

**Exploring differences in the immune
response triggered by clinically relevant
Candida spp.**



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À minha família

*“Recomeça...
Se puderes
Sem angústia
E sem pressa.
E os passos que deres,
Nesse caminho duro
Do futuro
Dá-os em liberdade.
Enquanto não alcances
Não descanses.
De nenhum fruto queiras só metade.*

*E, nunca saciado,
Vai colhendo ilusões sucessivas no pomar.
Sempre a sonhar
E vendo
O logro da aventura.
És homem, não te esqueças!
Só é tua a loucura
Onde, com lucidez, te reconheças...”*

Sísifo, Miguel Torga

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List of abbreviations

3-APA – 3-aminophthalate
ALT – Alanine aminotransferase
AMPs – Antimicrobial peptides
AP-1 – Activator protein 1
APCs – Antigen-presenting cells
Asn – Asparagine
AST – Aspartate aminotransferase
ATP – Adenosine triphosphate
AUC – Area under the curve
BCA – Bicinchoninic acid
BMDCs – Bone marrow-derived dendritic cells
BMDMs – Bone marrow-derived macrophages
BSA – Bovine serum albumin
CARD9 – Caspase recruitment domain-containing protein 9
cDNA – Circular DNA
CFUs – Colony-forming units
CGD – Chronic granulomatous disease
CLRs – C-type lectin receptors
CMPs – Common myeloid progenitor cells
CMV – Cytomegalovirus
DCs – Dendritic cells
DMEM – Dulbecco's modified Eagle medium
DMSO – Dimethyl sulfoxide
dNTP – Deoxyribonucleotide triphosphate
dsRNA – Double-stranded RNA
ECM – Extracellular matrix
ELISA – Enzyme-linked immunosorbent assay
FACS – Fluorescence-activated cell sorting
FBS – Fetal bovine serum
Foxp3 – Forkhead box P3
G-CSF – Granulocyte colony-stimulating factor
GITR – Glucocorticoid-induced TNFR-related protein
GITRL – GITR ligand
GM-CSF – Granulocyte-macrophage colony-stimulating factor
GPI – Glycosylphosphatidylinositol
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HK – Heat-killed
HPLC – High-performance liquid chromatography
HRP – Horseradish peroxidase
HSCs – Hematopoietic stem cells
ICAM-2 – Intercellular adhesion molecule 2
ICL1 – Isocitrate lyase 1

IFIT2 – Interferon-induced protein with tetratricopeptide repeats 2
IFN – Interferon
IL – Interleukin
IMDM – Iscove’s modified Dulbecco’s Medium
iNOS – Inducible nitric oxide synthase
IPA – Ingenuity pathway analysis
IRAK4 – interleukin-1 receptor-associated kinase
IRF3 – Interferon-regulated factor 3
IRGs – Interferon-regulated genes
ITAM – Immunoreceptor tyrosine-based activation motif
ITGB2 – Integrin subunit β 2
JAK – Janus kinase
LPS – Lipopolysaccharide
LRRs – leucine-rich repeats
LTA – Lymphotoxin- α
LTF – Lactotransferrin/lactoferrin
M-CSF – Macrophage colony-stimulating factor
MALT1 – Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK – Mitogen-activated protein kinase
MBL – Mannose-binding lectin
MDA5 – Melanoma differentiation-associated protein 5
MHC – Major histocompatibility complex
MOI – Multiplicity of infection
MPO – Myeloperoxidase
mRNA – Messenger RNA
NETs – Neutrophil extracellular traps
NF- κ B – Nuclear factor kappa light chain enhancer of activated B cells
NFAT – Nuclear factor of activated T-cells
NLRs – NOD-like receptors
NO – Nitric oxide
OPC – Oropharyngeal candidiasis
PAMPs – Pathogen-associated molecular patterns
PBMCs – Peripheral blood mononuclear cells
PBS – Phosphate-buffered saline
PCA – Principal component analysis
PKC – Protein kinase C
PMA – Phorbol 12-myristate 13-acetate
PMNs – Polymorphonuclear leukocytes
PRRs – Pattern-recognition receptors
RLRs – RIG-I-like receptors
RNAseq – RNA sequencing
RNS – Reactive nitrogen species
ROS – Reactive oxygen species
RPMI – Roswell Park Memorial Institute medium
RT-PCR – Reverse transcriptase PCR

RT-qPCR – Real-time quantitative PCR
Saps – Secreted aspartyl proteases
Ser – Serine
SNPs – Small nucleotide polymorphisms
SOD – Superoxide dismutase
STAT – Signal transducer and activator of transcription
SYK – Spleen tyrosine kinase
TCA – Tricarboxylic acid
TGF- β – Transforming growth factor β
Thr – Threonine
TLRs – Toll-like receptors
TMB – 3,3',5,5'-Tetramethylbenzidine
TNF – Tumour necrosis factor
TRIF – TIR-domain-containing adapter-inducing interferon- β
TSLP – Thymic stromal lymphopoietin
WT – Wild-type
YPD – Yeast extract peptone dextrose medium

Summary

Systemic candidiasis is a life-threatening disease that affects ~750,000 people every year and has unacceptably high mortality rates due to a lack of efficient therapeutics. *C. albicans* is the most common and virulent agent of systemic candidiasis, whereas less virulent *Candida* spp. such as *C. parapsilosis* are more easily cleared by the host and thus have a lower incidence in comparison with *C. albicans*. Here, through comparative transcriptomic analysis, I sought to identify differences in the immune response triggered by *C. albicans* and *C. parapsilosis*, in order to validate *C. parapsilosis*-specific targets for immune modulation during *C. albicans* infections. This was conducted to assess the impact of the identified targets in promoting *C. albicans* clearance and to determine their potential as immunotherapies for *C. albicans*-induced systemic candidiasis. Strikingly, I found that the *C. parapsilosis*-triggered macrophage immune response is dominated by the type I IFN signalling pathway, with IFN- β standing as the top upstream activator of this pathway. *In vitro* studies presented here reveal that IFN- β boosts the candidacidal activity of BMDMs towards *C. albicans* via a mechanism independent of ROS, NO and conventional AMPs. *In vivo* results indicate that administration of IFN- β has a negative effect on mice survival during *C. albicans* challenge. However, reduced kidney fungal burden was observed in mice injected with a high dose of IFN- β that survived the infection. Preliminary *in vivo* experiments revealed that administration of hetIL-15, a type I IFN-inducible cytokine complex, promotes splenic lymphocyte proliferation in *C. albicans*-injected mice, with a significant expansion of granzyme B-producing NK cell populations, and IFN- γ - and granzyme B-producing CD8⁺ T cells. *C. albicans* has been previously reported to inhibit macrophage IL-27 production, another type I IFN-inducible cytokine. I uncovered here that IL-27 inhibition is triggered by a soluble mediator, secreted during the *C. albicans* morphotypical transition to true hyphae. Moreover, I have also discovered a novel β -glucan-driven mechanism of IL-27 inhibition dependent on Dectin-1 signalling. However, it is still unclear whether *C. albicans* uses this mechanism to inhibit IL-27 production. This thesis presents the first comparative analysis of the macrophage immune response triggered by *C. albicans* and *C. parapsilosis*, and the collective results displayed here can be further explored to help devising new therapeutic approaches for treatment of life-threatening systemic candidiasis.

Chapter 1

General Introduction

1.1 Candida spp.

Candida defines a fungal genus from the phylum Ascomycota that comprises a wide variety of eukaryotic yeasts species that can be isolated from environmental, animal and human sources. *Candida* spp. frequently exist as mammal commensal fungi that constitute part of the normal microbiota of gut mucosa or genitourinary tract in healthy individuals, however these can frequently shift from being a harmless commensal to a disease-inducing pathogen when host immunity gets compromised^{1,2}. Indeed, more than seventeen different *Candida* spp. have been identified as aetiological agents of human infection, but the majority of *Candida* infections are caused by only five different species: *C. albicans*, *C. glabrata* and *C. parapsilosis*, *C. tropicalis* and *C. krusei*^{3,4}.

1.1.1 Epidemiology of systemic candidiasis

Most fungal infections are superficial, and common amongst the population with no significant associated morbidity and mortality^{5,6}. *Candida* spp. are the second most common agents of fungal infection after dermatophytes, and the majority of *Candida* infections are superficial and non-invasive, such as mucosal oral infections or vulvovaginal candidiasis, with varying incidence depending on socioeconomic and geographic factors, as well as cultural habits⁶. While invasive fungal infections are much less frequent than superficial mycoses, they are associated with high mortality rates and pose a much greater health concern, killing an estimated ~1.5 million people every year⁶. Life-threatening invasive candidiasis is estimated to affect ~750,000 people every year and is associated with extremely high mortality rates ranging from 46-75%^{6,7}. Moreover, invasive candidiasis and candidaemia are typically nosocomial infections, as most disease cases are acquired in hospital environments and in patients with long intensive care unit stays. This causes *Candida* to be the fourth leading cause of nosocomial bloodstream infections and the third in intensive care units in developed countries like the U.S.⁸⁻¹⁰. According to a meta-analysis study on the global epidemiology of fungal infections, only 39 countries have published data regarding the national burden of candidaemia. From these countries, the U.K. ranks as the country with the 9th highest candidaemia burden, with 5,142 cases reported in 2017 and an estimated burden rate of 8.1 cases per 100,000 citizens every year^{7,11}.

Over 90% of invasive candidiasis cases are caused by *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. krusei*. Of those, *C. albicans* stands as the most common infectious agent^{3,12,13}. Nevertheless, over the past two decades there has been a significant increase on the incidence of non-*albicans Candida* spp. isolated from infected patients suffering with candidaemia.

1.1.2 *Candida albicans*

C. albicans is the main *Candida* sp. responsible for infection in humans. Due to its incidence and clinical relevance, most scientific research is focused on *C. albicans*, therefore making it undoubtedly the best studied *Candida* sp. and setting it as a model yeast for studying *Candida*-host interactions. *C. albicans* is a polymorphic fungus that can adopt several different phenotypes^{14–17} (Figure 1.1). The most relevant morphologies observed during infection are the round ovoid-shaped budding yeast (white phenotype or blastoconidium) and true hyphae – long tubular *Candida* cells separated by non-constrictive septa originated from a single yeast. However, other phenotypes were also discovered such as: pseudohyphae – chains of adjacent elongated *Candida* cells separated by constrictive septa); chlamydospores – thick-walled spore-like structures; opaque phenotype – white elongated yeasts with unique pimples on their surface that are associated with sexual reproduction; and GUT (gastrointestinal induced transition) phenotype – a phenotype visually similar to opaque phenotype, but without surface pimples, that *C. albicans* adopts by downregulating virulence-associated genes in order to thrive as commensal in the large intestine of the host. In the human host, *C. albicans* yeast cells are mostly associated with commensalism and immune evasion, whilst true hypha is important for invasion and to drive pathology. Indeed, most pathways involved in this phenotypical transition are often required for virulence, and this is confirmed in mutant yeast-locked *C. albicans* strains, as these are typically much less virulent than WT strains, therefore providing a link between morphology and virulence^{16,18,19}. Nevertheless, yeast forms are also important for dissemination and carriage of *C. albicans* throughout the body during systemic infection^{16,20}. Although *C. albicans* stands as a model pathogen for the study of candidiasis, there are several differences between it and other related pathogenic *Candida* spp. that are reflected in species-specific virulence profiles and host responses.

1.1.3 *Candida glabrata*

C. glabrata is another yeast species capable of infecting humans that exists mainly in yeast morphology (Figure 1.1). Due to its inability to form pseudohyphae under normal conditions, *C. glabrata* was initially believed to be monomorphic and was classified in the *Torulopsis* genus, however it was later reclassified in the genus *Candida* when it was determined that the ability to form pseudohyphae was not a reliable distinguishing factor for species belonging to the genus²¹. Nevertheless, whilst most *Candida* spp. like *C. albicans* and *C. parapsilosis* are phylogenetically close to one another, *C. glabrata* is considered a “misnomer” as it is more closely related to the avirulent yeast *Saccharomyces cerevisiae* than it is to other *Candida* spp. In fact, unlike the diploid *C. albicans*, *C. glabrata* is a strictly haploid yeast, and whilst morphological transition is an important virulence trait of *C. albicans*, *C. glabrata* virulence mechanisms do not rely on morphology, as unlike other *Candida* spp., *C. glabrata* does not form pseudohyphae when grown at 37°C and only exists in yeast form during infections^{21,22}. From the non-*albicans* *Candida* spp. mainly isolated from candidiasis, *C. glabrata* is possibly the most notable and alarming one, as its prevalence has significantly increased possibly due to its resistance and tolerance to azoles, the most widespread antifungals used as a first line treatment or prophylaxis for invasive fungal infections, especially in old neoplastic patients undergoing fluconazole prophylactic treatment^{10,23,24}

1.1.4 *Candida parapsilosis*

C. parapsilosis is also a causative agent of *Candida* infections. Unlike *C. albicans*, *C. parapsilosis* invasive infections frequently happen without prior colonisation of the host and through horizontal transmission via contaminated external sources such as catheters or invasive medical devices²⁵. Indeed, *C. parapsilosis* can strongly adhere to these devices, where it can grow as extensive biofilms, which is possibly one of the main reasons why *C. parapsilosis* infections are more frequent in neonates than other *Candida* spp.^{3,25–27}. For a long time, *C. parapsilosis* isolates were separated into three groups, but further genetic studies have facilitated their classification into three distinct new species: *C. parapsilosis sensu stricto* (commonly addressed as *C. parapsilosis*), *C. metapsilosis* and *C. orthopsilosis*. However, of those three species, *C. parapsilosis sensu stricto* is responsible for the vast majority of clinical manifestations, and most laboratories do not make the distinction between species from the

C. parapsilosis complex due to lack of efficient and reliable commercial systems for discriminative analysis^{25,28}. *C. parapsilosis* can exist as yeast form or pseudohyphae, and the latter is associated the activation of the inflammatory response during infection^{25,29} (Figure 1.1).

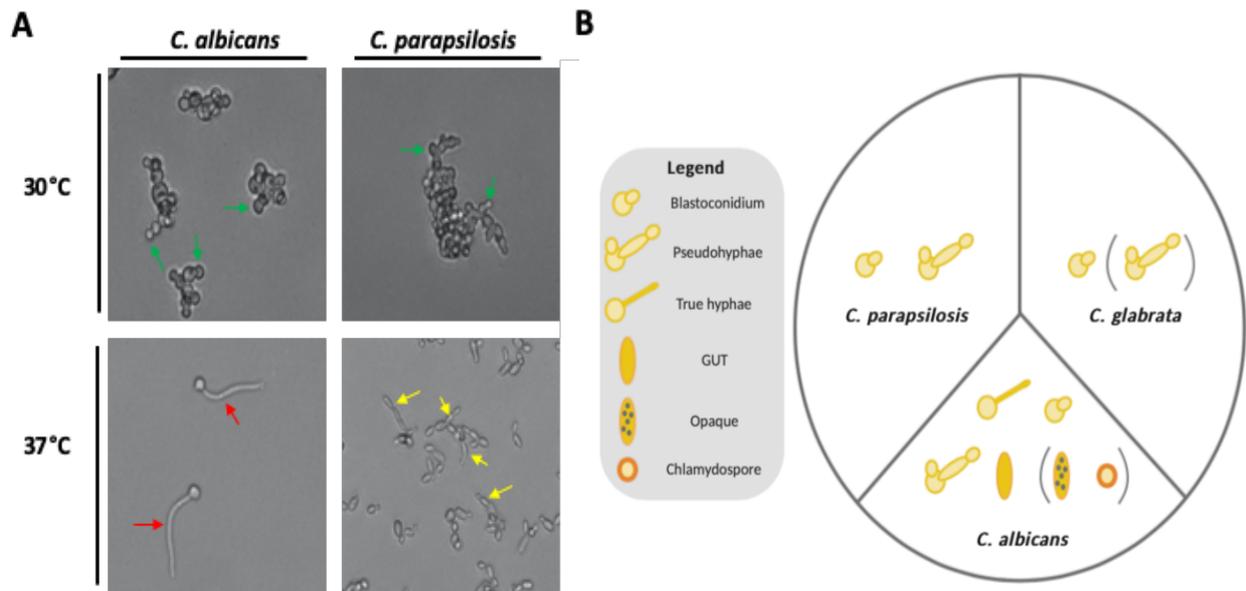


Figure 1.1 - *Candida* spp. different morphotypes. (A) *C. albicans* and *C. parapsilosis* were grown overnight in YPDB, washed twice with PBS and grown in RPMI-BMDM for 3 h at different temperatures. Cultures were then serially diluted, and pictures were taken using a 20X objective lens on an EVOS microscope. Green arrows point to yeast forms, red arrows point to true hyphae and yellow arrows to pseudohyphae. (B) Schematic of the different morphotypes observed in *C. albicans*, *C. parapsilosis* and *C. glabrata*, the main *Candida* spp. used in this study. Morphologies between grey brackets are not normally verified within the human host, and their impact during infection/ability to drive pathology is still poorly understood. (Figure 1.1-B was adapted from³⁰ and made using Biorender.com).

1.1.5 *Candida tropicalis*

C. tropicalis is another pathogenic *Candida* spp. originally isolated from a patient with fungal bronchitis in 1910^{31,32}. Phylogenetically, *C. tropicalis* is similar to *C. albicans* when compared with other clinically relevant *Candida* spp., and shares with it common phenotypical and biochemical traits, such as being able to exist as white and opaque phenotypes, and expressing a range of genes known to be involved in virulence and host invasion³². It frequently exists in pseudohyphae phenotype when infecting the human host, and like *C. albicans*, some *C. tropicalis* strains were also reported to form true hyphae, however, these do not show the same degree of filamentation as the former³²⁻³⁴. *C. tropicalis* is a commensal from the normal human microbiome, and can be found in skin, gastrointestinal, genitourinary and respiratory tracts. *C. tropicalis* is frequently isolated from patients with superficial and systemic *Candida* infections and is particularly common in patients suffering with neutropenia and leukaemia, and in individuals with damaged gastrointestinal mucosa or with unbalanced microbiota due to extensive use of antibiotics^{3,25,27,32,35}. Moreover, *C. tropicalis* is remarkably osmotolerant, as it can thrive in high salt concentrations. This can contribute for its persistence in saline environments, and to the expression of virulence, and antifungal resistance genes that can partially account for its natural resistance to frequently prescribed antifungal drugs such as azoles and echinocandins³².

1.1.6 *Candida krusei*

C. krusei is a pathogenic *Candida* spp. that can exist in yeast and pseudohyphae. *C. krusei* still remains largely understudied, in comparison with the other *Candida* spp. mentioned above, as it is not a frequently isolated agent of candidiasis. However, like the other four non-*albicans* species, its incidence is been rising over the past decade^{36,37}. Indeed, *C. krusei* is recently being mainly isolated from immunocompromised individuals, with special incidence in leukaemia patients or patients suffering with lymphocyte deficiencies³⁸. One of the reasons for its emerging prevalence is its intrinsic resistance to some antifungals such as azoles that are prescribed for treatment of systemic candidiasis, with many *C. krusei* infections being associated with prophylactic or therapeutic use of these antifungals^{37,39}.

1.1.7 *Candida* cell wall

Candida cells are protected by an outer layer called the fungal cell wall. The cell wall is a malleable and mechanically robust dynamic structure that is essential for the yeast cell viability, morphogenesis and pathogenesis, whose composition is tightly regulated in response to environmental and stress stimuli. Approximately one fifth of the *C. albicans* genome comprise genes involved in the biosynthesis and maintenance of the fungal cell wall, highlighting the importance of the organelle⁴⁰. Since the cell wall is the most extrinsic layer of the yeast cell, it is the first structure to come in contact with host cells, and therefore plays a pivotal role during infection. Indeed, most cell wall components are PAMPs (pathogen-associated molecular patterns) and a proper recognition of these by PRRs (pattern recognition receptors) on immune cells is vital to trigger an adequate host immune response during candidiasis. *Candida* cell wall is generally comprised of an outer layer of glycoproteins extensively modified with N- and O-linked carbohydrates (mannans or mannoproteins), in some cases containing glycosylphosphatidylinositol (GPI)-linked proteins, that comprise 30-40% of the fungal cell wall dry weight⁴¹ (Figure 1.2-A). Mannans can have very diverse structures depending on their type and degree of mannosylation. Mannose units can be incorporated into three different structures namely highly branched N-linked mannan: α -mannans attached to nitrogen atoms on asparagine (Asn) residues; O-mannans: mannose polymers covalently attached to atoms of oxygen from serine (Ser) or threonine (Thr) residues and phosphomannans: mannose units attached to phosphorus atoms that are bound to other α -mannans or glycolipids (Figure 1.2-B). The inner layer of the fungal cell wall is mainly comprised of β -glucans – polymers of glucose assembled into long chains – that account for 50-60% of the cell wall dry weight (Figure 1.2-A). β -(1,3)-glucan is the most abundant glucan subtype and serves as the main polysaccharide to which other cell wall components, like β -(1,6)-glucan are covalently attached to, making its synthesis vital for proper cell wall assembly and normal development of *Candida*^{42,43}. The innermost layer of the cell wall is composed mainly by chitin, a N-acetyl-D-glucosamine polymer that contributes for the overall integrity, correct formation and osmotic stability of the cell wall, and accounts for ~2% of its dry weight⁴³⁻⁴⁵ (Figure 1.2-A). Below the cell wall, the fungal plasma membrane contains chitin and β -glucan synthases that ensure the synthesis of cell wall components and its proper

maintenance, and ergosterol, the most abundant fungal sterol that regulates membrane permeability and fluidity⁴⁶ (Figure 1.2-A).

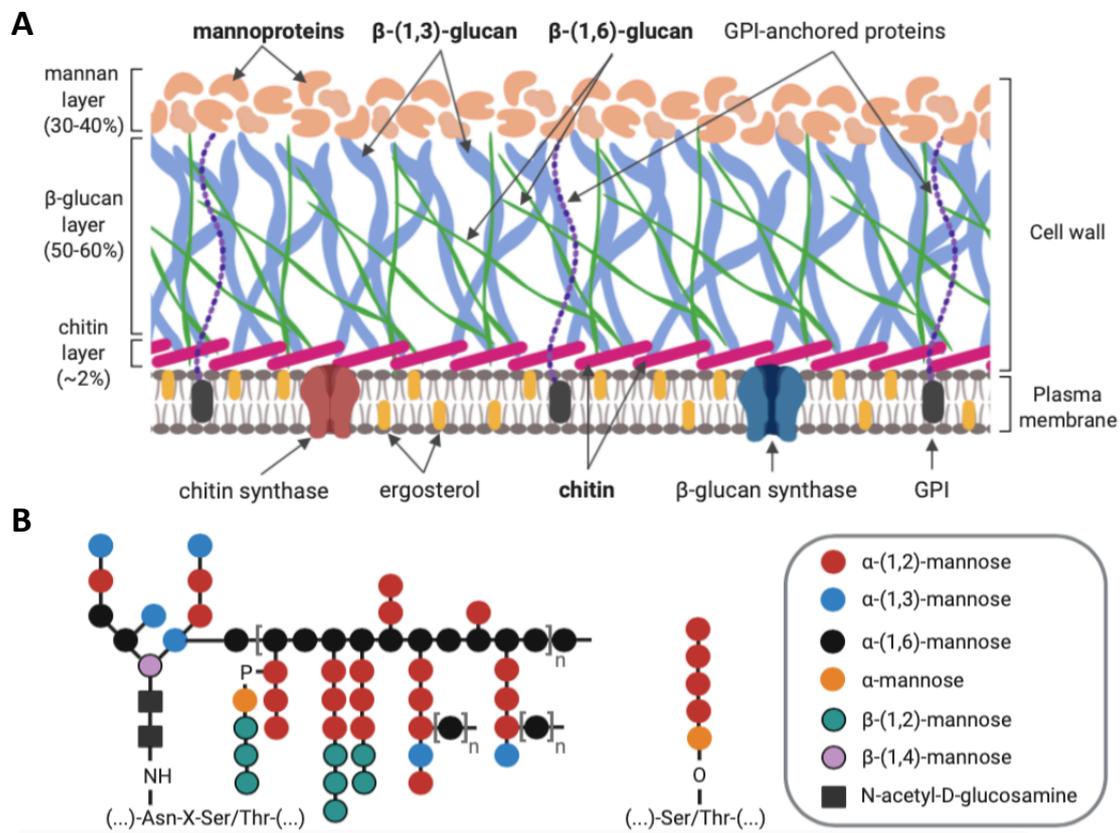


Figure 1.2 – Schematic of the *Candida* cell wall. (A) Illustration of the *Candida* spp. cell wall containing main structural carbohydrates and plasma membrane-associated proteins. (B) Schematic exemplifying the diversity of mannans. Adapted from^{41,47}. Images were made using Biorender.com.

1.1.8 *Candida* virulence mechanisms

The weakened or immunocompromised state of the host was long thought to be the sole driver for the establishment of opportunistic fungal infections. Nowadays, this concept does not explain the full picture and is not entirely reflective of the pathogenesis of candidiasis, as pathogenic fungi are endowed with a wide range of virulence factors that allow them to actively participate in the pathophysiology of the disease, and influence the pathogenicity of different *Candida* spp. Overall, these virulence strategies allow *Candida* to invade and survive within different host niches and to evade and withstand host defences during infection.

1.1.8.1 Polymorphism

Polymorphism defines the transition between yeast and hyphal/pseudohyphal growth forms. For most *Candida* species, both phenotypical forms are important for pathogenicity and for the onset of candidiasis^{16,18–20}. Moreover, there is a set of genes expressed during germination that code for virulence-associated factors not directly involved in hyphal formation *per se*. In *C. albicans* these include *HWP1* (hyphal wall protein 1), *ALS3* (agglutinin-like sequence protein 3), secreted aspartyl proteases like *sap4*, -5 and -6, and *ECE1* (extent of cell elongation 1). Hyphal growth can be triggered by different environmental stimuli. In case of *C. albicans*, these include such as alkaline pH, starvation, presence of serum proteins or N-acetyl-D-glucosamine, temperature, CO₂ and quorum sensing (a method of microbial chemical communication within *Candida* populations)¹⁷. Since it does not normally form pseudohypha, *C. glabrata* does not rely on this mechanism for virulence⁴⁸. Regarding *C. parapsilosis*, pseudohypha formation does not seem to greatly contribute for virulence during infection unlike the formation of true hypha contributes for *C. albicans* virulence⁴⁸.

1.1.8.2 Adhesion and biofilm formation

The ability to adhere to host surfaces is necessary for *Candida* colonisation and for triggering infection. Moreover, adherence to abiotic surfaces such as indwelling medical devices can also be the cause of bloodstream *Candida* infection²⁵. Adherence is mostly mediated by adhesins such as Als proteins, particularly Als3, and Hwp1 that are GPI-associated proteins present at the cell surface of *Candida*, and the importance of these proteins on the establishment of infection and progression of disease was already identified^{49–52}. Some adhesins like Als3 also act as invasins. These were shown to promote *Candida* endocytosis by epithelial and endothelial cells independently of fungal viability as a fungal invasion mechanism⁵³. Another important feature of these two families of adhesins is these proteins' contribution for the formation of *Candida* biofilms. These complex microbial structures are highly resistant to antifungals and can facilitate *Candida* infections by forming extensive fungal mats on mucosal surfaces which hinder *Candida* clearance by the host or on invasive medical devices like catheters and promote direct fungal dissemination into the host bloodstream¹⁷.

1.1.8.3 Secreted enzymes and toxins

Besides its ability to adhere to host surfaces, *Candida* also secrete an arsenal of hydrolases that facilitate cell penetration and invasion. These can be proteases, phospholipases or lipases. Secreted aspartyl proteases (Saps) are hydrolytic enzymes mostly involved, amongst other processes, in the acquisition of nutrients from exogenous proteins and were proposed to facilitate active penetration of host cells by *Candida*^{54,55}. Several different Sap-coding genes were discovered, however their expression varies depending on the *Candida* spp. and other conditions. *C. albicans* genome contains at least ten distinct Saps⁵⁶⁻⁵⁹, whereas only three Sap-coding genes were detected in *C. parapsilosis* genome⁶⁰. *C. glabrata* does not code for any Sap orthologs, however it produces 11 different surface-associated aspartyl proteases called yapsins that have similar function and were associated with its virulence. *C. glabrata* yapsins were shown to be important for adherence and survival during infection in both J774A.1 macrophage cell line and in an *in vivo* murine model of systemic candidiasis⁶¹⁻⁶³. *C. albicans* Sap1 to -6 and *C. parapsilosis* Sapp1 to -3 are the most broadly studied of these proteins and were shown to exert broadly deleterious effects on the immune response triggered during candidiasis^{60,64-68}.

Candida spp. use phospholipases to hydrolyse phospholipids that can yield fatty acids and a number of lipophilic intermediate molecules that play multiple roles in cell development, metabolism and signalling. However, a role for these enzymes in virulence was described, as pathogenic *Candida* spp. can use phospholipases to disrupt host cell membranes by hydrolysing ester linkages in glycerophospholipids^{69,70}. *C. albicans* codes for different types of phospholipases, but only Plb1 and Pld1 were shown to be relevant for its virulence in both systemic and oral candidiasis models respectively⁷¹⁻⁷⁴. *C. parapsilosis* and *C. glabrata* also secrete phospholipases, however their expression has great intra-species variation, with some clinical isolates not producing these enzymes at all. Therefore, a direct relation between phospholipase expression and virulence degree in these two species has not yet been established^{60,61}.

Extracellular lipases catalyse the hydrolysis of ester bonds in triacylglycerols and are used by *Candida* spp. mainly to digest lipids and acquire nutrients. However, these enzymes

were identified to play a role in pathogenic *Candida* spp. virulence, as they can contribute to adhesion to host tissue and cells, induce nonspecific hydrolysis of other cellular components along due to their additional phospholytic activities, and can also trigger inflammatory processes when targeting host immune cells⁷⁵. *C. albicans* codes for at least ten lipases (Lip1 to -10) however, to date only Lip8 was shown to contribute for *C. albicans* pathogenicity in a mouse model of systemic candidiasis⁷⁵. *C. parapsilosis* codes for four lipases (*CpLip1* to -4) however only *CpLip1* and -2 are secreted as functional enzymes and were implicated in its virulence and to contribute for successful host invasion^{60,76–78}. Unlike *C. albicans* and *C. parapsilosis*, no extracellular lipases were identified to play a role in virulence of *C. glabrata*^{34,79}.

Besides these enzymes, *C. albicans* also secretes candidalysin, a recently discovered fungal toxin generated from the parent polypeptide Ece1 (Figure 1.3). Candidalysin appears to be a distinct feature of *Candida* spp. capable of forming true hyphae, as *C. dubliniensis* and *C. tropicalis* were the only non-*albicans* species identified to express known *ECE1* orthologs, and are therefore predicted to also produce the toxin^{80,81}.

1.1.8.4 Metabolic plasticity

For efficient host invasion, *C. albicans* needs to form true hyphae, and produce an arsenal of virulence-associated molecules that demand a lot of energy. *C. albicans* uses glucose as the main preferred carbon source to fuel these metabolic and virulence-associated processes, and during infection, it focuses mainly on upregulating genes associated with glycolysis, fatty acid β -oxidation and the tricarboxylic acid (TCA) cycle to yield glucose and therefore generate enough energy. However, once in the bloodstream or inside the nutrient-deprived environment of the phagosome, *C. albicans* has evolved strategies of metabolic plasticity that allow it to adapt its metabolism to survive within such host niches. Under those conditions, *C. albicans* undergoes a metabolic shift from a highly glycolytic to a gluconeogenic state, by upregulating the expression of two key enzymes involved in the glyoxylate cycle, isocitrate lyase (*ICL1*) and malate synthase (*MLS1*)⁸². The glyoxylate cycle is a shunt conserved among bacteria, plants, fungi and nematodes, that allows *C. albicans* to bypass the CO₂-generating steps of the TCA cycle and permits the conservation of carbons to be used as substrates in gluconeogenesis. Isocitrate is transformed into glyoxylate and succinate

(through Icl1) and then glyoxylate is converted into malate (by Mls1). Malate dehydrogenase converts malate into oxaloacetate so it can be used for gluconeogenesis, therefore enhancing the produced glucose yield and allowing *C. albicans* to survive longer inside phagosomes^{82,83}

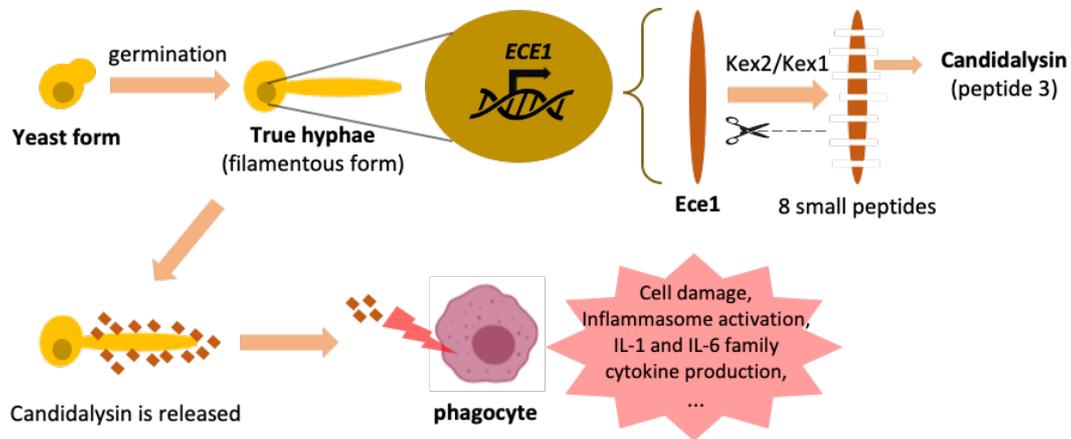


Figure 1.3 – Simplified schematic for candidalysin production. During hypha formation, *C. albicans* expresses *ECE1*, a gene that codes for a polypeptide composed of eight smaller peptides separated by lysine-arginine (K-R) residues. This peptide is a substrate for the protease Kex2 that will cleave Ece1 in the K-R motifs followed by Kex1 that will further remove the R residue in the C-terminal of the peptides, to generate eight small peptide products. The third peptide generated by this cleavage (Ece1-III_{62-93K}) is a cytolytic toxin named candidalysin that induces secretion of a myriad of cytokines and chemokines in epithelial cells and was found to be a molecular determinant of epithelial cell damage and host recognition of *C. albicans*^{84–86}. The immune mediators released from epithelial cells in response to the toxin will drive the local immune response towards a protective Th17 phenotype during mucocutaneous infection and promote renal neutrophil recruitment during systemic infection that will contribute for *Candida* clearance^{81,87,88}.

1.2 Immune response against *Candida* spp.

1.2.1 *Candida* spp. recognition by immune cells

The first essential step for mounting an efficient immune response against *Candida* is the recognition of fungal PAMPs by host cell PRRs. Proper PRR signalling triggered by PAMPs allows immune cells to respond to the invading pathogen and ultimately deploy an efficient immune response to fight it, while minimizing the collateral damage the host might suffer from it. Four different PRR families have been implicated in the recognition of *Candida*: C-type lectin receptors (CLRs), Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG I-like receptors (RLRs)^{89,90} (Figure 1.4).

1.2.1.1 Toll-like receptors (TLRs)

TLRs are type I transmembrane proteins characterised by having extracellular leucine-rich repeats (LRRs) motif domains responsible for PAMP recognition and a cytoplasmic Toll/IL-1 receptor domain (TIR) that triggers signalling transduction^{91,92}. Generally, all TLRs form heterodimers for signalling activation, except for TLR2 that heterodimerises with TLR1 and TLR6⁹³. All TLRs except TLR3 signal through the MyD88 adaptor molecule that upon ligand activation binds to TIR-domain at the TLRs cytoplasmic tail and recruits IRAK4, leading to phosphorylation of IRAK1⁹⁴. TRAF6 then joins the complex and activates the TAB (TAK1/TGF- β) complex that will in turn contribute for degradation of I κ B α , resulting in nuclear migration of NF- κ B to the nucleus, and can also activate AP-1 via MAPK leading to the expression of inflammatory cytokines^{92,95}. TLR3 (and not exclusively, TLR4) signals through TRIF which leads to recruitment of TBK1 and to phosphorylation and nuclear migration of IRF3 resulting in the production of type I IFN⁹⁶. TRIF-dependent signalling can also activate IRF7 and induce late-phase NF- κ B expression^{92,97}. Whilst TLRs are not the primary receptors driving *Candida* phagocytosis, they have been implicated in the initiation of the immune response after *Candida* infection⁹⁸. Even so, the overall dependence on TLR signalling for resistance against candidiasis is still controversial. Murine studies have revealed that MyD88-dependent signalling is essential for host resistance against *C. albicans*⁹⁹. However, IL-1R signalling, which requires MyD88, also proved to be protective^{100,101}. Therefore, the susceptibility of MyD88-deficient mice to *Candida* infection cannot easily be accounted to loss of TLR signalling. Furthermore, genetic studies have revealed that human *MYD88* deficiency does not

predispose immunocompetent patients to fungal diseases¹⁰². TRIF signalling was also implicated in the response against *Candida*¹⁰³, however its impact on conferring protection in either human or mice-induced candidiasis was not yet assessed. Nevertheless, several individual TLRs such as TLR2, -3, -4, -6, -7 and -9 have been implicated in host protection during candidiasis, with the contribution of each receptor generally depending on the *Candida* species, the morphotype, infection route and TLR cooperation⁹⁰. TLRs can either be located at the plasma membrane and contribute for the initial recognition of *Candida* PAMPs, or at the cytoplasm. Cytoplasmic TLRs are then recruited to *Candida*-containing phagosomes to trigger consequent signalling after recognition of fungal ligands not normally exposed at the surface of the pathogen.

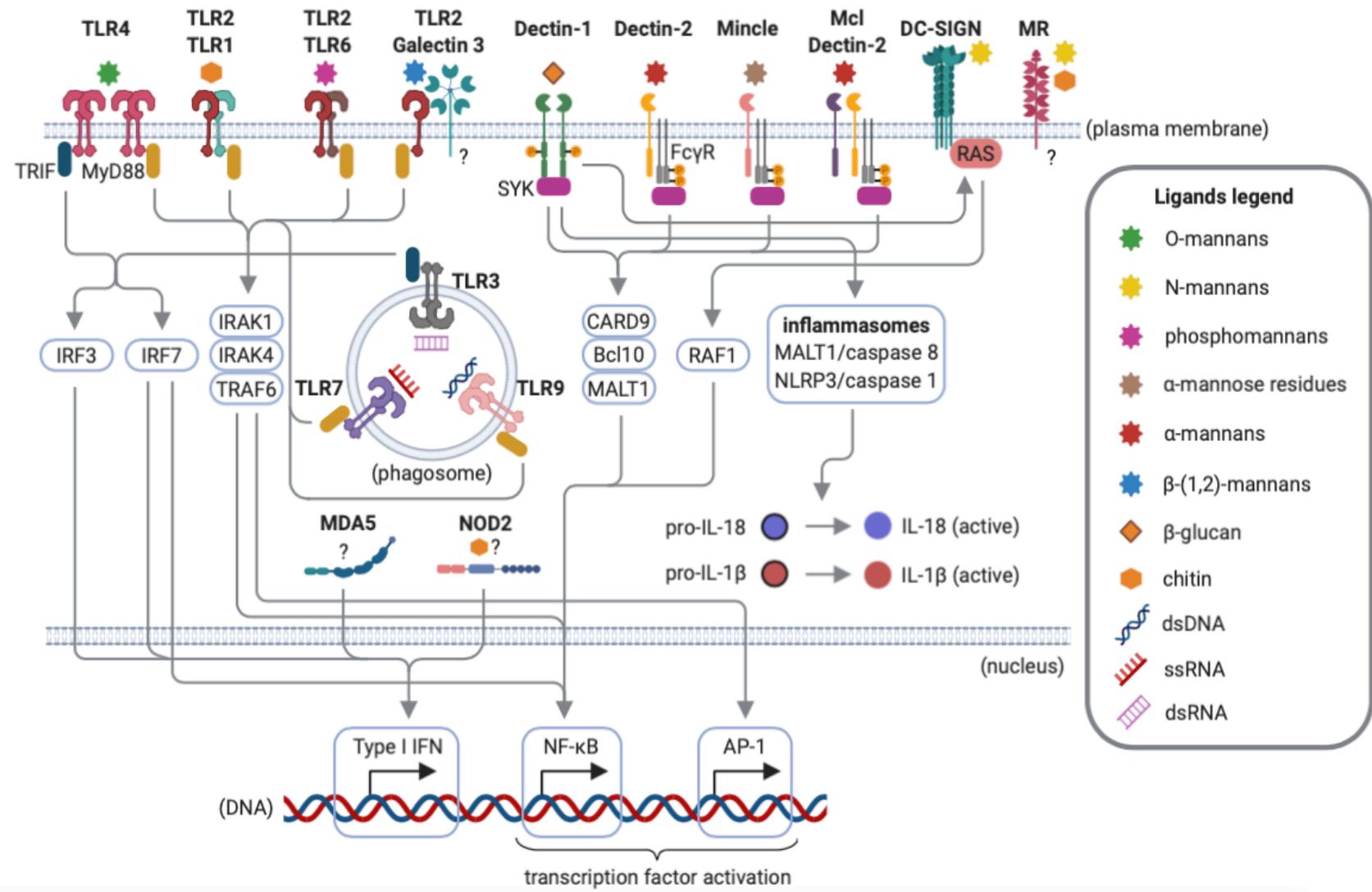


Figure 1.4 – Main PRRs responsible for *Candida* spp. recognition. Adapted from²⁶. Images were made using Biorender.com.

1.2.1.1.1 Plasma membrane TLRs

From the thirteen different TLRs identified mice, and the ten in humans, TLR2, -4, and -6 were the only plasmatic membrane TLRs associated with the recognition of *Candida* PAMPs¹⁰⁴. Most membranal TLRs can detect a variety of different ligands, including those completely unrelated with one another in structure, which suggests the involvement of other accessory proteins and/or other PRRs, in the discrimination of distinct ligands¹⁰⁵.

TLR2 was shown to form heterodimers with TLR6 to sense phosphomannans¹⁰⁶⁻¹⁰⁸, with Galectin-3 to bind β -(1,2)-mannans¹⁰⁹⁻¹¹¹, and with TLR1 to sense chitin oligomers¹¹². TLR2 signalling activation has been shown to ameliorate macrophage candidacidal activity towards *C. albicans* and to consequently impair its clearance¹¹³, but its role during systemic candidiasis is still controversial. Villamón and colleagues have revealed that TLR2-deficient mice are extremely susceptible to a high dose of *C. albicans* due to impaired neutrophil recruitment to infected sites and impaired ability to mount protective Th1 responses^{114,115}. However, Netea *et al.* have shown that TLR2 signalling is detrimental to the outcome of systemic candidiasis, as TLR2-deficient mice had impaired IL-10 synthesis and higher levels of IFN- γ , showed reduced Treg proliferation and increased macrophage candidacidal activity. Authors propose that *C. albicans* triggers TLR2 signalling as an immune evasion mechanism¹¹⁶. Lastly, another study has revealed that survival rate of TLR2-deficient mice against systemic *C. albicans* infection is not different from immunocompetent mice, although the TLR2 knock-out mice display slight but significantly lower fungal burden in the kidney¹¹⁷. Nevertheless, the same paper reports that following intragastric challenge with *C. albicans*, TLR2-deficient mice have reduced Th1 and increased Th2 polarisation, indicating that TLR2 signalling might confer protection in some mucosal forms of candidiasis¹¹⁷. There is contradictory literature concerning the impact of TLR2, which makes it difficult to define its exact role in candidiasis.

TLR6, another TLR that can form heterodimers with TLR2 and recognise phosphomannans, appears to have a dispensable role in *C. albicans*-induced systemic candidiasis, as both WT and TLR6-deficient mice have similar kidney fungal burden and survival rates towards infection¹¹⁸. However, TLR6 may have a cell-specific role in the recognition of *Candida* and subsequent immune response, as splenocytes from TLR6-deficient

mice produce significantly more IFN- γ and less IL-10 compared to splenocytes from immunocompetent mice, suggesting that TLR6 signalling might, to some degree, impair protective Th1 expansion during systemic infection¹¹⁸.

TLR4 recognises O-mannans⁴¹ and has been implicated in the recognition of *C. albicans* by macrophages, and to confer protection during systemic candidiasis by augmenting macrophages' candidacidal activity and by promoting secretion of chemoattractants that will induce neutrophil migration to kidneys of infected mice resulting in increased fungal clearance^{119,120}. Moreover, it was shown that TLR4 recognises *C. albicans* motifs in a strain-specific manner. Different *C. albicans* strains elicit distinct cytokine secretion profiles on human PBMCs, resulting in different susceptibility in murine models of systemic candidiasis, possibly due to inter-strain variations in expression of O-mannans¹²¹.

1.2.1.1.2 Phagosomal TLRs

The phagosomal TLR3, -7 and -9 recognise nucleic acid ligands present in the phagosome¹²²⁻¹²⁴ and therefore are mostly associated with antiviral responses, however reports have revealed a role for these in the recognition of *Candida*-derived molecules during phagocytosis.

TLR9 was shown to negatively regulate TNF, IL-6 and nitric oxide production in macrophages^{125,126}. Studies suggest that TLR9 might contribute to a deleterious Th2 response, as TLR9-deficient BMDCs (bone marrow-derived dendritic cells) produce less IL-12p40 upon *C. albicans* stimulation when compared to WT cells, and CD4⁺ T cells purified from *C. albicans*-infected TLR9^{-/-} mice produce enhanced levels of IL-4 and reduced levels of IFN- γ ^{117,127}. Nevertheless, literature indicates that TLR9 has either a redundant^{126,127} or detrimental¹¹⁷ role in murine models of disseminated *C. albicans* infections, depending on the strain.

TLR7 greatly contributes to IL-12p70 production in *C. albicans*-infected BMDCs, revealing a role of TLR7 in induction of protective Th1 responses. This also possibly explains why TLR7-deficient mice are more susceptible to disseminated *C. albicans* infections when compared to WT mice¹²³. Moreover, although it was observed to contribute to fungal

persistence in infected organs during systemic infection, TLR7 was shown to be required for IFN- β production by BMDCs infected with *C. glabrata*¹²².

Information on the impact of TLR3 in *C. albicans*-mediated immune response is scarce, as no reports have yet confirmed what the impact of *in vivo* TLR3 activation is during candidiasis. A role for TLR3 in host protection is best described in dendritic cells (DCs), where activation of TLR3-signalling can induce DC maturation and contribute to effective T-cell priming via the production of IL-12 and IFN- β ¹²⁴. One study has also shown that TLR3 mediates production of IL-8, a neutrophil chemoattractant, in *C. albicans*-infected endothelial cells¹²⁸. As endothelial invasion is a crucial step for *Candida* vascular infiltration and initiation of systemic infection, it is likely that IL-8 produced by these cells is a protective mechanism to prevent *Candida* bloodstream dissemination. Additionally, genetic studies revealed that patients with a TLR3 polymorphism are more susceptible to cutaneous candidiasis and their PBMCs have impaired ability to produce IFN- γ , IFN- β and TNF. However more *in vivo* studies are required to fully ascertain the immune mechanisms that lead to susceptibility^{129,130}.

1.2.1.2 C-type lectin receptors (CLRs)

CLRs define a family of heterogenous binding proteins present on the surface of host cells and are characterised by presence of at least one extracellular C-type lectin-like domain responsible for recognition of PAMPs. Different C-type lectin-like domains vary on their Ca²⁺-dependency and overall structure, which results in different carbohydrate affinities and broad ligand diversity across the family^{131,132}. The majority of CLRs rely on cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) domains for signalling, and this could either be achieved via their own cytoplasmic domains (such as Dectin-1 hemi-ITAM domain) or through ITAMs contained within cytoplasmic domains of co-receptors such as Fc γ R (like Mincle and Dectin-2). Phosphorylation of Dectin-1 hemi-ITAM domains after β -glucan binding and of ITAM domains from Fc γ R coupled with Mincle and Dectin-2 after recognition of α -mannose and α -mannans respectively, promotes SYK-dependent activation of the CARD9/Bcl10/MALT1 complex. This complex will lead mainly to NF- κ B activation, but can also trigger MAPK, NFAT, IRF-1 and IRF5-dependent gene expression^{89,133,134}. CLRs were long implicated to be main drivers of the immune response against *Candida*, due to their ability to

trigger pathways essential for successful antifungal responses and host protection^{89,135–137}. Besides membrane-bound receptors, other soluble CLRs such as mannose-binding lectin (MBL) were also implicated in anti-*Candida* immunity. Unlike membrane-bound CLRs, MBL cannot trigger direct cell-mediated signalling and affect gene expression, however due to its affinity to mannose-rich structures, it can act as a *Candida* opsonin, inhibiting its growth and facilitating its phagocytosis by immune cells¹³⁷. The impact of MBL during candidiasis is still understudied, however its administration during systemic *Candida* infection is associated with increased host resistance¹³⁸.

Signalling through Dectin-1 and Dectin-2 cluster CLRs is essential for protection against candidiasis as both *Card9*-deficient mice and humans with genetic defects in *CARD9* are highly susceptible to infection^{133,139–142}, however whether MR and DC-SIGN are necessary for normal host defence against *Candida* is still uncertain.

1.2.1.2.1 Dectin-1

Dectin-1 (*Clec7a*) is a type II transmembrane CLR with a single extracellular carbohydrate-recognition domain, that recognises β -1,3-glucans from the fungal cell wall in Ca^{2+} -independent manner. Particulate β -glucans are internalized through the formation of a phagocytic synapse, that is required for Dectin-1 activation when host cells encounter β -glucan-containing pathogens, whilst soluble β -glucans do not trigger phagocytic synapse formation and therefore are thought to exert an antagonist effect on Dectin-1 signalling¹⁴³. Besides signalling through the *CARD9*/*Bcl10*/*MALT1* complex like most CLRs, Dectin-1 can also activate RAS-Raf1 kinase pathway and both canonical (through *NLRP3*/caspase 1) and non-canonical (*MALT1*/caspase 8) inflammasomes, leading to maturation of *IL-1 β* and *IL-18*¹⁴⁴. Activation of Dectin-1 by fungal β -(1,3)-glucan contributes to the innate immune response through the secretion of cytokines such as *TNF*, *IL-6*, *IL-2*, *IL-10*, *IL-22* and chemokines like *CXCL2* and *CCL2*. Dectin-1 activation also augments cellular candidacidal activity by contributing for the production of reactive oxygen species (ROS) and the release of neutrophil extracellular traps (NETs)^{137,145,146}. Appropriate recognition and internalization of *Candida* via Dectin-1 by antigen-presenting cells (APCs) facilitates the presentation of fungal antigens to naïve T cells and contributes for the initiation of the adaptive immune response¹⁴⁴. Highly

immunogenic β -glucan is abundantly present in *Candida* cell walls, however *C. albicans* masks its β -glucan layers with layers of mannans to evade recognition by Dectin-1, as β -glucans are only exposed in bud scars in *C. albicans* yeast forms and not in hyphal forms¹³⁵ (Figure 1.2). Moreover, hyphae have structurally different β -glucans compared to yeasts, and are thought to have lower affinity to Dectin-1 and induce different signalling responses¹⁴⁷.

Despite its implication in the anti-*Candida* immune response, the role of Dectin-1 in some forms of candidiasis is still controversial. Human studies have shown that nonsense Dectin-1 mutations or polymorphisms predispose patients to various forms of mucocutaneous candidiasis^{148–150}. Indeed, Dectin-1 is associated with maintenance of mucosal health, especially in the gut, however murine studies have shown that Dectin-1 is not involved in controlling *C. albicans* gastrointestinal colonisation^{151,152}. The reason for these discrepancies between mice and humans is still unclear, however it is important to note that *C. albicans* is not a natural murine gut commensal¹⁵³, so humans may have developed evolutionary mechanisms of *Candida* tolerance in the gastrointestinal mucosa that are absent in mice.

During systemic candidiasis, Dectin-1 appears to be involved in efficient clearance of *C. parapsilosis*, *C. glabrata* and *C. tropicalis* in colonised organs, without affecting survival¹⁵⁴. Regarding *C. albicans*, its effect appears to be fungal strain-specific, and dependent on chitin content, as strains with higher chitin levels on the cell wall appear to impair Dectin-1 recognition. In infections with the latter, Dectin-1 does not improve survival nor fungal clearance in affected organs¹⁵⁵.

1.2.1.2.2 Dectin-2 cluster CLRs

A cluster of CLR genes located near the Dectin-1 locus (human chromosome 12, mice chromosome 6) include Dectin-2, Mincle and Mcl. Dectin-2 cluster CLRs are type II transmembrane receptors with a single carbohydrate recognition domain that were implicated in the immune response against *Candida* and whose expression appears to be restricted to myeloid cells¹³⁷.

Dectin-2 (*CLEC6A* in humans, *Clec4n* in mice) binds mannose-rich structures (α -mannans) that are present and exposed in all *C. albicans* morphotypes, although it appears to exhibit preferential recognition of hyphae rather than yeast forms^{156,157}. *In vivo* murine models revealed a protective role of Dectin-2 in systemic infection with *C. glabrata* and *C. albicans*, as Dectin-2 deficiency correlates with decreased survival and impaired *Candida* clearance in kidneys^{158–160}. In infections with the *C. albicans*, Dectin-2 was shown to contribute to protective Th17 responses by promoting IL-1 β and IL-23 production by DCs^{161,162}.

Mincle (Macrophage-inducible C-type lectin, *Clec4e*) binds α -mannose residues present in most cell wall mannans¹⁶³. Besides recognising *Candida* cell wall mannans, a role as a cell death sensor was also described for Mincle. Mincle can sense SAP130, a component released by host cells upon cell death and promote production of cytokines that drive neutrophil infiltration into damaged tissue¹⁶⁴. Mincle was shown to be present in the macrophage phagocytic synapse after *C. albicans* recognition, and although it has a redundant role in phagocytosis, it contributes to production of inflammatory cytokines during infection¹⁶⁵. Regarding candidiasis, *in vivo* studies have revealed that although Mincle does not affect mice survival after intravenous *C. albicans* challenge, it might play a role in effective *C. albicans* clearance in the kidney in a strain-specific manner^{160,165}.

Mcl (*Clec4d*, often referred to as Dectin-3), does not possess any known intracellular signalling domains, unlike Dectin-2 and Mincle. Although research states that it can activate SYK-mediated signalling, Mcl does not contain an arginine residue in its transmembrane domain that is usually required for coupling with Fc γ R. This makes it unlikely that Mcl/SYK-mediated signalling relies on Fc γ -chain¹⁶⁶, and probable that Mcl signalling is mostly achieved through collaboration with other CLRs such as Dectin-2 to boost α -mannan-triggered signalling and NF- κ B activation^{167,168}. Mcl was shown to be indispensable for resistance against systemic *C. albicans* infection, but while it significantly contributes for the secretion of TNF, IL-6, IL-17, IL-1 β , G-CSF, KC and CXCL2 in the kidneys of infected mice, the molecular and immunological mechanisms underpinning Mcl's dependence have yet to be fully elucidated¹⁶⁸.

1.2.1.2.3 DC-SIGN

DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin, *CD209* in humans) is a type II transmembrane receptor that possesses a single carbohydrate domain preferentially expressed in DCs, therefore making it an effective DC phenotypic marker. DC-SIGN is frequently found in tetrameric complexes at the cell membrane. These complexes increase the binding affinity of their carbohydrate binding domain for ligands with multiple mannose units, enabling it to recognise highly mannosylated structures like N-linked mannans of *Candida*^{169,170}. However, DC-SIGN, has incomplete ITAM in its cytoplasmic tail and it is thought not to directly induce cell responses, but to modulate other signalling pathways. As example, DC-SIGN can interacting with RAS pathway, leading to recruitment of Raf-1 and ultimately enhancing NF- κ B transcriptional rate^{137,171,172}

DC-SIGN also acts as an adhesion receptor, as it can interact with ICAM-2 on endothelial cells to induce DC migration, while also facilitating clustering with naïve T cells through ICAM-3 binding¹⁷¹. Mice do not express a single DC-SIGN ortholog, but 8 clustered homolog genes have been identified. However only two (SIGN-R1 and SIGN-R3) were reported to bind mannose moieties^{137,172}. This complicates the study of DC-SIGN during candidiasis using murine models, and most authors rely on *in vitro* studies. It was shown that DC-SIGN-mediated recognition of N-linked mannans is essential for human DC interaction and phagocytosis with *C. albicans*¹⁷³. Impairment of phagocytosis of *C. albicans* by DCs could result in inefficient antigen processing and cytokine production, and consequently impair the adaptive immune response towards *Candida*.

1.2.1.2.4 Mannose receptor (MR)

MR (Mannose receptor, *Cd206*) is a type I transmembrane protein expressed mainly by macrophages and DCs that has a short cytoplasmic domain lacking any known signalling motif and eight extracellular carbohydrate binding domains. MR contains two distinct lectin-binding domains, one that recognises carbohydrates like N-linked mannans, fucose or N-acetyl-D-glucosamine in a Ca²⁺-dependent manner, and another that binds sulphated acidic glycans in a Ca²⁺-independent mechanism^{137,174}. Upon ligand binding, MR is rapidly internalised in clathrin-coated vesicles and is frequently found inside early endosomes¹⁷⁴. This

characteristic allows MR to mediate phagocytosis of non-opsonised fungi after recognition of carbohydrates in their cell wall, as happens in *C. albicans*-infected macrophages^{135,175}. MR binds chitin and N-mannans, and since it lacks known signalling motifs, it is thought to mainly act as a scavenger receptor^{137,174}. However, MR can collaborate with other PRRs such as Dectin-1 and TLR2 to elicit signalling and induce protective Th17 responses during both *Paracoccidioides brasiliensis* and *C. albicans* infection^{176,177}. Although MR was implicated in *Candida* phagocytosis, murine *in vivo* studies revealed a redundant role for MR in conferring resistance against *C. albicans*¹⁷⁸.

1.2.1.3 Other PRRs

TLRs and CLRs are the two main PRR families involved in *Candida* recognition, however PRRs from other families have been implicated in the anti-*Candida* immune response.

NOD-like receptors (NLRs) are cytoplasmic PRRs characterized by having long leucine-rich repeats and a nucleotide-binding domain. *C. albicans* is known to activate the NLRP3 (NACHT, LRR, and PYD domains-containing protein 3) and MALT1/caspase 8 inflammasome, leading to cleavage of immature pro-IL-1 β and pro-IL-18 into functional cytokines that are critical for the control of fungal infections^{101,137,179}. NLRP3 is expressed in different cell types such as myeloid cells, B cells and epithelial cells, and reports suggest that it confers resistance to both mucocutaneous and disseminated candidiasis^{90,180–182}. Another NLR, NOD2 (Nucleotide oligomerization domain 2), was also implicated in the immune response against *Candida*, as it was shown to sense chitin fragments from *C. albicans* and contribute to production of IL-10 and IL-27 (downstream of IFN- β), by internalised *C. parapsilosis*^{183,184}. However, NOD2 appears to be dispensable for effective cytokine production by human PBMCs and in conferring resistance against candidiasis¹⁸⁵.

RIG-I-like receptors (RLRs) are cytosolic receptors expressed in a broad range of cells and have largely been implicated in the anti-viral response. RLRs have a DExD/H box RNA helicase domain with ATPase activity and are known to promote robust type I Interferon production¹³⁷. MDA5 (melanoma differentiation-associated protein 5, *IFIH1*) was implicated in the response against *C. albicans*¹⁸⁶, however the fungal ligand(s) that trigger MDA5

activation are still unknown, and the molecular pathways that lead to MDA5-mediated resistance during candidiasis are still poorly understood. The type I Interferon pathway was identified as central for host resistance against systemic candidiasis¹⁸⁷, but literature indicates that *Candida*-driven type I IFN production is mostly mediated by the TLR7/TLR9 activation/signalling^{184,188,189}. Nevertheless, it is possible that a yet unidentified *Candida*-triggered mechanism of type I IFN production mediated by MDA5 signalling contributes to a protective effect during infection.

1.2.2 Innate phagocyte responses against *Candida*

Innate phagocytes like neutrophils, monocytes, macrophages, DCs, and NK cells are important players during *Candida* infection as these are some of the first cells to come into contact with the invading pathogen and initiate the immune response upon infection. Following uptake of fungal pathogens, phagocytes are not only able to deploy mechanisms to kill it, these cells also generate fungal antigens that will help initiate adaptive immunity, secrete cytokines and chemokines that will bolster the local inflammatory response and recruit other immune cells to the infected site to contribute to fungal clearance.

1.2.2.1 Candidacidal mechanisms of innate phagocytes

Once *Candida* is engulfed by phagocytes, it is internalized in a phagosome that will undergo several vesicle fission and fusion events with endosomal components to form a phagolysosome. This acidic intracellular compartment is endowed with potent antimicrobial components that will ultimately lead not only to the killing and digestion of the microbe, but also help generate fungal antigens, that along with induced cytokines will facilitate activation of adaptive immunity¹⁹⁰. These antimicrobial mechanisms can be either oxidative (relying on oxygen-derived cytotoxic chemical compounds), or non-oxidative and rely on molecules that will have direct fungicidal or fungistatic effects.

1.2.2.1.1 Oxidative mechanisms

Oxidative mechanisms triggered against *Candida* spp. consist mostly on the production of reactive oxygen species or intermediates (ROS). This process is triggered by non-ingestible particles in the phagosome and leads to assembly of NOX2 NADPH oxidase (phox) in the

phagosomal membrane (Figure 1.5-A). Superoxide generated by NOX2, that has little to no toxicity on the *Candida* spp., is then subject to a series of chemical transformations that ultimately will generate toxic ROS with potent antifungal activity¹⁹¹⁻¹⁹⁴ (Figure 1.5-B and C). Possibly the most toxic and effective ROS against *Candida* are the metabolites generated from myeloperoxidase (MPO), such as hypiodous and hypochloric acid^{191,194,195}. Macrophages do not produce MPO, and its prime producers are neutrophils that store high concentrations of this enzyme in their azurophilic granules, and monocytes, where the enzyme is stored inside lysosomes. However, macrophages can uptake MPO released by these cells into the extracellular milieu via mannose receptors and transport it to the phagolysosome to aid in the neutralization of internalised *Candida* spp.^{136,196,197}.

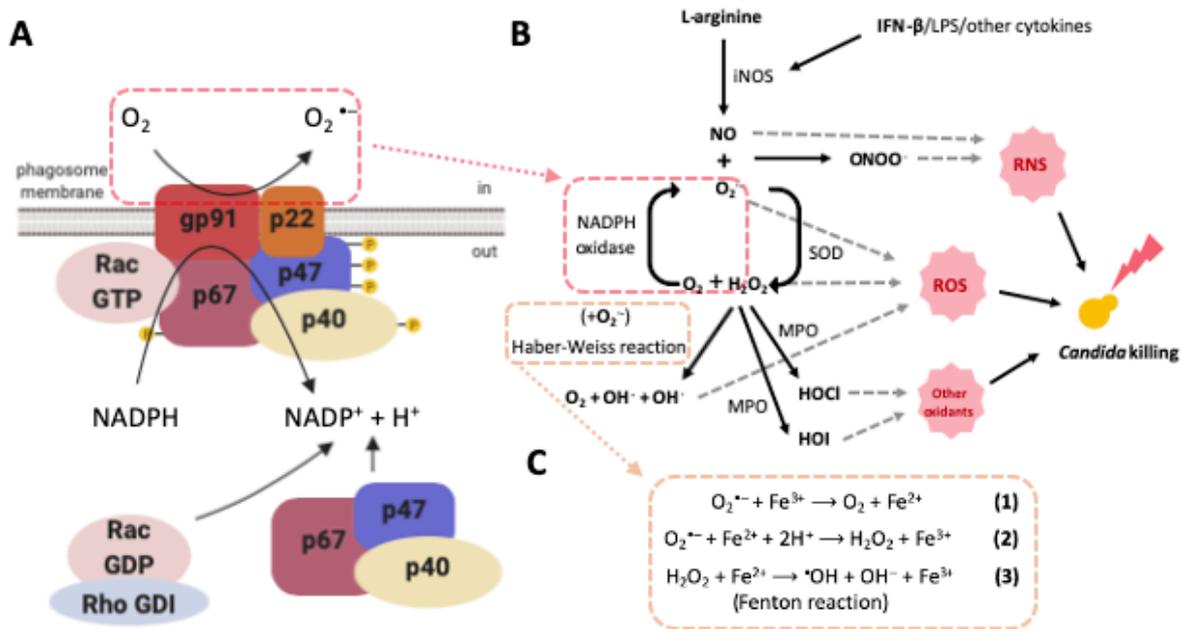


Figure 1.5 – Simplified schematic of oxidative mechanisms deployed by phagocytes upon *Candida* phagocytosis. (A) Assembly of NOX2 phagosomal NADPH oxidase complex. Organizer and activator proteins (p47^{phox} and p67^{phox} respectively) along with GTP-bound Rac (previously stabilized by Rho GDI) and p40^{phox} assemble in the cytosol and will translocate to phagosomal membrane where they will form a complex with the previously assembled heterodimer comprised of gp91^{phox} and p22^{phox}. Upon activation, the complex will generate superoxide anions from molecular oxygen inside the phagosome¹⁹⁸. (B) Inside the phagosome, superoxide can then react with nitric oxide produced by iNOS from L-arginine via antecedent stimuli like PRR activation or cytokine signalling^{194,199} to form peroxynitrite, or be transformed by superoxide dismutase (SOD) into hydrogen peroxide and molecular oxygen. In the presence of halides like chloride and iodide, myeloperoxidase (MPO) can transform hydrogen peroxide into other fungicidal oxidants like hypiodous or hypochlorous acid^{191,194,195}. In the presence of iron, superoxide and hydrogen peroxide can also be transformed through the Haber-Weiss reaction^{191–194}. (C) The Haber-Weiss is an iron-catalyzed reaction comprised of three steps. The first consists on the reaction of superoxide with ferric cations to generate molecular oxygen and ferrous cations. In the second step, ferrous cations react with superoxide and protons to generate ferric cations and hydrogen peroxide. The last step consists of a Fenton reaction, that in the presence of ferrous cations will transform hydrogen peroxide into highly toxic hydroxyl radicals. (Adapted from^{194,200,201}. Image A was made using Biorender.com)

Nitrosative mechanisms can be also considered as a type of oxidative killing mechanisms, as they rely on oxygen. These consist of the generation of reactive nitrogen species or intermediates (RNS) like nitric oxide (NO) (Figure 1.5-B). In macrophages, NO is mainly produced by the enzyme inducible nitric oxide synthase (iNOS), although other constitutively expressed nitric oxide synthases like eNOS and nNOS can also produce it in lower concentrations to support physiological functions²⁰². In the context of *Candida* spp. infections, NO by itself has no direct candidacidal effect²⁰³, however when it reacts with

superoxide produced during a respiratory burst it generates peroxynitrite that has potent candidacidal activity^{136,204}.

