Candida albicans suppression of neutrophil reactive nitrogen species is rescued by host Hif-1α *in vivo*

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Abstract

Candida spp. cause 750,000 cases per annum of invasive disease worldwide, with up to a 50% mortality rate. Poor efficacy of current antifungals, lack of vaccines and rising antifungal resistance rates point towards an urgent need to develop new therapies. *Candida albicans* is a human commensal fungus that can cause life-threatening invasive infection in immunocompromised individuals. *C. albicans* is able to manipulate host macrophages and neutrophils to escape phagosomal killing and has previously been shown to suppress reactive nitrogen species (RNS) production *in vitro*. However, the effects of *C. albicans* on RNS *in vivo* and the molecular and cellular mechanisms involved remain unclear. Using a zebrafish model, we aimed to characterise RNS suppression by *C. albicans in vivo*. We demonstrate that *C. albicans* suppressed neutrophil RNS both proximally and distally to the infection site in a partially active process, with heat-killed *C. albicans* not reducing RNS to the same extent as live fungi. Using a *car1*Δ mutant, we show that fungal arginase is partially responsible for the reduction in neutrophil RNS. Stabilisation of Hif-1α, a transcription factor with a key role in immune regulation, rescued neutrophil RNS production during *C. albicans* infection, leading to improved infection outcomes. The protective effect of Hif-1α stabilisation was neutrophil- and nitric oxide synthase-dependent. Together, these data demonstrate that Hif-1α stabilisation can restore the neutrophil RNS response in *C. albicans* infection, leading to improved infection outcomes, highlighting the potential of targeting Hif-1α and RNS in host directed therapies against fungal infections.

Introduction

Candida albicans is a commensal fungus, colonising 70% of healthy individuals (Witherden *et al.*, 2017), which can cause superficial mucosal infections with negligible mortality. However, *C. albicans* can also act as an opportunistic pathogen capable of causing life-threatening, invasive disease in immunocompromised individuals and neonates (Witherden *et al.*, 2017; Flores-Maldonado *et al.*, 2019; Pellon, Sadeghi Nasab and Moyes, 2020). *Candida* species are the 4th most common nosocomial infection in the USA and

account for up to 22% of health-care-associated bloodstream infections (Pfaller and Diekema, 2010; Magill *et al.*, 2014). There are approximately 750,000 cases of severe candidiasis worldwide per year and the estimated mortality rate is around 50% (Bongomin *et al.*, 2017; WHO, 2022). This high mortality, combined with the lack of fungal vaccines and rising rates of antifungal resistance, has resulted in *C. albicans* being designated as a critical priority fungal pathogen, with urgent research into new alternative therapies required (WHO, 2022).

C. albicans infections are initially detected by pattern recognition receptors, including Toll-like receptors, C-type lectin receptors and the Rig-I like receptor, MDA-5, resulting in a downstream pro-inflammatory response and innate immune cell recruitment (reviewed in Burgess, Condliffe and Elks, 2022). Although many immune cell types are recruited to the sites of *C. albicans* infection, neutrophils are one of the most potent killers of *C. albicans* and neutropenic patients are particularly at risk of invasive disease (Uzun *et al.*, 2001; Brown, 2011). Candidalysin, produced by hyphal *C. albicans*, is a particularly strong stimulator of CXCL1-mediated neutrophil recruitment (Drummond *et al.*, 2019). Following recruitment, neutrophils have multiple mechanisms of eliminating *C. albicans*, including the production of reactive oxygen (ROS) and nitrogen (RNS) species (Winterbourn *et al.*, 2006). Inhibition of inducible nitric oxide synthase (iNOS) leads to increased mortality in mouse models of *C. albicans* infections, showing the importance of RNS mediated fungicidal activity (Tillmann, Gow and Brown, 2011; Wagener *et al.*, 2017; Navarathna, Lionakis and Roberts, 2019).

C. albicans has a variety of mechanisms that can subvert the host innate immune response (Luo *et al.*, 2013). Additionally, *C. albicans'* ability to rapidly adapt to changes in environmental pH, metabolic flexibility and a robust stress response allows colonisation of a wide range of niches within the host (Mayer, Wilson and Hube, 2013). The morphotype yeast-to-hyphal transition is essential for host tissue invasion and limits innate immune sensing of *C. albicans* via masking of β-glucan (Gantner, Simmons and Underhill, 2005; van der Graaf *et al.*, 2005). *C. albicans* can suppress ROS and RNS production by macrophages *in vitro* (Wellington, Dolan and Krysan, 2009) with an, as yet, unidentified *C. albicans*

secreted compound responsible for RNS inhibition in murine macrophages (Collette, Zhou and Lorenz, 2014). However, there is currently a lack of *in vivo* observations on the effects of *C. albicans* on RNS and little is known about the molecular and cellular mechanisms underlying RNS suppression and whether it is possible to rescue this with potential therapeutic application.

Hypoxia inducible factor (HIF) proteins are important regulators of cellular responses to hypoxia, innate immunity and inflammation (McGettrick and O'Neill, 2020). In hypoxic conditions, HIF-1α proteins are stabilised, allowing entry into the nucleus, where they form heterodimers with HIF-1β. Consequent transcription of target genes regulate cellular metabolism, proliferation, migration, differentiation, angiogenesis and pro-inflammatory mediators (Krzywinska and Stockmann, 2018; Hammond, Lewis and Elks, 2020). HIF-1α stabilisation is associated with increased neutrophil survival, via NF-κB and promotes neutrophil extracellular trap formation (Walmsley *et al.*, 2005; McInturff *et al.*, 2012). We have previously shown that stabilisation of Hif-1α increases neutrophil RNS output, which is protective against *Mycobacterium marinum* infection *in vivo* (Elks *et al.*, 2013). Therefore, Hif-1α stabilisation represents a potential host directed therapy to target neutrophil RNS during fungal infections, such as *C. albicans*.

Zebrafish are a highly tractable *in vivo* model system to investigate the behaviours of innate immune cells during infection (Torraca and Mostowy, 2018). Zebrafish larvae are transparent, allowing the use of innate immune transgenic lines to directly observe neutrophil behaviours and interactions with pathogens (Lam *et al.*, 2004; Renshaw *et al.*, 2006). Adaptive immunity does not mature until 4 to 6 weeks post fertilisation, whereas innate immune cells are present from early larval stages, allowing examination of innate immune responses to infections without the presence of adaptive immunity (Lam *et al.*, 2004). Several infection sites have been used in zebrafish to model *C. albicans* infection, including localised infection into the hindbrain ventricle and systemic infection via injection into the caudal vein, allowing neutrophil behaviours to be observed to better our understanding of

host-*C. albicans* interactions in an *in vivo* context (Bergeron *et al.*, 2017; Gratacap *et al.*, 2017; Seman *et al.*, 2018).

