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 downregulation of RNS in human primary neutrophils and with clinical *Candida* spp. isolates, including emerging human pathogens *C. auris* and *C. glabrata*. Using a zebrafish *arginase 2* transgenic line and a *C. albicans car1*Δ mutant, we show that both host and fungal arginase contribute the reduction in neutrophil RNS. Despite pathogen downregulation, upregulation of neutrophil RNS via Hypoxia inducible factor (Hif)-1α stabilisation, was sufficient to improve *C. albicans* infection survival, dependent on the presence of neutrophils and Nitric oxide synthase 2 (Nos2). Finally, restoration of neutrophil RNS, via Hif-1α stabilisation, was synergistic with 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.

53 **Introduction**

54 *Candida albicans* (*C. albicans*) is a commensal fungus, colonising 70% of healthy
55 individuals (Witherden *et al.*, 2017) and can cause superficial mucosal infections with
56 negligible mortality. However, *C. albicans* can act as an opportunistic pathogen capable of
57 causing life-threatening, invasive disease in immunocompromised individuals (Witherden *et*
58 *al.*, 2017; Flores-Maldonado *et al.*, 2019; Pellon, Sadeghi Nasab and Moyes, 2020). There are
59 approximately 750,000 cases of severe candidiasis worldwide per year and the estimated
60 mortality rate is high, estimated to be around 50% (Bongomin *et al.*, 2017; WHO, 2022), likely
61 an underestimation due to poor surveillance mechanisms worldwide (Lamoth *et al.*, 2018;
62 Almeida, Rodrigues and Coelho, 2019). This high mortality, combined with a lack of fungal
63 vaccines and rising rates of antifungal resistance, has resulted in *C. albicans* being designated
64 as one of four critical priority fungal pathogens by the World Health Organization, with another
65 *Candida* spp., the emerging human pathogen *Candida auris*, also a critical priority pathogen
66 (WHO, 2022). Hence, urgent research into new therapies is required.

67 *C. albicans* is detected by pattern recognition receptors, including Toll-like receptors,
68 C-type lectin receptors and the Rig-I like receptor MDA-5, resulting in a downstream pro-
69 inflammatory response and innate immune cell recruitment (Burgess, Condliffe and Elks,
70 2022). Although many immune cell types are recruited to the sites of *C. albicans* infection,
71 neutrophils are one of the most potent killers of *C. albicans*, with neutropenic patients being
72 particularly at risk of invasive disease (Uzun *et al.*, 2001; Brown, 2011). Following recruitment,
73 neutrophils have multiple mechanisms of eliminating *C. albicans*, including the production of
74 reactive oxygen (ROS) and nitrogen (RNS) species (Winterbourn *et al.*, 2006).
75 Pharmacological inhibition of nitric oxide synthase (NOS) leads to increased mortality in *C.*
76 *albicans* infections in mice *in vivo* and reduced candidacidal activity in murine macrophages
77 and peritoneal cells *in vitro*, showing the importance of RNS-mediated fungicidal activity
78 (Rementería, García-Tobalina and Sevilla, 1995; Vazquez-Torres *et al.*, 1995; Vazquez-
79 Torres, Jones-Carson and Balish, 1996; Navarathna, Lionakis and Roberts, 2019). *C. albicans'*
80 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). As part of these mechanisms, *C. albicans* is able to subvert the host innate immune response, including ROS and RNS (Luo *et al.*, 2013). Despite *Candida* spp. being primarily controlled by neutrophils in human disease, most immune evasion studies have used 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* can suppress ROS and RNS production by macrophages *in vitro* (Wellington, Dolan and Krysan, 2009; Collette, Zhou and Lorenz, 2014). Culture supernatant also suppressed macrophage RNS to a lower degree implying secreted compounds are partially involved in RNS suppression (Collette, Zhou and Lorenz, 2014). How *Candida* spp. evade neutrophil RNS is a major knowledge gap, critical to understand 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 behaviours of innate immune cells during infection (Henry *et al.*, 2013; Speirs *et al.*, 2024; 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). Zebrafish models of *C. albicans* infection are well established, with a dose-dependent effect on zebrafish mortality (Chao *et al.*, 2010), and have been used to uncover important disease mechanisms (Rosowski *et al.*, 2018; Archambault *et al.*, 2019; Blair *et al.*, 2025). Furthermore, *C. albicans* is able to undergo the yeast-to-hyphae transition in zebrafish larvae, demonstrating a comparable pathogenesis to human infection (Chao *et al.*, 2010; Gratacap *et al.*, 2017; Seman *et al.*, 2018; Christou *et al.*, 2025).

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 (Hif)-1α increases neutrophil RNS output, which is protective against the bacteria *Mycobacterium marinum* infection in zebrafish (Elks *et al.*, 2013). HIF proteins are

important regulators of cellular responses to hypoxia, innate immunity and inflammation (McGettrick and O'Neill, 2020). HIF-1α proteins are stabilised in hypoxic conditions, or by TLR signalling in normoxia, triggering transcription of target genes that regulate cellular metabolism, proliferation, migration, differentiation, angiogenesis and pro-inflammatory mediators (Peyssonnaux *et al.*, 2005; Jantsch *et al.*, 2011; Krzywinska and Stockmann, 2018; Hammond, Lewis and Elks, 2020). Therefore, we set out to address whether Hif-1α stabilisation 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. Importantly, a similar dampening effect on RNS by *C. albicans* was also observed in human primary neutrophils. RNS suppression was not limited to laboratory strains of *C. albicans*; it was found during infection with *C. albicans* clinical isolates from non-invasive and invasive disease and with other *Candida* species. Heatkilled *C. albicans* was only partially able to decrease neutrophil RNS. Significantly, the ability of strains to reduce neutrophil RNS positively correlated with virulence *in vivo*. Both fungal arginase (car1) and host arginase induction were found to contribute to RNS reduction, with a *car1*Δ mutant having a reduced effect on neutrophil RNS and host arginase induced in zebrafish. Upregulation of host RNS production via Hif-1α stabilisation enhanced host control of *C. albicans* infection. Restoration of neutrophil RNS had an additive effect on host survival and clearance of *C. albicans* infection when combined with antifungals. Together, these data highlight RNS and Hif-1α as potential targets for host directed therapies against *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

136 University of Sheffield Local Ethical Review Panel. Human blood was collected from healthy
137 individuals following informed consent. Ethical approval was granted by the University of
138 Sheffield Research Ethics Committee (study reference number 031773).
139

140 **Zebrafish Husbandry**

141 Zebrafish were maintained in Home Office approved facilities in Biological Services
142 Unit (BSU) aquaria at the University of Sheffield, in accordance with standard protocols and
143 local animal welfare regulations. Adult fish were maintained at 28°C with a 14/10 hour
144 light/dark cycle. Larvae were maintained at 28°C with a 14/10 hour light/dark cycle in 1X E3
145 medium (E3 60X stock: 5.0 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$; diluted
146 to 1X in distilled water) with 0.001% Methylene Blue (Sigma-Aldrich) to prevent fungal growth
147 in the first 24 hours. From 24hpf, larvae were kept in E3 without Methylene Blue so that
148 *Candida* spp. infection was not impacted. The following zebrafish strains were used: nacre
149 (wild type), *Tg(mpx:GFP)i114* (Renshaw *et al.*, 2006), *Tg(lyz:nfsB.mCherry)sh260* (Buchan *et*
150 *al.*, 2019), *Tg(mpeg:nlsClover)*sh436 (Bernut *et al.*, 2019), *TgBAC(arg2:GFP)sh571*
151 (Hammond *et al.*, 2023).
152

153 ***Candida* spp. Culture**

154 *C. albicans* strains (Table 1) and *Candida* spp. clinical isolates (Table 2) were used in
155 this study. *C. albicans* was initially grown on YPD (4001022, MP Bio) plates at 28°C for 48
156 hours, then transferred to overnight liquid culture in YPD broth in a shaking incubator at 30°C
157 at 200 rpm. *C. albicans* was heat-killed by incubation in a heat block at 65°C for 1 hour
158 (Panpetch *et al.*, 2017). To denature fungal proteins, *C. albicans* was boiled at 100°C for 5
159 minutes.
160

161 **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	(Seman *et al.*, 2018)
***C. albicans* SN148 GFP**	SN148 *ENO1/ENO1-GFP::LEU2*	(Christou *et al.*, 2025)
***C. albicans* SC5314**	wt; clinical blood isolate	(Gillum, Tsay and Kirsch, 1984)
C. albicans car1Δ	As SC5314 but *car1Δcar1Δ*	(Schaefer *et al.*, 2020)
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	(Seman *et al.*, 2018)

Table 2: List of *Candida* spp. clinical isolates

Species	Name	Invasive/non-invasive	Sample Site	Source
Candida albicans	AJP4	Non-invasive	Vaginal sample	Provided by Dr David Partridge
Candida albicans	AJP5	Invasive	Blood sample	Collected at Sheffield Children's Hospital
Candida albicans	AJP9	Non-invasive	Tongue swab	Collected at Sheffield Children's Hospital
Candida albicans	AJP25	Invasive	Line tip sample	Collected at Sheffield Children's Hospital

Candida glabrata	AJP12	Invasive	Urine sample	Collected at Sheffield Children's Hospital
Candida parapsilosis	AJP22	Invasive	Urine sample	Collected at Sheffield Children's Hospital
Candida guilliermondii	AJP24	Non-invasive	Tracheal swab	Collected at Sheffield Children's Hospital
Candida auris	StG2	Invasive	Bloodstream	Provided by Dr Tihana Bicanic

164

165 ***C. albicans* Infections**

166 Overnight liquid *C. albicans* cultures were washed 3 times in phosphate buffered saline
167 (PBS) and counted on a haemocytometer. Washed cultures were resuspended in 10% PVP
168 (polyvinylpyrrolidone; Calbiochem) to achieve the desired inoculation. Infection dose for
169 systemic caudal vein infections were 200 colony forming units (cfu) in a 1nl injection volume,
170 100 cfu in 1nl for experiments with immune cell ablation or 500 cfu in 1nl in experiments with
171 antifungals.

172 For survival curves, zebrafish survival was monitored daily and dead zebrafish were
173 removed. Death was determined by a cessation of heartbeat/circulation or tissue degradation
174 post-mortem. At 4 days post infection (dpi; equivalent to 5 days post fertilisation, dpf), all
175 surviving zebrafish larvae were culled.

176

177 **RNA Injections**

178 Larvae were injected with dominant active *hif-1αb* variant RNA (DA1) or dominant
179 negative *hif-1αb* variant RNA in dH_2O with 10% phenol red (Sigma-Aldrich, to visualise
180 successful injection) at the one-cell stage, as previously described (Elks *et al.*, 2011). 10%
181 (v/v) phenol red in dH_2O (PR; Sigma-Aldrich) was used as a solvent control.

182

183 **Morpholino Injections**

184 A *csf3r* morpholino (Gene Tools) was used to deplete neutrophil populations (Ellett *et*
185 *al.*, 2011). A standard control morpholino (Gene Tools) used as a negative control. The
186 antisense morpholino oligonucleotide sequences were: *csf3r* – 5'-
187 GAAGCACAAGCGAGACGGATGCCAT-3'; control – 5'-
188 CCTCTTACCTCAGTTACAATTTATA-3'. *Csf3r* morpholino was functionally validated by
189 whole body neutrophil count at 2 dpf.

190

191 **F0 CRISPR-Cas9 Knockdown**

192 Two sgRNAs were designed to target the ATG/first exon of *nos2a* and *nos2b* to
193 knockdown zebrafish *nos2* genes by CRISPR-Cas9. Efficacy of knockdown of RNS production
194 was functionally validated by whole-body anti-nitrotyrosine staining. Tyrosinase (*tyr*) sgRNA,
195 targeting a pigment gene that plays no role in neutrophil RNS production, was used as a
196 negative control and to demonstrate Cas9 function (Isles *et al.*, 2019). The target sequences
197 were: *nos2a* – 5'-TTTCTCATTTTCAATGATAG-3'; *nos2b* – 5'-
198 GTTCGCTCTTGTGAGTGACC-3'; *tyr* – 5'-CCTGACCTCCTGAAGACCCC-3'. 25μM sgRNA
199 was co-injected with tracrRNA, 1 μl Cas9 and phenol red.