1.2.2.1.2 Non-oxidative mechanisms

The importance of non-oxidative mechanisms in the killing of *Candida* spp. is probably best demonstrated in patients suffering from chronic granulomatous disease (CGD) that contract fungal infections due to defective phagocyte NADPH oxidase assembly which translates in reduced production of ROS. Although these patients are more susceptible to candidiasis than healthy individuals, some patients are still able to clear the infection²⁰⁵. Moreover, studies using iNOS/gp91^{phox} double knock out mice revealed that these mice are highly susceptible to candidiasis, but their phagocytes do not show defective ability to kill *C. albicans in vitro*²⁰⁶.

1.2.2.1.2.1 Hydrolases

Hydrolases are a class of enzymes that can cleave covalent bonds using molecules of water. Some hydrolases are stored inside neutrophilic granules, macrophages and monocytes have antimicrobial properties and can contribute to pathogen elimination. These can either be delivered to the phagosome to help eliminate internalised pathogens or released into the extracellular milieu to act on uninternalized pathogens and promote chemotaxis of neighbouring cells. Probably the most broadly studied and efficient hydrolases implicated in killing *Candida* are lysozyme, mainly found inside lysosomes, and neutrophilic serprocidins such as cathepsin G and elastase present within neutrophil granules²⁰⁷⁻²¹⁰. Once inside the acidic environment of the phagolysosome, lysozyme will aid in the killing of internalised *Candida*, target of fungal cell wall components that will be released into the intracellular milieu and act as fungal antigens, while also inhibiting the production of Saps to greatly impair *C. albicans* virulence and fitness^{207,208}. Serprocidins are dependent on ROS to show their true antimicrobial activities and these can either be transported into the phagosome to kill internalized *Candida* or be expelled by neutrophils after NETosis. NETosis is a form of cell death in which the neutrophil “bursts” and releases NETs (neutrophil extracellular traps) that consist of a condensed chromatin decorated with antimicrobial compounds that can trap *Candida* in a mesh of cytotoxic compounds, ultimately leading to its killing²⁰⁹⁻²¹³.

1.2.2.1.2.2 Antimicrobial peptides (AMPs)

AMPs are small, soluble, naturally occurring molecules produced by a vast array of cells. Epithelial cells are the primary source of AMPs, however these can also be released by different innate phagocytes²¹⁴. Mammalian AMPs can exert a fungistatic or fungicidal effect against *Candida* spp. by disrupting the fungal cell wall, membrane permeabilization through formation of pores, or by inactivating cytoplasmic targets²¹⁵. Besides their direct antifungal activity, most AMPs can also act as alarmins by chemoattracting and activating neighbouring immune cells to the infection site²¹⁶.

Cathelicidins, or cathelicidin-derived peptides are small cationic and amphipatic molecules that have candidacidal activity against *C. albicans*^{136,217}. Secreted cathelicidins destabilize *Candida* spp. cell wall and the cytoplasmic membrane, leading to a breakdown of the membrane into vesicles and leakage of vital intracellular components, ultimately resulting in fungal death^{218,219}. Besides their direct candidacidal activity, human cathelicidin can bind to *C. albicans* cell wall impairing its adhesion to host cells, while also inhibiting biofilm formation^{220,221}.

Like cathelicidins, human and murine α - and β -defensins are small polypeptides with cationic charge²²². α -defensins seem to be constitutively expressed and stored within NK cells and neutrophil azurophilic granules, that will later fuse with the phagosome to aid in the elimination of intracellular pathogens. Conversely, expression of β -defensin is cytokine-inducible and often requires TLR-mediated signalling²²²⁻²²⁵. Similar to cathelicidins, defensins kill by disrupting the target microbial cell membrane through its permeabilization, leading to leakage of vital fungal components^{222,226}. Besides their direct antifungal potential against *Candida*, defensins can also activate and link both innate and adaptive immunity, by acting as monocyte and T cell chemoattractants^{222,227-230}.

Besides main conventional AMPs like cathelicidins and defensins, other molecules were identified to have AMP-like features and have proved to be effective against *Candida* spp. Lactotransferrin or lactoferrin (LTF) is an iron-binding glycoprotein secreted mainly by epithelial cells but is also present in neutrophilic granules^{231,232}. LTF also has antifungal activity

against multiple *Candida* spp. as it can disrupt the cell wall leading to fungal cell collapse and sequester iron inside host phagosomes as a nutritional immunity mechanism to restrict *Candida* growth and ameliorate infection^{233–238}. Its candidacidal activity is relatively low, however, it has a synergistic effect when administered in combination with some antifungal drugs due to its lower toxicity and ability to permeabilize fungal cells, thereby facilitating the delivery of antifungals that target *Candida* pathogens^{233,235}. Besides its direct AMP activity, LTF is also an immunomodulatory molecule. It can reduce ROS production, act as an antagonist for some PRRs and interfere with the expression of several immune mediators^{232,239–241}. Moreover, LTF can also affect the adaptive immune response by promoting migration of immature DCs to lymphoid organs during infection, promoting maturation of Th cells and enhancing differentiation of naïve B cells into effective antigen presenting cells^{232,242,243}.

Some chemokines and cytokines (such as CCL20, CXCL8, -14, -17 and TSLP (thymic stromal lymphopoietin)) share striking structural similarities between most classical AMPs. They can have direct antimicrobial activity against a variety of bacterial and fungal pathogens, including *C. albicans*, through a membrane-damage mechanism similar to those verified with convectional AMPs. These antimicrobial cytokines and chemokines are classified as kinocidins^{244–250}.

1.2.2.1.2.3 Perforin and cytolytic proteins

The primary antimicrobial strategy deployed by NK cells (and also CD8⁺ T cells) is the release of cytolytic proteins and enzymes present in their granules. These include perforin and granulysin that form oligomers and lead to the formation of large transmembrane pores that penetrate and damage the target cell generating a massive influx of water, and loss of vital cytosolic components, resulting in cell death²⁵¹. Besides lytic proteins, NK cell granules also include granzymes such as granzyme B, an enzyme that triggers target cell apoptosis via caspases 3 and 7 and greatly depends on the membrane permeability conferred by perforin and/or granulysin to be delivered to target cells^{252–255}. From these molecules previously mentioned, only perforin was proved to have a direct fungistatic effect against *C. albicans*, by inhibiting its filamentation and metabolic activity^{256,257}. Granzyme B does not have direct

candidacidal activity and it was proven to be dispensable for cytotoxicity, as NK cells lacking granzymes still have cytotoxic properties²⁵⁸. However, granzyme B-mediated apoptosis of *Candida*-infected cells will result in the release of other chemoattractants that will recruit other phagocytes and effector cells and will indirectly help to eliminate the fungal pathogen.

1.2.3 T cell responses against *Candida* spp.

T cells play a pivotal role in the host adaptive immune response against *Candida* as they provide both direct and indirect means of limiting fungal proliferation and disease progression. After processing *Candida* antigens, APCs will migrate to lymphoid tissues and present them to T cells via MHC complexes to generate antigen-specific acquired immunity. Moreover, cytokines secreted by APCs will also dictate the development of the T helper response triggered upon infection. Functionally, T cells can be grouped into two main subsets: cytotoxic T cells or CD8⁺ T cells and helper T cells or CD4⁺ T cells.

1.2.3.1 Helper T cells

CD4⁺ Helper T cells influence the immune response and the activity of different immune cells through secretion of a signature set of cytokines that will either boost, suppress or regulate inflammation against the fungal pathogen. CD4⁺ T cell responses are the predominant and possibly most important cell-mediated adaptive immune response against *Candida*, as evidenced by HIV⁺ patients that lack CD4⁺ T cells that are highly susceptible to various forms of candidiasis, and in T cell-depleted murine models, where adoptive transfer of CD4⁺ T cells greatly improved survival against disseminated *C. albicans*^{259,260}. Cytokines released by APCs during candidiasis will dictate the development of a specific Th subset and inhibit the proliferation of other subsets, ensuring the prevalence of only one Th response type and thereby maximising its potential²⁶¹.

1.2.3.1.1 T helper 1 (Th1) response

Th1 cells were long implicated in protection against multiple forms of candidiasis, mainly due to their ability to secrete large quantities of IFN- γ , the prime Th1 effector cytokine, and other relevant effector molecules such as IL-2, TNF and LTA²⁶². IL-12 and IL-18 produced by APCs and phagocytes are the main polarising cytokines and initiators of Th1 proliferation,

however, IFN- γ produced by Th1 cells can also promote upregulation of IL-12R β 2 via autocrine signalling, boosting the responsiveness of these cells to IL-12 and promoting prevalence of the Th1 phenotype^{261,263}. The master transcription factor of Th1 phenotype is T-bet, as it promotes *Ifng* transcription, and its overexpression in naïve CD4⁺ T cells results in Th1 differentiation. Moreover, T-bet knock-out results in Th2 and Th17 differentiation and consequently impaired Th1 immunity²⁶⁴. IFN- γ secreted by Th1 cells will boost the candidacidal function of phagocytes by enhancing ROS and NO production, promoting upregulation of relevant surface molecules such as MHC II, Fc γ R and integrins, contributing to the acidification of phagosomes and the release of antimicrobial molecules and promoting production of *Candida*-specific antibodies.^{259,262,265} The dependence on a Th1 response for protection against *Candida* infections is best described in systemic candidiasis. In systemic candidiasis models, IL-12 blockade impairs the development of resistance to infection, resulting in Th2 proliferation, and the depletion of IFN- γ renders mice highly susceptible to systemic infection^{266,267}.

1.2.3.1.2 T helper 2 (Th2) response

Th2 cells are associated with fungal persistence and they are broadly accepted to have a deleterious effect during candidiasis by dampening Th1 proliferation, inhibiting macrophage candidacidal functions and promoting *Candida* persistence and disease progression^{90,268}. IL-4 and IL-13 are the main Th2-polarising cytokines that will induce upregulation of GATA-3, the master Th2 transcription factor, in naïve T cells stimulating them to further secrete IL-4. IL-4 has a detrimental effect during invasive candidiasis by suppressing the uptake of *Candida* by innate phagocytes through induction of macrophage “M2” polarisation and impairment of mechanisms of oxidative killing^{265,269–271}. The impact of Th2 polarisation in systemic candidiasis was well documented using *in vivo* models. Unlike IFN- γ -deficient mice, IL-4-deficient animals are no more susceptible to *C. albicans* i.p. challenge than immunocompetent mice. Moreover, inhibition of IL-4 production in immunocompetent mice during the onset of Th2 proliferation was shown to restore antifungal immunity^{90,265,272}. Furthermore, GATA-3-overexpressing mice are also more susceptible to disseminated candidiasis due to significantly lower levels of IFN- γ produced by splenocytes, and impaired recruitment of macrophages to infected organs²⁷³.

1.2.3.1.3 T helper 17 (Th17) response

The protective effects of Th17 cells are achieved through secretion of effector cytokines such as IL-17A, IL-17F and IL-22. The main initial drivers of a Th17 response are IL-1 β , IL-6 and TGF- β that will upregulate the expression of ROR γ t, the master Th17 transcription factor, resulting in secretion of IL-17A, IL-17F and IL-22. However, IL-1 β , IL-6 and TGF- β *per se* are not sufficient to maintain Th17 development and continuous stimulation with these cytokines will lead to production of high levels of IL-10 that can dampen Th17-mediated inflammation²⁷⁴. IL-23 signalling was shown to be required for Th17 lymphocytes to achieve terminal differentiation and maintain a Th17 phenotype, and lack of IL-23 results in impaired migration of Th17 cells to lymphoid tissues and reduced secretion of Th17 effector molecules in infected sites^{274–276}. IL-17 promotes recruitment and proliferation of monocytes and neutrophils by increasing the local production of chemokines such as IL-8, CXCL1 and CCL2 and stimulating the release of hematopoietic cytokines G-CSF and GM-CSF that will lead to proliferation of local myeloid cells. There is strong evidence that Th17 responses protect against forms of mucocutaneous candidiasis by sustaining epithelial immunity, as IL-17 and IL-22 have chemotactic properties and bolster the production of AMPs which contribute to the maintenance and candidacidal properties of epithelial barriers^{277–281}. IL-17A also plays a role in conferring resistance against systemic candidiasis, as both IL-17A- and IL-17AR-deficient mice are more susceptible to infection compared with immunocompetent mice, possibly due to reduced neutrophil recruitment to infected tissues^{162,282,283}. However, excessive IL-17 release driven by IL-23 can amplify neutrophil influx to infected organs, which can exacerbate local inflammation and have a deleterious effect^{284,285}. Considering this, although Th17 was implicated to play a protective role in systemic candidiasis^{162,282,283}, the Th1 response seems to have a more prominent role in conferring resistance to infection^{90,282}.

1.2.3.1.4 Regulatory T cell (Treg) response

The Treg phenotype is associated with fungal persistence and immune homeostasis. IL-2 and TGF- β are the main Treg-polarising cytokines, indispensable for induction of Foxp3, the master Treg transcription factor^{286,287}. Tregs secrete IL-10 and TGF- β as effector cytokines. TGF- β will induce the expansion and differentiation of the naïve T cells on site into Tregs, which in turn will suppress Th1 and Th2 responses²⁸⁸. The link between Tregs and Th17 is still

controversial. Treg proliferation was shown to antagonise Th17 proliferation¹⁰³, however reports have shown that Tregs can also compete for IL-2, a negative regulator of Th17 development, resulting in prolonged IL-17A production during infection^{288–290}. The role of Tregs and their impact in candidiasis is not yet fully understood, however they are associated with fungal persistence, which is essentially detrimental for systemic infection, but can be beneficial in some forms of mucocutaneous candidiasis as tissue persistence can enhance local immunity, resulting in a durable and protective host response^{259,288}. Nevertheless, patients that suffer from immune dysregulation enteropathy polyendocrinopathy X-linked (IPEX) syndrome, who have reduced Treg cells as a consequence of *FOXP3* mutations, were reported to be generally more susceptible to *Candida* infections²⁹¹, suggesting that Tregs might be required for protection against candidiasis.

1.2.3.2 Cytotoxic T cells

Much like CD4⁺ helper T cells, CD8⁺ T cells can also differentiate into different cell subsets, namely Tc (cytotoxic T cell subsets), depending on the cytokine concentrations present in the environment. Tc17 cells act mostly at mucosal level, and are primed by TGF- β , IL-6 or IL-23 to secrete IL-17 and AMPs in order to sustain mucosal immunity. Tc2 cells, primed by IL-4, release high levels of IL-4, IL-5 and IL-10 thereby contributing to amelioration of inflammation. Tc1 cells are activated by IL-12 and IFN- γ , and besides secreting IFN- γ and TNF that contribute to inflammation and local phagocyte activation, Tc1 lymphocytes also exert similar antifungal functions as NK cells through the release of granzymes and perforin in response to *Candida*-infected host cells, which accounts for their direct candidacidal activity²⁹². Besides having cytotoxic effect against cells containing internalized *Candida*, CD8⁺ T cells were shown to have direct fungistatic activity against *C. albicans* hyphae, and to play a heightened role when CD4⁺ T cells are depleted, like in *Candida*-infected HIV⁺ positive patients, as their activity compensates for the reduced CD4⁺ T cell numbers to limit the spread of infection^{293,294}.

1.2.4 B cell responses against *Candida* spp.

Unlike T cells, that recognise specific invading pathogens and ultimately deploy mechanisms to disrupt it either directly or indirectly by activating other immune cells, B cells

focus mainly on producing pathogen-specific antibodies that can spread widely throughout the host body, penetrate infected tissues and opsonise pathogens²⁹⁵. Antibody-mediated opsonization of *Candida* spp. will facilitate its phagocytosis by innate immune cells via FcRs and indirectly through classical complement pathway activation²⁹⁶. The impact of B cells in systemic candidiasis is still controversial. Carrow and colleagues have shown that CBA/N mice and B cell-competent mice CBA/J mice are equally susceptible to primary and secondary systemic *C. albicans* challenge²⁹⁷, however Montagnoli *et al.* showed that although B cell-deficient μ MT mice on BALB/c background are more resistant to primary i.v. infection with *C. albicans*, they are more susceptible to a secondary infection compared with WT controls²⁹⁸. In the latter study, authors postulate that the increased susceptibility to re-infection observed in B cell-depleted mice is due to a failure to generate IL-10-producing DCs and to induce Treg proliferation, as opsonised *C. albicans* restored IL-10 production by DCs from μ MT mice *in vitro*²⁹⁸. Despite the conflictive results using animal models, case reports of patients with B cell lymphoma and/or receiving B cell depletion therapy via rituximab administration, a monoclonal antibody that targets the major B cell receptor CD20, evidence that these patients are more susceptible to fungal infections, including invasive candidiasis, suggesting the involvement of B cells in conferring protection towards the infection^{299–301}. Interestingly, besides their role as producers of pathogen-specific antibodies, B cells were recently reported to contribute for *C. albicans* control mainly by promoting MHC-II-mediated fungal antigen presentation to Th cells and to induce proliferation of Th1 and Th17 cells, in an antibody-independent mechanism³⁰².

1.3 Systemic candidiasis

Systemic candidiasis (or candidaemia) defines a disseminated bloodstream infection caused by *Candida* spp. that can have diverse clinical manifestations (Figure 1.6). Candidaemia results from invasive candidiasis i.e. the entrance of *Candida* spp. normally residing in the gut or mucocutaneous microbiota as a commensal yeast into the body. This can happen through the breach of epithelial barriers by the use of catheters or invasive medical devices (Figure 1.6-A), gastrointestinal mucosa inflammation, or gut surgery (Figure 1.6-B) or through impairment of the immune response at the mucosal level that leads to fungal proliferation and inefficient clearance^{26,303,304} (Figure 1.6-C). As a consequence of *Candida* spp. leakage from the gut, localized deep-seated infections such as peritonitis can also occur, without previous bloodstream colonisation^{26,304} (Figure 1.6-B). During candidaemia, *Candida* spp. can also enter the urine following kidney and/or bladder infection, and although less frequently, localized bladder and kidney invasion can also occur through genital infection and lead to candidaemia³⁰⁴ (Figure 1.6-D). As a result of the systemic diffusion of *Candida* spp., multiple organs can be affected leading to several types of deep-seated infections (Figure 1.6, grey boxes), acute disseminated candidiasis or, especially in neutropenic patients, to chronic disseminated candidiasis, where the host is unable to fully clear the pathogen. The ineffective *Candida* spp. clearance frequently culminates in a severe immune dysregulation at affected organs ultimately leading to mortality either by organ failure (severe sepsis) or cardiac decompensation (septic shock)³⁰⁵.

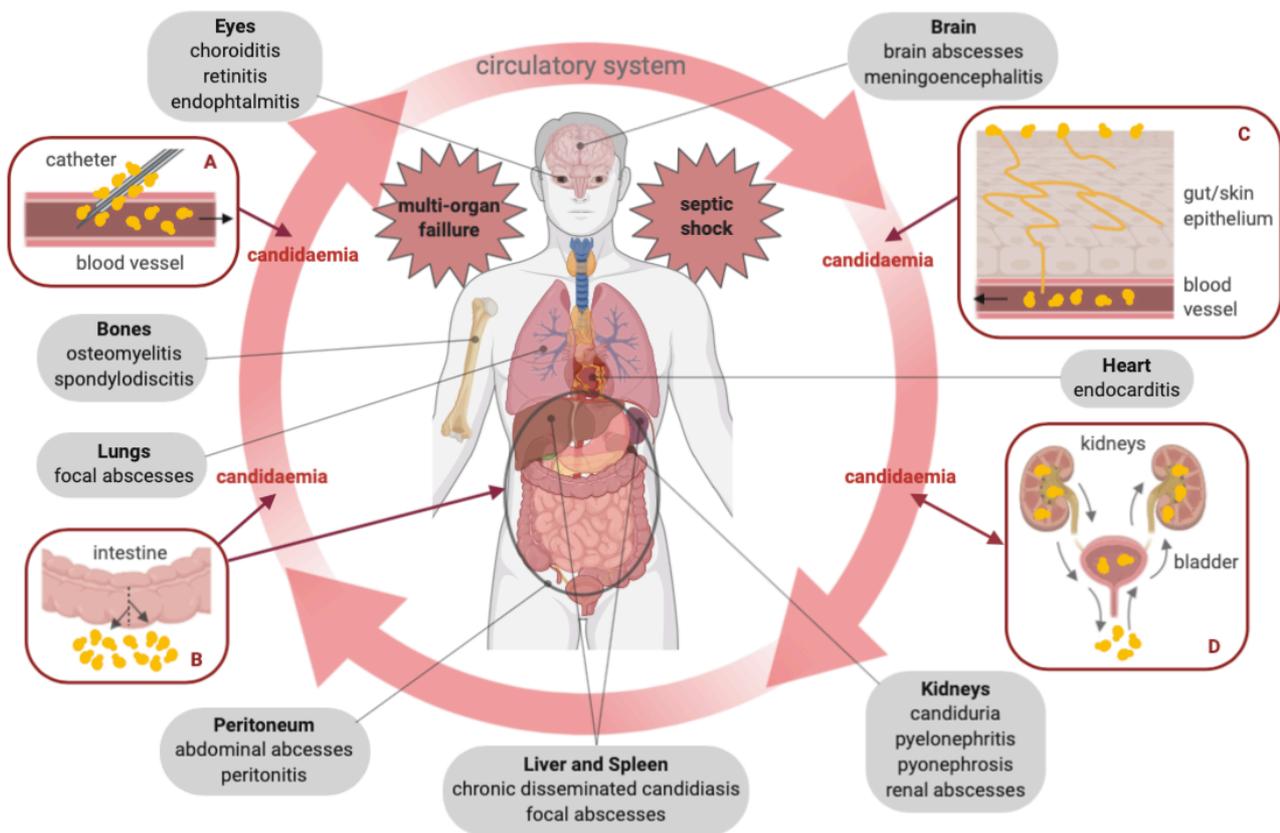


Figure 1.6 – Schematic of pathogenesis and clinical manifestations of systemic candidiasis. Adapted from^{26,304}. Figure was made using Biorender.com

1.3.1 Host immune response during systemic candidiasis

Most systemic candidiasis infections happen due to impaired immune restriction of *Candida* spp. in mucosal interfaces (especially in the gut) where these fungi usually exist as commensal organisms. In this setting, epithelial cells such as keratinocytes and fibroblasts are frequently the first line of defence, and disseminated candidiasis usually occurs when mucosal surfaces and epithelial barriers are breached²⁷⁹ (Figure 1.6). Resident mononuclear phagocytes are important in the early clearance of invading *Candida* spp. in the mucosa and when these are insufficient to control local infection, neutrophils are recruited to the site. Neutrophils are the most potent cells at killing *Candida* spp. and were shown to be indispensable for its clearance in damaged gastrointestinal tract and during mucosal infections^{306,307}. Once neutrophils mobilise to the infected site, they will be activated by granulopoietic cytokines produced by epithelial cells and APCs to secrete pro-inflammatory cytokines that will stimulate the secretion of TGF- β , IL-1 and IL-6 by local immune cells thereby polarising the local T cell response towards a protective Th17 phenotype^{306,307}.

When mucosal immunity fails to clear invading *Candida* spp., the pathogen can access the endothelium and disseminate throughout the circulatory system. Once in the bloodstream, circulating myeloid cells such as monocytes and neutrophils can still recognise *Candida* and deploy mechanisms to eliminate it, however these are frequently insufficient and unable to limit infection, especially in immunocompromised patients resulting in pathogen accumulation in the main target organs, followed by systemic infection (Figure 1.6). In humans, the lungs are most common target for metastatic infection, followed by the gastrointestinal tract, kidneys, liver, and spleen³⁰³. In mice, *Candida* generally has high tropism to the kidney, brain, spleen and liver, however it is in the kidney where the infection is most persistent²⁰. Once infection settles in these organs, especially in the kidneys, an appropriate leukocyte infiltration and local Th1 polarisation is essential for effective clearance of infection^{266,267}.

The leukocyte recruitment dynamics during systemic candidiasis vary depending on the affected organ. Monocyte and macrophage infiltration in the kidney, mostly mediated by CCR2 and CX3CR1 signalling respectively, is vital for deploying an early and effective innate immune response against the invading *Candida*^{305,308,309}. However, neutrophils are the main leukocytes recruited to *Candida*-infected tissues, with the exception of the brain, where microglia are the main innate immune cells controlling the infection^{20,310–312}. Indeed, neutrophils are essential for host defence during systemic candidiasis^{310,313}, and an orchestration between splenic monocytes, NK cells and renal neutrophils involving type I IFN, IL-15 and GM-CSF greatly contributes to fungal clearance by boosting neutrophil candidacidal activity³¹⁴ (Figure 1.7-A). Nevertheless, neutrophil action and infiltration in affected organs, especially late in the course of infection, is seen as a “double-edged sword” as the exacerbated release of secreted factors and pro-inflammatory cytokines by these cells can generate immunopathology, contribute to organ damage, lead to septic shock and organ dysfunction³¹¹. In line with this, corticosteroid administration is frequently necessary to avoid deleterious hyper-inflammation in neutropenic patients after neutrophil reconstitution^{305,315}.

Neutrophil mobilisation into the kidney is sluggish during the early stages of infection, and although delayed recruitment often results in inefficient immune response and fungal invasion into the renal parenchyma, neutrophil ablation or recruitment impairment through

inhibition of CCR1 in late stages of systemic candidiasis was shown to improve mice survival^{311,316–318}. Moreover, CXCR1 was shown to be dispensable for neutrophil infiltration in the kidneys but contributes for *Candida* clearance by promoting neutrophil degranulation and boosting candidacidal activity³¹⁹. Without affecting neutrophil infiltration and overall fungal burden, IL-17 (produced mainly by kidney epithelial cells) can also be detrimental due to induction of high levels of IL-1, TNF and IL-6 in neutrophils and other leukocytes that exacerbate the immune response and ultimately lead to damaging hyperinflammation^{285,317,318,320}. Interestingly, candidalysin can also promote detrimental neutrophil infiltration in the kidney, especially during early stages of infection. This leads to renal damage and immunopathology, which can contribute to the higher mortality observed in *C. albicans*- and *C. tropicalis*-induced systemic candidiasis compared with other *Candida* species⁸⁸.

Indeed, in agreement with epidemiology data, *in vivo* murine studies revealed that during systemic candidiasis, *C. albicans* is much more virulent than *C. glabrata* and *C. parapsilosis*, with *C. parapsilosis* being the species with the lowest virulence^{321–323}. This interspecies difference in virulence is not only due to the putative virulence factors characteristic of each *Candida* spp. but also due to their cell wall composition and architecture. The abundance and exposure of different cell wall PAMPs greatly influences *Candida* recognition by immune cells and the consequently released cytokines that will ultimately shape the immune response during infection and affect the outcome of disease.

Overall, and regardless of the *Candida* spp. driving the pathology, an appropriate balance and fine-tuning of the immune response is generally required for clearance of systemic candidiasis (Figure 17-B). However, the disease progression is complex, and varies greatly depending on the patient's condition, which makes it challenging to study using animal models and to devise a global treatment that will fit all infected patients. Therefore, a timely diagnosis and a proper understanding of the immunological state of infected patients during the onset and progression of systemic candidiasis is crucial for the deployment of proper and effective treatments.

1.3.2 Risk factors for systemic candidiasis

Treatments or conditions that impair host innate immunity and promote or facilitate *Candida* spp. tissue invasion or its direct dissemination to the bloodstream can stand as risk factors for systemic candidiasis. These include: the repeated or long-term use of broad-spectrum antibiotics, which results in increased proliferation of *Candida* spp. in the gut; disruption of gastrointestinal or skin barriers, either by invasive medical devices such as catheters or surgery that create an opening for *Candida* spp. to enter the bloodstream from mucocutaneous sites; medical immunosuppression such as chemotherapy, corticosteroid therapy or other treatments that can impair innate immune responses, hinder *Candida* spp. clearance in tissues and facilitate its invasion into internal organs. Immunocompromised patients such as diabetics, HIV⁺ patients and leukaemia patients are also at higher risk of contracting the disease, as well as the immunosenescent, i.e. the elderly population with age-related immune system impairments such as neutropenia^{26,27,296,303,316,324}.

Some primary immunodeficiency diseases can predispose patients to invasive candidiasis. Patients with chronic granulomatous disease (CGD), manifested either by an X-linked *CYBB* mutation or *CYBA*, *NCF1*, *NCF2* or *NCF4* autosomal recessive mutations are susceptible to invasive candidiasis due to impaired ROS generation mechanisms^{325,326}. Leukocyte adhesion deficiency type I, caused by autosomal recessive mutation of *CD18* gene (*ITGB2*) that codes for integrin beta-chain 2, is also associated with increased incidence of invasive candidiasis due to impaired neutrophil migration through endothelia and consequent reduction of recruitment to infection sites³²⁶. Patients with severe congenital neutropenia caused either by autosomal-dominant mutations in *ELA2* (neutrophil elastase-coding gene) or autosomal recessive mutations in *HAX1* (HCLS1-associated protein X-1, essential for maintaining mitochondria integrity and conferring protection against myeloid cell apoptosis) are also at higher risk of invasive candidiasis^{326,327}.

Genetic mutations in genes involved in PRR-signalling pathways can also predispose patients to infection or contribute to susceptibility. Individuals with genetic mutations on the CLR adaptor protein *CARD9* and the TLR-coding genes *TLR1*, *TLR2* and *TLR4* were identified at being at higher risk for contracting invasive candidiasis due to impaired *Candida* PAMPs-

triggered CLR and TLR signalling^{129,130,140–142,328–331}. Dectin-1 polymorphisms might also increase the probability of developing systemic candidiasis due to impaired IL-1 β and Th17 responses, that can culminate in increased *Candida* colonization and persistence in tissues¹⁵⁰.

Genetic variations in cytokine/chemokine-coding genes have also been identified as risk factors for disseminated candidiasis. A polymorphism in *IL4* predisposes patients to chronic disseminated candidiasis and although the phenotype that leads to susceptibility has yet to be elucidated, it was shown that *IL4* polymorphisms are also associated with susceptibility to recurrent vulvovaginal candidiasis due to reduced NO and MBL production^{328,332,333}. Individuals with SNPs for *IL10* and *IL12B*, although not directly associated with increased mortality, have impaired ability to clear candidaemia possibly due to low production of IL-10 and IFN- γ , and impaired ability to modulate Th1 and Th2 responses to fight infection³³⁴. A study revealed that patients in surgical ICU with a SNP in *TNF* are more susceptible to intra-abdominal candidiasis and possible posterior *Candida* bloodstream dissemination³³⁵. Mutations in chemokine receptors *CXCR1* and *CX3CR1* were also identified as risk factors for invasive candidiasis due to impaired neutrophil degranulation and mononuclear phagocyte recruitment respectively^{309,319}.

Smeeckens and colleagues found that SNPs in genes involved in type I IFN pathway are significantly associated with susceptibility to systemic candidiasis^{186,187}. This includes *CCL8* (MCP-2, a monocyte chemoattractant), *STAT1* (a major transcription factor involved in the expression of type I IFN-inducible genes), *SP110* (a transcriptional coactivator that is involved in the regulation of some transcription factors associated with innate immunity, such as NF- κ B³³⁶), *PSMB8* (a subunit of the immunoproteasome, which is involved in the generation of peptide antigens that will be presented by APCs to CD8⁺ T cells via MHC I³³⁷) and *IFITH* (MDA5)^{186,187}.

A genome-wide association study revealed three novel gene polymorphisms predicted to be associated with increased risk for development of candidaemia. These include *CD58* (lymphocyte function-associated antigen 3), an accessory cell adhesion molecule present on the surface of APCs known to bind to CD2 receptor on T cells and mediate their regulation and effector function^{338,339}, and that plays a yet unexplored role in *Candida* phagocytosis and

control of *C. albicans* hyphal growth³⁴⁰; *TAGAP* (T-cell activation RhoGTPase-activating protein) that was shown to be essential for effective clearance of *C. albicans* in target organs of systemic candidiasis in late stages of infection³⁴⁰; and the *LCE4A-Clorf68* locus, a gene cluster that comprises several elements involved in the maintenance of epithelial barrier function and integrity^{340,341}.

1.3.3 Current therapeutics for systemic candidiasis

The majority of current therapies prescribed for treatment of systemic candidiasis consist of the administration of antifungal drugs. However, current drugs have limited efficacy, are expensive, require long-term treatment regimes and can have undesirable interactions with other drugs, which poses a problem for some immunocompromised patients that are often polymedicated^{10,342,343}. Probably the biggest caveat for some current pharmacological therapies, especially with polyenes, are their toxicity and chance of side effects, which happens mostly due to the fact that, like the human host, *Candida* is a eukaryote, so most proteins that are targeted by antifungal drugs have a high degree of homology to those present in humans. The undesirable targeting of these human homologs by these antifungals can culminate in severe hepatotoxic and nephrotoxic side effects, necessitating controlled administration of these drugs as treatment often needs to be ceased due to toxicity-related side effects²⁶.

Current common antifungal treatments for invasive candidiasis consists mainly of three different drug classes (azoles, echinocandins and polyenes) that vary on their site of action. The prescription of different antifungal drugs varies depending on the severity of disease, the patient state and the *Candida* spp. detected. However, some *Candida* spp. are naturally resistant to these antifungals (e.g. *C. auris*, *C. krusei* and *C. glabrata* are generally resistant or less susceptible to azoles^{344–349}; *C. glabrata* is usually resistant to echinocandins^{350–352}; and *Candida lusitanae* is often resistant to polyenes^{26,353}), and/or can deploy mechanisms to resist or ameliorate antifungal action (e.g. *C. glabrata* can grow by taking up exogenous like mammalian cholesterol to evade azole treatment³⁵⁴; some pathogenic *Candida* spp. can upregulate chitin synthase expression to strengthen the fungal cell wall and reduce echinocandin susceptibility^{352,355,356}; *C. lusitanae* can trigger loss-of-function mutations in

erg3 resulting in reduced targetable ergosterol levels in the membrane in order to resist polyene action^{357–359}.

Furthermore, a timely and efficient diagnostic determination is necessary for effective treatment of systemic candidiasis, however diagnosis is challenging as there are no specific symptoms characteristic of invasive candidiasis when comparing with other systemic infections²⁶. The delay on antifungal treatment due to poor diagnosis often correlates with higher patient mortality, so patients at higher risk of systemic candidiasis are often subject to prophylactic treatment^{26,360,361}. However, no efficient treatments have been convincingly reported to date, as none improved patient survival^{26,362–364}. Moreover, drug prophylaxis may also promote antifungal resistance, potentially limiting future treatment options.

1.3.3.1 Immunotherapies

Immunotherapy conceptually consists on exploiting the host immune system by bolstering immunity towards the fungal pathogen, thereby facilitating its clearance by the infected host. This might improve the outcome of disease especially in immunocompromised patients. Immunotherapies consist of either: administration of vaccines made using *Candida*-derived antigens that can manipulate and generate host immunity towards infection; targeted activation of both innate and adaptive immune response elements towards *Candida* like phagocytes, T cells and antibodies; development or administration of auxiliary immune response components such as cytokines, synthetic antibodies or other biological components that can target *Candida* directly, negatively affecting its viability, or *Candida*-derived secreted pathogenic molecules^{365,366}. Immunotherapies can either replace current pharmacological treatments or be used in a synergistic combination with current antifungals in order to boost their efficacy.

1.3.3.1.1 Antibody therapy

Monoclonal antibodies (mAbs) are a promising therapeutic approach to treat invasive candidiasis due to their high antigen specificity and reproducibility. Since mAbs are highly specific to a target antigen, administration offers advantages over classic antifungal treatments as, theoretically, mAbs should not trigger any toxic side effects observed with some current antifungal drugs.

Mycograb and C28Y (a modified variant of Mycograb) are monoclonal antibodies (mAbs) that target fungal Hsp90, a fungal chaperone that facilitates folding and function of several proteins involved in *C. albicans* virulence, including drug resistance mechanisms³⁶⁷. Clinical trials have shown that Mycograb and C28Y have strong antifungal activity and exert a synergistic effect when administered in combination with amphotericin B in patients with invasive candidiasis, resulting in a more efficient *Candida* elimination, with no additional severe side effects detected^{368,369}.

MAb C7 is an antibody that binds to Als3 and enolase, a component of *Candida* spp. cell walls, and shows direct candidacidal activity as well as an ability to inhibit *C. albicans* adhesion and germination^{370,371}. In murine models of systemic candidiasis, C7 greatly improved mouse survival and contributed to significantly increased clearance of *C. albicans* in the brain and kidneys of infected mice³⁷². C7 stands as a promising mAb for anti-invasive candidiasis therapy, however a full assessment of its effect in human clinical trials is still needed to validate its effectiveness as a therapeutic.

1.3.1.2 Granulocyte therapy

Since neutropenia is one of the main risk factors for the development of systemic candidiasis, adoptive transfer of neutrophils from healthy donors to infected neutropenic patients stands as a possible treatment approach. Anecdotal clinical studies have revealed that granulocyte transfusion in cancer patients with systemic candidiasis is generally well tolerated and restores neutrophil levels and neutrophilic function, but it did not convincingly improve resolution of infection in those patients^{373,374}. A retrospective study showed that granulocyte transfusion resulted in reduced survival rate of patients with candidaemia compared with infected patients who did not receive transfusions³⁷⁵. Nevertheless, the same study has shown patients with leukaemia had better candidaemia-associated short-term survival when subject to granulocyte transfusion therapy, suggesting that granulocyte transfusion might be beneficial when administered in high-risk cancer patients with invasive candidiasis³⁷⁵. Overall, granulocyte transfusion has several limitations in the treatment of systemic candidiasis. These include the difficulty of *ex vivo* granulocyte preservation and maintenance of their original candidacidal functions after transfusion, the inconclusive

evidence of their efficacy as a therapy for systemic candidiasis, the risk of transfusion-related diseases like CMV infection, and the emergence of potential life-threatening side effects associated with the therapy^{373,375,376}.

1.3.1.3 T cell-based therapies

As most patients suffering from systemic candidiasis are highly immunocompromised and unable to mount an efficient adaptive response as the infection progresses. Adoptive transfer of either autologous naïve T cells or T cells from healthy compatible donors expanded by *ex vivo* stimulation with a specific fungal antigen could be used as a treatment to promote protective *Candida*-specific T-cell responses in infected patients. Stem cell transplantation recipients are extremely susceptible to systemic candidiasis due to the deleterious long-lasting immunosuppression after transplantation associated with an imbalance in Th1 and Th2 responses, where low levels of IFN- γ and high levels of Th2 effector cytokines are thought to be the main mediators of sustained disease³⁷⁷. In this setting, preclinical studies suggest that adoptive transfer of Th1 cells confer significant protection against invasive fungal infection^{378,379}. However, to my knowledge, no human clinical studies were performed yet to confirm its potential.

Another strategy to promote T cell-derived immunity during systemic candidiasis is via the use of DCs. Studies using animal models have shown that transplantation of DCs pulsed with either *C. albicans* yeasts or transfected with yeast-derived RNA can confer host resistance through surface expression of *Candida*-specific antigens and MHC II, as well as through robust secretion of IL-12, contributing for generation of protective Th1 responses^{380–382}.

Adoptive transfusion of cytotoxic T cells into immunocompromised patients can also be considered for treatment of systemic candidiasis, especially in HIV⁺ patients whose CD4⁺ T cells counts are low, and where CD8⁺ T play a heightened role in host protection^{293,294,383}. Moreover, it was reported that CD4⁺ T cells are dispensable for vaccination against other pathogenic fungi also capable of inducing systemic infection in humans, as CD8⁺ T cells can confer resistance via an MHC I-dependent mechanism³⁸⁴, and it is possible that a similar effect

could be verified during systemic candidiasis. Another interesting mechanism to boost cytotoxic T cell functions during systemic candidiasis is through the use of Dectin-1-expressing CAR (chimeric antigen receptor) T cells (D-CAR). In a model of invasive aspergillosis, adoptive transfer of D-CAR T cells in immunocompromised mice resulted in improved clearance of infection due to their high specificity to fungal β -glucan, robust release of IFN- γ and high cytotoxic activity³⁸⁵. Since β -glucan is a universal cell wall component of pathogenic fungi, it is likely that adoptive transfer of D-CAR T cells could also provide protection against systemic candidiasis.

Although some T cell-based adoptive transfer therapies look promising for treatment of systemic candidiasis, they are time-consuming and expensive, and it is highly unlikely that these therapies are going to be widely deployed in most clinical settings anytime soon.

1.3.1.4 Cytokine therapies

Much like monoclonal antibodies, cytokine therapies focus on bolstering the host immune response against *Candida* and most should not display the undesirable side effects observed with some antifungal drugs. Several major pro-inflammatory cytokines such as IL-1, IL-6 and TNF were implicated in conferring host resistance against systemic candidiasis in murine models^{101,386–389}, however their high pleiotropism and broad spectrum of action may induce severe inflammation-related immunopathology and trigger deleterious sepsis-like symptoms in the host, which is why most of them were not considered as therapies.

1.3.1.4.1 G-CSF

As neutropenia is a major risk determinant for the incidence of invasive candidiasis, G-CSF, a granulopoietic cytokine that stimulates proliferation and differentiation of neutrophils whilst promoting their candidacidal activity was considered for its treatment^{390,391}. Murine *in vivo* studies have revealed G-CSF therapeutic potential as it significantly contributes to host survival and effective clearance of *Candida* during systemic infection in both neutropenic and non-neutropenic mice^{392–398}. A meta-analysis study suggests that promoting G-CSF-driven neutrophilia is not a viable prophylaxis for preventing or enhancing the outcome of fungal infections³⁹⁹, however, anecdotal studies indicate that G-CSF has a beneficial effect in the

outcome of infection when used in combination with other antifungals such as amphotericin B and some azoles^{393,394,400}. Combinational antifungal therapy with G-CSF might be a viable therapy for systemic candidiasis, however more clinical studies and trials are required to confirm this hypothesis.

1.3.1.4.2 GM-CSF

Due to its ability to induce myeloid cell haematopoiesis, enhance *Candida* phagocytosis by granulocytes and to prolong neutrophil survival, GM-CSF also stands out as an interesting cytokine for therapy of invasive candidiasis^{314,398,401,402}. Administration of GM-CSF has a protective effect in both neutropenic and immunocompetent mice in *in vivo*-induced systemic candidiasis^{403,404}, and clinical studies have provided evidence for the therapeutic potential of GM-CSF in neutropenic patients^{405,406}. Moreover, like G-CSF, GM-CSF also appears to synergise with amphotericin B and azoles in the treatment of disseminated candidiasis^{407–409}. A phase IV clinical trial revealed that prophylactic treatment with GM-CSF in patients going through hematopoietic stem cell transplantation results in significantly lower invasive fungal disease-related mortality, evidencing that GM-CSF can be used to counteract the effects of neutropenia in these patients and reduce infection-related mortality rates in this setting⁴¹⁰.

1.3.1.4.3 IFN- γ

Multiple studies have shown the importance of IFN- γ in conferring resistance against systemic candidiasis, providing a solid conceptual basis for its immunotherapeutic potential^{262,265–267,411}. IFN- γ can be used as prophylactic treatment in patients with CGD as it was shown to partially contribute to ROS production in these patients, resulting in reduced incidence of severe infections^{412,413}. Additional anecdotal studies demonstrate the beneficial effects of IFN- γ therapy in disseminated candidiasis detected in patients with acute leukaemia^{414,415}. The resistance mechanisms conferred by IFN- γ administration in patients with invasive candidiasis were then revealed in a later study, that showed that IFN- γ partially restored cell-mediated immune function in those patients via induction of IL-1 β , TNF, IL-17 and IL-22⁴¹⁶. The collective results from these small studies highlight the potential of IFN- γ to be used as adjuvant antifungal therapy, however clinical trials are required to ascertain its value as treatment for systemic candidiasis.

1.3.1.5 Anti-*Candida* vaccine

An efficient, licensed vaccine for treatment of systemic candidiasis has yet to be developed, as any vaccine faces three main challenges. The first is bound to *Candida*'s phenotypic and genomic plasticity. Several hyphal-specific molecular targets have been proposed for vaccination, since hyphal morphology is deeply related to *Candida* pathogenesis^{417–420}. Nevertheless, vaccines that target hyphal-specific cell wall components would not be sufficient for effective protection, as yeast forms are also important for widespread blood and organ dissemination, while some species like *C. glabrata* do not rely on filamentation to cause disease^{16,20,22,420}. Another important factor is that apart from its morphological plasticity, some *Candida* spp. deploy genome plasticity mechanisms that allow it to adapt to different stress environments by downregulating the expression of key target proteins, which stands as an important mechanism of antifungal resistance as mentioned above. This same strategy could be deployed by *Candida* to limit the levels of cell wall proteins targeted by a vaccine, thereby reducing its efficacy.

The second main challenge is bound to the fact that *Candida* is a commensal, and therefore the human host has likely already developed evolutionary immune tolerance towards it, and it is when this fine-tuning between immunity and tolerance is disrupted that most *Candida*-associated pathologies arise^{259,288,420,421}. This evolutionary host tolerance that facilitates *Candida* commensalism likely hinders the development of efficient and protective immunological memory to pathogenic *Candida* spp. and ultimately contributes for the clinical settings of systemic candidiasis, especially as the detrimental disease outcomes are usually due to host-derived immunopathology rather than pathogen-driven host damage *per se*. Therefore, general targeting of this balance by vaccination or other therapies might have a deleterious effect rather than protective, i.e. contributing for disease progression or immunopathology.

The third and possibly the biggest challenge for vaccination is related to the weakened immune system of the heterogenous immunocompromised patients at higher risk for invasive candidiasis. Besides being less responsive to immunization, therapy for these patients should focus on understanding the immunological impairments of each patient that are bolstering

the severity of disease, and tailor any treatments to counteract these deficiencies⁴²⁰. This is the main reason it is highly unlikely that a “universal” protective vaccine for systemic candidiasis will ever be produced, e.g. a vaccine that is effective for infected patients with leukaemia-derived neutropenia might not be as effective for HIV⁺ patients with severe CD4⁺ T cell depletion.

Overall, novel treatments for systemic candidiasis are urgently required to reduce the unacceptably high mortality rates. Unlike antifungals, immunotherapy is a promising approach. Nevertheless, efficient immunotherapies are scarce and not very effective. Their lack of efficacy stems not only from the heterogenous comorbidities and immunological states of different patients suffering from systemic candidiasis, that greatly influence their responsiveness to current treatments, but also due to a poor understanding of the complex host immune response towards infection. Comparing how host immunity responds to infection with different *Candida* spp. with distinct virulence may permit the identification of novel immune mechanisms underlying both resistance and susceptibility to systemic candidiasis and facilitate the discovery of new targets for the design of more efficient immunotherapies.

1.4 Hypothesis

The main hypothesis of this PhD project was that the modulation of the host immune response triggered by the highly virulent species *C. albicans* to mirror the one induced by the low virulence species *C. parapsilosis* would contribute to a better clearance of infection. The results obtained in this project and displayed in this thesis could ultimately be used to help devising novel immunotherapies for treatment of life-threatening systemic candidiasis.

1.5 Aims of this thesis

- i) To identify and validate differences in the immune responses triggered by *C. albicans* and by *C. parapsilosis*;
- ii) To explore the impact of immune modulation using the validated targets during *in vitro* and *in vivo* *C. albicans* infections;
- iii) If proven to be beneficial, elucidate the molecular mechanisms underlying resistance or the enhanced candidacidal effect of the validated targets.

Chapter 2

Materials and Methods

2.1 Reagents

Tables detailing the different types of media and compounds used for cell stimulation in this study are included in the Appendix section (Chapter 7, Figures 7.1 and 7.2).

2.2 Fungal strains and culture conditions

A table including details on all wild-type *Candida* spp. and mutant *C. albicans* strains used in this thesis is included in the Appendix section (Chapter 7, Figures 7.3 and 7.4). *Candida* spp. were propagated and maintained in petri dishes containing YPD Agar medium (YPDA) (10 g/L yeast extract, 20 g/mL glucose, 20 g/L bacteriological peptone, 15 g/L agar) for 16 h at 30°C. After incubation, plates were kept at 4°C for up to 2 weeks. For experimental assays, *Candida* spp. were streaked from petri dishes, inoculated in 14 mL polypropylene tubes with vented caps containing 5 mL of YPD broth medium (YPD medium with no agar - YPDB) and grown for 16 h at 30°C in a shaking incubator at 250 rpm. *Candida* cultures were then transferred to a 50 mL conical centrifuge tube, centrifuged at 350 g for 5 min and pellets were washed 3 times with 20 mL PBS by centrifugation. *Candida* spp. density was then assessed using a haemocytometer, pellets were centrifuged and adjusted to 1×10^8 CFUs/mL in PBS. The suspension was then diluted 1:50 in RPMI-BMDM to 2×10^6 CFUs/mL, the yeast concentration was checked again using a haemocytometer and adjusted until correct. The *Candida* spp. suspension in RPMI-BMDM was then used for *in vitro* infection assays. (For *ex vivo* splenocyte stimulations, *Candida* spp. were resuspended in a different medium to a higher density. Further details are outlined in section 2.5.2.1.3).

2.3 Generation of host cells for *in vitro* experiments

Bone marrow-derived macrophages (BMDMs) from C57BL/6J mice were used for *in vitro* infection assays. BMDMs were obtained by seeding harvested mice bone marrow in an optimal medium for differentiation in the presence of M-CSF (Macrophage-Colony Stimulating Factor). M-CSF will stimulate HSCs (hematopoietic stem cells) in the bone marrow to proliferate into CMPs (common myeloid progenitor cells), that will in turn become myeloblasts, then monocytes and ultimately macrophages⁴²². For some experiments, BMDMs differentiated from bone marrow of mutant knock-out mice was used. For each experiment where mutant BMDMs were used, conditions with wild-type BMDMs differentiated from

bone marrow of age- and gender-matched C57BL/6J mice were included. A table with details on all knock-out bone marrow used in this thesis is included in the Appendix section (Chapter 7, Figure 7.5).

2.3.1 Bone marrow harvest

Bone marrow was harvested from femurs and tibias of both legs from WT or mutant mice by aseptically flushing the bones with sterile ice-cold PBS into a 50 mL conical centrifuge tube using a 10 mL syringe and a 25 G needle. Bone marrow was centrifuged at 350 g for 5 min at 4°C. After centrifugation, pellets were resuspended in 10 mL ice-cold PBS, the suspension was strained through a 100 µm cell strainer to remove muscle tissue and bone fragments and centrifuged again. Following centrifugation, supernatants were discarded and bone marrow was either processed and cultured straight away for macrophage differentiation or frozen for later use. To freeze bone marrow: the supernatant was discarded and the pellet containing bone marrow cells was resuspended in 1 mL of freezing medium (10% (v/v) DMSO in heat inactivated fetal bovine serum (FBS), sterile filtered). The suspension was transferred to cryovials, frozen overnight at -80°C inside a Mr. Frosty freezing container (Nalgene) and later transferred to liquid nitrogen tanks. Once needed, cryovials were quickly thawed in a water bath at 37°C, and used for bone marrow differentiation protocol (2.3.2.).

2.3.2 Bone marrow-derived macrophages (BMDMs) differentiation

To obtain BMDMs, bone marrow was either cultured immediately after being harvested as described in 2.3.1. or frozen bone marrow vials were thawed and cultured. For freshly harvested bone marrow, after the last washing step by centrifugation, supernatants were discarded and resuspended in ice-cold Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 5% (v/v) heat-inactivated horse serum (HS), 1% (v/v) PenStrep (100 U/mL penicillin, 100 µg/mL streptomycin), 2 µM L-glutamine and 10 mM HEPES (DMEM-BMDM). For frozen bone marrow, once thawed, cryovial contents (~1 mL) were diluted 1:10 in 9 mL of cold DMEM-BMDM and quickly washed by centrifugation at 350 g for 5 min at 4°C to dilute the cytotoxic DMSO in the freezing medium and minimize cell viability loss. After centrifugation, pellets were resuspended in fresh DMEM-BMDM. Viable bone marrow cell concentration was

assessed using a MUSE Cell Analyser (Merck). Cells were centrifuged again, pellets were resuspended at 7×10^6 viable cells/mL and 1 mL of the suspension was seeded in 145 mm² anti-pyrogenic-treated cell culture dishes containing DMEM-BMDM supplemented with 10 ng/mL recombinant murine M-CSF (PeproTech) in a final volume of 35 mL (2×10^5 bone marrow cells/mL). Cells were cultured for 3 days at 37°C in a humidified atmosphere with 5% CO₂. After 3 days in culture, 15 mL of fresh DMEM-BMDM with 10 ng/mL M-CSF was added to the plates. After 6 to 7 days of incubation, media was discarded, and dishes were washed with 20 mL of PBS at room temperature to remove non-adherent cells. Adherent differentiated BMDMs were detached from the bottom of the plate by incubation with 7 mL of 8 mg/L lidocaine (Sigma) in PBS for 4 min at 37°C inside the cell incubator. After the incubation period, plates were gently tapped to facilitate cell detaching and 7 mL of RPMI 1640 (Gibco) supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) PenStrep (100 U/mL penicillin, 100 µg/mL streptomycin) (RPMI-BMDM) was added to each plate to dilute the lidocaine and avoid loss of cell viability due to lidocaine cytotoxicity. The dishes were washed extensively with the cell suspension, contents were transferred to 50 mL conical centrifuge tubes and centrifuged at 350 g for 5 min at room temperature. Pellets were then resuspended in RPMI-BMDM and viable BMDM concentration was assessed using a MUSE Cell Analyser. BMDMs were centrifuged again and the pellet was resuspended in RPMI-BMDM to 2×10^6 viable BMDMs/mL for use *in vitro* assays.

2.4 *In vitro* infection assays

For all macrophage-*Candida* co-stimulation assays, BMDMs were seeded in either multiwell plates or anti-pyrogenic-treated culture dishes and incubated for 16 h in a cell incubator at 37°C in a humidified atmosphere with 5% CO₂ to ensure adherence to the wells/dishes before experimental assays. For experiments performed in flat 96-well plates, 100 µL of BMDM suspension in RPMI-BMDM were seeded per well, 600 µL for 24-well plates, 2 mL for 6 cm² cell culture dishes or 2.5 mL in 6-well plates. For stimulations, *Candida* spp. were prepared and resuspended at 2×10^6 viable CFUs/mL in RPMI-BMDM as described in section 2.2. Before stimulation, media was discarded, and *Candida* spp. was added to the cells in the same volume of media the cells were seeded in. This was done so that both cells and yeasts could be co-cultured at a multiplicity of infection of 1 *Candida* spp. colony-forming unit

(CFU) to 1 macrophage host cell (MOI 1:1). Co-cultures were incubated for varying times depending on the stimulation period length. For most infection assays using *Candida* spp., 2.5 µg/mL amphotericin B diluted in RPMI-BMDM was added to BMDM-*Candida* spp. cocultures after 2 h of stimulation. This was performed to neutralise (kill) *Candida* spp. while maintaining the integrity of fungal cells. Addition of the drug allowed macrophages to respond to pathogen stimulation for long periods of time, whilst minimizing cell viability loss, otherwise some *Candida* spp. (especially *C. albicans*) would start lysing BMDMs after ~3 h of coincubation.

2.4.1 RNA sequencing (RNAseq) and downstream analyses

To identify gene expression profiles induced by different *Candida* spp., an RNAseq experiment was performed. Samples used for RNAseq were prepared by Dr. Selinda Orr and Dr. Aiysha Thompson (Institute of Infection and Immunity, Cardiff University, UK). RNA sequencing was performed by Oxford Genomics Centre Samples, prior to the start of the candidate's doctoral studies.

2.4.1.1 RNA harvest

BMDMs were seeded in 6 cm² cell culture dishes and infected with *Candida* spp. at a MOI of 1:1 as described in section 2.2. 2.5 µg/mL amphotericin B was added to the plates after 2 h of stimulation. After each stimulation time point, media was removed, 1 mL of TRIzol (Life Technologies) was added to each plate and BMDMs were scraped using a disposable cell scraper. Cells were then collected and transferred to individual 1.5 mL centrifuge tubes, snap frozen in liquid nitrogen and stored at -80°C until further processing.

2.4.1.2 RNA purification and isolation

BMDMs were stimulated and RNA was harvested and frozen as described in section 2.4.1.1. RNA was purified using TRIzol and a RNeasy Mini Kit (Qiagen). Frozen vials containing RNA harvested in TRIzol were thawed at room temperature, 0.2 mL of chloroform was added to each vial, samples were vigorously shaken for 15 s and incubated for 3 min at room temperature. Vials were centrifuged at 12,000 g for 15 min at 4°C, the aqueous phase was transferred to new 1.5 mL microcentrifuge tubes and mixed with equal volumes of 70% (v/v)

ethanol in DEPC-treated water. Samples were then transferred into RNeasy spin columns and centrifuged for 15 s at 10,000 rpm (8,000 g), flow-through was discarded, and columns were washed with 350 μ L of RW1 buffer. Following another 15 s centrifugation at 10,000 rpm (8,000 g), sample RNA in the column was incubated with 80 μ L RNase-free DNase for 15 min at room temperature to remove DNA contaminants. Columns were washed again with 350 μ L of RW1 buffer and centrifuged for another 15 s centrifugation at 10,000 rpm (8,000 g). Flow-through was discarded and 0.5 mL of RPE buffer was added to the columns before another centrifugation at 10,000 rpm (8,000 g) for 2 min. Spin columns were placed in clean tubes and centrifuged at full speed for 1 min to eliminate any possible carryover of RPE buffer. Spin columns were placed in new 1.5 mL collection tubes. RNA in the columns was eluted in 30 μ L of RNase-free water, and RNA concentration of each sample was quantified using a NanoDrop 2000 (Thermo).

2.4.1.3 RNAseq analysis

RNA quality and quantity was assessed using Agilent 2100 Bioanalyser and an RNA Nano 6000 kit. RNA samples had RNA integrity numbers (RIN) of 9.9-10. Frozen RNA samples were then shipped to Oxford Genomics Centre. RNAseq libraries using PolyA selection were generated and sequenced using a 75-base paired end (2x75bp PE) dual index read format on the Illumin HiSeq4000 according to the manufacturer's instructions. Raw data resulting from RNAseq performed by Oxford Genomics Centre was then processed by Dr. Robert Andrews (Systems Immunity Research Institute, Cardiff University, UK). Reads were trimmed using Trimmomatic⁴²³ to remove any adaptor sequence from reads, and any reads with poor quality base calls. Sequencing quality was assessed used FastQC (Babraham Bioinformatics). Sequences were then mapped to the reference mouse genome (Mus_musculus.GRCm38) using STAR⁴²⁴. Reads per gene (gene expression) were calculated in featureCounts software⁴²⁵ using BAM files output from STAR as input, in combination with the mouse GTF files containing all exon coordinates (Mus_musculus.GRCm38.84.gtf). Read counts per gene were processed in R (The R Foundation for Statistical Computing), using the bioconductor package DESeq2⁴²⁶. Reads were processed using the standard workflow, to give normalised gene counts values used for clustering and downstream analyses. Additionally, lists containing normalised count values of differentially expressed genes between conditions, yielding log₂FC

expression changes, and associated adjusted p -values were generated for gene clustering, gene enrichment and pathway analysis. All downstream analysis from processed RNAseq dataset lists was performed by me, the PhD candidate, unless stated otherwise.

2.4.1.3.1 Principal Component Analysis (PCA) from RNAseq data

Principal Component Analysis (PCA) was performed by Dr. Robert Andrews using R, for each of the pair wise comparisons between different conditions set for RNAseq (e.g. *C. parapsilosis*-stimulated BMDMs at 10 h vs *C. parapsilosis*-stimulated BMDMs at 24 h). Lists containing significantly induced genes with a Benjamini-Hochberg-corrected adjusted p -value cut-off of 0.05 were generated and combined to give a list of significant genes for the study. The normalised counts for these genes were used to plot each sample as a function of gene expression using PCA. PCA showed replicate biological samples clustering together.

2.4.1.3.2 Scatter and volcano plots

To assess how the different *Candida* species influence gene expression in BMDMs after infection, multiple gene lists were generated. These lists comprised untreated control-normalised \log_2 FC values from genes obtained from RNAseq that fitted a cut-off of more than 10 reads per gene and adjusted p -value of 0.05. Different lists were generated for each pair wise comparisons between different stimulations with the different *Candida* spp. at the same timepoint (e.g. *C. parapsilosis*-stimulated BMDMs at 10 h vs *C. albicans*-stimulated BMDMs at 10 h). Scatter plots were then drawn on R using the ggplot2 package⁴²⁷ and genes were coloured differently according to their expression. To evidence type I Interferon pathway activation in BMDMs after stimulation with different *Candida* species, lists comprising adjusted p -values and untreated control-normalised \log_2 FC values for all the genes obtained from *Candida* sp. stimulation conditions were generated and used as input for volcano plots drawn on R using the ggplot2 package. Gene names from the generated lists were then inputted on INTERFEROME database⁴²⁸ to find type I Interferon-regulated genes (type I IRGs). Filtered type I IRGs were then coloured red on volcano plots. Scatter plots showed on this study were made by Dr. Robert Andrews. Volcano plots were also drawn by Dr. Robert Andrews using filtered gene datasets obtained on INTERFEROME by me, the PhD candidate.

2.4.1.3.3 Gene clustering, gene ontology and canonical pathway analysis

Lists generated from RNAseq data containing normalised gene count values of all genes that fitted a cut-off of more than 10 reads per gene, \log_2FC lower than -1.5 and higher than 1.5 and an adjusted p -value of 0.05 were used as input for all downstream RNAseq analyses. For gene ontology analyses, generic names for all the genes that fitted the referred cut-off above were used and filtered by using different Gene Ontology (GO) term annotation. Search analysis was performed using Princeton Generic Gene Ontology (GO) Term Finder (<https://go.princeton.edu>) (Lewis-Sigler Institute, Princeton University, New Jersey, USA) by setting a p -value cut-off of 0.01 with Bonferroni correction. Filtered gene lists were used as input and hierarchical clustering was performed on Genesis software. For gene clustering, normalised counts for each gene from the gene lists generated were z-transformed and k-means clustering was performed using Genesis software⁴²⁹. For canonical pathway analysis, individual gene lists generated from RNAseq data were run on Ingenuity Pathway Analysis system (IPA) (Qiagen) for core analysis and then overlaid with the global molecular network in the Ingenuity pathway knowledge base (IPKB). For the generation of heatmaps comprising all type I interferon regulated genes (type I IRGs) obtained from RNAseq, generated gene lists were used as input to run analysis on the INTERFEROME database. All heatmaps presented in this study were created using Genesis software.

2.4.2 Quantification of gene expression at RNA level

To quantify BMDM gene expression, total RNA previously purified as described in section 2.4.1.2. was isolated and converted to cDNA by Reverse Transcriptase PCR (RT-PCR) and later used as template for Real Time – quantitative PCR (RT-qPCR).

2.4.2.1 Reverse Transcriptase – PCR (RT-PCR)

Purified macrophage RNA was converted to cDNA by RT-PCR to be used for gene expression quantification by RT-qPCR. Reactions started with an initial concentration of 52.1 ng/mL of RNA, and conversion to cDNA was performed using TaqMan Reverse Transcription Reagents (Invitrogen). Reagent volumes are described in Figure 2.1.

