASinner to isolate homozygous deletion strains. Two independent homozygous deletion isolates, 17322 and 17323, were subjected to a battery of *in vitro* tests and found to behave identically. Therefore, isolate 17322 was analysed in all of the subsequent experiments.

Quantitative PCR. RNA was isolated from *C. albicans* cells using the MasterPure Yeast RNA Extraction kit (Epicentre Biotechnologies). RNA was treated with amplification grade DNase I (Invitrogen) and used for cDNA synthesis with the Masterscript kit (5 PRIME). The primer pairs (Table 3) were used in conjunction with SYBR Green

PCR Master Mix (Applied Biosystems) and twin.tec real-time 96-well PCR plates (Eppendorf) in an ABI 7300 Real-Time PCR system (Applied Biosystems). Dissociation curves were analysed for all reactions to verify single peaks/products. Expression levels were analysed using ABI 7300 System SDS software (Applied Biosystems).

Flow adhesion assay. The flow adhesion assay was performed as described previously (Grubb *et al.*, 2009) using the immortalized human microvascular endothelial cell line HMEC-1 (Ades *et al.*, 1992) (provided by F. J. Candal, Centers for Disease Control and Prevention, Atlanta, GA, USA). Briefly, HMEC-1 cells were cultured on sterilized glass slides in four-well plates for 72 h until confluent monolayers had formed. Any non-adherent cells were removed by washing with HBSS. The slides were then mounted in a parallel plate flow chamber (GlycoTech) and placed onto a 37 °C stage in an environmental chamber also maintained at 37 °C. *C. albicans* cells at 2 × 10⁶ ml⁻¹ were perfused through the flow chamber and over the endothelial cell monolayers using an automated syringe pump (Harvard Apparatus) for 15 min at a shear stress of 1.5 dynes cm⁻² (0.15 N m⁻²). Adhesion of *C. albicans* to endothelial cells was visualized using an integrated high-resolution digital camera (AxioCam MRm, Zeiss) with AxioVision 4.6 software (Imaging Associates) attached to a Zeiss Axiovert 200M inverted microscope. Images of at least three random fields of view (× 20 objective; area of the field analysed = 0.15 mm²) were taken after 15 min of flow. Each experiment was performed three times and at least three images were recorded per slide. Differences between samples were analysed using Student's *t* test.

Murine virulence assays. For injection, cultures of the *als3Δ* strain and a wild-type strain were grown overnight at 28 °C in YPD. Cells were harvested by centrifugation and washed three times in sterile pyrogen-free saline. Cells were counted using a haemocytometer, and appropriate dilutions were made so that the required dosage of cells could be injected in a final volume of 200 µl into the lateral tail veins of 6–8-week-old female BALB/c mice. Confirmation of the number and viability of cells present in the infecting inocula was performed by plate counts. Groups of five mice were used for each condition. Days on which the animals died were recorded; severely moribund animals were humanely sacrificed to minimize suffering and recorded as having died the following day. In all experiments, one kidney was processed for histopathology, whereas the other kidney, brain and spleen were homogenized and fungal loads determined by plating dilutions onto Sabouraud agar plates. All experiments were performed in accordance with institutional regulations in place at the University of Texas at San Antonio. Mice were allowed a 1 week acclimatization period before experiments were started.

The competition experiment was based on that described by Wu *et al.* (2007) and was performed using the GFP-labelled wild-type

Table 2. Plasmids used in this study

Plasmid	Reference or source
pSFS2	Reuss <i>et al.</i> (2004)
pMT3000	Paget <i>et al.</i> (1994)
pALHF	This study
pARHFouter	This study
pARHFinner	This study
pALHFS	This study
pASouter	This study
pASinner	This study

Table 3. Oligonucleotides used in this study

Underlined sequences indicate restriction enzyme recognition sites engineered into the oligonucleotides.