200

201 **Pharmacological Treatment of Zebrafish Larvae**

202 For pharmacological inhibition of nitric oxide synthase, larvae were treated with 200
203 μM N6-(1-Iminoethyl)-lysine (L-NIL) by immersion. dH_2O was used as a solvent control.

204 For antifungal survival curves and clearance assays, larvae were treated with either
205 5.0 μg/ml fluconazole in DMSO or 0.5 μg/ml caspofungin in dH_2O by immersion. DMSO or
206 dH_2O was used as a solvent control. At 2 dpi, larvae were transferred into fresh E3 and
207 treatment was re-administered.

208

209 **Anti-nitrotyrosine Staining**

210 1 dpi larvae (2 dpf) were fixed in 4% formaldehyde in PBS overnight at 4°C. Whole-
211 body nitrotyrosine levels were labelled using a rabbit polyclonal anti-nitrotyrosine antibody (06-
212 284; Merck Millipore) and were detected using Alexa-633 conjugated goat-anti-rabbit
213 secondary antibody (Invitrogen Life Technologies), as previously described (Elks *et al.*, 2013,
214 2014) in a T*g(mpx:GFP)i114* background to assess for neutrophil specific RNS.

215

216 **Microscopy and Quantification of Anti-nitrotyrosine Staining**

217 For *C. albicans* SC5314 and *car1*Δ*,* brightfield images were taken using an inverted
218 Leica DMi8 with a 40x lens and a Hamamatsu OrcaV4 camera. Stained larvae were imaged
219 on a Leica DMi8 inverted microscope with a Leica TCS-SPE line-scanning confocal for
220 imaging with a 40x 1.1NA water immersion lens. For quantification purposes, acquisition
221 settings were kept the same across the groups. Corrected fluorescence intensity was
222 calculated using FIJI measurements assessing the cell fluorescence of individual cells
223 corrected for cell size and background fluorescence of the image (Burgess *et al.*, 2010; Elks
224 *et al.*, 2013, 2014).

225

226 **Hyphal staging**

227 For assessment of fungal growth of *Candida* spp clinical isolates, which lack fluorescent
228 proteins, brightfield images were taken using an inverted Leica DMi8 with a 40x lens and a
229 Hamamatsu OrcaV4 camera. Classification of hyphal stages was done manually, based on a
230 previously described descriptive criteria (Thrikawala *et al.*, 2022). Criteria for hyphal staging
231 were:

232 1. Yeast: only spherical, yeast cells were observed. No signs of any hyphal growth.
233 2. Germinating: ovoid cells, resembling pseudohyphae or very short hyphae, were
234 observed.
235 3. Hyphal extension: a small number of short or medium length hyphae were observed.

236 4. Destructive hyphae: multiple invasive hyphae were observed. Majority of observed *C.*
237 *albicans* was in hyphal morphotype.
238

239 **Human neutrophils**

240 Human neutrophils were isolated by Plasma-Percoll density gradient centrifugation of
241 whole blood from healthy donors as previously described (Herman, Rahman and Prince, 2020).
242 0.5x 10^6 cells were added to individual wells in 96-well plates in 100μl volumes. Cells were
243 primed with 1 μg/ml lipopolysaccharides from *Escherichia coli* (LPS—Sigma, L8274).
244 Neutrophils were infected with an MOI of 1.0 or 0.5 *Candida albicans*, MOI 1.0 heat-killed
245 *Candida albicans* or uninfected (sterile RPMI media) and were incubated at 37°C, 5% CO_2 for
246 3 hours. Cells were cytocentrifuged onto microscope slides and fixed in 4% formaldehyde for
247 staining. Immunostaining was performed as described above.

248

249 **Microscopy of *C. albicans* Infection clearance**

250 Brightfield images were taken using an inverted Leica DMi8 with a 2.5x lens and a
251 Hamamatsu OrcaV4 camera. Surviving fish were classified as 'infection cleared' only if no
252 signs of remaining fluorescent *C. albicans* infection could be observed at 2.5x magnification.

253

254 **Statistical Analysis**

255 All data were analysed using GraphPad Prism 10.5.0 1 (GraphPad Software, La Jolla,
256 California, USA, www.graphpad.com). Nitrotyrosine staining data was analysed with two-tailed
257 Mann-Whitney test or Kruskal-Wallis test, with Dunn's multiple comparisons test. Survival
258 curves were analysed with Gehan-Breslow-Wilcoxon test, with Bonferroni correction. The p
259 values shown are: ns=not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001
260

261 **Results**

262 ***C. albicans* infection suppresses neutrophil RNS production below basal levels**

263 Live *C. albicans* caused a 90.0% reduction in the levels of RNS (visualised using an
264 anti-nitrotyrosine antibody) compared to mock infection (PVP) controls (Figure 1A-B),
265 indicating not only a lack of induction of RNS, but also a downregulation of basal RNS. We
266 have previously shown that RNS is predominantly found in neutrophils both in unchallenged
267 and mock infected (PVP) individuals (Elks *et al.*, 2014) and is upregulated after bacterial
268 challenge with *Mycobacterium marinum* (Elks *et al.*, 2013). This established that *C. albicans*
269 supressed neutrophil RNS *in vivo* in contrast to bacterial challenge. In mock infected (PVP)
270 controls there was a basal level of nitrotyrosine levels in neutrophils, as observed previously
271 (Elks *et al.*, 2013, 2014). We tested another bacterium, *Staphylococcus aureus* to determine
272 if induction of neutrophil RNS was limited to mycobacteria. *S. aureus* (SH1000) infection also
273 caused a significant increase in neutrophil nitrotyrosine fluorescence, corroborating previous
274 observations of increased RNS levels in response to mycobacterial infection (Figure S1; Elks
275 *et al.*, 2013). RNS reduction by *C. albicans* was not limited to a single strain as we infected
276 with a different *C. albicans* strain, SN148 GFP, and observed similar RNS suppression (Figure
277 S2). This allowed the use of TT21-dTomato and SN148 GFP interchangeably, depending on
278 the colour combinations required for each experiment. Zebrafish larvae infected with heat-
279 killed C. albicans TT21-dTomato had an intermediate level of RNS suppression, between the
280 PVP control and live C. albicans infection (Figure 1A-B). Hence, the full effect of neutrophil
281 RNS suppression is dependent on live C. albicans suggestive of partially active and passive
282 mechanisms.

283 To confirm our finding in humans, we performed anti-nitrotyrosine staining on human
284 primary polymorphonuclear neutrophils (PMNs) after *C. albicans* infection *in vitro*. Detectable
285 and robust RNS induction in human neutrophils was induced by priming with LPS which led
286 to a robust nitrotyrosine response after 3 hours in the absence of infection (PVP mock infected)
287 (Figure 1C-D). When *C. albicans* was added at an MOI of 1.0 nitrotyrosine was suppressed to
288 baseline unstimulated levels, despite the presence of LPS (Figure 1C-D). A lower MOI of 0.5

289 live *C. albicans* showed less of an RNS suppression compared to an MOI of 1.0 of live *C.*
290 *albicans*, indicating a dose dependent effect (Figure 1C-D). Similarly, an MOI of 1.0 of heat-
291 killed *C. albicans* exhibited lower RNS suppression compared to the same MOI of live *C.*
292 *albicans*. Together these data show that *C. albicans* suppresses RNS production in human
293 neutrophils as in zebrafish.

294 The laboratory reference strain SC5314 and strains derived from this (including SN148
295 and TT21) have inactivating missense mutations in RNAi component Argonaute and so may
296 behave differently to *C. albicans* strains found in the clinic (Iracane *et al.*, 2024). Therefore,
297 clinical isolates from invasive (AJP5 and AJP25) and non-invasive (AJP4 and AJP9) infections
298 were tested for neutrophil RNS suppression in zebrafish. Our clinical isolates lacked a
299 fluorescent protein marker, so brightfield microscopy was used to identify sites of infection in
300 anti-nitrotyrosine stained zebrafish larvae. Both invasive (AJP5 and AJP25) and non-invasive
301 (AJP4 and AJP9) isolates were able to significantly reduce neutrophil RNS compared to mock
302 infection (PVP) controls (Figure 1E-F). These observations demonstrate neutrophil RNS
303 suppression by *C. albicans* is conserved in clinical isolates, implying RNS suppression in *C.*
304 *albicans* infections may be a clinically relevant phenomenon.

305

306 **Full neutrophil RNS suppression is dependent on hypha-forming *C. albicans***

307 *C. albicans* morphological switch from yeast to hyphae is associated with increased
308 virulence and shifts in the transcriptome and secretome (Nantel *et al.*, 2002; Höfs, Mogavero
309 and Hube, 2016; Vaz *et al.*, 2021). Classification of hyphal growth by *C. albicans* TT21 and
310 clinical isolates in zebrafish revealed the isolates that caused greater neutrophil RNS
311 suppression (AJP9 and AJP25) (Figure 1E-F) exhibited greater hyphal growth *in vivo*
312 compared to those that reduced RNS to a lesser extent (AJP4 and AJP5) (Figure 2A-B). Based
313 on this, we hypothesised hyphal switching may be partially responsible for the suppression of
314 neutrophil RNS. To investigate this, 1 dpf zebrafish were infected with *C. albicans* TT21-
315 dTomato or yeast-locked strain *C. albicans* NRG1^{OEX}-dTomato (Seman *et al.*, 2018). Both *C.*

316 *albicans* NRG1^{OEX}-dTomato and heat-killed *C. albicans* NRG1^{OEX}-dTomato caused a modest
317 decrease in RNS compared to wildtype strains, with reductions comparable to heat-killed *C.*
318 *albicans* (Figure 2C-D). These data suggest that an important component of full RNS
319 suppression by *C. albicans* is hyphal switch-dependent, but that hyphal switch is not essential
320 for neutrophil RNS suppression.
321
322 **Host arginase is induced by *C. albicans***
323 Inducible nitric oxide synthase (iNOS; Nos2 in zebrafish) is the enzyme responsible for
324 RNS production. iNOS competes with the arginase enzyme for a shared substrate, L-arginine,
325 and arginase can competitively inhibit RNS production by iNOS (Bansal and Ochoa, 2003).
326 We investigated the impact of *C. albicans* infection on host arginase levels, as a potential
327 mechanism by which neutrophil RNS is reduced. PVP injected larvae had a low basal level of
328 *arg2:GFP* expression (Figure 3A-B). *Mycobacterium marinum* (used as a control for arginase
329 induction) caused a significant upregulation of *arg2:GFP* compared to PVP, as previously
330 described (Hammond et al., 2023). *C. albicans* TT21-dTomato caused a greater upregulation
331 in *arg2:GFP* expression compared to *M. marinum* indicating a robust host arginase
332 upregulation. *C. albicans* NRG1^{OEX}-dTomato also caused upregulation of *arg2:GFP* compared
333 to both PVP and *M. marinum* indicating this is a hyphal-independent process. Examination of
334 *arg2:GFP* expression in neutrophils (labelled by *mpx:GFP*) revealed *C. albicans* TT21-
335 dTomato caused an increase in the percentage of *arg2:GFP*+ neutrophils (41.59% of the
336 neutrophil population in field of view; Figure 4C-D) compared to PVP (2.01%) and *M. marinum*
337 (10.97%). Together, these results reveal *C. albicans* infection upregulates host arginase
338 expression in a subpopulation of neutrophils to a greater level than the professional bacterial
339 pathogen *M. marinum*.
340
341 ***C. albicans* derived arginase contributes to the suppression of neutrophil RNS**
342 Fungi are known to produce enzymes with similar structure and functions to vertebrate
343 enzymes. *C. albicans* produces its own version of arginase, *CAR1* (Schaefer *et al.*, 2020). We

344 hypothesised that *C. albicans* arginase interfered with the host RNS production, resulting in
345 neutrophil RNS suppression. 1 dpf zebrafish were infected with *C. albicans car1*Δ or its
346 parental strain *C. albicans* SC5314 and RNS production was measured at 1 dpi by anti-
347 nitrotyrosine staining. *C. albicans car1*Δ infection decreased neutrophil RNS production
348 compared to PVP (Figure 4A-B). *C. albicans car1*Δ caused an intermediate level of RNS
349 suppression between live *C. albicans* SC5314 and heat-killed *C. albicans* SC5314 (Figure 4A-
350 B), indicating that CAR1 contributes to the suppression of neutrophil RNS. Heat-killed *C.*
351 *albicans car1*Δ was not significantly different from heat-killed *C. albicans* SC5314. Infection
352 with *C. albicans car1Δ* resulted in significantly greater zebrafish larvae survival than infection
353 with *C. albicans* SC5314 (Figure 4C), suggesting RNS suppression by car1 is important for *C.*
354 *albicans* virulence *in vivo*.
355

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

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

366 When percentage zebrafish survival at 4 dpi were plotted against the percentage
367 reduction of RNS levels by *C. albicans* strains/isolates compared to PVP control, survival
368 negatively correlated with reduction in neutrophil RNS levels (Figure 5C. Full survival curves
369 for *Candida* spp. are in Figure S3 and S4.) (Pearson's correlation coefficient: -0.718, 95%
370 confidence interval: -0.208 – -0.921; $p<0.05$, R^2 value: 0.516). Therefore, a greater

371 suppression of host RNS levels is correlated with increased virulence in zebrafish infection *in*
372 *vivo*, indicating that suppression of neutrophil RNS is a potential virulence mechanism in
373 human disease-relevant *Candida* spp..

