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Candida spp. suppress neutrophil reactive nitrogen species to evade killing

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ABSTRACT *Candida albicans* is a human commensal that can cause life-threatening invasive infection in immunocompromised individuals. Human immunity to *C. albicans* infection is thought to be largely dependent on neutrophil reactive oxygen and nitrogen species (ROS/RNS) generation by neutrophils. Despite this, our understanding of innate immune killing and escape by *C. albicans* is primarily studied in macrophages, and the precise mechanisms of evasion are unclear in neutrophils. Here, we sought to determine the importance of neutrophil reactive nitrogen species (RNS) production during *C. albicans* infection *in vivo*. Using a zebrafish model, we found that *C. albicans* rapidly downregulated neutrophil RNS below basal levels during the first day post-infection, a time at which neutrophil RNS is upregulated in bacterial infections as an important host-defense mechanism, indicating fungal evasion of host neutrophils. We confirmed the downregulation of RNS in human primary neutrophils and with clinical *Candida* isolates, including emerging human pathogens *Candida auris* and *Candida glabrata*. Inducible nitric oxide synthase (iNOS; Nos2 in zebrafish), the enzyme responsible for RNS production, competes with the arginase enzyme for a shared substrate, L-arginine. Using a zebrafish *arginase2* transgenic line and a *C. albicans car1*Δ mutant, we showed that both host and fungal arginase contribute to the reduction in neutrophil RNS. Despite pathogen downregulation, upregulation of neutrophil RNS via hypoxia-inducible factor 1α (Hif-1α) stabilization was sufficient to improve host survival following *C. albicans* infection. Inhibition of Nos2 blocked the host protective effect of Hif-1α stabilization. Finally, restoration of neutrophil RNS via Hif-1α stabilization was additive to clinically relevant antifungal treatment, increasing survival and clearance of *C. albicans* infections. Together, these data demonstrate that restoration of the neutrophil RNS response in *C. albicans* infection improves infection outcomes, highlighting the potential of targeting Hif-1α and RNS in host-directed therapies against fungal infections.

IMPORTANCE *Candida albicans* is a fungus that normally lives harmlessly in the human body but can cause life-threatening infections in people with weakened immune systems. A key part of the body's defense against this fungus is neutrophils, immune cells that kill microbes using toxic molecules. However, how *Candida* avoids neutrophil defense is not well understood. Here, we used zebrafish and human immune cells to show that *Candida* suppresses an important neutrophil defense, reactive nitrogen species (RNS), during infection. Unlike bacteria, which trigger RNS, *Candida* reduces these protective molecules to below normal levels, helping its survival. This effect was also observed with other disease-causing *Candida* species. We went on to show that both the host and *Candida* contribute to this suppression. Importantly, boosting the neutrophil response improved survival and helped clear infection, especially when combined with standard antifungal drugs. These findings suggest new ways to support the immune system alongside existing treatments.

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Candida albicans (*C. albicans*) is a commensal fungus, colonizing 70% of healthy individuals (1) and can cause superficial mucosal infections with negligible mortality. However, *C. albicans* can act as an opportunistic pathogen capable of causing life-threatening, invasive disease in immunocompromised individuals (1–3). There are approximately 750,000 cases of severe candidiasis worldwide per year, and the estimated mortality rate is high, estimated to be around 50% (4, 5), likely an underestimation due to poor surveillance mechanisms worldwide (6, 7). This high mortality, combined with a lack of fungal vaccines and rising rates of antifungal resistance, has resulted in *C. albicans* being designated as one of four critical priority fungal pathogens by the World Health Organization, with another *Candida* species, the emerging human pathogen *Candida auris*, also a critical priority pathogen (5). Hence, urgent research into new therapies is required.

C. albicans is 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 (8). 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*, with neutropenic patients being particularly at risk of invasive disease (9, 10). Following recruitment, neutrophils have multiple mechanisms of eliminating *C. albicans*, including the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (11). Pharmacological inhibition of nitric oxide synthase (NOS) leads to increased mortality in *C. albicans* infections in mice *in vivo* and reduced candidacidal activity in murine macrophages and peritoneal cells *in vitro*, showing the importance of RNS-mediated fungicidal activity (12–15). iNOS, the enzyme responsible for RNS production, competes with the arginase enzyme for a shared substrate, L-arginine. Arginase can competitively inhibit RNS production by iNOS in leukocytes (16). *C. albicans* is able to subvert the host innate immune response, including ROS and RNS (17). Despite *Candida* spp. being primarily controlled by neutrophils in human disease, most immune evasion studies have focused on murine macrophage cells. This is, in part, because neutrophils are a more challenging cell type to study *in vitro* due to their short lifespan and ease of accidental activation. *C. albicans* has been shown to suppress ROS in murine macrophages and neutrophils and RNS production by macrophages *in vitro* (18, 19). Culture supernatant also suppressed macrophage RNS, although to a lower extent, implying secreted compounds are partially involved in RNS suppression (19). How *Candida* spp. evade neutrophil RNS is a major knowledge gap, critical to understanding the pathogenesis of human disease. However, to overcome challenges associated with studying neutrophils *in vitro*, we require *in vivo* animal models to understand the neutrophil response in intact tissues.

Zebrafish are a highly tractable *in vivo* model system to investigate the behaviors of innate immune cells during infection (20–23). Adaptive immunity does not mature until 4–6 weeks post-fertilization, whereas innate immune cells are present from early larval stages, allowing examination of innate immune responses to infections without the presence of adaptive immunity (22). Zebrafish models of *C. albicans* infection are well-established, with a dose-dependent effect on zebrafish mortality (24), and have been used to uncover important disease mechanisms (25–27). Furthermore, *C. albicans* is able to undergo the yeast-to-hyphae transition in zebrafish larvae, demonstrating a comparable pathogenesis to human infection (24, 28–30).

Stimulation of RNS has the potential to be a host-directed therapy to *C. albicans* infection that would complement existing antifungals. We have previously shown that hypoxia-inducible factor 1α (HIF-1α) increases neutrophil RNS output, which is protective against the bacterial *Mycobacterium marinum* infection in zebrafish (31). HIF proteins are important regulators of cellular responses to hypoxia, innate immunity, and inflammation (32), triggering transcription of target genes that regulate cellular metabolism, proliferation, migration, differentiation, angiogenesis, and pro-inflammatory mediators

(33–36). Therefore, we set out to address whether HIF-1α stabilization may be a potential therapeutic mechanism that can enhance neutrophil RNS during *C. albicans* infections.

Here, we show that *C. albicans* infection suppressed neutrophil RNS in zebrafish larvae, with a similar dampening effect in human neutrophils. The effect was observed with clinical strains and other *Candida* species, with the ability of strains to reduce neutrophil RNS positively correlated with virulence *in vivo*. Upregulation of host RNS production via Hif-1α stabilization enhanced host control of *C. albicans* infection, and this was additive with conventional antifungals. Together, these data highlight RNS and Hif-1α as potential targets for host-directed therapies against *C. albicans* infections.

MATERIALS AND METHODS

Zebrafish husbandry

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 h light/dark cycle. Larvae were maintained at 28°C with a 14/10 h light/dark cycle in 1× 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 1× in distilled water) with 0.001% Methylene Blue (Sigma-Aldrich) to prevent fungal growth in the first 24 h. From 24 hpf, larvae were kept in E3 without Methylene Blue so that *Candida* species infection was not impacted. The following zebrafish strains were used: nacre (wild type), *Tg(mpx:GFP)i114* (23), *Tg(lyz:nfsB.mCherry)sh260* (37), *Tg(mpeg:nlsClover)*sh436 (38), *TgBAC(arg2:GFP)sh571* (39), and *Tg(phd3:GFP)i144* (40).

Candida species culture

C. albicans strains (Table 1) and *Candida* species clinical isolates (Table 2) were used in this study. *C. albicans* was initially grown on YPD (4001022, MP Bio) plates at 28°C for 48 h and then transferred to overnight liquid culture in YPD broth in a shaking incubator at 30°C at 200 rpm. *C. albicans* was heat-killed by incubation in a heat block at 65°C for 1 h (41). To denature fungal proteins, *C. albicans* was boiled at 100°C for 5 min.

Zebrafish infection

Overnight liquid *C. albicans* cultures were washed three times in phosphate-buffered saline (PBS) and counted on a hemocytometer. Washed cultures were resuspended in 10% polyvinylpyrrolidone (PVP; Calbiochem) to achieve the desired inoculation. Infection dose for systemic caudal vein infections was 200 colony-forming units (cfu) in a 1 nL injection volume, 100 cfu in 1 nL for experiments with immune cell ablation, or 500 cfu in 1 nL in experiments with antifungals. Dosing was performed to achieve around 50% of zebrafish death by 4 days post-infection (dpi; equivalent to 5 days post-fertilization, dpf) (Fig. S1A available at https://doi.org/10.15131/shef.data.32630970), and injected cfu were validated by plating (Fig. S1B available at https://doi.org/10.15131/shef.data.32630970).