Oligonucleotide	Sequence	Source or reference
ALS3_LHF_UPS	5'- <u>GGG</u> CCCATGAGCAAACAATCCGAAG-3'	This study
ALS3_LHF_DS	5'- <u>CTCGAG</u> TCGCCCGTGCTTTTGAC-3'	This study
ALS3_RHF_outer_UPS	5'- <u>CCGCGG</u> TGTTGGCTACTAAGTTGAGGTG-3'	This study
ALS3_RHF_outer_DS	5'- <u>GAGCT</u> CACACCGAAACCCTCAATCAC-3'	This study
ALS3_RHF_inner_UPS	5'- <u>CCGCGG</u> CCATCATAGTCGCCTTTTAGA-3'	This study
ALS3_RHF_inner_UPS	5'- <u>GAGCT</u> CCAGCAACATTTTCTTGGACCT-3'	This study
ACT1-S	5'-ATGTGTAAAGCCGGTTTGGCCG-3'	Toyoda <i>et al.</i> (2004)
ACT1-A	5'-CCATATCGTCCCAGTTGGAAAC-3'	Toyoda <i>et al.</i> (2004)
ALS3_FOR	5'-CAACTTGGGTTATTGAAACAAAAACA-3'	Nailis <i>et al.</i> (2006)
ALS3_REV	5'-AGAAACAGAAACCCAAAGAACAACCT-3'	Nailis <i>et al.</i> (2006)
ECE1_FOR	5'-CCAGAAATTGTTGCTCGTGTTG-3'	Bassilana <i>et al.</i> (2005)
ECE1_REV	5'-CAGGACGCCATCAAAAACG-3'	Bassilana <i>et al.</i> (2005)
HWP1_FOR	5'-TCAGCCTGATGACAATCCTC-3'	Cleary <i>et al.</i> (2010)
HWP1_REV	5'-GCTGGAGTTGTTGGCTTTTC-3'	Cleary <i>et al.</i> (2010)
ALS1_FOR	5'-TTCTCATGAATCAGCATCCACAA-3'	Green <i>et al.</i> (2005)
ALS1_REV	5'-CAGAATTTTCACCCATACTTGGTTTC-3'	Green <i>et al.</i> (2005)
ALS5_FOR	5'-CTGCCGGTTATCGTCCATTTA-3'	Green <i>et al.</i> (2005)
ALS5_REV	5'-TTGATACTGGTTATTATCTGAGGGAGAAA-3'	Green <i>et al.</i> (2005)

(SC5314-GFP) and *als3Δ* mutant (173221) strains. Cells were counted using a haemocytometer, and equal numbers from each strain were mixed prior to injection. Groups of five mice were used for each sacrifice time point (3, 6 or 24 h post-injection). To assess the relative proportions of the two strains before and after infection, dilutions of the cells to be injected or homogenized tissues were plated onto Sabouraud agar plates to obtain the total fungal burden. Colonies from these plates were then replica-plated onto YPD agar plates containing 100 µg nourseothricin ml⁻¹ (Werner Bioagents) to identify the proportion of recovered cells that were nourseothricin-resistant (SC5314-GFP).

Histopathology. Kidneys excised from deceased or sacrificed mice were fixed in 10% buffered formalin and stored at 4 °C until required. Kidneys were embedded in paraffin, and tissue slices were cut and stained with Grocott–Gomori methenamine-silver (GMS) (Grocott, 1955) or haematoxylin and eosin (H&E) stain prior to microscopic evaluation.

RESULTS AND DISCUSSION

Phenotypic analysis of the deletion strain

Positional effects which alter *URA3* expression have a profound influence on the virulence of *C. albicans* (Brand *et al.*, 2004; Correia *et al.*, 2010). To avoid this potential pitfall, we used the *SAT1* flipper method to delete both copies of *ALS3* in the wild-type strain SC5314. To verify that the *in vitro* phenotype of our *als3Δ* null strain matched that reported previously, we grew it under hypha-inducing conditions and compared its growth with that of the wild-type parental strain. As expected, the wild-type, heterozygote and deletion strains all formed hyphae under inducing conditions such as growth in RPMI 1640 at 37 °C or on solid Spider medium (data not shown). As

reported for previous *als3Δ* mutants, our null strain was defective in its ability to form a biofilm on an elastomer surface *in vitro* (Fig. 1a) even though it was able to form hyphae. Furthermore, when material was removed from the well inoculated with the wild-type strain and examined microscopically the hyphal cells were densely packed and intertwined, whereas the *als3Δ* hyphae were more loosely associated and spread out in an open network (Fig. 1b). The heterozygote generally resembled the wild-type. These observations were in agreement with the phenotypes reported for deletion strains constructed by other groups using different methodologies (Nobile *et al.*, 2006; Zhao *et al.*, 2004).