Reagent	Quantity used (μL)
10X RT buffer	2.5
25 mM MgCl ₂	5.5
dNTP mix	5
Random Hexamer	0.625
OligoDT primers	0.625
RNAse Inhibitor	0.5
Multiscribe RT	0.625
Diluted purified RNA (52.1 ng/mL)	9.6
TOTAL VOLUME (per well)	25

Figure 2.1 – TaqMan Reverse Transcription Reagents (Invitrogen) reaction mix. Table describing reaction volumes used to generate cDNA from isolated RNA via RT-PCR

The reaction mix was then incubated on a Nexus Gradient MasterCycler thermocycler (Eppendorf) and one single PCR cycle at 25°C for 10 min, 48°C for 30 min and 95°C for 5 min was performed. cDNA was either kept on ice if RT-qPCR was to be performed on the same day or stored at -20°C for later use. Leftover RNA was snap frozen and stored at -80°C.

2.4.2.2 Real Time – quantitative PCR (RT-qPCR)

Relative expression of genes of interest in cDNA was determined by RT-qPCR using TaqMan primer probe assays. For each quantification experiment, *Hprt* was used as the normalising housekeeping gene. RT-qPCR reactions were performed by the TaqMan method using TaqMan Universal PCR Mastermix (Thermo) and TaqMan gene-specific primer probe assays (Thermo). The general RT-qPCR reaction mix is described in the Figure 2.2.

Reagent	Quantity used (μL)
2X Taqman Mastermix	5
20X Taqman gene probe	0.5
cDNA	1.5
DEPC-treated water	3
TOTAL VOLUME (per well)	10

Figure 2.2 – TaqMan RT-qPCR (Invitrogen) reaction mix. Table describing reaction volumes used to quantify gene transcripts from cDNA by RT-qPCR

TaqMan gene probes are coupled with a FAM label on 5' end and a minor groove binder (MGB) conjugated with a non-fluorescent quencher (NFQ) on the 3' end. During the denaturation step, the fluorescence emitted from FAM label is quenched by NFQ. Quenching

lasts as long as NFQ remains intact in the probe in proximity to the FAM label. On the annealing step, TaqMan probes bind to complementary sequences on the denatured cDNA sample, and during the polymerization (extension) step. Once Taq DNA polymerase reaches a TaqMan probe during DNA polymerisation, it cleaves both FAM label and NFQ from the probe, separating the fluorescent molecule from its quencher. FAM molecule will then emit light and fluoresce and this light will then be detected by the qPCR machine system. With each PCR cycle, more FAM label molecules will be cleaved, released from the gene probe and fluoresce. This increase in fluorescence is proportional the quantity of synthesized amplicon and will ultimately be used as surrogate measure to detect gene expression at transcriptomic level. The RT-qPCR experiment reactions were performed on QuantStudio 12K Flex Real-Time PCR system (Thermo) for 45 cycles under standard TaqMan incubation periods and temperatures. Results obtained from RT-qPCR are shown as *Hprt*-normalised relative fold change of a gene of interest during a stimulation condition from respective untreated controls using the $2^{-\Delta\Delta Ct}$ method.

2.4.3 *Candida* killing assays

Killing assays were performed to assess how stimulation with a compound of interest affects BMDMs ability to clear *Candida* spp. *in vitro*. BMDMs were seeded in 24-well plates as described in section 2.4. and pre-stimulated with 600 μ L of specific drug/cytokine for 24 h or 48 h. Following pre-stimulation, *Candida* spp. were added to BMDMs at a MOI 1:1 in 600 μ L without previous removal of any media on wells (final volume of 1.2 mL per well) and cells were stimulated for another 3 h. After stimulation, plates were centrifuged at 350 g for 5 min at room temperature, media was discarded, 300 μ L of 1% (v/v) Triton X-100 in PBS was added to each well and cells were scrapped using sterile P1000 tips. Contents were transferred to individual 1.5 mL centrifuge tubes, 300 μ L of PBS was added to each well and they were scraped again. Well contents were added to their respective tubes, that were properly vortexed and serially diluted 10X twice to 1:100 (100 μ L of scraped suspension into 900 μ L of PBS). 20 μ L of each neat and diluted suspensions were spotted in YPDA plates and incubated for 24 h at 37°C. After incubation, visible CFUs were counted and yeast viability was determined by comparing each stimulation condition where BMDMs were infected with

Candida spp. to their respective control where *Candida* spp. was incubated in the absence of BMDMs (unstimulated yeast control), using the following formula:

$$\text{Yeast killing (CFUs)(\%)} = 100 - \frac{\bar{x} CFUs_{\text{stimulation condition}} * 100}{\bar{x} CFUs_{\text{unstimulated yeast control}}}$$

2.4.4 Nitrite quantifications

Nitrite levels released by BMDMs into the media were quantified using the Griess test⁴³⁰, a spectrophotometric assay where nitrite accumulation in the cell culture media is quantified as a surrogate measure of nitric oxide production by cells by incubating the supernatants with modified Griess reagent (Sigma). Modified Griess reagent contains N-(1-Naphthyl)ethylenediamine dihydrochloride suspended in water and sulphanilamide in phosphoric acid. Once it is added to the cell culture media, sulphanilamide (a diazotizing reagent) will react to nitrite to form a transient diazonium salt intermediate, which will then react to N-(1-Naphthyl)ethylenediamine to form a stable azo compound that over time will accumulate in the medium and change its colour to an intense purple, that can be quantified by spectrophotometry⁴³¹.

BMDMs were seeded in a flat 96-well plate and stimulated with *C. albicans* or *C. parapsilosis* as described in 2.4. in the presence or absence of IFN- β or poly(I:C) at different concentrations for 24 h or 48 h. 2.5 $\mu\text{g}/\text{mL}$ amphotericin B was added to wells after 2 h of co-stimulation. After each stimulation period, plates were centrifuged at 350 g for 5 min and 50 μL of supernatant from each well was transferred to a new flat-bottom 96-well plate. A sodium nitrite solution was also prepared and serially diluted 1:2 in distilled water seven times, to generate a standards curve. 50 μL of each standard were also transferred in duplicate to the new 96-well plate alongside the supernatants. Both samples and standards were incubated with 50 μL of modified Griess reagent for 10 min at room temperature. Absorbance at 540 nm was measured using a Multiskan Spectrum plate reader (Thermo Scientific) and nitrite levels in the sample were then calculated from the standards curve.

2.4.5 Luminol assay for ROS detection

Luminol assays were performed to detect the production of ROS by BMDMs. Luminol (3-Aminophthalhydrazide, 5-Amino-2,3-dihydro-1,4-phthalazinedione) is added to macrophages and once it comes in contact with the reactive oxygen species superoxide and hydrogen peroxide forms the excited state of 3-APA (3-aminophthalate). The decay to lower excitation levels of 3-APA leads to photon emission and consequent production of light. The emitted chemiluminescence is then detected on a luminometer and it is used as a surrogate method of ROS production by cells^{432,433}.

BMDMs were seeded in 96-well plates with flat white bottom as described in section 2.4. and stimulated with 200 μL of 1,000 U/mL of IFN- β for 48 h. Following incubation, media was removed and 100 μL of 800 μM luminol in DMEM with no phenol red (Gibco) supplemented with 10 % (v/v) FBS, 2mM L-glutamine and 1% (v/v) PenStrep (DMEM-luminol) was added to each well. Cells were incubated for 30 min at 37°C followed by addition of 100 μL of the different *Candida* spp. at a MOI 1:1 or 400 ng/mL PMA (phorbol 12-myristate 13-acetate) in DMEM-luminol (positive control) was added on top. The plate was immediately transferred to a FLUOstar Omega luminescence plate reader (BMG) at 37°C and luminescence was measured in 8.7 min cycles for approximately 3 h (22 measurement cycles). For statistical analysis, area under the curve (AUC) values of total luminescence measured during the course of the experiment were calculated for every condition (except stimulations with PMA) by setting the values obtained on the first measurement cycle as baseline.

2.4.6 Detection of itaconate produced by BMDMs during infection

Itaconate produced by macrophages after stimulation with *Candida* spp. and IFN- β was detected by mass spectrometry. All co-stimulations, metabolite harvesting and protein quantifications for normalising itaconate readouts were performed by me, the PhD candidate, whereas the mass spectrometry quantification *per se* was performed by Dr. Erika Palmieri (National Cancer Institute, Frederick, MD, USA).

2.4.6.1 Sample preparation

BMDMs were seeded in 6-well plates as described in section 2.4. Media was removed and cells were stimulated with *C. albicans* and *C. parapsilosis* diluted in RPMI-BMDM at a MOI 1:1, and 2.5 mL of medium or IFN- β at final concentration of 1,000 U/mL was added on top (final volume of 5 mL per well). Plates were incubated for 48 h at 37°C and 2.5 μ g/mL amphotericin B was added after 2 h of incubation. Following stimulation, plates were centrifuged at 350 g for 5 min at 4°C, media was discarded, 500 μ L of ice cold 80% (v/v) methanol was added and each well was scraped using a disposable cell scraper. Well contents were transferred to individual tubes on ice, and wells were scraped a second time with fresh 500 μ L of 80% (v/v) methanol to ensure complete BMDM lysis and metabolite harvest. After scraping, lysates were again transferred to ice-cold tubes and were centrifuged at 12,000 g for 20 min at 4°C. After centrifugation, supernatants containing polar metabolites were transferred to new cryovials and snap frozen until used for quantification and pellets were stored at -80°C for later protein quantification.

2.4.6.2 Quantification of itaconate by mass spectrometry

For itaconate quantification, supernatants isolated as described in 2.4.6.2. were dried using a vacuum concentrator. Dried samples were resuspended in Milli-Q water and run in an Agilent 6410 Triple Quadrupole mass spectrometer interfaced with a 1200 Series HPLC quaternary pump (Agilent) for ESI-LC-MS/MS (Electrospray Ionization-Liquid Chromatography-tandem Mass Spectrometry) analysis in multiple reaction monitoring mode for detection of itaconate at m/z 129.0 > 85.1. Chromatographic resolution was obtained in reverse phase on Eclipse Plus C18 column (1.8 μ m; Agilent), with a flow rate set at 0.4 mL/min. Itaconate values were obtained from a calibration curve using four different concentrations of standard processed under the same conditions as the samples.

2.4.6.3 Protein quantification for normalisation of itaconate levels

To normalise itaconate expression, and express values of itaconate weight per weight of total protein levels produced by cells from each condition, total protein levels were quantified by the bicinchoninic acid (BCA) method. This method uses the combination of two reagents: A - sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate

in 0.1 M sodium hydroxide; and B – 4% (v/v) copper(II) sulphate. Once the sample is added to the reagent mix, proteins in the sample will reduce Cu^{2+} ions from copper sulphate to Cu^+ , that will in turn react with bicinchoninic acid and form a complex that will change the medium to a deep purple colour. This colour intensity shift is proportional to the concentration of protein present in the wells and is used as a surrogate measure of total protein concentration of a given sample⁴³⁴. To prepare samples for quantification, frozen pellets were resuspended in 100 μL 200 mM NaOH, heated up at 90°C for 20 min to facilitate solubilization and centrifuged at 14,000 rpm for 10 min. Supernatants were transferred to new microcentrifuge tubes and subjected to one freeze-thawing cycle at -80°C. Total protein was then quantified using the Pierce BCA Protein Assay Kit (Thermo): BCA reagent A and B were mixed in a ratio of 50:1, 80 μL of the mix was distributed to wells in a flat bottom 96-well plate and overlaid with 10 μL of sample diluted 1:2 (5 μL of sample + 5 μL PBS) or previously prepared BSA standards. The plate was incubated at 37°C for 30 min and absorbance at 562 nm was read using a Multiskan Spectrum plate reader (Thermo Scientific). Total protein concentration from samples was estimated from the BSA standards curve.

2.4.7 IL-27 blocking experiments

Chapter 5 of this thesis mostly comprises experiments designed to assess *Candida* spp. ability to block IFN- β and downstream IL-27 production by BMDMs. Two different experimental designs were conducted: “Direct stimulation experiments” where BMDM suspension were seeded in flat bottom 96-well plates as described in section 2.4., cells were directly stimulated with *Candida* spp. in the presence or absence of another stimulant for 24 h and IL-27 was detected in the cell culture medium; “Filtered supernatants experiments” where 600 μL of *Candida* spp. suspension in RPMI-BMDM prepared as described in 2.4. were seeded in 24-well plates and incubated for 24 h in the absence or presence of BMDMs to allow for the accumulation of *Candida* spp.- and/or BMDM-specific soluble mediators in the medium. Cell culture medium was harvested, sterile filtered through 0.22 μm membrane filters. Filtered supernatants were then added to freshly plated BMDMs alongside *C. parapsilosis* suspended in fresh RPMI-BMDM for another 24 h. This was performed to assess whether the previously accumulated soluble mediators in the medium were able to inhibit *C. parapsilosis*-induced IL-27 production. 2.5 $\mu\text{g}/\text{mL}$ of amphotericin B was added to wells to

neutralise *Candida* spp. at different stages depending on the experiment. In chapter 5 results section, schematics explaining experimental designs were included before the results of every new type of experiment were shown. Once samples were transferred to IL-27 ELISA plates, leftover supernatants were transferred to new U-bottom 96-well plates and stored at -80°C for future ELISA quantifications.

2.4.7.1 Heat killing *Candida* spp.

For some IL-27 blockade experiments, *C. albicans* was heat-killed (HK) prior to stimulation of BMDMs at different phenotypical states: “Yeast” (yeast form) or “hyphae” (true hyphae) phenotype. *C. albicans* was propagated for 16 h at 30°C in YPDB and washed three times with 20 mL PBS as described in section 2.2. To get HK yeast, *C. albicans* suspension was heat-killed in boiling water for 40 min and it was afterwards allowed to cool on ice for another 20 min. The suspension containing HK yeasts was centrifuged at 350 g for 5 min, resuspended in 20 mL PBS and *C. albicans* concentration was counted using a haemocytometer. The suspension was centrifuged again, adjusted to 1×10^8 CFUs/mL and diluted 1:50 in RPMI-BMDM as described in 2.2. To get *C. albicans* HK hyphae the process was similar, but before boiling, the suspension was centrifuged at 350 g for 5 min, pellets were resuspended in 5 mL RPMI-BMDM, transferred to new 14 mL polypropylene tubes with vented caps. and *C. albicans* was grown at 37°C for 3 h before boiling to ensure germination. Following the growth period, tube contents were transferred to new 50 mL centrifuge tubes and washed three times with 20 mL PBS by centrifugation. Following the last washing step, the tube containing suspended *C. albicans* was then boiled and processed as described above.

2.4.7.2 Enzyme-Linked Immunosorbent assays (ELISAs) for cytokine detection

To detect protein levels in a medium, sandwich ELISAs were performed. The principle of this type of ELISAs consists in the binding of an antigen in a sample to an antigen-specific capture antibody that is adhered to the bottom of a well. A biotinylated detection antibody is then added and will bind specifically to the antigen previously bound to the capture antibody forming a complex (“sandwich”) with adhered capture antibody-antigen-detection antibody. A solution containing streptavidin-horse radish peroxidase (HRP) is added to the wells, and streptavidin will then bind to biotin present on the Fc region of the detection

antibody to form conjugates, adding HRP to the complex. TMB (3,3',5,5'-Tetramethylbenzidine), a colourless chromogenic substrate of HRP is then added. The reaction of TMB with HRP will generate a blue compound and the quantity of product generated is proportional to the concentration of target antigen in each well. When appropriate, 1N H₂SO₄ is then added to wells to stop the reaction by acidifying the medium and inactivating HRP.

For this study, three different ELISA kits were used: Ready-SET-Go! Uncoated ELISA kits from eBioscience, DuoSet ELISA kits from R&D Systems and a High Sensitivity IFN- β ELISA kit from PBL. Whilst the sandwich ELISA assay principle is the same for all the different kits, these differ slightly on experimental methodology. For the description of each method, each kit will be referred to as eBioscience ELISA, R&D ELISA or PBL ELISA respectively. After each washing step, wells were dried by blotting the plates against absorbent paper (for PBL ELISA, plates were not blotted). All wash steps for both eBioscience and R&D ELISAs were performed using 200 μ L 0.05% (v/v) Tween 20 in PBS per well as washing solution and washing steps were completed using a HydroFlex microplate washer (Tecan). At the end of the assays, absorbance values were registered using a Multiskan Spectrum plate reader (Thermo Scientific). A table including all the different ELISA kits used for this study was included in the Appendix section (Chapter 7, Figure 7.7).

2.4.7.2.1 eBioscience ELISAs

For eBioscience ELISAs, half-well high binding 96-well plates were coated with 50 μ L of capture antibody diluted 1:250 in coating buffer (10X coating buffer diluted 1:10 in distilled water) to working concentration, and plates were incubated overnight at 4°C. Following incubation, wells were washed three times, and then blocked with 100 μ L reagent diluent (5X ELISA/ELISPOT diluent diluted 1:5 in distilled water) for 1 h. Plates were washed three times, 50 μ L of sample or previously prepared standards (provided with each kit and diluted according to kit recommendations) were added to their respective wells and plates were incubated overnight at 4°C. The next day, plates were washed five times, 50 μ L of detection antibody diluted 1:250 in reagent diluent were added to each well and plates were incubated for 1 h at room temperature. Following incubation, plates were washed three times, 50 μ L of

HRP-streptavidin conjugate diluted 1:250 in reagent diluent were distributed to each well and plates were incubated again for 30 min at room temperature. Plates were washed five times to remove HRP-streptavidin and 50 μ L of TMB substrate were added to wells. Once standard colour shift was appropriate for the generation of a standard curve, 25 μ L of 1N H₂SO₄ was added to wells to inactivate HRP, and absorbance was read at 450 nm with 570 nm absorbance values subtracted.

2.4.7.2.2 R&D ELISAs

Unlike eBioscience ELISAs, R&D antibodies dilutions are not standardised as both their stock and working concentrations vary depending on the target protein kit and lot. Henceforth, for the different R&D ELISA kits used, antibodies were diluted according to their respective kit recommendations. Half-well high binding 96-well plates were coated with 50 μ L of capture antibody diluted to working concentration in PBS, and plates were incubated overnight at 4°C. Following incubation, wells were washed three times, and then blocked with 100 μ L reagent diluent (0.1% (w/v) BSA in PBS) for 1 h. Plates were washed three times, 50 μ L of sample or previously prepared standards (provided with each kit and diluted according to kit recommendations) were added to their respective wells and plates were incubated overnight at 4°C. The next day, plates were washed five times, 50 μ L of detection antibody diluted to working concentration in reagent diluent were added to each well and plates were incubated for 2 h at room temperature. Following incubation, plates were washed three times, 50 μ L of HRP-streptavidin conjugate diluted 1:40 in reagent diluent to working concentration were distributed to each well and plates were incubated again for 20 min at room temperature. Plates were washed five times to remove HRP-streptavidin and 50 μ L of TMB substrate were added to wells. Once standard colour shift was appropriate for the generation of a standard curve, 25 μ L of 1N H₂SO₄ was added to wells to inactivate HRP, and absorbance was read at 450 nm with 570 nm absorbance values subtracted.

2.4.7.2.3 PBL ELISAs

Unlike eBioscience and R&D ELISA kits, PBL ELISA kit included a plate already coated with capture antibody. Antibody solution containing detection antibody, sample diluent, HRP solution, TMB substrate and stop solution were all provided with the ELISA kit and prepared

according to kit and lot recommendations. All the washing steps performed for this ELISA were done by hand. The coated plate was removed from the fridge, 50 μ L of sample diluent was distributed to every well and overlaid with 50 μ L of sample or previously prepared standards according to kit recommendations. Plate was sealed and incubated at room temperature for 1 h shaking at 650 rpm. Following incubation, wells were washed four times with 250 μ L of wash solution, 50 μ L of antibody solution was added to each well and the plate was incubated at room temperature shaking at 650 rpm for 30 min. Afterwards, wells were washed again four times with 250 μ L of washing solution, 50 μ L of HRP previously diluted according to kit guidelines was added to each well and plate was incubated at room temperature shaking at 650 rpm for 10 min. Once the incubation was over, the plate was washed four times with 250 μ L of washing solution, 100 μ L of TMB substrate was distributed to each well and the plate was incubated in the dark for 10 min at room temperature (no shaking). Following incubation, 100 μ L of stop solution was added and absorbance at 450 nm was read after 5 min.

2.4.7.3 Luminex assays for cytokine detection

For the indicated experiments, to detect both IFN- α and IFN- β in frozen supernatants a ProcartaPlex mouse IFN- α /IFN- β 2-plex Multiplex Immunoassay (Invitrogen) luminex xMAP assay was performed. All cell stimulations and prior acquisition of samples were performed by me, the PhD candidate, but the Luminex assay was performed by Dr. Magdalena Czubala. Luminex is a fluorescence covalent microbead immunosorbent assay that allows for the possible quantification of a myriad of analytes in a sample. Samples are added to a mixture of colour-coded beads coated with capture antibodies that will bind specifically to an antigen present within a sample. Antigen-specific biotinylated detection antibodies are then added to form a bead-capture antibody-sample-biotinylated detection antibody “sandwich”. Streptavidin-conjugated fluorophores are then added, causing the fluorophores to bind to Fc region of the detection antibodies. Beads are then ran on a flow-based detection machine with two coupled lasers, one that will detect a specific bead and determine which protein it is meant to quantify and another one which will measure the intensity of the fluorophore signals bound to the bead complex. The combination of the two lasers will allow the accurate quantification of specific antigen analytes present in a sample⁴³⁵.

Before starting the assay, 96-well plates containing frozen supernatant samples were removed from -80°C to the fridge at 4°C to allow the samples to thaw. All solutions, antibodies and standards used in this assay were provided and prepared according to kit recommendations. Meanwhile, the vial containing the magnetic beads coated with target antigen-specific capture antibodies were vortexed for 30 s and 50 µL of the mix was distributed to wells of a flat-bottom 96-well plate provided with the kit. The plate was inserted in a hand-held magnetic plate washer which pulls and holds all the magnetic beads to the bottom of the wells. The beads were allowed to accumulate at the bottom of each well for 2 min. The liquid was removed by quickly inverting the plate over a waste container. The plate remained in the magnetic plate washer throughout the washing. 150 µL of wash buffer 1X was added to wells for 30 s and beads were washed by inversion. The plate was removed from the magnetic plate washer, 50 µL of sample or previously prepared standards were added to the dedicated wells, plate was sealed with a provided plate seal and incubated at room temperature for 2 h at 500 rpm orbital shaking. Following incubation, plate was docked to magnetic plate washer and washed 3 times as described above. After the last wash, plate was removed from magnetic plate washer and 25 µL of detection antibody mixture (1X) was added to each well. Plate was sealed and incubated at room temperature for 30 min at 500 rpm orbital shaking. After incubation, plate was docked again into magnetic plate washer and washed three times as described before. 50 µL of SAPE (Streptavidin-PE) solution was added to wells, and plate was incubated for 30 min at room temperature at 500 rpm, followed by three washes as described above. 120 µL of reading buffer was added to each well and plate was incubated for 5 min at room temperature at 500 rpm orbital shaking. Plate was run on a Luminex 200 instrument (Luminex) set up on RP1 low target value settings. Quantified protein concentrations in each sample were then calculated from established standard curves.

2.5 *In vivo* assays

To test the impact of certain cytokines in the progression of candidaemia, *in vivo* experiments using murine model of systemic candidiasis were performed.

2.5.1 Mice and ethics statement

C57BL/6J mice used for all experiments in this thesis were either bought from Charles River or bred from in-house colonies and were maintained and handled according to institutional and U.K. Home Office guidelines. Animals used for all *in vivo* experiments were age- and gender-matched. All *in vivo* experimental procedures presented in this thesis were performed in strict accordance with the Project License and procedures that were approved by Cardiff University Animal Welfare and Ethical Review Body and the U.K. Home Office. The animal care and use protocol adhered to the Animals (Scientific Procedures) Act 1986.

2.5.2 *In vivo C. albicans* infection

In preparation for *in vivo* experiments, female C57BL/6J aged 7-13 weeks were co-housed in scintainer cages containing sterile bedding, water and mouse chow. The cages were kept under 12h:12h light:dark cycles, under controlled temperature (21-24°C) and humidity (50-60%) inside ventilated scintainers. Before being injected, cages containing the mice were set at an incubator at 37°C for 20 min, to facilitate vasodilation for posterior injection. For *in vivo* infections, the tail-vein injection model was used and carried out as previously described⁴³⁶ with slight modifications. *C. albicans* was propagated and washed as described in 2.2, yeasts were resuspended in 0.01% (w/v) low-endotoxin BSA in PBS at 1.5×10^6 CFUs/mL, and administered via i.v. injection in a final volume of 100 μ L (1.5×10^5 CFUs per mouse). Different cytokines were also administered during the course of the infection alongside initial *C. albicans* injection. These were either administered i.v. at the desired dose in 100 μ L or i.p. in volumes ranging from 100-400 μ L. depending on the cytokine and the experiment design. After initial injection, and until the end of the experiment, mice were weighed daily and examined twice a day where they were scored according to the standards of an adapted animal distress scoring sheet⁴³⁷. Mice were terminated immediately once they reached a humane end point (20% initial weight loss or once they have achieved a critical distress score). An example of the adapted animal distress scoring sheet used in all *in vivo* experiments is included in the Appendix section (Chapter 7, Figure 7.9). All i.v. injections were performed by Dr. Selinda Orr.

2.5.2.1 Tissue harvesting

Mice were sacrificed under aseptic conditions by CO₂ asphyxiation and termination was confirmed by dislocation of the neck at the end of the experiment or at the humane end point. Blood was drawn by cardiac puncture using a 1 mL syringe coupled with a 25 G needle and transferred to SST microtainer tubes. Using sterile scissors and tweezers, an incision was made in the abdomen and the peritoneal cavity was opened to expose internal organs. The left kidney was harvested and stored inside a previously weighed 14 mL vented-cap polypropylene tube and the spleen was harvested into a 15 mL centrifuge tube containing 3 mL PBS. An incision was then made at the base of the skull to remove the skin and expose the cranium. Once the skull was exposed, incisions across the interparietal bone and through the sagittal suture were performed, the right parietal bone was lifted, the right hemisphere of the brain was harvested and placed in a previously weighed 14 mL vented-cap polypropylene tube. After blood, spleen, left kidney and right brain were collected, tubes containing left kidney and right brain were reweighed to calculate the organ's weight and 1 mL of PBS was added to submerge the organs. All organs were kept on ice until processing.

2.5.2.1.1 Blood processing and serum isolation for cytokine detection

To detect levels of circulating cytokines, serum was isolated from the collected blood. To do so, blood was harvested into SST (Serum Separating Tubes) microtainer tubes that contain a polymer gel in the bottom. The tubes were centrifuged at 10,000 rpm (10,621 g) for 10 min at 4°C and after centrifugation the polymer gel allowed the separation of the serum (on top) from coagulants and other blood components that were seeded in the bottom of the tube. Serum was then transferred to new microcentrifuge tubes and SST microtainer tubes were discarded. Serum was either transferred in a volume of 50 µL per well to previously blocked and washed ELISA plates for the detection of a specific cytokine, or frozen at -80°C for later use.

2.5.2.1.2 Organ homogenisation to evaluate fungal burden

To evaluate fungal burden in kidney and brain of mice after i.v. challenge with *Candida* spp., the organs were first homogenised using an electric mechanical homogenizer. To wash the homogenizer, two 50 mL centrifuge tubes were filled with 30 mL of 70% (v/v) ethanol and

another two with 30 mL PBS, homogeniser was submerged first in a tube with 70% (v/v) ethanol, turned on for 5 s, transferred to a tube with PBS and turned on again for another 5s. Two washing cycles were performed before the organs were blended and between every different organ to minimize crossed-contamination of *Candida* spp. between organs. Washing tubes were discarded and renewed every 5-6 organs. Organs were blended under aseptic conditions for a minimum of 5 s and until properly homogenized. Homogenates were then transferred to individual microcentrifuge tubes and kept on ice. Tube contents were then vortexed and serially diluted 10X until 1:1,000 in previously prepared tubes filled with PBS (100 μ L of suspension + 900 μ L PBS), and 20 μ L of neat homogenate, 1:10, 1:100 and 1:1000 dilutions from each organ were spotted in duplicate in a single YPDA plate containing 50 μ g/mL chloramphenicol (YPDA-chloramphenicol). Plates were allowed to dry for a few minutes and were then transferred to an incubator at 37°C. For experiments where mice were injected with *C. albicans*, plates were incubated for 24 h, and with *C. parapsilosis* for 48 h. After incubation, CFUs were counted.

2.5.2.1.3 Splenocyte experiments

To evaluate how the administered hetIL-15 affects splenic adaptive immune responses during systemic candidiasis, an *ex vivo* experiment using murine splenocytes were performed.

2.5.2.1.3.1 Splenocyte isolation

To isolate splenocytes, PBS was poured off the spleens and the organs were transferred to a 100 μ m strainer placed on top of a new 50 mL centrifuge tube. Spleens were then smashed through the strainer into the 50 mL tube using a 5 mL syringe plunger (necrotic parts of the spleen were not smashed and discarded). Smashed splenic pulp retained on the strainer was washed through the strainer with PBS using a sterile Pasteur pipette. Once the strainer was properly washed, suspension collected in the tube was centrifuged at 350 g for 5 min at 4°C. Following centrifugation, supernatants were discarded, and pellets were resuspended in 1 mL 1X ACK lysis buffer to lyse erythrocytes present in the splenic tissue. The suspension was incubated at room temperature for 0.5 – 1 min, 9 mL of ice-cold IMDM (Gibco) supplemented with 10% (v/v) FBS (HI), 1% (v/v) PenStrep, 2 mM L-glutamine and 5 mM of β -mercaptoethanol (IMDM-spleen) was added to dilute the lysis buffer and minimize

splenocyte viability loss and the sample was centrifuged again. Supernatants were discarded, pellets were washed twice with 10 mL ice-cold IMDM-spleen by centrifugation to remove dead cells and cell debris. Cells were counted using a MUSE Cell Analyser and resuspended at 1×10^7 viable cells/mL in IMDM-spleen to be used in following experiments.

2.5.2.1.3.2 Splenocyte staining for flow cytometry analysis

Flow cytometry analysis was performed on mice spleens to evaluate the levels of different splenic cell populations and their specific cytokine production profiles in order to determine how *C. albicans* and hetIL-15 affect splenic T cell responses. 100 μ L of splenocyte suspensions prepared as described in 2.5.2.1.3.1. were seeded in triplicate in a U-bottom 96-well plate. For each spleen, cells seeded in one well were stimulated with 10 μ g/mL brefeldin A in IMDM-spleen and the other two wells were stimulated with a mix of 10 μ g/mL brefeldin A, 100 ng/mL PMA and 1 μ g/mL ionomycin in IMDM-spleen for 4 h at 37°C. Brefeldin A was added to block vesicle formation on the cells' Golgi complex and endoplasmic reticulum. Brefeldin A inhibits the transport of cytokines in these organelles towards the cell membrane for exocytosis, leading to their accumulation in the intracellular milieu⁴³⁸. PMA and ionomycin (a calcium ionophore isolated from *Streptomyces globatus*) are synergistic activators of Protein Kinase C (PKC) and downstream inducers of NF- κ B and NFAT pathways. The induction of these pathways will lead to T cell activation and augmented cytokine production. Usage of PMA/ionomycin in combination with brefeldin A facilitates intracellular cytokine staining and later detection by flow cytometry⁴³⁹. After 4 h stimulation and throughout the staining process, the plate was kept cold at all times either in the fridge or on ice. All washing solutions, buffers or diluted antibodies were added to cells ice cold. After each centrifugation step, supernatants were discarded. The plate was centrifuged at 350 g for 5 min at 4°C, supernatants were removed, and cells were washed with 150 μ L cold PBS by centrifugation. After the PBS wash, cells were incubated with 100 μ L of 1X Live/Dead Fixable Aqua stain in PBS (Life Technologies) for 15 min. This solution contains an aqua-fluorescent dye that will bind to free amines. In cells with compromised membranes the dye will bind amines present on the cell membrane and on the cells' interior, yielding intense fluorescence. In viable cells the dye will only bind amines present on the cell surface, resulting in a less intense fluorescence. This difference in fluorescence intensity between non-viable and viable cells is

typically greater than 50-fold, allowing the easy discrimination between the two. Following incubation with Live/Dead stain, plates were centrifuged, and pelleted cells were washed first with 100 μ L PBS and then with 150 μ L FACS buffer (0.05% (w/v) sodium azide and 0.1% (w/v) BSA in PBS). Cells were then blocked with 20 μ L FACS block containing an Fc γ R block (4 mg/mL anti-Fc γ R (2.4G2 clone) and 5% (v/v) rabbit serum in FACS buffer) for 15 min. A blocking antibody for Fc γ R was included to block non-antigen-specific binding of fluorophore-coupled antibodies during staining steps. After the blocking step, 20 μ L of diluted surface staining antibodies in FACS buffer at 2X working concentration was added on top of the FACS block and cells were incubated for 30 min. After staining, 150 μ L of FACS buffer was added to wells and plates were centrifuged at 350 g for 5 min at 4°C. Supernatants were removed, and pelleted cells were incubated for 20 min with 100 μ L of Cytofix/Cytoperm (BD) to ensure fixation of cells stained with surface antibodies and membrane permeabilization, necessary for intracellular staining. Cells were then washed twice with 100 μ L of Perm/Wash buffer (BD) by centrifugation. Cytofix/Cytoperm solution contains not only formaldehyde as a fixative agent, but also saponin to permeabilize cellular membranes. Saponin-mediated permeabilization is a reversible process⁴⁴⁰, and cells were washed with Perm/Wash after the Cytofix/Cytoperm step, as Perm/Wash also contains saponin, which ensured membranes were still permeable at the time the intracellular staining antibodies were added. Following the washing step, cells were blocked again with 20 μ L of FACS block for 15 min and then 20 μ L of previously diluted intracellular staining antibodies in FACS buffer at 2X working concentration was added on top. The plate was then incubated for another 30 min. After incubation, 100 μ L of Perm/Wash solution was added to each well, plates were centrifuged again, and pellets were resuspended in 150 μ L of Cytofix (BD) and kept in the fridge for a maximum of 5 days until they were run on a flow cytometer. For flow cytometry analysis, samples were transferred to FACS tubes, diluted with FACS buffer if necessary, and run on a FACS Canto II (BD) flow cytometer using FACSDiva software (BD). Flow cytometry data was then analysed using FlowJo software (BD).

2.6 Statistical analyses

Data in all graphs included in this study are presented as means \pm SEM. Different statistical analyses were performed depending on experimental design and nature of the

presented data. For statistical analysis of *in vivo* fungal burden results, raw CFU data was transformed using $Y=\sqrt{Y+0.5}$ ⁴⁴¹. Statistical analysis was performed using GraphPad Prism 7 software and statistical significance was set at * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$. In some graphs, “ns” was included to highlight when statistical significance between datasets was not achieved.

Chapter 3

Transcriptomic analysis of the macrophage immune response triggered by *Candida* spp.

3.1 Introduction

Systemic candidiasis is a major health concern, which has an unacceptably high mortality rate^{5-7,11}. Many patients succumb to infection due to an unbalanced and exacerbated pathogen-induced immune response. Currently available therapeutics are often insufficient to avert this outcome, and immunosuppressive therapy is often prescribed to these patients in an effort to ameliorate excessive inflammation^{305,315}. The challenges in treatment for systemic candidiasis stem not only from the insufficient pharmacological therapies available, but also due to the poor understanding of host immune responses to different infection-causing *Candida* spp. Indeed, during systemic candidiasis, an adequate host immune response is vital to curtail infection, as immunocompromised patients are at a much higher risk of contracting and succumbing to candidiasis compared to immunocompetent individuals. As these patients often fail to trigger protective immune responses to limit infection, personalized immunotherapy might be a suitable treatment approach.

One of the main aims of this thesis is to better understand how host immunity differs in response to various clinically relevant *Candida* spp. with differing virulence. Arendrup and colleagues grouped eight different *Candida* spp. isolated from patients suffering from candidaemia into three virulence groups according to their *in vivo* pathogenicity in mice³²¹. Group I (*C. albicans* and *C. tropicalis*) comprises highly virulent species that induced the highest mortality rates, higher number of infected kidneys, kidney fungal burden and inflammation. Group II (*C. glabrata*, *C. lusitaniae* and *C. kefyr*) includes species with intermediate virulence, and Group III (*C. parapsilosis*, *C. krusei* and *C. guilliermondii*) consists of species with the lowest virulence³²¹. This results chapter describes a transcriptomic analysis evaluating how macrophages respond to infection induced by *Candida* spp. belonging to these different virulence groups: *C. parapsilosis* (Group III), *C. glabrata* (Group II) and *C. albicans* (Group I)

Transcriptomics (analysis of a complete set of transcripts produced by cells or tissues) enables the determination of actively expressed genes and transcripts under various conditions at single cell, tissue or organism level. Understanding and identifying

transcriptomic variations between pathogens with various levels of virulence can be explored to find relevant genes/pathways associated with fungal clearance and host resistance. Transcriptomics can also be used to identify potential biomarkers and/or novel therapeutic targets for disease treatment.

“First generation” transcriptomic sequencing assays, like the Sanger method, were broadly used in the past and dominated the field for years. However, these platforms were expensive, required high amounts of initial sample and the analysis was time consuming⁴⁴². Next-generation sequencing (NGS) assays are considerably cheaper, faster, require less RNA and allow for more accurate transcript quantification when compared to older platforms. RNAseq offers advantages over other hybridization-based methods like microarrays such as: permitting the quantification of novel transcripts that are not mapped to a genome, detection of gene expression on a wider dynamic range with higher specificity and sensitivity and the ability to detect both coding and non-coding RNAs^{443,444}.

Multiple transcriptomic studies have been conducted to evaluate gene expression during *Candida* infection in host cells focusing on the pathogen transcriptome^{445–452}, the host transcriptome^{451,453–457}, or both^{458–462}. Reports comparing the transcriptomes of distinct *Candida* spp. when subject to diverse stimuli were also published^{463,464}, however, to my knowledge there are no comparative studies looking at the transcriptome of phagocytes after stimulation with different *Candida* spp.

3.2 Aims of this chapter

The main hypothesis addressed in this chapter is that the host immune response varies between *Candida* spp. with distinctive virulence. Here, various RNAseq analyses were conducted in order to compare the macrophage transcriptome induced by different *Candida* spp. and identify the main immune pathways exclusively activated by the distinct species studied. For that purpose, the following aims were set:

- I. Assess how *Candida* spp. with differing virulence affect the macrophage transcriptome;
- II. Identify and validate activation of major immune pathways specifically triggered by low virulent *Candida* spp.

3.3 Results

3.3.1 *Candida* spp. with differing virulence induce distinct gene expression in a species-specific manner

Before focusing on genes involved in the immune response *per se*, I first aimed to evaluate how the different *Candida* spp. used in this study affected the general transcriptome of bone marrow-derived macrophages (BMDMs). Prior to the start of my doctoral studies, *in vitro* stimulations and posterior RNA isolation and processing were performed by Drs. Selinda Orr and Aiysha Thompson, whilst the initial post-sequencing analysis was performed by Dr. Robert Andrews. Using data obtained from RNAseq analysis, fold-change expression values of each detected gene obtained from *Candida* infection conditions were calculated from their respective untreated control at the same timepoint ($\log_2(\text{FC})$). Expression values for genes induced by the different *Candida* spp. were then plotted against each other in scatter plots. Dots representing different genes were coloured differently depending on their expression specificity (Figure 3.1)

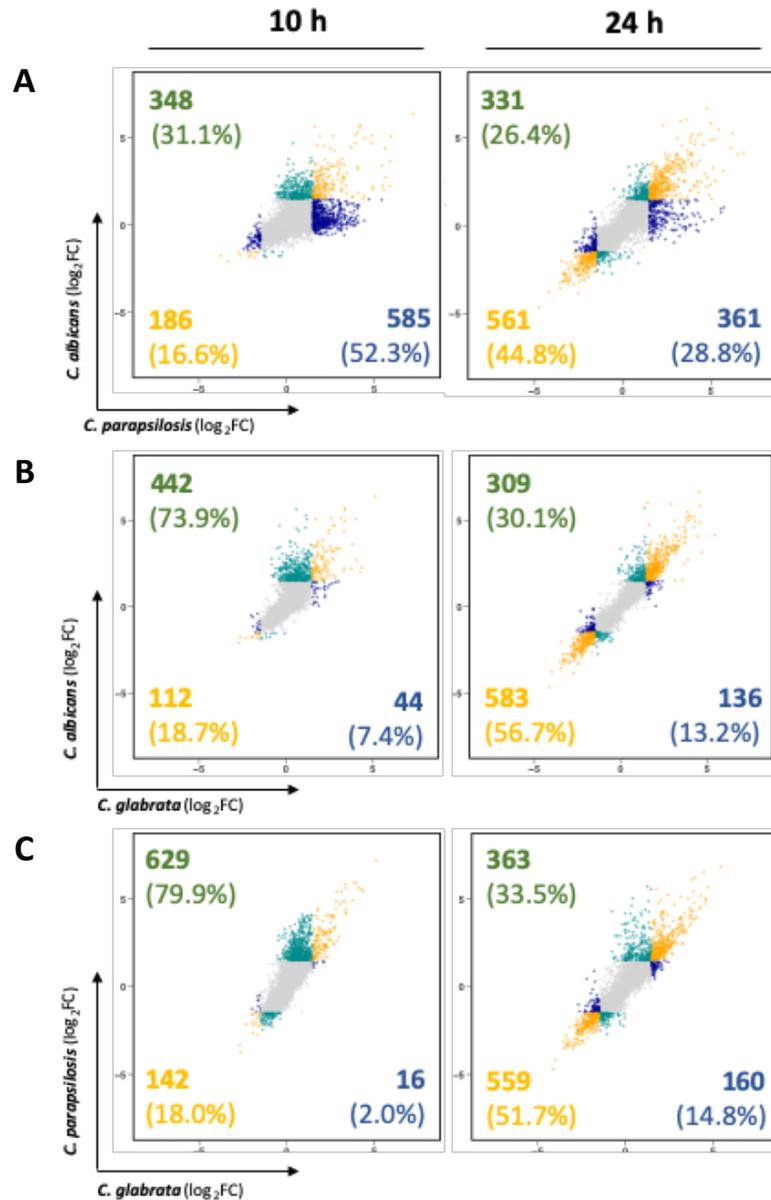


Figure 3.1 – Distinct *Candida* spp. elicit differential gene expression in BMDMs. Normalised gene expression values (\log_2FC) detected in BMDM stimulated with *C. parapsilosis*, *C. glabrata* and *C. albicans* were obtained by comparing each infection condition with their respective untreated control at the same timepoint (A: *C. albicans* versus *C. parapsilosis*; B: *C. albicans* versus *C. glabrata*; C: *C. parapsilosis* versus *C. glabrata*). Genes with an adjusted p-value ≤ 0.05 for each *Candida* spp. stimulation condition at the same timepoint, were then plotted against other. In each scatter plot graph, green dots represent genes that are either significantly upregulated ($\log_2(FC) \geq 1.5$) or downregulated ($\log_2(FC) \leq -1.5$) by infection with the *Candida* sp. plotted on the Y axis and not significantly changed by stimulation with the *Candida* sp. plotted on the X axis. Blue dots represent genes that are either significantly upregulated or downregulated by infection with the *Candida* sp. plotted on the X axis and not significantly changed by stimulation with the *Candida* sp. plotted on the Y axis. Yellow dots represent genes that are significantly upregulated or downregulated during infection with both *Candida* spp. plotted. Grey dots represent non-significantly regulated genes ($\log_2(FC)$ higher than -1.5 and lower than 1.5). In each graph, the coloured numbers refer to the number of both up- and downregulated genes that meet the significance threshold and the percentages these numbers represent out of the total significantly regulated genes. Scatter plots were drawn and analysed by Dr. Robert Andrews using R.

Results show that *C. albicans* (Group I) and *C. parapsilosis* (Group III) induced expression of the highest number of species-specific genes (Figure 3.1-A). By comparing the *C. albicans*-induced and *C. parapsilosis* transcriptomes after 10 h stimulation, 186 genes (16.6% of all significantly regulated genes) were found to be significantly regulated by both strains. Interestingly, there were also a high number of genes whose expression was exclusively induced by a specific *Candida* sp.: 348 genes (31.1% of significant transcriptome) were exclusively regulated by *C. albicans* and 585 genes (52.3%) by *C. parapsilosis*. This trend was also observed after 24 h of stimulation with 331 genes (26.4%) being specifically regulated by *C. albicans* and 361 genes (28.8%) by *C. parapsilosis* (Figure 3.1-A). Compared with *C. albicans* and *C. parapsilosis*, *C. glabrata* (group II) treated BMDMs displayed less transcriptomic modification (Figure 3.1-B and -C), with species-specific gene regulation ranging from 44 to 16 genes (7.4% to 2% of all significantly regulated genes) detected after comparison with the other *Candida* spp. at 10 h post treatment. Interestingly, this increased to 136 to 160 genes (13.2% to 14.8% of all significantly regulated genes) after 24 h stimulation, depending on comparison (Figure 3.1-B and -C).

I then decided to assess if this species-specific differential gene expression could include genes that code for molecules that can shape the immune response. For this purpose, a list containing genes significantly induced by *C. albicans* and *C. parapsilosis*, with a $\log_2(\text{FC})$ lower than -1.5 and higher than 1.5 and adjusted p-value ≤ 0.05 was drawn and filtered using gene ontology (GO) terms to find protein coding transcripts with cytokine, chemokine and growth factor activity (Figure 3.2).

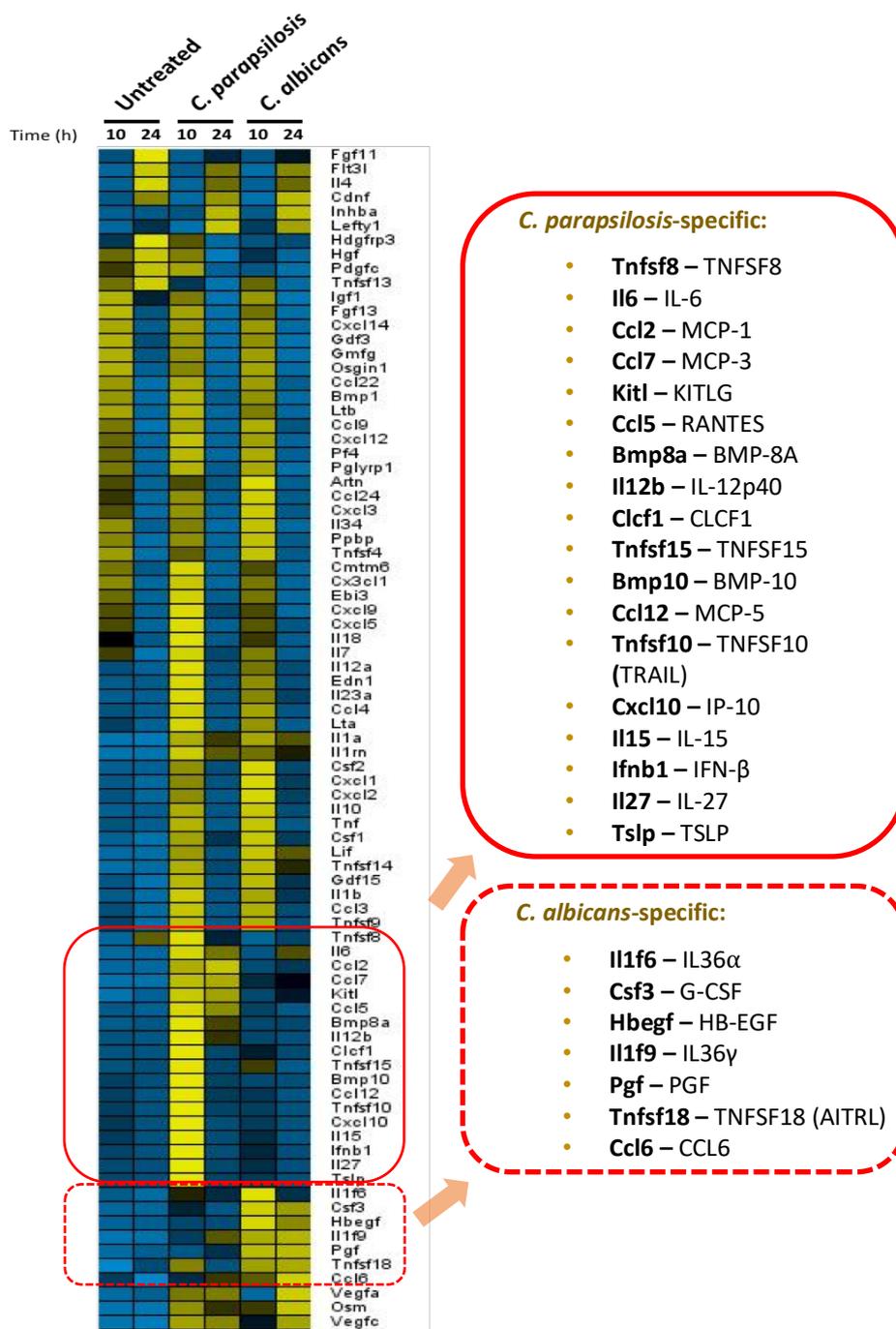


Figure 3.2 – *C. albicans* and *C. parapsilosis* induce differential expression of Immune mediators by BMDMs at RNA level. Genes obtained from RNAseq analysis were filtered using GO term annotations for protein coding genes with cytokine (GO:0005125), chemokine (GO:0008009) and growth factor (GO:0008083) activity. Hierarchical clustering on the filtered genes was performed using Genesis software. On the heatmap, the solid-line box highlights genes specifically induced by *C. parapsilosis*, while the dashed box highlights genes induced specifically by *C. albicans*. On the right, inside the enlarged boxes are displayed the names of all the differentially induced genes in bold, with the names of the respective proteins they code for on the right. On the heatmap, yellow means upregulation, blue means downregulation.

Results revealed that expression of immune mediators varies depending on the *Candida* spp. used for stimulation. In regards to cytokine-, chemokine- and growth factor-coding genes, hierarchical clustering of the filtered genes indicated that whilst the majority of genes are “similarly” expressed by both *Candida* spp., there are smaller gene sets that appear to be exclusively upregulated by specific *Candida* spp. *C. parapsilosis* is predicted to exclusively induce expression of eighteen different immune mediators, and a smaller set of seven genes is predicted to be triggered exclusively by *C. albicans*.

3.3.2 Type I IFN pathway is strongly activated in BMDMs stimulated with *C. parapsilosis* but not with *C. albicans*

A number of cytokines, chemokines and growth factors were shown to be differently regulated by *C. parapsilosis* and *C. albicans* at the RNA level, suggesting that the two *Candida* spp. induce distinct immune responses in BMDMs. To further validate this, I aimed to identify the different pathways activated by the two *Candida* spp., however, the large number of genes obtained from RNAseq made data interpretation challenging, therefore I performed k-means clustering to group genes sharing similar expression patterns and to facilitate the analysis. For this purpose, significantly induced genes ($\log_2(\text{FC})$ lower than -1.5 and higher than 1.5, adjusted p-value ≤ 0.05) from all the conditions tested in the RNAseq initial dataset were used for k-means clustering (Figure 3.3).

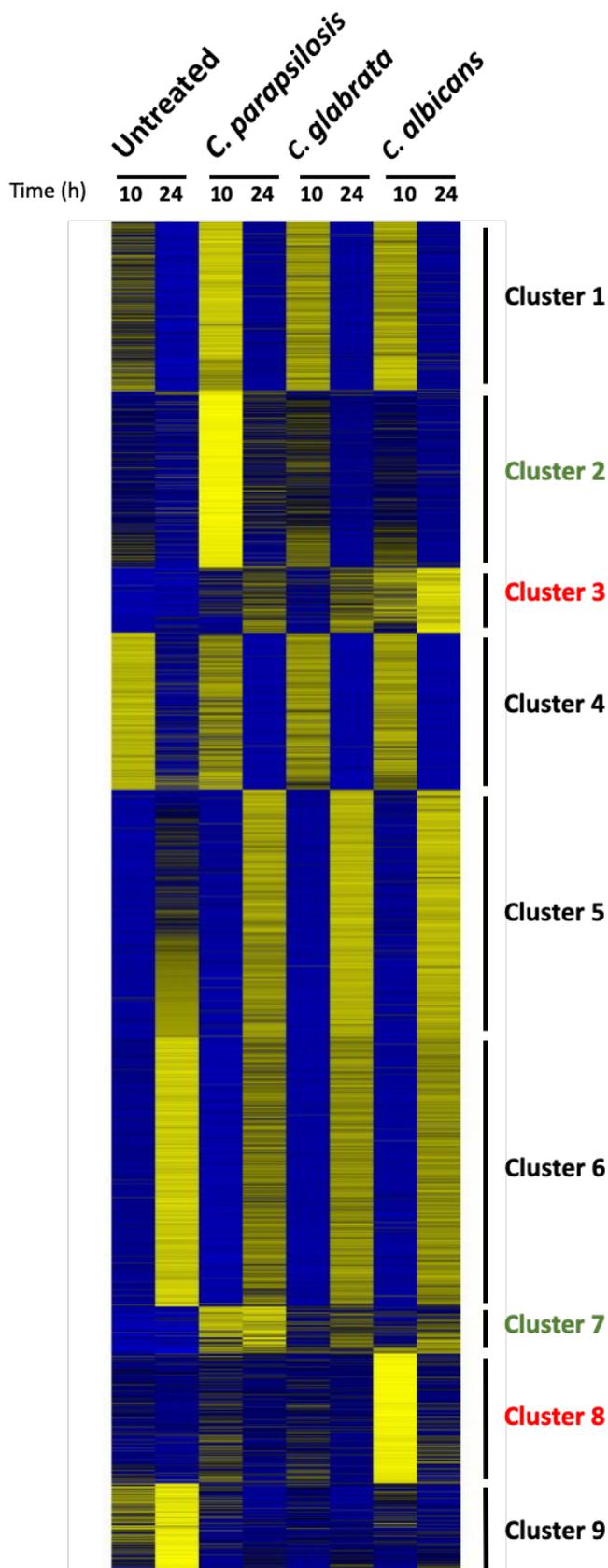


Figure 3.3 – Genes that share similar expression patterns are induced in BMDMs after *Candida* spp. stimulation. Nine gene clusters were generated by k-means clustering of genes obtained from RNAseq that fitted a cut-off of $\log_2(\text{FC}) \geq 1.5$ and adjusted p -value ≤ 0.05 . K-means clustering and heatmap were created using Genesis software. Clusters in green (clusters 2 and 7) comprise mostly genes that are exclusively upregulated during *C. parapsilosis* stimulations, and clusters in red (clusters 3 and 8) during *C. albicans* stimulations. In the heatmap, yellow means upregulation, blue means downregulation.

K-means clustering groups genes from a specific dataset that share similar expression patterns into a number of clusters previously set. To select an ideal cluster number for posterior analysis, initially a high number of clusters was set (fifteen) and main expression trends were identified in each obtained cluster. Seven main trends were observed by analysing the expression graphs from each of the initial fifteen clusters generated (data not shown). The number of resulting clusters was then reduced until one of these identified trends were lost, which happened when eight clusters were generated. Using this observation as a guide, the ideal number of clusters set for this analysis was nine.

These nine different clusters revealed the following main expression patterns: Clusters 1 and 4 comprise mostly genes upregulated at 10 h stimulations and downregulated at 24 h; Cluster 2 includes genes upregulated with *C. parapsilosis* stimulations at 10 h; Cluster 3 includes genes downregulated in untreated controls and upregulated during *C. albicans* stimulations at 24 h; both Cluster 5 and 6 comprise genes downregulated at 10 h stimulations and upregulated at 24 h; Cluster 7 includes genes downregulated in untreated controls and upregulated during *C. parapsilosis* stimulations at both 10 h and 24 h; Cluster 8 includes genes upregulated with *C. albicans* stimulations at 10 h; Cluster 9 comprises genes upregulated in untreated controls at 24 h.

Clusters comprising genes whose expression is mostly specifically upregulated during either *C. parapsilosis* (Clusters 2 and 7) or *C. albicans* (Clusters 3 and 8) infection were then analysed on Ingenuity Pathway Analysis (IPA) software to determine the main canonical pathways associated with each cluster (Figure 3.4)

<i>Candida</i> sp. specificity	Cluster	Top 5 canonical pathways	$-\log_{10}$ (p-value)	Molecules
<i>C. parapsilosis</i>	2	Interferon Signalling	10.6	IFI35, IFIT1, IFIT3, IFITM3, IFNB1, ISG15, OAS1, SOCS1, STAT1, STAT2, TAP1
		Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	10.3	CCL5, DDX58, EIF2AK2, IFIH1, IFNB1, IL12B, IL18, IRF7, NOD1, OAS1, Oas1b, Oas1d, Oas1h, OAS2, OAS3, RIPK2, TLR3, TLR7, TLR9
		Role of Hypercytokinemia/hyperchemokinememia in the Pathogenesis of Influenza	8.29	CCL2, CCL5, CCR5, CXCL10, IFNB1, IL12B, IL15, IL18, IL1RN
		Activation of IRF by Cytosolic Pattern Recognition Receptors	6.88	DDX58, DHX58, IFIH1, IFIT2, IFNB1, IRF7, ISG15, STAT1, STAT2, ZBP1
		Communication between Innate and Adaptive Immune Cells	5.61	CCL5, CXCL10, IFNB1, IL12B, IL15, IL18, IL1RN, TLR3, TLR7, TLR9
	7	Graft-versus-Host Disease Signalling	5.6	CD86, HLA-A, HLA-F, IL1A, IL6
		Communication between Innate and Adaptive Immune Cells	5.55	CCR7, CD86, HLA-A, HLA-F, IL1A, IL6
		Hepatic Fibrosis / Hepatic Stellate Cell Activation	5.09	CCR7, COL9A3, IL1A, IL1R2, IL6, MMP2, VEGFA, VEGFC
		Allograft Rejection Signalling	4.8	CD86, H2-M2, H2-T22, HLA-A, HLA-F
		Granulocyte Adhesion and Diapedesis	4.53	Ccl2, Ccl7, CLDN2, IL1A, IL1R2, MMP2, MMP3
<i>C. albicans</i>	3	eNOS Signalling	3.5	ADCY6, AQP3, BDKRB2, HSPA1A/HSPA1B, Hspa1b, IRS2, PGF
		IL-10 Signalling	2.63	ARG2, CCR1, IL1R1, IL36G
		STAT3 Pathway	2.443	CISH, IGF1R, SOCS2, TGFBR3
		Huntington's Disease Signalling	2.4	CACNA1B, CPLX2, GNG4, HSPA1A/HSPA1B, Hspa1b, IGF1R, IRS2
		Serotonin Receptor Signalling	2.34	ADCY6, HTR2A, HTR7
	8	Hematopoiesis from Multipotent Stem Cells	4.09	CSF1, CSF2, CSF3
		Hematopoiesis from Pluripotent Stem Cells	3.75	CSF1, CSF2, CSF3, LIF
		Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	3.27	CSF1, CSF2, HLA-A, IL36A, SLAMF1
		Atherosclerosis Signalling	2.63	COL5A3, CSF1, F3, IL36A, TNFSF14
		Role of Tissue Factor in Cancer	2.55	CSF1, CSF2, EGR1, F3, HBEGF

Figure 3.4 – Activation of different canonical pathways in *C. albicans* and *C. parapsilosis*-specific clusters. IPA core analysis was performed using individual lists with genes from each *Candida* specific clusters and then overlaid with the global molecular network in the Ingenuity pathway analysis knowledge base (IPKB). Results display the main canonical pathways predicted to be activated in each cluster according to *p*-value as well as the molecules within each cluster that belong to these pathways.

From *C. parapsilosis*-specific clusters, Cluster 2 is comprised mostly of genes that code for proteins associated with viral recognition by host cells (e.g. TLR7, TLR9, NOD1, IRF7), proteins related to an Interferon signature like transcription factors (e.g. STAT1, STAT2), cytokines and chemokines involved in the chemotaxis of leukocytes and monocytes (CXCL10 and CCL5), T and B cell-activating cytokines (e.g. IFN- β , IL-12p40, IL-15) (Figure 3.3 and 3.4). Interestingly, Cluster 7 also appears to be enriched in genes associated with the link between innate and adaptive immunity like MHC class I complex proteins (e.g. HLA-A, HLA-F) (Figure 3.3 and 3.4). Regarding *C. albicans*-specific clusters, Cluster 3 comprises genes coding for proteins involved in eNOS signalling (e.g. PGF, ADCY6 and BDKRB2), genes responsible for inhibition of JAK2-dependent phosphorylation of STAT3 (e.g. SOCS2, CISH) and genes produced as a consequence of IL-10 signalling activation (e.g. ARG2, CCR1) (Figure 3.3 and 3.4). Cluster 8 is enriched for genes mostly associated with haematopoiesis (e.g. M-CSF, GM-CSF and G-CSF) (Figure 3.3 and 3.4).

A lot of genes in Cluster 2, which includes mainly genes upregulated during *C. parapsilosis* infection at 10 h post stimulation, are associated with type I Interferon (IFN) induction (e.g. STAT1, STAT2, IRF7, ISG15) and downstream signalling (e.g. IFIT1, IFIT3, SOCS1). Also, many cytokines and chemokines present in this cluster (e.g. IL-15, IL-27, CXCL10 and CCL5) are known to be induced by type I IFN⁴⁶⁵⁻⁴⁶⁷. These molecules, along with the presence of the gene that codes for IFN- β (IFNB1), suggested that type I IFN signalling pathway was strongly activated in BMDMs after stimulation with *C. parapsilosis*, whilst the same did not appear to be case for BMDMs that were stimulated with *C. albicans*. To confirm this, using the data obtained from RNAseq, I decided to evaluate how the two *Candida* spp. induced the expression of type I IFN-regulated genes (IRGs), as a surrogate indicator of type I IFN signalling pathway activation. For that purpose, individual lists containing adjusted p -value and $\log_2(\text{FC})$ expression values were generated for genes expressed by BMDMs during *C. albicans* and *C. parapsilosis* infection after 10 h and 24 h stimulation. Each list was then filtered on the INTERFEROME database to find type I IRGs known to be expressed by BMDMs. Volcano plots were drawn for each condition, and type I IRGs are represented by red dots (Figure 3.5).

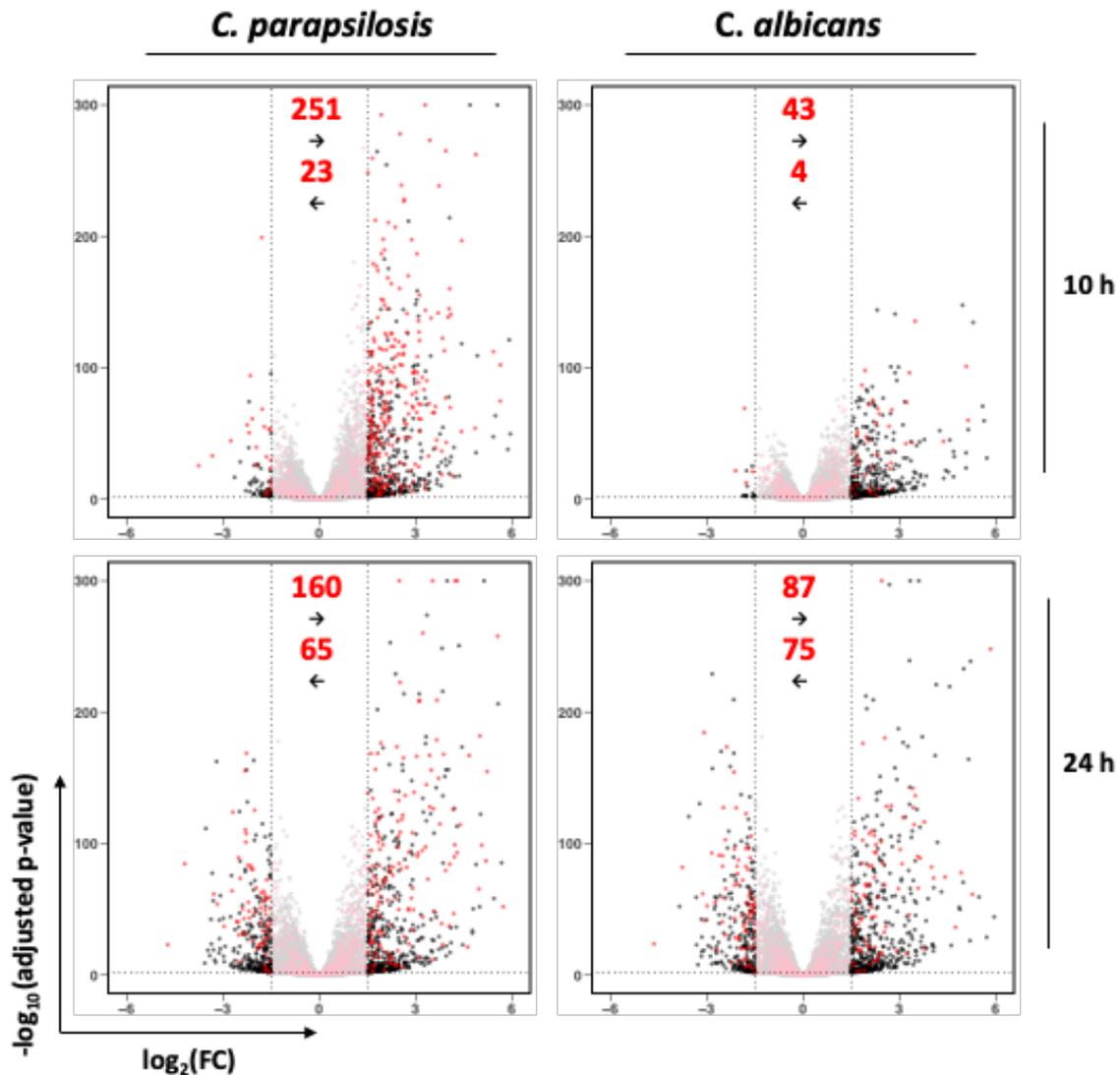


Figure 3.5 – *C. parapsilosis* induces significant expression of a higher number of type I IFN-regulated genes by BMDMs compared with *C. albicans*. Volcano plots were drawn using lists of adjusted p-values and normalised gene expression values ($\log_2(\text{FC})$) from BMDMs stimulated with *C. parapsilosis* and *C. albicans*. These lists were generated by comparing each infection condition with their respective untreated control at the same timepoint. Dashed lines mark the set significance cut-offs (adjusted p -value of 0.05, $\log_2(\text{FC}) = -1.5$ and $\log_2(\text{FC}) = 1.5$). Genes were filtered using the INTERFEROME database (www.interferome.org) and type I IRGs known to be expressed by BMDMs present in each graph were highlighted in red. Red numbers represent the amount of significantly upregulated (\rightarrow) and downregulated (\leftarrow) type I IRGs in each graph. Volcano plots were drawn by Dr. Robert Andrews using R.

