



IN VITRO EVALUATION OF INNATE IMMUNITY EFFECTS ON *Candida glabrata* CELL WALL PROPERTIES AND HOST-PATHOGEN INTERACTIONS

By

SUBATRRA NAIR A/P BALAKRISHNAN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment
of the requirement for the Degree of Master of Science

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September 2024

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Human fungal infections vary from other microbial infections in many aspects, owing to their highly varied disposition and infectivity against a wide range of human cell types. *Candida* species are the leading cause of invasive candidiasis with *Candida glabrata* being the second most frequently isolated *Candida* species from intensive care unit patients. *Candida glabrata* is an opportunistic pathogen that causes a wide range of infections, often linked to antifungal resistance. Efforts to develop new antifungals have struggled to keep up with rising resistance, a major concern for clinicians. During infection, *C. glabrata* interacts with innate immune cells like macrophages, monocytes, and neutrophils, which serve as the first line of defense. It has been shown that *C. glabrata* can survive and even proliferate inside macrophages, producing relatively minimal impact on immunological response and cytokine production. This study concentrated on the *C. glabrata* cell wall, as fungal cell wall is the primary site of contact with host cells of the innate immune system. Exposure of human THP-1 macrophages impacted on *C. glabrata* cell wall biochemistry,

biophysical cell wall properties, antifungal susceptibility as well as impact on immune response and recognition. Upon exposure to THP-1 macrophages, the cell wall architecture of *C. glabrata* was modulated, demonstrated by a reduction in the β -glucan and chitin layers, along with an increase in the mannan layer. The increase in the mannan layer led to alterations in cell surface hydrophobicity, cell adhesion, and cell wall porosity of *C. glabrata*. Moreover, the increase in the mannan layer shielded the fungus from antifungal treatment, contributing to its protection and concealment. This led to a significant enhancement of antifungal resistance in *C. glabrata* upon exposure to THP-1 macrophages. In addition, the immune response and recognition upon macrophage exposure was subsequently explored using human magnetic Luminescence assay for the cytokine quantification, flow cytometry analysis for the phagocytosis assay as well as CFU counting for the killing assay. Collectively, data from both phagocytosis and killing assays demonstrated that exposure to human THP-1 macrophages made *C. glabrata* more pathogenic and harder to be killed, due to the cell wall modifications.

Keywords: antifungal resistance, *Candida glabrata*, cell wall architecture, immune evasion strategy, innate immunity

SDG: GOAL 3: Good Health and Well-being

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**PENILAIAN IN VITRO KESAN SISTEM IMUN SEMULA JADI KE ATAS
SIFAT DINDING SEL *Candida glabrata* DAN INTERAKSI ANTARA
PERUMAH DAN PATOGEN**

Oleh

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Jangkitan kulat manusia berbeza daripada jangkitan mikroorganisma lain dalam pelbagai aspek, disebabkan oleh sifat mereka yang sangat berbeza dan kebolehan untuk menjangkiti pelbagai jenis sel manusia. Spesies *Candida* merupakan punca utama kandidiasis invasif di mana *Candida glabrata* merupakan spesies *Candida* kedua yang paling kerap ditemui daripada pesakit unit rawatan rapi. *Candida glabrata* adalah patogen oportunistik manusia yang terkenal yang menyebabkan pelbagai penyakit yang melibatkan jangkitan superfisial hingga sistemik yang sering dikaitkan dengan peningkatan kegagalan rawatan. Usaha terkini untuk membangunkan antikulat baru tidak mampu mengekalkan tahap peningkatan rintangan antikulat yang pantas, yang telah menjadi kebimbangan utama di kalangan doktor. Semasa jangkitan berlaku, *C. glabrata* berinteraksi dengan sel-sel sistem imun semula jadi, seperti makrofaj, monosit, dan neutrofil, yang melibatkan reaksi terawal melawan patogen yang menyerang. Kajian membuktikan bahawa, *C. glabrata* boleh bertahan hidup dan berkembang biak di dalam makrofaj, dengan memberikan kesan minimal terhadap

reaksi imunologi dan pengeluaran sitokin. Kajian ini secara keseluruhan, memberi tumpuan kepada dinding sel *C. glabrata*, kerana dinding sel kulat adalah tempat utama pertemuan dengan sel perumah sistem imun semula jadi. Pendedahan makrofaj manusia THP-1 memberi kesan kepada sifat biokimia dinding sel *C. glabrata*, sifat sifat biofisikal dinding sel, kerentanan antikulat serta kesan terhadap respons imun dan pengelakan. Apabila terdedah kepada makrofaj THP-1, reka bina dinding sel *C. glabrata* telah dimodulasi, ditunjukkan dengan pengurangan dalam lapisan β -glukan dan kitin, bersama-sama dengan peningkatan dalam lapisan mannan. Peningkatan dalam lapisan mannan membawa kepada perubahan dalam hidrofobisiti permukaan sel, lekatan sel, dan keliangan dinding sel *C. glabrata*. Selain itu, peningkatan dalam lapisan mannan melindungi kulat daripada rawatan antikulat, menyumbang kepada perlindungan dan penyembunyian. Ini membawa kepada peningkatan ketara rintangan antikulat dalam *C. glabrata* apabila terdedah kepada makrofaj THP-1. Di samping itu, tindak balas imun dan pengiktirafan terhadap pendedahan makrofaj kemudiannya diterokai menggunakan ujian Luminex magnet manusia untuk kuantifikasi sitokin, analisis sitometri aliran untuk ujian fagositosis serta pengiraan CFU untuk ujian pembunuhan. Secara kolektif, data daripada kedua-dua fagositosis dan ujian membunuh menunjukkan bahawa pendedahan kepada makrofaj manusia THP-1 menjadikan *C. glabrata* lebih patogenik dan lebih sukar untuk dibunuh, disebabkan oleh pengubahsuaian dinding sel.

Kata Kunci: rintangan antikulat, *Candida glabrata*, dinding sel, strategi pengelakan imun, imun semula jadi

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LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
AIDS	Acquired immunodeficiency syndrome
ATCC	American Type Culture Collection
BSI	Bloodstream infection
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming units
CLSI	Clinical and Laboratory Standard Institute
CLR	C-type lectin receptors
CWPs	Cell wall proteins
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
FBS	Foetal bovine serum
GM-CFS	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
ICU	Intensive care unit
MATH	Microbial adhesion to hydrocarbon
OD	Optical density
OPC	Oropharyngeal candidiasis
PBS	Phosphate buffered saline
PMN	Polymorphonuclear neutrophils
RPMI	Roswell Park Memorial Institute

SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SD	Standard deviation
SOT	Solid organ transplantation
TEM	Transmission electron microscopy
UTI	Urinary tract infections
UV	Ultraviolet
VVC	Vulvovaginal candidiasis
WHO	World Health Organisation
YPD	Yeast-peptone-dextrose

CHAPTER 1

INTRODUCTION

1.1 Introduction

Invasive fungal infections have been on the rise since the late 20th century with species belonging to the *Candida* genus being more prevalent (43–75%) (Bitar et al., 2014). Among those species, *Candida albicans* stands out as the most commonly identified causative agent of these infections however *C. glabrata* has been frequently isolated from invasive candidiasis patients as well as linked to higher patient mortality (Boan & Gardam, 2019; Hu et al., 2019). The rising occurrence of infections involving *C. glabrata* and other non-*albicans* species is indirectly linked to advancements in various technologies and contemporary medical interventions. These factors encompass organ transplantation, the use of diverse medical devices such as urinary and vascular catheters, intrauterine devices, pacemakers, prosthetic valves, and the extensive application of broad-spectrum antibiotic treatments (Aldardeer et al., 2020; Sganga et al., 2019). In reality, invasive candidiasis caused by *C. glabrata* leads to significant morbidity and mortality rates ranging from 40–60%, possibly attributed to *C. glabrata* intrinsic low susceptibility to commonly used azoles (Timmermans et al., 2018). *Candida glabrata* can also develop resistance to other antifungal classes, resulting in poor treatment outcomes and limited options (Alastruey-Izquierdo et al., 2015).

Besides, *C. glabrata* has traditionally been recognized not only as a commensal but also an opportunistic fungus within the digestive system (Li et al., 2021). Similar to *C. albicans*, it has the potential to pose a lethal threat in immunocompromised patients

(Gabaldón & Fairhead, 2019). *Candida glabrata* is a haploid yeast that lacks the ability to transition into true hyphal growth, instead strictly maintaining its yeast form under the majority of conditions (Sassani et al., 2016). This is notably different from *C. albicans*, where the dimorphic switch is considered one of the most crucial virulence factors (Jacobsen et al., 2010a). Even without this morphological adaptability, *C. glabrata* happens to be a successful pathogen, well-suited to the human host. The yeast can attach to host cells and form biofilms, a mechanism aided by a substantial group of glycosylphosphatidylinositol-linked epithelial adhesins (Epa proteins) and potentially other adhesin-like cell-wall-anchored proteins (De Groot et al., 2008a).

Moreover, *C. glabrata* also possesses a notable intrinsic stress tolerance, allowing the fungus to endure extended periods of starvation and resist oxidative stress (Kraneveld et al., 2011). In vitro, *C. glabrata* demonstrates resilience against interactions with host immune cells, particularly macrophages (Kramer et al., 2006). A substantial portion of phagocytosed *C. glabrata* cells can survive and even proliferate within both human and murine macrophages (Cuéllar-Cruz et al., 2014). The survival of microbial pathogens within a host is contingent upon effective immune evasion strategies. Given the multifaceted nature of host defence mechanisms, particularly those involving innate immune effector cells like macrophages, various evasion strategies have evolved among bacterial and fungal pathogens (Seider et al., 2010; Smith & May, 2013). Within macrophages, *C. glabrata* has been demonstrated to disrupt the phagosomal maturation process, generation of reactive oxygen species as well as the cytokine production (Seider et al., 2011a). Not only that, *C. glabrata* also utilises strategies such as inducing autophagy, reprogramming metabolic gene transcription to thrive in the nutrient-deficient macrophage environment, and reshaping its chromatin

architecture to cope with DNA damage stress, all aimed at facilitating replication within macrophages (Kaur et al., 2007a; Roetzer et al., 2010a).

Besides, phagocytosis and the secretion of proinflammatory cytokines are initiated when the host immune system identifies the components of the fungal cell wall, particularly the β -glucan or chitin (Alves et al., 2020a). The inner skeletal layer of *C. glabrata* cell wall is made up of chitin, β -1,3-glucan, and β -1,6- glucan, which is connected to an outer layer composed of highly mannosylated proteins and phospholipomannan (Wheeler & Fink, 2006a). Here, β -1,3-glucan is crucial for immune detection, as it is recognized by the dectin-1 receptor on macrophages, being the most immunogenic component of the fungal cell wall. Disruption of the cell wall integrity and exposure of β -1,3-glucan lead to dectin-1 binding, triggering recognition and prompting macrophage activation along with the release of cytokines like TNF- α (G. D. Brown et al., 2003a). Despite significant advances in understanding *C. glabrata* pathogenicity, there remain several research gaps related to how its cell wall modifications contribute to immune evasion and antifungal resistance. These gaps hinder the development of more effective treatment strategies and a deeper understanding of its interaction with the host immune system. As fungal cell wall is the primary site of contact with host cells of the innate immune system, it is envisaged that the knowledge on how innate immune cells bring changes in *C. glabrata* cell wall compositions, and thus antifungal resistance and immune recognition, could be beneficial for a better appreciation and understanding of this complex human fungal pathogen.

1.2 Research Hypothesis

It is hypothesised that the interaction between *C. glabrata* and innate immune cells plays a crucial role in modifying its cell wall, thereby influencing its cell wall properties, antifungal susceptibility, and host-pathogen interactions.

H₀: Interaction of *C. glabrata* with innate immune cells has no significant impact on its fungal cell wall modifications, antifungal susceptibility, or host-pathogen interactions.

H₁: Interaction of *C. glabrata* with innate immune cells has a significant impact on its fungal cell wall modifications, antifungal susceptibility, and host-pathogen interactions.

1.3 Objectives

General objective:

To elucidate the impact of innate immunity on *C. glabrata* cell wall properties and host pathogen interactions.

Specific objectives:

1. To determine the impact of macrophages exposure on cell wall biochemistry and biophysical properties of *C. glabrata*.
2. To determine the impact of macrophages exposure on the antifungal susceptibility of *C. glabrata*.
3. To determine the impact of macrophages exposure on *C. glabrata* recognition and host-pathogen interactions.

CHAPTER 2

LITERATURE REVIEW

2.1 *Candida* spp.

The *Candida* genus comprises a polyphyletic collection of fungal species, including closely related organisms such as *C. albicans*, *C. tropicalis*, and *C. parapsilosis*, as well as more distantly related species like *C. glabrata* and *C. krusei* (Butler et al., 2009a). Despite their evolutionary divergence, different species within this genus have acquired the ability to thrive in various regions of the human body. They have demonstrated proficiency in evading immune detection and adjusting to host reactions, establishing themselves as essential elements of the typical microbial community (Mavor et al., 2005a). Moreover, *Candida* species frequently inhabit the gastrointestinal tract, oral and vaginal cavity in individuals with good health (Brian Mochon et al., 2010). At the macroscopic level, *Candida* spp. like *C. glabrata* exhibit round colonies in cream or white colour when grown on yeast peptone dextrose agar (YPD) or Sabouraud dextrose agar (SDA) agar at temperatures ranging from 25°C to 37 °C. The texture of these colonies varies, presenting as smooth, glistening, dry, or wrinkled and dull, depending on the species of *Candida* (Mayer et al., 2013). Moreover, *Candida* spp. have the ability to exhibit three primary cellular forms: yeast, pseudohyphae, and hyphae, with variations observed among different species. Yeasts are individual cells characterised by an oval shape, capable of displaying both axial and bipolar budding patterns (Sudbery et al., 2004a). While pseudohyphae and hyphae are commonly referred to as "filamentous" morphologies due to their characteristic growth pattern, where cells elongate in a polarised manner and are connected end to end. Pseudohyphal cells typically have an ellipsoidal shape (i.e., wider at the centre

than at the ends) with constrictions at the septal junctions. In contrast, hyphal cells are distinguished by their parallel sides, consistent width, and complete septa without constrictions. Unlike pseudohyphae, hyphal cells possess pores in their septa that facilitate communication between cells (S. Kumar et al., 2022). Although the majority of *Candida* spp. adopt yeast and pseudohyphal forms, only three *Candida* spp; *C. albicans*, *C. tropicalis*, and *C. dubliniensis* can exhibit the ability to form true hyphae (Thompson et al., 2011).

Traditionally, *Candida* species were classified as yeasts that exhibit hyphal or pseudohyphal growth patterns and do not produce sexual spores. Nevertheless, the advent of molecular DNA sequencing techniques has unveiled the vast diversity of *Candida* species within the Hemiascomycetes group, which also includes the well-known model yeast *Saccharomyces cerevisiae* (Wisplinghoff et al., 2004a). With the exclusion of *C. glabrata* and *C. krusei*, the most clinically significant *Candida* spp. group together within a singular clade. Within this clade, these species share a modified genetic code where CUG codons encode serine instead of the universally encoded leucine (Butler et al., 2009b). Within the *Candida* clade, there are two separate sub-clades: one consists of haploid species, including less common pathogens like *C. lusitaniae*, and the other includes diploid species such as *C. albicans* and *C. parapsilosis*, which are more frequently linked to pathogenic traits (Figure 2.1) (Mavor et al., 2005b).

colored on SDA, resembling those of other *Candida* species, albeit smaller in size, often appearing quite diminutive. Significantly, the cells of *C. glabrata* (1–4 µm) are notably smaller than those of *C. albicans* cells (4–6 µm) (Ciurea et al., 2020). In CHROMagar identification, *C. glabrata* display pink colour colonies. *C. glabrata* is only capable in fermenting and assimilating glucose and trehalose (Kaur et al., 2005). Lastly, when considering its genetic characteristics, *C. glabrata* stands out due to its haploid genome, differing from the diploid genome of *C. albicans* and the other non-*Candida albicans* *Candida* (NCAC) species (Rodrigues et al., 2014a).

Sequencing of the genome of *C. glabrata* (CBS138/ American Type Culture Collection, ATCC2001) unveiled its shared ancestry with *S. cerevisiae* (Dujon et al., 2004). *Candida glabrata* and *S. cerevisiae* exhibit a comparable GC content of approximately 38% and demonstrate a substantial likeness in the amino acid sequences of orthologous proteins (Dujon et al., 2004). *Candida glabrata* has undergone a greater gene loss in comparison to *S. cerevisiae*, minimizing duplication events and leading to the elimination of specific metabolic pathways. These pathways include those crucial for galactose metabolism (*GAL1*, *GAL7*, *GAL10*), phosphate metabolism (*PHO3*, *PHO5*, *PHO11*, *PHO12*), nitrogen metabolism (*DAL1*, *DAL2*), sulfur metabolism (*SAM4*), and pyridoxine biosynthesis (*SNO1*, *SNO2*, *SNO3*) (Butler et al., 2004). Nonetheless, *C. glabrata* appears to retain most of the homologous genes crucial for mating present in *S. cerevisiae*, which include pheromone and receptor genes (Muller et al., 2008).

Candida glabrata was long thought to be a comparatively non-pathogenic saprophyte of healthy individual's natural flora and was not often linked to major health problems. Nevertheless, the prevalence of mucosal and systemic infections linked to *C. glabrata*

has significantly increased due to the extensive utilisation of immunosuppressive therapies and broad-spectrum antibiotic treatments (Tortorano et al., 2006). Furthermore, research suggests that invasive candidiasis resulting from *C. glabrata* is primarily associated with patients with solid tumors and individuals undergoing solid organ transplantation (SOT) (Pfaller et al., 2014). Given that cancer patients, transplant recipients, as well as AIDS patients all have reduced T-cell functioning, it is possible that T cells help in the prevention of *C. glabrata* infections in healthy people (Desai et al., 2011a).

2.1.2 Structure of *Candida glabrata*

The dynamic fungal cell walls play a vital role in cell viability, morphogenesis, and pathogenesis. Apart from their role as the outer layer, the cell wall acts as a dynamic organelle with a composition that greatly influences the ecology of the fungus. This composition is intricately regulated in response to environmental conditions and external stresses (Lesage & Bussey, 2006a). The significance of the cell wall is highlighted by the fact that approximately one-fifth (20%) of the yeast genome is devoted to the synthesis of this essential structure (Karkowska-Kuleta et al., 2020). Many elements of the cell wall are conserved among different fungal species, while some are exclusive to particular species. The cell wall is notable for its role as a cellular component that demonstrates significant phenotypic diversity and adaptability (Coronado et al., 2007). Besides, the cell wall is organised into distinct layers, with the most inner layer serving as a more conserved structure upon which the subsequent layers are deposited. This arrangement can differ among various fungal species. The cell wall of *Candida* is crucial for its resilience across different epithelia. Its distinctive composition, featuring polysaccharides such as chitin, glucans and mannans (Figure

2.2) and hydrolytic enzymes, bolsters its ability to withstand dislodging forces of both physical and chemical nature within host niches (Jayatilake, 2011).

Within *C. glabrata*, these polysaccharides form two layers: an internal skeletal layer consisting of chitin and β -1,3-linked glucan, and an external layer of mannan and cell wall proteins attached to the skeletal layer via a glycosylphosphatidylinositol (GPI) remnant (Dünkler et al., 2005). According to a study conducted by (De Groot et al., 2008b) on the ultrastructure of *C. glabrata* cell wall using electron microscopy, it was reported that the cells of *C. glabrata* are enclosed by a wall measuring 100 nm to 200 nm in thickness. The inner section of the wall appears semi-transparent and it is enveloped by a denser layer visible under electron microscopy. The configuration of the cell wall undergoes modifications throughout the cell cycle, particularly during cytokinesis. Additionally, in *C. glabrata*, the glucan network is less dense, and the external cell wall layer is rich in mannoproteins that create pathogen-associated molecular patterns (PAMPs). These PAMPs are recognized by distinct innate immune receptors known as pathogen recognition receptors (PRRs) (Davidson et al., 2018). Indeed, the β -glucans present in the cell wall of *C. glabrata* might be better shielded from host immune detection by the receptor dectin-1 when compared to the situation in *C. albicans* (Wheeler & Fink, 2006b).

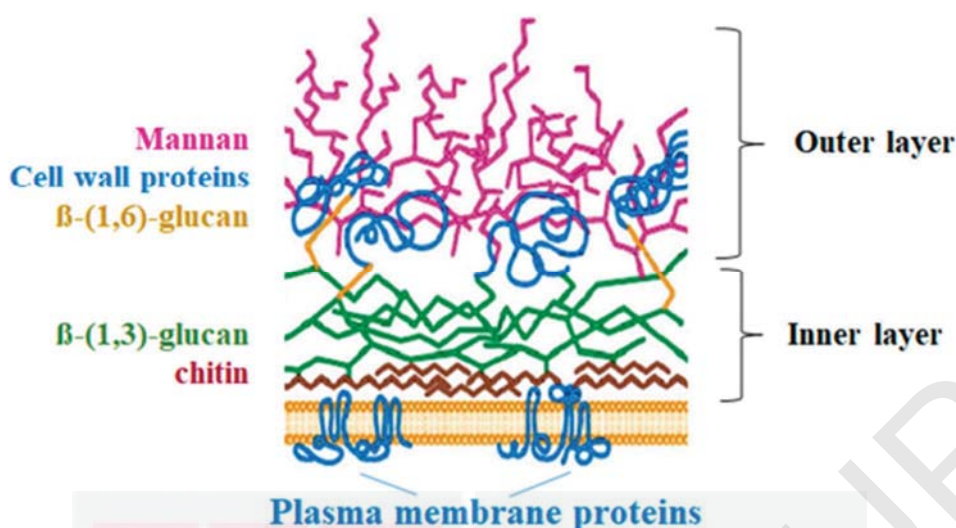


Figure 2.2: A schematic representation illustrating the composition of the primary components of the fungal cell wall in *Candida* yeasts (Jiang et al., 2022)

Due to their close phylogenetic relationship and unique features compared to other pathogenic *Candida* species, *C. glabrata* and *S. cerevisiae* showed similarities in the length and composition of side chains in their mannans. Specifically, the triose side chain mainly consisted of β -1,2-linked mannose residues at the non-reducing end (Dodgson et al., 2003). On the other hand, in the *C. albicans* serotype A mannan, the β -1,2-linked mannose residue is part of the side chains ranging from mannopentaose to mannoheptaose (Shibata et al., 2007). While there is variability in mannan structure, the core biosynthetic processes appear to be preserved. This conservation simplifies the study of *C. glabrata* glycosylation. N-glycans, a key form of mannoprotein modification, exhibit a core structure that remains uniform across eukaryotes (Hall & Gow, 2013). Glycopeptides commonly contain numerous sizable N-glycans, adding 50,000 to 100,000 Da to the mannoproteins size. The phosphorylation of the mannosyl side chains introduces a negative surface charge to yeast cells (Lesage & Bussey, 2006b). Although N-chain elongation is not essential for cell wall formation, the lack of outer chains in *MNN9* mutants increases cell wall permeability and reduces integrity (Orlean, 2012). Furthermore, N-linked protein glycosylation occurs in two phases,

starting with the assembly of the core oligosaccharide structure at the endoplasmic reticulum membrane (Aebi, 2013). The finalised core structure is a branched oligosaccharide made up of Glc3Man9GlcNAc2 units, transferred intact from its lipid anchor to asparagine residues on a growing peptide. Following this, the attached oligosaccharide is trimmed down to yield Man8GlcNAc2 (Thak et al., 2020). The following step in the N-linked glycosylation process occurs in the Golgi complex, where *OCH1* introduces a single α -1,6-linked mannose to the Man8GlcNAc2 core (Hernández-Chávez et al., 2019). Subsequently, proteins can develop a core-type structure by adding two more mannose units or a more complex α -1,6-linked backbone structure, featuring branching α -1,2- and α -1,3-mannoses (Lussier et al., 2000). The participation of *C. glabrata* in host-pathogen interactions is primarily linked to α -linked mannose residues, which interact with receptors like the mannose receptor, mannan-binding protein, Dectin-2, and the DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) (Cambi et al., 2008).

In contrast, polysaccharides such as chitin and glucan are synthesized at the plasma membrane by transmembrane enzymatic complexes. Initially transported to the plasma membrane in an inactive state through secretory vesicles, these complexes become active upon integration into the plasma membrane. This process differs from the synthesis of mannans and other glycoconjugates, which occur in the endoplasmic reticulum and Golgi (Gow et al., 2017). Prominent chitin-rich areas are noted at the septa, bud necks, and bud scars in *C. glabrata*. Additionally, the relatively low levels of alkali-insoluble glucans indicate a reduced presence of glucan-chitin cross-links, accounting for around 20% of the cell dry weight (Xu et al., 2021). In chitin synthesis, UDP-N-acetylglucosamine acts as the substrate. Chitin is composed of linear chains

of β -1,4-N-acetylglucosamine and serves as one of the oldest structural polysaccharides in fungal cell walls. Various families of chitin synthases, with molecular weights ranging from 100 to 130 kDa, have been identified to enhance the bioinformatic understanding of chitin synthesis (Rajendran et al., 2014). Moreover, chitin biosynthesis is a potential mechanism influencing virulence and resistance to antifungal treatments, as chitin plays a vital role in maintaining the structural integrity of the cell wall. Increased chitin levels restrict drug penetration through the cell wall (Perlin, 2014).

Typically, glucans are organised as triple helical polymers, playing a role in preserving the cell wall rigidity (Nakashima et al., 2018). The β -1,3-glucan is believed to establish a lattice support structure to which other molecules attach. Having an average degree of polymerization of 1,500, this corresponds to a molecular weight of 240,000 and a maximum fiber length of around 600 nm. This length is approximately three to six times the average cell wall thickness, representing roughly 1/10 of a cell circumference (Shiradhane et al., 2018). Glucans are synthesized by a glucan synthase complex bound to the plasma membrane. This complex uses UDP-glucose as a substrate to release linear β -1,3-glucan chains through the membrane into the cell wall. These chains can then be acted upon by various trans-glycosidase enzymes (Douglas, 2000). The protein complex consists of at least two proteins: (i) the presumed catalytic subunit, encoded by the gene(s) FKS/GSC, and (ii) a regulatory subunit encoded by *RHO1*, with a molecular weight of around 20 kDa. The FKS/GSC subunits are targeted by the echinocandin class of antifungal medications and the recently discovered plant metabolite poacic acid (Schuster et al., 2016). Conversely, β -1,6-glucan is believed to function as binding sites for chitin, mannan, and mannoproteins. Moreover, they might

act to connect neighboring β -1,3-glucan polymer strands. However, the precise manner of attachment to these carbohydrates has not been structurally defined, except for the suggestion that mannoproteins could be linked via a GPI anchor (Lowman et al., 2011a). Furthermore, in pathogenic fungi, there are fewer glucan synthase genes in comparison to chitin synthase genes (Mouyna et al., 2004).

2.2 *Candida glabrata* (*Nakaseomyces glabrata*) in human diseases

Candida glabrata is a significant opportunistic fungal pathogen in humans, responsible for both superficial and systemic infections, especially in immunocompromised individuals. As the second most common cause of candidemia, it is crucial to gain a comprehensive understanding of its virulence and mechanisms of antifungal resistance. In contrast to the dimorphic nature of *C. albicans*, where filamentation is a key virulence factor, *C. glabrata* has a haploid genome and lacks the ability for filamentous growth (Nace et al., 2009). Managing *C. glabrata* infections clinically poses challenges due to its inherent high resistance to various antifungal medications, particularly azoles. Prolonged and prophylactic treatments often result in the emergence of antifungal resistance, diminishing the efficacy of therapeutic approaches (Tscherner et al., 2011).

2.2.1 Candidiasis

Candidiasis is a fungal infection caused by various opportunistic yeast pathogens belonging to the *Candida* genus. It can affect individuals of all ages and commonly arises in populations with identifiable risk factors. Candidiasis encompasses a range of conditions, ranging from mild superficial infections such as vulvovaginal candidiasis (VVC), oropharyngeal candidiasis (OPC), and cutaneous candidiasis, to potentially life-threatening invasive or systemic infections like bloodstream infections (candidemia) and deep-seated candidiasis. Deep-seated candidiasis often presents as intra-abdominal abscesses, peritonitis, and osteomyelitis, which may occur with or without candidemia (Pappas et al., 2018a). Host factors such as reduced digestive secretions, dietary conditions, nutrient deficiencies, compromised immune system and liver function, medications, prolonged antibiotic usage, and changes in bowel flora are thought to significantly elevate the risk of candidiasis (Martins et al., 2014). Although over 30 *Candida* spp. are identified as capable of causing infections, only five *Candida* species, namely *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* are frequently isolated from patients with candidiasis (Lim et al., 2012). Despite the increasing prevalence of candidiasis attributed to NCAC species, the primary cause of candidiasis, irrespective of clinical setting and geographic location, remains to be *C. albicans* (Sudbery et al., 2004b). Moreover, *Candida* species are the fourth most frequent cause of nosocomial bloodstream infections (BSI), accounting for 8% to 10% of all hospital-acquired BSIs in the USA. This carries a significant economic burden and the estimated annual expenditure for managing the disease in the United States alone is in the range of USD 2-4 billion (Pfaller & Diekema, 2007a). While in Malaysia, there is a growing trend in the *Candida* spp causing infections including the

emergence of new species that were not associated with infections in the past at where in 2018, a fatal case of *C. auris* infection was reported in a patient with neutropaenia at Kampar hospital, Perak (Mohd Tap et al., 2018).