374

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

377 Hif-1α is a transcription factor with known roles in regulation of innate immunity
378 (McGettrick and O'Neill, 2020). We have previously shown stabilised Hif-1α (using injection of
379 a dominant active variant RNA) is protective against *M. marinum* infection *in vivo* by increasing
380 neutrophil RNS levels (Elks *et al.*, 2013; Hammond, Lewis and Elks, 2020). We therefore
381 hypothesised that stabilisation of Hif-1α would restore neutrophil RNS in *C. albicans* infection
382 and increase host survival.

383 1 cell stage zebrafish larvae were injected with water/10 % phenol red (solvent) control
384 (PR), dominant negative *hif-1α* (DN *hif-1α*) or dominant active *hif-1α* (DA *hif-1α*) RNA (as
385 previously described; (Elks *et al.*, 2011)), and subsequently infected systemically with *C.*
386 *albicans* TT21-dTomato infection at 30 hpf. DA *hif-1α* larvae had significantly greater survival
387 over the first 4 days post infection than both PR and DN *hif-1α* larvae (Figure 6A),
388 demonstrating that Hif-1α stabilisation is host-protective in *C. albicans* infection.

389 Neutrophils play an essential role in innate immune control of invasive candidiasis
390 (Desai and Lionakis, 2018). In zebrafish neutrophils are responsible for the killing of *C.*
391 *albicans*, whereas macrophages internalise but do not kill (Brothers *et al.*, 2013). Therefore,
392 we hypothesised that the protective effect of Hif-1α stabilisation would be mediated by
393 neutrophils rather than macrophages. Firstly, to assess the impact of Hif-1α stabilisation on
394 macrophage-mediated immunity, we depleted the macrophage population by injection of
395 clodronate liposomes (van Rooijen and Hendrikx, 2010) at 30 hpf (or PBS liposomes as a
396 negative control) leading to a significant depletion of the macrophage population (Figure S5).
397 At 30 hpf, PR or DA *hif-1α* larvae were injected into the caudal vein with clodronate liposomes

398 or PBS liposomes followed by infection with 100cfu *C. albicans* TT21-dTomato at 2 dpf. Both
399 groups of PBS liposome-injected larvae had greater survival than their clodronate liposome,
400 macrophage-depleted, siblings. DA *hif-1α* was host protective both with or without depletion
401 of macrophages, with DA *hif-1α* + clodronate liposome larvae having greater survival than PR
402 + clodronate liposome larvae (Figure 6B; $p<0.0001$), indicating that the host-protective effect
403 of Hif-1α stabilisation is not dependent on the presence of macrophages. Next, we injected 1
404 cell stage zebrafish larvae with a *csf3r* morpholino (Ellett *et al.*, 2011) to deplete neutrophil
405 numbers by over 60% in 2 dpf zebrafish larvae (Figure S6). *csf3r* morpholino injection led to
406 a significant decrease in survival of 100 cfu *C. albicans* infected larvae in both PR and DA *hif-*
407 *1α* larvae (Figure 6C). DA *hif-1α* + *csf3r* larvae had lower survival compared to PR + *csf3r*
408 larvae, showing a loss of the protective effect of Hif-1α stabilisation in neutrophil ablated larvae
409 (Figure 6C), indicating that the protective effect of Hif-1α stabilisation is neutrophil-dependent.
410 DA *hif-1α* did not alter the recruitment of neutrophils to the site of a local hindbrain *C.*
411 *albicans* infection compared to PR injected controls (Figure S7), therefore we hypothesised
412 that the host protective effect of Hif-1α stabilisation in *C. albicans* infection was due to
413 increased neutrophil RNS production. DA *hif-1α* doubled the levels of nitrotyrosine compared
414 to PR in mock infected (PVP) larvae (Figure 7A-B) similar to previous observations (Elks *et*
415 *al.*, 2013). While *C. albicans* reduced nitrotyrosine levels in PR control injected larvae, DA *hif-*
416 *1α C. albicans* infected larvae had high levels of neutrophil nitrotyrosine compared to mock
417 infected (PVP) DA1 controls (Figure 7A-B).
418 To demonstrate that the increase in RNS after Hif-1α stabilisation is responsible for
419 the host protective effect in *C. albicans* infection, we inhibited production of RNS both
420 pharmacologically, using L-NIL, and genetically, using *nos2a+b* double CRISPR-Cas9
421 knockdown ("CRISPants"; Moore *et al.*, 1994; Isles *et al.*, 2019). L-NIL treatment did not
422 impact the growth of *C. albicans in vitro* (Figure S8). Following infection with *C. albicans*, PR
423 + L-NIL-treated larvae had reduced survival compared to PR + dH_2O (drug solvent control)-
424 treated larvae (Figure 7C), indicating a role of RNS in *C. albicans* control. DA *hif-1α* + L-NIL-
425 treated larvae did not have a significant survival advantage compared to either PR + dH_2O or

426 PR + L-NIL groups (Figure 7C), demonstrating that iNOS inhibition is sufficient to remove the
427 host protective effect of Hif-1α stabilisation. *Nos2a+b* double CRISPants were shown to inhibit
428 nitrotyrosine production in mock infected zebrafish (Figure S9). DA *hif-1α* with *nos2a+b*
429 CRISPants no longer had increased survival following *C. albicans* infection compared to PR
430 + *tyr* sgRNA (*tyrosinase* CRISPant control; Isles *et al.*, 2019) larvae, supporting that Nos2 is
431 required for Hif-1α stabilisation dependent host protection (Figure 7D). Together these data
432 demonstrate that the Hif-1α stabilisation is host-protective in *C. albicans* infection via a
433 neutrophil- (Figure 6) and RNS-dependent (Figure 7) mechanism.
434

435 **Increased neutrophil RNS has an additive host protective effect when used alongside**
436 **antifungals**

437 We next investigated the potential of Hif-1α stabilisation to act as an adjunctive therapy
438 alongside established antifungals. At 1 dpf, PR/DA *hif-1α* larvae were injected into the caudal
439 vein with 500 cfu *C. albicans* TT21-dTomato then treated by immersion with 5.0 µg/ml
440 fluconazole or 1.0 µg/ml caspofungin. Both fluconazole (Figure S10) and caspofungin (Figure
441 S11) treatment had dose dependent effects on zebrafish larval survival post *C. albicans*
442 infection. Fluconazole significantly increased host survival in both PR and DA *hif-1α* larvae
443 (Figure 8A). DA *hif-1α* + fluconazole had increased survival compared to PR + fluconazole
444 and DA *hif-1α* + DMSO. Combination of DA *hif-1α* + caspofungin similarly increased survival
445 compared to PR + caspofungin (Figure 8B) and DA *hif-1α* + dH_2O (Figure 8B), demonstrating
446 that combination of Hif-1α stabilisation with fluconazole or caspofungin has an additive effect
447 on survival.

448 Surviving larvae were imaged at 4 dpi to quantify clearance of *C. albicans* infection
449 (Figure 8C-F). 27% of DA *hif-1α* + fluconazole larvae had completely cleared infection at 4 dpi
450 (Figure 8C-D), greater than both PR alone (4%) and PR + fluconazole (8%). 22% of larvae
451 treated with DA *hif-1α* + caspofungin cleared infection (Figure 8E-F) compared to 3% of PR-

452 treated larvae and 4% of larvae treated with PR and caspofungin. Hence, Hif-1α stabilisation
453 also has an additive effect on clearance when combined with fluconazole or caspofungin.

454

455 **Discussion**

456 The increasing prevalence of antifungal resistance worldwide highlights the
457 importance of discovering new antifungal targets or host-directed therapies capable of
458 improving disease outcomes. Here, we have shown that *Candida* spp. suppression of
459 neutrophil RNS is an important factor in their virulence *in vivo* and that restoration of neutrophil
460 RNS represents a potential host directed therapy for difficult-to-treat *C. albicans* infection.

461 We found that *C. albicans* can suppress neutrophil RNS production in zebrafish *in vivo*
462 and in human primary neutrophils *in vitro*. *C. albicans* has been shown to suppress RNS
463 production in murine bone marrow derived macrophages *in vitro* (Collette, Zhou and Lorenz,
464 2014), which is supported by a variety of sources demonstrating suppression of ROS by *C.*
465 *albicans in vitro* (Frohner *et al.*, 2009; Wellington, Dolan and Krysan, 2009). While previous
466 publications have demonstrated RNS suppression in macrophages, our data demonstrates an
467 effect in neutrophils in a live tissue-setting. Human alveolar macrophages are poor RNS
468 producers (Muijsers *et al.*, 2001) and *C. albicans* is primarily controlled by neutrophils in
469 human disease (Gazendam *et al.*, 2016), therefore we chose to dissect the mechanisms
470 behind RNS suppression in neutrophils in the tractable *in vivo* zebrafish model.

471 Here, we identify that RNS suppression is partially dependent on live, hyphae-forming
472 *C. albicans*. The hyphal morphotype is highly associated with a shift in transcription and
473 increased virulence (Sudbery, 2011; Desai, 2018), which may trigger expression of protein(s)
474 necessary for RNS suppression by *C. albicans*. Proteomic analysis of supernatant from hyphal
475 *C. albicans in vitro* identified 301 secreted proteins specific to hyphae (Vaz *et al.*, 2021).
476 Further examination and characterisation of these proteins could point towards candidates
477 involved in active RNS suppression. However, RNS suppression was still observed in non-
478 hyphae-forming, non-albicans *Candida* spp. isolates (*C. glabrata, C. parapsilosis, C.*

479 *guilliermondii*), suggesting that the presence of hyphae themselves is not essential for RNS
480 suppression.

481 Host and *C. albicans* derived arginase also contribute to the suppression of neutrophil
482 RNS production. CAR1 is the only known arginase gene in the *C. albicans* genome, encoding
483 a cytosolic arginase (Schaefer *et al.*, 2020). Fungal arginase may be interfering with the host
484 arginase-iNOS balance, resulting in reduced iNOS activity and RNS suppression. The modest
485 decrease in neutrophil RNS production after infection with the CAR1 mutant suggests that *C.*
486 *albicans* arginase production is not the sole RNS reducing mechanism. Our findings suggest
487 that multiple, additive mechanisms of neutrophil RNS suppression by *C. albicans* may underlie
488 their success at the large overall suppression of neutrophil RNS observed. *C. albicans* SOD5
489 has been associated with degradation of antimicrobial ROS *in vitro* (Frohner *et al.*, 2009), *C.*
490 *albicans* chitin induces host arginase-1 activity, decreasing RNS production (Wagener *et al.*,
491 2017), *YHB1* is able to detoxify RNS (Hromatka, Noble and Johnson, 2005) and Collette *et al.*
492 identified an unknown, small secreted molecule capable of suppressing RNS (Collette, Zhou
493 and Lorenz, 2014). The presence of chitin may account for the partial suppression of RNS by
494 heat-killed *C. albicans* that we observed. Further investigation could aim to establish which
495 are the most important mechanisms and effector proteins involved in RNS suppression in
496 neutrophils.