This analysis revealed that *C. parapsilosis* triggers upregulation of a higher number of type I IRGs in BMDMs than *C. albicans* (Figure 3.5). Although this was observed at both timepoints tested, this was more evident at the 10 h timepoint, where the number of significantly upregulated type I IRGs by *C. parapsilosis* (251 genes) was almost six-fold higher than the ones induced by *C. albicans* (43 genes).

Since IPA analysis predicted the activation of type I IFN signalling pathway in BMDMs by *C. parapsilosis* and not *C. albicans*, and *C. parapsilosis*-induced transcriptome appears to be richer in type I IRGs, next I decided to validate activation of this pathway (Figure 3.6). Therefore, I quantified the expression of IFN- α (*Ifna4*) and IFN- β (*Ifnb1*), the main type I IFN cytokines responsible for activating type I IFN signalling pathway, in BMDMs after stimulation with *Candida* spp. by RT-qPCR

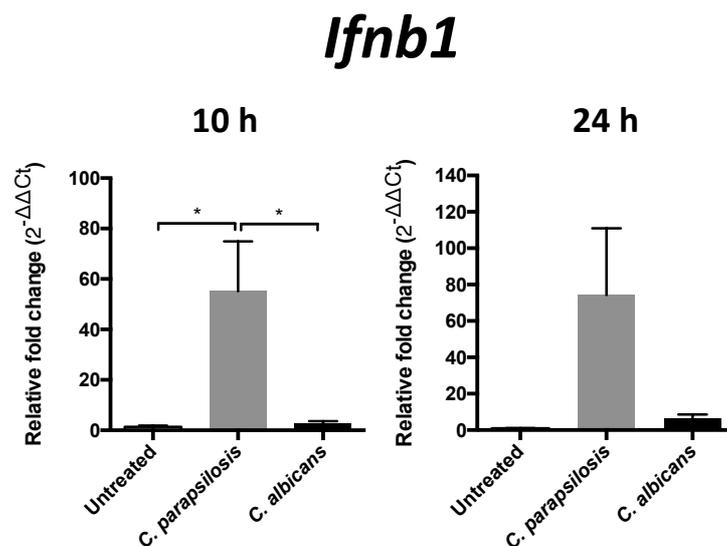


Figure 3.6 – IFN- β gene is highly upregulated in *C. parapsilosis*-stimulated macrophages. *C. parapsilosis* and *C. albicans* were added to BMDMs for 10 h (left graph) or 24 h (right graph). Macrophage RNA was extracted and converted to cDNA, and *Ifnb1* expression was quantified by RT-qPCR using *Hprt1* as the normalising housekeeping gene. Data is represented as relative fold change of *Ifnb1* ($2^{-\Delta\Delta Ct}$) in each *Candida* spp. stimulation conditions compared with the normalised untreated control at the same time point. Results are displayed as means \pm SEM, n=3, One-way ANOVA with Tukey post-test, * $p < 0.05$.

Unlike IFN- β (*Ifnb1*), very high to no Ct values were detected after 45 PCR cycles for IFN- α (*Ifna4*), and no clear differences in gene expression were detected in *Candida* spp.-stimulated cells versus untreated controls (data not shown). At both timepoints tested (10 h and 24 h) *Ifnb1* was highly upregulated in *C. parapsilosis*-stimulated BMDMs, but not during *C. albicans* stimulation when compared to untreated controls (Figure 3.6). *Ifnb1* expression was higher at 24 h (83.26 ± 44.84 fold change) than at 10 h (47.02 ± 10.04 fold change). Although significance was verified at 10 h, it was not achieved at 24 h, however, the results obtained at both timepoints show a clear biological trend of increased expression of this gene in BMDMs after stimulation with *C. parapsilosis*. IFN- β expression triggered by *C. albicans* was

remarkably lower than that induced by *C. parapsilosis* and not statistically significant (10 h: 2.65 ± 1.75 fold change; 24 h: 6.39 ± 3.72 fold change).

3.4 Discussion

C. albicans, *C. parapsilosis* and *C. glabrata* each cause systemic candidiasis, however their incidence rates vary wildly^{3,12,13,25,27,35}, and disease outcome also varies depending on the species. Whilst *C. albicans*-induced systemic candidiasis is associated with poor clearance, especially from the kidney, and a high degree of inflammation during later stages of infection, *C. glabrata*- and *C. parapsilosis*-induced candidaemia is usually associated with more effective clearance, and reduced kidney inflammation^{154,304}. These outcomes are greatly influenced not only by the intrinsic virulence of each fungal pathogen but also by the cytokine milieu and immune pathways triggered during the onset of disease. Indeed, the set of immune mediators secreted by innate phagocytes upon initial contact with invading *Candida* is critical as they can shape the immune response necessary for efficient pathogen clearance. On the other hand, an inappropriate response can culminate in pathogen survival, promote dissemination and trigger excessive inflammatory progression of disease⁴⁶⁸. Therefore, understanding how *Candida* spp. with differing virulence influence the immune pathways triggered upon infection can provide an insight into the factors underlying *Candida* pathogenicity and can ultimately be explored to identify potential therapeutic targets for systemic candidiasis.

3.4.1 ***Candida* spp. with differing virulence induce distinct gene expression in a species-specific manner**

Monocytes and macrophages are some of the first cells to recognise *Candida* spp. and initiate host immunity during infection. Although these cells are not as efficient as granulocytes at eliminating *Candida*, macrophages are the main producers of cytokines and chemokines during early infection making them particularly important in inducing cell-cell signalling and initiating inflammation⁴⁵⁶.

RNAseq was performed to track differences in the BMDM transcriptome after infection with the *Candida* spp. selected for this study. Results revealed that besides sharing commonly up- and down-regulated genes, there are sets of genes that are exclusively induced by the different *Candida* spp. (Figures 3.1 and 3.3). This was particularly evident in infections with *C. albicans* and *C. parapsilosis*. *C. glabrata* did not induce expression of a large set of exclusively regulated genes. This finding fits with previously published data regarding the virulence of

these species³²¹, as *C. albicans* and *C. parapsilosis* are on opposite ends of the virulence spectrum and they induce the highest number of exclusively regulated genes. Whilst *C. glabrata* has intermediate virulence, and the data suggests that the *C. glabrata*-induced transcriptome comprises mostly genes that are also significantly regulated during *C. albicans* and *C. parapsilosis* infection.

In a similar manner, comparative RNAseq studies revealed substantial differences in the macrophage transcriptome triggered by *M. tuberculosis*, a highly virulent causative agent of tuberculosis, and *M. bovis*, a low virulence species with lower incidence and significantly less infectious in immunocompetent hosts, and between *M. tuberculosis* strains with differing virulence^{469,470}. Unlike *M. bovis*, *M. tuberculosis* is highly infectious in humans but not in bovines where *M. tuberculosis* is unable to sustain infection and shows reduced pathology compared with *M. bovis*. Comparative transcriptomic analysis revealed that *M. bovis* induces much stronger activation of cytosolic DNA-sensing pathways, production of type I IFN and activation of membranal type VII secretion systems in bovine macrophages compared with *M. tuberculosis*, as a mechanism to rupture the phagosome and facilitate its escape from macrophages, which accounts for its prevalence and pathogenicity within bovine hosts⁴⁷⁰. Another comparative study showed that the avirulent *M. tuberculosis* H37Ra strain induces higher expression of SLC7A2 in macrophages, a gene involved in NO production pathway, compared with the virulent H37Rv strain revealing its importance in regulating the intracellular survival of *M. tuberculosis* and host resistance⁴⁶⁹. These studies revealed that, despite their phylogenetic similarity, pathogens with differing virulence trigger distinct host immune responses similar to my findings with different *Candida* spp. especially with *C. albicans* and *C. parapsilosis*.

My results revealed that *C. parapsilosis* induces higher number of differentially expressed immune mediator transcripts than *C. albicans*. Some of these findings confirm previously published data. For examples, *C. parapsilosis* was shown to induce higher levels of IL-6, IFN- β , IL-27 and IL-12 than *C. albicans* in phagocytes^{154,184,453,471}. Transcriptomic analysis performed by Németh and colleagues revealed robust induction of CCL7, CCL5 (chemoattractants for monocytes and T cells, respectively) and IL-15 in *C. parapsilosis*-infected J77A cells, however they showed the opposite results for CCL2⁴⁵³. CCL2 was

described to be predominantly induced by *C. albicans* and not *C. parapsilosis* in multiple infection models^{453,472,473}, but my research indicates the reverse in BMDMs. The reason for this is unclear, however multiple factors such as the usage of murine primary cells over cell lines and longer stimulation timepoints for my experiments might explain the differences between the studies. Moreover, CCL2 production in monocytes stimulated with *C. albicans* was shown to depend on hyphal formation and length⁴⁷⁴ and in these experiments *Candida* cells were inactivated with amphotericin B after 2 h of co-stimulation, which stopped filamentation and did not lead to formation of extensive hypha.

Some *C. parapsilosis*-specific immune mediators detected in my analyses have previously been implicated in the immune response against other *Candida* spp. TNFSF8 was implicated in generation of Th2 cell responses and generation of memory CD8⁺ T cells⁴⁷⁵ and was shown to play a role in the susceptibility of patients with burn injuries to *C. albicans* infections⁴⁷⁶. KITL (stem cell factor or c-kit ligand) is a hematopoietic cytokine involved in the proliferation of mast cells^{477,478} that was implicated in the immune response against *C. albicans*^{479–482}. CXCL10 (IP-10) is an interferon-inducible chemokine that promotes chemotaxis of Th1 cells and NK cells^{483,484} and was implicated in mucocutaneous *C. albicans* and *C. tropicalis* infections as the fungi were shown to block its production in keratinocytes as a mechanism to inhibit Th1 lymphocyte recruitment to infected sites⁴⁸⁵. *C. albicans* was shown to induce production of TSLP, a chemokine related to IL-7 strongly associated with promotion of Th2 proliferation, in DCs via Dectin-1 signalling, however the biological relevance of its production in the context of candidiasis was not assessed⁴⁸⁶. Nevertheless, these previous observations were done in different cell types other than BMDMs. The ability of *C. parapsilosis* to trigger expression of these genes in those cell types and their impact on *C. parapsilosis*-triggered infection has yet to be assessed.

Other *C. parapsilosis*-specific immune mediators were implicated in several immunological processes, however their impact on *Candida* infections was not yet studied. Bone morphogenetic proteins like BMP-8A are growth factors from the TGF- β family that are involved in CD4⁺ T cell activation and homeostasis and production of IFN- γ by CD8⁺ T cells⁴⁸⁷. CLCF1 (cardiotrophin-like cytokine factor 1) is extensively studied as a neurotrophic factor, however it was shown to exert immunomodulatory effects such as promoting B cell expansion

and humoral responses and affecting leukocyte hematopoiesis⁴⁸⁸. TNFSF15 (or TNF-like ligand 1A) exerts pleiotropic effects on adaptive immunity and was implicated in different Th responses such as synergising with IL-12 to promote IFN- γ production and Th1 polarisation, promoting deleterious Th2 responses and contributing to lung inflammation in mice models of asthma, negatively regulating Th17 proliferation independently of IL-2 production and inducing proliferation of Tregs in lymphoid organs of the small intestine⁴⁸⁹.

C. albicans-specific cytokines IL-36 α , IL-36 γ and G-CSF were shown to confer epithelial protection during mucocutaneous candidiasis by promoting protective IL-23 secretion and granulopoiesis, and their expression was shown to be triggered by candidalysin^{85,87,490}. As ECE1, the polypeptide precursor of candidalysin is only expressed by *C. albicans*, *C. tropicalis* and *C. dubliniensis*^{80,81}, it explains why these immune mediators were not strongly induced in *C. parapsilosis* stimulations. From the other *C. albicans*-specific immune mediators, only CCL6 (C10), an IL-4, IL-10 and IL-13-inducible monocyte/macrophage and neutrophil chemoattractant that signals through CCR1, was reported to be induced by *C. albicans*. CCL6 is one of the main CCR1 ligands produced during *C. albicans*-induced systemic candidiasis by kidney cells and along with CCL3, CCL5, CCL7 and CCL9 it contributes to neutrophil-driven immunopathology during infection^{317,318}.

The other immune mediators specifically induced by *C. albicans* were shown to influence host immunity however their impact during *Candida* infections remains understudied. HB-EGF (heparin-binding growth factor) is a growth factor from the EGF family that is mainly involved in several developmental processes. Not much is known regarding the effect of HB-EGF on host immunity, however reports have shown that it can induce an M2-like polarisation in macrophages and exert anti-inflammatory effects^{491,492}. Nevertheless, the M1/M2 model oversimplifies macrophage heterogeneity, as macrophage activation is reflective of a broad spectrum of changes that cannot be properly described using this binary terminology^{493,494}.

PGF (placental growth factor) is mostly involved in angiogenesis and endothelial cell proliferation, however it was shown to exert pleiotropic effects in host immunity such as promoting monocyte chemotaxis and production of pro-inflammatory cytokines, inhibiting

maturation, MHC II expression and IL-12, IL-8 and TNF production in DCs and impairing cytotoxic functions and IFN- γ production by NK cells⁴⁹⁵. TNFSF18 (GITR) is a member of the TNF superfamily that acts mainly on Tregs to negatively modulate their cell suppressor activity and to promote a prevalent Th1 or Th17 response^{496,497}.

3.4.2 Type I IFN pathway is strongly activated in BMDMs stimulated with *C. parapsilosis* and not with *C. albicans*

Transcriptomic analysis of each individual *Candida* sp.-specific clusters facilitated identification of host immune pathways exclusively activated by *C. albicans* and *C. parapsilosis*. *C. albicans*-species clusters comprised genes mostly involved in granulopoiesis, which aligns with published literature as *C. albicans* was shown to induce secretion of these factors such as GM-CSF (*CSF2*)^{314,498–500} and G-CSF (*CSF3*)^{100,281,500–502} during infections. These factors enhance proliferation and boost candidacidal activities of neutrophils and monocytes at infected sites^{391,503,504}. Moreover, β -glucan from *C. albicans* was also shown to promote monocyte to macrophage differentiation partially via M-CSF (*CSF1*) release⁵⁰⁵. Besides expression of hematopoietic genes, *C. albicans* induces expression of genes involved in the IL-10 pathway such as IL-10, arginase-2 (*ARG2*) and CCR1. This is in accordance with published reports that have previously shown that *C. albicans* triggers higher levels of IL-10 compared with *C. parapsilosis* in macrophages^{154,183,506}, as a mechanism to induce M2 polarisation which greatly impairs macrophage phagocytosis and candidacidal activity, promoting *C. albicans* survival during infection^{183,506}.

Unlike, *C. albicans*, the *C. parapsilosis*-specific immune response appears to be dominated by a type I Interferon signalling signature. Several *C. parapsilosis*-induced nucleic acid-sensing PRRs such as TLR3, -7, -9, NOD1 and MDA5 (*Ifih1*) are known to induce type I IFN^{507–510}, and transcription factors STAT1, STAT2, IRF7 are triggered by type I IFN signalling^{511,512}. Moreover, cytokines IL-15 and IL-18^{513,514} and chemokines CCL2, CCL5 and CXCL10^{515–519} are known to be type I IFN-regulated genes (IRGs). The activation of type I IFN signalling was then supported by the fact that the global transcriptome of *C. parapsilosis*-infected BMDMs includes a much higher number of type I IRGs comparatively to *C. albicans* and was confirmed by validation of IFN- β as the main activator of said pathway. These results

corroborate previous findings published by our group, as *C. parapsilosis* (and not *C. albicans*) was found to induce a robust, phagocytosis-dependent IFN- β production via TLR7 and NOD2-mediated signalling in BMDMs¹⁸⁴. This was the first study to describe *C. parapsilosis* as an inducer of innate type I IFN production in macrophages, however the literature is controversial about the ability of *C. albicans* and other *Candida* spp. to induce IFN- β , and the associated mechanism of its production.

Bourgeois and colleagues found that *C. glabrata*, *C. albicans* and *C. dubliniensis* trigger production of IFN- β in BMDCs, with *C. glabrata* inducing much higher levels than the other two, in a TLR7/MyD88 and IRF1-dependent manner¹⁸⁸. IFN- β was not detected at the mRNA level in *C. glabrata*-infected BMDMs after 4 h of stimulation in their study, however it is important to note that these authors worked with GM-CSF-derived macrophages that are naturally less responsive to type I IFN than M-CSF-BMDMs due to a lower basal expression of genes involved in its signalling pathway such as STAT1, TLR7, TLR3, and IRF7^{188,520}. As authors showed in BMDCs that *C. glabrata*-driven IFN- β production is greatly augmented via an IRF7- and STAT1-dependent autocrine/paracrine positive feedback loop¹⁸⁸, the reduced basal expression of these key transcription factors in GM-CSF-BMDMs can be the reason why IFN- β transcripts were not significantly detected after such a short stimulation period with *C. glabrata* (4 h). Moreover, GM-CSF-BMDMs are not an ideal macrophage model to study *Candida*-host interactions, as M-CSF is the main, most abundant cytokine in circulation that induces macrophage differentiation⁵²¹, which makes *in vitro* results using M-CSF-BMDMs more reflective of *in vivo Candida* infections. Biondo *et al.* showed that *C. albicans* also induces production of IFN- β in BMDCs in a TLR7/TLR9/MyD88- and IRF1/3/7- dependent fashion¹²². They tested three different live *C. albicans* strains (but not SC5413 strain), *C. glabrata* and *C. tropicalis* but IFN- β levels detected were minimal (~20 pg/mL) regardless of the species and MOI tested. Authors did not detect IFN- β transcripts in M-CSF-BMDMs infected with *C. albicans* ATCC 90028 for 10 h even at an MOI of 2:1¹²², which did not happen in my experiments, as low levels of IFN- β mRNA were detected with *C. albicans* stimulations at that same timepoint (although not significant when compared with the untreated control). Nevertheless, it is possible that is reflective of unidentified strain-specific differences in fungal ligand levels and exposure between *C. albicans* ATCC 90028 and SC5314. Del Fresno and

colleagues showed that heat-killed *C. albicans* and curdlan can trigger IFN- β production in BMDCs but in a Dectin-1/Dectin-2/Syk/CARD9/IRF5-dependent manner, suggesting that fungal β -glucan is the trigger for its induction, independently of yeast viability¹⁸⁹. Nevertheless, the authors did not test the ability of live *C. albicans* to induce IFN- β , and the heat-killing process sheds the mannan layer that normally masks β -glucans on the cell wall⁵²². This makes the β -glucans readily available for detection by Dectin-1 and therefore amplifies Dectin-1-mediated signalling, which does not happen in infections with live *C. albicans* as β -glucans are only exposed in low amount at yeast bud scars¹³⁵. Using naïve human PBMCs, Smeekens *et al.* revealed that live *C. albicans* UC 820 stimulated the secretion of robust levels of IFN- β , however PBMCs comprise mixed populations of lymphocytes (70-90%) and leukocytes (10-20%) and the authors did not identify the specific cell type(s) responsible for its production^{187,523}.

Overall, regardless of the *Candida* spp. tested, most literature indicates that BMDCs produce higher levels of IFN- β compared with macrophages, although results from our group suggest the opposite¹⁸⁴. It is challenging to explain such heterogeneity, as the reason behind the ability of different *Candida* spp. to trigger IFN- β in different myeloid cells is not yet fully understood. However, DCs were shown to exhibit higher phagocytic rate towards *C. albicans* than macrophages mainly due to their higher expression of MR, a *Candida* phagocytic receptor that recognises N-linked mannans in the cell wall^{135,175,524}. This is relevant in the context of IFN- β induction by live *Candida* spp. considering that it was shown to be dependent on phagocytosis^{98,122,184}. Furthermore, *C. parapsilosis* is internalised at a higher rate than *C. albicans* in both macrophages and neutrophils^{77,525}, and although the reason why is also unclear, it could possibly be due to the fact that *C. parapsilosis* cell wall is richer in mannans compared to both *C. albicans* and *C. glabrata* which could contribute for its ability to induce higher levels of IFN- β ^{154,524}. This suggests that the abundance and exposure of different fungal ligands responsible for IFN- β induction verified in different *Candida* spp. strains can influence activation of this pathway in host cells and could potentially account for the different levels detected. Moreover, “M2” macrophage polarisation induced by *C. albicans* greatly impairs phagocytosis which might contribute in part for the differences observed in my research^{183,506}.

3.5 Conclusions

The results presented in this chapter revealed that macrophages respond differently to *Candida* spp. during infection and that the immune response triggered appears to be reflective of *Candida* spp. virulence. My research suggests that whilst the *C. albicans*-induced immune response appears to be dominated by an IL-10-triggered signalling and candidalysin-induced cytokine production (although this still needs further validation), the response induced during *C. parapsilosis* infections appears to be predominantly controlled by a type I IFN signalling activation, triggered by a strong IFN- β production, which was not observed with *C. albicans*.

Data presented here fulfilled the aims and confirmed the hypothesis set for this chapter, as it clarified that *C. parapsilosis* and *C. albicans* trigger distinct immune responses in macrophages and facilitated the validation of type I IFN pathway as a target for immune modulation in infections caused by highly pathogenic *C. albicans*.

Chapter 4

Investigating the candidacidal properties of
type I Interferon

4.1 Introduction

Macrophages play an important role during systemic candidiasis. Tissue-resident macrophages function as a first line of defence and can protect hosts by recognising invading *Candida* at early stages of infection, preventing host dissemination and initiating host immunity^{526,527}. Chemoattractants secreted as a consequence of this initial recognition will then recruit circulating inflammatory monocytes and monocyte-derived macrophages to the infected site to aid in the killing of the pathogen. When phagocytes and tissue immunity fail to limit *Candida* proliferation, the pathogen reaches the host bloodstream and invades target organs. Tissue-resident macrophages in the main target organs such as the spleen, liver and kidney can also protect the host by deploying antifungal mechanisms to kill the invading pathogen and promote recruitment of other phagocytes to help clear the infection^{20,457,528}.

Most antifungal mechanisms deployed by macrophages in response to *C. albicans* are induced and regulated by pleiotropic pro-inflammatory cytokines, like Interferons. Interferons (IFNs) are a family of cytokines that mediate diverse cellular responses. These cytokines, first discovered in 1957, were initially described as an “activity” that was secreted by cells during a viral infection in order to protect other neighbouring cells from being infected^{529,530}. Therefore, they were named after their ability to induce the production of a myriad of antiviral gene products in order to limit viral replication and consequent spread of infection^{529,530}. IFNs are found in all nucleated cells in mammals, and almost all cell types in the human body can produce them in response to stimulation of an array of cytosolic and transmembrane receptors⁵³¹.

Type I IFN is a family that comprises almost 20 members, that vary between mammalian species. The only type I IFNs expressed in both human and mice are IFN- α , - β , - ω , - κ and - ϵ with the former two being the most well-characterized⁵³²⁻⁵³⁴. IFN- α is coded by 14 different genes, resulting in a vast array of isoforms with different subtype-specific functions, whilst IFN- β is coded by a single gene^{511,532}. All these cytokines are agonists of the ubiquitously expressed heterodimeric receptor composed of IFNAR1 and IFNAR2 called type I IFN-receptor or IFN- α/β R. They signal through TYK2 and JAK1 to induce the formation of STAT1-STAT2 heterodimers. These STAT molecules then associate with other Interferon regulatory factors

(IRFs) to form heterodimeric transcription factors that will induce the transcription of IFN-inducible genes⁵¹¹. Type I IFN has three major functions: to induce antimicrobial states in both infected cells and neighbouring naïve cells in order to limit the proliferation and spreading of invading pathogens; to modulate and fine-tune the innate immune response in order to promote antigen presentation and NK cell functions while limiting exacerbated inflammation and cytokine production; to induce the production of antigen-specific-T and -B cell responses and therefore activate the adaptive immune response⁵³⁵.

Regarding systemic candidiasis, the type I IFN pathway was identified as critical in conferring protection against infection as patients with polymorphisms in genes involved in this pathway are more susceptible to the disease, possibly through shifting from a protective Th1 response towards a Th17 phenotype¹⁸⁷. However, *in vivo* reports and literature in general are conflicting regarding the role of type I IFN in systemic candidiasis, as studies provide evidence that it can be either detrimental^{188,536–538} or beneficial towards infection resolution^{122,189,314}, making it difficult to ascertain its exact role in the disease.

The primary objectives of this PhD project were to identify differences in the immune response triggered in macrophages by *Candida* spp. with distinct virulence. Subsequently, it was planned to assess if modulation of the host immune response towards highly virulent species *C. albicans* to mirror the one induced by the low virulence species *C. parapsilosis* would result in improved *C. albicans* clearance and infection outcome. Results displayed in the previous chapter revealed that the immune response deployed by BMDMs after *C. parapsilosis* challenge is dominated by an IFN- β -driven signalling pathway activation, while this was not observed in *C. albicans*-infected BMDMs.

In this chapter, I have focused on exploring some of the consequences of engaging type I IFN signalling pathway in BMDMs *in vitro* and *in vivo* during *C. albicans* infection and determining its candidacidal potential.

4.2 Aims

This chapter addresses the hypothesis that activation of type I IFN signalling during *C. albicans* challenge is beneficial to the outcome of infection, resulting in improved fungal clearance. Here, various *in vitro* and *in vivo* experiments were conducted to test this hypothesis and to fulfil the following aims:

- I. To trigger type I Interferon signalling pathway in BMDMs during highly virulent species *C. albicans* infection;
- II. To assess the impact of type I IFN signalling pathway activation and elucidate its possible candidacidal mechanism in BMDMs during *C. albicans* infection;
- III. To assess the impact of type I IFN signalling pathway activation *in vivo* using a mouse model of systemic candidiasis.

4.3 Results

4.3.1 Type I Interferon administration in BMDMs shows candidacidal effect against *C. albicans* and boosts nitric oxide release

Since an IFN- β -dominated type I Interferon signalling pathway is strongly induced by low virulent species *C. parapsilosis* in BMDMs and not by the highly virulent species *C. albicans* (Chapter 3), I decided to activate this pathway and explore its impact in macrophages during infection with *C. albicans*. Double-stranded RNA (dsRNA) is known to trigger the production of type I Interferon in macrophages and dendritic cells (DCs) via TLR3 activation^{539–541}. Thus, I stimulated macrophages with polyinosinic:polycytidylic acid (poly(I:C)), a dsRNA mimetic drug, to induce type I Interferon⁵⁴¹ and evaluate its effect on the candidacidal activity of BMDMs during *C. albicans* challenge (Figure 4.1).

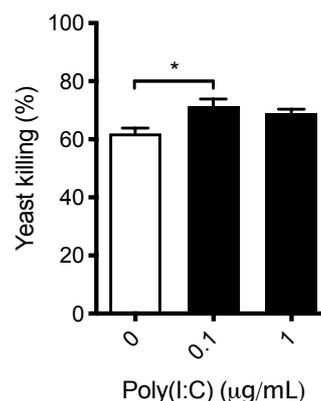


Figure 4.1 – Poly(I:C) enhances BMDMs ability to kill *C. albicans*. BMDMs were pre-stimulated with poly(I:C) for 48 h and following pre-stimulation, *C. albicans* was added to BMDMs for 3 h. BMDMs were then lysed and scraped, well contents were diluted and plated on YPDA to evaluate yeast viability by counting CFUs. No amphotericin B was added for these assays. Statistical analysis was performed by individually comparing the conditions where the cells were treated with *C. albicans* and poly(I:C), with their respective control where cells were stimulated with the pathogen in the absence of poly(I:C). Results are presented as means \pm SEM of $n=4$. Repeated Measures One-way ANOVA with Tukey post test, * $p<0.05$.

After 48 h of pre-stimulation, 0.1 $\mu\text{g/mL}$ poly(I:C) significantly boosted the ability of BMDMs to clear *C. albicans* (71.32 \pm 5.13 % killing) by 9% compared with the killing observed in the absence of the drug (62.00 \pm 3.69 % killing). At the highest concentration tested, a slightly enhanced killing was also observed (68.98 \pm 1.42 % killing) but statistical significance was not achieved.

As poly(I:C) enhanced macrophage candidacidal activity (Figure 4.1), I then investigated the molecular mechanism responsible for this. Poly(I:C) is known to elicit type I IFN production, and type I IFN induces expression of iNOS, the enzyme responsible for production of NO, an antimicrobial molecule^{199,541}. Therefore, I hypothesized that the enhanced killing of *C. albicans* induced by poly(I:C) in BMDMs was due to increased production of NO. To investigate this, I assessed the expression of *Nos2*, the gene that codes for iNOS, in BMDMs stimulated by *C. albicans* or *C. parapsilosis* (Figure 4.2).

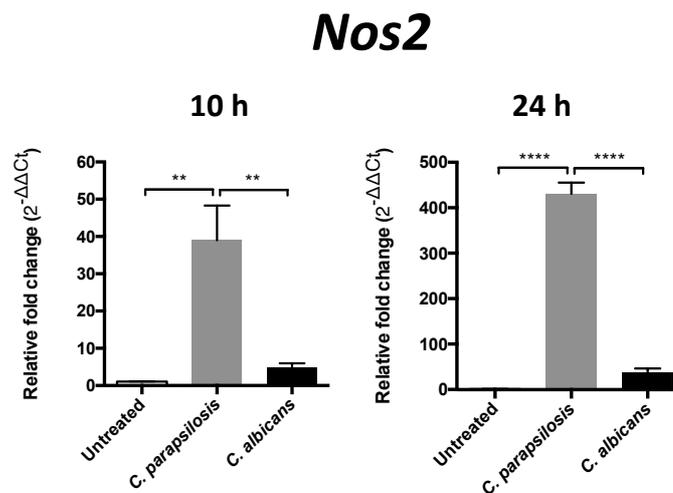


Figure 4.2 – iNOS gene is highly upregulated in *C. parapsilosis*-stimulated macrophages. *C. parapsilosis* and *C. albicans* were added separately to BMDMs for 10 h (left graph) or 24 h (right graph). Macrophage RNA was extracted and converted to cDNA, and *Nos2* expression was quantified by RT-qPCR using *Hprt1* as the normalising housekeeping gene. Data is represented as relative fold change of *Nos2* compared with the untreated control at the same time point. Results are displayed as means ± SEM, n=3, One-way ANOVA with Tukey post-test, ** $p < 0.01$, *** $p < 0.001$.

At both timepoints tested, iNOS expression was significantly elevated at RNA level in BMDMs stimulated with *C. parapsilosis*, but not with *C. albicans* (Figure 4.2). At 10 h, *Nos2* levels were 38 times higher (38.34 ± 8.696 fold change) in *C. parapsilosis*-stimulated macrophages, whilst only 5 times higher (4.670 ± 1.034 fold change) in *C. albicans* stimulations compared to the untreated control. Expression was highest after 24 h stimulation in BMDMs stimulated with *C. parapsilosis* with a 451 fold expression (450.5 ± 89.11 fold change) of iNOS, and is significantly different than the 35 fold expression (35.23 ± 2.305 fold change) induced by *C. albicans* at the same timepoint. These results demonstrate that *C. parapsilosis* significantly induces higher expression of iNOS in BMDMs compared to *C. albicans*.

As I have shown that poly(I:C), a drug that induces production of type I IFN, boosts the ability of BMDMs to kill *C. albicans*, and since the type I IRG *Nos2* is upregulated in *C. parapsilosis*-stimulated BMDMs due to strong type I IFN signalling pathway induction, I hypothesized that the enhanced candidacidal effect observed in poly(I:C)-stimulated BMDMs could be due to higher levels of NO. I then decided to quantify NO production by BMDMs stimulated by poly(I:C) in the presence of the two *Candida* species (Figure 4.3).

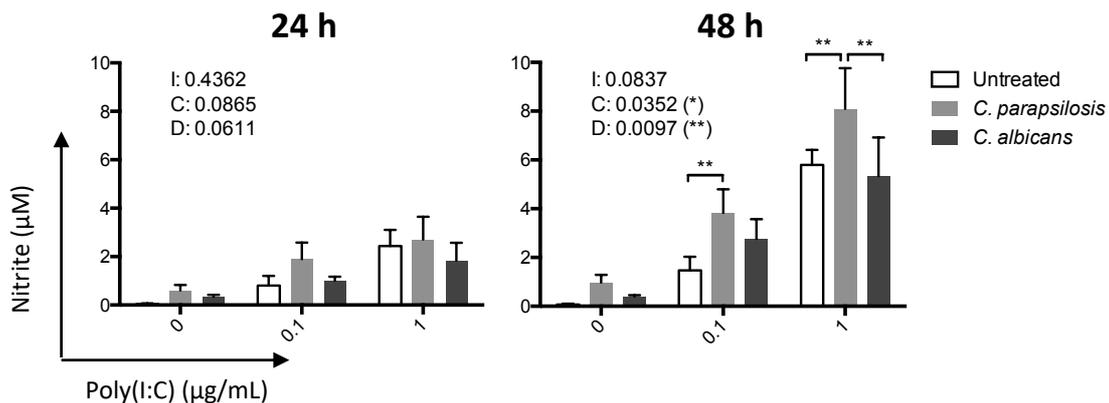


Figure 4.3 – Poly(I:C) elicits production of NO by BMDMs during *C. albicans* and *C. parapsilosis* stimulations. BMDMs were stimulated with *C. albicans* or *C. parapsilosis*, in the presence of poly(I:C) for 24 h (left graph) and 48 h (right graph). Nitrite levels in the supernatants were measured using the Griess test. Results are displayed as means \pm SEM, $n=3$, and data were analysed by Two-way Repeated Measures ANOVA with Tukey post tests to determine the impact of the different *Candida* species tested and of the poly(I:C) treatment on the release of nitric oxide by BMDMs. Significance values (p -values) are represented as: “I” – interaction between *Candida* species and poly(I:C); “C” – impact of different *Candida* species tested; “D” – effect of different concentrations of poly(I:C). Tukey post tests were performed to assess the impact of different *Candida* spp. on the release of nitric oxide when BMDMs are stimulated with a specific poly(I:C) dose. * $p<0.05$, ** $p<0.01$.

NO produced by BMDMs was quantified using the Griess test. The NO molecule is highly volatile and reactive, so this assay, first described in 1879, measures the concentration of nitrite ion (NO_2^-), one of the two primary breakdown products of NO in a solvent, in this case, the cell culture media, as a surrogate measurement of NO production by cells after stimulation⁴³⁰. At 24 h, minimal levels of nitrite were detected after incubation with poly(I:C). These levels trend slightly higher when macrophages are incubated in the presence of the highest dose of the drug tested (1 $\mu\text{g/mL}$). The highest concentration of nitrite was detected in *C. parapsilosis*-stimulated BMDMs ($1.733 \pm 0.6119 \mu\text{M}$), but with no significant differences in nitrite production being induced by the different *Candida* species. After 48 h stimulation, however, there was a clear dose-dependent trend of increased nitrite levels produced by cells

incubated with poly(I:C). Peak production occurred with the highest concentration of poly(I:C) tested. At this timepoint, the production of NO was found to be not only dependent on stimulation with poly(I:C) but also on the *Candida* species. When in the presence of 0.1 µg/mL poly(I:C), macrophages produce more NO when stimulated with *C. parapsilosis* (3.833 ± 0.960 µM) then when left uninfected (1.467 ± 0.561 µM). These levels are also higher than those induced by *C. albicans* (2.767 ± 0.801 µM) although not statistically significant. Stimulation with 1 µg/mL poly(I:C) induced the highest levels of NO, with maximum levels detected in BMDMs stimulated with *C. parapsilosis* (8.1 ± 1.662 µM). With the highest concentration of the drug, the nitrite levels were significantly higher in *C. parapsilosis*-infected BMDMs than in uninfected cells (5.8 ± 0.611 µM) and in *C. albicans*-infected BMDMs (5.333 ± 1.586 µM). Furthermore, *C. albicans* stimulation alongside poly(I:C) does majorly affect the poly(I:C)-mediated induction of NO.

Poly(I:C) was found to enhance production of NO by BMDMs in a dose- and *Candida* sp.-dependent way. By analysing the results displayed in Figure 4.3 and the killing assay results (Figure 4.1), I have postulated that NO induced by poly(I:C) could be the antimicrobial compound responsible for the enhanced killing of *C. albicans* by BMDMs. As mentioned above, even though poly(I:C) induces expression of type I IFN, it does not directly engage the Interferon- α/β receptor (IFNAR1/2), unlike type I IFNs. The upstream activation of TLR3 by poly(I:C) could induce other molecules that could potentially exert antimicrobial effects against *C. albicans* that may not be type I IFN-dependent. To rule out this possibility, I stimulated BMDMs directly with IFN- β and performed the same killing assays with *C. albicans* and Griess tests to measure NO production (Figure 4.4).

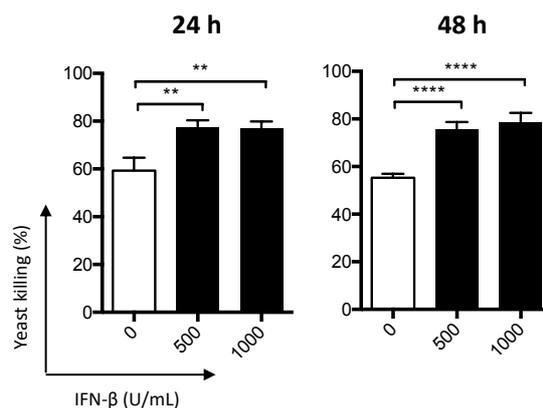


Figure 4.4 – IFN-β enhances BMDMs ability to kill *C. albicans*. BMDMs were pre-stimulated with IFN-β for 24 h (left graph) or 48 h (right graph), following pre-stimulation, *C. albicans* was added to BMDMs for 3 h. BMDMs were then lysed and scraped, well contents were diluted and plated on YPDA to evaluate killing by counting CFUs. No amphotericin B was added for these assays. Statistical analysis was performed by comparing BMDMs stimulated with *C. albicans* and IFN-β with their respective control where BMDMs were incubated with the pathogen in the absence of IFN-β. Results are presented as means ± SEM of at least n=3. Repeated Measures One-way ANOVA with Tukey post-test, ** $p < 0.01$, **** $p < 0.0001$.

Like poly(I:C), IFN-β also boosted BMDMs candidacidal activity against *C. albicans*, however this enhanced killing was much higher in BMDMs stimulated with IFN-β (Figure 4.4) than with poly(I:C) (Figure 4.1). At both timepoints, stimulation with the two different concentrations of IFN-β resulted in significantly higher killing of *C. albicans* by BMDMs. Pre-stimulation with both 500 and 1000 U/mL IFN-β for 24 h resulted in 18 % enhanced killing (500 U/mL: 77.41 ± 2.925 %; 1000 U/mL: 77.2 ± 2.644 %) compared to the control where BMDMs were infected with *C. albicans* in the absence of IFN-β (59.29 ± 5.386 %). This enhanced killing was more evident in macrophages primed for 48 h, with 500 U/mL IFN-β boosting BMDMs ability to kill *C. albicans* by 21 % (75.82 ± 2.882 %) and 1000 U/mL by 24 % (78.74 ± 3.789 %) compared to the control (0 U/mL IFN-β) at the same timepoint (55.31 ± 1.635 %).

Although not tested at the same concentrations, results presented in Figure 4.4 imply that macrophages stimulated with IFN-β display superior candidacidal activity compared to poly(I:C). Since poly(I:C) stimulation resulted in elevated production of NO by BMDMs (Figure 4.3), I next decided to assess NO production in BMDMs stimulated with IFN-β in the presence of the two *Candida* spp. using the Griess test (Figure 4.5).

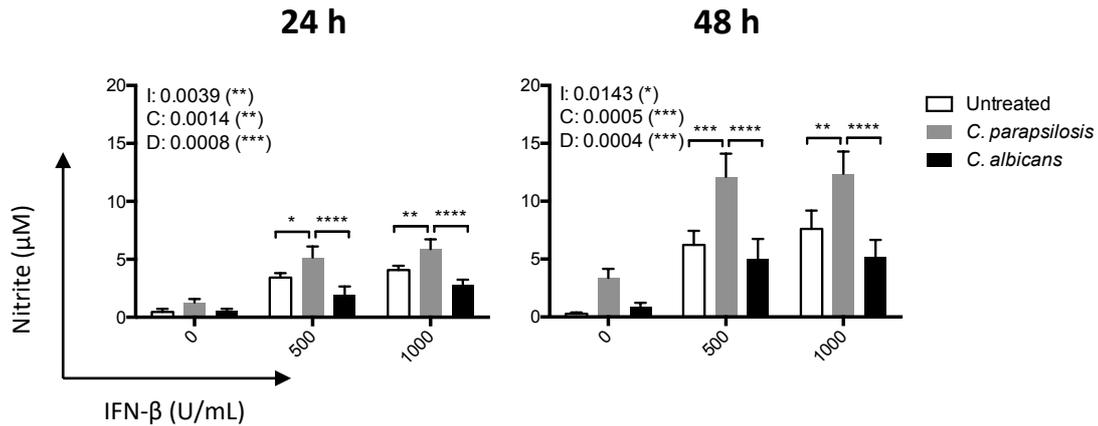


Figure 4.5 – IFN-β elicits production of NO by BMDMs during *C. albicans* and *C. parapsilosis* stimulations. BMDMs were stimulated with *C. albicans* or *C. parapsilosis*, in the presence of IFN-β for 24 h (left graph) and 48 h (right graph). Nitrite levels in the supernatants were measured using the Griess test. Results are displayed as means ± SEM, n=5, and data were analysed by Two-way Repeated Measures ANOVA with Tukey post-tests to determine the impact of the different *Candida* species tested and of the IFN-β treatment on the release of nitric oxide by BMDMs. Significance values (*p*-values) are represented as: “I” – interaction between *Candida* species and IFN-β; “C” – impact of different *Candida* species; “D” – Effect of IFN-β stimulation. Tukey post-hoc tests were performed to assess the impact of different *Candida* spp. on the release of nitric oxide when BMDMs are stimulated with a specific IFN-β dose. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

At both timepoints, higher levels of nitrite were detected in the media of cells stimulated with either concentration of IFN-β, regardless of the presence of *Candida* spp. (Figure 4.5). At 24 h, maximal production of nitrite was observed in *C. parapsilosis*-stimulated BMDMs in the presence of 1000 U/mL IFN-β (12.378 ± 1.909 µM). At this timepoint, macrophages stimulated with *C. parapsilosis* produced significantly higher levels of NO than uninfected BMDMs or BMDMs stimulated with *C. albicans* in the presence of both 500 U/mL (Untreated: 3.420 ± 0.387 µM; *C. parapsilosis*: 5.160 ± 0.943 µM; *C. albicans*: 1.960 ± 0.7 µM) and 1000 U/mL IFN-β (Untreated: 4.080 ± 0.360 µM; *C. parapsilosis*: 5.880 ± 0.847 µM; *C. albicans*: 2.820 ± 0.424 µM). Nitrite levels were highest after 48 h stimulation. At both the 24 h and 48 h timepoints, stimulation with both concentrations of IFN-β led to higher levels of NO production by BMDMs, with *C. parapsilosis*-infected BMDMs releasing more nitrite to the media than uninfected BMDMs or *C. albicans*-stimulated BMDMs at both 500 U/mL (Untreated: 6.232 ± 1.207 µM; *C. parapsilosis* : 12.107 ± 2.010 µM; *C. albicans*: 5.038 ± 1.699 µM) and 1000 U/mL IFN-β (Untreated: 7.608 ± 1.589 µM; *C. parapsilosis*: 12.378 ± 1.909 µM; *C. albicans*: 5.166 ± 1.496 µM). Moreover, similarly to what was observed with poly(I:C)

(Figure 4.3), *C. albicans* stimulation alongside IFN- β does not significantly affect the IFN- β -inducible NO production in BMDMs.

These results shown that IFN- β stimulation leads to enhanced production of nitrite in BMDMs, and that IFN- β augments the ability of these cells to kill highly virulent *C. albicans* cells (Figure 4.4). This data fit with the theory that IFN- β -induced NO could be the antimicrobial molecule responsible for enhanced antifungal activity against *C. albicans*. Since I hypothesized that this antimicrobial compound is responsible for the antifungal effect of IFN- β observed in BMDMs, I decided to perform the same type of killing assays using *C. parapsilosis*-infected BMDMs pre-stimulated with IFN- β , to assess if a similar killing trend observed with *C. albicans* was also verified against *C. parapsilosis* (Figure 4.6).

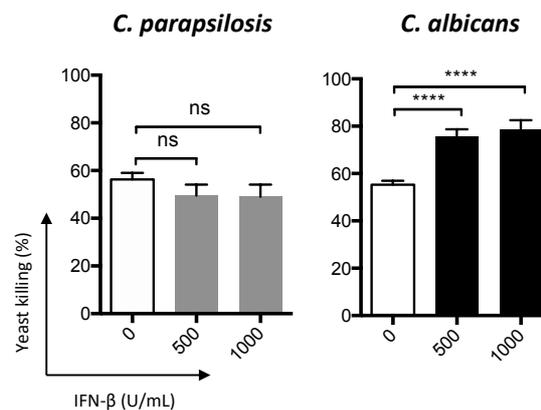


Figure 4.6 – IFN- β enhances BMDMs ability to kill *C. albicans*, but not *C. parapsilosis*. BMDMs were pre-stimulated with IFN- β for 48 h and following pre-stimulation were challenged with *C. parapsilosis* (left graph) or *C. albicans* (right graph) for 3 h. BMDMs were then lysed and scraped, well contents were diluted and plated on YPDA to evaluate killing by counting CFUs. No amphotericin B was added for these assays. Statistical analysis was performed by comparing BMDMs stimulated with *Candida* spp. and IFN- β with their respective control where BMDMs were incubated with the *Candida* spp. in the absence of IFN- β . Results are presented as means \pm SEM of n=5. Repeated Measures One-way ANOVA with Tukey post-test, ns - no statistical significance, **** p <0.0001. (The data on the right graph with the results on *C. albicans* killing is the same as that presented in the right graph in Figure 4.4).

Unlike *C. albicans*, pre-stimulation with IFN- β does not increase the ability of BMDMs to kill *C. parapsilosis* (Figure 4.6). In fact, after 48 h of incubation, IFN- β at both concentrations tested led to a slight non-significant decrease in killing of *C. parapsilosis* (500 U/mL: 49.51 \pm 4.605 %; 1000 U/mL: 49.07 \pm 5.057 %) compared with the baseline control (0 U/mL: 56.27 \pm 2.811 %). Since stimulation with *C. parapsilosis* alone already elicits the production of high

levels of IFN- β by BMDMs, the addition of IFN- β could be inducing a detrimental effect on cell viability in this experiment setting.

4.3.2 Candidacidal mechanism of IFN- β against *C. albicans* is non-nitrosative and non-oxidative

As mentioned above, in macrophages, NO is mainly produced by the enzyme inducible nitric oxide synthase (iNOS), which converts L-arginine into NO and citrulline⁵⁴². To prove the initial theory that NO is the antimicrobial molecule responsible for the enhanced candidacidal activity of BMDMs incubated with IFN- β against *C. albicans*, I performed killing assays using iNOS-deficient BMDMs primed with IFN- β for 48 h following infection with *C. albicans* (Figure 4.7-B). First, I wanted to confirm that these knock-out macrophages for *Nos2* (iNOS-coding gene) were unable to produce NO after stimulation with IFN- β and *C. albicans* (Figure 4.7-A).

As expected, iNOS-deficient BMDMs largely failed to generate detectable levels of nitrite after stimulation with both *C. albicans* and IFN- β , with minimal production detected in iNOS-deficient cells in the presence of 1000 U/mL IFN- β ($0.336 \pm 0.330 \mu\text{M}$) (Figure 4.7-A). Nitrite levels measured in the media of these knock-out BMDMs infected with *C. albicans* are significantly lower or not detectable compared to the levels produced by WT BMDMs (Figure 4.7-A). Moreover, killing assay results showed that, like WT macrophages, *Nos2*^{-/-} macrophages also displayed enhanced candidacidal activity against *C. albicans* when primed with IFN- β at both concentrations tested (Figure 4.7-B). In WT BMDMs, the killing profiles were comparable to those shown previously (Figure 4.3), with pre-stimulation with 500 U/mL IFN- β resulting in 19% enhanced killing ($74.213 \pm 3.498 \%$) and 22% with 1000 U/mL IFN- β ($77.381 \pm 3.353 \%$) compared to the control ($55.306 \pm 2.402 \%$). In iNOS-deficient cells, surprisingly, a similar antifungal effect against *C. albicans* exerted by IFN- β was observed, with 500 U/mL IFN- β enhancing killing by 18 % ($70.968 \pm 4.918 \%$) and 1000 U/mL by 23 % ($76.442 \pm 1.562 \%$) compared to the respective unstimulated control ($52.72 \pm 1.861 \%$). Statistical analysis evidence that in these experiments, the enhanced killing is due to the cytokine-induced effect, with no significant influence of the genotype of the macrophages. These results disproved my initial hypothesis, since NO is not the main player responsible for the

candidacidal mechanism induced by type I IFN activation of BMDMs during *C. albicans* challenge.

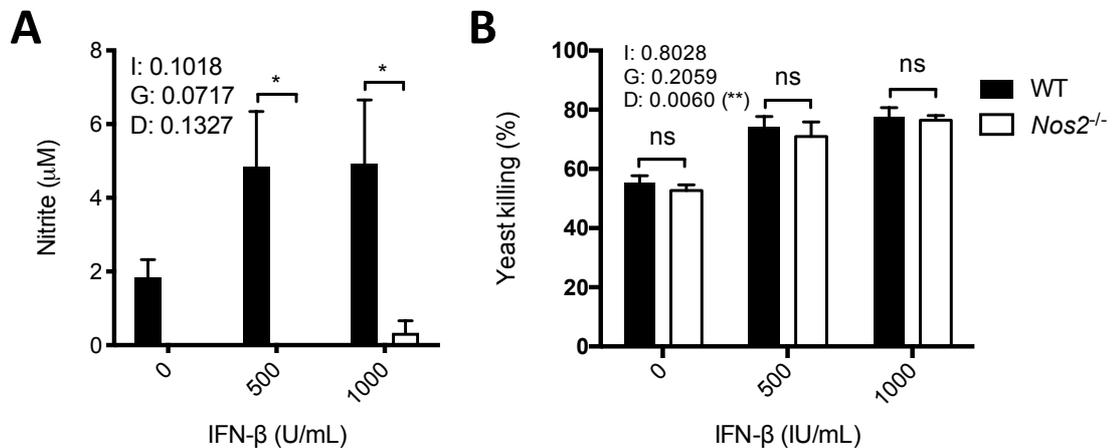


Figure 4.7 – The candidacidal effect of IFN-β is independent of NO production. (A) Wild-type (“WT”, black bars) and iNOS-deficient BMDMs (“*Nos2*^{-/-}”, blank bars) were stimulated with *C. albicans* in the presence of IFN-β for 48 h. Nitrite levels in the supernatants were measured using the Griess test. (B) Wild-type BMDMs or iNOS-deficient BMDMs were pre-stimulated with IFN-β for 48 h. Following pre-stimulation, *C. albicans* was added to cells for 3 h. BMDMs were then lysed and scraped, well contents were diluted and plated on YPDA to evaluate killing by counting CFUs. (A-B) No amphotericin B was added for these assays. Results are displayed as means ± SEM and data were analysed by Two-way Repeated Measures ANOVA with Tukey post tests to determine: (A) the effect of the macrophage genotype and of IFN-β treatment on the release of nitric oxide by BMDMs. Significance values (p-values) are represented as “I” – interaction between the presence of *Nos2* gene in macrophages and stimulation with IFN-β, “G” – presence of *Nos2* gene in macrophages, “D” - effect of different concentrations of IFN-β. Tukey post-hoc tests were performed to assess the impact of the presence of *Nos2* gene in BMDMs when stimulated with *C. albicans*, on the release of nitric oxide upon incubation with a specific IFN-β dose; (B) the impact of both IFN-β and presence of *Nos2* in macrophages (i.e. expression of iNOS) on the ability of BMDMs to kill *C. albicans*. Significance values (p-values) are represented as “I” – interaction between the presence of *Nos2* in macrophages and stimulation with IFN-β, “G” - presence of *Nos2* gene in macrophages, “D” – effect of different concentrations of IFN-β. Tukey post-hoc tests were performed to check if macrophages lacking iNOS expression displayed impaired killing of *C. albicans* compared to WT cells after pre-stimulation with a specific IFN-β dose. A and B: n=3. ns – no statistical significance, **p*<0.05, ***p*<0.01.

Besides nitrosative mechanisms, macrophages can also deploy oxidative mechanisms to eliminate internalized pathogens. As type I IFN signalling triggers STAT1 activation which will lead to production of ROS in activated macrophages via p38 MAPK/STAT1/ROS pathway^{543,544}, I then decided to assess if ROS production by BMDMs could be the main player responsible for the enhanced killing observed in BMDMs after IFN-β stimulation. To that end, a luminol assay, a type of chemiluminescence assay for the detection of major ROS species was performed (Figure 4.8).

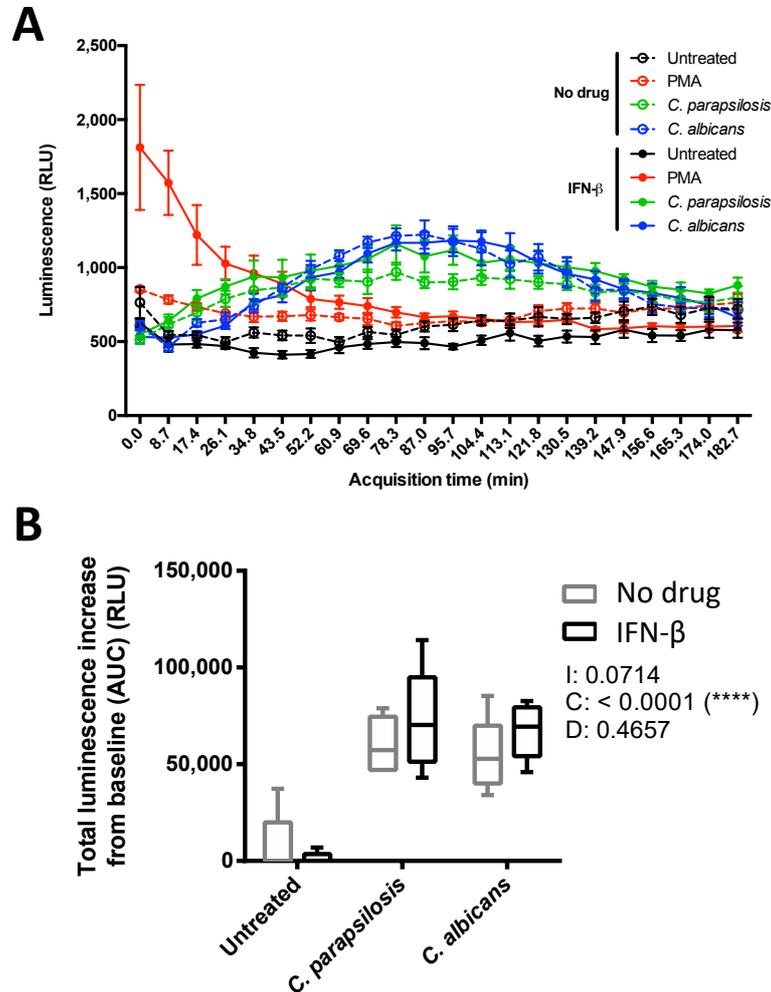


Figure 4.8 – IFN-β does not affect ROS production by BMDMs during *Candida* spp. infection. WT BMDMs were pre-stimulated with 1,000 U/mL IFN-β for 48 h. Following pre-stimulation, *Candida* spp. were added to BMDMs and a luminol assay was performed to detect ROS production over a time course of 3 h (3.045 h, 22 measurement cycles). No amphotericin B was added for these assays. Top graph (A) shows total luminescence values detected during the course of the luminol assay. Bottom graph (B) shows area under the curve (AUC) values for each condition acquired by setting luminescence values detected on the initial cycle (0.0 min) as a baseline. In both graphs results are shown as means ± SEM, n=5. In graph B, Two-way Repeated Measures ANOVA with Sidak’s multiple comparisons post-test was performed to evaluate the impact of stimulation with both IFN-β and different *Candida* spp. on the generation of ROS by BMDMs. Significance values (p-values) are represented as: “I” – interaction between different *Candida* spp. and IFN-β stimulation; “C” – impact of stimulation with the different *Candida* species; “D” - effect of IFN-β stimulation. ****p<0.0001.

For the luminol assay, BMDMs were primed with IFN- β for 48 h, incubated with luminol for 30 min to maximize cellular uptake, all other stimulants, i.e. PMA, *C. albicans* or *C. parapsilosis* were added to the wells and the plate was immediately placed inside a luminometer and measurements were initiated (Figure 4.8). This plate transference was done as fast as possible, mostly because ROS release can be a very rapid process and maximal peak release can easily be missed in some conditions, especially from positive control conditions. Luminescence was detected in 8.7 min cycles (time necessary for the plate reader to register data all the wells set for this experiments' layout) for 22 cycles that accounts for a total of approximately 3 h of detection of ROS which matches the stimulation period set for the killing assays using IFN- β . Results are expressed as relative luminescence units (RLU).

Figure 4.8-A shows the raw readings registered for the 22 cycles in these experiments. Uninfected BMDMs (Untreated) produced the lowest levels of ROS of all the conditions tested with maximal production registered at the initial cycle (0 min – 763.733 \pm 106.191 RLU) and minimal at cycle 8 (60.9 min – 495.333 \pm 36.324 RLU), with IFN- β only slightly changing ROS release in these cells (minimal: 43.5 min – 411.933 \pm 25.403 RLU; maximal: 625.933 \pm 27.681 RLU). PMA was included in these experiments as a positive control. Compared with untreated macrophages, PMA by itself led to an insubstantial change in the production of ROS by BMDMs stimulated in the absence of IFN- β with maximal release at the first cycle (0 min – 851.933 \pm 15.189 RLU) and minimal at cycle 10 (78.3 min – 607.4 \pm 24.015 RLU). IFN- β stimulation induced production of higher levels of ROS by PMA-stimulated macrophages, with the highest levels registered in these experiments at the first cycle (0 min – 1812.4 \pm 422.178 RLU) (minimal: cycle 17; 139.2 min - 584.133 \pm 12.819 RLU). Overall, *Candida* spp.-infected macrophages display higher ROS values than untreated BMDMs, independently of IFN- β stimulation, and these values do not change significantly with cytokine stimulation. BMDMs stimulated with *C. parapsilosis* in the absence of IFN- β have maximal production of ROS at cycle 10 (78.3 min – 969.133 \pm 51.807 RLU) and minimal release at the initial cycle (0 min – 512.467 \pm 27.017 RLU) and stimulation with IFN- β leads to maximal production also at cycle 10 (78.3 min – 1158.933 \pm 125.776 RLU) and minimal at first cycle (0 min – 548.667 \pm 29.124 RLU). *C. albicans*-infected BMDMs in the absence of IFN- β stimulation showed a maximal production of ROS at cycle 11 (87 min – 1223.4 \pm 95.584 RLU) and minimal at cycle 2 (8.7 min

– 462 ± 25.117 RLU), IFN- β induced ROS production peak at cycle 12 (95.7 min – 1183.067 ± 78.848 RLU) and minimal production was detected at cycle 2 (8.7 min – 527.113 ± 45.945 RLU).

For statistical analysis and to truly assess if IFN- β significantly altered ROS produced during the 3 h course of *Candida* spp. infection, area under the curve (AUC) values were calculated for every condition set for this experiment and plotted together in the same graph (Figure 4.8-B). For this analysis, conditions where BMDMs were stimulated with PMA were not included as PMA was only used as a positive control to check if macrophages were indeed capable of responding to a respiratory burst-inducing stimulus. AUC values were calculated by setting luminescence values registered on the initial cycle (0 min) as baseline, and only values higher than the ones detected on that cycle were considered for the calculation. Since ROS levels on PMA-stimulated macrophages, especially in the presence of IFN- β , were highest at the beginning of the 3 h reading experiment, AUC values obtained by this method would be very low or non-existent and would not reflect the biology of these results. As was verified in Figure 4.8-A, *Candida* spp.-infected macrophages produce more ROS than uninfected macrophages, independently of addition of IFN- β . BMDMs infected with *C. parapsilosis* show similar total luminescence values when stimulated in the absence (60120.4 ± 6327.298 RLU) or presence of IFN- β (72541.6 ± 11799.760 RLU), and a similar result was verified with *C. albicans* (No drug: 54536.8 ± 8491.994 RLU; IFN- β : 67313 ± 6311.8 RLU). Statistical analysis revealed that only the different *Candida* spp., and not IFN- β , are able to significantly influence the production of ROS by BMDMs.

These results showed that IFN- β does not significantly alter ROS production in BMDMs during both *C. albicans* and *C. parapsilosis* challenge, which suggested that ROS are not the main player responsible for the enhanced *C. albicans* killing observed in macrophages after IFN- β stimulation.

4.3.3 The IFN- β -inducible candidacidal effect does not involve blockade of *C. albicans* glyoxylate cycle

Once internalised by macrophages, *C. albicans* adapts its metabolism in order to survive in the hostile, nutrient-deprived environment of the phagosome. *C. albicans* enters the glyoxylate cycle to withstand carbon starvation in the phagosome and bypass the steps of the TCA cycle that would use carbon to generate CO₂ in order to conserve these elements for use in the production of glucose^{83,545,546}. Itaconate is produced in activated macrophages during certain infections⁵⁴⁷ by an enzyme coded by the mammalian immune responsive gene 1 (IRG1), a gene highly expressed during inflammation^{548,549}, and has long been identified as an inhibitor of ICL1 (isocitrate lyase), the enzyme responsible for entrance in the glyoxylate cycle^{83,550–552}. Type I and type II IFNs were recently identified as potent inducers of IRG1 expression in macrophages^{549,553}. Based on these findings, I hypothesized that type I IFN pathway activation would affect the ability of *C. albicans* to enter the glyoxylate cycle, which could account for the candidacidal effect observed with IFN- β . (Figure 4.9).