To confirm that there was no *ALS3* expression in the mutant, RNA was isolated from filamentous cultures and used for quantitative real-time PCR analysis. This showed that hyphae formed by the null strain lacked *ALS3* transcript, while in the wild-type strain, *ALS3* was abundant (Fig. 2). An intermediate amount of *ALS3* transcript was present in the heterozygote. We also examined the expression of two other hypha-specific genes, *ECE1* and *HWP1*. Transcription of these genes in the mutant strain was not markedly different from that seen in the wild-type strain or the *ALS3* heterozygote, thus confirming that, except for an absence of *ALS3*, the hyphae formed by the null strain induced other hyphal genes as expected (Fig. 2). It has been shown elsewhere that Als1p and Als3p have some functional redundancy during biofilm formation in an intravenous rat catheter model (Nobile *et al.*, 2008), while Als1p, Als3p and Als5p all contain amino acid sequences predicted to allow amyloid formation (Otoo *et al.*, 2008). Since it was possible that deletion of *ALS3* resulted in compensatory elevated

Fig. 1. Biofilm formation of the *als3Δ* null strain. *C. albicans* strains were incubated with silicone squares in Spider medium at 37 °C for 48 h. The wild-type parental strain (SC5314) and the *ALS3/als3Δ* heterozygote (173) formed a biofilm on the silicone square, whereas the *als3Δ* null strain (17322) grew as a mixture of loose cells and smaller clumps floating in the medium (a). Hyphae formed by the wild-type strain were densely packed and intertwined, but the *als3Δ* hyphae appeared to be loosely associated and formed an open network (b). Hyphae of the heterozygote appeared less tangled and clumped than those of the wild-type strain. These experiments were repeated three times with similar results.

expression of other ALS family members, particularly *ALS1* and *ALS5*, we examined transcription of these two genes in the *ALS3/als3Δ* heterozygote and the *als3Δ* null strain. There was some variation in the expression of *ALS1* and *ALS5* between the different replicate hyphal cultures but there was clearly no trend of *ALS1* or *ALS5* transcriptional upregulation to compensate for decreasing levels of *ALS3* transcript in the heterozygote and *als3Δ* null strains

(Fig. 2). This suggests that the expression of these genes is not upregulated to compensate for the loss of Als3p.

Adhesion to endothelial cells under conditions of flow

Adhesion of *C. albicans* to both itself and host cells is critical for the establishment of biofilms and fungal

Fig. 2. Quantitative real-time PCR analysis. RNA was isolated from cells grown in RPMI 1640 at 37 °C. Light-grey bars, transcript levels in the wild-type strain; hatched bars, levels in the heterozygote; dark-grey bars, levels in the *als3Δ* mutant. Levels were normalized by *ACT1* and expressed relative to the wild-type control; therefore, the results for the latter are set at 1.0. The experiment was carried out in triplicate and error bars represent SEM. The asterisk indicates that no *ALS3* transcript was detected in the deletion strain.

infection. We previously tested the influence of morphology on the ability of *C. albicans* to adhere to human endothelial cells under flow, and observed that yeast cells adhere in greater numbers to these host cells than hyphae or pseudohyphae (Grubb *et al.*, 2009), consistent with our results showing that yeast cells are able to escape the bloodstream and invade deep tissues (Saville *et al.*, 2003). We used microvascular-derived endothelial cells (HMEC-1) in our study because it is the microvascular endothelium at which most adhesion and extravasation occur *in vivo* (Fiebig *et al.*, 1991). During a disseminated infection, *Candida* cells are most likely to encounter cell surface molecules expressed by the microvascular endothelium, and so we felt that this model represented the best system to analyse *C. albicans* adherence. Since Als3p is not found on the surface of yeast cells (Coleman *et al.*, 2009), it was not expected that a lack of Als3p would affect the ability of the cells to adhere to microvascular endothelial cells under flow. This proved to be the case (Fig. 3), with no significant difference in the adhesion of wild-type and *als3Δ* mutant yeast cells ($P=0.172$). The abundance of Als3p in the hyphal cell wall (Coleman *et al.*, 2009), and its importance for the formation of normal biofilms *in vitro* (Nobile *et al.*, 2006; Zhao *et al.*, 2006), suggested that cells lacking Als3p might be deficient in their ability to adhere to the endothelial cells. However, in our flow model, the hyphae of the *als3Δ* strain adhered as well as their wild-type counterparts ($P=0.251$), although in both strains hyphae adhered less well than yeast cells (Fig. 3). These results reinforce our previous observations that yeast cells adhere more efficiently than hyphae to endothelial cells.

