Investigation of *Candida albicans* Suppression of Neutrophil Reactive Nitrogen Species in Zebrafish Larvae

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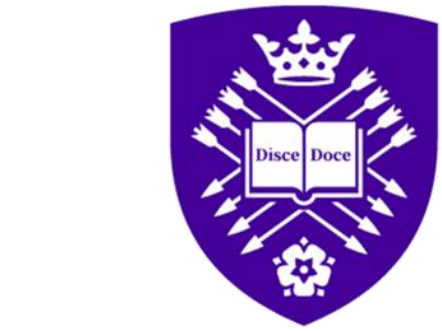

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Abstract

Though often a harmless commensal, *C. albicans* causes frequent mucosal infections and can cause life-threatening, invasive infections, especially in immunocompromised individuals. Greater understanding of *C. albicans* pathogenesis *in vivo* is required. I investigated *C. albicans* interactions with neutrophils, using zebrafish *in vivo* models, aiming to unravel mechanisms of *C. albicans* pathogenesis and host-pathogen interactions.

Reactive nitrogen species (RNS) are a critical host antimicrobial to kill *C. albicans*. I showed *C. albicans* suppresses neutrophil RNS levels in zebrafish. Heat-killed *C. albicans* caused an intermediate degree of RNS suppression, implying a passive mechanism for RNS suppression. Live *C. albicans* suppressed neutrophil RNS proximal to the site of infection, and also distally. My data support prior observations of *C. albicans* suppression of mammalian macrophage RNS *in vitro*.

I investigated mechanisms underlying neutrophil RNS suppression by *C. albicans in vivo*. *C. albicans* hyphae and arginase (car1) were revealed to have a role in RNS suppression.

Neutrophil RNS suppression was observed across a selection of *C. albicans* and non-albicans *Candida* spp. clinical isolates. RNS suppression by *Candida* spp. correlated with virulence in zebrafish *in vivo*, suggesting RNS suppression is important for *Candida* spp. pathogenesis in human disease.

Hif-1α is a potential host directed therapy (HDT) target, known to increase neutrophil RNS production in bacterial infection. However, the role of Hif-1α is poorly characterised in *C. albicans* infections. Hif-1α stabilisation protected against *C. albicans* infection in zebrafish *in vivo*, via a neutrophil-mediated, RNS-dependent mechanism, rescuing neutrophil RNS levels after *C. albicans* infection. Combination of Hif-1α with antifungals had an additive effect on zebrafish survival and clearance of *C. albicans* infection. Hence, Hif-1α is a potential target for a HDT for *C. albicans* infections.

This thesis demonstrated *C. albicans* suppression of neutrophil RNS *in vivo*, a potentially important strategy in *Candida* pathogenesis, and highlighted the potential of targeting Hif-1α and RNS in HDTs against *C. albicans* infections.

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Figure 0.1: Millie and Maddie

(A) Millie and Maddie running in the snow at Haytor, Dartmoor, on Christmas Day 2010

(B) Millie waiting patiently for her dinner

(C) Maddie waiting, less patiently, for her dinner

Abbreviation list

AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
BMDM	Bone marrow derived macrophage
CARD	Caspase activation and recruitment domain
CDC	Centre for Disease Control and Prevention
cDNA	Coding DNA
cfu	Colony forming units
CHT	Caudal haematopoietic tissue
CLP	Caecal ligation and puncture
CLR	C-type lectin receptor
CMC	Chronic mucocutaneous candidiasis
CR	Complement receptor
CRISPR	Clustered regularly interspaced short palindromic repeats
Csf3r	Colony stimulating factor 3 receptor
Csf3a	Colony stimulating factor 3a
CSP	Caspofungin
DA1	Dominant active Hif-1α
DAMP	Damage associated molecular pattern
DEPC	Diethyl pyrocarbonate
dH_2O	Distilled H_2O
DMOG	Dimethyloxalylglycine
DMSO	Dimethyl sulfoxide
DN1	Dominant negative Hif-1α
DNA	Deoxyribonucleic acid
dpf	Days post fertilisation
dpi	Days post infection
DTPA	Dipropylenetriamine
ED50	Effective dose for 50% population
eNOS	Endothelial nitric oxide synthase
FBS	Foetal bovine serum
FIH	Factor inhibiting HIF
FLC	Fluconazole
G-CSF	Granulocyte colony-stimulating factor

gDNA	Genomic DNA
GFP	Green fluorescent protein
GlcNAc	N-Acetylglucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
gRNA	Guide RNA
HDT	Host directed therapy
HIF	Hypoxia inducible factor
HIV	Human immunodeficiency virus
hpf	Hours post fertilisation
hpi	Hours post infection
HRE	Hypoxia responsive element
HSC	Haematopoietic stem cell
ICU	Intensive care unit
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
iNOS	Inducible nitric oxide synthase
LD50	Lethal dose for 50% population
L-NIL	N^6-(1-Iminoethyl)-lysine, dihydrochloride
L-NMMA	N-Monomethyl-L-arginine
LPS	Lipopolysaccharide
LTA4H	Leukotriene A4 hydrolase
LTB_4	Leukotriene B4
MARCO	Macrophage receptor with collagenous domain
MAVS	Mitochondrial antiviral-signaling
MBL	Mannan-binding lectin
MDSC	Myeloid-derived suppressor cell
MIC	Minimum inhibitory concentration
Mo	Morpholino
mRNA	Messenger RNA
MTL	Mating-type-like
MYD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate

NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NT	Nitrotyrosine
OD	Optical density
PAD4	Peptidylarginine deiminase 4
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PBSTx	PBS-0.4% Triton X-100
PCR	Polymerase chain reaction
PD	Programmed death
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PHD	Prolyl hydroxylases domain enzymes
PMN-DC	Polymorphonuclear-dendritic cell
PR	Phenol red
PRR	Pattern recognition receptor
PVP	Polyvinylpyrrolidone
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
RNA	RNA interference
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RTD-1	Rhesus theta defensin-1
RVVC	Recurrent vulvovaginal candidiasis
Saps	Secreted aspartyl proteinases
SEM	Standard error of the mean
siCSP	Sub-inhibitory caspofungin
SOD	Superoxide dismutase
SS	Sheep serum
STAT	Signal transducers and activators of transcription

syk	Spleen tyrosine kinase
Th	T helper
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TRIM	Tripartite motif-containing protein
tRNA	Transfer RNA
UK	United Kingdom
USA	United States of America
USD	United States Dollars
VHL	von Hippel-Lindau protein
WHO	World Health Organisation
YPD	Yeast peptone dextrose

Declaration

I, the author, confirm that the Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not previously been presented for an award at this, or any other, university.

Part of the work discussed in this thesis has been published in various publications, listed below:

- Burgess, T. B., Condliffe, A. M., & Elks, P. M. (2022). A Fun-Guide to Innate Immune Responses to Fungal Infections. *Journal of Fungi*, *8*(8), 805. https://doi.org/10.3390/jof8080805
- Burgess, T. B., Hammond, F. R., Szkuta, P. T., Lewis, A, Christou, S, Ayscough, K. R., Johnston, S. A., Condliffe, A. M., & Elks, P. M. (2023). *Candida albicans* suppression of neutrophil reactive nitrogen species is rescued by host Hif-1α *in vivo*. bioRxiv (Preprint), 2023.11.02.565128. https://doi.org/10.1101/2023.11.02.565128

Part of the work in this thesis was carried out by other individuals. Their names, and specific contributions, are listed below:

- Dr Ffion Hammond – previous PhD student in Elks lab
    - Figure 6.2: Hif-1α is protective against *C. albicans* infection *in vivo*
    - Figure 6.3: Hif-1α stabilisation protects against *C. albicans* infection *in vivo* in the absence of macrophages
    - Figure 6.10: Hif-1α stabilisation restores neutrophil RNS level in *C. albicans* infection
- Imogen Ashcroft and Shami Vahivatdar – undergraduate medical students, carrying out SSC project under my supervision
    - Figure 5.2B,C: *C. albicans* clinical isolates grow at 30 °C *in vitro*
    - Figure 5.6: Non-albicans *Candida* spp. clinical isolates grow at 28 °C and 37 °C *in vitro*

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1. Introduction

1.1 The Fungal Kingdom

Fungi are a highly diverse kingdom of eukaryotic organisms, comprised of microorganisms (such as yeasts and moulds) and macroscopic mushrooms. It is estimated there are between 2.2 and 3.8 million fungal species (Sun, Hoy and Heitman, 2020). Fungi are heterotrophic and non-photosynthetic, so obtain their nutrients from external sources, often by secretion of digestive enzymes into the environment (Buckley, 2008). Fungi have previously been characterised by the presence of chitin in the cell wall and are associated with a range of traits not necessarily exhibited by all fungi, including polarised multicellular growth, fruiting body development and dimorphism (Buckley, 2008; Nagy *et al.*, 2017). However, Richards *et al.* argue that there is no defining characteristic of fungi, with features used to define the fungal kingdom only partially present in fungi or present in non-fungal organisms, such as protists (Richards, Leonard and Wideman, 2017).

Fungi have a diverse range of functions, reflective of their genomic and phenotypic heterogeneity. In the environment, fungi are primarily decomposers of organic material, with roughly 50% of dead plant and animal matter in tropical rainforests degraded by fungi (Buckley, 2008). Many fungi establish symbiotic relationships with plant roots, known as mycorrhizae. Over 250,000 plant species are associated with mycorrhizal fungi, which facilitate nutrient and water uptake by plants, promote seedling establishment, and transfer nutrients between connected plants (Buckley, 2008; Martin and van der Heijden, 2024). The most obvious function of fungi to many will be as a food source: global consumption of mushrooms is roughly 12.7 million tonnes, with the UK mushroom market worth $1.27 billion USD per year (Shirur, Barh and Annepu, 2021; Grand View Research, 2024). Microscopic fungi are also used in food production – the most well-known being the use of baker's yeast (*Saccharomyces cerevisiae*) for the production of bread, as well as fermentation of beer, wine and cheese (Dupont *et al.*, 2017). Some fungi have applications in medicine. The first antibiotic discovered (penicillin) was isolated from a fungus: *Penicillium rubens* (Aminov, 2010; Houbraken, Frisvad and Samson, 2011). Lovastatin (to treat high blood cholesterol) is derived from *Aspergillus terreus* and polysaccharide-K (used as an adjuvant to treat some cancers) is derived from *Trametes versicolor* (Manzoni and Rollini, 2002; Fritz *et al.*, 2015). Fungi that produce psychoactive agents (eg. psilocybin) are used both in indigenous ceremonies and recreationally (Van Court *et al.*, 2022).

Not all fungi have beneficial effects. Many mushrooms are poisonous if ingested, capable of causing gastrointestinal disturbances, hallucinations, severe organ failure and death (El-Ramady *et al.*, 2022). Some fungi can cause disease in plants, animals and humans. Of the millions of fungal species, less

than 300 species are able to cause disease in humans, with the vast majority of cases caused by fungi from 4 genera: *Aspergillus, Candida, Cryptococcus* and *Pneumocystis* (Thambugala *et al.*, 2024).

1.2 Fungi: A rising threat

Fungal disease can range from minor, superficial infections (approximately 1 billion cases worldwide) to severe or life threatening conditions, such as severe invasive candidiasis and chronic pulmonary aspergillosis (Bongomin *et al.*, 2017). It has been estimated that up to 6.55 million people worldwide have a life-threatening fungal infection each year, resulting in approximately 3.75 million deaths, of which 2.55 million are directly attributable to fungal infection (i.e. no comorbidity which may have been more responsible for death; Denning, 2024). This represents a substantial increase from the previously accepted figure of 1.7 million deaths annually (Bongomin *et al.*, 2017). Denning acknowledges their estimate of 3.75 million deaths from fungal infections is a crude, and potentially inaccurate, approximation – a reflection of substantial data gaps, poor international surveillance of fungal infections and imprecise diagnosis (Almeida, Rodrigues and Coelho, 2019; WHO, 2022; Denning, 2024a). Fungal infections have a vast burden beyond mortality. Fungal keratitis leads to blindness in over 600,000 eyes every year (Brown *et al.*, 2021), while there are 11.5 million people affected by fungal asthma annually and 2.1 billion cases of fungal skin disease (GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017; Denning, 2024a).

While the burden of fungal disease is large, awareness within the general public is low, with less than a third of surveyed Americans being aware of fungal diseases (Benedict, Molinari and Jackson, 2020). A consequence of the lack of awareness is that fungal infections receive relatively little attention and resources from researchers and policymakers worldwide (Bongomin *et al.*, 2017; WHO, 2022). For example, it is estimated that for every individual that dies of meningococcal (bacterial) meningitis, $2,458 USD is spent on research, while only $31 USD is spent per individual that dies of cryptococcal (fungal) meningitis, despite being responsible for 20 times the number of deaths (Rodrigues and Albuquerque, 2018). While other factors may influence this funding allocation (including the demographic affected by meningococcal meningitis infections, epidemiology of cryptococcal meningitis and confounding co-infections), this discrepancy highlights the underfunding of fungal infection research.

Fungal infections are an increasing global health concern: *Candida auris* outbreaks have been observed in China, the UK and the USA, while India experienced a major surge in cases of mucormycosis associated with COVID-19, with high mortality (Adams *et al.*, 2018; Rhodes *et al.*, 2018; H. Du *et al.*, 2020; Muthu *et al.*, 2021, 2022; Bing *et al.*, 2024). In October 2022, the WHO produced its first report on fungal priority pathogens, representing the first global effort to prioritise

fungal pathogen research and coordinate strategies for policymakers and public health officials (WHO, 2022). Significant strengthening of surveillance networks, investment in antifungal research, improved diagnostics and robust public health policies are required to ensure global systems are prepared for future fungal outbreaks (Haldane *et al.*, 2021; Baker *et al.*, 2022; Jafarlou, 2024).

1.3 Candida albicans

1.3.1 The *Candida* genus

The *Candida* genus refers to approximately 200 species of yeast (Brandt and Lockhart, 2012). *Candida* spp. are part of the *Ascomycota* phylum – the largest fungal phylum, which also includes *Aspergillus* spp., *Saccharomyces* spp. and *Penicillium* spp.(Kurtzman, Fell and Boekhout, 2011). Most *Candida* spp. are harmless and are common components of a healthy microbiome (Nash *et al.*, 2017; Wu *et al.*, 2020). Roughly 30 species of *Candida* spp. can cause human infection (Miceli, Díaz and Lee, 2011). Some *Candida* spp. are of extreme clinical importance. Of the 19 fungal priority pathogens listed by the WHO, 6 are *Candida* spp.: *Candida albicans* and *Candida auris* are critical priority, *Candida glabrata, Candida parapsilosis* and *Candida tropicalis* are high priority and *Candida krusei* is medium priority (WHO, 2022). The most prevalent *Candida* spp. causing disease in humans is *C. albicans* (Pappas *et al.*, 2018). A selection of non-albicans *Candida* spp. are explored later (see 1.4 Non-albicans *Candida* spp.).

1.3.2 *C. albicans* epidemiology

While there are at least 15 distinct *Candida* spp. that can cause human disease, the most prevalent is *Candida albicans* (Pappas *et al.*, 2018). *C. albicans* is typically a commensal fungus, present in the microbiome of approximately 70% of the healthy population (Witherden *et al.*, 2017). In the majority of cases, *C. albicans* infections are limited to superficial infections, such as skin infections or thrush, which have high morbidity but very low mortality. Globally, it is estimated there are 1 billion cases of superficial fungal infection, 134 million cases of recurrent vulvovaginal candidiasis (RVVC) and 2 million cases of oral candidiasis annually (Bongomin *et al.*, 2017).

In at-risk individuals, commensal colonisation or superficial infections can progress to severe, systemic candidiasis (Pellon, Sadeghi Nasab and Moyes, 2020). Comorbidity appears to be the greatest risk factor for invasive candidiasis, particularly HIV/AIDS, neutropenia, cancer and stem cell transplantation (Pappas *et al.*, 2018; Thomas-Rüddel *et al.*, 2022). Prolonged use of broad spectrum antibiotics, use of central venous catheters, total parental nutrition and longer stays in intensive care units have also been associated with increased risk of invasive candidiasis (Zhang *et al.*, 2020; Thomas-Rüddel *et al.*, 2022). Hence, *Candida* spp. are the fourth most common nosocomial systemic infections in the USA, with a 45-75% mortality (Pfaller and Diekema, 2010; Brown, Denning and

Levitz, 2012). There appears to be bimodal incidence with age, with both neonates and the elderly being most at risk (Bongomin *et al.*, 2017; UK Health Security Agency, 2023)

The exact global burden of invasive candidiasis is not currently known. Few countries carry out population-based surveillance of candidiasis, with no population-based data available from Africa, Asia, the Middle East or Latin America (Lamoth *et al.*, 2018). Admission data from individual institutions within these regions does exist, but lack a consistent methodology, making comparisons or extrapolation difficult (Kreusch and Karstaedt, 2013; Santolaya *et al.*, 2014; Lamoth *et al.*, 2018). Because of this, only crude estimates of the global number of invasive candidiasis cases exist, varying from 750,000 cases to 1.56 million cases annually (Bongomin *et al.*, 2017; Denning, 2024a). Mortality rates also vary depending on the study. Denning *et al.* estimates 995,000 deaths from invasive candidiasis globally, giving a 63.6% mortality rate – though the accuracy of this figure has been questioned (Denning, 2024a; Ikuta, Meštrović and Naghavi, 2024). The WHO estimates invasive candidiasis mortality ranges from 20% to 50% (WHO, 2022). A retrospective study of 391 candidaemia patients across a 10 year period in Germany observed a 47.3% mortality at 28 days post positive culture and 59.8% mortality at 180 days post positive culture (Schroeder *et al.*, 2020). However, long term mortality was likely impacted by comorbidity in these patients, potentially inflating the observed mortality rate. A recent multinational observational study, from 2018 to 2019, monitored 171 candidaemia patients, with matched controls, from 28 hospitals in 12 European countries. Mortality was 40.4% in candidaemia patients, though mortality of matched controls was 22.2%, suggesting an attributable mortality of 18.2% for candidaemia (Salmanton-García *et al.*, 2024). Species specific mortality ranged from 32.1% for *C. albicans* (7.7% attributable mortality) to 72.7% mortality for *C. tropicalis* (63.6% attributable mortality). While matched controls allow an attributable mortality to be calculated, this does not account for variability in disease progression and relied on subjective clinical determination by participant researchers, which could be confounding factors.

1.3.3 *C. albicans* cell biology

C. albicans is a diploid, unicellular fungus, commonly found in the microbiome. (Bennett and Turgeon, 2016). *C. albicans* grows in 3 distinct forms: yeast, pseudohyphae and hyphae (Figure 1.1; Sudbery, 2011). The spherical, unicellular yeast form colonises mucosal surfaces and is well tolerated by the immune system. Contrastingly, hyphae are long, tube-like filaments extending out of a spherical yeast cell. Hyphae are associated with increased virulence, given their ability to invade and damage epithelial and endothelial cells (Dalle *et al.*, 2010). Pseudohyphae have a similar tubular appearance to hyphae but are actually formed of several attached, elongated cells, allowing branching to occur (Sudbery, 2011).The yeast-to-hyphae transition is tightly regulated by a variety of

transcription factors. *NRG1* is a key negative regulator of hyphal morphogenesis, with *C. albicans nrg1Δ* strains forming hypha under all tested growth conditions (Braun, Kadosh and Johnson, 2001; Homann *et al.*, 2009). Nrg1 is a DNA binding protein, which binds to Nrg1 Response Elements (NREs) in the promoter regions of genes involved in hyphal development (Murad *et al.*, 2001). Nrg1 subsequently binds to Tup1, which represses transcription by preventing binding of RNA polymerase to the promoter region. Tup1 interactions with other DNA binding proteins, such as Mig1 and Rfg1, also play a role in suppression of hyphae formation (Braun, Kadosh and Johnson, 2001; Murad *et al.*, 2001; Kebaara *et al.*, 2008). Tup1-mediated suppression of hyphae formation is promoted by quorum sensing via farnesol signalling (Kebaara *et al.*, 2008; Oliveira *et al.*, 2021). Quorum sensing is a system of cell-to-cell communication, allowing coordination of biofilm formation, hyphae formation and virulence by *C. albicans*, via secreted quorum sensing molecules, including farnesol (Zawrotniak, Wojtalik and Rapala-Kozik, 2019). Conversely, Swi1 and Snf2 have been demonstrated to form a chromatin remodelling complex that, when recruited to promoter regions of hyphal development genes, facilitates RNA polymerase binding and promotes expression of hyphae-specific genes (Mao *et al.*, 2006, 2008).

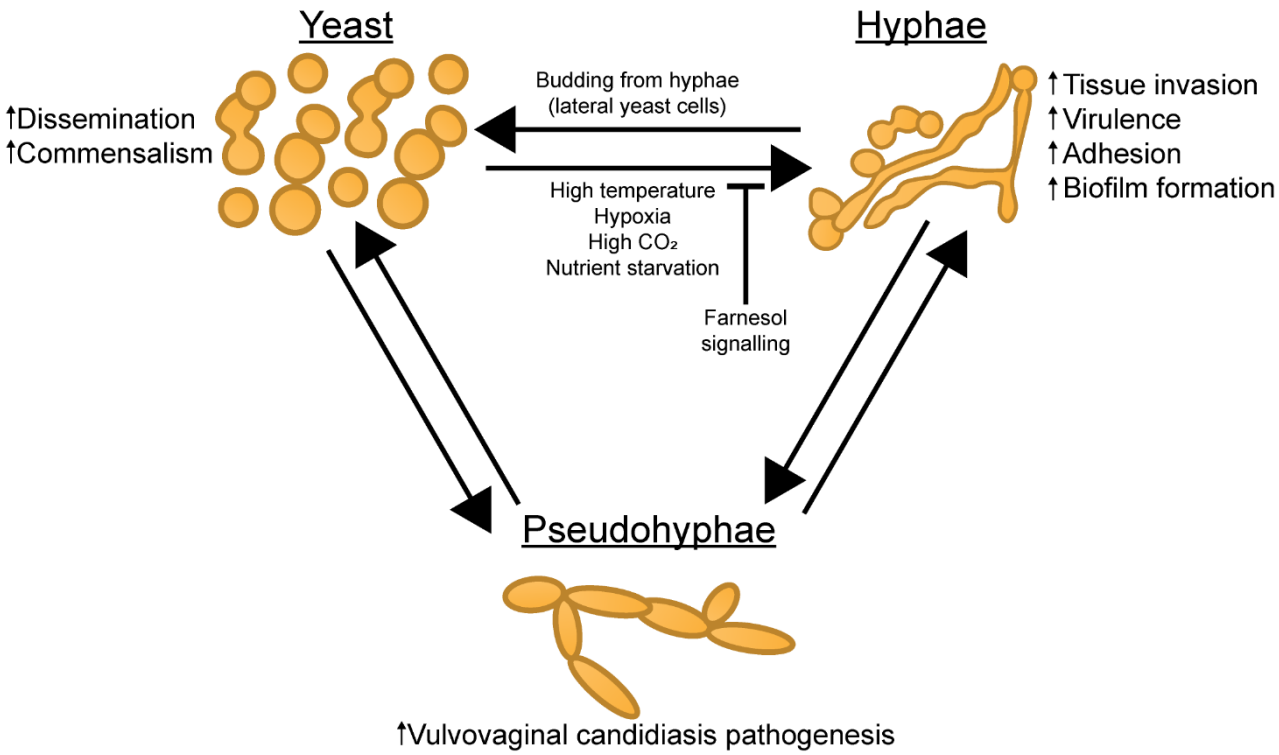

Figure 1.1: *C. albicans* morphologies

Diagram showing the different *C. albicans* morphologies. Yeast cells are most associated with commensalism and increased efficiency of dissemination (Sudbery, 2011; Seman *et al.*, 2018). Yeast-to-hyphae transition can be induced by a range of environmental factors and inhibited by quorum sensing by farnesol (Desai, 2018; Oliveira *et al.*, 2021). Hyphae are associated with tissue invasion and increased virulence *in vivo*, with upregulation of genes involved in adhesion, biofilm formation and upregulation of hyphae-specific virulence factors (Sudbery, 2011; Moyes *et al.*, 2016; Desai, 2018). Hyphae may revert to yeast while in biofilms. Newly formed yeast cells bud off of hyphae, becoming lateral yeast cells, which facilitate dissemination (Uppuluri *et al.*, 2018; Wakade and Krysan, 2021). Yeast can be induced to form pseudohyphae by the same environmental factors that induce hyphae formation. The role of pseudohyphae is poorly characterised but pseudohyphae are suggested to be the dominant morphotype in vulvovaginal candidiasis pathogenesis (Roselletti *et al.*, 2019).

C. albicans can undergo further phenotypic switching, known as white-opaque switching (Soll, 2024). This phenotypic switching was first noted in 1987, following observations of a "white" phenotype, the 'default' state in which colonies appeared as white hemispheres, and an "opaque" phenotype, in which colonies were larger, flatter and opaque or grey (Slutsky *et al.*, 1987). Difference in colony appearance is the consequence of changes in cellular morphology, with white cells being small and spherical, while opaque cells are elongated and 2-3 times the volume of white cells. Phenotypic switching between white and opaque cells is reversible (Anderson and Soll, 1987; Slutsky *et al.*, 1987). The implications of white and opaque phenotypes extend beyond cellular appearance. Opaque cells were more susceptible to neutrophil killing *in vitro* and to killing by hydrogen peroxide *in vitro* than white cells (Kolotila and Diamond, 1990). Counterintuitively, exposure to neutrophils promoted *C. albicans* to switch from a white to opaque phenotype, which was more susceptible to killing (Kolotila and Diamond, 1990). Comparison of *C. albicans* phenotypes in a mouse model revealed white and opaque cells have specific niche preferences, with opaque cells able to colonise multiple organs, outcompeting white cells in the heart and spleen, but white cells being the dominant colonisers of the kidney (Takagi *et al.*, 2019). Persistent infection in mice favoured white cells in the kidney, with *C. albicans* not recovered from any other organ and almost no opaque cells detected. Intestinal colonisation also favoured white cells, with opaque cells observed to undergo phenotypic switching to white cells (Takagi *et al.*, 2019). Opaque cells were also less virulent than white cells in a murine model of oropharyngeal candidiasis (Solis *et al.*, 2018). White and opaque cells had no difference in hyphae formation during coculture with human oral epithelial cells *in vitro* (OKF6/TERT-2 cell line), but opaque cells did have reduced expression of Als3 invasin compared to white cells, which was correlated with a reduced adherence and invasion of oral epithelial cells *in vitro* (Solis *et al.*, 2018).

C. albicans is a parasexual organism, capable of replication both by sexual reproduction and asexual reproduction through asymmetric budding of yeast cells (Berman, 2006). Asexual budding of yeast cells is the most common method of replication, with sexual reproduction not thought to be possible in *C. albicans* until the discovery of the *MTL* locus (Hull and Johnson, 1999; Soll, 2024). The *MTL* locus contains 2 idiomorphs: **a** and α (Figure 1.2A). *C. albicans* with a heterozygous *MTL* genotype (**a**/α) are locked in the white cell phenotype and unable to sexually reproduce. Transitioning to a homozygous *MTL* genotype (**a**/**a** or α/α) by homozygosis allows white-opaque switching, which facilitates sexual reproduction as opaque cells are approximately one million times more efficient at undergoing sexual reproduction compared to white cells (Miller and Johnson, 2002). *C. albicans* exposed to fluconazole *in vitro* had increased incidence of a homozygous *MTL* genotype, suggesting fluconazole-mediated stress may promote homozygosis (Ou *et al.*, 2017). This may facilitate *C.*

albicans mating, allowing the development of increased fluconazole resistance via genetic recombination in progeny cells. Sexual reproduction can be homothallic or heterothallic (Figure 1.2B,C). Heterothallic reproduction allows recombination between daughter cell nuclei, introducing the possibility of development of competitive advantages (Forche *et al.*, 2008; Usher, 2019). Homothallic reproduction appears to be driven by mutation of *BAR1*, which leads to aberrant secretion of mating pheromones, or environmental stresses, such as glucose starvation and oxidative stress (Alby, Schaefer and Bennett, 2009; Guan *et al.*, 2019). *In vitro* glucose depletion was recently demonstrated to promote sexual reproduction by white cells by activating the pheromone MAPK pathway, revealing the current dogma that opaque cells are critical for mating may be bypassed under certain environmental conditions (Guan *et al.*, 2023). Progeny cells are initially tetraploid, which is highly unstable, and return to a diploid state via random chromosome loss (Forche *et al.*, 2008). *C. albicans ofr1Δ* is able to undergo homothallic mating with an **a**/α *MTL* genotype, specifically when grown on GlcNAc media but not on glucose media, suggesting *OFR1* has an uncharacterised, carbon source-dependent role in regulation of *C. albicans* mating (Sun *et al.*, 2016).

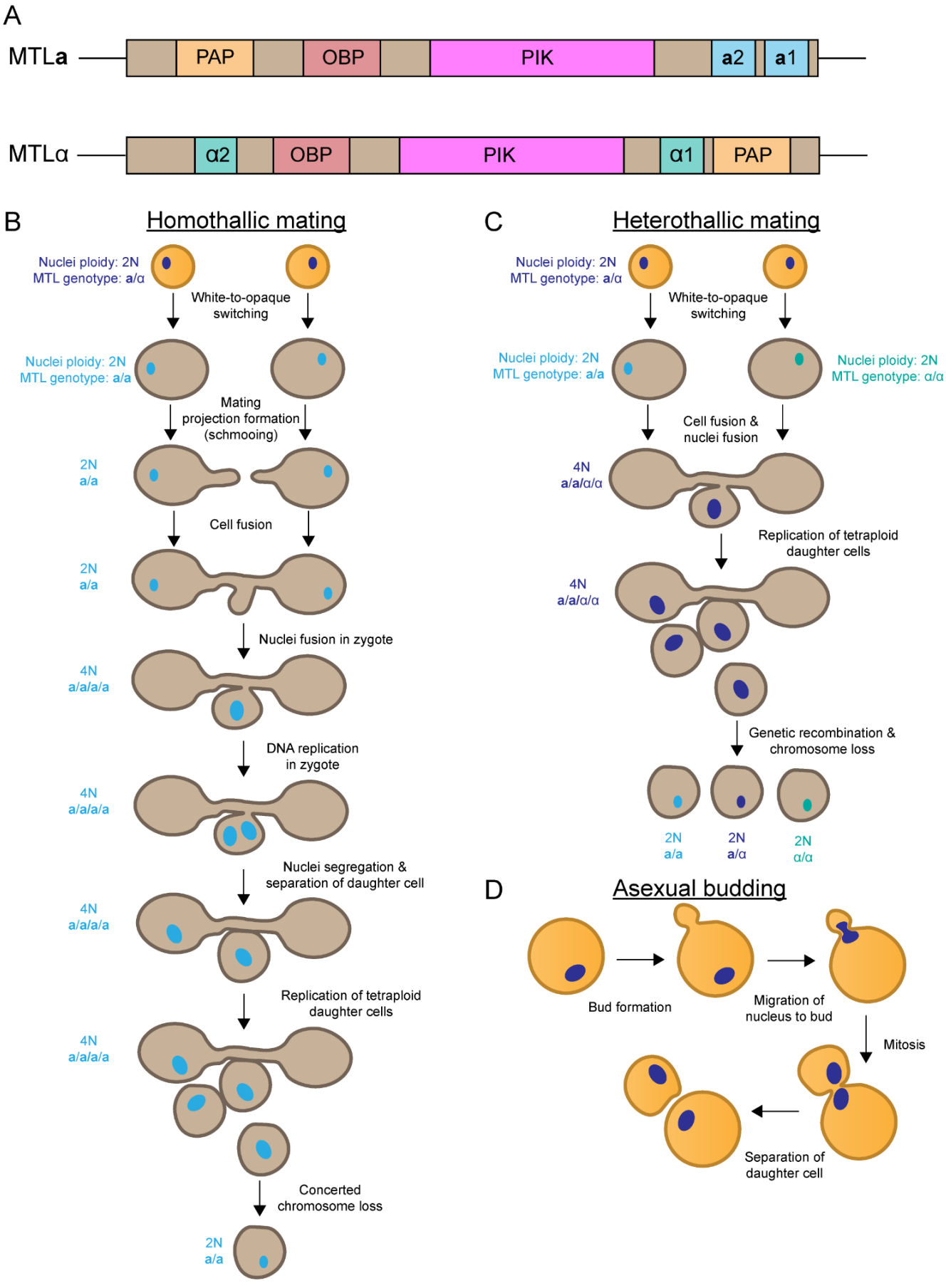
A
MTLa
PAP
OBP
PIK
a2
a1
MTLα
α2
OBP
PIK
α1
PAP
B
Homothallic mating
Nuclei ploidy: 2N
MTL genotype: a/α
White-to-opaque switching
Nuclei ploidy: 2N
MTL genotype: a/a
Mating projection formation (schmooing)
2N
a/a
Cell fusion
2N
a/a
Nuclei fusion in zygote
4N
a/a/a/a
DNA replication in zygote
4N
a/a/a/a
Nuclei segregation & separation of daughter cell
4N
a/a/a/a
Replication of tetraploid daughter cells
4N
a/a/a/a
Concerted chromosome loss
2N
a/a
C
Heterothallic mating
Nuclei ploidy: 2N
MTL genotype: a/α
White-to-opaque switching
Nuclei ploidy: 2N
MTL genotype: a/a
Nuclei ploidy: 2N
MTL genotype: α/α
Cell fusion & nuclei fusion
4N
a/a/α/α
Replication of tetraploid daughter cells
4N
a/a/α/α
Genetic recombination & chromosome loss
2N
a/a
2N
a/α
2N
α/α
D
Asexual budding
Bud formation
Migration of nucleus to bud
Mitosis
Separation of daughter cell

Figure 1.2: *C. albicans* sexual and asexual reproduction.

(A) Schematic representation of *C. albicans MTL* locus.

(B) Diagram of homothallic sexual reproduction by *C. albicans*. White cells with a heterozygous *MTL* genotype undergo homozygosis, which facilitates switching to an opaque phenotype (Soll, 2024). Pheromone signalling between *C. albicans* cells induces schmooing – formation of specialised projections (called a schmoo), which extend and fuse to form a bridge between cells and a mating zygote (Bennett, 2015). Nuclei migrate to the zygote, fuse into a single tetraploid nucleus and then undergoes DNA replication. One tetraploid nucleus migrates back to the parent cell, while the other tetraploid nucleus remains in the daughter cell and continues to replicate. Tetraploid daughter cells continue to bud and replicate asexually. Tetraploid daughter cells eventually return to a diploid state through concerted chromosome loss (Bennett, 2015; Usher, 2019; Soll, 2024). The mating process, including schmooing, takes approximately 2 hours.

(C) Diagram of heterothallic sexual reproduction by *C. albicans*. Repeated steps from (B) have been shortened for simplicity. Recombination in tetraploid daughter cells leads to introduction of genetic diversity (Alby, Schaefer and Bennett, 2009).

(D) Diagram of asexual reproduction by *C. albicans.* A small bud forms at part of the *C. albicans* cell membrane, initially expanding via apical growth (Staebell and Soll, 1985). The diploid nucleus migrates to the growing bud and undergoes DNA replication. The daughter cell then splits from the parent cell, each containing a single diploid nucleus (Molero *et al.*, 1998; Berman, 2006).

C. albicans is also part of the CUG clade of fungi – a group of fungal species in which the CUG codon codes for serine, rather than leucine (Santos and Tuite, 1995; Berman, 2012). In *C. albicans*, and some other *Candida* spp., CUG is translated as serine 95-97% of the time and as leucine 3-5% of the time (Gomes *et al.*, 2007). The intentional mistranslation of CUG is driven by an atypical Seryl-$tRNA_{CAG}$, which is recognised by both seryl- and leucyl-tRNA synthetases, allowing attachment of either serine or leucine to the amino acid chain (Santos, Keith and Tuite, 1993). This was suggested to introduce a high level of phenotypic diversity into the *C. albicans* proteome, facilitating evolution and evasion of immune responses through cell surface variability (Gomes *et al.*, 2007; Miranda *et al.*, 2013). However, high-resolution mass spectrometry based proteogenomics of *C. albicans* clinical isolates did not observe the previously reported values of CUG mistranslation as leucine. Mistranslation of CUG was observed at comparable rates to mistranslation of other codons, suggesting the role of CUG mistranslation in generating phenotypic diversity has previously been exaggerated (Mühlhausen *et al.*, 2021).

The *C. albicans* cell wall is a two-layered structure, essential for morphological integrity and protection from physical and chemical stresses (Figure 1.3; Garcia-Rubio *et al.*, 2020). The inner layer of the cell wall is primarily composed of chitin and β(1,3)-glucan. β(1,6)-glucan is also present in the cell wall inner layer and is responsible for linking chitin, β(1,3)-glucan and proteins in the cell wall (Iorio *et al.*, 2008; Gow, Latge and Munro, 2017). Whereas chitin was previously believed to form a distinct layer adjacent to the cell membrane, recent analysis and transmission electron microscopy has suggested chitin is distributed non-continuously throughout the inner layer of the cell wall (Lenardon *et al.*, 2020). It was hypothesised the rigid chitin microfibrils are connected by soft, amorphous chains, providing structural integrity to the cell wall while ensuring the cell wall is flexible enough to allow rapid expansion or contraction in response to stresses (Ene *et al.*, 2015; Lenardon *et al.*, 2020). Cell wall proteins, primarily GPI-anchored proteins, are distributed throughout the inner layer of the cell wall. 90% of GPI-anchored proteins are attached to β(1,3)-glucan and the remaining 10% are bound to chitin (Kapteyn *et al.*, 2000). The outer layer of the cell wall is predominantly composed of *N*-linked manno-oligosaccharide chains. These mannans are not rigid, so do not contribute to cell shape, but do influence *C. albicans* antifungal resistance and evasion of innate immunity (see 1.5.5 *C. albicans* mechanisms of evading immune response)(Gow and Hube, 2012; Garcia-Rubio *et al.*, 2020). The *C. albicans* cell wall surrounds the plasma membrane, a lipid bilayer containing various proteins. Whereas mammalian cell membranes contain cholesterol, ergosterol is the major sterol component of the fungal cell, used to maintain membrane fluidity and integrity (Dupont *et al.*, 2012; Rodrigues, 2018). The fungal cell membrane also contains various proteins, several of which have roles in cell wall synthesis and maintenance, such as β(1,3)-glucan synthase

(Cabezón *et al.*, 2009). Other plasma membrane proteins are virulence factors (Cabezón *et al.*, 2009). The *C. albicans* cell wall and plasma membrane are common targets of antifungals, owing to the importance of the cell wall for *C. albicans* integrity and the presence of unique targets without mammalian orthologs (see 1.3.5 *C. albicans* treatments; Hasim and Coleman, 2019).

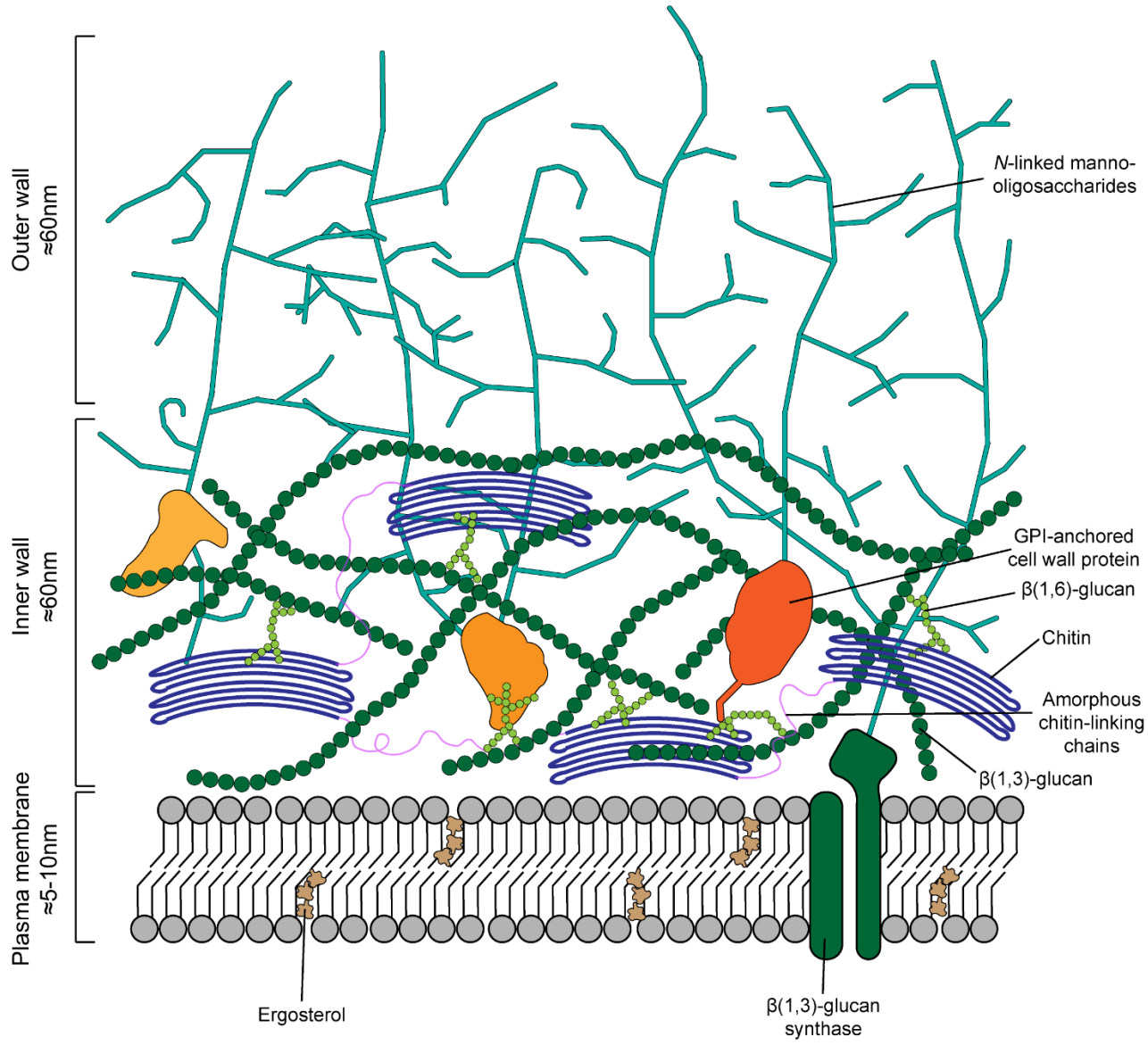

Figure 1.3: *C. albicans* plasma membrane and cell wall structure

Diagram of *C. albicans* plasma membrane and cell wall (not to scale). Plasma membrane is made of a phospholipid bilayer containing regions of ergosterol to retain membrane fluidity (Rodrigues, 2018). Proteins, such as β(1,3)-glucan synthase, reside in the plasma membrane (Cabezón *et al.*, 2009; Zhao *et al.*, 2023). The inner cell wall is mostly made up of chitin and β(1,3)-glucan. Chitin microfibrils are linked by soft, amorphous chains, allowing the cell wall to retain flexibility (Lenardon *et al.*, 2020). β(1,6)-glucan forms links between chitin and β(1,3)-glucan. A variety of different proteins reside in the inner cell wall, which may be linked to the plasma membrane, chitin or β(1,3)-glucan (Kapteyn *et al.*, 2000). The outer cell wall is composed of *N*-linked manno-oligosaccharide chains (Garcia-Rubio *et al.*, 2020; Lenardon *et al.*, 2020). Figure adapted from (Lenardon *et al.*, 2020)

1.3.4 *C. albicans* pathogenesis

Many systemic *C. albicans* infections are believed to originate in the gut as commensal fungi, from where they breach the intestinal epithelium and disseminate around the body (Miranda *et al.*, 2009). This can be influenced by host factors, such as abdominal surgery damaging the epithelium, immunocompromised status, microbiome imbalance, trauma and chemotherapy (Sprague, Kasper and Hube, 2022). The major *C. albicans* route for translocating the intestinal epithelium is active penetration by hyphae (Dalle *et al.*, 2010; Sprague, Kasper and Hube, 2022). Infection of C2BBe1 intestinal epithelial cells with *C. albicans in vitro* demonstrated translocation of *C. albicans* across an epithelium was strongly associated with hyphal growth and cellular damage. However, translocation of *C. albicans* was not completely hyphae-dependent, as *C. albicans hgcΔ* (a yeast-locked mutant) was still able to translocate across the epithelium *in vitro*, though to a lesser degree than wild type *C. albicans* (Allert *et al.*, 2018). Deletion of candidalysin (a hyphae-specific cytolytic peptide) abolished epithelial translocation by *C. albicans* and significantly reduced damage to epithelial cells, but hyphae formation was unimpaired. Interestingly, the yeast-locked *C. albicans hgcΔ* mutant, capable of translocation, continued to express *ECE1* (candidalysin) and candidalysin has been shown to disrupt epithelial integrity in an intestinal organoid *in vitro* model (Allert *et al.*, 2018; Morelli and Queiroz, 2025). Together, this suggests candidalysin is the critical factor for gut epithelium translocation. Scanning electron microscopy of HeLa and Caco-2 cells infected with *C. albicans* revealed a method of epithelial translocation by hyphae without cell damage (Lachat *et al.*, 2022). Invading hyphae were enveloped by host membranes, forming a tunnel that facilitates trans-cellular invasion by *C. albicans* (Lachat *et al.*, 2022). Whether this is a *C. albicans*-induced process to minimise immune cell activation or a host strategy to prevent cellular damage is unknown. Further validation in more physiologically relevant models of *C. albicans* infection is required. *C. albicans* may also induce endocytosis by host cells to facilitate tissue invasion via binding of Als3 to host cell cadherins (Phan *et al.*, 2007). In OKF6/TERT-2 oral epithelial cells *in vitro*, *C. albicans* induced formation of a multi-protein receptor complex, composed of c-Met, EGFR and E-cadherin, which binds to *C. albicans* Als2 and Hyr1 to facilitate endocytosis into the oral epithelial cells (Phan *et al.*, 2023).

While hyphae are critical for tissue invasion, the yeast morphotype is more able to disseminate around the body. A yeast-locked *C. albicans* mutant ($NRG1^{OEX}$-NEON) had significantly greater dissemination from yolk infection in zebrafish at 24, 30 and 44 hours post infection, but had reduced mortality relative to wild-type (Seman *et al.*, 2018). Mice were given a bloodstream infection with *C. albicans* SSY50-B, which carries a *tet-NRG1* allele (Saville *et al.*, 2003). This strain is yeast-locked in normal conditions, but in the presence of doxycycline, *tet-NRG1* expression is inhibited, allowing

normal yeast-to-hyphae transition. Doxycycline treatment had no impact on *C. albicans* burden in the kidney, brain and spleen at 6 hours post infection. At 3 days post infection, mice that had not received doxycycline had greater *C. albicans* burden in the kidney and spleen than doxycycline treated-mice, with no difference in the brain burden, further demonstrating the ability of yeast-locked *C. albicans* to extravasate from circulation to colonise diverse tissues (Saville *et al.*, 2003). Macrophages and neutrophils were observed to facilitate dissemination of *C. albicans* yeast via a 'Trojan horse' mechanism in zebrafish *in vivo*, which was also possible in the absence of blood flow (Scherer *et al.*, 2020). However, blood flow dissemination of *C. albicans* without phagocytic involvement was also possible, showing a redundancy in the mechanisms *C. albicans* uses to spread throughout the body (Scherer *et al.*, 2020).

Following tissue invasion and dissemination, *C. albicans* has a range of virulence factors to aid pathogenicity. The hyphal morphotype plays a key role in virulence, with yeast-locked *C. albicans* mutants being hypovirulent in a mouse *in vivo* model (Murad *et al.*, 2001). Candidalysin, encoded by *ECE1*, is a key hyphae-specific virulence factor. *C. albicans ece1Δ* caused reduced cell damage compared to wild type *C. albicans* in a zebrafish swim bladder infection (Moyes *et al.*, 2016). Secreted candidalysin self-assembles into large polymers, which insert into host cell membranes, forming a pore that causes membrane permeabilisation and cell lysis (Russell *et al.*, 2022; Schaefer *et al.*, 2024). Secreted aspartic proteases (Saps) have versatile proteolytic functions in *C. albicans* pathogenesis, including degradation of host collagen, laminin, immunoglobulins, clotting factors and IL-1β (Naglik *et al.*, 2004). Disruption of Sap1-3 reduced proteolytic activity *in vitro* and reduced virulence in mouse and guinea pig *in vivo* models, though changes in proteolytic activity and virulence did not directly correlate (Hube *et al.*, 1997). *C. albicans sap6Δ* mutants exhibited severely impaired tissue damage and tissue invasion in mice *in vivo* (Felk *et al.*, 2002). Sap6 also acts as an adhesin and promotes formation of large aggregates between *C. albicans* hyphae, but not with yeast, and adhesion of these aggregates to oral epithelial cells *in vitro*. These Sap6-mediated hyphal aggregates may play a role in virulence (Kumar *et al.*, 2015). Other adhesins play a role in virulence. Hgc1 promotes adhesion of *C. albicans* to the endothelium during circulation (Wilson and Hube, 2010). Als3 has multiple functions: as an adhesin by binding to endothelial and epithelial cells; as an invasion by inducing endocytosis; as a ferritin receptor used for iron scavenging; and in biofilm formation through binding to *C. albicans* Hwp1 (Liu and Filler, 2011).

C. albicans biofilms act as an important virulence factor. Across multiple studies, biofilm formation has been strongly associated with candidiasis mortality (Tumbarello *et al.*, 2007; Rajendran *et al.*, 2016; Vitális *et al.*, 2020). Human serum significantly upregulated biofilm-associated genes *in vitro* and stimulated increased biofilm formation *in vitro*, potentially explaining the propensity of *C.*

albicans to form biofilms in central venous catheters and in human infection (Samaranayake *et al.*, 2013). Several transcription factors regulate biofilm formation, including Bcr1, Tec1, Efg1, Rob1 and Brg1 (Mayer, Wilson and Hube, 2013). Mature biofilms confer protection from antifungals, with the enhanced extracellular matrix believed to prevent antifungals from physically reaching *C. albicans* cells, limiting their toxicity (Vediyappan, Rossignol and d'Enfert, 2010). Finally, biofilms protect *C. albicans* from neutrophil killing and do not induce production of reactive oxygen species (ROS) *in vitro* (Xie *et al.*, 2012). Further virulence factors that specifically contribute to evasion of host immune responses are explored later (see 1.5.5 *C. albicans* mechanisms of evading immunity).

1.3.5 *C. albicans* treatments

Fungal infections are treated with antifungal drugs, of which there are 3 main classes: polyenes, azoles and echinocandins (Chang *et al.*, 2017). Polyenes bind to ergosterol in the fungal cell membrane, preventing ergosterol-dependent membrane fusion and endocytosis (Ghannoum and Rice, 1999; Van Leeuwen, Golovina and Dijksterhuis, 2009). Binding of amphotericin B to ergosterol can also trigger formation of pores in the fungal membrane, allowing efflux of cellular components and causing cell death (Gray *et al.*, 2012; Lakhani, Patil and Majumdar, 2019). Similarly, azoles, such as fluconazole, inhibit lanosterol-14-α-demethylase, preventing the conversion of lanosterol to ergosterol in the fungal cell membrane. This restricts fungal growth and increases cell membrane permeability, which can cause cell lysis (Ghannoum and Rice, 1999; Herrick, Patel and Hashmi, 2024). Echinocandins non-competitively bind to β(1,3)-glucan synthase, preventing formation of β(1,3)-glucan, resulting in fungal cell wall abnormalities, growth inhibition and cell lysis due to osmotic stress (Szymański *et al.*, 2022).

Clinical guidance for treatment of invasive *C. albicans* infections varies by region and presence of underlying conditions. Whittington NHS trust guidelines state first line therapy for invasive candidiasis is intravenous fluconazole for non-neutropenic patients or intravenous anidulafungin for neutropenic patients (Whittington Health NHS Trust, 2019), whereas other NHS trust guidelines suggest intravenous anidulafungin or liposomal amphotericin B, with no difference stated for neutropenic or non-neutropenic patients (Doncaster and Bassetlaw Teaching Hospitals, 2022; Norfolk and Norwich University Hospitals, 2024). Guidelines by the Infectious Disease Society of America suggest echinocandins as first line therapy for invasive *Candida* infections (Pappas *et al.*, 2016). However, compliance with these guidelines is questionable, with one study revealing 29.6% of candidaemia patients, in hospitals from 9 US states, received fluconazole as a first line therapy instead of an echinocandin (Gold *et al.*, 2021). Whether this non-compliance was due to lack of awareness of guidelines or clinical judgment is unclear. Regional guideline variations may be a reflection of local resistance profile or preference of local infectious disease consultants.

Robust studies comparing the efficacy of candidiasis treatments are limited. In 135 patients with confirmed *C. albicans* invasive candidiasis, anidulafungin had a significantly greater global response than fluconazole at all measured time points, when both drugs were administered intravenously (Reboli *et al.*, 2011). Median time to negative blood cultures was 3 days quicker with anidulafungin treatment than with fluconazole treatment. 6-week survival was not significantly different between groups, however, anidulafungin treatment had significantly fewer deaths within 24 hours of the end of treatment than fluconazole treatment (4 vs 13). A greater number of fluconazole treated patients had to withdraw from the trial than anidulafungin treated patients, due to observed failure of treatment, adverse events or worsening clinical status, which may further imply inferiority of fluconazole treatment (Reboli *et al.*, 2011). An alternative study in US hospitals revealed candidaemia patients treated with an echinocandin were more likely to die than patients treated with fluconazole (Gold *et al.*, 2021). Active surveillance programmes across a 13 year period in 14 hospitals in France also observed increased mortality in patients treated with echinocandins compared to patients treated with fluconazole (Bretagne *et al.*, 2021). However, both of these studies note this is likely a consequence of disease progression, as critically ill patients are more likely to be treated with an echinocandin (Leroy *et al.*, 2016; Bretagne *et al.*, 2021; Gold *et al.*, 2021). Furthermore, these studies were not restricted to candidiasis caused by *C. albicans*. Isavuconazole and caspofungin treatment had no difference in mortality or incidence of adverse events (Kullberg *et al.*, 2019). 60.3% isavuconazole treated patients met the criteria for successful treatment, compared to 71.1% caspofungin treated patients. Isavuconazole was determined noninferior to caspofungin, as the difference in treatment success did not meet prespecified criteria (Kullberg *et al.*, 2019).

Because fungi are eukaryotes, identification of specific antifungal targets that do not harm host cells is challenging, making development of new antifungals slow (Perfect, 2017; Rauseo *et al.*, 2020). In the last 15 years, only 3 new antifungals have been approved: isavuconazole in 2015, ibrexafungerp in 2021 and rezafungin in 2024, though isavuconazole and rezafungin are just new variants of an azole and echinocandin respectively (Daele *et al.*, 2019; Phillips, Rocktashel and Merjanian, 2023; MHRA, 2024). Ibrexafungerp is the first triterpenoid, a novel class of antifungal that non-competitively inhibits β(1,3)-glucan synthase by a similar, but different, mechanism to echinocandins (Jallow and Govender, 2021). Fosmanogepix is another novel broad-spectrum antifungal, currently in clinical trials (Almajid *et al.*, 2024). Fosmanogepix is a pro-drug, which is converted to its active form, manogepix, by phosphatase enzymes. Manogepix inhibits to Gwt1, a fungal GPI-anchored cell wall transfer protein that usually catalyses inositol acylation of GPI (Pappas *et al.*, 2023). Inhibition of Gwt1 by manogepix suppressed *C. albicans* hyphae formation, adhesion and biofilm formation (Watanabe *et al.*, 2012). A small phase 2 trial of fosmanogepix in patients with candidaemia had 80%

treatment success, with 85% 30-day survival and no treatment related adverse events reported (Pappas *et al.*, 2023)

1.3.6 Antifungal resistance in *C. albicans*

While resistance in *C. albicans* specifically is currently relatively low around the world, antifungal resistance rates are increasing annually. In 2018-19, 3.5% of *C. albicans* isolates from Leeds were resistant to fluconazole, which rose to 9.6% *C. albicans* isolates by 2020-21 (Ratner *et al.*, 2025). A systematic review of the resistance profile of *C. albicans* isolated from human oral cavities revealed antifungal resistance rates varied from 0% for amphotericin B and 0.16% for nystatin up to 28.56% for econazole (Kessler *et al.*, 2022). Development of antifungal resistance can be tracked in individuals with recurrent *Candida* infections. *C. albicans* isolates were taken from a patient with recurring oropharyngeal and oesophageal candidiasis, from shortly after the patient's stem cell transplantation in 2006 up to their death in 2011 (Jensen *et al.*, 2015). Within 2 years, the fluconazole minimum inhibitory concentration (MIC) of isolates rose from 0.125 mg/L to 16 mg/L. MIC of caspofungin rose from 0.06 mg/L to >32 mg/L across a 4 year period. By 2011, *C. albicans* isolates exhibited pan-azole resistance, echinocandin resistance and amphotericin B resistance, stated to be the first example of multidrug resistance emerging *in vivo* in *C. albicans* (Jensen *et al.*, 2015). Increasing resistance did not correlate well with virulence in *Galleria mellonella* infection, suggesting multidrug resistance arose without significant fitness costs (Jensen *et al.*, 2015). Rapid emergence of resistance concurrently around the globe highlights an overwhelming need to develop novel treatments for fungal infections.

C. albicans has multiple mechanisms of azole resistance. *ERG11* encodes lanosterol-14-α-demethylase, the target protein of azoles, such as fluconazole. Mutations of *ERG11* have been linked with azole resistance, supposedly by causing amino acid substitutions that reduce the affinity of azoles for Erg11 (White, Marr and Bowden, 1998). 19 different amino acid substitutions were detected in 10 fluconazole resistant *C. albicans* isolates, though no link between specific substitutions and level of fluconazole resistance was shown (Feng *et al.*, 2010). Structural analysis of Erg11 mutants revealed azole resistance is conferred by changes in missense mutations resulting in changes in the size of the catalytic pocket of Erg11 (Debnath and Addya, 2014). It was suggested increases in size of the catalytic pocket may reduce the affinity of azoles, while decreases in size of the catalytic pocket may make it inaccessible. Fluconazole resistance was correlated in tested *C. albicans* isolates with predicted changes in catalytic pocket size, based on their amino acid substitutions (Debnath and Addya, 2014). *ERG3* mutations have also been linked to azole resistance. Typically, azole treatment causes accumulation of 14-α-methylated sterol intermediates, which Erg3 converts to toxic sterol 14-α-methylergosta-8,24(28)-dien-3β,6α-diol, resulting in cellular toxicity

(Whaley *et al.*, 2017). Mutations causing loss of function in Erg3 prevent accumulation of these toxic metabolites, causing resistance to azoles (Sanglard *et al.*, 2003; Martel *et al.*, 2010). However, *ERG3* mutations have also been associated with reduced virulence in a mouse model of invasive candidiasis, suggesting there is a fitness cost to gaining azole resistance by this mechanism (Hirayama *et al.*, 2020). Overexpression of drug efflux pumps is a further mechanism of azole resistance in *C. albicans*. Deletion of *MDR1* in a fluconazole resistant *C. albicans* clinical isolate led to loss of the resistant phenotype (Hiller, Sanglard and Morschhäuser, 2006). In a range of *C. albicans* clinical isolates from vulvovaginal candidiasis, upregulation of *CDR1* and *MDR1* was strongly correlated with fluconazole resistance and with progression to RVVC (Esfahani *et al.*, 2024).

Resistance to echinocandins is less common and the primary known mechanism of echinocandin resistance is mutation of *FKS1* – a gene encoding the catalytic subunit of the β(1,3)-glucan synthase complex (Lee, Robbins and Cowen, 2023). Sequencing of 4 caspofungin-resistant *C. albicans* isolates identified mutations in *FKS1*-S645. Introduction of a single *FKS1*-P645 allele to a previously susceptible lab strain of *C. albicans* (CAI4) conferred resistance to caspofungin, suggesting this single point mutation alone is sufficient for caspofungin resistance (Park *et al.*, 2005). It is believed this S645P mutation reduces the affinity to echinocandins (Park *et al.*, 2005; Sawadogo *et al.*, 2019)

C. albicans may also display antifungal tolerance – a phenotype that is distinct from antifungal susceptibility or resistance. Antifungal tolerance is defined as a subpopulation of cells that have an antifungal susceptible genotype in resting conditions but are able to grow slowly at inhibitory drug concentrations (Rosenberg *et al.*, 2018). Isolates taken from patients with persistent *C. albicans* infections were more likely to display a tolerance phenotype than *C. albicans* isolates that were cleared by a single course of fluconazole treatment (Rosenberg *et al.*, 2018). 69.2% of tested *C. albicans* strains displayed temperature-dependent tolerance *in vitro*, including *C. albicans* SC5314, while 23.3% strains were tolerant at all tested temperatures (30 °C, 37 °C and 39 °C; Yang *et al.*, 2023). The mechanisms underlying tolerance require further investigation, particularly *in vivo*.

1.4 Non-albicans *Candida* spp

1.4.1 *Candida auris*

*Candida auris (*recently renamed *Candidozyma auris*) was first identified in Japan in 2009 (Satoh *et al.*, 2009; Liu *et al.*, 2024). Since its discovery 16 years ago, *C. auris* has been detected around the world, being isolated from patients in at least 40 countries across 6 continents and causing outbreaks in China, UK and USA (Adams *et al.*, 2018; Rhodes *et al.*, 2018; H. Du *et al.*, 2020; Bing *et al.*, 2024). *C. auris* has emerged as a globally significant pathogen, being designated at a critical priority fungal pathogen by the WHO (WHO, 2022; Chowdhary, Jain and Chauhan, 2023). Poor

surveillance mechanisms mean that global case numbers of *C. auris* infections are not known. The CDC reported 4514 cases of *C. auris* infections in the USA in 2023, representing a 54% increase in cases since 2022 and a 8751% increase since 2016 (CDC, 2024). A report of ICU patients in India with candidaemia revealed *C. auris* was the leading causative agent (Shastri *et al.*, 2020).

C. auris was named *auris* after the latin word for 'ear', where it was first isolated (Satoh *et al.*, 2009). *C. auris* is able to grow at temperatures up to 42 °C, higher than other *Candida* spp., which may aid its pathogenicity in humans (Satoh *et al.*, 2009; H. Du *et al.*, 2020). It was previously believed that *C. auris* is unable to form hyphae. This hypothesis was supported by genome analysis, which revealed *C. auris* lacks *HWP1* (Hyphal wall protein 1) and *ECE1* (Candidalysin) genes – genes that are highly expressed by *C. albicans* hyphae (Muñoz *et al.*, 2018). However, Yue *et al.* demonstrated that passage through a mouse host led to a filamentous phenotype: elongated *C. auris* cells that resemble *C. albicans* true hyphae but had some different morphological characteristics, including a greater number of vacuoles and a preference for growing at lower temperatures (Yue *et al.*, 2018). Strangely, *EFG1* (Enhanced filamentation growth protein 1), essential for hyphal growth in *C. albicans*, is downregulated in filamentous *C. auris* cells (Yue *et al.*, 2018). Filamentous growth of *C. auris* has since been observed widely (Bravo Ruiz *et al.*, 2020; Fan *et al.*, 2021; Santana and O'Meara, 2021; Bryak *et al.*, 2024). Fan *et al.* proposed *C. auris* exists in 4 different morphologies: typical yeast, filamentous, elongated and aggregated (Fan *et al.*, 2021). Hsp90 has been implicated as a repressor of filamentous growth in *C. auris,* as well as having a role in azole tolerance (Kim *et al.*, 2019). The exact mechanism of filamentation regulation in *C. auris* and the biological and clinical significance of different *C. auris* morphologies remains unclear.

Understanding of the innate immune response to *C. auris* is uncertain and often conflicting. *C. auris* elicited a stronger pro-inflammatory cytokine response by human peripheral blood mononuclear cells than *C. albicans* and was less virulent in a mouse infection model (Bruno *et al.*, 2020). Contrastingly, Wang *et al.* claimed *C. auris* induces a less potent innate immune response than *C. albicans* by murine bone-derived macrophages and in a murine *in vivo* infection model (Y. Wang *et al.*, 2022). These differences may be explained by the difference in cell type used or heterogeneity between the different strains of *C. auris* used. Although differences have been observed in the cytokine profile elicited by *C. auris* infection, multiple studies have corroborated that *C. auris* has reduced virulence compared to *C. albicans* in a murine infection model (Ben-Ami *et al.*, 2017; Wang *et al.*, 2018; Bruno *et al.*, 2020; Y. Wang *et al.*, 2022). In contrast, in a zebrafish swim bladder infection model, *C. auris* was significantly more virulent than *C. albicans* and fish infected with *C. auris* had a greater fungal burden at 96 hpi than fish infected with *C. albicans* (Pharkjaksu *et al.*, 2021). Coculture with human neutrophils revealed *C. auris* is resistant to neutrophil killing.

Furthermore, human neutrophils did not produce NETs when exposed to *C. auris*, but do in the presence of *C. albicans* (Johnson *et al.*, 2018). Phagocytosis of *C. auris* by neutrophils was also reduced compared to *C. albicans*, and this was observed across multiple *C. auris* strains from different clades (Johnson *et al.*, 2018; Horton *et al.*, 2021). *C. auris* infection stimulated reduced neutrophil recruitment compared to *C. albicans*, with approximately 50% fewer neutrophils being recruited to *C. auris* in zebrafish hindbrain infection at 4 hpi. Neutrophils recruited to *C. auris* in zebrafish appeared viable but did not produce NET-like structures, whereas neutrophils recruited to *C. albicans* appeared to undergo NETosis (Johnson *et al.*, 2018). Examination of the *C. auris* cell wall revealed increased abundance of *N*-linked mannans in the cell wall compared to *C. albicans*, which was driven by *C. auris PMR1* and *VAN1*. Mutation of *PMR1* and *VAN1* resulted in reduced cell wall mannan, which was compensated for by an increase in cell wall glucan (Horton *et al.*, 2021). *C. auris pmr1Δ* and *van1Δ* also had increased neutrophil recruitment in zebrafish hindbrain infection, reduced fungal burden in zebrafish and increased phagocytosis and killing by human neutrophils *in vitro* (Horton *et al.*, 2021). These results reveal a mechanism for *C. auris* evasion of neutrophil responses by human and zebrafish neutrophils but do not explain why *C. auris* appears to lack virulence in murine infection models. It has been suggested difference in host species neutrophil receptors may be responsible for the ability of mice to control *C. auris* infection (Ferwerda *et al.*, 2009; Li *et al.*, 2011; Johnson *et al.*, 2018). Regardless, further work is required to decipher the full interaction between *C. auris* and host innate immune cells.

Unlike *C. albicans, C. auris* is not a common component of the gut microbiota (H. Du *et al.*, 2020; Chowdhary, Jain and Chauhan, 2023; Tharp *et al.*, 2023). However, *C. auris* is highly competent at colonising the skin. Although skin is initially resistant to *C. auris* colonisation due to the prevalence of *Malassezia* species, once established on the skin, *C. auris* is fairly stable (Proctor *et al.*, 2021). Huang *et al.* reported that *C. auris* is able to persistently colonise the skin, while also penetrating the deeper skin tissue (Huang *et al.*, 2021). It is believed this skin colonisation may facilitate the spread of *C. auris* infections within a hospital environment (Alanio *et al.*, 2022; Chowdhary, Jain and Chauhan, 2023). *C. auris* is able to survive on a variety of surfaces for over 3 weeks (Dire *et al.*, 2023), which may further enable nosocomial spread of infection. There is steadily accumulating evidence that healthcare environments are the primary site of transmission (Chowdhary, Sharma and Meis, 2017; Kappel *et al.*, 2024). Retrospective analysis of *C. auris* outbreaks in London hospitals provided evidence of likely inter- and intra-hospital transmission, with temperature probes suspected to cause at least 4 transmissions (Kappel *et al.*, 2024).

C. auris isolates have a high level of antifungal resistance. Analysis of isolates from 54 patients, sourced from Pakistan, India, South Africa and Venezuela, revealed 93% of *C. auris* isolates were

resistant to fluconazole, 54% resistant to voriconazole, 35% resistant to amphotericin B, 7% resistant to echinocandins and 6% resistant to flucytosine (Lockhart *et al.*, 2017). 41% isolates were resistant to 2 or more classes of antifungals. Mortality within this cohort of patients was 59%, indicating the danger posed by these highly resistant infections. An alternative analysis of 350 isolates from Indian hospitals found 90% of isolates were fluconazole resistant and 25% were resistant to 2 or more classes of antifungals (Chowdhary *et al.*, 2018). The prevalence of multidrug resistance means there is now a lack of treatment options for *C. auris* infections, making *C. auris* infections a profound clinical challenge (De Gaetano *et al.*, 2024).

1.4.2 *Candida glabrata* (*Nakaseomyces glabrata*)

C. glabrata is estimated to account for 15-25% of cases of invasive candidiasis (Hassan, Chew and Than, 2021) and has been designated a high priority fungal pathogen (WHO, 2022). *C. glabrata* has been proposed to be renamed to *Nakaseomyces glabrata* numerous times (or to *Nakaseomyces glabratus* in 2022), though this remains controversial (de Hoog *et al.*, 2023; Denning, 2024b). *C. glabrata* is a common cause of candidiasis, so renaming could lead to confusion in a clinical setting and dilute public health messaging around the significance of *Candida* spp. On the other hand, based on phylogenetic distance, *C. glabrata* is more distantly related to *C. albicans* than humans are to snakes (Katsipoulaki *et al.*, 2024).

Whereas *C. albicans* is dimorphic, *C. glabrata* is unable to undergo the yeast-to-hyphal transition (Hassan, Chew and Than, 2021). Under prolonged exposure to high CO_2 concentrations, *C. glabrata* is able to form aggregated chains of pseudohyphal cells (Sasani *et al.*, 2016). The lack of true hyphae for tissue invasion means *C. glabrata* has very different virulence mechanisms from *C. albicans*. Epithelial adhesins, encoded by *EPA* genes, have been heavily implicated in *C. glabrata* virulence. *C. glabrata* isolates have between 17 and 23 Epa proteins (Frías-De-León *et al.*, 2021). *EPA6*, an adhesin associated with urinary tract infections, has been shown to be critical for biofilm formation (Domergue *et al.*, 2005; Iraqui *et al.*, 2005). *GCN5* and *MSS11* have both been demonstrated to regulate *EPA6*, suggesting some redundancy in regulation of virulence factors. *C. glabrata gcn5Δ* and *C. glabrata mss11Δ* were shown to have reduced virulence in macrophages *in vitro* and *Galleria mellonella in vivo*, respectively (S. Yu *et al.*, 2022; Wang *et al.*, 2024). *GCN5* also had a divergent function, with *C. glabrata gcn5Δ* having reduced azole and echinocandin resistance (S. Yu *et al.*, 2022). Yapsin family proteases, encoded by *YPS* genes, have also been demonstrated to have a key role in *C. glabrata* virulence (Kaur, Ma and Cormack, 2007). *Yps* are GPI-linked aspartyl proteases, hypothesised to remodel the cell surface by removal of other GPI-linked cell wall proteins, which is believed to regulate *C. glabrata* virulence. 11 *YPS* genes have been identified, of which 8 are unique to *C. glabrata.* Mutation of all *YPS* genes results in an inability for *C. glabrata* to form biofilms,

though reintroduction of *YPS1* alone is sufficient to restore biofilm formation *in vitro* (Rasheed, Battu and Kaur, 2018). *Yps* proteins have also been associated with modulating the secretome of *C. glabrata,* with *Yps1* and *Yps7* also shown to be part of the secretome (Rasheed, Kumar and Kaur, 2020). *C. glabrata ypsΔ* mutants stimulated greater production of NO by macrophages *in vitro* than wild-type *C. glabrata*, which may hint at a wider role for Yps proteases in modulation of host immune responses (Kaur, Ma and Cormack, 2007).

1.4.3 *Candida parapsilosis*

Designated a high priority fungal pathogen, *Candida parapsilosis* is one of the most common non-albicans causes of invasive candidiasis: *C. parapsilosis* ranks 3rd in the UK, USA and Australia, 2nd in Spain, China and Japan and 1st in Venezuela and Colombia as causes of candidaemia in ICU patients (Tóth *et al.*, 2019). *C. parapsilosis* is a member of the *C. parapsilosis* complex, which also consists of *C. parapsilosis, Candida orthopsilosis* and *Candida metapsilosis*, formerly known as *C. parapsilosis* Group I, II and III (Tavanti *et al.*, 2005). The 3 species are phenotypically indistinguishable. Examination of *C. parapsilosis* isolates across a 20-year period detected 3.9% of isolates were fluconazole resistant (Pfaller *et al.*, 2019). Smaller single-centre studies have found higher levels of fluconazole resistance: from 7.5% *C. parapsilosis* isolates in a hospital outbreak in France to 54% *C. parapsilosis* isolates in an outbreak in Mexico and 68% of *C. parapsilosis* isolates from a Brazilian hospital (Corzo-Leon *et al.*, 2021; Fekkar *et al.*, 2021; Thomaz *et al.*, 2021). In neonatal patients in South Africa, 54% *C. parapsilosis* isolates were fluconazole resistant and 14% isolates were voriconazole resistant (Magobo *et al.*, 2017). Hence, *C. parapsilosis* antifungal resistance is highly variable, which may be a reflection of local infection control policy, azole use, antimicrobial stewardship practices and regionalised spread of fluconazole resistant clones (Escribano and Guinea, 2022).

C. parapsilosis was originally most associated with infections in neonates, particularly neonates born prematurely or with low birth weight (Tóth *et al.*, 2019; Govrins and Lass-Flörl, 2024). The reasons for the affinity of *C. parapsilosis* for neonatal infection are not understood. *C. parapsilosis* is a common component of the skin and oral microbiome in healthy term-born infants (Ward *et al.*, 2018). *C. parapsilosis* is a minor component of the adult microbiome and expansion of *C. parapsilosis* in the microbiome was observed to precede development of invasive candidiasis (Zhai *et al.*, 2020). Increased prevalence of *C. parapsilosis* in the mycobiome of pre-term infants was observed, though this study lacked full-term infant controls so could only compare *C. parapsilosis* prevalence in pre-term infants with previously published values in full-term infants, meaning experimental differences could be responsible for variation in observed *C. parapsilosis* prevalence. *C. parapsilosis* was the most abundant fungi in infants born <31 weeks (James *et al.*, 2020). Mycobiome testing was done on

faecal samples taken at 6-18 months old, suggesting differences in *C. parapsilosis* incidence persist throughout early life. However, it is unclear when *C. parapsilosis* establishes its position in the developing mycobiome. The authors hypothesised pre-term infants acquired *C. parapsilosis* from the hospital environment, rather than vertical transfer from the mother. It may be that pre-term infants have greater *C. parapsilosis* prevalence in the mycobiome because of the time spent in neonatal ICUs, though this does not explain why *C. parapsilosis* becomes dominant in the mycobiome rather than other nosocomial fungi, such as *C. albicans*. Recently, there has been a shift in epidemiology, with *C. parapsilosis* infections becoming increasingly common in adult ICU patients (Brescini *et al.*, 2022).

C. parapsilosis is unable to form true hyphae but can form pseudohyphae (Tóth *et al.*, 2019). The role of pseudohyphae in virulence is poorly characterised. Laffey & Butler identified 4 *C. parapsilosis* phenotypes: crepe, concentric, crater and smooth. Crepe and concentric cells were almost entirely pseudohyphal; crater and smooth cells were almost entirely yeast. They demonstrated concentric (pseudohyphal) *C. parapsilosis* was significantly better at forming biofilms *in vitro* than other phenotypes, implying a role for pseudohyphae in biofilm formation (Laffey and Butler, 2005). However, *C. parapsilosis hst1Δ/Δ*, unable to form pseudohyphae, had no difference in virulence in *G. mellonella* infection compared to wild type *C. parapsilosis* (Rupert and Rusche, 2022). No correlation was observed between pseudohyphal length or spatial orientation and engulfment time by macrophages *in vitro*, suggesting pseudohyphae do not play a role in delaying immune clearance (Tóth *et al.*, 2014). Tóth *et al.* aimed to identify other virulence factors in *C. parapsilosis*. 3 genes were identified with regulatory roles in virulence: *CPAR2_100540* had a role in iron acquisition and protecting against oxidative stress; *CPAR2_200390* regulated cell wall homeostasis, morphogenesis and biofilm formation; *CPAR2_303700* affected viability, growth rate, adhesion and susceptibility to hydrogen peroxide (Tóth *et al.*, 2018). Counter to its pathogenic role, *C. parapsilosis* caused negligible cell damage to premature intestinal epithelial cells *in vitro* and reduced hyphal invasion and cell damage by *C. albicans* when cocultured, counter-intuitively demonstrating a protective role for *C. parapsilosis* (Gonia *et al.*, 2017). *C. parapsilosis* supernatant alone was able to reduce the virulence of *C. albicans* in a zebrafish swim bladder infection model. There is a complicated picture around *C. parapsilosis* pathogenesis that needs unravelling.

1.4.4 *Candida guilliermondii*

C. guilliermondii (also known as *Meyerozyma guilliermondii*, or previously as *Pichia guilliermondii*) is a relatively uncommon pathogen. Incidence of candidaemia due to *C. guilliermondii* ranges from 1 to 3%, depending on the region (Hirayama *et al.*, 2018). *C. guilliermondii* is most associated with chronic onychomycosis: infection of the nail plate or nail bed (Savini *et al.*, 2011; Merad *et al.*, 2021).

The *C. guilliermondii* complex refers to a group of phenotypically and morphologically indistinguishable *Candida* species, including *Candida guilliermondii, Candida fermentati, Candida carpophila* and *Candida xestobii* (Hirayama *et al.*, 2018). While *C. guilliermondii* is currently fairly rare, it is considered an emerging nosocomial pathogen.

C. guilliermondii has increased levels of antifungal resistance compared to *C. albicans*. Savini *et al.* previously reported 75.2% *C. guilliermondii* was sensitive to fluconazole, compared to 97.8% *C. albicans*. *C. guilliermondii* also has reduced sensitivity to echinocandins (Savini *et al.*, 2011). Analysis of *C. guilliermondii* isolates from 22 patients from Spain revealed *C. guilliermondii* had a significantly greater Minimum Inhibitory Concentration (MIC) compared to *C. albicans* for all tested antifungals. *C. guillidermondii* MICs were 2.0 μg/ml for anidulafungin, 16.0 μg/ml for fluconazole and 1.0 μg/ml for amphotericin B, compared to 0.031 μg/ml, 0.25 μg/ml and 0.125 μg/ml, respectively, for *C. albicans* isolates (Marcos-Zambrano *et al.*, 2017). A similar study of *C. guilliermondii* isolates in Mexico revealed 31% isolates were resistant to caspofungin and 1 isolate was resistant to fluconazole and caspofungin (Castillo-Bejarano *et al.*, 2020). *C. guilliermondii* is unable to form true hyphae (pseudohyphae can be observed under certain conditions) but does undergo phenotypic switching between white and brown phenotypes (Papon *et al.*, 2013; Lastauskienė *et al.*, 2015). Brown phenotype *C. guilliermondii* exhibited 20 times greater resistance to amphotericin B and a 2 times greater resistance to acetic acid, showing phenotypic switching plays a role in antifungal resistance in *C. guilliermondii* (Lastauskienė *et al.*, 2015). Phenotypic switching occurs at a relatively low frequency, but survival of brown phenotype *C. guilliermondii* may explain the reoccurrence of chronic infections following antifungal treatment. Unlike *C. albicans*, *C. guilliermondii* is widely distributed in nature. *C. guilliermondii* is frequently isolated from soil, plants, insects, seawater, the atmosphere and trees, as well as being a component of the human microbiome (Savini *et al.*, 2011). It could be hypothesised that prolonged environmental exposure to antifungals has resulted in increased evolution of antifungal resistance.