Here, we show that *C. albicans* infection suppressed neutrophil RNS in zebrafish. RNS suppression by *C. albicans* was shown to occur both locally and distally to the infection site, and was a partially active process, with heat-killed *C. albicans* having a reduced effect on neutrophil RNS. Fungal arginase (*car1*) plays a role in RNS reduction, with a *car1*Δ mutant having a reduced effect on neutrophil RNS. Furthermore, we show that it is possible to restore host RNS production via Hif-1α stabilisation, enhancing host control of *C. albicans* infection. Together, this work highlights Hif-1α and RNS as potential targets for host directed therapies against invasive *C. albicans* infections.

Methods

Ethics Statement

Zebrafish were handled in accordance with Animals (Scientific Procedures) Act 1986, under Project Licenses P1A4A7A5E and PP7684817. Ethical approval was granted by the University of Sheffield Local Ethical Review Panel.

Zebrafish

Zebrafish were maintained in Home Office approved facilities in Biological Services Unit (BSU) aquaria at the University of Sheffield, in accordance with standard protocols and local animal welfare regulations. Adult fish were maintained at 28°C with a 14/10 hour light/dark cycle. Embryos were maintained at 28 °C with a 14/10 hour light/dark cycle in 1X E3 medium (E3 60X stock: 5.0 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$; diluted to 1X in distilled water) with 10-5% Methylene Blue (Sigma-Aldrich). Methylene Blue was added to prevent fungal growth. The following zebrafish strains were used: nacre (wild type), *Tg(mpx:GFP)i114* (Renshaw *et al.*, 2006), *Tg(lyz:nfsB.mCherry)sh260* (Buchan *et al.*, 2019), Tg(*mpeg:nlsClover*)sh436 (Bernut *et al.*, 2019).

Fungal Strains

Several *C. albicans* strains were used in this study (Table 1). *C. albicans* was initially grown on YPD (4001022, MP Bio) plates at 28°C for 48 hours, then transferred to overnight liquid culture in YPD broth in a shaking incubator at 30°C at 200 rpm.

Table 1: List of *C. albicans* strains used in this study

Strain	Genotype	Reference
***C. albicans* TT21-dTomato**	*ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434::URA3-tetO ENO1/eno1::ENO1 tetR-ScHAP4AD-3XHA-ADE2* pENO1-dTomato-NATR	(Seman *et al.*, 2018)
***C. albicans* SN148 GFP**	SN148 *ENO1/ENO1-GFP::LEU2*	(Christou, Ayscough and Johnston, 2023)
***C. albicans* SC5314**	wt; clinical blood isolate	(Gillum, Tsay and Kirsch, 1984)
C. albicans car1Δ	As SC5314 but *car1Δcar1Δ*	(Schaefer *et al.*, 2020)

RNA Injections

Embryos were injected with dominant active *hif-1αb* variant RNA (DA1) or dominant negative *hif-1αb* variant RNA at the one-cell stage, as previously described (Elks *et al.*, 2011). Phenol Red (PR; Sigma-Aldrich) was used as a vehicle control.

Morpholino Injections

A *csf3r* morpholino (Gene Tools) was used to deplete neutrophil populations. A standard control morpholino (Gene Tools) used as a negative control. The antisense morpholino oligonucleotide sequences were: *csf3r* – 5'-GAAGCACAAGCGAGACGGATGCCAT-3'; control – 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

F0 CRISPR-Cas9 Knockdown (CRISPant)

Two sgRNAs were designed to target the ATG/first exon of *nos2a* and *nos2b* to knockdown iNOS and RNS production. Efficacy of knockdown of RNS production was functionally validated by anti-nitrotyrosine staining. Tyrosinase (*tyr*) sgRNA was used as a negative control (Isles *et al.*, 2019). The target sequences were: *nos2a* – 5'-TTTCTCATTTTCAATGATAG-3'; *nos2b* – 5'-GTTCGCTCTTGTGAGTGACC-3'; *tyr* – 5'-CCTGACCTCCTGAAGACCCC-3'. 25µM sgRNA was co-injected with tracrRNA, 1:2 diluted Cas9 and phenol red.

C. albicans Infections

Overnight *C. albicans* cultures were washed 3 times in phosphate buffered saline (PBS) and counted on a haemocytometer. Washed cultures were resuspended in 10% PVP (polyvinylpyrrolidone; Calbiochem) to achieve the desired inoculation. Infection dose for systemic caudal vein infections were 100 colony forming units (cfu) or 200 cfu. Infection dose for localised hindbrain infections was 20 cfu. *C. albicans* was heat-killed by incubation in a heat block at 65°C for 1 hour (Panpetch *et al.*, 2017). To denature fungal proteins, *C. albicans* was boiled at 105°C for 5 minutes.

For survival curves, zebrafish survival was monitored daily and dead zebrafish were removed from the Petri dish. Death was determined by a lack of heartbeat/circulation or tissue degradation post mortem. At 4 days post infection (dpi; equivalent to 5 days post fertilisation, dpf), all surviving zebrafish larvae were culled.

Anti-nitrotyrosine Staining

1 dpi embryos were fixed in 4% paraformaldehyde in PBS overnight in 4°C. Nitrotyrosine levels were labelled using a rabbit polyclonal anti-nitrotyrosine antibody (06-284; Merck Millipore) and were detected using Alexa-633 conjugated goat-anti-rabbit secondary antibody (Invitrogen Life Technologies) imaged using line-scanning confocal microscopy, as previously described (Elks *et al.*, 2013, 2014).

Microscopy and Quantification of Anti-nitrotyrosine

For *C. albicans* SC5314 and *car1*Δ*,* brightfield images were taken using an inverted Leica DMi8 with a 40x lens and a Hamamatsu OrcaV4 camera. Stained embryos were imaged on a Leica DMi8 inverted microscope with a Leica TCS-SPE line-scanning confocal for imaging with a 40x lens. For quantification purposes, acquisition settings were kept the same across the groups. Corrected fluorescence intensity was calculated using Image J measurements assessing the cell fluorescence of individual cells corrected for cell size and background fluorescence of the image (Elks *et al.*, 2013, 2014).