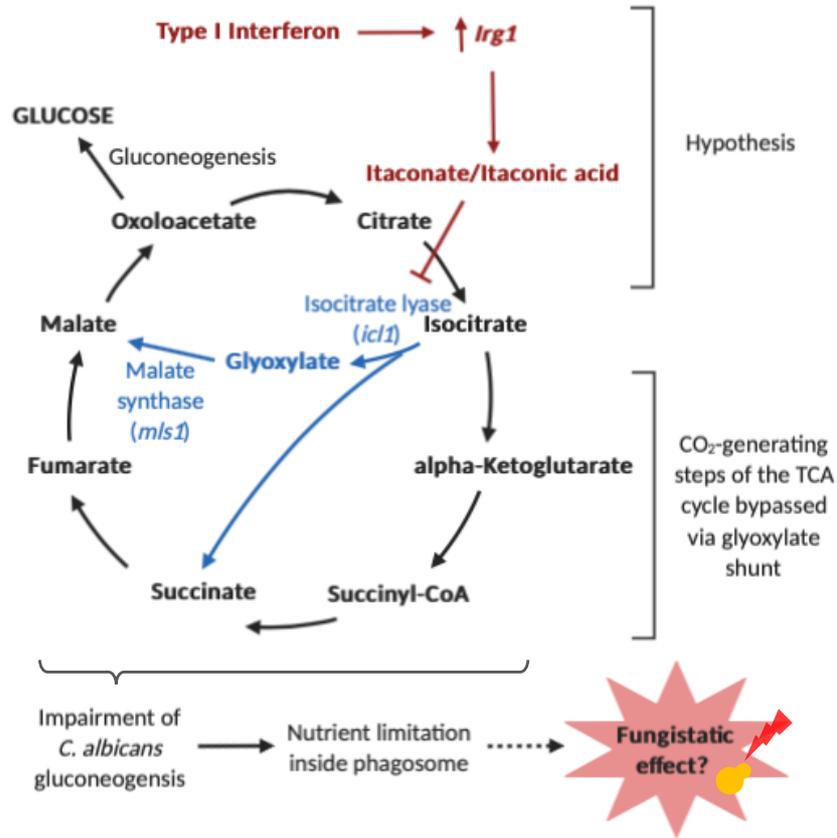


Figure 4.9 – Schematic of the glyoxylate cycle inhibition hypothesis. A simplified version of the TCA cycle is presented (bold black) alongside the glyoxylate cycle (blue) known to be triggered by *C. albicans* during phagocytic stress. The theory of macrophage IFN- β -mediated blockade of the glyoxylate cycle via itaconate production is highlighted in red. (Adapted from^{82,83}. Image was made using Biorender.com).

I hypothesized that the enhanced *C. albicans* killing in BMDMs with IFN- β stimulation was due to upregulation of murine *Irg1* expression which would result in augmented production of itaconate. Once inside the phagosome, itaconate would act on internalised *C. albicans* and block fungal ICL1. Inhibition of ICL1 would block entrance to the glyoxylate shunt and impair *C. albicans* carbon-conservation mechanism, resulting in reduced gluconeogenesis that would greatly affect the yeast's ability to sustain molecular and metabolic processes necessary for survival inside (or escape from) the nutrient-deprived hostile environment of the phagosome. This phenomenon could possibly result in a substantial fitness reduction of the yeast and exert a consequent fungistatic effect, which could ultimately culminate in improved fungal clearance by macrophages (Figure 4.9).

To investigate this theory, I quantified the expression of IRG1 and measured itaconate levels produced by BMDMs after challenge with *C. parapsilosis* or *C. albicans* in the presence of IFN- β (Figure 4.10).

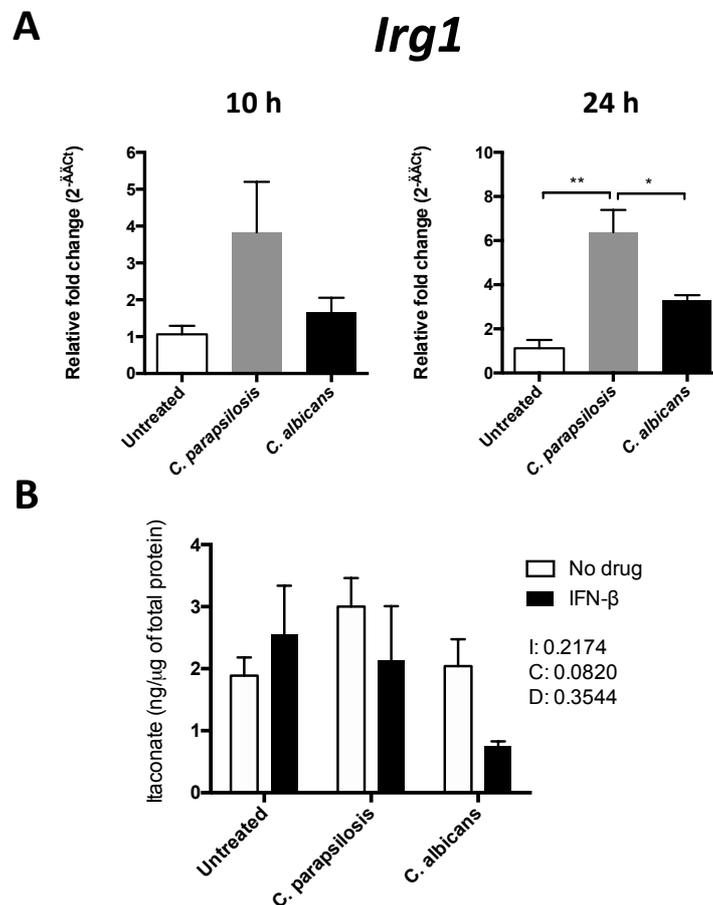


Figure 4.10 – IFN- β does not appear to enhance itaconate production in BMDMs stimulated with *Candida* spp. (A) *C. parapsilosis* and *C. albicans* were added separately to BMDMs for 10 h (left graph) or 24 h (right graph). Macrophage RNA was extracted and converted to cDNA, and *Irg1* expression was quantified by RT-qPCR using *Hprt1* as the normalising housekeeping gene. Data is represented as relative fold change of *Irg1* ($2^{-\Delta\Delta C_t}$) in each sample compared with the untreated control at the same time point. One-way ANOVA with Tukey post-test. (B) BMDMs were stimulated with *C. albicans* or *C. parapsilosis* in the absence or presence of 1,000 U/mL IFN- β for 48 h. Cells were lysed and scraped twice with ice-cold 80% methanol to extract metabolites in the samples. Following processing and drying, lysates were resuspended in Milli-Q-purified water and Itaconate in the samples was quantified by ESI-LC-MS/MS. Absolute itaconate levels in samples were estimated from an established calibration curve. Two-way Repeated Measures ANOVA with Sidak’s multiple comparison post-test was performed to assess the impact of both challenge with different *Candida* spp. and of IFN- β on the production of itaconate. Significance values (p-values) are represented as: “I” – interaction between stimulation with *Candida* spp. and IFN- β ; “C” – impact of stimulation with different *Candida* spp.; “D” – effect of IFN- β stimulation. Results are displayed as means \pm SEM. A and B: n=3. * p <0.05, ** p <0.01.

Results show a clear upregulation of *Irg1* in BMDMs stimulated with *C. parapsilosis* (Figure 4.10-A). At 10 h, although not statistically significant, *Irg1* expression after *C. parapsilosis* stimulation is almost four times higher than the untreated control (3.83 ± 1.37 fold change), whereas *C. albicans* only induced two-fold higher expression (1.67 ± 0.39 fold change). *Irg1* expression was overall higher after 24 h of stimulation, with a six-fold higher (6.38 ± 1.01 fold change) expression detected in *C. parapsilosis*-stimulated BMDMs and a three-fold induction stimulated by *C. albicans* (3.27 ± 2.13 fold change) compared to the untreated control. Higher expression of *Irg1* in *C. parapsilosis*-stimulated BMDMs was only statistically significant at 24 h, although it was also somewhat increased with *C. parapsilosis* at 10 h. *Irg1* is only significantly upregulated at RNA level during *C. parapsilosis* stimulation but not with *C. albicans*.

Overall, *C. parapsilosis* induced higher levels of itaconate produced in macrophages than *C. albicans* (Figure 4.10-B). In uninfected macrophages (untreated), IFN- β -stimulated BMDMs release similar itaconate levels (2.54 ± 0.79 ng/ μ g) compared with unstimulated BMDMs (1.89 ± 0.29 ng/ μ g). In macrophages infected with *Candida* spp., however, lower levels of itaconate were detected in BMDMs stimulated with IFN- β . In *C. parapsilosis*-stimulated macrophages, itaconate concentrations decreased from 3.00 ± 0.29 ng/ μ g to 2.12 ± 0.88 ng/ μ g in the presence of IFN- β . This decrease was also verified in *C. albicans*-infected macrophages with 2.04 ± 0.44 ng/ μ g itaconate detected in the absence of IFN- β and 0.75 ± 0.08 ng/ μ g when IFN- β was added. Results revealed that, unexpectedly, IFN- β does not lead to enhanced production of itaconate by macrophages. Whilst IFN- β led to a small but not significant increase of itaconate levels in uninfected macrophages, lower levels of itaconate were detected in macrophages treated with IFN- β in combination with *Candida*.

These results indicate that IFN- β does not lead to production of significantly higher levels of itaconate during *Candida* spp. infections. I then performed killing assays with IFN- β using *Irg1*-deficient BMDMs, which are incapable of producing itaconate, and an *icl1*-deficient *C. albicans* strain that is incapable of activating the glyoxylate to further confirm this finding. (Figure 4.11)

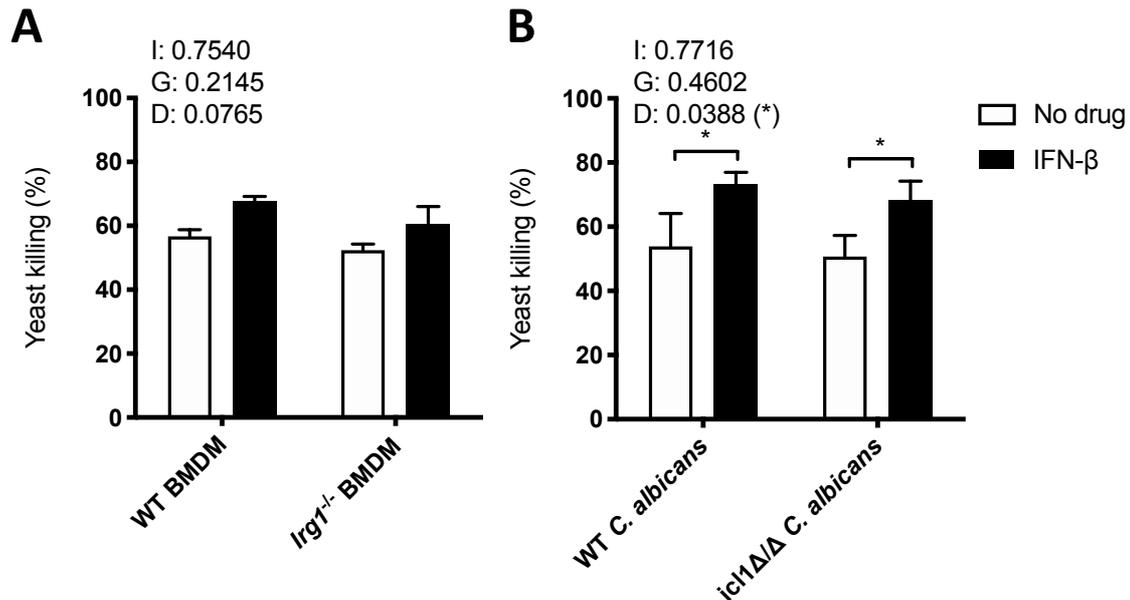


Figure 4.11 – The itaconate-glyoxylate axis is not responsible for the IFN-β candidacidal effect. (A) Wild-type BMDMs (“WT BMDM”) or *Irg1*-deficient BMDMs (“*Irg1*^{-/-} BMDM”) were pre-stimulated with 500-1,000 U/mL IFN-β for 48 h. Following pre-stimulation, WT *C. albicans* was added to cells for 3 h. (B) Wild-type BMDMs were pre-stimulated with 1,000 U/mL IFN-β for 48 h. Following pre-stimulation, *C. albicans* SC5314 (“WT *C. albicans*”) or *icl1*-deficient *C. albicans* (“*icl1Δ/Δ C. albicans*”) were added to cells for 3 h. BMDMs were then lysed and scraped, well contents were diluted and plated on YPDA to evaluate killing by counting CFUs. No amphotericin B was added for these assays. Results are displayed as means ± SEM, and data was analysed by Two-way Repeated Measures ANOVA with Sidak’s multiple comparison post-test to determine the impact of: (A) both IFN-β and presence of *Irg1* gene in macrophages (i.e. expression of *Irg1*, and itaconate production) on the ability of BMDMs to kill *C. albicans*. Significance values (p-values) are represented as “I” – interaction between the presence of *Irg1* gene in macrophages and stimulation with IFN-β, “G” - presence of *Irg1* gene in macrophages, “D” – effect of pre-stimulation with IFN-β; (B) both pre-stimulation of BMDMs with IFN-β and the presence of *icl1* in *C. albicans* (i.e. expression of *icl1*, and consequent ability of the yeast to produce isocitrate lyase) on the ability of BMDMs to kill *C. albicans*. Significance values (p-values) are represented as “I” – interaction between the presence of *icl1* in *C. albicans* and BMDM pre-stimulation with IFN-β, “G” - presence of *icl1* in *C. albicans*, “D” – effect of BMDM pre-stimulation with IFN-β. A: n=3; B: n=4. **p*<0.05.

In the absence of IFN-β, both wild-type and *Irg1*^{-/-} BMDMs show similar killing of *C. albicans*: wild-type macrophages display 57% killing ability (56.74 ± 2.03 %) and *Irg1*-deficient macrophages only show a small 5% decrease (52.39 ± 1.89 %) in their ability to kill *C. albicans*. Pre-stimulation with IFN-β led to an 11% increase of fungal killing in wild-type macrophages (67.62 ± 1.54 %) and to an 8% increased killing in *Irg1*-deficient BMDMs (60.490 ± 5.53 %). Despite not being statistically significant, IFN-β stimulation led to a trend of enhanced killing of *C. albicans* in both wild-type and *Irg1*-deficient cells, suggesting that the candidacidal

properties of IFN- β in macrophages are not due to IRG1 expression and consequent itaconate production.

WT BMDMs infected with WT or *icl1*-mutant *C. albicans* show similar killing profiles in the absence of IFN- β with 53.91 ± 10.25 % killing for wild-type *C. albicans* and 50.71 ± 6.57 % for *icl1*-deficient *C. albicans*. Pre-stimulation with IFN- β boosts candidacidal activity against wild-type *C. albicans* by 19% (73.24 ± 3.74 % killing) and *icl1*-deficient *C. albicans* by 18% (68.24 ± 5.94 % killing). Statistical analysis showed that the enhanced candidacidal activity observed in these experiments is due to IFN- β stimulation alone, with no significant involvement of the yeast's genotype, i.e. expression of *icl1*.

Although *Irg1* is upregulated in *C. parapsilosis*-infected BMDMs (Figure 4.10-A), itaconate production was not found to be elevated in macrophages stimulated with *C. albicans* in the presence of IFN- β (Figure 4.10-B). Macrophages deficient in *Irg1* and consequently unable to produce itaconate, show *C. albicans* killing profiles similar to wild-type macrophages following stimulation with IFN- β (Figure 4.11-A). Similar enhanced killing trends were also observed in BMDMs primed with IFN- β after challenge with both wild-type and *icl1*-deficient *C. albicans* (Figure 4.11-B). The collective results in this section, disproved the proposed IFN- β –itaconate-glyoxylate cycle hypothesis.

4.3.4 Conventional AMPs are not responsible for the candidacidal effect of IFN- β

Since the candidacidal effect of IFN- β is not mediated by nitrosative or oxidative mechanisms, or by blockade of glyoxylate cycle metabolic shift of the yeast, I hypothesized that IFN- β enhanced the killing ability of BMDMs by inducing upregulation of AMPs that would directly affect fungal viability. To test this hypothesis, I measured the production of some conventional AMPs by BMDMs during *Candida* spp. stimulation and assessed whether IFN- β induced their upregulation during infection.

I quantified expression of five different AMPs by BMDMs during *Candida* and IFN- β co-stimulation: Camp, β -defensins 1 and 2, LTF and TSLP. These genes were all shown to have direct candidacidal activity against *C. albicans*^{136,218,221,224,229,249,554}. *Camp* codes for the

protein precursor of cathelicidin-related antimicrobial peptide (CRAMP), the murine homolog of human LL-37^{218,221}. *Defb1* and *Defb2* code for murine β -defensin 1 and β -defensin 2 respectively. These two AMPs are produced by activated human macrophages and were shown to directly induce *C. albicans* death^{224,229}. *Ltf* codes for lactotransferrin (LTF), a molecule that besides having a direct candidacidal activity, also plays a role in iron limitation inside the phagosome^{136,554}. *Tslp* codes for thymic stromal lymphopoietin (TSLP) a cytokine with AMP properties mainly produced by epithelial cells²⁴⁹ (Figure 4.12).

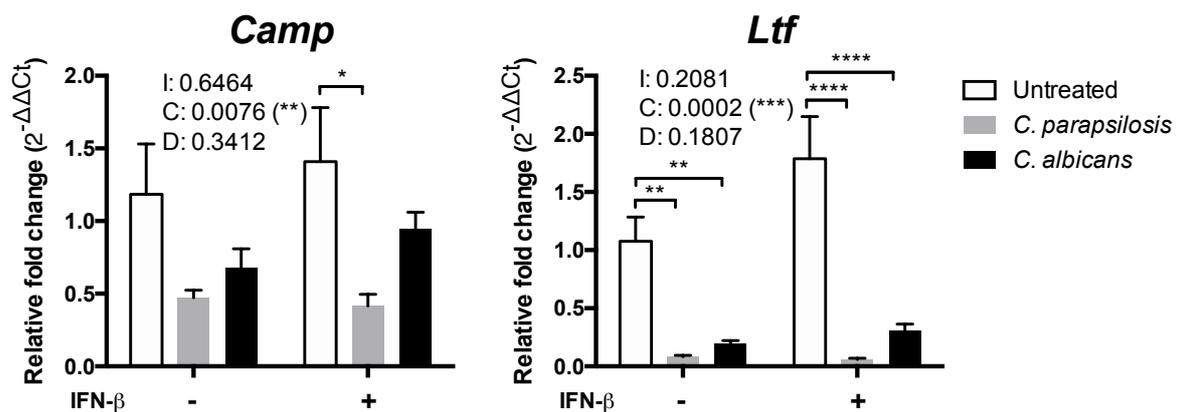


Figure 4.12 – IFN- β does not induce upregulation of cathelicidin antimicrobial peptide and lactotransferrin at RNA level in BMDMs challenged with *Candida* spp. BMDMs were stimulated with *C. albicans* or *C. parapsilosis* in the absence or presence of 1,000 U/mL IFN- β for 48 h. Macrophage RNA was extracted and converted to cDNA. Cathelicidin antimicrobial peptide (*Camp*) and lactotransferrin (*Ltf*) gene expression was then quantified by RT-qPCR using *Hprt1* as the normalising housekeeping gene. Data is represented as relative fold change ($2^{-\Delta\Delta Ct}$) of either *Camp* or *Ltf* in each sample compared with its respective untreated control. Results are displayed as means \pm SEM, n=4, Two-way Repeated Measures ANOVA with Sidak's multiple comparison post-test was performed to determine the impact of both IFN- β stimulation and challenge with different *Candida* spp. tested on the expression of either *Camp* or *Ltf*. "I" – interaction between stimulation with different *Candida* species and stimulation with IFN- β ; "C" – impact of stimulation with different *Candida* species; "D" – impact of IFN- β stimulation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

Stimulation with IFN- β alone led to a slight but insignificant upregulation of both *Camp* (1.41 ± 0.37 fold change) and *Ltf* (1.79 ± 0.36 fold change). Both *Candida* spp. tested induced a downregulation of *Camp*, with *C. parapsilosis* inducing a 0.47 ± 0.05 fold change and *C. albicans* inducing a 0.68 ± 0.13 fold change in expression. IFN- β stimulation did not restore the reduced *Camp* gene expression triggered by *Candida* spp., with *C. parapsilosis* showing a 0.42 ± 0.08 fold change and *C. albicans* a 0.95 ± 0.11 fold change. Regarding *Ltf*, both *C. parapsilosis* (0.08 ± 0.01 fold change) and *C. albicans* (0.19 ± 0.03 fold change) induced

significantly lower *Ltf* gene expression in macrophages. IFN- β stimulation also did not rescue the downregulation of *Ltf* with *C. parapsilosis* showing a 0.06 ± 0.01 fold change and *C. albicans* a 0.31 ± 0.06 fold change. Statistical analysis revealed that both *Camp* and *Ltf* expression is only significantly altered by stimulation with the different *Candida* spp. and not by IFN- β (Figure 4.12).

The expression of other AMPs like beta-defensins 1 and -2 (*Defb1* and *Defb2*) and thymic stromal lymphopoietin (*Tslp*) was also measured by the same technique, however very high to no Ct values were detected after 45 qPCR cycles. In the few conditions and experiments where high Ct values were detected, no clear differences in gene expression were detected after stimulation with different *Candida* spp. or with IFN- β compared with untreated controls (data not shown).

Results from this section suggest that the conventional AMPs CRAMP, LTF, beta-defensins 1 and -2 and TSLP are not the players responsible for the enhanced candidacidal effect verified with IFN- β .

4.3.5 Administration of IFN- β has a detrimental effect during systemic candidiasis

To this point, I thoroughly examined the candidacidal effect of type I IFN *in vitro*, however these experiments were not sufficient to fully explain the molecular mechanisms driving the anti-*Candida* effect observed. Therefore, I decided to test if activation of the type I IFN pathway could improve the outcome of systemic candidiasis and possibly facilitate discovery of the molecular mechanism(s) responsible for the observed candidacidal effect of type I IFN using an *in vivo* murine model of the disease.

For this purpose, naïve mice were intravenously challenged with a high dose of *C. albicans* and with different doses of IFN- β at day 0, and animals were monitored for 7 days. Survival rates based on humane end point and fungal burden in the main target organs (kidneys and brain) were then quantified (Figure 4.13).

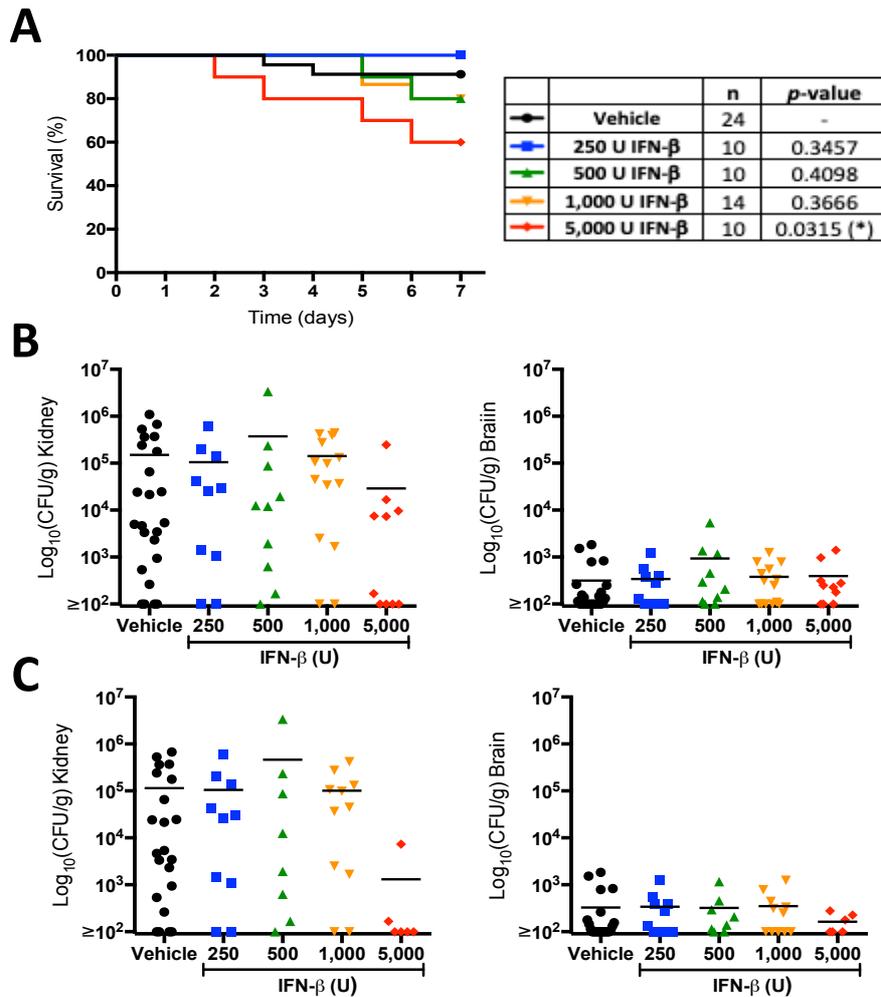


Figure 4.13 – IFN-β administration during *C. albicans*-induced systemic candidiasis increases susceptibility to disease. Naïve C57Bl6/J mice were intravenously challenged with 1.5×10^5 *C. albicans* CFUs and different concentrations of IFN-β and monitored for 7 days. Total number of mice used in each condition and *p*-values for survival curves of IFN-β-injected mice compared to mice injected with vehicle (0.01% BSA in PBS) obtained by log-rank test are shown (A). Kidney (left graphs) and brain (right graphs) fungal burden after infection was quantified for all mice used in these experiments (B) and for mice that survived the 7-day infection with *C. albicans* (C). Each symbol represents one mouse. (C) Vehicle: n=22; 250 U: n=10; 500 U: n=8; 1,000 U: n=11; 5,000 U: n=6. For statistical analysis of fungal burden graphs, one-way ANOVAs with Tukey post-hoc tests were performed. Graphs show cumulative data of four independent experiments. **p*<0.05

Figure 4.13-A shows that high concentrations of IFN-β had a detrimental effect on mice survival during the course of candidaemia. This effect was statistically significant in mice injected with the highest concentration tested (5,000 U IFN-β) where only 60% of mice survived the infection. In Figure 4.13-B, fungal burden results obtained from all mice indicate that IFN-β does not significantly improve clearance of *C. albicans* in kidneys and brain. Interestingly, and although not statistically significant, mice that survived the 7-day course of

infection showed a somewhat reduced fungal burden in the kidney compared with other mice injected with vehicle or lower doses of IFN- β as shown in Figure 4.13-C.

Overall, these results suggest that while activation of type I IFN signalling pathway may slightly improve fungal clearance, it increases mortality during systemic candidiasis.

4.3.6 HetIL-15 promotes splenic T cell expansion and enhances IFN- γ and granzyme B production during systemic candidiasis

IFN- β administration did not improve disease outcome during systemic candidiasis, in fact it increased mortality (Figure 4.13-A). This was possibly due to excessive inflammation exerted as a consequence of IFN- β administration, as IFN- β did not increase organ fungal burden (Figure 4.13-B), and mice injected with high dose IFN- β that survived the infection displayed slightly reduced *Candida* loads (Figure 4.13-C). To try and overcome the deleterious effect of IFN- β administration on mice survival, and to continue assessing if type I IFN signalling might improve the outcome of infection, I decided to assess the impact of other cytokines downstream of type I IFN.

IL-15 is a type I IRG that plays a protective role during systemic candidiasis³¹⁴, and my RNAseq data indicates that it is specifically induced by *C. parapsilosis*-stimulated but not by *C. albicans* in BMDMs (Figure 3.2). IL-15 is produced by macrophages and many other cell types in response to infectious agents, and shown to act mainly in lymphocytes as it controls growth and differentiation of T and B lymphocytes, activation of Natural Killer (NK) and phagocytic cells. IL-15 exists as a heterodimer with IL-15R α (hetIL-15) in circulation, and its heterodimeric form has superior bioactivity than the monomer^{555,556}. For these experiments, I have used a chimeric heterodimeric IL-15:IL-15R α -Fc complex kindly provided by Dr. Cristina Bergamaschi that was purified from supernatants of transfected clonal HEK293 cell lines as described before⁵⁵⁷. Briefly, HEK293 cells were co-transfected with both IL-15 and IL-15R α -Fc coding-plasmids, and once secreted these monomers will associate with each other in the cytoplasm to form the heterodimeric complex that is then purified. The IL-15R α -Fc plasmid codes for IL-15R α fused with an Fc fragment region of human IgG1, that will yield a more

stable form of hetIL-15 that can act as a cell-associated cytokine *in vivo* by mimicking IL-15 trans-presentation⁵⁵⁷.

Initially, I assessed if administration of hetIL-15 would improve the outcome of systemic candidiasis *in vivo*. For this purpose, naïve mice were intravenously challenged with *C. albicans*, intraperitoneally injected with a single dose of 3 µg hetIL-15, a dose previously tested in naïve uninfected mice⁵⁵⁶ and monitored for 4 days. Mice survival rates, organ fungal burden (kidneys and brain) and serum cytokine (IL-12p40 and IL-6) levels were analysed (Figure 4.14).

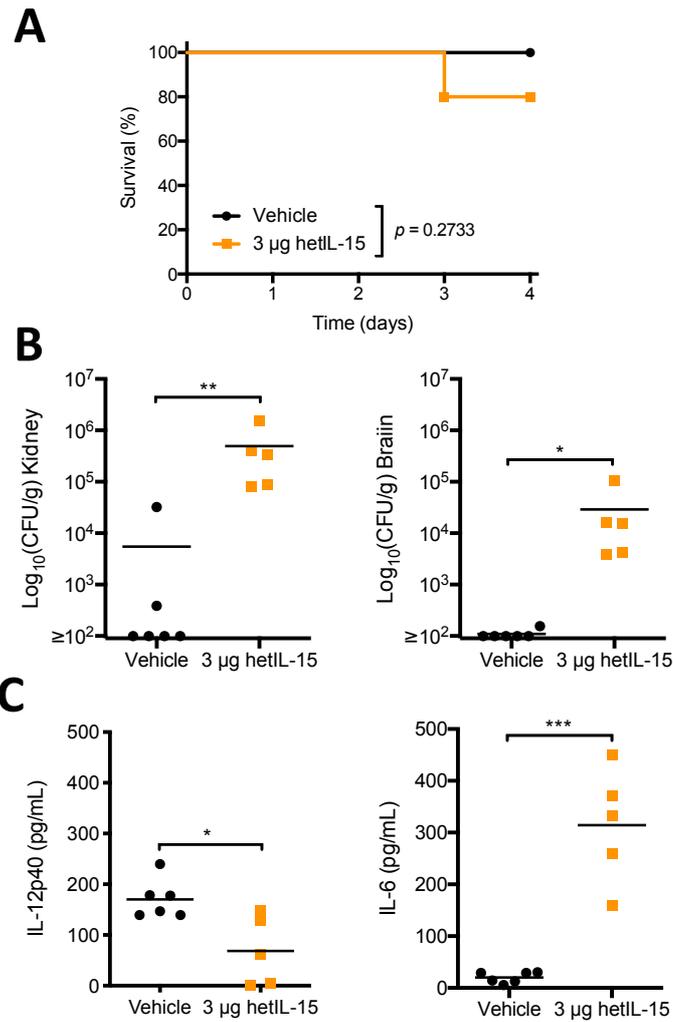


Figure 4.14 – Administration of hetIL-15 during *C. albicans*-induced systemic candidiasis leads to increased fungal burden and altered serum levels of IL-12p40 and IL-6. Naïve C57Bl6/J mice were intravenously challenged with 1.5×10^5 *C. albicans* CFUs and injected intraperitoneally with 3 µg hetIL-15 and monitored for 4 days. Survival curves with log-rank test results are shown (A). Kidney (right graph) and brain (left graph) fungal burden after infection were evaluated (B). IL-12p40 (left graph) and IL-6 (right graph) levels in the serum were quantified by ELISA (C). In both fungal burden (B) and cytokine measurement graphs (C), each dot represents a mouse, and for statistical analysis of both sets of results, one-way ANOVAs with Tukey post-hoc tests were performed. Graphs display data from one independent experiment. Vehicle: six mice (n=6); HetIL-15: five mice (n=5). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Mice injected with hetIL-15 displayed slightly lower but not significantly lower survival rate (86.7%) than vehicle-injected mice (Figure 4.14-A). Nevertheless, hetIL-15 contributed to significant fungal persistence in kidneys and brains of infected mice injected compared with vehicle-treated mice (Figure 4.14-B). Lower levels of IL-12p40 and higher levels of IL-6 were also detected in the serum of hetIL-15-injected mice (Figure 4.14-C) suggesting that hetIL-15

at the dose tested induced a deleterious effect by impairing *C. albicans* clearance. It is possible that hetIL-15 may impair Th1 polarisation due to reduced levels of circulating IL-12p40.

HetIL-15 produced by innate phagocytes was shown to contribute to initiation of adaptive immunity by activating and enhancing proliferation of splenic T cells and NK cells^{314,556}. The hetIL-15 concentration tested in this experiment was excessive, as hetIL-15-injected mice showed clear signs of distress compared with vehicle-injected. This dose of hetIL-15 was previously proved to be safe and well tolerated when administered in naïve mice monitored for 4 days⁵⁵⁶, however its impact on mice survival was never assessed during *Candida* systemic infections. *Post-mortem* dissection revealed that mice had remarkably enlarged spleens with clear signs of necrosis, evidencing exacerbated overactivation of splenocytes by that excessive dose and making it difficult to ascertain the impact of hetIL-15 administration during systemic candidiasis. Therefore, I decided to perform another *in vivo* experiment using a lower dose of the heterodimer to ensure mice survival and assess the impact of hetIL-15 in shaping splenic responses including CD8⁺ T cell and NK cell activation during infection whilst minimising detrimental hyper-inflammation and bypassing its detrimental effect previously observed (Figure 4.15).

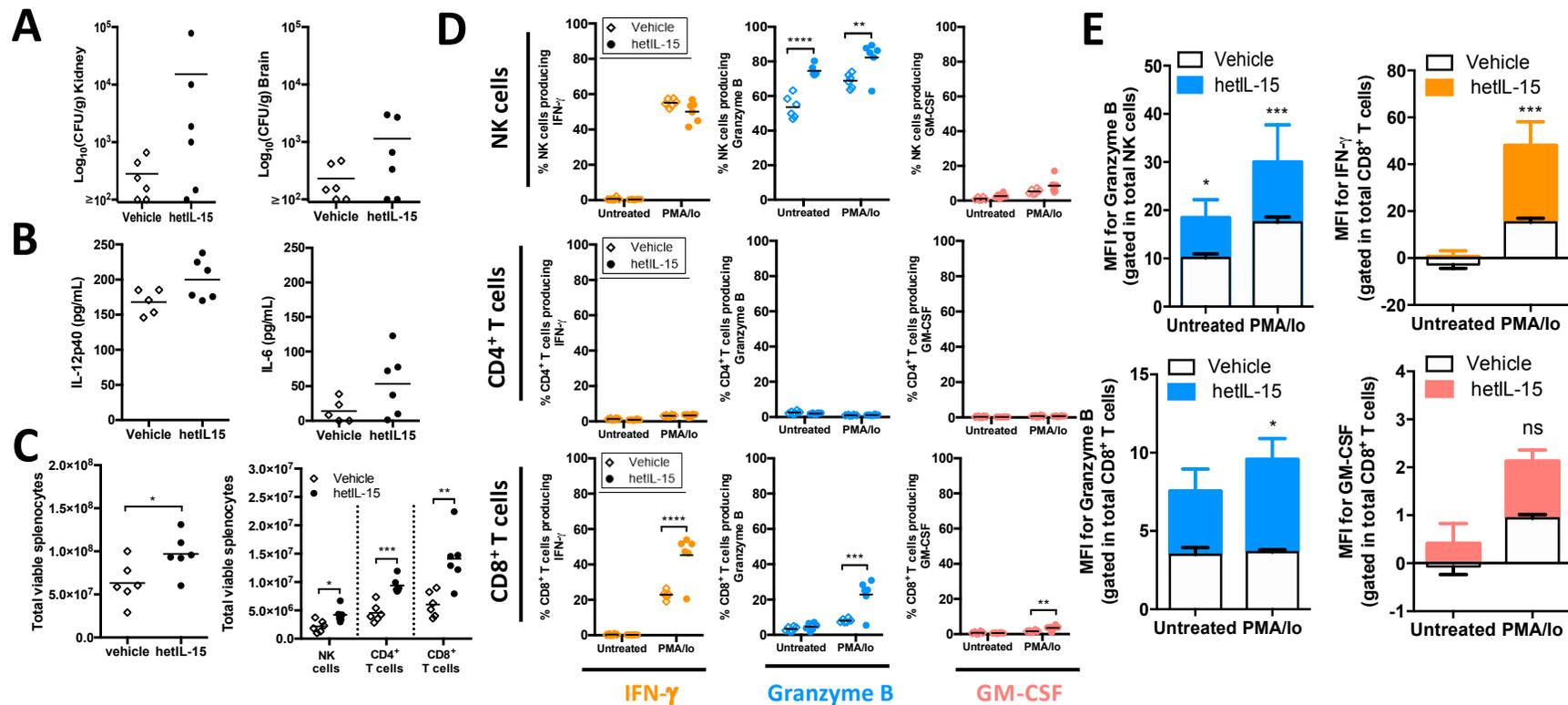


Figure 4.15 – HetIL-15 modulates splenic responses during *C. albicans* in vivo infections. Mice were intravenously challenged with *C. albicans*, i.p. injected with 1 μ g hetIL-15 in 100 μ L 0.01% BSA in PBS and monitored for four days. (A) Fungal burden was assessed in kidneys and brains of infected mice. (B) IL-12p40 and IL-6 levels in the serum of infected mice were detected. (C) Total viable splenocyte numbers from each harvested spleen were counted using a MUSE cell counter (left graph) and total numbers of NK cells, CD4⁺ and CD8⁺ T cells were measured by flow cytometry (right graph). (D and E) Splenocytes from each mouse were left unstimulated or stimulated with PMA/ionomycin for 4 h and IFN- γ -, granzyme B- and GM-CSF-producing NK cells, CD4⁺ or CD8⁺ T cells were detected by flow cytometry. For images A, B and C, data from every graph is presented as means. Statistical analysis on data from the right graph of image C was performed separately for every cell type and graphs were then compiled together in a single graph. Data from image D graphs are presented as means of % cells producing cytokines. Data from image E graphs are presented as means \pm SEM of the MFI variation calculated as the difference between MFI(ungated isotype) and MFI(ungated cytokine stain). Unpaired t-test were performed for statistical analysis of data from images A, B and C, and One-way ANOVAs with Tukey post-hoc tests for data from images D and E. Dots represent data from one mouse, and results were obtained from one independent experiment with six mice per group (n=6). ns – not significant, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.

Although not significant, higher fungal loads were still observed in kidneys and brain of hetIL-15-injected mice compared with mice injected with vehicle (Figure 4.15-A). Nevertheless, all mice survived the course of infection unlike the previous experiment. Mice injected with hetIL-15 had slightly higher, but not significant, levels of circulating IL-12p40 compared with vehicle-injected mice, and 1 μ g hetIL-15 induced lower production of circulating IL-6 levels compared with results obtained with the higher dosage (Figure 4.15-B). HetIL-15-injected mice had a robust proliferation of splenocytes compared with mice treated with vehicle Figure 4.15-C. Flow cytometry analysis revealed that hetIL-15 induced a significant expansion of splenic NK cells, CD4⁺ T cells and CD8⁺ T cells (Figure 4.15-C). Production of IFN- γ , granzyme B and GM-CSF was assessed in these populations. HetIL-15 induced expansion of granzyme B-producing NK cells, and CD8⁺ T cells producing IFN- γ , granzyme B and GM-CSF (Figure 4.15-D). However, MFI variation of the cytokine channel from these populations revealed that GM-CSF production from CD8⁺ T cells is not significant (Figure 4.15-E). Nevertheless, this is a result obtained from one independent experiment, and further experimentation is required to fully validate these findings. (Gating strategy for this flow cytometry experiment, as well as representative plots from all populations analysed are included in the Appendix section (Chapter 7)).

4.4 Discussion

Current antifungal therapeutics for systemic candidiasis are insufficient. Antifungal drugs have limited efficacy, can interact with other drugs and be associated with severe toxicity. There is a limited repertoire of effective antifungal drugs, and development of novel classes of antifungals is urgently necessary to reduce the alarming mortality rates associated with systemic candidiasis, but research is lagging. This is mostly due to the difficulty underlying the identification of efficient fungal-specific drug targets, as fungal pathogens and human host cells are both eukaryotic and therefore share some homology in most metabolic and signal transducing pathways, causing some antifungal drugs to target host cells with considerable affinity⁵⁵⁸. Moreover, the detrimental outcomes of the disease stem mostly from an inadequate and/or exacerbated immune response towards the invading *Candida* spp. often culminating in fatal immunopathology. This evidences that manipulation of host immunity to strengthen or activate an adequate immune response against *Candida* during infection might pose as an interesting and viable therapy for systemic candidiasis or be used as adjuvant to boost the efficacy of current antifungal treatments.

4.4.1 **Type I Interferon administration in BMDMs shows candidacidal effect against *C. albicans* and boosts nitric oxide release**

Initially, I showed that activation of type I IFN signalling in BMDMs either via poly(I:C) or IFN- β stimulation resulted in the same trends of enhanced killing of *C. albicans*, and increased production of NO. Poly(I:C) and IFN- β are known to elicit robust nitric oxide production via iNOS expression in macrophages^{199,541,559}. During *in vitro* infection, *C. parapsilosis* elicited higher levels of NO than *C. albicans*, which was expected due to its ability to induce robust expression of IFN- β . Moreover, *C. albicans* was shown to inhibit NO production through cell wall chitin that will in turn induce expression of arginase-1 by the macrophage, an enzyme that will compete with iNOS for the substrate L-arginine^{506,560}. Although it was not yet proved whether *C. parapsilosis* can deploy similar mechanisms, this could have also contributed to the lower levels of NO observed with *C. albicans*.

Literature is controversial regarding the ability of type I IFN signalling activation to enhance the ability of macrophages to kill *C. albicans*. It was revealed that IFN- β enhances the candidacidal effect of BMDCs and bone marrow cells against *C. albicans* ATCC 90028 and SC5314 respectively^{122,538}, however regarding macrophages this affect appears to depend on their origin. IFN- β was shown to impair the candidacidal activity of peritoneal macrophages from BALB/c and severe combined immunodeficient mice against *C. albicans* B311^{561,562}, and Majer and colleagues supported this by showing that abrogation of IFNAR1 does not alter peritoneal macrophages ability to kill *C. albicans* SC5314⁵³⁷, however in the latter study, the authors did not measure type I IFN production by macrophages upon *C. albicans* stimulation. To my knowledge, this was the first time IFN- β was shown to enhance BMDMs candidacidal activity towards *C. albicans*, but whether the same effect is verified in other murine macrophage types and with other *C. albicans* strains was not assessed here.

Interestingly, the same candidacidal effect exerted by IFN- β in BMDMs against *C. albicans*, was not verified during *C. parapsilosis* infection. *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) was shown to induce RIPK1-dependent necroptosis, a highly inflammatory form of cell death, in BMDMs during infection through production of IFN- β ⁵⁶³. Moreover, TNFSF10 (TRAIL), a cytokine that my research predicts to be exclusively upregulated in *C. parapsilosis*-infected BMDMs, was shown to induce necrotic cell death via RIPK1⁵⁶⁴. As *C. parapsilosis per se* is able to elicit strong induction of IFN- β , it is possible that overactivation of type I IFN signalling exerted a deleterious effect on BMDMs viability and affected its candidacidal activity. Nevertheless, only *Candida*, and not macrophage viability was assessed in these killing assays for this hypothesis to be validated. IFNAR1-deficient BMDMs should be used to confirm the candidacidal impact of type I IFN signalling in *C. parapsilosis*-infected BMDMs.

Untreated BMDMs displayed similar ability to kill *C. albicans* and *C. parapsilosis*. This result was unexpected, as previous reports have revealed that macrophages are more efficient at killing *C. parapsilosis* than *C. albicans in vitro*^{193,565}, and that macrophages phagocytise higher number of *C. parapsilosis* yeasts compared with *C. albicans*^{77,184}. Nevertheless, these authors tested a higher MOI (4-10 macrophages to 1 yeast cell) for similar

or shorter interaction periods than I used in these experiments, so it is possible that killing differences between the two *Candida* spp. would be observed at similar MOIs or at longer incubation timepoints.

4.4.2 Candidacidal mechanism of IFN- β against *C. albicans* is non-nitrosative and likely non-oxidative

The mechanism of *C. albicans* killing was examined in this chapter. Given that nitric oxide has been reported to be a potent anti-microbial agent, its effects were examined in bone marrow derived macrophages using genetically modified mice. Surprisingly, iNOS-deficient BMDMs displayed the same killing profile against *C. albicans* as wild-type BMDMs, suggesting that the antifungal mechanism induced by the cytokine is NO-independent. These results support a previous study where iNOS deletion did not hinder *in vitro* clearance of *C. albicans* by naïve BMDMs⁵⁶⁶. Although macrophages produce NO as an antimicrobial nitrosative mechanism, the NO molecule by itself has little to no antimicrobial activity, however, in the presence of ROS, the molecule reacts with superoxide and generates peroxynitrite that has potent antifungal activity^{136,194}. Therefore, the production of NO by itself as an antifungal compound is most likely ineffective if not accompanied by a matching respiratory burst.

Since NO depletion had no effect, I next examined the production of other reactive oxygen species by using luminol which can be converted to a fluorescent derivative by multiple reactive oxygen species, including hydrogen peroxide, peroxynitrite and superoxide. Overall, my research revealed that IFN- β did not significantly enhance ROS production in uninfected BMDMs. Type I IFN is known to induce upregulation of NOX2 via STAT1 that promotes ROS production in BMDMs⁵⁶⁷. Moreover, ROS can also trigger phosphorylation of STAT1 in activated macrophages which augments the respiratory burst via a positive feedback loop^{543,544}. ROS production can cause deleterious oxidative damage to host cells, and so they need to deploy mechanisms to scavenge ROS and ameliorate this damage. In macrophages this can be mediated by MST1/2 kinases, that are triggered by cytosolic ROS and that stabilize NRF2, a transcription factor that induces the production of antioxidants⁵⁶⁸. Other mechanisms included glutathione redox, superoxide dismutase and catalase⁵⁶⁹. In my experiments,

BMDMs were stimulated with IFN- β for a long time (48 h), therefore it is possible that macrophages have already deployed antioxidant mechanisms to counteract ROS elicited by the cytokine and/or that expression of NADPH oxidase elements was already “switched-off” explaining why no significant differences were detected with IFN- β . Another possible mechanism for this would be the reported negative effect of M-CSF on NOX complex components in M-CSF-derived BMDMs⁵⁷⁰.

PMA is a protein kinase C agonist that rapidly stimulates NOX2 activity⁵⁷¹ and was used here as a positive control. PMA stimulation produced a detectable spike in ROS production in presence of the IFN- β , however this was not identified in untreated cells, which gives further evidence for absence of NOX components or lack of other signalling mediators. Biondo *et al.* obtained similar results using BMDCs, where high levels of superoxide were only generated after IFN- β stimulation in PMA-primed BMDCs¹²². A possible explanation is that IFN- β is able to induce NADPH oxidase components such as NOX2, which along with PMA stimulation allows prolonged ROS release by macrophages.

Interestingly, although PMA stimulation was not able to induce ROS in untreated cells, a detectable burst in ROS was seen after incubation with *C. albicans* and *C. parapsilosis*. Given that the peak of this production did not occur until 90 mins after stimulation, it is possible that NOX components were produced as a result of fungal detection, or that a different signalling pathway other than protein kinase C is utilised. Remarkably, *C. albicans* and *C. parapsilosis* induced similar production of ROS levels in BMDMs. This is consistent with conflicting reports in the literature, where the ability to induce ROS production seems to be dependent on the host cell type. Tóth and colleagues published that *C. albicans* induces higher levels of ROS compared with *C. parapsilosis* in THP-1 cells, however Wellington and colleagues revealed that *C. albicans* is able to block ROS production in J774 cells^{29,572}. To my knowledge, no other studies compared ROS release between *C. albicans* and *C. parapsilosis* in BMDMs, making it difficult to elucidate the differences observed between primary cells and cell lines. However, it is known that *C. albicans* deploys mechanisms to resist oxidative stress such activation as activation of CAP1 transcription factor that will induce expression of antioxidants, and through production of ROS detoxifying enzymes like catalases and

superoxide dismutase^{546,573}. Like *C. albicans*, *C. parapsilosis* can also express those enzymes^{463,574}, which could explain the similar response observed in both *Candida* species.

4.4.3 The IFN- β -inducible candidacidal effect is independent of IRG1 and itaconate production

Given that ROS cannot explain the candidacidal effects observed with IFN- β , I next examined other cytotoxic mediators. Recently, it has been reported that itaconic acid produced via IRG1 can inhibit the glyoxylate cycle in microbes^{553,575}, leading to their death. *Candida* species also possess this cycle and macrophages can produce itaconic acid via IRG1^{82,548}.

When examined, *C. parapsilosis* induced higher expression of IRG1 RNA than *C. albicans*. This was expected as *C. parapsilosis* induces IFN- β production in BMDMs, and *Irg1* is a type I IRG^{553,576}. Following this logic, IFN- β administration was expected to contribute to higher levels of itaconate, however, no statistically significant changes were recorded with IFN- β , except perhaps lower in *C. albicans* in the presence of IFN- β . Given that IRG1 RNA was higher with IFN- β treatment, this suggests that this is not directly correlated to IRG1 protein function. Itaconate can also induce production of ATF3, a global negative regulator of immune activation, via NRF2 that was shown to suppress type I interferon signalling⁵⁷⁶⁻⁵⁷⁸. This could have triggered a negative feedback mechanism and explain why no significant differences were observed. Additionally, itaconate production has also been linked to a broken mitochondrial TCA cycle in activated macrophages after TLR4-signalling. Although O-linked mannans from *Candida* cell wall can activate TLR4, in the absence of these mitochondrial metabolic alterations, it is unlikely itaconic acid can be effectively produced^{41,579}.

Without IFN- β -treatment, stimulation with *C. parapsilosis* led to higher levels of itaconate than *C. albicans*. The reason for this is unclear, however it was reported that some bacteria species like *Yersinia pestis* and *Pseudomonas aeruginosa*, produce enzymes capable of itaconate degradation in order to override inhibition of glyoxylate cycle and survive within the phagocyte⁵⁸⁰. Some *Aspergillus* spp. are also able to produce enzymes capable of degrading itaconate⁵⁸¹. It is unclear if *Candida* spp. can also produce orthologous enzymes

during infection, but if *C. albicans* can deploy these mechanisms and not *C. parapsilosis* it could explain the differences in itaconate production observed between the two species. Unstimulated macrophages do not normally produce such as high levels of itaconate. It is possible that the amphotericin B batch used for these experiments had some endotoxin contamination, as in parallel experiments, untreated BMDMs had high background levels of TNF and IL-27 (data not shown).

Despite these caveats, *Candida* killing assays using *Irg1*-deficient BMDMs and *icl1*-deficient *C. albicans* confirm that host IRG1 or yeast ICL1 expression do not affect IFN- β candidacidal properties. Comparatively, silencing *Irg1* expression in macrophages impairs clearance of bacterial species that code for ICL1 like *Salmonella enterica*⁵⁴⁸, however the same does not appear to be true for *C. albicans*-infected BMDMs. Interestingly, unlike what I have shown with *C. albicans*, deletion of *C. glabrata icl1* makes it more susceptible to killing by RAW264.7 macrophages⁵⁸². Whilst *C. albicans* usually escapes or rapidly induces killing of macrophages after internalization, *C. glabrata* has stealth mechanisms to persist longer inside these cells without lysing them^{22,583}. Living for such a prolonged time inside macrophages requires a strict control of metabolism for nutrient preservation, and in the case of *C. glabrata*, *Icl1* appears to be essential for that purpose and to resist macrophage action. Ablation of *ICL1* ortholog (*acuD*) of *A. fumigatus*, does not have a detrimental effect in models of invasive aspergillosis, suggesting that the mould does not rely on isocitrate lyase-mediated metabolic flexibility to thrive during infection^{584,585}. However, *Icl1* and glyoxylate cycle were shown to be highly associated to *C. albicans* virulence in a mice model of systemic candidiasis, as mice are much more resistant to *ICL1*-null mutants than wild type *C. albicans*⁸². Systemic candidiasis is however a complex disease, that involves a myriad of other immune cell types other than macrophages. Although it was proved that *C. albicans* triggers the upregulation of *ICL1* for entrance in the glyoxylate cycle when co-incubated with macrophages and neutrophils^{82,586,587}, my results suggest that *I1* expression by the fungal pathogen does not affect macrophages' ability to eliminate it after 3 h of co-incubation *in vitro*.

These collective results suggested that the IFN- β candidacidal mechanism is independent of metabolic restriction of *C. albicans* via blockade of the glyoxylate cycle.

4.4.4 Conventional AMPs are not responsible for the candidacidal effect of IFN- β

Expression of β -defensins 1 and -2 and TSLP was not detected at RNA level. Although RNAseq data did not suggest that β -defensin 1 (*Defb1*) and -2 (*Defb2*) were upregulated during *Candida* spp. infection, these two AMPs were quantified, as these are highly expressed by macrophages and blood monocytes and were shown to have strong candidacidal activity against *C. albicans*. β -defensins were also found to be induced in macrophages and kidney cells by pro-inflammatory cytokines and LPS^{224,588}, however, its expression was not detected in *Candida*-infected BMDMs. A similar result was reported in reconstituted human oral epithelia, where *C. albicans* inhibits β -defensin transcription after 24 h of infection partially through production of Sap1-3⁵⁸⁹. As *C. parapsilosis* also codes for Sap1-3 orthologs⁶⁰, it could explain why these were not detected with both *Candida* spp. Elder and colleagues revealed that TSLP is secreted by monocyte-derived DCs upon exposure of *C. albicans* in a Dectin-1-dependent manner⁵⁹⁰, however the authors measured TSLP at protein level and infected DCs with double the amount of *Candida* per cells used in my research. It is likely that TSLP detection was not detected at the RNA level in BMDMs because transcription was already “shut down” after 24 h of stimulation at the MOI tested.

IFN- β stimulation by itself did not lead to enhanced levels of *Camp* in uninfected cells, and a slight increase in *Ltf* expression was detected. However, stimulation with both *C. albicans* and *C. parapsilosis* induced downregulation of both AMPs at transcript level, regardless of the presence of cytokine, suggesting that IFN- β does not directly influence *Camp* and *Ltf* expression in BMDMs. Both *Camp* and *Ltf* expression can regulate production of type I IFN. *Camp*-deficient RAW264.7 macrophages and BMDCs show reduced levels of IFN- α after activation with the TLR9 agonist CpG⁵⁹¹, and LL-37 (human ortholog of *Camp*) boosts IFN- α production in pDCs by facilitating recognition of RNA by TLR7 and TLR8⁵⁹². Lactoferrin induces production of type I IFN in peritoneal macrophages⁵⁹³ and oral administration of lactoferrin is also known to boost type I IFN levels in circulation and specific organs^{594,595}.

Although it is known that both AMPs are involved in production of type I interferon, literature on the ability of type I IFN to induce production of those AMPs is scarce. Recently,

C. glabrata-induced type I IFN production was shown to downregulate LTF expression in splenocytes during systemic infection⁵⁹⁶. *C. parapsilosis* results fit this finding as it induces IFN- β production in BMDMs, however a downregulation was also verified in *C. albicans*-stimulated BMDMs that do not produce type I IFN. The reason for this is unclear, however there is a heterogeneous number of different cells that influence the immune response in the spleen during systemic candidiasis, making it difficult to link that finding to a specific cell type. Regarding *Camp*, it was unexpected that IFN- β did not alter its expression as type I interferon has been suggested to indirectly suppress LL-37 release in human monocytes/macrophages^{597,598}.

Both *Candida* spp. have downregulated expression of the two AMP-coding genes. *C. albicans* stimulation was shown not to significantly affect *Camp* expression in neutrophils⁴⁵⁹, but research has revealed that it can ameliorate LL-37 action by shedding MSB2 fragments^{219,599}. Although this was not reported by *C. parapsilosis*, according to *Candida* Genome database⁵⁷⁴, *C. parapsilosis* is predicted to code for an ortholog of MSB2, and therefore it might be able to deploy the same mechanism. Moreover, *C. albicans* SAPs can also cleave and inactivate LL-37, and as *C. parapsilosis* can also code for Sap orthologs it might be able to deploy the similar mechanisms^{60,600}. Nevertheless, these mechanisms are post-translational, and cannot explain the downregulation verified.

4.4.5 Administration of IFN- β is associated with a detrimental effect during systemic candidiasis

My results show that administration of IFN- β does not improve the outcome of murine systemic candidiasis. Type I IFN signalling was shown to promote host resistance against infection caused by bacterial species such as *E. coli* and *S. pneumoniae*⁶⁰¹, and even fungal species like *C. neoformans*⁶⁰². A protective role of type I IFN during invasive pulmonary aspergillosis was also described^{603–606}. However, for other pathogens such as *M. tuberculosis* and *S. aureus* type I IFN was reported to have a detrimental effect^{607–611}. Literature regarding the role of type I IFN during systemic candidiasis is heterogeneous and controversial.

Some studies corroborate my findings. Jensen and colleagues revealed that poly(I:C)-triggered type I IFN production in BALB/c mice during systemic candidiasis induced by *C. albicans* B311 (1×10^4 CFUs) is detrimental to infection as it impairs fungal clearance in spleens, livers and kidneys of infected mice by suppressing the candidacidal activity of macrophages⁵⁶². Guarda's findings line up with this, as both poly(I:C) administration and type I IFN signalling worsen susceptibility of C57BL6/J mice towards *C. albicans* (2.5×10^5 CFUs; unspecified strain) by suppressing IL-1 signalling and inflammasome activation⁵³⁶. Using IFNAR-1 deficient mice and *C. albicans* SC5314 (1×10^5 CFUs), Majer *et al.* reported that type I IFN is detrimental for survival against systemic candidiasis by promoting fatal immunopathology driven by excessive recruitment of inflammatory DCs and neutrophils to infected kidneys in early stages of infection⁵³⁷. A different article from the same group also revealed that type I IFN promotes *C. glabrata* ATCC2001 persistence in spleens, brains and livers of infected mice (5×10^7 CFUs per 25 g of mouse weight)¹⁸⁸. My results are similar to those reported in Stawowczyk *et al.* where i.p. administration of 100,000 U IFN- β in C57BL/6N mice before i.v. challenge of *C. albicans* SC5314 (2.5×10^5 CFUs) increased the susceptibility of mice towards infection and resulted in reduced fungal clearance in kidneys. In this article, authors suggest that type I IFN contributes to susceptibility through downstream IFIT2 production that impairs phagocytosis, production of ROS by myeloid cells and synthesis of phagocyte chemoattractants in the kidneys of infected mice⁵³⁸.

On the other hand, other reports have proved that type I IFN signalling is beneficial to systemic candidiasis. Using IFNAR1-deficient mice (C57BL6/J background) Biondo and colleagues showed that type I IFN signalling improves survival and kidney fungal clearance during systemic candidiasis induced by *C. albicans* ATCC 90028 (1×10^4 CFUs) possibly through its ability to boost DCs candidacidal activity¹²². Del Fresno *et al.* obtained similar results using the same mouse model and *C. albicans* SC5314 (1×10^5 CFUs), proposing that type I IFN signalling is required for protective neutrophil recruitment to the kidneys and effective *Candida* clearance¹⁸⁹. Dominguez-Andres and colleagues proposed that type I IFN production by myeloid phagocytes is necessary for triggering protective signalling cascades in spleen and kidneys of C57BL67/J mice that will ultimately boost the candidacidal activity of kidney neutrophils towards *C. albicans* SC5314 (1×10^5 CFUs)³¹⁴.

The conflicting literature makes it hard to underpin the effect of type I IFN during infection. Although my results suggest that IFN- β is deleterious to mice survival during systemic candidiasis, no significant differences in fungal burden were verified, although a slightly decreased fungal load was detected in kidneys of mice injected with the 5,000 U IFN- β that survived the infection. IFN- β administration can lead to excessive monocyte and DC activation and recruitment to the kidneys where they can contribute to immunopathology, and this effect might be rescued using pioglitazone, a pharmacological agonist of PPAR- γ receptors that impairs trafficking of inflammatory phagocytes and suppresses fatal immunopathology during *C. albicans*-induced systemic candidiasis⁵³⁷. If the deleterious effect observed was due to immunopathology, co-administration of IFN- β and pioglitazone during infection could ameliorate excessive inflammation triggered by IFN- β whilst improving fungal clearance and mice survival.

4.4.6 HetIL-15 promotes splenic T cell expansion and enhances IFN- γ and granzyme B production during systemic candidiasis

As IFN- β administration proved to be detrimental for the outcome of systemic candidiasis, I have decided to assess the effect of IL-15, a cytokine induced downstream of IFN- β as a consequence of type I IFN signalling activation^{314,612}. Initial *in vivo* experiment using a high dose of hetIL-15 (3 μ g) did not improve fungal clearance of *C. albicans* in kidneys and brains neither improved mice survival. Moreover, lower levels of circulating IL-12p40 and higher levels of IL-6 in detected in infected mice suggest that at that concentration, hetIL-15 might have ameliorated Th1 polarisation and contributed for immunopathology which would explain the reduced survival rates and higher fungal burdens^{613,614}. Moreover, other studies have suggested that exacerbated production of IL-15 can have deleterious effects such as the development of leukaemia through robust chromosomal instability originated in NK and NKT cells, however in some NK cell-derived cancers this can be ameliorated through an appropriate dosage of IL-15^{615,616}. Indeed, IL-15 production was shown to be necessary for NK cell development and activation during systemic candidiasis³¹⁴, and so it is possible that in my first experiment, 3 μ g hetIL-15 was a detrimental excessive dose.

I then decided to repeat the same experiment, but with a lower dose of hetIL-15 (1 µg) and look at how it affects splenic adaptive responses during *C. albicans* challenge. In this experiment, all mice survived the infection, and compared with the previously tested dosage, lower organ fungal burdens and circulating IL-6 levels were detected. Besides, slightly higher (not significant) IL-12p40 levels were detected this time in hetIL-15-injected mice compared with vehicle-injected mice, which supports my previous theory that 3 µg hetIL-15 was an excessive dose in this model of disease. Looking at the spleen dynamics, administration of hetIL-15 led to significantly enlarged spleens and robust expansion of NK cells, CD4⁺ and CD8⁺ T cells compared to controls, which lines up with literature^{557,617,618}. Looking at cytokine production from these populations, hetIL-15 also induced robust production of IFN-γ by CD8⁺ T cells and granzyme B from NK cells and CD8⁺ T cells as was also previously reported⁶¹⁷ suggesting that hetIL-15 boosts the cytotoxic activity of splenocytes. However, this did not translate to efficient clearance of *Candida* in the kidneys and brains. CD8⁺ T cells play a role in clearance of *Candida* during systemic infection^{292,293}, however, Dominguez-Andres and colleagues reported that IL-15-driven GM-CSF production by splenic NK cells is essential for boosting the candidacidal activity of kidney neutrophils and promote *Candida* clearance³¹⁴. In this last *in vivo* experiment, only a slight, not significant production of GM-CSF by CD8⁺ T cells was detected with hetIL-15, but not by NK cells. The reason for this is unclear, as it was proven that *C. albicans* can trigger its production via type I IFN-driven IL-15 release in monocytes³¹⁴, and that IL-15-releasing DCs can trigger GM-CSF release by NK cells⁶¹⁹. It is possible that GM-CSF release was boosted by hetIL-15 but was not detected by splenic NK cells after four days of administration because its effect was already absent. Dominguez-Andres also found that IFN-γ production by NK cells peaked after 24 h of infection and its levels dropped significantly after 36 h where they were comparable to the those detected in uninfected NK cells³¹⁴. It is possible that significant differences in production of both GM-CSF and IFN-γ by NK cells could be detected 24 h after hetIL-15 injection. Nevertheless, in my pilot experimental setting, hetIL-15 does not appear to contribute for fungal clearance after four days of infection.

4.5 Conclusions

Macrophages are key phagocytes in initiating an adequate immune response against *C. albicans* and are important players in the elimination and restriction of *Candida* proliferation in several host niches during systemic candidiasis. Bolstering these cells' candidacidal ability might stand as a novel therapeutic approach to promote clearance of *C. albicans* and improve the onset of candidaemia.

In this chapter it was revealed that IFN- β stimulation boosts macrophage candidacidal activity against *C. albicans*, and although it elicits a robust NO production, it was evidenced that type I IFN signalling-induced candidacidal mechanism does not depend on NO and ROS generation, and conventional AMP production which are major components of the antifungal defence mechanism deployed by phagocytes. Moreover, IFN- β also does not appear to affect *C. albicans* metabolic plasticity and to inhibit the pathogen's ability to use the glyoxylate cycle, a recently discovered, virulence-associated strategy deployed by *C. albicans* to survive inside macrophages.

IFN- β promotes *C. albicans* clearance by BMDMs *in vitro*, however, *in vivo* experiments suggest that its administration during systemic candidiasis has a detrimental effect as mice are more susceptible to disease when injected with a high dose of IFN- β , possibly due to deleterious cytokine-driven immunopathology. Nevertheless, other cytokines induced downstream of type I IFN signalling might contribute to a better outcome of the pathology while bypassing the detrimental effects exerted by IFN- β . Following this rationale, hetIL-15 was tested, and was shown to augment splenic immunity by inducing proliferation of NK cells, CD4⁺ and CD8⁺ T cell production during systemic candidiasis. This was accompanied by a robust granzyme B production by NK cells and CD8⁺ T cells and increased IFN- γ production by CD8⁺ T cells. This suggested that hetIL-15 augments cytotoxic activity of splenocytes, however no significant differences were observed in fungal clearance of *C. albicans* in main target organs of disease. Nevertheless, it is possible that this was due to an excessive dose of hetIL-15, that caused CD8⁺ T cells and NK cells to excessively kill macrophages and other phagocytes necessary for effective clearance of *C. albicans*, and further experiments with longer time

courses and lower hetIL-15 doses need to be conducted to assess if it can be beneficial to the outcome of infection.