TABLE 1 List of *C. albicans* strains

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	(29)
C. albicans SN148 GFP	SN148 *ENO1/ENO1-GFP::LEU2*	(30)
C. albicans SC5314	wt; clinical blood isolate	(42)
*C. albicans car1*Δ	As SC5314 but *car1*Δ*car1*Δ	(43)
C. albicans NRG1^{OEX}*-dTomato*	e2::hisG/ade2::hisG *ura3::imm434/ura3::imm434::URA3-tetO-NRG1 ENO1/eno1*::ENO1 tetR-ScHAP4AD-3XHA-ADE2 pENO1-dTomato-NATR	(29)
*C. albicans apm4*Δ/Δ	As SN148 but *apm4*Δ*apm4*Δ	(30, 44)

TABLE 2 List of *Candida* species clinical isolates

Species	Name	From invasive or non-invasive disease	Sample site	Source
Candida albicans	AJP4	Non-invasive	Vaginal sample	Sheffield Teaching Hospital's Trust
Candida albicans	AJP5	Invasive	Blood sample	Sheffield Teaching Hospital's Trust
Candida albicans	AJP9	Non-invasive	Tongue swab	Sheffield Teaching Hospital's Trust
Candida albicans	AJP25	Invasive	Line tip sample	Sheffield Teaching Hospital's Trust
Candida glabrata	AJP12	Invasive	Urine sample	Sheffield Teaching Hospital's Trust
Candida parapsilosis	AJP22	Invasive	Urine sample	Sheffield Teaching Hospital's Trust
Candida guilliermondii	AJP24	Non-invasive	Tracheal swab	Sheffield Teaching Hospital's Trust
Candida auris	StG2	Invasive	Bloodstream	City St George's University of London

For survival curves, zebrafish survival was monitored daily, and dead zebrafish were removed. Death was determined by a cessation of heartbeat/circulation or tissue degradation post-mortem. At 4 dpi, all surviving zebrafish larvae were culled.

For *Staphylococcus aureus* experiments, 100 cfu of strain SH1000 was injected into the caudal vein in a 1 nL injection volume, as previously described (45).

RNA injections

Larvae were injected with dominant active *hif-1αb* variant RNA (DA *hif-1α*) or dominant-negative *hif-1αb* variant (DN *hif-1α*) RNA in dH_2O with 10% phenol red (Sigma-Aldrich, to visualize successful injection; 1 nL total injection volume) at the one-cell stage, as previously described (46); 10% (vol/vol) phenol red in dH_2O (PR; Sigma-Aldrich) was used as a solvent control.

Morpholino injections

A *csf3r* morpholino (Gene Tools) was used to deplete neutrophil populations (47). A standard control morpholino (Gene Tools) was used as a negative control. The antisense morpholino oligonucleotide sequences were as follows: *csf3r*, 5′-GAAGCACAAGCGAGAC GGATGCCAT-3′, and control, 5′-CCTCTTACCTCAGTTACAATTTATA-3′. *csf3r* morpholino was functionally validated by whole-body neutrophil count at 2 dpf.

F0 CRISPR-Cas9 knockdown

Two sgRNAs were designed to target the ATG/first exon of *nos2a* and *nos2b* to knock down zebrafish *nos2* genes by CRISPR-Cas9. Efficacy of knockdown of RNS production was functionally validated by whole-body anti-nitrotyrosine staining. Tyrosinase (*tyr*) sgRNA, targeting a pigment gene that plays no role in neutrophil RNS production, was used as a negative control and to demonstrate Cas9 function (48). The target sequences were as follows: *nos2a*, 5′-TTTCTCATTTTCAATGATAG-3′; *nos2b*, 5′-GTTCGCTCTTGTGAGTG ACC-3′; and *tyr*, 5′-CCTGACCTCCTGAAGACCCC-3′.

In total, 25 µM sgRNA was co-injected with tracrRNA, Cas9 protein, and phenol red in a total injection volume of 1 nL.

Pharmacological treatment of zebrafish larvae

For pharmacological inhibition of nitric oxide synthase, larvae were treated with 200 µM N6-(1-iminoethyl)-lysine (L-NIL) by immersion. dH_2O was used as a solvent control (31). For pharmacological stabilization of Hif-α, larvae were treated with 5.0 µM FG4592 (Roxadustat, Selleckchem) by immersion. DMSO was used as a solvent control (49).

For antifungal survival curves and clearance assays, larvae were treated with either 5.0 µg/mL fluconazole in DMSO or 1.0 µg/mL caspofungin in dH_2O by immersion. DMSO or dH_2O was used as a solvent control. At 2 dpi, larvae were transferred into fresh E3, and treatment was re-administered.

Anti-nitrotyrosine staining

One day post-infection, larvae (2 dpf) were fixed in 4% formaldehyde in PBS overnight at 4°C. Whole-body nitrotyrosine levels were labeled 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), as previously described (31, 50) in a T*g(mpx:GFP)i114* background to assess for neutrophil-specific RNS.

Microscopy and quantification of anti-nitrotyrosine staining

For *C. albicans* SC5314 and *car1*Δ, brightfield images were taken using an inverted Leica DMi8 with a 40× lens and a Hamamatsu OrcaV4 camera. Stained larvae were imaged on a Leica DMi8 inverted microscope with a Leica TCS-SPE line-scanning confocal for imaging with a 40 × 1.1 NA water immersion lens. For quantification purposes, acquisition settings were kept the same across the groups. Corrected fluorescence intensity was calculated using FIJI measurements assessing the cell fluorescence of individual neutrophils corrected for cell size and background fluorescence of the image (31, 50, 51).

Hyphal staging

For assessment of fungal growth of *Candida* species clinical isolates, which lack fluorescent proteins, brightfield images were taken using an inverted Leica DMi8 with a 40x lens and a Hamamatsu OrcaV4 camera. Classification of hyphal stages was done manually, with the experimenter blinded to experimental groups, based on a previously described descriptive criteria (52). Criteria for hyphal staging were as follows.

1. Yeast: only spherical, yeast cells were observed. No signs of any hyphal growth.
2. Germinating: ovoid cells, resembling pseudohyphae or very short hyphae, were observed.
3. Hyphal extension: a small number of short- or medium-length hyphae were observed.
4. Destructive hyphae: multiple invasive hyphae were observed. The majority of observed *C. albicans* was in hyphal morphotype.

Human neutrophils

Human neutrophils were isolated by Plasma-Percoll density gradient centrifugation of whole blood from healthy donors as previously described (53); 0.5×10^6 cells were added to individual wells in 96-well plates in 100 µL volumes. Cells were primed with 1 µg/mL lipopolysaccharides (LPS) from *Escherichia coli* (Sigma, L8274). Neutrophils were infected with *Candida albicans* at an MOI of 1.0 or 0.5, with heat-killed *C. albicans* at an MOI of or 1.0, left uninfected (sterile RPMI media), and were incubated at 37°C, 5% CO_2 for 3 h. Cells were cytocentrifuged onto microscope slides and fixed in 4% formaldehyde for staining. Immunostaining was performed as described above.

Microscopy of *C. albicans* infection clearance

Brightfield images were taken using an inverted Leica DMi8 with a 2.5× lens and a Hamamatsu OrcaV4 camera. Surviving fish were classified as "infection cleared" only

