Macrophages *Candida albicans* infection upon bacterial endotoxin priming

Vânia Filipa Dos Santos Moreira

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DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

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Abstract

Candida albicans is the most prevalent fungal species in human fungal infections, mostly among the immunocompromised population, including patients suffering from severe sepsis, in which the incidence of invasive fungal infection has been increasing. In these situations, the immunological deficiency allows the fungi to overcome the protective host defense mechanisms.

However, the influence of sepsis, which induces a severe inflammatory response, in the outcome of *C. albicans* infection is an interesting field. In these cases does the fungal disease result from a lack of recognition by the innate immune system or overreaction of the inflammatory response?

The main goal of this work was to study the effect of macrophages priming by bacterial endotoxin (LPS), the major inflammatory mediator, in the outcome of *C. albicans* infection. In particular, the viability of infected macrophages and internalized yeast, *C. albicans* escape mechanisms and the inflammatory response mediated by the macrophages release of cytokine.

The main conclusion of this study is that a short, but severe exposure of the macrophages to bacterial endotoxin, does not enhance the immune response to a subsequent *C. albicans* infection. First, an increase in the proinflammatory cytokine TNF-α was detected when macrophages were stimulated with LPS for one hour and then infected with *C. albicans*. Then, a slight decrease in the viability of internalized fungi was observed, and cellular phagolysosome activated. However, and most importantly, macrophages viability decreases slightly in this situation.

Understanding the biological mechanisms associated with the fungal infection upon a severe inflammatory response, would be a major contribution to the development of efficient treatment of invasive fungal infections among patients with severe sepsis.

Key-words: Lipopolysaccharide LPS / bacterial endotoxin; macrophages; *Candida albicans*; Heat-killed *Escherichia coli*

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Resumo

Candida albicans é o microorganismo mais prevalente nos casos de infecções fúngicas em humanos, principalmente entre a população de doentes immunocomprometidos, incluindo doentes com septicémia, nos quais a incidência de infecções por fungos têm vindo a aumentar. Nestas situações, a deficiência imunológica permite ao fungo suprimir os mecanismos de defesa do hospedeiro.

A influência do estado de septicémia, que inclui uma resposta inflamatória generalizada e severa, no desfecho da infecção fúngica, é uma área pouco estudada. Nestes casos, a infecção fúngica pode resultar da falta de reconhecimento pelo sistema imunitário ou uma resposta inflamatória aguda.

Este trabalho teve como objectivo estudar o efeito de macrófagos pré-activados com lipopolissacárido bacteriano, um agente inflamatório, na resposta do hospedeiro à infecção por *C. albicans*. Em particular, a viabilidade dos macrófagos, das leveduras internalizadas e a resposta inflamatória mediada pela libertação de citocinas do macrófago.

A principal conclusão tirada deste estudo é que uma exposição aguda, embora curta, dos macrófagos à endotoxina de *Escherichia coli*, não aumenta a resposta imunitária à infecção fúngica. Em primeiro lugar, foi detectado um aumento na produção da citocina TNF-α quando o macrófago é estimulado, durante uma hora, com LPS e seguidamente infectado com *C. albicans*. Um aumento da viabilidade do número de leveduras internalizadas pelos macrófagos foi observado. Do mesmo modo, o fagolisossoma parece mais activado. Contudo, e muito importante, nestas condições a viabilidade dos macrófagos sofre um ligeiro decréscimo.

Compreender os mecanismos biológicos associados a infecções por fungos, aquando de uma resposta inflamatória aguda, seria uma importante contribuição no desenvolvimento de tratamentos mais eficazes destas infecções em doentes com septicémia.

Palavras-chave: Lipopolissacárido LPS/ endotoxina; macrófagos; *Candida albicans*; *Escherichia coli* inactivada

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List of Abbreviations

Als- agglutinin-like sequence

ATP- Adenosine triphosphate

Ca- *Candida albicans*

CFU- Colony forming unit

CLR- C-type lectin receptors

Cph 1 - Candida pseudohyphal regulator (transcription factor)

CWP - Cell wall proteins

DMEM- Dulbecco's modified eagle medium

DNA- Deoxyribonucleic acid

EAP1- Enhanced adherence to polystyrene protein

ELISA- Enzyme-Linked Immunosorbent Assay

FBS- Fetal bovine serum

IL- Interleukin

GlcNAc- N-acetylglucosamine

GPI- Glycosylphosphatidylinositol

HIV- Human Immunodeficiency Virus

HKEc- Heat Killed *Escherichia coli*

LBP -LPS-Binding Protein

LPS- Lipopolysaccharide

Mϕ- Macrophages

MAPK- Mitogen-activated protein kinase

MBL- Mannose-binding lectin

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MKP1- MAPK phosphatase

MOI- Mulplicity of infection

MMR- macrophage mannose receptor

NF-κB- Nuclear factor κB

NLR- NOD-like Receptors

NLRP3- NLR family, pyrin 3

PAMP- Pathogen-associated molecular patterns

PBS- Phosphate buffered saline

PRR- Pattern-recognition receptors

ROS- Reactive oxygen species 1

RPMI- Cell culture medium developed in Roswell Park Memorial Institute

SEM- Standard error of the means

TLR- Toll-Like Receptor

TNF-α- Tumor Necrosis Factor alpha

YPD - Yeast Extract Peptone Dextrose

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Introduction

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1 Introduction

The frequency of invasive fungal infections among patients with severe sepsis has been increasing proportionally with the raise of immunocompromised patients. In these cases *C. albicans* is the most prevalent opportunistic pathogen. Although, this yeast is part of the microbial flora, when the immune system is depressed, it becomes virulent. So, it is extremely important to study the dynamic and complex relationship between the fungi and the immune system.

The main aim of this work was to study the infection of *C. albicans* by phagocytic cells, when the immune system is already under stress. Particularly, the effect of the macrophages exposure to endotoxin, in the outcome of fungal infection.

In this section, a brief literature review is introduced. Firstly, focus on *C. albicans* characteristics then, the response of the innate immune system to a fungal infection. Thirdly, the bacterial endotoxin and the resulting immune response. Finally, the interaction between fungi and bacteria components. Each of these will be discussed in turn.

1.1 *Candida albicans* a threat for imunocompromised patients

Candida albicans is a fungal opportunistic pathogen living in a commensal relationship with healthy individuals. Member of the human gastrointestinal and genitourinary normal flora, *C. albicans* isn´t harmful to the host unless the immune system becomes impaired. In these cases, commensalism will develop towards extensive colonization of mucosal surfaces and fungal dissemination. *Candida* spp. are still the most common pathogen in nosocomial infections, and *Candida albicans* is by far the most common fungi isolated from patients (Fridkin & Jarvis, 1996; Gould, 2011).

The disease, caused by an abnormal colonization of this organism in the human body, is called candidiasis. It can be classified as: mucocutaneous candidiasis, oropharyngeal candidiasis, cutaneous candidiasis or candidaemia depending where and how the fungal spread occurs (Berman & Sudbery, 2002; d'Enfert, 2009; Romani, 2011)

To treat the disease, several drugs can be administrated: polyenes, (amphotericin B–based preparations and nystatin), azole antifungal agents, including imidazoles and triazoles, allylamines, (terbinafine, antimetabolites, that includes 5-fluorocytosine (5-

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FC)), and echinocandins, with three drugs approved for human use, caspofungin, anidulafungin and micafungin (Murray, Kobayashi, Pfaller, & Rosenthal, 1990; Gould, 2011). Azoles and allylamines target is the synthesis of ergosterol present in the fungal membranes. Polyenes bind to ergosterol in the fungal cytoplasmatic membrane and forms spanning pores, leading to cell death. Finally, the echinocandin antifungal agents inhibit non-specifically the enzyme 1,3-β-glucan synthase therefore, suppressing the synthesis of β-glucan in the fungal wall. 5-FC inhibits both DNA and protein synthesis.

However there are reports of clinical cases of antifungal resistance of *C. albicans*, so resistant strains have been studied (Holmes et al., 2012; Schuetzer-muehlbauer et al., 2003). Choice of therapy is based on published guidelines as (Pappas et al., 2004).

1.1.1 *C. albicans*: the Human commensal fungi

Candida albicans cells can present different morphologies and structures: yeast or blastophore, pseudohyphae, true hyphae and chlamydospores. The prevalence of each morphology *in vitro* is determined by growth media composition. The yeast-to-hypha phenotypic switch has been mentioned as a virulence factor since, hyphal morphology has been associated with increased ability to adhere and penetrate mucosal surfaces and spread through the host (Gow, 1997).

The yeast and pseudohyphae morphology are easily distinguishable. The yeast form is a recognizable spherical or oval cell able to reproduce asexually by budding (Romani, 2011). The pseudohyphal morphology is characterized as yeast cells where budding occurs as a synchronized process in which cytokinesis does not occur. Thus, the daughter cells remain attached to the mother cell and the resulting structure is constricted at the septal junctions. This morphology is observed in C. albicans although its relevance to the host's immunological response remains elusive (Gow, Veerdonk, Brown, & Netea, 2011).

On the other hand, hyphae are long tubes germinating from the yeast cell with parallel sides and no constrictions, also called the apical cell. During cytokinesis the cytoplasm is not partitioned equally, apical cells receive more cytoplasm from the mother cell than the sub-apical cell. This region of the fungal cell doesn't replicate until it can regenerate sufficient cytoplasm to re-enter the cell cycle, so it remains arrested in the cell cycle. It contains one nucleus and it is largely vacuolated. Studies have reported that this morphological change can be a response to nutritional deprivation, as a way to reach nutrients (P. E. Sudbery, 2001; Gow, 1997). This morphology can be induced in

experiments by the presence of serum, high temperature or acidic pH among other factors. Lastly, chlamydospores are structures occasionally formed at the tip of hypha or pseudohyphal cells. These thick-walled round cells occur in response to unfavorable growth conditions. In vitro, can be induced if incubated in hydrocarbonate rich medium (Berman & Sudbery, 2002) and are asexual reproductive structures.

The fungal cell wall maintains the microorganism integrity and is responsible for the interaction of other cells or surfaces. This strong, yet plastic structure is constituted by the inner layer, comprised mainly by glucan and chitin, and matrix (outer layer) with glycosylated proteins. The inner layer core structure of the cell wall is formed by β-(1,3)-glucan covalently attached to β-(1,6)-glucan and chitin ((β-1,4)-linked polymer of N-acetylglucosamine (GlcNAc)). This resulting network has its polymers attached to polysaccharide chains by hydrogen bonds, forming strong microfibrilis.