Although all three aims set for this chapter were addressed, whilst IFN- β was proved to improve the outcome of infection in BMDMs, its candidacidal mechanism was not yet fully elucidated here. Moreover, my research has not permitted a definitive conclusion regarding the impact of type I IFN pathway in systemic *in vivo* infection, and more experimentation is required to truly ascertain its effect. The identification of the IFN- β -driven molecular pathways that endow macrophages with robust anti-*Candida* activity is of interest. Once identified, their *in vivo* induction might bypass the detrimental effects observed with IFN- β administration and improve the outcome of systemic candidiasis. Ultimately, this can be explored for the development of a novel class of antifungals for treatment of systemic candidiasis and be used as an alternative or complement to some toxic and ineffective antifungals currently prescribed for treatment of its treatment.

4.6 Future experiments

Here, experiments designed to assess IFN- β production by *Candida* spp. and to elucidate the candidacidal mechanism of type I IFN were conducted in murine BMDMs. Assessing if the same results are verified in other primary tissue-resident macrophages such as peritoneal macrophages and in human monocytes and monocyte-derived macrophages is of interest as it could add clinical relevance to these findings. Moreover, assessing if similar results would be obtained using different *C. albicans* and *C. parapsilosis* strains would further strengthen my data.

Although my results provide strong evidence that NO is not involved in the BMDM enhanced candidacidal effect caused by type I IFN, the same cannot be said about ROS. To support my results, killing assays could be performed using a mix of ROS scavengers or NOX2-deficient BMDMs in the presence of IFN- β .

Only selected AMPs were quantified here in *Candida* spp.- and IFN- β -stimulated BMDMs to test if these are the molecular players responsible for the enhanced type I IFN-

triggered candidacidal effect. Other AMPs should be detected to further elucidate this mechanism. Moreover, some chemokines induced by type I IFN have AMP-like structure and direct antimicrobial activity (kinocidins), and it would be interesting to assess if some of these purified chemokines have direct candidacidal effect against *C. albicans*. Moreover, type I IFN signalling might promote phagosome acidification and production of phagosomal hydrolases which could account for its candidacidal activity, however this was not assessed here.

My *in vivo* experiments showed that administration of type I IFN itself does not improve the outcome of *C. albicans*-induced systemic candidiasis possibly due to excessive inflammation triggered by the cytokine. It would be interesting to assess if this deleterious immunopathology could be overcome by co-administration of IFN- β and other inflammation suppressants such as pioglitazone. Administration of hetIL-15 induced downstream of IFN- β boosted cytotoxic effect of splenic lymphocytes but did not result in improved *Candida* clearance. Different concentrations of hetIL-15 should be tested during infection and in longer *in vivo* experiments, to truly assess if it can contribute for resistance against systemic candidiasis. Moreover, administration of other type I IFN-inducible cytokines induced downstream of IFN- β should also be tested.

Chapter 5

Elucidating the *C. albicans*-mediated mechanism of IL-27 inhibition in macrophages

5.1 Introduction

During systemic candidiasis, cytokines from the IL-12 family are important in conferring host resistance due to their ability to link host innate immunity with the development of adaptive immune responses. IL-27 is a member of the IL-12 family produced mainly by myeloid cells that exerts broad immunomodulatory effects in the immune system^{620,621,630,631,622–629}.

The effect of IL-27 during *Candida* spp. infection was only recently studied by our group. We have shown that some *Candida* spp. like *C. parapsilosis*, *C. tropicalis* and *C. glabrata*, but not *C. albicans* can induce IL-27 production in BMDMs, with *C. parapsilosis* inducing much higher levels than the former species. Since IL-27 is type I IRG induced downstream of type I interferon signalling, IFN- β contributed to production of high levels of IL-27 by infected BMDMs¹⁸⁴. We have also found that whilst *C. albicans* is unable to induce IL-27, possibly due to inability to trigger IFN- β production to the same extent as *C. parapsilosis*, *C. albicans* is also able to actively block IL-27 production via an unidentified soluble mediator¹⁸⁴. Moreover, we have revealed that impairment of IL-27 signalling confers resistance against *C. parapsilosis* as IL-27R α -deficient mice display lower kidney fungal burden after infection with minimal inflammatory infiltrates detected¹⁸⁴, but on the other hand, IL-27 signalling abrogation does not have an apparent effect on *C. albicans*-induced systemic candidiasis¹⁸⁴.

This chapter's main focus is to understand how *C. albicans* is able to block IL-27 production in BMDMs, and if the pathogen is also able to block IFN- β production, upstream of IL-27. For that end, in here I have designed experiments to elucidate the *C. albicans*-mediated IL-27 inhibitory mechanism.

5.2 Aims of this chapter

The main hypothesis set for this chapter is that *C. albicans* is able to block *C. parapsilosis*-induced IFN- β and IL-27 production via a virulence-related specific soluble mediator. Throughout this chapter, *in vitro* experiments were conducted to address the following aims:

- I. To assess whether *C. albicans* is able to inhibit *C. parapsilosis*-induced IFN- β production, upstream of IL-27, in BMDMs;
- II. To elucidate the *C. albicans*-mediated mechanism of IL-27 inhibition in BMDMs.

5.3 Results

5.3.1 *C. albicans* blocks *C. parapsilosis*-induced IFN- β and IL-27 via a secreted molecule.

Since IL-27 is a type I IRG and is induced downstream of IFN- β signalling, I hypothesized that the previously reported IL-27 blockade happened due an upstream blockade of IFN- β induced by *C. albicans* that would result in reduced IL-27 levels. To test this hypothesis and to understand how *C. albicans* blocks IL-27, *C. albicans* was incubated for 24 h in the absence or presence of BMDMs to allow for the accumulation of soluble mediators in the cell culture medium, supernatants were then sterile filtered to remove non-soluble material (such as live fungi and large cellular debris) from the media and were then added to freshly plated BMDMs alongside *C. parapsilosis*. Following another 24 h incubation, IL-27 and IFN- β levels were then detected in the supernatants (Figure 5.1).

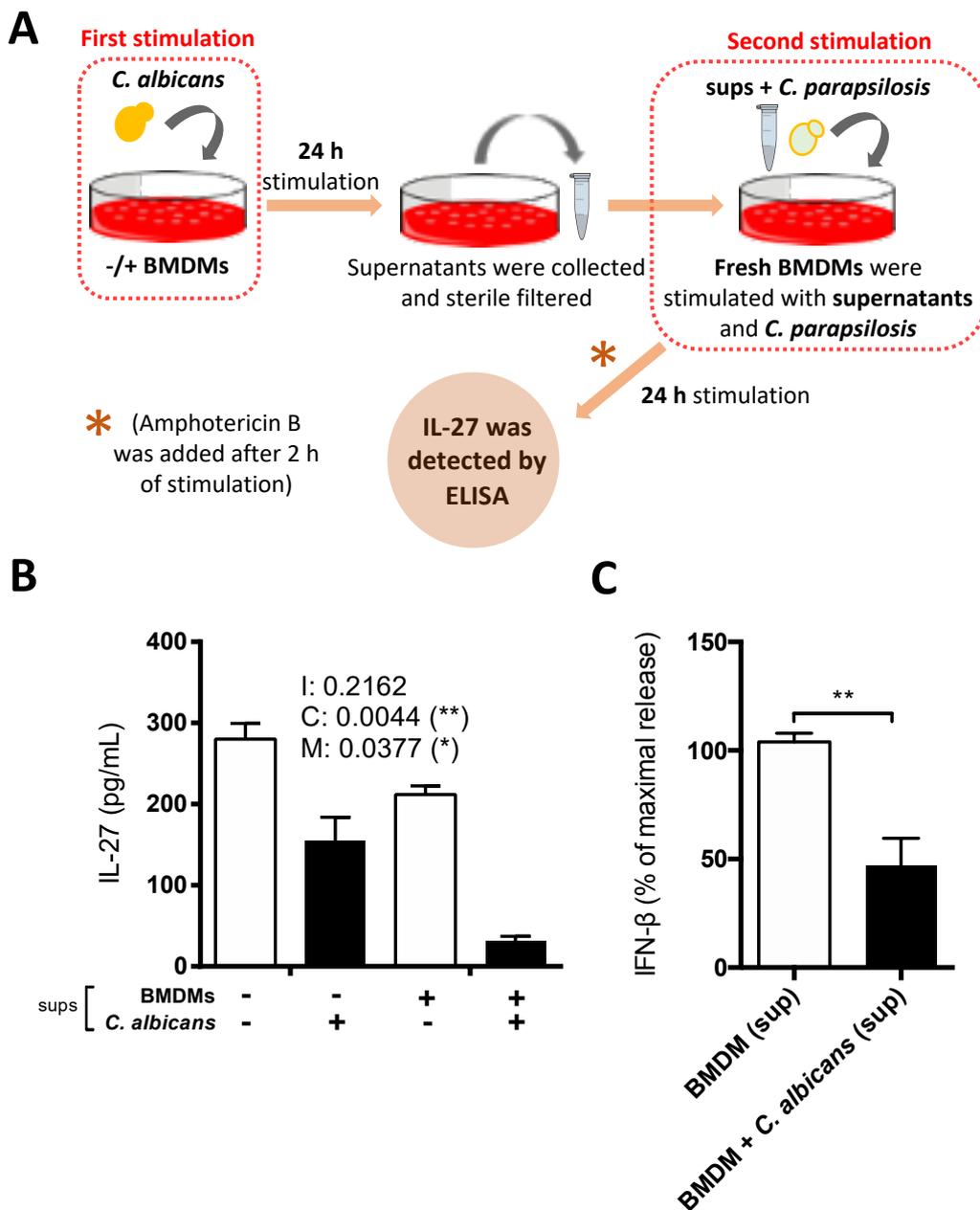


Figure 5.1 – Supernatants from *C. albicans*-stimulated BMDMs block *C. parapsilosis*-induced IFN- β and IL-27 production. (A) Schematic of experimental design: *C. albicans* was resuspended in cell culture medium and incubated in the presence (“+BMDMs”) or absence (“-BMDMs”) of BMDMs. After 24 h incubation, supernatants were collected, sterile filtered and added to fresh BMDMs with *C. parapsilosis*. IFN- β and IL-27 levels produced by macrophages were detected after another 24 h stimulation. Amphotericin B was added after 2 h co-stimulation of BMDMs with supernatants and *C. parapsilosis*. (B) IL-27 levels were measured by ELISA. Data is represented as means \pm SEM, n=3, Two-way Repeated Measures ANOVA with Sidak post-hoc test. I: Interaction between +/- *C. albicans* in supernatants and supernatants from +/- BMDMs in the first 24 h of incubation; C: effect of the initial presence of *C. albicans*, M: effect of the initial presence of BMDMs. (C) IFN- β levels were detected by Luminex assay or ELISA. Raw data was converted to percentage of maximal release of IFN- β from the conditions “BMDM (sup)”. Data is presented as means \pm SEM, n=5, two-tailed paired t-test. * p <0.05, ** p <0.01.

BMDMs stimulated with both *C. parapsilosis* and filtered conditioned medium (-BMDMs sups) produced high levels of IL-27 (280.2 ± 19.5 pg/mL) (Fig 5.1B). Stimulation with supernatants from *C. albicans* cultured in the absence of BMDMs resulted in a ~45% inhibition of *C. parapsilosis* induced IL-27 levels in BMDMs (154.3 ± 29.5 pg/mL). Macrophages stimulated with *C. parapsilosis* and supernatants from uninfected BMDMs also produced high levels of IL-27 (211.8 ± 10.7 pg/mL), however, addition of supernatants from *C. albicans* co-cultured with BMDMs alongside *C. parapsilosis* led to higher reduction of ~85% in IL-27 levels (31.6 ± 5.7 pg/mL). Interestingly, conditioned media obtained from *C. albicans*-infected BMDMs led to significantly lower levels of IL-27 produced by BMDMs after stimulation with *C. parapsilosis* compared with conditioned media obtained from *C. albicans* alone. Therefore, statistical analysis revealed that in these experiments, IL-27 production is influenced not only by the presence of *C. albicans* in the conditioning supernatant cultures ($p=0.0044$) but also by the presence of macrophages in the first 24 h of stimulation when co-incubated alongside *C. albicans* ($p=0.0377$).

I have also determined if *C. albicans* conditioned media was able to block IFN- β production. For that end, IFN- β levels in second culture supernatants were measured by two different techniques: ELISA, and Luminex. To compile data obtained via the two different quantification methods in the same graph, results were converted to percentage of maximal release. To do so, for each individual experiment, IFN- β levels were calculated by setting the values detected in macrophages stimulated with *C. parapsilosis* + supernatants from uninfected BMDMs (positive control, condition with maximal release) as 100%. Figure 5.1-C shows the results from IFN- β quantifications. Stimulation with supernatants from BMDMs-*C. albicans* co-cultures led to a significant ~55% reduction of IFN- β levels produced by *C. parapsilosis*-infected BMDMs. (BMDM (sup): 103.9 ± 4.1 %; BMDM + *C. albicans* (sup): 47.0 ± 12.6 %). Results indicate that, like IL-27, *C. albicans* supernatants are also able to block IFN- β production.

The experimental design used for experiments shown on Figure 5.1-A differed slightly from the one used by Patin and colleagues, who only used supernatants from BMDMs co-incubated with *C. albicans* and have not tested supernatants from *C. albicans* incubated in

the absence of BMDMs in the first 24 h of stimulation. Moreover, in those experiments, authors have added amphotericin B to neutralise *C. albicans* growth after 2 h of co-stimulation¹⁸⁴. This allowed macrophages to respond to pathogen stimulation while minimising macrophage viability loss. Therefore, most BMDMs would still be alive and viable after the initial 24 h of co-stimulation with the pathogen. This way however, amphotericin B was present in the supernatants after filtration and it could affect *C. parapsilosis* growth in the second stimulation step when supernatants (containing amphotericin B in this case) and *C. parapsilosis* are added to freshly plated BMDMs. For this reason, in experiments shown in Figure 5.1, amphotericin B was not added in the first 24 h of stimulation. This allowed *C. albicans* to grow unhindered when added to BMDMs, and naturally after 24 h the fungal pathogen would kill all macrophages in the well. As a consequence of cell lysis, macrophage intracellular components and other immune mediators would be released in to the medium, and it was possible that in these experiments the IL-27 inhibition verified could be an effect of these components released by dead macrophages and not from a mediator induced from *C. albicans*. To evaluate if this was the case, similar IL-27 blockade experiments were performed with addition, or not, of amphotericin B in the first 24 h of stimulation (Figure 5.2)

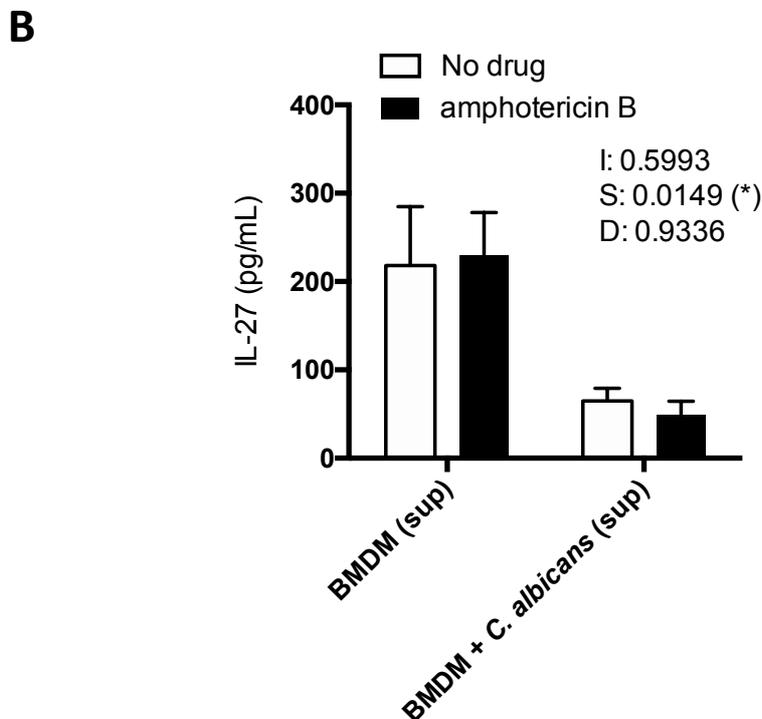
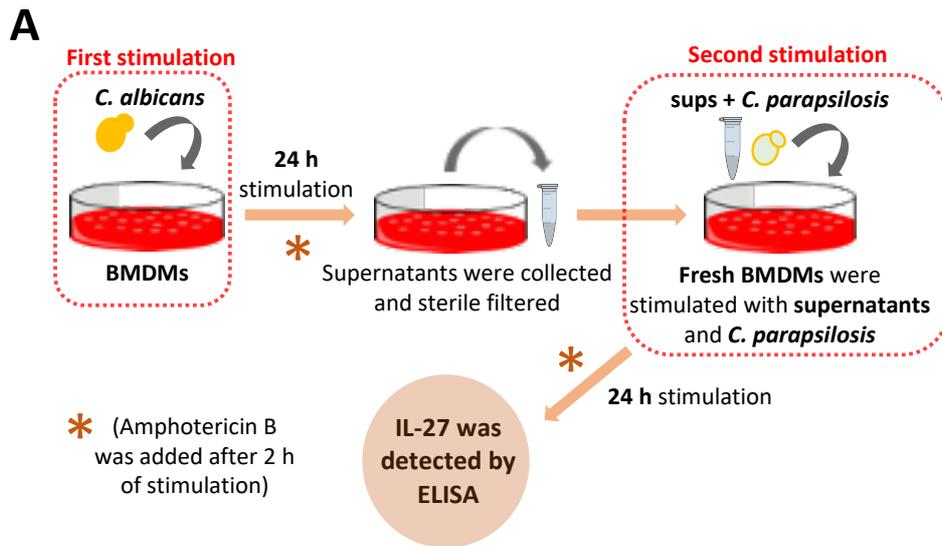


Figure 5.2 – Amphotericin B-mediated neutralisation of *C. albicans* during initial co-stimulation with BMDMs does not affect blockade of *C. parapsilosis*-induced IL-27 production. (A) Schematic of experimental design: *C. albicans* was co-cultured with BMDMs for 2 h, followed by addition (“amphotericin B”) or not (“No drug”) of amphotericin B (2.5 $\mu\text{g}/\text{ml}$) for another 22 h. Following 24 h stimulation, supernatants were collected, sterile filtered and added to fresh BMDMs with *C. parapsilosis* for another 24 h. Amphotericin B was added again after 2 h co-stimulation of BMDMs with supernatants and *C. parapsilosis*. IL-27 levels produced by macrophages were detected by ELISA. (B) IL-27 levels detected after the second stimulation described in A. Data is presented as means \pm SEM, $n=5$, Two-way Repeated Measures ANOVA with Sidak post-hoc test. I: interaction between supernatants from BMDMs $-/+$ *C. albicans* and neutralisation of *C. albicans* using amphotericin B; S: effect of different supernatants tested; D: impact of $-/+$ amphotericin B to neutralise *C. albicans*. * $p<0.05$.

Stimulation with *C. parapsilosis* and conditioned media obtained from filtered supernatants collected from uninfected BMDMs elicited production of high levels of IL-27 in fresh BMDMs, regardless of the presence (230.1 ± 48.1 pg/mL) or absence (218.1 ± 66.9 pg/mL) of amphotericin B. Stimulation of fresh BMDMs with *C. parapsilosis* and conditioned media obtained from supernatants collected from *C. albicans*-infected BMDMs in the presence of amphotericin B (49.8 ± 14.8 pg/mL) led to similar inhibition of *C. parapsilosis*-induced IL-27 as observed with conditioned media collected from *C. albicans*-infected BMDMs cultured in the absence of the drug (65.0 ± 14.3 pg/mL) (Inhibition of IL-27 - No drug: ~70%, amphotericin B: ~78%). Statistical analysis revealed that in these experiments, IL-27 production is only influenced by the presence of *C. albicans* in supernatants ($p=0.0149$) and not by addition of amphotericin B the first 24 h of stimulation ($p=0.9336$).

Results from the preceding experiments suggest that IL-27 inhibition is caused by a secreted molecule from *C. albicans* and is not a consequence of *C. albicans*-induced BMDM lysis in the first 24 h of stimulation. To further validate this theory and to assess if this blockade was dependent on the direct contact of *C. albicans* with BMDMs, a new experimental design was defined using three different 24 h incubation steps. These involved two sterile filtrations of supernatants before addition to BMDMs with *C. parapsilosis* to detect IL-27: *C. albicans* was resuspended in cell culture medium and incubated for 24 h in the absence of BMDMs ("First stimulation"), the supernatants were sterile filtered and added to BMDMs for another 24 h ("Second stimulation"), supernatants from stimulated BMDMs were sterile filtered again and added to freshly plated macrophages alongside *C. parapsilosis* for another 24 h ("Third stimulation"). In this setting, *C. albicans* was never in direct contact with the macrophages and therefore macrophages would not be killed as a result of this contact (Figure 5.3-A). In parallel, and for comparison purposes, a different set of BMDMs were stimulated with *C. albicans* for 24 h, supernatants were sterile filtered and added to fresh BMDMs with *C. parapsilosis* as in previous experiments. In this case, supernatants were only filtered once and *C. albicans* was allowed to have direct contact with BMDMs (Figure 5.1-A, conditioned media obtained from *C. albicans* co-incubated with BMDMs).

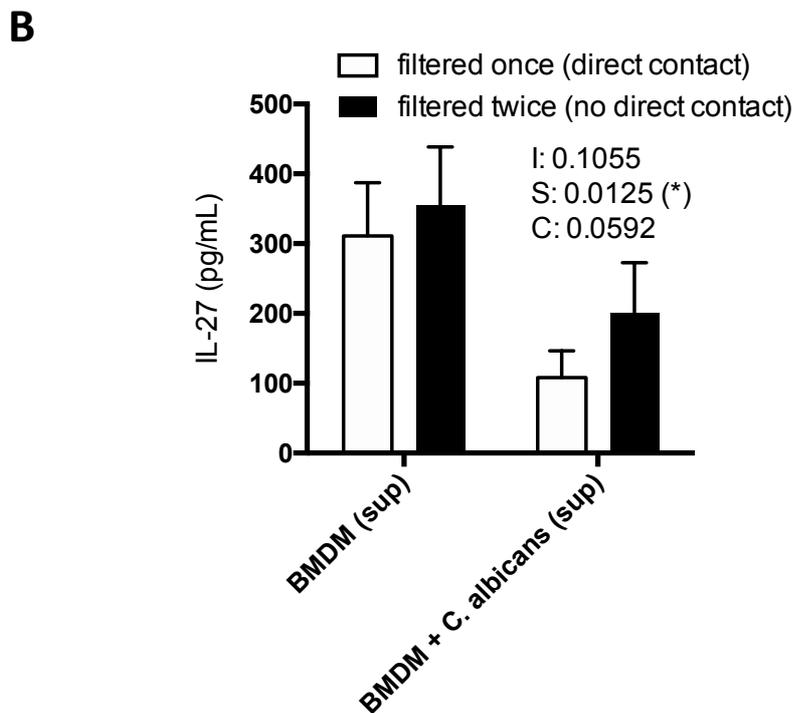
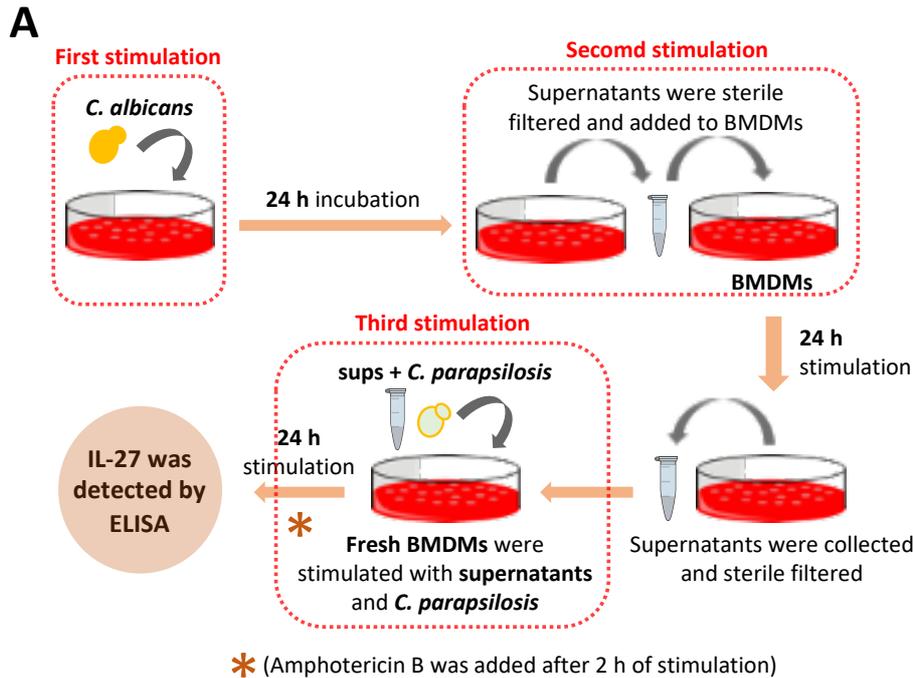


Figure 5.3 – *C. albicans*-mediated IL-27 blockade is not dependent on direct contact of the pathogen with BMDMs. (A) Schematic of experimental design: *C. albicans* was resuspended in cell culture medium and incubated for 24 h. Supernatants were collected, sterile filtered and added to BMDMs. Following 24 h stimulation, supernatants were collected, sterile filtered again and added to fresh BMDMs alongside *C. parapsilosis*. IL-27 levels produced by macrophages were detected after another 24 h stimulation by ELISA. Amphotericin B was added 2 h after stimulation with *C. parapsilosis* and supernatants. (B) IL-27 levels detected after the second stimulation described in A. Data is presented as means \pm SEM, n=5, Two-way Repeated Measures ANOVA with Sidak post-hoc test. I: interaction between the different supernatants tested and *C. albicans* initial direct contact; S: effect of different supernatants tested; C: impact of initial contact of *C. albicans* with BMDMs to generate the IL-27 blocking signal. * $p < 0.05$.

Figure 5.3-B reveals the results obtained from these experiments. In experiments involving direct contact of BMDMs with *C. albicans* (white bars), stimulation with conditioned media obtained from *C. albicans* supernatants (108.3 ± 38.2 pg/mL) led to a ~65% reduction of *C. parapsilosis*-triggered IL-27 levels compared with stimulations using conditioned media from uninfected BMDMs (310.9 ± 76.6 pg/mL). In experiments where supernatants were filtered twice and no direct contact of *C. albicans* with BMDMs has occurred, *C. albicans* supernatants (201.3 ± 71.5 pg/mL) led to a ~45% reduction of *C. parapsilosis*-induced IL-27 levels compared with stimulations using *C. parapsilosis* and supernatants from uninfected BMDMs (355.5 ± 83.1 pg/mL). Although the extent of IL-27 inhibition is slightly different depending on whether *C. albicans* has directly contacted with BMDMs or not in the first 24 h of stimulation, *C. albicans* supernatants obtained from both methods were able to significantly inhibit IL-27 production.

The collective results obtained from Figures 5.1 to 5.3 led me to formulate a theory for the *C. albicans*-mediated blockade of *C. parapsilosis*-induced IFN- β and IL-27 production. A scheme summarising this theory is presented in Figure 5.4

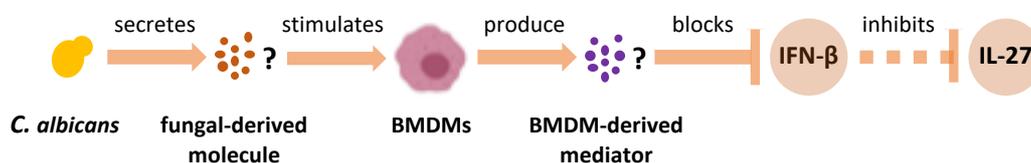


Figure 5.4 – Theory of IFN- β blockade mechanism induced by *C. albicans* during stimulation with BMDMs. Hypothesis: IL-27 inhibition is caused by a soluble mediator secreted by *C. albicans*, that once in contact with BMDMs will induce the production of a secondary macrophage-derived mediator responsible for blocking IFN- β production. This blockade would translate in lower levels of IL-27 produced downstream of IFN- β .

I have hypothesized that this blocking mechanism would be orchestrated by two different mediators: a primary *C. albicans*-derived secreted molecule, and a secondary BMDM-derived mediator. My theory postulates that *C. albicans* secretes a soluble molecule, that will stimulate BMDMs to produce a cell-derived mediator that will be responsible for blocking IFN- β production. *C. parapsilosis* induces IL-27 production in BMDMs via phagocytosis and activation of TLR7-MyD88 and NOD2 signalling, promoting expression of both IFN- β and IL-27¹⁸⁴. IFN- β would then be secreted and signal through IFNAR1/2 leading

to continuous production of both cytokines in a positive feedback loop. Blockade of IFN- β production and consequent signalling via IFNAR1/2 would break the feedback loop and result in reduced levels of IFN- β . This would be reflected by lower levels of IL-27 downstream of type I IFN signalling.

5.3.2 Cytokines specifically induced by *C. albicans* are not the BMDM-derived mediator responsible for IL-27 inhibition

I have hypothesized that the secondary BMDM-derived mediator could be a cytokine, chemokine or growth factor produced by BMDMs in response to *C. albicans* secreted molecule (primary signal). Taking this into consideration, I then postulated that this secondary signal could be one of the immune mediators triggered specifically by *C. albicans* stimulation in BMDMs and not by *C. parapsilosis* previously found in the RNAseq analysis performed on Chapter 3 (Figure 3.2, dashed box). To test this hypothesis, BMDMs were stimulated with *C. parapsilosis* and the *C. albicans*-specific immune mediators individually and IL-27 levels were later detected in cell culture medium (Figure 5.5).

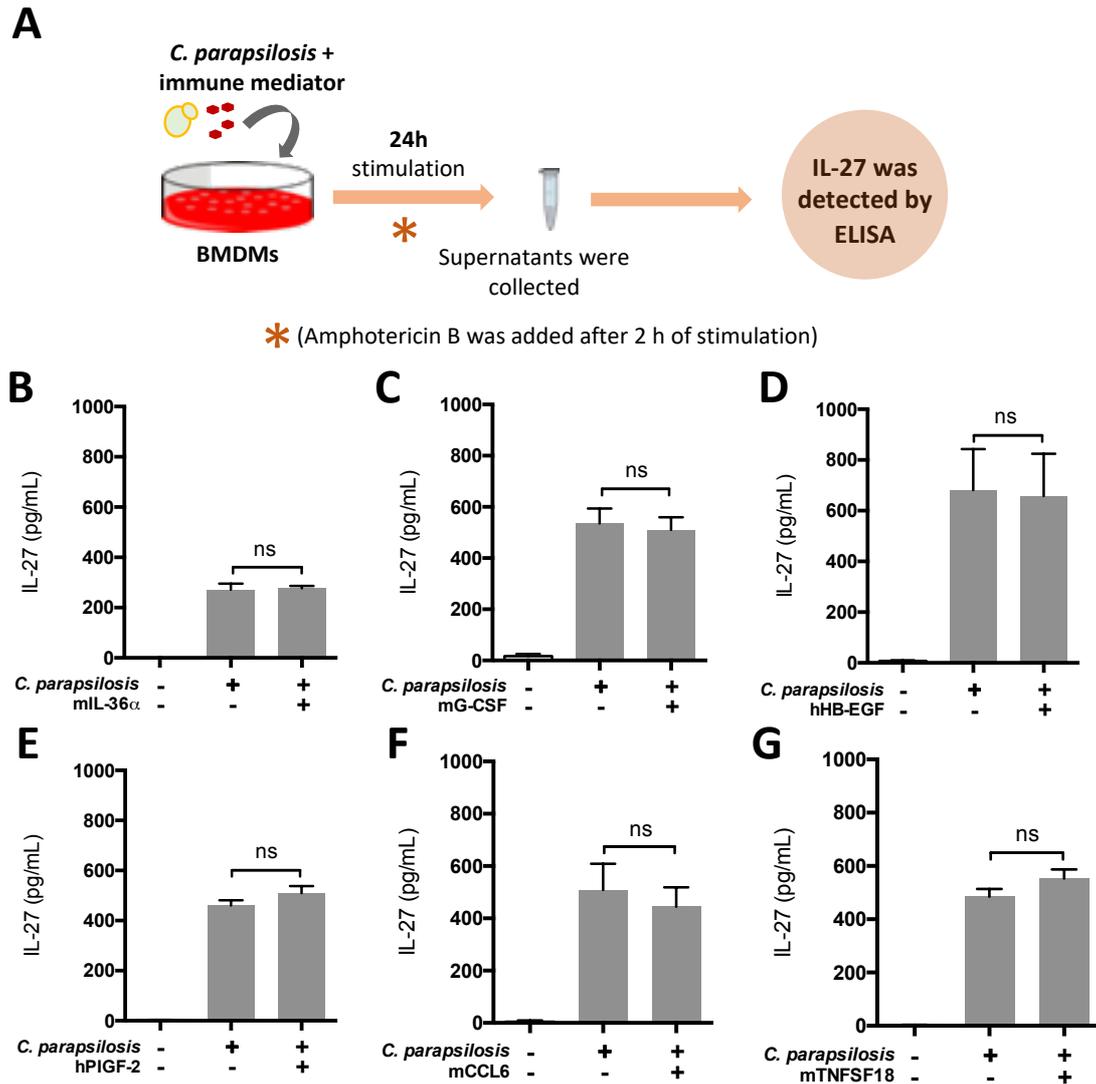


Figure 5.5 – Immune mediators specifically induced by *C. albicans* do not block IL-27 production triggered by *C. parapsilosis*. (A) Schematic of experimental design: BMDMs were stimulated with either *C. parapsilosis* and 500 ng/mL of recombinant murine IL-36 α (B), murine G-CSF (C), human HB-EGF (D), human PIGF-2 (E), murine CCL6 (F) or murine TNFSF18 (G) for 24 h. Amphotericin B was added after 2 h of co-stimulation. IL-27 levels in the supernatants were detected by ELISA. Data is presented as means \pm SEM. B and C: n=3; D, E and F: n=4; G: n=5. Ordinary One-way ANOVA with Tukey post-hoc test. ns – not statistically significant.

Stimulation of *C. parapsilosis*-infected BMDMs with murine IL-36 α (*Il1f6*) did not result in significantly lower levels of IL-27 (276.7 ± 9.3 pg/mL) compared with BMDMs infected in the absence of IL-36 α (270.8 ± 24.6 pg/mL). According to the immune mediators heatmap (Figure 3.2), IL-36 γ (*Il1f9*), another IL-36 isoform that was specifically induced in BMDMs by *C. albicans*, was not tested, however all four isoforms of IL-36 are known to signal through the same IL-36R receptor⁶³², therefore IL-36 α was used as representative cytokine of the IL-36

family. (Figure 5.5-C) Addition of murine G-CSF (*Csf3*) had also not significantly influenced production of *C. parapsilosis*-induced IL-27 (510.1 ± 49.1 pg/mL) versus *C. parapsilosis* stimulations alone (534.8 ± 58.1 pg/mL). (Figure 5.5-D) Addition of human HB-EGF (*HBEGF*, human ortholog of murine *Hbegf*) in BMDMs stimulated with *C. parapsilosis* did not result in significantly lower levels of IL-27 detected (657.5 ± 166.9 pg/mL) compared with BMDMs stimulated with *C. parapsilosis* in its absence (681.1 ± 162.1 pg/mL). (Figure 5.5-E) Stimulation with human PIGF-2 (*PGF*, human ortholog of murine *Pgf*) and *C. parapsilosis* did not trigger the production of significantly different levels of IL-27 (509.8 ± 27.9 pg/mL) compared with *C. parapsilosis* stimulations alone (460.3 ± 21.2 pg/mL). (Figure 5.5-F) Stimulation with *C. parapsilosis* and murine CCL6 (*Ccl6*) similarly did not block IL-27 production (444.6 ± 74.2 pg/mL) compared with stimulation with just *C. parapsilosis* (509.1 ± 99.3 pg/mL). (Figure 5.5-G) Lastly, murine TNFSF18 (*Tnfsf18*) did also not inhibit *C. parapsilosis*-induced IL-27 production (*C. parapsilosis* + mTNFSF18: 553.2 ± 33.8 pg/mL; *C. parapsilosis*: 484.6 ± 29.3 pg/mL).

Results revealed that none of the immune mediators specifically induced by *C. albicans* and not *C. parapsilosis* (Figure 3.2) are able to trigger inhibition of IL-27, suggesting that none of these are the secondary BMDM-derived immune mediator responsible for its blockade.

5.3.3 Preliminary investigations suggest that the *C. albicans*-derived secreted molecule is not delivered in extracellular vesicles

C. albicans is able to secrete extracellular vesicles (EVs) that can contain virulence-associated components. These structures can be used by the fungus as an alternative transportation mechanism to deliver macromolecules across the cell wall to target cells⁶³³.

I decided to assess if the *C. albicans*-derived secreted molecule (primary signal of IL-27 inhibition) was delivered inside EVs. To this end, *C. albicans* was resuspended in cell culture medium and incubated for 24 h. Following incubation, supernatants were sterile filtered, collected and ultracentrifuged at 100,000 g for 2 h at 4°C. This resulted in the pelleting of all EVs present in the medium after ultracentrifugation, whilst other smaller, conventionally secreted proteins would still be suspended in the supernatants⁶³⁴. After ultracentrifugation,

the supernatants were harvested to new tubes and the pellets were resuspended in fresh cell culture medium to the same initial volume prior to ultracentrifugation. This ensured that any deposited EVs would be at the same concentration as they initially were before this process and allowed for direct comparison between both fractions obtained after ultracentrifugation. Supernatants and resuspended pellets were then added separately to BMDMs with *C. parapsilosis* and IL-27 production was detected after 24 h of co-stimulation (Figure 5.6)

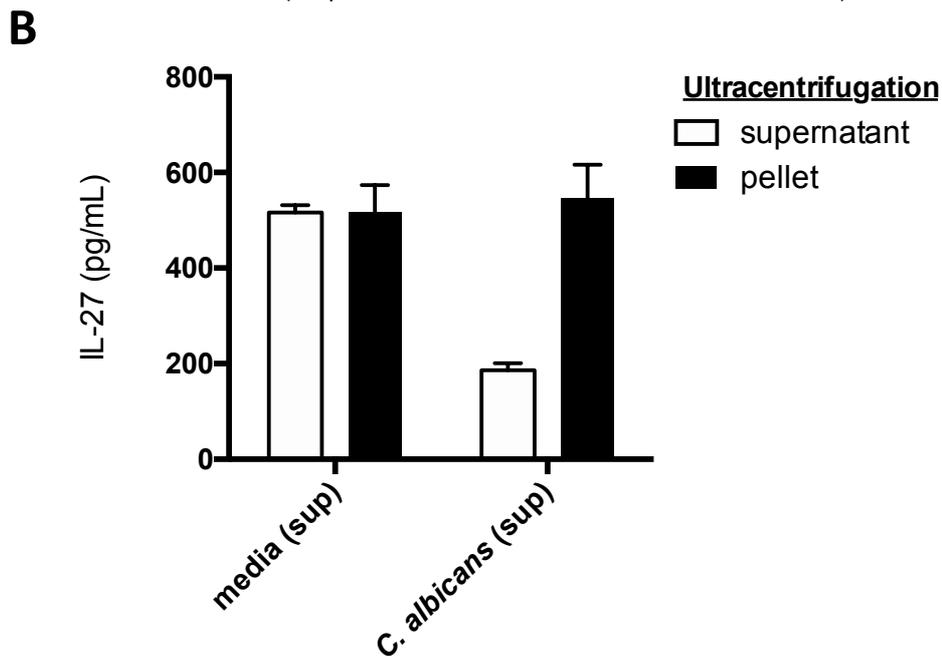
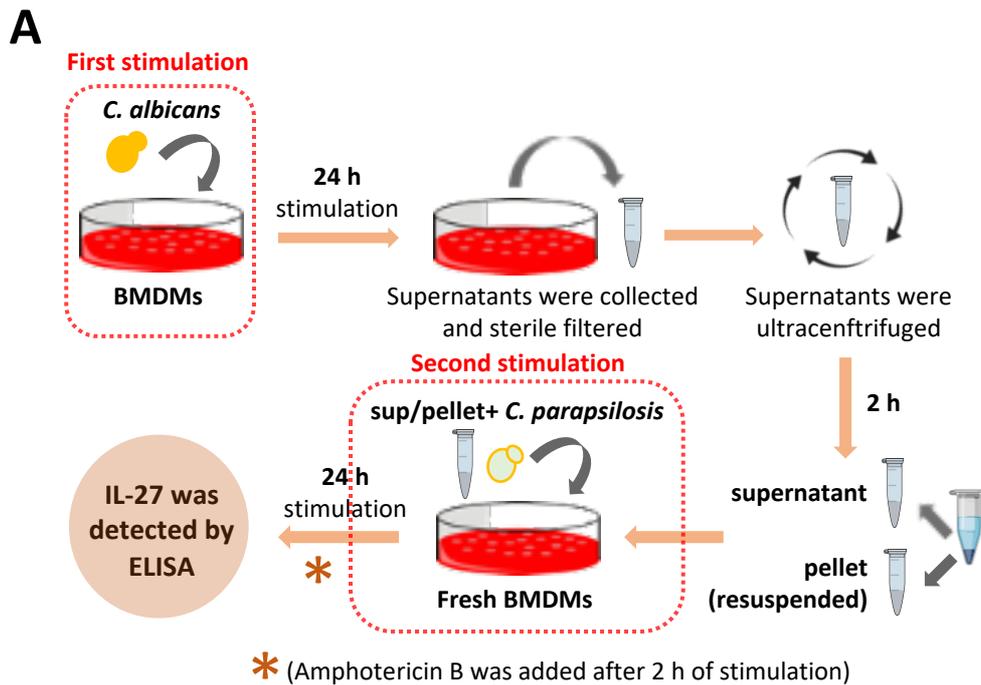


Figure 5.6 – *C. albicans* extracellular vesicles do not appear to block *C. parapsilosis*-induced IL-27. (A) Schematic of experimental design: *C. albicans* was resuspended in cell culture medium and incubated for 24 h. Supernatants were collected, sterile filtered and ultracentrifuged for 2 h. Supernatants obtained after centrifugation were collected and the pellets containing extracellular vesicles from *C. albicans* were resuspended in cell culture medium to the initial volume before ultracentrifugation. Supernatants (white bars) and resuspended pellets (black bars) were added separately to BMDMs alongside *C. parapsilosis* and incubated for 24 h. Amphotericin B was added after 2 h of co-stimulation with *C. parapsilosis*. IL-27 levels produced by BMDMs were detected by ELISA. (B) IL-27 levels detected after the second stimulation described in A. Data is presented as means \pm SEM of technical triplicate values of one independent experiment. n=1.

Figure 5.6-B demonstrate the results obtained in the experiment using ultracentrifuged supernatants and pellets. Stimulation with *C. parapsilosis* and supernatants from *C. albicans* resulted in a reduction of ~64% of IL-27 levels produced by BMDMs (186.4 ± 14.8 pg/mL) compared with the control condition where BMDMs were stimulated with *C. parapsilosis* and just cell culture medium (516.1 ± 15.7 pg/mL). This reduction, however, was not verified in stimulations using resuspended pellets. BMDMs stimulated with *C. parapsilosis* and resuspended pellets obtained from ultracentrifuged media (517.4 ± 56.1 pg/mL) produced similar IL-27 levels as BMDMs stimulated with *C. parapsilosis* and resuspended pellets from *C. albicans* (546.3 ± 69.9 pg/mL). Since IL-27 blockade was only verified with supernatant fractions not with resuspended pellets from *C. albicans*, it is likely that the *C. albicans* secreted molecule is not delivered inside EVs. However, Figure 5.6-B only presents results from one independent experiment, and more experimentation is required to confirm this.

5.3.4 IL-27 blockade is dependent on *C. albicans* germination and formation of true hyphae.

To facilitate the discovery of the secreted molecule responsible for IL-27 blockade, I decided to test if other non-*albicans* *Candida* spp. were also able to inhibit the *C. parapsilosis*-induced IL-27 levels. (Figure 5.7)

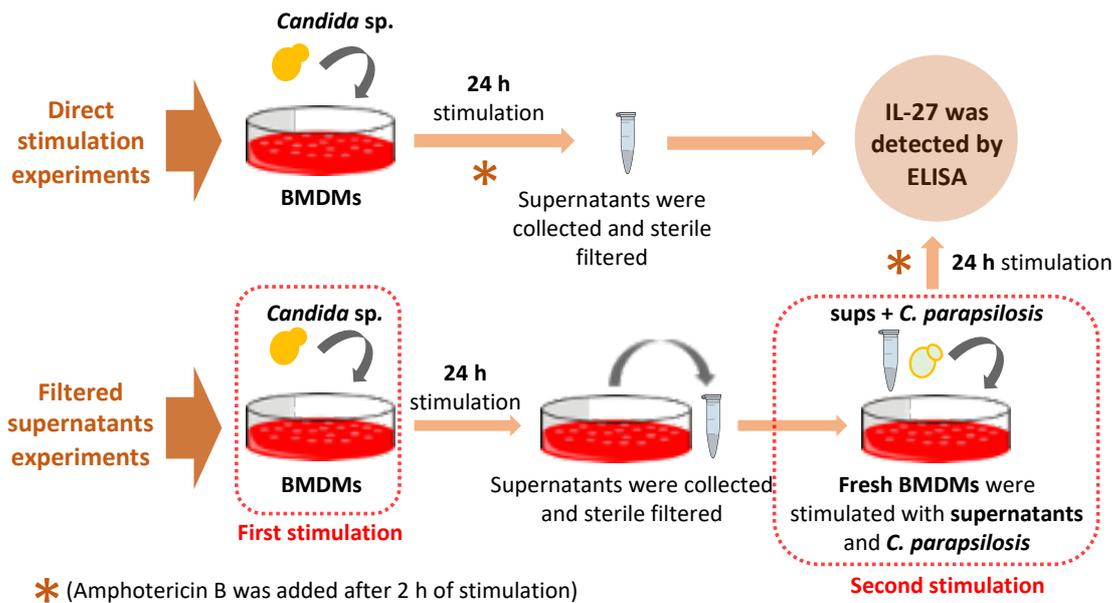


Figure 5.7 – Schematic of experimental design for IL-27 blockade experiments using different *Candida* spp. Two different experiments were conducted: Direct stimulation experiments: BMDMs were directly stimulated with *Candida* spp. and IL-27 levels were measured in the cell culture media after 24 h of co-stimulation by ELISA; Filtered supernatants experiments: BMDMs were co-cultured with *Candida* spp. for 24 h, supernatants were collected, sterile filtered and added to fresh BMDMs alongside *C. parapsilosis* for another 24 h before measuring IL-27 in the media by ELISA. For the direct stimulation experiments amphotericin B was added after 2 h of co-stimulation with *Candida* spp., while for the filtered supernatant experiments amphotericin B was only added during the last 24 h of stimulation when BMDMs were stimulated with *C. parapsilosis* and the filtered supernatants.

Each *Candida* spp. was added to BMDMs and IL-27 levels were detected after 24 h of co-stimulation (“Direct stimulation experiments”). This was performed to confirm if these different *Candida* spp., were able to directly induce IL-27 levels. In parallel, BMDMs were also stimulated with these *Candida* spp. for 24 h to allow for accumulation of any mediators capable of IL-27 inhibition. These supernatants were then sterile filtered and added with *C. parapsilosis* to freshly plated macrophages. IL-27 levels were then measured by ELISA after another 24 h of co-incubation to assess if these *Candida* spp. were also able to block cytokine production via a soluble mediator.

The first non-*albicans* species tested was *C. glabrata*. Although *C. glabrata* is considered a low virulence haploid species that only grows in yeast form, *C. glabrata* is a growing concern as it is frequently isolated from patients suffering from candidaemia, though with lower incidence compared with *C. albicans*^{3,13,321} (Figure 5.8).

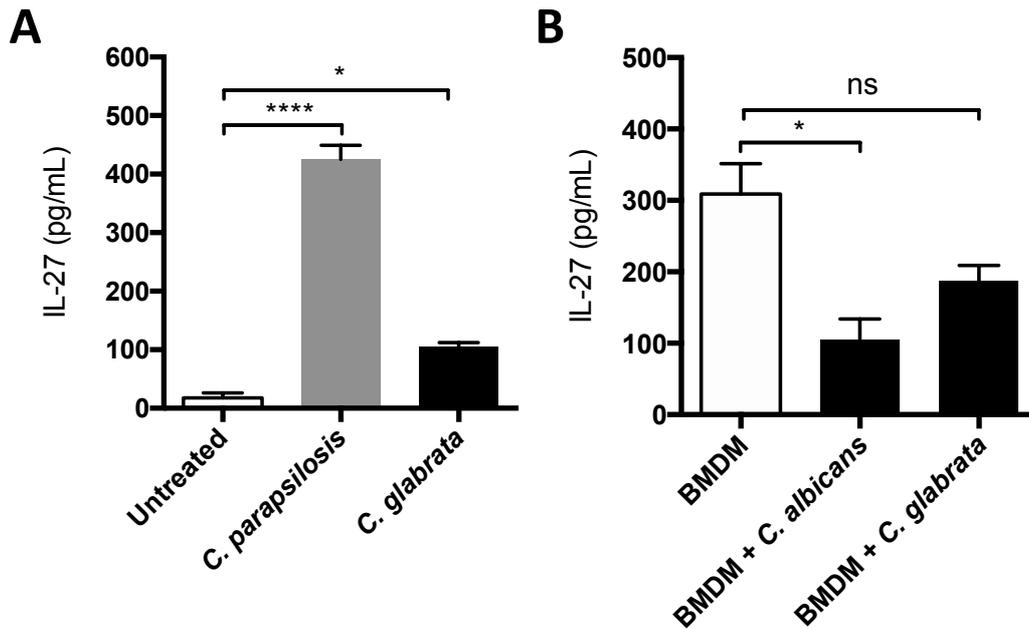


Figure 5.8 – Supernatants from BMDMs stimulated with *C. glabrata* do not inhibit *C. parapsilosis*-induced IL-27 production to the same extent as *C. albicans*. (A) BMDMs were stimulated with *C. parapsilosis* or *C. glabrata* for 24 h. Amphotericin B was added after 2 h of co-stimulation. (B) BMDMs were stimulated with *C. albicans* or *C. glabrata* for 24 h, supernatants were collected, sterile filtered and added to fresh BMDMs alongside *C. parapsilosis* for another 24 h. Amphotericin B was added after 2 h of macrophage co-stimulation with the filtered supernatants and *C. parapsilosis*. IL-27 in the cell culture medium was detected by ELISA. Data is presented as means ± SEM. A and B: n=3. Ordinary One-way ANOVA with Tukey post-hoc test. ns – not statistically significant, * $p < 0.05$, **** $p < 0.0001$.

Direct stimulation of BMDMs with *C. glabrata* led to production of significantly higher levels of IL-27 (104.0 ± 8.4 pg/mL) compared with the untreated control (17.9 ± 8.1 pg/mL). This production, however, was low compared to the IL-27 levels induced by *C. parapsilosis* stimulation (425.4 ± 24.0 pg/mL) (Figure 5.8-A). Supernatants from *C. glabrata*-infected BMDMs led to reduced (but not statistically significant) levels of *C. parapsilosis*-induced IL-27 (187.3 ± 21.7 pg/mL) compared with the levels detected in BMDMs stimulated with *C. parapsilosis* and uninfected BMDMs (308.9 ± 42.4 pg/mL) (Figure 5.8-B). This inhibition, however, was not as robust as the one observed in BMDMs stimulated with *C. parapsilosis* and supernatants from *C. albicans* co-stimulated with BMDMs (104.4 ± 29.7 pg/mL).

The next non-*albicans* species tested was *C. tropicalis*, another less prevalent agent of systemic candidiasis. Unlike *C. glabrata*, *C. tropicalis* is a diploid fungus that can grow as either yeast or pseudohyphae, although some reports have shown that some strains can also form

true hyphae when the right conditions are met^{33,34}. For these experiments, two *C. tropicalis* clinical isolates strains were tested: AM2007/0112 and SCS74663 (Figure 5.9).

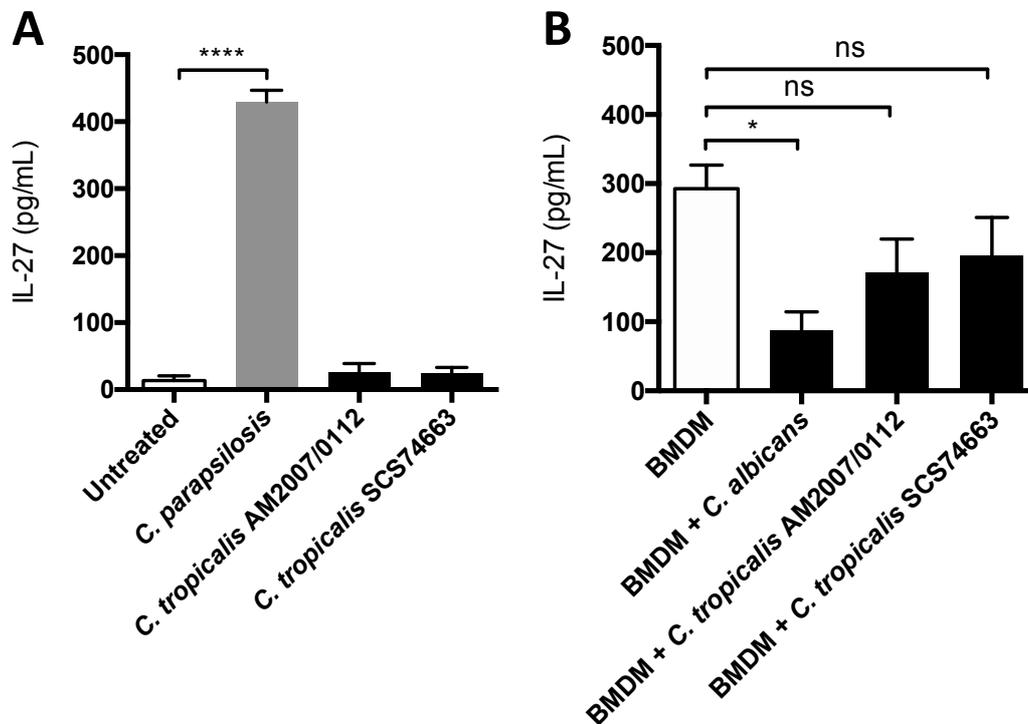


Figure 5.9 – Supernatants from BMDMs stimulated with *C. tropicalis* do not inhibit *C. parapsilosis*-induced IL-27 production to the same extent as *C. albicans*. (A) BMDMs were stimulated with *C. parapsilosis* or two different strains of *C. tropicalis* for 24 h. Amphotericin B was added after 2 h of co-stimulation. (B) BMDMs were stimulated with *C. albicans* or two different strains of *C. tropicalis* for 24 h, supernatants were collected, sterile filtered and added to fresh BMDMs alongside *C. parapsilosis* for another 24 h. Amphotericin B was added after 2 h of macrophage co-stimulation with the filtered supernatants and *C. parapsilosis*. IL-27 in the cell culture medium was detected by ELISA. Data is presented as means ± SEM. A and B: n=4. Ordinary One-way ANOVA with Tukey post-hoc test. ns – not statistically significant, *p<0.05, ****p<0.0001.

Direct stimulation of BMDMs with both *C. tropicalis* AM2007/0112 (26.4 ± 12.6 pg/mL) and SCS74663 (25.3 ± 7.9 pg/mL) strains did not result in significant induction of IL-27 compared with the negative control (Untreated: 13.4 ± 7.3 pg/mL) (Figure 5.9-A). Supernatants from BMDMs infected with both AM2007/0112 (171.6 ± 48.1 pg/mL) and SCS74663 (195.0 ± 56.0 pg/mL) strains led to reduced levels of *C. parapsilosis*-triggered IL-27 compared to the control condition where BMDMs were stimulated with *C. parapsilosis* and supernatants from uninfected BMDMs (292.5 ± 34.1 pg/mL) (Figure 5.9-B). However, these reductions caused by supernatants from both *C. tropicalis* strains were not statistically significant and IL-27 levels were not as low as the ones observed with *C. albicans* supernatants (87.3 ± 27.1 pg/mL).

The last non-*albicans* *Candida* species tested was *C. dubliniensis*. From a phylogenetic point, *C. dubliniensis* is closely related to *C. albicans*, and both species share many phenotypical traits like the ability to produce true hyphae^{635,636}. Although *C. dubliniensis* is considered less virulent than *C. albicans* and while it is mostly isolated from oropharyngeal candidiasis (OPC), it was also identified as a causative agent of systemic candidiasis⁶³⁷ (Figure 5.10).

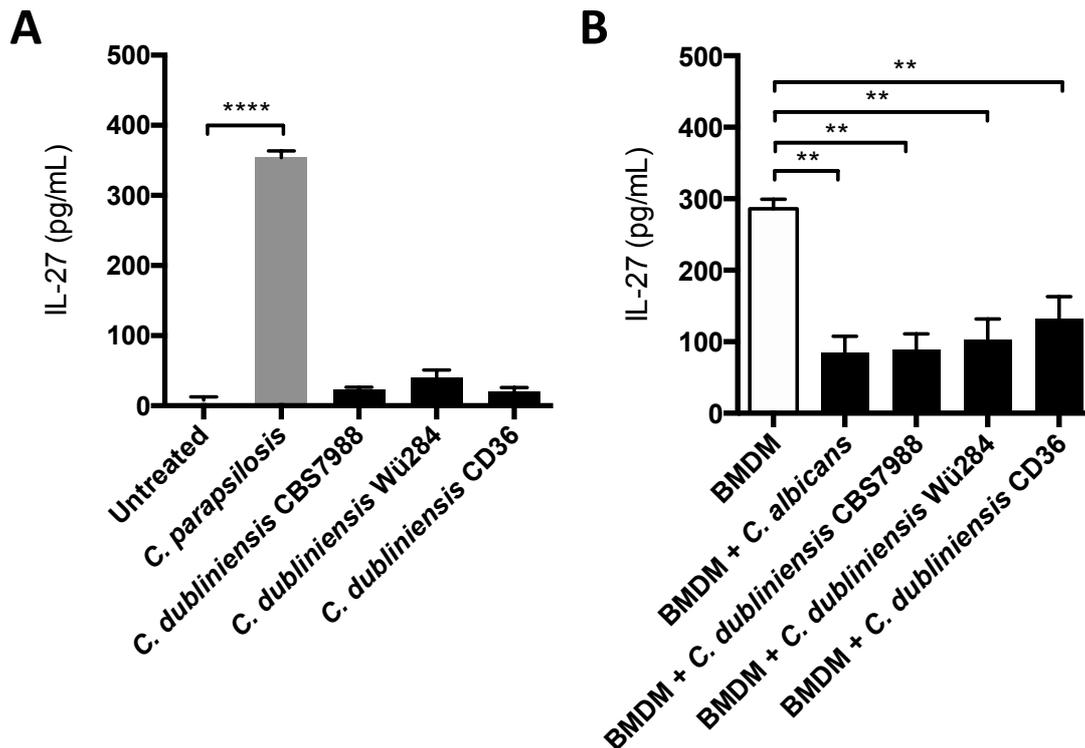


Figure 5.10 – Supernatants from BMDMs stimulated with *C. dubliniensis* inhibit *C. parapsilosis*-induced IL-27 production to the same extent as *C. albicans*. (A) BMDMs were stimulated with *C. parapsilosis* or three different strains of *C. dubliniensis* for 24 h. Amphotericin B was added after 2 h of co-stimulation. (B) BMDMs were stimulated with *C. albicans* or three different strains of *C. dubliniensis* for 24 h, supernatants were collected, sterile filtered and added to fresh BMDMs alongside *C. parapsilosis* for another 24 h. Amphotericin B was added after 2 h of macrophage co-stimulation with the filtered supernatants and *C. parapsilosis*. IL-27 in the cell culture medium was detected by ELISA. Data is presented as means ± SEM. A and B: n=3. Ordinary One-way ANOVA with Tukey post-hoc test. **p<0.01, ****p<0.0001.

Direct stimulation of BMDMs with the three different *C. dubliniensis* strains tested: CBS7988 (23.0 ± 3.4 pg/mL), Wü284 (40.6 ± 10.4 pg/mL) and CD36 (20.5 ± 5.7 pg/mL), did not result in significantly higher levels of IL-27 produced compared with the untreated control (7.9 ± 4.6 pg/mL) (Figure 5.10-A). Supernatants from BMDMs infected with *C. dubliniensis* CBS7988 (88.3 ± 22.6 pg/mL), Wü284 (102.5 ± 29.5 pg/mL) and CD36 (132.7 ± 30.6 pg/mL)

strains all induced a statistically significant reduction of *C. parapsilosis*-induced IL-27 levels produced versus stimulations with *C. parapsilosis* and media from uninfected BMDMs (286.1 ± 13.3 pg/mL) (Figure 5.10-B). Moreover, statistical analysis revealed that IL-27 blockade caused by supernatants from each *C. dubliniensis* strains is not significantly different than the one induced by *C. albicans* supernatants (84.0 ± 23.5 pg/mL).

From all the non-*albicans* species tested, only supernatants from BMDMs stimulated with the three different *C. dubliniensis* strains were found to significantly inhibit IL-27 production in *C. parapsilosis*-infected BMDMs to a similar extent as *C. albicans*. An important virulence characteristic shared by both *C. albicans* and *C. dubliniensis* is that they are the only *Candida* spp. known to normally be able to grow in true hyphae form⁶³⁶. As mentioned before, some strains of *C. tropicalis* were reported to also be able to form true hyphae, however this only happens in a few strains when grown under specific conditions^{33,34}. I then assessed if blockade of IL-27 was dependent on yeast germination and formation of true hyphae. For this purpose, I have performed experiments (in the similar design as detailed in Figure 5.7) using yeast-locked *C. albicans* mutants that are unable to germinate, and heat-killed (HK) wild-type *C. albicans* SC5413 at both yeast and hyphal phenotypes. Two different *C. albicans* mutants were tested: *flo8*-deficient and *egf1/cph1*-double knock-out *C. albicans*. These genes all code for transcription factors that regulate *C. albicans* dimorphic switch and germination^{18,19} (Figure 5.11).

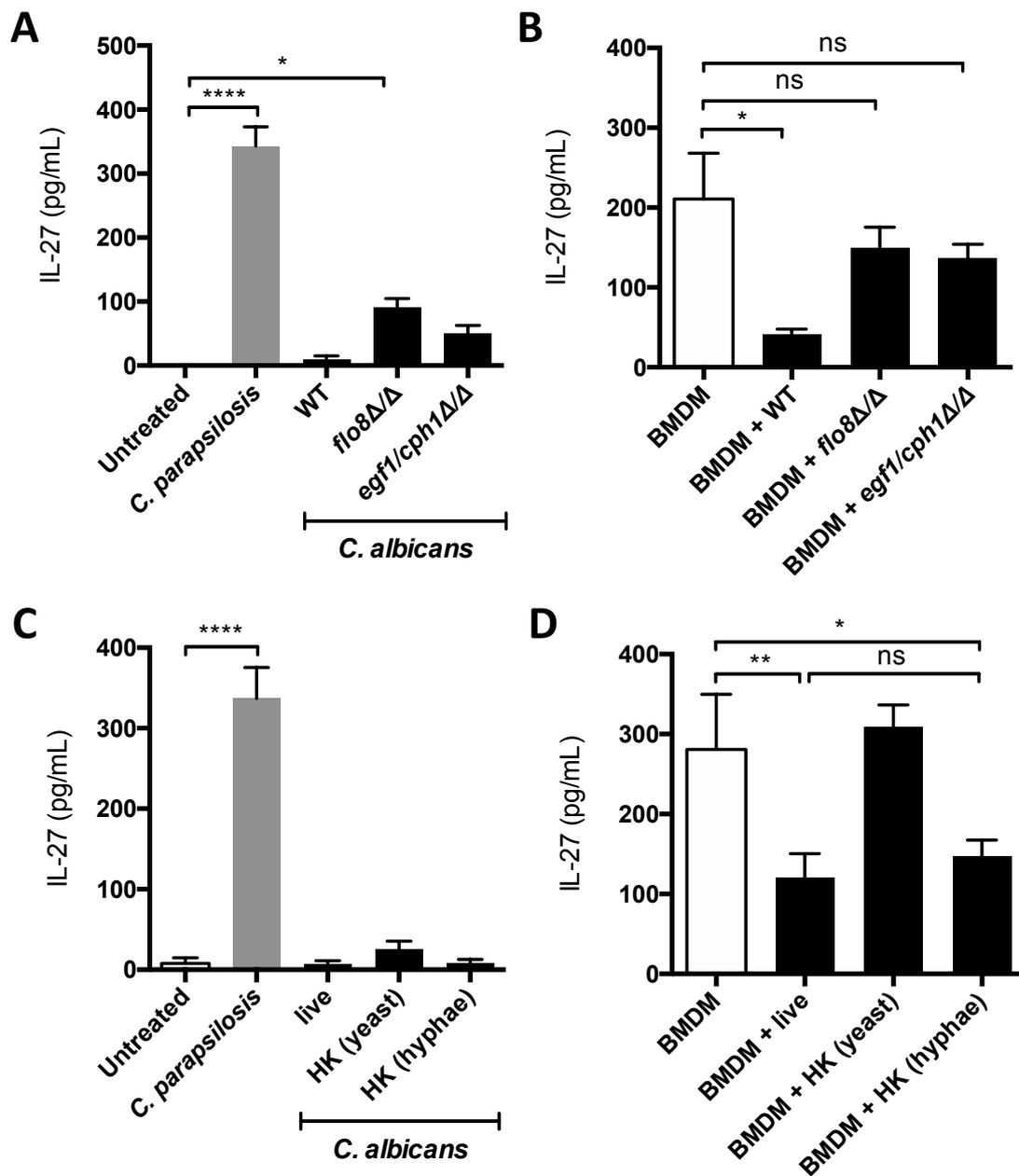


Figure 5.11 – *C. albicans*-mediated IL-27 inhibition is dependent on yeast germination and switch to hyphal phenotype. (A and C) BMDMs were stimulated with wild-type *C. albicans* (“WT”, BWP17+Clp30 isogenic strain) or two different yeast-locked *C. albicans* mutant strains (“*flo8Δ/Δ*” and “*egf1/cph1Δ/Δ*”) (A) and with live or heat-killed *C. albicans* SC5314 at yeast (“HK (yeast)”) or hyphal (“HK (hyphae)”) phenotype (C) for 24 h. Amphotericin B was added after 2 h of co-stimulation. (B and D) BMDMs were stimulated with wild-type *C. albicans* or two different yeast-locked *C. albicans* mutant strains (B) and with live or heat-killed *C. albicans* SC5314 at yeast or hyphal phenotype (D) for 24 h, supernatants were sterile filtered and added to fresh BMDMs alongside *C. parapsilosis* for another 24 h. Amphotericin B was added after 2 h of macrophage co-stimulation with the filtered supernatants and *C. parapsilosis*. IL-27 in the cell culture medium was detected by ELISA. Data is presented as means \pm SEM. A, B and C: n=3, D: n=4. Ordinary One-way ANOVA with Tukey post-hoc test. ns – not statistically significant, * p <0.05, ** p <0.01, **** p <0.0001.