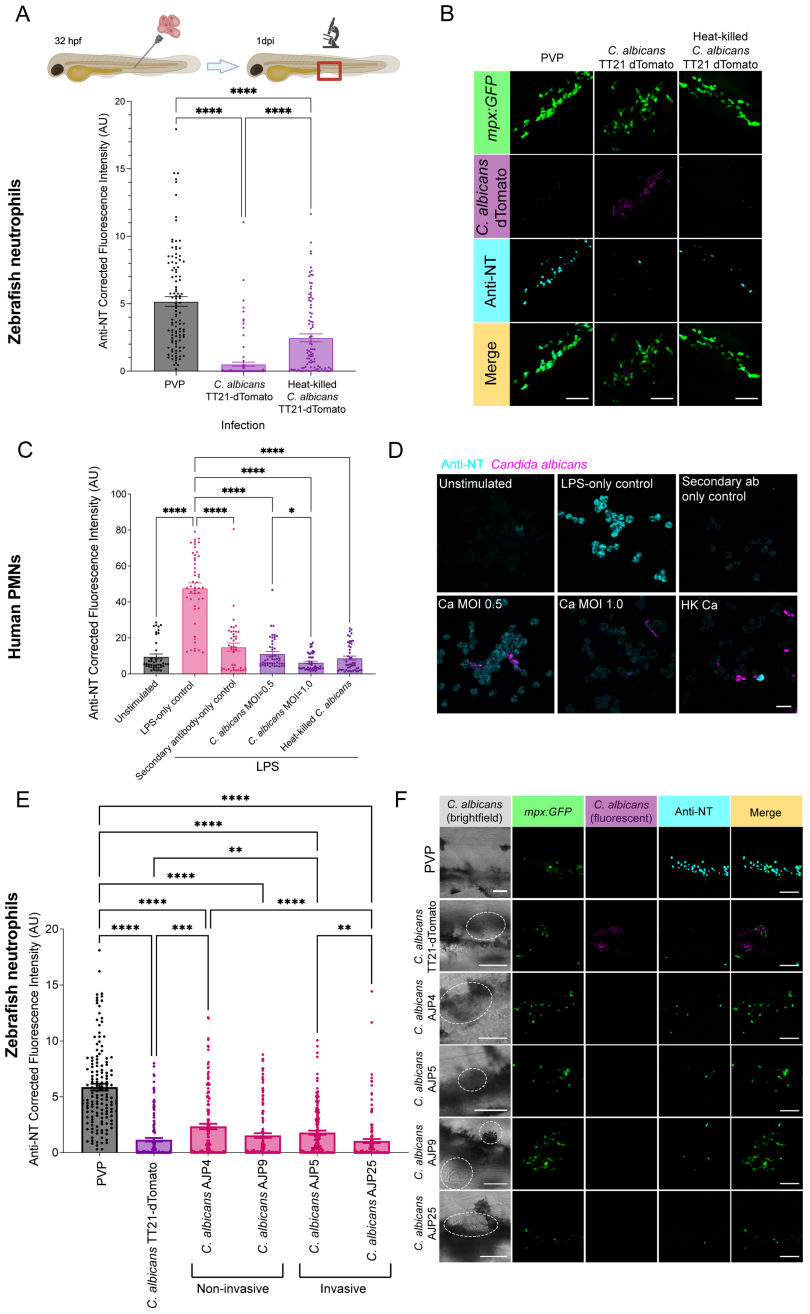

FIG 1 *C. albicans* suppressed neutrophil RNS production below resting levels. (A) Schematic of experiment: 1 dpf zebrafish were injected with PVP or *C. albicans* into the caudal vein. At 1 dpi, larvae were fixed and stained with anti-nitrotyrosine primary antibody and goat anti-rabbit Alexa-633 secondary antibody. Zebrafish were then imaged at the site of infection in the caudal hematopoietic tissue (CHT), and anti-nitrotyrosine fluorescence was quantified. The graph shows anti-nitrotyrosine fluorescence at 1 dpi following injection of PVP or 200 cfu of 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 to 18 fish, obtained from three independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, and then Dunn's multiple comparisons test. *P* values are shown: ****$P < 0.0001$. (B) Representative images of PVP, *C. albicans* TT21-dTomato, and heat-killed *C. albicans* TT21-dTomato-infected zebrafish larvae at 1 dpi. Scale bars = 50 µm. (C) Corrected fluorescence intensity of PMNs fixed at 3 h post-LPS treatment and stained with anti-nitrotyrosine antibody (in arbitrary units [AU]). Non-LPS-stimulated resting neutrophils were used as unstimulated controls (gray bar). LPS-stimulated

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Fig 1 (Continued)

neutrophils were included as controls, either stained with primary (anti-NT antibody) and secondary antibody (goat-anti-rabbit Alexa-633) (LPS-only control), or secondary alone (Secondary antibody-only control) (pink bars). Neutrophils infected with an MOI of 0.5 and 1.0 live *C. albicans* and heat-killed *C. albicans* all show a decrease in nitrotyrosine in the presence of LPS compared to LPS-only controls (purple bars). N = 42–48 neutrophils from two independent experiments and donors. (D) Representative images of human PMNs stained with anti-nitrotyrosine (anti-NT). Scale bars = 50 µm. *P < 0.05, ****P < 0.0001. (E) Anti-nitrotyrosine fluorescence at 1 dpi following injection of PVP, *C. albicans* TT21-dTomato, or *C. albicans* clinical isolates into the caudal vein at 30 hpf. N = 144 neutrophils from 24 fish, obtained from three independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test and then Dunn's multiple comparisons test. P values shown: **P < 0.01, ***P < 0.001, ****P < 0.0001. (F) Representative images of PVP, *C. albicans* TT21-dTomato, *C. albicans* AJP4, *C. albicans* AJP5, *C. albicans* AJP9, and *C. albicans* AJP25 infected zebrafish larvae at 1 dpi. White dotted circles show *C. albicans*. Scale bars = 50 µm.

if no signs of remaining fluorescent *C. albicans* infection could be observed at 2.5× magnification.

Statistical analysis

All data were analyzed using GraphPad Prism 10.5.0 1 (GraphPad Software, La Jolla, CA, USA, https://www.graphpad.com/). Nitrotyrosine staining data were analyzed with a two-tailed Mann-Whitney test or Kruskal-Wallis test, with Dunn's multiple comparisons test. Survival curves were analyzed with the 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 below basal levels

Live *C. albicans* caused a 90.0% reduction in the levels of RNS (visualized using an anti-nitrotyrosine antibody) compared to mock infection (PVP) controls (Fig. 1A and B), indicating not only a lack of induction of RNS, but also a downregulation of basal RNS. We have previously shown that RNS is predominantly found in neutrophils both in unchallenged and mock-infected (PVP) individuals (50) and is upregulated after bacterial challenge with *Mycobacterium marinum* (31). This established that *C. albicans* suppressed neutrophil RNS *in vivo* in contrast to bacterial challenge. In mock-infected (PVP) controls, there was a basal level of nitrotyrosine in neutrophils, as observed previously (31, 50). We tested another bacterium, *Staphylococcus aureus,* to determine if induction of neutrophil RNS was limited to mycobacteria. *S. aureus* (SH1000) infection also caused a significant increase in neutrophil nitrotyrosine fluorescence (Fig. S2 available at https://doi.org/10.15131/shef.data.32630970), corroborating previous observations of increased RNS levels in response to mycobacterial infection (31). RNS reduction by *C. albicans* was not limited to a single strain, as we infected with a different *C. albicans* strain, SN148 GFP, and observed similar RNS suppression (Fig. S3 available at https://doi.org/10.15131/shef.data.32630970). This allowed the use of TT21-dTomato and SN148 GFP interchangeably, depending on the color combinations required for each experiment. Zebrafish larvae infected with heat-killed C. albicans TT21-dTomato had an intermediate level of RNS suppression, between the PVP control and live C. albicans infection (Fig. 1A and B). These data indicate that the full effect of neutrophil RNS suppression is dependent on live *C. albicans* but can be suppressed to a lesser extent by heat-killed *C. albicans*, suggestive of a combination of active and passive mechanisms.

To confirm our finding in humans, we performed anti-nitrotyrosine staining on human primary polymorphonuclear neutrophils (PMNs) after *C. albicans* infection *in vitro*. Detectable and robust RNS induction in human neutrophils was induced by priming with

LPS, which led to a robust nitrotyrosine response after 3 h in the absence of infection (LPS mock-infected) (Fig. 1C and D). When *C. albicans* was added at an MOI of 1.0, nitrotyrosine was suppressed to baseline unstimulated levels, despite the presence of LPS (Fig. 1C and D). At a lower MOI of 0.5, live *C. albicans* showed less RNS suppression compared to an MOI of 1.0 of live *C. albicans*, indicating a dose-dependent effect (Fig. 1C and D). Similarly, an MOI of 1.0 of heat-killed *C. albicans* exhibited lower RNS suppression compared to the same MOI of live *C. albicans*. Together, these data show that *C. albicans* suppresses RNS production in human neutrophils as in zebrafish.

The laboratory reference strain SC5314 and strains derived from this (including SN148 and TT21) have inactivating missense mutations in RNAi component Argonaute, and hence, they may behave differently from *C. albicans* strains found in the clinic (54). Therefore, clinical isolates from patients with invasive (AJP5 and AJP25) and non-invasive (AJP4 and AJP9) disease were tested for neutrophil RNS suppression in zebrafish. Our clinical isolates lacked a fluorescent protein marker; hence, brightfield microscopy was used to identify sites of infection in anti-nitrotyrosine stained zebrafish larvae. Isolates from both invasive (AJP5 and AJP25) and non-invasive (AJP4 and AJP9) disease in patients were able to significantly reduce zebrafish neutrophil RNS compared to mock infection (PVP) controls (Fig. 1E and F). These observations demonstrated that neutrophil RNS suppression by *C. albicans* is conserved in clinical isolates, implying that RNS suppression in *C. albicans* infections may be a clinically relevant phenomenon.

Full neutrophil RNS suppression is partially dependent on *C. albicans* hyphae formation

C. albicans morphological switch from yeast to hyphae is associated with increased virulence and shifts in the transcriptome and secretome (55–57). Classification of hyphal growth by *C. albicans* TT21 and clinical isolates in zebrafish revealed that the isolates associated with greater neutrophil RNS suppression (AJP9 and AJP25) (Fig. 1E and F) also exhibited greater hyphal growth *in vivo* compared to those that reduced RNS to a lesser extent (AJP4 and AJP5) (Fig. 2A and B). Based on this correlation, we hypothesized that hyphal switching may be partially responsible for the suppression of neutrophil RNS. To investigate this, 1 dpf zebrafish were infected with *C. albicans* TT21-dTomato or yeast-locked strain *C. albicans* NRG1^{OEX}-dTomato (29). Both *C. albicans* NRG1^{OEX}-dTomato and heat-killed *C. albicans* NRG1^{OEX}-dTomato caused a modest decrease in RNS compared to wild-type strains, with reductions comparable to heat-killed *C. albicans* (Fig. 2C and D). These data suggest that full neutrophil RNS suppression by *C. albicans* is partially hyphal switch-dependent.

During hypha formation, synthesis of cell wall components, such as chitin, is upregulated (58). To determine whether *C. albicans* chitin is involved in RNS suppression, a strain lacking AP-2 (*apm4*Δ/Δ), which has higher chitin levels but decreased virulence in zebrafish due to defective hyphae formation (30), was investigated. Despite defects in hypha formation, *apm4*Δ/Δ suppressed RNS to similar levels as wild-type *C. albicans*, suggestive of a role for the cell wall component chitin in RNS suppression (Fig. S4 available at https://doi.org/10.15131/shef.data.32630970).

Host arginase is induced by *C. albicans*

Inducible nitric oxide synthase (iNOS; Nos2 in zebrafish) is the enzyme responsible for RNS production. iNOS competes with the arginase enzyme for a shared substrate, L-arginine, and arginase can competitively inhibit RNS production by iNOS (16). We investigated the impact of *C. albicans* infection on host arginase levels. PVP-injected larvae had a low basal level of *arg2:GFP* expression (Fig. 3A and B). *Mycobacterium marinum* (used as a positive control for arginase induction) caused a significant upregulation of *arg2:GFP* compared to PVP, as previously described (39). *C. albicans* TT21-dTomato caused a greater upregulation in *arg2:GFP* expression compared to *M. marinum,* indicating a robust host arginase upregulation. *C. albicans* NRG1^{OEX}-dTomato also caused upregulation of *arg2:GFP* compared to both PVP and *M. marinum,* indicating

that this is a hyphal-independent process. Examination of *arg2:GFP* expression in neutrophils (labeled by *mpx:GFP*) revealed *C. albicans* TT21-dTomato caused an increase in the percentage of *arg2:GFP* + neutrophils (41.59% of the neutrophil population in the field of view; Fig. 4C and D) compared to PVP (2.01%) and *M. marinum* (10.97%). Together, these results reveal *C. albicans* infection upregulates host arginase expression in a subpopulation of neutrophils to a greater level than the professional bacterial pathogen *M. marinum*.