Statistical Analysis

All data were analysed using GraphPad Prism 9.5.1 1 (GraphPad Software, La Jolla, California, USA, www.graphpad.com). Nitrotyrosine staining data was analysed with Kruskal-Wallis test, with Dunn's multiple comparisons test. Nitrotyrosine staining data for proximal and distal RNS levels was analysed with 2-way ANOVA, with Sidák's multiple comparison test. Survival curves were analysed with Gehan-Breslow-Wilcoxon test, with Bonferroni correction. The p values shown are: ns=not significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$

Results

C. albicans infection suppresses neutrophil RNS production

C. albicans has previously been demonstrated to modulate host innate immune responses, by suppressing production of ROS and RNS by macrophages *in vitro* (Wellington, Dolan and Krysan, 2009; Collette, Zhou and Lorenz, 2014). In zebrafish, we have previously shown that RNS, identified using an anti-nitrotyrosine antibody, is predominantly produced in neutrophils, both in resting/mock infected individuals (Elks *et al.*, 2014) and to an elevated level after bacterial infection with *Mycobacterium marinum* (Elks *et al.*, 2013). We aimed to establish if *C. albicans* is capable of modulating neutrophil RNS in an *in vivo* zebrafish model. 1 day post fertilisation (dpf) zebrafish larvae were infected in the caudal vein with either live *C. albicans*, heat killed *C. albicans* or a mock infected (PVP) control. At 24 hours post infection (hpi), zebrafish larvae were fixed and stained with an anti-nitrotyrosine antibody as a measure of host neutrophil RNS production. In mock infected (PVP) controls there was a basal level of anti-nitrotyrosine levels in neutrophils (Elks *et al.*, 2013, 2014). Compared to the PVP control, *C. albicans* TT21-dTomato caused a 90.0% ($p<0.0001$) reduction in the corrected fluorescence intensity of nitrotyrosine staining (Figure 1A-B). Heat-killed *C. albicans* TT21-dTomato had an intermediate level of RNS suppression, between PVP (PVP=5.17, heat-killed *C. albicans* TT21-dTomato=2.47; $p<0.0001$) and live *C. albicans* infection (*C. albicans* TT21-dTomato=0.52, heat-killed *C. albicans* TT21-dTomato=2.47; $p<0.0001$). Similar phenotypes of RNS suppression were observed in infection with live and heat-killed *C. albicans* SN148 GFP, suggesting the effect is not strain specific (Figure S1). Hence, we used these fluorescence strains interchangeably, depending on the colour combinations required for each experiment. Together these data show that *C. albicans* suppresses neutrophil RNS production *in vivo*, while heat-killed *C. albicans* is capable of generating an intermediate level of RNS suppression.

Suppression of neutrophil RNS by *C. albicans* occurs both proximally and distally to the infection site

To determine whether neutrophils with reduced RNS need to be proximal to *C. albicans* infection, a local hindbrain ventricle *C. albicans* infection was performed, with RNS production measured both at the site of infection in the hindbrain and a distal, uninfected site in the caudal haematopoietic tissue (CHT). *C. albicans* caused a significant decrease in nitrotyrosine levels in both proximal neutrophils in the hindbrain (PVP=2.38, *C.* albicans=0.52; $p<0.0001$) and distal neutrophils in the CHT (PVP=2.59, *C.* albicans=0.71; $p<.0001$) compared to PVP control (Figure 2A-C). Hence, live *C. albicans* is capable of causing both a proximal and distal suppression of neutrophil RNS, suggestive of the influence of a secreted factor. Heat-killed *C. albicans* caused an intermediate RNS reduction in the hindbrain but had no effect on RNS in distal neutrophils in the CHT region, suggesting that multiple mechanisms may reduce neutrophil RNS that differ between proximal and distal regulation. Boiled *C. albicans* had no effect on neutrophil RNS at either location, implying that a secreted factor regulating neutrophil RNS may be a protein (that does not function once denatured). Together, these results suggest that *C. albicans* may have multiple mechanisms of neutrophil RNS suppression, some which act locally and others which act more distally.

***C. albicans* arginase, CAR1, plays a role in the suppression of neutrophil RNS**

Host RNS levels are controlled by a balance of two enzymes that share the substrate L-arginine, iNOS and arginase. iNOS is the enzyme responsible for RNS production and arginase, shunts the metabolism of L-arginine away from RNS towards L-ornithine production (Bansal and Ochoa, 2003). Fungi are known to produce enzymes with similar functions in nitric oxide production, for example C. albicans produces its own version of arginase, *CAR1* (Schaefer *et al.*, 2020). We therefore hypothesised that *C. albicans* arginase interferes with the host arginase-iNOS axis, resulting in neutrophil RNS suppression. 1 dpf zebrafish were infected with *C. albicans car1*Δ or its parental strain *C. albicans* SC5314 and

RNS production was measured at 24 hpi by anti-nitrotyrosine staining. *C. albicans car1*Δ infection decreased neutrophil RNS production compared to PVP ($p<0.0001$) (Figure 3A-B). The RNS produced after live *C. albicans car1*Δ infection was significantly different from live *C. albicans* SC5314 ($p<0.05$), heat-killed *C. albicans* SC5314 ($p<0.0001$) and heat-killed *C. albicans car1*Δ ($p<0.0001$), Heat-killed *C. abicans car1*Δ was not significantly different from heat-killed *C. albicans* SC5314 ($p>0.9999$), suggesting an active component is responsible for differences observed in live *C. albicans*. *C. albicans car1*Δ caused an intermediate level of RNS suppression between live *C. albicans* SC5314 and heat-killed *C. albicans* (Figure 3A-B), indicating that *CAR1* plays a partial role in suppression of neutrophil RNS.

Hif-1α is protective against *C. albicans* infection in a neutrophil-dependent manner

Hif-1α is a transcription factor with known roles in regulation of innate immunity (McGettrick and O'Neill, 2020) and has previously been shown to be protective against *Mycobacterium marinum* infection *in vivo* by increasing neutrophil RNS levels (Elks *et al.*, 2013; Hammond, Lewis and Elks, 2020). We aimed to stabilise Hif-1α in *C. albicans* infected zebrafish embryos, in order to restore innate immune responses and increase host survival in *C. albicans* infection.