497 Neutrophil RNS suppression was observed in infection with various *C. albicans*
498 laboratory strains and clinical isolates alongside non-albicans *Candida* spp. clinical isolates.
499 This suggests that RNS suppression could be conserved widely across clinically relevant
500 *Candida* spp. Importantly, *Candida* spp. virulence correlated with neutrophil RNS suppression
501 *in vivo* indicating that neutrophil RNS suppression may be a virulence factor. Virulence is
502 determined by a wide range of factors, not solely neutrophil RNS suppression (Ghannoum
503 and Abu-Elteen, 1990), any of which could be differentially regulated in these *Candida* spp.
504 isolates. It was interesting to note that the emerging human pathogen *C. auris* was able to
505 efficiently suppress neutrophil RNS in zebrafish, despite only been identified as a human
506 pathogen since 2009 (Lockhart *et al.*, 2017), suggesting that this immune evasion mechanism

507 may have evolved in the environment (Casadevall, Kontoyiannis and Robert, 2019).
508 Suppression of neutrophil RNS by environmental strains may act as a useful proxy for
509 determining whether *Candida* spp. or strains have the potential to be virulent in humans.

510 We found that upregulation of RNS, via Hif-1α stabilisation, has a protective effect in
511 *C. albicans* infection in zebrafish larvae, via a neutrophil-mediated, RNS-dependent
512 mechanism. Neutrophils are the primary innate immune cell responsible for killing *Candida*
513 *spp.* (Brown, 2011). Neutrophil suppression is therefore an important mechanism of *C.*
514 *albicans* pathogenesis that, if restored, could represent an exciting therapeutic opportunity.
515 RNS is well characterised as being candidacidal (Rementería, García-Tobalina and Sevilla,
516 1995; Vazquez-Torres *et al.*, 1995; Tillmann, Gow and Brown, 2011; Navarathna, Lionakis
517 and Roberts, 2019) and NO-releasing nanoparticles inhibited the growth of *C. albicans in vitro*
518 and *in vivo* (Macherla *et al.*, 2012). Hence, upregulation of neutrophil RNS production is a
519 potential mechanism that could be targeted therapeutically to improve immune candidacidal
520 activity. We have previously demonstrated Hif-1α stabilisation protects against *M. marinum*
521 infection in a similar manner (Elks *et al.*, 2013), suggesting Hif-1α stabilisation could offer
522 protective effect against multiple and different groups of pathogens (bacteria and fungi) where
523 neutrophil-mediated immune responses are dampened during pathogenesis. Further to this,
524 we showed restoration of neutrophil RNS had an additive effect on survival and clearance
525 when combined with traditional antifungals. Early fungicidal activity by neutrophils prior to
526 hyphal growth is vital for subsequent clearance and resolution of *C. albicans* infection, with
527 early phagocytosis a strong prognostic indicator of survival (Bergeron *et al.*, 2017; Zhu *et al.*,
528 2023). As a primarily fungistatic agent (Vasicek *et al.*, 2014), fluconazole may help to restrict
529 initial growth of *C. albicans*, allowing neutrophils with increased RNS production to kill *C.*
530 *albicans,* resulting in greater survival and clearance. Conversely, caspofungin predominantly
531 has a fungicidal effect (Letscher-Bru and Herbrecht, 2003; Szymański *et al.*, 2022), though
532 increased neutrophil RNS production may also facilitate earlier killing of *C. albicans* in
533 combination and allow lower doses of these poorly tolerated drugs for shorter treatment
534 periods.

535 Our data shows that *Candida* spp. can efficiently suppress neutrophil RNS as a
536 virulence mechanism and that Hif-1α stabilisation is able to overcome this, conferring a
537 neutrophil-mediated, RNS-dependent protective effect *in vivo*. Neutrophil RNS restoration has
538 the potential as an adjunctive host-directed therapeutic strategy to aid in the treatment of
539 *Candida* spp. infections.
540

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548

549 **Author Contributions**

550 Conceived and designed the experiments: TBB, FRH, PTS, AL, SC, SAJ, AMC, PME.
551 Performed the experiments: TBB, FRH, PTS, AL. Generation/provision of *Candida spp*. strains:
552 SC, TB KRA, DGP. Analysed the data: TBB, FRH, PTS, AL, AMC, PME. Wrote and drafted
553 the manuscript: TBB, FRH, PTS, AL, SAJ, AMC, PME.
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Competing Interests

The authors declare that they have no conflict of interest.

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799

800

801 **Figure Legends:**

802 **Figure 1: *Candida albicans* suppresses neutrophil reactive nitrogen species (RNS)**
803 **production below resting levels.**
804 (A) Schematic of experiment: 1 dpf zebrafish were injected with PVP or *C. albicans* into the
805 caudal vein. At 1 dpi, larvae were fixed and stained with anti-nitrotyrosine primary antibody
806 and goat-anti-rabbit Alexa-633 secondary antibody. Zebrafish were then imaged at the site of
807 infection in the caudal haematopoietic tissue (CHT) and anti-nitrotyrosine fluorescence was
808 quantified. Graph shows anti-nitrotyrosine fluorescence at 1 dpi following injection of PVP, live
809 *C. albicans* TT21-dTomato or heat-killed *C. albicans* TT21-dTomato into the caudal vein at 30
810 hpf. N=96-108 neutrophils from 16-18 fish, obtained from 3 independent experiments. Error
811 bars show SEM. Statistical significance determined by Kruskal-Wallis test, then Dunn's
812 multiple comparisons test. P values shown: ****p<0.0001.

813 (B) Representative images of PVP, *C. albicans* TT21-dTomato and heat-killed *C. albicans*
814 TT21-dTomato infected zebrafish larvae at 1 dpi. Scale bars = 50 µm.

815 (C) Corrected fluorescence intensity of PMNs fixed at 3 hours post LPS treatment and stained
816 with anti-nitrotyrosine antibody (in arbitrary units, AU). Non-LPS stimulated resting neutrophils,
817 LPS stimulated neutrophils and a secondary antibody only groups were included as controls.
818 Neutrophils infected with an MOI of 0.5 and 1.0 live *C. albicans* and heat-killed *C. albicans* all
819 show a decrease in nitrotyrosine in the presence of LPS compared to LPS-only controls. N=42-
820 48 neutrophils from 2 independent experiments and donors.
821 (D) Representative images of human PMNs stained with anti-NT. Scale bars = 50 µm. *p<0.05,
822 ****p<0.0001.
823 (E) Anti-nitrotyrosine fluorescence at 1 dpi following injection of PVP, *C. albicans* TT21-
824 dTomato or *C. albicans* clinical isolates into the caudal vein at 30 hpf. N=144 neutrophils from
825 24 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical
826 significance determined by Kruskal-Wallis test, then Dunn's multiple comparisons test. P
827 values shown: **p<0.01, ***p<0.001, ****p<0.0001.

828 (F) Representative images of PVP, *C. albicans* TT21-dTomato, *C. albicans* AJP4, *C. albicans*
829 AJP5, *C. albicans* AJP9 and *C. albicans* AJP25 infected zebrafish larvae at 1 dpi. White dotted
830 circles show *C. albicans.* Scale bars = 50 µm.

831

832 **Figure 2: Robust RNS suppression is associated with hyphae-forming *C. albicans***
833 (A) Hyphal classification of zebrafish larvae infected with range of *C. albicans* isolates at 1 dpi.
834 N=24 fish, obtained from 3 independent experiments.

835 (B) Representative images of hyphal classification in 1 dpi zebrafish larvae infected with *C.*
836 *albicans* TT21-dTomato or *C. albicans* clinical isolate. Images are labelled with the *C. albicans*
837 isolate. White dotted circles show regions of fungal growth. Scale bar = 50 µm.

838 (C) Anti-nitrotyrosine fluorescence at 1 dpi following injection of PVP, *C. albicans* TT21-
839 dTomato, heat-killed *C. albicans* TT21-dTomato*, C. albicans* NRG1^{OEX}-dTomato or heat-killed
840 *C. albicans* NRG1^{OEX}-dTomato into the caudal vein. N=120 neutrophils from 20 fish, obtained
841 from 3 independent experiments. Error bars show SEM. Statistical significance determined by
842 Kruskal-Wallis test, with Dunn's multiple comparisons tests. *p<0.05, ****p<0.0001.
843 (D) Representative images of PVP, *C. albicans* TT21-dTomato, heat-killed *C. albicans* TT21-
844 dTomato*, C. albicans* NRG1^{OEX}-dTomato or heat-killed *C. albicans* NRG1^{OEX}-dTomato
845 infected larvae at 1 dpi. Scale bars = 50 µm.

846

847 **Figure 3: *C. albicans* infection stimulates host *arginase 2* expression.**
848 (A) Corrected fluorescence intensity of *Tg(arg2:GFP)* zebrafish larvae at 1 dpi following
849 injection of PVP, *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato or
850 *M. marinum* Crimson into the caudal vein at 30 hpf. For PVP, *C. albicans* TT21-dTomato and
851 *C. albicans* NRG1^{OEX}-dTomato: n=84-108 cells from 14-18 fish, obtained from 3 independent
852 experiments. For *M. marinum*: n=48 cells from 8 fish, obtained from 2 independent

853 experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test,
854 then Dunn's multiple comparisons test. P values shown: **p<0.01, ****p<0.0001
855 (B) Representative images of *Tg(arg2:GFP)* zebrafish embryos injected with PVP, *C. albicans*
856 TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato or *M. marinum* Crimson at 24hpi. White arrow
857 heads point towards neutrophils. Yellow arrow heads point towards *C. albicans*. Scale bars =
858 50 µm.
859 (C) Number of *lyz:nfsB.mCherry*-expressing cells that are *arg2:GFP*+ and *arg2:GFP-,*
860 following infection with *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato, *M.*
861 *marinum* Crimson or PVP. N=113-199 cells from 12 zebrafish, obtained from 2 independent
862 experiments.
863 (D) Percentage of *lyz:nfsB.mCherry*-expressing neutrophils that are *arg2:GFP*+ and
864 *arg2:GFP-,* following infection with *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato,
865 *M. marinum* Crimson or PVP. N=113-199 cells from 12 zebrafish, obtained from 2 independent
866 experiments.
867

868 **Figure 4: *C. albicans car1* contributes to suppression of neutrophil RNS.**
869 (A) Anti-nitrotyrosine fluorescence at 1 dpi following injection of PVP, *C. albicans* SC5314*,*
870 heat-killed *C. albicans* SC5314*, C. albicans car1*Δ or heat-killed *C. albicans car1*Δ into the
871 caudal vein. N=144 neutrophils from 24 fish, obtained from 3 independent experiments. Error
872 bars show SEM. Statistical significance determined by Kruskal-Wallis test, with Dunn's
873 multiple comparisons tests. *p<0.05, ****p<0.0001.
874 (B) Representative images of anti-nitrotyrosine stained larvae. Dashed lines show *C. albicans*.
875 Scale bars = 50 µm.
876 (C) 1 dpf nacre larvae were injected into the caudal vein with 200 cfu *C. albicans* SC5314 or
877 *C. albicans car1Δ*. Larval survival was measured daily up to 4 dpi. n=180 fish, obtained from
878 3 independent experiments. Error bars show SEM. Statistical significance determined by
879 Gehan-Breslow-Wilcoxon test, with Bonferroni correction. P values shown: ****p<0.0001

880

881 **Figure 5: Non-albicans *Candida* spp. suppress neutrophil RNS.**

882 (A) Anti-nitrotyrosine fluorescence at 1 dpi following injection of PVP, *C. albicans* TT21-
883 dTomato, *C. glabrata* AJP12, *C. parapsilosis* AJP22, *C. guilliermondii* AJP24 or *C. auris* StG2
884 into the caudal vein. N=144 neutrophils from 24 fish, obtained from 3 independent experiments.
885 Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, with Dunn's
886 multiple comparisons tests. * indicates difference compared to PVP. † indicates difference
887 compared to *C. albicans* TT21-dTomato. ‡ indicates difference compared to *C. glabrata*
888 AJP12. P values shown: **p<0.01, ***p<0.001, ****p<0.0001.