1.5 Host-*C. albicans* Interactions

1.5.1 Components of immunity

The immune system is broadly composed of two parts: innate immunity and adaptive immunity. The innate immune system forms the initial defence against pathogens. Innate immunity consists of a wide range of cells and tissues, from mucosal membranes and skin, which form physical barriers to prevent pathogen entry, to sophisticated phagocytic cells, such as neutrophils and macrophages (Janeway *et al.*, 2001). Innate immune cells develop in the bone marrow from haematopoietic stem cells (HSCs). HSCs can differentiate into myeloid or lymphoid progenitor cells. Further differentiation

of myeloid progenitor cells gives rise to the majority of innate immune cells: neutrophils, basophils, eosinophils and monocytes, which can further differentiate into dendritic cells and macrophages. Lymphoid progenitor cells predominantly differentiate into adaptive immune cells, but can also differentiate into innate lymphocytes: natural killer cells and innate lymphoid cells (Carpenter and O'Neill, 2024). Innate immunity is typically thought of as lacking immune memory, though this dogma has been challenged in recent years by the observation of trained innate immunity (Netea *et al.*, 2020).

Adaptive immunity confers pathogen-specific, long-lasting protection. This takes considerably more time than innate immune responses, with adaptive immune responses taking 4-7 days to develop (Janeway *et al.*, 2001). The adaptive immune system can be divided into two components: T cells and B cells (Janeway *et al.*, 2001; Chi, Pepper and Thomas, 2024). Lymphoid progenitor cells in the bone marrow differentiate into immature T cells, which migrate to the thymus for selection and maturation. In the thymus, T cells are educated to recognise self-antigens, with auto-reactive T cells removed, and express either CD4 or CD8 coreceptors (Fang *et al.*, 2018; Sun *et al.*, 2023). CD4+ T cells (T helper cells) primarily coordinate cytokine and cellular responses to pathogens, while CD8+ T cells (cytotoxic T cells) induce cell death of target cells with intracellular pathogens and form memory T cells for long-lasting protection (Sun *et al.*, 2023). Lymphoid progenitor cells can also differentiate into immature B cells (Fang *et al.*, 2018). Presentation of foreign antigens to immature B cells triggers differentiation and maturation. B cells undergo somatic hypermutation, in which B cells intentionally introduce point mutations into the variable region of immunoglobulin genes to produce an antibody with greater affinity for the antigen (Chi, Pepper and Thomas, 2024). This forms plasma B cells, which produce high quantities of antibodies to opsonise pathogens and neutralise toxins. A subset of mature B cells become memory B cells, which ensure long-lasting protection (Janeway *et al.*, 2001; Chi, Pepper and Thomas, 2024). Both innate and adaptive immune responses are required for full protection against pathogens. The specific mechanisms used by the immune system to respond to *C. albicans* infections are explored below (see 1.5.2 Innate immune response to *C. albicans* and 1.5.3 Adaptive immune response to *C. albicans*).

1.5.2 Innate immune response to *C. albicans*

1.5.2.1 Barriers to entry

The first components of our protection against invading fungi are the physical and anatomical barriers that prevent entry of pathogens, primarily skin and mucosal membranes (Drummond, Gaffen, *et al.*, 2015).

The skin is colonised by a range of commensal microorganisms, the main fungal species being members of the *Malassezia* genus (Findley *et al.*, 2013; de Hoog *et al.*, 2017). *Candida* spp. are relatively minor components of the skin microbiota (Findley *et al.*, 2013). Tight junctions in the epithelia form a physical barrier to fungal entry, while *C. albicans* colonisation on the skin has been shown to be controlled by skin-resident dendritic cells (Igyártó *et al.*, 2011; Drummond, Gaffen, *et al.*, 2015). There are at least three different subsets of skin-resident dendritic cells: Langerhans cells, Langerin$^+$ dermal dendritic cells and classic dermal dendritic cells. In a *C. albicans* skin infection mouse model, Langerin$^+$ dermal dendritic cells promoted the development of Th1 cells, while inhibiting Th17 cell development. Absence of Langerin$^+$ dermal dendritic cells led to an absence of Th1 cells. Concurrently, Langerhans cells stimulated Th17 cell development, suggesting Langerhans cells and Langerin$^+$ dermal dendritic cells are using unique and opposing responses to promote clearance of *C. albicans* skin infections (Igyártó *et al.*, 2011). Indwelling central venous lines are a major source of invasive *Candida* infections, providing a direct route for *Candida* spp. on the skin to bypass these barriers and enter the body (Lass-Flörl *et al.*, 2024).

C. albicans is a relatively common commensal in the gastrointestinal tract, present in the gut microbiome of 70% of healthy individuals (Underhill and Iliev, 2014; Witherden *et al.*, 2017). As with the skin, tight junctions and adherens junctions maintain the integrity of the epithelial barrier (Tong and Tang, 2017). Mucin glycoproteins are the primary component of gastrointestinal mucus. Exposure of *C. albicans* to various mucins *in vitro* suggested mucins suppress *C. albicans* hyphae formation, reduce *C. albicans* adhesion to epithelial cells and suppress formation and maturation of *C. albicans* biofilms (Kavanaugh *et al.*, 2014). *C. albicans* proteases are able to degrade mucins, suggesting this is an ongoing competitive interaction (Colina *et al.*, 1996). Phagocytic cells patrol the lamina propria to ensure any *C. albicans* that crosses the gut epithelium are detected and killed (Tong and Tang, 2017). Gastrointestinal commensals are believed to be the site of origin of many systemic candidiasis infections, suggesting these defences are not always successful, especially in immunocompromised patients, patients treated with broad spectrum antibiotics and abdominal surgery patients (Miranda *et al.*, 2009; Lass-Flörl *et al.*, 2024).

1.5.2.2 Neutrophils

Neutrophils are critical for the anti-*Candida* immune response (summarised in Figure 1.4; Brown, 2011). Neutrophil recruitment to *C. albicans* is driven by detection of pathogen/damage associated molecular patterns (PAMP/DAMPs) by pattern recognition receptors (PRRs) and subsequent inflammatory cytokine/chemokine production (summarised in Table 1.1; modified from Burgess, Condliffe and Elks, 2022). Several toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are critical for the recognition of *C. albicans* PAMPs (summarised in Table 1.1; modified from Burgess,

Condliffe and Elks, 2022). Mincle, a CLR, binds α-mannose and other fungal cell wall components, resulting in pro-inflammatory signalling and recruitment of neutrophils (Yamasaki *et al.*, 2009; Patin, Thompson and Orr, 2019). Candidalysin, produced by hyphal *C. albicans*, appears to be a potent stimulator of innate immune responses in mucosal, central nervous system and systemic infections (Richardson *et al.*, 2018; Drummond *et al.*, 2019; Swidergall *et al.*, 2019). Candidalysin stimulates IL-1β production via a CARD9-dependent mechanism, which in turn leads to CXCL1-mediated recruitment of neutrophils (Drummond *et al.*, 2019). TRAF1 (induced by pro-inflammatory cytokine TNF) inhibits CXCL1 in *C. albicans* infection (Carpentier and Beyaert, 1999; Bai *et al.*, 2020), suggesting a regulatory role for TRAF1 to prevent excess neutrophil recruitment and activation. Epidermal growth factor receptor (EGFR) may also be key for immune responses to candidalysin. Inhibition of EGFR in mouse models of oral candidiasis reduces IL-1β and CXCL1 production (Ho *et al.*, 2019; Swidergall *et al.*, 2021), highlighting a role for EGFR in induction of CXCL1-mediated neutrophil recruitment. Neutrophil recruitment and survival are also reduced by EGFR inhibition, providing strong evidence of the link between candidalysin, EGFR and CXCL1-mediated neutrophil recruitment. IL-33 is another key mediator of neutrophil recruitment (Le *et al.*, 2012) and IL-33 knockout mice have increased mortality in *C. albicans* infection. Based on *in vitro* primary cell models, IL-33 operates via IL-23 and GM-CSF to promote phagocytosis by neutrophils (Nguyen *et al.*, 2022). IL-33 also suppresses IL-10 expression, resulting in superior fungicidal activity by neutrophils *in vitro*. IL-10 expression has been associated with persistent *C. albicans* infection in other *in vitro* data (Alvarez-Rueda *et al.*, 2020), which may be due to reduced neutrophil activity. IL-23 has additional mechanisms of aiding antifungal immunity. IL-23 deficient mice have increased myeloid cell apoptosis, resulting in reduced survival in systemic *C. albicans* infection (Nur *et al.*, 2019). Interestingly, this occurred independent of IL-17 and was unique to fungal infections.

Table 1.1: Pattern recognition receptors in *C. albicans* infection

Pattern Recognition Receptor	Localisation	Cell Expression	Adaptor Proteins	Effectors	Pathogen/Damage Associated Molecular Patterns Recognised	References
TLR2	Plasma membrane	Monocytes, macrophages, dendritic cells, mast cells, neutrophils	MyD88, Mal	NF-κB, TNF, TGFβ, IL-10, IL-12, IFNγ	Phospholipomannan, β-glucans	(Netea et al., 2008; Chai et al., 2009; Patin, Thompson and Orr, 2019; Jannuzzi et al., 2020)
TLR4	Plasma membrane, endosome membrane	Monocytes, macrophages, dendritic cells, mast cells, neutrophils, B cells, intestinal epithelium	MyD88, Mal, TRIF, TRAM	NF-κB, TNF, IL-8, Type I IFN	O-linked mannosyl, Mannan, Glucuronoxylomannan	(Netea et al., 2008; Chai et al., 2009; Patin, Thompson and Orr, 2019; Jannuzzi et al., 2020)
TLR7	Endosome membrane	Monocytes, macrophages, dendritic cells, B cells	MyD88	IFN-β, Type I IFN	ssRNA	(Netea et al., 2008; Bourgeois et al., 2011; Patin, Thompson and Orr, 2019; Jannuzzi et al., 2020)
TLR9	Endosome membrane	Monocytes, macrophages, dendritic cells, B cells	MyD88	NF-κB, IL-12, TNFα	Unmethylated DNA with CpG motif	(Netea et al., 2008; Ramirez-Ortiz et al., 2008; Kasperkovitz et al., 2011; Patin, Thompson and Orr, 2019; Jannuzzi et al., 2020)
Dectin-1	Plasma membrane	Monocytes, macrophages, dendritic cells, neutrophils, mast cells, some T cells	hemITAM	IL-2, IL-6, IL-10, IL-23	β-1,3-glucans	(Netea et al., 2008; Patin, Thompson and Orr, 2019; Jannuzzi et al., 2020)

Dectin-2	Plasma membrane	Monocytes, macrophages, dendritic cells, neutrophils	ITAM-FcRγ	TNFα	Mannose	(Netea et al., 2008; Patin, Thompson and Orr, 2019; Jannuzzi et al., 2020)
Mincle	Plasma membrane	Monocytes, macrophages, dendritic cells, neutrophils, mast cells, some B cells	ITAM-FcRγ	NF-κB, IL-1, IL-6, IL-10 IL-12, IL-23	α-mannose, glyceroglycolipid, mannosyl fatty acids, MSG/gpA	(Richardson and Williams, 2014; Patin, Thompson and Orr, 2019; Jannuzzi et al., 2020)
DC-SIGN	Plasma membrane	Macrophages, dendritic cells, activated B cells	LSP1	IL-10	Mannose, N-linked mannans, galactomannans	(Netea et al., 2008; Patin, Thompson and Orr, 2019; Jannuzzi et al., 2020)
Mannose Receptor	Plasma membrane	Macrophages, Kupffer cells, endothelial cells	Associated with FcRγ and GBR2, exact mechanism unknown	TNF, IL-1β	Mannose, α-glucans, chitin	(Fradin, Poulain and Jouault, 2000; Netea et al., 2008; Rajaram et al., 2017; Patin, Thompson and Orr, 2019)
MDA5	Cytoplasm	Monocytes, macrophages, dendritic cells, B cells, epithelial cells, endothelial cells, fibroblasts	CARDs, MAVs	NF-κB, Type I IFN, Type III IFN, TNFα, IL-12,	dsRNA	(Patin, Thompson and Orr, 2019; Wang et al., 2020)

Neutrophils have been shown to coordinate their migration to sites of infection through a process called neutrophil swarming (Lämmermann *et al.*, 2013). Swarming inhibits the growth of several fungal pathogens *in vitro*: *C. albicans, C. auris, Candida glabrata, C. neoformans* and *A. fumigatus* (Sun and Shi, 2016; Hopke *et al.*, 2020; Hind *et al.*, 2021). Swarms were smaller for yeast-locked *C. albicans* and *C. glabrata* (which are unable to hyphate) compared to wild type *C. albicans.* This could

imply a potential correlation between hyphae formation and neutrophil swarming, which requires further investigation. Swarming in fungal infections is dependent on the leukotriene lipid mediator LTB_4, meaning it operates by the same mechanism as swarming in other infections or injury (Lämmermann, 2016; Sun and Shi, 2016; Lee *et al.*, 2018). LTB_4 has further implications in *C. albicans* neutrophil responses, beyond swarming. Leukotriene A4 hydrolase (LTA4H) is the hydrolase and aminopeptidase enzyme that catalyses conversion of LTA_4 to LTB_4, while BLT1 is the receptor for LTB_4 (Xin *et al.*, 2024). *Lta4h*$^{-/-}$ mice had significantly reduced survival and increased fungal burden in *C. albicans* infection. Neutrophils isolated from *Lta4h*$^{-/-}$ mice had reduced phagocytosis and *C. albicans* killing *in vitro*. These observations were replicated in neutrophils isolated from *Blt1*$^{-/-}$ mice. The *in vitro* effects of *Lta4h* knockout could be abrogated by the presence of exogenous LTB_4, suggesting LTB_4 has a crucial role in regulating neutrophil phagocytosis and killing in *C. albicans* infection (Xin *et al.*, 2024).

Following migration to sites of infection, neutrophils have several mechanisms to eliminate fungal pathogens, including phagocytosis and degradation in the phagolysosome, degranulation, production of reactive oxygen species (ROS), production of RNS (fully explored in 1.5 Reactive nitrogen species) and neutrophil extracellular trap (NET) release. Neutrophils produce granules containing a range of bactericidal and fungicidal effectors, including myeloperoxidase, cathepsins, defensins and lactoferrin (Lacy, 2006) that can be directed to the phagosome. The extracellular secretion of effectors by degranulation also leads to fungal killing and is preferentially used in *Candida* infections with pseudo-hyphae (Diamond, Krzesicki and Jao, 1978). Degranulation was dependent on CXCR1 in *C. albicans* infection in a mouse model, demonstrating a novel function of murine CXCR1, which correlates with evidence that human CXCR1 promotes oxidative and non-oxidative bactericidal activity by neutrophils (Hartl *et al.*, 2007; Swamydas *et al.*, 2016). Neutrophils also produce ROS, such as superoxide or hydrogen peroxide, which can be used intracellularly to kill phagocytosed fungi, or extracellularly to target hyphae (Winterbourn *et al.*, 2006; Dupré-Crochet, Erard and Nüβe, 2013). The transcriptional response of human neutrophils to *C. albicans* infection was measured by RNA-seq after 60 minutes of coculture *in vitro* (Niemiec *et al.*, 2017). Key pro-inflammatory mediators were upregulated, including *NFκB, IL-1β, NLRP3* and *CXCL1*. While most genes were not significantly upregulated until 30 or 60 minutes post infection, *MAP3K9* and *APLN* were substantially upregulated within 15 minutes of infection (Niemiec *et al.*, 2017).

Neutrophils are capable of expelling chromatin covered in antimicrobial proteins, leading to entrapment and killing of extracellular pathogens (Urban *et al.*, 2009). These neutrophil extracellular traps (NETs) contribute to neutrophil control of *C. albicans* and reduce fungal burden in *C. albicans in vitro* human neutrophil models (Urban *et al.*, 2006, 2009; Hopke *et al.*, 2020) and may be involved in

swarm initiation (Hopke *et al.*, 2020; Isles *et al.*, 2021). NETs have also been demonstrated to directly stimulate Th17 cell differentiation, via TLR2 and RORγt, corresponding with increasing IL-17 and GM-CSF production (Wilson *et al.*, 2022). IL-17 and GM-CSF both stimulate neutrophil activity in *C. albicans* infection (Mengesha and Conti, 2017; Nguyen *et al.*, 2022), creating a feedback loop, in which NETs promote an adaptive immune response and additional neutrophil activity. A subpopulation of neutrophils also produce IL-17, which may further feed into this positive feedback loop (Taylor *et al.*, 2014). Recent evidence suggests NETosis induced by *C. albicans* can occur independent of Peptidylarginine deiminase 4 (PAD4), contradicting established literature that NETosis is PAD4-dependent (Rohrbach *et al.*, 2012; Guiducci *et al.*, 2018; Tatsiy and McDonald, 2018; Thiam *et al.*, 2020). Further research is needed to clarify whether PAD4-independent NETosis is a non-canonical mechanism of NETosis for all stimuli or is a phenomenon unique to fungal infections. Despite their fungicidal role, NETs may have an overall detrimental effect on the host. NET proteins intended to eliminate *C. albicans* have been observed bound to, but not killing, *C. albicans* and inducing apoptosis of host cells (Karkowska-Kuleta *et al.*, 2021).

1.5.2.3 Macrophages

The primary mechanism of pathogen clearance by macrophages is phagocytosis (Figure 1.4). Macrophage PRRs or Fc receptors bind to fungal PAMPs/DAMPs or opsonising antibodies respectively, triggering engulfment of the fungus (Gilbert, Wheeler and May, 2015). Two mechanisms of fungal engulfment have been described: zipper phagocytosis and coiling phagocytosis (Griffin *et al.*, 1975; Rittig *et al.*, 1998). Following investigation in mouse knockout models, Anion Exchanger 2, a Cl^-/HCO_3^- exchanger in the macrophage plasma membrane, has been suggested as a critical regulator of *C. albicans* engulfment. *Ae2* knockout mouse macrophages were less able to phagocytose zymosan than wild type macrophages *in vitro*, which correlated with impaired TNFα production (Urso *et al.*, 2016). TNFα typically stimulates neutrophil recruitment to *C. albicans* infection, enhances phagocytosis and interferes with *C. albicans* biofilm formation, so impaired TNFα production would impede these responses (Filler, Yeaman and Sheppard, 2005; Rocha *et al.*, 2017). Furthermore, *Ae2* knockout macrophages had increased intracellular pH, which inhibited Dectin-1 expression. Together, this resulted in impaired phagocytosis and killing of *C. albicans* by *Ae2* knockout murine macrophages (Urso *et al.*, 2016). Engulfed *C. albicans* are held in the phagosome, which fuses with the lysosome to form a phagolysosome. Acidification of the phagolysosome allows fungal degradation by acid-dependent proteases (such as Cathepsin D), combined with fungal killing by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Schuit, 1979; Brown, 2011; Gilbert, Wheeler and May, 2015).

Vomocytosis (also referred to as nonlytic exocytosis) is the expulsion of phagocytosed particles without degradation into the extracellular environment and occurs in macrophages *in vitro* in *C. albicans* and *C. neoformans* infection (Ma *et al.*, 2006; Bain *et al.*, 2012). Both the pathogen and macrophage remain intact and viable after vomocytosis (Bain *et al.*, 2012). MARCO (Macrophage Receptor with Collagenous domain) is a scavenger receptor on the macrophage plasma membrane. *Marco*$^{-/-}$ murine macrophages had increased vomocytosis of yeast-locked *C. albicans*, from roughly 3% of wild type macrophages experiencing at least one vomocytosis event to over 30% of *Marco*$^{-/-}$ macrophages (Onyishi *et al.*, 2024). MARCO may modulate *C. albicans* vomocytosis by an unknown mechanism. It is unclear whether vomocytosis is a *C. albicans*-driven strategy of immune evasion, a host-driven process to allow training of macrophages or a result of faulty macrophage-to-macrophage transfer (known as dragotcytosis; Dragotakes, Fu and Casadevall, 2019).

Phagocytosis of *C. albicans* can trigger a yeast-to-hyphae transition, leading to macrophage killing through mechanical piercing by hyphae or induction of pyroptosis, allowing escape of *C. albicans* (Uwamahoro *et al.*, 2014). To counteract this, macrophages are able to fold phagocytosed fungal hyphae at septal junctions, resulting in significantly reduced hyphal growth and disruption to the cell wall (Bain *et al.*, 2021). This represents a previously uncharacterised macrophage function, though exactly how much hyphal folding contributes to fungal clearance, and whether this occurs *in vivo*, is unknown.

Phagocytosis of *C. albicans* is not always possible: hyphae may become too long to phagocytose (Lewis *et al.*, 2012). Inability to phagocytose *C. albicans* typically leads to 'frustrated phagocytosis', a process in which there is downregulation of phagocytosis mechanisms and a strong inflammatory response, mediated by IL-1β (Oghiso and Kubota, 1986; Takemura *et al.*, 1986).

Macrophages are a highly heterogenous population, existing on a spectrum of behaviours between M1, pro-inflammatory phenotypes and M2, wound healing phenotypes (Atri, Guerfali and Laouini, 2018). Proteomic analysis revealed a pro-inflammatory to wound healing phenotypic switch in *C. albicans* infection (Reales-Calderón *et al.*, 2014). Stimulating an M1 phenotype led to decreased fungal burden and increased survival of mouse models in *C. albicans* infection (Gao *et al.*, 2020). This reveals a mechanism to avert pro-inflammatory macrophage polarisation to the detriment of the host, driven by interactions with *C. albicans*. It could be possible to improve outcomes of *C. albicans* infection by therapeutically promoting M1 macrophage polarisation, though this must be done with caution to prevent excess, harmful inflammation.

1.5.2.4 Other components of innate immune response

The complement system is a series of proteins which, when activated, trigger pathogen opsonisation, release of chemotactic factors and formation of the membrane attack complex in the pathogen cell membrane (Müller-Eberhard, 1988; Tsoni *et al.*, 2009). There are three main routes of complement activation: the classical, alternative and lectin pathways. Complement is crucial in responses to fungal infection, particularly the alternative complement pathway, with complement-deficient mouse and guinea pig models being more vulnerable to *Candida* infections (Gelfand *et al.*, 1978; Böttger *et al.*, 1986; Tsoni *et al.*, 2009). CR3 is a common β subunit of β2 integrin complement receptors and has been suggested to be the major receptor for detection of β-glucan by neutrophils, ahead of Dectin-1 (van Bruggen *et al.*, 2009). CR3 binds directly to β-glucan, triggering phagocytosis of β-glucan-bearing particles (van Bruggen *et al.*, 2009; O'Brien *et al.*, 2012). Binding of CR3 to β-glucan in *C. albicans* infection initiates a complex, temporally regulated pathway that differentially upregulates either neutrophil swarming or NETosis (Johnson *et al.*, 2017). CR3-mediated neutrophil phagocytosis of *C. albicans* is regulated by BLT1 – the receptor for LTB_4, a potent chemotactic mediator associated with neutrophil swarming (Xin *et al.*, 2024).

Innate lymphoid cells (ILCs) are a relatively recently discovered component of innate immunity and a rapidly emerging area of research. ILCs are primarily tissue-resident, cytokine-producing cells, derived from lymphoid progenitor cells but lacking T cell receptors (Vivier *et al.*, 2018). ILCs are split into several groups, based on their functional similarity to T cells, with ILC3s (similar to Th17 cells) having the most relevance to fungal infections (Ebbo *et al.*, 2017; Vivier *et al.*, 2018). In a mouse model of oropharyngeal candidiasis, ILCs in the oral mucosa were the primary source of pro-inflammatory IL-17 during *C. albicans* infection, acting as the first line of defence in the antifungal response. Depletion of ILCs increased susceptibility to *C. albicans* infection, with increased fungal burden and greater reduction in body weight (Gladiator *et al.*, 2013). ILC3s highly express the antibody Fc receptor Fcγ. Deletion of *Fcer1g* specifically in ILC3s in mice (conditional knockout by Cre-lox system) infected with *C. albicans* led to reduced survival and increased *C. albicans* burden (C. Huang *et al.*, 2024). *Fcer1g*-deficient ILC3s had significantly reduced expression of multiple pro-inflammatory genes, including *Il17a, Il22* and *Ccr7*. Exogenously added IL-17A partially rescued survival of mice with FcεR1γ-deficient ILC3s, suggesting FcεR1γ-mediated immunity is IL-17A-dependent in *C. albicans* infection (C. Huang *et al.*, 2024). This supports previous work that showed a critical role for IL-17A and IL-17F, produced by ILC3s, in clearance of *C. albicans* infection in the epidermis (Iwasawa *et al.*, 2022).

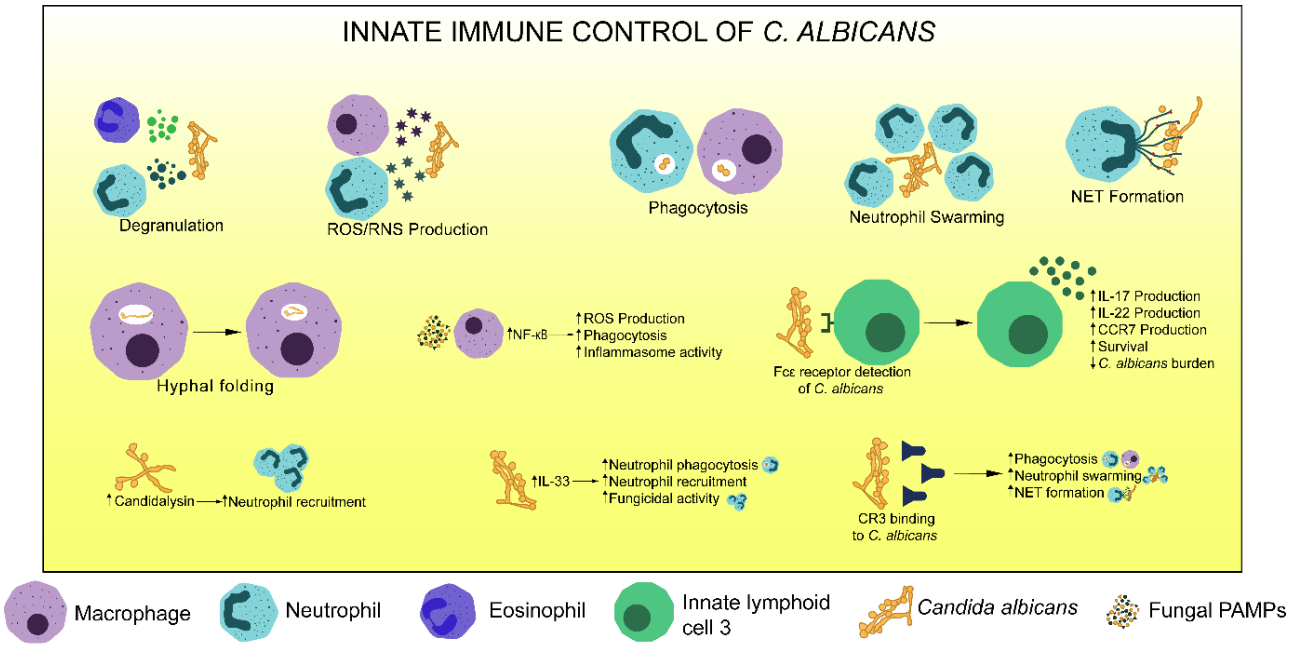

Figure 1.4: Innate immune response to *C. albicans* infection

Summary diagram of innate immune responses to *C. albicans.* Innate immune cells can kill *C. albicans* by degranulation, ROS/RNS production and phagocytosis . Neutrophils may undergo NETosis to kill *C. albicans* or coordinate their migration to carry out neutrophil swarming. Recognition of fungal ligands, such as candidalysin, stimulates production of IL-1β, triggering neutrophil recruitment (Drummond *et al.*, 2019). Increased expression of IL-33 in C. albicans infection triggers neutrophil recruitment and phagocytosis (Nguyen *et al.*, 2022). Macrophages can kill *C. albicans* hyphae by folding, causing mechanical damage to *C. albicans.* ILC3s produce pro-inflammatory cytokines in response to Fc receptor signalling, resulting in an inflammatory response and decrease in *C. albicans* burden (Gladiator *et al.*, 2013; C. Huang *et al.*, 2024). Complement protein CR3 binding to *C. albicans* trigger increased phagocytosis, neutrophil swarming and NETosis (Johnson *et al.*, 2017). Figure adapted from (Burgess, Condliffe and Elks, 2022).

1.5.3 Adaptive immune response to *C. albicans*

Innate immunity alone may not be sufficient to clear *C. albicans* infection, in which case a specific adaptive immune response develops. Although they are an innate immune cell, dendritic cells play a crucial role in linking innate and adaptive immune responses (Banchereau and Steinman, 1998). Dendritic cells differentiated from human peripheral blood monocytes, were shown to phagocytose and kill *C. albicans in vitro*, as well as stimulating T cell proliferation via presentation of *C. albicans*

antigens (Newman and Holly, 2001). Conditional knockout of FAM21 (involved in actin polymerisation and endosomal trafficking) in murine CD11c+ dendritic cells revealed reduced *C. albicans* phagocytosis *in vitro* and reduced survival in *C. albicans* infection *in vivo* (Kulkarni *et al.*, 2023). Antigen presentation of proteins was reduced in FAM21-deficient dendritic cells, though presentation of smaller peptides that did not require uptake and processing, was not affected, suggesting FAM21 has a role in antigen uptake (Kulkarni *et al.*, 2023). Dendritic cells elicit different responses depending on whether they ingest *C. albicans* yeast or hyphae, with yeast stimulating IL-12 production and priming of Th1 cells, while hyphae induced IL-4 production (d'Ostiani *et al.*, 2000). *C. albicans* yeast were also shown to stimulate Th17 cells, dependent on IL-6 secreted by Langerhans cells (specialised dermal dendritic cells), following binding to Dectin-1 (Kashem *et al.*, 2015). However, Langerhans cells did not stimulate Th17 cell development following exposure to *C. albicans* hyphae (Kashem *et al.*, 2015). Further to this, exposure to *C. albicans* hyphae stimulated significantly greater IL-33 production by dendritic cells *in vitro* than exposure to *C. albicans* yeast (Oh *et al.*, 2022). This increased IL-33 production was related to size of the stimulus: the physically larger *C. albicans* hyphae caused longer duration of Dectin-1 signalling, stimulating greater *Il33* expression. Increased IL-33 levels, due to hyphae exposure, triggered development of Th9 cells: a skin-tropic subset of CD4+ T helper cells that transiently secrete IL-9 in response to *C. albicans*, stimulating upregulation of pro-inflammatory cytokines by other T cell populations (Schlapbach *et al.*, 2014; Oh *et al.*, 2022). Together, these results show coordination of complex differential adaptive immune responses to yeast and hyphae by dendritic cells.

T cells can be split into 2 general groups: CD4+ T helper cells and CD8+ cytotoxic T cells, both of which have roles in antifungal immunity (Richardson and Moyes, 2015). CD4+CD8+ double positive T cells have been observed in *C. albicans* infection, though their exact function is unknown (Misme-Aucouturier *et al.*, 2019). Vaccination of mice with recombinant *C. albicans* Als3p protein induced Th1, Th17 and Th1/17 cell development, which was associated with increased phagocyte recruitment and increased survival in *C. albicans* infection *in vivo* (Lin *et al.*, 2009). Th17 cells conferred long term protection to *C. albicans* infection of the oral mucosa in mice and transplantation of CD4+ T cells into *Rag1*$^{-/-}$ mice (lacking T and B cells) accelerated clearance of oral *C. albicans* infection (Hernández-Santos *et al.*, 2013). Th17 cell differentiation is regulated by MBL (Mannan-binding lectin), potentially via IL-6 and TGF-β signalling (F. Wang *et al.*, 2022). Defects in Th17 cell development and function are associated with chronic mucocutaneous candidiasis (CMC; explored further in 1.4.4 Defects in immune response to *C. albicans*), persistent *Candida* infections of the mucous membranes, occasionally with systemic involvement, demonstrating the importance of Th17 cells in anti-*Candida* immunity (Okada *et al.*, 2016). CMC and systemic candidiasis are also

common in HIV infection, when CD4+ T cell counts fall below a threshold value (<200 cells/µl; Anwar, Malik and Subhan, 2012). CD8+ T cells are also important in mediating immunity to *Candida* spp. CD8+ T cells have been shown to inhibit growth of *C. albicans* hyphae *in vitro*, but their wider role is less well characterised than CD4+ T cells (Beno, Stöver and Mathews, 1995). A clinical study revealed invasive candidiasis patients had reduced CD8+ T cell counts compared to ICU patients without invasive candidiasis (Zhang *et al.*, 2021). Reduced numbers of CD8+ T cells was recapitulated in a mouse model of *C. albicans* infection. *C. albicans* was demonstrated to activate the mTOR signalling pathway, increasing expression of Eomes in CD8+ T cells, resulting in T cell exhaustion (Zhang *et al.*, 2021). T cell exhaustion has been observed elsewhere in *C. albicans* infection, with invasive candidiasis patient T cells expressing high levels of PD-1. PD-1 expression correlated with patient mortality from invasive candidiasis, suggesting this could be targeted therapeutically (Mellinghoff *et al.*, 2022).

Historic studies have suggested that B cells do not play a significant role in *C. albicans* immunity, with B cell deficient mouse models having no increase in susceptibility to *C. albicans* infection *in vivo* (Sinha, Prasad and Monga, 1987; Bistoni *et al.*, 1988). More contemporary investigations suggested *C. albicans* hyphae, but not yeast, directly activate B cells via TLR2 and MyD88 signalling (Ferreira-Gomes *et al.*, 2021). This stimulates IL-6 secretion, promoting Th17 cell development, suggesting B cells may work indirectly to protect against *C. albicans* infection (Ferreira-Gomes *et al.*, 2021). Intestinal IgA, produced by mature B cells, targets *C. albicans* adhesins, suppressing hyphae formation, promoting a commensal *C. albicans* phenotype (Ost *et al.*, 2021).

1.5.4 Defects in innate immune response to *C. albicans*

Immunocompromised patients, particularly neutropenic patients, are most at risk of candidiasis, whether their immune defects are caused by HIV/AIDS, immunosuppressive drugs, old age or genetic disorders (Yapar, 2014). As noted above, CMC is recurrent or persistent infections of mucosal membranes caused by *Candida* species, primarily by *C. albicans* (Okada *et al.*, 2016). Severity of CMC is highly variable and dependent on the anatomical location of *C. albicans* infection: from soreness around the mouth and ulceration in oral cavity infections to abdominal pain and severe diarrhoea in digestive tract infections (Humbert *et al.*, 2018). Although progression of CMC to systemic candidiasis is relatively rare, acquisition of invasive candidiasis in intensive care units is fairly common (7.07 episodes per 1000 admissions) and has a 42% 30-day mortality (Bassetti *et al.*, 2019).

Susceptibility to recurrent *Candida* infection in CMC is caused by various host genetic mutations, the most common being STAT1 gain of function mutations (Figure 1.5; Maródi *et al.*, 2012). These

mutations increase STAT1 responses to IFNα, IFNβ, IFNγ and IL-27, causing repressed development of IL-17 T cells and susceptibility to mucosal *Candida* infection (Lanternier *et al.*, 2013). IL-17 is a key regulator of antifungal immunity. IL-17 knockout mice have high susceptibility to *C. albicans* infection and reduced levels of neutrophil-recruiting chemokines (Kao *et al.*, 2004; Huppler *et al.*, 2014). A strong association has been demonstrated between candidiasis and use of IL-17 inhibitors (used in treatment of several inflammatory diseases), with significant downregulation of 9 pro-inflammatory cytokines or neutrophil-recruiting chemokines (Davidson *et al.*, 2022). A recent clinical study demonstrated 16 CMC patients had lower serum IL-17 levels than healthy controls (Chimenz *et al.*, 2022), supporting previous evidence that IL-17 is impaired in CMC patients (Puel *et al.*, 2012). IL-17 defects have been attributed to STAT1 gain of function mutations, IL-17 mutations and anti-IL-17 autoantibodies (Kisand *et al.*, 2010; Puel *et al.*, 2010, 2012; Bader *et al.*, 2012).

An alternative genetic mutation underlying CMC is autosomal recessive CARD9 deficiency (Figure 1.5; Corvilain, Casanova and Puel, 2018). CARD9 is an adaptor protein utilised by a variety of CLRs, such as Dectin-1, to stimulate NF-κB signalling. CARD9 deficiency has been demonstrated to cause reduced cytokine production by human patient peripheral blood mononuclear cells and impaired neutrophil recruitment in *in vivo* mouse models, leading to increased susceptibility to systemic candidiasis (Drummond *et al.*, 2011; Drummond, Collar, *et al.*, 2015). While several mutant CARD9 alleles associated with CARD9 deficiency have been revealed (Corvilain, Casanova and Puel, 2018), less established is the effect other gene mutations may have on CARD9 expression. Ovarian tumour deubiquitinase family member 1 (OTUD1) has recently been identified as a positive regulator of CARD9. OTUD1 deubiquitinates ubiquitinated CARD9, leading to CARD9 activation. Infection of OTUD1 homozygous knockout mice with *C. albicans* led to reduced mouse survival and increased fungal burden in kidney, lung and spleen slices compared to wild type mice (Chen *et al.*, 2021). OTUD1 mutations may cause susceptibility to *C. albicans* infection through CARD9 deficiency, highlighting a novel cause of CMC and introducing a potential therapeutic target for treatment of CARD9 deficiency.

Given its key role in NF-κB signalling, MyD88 defects have been associated with susceptibility to a range of infectious diseases (Figure 1.5; Picard, Casanova and Puel, 2011). MyD88 knockout mice are highly susceptible to *C. albicans* infection, with increased mortality and fungal burden (Villamón *et al.*, 2004). In a zebrafish wound model, MyD88 deficiency caused reduced recruitment of both local and distant neutrophils, which was maintained over 6 hours (Hu *et al.*, 2021). MyD88 deficiency, however, did not impair neutrophil activation, which was dependent on MAVS signalling. Although only studied in a wound model, if this mechanism is maintained in fungal infection, it would suggest

patients with MyD88 deficiency have impaired neutrophil recruitment (but no defects in neutrophil activation), which causes increased susceptibility to *C. albicans* infection.

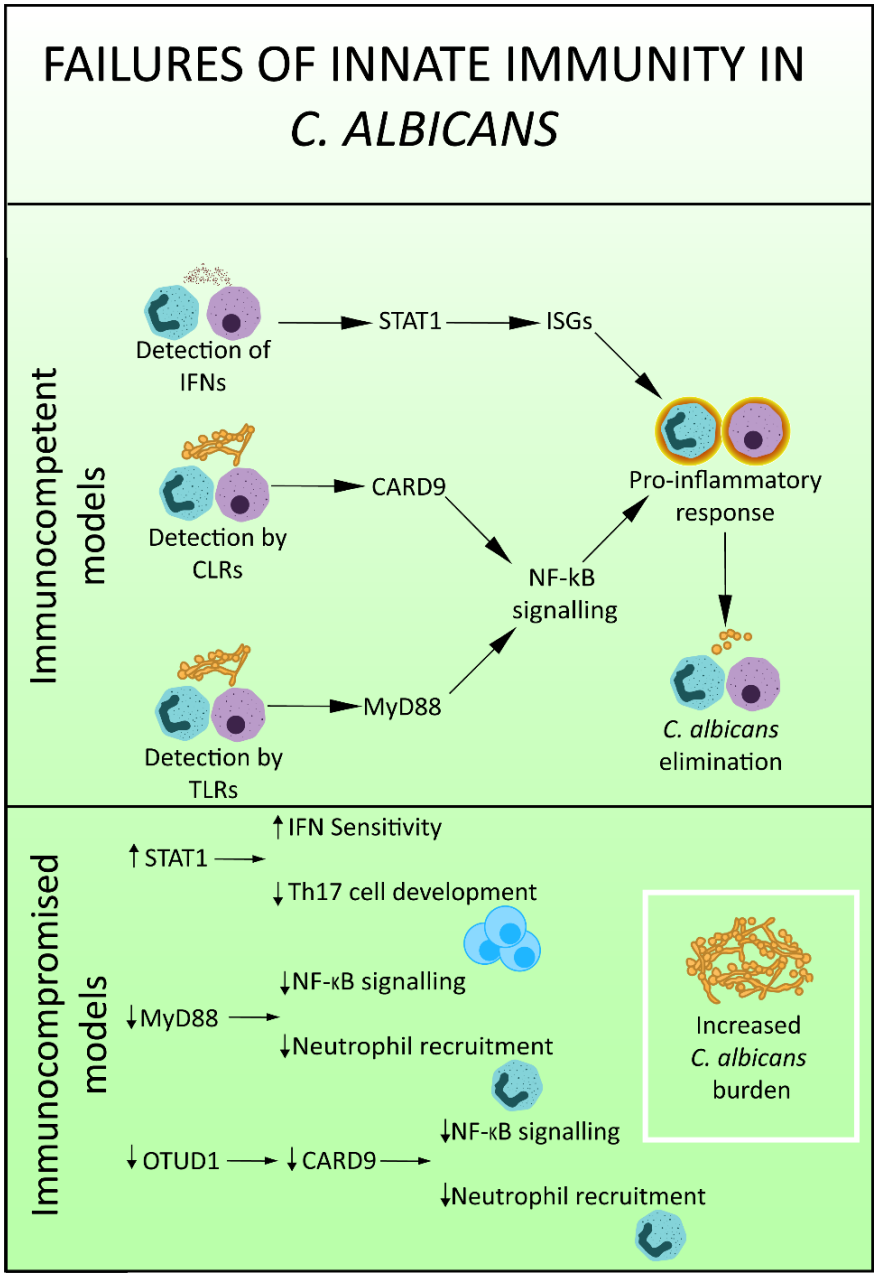

Figure 1.5: Defects in innate immune response to *C. albicans*

Examples of specific failures of innate immunity which lead to susceptibility to *C. albicans* infection. STAT1 gain of function mutations increase sensitivity to IFNs, leading to increased susceptibility to *C. albicans* infections (Lanternier *et al.*, 2013). OTUD1 or MyD88 deficiencies result in reduced transcription of NF-κB, resulting in reduced inflammatory response and increased *C. albicans* burden (Chen *et al.*, 2021; Hu *et al.*, 2021). Figure adapted from (Burgess, Condliffe and Elks, 2022).

1.5.5 *C. albicans* mechanisms of evading immunity

C. albicans has developed multiple mechanisms of evading host immune responses (Figure 1.6; Luo, Skerka, *et al.*, 2013). Greater understanding of these *C. albicans* evasion strategies may reveal new potential therapeutic targets.

Remodelling of the *C. albicans* cell wall can reduce immune recognition. *O*-mannan polysaccharides in the outer cell wall mask β-glucan, with loss of *O*-mannans leading to increased recognition by Dectin-1 and enhanced phagocytosis of *C. albicans* (Bain *et al.*, 2014). Similarly, β(1,3)-glucan in the cell wall is masked by phosphatidylserine. Mutation of Cho1, a phosphatidylserine synthase, increased the exposure of β(1,3)-glucan in the cell wall, resulting in greater Dectin-1 binding and increased release of TNF-α by macrophages *in vitro* (Davis *et al.*, 2014). However, *C. albicans cho1Δ* was not more susceptible to killing by RAW264.7 macrophages or human neutrophils *in vitro,* suggesting β-glucan masking reduces innate immune cell recruitment and cytokine production without affecting susceptibility to immune cell killing (Davis *et al.*, 2014). β-glucan masking occurs as a response to a diverse range of host signals suggestive of immune cell attack, such as hypoxia, growth on lactate or glycerol or limitation of copper or iron (Pradhan *et al.*, 2019). The mechanisms for β-glucan masking vary with the inducing signal: hypoxia-induced β-glucan masking is dependent on mitochondrial ROS signalling, whereas iron-related β-glucan masking is mitochondrial ROS-independent and mediated by Ftr1 and PKA (Pradhan *et al.*, 2019). Farnesol, a *C. albicans* quorum sensing molecule, is responsible for inducing β-glucan masking in response to changes in environmental pH (Cottier *et al.*, 2019).

Phagocytosis is the primary mechanism by which macrophages aim to control *C. albicans* infections. However, *C. albicans* has been observed to escape from macrophages, using a variety of strategies. Live imaging of murine bone marrow derived macrophages (BMDMs) and RAW264.7 cells *in vitro* observed macrophage cell death (Uwamahoro *et al.*, 2014). Macrophage death occurred in 2 phases: an early phase (which was absent from RAW264.7 cells) and a late phase. *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs had increased resistance against the early phase of cell death but not the late phase, suggesting pyroptosis may be responsible for early macrophage death. Late phase macrophage death required *C. albicans* hyphae, so this may be caused by mechanical piercing of macrophages by hyphae (Uwamahoro *et al.*, 2014). *C. albicans mmm1Δ* had impaired macrophage killing. Wild type *C. albicans* treated with an NLRP3 inhibitor (to inhibit inflammasomes) had the same level of macrophage killing as *C. albicans mmm1Δ* in the first 9 hours of co-culture, suggesting *C. albicans* MMM1 protein has a role in inducing macrophage NLRP3-dependent pyroptosis (Tucey *et al.*, 2016). *C. albicans mmm1Δ* had slower hyphal growth than wild type *C. albicans in vitro*. Hence, the proposed mechanism was that MMM1 promotes hyphal elongation and cell wall remodelling, which

facilitates rapid inflammasome activation and induction of pyroptosis to allow escape from macrophages (Tucey *et al.*, 2016). Gasdermin D is a key mediator of pyroptosis, via formation of a pore in the macrophage plasma membrane (Sborgi *et al.*, 2016). Gasdermin D pore formation is involved in *C. albicans* escape from macrophages *in vitro* and inhibition of gasdermin D protects against *C. albicans* infection in mice *in vivo* (Ding *et al.*, 2021). NLRP3 inflammasome activation *in vitro* has been observed in response to *C. albicans* Als3 and candidalysin (both hyphae-specific proteins), implying redundancy in the mechanisms used to escape from macrophages by pyroptosis (Kasper *et al.*, 2018; Rogiers *et al.*, 2019; Zhou *et al.*, 2025). Olivier *et al.* suggested 3 strategies of *C. albicans* inducing cell death to escape from macrophages: gasdermin D-mediated pyroptosis, induction of extracellular traps and formation of candidalysin pores in the macrophage membrane (Olivier *et al.*, 2022).

Biofilm formation plays an important role in evading the immune response to *C. albicans*. Migration of J774.1 macrophages was significantly slower through 3D *C. albicans* biofilms compared to migration towards planktonic *C. albicans* (Alonso *et al.*, 2017). *C. albicans* in biofilms were resistant to killing by human neutrophils *in vitro* (Xie *et al.*, 2012). Furthermore, biofilms affect neutrophil ROS production: neutrophil ROS production after 1 hour coculture with mature *C. albicans* biofilms was significantly lower than neutrophil ROS production with early biofilms (Xie *et al.*, 2012).

β-glucan binding to Dectin-1 was revealed to upregulate PD-L1 expression in human and murine neutrophils *in vivo*. Increased PD-L1 led to a subsequent increase in CXCL1 and CXCL2 expression – chemoattractant cytokines. Further analysis using a mouse *in vivo* model showed bloodstream *C. albicans* infection induced CXCL1 and CXCL2 expression in neutrophils in the bone marrow, causing retention of neutrophils in the bone marrow and inhibiting neutrophil migration into the circulation (Y. Yu *et al.*, 2022). Another mechanism of immune evasion is *C. albicans* secretion of aspartyl proteases, of which one is Sap6. Neutrophils endocytose Sap6, resulting in reduced ROS production and NETosis *in vitro*, via Sap6-mediated proteolytic degradation of host NADPH oxidase (Zawrotniak *et al.*, 2025). Preincubation of neutrophils with Sap6 led to impaired neutrophil killing of *C. albicans*. However, the contribution of Sap6 to host ROS suppression, compared to other *C. albicans* proteins, was not evaluated with a *C. albicans sap6Δ* mutant, nor was the effect quantified *in vivo* (Zawrotniak *et al.*, 2025). A forward screen of 131 *C. albicans* mutants in zebrafish identified 7 mutations with reproducible virulence defects that could be partially complemented by reinserting the mutated gene: 5 strains affecting morphogenesis and 2 strains affecting infectivity (Blair *et al.*, 2025). For example, *C. albicans nmd5Δ* had increased levels of phagocytosis and increased survival in zebrafish larval infection, suggesting Nmd5 has a biologically relevant role in evasion of phagocytosis *in vivo* (Blair *et al.*, 2025).

Adaptation to host oestrogen has elicited an immune evasion strategy that inhibits part of the complement pathway. Ebp1 (oestrogen binding protein 1) negatively regulates *bcr1* expression, encoding a protein that regulates cell wall composition and promotes biofilm formation (Nobile and Mitchell, 2005). Host oestrogen inhibits *C. albicans* Ebp1, leading to an increase in Bcr1 and a subsequent increase in Gpd2 levels. Gpd2, glycerol-3-phosphate dehydrogenase 2, is a metabolism regulation protein identified to bind to host complement proteins (Ansell *et al.*, 1997; Luo, Hoffmann, *et al.*, 2013). Gpd2 translocates to the *C. albicans* cell surface, where it binds with Factor H complement protein, inhibiting the alternative complement system (Kumwenda *et al.*, 2022). In a zebrafish *C. albicans* infection model, exogenous oestrogen treatment resulted in reduced zebrafish survival, which was recapitulated in zebrafish infection with *C. albicans* overexpressing *GPD2* (Kumwenda *et al.*, 2022). Women with RVVC were revealed to have fewer circulating neutrophils than healthy women and neutrophils from women with RVVC had reduced ability to kill *C. albicans in vitro* compared to neutrophils from healthy women (Consuegra-Asprilla *et al.*, 2024). Oestrogen levels in these women were not measured, so it is unclear whether the observed differences are caused by elevated oestrogen levels or a *C. albicans* factor.

C. albicans interactions with other organisms can facilitate immune evasion. *Staphylococcus aureus* was observed to adhere to the surface of *C. albicans* hyphae *in vitro*, resulting in reduced efficacy of NET-mediated killing on *C. albicans* (Jing *et al.*, 2024). Adhesion to *C. albicans* hyphae protected *S. aureus* from neutrophil phagocytosis, revealing a synergistic relationship between these organisms to evade neutrophil activity. Furthermore, *S. aureus* upregulated hyphae-specific and proliferation genes in *C. albicans*, suggesting co-infection with *S. aureus* may promote infection by *C. albicans* (Jing *et al.*, 2024). Co-infection of *Pseudomonas aeruginosa* and *C. albicans* in a zebrafish model led to increased *C. albicans* burden and epithelial invasion compared to *C. albicans* infection alone, suggesting *P. aeruginosa* promotes *C. albicans* virulence (Bergeron, Seman, *et al.*, 2017). While a *C. albicans*/*P. aeruginosa* dual species biofilm enhanced meropenem tolerance of *P. aeruginosa, C. albicans* was made more susceptible to amphotericin B, via *P. aeruginosa*-mediated downregulation of *C. albicans* detoxification enzymes (Alam *et al.*, 2020, 2023). Hence, co-infections may not always act synergistically to facilitate *C. albicans* evasion of immune responses. Overall, the role of co-infections is an emerging area of research, with many as-yet unanswered questions.

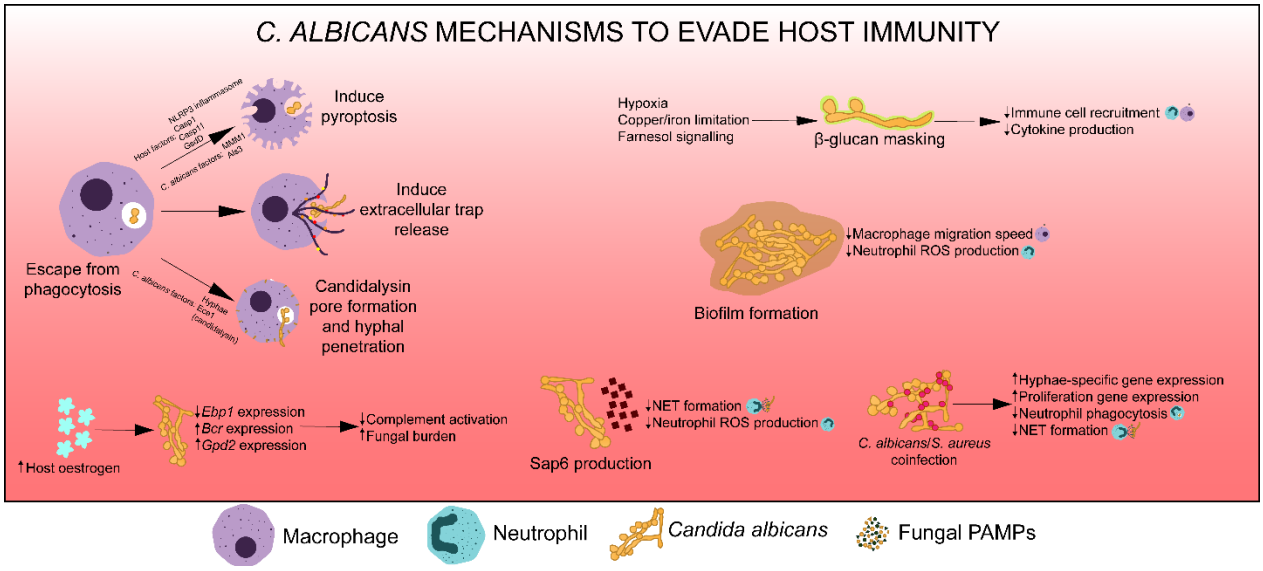

Figure 1.6: *C. albicans* mechanisms to evade host immunity

Examples of *C. albicans* methods to evade host immune responses. *C. albicans* has 3 separate strategies to escape from phagocytosis: induction of pyroptosis, induction of extracellular trap release and candidalysin pore formation (Olivier *et al.*, 2022). β-glucan masking can prevent immune cell recruitment and production of pro-inflammatory cytokines (Bain *et al.*, 2014; Pradhan *et al.*, 2019). Host oestrogen increases Gpd2 on the *C. albicans* cell wall, which binds to host Factor H to inhibit the alternative complement system (Kumwenda *et al.*, 2022).

1.6 Reactive nitrogen species

Reactive nitrogen species (RNS) is a collective term for a diverse group of antimicrobial compounds that are all derived from nitric oxide (NO; Martínez and Andriantsitohaina, 2009). NO is produced by nitric oxide synthase (NOS), which converts L-arginine into L-citrulline and NO (Babić and Peyrot, 2019; Andrés *et al.*, 2022). NO is an uncharged, free radical, so has a single unpaired electron, making it highly reactive (Bloodsworth, O'Donnell and Freeman, 2000). NO reacts with O_2 or superoxide to generate RNS, such as peroxynitrite ($ONOO^-$), nitrite (NO_2^-), nitrate (NO_3^-), dinitrogen trioxide (N_2O_3) and nitrosoperoxycarbonate (CNO_5^-;(Fang, 2004; Martínez and Andriantsitohaina, 2009; Adams, Franco and Estevez, 2015). NO radicals alone can also bind to pathogen targets to have antimicrobial effects, so NO can be considered an RNS in the context of infection (Pacelli *et al.*, 1995; Schapiro, Libby and Fang, 2003).

There are 3 NOS isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). eNOS and nNOS are constitutively expressed, whereas iNOS expression is induced by infection or inflammation (Förstermann and Sessa, 2012). eNOS and nNOS are expressed in endothelial cells and

specific neurons respectively, with the NO synthesised by eNOS used to regulate vasodilation and NO synthesised by nNOS used in synaptic plasticity or as an atypical neurotransmitter (Drew and Leeuwenburgh, 2002; Förstermann and Sessa, 2012). NO production by eNOS and nNOS is tightly regulated by intracellular Ca^{2+} signalling, via a calmodulin-dependent mechanism (Salerno *et al.*, 1997).

iNOS is most abundantly expressed in innate immune cells, but low-level expression can be induced in virtually any cells by pro-inflammatory cytokines, such as IL-1β, IFNs and TNFα (Xie, Kashiwabara and Nathan, 1994; Taylor and Geller, 2000; Fang, 2004). In mouse models, macrophages are the greatest producers of RNS, whereas, in humans, neutrophils are the greatest producers of RNS (Muijsers *et al.*, 2001; Munder *et al.*, 2005).

RNS are highly antimicrobial, so have an important role in innate immunity, though RNS can also have indirect effects on cell signalling: peroxynitrite-mediated tyrosine nitration prevents tyrosine phosphorylation and targets proteins towards degradation pathways (Gow *et al.*, 1996; Patel *et al.*, 1999; Fang, 2004; Andrés *et al.*, 2022). Importantly for my work, RNS are fungicidal. Co-incubation of *C. albicans* with murine peritoneal macrophages revealed RNS are critical for candidacidal activity, with 83.8% reduction of candidacidal activity when RNS production was inhibited (Rementería, García-Tobalina and Sevilla, 1995). Specifically, Vazquez-Torres *et al.* suggested peroxynitrite is the primary RNS responsible for candidacidal activity (Vazquez-Torres, Jones-Carson and Balish, 1996). L-NMMA (a NOS inhibitor) treatment reduced NO levels produced by rat neutrophils by 50% and was correlated with a 50% decrease in candidacidal activity (Fierro *et al.*, 1996). Administration of exogenous RNS has repeatedly been shown to kill *C. albicans,* and act synergistically with antifungals to kill *C. albicans,* confirming the candidacidal activity of RNS (McElhaney-Feser, Raulli and Cihlar, 1998; Macherla *et al.*, 2012; Heilman *et al.*, 2013; Stasko *et al.*, 2018; Pandey *et al.*, 2025). Inhibition of phagocytosis, using cytochalasin B, substantially reduced candidacidal activity by rat neutrophils, without affecting NO levels, suggesting RNS-dependent killing of *C. albicans* is predominantly intracellular (Fierro *et al.*, 1996). Nitrite is a component of sweat, which can become acidified by the low pH environment on the skin. Acidified nitrite was effectively able to kill *C. albicans in vitro*, though this was tested at nitrite concentrations above those that would be present in sweat (Weller *et al.*, 2001). In a murine model of oral candidiasis, reduced nitrite levels in the saliva correlated with greater oral *C. albicans* burden, suggesting saliva nitrite was candidacidal (Elahi *et al.*, 2001). An observational study of human oral candidiasis patients noticed increased saliva nitrite levels in oral candidiasis patients compared to uninfected individuals, suggesting salivary RNS can be upregulated in response to fungal infection (Hillestad *et al.*, 2005). RNS appear to synergise with ROS to kill *C. albicans*, with a synergistic effect on inhibition of *C. albicans* growth observed *in vitro* with

combination of hydrogen peroxide and an NO donor (DPTA NONOate; Kaloriti *et al.*, 2012). Combination of RNS with osmotic stress had a more modest increase in *C. albicans* growth inhibition and had no effect on inhibition of *C. glabrata* (Kaloriti *et al.*, 2012). In response to RNS, *C. albicans* has developed multiple strategies to tolerate nitrosative stress (fully explored in 3.1.1 *C. albicans* defences against RNS; Brown, 2011). Modulation of host RNS, to overcome *C. albicans* evasion strategies, could be explored as a potential host-directed therapy for treatment of *C. albicans* infections.

1.7 Host-directed therapies

There are many issues concerning current antifungal treatment: toxicity of antifungals, low effectiveness, slow development of new antifungals and the rise of antifungal resistance (Ademe, 2020). An alternative approach to treating infectious disease is targeting the immune system, which should be more resilient against emerging drug resistance. Host directed therapies (HDTs) stimulate host cellular pathways and activate immune responses to aid clearance of pathogens (Zumla et al., 2016). HDTs have been proposed as an adjunctive therapy alongside current antifungals (Armstrong-James et al., 2017).

G-CSF is an endogenous signalling molecule that induces formation of granulocyte precursor cells, which will later differentiate into mature neutrophils. GM-CSF has a similar role in immune cell development, but with a wider spectrum of activity (Mehta, Malandra and Corey, 2015; Damiani et al., 2020). G-CSF/GM-CSF treatment aids clearance of *C. albicans* and *A. fumigatus* in *in vitro*, rabbit and mouse models (Kullberg et al., 1999; Gonzalez et al., 2001; Dongari-Bagtzoglou and Kashleva, 2003). In 2 separate cases of relapsing *C. albicans* meningoencephalitis in CARD9-deficient patients, treatment with either G-CSF or GM-CSF resulted in complete clinical remission (Gavino et al., 2014; Celmeli et al., 2016). In another case, a paediatric patient with a history of CMC and CARD9 deficiency was successfully treated for invasive *C. albicans* infection by a combination of G-CSF and antifungals (Du et al., 2020). Treatment with G-CSF restored neutrophil swarming and fungal killing in cirrhosis-patient neutrophils to similar levels to healthy neutrophils, suggesting G-CSF treatment can enhance neutrophil function in cirrhosis patients, who are at increased risk of severe fungal infection (Knooihuizen *et al.*, 2021). Therefore, G-CSF/GM-CSF shows potential for treating *C. albicans* infections in immunodeficient patients.

IFNγ is another endogenous cytokine that has been used as an adjunctive therapy in invasive fungal infections, though rarely for *C. albicans* infections and more widely in *Aspergillus* spp. infections (Kelleher *et al.*, 2006; Delsing *et al.*, 2014; Danion *et al.*, 2020). 6 invasive candidiasis patients were treated with adjunctive IFNγ, alongside standard antifungal therapy. Treatment was successful in all

candidiasis patients, with patients exhibiting increased IL-17 and IL-22 levels compared to patients not treated with IFNγ (Delsing *et al.*, 2014). Direct antifungal activity of IFNγ and ability of IFNγ to enhance the efficacy of amphotericin B was tested *in vitro*, revealing IFNγ has no direct antifungal effect on *C. albicans* and did not enhance amphotericin B efficacy against *C. albicans* but IFNγ did have a direct antifungal effect on *A. fumigatus* and increased the efficacy of amphotericin B against *A. fumigatus* (El-Khoury *et al.*, 2017).

Other HDTs remain in earlier stages of development. Targeting spleen tyrosine kinase (syk) was suggested as a potential HDT after syk was shown to be critical for protection against *C. albicans* infection in mice models, through regulation of neutrophil responses (Negoro et al., 2020; Zajta et al., 2021). However, while syk inhibitors have been developed and approved for clinical use (Markham, 2018), to the best of my knowledge, there are currently no pharmacological syk stimulators for experimental or clinical use, introducing significant barriers to further investigation of syk as a potential HDT.

Investigations into Trim72 (tripartite motif-containing protein 72) as a HDT are at a similar early stage. Treatment of *C. albicans* infected mice with exogenous Trim72 led to increased survival. Correspondingly, *Trim72*$^{-/-}$ mice had increased susceptibility to *C. albicans* infection (Tan *et al.*, 2024). Trim72 was shown to enhance macrophage migration to *C. albicans* by increasing CCL2 production via NF-κB signalling. The protective effect of exogenous Trim72 was abolished both by the depletion of macrophages and by inhibition of NF-κB signalling, confirming the mechanism of protection (Tan *et al.*, 2024).

Rhesus theta defensin-1 (RTD-1) is an antimicrobial peptide, with potent antifungal properties in both *in vitro* and *in vivo* mouse models. RTD-1 also promotes neutrophil recruitment and reduces TNF, IL-1β and IL-17 production in *C. albicans* infected mice (Basso et al., 2018, 2019). Furthermore, RTD-1 suppresses pro-inflammatory cytokines in in vitro and in vivo mouse models, reducing host damage, improving long term outcomes and improving pathogen clearance (Tongaonkar et al., 2015; Beringer et al., 2016; Bensman et al., 2017; Jayne et al., 2018). Hence, RTD-1 represents a promising new class of therapy, capable of modulating host responses to improve long term outcomes to infection, while also having a direct antifungal effect.

HIF-1α (Hypoxia Inducible Factor) stabilisation has also been proposed as a HDT for *C. albicans* infections, though is at an early stage of investigation (explored fully in 6.1.2 Hypoxia inducible factor (HIF) as a host directed therapy). Hif-1α stabilisation is protective against *Mycobacterium marinum* infection in zebrafish larvae via IL-1β, which stimulates antimicrobial nitric oxide production (Elks *et al.*, 2013; Ogryzko *et al.*, 2019). In addition, Hif-1α deficient mice have been shown to be more

susceptible to *C. albicans* infection (Li *et al.*, 2018a). Hif-1α stabilisation has potential as a HDT for treatment of fungal infections but requires appropriate *in vitro* models that allow both genotypic and phenotypic characterisation of the effects of Hif-1α stabilisation on fungal infection and immune cell behaviour.

1.8 Hypoxia inducible factor

1.8.1 Overview of Hypoxia inducible factor signalling

Hypoxia inducible factor (HIF) proteins are most associated with regulation of the cellular responses to hypoxia, though they also have roles in inflammatory conditions and cancer (Yuan *et al.*, 2024). HIF proteins are composed of an α and β subunit. α subunits (HIF-1α, HIF-2α, HIF-3α) are evolutionarily conserved transcription factors, that are regulated post-transcriptionally by oxygen-dependent prolyl hydroxylases domain enzymes (PHDs) and factor inhibiting HIF (FIH; Taabazuing, Hangasky and Knapp, 2014). β subunits (HIF-1β, HIF-2β, HIF-3β) are constitutively expressed and contain DNA binding domains, allowing binding to hypoxia responsive elements (HREs) in target genes (Krzywinska and Stockmann, 2018). In normal oxygen conditions, HIF-α proteins are transcribed, then hydroxylated by PHD and FIH, triggering binding by von Hippel-Lindau protein (VHL), ubiquitination and proteasomal degradation (Hammond, Lewis and Elks, 2020). In hypoxia, PHD and FIH are unable to bind and hydroxylate HIF-α. This allows HIF-α to form heterodimers with HIF-β, which enter the nucleus, bind to HREs and activate transcription of target genes (Figure 1.7). Downstream effects of HIF signalling include regulation of cellular metabolism, proliferation, migration, differentiation promotion of cell survival and angiogenesis (Krzywinska and Stockmann, 2018). The different HIF-α isoforms coordinate these varying downstream effects and can have synergistic or opposing roles (Yuan *et al.*, 2024). Both HIF-1α and HIF-2α stimulate increased expression of *VEGF* and *EPO*, genes involved in angiogenesis and red blood cell production, respectively (Hu *et al.*, 2003). However, HIF-1α promotes iNOS expression, whereas HIF-2α induces arginase expression – enzymes that have opposing functions in inflammation (Takeda *et al.*, 2010). HIF-3α is considered an inhibitory element, but its role is far less characterised than HIF-1α or HIF-2α and is much less widely/more lowly expressed (Yuan *et al.*, 2024).

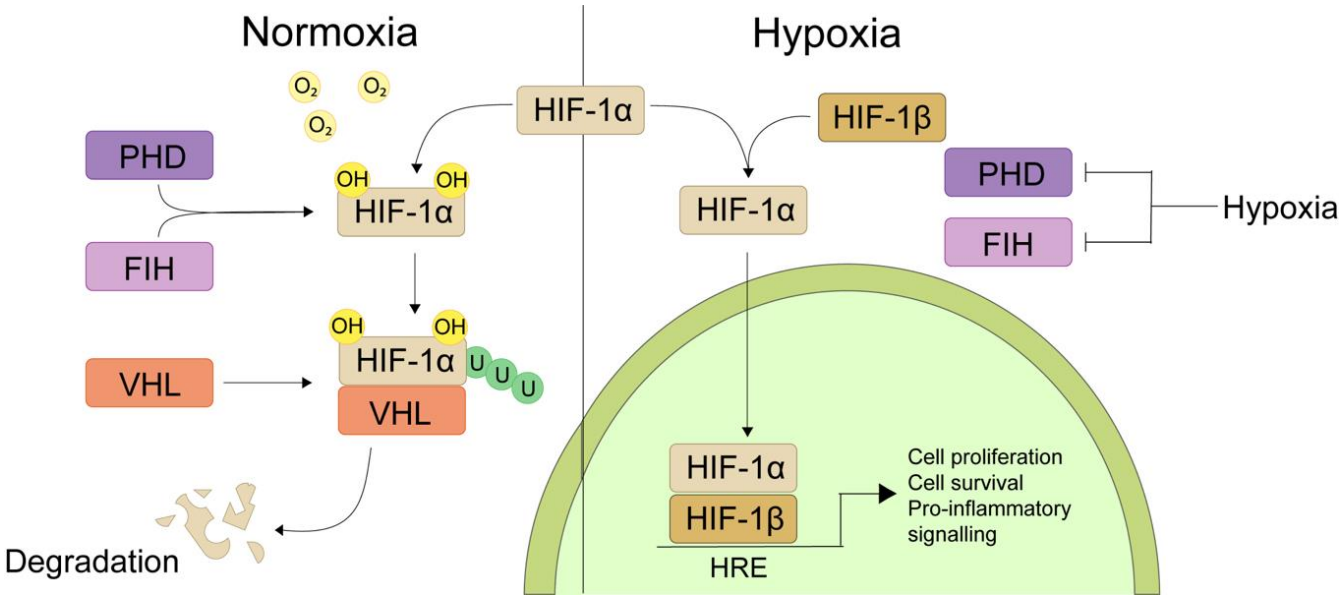

Figure 1.7: Hypoxia inducible factor signalling

In normoxia, HIF-1α is hydroxylated by PHD and FIH, allowing VHL binding and ubiquitination. This leads to proteasomal degradation of HIF-1α. In hypoxia, lack of oxygen inhibits PHD and FIH. This allows HIF-1α to migrate to the nucleus and form a heterodimer with HIF-1β, triggering transcription of gene targets. Diagram based on (Hammond, Lewis & Elks, 2020).

1.8.2 Role of Hypoxia inducible factor in immunity

Infection triggers localised hypoxia, alongside low glucose levels, high lactate concentrations and increased free oxygen radicals (Schor, Vaday and Lider, 2000; Saadi, Wrenshall and Platt, 2002; Krzywinska and Stockmann, 2018). The hypoxia observed in infection is a physiological response to pathogen proliferation and increased oxygen consumption by migrating immune cells, and is maintained without causing cell death (Taylor and Colgan, 2017). This localised hypoxia triggers activation of HIF signalling. HIF-1α can also be stabilised by TLR signalling, via MYDD88-dependent NF-κB activity, inducing a subset of pro-inflammatory genes to a significantly greater degree than hypoxia-induced HIF-1α signalling (Peyssonnaux *et al.*, 2005; Jantsch *et al.*, 2011).

HIF proteins are critical regulators of immune responses and inflammation. Immunohistochemistry of human skin biopsies revealed HIF-1α is activated in response to infection with a wide range of pathogens, including *Staphylococcus aureus,* varicella zoster virus, *Tinea rubrum, C. albicans* and *Leishmania donovani*, suggesting HIF-1α activation is a general response to infection and not pathogen-specific (Werth *et al.*, 2010).

Macrophages from HIF-1α deficient mice exhibited decreased bactericidal activity, while activation of HIF-1α promoted macrophage control of bacterial infection (Peyssonnaux *et al.*, 2005). Peyssonnaux *et al.* also demonstrated bacterial exposure can be a greater stimulus for HIF-1α stabilisation than hypoxia. Furthermore, HIF-1α stabilisation increases neutrophil survival, via NF-κB activity, and promotes NET formation (Walmsley *et al.*, 2005; McInturff *et al.*, 2012). Conversely, NET formation is significantly reduced in hypoxia (Branitzki-Heinemann *et al.*, 2016). Hence, HIF-1α signalling may trigger downstream effectors that upregulate NET formation but the physiological relevance of this must be questioned, owing to the hypoxic environmental conditions during infection.

In a zebrafish model, Hif-1α stabilisation has been shown to be protective against systemic infection with *Mycobacteria marinum* (Elks *et al.*, 2013). Hif-1α signalling stimulates IL-1β production, which promotes nitric oxide production by neutrophils, leading to a reduced bacterial burden (Ogryzko *et al.*, 2019). The induction of nitric oxide production by HIF-1α stabilisation has also been observed in mouse models of tuberculosis infection (Braverman and Stanley, 2017). However, increased HIF-1α levels have also been associated with excess inflammation, host damage and disease progression in the later stages of tuberculosis infection (Domingo-Gonzalez *et al.*, 2017). Similarly, in SARS-CoV-2 infection, viral proteins upregulate HIF-1α, resulting in an excessive pro-inflammatory response and increased susceptibility to SARS-CoV-2 infection (Tian *et al.*, 2021).

HIF-1α has been implicated in fungal immunity. In a murine model of *A. fumigatus* infection, HIF-1α knockout mice had increased lung *A. fumigatus* burden and greater hyphal growth compared to wild type mice (da Silva-Ferreira *et al.*, 2022). HIF-1α knockout mice had impaired neutrophil recruitment to the site of infection and increased neutrophil death, suggesting a role for HIF-1α in regulating the neutrophil response to fungal infections (da Silva-Ferreira *et al.*, 2022).

HIF-1α also plays a role in trained immunity to fungal stimuli. Detection of β-glucan (a fungal cell wall component) by Dectin-1 was shown to trigger phosphorylation of Akt1 in human monocytes. Akt1 phosphorylation triggered increased phosphorylation of mTOR, which led to increased HIF-1α signalling. HIF-1α promoted a shift in monocyte metabolism towards aerobic glycolysis, resulting in a 'trained' phenotype, in which monocytes had quicker production of pro-inflammatory cytokines and enhanced phagocytosis (Cheng *et al.*, 2014). The role of HIF-1α in trained immunity was validated using a mouse *in vivo* model, primed with β-glucan then infected with a lethal dose of *S. aureus.* HIF-1α knockout mice lost the protective effect of β-glucan priming, demonstrating this model of trained immunity is HIF-1α-dependent (Cheng *et al.*, 2014). β-glucan is not present in the *S. aureus* cell membrane, suggesting Hif-1α-dependent trained immunity generated by β-glucan priming may

confer non-specific protection. LPS-induced immune memory in microglia was also shown to be regulated by HIF-1α (Dong *et al.*, 2024). Whole *C. albicans* is also capable of generating trained immunity, with pre-treatment with a sublethal dose of *C. albicans* increasing survival in mice later infected with a lethal dose of *C. albicans*, presumably operating by the same HIF-1α-dependent mechanism, though this was not confirmed (Cheng *et al.*, 2014).

1.9 Zebrafish (*Danio rerio*)

1.9.1 Overview of zebrafish *in vivo* model

Zebrafish (*Danio rerio*) larvae are highly effective *in vivo* models of human disease. Several zebrafish characteristics give them inherent advantages as an *in vivo* model: high fecundity, external fertilisation and development, small size and rapid development (Goldsmith and Jobin, 2012; Mastrogiovanni *et al.*, 2024). Compared to other *in vivo* models, such as mice, zebrafish larvae are relatively high throughput, easier to maintain, less labour intensive and less expensive (Goldsmith and Jobin, 2012). Pharmacological treatment of zebrafish larvae is a simple process, with most small molecule drugs being able to be administered via immersion in the zebrafish media (Goldsmith and Jobin, 2012). Under the Animals (Scientific Procedures) Act 1986, zebrafish only become protected at 5.2 days post fertilisation (dpf), the point they become capable of independent feeding when incubated at 28 °C (UK Government, 1986). Therefore, use of zebrafish larvae under 5.2 dpf in research constitutes a partial replacement of protected animals (NC3Rs, 2023). Furthermore, zebrafish have a fully sequenced genome, which has a high homology with the human genome, including non-coding regions (Shin *et al.*, 2005; Goldsmith and Jobin, 2012). Approximately 70% human genes have at least one zebrafish orthologue, including 84% of human disease-associated genes (Barbazuk *et al.*, 2000; Howe *et al.*, 2013).

Zebrafish are highly genetically tractable, with advancements in gene editing technology offering an increasing number of editing techniques (Y. Li *et al.*, 2021). Historically, morpholinos have been used widely in zebrafish research to effectively knockdown gene expression (Bedell, Westcot and Ekker, 2011). Morpholinos are synthetic oligonucleotides that bind to complementary strands of mRNA, preventing their translation or splicing (Stainier *et al.*, 2017). Morpholinos were widely adopted by zebrafish researchers owing to their high efficacy, low production costs and ease of use (Summerton and Weller, 1997; Stainier *et al.*, 2017). Increased sophistication of morpholino knockdown has been observed, with photo-activated and photo-cleavable morpholinos allowing activation or deactivation of morpholino knockdown temporally and spatially by UV exposure (Tallafuss *et al.*, 2012). However, following over a decade of use, concerns have arisen about the use of morpholinos. Prolonged storage in aqueous solution can reduce morpholino activity and increase toxicity, though this is

morpholino-specific, with some retaining efficacy for over 7 years (Bill *et al.*, 2009; Bedell, Westcot and Ekker, 2011). Off-target effects are a potential weakness of morpholino use. Non-specific neural defects have been observed in 15-20% morpholino experiments in zebrafish, which is believed to be caused by morpholino activation of p53, triggering p53-induced apoptosis (Robu *et al.*, 2007; Eisen and Smith, 2008). Morpholino p53 induction was also observed to induce expression of hindbrain boundary markers, suggesting off-target effects of morpholinos include non-specific upregulation of genes, not just non-specific cell death (Gerety and Wilkinson, 2011). These off-target effects may confound results. Finally, morpholino effects can wear off as the morpholino degrades, which can happen from 50 hours post injection (Bedell, Westcot and Ekker, 2011).