2.2.1.1 Superficial candidiasis

The most prevalent type of candidiasis is superficial candidiasis, primarily influencing the mucous membranes of the human oral cavity and the vagina, where *Candida* resides as a commensal organism. Besides, superficial candidiasis is a common and manageable condition frequently encountered in routine clinical settings, although they may act differently in immunosuppressed patients (Hayet al.,1999). Predisposing factors often elevate the susceptibility to superficial infections like OPC and cutaneous candidiasis, but this is not the case for VVC. OPC emerges as one of the initial clinical indicators of underlying human immunodeficiency virus (HIV) infection. It occurs in 50% to 95% of individuals with acquired immunodeficiency syndrome (AIDS) (Rabeneck et al., 1993). Furthermore, prior to the introduction of highly active antiretroviral therapy (HAART), 90% of AIDS patients suffered from oropharyngeal candidiasis (OPC). Even with HAART, AIDS patients still exhibit a significant rate of oral *Candida* colonization (80%), with more than 30% experiencing symptomatic OPC (M. A. Thompson et al., 2010).

VVC impacts 75% of female population at least once in their lifetime, primarily during the reproductive years (Ciurea et al., 2020). Furthermore, recurrent VVC (RVVC), characterised by more than three episodes of VVC per year, impacts nearly 8% of women worldwide (Denning et al., 2018). Unlike OPC, RVVC primarily affects immunocompetent and generally healthy women, which explains why the global

burden of VVC is considerably higher compared to other forms of candidiasis (Fidel, 2002). Rough estimates suggest that invasive candidiasis accounts for approximately 700,000 cases annually worldwide, OPC contributes to around 15.5 million infections each year, and RVVC alone is linked to an estimated 140 million cases annually (De Bernardis et al., 2018). Although VVC is not a life-threatening disease, the sheer magnitude of the disease burden leads to approximately \$1.8 billion in annual medical cost. Moreover, the economic impact, attributed to lost work hours, has been recently extrapolated to reach an additional \$1 billion per year in the United States alone (Bongomin et al., 2017). Although *C. albicans* is responsible for more than 90% of VVC cases, NCAC species have been recognised as emerging causative agents. In certain cases, the prevalence of NCAC species is notably high, surpassing 50% (Kennedy & Sobel, 2010). As for the management of uncomplicated VVC, the common over-the-counter (OTC) azoles such as clotrimazole, miconazole and tioconazole are effective (Marion K et al., 2004).

On the other hand, cutaneous candidiasis affects individuals of all ages, constituting approximately 1% of all outpatient visits and 7% of all inpatient visits to dermatological clinics (Taudorf et al., 2019). These infections are frequently preceded by local skin conditions, including heightened humidity, occlusion, compromised skin barriers, and changes in microbial flora. Furthermore, other recognised risk factors encompass immunosuppression due to medical conditions, endocrine disorders, or compromised blood flow (Rosso & Kircik, 2013). Usually, cutaneous candidiasis appears as a secondary infection on top of an existing medical condition or, in cases of widespread cutaneous candidiasis, may occasionally present as primary skin infections in infants. These infections typically respond well to topical treatments such

as azoles, terbinafine cream, or nystatin. Oral antifungal therapy is typically reserved for extensive disease. In situations like interdigital candidiasis or intertrigo, antifungal treatment may will not always be effective, but regular cleansing with an antiseptic drying agent like potassium permanganate solution can sometimes be more beneficial (Kelly, 2012).

2.2.1.2 Invasive candidiasis

Invasive candidiasis, alternatively known as systemic or haematogenously disseminated candidiasis, encompasses the infiltration and dissemination of *Candida* throughout various organs (deep-seated candidiasis), including the brain, kidneys, heart, lungs, and liver, via the bloodstream (candidemia) (Potrykus et al., 2013). The occurrence of invasive candidiasis varies with age, reaching its highest rates at the extremes of age. Table 2.1 provides a summary of the associated risk factors.

Table 2.1: Risk Factors for invasive candidiasis.

Critical illness, with particular risk among patients with long-term ICU stay
Abdominal surgery, with particular risk among patients who have anastomotic leakage or have had repeat laparotomies
Acute necrotising pancreatitis
Haematologic malignant disease
Solid-organ transplantation
Solid-organ tumours
Neonates, particularly those with low birth weight, and preterm infants
Use of broad-spectrum antibiotics
Presence of central vascular catheter, total parenteral nutrition
Haemodialysis
Glucocorticoid use or chemotherapy for cancer
Candida colonisation, particularly if multifocal (colonization index >0.5 or corrected colonisation index >0.4)

[Adapted from (Cleveland et al., 2015)]

According to (Soulountsi et al., 2021), *C. albicans* stands out as the most prevalent species globally, accounting for an average of 66% of all *Candida* spp. Moreover, dominance of *C. albicans* varies worldwide, ranging from 37% in Latin America to 70% in Norway. Nevertheless, NCAC species such as *C. glabrata* (46.4%), *C. parapsilosis* (24.7%) and *C. tropicalis* (13.9%) are on the rise in terms of prevalence as well (Pfaller & Diekema, 2007).

Candidemia has emerged as a serious concern in critical care medicine, attributed to the rising prevalence of immunocompromised patients (Tsay et al., 2020). The five most prevalent pathogens are *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* that responsible for over 90% of invasive candidiasis, with the majority presenting as candidaemia (Pappas et al., 2015). Undoubtedly, candidemia is the fourth most prevalent cause of nosocomial BSI and ranks as the third most common cause of bloodstream infections in the ICU in the USA (Lockhart et al., 2014). The occurrence of candidemia has been consistently rising in recent years, attributable to increased intensive care, heightened utilisation of invasive procedures, intravascular catheters, and greater reliance on antifungals (Hassan et al., 2009). Moreover, candidemia results in a mortality rate of 15–35% among adults and 10–15% among neonates, with each episode incurring a hospitalisation cost of around US\$4000 (Fridkin et al., 2005). In a recent one-year population-based investigation carried out in Spain involving 29 hospitals and 773 cases, the prevalence of candidemia has been reported to be 8.1 cases per 100,000 inhabitants and rate aligns with findings from other European studies (Puig-Asensio et al., 2014). According to Asmundsdottir et al. (2013), the European surveillance studies indicate that the occurrence of *Candida* bloodstream infections (BSI) varies, with an incidence ranging from approximately 3 to 8.6 per 100,000

population annually. In addition, prolonged hospitalisations caused by candidemia impose a considerable economic burden. The management of candidemia and invasive candidiasis comes with substantial costs (Wan Ismail et al., 2020)(Slavin et al., 2004).

Due to the elevated morbidity and mortality linked with delayed treatment, various strategies have been introduced to mitigate the detrimental effects of candidemia in critically ill patients (Morrell et al., 2005). Besides the prophylactic administration of antifungal drugs in specific clinical scenarios, ICU physicians might employ an empirical approach, depending on signs, symptoms, fungal biomarkers, and particular risk factors for candidemia when no identifiable pathogen is present (León et al., 2014). Furthermore, upon the diagnosis of candidemia, it is crucial to promptly execute appropriate source control measures for the infection, including catheter removal, drainage, and debridement (Bassetti et al., 2014). In past years, substantial research has been conducted on antifungal medications targeting *Candida* at where currently the most frequently employed antifungal drugs for treating candidemia include echinocandins (such as caspofungin, micafungin, and anidulafungin), azoles (including fluconazole and voriconazole), and amphotericin B (Gafer-Gvili et al., 2008). Echinocandins seem to exhibit effectiveness comparable to, and better tolerance than, formulations of amphotericin B. (Bassetti et al., 2019; Reboli et al., 2007).

In addition, systemic candidiasis denotes end-organ disease resulting from haematogenous seeding, as any *Candida* infection carried by the bloodstream can potentially infect organs through circulation (Rodrigues et al., 2019). In the United States, BSI caused by *Candida* spp are common, comprising 8%–15% of sepsis cases (Wisplinghoff et al., 2004b). Additionally, the increasing population of individuals

with impaired mucosal integrity and innate immune function contributes to this trend. Notably, the kidney emerges as the primary target organ in candidemia, resulting in renal failure as a significant pathophysiological manifestation of *Candida* sepsis (Spellberg et al., 2005). In addition, the condition encompassing both candidemia and deep-seated candidiasis, may arise after gastrointestinal surgery, facilitating the penetration of the fungus through the mucosa (Morad et al., 2018). Concurrently, neutropenia induced by chemotherapy or immunosuppression in solid-organ or haematological stem cell transplant patients hampers the immune system's ability to recognise and clear *Candida* cells, ultimately resulting in disseminated infections (Safdar & Armstrong, 2011). On the other hand, in the case of severe acute pancreatitis (SAP), the extent of necrosis is positively correlated with infection, as necrotic tissue facilitates the translocation of microbes from the gastrointestinal tract. Not only that, in patients with SAP, fungal infections are linked to higher morbidity compared to cases of isolated bacterial infection (Vege et al., 2009). Furthermore, data obtained from postmortem investigations over a 12-year period revealed that 54% of patients with positive blood cultures for *C. albicans* displayed invasive candidiasis characterized by deep-organ lesions in the kidneys (80%), brain (52%), and heart (48%) (Potrykus et al., 2013). This highlights the susceptibility of all tissues to *Candida* infections. A study by Lewis et al. (2013), reported an upsurge in the occurrence of systemic candidiasis and the presence of *C. albicans* in various anatomical sites, including the lung (79%), gastrointestinal tract (35%), kidney (34%), liver (20%), and spleen (19%) in autopsies of cancer patients. Importantly, individuals with persistent neutropenia are at a higher risk of renal and cardiac invasion.

Timely detection of candidemia as well as deep-seated candidiasis, and the initiation of treatment with suitable antifungal medications undeniably reduce morbidity and mortality (Morgan et al., 2005). Current international guidelines strongly advocate for the use of echinocandins as the primary treatment for deep seated candidiasis in all adults due to the growing prevalence of infections attributed to azole-resistant NCAC species (Lortholary et al., 2014). Overall, resistance to voriconazole was observed in 3% of all isolates and in 8% of *C. glabrata* isolates. Those resistant to voriconazole also showed resistance to fluconazole. It is important to highlight that all isolates were susceptible to caspofungin. (Lockhart et al., 2011). Not only that, due to their effectiveness, favourable safety profile, and minimal drug–drug interactions, echinocandins are the preferred choice for initial therapy in deep seated candidiasis patients as well (Pappas et al., 2009).

2.2.2 Antifungal resistance in *Candida glabrata*

The increased mortality rate seen in instances of invasive *C. glabrata* infections is linked to a mix of low inherent susceptibility and natural resistance to fluconazole, as demonstrated in a significant number of clinical isolates of this fungus (Israel et al., 2019). This is mainly due to the widespread use of azoles as the primary prophylactic treatment for fungal infections because of their cost-effectiveness. Azoles are also commonly used as the second-line option for invasive infections caused by different *Candida* species, contributing to the emergence of cross-resistance to other azoles. Consequently, the initial suggestion in the guidelines of the Infectious Diseases Society of America is to contemplate treatment with echinocandins for patients with candidemia who are at risk of contracting infections caused by *C. glabrata* (Smyth et al., 2018). As a matter of fact, in certain European nations like Poland, up to 22% of

isolates have been reported to exhibit resistance to fluconazole, while resistance to echinocandins is practically non-existent (Szweda et al., 2015). In contrast, countries like Australia as well as Germany have documented elevated rates of resistance to both fluconazole (22.8–38%) and echinocandins (17.1–48%) (Aldejohann et al., 2021).

For many years, azoles constitute the most extensively utilised class of antifungal medications. Azoles act on the lanosterol 14- α -demethylase, which is encoded by the *ERG11*. By inhibiting this enzyme, it effectively prevents the formation of ergosterol, a crucial component of the fungal membrane (Odds et al., 2003; Vanden Bossche et al., 1995). Compared to other *Candida* species, *C. glabrata* displays inherently lower susceptibility to azoles. Moreover, extended and prophylactic utilization of azoles often results in the development of clinically resistant strains of *C. glabrata*. In *C. albicans*, one of the mechanisms contributing to azole resistance entails the overexpression or mutation of the azole target ERG11 (Vermitsky & Edlind, 2004). Nevertheless, resistance does not appear to be mediated by either overexpression or mutations of *ERG11* in azole-resistant *C. glabrata* (Sanguinetti et al., 2005). Resistance is not primarily driven by mutations in the target enzyme but rather by the increased expression and notable upregulation of drug efflux pumps, notably the ABC (ATP-binding cassette) transporter family and the major facilitator superfamily (Tome et al., 2018). Three ABC transporters play a crucial role in azole resistance in *C. glabrata*: *CDR1*, *CDR2* (*PDH1*), and *SNQ2* (Bennett et al., 2004; Torelli et al., 2008). Moreover, another fungal ABC transporter called *AUS1* has been linked to sterol uptake in yeast and may play a role in the inherent low susceptibility of *C. glabrata* to azoles, although the exact mechanisms are not fully understood (Sipos & Kuchler, 2006). The inhibitory impact of fluconazole on *C. glabrata* growth is lessened by the

presence of serum in the medium, possibly due to *C. glabrata* ability to absorb cholesterol (a sterol) from the serum. This effect is dependent on *AUS1*, indicating a potential role for *AUS1* in sterol uptake in *C. glabrata* (Nakayama et al., 2000). A study by H. Nakayama et al. (2007), revealed that *AUS1*-mediated cholesterol absorption from the medium could compensate for the absence of membrane ergosterol caused by *ERG11* inhibition, potentially contributing to *C. glabrata* reduced susceptibility to azoles.

On the other hand, polyenes are fungicidal antifungal agents in use for over 50 years, that function by intercalating into membranes containing ergosterol, primarily the plasma membrane. This intercalation leads to the formation of pores, causing the leakage of cellular components, disruption of ion and electrical gradients, and thus causing cell death (Ellis, 2002). Study conducted by Goemaere et al., (2018), suggests that clinical isolates of different *Candida* species, including *C. glabrata*, exhibit resistance or reduced susceptibility to amphotericin B, a significant polyene antifungal. This reduced susceptibility to amphotericin B seems to be linked to a decrease in ergosterol content in the plasma membrane, which correlates with lower susceptibilities to this antifungal agent (Gray et al., 2012). A comprehensive understanding of the mechanisms underlying the diminished susceptibility to polyenes in *C. glabrata* is crucial. The initial molecular explanation for reduced susceptibility to amphotericin B in *C. glabrata* involved a missense mutation (*C198F*) or a nonsense mutation (at *GLN332*) in *ERG6*, responsible for encoding C-24 methyltransferase (Vandeputte et al., 2008). This enzyme converts zymosterol to fecosterol in the ergosterol biosynthesis pathway (Vandeputte et al., 2007). Additionally, instances of resistance in *C. glabrata* have been associated with *ERG11* deletion or mutations in

ERG1, *ERG2*, and *ERG11*. The absence of enzyme activities encoded by *ERG6* and *ERG2* results in the accumulation of zymosterol and fecosterol, respectively (Hull et al., 2012). Zymosterol and fecosterol are capable of supporting fungal cell growth, and the absence of ergosterol in the *Candida* cell membrane provides resistance to amphotericin B (Young et al., 2003).

Another main class of antifungal drugs are echinocandins that act as inhibitors of the *FKS1/FKS2* β -(1,3)-glucan synthases, pivotal enzymes in the synthesis of β -1,3-glucan, a fundamental component of the cell wall in all fungi (Douglas, 2001). Clinically approved echinocandins drugs such as caspofungin, anidulafungin, and micafungin function as non-competitive inhibitors of *FKS1/FKS2* at where the drug exerts a fungicidal action by disrupting the integrity and structural organisation of the cell wall (Perlin, 2007). Therefore, mutations in *FKS1* and *FKS2*, which encode the catalytic subunits of the β -1,3-glucan synthases, are responsible for conferring echinocandin resistance in *C. glabrata* (Cleary et al., 2008; Thompson et al., 2008). Moreover, the identification of approximately 11 novel mutations in *FKS1* or *FKS2* from clinical isolates of *C. glabrata* has been associated with diminished susceptibility to echinocandins. Nonetheless, the reduced enzymatic activities observed in mutant variants of *FKS1/2* may impact their fitness within the host, potentially contributing to the low frequency of echinocandin resistance (Garcia-Effron et al., 2009). For instance, both clinical isolates and laboratory strains of *C. albicans* exhibit efflux-mediated resistance to caspofungin due to ectopic overexpression of the *CDR2* ABC transporter (Schuetzner-Muehlbauer et al., 2003).

2.3 Pathogenesis of *Candida glabrata*

The lack of pathogenicity in animal models for *C. glabrata* shows that it possesses few virulence attributes (Jabra-Rizk et al., 2016). However, contradictory evidence lies in the high mortality rate and the swift spread of the disease, where in reality, there are only limited studies reported on the virulence of *C. glabrata* (Haynes, 2001). On the other hand, *C. albicans* is recognized for harboring multiple virulence factors that enhance its pathogenicity. These factors encompass adherence to epithelial and endothelial cells, proteinase synthesis, formation of hyphae and pseudohyphae, phenotypic switching, synthesis of phospholipase, as well as antigenic modulation arising from pseudohyphae formation. (Jayatilake et al., 2006a). In addition, it is believed that if *C. glabrata* exhibits low virulence, the absence of hypha formation could be a contributing factor. Hypha formation is acknowledged as a mechanism that enhances adherence, tissue invasion, proteolytic enzyme elaboration, and antigen modulation in *C. albicans* (Silva et al., 2012a).

2.3.1 Pathogenic attributes of *Candida glabrata*

The primary step in *Candida* pathogenesis involves adhering to host surfaces for initial colonisation. Adherence plays a crucial role in the organism's persistence within the host and is deemed essential for the establishment of disease (Henriques et al., 2002).

Moreover, *Candida* species can adhere to medical device surfaces and form biofilms.

Factors like the composition of cell wall proteins and the physicochemical properties of the cell surface have been recognized as influential factors in adhesion (Chaffin, 2008). Proteins on the surface of *Candida* responsible for specific adherence are referred to as adhesins (De Las Peñas et al., 2003). Within *C. glabrata*, a notable set

of adhesins is determined by the EPA (epithelial adhesin) gene family. The general structure of Epa proteins closely resembles that of the Als (agglutinin-like sequence) proteins found in *C. albicans*, and it has been established that *EPA1p* acts as a calcium-dependent lectin, binding to glycol conjugates containing N-acetyl lactosamine (de Groot et al., 2013). Furthermore, even though *EPA6* was not expressed *in vitro*, its expression elevates during *in vivo* urinary tract infection, indicating the ability of *C. glabrata* to adapt to diverse environmental conditions (Koester & Johnston, 2005). In addition, these genes provide the instructions for GPI-anchored cell wall proteins capable of binding to carbohydrates on host cells. Moreover, *C. glabrata* strains with specific mutations exhibit a noticeable enhancement in their organ colonisation ability (Rodrigues et al., 2014b). Significantly, both *C. albicans* and *S. cerevisiae* lack two GPI-anchored cell wall proteins, *PWP7P* and *AEDIP*. Deleting these proteins underscores their essential role in *C. glabrata* adherence (Desai et al., 2011b). This implies that *C. glabrata* has evolved distinctive mechanisms for a more specialised interaction with host cells (Riera et al., 2012).

On the other hand, the well-established notion that cells which can effectively form biofilms are more resistant to antimicrobial agents than mobile, planktonic cells also hold true for *C. glabrata* biofilm production. Indeed, *C. glabrata* capacity to generate biofilms plays a crucial aspect in its virulence mechanisms (Rodrigues et al., 2014b). Research on *Candida* colonisation on denture surfaces has validated that the biofilm formation of *C. glabrata* is stimulated by heightened serum environments, contributing to its prevalence in oral cavity-related infections (Li et al., 2007). Remarkably, when compared to other *Candida* strains, *C. glabrata* demonstrates the least metabolic activity in biofilms, despite harboring the highest count of cultivatable

cells within the biofilm (Figure 2.3) (Cavalheiro & Teixeira, 2018). Additionally, *C. glabrata* biofilms exhibit lower overall biomass but higher levels of proteins and carbohydrates compared to other *Candida* species. Furthermore, the biofilm architecture of *C. glabrata* is characterized by densely packed blastoconidia, lacking pseudohyphae or hyphae (Jayatilake et al., 2006b).

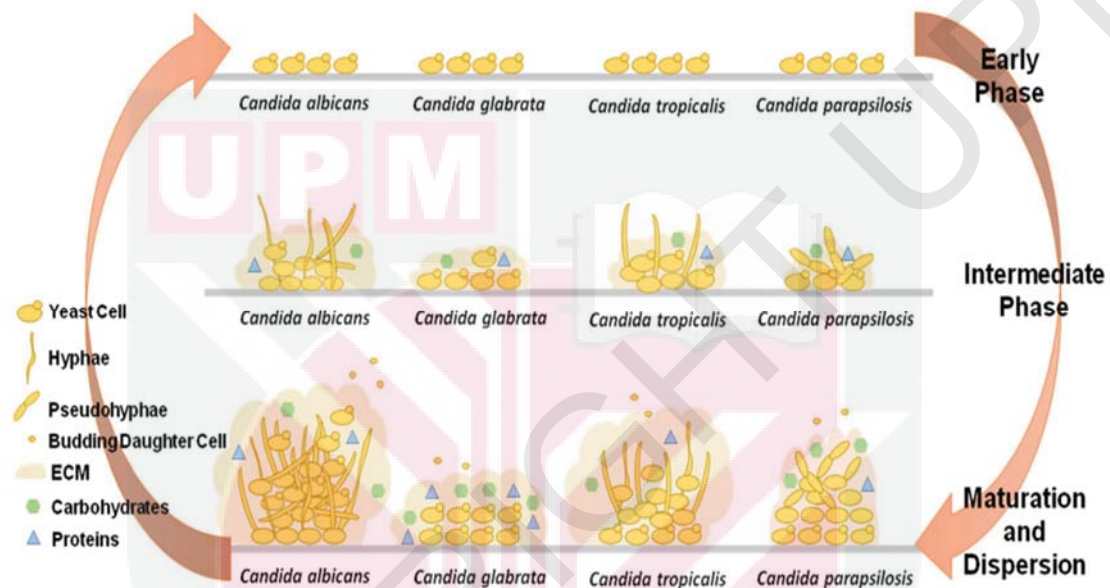


Figure 2.3: Comparative schematics depicting the biofilm formation by *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* (Cavalheiro & Teixeira, 2018)

Moreover, the virulence factors exhibited by *Candida* species include not only adhesion and biofilm formation but also the ability to degrade host tissues, possibly through the release of hydrolytic enzymes into the nearby environment. The collective secretion of enzymes such as proteases, phospholipases, lipases, and hemolysins assist the yeasts in their survival and proliferation within macrophages, facilitating the breakdown of the epithelium. Unlike some other *Candida* species, *C. glabrata* does not produce proteases. (Silva et al., 2010). However, they are capable of producing and releasing extracellular phospholipases, diverse enzymes that facilitate interaction and destruction within the host mucus (Ghannoum, 2000). Phospholipases can break down phospholipids into fatty acids, and their production disrupts the integrity of cell

membranes, affecting their structure and function as well as exposes receptors that facilitate adherence (Kalkanci et al., 2012). Moreover, the phospholipases released by *C. glabrata* are similar to two of the phospholipases generated by *C. albicans*, namely phospholipase B (PLB) and lyso-phospholipase (Ghannoum, 2000). Besides, lipases play a role in the hydrolysis of triacylglycerols which is also a process undertaken by *C. glabrata*. While, in *C. albicans*, ten genes responsible for lipases have been identified, and studies have indicated that mutants lacking these lipases exhibit significantly reduced virulence in a murine intravenous infection model (Sikora et al., 2011).

2.4 Innate immunity

The human immune system is categorised into innate and adaptive components, each serving distinct purpose and roles. The adaptive aspect centers on two specialized cell types: T cells and B cells. Each lymphocyte carries a distinct structurally unique receptor, leading to a vast and varied repertoire of antigen receptors across the entire lymphocyte population (Uslan et al., 2000). On the contrary, the effector mechanisms of innate immunity, encompassing antimicrobial peptides, phagocytes, and the alternative complement pathway, are promptly activated right away following infection and quickly stop the pathogen's ability to replicate (Castrejón-Jiménez et al., 2022). Besides, cells of both haematopoietic and non-haematopoietic origin are responsible for innate immune protection. Hematopoietic cells involved in innate immune reactions include macrophages, dendritic cells, mast cells, neutrophils, eosinophils, natural killer (NK) cells, as well as NK/T cells. In addition to hematopoietic cells, the skin and epithelial cells in the respiratory, gastrointestinal, and genitourinary tracts also demonstrate innate immune activity (Janeway & Medzhitov,

2002). Moreover, in order to enhance these cellular defences, innate immunity also incorporates a humoral component encompassing well-characterised elements like complement proteins, lipopolysaccharides (LPS) binding protein, C-reactive protein, pentraxins, collectins, and antimicrobial peptides, including defensins. Circulating proteins within innate immunity play a dual role in microbial detection and effector mechanisms, aiding in the efficient clearance of infections (Pancer & Cooper, 2006).

The innate immune system significantly influences the dynamics of host-fungi interaction and provides protection against fungal diseases through direct fungal elimination, influencing microbial communities that impact fungal commensalism, and shaping the adaptive immune response such as Th1 and Th17 polarisation. These diverse functions are primarily achieved through receptor-mediated recognition of fungal determinants and the detection of geometric cues like fungal size (Branzk et al., 2014). Besides, fungal ligands trigger various PRRs, such as C-type lectin receptors (CLRs), toll-like receptors (TLRs), as well as NOD-like receptors (NLRs) (Lionakis et al., 2017). Members within the CLRs family possess a conserved C-type lectin-like domain (CTLCD), which binds to carbohydrates or Ca^{2+} (Zelensky & Gready, 2005). Numerous CLRs have been documented, but the extensively researched CLRs crucial in antifungal defense include Dectin-1, the Dectin-2 cluster (comprising Dectin-2, Mincle, and Mcl), the mannose receptor (MR), and DC-specific ICAM-3 grabbing non-integrin (DC-SIGN), predominantly found on myeloid cells (Vautier et al., 2012). In brief, Dectin-2, Mincle, and Mcl form associations with the immunoreceptor tyrosine-based activation motif (ITAM)-containing $\text{Fc}\gamma$ signaling chain, whereas Dectin-1 features a hemITAM in its cytoplasmic tail (Geijtenbeek & Gringhuis, 2016). Dectin-1 also interacts with β -glucans found in diverse fungal pathogens. The

signaling of Dectin-1 is vital in multiple antifungal reactions, including phagocytosis, production of ROS, cytokine release, activation of the inflammasome, and the stimulation of Th1 and Th17 responses (Bryant et al., 2015). As for the TLRs, it is the most extensively studied family of PRRs involved in pathogen recognition. These evolutionarily conserved transmembrane proteins recognize pathogens both outside and inside cells, within endosomes and lysosomes. TLRs consist of N-terminal leucine-rich repeats (LRRs) and a C-terminal Toll/IL-1R homology (TIR) domain (Takeuchi & Akira, 2010). Additionally, TLRs transmit signals through adapter proteins such as Myeloid Differentiation Primary Response 88 (MyD88), MyD88 Adapter-Like (Mal), TIR-Domain-Containing Adapter-Inducing IFN- β (TRIF), and TRIF-Related Adaptor Molecule (TRAM) (Kawasaki & Kawai, 2014). On the other hand, NLRs constitute another component of the innate immune system, capable of recognising PAMPs originating from internalised microbial components (Van De Veerdonk et al., 2015). NLRs are structured with a C-terminal ligand-sensing array of LRR, a central oligomerization NACHT domain, and an N-terminal protein-protein domain (CARD or pyrin domain). These NLRs are typically classified into two primary family groups: NOD1/2 and the inflammasome-forming NLRs (Zhong et al., 2013). Inflammasome multiprotein complexes typically consist of an NLR, such as NLRP3 or NALP3, ASC, and Caspase proteins. Activation of the inflammasome leads to Caspase-mediated cleavage of pro-IL-1 β and pro-IL-18 into their functional forms, IL-1 β and IL-18 (Zhong et al., 2013). Both IL-1 β and IL-18 have been identified as crucial factors in the control of fungal infections (Stuyt et al., 2002).

2.4.1 *Candida glabrata* interaction with innate immune cells

The interplay between the host and pathogenic fungi plays a pivotal role in determining the outcome of an infection or disease (Bourgeois & Kuchler, 2012). Throughout its commensal and pathogenic phases, *C. glabrata* is believed to engage with various host cells, including epithelial, endothelial, and immune cells (Brunke et al., 2010). Models employed for studying the pathogenesis of *C. glabrata*, both *in vivo* and *in vitro*, encompass *Mus musculus* (mice), *D. melanogaster* (fruit fly), and *Galleria mellonella* (wax moth). Moreover, *in vitro* models include cell lines of epithelial and endothelial cells, macrophages, human neutrophils, as well as reconstructed oral epithelia (Ho & Haynes, 2015; Kaur et al., 2007b; Zhang et al., 2016).