The outer layer is comprises by glycosylated proteins, amino-acids chains with O- and N-linked mannose polymers, mainly glycosylphosphatidylinositol (GPI). These cell wall proteins (CWP) attach to the inner layers chitin or β-(1,3)-glucan chains linked by β-1,6-glucan components. Their content in carbohydrates, as mannose, account up to 90% of their molecular mass (Gow et al., 2011; Netea, Brown, Kullberg, & Gow, 2008).
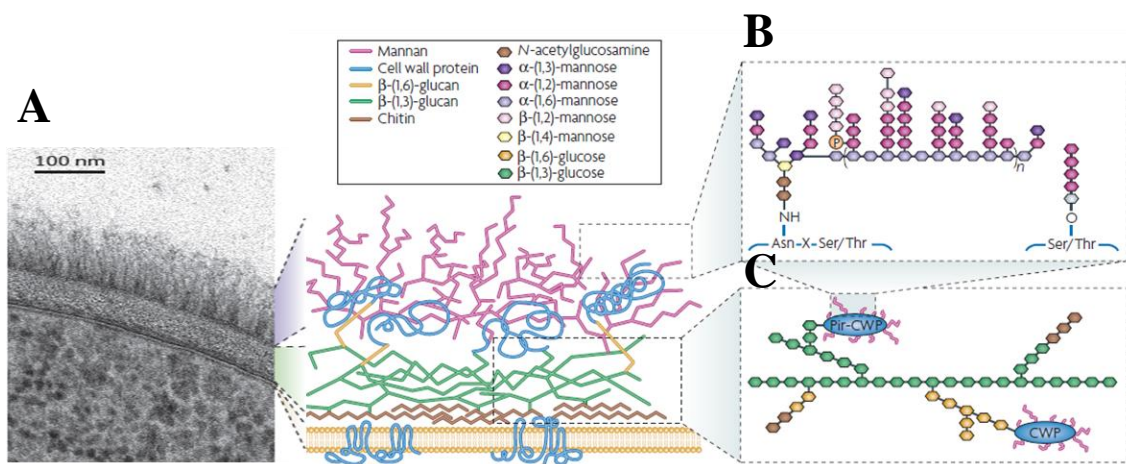
Fig. 1 Candida albicans cell wall structure. A) A electronic microscopy picture of the yeasts cell with amplification of the cell wall. Schematics illustrations show the distribution of the main componetes of the inner and outer layer of the cell wall. B) The outer layer is mainly comprised by (1,3)-glucan and chitin polysaccharides. C) The inner layer has CWP attached to mannan rich structures by N-acetylglucosamine residues. Illustration adapted from Netea, et al., (2008) and Gow, et al., (2011).

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As mentioned above, during the morphogenic switching changes in the cell wall occur, mainly in the mannans structure of CWPs. The expression of CWPs is highly regulated during this switch. Genes such as hyphal wall protein 1 (Hwp1), hyphally regulated protein 1 (Hyr1) and agglutinin-like sequence 3 (Als3) are upregulated while hyphal growth (Netea et al., 2008).

Although the differences between the components of yeasts cell wall and hyphae are not yet certain, data indicate that hyphae phenotype contains fewer phosphodiester-linked β-1,2-monno-oligosaccharides than CWPs in yeast cells. Also, hyphal cells contain 3-5 times more chitin than yeast cells and different β-glucan exposure content, (Gow et al., 2011; Netea et al., 2008).

Purified chitin from *C. albicans* blocks the recognition of yeast cells by the innate immune system, as yeast cells have more chitin than hyphal cells; this blockage is pointed out as evidence that immune recognition of yeast and hyphae is different (Mora-Montes, et al., 2011). In the host-fungal interactions, the fungal cell wall components constitute the called Pathogen Associated Molecular Pattern (PAMP's) and are recognized by host cells receptors called Pattern-Recognition Receptors (PRR), being responsible for the response of the host to its presence.

1.1.2 *C. albicans* virulence factors

Virulence is a complex, dynamic and changeable phenomenon that includes both host and microbial factors. Virulence factors are the components that promote a successful invasion of the host tissues. *C. albicans* main virulence factors are: the cell wall components associated with dimorphic transition; adhesion to epitelium, the cell hydrophobicity and enzyme production and secretion (Fernández-Arenas et al., 2008; McCullough, Ross, & Reade, 1996).

Round yeast cells when exposed to certain stimuli evaginate elongating structures from the cell surface to in order that true hyphal is formed. This first filamentous structure is called germ tube (Berman, 2006; P. Sudbery, Gow, & Berman, 2004).

Hyphal growth in *C. albicans* can be induced by different environmental triggers, for example: acidic pH, low nitrogen, hypoxia, CO_2, high temperature, nutritional starvation and others. As to this, each resulting fungal hyphae has possibly different cell wall proteins and immunological properties depending in which stimuli triggered the hyphal growth (Berman & Sudbery, 2002; Gow et al., 2011).

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The association of *C. albicans* virulence with the ability of the fungal cell to switch from yeast to hyphal morphology is widely recognized. In Berman & Sudbery (2002) the authors wrote: "it is generally thought that hyphal cells expressing cell-wall proteins that facilitate adhesion to human tissues are important for tissues invasion, as well as for escape from phagocytosis mediated by neutrophils or macrophages". Some years later, Gow et al. (2011) describes in detail the host invasion process. The steps associated with how this fungal organism invades the host tissue are: adhesion and colonization of the epithelium; penetration and invasion by hyphae; vascular dissemination that involves hyphal penetration of blood vessels; seeding of yeast cells into the bloodstream, and, finally, endothelial colonization and penetration of other tissues during candidaemia.

The adhesion process is pointed as a virulence factor because allows the microorganism to establish cell to cell relationships enabling the fungus to survive hostile environments. To adhere to the mucosal surface, the hyphal development seems crucial (McCullough et al., 1996).

The cell wall adhesin EAP1 encodes a (GPI)-anchored glucan-cross-linked cell wall protein that mediates adhesion of *C. albicans.* The mechanism behind the cell-cell adhesion is the capability of the fungal cell is to cleave the GPI-linkage and then transfer this anchor and attached protein to form a covalent linkage with β-glucan in its cell wall. The cell wall proteins Als1 and Als3, upregulated during hyphal growth, might also function in surface attachment (Finkel & Mitchell, 2010).

The enzymes produced by the yeast form may function in two major ways, tissues invasion and colonization. These proteinases are capable of degrading mucous membranes immunoglobulin and some of them have keratinolytic or collagenolytic activity. Because proteinase-deficient mutant strains have been described to be considerably less lethal than proteolytic parent strains in animal model, this characteristic remains as a possible virulence factor although the mechanism by which *Candida* proteinases promote adherence is yet to be known. (McCullough, Ross, & Reade, 1996)

1.2 Immune system and *C. albicans* recognition

Microorganisms have co-evolved with their mammalian hosts over millions of years. This suggests the existence of a complex mechanism for immune surveillance in the host and sophisticated fungal strategies to antagonize immunity.

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The immune system establishes a series of pro- and anti-inflammatory events to maintain a suitable host-fungus relationship, the disruption of which can have pathological consequences. Romani, (2011) proposes two main strategies of the host immune response to fungi: resistance (the ability to limit fungal activity) and tolerance, (the ability to limit the host damage caused by the immune response or other mechanisms).

1.2.1 Recognition of pathogenic *C. albicans* by macrophages

The task of recognizing and killing an invading pathogen and activating the host response is accomplished by phagocytic cells: monocytes, macrophages, dendritic cells, neutrophil and T-Cell.

Macrophages are polymorphonuclear leucocytes cells from the innate immune system. They assume an important role in fungal host defense due to their armamentarium of highly potent bactericidal effector mechanisms. This includes: ability to adhere, to phagocytose and to kill the engulfed microorganisms via the subsequent production of toxic oxygen radicals in the phagolysosomes (Elhelu, 1983; Thiel, Caldwell, & Sitkovsky, 2003).

These cells, present in the normal adult blood, are differentiated from a haemopoietic stem cell in the bone marrow. The differentiation process occurs due to growth factors action. The differentiation of the haemopoietic stem cell gives rise to the mieloide progenitor cell, than the monocyte and finally the macrophage (Cavill, 2002). The macrophages can be found in many nonlymphoid organs of the human body: liver, lung, nervous system, epidermis and others.

The recognition of the invasive pathogens, by the macrophages, is carried out by the PRR present in its membrane (Fig. 2). Many classes of PRRs have been implicated in the recognition of *C. albicans* PAMPs and the induction of innate host responses: the Toll-like receptors (TLRs), the C-type lectin receptors (CLRs) and the NOD-like receptors (NLRs) (Gow et al., 2011).

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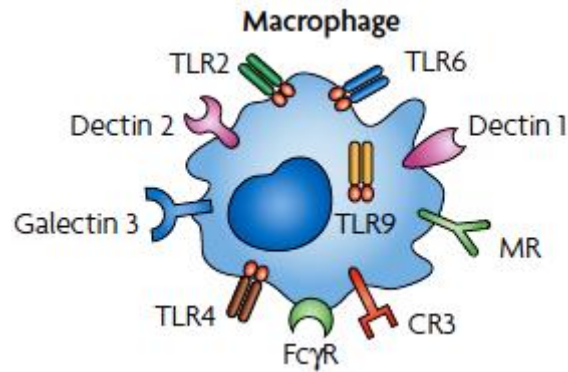
Fig. 2 Mammalian macrophages pattern-recognition receptors. In a sense, most macrophages PPR seem to be involved in *C. albicans* recognition. Toll-Like receptors (TLR2, TLR4), Mannose receptor (MR), Dectin-2 attach to mannan type structures. Dectin-1 recognizes β-glucan structures, galectin 3 recognizes β-mannosides in the intestinal mucosa. Adapted from (Netea et al., 2008).

Mammalian TRLs have leucine-rich repeats in the extracellular area and a Toll interleukin-1 (IL-1) receptor (TIR) domain, located in the cytoplasmic space. The former is responsible for the recognition of PAMPs, and the latter is responsible for inducing host responses. The receptors involved in *C. albicans* recognition and induction of pro-inflammatory induction *in vitro* are TLR2 and TLR4. TLR2 recognizes phospholipomannan and the O-linked mannan has been shown to be recognized by TLR4 (Cheng, Joosten, Kullberg, & Netea, 2012). Experiments in vivo show Knockout mice deficient in TRL2 or TLR4 have an altered susceptibility to disseminated candidiasis, suggesting an important role in the innate host response (Gow et al., 2011).

CLRs mostly recognize polysaccharide structures of microorganisms. *C. albicans* is recognized by several CLRs including decitin 1 (CLEC7A), decitin 2 (CLEC6A), macrophage mannose receptor 1 (MMR), dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN; also known as CD209), macrophage-inducible C-type lectin (MINCLE; also known as CLEC4E) and the circulating mannose-binding lectin (MBL; also known as MBPC) (Cheng et al., 2012). MMR recognize N-linked mannans from *C. albicans*. It is unclear whether MMR has a role in phagocytosis, but it is thought to mediate intracellular signals that lead to cytokine production (Cheng et al., 2012). Dectin-1 is the major recognizer of fungal β-glucan structures in macrophages (Brown, et al., 2002, in (Cheng et al., 2012)). Dectin -2 recognizes mannose-rich structures and induces the production of the cytokine Tumor Necrose Factor (TNF-α) by interacting with another receptor Fcγ. This cytokine production occurs in response to *C. albicans* hyphal growth (Gow et al., 2011). DC-SIGN receptor is involved in the recognition of N-

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linked mannans from *C. albicans* and the uptake of *C. albicans* yeast cells (Gow et al., 2011). Galactin 3 mediates the recognition of *C. albicans* β-mannosides in the intestinal mucosa. Finally, MBL (Mannose-binding lectin) a soluble CLR, mediates Candida opsonization and uptake via binding to Candida mannan and to the surface C1q receptor on the phagocyte (Cheng et al., 2012).