CRISPR-Cas9 is now the preferred method of mutagenesis in zebrafish experiments (Li *et al.*, 2016). Guided to a specific gene by a complementary strand of guide RNA (gRNA), Cas9 creates double stranded breaks in DNA, resulting in deletion of a region of the gene and functional knockdown. CRISPR-Cas9 can also be used to knock in genes, such as a gene encoding a fluorescent protein, create stable transgenic zebrafish lines and modulate transcription without deleting a gene via CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi; Li *et al.*, 2016; Liu *et al.*, 2019). CRISPR-Cas9 mutagenesis can result in a mosaic phenotype, in which, within a single zebrafish, some cells inherit the CRISPR-induced mutation and some cells have a wild type genotype. Efficiency of knockdown can be increased by incubating single cell zebrafish embryos at 12 °C, instead of 28 °C, which prolongs the single cell stage and provides more time for mutagenesis to occur (Terzioglu *et al.*, 2020). Off-target effects are still an issue in CRISPR-Cas9 mutagenesis, as Cas9 can tolerate a certain degree of mismatch between gRNA and genomic DNA (Hsu *et al.*, 2013; Guo *et al.*, 2023). *In silico* tools can be used to predict potential off-target effects, allowing design of gRNAs with minimal predicted off-target effects. However, these tools have limited sensitivity and cannot account for low probability stochastic off-target effects (Bao *et al.*, 2021; Guo *et al.*, 2023). Modification of Cas9 protein has led to the creation of hyper accurate Cas9 (hypaCas9), which has higher on-target activity and reduced off-target effects compared to standard Cas9 (Chen *et al.*, 2017).

1.9.2 Zebrafish infection models

The complexity of immune cell interactions within a host environment cannot be recapitulated *in vitro*, meaning *in vivo* models remain a critical aspect of infectious disease research (Shi *et al.*, 2019). Zebrafish larvae are highly effective models for investigating innate immune responses to pathogens. Adaptive immunity does not mature until 4 to 6 weeks post fertilisation, whereas innate immunity is present from early embryonic stages, allowing examination of innate immune responses without interference from the adaptive immune system (Lam *et al.*, 2004). *Ex vivo* embryonic development and transparent embryos means fluorescent live imaging is possible, allowing

observation of innate immune cell interactions with pathogens (Lam *et al.*, 2004). Generation of a neutrophil reporter line (*Tg(mpx:GFP)i114*) labelled with green fluorescent protein (GFP) has allowed tracking of neutrophil behaviours in real time (Renshaw *et al.*, 2006). Innate immune signalling pathways (including HIF signalling) are highly conserved between mammals and zebrafish, making the model highly relevant to human immunity (Stein *et al.*, 2007). Zebrafish have 6 known orthologs of HIF-α – based on sequence homology, the most closely related zebrafish ortholog to human HIF-1α is Hif-1αb (Hampton-Smith and Peet, 2009; Rytkönen *et al.*, 2013). Zebrafish Hif signalling can be manipulated by well validated pharmacological and genetic interventions, making zebrafish an effective model to study Hif signalling (Elks *et al.*, 2015). Furthermore, RNS roles are widely conserved in zebrafish, though there are some differences. Zebrafish have orthologs of nNOS (nos1) and iNOS (nos2) but do not have a known eNOS ortholog (Holmqvist *et al.*, 2000; Y. Huang *et al.*, 2024). As with humans, RNS is predominantly produced by neutrophils in zebrafish larvae, in both mock PVP infection and at elevated levels in *Mycobacteria marinum* infection; whereas in mice, macrophages are the immune cell that most abundantly produces RNS (Muijsers *et al.*, 2001; Munder *et al.*, 2005; Elks *et al.*, 2013, 2014). Hence, zebrafish are an effective model for investigating host neutrophil RNS levels, which may more closely represent human neutrophil RNS than mice immune cells, in this specific regard.

Zebrafish can be used to model *C. albicans* infection. Chao *et al.* revealed intraperitoneal *C. albicans* infection kills zebrafish in a dose dependent manner (Chao *et al.*, 2010). Importantly, *C. albicans* underwent a yeast-to-hyphae transition, which was associated with increased virulence, demonstrating a comparable pathogenesis to human infection (Chao *et al.*, 2010). Multiple infection sites can be used in zebrafish to model *C. albicans* infection, including the hindbrain, swim bladder and yolk (Bergeron, Barker, *et al.*, 2017; Gratacap *et al.*, 2017; Seman *et al.*, 2018). These different infection sites allow examination of the effect of systemic *C. albicans* infections on immune responses, dissemination of *C. albicans* from a specific site, immune cell recruitment and tissue-specific immune responses and pathology (Rosowski *et al.*, 2018). The optical transparency of zebrafish larvae is a particular advantage in this model, as *C. albicans* can be directly observed at high magnifications without the need for fluorophores (Chao *et al.*, 2010). Fluorescent *C. albicans* strains do facilitate easier examination of host-*Candida* interactions and at lower magnification (Seman *et al.*, 2018). The ease of drug administration makes a *C. albicans* zebrafish infection model ideal for screening anticandidal drugs, with survival and fungal burden being easily measured outputs to evaluate the success of treatments (Kulatunga *et al.*, 2019).

1.10 Hypothesis & aims

C. albicans can suppress ROS and RNS production by macrophages *in vitro* (Wellington, Dolan and Krysan, 2009) with an, as yet, unidentified *C. albicans* secreted compound responsible for RNS inhibition in murine macrophages (Collette, Zhou and Lorenz, 2014). However, there is currently a lack of *in vivo* observations on the effects of *C. albicans* on RNS and little is known about the molecular and cellular mechanisms underlying RNS suppression and whether it is possible to rescue this with potential therapeutic application.

In this PhD project, I examined the hypothesis: ***Candida albicans* suppresses neutrophil RNS levels *in vivo* and this can be targeted therapeutically to improve host infection outcomes.**

In order to test this hypothesis, several aims were addressed:

- Aim 1: To observe neutrophil RNS suppression by *C. albicans* in zebrafish *in vivo*
    a. Develop tools to observe *C. albicans* infection *in vivo*
    b. Develop tools to observe *C. albicans* suppression of neutrophil RNS *in vivo*
    c. Validate suppression of neutrophil RNS *in vivo* with multiple strains of *C. albicans*
- Aim 2: To examine the mechanism underlying *C. albicans* mediated suppression of host RNS production
    a. Characterise host factors involved in *C. albicans* mediated suppression of host RNS production
        i. Examine the role of host pattern recognition receptors in neutrophil RNS suppression by inhibiting adaptor proteins
        ii. Characterise cytokine profile in response to *C. albicans* infection *in vivo*
    b. Determine pathogen factors involved in *C. albicans* mediated suppression of host RNS production
        i. Inspect the incidence of hyphae formation by *C. albicans in vivo*
        ii. Explore the role of hyphae in RNS suppression using yeast-locked *C. albicans* mutants
        iii. Examine the role for fungal arginase in neutrophil RNS suppression
- Aim 3: Assess the clinical relevance of neutrophil RNS suppression by *C. albicans*
    a. Observe neutrophil RNS suppression by *C. albicans* clinical isolates
    b. Explore factors contributing to varying RNS suppression phenotypes by *C. albicans* clinical isolates
    c. Observe neutrophil RNS suppression by non-albicans *Candida* spp. clinical isolates

    d. Explore factors contributing to varying RNS suppression phenotypes by non-albicans *Candida* spp. clinical isolates

- Aim 4: To improve the innate immune response to *C. albicans* infection by therapeutically targeting *C. albicans*-mediated suppression of host RNS levels
    a. Validate techniques to modulate host *hif-1αb*
    b. Demonstrate ability of Hif-1α stabilisation to protect against *C. albicans* infection *in vivo*
    c. Characterise mechanisms by which Hif-1α stabilisation has a protective effect in *C. albicans* infection
    d. Explore the use of Hif-1α stabilisation in combination with traditional antifungals to have synergistic effects

2. Materials & methods

2.1 Ethics

All experiments were performed in accordance with Animals (Scientific Procedures) Act 1986, under relevant project license (PPL number: PP7684817) and personal license (PIL number: I03638860). Ethical approval was granted by the University of Sheffield Local Ethical Review Panel.

2.2 Zebrafish Husbandry

2.2.1 Adult zebrafish maintenance

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.

Table 2.1: List of transgenic zebrafish lines used in this thesis

Zebrafish line	Allele number	Labelling	Reference
Tg(lyz:nfsB.mCherry)	sh260	Neutrophils	(Buchan *et al.*, 2019)
Tg(mpx:GFP)	i114	Neutrophils	(Renshaw *et al.*, 2006)
TgBAC(tnfα:GFP)	pd1028	*tnfα* expressing cells	(Marjoram *et al.*, 2015)
TgBAC(il-1β:GFP)	sh445	*il-1β* expressing cells	(Ogryzko *et al.*, 2019)
TgBAC(arg2:GFP)	sh571	*arg2* expressing cells	(Hammond *et al.*, 2023)
Tg(phd3:GFP)	i144	*phd3* gene expression	(Santhakumar *et al.*, 2012)
Tg(mpeg:nlsClover)	sh436	Macrophages (nuclei marker)	(Bernut *et al.*, 2019)

2.2.2 Embryo maintenance

Embryos were maintained in Petri dishes (maximum 60 embryos per dish) in 1X E3 solution (E3 60X stock: 5.0 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$; diluted to 1X in distilled water) with 10^{-5}% Methylene Blue (Sigma-Aldrich). Methylene Blue was added to prevent fungal growth.

At approximately 7 hpf (hours post fertilisation), Petri dishes were checked and any unfertilised or dead embryos were removed. At 26-28 hpf, zebrafish were dechorionated using jeweller's tweezers (Sigma-Aldrich) and chorions and debris were removed.

2.2.3 Power calculations

Power calculations were carried out by Dr Philip Elks for a prior grant application, based on experiments conducted on infection of zebrafish with *Mycobacterium marinum*.

Corrected fluorescence intensity of either anti-nitrotyrosine staining or cytokine fluorescence is powered to detect a difference of 20% in size (with 80% confidence) using a sample size of 63 (e.g. 6 cells in 11 larvae, spread across 3 experimental repeats). Survival experiments are powered to detect a difference of 20% in effect size, (using Chi squared sample size estimation with 80% confidence) using a sample size of 99 larvae per group (e.g. 33 larvae per group, with 3 experimental repeats).

2.3 Genetic and pharmacological treatment of zebrafish

2.3.1 Preparation of microinjection needles

Microinjection needles were made by pulling glass capillaries (WPI) using a P1000 micropipette puller (Sutter Instruments).

2.3.2 RNA Injections

Embryos were injected with 1 nl 100 ng/µl dominant active *hif-1αb* variant RNA (DA1) or 1 nl 100 ng/µl dominant negative *hif-1αb* variant RNA at the one-cell stage, as previously described (Table 2.2; Elks *et al.*, 2011). H*if-1αb* is the closest related zebrafish homolog to human Hif-1α by sequence homology (Hampton-Smith and Peet, 2009; Rytkönen *et al.*, 2013). Working concentrations of DA1 and DN1 were prepared dilution of a stock concentration DA1/DN1 (≈4000 ng/µl) in 10% Phenol red in DEPC-H_2O (PR; Sigma-Aldrich). DA1/DN1 stocks were stored at -80 °C. 1 nl PR was used as a negative control.

Table 2.2: Hif-1α constructs

	Full name	Hif variant	PCR amplification primers		Concentration used (ng/µl)	Reference
			Forward	Reverse		
DA1	Dominant active Hif-1α	*Hif-1αb*	5'-AGCAGGCGACACTGTGTTGGCAGCTGCAGCAGGCGA-3'	5'-TCGCCTGCTGCAGCTGCCAACACAGTG-3'	100	(Elks *et al.*, 2011)
DN1	Dominant negative Hif-1α	*Hif-1αb*	5'-ACCATGGATACTGGAGTTGTCAC-3'	5'-ATCGATTCAAGAATTCTTGGGGTTGTA-3'	100	(Elks *et al.*, 2011)

2.3.3 Generation of zebrafish CRISPants

CRISPR guide RNAs (gRNAs; Table 2.3) were designed using CHOPCHOP (https://chopchop.cbu.uib.no/)(Labun *et al.*, 2016, 2019). 100 µM stock concentrations of gRNA were stored at -80 °C. gRNA complex was formed by mixing 1 µl gRNA with 1 µl tracrRNA and incubating at 95 °C for 5 minutes. gRNA complex was stored overnight at -20 °C. Prior to injections, 1 µl Cas9 was added to gRNA complex and incubated at room temperature for 10 minutes. 1 µl 10% PR in DEPC-H_2O was added to Cas9-gRNA complex. 1 cell stage zebrafish embryos were injected into the centre of the yolk with 1 nl Cas9-gRNA complex, then incubated at 28 °C. *Tyr* gRNA was used as a negative control for experiments (Isles *et al.*, 2021) and as a positive control, with an observable phenotype, to validate successful CRISPR knockdown. *Card9* knockdown was validated by PCR and restriction digest (2.5 Molecular techniques). Efficacy of *nos2a/nos2b* CRISPR knockdown of iNOS, and subsequently RNS production, was functionally validated by anti-nitrotyrosine staining (2.6 Antibody staining of zebrafish embryos).

Table 2.3: CRISPR target sequences in this chapter

Target gene/guide name	Target Sequence
tyr	5'- GGACTGGAGGACTTCTGGGGAGG-3'
Nos2a	5'- TTTCTCATTTTCAATGATAG-3'
Nos2b	5'-GTTCGCTCTTGTGAGTGACC-3'
*card9*_16	5'-TCAAGACCATTGAGCCCTCAAGG-3'
*card9*_113	5'-GTGCAAGGTGCTGAGCAGCGAGG-3'
*card9*_119	5'-GATCTACAATGATCCAAGTTTGG-3'

Two gRNAs were designed to target the ATG/first exon of *nos2a* and *nos2b* to knockdown iNOS, and subsequently RNS, production. Tyrosinase (*tyr*) gRNA was used as a negative control (Isles et al 2019). The target sequences were: *nos2a* – 5'- TTTCTCATTTTCAATGATAG-3'; *nos2b* – 5'-GTTCGCTCTTGTGAGTGACC-3'; *tyr* – 5'- CCTGACCTCCTGAAGACCCC-3'. 100 µM stock concentrations of gRNA were stored at -80 °C. gRNA complex was formed by mixing 1 µl gRNA with 1 µl tracrRNA and incubating at 95 °C for 5 minutes. gRNA complex was stored overnight at -20 °C. Prior to injections, 1 µl Cas9 was added to gRNA complex and incubated at room temperature for 10 minutes. 1 µl 10% phenol red in DEPC-H_2O was added to Cas9-gRNA complex. 1 cell stage zebrafish embryos were injected into the centre of the yolk with 1 nl Cas9-gRNA complex, then incubated at 28 °C.

2.3.4 Morpholino injections

All morpholinos were diluted in dH_2O to a 1.0 mM stock concentration and stored at room temperature. Morpholinos were diluted to the desired concentration (Table 2.4) in 10% phenol red in dH_2O. Embryos were injected with 1 nl morpholino at the one-cell stage.

Csf3r morpholino was functionally validated by full body neutrophil count. *Myd88* morpholino was validated by generation of cDNA, PCR and gel electrophoresis.

Table 2.4: Morpholinos used in this thesis

Gene	Sequence	Injected concentration (µM)	Reference
Standard Control Morpholino	5'-CCTCTTACCTCAGTTACAATTTATA-3'	500-750	Gene tools
csf3r	5'-GAAGCACAAGCGAGACGGATGCCAT-3'	500	(Ellett *et al.*, 2011)
myd88	5'-GTTAAACACTGACCCTGTGGATCAT-3'	750	(Bates *et al.*, 2007)

2.3.5 Pharmacological treatment of zebrafish

Pharmacological treatment of zebrafish larvae was delivered by immersion in E3 solution. Solvent controls (dH_2O or DMSO) were used as appropriate.

For pharmacological inhibition of nitric oxide synthase, embryos were treated with 200 µM L-NIL (N^6-(1-Iminoethyl)-lysine, dihydrochloride; Merck) by immersion. dH2O was used as a vehicle control.

For antifungal survival curves and clearance assays, embryos were treated with either 5.0 µg/ml fluconazole in DMSO, 1.0 µg/ml caspofungin in dH_2O or 0.5 µg/ml caspofungin in dH_2O by immersion. DMSO or dH_2O was used as a solvent control. At 2 dpi, embryos were transferred into fresh E3 and treatment was re-administered.

For pharmacological stabilisation of Hif-1α, embryos were treated with 2.5 µM FG4592 in DMSO and transferred into fresh E3 after 24 hours of treatment.

2.3.6 Clodronate liposome injection

1 dpf zebrafish embryos were injected into the caudal vein with 1 nl clodronate liposomes (clodronateliposomes.com). PBS liposomes were used as a negative control. Zebrafish injected with liposomes were infected with 100 cfu *C. albicans* via the duct of Cuvier at 2 dpf.

Macrophage depletion by clodronate liposomes was validated by full body macrophage count in *Tg(mpeg:nlsClover)* zebrafish embryos at 2 dpf.

2.4 *C. albicans* culture and zebrafish infection

2.4.1 *C. albicans* culture and injection

Various *C. albicans* laboratory strains (Table 2.5) and *Candida* spp. clinical isolates (Table 2.6) were used in this thesis. *C. albicans* was cultured from glycerol stocks, stored at -80 °C. Under sterile conditions, a culture loop was dipped into glycerol stocks of *C. albicans* and used to streak across YPD agar plates (4001022, MP Bio). YPD plates with *C. albicans* laboratory strains were cultured for 2 days at 28 °C or 37 °C for *Candida* spp. clinical isolates. The day before a planned injection, several *C. albicans* colonies were transferred to a falcon tube containing 10 ml YPD broth. Liquid cultures were incubated overnight at 30 °C in a shaking incubator at 200 rpm.

1 ml of overnight *C. albicans* culture was transferred to a 1.5 ml Eppendorf tube. The sample was spun at 3000 rpm for 3 minutes, then the supernatant removed and pellet resuspended in 1 ml PBS. The PBS wash was repeated twice. From the washed *C. albicans* culture, 5 µl was taken and added to 95 µl PBS, to create a 1:20 dilution. 10 µl was placed into a haemocytometer (Hawksley) and counted. The total number of colony forming units (cfu) in the 1 ml washed *C. albicans* was calculated using the following equation:

$$\text{cfu/ml} = \text{Number of counted cells} \times 20 \times 10^4$$

This value was divided by 10^9 to determine the volume required to create a 1000 cfu/nl stock. 1 ml washed *C. albicans* was washed once more and resuspended in the calculated volume of PBS to create a 1000 cfu/nl solution. *C. albicans* was diluted further in 10% PVP in PR to achieve the desired infection concentration. 1 nl was injected with an infection dose of 20 cfu for hindbrain infections and 200 cfu or 500 cfu for caudal vein injections. All *C. albicans* injections took place at 30-35 hpf.

Table 2.5: List of *C. albicans* laboratory strains used in this thesis

Strain	Parental Strain	Genotype	Reference
C. albicans TT21-dTomato	SN52	*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	SN148 *ENO1/ENO1-GFP::LEU2*	(Christou, Ayscough and Johnston, 2023)
C. albicans SC5314	-	wt; clinical blood isolate	(Gillum, Tsay and Kirsch, 1984)
C. albicans car1Δ	SC5314	As SC5314 but *car1Δcar1Δ*	(Schaefer *et al.*, 2020)
C. albicans NRG1^{OEX}-dTomato	SN52	*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 2.6: List of *Candida* spp. clinical isolates used in this thesis

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

2.4.2 *C. albicans* glycerol stock preparation

C. albicans used to make glycerol stocks were cultured as described (2.4.1 *Candida albicans* culture). 500 µl *C. albicans* in YPD broth was transferred into a fresh cryotube, with 500 µl glycerol. The solution was mixed by pipetting, then stored at -80 °C until required.

2.4.3 *M. marinum* culture and injection

M. marinum (no. BAA-535; ATCC) containing a psMT3-mCherry vector (van der Sar *et al.*, 2009) was grown on 7H10 Middlebrook (BD 262710) agar plates, supplemented with Middlebrook OADC (SLS 211886) and 50 µg/ml hygromycin (Sigma H0654). Prior to injections, *M. marinum* mCherry was transferred into liquid culture in 7H9 Middlebrook (BD 271310) growth media, supplemented with Middlebrook ADC (SLS 211887) and 50 µg/ml hygromycin. Liquid cultures of *M. marinum* were cultured overnight at 28 °C and allowed to reach the log-phase of growth. *M. marinum* culture was determined to be in log-phase of growth using a spectrophotometer, when OD_{600nm} = 0.6-1.0. *M. marinum* culture was washed 3 times in PBS and resuspended in 10% PVP in PR to the desired infection dose. 100 cfu *M. marinum* mCherry was injected into the caudal vein of zebrafish at 30-35 hours post fertilisation.

2.4.4 Growth curves

200 µl from an overnight *C. albicans* culture was added to 20 ml YPD in a 50 ml falcon tube. 800 µl of new *C. albicans* culture was added to a glass cuvette and OD_{600nm} was measured using a spectrophotometer. OD_{600nm} of *C. albicans* culture at start of a growth curve should be 0.1-0.2. Drug treatments were added to *C. albicans* culture to the desired concentration. YPD with no drug treatment and YPD with DMSO were used as negative controls. Cultures were incubated in a shaking incubator at 30 °C and at 200 rpm. OD_{600nm} was measured every hour for 10 hours. At OD_{600nm} >1.0, the spectrophotometer loses accuracy. To overcome this, 100 µl *C. albicans* culture was removed and diluted in 900 µl YPD broth. The resulting OD_{600nm} measurement was multiplied by 10.

2.4.5 Validation of *C. albicans* microinjection doses

C. albicans was prepared and diluted to desired infection dose as described above. Microinjection needles were loaded with *C. albicans* diluted to 20, 50, 200 or 500 cfu/nl, in 10% PVP in PR. 1 nl *C. albicans* was injected onto a YPD plate then incubated at 28 °C. After 48 hours incubation, colonies were counted.

2.4.6 Heat-killing and boiling *C. albicans*

C. albicans strains were prepared and washed in PBS, as described above (see 2.4.1 *C. albicans* culture and injection). In 1.5 ml microcentrifuge tubes, *C. albicans* diluted in 1ml PBS was heat killed by incubating in a heat block at 60 °C for 1 hour (Panpetch *et al.*, 2017). *C. albicans* was heat killed to

inactivate *C. albicans*, while leaving surface proteins intact. C*. albicans* was boiled by using the above protocol for heat killing, followed by incubation in a heat block at 105 °C for 5 minutes. *C. albicans* was boiled to denature all proteins. Following incubation, *C. albicans* was centrifuged at 3000 rpm for 3 minutes, supernatant was removed and *C. albicans* was diluted to an appropriate infection dose in 10% PVP in PR.

Heat killing protocol was validated by microinjection of 1 nl heat killed *C. albicans* onto YPD plates. Plates were incubated at 28 °C for 48 hours, then colonies were counted.

2.4.7 Monitoring zebrafish survival

For survival curves, zebrafish mortality was measured once daily. Dead zebrafish were removed from the petri dish and inactivated in bleach and Virkon solution. Death was determined by lack of heartbeat, cessation of circulation or large tissue degradation post mortem. At 4 dpi (5 dpf), all surviving zebrafish larvae were anaesthetised in Tricaine (MS222) and then culled in bleach and Virkon solution.

2.5 Molecular techniques

2.5.1 Preparation of Hif-1α RNA

2.5.1.1 DNA linearisation

Reaction mixture was prepared containing 10 µl 10x CutSmart buffer, 10 µg DNA midi, DEPC-treated water up to total volume of 99 µl and 1 µl Not1 restriction enzyme. Sample was incubated in a heat block at 37 °C for 2 hours. DNA product was then purified using QIAquick PCR purification kit (Qiagen). 500 µl buffer PB was added to sample and mixed with pipette. Sample was then transferred to a QIAquick column within a 2 ml collection tube and centrifuged for 1 minute at 14,800 rpm at room temperature. Column was transferred to a fresh collection tube and 750 µl buffer PE added to column. Sample was centrifuged again with same settings and column was transferred to a fresh collection tube. 30 µl buffer EB was added to column, allowed to stand for 1 minute then centrifuged for 1 minute. DNA linearisation was checked by gel electrophoresis, comparing to a 1kB Hyperladder (Bioline) and non-linearised DNA. Linearised DNA samples were stored at -20 °C.

2.5.1.2 RNA Preparation

RNA for injection into one-cell stage zebrafish embryos was prepared using an Ambion mMessage SP6 kit (Ambion; AM1340). A reaction mixture was prepared containing 10 µl 2x NTP/CAP, dH_2O up to a total volume of 18 µl, 1 µg linearised DNA template and 2 µl 10x reaction buffer. Reaction mixture was mixed and centrifuged at 14,000 rpm for 1 minute. 2 µl SP6 enzyme µl was added to the sample, which was incubated in a heat block at 37 °C for 2 hours. 1 µl Turbo DNase was added and

sample was incubated at 37 °C for a further 30 minutes to remove any remaining template DNA. Sample was stored at -20 °C for at least 20 minutes for more efficient DNA precipitation. 480 µl dH_2O then 500 µl acid:phenol:chloroform ($CHCl_3$; Ambion AM9720) was added to sample, which was then vortexed for 2 minutes. Sample was then centrifuged at 10,000 g for 10 minutes at 4 °C. The upper phase of sample was removed (approximately 400 µl) and placed in an Amicon Ultracel 100k filter (Millipore UFC510024) within a collection tube. Sample was centrifuged at 14,000 g for a further 10 minutes at room temperature. The filter was placed upside-down in a fresh collection tube and centrifuged at 1000 g for 2 minutes at room temperature. RNA concentration was measured using a Nanodrop spectrophotometer (ND1000), with an expected concentration of 4000 ng/ µl. RNA was stored at -80 °C.

2.5.2 RNA extraction

Zebrafish embryos were anaesthetised, transferred to an RNAse-free microcentrifuge tube then snap frozen in dry ice for 10 minutes. Samples were homogenised in 500 µl Trizol (Invitrogen) and left at room temperature for 5 minutes. To ensure zebrafish were fully homogenised, samples were transferred to QIAshredder column (QIAgen) and centrifuged at 14,800 rpm (16,162 g) for 2 minutes. 100 µl chloroform was added to samples, which were then left at room temperature for 3 minutes, then centrifuged at 14,800 rpm for 5 minutes. The aqueous phase was removed and transferred to a clean RNAse-free Eppendorf tube. 250 µl isopropanol was added and the tube was vortexed for 1 minute. Samples were left at room temperature for 10 minutes, before being centrifuged at 14,800 rpm for 15 minutes at 4 °C. Supernatant was removed and 1 ml 75% ethanol was added to tube. Samples were centrifuged at 14,800 rpm for 5 minutes at 4 °C and supernatant removed. Pellet was left to air dry for 5 minutes, before being resuspended in 20 µl dH_2O and stored at -20 °C.

2.5.3 cDNA conversion

cDNA synthesis used a Superscript II Reverse Transcriptase kit (ThermoFisher Scientific). A reaction mixture of 1 µl 500 µg/ml Oligo(dT)12-18, 1 µl 10 mM dNTP mix, 2 µg RNA and dH2O up to 12 µl was made. The reaction mixture was incubated at 65 °C then cooled briefly on ice. 4 µl 5x First Strand Buffer and 2 µl 0.1M DTT was added to the reaction mixture, which was then incubated at 42 °C for 2 minutes. 1 µl Superscript II Reverse Transcriptase was added to the reaction mixture and mixed gently by pipetting. Reaction mixture was incubated at 42 °C for 50 minutes, then at 70 °C for 15 minutes to inactivate. Samples were then stored at -20 °C.

2.5.4 gDNA extraction

1 dpf zebrafish embryos were dechorionated and anaesthetised in Tricaine (MS222). Individual embryos were transferred to a 96 well v-bottom plate and excess E3 removed. 100 µl 50 mM NaOH

was added to each well. Samples were sealed with parafilm, then heated to 95 °C for 30 minutes. Samples were allowed to cool to room temperature and 10 µl 1.0 M Tris-HCl pH8.0 was added to each well. Samples were stored at -20 °C.

2.5.5 PCR amplification

10 µl reaction mixes were prepared for PCRs, composing of 1 µl cDNA/gDNA, 2 µl 5x Firepol DNA polymerase master mix, 0.5 µl 10 mM forward primer, 0.5 µl 10 mM reverse primer and 6 µl dH_2O (for primer sequences, see Table 2.7). No DNA controls were included in all PCRs, replacing DNA with dH_2O. PCR cycles were carried out as detailed in Table 2.8, unless otherwise stated. Upon completion of PCR cycle, samples were held at 4 °C and then stored at -20 °C. Housekeeping gene *ef1* was used as a positive control (McCurley and Callard, 2008).

Table 2.7: Primer sequences used in this thesis

Myd88	Forward primer	5′-TCTTGACGGACTGGGAAACTCG-3′
	Reverse primer	5′-GATTTGTAGACGACAGGGATTAGCC-3′
Card9	Forward primer	5’-GTTTTTGAGGTGGAGGAAGATG-3’
	Reverse primer	5’-AGAACTCTTACCAACTTTGCGG-3’
Ef1	Forward primer	5’-CAGCTGATCGTTGGAGTCAA-3’
	Reverse primer	5’-TGTATGCGCTGACTTCCTTG-3’

Table 2.8: PCR cycles used in this thesis for Firepol reactions

Initial denaturation	95 °C		5 minutes
Variable cycles	95 °C		30 s
	Myd88	58 °C	30 s
	Card9	55 °C	
	72 °C		45 s
Final extension	72 °C		10 minutes
Storage	4 °C		Hold

2.5.6 Restriction digest

Following PCR amplification, 0.25 µl restriction enzyme (NEB; Table 2.9) was added to PCR reaction mix. As per supplier instructions, samples were incubated at 37 °C for 1 hour. Samples were stored at -20 °C.

Table 2.9: Restriction enzymes used in this thesis

Target gene/guide name	Restriction enzyme
*card9*_16	Hpy188III
*card9*_113	BlpI
*card9*_119	AlwI

2.5.7 Gel electrophoresis

For validation of *myd88* morpholino, 10 µl samples and 8 µl 1 kB Hyperladder (Bioline) were run for 50 minutes on a 2.0% TAE agarose gel at 100 V.

For validation of *card9* CRISPR knockdown, 10 µl samples and 8 µl 100 bp ladder (NEB) were run for 1 hour 20 minutes on a 1.5% TAE agarose gel at 100 V.

2.6 Antibody staining of zebrafish embryos

2.6.1 Fish Fixation

Fixation of zebrafish embryos took place at 24 hpi (2 dpf). Zebrafish were anaesthetised with Tricaine. Up to 15 zebrafish were transferred to 1.5 ml Eppendorf tubes, then 1 ml 4% paraformaldehyde (PFA, diluted in PBS) with 0.4% Triton X-100 was added. Samples were incubated at 4 °C for 24 hours. Following fixation, PFA-0.4% Triton X-100 was removed and samples were washed twice for 2 minutes in 1 ml PBS-0.1% Tween20. Samples were stored at 4 °C in 1 ml PBS-0.1% Tween20 until antibody staining took place.

2.6.2 Anti-nitrotyrosine antibody staining

All steps were completed on a rocking shaker at 40 rpm, for gentle agitation, except for primary antibody incubation, which used an orbital shaker at 100 rpm. Fixed embryos were washed four times for 5 minutes in 1 ml PBS-0.4% Triton X-100 (Thermo Scientific; PBSTx). Embryos were then treated for 30 minutes at room temperature with 10 µg/ml Proteinase K (Sigma-Aldrich) diluted in 1 ml PBSTx, before a further four rounds of washing for 10 minutes in 1 ml PBSTx. Embryos were blocked for 2 hours at room temperature in 1 ml 5% sheep serum (Stratech) in PBSTx (5% SS:PBSTx). Following blocking, embryos were incubated at 4 °C overnight in 200 µl 1:250 primary anti-nitrotyrosine antibody (Millipore) diluted in 5% SS:PBSTx. The next morning, samples were washed

four times for 10 minutes in 1 ml PBSTx, then blocked for 1 hour at room temperature in 5% SS:PBSTx. Embryos were treated with 200 µl 1:500 goat-anti-rabbit-633 antibody (Molecular Probes, A21071) diluted in 5% SS:PBSTx for 2 hours at room temperature in the dark. Samples were washed four times for 20 minutes in the dark with PBSTx, then post fixed in 1 ml 4% PFA-0.4% Triton X-100 for 20 minutes in the dark. Samples were briefly washed in 1 ml PBSTx to remove PFA. Prior to microscopy, samples were stored in 1 ml PBSTx in the dark at 4 °C for up to 1 week.

2.7 Zebrafish microscopy

2.7.1 Mounting fish

For confocal imaging, zebrafish were anaesthetised in 0.168 mg/ml Tricaine. 10-20 zebrafish were transferred to one well of µ-Slide 4 well chambered coverslip (Ibidi) and excess fluid was removed. Approximately 500 µl 1.0% low melting agar gel, containing 0.168 mg/ml Tricaine, was added to well and zebrafish were positioned as desired using extended length gel loading tip. Once set, agarose was covered in approximately 500 µl E3 containing Tricaine to prevent dehydration.

2.7.2 Imaging and quantification of anti-nitrotyrosine staining

Anti-nitrotyrosine stained embryos were mounted (see 2.7.1 Mounting fish) and imaged on a Leica DMi8 inverted microscope with a Leica TCS-SPE line-scanning confocal with a 40x 1.1NA water immersion lens. For quantification purposes, acquisition settings were kept the same across the groups. For *C. albicans* SC5314 and *car1*Δ, which lacked fluorescent proteins, brightfield images were taken using an inverted Leica DMi8 with a 40x lens and a Hamamatsu OrcaV4 camera.

Anti-nitrotyrosine staining was quantified using Image J measurements. In each zebrafish, the 6 brightest regions of staining that co-localised with neutrophils were quantified by drawing circles around the region of staining and calculating the intensity density. 3 blank regions in each fish were also quantified and a mean average calculated to establish the level of background fluorescence. Corrected fluorescence intensity was calculated for each region of staining using the following equation:(Burgess *et al.*, 2010; Elks *et al.*, 2013).

$$\text{Corrected fluorescence intensity} = \text{Intensity density} - (\text{Mean background} \times \text{Area of stained region})$$

2.7.3 Imaging and quantification of cytokine fluorescence

Embryos were mounted (2.7.1 Mounting fish) and imaged on a Leica DMi8 inverted microscope with a Leica TCS-SPE line-scanning confocal with a 40x 1.1NA water immersion lens.
Corrected fluorescence intensity was calculated using Image J measurements (assessing the cell fluorescence of individual cells corrected for cell size and background fluorescence of the image), as previously described (Burgess *et al.*, 2010; Elks *et al.*, 2013).

For counting the number of *lyz:mCherry+/il-1β:eGFP+* cells and *arg2:GFP+/lyz:mCherry+* cells, images were analysed by moving through z-stacks and searching for cells with colocalisation.

2.7.4 Hyphal staging

For assessment of fungal growth of *C. albicans* TT21-dTomato, confocal images were taken using a Leica DMi8 inverted microscope with a Leica TCS-SPE line-scanning confocal with a 40x 1.1NA water immersion lens. Classification of hyphal stages was done manually, based on a previously described protocol (Thrikawala *et al.*, 2022). Criteria for hyphal staging was:

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 – small number of short or medium length hyphae were observed.
4. Destructive hyphae – multiple, spread-out invasive hyphae were observed. Majority of observed *C. albicans* was in hyphal morphotype.

2.7.5 Imaging and quantification of *phd3:GFP* fluorescence

For *phd3:GFP* quantification, brightfield and fluorescent images on the widefield microscope were taken at 1 dpi on an inverted Leica DMi8 with 2.5× objective lens, using a Hamamatsu Orca v4 camera.

2.7.6 Imaging and quantification of neutrophil behaviours

For quantification of neutrophils, 1 dpi *Tg(mpx:GFP) i114* zebrafish embryos were observed using a HC PL PLAN 40x0.07 immersion lens on an inverted Leica DMi8 microscope. All neutrophils within the hindbrain were counted.

For quantification of internalised *C. albicans*, confocal images were taken of a single site of interest at the site of *C. albicans* infection. Within this field of view, the number of neutrophils with internalised *C. albicans* were counted. This field of view was also used for quantification of spacious and tight phagosomes and folded and non-folded hyphae. A spacious phagosome was defined as any phagosome containing *C. albicans* in which *C. albicans* did not make contact with any part of the phagosomal membrane. Tight phagosomes were phagosomes containing *C. albicans* in which *C. albicans* made contact with any part of the phagosomal membrane. Hyphae were classified as folded if a single, continuous hypha was observed to be at an acute angle (<90°) to itself (Bain *et al.*, 2021).

2.7.7 Microscopy of Clearance of *C. albicans* Infection

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

if no signs of remaining fluorescent *C. albicans* infection could be visualised, based on observation at 2.5x magnification.

2.8 Data Analysis

All data were plotted and analysed using GraphPad Prism 9.5.1.1 and 10.4.1 (GraphPad Software, La Jolla, California, USA, www.graphpad.com). Specific statistical test used is detailed in the relevant chapters and figure legends. P values shown: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

For Hif-1α and antifungal survival curves, potential synergy was calculated with a previously published equation (Demidenko and Miller, 2019). A drug independence curve was calculated, using the equation:

$$S_{AB}(t) = 1 - \big(1 - S_A(t)\big)\big(1 - S_B(t)\big)$$

$S_{AB}(t)$ is hypothetical survival if combination of Hif1α stabilisation and antifungals has synergistic effect

$S_A(t)$ is survival with Hif1α stabilisation

$S_B(t)$ is survival with antifungal treatment

Combination of Hif-1α stabilisation with antifungals was deemed to have a synergistic effect if the survival curve was greater than $S_{AB}(t)$.

3. *Candida albicans* suppresses neutrophil RNS production *in vivo*

3.1 Introduction

3.1.1 *C. albicans* defences against RNS

Survival of fungal pathogens within the host relies on the ability to evade innate immune responses to grow and disseminate. *C. albicans* has, therefore, developed several mechanisms that allow it to persist in the presence of oxidative and nitrosative stress (Missall, Lodge and McEwen, 2004; Brown, Haynes and Quinn, 2009). DTPA NONOate is an NO donor, which spontaneously dissociates in a pH-dependent manner to release NO. *C. albicans cta4* mutant had increased susceptibility to DPTA NONOate *in vitro*, while *cwt1* mutant had increased resistance to DPTA NONOate (Sellam *et al.*, 2012). Cwt1p was revealed to be a repressor of Yhb1 flavohaemoglobin, which has previously been demonstrated to detoxify NO by converting to less toxic NO_3^- (Gardner *et al.*, 1998; Sellam *et al.*, 2012). From a negligible level of basal expression, exposure to NO induces a significant upregulation of Yhb1 by *C. albicans in vitro*, suggesting an inducible response to nitrosative stress (Ullmann *et al.*, 2004). *C. albicans yhb1* mutant has reduced virulence compared to wild type strains in murine infection models (Ullmann *et al.*, 2004; Hromatka, Noble and Johnson, 2005). However, inhibition of murine NO production did not restore *C. albicans yhb1Δ* virulence, despite a hyperfilamentous phenotype, suggesting the attenuated virulence may relate to factors additional to the reduced ability to detoxify host NO (Hromatka, Noble and Johnson, 2005).

Hog1 is a stress-activated protein kinase, which is phosphorylated in response to a diverse range of stresses, including osmotic, heavy metal and oxidative stress (Smith *et al.*, 2004). Phosphorylation of Hog1 has traditionally been used as a proxy for activation of Hog1 (Klipp *et al.*, 2005). Crucially, Hog1 stimulates stress-specific transcriptional responses, facilitating a nuanced and highly co-ordinated stress response (Enjalbert *et al.*, 2006). *C. albicans hog1Δ* was highly sensitive to NO *in vitro*, contradicting previous dogma that Hog1 does not contribute to the nitrosative stress response (Herrero-de-Dios *et al.*, 2018). In response to nitrosative stress, Hog1 is oxidised, rather than phosphorylated, leading to induction of nitrosative stress-specific transcription, promoting resistance to RNS, resistance to phagocytic killing *in vitro* and increased virulence in *Galleria mellonella in vivo* infection (Herrero-de-Dios *et al.*, 2018). Stress regulation by Hog1 is conserved in *C. auris*, suggesting any attempts to target *C. albicans* Hog1 therapeutically would also be effective against *C. auris* (Shivarathri *et al.*, 2024).

Further investigation of *C. albicans* nitrosative stress responses revealed *C. albicans* can suppress nitric oxide synthesis by RAW264.7 macrophage-like cells and murine BMDMs *in vitro* (Collette, Zhou and Lorenz, 2014). Whereas other nitrosative stress responses have focused on detoxification of

RNS, these findings revealed a *C. albicans* mechanism of modulating host immune responses by inhibiting NO synthesis. NO inhibition was revealed to be dependent on direct contact between *C. albicans* and macrophages and was mediated by an unknown, secreted compound (Collette, Zhou and Lorenz, 2014). Dr Ffion Hammond (previous PhD student in Elks lab) similarly observed that *C. albicans* infection in zebrafish reduced host RNS levels below the basal level in uninfected zebrafish embryos (Hammond, PhD thesis, 2022). Hence, *C. albicans* has an uncharacterised mechanism of suppressing host RNS levels, which has been observed both *in vitro* and *in vivo*.

3.1.2 Hypothesis and aims

Neutrophils are critical for control of *C. albicans* infections by the innate immune system (Brown, 2011). Hence, any modulation of neutrophil activity by *C. albicans* would likely have significant impacts on infection outcomes. RNS, including NO, are critical for candidacidal activity and reduction of RNS levels results correlates with a reduction of *C. albicans* killing (Rementería, García-Tobalina and Sevilla, 1995). Therefore, *C. albicans* mechanisms that negatively modulate neutrophil or RNS activity warrant further investigation.

C. albicans has previously been demonstrated to inhibit murine RAW264.7 macrophage NO synthesis *in vitro* (Collette, Zhou and Lorenz, 2014). However, the effect of *C. albicans* on RNS *in vivo* and the molecular and cellular mechanisms involved remain unclear. Collette *et al*. used LPS/IFNγ-stimulated murine RAW264.7 and J774A.1 macrophages in their investigations as, in mice, it is primarily macrophages that produce high levels of RNS (Munder *et al.*, 2005). However, in the zebrafish model, RNS is predominantly produced by neutrophils, both in mock infection and at elevated levels in *M. marinum* infection (Elks *et al.*, 2013, 2014). Human neutrophils are also known to produce higher levels of RNS than macrophages (Muijsers *et al.*, 2001), suggesting investigating neutrophil RNS production in zebrafish may be a more appropriate model for the human RNS response than *in vitro* murine macrophages.

I, therefore, investigated the impact of *C. albicans* on neutrophil RNS levels in zebrafish *in vivo*, based on the hypothesis that: ***C. albicans* suppresses neutrophil RNS levels in a zebrafish *in vivo* infection model.**

To examine this hypothesis, the following aims were addressed:

- Demonstrate neutrophil RNS suppression by *C. albicans in vivo*
- Investigate whether RNS suppression requires live *C. albicans*
- Examine if RNS suppression is a local or systemic phenomenon, using a localised hindbrain infection model and imaging RNS at proximal and distal sites.

3.2 Results

3.2.1 *C. albicans* TT21-dTomato suppresses neutrophil RNS production *in vivo*

C. albicans has previously been demonstrated to suppress ROS and RNS production in macrophages *in vitro* (Wellington, Dolan and Krysan, 2009; Collette, Zhou and Lorenz, 2014). However, there has been a lack of *in vivo* data on the effect of *C. albicans* on ROS and RNS production. *In vitro* observations lack the complex microenvironment and host intercellular interactions of an *in vivo* system, leaving questions about the biological significance of previous observations. Furthermore, neutrophils are the key innate immune cell involved in control of *C. albicans* infections, so it is necessary to confirm whether RNS suppression occurs in neutrophils or is limited to macrophages only (Brown, 2011). To establish if *C. albicans* can suppress RNS production *in vivo*, a zebrafish *C. albicans* infection model was used (Chao *et al.*, 2010). Dr Ffion Hammond (former PhD student) previously demonstrated that *C. albicans* SN148 GFP can cause suppression of neutrophil RNS in a zebrafish *in vivo* infection model (Hammond PhD thesis, 2022). I aimed to robustly repeat this experiment in my own hands. Based on the previously used protocol, 1 dpf *Tg(lyz:nfsB.mCherry)* zebrafish were injected with 500 cfu *C. albicans* SN148 GFP into the caudal vein to generate a systemic infection. 10% PVP in PR was used as a mock injection control. At 24 hpi, zebrafish were fixed in 4% PFA and stained with an anti-nitrotyrosine antibody as an indirect measure of RNS.

PVP injected embryos had detectable, basal anti-NT (anti-nitrotyrosine) levels (Figure 3.1). Anti-NT fluorescence was co-localised with *lyz:mCherry* fluorescence, showing RNS production by neutrophils – consistent with previous literature (Elks *et al.*, 2013, 2014). Infection with *C. albicans* SN148 caused a significant decrease in anti-NT levels compared to PVP injected fish, from 5.17 in PVP controls to 0.78 in *C. albicans* SN148 infected zebrafish (Figure 3.1; $p<0.0001$), representing an 84.9% reduction in anti-NT levels. This is greater than previous reports: Dr Hammond observed a 64.2% reduction in anti-NT levels due to *C. albicans* SN148 infection at 24 hpi (Hammond PhD thesis, 2022).Variation between antibody staining or primary antibody stock may have contributed to these small differences. While the effect size was greater than expected, observation of neutrophil RNS suppression by *C. albicans* SN148 GFP shows my ability to repeat this protocol and the reproducibility of this observation.

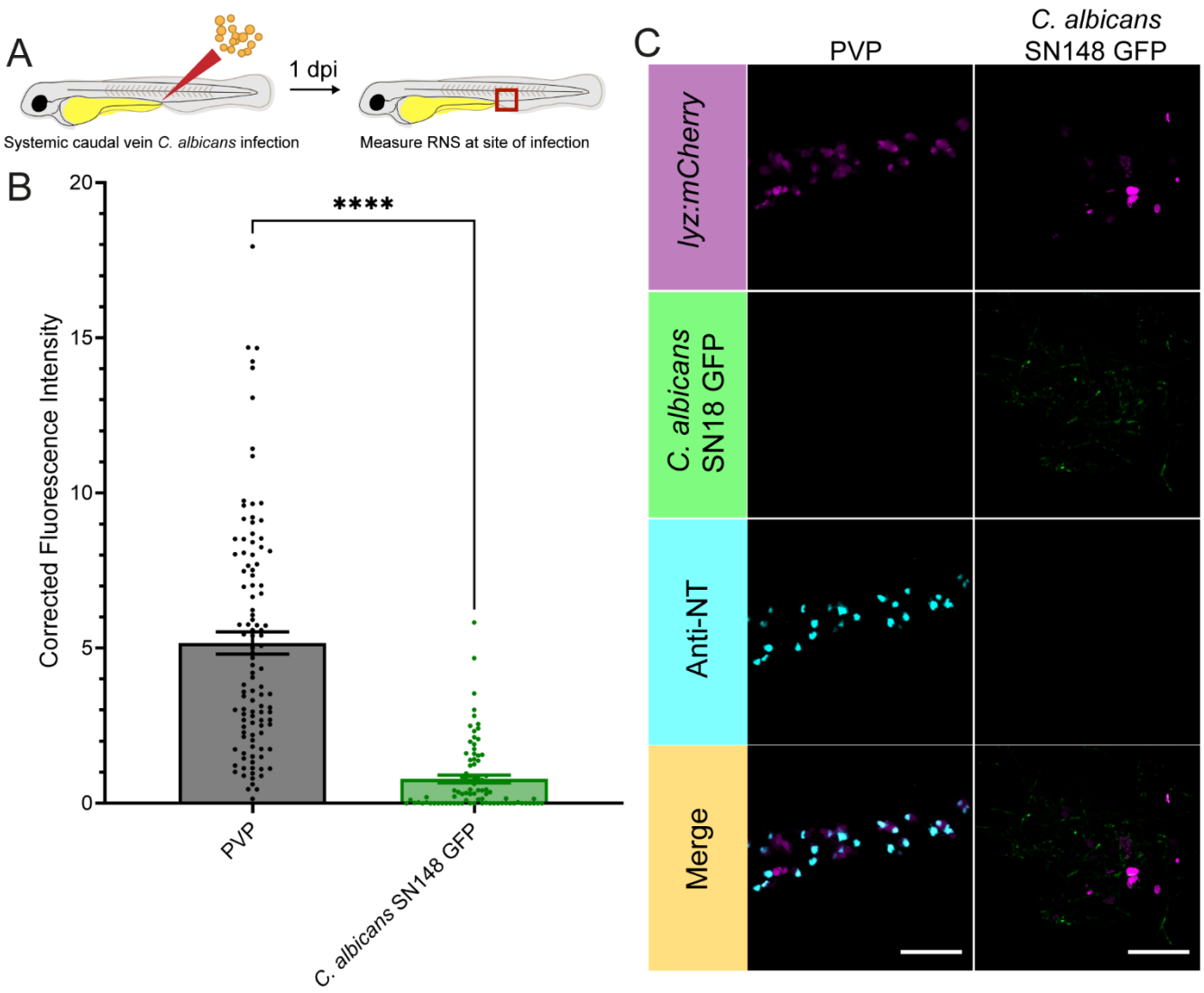

Figure 3.1: *C. albicans* SN148 GFP suppresses neutrophil RNS *in vivo*.

(A) 1 dpf zebrafish were injected with PVP or *C. albicans* SN148 GFP 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 haematopoietic tissue (CHT) and anti-nitrotyrosine fluorescence was quantified.

(B) Anti-nitrotyrosine fluorescence at 24 hpi following injection of PVP or *C. albicans* SN148 GFP into the caudal vein at 30 hpf. N=84-108 neutrophils from 14-18 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by two-tailed Mann-Whitney test. P values shown: ****p<0.0001.

(C) Representative images of PVP and *C. albicans* SN148 GFP infected zebrafish embryos at 24 hpi. Scale bars = 50 µm

Having demonstrated RNS suppression by *C. albicans* SN148 GFP, I aimed to establish whether RNS suppression is unique to this strain. This required the selection of an alternative laboratory reference strain. *C. albicans* TT21-dTomato is a strongly hypha producing strain that is widely used within the field (Seman *et al.*, 2018). Furthermore, *C. albicans* TT21-dTomato has a different parental lineage to *C. albicans* SN148 GFP: *C. albicans* TT21-dTomato is *C. albicans* THE21 (itself derived from *C. albicans* CAI8), while *C. albicans* SN148 GFP is derived from *C. albicans* SN152 (Seman *et al.*, 2018; Christou, Ayscough and Johnston, 2023). These divergent lineages may have allowed introduction of genetic differences responsible for differences in their ability to suppress host RNS. Therefore, *C. albicans* TT21-dTomato was a logical alternative strain to investigate.

An optimum dose for *C. albicans* TT21-dTomato infection of zebrafish embryos needed to be established to ensure the robustness of subsequent experiments. As *C. albicans* TT21-dTomato is highly hypha producing, and, therefore, hyper-pathogenic, infection doses lower than used for *C. albicans* SN148 GFP were considered. 1 dpf nacre zebrafish embryos were infected with 50, 200 or 500 cfu *C. albicans* TT21-dTomato via microinjection of the caudal vein. PVP was used as a mock injection control. Mortality was used as a measure of infection outcome, with zebrafish survival monitored daily until 4 dpi. To validate the infection doses used, microinjection needles loaded with 20, 50, 200 or 500 cfu/nl *C. albicans* injected 1 nl onto YPD agar plates. Colonies of *C. albicans* on YPD plates were counted after 48 hours incubation at 28 °C.

Upon counting *C. albicans* colonies on YPD plates, the average colonies observed was slightly lower than expected for all infection doses, except 500 cfu/nl (Figure 3.2A; 20 cfu/nl = 6.3 colonies, 50 cfu/nl = 28.2 colonies, 200 cfu/nl = 162.3 colonies, 500 cfu/nl = 499.8 colonies). Given all observed colonies were within the same order of magnitude of the expected infection dose, the method of *C. albicans* preparation and microinjection into zebrafish was deemed valid.

Survival of zebrafish infected with *C. albicans* was monitored for 4 days. Zebrafish infected with the highest dose (500 cfu) of *C. albicans* had the lowest level of survival – 35.4% at 4 dpi (Figure 3.2B). 50 cfu and 200 cfu had greater levels of survival (82.3% and 50.8% respectively), demonstrating dose dependent survival. 200 cfu was selected as the optimum infection dose for all future systemic infections as it had 4 dpi survival closest to 50%. Although 7 (5.4%) PVP injected zebrafish died at 2 dpi, this was not an abnormal level of death and all infection survival curves demonstrated significant difference from the control (50 cfu/nl: $p<0.01$, 200 cfu/ml: $p<0.001$, 500 cfu/nl: $p<0.001$). Across all infection doses, the greatest level of death was between 1 dpi and 2 dpi, suggesting this is a critical time period for host control of *C. albicans* TT21-dTomato infection.

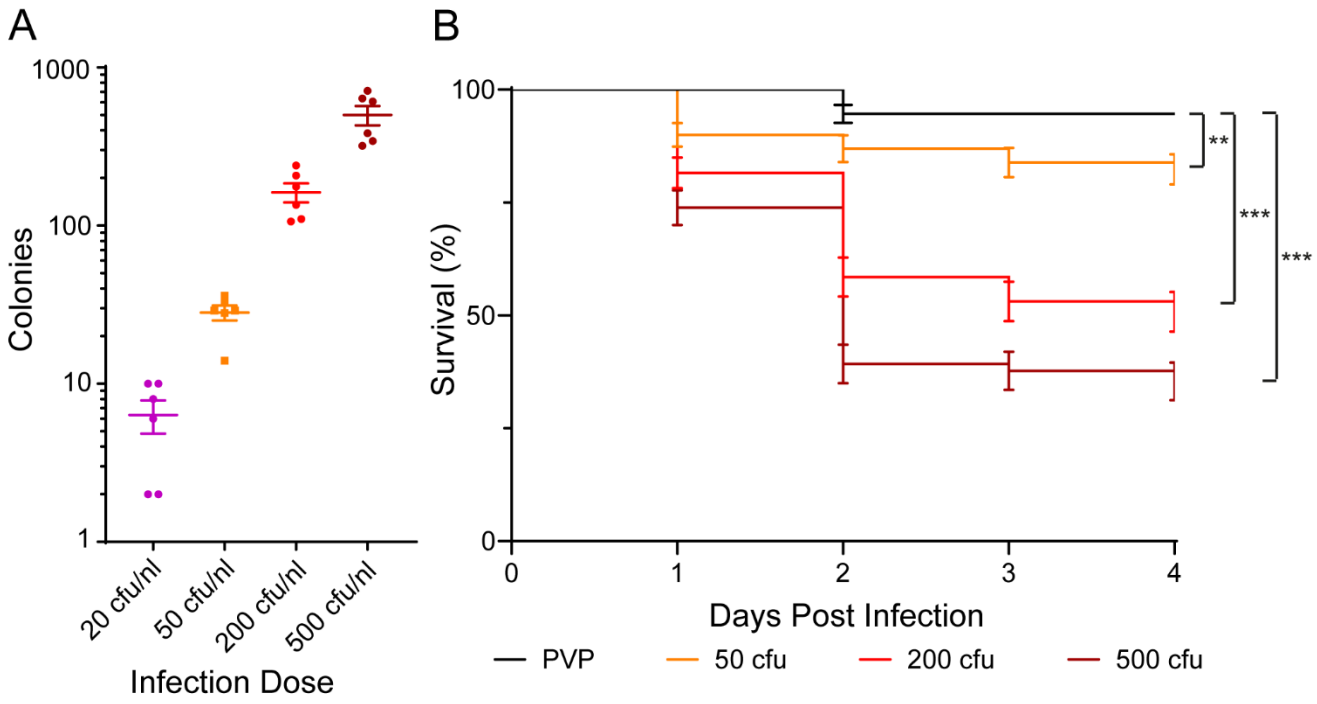

Figure 3.2: *C. albicans* infected zebrafish embryos have dose dependent survival.

(A) Infection doses were validated by microinjection of 20, 50, 200 and 500 cfu *C. albicans* onto a YPD agar plate and counting of colonies grown after 48 hours incubation at 28 °C. n=2 independent experiments, each experiment was performed in technical triplicate.

(B) 1 dpf nacre zebrafish embryos were injected into the caudal vein with PVP, 50, 200 and 500 cfu *C. albicans* TT21-dTomato. Mortality was measured daily up to 4 dpf. n=130 fish, obtained from 3 independent experiments. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. P values shown are: **p<0.01, ***p<0.001

With an optimum infection dose determined, I next aimed to investigate whether *C. albicans* TT21-dTomato causes suppression of neutrophil RNS levels. To avoid fluorophore clashes, *Tg(mpx:GFP)* i114 zebrafish were used as an alternative neutrophil marker to *Tg(lyz:nfsB.mCherry)*(Renshaw *et al.*, 2006). 1 dpf *Tg(mpx:GFP)* zebrafish embryos were injected into the caudal vein with 200 cfu *C. albicans* TT21-dTomato to generate a systemic infection. PVP was used as a mock infection and solvent control. At 24 hpi, embryos were fixed in 4% PFA and stained with anti-nitrotyrosine antibody.

PVP injected zebrafish had a basal level of anti-NT staining, as has previously been shown (Elks *et al.*, 2013, 2014). *C. albicans* TT21-dTomato caused a 90.0% decrease in anti-NT levels compared to PVP,

from 5.17 in uninfected, PVP controls to 0.52 in *C. albicans* TT21-dTomato infected zebrafish (Figure 3.3; p<0.0001). This was similar to the 84.9% reduction in anti-NT levels observed in *C. albicans* SN148 GFP infected embryos. These data shows that *C. albicans* TT21-dTomato causes suppression of neutrophil RNS levels, demonstrating the phenomenon is not unique to *C. albicans* SN148 GFP.

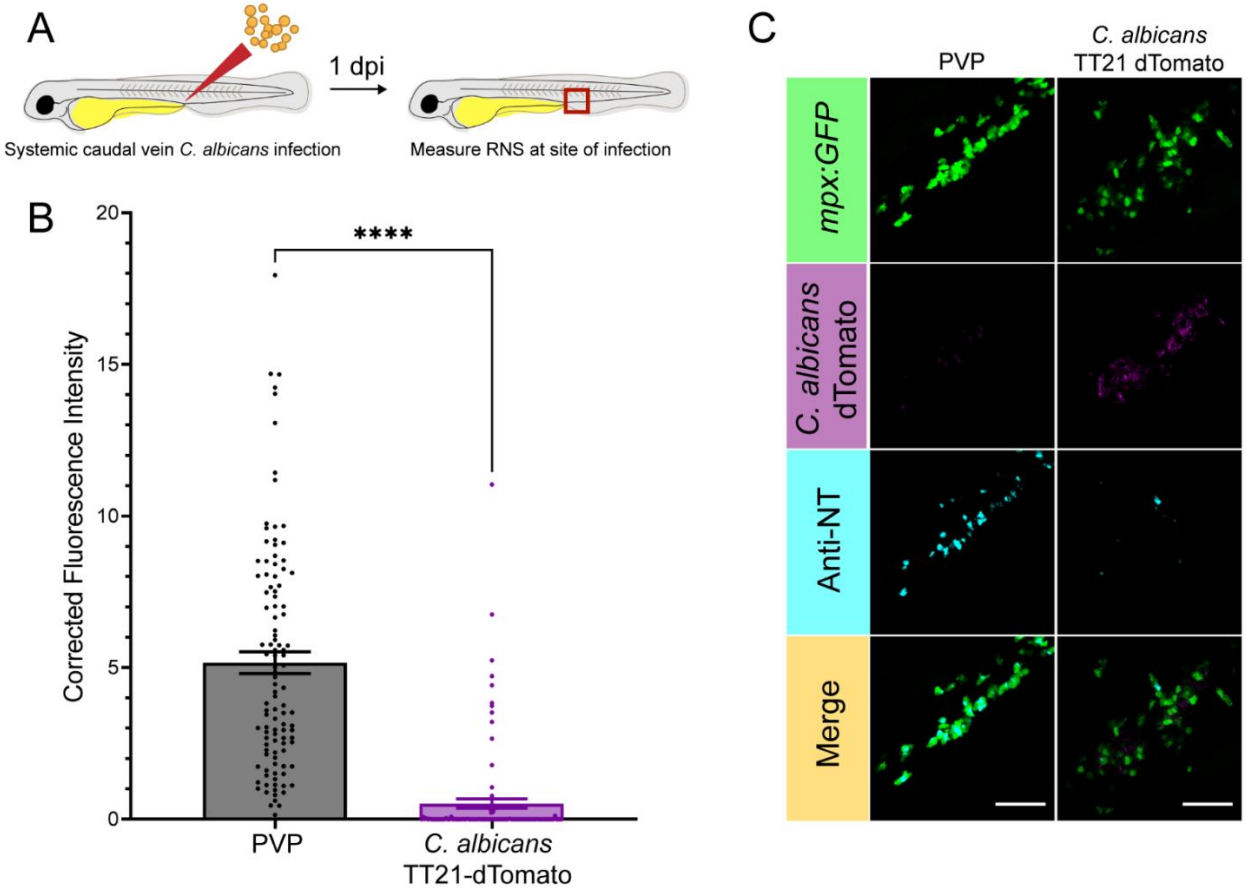

Figure 3.3: *C. albicans* TT21-dTomato suppresses neutrophil RNS production *in vivo.*

(A) 1 dpf zebrafish were injected with PVP or *C. albicans* TT21-dTomato 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 haematopoietic tissue (CHT) and anti-nitrotyrosine fluorescence was quantified.

(B) Anti-nitrotyrosine fluorescence at 24 hpi following injection of PVP or *C. albicans* TT21-dTomato into the caudal vein at 30 hpf. N=108 neutrophils from 18 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by two-tailed Mann-Whitney test. P values shown: ****p<0.0001.

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

3.2.2 Heat killed *C. albicans* has an intermediate effect on RNS production

In order to investigate whether active mechanisms are involved in *C. albicans* suppression of host RNS production, I aimed to produce heat killed *C. albicans*. Heat-killed *C. albicans* has been inactivated but proteins should remain intact as the temperature is not high enough for denaturation (Tansey and Brock, 1972). Hence, observation of RNS suppression by heat killed *C. albicans* could indicate that the effect is mediated by a cell surface protein, while lack of RNS suppression by heat killed *C. albicans* may suggest the effect requires a process mediated by a living pathogen. *C. albicans* was heat killed by incubation at 60 °C for 1 hour (Panpetch *et al.*, 2017). I validated this method of heat killing by injecting 1 nl of live and heat killed 200 cfu/nl *C. albicans* TT21-dTomato or 500 cfu/nl *C. albicans* SN148 GFP onto YPD agar plates. Colonies were counted after 24 hours incubation at 28 °C. PVP was included as a negative control.

No fungal growth was observed on plates injected with PVP, confirming no contamination in the PVP stock. Live *C. albicans* TT21-dTomato led to an average of 72 colonies, lower than expected (Figure 3.4). Culture of *C. albicans* SN148 GFP led to an average of 250 colonies after 48 hours, half of the expected level of growth. No fungal growth was observed on plates injected with heat killed *C. albicans* TT21-dTomato or heat killed *C. albicans* SN148 GFP, validating this method of heat killing.

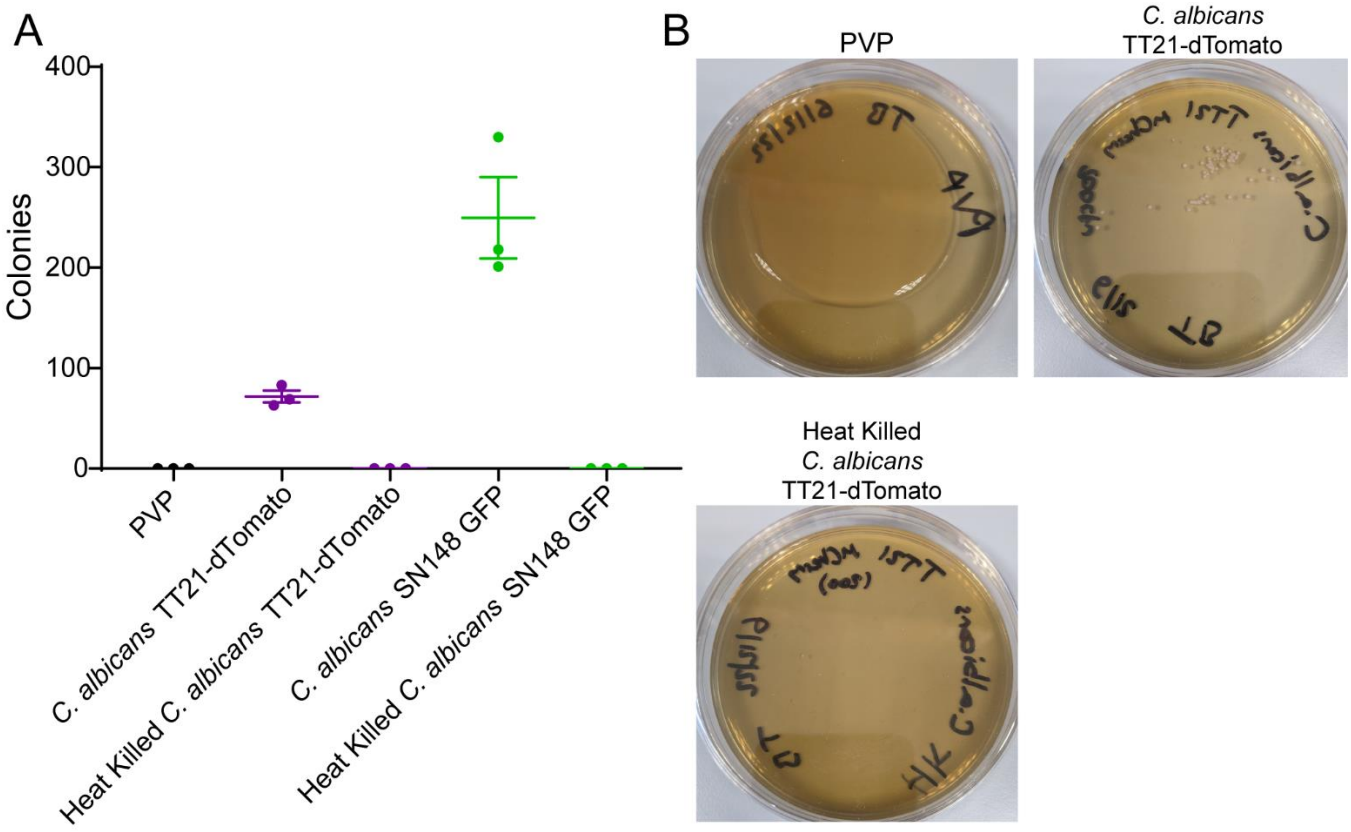

Figure 3.4: *C. albicans* TT21-dTomato and *C. albicans* SN148 GFP is heat killed at 60° C.

(A) Heat killing protocol was validated by microinjection of 200 cfu *C. albicans* TT21-dTomato, heat killed *C. albicans* TT21-dTomato, 500 cfu *C. albicans* SN148 GFP or heat killed *C. albicans* SN148 GFP. PVP was included as a negative control. N=3 independent experiments. No statistics performed.

(B) Representative images of PVP, 200 cfu *C. albicans* TT21-dTomato and heat killed *C. albicans* TT21-dTomato following 48 hours incubation on YPD media at 28 °C.

With a validated method of heat killing *C. albicans*, I next sought to examine the effect of heat killed *C. albicans* on RNS levels *in vivo*. 1 dpf *Tg(lyz:nfsB.mCherry)* zebrafish were infected with 500 cfu *C. albicans* SN148 GFP or heat killed *C. albicans* SN148 GFP into the caudal vein to generate a systemic infection. PVP was used as a mock injection control, while live *C. albicans* functioned as a positive control. At 24 hpi, zebrafish were fixed in 4% PFA and stained with an anti-nitrotyrosine antibody as an indirect measure of RNS.

Infection with *C. albicans* SN148 caused a significant decrease in anti-NT levels compared to PVP injected fish, from 5.17 in PVP controls to 0.78 in *C. albicans* SN148 infected zebrafish (Figure 3.5; $p<0.0001$). Heat killed *C. albicans* SN148 caused a 43.3% decrease in anti-NT compared to PVP (Heat

killed *C. albicans* SN148=2.93; $p<0.0001$). Anti-NT levels produced by embryos infected with heat killed *C. albicans* SN148 GFP were greater than anti-NT levels following live *C. albicans* SN148 GFP infection: approximately 3.7-times larger ($p<0.0001$). Thus, heat killed *C. albicans* caused an intermediate degree of RNS suppression, which may suggest a combination of active (living *C. albicans*-dependent) and passive mechanisms of RNS suppression.

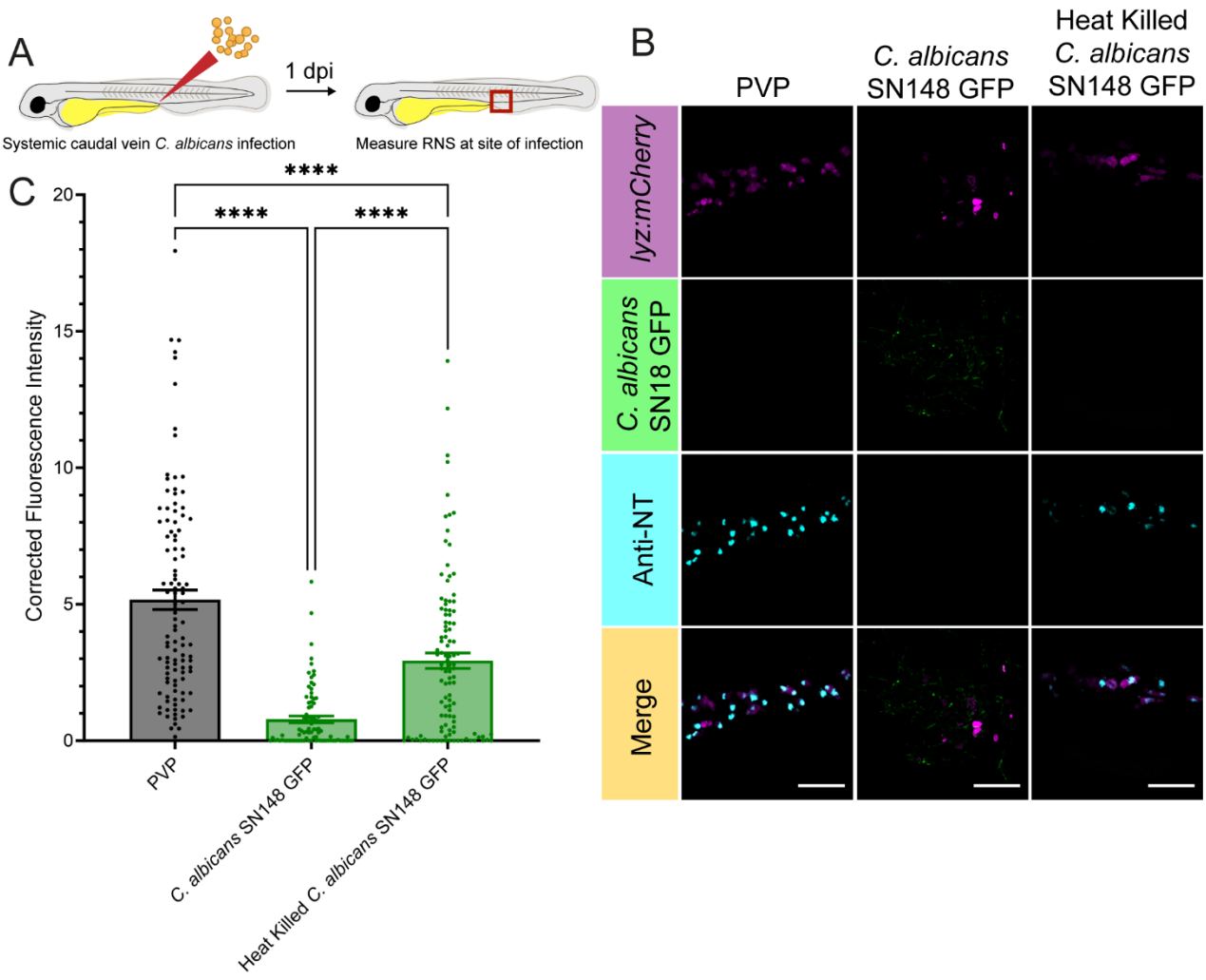

Figure 3.5: *C. albicans* SN148 GFP suppresses neutrophil RNS *in vivo*.

(A) 1 dpf zebrafish were injected with PVP, *C. albicans* SN148 GFP or heat-killed *C. albicans* SN148 GFP 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 haematopoietic tissue (CHT) and anti-nitrotyrosine fluorescence was quantified.

(B) Anti-nitrotyrosine fluorescence at 24 hpi following injection of PVP, live *C. albicans* TT21-dTomato or heat-killed *C. albicans* TT21-dTomato into the caudal vein at 30 hpf. N=84-108 neutrophils from 14-18 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, then Dunn's multiple comparisons test. P values shown: ****p<0.0001.

(C) Representative images of PVP, *C. albicans* SN148 GFP and heat killed *C. albicans* SN148 GFP infected zebrafish embryos at 24 hpi. Scale bars = 50 µm.

Anti-NT staining was repeated with in zebrafish embryos injected with live and heat killed *C. albicans* TT21-dTomato. *C. albicans* TT21-dTomato decreased anti-NT levels to 0.52, compared to 5.17 in uninfected, PVP controls, representing a 90.0% decrease (Figure 3.6; $p<0.0001$). Heat-killed *C. albicans* TT21-dTomato caused a 52.2% decrease in anti-NT compared to PVP (PVP=5.17, heat-killed *C. albicans* TT21-dTomato=2.47; $p<0.0001$). Anti-NT levels with live *C. albicans* were 79.0% lower than anti-NT levels with heat killed *C. albicans* TT21-dTomato ($p<0.0001$). Heat-killed *C. albicans* TT21-dTomato and heat killed *C. albicans* SN148 GFP both caused approximately a 50% decrease in neutrophil anti-NT levels, suggesting no differences exist between the ability of each *C. albicans* strain to suppress neutrophil RNS levels. Because no significant differences in RNS depletion exists between strains, *C. albicans* TT21-dTomato was selected as the default strain for future experiments as it is more widely used and better characterised than *C. albicans* SN148 GFP (Seman *et al.*, 2018).

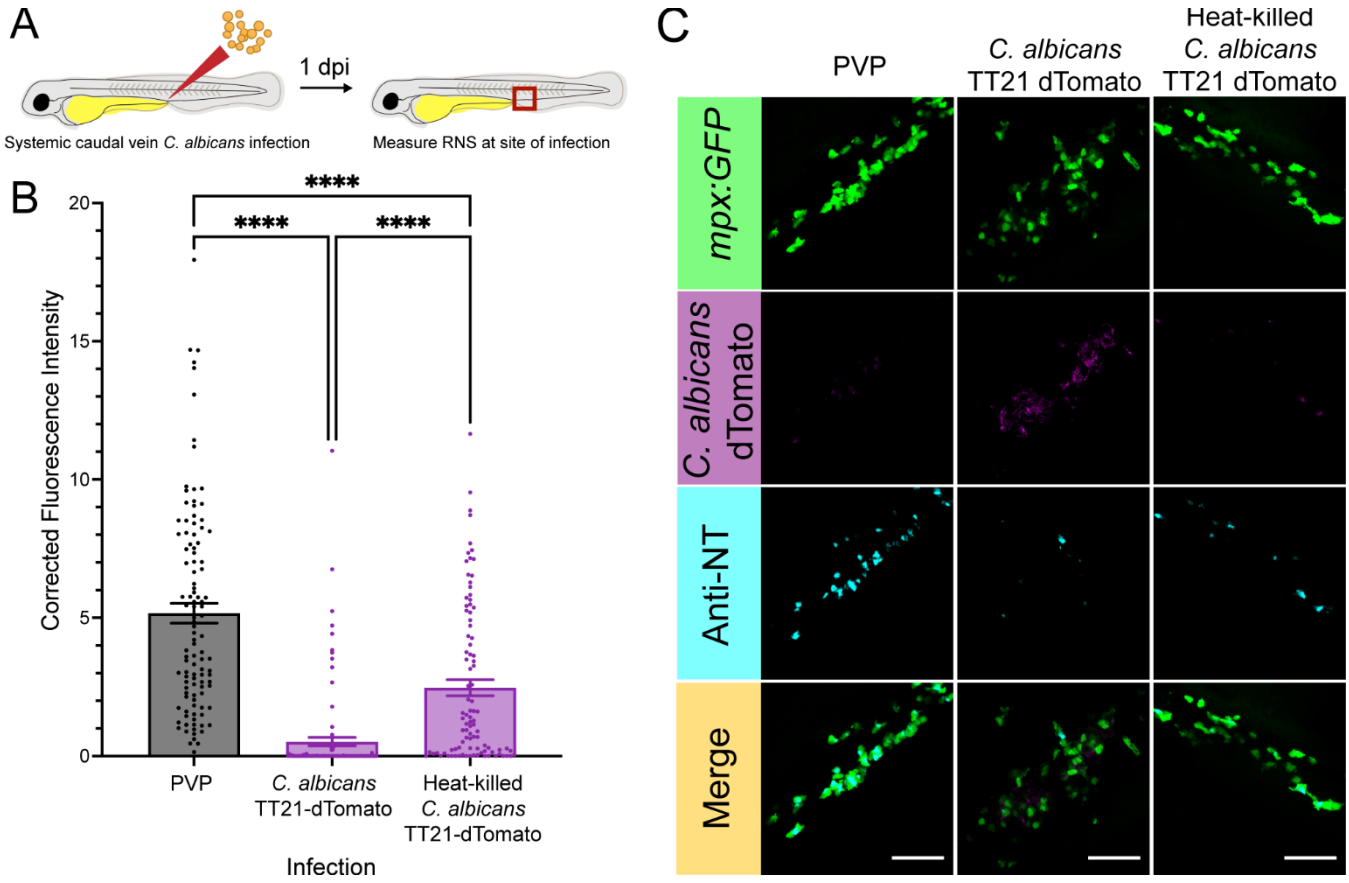

Figure 3.6: *C. albicans* TT21-dTomato suppresses neutrophil RNS production *in vivo.*

(A) 1 dpf zebrafish were injected with PVP, *C. albicans* TT21-dTomato or heat-killed *C. albicans* TT21-dTomato 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 haematopoietic tissue (CHT) and anti-nitrotyrosine fluorescence was quantified.

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

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

3.2.3 Suppression of neutrophil RNS occurs proximally and distally to *C. albicans* infection

Heat killed *C. albicans* causes an intermediate degree of neutrophil RNS suppression, suggesting RNS suppression by *C. albicans* is partially dependent on active processes in living *C. albicans*. I hypothesised that *C. albicans* may be secreting or releasing a factor to cause RNS suppression, which may be an active or passive process. Based on this, I reasoned that secretion of a factor by *C. albicans* could lead to an RNS suppression phenotype at distal sites. To investigate this hypothesis, I used a localised hindbrain ventricle infection model. 1 dpf *Tg(mpx:GFP)* zebrafish embryos were injected with 20 cfu live *C. albicans* TT21-dTomato, heat killed *C. albicans* TT21-dTomato or boiled *C. albicans* TT21-dTomato into the hindbrain ventricle. While heat killed *C. albicans* will have intact proteins, boiled *C. albicans* will have predominantly denatured proteins (Kim *et al.*, 2000). PVP was used as a mock injection control. RNS production was measured at 24 hpi, both at the site of infection in the hindbrain and at a secondary distal location in the caudal haematopoietic tissue (CHT), where no *C. albicans* infection was present (Figure 3.7A).