889 (B) Representative images of PVP, *C. albicans* TT21-dTomato, *C. glabrata* AJP12, *C.*
890 *parapsilosis* AJP22, *C. guilliermondii* AJP24 or *C. auris* StG2 infected larvae at 1 dpi. Scale
891 bars = 50 µm.

892 (C) Reduction in RNS levels compared to PVP control plotted against zebrafish survival at
893 4dpi, following systemic infection with 200 cfu *Candida* spp. Full survival curves for *Candida*
894 spp. are in Figures S4 and S5. Each plotted point is based on 3 independent repeats of
895 relevant experiment. Line of best fit and 95% confidence error lines are shown. Pearson's
896 correlation coefficient and R^2 value were calculated.

897

898 **Figure 6: Hif-1α stabilisation is protective against *C. albicans* infection.**

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

904 (B) Survival curve of 100 cfu *C. albicans* TT21-dTomato infected zebrafish larvae following
905 injection with DA1 or PR, then treatment with PBS or clodronate liposomes. Mortality was

906 measured daily. N=102-103 fish, obtained from 3 independent experiments. Error bars show
907 SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test with Bonferroni
908 correction. ****p<0.0001.
909 (C) Survival curve of 100 cfu *C. albicans* TT21-dTomato infected zebrafish larvae following
910 injection with PR or DA1 and control or *csf3r* morpholino. Mortality was measured daily. N=135
911 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance
912 determined by Gehan-Breslow-Wilcoxon test with Bonferroni correction. *p<0.05, **p<0.01.
913
914 **Figure 7: The host protective effect of Hif-1α stabilisation is RNS-dependent.**
915 (A) Anti-nitrotyrosine fluorescence at 1 dpi in PR and DA1 injected fish, following injection of
916 PVP or 500 cfu *C. albicans* SN148 GFP. N=108 neutrophils from 18 fish, obtained from 3
917 independent experiments. Error bars show SEM. Statistical significance determined by
918 Kruskal-Wallis test, with Dunn's multiple comparisons test. ***p<0.001, ****p<0.0001
919 (B) Representative images of 1 dpi Tg(*lyz:NTRmCherry*) zebrafish larvae injected with PR or
920 DA1 and infected with 500 cfu *C. albicans* SN148 GFP or PVP. Scale bars = 50 µm.
921 (C) Survival curve of 200 cfu *C. albicans* TT21-dTomato infected zebrafish larvae following
922 injection with PR or DA1, then treatment with dH_2O or L-NIL. N=135 fish, obtained from 3
923 independent experiments. Error bars show SEM. Statistical significance determined by
924 Gehan-Breslow-Wilcoxon test, with Bonferroni correction. **p<0.01, ***p<0.001.
925 (D) Survival curve of 200 cfu *C. albicans* TT21-dTomato infected zebrafish larvae following
926 injection with PR or DA1 and *tyr* or *nos2a+b* sgRNA. N=135 fish, obtained from 3 independent
927 experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-
928 Wilcoxon test, with Bonferroni correction. **p<0.01, ***p<0.001.
929
930 **Figure 8: Hif-1α stabilisation has an additive effect with antifungals.**
931 (A) Survival of *C. albicans* TT21-dTomato infected zebrafish larvae following injection with
932 dominant active *hif-1α* (DA1) or phenol red (PR) control. Larvae were treated with 5.0 µg/ml

933 fluconazole or DMSO solvent control. Mortality was measured daily. N=135 fish, obtained from
934 3 independent experiments. Error bars show SEM. Statistical significance determined by
935 Gehan-Breslow-Wilcoxon test with Bonferroni correction. **p<0.01, ****p<0.0001

936 (B) Survival of *C. albicans* TT21-dTomato infected zebrafish larvae following injection with
937 DA1or PR control. Larvae were treated with 1.0 µg/ml caspofungin or dH_2O solvent control.
938 Mortality was measured daily. N=135 fish, obtained from 3 independent experiments. Error
939 bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test with
940 Bonferroni correction. **p<0.01, ****p<0.0001

941 (C) Clearance of *C. albicans* infection at 4 dpi by zebrafish larvae following injection with DA1
942 or PR control and treatment with 5.0 µg/ml fluconazole or DMSO solvent control. N=3
943 independent experiments, 135 fish per group. Error bars show Standard Deviation. Statistical
944 significance determined by one-way ANOVA, with Tukey's multiple comparisons test. *p<0.05,
945 **p<0.01

946 (D) Infection outcomes of *C. albicans* TT21-dTomato infected zebrafish larvae at 4 dpi,
947 following injection with DA1 or PR and treatment with 5.0 µg/ml fluconazole or DMSO solvent
948 control. N=3 independent experiments, 135 fish per group.

949 (E) Clearance of *C. albicans* infection at 4 dpi by zebrafish larvae following injection with DA1
950 or PR control and treatment with 1.0 µg/ml caspofungin or dH_2O solvent control. N=3
951 independent experiments, 135 fish per group. Error bars show Standard Deviation. Statistical
952 significance determined by one-way ANOVA, with Tukey's multiple comparisons test.

953 (F) Infection outcomes of *C. albicans* TT21-dTomato infected zebrafish larvae at 4 dpi,
954 following injection with DA1 or PR and treatment with 1.0 µg/ml caspofungin or dH_2O solvent
955 control. N=3 independent experiments, 135 fish per group.

956

Figure 1

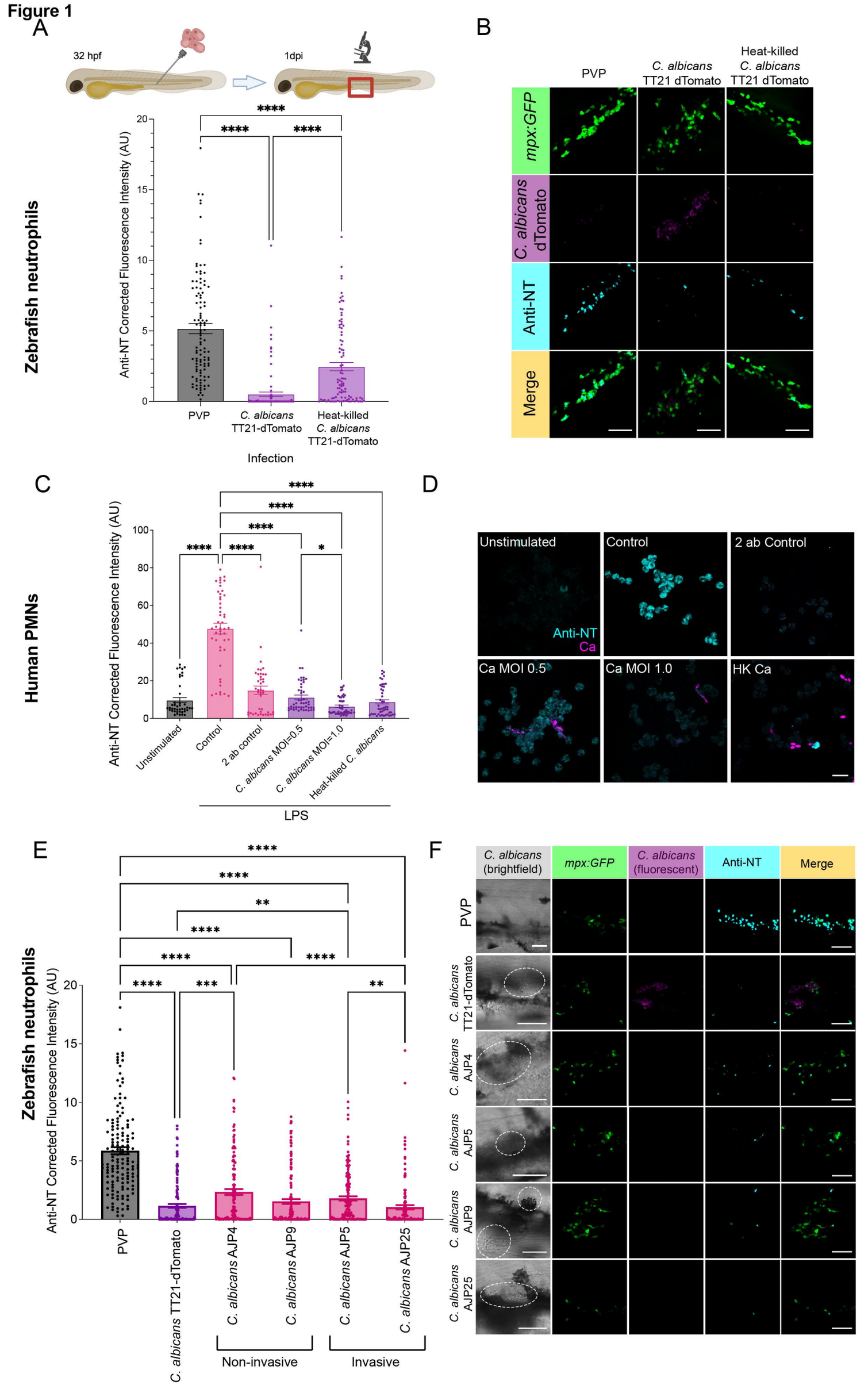

A
32 hpf
1dpi
Zebrafish neutrophils
Anti-NT Corrected Fluorescence Intensity (AU)
PVP
C. albicans TT21-dTomato
Heat-killed C. albicans TT21-dTomato
Infection
B
PVP
C. albicans TT21 dTomato
Heat-killed C. albicans TT21 dTomato
mpx:GFP
C. albicans dTomato
Anti-NT
Merge
C
Human PMNs
Anti-NT Corrected Fluorescence Intensity (AU)
Unstimulated
Control
2 ab control
C. albicans MOI=0.5
C. albicans MOI=1.0
Heat-killed C. albicans
LPS
D
Unstimulated
Control
2 ab Control
Anti-NT
Ca
Ca MOI 0.5
Ca MOI 1.0
HK Ca
E
Zebrafish neutrophils
Anti-NT Corrected Fluorescence Intensity (AU)
PVP
C. albicans TT21-dTomato
C. albicans AJP4
C. albicans AJP9
C. albicans AJP5
C. albicans AJP25
Non-invasive
Invasive
F
C. albicans (brightfield)
mpx:GFP
C. albicans (fluorescent)
Anti-NT
Merge
PVP
C. albicans TT21-dTomato
C. albicans AJP4
C. albicans AJP5
C. albicans AJP9
C. albicans AJP25