The NOD- like receptors are located inside the cytoplasm and can trigger an innate immune response to intracellular pathogens for instance, the endogenous inflamossome (NLRP3). It is a protein complex which, triggered by PAMPs from microorganisms or an intracellular danger signal, for instance ATP release from epithelial damaged cells, activates caspases 1 inducing the production of pro-inflamatory cytokines as interleucine-1β (Gow et al., 2011; Hise et al., 2009).

As shown in Fig. 3, the recognition of PAMPs by PRR leads to the activation of several pathways and the production of cytokines as IL-1β and TNF-α.
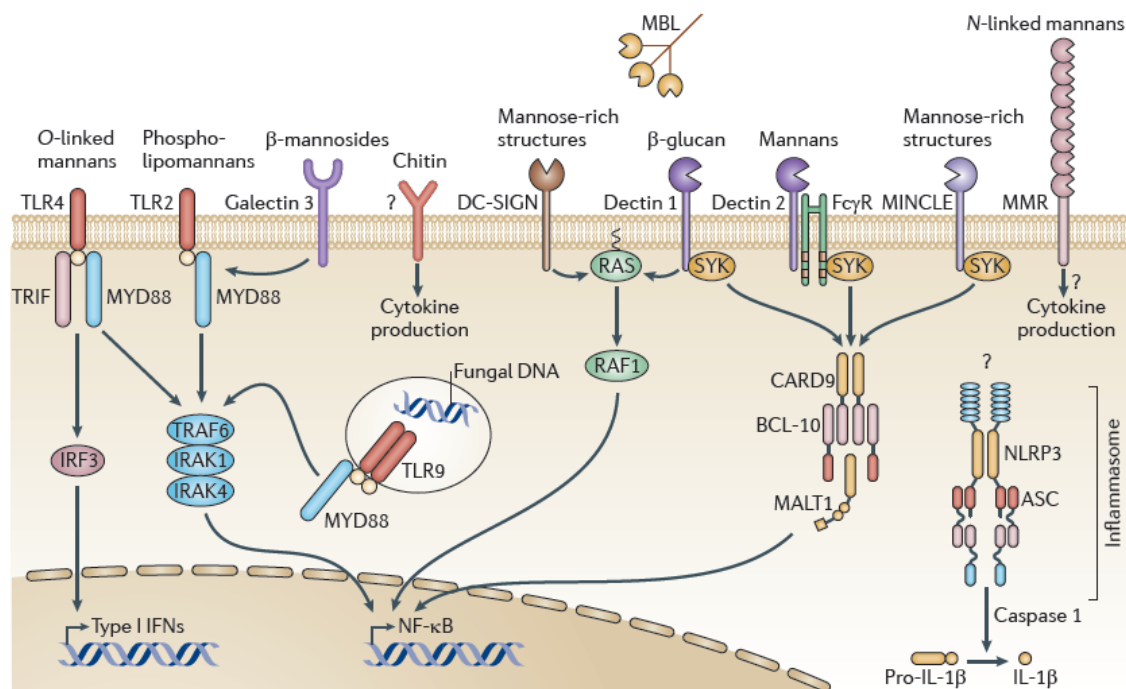

Fig. 3 Recognition of *C. albicans* PAMPs by the macrophages PRR. Mannan structures are recognized by TRL4, TLR2, Galectin 3, DC-SIGN, Dectin-2 and MMR, inducing several pathway leading to the production of interferons, nuclear factor-κB (NF- κB)and probably the activation of the NOD-like receptor pyrin domain-containing 3 (NLRP3) inflamossome. Adapted from Gow et al., (2011).

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1.2.2 Macrophages defenses against Candida infection

Immune system response against fungal infection has many allied. Starting by the physical barriers, than phagocytosis where the immune cell degrades the engulfed pathogen and soluble factors present in the blood stream. This way, most fungal infections don´t disseminate through the body causing disease. Phagocytic cells, as macrophages, are the most effective weapon against fungal attack. After *C. albicans* recognition the following phagocytic process occurs by oxidative and non-oxidative mechanisms.

Although, the innate immune system has an efficient army, as mentioned before, in imunodepressed individuals it is not efficient and fungal dissemination occurs.

1.2.3 *C. albicans* response to macrophages internalization

After the macrophages PRR recognize and internalize the pathogen, *C. albicans* yeast form starts to grow a germ tube. While undergoing this hyphal growth, the yeasts transcriptional profile changes. A dramatic reprogramming of transcription in fungi occurs. In a first response to endocycitosis the microorganism switches its transcriptome to a starvation mode, metabolically gluconeogénese and fatty acid degradation is activated, and translation is dowregulated. In a later phase, when the hypha is escaping from the macrophage, the fungal cells quickly resume glycolytic growth(Lorenz, Bender, & Fink, 2004).

1.2.4 *C. albicans* and hosts perfect relationship Vs Invasion

As described before, *C. albicans* usually lives peacefully in the host without causing disease, and this equilibrium is only ruptured when the host's immune system is compromised. So what are the mechanisms used by the fungi and the host to maintain a healthy relationship?

During commensal stage, C-Jun is independent of fungal morphology and leads to Nuclear factor κB (NF-κB) activation but not to the production of proinflammatory cytokines. However, the activation of MKP1 and c-Fos, is dependent on hyphal growth and the increased fungal population thus, inducing a potent inflammatory response (Fig. 4)(Cheng et al., 2012).

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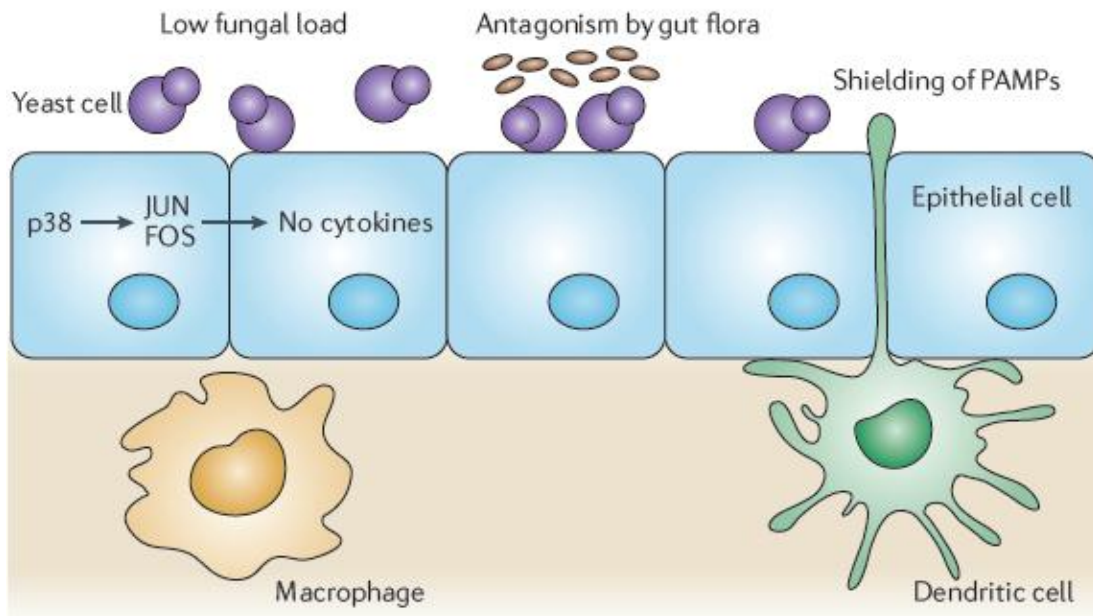
Fig. 4 Colonization of mucosal surfaces by _Candida albicans_. The presence of a normal amount of yeast cells does not induce epithelium cell damage. Thus, no cytokines are produced by the immune cells. In this situation, the fungus PAMPs are hidden and the other microorganism from the bacterial flora act as repressors of hyphal growth. Adapted from Gow et al., (2011).

As known, in the tracts colonized by _C. albicans_, other microorganism can be found. Morales & Hogan, (2010) points out beneficial fungi-bacteria interactions as a method to control fungi spread. For instance, lactic acid bacteria compete with _C. albicans_ for adhension sites in the intestinal and female reproductive tract cell surface. This mixed population inhibits fungal spread, so _C. albicans_ invasion and dissemination is controlled.

An issue concerning this topic is the use of broad spectrum antibiotics, where microbial flora equilibrium is disruptured leading to colonization. In case of bacterial infection an answer for the problem could be the administration of specific targeted drugs for the pathogen, this way the beneficial bacterial-fungal interactions are not disturbed (Morales & Hogan, 2010).

When _C. albicans_ turns to an opportunistic virulent organism the aim is to invade mucosal tissue and spread through bloodstream. The process of crossing the tissue surface can be accomplished either by endocytosis or direct penetration of the epithelium Fig. 5. In the former mechanism, fungal cells are internalized by the epithelium where hyphal cells are thought to induce endocytosis rather than yeast due to the hyphal CWP Adenesin 3 (Als3) adherence to epithelium. In the latter manner, the production of lytic enzymes such as secreted aspartyl proteinase by hyphae seems to

be an effective process for tissue penetration (Gow et al., 2011). *C. albicans* is also capable of downregulating epithelial TLR4 expression hence, the epithelial cell becomes more vulnerable to fungal infection (Cheng et al., 2012).
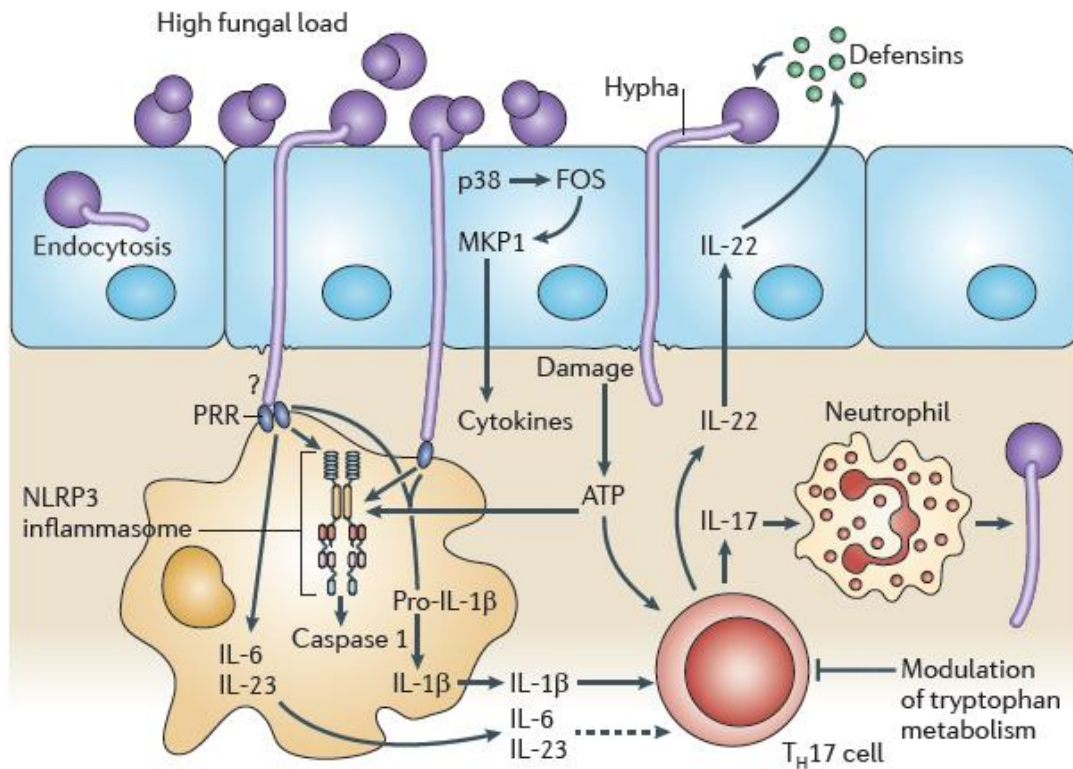
Fig. 5 Invasion of the epithelium by *C. albicans* hyphae. The filamentous form of the fungi can enter the mucosal surface by two different processes: endocytosis or mucosal penetration. The resulting damaged cell releases ATP that is recognized by the immune system as a danger signal. Thus, immune cells as macrophages, T-cell Helper or neutrophils produce cytokines as an inflammatory response. Adapted from Gow et al., (2011).