PVP injected embryos had a basal anti-NT level in neutrophils in the hindbrain and the CHT (Figure 3.7B,C). Anti-NT levels in the hindbrain and CHT were not statistically different, suggesting there is no difference in basal RNS in neutrophils in different tissues. Live *C. albicans* TT21-dTomato caused a significant decrease in anti-NT levels in both proximal neutrophils in the hindbrain (PVP=2.38, C. albicans=0.52; $p<0.0001$) and distal neutrophils in the CHT (PVP=2.59, C. albicans=0.71; $p<.0001$) compared to PVP control (Figure 3.7B,C). This was a 78.2% reduction in anti-NT levels in neutrophils in the hindbrain and 72.6% reduction in anti-NT levels in neutrophils in the CHT, revealing a similar magnitude of effect proximally and distally by live *C. albicans.* Hence, live *C. albicans* is capable of causing both a proximal and distal suppression of neutrophil RNS, suggestive of the influence of a secreted factor. Heat killed *C. albicans* TT21-dTomato caused a 40.1% reduction in anti-NT levels, compared to PVP, in the hindbrain (Figure 3.7B,C; $p<0.05$). This was a reduction in anti-NT levels significantly less than caused by live *C. albicans* TT21-dTomato infection ($p<0.05$), demonstrating the intermediate RNS suppression phenotype. Heat killed *C. albicans* TT21-dTomato infection caused anti-NT levels in the CHT that were not significantly different from distal anti-NT levels in mock infected larvae, suggesting that heat killed *C. albicans* TT21-dTomato had no effect on RNS in distal neutrophils. This may further imply a secreted factor by live *C. albicans* is responsible for RNS downregulation. Neutrophil anti-NT levels with boiled *C. albicans* TT21-dTomato were 2.23 in the hindbrain, which was not significantly different from embryos injected with PVP. In the CHT, neutrophil anti-NT levels were 2.39 with boiled *C. albicans* TT21-dTomato, showing no difference in anti-NT levels in proximal or distal neutrophils. The absence of RNS suppression by boiled *C. albicans* at both the proximal and distal site imply that the hypothesised secreted factor may be a protein,

which does not function once denatured. Together, these results suggest that *C. albicans* may have multiple mechanisms of neutrophil RNS suppression: a secreted protein that is able to act distally and a secondary, passive mechanism of RNS suppression responsible for proximal RNS suppression by heat killed *C. albicans*.

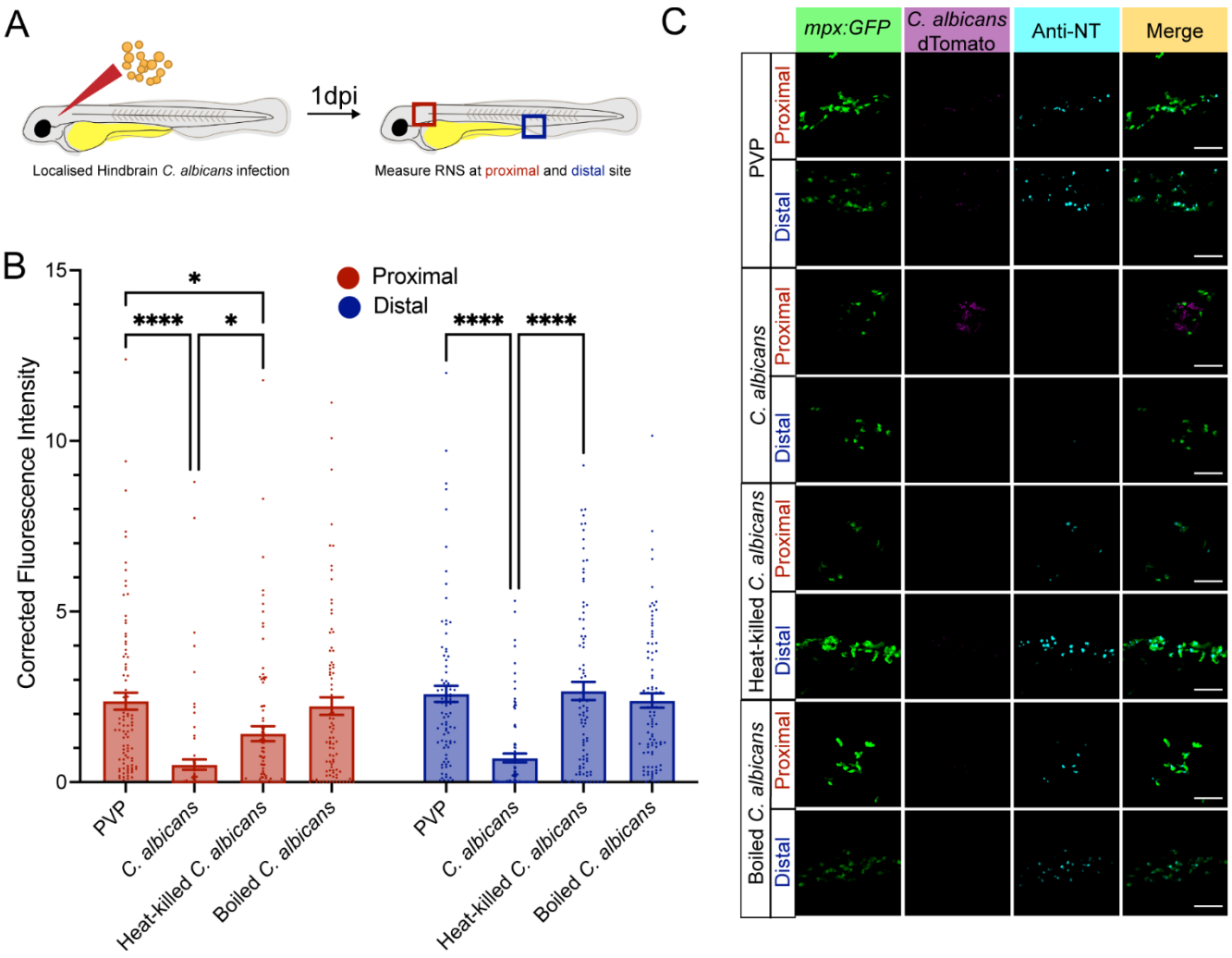

Figure 3.7: *C. albicans* suppression of RNS occurs proximally and distally to site of infection.

(A) 1 dpf zebrafish were injected with PVP, *C. albicans* TT21-dTomato, heat-killed *C. albicans* TT21-dTomato or boiled *C. albicans* TT21-dTomato, into the hindbrain ventricle. At 24 hpi, embryos were fixed and stained with anti-nitrotyrosine primary antibody and goat-anti-rabbit Alexa-633 secondary antibody. Zebrafish were then imaged at the hindbrain and CHT regions and anti-nitrotyrosine fluorescence was quantified.

(B) Anti-nitrotyrosine fluorescence at 24 hpi following injection of PVP, *C. albicans* TT21-dTomato, heat-killed *C. albicans* TT21-dTomato or boiled *C. albicans* TT21-dTomato into the hindbrain ventricle. N=90 neutrophils from 15 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by 2-way ANOVA, then Sidák's multiple comparisons test. ns=not significant, *p<0.05, ****p<0.0001

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

3.3 Discussion

In this chapter, I observed neutrophil RNS suppression by *C. albicans* using a zebrafish *in vivo* infection model, bringing light to a fungal strategy for modulating host innate immunity. RNS are a group of highly potent antimicrobial compounds, including nitric oxide, nitrogen dioxide and peroxynitrite, that are typically vital for the innate immune response to pathogens (Warris and Ballou, 2019). Evolution as a commensal fungus in a variety of human niches has led to *C. albicans* developing a range of stress responses (Brown *et al.*, 2014). Neutrophil RNS suppression could be the latest example of a *C. albicans* protective behaviour that has a subsequent impact on virulence in the host.

I demonstrated that *C. albicans* can suppress neutrophil RNS production *in vivo*. This supports previous *in vitro* literature (Chinen *et al.*, 1999; Schröppel *et al.*, 2001; Collette, Zhou and Lorenz, 2014). Collette *et al.* showed *C. albicans* suppresses NO production by macrophages *in vitro* (Collette, Zhou and Lorenz, 2014). Collette and colleagues observed approximately a 90% decrease in NO synthesis by RAW264.7 macrophages and bone marrow derived macrophages at a 0.1:1 *Candida*:macrophage ratio, while Chinen *et al.* saw roughly a 65% decrease in macrophage RNS levels at the same *Candida*:macrophage ratio. However, the authors looked at different time points: Collette *et al.* measured nitrite concentration after 24 hours of coculture (the same time point I looked at), while Chinen *et al.* measured nitrite after 48 hours of *C. albicans* exposure. The lower level of RNS suppression at the later time point used by Chinen *et al.* could suggest recovery of the macrophage RNS response over time, or simply be the result of biological variance and the different *C. albicans* strain and murine macrophages used. Across different *C. albicans* strains and different experiments, I observed between a 78% and a 90% decrease in neutrophil RNS levels *in vivo*; Ffion Hammond previously reported a 64% reduction in neutrophil RNS levels in *C. albicans* infection *in vivo* (Hammond (thesis), 2022). Due to the nature of *in vivo* infection, it is not possible to accurately estimate the *Candida*:immune cell ratio for comparison with the *in vitro* literature. My *in vivo* observations saw similar percentage decreases in RNS levels to Collette *et al.*, while Dr Hammond observed similar RNS suppression to Chinen *et al.*, suggesting *C. albicans* can efficiently suppress RNS levels both *in vitro* and *in vivo*. While previous publications have highlighted RNS suppression in macrophages, my data demonstrates this effect in neutrophils. Human neutrophils produce higher levels of RNS than macrophages (Muijsers *et al.*, 2001), as is the case in zebrafish larvae (Elks *et al.*, 2013), suggesting my findings may better reflect the human RNS response to *C. albicans,* making zebrafish a useful *in vivo* model to investigate the neutrophil immune response in these difficult to study, short-lived cells.

Suppression of macrophage ROS by *C. albicans in vitro* has been shown elsewhere (Frohner *et al.*, 2009; Wellington, Dolan and Krysan, 2009). Formation of RNS and ROS occurs via separate enzymatic pathways (Warris and Ballou, 2019), so the ability of *C. albicans* to modulate both of these highly potent antimicrobial effectors suggests a plethora of immunosuppressive strategies that *C. albicans* has developed. This hypothesised redundancy of evasion strategies could ensure that loss of any single pathway does not overly impact *C. albicans* virulence.

Heat-killed *C. albicans* was revealed to have an intermediate effect on neutrophil RNS levels, reducing RNS compared to uninfected controls, but not to the same extent as live *C. albicans*. This finding conflicts with Chinen *et al.*, who did not observe any suppression of macrophage NO levels by heat-killed *C. albicans* after 48 hours of exposure (Chinen *et al.*, 1999). As heat killed *C. albicans* cannot replicate, it is possible that macrophages were able to clear sufficient numbers of heat killed *C. albicans* within 48 hours, allowing macrophage NO production to recover. Chinen *et al.* do not make any mention of potential clearance of live or heat killed *C. albicans* in their experiments. Collette *et al.* observed a 10-20% suppression of macrophage NO by heat killed *C. albicans* after 24 hours, which was independent of the *Candida*:macrophage ratio (Collette, Zhou and Lorenz, 2014). This is substantially lower than the 43-52% reduction in neutrophil RNS levels that I observed *in vivo* from heat killed *C. albicans*. I validated my method of heat killing, showing no fungal growth after 48 hours culture of heat killed *C. albicans*, suggesting the difference in effect size is not caused by supposedly heat killed *C. albicans* actually being alive. Collette *et al.* used a similar protocol for heat killing (incubation at 65 °C for 20 minutes) so this is also unlikely to be a cause of difference.

Furthermore, I showed *C. albicans* suppression of neutrophil RNS occurs both proximally at the site of infection and also at distal sites, where no infection was observed. Investigations using *in vitro* models have been unable to fully answer questions about host-*C. albicans* interactions in the context of a whole organism. Previous work has suggested direct contact between *C. albicans* and macrophages is required for suppression of macrophage NO, with *C. albicans* having no effect on macrophage NO production when separated by a Transwell barrier (Collette, Zhou and Lorenz, 2014). The findings of this thesis do not preclude a suppression effect from direct contact between *C. albicans* and neutrophils, but the evidence strongly suggests that neutrophils with depleted RNS do not need to have phagocytosed *C. albicans*. To integrate these conclusions, I hypothesise direct contact between *C. albicans* and neutrophils triggers secretion of a protein by *C. albicans*, which is able to spread via the circulation and have an immunosuppressive effect on proximal and distal neutrophils. The *in vivo* zebrafish infection model suggests that *C. albicans* has a secreted mechanism of RNS suppression, which is capable of having effects large distances (millimetres) across an organism. A caveat of these conclusions is that it is possible that live *C. albicans* could have

disseminated from the hindbrain to the CHT, suppressed neutrophil RNS, and been phagocytosed and degraded prior to fixation and imaging, with the suppressive effect remaining after *C. albicans* was cleared. *C. albicans* has been shown to be capable of disseminating from the hindbrain in low amounts by 24 hpi, with disseminated *C. albicans p*rimarily observed in dorsal regions (Brothers, Newman and Wheeler, 2011). The authors did not quantify how often dissemination occurs, preventing comparison with my data. In my experiments, a minority of *C. albicans* hindbrain infections had disseminated to distal sites and zebrafish with any observable disseminated *C. albicans* were not included for analysis of proximal and distal neutrophil RNS. However, this does not preclude the possibility of disseminated *C. albicans* being cleared prior to observation. It is, however, unlikely that a small amount of disseminated *C. albicans* would be solely responsible for such a large reduction (72.6% decrease compared to PVP) in distal neutrophil RNS. The zebrafish hindbrain ventricle becomes sealed after 48 hpf (Kimmel *et al.*, 1995; Haddon and Lewis, 1996), so this experiment could be repeated at a later time point to ensure dissemination within 24 hours is not possible, though this would likely impact the recruitment of neutrophils to the hindbrain. Alternatively, neutrophils recruited to *C. albicans* infection in the hindbrain could have reverse migrated to the CHT, giving the appearance of a distal effect on RNS levels. Separate populations of neutrophils in the zebrafish head and CHT regions have previously been reported, though more contemporary investigations suggested both populations of neutrophils are equally likely to be recruited to the site of inflammation, regardless of location (García-López *et al.*, 2023). Time lapse imaging of neutrophil migration to and from the hindbrain in *C. albicans* infection would reveal whether reverse migration is occurring.

Heat killed *C. albicans* had an intermediate effect on RNS levels of proximal neutrophils but caused no suppression of neutrophil RNS levels distally. Heat killed *C. albicans* has mostly intact cell surface components, however, it is unable to secrete proteins, carry out active processes, such as transcription or translation. Thus, neutrophil RNS suppression by heat killed *C. albicans* is likely caused by a cell surface component that is constitutively part of the cell surface prior to exposure to host cells. Chitin is a main structural component of the *C. albicans* cell wall and has previously been associated with reduced NO production, so could be responsible for this effect (Gow, Latge and Munro, 2017; Wagener *et al.*, 2017). The greater RNS suppression caused by live *C. albicans* may then be the result of an additional secreted protein, potentially triggered by direct contact with host immune cells. An alternative explanation could be that heat killing is partially denaturing fungal cell surface proteins, reducing the activity of the hypothesised secreted protein. Alternatively, heat killing may be damaging the fungal cell wall, resulting in exposure of chitin, which is known to decrease RNS production by inducing host arginase-1 activity (Wagener *et al.*, 2017). Boiled *C.*

albicans had no effect on neutrophil RNS levels in proximal or distal neutrophils. Boiling causes denaturation of proteins. Hence, it is likely to be a protein that is responsible for the proximal and distal suppression of neutrophil RNS. An alternate explanation could be that the hypothesised factor is a non-protein component of the cell wall, which is similarly disrupted by heat-killing and boiling. A non-protein cell wall component may be shed, as opposed to secreted, to impact host neutrophil RNS levels proximally and distally.

The intermediate phenotype of RNS suppression by heat killed *C. albicans* could hint at multiple mechanisms of neutrophil RNS suppression by *C. albicans*: a secreted protein that causes proximal and distal RNS suppression and a cell surface protein that causes a lesser degree of proximal RNS suppression. Current literature supports the idea of multiple mechanisms of immune suppression by *C. albicans*. *C. albicans* Sod4 and Sod5 has been associated with degradation of antimicrobial ROS *in vitro* (Frohner *et al.*, 2009). Sod4/5 are Superoxide Dismutases (SODs), GPI-anchored cell wall proteins. *C. albicans sod4Δ* and *sod5Δ* showed an accumulation of ROS in BMDMs and were hypersusceptible to killing by BMDMs. The authors revealed Sod4/5 degrade extracellular ROS, without impacting intracellular BMDM ROS, highlighting a defence mechanism by *C. albicans*, as opposed to a hostile virulence factor designed to antagonise host cells. Yhb1 is suggested to similarly detoxify NO, with *C. albicans yhb1Δ/yhb1Δ* mutants being hypersensitive to killing by DPTA NONOate *in vitro* compared to wild type strains (Hromatka, Noble and Johnson, 2005). *C. albicans* strains were exposed to DPTA NONOate, as a pharmacological proxy of endogenous NO produced by immune cells, to allow precise control of NO concentration. The *C. albicans yhb1Δ/yhb1Δ* mutant had significantly reduced virulence in a mouse infection model. However, *C. albicans yhb1Δ/yhb1Δ* infection had equal virulence in NOS2 knockout mice (unable to produce NO) as wild type mice, suggesting Yhb1 has an additional role in virulence beyond detoxification of NO (Hromatka, Noble and Johnson, 2005).

Chitin has also been implicated in modulation of host immune responses. *C. albicans* chitin was demonstrated to induce arginase-1 expression and arginase activity in human monocyte-derived macrophages *in vitro* (Wagener *et al.*, 2017). Arginase is a ureohydrolase enzyme that competes with iNOS for a substrate and negatively regulates iNOS activity (see 4.1.3 Arginase in immunity; Takeda *et al.*, 2010). Arginase-1 expression was measured by immunoblotting, while arginase activity was measured using a standard assay kit, based on conversion of arginine to urea. Purified chitin alone caused a comparable increase in arginase activity to *C. albicans*, suggesting chitin is the primary inducer of host arginase in human macrophages. In contrast, chitin alone caused a modest decrease in macrophage NO synthesis compared to live *C. albicans* (Wagener *et al.*, 2017), suggesting that chitin-mediated induction of host arginase is not the only mechanism of macrophage

NO suppression. Flow cytometry of human macrophages exposed to chitin revealed a polarisation towards an anti-inflammatory M2 phenotype that was not observed following exposure to untreated *C. albicans* (Wagener *et al.*, 2017). Hence, chitin promotes host arginase activity, resulting in a shift to an anti-inflammatory phenotype. Presumably, the pro-inflammatory population of macrophages is maintained after exposure to whole *C. albicans* due to the induction of pro-inflammatory cytokines by detection of PAMPs. The above papers implicate proteins and polysaccharides that are components of, or are bound to, the *C. albicans* cell surface. Collette *et al.* concluded macrophage NO suppression was being driven by an unknown secreted compound (Collette, Zhou and Lorenz, 2014). The hypothesised compound was suggested to be < 3 kDa, aqueous and heat-stable, though the authors were not able to propose the identity of this compound. The diversity of proteins and genes associated with decreased RNS production correlates with my hypothesis that multiple mechanisms of host RNS suppression exist. Further investigation could aim to establish which, if any, mechanism contributes the most to host RNS suppression, and if any mechanisms can be targeted to abrogate the suppressive effect by *C. albicans*.

Suppression of neutrophil RNS in the CHT points towards a secreted compound that is able to spread around the body, potentially via the circulatory system. The requirement for circulation could be confirmed in zebrafish larvae using a *tnnt2a* morpholino, which results in non-contractile embryonic hearts, so prevents blood flow (Sehnert *et al.*, 2002). If distributed by the circulatory system, there is the potential to detect and measure presence of a secreted compound in the blood. Pathogen-specific secreted compounds are a rich source for potential biomarkers of infection (Kalita *et al.*, 2023). Identification of a novel biomarker, specific to *C. albicans* infections, could help to meet the urgent need for superior methods of diagnosing candidiasis (WHO, 2022). To identify potential diagnostic biomarker candidates, Vaz *et al.* used mass spectrometry to analyse the *C. albicans* hyphal secretome (Vaz *et al.*, 2021). 301 hyphal secreted proteins were identified, of which 7 were categorised as highly immunoreactive and potential antibody-based diagnostic biomarker candidates. More work is needed to validate these candidates as diagnostic biomarkers. Of the 301 identified proteins, 111 have not previously been described as being part of the hyphal secretome and some were previously described as being cell surface proteins (Vaz *et al.*, 2021). Examination of the secretome produced by Vaz and colleagues may help point towards candidates involved in RNS suppression. Further research into the specific molecule responsible for suppressing neutrophil RNS distally is required to determine its suitability to act as a biomarker of *C. albicans* infection.

The observation of distal neutrophil RNS suppression raises new questions about the potential role of *C. albicans* in the development of polymicrobial co-infections in immunocompromised patients. *C.*

albicans is a common commensal fungus in the gut (Kumamoto, Gresnigt and Hube, 2020). Suppression of distal neutrophil RNS by a compound secreted by *C. albicans* in the gut could, hypothetically, allow other pathogens to establish an infection. There is evidence of commensal microorganisms having systemic effects. Trimethylamine N-oxide metabolites produced by the gut microbiota have been implicated in chronic kidney diseases and progression of coronary artery disease (Wilson Tang *et al.*, 2015). CsgA secreted by *Escherichia coli* in the gut of mice forms CsgA aggregates extracellularly, helping to form biofilms and mediating adhesion to epithelial cells. CsgA secretion also promotes aggregation of host αSyn, which is associated with neurodegenerative disorders, such as Parkinson's disease (Sampson *et al.*, 2020). Regarding immunity, *Bacteroides fragilis* produces polysaccharide A, which binds to TLR2 on regulatory T cells, suppressing T_H17 cell activity and promoting immune tolerance (Round *et al.*, 2011; Ivanov and Honda, 2012). There is evidence that already points towards *Candida* spp. in the gut microbiome having distal effects. *Candida*-derived prostaglandin E2 (PGE2) is able to downregulate IL-18 production by bronchial epithelial cells *in vitro* and downregulate TNF-α production by murine splenocytes *in vitro* (Noverr *et al.*, 2001). Kim *et al.* later demonstrated fungal PGE2 is able to reach the lung, via the bloodstream, in a mouse model and drive murine lung macrophages into an M2, anti-inflammatory phenotype *in vivo* (Kim *et al.*, 2014). Gut *Candida*-derived PGE2 has further been associated with development of allergic inflammation in the lung (Kim *et al.*, 2014; Underhill and Iliev, 2014). The *C. albicans* quorum sensing molecule farnesol has been shown to modulate human immune cell phenotype *in vitro*. Farnesol increased serine palmitoyltransferase activity and inhibited dihydroceramide desaturase in human monocyte-derived dendritic cells *in vitro*, resulting in altered sphingolipid composition in dendritic cell membranes. This resulted in impaired mitochondrial respiration, reduced ability of dendritic cells to secrete IFN-β and reduced ability to induce IFN-γ production by T cells, demonstrating an immunosuppressive effect by *C. albicans* (Batliner *et al.*, 2024). Together, these examples demonstrate that systemic immunomodulation by a component of the microbiome, including *C. albicans*, is theoretically possible. The data in this chapter reveals suppression of distal neutrophil RNS. Additional work is required to characterise whether this is systemic immunosuppression and the biological significance of distal RNS suppression for the innate immune system's ability to control other pathogens.

In conclusion, *C. albicans* is able to suppress neutrophil RNS levels in a zebrafish *in vivo* infection model. This result marks the first time suppression of host RNS by *C. albicans* has been shown *in vivo*, highlighting that this observation is not solely an *in vitro* event. Use of the zebrafish *in vivo* model allows wider questions to be asked about the biological significance of RNS suppression, within the context of a complex host system. Heat killed *C. albicans* caused an intermediate level of

neutrophil RNS suppression. Live *C. albicans* was able to suppress neutrophil RNS levels at distal neutrophils, implying the presence of a secreted factor. Future work to identify the secreted factor could involve mass spectrometry of *C. albicans* secreted proteins and shed factors. However, this was deemed not feasible in this model, due to the technical difficulty of zebrafish larvae exsanguination to obtain secreted factors that enter the host circulatory system. Further questions remain about the mechanisms underlying *C. albicans* suppression of neutrophil RNS, which are more suitable for investigation with the zebrafish *in vivo* model, and whether this is a clinically relevant observation.

4. Investigating the mechanisms underlying neutrophil RNS suppression by *C. albicans in vivo*

4.1 Introduction

4.1.1 Host factors involved in *C. albicans* modulation of innate immunity

C. albicans can modulate host innate immunity, which is often mediated by binding of *C. albicans* proteins to host factors (see 1.5.5 *C. albicans* mechanisms of evading immunity and 3.1.1 *C. albicans* defences against RNS). Host TLR is a common target of *C. albicans* PAMPs (Figure 4.1). TLR2 was associated with anti-inflammatory IL-10 production and reduced pro-inflammatory IFNγ production by murine spleen cells exposed to *C. albicans* (Netea *et al.*, 2004). TLR2-mediated signals prolong the survival of Treg cells *in vitro*, which was associated with increased susceptibility to *C. albicans* infection in mice *in vivo* (Netea *et al.*, 2004). TLR2 knockout mice had increased survival in *C. albicans* infection compared to wild type mice, demonstrating how *C. albicans* modulation of host immunity via TLR2 has biologically significant impacts on host infection outcomes (Netea *et al.*, 2004). *C. albicans* chitin induces host IL-10 production via mannose receptor, TLR9 and NOD2, which was able to dampen LPS-induced inflammation in mice *in vivo* (Wagener *et al.*, 2014). *C. albicans* chitin also binds to host platelet TLR8, which decreases intracellular Ca^{2+} in platelets, leading to reduced platelet aggregation and reduced platelet adhesion to neutrophils and to *C. albicans* (Leroy *et al.*, 2019). Similarly, *C. albicans* β(1,3)-glucan suppressed platelet aggregation and platelet adhesion to neutrophils via TLR4 *in vitro* (Vancraeyneste *et al.*, 2016). These mechanisms of preventing platelet adhesion may facilitate *C. albicans* escape from immune cells (Vancraeyneste *et al.*, 2016; Leroy *et al.*, 2019).

Dectin-1 may also be targeted by *C. albicans* to enable modulation of host immunity. Binding of β(1,3)-glucan to Dectin-1 inhibited neutrophil migration from the bone marrow into circulation in mice *in vivo* via upregulation of PD-L1 (Y. Yu *et al.*, 2022). Induction of PD-L1 expression on macrophages by *C. neoformans* has been shown to suppress macrophage activation and promote T cell exhaustion (Shindo *et al.*, 2017; Che *et al.*, 2018). Whether *C. albicans* induction of PD-L1 on neutrophils has similar consequences for immune cell activity and exhaustion is unknown. Zebrafish do not have an exact homolog for Dectin-1, but do respond to stimulation by β(1,3)-glucan, with 2 putative CLRs identified that may carry out the role of Dectin-1: Sclra and Clec4c (Petit *et al.*, 2019; Glass, Robinson and Rosowski, 2025).

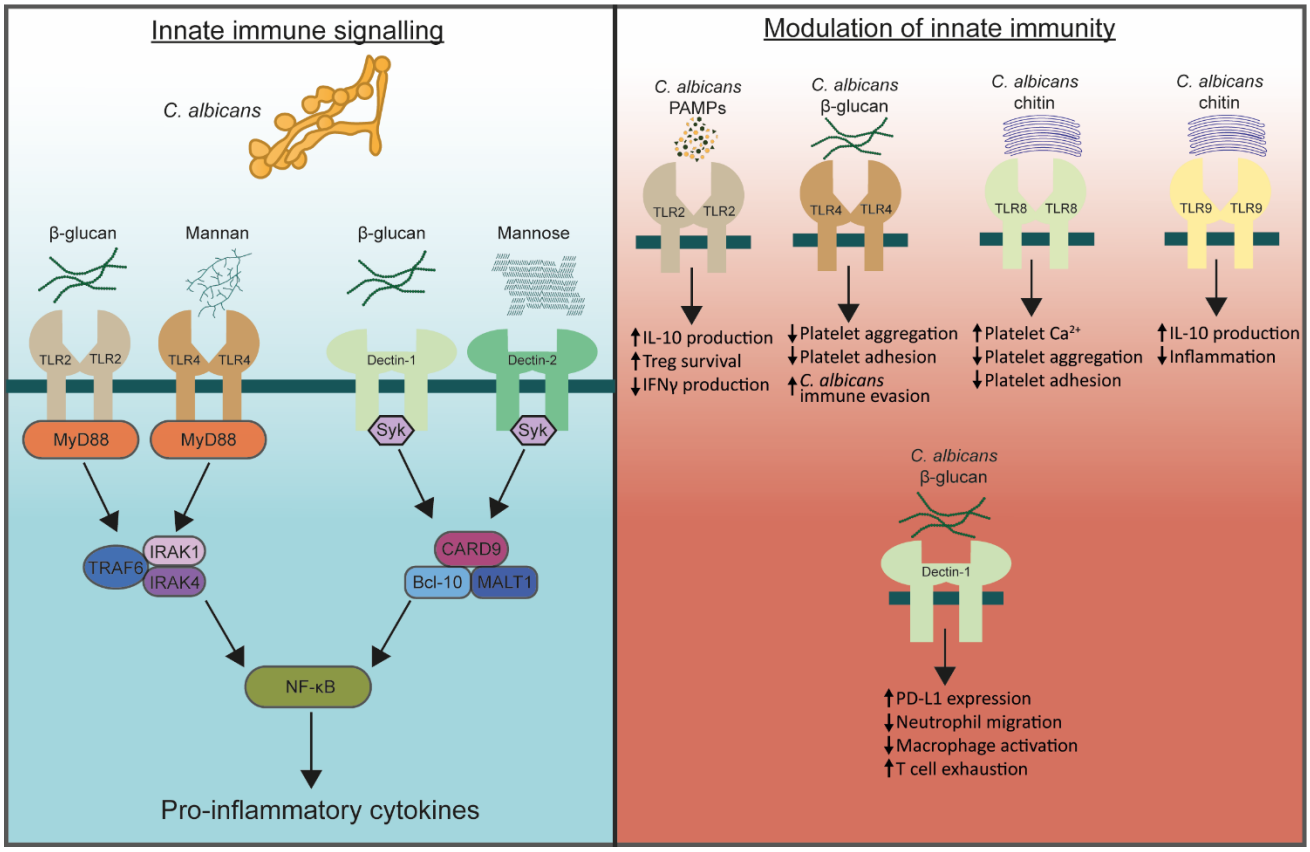

Figure 4.1: *C. albicans* modulation of host innate immune signalling

C. albicans PAMPs (such as β-glucan, mannan and mannose) are detected by TLRs and CLRs. TLR stimulation triggers a signalling cascade, via the MyD88 adaptor protein, resulting in activation of the NF-κB transcription factor and production of pro-inflammatory cytokines (Patin, Thompson and Orr, 2019). Dectin signalling operates via Syk and Card9 adaptor proteins, stimulating NF-κB transcription factor and production of pro-inflammatory cytokines. *C. albicans* PAMPs can also modulate host immune responses, resulting in production of anti-inflammatory cytokines, reduced platelet aggregation and adhesion, impaired neutrophil migration and reduced macrophage activation, all of which facilitate immune evasion by *C. albicans* (Netea *et al.*, 2004; Vancraeyneste *et al.*, 2016; Leroy *et al.*, 2019; Y. Yu *et al.*, 2022).

4.1.2 *C. albicans* hyphae

C. albicans hyphae are important virulence factors, with roles in tissue invasion, virulence and immune evasion (Sudbery, 2011). Upon initial phagocytosis of *C. albicans* yeast by macrophages, *C. albicans* undergoes an extreme transcriptional change, with an upregulation of nutrient acquisition processes, such as gluconeogenesis, and a downregulation of translation components (Lorenz, Bender and Fink, 2004). *C. albicans* yeast phagocytosis by neutrophils stimulated a different early transcriptional response, with *C. albicans* methionine and arginine synthetic pathways upregulated (Rubin-Bejerano *et al.*, 2003). The late *C. albicans* response to macrophage phagocytosis was to restore glycolysis metabolism and promote the yeast-to-hyphae morphological transition, to facilitate escape from the macrophage phagosome (Lorenz, Bender and Fink, 2004). *C. albicans* was shown to neutralise the acidic environment of the macrophage phagosome in RAW264.7 cells *in vitro*, via an stp2-dependent mechanism, which facilitated hyphae formation (Vylkova and Lorenz, 2014). *STP2* is a transcription factor that regulates expression of multiple amino acid permeases, which are vital for amino acid synthesis. Given this role, *STP2* could be upregulated in the early response to phagocytosis, to facilitate nutrient synthesis and neutralise phagosome pH, enabling transition to the hyphal morphology. To support this hypothesis, *C. albicans stp2Δ* was unable to form hyphae in acidic conditions *in vitro* and was unable to escape phagocytosis by RAW264.7 (macrophage-like) cells *in vitro* (Vylkova and Lorenz, 2014). *C. albicans* hyphae promoted fungal escape by inducing macrophage pyroptosis and by mechanical piercing of the macrophage membrane (Uwamahoro *et al.*, 2014). Hyphae alone are not sufficient for inducing macrophage pyroptosis: *C. albicans hog1Δ* had normal hyphae formation but was unable to induce pyroptosis of murine bone marrow derived macrophages (O'Meara *et al.*, 2018). Hog1 is a stress-activated protein kinase, which regulates *C. albicans* cell wall remodelling, suggesting Hog1-regulated cell wall components may be responsible for inducing macrophage pyroptosis (O'Meara *et al.*, 2018).

C. albicans hyphae are associated with expression of a specific subset of hyphae-specific genes, with 301 secreted hyphal proteins identified (Murad *et al.*, 2001; Sudbery, 2011; Vaz *et al.*, 2021). Hyphae also exhibit a different transcriptional response to stimulation by neutrophils compared to yeast (Niemiec *et al.*, 2017). Candidalysin (*ECE1*) is a hyphae-specific virulence factor. Polymerisation of candidalysin forms cytolytic pores, which insert into immune cell membranes to cause membrane permeabilisation, cell lysis and *C. albicans* escape (Olivier *et al.*, 2022; Russell *et al.*, 2022; Schaefer *et al.*, 2024). One of these hyphae-specific genes could be responsible for suppression of neutrophil RNS by *C. albicans.*

4.1.3 Arginase in immunity

Arginase is a ureohydrolase, which catalyses the conversion of L-arginine to L-ornithine and urea (Wu and Morris, 1998). There are 2 primary physiological functions for arginase: final step of ammonia detoxification by producing urea and production of ornithine as a precursor for proline and polyamine synthesis (Wu and Morris, 1998; Caldwell *et al.*, 2018). Ammonia detoxification is required to convert toxic ammonia into nontoxic, water-soluble urea, which can be easily excreted as a component of urine. Failure to detoxify ammonia results in hyperammonaemia, which can cause neurological impairment, liver toxicity and death (Deignan, Cederbaum and Grody, 2008). Proline is necessary for collagen formation, while polyamines have essential roles in cell growth, proliferation, wound healing and tissue repair (Lange *et al.*, 2004; Morris, 2009; Caldwell *et al.*, 2018).

Arginase competes with inducible nitric oxide synthase for a common substrate – L-arginine (Bansal and Ochoa, 2003; Takeda *et al.*, 2010). Arginase and iNOS negatively regulate each other through competitive inhibition and at a genetic level (Modolell *et al.*, 1995). Hence, induction of arginase activity results in inhibition of iNOS (and a subsequent reduction in RNS levels) and vice versa.

Humans have two isoforms of the arginase gene: *ARG1* and *ARG2* (Li *et al.*, 2022). There is approximately 60% sequence homology between *ARG1* and *ARG2*, with 100% homology in the catalytic domains (Vockley *et al.*, 1996). *ARG1* is primarily expressed in the liver, with its main function being ammonia detoxification (Li *et al.*, 2022). *ARG1* expression has been observed in the pancreas, skin, intestine and lung, though its function in these tissues is unknown (Hochstedler *et al.*, 2013). *ARG2* is highly expressed in the kidneys and pancreas, with lower levels of expression in other tissues, including the brain, spinal cord and intestines (Choi *et al.*, 2012). *ARG2* is believed to be an inducible isoform of arginase, involved in polyamine and proline synthesis and impacting on nitric oxide synthase activity (Vockley *et al.*, 1996). Arginase has been implicated in other cellular functions, such as cellular senescence, apoptosis and autophagy (Li *et al.*, 2022).

Arginase expression has been observed in immune cells. *ARG1* expression has been observed in murine macrophages and is considered an anti-inflammatory marker in these cells, owing to the role of arginase-expressing macrophages in wound repair (Campbell *et al.*, 2013; Pourcet and Pineda-Torra, 2013). Anti-inflammatory IL-10 induced *ARG2* expression in murine bone marrow derived macrophages *in vitro*, triggering a downregulation of HIF-1α and IL-1β (Dowling *et al.*, 2021). Hence, Arg2 is involved in resolution of inflammation in murine macrophages. Human neutrophils constitutively express *ARG1*, with arginase stored within neutrophilic granules until neutrophils are activated and arginase is released (Munder *et al.*, 2005; Jacobsen *et al.*, 2007). ARG1 is inactive

inside the granule but is activated by the change in pH following release into the extracellular environment via degranulation (Rotondo *et al.*, 2011). ARG1 secreted by human neutrophils was able to suppress T cell proliferation *in vitro*, demonstrating an immunosuppressive effect of neutrophil arginase (Rotondo *et al.*, 2011). Furthermore, cleavage of ARG1 by NETs increased the enzymatic activity of ARG1 *in vitro*, enhancing suppression of T cell proliferation (Canè *et al.*, 2023). Neutrophil arginase-1 release has also been linked to induction of apoptosis in cancer cells *in vitro* (García-Navas, Gajate and Mollinedo, 2021).

C. albicans produces its own arginase – *CAR1* (Schaefer *et al.*, 2020). While three genes were identified as putative arginase or arginase-like genes based on sequences, only one was confirmed as a true arginase (*CAR1*), with the other two believed to be agmatinase and guanidinobutyrase (ureohydrolase family members; Schaefer *et al.*, 2020). *CAR1* was revealed to be vital for arginine-induced hyphae formation *in vitro*, with *C. albicans car1Δ* having impaired hyphae formation on arginine-supplemented media but normal hyphae formation on ornithine- and urea-supplemented media (Schaefer *et al.*, 2020).

4.1.4 Hypothesis and aims

I demonstrated *C. albicans* suppresses neutrophil RNS in zebrafish *in vivo* (Chapter 3), corroborating observation of murine macrophage NO suppression by *C. albicans in vitro* (Collette, Zhou and Lorenz, 2014). However, the molecular mechanisms underlying this effect remain unclear: it is unknown what *C. albicans* product(s) are responsible for host RNS suppression or which host factors are targeted by *C. albicans* to drive RNS suppression. I investigated the mechanisms underlying neutrophil RNS suppression by *C. albicans*, with the hypothesis **"*C. albicans* suppression of neutrophil RNS is mediated through host pattern recognition receptors and is hyphae- and *C. albicans* arginase-dependent".**

To examine this hypothesis, the following aims were addressed:

- Investigate host factors involved in *C. albicans* suppression of neutrophil RNS, using morpholinos and CRISPR/Cas9 to knockdown host pattern recognition receptor adaptor proteins (*myd88, card9*)
- Examine the role of the hyphal morphotype on neutrophil RNS suppression
- Explore the host cytokine response to *C. albicans* infection *in vivo*
- Examine the role of *C. albicans* arginase on neutrophil RNS suppression

4.2 Results

4.2.1 Suppression of neutrophil RNS is not TLR-dependent

I aimed to investigate the mechanisms underlying *C. albicans* suppression of neutrophil RNS levels *in vivo*. To begin, I chose to explore host components that may be involved in neutrophil RNS suppression. Host cells have a range of PRRs that are used to detect *C. albicans* infections and instigate the immune response (Burgess, Condliffe and Elks, 2022). I hypothesised that a *C. albicans* protein may be exploiting host recognition pathways to reduce RNS levels.

TLR2-deficient murine macrophages have previously been demonstrated to have increased candidacidal activity (Netea *et al.*, 2004). Meanwhile, *Cryptococcus neoformans* has a mechanism of modulating macrophage behaviour and suppressing NO production, which is TLR4-dependent (Dang *et al.*, 2022). It is possible that *C. albicans* suppression of neutrophil RNS *in vivo* operates via a similar host TLR-dependent mechanism. To investigate this, a *myd88* e2i2 splice morpholino was used to knockdown host MyD88, disrupting TLR signalling (apart from TLR3, which acts independently of Myd88)(Fitzpatrick *et al.*, 2020).

Embryos injected with the control morpholino had a DNA band at approximately 520 bp (Figure 4.2B), demonstrating successful cDNA conversion and PCR amplification with the *myd88* primers. Embryos injected with *myd88* morpholino mostly lost the 520 bp DNA band, though a small band was still visible, indicating successful knockdown. A thicker band was observed that was approximately 600 bp. The *myd88* e2i2 splice blocking morpholino targets the splice site at the 3' end of exon 2, causing fusion of exon 2, intron 2 and exon 3, resulting in an altered sequence of greater size, as was observed previously and in my experiment (Bates *et al.*, 2007; Gaines, 2022). Embryos injected with *myd88* morpholino had a third DNA band present, roughly 650-700 bp, which was not observed in the reference image (Figure 4.2A).This band could have been caused by non-specific amplification of random segments of DNA, though this would not explain why the band was only observed with *myd88* morpholino.

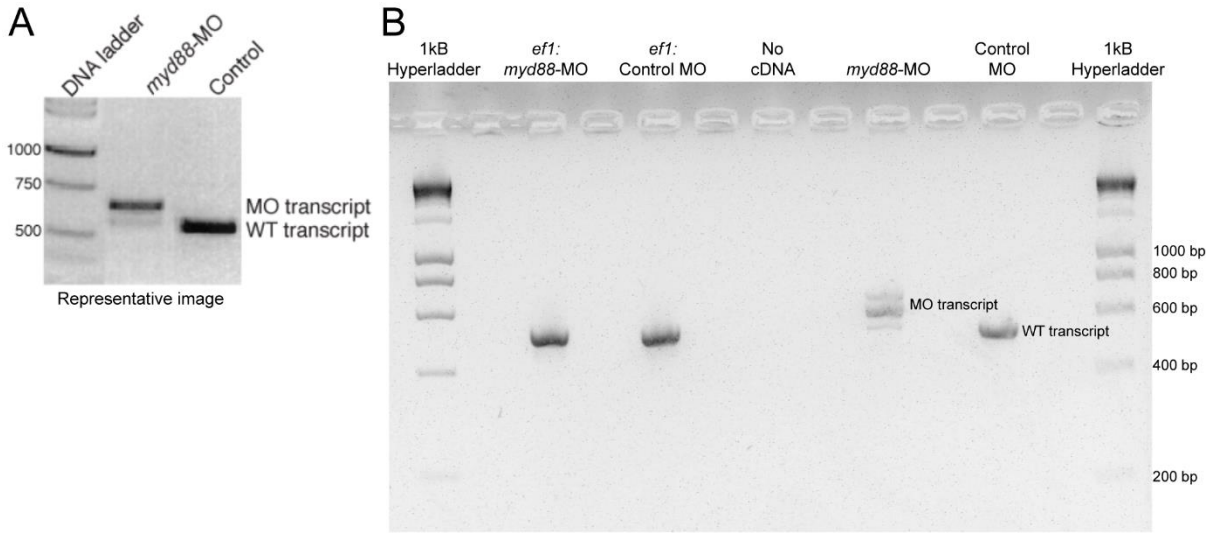

Figure 4.2: *myd88* e2i2 splice morpholino causes knockdown of host *myd88*.

(A) Expected band sizes for successful and unsuccessful knockdown of MyD88 with the *myd88* e212 morpholino. Taken from (Bates *et al.*, 2007).

(B) 2% TAE agarose gel of embryo cDNA injected with *myd88* morpholino or standard control morpholino. *Ef1* and no cDNA controls were included.

Having successfully demonstrated knockdown of host *myd88*, morpholino injections were repeated and 1 dpf *Tg(mpx:GFP)* i114 zebrafish were injected into the caudal vein with 200 cfu *C. albicans* TT21-dTomato or PVP. Fixation and anti-nitrotyrosine staining took place at 24 hpi.

With the control morpholino, PVP-injected embryos had a basal anti-NT levels, which were significantly reduced by *C. albicans* infection (Figure 4.3; $p<0.0001$). Embryos injected with *myd88* morpholino and PVP also had a basal anti-NT level, which was statistically similar to embryos injected with the control morpholino and PVP. Embryos injected with *myd88* morpholino and *C. albicans* had reduced anti-NT levels, which were significantly lower than control morpholino + PVP and *myd88* + PVP embryos ($p<0.0001$ for both). *C. albicans* infected embryos injected with control or *myd88* morpholino were not significantly different, suggesting host *myd88* does not play a role in RNS suppression by *C. albicans in vivo.*

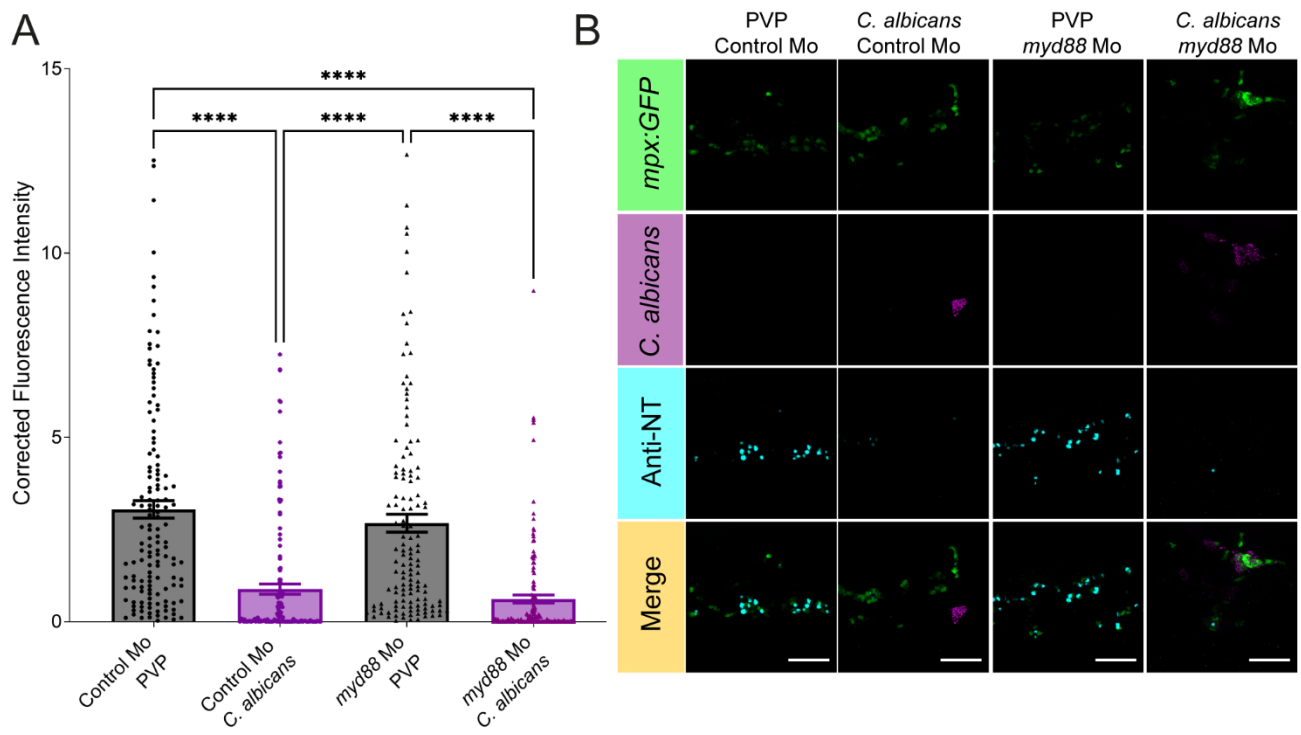

Figure 4.3: RNS suppression by *C. albicans* is not host *myd88*-dependent

(A) Anti-nitrotyrosine fluorescence at 24 hpi following injection of control or *myd88* morpholino and injection into the caudal vein of PVP or *C. albicans* TT21-dTomato. N=132-144 neutrophils from 22-24 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, with Dunn's multiple comparisons tests. P values shown: ****p<0.0001.

(B) Representative images of PVP or *C. albicans* TT21-dTomato infected embryos, injected with control or *myd88* morpholino, at 24 hpi. Scale bars = 50 µm.

While it does not appear to play a role in RNS suppression, host *myd88* may still be important in the anti-Candida immune response. To further investigate the significance of *myd88* in *C. albicans* infections, embryos injected with control or *myd88* morpholino were injected into the caudal vein with 200 cfu *C. albicans* TT21-dTomato.

Control morpholino embryos had 30.4% survival at 4 dpi, while *myd88* morpholino embryos had 35.6% survival at 4 dpi (Figure 4.4). There was no statistical difference between control and *myd88* morpholino survival, suggesting host *myd88* may not play a significant role in the early innate immune response to *C. albicans* infections *in vivo*.

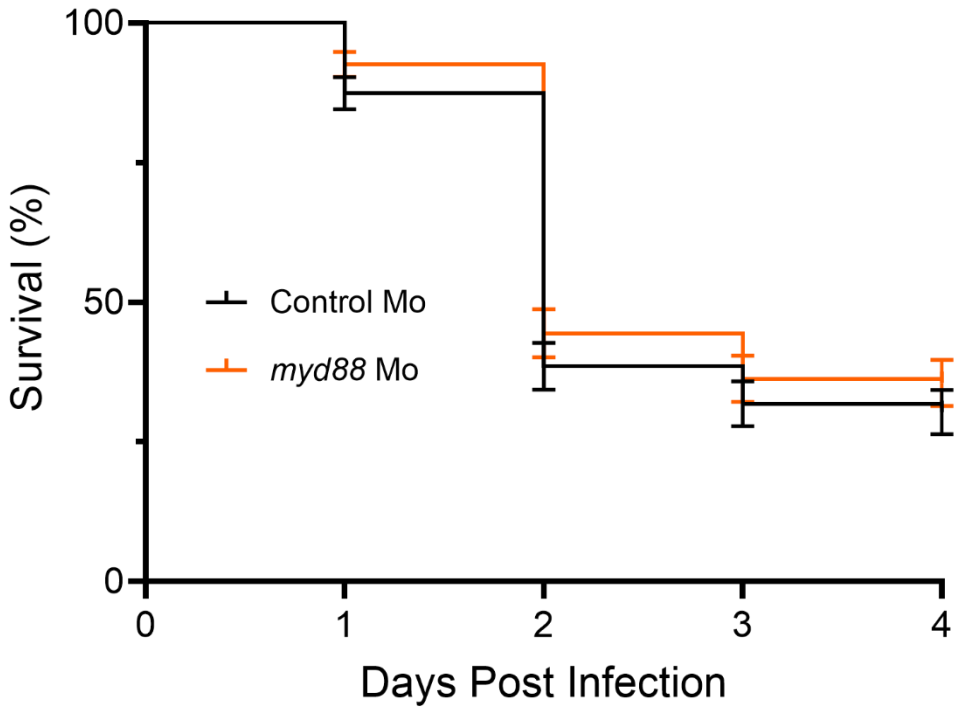

Figure 4.4: *myd88* morpholino has no effect on survival in *C. albicans* infection

1 cell stage nacre embryos were injected with control or *myd88* morpholino, then injected into the caudal vein with 200 cfu *C. albicans* TT21-dTomato at 1 dpf. Mortality was measured daily up to 4 dpi. n=135 fish, obtained from 3 independent experiments. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. No significant difference observed.

No link was identified between host *myd88* and RNS suppression by *C. albicans*, suggesting neutrophil RNS suppression by *C. albicans* is not host TLR-dependent. An alternative host factor that may be implicated in RNS suppression by *C. albicans* is CLRs, such as Dectin-1. CLR proteins have been proposed to have an essential role in the *C. albicans* immune response (Taylor *et al.*, 2007; Saijo *et al.*, 2010; Zhu *et al.*, 2013). Zebrafish do not have an exact homolog for Dectin-1, but do have 2 putative CLRs identified that may carry out the role of Dectin-1: Sclra and Clec4c (Petit *et al.*, 2019; Glass, Robinson and Rosowski, 2025)

I hypothesised that *C. albicans* may manipulate host RNS production through CLR signalling. To investigate the role of CLRs in RNS suppression by *C. albicans,* I aimed to knockdown zebrafish *card9* using CRISPR/Cas9. Card9 is a key adaptor protein for CLR proteins and is essential for mediating the antifungal immune response (Gross *et al.*, 2006; Bi *et al.*, 2010; Lionakis and Holland, 2013).

Three different gRNAs were designed to target *card9* exon 1, which encodes a functional, CARD9 domain at the N terminus: *card9*_16, *card9*_113 and *card9*_119 (Bertin *et al.*, 2000). One of these gRNAs was based on a previously targeted sequence (Brewer *et al.*, 2022; *card9*_113), while the other two were novel target sequences. 1 cell stage *Tg(mpx:GFP)* i114 embryos were injected with individual gRNAs, Cas9 and tracrRNA. Another group of embryos was co-injected with all three gRNAs, in order to check for potential synergistic action. Tyrosinase (*tyr*), a key gene in melanocyte development and pigmentation but not in innate immunity (Camp and Lardelli, 2001), gRNA was used as a positive control for successful CRISPR/Cas9 mediated knockdown, by observation of loss of pigment in embryos (Isles *et al.*, 2021). At 2 dpf, gDNA was extracted and amplified by PCR. *Card9* gene knockdown was then validated by restriction digest.

Without restriction digest, *tyr* injected embryos had a single DNA band, showing successful PCR amplification of DNA segment (Figure 4.5A-C). Restriction digest of *tyr* gDNA by Hyp188III led to appearance of 2 DNA bands of lower molecular weight (Figure 4.5A). A 3rd DNA band was present, of similar size to undigested DNA, suggesting restriction digest was not 100% efficient. Injection of *card9*_16 gRNA did not lead to loss of Hyp188III digest site, demonstrated by the presence of 2 DNA bands (Figure 4.5A). Digest of *tyr* gDNA by BIp1 led to appearance of a thick band at a lower size than undigested DNA, as well as a band remaining present at the same size as undigested DNA (Figure 4.5B). Injection of *card9*_113 gRNA did not lead to loss of BIp1 digest site (Figure 4.5B). Digest of *tyr* gDNA by AIwI led to appearance of 2 DNA bands of lower molecular weight (Figure 4.5C). Injection of *card9*_119 gRNA did not lead to loss of Hyp188III digest site, demonstrated by the presence of 2 DNA bands (Figure 4.5C). Loss of pigmentation in *tyr* injected embryos (Figure 4.5E) confirmed Cas9 protein used was functional. Hence, the designed *card9* gRNAs were unable to cause disruption of host *card9* gene. Alternative methods of knocking down *card9* should be considered for future work.

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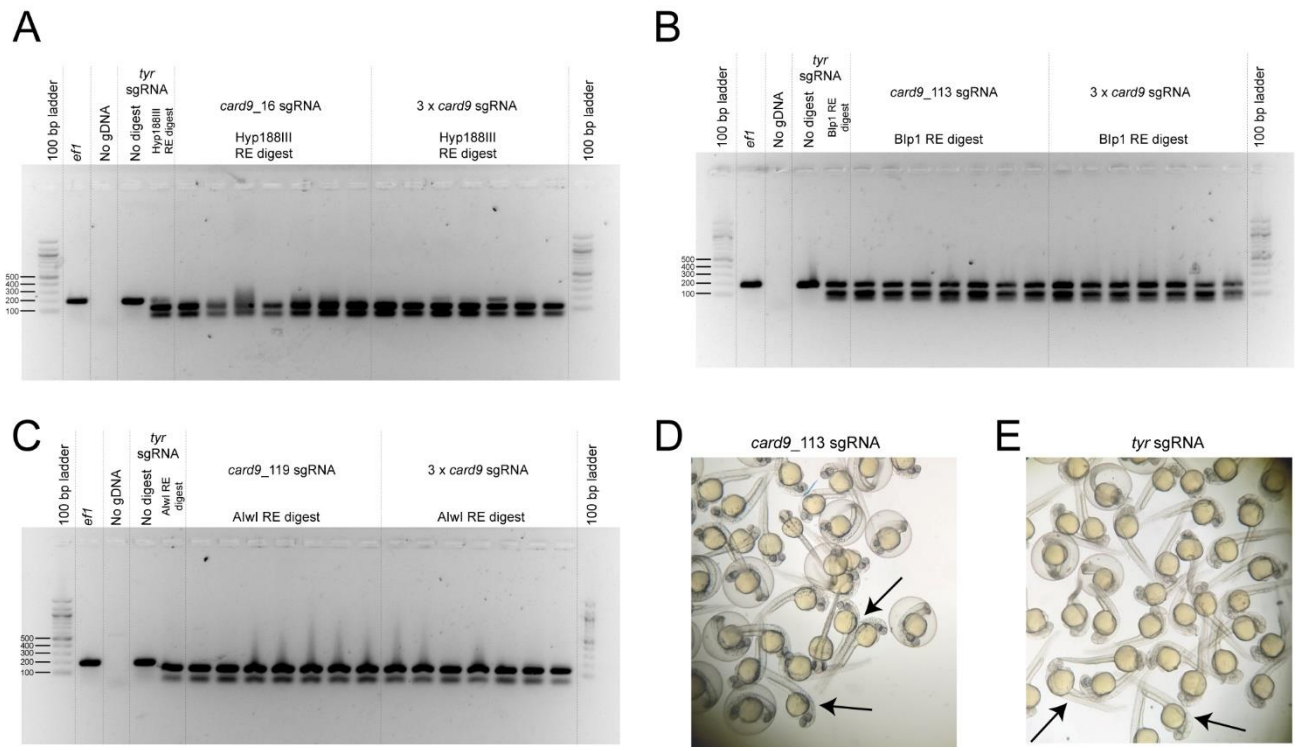

Figure 4.5: *card9* gRNA **does not cause loss of restriction digest site.**

(A) 1.5% TAE agarose gel of embryo gDNA injected with *card9*_16 gRNA, 3 *card9* gRNAs or *tyr* control gRNA. Restriction digest by Hyp188III carried out to identify loss of restriction site. *Ef1* and no gDNA controls were included.

(B) 1.5% TAE agarose gel of embryo gDNA injected with *card9*_113 gRNA, 3 *card9* gRNAs or *tyr* control gRNA. Restriction digest by BIp1 carried out to identify loss of restriction site. *Ef1* and no gDNA controls were included.

(C) 1.5% TAE agarose gel of embryo gDNA injected with *card9*_119 gRNA, 3 *card9* gRNAs or *tyr* control gRNA. Restriction digest by AIwI carried out to identify loss of restriction site. *Ef1* and no gDNA controls were included.

(D) Representative image of 1 dpf *Tg(mpx:GFP)* embryos injected with *card9*_113 gRNA. Arrows show pigmentation of zebrafish.

(E) Representative image of 1 dpf *Tg(mpx:GFP)* embryos injected with *card9*_113 gRNA. Arrows show loss of pigmentation of zebrafish.

4.2.2 Active suppression of neutrophil RNS is hyphae-dependent

C. albicans is a dimorphic fungus, capable of switching from a yeast to a hyphal morphology (Sudbery, 2011). *C. albicans* hyphae are associated with increased virulence, manifesting as secretion of different virulence factors (primarily candidalysin), masking of β-glucan and mechanical damage to surrounding tissues (Sudbery, 2011). This hyphal switch could be responsible for suppression of host RNS in *C. albicans* infection.

As an initial investigation of the role of hypha in RNS suppression, the ability of *C. albicans* TT21-dTomato to form hyphae *in vivo* was assessed. Fluorescent images of *C. albicans* infected zebrafish embryos at 24 hpi were analysed and classified by degree of fungal growth, based on a previously described protocol (Thrikawala *et al.*, 2022). 38.9% *C. albicans* TT21-dTomato infections were solely in the yeast morphotype (Figure 4.6), while 27.8% of infections were germinating – transitioning from yeast to the hyphal morphology. 11.1% *C. albicans* TT21-dTomato infections were in the hyphal extension stage (Figure 4.6). Finally, 22.2% of infections were in the destructive hyphae stage at 24 hpi. Combined, 33.3% *C. albicans* TT21-dTomato infections had developed into invasive hyphae, indicating *C. albicans* TT21-dTomato is able to produce hyphae *in vivo* in zebrafish larvae at 28°C, replicating previous literature (Seman *et al.*, 2018).

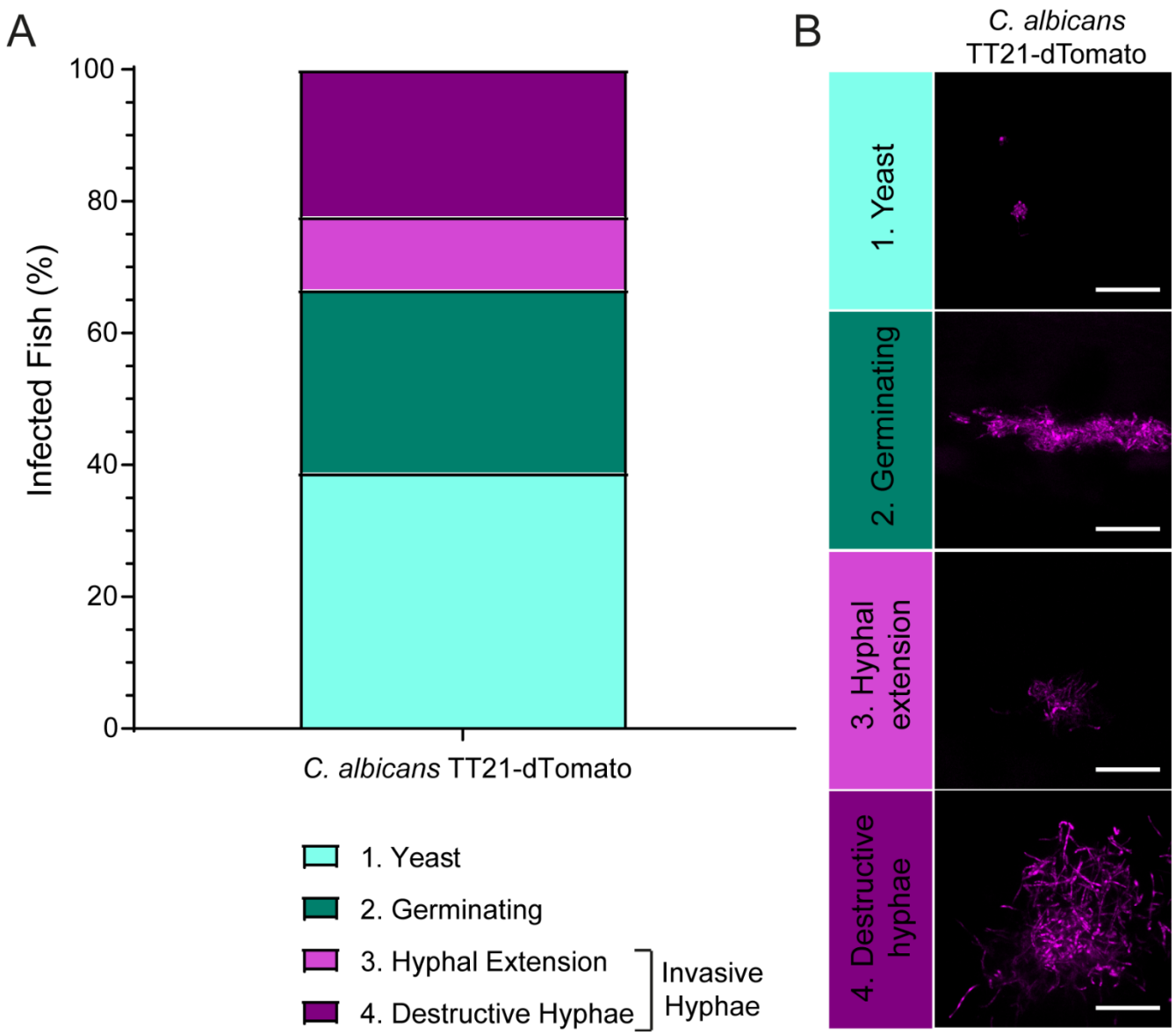

Figure 4.6: *C. albicans* TT21-dTomato switches to hyphal morphotype *in vivo*.

(A) Hyphal severity of zebrafish embryos infected with *C. albicans* TT21-dTomato at 24 hpi. N=18 fish, obtained from 3 independent experiments.

(B) Representative images of hyphal severity staging in 1 dpi zebrafish embryos infected with *C. albicans* TT21-dTomato. Scale bar = 50 µm

The above results (Figure 4.6) confirm *C. albicans* TT21-dTomato is able to form hyphae in zebrafish *in vivo* infection but does not demonstrate any link between hyphal formation and RNS suppression by *C. albicans*. To properly investigate the role of hyphae in host RNS suppression, 1 dpf *Tg(mpx:GFP)* i114 zebrafish embryos were injected into the caudal vein with PVP, *C. albicans* TT21-dTomato or *C. albicans* NRG1^{OEX}-dTomato, as well as heat killed *C. albicans* strains. *C. albicans* NRG1^{OEX}-dTomato has an extra copy of *NRG1*, a transcription factor known to be a negative regulator of the yeast-to-hypha transition (Murad *et al.*, 2001). Constitutive overexpression of *NRG1,* driven by the *URA3* promoter, leads to a yeast-locked phenotype, as well as an inability to form mature biofilms (Braun, Kadosh and Johnson, 2001; Uppuluri *et al.*, 2010).

C. albicans TT21-dTomato caused a 53.1% decrease in anti-NT levels compared to PVP (Figure 4.7; $p<0.0001$), a more modest suppressive effect than previous experiments. Heat killed *C. albicans* TT21-dTomato caused a small, non-significant decrease in anti-NT levels compared to PVP (PVP=3.29, Heat killed *C. albicans* TT21-dTomato=2.98), which was also inconsistent with previous experiments. *C. albicans* NRG1^{OEX} and heat killed *C. albicans* NRG1^{OEX} caused a 24.0% ($p<0.05$) and 22.4% ($p<0.05$) decrease in anti-NT levels, respectively, compared to PVP . For both live and heat killed *C. albicans* NRG1^{OEX}, anti-NT levels were greater than *C. albicans* TT21-dTomato ($p<0.05$ for both) but not significantly different from heat killed *C. albicans* TT21-dTomato. Live and heat killed *C. albicans* NRG1^{OEX} did not have significantly different anti-NT levels compared to each other (*C. albicans* NRG1^{OEX}=2.50, heat killed *C. albicans* NRG1^{OEX}=2.55). These results suggest *C. albicans* hyphae may be responsible for at least part of the active component of neutrophil RNS suppression, carried out by live *C. albicans*.

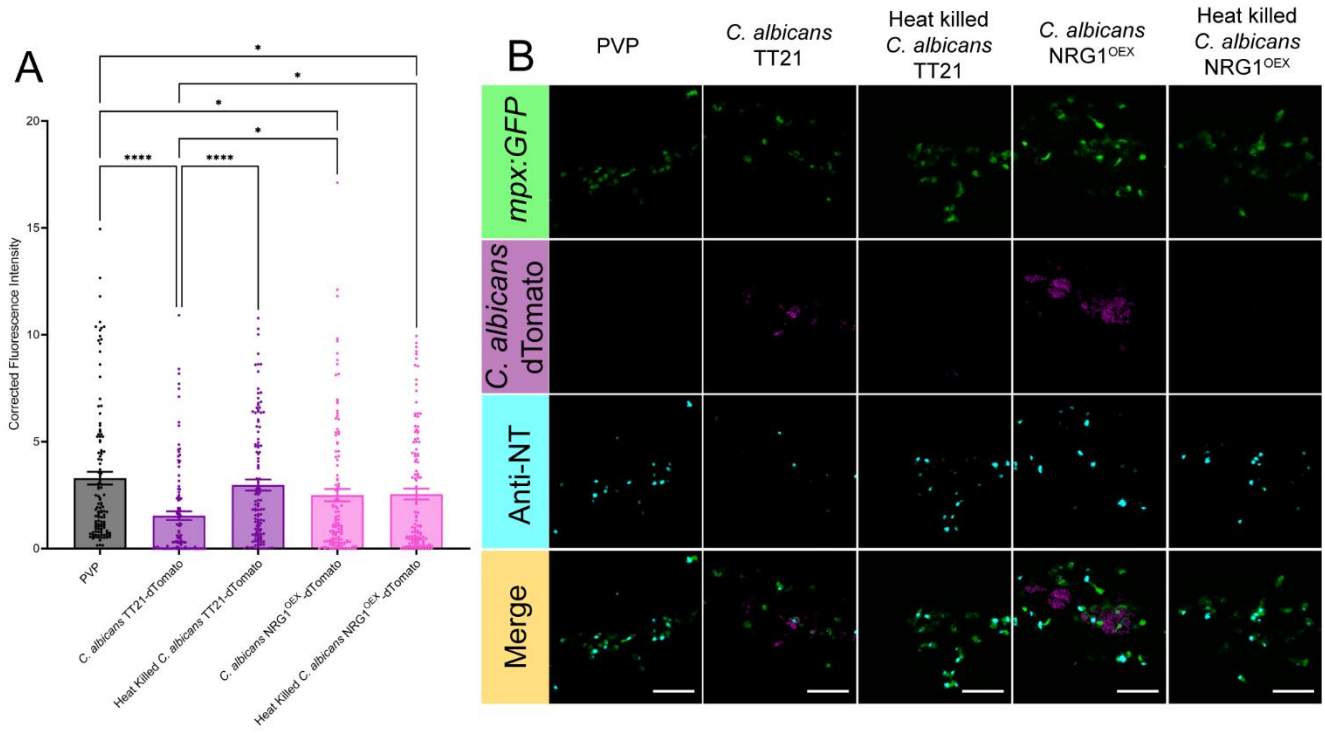

Figure 4.7: *C. albicans* NRG1^{OEX} do not reduce neutrophil RNS to the same extent as wild type *C. albicans.*

(A) Anti-nitrotyrosine fluorescence at 24 hpi 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 3 independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, with Dunn's multiple comparisons tests. P values shown: *p<0.05, ****p<0.0001.

(B) 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 embryos at 24 hpi. Scale bars = 50 µm.

4.2.3 *C. albicans* does not cause general pro-inflammatory suppression

RNS are cytotoxic, antimicrobial effectors at the end of a pro-inflammatory cascade (Warris and Ballou, 2019). It is unclear whether *C. albicans* suppression of neutrophil RNS levels is directed specifically at RNS production, or whether it is a more general suppression of pro-inflammatory responses. Therefore, I aimed to establish whether *C. albicans* suppression of neutrophil RNS was targeted specifically to RNS or whether this suppression was directed at pro-inflammatory cytokines upstream to RNS in the pathway.

TNF-α is a pro-inflammatory cytokine, primarily expressed by macrophages, previously identified to induce formation of RNS (Giroir *et al.*, 1992; Parameswaran and Patial, 2010; Sedger and McDermott, 2014; Blaser *et al.*, 2016). To investigate the impact of *C. albicans* infection on TNFα production, *TgBAC(tnfα:GFP)* zebrafish were injected into the caudal vein with PVP, *C. albicans* TT21-dTomato or *C. albicans* NRG1^{OEX}-dTomato at 30 hpf. Inclusion of the yeast-locked strain, *C. albicans* NRG1^{OEX}-dTomato, allowed comparison of the effect of hyphal and yeast morphologies on the cytokine response.

At 24 hpi, zebrafish were mounted and imaged to allow quantification of *tnfα:GFP* expression. PVP elicited a basal level of *tnfα:GFP* expression (Figure 4.8). *C. albicans* infection, with both strains, stimulated a significant increase in *tnfα:GFP* expression compared to the PVP control. *C. albicans* TT21-dTomato generated approximately a 4-fold increase compared to PVP (PVP = 2.4; *C. albicans* TT21-dTomato = 10.0; $p<0.0001$), whereas *C. albicans* NRG1^{OEX}-dTomato elicited almost a 3-fold increase (PVP = 2.4; *C. albicans* NRG1^{OEX}-dTomato = 6.8; $p<0.0001$). Hence, *C. albicans* infection stimulates *tnfα* expression *in vivo*. *C. albicans* TT21-dTomato caused significantly greater levels of *tnfα:GFP* expression than *C. albicans* NRG1^{OEX}-dTomato, implying the hyphal morphotype causes a greater pro-inflammatory response than yeast.

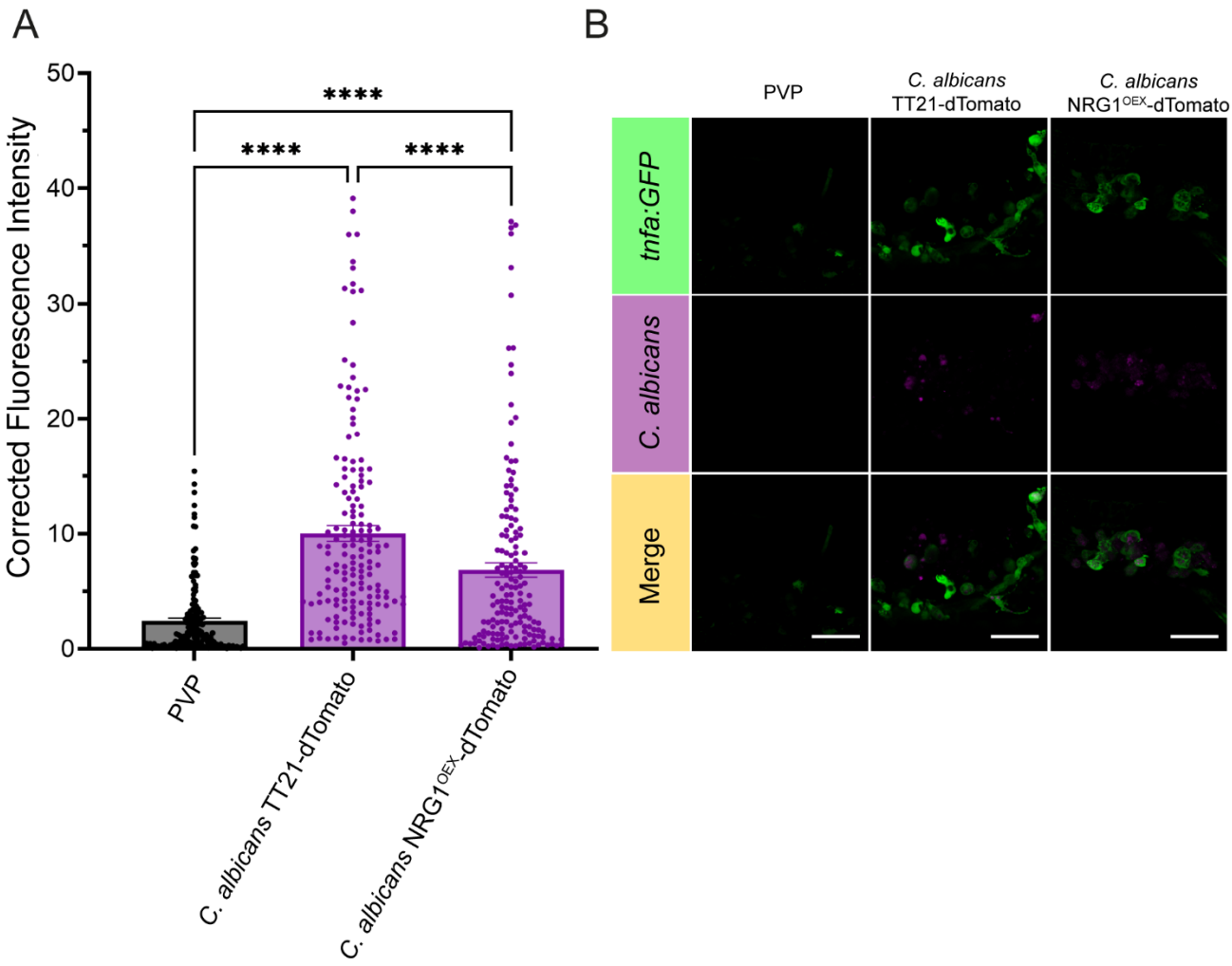

Figure 4.8: *C. albicans* infection increases TNFα production in zebrafish larvae.

(A) Corrected fluorescence intensity of *Tg(tnfα:GFP)* pd1028 zebrafish larvae at 24 hpi following injection of PVP, *C. albicans* TT21-dTomato or *C. albicans* NRG1^{OEX}-dTomato into the caudal vein at 30 hpf. N=162 cells from 27 zebrafish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, then Dunn's multiple comparisons test. ****p<0.0001.

(B) Representative images of *Tg(tnfα:GFP)* zebrafish at 24 hpi following injection of PVP, *C. albicans* TT21-dTomato or *C. albicans* NRG1^{OEX}-dTomato. Scale bars=50µm

IL-1β was also investigated as an additional pro-inflammatory cytokine, owing to its important role in the innate immune response (Dinarello, 1996). Inhibition of IL-1β has previously been associated with reduced RNS levels *in vivo,* in *M. marinum* infected and uninfected zebrafish, demonstrating a causal link between IL-1β expression and RNS levels (Ogryzko *et al.*, 2019). 1 dpf *TgBAC(il-1β:eGFP)xTg(lyz:mCherry)* embryos were injected with PVP, *C. albicans* TT21-dTomato or *C. albicans*

NRG1^{OEX}-dTomato into the caudal vein. Adult *TgBAC(il-1β:eGFP)* zebrafish were homozygous so were outcrossed with *Tg(lyz:mCherry)* zebrafish to ensure *il-1β:eGFP+* embryos had equal copies of the transgene and to allow examination of *il-1β:eGFP* expression specifically in neutrophils.

PVP injected embryos exhibited a basal level of *il-1β:eGFP* expression (Figure 4.9A). *C. albicans* infection stimulated a large increase in *il-1β:eGFP* expression, with a 5-fold increase caused by *C. albicans* TT21-dTomato compared to PVP (PVP = 0.79; *C. albicans* TT21-dTomato = 4.06; p<0.0001). *C. albicans* NRG1^{OEX}-dTomato stimulated just over a 4-fold increase in *il-1b:eGFP* expression compared to PVP (PVP = 0.79; *C. albicans* NRG1^{OEX}-dTomato = 3.38; p<0.0001). In this experiment, there was no significant different difference in *il-1β:eGFP* levels between *C. albicans* TT21-dTomato and *C. albicans* NRG1^{OEX}-dTomato (Figure 4.9A; p=0.5426).