Fig. 3. Adhesion under conditions of flow. *C. albicans* cells were perfused through a parallel plate flow chamber over glass slides coated with confluent HMEC-1 monolayers. Adhesion of *C. albicans* to the microvascular endothelial cells was visualized using an inverted fluorescence microscope with an integrated high-resolution digital camera. The area of the analysed field was 0.15 mm². Results are presented as the total number of adherent cells per square millimetre. The experiment was carried out in triplicate and error bars represent SEM.

Virulence of the *als3Δ* null strain in a murine model of systemic candidiasis

The role of Als3p in virulence has been examined using a variety of *in vitro* methods. Strains lacking Als3p have been found to be impaired in their ability to damage different cell types grown in culture (Almeida *et al.*, 2008; Zhao *et al.*, 2004). Furthermore, *als3Δ* strains have been reported to be deficient in ferritin binding *in vitro* (Almeida *et al.*, 2008) and to show decreased endocytosis by human cells (Phan *et al.*, 2007). The results of these assays have prompted some researchers to suggest that Als3p plays a key role in *C. albicans* virulence. However, direct examination of the virulence of a strain lacking Als3p *in vivo* using the systemic infection model has yet to be reported. We therefore tested our *als3Δ* null strain in the commonly used murine model of haematogenously disseminated candidiasis. Contrary to the implications of earlier studies, deletion of *ALS3* did not impair the virulence of the mutant strain compared with its wild-type parent (Fig. 4a). This does not rule out the possibility that Als3p plays a role in the damage caused to host cells, as previous *in vitro* tests suggest, but it is clearly not a major determinant in the outcome of a systemic infection.

To assess the phenotype of the strain growing within the tissues, we performed a histological analysis of infected kidneys retrieved from the deceased mice. The mutant strain was able to form abundant hyphae within the kidneys (Fig. 4b) and showed no deficiency in its ability to invade or to damage host tissue. Moreover, neutrophil infiltration was readily apparent in tissues infected with either the wild-type or the *als3Δ* strain (Fig. 4c). In agreement with recent results using a neonatal intraperitoneal infection model (Tsai *et al.*, 2011), we found that infections with the wild-type or mutant strain resulted in efficient dissemination, with similar fungal burdens in the brain, kidney and spleen (data not shown). After earlier studies showing that yeast-form cells can disseminate, and our finding that *als3Δ* yeast or hyphae can adhere to endothelial cells under flow as efficiently as a wild-type strain, it was perhaps not surprising to find abundant *C. albicans* cells in the kidneys. However, if as previously reported, *als3Δ* cells are deficient for ferritin assimilation *in vivo*, this does not appear to impair their ability to proliferate within host tissues.

In order to examine the kinetics of the infection process more closely, we performed a competition assay. Equal numbers of wild-type and *als3Δ* null yeast cells were injected into mice, and the proportions of each strain residing in the kidney, spleen and brain were measured at 3, 6 and 24 h post-infection. The relative proportions of the wild-type and *als3Δ* cells recovered from the organs matched those present in the infecting inoculum (Fig. 5). Only the 24 h spleen sample showed a significant deviation from the inoculum ($P<0.05$), with a greater proportion of *als3Δ* cells being recovered from the infected tissues. These results reinforce our observations that the *als3Δ* strain is