1 cell stage zebrafish embryos were injected with phenol red (solvent control, PR), dominant negative *hif-1α* (DN1) or dominant active *hif-1α* (DA1) RNA, then infected systemically with *C. albicans* TT21-dTomato infection at 30 hpf. Mortality was measured over the following 4 days. DA1 injected embryos had significantly greater survival across 4 days than both PR ($p<0.01$) and DN1 ($p<0.001$) injected larvae (Figure 4A-B), suggesting Hif-1α stabilisation is host protective in *C. albicans* infection.

Neutrophils play an essential role in innate immune control of invasive candidiasis (Desai and Lionakis, 2018). Brothers *et al.* showed neutrophils are responsible for killing of *C. albicans*, whereas macrophages internalised, but did not kill, *C. albicans in vivo* (Brothers *et al.*, 2013). Therefore, we hypothesised that the protective effect of Hif-1α stabilisation was

mediated by neutrophils. To assess the impact of Hif-1α stabilisation on macrophage-mediated immunity, we depleted the macrophage population by injection of clodronate liposomes (van Rooijen and Hendrikx, 2010) at 30 hpf or PBS liposomes as a negative control, leading to a significant depletion of the macrophage population (Figure S3). 1 cell stage embryos were injected with DA1 or PR control. At 30 hpf, embryos were injected into the caudal vein with clodronate liposomes or PBS liposomes followed by infection with 100cfu *C. albicans* TT21-dTomato at 2 dpf. Both groups of PBS liposome injected larvae had greater survival than clodronate liposome, macrophage depleted, siblings. DA1 was protective in both the presence or depletion of macrophages, with clodronate liposome larvae having greater survival than PR clodronate liposome embryos (Figure 4C; $p<0.0001$), indicating that the host-protective effect of Hif-1α stabilisation is not dependent on the presence of macrophages.

Next, we aimed to establish if neutrophils were responsible for the protective effect of Hif-1α stabilisation in *C. albicans* infection. We injected 1 cell stage zebrafish embryos with a *csf3r* morpholino (Ellett *et al.*, 2011) to deplete neutrophil numbers in 2 dpf zebrafish larvae (Figure S4). *Csf3r* morpholino injection led to a significant decrease in survival of 100cfu *C. albicans* infected larvae, in both PR and DA1 injected fish (Fig 4D). DA1 + *csf3r* larvae had lower survival compared to PR + *csf3r* larvae, showing a loss of the protective effect of Hif-1α stabilisation in neutrophil ablated larvae (Figure 4D) and demonstrating that the protective effect of Hif-1α stabilisation is neutrophil dependent.

Host protective effect of Hif-1α is RNS-dependent

Hif-1α stabilisation has previously been shown to increase RNS production in zebrafish neutrophils (Elks *et al.*, 2013). We hypothesised that the host protective effect of Hif-1α stabilisation in *C. albicans* infection was due to increased neutrophil RNS production. Therefore, we investigated the effect of dominant active *hif-1α* (DA1) on neutrophil behaviours during *C. albicans* infection. DA1 did not alter the recruitment of neutrophils to the site of a local *C. albicans* infection compared to phenol red (PR) injected controls (Figure

S5), implying a change in neutrophil behaviour, not neutrophil numbers, is responsible for the protective effect of Hif-1α stabilisation. In anti-nitrotyrosine stained larvae, DA1/PVP caused a doubling in nitrotyrosine levels compared to PR/PVP, mock injected larvae (Figure 5A-B; PR/PVP=16.36, DA1/PVP=33.25; $p<0.0001$) as previously described (Elks *et al.*, 2013). While *C. albicans* reduced nitrotyrosine levels in PR control injected embryos, DA1 injection restored nitrotyrosine levels in *C. albicans* infected larvae to comparable levels of uninfected larvae (Fig 5A-B).

To demonstrate that this restoration of RNS in Hif-1α stabilisation is responsible for the host protective effect in *C. albicans* infection, we inhibited production of RNS pharmacologically, using L-NIL, and genetically, using *nos2a+b* (zebrafish iNOS) double CRISPR-Cas9 knockdown ("CRISPants"; Moore *et al.*, 1994; Isles *et al.*, 2019). L-NIL treatment did not impact the growth of *C. albicans* outside of the host *in vitro* (Figure S6). PR + L-NIL larvae had reduced survival compared to PR + dH_2O (drug solvent control) treated larvae (Figure 5C), demonstrating the importance of RNS in *C. albicans* control. DA1 + L-NIL treated larvae did not have a significant survival advantage compared to either PR + dH_2O or PR + L-NIL groups (Figure 5C), indicative of iNOS inhibition removing the host protective effect of Hif-1α stabilisation. *Nos2ab* double CRISPants were shown to inhibit nitrotyrosine production in PVP injected zebrafish (Figure S7). DA1 with *nos2ab* CRISPants no longer had increased survival compared to PR + *tyr* sgRNA (*tyrosinase* CRISPant control; Isles *et al.*, 2019) larvae, supporting that RNS production by *nos2* is responsible for the host protective effect of Hif-1α stabilisation (Figure 5D). Together these data demonstrate that the Hif-1α stabilisation is host-protective via a neutrophil- (Fig 4) and RNS-dependent (Fig 5) mechanism.

Discussion

The increasing prevalence of antifungal resistance worldwide highlights the importance of discovering new antifungal targets or host directed therapies capable of improving disease outcomes. Here, we have shown that Hif-1α stabilisation represents a

potential host directed therapy capable of restoring the neutrophil RNS response to *C. albicans* infection.

We first demonstrated that *C. albicans* can suppress neutrophil RNS production *in vivo*. Collette *et al.* previously showed *C. albicans* suppresses RNS production in murine bone marrow derived macrophages *in vitro* (Collette, Zhou and Lorenz, 2014), which is supported by a variety of sources showing suppression of ROS by *C. albicans in vitro* (Frohner *et al.*, 2009; Wellington, Dolan and Krysan, 2009). While previous publications have demonstrated RNS suppression in macrophages, our data demonstrates an effect in neutrophils. Human neutrophils produce higher levels of RNS than macrophages (Muijsers *et al.*, 2001), as is the case in zebrafish larvae, suggesting our findings may reflect the human RNS response to *C. albicans* making zebrafish a useful *in vivo* model to investigate the immune response in these difficult to study *ex vivo*, short-lived cells.