As *C. albicans* hyphae invade the cell surface, the immune system activates a cascade in response to the pathogens invasion. For this, cytokines are produced, Mitogen-activated protein kinases (MAPK) phosphatase 1 (MKP1) is activated and ATP is released from the damaged cell as adanger signal to the immune system (Fig. 5).

As described before, macrophages recognize the hyphae PAMPs by PRRs. Inside, the inflamossome is activated, and new inter leukines-1β (IL-β) is produced.

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Other immune cells also have a role in this process as T-cells and neutrophils. For instance, the cytokine produced by the T-cell induces the release of molecules that act as a antifungal defense mechanism, so called defensins (Gow et al., 2011).

1.2.5 *C. albicans* escape mechanisms

In the past years fungal research have published data indicating that fungal organisms evolved strategies to escape from PRR recognition or to use it in their own advantage to evade the host response (Netea et al., 2008).

In the mammalian host, inappropriate or premature activation of immunomodulatory receptors can be used as an escape pathway by *C. albicans*. As described by Cheng et al., (2012) after the yeast is internalized by the macrophage, the CO_2 produced in the process, allows the activation of the transcription factor of hyphal growth Efg1p so, the hyphae formed lysis the macrophages membrane and *C. albicans* escapes from the cell (Berman & Sudbery, 2002).
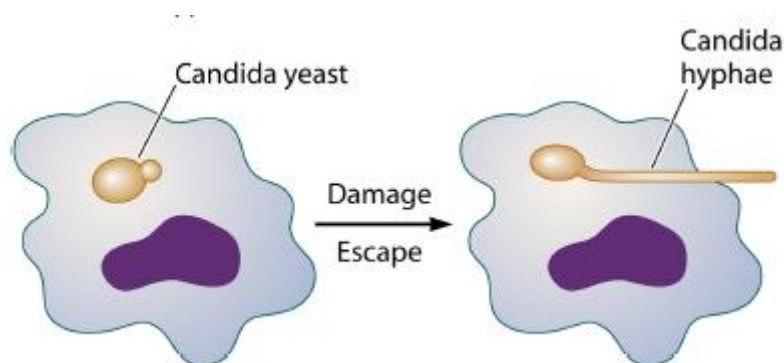
Fig. 6 *C. albicans* escape from the macrophage. After internalized, phenotypic switching occurs. Hyphal growth is induced and the resulting hypha is able to disrupt from the macrophage. Adapted from Cheng et al., (2012).

Hyphal growth, escape from macrophage, and macrophage evasion has been described in Mckenzie et al., (2010). Scanning electron and live video microscopy were performed in *C. albicans* infected J774.1 murine macrophages. They were able to observe the elongation, stretching and piercing of the macrophages cell. However, in a very low rate, the exocytosis can occur without the lysis of the macrophage. Bain et al., (2012) using the same cell line observed the host undergoing mitosis immediately after the hyphal evasion, so, the hyphal growth inside the macrophage does not induce macrophages death.

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Another possible escape mechanism for *C. albicans* is the prevention of its PAMPs recognition. If CWP as mannans and glucans are not exposed to the Dectin-1 macrophage receptor the yeast is able to act as a silent intruder (Fig. 7) (Cheng et al., 2012; Netea et al., 2008).
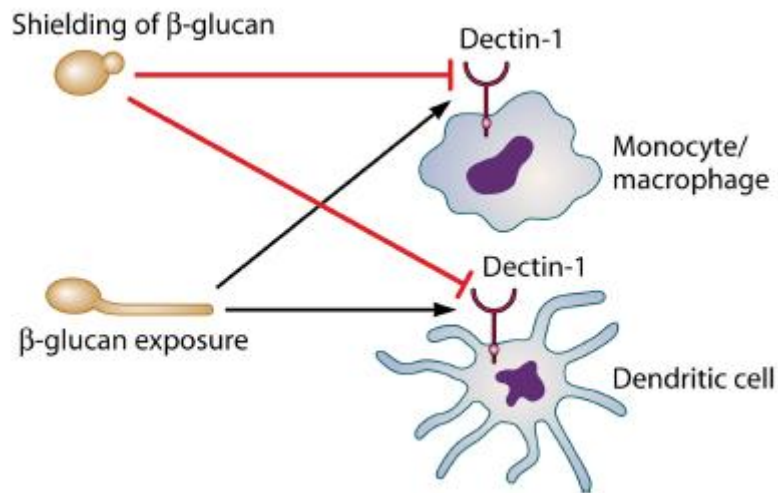
Fig. 7 Evasion strategy of *C. albicans***.** Shielding of the fungus PAMPs thus, inhibiting the recognition of macrophages PRR. Adapted from Cheng et al., (2012).

Another way for escaping the immune cells is if *C. albicans* inhibits phagocytosis. This is possible by suppressing the production of reactive oxygen species (ROS) or inhibiting the formation of the macrophages phagolysosome (Cheng et al., 2012). Fernández-Arenas et al., (2008) points out that the inhibition of phagosomes containing a yeast cell fusion with the lysosome, is an alternative evasion stratagie. Hence, the yeast inside the phagossome will not be killed and can escape the macrophage. Moreover, the fungal structure can leave the macrophage by a non-lytic exocytosis. This way, the fungus leaves the macrophage surrounded by phagosomal membrane becoming undetectable for the immune system (Fernández-Arenas, et al., 2008).

As a commensal microorganism, *C. albicans* struggles with the host immune system continuously. When the immunological balance between fungal commensalism and host immunity, is disrupted, the fungi implies its evasion strategies to escape the host defenses.

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1.3 *Escherichia Coli* endotoxin - Lipopolysaccharide

E. coli is a rod-shaped gram-negative bacteria that belongs to the Enterobacteriaceae family. It is one of the most prevalent microorganism of the human's intestinal flora and the most frequent bacteria agent of human disease.

The bacterial motif lipopolysacaharide (LPS) is a major component of the outer layer of gram-negative bacteria outer membrane. It is composed by an O-side chain, lipid A and a core oligosaccharide (Fig. 8). The lipid A is usually referred as the bacterial endotoxin, when exposed, stimulates the hosts immune system, so it responsible for the inflammatory activity mentioned previously. Its structure can differ between species and strains as a response to different environmental stimuli and plasma membrane deficiency (Ruiz, Kahne, & Silhavy, 2009).

The main function of LPS in the outer membrane of gram-negative bacteria is to confer low fluidity. This is possibly due to the fatty acid chains of lipid A that are highly saturated allowing a tight packing. This property of LPS enables the outer membrane to prevent the rapid diffusion of small hydrophobic molecules giving the microorganism an increased resistance to hydrophobic antibiotics regarding Gram-positive bacteria (Ruiz et al., 2009).

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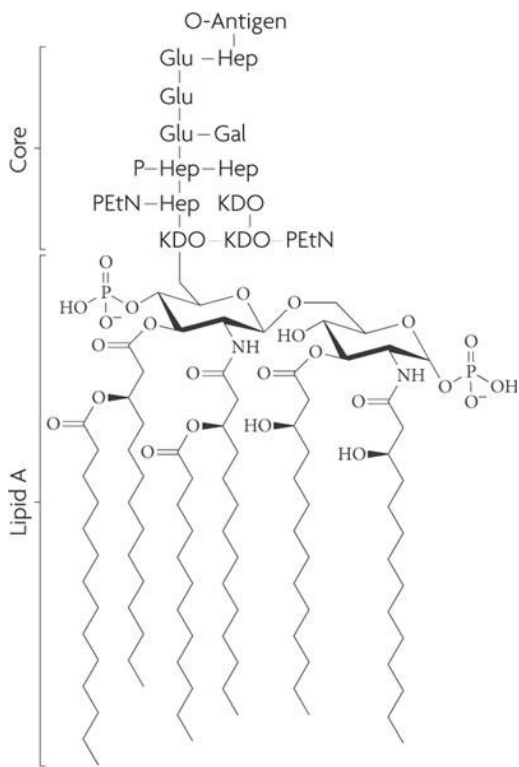
Fig. 8 *Escherichia coli* LPS structure. Adapted from Ruiz, Kahne, & Silhavy, (2009).

LPS is also the major cause of sepsis among humans. The noxious effects to host cells leads to a acute disseminated inflammation state with organ failure and death. If exposed to a sublethal concentration, the result can be an initial state of tolerance but then a severe inflammatory response is subsequent.

LPS is recognized by Toll-like receptors, particularly TLR4 (Lu, Yeh, & Ohashi, 2008) inducing the production of proinflammatory cytokines, such as TNF-α, IL-1 and IL-6, and nitric oxide, through the activation of the p38 kinase pathway. (Dobrovolskaia & Vogel, 2002).

Once introduced in the eukaryotic host, LPS binds to LBP (LPS-binding protein) free in the plasma. This complex then binds to the cell surface receptor CD14 in the macrophage. Next, LPS is transferred to transmembrane signaling complex toll-like receptor 4 (TLR4) MD-2, triggering the release of inflammatory cytokines (Guha & Mackman, 2001).

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1.4.1 Interactions between bacteria and fungi

As mentioned, *C. albicans* is the major fungal opportunistic pathogen due to its capacity to evade from the innate immune system. In addition, it is capable of interacting with other microorganisms in order to survive in a hostile environment, other species of fungi or bacteria.

Bacteria and fungi can be associated in polymicrobial communities or in symbiotic relationships as mixed biofilmes in the oral cavity.

Regarding the interkingdom interactions of *C. albicans*, several relationships have been described. For instance, nutritional conditions have been shown to modulate coadherence between this fungus and oral bacteria (Peleg, Hogan, & Mylonakis, 2010). The quorum sensing farnesol inhibits hyphal growth in *C. alibicans* but modulates the expression of virulence genes in *Pseudomonas aeruginosas.* The gram positive bacterial peptidoglican induces *C. albicans* hyphal growth (Xu et al., 2008; in Gow et al., 2011). Also, the presence of *C. albcans* has been shown to significantly enchance *Staphylococcus aureus* biofilm formation and its resistence to vacomycin in serum (Frey-Klett et al., 2011). *E.coli* LPS was demonstrated to influence the physiology of *Saccharomyces cerevisiae*, by modulating the Hog1 pathway, an homolog of p38 (Marques, Rodrigues, de Magalhães-Sant'ana, & Gonçalves, 2006).

The interest for bacterial-fungal biofilm structures has been increasing in the clinical context due to the prevalence of mixed infections and presence in medical devices. In the latter cases, microorganisms, both yeast and bacteria typically show enhanced resistance to antibiotic therapies.