Neutrophils play a crucial role in the host defence mechanism against fungal infections, but their specific role in controlling *C. glabrata* infections is not thoroughly explored (Netea et al., 2015). Study conducted by Johnson et al. (2017) have reported that human neutrophils produce neutrophil extracellular traps after engulfing *C. glabrata* cells. Neutrophils activated by *C. glabrata* release monocyte chemoattractants such as MIP-1 α and MIP-1 β , resulting in heightened migration of monocytes to the location of the interaction between neutrophils and *C. glabrata* (Duggan et al., 2015). Besides, the impact of the neutrophil environment on *C. glabrata* has been examined, revealing upregulation in genes related to oxidative stress, gluconeogenesis, glyoxylate cycle, as well as methionine metabolism (Fukuda et al., 2013).

On the other hand, the importance of NK cells, which are the effector lymphatic cells of the innate and adaptive immune systems in the fight against *C. glabrata* infection

has been brought to light by a recent study Vitenshtein et al., (2016). The study has reported that the adhesins EPA1, EPA6, and EPA7 were all demonstrated to be distinctly identified by the NKp46/NCR1 receptor of the NK cells, leading to the elimination of disseminated *C. glabrata* infection. In addition, murine dendritic cells have been documented to generate IFN- β via the toll-like receptor TLR7, highlighting the significance of interferon type I (IFN-I) signaling in modulating the host response to *C. glabrata* infection (Bourgeois et al., 2011).

Meanwhile, macrophages belonging to the monocytic lineage, are professional phagocytes and integral components of the innate immune system, serving as a frontline defence against invading microbes. These cells are prevalent in nearly all tissues and particularly abundant at mucosal surfaces (Pollard, 2009). In spite of engulfing and eliminating the fungal pathogen, macrophages also play a role in recruiting other immune cells to the infection site through the production of cytokines and chemokines (Seider et al., 2011b). *C. glabrata* can survive and replicate within both human and murine macrophages without causing any detrimental effects on the macrophages (Rai et al., 2012). Furthermore, infection with *C. glabrata* did not notably activate any MAPK pathways, such as Erk1/2 (Extracellular signal-regulated kinases), SAPK/JNK (Stress-activated protein kinases/Jun amino-terminal kinases), and NF- κ B signaling (Kasper et al., 2014). Furthermore, upon *C. glabrata* infection, the spleen tyrosine kinase, Syk, undergoes phosphorylation, leading to the NLRP3 inflammasome-dependent formation of IL-1 β in human THP-1 macrophages (Serda et al., 2002). The family of eleven cell surface-associated proteases (CgYapsins) has been demonstrated to play a crucial role in regulating the activation of the Syk pathway.

The absence of these proteases resulted in heightened IL-1 β release and the subsequent killing of *C. glabrata* cells (Figure 2.4) (Rasheed et al., 2018a).

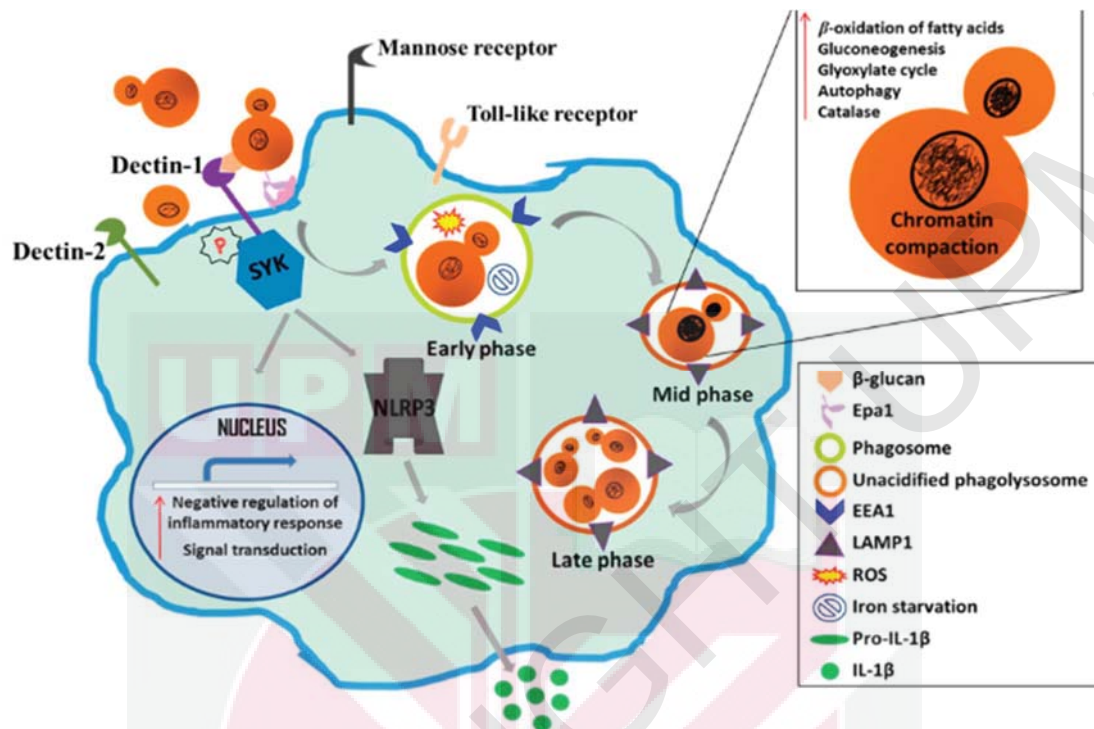


Figure 2.4: A schematic diagram depicting key aspects of the interaction between *C. glabrata* and macrophages (K. Kumar et al., 2019)

2.4.2 *Candida glabrata* immune evasion strategy

Throughout their evolutionary process, *C. glabrata* have developed strategies enabling them to thrive within the diverse niches provided by human hosts. This species requires swift and synchronized adjustments in their metabolism and physiology to evade immune surveillance and acclimates to challenging or dynamically shifting microenvironments (Alves et al., 2020b). These adaptive strategies enable the fungus to endure conditions marked by nutrient constraints, antimicrobial peptide presence, pH variations, oxygen deprivation, change in temperature, as well as exposure to oxidative, nitrosamine, and cationic stress (Pais et al., 2019).

The fungal cell wall is crucial in initiating the early phases of host-fungus interaction, enabling mutual recognition and triggering signal transduction via host receptors (Seider et al., 2014). Fungal pathogens frequently employ the masking of immunostimulatory cell wall components such as β -glucan during interactions with immune cells in order to evade detection, macrophage activation and phagocytosis. The recognition and activation of the host's immune system hinge on the accessibility to β -glucan and chitins present in the fungal cell wall (Silva et al., 2012b). The immunological recognition pathway in *C. glabrata* is greatly impacted by β -1,3-glucan and is aided by the dectin-1 receptor on macrophages (G. D. Brown et al., 2003b). For instance, deletion mutants that disrupt cell wall integrity and modify to expose the immunostimulatory cell wall components elicited a more robust inflammatory response from macrophages. This was evident through increased TNF- α secretion, frequently accompanied by a heightened oxidative burst, and, in most instances, the deletion mutants were more effectively eliminated by macrophages (Kaur et al., 2007c). This observation was also reported by West et al. (2013) and Seider et al. (2014) for deletion mutants that lacked cell surface-associated *C. glabrata* proteases (yapsins) and had defects in protein glycosylation.

Next, following the process of phagocytosis, one key antimicrobial mechanism employed by macrophages involves transporting the engulfed microorganism to a phagolysosome. Numerous intracellular pathogens, including fungi, have developed strategies to impede phagosome maturation, thereby establishing a more favourable environment within macrophages (Flannagan et al., 2009; Gilbert et al., 2015). A study conducted by Seider et al., (2011c) have reported that *C. glabrata* inhabits a modified phagosomal compartment, preventing the complete maturation and acidification of the

phagosome. Phagosomes containing *C. glabrata* exhibit markers associated with early and late endosomal stages (EEA1 and LAMP-1, respectively) but lack markers indicative of the phagolysosomal stage (cathepsin D, proteolytic activity, lysosomal fusion). Similarly, while the phagosomal proton pump V-ATPase appears to be available in these compartments, phagosomes containing viable yeasts show only weak acidification. Besides that, the utilisation of amino acids as carbon sources by *C. glabrata* and the subsequent release of ammonia have been linked to environmental alkalinisation and the neutralisation of phagosome pH (Vylkova & Lorenz, 2014). Not only that, both the putative *C. glabrata* α -1,6-mannosyltransferase MNN10 and the related mannosyltransferase MNN11 play a crucial role *in vitro* environmental alkalinisation as well as in the hindrance of phagosome acidification. This implies that the (MNN10/11-dependent) alkalinisation serves as a fungal strategy to alter the pH of the phagosome (West et al., 2013). Moreover, a study conducted by Rai et al., (2015) has suggested the involvement of phosphatidylinositol 3-kinase-mediated signalling in modifying phagosomes. Macrophages infected with *C. glabrata* mutants lacking the two-class III phosphoinositide 3-kinase (PI3K) subunit-encoding genes *VPS15* and *VPS34* showed a slightly increased number of acidified phagosomes.

Furthermore, one of the key components of the macrophage anti-microbial response is the generation of reactive oxygen species (ROS). The macrophage NADPH oxidase complex, formed on both cytoplasmic and phagosomal membranes, generates superoxide, hydroxyl anions, and hydroxyl radicals (Segal et al., 2012). *Candida glabrata* exhibits a significantly elevated intrinsic resistance to oxidative stress when compared to *C. albicans* and *S. cerevisiae*. This resistance is attributed partly to the expression of a highly effective catalase, *CTA1* (Cuéllar-Cruz et al., 2008). The

activation of a *CTAI*-reporter gene is triggered after macrophage phagocytosis, indicating exposure of *C. glabrata* to oxidative stress within macrophages and the role of *CTAI* in the oxidative stress response (Roetzer et al., 2010b). Moreover, the fundamental reaction to oxidative stress in an *in vitro* setting encompasses thioredoxin peroxidases (TSA1 and TSA2), thioredoxin reductases (TRR1, TRR2), thioredoxin cofactor (TRX2), glutathione peroxidase (GPX2), as well as superoxide dismutases (SOD1 and SOD2) (Jandric & Schüller, 2011). A study conducted by (Briones-Martin-Del-Campo et al., 2015) has also suggested that superoxide dismutases are essential not only for shielding against oxidative damage but also play a crucial role in metabolism, biosynthesis of lysine, DNA protection, and the aging process in *C. glabrata*.

Finally, limiting micronutrients proves to be an efficient method for managing the proliferation of pathogens (Martínez-Pastor & Puig, 2020; Riedelberger et al., 2020). This is particularly relevant for micronutrients like zinc and iron, crucial for the growth of intracellular pathogens such as *C. glabrata* (Pappas et al., 2018b). Under such limitations, *C. glabrata* has devised strategies to acquire micronutrients, enabling its proliferation, survival, and heightened resistance to phagocytosis (Alves et al., 2020b). Indeed, *C. glabrata* demonstrates the ability to acquire free iron and extract iron from iron-binding proteins such as hemoglobin, ferritin, and transferrin. The siderophore-iron transporter SIT1 plays a crucial role in facilitating iron uptake, enabling the microorganism to resist phagocytosis and thrive within host macrophages by utilizing intracellular iron stores. Additionally, interferons, particularly IFN-I, have been observed to play a key role in promoting the persistence of *C. glabrata* in organs like the brain, liver, and spleen in murine models by disrupting cellular iron balance,

thereby aiding the pathogen in acquiring iron (Riedelberger et al., 2020). Additionally, *C. glabrata* can elude immunological scrutiny by sequestering zinc in vacuoles to regulate zinc homeostasis. Not only that, the deletion of ZRC1 in *C. glabrata* (which is also implicated in the regulation of zinc sequestration in *S. cerevisiae*) results in a survival deficiency and proves vital for immune evasion (Riedelberger, Penninger, Tscherner, Hadriga, et al., 2020). Also, IFN-I plays a crucial role in the modulation of zinc homeostasis by lowering plasma concentrations of this micronutrient. It achieves this by triggering the expression of hepatic metallothionein, which sequesters zinc for cytoplasmic transport during intracellular zinc mobilisation. Research shows that after infection with *C. glabrata* in both *in vitro* and *in vivo* models, IFN-I suppresses the expression of the metallothionein gene (Riedelberger et al., 2020).

CHAPTER 3

METHODOLOGY

3.1 Strain and growth conditions

Candida glabrata reference strain (ATCC 2001) was used in this study. Standard culture media were used, including Sabouraud dextrose agar (SDA) (Oxoid, UK): dextrose (4%, w/v), peptone (1%, w/v), agar (1.5%, w/v) and Sabouraud dextrose broth (SDB) (Oxoid, UK): dextrose (2%, w/v), meat peptone (0.5% w/v) and casein peptone (0.5% w/v). *Candida glabrata* was routinely grown in SDA, at where it was streaked from the glycerol stock culture and then incubated at 37°C for 24 hours. From the homogeneous culture of *C. glabrata*, a solitary colony was meticulously selected with an inoculation loop and moved into 5 ml of SDB. Subsequently, the *C. glabrata* broth culture was placed in a shaking incubator (N-Biotek, South Korea) at 37°C and 180 rpm for 24 h. After 24h incubation, the cultures were adjusted to an optical density at 600 nm (OD₆₀₀) of 0.1 in SDB before any assays to be conducted. For the mid-term storage of the culture in glycerol stock, 500 µL of *C. glabrata* culture was combined with 500 µL of 40% (v/v) glycerol to achieve a final concentration of 20% (v/v). The culture glycerol stocks were stored at -80°C for later utilization. In order to assure the viability of *C. glabrata*, the stocks were replenished every six months (Chew et al., 2019a).

3.2 Cell culture

THP-1 monocyte (ATCC TIB-202) was purchased from American Type Culture Collection (ATCC, USA). Base medium for the cell line, Roswell Park Memorial Institute (RPMI-1640) (Thermo Fisher Scientific, USA) medium was used and the complete RPMI medium was prepared by adding 2-mercaptoethanol (BME) (Sigma-Aldrich, USA) to a final concentration of 0.05 mM and foetal bovine serum (FBS) (Thermo Fisher Scientific, USA) to a final concentration of 10% (v/v) to the base medium.

3.2.1 Subculture of THP-1 cells

Routinely, THP-1 cell line was expanded by replacing the complete RPMI medium every two to three days and regular media changes was done by centrifuging and resuspending the cells in 20 ml of fresh RPMI medium every seven days in T-75 cell culture flasks to maintain cell health and avoid toxic metabolite accumulation. Culture was then incubated in a humidified, 5% (v/v) CO₂ incubator at 37°C (Galaxy 170 R/S CO₂ Incubator). The number of cells was maintained at a concentration of 8×10^5 to 1×10^6 cells/ml. The Neubauer counting chamber method was used for cell counting and the viable cells were determined by Trypan blue exclusion. Besides, the cell line was maintained with its passage number within the range of 15 to 20.

3.2.2 Cryopreservation

Single cell suspension (1×10^6 cells/ml) was prepared in complete RPMI medium supplemented with 5% (v/v) dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA). Then, 1 ml of cell suspension was transferred into cryovials and kept at 4°C for 3 h and let it to cool down slowly and then kept at -20°C. Subsequently, the vials were agitated and placed in a -80°C freezer overnight. The following day, the vials were moved to the liquid nitrogen tank for long term storage.

3.3 Differentiation of THP-1 cells

Upon reaching 80% of confluency, the cells were seeded at a seeding density of 1×10^6 cells/ml and stimulated with 10 ng/ml phorbol 12-myristate-13-acetate (PMA) (Sigma-Aldrich, USA) for 72 h to differentiate them into macrophages. Following the PMA stimulation, the cell supernatants were collected to determine the undifferentiated THP-1 cells (suspension cells). The differentiated THP-1 cells (adherent cells) were detached from the T-25 cell culture flask with 5 ml of Accutase (Nacalai Tesque, Japan) incubation for 10 min and then scraped by using a sterile cell scraper. Following that, Trypan blue staining was used to count the live adherent cells under an inverted microscope (Ozleyen et al., 2021). THP-1 macrophages were routinely prepared by adding PMA to THP-1 monocytes every time before conducting any assays to ensure its viability and efficiency.

3.3.1 Flow cytometry analysis of THP-1 differentiation

All remaining procedures were carried out at 4°C after the THP-1 cells were collected on ice. After centrifuging the cells, the THP-1 cells were reconstituted in Fluorescent Activated Cell Sorting (FACS) buffer (0.1% azide, 2% FBS in PBS) supplemented with 11 µg/mL IgG. Subsequently, the cells were labeled with PerCP/Cy5.5 anti-mouse/human CD11b antibody (Biolegend, #101227), fixed with 2% (w/v) paraformaldehyde (PFA), and examined using flow cytometry on a FACS Calibur instrument. (Starr et al., 2018a). The data collected was then analysed using BD FACSDiva software.

3.3.2 *Candida glabrata* infection of THP-1 macrophages

Overnight culture of *C. glabrata* was washed three times with phosphate buffered saline (PBS) and resuspended in SDB. The number of *C. glabrata* was adjusted to 1×10^5 cells/ml for co-incubation. On the other hand, THP-1 macrophages were washed with PBS and detached from flask using Accutase. Collected THP-1 macrophages were counted using a haemocytometer to standardise the number of cells (1×10^6 cells/ml) for co-incubation. *C. glabrata* was co-incubated with THP-1 macrophages at a *Candida*-macrophage ratio of 1:10 in a 6-well plate. The plate was incubated at 37°C in a CO₂ incubator for 2 h, allowing the *C. glabrata* to be phagocytosed. After 2 h of co-incubation, the non-engulfed *C. glabrata* were removed and the wells were top up with 2 ml of fresh complete medium, leaving only adherent THP-1 macrophages with engulfed *C. glabrata* in the wells. The 6-well plate was then further incubated for another 48 h (Barker et al., 2005). After 48 h of co-incubation, the phagocytosed *C.*

glabrata was harvested out of the THP-1 macrophages by lysing the THP-1 macrophages using ice-cold sterile deionized water. Ice-cold sterile deionized water was used to carefully lyse the THP-1 macrophages without damaging the phagocytosed *C. glabrata* cells, ensuring better recovery of the yeast for further analysis. The phagocytosed *C. glabrata* were then plated on SDA agar and the colony forming unit (CFU) was determined after incubating the plates at 37°C for 24 h (Ng et al., 2015). The survival ratio of phagocytosed *C. glabrata* cells was determined by applying the formula below:

$$\text{Survival ratio} = \frac{\text{CFU of 24 h sample}}{\text{CFU of 2 h control}} \times 100\%$$

3.4 *Candida glabrata* cell wall biochemistry

3.4.1 Visualisation of cell wall architecture

Transmission electron microscopy (TEM) was used to visualise the cell wall architectures, the thickness of outer and inner cell wall layers of *C. glabrata* that has been exposed to THP-1 macrophage. Briefly, upon 48 h of co-incubation with THP-1 macrophage, *C. glabrata* cells were harvested (refer to section 3.3.2), washed twice with PBS, pH 7.4 and resuspended in fresh complete RPMI medium. The sample was then centrifuged and the supernatant was removed. The sample was fixed in 2.5% of glutaraldehyde for 6 h at 4°C. Subsequently, fixative was removed and horse serum was added to the sample and incubated for 24 h. After 24 h of incubation, the clotted sample was diced into 3 mm³ and fixed with 4% glutaraldehyde for another 2 h at 4°C. The specimen underwent a series of steps: initially washed thrice with 0.1M sodium

cacodylate buffer for 10 minutes per wash. Subsequently, it was post-fixed with 1% (w/v) osmium tetroxide for 2 hours at 4°C, followed by another three washes with 0.1M sodium cacodylate buffer. Next, dehydration occurred using a gradient of acetone concentrations: 35% (v/v) for 10 minutes, 50% (v/v) for 10 minutes, 75% (v/v) for 10 minutes, 95% (v/v) for 10 minutes, and thrice with 100% (v/v) for 15 minutes each. The sample was then infiltrated with a resin:acetone mixture (1:1) for an hour, followed by a resin:acetone mixture (3:1) for two hours, pure resin overnight, and again pure resin for two hours. Subsequently, the sample was embedded in BEEM capsules filled with pure resin and allowed to polymerize in an incubator at 60°C for 48 hours. Ultrathin sections were sliced using an EM UC7 ultramicrotome to a thickness of 80 nm. The cell wall structures of the sample were visualized and captured using a JEM-2100F field emission electron microscope (JEOL, JAPAN). The thickness of the outer and inner cell wall layers of *C. glabrata* was measured by averaging twenty measurements from each cell (n=10) using ImageJ software (Schneider et al., 2012).

3.4.2 Flow cytometry analysis of cell wall components

Upon co-incubation with THP-1 macrophage, *C. glabrata* cells were harvested (refer to section 3.3.2), washed twice with PBS, pH 7.4 and resuspended in RPMI medium. The sample was then transferred to FlowTubes (a polystyrene round bottom tube, Corning, USA) and centrifuged at 0.8 RCF for 2 min and the supernatant was removed. All tubes were reconstituted with 1 mL of chilled flow cytometry (FC) blocking solution and left to incubate for 30 minutes at room temperature in a shaking incubator. Following this, the sample underwent centrifugation and was washed three times with

1 mL of cold FC washing solution. The cells were then harvested by centrifugation. Subsequently, the cell wall components were stained sequentially (β -glucan > mannan > chitin). To stain β -glucan, the cells were suspended in 100 μ l of Fc: Dectin-1 protein in FC blocking solution ([Fc (human): Dectin-1 (mouse) (recombinant), Adipogen] diluted to a final concentration of 1 μ g/ml) and incubated on ice for 60 minutes to allow for the specific binding of β -glucans. Dectin-1 exhibits a strong affinity for β -1,3-glucan, making it an effective tool for selectively identifying and staining this component in the fungal cell wall (Cai et al., 2018). Following an hour of incubation, the cells were centrifuged and washed three times with 1 mL of cold FC washing solution. The cells were again resuspended in 200 μ l of 1:200 Alexa Fluor 488 in FC blocking solution and incubated for 45 min on ice. Cells were then centrifuged as well as washed thrice with FC washing solution. Subsequently, for the staining of mannan layer, the tubes were wrapped in aluminium foil before proceeding. The cells were reconstituted with 1 mL of ConcanavalinA Texas Red Conjugate (ConA-TRed) solution from Thermo Fisher Scientific, USA, in 1x PBS adjusted to a final concentration of 25 μ g/mL. They were then incubated at room temperature for 45 minutes in a shaking incubator. Cells were then collected by centrifugation and washed once in 1x PBS and resuspended again in 1x PBS. Next, the cells were stained for chitin with 500 μ l of Calcofluor White (CFW, fluorescent brightener 28; Sigma-Aldrich, USA) and incubated at room temperature for 10 min in dark place. Finally, fixation of the staining on the cell wall components was done by resuspending the cell pellet with 1 mL of 2% PFA and further incubated for 10 min (Nogueira MF, 2017). The sample was then centrifuged, resuspended in PBS and then analysed by flow cytometry. Analysis of the results were done using FCS software and Kaluza analysis software.

3.5 Biophysical properties of *Candida glabrata* cell wall

3.5.1 Cell surface hydrophobicity assay

Cell surface hydrophobicity (CSH) was assessed using the microbial adhesion to hydrocarbons (MATH) assay. In brief, freshly harvested *C. glabrata* cells were washed twice with PBS and the number of cells was adjusted to OD_{600nm} of 0.1 in PBS (A₀). Next, 0.4 mL of n-hexadecane (Sigma-Aldrich, USA) was added to 3 mL of *C. glabrata* suspension and vortexed vigorously in order to allow phase separation for 10 min at 30°C. Subsequently, the OD_{600nm} of the aqueous phase was measured (A₁) and the percentage of hydrophobicity was calculated using the formula below (Silva-Dias et al., 2015) :

$$\text{Hydrophobicity (\%)} = 1 - \left[\frac{A_1}{A_0} \right] \times 100\%$$

3.5.2 Cell adhesion assay

Freshly harvested *C. glabrata* cells were washed twice resuspended in PBS. A total volume of 2 ml *C. glabrata* cell suspension (1x10⁵ cells/ml) was added to each well of a 12-well plate (non-treated polystyrene, Corning, USA) and incubated for 1 h at 37°C. The cell suspension was removed from the wells and then the wells were washed three times with 1 mL of PBS. Using a cell scraper, the adhered cells were scraped into 1 mL of PBS, and the OD_{600nm} of the suspension was quantified. The suspension was also diluted and plated onto SDA agar for CFU counting (Ene et al., 2012a).

3.5.3 Cell wall porosity assay

Cell wall porosity assay was modified from a protocol previously described (De Nobel et al., 1990a). In summary, newly harvested *C. glabrata* cells underwent two washes with dH₂O. Subsequently, aliquots of 1 x 10⁵ cells/ml were cultured for 30 minutes at 30°C in a shaking incubator in 1 mL of either 10 mM Tris-HCl pH 7.4 (serving as the control buffer), the same buffer containing 5 mg/ml DEAE-dextran (500 kDa), or the buffer with 15 mg/ml poly-L-lysine (50 kDa) (Liu et al., 2019). Cells were then centrifuged at 10000 x g for 2 min and absorbance of the supernatants were measured at 260 nm. Relative porosity was calculated using the following formula:

$$\text{Relative porosity (\%)} = 100 \times \left[\frac{\text{ADEAE-ABUFFER}}{\text{APOLY-L-LYSINE-ABUFFER}} \right]$$

3.6 Antifungal susceptibility assay

Minimum inhibitory concentrations (MIC) of antifungals including amphotericin B, fluconazole and caspofungin against *C. glabrata* ATCC 2001 was determined using the broth microdilution method described in M27-A2, a guideline by the Clinical and Laboratory Standards Institute (CLSI, 2002). As for quality control in the broth microdilution procedures, *Candida parapsilosis* ATCC 22019 (ATCC, USA) strain was used. The MIC of amphotericin B (Sigma-Aldrich, USA), fluconazole (Tokyo Chemical Industry, Japan) and caspofungin (MedChemExpress, USA) was determined to be 0.5 µg/ml, 8 µg/ml, and 0.5 µg/ml respectively. Antifungal susceptibility assay was carried out in order to determine the antifungal susceptibility of *C. glabrata* that have been exposed to THP-1 macrophages. Briefly, freshly harvested *C. glabrata* (refer to section 3.3.2) was centrifuged and resuspended in RPMI medium. Using a

hemacytometer, the yeast cells were counted and adjusted to the count of 1×10^5 cells/ml. In a 96-well plate (Corning, USA), 100 μ l of *C. glabrata* cell suspension was mixed together with 100 μ l of the specified concentration of antifungals as mentioned earlier and then cultured for 24 h at 37°C. After the 24-hour incubation period, the sample was collected and reconstituted in 1 ml of PBS. The cell suspension was subsequently diluted in a series, and 100 μ l of each dilution was spread onto SDA agar plates and incubated for 24 hours at 37°C. Following incubation, the resulting colonies were enumerated, and the CFU were calculated. The viability of the antifungal-treated *C. glabrata* compared to the untreated control was determined as described below. (Ng et al., 2015):

$$\text{Viability of } C. \text{ glabrata} = \left[\frac{\text{CFU of antifungal treated sample}}{\text{CFU of untreated sample}} \right] \times 100\%$$

3.7 Impact on immune response and recognition

3.7.1 Cytokine analysis

The concentration of cytokines (TNF- α , IL-6, IL-4, and IL-10) produced by the THP-1 macrophages in response to *C. glabrata* were determined by Luminex discovery assay (LXSAHM-4; Minneapolis, USA) using Human premixed multi-analyte kit (R&D Systems, USA) with beads from Luminex Corporation (Austin, USA) coated with antibodies in-house. Analysis of beads was performed on a Bio-Plex™ 200 system (Bio-Rad Laboratories, Inc., USA) and the data was analysed using Bio-Plex Manager™ software version 6.1 (Bio-Rad Laboratories, Inc., USA) (Bai et al., 2019). Briefly, for the sample preparation, 1×10^6 cells/ml of THP-1 macrophages were cultured together with freshly harvested *C. glabrata* (control) and *C. glabrata* (that has

prior exposure with THP-1 macrophages) to the yeast to cell ratio of 10:1 for 12 h and 24h at 37°C in a 6-well plate. The cell culture supernatants were then collected at both time points and stored at -80°C.