C. albicans-derived arginase contributes to the suppression of neutrophil RNS

C. albicans produces its own version of arginase, *CAR1* (43). We hypothesized that *C. albicans* arginase interfered with the host RNS production, 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 1 dpi by anti-nitrotyrosine staining. *C. albicans car1*Δ infection decreased neutrophil RNS production compared to PVP (Fig. 4A and B). *C. albicans car1*Δ caused an intermediate level of RNS suppression between live *C. albicans* SC5314 and heat-killed *C. albicans* SC5314 (Fig. 4A and B), indicating that CAR1 contributes to the suppression of neutrophil RNS. Heat-killed *C. albicans car1*Δ was not significantly different from heat-killed *C. albicans* SC5314. Infection with *C. albicans car1*Δ resulted in significantly greater zebrafish larvae survival than infection with *C. albicans* SC5314 (Fig. 4C), suggesting that RNS suppression by car1 is important for *C. albicans* virulence *in vivo*.

The level of neutrophil RNS suppression by *Candida* spp. correlates with virulence

C. albicans is the leading causative agent of candidiasis, but there are many other clinically important *Candida* spp. We investigated the effect of a range of *Candida* species clinical isolates on neutrophil RNS levels *in vivo*. Clinical isolates of *C. parapsilosis* (AJP22), *C. guilliermondii* (AJP24), and *C. auris* (StG2) all robustly reduced neutrophil RNS compared to PVP controls, but to a lesser extent than a laboratory strain of *C. albicans* (Fig. 5A and B). *C. glabrata* AJP12 caused a more modest decrease in neutrophil RNS compared to *C. albicans*. (Fig. 5A and B). Together, these results show that neutrophil RNS suppression is conserved across *Candida* species clinical isolates but that levels of RNS suppression can vary between species.

Intriguingly, when percentage zebrafish survival at 4 dpi was plotted against the percentage reduction of RNS levels by *C. albicans* strains/isolates compared to PVP control, survival negatively correlated with reduction in neutrophil RNS levels (Fig. 5C. Full survival curves for *Candida* spp. are in Fig. S5 and S6 (available at https://doi.org/10.15131/shef.data.32630970) (Pearson's correlation coefficient: −0.718, 95% CI: −0.208 to −0.921; $P < 0.05$, R^2 value: 0.516). Therefore, a greater suppression of host RNS levels is correlated with increased virulence in zebrafish infection *in vivo*, indicating that suppression of neutrophil RNS is a potential virulence mechanism in human disease-relevant *Candida* spp.

Increased neutrophil RNS, via Hif-1α stabilization, is protective against *C. albicans* infection

Hif-1α is a transcription factor with known roles in regulation of innate immunity (32). We have previously shown that stabilized Hif-1α (using injection of a dominant active variant RNA) is protective against *M. marinum* infection *in vivo* by increasing neutrophil RNS levels (31, 36). We therefore hypothesized that stabilization of Hif-1α would restore neutrophil RNS in *C. albicans* infection and increase host survival.

One-cell-stage zebrafish larvae were injected with water/10% phenol red (solvent) control (PR), dominant-negative *hif-1*α (DN *hif-1*α), or dominant-active *hif-1*α (DA *hif-1*α) RNA (as previously described (46)) and subsequently infected systemically with *C. albicans* TT21-dTomato infection at 30 hpf. DA *hif-1*α larvae had significantly greater

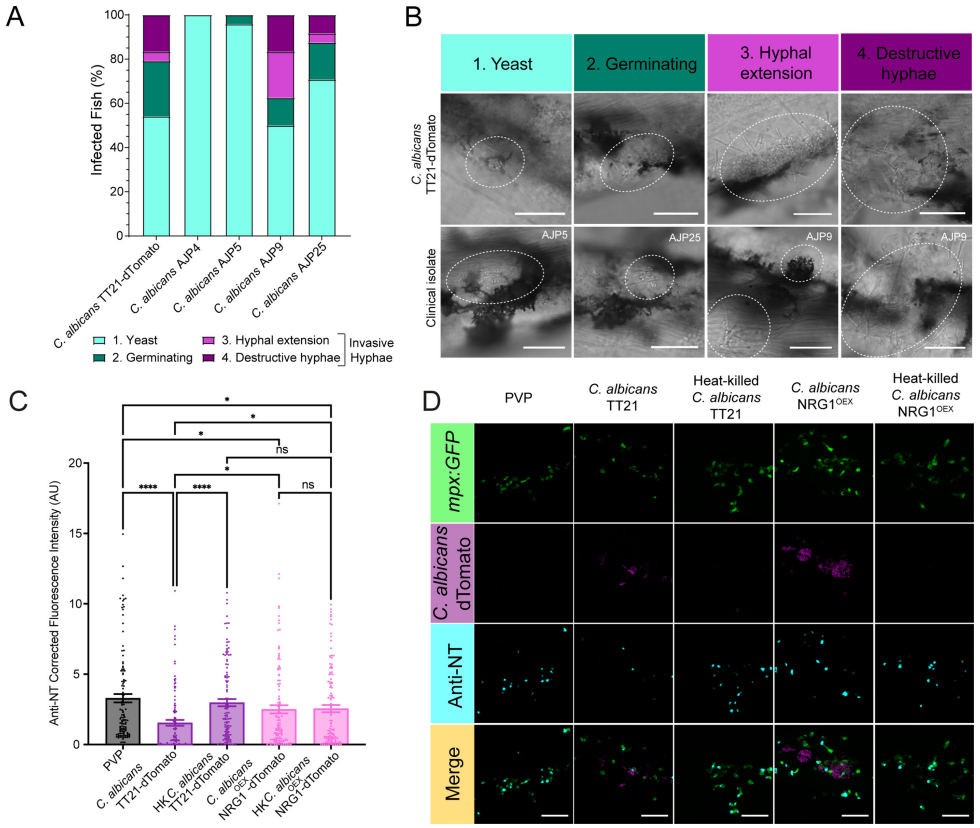

FIG 2 Robust RNS suppression was associated with hypha-forming *C. albicans*. (A) Hyphal classification of zebrafish larvae infected with a range of *C. albicans* isolates at 1 dpi. *N* = 24 fish, obtained from three independent experiments. (B) Representative images of hyphal classification in 1 dpi zebrafish larvae infected with *C. albicans* TT21-dTomato or a *C. albicans* clinical isolate. Images are labeled with the *C. albicans* isolate. White-dotted circles show regions of fungal growth. Scale bar = 50 µm. (C) Anti-nitrotyrosine fluorescence at 1 dpi following injection of PVP, *C. albicans* TT21-dTomato, heat-killed *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato, or heat-killed *C. albicans* NRG1^{OEX}-dTomato into the caudal vein. *N* = 120 neutrophils from 20 fish, obtained from three independent experiments. Error bars show SEM. Statistical significance was determined by Kruskal-Wallis test, with Dunn's multiple comparisons tests. **P* < 0.05, *****P* < 0.0001. (D) Representative images of PVP, *C. albicans* TT21-dTomato, heat-killed *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato or heat-killed *C. albicans* NRG1^{OEX}-dTomato infected larvae at 1 dpi. Scale bars = 50 µm.

survival over the first 4 days post-infection than both PR and DN *hif-1*α larvae (Fig. 6A), demonstrating that Hif-1α stabilization is host-protective in *C. albicans* infection. *C. albicans* infection was not sufficient to upregulate the *Tg(phd3:GFP)i144* Hif reporter transgenic line (40), which may indicate why DN *hif-1*α larvae did not affect zebrafish survival post-*C. albicans* infection (Fig. S7 available at https://doi.org/10.15131/shef.data.32630970).

Neutrophils play an essential role in innate immune control of invasive candidiasis (59). In zebrafish, neutrophils are responsible for the killing of *C. albicans*, whereas macrophages internalize but do not kill (60). Therefore, we hypothesized that the protective effect of Hif-1α stabilization would be mediated by neutrophils rather than macrophages. First, to assess the impact of Hif-1α stabilization on macrophage-mediated immunity, we depleted the macrophage population by injection of clodronate liposomes (61) at 30 hpf (or PBS liposomes as a negative control), leading to a significant depletion of the macrophage population (Fig. S8 available at https://doi.org/10.15131/shef.data.32630970). At 30 hpf, PR or DA *hif-1*α larvae were injected into the caudal vein with clodronate liposomes or PBS liposomes, followed by infection with 100 cfu *C. albicans* TT21-dTomato at 2 dpf. Both groups of PBS liposome-injected larvae had greater