Among the mixed infections comes *C. albicans* fungal infection during LPS induced severe sepsis, these cases have been increasing over the past years. Xie et al., (2008), conducted a study where the impact of invasive fungal infections had on the outcome of critically ill surgical patients with severe sepsis was evaluated. The main findings were that *C. albicans* was the most prevalent pathogen, among patients with severe sepsis and fungal infections. Also, the mortality rate is higher in these cases if compared with septic patients with no fungal infection.

A study conducted in mice suggests that the administration of LPS in order to the organism develop tolerance, enhances survival after disseminated fungal infection with *Cryptococcus neoformans* (Rayhane, Fitting, Lortholary, Dromer, & Cavaillon, 2000).

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1.5 Aims

The main aim of this work was to study the effect of macrophages stimulation by *E. coli* LPS and heat killed *E. coli* (bacterial endotoxin) in the outcome *C. albicans* infection. This study included the assessment of macrophages response to the infection, the cytokine production, the viability of the internalized yeasts as a measure of the phagocytic efficiency, and finally the macrophages viability.

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Materials and Methods

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2   Materials and Methods

2.1   Microorganisms culture media and solutions

2.1.1   *Candida albicans* growth conditions

The wild-type *C. albicans* strain (YP0037) used in this study is part of the Pathogenic Yeast Collection of the Institute of Microbiology from the Faculty of Medicine of the University of Coimbra.

Yeasts were grown at 30ºC, on solid Yeast Extract Peptone Dextrose (YPD) medium. This medium was prepared with: 0.5 % Bacto Yeast Extract (Panreac-Cultimed, 403687); 1% Peptone, Bacteriological (Panreac-Cultimed, 403695); 2% Glucose (Dextrose; Corn Sugar, Sigma, G8270); and 2% Agar Technical (Panreac-Cultimed, 401792)). The solution was sterilized by autoclaving at 121ºC with pressure 1.2 atm for 20 min and distributed onto petri dishes.

2.1.2   Yeast cells harvesting

For infection assays, a 24 h grown culture was harvested and suspended in 1 mL of PBS (Phosphate buffer saline buffer: 10mM Na_2PO_4; 1.8mM KH_2PO_4; 137mM NaCl; 2.7mM KCl at pH 7.4), centrifuged at 16060g for 2min then ressuspended in cold PBS. This procedure was performed twice.

For cell number count a haemocytometer was used. For this purpose the sample was diluted 100x.

2.1.3   *E. coli* growth and inactivation

The strain of *E. coli* used in this study was isolated from a human specimen. Bacteria were grown in Columbia medium during 24 h at 37ºC. Colonies were suspended in distilled water until a cell density of 0.5 McFarland units (1.5×10^8 CFU/mL) (Zamora & Pérez-Garcia, 2012). The initial suspension was diluted twice by 1:10 serial dilutions obtaining 1.5×10^5 CFU/mL.

The procedure to obtain heat-killed *E. coli* (HKEc) was adapted from Empey, Hollifield, & Garvy, (2007). Briefly, 1 mL aliquots were boiled for 5 min to expose the endotoxin in the outer membrane, cooled and spun up at 16060g for 10 min; then resuspended in

900 µL of distilled water. This process was repeated twice. The ultimate suspension was kept frozen in RPMI medium at -20ºC, for further use.

To verify if the bacteria had been efficiently killed, a sample of the heat-killed *E. coli* was spotted in YPD solid medium next to non-treated suspensions of the two dilutions. After 48 h at 37ºC, no bacterial growth was observed comparing with the non-treated samples observed in the other spots.

2.1.4 Effect of LPS and HKEc on *C. albicans* viability

The effect of a commercial LPS and of HKEc on the growth capacity of *C. albicans* was assessed by incubating a yeast cell suspension. Yeasts cells were grown overnight in YPD Agar and harvested as described in section 2.1. The yeast suspension, was incubated with LPS (Sigma, L8274; commercial, *E. Coli* Serotype 026:B6; L-8274, Sigma-Aldrich) 0.1 µg/mL or 1 µg/mL, and HKEc for 1.5 h, 3 h and 6 h. HKEc prepared and collected as described under 2.1.3.

After each incubation time, serial dilutions were performed and 5 µL of each dilution were spotted in YPD agar and grown for 2 days at 30ºC. Also, 100 µL aliquots were plated in YPD agar for Colony Forming Units (CFU's) counting. The plates were incubated at 30ºC and CFU'S counted after 3 days.

2.1.5 *C. albicans* hyphal growth after incubation with LPS and HKEc

The aim of this task was to verify if LPS or HKEc had any effect in *C. albicans* germ tube formation, i. e. in *C. albicans* yeast-to-hypha transition.

The yeast cells were prepared as described in section 2.1.2 and HKEc as described in section 2.1.3.

For germ tube assessment 1×10^{6} yeast per mL were suspended in 1 mL of RPMI medium (Sigma R4130) supplemented with 10% of inactive Fetal Bovine Serum, 23.8 mM of sodium bicarbonate, 50 mM of glucose) with 0.1µg LPS, 1 µg LPS and HKEc, and grown at 37ºC, with 180 rpm rotation.

The percentage of germ tube formation was assayed by haemocytometer counting established standards (Berman, 2006; P. Sudbery et al., 2004), and expressed as the ratio between number of filamentous yeast and total cell number.

2.2 Infection assays

2.2.1 Cell line and culture conditions

The cell line RAW 264.7 murine macrophages, was purchased through Sigma European collection of cell cultures (ECACC [91062702]). The cell line grew in $75cm^2$ Corning® flasks with Dulbecco's Modified Eagle Medium (DMEM sigma's D5648) supplemented with 10% of active Fetal Bovine Serum (FBS), 10mM HEPES, 12mM Sodium Bicarbonate and 11mg/ml sodium piruvate. The cells grew at 37ºC with 5% CO_2 up to 80% confluency and split 1:5 every 2 days (number of generations < #18).

2.2.2 Cell harvest

For infection assays, the macrophages (Mϕ) were plated in multi-wells 18 h before infection with appropriate cell number to allow the cells to grow and duplicate. Cells were removed mechanically from the flask, by scrapping, and cell number assayed. For this, trypan blue 0,4% (SIGMA, T8154) was diluted in a range 2x (10 µL cell suspension + 10 µL trypan blue) and only viable cells were counted in the haemocytometer. The cells were supplemented with RPMI medium.

2.2.3 Macrophage co-infection assay with *C. albicans*, LPS, and HKEc

Yeast and macrophages were harvested as described in section 2.1.2 and 2.2.2 respectively. RAW 264.7 macrophages were incubated with 0.1 µg/mL of LPS or HKEc (1:1 HKEc) 1 h and 24 h before infection, in appropriate cell culture clusters at 37ºC 5% CO_2.

After stimulating the macrophages, the medium was removed and fresh RPMI medium was with yeast cells. Co-cultures were incubated at 37ºC and 5% CO_2 during several time-periods. The multiplicity of infection (MOI) used in all experiments was 1:1.

2.2.4 *C. albicans* viability assay

This experiment was performed to verify the yeast cell viability upon exposure to stimulated macrophages, as a measure of the phagocytic efficiency of macrophages.

For the yeasts viability assessment, $1x10^5$ Mϕ/mL were plated in 96 well cell culture cluster (Costar®, Corning Inc.) as described in 2.2.2 and infected with yeast as

mentioned in section 2.2.3. After the infection assay, the medium was collected together with the yeast cells, labeled as non-adherent yeasts. Then, to each well, 200 µL of distilled water and 50 µL of 0.5 % of Triton X-100 (BDH, Prod 30632) was added, to harvest the adhered cells (macrophages and internalized yeasts), by scrapping with a 200 µL pipette tip. The collected suspension was added to another 250 µL of distilled water, for release of internalized *C. albicans*. These samples were named as adherent yeasts.

Both non-adherent and adherent samples were diluted, and 100 µL of each was plated in YPD solid medium in triplicate. These plates were grown at 30ºC for 3 days before CFU counting.

2.2.5 Influence of LPS- and HKEc-stimulated macrophages in *C. albicans* infection

In order to study the interaction between macrophages stimulated with LPS and HKEc and the yeast cells a differential fluorescence staining protocol in 1:1 co-cultures that allows the distinction between total yeast cells (green), and internalized yeasts attacked by phagolysosomes (yellowish to reddish) and a colony forming unit (CFU) assay estimating both the yeast cells that were internalized and the yeast cells that were attached or internalized by macrophages.

Macrophages were harvested as described in section 2.2.2 and $2.5{\times}10^5$ Mɸ/mL (viable macrophages) were seeded in 12 well cell culture cluster (Costar®, Corning Inc.) with 16 mm cover slips.

On the day of the infection, yeast cells were collected as described in 2.1.2 and labeled with 2 µM Oregon Green® 488 (Life Technologies, O6185) for 1h at 30ºC in the dark. Macrophages were stained with 300 mM of Lysotracker® red (Invitrogen, Molecular Probes, L7528-20x50µL) for 1 h at 37ºC with 5% CO_2. Then, 700 µL of the medium was removed and macrophages were incubated with 0.1 µg/mL LPS and HKEc for 1 h, also in the dark.

After 1 h of stimulation with LPS and HKEc, macrophages were infected with *C. albicans* previously labeled with Oregon Green and co-cultures were then incubated for 3 h at 37ºC and 5% CO_2.

After 3 h of co-infection, performed as described in 2.2.3, cells were washed twice with PBS and fixed with 4% formaldehyde solution (36. 5 -38%, Sigma Aldrich, F8775) for

13 min at room temperature. The cover slips were washed again three times with cold PBS, and the coverslips were mounted with fluorescence mounting medium (Dako, S3023).

Images were acquired in a Observer Z1 microscope (Carl Zeiss, Jena, Germany).

2.2.6 Macrophage Viability assay

With the purpose of studying the effect of the several infection assays in the macrophages viability, trypan blue dye was used, since this dye probes for dead cells. As these cells have a ruptured membrane, the vital stain enters the non-viable cell distinguishing living cells from dead cells.

After 1.5 h, RPMI medium with non- adherent/non-internalized cells was removed to allow full ingestion of the remaining yeast. Next, new medium was added and incubated again at 37°C in 5% CO_2 for 6h.

For cell counting, a haemocytometer and a (NIKON LWD 0.52) phase contrast microscope was used.

2.2.7 Inflammatory response of infected macrophages

In order to quantify the amount of IL-1β and TNF-α released during the macrophages infection and estimate the inflammatory response of the macrophage, two separate ELISA assays were performed. Commercial kits were used, the Mouse TNF alpha ELISA Ready-SET-Go!® and Mouse IL-1 beta ELISA Ready-SET-Go!®.

For this experiment, 2.5×10^5 Mφ/mL were grown and plated in 12 well cell culture cluster (Costar®, Corning Inc.) as mentioned in section 2.2.2. Yeast cells were harvested as described in section 2.1.2 and the macrophages stimulation and subsequent infection was performed as mentioned in section 2.2.3.

After a 3 h-period of infection supernatants of each well were collected (1mL), kept in ice, centrifuged at 14825g for 10 min at 4°C, and 500 µL of the supernatant stored at -80°C for further use.