3.7.2 Phagocytosis assay

In this assay, flow cytometry was employed to determine the percentage uptake of *C. glabrata* by THP-1 macrophages at a *Candida*-macrophage ratio of 1:10. The experiment was carried out according to the method previously described by Shu et al. (2022). As for the sample preparation, the infected THP-1 macrophages (infected with *C. glabrata* that been prepared as in section 3.3.2) were washed thrice by rinsing the cell monolayers with 1x PBS, followed by detaching the cells using 1x Accutase and incubated at 37°C for 10 min. The cell suspension was then collected and centrifuged at 400 x g for 5 min. The cell pellets were then resuspended in 1 ml of FC blocking solution and incubated for 30 min at room temperature in a shaking incubator. Upon incubation, the tubes were then centrifuged again at 0.8 RCF for 2 min and cells were then washed thrice by resuspending in 1 ml of FC washing solution. Cells were then collected by centrifugation and resuspended in 5 ml of fresh PBS/BSA containing 11 µg/ml of IgG and further incubated for 20 min. Cells were again centrifuged and supernatant was discarded. Following that, staining procedure was done under dark condition. As for staining the *C. glabrata* that resides inside the macrophages, 500 µl of 25 µg/ml Calcofluor white was added and incubated for 10 minutes. The cells were then centrifuged again to remove the supernatant and added with 100 µl of FITC anti-mouse/human CD11b antibody (BioLegend, #101206) for 30 min on ice in order to stain the THP-1 macrophages. The cells were then centrifuged to remove the

supernatant and then proceeded to the last step which was to fix the staining. Fixation was done by resuspending the pellet with 1 ml of 2% PFA and incubated for 10 min. Finally, the cells were then centrifuged and resuspended in PBS and the content was transferred into FC tubes. Analysis of the results were done using FCS software and Kaluza analysis software version 2.1.

3.7.3 Macrophage killing assay

Macrophage killing assay was conducted as previously described by Li et al., (2019, 2023). Briefly, 1×10^6 cells/ml of THP-1 macrophages were cultured with freshly harvested *C. glabrata* (control) and *C. glabrata* (that has been prior exposed with THP-1 macrophages) to the yeast to cell ratio of 10:1 for 24 h at 37°C. The co-incubated samples were lysed with the addition of ice cold dH₂O for 10 min. Subsequently, the samples were diluted in PBS and plated on SDA and further incubated at 37°C for 24 h. After 24 h of incubation, the viability of *C. glabrata* was determined by CFU counting.

CHAPTER 4

RESULTS

4.1 Culturing THP-1 monocytes

4.1.1 Morphology of THP-1 cells

Morphology of THP-1 cells upon receiving and reviving THP-1 ATCC (TIB-202) vial was observed under inverted microscope. The images revealed the typical characteristics of THP-1 cell line where it was observed to be single, round-shaped and suspended (Figure 4.1). These lymphoblast-like cells are suspension cells and floating cells are viable.

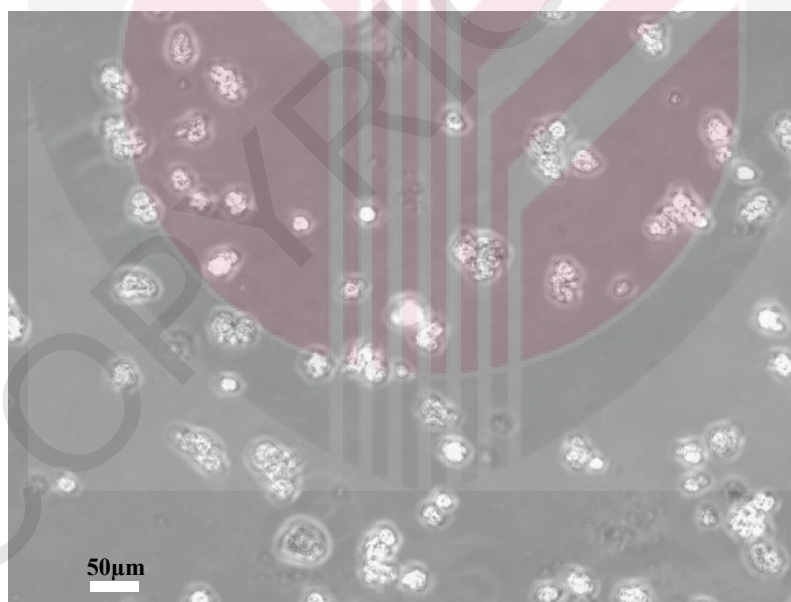


Figure 4.1: Photomicrograph (original magnification at 400x) showing morphology of THP-1 monocytes at low density of cells (2.0×10^5 viable cells/mL) under inverted microscope.

4.1.2 Differentiation of THP-1 monocytes

The reduction in the nucleocytoplasmic ratio followed by an increase in cytoplasmic volume has been associated to the morphological characteristics of THP-1 cells following differentiation (Sokol et al., 1987). As anticipated, upon PMA treatment the differentiated THP-1 cells showed much greater cytoplasmic volume and reduced nucleocytoplasmic ratio in relative to the monocytes (Figure 4.2). At where, the THP-1 monocytes (Figure 4.2 A) are generally smaller, rounder, and exhibit a more uniform shape, whereas differentiated macrophages (Figure 4.2 B) are larger and have a more irregular morphology. Besides, PMA treatment also enhanced the adherence of the treated cells in comparison with the monocytes (data not shown).

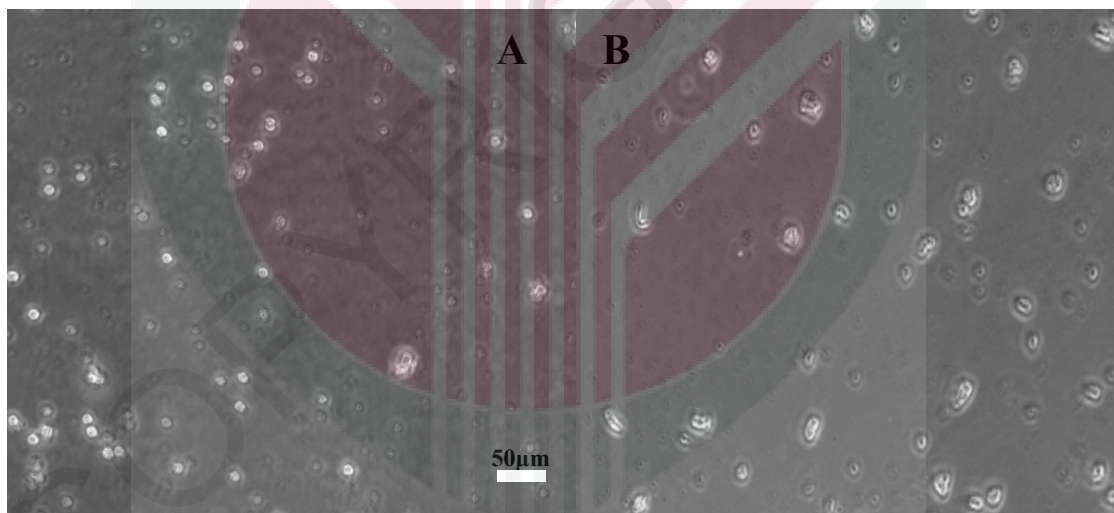


Figure 4.2: Photomicrographs (original magnification at 400x) showing the (a) THP-1 monocytes and (b) THP-1 differentiated macrophages under inverted microscope.

4.1.2.1 Flow cytometry analyses upon THP-1 differentiation

In addition, to confirm the differentiation of THP-1 monocytes into macrophages induced by PMA, flow cytometry analyses were conducted to look into the features of macrophage differentiation such as enhanced granularity, increased autofluorescence as well as increase in surface marker expression (Daigneault et al., 2010a). As for enhanced granularity, it is demonstrated by the increase in side scatter (SSC) on flow cytometry (Figure 4.3) where the SSC-A mean for THP-1 differentiated macrophages are higher than the monocytes. An additional distinguishing feature of macrophage differentiation is the existence of autofluorescence, which is notable in differentiated macrophages like alveolar macrophages but is limited in other myeloid cell varieties such as monocytes and dendritic cells (Maarten et al., 1994). The unstained THP-1 differentiated macrophages shows increased autofluorescence on flow cytometry compared to the monocytes which confirms successful macrophage differentiation (Figure 4.4).

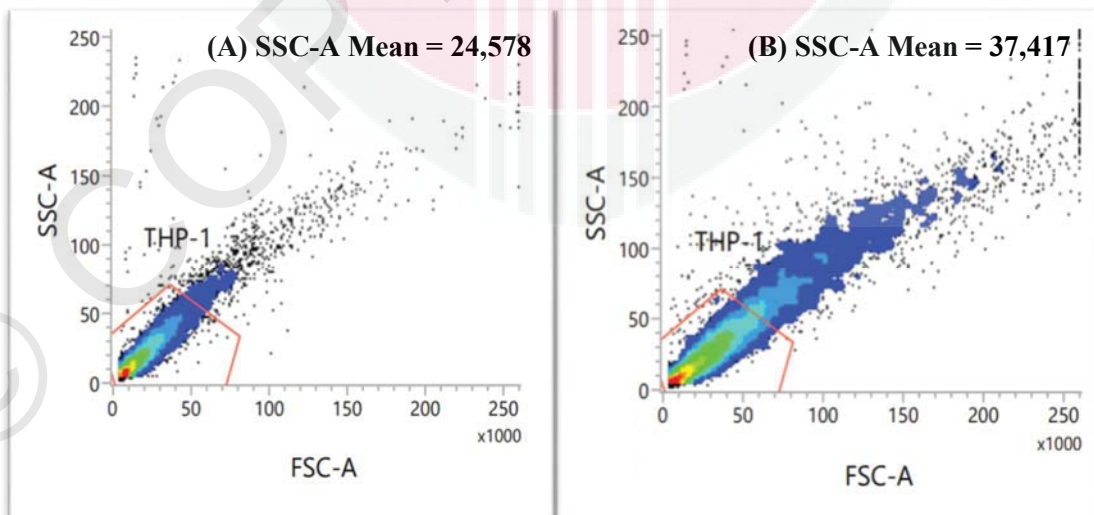


Figure 4.3: Increased in complexity upon THP-1 differentiation. Side light scatter and forward light scatter plot with the mean fluorescence intensity shown in the upper right-hand corner for (A) THP-1 monocytes and (B) THP-1 differentiated macrophages.

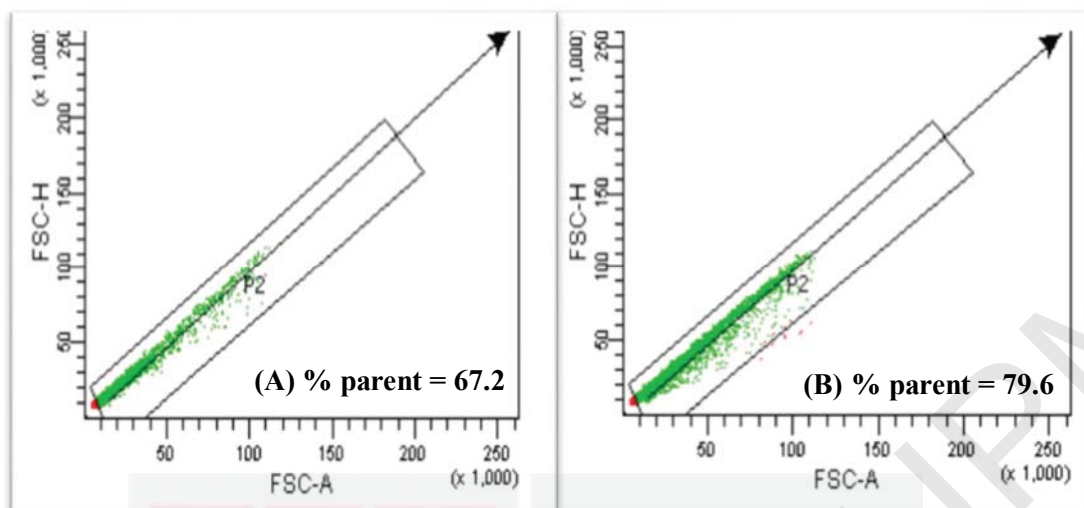


Figure 4.4: Increased in autofluorescence upon THP-1 differentiation. Forward light scatter plot with the % parent value shown in the upper-right hand corner for (A) unstained THP-1 monocytes and (B) unstained THP-1 differentiated macrophages.

As for the THP-1 macrophage surface marker expression, CD11b expression level was observed to be multiplied upon differentiation supporting the terminal differentiation on THP-1 monocytes after PMA treatment (Figure 4.5).

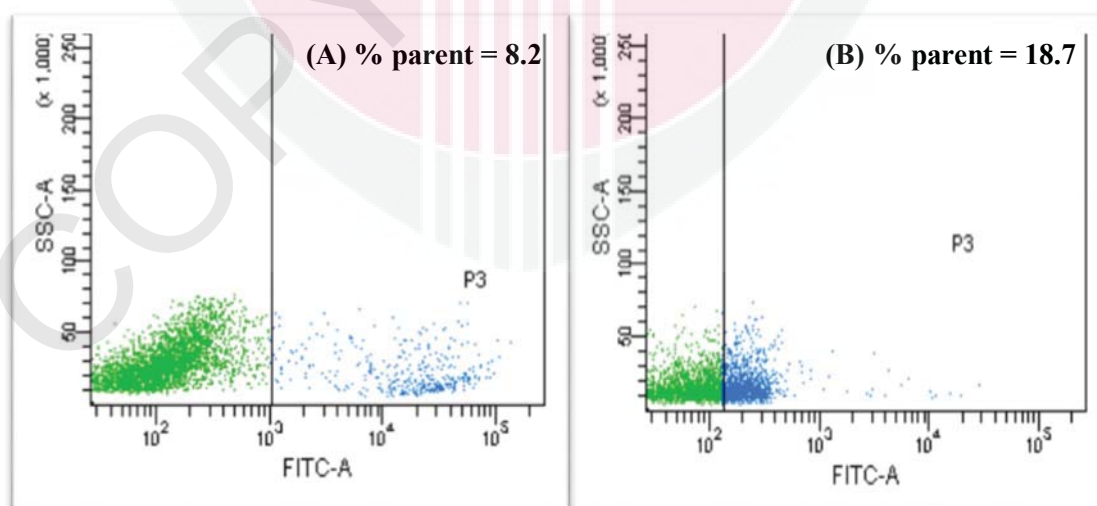


Figure 4.5: CD11b marker expression on (A) THP-1 monocytes and (B) THP-1 differentiated macrophages with % parent value shown on the upper right-hand corner.

4.1.2.2 Optimization of THP-1 macrophages and *Candida glabrata* co-culture conditions

In order to mimic the host and pathogen interactions environment, the co-culture conditions of THP-1 macrophages with *C. glabrata* was optimised from the aspects of fungus-macrophage ratio and duration of co-incubation. From the results obtained, for the cell ratio, 10:1 ratio of THP-1 macrophages to *C. glabrata* was chosen and fixed prior to conducting every other assays later. Besides, for the period of co-incubation, it was optimized to be 48 h of co-incubation upon *C. glabrata* being phagocytosed by THP-1 macrophages. The cell ratio and co-incubation duration was set based on the percentage of survival ratio of the *C. glabrata* upon phagocytosis (Figure 4.6).

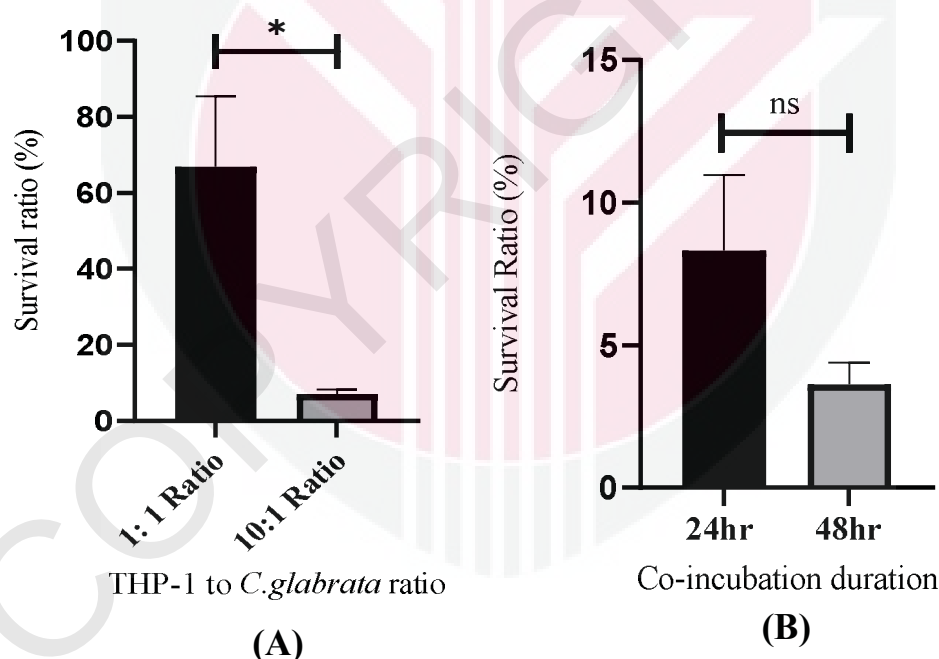


Figure 4.6: Optimisation of THP-1 macrophages and *C. glabrata* co-culture conditions in (A) THP-1 macrophages and *C. glabrata* ratio and (B) duration of co-incubation. The findings are displayed as means $\pm$ standard deviation. Statistical significance was denoted by * for $p < 0.05$ and by ns for $p > 0.05$, indicating significance and insignificance, respectively, compared to the *C. glabrata* control. The results obtained are the mean of triplicates from three independent experiments.

4.2 *Candida glabrata* cell wall biochemistry

4.2.1 Transmission electron microscopy (TEM) observation on *Candida glabrata* cell wall architectures

The cell wall architecture of *C. glabrata* cells grown exposed to human cell line, THP-1 macrophages or without any immune exposure was examined by transmission electron microscopy (TEM). The results obtained unveiled significant variances in cell wall composition, including the thickness of the inner (β -glucan and chitin) and outer cell wall layer (mannan), along with its density (refer to Figure 4.7). The inner cell wall layer of THP-1 exposed *C. glabrata* cells were thinner where the β -glucan and chitin layer happened to be significantly reduced. Conversely, the thickness of the outer layer (mannan fibrils) of THP-1 exposed *C. glabrata* dramatically increased and displayed denser structures (Figure 4.8). In terms of observing the cell membrane layer, it cannot be visualized under the current TEM magnification (25000x), as a higher magnification is necessary for adequate observation (50,000x to 200,000x) (Baxa, 2018).

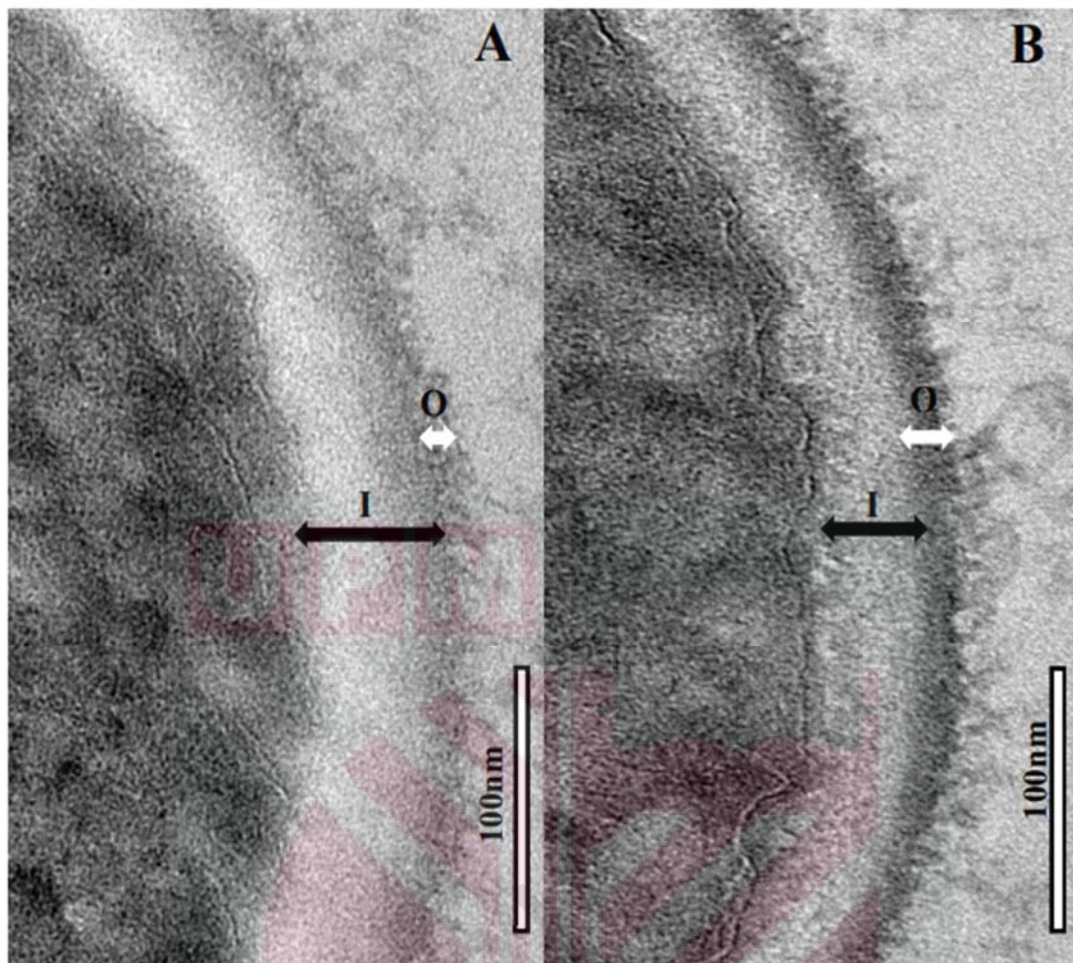


Figure 4.7: Exposure of THP-1 macrophages induces changes in the ultrastructure of the *C. glabrata* cell wall. Representative TEM images illustrating the cell wall structures of *C. glabrata* following exposure to THP-1 macrophages, where (I) represents the inner cell wall layer (β -glucan and chitin) and (O) represents the outer cell wall layer (mannan). Scale bar represents 100nm (original magnification at 25000x). (A) *C. glabrata* control, (B) *C. glabrata* that has been exposed with THP-1 macrophages.

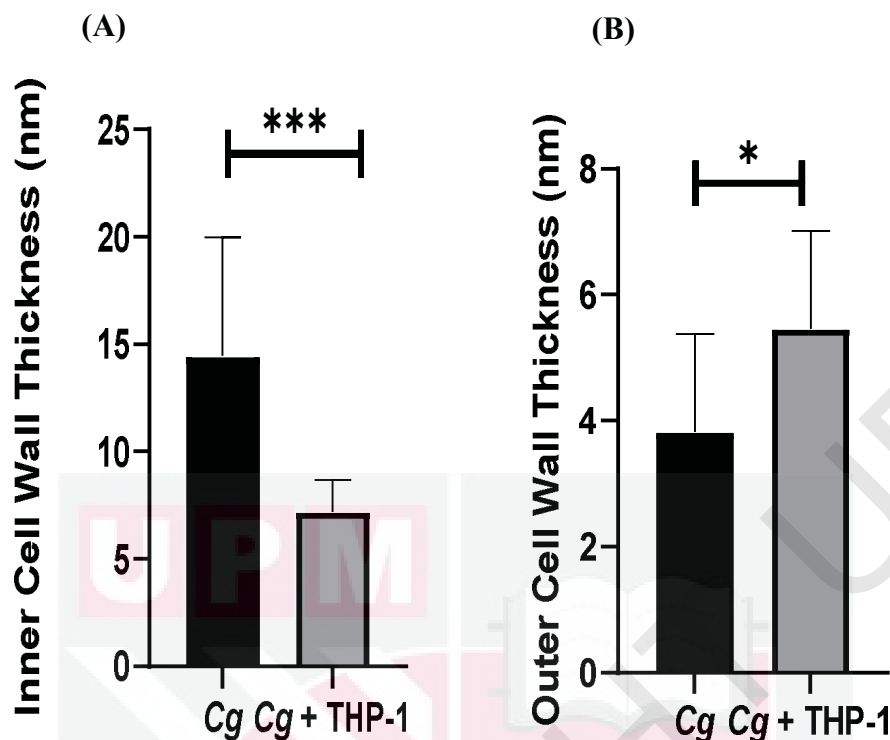


Figure 4.8: Exposure to THP-1 macrophages leads to changes in the thickness of the *C. glabrata* cell wall. (A) Quantification of the inner cell wall thickness. (B) Quantification of the outer cell wall thickness. The data is depicted as means $\pm$ standard deviation derived from 10 individual cells, averaging 20 measurements taken from the cell periphery of each *C. glabrata* cell. Statistical significance was indicated by * for $p < 0.05$ and * for $p < 0.001$ compared to the *C. glabrata* control group not exposed to THP-1 macrophages.**

4.2.2 Flow cytometry analysis on *Candida glabrata* cell wall components

A triple-staining technique was employed to fluorescently mark the distinct layers of the *C. glabrata* cell wall: chitin, β -glucans, and mannans. Chitin was specifically labeled using Calcofluor White (CFW). β -glucans were identified by protein Fc-Dectin1 conjugated anti-human Alexa Fluor 488, while mannans were labelled by Concanavalin A conjugated Alexa Fluor 647. Fungal cells exposed to THP-1 macrophages and unstressed controls were stained for all three cell wall components and then underwent quantitative assessment of each component using flow cytometry. The fluorescent dyes used exhibit minimal spectral overlap, enabling concurrent

analysis. Figure 4.9 illustrates the distinct alterations observed in the cell wall composition of chitin, β -glucan and mannan. Upon THP-1 exposure, *C. glabrata* showed a significant increase in mannan levels (2-fold) when compared to unstressed cells. Meanwhile, the β -glucan layer, which constitutes the immunogenic component of *C. glabrata* cell wall, was significantly reduced (by 0.5-fold) following exposure to THP-1 macrophages. Chitin, being a more stable and robust component of the cell wall, is less likely to undergo rapid fluctuations during immune exposure (H. E. Brown et al., 2020), therefore no significant changes in the chitin layer levels beyond the standard deviation were observed.

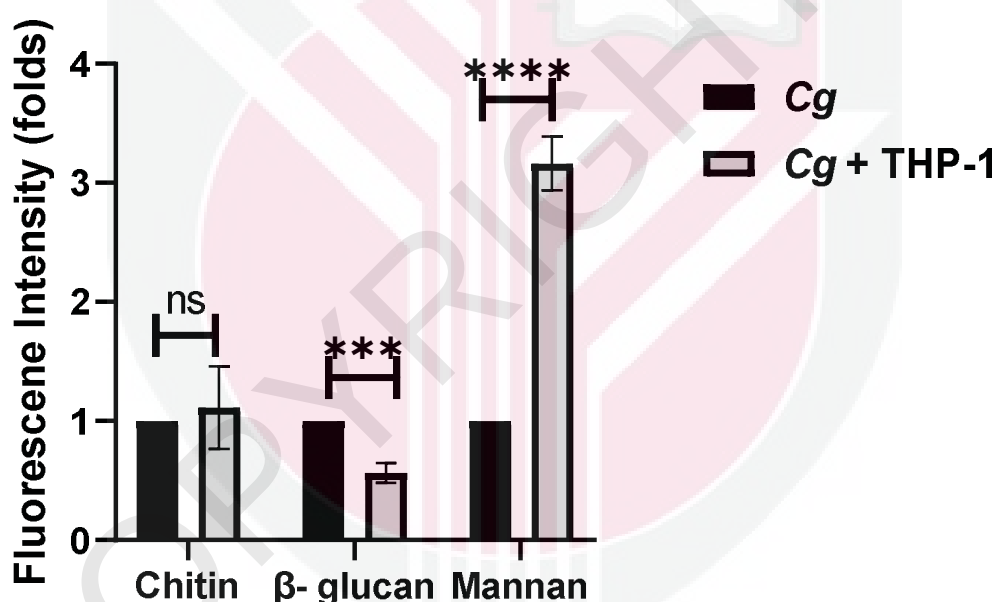


Figure 4.9: Quantitative analysis of cell wall components in *C. glabrata* by flow cytometry. Quantification is presented as fold changes in fluorescence intensity, with the unstressed control sample set to 1. The displayed data represent the outcomes of three separate replicates, with error bars illustrating the standard deviation (SD). Horizontal bars with the asterisk mark significant differences between the *C. glabrata* that has been exposed with THP-1 macrophage and control (ns $p > 0.05$, *** $p < 0.001$ and **** $p < 0.0001$)

4.3 Biophysical cell wall properties

4.3.1 Cell surface hydrophobicity assay

Considering the significant influence on cell wall ultrastructure, it was predicted that exposure of human macrophage cell line, THP-1 macrophages could also influence the biophysical properties of the *C. glabrata* cell wall. Cell surface hydrophobicity was examined using the microbial adhesion assay to hydrocarbons (MATH) assay. Hydrophobicity is another characteristic that is usually related with biofilm formation. *Candida glabrata* that has been exposed to THP-1 macrophages displayed higher percentage value of cell surface hydrophobicity (Figure 4.10).