The experiment was later repeated, with a *M. marinum* Crimson infection group included. *M. marinum* has previously been demonstrated to upregulate *il-1β:eGFP* expression *in vivo* (Ogryzko *et al.*, 2019). Hence, inclusion of *M. marinum* allows comparison of the impact of *C. albicans* on *il-1b:eGFP* expression with a positive control. As with the previous experiment, PVP elicited a basal level of *il-1β:eGFP* expression (Figure 4.9B). *C. albicans* TT21-dTomato stimulated over an 11-fold increase in *il-1β:eGFP* expression, from 0.82 in PVP injected embryos to 9.14 (Figure 4.9B; p<0.0001). *C. albicans* NRG1^{OEX}-dTomato increased *il-1β:GFP* expression to 6.00, which was significantly greater than PVP (p<0.0001). *Il-1b:GFP* expression in *C. albicans* NRG1^{OEX}-dTomato infection was significantly lower than *C. albicans* TT21-dTomato (Figure 4.9B; p<0.001), which did not match with results from the previous experiment (Figure 4.9A). *M. marinum* Crimson *il-1b:GFP* levels were 5.66, which was approximately a 7-fold increase compared to PVP (p<0.0001). *M. marinum* Crimson *il-1β:GFP* levels were significantly lower than *C. albicans* TT21-dTomato (p<0.01) but were not significantly different to *C. albicans* NRG1^{OEX}-dTomato (p>0.9999). Across both experiments and all infection groups, there were relatively few examples of *il-1β:eGFP* being co-localised with *lyz:mCherry; il-1β:eGFP* expression was mostly not co-localised with *lyz:mCherry* expression (Figure 4.9C), indicating *il-1β:eGFP* was primarily being expressed in immune cells that were not neutrophils. Together, both experiments reveal *C. albicans* increases *il-1b:GFP* expression, demonstrating induction of a pro-inflammatory response. *C. albicans* TT21-dTomato generated *il-1β:GFP* expression approximately 1.5-times greater than *M. marinum* Crimson, whereas *C. albicans* NRG1^{OEX}-dTomato was not significantly different from *M. marinum*, implying the hyphal morphotype causes a greater inflammatory response than *C. albicans* yeast or *M. marinum.*

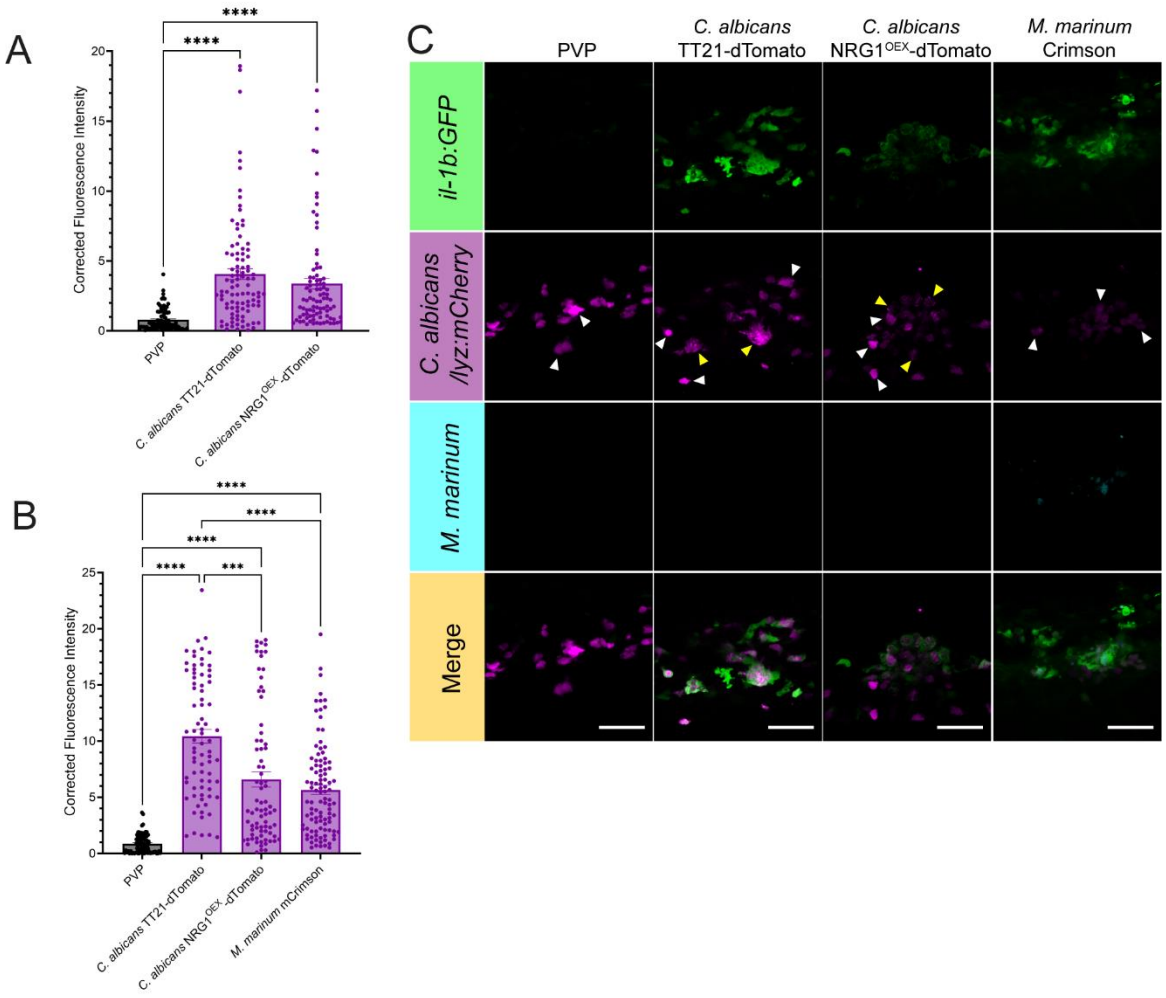

Figure 4.9: *C. albicans* infection stimulates *il-1β:eGFP* production in zebrafish larvae.

(A) Corrected fluorescence intensity of *Tg(il-1β:GFP)xTg(lyz:mCherry)* zebrafish larvae at 24 hpi following injection of PVP, *C. albicans* TT21-dTomato or *C. albicans* NRG1^{OEX}-dTomato into the caudal vein at 30 hpf. N=90-96 cells from 15-16 zebrafish, obtained from 2 independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, then Dunn's multiple comparisons test. P values shown: ****p<0.0001.

(B) Corrected fluorescence intensity of *Tg(il-1β:GFP)xTg(lyz:mCherry* zebrafish larvae at 24 hpi following injection of PVP, *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato or *M. marinum* Crimson into the caudal vein at 30 hpf. N=96-102 cells from 16-17 zebrafish, obtained from 2 independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, then Dunn's multiple comparisons test. P values shown: ***p<0.001, ****p<0.0001.

(C) Representative images of *Tg(il-1β:GFP)xTg(lyz:mCherry)* zebrafish at 24 hpi following injection of PVP, *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato or *M. marinum* Crimson. White arrow heads point towards neutrophils. Yellow arrow heads point towards *C. albicans*. Scale bars = 50 µm

In the previous experiments, the pro-inflammatory cytokine response induced by *C. albicans* has primarily been observed in cells that are likely macrophages. Conversely, *C. albicans* has been demonstrated to suppress RNS production in neutrophils. It, therefore, remains possible that *C. albicans* is having a broader immunosuppressive effect on cytokines and inflammatory effectors, but this effect is confined to neutrophils. To investigate the impact of *C. albicans* on neutrophil cytokine expression, I aimed to quantify the number of neutrophils that express *il-1β:eGFP* in *C. albicans* infection. Using the previously taken images, I quantified the number of *il-1β:eGFP*-expressing cells that were either *lyz:mCherry*+ (neutrophils) or *lyz:mCherry*- (not neutrophils). The level of *il-1β:eGFP* expression was not considered in these cell counts.

No *lyz:mCherry+* cells were observed following PVP injection, suggesting IL-1β is not expressed in neutrophils basally (Figure 4.10). In *C. albicans* TT21-dTomato infection, 36 *il-1β:eGFP*-expressing cells were *lyz:mCherry*+, 13.64% of the total population of *il-1β:eGFP*-expressing cells (Figure 4.10B). In comparison, 10.04% of *il-1β:eGFP*-expressing cells were *lyz:mCherry*+ in *C. albicans* NRG1^{OEX}-dTomato infection and 9.95% were *lyz:mCherry*+ in *M. marinum* Crimson infection. The similar percentages of *il-1β:eGFP*-expressing cells that are neutrophils (*lyz:mCherry*+) in *C. albicans* and *M. marinum* infection implies that *C. albicans* is not suppressing neutrophil *il-1β* expression specifically. 264 cells in total expressed *il-1β:eGFP* in *C. albicans* TT21-dTomato infection, more cells than in embryos infected with *C. albicans* NRG1^{OEX}-dTomato and *M. marinum* Crimson. Paired with the observed increased *il-1β:eGFP* levels (Figure 4.10), this further suggests that the hyphal *C. albicans* elicits a greater pro-inflammatory response than yeast or *M. marinum.*

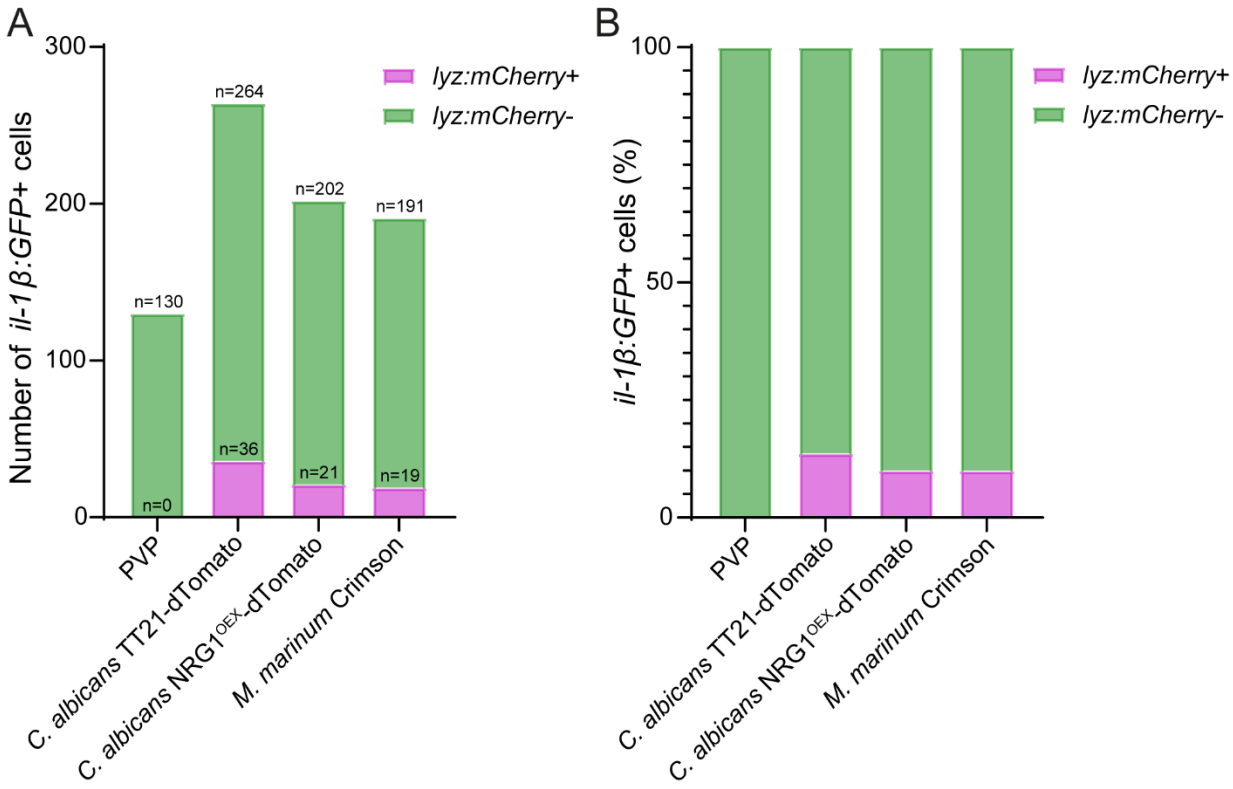

Figure 4.10: *C. albicans* infection stimulates *il-1β:eGFP* expression in neutrophils.

(A) Number of *il:1β:eGFP*-expressing cells that are *lyz:nfsB.mCherry*+ and *lyz:nfsB.mCherry-,* following infection with *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato, *M. marinum* Crimson or PVP. N=130-264 cells from 16 zebrafish, obtained from 2 independent experiments.

(B) Percentage of *il:1β:eGFP*-expressing cells that are *lyz:nfsB.mCherry*+ and *lyz:nfsB.mCherry-,* following infection with *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato, *M. marinum* Crimson or PVP. N=130-264 cells from 16 zebrafish, obtained from 2 independent experiments.

4.2.4 *C. albicans* arginase (CAR1) plays a role in neutrophil RNS suppression

To further investigate mechanisms of RNS suppression, *C. albicans* interference with host arginase was considered as a potential mechanism underlying *C. albicans* suppression of neutrophil RNS. Production of host RNS is driven by iNOS, which converts L-arginine to L-citrulline and produces nitric oxide – a key RNS. Arginase competes with iNOS for the same substrate, L-arginine, shunting metabolism towards polyamine production for use in tissue repair (Bansal and Ochoa, 2003; Takeda *et al.*, 2010). *C. albicans* produces its own version of arginase, *CAR1* (Schaefer *et al.*, 2020). I, therefore, hypothesised that *C. albicans* arginase may be interfering with the host arginase-iNOS axis, resulting in decreased RNS production.

I investigated the impact of *C. albicans* infection on host arginase levels by generating systemic *C. albicans* infections in *Tg(arg2:GFP)* zebrafish. *C. albicans* has previously been demonstrated to upregulate *arg2:GFP* in a subpopulation of neutrophils in zebrafish (Hammond *et al.*, 2023), however, this was not fully quantified. 1 dpf *Tg(arg2:GFP)* zebrafish embryos were injected into the caudal vein with 200 cfu *C. albicans* TT21-dTomato, *C. albicans* NRG1^{OEX}-dTomato or PVP. A *Mycobacterium marinum* infected group were also included, to allow comparison with a pathogen known to stimulate arginase expression (Hammond *et al.*, 2023). At 24 hpi, embryos were mounted, imaged and *arg2:GFP* fluorescence was quantified.

PVP injected embryos had a low basal level of expression (Figure 4.11A). *M. marinum* caused a slight, but significant, upregulation in *arg2:GFP* compared to PVP (PVP=0.46, *M. marinum*=0.89; $p<0.01$), as has been previously reported (Hammond *et al.*, 2023). *C. albicans* TT21-dTomato caused a significant upregulation in *arg2:GFP* expression compared to PVP (TT21-dTomato=2.25; $p<0.0001$) and compared to *M. marinum* ($p<0.0001$). *C. albicans* NRG1^{OEX}-dTomato (NRG1=1.82) also caused upregulation of *arg2:GFP* compared to both PVP ($p<0.0001$) and *M. marinum* ($p<0.01$). Although *C. albicans* TT21-dTomato increased *arg2:GFP* to a greater extent than *C. albicans* NRG1^{OEX}-dTomato, this was not a statistically significant difference. Hence, *C. albicans* is able to significantly upregulate *arg2:GFP*, more so than *M. marinum*, indicating that *C. albicans* infections are stimulating expression of a host anti-inflammatory gene.

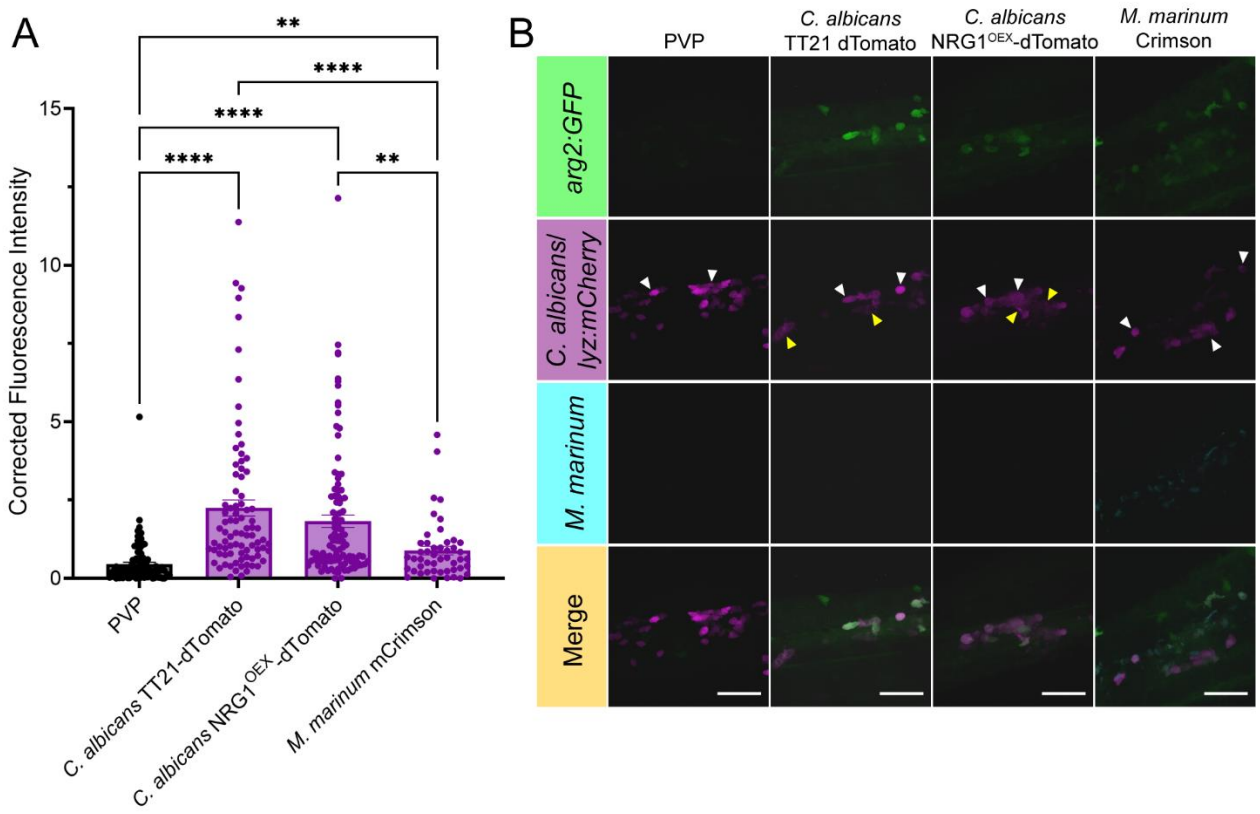

Figure 4.11: *C. albicans* infection induces *arg2:GFP* expression.

(A) Corrected fluorescence intensity of *Tg(arg2:GFP)* zebrafish larvae at 24 hpi 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 14-18 fish, obtained from 3 independent experiments. For *M. marinum*: n=48 cells from 8 fish, obtained from 2 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 arrow heads point towards neutrophils. Yellow arrow heads point towards *C. albicans.* Scale bars = 50 µm.

Hammond *et al.* previously demonstrated it is primarily neutrophils that upregulate *arg2:GFP* expression during infection (Hammond *et al.*, 2023). I aimed to quantify the number of neutrophils that express *arg2:GFP* during *C. albicans* infection, in order to examine whether *C. albicans* stimulates a greater *arg2* response than other pathogens. As it was used by Hammond and colleagues, *M. marinum* was selected as the positive control for comparison with another pathogen.

In PVP injected embryos, 2.01% of neutrophils expressed *arg2:GFP* (Figure 4.12B). This could represent a small subpopulation of neutrophils that basally express *arg2*. Alternatively, *arg2:GFP* may have been upregulated as part of the wound response to microinjection. PVP injected embryos had a greater number of neutrophils in total than *C. albicans* or *M. marinum* infected embryos (Figure 4.12A). A possible explanation is that in PVP injected embryos, *arg2:GFP+* neutrophils were counted at the caudal haematopoietic tissue (CHT), where immune cells are produced. In *C. albicans* and *M. marinum* infected embryos, *arg2:GFP+* neutrophils were counted at the site of infection, which may have been away from the CHT so required neutrophils to migrate. In *C. albicans* TT21-dTomato infected embryos, 41.59% of neutrophils expressed *arg2:GFP*; 31.20% of neutrophils in *C. albicans* NRG1^{OEX}-dTomato were *arg2:GFP*+. Both *C. albicans* strains caused a sizable increase in the number, and percentage, of *arg2:GFP*+ neutrophils compared to PVP and *M. marinum*, adding to the evidence that *C. albicans* infections are promoting an anti-inflammatory phenotype in a subpopulation of neutrophils. 10.97% of neutrophils in *M. marinum* infected embryos were *arg2:GFP+*. This was substantially lower than the previously reported figure of 31.7% neutrophils being *arg2:GFP+* in *M. marinum* infection at 1 dpi (Hammond *et al.*, 2023).

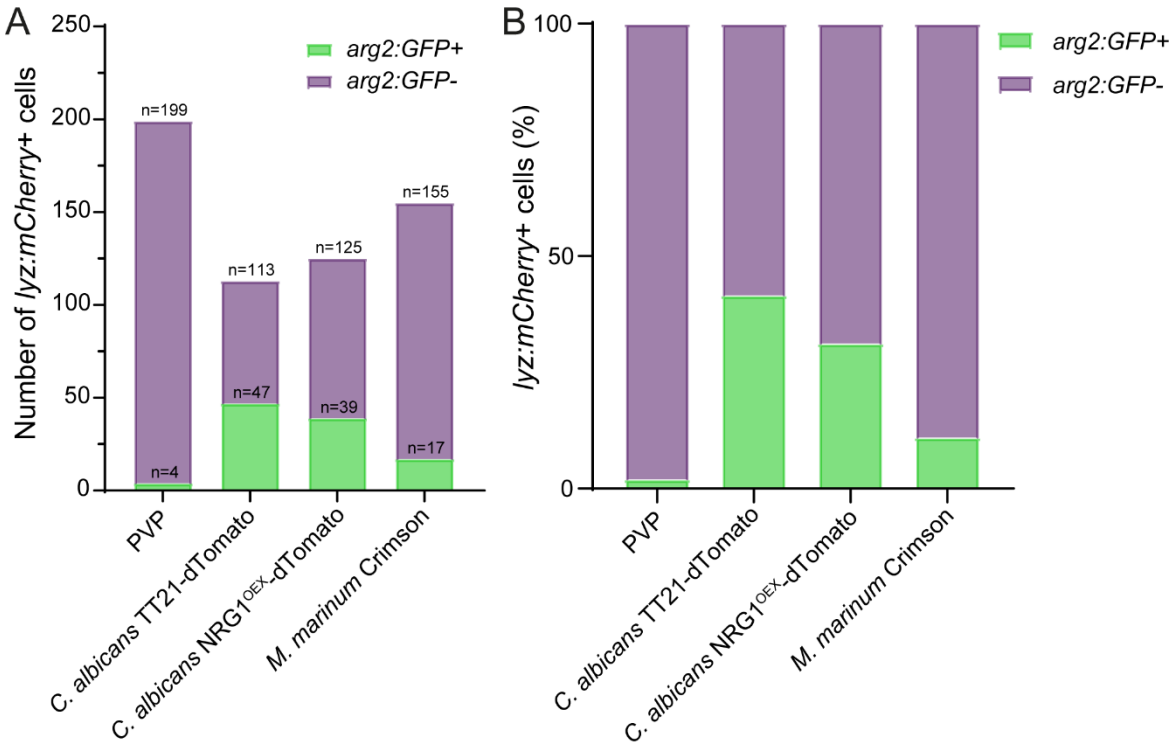

Figure 4.12: *C. albicans* infection increases the number of *arg2:GFP*+ neutrophils.

(A) 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 2 independent experiments.

(B) Percentage 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 2 independent experiments.

While *C. albicans* infection can upregulate host arginase expression and the number of neutrophils expressing arginase, it is not clear whether *C. albicans*-arginase is responsible for this upregulation or whether this upregulation is responsible for suppression of host-RNS production. *CAR1* is the only arginase known to be produced by *C. albicans* (Schaefer *et al.*, 2020). The impact of *C. albicans* arginase on host RNS production was investigated by infecting 1 dpf *Tg(mpx:GFP)* zebrafish with *C. albicans car1*Δ or its parental strain *C. albicans* SC5314 as the appropriate control. RNS production was measured at 24 hpi by anti-nitrotyrosine staining.

C. albicans SC5314 caused a 95.0% decrease in RNS production compared to PVP (Figure 4.13; $p<0.0001$), showing potent RNS suppression. Heat killed *C. albicans* SC5314 caused an intermediate level of RNS suppression (64.8% decrease compared to PVP), as seen with other heat killed strains. Heat killed *C. albicans car1Δ* caused a 59.0% decrease compared to PVP ($p<0.0001$) and was not

significantly different from heat killed *C. albicans* SC5314 (p>0.9999). *C. albicans car1*Δ infection caused a 73.7% decrease in neutrophil RNS production compared to PVP (p<0.0001). This was significantly greater than RNS production following *C. albicans* SC5314 infection (p<0.05) but still lower than RNS production with heat killed *C. albicans* SC5314 (p<0.0001). Together, this suggests that *CAR1* has a role in *C. albicans* suppression of host RNS but *C. albicans* must be alive for *CAR1* to have an effect. This may imply *CAR1* is involved in an active process or secretion mechanism.

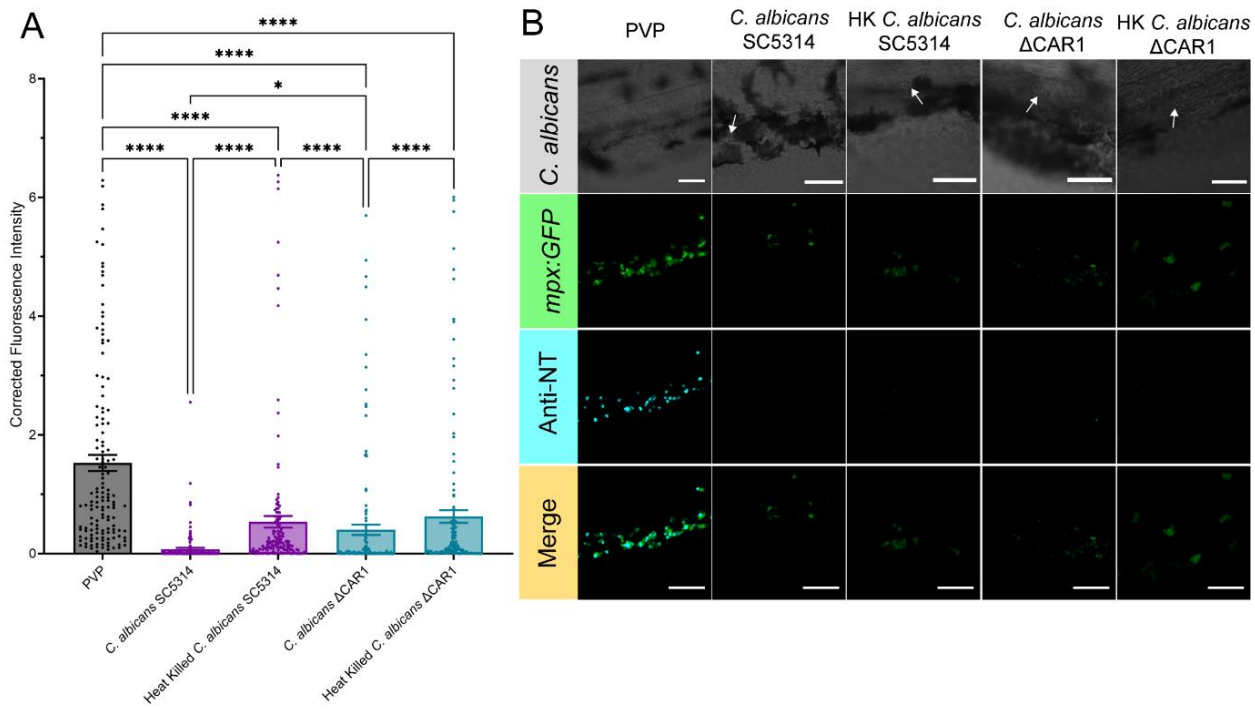

Figure 4.13: *C.* albicans car1 is partially responsible for host RNS suppression

(A) Anti-nitrotyrosine fluorescence at 24 hpi following injection of PVP, *C. albicans* SC5314, heat-killed *C. albicans* SC5314, *C. albicans car1Δ* or heat-killed *C. albicans car1Δ* into the caudal vein. N=144 neutrophils from 24 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, with Dunn's multiple comparisons tests. *p<0.05, ****p<0.0001.

(B) Representative images of PVP, *C. albicans* SC5314, heat-killed *C. albicans* SC5314*, C. albicans car1Δ* or heat-killed *C. albicans car1Δ* infected embryos at 24 hpi. Arrows show *C. albicans* identified on brightfield images. Scale bars = 50 μm.

Having demonstrated that *C. albicans car1Δ* suppresses host RNS production less than *C. albicans* SC5314, I next wanted to establish whether this has an observable effect on zebrafish survival. 1 dpf nacre embryos were injected with 200 cfu *C. albicans* SC5314 or *C. albicans car1Δ* via the caudal vein. Mortality was monitored daily up to 4 dpi.

C. albicans SC5314 had 30.6% survival at 4 dpi, with the greatest mortality occurring between 0 and 1 dpi (Figure 4.14). *C. albicans car1Δ* had 54.4% survival at 4 dpi, significantly greater than *C. albicans* SC5314 ($p<0.0001$), with the greatest mortality occurring between 1 and 2 dpi. Hence, *C. albicans car1Δ* had reduced virulence *in vivo* compared to a wild type isolate.

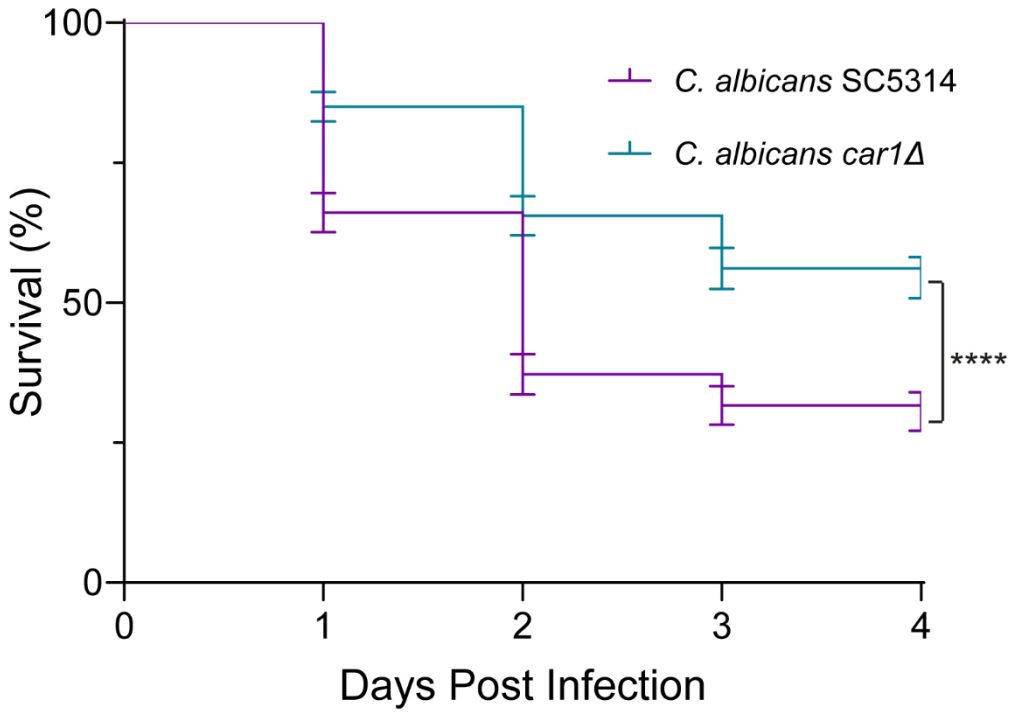

Figure 4.14: Zebrafish infected with *C. albicans car1Δ* have greater survival than zebrafish infected with *C. albicans* SC5314.

1 dpf nacre embryos were injected into the caudal vein with 200 cfu *C. albicans* SC5314 or *C. albicans car1Δ*. Mortality was measured daily up to 4 dpi. n=180 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. P values shown: ****p<0.0001

4.3 Discussion

In this chapter, I explore mechanisms underlying *C. albicans* suppression of neutrophil RNS levels, demonstrating a role for *C. albicans* arginase in modulating neutrophil phenotype and a role for hyphae in active RNS suppression by live *C. albicans*.

C. albicans infection was shown to induce host neutrophil arginase expression *in vivo*, to a greater degree than *M. marinum* infection. Wild type *C. albicans* caused greater arginase upregulation than yeast locked *C. albicans,* suggesting a role for hyphae in arginase induction. Wagener *et al.* reported a 40% increase in arginase enzyme activity by human monocyte-derived macrophages *in vitro* following *C. albicans* infection, with a 7.43-fold increase in Arg-1 protein level (Wagener *et al.*, 2017). Similarly, Schaefer *et al.* described approximately a 40% increase in arginase activity by murine RAW274.6 macrophages *in vitro* when stimulated with wild type *C. albicans* (Schaefer *et al.*, 2020). I observed a 4.89-fold increase in arginase levels with *C. albicans* TT21-dTomato infection *in vivo* and a 3.96-fold increase in arginase levels with *C. albicans* NRG1^{OEX}-dTomato infection, greater than the reported increase in arginase enzyme activity but less than the reported increase in arginase protein level (Wagener *et al.*, 2017; Schaefer *et al.*, 2020). I observed *arg2:GFP* expression, which may not directly correlate with enzyme activity or protein level, which could explain the differences in our observations. Further explanations could be the difference in model investigated (macrophages *in vitro* vs neutrophils *in vivo*), comparative infection doses or the arginase isoform studied: Wagener and colleagues studied arginase-1 responses, whereas I looked at arginase-2 levels. Schaefer *et al.* did not specify which arginase isoform they measured. Mammalian cells have both arginase-1 and arginase-2 isoforms, though arginase-1 is the most widely studied in macrophage polarisation (Rath *et al.*, 2014; Hammond *et al.*, 2023). In zebrafish, *arg2* is more highly expressed in immune cells than *arg1*, making it the more appropriate isoform to study (Hammond *et al.*, 2023). Furthermore, *C. albicans* infection increased the number of neutrophils that express arginase. In wild type *C. albicans* infection, 41.59% of neutrophils expressed arginase, while 31.20% of neutrophils expressed arginase in yeast-locked *C. albicans* infection. This demonstrates a sizable upregulation in arginase expression induced by *C. albicans* compared to PVP (vehicle control) and *M. marinum* (pathogen control). I observed *M. marinum* infection stimulated arginase expression in 10.97% of neutrophils, substantially lower than the previously observed 31.7% of neutrophils (Hammond *et al.*, 2023). Infection dose in both instances was 100 cfu *M. marinum*, so was unlikely a factor in the discrepancy. The time point considered (24 hpi) was also the same in both instances. Differences in arginase expression in *M. marinum* infection could be the result of standard variation in zebrafish *in vivo* model. Compared to values previously published by Hammond *et al.*, yeast locked *C. albicans* induced arginase to the same degree as *M. marinum,* with wild type *C. albicans* still increasing

arginase expression to a greater degree than *M. marinum* (Hammond *et al.*, 2023). Together, these data propose *C. albicans* upregulates host arginase expression, driving neutrophils towards an anti-inflammatory phenotype.

C. albicans may actively be driving arginase expression in neutrophils as a survival strategy. Wagener *et al.* demonstrated *C. albicans* chitin alone is sufficient to increase macrophage arginase activity (Wagener *et al.*, 2017). While purified chitin increased arginase activity to a similar degree as live *C. albicans*, chitin did not reduce macrophage NO synthesis as much as live *C. albicans*. This suggests arginase induction by chitin contributes towards macrophage NO suppression but is not entirely responsible. While Wagener and colleagues demonstrated chitin is sufficient for macrophage arginase induction, they did not demonstrate chitin is necessary for this effect, leaving room for additional mechanisms of macrophage arginase induction.

An alternative explanation for these arginase-expressing neutrophils is neutrophil heterogeneity. Owing to their short lifespan, inability to divide and the technical difficulty in studying this cell type, neutrophils were previously believed to be a homogenous population of terminally differentiated cells with definitive, pro-inflammatory functions (Ng, Ostuni and Hidalgo, 2019; Ganesh and Joshi, 2023). Mounting evidence of different neutrophil subtypes is rapidly shifting this perception, though there is a lack of clarity on the classification and function of neutrophil subtypes and the molecular basis of heterogeneity. Using healthy mice, Ballesteros *et al.* identified distinct transcriptional profiles and phenotypes of neutrophils based on the tissues they reside in, suggesting tissue environments may be driving neutrophil reprogramming (Ballesteros *et al.*, 2020). Xie *et al.* identified 5 subtypes of developing neutrophils in murine bone marrow and a further 3 distinct subpopulations of mature neutrophils in murine peripheral blood (Xie *et al.*, 2020). Neutrophil heterogeneity has also been observed in fungal infection. A subset of neutrophils are able to transdifferentiate into neutrophil-dendritic cell hybrids (PMN-DCs) that express typical neutrophil markers, as well as MHC class II, CD11c and other dendritic cell markers (Matsushima *et al.*, 2013). Intravenous infection of *C. albicans* in mice increased the number of PMN-DCs present and PMN-DCs were more effective at killing *C. albicans* than conventional neutrophils *in vitro* and *in vivo* (Fites *et al.*, 2018). PMN-DCs were shown to be capable of antigen presentation to T cells following *C. albicans* exposure, revealing a neutrophil subpopulation that develops a dual function as antigen-presenting cells and traditional neutrophil antimicrobial roles (Fites *et al.*, 2018). Myeloid-derived suppressor cells (MDSCs) are innate immune cells that suppress T cell responses, with distinct neutrophilic and monocytic subsets (Gabrilovich and Nagaraj, 2009). *C. albicans* induced neutrophilic MDSCs to suppress T cell proliferation and Th2 responses. In contrast, monocytic MDSCs were not induced and conventional neutrophils had no impact on T cell function (Rieber *et al.*, 2015).

Interestingly, *C. albicans* induction of neutrophilic MDSCs, and subsequent T cell suppression, was protective against invasive candidiasis in a mouse model. The authors speculated that neutrophil MDSC driven T cell suppression may help to dampen pathogenic hyperinflammation by NK and Th17 cells (Rieber *et al.*, 2015). This highlights an anti-inflammatory function of neutrophilic cells in fungal infection. MDSCs are characterised by increased arginase expression, which enhances L-arginine metabolism, depleting this amino acid from the local environment, resulting in inhibition of T cell proliferation (Gabrilovich and Nagaraj, 2009). Furthermore, neutrophilic MDSCs express high levels of ROS but low levels of NO. It is possible the *arg2*-expressing neutrophils I observed may be similar to neutrophilic MDSCs. This could imply that the arginase expression I observed is an intentional host mechanism, designed to prevent hyperinflammation, as opposed to a *C. albicans* strategy to mitigate inflammation and promote its own survival. MDSCs from cancer patients express lysozyme (Savardekar *et al.*, 2024), suggesting these cells could appear *lyz+ in vivo*. However, to the best of my knowledge, MDSCs have never been investigated in zebrafish. Larval zebrafish lack an adaptive immune system, so further investigation would be beyond the scope of the larval zebrafish model. *C. albicans* arginase was revealed to have a role in suppression of host neutrophil RNS levels. *CAR1* is the only known arginase-encoding gene in the *C. albicans* genome, encoding a cytosolic arginase (Schaefer *et al.*, 2020). In mammals, arginase competes with nitric oxide synthase (NOS) for a common substrate: L-arginine (Rath *et al.*, 2014). Hence, increased arginase activity leads increased consumption of L-arginine, consequently leading to decreased NOS activity due to competitive inhibition. *CAR1* expression was induced in *C. albicans* hyphae, but repressed in yeast, following interactions with neutrophils (Niemiec *et al.*, 2017), which may suggest *CAR1* play a larger role in suppression of host RNS by hyphal *C. albicans*. I hypothesise that *C. albicans* arginase consumes L-arginine in the local microenvironment, leading to a shortage of L-arginine for host iNOS and a subsequent reduction in host RNS levels. This may synergise with induction of host arginase expression by *C. albicans* to lead to a large suppression of neutrophil RNS levels. *C. albicans car1Δ* had a reduced, but not completely abrogated, effect on neutrophil RNS levels, suggesting *C. albicans* arginase production may not be the only or the predominant RNS reducing mechanism. Additive strategies of neutrophil RNS suppression by *C. albicans* may underlie their success at the large overall suppression of neutrophil RNS observed locally and distally. A further consideration is *C. albicans* may have redundant mechanisms of arginine metabolism. *C. albicans car1Δ* was able to grow on arginine media (Schaefer *et al.*, 2020), suggesting *C. albicans* has alternative arginase-independent pathways for arginine metabolism. *Kluyveromyces lactis* converts arginine to ketoarginine, which is then decarboxylated and oxidised to form 4-guanidinobutyrate, a precursor to GABA (Romagnoli *et al.*, 2014). *C. albicans* may have a similar pathway of arginine catabolism, by

producing ketoarginine. The enzyme responsible for non-canonical arginine catabolism in *K. lactis* (*KlGBU1*) has a *C. albicans* homolog: *GBU19* (orf19.3418; Schaefer *et al.*, 2020). *GBU19* has been described as a guanidinobutyrase gene in *C. albicans,* predicted to be secreted, but may have additional, uncharacterised activities.

C. albicans car1Δ had greater survival at 4 dpi compared to wild type *C. albicans* in zebrafish larvae. The *car1Δ* mutant strain had greater neutrophil RNS levels than wild type *C. albicans*, which may be responsible for the difference in survival *in vivo*. However, in a *Galleria mellonella* infection model, *C. albicans car1Δ* had no difference in virulence compared to wild type *C. albicans* (Schaefer *et al.*, 2020). While *G. mellonella* haemocytes produce NO as part the immune response, melanisation is the principle method of antimicrobial defence for *G. mellonella* (Krishnan, Hyrsl and Simek, 2006; Pereira *et al.*, 2020). Hence, the presence or absence of RNS suppression by *C. albicans* may make no difference to virulence in the *G. mellonella* infection model, as melanisation can still occur regardless. A further caveat on these results may be the ability of *C. albicans car1Δ* to form hyphae. *In vitro*, *C. albicans car1Δ* is unable to grow hypha on arginine media but can form hypha on media with ornithine and urea (Schaefer *et al.*, 2020). I observed hyphae in *C. albicans car1Δ* infection *in vivo*, but to a lesser extent than *C. albicans* SC5314 and this was not formally quantified.

C. albicans infection triggered increased *tnfα:GFP* expression, demonstrating stimulation of a pro-inflammatory host response. TNFα is primarily expressed by macrophages and monocytes in humans (Sedger and McDermott, 2014). TNFα stimulates chemokine expression, leading to recruitment of neutrophils, enhanced phagocytosis and fungicidal activity (Cannom *et al.*, 2002). TNF-/- mice were highly susceptible to *C. albicans* infection, showing the importance for TNFα for the anti-*Candida* immune response (Marino *et al.*, 1997). TNFα may also have direct effects on *C. albicans*: Rocha *et al.* showed TNFα blocks biofilm formation by *C. albicans in vitro.* However, *in vitro* growth of planktonic *C. albicans* was not affected, suggesting TNFα interferes with components of the extracellular biofilm matrix (Rocha *et al.*, 2017). This investigation used TNFα concentrations equivalent to those used on mammalian cells *in vitro*, suggesting the biofilm blocking by TNFα may be biologically relevant. However, the effect of TNFα on *C. albicans* biofilm formation has not been shown *in vivo*.

Furthermore, *C. albicans* stimulated increased IL-1β levels, led to a greater number of IL-β-expressing cells and increased the percentage of IL-1β-expressing neutrophils compared to PVP and *M. marinum* controls. IL-1β expression has also been shown to be stimulated by *C. albicans* infection of a mouse model, with IL-1β levels significantly increasing up to 24 hpi (Cannom *et al.*, 2002). IL-1β-/- mice infected with *C. albicans* had significantly lower survival compared to control mice. Further to this, IL-1β knockout mice had reduced neutrophil recruitment compared to control mice *in vivo*,

but had no difference in neutrophil-mediated damage to *C. albicans in vitro*. Hence, IL-1β appears to be important for neutrophil recruitment in *C. albicans* infection but not for stimulating candidacidal activity in a mouse model (Vonk *et al.*, 2006). Contrary to this observation, Ogryzko *et al.* showed IL-1β knockdown inhibits neutrophil NO production in *M. marinum* infection of zebrafish larvae (Ogryzko *et al.*, 2019). These opposing observations could be explained by the fact murine macrophages produce NO to a greater degree than murine neutrophils, so inhibition of NO, by inhibiting IL-1β, has no impact on neutrophil candidacidal activity (Muijsers *et al.*, 2001). In zebrafish, neutrophils are the dominant producers of NO, so inhibition of IL-1β has an observable phenotype in these cells (Elks *et al.*, 2013, 2014; Ogryzko *et al.*, 2019). Together, the increased levels of TNFα and IL-1β in *C. albicans* infection *in vivo* suggest host RNS suppression is specific to RNS and not downstream of a wider suppression of inflammation.

I also identified that the active component of RNS suppression is hyphae-dependent. The hyphal morphotype is highly associated with a shift in transcription and increased virulence (Sudbery, 2011; Desai, 2018), which may trigger expression of a protein necessary for active RNS suppression by *C. albicans*. Proteomic analysis by Vaz and colleagues identified 301 secreted proteins specific to hyphae (Vaz *et al.*, 2021). Further examination and characterisation of these proteins could point towards candidates involved in active RNS suppression. Collette *et al.* previously suggested direct contact between *C. albicans* and macrophages is required to initiate secretion of the compound responsible for suppression of host NO (Collette, Zhou and Lorenz, 2014). Niemiec *et al.* investigated the effect of neutrophil-*Candida* interactions on the *C. albicans* transcriptome. Exposure to neutrophils caused a significant change in transcriptome of *C. albicans* yeast and hyphae: genes involved in acetate and carboxylic acid catabolism upregulated, while genes involved in glycolysis and monocarboxylic acid metabolism were downregulated (Niemiec *et al.*, 2017). Arginine metabolism was differentially regulated, with *CAR1/2* repressed in yeast and induced in hyphae and *ARG1/3* induced in yeast and repressed in hyphae. Cross-referencing of *C. albicans* genes upregulated by interactions with neutrophils and components of the hyphal secretome identified 3 genes: *ZTA1, FDH12* and orf19.5250 (Niemiec *et al.*, 2017; Vaz *et al.*, 2021). *ZTA1* (ζ-crystallin) is predicted to be a quinone oxidoreductase and has a role in protection from endogenous oxidative stress. *C. albicans zta1Δ* mutants were more susceptible to neutrophil killing *in vitro* but had no difference in susceptibility to ROS *in vitro* (Gandra *et al.*, 2023). This suggests *ZTA1* is involved in an alternative method of immune evasion, which may be suppression of neutrophil RNS. *FDH12,* also called *FDH1*, is formate dehydrogenase and is involved in oxidative metabolism (Doedt *et al.*, 2004). Orf19.5250 is an uncharacterised protein with unknown function. It is possible that one of these proteins has a role in neutrophil RNS suppression. A key caveat on these observations is that I

observed a lower level of RNS suppression by *C. albicans* than in previous experiments – a 53.1% decrease in RNS levels compared to approximately a 90.0% decrease. Furthermore, heat killed *C. albicans* TT21-dTomato did not cause an intermediate level of RNS suppression in this experiment. Given the higher than expected RNS levels for wild type *C. albicans*, it is possible that yeast-locked *C. albicans* also induced higher host RNS levels and the ability of yeast-locked *C. albicans* to suppress host RNS is being underestimated.

C. albicans was able to undergo the yeast-to-hyphae transition in zebrafish embryos at 28 °C. I observed invasive hyphae in 33.3% of *C. albicans* TT21-dTomato infected zebrafish at 24 hpi, with 38.9% of *C. albicans* infections solely in the yeast morphotype. In contrast, Seman *et al.* observed 21.7% of *C. albicans* infections in zebrafish were solely yeast at 30 hpi, 24.5% yeast at 44 hpi and 67.5% yeast at 68 hpi (Seman *et al.*, 2018). I incubated my zebrafish at 28 °C, whereas Seman *et al.* incubated their zebrafish at 21 °C. This could suggest *C. albicans* preferentially forms hyphae at lower temperatures, though this would conflict previous literature that shows 37 °C is the optimum temperature for hyphae formation (Shapiro *et al.*, 2009, 2012; Desai *et al.*, 2015). An alternative explanation may be a reduction in zebrafish immune activity at 21 °C, permitting uncontrolled fungal growth. However, the authors claim the reduced temperature had no effect on host immune function, based on similar full body neutrophil count and neutrophil recruitment to a tail wound at 21 °C, 28 °C and 33 °C (Seman *et al.*, 2018). Multiple other differences existed between the experiments that may be responsible for the discrepancy in hyphae formation: Seman *et al*. injected 20 cfu into the yolk (a nutrient rich environment) at the prim-22 stage (approximately 36 hpf), whereas I injected 200 cfu into the caudal vein to generate a systemic infection at 30-35 hpf.

Finally, *C. albicans* suppression of host RNS levels was shown to not be host TLR-dependent in a zebrafish model. TLRs have traditionally been associated with a protective effect in the antifungal immune response (Netea *et al.*, 2015; Burgess, Condliffe and Elks, 2022). *C. albicans* Sel1 drives a pro-inflammatory cytokine response via TLR2/4 (Wang *et al.*, 2019). A cohort of patients with TLR3 L412F mutant all had increased susceptibility to chronic candidiasis. Expression of the TLR3 L412F variant on HEK-293 cells *in vitro* revealed reduced activation of NF-κB signalling by this TLR3 variant, suggesting dysfunctional TLR3 signalling is responsible for susceptibility to candidiasis in these patients (Nahum *et al.*, 2011). Syk-deficient neutrophils are known to have poor candidacidal activity. LPS was used to trigger TLR signalling pathways in syk-deficient murine neutrophils *in vitro*, resulting in a partial restoration of neutrophil function and improved candidacidal activity through phagocytosis, degranulation and neutrophil swarming (Viens *et al.*, 2022). LPS priming did not increase the ability of syk-deficient neutrophils to produce ROS, suggesting ROS induction involves a syk-dependent mechanism or is independent of TLR signalling in murine neutrophils. Restoration of

neutrophil candidacidal activity by LPS priming was recapitulated in human *ex vivo* neutrophils treated with kinase inhibitors (Viens *et al.*, 2022). Hence, TLR activation can play an important role in stimulating neutrophil activity against *C. albicans* infections in a syk-independent manner.

Vaccination against *C. albicans*, using *C. albicans* extracellular vesicles, was shown to induce protection in a mouse model that was dependent on TLR4 (Honorato *et al.*, 2024). However, there is precedent for pathogens acting via TLRs to manipulate immune responses: *Bacteroides fragilis* binds to TLR2 to promote immune tolerance and *Cryptococcus neoformans* CPL1 binds to TLR4 to promote M2 macrophage polarisation (Round *et al.*, 2011; Ivanov and Honda, 2012; Dang *et al.*, 2022). TLR2 knockout mice were shown to be more resistant to disseminated *C. albicans* infection than wild type mice, as *C. albicans* can induce release of anti-inflammatory IL-10 via TLR2 (Netea *et al.*, 2004). *C. albicans* chitin also promotes IL-10 release via TLR9, dampening LPS-driven inflammation in mice *in vivo* (Wagener *et al.*, 2014). Chitin was further revealed to prevent platelet adhesion aggregation via TLR8, which was speculated to aid *C. albicans* immune evasion (Leroy *et al.*, 2019). These conflicting roles of TLR signalling in *C. albicans* infection reveal a complex interplay of pro- and anti-inflammatory signalling. No effect on *C. albicans* suppression of neutrophil RNS was observed in zebrafish injected with *myd88* morpholino, suggesting RNS suppression occurs independent of the complex TLR-*Candida* interactions. No difference in survival was observed in control and *myd88* morpholino zebrafish, implying TLR signalling does not play a large role in innate immune responses to *C. albicans* infections in zebrafish *in vivo*. This may suggest other PRRs have a more important role in early anti-*Candida* immunity *in vivo*. Although I demonstrated morpholino knockdown of *myd88* at an RNA level, no data has been included to demonstrate absence of functional Myd88 protein. It is possible that, despite the splice variant morpholino, some functional Myd88 protein remains, meaning the true phenotype of *myd88* knockdown was not observed.

CARD9 was also investigated as a host factor involved in *C. albicans* suppression of neutrophil RNS. The designed CRISPR gRNAs were unable to cause successful knockdown of CARD9 when injected individually or when co-injected. This experiment took place at the end of my PhD, so there was insufficient time to design and test new CRISPR guides. Alternatively, future investigations could use a stable Card9 knockout zebrafish line, recently produced by the Zebrafish Mutation Project (Glass, Robinson and Rosowski, 2025). Glass and colleagues validated that Card9 knockout inhibits CLR signalling in zebrafish, confirming the rationality behind targeting Card9 in my experiments. CLRs are well documented as being critical for antifungal immunity (Hardison and Brown, 2012). However, Marakala *et al.* noticed the importance of Dectin-1 for anti-*Candida* immunity varies with different *C. albicans* strains. Dectin-1 knockout mice were hypersusceptible to *C. albicans* SC5314 infection but had similar survival to wild type mice when infected with *C. albicans* ATCC18804. Dectin-1 knockout

mice also had reduced pro-inflammatory cytokine production with *C. albicans* SC5314 compared to wild type mice, whereas no differences were observed in *C. albicans* ATCC18804 infection (Marakalala *et al.*, 2013). The authors believed the differences in immune responses was caused by variations in the cell wall composition of different *C. albicans* strains. It is possible that these cell wall variations are also responsible for mediating neutrophil RNS suppression via CLRs. The investigations in this thesis have solely used *C. albicans* strains derived from *C. albicans* SC5314. Given the possibility for cell wall differences, further investigations should check whether neutrophil RNS suppression is conserved across different *C. albicans* strains.

In this chapter, I aimed to investigate mechanisms underlying *C. albicans* suppression of neutrophil RNS *in vivo*. I observed an increase in neutrophils expressing arginase and neutrophils expressing IL-1β, suggesting divergent pro- and anti-inflammatory responses to *C. albicans* and implying neutrophil heterogeneity. *C. albicans* arginase (*CAR1)* was shown to have a role in neutrophil RNS suppression, though it is unclear whether this is through induction of host arginase and an anti-inflammatory response or consumption of local arginine to prevent RNS synthesis. *C. albicans* hyphae were revealed to be key for the active component of host RNS suppression. There may be a hyphal secreted compound partially responsible for RNS suppression. Further investigation is required to identify the host factors involved in RNS suppression. Additionally, different strains of *C. albicans* should be explored to reveal whether host RNS suppression is conserved across multiple *C. albicans* strains.

5. Suppression of neutrophil RNS levels is clinically relevant and conserved across *Candida* spp.

5.1 Introduction

5.1.1 *C. albicans* clinical isolates

The majority of pathogen research is conducted using laboratory reference strains: standardised strains of a pathogen, often with fully sequenced genomes. Use of reference strains ensures consistency and, owing to the characterised genome, facilitates better genetic understanding of measured phenotypes (Davel *et al.*, 2019; Ceballos-Garzon *et al.*, 2025). *C. albicans* SC5314, and its derivatives, is the primary reference strain used for studying *C. albicans* (Iracane *et al.*, 2024). Strain SC5314 was isolated from a patient with systemic *Candida* infection in New York, USA (Odds, Brown and Gow, 2004). *C. albicans* SC5314 first appeared in publications in the 1960s and was widely used by scientists at E.R. Squibb (now Bristol-Myers Squibb) throughout the 1980s (Maestrone and Semar, 1968; Meyers *et al.*, 1968; Odds, Brown and Gow, 2004). Fonzi and Irwin used targeted mutagenesis of *C. albicans* SC5314 to remove the *URA3* locus, creating an array of daughter strains that subsequently were the basis for most transgenic strains and genetic research into *C. albicans* (Fonzi and Irwin, 1993; Odds, Brown and Gow, 2004). *C. albicans* SC5314 was the first *C. albicans* strain to have a fully sequence genome, chosen because of its widespread use in molecular and host-pathogen interaction studies (Jones *et al.*, 2004).

However, strain SC5314 may not be the most accurate representation of the wider spectrum of *C. albicans* isolates. SC5314 was selected as a reference strain for arbitrary reasons, and some have argued overreliance on *C. albicans* SC5314 has resulted in observation biases, preventing a broad understanding of *C. albicans* biology. *C. albicans* SC5314 was revealed to have a defective variant of *AGO1* – a *C. albicans* homolog of Argonaute, which is used in RNA interference (RNAi). The widespread use of the *C. albicans* SC5314 *AGO1*-K361 variant led to a longstanding dogma that *C. albicans* did not have a functional RNAi system. However, 97% of *C. albicans* clinical isolates have an *AGO1*-E361 variant, which is RNAi active (Iracane *et al.*, 2024). Hence, *C. albicans* SC5314 is unsuitable to study RNAi pathways, which are present in 97% of clinical isolates, revealing a fundamental flaw in the most widely used *C. albicans* reference strain. In other fungi, RNAi has important roles in genome integrity, defence against mycoviruses, stress responses, drug tolerance and virulence (Lax *et al.*, 2020). Hence, regulation of stress responses and virulence in *C. albicans* SC5314 may not be representative of regulation of these phenotypes in *C. albicans* clinical isolates, which may have restricted the detection of potential therapeutic targets. Other differences have been observed between *C. albicans* reference strains and clinical isolates. Biofilm formation was

compared across a range of *C. albicans* reference strains and clinical isolates. While 3 reference strains had comparable biofilm formation to the tested clinical isolates, *C. albicans* SC5314 (ATCC MYA-2876), *C. albicans* B311 (ATCC 32354) and *C. albicans* X657 (ATCC 90234) had significantly greater biofilm formation than the clinical isolates, based on *C. albicans* monoculture *in vitro* (Alnuaimi *et al.*, 2013). This reveals a heterogeneity between reference strains and clinical isolates and between different reference strains. Examination of filamentation *in vitro* by clinical isolates showed almost all *C. albicans* clinical isolates showed severe filamentation defects on a range of solid media (YPD, 10% FBS with glucose, Lee's media and RPMI media) compared to SC5314 (Brandquist *et al.*, 2023). No correlation was observed between filamentation and clades or whether the isolate caused systemic or non-systemic infections. However, hyphae formation *in vivo* was not considered in this investigation, which may have revealed more nuanced variations in filamentation phenotype. *C. albicans* SC5314 is highly invasive and pathogenic compared to other strains. Glazier *et al.* investigated genetic causes of this hypervirulence. *C. albicans rob1Δ/Δ* mutants have previously been shown to have reduced filamentation *in vitro* (Homann *et al.*, 2009). Glazier *et al.* revealed SC5314 is heterozygous at *ROB1*, with a *ROB1*-S946 allele and a *ROB1*-P946 allele (Glazier *et al.*, 2023). All of the other 224 analysed isolates were homozygous for *ROB1*-P946, revealing a unique mutation in *C. albicans* SC5314. *C. albicans* SC5314 $ROB1^{P946}$*/rob1Δ* (lacking the *ROB1*-S946 allele) had reduced hyphae formation *in vitro* and reduced biofilm formation *in vitro* and *in vivo*. The *ROB1*-S946 allele is a phenotypic gain-of-function mutation only present in *C. albicans* SC5314, while *ROB1*-P946 is phenotypically less active but more prevalent allele (Glazier *et al.*, 2023). 2150 genes were differentially expressed in a *C. albicans rob1Δ/Δ* mutant, suggesting the phenotypically more active *ROB1*-S946 allele could be influencing a wide range of downstream targets in SC5314 (Nobile *et al.*, 2012). Together, these findings reveal the shortcomings of using *C. albicans* SC5314 for the majority of *Candida* research and highlight the need to validate major findings in a range of clinical isolates.

5.1.2 Hypothesis & Aims

In the previous chapters, *C. albicans* has been shown to suppress neutrophil RNS production *in vivo*. However, these experiments have used laboratory reference strains derived from *C. albicans* SC5314 – a strain used widely for molecular research historically and that may not accurately reflect the wider population of *C. albicans* isolates (Iracane *et al.*, 2024). Furthermore, invasive candidiasis is caused by *Candida* spp. other than *C. albicans*: 6 *Candida* spp. are included in the WHO fungal priority pathogens list (WHO, 2022). These non-albicans *Candida* spp. represent an understudied but clinically important group of pathogens. Suppression of neutrophil RNS is currently a gap in our

knowledge and may be a clinically relevant observation that could impact pathogenesis of *C. albicans* and other *Candida* spp.

I hypothesised that **neutrophil RNS suppression *in vivo* is conserved across *C. albicans* clinical isolates and across non-albicans *Candida* spp.** To address this hypothesis, the following aims were addressed:

- Determine whether *C. albicans* clinical isolates are capable of neutrophil RNS suppression *in vivo*
- Compare differences in RNS suppression by *C. albicans* clinical isolates with virulence *in vivo*
- Explore whether non-albicans *Candida* spp. clinical isolates are capable of neutrophil RNS suppression *in vivo*
- Compare differences in RNS suppression by non-albicans *Candida* spp. clinical isolates with virulence *in vivo*

5.2 Results

5.2.1 RNS suppression by *C. albicans* is conserved across clinical isolates

I aimed to investigate whether neutrophil RNS suppression is conserved across a range of *C. albicans* clinical isolates. Four *C. albicans* clinical isolates were selected for these experiments: two sourced from patients with invasive candidiasis and two from non-invasive sampling (AJP isolates, Table 2.6). *Tg(mpx:GFP)* i114 zebrafish embryos were injected into the caudal vein with 200 cfu *C. albicans* TT21-dTomato, *C. albicans* AJP4, *C. albicans* AJP5, *C. albicans* AJP9, *C. albicans* AJP25 or PVP (as a negative control). At 24 hpi, embryos were fixed in 4% PFA and stained with an anti-nitrotyrosine antibody as an indirect measure of RNS production. *C. albicans* clinical isolates lacked a fluorophore, so brightfield microscopy was used to identify sites of infection in stained embryos.

C. albicans TT21-dTomato caused an 80.2% decrease in anti-NT levels compared to PVP injected embryos (Figure 5.1; PVP=5.87, heat-killed *C. albicans* TT21-dTomato=1.16; $p<0.0001$). All *C. albicans* clinical isolates caused a decrease in anti-NT levels compared to PVP, but to varying degrees. *C. albicans* AJP4 caused a 60.0% decrease compared to PVP (*C. albicans* AJP4 =2.35; $p<0.0001$), while *C. albicans* AJP5 caused a 69.4% decrease in anti-NT levels compared to PVP (*C. albicans* AJP5=1.80; $p<0.0001$). Both *C. albicans* AJP4 and *C. albicans* AJP5 caused less suppression of anti-NT levels than *C. albicans* TT21-dTomato ($p<0.001$ and $p<0.01$ respectively), suggesting these clinical isolates may be less potent at suppressing RNS levels than the laboratory reference strain.

C. albicans AJP9 caused a 73.9% decrease in anti-NT levels compared to PVP (Figure 5.1; *C. albicans* AJP9=1.53; $p<0.0001$), but was not significantly different from *C. albicans* TT21-dTomato. *C. albicans* AJP25 caused an 82.3% decrease in anti-NT levels compared to PVP (*C. albicans* AJP25=1.04; $p<0.0001$), which was greater than, but not significantly different to, the RNS suppression caused by *C. albicans* TT21-dTomato. There appeared to be no obvious link between the anti-NT levels and whether an isolate was invasive (AJP5 and AJP25) or non-invasive (AJP4 and AJP9). These observations demonstrate that RNS suppression by *C. albicans* is not unique to *C. albicans* SC5314 derivatives and is conserved more widely across *C. albicans* isolates, implying RNS suppression in *C. albicans* infections may be a clinically relevant phenomenon.

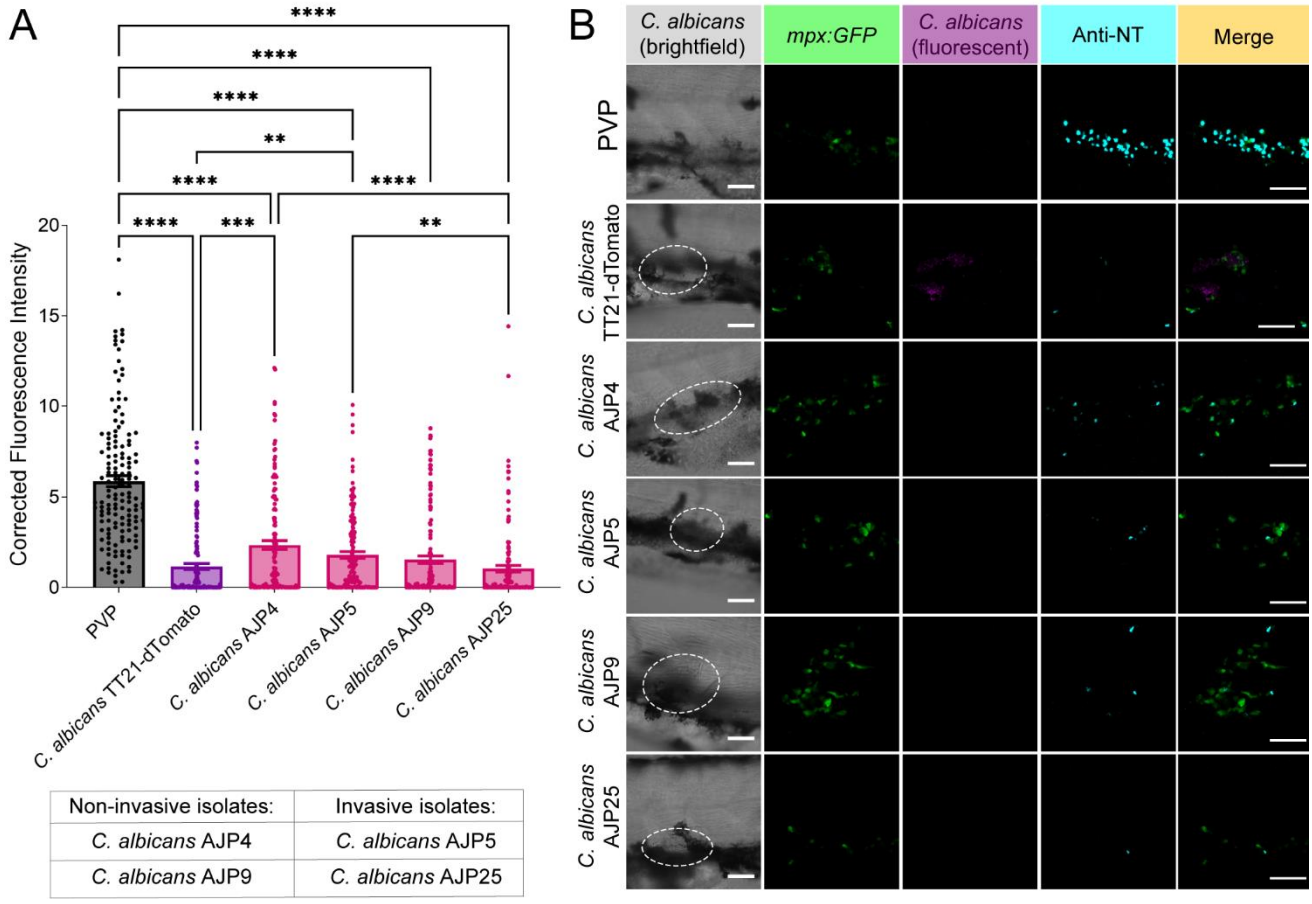

Figure 5.1: *C. albicans* clinical isolates suppress neutrophil RNS production.

(A) Anti-nitrotyrosine fluorescence at 24 hpi following injection of PVP, *C. albicans* TT21-dTomato, *C. albicans* AJP4, *C. albicans* AJP5, *C. albicans* AJP9 or *C. albicans* AJP25 into the caudal vein at 30 hpf. N=144 neutrophils from 24 fish, obtained from 3 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<0.001, ****p<0.0001.

(B) Representative images of PVP, *C. albicans* TT21-dTomato, *C. albicans* AJP4, *C. albicans* AJP5, *C. albicans* AJP9 and *C. albicans* AJP25 infected zebrafish embryos at 24 hpi. White dotted circles show *C. albicans.* Scale bars = 50 µm.

Neutrophil RNS suppression by *C. albicans* was conserved across different clinical isolates, to varying degrees. I aimed to investigate potential causes of the different levels of RNS suppression by *C. albicans* clinical isolates. First, I compared the ability of *C. albicans* clinical isolates to grow at different temperatures *in vitro*. *C. albicans* strains, with a starting OD_{600nm} of 0.1-0.2, were grown in YPD media at 30 °C in a shaking incubator at 200 rpm. OD_{600nm} was measured every hour for 10 hours, as a measure of *C. albicans* growth. Fungal growth *in vitro* was analysed at 3 temperatures:

28 °C (zebrafish incubation temperature), 30 °C (standard laboratory growth conditions for *C. albicans*) and 37 °C (human body temperature; work for 28 °C and 37 °C carried out by Imogen Ashcroft and Shami Vahivatdar, two undergraduate Medical SSC students, under my supervision).

At 30 °C, *C. albicans* TT21-dTomato (the lab reference strain) had the greatest amount of growth after 9 hours (Figure 5.2A; OD_{600nm}=7.705), which was slightly greater than the amount of growth by *C. albicans* AJP25 (OD_{600nm}=7.295). *C. albicans* AJP4 and AJP5 had the lowest amount of growth at 9 hours, with OD_{600nm} of 3.920 and 2.990 respectively. *C. albicans* AJP9 had an intermediate level of growth after 9 hours (OD_{600nm}=6.015). At 28 °C, a similar pattern was observed, with *C. albicans* TT21-dTomato having the greatest amount of growth and *C. albicans* AJP5 having the least amount of growth (Figure 5.2B). Across all isolates, the level of growth at 28 °C was lower than at 30 °C. At 37 °C, *C. albicans* AJP25 had the greatest growth after 5 hours (Figure 5.2C; OD_{600nm}=1.985) and *C. albicans* AJP5 had the least growth (OD_{600nm}=0.782). Across all isolates, the level of growth at 37 °C was greater than at 30 °C. Together, this demonstrates *C. albicans* clinical isolates are able to grow *in vitro* at standard growth conditions for laboratory strains and at zebrafish incubation temperature. Clinical isolates also grow in a temperature-dependent manner *in vitro*.

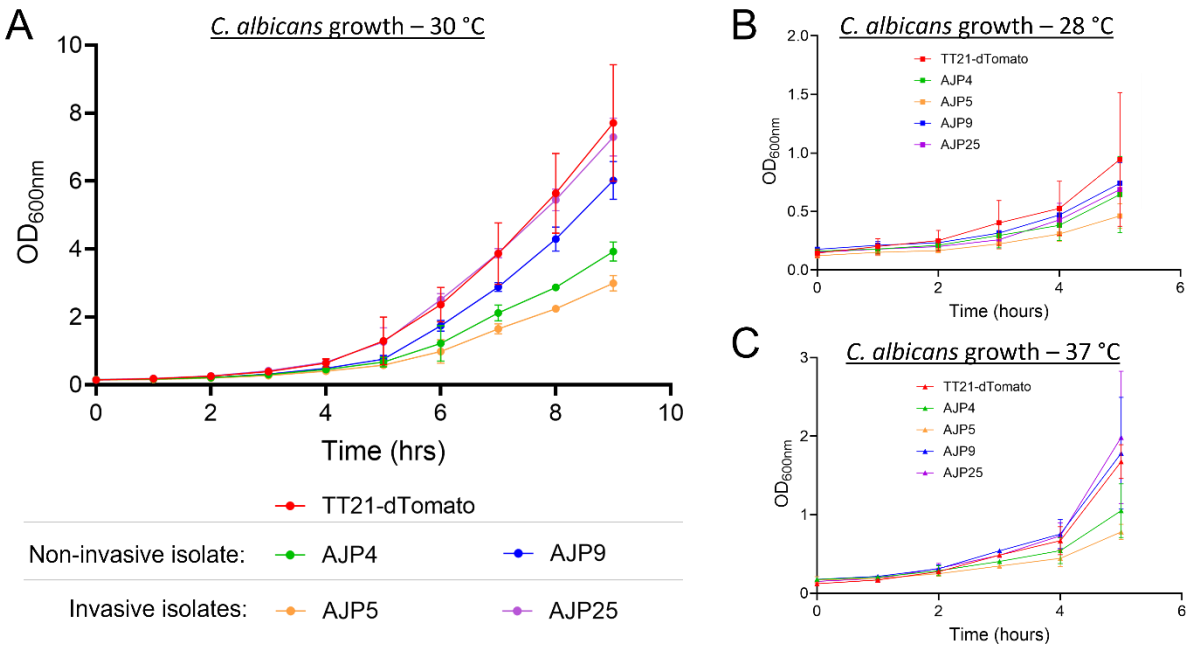

Figure 5.2: *C. albicans* clinical isolates grow at 30 °C *in vitro*.

(A) *C. albicans* clinical isolates were grown in aliquots of YPD liquid media. *C. albicans* TT21-dTomato in YPD media was used as a positive control. Cultures were grown in 20 ml volumes at 30 °C shaking at 200 rpm. Optical density (OD_{600nm}) of *C. albicans* cultures was measured every hour for 10 hours. N=2 independent experiments. Error bars show standard deviation.

(B) *C. albicans* clinical isolates were grown in aliquots of YPD liquid media. *C. albicans* TT21-dTomato in YPD media was used as a positive control. Cultures were grown in 20 ml volumes at 28 °C shaking at 200 rpm. Optical density (OD_{600nm}) of *C. albicans* cultures was measured every hour for 6 hours. N=3 independent experiments. Error bars show standard deviation. Measurements taken by Imogen Ashcroft and Shami Vahivatdar under my supervision.

(C) *C. albicans* clinical isolates were grown in aliquots of YPD liquid media. *C. albicans* TT21-dTomato in YPD media was used as a positive control. Cultures were grown in 20 ml volumes at 37 °C shaking at 200 rpm. Optical density (OD_{600nm}) of *C. albicans* cultures was measured every hour for 6 hours. N=3 independent experiments. Error bars show standard deviation. Measurements taken by Imogen Ashcroft and Shami Vahivatdar under my supervision.

To further examine potential causes of varying RNS suppression, I examined the ability of *C. albicans* clinical isolates to form hyphae in *in vivo* zebrafish infection. Brightfield images of *C. albicans* infected zebrafish embryos at 24 hpi were analysed and classified by degree of fungal growth, based on a previously described protocol (Thrikawala *et al.*, 2022).

54.1% *C. albicans* TT21-dTomato infections were solely in the yeast morphotype (Figure 5.3), while 25.0% infections were germinating, 4.2% were in hyphal extension and 16.7% were destructive hyphae. *C. albicans* AJP4 and AJP5 infections were 100.0% and 95.8% classified as yeast at 24 hpi. These isolates only being present in the yeast morphotype at 24 hpi may be explained by their slower growth rate (Figure 5.2) compared to the reference strain. 50.0% *C. albicans* AJP9 infections were 50.0% yeast, 12.5% were germinating, 20.8% were in hyphal extension and 16.7% were destructive hyphae (Figure 5.3), indicating a similar proportion of infections remaining in the yeast morphotype, but a more rapid progression to hyphal stages once the yeast-to-hyphal transition occurs. *C. albicans* AJP25 infections were 70.8% yeast, 16.7% germinating, 4.2% hyphal extension and 8.3% destructive hyphae.

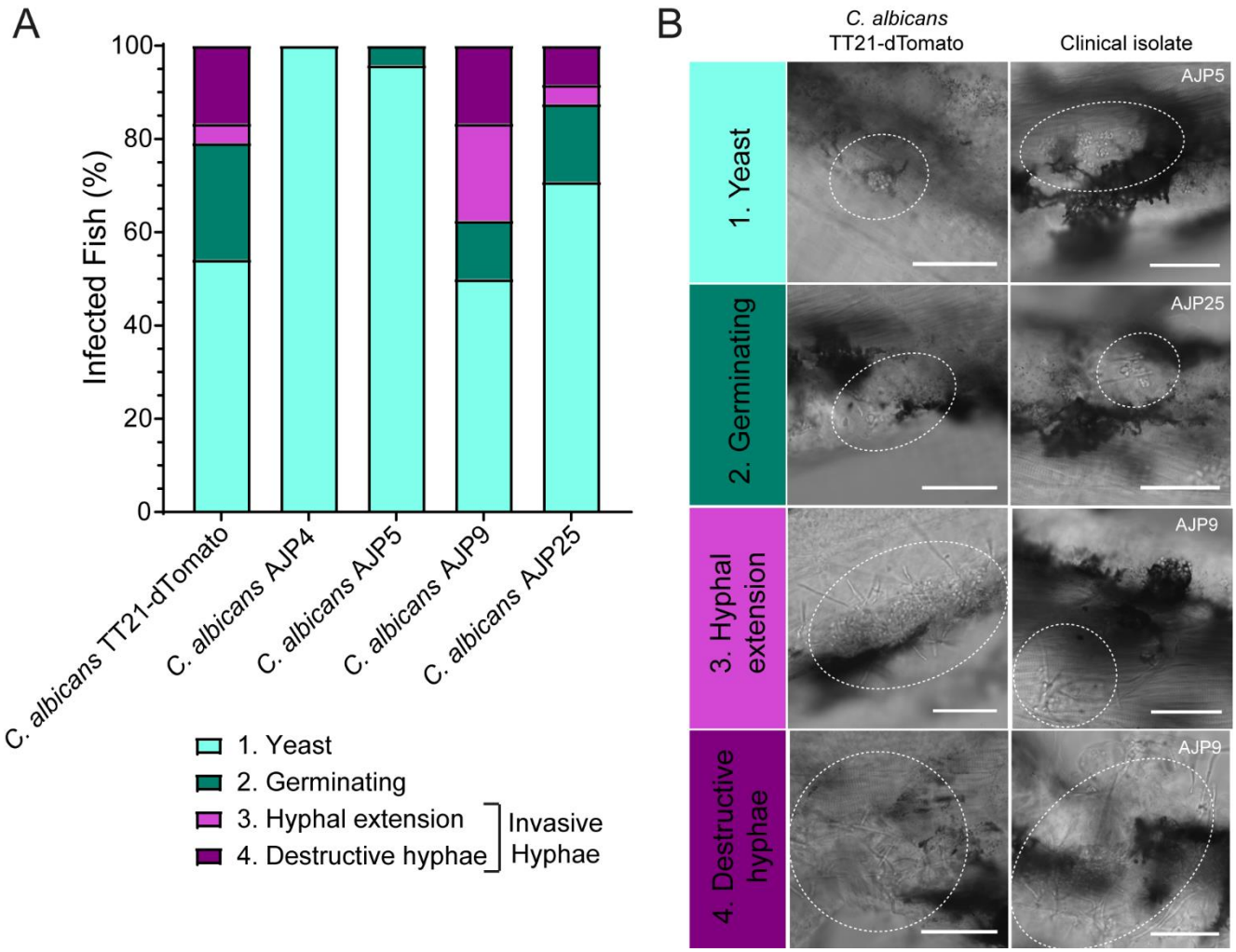

Figure 5.3: *C. albicans* clinical isolates hyphal severity.