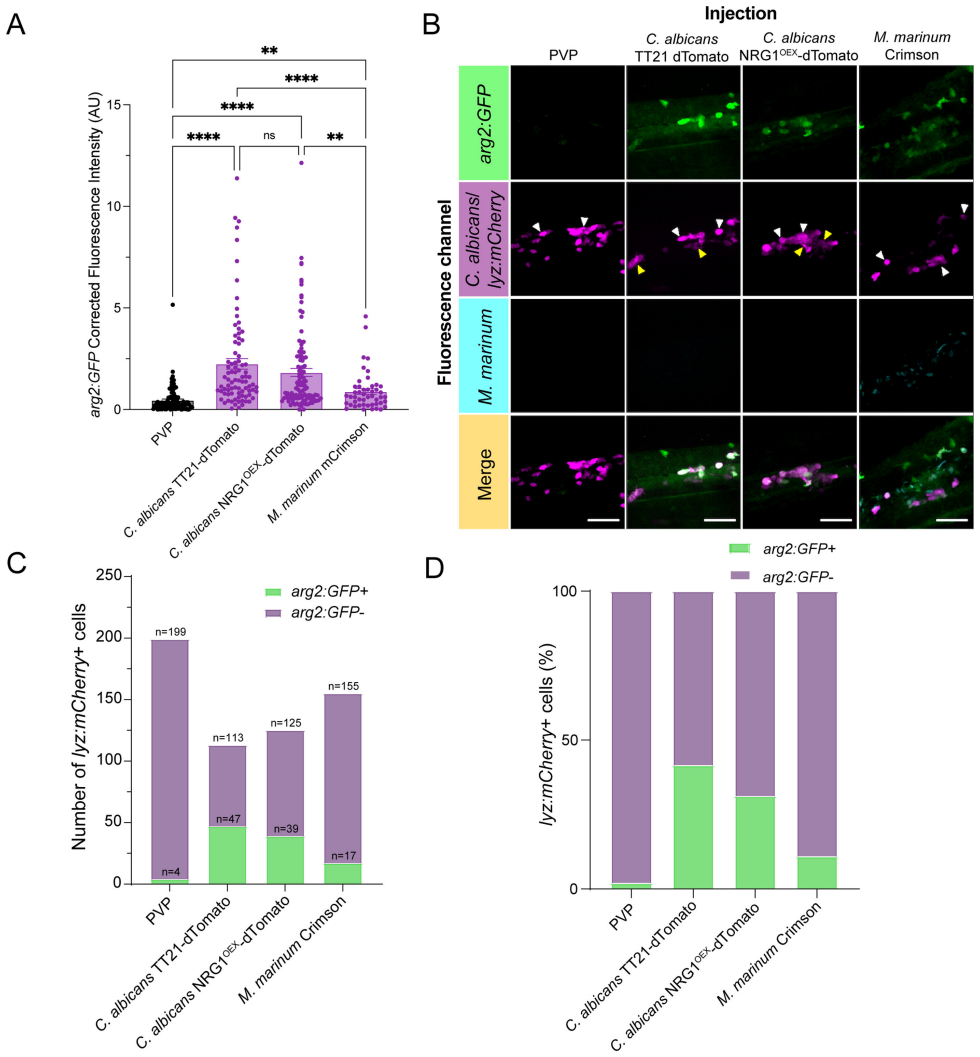

FIG 3 *C. albicans* infection stimulated host *arginase* 2 expression. (A) Corrected fluorescence intensity of *Tg(arg2:GFP)* zebrafish larvae at 1 dpi following injection of PVP, *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato, or *M. marinum* Crimson into the caudal vein at 30 hpf. For PVP, *C. albicans* TT21-dTomato, and *C. albicans* NRG1^{OEX}-dTomato: n = 84–108 cells from 14 to 18 fish, obtained from three independent experiments. For *M. marinum*: n = 48 cells from eight fish, obtained from two independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, then Dunn's multiple comparisons test. P values shown: **P < 0.01, ****P < 0.0001. (B) Representative images of *Tg(arg2:GFP)* zebrafish embryos injected with PVP, *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato, or *M. marinum* crimson at 24 hpi. White arrowheads point toward neutrophils. Yellow arrowheads point toward *C. albicans*. Scale bars = 50 µm. (C) Number of *lyz:nfsB.mCherry*-expressing cells that are *arg2:GFP*+ and *arg2:GFP*–, following infection with *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato, *M. marinum* Crimson or PVP. N = 113–199 cells from 12 zebrafish, obtained from two independent experiments. (D) Percentage of *lyz:nfsB.mCherry*-expressing neutrophils that are *arg2:GFP*+ and *arg2:GFP*–, following infection with *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato, *M. marinum* Crimson, or PVP. N = 113–199 cells from 12 zebrafish, obtained from two independent experiments.

survival than their clodronate liposome, macrophage-depleted, siblings. DA *hif-1*α was host protective both with or without depletion of macrophages, with DA *hif-1*α + clodronate liposome larvae having greater survival than PR + clodronate liposome larvae (Fig. 6B; P < 0.0001), indicating that the host-protective effect of Hif-1α stabilization is not dependent on the presence of macrophages. Next, we injected one-cell stage zebrafish larvae with a *csf3r* morpholino (47) to deplete neutrophil numbers by over 60% in 2 dpf zebrafish larvae (Fig. S9 available at https://doi.org/10.15131/shef.data.32630970). *csf3r*

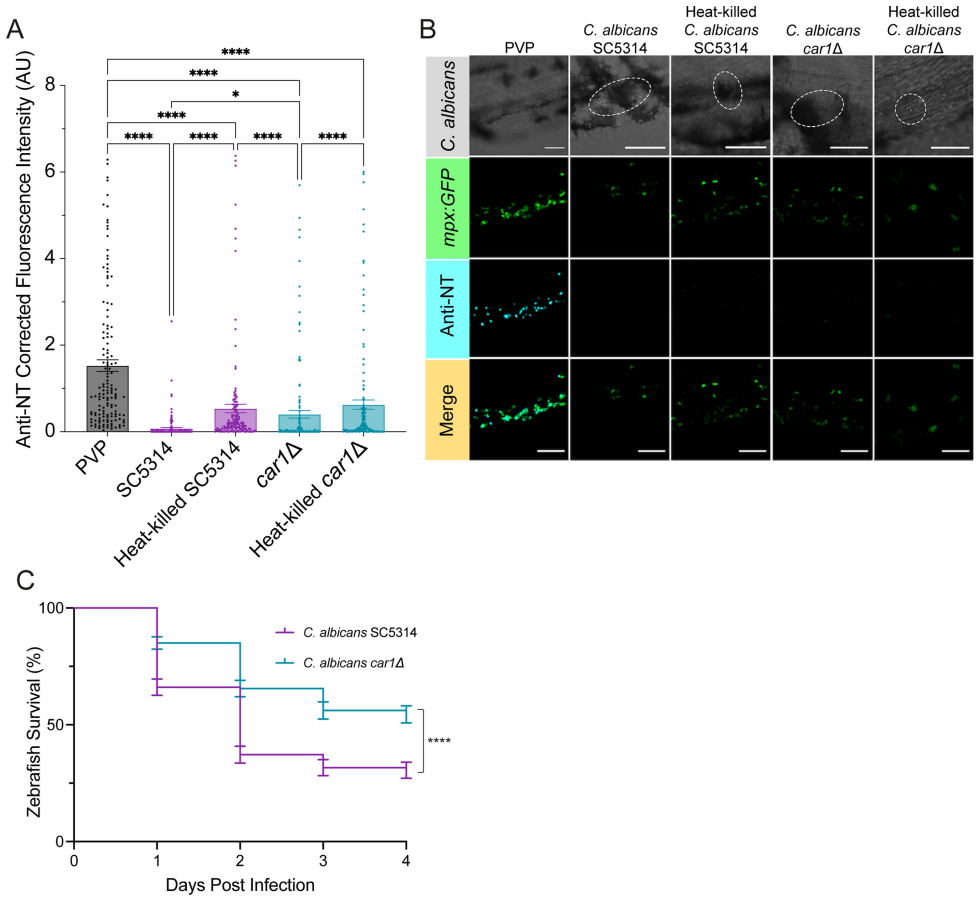

FIG 4 *C. albicans car1* contributed to suppression of neutrophil RNS. (A) Anti-nitrotyrosine fluorescence at 1 dpi following injection of PVP, *C. albicans* SC5314, heat-killed *C. albicans* SC5314, *C. albicans car1*Δ, or heat-killed *C. albicans car1*Δ into the caudal vein. *N* = 144 neutrophils from 24 fish, obtained from three 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 larvae. Dashed lines show *C. albicans*. Scale bars = 50 µm. (C) One dpf nacre larvae were injected into the caudal vein with 200 cfu *C. albicans* SC5314 or *C. albicans car1*Δ. Larval survival was measured daily up to 4 dpi. *N* = 180 fish, obtained from three independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. *P* values shown: *****P* < 0.0001

morpholino injection led to a significant decrease in survival of 100 cfu *C. albicans*-infected larvae in both PR and DA *hif-1*α larvae (Fig. 6C). DA *hif-1*α + *csf3*r larvae had lower survival compared to PR + *csf3r* larvae, showing a loss of the protective effect of Hif-1α stabilization in neutrophil-ablated larvae (Fig. 6C), indicating that the protective effect of Hif-1α stabilization is neutrophil-dependent.

DA *hif-1*α did not alter the recruitment of neutrophils to the site of a local hindbrain *C. albicans* infection compared to PR-injected controls (Fig. S10 available at https://doi.org/10.15131/shef.data.32630970). Therefore, we hypothesized that the host protective effect of Hif-1α stabilization in *C. albicans* infection was due to increased neutrophil RNS production. DA *hif-1*α doubled the levels of nitrotyrosine compared to PR in mock-infected (PVP) larvae (Fig. 7A and B), similar to previous observations (31). While *C. albicans* reduced nitrotyrosine levels in PR control-injected larvae, DA *hif-1*α *C. albicans*-infected larvae had high levels of neutrophil nitrotyrosine compared to mock-infected (PVP) DA1 controls (Fig. 7A and B).