Figure 5.11-A and -B display data from stimulations using yeast-locked *C. albicans* and Figure 5.11-C and -D comprise data obtained in experiments using HK *C. albicans*. (Figure 5.11-A) Direct stimulation of BMDMs with *egf1/cph1*-double knock-out *C. albicans* ("*egf1/cph1* Δ/Δ ") did not lead to production of significant levels of IL-27 (49.6 ± 13.0 pg/mL) compared with the untreated control (0 pg/mL). *Flo8*-deficient *C. albicans* (*flo8* Δ/Δ), however, was able to significantly trigger production of IL-27 in macrophages (90.6 ± 14.3 pg/mL) although these levels were not as high as those induced by *C. parapsilosis* (343.1 ± 30.2 pg/mL). (Figure 5.11-B) Unlike supernatants from BMDMs infected with wild-type *C. albicans* (41.9 ± 5.9 pg/mL), stimulation with supernatants from BMDMs infected with both *flo8*-deficient (149.5 ± 26.0 pg/mL) and *egf1/cph1*-double knock-out *C. albicans* (136.5 ± 17.8 pg/mL) did not result in a significant reduction of *C. parapsilosis*-induced IL-27 levels in BMDMs compared with the condition where BMDMs were stimulated with both *C. parapsilosis* and supernatants from uninfected macrophages (210.9 ± 57.4 pg/mL). (Figure 5.11-C) BMDMs stimulated with live (7.3 ± 3.8 pg/mL) or HK *C. albicans* in both yeast (24.9 ± 10.8 pg/mL) and hyphal form (7.8 ± 4.9 pg/mL) do not induce significant production of IL-27 compared with untreated BMDMs (8.0 ± 6.8 pg/mL). (Figure 5.11-D) Supernatants from BMDMs stimulated with live (121.3 ± 16.8 pg/mL) or HK *C. albicans* in hyphal form (147.5 ± 11.6 pg/mL), but not HK *C. albicans* in yeast form (309.3 ± 15.7 pg/mL), led to significant reduction of levels of *C. parapsilosis*-induced IL-27 production compared with macrophages treated with supernatants from uninfected BMDMs (281.0 ± 39.8 pg/mL).

Collective results obtained from experiments presented in this section 5.3.4. indicate that production of the secreted molecule responsible for *C. albicans*-mediated blockade of IL-27 is dependent on the ability of the fungus to switch from yeast to true hypha phenotype. My results also suggest that the ability to inhibit IL-27 production is not limited to *C. albicans*, but also to other *Candida* spp. capable of this phenotype (i.e. *C. dubliniensis*).

5.3.5 Candidalysin is not the *C. albicans*-derived secreted molecule responsible for IL-27 inhibition

Candidalysin is a novel fungal toxin produced by *C. albicans* hyphal cells that was shown to be important for inducing cell damage and triggering inflammation in host cells^{84–86}. *C. dubliniensis* hyphal cells were also shown to trigger a MAPK/c-Fos induced response in oral epithelial cells, similar to what was verified with *C. albicans* hypha, characteristic of a candidalysin signature^{85,638}. *C. dubliniensis* also expresses *ece1*, the polypeptide precursor of candidalysin, after germination and formation of true hyphae, and so is predicted to also produce candidalysin. Since both *Candida* species were able to significantly block IL-27 production by BMDMs, I decided to assess whether candidalysin is the secreted molecule responsible for IL-27 inhibition. To do so, experiments with the same design as those described in Figure 5.7 were conducted using different mutant *C. albicans* strains: an *ece1*-deficient mutant (“*ece1* Δ/Δ ”) where the full ECE1 gene was knocked-out of *C. albicans* genome, a candidalysin-deficient mutant (“*ece1* Δ/Δ +*ECE1* $\Delta_{184-279}$ ”) where the full *ece1* gene was knocked-out, and reintegrated back into *C. albicans* genome after removal of the gene sequence that codes for peptide 3 (candidalysin, Ece1-III₆₃₋₉₃), and a revertant mutant strain (“*ece1* Δ/Δ +*ECE1*”) where *ece1* was knocked-out and the full extent of the gene was reintegrated back in *C. albicans* genome (Figure 5.12).

Direct stimulation of BMDMs with candidalysin-deficient (“*ece1* Δ/Δ +*ECE1* $\Delta_{184-279}$ ”) mutant (9.3 ± 5.5 pg/mL), *ece1*-deficient (“*ece1* Δ/Δ ”) mutant (12.8 ± 2.9 pg/mL) and revertant (“*ece1* Δ/Δ +*ECE1*”) mutant strains (12.3 ± 6.9 pg/mL) did not induce production of significant levels of IL-27 compared with the untreated control (3.5 ± 3.5 pg/mL) (Figure 5.12-A). Supernatants from BMDMs stimulated candidalysin-deficient (88.6 ± 17.6 pg/mL), *ece1*-deficient (101.1 ± 27.5 pg/mL), and revertant (68.7 ± 24.5 pg/mL) mutant *C. albicans* all induce a significant inhibition of *C. parapsilosis*-induced IL-27 similar to the one observed with supernatants from WT *C. albicans* (70.5 ± 20.5 pg/mL) compared with the positive control where BMDMs were stimulated with *C. parapsilosis* and supernatants from uninfected BMDMs (390.3 ± 52.8 pg/mL) (Figure 5.12-B).

Results displayed on Figure 5.12 suggest that candidalysin is not the molecule secreted by *C. albicans* that is responsible for inhibition of *C. parapsilosis*-induced IL-27.

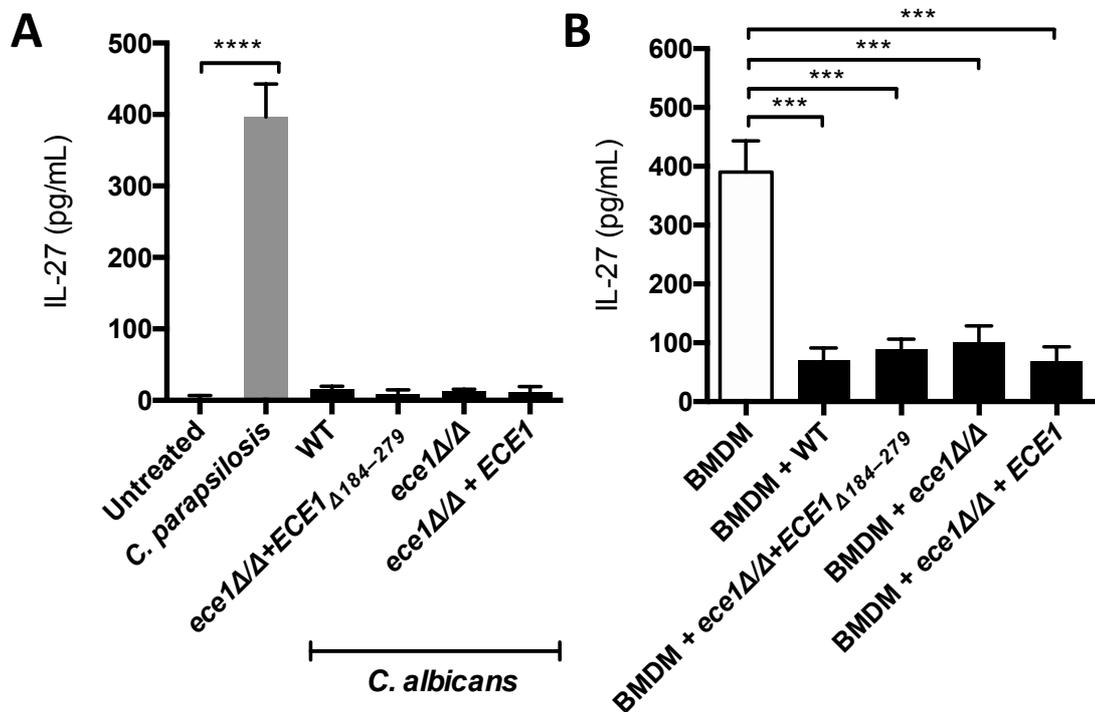


Figure 5.12 – Supernatants from BMDMs stimulated with candidalysin-deficient mutant *C. albicans* inhibit *C. parapsilosis*-induced IL-27 production. (A) BMDMs were stimulated with *C. parapsilosis*, wild-type *C. albicans* (“WT”, BWP17+Clp30 strain), a candidalysin-deficient *C. albicans* strain (“*ece1Δ/Δ+ECE1_{Δ184-279}*”), an *ece1*-deficient *C. albicans* strain (“*ece1Δ/Δ*”) or a revertant strain (“*ece1Δ/Δ+ECE1*”) for 24 h. Amphotericin B was added after 2 h of co-stimulation. (B) BMDMs were stimulated with wild-type *C. albicans*, candidalysin-deficient *C. albicans* strain, *ece1*-deficient *C. albicans* strain or a revertant strain for 24 h, supernatants were collected, sterile filtered and added to fresh BMDMs alongside *C. parapsilosis* for another 24 h. Amphotericin B was added after 2 h of macrophage co-stimulation with the filtered supernatants and *C. parapsilosis*. IL-27 in the cell culture medium was detected by ELISA. Data is presented as means ± SEM. A: n=3, B: n=4. Ordinary One-way ANOVA with Tukey post-hoc test. *** $p < 0.001$, **** $p < 0.0001$.

5.3.6 Dectin-1 signalling activation via particulate β -glucan polymers blocks *C. parapsilosis*-induced IL-27 production

Results from section 5.3.5. revealed that candidalysin is not the secreted molecule responsible for mediation of *C. albicans* mediated IL-27 blockade in *C. parapsilosis*-treated BMDMs. Previous results suggest that this fungal-derived mediator is secreted by *C. albicans* as a consequence of the morphological switch from yeast to true hyphae (Figure 5.12-B). Data also indicates that viability of *C. albicans* is not strictly necessary for inhibition of IL-27 to occur since stimulation of macrophages with HK *C. albicans* in hyphal form is able to induce said inhibition (Figure 5.11-D). The capacity of *C. albicans* to switch between these different morphologies is linked to its virulence: yeast phenotypes are associated with commensalism and hyphae are associated with tissue invasion, cell damage and parasitism. Different *C. albicans* morphotypes play an important role in determining the cytokine profile and immune response triggered in phagocytes⁶³⁹. This is due not only to a differential production of proteins and enzymes by *C. albicans* in different phenotypes that will affect cytokine production and consequent immune response, but also possibly due to conformational changes of the cell wall of the fungus and different exposure of cell wall carbohydrates acting as PAMPs depending on *C. albicans* morphogenetic form¹⁰⁹.

The three main structural carbohydrates found in the fungal cell wall are chitin, mannans and β -glucans^{41,47}. Some of these carbohydrates like mannans and some forms of β -glucan are water soluble and known to be released into synthetic media⁶⁴⁰. Since these carbohydrates are immunogenic, I assessed if these could be the secreted molecule(s) responsible for blocking *C. parapsilosis*-induced IL-27. These carbohydrates exist mostly as branched polymers, however macrophages are able to discriminate between those and their monomeric forms by differential PRR sensing. I then stimulated macrophages with different carbohydrates alongside *C. parapsilosis* and assessed if these could block IL-27 synthesis. Released IL-27 levels were detected in the cell culture medium after 24 h of co-stimulation by ELISA (Figure 5.13).

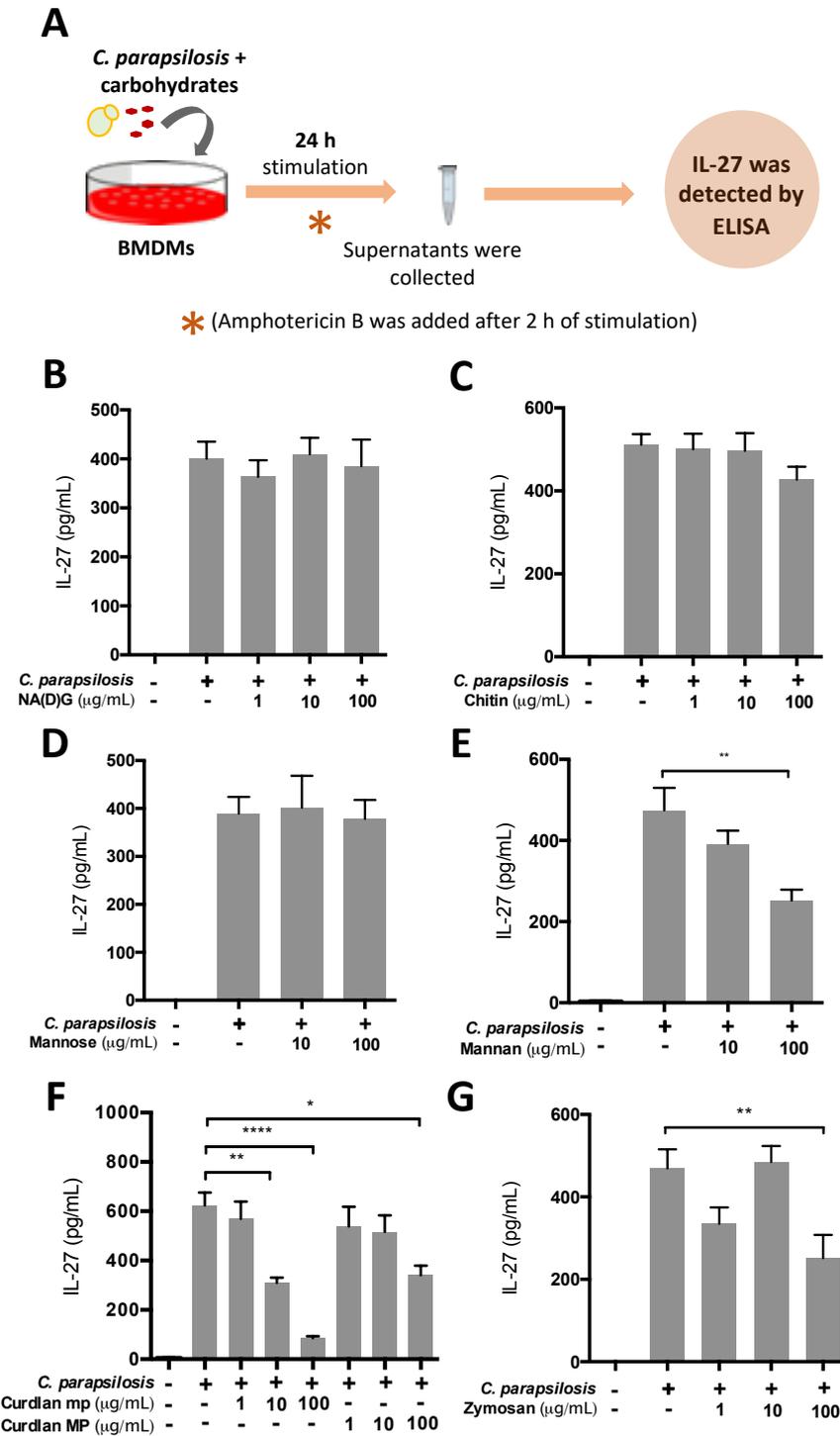


Figure 5.13 – Mannan and particulate β -glucan polymers inhibit *C. parapsilosis*-induced IL-27 production. (A) Schematic of experimental design: BMDMs were stimulated with *C. parapsilosis* and mannose (B), mannan from *S. cerevisiae* (C), N-acetyl-D-glucosamine (D), chitin from shrimp shell (E), curdlan micro- ("Curdlan mp") or macroparticles ("Curdlan MP") (F) or zymosan (G) for 24 h. Amphotericin B was added after 2 h of co-stimulation. IL-27 in the cell culture medium was detected by ELISA. Data is presented as means \pm SEM. B and D: n=3, C and F: n=4, E: n=5, G: n=7. Ordinary One-way ANOVA with Tukey post-hoc test. * p <0.05, ** p <0.01, **** p <0.0001.

Figure 5.13-B and -C shows the results obtained from stimulations using chitin monomers (N-acetyl-D-glucosamine) and chitin polymers isolated from shrimp shells. (Figure 5.13-B) stimulations with N-acetyl-D-glucosamine did not induce significant reduction of *C. parapsilosis*-induced IL-27. The lowest levels were detected with 1 µg/mL N-acetyl-D-glucosamine (363.3 ± 34.0 pg/mL) however they are not significantly different to the levels induced by stimulations with *C. parapsilosis* in the absence of the chitin monomers (400.6 ± 34.5 pg/mL). (Figure 5.13-C) Chitin polymers isolated from shrimp shells also did not significantly inhibit IL-27 levels compared with the conditions where BMDMs were incubated with *C. parapsilosis* in the absence of chitin (511.1 ± 25.5 pg/mL). The lowest levels were detected with the highest concentration tested (100 µg/mL) (427.2 ± 31.2 pg/mL). Figures 5.14-D and -E show the results obtained from stimulations using mannan monomers (mannose) and mannan isolated from *S. cerevisiae*. (Figure 5.13-D) Stimulation with mannose did not result in a significant reduction of IL-27 levels in BMDMs compared with *C. parapsilosis* stimulations alone (389.6 ± 34.5 pg/mL), with the lowest levels detected with 100 µg/mL mannose (378.3 ± 39.3 pg/mL). (Figure 5.13-E) Unlike mannose, stimulation with mannan polymers have resulted in a dose-dependent IL-27 inhibition with 100 µg/mL mannan inducing significantly lower levels of the cytokine (252.1 ± 26.5 pg/mL) compared with stimulations with *C. parapsilosis* in the absence of mannan (474.6 ± 54.9 pg/mL). Figures 5.14-F and -G display results obtained in experiments using curdlan – a neutral, water insoluble, particulate β-(1,3)-glucan polymer isolated from the bacterium *Alcaligenes faecalis*⁶⁴¹; and zymosan – a product of *S. cerevisiae* fungal cell wall composed mostly of β-(1,3)- and β-(1,6)-glucans. (Figure 5.13-F) In these experiments, different sized curdlan particles (micro- and macro-particles) were tested. Stimulation with both micro- and macro-particles of curdlan led to a dose-dependent inhibition of IL-27. Significant differences in IL-27 levels were detected with 10 µg/mL (309.8 ± 20.9 pg/mL) and 100 µg/mL (85.2 ± 8.0 pg/mL) curdlan micro-particles, and with 100 µg/mL curdlan macro-particles (341.1 ± 38.2 pg/mL) compared BMDM stimulation with *C. parapsilosis* in the absence of curdlan (623.4 ± 52.5 pg/mL). (Figure 5.13-G) Stimulations with 100 µg/mL zymosan (251.4 ± 56.2 pg/mL) also resulted in significant reduction of IL-27 produced by BMDMs compared with *C. parapsilosis* alone (469.2 ± 46.5 pg/mL). Unlike curdlan, zymosan induced a “bell-shaped” curve inhibition of IL-27 since stimulation with the lowest dose tested (1 µg/mL) also resulted in lower levels of IL-27 (334.8

± 39.6 pg/mL), although not significant, whereas the intermediated concentration tested does not appear to induce a blockade ($10 \mu\text{g/mL}$: 484.7 ± 39.0 pg/mL).

Results shown on Figure 5.13 suggest that both mannan and β -glucan particles are able to inhibit production of IL-27 when added in combination with *C. parapsilosis*. However, these particles are PAMPs that signal through the same PRRs that could be involved in *C. parapsilosis* recognition by BMDMs. Binding of these particles to their respective PRRs could block BMDM recognition of *C. parapsilosis* and therefore result in reduced phagocytosis of the yeast. This is particularly important in this experimental setting since the induction of IL-27 by BMDMs was shown to be dependent on *C. parapsilosis* phagocytosis and formation of phagosome¹⁸⁴. TNF and IFN- β were shown to act synergistically to induce production of IL-27 in BMDMs¹⁸⁴. To assess if mannan and curdlan-mediated reduction of IL-27 was independent of competition for the same PRRs as *C. parapsilosis* PAMPs, BMDMs were stimulated in the absence of *C. parapsilosis* with a TNF and IFN- β mix in combination with the carbohydrates and IL-27 levels were detected after 24 h of co-stimulation (Figure 5.14).

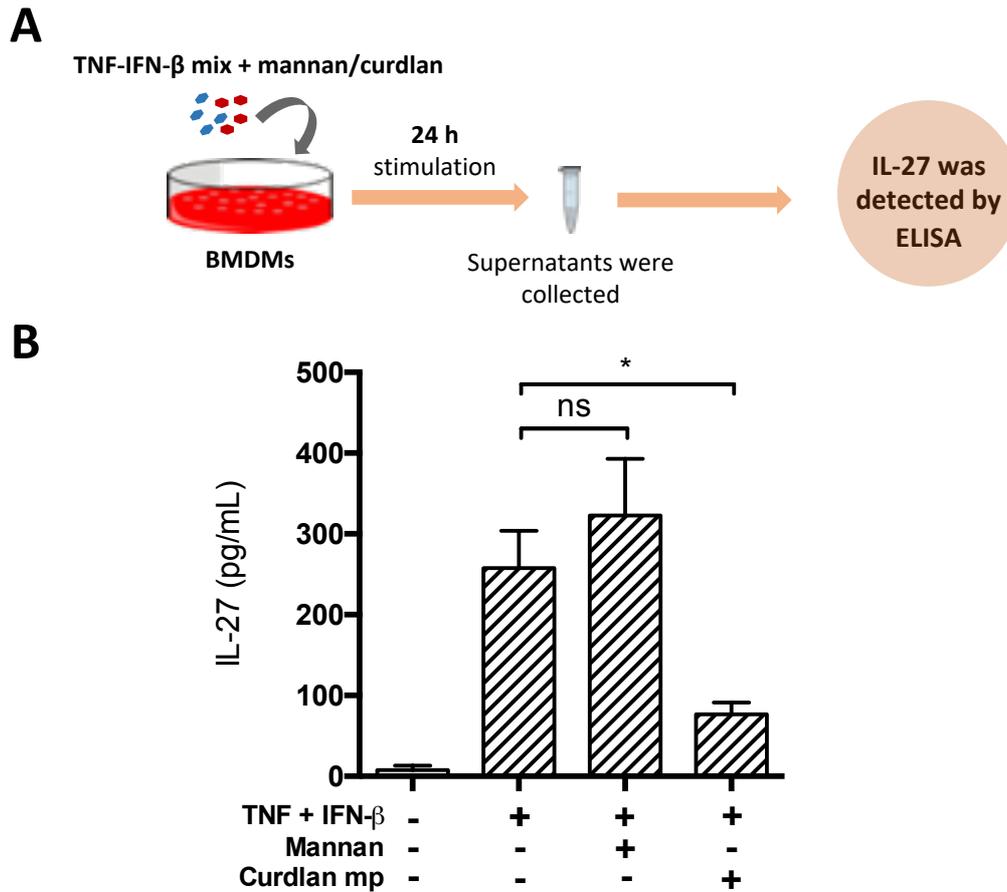


Figure 5.14 – Particulate β -glucan polymers, and not mannan, block IL-27 induced via TNF–IFN- β mix. (A) Schematic of experimental design: BMDMs were stimulated with a mix of recombinant murine TNF and IFN- β (10 ng/mL TNF + 200 U/mL IFN- β) and 100 μ g/mL of either mannan isolated from *S. cerevisiae* or curdian micro-particles for 24 h. IL-27 in the cell culture medium was detected by ELISA. (B) Experiment results. Data is presented as means \pm SEM. n=5. Ordinary One-way ANOVA with Tukey post-hoc test. ns – not statistically significant, *p<0.05.

Figure 5.14-B shows the results obtained in experiments using TNF–IFN- β mix in combination with mannan or curdian micro-particles. Stimulation with TNF–IFN- β mix triggered the production of IL-27 by BMDMs (257.6 \pm 46.4 pg/mL). Addition of 100 μ g/mL mannan did not lead to significant changes of IL-27 levels produced by the cytokine mix (322.6 \pm 70.3 pg/mL), however, stimulation with 100 μ g/mL curdian micro-particles resulted in a significant inhibition of IL-27 (76.5 \pm 14.6 pg/mL).

Results displayed in Figure 5.14-B revealed that only curdian is able to inhibit the levels of IL-27 generated by stimulation with TNF–IFN- β mix. This suggests that unlike curdian, mannan is possibly competing with *C. parapsilosis* for the PRRs, and that the reduced IL-27

levels detected after co-stimulation with *C. parapsilosis* and mannan (Figure 5.14-E) could be due to impaired recognition and consequently reduced phagocytosis of *C. parapsilosis* by macrophages.

Mannans can be recognised by different PRRs depending on their conformation, with Mincle and Dectin-2 being some of the CLRs mainly responsible for recognition of α -linked N-mannans^{93,639}. β -glucan is mainly recognised by Dectin-1 in macrophages^{93,642}. To assess if PRR signalling triggered by both carbohydrates had an effect on reducing IL-27 levels induced by *C. parapsilosis*, Mincle/Dectin-2 double knock-out BMDMs were co-stimulated with *C. parapsilosis* and mannan, and Dectin-1-deficient BMDMs were stimulated with *C. parapsilosis* and curdlan micro-particles for 24 h. IL-27 levels in the cell culture media was detected by ELISA (Figure 5.15).

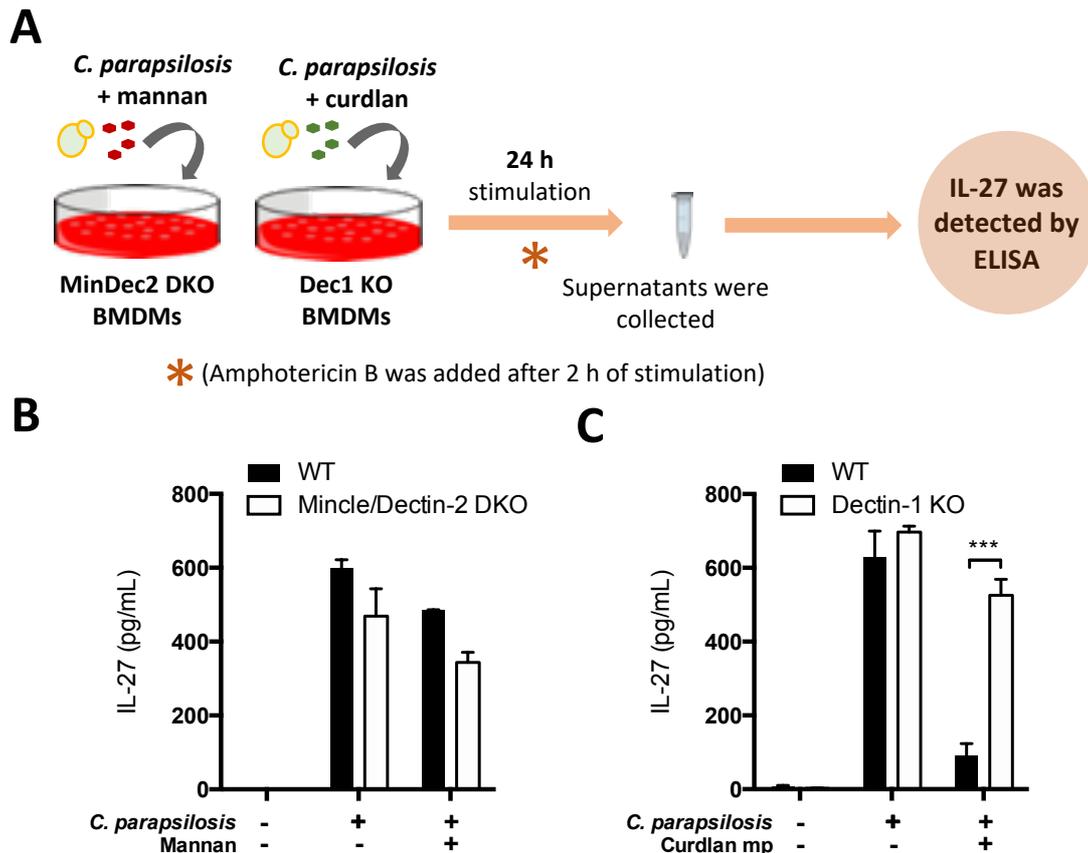


Figure 5.15 – Inhibition of IL-27 via particulate β -glucan polymers is dependent on Dectin-1 signalling. (A) Schematic of experimental design: Wild-type (“WT”) or Mincle and Dectin-2-deficient (“Mincle/Dectin-2 DKO”) BMDMs were stimulated with *C. parapsilosis* in the presence or absence of 100 $\mu\text{g}/\text{mL}$ mannan isolated from *S. cerevisiae* for 24 h (B). Wild-type (“WT”) or Dectin-1-deficient (“Dectin-1 KO”) BMDMs were stimulated with *C. parapsilosis* in the presence or absence of 100 $\mu\text{g}/\text{mL}$ curdlan micro-particles for 24 h (C). Amphotericin B was added after 2 h of co-stimulation. IL-27 in the cell culture medium was detected by ELISA. Data is presented as means \pm SEM. B and C: $n=3$, Two-way Repeated Measures ANOVA with Sidak post-hoc test to evaluate for differences in IL-27 release between wild-type cells and CLR-deficient cells after stimulation with both *C. parapsilosis* and different polysaccharides particles. *** $p<0.001$.

Figures 5.15-B and -C show the results from experiments using CLR-deficient macrophages stimulated with *C. parapsilosis* and their respective carbohydrate ligands. (Figure 5.15-B) Stimulation of Mincle/Dectin-2-deficient macrophages with *C. parapsilosis* in the absence of mannan led to lower levels of IL-27 (469.0 ± 73.9 pg/mL) compared with WT macrophages (598.4 ± 23.5 pg/mL) (although not statistically significant). Addition of mannan resulted in similar lower levels (not significant) of IL-27 detected by both WT (484.9 ± 1.6 pg/mL) and double knock-out BMDMs (343.4 ± 27.9 pg/mL). (Figure 5.15-C) Stimulation of Dectin-1-deficient BMDMs with *C. parapsilosis* in the absence of curdlan resulted in a similar production of IL-27 (696.9 ± 16.0 pg/mL) compared with WT BMDMs (627.8 ± 71.7 pg/mL).

Addition of curdlan resulted in a reduction of IL-27 levels produced by both types of macrophages, however the IL-27 levels produced by WT BMDMs (89.6 ± 34.1 pg/mL) were significantly lower than the ones detected by Dectin-1-deficient macrophages (525.5 ± 43.7 pg/mL).

Regarding mannan, results presented on Figure 5.15-B are consistent with the ones obtained in Figure 5.13-E and suggest that mannan does not trigger inhibition of IL-27. Figure 5.15-B suggests that the reduced *C. parapsilosis*-induced IL-27 levels observed with mannan stimulation are probably not due to Mincle and Dectin-2 signalling activation, but possibly due to an impairment of *C. parapsilosis* recognition by BMDMs caused by mannan blockade of PRRs responsible for yeast recognition and consequent phagocytosis. Nonetheless, more experiments need to be performed to verify this. Figure 5.15-C results revealed that curdlan microparticles are able to block *C. parapsilosis*-induced IL-27 in a Dectin-1-dependent manner.

5.4 Discussion

Previous work from our group has shown that induction of IL-27 by macrophages was exclusive to some less virulent non-*albicans* species with *C. parapsilosis* being the *Candida* spp. capable of inducing the highest levels of this cytokine. In the context of *C. parapsilosis* infection, IL-27 production was shown to be dependent on type I IFN signalling pathway activation via IFN- β in a phagocytosis-dependent manner. Interestingly, in the same report, it was also shown that although *C. albicans* is not able to induce production of IL-27, it is able to actively block it via a soluble mediator¹⁸⁴.

Results revealed in chapter 4 revealed that IFN- β enhances macrophages candidacidal ability against *C. albicans*. Therefore, I was particularly interested in this finding: if the *C. albicans*-mediated blockade of IL-27 was happening upstream at IFN- β level, it could pose as an interesting, novel mechanism deployed by *C. albicans* for immune evasion and survival inside macrophages.

This chapter focused on assessing *C. albicans* potential to block IFN- β and IL-27 and to elucidate the molecular mechanism of this inhibition. The impairment of this inhibitory ability of *C. albicans* could promote the production of type I IFN by macrophages, as verified with low virulence non-*albicans* species and contribute to a better killing of the pathogen as was shown in the previous results chapter.

5.4.1 ***C. albicans* blocks *C. parapsilosis*-induced IFN- β and IL-27 via a secreted molecule**

I have shown here that besides *C. albicans* being able to trigger inhibition of *C. parapsilosis*-induced IL-27 production, which is consistent with previous findings, it is also able to block IFN- β , an upstream regulator of IL-27 production by *C. parapsilosis*¹⁸⁴. My experiments revealed that IL-27 inhibition was stronger with supernatants from *C. albicans* initially co-cultured with BMDMs, compared with *C. albicans* cultured in their absence. Considering this, I have postulated that the IL-27 inhibition is a two-step process that involves two mediators: a primary fungal-derived signal that consists of a soluble mediator released

by *C. albicans* to the medium, independently of the presence of BMDMs, and a secondary macrophage-derived signal that would be produced by BMDMs upon contact with the fungal-derived mediator, that would induce the blockade.

No other fungal species is known to actively block type I IFN production in macrophages, however, this is not true for some viral and bacterial pathogens. Both pDCs and macrophages were shown to produce type I IFN after recognition of HIV-infected cells via TLR7 and TLR9^{643,644}. HIV is able to block type I IFN production by these cells by inhibiting STAT1 phosphorylation and block its migration to the nucleus via viral accessory proteins⁶⁴⁵. Kaposi's sarcoma-associated herpesvirus (KSHV) is also able to block type I IFN induction via production of viral RIF (product of *orf10*). The latter leads to formation of complexes with Jak1, Tyk2, STAT2 and IFNAR1/2 at the receptors' site in the membrane which will block kinase activity of Tyk2 and Jak1⁶⁴⁶. Hepatitis C virus (HCV) is also capable of inhibiting type I IFN genes in hepatocytes via miR-373-mediated targeting of Jak1 and IRF9⁶⁴⁷. Other virus-derived inhibitors of the type I IFN response were already identified, mainly in RNA-viruses⁶⁴⁸, however their blockade is triggered by viral proteins not expressed by fungi, and therefore cannot be considered as possible molecules to explain the IFN- β /IL-27 inhibition verified with *C. albicans*. *M. tuberculosis* inhibits STAT-1 activation resulting in reduced IFN- α production upon infection by monocytes and alveolar macrophages. The pathogen inhibits type I IFN autocrine signalling in BMDMs by blocking phosphorylation of Tyk2 and Jak1 rendering the kinases unable to phosphorylate STAT1/2 and therefore block its migration to the nucleus. This poses as a mechanism to inhibit genes involved in host defence against the pathogen like iNOS and chemokines which were proven to be essential for host resistance to the infection^{649,650}. Much like what was observed with *C. albicans* and *C. parapsilosis*, the ability of *M. tuberculosis* to inhibit type I IFN production appears to be a feature of virulence. Unlike *M. tuberculosis*, the less virulent mycobacterial species *M. bovis* Bacillus Calmette-Guérin (*M. bovis* BCG) that does not normally cause disease in the immunocompetent host, is not able to inhibit IFN- α ⁶⁴⁹.

5.4.2 Cytokines specifically induced by *C. albicans* are not the BMDM-derived mediator responsible for IL-27 inhibition

None of the *C. albicans*-specific cytokines tested was responsible for the inhibition of IL-27. From those, TNFSF18 (GITRL) was probably the strongest candidate to be the macrophage-derived mediator responsible for IL-27 blockade. Splenocytes deficient for GITR (TNFSF18 receptor) were shown to express higher levels of MyD88 compared to WT cells⁶⁵¹, suggesting that GITR activation can downregulate expression of the adaptor protein, and consequently impair TLR-mediated signalling. Although this was not proven with innate phagocytes, since my data has predicted that TNFSF18 is upregulated during *C. albicans* infections, it was tempting to assume that the mechanism of IL-27 inhibition deployed by the pathogen could be due to a reduction of MyD88 levels caused by GITR signalling activation that would impair TLR7-mediated production of IFN- β and IL-27. However, results obtained suggest this was not the case. GITR is mostly expressed in T cells, and although macrophages also express it at low levels, unlike CD8⁺ and CD4⁺ T cells, these cells do not rapidly upregulate GITR expression upon activation to bolster TNFSF18 signalling, which could explain why IL-27 inhibition was not observed in BMDMs⁴⁹⁶.

5.4.3 *C. albicans*-derived secreted molecule is apparently not delivered in extracellular vesicles

Extracellular vesicles (EVs) can be used by pathogenic fungi as virulence bags to transport immunogenic molecules. Some of these like include highly immunogenic molecules like Saps^{633,652} that could be responsible for IL-27 inhibition. Besides, EV stimulation elicits cytokine production in BMDMs from which the macrophage-derived secondary mediator responsible for IL-27 inhibition could be included⁶³³. If EVs were playing a part in the delivery of the primary mediator, purification and analysis of the components present inside these structures could facilitate its identification. However, my results indicate that EVs within *C. albicans* supernatants do not trigger said inhibition, suggesting that the fungal derived mediator is being conventionally secreted into the medium. Nevertheless, this is a result inferred from one independent experiment, and further repeats need to be performed to validate this finding.

5.4.4 IL-27 blockade is dependent on *C. albicans* germination and formation of true hyphae.

As mentioned above, the ability of *M. tuberculosis* to inhibit type I IFN in monocytes and macrophage as an immune evasion strategy is a virulence trait, as the low virulence *M. bovis* BCG is not able to induce blockade⁶⁴⁹. I have postulated that a similar effect could be verified with *Candida* spp. From other non-*albicans* clinically relevant *Candida* spp. tested, only supernatants from *C. dubliniensis*, were shown to inhibit *C. parapsilosis*-induced IL-27 to the same extent as *C. albicans*. This finding and results obtained in experiments using supernatants from both yeast-locked *C. albicans* and HK *C. albicans* revealed that IL-27 blockade is dependent on the phenotypical switch from yeast to true hypha.

C. tropicalis was also reported to form true hypha, but surprisingly it did not trigger significant inhibition of IL-27. *C. tropicalis* filamentation degree however differs from *C. albicans*, as *C. tropicalis* hypha lengths are much shorter than those observed with *C. albicans* grown in similar conditions⁶⁵³. Moreover, the ability of *C. tropicalis* to normally exist in this phenotype is strain-specific, and it is highly influenced by the physicochemical compositions of the medium³³. Microscopical observation of both *C. tropicalis* strains grown in RPMI-BMDM used in this study revealed these exist mainly in pseudohyphal form, unlike *C. albicans* and *C. dubliniensis* that existed mainly in true hypha phenotype. This could explain why no significant IL-27 inhibition occurred with *C. tropicalis* supernatants.

5.4.5 Candidalysin is not the *C. albicans*-derived secreted molecule responsible for IL-27 inhibition

Since the IFN- β /IL-27 inhibition was shown to be mediated by a fungal-derived secreted molecule, I have postulated that this molecule could be coded by a virulence-associated gene exclusively produced by *Candida* spp. during the transition from yeast to hyphal form.

Candidalysin expression appears to be a distinct feature of *Candida* spp. capable of forming true hyphae, as *C. dubliniensis* and *C. tropicalis* were the only non-*albicans* species identified to express known *ece1* orthologs, and therefore predicted to produce the toxin^{80,81}. Moreover, *ECE1* is highly expressed during hypha formation and knocking out genes that

contribute to this phenomenon like *flo8* and *cph1* was shown to inhibit expression of *ece1* in *C. albicans*, and inhibit candidalysin synthesis^{19,654}, making it a strong candidate to be the secreted molecule responsible for IL-27. However, my results suggested that candidalysin or all the other seven peptides originated from ECE1 are not the secreted molecule responsible for the blockade, as either candidalysin-deficient or *ece1*-deficient *C. albicans* block IL-27 induction to the same extent as the WT strain.

5.4.6 Dectin-1 signalling activation blocks *C. parapsilosis*-induced IL-27 production

Despite the repertoire of cytokines host cells can produce after infection with *C. albicans*, the fungus can deploy mechanisms to evade the immune response and hijack cytokine production by immune cells. The fungal cell wall plays an important part in this evasion of the host response. Most cell wall components are PAMPs and a proper recognition of these by PRRs of immune cells is vital to trigger an adequate host immune response¹⁴. Considering this, I then postulated that the fungal-derived mediator responsible for IL-27 inhibition could be a component shed from *C. albicans* cell wall into the medium, and therefore tested if any of the three main carbohydrates present in the fungal cell could inhibit *C. parapsilosis*-induced IL-27 synthesis.

Stimulation with either N-acetyl-D-glucosamine and chitin did not result in inhibition of *C. parapsilosis*-triggered IL-27 levels. Chitin exists as long biopolymers, and upon infection, host chitinases (enzymes responsible for the degradation of chitin) will degrade it into smaller molecules. The different size of these molecules will influence the immune response triggered by macrophages. Ultrapurified fungal chitin and large sized chitin polymers are biologically inert and were shown not to directly stimulate production of cytokines. Intermediate fragments from 40-70 μm size were able to stimulate TNF production in a phagocytosis-independent mechanism⁶⁵⁵. Fungal-derived small chitin particles from a size resembling what is likely to be found in the host natural environment (1-10 μm) can induce either TNF or IL-10 secretion depending on its concentration¹⁸³. Besides not being of fungal origin, chitin preparations used here contained particles from different sizes ranging from 10 to 400 μm , with an average size of $\sim 200 \mu\text{m}$. Since cells do not react to large chitin polymers, it is possible

that no inhibitory effect was observed with chitin due to their big sizes. To rule out this possibility, the same experiments should be performed using *C. albicans*-purified small chitin particles.

My research shows that stimulation with mannan and β -glucan particles like zymosan and curdlan resulted in reduced levels of IL-27 induced by *C. parapsilosis*. This finding was interesting as these play different roles in as PAMPs. β -(1,3)-glucan is highly immunogenic, and its recognition by Dectin-1 leads to phagocytosis and consequent production of cytokines that are vital for effective clearance of *C. albicans*^{656,657}. Mannans can also be recognised by macrophages and trigger cytokine secretion⁴¹, however they are mostly associated with stealth as they conceal the intrinsic β -glucan layer of the cell wall from PRR recognition to ameliorate the strength of the pro-inflammatory response¹⁰⁹.

Since curdlan is known to hydrate and form gels, it can exist as large aggregated macro-particles (~100 μ m) and due to its size, macrophages are often unable to internalise them resulting in a process deemed "frustrated phagocytosis"⁶⁵⁸. To avoid this phenomenon, and in case the curdlan-driven IL-27 inhibition was dependent on particle phagocytosis, curdlan particles were sonicated which destroyed these aggregates generating smaller sized micro-particles⁶⁵⁸. This augmented the signal necessary for IL-27 inhibition since micro-particles are ten times more effective at blocking IL-27 production than macro-particles. This finding hinted that curdlan-induced IL-27 inhibition could be dependent on particle phagocytosis. Zymosan-induced IL-27 inhibition was not as strong as the one induced by curdlan microparticles. This could probably be due to an increased uptake of curdlan micro-particles due to their smaller size compared to zymosan. Moreover, a bell-shaped curve of IL-27 levels was observed with zymosan stimulations, where inhibition was not observed with the intermediate concentration tested (10 μ g/mL). The reason for this is unclear, moreover, it is known that besides Dectin-1, zymosan also agonises TLR2 and TLR6^{93,659,660} and differential activation of these receptors might, to some extent, rescue the inhibitory effect.

TNF was shown to potentiate IL-27 induction when used in combination with IFN- β ¹⁸⁴. Although this molecular mechanism was not yet elucidated, it could be due to an NF-kB and

MAPK-dependent activation of IRF1 as a consequence of TNF-mediated signalling that amplified STAT1 signalling exerted by IFN- β ⁶⁶¹. Using a mix of TNF and IFN- β to induce IL-27 production in uninfected BMDMs, I have assessed that β -glucan and not mannan can induce IL-27 blockade in this setting. This suggested that only β -glucan was truly responsible for this inhibition and that in stimulations with *C. parapsilosis*, mannan was possibly blocking PRR binding and *C. parapsilosis* recognition, therefore reducing yeast uptake by BMDMs, which is essential for IL-27 production¹⁸⁴.

Experiments using PRR-deficient BMDMs are in alignment with this. A reduced IL-27 production was verified in Mincle/Dectin-2-deficient versus WT suggests that these two PRRs are involved in recognition and internalisation of yeast. Addition of mannan resulted in reduction of IL-27 levels, however this trend was conserved regardless of the BMDM phenotype. Purified mannan was shown to inhibit TLR4-dependent synthesis of IL-6 and TNF during infections with live *C. albicans* by antagonising mannan-sensing PRRs and impairing the recognition of mannans in *C. albicans* cell wall⁴¹. Moreover, yeast-derived mannan and mannose were shown to impair *C. parapsilosis* phagocytosis in a similar fashion⁶⁶². This fitted with my hypothesis that mannan-driven reduced levels of IL-27 was caused due to impairment of *C. parapsilosis* phagocytosis. Nevertheless, due to its diverse forms, a modest number of PRRs besides Mincle and Dectin-2 were implicated in mannan recognition^{41,93,109-111}, and the impact of these PRRs in this setting has yet to be explored.

A significant reduction of *C. parapsilosis*-induced IL-27 was verified in WT cells versus Dectin-1 knock-out cells. Unlike what was verified with Mincle/Dectin-2-deficient cells challenged with *C. parapsilosis*, Dectin-1-deficient BMDMs produce similar levels of IL-27 when challenged with the yeast. This aligns with previous research, as Dectin-1 was shown to be dispensable for phagocytosis of *C. parapsilosis* by neutrophils⁵²⁵ and confirms that unlike mannan, curdlan exerts a truly inhibitory effect of IL-27. Moreover, IL-27 levels detected in Dectin-1 knock-out macrophages stimulated with *C. parapsilosis* and curdlan were significantly lower than the ones observed when the same cells are stimulated only with *C. parapsilosis*. This was an interesting finding as it suggests that other receptors able to

recognise curdlan like TLR4^{663,664} or β -glucan like TLR2⁹³, might also contribute to some extent for the curdlan-mediated IL-27 inhibition.

Although β -glucans in yeast morphotypes are shielded by the mannan layer from Dectin-1 recognition, Dectin-1 is able to recognise hyphal β -glucans¹⁰⁹. This suggests that β -glucan is more accessible on the hyphal surface, and that a shorter mannan layer and/or a lower abundance of mannan are probably responsible for this effect^{665,666}. Moreover, *C. albicans* hyphae are richer in β -glucan than yeasts⁶³⁹ and heat-killing process is known to expose β -glucans in *C. albicans* cell wall⁵²². Since β -glucan particles can inhibit IL-27 produced by *C. parapsilosis*, this could explain why in previous experiments supernatants stimulated with HK *C. albicans* in hyphal form induced a blockade similar to what was registered with germinated live *C. albicans*. Nevertheless, other hypha inactivation methods that do not alter the normal cell wall composition such as thimerosal-mediated fungal killing should be tested.

5.5 Conclusions

Results presented in this chapter confirm and complement a previously reported *C. albicans*-driven mechanism for IL-27 inhibition, indicating that it is a fungal molecule-driven blockade, dependent on yeast-to-true hyphae phenotype transition. Moreover, my research also revealed that this inhibition is not limited to *C. albicans*, as *C. dubliniensis* is also able to trigger it to a similar extent. IL-27 induction by *C. parapsilosis* in BMDMs is dependent on type I IFN signalling activation, and I have shown here that besides being able to block IL-27, *C. albicans* is also able to inhibit IFN- β synthesis, which is a novel finding. Considering this, it is tempting to assume that the IL-27 inhibition observed with the *C. albicans*-derived mediator happens due to an upstream blockade of IFN- β which translates in reduced levels of secreted IL-27, however further experimentation is required to ascertain this.

The recently identified candidalysin, linked to *C. albicans*-induced damage and inflammation activation, has been subject to a lot of studying. Interestingly, my research revealed that candidalysin (or other ECE1-derived peptides) are not responsible for triggering said inhibition. Moreover, cytokines specifically induced in BMDMs by *C. albicans* (most of them induced by candidalysin) were not found to inhibit *C. parapsilosis*-induced IL-27 production, suggesting that the toxin is not playing a part in the *C. albicans*-mediated blockade of IL-27.

My research has also revealed that β -glucan particles can trigger inhibition of IL-27 induced by *C. parapsilosis* in a Dectin-1-dependent manner. However, whether a form of *C. albicans* cell wall β -glucan not produced by *C. parapsilosis* is the secreted fungal-derived mediator was not addressed here. Furthermore, the *C. albicans*-derived molecule responsible for IFN- β and IL-27 blockade is soluble, as it was still retained in the supernatants after sterile filtration through 0.22 μm mesh filters. Curdlan and zymosan particles sizes, that I have shown to inhibit IL-27, are bigger than the 0.22 μm cut-off and would be retained in the filter and not be present in the supernatants after filtration. Although it is known that *C. albicans* cell wall has soluble forms of β -glucan, it is uncertain whether these could be triggering the IL-27 blockade, as Dectin-1-mediated phagocytosis and consequent signalling only happens with particle β -glucans, whereas soluble β -glucans exert mostly an antagonistic effect of the

receptor. This could indicate that Dectin-1 agonism by β -glucan particles is a different, though analogous, IL-27 inhibitory mechanism. Although this would be an interesting finding, if proven to be true, it does not elucidate what is the *C. albicans*-derived soluble molecule initially identified as being the driver of IFN- β and IL-27 inhibition. Therefore, further experimentation is required to ascertain this. Figure 5.16 summarises the collective results obtained in this chapter.

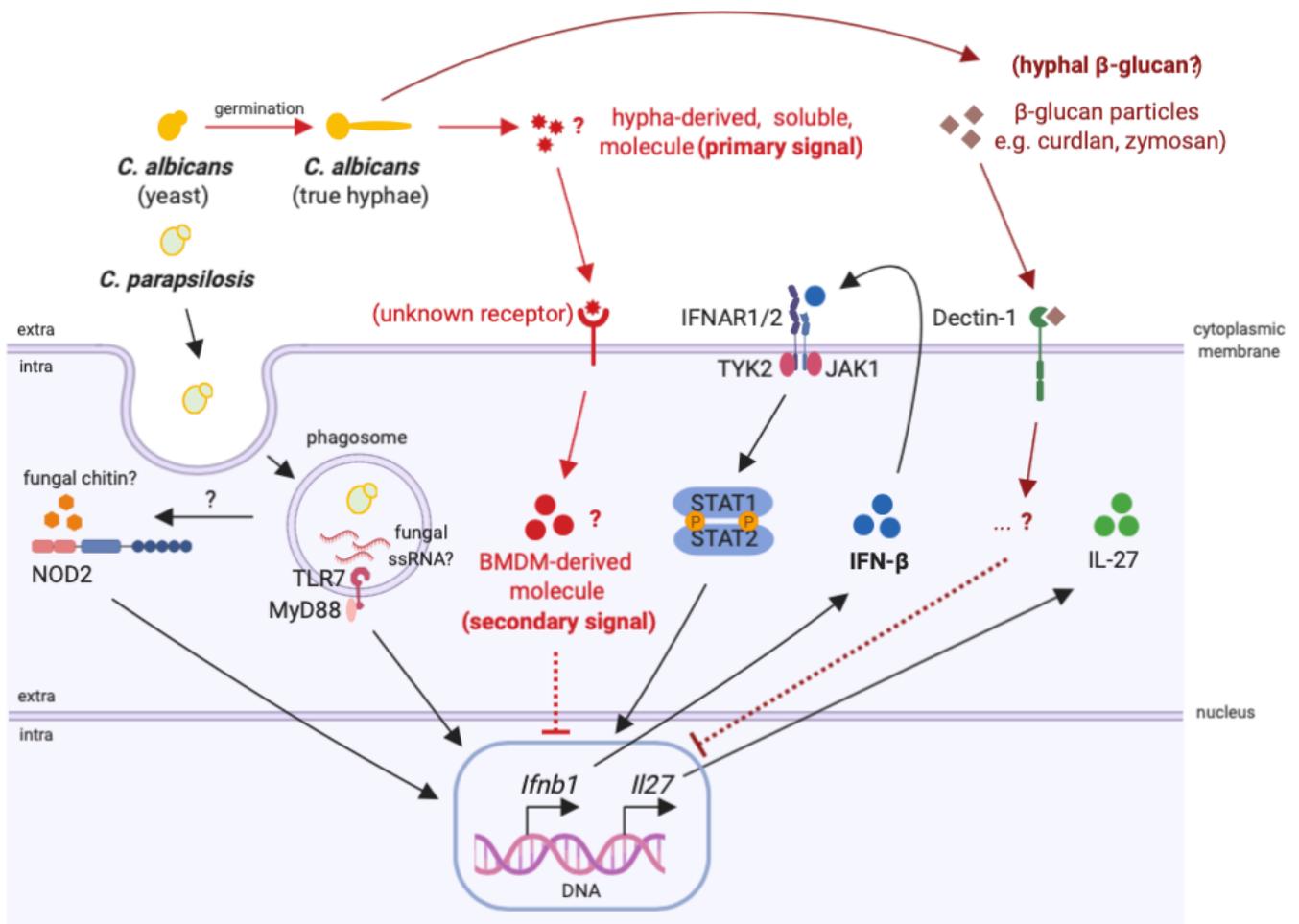


Figure 5.16 – Summary of IFN- β inhibition experiments in this chapter with the possible IFN- β /IL-27 inhibition mechanisms. *C. parapsilosis* induces IFN- β production in BMDMs in a phagocytosis-dependent process that requires TLR7/MyD88-mediated signaling triggered by fungal ssRNA in the phagosome and NOD2 activation in the cytosol, possibly by *C. parapsilosis* chitin. IL-27 transcription is then induced downstream of IFN- β . Through IFNAR1/2, IFN- β will then activate STAT1/2 heterodimers and augment IFN- β and IL-27 levels produced by BMDMs. Upon germination and formation of true hyphae, *C. albicans* secretes a soluble mediator that is able to block IFN- β production and consequently reduce IL-27 levels. This inhibition is also achieved through β -glucan particles via a Dectin-1 dependent mechanism, possibly through *C. albicans* hyphal β -glucan. Image created with biorender.com

5.6 Future experiments

The IFN- β and IL-27 inhibitory mechanisms discussed in this chapter could be of clinical relevance. These findings however were only discovered in BMDMs and should be validated in both murine and human primary monocytes and macrophages.

Other strains of *C. parapsilosis* were shown to induce IL-27 production, and in the case of *C. dubliniensis*, different strains were shown here to be able to similarly block its production. However, only one strain of *C. albicans* was tested for its ability to block IL-27 production. Testing other *C. albicans* strains, including ones with attenuated virulence, could be interesting and may further strengthen the link between the ability to inhibit IFN- β /IL-27 and virulence.

Although my research has contributed to a better understanding of the dynamics underlying the IFN- β /IL-27 inhibitory mechanism, the two proposed mediators responsible for this process have not been yet identified. Regarding the *C. albicans*-derived mediator, besides soluble cell wall β -glucan and hyphal cell-specific β -glucans, other proteins specifically produced by hyphal cells are interesting candidates to be the components responsible for inhibition such as Saps and Hwp1. Moreover, the eicosanoid metabolite prostaglandin E2 (PGE₂) produced by *C. albicans* was shown to block both CXCL10, a type I IRG, and IFN- α production by keratinocytes and pDCs respectively^{485,667}, and stands as an interesting candidate to be the soluble mediator responsible for IFN- β /IL-27 blockade. Nevertheless, fractionating the components present in *C. albicans* supernatants by density, and assessing which fractions can induce the blockade of *C. parapsilosis*-induced IL-27 could greatly facilitate the discovery of the fungal mediator and also reveal if it is either a protein or a soluble form of cell wall carbohydrate.

Regarding the BMDM-derived mediator, discovery of which specific IL-27 induction pathway component activated by *C. parapsilosis* that is being targeted for inhibition, i.e. TLR7, NOD2 or type I IFN pathways is essential and would facilitate the discovery of the secondary mediator. Several negative regulators of type I IFN production were identified^{668,669}. These can act at different levels, including blockade at cytokine level, antagonising or affecting

IFNAR1/2 function, impairing the function of transcription factors like STAT1, or even affect TLR signalling. An example of these negative regulators is miR-146a. This non-coding RNA can inhibit IRAKs and TRAF6 that are associated with TLR-MyD88 signalling. TRAF6 was also implicated in NOD2 signalling as a link for induction of NF- κ B^{94,104,670-672}. Interestingly, both HK *C. albicans* and curdlan can trigger production of miR-146a in murine and human macrophages^{673,674}, making it an interesting candidate to be the secondary BMDM-derived mediator, and explain the Dectin-1-dependent IL-27 inhibition I have observed with curdlan.

Chapter 6

General Discussion

Systemic candidiasis is a major health concern, as it affects a significant number of patients, especially in the U.K. and is associated with unacceptably high mortality^{5-7,11}. Most patients succumb to infection due to pathogen-induced immunopathology that happens due to an improper and unbalanced immune response deployed by the host and current available therapeutics are often insufficient to avert this outcome. The main antifungals primarily prescribed for treatment of systemic candidiasis have low efficacy and immunosuppressive therapy is often prescribed in an effort to ameliorate excessive deleterious inflammation^{10,305,315,342,343}. The challenges of treatment for systemic candidiasis stem not only from the insufficient pharmacological therapies available, but also due to the poor understanding of how host immunity responds towards different *Candida* spp. capable of inducing infection. This is particularly important in systemic candidiasis, as immunodepressed patients are at a much higher risk for contracting the disease compared with immunocompetent individuals^{26,27,296,303,316,324,675}. Thus, the development of novel immunotherapies administered in a personalized medicine approach might be a suitable treatment option.

The main premise of this thesis was to focus on the immune response triggered by pathogenic *Candida* spp. with distinct virulence and elucidate some of the differences in the immune response underlying host resistance against the low virulence species. Potential targets were identified and the impact of modulating these targets was then explored in the context of infections caused by the highly virulent species *C. albicans* in order to assess their therapeutic potential.

6.1 *C. albicans* versus *C. parapsilosis* immune responses

Although several *Candida* spp. are known, only a select few were identified as aetiological agents of systemic candidiasis. From those, *C. albicans* still stands as the most frequently isolated pathogen^{3,12,13}, and even though species like *C. parapsilosis* are less common, their incidence has been rising in recent years. *C. parapsilosis* infections are more frequent in neonates possibly due to transmission of the yeast by health care workers, and in patients carrying indwelling medical devices, to which *C. parapsilosis* frequently adheres to forming extensive biofilms^{3,25-27}. Both epidemiologic and *in vivo* studies evidence that *C.*

albicans and *C. parapsilosis* virulence positively correlates with their incidence rates in systemic candidiasis as *C. albicans*-driven candidaemia-associated mortality is estimated to be approximately 44.8%, whereas *C. parapsilosis* is only 28.5%²⁵. Moreover, in most *in vivo* murine studies, whilst *C. albicans*-infected immunocompetent mice succumb to infection, they are able to survive and clear *C. parapsilosis* more effectively even when injected in higher doses^{154,321,323,676}.

C. albicans and *C. parapsilosis* differ greatly in their biology and virulence attributes which can account for the different incidence rates and pathogenicity. As an example, hydrolytic enzymes produced by *Candida* greatly contribute to host tissue damage by disrupting cell membranes, degrading ECM proteins, and promoting fungal adhesion, biofilm formation and cell survival, ergo facilitating host invasion and tissue infiltration^{54,55}. *C. albicans* encodes for more secreted aspartyl proteases (Saps), lipases and phospholipases^{56,57,59,71–75} than *C. parapsilosis*^{60,61,76,78,677}, and unlike the latter, *C. albicans* can form true hyphae which could partly account for its increased ability to penetrate host tissues, evade phagocyte digestion and induce systemic infection when compared with *C. parapsilosis*^{16,18–20,323,678}. Furthermore, *C. parapsilosis* isolates obtained from mucocutaneous sites express higher levels of adhesion molecules that along with its cell surface hydrophobicity correlates with its strong adherence to polystyrene surfaces present and epithelial cells⁶⁰, which could explain why *C. parapsilosis* infections frequently originate from biofilms detected in invasive medical devices without previous colonisation of the host.

To initially identify differences in the immune response that might underly the distinct pathogenicity between the two species, a comparison between the macrophage transcriptome activated during *C. albicans* and *C. parapsilosis* stimulations was performed. This analysis revealed marked differences between the immune response triggered by the two pathogens. *C. albicans* promoted an immune response that is exclusively dominated by anti-inflammatory IL-10-induced signalling and by elements known to be triggered by candidalysin such as IL-36 α , IL-36 γ and G-CSF^{85,87,490}. *C. albicans* induces the secretion of chitinases during macrophage phagocytosis, which digest fungal cell wall chitin into small fragments that are shed into the environment. Released chitin will then be taken up by MR in macrophages and induce IL-10 secretion via TLR9/NOD2 that will promote alternative

macrophage polarisation^{183,506}. As alternatively polarised macrophages have impaired ability to clear *C. albicans*, this represents a *C. albicans* immune evasion strategy to survive macrophage attack and thrive intracellularly until it escapes by inducing pyroptosis or by germinating and piercing through the host cell membrane^{183,506}. *C. parapsilosis* cell wall contents and architecture differ from *C. albicans*. *C. albicans* β -glucan layer is in closer proximity to the cell membrane compared with *C. parapsilosis*, and is shielded by the mannan layer, whereas both chitin and β -glucans are closer to the cell wall surface in *C. parapsilosis* and more exposed. Moreover, *C. parapsilosis* wall is more porous than *C. albicans*, and its N-linked mannans are shorter, which can make immunogenic PAMPs present in deeper layers of the cell wall such as chitin and β -glucan more accessible for recognition by host cell PRRs^{471,655,656}. Although chitin might be more accessible in *C. parapsilosis*, we have shown that the *C. parapsilosis* cell wall has a much lower chitin content compared to *C. albicans*¹⁵⁴, and it is still unknown whether *C. parapsilosis* can induce chitinase production in macrophages to the same extent as *C. albicans*. This could potentially explain why a similar IL-10 signature was not detected in *C. parapsilosis*-stimulated macrophages. It was not fully addressed here if *C. albicans*-induced expression of IL-36 α , IL-36 γ and G-CSF is fully dependent on candidalysin production, however, if proven to be true it could explain why these genes were not strongly induced in *C. parapsilosis*, as the latter does not express *ece1*, and therefore it does not produce candidalysin^{80,81}.

The type I IFN signalling pathway was identified as the dominant immune pathway exclusively induced by *C. parapsilosis* in BMDMs and it was validated as a target for immune modulation during *C. albicans* infections. Comparison of different reports does not allow for a definitive discrimination of which *Candida* spp. and strains are capable of inducing type I IFN production. My research and findings from our group are the first reporting that *C. parapsilosis* is able to induce type I IFN in BMDMs, whilst *C. albicans* does not¹⁸⁴, however other reports contradict our findings, where they showed that *C. albicans*^{122,187,188,314}, *C. glabrata* and *C. dubliniensis*¹⁸⁸ can also induce its production. Initially I hypothesized that *C. albicans* ability to induce type I IFN production could be a strain-specific effect. However, different *C. albicans* strains were proved to be able to trigger type I IFN production^{122,187} and

other papers have reported similar findings with *C. albicans* SC5314, the same strain used in my research which I have showed not to induce significant production of type I IFN^{188,314}.