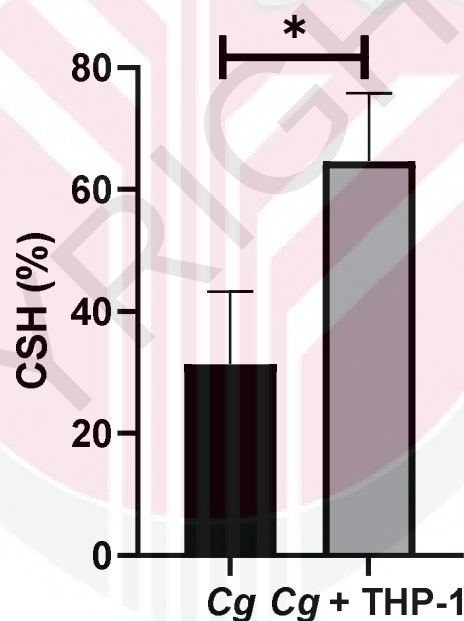


Figure 4.10: *Candida glabrata* hydrophobicity was measured according to MATH assay. Results are presented as means $\pm$ SD. * $p < 0.05$ was considered statistically significant relative to *C. glabrata* control. The results obtained are the mean of triplicates from three independent experiments.

4.3.2 Cell adhesion assay

Adhesion plays a pivotal role in the virulence profile of *C. glabrata*, significantly contributing to the colonisation of tissues and the formation of biofilms on medical devices implanted in hospital patients (Ciurea et al., 2020). The percentage of relative adhesion of *C. glabrata* that has been exposed to THP-1 macrophages were reported to be higher indicating that it is more virulent than the *C. glabrata* control despite the fact that the results are statistically insignificant (Figure 4.11).

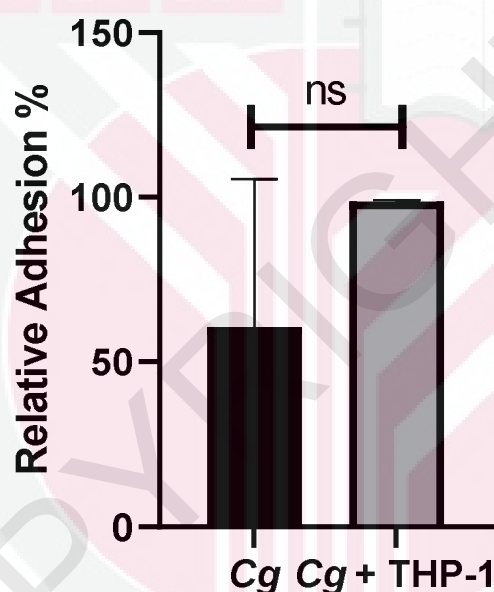


Figure 4.11: Relative adhesion of *C. glabrata* cells to a non-treated polystyrene plastic surface. Results are presented as means $\pm$ SD. ns $p > 0.05$ was considered statistically insignificant relative to *C. glabrata* control. The results obtained are the mean of triplicates from three independent experiments.

4.3.3 Cell wall porosity assay

The porosity of *C. glabrata* cell wall was examined by employing an assay that relies on the leakage of UV-absorbing compounds induced by polycations from the cells. In this assay, the leakage caused by small polycations (e.g., poly-L-lysine, known to induce leakage irrespective of cell wall porosity) is contrasted with the release prompted by large polycations (like DEAE-dextran, leading to limited leakage depending on the cell wall's porosity level) (De Nobel et al., 1990b). The cell wall *C. glabrata* that has been exposed to THP-1 macrophages was significantly less porous than the *C. glabrata* control proving the fact that immune cell exposure has major impact upon biophysical properties of the *C. glabrata* cell wall (Figure 4.12).

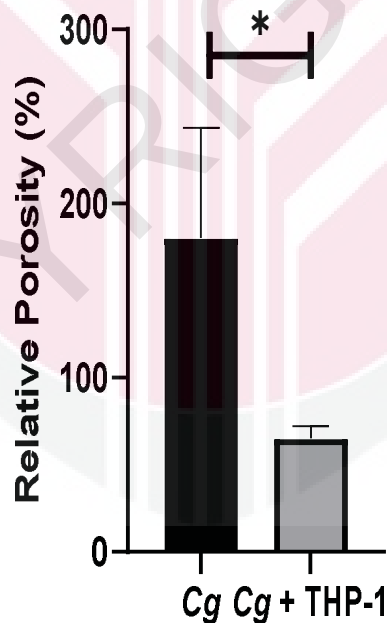


Figure 4.12: The relative cell wall porosity of THP-1 exposed *C. glabrata* cells conducted using polycation induced leakage of UV-absorbing compounds. Results are presented as means $\pm$ SD. * $p < 0.05$ was considered statistically significant relative to *C. glabrata* control. The results obtained are the mean of triplicates from three independent experiments.

4.4 Antifungal susceptibility assay

To investigate the impact of human macrophage cell line, THP-1 macrophages on the susceptibility of *C. glabrata* to antifungal drug, the capacity of *C. glabrata* cells exposed to THP-1 macrophages or without any immune cell exposure to withstand caspofungin, fluconazole and amphotericin B was examined using a broth microdilution assay. Importantly, THP-1 macrophage exposure decreased the sensitivity of *C. glabrata* cells to caspofungin, fluconazole, and amphotericin B, exhibiting varying levels of resistance to their fungicidal effects. The *C. glabrata* cells exposed to THP-1 macrophages demonstrated increased resistance to all three antifungals examined. Hence, findings from this study indicate that *C. glabrata* cells exposed to THP-1 macrophages in vitro may exhibit reduced sensitivity to antifungal treatments.

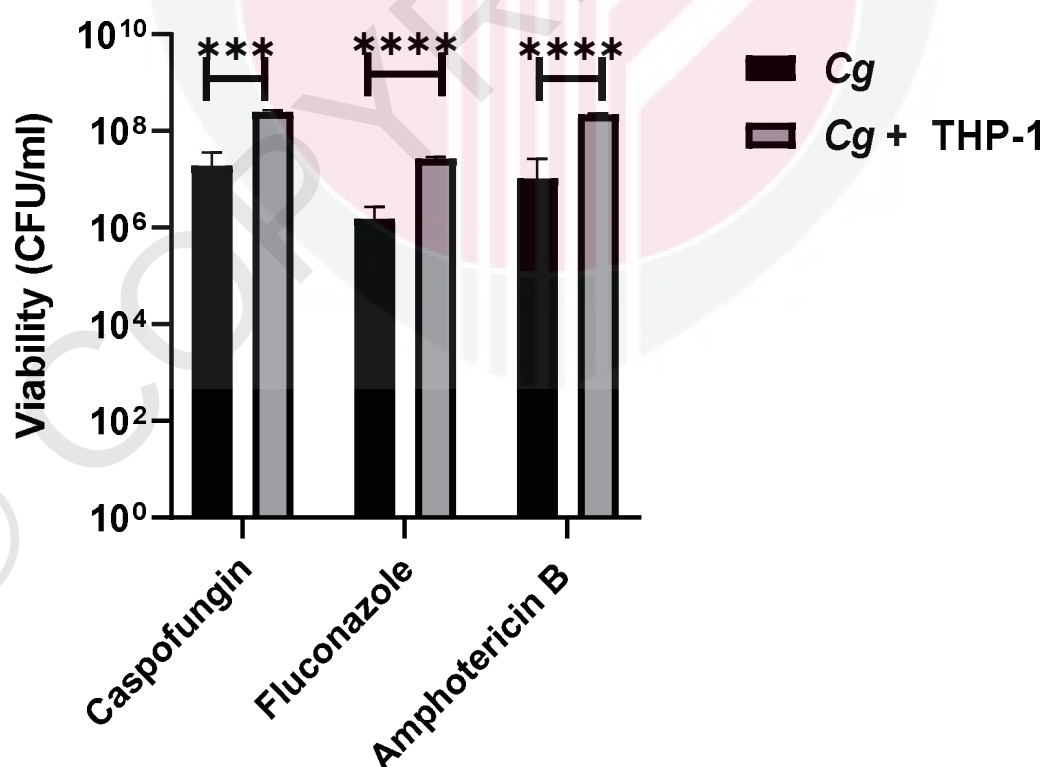


Figure 4.13: Exposure of THP-1 macrophages reduces the susceptibility of *C. glabrata* to caspofungin, fluconazole and amphotericin B. Results presented as means $\pm$ SD. * $p < 0.001$ and **** $p < 0.0001$ were considered statistically significant relative to *C. glabrata* control cells. Each experiment was performed in triplicate, and every individual experiment was repeated three times.**

4.5 Impact on immune response and recognition

4.5.1 Cytokine analysis

THP-1 macrophages exposed *C. glabrata* was examined whether it has an impact on the immune response and recognition, by measuring the release of pro- and anti-inflammatory cytokines. To test this, the levels of TNF- α , IL-6, IL-10 and IL-4 were measured in culture media of either uninfected THP-1 cells or THP-1 macrophages infected with *C. glabrata* control and THP-1 macrophages with *C. glabrata* that has prior immune exposure. As shown in Figure 4.14, *C. glabrata* infection led to no significant production in any pro- or anti-inflammatory cytokines in THP-1 macrophages except for IL-6.

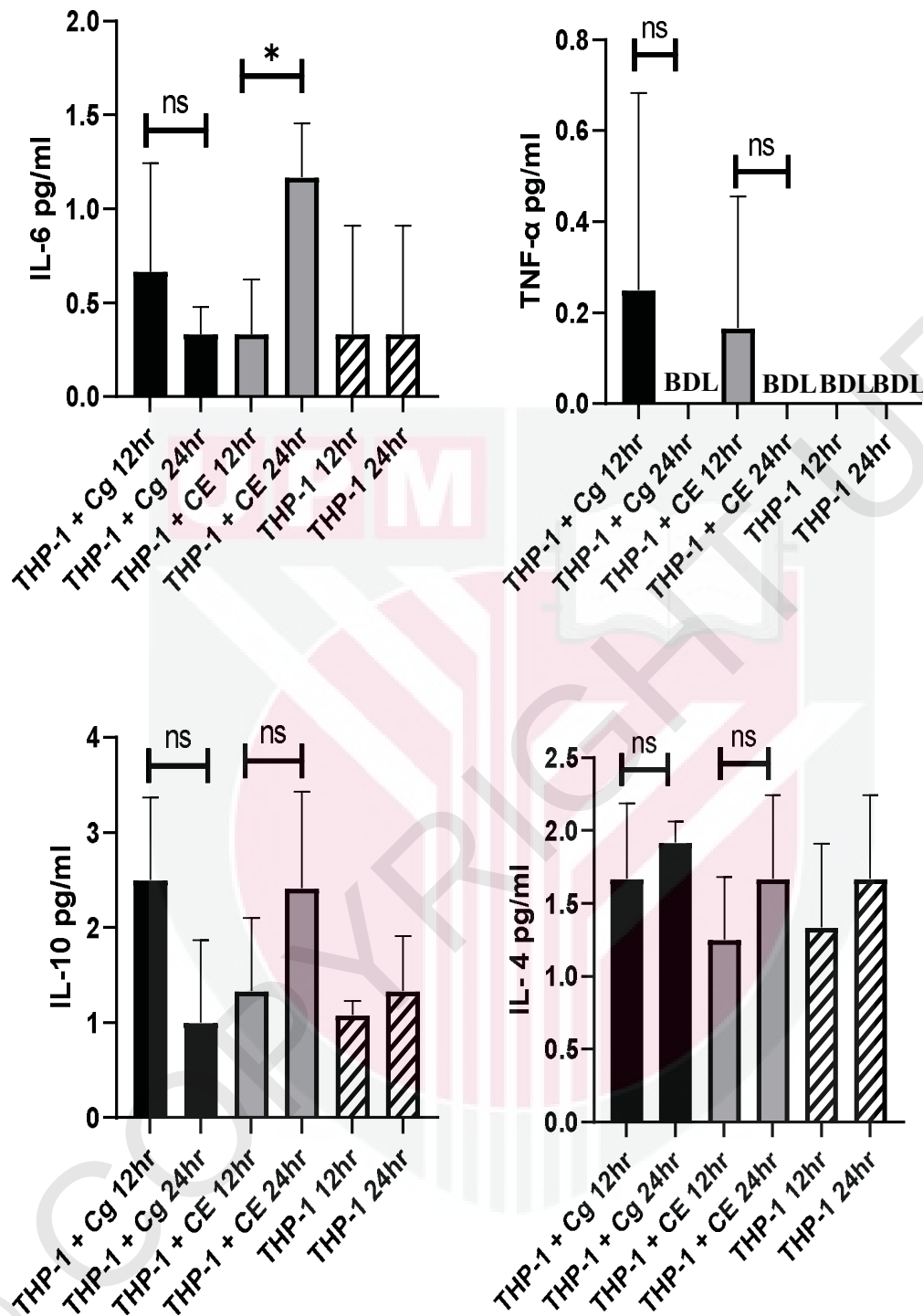


Figure 4.14: THP-1 macrophages exposure on *C. glabrata* suppresses the production of the pro- and anti-inflammatory cytokines. Expression of cytokines was evaluated in 100μL of culture medium using Human premixed multi-analyte kit® and determined by Luminex discovery assay. Values are presented as mean ± standard deviation. (ns $p > 0.05$, * $p < 0.05$). BDL = below the detection limit (TNF-α:7.41 pg/mL).

4.5.2 Phagocytosis assay

THP-1 macrophages infected by *C. glabrata* control and *C. glabrata* that has been exposed to THP-1 macrophages priorly at an MOI of 10 were used for flow cytometry. The percentage in uptake of intracellular *C. glabrata* yeasts were determined at 48 h of co-culture (Figure 4.15).

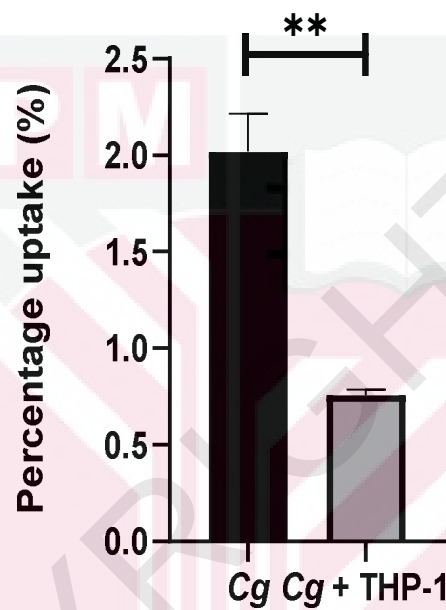


Figure 4.15: The uptake percentage of *C. glabrata* cells by THP-1 macrophages was assessed using flow cytometry. The average percentages of positive yeast cells $\pm$ standard deviation from three sets of independent experiments were presented. Asterisks denote data that exhibited significant differences (** $p < 0.01$) based on Student's t-test (unpaired).

4.5.3 Macrophage killing assay

As shown in Figure 4.16, the CFUs of *C. glabrata* cells get killed by THP-1 macrophages significantly reduced when the *C. glabrata* had a prior THP-1 exposure resulting in high viability of the cells. The results obtained complements the hypothesis made which was upon THP-1 prior exposure, *C. glabrata* will employ an immune evasion strategy and elicit the immune recognition and thus the number of *C. glabrata* getting killed is lower.

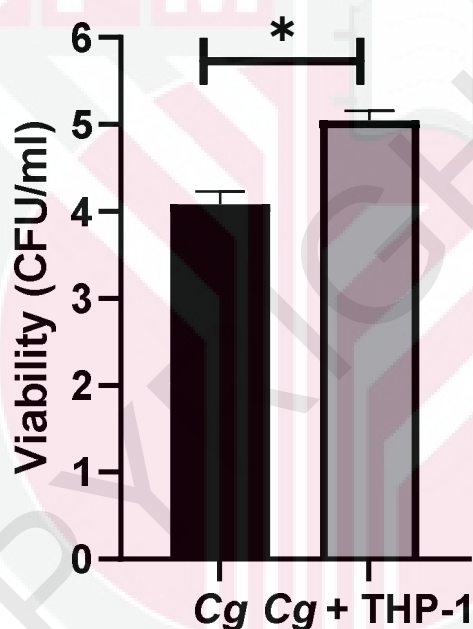


Figure 4.16: THP-1 cells killing assay. The study was conducted in triplicate, and the results are presented as mean $\pm$ standard deviation. Statistical analysis was performed using Student's t-test. (* $p < 0.05$).

CHAPTER 5

DISCUSSION

5.1 THP-1 ATCC (TIB-202)

THP-1 is a human monocytic cell line which was initially derived from the peripheral blood of a 1-year-old male patient diagnosed with acute monocytic leukaemia (Tsuchiya et al., 1980). This cell line has been extensively employed in the study of immunological responses wherein the cells exhibit both monocyte and macrophage-like characteristics (Daigneault et al., 2010b). THP-1 cells exhibit characteristics resembling both differentiation and morphology akin to primary monocytes. Specifically, the morphological attributes of individual, spherical, and suspended THP-1 cells transition into flat and amoeboid-shaped macrophages upon differentiation (Qin, 2012). Compared to primary monocytes, the THP-1 cell line exhibits a more consistent genetic profile due to its derivation from a singular origin. This genetic homogeneity minimizes the variability in cell characteristics that typically arise during the transformation of monocytes into macrophages (Pauls et al., 2018).

5.1.1 Differentiation of THP-1 monocytes

Standard protocols are commonly utilized to prompt macrophage differentiation in monocytic cell lines; however, achieving a fully differentiated macrophage phenotype has not been a primary focus in prior research endeavors. A study conducted by Richter et al, (2016) , reported that the rearrangement of the kinome and the rewiring of MAPK pathways are indicative of monocyte-to-macrophage differentiation. Additionally, it was also identified that the protein kinase TAK1 as a central signalling hub and master regulator governing macrophage function. In this study, several macrophage

characteristics related to differentiation in THP-1 cells was confirmed when subjected to PMA before using the cells for the rest of the assays. These characteristics include the reduction in the nucleocytoplasmic ratio followed by an increase in cytoplasmic volume, enhanced granularity, increased autofluorescence as well as the increase in surface marker expression. Generally, macrophages are five to ten-fold larger in size compared to monocytes, therefore upon PMA treatment, an increase in cytoplasmic volume was observed in the differentiated THP-1 macrophages (Figure 4.2). Moreover, treatment with PMA induces a macrophage-like phenotype characterized by enlarged cells that exhibit increased complexity. This transformation of THP-1 cells towards a more granular phenotype under PMA influence is evident through heightened complexity (SSC) as observed in Figure 4.3. Additionally, autofluorescence refers to the intrinsic emission of light by biological structures like mitochondria and lysosomes after absorbing light and upon THP-1 differentiation, and the unstained THP-1 macrophages emitted more autofluorescence in comparison to the THP-1 monocytes (Figure 4.4). Besides, CD11b is a macrophage marker that was reported to be upregulated during differentiation (Duan et al., 2016) and as anticipated upon PMA treatment, the expression of CD11b marker was reported to be increased (Figure 4.5).

5.1.2 Co-culture conditions of THP-1 macrophages and *Candida glabrata*

Some studies have shown that innate immune cell (THP-1 human macrophages) exposure affect the host-pathogen interaction of *C. glabrata* due to the ability of *C. glabrata* that can survive and even replicate inside the macrophage while causing surprisingly low damage and cytokine release (Rai et al., 2013; Starr et al., 2018b). Immune evasion strategies employed by *C. glabrata* is known to be essential to evade

or elicit the immune response (Kasper et al., 2015). However, the impact of innate immune cell exposure on cell wall properties, antifungal susceptibility and host-pathogen interactions has not been addressed. Therefore, the effect of innate immunity on *C. glabrata* cell wall properties & host-pathogen interactions were examined in this study.

To establish a balanced environment simulating interactions between host and pathogen, the co-culture conditions of THP-1 macrophages with *C. glabrata* were optimised based on the fungus-macrophage ratio and duration of co-incubation upon phagocytosis. As for the fungus-macrophage ratio, 10:1 of THP-1 macrophages to *C. glabrata* was reported to be optimum as the survival ratio of *C. glabrata* was more reflective of the condition in patients with invasive candidiasis. This was to maintain the capability of THP-1 macrophages to carry out phagocytosis efficiently without getting exposed to overwhelming number of *C. glabrata* at a time. Secondly, the survival ratio of *C. glabrata* after it has been exposed to THP-1 for 48 h is lesser than 24 h of exposure. Therefore, it was postulated that more *C. glabrata* were killed by macrophages after 48 h of co-incubation and the changes induced by this time point might be more significant than 24 h co-incubation.

5.2 *Candida glabrata* cell wall biochemistry

The fungal cell wall serves as a crucial organelle, offering structural integrity and regulating permeability to maintain periplasmic protein retention. This serves as a protective barrier against host-damaging enzymes and physical strain, playing a crucial role in the interactions between the host and fungus that facilitate the onset of disease (Gow & Lenardon, 2023). Serving as the initial contact point between host and yeast

cells, the cell wall facilitates interactions with the external environment via fungal adhesins and host receptors (Garcia-Rubio et al., 2020).

Several factors, such as carbon sources, pH variations, and micronutrient restrictions, trigger cell wall remodelling in *C. glabrata* (Chew et al., 2019b; Sherrington et al., 2017). Exposure of innate immune cells (THP-1 macrophages) also induces changes in cell wall architecture of *C. glabrata* (Figure 4.7 and Figure 4.8). Findings from this research reveal that when THP-1 macrophages are exposed to it, the cell wall of *C. glabrata* shows a decrease in the thickness of the inner cell wall and an increase in the thickness of the outer mannan layer. A thicker outer layer of mannan in the cell wall is commonly linked to better shielding of the inner glucan and chitin layers. One plausible theory suggests that immune cell exposure triggers changes in the cell wall, modifying the mannan structure and concealing the inner cell wall layer, which is essential for immune response activation. Additionally, the elongated mannan fibrils seen in the outer cell wall could be due to shifts in the balance between extending and branching mannan side chains, influenced by environmental signals that impact the structure of mannan fibrils (Lowman et al., 2011b). Certainly, the initiation of the cell wall integrity pathway is a common response to environmental pressures in human fungal pathogens (Munro et al., 2007; Yoshio Okawa & Kougi Goto, 2006). Moreover, the intensity of outer mannan layer in Figure 4.7 for *C. glabrata* that has prior THP-1 exposure observed to be more intense compared to *C. glabrata* control. As per Hawkes (2017), variations in mass or thickness within the sample can alter the amplitude of transmitted electron waves. Areas with differing mass or thickness will engage differently with the electron beam, leading to disparities in electron intensity and contrast that are evident in the generated images. Therefore, the increased intensity of the thicker outer mannan layer in Figure 4.7B is attributed to its more densely packed

arrangement. The mannan layer's density and arrangement play a crucial role in concealing or exposing β -glucan, impacting the nanoscale geometry and interactions within the cell wall.

The immunogenicity of β -glucans arises from their molecular configuration, allowing them to be recognized as PAMPs by PRRs present on neutrophils, macrophages, and dendritic cells, such as toll-like receptors, dectin-1, CR3, and CD5. These engagements trigger intracellular signaling cascades, prompting the production of immune molecules that govern both innate and adaptive immune responses (Vetvicka et al., 2020). The somewhat limited quantities of alkali-insoluble glucans in *C. glabrata* imply fewer connections between glucan and chitin, making up around 20% of the cell's dry weight. This suggests a less dense glucan framework and a tightly packed outer layer of mannoproteins inherent to *C. glabrata*. As a result, β -glucans present in the cell wall could potentially provide efficient concealment from host immune detection facilitated by the dectin-1 receptor (De Groot et al., 2008c; Rodríguez-Pena et al., 2010). Therefore, masking the inner glucan layer has been one of the immune evasion strategies used by *C. glabrata* which also concurrently proved in our flow cytometry analysis data (Figure 4.9) that records increase in MFI folds in mannan and significant reduction in the inner cell wall layer.

5.3 Biophysical cell wall properties of *Candida glabrata*

Besides cell wall biochemistry, the biophysical cell wall properties of *C. glabrata* also have been studied in this study by conducting the cell surface hydrophobicity (CSH) assay, cell adhesion assay as well as cell wall porosity assay. The primary stage of *Candida* infection begins with the attachment to host surfaces, a critical process for the initial colonization and subsequent biofilm development. Microbial adhesion to surfaces involves multiple elements, such as the presence of particular cell surface glycoproteins (Nobile et al., 2006). Yeast cell surface hydrophobicity (CSH) contributes to the adherence process of yeasts, attributed to the presence of hydrophobic proteins (Epa family) within the cell wall matrix beneath an outer fibrillary layer (Hazent, 1989; Naglik et al., 2003). Based on Figure 4.10, the results shows that the *C. glabrata* that has been exposed to THP-1 macrophages have higher percentage of cell surface hydrophobicity compared to the *C. glabrata* control. Comparatively speaking, hydrophobic cells have a stronger propensity to co-adhere than hydrophilic ones (Luo & Samaranayake, 2002). Therefore, upon exposure to THP-1 macrophages, *C. glabrata* changes to display more virulent characteristics i.e., adhering with increased CSH, which is the first step in biofilm formation. The highly hydrophobic cells exhibit greater tendencies to adhere and cluster, resulting in the formation of denser biofilms. These biofilms are more resistant to antifungal treatments and immune system attacks. The protective nature of biofilms shields fungal cells, contributing to chronic infections and complicating treatment outcomes (Frías-De-león et al., 2021).

Consistent with CSH assay, the results from cell adhesion assay (Figure 4.11) also shows the same trend with *C. glabrata*. The primary adhesin family involved in *C.*

glabrata virulence is the EPA (Epithelial Adhesin) family, which consists of several genes (*EPA1*, *EPA6* and *EPA7*) that promote adherence to host tissues, a critical step for colonization and infection (Rodrigues et al., 2017). The relative adhesion of *C. glabrata* exposed to THP-1 macrophages was found to be higher than the *C. glabrata* control. Plastic surfaces and yeast cells, carry a negative surface charge, which typically implies a repulsive force (Klotz et al., 1985). Nevertheless, *C. glabrata* has been observed to adhere effectively to abiotic surfaces. The adhesion strength of *C. glabrata* cells is significantly weaker towards a hydrophilic surface compared to a hydrophobic surface (Luo & Samaranayake, 2002). In line with these findings, several studies have noted a strong association between the increased adherence capability of *C. glabrata* cells to abiotic surfaces and a high CSH (El-Kirat-Chatel et al., 2015). Therefore, adherence to an inert material is facilitated through hydrophobic interactions, commonly referred to as van der Waals forces (De Groot et al., 2008d; Gómez-Molero et al., 2015).

On the other hand, a measure of cell wall structure is its porosity, which indicates the ability for molecular movement within the wall and is associated with the spacing between wall polysaccharides. Porosity is linked to the digestibility of the cell wall and its efficiency in saccharification (Stephanopoulos, 2007). As for the cell wall porosity assay conducted, it was observed that the relative porosity of the *C. glabrata* that has been exposed to THP-1 macrophages was significantly lower than the *C. glabrata* control, demonstrating that the immune cell exposure significantly influences the biophysical properties of the *C. glabrata* cell wall. The decreased porosity observed in THP-1-exposed *C. glabrata* correlated with the heightened presence of mannan fibrils (Figure 4.8), aligning with past findings linking increased porosity to shorter mannan side chains (De Nobel et al., 1990b). A robust mannan layer can form

a dense, protective barrier that may decrease porosity and restrict the diffusion of substances, including antifungal agents (Lima et al., 2019).

5.4 Antifungal susceptibility assay

The sensitivity of *C. glabrata* that been exposed to immune cells and *C. glabrata* control to antifungal drugs were compared. Caspofungin, an echinocandin that hinders β -1,3-glucan synthase; amphotericin B, a polyene that interferes with plasma membrane function by binding to ergosterol; and fluconazole, an azole that obstructs the conversion of lanosterol to ergosterol by binding to fungal cytochrome P-450 (Ene et al., 2012b) were evaluated. Antifungal susceptibility testing conducted through broth microdilution assays demonstrated a heightened tolerance of the *C. glabrata* that has been exposed to THP-1 macrophages to all three class of antifungals; echinocandin, polyene and azole compared to the *C. glabrata* control (Figure 4.13). This reduced antifungal activity may be associated with cell wall porosity, as a less porous cell exposed to immune cells might restrict the entry of these drugs, contributing to resistance. The cell wall functions as a protective barrier, and its porosity influences the ease with which antifungal agents can penetrate the cell (G. D. Brown et al., 2018).

The emergence of echinocandin resistance in *Candida* species typically necessitates prolonged or recurrent exposure to drugs, although it can also arise quickly after beginning treatment (Fekkar et al., 2014). During treatment, acquired resistance arises and entails alterations in amino acids within critical regions of the Fks subunits of glucan synthase. The action of echinocandins triggers diverse cellular stress response pathways, leading to the development of drug-adapted persister states that can eventually give rise to FKS-resistant mutants (Perlin, 2015). In this study, the results

from Figure 4.13 shows that, upon THP-1 macrophage exposure, the *C. glabrata* strain observed to be more resistant towards caspofungin drug compared to the *C. glabrata* control with no immune cell exposure. This observation can be supported by the TEM observation (Figure 4.7) as well as the flow cytometry quantification of cell wall components (Figure 4.8) where the β -glucan layer of the *C. glabrata* upon THP-1 exposure reported to be reduced significantly and observed to be masked by the thicker outer mannan layer. The changes are likely to increase the cell resistance towards caspofungin since this drug specifically targets the β -1,3-glucan synthase as its mode of action. According to Wagner et al., (2023), decrease in β -glucan levels might influence alterations in cell wall structure and makeup, consequently impacting *Candida* susceptibility to caspofungin and potentially causing resistance to develop. Besides, thinner β -glucan layers also may reduce the drug's ability to bind effectively to the cell wall, leading to decreased susceptibility.