Fig. 4. Virulence of the *als3Δ* null strain. (a) Approximately 2.8×10^5 *C. albicans* yeast cells of the wild-type SC5314 and 2.7×10^5 yeast cells of the *als3Δ* strain (left panel) or 3.7×10^5 *C. albicans* yeast cells of the wild-type SC5314 and 3.2×10^5 yeast cells of the *als3Δ* strain (right panel) were injected into the lateral tail veins of 6–8-week-old female BALB/c mice. Deletion of *ALS3* did not change the virulence properties of the parental wild-type strain using this model ($P=0.696$ or $P=0.570$). Examination of kidneys retrieved from infected mice revealed (b) extensive *C. albicans* growth containing hyphal cells in infections by both the wild-type control and the *als3Δ* deletion strain (GMS staining), and (c) infiltration of numerous neutrophils (H&E staining).

equally able to disseminate from the bloodstream as a wild-type strain and is equally able to cause disease.

Several earlier studies have examined the interactions of strains lacking Als3p with human cells *in vitro* and found that *als3Δ* null strains are defective in their ability to invade and damage reconstituted tissue culture models and cell monolayers (Almeida *et al.*, 2008; Zhao *et al.*, 2004). However, our results indicate that Als3p is not required for *C. albicans* cells to leave the bloodstream, invade the deep organs or cause a lethal disease in mice. The fact that the

survival curves produced by the wild-type and the *als3Δ* mutant strains are indistinguishable suggests that Als3p plays a much more subtle role in pathogenesis than might be inferred from studies focused on the ability of fungal cells to damage human cells in a static environment. Interestingly, Als3p and several other cell wall proteins have recently been shown not to be required for *C. albicans* to be taken up by murine macrophages *in vitro*, nor does a lack of Als3p impair the ability of the fungus to kill these macrophages (McKenzie *et al.*, 2010). Examining the various models and conditions used to characterize the

Fig. 5. Competitive fitness of the *als3Δ* null strain. (a) A total inoculum of 5.7×10^6 *C. albicans* yeast cells (3 and 6 h groups) or 2.5×10^6 yeast cells (24 h group) was injected into the lateral tail veins of 6–8-week-old female BALB/c mice for timed sacrifice competition experiments. Results are presented as the mean proportion of recovered cells that were nourseothricin-resistant. Inocula consisted of roughly equal proportions of wild-type (light-grey bars) and *als3Δ* (dark-grey bars) cells. Cells recovered from homogenized tissues were plated onto Sabouraud agar plates to obtain the total fungal burden. Colonies ($n=849$ – 1788) from these plates were then replicated onto YPD agar plates containing $100 \mu\text{g}$ nourseothricin ml^{-1} to identify the proportion that were resistant. Error bars, SD. Significance was assessed by the chi-squared test. A *P* value of <0.05 was considered significant. (b) In each group, fungal burden is clustered around the median. Overall fungal burden in the organs was similar between time points, with the exception of the 24 h kidney sample. However, this sample still fell within the range of burdens seen at the same time point in previous experiments (Cleary *et al.*, 2010).

role of Als3p, it is apparent that this protein is important in *C. albicans* adhesion; however, it is equally clear that its role is not general in nature but particular to specific types of adhesion. Under flow conditions, it appears that Als3p is not required for *C. albicans* hyphae to bind to endothelial cells, but it is vital for the formation of normal biofilms in which hyphae intertwine in a complex 3D structure (Zhao *et al.*, 2006). Therefore, it seems likely that the most important role for Als3p is in the adhesion of *C. albicans* cells to other *C. albicans* cells, particularly during biofilm

formation. In fact, it has recently been suggested that cell wall adhesins may have evolved from a mating agglutination system (Nobile *et al.*, 2008; Soll, 2008) and that biofilm formation by white cells in response to α -pheromone is an important component of *C. albicans* mating (Daniels *et al.*, 2006; Sahni *et al.*, 2009, 2010). *C. albicans* can inhabit many niches in the host, and thus encounters a variety of cell types and environmental conditions. Whilst *in vitro* models recapitulating some of these conditions have implied a broad role for Als3p in *C.*

albicans virulence, our results indicate that its role may be more specific than general, since the deletion strain showed no defect in virulence in the widely used haematogenously disseminated murine infection model.

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