We showed *C. albicans* suppression of neutrophil RNS occurs both proximally at the site of infection and also at distal sites. Investigations using *in vitro* models have been unable to fully answer questions about host-*C. albicans* interactions in the context of a whole organism. Previous work has suggested direct contact between *C. albicans* and macrophages is required for RNS suppression and that an unidentified secreted factor plays a role (Collette, Zhou and Lorenz, 2014). Our findings do not preclude a suppression effect from direct contact between *C. albicans* and neutrophils, but suggest that direct contact is not a requirement and that suppression involves the secretion of a factor, allowing suppression of distal neutrophil RNS. Our *in vivo* model suggests that *C. albicans* has a secreted mechanism of RNS suppression which is capable of having effects large distances (millimetres) across an organism. This raises new questions about the role of *C. albicans* in the development of polymicrobial coinfections in immune compromised patients. This secreted factor is likely to be a protein as boiling *C. albicans*, resulting in denaturation of proteins, inhibits the distal suppression of neutrophil RNS. Pathogen-specific secreted compounds are a rich source for potential biomarkers of infection (Stastna and Van Eyk, 2012). Further research into the specific molecule responsible for suppressing neutrophil

RNS distally is required to determine its suitability to act as a biomarker of *C. albicans* infection. The intermediate phenotype of RNS suppression by heat-killed *C. albicans* hints at multiple mechanisms of neutrophil RNS suppression by *C. albicans*. Current literature supports the idea of multiple mechanisms of suppression. *C. albicans* SOD5 has been associated with degradation of antimicrobial ROS *in vitro* (Frohner *et al.*, 2009), *C. albicans* chitin induces host arginase-1 activity, decreasing RNS production (Wagener *et al.*, 2017), *YHB1* is able to detoxify RNS (Hromatka, Noble and Johnson, 2005) and Collette *et al.* identified an unknown, small secreted molecule capable of suppressing RNS (Collette, Zhou and Lorenz, 2014). The diversity of proteins and genes associated with decreased RNS production correlates with our hypothesis that multiple mechanisms of RNS suppression exist. Further investigation could aim to establish which, if any, mechanism contributes the most to host RNS suppression, and if any mechanisms can be targeted to downregulate the suppressive effect. Alternative explanations may be that heat-killing is partially denaturing fungal proteins, reducing activity of the hypothesised secreted protein, or that heat-killing is damaging the fungal cell wall, resulting in exposure of chitin, which is known to decrease RNS production (Wagener *et al.*, 2017).

Here, we identify that *C. albicans* arginase is one such mechanism involved in the suppression of host RNS production. *CAR1* is the only known arginase gene in the *C. albicans* genome, encoding a cytosolic arginase (Schaefer *et al.*, 2020). Fungal arginase may be interfering with the host arginase-iNOS balance, resulting in reduced iNOS activity and RNS suppression. The modest decrease in neutrophil RNS production after infection with the *CAR1* mutant suggests that *C. albicans* arginase production may not be the predominant RNS reducing mechanism, but additive strategies of neutrophil RNS suppression by *C. albicans* may underlie their success at the large overall suppression of neutrophil RNS observed locally and distally.

We have demonstrated that Hif-1α stabilisation has a protective effect in *C. albicans* infection in zebrafish embryos, via a neutrophil-mediated, RNS-dependent mechanism. Neutrophils are the primary innate immune cell responsible for killing *Candida spp.* (Brown,

2011), supporting our conclusion that neutrophil suppression is an important strategy of *C. albicans* during infection that, if rescued, could represent an exciting therapeutic opportunity. RNS has well characterised, highly candidacidal effects (Rementería, García-Tobalina and Sevilla, 1995; Vazquez-Torres *et al.*, 1995; Tillmann, Gow and Brown, 2011; Navarathna, Lionakis and Roberts, 2019). Similarly, Macherla *et al.* revealed NO-releasing nanoparticles inhibit the growth of *C. albicans in vitro* and *in vivo* (Macherla *et al.*, 2012). Hence, upregulation of neutrophil RNS production, following Hif-1α stabilisation, is a potential mechanism that could be targeted therapeutically to improve immune candidacidal activity. We have previously demonstrated Hif-1α stabilisation protects against *M. marinum* infection in a similar manner (Elks *et al.*, 2013), suggesting Hif-1α stabilisation could offer protective effect against multiple and different groups of pathogens (fungi and bacteria) where neutrophil-mediated immune responses are subverted.

Our data indicates that Hif-1α stabilisation is able to overcome *C. albicans* suppression of neutrophil RNS, conferring a neutrophil-mediated, nitric oxide-dependent protective effect *in vivo*. Hence, Hif-1α stabilisation has potential as a host directed therapeutic strategy to aid in the treatment of *C. albicans* infections, following further validation in human systems. We also begin to determine the mechanisms underlying RNS suppression by *C. albicans*. Identification of specific proteins, genes or compounds responsible for RNS suppression could point towards new potential antifungal targets or prospective biomarkers to aid the diagnosis and treatment of *C. albicans* infections.