Recent discoveries indicate that amphotericin B (polyenes) exerts its fungicidal action through various mechanisms. Specifically, by inducing ergosterol sequestration, leading to changes in the cell membrane that ultimately result in cell death (Palacios et al., 2007). Additionally, amphotericin B also triggers a notable accumulation of reactive oxygen species (ROS), a phenomenon associated with cellular damage, induction of apoptosis, and eventual cell death (Phillips et al., 2003). In contradictory, a recent study conducted by Mesa-Arango et al., (2016) discovered that, amphotericin B resistant strains present an enlarged cell wall and these alterations are associated with heightened detection of cell wall β -1,3-glucans in amphotericin B resistant yeast strains and with elevated immune activation compared to susceptible isolated. Despite reporting a decrease in inner cell wall layer (Figure 4.8), the resistance of *C. glabrata* that has been exposed to THP-1 macrophages towards all the antifungals (Figure 4.13)

in this study might be due to the successful β -glucan masking strategy by an increase in outer mannan layer that employed by *C. glabrata* upon immune exposure to evade cell death.

As for the fluconazole used, mechanisms such as ergosterol biosynthesis modification, antifungal drug efflux, and alteration in the drug target's affinity are proposed as contributing factors to resistance against azole antifungal drugs (De Marañon et al., 1996). A study conducted by Angiolella et al., (2002), reported the relationship between the fluconazole and glucan associated proteins. In drug-susceptible strains, the exposure to fluconazole led to a significant reduction in the presence of enolase and one form of phosphoglycerate mutase in the cell wall, accompanied by an increase in the abundance of two glucanases and a higher amount of polydisperse, highly glycosylated material. Overall, there was a relative increase in the glycosylated components and enzymes related to β -glucan metabolism. As β -glucanases may possess transglycosylase activities as well, the observed enrichment could indirectly indicate consistent rearrangements and modifications of the cell wall structure (Stubbs et al., 1999). This finding also aligns with previous reports suggesting the influence of fluconazole on cell wall β -glucanases, which is associated with reduced resistance to phagocytosis (Hazen et al., 2000).

5.5 Impact on immune response and recognition

Subsequently, in order to investigate whether prior THP-1 macrophages exposure has an impact on immune response and recognition of *C. glabrata*, cytokine analysis, phagocytosis assay as well as macrophage killing assay were employed. Two pro-inflammatory cytokines; IL-6, TNF- α and two anti-inflammatory cytokines; IL-10 and IL-4 were analysed using Human premixed multi-analyte kit. The Luminex discovery

run was successful since the standard curve has an R² value more than 0.995, with a percentage of recovery that falls within the range (70% - 130%) and percentage of CV of replicate that is less than 20%. The results obtained (Figure 4.14) shows a common pattern for IL-6 and IL-10, where the THP-1 that has been co-incubated with *C. glabrata* control for 12 h records a higher cytokine release compared to the THP-1 infected with the same control for 24 h despite the reading are statistically insignificant. This might be due to the accelerated immune reaction upon infection that subside after passing the half-life of the cytokine (IL-6 = 15.5 h and IL-10 = 4.6 h). While on the other hand, THP-1 that was co-incubated with *C. glabrata* that has prior immune exposure tend to have a slower start but happen to release more cytokine at 24 h which was significant for IL-6 but insignificant for IL-10. This phenomenon might be due to increase in outer mannan layer that mask the immunogenic part of the *Candida*, β -glucan (Figure 4.7) that might hinder the immune recognition as well as the immune response. As for the IL-4, the same pattern as IL-6 and IL-10 was observed for the THP-1 co-incubated with *C. glabrata* control but not for the *C. glabrata* that has prior immune exposure. As for the TNF- α , the results showed more reading that was below the detection limit. Despite the fact that *C. glabrata* infection generally reported to have no significant production of any cytokine in THP-1 macrophages, a study conducted by (Rasheed et al., 2018b) have second that but also reported a 1.4-fold higher production of IL-1 β in WT-infected macrophages compared with uninfected macrophages. Besides, research conducted by (Seider et al., 2011d) have also reported that *C. glabrata* infection led to significant intracellular proliferation but no evident macrophage damage or apoptosis reported and the production of reactive oxygen species was suppressed. Moreover, apart from GM-CSF, levels of both pro-inflammatory and anti-inflammatory cytokines exhibited only minor increases. In

addition, a study by Li and colleagues (Li et al., 2007) have also reported that except for GM-CSF, *C. glabrata* did not trigger any proinflammatory cytokine response in oral epithelial cells similar to that of *C. albicans* in mixed oral infection model. Therefore, no significant production in cytokine upon *C. glabrata* infection observed in this study might be due to the immune evasion strategies employed by *C. glabrata* that enable them to elicit the immune response.

As for the phagocytosis assay conducted, Figure 4.15 shows significant reduced percentage uptake by THP-1 macrophages in *C. glabrata* that has prior exposure with THP-1 macrophages compared to *C. glabrata* control. This might be due to the immune evasion strategy employed by *C. glabrata* that upon a prior immune exposure they alter its cellular structure i.e., cell wall and avoid being engulfed by the macrophages in the upcoming phagocytosis. In addition, some researchers also speculate that *C. glabrata* may preferentially undergo cellular uptake and proliferation within macrophages as an immune evasion strategy to evade immediate elimination by neutrophils (Jacobsen et al., 2010b). Besides, a sequence of studies has provided insight into how modifications in *Candida* cell wall composition, morphogenesis, and the activation state of macrophages influence the overall phagocytic uptake process (McKenzie et al., 2010; Mora-Montes et al., 2011). Recent study conducted by Lewis and co-authors (Lewis et al., 2012) have also reported that *Candida* strains lacking O-linked and N-linked mannan are taken up at higher rates by macrophages and that goes well together with the findings in this study (Figure 4.8 and Figure 4.15) where the *C. glabrata* that have been exposed to THP-1 macrophages has higher mannan level and lower percentage uptake by macrophages.

The macrophage killing assay conducted shows the same trend as the phagocytosis assay where the viability of *C. glabrata* which has a prior immune cell exposure happened to be resisting killing by macrophages compared to *C. glabrata* control. Apart from implementing intracellular survival and blocking phagosomal acidification as an evasion strategy, the *C. glabrata* with prior THP-1 macrophage exposure also shows modification in cell wall components as previously discussed in Section 5.2 where a study conducted by Netea and colleagues (Netea et al., 2008) reported that while cell wall mannans can conceal the recognition of underlying β -glucans within the cell wall, they also play a role in immune recognition and that they are essential for phagocytosis and influence the rate of macrophage killing once engulfed into macrophage phagosomes. Moreover, a decrease in cell wall O-mannan has been documented to lead to the exposure of β -glucan in the inner cell wall, enhancing recognition by Dectin-1, a factor associated with enhanced phagosome maturation (Bain et al., 2014). Therefore, enhanced cell wall mannan in *C. glabrata* that has been exposed to THP-1 macrophages are better at resisting killing by the macrophages and survive the immune attack.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1 Conclusion

This study confirmed that, upon immune cell (THP-1 macrophages) exposure, the cell wall composition, antifungal susceptibility and host-pathogen interaction of *C. glabrata* changes by altering the fungus into a more resistant strain, making it harder to be killed. This finding goes together with the reports in literature that mentions *C. glabrata* employs an immune evasion strategy to evade the host innate immune response which also makes the fungus harder to be treated clinically. The findings from this study imply that, as *C. glabrata* interacts with THP-1 macrophages, it modifies its cell wall biochemistry by thickening the outer mannan layer while decreasing the inner β -glucan and chitin layers. These changes affect the immunogenicity of the cell, making it less recognisable to host immune cells and consequently delaying the innate immune response. Moreover, the study also indicates alterations in the biophysical properties of the *C. glabrata* cell wall following exposure to THP-1 macrophages, resulting in increased hydrophobicity, enhanced adhesion, and reduced porosity. These changes contribute to increased virulence and immune response elicitation by the cell. Furthermore, the data generated from this study can enhance our comprehension of the differences in antifungal susceptibility between *C. glabrata* encountering antifungals for the first time and those with previous immune exposure. These variances are attributed to alterations in the cell wall composition, suggesting a need for further investigation into targeting the outer cell wall for antifungal drug purposes. In addition, the analysis of data from assays such as the macrophage killing assay, phagocytosis assay, and cytokine expression has provided

valuable insights into how immune response and recognition of *C. glabrata* are influenced by prior immune exposure compared to no prior exposure. Upon previous exposure to THP-1 macrophages, it is noted that *C. glabrata* elicits a distinct immune response characterized by a reduced rate of *C. glabrata* killing and a significantly lower percentage of uptake compared to the control group of *C. glabrata*. Based on the findings obtained in this study, it is concluded that this project has achieved its primary objective which is to elucidate the impact of innate immunity on *C. glabrata* cell wall properties and host-pathogen interactions.

6.2 Study limitations and recommendations for future research

The findings in this study present numerous opportunities for conducting future research in various avenues. The alterations noted in the cell wall composition, antifungal susceptibility, and host-pathogen interaction of *C. glabrata* following prior exposure to immune cells, as revealed in this study, provide valuable insights. However, these observations may not fully capture the genetic basis of these observable traits. Therefore, it would be worthwhile to conduct additional research involving genotypic analysis to explore the organism genetic composition, especially the key genes responsible for distinct traits and characteristics. In particular, the roles of antifungal resistance genes and associated cellular pathways have yet to be explored.

Additionally, the correlation observed between the heightened outer mannan layer in the cell wall and antifungal resistance in *C. glabrata* following prior immune exposure suggests that a thicker outer cell wall layer can impede antifungal agents from accessing the glucan layer or cell membrane efficiently. Consequently, focusing on distinctive cell wall components such as mannan in *Candida* species offers a promising

approach for creating innovative antifungal treatments that can efficiently target critical structures within the cell wall to combat fungal infections.



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APPENDICES

APPENDIX A

PREPARATION OF SOLUTIONS AND REAGENTS

Sabouraud dextrose agar (SDA)

Dissolve 65.0 g in 1 L of distilled water. Heat in boiling water and stir regularly until fully dissolved. Autoclave at 121°C for 15 minutes. Cool the agar to approximately 50°C to 60°C before pouring it into petri dishes. Let it solidify and store at 4°C until needed.

Sabouraud dextrose broth (SDB)

Dissolve 30.0 g in 1 L of distilled water. Blend the powder by shaking or gentle heating. Autoclave at 121°C for 15 minutes. Let the broth cool to room temperature and store at 4°C until needed.

1× Phosphate buffer saline (PBS)

Combine 1 part of 10× PBS (1st Base, Singapore) with 9 parts of distilled water. Gently invert multiple times to ensure thorough mixing.

Roswell Park Memorial Institute (RPMI) (MEDIA FOR MIC TEST)

Add a bottle of RPMI 1640 powder medium into autoclaved water. Stir until completely dissolved without heating. Rinse the RPMI powder bottle to remove any remaining powder and add it to the solution. Adjust the pH to around 0.1-0.3 units below the desired pH using 1N HCl or 1N NaOH. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns. Aseptically dispense the medium into sterile containers and store at 4°C.

APPENDIX B

CALIBRATION & VERIFICATION REPORT OF CYTOKINE ANALYSIS

Luminex

Calibration and Verification Report

20-Dec-23 2:24:46PM

Date/Time: 20-Dec-23 through 20-Dec-23

CAL1

Date	Time	Serial	User	Version		Lot	Pressure	Status
				Firmware	LXR			
12/20/2023	10:49 AM	LX100150414 01		2.6.1	2.1.495.0	B98559	7.93	Pass
Channel	Temp	Volt	DAC	R-Val	Result	Target	Status	
DD	24.39	93.34	1,499	689	10,029.00	10,100.00	Pass	
CL1	24.39	111.28	1,787	1,110	3,522.25	3,518.00	Pass	
CL2	24.83	114.89	1,845	1,233	3,674.73	3,669.00	Pass	
Final Result Code		The operation completed successfully. (0x00000000)						

Luminex

Calibration and Verification Report

20-Dec-23 2:24:49PM

MagCAL1

Date	Time	Serial	User	Version		Lot	Pressure	Status
				Firmware	LXR			
12/20/2023	10:49 AM	LX100150414 01		2.6.1	2.1.495.0	B98832	7.93	Pass
Channel	Temp	Volt	DAC	R-Val	Result	Target	Status	
DD	24.39	62.96	1,011	358	5,024.00	4,951.00	Pass	
CL1	24.39	111.28	1,787	1,110	3,518.94	3,518.00	Pass	
CL2	24.83	114.89	1,845	1,233	3,676.33	3,669.00	Pass	
Final Result Code		The operation completed successfully. (0x00000000)						

Luminex

Calibration and Verification Report

20-Dec-23 2:24:49PM

CAL2

ALL

Date	Time	Serial	User	Version		Lot	Pressure	Status
				Firmware	LXR			
12/20/2023	10:50 AM	LX1001504 1401		2.6.1	2.1.495.0	B98831	7.93	Pass
Channel	Temp	Volt	DAC	Result		Target		Status
RP1 (1)	25.91	517.46	2,119	3,702.93		3,698.00		Pass
RP1 (2)	25.91	628.82	2,575	16,859.30		16,825.90		Pass
Final Result Code		The operation completed successfully. (0x00000000)						

Calibration and Verification Report

20-Dec-23 2:24:49PM

Classification: CON1
System Status: Pass

Date 12/20/2023
Time 10:50 AM
Instrument Type LX100LX200
Serial No. LX10015041401
User xPONENT for LX100LX200 3.1.971.0
Software Version 2.6.1
Firmware Version 2.1.495.0
LXR Version 7.93
Pressure 25.91
Temperature 899503
Lot Number 12-Jan-25 12:00:00AM
Lot Expiration

Doublet Discriminator Peak

Reference	Target	Lower Tolerance	Upper Tolerance	Actual	Status
R1	10902	9212	12592	10475	Pass
R2	10948	9426	12470	10891	Pass
R3	11554	9382	13726	10896	Pass
R4	10411	8756	12066	10052	Pass
R5	10909	9229	12589	10460	Pass

Counts (microsphere events)

Reference	Target (Z)	Actual	Status
R1	1,000	1,000	Pass
R2	1,000	1,002	Pass
R3	1,000	1,028	Pass
R4	1,000	1,183	Pass
R5	1,000	1,004	Pass

RP1 Background (mean)

Reference	Tolerance (S)	Actual	Status
R1	100.0	1.1	Pass
R2	100.0	1.1	Pass
R3	100.0	1.7	Pass
R4	100.0	2.2	Pass
R5	100.0	1.6	Pass

Mean Fluorescent Intensity

Reference	CL1 Mean			CL2 Mean		
	Target	Lower Tolerance	Upper Tolerance	Target	Lower Tolerance	Upper Tolerance
R1	735.0	674.0	796.0	216.0	198.7	233.3
R2	72.2	46.6	97.8	71.3	65.5	77.1
R3	22927.0	20680.2	25173.8	5686.0	5033.3	6288.7
R4	2353.0	2150.6	2555.4	17389.0	16276.1	18501.9
R5	229.0	200.4	257.6	5790.0	5448.4	6131.6
						5735.7

Percent Coefficient of Variation

Reference	CL1 %CV			CL2 %CV		
	Tolerance (S)	Actual	Status	Tolerance (S)	Actual	Status
R1	8.5	4.5	Pass	9.6	4.6	Pass
R2	9.5	6.3	Pass	9.8	5.9	Pass
R3	7.6	4.9	Pass	9.1	5.1	Pass
R4	7.7	5.0	Pass	8.6	5.4	Pass
R5	8.7	5.0	Pass	9.1	4.8	Pass

Calibration and Verification Report

20-Dec-23

2:24:49PM

Classification: **MagCON1**
System Status: **Pass**

Date: 12/20/2023
Time: 10:51 AM
Instrument Type: LX100LX200
Serial No.: LX10015041401
User: sPONENT for LX100LX200 3.1.971.0
Software Version: 2.6.1
Firmware Version: 2.1.495.0
LXR Version: 7.93
Pressure: 25.91
Temperature: B68227
Lot Number: 10-Oct-24 12:00:00AM
Lot Expiration:

Doublet Discriminator Peak

Reference	Target	Lower Tolerance	Upper Tolerance	Actual	Status
R1	5344	4516	6172	5442	Pass
R2	5367	4621	6113	5237	Pass
R3	5664	4599	6729	5445	Pass
R4	5250	4415	6085	5028	Pass
R5	5348	4524	6172	5230	Pass

Counts (microsphere events)

Reference	Target (Z)	Actual	Status
R1	1,000	1,015	Pass
R2	1,000	1,000	Pass
R3	1,000	1,040	Pass
R4	1,000	1,139	Pass
R5	1,000	1,053	Pass

RP1 Background (mean)

Reference	Tolerance (S)	Actual	Status
R1	100.0	1.1	Pass
R2	100.0	1.1	Pass
R3	100.0	1.8	Pass
R4	100.0	1.9	Pass
R5	100.0	1.6	Pass

Mean Fluorescent Intensity

Reference	CL1 Mean			CL2 Mean		
	Target	Lower Tolerance	Upper Tolerance	Target	Lower Tolerance	Upper Tolerance
R1	735.0	674.0	796.0	216.0	198.7	233.3
R2	72.2	46.6	97.8	71.3	65.5	77.1
R3	22927.0	20880.2	25173.8	5686.0	5083.3	6288.7
R4	2377.0	2172.6	2581.4	17158.0	16059.9	18256.1
R5	229.0	200.4	257.6	5790.0	5448.4	6131.6

Percent Coefficient of Variation

Reference	CL1 %CV			CL2 %CV		
	Tolerance (S)	Actual	Status	Tolerance (S)	Actual	Status
R1	8.5	5.2	Pass	9.6	5.5	Pass
R2	9.5	6.3	Pass	9.8	5.7	Pass
R3	7.6	5.1	Pass	9.1	5.5	Pass
R4	7.7	5.0	Pass	8.6	5.2	Pass
R5	8.7	5.0	Pass	9.1	4.7	Pass

Calibration and Verification Report

20-Dec-23 2:24:49PM

Reporter: CON2
System Status: Pass
Date 12/20/2023
Time 10:51 AM
Instrument Type LX100LX200
Serial No LX10015041401
User
Software Version xPONENT for LX100LX200 3.1.971.0
Firmware Version 2.6.1
LXR Version 2.1.495.0
Pressure 7.93
Temperature 25.91
Lot Number B99504
Lot Expiration 12-Jan-25 12:00:00AM

RP1 Mean Fluorescent Intensity (MFI)

Reference	Target	Lower Tolerance	Upper Tolerance	Actual	Status
Low RP1 Setting					
R1	32.2	27.4	37.0	32.6	Pass
R2	186.0	167.4	204.6	186.7	Pass
R3	1923.0	1730.7	2115.3	1927.7	Pass
R4	15968.0	14371.2	17564.8	16191.1	Pass

Counts (microsphere events)

Reference	Target ($\geq$)	Actual	Status
Low RP1 Setting			
R1	1,000	1,172	Pass
R2	1,000	1,000	Pass
R3	1,000	1,118	Pass
R4	1,000	1,078	Pass

RP1 Percent (%) Coefficient of Variation (%)

Reference	Tolerance ($\leq$)	Actual	Status
Low RP1 Setting			
R1	28.0	20.7	Pass
R2	15.0	11.6	Pass
R3	13.0	9.7	Pass
R4	12.0	9.4	Pass

RP1 Linearity - Average Residual Percent (%)

Reference	Tolerance ($\leq$)	Actual	Status
Low RP1 Setting			
R1 - R4	2.0	0.1	Pass

APPENDIX C

DATA INTERPRETATION REPORT OF CYTOKINE ANALYSIS

Data Interpretation Report

Batch Name: Subatrra UPM LXSAM04 20231220
Protocol Name: Subatrra UPM LXSAM04 20231124
Protocol Version: 1
Test Name: IL-4

$R^2 = 0.994769668954082$

Sum of Residuals = -372.780599125905

Average Residuals = -31.0650499271588

Relative Sum of Squares Absolute = 45792.3014120972

Relative Sum of Squares Relative = 8.57942787021472

Standard Error of the Estimate = 1.1070829024458

Adjusted Coefficient of Multiple Determination = 0.991780908356415

Coefficient a = 4.51327799816084

Coefficient b = 179593.533547434

Coefficient c = 17.0813942455932

Coefficient d = -0.2718137597328

Coefficient f = 16.8641369046798

Data Interpretation Report

Batch Name: Subatrra UPM LXSAM04 20231220
Protocol Name: Subatrra UPM LXSAM04 20231124
Protocol Version: 1
Test Name: IL-6

$R^2 = 0.988804929325374$

Sum of Residuals = 10.9987280938943

Average Residuals = 0.916560674491192

Relative Sum of Squares Absolute = 3369.42966288394

Relative Sum of Squares Relative = 23.9725083589437

Standard Error of the Estimate = 1.85057938259283

Adjusted Coefficient of Multiple Determination = 0.98240774608273

Coefficient a = -0.381469828394156

Coefficient b = 2199300.83541326

Coefficient c = 524034.215198864

Coefficient d = -1.79350341178171

Coefficient f = 0.551998742808599

Data Interpretation Report

Batch Name: Subatrra UPM LXSAM04 20231220
Protocol Name: Subatrra UPM LXSAM04 20231124
Protocol Version: 1
Test Name: IL-10

$R^2 = 0.997838642100068$

Sum of Residuals = 33.985436605424

Average Residuals = 2.83211971711867

Relative Sum of Squares Absolute = 11519.4680516785

Relative Sum of Squares Relative = 6.32685735520965

Standard Error of the Estimate = 0.950703300209876

Adjusted Coefficient of Multiple Determination = 0.996603580442965

Coefficient a = -0.248610131786894

Coefficient b = 3922.09967357355

Coefficient c = 515.517269157796

Coefficient d = -6.59826142443763

Coefficient f = 0.166617760944485

Data Interpretation Report

Batch Name: Subatrra UPM LXSAM04 20231220
Protocol Name: Subatrra UPM LXSAM04 20231124
Protocol Version: 1
Test Name: TNF-alpha

$R^2 = 0.997489727021552$

Sum of Residuals = -28.6197409236869

Average Residuals = -2.38497841030725

Relative Sum of Squares Absolute = 24528.1294847795

Relative Sum of Squares Relative = 155.05238351687

Standard Error of the Estimate = 4.706414824727

Adjusted Coefficient of Multiple Determination = 0.996055285319581

Coefficient a = 4.03839895670245

Coefficient b = 14925079.6285578

Coefficient c = 1058.09013978264

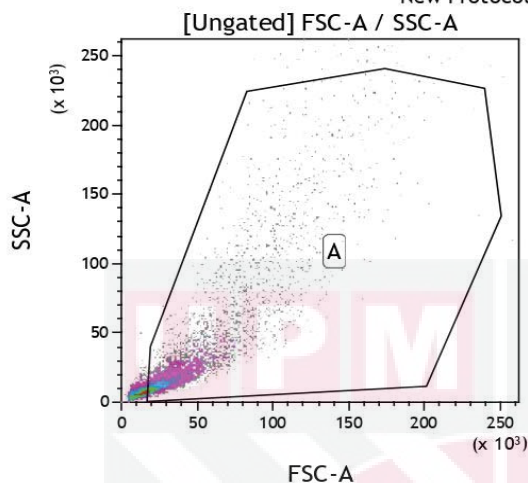
Coefficient d = -0.144065283557981

Coefficient f = 11.6813111932079

APPENDIX D

FLOW CYTOMETRY ANALYSIS OF *Candida glabrata* CELL WALL COMPONENTS

Specimen_001_Cg R1_004
New Protocol 1 - Plot Sheet 1

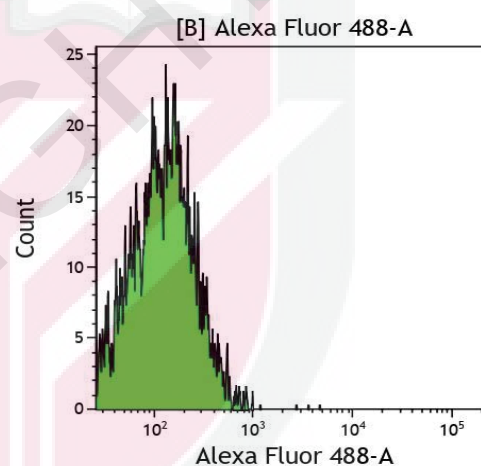
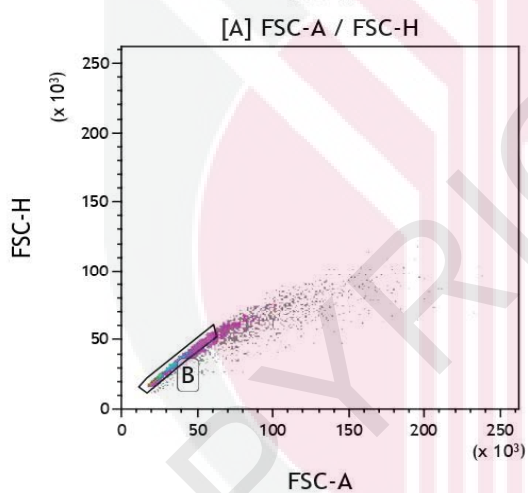


Cg R1: *C. glabrata* control (1st Rep)

Alexa Fluor 488-A = β -glucan

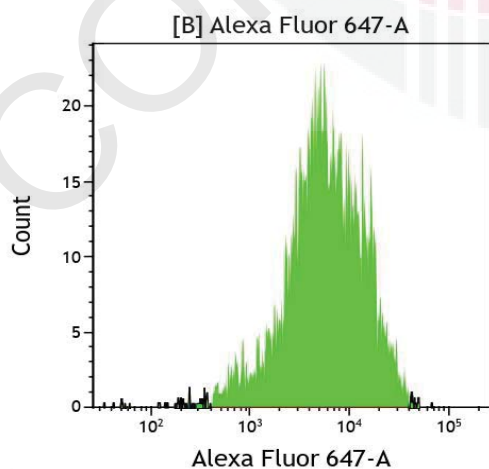
Alexa Fluor 647 = Mannan

BV421 = Chitin



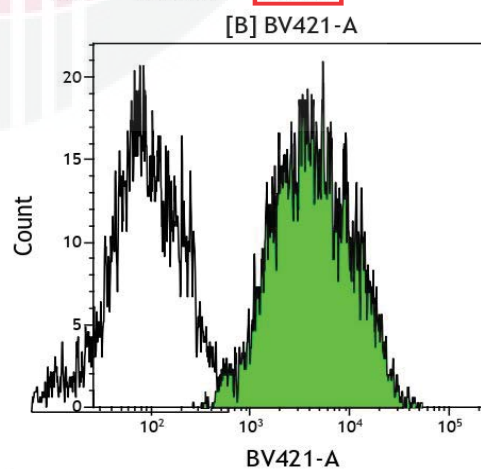
Gate X-A-Mean X-G-Mean

All 297.48 97.10



Gate X-A-Mean X-G-Mean

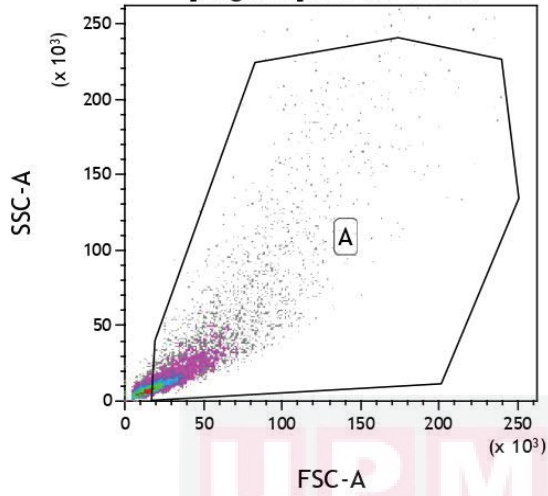
All 7,590.20 4,632.64



Gate X-A-Mean X-G-Mean

All 6,062.83 4,117.44

[Ungated] FSC-A / SSC-A



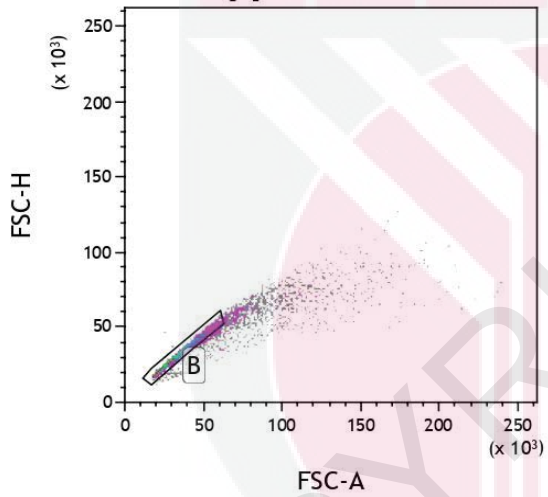
Cg R2: *C. glabrata* control (2nd Rep)