To demonstrate that the increase in RNS after Hif-1α stabilization is responsible for the host protective effect in *C. albicans* infection, we inhibited production of RNS both pharmacologically, using L-NIL, and genetically, using *nos2a* + *b* double CRISPR-Cas9

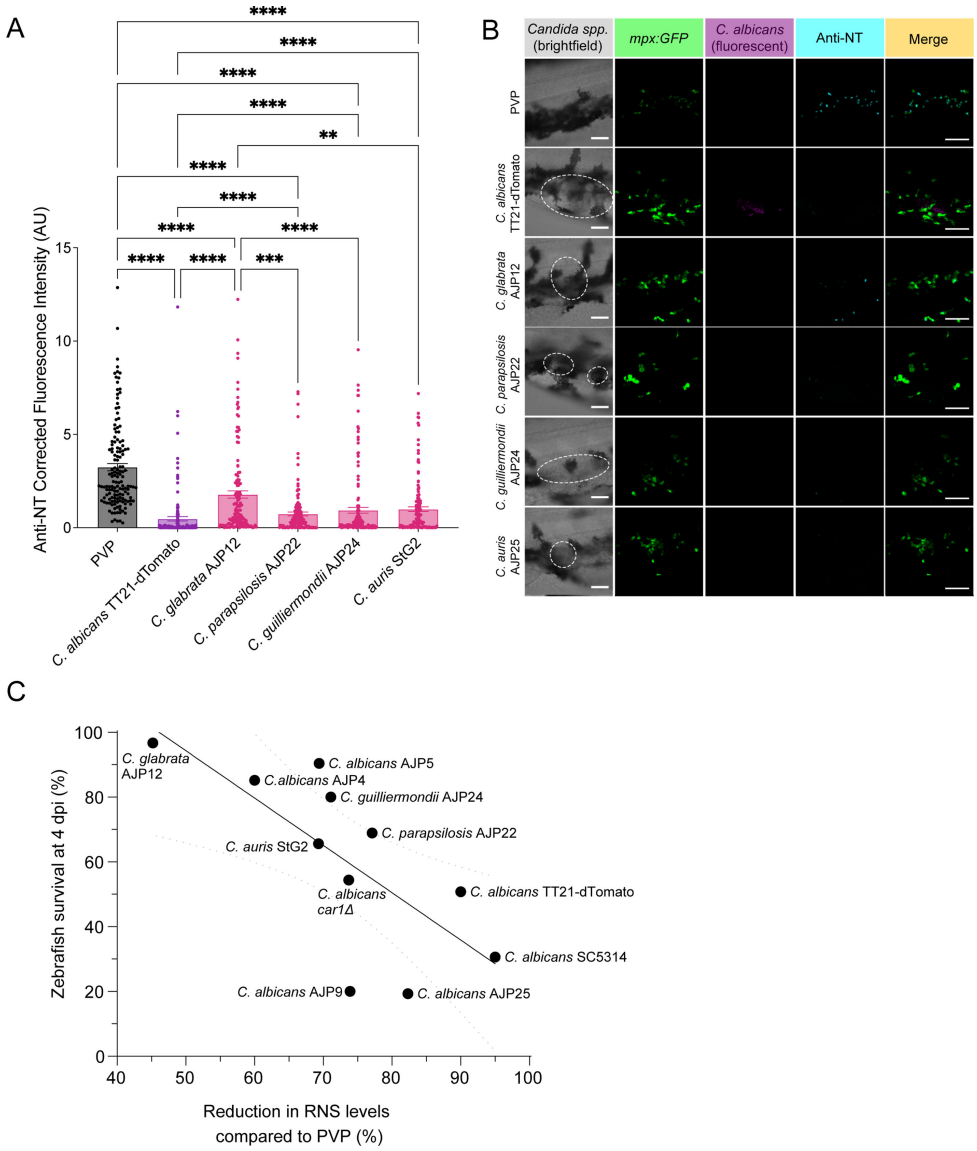

FIG 5 Non-albicans *Candida* spp. suppressed neutrophil RNS. (A) Anti-nitrotyrosine fluorescence at 1 dpi following injection of PVP, *C. albicans* TT21-dTomato, *C. glabrata* AJP12, *C. parapsilosis* AJP22, *C. guilliermondii* AJP24, or *C. auris* StG2 into the caudal vein. $N = 144$ neutrophils from 24 fish, obtained from three independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, with Dunn's multiple comparisons tests. * indicates difference compared to PVP. † indicates difference compared to *C. albicans* TT21-dTomato. ‡ indicates difference compared to *C. glabrata* AJP12. *P* values shown: $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (B) Representative images of PVP, *C. albicans* TT21-dTomato, *C. glabrata* AJP12, *C. parapsilosis* AJP22, *C. guilliermondii* AJP24, or *C. auris* StG2 infected larvae at 1 dpi. Scale bars = 50 µm. (C) Reduction in RNS levels compared to PVP control plotted against zebrafish survival at 4 dpi, following systemic infection with 200 cfu *Candida* spp. Full survival curves for *Candida* spp. are in . Each plotted point is based on three independent repeats of the relevant experiment. Line of best fit and 95% confidence error lines are shown. Pearson's correlation coefficient and R^2 value were calculated.

knockdown ("CRISPants" [48, 62]). L-NIL treatment did not impact the growth of *C. albicans in vitro* (Fig. S11 available at https://doi.org/10.15131/shef.data.32630970). Following infection with *C. albicans*, PR + L-NIL-treated larvae had reduced survival compared to PR + dH_2O (drug solvent control)-treated larvae (Fig. 7C), indicating a role of RNS in *C. albicans* control. DA *hif-1*α + L-NIL-treated larvae did not have a significant survival advantage compared to either PR + dH_2O or PR + L NIL groups (Fig. 7C),

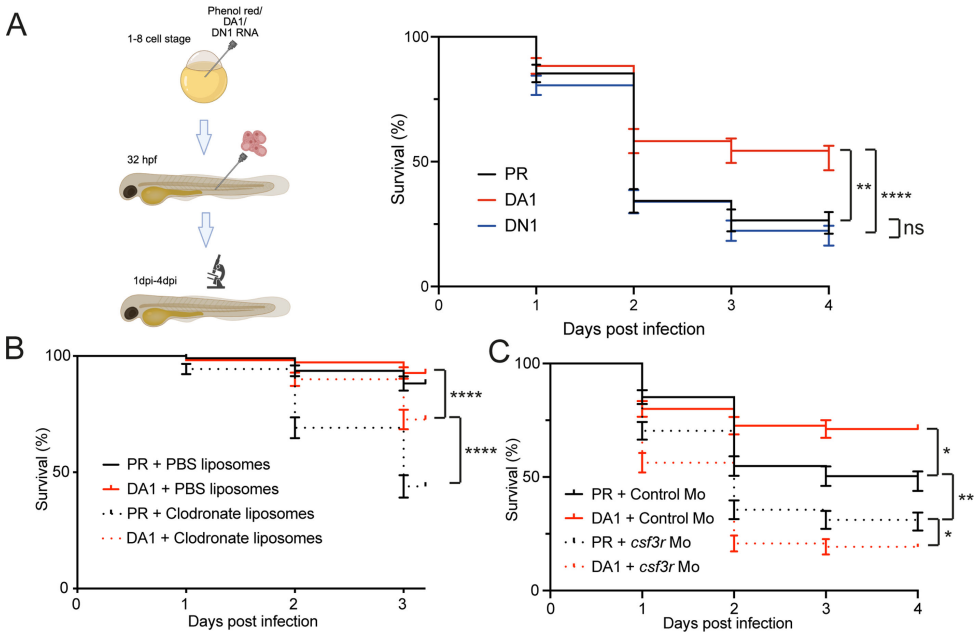

FIG 6 Hif-1α stabilization was protective against *C. albicans* infection. (A) Survival of *C. albicans* TT21-dTomato-infected zebrafish larvae following injection with DA1, DN1 RNA, or phenol red (PR) control. Mortality was measured daily. *N* = 102–103 fish, obtained from three independent experiments. Error bars show SEM. Statistical significance determined by the Gehan-Breslow-Wilcoxon test with Bonferroni correction. ***P* < 0.01, *****P* < 0.0001. (B) Survival curve of 100 cfu *C. albicans* TT21-dTomato-infected zebrafish larvae following injection with DA1 or PR, then treatment with PBS or clodronate liposomes. Mortality was measured daily. *N* = 102–103 fish, obtained from three independent experiments. Error bars show SEM. Statistical significance determined by the Gehan-Breslow-Wilcoxon test with Bonferroni correction. *****P* < 0.0001. (C) Survival curve of 100 cfu *C. albicans* TT21-dTomato-infected zebrafish larvae following injection with PR or DA1 and control or *csf3r* morpholino. Mortality was measured daily. *N* = 135 fish, obtained from three independent experiments. Error bars show SEM. Statistical significance determined by the Gehan-Breslow-Wilcoxon test with Bonferroni correction. **P* < 0.05, ***P* < 0.01.

demonstrating that iNOS inhibition is sufficient to remove the host protective effect of Hif-1α stabilization. *Nos2a+b* double CRISPants were shown to inhibit nitrotyrosine production in mock-infected zebrafish (Fig. S12 available at https://doi.org/10.15131/shef.data.32630970). DA *hif-1*α with *nos2a+b* CRISPants no longer had increased survival following *C. albicans* infection compared to PR + *tyr* sgRNA (*tyrosinase* CRISPant control [48]) larvae, supporting that Nos2 is required for Hif-1α stabilization-dependent host protection (Fig. 7D). Together, these data demonstrate that Hif-1α stabilization is host-protective in *C. albicans* infection via a neutrophil- (Fig. 6) and RNS-dependent (Fig. 7) mechanism.