Figures and Legends

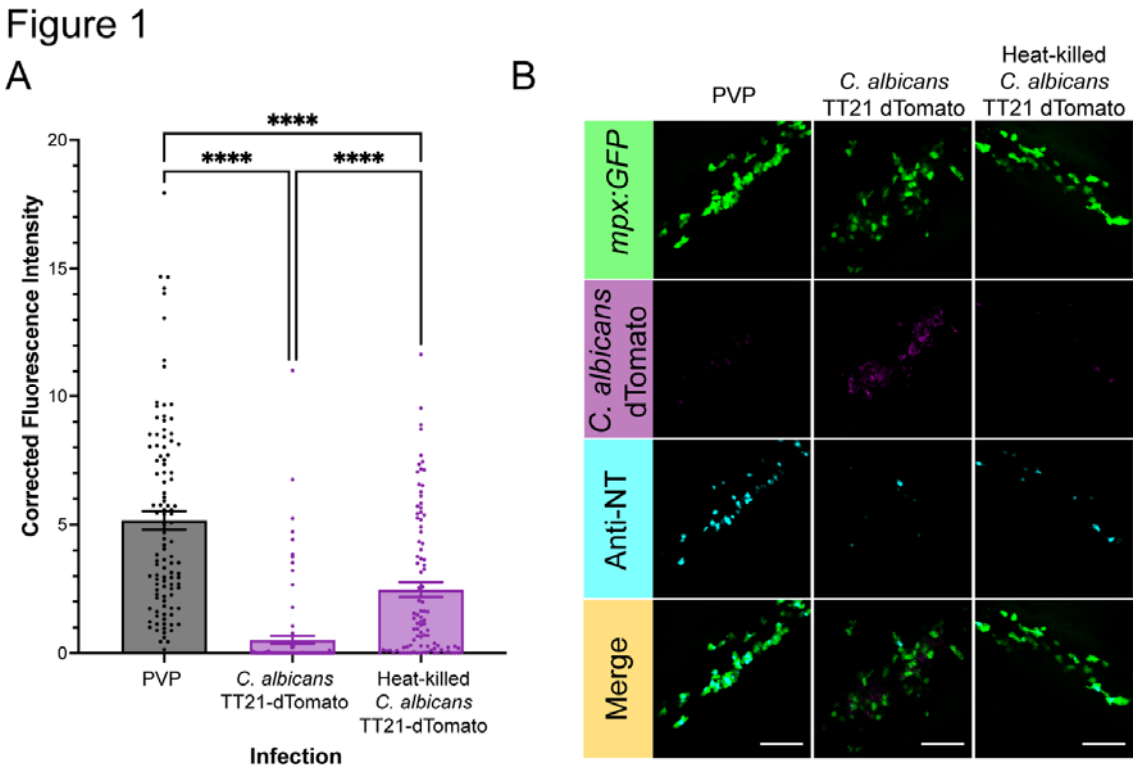

Figure 1: *C. albicans* suppressed neutrophil RNS production *in vivo*.

(A) Anti-nitrotyrosine fluorescence at 24 hpi following injection of PVP, live *C. albicans* TT21-dTomato or heat-killed *C. albicans* TT21-dTomato into the caudal vein at 30 hpf. N=96-108 neutrophils from 16-18 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, then Dunn's multiple comparisons test. ****$p<0.0001$.

(B) Representative images of PVP, *C. albicans* TT21-dTomato and heat-killed *C. albicans* TT21-dTomato infected zebrafish embryos at 24 hpi. Scale bars = 50 µm.

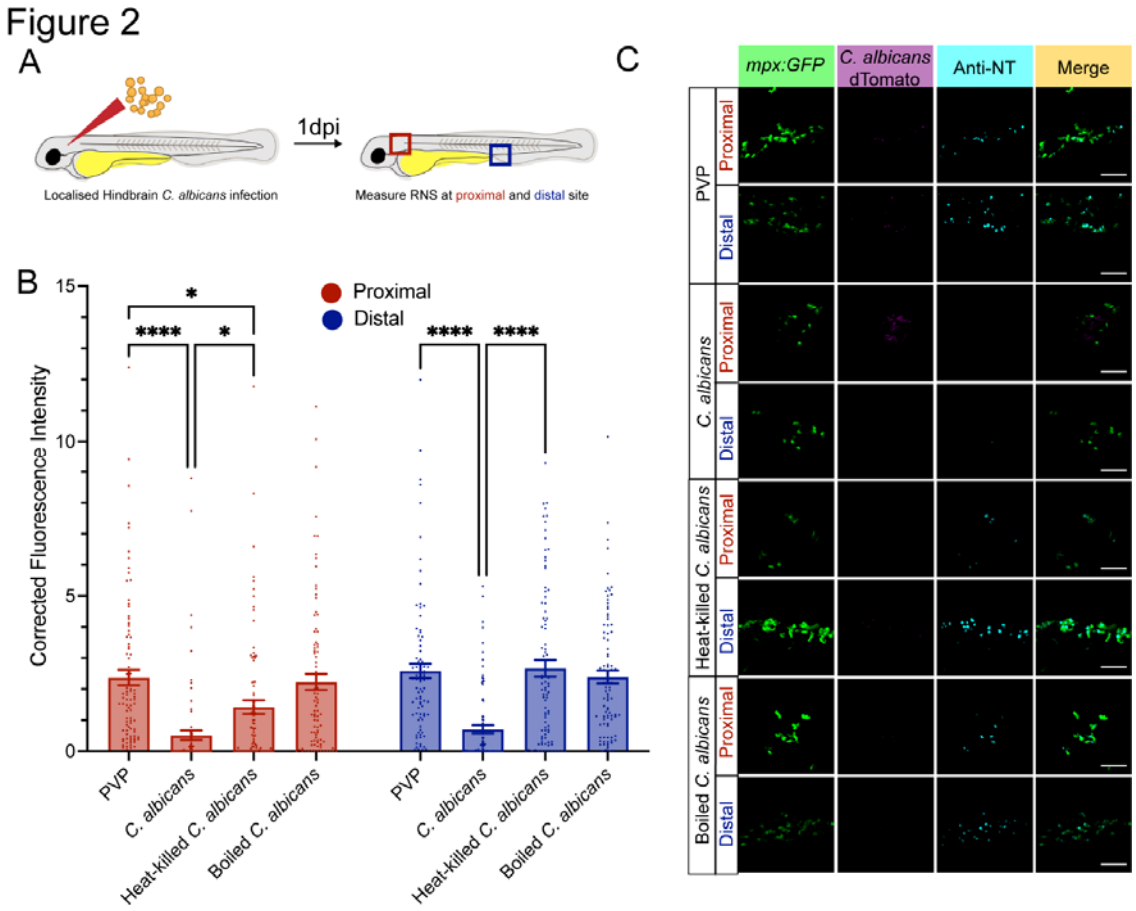

Figure 2: *C. albicans* suppression of RNS occurs proximally and distally to site of infection.

(A) 1 dpf zebrafish were injected with PVP, *C. albicans,* heat-killed *C. albicans* or boiled *C. albicans*, into the hindbrain ventricle. At 24 hpi, embryos were fixed and stained with anti-nitrotyrosine primary antibody and goat-anti-rabbit Alexa-633 secondary antibody. Zebrafish were then imaged at the hindbrain and CHT regions and anti-nitrotyrosine fluorescence was quantified.

(B) Anti-nitrotyrosine fluorescence at 24 hpi following injection of PVP, *C. albicans,* heat-killed *C. albicans* or boiled *C. albicans* into the hindbrain ventricle. N=90 neutrophils from 15 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by 2-way ANOVA, then Sidák's multiple comparisons test. ns=not significant, *p<0.05, ****p<0.0001

(C) Representative images of anti-nitrotyrosine stained 24 hpi zebrafish embryos at proximal and distal sites.