Alexa Fluor 488-A = β -glucan

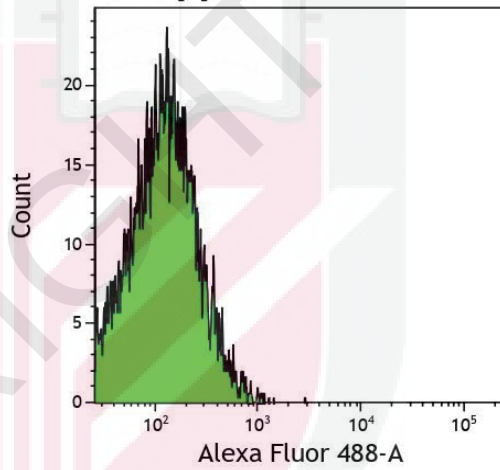
Alexa Fluor 647 = Mannan

BV421 = Chitin

FSC-A
[A] FSC-A / FSC-H



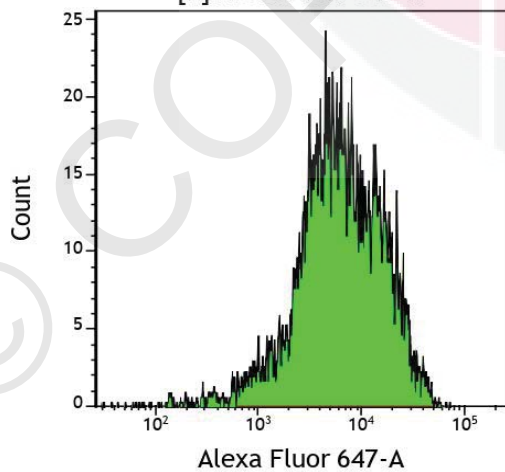
[B] Alexa Fluor 488-A



Gate X-A-Mean X-G-Mean

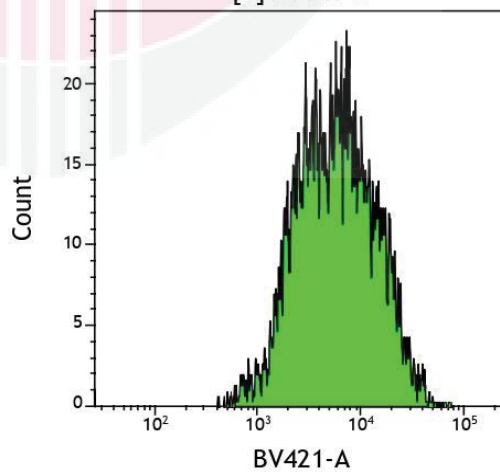
All 181.04 90.74

[B] Alexa Fluor 647-A



Alexa Fluor 647-A

[B] BV421-A



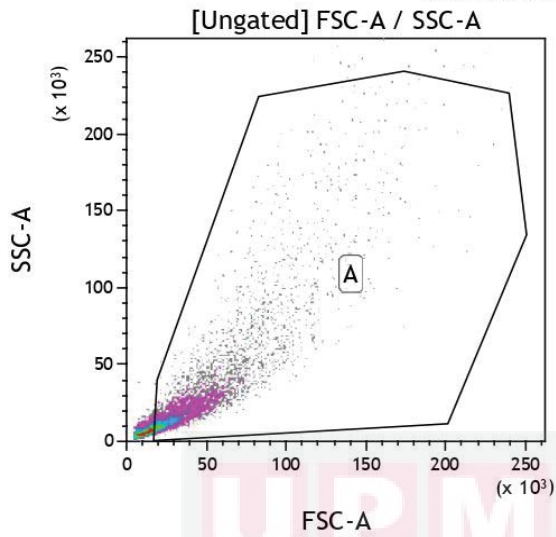
BV421-A

Gate X-A-Mean X-G-Mean

All 8,423.84 4,625.42

Gate X-A-Mean X-G-Mean

All 8,073.48 5,648.03

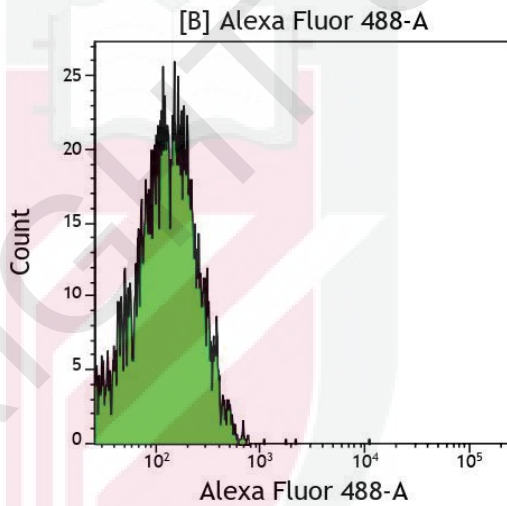
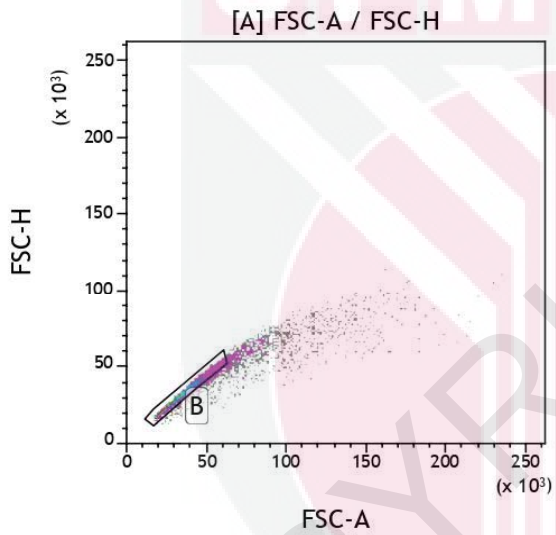


Cg R3: *C. glabrata* control (3rd Rep)

Alexa Fluor 488-A = β -glucan

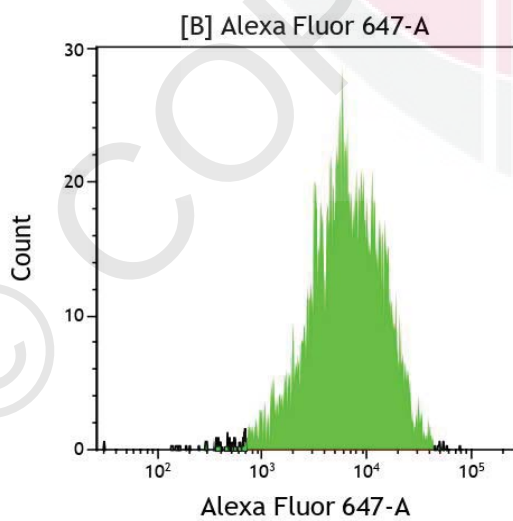
Alexa Fluor 647 = Mannan

BV421 = Chitin



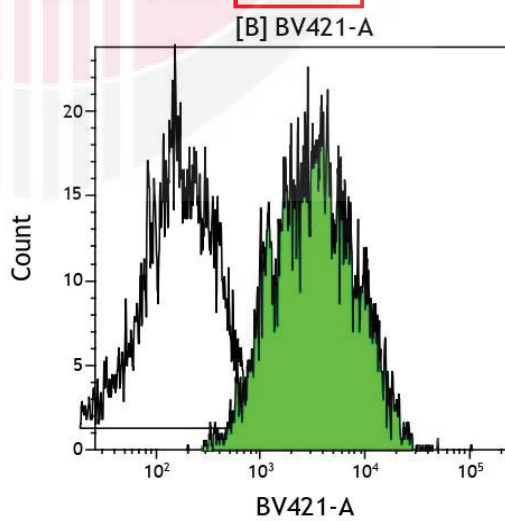
Gate X-A Mean X-G Mean

All 137.68 103.34



Gate X-A Mean X-G Mean

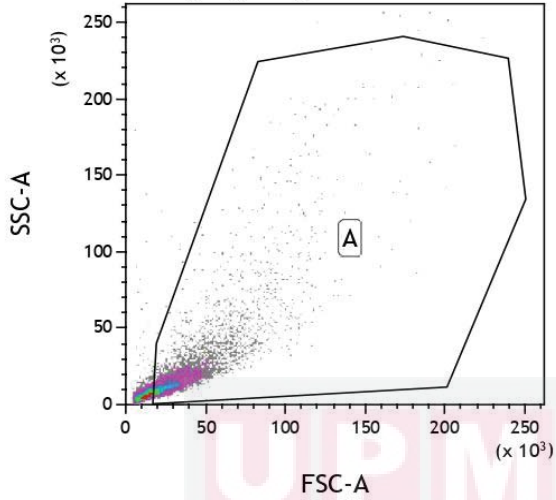
All 8,288.18 5,826.30



Gate X-A Mean X-G Mean

All 4,744.96 3,234.67

[Ungated] FSC-A / SSC-A



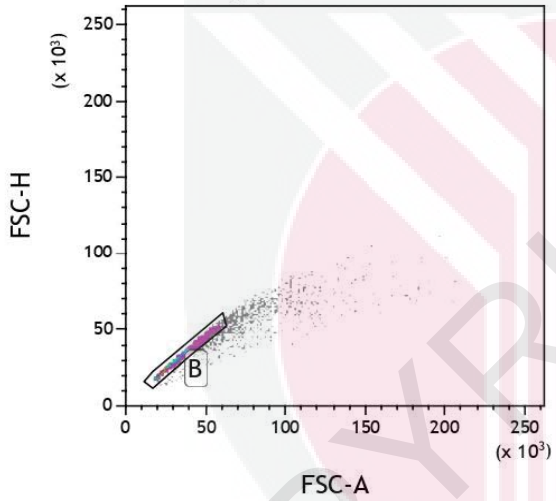
CE R1: Cg + THP-1 (1st Rep)

Alexa Fluor 488-A = β -glucan

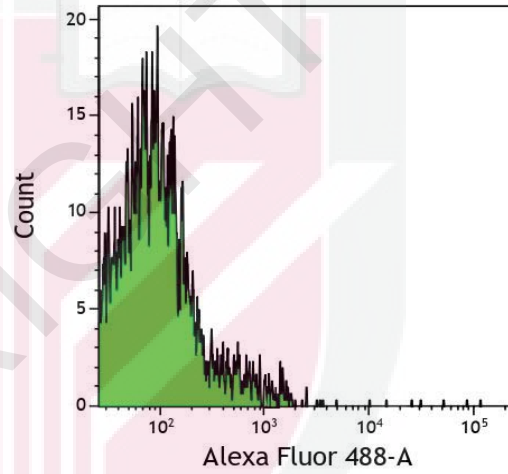
Alexa Fluor 647 = Mannan

BV421 = Chitin

[A] FSC-A / FSC-H



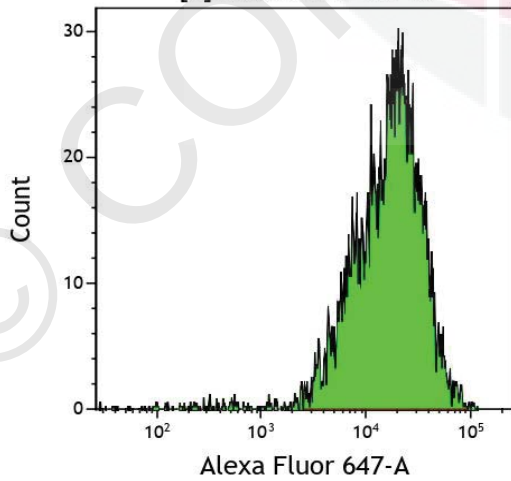
[B] Alexa Fluor 488-A



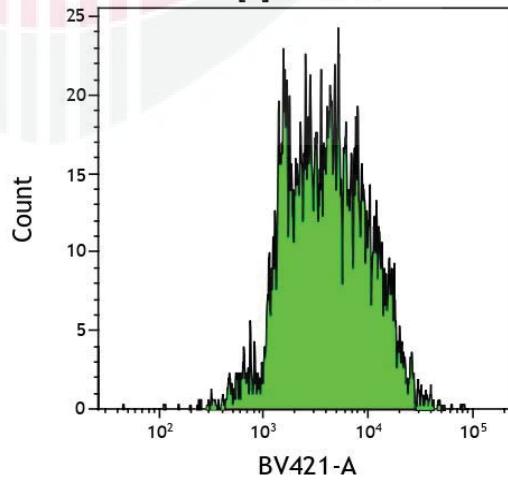
Gate X-A Mean X-G Mean

All 151.71 57.48

[B] Alexa Fluor 647-A



[B] BV421-A

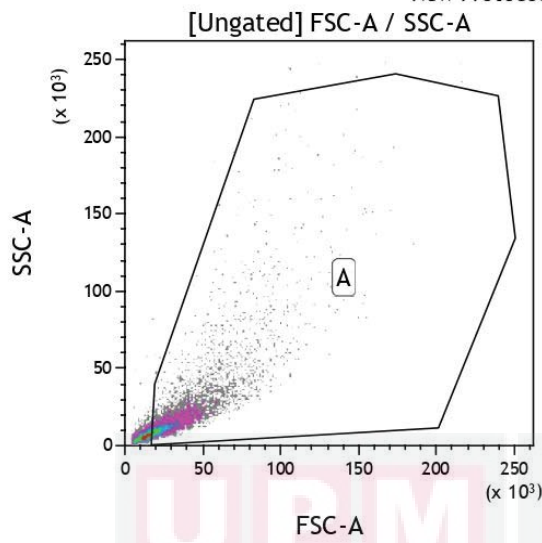


Gate X-A Mean X-G Mean

All 19,516.08 13,824.02

Gate X-A Mean X-G Mean

All 5,908.46 3,942.43

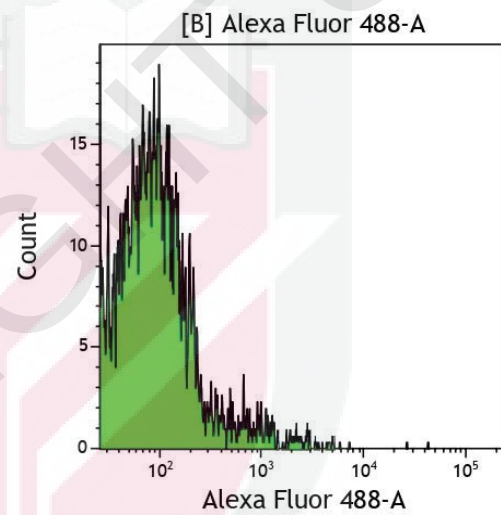
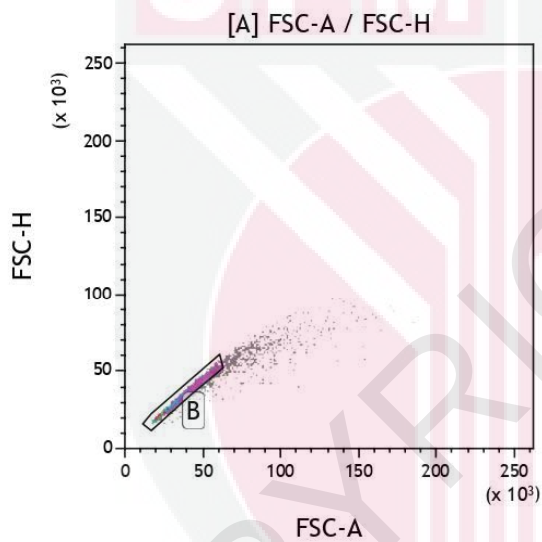


CE R2: Cg + THP-1 (2nd Rep)

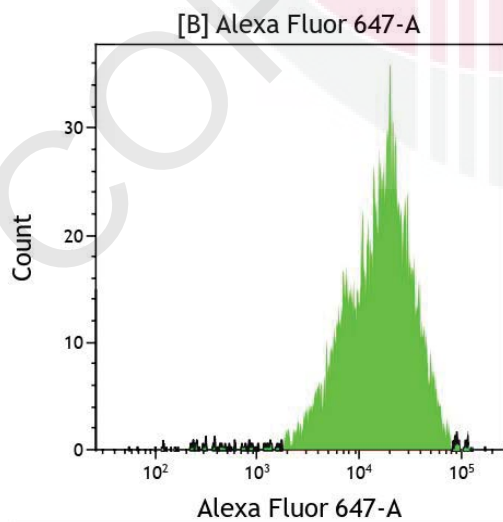
Alexa Fluor 488-A = β -glucan

Alexa Fluor 647 = Mannan

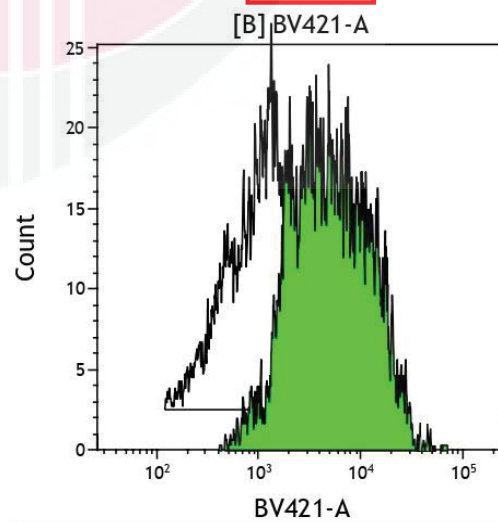
BV421 = Chitin



Gate	X-Mean	X-GMean
All	676.91	57.26

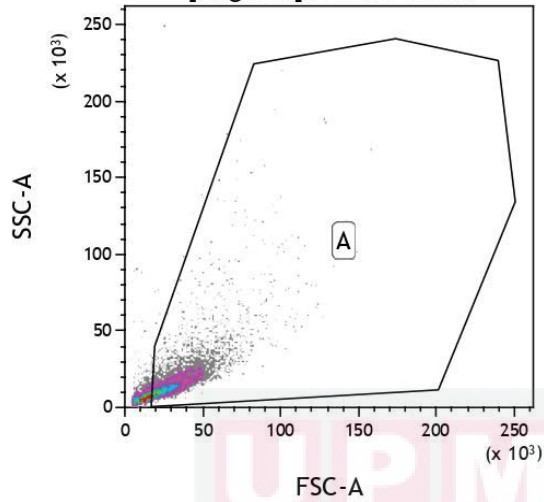


Gate	X-Mean	X-GMean
All	19,307.33	14,303.86



Gate	X-Mean	X-GMean
All	7,772.95	4,950.50

[Ungated] FSC-A / SSC-A



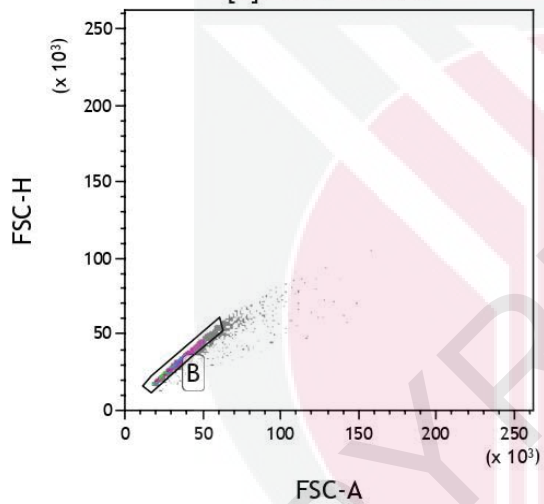
CE R3: *Cg* + THP-1 (3rd Rep)

Alexa Fluor 488-A = β -glucan

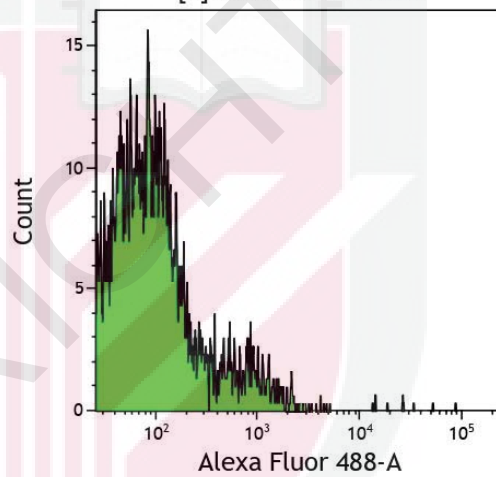
Alexa Fluor 647 = Mannan

BV421 = Chitin

[A] FSC-A / FSC-H



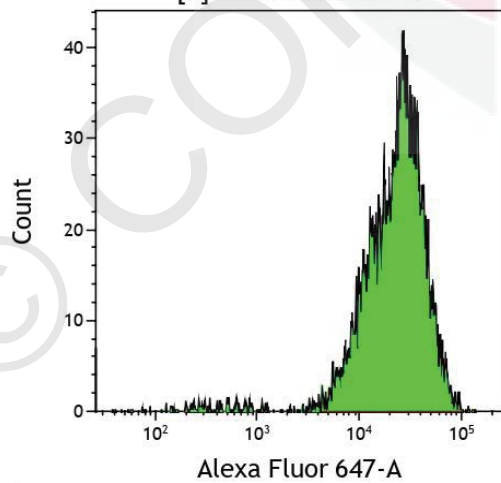
[B] Alexa Fluor 488-A



Gate X-A Mean X-G Mean

	X-A Mean	X-G Mean
All	974.87	49.21

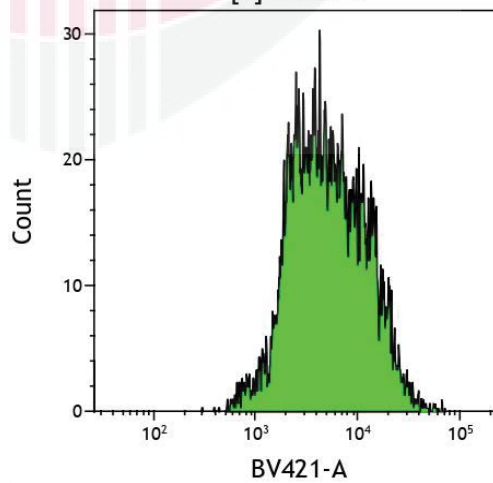
[B] Alexa Fluor 647-A



Alexa Fluor 647-A

	X-A Mean	X-G Mean
All	25,937.80	19,897.92

[B] BV421-A

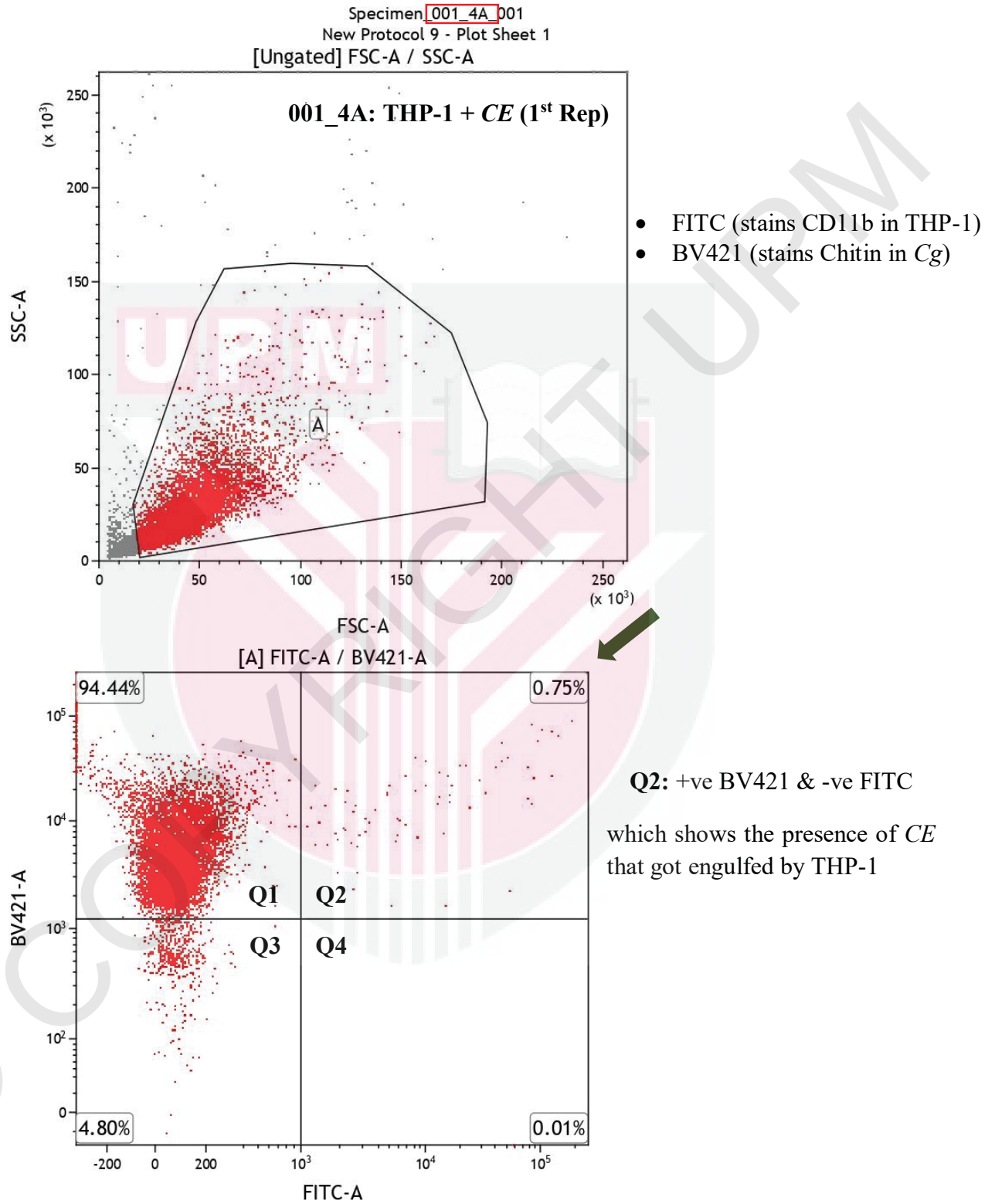


BV421-A

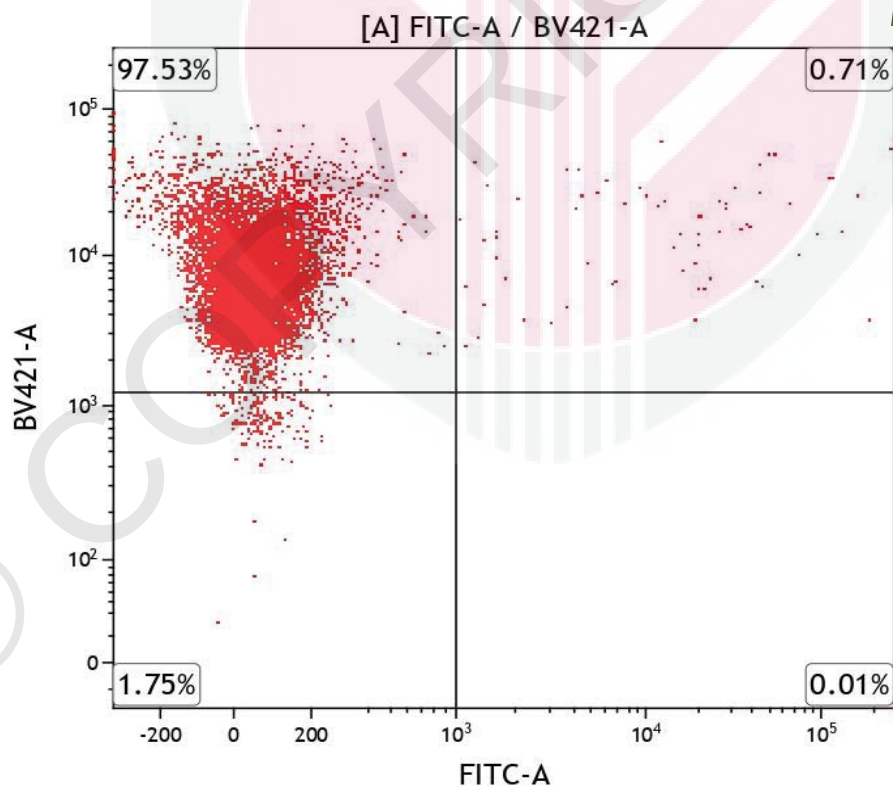
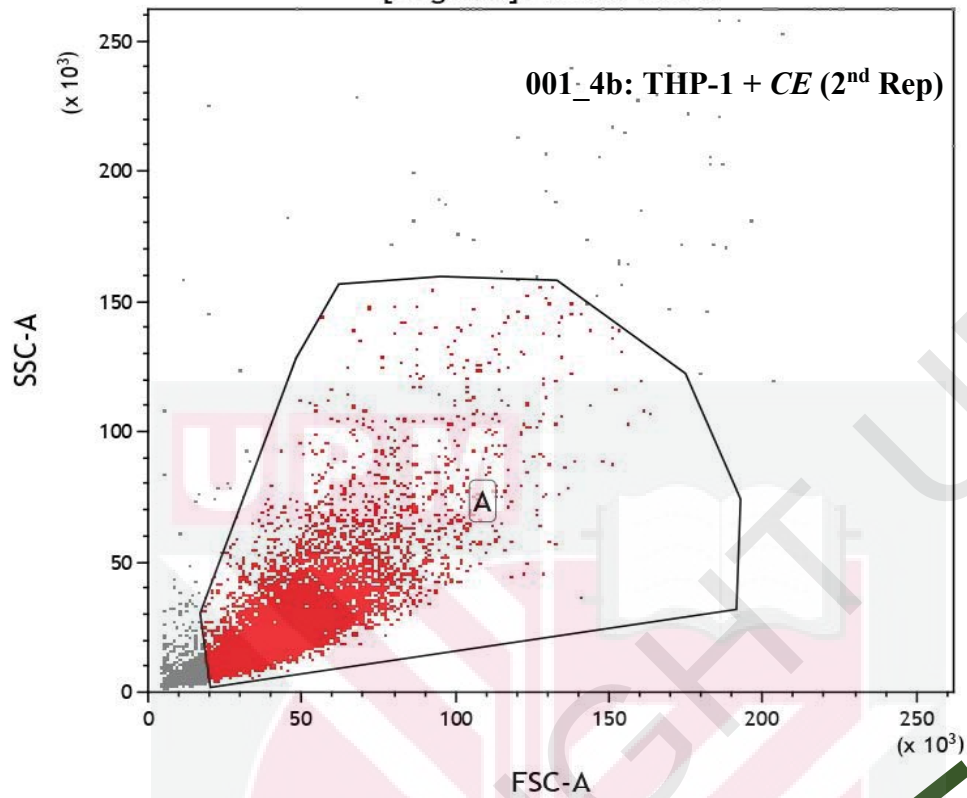
	X-A Mean	X-G Mean
All	6,983.53	5,050.11