The ELISA assay was performed according to the manufacter's instructions. Data was obtained by reading the plate in a (SPECTRA MAX PLUS 384) spectrometer with the SoftMax 5.2 software at wavelength 450nm and 570nm. The absorvance of the samples were acquired at a wavelength of 570 nm and subtracted from that obtained at

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a wavelength of 450nm. Cytokine concentration values were interpolated from a three order polymonial curve obtained from two-fold serial dilutions of the top standard included in the kit.

2.3 Statistical Analysis

Statistical analysis was performed on GraphPad Prism 5th edition. Statistical significance within groups was analysis by a 1way ANOVA test with Bonferroni's Muitiple comparison test performed.

As to analysis between groups, a 2way ANOVA with Bonferroni's Muitiple comparison test was performed in which each column was compared with the control column. In this case when performed, the control column was infection of macrophages with *C. albicans* (Mϕ + Ca). Statistical significance was considered when p<0,05. All results are shown as mean ±SEM for independent experiments.

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Results

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3 Results

3.1 Effect of LPS and HKEc on *C. albicans* viability

LPS is a bacterial endotoxin, present in *E. coli*'s outer membrane, capable of inducing a severe inflammatory response.

Before studying the impact of LPS and of inactivated *E. coli* cells in the macrophage-*C. albicans* interaction an assay was performed to understand whether LPS and HKEc had any effect on the yeast growth and viability, for further understanding the dynamics in the infection assays.

For this, yeast suspensions were spotted in YPD agar after 0 h; 3 h and 6 h of incubation at 37ºC and 180 rpm and 100 µL aliquots plated also in YDP agar for CFU counting. Yeast colonies were allowed to grow during 72 h at 30ºC.
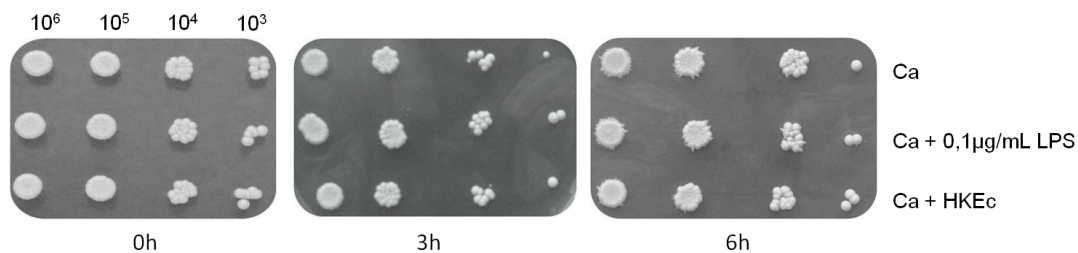

Fig. 9 HKEc and LPS effect in *C. albicans* growth and colony morphology. A spot dilution assay was used. Pictures were acquired by a digital camera with 7.2 megapixels, 72 h after incubation at 30ºC. The photos are representative images of 3 independent assays.
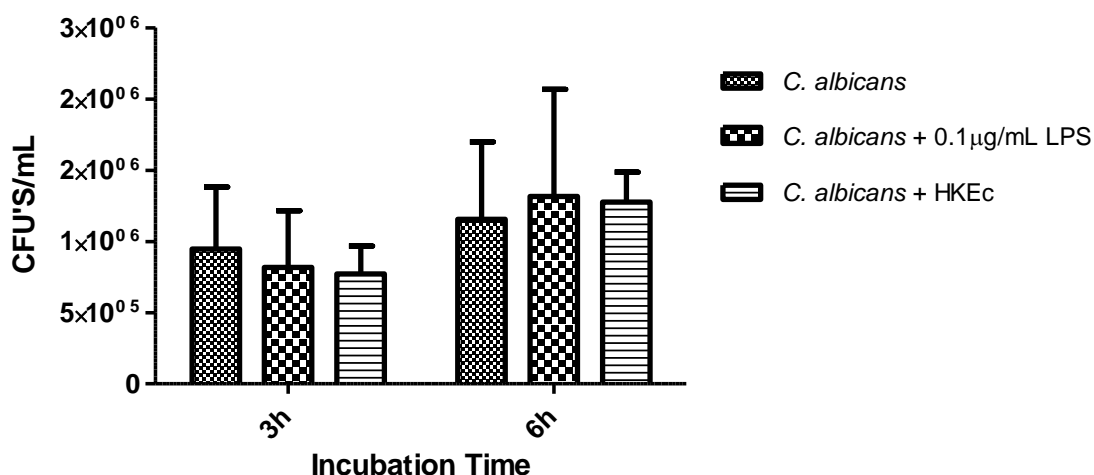
Fig. 10 *C. albicans* viability upon LPS and HKEc exposure. Yeast suspensions were incubated during 3 h and 6h with LPS 0.1 ug/mL and HKEc (1.5 ×10^5 bacterial cells). Viability was assessed by a colony forming unit assay. This data represents three independent experiments. Results are presented as means ±SEM.

The incubation of *C. albicans* with LPS or HKEc, has no effect in its growth or colony morphology, comparing each treated row with the non treated colonies (Ca) (**Fig. 9**). As to the fungus viability, Fig. 10 indicates a slight decrease in CFU number when the yeast is incubated with LPS and HKEc at 3 h incubation, and a increase after 6 h comparatively to *C. albicans* with no endotoxin. However, neither are confirmed by the spot dilution assay or have statistic significance.

3.2 *C. albicans* hyphal growth in RPMI medium

In *C. albicans*,the yeast-to-hypha morphological transition is considered a virulence factor. This transition begins with the formation of a structure called the germ tube, initiating the switch to filamentous morphology, either pseudo-hypha or hypha. So, in order to verify if LPS and HKEc influenced hyphal growth in *C. albicans* the ratio of cells with filamentous growth was determined.
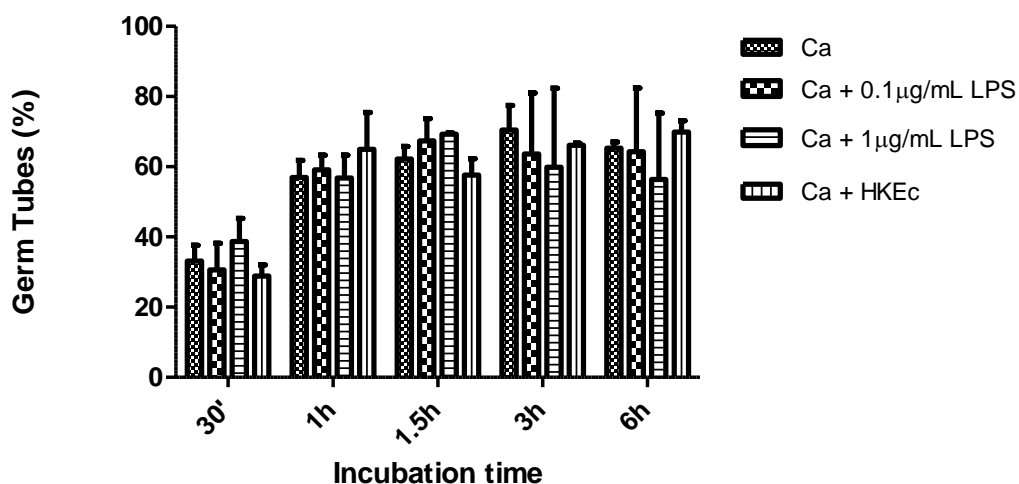
Fig. 11 Percentage of germ tube formation in *C. albicans.* Cells were incubated with: 0.1µg mL^{-1}, 1µg/mL LPS and HKEc for 30 min to 6 h. 1×10^6 yeasts per mL were incubated in RPMI medium, at 37ºC and 180rpm, with the referred compounds and filamentous cells counted after each incubation time. The results represent three independent experiments and are presented as mean ±SEM.

As described in chapter 1, environmental factors induce hyphal growth in *C. albicans.* Under these conditions, germ tube formation starts immediately after the incubation of yeast with RPMI medium at 37ºC. As demonstrated in **Fig. 11**, after 30 minutes of incubation, approximately 35% of the yeast population exhibits a germ tube. During the first hour, there is an increase of filamentous cells, and the percentage of germ tube remains in a range of 70% of the yeasts for the next five hours. The presence of LPS, either 0.1µg mL^{-1}, 1µg/mL, or HKEc did not affected the formation of germ tube, so the transition of *C. albicans* cells to a filamentous form is not affected by bacterial endotoxin.

3.3 *Candida albicans* resistance to phagcytosis

In order to ascertain if macrophages previously stimulated, are capable of killing the internalized yeast compared to non-activated macrophages, *C. albicans* viability was assessed in previously primed macrophages and in resting macrophages. For this, Colony Forming Units of the yeast were counted after suspensions were plated in YPD agar and grown for 3 days at 30ºC.

Fig. 12 indicates that the number of viable yeast cells remaining in the supernatant during infection decreases with time. After 3h, number of CFU's counted is reduced by half regarding 1.5 h.

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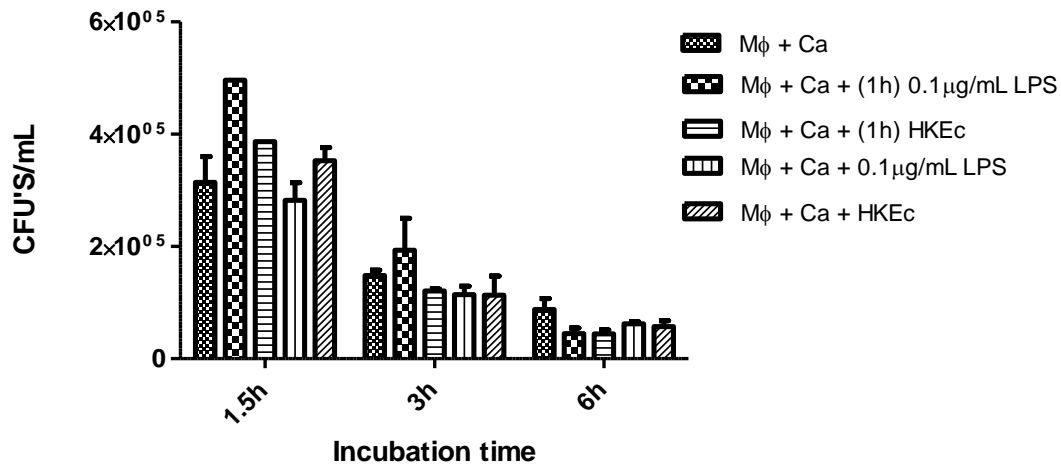
Fig. 12 *C. albicans* cells non-adherent to macrophages. The CFUs in the supernatants of co-cultures were quantified after 1.5 h, 3 h and 6 h of infection at 37ºC and 5% CO_2. Supernatants were plated in YPD plates and grown at 30ºC and colonies counted after 3 days. The results are introduced as means ±SEM, n=3.

The analysis of CFU's adherent to macrophages (Fig. 13) showed that macrophages efficiently internalize yeasts in the first 1.5 h of infection, as the surviving yeast population during the following 1.5 h, remains in the same range. After 6 h of infection, the fungi is able to duplicate inside the macrophage. When macrophages are activated for 1h with 0.1μg mL^{-1} of LPS or HKEc before infection, and co-incubated with bacterial endotoxin and yeast, a slight decrease in the yeast cells internalized is noticed in each hour of infection.