Figure 3

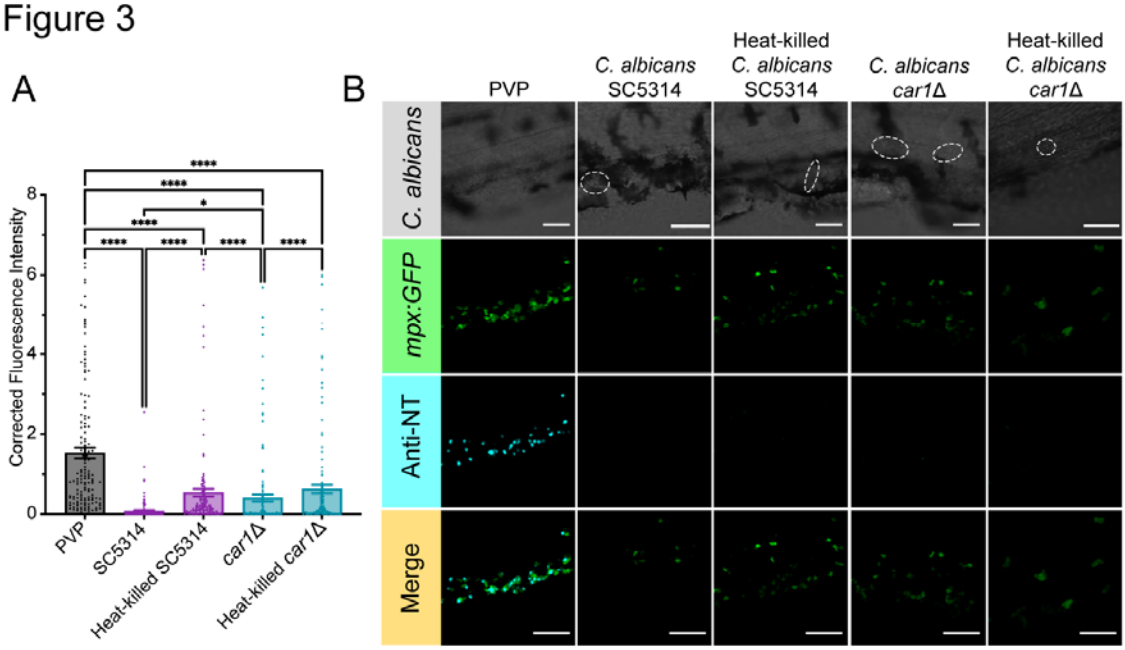

Figure 3: *C. albicans car1*Δ has a role in suppression of neutrophil RNS.

(A) Anti-nitrotyrosine fluorescence at 24 hpi following injection of PVP, *C. albicans* SC5314, heat-killed *C. albicans* SC5314, *C. albicans car1*Δ or heat-killed *C. albicans car1*Δ into the caudal vein. N=144 neutrophils from 24 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, with Dunn's multiple comparisons tests. *p<0.05, ****p<0.0001.

(B) Representative images of anti-nitrotyrosine stained embryos. Dashed lines show *C. albicans*. Scale bars = 50 µm.

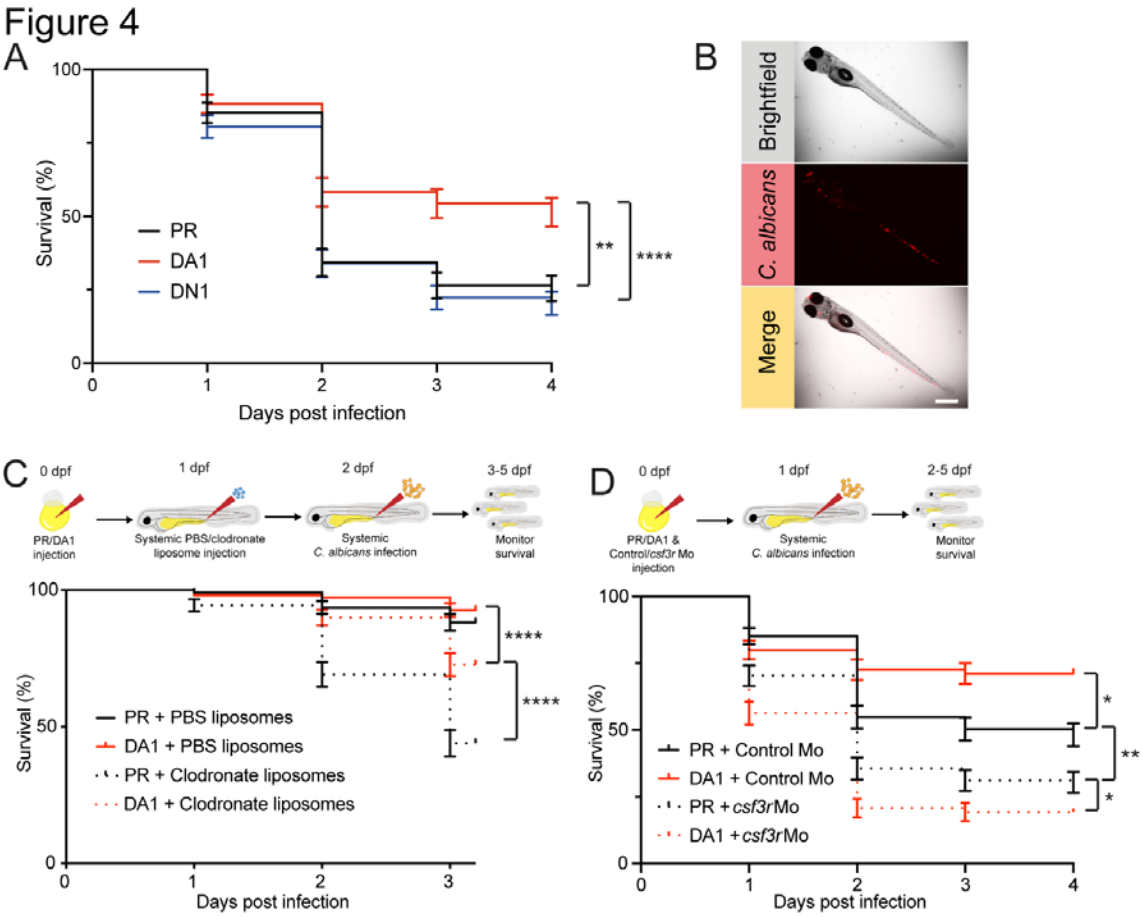

Figure 4: Hif-1α stabilisation is protective against *C. albicans* infection.