Hif-1α stabilization has an additive host protective effect when used alongside antifungals

We next investigated the potential of Hif-1α stabilization to act as an adjunctive therapy alongside established antifungals. At 1 dpf, PR/DA *hif-1*α larvae were injected into the caudal vein with 500 cfu *C. albicans* TT21-dTomato and then treated by immersion with 5.0 µg/mL fluconazole or 1.0 µg/mL caspofungin. Both fluconazole (Fig. S13 available at https://doi.org/10.15131/shef.data.32630970) and caspofungin (Fig. S14 available at https://doi.org/10.15131/shef.data.32630970) treatment had dose-dependent effects on zebrafish larval survival post-*C. albicans* infection. Fluconazole significantly increased host survival in both PR and DA *hif-1*α larvae (Fig. 8A). DA *hif-1*α + fluconazole had increased survival compared to PR + fluconazole and DA *hif-1*α + DMSO. Combination of DA *hif-1*α + caspofungin similarly increased survival compared to PR + caspofungin

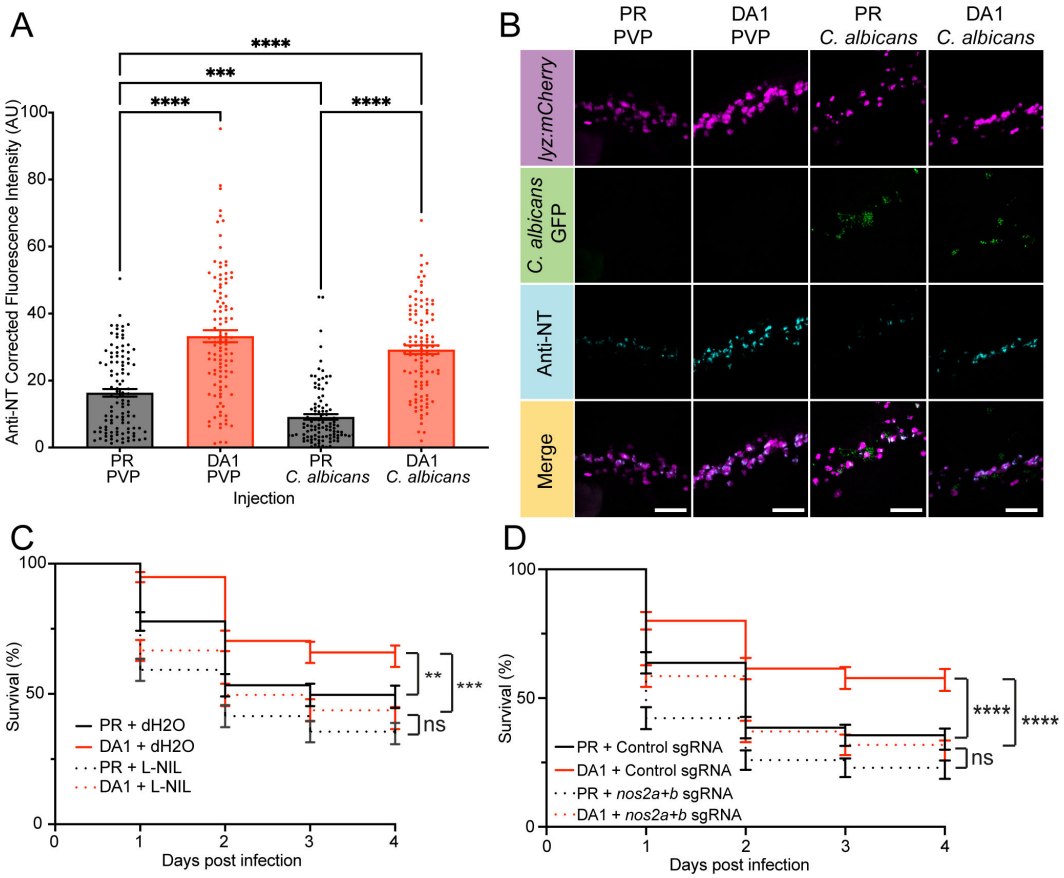

FIG 7 The host protective effect of Hif-1α stabilization was RNS-dependent. (A) Anti-nitrotyrosine fluorescence at 1 dpi in PR- and DA1-injected fish, following injection of PVP or 500 cfu *C. albicans* SN148 GFP. *N* = 108 neutrophils from 18 fish, obtained from three independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, with Dunn's multiple comparisons test. ***$P < 0.001$, ****$P < 0.0001$. (B) Representative images of 1 dpi Tg(*lyz:NTRmCherry*) zebrafish larvae 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 larvae following injection with PR or DA1, then treatment with dH_2O or L-NIL. *N* = 135 fish, obtained from three 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 larvae following injection with PR or DA1 and *tyr* or *nos2a+b* sgRNA. *N* = 135 fish, obtained from three independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. **$P < 0.01$, ***$P < 0.001$.

(Fig. 8B) and DA *hif-1*α + dH_2O (Fig. 8B), demonstrating that the combination of Hif-1α stabilization with fluconazole or caspofungin has an additive effect on survival.

Surviving larvae were imaged at 4 dpi to quantify the clearance of *C. albicans* infection (Fig. 8C through F); 27% of DA *hif-1*α + fluconazole larvae had completely cleared infection at 4 dpi (Fig. 8C and D), greater than both PR alone (4%) and PR +fluconazole (8%). In total, 22% of larvae treated with DA *hif-1*α + caspofungin cleared infection (Fig. 8E and F) compared to 3% of PR-treated larvae and 4% of larvae treated with PR and caspofungin. Hence, Hif-1α stabilization also has an additive effect on clearance when combined with fluconazole or caspofungin.

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 *Candida* species suppression of neutrophil RNS is an important factor in their virulence *in vivo,* and that restoration of neutro-

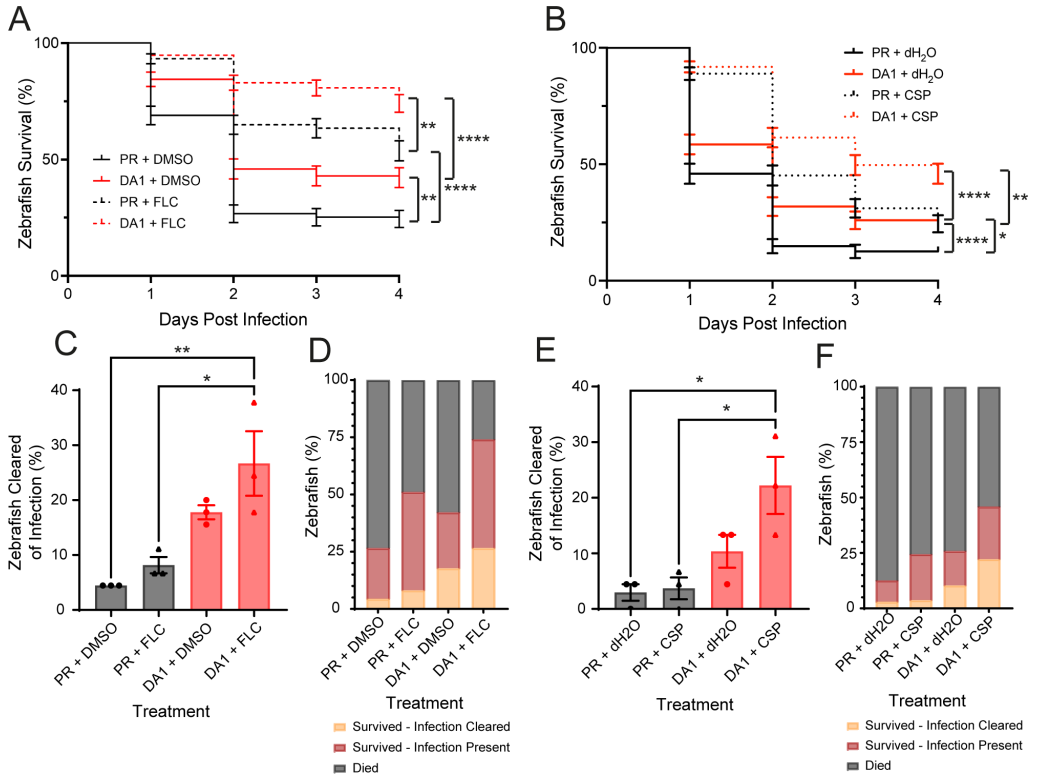

FIG 8 Hif-1α stabilization had an additive effect with antifungals. (A) Survival of *C. albicans* TT21-dTomato-infected zebrafish larvae following injection with dominant active *hif-1*α (DA1) or phenol red (PR) control. Larvae were treated with 5.0 µg/mL fluconazole or DMSO solvent control. Mortality was measured daily. *N* = 135 fish, obtained from three independent experiments. Error bars show SEM. Statistical significance was determined by Gehan-Breslow-Wilcoxon test with Bonferroni correction. $^{**}P < 0.01$, $^{****}P < 0.0001$ (B) Survival of *C. albicans* TT21-dTomato-infected zebrafish larvae following injection with DA1 or PR control. Larvae were treated with 1.0 µg/mL caspofungin or dH_2O solvent control. Mortality was measured daily. *N* = 135 fish, obtained from three independent experiments. Error bars show SEM. Statistical significance was determined by Gehan-Breslow-Wilcoxon test with Bonferroni correction. $^{**}P < 0.01$, $^{****}P < 0.0001$. (C) Clearance of *C. albicans* infection at 4 dpi by zebrafish larvae following injection with DA1 or PR control and treatment with 5.0 µg/mL fluconazole or DMSO solvent control. *N* = 3 independent experiments, 135 fish per group. Error bars show Standard Deviation. Statistical significance was determined by one-way ANOVA, with Tukey's multiple comparisons test. $^{*}P < 0.05$, $^{**}P < 0.01$ (D) Infection outcomes of *C. albicans* TT21-dTomato infected zebrafish larvae at 4 dpi, following injection with DA1 or PR and treatment with 5.0 µg/mL fluconazole or DMSO solvent control. *N* = 3 independent experiments, 135 fish per group. (E) Clearance of *C. albicans* infection at 4 dpi by zebrafish larvae following injection with DA1 or PR control and treatment with 1.0 µg/mL caspofungin or dH_2O solvent control. *N* = 3 independent experiments, 135 fish per group. Error bars show standard deviation. Statistical significance was determined by one-way ANOVA, with Tukey's multiple comparisons test. (F) Infection outcomes of *C. albicans* TT21-dTomato infected zebrafish larvae at 4 dpi, following injection with DA1 or PR and treatment with 1.0 µg/mL caspofungin or dH_2O solvent control. *N* = 3 independent experiments, 135 fish per group.

phil RNS represents a potential host-directed therapy for difficult-to-treat *C. albicans* infection.