(A) Hyphal severity of zebrafish embryos infected with range of *C. albicans* isolates at 24 hpi. N=24 fish, obtained from 3 independent experiments

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

Having observed differences in RNS suppression by *C. albicans* clinical isolates, I next aimed to investigate whether these differences in RNS suppression correlate with virulence in a zebrafish infection model. 1 dpf embryos were injected into the caudal vein with 200 cfu *C. albicans* clinical isolates, *C. albicans* TT21-dTomato or PVP.

C. albicans AJP4 and AJP5 had 85.2% and 90.4% survival ,respectively, at 4 dpi, significantly greater than the 37.0% survival rate of *C. albicans* TT21-dTomato at 4 dpi (Figure 5.4). Unlike the laboratory reference strains and other clinical isolates, *C. albicans* AJP4 and AJP5 had the greatest level of death between 3 dpi and 4 dpi, suggesting virulence may be delayed in these isolates. Both AJP4 and AJP5 had slower growth *in vitro*, which may explain this delayed virulence. *C. albicans* AJP9 and AJP25 had 20.0% and 19.3% survival at 4 dpi, significantly lower than *C. albicans* TT21-dTomato. As with the reference strain, the greatest amount of death was observed between 1 dpi and 2 dpi. There was no correlation between zebrafish survival and whether the clinical isolate was invasive (AJP5 and AJP25) or non-invasive (AJP4 and AJP9).

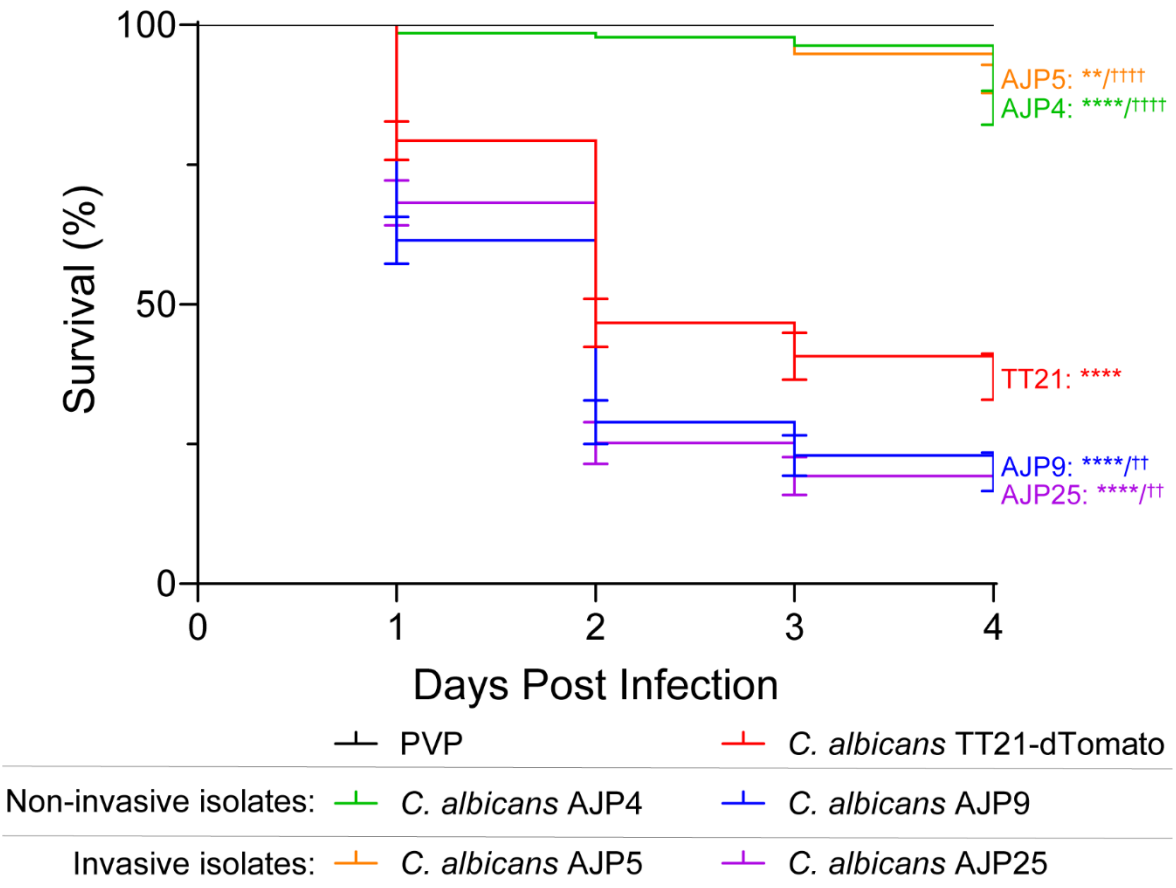

Figure 5.4: *C. albicans* clinical isolates have varying levels of virulence in zebrafish.

1 dpf nacre embryos were injected into the caudal vein with 200 cfu *C. albicans* TT21-dTomato, AJP4, AJP5, AJP9, AJP25 or PVP. Mortality was measured daily up to 4 dpi. n=135 fish, obtained from 3 independent experiments. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. * indicates difference compared to PVP. † indicates difference compared to *C. albicans* TT21-dTomato. P values shown: **/††p<0.01, ****/††††p<0.0001.

5.2.2 Suppression of neutrophil RNS is conserved in non-albicans *Candida* spp.

Although *C. albicans* is the leading causative agent of candidiasis, there are many other clinically important *Candida* spp. There are 6 *Candida* spp. in the WHO fungal priority pathogens list: *C. albicans, Candida auris, Candida* (*Nakaseomyces*) *glabrata, Candida tropicalis, Candida parapsilosis* and *Candida krusei* (WHO, 2022). I aimed to investigate the effect of a range of *Candida* spp. on neutrophil RNS levels, in order to determine if RNS suppression is unique to *C. albicans* and assess the wider clinical relevance of RNS suppression.

I selected of 4 *Candida* spp. clinical isolates: *C. glabrata* AJP12, *C. parapsilosis* AJP22, *C. guilliermondii* AJP24 and *C. auris* StG2 (Table 2.6). *C. glabrata, C. parapsilosis* and *C. auris* are high or critical priority fungal pathogens, responsible for a large percentage of non-albicans candidiasis. *C. guilliermondii* is rarer than the other selected isolates but was included because of its greater prevalence in the environment compared to other *Candida* spp. and as it is less well studied than other *Candida* spp. 1 dpf *Tg(mpx:GFP)* embryos were injected with 200 cfu *C. albicans* TT21-dTomato, *C. glabrata* AJP12, *C. parapsilosis* AJP22, *C. guilliermondii* AJP24, *C. auris* StG2 or PVP into the caudal vein to generate a systemic infection. Embryos were fixed at 24 hpi and stained with anti-nitrotyrosine antibody to allow quantification of neutrophil RNS production.

C. albicans TT21-dTomato infection caused an 85.2% decrease in neutrophil anti-NT levels, from 3.25 in PVP injected embryos to 0.48 (Figure 5.5; $p<0.0001$), corroborating my previous observations (see Chapter 3 and Figure 5.1). *C. glabrata* AJP12 reduced anti-NT levels to 1.78 (45.2% decrease compared to PVP; $p<0.0001$). This was significantly lower anti-NT suppression than all other *Candida* spp. isolates. *C. parapsilosis* AJP22 caused a 77.1% decrease in anti-NT levels compared to PVP, from 3.25 to 0.74 ($p<0.0001$). This was significantly less than anti-NT levels with *C. glabrata* ($p<0.001$), but significantly greater than anti-NT levels in *C. albicans* infected embryos ($p<0.0001$). *C. guilliermondii* AJP24 and *C. auris* StG2 had similar effects on neutrophil anti-NT levels, causing a 71.1% ($p<0.0001$) and 69.3% ($p<0.0001$) decrease in anti-NT levels, respectively, compared to PVP. Anti-NT levels in embryos infected with *C. guilliermondii* AJP24 and *C. auris* StG2 was significantly greater than embryos infected with *C. albicans* TT21-dTomato ($p<0.0001$ for both). *C. guilliermondii* AJP24 caused lower anti-NT levels than *C. glabrata* AJP12 (*C. guilliermondii* = 0.94, *C. glabrata* = 1.78; $p<0.0001$), as did *C. auris* StG2 (*C. auris* = 1.00, *C. glabrata* = 1.78; $p<0.01$). Together, these results show that neutrophil RNS suppression is conserved across *Candida* spp. clinical isolates, but that levels of RNS suppression vary between species.

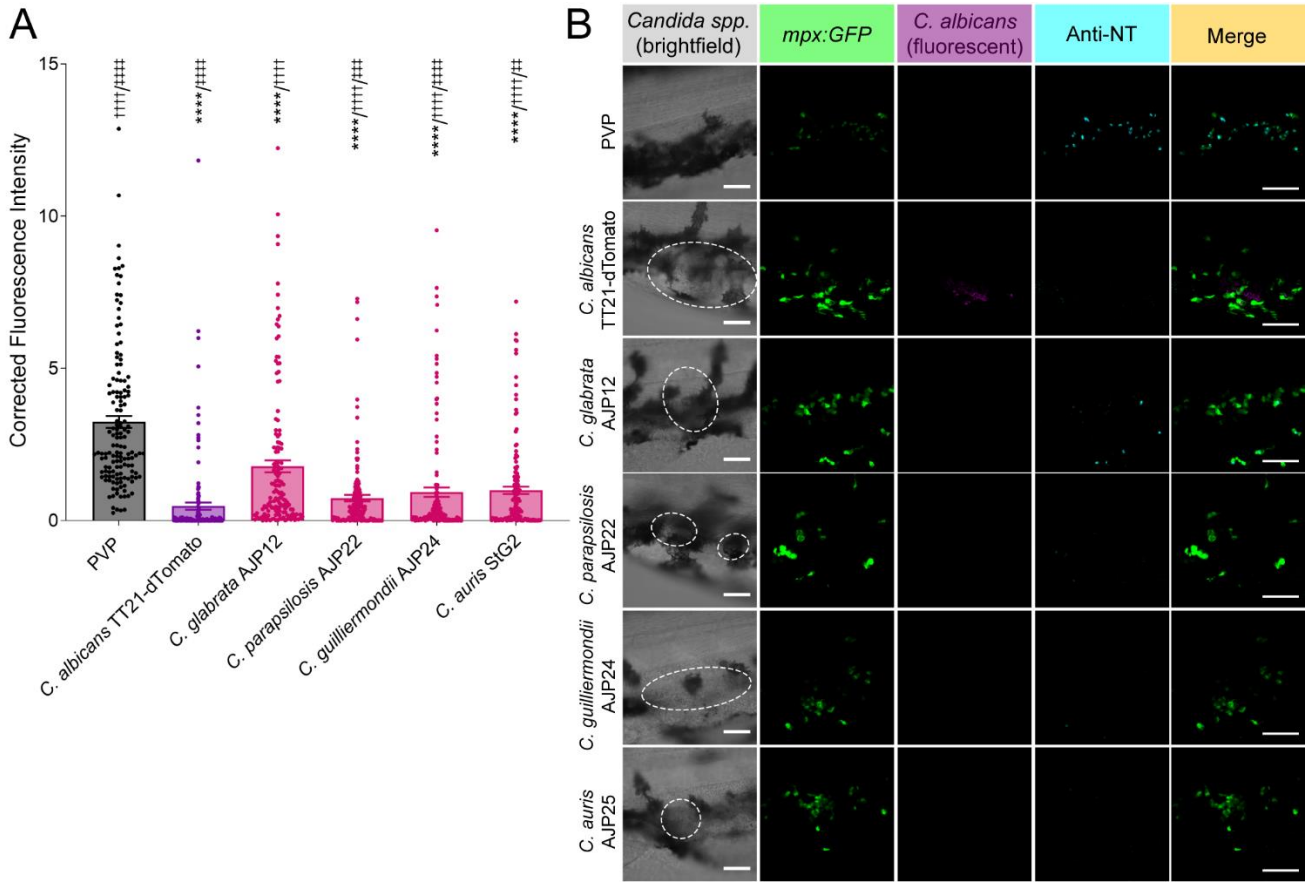

Figure 5.5: Non-albicans *Candida* spp. suppress neutrophil RNS levels.

(A) Anti-nitrotyrosine fluorescence at 24 hpi 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 3 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 embryos at 24 hpi. Scale bars = 50 µm.

As with previous clinical isolates, I aimed to explore potential reasons for differences in RNS suppression by *Candida* spp. clinical isolates. I first aimed to assess the ability of selected isolates to grow *in vitro* at relevant temperatures. From a starting OD_{600nm} of 0.1-0.2, isolates were grown in YPD media at 28 °C (zebrafish incubation temperature) in a shaking incubator at 200 rpm. OD_{600nm} was measured every hour for 7 hours, as a measure of *Candida* spp. growth. Isolates were also cultured at 37 °C (work carried out by Imogen Ashcroft and Shami Vahivatdar under my supervision).

At 28 °C, *C. albicans* TT21-dTomato and *C. glabrata* AJP12 had similar OD_{600nm} (1.703 and 1.713, respectively), suggesting similar growth rates (Figure 5.6A). This was greater than the OD_{600nm} for *C. guilliermondii* AJP24 (1.175), *C. parapsilosis* AJP22 (1.025) and *C. auris* StG2 (0.774), revealing slower growth rates in these isolates. *C. glabrata* AJP12 also had the greatest growth at 37 °C (Figure 5.6B; OD_{600nm}=5.853). Unlike at 28 °C, the growth of *C. glabrata* AJP12 was much greater than *C. albicans* TT21-dTomato at 37 °C (*C. albicans* TT21-dTomato OD_{600nm}=4.210). Growth of the remaining non-albicans *Candida* spp. at 37 °C was substantially lower: *C. parapsilosis* AJP22 OD_{600nm}=2.043, *C. guilliermondii* AJP24 OD_{600nm}=1.550 and *C. auris* StG2 OD_{600nm}=1.613. Across all isolates, growth was greater at 37 °C than at 28 °C.

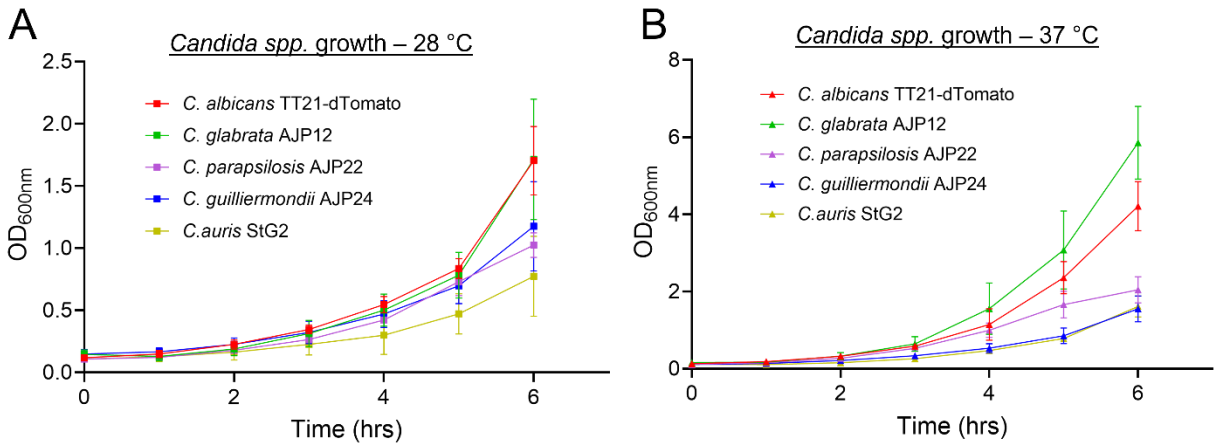

Figure 5.6: Non-albicans *Candida* spp. clinical isolates grow at 28 °C and 37 °C *in vitro.*

(A) *Candida* spp. clinical isolates were grown in aliquots of YPD liquid media. *C. albicans* TT21-dTomato in YPD media was used as a positive control. Cultures were grown in 20 ml volumes at 28 °C shaking at 200 rpm. Optical density (OD_{600nm}) of *Candida* spp. cultures was measured every hour for 7 hours. N=3 independent experiments. Error bars show standard deviation. Work done by Imogen Ashcroft and Shami Vahivatdar under my supervision.

(B) *Candida* spp. clinical isolates were grown in aliquots of YPD liquid media. *C. albicans* TT21-dTomato in YPD media was used as a positive control. Cultures were grown in 20 ml volumes at 37 °C shaking at 200 rpm. Optical density (OD_{600nm}) of *Candida* spp. cultures was measured every hour for 7 hours. N=3 independent experiments. Error bars show standard deviation. Work done by Imogen Ashcroft and Shami Vahivatdar under my supervision.

Next, I aimed to investigate whether the differences in RNS suppression correlated with virulence. 1 dpf nacre embryos were injected with 200 cfu *C. albicans* TT21-dTomato, *C. glabrata* AJP12, *C. parapsilosis* AJP22, *C. guilliermondii* AJP24, *C. auris* StG2 or PVP into the caudal vein. Mortality was monitored daily up to 4 dpi.

All embryos injected with PVP survived up to 4 dpi. *C. albicans* TT21-dTomato had 20.0% survival at 4 dpi – higher than expected at that infection dose (Figure 5.7A). *C. glabrata* AJP12 had 96.7% survival at 4 dpi, which was not significantly different from PVP (Figure 5.7A,B). Although 200 cfu *C. glabrata* AJP12 was able to suppress neutrophil RNS production, *C. glabrata* AJP12 appears to lack virulence at this infection dose. *C. parapsilosis* AJP22 had 68.9% survival at 4 dpi (Figure 5.7A,C), which was significantly less than PVP ($p<0.0001$) and greater than *C. albicans* TT21-dTomato ($p<0.0001$).

Embryo survival in *C. guilliermondii* infection was 80.0% (Figure 5.7A,D) and 65.6% in *C. auris* infection (Figure 5.7A,E) at 4 dpi. Across all non-albicans *Candida* spp. isolates, the greatest mortality occurred between 3 dpi and 4 dpi, except for *C. parapsilosis,* for which the greatest mortality was observed between 2 dpi and 3 dpi. This is later than *C. albicans* TT21-dTomato, which had greatest mortality between 1 dpi and 2 dpi. It is possible the non-albicans *Candida* spp. isolates experienced a lag phase in their growth *in vivo,* reflecting their poor adaptation to growth in zebrafish, which delayed mortality until beyond the timeframe investigated in this experiment.

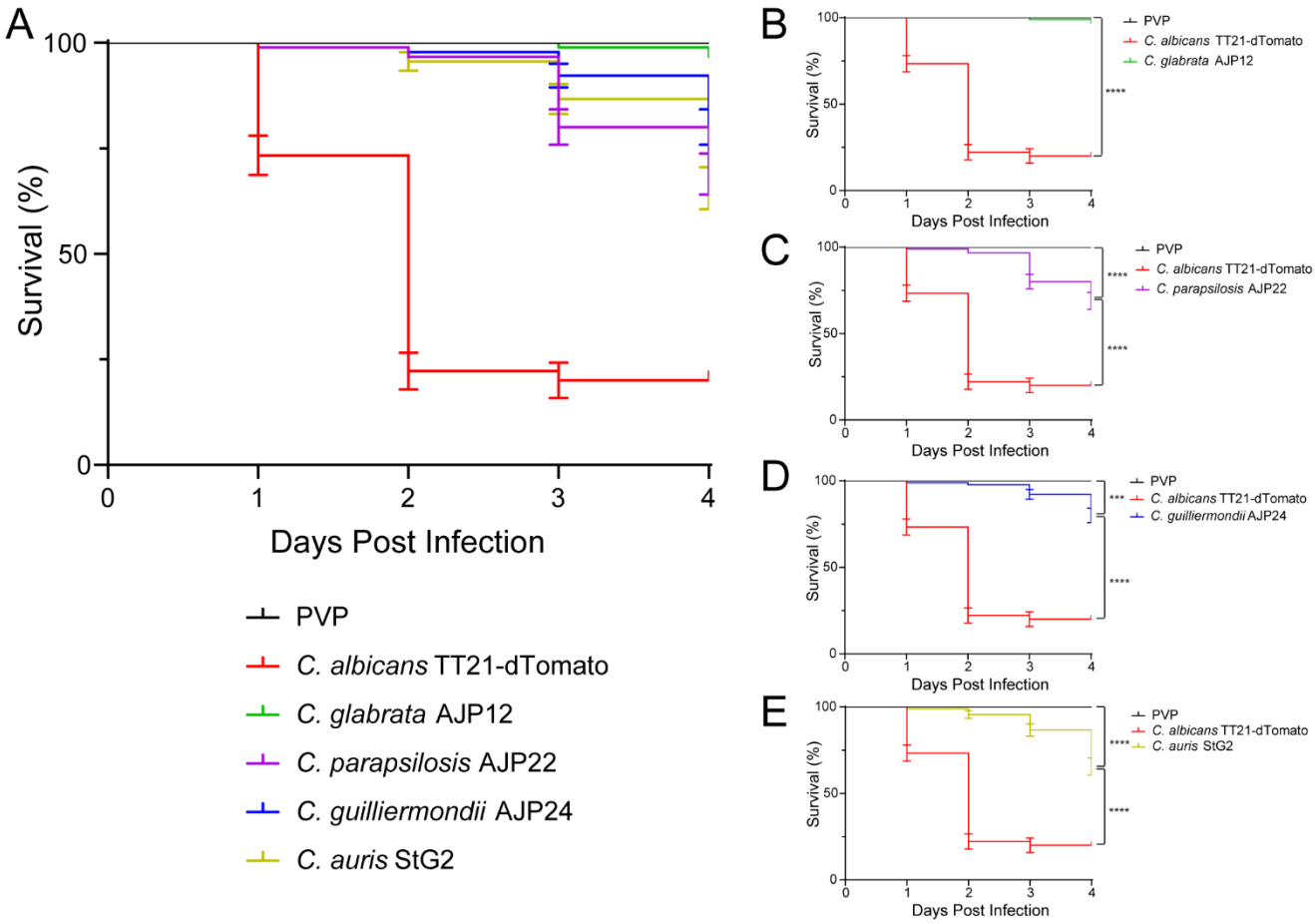

Figure 5.7: Non-albicans *Candida* spp. clinical isolates have varying levels of virulence in zebrafish.

(A) 1 dpf nacre embryos were injected into the caudal vein with 200 cfu *C. albicans* TT21-dTomato, *C. glabrata* AJP12, *C. parapsilosis* AJP22, *C. guilliermondii* AJP24, *C. auris* StG2 or PVP. Mortality was measured daily up to 4 dpi. n=90 fish, obtained from 2 independent experiments. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. Statistical differences only shown on (B-E) for simplicity.

(B) Survival curve from (A) showing only PVP, *C. albicans* TT21-dTomato and *C. glabrata* AJP12. n=90 fish, obtained from 2 independent experiments. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. P values shown: ****p<0.0001

(C) Survival curve from (A) showing only PVP, *C. albicans* TT21-dTomato and *C. parapsilosis* AJP22. n=90 fish, obtained from 2 independent experiments. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. P values shown: ****p<0.0001

(D) Survival curve from (A) showing only PVP, *C. albicans* TT21-dTomato and *C. guilliermondii* AJP24. n=90 fish, obtained from 2 independent experiments. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. P values shown: ***p<0.001, ****p<0.0001

(E) Survival curve from (A) showing only PVP, *C. albicans* TT21-dTomato and *C. auris* StG2. n=90 fish, obtained from 2 independent experiments. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. P values shown: ****p<0.0001

To attempt to overcome a potential lag phase in isolate growth *in vivo*, the survival curves were repeated using a greater infection dose of non-albicans *Candida* spp. isolates. 1 dpf nacre embryos were injected with 200 cfu *C. albicans* TT21-dTomato, 500 cfu *C. glabrata* AJP12, *C. parapsilosis* AJP22, *C. guilliermondii* AJP24, *C. auris* StG2 or PVP into the caudal vein.

All embryos injected with PVP survived up to 4 dpi. Survival of *C. albicans* TT21-dTomato infected embryos at 4 dpi was 51.1%, with the greatest mortality observed between 1 dpi and 2 dpi (Figure 5.8A). *C. glabrata* had reduced survival at 500 cfu, compared to 200 cfu, with 83.3% embryos surviving at 4 dpi – significantly less than PVP (Figure 5.8B; p<0.001). Hence, *C. glabrata* does display virulence *in vivo* at a higher infection dose (500 cfu). 70.0% embryos infected with *C. parapsilosis* AJP22 survived up to 4 dpi (similar to the survival rate of 200 cfu *C. parapsilosis* infection), while 4 dpi survival in *C. guilliermondii* AJP24 infected embryos was 65.6%. Survival of *C. auris* StG2 infected embryos at 4 dpi was 56.7%, which was not significantly different from *C. albicans* TT21-dTomato. For *C. parapsilosis* AJP22, *C. guilliermondii* AJP24 and *C. auris* StG2, the greatest mortality occurred after 2 dpi, later than *C. albicans* TT21-dTomato, suggesting a lag phase in the *in vivo* growth may still be occurring at the greater infection dose.

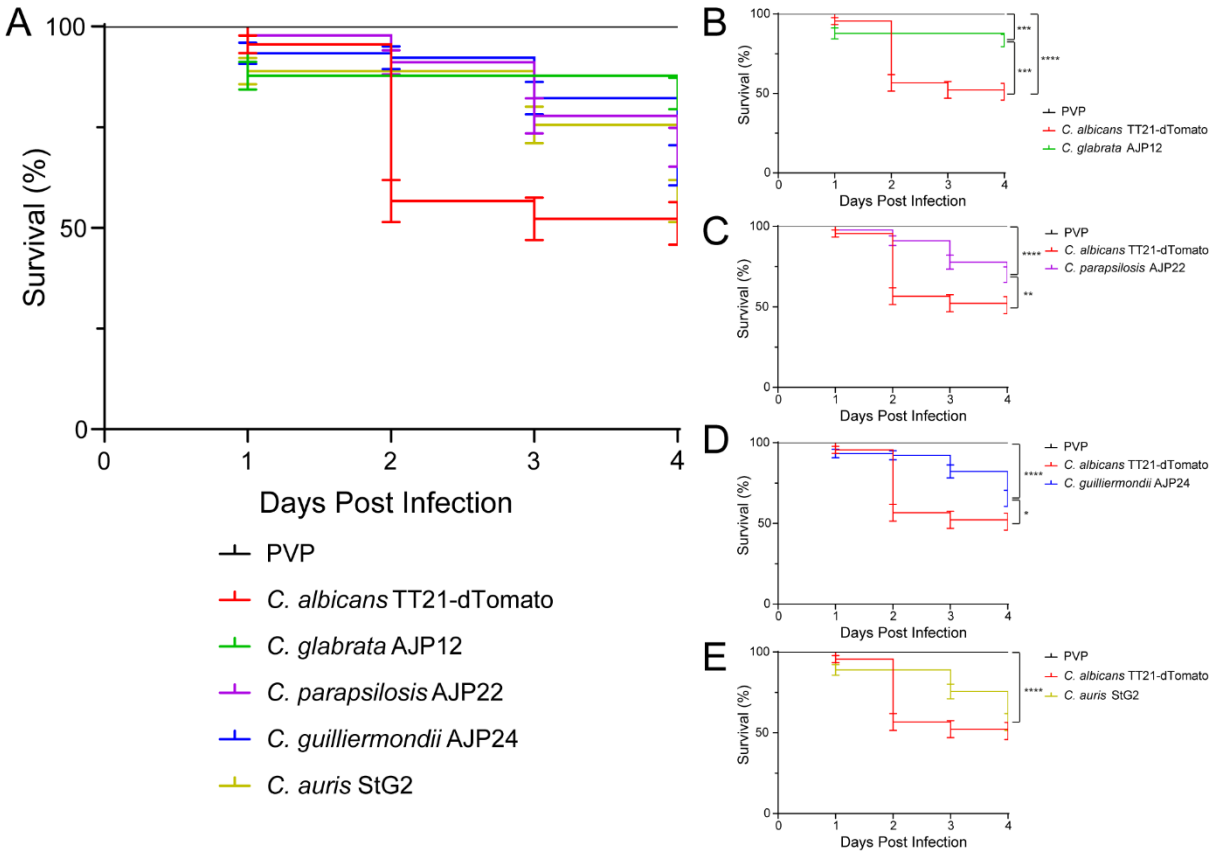

Figure 5.8: Non-albicans *Candida* spp. clinical isolates have varying levels of virulence.

(A) 1 dpf nacre embryos were injected into the caudal vein with 200 cfu *C. albicans* TT21-dTomato, 500 cfu *C. glabrata* AJP12, *C. parapsilosis* AJP22, *C. guilliermondii* AJP24, *C. auris* StG2 or PVP. Mortality was measured daily up to 4 dpi. n=90 fish, obtained from 2 independent experiments. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. Statistical differences only shown on (B-E) for simplicity.

(B) Survival curve from (A) showing only PVP, 200 cfu *C. albicans* TT21-dTomato and 500 cfu *C. glabrata* AJP12. n=90 fish, obtained from 2 independent experiments. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. P values shown: ***p<0.001, ****p<0.0001

(C) Survival curve from (A) showing only PVP, 200 cfu *C. albicans* TT21-dTomato and 500 cfu *C. parapsilosis* AJP22. n=90 fish, obtained from 2 independent experiments. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. P values shown: **p<0.01, ****p<0.0001

(D) Survival curve from (A) showing only PVP, 200 cfu *C. albicans* TT21-dTomato and 500 cfu *C. guilliermondii* AJP24. n=90 fish, obtained from 2 independent experiments. Statistical significance

determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. P values shown: *p<0.05, ****p<0.0001

(E) Survival curve from (A) showing only PVP, 200 cfu *C. albicans* TT21-dTomato and 500 cfu *C. auris* StG2. n=90 fish, obtained from 2 independent experiments. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. P values shown: ****p<0.0001

5.2.3 *Candida* spp. suppression of RNS correlates with virulence *in vivo*

Neutrophil RNS suppression and virulence in zebrafish infection *in vivo* was observed across a range of *C. albicans* laboratory strains and *Candida* spp. clinical isolates, to varying degrees. Given this inter-strain variation in virulence *in vivo* and neutrophil RNS suppression, I examined the potential for a correlation between virulence *in vivo* and the degree of neutrophil RNS suppression by *Candida* spp.

The reduction in RNS levels (measured as anti-NT level) compared to the relevant PVP control was plotted against zebrafish survival at 4 dpi following caudal vein injection of *Candida* spp. 4 dpi survival of 200 cfu non-albicans *Candida* spp. was used as anti-NT levels were measured in 200 cfu infections. Zebrafish survival at 4 dpi negatively correlated with reduction in neutrophil RNS levels (Figure 5.9). A Pearson's correlation coefficient was calculated at -0.718 (95% confidence interval: -0.208 – -0.921; $p<0.05$), indicative of a negative correlation. R^2 value was calculated at 0.516, demonstrating 51.6% of the variation in virulence can be explained by the variation in neutrophil RNS suppression. Hence, increased ability to suppress host RNS levels is correlated with increased virulence in zebrafish infection *in vivo*, suggesting *C. albicans* suppression of RNS may be an important virulence factor in this model.

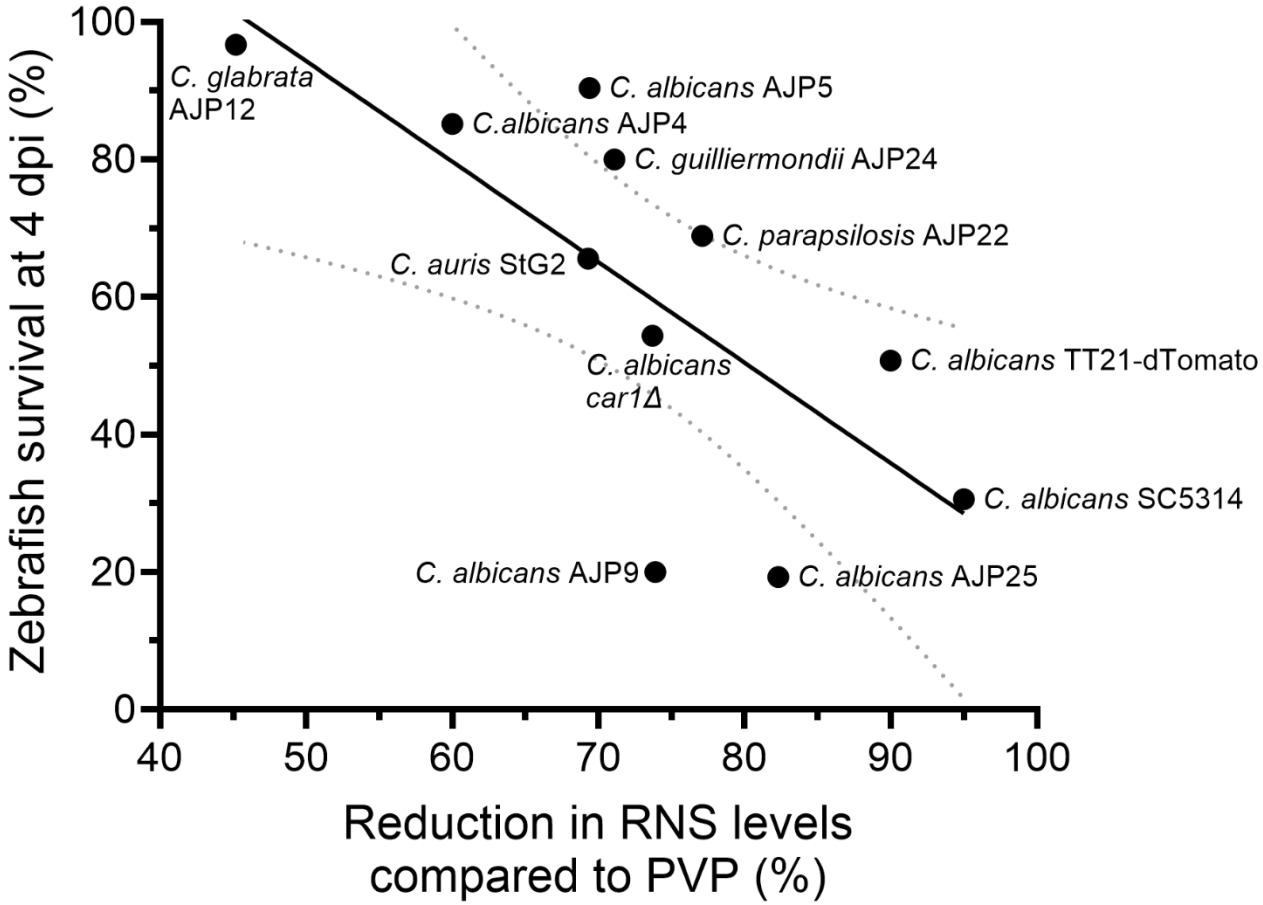

Figure 5.9: *Candida* spp. reduction of host RNS negatively correlates with zebrafish survival

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

5.3 Discussion

In this chapter, I demonstrated suppression of neutrophil RNS levels is conserved across a range of *C. albicans* and other *Candida* spp. clinical isolates. These results show that neutrophil RNS suppression by *Candida* spp. is well conserved across species, and is potentially a clinically relevant phenomenon that increases virulence of these species.

I showed *C. albicans* clinical isolates are able to suppress neutrophil RNS *in vivo*. No correlation was observed between an isolates ability to suppress neutrophil RNS and whether the isolate came from invasive or non-invasive disease. Thewes *et al*. aimed to characterise phenotypic and genotypic differences between an invasive and non-invasive laboratory strains of *C. albicans*. While *C. albicans* SC5314 (invasive) had greater ability to invade host tissue with hyphae than *C. albicans* ATCC10231 (non-invasive), no significant genetic differences between the strains were observed. Transcriptional profiling revealed non-invasive *C. albicans* ATCC10231 had significantly higher expression of 79 genes, including genes associated with oxidative stress, nitrogen metabolism and carbon metabolism compared to invasive *C. albicans* SC5314. *C. albicans* ATCC10231 also had higher expression of 24 genes with unknown function (Thewes *et al.*, 2008). These results suggest an invasive or non-invasive phenotype is not caused by the presence or absence of certain genes, but by differential expression of genes, though the mechanism underlying this differential expression is unknown. This investigation solely considered laboratory reference strains, which may not accurately reflect genotypic variations in clinical isolates. There have been many attempts to characterise genotypic differences related to the invasiveness of clinical isolates, with conflicting results. Luu *et al.* showed the genotype of *C. albicans* isolates obtained from the oral mucosa and from the bloodstream had no significant differences, suggesting no correlation between genotype and invasiveness (Luu *et al.*, 2001). In contrast, Karahan *et al.* demonstrated a correlation between genotype and invasiveness of *C. albicans* isolates, with the absence of transposable group I intron in the 25S rDNA gene being associated with increase invasiveness (Karahan *et al.*, 2004). Sampaio *et al.* investigated longitudinal adaptations of *C. albicans* by obtaining *C. albicans* isolates from a single immunocompromised patient with recurrent candidaemia over the course of 4 months while treated with fluconazole. Genotyping confirmed that all isolates obtained were the same strain of *C. albicans.* Using a mouse model of systemic candidiasis, the authors showed the virulence of isolates steadily decreased over the 4-month period. The authors proposed the initially virulent strain of *C. albicans* progressed to a disseminated infection upon the patient becoming immunocompromised, and then adapted to become less virulent and favour commensalism, as a strategy to promote its own survival (Sampaio *et al.*, 2010). A pathogen modulating its own virulence further may suggest that the occurrence of invasive disease, as opposed to non-invasive disease or commensalism, is reliant on host factors

('evolutionary pressures') rather than any specific genotypic variations in *C. albicans*. Epigenetic plasticity has been attributed as a key mechanism by which *C. albicans* can modulate gene expression and readily adapt to different environmental niches (Freire-Benéitez *et al.*, 2016). Hence, the clinical isolates used in my experiments may be differentially regulating their RNS suppressive mechanisms, potentially through epigenetic changes, resulting in the varying phenotype.

Although genotypic differences between invasive and non-invasive *C. albicans* clinical isolates have not been observed at a population level, this does not preclude the possibility of genotypic differences or polymorphic variation in the specific isolates I used in this chapter. No sequencing data was provided with the *C. albicans* clinical isolates used in this thesis. Transcriptional profiling and proteome analysis of *C. albicans* clinical isolates could help identify potential causes for the varying effect on host RNS levels in future studies.

In vitro growth of *C. albicans* clinical isolates was explored as a potential cause of the varying effect on host RNS levels. A correlation between *in vitro* growth rate and host RNS levels was observed, with isolates with lower *in vitro* growth rates (*C. albicans* AJP4/AJP5) having greater host RNS levels during infection. *C. albicans* AJP4 and AJP5 also had lower occurrence of invasive hyphae quantified *in vivo* than the other clinical isolates and laboratory strain. The exact link between *in vitro* growth rate and *in vivo* hyphal formation is unclear. Hornby *et al.* aimed to explore the causes of the inoculum size effect in *C. albicans*: the concept that, in identical conditions, high infection doses result in preferential growth of yeast, while low infection doses lead to preferential growth of hyphae (Hornby *et al.*, 2001). They revealed quorum sensing plays a role in *C. albicans* regulation hyphal formation (Hornby *et al.*, 2001). Farnesol, secreted by *C. albicans*, blocks degradation of fungal Nrg1 and Cup9. Inhibition of farnesol allows Nrg1 and Cup1 degradation, resulting in hyphal initiation (Lu *et al.*, 2014). In *C. albicans*, CUG codons are primarily transcribed as serine (95-97% of the time), as opposed to leucine in most other organisms (Miranda *et al.*, 2013). Recombinant *C. albicans* strains with 20-98% leucine at CUG codons had increased hyphal formation under non-inducing conditions, due to reduced farnesol production (Oliveira *et al.*, 2021). These observations suggest low growth may not be responsible for the lack of hyphal growth by *C. albicans* AJP4 and AJP5. However, neither Hornby nor Lu examined the influence of the host microenvironment on quorum sensing and induction of hyphal formation, as they solely conducted their experiments *in vitro*. Wakade *et al.* compared hyphae formation *in vitro* and in a mouse ear *in vivo* model. The 4 tested strains (including *C. albicans* SC5314) had similar filamentation phenotypes *in vivo*, but had greater variation in filamentation phenotype *in vitro*, suggesting *in vitro* and *in vivo* hyphae formation are not directly correlated (Wakade *et al.*, 2021). Furthermore, differential regulation of hyphae formation *in vitro* and *in vivo* was revealed, with *EFG1* and *BRG1* being important in both

models but *UME6* and *BCR1* being required for *in vitro* filamentation but not critical for *in vivo* filamentation (Wakade *et al.*, 2021). The authors suggested *C. albicans* filamentation phenotypes and transcriptional regulation vary across different niches, making it difficult to relate *in vitro* growth rates to zebrafish *in vivo* hyphae formation, and subsequently to host RNS suppression, in my experiments. Furthermore, any potential links between *in vitro* growth and *in vivo* hyphae formation in my experiments are complicated by the presence of the host immune response, which may have a disproportionately greater impact on slower growing isolates that are unable to establish a foothold before recruitment of innate immune cells. While it is logical that low *in vitro* growth would result in reduced fungal growth and low hyphae formation *in vivo*, the experiments in this chapter were insufficient to directly show a causal link.

C. albicans clinical isolates had varying filamentation phenotypes *in vivo* compared to *C. albicans* TT21-dTomato, with AJP4 and AJP5 having reduced hyphal formation, AJP25 having similar hyphal formation to TT21-dTomato and AJP9 having greater hyphal formation than TT21-dTomato. Brandquist *et al.* previously showed most *C. albicans* clinical isolates had reduced hyphae formation compared to *C. albicans* SC5314 *in vitro* (Brandquist *et al.*, 2023). Only 3 isolates had comparable filamentation phenotypes to *C. albicans* SC5314 on solid media. The lack of *in vivo* examination in Brandquist's study makes it difficult to directly infer how their results may relate to mine. Wakade *et al.* revealed non-related *C. albicans* strains had similar filamentation phenotypes in a mouse ear *in vivo* model, though this investigation used different laboratory strains, not clinical isolates (Wakade *et al.*, 2021). I observed clinical isolates with lower, similar and greater hyphae formation than the reference strain. No correlation was observed between hyphae formation and invasive/non-invasive isolates, which is supported by contemporary literature (Brandquist *et al.*, 2023). However, only 4 clinical isolates were used in my *in vivo* experiments, which is too small a sample size to make meaningful conclusions about hyphae formation in clinical isolates compared to reference strains. In addition, temperature is a regulator of hyphae formation (Mukaremera *et al.*, 2017), so the observed hyphal phenotypes are only relevant for 28 °C and may not be reproduced at 37 °C. It is possible the isolates with greater hyphal formation (AJP9 and AJP25) are better adapted to growing at 28 °C, facilitating the yeast-to-hyphal transition at this lower temperature. Human body temperatures can vary considerably depending on the body site, health status and ambient temperature (White *et al.*, 2011). AJP9 and AJP25 were isolated from the tongue (33 °C) and a line tip sample (28-36 °C, depending on the site, depth of the line tip and ambient temperature), respectively. AJP4 was a vaginal sample (36-37 °C;Fugl-Meyer, Sjögren and Johansson, 1984) and AJP5 was a blood sample (38 °C; Institute for Quality and Efficiency in Healthcare (IQWiG), 2023). Metabolic profiling of *Candida* spp. isolates sourced from the blood, respiratory tract and vagina revealed clustering of

isolate metabolomes based on their isolation sites, suggesting *Candida* spp. are able to adapt their metabolic phenotype to suit their local environment (Oliver *et al.*, 2020). Given their sites of isolation, it is possible AJP9 and AJP25 are better adapted to growth at lower temperatures, leading to increased hyphal formation at 28 °C, though this is entirely speculative.

C. albicans AJP4 and AJP5 lacked virulence in zebrafish *in vivo* infection compared to other clinical isolates and the reference strain. Hence, a correlation between *in vitro* growth, hyphae formation, host RNS suppression and virulence was observed. A likely reason for the low virulence of *C. albicans* AJP4 and AJP5 *in vivo* is slow growth, resulting in a low fungal burden, which could be controlled by the host immune system. However, this assumes that *in vitro* fungal growth is directly proportional to *in vivo* growth for these isolates. To confirm this hypothesis, *in vivo* growth of *C. albicans* clinical isolates could be estimated by culturing 1 dpi, 2 dpi, 3 dpi and 4 dpi *C. albicans*-infected zebrafish embryos on YPD plates and counting the number of *C. albicans* colonies formed. No correlation was observed between virulence and invasive/non-invasive isolates. Varying virulence of *C. albicans* clinical isolates in *in vivo* models has been consistently observed in other literature (Wingard *et al.*, 1982; Hu, Farah and Ashman, 2006; Ells *et al.*, 2014). Hyphae are associated with increased virulence (Dalle *et al.*, 2010), so it is not overly speculative to suggest greater hyphal formation is responsible for increased virulence in *C. albicans* TT21-dTomato, AJP9 and AJP25. It is more difficult to propose a causal link between host RNS suppression and virulence. Isolates with reduced host RNS suppression (greater RNS levels) showed a correlation with decreased virulence, though this is confounded with *in vitro* growth rate and hyphal formation. To demonstrate causality between RNS suppression and virulence, I first need to establish how *C. albicans* suppresses host RNS and then measure survival of zebrafish infected with *C. albicans* mutants lacking the ability to suppress host RNS.

Suppression of host RNS was conserved across different *Candida* spp. isolates, to varying degrees. *C. auris, C. parapsilosis* and *C. guilliermondii* all had similar levels of host RNS suppression – slightly less than *C. albicans*. These isolates all had lower *in vitro* growth than *C. albicans* TT21-dTomato, with *C. auris* having the lowest *in vitro* growth rate. Hence, either *in vitro* growth rate is not correlated with host RNS suppression, *in vitro* and *in vivo* growth are not correlated or *C. auris, C. parapsilosis* and *C. guilliermondii* have a more potent mechanism of suppressing host RNS than *C. albicans*, overcoming their lower *in vitro* growth. *C. glabrata* had equivalent *in vitro* growth to *C. albicans* TT21-dTomato but had significantly greater neutrophil RNS levels. This further suggests *in vitro* fungal growth is not associated with host RNS suppression and reveals *C. glabrata* may be less able to suppress host RNS levels than other *Candida* spp. An important caveat on this conclusion is that only 1 representative isolate of each non-albicans *Candida* spp. was used, so this may not be representative of RNS suppression by a wider population of non-albicans *Candida* spp.

Candida spp. virulence correlated with neutrophil RNS suppression *in vivo*. While this suggests neutrophil RNS suppression is an important virulence factor in determining zebrafish mortality, there are several confounding factors to weaken this conclusion. Virulence is determined by a wide range of factors, not solely neutrophil RNS suppression (Ghannoum and Abu-Elteen, 1990). Differential regulation of any virulence factor in these *Candida* spp. strains could be affecting virulence in zebrafish *in vivo*, which was not accounted for in this analysis. *Candida* spp. clinical isolates were not adapted for growth at 28 °C, whereas *C. albicans* TT21-dTomato was. These potential differences in *in vivo* growth could affect virulence in zebrafish. Only 11 strains were included in this analysis. Inclusion of a greater number of data points may reveal a stronger correlation between *Candida* spp. virulence and RNS suppression.

Human neutrophils *in vitro* were able to inhibit *C. albicans* growth by 75% but had no impact on *C. auris*, demonstrating *C. auris* is highly resistant to neutrophil killing *in vitro* (Johnson *et al.*, 2018). Measurement of neutrophil ROS production revealed *C. auris* induced lower neutrophil ROS production than *C. albicans* or PMA (phorbol 12-myristate 13-acetate). *C. auris* did not reduce neutrophil ROS production when combined with PMA, suggesting *C. auris* does not suppress ROS production but has mechanisms to avoid inducing neutrophil ROS production (Johnson *et al.*, 2018). Alternatively, *C. auris* suppression of ROS may operate via a separate pathway to PMA-induction of ROS, disguising the suppressive effect. Day *et al.* showed *C. auris* is highly resistant to hydrogen peroxide *in vitro* compared to *C. albicans.* Resistance to hydrogen peroxide by *C. auris* was dependent on Hog1 – a stress activated protein kinase that is highly conserved between fungal species (Nikolaou *et al.*, 2009; Day *et al.*, 2018). *C. auris hog1Δ* mutant strain had reduced virulence in a *C. elegans* infection model, though Hog1 homologs in other *Candida* spp. have wider roles in regulating virulence (such as regulating yeast-to-hyphae transition and white-opaque switching in *C. albicans*), so this reduction in virulence may not be entirely due to reduced ROS tolerance (Alonso-Monge *et al.*, 2003; Enjalbert *et al.*, 2006; Liang *et al.*, 2014; Day *et al.*, 2018). *C. auris hog1Δ* was also more susceptible to killing by murine bone-marrow-derived macrophages and neutrophils. Neutrophil hydrogen peroxide production was greater with *C. auris hog1Δ* than wild type *C. auris*, implying Hog1 may regulate methods to avoid inducing neutrophil ROS production as well as promoting tolerance (Shivarathri *et al.*, 2024). *C. auris hog1Δ* had increased β-glucan and decreased chitin in the cell wall. In *C. albicans*, β-glucan induces a pro-inflammatory response and chitin promotes arginase-1 expression and an anti-inflammatory phenotype in macrophages (Vautier, MacCallum and Brown, 2012; Wagener *et al.*, 2017). Therefore, increased neutrophil hydrogen peroxide production following exposure to *C. auris hog1Δ* may be the result of an increased pro-inflammatory response due to remodelling of the *C. auris* cell wall. While Johnson *et al.* and

Shivarathri *et al*. demonstrated *C. auris* can avoid inducing neutrophil hydrogen peroxide production, I observed a suppression of host RNS levels below the basal level, suggesting a more potent mechanism for *C. auris* suppression of host RNS than evading ROS.

The stress sensitivity of *Candida* spp. was monitored by Priest and Lorenz by measuring *in vitro* growth rate while exposed to stresses. *C. parapsilosis* was shown to be tolerant to hydrogen peroxide, with no difference in doubling time compared to non-stressed *C. parapsilosis,* but was highly sensitive to NONOate, a nitric oxide donor (Priest and Lorenz, 2015). *C. guilliermondii* was highly sensitive to both hydrogen peroxide and NONOate, with no fungal growth observed. Furthermore, coincubation of *C. parapsilosis* with RAW264.7 macrophage-like cell lines revealed *C. parapsilosis* induces a significant increase in macrophage NO production (Priest and Lorenz, 2015). The same phenotype was observed with *C. guilliermondii,* though *C. guilliermondii* induced macrophage NO slightly less than *C. parapsilosis.* This directly contrasts my results, in which I observed suppression of host RNS levels by *C. parapsilosis* and *C. guilliermondii*. Priest and Lorenz noted 1:10 *Candida*:macrophage ratio induced RAW264.7 macrophage NO to a lesser degree than 1:100 *Candida*:macrophage ratio, suggesting increased proportions of *C. parapsilosis* or *C. guilliermondii* may result in NO suppression. However, a 1:10 *Candida*:macrophage ratio is considerably greater than the *Candida*:immune cell ratio that would be present in my zebrafish *in vivo* model, in which RNS suppression was observed. An alternative reasons for the opposing observations could be inter-strain variation. The use of RAW264.7 macrophages is not considered a potential reason for the opposing observations as Collette *et al.* previously showed suppression of macrophage NO by *C. albicans* using RAW264.7 macrophages (Collette, Zhou and Lorenz, 2014). Priest and Lorenz observed approximately a 10% decrease in macrophage NO production by *C. albicans* compared to controls, whereas Collette *et al.* observed a 90% decrease in macrophage NO production by *C. albicans* at the same *Candida:*macrophage ratio. A change in experimental set up by the same lab may be responsible for the reduced phenotype observed and could explain why Priest and Lorenz did not observe NO suppression by *C. parapsilosis* and *C. guilliermondii*.

C. glabrata caused a lesser degree of host RNS suppression than the other *Candida* spp. Previous investigations into host nitrosative and oxidative responses to *C. glabrata* have had inconsistent results. Kaur *et al.* showed *C. glabrata* BG2 does not stimulate an increase in macrophage NO production by murine J774A.1 macrophages. Crucially, Kaur *et al.* did not observe macrophage NO suppression by *C. glabrata* as NO production for macrophages exposed to *C. glabrata* and IFNγ was the same as macrophages stimulated by IFNγ alone (Kaur, Ma and Cormack, 2007). These investigations, and mine, only considered the effect of *C. glabrata* on immune cell RNS production at a single time point. Muñoz-Duarte *et al.* explored NO production in *C. glabrata* infection at multiple

time points, though they carried out their investigations in MG-63 osteoblasts: a mesenchymal-derived cell type that is not usually infected by *Candida* spp*. C. glabrata* CBS138 increased NO production by human MG-63 osteoblasts *in vitro* after 1 hour of coincubation – the same time point used by Kaur *et al.* (Muñoz-Duarte *et al.*, 2016). Osteoblast NO production was maintained at the same level after 3 hours and 6 hours of incubation with *C. glabrata* CBS163. A *C. glabrata* clinical isolate caused a much greater increase in osteoblast NO production at 1 hour and 3 hours but NO production fell below the levels induced by the reference strain at 6 hours (though still slightly greater than NO production by control osteoblasts). I looked at neutrophil RNS production at 24 hpi, so these results do not necessarily contradict mine. It is possible that *C. glabrata* stimulates an initial increase in host RNS production, that is later reduced and suppressed as *C. glabrata* adapts to overcome the nitrosative stress. To confirm these findings, I could investigate the effect of *Candida* spp*.* on neutrophil RNS in zebrafish larvae at multiple time points.

C. albicans TT21-dTomato was more virulent in the zebrafish model than non-albicans *Candida* spp*.* clinical isolates. *C. albicans* TT21-dTomato is a hypervirulent, strongly hyphae producing strain, which explains its high virulence in the *in vivo* infection model (Seman *et al.*, 2018). Furthermore, non-albicans *Candida* spp*.* clinical isolates are not well adapted to growth at 28 °C as they were isolated from human patients, so isolates may have become adapted to growth at approximately 37 °C. This could be overcome by incubating infected zebrafish larvae at a higher temperature. Adult zebrafish can tolerate a wide range of temperatures, from 6 °C to 38 °C, but optimum temperature for growth of zebrafish larvae is 28 °C (López-Olmeda and Sánchez-Vázquez, 2011; Scott and Johnston, 2012; Jørgensen, 2020; Haverinen *et al.*, 2021). During spring viraemia carp virus infection, adult zebrafish incubated at 22 °C developed signs of skin haemorrhaging by 3 dpi, with 80% of fish having signs of skin haemorrhaging by 5 dpi. In contrast, adult zebrafish incubated at 28 °C never exhibited any signs of skin haemorrhaging and were able to clear the viral infection (Boltaña *et al.*, 2013). Lam *et al.* investigated the effect of increased temperature on zebrafish neutrophil function by incubating zebrafish larvae for 1 hour at 38 °C. Neutrophil recruitment to *Staphylococcus aureus* infection was reduced at 2 hpi, though there was no difference in zebrafish survival. Similarly, neutrophil recruitment to a tail fin wound was reduced at 4 hpw by pre-incubation at 38 °C (Lam, Harvie and Huttenlocher, 2013). Temporary incubation at 38 °C also triggered a change in neutrophil gene expression, primarily in heat shock proteins, which may have further impacts on neutrophil behaviour. Long term incubation of zebrafish larvae would introduce additional complications: developmental malformations were observed in zebrafish larvae incubated at 32.5 °C by 24 hpf, with malformations becoming more pronounced in embryos incubated at 34.5 °C and 36.5 °C (Pype *et al.*, 2015). Hence, zebrafish immune function operates on a bell-shaped curve, with 28 °C the optimum

temperature for immune activity (Scharsack and Franke, 2022). Therefore, substantial increases in incubation temperature to aid fungal growth could have detrimental effects on the ability of zebrafish innate immune cells to control infection, further contributing to increased virulence and adding caveats to any conclusions made.

Temperature is a regulator of gene expression in *C. albicans*. Increases in temperature have been associated with phenotypes ranging from increased susceptibility to caspofungin to increased hyphae formation, increased host cell adhesion and virulence (Desai *et al.*, 2015; Leach *et al.*, 2016; Zheng *et al.*, 2023). Increasing zebrafish incubation temperature is not a feasible option to examine *Candida* spp., due to the consequences for host condition and immune cell function. There are other options to explore *Candida* spp. behaviour and gene expression that usually happens at higher temperatures. One option would be to condition *Candida* spp. clinical isolates to grow optimally at 28 °C. Leach *et al.* demonstrated thermal adaptation by *C. albicans*. Increasing incubation temperature from 30 °C to 37 °C caused upregulation of Hsp1 by *C. albicans* but Hsp1 returned to basal levels within 20 minutes, suggesting an adaptation to the temperature increase (Leach *et al.*, 2012). *C. albicans* also showed adaptation to 42 °C, with Hsp1 returning to basal levels within 2 hours. Schwiesow *et al.* aimed to show sustained thermotolerance by serially passaging *Cryptococcus neoformans* environmental isolate, steadily increasing the incubation temperature. After 38 passages, *C. neoformans* had adapted into genetically distinct strains that were more able to grow at 35 °C than the original isolate, showing increased thermotolerance (Schwiesow, Elde and Hilbert, 2024). However, thermally adapted strains had further unpredictable phenotypic changes, with one thermally adapted strain forming more titan cells than the original isolate and two thermally adapted strains being unable to form titan cells. Fluconazole resistance also changed unpredictably and in opposing ways in thermally adapted strains (Schwiesow, Elde and Hilbert, 2024). Serial passage of *Candida* spp. clinical isolates may increase growth at 28 °C but could also induce unpredictable changes in virulence factor expression, yeast-to-hyphae transition or white opaque switching, having unknown impacts on *Candida* spp. behaviour and virulence and confounding experimental conclusions. Furthermore, previous publications have demonstrated thermal adaptation to increased temperatures. There is no guarantee these protocols would be as effective for adapting *Candida* spp. clinical isolates to grow at a lower temperature. Beyond incubating zebrafish at increased temperatures or adapting *Candida* spp. to lower temperatures, a further alternative could be using the Arabian Killifish as an infection model. Arabian Killifish (*Aphanius dispar*) can tolerate temperatures up to 40 °C for up to 12 days and incubation of newly fertilised embryos at 34 °C caused no malformations compared to embryos incubated at 28 °C (Alsakran *et al.*, 2024; Minhas *et al.*, 2024). As with zebrafish, killifish embryos are transparent,

allowing live imaging of host-pathogen interactions (Alsakran *et al.*, 2024). A *C. albicans* infection model in killifish has already been established (Minhas *et al.*, 2024). However, to the best of my knowledge, there is no publicly available genome database for *A. dispar* and there are no published fluorescent immune cell transgenic *A. dispar* lines. Further development of these genetic resources and characterisation of the killifish model is required before it would be practical to replace zebrafish as a *in vivo* infection model with killifish.

The results presented in this chapter reveals neutrophil RNS suppression is conserved across *C. albicans* clinical isolates and a range of non-albicans *Candida* spp., highlighting the possibility that RNS suppression by *Candida* spp. is a clinically relevant phenomenon. Many of the patients that develop invasive candidiasis, such as HIV/AIDS patients, have dysfunctional adaptive immune systems, so rely entirely on innate immunity for infection control. Mechanisms that suppress innate immune responses, especially neutrophils, which are key for immune control of *C. albicans*, may explain why these patients are so susceptible to invasive candidiasis. The revelation that neutrophil RNS suppression by *Candida* spp. is clinically relevant also brings about an opportunity to design new therapeutics that target this mechanism, either by inhibiting the *Candida* protein responsible for RNS suppression or targeting host immune cells to overcome RNS suppression.

6. Restoration of host RNS via Hif-1α stabilisation is host protective in *C. albicans* infection

6.1 Introduction

6.1.1 Combination antifungal therapy

Relatively poor efficacy of antifungal drugs and rising rates of antifungal resistance have led to a pressing need to develop new therapeutic alternatives for treatment of fungal infections (Burgess, Condliffe and Elks, 2022). Combination antifungal therapy represents a potential way to improve efficacy of antifungal treatment, reduce toxicity by using lower doses and slow the development of antifungal resistance (Johnson and Perfect, 2010; Shrestha, Fosso and Garneau-Tsodikova, 2015). Combination of posaconazole and caspofungin had a synergistic effect on *C. albicans* killing *in vitro*, with significantly lower MIC of both antifungals when combined, and on survival on *C. albicans* infected mice *in vivo* (Chen *et al.*, 2013). A clinical trial involving 219 candidaemic patients revealed fluconazole and amphotericin B combination therapy increased treatment success rate and clearance of *Candida* spp. in the bloodstream compared to fluconazole alone, though had no impact on mortality (Rex *et al.*, 2003). A significant barrier to the wider use of combination antifungal therapy is the adverse effects associated with antifungals, combined with the lack of rigorous clinical trials, meaning combination antifungal therapy tends to be reserved for last-resort, salvage therapy (Jacobs and Chaturvedi, 2024).

Traditional antifungals may also be combined with other drugs to improve infection outcomes. Fluconazole, voriconazole, itraconazole and posaconazole all demonstrated synergistic inhibition of *C. albicans* growth *in vitro* when combined with derivatives of tobramycin – an aminoglycoside antibiotic (Shrestha, Fosso and Garneau-Tsodikova, 2015). Antifungals may also be combined with host directed therapies (HDTs). G-CSF/GM-CSF are endogenous signalling molecules that promote maturation proliferation and activation of innate immune cells and can be used as HDTs (Mehta, Malandra and Corey, 2015; Sam *et al.*, 2018). Adjunctive GM-CSF therapy, alongside voriconazole, led to complete clinical remission of *C. albicans* meningoencephalitis in a patient with CARD9-deficiency (Gavino *et al.*, 2014). G-CSF, fluconazole and amphotericin B co-administration resulted in successful treatment in a similar case of *C. albicans* meningoencephalitis in a CARD-deficient patient (Celmeli *et al.*, 2016). G-CSF adjunctive therapy has also been successful in a paediatric, CARD9-deficient patient with invasive candidiasis (B. Du *et al.*, 2020a). IFNγ adjunctive therapy has also successfully been used to treat patients with invasive candidiasis (Delsing *et al.*, 2014). Combination of HDTs with antifungals in a clinical setting has largely been restricted to individual cases or small

pilot studies. Larger randomised trials of adjunctive HDTs are required to validate this promising avenue of combination therapy, alongside increased pre-clinical research on potential new HDTs.

6.1.2 Hypoxia inducible factor (HIF) as a host directed therapy

Hypoxia inducible factor (HIF) has well characterised roles for modulating innate immune function in response to infection and inflammation (Hammond, Lewis and Elks, 2020). Infection can create an environment of localised hypoxia, reducing function of PHD enzymes and leading to stabilisation of HIF-α subunits. HIF-1α can also be stabilised by TLR signalling in the absence of hypoxia, via MYD88-dependent NF-κB activity, inducing a subset of pro-inflammatory genes to a significantly greater degree than hypoxia-induced HIF-1α signalling (Peyssonnaux *et al.*, 2005; Jantsch *et al.*, 2011). HIF-α subsequently binds to HIF-β subunits, which then translocate to the nucleus to induce transcription of downstream targets (Werth *et al.*, 2010; Hammond, Lewis and Elks, 2020; McGettrick and O'Neill, 2020). HIF-1α and HIF-2α isoforms have an overlapping but distinct set of target genes. One key difference is HIF-1α being associated with increased iNOS transcription and HIF-2α being associated with increased arginase transcription (Takeda *et al.*, 2010; Thompson *et al.*, 2014). HIF-α isoforms represent attractive targets for immunomodulation to improve host ability to respond to infections.

Hif-1α modulation has been studied as a potential HDT for treatment of bacterial infections. Hif-1α stabilisation has previously been shown to be protective against *M. marinum* infection in zebrafish embryos, via IL-1β-dependent upregulation of RNS (Elks *et al.*, 2013, 2014; Ogryzko *et al.*, 2019). The protective effect of Hif-1α stabilisation in *M. marinum* infection is maintained in a zebrafish comorbid model with a tail fin wound (Schild *et al.*, 2020). Pharmacological stabilisation of HIF-1α, using AKB-4924, enhanced the bactericidal activity of human U937 phagocytes against *S. aureus in vitro*. AKB-4924 treatment led to greater phagocyte killing of *S. aureus* than mimosine, suggesting AKB-4924 may be more potent than other HIF-1α stabilisers (Okumura *et al.*, 2012). Both AKB-4924 and mimosine stabilise Hif-1α by inhibiting PHD enzymes, suggesting the greater phagocytic killing is due to greater potency and not because of a different mechanism (Warnecke *et al.*, 2003; Marks *et al.*, 2015). Hif-1α stabilisation also reduced lesion size and bacterial burden in the lung in mice infected with *S. aureus*, recapitulating previous observations that used mimosine as a HIF-1α stabiliser (Zinkernagel *et al.*, 2008; Okumura *et al.*, 2012). HIF-1α stabilisation was further shown to have protective effects against *Clostridium difficile* and *Pseudomonas aeruginosa* infections in mice *in vivo* and reduced lesion size in mice with polymicrobial periodontitis (Hirota *et al.*, 2010; Schaible *et al.*, 2013; Hirai *et al.*, 2018). Modulation of HIF-2α also has host protective effects, though HIF-2α is less studied than HIF-1α in the context of infection. Inhibition of Hif-2α in zebrafish embryos increased neutrophil RNS levels and reduced *M. marinum* burden (Elks *et al.*, 2013). HIF-2α-deficient murine neutrophils had increased apoptosis and reduced inflammation in LPS-mediated lung injury

in vivo, but had no change in neutrophil chemotaxis or phagocytosis (Thompson *et al.*, 2014). While this resulted in reduced lung tissue damage in this model of inflammation, it is unclear whether increased neutrophil apoptosis (due to HIF-2α deficiency) would have beneficial effects in the context of infection.

Fungal infections have been shown to create hypoxic microenvironments and to stimulate HIF-1α activation (Grahl and Cramer, 2010; Werth *et al.*, 2010). Immunohistochemistry of human skin biopsies revealed increased HIF-1α levels in response to infection with *Tinea rubrum* and *C. albicans*. HIF-1α activation was observed in keratinocytes, dermal capillaries, macrophages and neutrophils (Werth *et al.*, 2010). *C. albicans* burden was significantly increased, and TNFα levels decreased, in HIF-1α knockout mice, suggesting a protective role for HIF-1α in *C. albicans* infection *in vivo* (Li *et al.*, 2018b). Pharmacological stabilisation of HIF-1α in mice led to a significant decrease in *C. albicans* colonisation of the gut, an effect that was ablated in mice with *Hif1α* deleted from the intestinal epithelium (Fan *et al.*, 2015). HIF-1α stabilisation also led to significantly greater survival in disseminated *C. albicans* infection compared to untreated controls and mimosine-treated mice with *Hif1α* deleted from the intestinal epithelium. However, disseminated infection was generated by cyclophosphamide-induced damage to the gut epithelium (Fan *et al.*, 2015). This likely resulted in unequal amounts of *C. albicans* generating systemic infection, as HIF-1α stabilisation reduced *C. albicans* colonisation, which may have inflated the mortality in control mice. Regardless, HIF-1α stabilisation has been demonstrated to have potential as a host directed therapy for treatment of *C. albicans* infections, warranting further investigation in *in vivo* models and characterisation of the mechanisms underlying any protective effect.

6.1.3 Hypothesis & Aims

In previous chapters, I have demonstrated the ability of *C. albicans* to suppress neutrophil RNS levels *in vivo*, reducing the ability of host innate immunity to respond to and resolve infection. Hif-1α stabilisation has previously been shown to increase neutrophil RNS levels in uninfected zebrafish embryos, suggesting Hif-1α stabilisation may have potential as a host directed therapy for the treatment of *C. albicans* infections (Elks *et al.*, 2013).

I hypothesised that **"Hif-1α stabilisation will have a host protective effect against *C. albicans* infection *in vivo*".** To address the hypothesis, the following aims were addressed:

- Examine the effect of Hif-1α stabilisation on zebrafish survival in *C. albicans* infection
- Explore the mechanisms underlying the protective effect of Hif-1α stabilisation on *C. albicans* infection

- Assess the ability of Hif-1α stabilisation to have synergistic effects on zebrafish survival and fungal clearance in *C. albicans* infection when combined with traditional antifungals

6.2 Results

6.2.1 Hif-1α stabilisation is protective against *C. albicans* infection

To investigate Hif-1α signalling as a potential host directed therapy for treatment of *C. albicans* infections, a reliable method of inducing Hif-1α signalling was required. I aimed to demonstrate DA1 (dominant active Hif-1αb variant RNA) is reliably able to upregulate Hif-1α targets in zebrafish embryos. Injection of 1 cell stage zebrafish embryos with DA1 has previously been used to stabilise Hif-1α (Elks *et al.*, 2013). *Tg(phd3:GFP) i144* zebrafish embryos were used as a reporter of Hif-1α activity, as *phd3* is the most upregulated target of Hif-1α signalling (Santhakumar *et al.*, 2012). 1 cell stage Tg(*phd3:GFP*)i144 zebrafish embryos were injected with DA1, then examined at 2 dpf for GFP fluorescence to validate the ability of DA1 to upregulate Hif-1α signalling targets in my hands (D'Angelo *et al.*, 2003; Santhakumar *et al.*, 2012). Phenol red (PR; solvent control) and DN1 (dominant negative hif-1α RNA) injected zebrafish were used as negative controls.

The negative control groups had an average *phd3:GFP* pixel count of 2 for PR and 8 for DN1, meaning zebrafish displayed almost no *phd3:GFP* fluorescence (Figure 6.1). Hence, there was a lack of *phd3:GFP* transcription, which suggests there was negligible Hif-1α signalling in resting state or when hif-1α was downregulated by DN1. Zebrafish injected with DA1 had an average *phd3:GFP* pixel count of 1001, significantly greater than both negative controls (Figure 6.01; $p<0.0001$). Hence, DA1 is able to stimulate *phd3:GFP* transcription, suggesting DA1 has promoted Hif-1α signalling. These results demonstrate successful Hif-1α stabilisation using DA1 RNA.

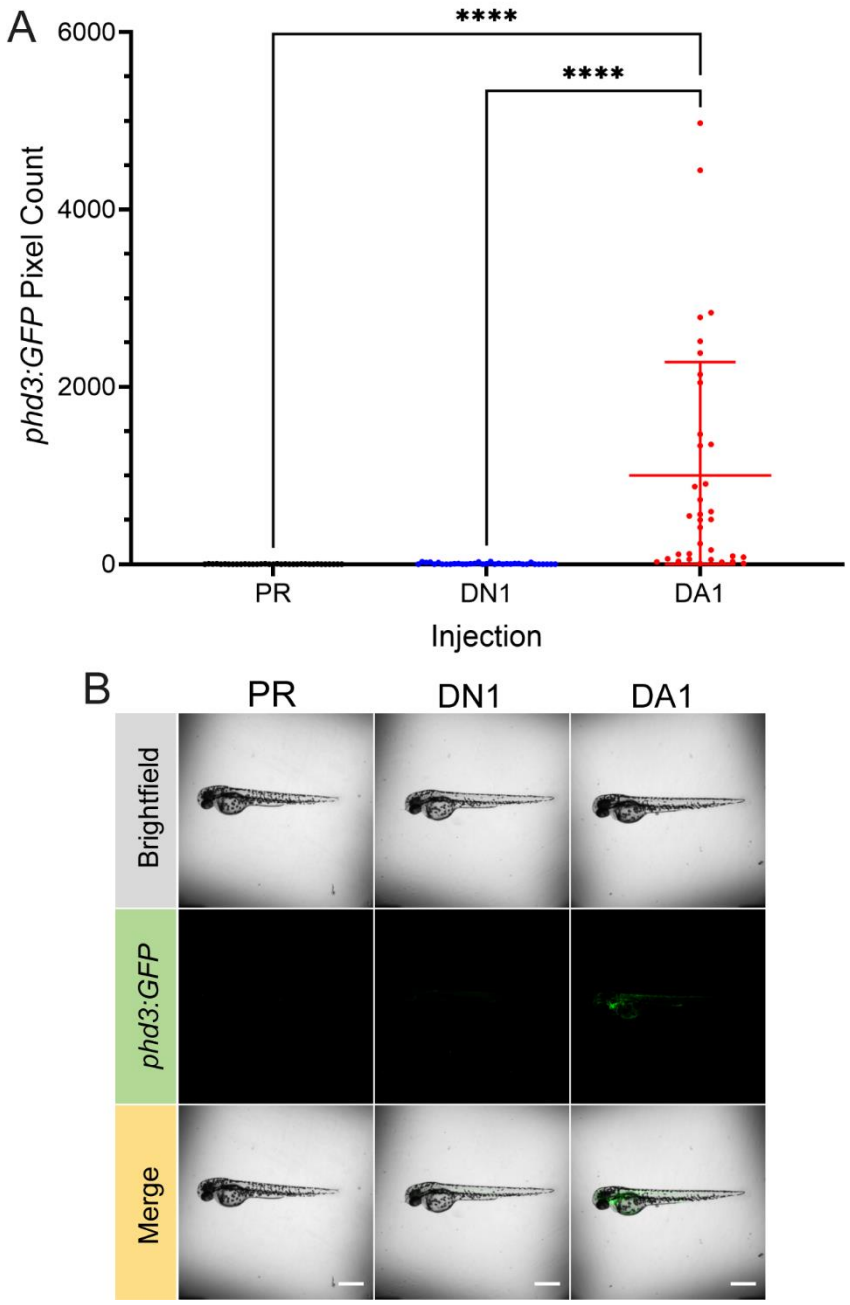

Figure 6.1: DA1 upregulates *phd3*:GFP expression.

(A) Pixel count of Tg(*phd3*:GFP) zebrafish embryos injected with DA1, DN1 or PR. N=35 fish, obtained from 2 independent experiments. Error bars show standard deviation. Statistical significance determined by one-way ANOVA, with Tukey's multiple comparisons test. P values shown: ****p0.0001

(B) Representative images of *Tg(phd3:GFP)* zebrafish embryos injected with PR, DA1 or DN1 at 2 dpf. Scale bar = 2 mm.

With a reliable method of Hif-1α stabilisation, I next aimed to upregulate innate immune responses and increase host survival in *C. albicans* infection by stabilising Hif-1α in *C. albicans* infected zebrafish embryos. Dr Ffion Hammond (former PhD student in Elks lab) injected 1 cell stage zebrafish embryos with PR, DN1 or DA1, then injected 200 cfu *C. albicans* TT21-dTomato into the caudal vein at 30 hpf. Mortality was measured over the following 4 days.

25.5% PR injected embryos survived up to 4 dpi (Figure 6.2). DN1 injected embryos had 20.4% survival at 4 dpi, which was not statistically different from 4 dpi survival in PR embryos. DA1 injected embryos had significantly greater survival across 4 days than both PR ($p<0.01$) and DN1 ($p<0.001$) injected larvae, with 51.5% survival at 4 dpi (Figure 6.2). The greatest amount of mortality across all groups occurred between 1 dpi and 2 dpi. At 1 dpi, all survival curves were relatively similar, with 85.3%, 81.4% and 88.3% survival for PR, DN1 and DA1, respectively. By 2 dpi, survival of DA1 injected embryos was 23.9% greater than PR injected embryos, suggest the protective effect of Hif-1α stabilisation is most effective between 1 dpi and 2 dpi. Together, these results highlight Hif-1α stabilisation is host protective in *C. albicans* infection.

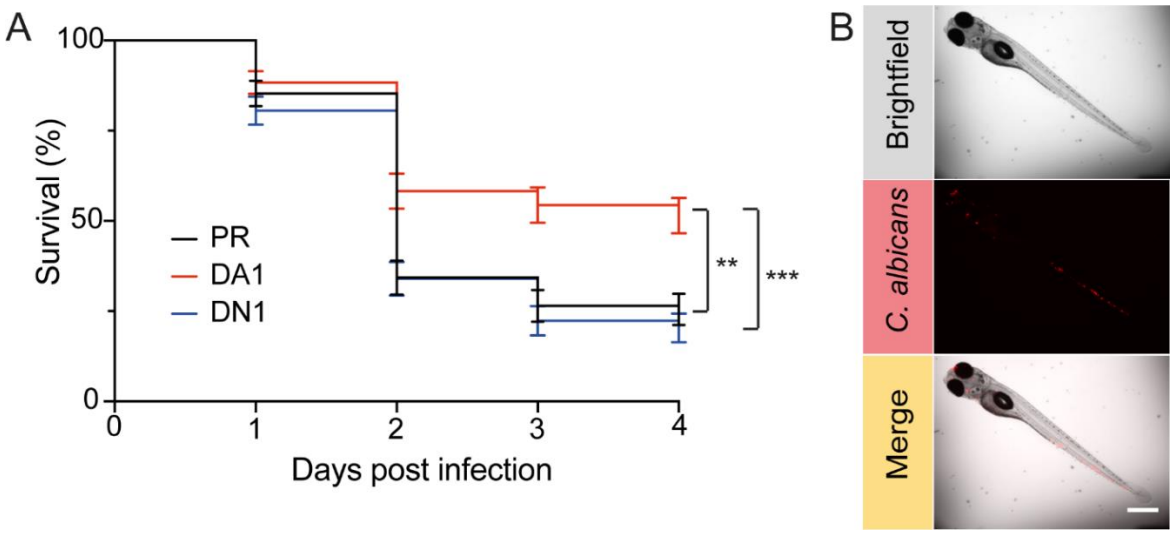

Figure 6.2: Hif-1α is protective against *C. albicans* infection *in vivo*.

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

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

6.2.2 Protective effect of Hif-1α stabilisation is neutrophil- and RNS dependent

I aimed to investigate the mechanisms underlying the protective effect of Hif-1α stabilisation. Macrophages have been shown to internalise, but not kill, *C. albicans* in zebrafish embryos, with *C. albicans* able to divide within macrophages (Brothers, Newman and Wheeler, 2011). I hypothesised that Hif-1α stabilisation may stimulate candidacidal activity by macrophages.