Figure 2

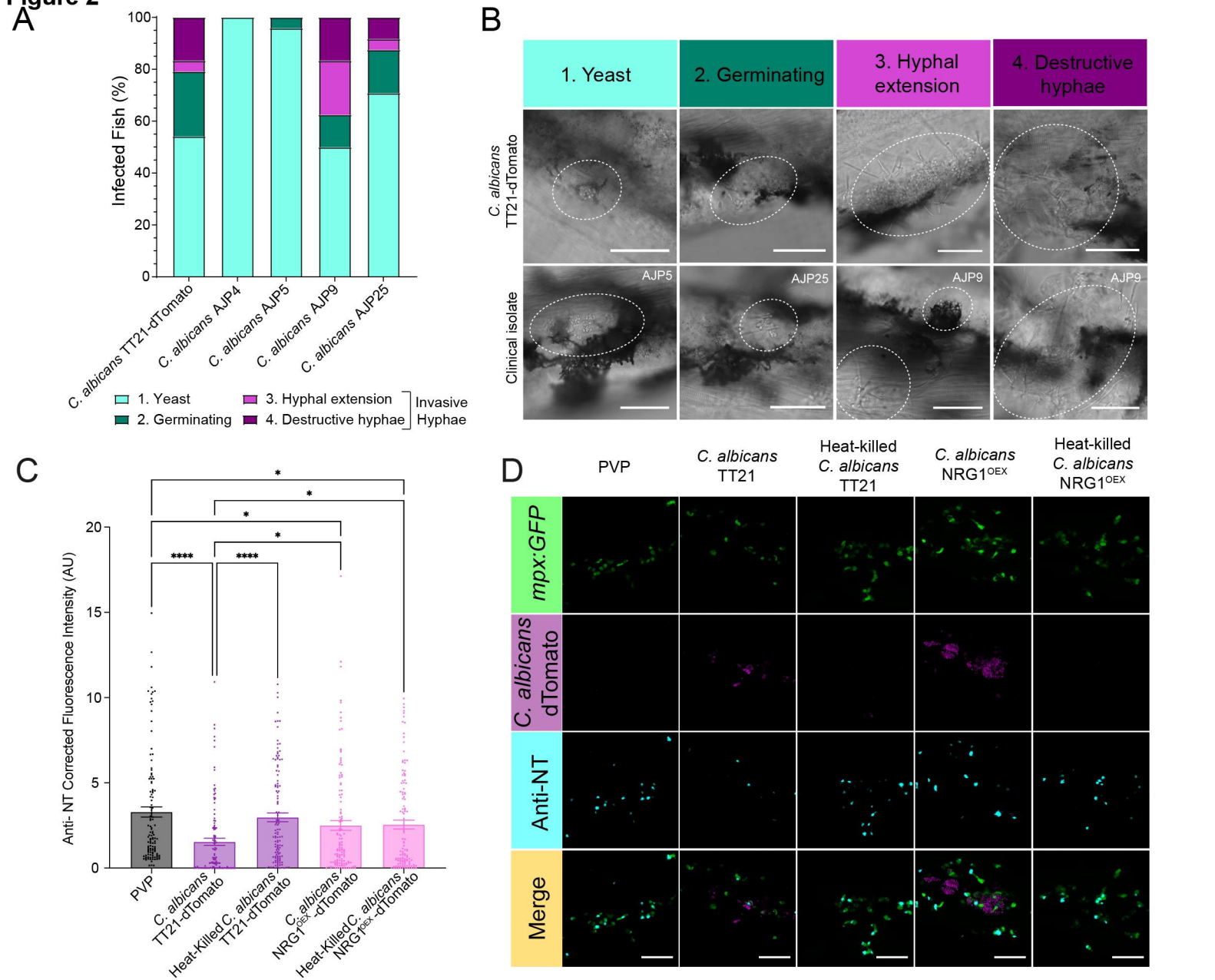

A
Infected Fish (%)
100
80
60
40
20
0
C. albicans TT21-dTomato
C. albicans AJP4
C. albicans AJP5
C. albicans AJP9
C. albicans AJP25
1. Yeast
2. Germinating
3. Hyphal extension
4. Destructive hyphae
Invasive Hyphae
B
1. Yeast
2. Germinating
3. Hyphal extension
4. Destructive hyphae
C. albicans TT21-dTomato
Clinical isolate
AJP5
AJP25
AJP9
AJP9
C
Anti- NT Corrected Fluorescence Intensity (AU)
20
15
10
5
0
****
****
*
*
*
*
PVP
C. albicans TT21-dTomato
Heat-Killed C. albicans TT21-dTomato
C. albicans NRG1OEX-dTomato
Heat-Killed C. albicans NRG1OEX-dTomato
D
PVP
C. albicans TT21
Heat-killed C. albicans TT21
C. albicans NRG1OEX
Heat-killed C. albicans NRG1OEX
mpx:GFP
C. albicans dTomato
Anti-NT
Merge

Figure 3

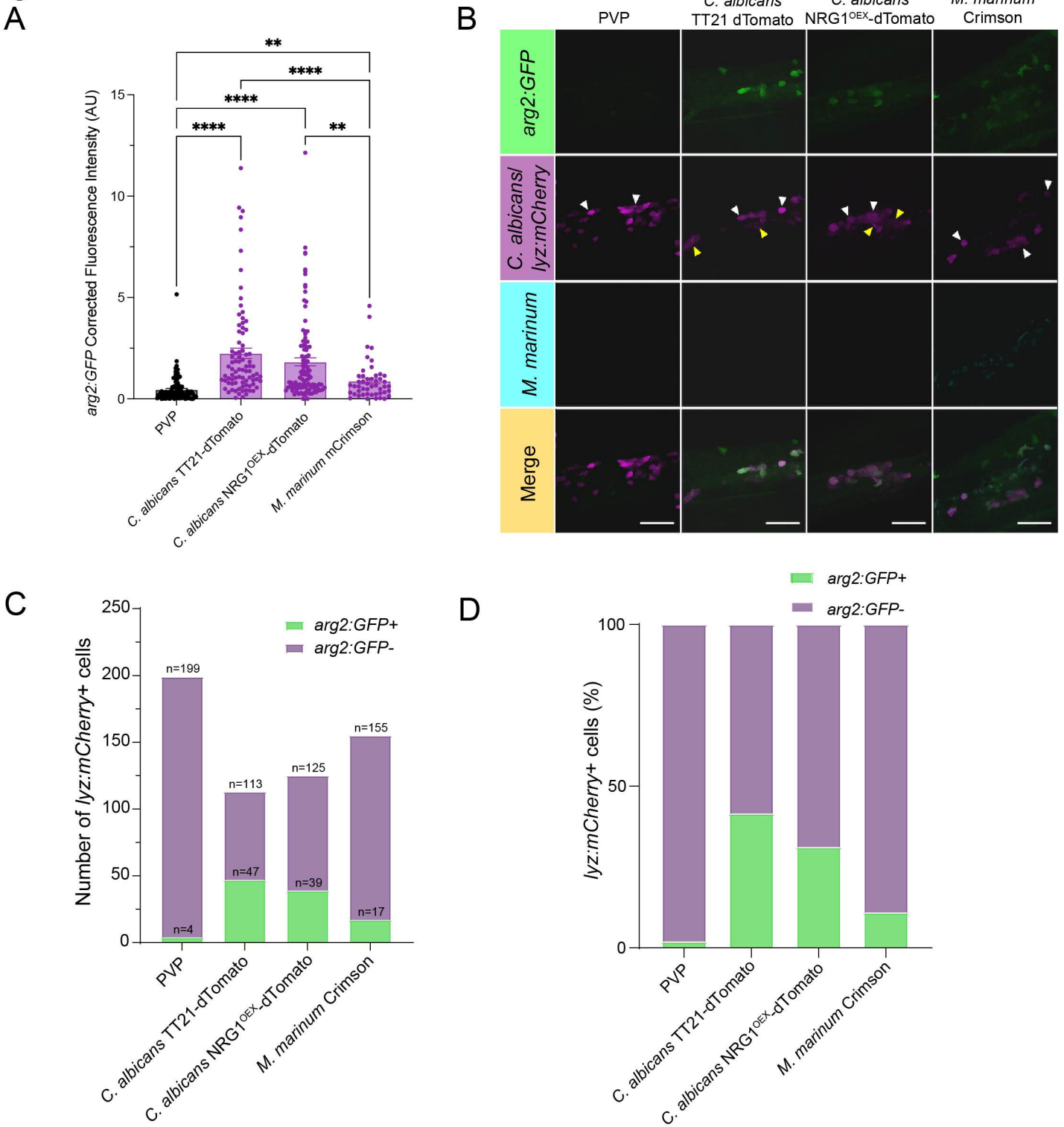
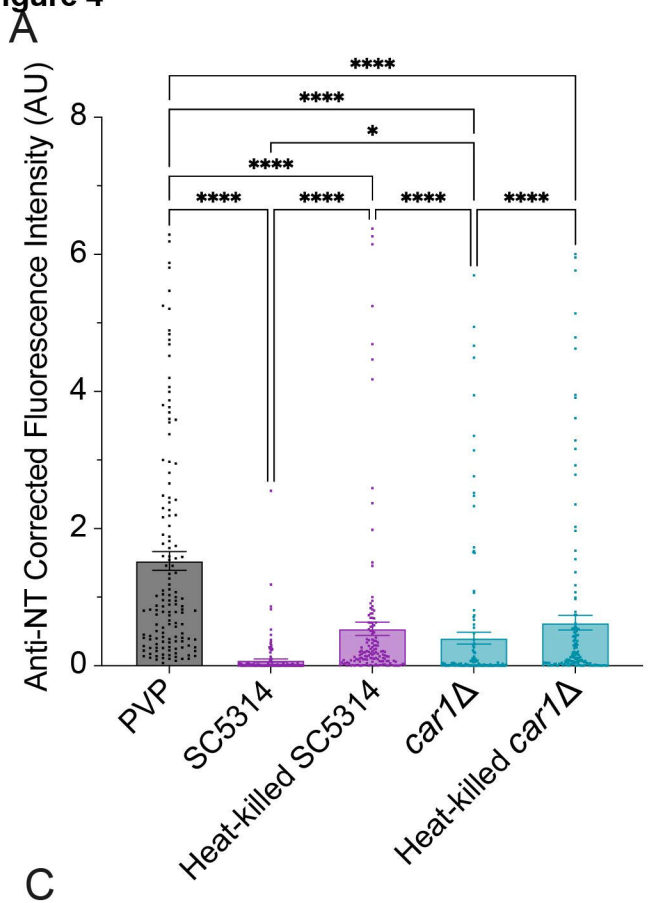
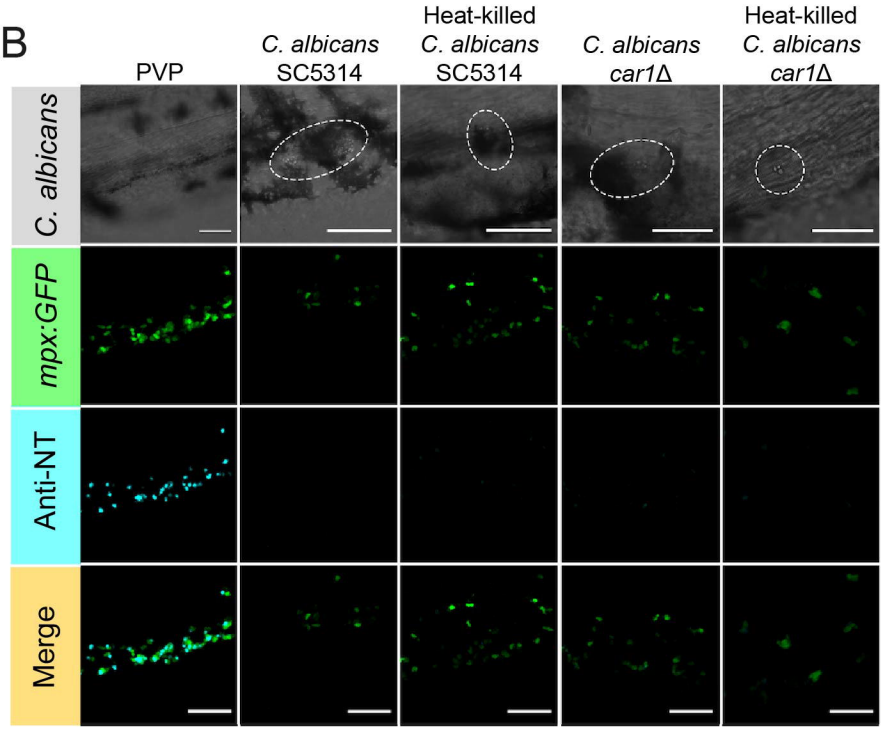