Fig. 13 *C. albicans* internalized by macrophages. Yeast colonies were plated in YPD agar and grown for 3 days at 30ºC. The results represent means ± SEM of three independent experiments.

This data suggests that, during infection, yeast adhere to the macrophage, are internalized, but, since macrophages are not able to efficiently kill them, yeast cells multiplicate inside the macrophage leading to a yeast population slight increase.
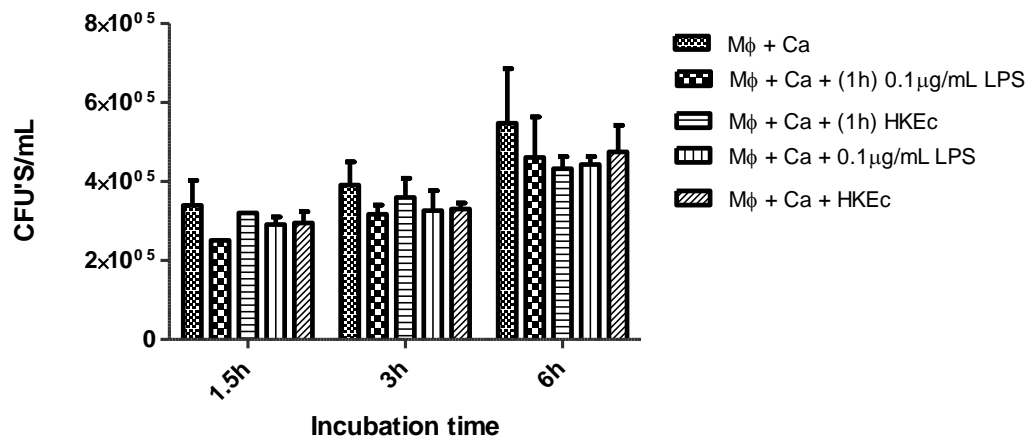
However, there is a decrease in *C. albicans* viability when the macrophage is exposed to LPS and HKEc, previously to fungal infection. Also, macrophages incubation with simultaneously with the yeast and LPS or HKEc with no prior stimuli.

3.4 RAW264.7 Macrophages Viability

Phagocytic cells, and particularly macrophages, are part of the first line of defense against pathogen invasion. Their primary function is to engulf the pathogen. However, the pathogens also developed strategies to escape phagocytosis. In these cases, does the escape involve macrophages lysis?

With the purpose of studying the effect of yeast infection in macrophage's viability, when previously stimulated with LPS and HKEc and co-infected with bacterial endotoxin, the numbers of viable and non-viable cells were counted and cell viability calculated. Fig. 14 show more than 80% of cellular viability non-infected macrophages. The percentage of cell death is due to the technique used.

The results demonstrate a decrease in the cells viability when macrophages are stimulated during 1 h with LPS previously to *C. albicans* infection. On the other hand, macrophages viability is not significantly affected by *C. albicans* infection or co-infection of the yeast and LPS or HKEc, nor fungal infection during HKEc exposure.
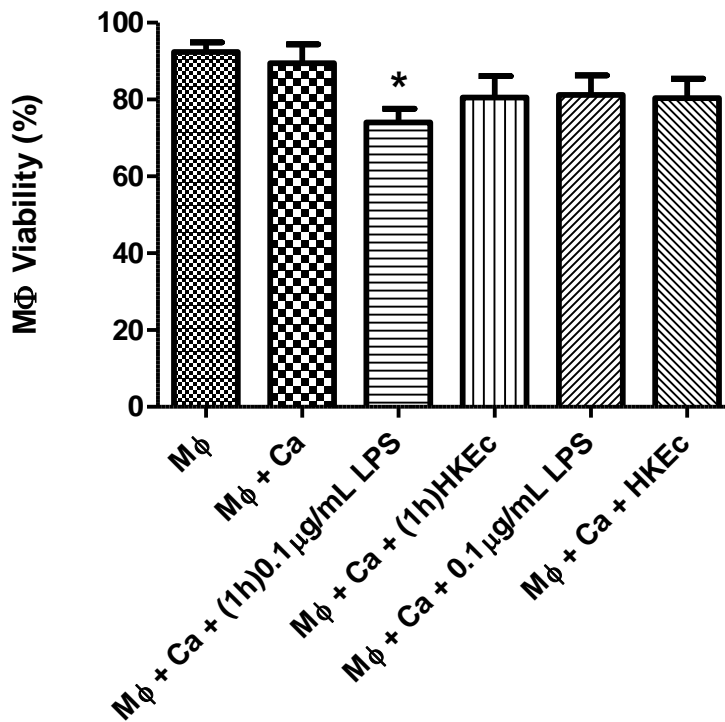
Fig. 14 Macrophages viability. Macrophages were activated with LPS and HKEc for 1 h before infection with *C. albicans*, (remaining the LPS and HKEc in the well) and also co-infected with no previous stimuli. The experiment was conducted for 6 h at 37°C and 5% CO_2. The results are presented as means ± SEM three independent experiments.

3.5   Inflammatory response – quantification of TNF-α and IL-1β by ELISA

When macrophages are exposed to a pathogen, the response involves the production of proinflammatory cytokines as TNF-α and IL-1β. The release of TNF-α results from the recognition of *C. albicans* PAMPs by TLRs and IL-1β to activation of inflammossome and caspase 1 activity.

To quantify the amount of TNF-α IL-1β released by macrophages when infected with yeast, an ELISA was performed. During macrophages infection, there is a significantly higher release of TNF-α then IL-1β (Fig. 15 A and B). However, the maximal production of both cytokines occurs when macrophages are co-infected with *C. albicans* and LPS after previously activated with 0.1 µg/mL of LPS for one hour, (Mɸ + 1 h 0.1 µg/mL LPS + Ca), 15 pg/mL of IL-1β and 862pg/mL of TNF-α.

Fig. 15 A) shows that the production of IL-1β by macrophages is slightly increased when infected with yeast, 7.15 pg/mL to 11.84 pg/mL. Also, if previously activated with LPS, the subsequent co-infection with yeast and LPS induces the macrophages to

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release more IL-1β 14.85 pg/mL. However, the activation itself with LPS, for 1 h, has no significant release of cytokine. Regarding the production of cytokine when exposed to HKEc, the incubation during 1 h has no significant production of IL-1β, and the afterward co-infection with yeast and HKEc has no increased production of cytokine comparing with macrophages and *C. albicans*, as expected.

On the other hand, macrophages produce larger amounts of TNF-α, as illustrated in Fig. 15 B). When stimulated 1h with 0.1 μg/mL of LPS, 468.28 pg/mL of cytokine was released, and 1h activation with HKEc produced 369.84 pg/mL. Significantly larger amounts than macrophages infected with yeast (277.24 pg/mL). The co-infection of macrophages with yeast and LPS or HKEc, during 3 h, following the one hour activation, shows an increase in TNF-α release in both cases (862.50 pg/mL and 462,958, respectively) if compared with macrophages with yeast only, 277.24 pg/mL.
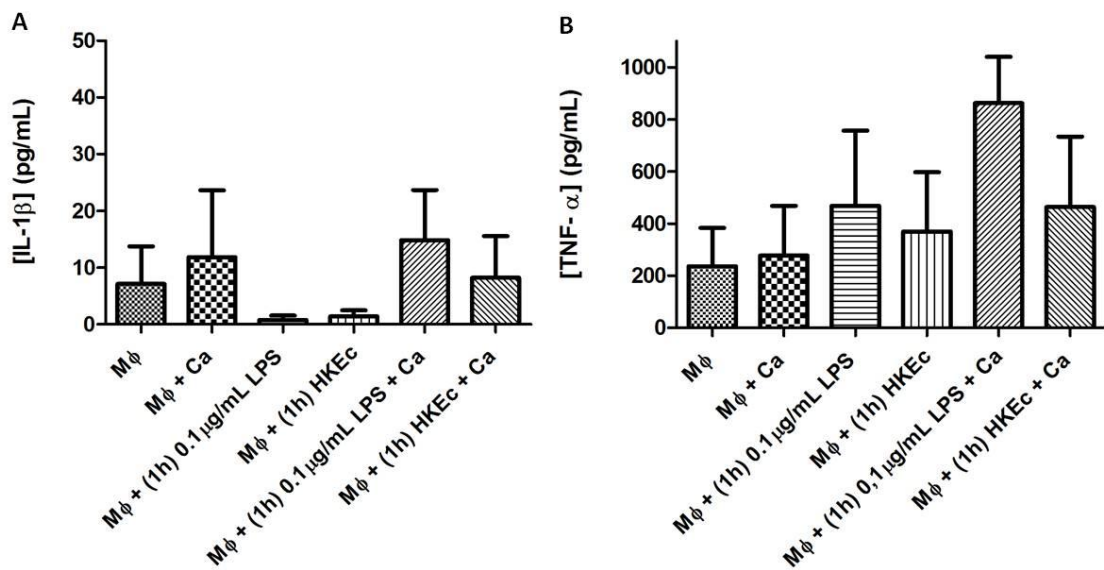
Fig. 15 Production of TNF – and IL1 by RAW 264.7 macrophages. Quantification of IL-1β (A) and TNF-α (B) obtained from macrophages (Mϕ), macrophages with *C. albicans* for 3 h (Mϕ + Ca), macrophages incubated with 0.1μg/mL LPS for 1 h (Mϕ + (1h) 0.1μg/mL LPS) , macrophages and HKEc for 1 h (Mϕ + (1h) HKEc), and macrophages co-incubated with LPS/HKEc and yeast for 3 h after 1 h stimulation with LPS or HKEc (Mϕ + (1h) 0.1μg/mL LPS + Ca); (Mϕ + (1h) HKEc + Ca). The infection lasted 3 h. The results are presented as mean ± SEM (n=3).