APPENDIX E

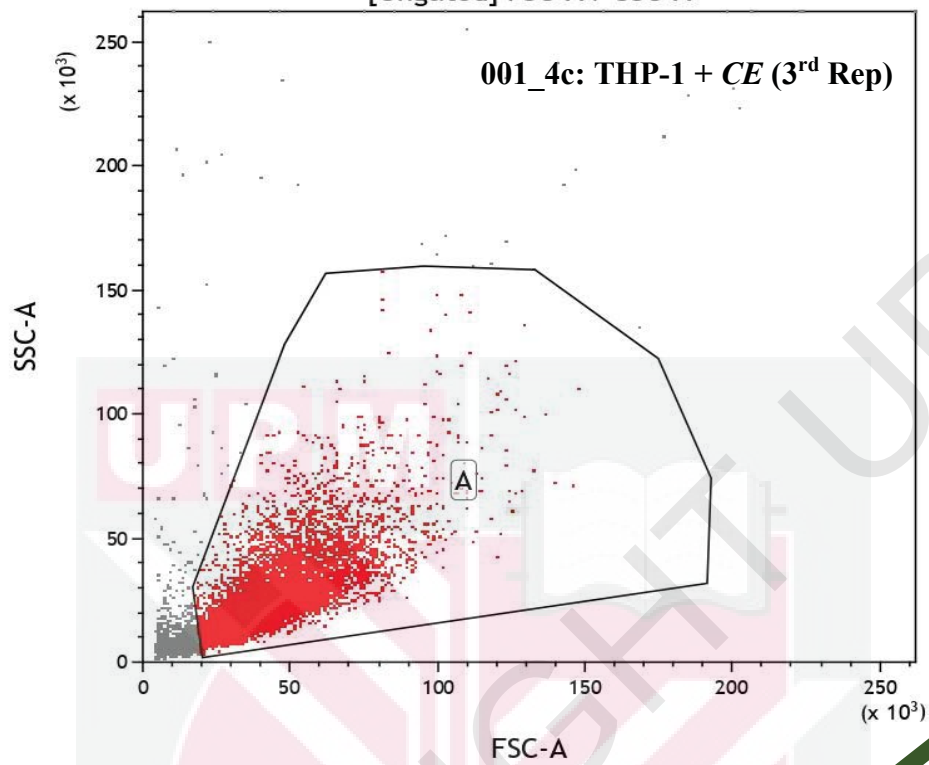
FLOW CYTOMETRY ANALYSIS (PHAGOCYTOSIS ASSAY)



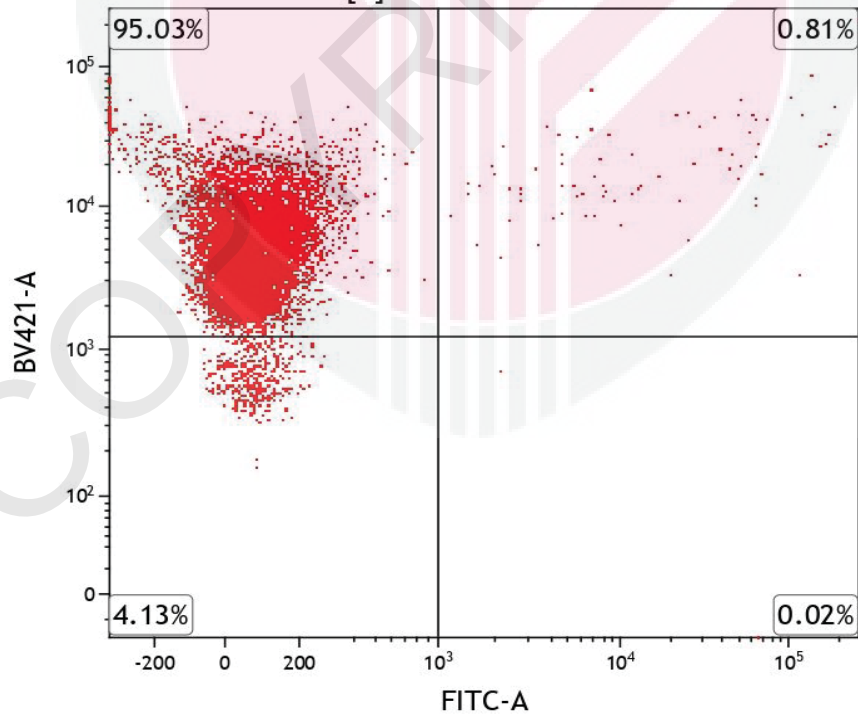
Specimen_001_4b_002
New Protocol 9 - Plot Sheet 1
[Ungated] FSC-A / SSC-A



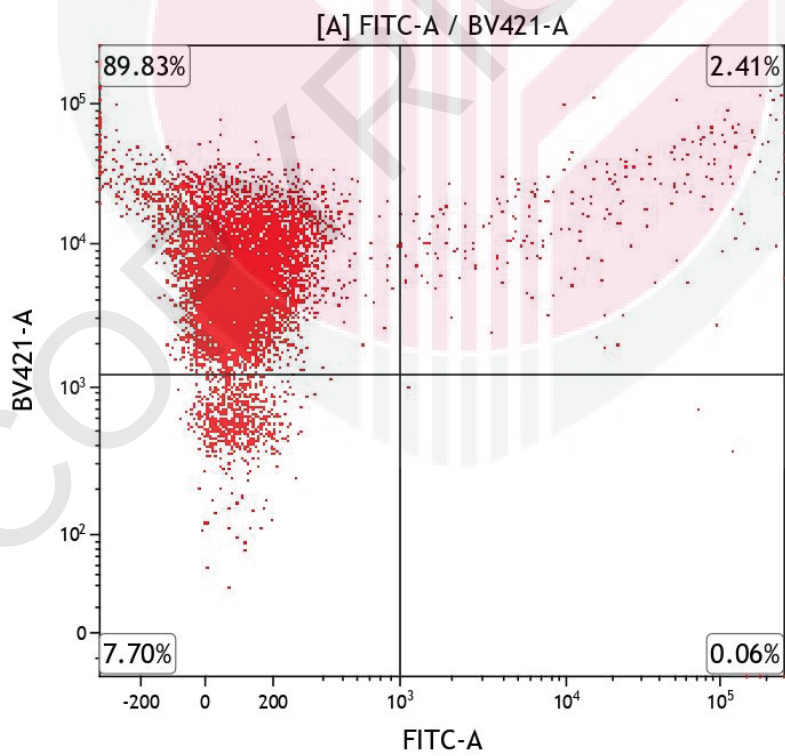
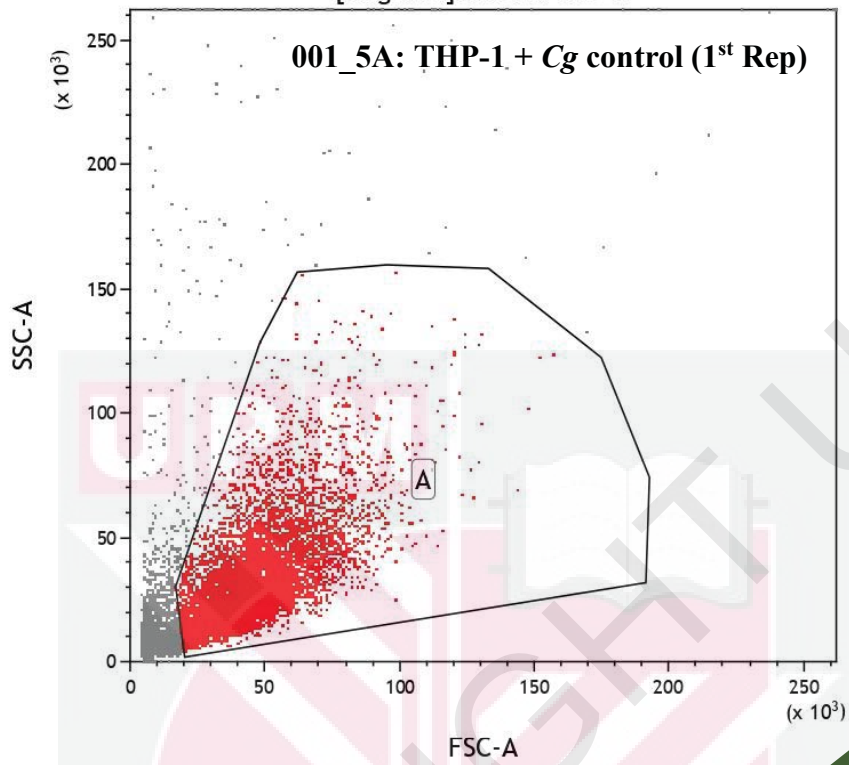
Specimen_001_4c_003
New Protocol 9 - Plot Sheet 1
[Ungated] FSC-A / SSC-A



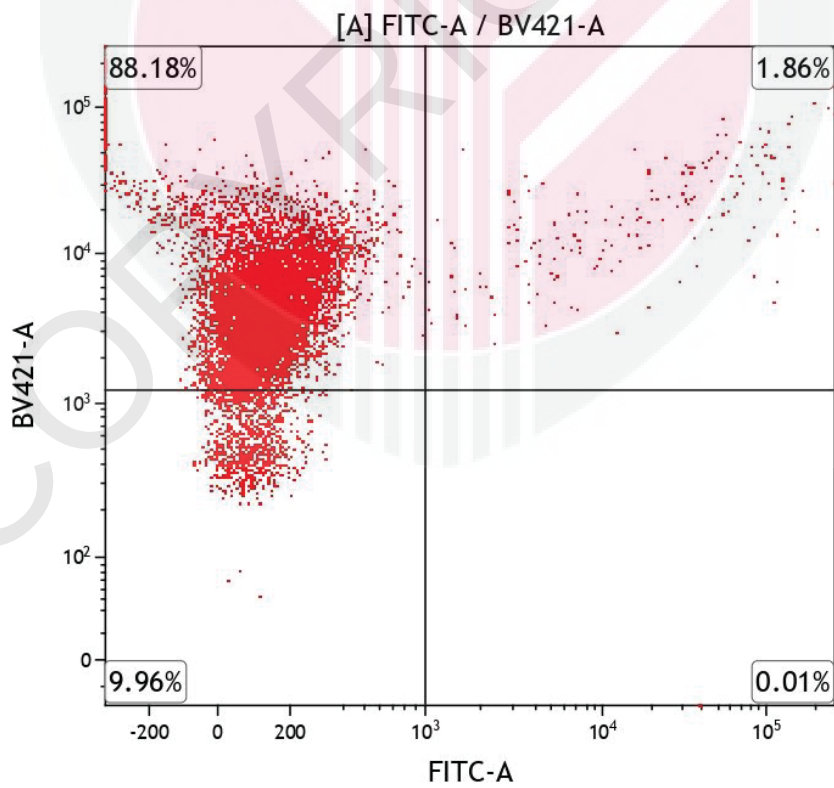
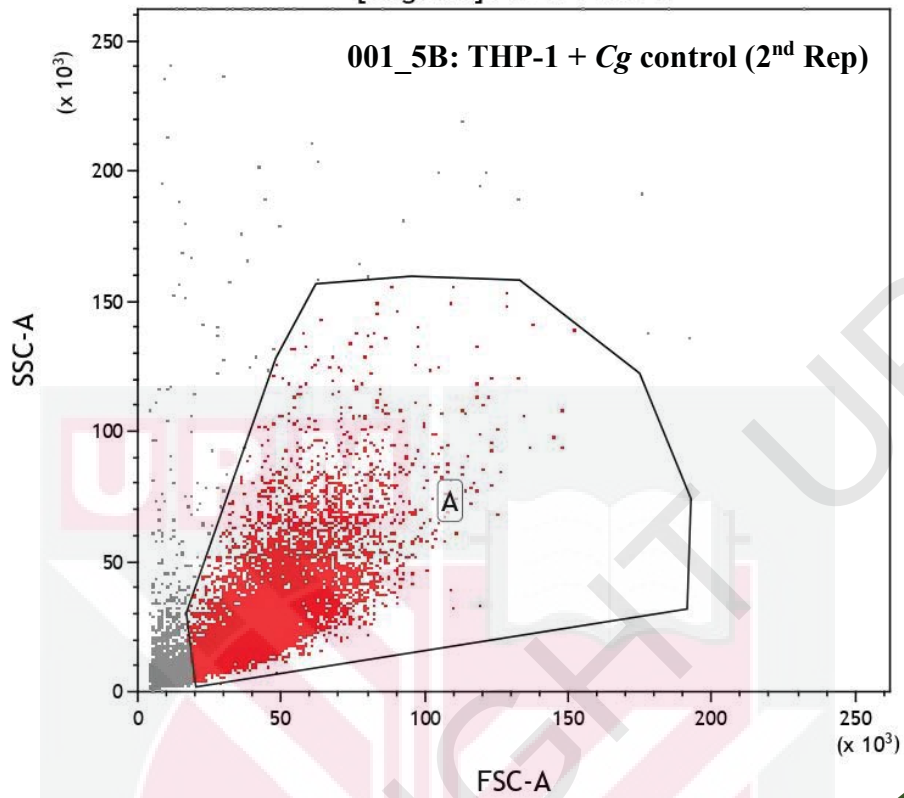
[A] FITC-A / BV421-A



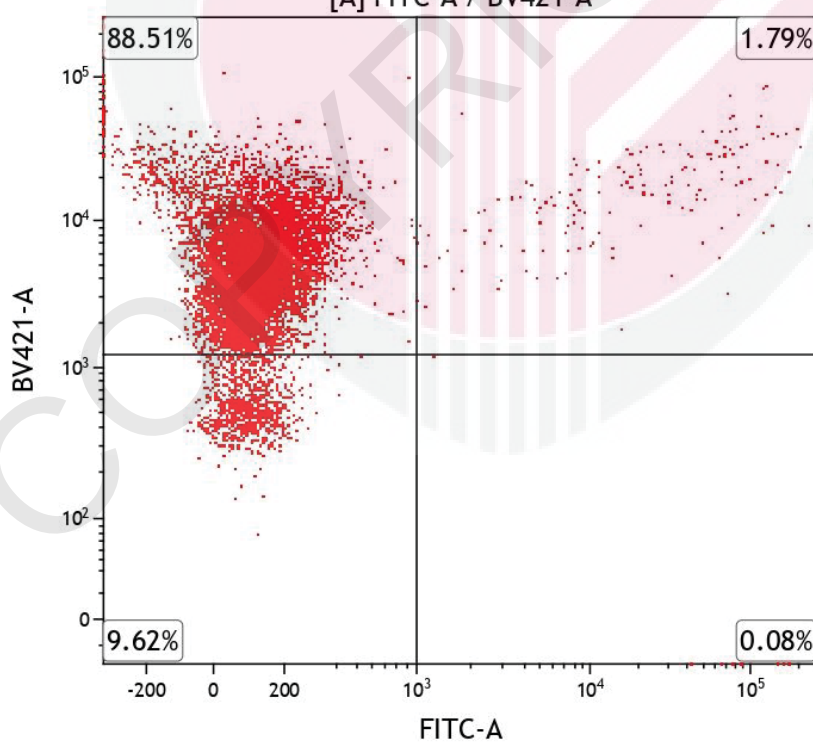
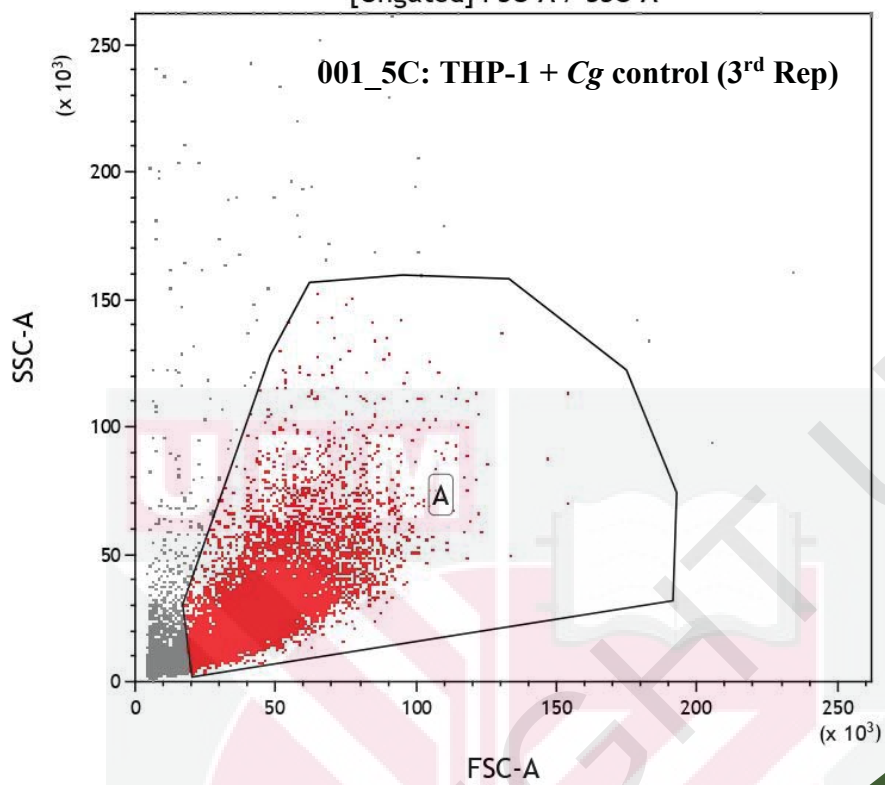
Specimen_001_5A_005
New Protocol 9 - Plot Sheet 1
[Ungated] FSC-A / SSC-A



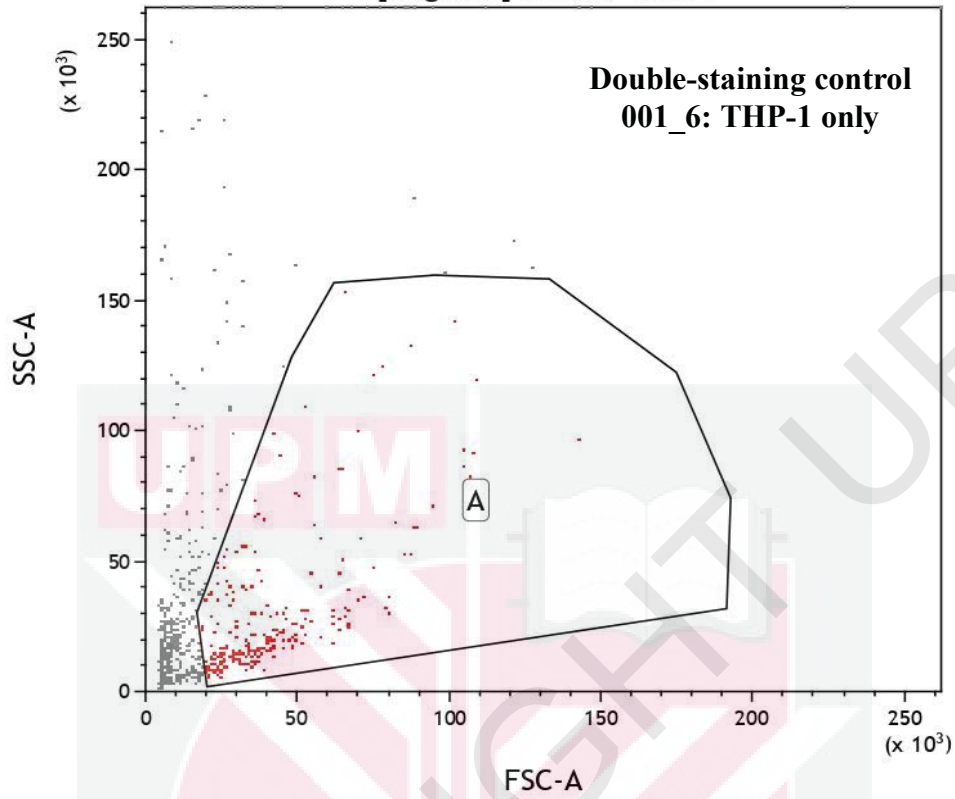
Specimen_001_5B_006
New Protocol 9 - Plot Sheet 1
[Ungated] FSC-A / SSC-A



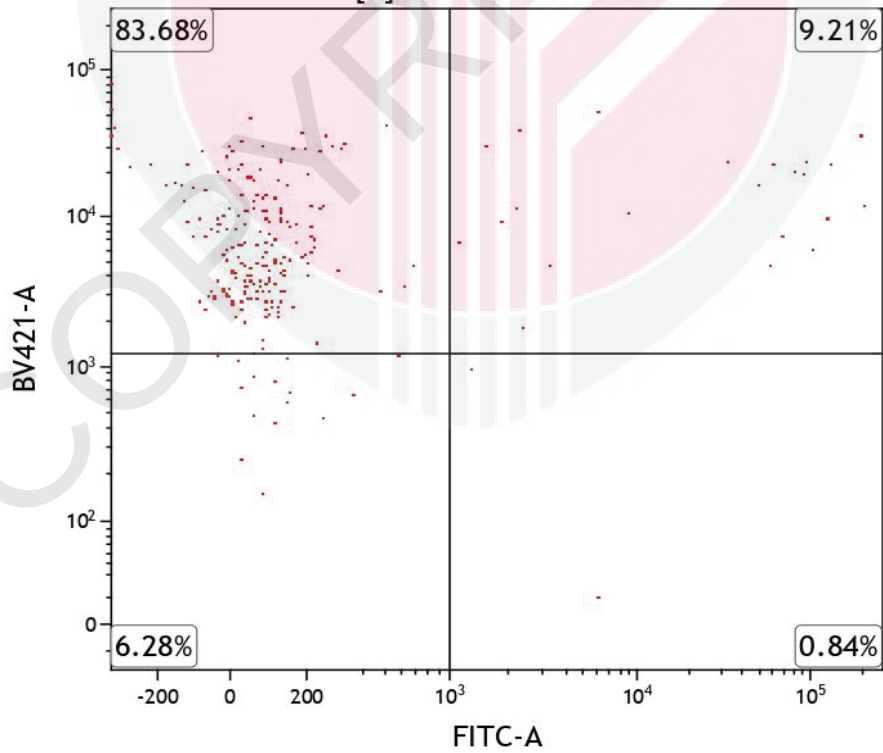
Specimen_001_5C_007
New Protocol 9 - Plot Sheet 1
[Ungated] FSC-A / SSC-A



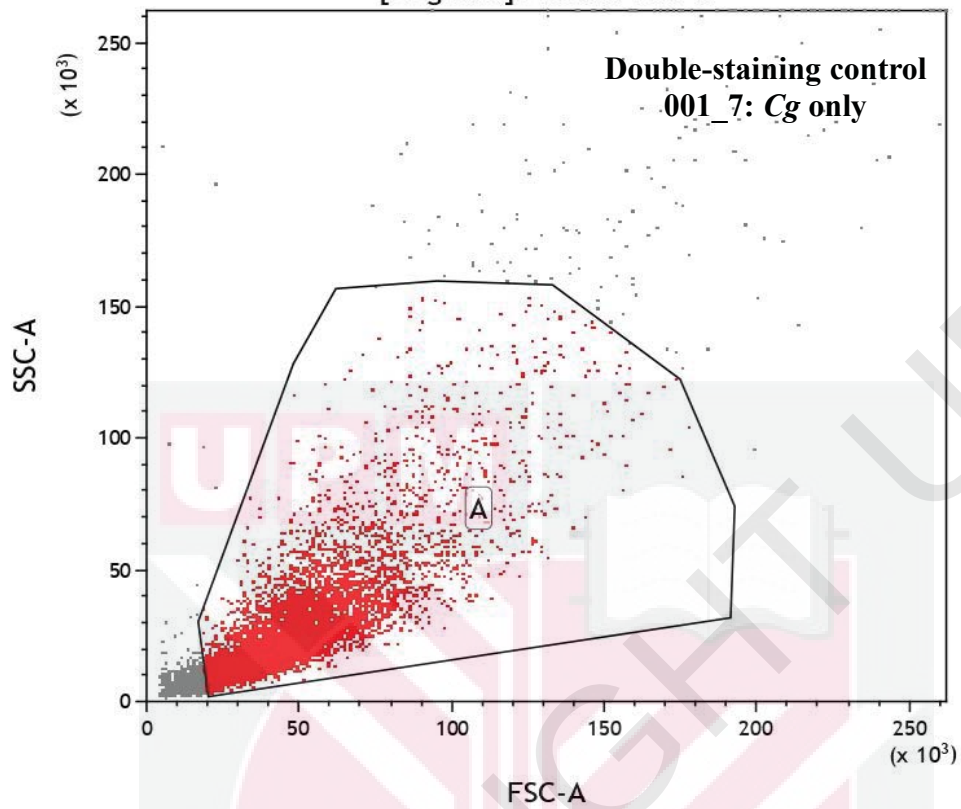
Specimen_001_6_008
New Protocol 9 - Plot Sheet 1
[Ungated] FSC-A / SSC-A



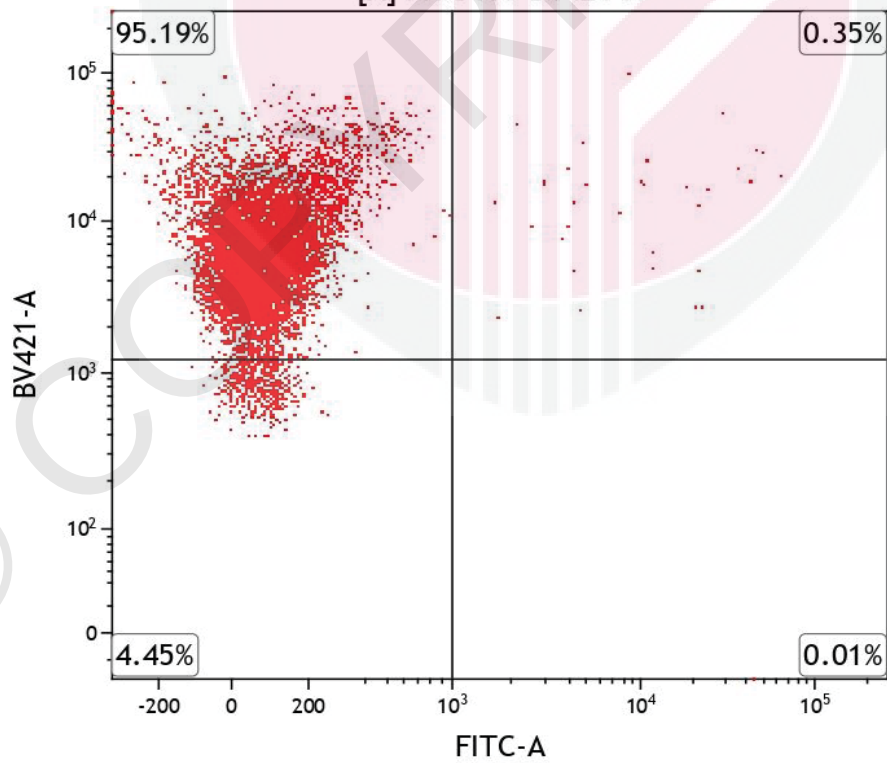
[A] FITC-A / BV421-A



Specimen_001_7_009
New Protocol 9 - Plot Sheet 1
[Ungated] FSC-A / SSC-A



[A] FITC-A / BV421-A



LIST OF PUBLICATIONS

Subatrra Nair Balakrishnan, Haizat Yamang, Michael C. Lorenz, Chew Shu Yih, Leslie Than Thian Lung 2022. Role of vaginal mucosa, host immunity and microbiota in vulvovaginal candidiasis. *Journal of Pathogens*. (Published)