A
arg2:GFP Corrected Fluorescence Intensity (AU)
15
10
5
0
**
****
****
****
**
PVP
C. albicans TT21-dTomato
C. albicans NRG1OEX-dTomato
M. marinum mCrimson
B
PVP
C. albicans TT21 dTomato
C. albicans NRG1OEX-dTomato
M. marinum Crimson
arg2:GFP
C. albicans/ lyz:mCherry
M. marinum
Merge
C
Number of lyz:mCherry+ cells
250
200
150
100
50
0
arg2:GFP+
arg2:GFP-
n=199
n=4
n=113
n=47
n=125
n=39
n=155
n=17
PVP
C. albicans TT21-dTomato
C. albicans NRG1OEX-dTomato
M. marinum Crimson
D
arg2:GFP+
arg2:GFP-
lyz:mCherry+ cells (%)
100
50
0
PVP
C. albicans TT21-dTomato
C. albicans NRG1OEX-dTomato
M. marinum Crimson

Figure 4


A
Anti-NT Corrected Fluorescence Intensity (AU)
0
2
4
6
8
****
****
*
****
****
****
****
****
PVP
SC5314
Heat-killed SC5314
car1Δ
Heat-killed car1Δ


B
PVP
C. albicans SC5314
Heat-killed C. albicans SC5314
C. albicans car1Δ
Heat-killed C. albicans car1Δ
C. albicans
mpx:GFP
Anti-NT
Merge

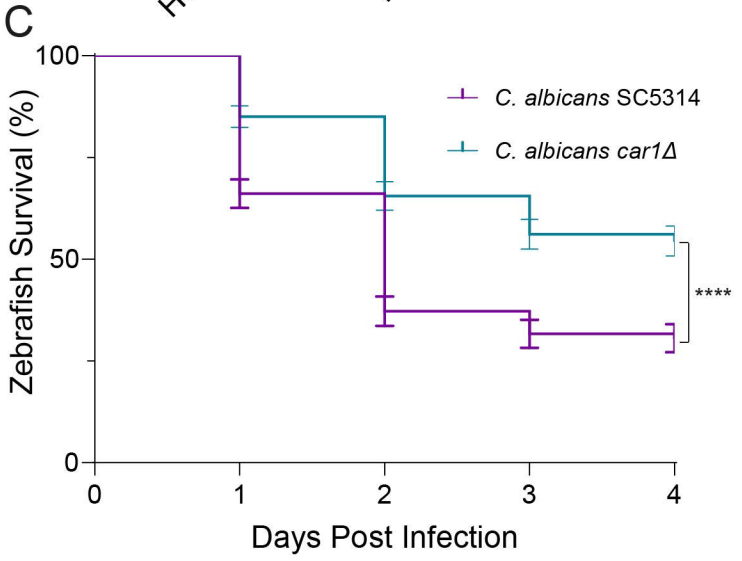
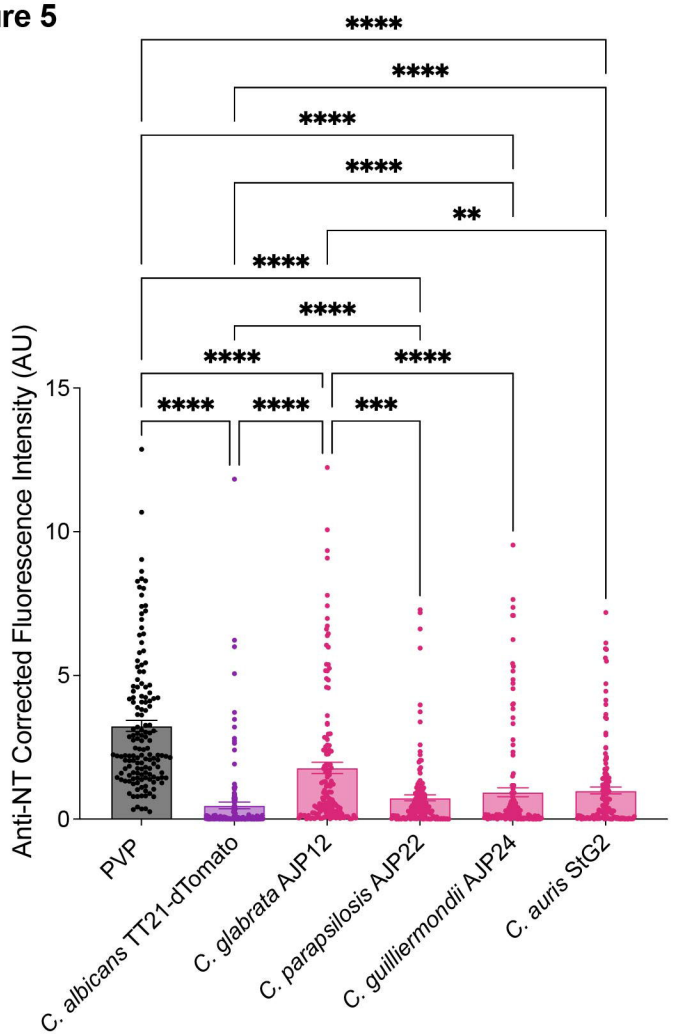
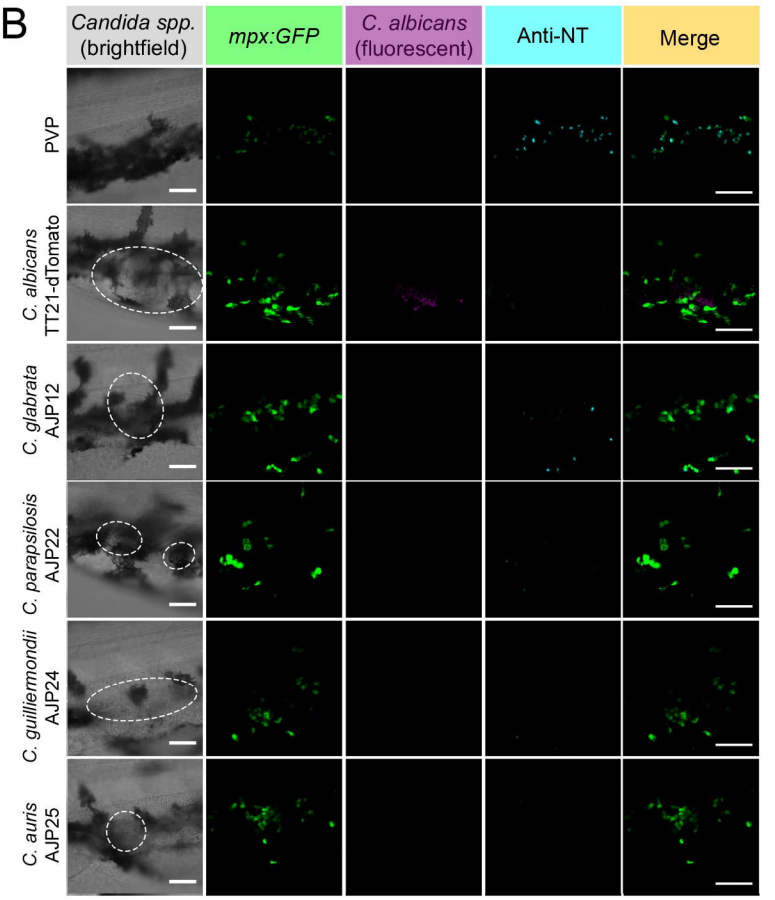

C
Zebrafish Survival (%)
0
50
100
C. albicans SC5314
C. albicans car1Δ
****
0
1
2
3
4
Days Post Infection

Figure 5


A
Anti-NT Corrected Fluorescence Intensity (AU)
15
10
5
0
****
**
***
PVP
C. albicans TT21-dTomato
C. glabrata AJP12
C. parapsilosis AJP22
C. guilliermondii AJP24
C. auris StG2


B
Candida spp. (brightfield)
mpx:GFP
C. albicans (fluorescent)
Anti-NT
Merge
PVP
C. albicans TT21-dTomato
C. glabrata AJP12
C. parapsilosis AJP22
C. guilliermondii AJP24
C. auris AJP25

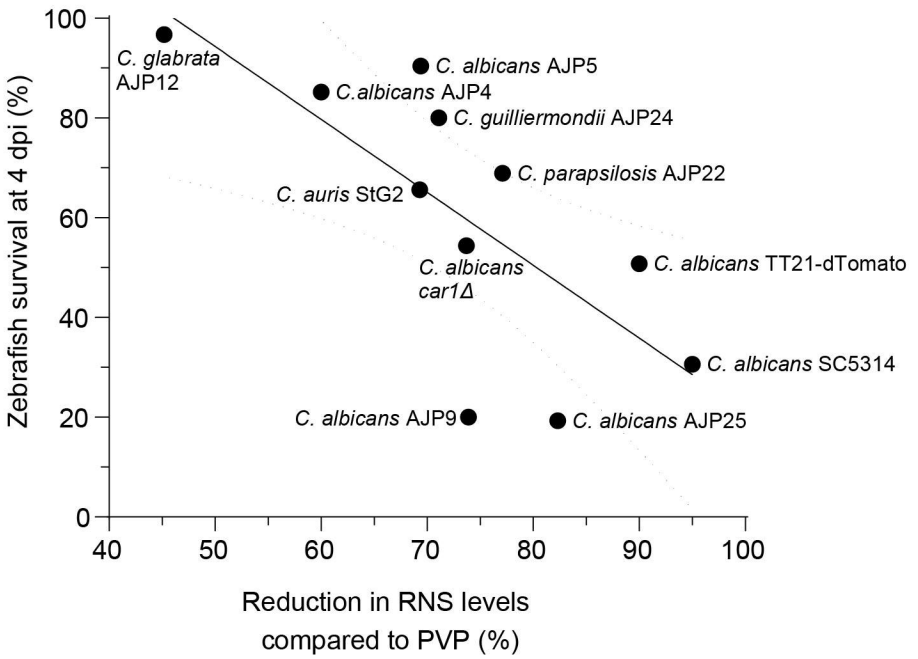

C
Zebrafish survival at 4 dpi (%)
100
80
60
40
20
0
40
50
60
70
80
90
100
Reduction in RNS levels compared to PVP (%)
C. glabrata AJP12
C. albicans AJP5
C.albicans AJP4
C. guilliermondii AJP24
C. parapsilosis AJP22
C. auris StG2
C. albicans car1Δ
C. albicans TT21-dTomato
C. albicans SC5314
C. albicans AJP9
C. albicans AJP25