To investigate the impact of Hif-1α stabilisation on macrophages, Dr Ffion Hammond (former PhD student) injected 1 dpf *Tg(mpeg:nlsClover)* zebrafish embryos with clodronate liposomes. Clodronate liposomes are internalised by macrophages, causing cell death, but are not internalised by non-macrophage phagocytic cells (van Rooijen and Hendrikx, 2010). PBS loaded liposomes were used as a negative control. Macrophage ablation was validated by full body macrophage count at 2 dpf (1 dpi).

Embryos injected with PBS liposomes had 213 macrophages on average at 2 dpf (Figure 6.3A,B). Injection of clodronate liposomes decreased full body macrophage count to 37 macrophages at 2 dpf (Figure 6.3A,B), demonstrating an 83% reduction in macrophage population ($p<0.0001$). Hence, clodronate liposomes are able to substantially deplete, but not completely ablate, the zebrafish macrophage population *in vivo*.

To assess the impact of Hif-1α stabilisation on macrophage-mediated immunity, 1 cell stage embryos were injected with DA1 or PR control. At 1 dpf, embryos 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. A lower infection dose of *C. albicans* was used for systemic infections to compensate for the immunocompromised nature of these embryos.
Both groups of PBS liposome injected larvae had greater survival than clodronate liposome, macrophage depleted, siblings. DA1 was protective in both the presence or depletion of macrophages, with clodronate liposome larvae having greater survival than PR clodronate liposome embryos (Figure 6.3C; $p<0.0001$), indicating that the host-protective effect of Hif-1α stabilisation is not dependent on the presence of macrophages.

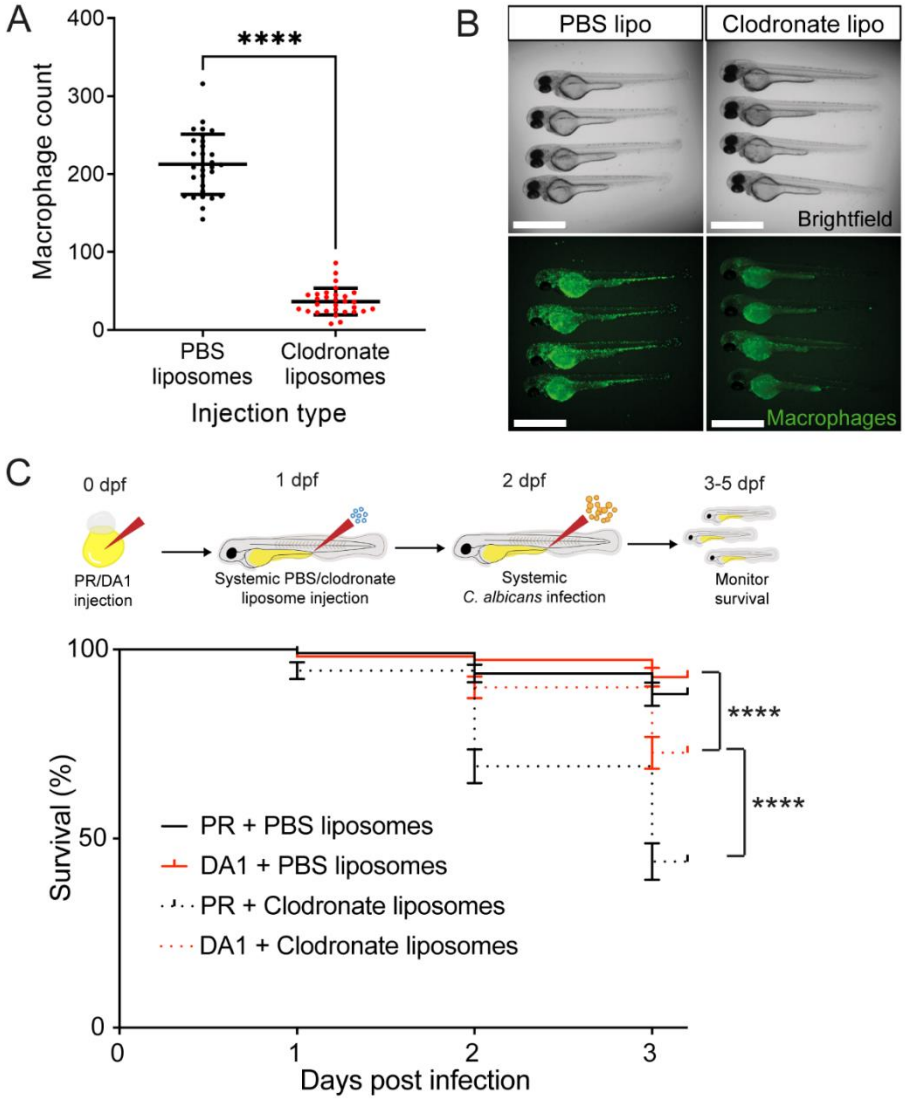

Figure 6.3: Hif-1α stabilisation protects against *C. albicans* infection *in vivo* in the absence of macrophages.

(A) Total body macrophage count at 2 dpf (1 dpi). N=30 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by unpaired T test. P values shown: ****p<0.0001.

(B) Representative images of 2 dpf (1 dpi) Tg(*mpeg:nlsClover*) zebrafish embryos following injection with PBS or clodronate liposomes. Scale bar = 2 mm.

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

All work in this figure was carried out by Dr Ffion Hammond

Given the importance of neutrophils in the anti-*Candida* immune response, I next hypothesised that the protective effect of Hif-1α stabilisation may be neutrophil-dependent. To investigate this hypothesis, I ablated the zebrafish neutrophil population by injecting 1 cell stage *Tg(mpx:GFP)* zebrafish with 500 µM *csf3r* morpholino (Ellett *et al.*, 2011). *Csf3r* (colony stimulating factor 3 receptor) encodes for the receptor for Csf3a (colony stimulating factor 3a; orthologous to human CSF), which plays a key role in development of neutrophil precursor cells into neutrophils (Panopoulos and Watowich, 2008; Ellett *et al.*, 2011; Meier *et al.*, 2022). Hence, *csf3r* knockdown prevents Csf3a signalling, halting development of mature, functional neutrophils. Neutrophil depletion was validated at 2 dpf by full body neutrophil count. Zebrafish injected with standard control morpholino had 190 neutrophils on average, whereas *csf3r* injected zebrafish had 70 neutrophils on average (Fig 6.4; p<0.0001). Hence, *csf3r* morpholino causes a significant depletion of the zebrafish neutrophil population at 2 dpf.

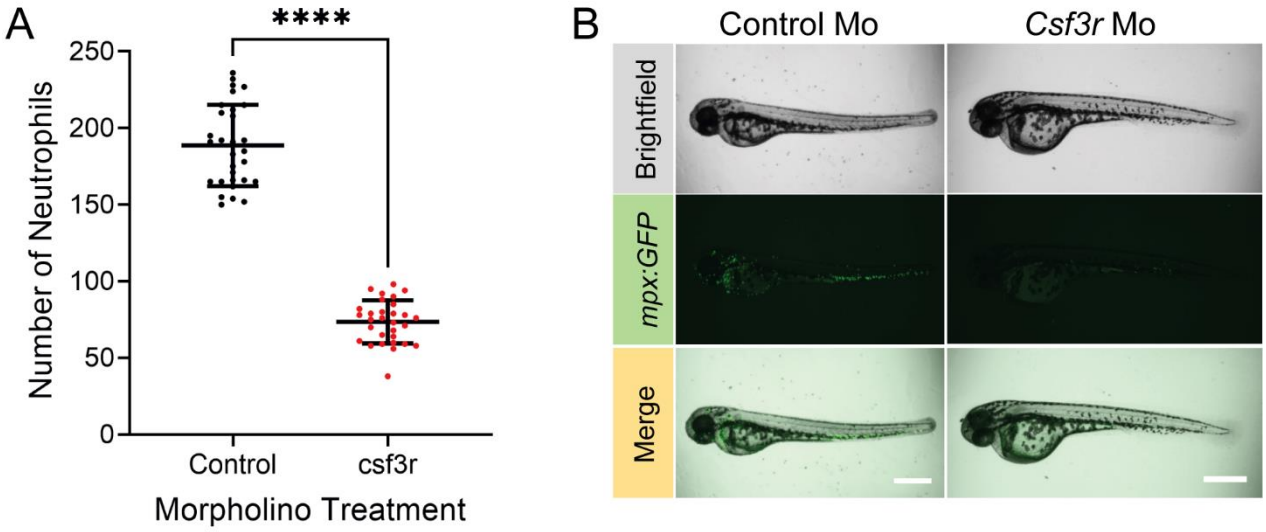

Figure 6.4: *csf3r* morpholino depletes the zebrafish neutrophil population.

(A) Total body neutrophil count at 2 dpf. N=30 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by unpaired T test. P values shown: ****p<0.0001.

(B) Representative images of 2 dpf *Tg(mpx:GFP)* i114 zebrafish embryos following injection with Control or *csf3r* morpholino. Scale bar = 1 mm

Survival curves were then carried out to assess the role of neutrophils in Hif-1α stabilisation. 1 cell stage nacre embryos were co-injected with DA1 or PR, and *csf3r* morpholino or control morpholino.

1 dpf zebrafish were then injected into the caudal vein with 100 cfu *C. albicans* TT21-dTomato. As with macrophage depleted zebrafish, a lower dose of *C. albicans* was used to account for the immunocompromised nature of zebrafish with a depleted neutrophil population.

PR + Control Mo zebrafish embryos had 48.1% survival at 4 dpi, a similar level of survival to previous experiments that used a greater infection dose (200 cfu). Loss of neutrophils in PR injected zebrafish (PR + *csf3r*) resulted in 30.4% survival at 4 dpi (Figure 6.5), showing a significant decrease in survival compared to PR injected fish with a full neutrophil population (PR + Control; $p<0.01$). This observation further highlights the importance of neutrophils in the anti-*Candida* immune response. DA1 + Control Mo zebrafish embryos had 71.1% survival at 4 dpi, significantly greater than survival of PR + Control zebrafish ($p<0.05$), demonstrating the protective effect of Hif-1α stabilisation. DA1 + *csf3r* embryos had reduced zebrafish survival (19.3%) compared to PR + *csf3r* embryos ($p<0.05$), showing a loss of the protective effect of Hif-1α stabilisation in the absence of neutrophils. Hence, the protective effect of Hif-1α stabilisation is neutrophil-dependent.

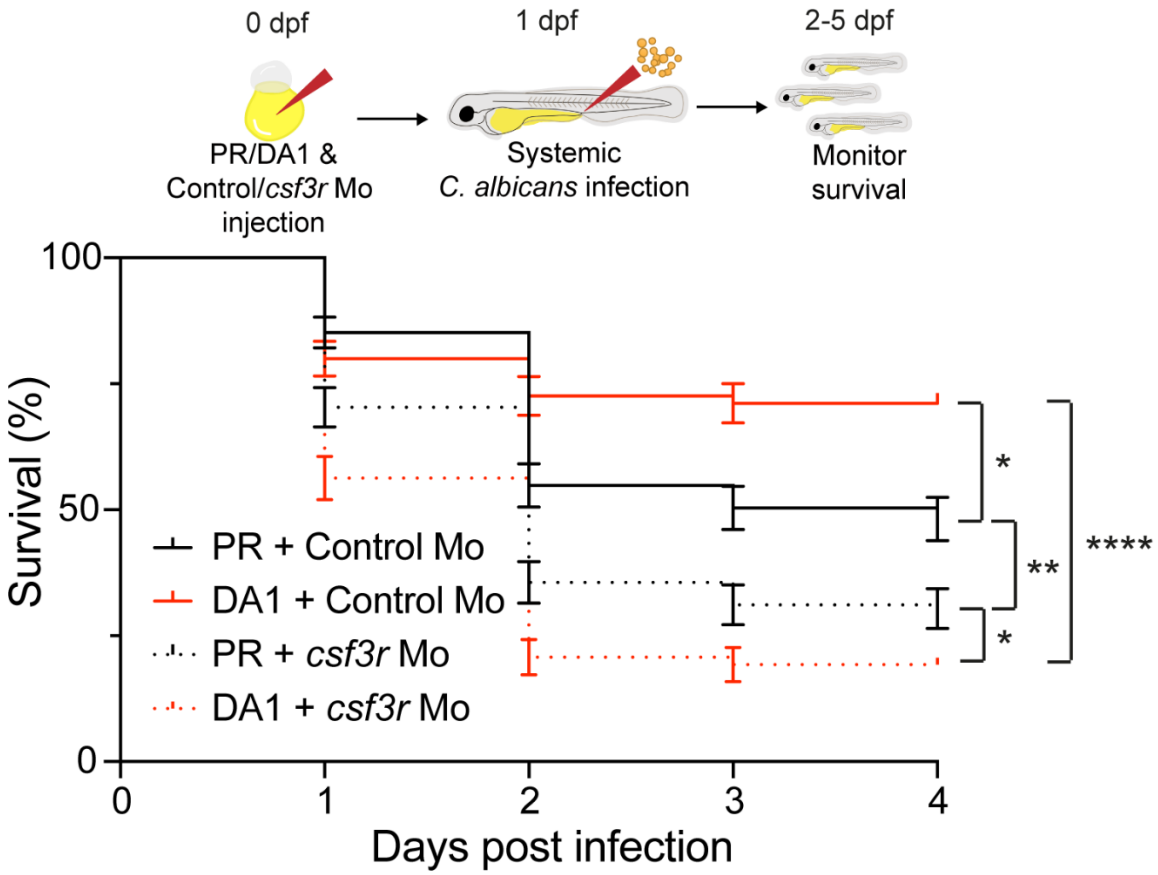

Figure 6.5: Protective effect of Hif-1α stabilisation is lost in larvae with a depleted neutrophil population.

Survival curve of 100 cfu *C. albicans* TT21-dTomato infected zebrafish embryos following injection with PR or DA1 and control or *csf3r* morpholino. Mortality was measured daily. N=135 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test with Bonferroni correction. P values shown: *p<0.05, **p<0.01, ****p<0.0001

Neutrophils are a critical for innate immune control of *C. albicans* and the protective effect of Hif-1α stabilisation in *C. albicans* infection is neutrophil-dependent. I aimed to characterise the mechanism underlying the protective effect of Hif-1α stabilisation *in vivo*. Full body neutrophil counts of zebrafish embryos injected with DA1 revealed Hif-1α stabilisation did not increase the total neutrophil population in uninfected embryos (Elks *et al.*, 2013). However, previous studies did not quantify the effect of Hif-1α stabilisation on neutrophil recruitment to the site of infection. I aimed to quantify neutrophil recruitment to *C. albicans* infection in the hindbrain at 24 hpi in embryos injected with PR and DA1. DN1 was also included as a negative control for RNA injection.

In PVP injected zebrafish, there was no difference in neutrophil recruitment to the hindbrain infection in embryos injected with PR (4 neutrophils) and embryos injected with DN1 (4 neutrophils; Figure 6.6). DA1 larvae mock infected with PVP recruited 8 neutrophils to the hindbrain infection, a significant increase compared to PR ($p<0.01$) and DN1 ($p<0.01$). *C. albicans* infection stimulated increased neutrophil recruitment in all groups (Figure 6.6). In *C. albicans* infected zebrafish, PR injected embryos recruited 16 neutrophils, DN1 injected embryos recruited 18 neutrophils and DA1 injected embryos recruited 19 neutrophils (Figure 6.6). There were no significant differences between groups infected with *C. albicans*, revealing Hif-1α stabilisation did not increase neutrophil recruitment to *C. albicans* infection in the hindbrain.

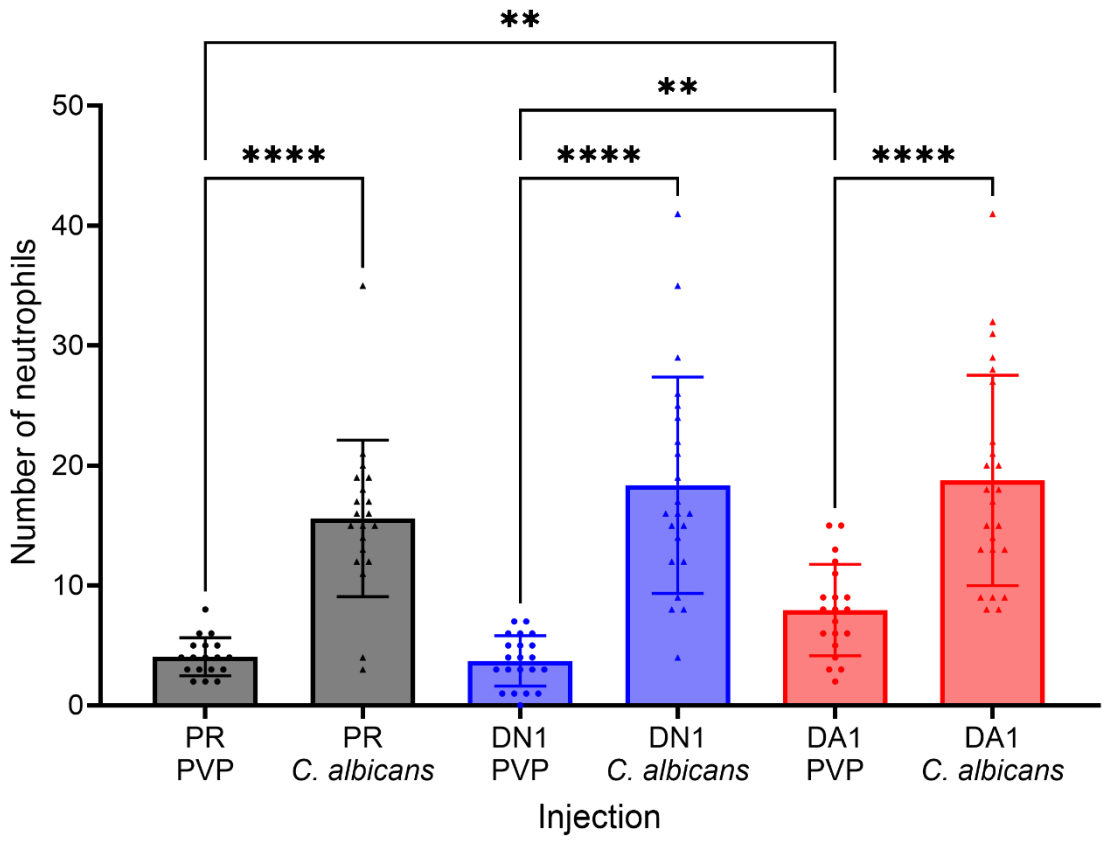

Figure 6.6: Hif-1α stabilisation does not increase neutrophil recruitment to *C. albicans* infection.

Number of neutrophils recruited to site of injection at 24 hpi, following injection with PVP or 20 cfu *C. albicans* TT21 dTomato into the hindbrain ventricle. Embryos were previously injected with PR, DN1 or DA1. N=18-24 fish, obtained from 3 independent experiments. Statistical significance determined by Brown-Forsythe and Welch ANOVA with Dunnett's T3 multiple comparisons test. P values shown: **p<0.01, ****p<0.0001.

The next potential mechanism for the protective effect of Hif-1α stabilisation I investigated was phagocytosis. Internalisation of *C. albicans* within phagocytes prevents the yeast-to-hyphal transition, limiting the virulence of *C. albicans* and preventing mortality (Brothers *et al.*, 2013). I, therefore, hypothesised that Hif-1α stabilisation promotes phagocytosis of *C. albicans* by neutrophils. I examined this hypothesis by quantifying the percentage of neutrophils with internalised *C. albicans* at 24 hpi.

20.4% of neutrophils in PR injected zebrafish had internalised *C. albicans* at 24 hpi (Figure 6.7). DN1 injected zebrafish had internalised *C. albicans* in 29.9% of neutrophils at 24 hpi, though this was not significantly different from PR injected zebrafish. 12.8% of neutrophils in DA1 injected embryos had internalised *C. albicans*, which was significantly less than DN1 ($p<0.01$) but not significantly different to PR. Across all 3 groups, there was a high level of variation. The protective effect of Hif-1α stabilisation in *C. albicans* infection does not appear to be caused by changes in internalisation of *C. albicans* by neutrophils.

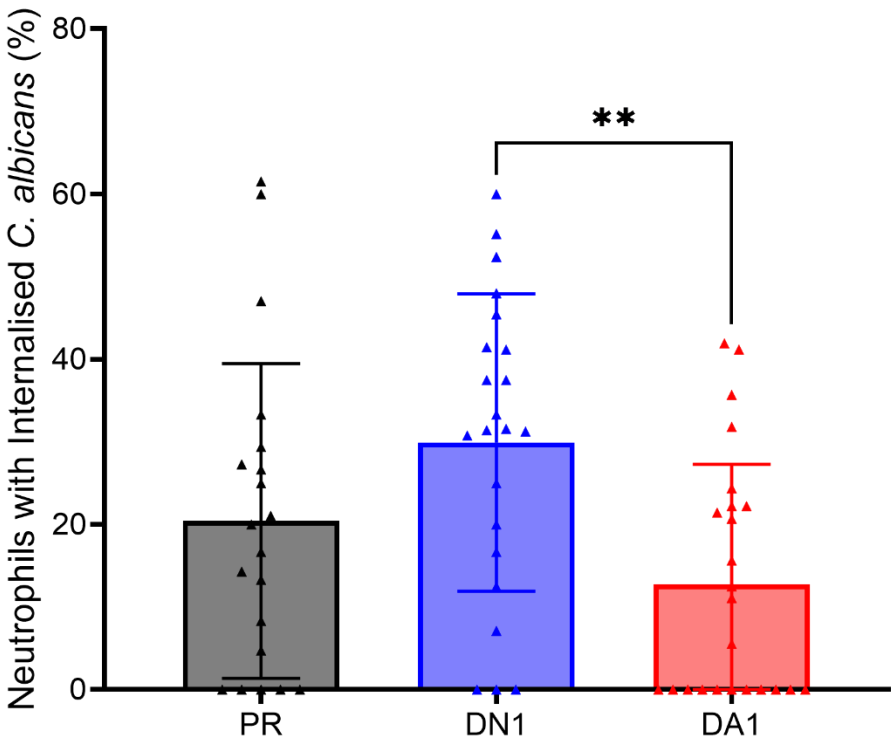

Figure 6.7: Hif-1α stabilisation does not increase internalisation of *C. albicans* by neutrophils.

Percentage of neutrophils with internalised *C. albicans* at 24 hpi, following injection with 20 cfu *C. albicans* TT21 dTomato into the hindbrain ventricle. Embryos were previously injected with PR, DN1 or DA1. N=20-24 fish, obtained from 3 independent experiments. Error bars show standard deviation. Statistical significance determined by Kruskal-Wallis test, with Dunn's multiple comparisons test. P values shown: **p<0.01.

While quantifying neutrophil internalisation of *C. albicans*, an incidental observation of different sized phagosomes was made. *C. albicans* was contained in 2 distinct types of phagosomes: spacious phagosomes, in which there was little or no contact between *C. albicans* and the phagosomal membrane, and tight phagosomes, in which the phagosomal membrane is in close contact with the fungi. Spacious phagosomes have been observed in immune cell responses to other pathogens (Alpuche-Aranda *et al.*, 1994; Schnettger *et al.*, 2017; Prajsnar *et al.*, 2021). I aimed to quantify the occurrence of spacious phagosomes in *C. albicans* infection in zebrafish embryos with, and without, stabilised Hif-1α.

In PR injected embryos, there was an average of 0 spacious phagosomes and 5 tight phagosomes within the field of view around the site of infection (Figure 6.8A). Within each larvae, 9.6% of phagosomes were spacious phagosomes based on mean average (Figure 6.8B), although the mean

was severely impacted by a few outliers and the median average was 0.0% spacious phagosomes. 14.2% phagosomes in DN1 larvae were spacious phagosomes, with an average of 1 spacious phagosome and 7 tight phagosomes. In DA1 injected larvae, 14.8% phagosomes were spacious, with 1 spacious phagosome and 8 tight phagosomes on average. Across all injection groups, the number of tight phagosomes were significantly greater than the number of spacious phagosomes (Figure 6.8A; $p<0.05$). No significant differences were observed between any groups for the percentage of phagosomes that were spacious phagosomes. These results indicate spacious phagosomes make up a minority of the phagosomes formed when *C. albicans* is internalised by neutrophils and that Hif-1α stabilisation has no impact on the occurrence of spacious phagosomes.

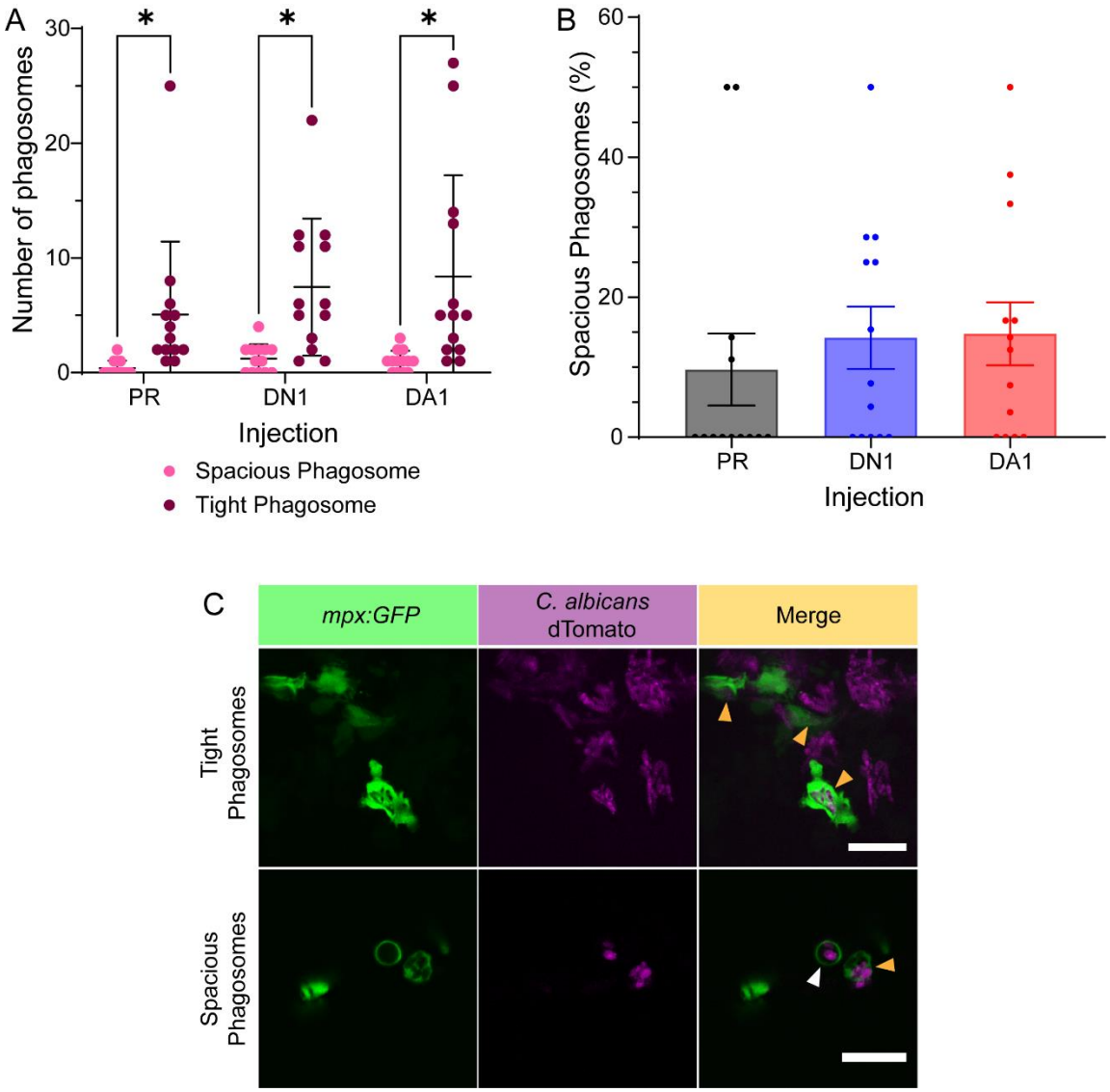

Figure 6.8: Hif-1α stabilisation does not affect spacious phagosome formation.

(A) Number of tight and spacious phagosomes in *C. albicans* TT21-dTomato infection in larvae injected with PR, DN1 or DA1 at 24 hpi. N=13 fish, obtained from 2 independent experiments. Error bars show standard deviation. Statistical significance determined by multiple paired Wilcoxon tests, with False Discovery Rate correction. P values shown: *p<0.05

(B) Percentage of total phagosomes that are spacious phagosomes in in *C. albicans* TT21-dTomato infection in larvae injected with PR, DN1 or DA1 at 24 hpi. N=13 fish, obtained from 2 independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, with Dunn's multiple comparisons test. No significant differences found.

(C) Representative images of tight and spacious phagosomes in *C. albicans* TT21-dTomato infected embryos. White arrowheads show *C. albicans* internalised within a spacious phagosome. Orange arrowheads show *C. albicans* internalised within a tight phagosome.

A further incidental observation was made while quantifying *C. albicans* internalisation by neutrophils: the appearance of seemingly folded *C. albicans* hyphae. Folding of *C. albicans* hyphae has previously been observed *in vitro*, as a macrophage strategy to inhibit hyphal growth, kill hyphae and prevent phagosomal escape (Bain *et al.*, 2021). However, folding of hyphal *C. albicans* has not been observed *in vivo*, to the best of my knowledge. I aimed to quantify the occurrence of folded *C. albicans* hyphae in *C. albicans* infection in zebrafish embryos with, and without, stabilised Hif-1α.

In PR injected larvae, within the field of view around the site of infection, there was an average of 0 folded *C. albicans* hyphae and 2 non-folded *C. albicans* hyphae internalised in neutrophils (Figure 6.9A). 1.4% observed *C. albicans* hyphae were folded in PR injected larvae (Figure 6.9B). 27.1% *C. albicans* hyphae, within the field of view, were folded in DN1 injected larvae, with an average of 1 folded and 3 non-folded *C. albicans* hyphae (Figure 6.9). In DA1 injected larvae, 22.4% observed *C. albicans* hyphae were folded: an average of 1 folded hypha and 3 non-folded hyphae (Figure 6.9). No significant differences were observed between any groups for the percentage of internalised *C. albicans* hyphae that were folded hyphae. While further characterisation is required to validate that these folded *C. albicans* hyphae are being folded by host neutrophils, these results suggested Hif-1α does not affect the incidence of folded *C. albicans* hyphae.

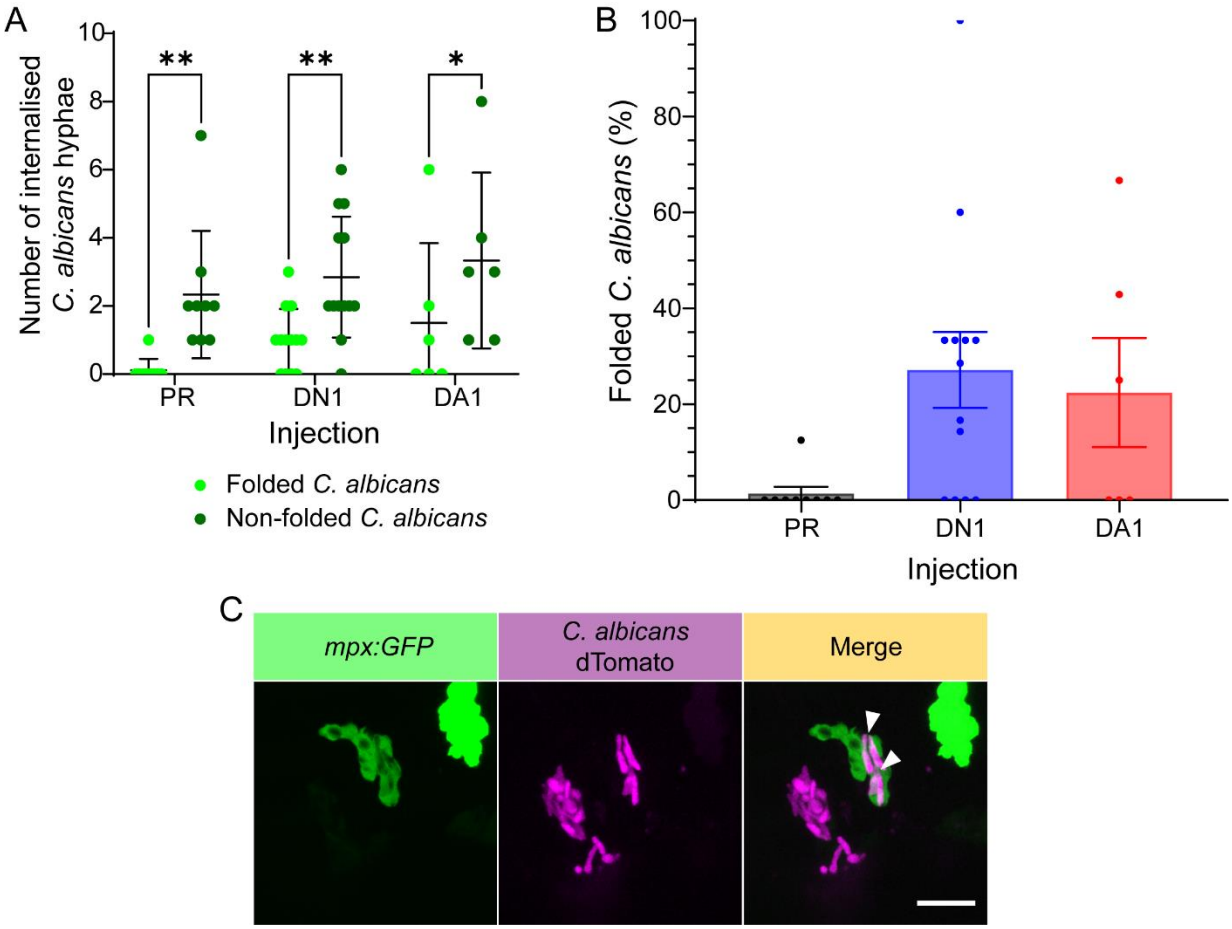

Figure 6.9: DA1 does not affect folding of internalised *C. albicans*

(A) Number of folded and non-folded *C. albicans* hyphae held within neutrophil phagosomes in *C. albicans* TT21-dTomato infection in larvae injected with PR, DN1 or DA1 at 24 hpi. N=6-13 fish, obtained from 2 independent experiments. Error bars show standard deviation. Statistical significance determined by multiple paired Wilcoxon tests, with False Discovery Rate correction. P values shown: *p<0.05, **p<0.01

(B) Percentage of total internalised *C. albicans* hyphae that are folded hyphae in *C. albicans* TT21-dTomato infection in larvae injected with PR, DN1 or DA1 at 24 hpi. N=6-13 fish, obtained from 2 independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, with Dunn's multiple comparisons test. No significant differences found.

(C) Representative images of folded hyphae in *C. albicans* TT21-dTomato infected embryos. White arrowheads show folded *C. albicans* hyphae internalised within a neutrophil.

To further investigate the mechanisms underlying Hif-1α stabilisation, neutrophil RNS levels were considered. *C. albicans* suppresses neutrophil RNS levels, so restoration of host RNS may lead to improved innate immune control of infection. Dr Ffion Hammond injected *Tg(lyz:mCherry)* embryos with PR or DA1, then infected embryos with 500 cfu *C. albicans* SN148 GFP or PVP at 30 hpf. At 24 hpi, embryos were fixed and stained with anti-nitrotyrosine antibody, allowing anti-NT levels to be measured.

DA1 + PVP caused approximately a doubling in anti-NT levels compared to PR + PVP, mock injected embryos (Figure 6.10; PR+PVP=16.36, DA1+PVP=33.25; $p<0.0001$). This correlates with previous observations that Hif-1α stabilisation can increase neutrophil RNS levels in uninfected zebrafish (Elks *et al.*, 2013). Anti-NT levels in PR + *C. albicans* embryos were 9.15, a 44.1% decrease compared to PR + PVP ($p<0.001$), demonstrating suppression of neutrophil RNS levels by *C. albicans* (Figure 6.10). DA1 + *C. albicans* had significantly greater anti-NT levels than PR + PVP ($p<0.0001$) and PR + *C. albicans* ($p<0.0001$) embryos. Anti-NT levels in DA1 + *C. albicans* were 29.20, which was not significantly different from DA1 + PVP (Figure 6.10). Therefore, Hif-1α stabilisation can restore neutrophil RNS levels in *C. albicans* infected embryos to comparable levels of uninfected embryos with stabilised Hif-1α, overcoming *C. albicans* suppression of RNS.

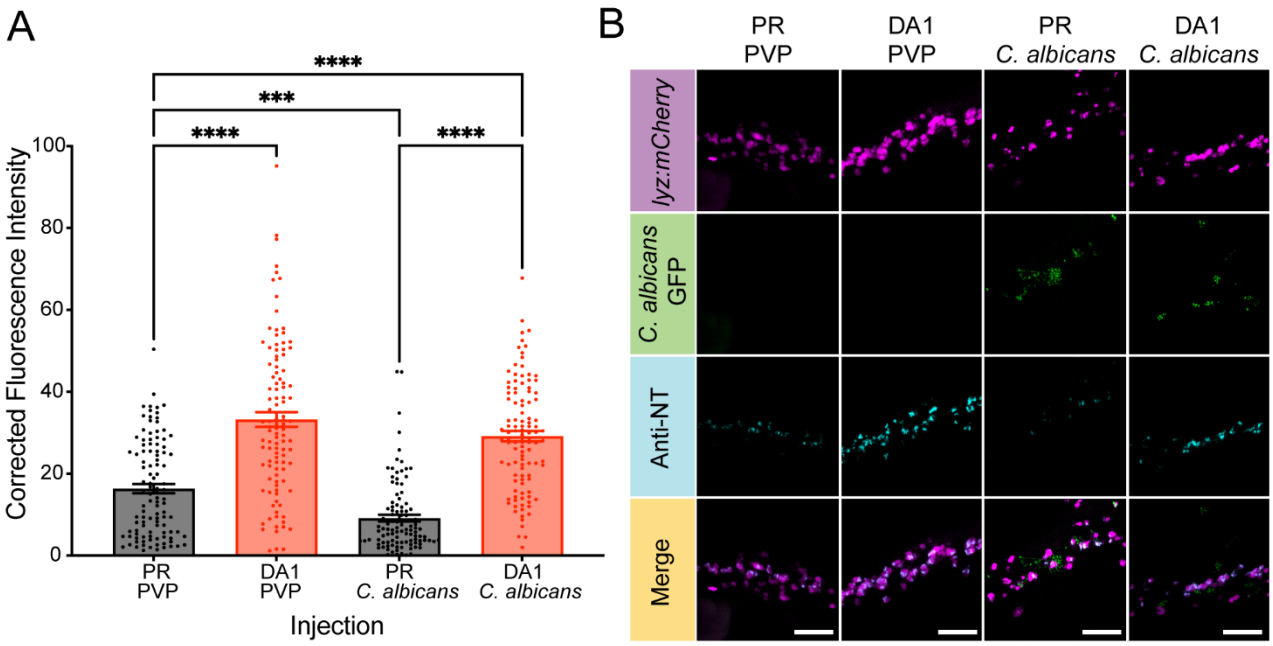

Figure 6.10: Hif-1α stabilisation restores neutrophil RNS level in *C. albicans* infection.

(A) Anti-nitrotyrosine fluorescence at 24 hpi in PR and DA1 injected fish, following injection of PVP or 500 cfu *C. albicans* SN148 GFP. N=108 neutrophils from 18 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, with Dunn's multiple comparisons test. P values shown: ***p<0.001, ****p<0.0001. Work carried out by Ffion Hammond.

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

I aimed to pharmacologically inhibit RNS production, in order to confirm restoration of neutrophil RNS is responsible for the protective effect of Hif-1α stabilisation. L-NIL is a commercially available, well-validated iNOS inhibitor, preventing production of nitric oxide (Moore *et al.*, 1994). Prior to using L-NIL *in vivo*, I needed to demonstrate L-NIL had no adverse effects on *C. albicans* growth *in vitro*.

C. albicans grown in YPD alone had an OD_{600nm} of 5.985 after 9 hours incubation (Figure 6.11). *C. albicans* grown in the presence of DMSO and L-NIL grew to similar levels, with OD_{600nm} of 6.120 and 6.225, respectively. No significant differences in growth rate were detected, suggesting neither L-NIL nor DMSO (solvent control) have any impact on growth of *C. albicans in vitro*.

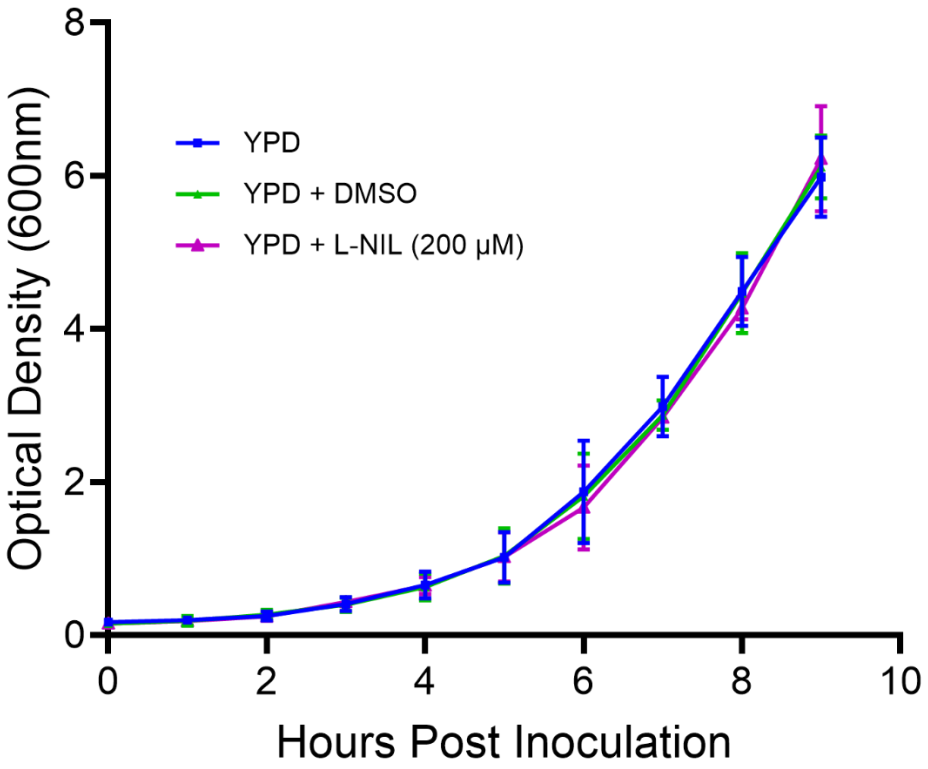

Figure 6.11: L-NIL does not impact growth of *C. albicans in vitro.*

C. albicans TT21-dTomato was grown in aliquots of YPD media alone, YPD + DMSO or YPD + L-NIL. Cultures were grown in 20 ml volumes at 30 °C shaking at 200 rpm. Optical density (OD_{600nm}) of *C. albicans* cultures was measured every hour for 10 hours. N=2 independent experiments. Error bars show standard deviation.

Survival curves were carried out on embryos injected with PR or DA1. Zebrafish embryos were infected with 200 cfu *C. albicans* TT21-dTomato into the caudal vein, then treated with 200 µM L-NIL or dH_2O (solvent control). Mortality was monitored up to 4 dpi.

PR + dH_2O treated embryos had 48.9% survival at 4 dpi. PR + L-NIL embryos had reduced survival (34.8% at 4 dpi) compared to PR + dH_2O embryos (Figure 6.12; $p<0.05$), demonstrating the importance of RNS in *C. albicans* control. 64.4% of DA1 + dH_2O embryos survived up to 4 dpi, significantly greater than PR + dH_2O ($p<0.01$), further displaying the protective effect of Hif-1α stabilisation. Survival of DA1 + L-NIL treated embryos at 4 dpi was 40.7%. DA1 + L-NIL treated larvae did not have a significant survival advantage compared to either PR + dH_2O or PR + L-NIL groups (Figure 6.12), indicative of iNOS inhibition by L-NIL removing the host protective effect of Hif-1α stabilisation.

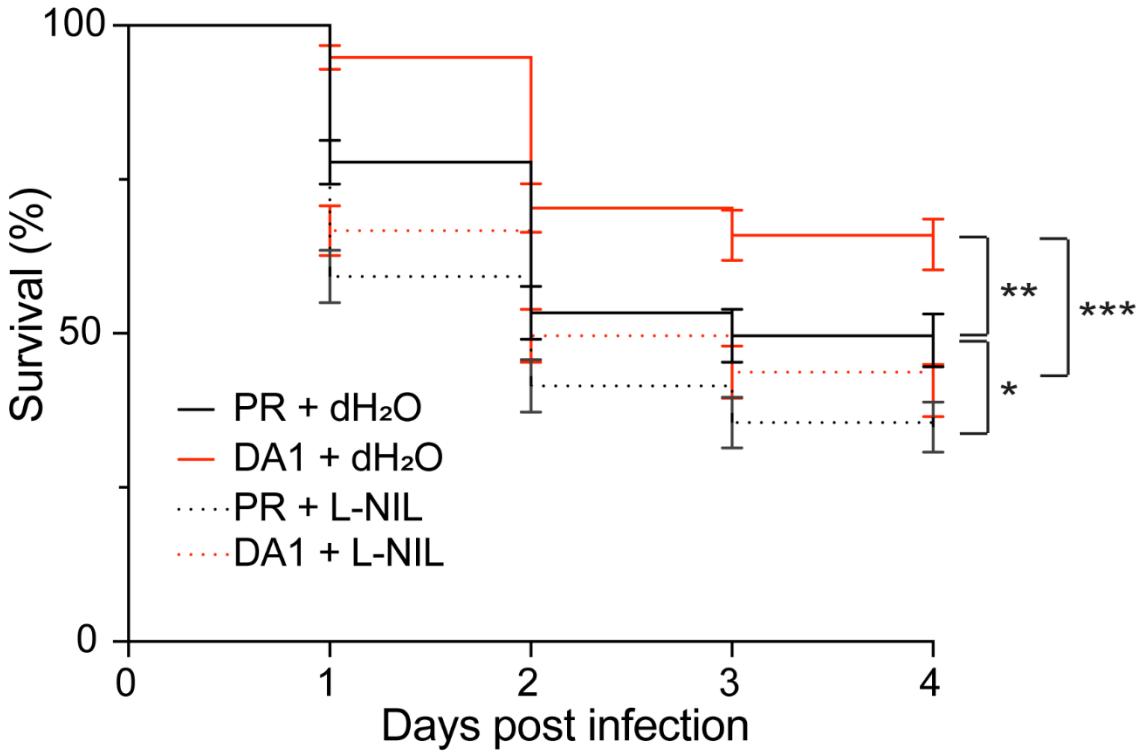

Figure 6.12: Protective effect of Hif-1α stabilisation lost with L-NIL treatment.

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

To confirm the importance of RNS in the protective role of Hif-1α stabilisation, I aimed to genetically inhibit RNS formation, using CRISPR/Cas9-mediated knockdown of *nos2a/nos2b*. Zebrafish iNOS is encoded by *nos2*, of which there are 2 isoforms: *nos2a* and *nos2b* (Lepiller *et al.*, 2009). To achieve successful CRISPR/Cas9 knockdown of zebrafish RNS levels, both *nos2a* and *nos2b* isoforms must be targeted (Szkuta, 2020). I initially aimed to validate the use of CRISPR-Cas9 mediated knockdown of *nos2a/nos2b* in my own hands by measuring neutrophil anti-NT levels.

Anti-NT levels in PR + *tyr* embryos were 2.63. DA1 + *tyr* anti-NT was increased to 3.03, showing a 15% increase compared to PR + *tyr* embryos, though this was not significant (Figure 6.13). Anti-NT levels in PR + *nos2a/nos2b* embryos were 1.01, a 62% decrease in anti-NT levels compared to PR + *tyr* (p<0.0001). DA1 + *nos2a/nos2b* anti-NT was reduced 51% compared to PR + *tyr* (1.01; p<0.0001). Hence, CRISPR/Cas9 knockdown of *nos2a/nos2b* is able to reduce host RNS levels, though not to the same degree as has previously been shown (Szkuta, PhD Thesis, 2020).

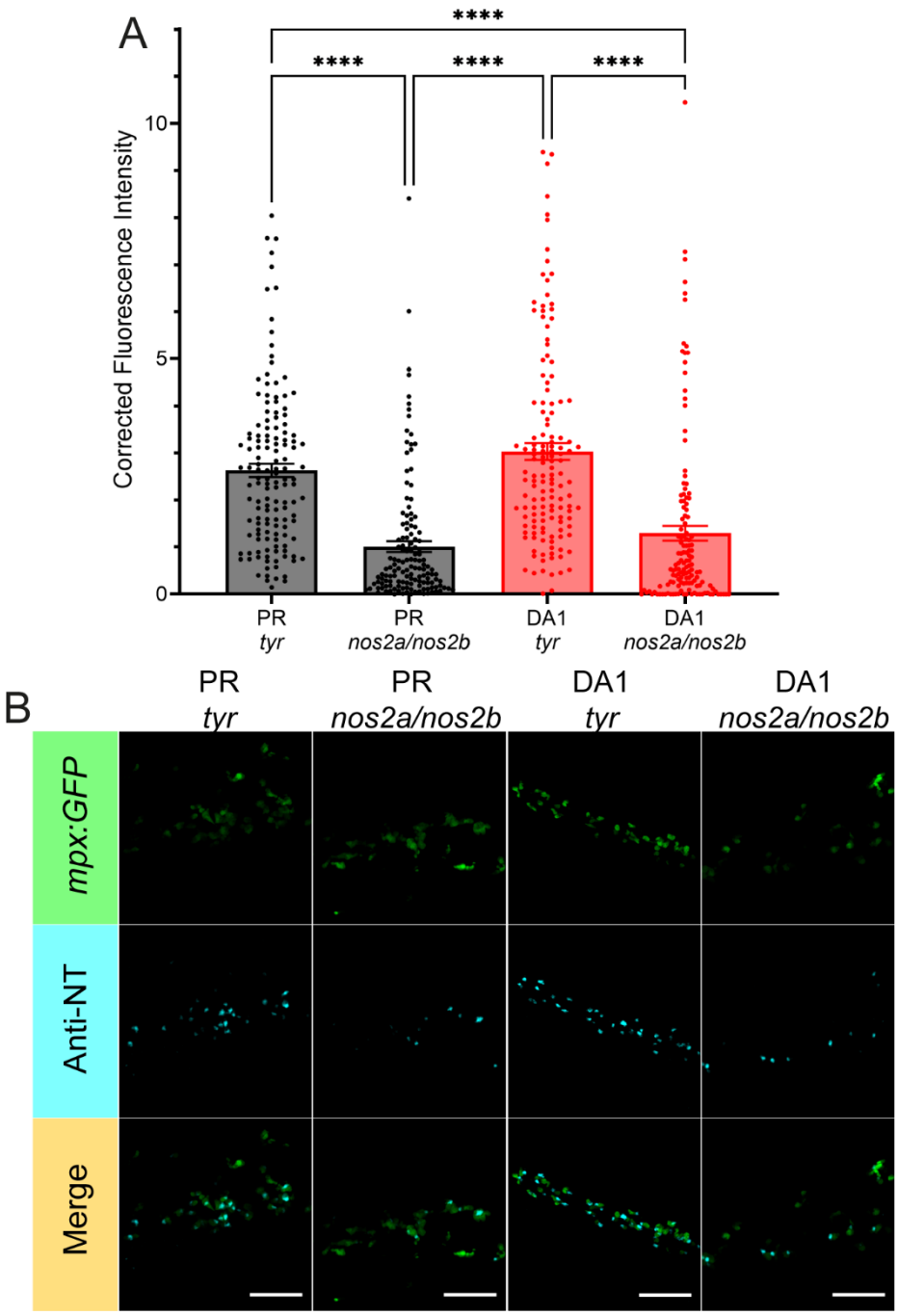

Figure 6.13: *nos2a/nos2b* knockdown reduces host RNS levels.

(A) Anti-nitrotyrosine fluorescence at 24 hpi following injection of PR or DA1 and *tyr* or *nos2a/nos2b* gRNA at the 1 cell stage. N=138 neutrophils from 23 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Kruskal-Wallis test, then Dunn's multiple comparison test. P values shown: ****p<0.0001.

(B) Representative images of PR + *tyr*, PR + *nos2a/nos2b*, DA1 + *tyr* and DA1 + *nos2a/nos2b* injected zebrafish embryos at 24 hpi. Scale bars = 50 µm.

Next, I conducted survival curves of *C. albicans* infected zebrafish with *nos2a/nos2b* knockdown. Embryos injected with PR or DA1 and *tyr* or *nos2a/nos2b* at the 1 cell stage were infected with 200 cfu *C. albicans* TT21-dTomato at 30 hpf. Survival was monitored daily up to 4 dpi.

Survival of PR + *tyr* embryos at 4 dpi was 34.1% (Figure 6.14). PR + *nos2a/nos2b* embryos had significantly reduced survival (22.2%; p<0.01), demonstrating the importance of *nos2* for the anti-*Candida* immune response. DA1 + *tyr* embryos had 57.0% survival at 4 dpi, significantly greater than PR + *tyr* (p<0.0001). 4 dpi survival of DA1 + *nos2a/nos2b* embryos was 29.6%, which was not significantly different from PR + *tyr* or PR + *nos2a/nos2b* embryos. The protective effect of Hif-1α stabilisation was lost in DA1 + *nos2a/nos2b* embryos, providing further evidence that the protective effect of Hif-1α stabilisation is RNS-dependent.

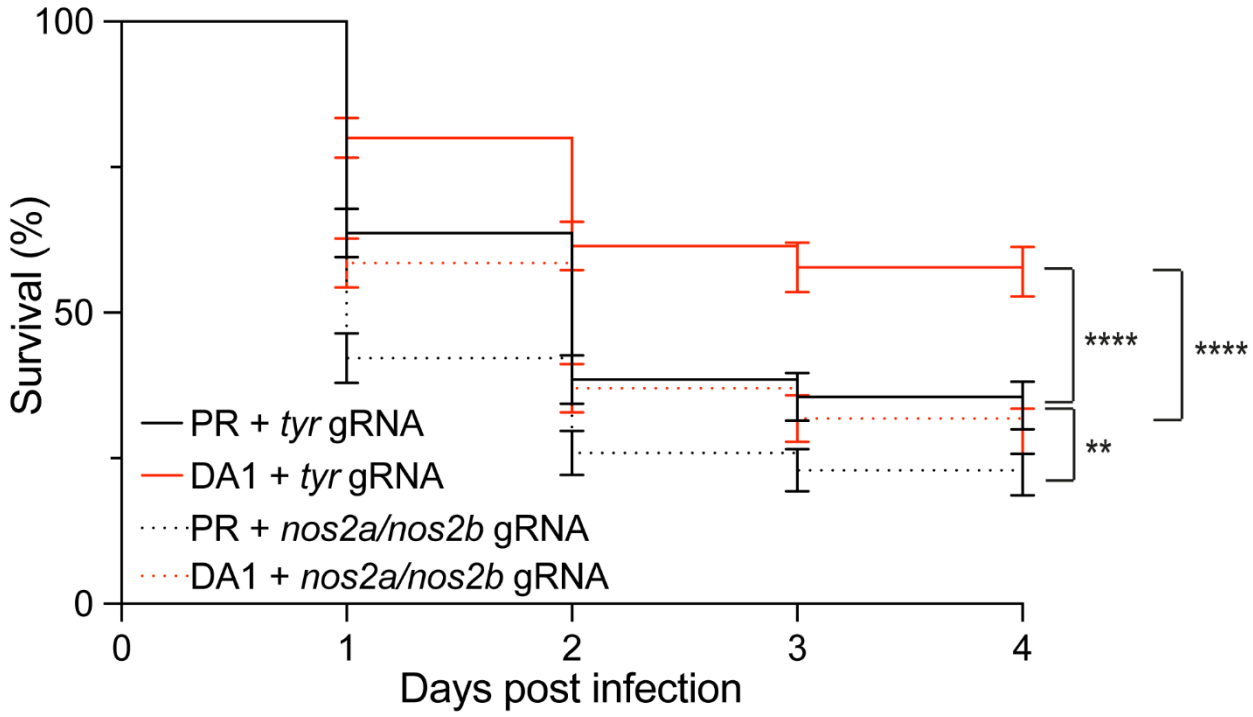

Figure 6.14: Protective effect of Hif-1α stabilisation lost with *nos2a/nos2b* knockdown.

Survival curve of 200 cfu *C. albicans* TT21-dTomato infected zebrafish embryos following injection with PR or DA1 and *tyr* or *nos2a/nos2b* gRNA. N=135 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test, with Bonferroni correction. P values shown: **p<0.01, ****p<0.0001.

6.2.3 Hif-1α stabilisation has an additive effect when combined with traditional antifungals

After demonstrating the mechanism underlying the protective effect of Hif-1α stabilisation, I aimed to explore the potential of Hif-1α to act as an adjunctive therapy alongside traditional antifungals. Fluconazole was selected as the first antifungal to study as it is widely used clinically, due to its wide antifungal spectrum, high bioavailability and relatively low toxicity (Lu *et al.*, 2021). Furthermore, combination therapy with fluconazole has been proposed as a promising avenue for synergistic interactions to treat *C. albicans* infections (Lu *et al.*, 2021).

To accomplish this, I first had to determine an optimum concentration of fluconazole to use in future experiments. To determine a suitable concentration of fluconazole, a logarithmic range of concentrations were trialled in zebrafish embryos infected via the caudal vein with 500 cfu *C. albicans* TT21-dTomato. A greater infection dose of *C. albicans* was used compared to previous experiments due to the potency of fluconazole. Only 15.6% of DMSO treated zebrafish embryos survived up to 4 dpi (Figure 6.15). This was highly similar to the amount of survival observed with the lowest dose of fluconazole (8.9%; 1.0 µg/ml). 4 dpi survival of 5.0 µg/ml and 10.0 µg/ml fluconazole was 22.2% and 57.8%, respectively. 50 µg/ml fluconazole treatment led to 75.6% zebrafish embryos surviving at 4 dpi and 100 µg/ml fluconazole resulted in 88.9% zebrafish embryos surviving at 4 dpi (Figure 6.15). Together, these results demonstrate fluconazole has a dose dependent effect on zebrafish survival in *C. albicans* infection. 5.0 µg/ml was determined as the optimum concentration of fluconazole for later experiments, as the shape of the survival curve most closely resembled that of an ED50 survival curve.

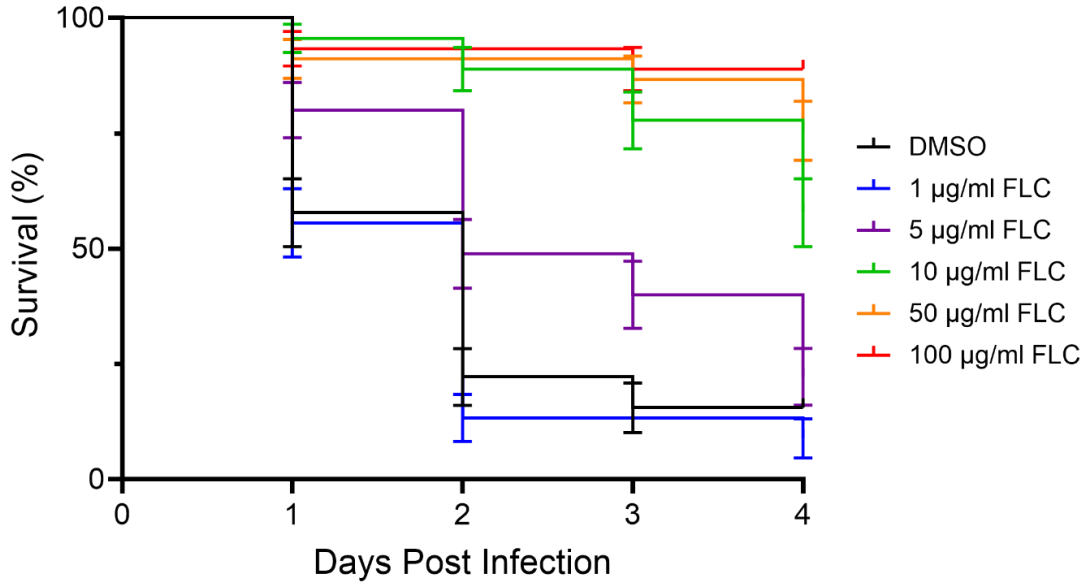

Figure 6.15: Fluconazole has a dose dependent effect on survival in *C. albicans* infection.

Survival of 500 cfu *C. albicans* TT21-dTomato infected zebrafish embryos treated with a range of fluconazole concentrations or DMSO vehicle control. N=45 fish, obtained from 1 independent experiment. Error bars show SEM. No statistical tests were performed.

1 cell stage zebrafish embryos were injected with PR or DA1. At 1 dpf, embryos were injected into the caudal vein with 500 cfu *C. albicans* TT21-dTomato to generate a systemic infection. Embryos were then treated by immersion with 5.0 µg/ml fluconazole or DMSO solvent control. Survival was monitored daily and dead embryos removed. At 2 dpi, surviving embryos were transferred to fresh E3 media and treatment was readministered.

In PR injected embryos, fluconazole (FLC) treatment significantly increased survival from 24.4% in DMSO treated embryos to 54.1% (Figure 6.16; $p<0.0001$), demonstrating the protective effect of fluconazole in zebrafish *C. albicans* infection. 42.2% DA1 + DMSO embryos survived up to 4 dpi, a significantly greater level of survival than PR + DMSO embryos (Figure 6.16; $p<0.01$). 74.1% DA1 + FLC embryos survived at 4 dpi compared to 42.2% DA1 + DMSO embryos ($p<0.0001$) and 54.1% PR + FLC embryos ($p<0.01$). DA1 + FLC survival was analysed for a synergistic effect, using a previously described protocol (Demidenko and Miller, 2019). To be synergistic, DA1 + FLC survival must be greater than the Drug Independence curve. The DA1 + FLC and Drug Independence curves are similar (Figure 6.16B), implying there is not a synergistic interaction between Hif-1α stabilisation and

fluconazole. The combination of Hif-1α stabilisation and fluconazole does have an additive effect on survival.

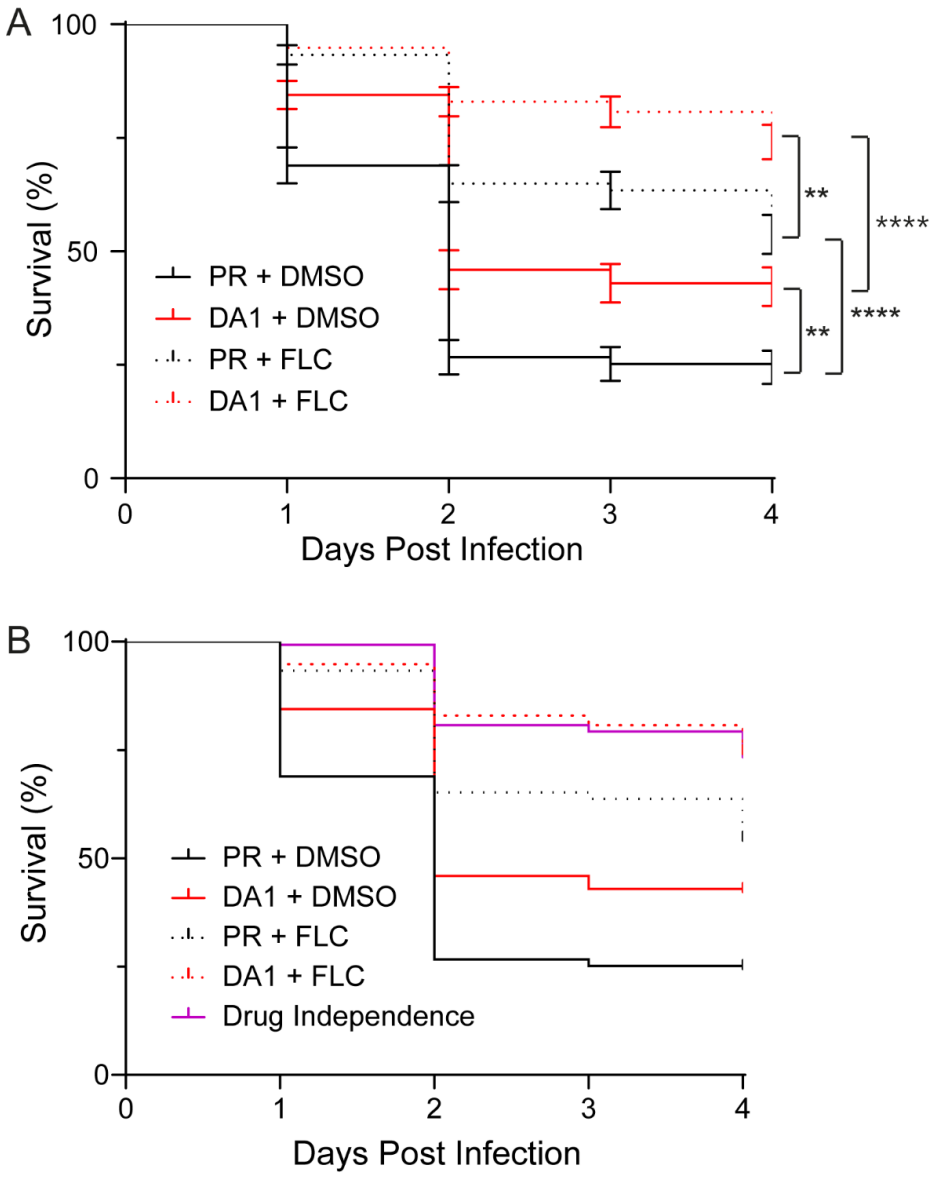

Figure 6.16: Combination of Hif-1α stabilisation and fluconazole has an additive effect on survival in *C. albicans* infection.

(A) Survival of C. albicans TT21-dTomato infected zebrafish embryos following injection with DA1 or PR. Embryos were treated with 5.0 µg/ml fluconazole or DMSO solvent control. Mortality was measured daily. N=135 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test with Bonferroni correction. P values shown: **p<0.01, ****p<0.0001

(B) Drug Independence curve was calculated and plotted, as previously described (2.8 Data analysis; Demidenko and Miller, 2019), to act as a statistical determination of synergy.

Clearance was also used as a measure of infection outcome, in order to understand the ability of Hif-1α stabilisation to lead to complete resolution of *C. albicans* infection. At 4 dpi, all surviving embryos were imaged to quantify clearance. Fluconazole treatment alone did not have a significant effect on clearance compared to PR + DMSO embryos (Figure 6.17). DA1 + DMSO had greater clearance (17.8%) compared to PR + DMSO (4.4%) and PR + FLC (8.2%), though this difference was not statistically significant. DA1 + FLC has 26.7% clearance at 4 dpi, greater than PR + DMSO ($p<0.01$), PR + FLC ($p<0.05$) and DA1 + DMSO (ns). These results suggest Hif-1α stabilisation increases clearance of *C. albicans* infection, with the combination of Hif-1α stabilisation and fluconazole having an additive effect on *C. albicans* clearance in zebrafish.

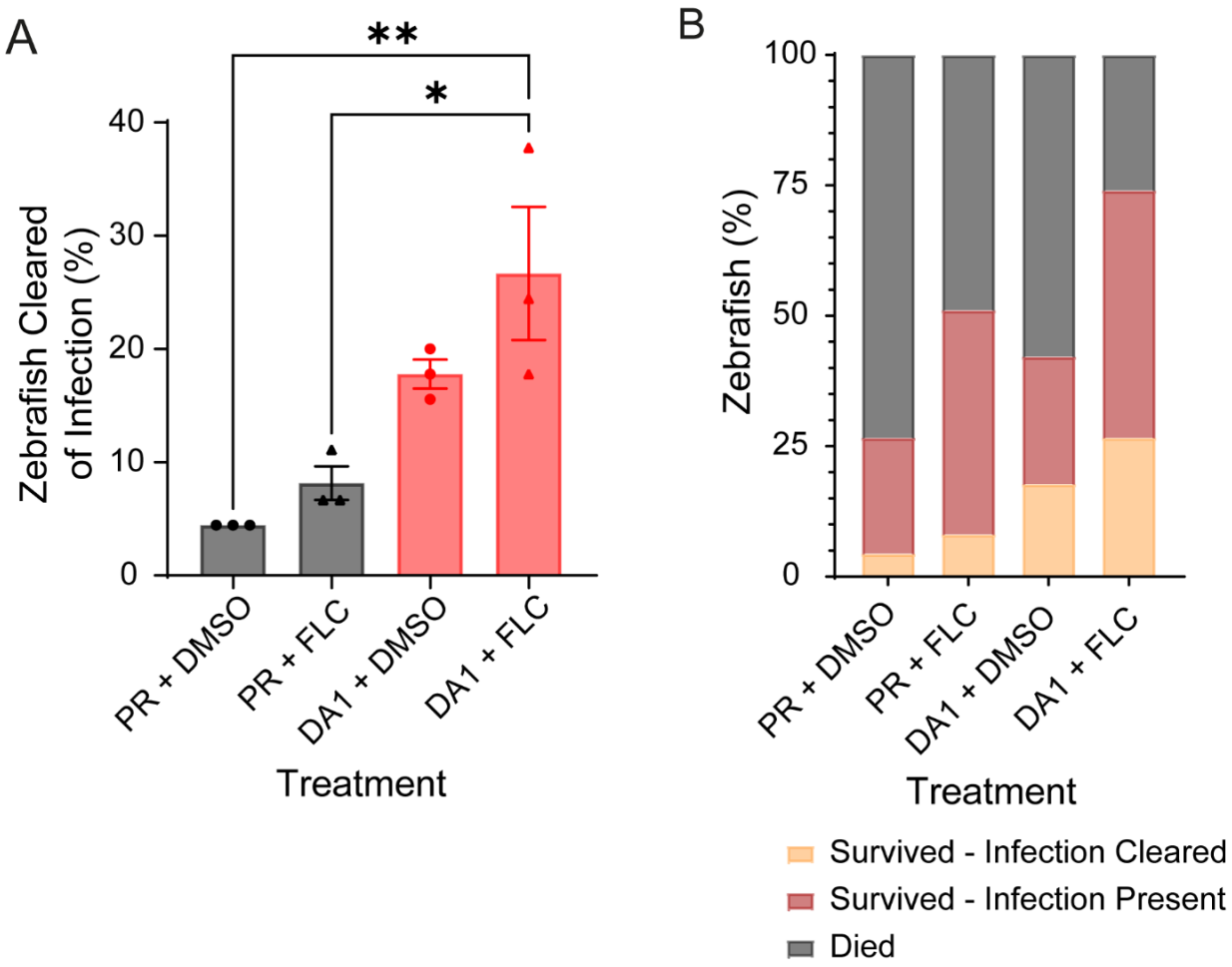

Figure 6.17: Combination of Hif-1α stabilisation and fluconazole has an additive effect on clearance of *C. albicans* infection.

(A) Clearance of *C. albicans* TT21-dTomato infection at 4 dpi by zebrafish embryos 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. Error bars show Standard Deviation. Statistical significance determined by one-way ANOVA, with Tukey's multiple comparisons test. P values shown: *p<0.05, **p<0.01

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

To better characterise potential combination therapies with Hif-1α stabilisation, I investigated a second antifungal: caspofungin. Echinocandins, such as caspofungin, are regularly used as first-line treatment for *C. albicans* infections (Bienvenu *et al.*, 2020). Caspofungin has a different mechanism of action to fluconazole, leaving potential for synergistic action with Hif-1α stabilisation (Vasicek *et al.*, 2014; Szymański *et al.*, 2022).

An optimum dose of caspofungin (CSP) was determined by treating zebrafish infected with 500 cfu *C. albicans* TT21-dTomato with varying doses of caspofungin. Embryos treated with H_2O (solvent control) had 10.0% survival at 4 dpi. Multiple doses of caspofungin did not appear to have a protective effect, with 18.3%, 11.7% and 15.0% survival at 4 dpi for 0.01 µg/ml, 0.1 µg/ml and 0.5 µg/ml caspofungin, respectively (Figure 6.18). 1.0 µg/ml caspofungin treated embryos had 38.3% survival and 5.0 µg/ml caspofungin treated embryos had 76.7% survival at 4 dpi (Figure 6.18). Therefore, caspofungin can protect against *C. albicans* infection in zebrafish *in vivo*. 1.0 µg/ml caspofungin was selected as the optimum dose for experiments going forward.

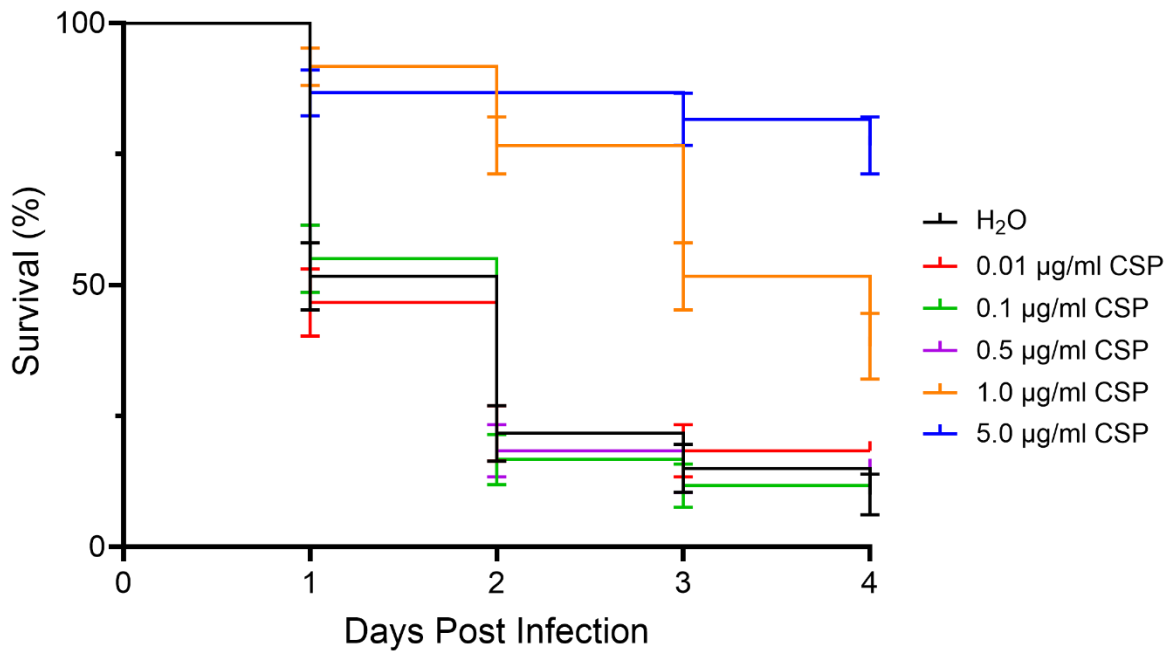

Figure 6.18: Caspofungin has a dose dependent effect on survival in *C. albicans* infection.

Survival of 500 cfu *C. albicans* TT21-dTomato infected zebrafish embryos treated with a range of caspofungin concentrations or dH_2O solvent control. N=60 fish, obtained from 1 independent experiment. Error bars show SEM. No statistical tests were performed.

1 cell stage zebrafish embryos were injected with PR or DA1. At 1 dpf, embryos were injected into the caudal vein with 500 cfu *C. albicans* TT21-dTomato to generate a systemic infection. Embryos were then treated by immersion with 1.0 μg/ml caspofungin or dH_2O solvent control. Survival was monitored daily up to 4 dpi. At 2 dpi, surviving embryos were transferred to fresh E3 media and treatment was readministered.

PR + dH_2O embryos had 12.6% survival at 4 dpi. Caspofungin (CSP) treatment in PR injected zebrafish had a protective effect, with 4 dpi survival of PR + CSP embryos being 24.4%, significantly greater than PR + dH_2O (Figure 6.19; $p<0.0001$). 25.9% DA1 + dH_2O embryos survived up to 4 dpi, showing the protective effect of Hif-1α stabilisation. Survival of DA1 + CSP embryos at 4 dpi was 45.9%, significantly greater than DA1 + dH_2O ($p<0.0001$) and PR + CSP ($p<0.01$). Caspofungin treated embryos had substantially higher survival at 1 dpi than embryos treated with dH_2O, with all embryos having similar rates of mortality from 2 dpi onwards, suggesting caspofungin treatment is most effective in the early stages of *C. albicans* infection. DA1 + CSP almost entirely overlapped with the Drug Independence curve (Figure 6.19B), suggesting a lack of synergy between Hif-1α stabilisation and caspofungin. Hence, combination of Hif-1α stabilisation and caspofungin has an additive effect on survival in *C. albicans* infection.

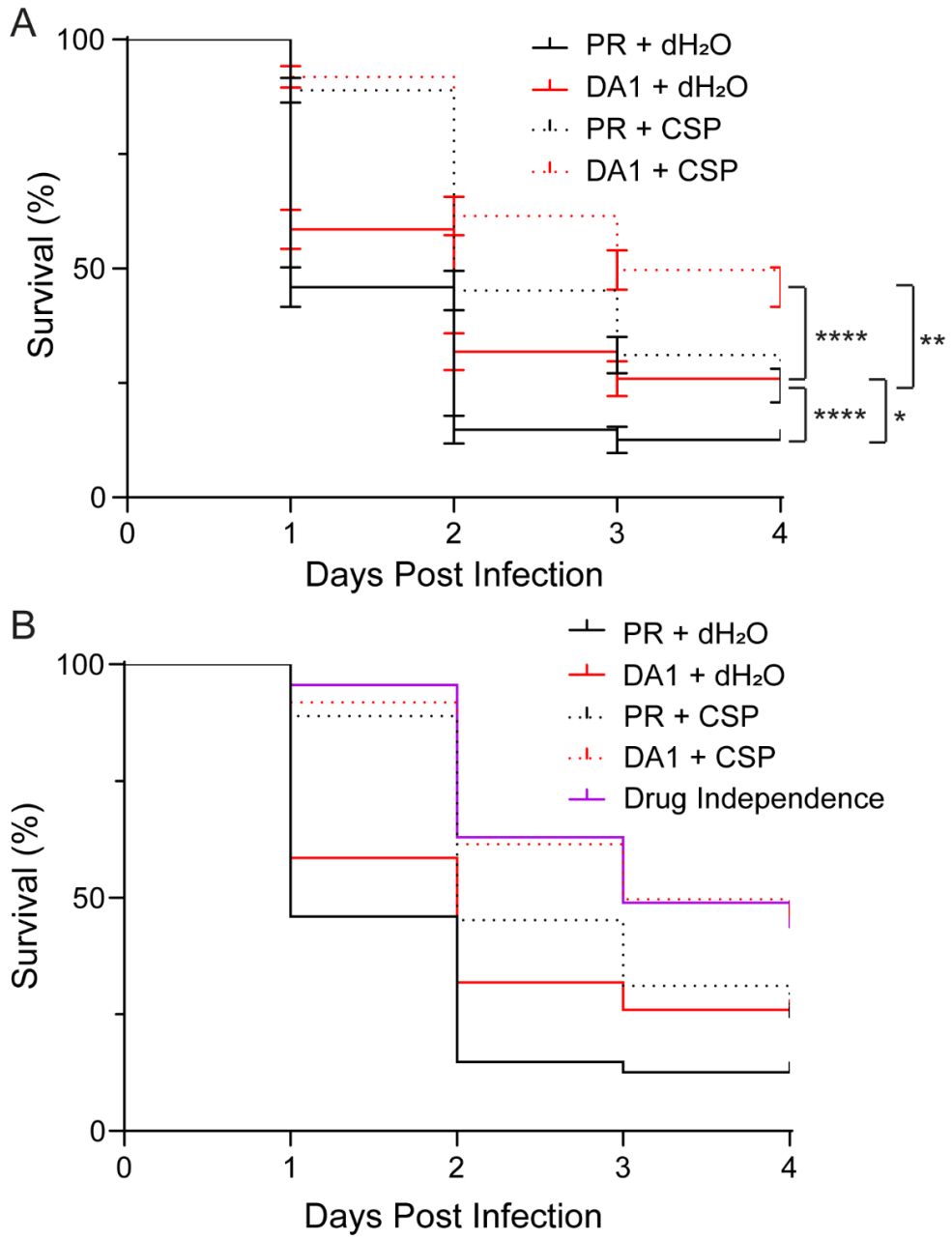

Figure 6.19: Combination of Hif-1α stabilisation and caspofungin has an additive effect on zebrafish survival in *C. albicans* infection.