The reason for *C. parapsilosis* to induce higher levels of IFN- β production in macrophages compared to *C. albicans* has not yet been explored. Our group and others have shown that the ability of *Candida* to trigger type I IFN production is dependent on phagocytosis^{122,184,188}. *C. parapsilosis* is internalized by macrophages at a faster rate than *C. albicans*^{77,525} and macrophage phagosomes frequently contain a higher number of *C. parapsilosis* yeasts than *C. albicans*^{77,184}, which could potentially result in an increased concentration of fungal ligands recognised by TLR7 and NOD2. This could consequently potentially explain the higher levels of IFN- β detected in response to *C. parapsilosis*. In addition, following phagocytosis, *C. albicans* germinates forming extensive hypha that can rupture the phagosome facilitating its escape¹⁹⁰. This induces rapid activation of the NLRP3 inflammasome which contributes to macrophage pyroptosis, a highly inflammatory form of cell death where the cell bursts releasing copious amounts of pro-inflammatory cytokines^{679,680}. Conversely, upon macrophage internalisation, *C. parapsilosis* can thrive and even replicate inside phagosomes for prolonged periods of time without inducing cell lysis⁷⁷. This prolonged internalisation period observed with *C. parapsilosis*, along with possible yeast cell proliferation, might augment and sustain TLR7/NOD2-mediated signalling, thereby contributing to the robust levels of secreted IFN- β . It is also possible that *C. parapsilosis* and not *C. albicans* has a species-specific mechanism of nucleic acid release whilst inside the phagosome that contributes to increased activation of these PRRs, however this has not yet been explored.

Regarding the general immune response triggered by both pathogens, our group has evidence that *C. parapsilosis* induces a higher pro-inflammatory response in phagocytes compared with *C. albicans*, with higher levels of IL-12, IL-6 and TNF being produced (unpublished preliminary results). *C. albicans*-induced immune response tends to be more anti-inflammatory, with high levels of IL-10 and lower levels of IL-12 and IL-6 secreted¹⁵⁴. *C. albicans* also induces higher levels of IL-1 β than *C. parapsilosis*, possibly due to its ability to activate the NLRP3 inflammasome and due to changes in differences in fungal cell wall structure. O-linked mannans that drive IL-1 β production in phagocytes upon recognition by

Dectin-1 and TLR4 are masked in *C. parapsilosis* cell wall by N-linked mannans, which can hinder their recognition, but not in *C. albicans*, which could explain the differences observed between the two species^{177,681}.

Whilst the *C. parapsilosis*-triggered immune response in phagocytes is more pro-inflammatory than *C. albicans*, the *in vivo* systemic response tends to be opposite. In regards to adaptive Th responses, *C. albicans* infection induces stronger IFN- γ and IL-17 production than *C. parapsilosis* both in PBMCs and in *ex vivo* splenocytes. Although the *in vivo* responses triggered by both pathogens were never directly compared, this suggests that the latter triggers less Th1 and Th17 polarisation during *in vivo* infections, which along with release of other chemoattractants will translate in reduced and sustained recruitment and activation of phagocytes in infected sites^{154,682}. Furthermore, *post-mortem* organ analysis of systemically infected mice shows that *C. albicans* widely disseminates into host tissues during systemic infections inducing a high degree of inflammation and tissue damage. In contrast, *C. parapsilosis* is not as easily detected within tissues even when mice are injected with high inocula, and infected tissues frequently show only mild inflammation^{60,160,184}. The reason for such differences in organ infiltration and inflammation might be due to the species different ability to penetrate host tissues and the fact that *C. parapsilosis* tends to trigger a delayed and tolerogenic systemic response that facilitates its survival inside host cells. This strategy might avoid excessive systemic inflammation and reduced recognition by local tissue resident cells and take advantage of phagocytes as vehicles for dissemination throughout the host instead of relying on damaging tissue penetration, which can drive excessive inflammation. Indeed, following macrophage uptake, *C. parapsilosis* was shown to impair phagosome maturation through the action of species-specific Saps and lipases, however the mechanism responsible for this phenomenon is still unknown^{78,683}. Although this was not explored here, it is possible that the discovered IFN- β promotes phagosome maturation and acidification in BMDMs, which could account for its candidacidal activity. If this is true, it is possible that unlike *C. albicans*, *C. parapsilosis* can deploy mechanisms to inhibit or override this IFN- β -induced phagosome maturation^{78,683}, and therefore explain why, no differences in killing were observed in IFN- β -primed macrophages when infected with *C. parapsilosis*.

6.2 IFN- β as a possible treatment for systemic candidiasis

Type I IFN has a broad spectrum of action in the immune response and has been implicated in several pathological settings. Type I IFN produced by innate immune cells will ultimately trigger MAPK pathways, NF- κ B and one or more IRFs that will not only bolster type I IFN production but also the secretion of different pro-inflammatory cytokines that will drive inflammation and exert a broad spectrum of effects at the adaptive immunity level^{507,684}. These effects include the promotion of B cell memory and antibody production^{685,686} and robust antigen-specific T cell proliferation. Regarding the latter, type I IFN acts as an activation signal to boost survival of proliferating T cells and supports Th1 differentiation through STAT4 signalling and IFN- γ production in a similar fashion as IL-12^{687,688}. Moreover, type I IFN can also promote induction of CD8⁺ T cells by enhancing MHC I-driven antigen presentation in DCs⁶⁸⁹. However, many other different effects on host immunity induced by type I IFN have been reported, especially during infectious diseases however these seem to be highly dependent on the type of infection.

Besides being used as a treatment for some viral infections such as hepatitis, herpes zoster and HPV^{690,691}, type I IFN is also used as treatment for multiple sclerosis. Multiple sclerosis (MS) is a neurodegenerative auto-immune disease that is thought to develop as a result of inflammation in the neuronal tissue driven by infiltration of inflammatory cells in the central nervous system that exacerbate local inflammation⁶⁹². Although a cure is not available, IFN- β is currently used as treatment for MS as it was shown to be neuroprotective and to significantly shorten MS relapses⁶⁹². The protective mechanism exerted by IFN- β in this setting is complex and not yet fully understood, however it is thought mainly to involve amelioration of cerebral inflammation by increasing the production of IL-10 and IL-4 via Th2 polarisation and to inhibit Th1 and Th17 proliferation that are believed to contribute to a detrimental influx and activation of inflammatory phagocytes towards the CNS and therefore the progression of disease⁶⁹².

Type I IFN is promptly produced upon viral infections as a host mechanism to inhibit viral replication and to activate a protective anti-viral state in neighbouring immune cells that limits infection before other humoral and adaptive immune mechanisms are deployed⁶⁹³.

While type I IFN is required to limit viral infections, it can also exert detrimental effects on cell viability and function. Type I IFN can inhibit cell proliferation mainly through upregulation of CDK inhibitors, resulting in G1 cell cycle arrest⁶⁹⁴, and contribute to cell apoptosis by indirectly enhancing AIM2-mediated inflammasome activation^{695,696}. The latter will result in upregulation of RIG-I and MDA5, whose signalling culminates in mitochondrial apoptosis via MAVS adaptor protein⁶⁹⁷. Type I IFN can also facilitate apoptosis upon TLR3 and TLR4 signalling activation by antagonising TRIF-dependent phosphorylation and consequent proteasome degradation of p27^{kip1}, a cell cycle inhibitor^{698,699}. This shows that although it can contribute to beneficial inflammation and infection resolution, type I IFN can act as a “double-edged sword” as its excessive production triggered either by different pathogens, or by DNA released from dying cells can be cytotoxic, impair infection resolution and even contribute to other inflammation-associated pathologies⁷⁰⁰. This negative effect was probably best described in PBMCs from chronic HIV⁺ patients, where excessive type I IFN produced mainly by pDCs contributes to T cell dysregulation by inhibiting proliferation of both protective CD4⁺ and CD8⁺ T cells⁷⁰¹.

Besides viral infections, type I IFN has also been implicated in some bacterial infections, however, in some settings its production can either be beneficial and contribute to their resolution or, unlike most viral infections, detrimental and promote pathogen persistence and tissue damage depending on the microbial species. In infections with *Chlamydia spp.* and *Legionella pneumophila*, type I IFN was shown to contribute for bacterial clearance by limiting bacterial replication inside host cells^{532,702–704}. In infections with the latter, type I IFN contributes for macrophage-mediated clearance of *L. pneumophila* through induction of an “M1-like” polarisation and robust nitric oxide production via IRG1-mediated itaconate production that restricts bacterial growth^{553,704}. In murine models of bacterial infection, macrophage secretion of type I IFN as a consequence of TLR7 recognition of group B streptococcal species results in robust production of IFN- γ and TNF that greatly contributes for bacterial clearance *in vivo* and mice survival^{601,705}. In *Helicobacter pylori* mucosal infections, NOD1 recognition of bacterial ligands drives type I IFN production that triggers production of CXCL10 which in turn facilitates protective T cell infiltration in infected tissues^{509,706}. Type I IFN-induced CXCL10 production was also shown to contribute for survival in models of polymicrobial sepsis by promoting neutrophil recruitment and function⁷⁰⁷.

However, type I IFN can also have detrimental effects by suppressing host immunity during some bacterial infections. During *Listeria monocytogenes* and *Francisella tularensis* infections, type I IFN production can decrease mice survival by promoting cell apoptosis and suppressing protective IFN- γ and IL-17 secretion^{708–715}. Studies have shown that type I IFN production during *M. tuberculosis* infection is necessary for host clearance of the pathogen in the lungs^{649,650}. However, other reports have indicated that excessive type I IFN production in this setting can also impair protective Th1 responses and exert anti-inflammatory effects via IL-10 production that can dampen *M. tuberculosis* clearance^{608,609}.

Regarding fungal infections, the role of type I IFN appears to vary depending on the disease and pathogen driving the infection. Nevertheless, unlike viral and bacterial infections, this topic is still greatly understudied, and, in most cases, there is heterogenous and contradictory literature making it difficult to ascertain the definitive role of type I IFN in certain mycoses.

During *A. fumigatus* infection, pDCs recruited to infected lungs produce type I IFN that contributes for fungal clearance and mice survival⁶⁰⁶. Lung epithelial cells also produce type I IFN after early recognition of *A. fumigatus* resting conidia which drives CXCL10 production and thus may contribute for recruitment of monocytes and other immune cells to the respiratory tract and consequent curtailment of infection⁶⁰³. Although conventional DCs were shown not to produce type I IFN upon *A. fumigatus* challenge, treatment of these cells with IFN- β significantly enhanced IL-12 production resulting in protective Th1 polarisation when co-cultured with purified CD4⁺ T cells⁷¹⁶. Exogenous activation of type I IFN signalling was also shown to confer remarkable protection during *Aspergillus* spp. infection in a murine model of CGD by facilitating neutrophil infiltration to infected lungs, indicating its potential as an immunotherapy agent for aspergillosis⁶⁰⁴.

Several reports have shown that type I IFN has a protective effect in cryptococcosis. During *C. neoformans* and *C. gattii* infections, type I IFN induction prolongs mice survival and contributes to fungal clearance in the lungs and brain mainly through induction of rapid and sustained influx of protective Ly6C^{high} monocytes and neutrophils as a result of robust Th1 and Th17 polarisation^{717–720}. Nevertheless, a different study also showed that in early phases

of infection, type I IFN can be detrimental and contribute for *C. neoformans* lung colonisation. This happens via inhibition of IL-4-mediated production of mucins – proteins that contribute for the production of mucus and consequent mucocilliary clearance of pathogens in the respiratory airway⁷²¹ – suggesting that Th2 phenotype can to some degree contribute for host protection during the early onset of pulmonary cryptococcosis⁶⁰².

As discussed previously in Chapter 4, it is challenging to define a concrete role for type I IFN in *C. albicans*-induced systemic candidiasis. Upstream induction of type I IFN during systemic candidiasis via poly(I:C) was shown to be detrimental to infection^{536,562} mainly due to IL-10 and STAT1 induction downstream of type I IFN signalling pathways. These suppress IL-1 synthesis and inflammasome activation respectively, which is essential for effective *C. albicans* clearance and survival of mice during infection, mainly by promoting infiltration of neutrophil and Ly6C⁺ monocytes to infected sites^{101,536}. Moreover, IL-10 produced as a consequence of type I IFN signalling can also polarise macrophages to an “M2-like” phenotype thereby impairing their ability to clear *Candida* and contribute for infection progression⁵⁶². Live *Candida*-driven type I IFN production appears to be mainly dependent on phagocytosis and TLR7/TLR9 and NOD2 activation^{122,184,188}, and although poly(I:C) signalling also converges in type I IFN production it does so via TLR3 or MDA5 signalling⁷²². This poly(I:C)-triggered signalling might also lead to the production of other cytokines and chemokines that would not normally be produced during fungal infection and via TLR7/TLR9/NOD2 activation that could contribute in part for the deleterious effect reported during systemic candidiasis.

Published *in vivo* studies that used *Ifnar1*-deficient mice to assess the impact of type I IFN signalling in systemic infections are also difficult to interpret. Using this mutant murine model, Majer and colleagues’ findings corroborate what was previously reported using poly(I:C). They postulated that type I IFN is detrimental for the outcome of *C. albicans* SC5314-induced systemic candidiasis due to excessive production of chemokines CCL2 and CXCL1. These drive excessive accumulation of Ly6C^{high} monocytes and neutrophils to kidney, resulting in fatal immunopathology^{536,537,562}. Indeed, although phagocytes like neutrophils and macrophages are essential for effective clearance of systemic candidiasis^{310,723}, the detrimental outcomes of systemic candidiasis are mostly driven by excessive infiltration of phagocytes, mainly neutrophils, in infected organs at late stages of infection. Excessive

phagocyte recruitment will exacerbate local inflammation and greatly contribute for organ dysfunction and septic shock^{305,311,315}. This was best evidenced in neutropenic patients with candidaemia undergoing neutrophil reconstitution, where corticosteroid therapy is frequently prescribed to avoid exacerbated inflammation^{305,315}. Some *in vivo* studies are also in line with this as they showed that excessive Th17-mediated neutrophil chemotaxis increased susceptibility towards the infection^{284,285}. Nevertheless, Biondo *et al.* obtained opposite results as they reported that type I IFN signalling protects mice against *C. albicans* ATCC 90028 infection by boosting the candidacidal activity of DCs towards infection, although this protective mechanism was not thoroughly explored¹²². Based on the data from Majer and Biondo^{122,537}, it would be tempting to speculate that type I IFN exerts different effects during systemic infections that could ultimately be associated with protection or susceptibility depending on the *C. albicans* strain driving the disease. However, del Fresno and colleagues confirmed this was not the case, as the authors used the same *C. albicans* SC5314 strain and dose as Majer⁵³⁷, but showed the opposite results. They found that type I IFN contributed to resistance to infection in WT compared to *Ifnar1*^{-/-} mice by bolstering secretion of CXCL1 and CXCL2 that resulted in enhanced recruitment of T cells, NK cells, B cells, DCs and neutrophils to infected kidneys and enhanced *C. albicans* clearance¹⁸⁹.

My *in vivo* research revealed that type I IFN administration is unlikely to be a viable treatment for *C. albicans*-induced systemic candidiasis. This was corroborated by a study that showed that administration of type I IFN prior to *C. albicans* challenge leads to increased susceptibility and kidney fungal burden⁵³⁸. Nevertheless, it is important to note that the authors injected mice with 100,000 U IFN- β , a dose 20x higher than the highest dose I have tested in my experiments and assessed kidney fungal burden 48 h after *C. albicans* inoculation. Although this was not thoroughly assessed, it is possible that such high doses had a detrimental effect on mice survival due to enhanced immunopathology, as was previously described with type I IFN signalling activation^{536,537,562}. However, my research showed that mice that survived the seven-day course of infection when injected with the highest dose of IFN- β (5,000 U) displayed lower kidney fungal burden compared with mice in other groups, suggesting that despite the possible exacerbated inflammation caused by type I IFN signalling activation, IFN- β contributed to some extent for *C. albicans* clearance in the kidneys. It is possible that this effect was caused due to an augmented macrophage candidacidal activity

driven by IFN- β as I have shown in BMDMs *in vitro* (Chapter 4) and possibly through activation of a protective systemic pathway that bolstered the ability of kidney neutrophils to clear *C. albicans*³¹⁴.

Taking into consideration my research and current literature, IFN- β administration *per se* might not be a viable treatment for systemic candidiasis. IFN- β is highly pleiotropic as besides being produced by most human cell types, it exerts multiple different effects in a broad spectrum of cell types⁷²⁴. Although my results have shown that type I IFN increases macrophage candidacidal ability towards *C. albicans* which could contribute for its clearance *in vivo*, it is likely that type I IFN signalling activation in other cell types during systemic candidiasis can trigger detrimental pathways and lead to production of several other mediators that can culminate in damaging hyper-inflammation. Nevertheless, it is possible that when used in combination with other drugs that ameliorate excessive phagocyte recruitment to infected kidneys like pioglitazone⁵³⁷, IFN- β might contribute for enhanced clearance of *C. albicans* whilst avoiding excessive inflammation and immunopathology triggered as a consequence of excessive type I IFN signalling pathway activation. Moreover, identification and targeting of the molecular mechanisms downstream of type I IFN signalling that are responsible for this enhanced candidacidal effect might be explored as treatment for systemic candidiasis, and bypass the deleterious effects exerted by IFN- β *in vivo*.

6.3 IL-15 as a possible treatment for systemic candidiasis

IL-15 exerts similar functions with IL-2 due to its structural similarities as well as sharing one of its receptor subunits for signalling (IL-2R β), however it is able to target a wider range of cells and tissues and also has distinct immunomodulatory properties^{725,726}. IL-15 binds to either soluble or membranal IL-15R α to trigger high affinity *trans* signalling in neighbouring cells expressing IL-15 receptor complex (IL-15/IL-2R β and common γ chain (γ_c)), which activates STAT3-, STAT5- and NF- κ B-mediated gene expression^{727,728}. During systemic infections, IL-15 is mainly produced by monocytes, macrophages and DCs in response to several stimuli, including type I IFN^{729,730}. IL-15 acts mostly on lymphocytes and helps in eliminating invading pathogens by contributing to the development, differentiation and activation of NK cells that then produce IFN- γ , TNF and GM-CSF^{727,731}. IL-15 can also stimulate

the development of the adaptive immune response by acting as a T cell chemoattractant, promoter of CD4⁺ and CD8⁺ T cells proliferation and by favouring Th1 proliferation⁷²⁷.

Like IFN- β , IL-15 has also been implicated in multiple disease settings. IL-15 can be considered a dangerous inflammatory cytokine as it can exacerbate T cell-driven auto-immune diseases due to its ability to facilitate survival of CD8⁺ T cells and impair immune-tolerance⁷³². Rheumatoid arthritis (RA) is a complex heterogenous auto-immune disease characterised, amongst other phenomena, by excessive infiltration of lymphocytes in the synovial tissue and aberrant cytokine regulation resulting in secretion of abnormally high levels of pro-inflammatory mediators in the joints⁷³³. Multiple reports suggest that IL-15 contributes to the RA pathology. Cells isolated from inflamed synovial tissue strongly express IL-15⁷³⁴, which stimulates CD4⁺ and CD8⁺ T cell migration to the inflamed tissue and can then activate T cells which will in turn stimulate TNF release from macrophages, thereby exacerbating local inflammation and contributing to the pathogenesis of RA^{735,736}. IL-15 has also been reported to play a pathogenic role in lymphocytic cancers such as adult T-cell leukaemia and cutaneous T cell lymphoma, by facilitating aberrant T cell proliferation and contributing to tumour expansion^{615,737}.

IL-15 has also been implicated in multiple microbial infections. In most bacterial infections, IL-15 was shown to play a protective role mainly through a synergetic effect with IL-12 that activates NK cells, $\gamma\delta$ T cells and NKT cells to produce IFN- γ and subsequent induction of Th1 proliferation⁷³⁸. During *E. coli* infections, IL-15 production by macrophages is thought to contribute for protective proliferation of $\gamma\delta$ T cells and antibody production that is critical for bacterial clearance^{739,740}. Macrophages and DCs were shown to produce high levels of IL-15 during *Salmonella* infection that along with IL-12 and IL-18 contribute for NK cell, T cell and $\gamma\delta$ T cell proliferation and protective IFN- γ production⁷³⁸.

Compared with bacterial infections, publications on the impact of IL-15 in fungal infections are scarce, and its role in these diseases is still greatly understudied. *C. neoformans* triggers IL-15 production from monocytes, which exerts potent anticryptococcal activity by stimulating proliferation of CD8⁺ T cells and by upregulating granulysin expression⁷⁴¹. IL-15 contributes to the ability of granulocytes to damage *A. fumigatus* by increasing ROS

production and bolsters IL-8 production which can recruit neutrophils to infected sites and contribute for *A. fumigatus* clearance during infection⁷⁴². In *in vivo* models of *Aspergillus*-induced lung allergy, IL-15 was shown to exert beneficial effects by inhibiting Th2 proliferation in the lungs and consequent production of IL-4 which is implicated in the pathogenesis of airway inflammation, and by stimulating IL-10-producing Treg expansion that will ameliorate lung inflammation and airway obstruction⁷⁴³. Conversely, during *Aspergillus*-induced eosinophilic esophagitis (an oesophagus inflammation triggered by exacerbated recruitment of eosinophils), IL-15 has a pathological effect as neutralisation of IL-15 reduces eosinophilia and contributes to disease resolution. This possibly happens via impairment of deleterious IL-15-driven Th2 polarisation, which prevents eosinophil apoptosis and production of the eosinophil chemoattractant eotaxin⁷⁴⁴. Regarding *C. albicans* infections, IL-15 was shown to increase granulocytes and monocytes candidacidal activity⁷⁴⁵. Recently, IL-15 was shown to play a protective role during *C. albicans* systemic infection. IL-15 produced as a consequence of type I IFN signalling activation in inflammatory monocytes stimulates GM-CSF production by NK cells which in turn will enhance kidney neutrophils candidacidal activity and contribute for fungal clearance and disease resolution³¹⁴.

Because of its ability to strongly induce NK cell and T cell proliferation and activation, IL-15 has been proposed as a therapeutic approach to some cancers due to its antitumor activity⁷⁴⁶. Furthermore, IL-15 therapy was also considered for AIDS treatment due to its ability to promote expansion and migration of virus-specific MHC-expressing CD8⁺ T cells to lymph nodes where HIV-infected cells are persistent, and by stimulating NK cell expression of granzyme B and perforin which primes them to lyse HIV-infected cells^{617,747}.

My initial *in vivo* experiment using a high dose of hetIL-15, resulted in increased susceptibility of mice towards *C. albicans* systemic infection. This was possible due to an excessive dosage of hetIL-15 administrated, as hetIL-15 is frequently referred to a “superagonist” due to its ability to increase IL-15-mediated signalling activation by 50-fold compared with the monomeric version of the cytokine⁷⁴⁸. Indeed, previous reports shown that hetIL-15 administration in mice at doses as high as 3 µg for 4-day course experiments was well tolerated and did not affect mouse survival⁵⁵⁶, however this dose was never tested in the context of systemic candidiasis. The detrimental effect observed with hetIL-15 in *C.*

albicans-infected mice could possibly have happened not only due to overactivation and recruitment of NK cells and CD8⁺ T cells, but also due to excessive inflammatory cell recruitment as IL-15 was also shown to bolster IL-8 and CCL2 production that can attract neutrophils and monocytes respectively to infected organs and contribute for detrimental hyper-inflammation^{731,749}. Moreover, IL-15 can also inhibit neutrophil apoptosis and promote neutrophilic function⁷³¹, and protect CD4⁺ and CD8⁺ T cells from the suppressive effect of Tregs that could result in augmented pro-inflammatory immune responses and potentially exert a deleterious effect during systemic candidiasis⁷⁵⁰. Using a lower dose of hetIL-15, a robust expansion and activation of NK cells and CD8⁺ T cells was observed. An enhanced production of IFN- γ by CD8⁺ T cells and granzyme B by both CD8⁺ T cells and NK cells was also detected, however, it did not translate in effective *C. albicans* clearance in the systemic model. The increased levels of IFN- γ could have activated phagocytes to kill *Candida*, however it is possible that the higher levels of granzyme B produced by NK cells and CD8⁺ T cells contributed for the apoptosis of other *Candida*-containing phagocytes, therefore hindering its clearance in main target organs of infection. Although this was not assessed, it could explain why no significant differences in fungal burden were observed between vehicle- and hetIL-15-injected mice. Moreover, in my pilot experiment, no significant levels of GM-CSF produced by NK cells were detected, unlike what was previously published³¹⁴. Reduced GM-CSF could result in reduced kidney neutrophil activation and consequently impaired ability to clear *C. albicans*. Besides, it is important to note that even the lower dose tested induced a marked expansion of mice spleens that could potentially have a pathological effect in long term infections. Taking these results into account it is not easy to define if hetIL-15 administration can exert a protective role in systemic candidiasis. Nevertheless, only two pilot *in vivo* experiments were conducted here using hetIL-15, and its therapeutic potential can be more thoroughly assessed by repeating these experiments, and by conducting longer experiments with different lower doses of hetIL-15.

6.4 The role of IL-27 in systemic candidiasis

As previously mentioned in Chapter 5, IL-27 plays an important role mainly as a crosstalk regulator between innate and adaptive immune responses. IL-27 signalling induces JAK-STAT, mTOR, ERK and MAPK pathways and multiple studies have shown its ability to regulate Th1,

Th2, Th17 and Treg responses^{620,621,754–757,622,623,626,627,629,751–753}. Due to its main ability to modulate Th responses, IL-27 has been implicated in multiple disease settings.

Atherosclerosis is a chronic vascular disease characterised by the accumulation of low-density lipoproteins in the arterial walls leading to the formation of plaques that block circulation. Cytokines secreted by Infiltrating leukocytes in the endothelium contribute to plaque formation and disease progression⁷⁵⁸. IL-27 was shown to exert a protective effect in atherosclerosis mainly by contributing to recruitment of IFN- γ -producing Th cells, macrophages and DCs which will take up modified low-density lipoprotein and impair plaque formation^{759,760}. IL-27 was also implicated in psoriasis, a cutaneous auto-immune disorder characterised by hyperproliferation of epidermal cells and high degree of epidermis inflammation. Psoriasis is caused partially by a marked infiltration of T cells, neutrophils and macrophages in the epidermis which are thought to be mainly mediated by detrimental Th1 and Th17 overactivation in the skin⁷⁶¹. Skin cells isolated from psoriatic lesion produce abnormally high levels of IL-27^{761,762} that primes keratinocytes to upregulate CXCL9, -10 and -11 expression which then contributes to Th1 cells infiltration and for the progression of skin inflammation^{761,763}. Indeed, IL-27 and IFN- γ positively regulate each other's expression as IL-27 derived from APCs induces IFN- γ production by T cells and in turn IFN- γ -mediated signalling activation in APCs will boost IL-27 production. This positive feedback is thought to exacerbate the epidermal inflammation characteristic of psoriasis^{624,752,753,764,765}. This IL-27/IFN- γ synergy is thought to constitute a host regulatory mechanism to prevent Th17 proliferation and consequent granulocyte recruitment^{766–769}. Besides its detrimental effects, IL-27 was also shown to exert protective anti-inflammatory effects as it inhibits TNF-induced production of IL-1 α and CCL20 from keratinocytes⁷⁷⁰. TNF was shown to contribute to psoriasis pathogenesis mainly by contributing to detrimental Th17 proliferation⁷⁷⁰, and by inducing CCL20 production, a Th17 chemoattractant^{771,772}, therefore IL-27 can also to some extent prevent the progression of psoriasis by impairing CCL20-mediated recruitment of Th17 cells into psoriatic lesions. As *in vivo* models suggest that Th17 proliferation is a determinant of joint inflammation and RA pathogenicity^{773,774}, IL-27 as a negative regulator of Th17 seems to play a protective role in RA pathology. *In vivo* models and human genetic studies evidenced that patients with IL-27 polymorphisms are frequently at higher risk of developing RA^{775,776}.

IL-27 has also been implicated in some infectious diseases. During *M. tuberculosis* infection, IL-27-signalling was shown to exert a detrimental effect in tuberculosis by contributing to bacterial colonisation and reduced production of TNF and IL-12p40 which dampen Th1 activation and consequent macrophage effector functions⁷⁵⁶. Similarly, during *Toxoplasma gondii* infections, decreased Th1 polarisation driven by IL-27 signalling protects the host against lethal hyper-inflammation and T cell-driven pathology^{755,777}. In *Clostridium difficile*-induced colitis, IL-27 was shown to exert a protective effect by downregulating IL-17 and IL-23 production, and upregulating IL-10 and IFN- γ in the gut which ameliorates excessive tissue inflammation and ultimately reduces mortality in murine models of infection⁷⁷⁸. The impact of IL-27 treatment in human myeloid cells infected with *Salmonella typhimurium* was also recently assessed. IL-27 stimulation was shown to significantly enhance IL-12p40, IL-6 and TNF production by monocytes and macrophages which is thought to contribute for induction of protective adaptive immune responses and bacterial clearance⁷⁷⁹.

The role of IL-27 during some parasitic infections appears complex and dependent on the infectious agent. IL-27 was shown to promote resistance against *Trypanosoma cruzi* and *Leishmania major* infections^{757,780}. During *T. cruzi* infections, IL-27 signalling is associated with dampened Th2 responses and enhanced Th1 proliferation, whereas during *L. major* infection, IL-27 not only inhibits Th2 responses but also Th1^{757,780}. Conversely, during *Leishmania infantum*-induced visceral leishmaniasis, IL-27 was shown to have a detrimental effect by impairing protective Th1 proliferation and suppressing IL-17-mediated neutrophil infiltration in the gut, favouring parasite colonisation^{781,782}. Similar results were obtained in *Leishmania donovani* infections, where IL-27 was shown to impair infection resolution by inhibiting protective Th1 responses. However, in this setting, IL-27 was shown to play a critical role in ameliorating detrimental liver inflammation during the acute phase of visceral leishmaniasis and to protect the host against severe liver immunopathology⁷⁸³. IL-27 was also shown to confer protection against malaria. Patients severely infected with malaria have low IL-27 levels in circulation and display elevated pro-inflammatory responses⁷⁸⁴. *In vivo* models of malaria have shown that IL-27 protects against infection by promoting IL-10 production and by limiting excessive T cell responses thereby ameliorating immunopathology^{785,786}.

IL-27 is still greatly understudied in the context of fungal infections. *A. fumigatus* was shown to induce expression of low levels of IL-27 in later stages of DC infection compared with other Th1-polarising cytokines such as IL-12 and IL-23, suggesting that IL-27 is not the main driver of protective Th1 responses against invasive aspergillosis⁷⁸⁷. However, a later study revealed that IFN- β -treated DCs produce IL-27 during *A. fumigatus* challenge⁷¹⁶, however its protective role has yet to be assessed. Conversely, IL-27 was shown to exert a detrimental effect during *C. neoformans* infection by contributing to Th2 proliferation which promotes lung fungal colonisation and shortens host survival⁷⁸⁸. The impact of IL-27 on *Candida* infections was only recently studied by our group. As mentioned previously in Chapter 5, our group has found that IL-27 production in BMDMs is triggered by a select number of *Candida* spp. with *C. parapsilosis* inducing the most robust secretion¹⁸⁴. *C. albicans* does not induce the production of IL-27 in BMDMs and interestingly, our group has discovered that *C. albicans* is able to actively block IL-27 via a soluble mediator-triggered mechanism¹⁸⁴ which I attempted to elucidate in Chapter 5. To complement these *in vitro* findings, using *Il27ra*-deficient mice our group has also discovered that IL-27 plays a detrimental role during *C. parapsilosis*-induced candidaemia by impairing protective IFN- γ and IL-17 T cell responses which hindered fungal clearance¹⁸⁴.

In contrast to *C. parapsilosis*, *Il27ra*-deficient mouse susceptibility to *C. albicans*-induced candidaemia was comparable to WT mice¹⁸⁴. *C. albicans* does not induce IL-27 production in macrophages and IL-27 production in the course of *C. albicans*-induced systemic disease might be minimal, if detectable at all. Whilst the *Il27ra*-deficient mouse model showed that abrogation of IL-27-mediated signalling does not significantly affect the outcome of *C. albicans*-induced systemic candidiasis¹⁸⁴, it does not permit a thorough determination of its therapeutic potential in the context of *C. albicans*-driven systemic infections. *C. albicans* was shown to induce stronger Th1 and Th17 polarisation in PBMCs and *in vivo* compared with *C. parapsilosis*^{154,682}. The poor outcomes of systemic candidiasis induced by *C. albicans* are mainly driven by exacerbated inflammation induced by excessive neutrophil activation and recruitment into infected organs in later stages of infection^{310,311,313,316-319}, and IL-17 production by kidney cells as a result of Th17 polarisation can greatly contribute to this phenomenon^{284,285,317,318}. Moreover, although a Th1 response was shown to be essential for effective *C. albicans* clearance during systemic candidiasis^{266,267},

the elevated levels of IFN- γ secreted by Th1 cells might over activate phagocytes in the kidneys of infected mice and contribute, to some extent, to detrimental hyper-inflammation. For example, besides activating local phagocytes, IFN- γ triggers neutrophil NETosis⁷⁸⁹ and the consequent release of copious levels of pro-inflammatory immune mediators that once in the kidney milieu might significantly contribute to tissue immunopathology.

Taking the previous information into account, and assuming that IL-27 signalling activation during *C. albicans* systemic infection will exert similar effects on host immunity as was observed with *C. parapsilosis*¹⁸⁴, it is possible that the *C. albicans*-mediated inhibition of IL-27 production observed in macrophages might contribute in part for the detrimental immunopathology observed during systemic infection. Moreover, although this was not explored in this thesis, and none of the data presented here truly corroborate the following hypothesis, it is possible that IL-27 administration in later stages of *C. albicans*-triggered systemic candidiasis might exert a protective effect by dampening excessive Th1 and Th17 activation during infection. This could consequently contribute for fungal clearance and host survival whilst ameliorating deleterious hyper-inflammation. Nevertheless, further experiments are required to test this hypothesis and to fully elucidate the mechanism of IL-27 blockade explored in this thesis. Once properly addressed, these would allow for a more accurate assessment of the potential of IL-27 to be used as treatment against *C. albicans*-induced candidaemia.

6.5 Conclusion

Understanding the distinct host immune responses triggered by different *Candida* spp. during systemic candidiasis can provide novel insight to novel molecular pathways associated with host resistance and accelerate the discovery of novel immunotherapies for the treatment of life-threatening systemic candidiasis. Nonetheless, this strategy is still greatly underexplored as most studies revolving around *Candida* pathogenicity and host response triggered during candidiasis tend to focus on *C. albicans* as a standard *Candida* pathogen model, whilst neglecting how other *Candida* spp. with lower incidence and virulence affect host immunity dynamics.

This thesis describes the first comparative transcriptomic analysis of the macrophage immune response towards *C. albicans* and *C. parapsilosis*, two different clinically relevant *Candida* spp. with distinct virulence attributes. Besides revealing that *C. albicans* and *C. parapsilosis* induce different transcriptomic responses in macrophages, results displayed here also show that the macrophage immune response triggered by *C. glabrata*, shares high degree of similarity between the immune responses induced by *C. albicans* and *C. parapsilosis*. Whilst the *C. albicans*-induced immune response appears to be exclusively dominated by anti-inflammatory IL-10-triggered signalling and candidalysin-driven cytokine production, the immune response induced in macrophages by the low virulence species *C. parapsilosis* is dominated by a type I IFN signalling signature.

Modulation of the macrophage immune response towards *C. albicans* by activating the type I IFN signalling pathway increased the ability of macrophages to kill *C. albicans* although the candidacidal mechanism responsible for this is yet to be fully elucidated. *In vivo* experiments, however revealed that administration of both type IFN- β and hetIL-15, also triggered by *C. parapsilosis* in macrophages, during *C. albicans in vivo* infections do not have a direct beneficial effect on systemic candidiasis, but these merit further investigation.

The previously discovered ability of *C. albicans* to block IL-27, a type I IFN-regulated cytokine, in macrophages was also explored here. My research revealed that *C. albicans* is able to block IL-27 via a soluble mediator that is secreted during hyphal morphological transition. A similar IL-27 blockade was also verified with β -glucan particles in a Dectin-1-dependant mechanism, however whether β -glucan is the *C. albicans*-derived molecule responsible for the fungal-mediated inhibition has not been elucidated. Furthermore, I also showed that *C. albicans* can inhibit IFN- β production induced by *C. parapsilosis* in macrophages in a similar fashion to that observed with IL-27, which could indicate a connection between the two cytokines. If this is true, the reduced levels of type I IFN-inducible IL-27 detected in macrophage stimulations using *C. albicans* conditioned media, might be a consequence of the upstream blockade of IFN- β , however this hypothesis still needs further validation.

Overall my research provided new insight into the host immune response triggered by different *Candida* pathogens and explored the therapeutic potential of type I IFN signalling pathway in the treatment of *C. albicans*-induced systemic candidiasis. Nevertheless, this thesis also left some questions that need to be addressed: Why does *C. parapsilosis* induce a robust type I IFN response during infection but not *C. albicans*? How does IFN- β boost macrophage candidacidal activity towards *C. albicans*? Can this be explored during systemic infections to enhance *C. albicans* clearance *in vivo*? What are the molecular mechanisms driving blockade of IFN- β and IL-27 mediated by *C. albicans*? Does *C. albicans* inhibit IFN- β and IL-27 synthesis during infection as an immune evasion strategy?

Furthermore, as the main goal of the transcriptomic analyses performed here was to identify *C. parapsilosis*-specific pathways exclusively activated during macrophage stimulations to further assess the candidacidal potential of their activation during *C. albicans* infections, the *C. albicans*- and *C. glabrata*-specific immune responses were not thoroughly explored here. Nonetheless, it would be interesting to validate the main immune pathways triggered by *C. albicans* and *C. glabrata* and to perform a more detailed and comprehensive analysis about how the immune responses differ between those three pathogens.

Answering the previous questions and performing a thorough comprehensive analysis of the macrophage immune response induced by *C. albicans* and *C. glabrata* could potentially pave the way towards a better understanding of host immunity dynamics during systemic *Candida* infections. This in turn could ultimately help devising new therapeutic approaches to improve patient recovery during life-threatening systemic candidiasis and reduce the unacceptably high mortality rates associated with this disease.

Chapter 7

Appendix

Reagent	Reference	Obtained from
agar	A1296	Sigma
amphotericin B	15290-018	Life Technologies
brefeldin A	420601	Biolegend
chitin (shrimp shell)	C7170	Sigma
chloramphenicol	C0378	Sigma
curdian particles	-	Phil Taylor (Cardiff University, UK)
D-(+)-glucose	G8270	Sigma
FBS	11573397	Fisher
Griess reagent (modified)	G4410	Sigma
HEPES	15630-056	Life Technologies
hHB-EGF	100-47	Peptotech
horse serum	16050122	Life Technologies
ionomycin	I0634	Sigma
L-glutamine	25030024	Life Technologies
lidocaine	L5647	Sigma
luminol	123072	Sigma
mannan (<i>S. cerevisiae</i>)	M7504	Sigma
mannose	1375182	Sigma
mCCL6	250-06	Peptotech
mG-CSF	250-05	Peptotech
mIFN- β	12410	Biotechne
mIL-15/IL-15R α (hetIL-15)	-	Cristina Bergamaschi (NCI-Frederick, USA)
mIL-36 α	-	Martin Stacey (University of Leeds, UK)
mIL-36RA	-	Martin Stacey (University of Leeds, UK)
mM-CSF	315-02	Peptotech
mPIGF-2	465-PL-010/CF	R&D Systems
mTNF	315-01A	Peptotech
mTNFSF18	2177-GL-025/CF	R&D Systems
N-acetyl-D-glucosamine	A3286	Sigma
PenStrep	15140122	Life Technologies
peptone	91249	Sigma
PMA	P8139	Sigma
poly(I:C)	tlrl-pic	Invivogen
rabbit serum	R9759	Sigma
TRIzol	15596018	Life Technologies
yeast extract	Y1625	Sigma
zymosan	Z 4250	Sigma

Figure 7.1 – Reagents used in this thesis

Medium	Constituent	Quantity/concentration	Company
YPDB (for yeast propagation)	D-(+)-Glucose	20 g/L	Sigma
	Peptone	20 g/L	Sigma
	Yeast Extract	10 g/L	Sigma
YPDA (for yeast propagation)	(same as YPDB)		
	Agar	15 g/L	VWR
YPDA-chloramphenicol (for in vivo CFUs)	(same as YPDA)		
	Chloramphenicol	50 µg/mL	Sigma
DMEM-BMDM (for BMDM differentiation)	DMEM	1X	Gibco
	Feat bovine serum (HI)	10%	Gibco
	Horse serum (HI)	5%	Gibco
	L-glutamine	1% (2 mM)	Gibco
	PenStrep (penicillin-streptomycin)	1% (100 U/mL – 100 µg/mL)	Gibco
	HEPES	1% (10 mM)	Gibco
	M-CSF	10 ng/mL	Peprotech
RPMI-BMDM (for infection assays)	RPMI 1640	1X	Gibco
	Feat bovine serum (HI)	10%	Gibco
	PenStrep (penicillin-streptomycin)	1% (100 U/mL – 100 µg/mL)	Gibco
DMEM-luminol (for luminol assays)	DMEM (no phenol red)	1X	Gibco
	Feat bovine serum (HI)	10%	Gibco
	PenStrep (penicillin-streptomycin)	1% (100 U/mL – 100 µg/mL)	Gibco
	L-glutamine	1% (2 mM)	Gibco
Freezing medium (for cell cryopreservation)	Feat bovine serum (HI)	1X	Gibco
	DMSO	10%	Fisher
IMDM-spleen (for splenocyte culture)	IMDM	1X	Gibco
	Feat bovine serum (HI)	10%	Gibco
	PenStrep (penicillin-streptomycin)	1% (100 U/mL – 100 µg/mL)	Gibco
	L-glutamine	1% (2 mM)	Gibco
	β-mercaptoethanol	5 mM	Fisher
FACS buffer (for flow cytometry)	PBS	1X	Gibco
	Bovine serum 2albumin	0.1%	Sigma
	Sodium azide	0.05%	Sigma
FACS block (for flow cytometry)	(same as FACS buffer)		
	Rabbit serum	5%	Gibco
	Anti-FcγR (2.4G2)	4 mg/mL	(homemade)

Figure 7.2 – Media and solutions used in this thesis

Species	Strain	Donated by
<i>C. albicans</i>	SC5413	Donna MacCallum (University of Aberdeen)
<i>C. dubliniensis</i>	CBS7988	Sascha Brunke (Hans Knöll Institute – Jena)
	Wü284	Sascha Brunke (Hans Knöll Institute – Jena)
	CD36	Sascha Brunke (Hans Knöll Institute – Jena)
<i>C. glabrata</i>	SCS74761	Donna MacCallum (University of Aberdeen)
<i>C. parapsilosis</i>	SCSB5882	Donna MacCallum (University of Aberdeen)
<i>C. tropicalis</i>	SCS74663	Donna MacCallum (University of Aberdeen)
	AM2007/0112	Donna MacCallum (University of Aberdeen)

Figure 7.3 – Different WT *Candida* strains used in this thesis

Strain ID	Isogenic strain	Notes	Donated by [Reference]
<i>ece1Δ/Δ</i>	BWP17	Ece1-deficient mutant	David Moyes (King's College, UK) [A]
<i>ece1Δ/Δ + ECE1</i>	BWP17	Revertant strain (<i>ece1</i>)	David Moyes (King's College, UK) [A]
<i>ece1Δ/Δ + ECE1_{Δ184-279}</i>	BWP17	Candidalysin (peptide 3)-deficient mutant	David Moyes (King's College, UK) [A]
<i>egf1/cph1Δ/Δ</i>	CAI4	Yeast-locked mutant	David Moyes (King's College, UK) [B]
<i>flo8Δ/Δ</i>	BWP17	Yeast-locked mutant	David Moyes (King's College, UK) [C]
<i>icl1Δ/Δ</i>	CAI4	Icl1-deficient mutant	Mike Lorenz (University of Texas, USA) [D]

Figure 7.4 – Different mutant *C. albicans* strains used in this thesis.

Strain references: A – ⁸⁵; B – ¹⁸; C – ¹⁹; D – ⁷⁹⁰

Genotype	Knocked out protein(s)	Source/generation method [Reference]
<i>Clec4e^{-/-}/Clec4n^{-/-}</i>	Mincle/Dectin-2	Generated via Crispr-Cas9 technology by insertion of LoxP and 3 stop codons on <i>Clec4n</i> (Dectin-2) in <i>Clec4e</i> (Mincle) KO mice. Mincle KO mice were obtained from JAX-MMRRC (USA) [A].
<i>Clec7a^{-/-}</i>	Dectin-1	Obtained from Phil Taylor (Cardiff University, UK) [B]. Backcrossed 11 generations to C57BL/6J background.
<i>Irg1^{-/-}</i>	IRG1	Bone marrow obtained from Daniel McVicar (NIH – Frederick, USA) [C].
<i>Nos2^{-/-}</i>	iNOS	Bone marrow obtained from Daniel McVicar (NIH – Frederick, USA) [D].

Figure 7.5 – Mutant mice bone marrow used in this thesis.

References: A – ¹⁶⁵; B – ⁶⁴²; C – ⁵⁴⁷; D – ⁷⁹¹.

Target transcript	Coded protein	Species	Reference	Company
<i>Camp</i>	Cathelicidin antimicrobial peptide	Mouse	Mm00438285_m1	Thermofisher
<i>Defb1</i>	β -defensin 1	Mouse	Mm00432803_m1	Thermofisher
<i>Defb2</i>	β -defensin 2	Mouse	Mm00657074_m1	Thermofisher
<i>Hprt</i>	HPRT	Mouse	Mm03024075_m1	Thermofisher
<i>Ifna4</i>	IFN- α isoform 4	Mouse	Mm00833969_s1	Thermofisher
<i>Ifnb1</i>	IFN- β	Mouse	Mm00439552_s1	Thermofisher
<i>Irg1</i>	IRG1	Mouse	Mm01224532_m1	Thermofisher
<i>Ltf</i>	Lactotransferrin	Mouse	Mm00434787_m1	Thermofisher
<i>Nos2</i>	iNOS	Mouse	Mm00440502_m1	Thermofisher
<i>Tslp</i>	Thymic stromal lymphopoietin	Mouse	Mm01157588_m1	Thermofisher

Figure 7.6 – RT-qPCR probes used in this thesis

Target protein	Target species	Reference	Company
IFN- β	Mouse	42410-1	PBL
IL-12p40	Mouse	88-7120-77	eBiosciences
IL-27	Mouse	88-7274-88	eBiosciences
IL-6	Mouse	88-7064-77	eBiosciences

Figure 7.7 – ELISA kits used in this thesis

Antibody target	Fluorophore	Isotype control	Working concentration ($\mu\text{g}/\text{mL}$)	Clone	Company
B220	FITC	Rat IgG2a	5	RA3-6B2	Biolegend
CD11c	PE/Cy7	Hamster IgG	2	N418	Biolegend
CD3e	Pacific Blue	Hamster IgG	5	145-2C11	Biolegend
CD4	PE/Cy7	Rat IgG2a	2	RM4-5	Biolegend
CD4	PerCP/Cy5.5	Rat IgG2a	2	RM4-5	Biolegend
CD8a	APC/Cy7	Rat IgG2a	1	YTS156.7.7	Biolegend
CD8a	PE	Rat IgG2a	1	53-6.7	Biolegend
FcyR	-	-	4000	2.4G2	(homemade)
GM-CSF	PerCP/Cy5.5	Rat IgG2a	5	MP1-22E9	Biolegend
Granzyme B	PE	Mouse IgG1	10	QA16A02	Biolegend
IFN- γ	FITC	Rat IgG1	5	XMG1.2	Biolegend
NK1.1	APC	Mouse IgG2a	2	PK136	Biolegend

Figure 7.8– Antibodies used in this thesis

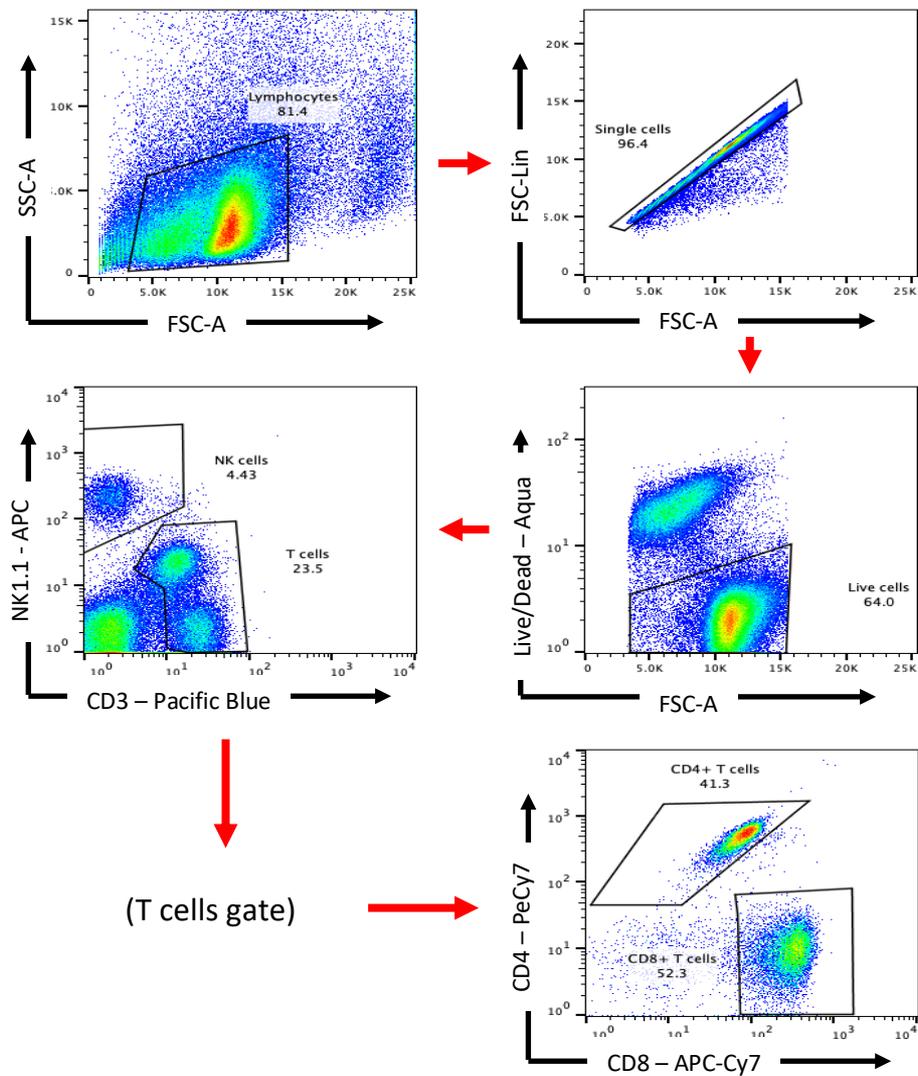


Figure 7.10 – Flow cytometry gating strategy to evaluate T responses in spleens of *C. albicans*-challenged mice. Lymphocytes were gated based on FSC and SSC, doublets were excluded by gating on single cells and viable single cells were discriminated using Live/Dead Fixable Aqua stain. NK cells were then discriminated as NK1.1⁺CD3⁻ cells, and a gate was set on NK1.1⁻CD3⁺ T cell populations to then discriminate between NK1.1⁻CD3⁺CD4⁺CD8⁻ Th cells and NK1.1⁻CD3⁺CD4⁻CD8⁺ cytotoxic T cells

NK cells

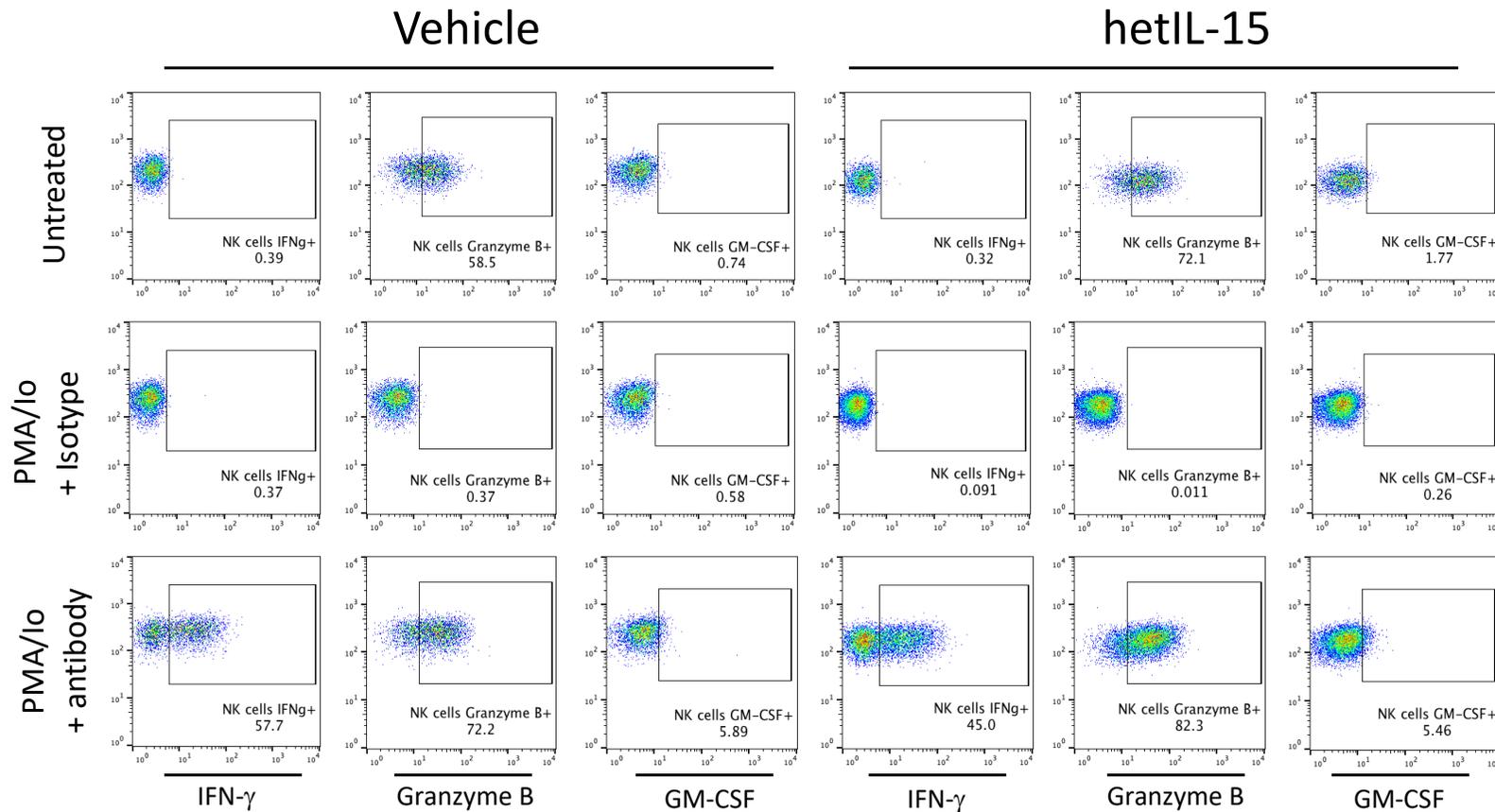


Figure 7.11 – Representative flow cytometry plots of NK cells gating strategy for cytokine detection. Mice were intravenously challenged 1.5×10^5 *C. albicans* CFUs and i.p. injected with $1 \mu\text{g}$ hetIL-15 in $100 \mu\text{L}$ 0.01% BSA in PBS. Four days later splenocytes were harvested and left unstimulated or re-stimulated with PMA/ionomycin for 4 h. NK cells producing IFN- γ , granzyme B and GM-CSF were then detected by flow cytometry. Flow plots are representative of 6 mice per group and data is representative of one independent experiment.

CD4⁺ T cells

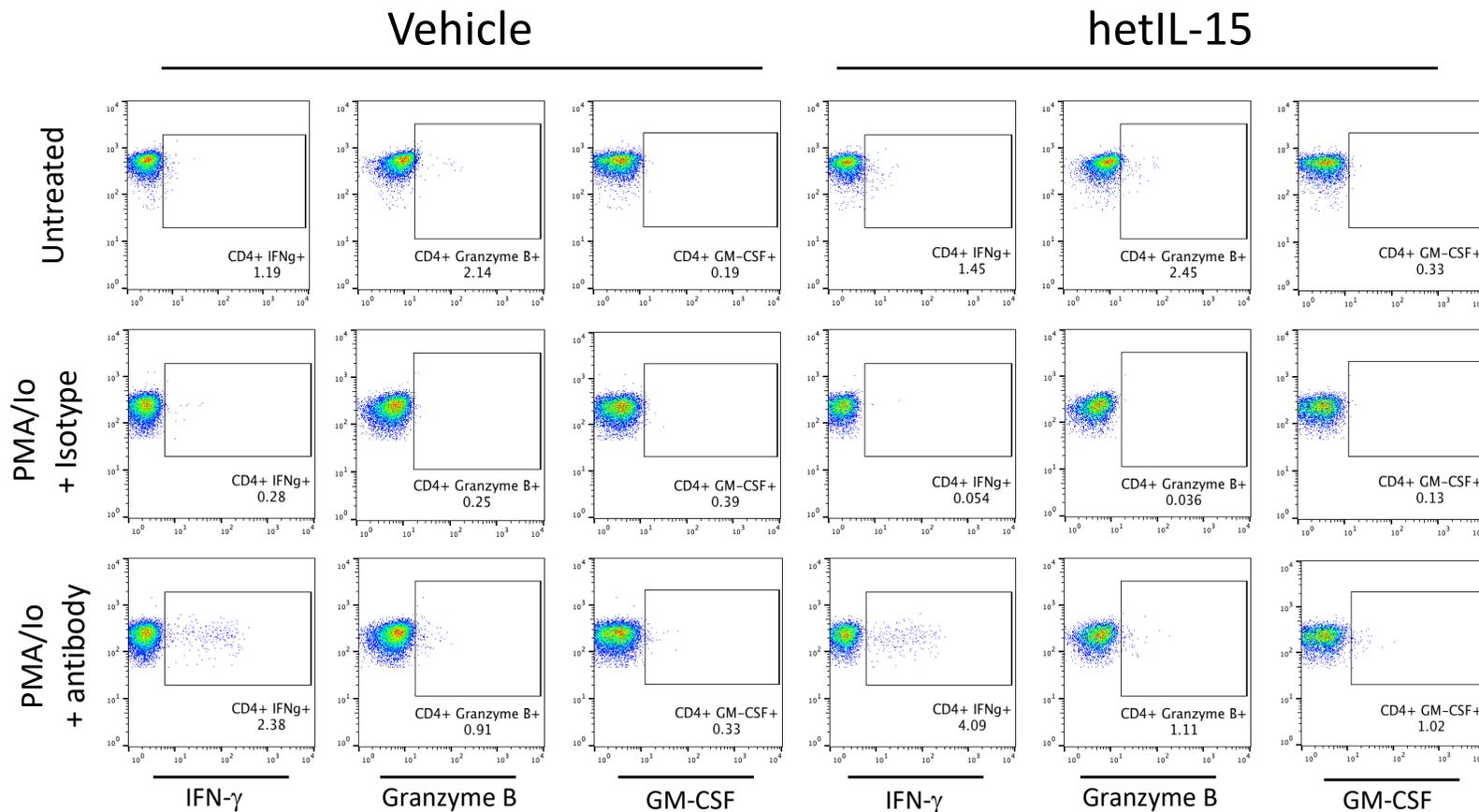


Figure 7.12 – Representative flow cytometry plots of CD4⁺ T cells gating strategy for cytokine detection. Mice were intravenously challenged 1.5×10^5 *C. albicans* CFUs and i.p. injected with $1 \mu\text{g}$ hetIL-15 in $100 \mu\text{L}$ 0.01% BSA in PBS. Four days later splenocytes were harvested and left unstimulated or re-stimulated with PMA/ionomycin for 4 h. CD4⁺ T cells producing IFN- γ , granzyme B and GM-CSF were then detected by flow cytometry. Flow plots are representative of 6 mice per group and data is representative of one independent experiment.

CD8⁺ T cells

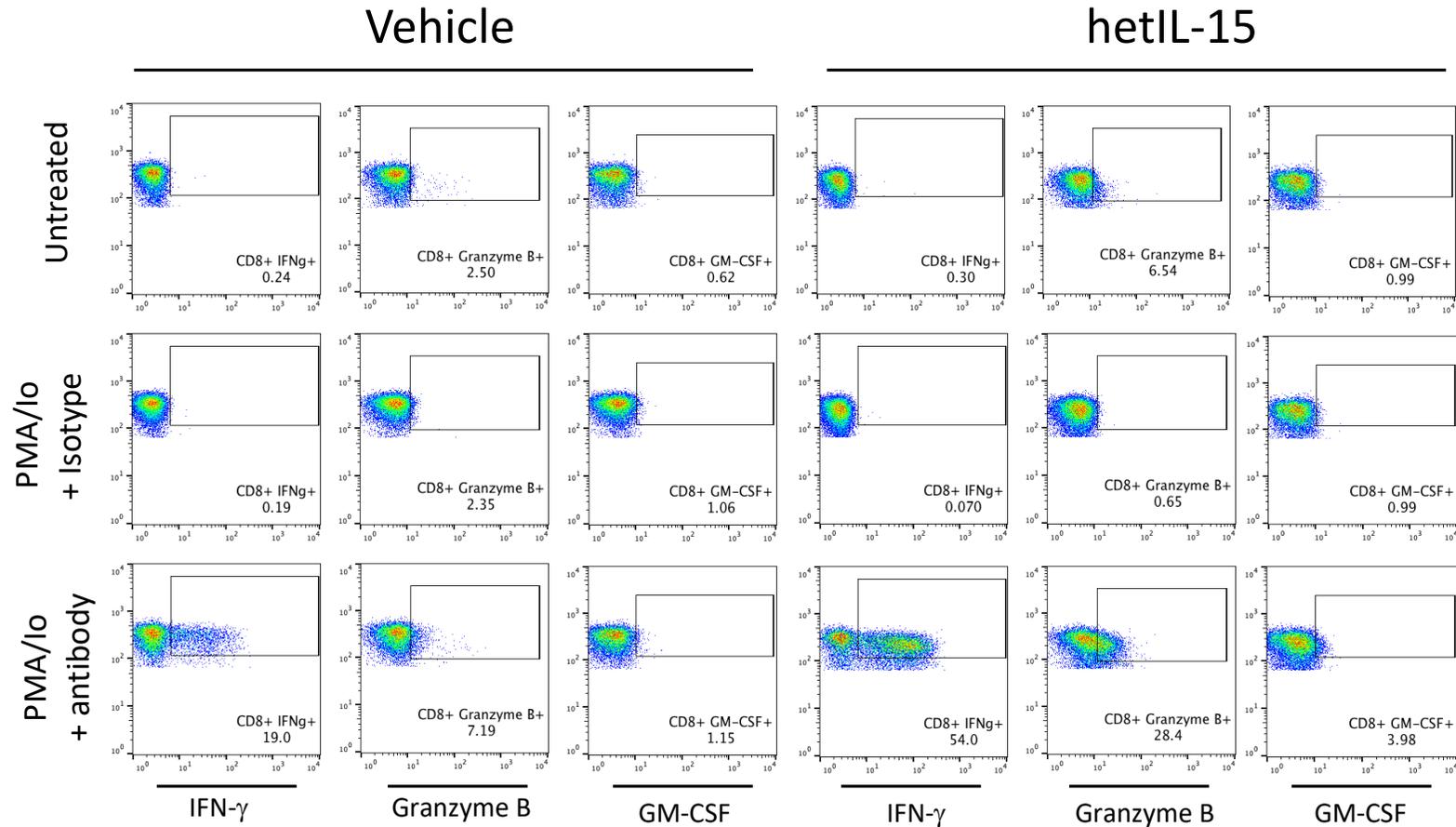


Figure 7.13 – Representative flow cytometry plots of CD8⁺ T cells gating strategy for cytokine detection. Mice were intravenously challenged 1.5×10^5 *C. albicans* CFUs and i.p. injected with $1 \mu\text{g}$ hetIL-15 in $100 \mu\text{L}$ 0.01% BSA in PBS. Four days later splenocytes were harvested and left unstimulated or re-stimulated with PMA/ionomycin for 4 h. CD8⁺ T cells producing IFN- γ , granzyme B and GM-CSF were then detected by flow cytometry. Flow plots are representative of 6 mice per group and data is representative of one independent experiment.

Chapter 8

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