We found that *C. albicans* can suppress RNS production in zebrafish neutrophils *in vivo* and in human primary neutrophils *in vitro*. *C. albicans* has been shown to suppress RNS production in murine bone marrow-derived macrophages *in vitro* (19), which is supported by a variety of sources demonstrating the suppression of ROS by *C. albicans in vitro* (18, 63). While previous publications have demonstrated RNS suppression in macrophages, our data demonstrate an effect in neutrophils in a live tissue setting. Human macrophages are poor RNS producers compared to mouse macrophages (64, 65), and *C. albicans* is primarily controlled by neutrophils in human disease (66). Therefore, we

chose to dissect the mechanisms behind RNS suppression in neutrophils in the tractable *in vivo* zebrafish model.

Here, we identify that RNS suppression is partially dependent on live, hypha-forming *C. albicans*. The hyphal morphotype is highly associated with a shift in transcription and increased virulence (67, 68), which may trigger the expression of protein(s) necessary for RNS suppression by *C. albicans*. Proteomic analysis of supernatant from hyphal *C. albicans in vitro* identified 301 secreted proteins specific to hyphae (57). Further examination and characterization of these proteins could point toward candidates involved in active RNS suppression. However, RNS suppression was still observed in non-hypha-forming, non-albicans *Candida* species isolates (*C. glabrata, C. parapsilosis,* and *C. guilliermondii*), suggesting that the presence of hyphae is not essential for RNS suppression.

C. albicans-derived arginase also contributed to the suppression of neutrophil RNS production. CAR1 is the only known arginase gene in the *C. albicans* genome, encoding a cytosolic arginase (43). CAR1 has been shown to be important in the hyphal morphogenesis response to arginine and for virulence in murine infection models. Our findings indicate fungal arginase could be interfering with the host arginase-iNOS balance, resulting in reduced iNOS activity and RNS suppression. Contrary to this, *C. albicans car1*Δ infection in a murine macrophage model did not influence RNS synthesis (43). As CAR1 is not considered a secreted protein, residing in the cytosol, the mechanism of how it would interact with neutrophil RNS is unclear. However, other immunogenic *C. albicans* enzymes have been shown to be released into the extracellular space during stress or death (69), which may be a potential point of interaction between CAR1 and neutrophil RNS production during *in vivo* pathogenesis. The modest decrease in neutrophil RNS production after infection with the CAR1 mutant suggests that *C. albicans* arginase production is not the sole RNS-reducing mechanism. A caveat to this experiment is that the complemented strain of the *car1*Δ *C. albicans* strain was not used; therefore, we do not control for any artefactual chromosomal changes that may have occurred during *car1*Δ deletion. Heat-killed *C. albicans* was able to partially suppress neutrophil RNS, suggesting that mechanisms may be partially passive. The presence of chitin may account for the partial suppression of RNS by heat-killed *C. albicans* that we observed, with *C. albicans* chitin shown to induce host arginase-1 activity, decreasing RNS production (70). Our findings suggest that multiple, additive mechanisms of neutrophil RNS suppression by *C. albicans* may underlie their success at the large overall suppression of neutrophil RNS observed. There are several potential *C. albicans* candidate molecules that may be involved in this process. *C. albicans* SOD5 has been associated with degradation of antimicrobial ROS *in vitro* and is found on the cell surface allowing interaction with the extracellular environment (63). *C. albicans sod5*Δ/Δ have decreased viability in the presence of macrophages (63). *C. albicans YHB1* is able to detoxify RNS (71); however, it is thought to be non-secreted with a cytosolic and mitochondrial localization. Hence, similar to CAR1, the mechanism of extracellular interaction is as yet unclear. *C. albicans* strains with deleted YHB1 are hypersensitive to RNS and are hypofilamentous (71). Collette et al. identified an unknown, small secreted molecule from *C. albicans* capable of suppressing RNS (19). Interestingly, RNS inhibition in mouse macrophages required direct C. albicans interaction, with RNS inhibition lost using transwell plates of 0.4 µm pores. However, the inhibitory molecule(s) was secreted, as co-culture supernatant was able to decrease RNS levels (19). They identified that the compound was small (less than 3 kDa in size), aqueous and heat-stable, a profile which fits the physical characteristics of many tissue culture media ingredients, impeding identification using standard *in vitro* methods. We have yet to elucidate whether RNS suppression is a fully active process that has evolved to evade immune cells, either incidentally via evolution of predation in the soil by amoebae (72) or as an intentional immune evasion strategy, or whether it is partially a passive process. Further investigation could aim to establish which are the most important mechanisms and effector proteins involved in RNS suppression in neutrophils.

Neutrophil RNS suppression was observed in infection with various *C. albicans* laboratory strains and clinical isolates alongside non-albicans *Candida* species clinical isolates. This suggests that RNS suppression could be conserved widely across clinically relevant *Candida* spp. Importantly, *Candida* species virulence correlated with neutrophil RNS suppression *in vivo,* indicating that neutrophil RNS suppression may be a virulence factor. Virulence is determined by a wide range of factors, not solely neutrophil RNS suppression (73), any of which could be differentially regulated in these *Candida* species isolates. With differences in virulence, there may be differences in disease pathogenesis that could influence RNS suppression that are challenging to measure without fluorescent labeling; for example, different fungal burden may have an impact on the level of RNS at the time points measured. It was interesting to note that the emerging human pathogen *C. auris* was able to efficiently suppress neutrophil RNS in zebrafish, despite only being identified as a human pathogen since 2009 (74), suggesting that this immune evasion mechanism may have evolved in the environment (75). Suppression of neutrophil RNS by environmental strains may act as a useful proxy for determining whether *Candida* spp. or strains have the potential to be virulent in humans.

We found that upregulation of RNS, via Hif-1α stabilization, has a protective effect in *C. albicans* infection in zebrafish larvae, via a neutrophil-mediated, RNS-dependent mechanism. Neutrophils are the primary innate immune cell responsible for killing *Candida* spp. (10). Neutrophil activity against *Candida* spp. is considered strong, as evidenced by high mortality in neutropenic patients (76). They are able to perform intracellular killing (66) alongside extracellular killing via NET (neutrophil extracellular trap) release (77). However, our data indicate not only suppression of a protective neutrophil mechanism but also suggest that the neutrophil response to *Candida* spp. can be improved. Neutrophil RNS suppression is therefore an important mechanism of *C. albicans* pathogenesis that, if restored, could represent an exciting therapeutic opportunity. RNS is well characterized as being candidacidal (12, 13, 15, 78) and enhances NET release (79). NO-releasing nanoparticles inhibited the growth of *C. albicans in vitro* and *in vivo* (80). Hence, upregulation of neutrophil RNS production is a potential mechanism that could be targeted therapeutically to improve immune candidacidal activity. We have previously demonstrated that Hif-1α stabilization protects against *M. marinum* infection in a similar manner (31), suggesting that Hif-1α stabilization could offer a protective effect against multiple and different groups of pathogens (bacteria and fungi) where neutrophil-mediated immune responses are dampened during pathogenesis.

We further showed that Hif-1α stabilization had an additive effect on survival and clearance when combined with traditional antifungals. Early fungicidal activity by neutrophils prior to hyphal growth is vital for subsequent clearance and resolution of *C. albicans* infection, with early phagocytosis a strong prognostic indicator of survival (81, 82). As a primarily fungistatic agent (83), fluconazole may help to restrict initial growth of *C. albicans*, allowing neutrophils with increased RNS production to kill *C. albicans,* resulting in greater survival and clearance. Conversely, caspofungin predominantly has a fungicidal effect (84, 85), although increased neutrophil RNS production therapeutically may also facilitate earlier killing of *C. albicans* in combination and allow lower doses of these poorly tolerated drugs for shorter treatment periods. Exactly when and how combination therapy should be administered to patients requires follow-on studies.

Our data show that *Candida* spp. can efficiently suppress neutrophil RNS as a virulence mechanism and that Hif-1α stabilization is able to overcome this, conferring a neutrophil-mediated, RNS-dependent protective effect *in vivo*. Neutrophil RNS restoration has the potential as an adjunctive host-directed therapeutic strategy to aid in the treatment of *Candida* species infections.

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ETHICS APPROVAL

Zebrafish were handled in accordance with the Animals (Scientific Procedures) Act 1986, under Project Licenses P1A4A7A5E and PP7684817. Ethical approval was granted by the University of Sheffield Local Ethical Review Panel. Human whole blood was collected from healthy individuals following informed consent. Ethical approval was granted by the University of Sheffield Research Ethics Committee (study reference number 031773).

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