(A) Survival of C. albicans TT21-dTomato infected zebrafish embryos following injection with DA1 or PR. Embryos were treated with 1.0 µg/ml caspofungin or dH_2O solvent control. Mortality was measured daily. N=135 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test with Bonferroni correction. P values shown: **p<0.01, ****p<0.0001

(B) Drug Independence curve was calculated and plotted, as previously described (Demidenko and Miller, 2019), to act as a statistical determination of synergy.

The effect of combining Hif-1α stabilisation and caspofungin on clearance of *C. albicans* infection was also investigated. Surviving embryos at 4 dpi were screened for any signs of remaining *C. albicans* TT21-dTomato infection. 3.0% PR + dH_2O embryos cleared the *C. albicans* infection, statistically similar to PR + CSP, in which 3.7% embryos cleared the infection (Figure 6.20). DA1 + dH_2O embryos had 10.4% clearance, which was greater than, but not significantly different to, PR injected embryos. Combination of DA1 and caspofungin increased clearance compared to PR + dH_2O and PR + CSP (DA1+CSP=22.2%; $p<0.05$ for both). Hif-1α stabilisation alone appears to have a positive trend towards clearance of *C. albicans* infection but combination of Hif-1α stabilisation with caspofungin has an additive effect on clearance.

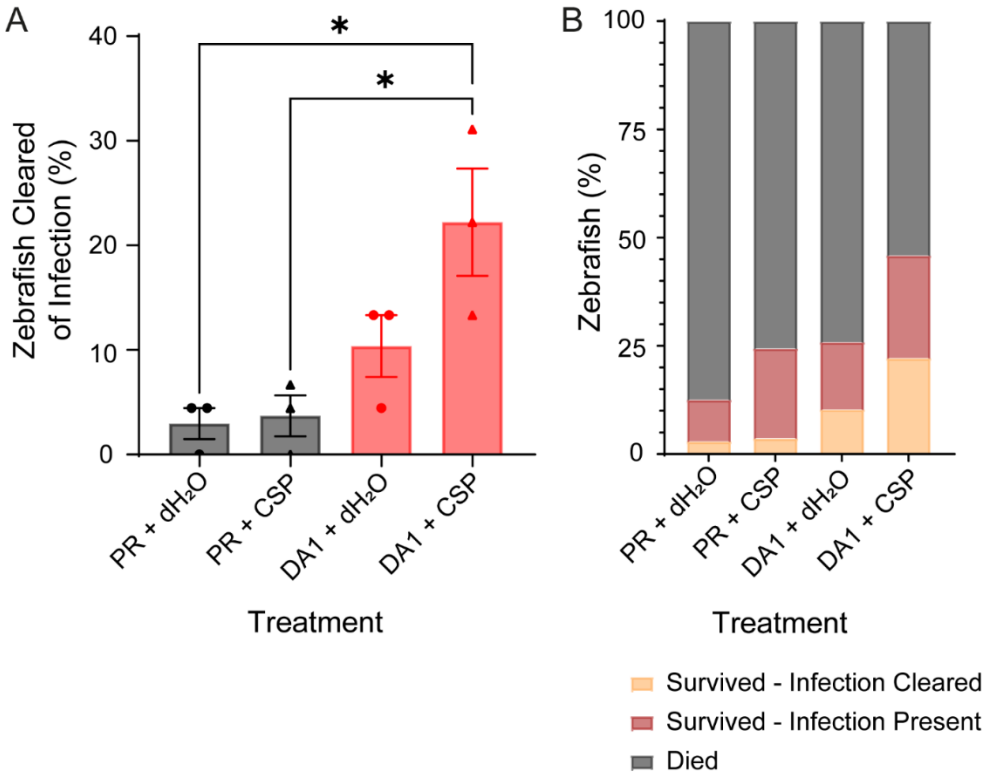

Figure 6.20: Combination of Hif-1α stabilisation and caspofungin has an additive effect on clearance of *C. albicans* infection.

(A) Clearance of *C. albicans* TT21-dTomato infection at 4 dpi by zebrafish embryos 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. Error bars show Standard Deviation. Statistical significance determined by one-way ANOVA, with Tukey's multiple comparisons test. P values shown: *p<0.05, **p<0.01

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

Sub-inhibitory concentrations of caspofungin have previously been shown to cause hyphal-biased unmasking of *C. albicans* β-glucan (Wheeler *et al.*, 2008), leading to greater recognition by Dectin-1. β-glucan masking is associated with reduced neutrophil recruitment and reduced phagocytosis in murine *in vivo* and *in vitro* models (Ballou *et al.*, 2016; Pradhan *et al.*, 2018). This suggests β-glucan unmasking may lead to greater immune cell recruitment. Based on this, I hypothesised that sub-inhibitory concentrations of caspofungin would combine with Hif-1α stabilisation to have a synergistic effect on survival and clearance, due to increased immune cell recruitment.

Based on the previous caspofungin dose survival curve (Figure 6.18), 0.5 µg/ml caspofungin was selected as a sub-inhibitory dose. Embryos injected with PR or DA1 were infected with 500 cfu *C. albicans* TT21-dTomato, then treated with sub-inhibitory caspofungin (siCSP) or dH_2O solvent control. Mortality was monitored daily up to 4 dpi.

20.0% PR + dH_2O embryos survived up to 4 dpi, while 26.7% PR + siCSP embryos survived up to 4 dpi (Figure 6.21; ns). There was no significant difference in survival in PR injected zebrafish treated with dH_2O or sub-inhibitory caspofungin, demonstrating the concentration used was sub-inhibitory. DA1 + dH_2O embryos had 36.3% survival at 4 dpi, which was significantly greater than PR + dH_2O ($p<0.05$) but not significantly different from PR + siCSP. Similarly, DA1 + siCSP survival was significantly greater than PR + dH_2O ($p<0.001$) but not different from PR + siCSP (Figure 6.21). 41.5% DA1 + siCSP embryos survived up to 4 dpi, which was greater than, but not significantly different from, DA1 + dH_2O, suggesting sub-inhibitory concentrations of caspofungin do not provide an additional protective effect when combined with Hif-1α stabilisation.

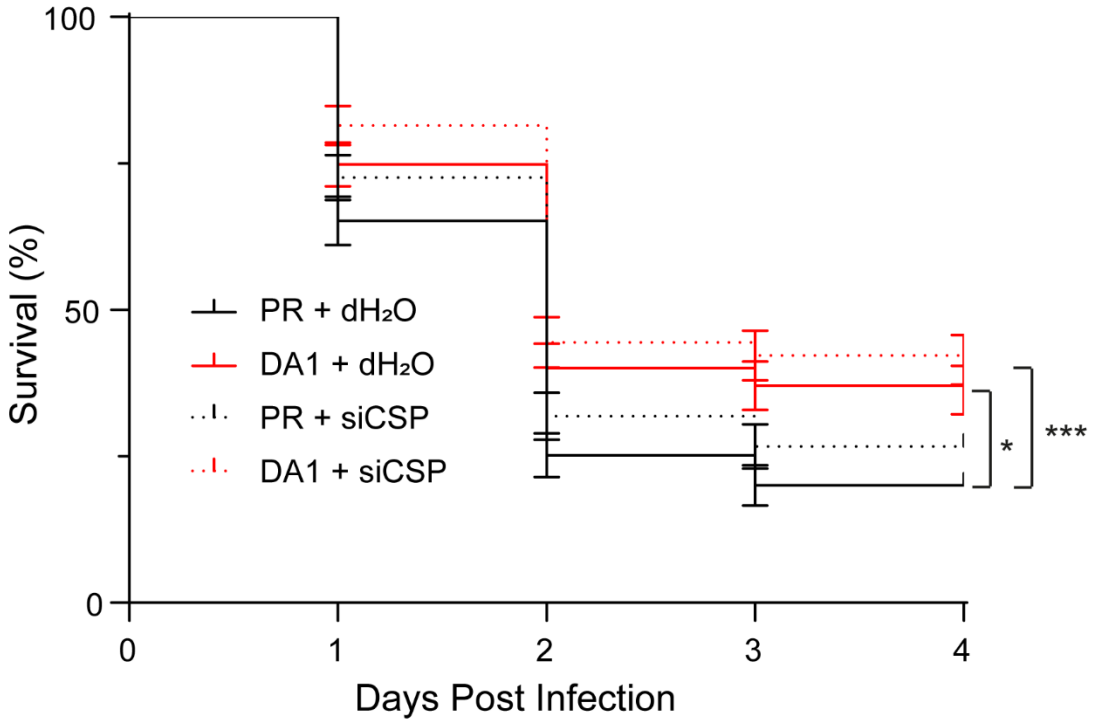

Figure 6.21: Combination of Hif-1α stabilisation and sub inhibitory caspofungin has no effect on survival in *C. albicans* infection.

Survival of C. albicans TT21-dTomato infected zebrafish embryos following injection with DA1 or PR. Embryos were treated with 0.5 µg/ml caspofungin or dH_2O solvent control. Mortality was measured daily. N=135 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test with Bonferroni correction. P values shown: *p<0.05, ***p<0.001

Although sub-inhibitory concentrations of caspofungin did not have an additive effect on survival when combined with Hif-1α stabilisation, I still investigated the effect of sub-inhibitory caspofungin on clearance of *C. albicans* infection *in vivo*. Clearance of *C. albicans* infection was observed in 3.7% PR + dH_2O embryos and 4.4% PR + siCSP embryos (Figure 6.22). In DA1 injected embryos, dH_2O treatment led to clearance in 14.8% embryos, while siCSP treatment resulted in clearance of *C. albicans* infection in 16.3% embryos (Figure 6.22). Hif-1α stabilisation appeared to promote clearance of *C. albicans* infection *in vivo*, though no statistically significant differences were observed between any group. Therefore, sub-inhibitory caspofungin does not have an additive effect on clearance of *C. albicans* infection when combined with Hif-1α stabilisation.

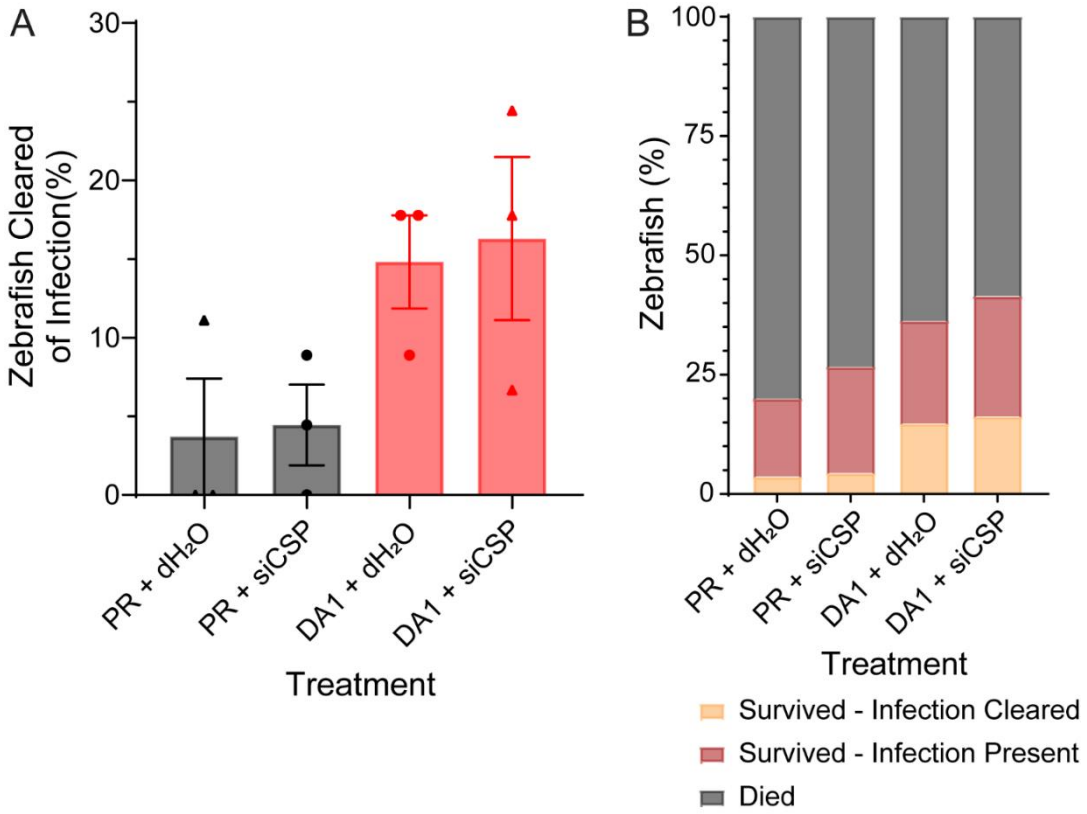

Figure 6.22: Combination of Hif-1α stabilisation and sub inhibitory caspofungin has no effect on clearance of *C. albicans* infection.

(A) Clearance of *C. albicans* TT21-dTomato infection at 4 dpi by zebrafish embryos following injection with DA1 or PR and treatment with 0.5 µg/ml caspofungin or dH_2O solvent control. N=3 independent experiments, 135 fish per group. Error bars show Standard Deviation. Statistical significance determined by one-way ANOVA, with Tukey's multiple comparisons test.

(B) Infection outcomes of *C. albicans* TT21-dTomato infected zebrafish embryos at 4 dpi, following injection with DA1 or PR and treatment with 0.5 µg/ml caspofungin or dH_2O vehicle control. N=3 independent experiments, 135 fish per group.

To further characterise Hif-1α stabilisation as a potential HDT for the treatment of *C. albicans* infections, I investigated a pharmacological method of Hif-1α stabilisation. FG4592 (Roxadustat) is a PHD inhibitor, which is used clinically as a HIF-1α stabiliser for the treatment of anaemia (Dhillon, 2019). I hypothesised pharmacological stabilisation of Hif-1α, with FG4592, would have a protective effect in *C. albicans* infection *in vivo* and have an additive effect in combination with fluconazole.

DMSO treatment alone led to 22.2% survival at 4 dpi, which was similar to the 25.2% FG4592-treated larvae that survived up to 4 dpi (Figure 6.23). Hence, FG4592 did not have a protective effect

in *C. albicans* infection. Fluconazole-treated larvae had 63.0% survival at 4 dpi, significantly greater than DMSO-treated larvae (Figure 6.23; p<0.0001). Larvae treated with both FG4592 and fluconazole had 65.9% survival at 4 dpi. This was not significantly greater survival than fluconazole-treated larvae, indicating the combination of FG4592 and fluconazole does not have an additive effect on zebrafish larvae survival in *C. albicans* infection.

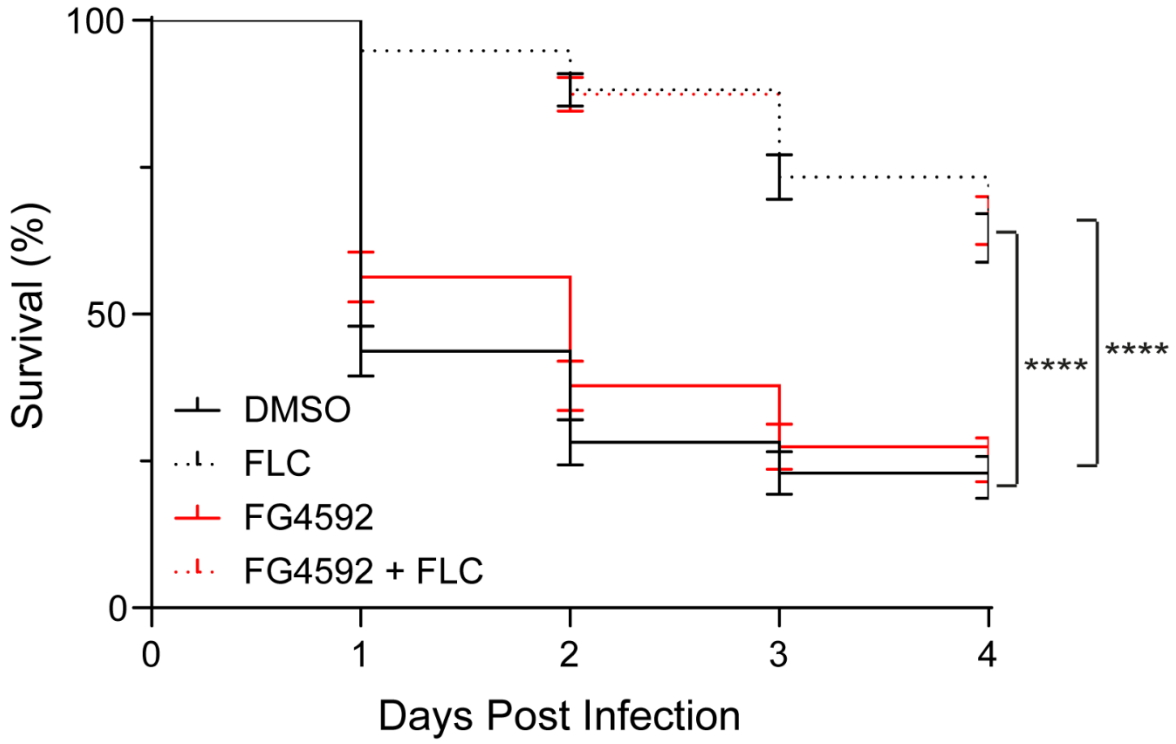

Figure 6.23: Combination of FG4592 and fluconazole has no effect on survival in *C. albicans* infection.

Survival of C. albicans TT21-dTomato infected zebrafish embryos treated with DMSO solvent control, 5.0 µg/ml fluconazole, 2.5 µM FG4592 or 5.0 µg/ml fluconazole and 2.5 µM FG4592. Mortality was measured daily. N=135 fish, obtained from 3 independent experiments. Error bars show SEM. Statistical significance determined by Gehan-Breslow-Wilcoxon test with Bonferroni correction. P values shown: ****p<0.0001

Although FG4592 did not have an effect on zebrafish survival in *C. albicans* infection, I investigated a potential effect on *C. albicans* clearance *in vivo*. Clearance in DMSO-treated larvae was 4.4%, while clearance in fluconazole-treated larvae was 5.9% (Figure 6.24). FG4592 treatment led to 13.7% larvae clearing the *C. albicans* infection. Combination of FG4592 and fluconazole led to 13.7% clearance (Figure 6.24). Despite the trend towards greater clearance with FG4592 treatment, there were no significant differences between any group, suggesting FG4592 alone and combination of FG4592 with fluconazole does not increase clearance of *C. albicans* infection *in vivo*.

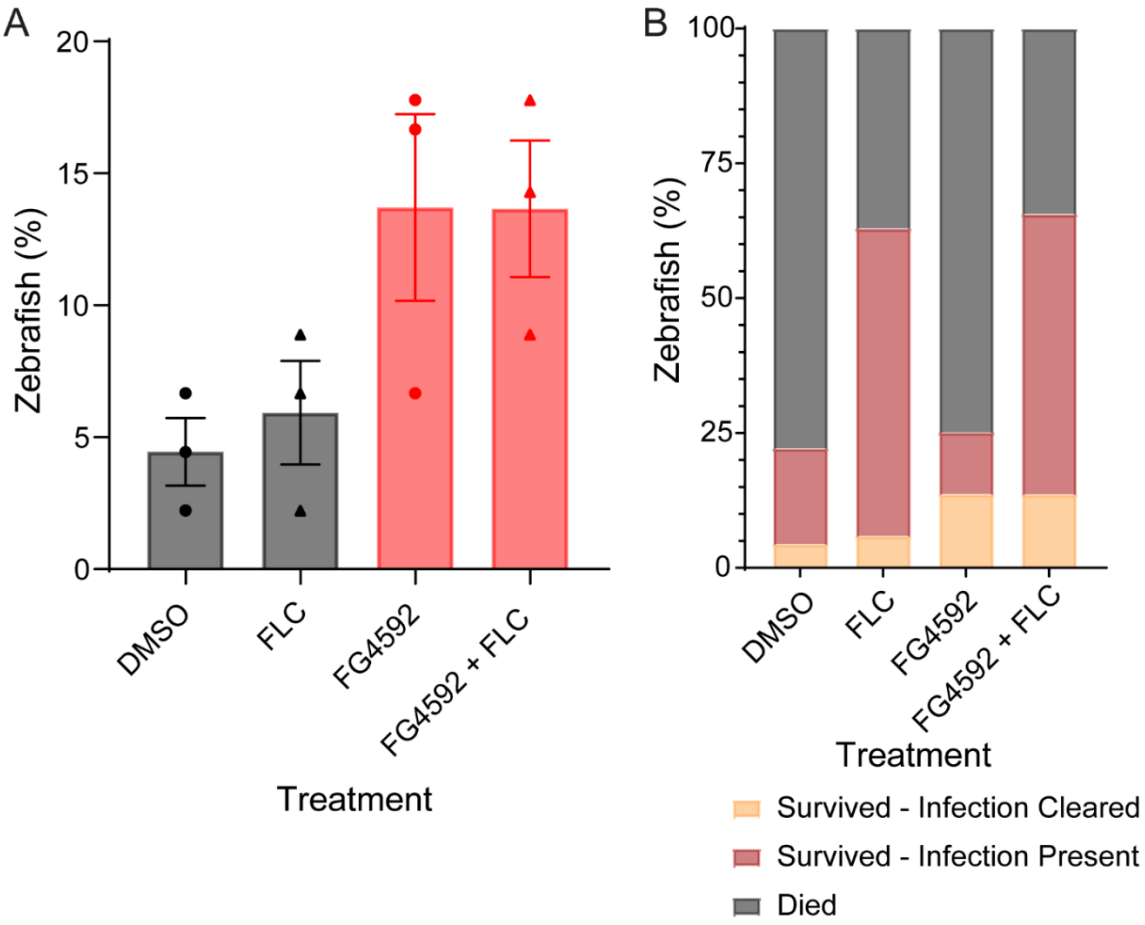

Figure 6.24: Combination of FG4592 and fluconazole has no effect on clearance of *C. albicans* infection.

(A) Clearance of *C. albicans* TT21-dTomato infection at 4 dpi by zebrafish embryos following treatment with DMSO solvent control, 5.0 µg/ml fluconazole, 2.5 µM FG4592 or 5.0 µg/ml fluconazole and 2.5 µM FG4592. N=3 independent experiments, 135 fish per group. Error bars show Standard Deviation. Statistical significance determined by one-way ANOVA, with Tukey's multiple comparisons test.

(B) Infection outcomes of *C. albicans* TT21-dTomato infected zebrafish embryos at 4 dpi, following treatment with DMSO solvent control, 5.0 µg/ml fluconazole, 5.0 µM FG4592 or 5.0 µg/ml fluconazole and 5.0 µM FG4592. N=3 independent experiments, 135 fish per group.

6.3 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. I have shown that Hif-1α stabilisation represents a potential host directed therapy capable of restoring the neutrophil RNS response to *C. albicans* infection, which may have further improvements in infection outcomes when combined with traditional antifungals.

I demonstrated that Hif-1α stabilisation has a protective effect in *C. albicans* infection in zebrafish embryos, via a neutrophil-mediated, RNS-dependent mechanism. Neutrophils are the primary innate immune cell responsible for killing *Candida* spp*.,* with neutropenic patients at high risk for developing invasive candidiasis (Brown, 2011; Desai, van de Veerdonk and Lionakis, 2018). This supports the conclusion that neutrophil RNS suppression is an important strategy of *C. albicans* during infection that could represent an exciting therapeutic target.

Hif-1α stabilisation has potential to act as a host directed therapy for treatment of *C. albicans* infections, by upregulating neutrophil RNS levels and improving immune candidacidal activity. Non-albicans *Candida* spp*.* also suppress neutrophil RNS production in zebrafish larvae (see Chapter 5), introducing the possibility that RNS upregulation by Hif-1α stabilisation could be a potential treatment for candidiasis caused by a range of *Candida* spp*.* NO-releasing nanoparticles restricted *C. albicans* growth *in vitro* and on burn lesions on mice *in vivo*, demonstrating exogenous RNS can be used therapeutically to treat *C. albicans* infections (Macherla *et al.*, 2012). Genetic (DA1) and pharmacological (DMOG; Dimethyloxalyl glycine) Hif-1α stabilisation has previously been shown to protect against *M. marinum* infection in zebrafish by an RNS-dependent mechanism (Elks *et al.*, 2013; Ogryzko *et al.*, 2019), suggesting Hif-1α stabilisation could offer protective effects multiple and different groups of pathogens (fungi and bacteria) where neutrophil-mediated immune responses are subverted. Hif-1α deficiency in murine macrophages resulted in reduced expression of pro-inflammatory cytokines when stimulated by LPS or curdlan (a D-glucose homopolymer and cellular component of *C. albicans*) *in vitro* (Li *et al.*, 2018b). This phenotype was matched *in vivo* when mice were infected with *Listeria monocytogenes* or *C. albicans*, with reduced pro-inflammatory cytokine expression and increased bacterial/fungal burden (Li *et al.*, 2018b). Activation of Hif-1α using $CoCl_2$ increased TNFα production by murine and human macrophages *in vitro* and reduced *C. albicans* burden *in vitro* (Li *et al.*, 2018b). Although the exact mechanism is contested, the most common hypothesis is that $CoCl_2$ stabilises Hif-1α/Hif-2α by replacing Fe^{2+} in PHD with Co^{2+}, rendering PHD enzymes inactive and allowing an accumulation of Hif-1α/Hif-2α (Muñoz-Sánchez and Chánez-Cárdenas, 2019). $CoCl_2$ stabilises Hif-1α significantly more than DMOG *in vitro* at equal concentrations (Borcar *et al.*, 2013). However, $CoCl_2$ inhibits lateral line function in zebrafish,

potentially affecting behaviour, and causes toxicity with prolonged use (Janssen, 2000; Stewart, Johansen and Liao, 2017). $CoCl_2$ has previously been associated with thyroid dysfunction and goitre formation when used in humans, making it unsuitable for investigation as a potential HDT to treat *C. albicans* infections in humans. Acetate, a short chain fatty acid, has also been implicated in Hif-1α-targeting therapies. Acetate has protective effects against *Klebsiella pneumoniae* and *Streptococcus pneumoniae in vitro* by enhancing bactericidal macrophage activity (Galvão *et al.*, 2018; Sencio *et al.*, 2020). Further investigation revealed acetate stimulates increased NO production by murine alveolar macrophages in the presence of *S. pneumoniae* but had no effect on NO levels in the absence of infection, suggesting acetate enhances the host effect rather than inducing it by itself (Machado *et al.*, 2022). Macrophage metabolism shifted towards glycolysis in the presence of acetate, increasing Hif-1α expression, which stimulated increased levels of IL-1β, triggering NO production. Increased Hif-1α expression by murine alveolar macrophages occurred in the presence and absence of *S. pneumoniae.* Simultaneously, acetate stimulated IL-1β production via NLRP3 inflammasome modulation, further enhancing NO production (Machado *et al.*, 2022). These experiments reveal a role for acetate as a potential host directed therapy, by Hif-1α-mediated upregulation of host RNS production. Butyrate, an alternative short chain fatty acid, was also shown to increase NO production by J774.16 macrophages exposed to *C. albicans* and *Cryptococcus neoformans in vitro*, enhancing fungal killing by macrophages (Nguyen *et al.*, 2011). Conversely, acetate was shown to reduce NO production by RAW264.7 macrophages *in vitro* stimulated by LPS (Liu *et al.*, 2012). Acetate drove LPS-stimulated murine BMDMs towards an anti-inflammatory phenotype by restricting glycolysis metabolism *in vitro*, which could be reversed, and a pro-inflammatory state restored, by pharmacological Hif-1α stabilisation with DMOG (Li *et al.*, 2024). Furthermore, butyrate reduced TNFα expression and NO production by LPS-stimulated rat neutrophils *in vitro* and promoted an anti-inflammatory phenotype in colonic lamina propria macrophages *in vitro* (Vinolo *et al.*, 2011; Chang *et al.*, 2014). These opposing conclusions could be explained by differences in cell type used or the difference between LPS stimulation and exposure to whole pathogens, which have a wider range of PAMPs. In a caecal ligation and puncture (CLP) mouse model of polymicrobial sepsis, administration of acetate improved mouse survival and reduced expression of pro-inflammatory cytokines (Li *et al.*, 2024). Hif-1α stabilisation reversed the suppression of pro-inflammatory cytokines, leading to increased levels of TNFα, IL-6 and IL-1β, and reduced survival in CLP mice treated with acetate, revealing nuanced roles for acetate and Hif-1α in sepsis that counter their roles in monomicrobial, non-sepsis infection models. SCFAs, such as acetate, have wide-ranging, complex immunomodulatory functions. For example, SCFAs have been shown to inhibit inflammasome activation, inhibit pro-inflammatory cytokine production and promote Treg cell

formation (Corrêa-Oliveira *et al.*, 2016; Liu *et al.*, 2023). SCFAs can also affect other tissues and cause adverse events, such as increased lipid accumulation, disruption of the gut epithelium (risking development of opportunistic infections), hyperglycaemia, hypotension, metabolic alkalosis (Keshaviah, 1982; Veech and Gitomer, 1988; Xiong *et al.*, 2022; Gao *et al.*, 2024). Hence, SCFAs may not be suitable as a potential HDT for treatment of *C. albicans* infections.

The host protective effect of Hif-1α stabilisation was revealed to be RNS-dependent following genetic and pharmacological inhibition of RNS synthesis. RNS has well characterised, highly candidacidal effects, making it logical that restoration of neutrophil RNS levels would improve the host's ability to respond to *C. albicans* infections (Rementería, García-Tobalina and Sevilla, 1995; Vazquez-Torres *et al.*, 1995; Tillmann, Gow and Brown, 2011; Navarathna, Lionakis and Roberts, 2019). Genetic inhibition of RNS formation was achieved by CRISPR/Cas9-mediated knockdown of *nos2a/nos2b*. Functional validation of *nos2a/nos2b* knockdown, by measuring anti-NT levels, revealed *nos2a/nos2b* knockdown was substantially less effective in my hands than has previously been shown (Szkuta, 2020). I used a different CRISPR/Cas9 preparation protocol, which has been shown to result in more efficient integration of gRNA. The reduced efficacy of my *nos2a/nos2b* knockdown suggests this protocol may not be effective for the gRNAs I used. Alternatively, CRISPR/Cas9 efficiency could have been reduced by freeze-thaw degradation of gRNAs or Cas9 protein (Farboud *et al.*, 2018). The protective effect of Hif-1α stabilisation was still abrogated with inefficient *nos2a/nos2b* knockdown, suggesting RNS formation was sufficiently inhibited to eliminate RNS-mediated protection in *C. albicans* infection.

Alternative genetic methods of RNS inhibition have been published that could be explored, including other *nos2a/nos2b* gRNAs, stable knockout zebrafish lines and morpholinos (Hall *et al.*, 2012; Rochon *et al.*, 2023; Y. Huang *et al.*, 2024; Yu *et al.*, 2024).

The *csf3r* morpholino was used to deplete the neutrophil population, to demonstrate the protective effect of Hif-1α stabilisation is neutrophil-dependent. A weakness of morpholinos is the risk of toxicity and off-target effects, raising the possibility of confusion between what is and is not a genuine morpholino phenotype (Bedell, Westcot and Ekker, 2011). The *csf3r* morpholino used is well characterised and widely used, suggesting the observed phenotype is genuine (Ellett *et al.*, 2011, 2018; Middel *et al.*, 2016; Klems *et al.*, 2020). A stable *csf3r* knockout zebrafish line has been generated, which provides an alternative to the *csf3r* morpholino (Pazhakh *et al.*, 2017). Furthermore, this would allow examination of the importance of neutrophils in *C. albicans* infection over a longer period of time, as neutrophil depletion is persistent and not abrogated following breakdown of a morpholino.

Clodronate liposomes were used to ablate the macrophage polarisation in zebrafish embryos, revealing the protective effect of Hif-1α stabilisation is not macrophage-dependent. Investigation of clodronate liposomes in a mouse model revealed, as well as depleting the macrophage population, clodronate liposomes were taken up by neutrophils, resulting in impaired neutrophil function. Clodronate liposomes caused reduced phagocytosis, reduced ROS production, impaired NET formation and reduced swarming of neutrophils, described as functional stunning of neutrophils (Culemann *et al.*, 2023). Depletion of neutrophil number was not observed with clodronate liposomes in this study or other studies (Bojarczuk *et al.*, 2016; Culemann *et al.*, 2023). In my experiments, mortality of clodronate liposome injected embryos may have been increased by functional stunning of neutrophils, reducing neutrophil ability to eliminate *C. albicans* infection. The protective effect of Hif-1α stabilisation was maintained in embryos injected with clodronate liposomes, suggesting Hif-1α stabilisation may be able to overcome the functional arrest caused by clodronate liposomes. *Spi1* (previously called *pu1*) morpholino injection has been used as an alternative method of macrophage depletion in zebrafish (Rhodes *et al.*, 2005; Tenor *et al.*, 2015). Low concentrations of *spi1* morpholino have no characterised impacts on neutrophil function, which would provide a better representation of the importance of macrophages in *C. albicans* infection in zebrafish embryos. However, high concentrations of *spi1* morpholino are known to cause depletion of neutrophils, as well as macrophages, making high concentrations unsuitable for this experiment (Tenor *et al.*, 2015).

I showed that genetic Hif-1α stabilisation has an additive effect on survival and clearance when combined with traditional antifungals. Fluconazole and caspofungin were used in these experiments as they are different classes of antifungal, so have different mechanisms of action, and are 2 of the most widely used antifungals for *C. albicans* infections (Vasicek *et al.*, 2014; Bienvenu *et al.*, 2020; Lu *et al.*, 2021). Fluconazole is a primarily fungistatic agent, which binds to lanosterol 14α-demethylase, preventing conversion of lanosterol to ergosterol, thus disrupting fungal membrane synthesis (Pasko, Piscitelli and Van Slooten, 1990; Vasicek *et al.*, 2014; Campoy and Adrio, 2017). I hypothesise the fungistatic activity of fluconazole restricts initial growth of *C. albicans* in the zebrafish candidiasis model, allowing neutrophils with increased RNS production (due to Hif-1α stabilisation) to kill *C. albicans*, resulting in the observed increase in survival and clearance. Using a mouse *in vivo* model of candidiasis, Zhu *et al.* revealed a complement-dependent first wave of neutrophil recruitment that is key for determining *C. albicans* infection outcome. Adhesin knockout mice, that lacked fungicidal activity in the first wave of neutrophil recruitment, had decreased survival in *C. albicans* infection compared to wild type mice (Zhu *et al.*, 2023). Hence, early fungicidal activity by neutrophils is vital for subsequent clearance and resolution of *C. albicans* infection. Phagocytosis in the early stages of

C. albicans infection in zebrafish was correlated with mortality, with high levels of early phagocytosis being a strong prognostic indicator of survival (Brothers *et al.*, 2013; Bergeron, Barker, *et al.*, 2017). Restricting *C. albicans* growth by fluconazole treatment may extend the early infection window, giving more time for neutrophil recruitment and fungicidal activity. On the other hand, caspofungin predominantly has a fungicidal effect, mediated through non-competitive inhibition of β(1,3)-D-glucan synthase. This prevents β(1,3)-D-glucan synthesis, impacting the integrity of the fungal cell wall and leading to cell death due to intracellular osmotic pressure (Letscher-Bru and Herbrecht, 2003; Szymański *et al.*, 2022). Early fungicidal activity by caspofungin may also restrict initial expansion of *C. albicans* infection in zebrafish embryos, having an additive effect on *C. albicans* killing with neutrophils with restored RNS levels and reducing the risk of mortality.

Disappointingly, pharmacological Hif-1α stabilisation, with FG4592, did not have an effect on survival or clearance of *C. albicans* infections *in vivo*, nor did FG4592 have an additive effect when combined with fluconazole. A positive trend in increased *C. albicans* clearance with FG4592 treatment was observed, so further optimisation of drug treatment (longer treatment duration or increased concentration) may lead to observation of an effect. An alternative pharmacological method of Hif-1α stabilisation may be required. FG4592 (Roxadustat) was selected as a pharmacological method of Hif-1α stabilisation as it has been approved in the UK for treating anaemia in chronic kidney disease, raising the possibility of drug repurposing (NICE, 2022). FG4592 inhibits HIF prolyl hydroxylases, preventing hydroxylation of HIF-α subunits and subsequent degradation (Zhu *et al.*, 2022). This stabilises HIF-1α and HIF-2α. HIF-1α and HIF-2α signalling both promote *VEGF* and *EPO* expression to aid with resolution of anaemia (Hu *et al.*, 2003; Zhu *et al.*, 2022). However, HIF-1α and HIF-2α have opposing effects on RNS production by immune cells, with HIF-1α promoting iNOS activity and HIF-2α stimulating arginase expression (Takeda *et al.*, 2010; Elks *et al.*, 2013). Hence, stabilisation of both HIF-1α and HIF-2α by FG4592 may not mimic the RNS-inducing effects of genetic stabilisation of Hif-1α alone. Roxadustat has been suggested to suppress T cell proliferation *in vitro* and roxadustat clinical trials observed higher incidence of urinary tract infections and pneumonia in roxadustat treated anaemia patients compared to epoetin alfa treated (standard treatment) anaemia patients (Chen *et al.*, 2019; Akizawa *et al.*, 2020; Eleftheriadis *et al.*, 2020). Therefore, alternative pharmacological methods of stabilising HIF-1α alone may be more suitable for investigation as a HDT for treatment of *C. albicans* infections, such as mimosine or acetate, though these have issues with toxicity (Zinkernagel *et al.*, 2008; Machado *et al.*, 2022)

Sub-inhibitory concentrations of caspofungin did not have an additive effect on survival or clearance of *C. albicans* infections when combined with genetic Hif-1α stabilisation. Sub-inhibitory concentrations of caspofungin have been shown to unmask β-glucan in the *C. albicans* cell wall,

increasing recognition by innate immune cells (Wheeler *et al.*, 2008; Wagner *et al.*, 2023). Hopke *et al.* demonstrated that neutrophils unmask *C. albicans* β-glucan by NET-mediated cell wall damage *in vitro* and in mice *in vivo* (Hopke *et al.*, 2016). Upon damage to the cell wall by NETs, *C. albicans* actively unmasks β-glucan itself and increases chitin deposition at the site of damage, aiming to strengthen the cell wall at the site of neutrophil attack (Hopke *et al.*, 2016). While the exposure of β-glucan and chitin in the cell wall are a *C. albicans* strategy to protect against host immune attack, they have an unintended consequence of increasing host immune recognition. Prolonged *C. albicans* infection in zebrafish embryos over 4 days would allow ample time for neutrophil-mediated β-glucan unmasking, which may disguise any host protective effects of β-glucan unmasking caused by sub-inhibitory concentrations of caspofungin. Examination of neutrophil recruitment at an earlier time point may provide more detail over whether sub-inhibitory caspofungin is responsible for any improvements in the host immune response, and whether this could improve initial infection control when combined with Hif-1α stabilisation.

Hif-1α stabilisation did not cause any changes in neutrophil recruitment to *C. albicans* infection in the hindbrain compared to control embryos. Neutrophil recruitment to the hindbrain in *C. albicans* infection has previously been quantified (Brothers *et al.*, 2013; Johnson *et al.*, 2018). Brothers *et al.* showed an average of 12 phagocytes recruited to the hindbrain in *C. albicans* infection at 4 hpi, though this figure includes neutrophils and macrophages. Johnson *et al.* observed approximately 13 neutrophils recruited to the hindbrain in *C. albicans* infection at 4 hpi. Both of these studies observed fewer neutrophils in the hindbrain than I did in PR, DN1 or DA1 injected embryos (16, 18 and 19 neutrophils at 24 hpi, respectively). The earlier time point (4 hpi) used by previous studies compared to this project (24 hpi) is likely responsible for the lower level of neutrophil recruitment observed, despite Johnson *et al*. using a greater infection dose (30-50 cfu). Brothers *et al.* used a lower infection dose than my project (10 cfu compared to 20 cfu) but their values were inflated by including macrophages in their quantification of phagocyte recruitment. Mock infection, with PVP, in Hif-1α stabilised embryos had higher neutrophil recruitment to the hindbrain than control embryos. Previous quantification of neutrophil recruitment to a tail fin wound revealed pharmacological Hif-1α stabilisation had no effect at 6 hpw and 48 hpw, but increased neutrophil numbers at 24 hpw (Elks *et al.*, 2011). Hif-1α stabilisation promoted neutrophil retention between 6 hpw and 48 hpw and delayed inflammation resolution. Neutrophil numbers in the hindbrain may be elevated in mock infection with Hif-1α stabilisation due to a similar retention of neutrophils and delayed resolution of the inflammation caused by microinjection. To speculate on this, no difference may have been observed in neutrophil numbers in the hindbrain in *C. albicans* infected embryos because the pro-

inflammatory stimulus (in this case, *C. albicans*) was still present so inflammation was ongoing in all groups, meaning delay of resolution by Hif-1α stabilisation could not happen.

Hif-1α stabilisation did not cause any difference in *C. albicans* internalisation by neutrophils at 24 hpi. Bergeron *et al.* previously quantified phagocytosis of *C. albicans* in the hindbrain, showing approximately 10% of *C. albicans* cells were internalised in neutrophils at 4 hpi (Bergeron, Barker, *et al.*, 2017). However, this study did not quantify the percentage of neutrophils with internalised *C. albicans*, making direct comparisons to my results difficult. Another study estimated 33% of phagocytes contained internalised *C. albicans* at 4 hpi (Brothers *et al.*, 2013). This is greater than the 20.4% neutrophils with internalised *C. albicans* in hindbrain infection that I observed at 24 hpi, though relatively similar to the 29.9% neutrophils with internalised *C. albicans* in embryos injected with DN1. Bergeron *et al.* suggested the level of phagocytosis at an early stage of infection is more predictive of the outcome of infection, so modifying my experiments to quantify *C. albicans* internalisation at an earlier time point may produce more clinically relevant data. A difference in *C. albicans* internalisation was observed between DN1 and DA1 injected embryos, though neither group was significantly different from PR embryos. This could suggest a subtle role for Hif-1α in phagocytosis, in which Hif-1α stabilisation leads to a small decrease in *C. albicans* internalisation and inhibition of Hif-1α signalling leads to a small increase in internalisation by neutrophils. This would conflict with previous observations that hypoxia leads to a Hif-1α-dependent increase in phagocytosis of *Escherichia coli* by RAW264.7 macrophages *in vitro* and murine peritoneal macrophages *in vivo* (Anand *et al.*, 2007). Another explanation could be that Hif-1α stabilised neutrophils appear to have fewer internalised *C. albicans* as they have elevated RNS levels, so are more efficiently killing internalised *C. albicans*. To validate this hypothesis, time lapse microscopy of early stages of *C. albicans* infection in Hif-1α stabilised zebrafish embryos is required, allowing direct, real-time observation of *C. albicans* engulfment by neutrophils and the fate of internalised *C. albicans*. This would also allow authentication that internalised *C. albicans* is being phagocytosed by neutrophils, and not a *C. albicans*-mediated strategy.

Internalisation of *C. albicans* in neutrophils was observed in both tight and spacious phagosomes, though Hif-1α stabilisation did not cause any difference in the incidence of spacious phagosomes. There is conflicting literature around spacious phagosomes. In *Salmonella* infection, spacious phagosomes have been associated with increased susceptibility to infection and are bacterial-induced structures to aid bacterial proliferation (Alpuche-Aranda *et al.*, 1994, 1995; Rosales-Reyes *et al.*, 2012). Similarly, spacious phagosomes in neutrophils in *Staphylococcus aureus* infection are non-acidified, providing an intracellular niche for bacterial survival (Prajsnar *et al.*, 2021). Dr Natalia Hajdamowizc (former PhD student in Condliffe lab) revealed hypoxia in *S. aureus* infection *in vitro*

reduced the number of spacious phagosomes, implying a potential link between spacious phagosome formation, hypoxia and Hif signalling (personal communication from Dr Philip Elks and Professor Alison Condliffe). Conversely, spacious phagosomes appear to restrict replication of *Mycobacteria tuberculosis* as part of a host driven mechanism (Schnettger *et al.*, 2017). This suggests the function of spacious phagosomes in host-pathogen interactions may be pathogen specific. Spacious phagosomes have been observed by transmission electron microscopy of RAW macrophages infected with *C. albicans*, though they were referred to as loose phagosomes and no comment was made on their potential function (Fernández-Arenas *et al.*, 2009). Phagosomes containing *C. albicans* recruit additional lysosomes in response to intraphagosomal growth, increasing the size of the phagosome and preventing fungal escape (Westman *et al.*, 2020). Spacious phagosomes in *C. albicans* infection may be phagosomes that have recently expanded in size due to lysosome recruitment and fusion. However, Westman *et al.* primarily observed lysosome recruitment to phagosomes as a response to hyphal extension within the phagosome, whereas many of the spacious phagosomes I observed only contained yeast. Time lapse microscopy may provide more clues when and why spacious phagosomes form in *C. albicans* infection and what the outcome for *C. albicans* contained in spacious phagosomes is compared to tight phagosomes. Given the lack of effect by Hif-1α stabilisation, this fell beyond the scope of this project.

Folded *C. albicans* hyphae were observed in neutrophils, potentially the first observation of folded hyphae *in vivo*. Folding of *C. albicans* hyphae has previously only been observed by macrophages *in vitro*, revealing a Dectin-1 mediated actin-dependent mechanism (Bain *et al.*, 2021). Bain *et al.* observed 30-50% of internalised hyphae having some degree of folding – greater than the percentage of folded hyphae I observed. This may be due to the difference in cell type (neutrophils *in vivo* vs macrophages *in vitro*) and *in vivo* observation of macrophages may reveal an increased percentage of folded *C. albicans* hyphae. A limitation of this study is the use of a single time point, meaning it is not possible to conclude *C. albicans* hyphae were folded by neutrophils. Time lapse microscopy would confirm whether host innate immune cells are responsible for hyphal folding and, potentially, allow examination of the biological significance of hyphal folding.

Within this chapter, I have demonstrated Hif-1α stabilisation protects against *C. albicans* infection *in vivo*, via a neutrophil-mediated, RNS-dependent mechanism. Hif-1α stabilisation had an additive effect on zebrafish survival and clearance of *C. albicans* infection when combined with traditional antifungals. Hence, Hif-1α stabilisation has potential as an adjunctive host directed therapeutic strategy to aid in the treatment of *C. albicans* infections, following further validation in human systems.

7. General Discussion

7.1 Summary of findings

In this thesis, I investigated the neutrophil response to *Candida* spp. infections, using zebrafish *in vivo* models (summarised in Figure 7.1) to reveal a *C. albicans* mechanism of suppressing host neutrophil RNS levels. RNS suppression was observed in *Candida* spp. clinical isolates, hinting at the relevance of RNS suppression for human disease. Hif-1α stabilisation was identified as a potential host directed therapy (HDT) for the treatment of *C. albicans* infections, via modulation of neutrophil RNS production.

Firstly, I observed neutrophil RNS suppression by *C. albicans* in zebrafish *in vivo*, expanding on an observation previously made in our lab by Dr Ffion Hammond (Hammond (thesis), 2022). In control groups, RNS production was co-localised with neutrophils, corroborating previous work that demonstrated neutrophils are the main RNS-producing cells in zebrafish (Elks *et al.*, 2013). I observed neutrophil RNS suppression by an alternative laboratory strain of *C. albicans*, suggesting the phenomenon is not unique to *C. albicans* SN148 GFP. Furthermore, in both laboratory strains, I observed an intermediate degree of neutrophil RNS suppression by heat-killed *C. albicans*, suggesting the presence of an active mechanism of RNS suppression by live *C. albicans* and a passive mechanism of RNS suppression responsible for the intermediate phenotype by heat-killed *C. albicans*. This work builds on previous *in vitro* experiments that demonstrated suppression of NO production by murine macrophages following exposure to *C. albicans* laboratory strains (SC5314 and RK9232; Chinen *et al.*, 1999; Collette, Zhou and Lorenz, 2014; Wagener *et al.*, 2017). A hindbrain *C. albicans* infection model was used to further examine neutrophil RNS suppression, revealing live *C. albicans* can cause neutrophil RNS suppression at distal sites where no *C. albicans* infection is present, suggesting a secreted factor that can move across the zebrafish, potentially via the circulation. Previous experiments suggested direct contact between *C. albicans* and immune cells is required for suppression of murine macrophage NO (Collette, Zhou and Lorenz, 2014). My work does not preclude this observation but may suggest direct *C. albicans*-neutrophil contact promotes secretion of a *C. albicans* factor, which can then have proximal and distal effects.

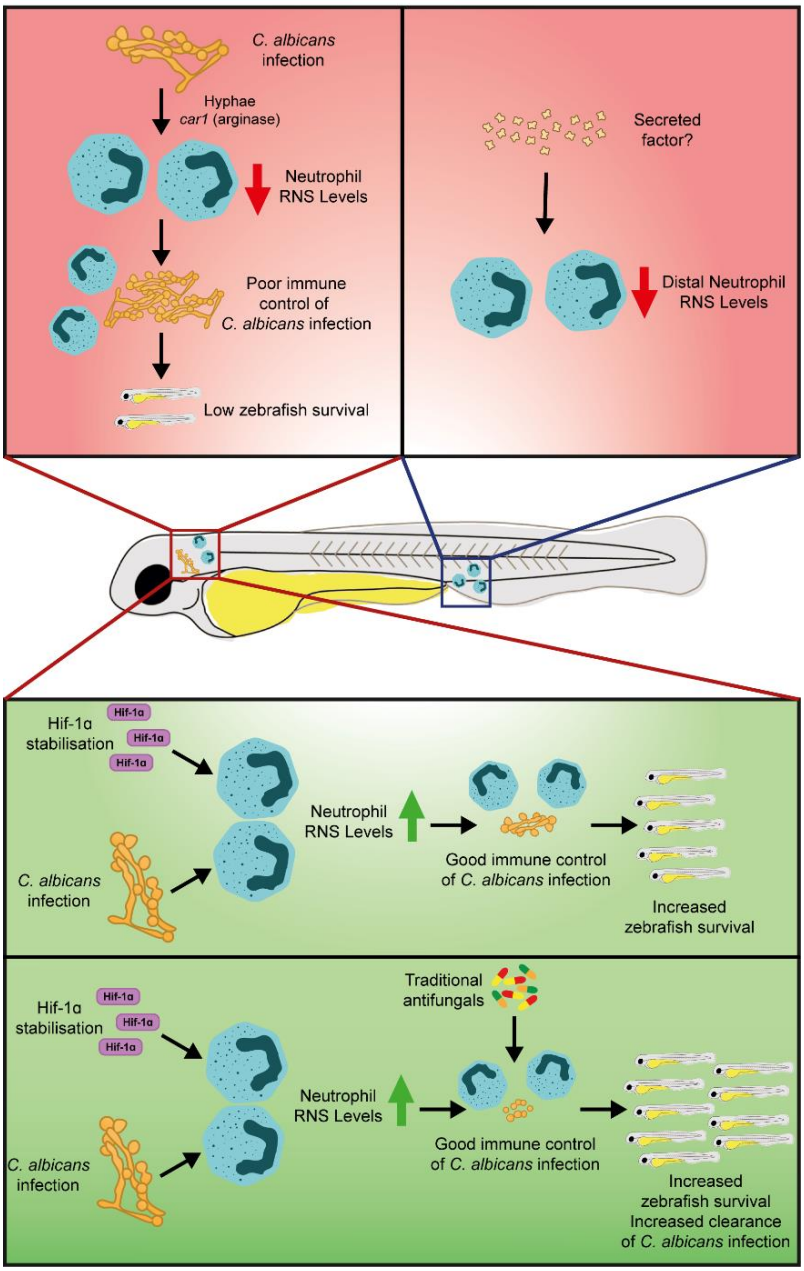

Figure 7.1: *C. albicans* suppresses neutrophil RNS *in vivo*, which can be rescued by host Hif-1α stabilisation.

C. albicans infection suppresses neutrophil RNS levels at the site of infection, which is, at least partially, mediated by *C. albicans* hyphae and *C. albicans* car1 (arginase). RNS suppression leads to poor neutrophil control of *C. albicans*, resulting in high levels of zebrafish mortality. Suppression of RNS in distal neutrophils also occurs, presumably caused by interactions with an, as yet unidentified, secreted or released *C. albicans* factor. Hif-1α stabilisation is able to overcome neutrophil RNS suppression by *C. albicans*, increasing zebrafish survival. Combination of Hif-1α stabilisation with traditional antifungals has an additive effect on zebrafish survival in *C. albicans* infection and on clearance of *C. albicans* infection.

Attempts were made to identify host factors involved in *C. albicans* suppression of neutrophil RNS. Neutrophil RNS suppression by *C. albicans* was revealed to be TLR-independent, following ablation of TLR signalling via *myd88* knockdown. Zebrafish larvae survival in *C. albicans* infection was not impacted by *myd88* knockdown, suggesting TLR signalling may not be as vital for the early innate immune response to *C. albicans* infections as previously believed (Netea *et al.*, 2015; Burgess, Condliffe and Elks, 2022). I attempted to investigate the role of host CLRs in suppression of neutrophil RNS by *C. albicans*. However, CRISPR-Cas9 mutagenesis of *card9* was unsuccessful, meaning I did not have a reliable method of inhibiting CLR signalling. Hence, the host factors involved in *C. albicans* suppression of neutrophil RNS remain unclear. A stable Card9 knockout zebrafish line, confirmed to ablate CLR signalling, has recently been produced, which would allow investigation of whether *C. albicans* host RNS suppression is CLR-dependent (Glass, Robinson and Rosowski, 2025).

The role of *C. albicans* hyphae in neutrophil RNS suppression was examined. *C. albicans* hyphae are associated with increased virulence, tissue invasion and a change in transcriptome and secretome (Sudbery, 2011; Seman *et al.*, 2018; Vaz *et al.*, 2021). I demonstrated live yeast-locked *C. albicans* stimulate the same level of neutrophil RNS suppression as heat-killed wild type *C. albicans* and heat-killed yeast-locked *C. albicans*, which was less than the degree of RNS suppression caused by live wild type *C. albicans*. Hence, the hyphal morphotype may be required for the greater degree of RNS suppression in wild type, lab strain, *C. albicans*, suggesting a hyphae-specific protein or a protein that is enriched in the hyphal cell wall may drive the active component of RNS suppression.

I investigated host cytokine expression in *C. albicans* infection. Live and heat-killed *C. albicans* both caused upregulation of TNFα and IL-1β production, demonstrating *C. albicans* infection stimulates a pro-inflammatory response from an early time point. TNFα and IL-1β production was greater in wild type *C. albicans* infection than yeast-locked *C. albicans* infection, corroborating previous evidence that hyphae are a strong driver of inflammation (d'Ostiani *et al.*, 2000; van der Graaf *et al.*, 2005; Sudbery, 2011). These results suggest RNS suppression is directed specifically to RNS production, as opposed to a general suppression of pro-inflammatory responses.

Arginase was investigated as a potential factor involved in neutrophil RNS suppression. *C. albicans* infection induced an increased host arginase expression and an increase in the percentage of arginase-expressing neutrophils. This could suggest *C. albicans* infection is able to drive neutrophils towards an anti-inflammatory phenotype. Induction of arginase expression in human macrophages has been observed *in vitro*, driven by *C. albicans* chitin, which was associated with a modest decrease in NO production (Wagener *et al.*, 2017). Upregulation of host arginase expression may be

a mechanism of RNS suppression by *C. albicans*. I also chose to explore the effect of *C. albicans* arginase (car1) on host RNS suppression. Mutation of *C. albicans* arginase reduced the degree of RNS suppression, suggesting *C. albicans* arginase could be involved in host RNS suppression. Mutation of *C. albicans* arginase increased zebrafish survival in *C. albicans* infection, though it is not clear how much this can be attributed to reduced RNS suppression and how much could be a consequence of reduced hyphae formation.

I inspected the ability of *C. albicans* clinical isolates to cause neutrophil RNS suppression. Four *C. albicans* clinical isolates were able to suppress neutrophil RNS *in vivo* to varying degrees. No obvious relationship was observed between the degree of RNS suppression and whether the isolate was from invasive or non-invasive disease. *C. albicans* clinical isolates that were more able to form hyphae in zebrafish at 28 °C were associated with increased RNS suppression, and increased mortality, further establishing a link between hyphae formation and RNS suppression. A selection of non-albicans *Candida* spp. clinical isolates (*C. glabrata, C. guilliermondii, C. parapsilosis* and *C. auris*) were able to suppress neutrophil RNS to varying degrees. Analysis of the RNS suppression and virulence of each *Candida* spp. revealed a correlation between RNS suppression and virulence, with a greater suppression of neutrophil RNS being associated with reduced zebrafish survival. These results indicate neutrophil RNS suppression is observed in *Candida* spp. clinical isolates, suggesting RNS suppression could be relevant to human candidiasis and RNS suppression could be an important feature of *Candida* spp. pathogenesis.

Hif-1α was investigated as a potential target for immunomodulation of the neutrophil response to *C. albicans* infections. Immunomodulation by HDTs could present new therapeutic options for treatment of *C. albicans* infections, which potentially limit the development of antifungal resistance (Armstrong-James *et al.*, 2017). Previous work by Dr Ffion Hammond revealed Hif-1α stabilisation protects against *C. albicans* infection *in vivo*, via a macrophage-independent mechanism. I, subsequently, showed the protective effect of Hif-1α stabilisation in *C. albicans* infection is neutrophil-mediated and RNS-dependent, with Hif-1α stabilisation rescuing RNS production by neutrophils. No role was discovered for spacious phagosomes or hyphal folding in Hif-1α-mediated protection in *C. albicans* infection. Combination of Hif-1α stabilisation with fluconazole and caspofungin had an additive effect on both survival and clearance of *C. albicans* infection. These results highlight the potential of targeting Hif-1α as a HDT for the treatment of *C. albicans* infections. This adds to previous data showing the importance of Hif-1α *in vitro* and in murine models of *C. albicans* infection (Fan *et al.*, 2015; Li *et al.*, 2018a). Further work is required to identify reliable pharmacological methods of Hif-1α stabilisation that cause a protective effect against *C. albicans* infection.

There are some important limitations on the work in this thesis. Zebrafish larvae are incubated at 28 °C, whereas invasive *C. albicans* infections in humans will generally be around 37 °C (Chao *et al.*, 2010; White *et al.*, 2011; Jørgensen, 2020). Many *C. albicans* genes, including those involved in hyphae formation, are thermally regulated (Enjalbert, Nantel and Whiteway, 2003; Leach *et al.*, 2012, 2016). Hence, *C. albicans* gene expression in zebrafish larvae at 28 °C may not accurately represent gene expression at 37 °C, potentially impacting virulence, degree of RNS suppression and hyphae formation (particularly by *Candida* spp. clinical isolates). Increasing the temperature of zebrafish incubation would have negative impacts on host immune function: incubation at 32.5 °C leads to developmental malformations in zebrafish larvae (Pype *et al.*, 2015; Scharsack and Franke, 2022). Meanwhile, thermal conditioning of *C. albicans* to grow at lower temperatures can have unpredictable effects on phenotype (Leach *et al.*, 2012; Schwiesow, Elde and Hilbert, 2024). Larval zebrafish that have completed early embryogenesis (>2 dpf) may be more resistant to incubation at high temperatures, though this has not been characterised. Incubation of >2 dpf zebrafish at 30 °C could provide more temperature-relevant information about *C. albicans* pathogenesis *in vivo*, though this would require zebrafish to be taken beyond the point of legal protection (5.2 dpf). Arabian killifish could be used as an alternative model, as they can tolerate temperatures up to 40 °C. However, further characterisation of the killifish genome, RNS in killifish and development of transgenic lines is required for this to be a viable alternative to zebrafish for investigating host factors involved in *C. albicans* interactions with innate immune cells (Alsakran *et al.*, 2024; Minhas *et al.*, 2024).

Although immune pathways are highly conserved between zebrafish and humans, there are some differences in zebrafish and human immunity that introduce limitations to this investigation (Barbazuk *et al.*, 2000; Lam *et al.*, 2004). In the zebrafish larvae, primitive neutrophils are produced by primitive haematopoiesis up to 35 hpf (Myllymäki, Yu and Feng, 2022). Primitive neutrophils have almost no phagocytic activity and were not observed to form NETs in response to *E. coli* infection (Le Guyader *et al.*, 2008). Fully mature neutrophils are first observed from 35 hpf in zebrafish larvae (Le Guyader *et al.*, 2008). I infected zebrafish larvae with *C. albicans* at 30-35 hpf, meaning early neutrophils recruited to the site of infection would have been primitive neutrophils, potentially impacting the early immune response. Furthermore, zebrafish larvae lack an adaptive immune system (Lam *et al.*, 2004). While this allowed examination of *C. albicans*-neutrophil interactions without interference from adaptive immune cells, total absence of adaptive immunity is not reflective of human infection and removed the potential to examine the effects of *C. albicans* and Hif-1α stabilisation on adaptive immune cells.

Drug penetrance in zebrafish is a further potential limitation. Most small molecule drugs can easily be administered to zebrafish larvae via immersion in E3 media (Goldsmith and Jobin, 2012). However, drug penetrance is poorly characterised in zebrafish larvae and the relationship between treatment concentration and internal concentration is unknown (Habjan *et al.*, 2024). Analysing drug levels in zebrafish blood is possible but highly labour-intensive and require specialist equipment, making these techniques impractical for standard use (Habjan *et al.*, 2024). Using these techniques, blood concentration of isoniazid and paracetamol was shown to be 20% and 10%, respectively, of the external concentration, suggesting poor penetrance of these compounds (van Wijk *et al.*, 2019). Drug penetrance increased with age up to 3 dpf, with compound uptake initially relying on passive diffusion through the skin and then being complemented by uptake through the gastrointestinal tract from 3 dpf (Zhang *et al.*, 2015; Habjan *et al.*, 2024). Furthermore, drug penetrance can be influenced by genetic variability between larvae, having unpredictable effects on experiment outcomes (Wiley, Redfield and Zon, 2017). Poor drug penetrance could have been responsible for the lack of observed phenotype following treatment with siCSP and FG4592. Other differences in the pharmacokinetic profiles of zebrafish may impact the translation of my findings into mammalian *in vivo* models.

Some individual experiments had limitations worth considering. *Myd88* morpholino knockdown would disrupt signalling by all TLRs, except TLR3 (Fitzpatrick *et al.*, 2020). Hypothetically, RNS suppression could be mediated via TLR3 signalling, which is not disrupted by *myd88* knockdown. While TLR3 is dogmatically believed to recognise dsRNA (produced by viruses but not fungi), TLR3 has been shown to induce pro-inflammatory cytokine expression in primary human endothelial cells *in vitro* and patients with TLR3 mutations had increased susceptibility to cutaneous candidiasis (Alexopoulou *et al.*, 2001; Müller *et al.*, 2007; Nahum *et al.*, 2011). Although specific criteria were set, there was an element of subjectivity in hyphal staging experiments. Experiments with *C. albicans car1Δ* are confounded by the reduced ability of *C. albicans car1Δ* to form hyphae (Schaefer *et al.*, 2020), which had previously been shown to play a role in neutrophil RNS suppression (see 4.2.2 Active suppression of neutrophil RNS is hyphae-dependent).

7.2 Potential impact

7.2.1 Understanding of *Candida* spp. infections

On the WHO Fungal Priority Pathogens List, *C. albicans* and *C. auris* are critical priority, and *C. glabrata* and *C. parapsilosis* are high priority (WHO, 2022). Improving our understanding of the pathogenesis underlying these *Candida* spp. infections could help to improve management of these infection and identify new therapeutic targets.

The principal observation of my thesis was that *C. albicans* suppresses neutrophil RNS levels in zebrafish larvae. RNS suppression was also observed by a variety of *Candida* spp. clinical isolates (four *C. albicans* isolates, *C. glabrata, C. guilliermondii, C. parapsilosis* and *C. auris*), suggesting RNS suppression could be a phenomenon relevant to human infections, pending validation in primary human neutrophils *in vivo*. Candidiasis patients could be considered to have suppressed innate immune function, so benefit from wider use of adjunctive therapies that improve neutrophil function, such as G-CSF/GM-CSF (Mehta, Malandra and Corey, 2015; B. Du *et al.*, 2020b). Furthermore, identification of the *C. albicans* factor responsible for neutrophil RNS suppression could reveal potential therapeutic targets for development of new antifungals.

I observed *C. albicans* caused suppression of neutrophil RNS at a distal site, where no infection was present, implying the secretion of a *C. albicans* factor to have distal effects. This could have clinical implications for commensal *C. albicans* and development of other infections. 70% of healthy individuals have *C. albicans* present in the gut microbiome (Witherden *et al.*, 2017). *C. albicans* in the gut microbiome could secrete a factor that disseminates around the body and causes neutrophil RNS suppression at distal sites. Distal RNS suppression by microbiome *C. albicans* could be possible, as gut-derived *Candida* spp*.* prostaglandin E2 has been associated with reprogramming alveolar macrophages and allergic lung inflammation in a mouse *in vivo* model (Kim *et al.*, 2014). Circulating neutrophils isolated from vulvovaginal candidiasis patients had reduced fungicidal activity compared to healthy neutrophils, revealing a potential case of suppression of distal neutrophil function by *C. albicans* in human infection (Consuegra-Asprilla *et al.*, 2024). However, only a correlation was shown and an alternative explanation could be that individuals with reduced neutrophil fungicidal activity are more susceptible to vulvovaginal candidiasis. Antibiotic treatment facilitates increased *C. albicans* gut colonisation in a mouse model and in human patients (Gutierrez *et al.*, 2020; Seelbinder *et al.*, 2020). Human patients treated with antibiotics had an average 7-fold increase in microbiome *C. albicans* and up to a 42% increase in abundance (Seelbinder *et al.*, 2020). This expanded prevalence could mean *C. albicans* reaches a high enough threshold to produce sufficient RNS-suppressing factor to have an observable effect in distal neutrophils. The suppression of RNS in distal neutrophils could potentially lead to poorer innate immune control of distal infections. This could hypothetically be relevant to tuberculosis infections: 25% of pulmonary tuberculosis patients in Asia and Africa had *Candida* spp*.* co-infection, which rose to 40% in pulmonary tuberculosis patients in India, so *Candida*-tuberculosis co-infections make up a large cohort of patients, who may have defective neutrophil responses due to *Candida* spp*.* suppression of neutrophil RNS (Kali *et al.*, 2013; Hadadi-Fishani, Shakerimoghaddam and Khaledi, 2020).

7.2.2 Host directed therapies

Increasing rates of antifungal resistance and poor efficacy of existing antifungals highlights the urgent need for new therapeutic alternatives to treat *C. albicans* infections (WHO, 2022). Host directed therapies (HDTs) target the host immune system, rather than directly targeting the pathogen (Zumla *et al.*, 2016). Stimulation of host immune pathways by HDTs could bypass the development of antifungal resistance by *C. albicans*, due to reduced use of antifungals and increased killing of *C. albicans* by immune cells.

Hif-1α stabilisation was demonstrated to promote RNS production by neutrophils, overcoming suppression of RNS by *C. albicans,* resulting in reduced *C. albicans* mortality. Combination of Hif-1α stabilisation with traditional antifungals had an additive effect on zebrafish survival in *C. albicans* infection and clearance of *C. albicans*. Hence, in a zebrafish model of *C. albicans* infection, Hif-1α stabilisation has shown potential as a HDT. This supports prior evidence of Hif-1α being an effective therapeutic target as a HDT in bacterial infections. Hif-1α stabilisation had protective effects against *M. marinum* infection in zebrafish larvae, *S. aureus* infection *in vitro*, and *S. pneumoniae* infection *in vitro* (Zinkernagel *et al.*, 2008; Elks *et al.*, 2013; Machado *et al.*, 2022). Further characterisation of the effect of HIF-1α stabilisation on human primary neutrophils *in vitro* in *C. albicans* infection would provide further evidence of the potential of targeting HIF-1α for a HDT. Some challenges still exist in developing Hif-1α stabilisation as a HDT. In my experiments, FG-4592 (a pharmacological Hif-1α stabiliser, also called Roxadustat) did not have a protective effect in *C. albicans* infection when administered alone, nor did it have an additive effect when combined with fluconazole. Zebrafish larvae were treated with FG-4592 for 24 hours, then moved into fresh E3 media without treatment, due to concerns about toxicity with prolonged FG-4592 treatment. Further optimisation of this protocol and longer treatment durations may have led to observation of a protective effect from FG-4592. Repurposing of Roxadustat (currently used to treat anaemia) as a HDT for treatment of *C. albicans* infections is complicated by its mechanism of action. Roxadustat stabilises both Hif-1α and Hif-2α, leading to opposing effects on induction of RNS production, which may have been responsible for a protective effect not being observed with FG4592 treatment in *C. albicans*-infected zebrafish (Takeda *et al.*, 2010; Zhu *et al.*, 2022). Roxadustat has been suggested to suppress T cell proliferation *in vitro* and roxadustat clinical trials observed higher incidence of urinary tract infections and pneumonia in roxadustat-treated anaemia patients compared to epoetin alfa treated (standard treatment) anaemia patients (Chen *et al.*, 2019; Akizawa *et al.*, 2020; Eleftheriadis *et al.*, 2020). Alternative pharmacological methods of stabilising HIF-1α alone may need to be investigated as a HDT for treatment of *C. albicans* infections. VH298 and an iridium(III) metal complex 1a have been suggested as potential Hif-1α stabilisers, via inhibition of the VHL-Hif-1α interaction (Frost *et*

al., 2016; G. Li *et al.*, 2021). However, VH298 still leads to increased Hif-2α activity, so would presumably have similar issues to Roxadustat (Frost *et al.*, 2016). Upregulation of Hif-1α signalling without concurrent upregulation of Hif-2α is a challenge for Hif-1α as a potential HDT target. One solution could be coadministration of a Hif-1α stabiliser (such as Roxadustat) with a Hif-2α inhibitor, to stimulate Hif-1α-mediated RNS production and inhibit Hif-2α-mediated arginase production. MK6482 (named belzutifan) is a HIF-2α inhibitor, which binds to proline residues in HIF-2α to prevent dimerisation with HIF-β (Toledo *et al.*, 2022). The effects of MK6482 on HIF-1α activity have not been reported to the best of my knowledge.

7.3 Future avenues of research

7.3.1 Determining the mechanism of *C. albicans* suppression of neutrophil RNS

This thesis has developed our knowledge of *C. albicans* interactions with neutrophils, revealing a mechanism of host immune suppression that is conserved in *Candida* spp. clinical isolates. However, this project was not exhaustive and several gaps in knowledge still remain.

Hyphae formation (Figure 4.7) and *C. albicans* arginase (*car1;* Figure 4.14) were associated with host RNS suppression in my experiments. Zebrafish embryos could be injected with arginase alone to validate its ability to suppress neutrophil RNS. Alternatively, *C. albicans* could be pre-treated with an arginase inhibitor (such as nor-NOHA) prior to injection into zebrafish larvae. Questions remain about whether other *C. albicans* factors have a role in neutrophil RNS suppression and what those factors might be. Measuring RNS levels in zebrafish injected with β-glucan, chitin or conditioned media could reveal other factors potentially involved in neutrophil RNS suppression. An iNOS antibody could be used in *C. albicans* infection *in vivo* to establish whether RNS suppression occurs at the enzymatic level (Elks *et al.*, 2013). A *tnnt2a* morpholino could be used in zebrafish to inhibit cardiac function, revealing whether distal RNS suppression is blood flow-dependent (Sehnert *et al.*, 2002). Time lapse microscopy could rule out the possibility of reverse migration of neutrophils recruited to *C. albicans* infection in the hindbrain back to the caudal haematopoietic tissue. Spatial transcriptomics would allow characterisation of the transcriptome throughout the zebrafish larvae in *C. albicans* infection, revealing nuances in cytokine response that were obscured by promoter driven transgenic lines (Qian and Weinstein, 2025).

Zebrafish larvae are a highly effective *in vivo* model and are relatively high-throughput compared to mammalian *in vivo* models. However, mammalian *in vivo* models may be more relevant to human infections. Examination of neutrophil behaviour in mammalian *in vivo* models is difficult but can be done with complex techniques. Intravital 2-photon microscopy has been used to directly observe *C. albicans* infection in a mouse kidney *in vivo,* and observe neutrophil recruitment to *C. albicans*

infection and phagocytosis of *C. albicans* in murine kidneys *in vivo* (Desai *et al.*, 2023; Silao *et al.*, 2023). Human skin blister models of pathogen-driven inflammation have also been used to examine neutrophil recruitment and cytokine production in response to heat-killed pathogens (Motwani *et al.*, 2018). Translating my observations of neutrophil RNS suppression by *C. albicans in vivo* into a primary human neutrophil *in vitro* model would also increase the relevance of my findings. DAF-FM staining could be used to quantify primary human neutrophil RNS production *in vitro* (Kojima *et al.*, 1998; Lewis, Matzdorf and Rice, 2016).

An *in vitro* model with faster experiment times would allow easier screening of *C. albicans* mutants, allowing identification of factors involved in RNS suppression. Generation of CRISPR interference (CRISPRi) systems in *C. albicans* has facilitated the creation of *C. albicans* mutant libraries in a far less time-consuming manner than traditional *C. albicans* mutagenesis (Wensing *et al.*, 2019; R. Wang *et al.*, 2022). These CRISPRi *C. albicans* mutant libraries could be screened in neutrophils *in vitro* to search for mutations with ablated RNS suppression. If a CRISPRi screen was able to identify specific proteins responsible for neutrophil RNS suppression, a microfluidics chip could be used to screen drugs capable of targeting this protein to inhibit RNS suppression (Qiang *et al.*, 2019). Phagosome acidification and proteolytic degradation can be observed in real time, which would reveal if RNS suppression occurs in the phagosome or prior to phagocytosis, and the impact of RNS suppression on *C. albicans* killing by neutrophils and phagosomal escape (Méndez-Alejandre *et al.*, 2023). Phagosome isolation and proteomic analysis would expose any modifications to phagosome composition induced by *C. albicans* (Dueñas *et al.*, 2023). Collection and analysis of neutrophils from patients with invasive candidiasis would provide further clinically relevant information on neutrophil function, epigenetic alterations and transcriptional profile.

7.3.2 Further investigation of HIF-1α as a host directed therapy

HIF-1α has potential as a HDT for treatment of *C. albicans* infections but requires further investigation. Further analysis of the mechanisms underlying the protective effect of Hif-1α stabilisation could be investigated. HIF-1α has previously been indicated to influence NET formation and neutrophil retention at the site of inflammation (Elks *et al.*, 2011; McInturff *et al.*, 2012). Although I did not see an effect of Hif-1α stabilisation on neutrophil recruitment in *C. albicans* infection at a single time point, time lapse microscopy would provide greater detail on the dynamics of neutrophil recruitment, retention and fate in *C. albicans* infection with Hif-1α stabilisation. The effects of Hif-1α stabilisation on NET formation in *C. albicans* infection could be observed using a *Tg(mpx:H2Bcerulean-P2A-mKO2CAAX)gl29* reporter line (Manley *et al.*, 2020; Isles *et al.*, 2021).

In addition, a protective effect from a pharmacological method of Hif-1α stabilisation in *C. albicans* infection *in vivo* must be demonstrated. This could come from optimisation of FG-4592 treatment or from alternative Hif-1α stabilisers, such as VH298, mimosine or acetate (Zinkernagel *et al.*, 2008; Machado *et al.*, 2022). Optimisation may need to include co-treatment with a Hif-2α inhibitor (such as TCS-7009 or belzutifan), to overcome non-specific stabilisation of Hif-1α and Hif-2α isoforms, leading to opposing effects on RNS regulation (Takeda *et al.*, 2010; Elks *et al.*, 2013; Scheuermann *et al.*, 2013; Toledo *et al.*, 2022).

A reliable pharmacological method of HIF-1α stabilisation would allow translation to a human primary neutrophil model – an important step to demonstrate relevance to human infections. Hif-1α stabilisation could also be explored as a HDT for other *Candida* spp*.,* such as *C. auris*.

7.4 Conclusion

In conclusion, in this thesis, I investigated the interactions between neutrophils and *C. albicans* in a zebrafish *in vivo* model, revealing a *C. albicans* strategy for suppressing neutrophil RNS production for the first time. Suppression of RNS by *C. albicans* occurred in distal neutrophils, implying a secreted factor capable of generating systemic immune suppression of neutrophils. Host factors involved in *C. albicans* suppression of RNS were explored but none were identified. Investigation of *C. albicans* factors involved in RNS suppression suggested a role for hyphae and *C. albicans* arginase. RNS suppression was conserved across a selection of *C. albicans* and non-albicans *Candida* spp*.* clinical isolates, hinting at the clinical relevance of this phenomenon. Hif-1α stabilisation was able to overcome *C. albicans* suppression of neutrophil RNS, conferring a neutrophil-mediated, RNS-dependent protective effect *in vivo*. Combination of Hif-1α stabilisation with traditional antifungals had an additive effect on zebrafish survival in *C. albicans* infection and on clearance of *C. albicans* infection. Therefore, the work in this thesis has highlighted an important *C. albicans* virulence strategy, which could ultimately be a new potential therapeutic target if the factor(s) responsible for RNS suppression can be identified, aiding the treatment of *C. albicans* infections. Hif-1α stabilisation has potential to act as a HDT for the treatment of *C. albicans* infections, pending further validation of efficacy and safety in human systems.

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