Figure 6

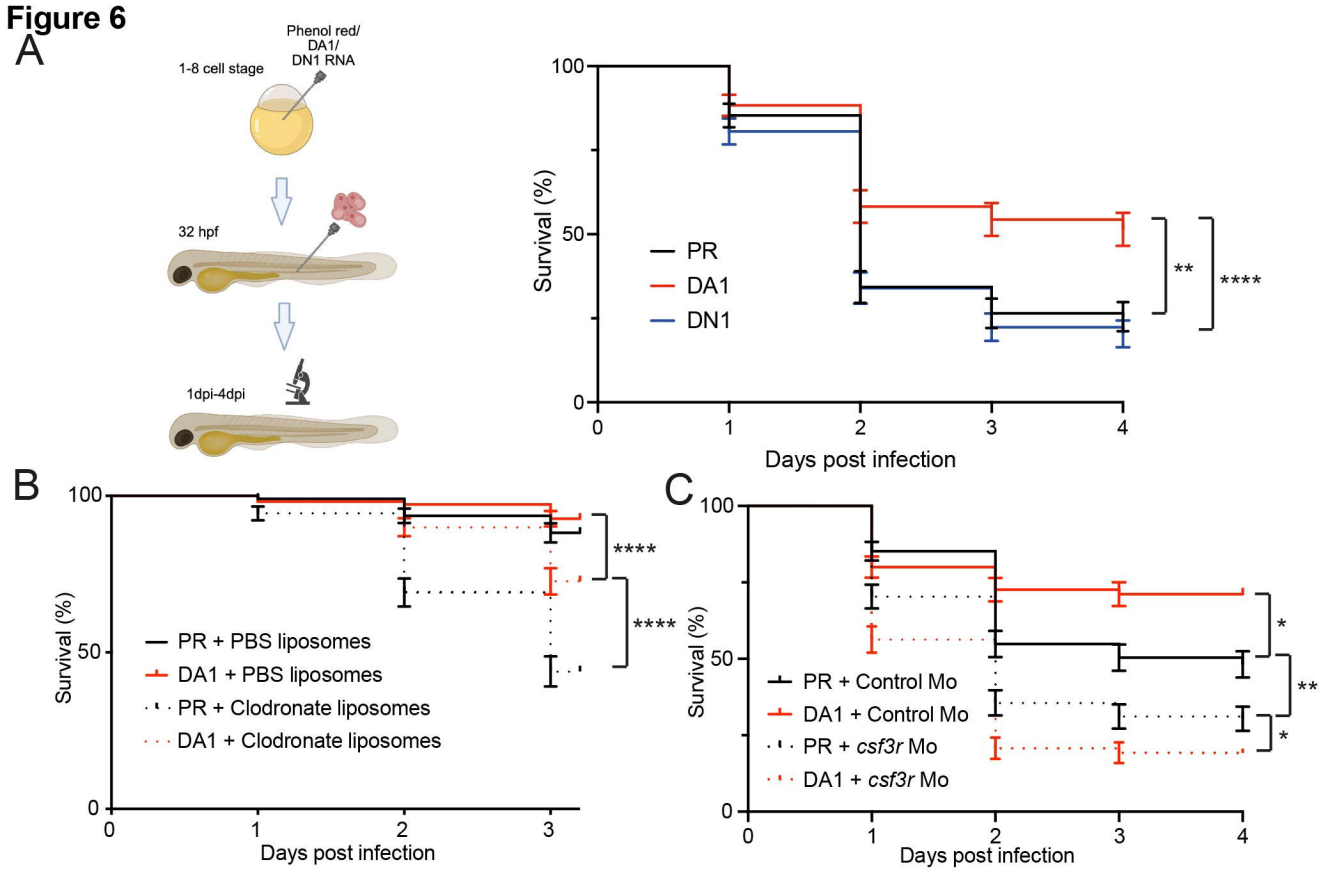

A
Phenol red/
DA1/
DN1 RNA
1-8 cell stage
32 hpf
1dpi-4dpi
Survival (%)
Days post infection
PR
DA1
DN1
**
****
B
PR + PBS liposomes
DA1 + PBS liposomes
PR + Clodronate liposomes
DA1 + Clodronate liposomes
****
****
C
PR + Control Mo
DA1 + Control Mo
PR + csf3r Mo
DA1 + csf3r Mo
*
**
*

Figure 7

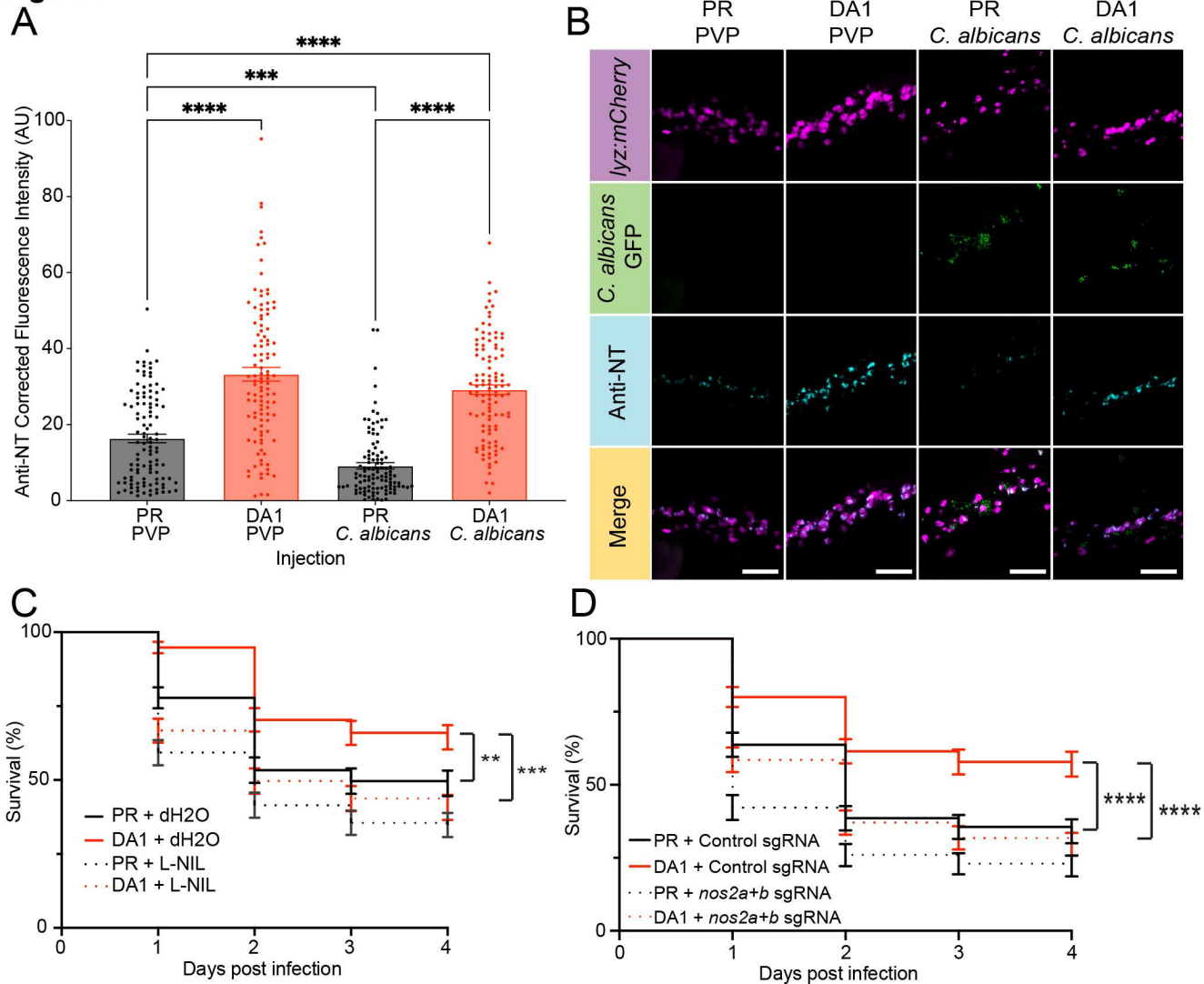
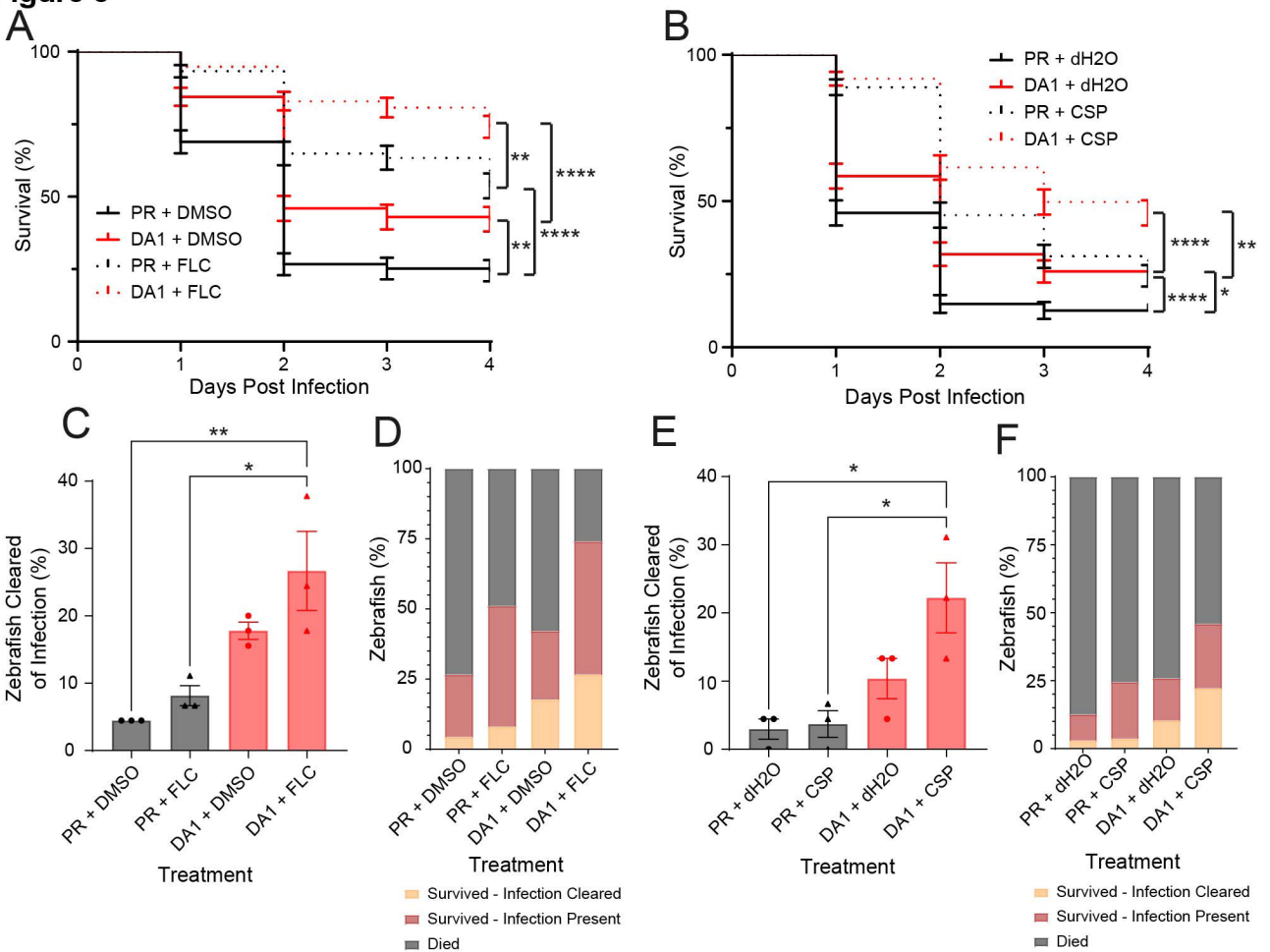

A
****
***
****
****
Anti-NT Corrected Fluorescence Intensity (AU)
100
80
60
40
20
0
PR PVP
DA1 PVP
PR C. albicans
DA1 C. albicans
Injection
B
PR PVP
DA1 PVP
PR C. albicans
DA1 C. albicans
lyz:mCherry
C. albicans GFP
Anti-NT
Merge
C
Survival (%)
100
50
0
PR + dH2O
DA1 + dH2O
PR + L-NIL
DA1 + L-NIL
**
***
0
1
2
3
4
Days post infection
D
Survival (%)
100
50
0
PR + Control sgRNA
DA1 + Control sgRNA
PR + nos2a+b sgRNA
DA1 + nos2a+b sgRNA
****
****
0
1
2
3
4
Days post infection

Figure 8


A
Survival (%)
Days Post Infection
PR + DMSO
DA1 + DMSO
PR + FLC
DA1 + FLC
**
****
**
****
B
Survival (%)
Days Post Infection
PR + dH2O
DA1 + dH2O
PR + CSP
DA1 + CSP
****
**
****
*
C
Zebrafish Cleared of Infection (%)
Treatment
PR + DMSO
PR + FLC
DA1 + DMSO
DA1 + FLC
**
*
D
Zebrafish (%)
Treatment
PR + DMSO
PR + FLC
DA1 + DMSO
DA1 + FLC
Survived - Infection Cleared
Survived - Infection Present
Died
E
Zebrafish Cleared of Infection (%)
Treatment
PR + dH2O
PR + CSP
DA1 + dH2O
DA1 + CSP
*
*
F
Zebrafish (%)
Treatment
PR + dH2O
PR + CSP
DA1 + dH2O
DA1 + CSP
Survived - Infection Cleared
Survived - Infection Present
Died