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3.6   Interaction between activated macrophages and *C. albicans*

With the purpose of understanding the macrophages response to fungal infection upon stimulation with bacterial endotoxin, regarding its morphology and phagolysosome activation, a differential fluorescence staining protocol in 1:1 co-cultures that allows the distinction between total yeast cells (green), and internalized yeasts attacked by phagolysosomes (yellowish to reddish)
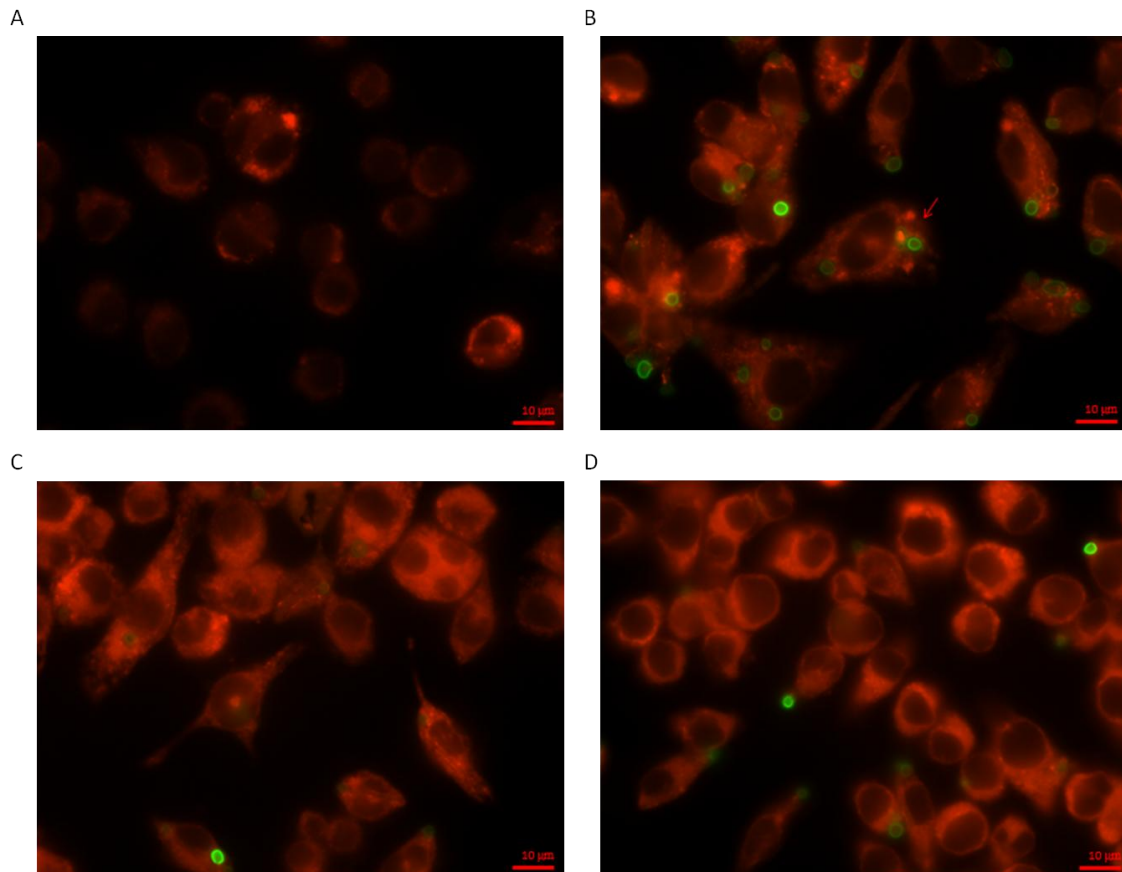
Fig. 16 Macrophages interaction with *C. albicans*. A) Macrophages; B) macrophages and *C. albicans*; C) macrophages stimulated 1 h with 0.1μg/mL LPS followed by 3 h infection with yeast *plus* LPS; D) macrophages stimulated 1 h with HKEc followed by 3 h co-infection with yeast *plus* HKEc. Red arrow indicates a macrophage with yeast cells internalized. MOI 1:1. Scale bar indicates 10μm. Images acquired in an Observer Z1 microscope (Carl Zeiss, Jena, Germany) with an objective Plan ApoChromt 63x. Representation of 50 pictures per condition.

When macrophages are not stimulated they appear as round cells (Fig. 16 A). But when exposed to *C. albicans* yeast cells a morphological change is observed, with cells exhibiting a stretched and elongated form (Fig. 16 B).

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Moreover, a stronger reddish color, Fig. 16 C and D, indicates that the macrophage is more activated in what concerns phagolysosome activation. This may lead to the observed decrease in green yeast cells in these conditions, i.e. a lower number of internalized viable yeast cells.

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Discussion

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4 Discussion

Over the past years, fungal opportunistic infections have been increasing among the imunocompromised: HIV-infected patients, cancer patients receiving chemotherapy, or patients that undergone organ transplant. Also, the number of opportunistic fungal infections has increased in patients with severe sepsis, and the impact of the fungal infection in the outcome of severe sepsis is not well understood. *C. albicans* is the major fungal pathogen, and invasive infection by this yeast in patients with severe sepsis leads to higher rate of mortality (Xie et al., 2008).

The aim of this work was to study the outcome of the fungal infection when the innate immune system is dealing with the main agent of sepsis - bacterial endotoxin. For this, an in vitro infection model was built, with a murine macrophage cell line - RAW 264.7, representing the first line of defense against pathogens. The macrophages inflammatory response was stimulated either with a commercial *E. coli* LPS or heat killed *E. coli* cells (HKEc). In this model of inflammatory conditions it was assessed *C. albicans* infection outcome: phagocytosis and survival inside the macrophage and the macrophages viability during infection.

At first glance it could not be discarded a possible direct effect of bacterial endotoxin (either LPS or HKEc) on the *C. albicans* growth, morphology and viability. In fact, this would affect the outcome of the in vitro macrophages infection with *C. albicans* in the presence of these bacterial elements. The influence of bacteria in fungal cell physiology has been described in several specific habitats of the human body. *C. albicans* and bacteria coexiste in many compartments and it is known that the imbalance between the two groups of microrganisms can lead to the yeast overgrowth (Payne et al., 2003). The opposite of this peaceful co-existence between yeast and bacteria is mixed infection that results in infectious diseases where both pathogens employ harmful effects to the host. An example of mixed infection is the respiratory infection occurring in cystic fibrosis patients, where *C. albicans* and *Pseudomonas aeruginosa* persist and influence the outcome of the infection (Chotirmall ;Greene, Catherine M; McElvaney, 2010). *P. aeruginosa* colonizes the surface of *C. albicans* hyphae leading to massive killing of the fungus, while the yeast form is resistance to the attack by the bacteria (Hogan and Kolter, 2002). During this work, as shown in

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figures 9,10 and 11, no evidence was found that bacterial endotoxin (*E. coli* LPS or HKEc), influence *C. albicans* viability or morphology. It is worth mentioning that these assays were performed with *C. albicans* in the yeast form and the noxious effects of *P. aeruginosa* were only sensed by the hyphal-*C. albicans* form (Hogan and Kolter, 2002; Brand et al., 2008). The absence of an effect of the bacterial (*E. coli*) endotoxin was also observed in the yeast *Saccharomyces cerevisiae*, with no effect in growth in a wild-type strain. However, in a *S. cerevisiae* hogΔ mutant for the gene *HOG1*, the homologue of mammalian cells p38 (Marques, Rodrigues, de Magalhães-Sant'ana, & Gonçalves, 2006) it was observed a decrease in viability. The absence of a response found in the present work might change if *C. albicans* in the hyphal form was used (Brand et al., 2008) and it would be interesting to test the response of a *C. albicans* mutant in the Hog1 protein.

In what regards the impact of priming the macrophages with bacterial endotoxin (LPS or HKEc) in the outcome of *C. albicans* infection assays, it was found that the viability of the yeast cells internalized by macrophages, was slightly lower than that observed when *C. albicans* was infecting non-primed macrophages. It is a fact that phagocyted *C. albicans* cells retain their ability to divide, inside the phagosome (Romani, 2011), meaning that during in vitro infection assays there are yeast cells growing and dividing while others are killed by the phagocytic machinery. The observed lower viability of yeast cells in primed macrophages indicates that the macrophages are more activated and can antagonize more efficiently the mechanisms of *C. albicans* resistance to phagocytosis. This result was confirmed using a differential fluorescence microscopic method where Lysotracker Red, a weak base that it is only partially protonated at the cell pH, but once protonated, it remains trapped in acidified organelles such as phagolysosome (pH<5.5-6), clearly showed that primed macrophages have the highest phagolysosome activation (reddish), in particular LPS-primed macrophages infected with *C. albicans*. Also, in this microscopic images it is possible to observe that less yeast cells are viable since the probe used, Oregon Green, dyes green viable yeast cells and only few appear in the preparations. This confirms the results obtained before in a study that concluded that this higher efficiency in killing *C. albicans* is related to increased density of dectin-1 at the surface of host cells, as to this PRR recognizes *C. albicans* β-glucan structures (Rogers, Williams, Feng, Lewis, & Wei, 2013). Using the differential fluorescence probing for viable yeast and for phagolysosome activation it seems that HKEc were less efficient "preparing" the macrophage to respond to *C. albicans* than LPS.

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Macrophages are innate immune cells that while responding decrease their viability, due to the production of deleterious factors aiming to kill the invaders that are also toxic to the cell (Romani, 2011). Nevertheless, some microbes, *C. albicans* included, are able to supress this effect and resist phagocytic attack and undergo a transcriptional change, as described by Lorenz, Bender, & Fink, (2004). This change is towards the development of a germ tube in order to escape the macrophage (Cheng et al., 2012). *C. albicans* exocytosis from macrophages can occur by a non-lytic process (Bain et al., 2012; Fernández-arenas et al., 2008) that does not result in macrophages death. In this work it was observed that macrophages RAW264.7 were less viable when, previously to *C. albicans* infection, were stimulated with 0.1µg/mL of LPS. As to if fungal infection kills the macrophage when escaping, this data indicates no decrease in macrophages viability when exposed to the fungus alone, a result concordant with that described by others (Bain et al.e, 2012). However, it should be noticed that there is a limitation regarding the technique used in the present work, since the cell scraping process induces a 10 % death among the macrophages population. These results could be complemented by another method as flow cytometry specifically fluorescence-activated cell sorting (FACS) using a differential staining with propidium iodide in which killed *C. albicans* would be stained and non-living *C. albicans* as described in Fernández-arenas et al., (2008).

Macrophages respond to the presence of pathogens by changing their morphology, by including this invader in phagosomes but also trigger the secretion of signaling molecules that can have microbicidal effects or recruit other cells of the immune system. This is the case of cytokines, molecules that are recognized and activate other cells in order to reinforce the pathogenic attack. In this study two of those were quantified using ELISA, IL-1β and TNF-α. The results obtained showed that there is an overall increase of cytokines secretion when the macrophages were primed with LPS or HKEc when compared with macrophages that were only infected with *C. albicans*. Nevertheless, samples were collected at 3 h of infection leading to very low concentrations of cytokines, with a high dispersion of results and difficulty in the statistical significance of the differences observed. To overcome this limitation a more efficient collection of supernatants should be tested and these findings could be complemented by quantifying the expression of the genes that code for the cytokynes measured.

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Conclusion

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5 Conclusion

This work main goal was to study the effect of macrophages priming with bacterial endotoxin in the outcome of *C. albicans* infection, the major fungal pathogen.

This *in vitro* model allowed the comparison between macrophages infected with *C. albicans*, and the response of LPS-primed macrophages infected with *C. albicans*, in order to assess the outcome of fungal infection. In fact, primed macrophages appeared more activated and engulf successfully the pathogen. However, they were unable to efficiently eradicate the fungal infection. Yeast cells viability is slightly decreased, when compared with the viability of yeasts internalized by non-primed macrophages. Nevertheless, the yeast population is not significantly reduced. Furthermore, macrophages viability is also affected, when macrophages are primed with LPS, indicating that the co-infection might be a stressful situation for the cell. Finally, the inflammatory response is enhanced, an increase in TNF-α production was observed.

In conclusion, pre-activating macrophages with bacterial endotoxin, seems to trigger the machinery to kill the pathogen but fails in completing the task.

The success of this *in vitro* model of a situation of inflammation due to endotoxin exposure followed by a *C. albicans* infection, indicates that it can be used for further studies such as increasing the period of LPS exposure before *C. albicans* infection and this way increasing the cytokines signals (either measured by ELISA or by real time PCR) and influence over macrophage function. The evaluation of macrophages ability to kill fungal cells and the mechanisms that are activated in primed macrophages when compared to resting macrophages will highlight the machinery that can be activated to increase the efficiency of macrophages to clear internalize *C. albicans* cells.

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