(A) Survival of *C. albicans* TT21-dTomato infected zebrafish embryos following injection with dominant active *hif-1α* (DA1), dominant negative *hif-1α* (DN1) RNA or phenol red (PR) solvent control. Mortality was measured daily. N=102-103 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test with Bonferroni correction. **p<0.01, ****p<0.0001.

(B) Representative image of 200 cfu *C. albicans* TT21-dTomato infected zebrafish embryo at 24 hpi. Scale bar = 1000 µm.

(C) Survival curve of 100 cfu *C. albicans* TT21-dTomato infected zebrafish embryos following injection with DA1 or PR, then treatment with PBS or clodronate liposomes. Mortality was measured daily. N=102-103 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test with Bonferroni correction. ****p<0.0001.

(D) Survival curve of 100 cfu *C. albicans* TT21-dTomato infected zebrafish embryos following injection with PR or DA1 and control or *csf3r* morpholino. Mortality was measured daily. N=135 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test with Bonferroni correction. *p<0.05, **p<0.01.

Figure 5

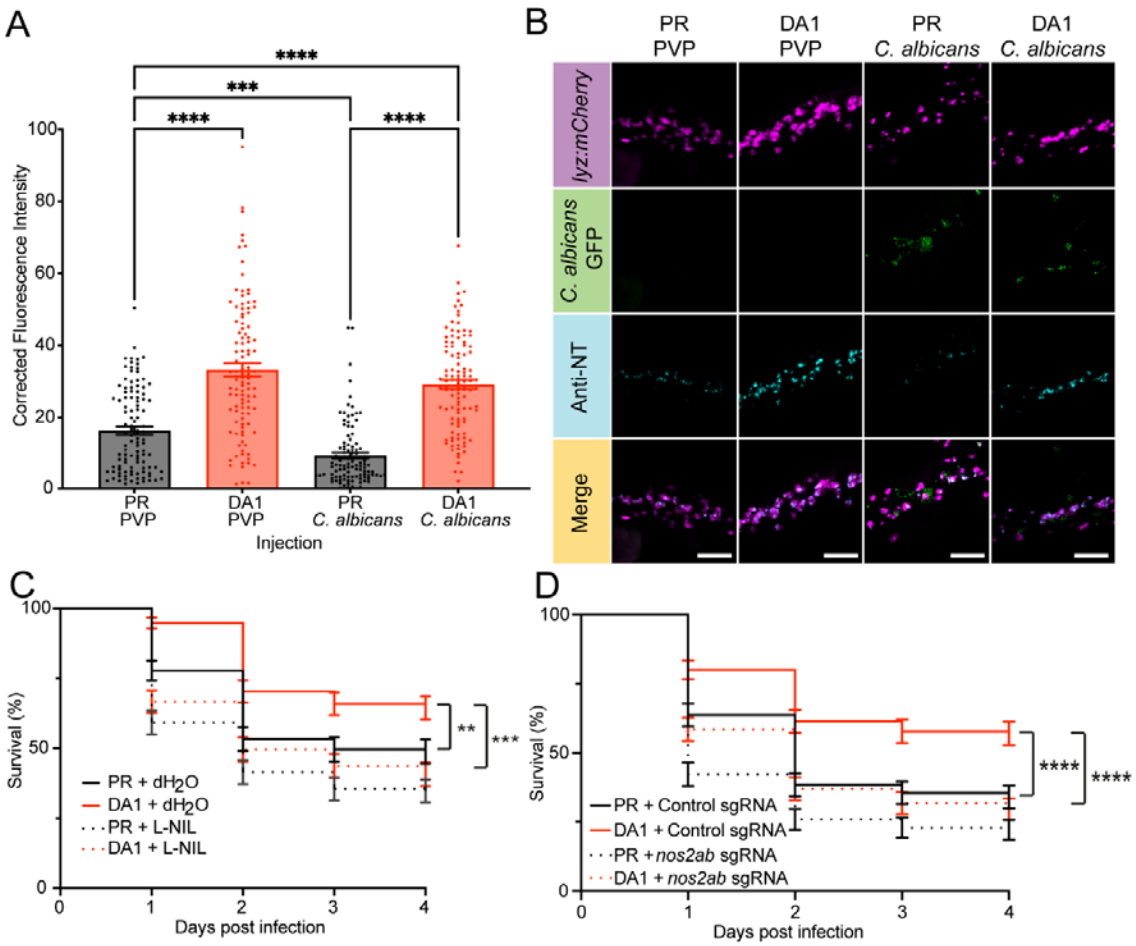

Figure 5: The host protective effect of Hif-1α stabilisation is RNS-dependent.

(A) Anti-nitrotyrosine fluorescence at 24 hpi in PR and DA1 injected fish, following injection of PVP or 500 cfu *C. albicans* SN148 GFP. N=108 neutrophils from 18 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by

Kruskal-Wallis test, with Dunn's multiple comparisons test. ns=not significant, ***p<0.001, ****p<0.0001

(B) Representative images of 1 dpi Tg(*lyz:NTRmCherry*) zebrafish embryos injected with PR or DA1 and infected with 500 cfu *C. albicans* SN148 GFP or PVP. Scale bars = 50 µm.

(C) Survival curve of 200 cfu *C. albicans* TT21-dTomato infected zebrafish embryos following injection with PR or DA1, then treatment with dH_2O or L-NIL. N=135 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. **p<0.01, ***p<0.001.

(D) Survival curve of 200 cfu *C. albicans* TT21-dTomato infected zebrafish embryos following injection with PR or DA1 and *tyr* or *nos2ab* sgRNA. N=135 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. **p<0.01, ***p<0.001.

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Author Contributions

Conceived and designed the experiments: TBB, FRH, PTS, AL, SC, AMC, PME. Performed the experiments: TBB, FRH, PTS, AL. Generation of Candida strains: SC, KRA, SAJ. Analysed the data: TBB, FRH, PTS, AL, AMC, PME. Wrote and drafted the manuscript: TBB, FRH, PTS, AL, AMC, PME.

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Competing Interests

The authors declare that they have no conflict of interest.

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