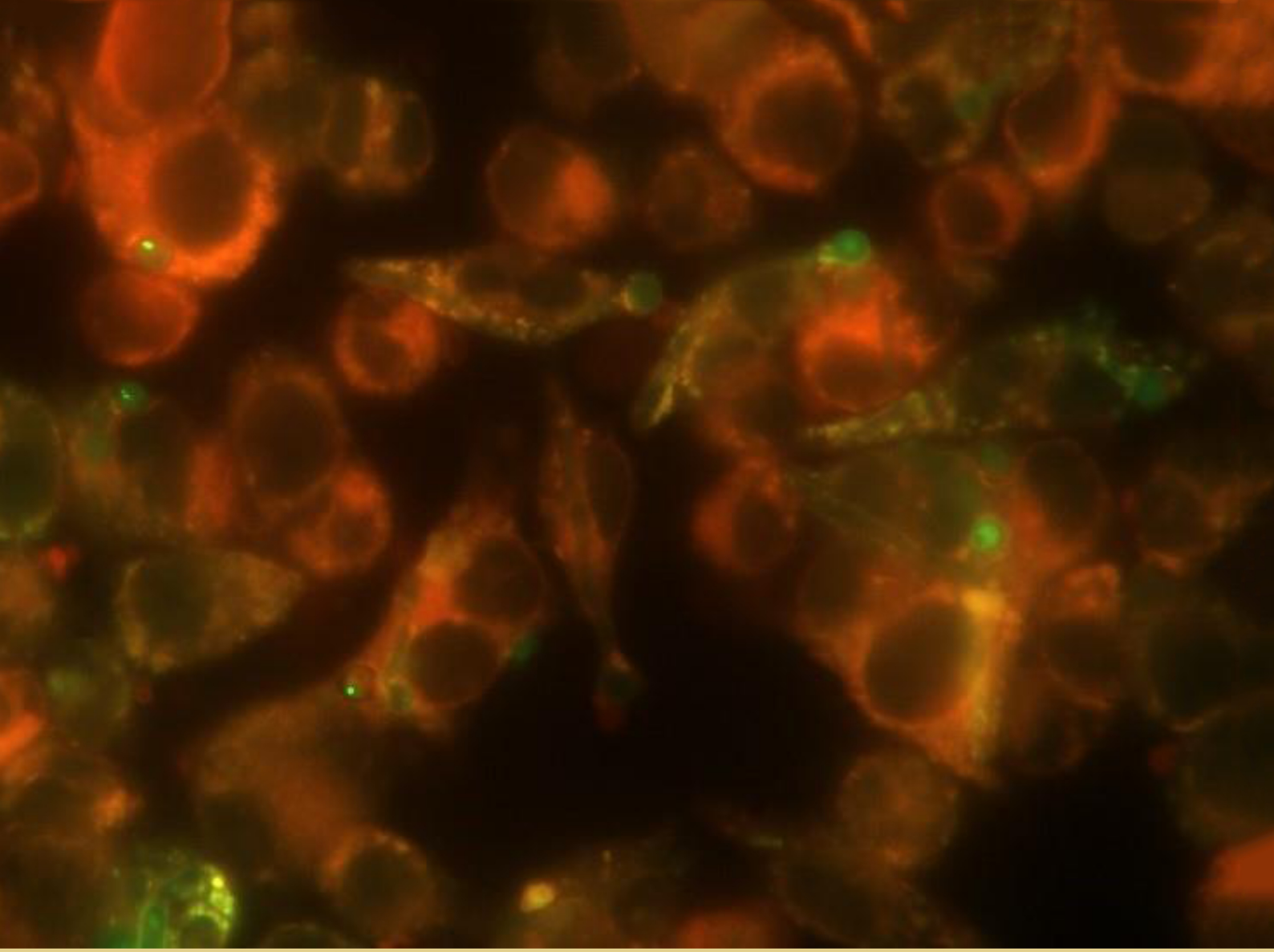

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Modulation of adenosine in the persistence of *Candida albicans* inside macrophages

Tese de Mestrado em Bioquímica, sob orientação cila Professora Doutora Teresa Gonçalves (Universidade de Coimbra) e apresentado à Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Julho 2016

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Universidade de Coimbra

Modulation of adenosine in the persistence of *Candida albicans* inside macrophages

Dissertação apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra para obtenção do Grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Teresa Maria Fonseca Oliveira Gonçalves (Universidade de Coimbra) e da Professora Doutora Paula Maria de Melim Vasconcelos de Vitorino Morais (Universidade de Coimbra).

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$A_{2A}R$ – A_{2A} Receptors

ADO - Adenosine

ADP – Adenosine Diphosphate

ALS3 – Agglutinin like Sequence 3

AMP – Adenosine Monophosphate

ATP – Adenosine Triphosphate

cAMP - 3'-5'-cyclic adenosine monophosphate

CD39 – Nucleoside Triphosphate Diphosphohydrolase

CD73 – 5'- Ribonucleotide Phosphohydrolase

CFU – Colony Formatting Units

CLRs – C-type Lectin Receptors

CNTs – Concentrative Nucleoside Transporters

CWP – Cell Wall Proteins

DAMPs – Damage Associated Molecular Patterns

DIP – Dipyridamole

ENTs – Equilibrative Nucleoside Transporters

IL-1β - Interleukin-1β

IL-10 – Interleukin-10

LPS – Lipopolysaccharides

MOI - Multiplicity of Infection

NLRs – NOD like Receptors

NT – Nucleoside Transporters

PAMPs - Pathogen-Associated Molecular Patterns

PBS - Phosphate Buffered Saline

PKA – Protein Kinase A

PRRs - Pattern-Recognition Receptors

ROS - Reactive Oxygen Species

SAPs – Secreted Aspartyl Proteinases

TLR – Toll like Receptors

TNF-α – Tumor Necrosis Factor α

YPD - Yeast Potato Dextrose

Abstract

With the development in today's society, the emergence of new, more drastic methods for treating diseases such as cancer, that weaken the immune system, leaving it more susceptible to opportunistic infections. *C. albicans* is a good example of a microorganism which takes advantage of the compromised immune system. As a commensal it is harmless and can be found in the microbiota of almost every human. However, changes in the host organism stimulate the passage of the fungus to opportunistic pathogen. Many factors influence the survival of this organism even in unfavorable environments for the same. Escape mechanisms of the host immune system is one of *C. albicans* strategies to avoid elimination. The modulation of the inflammatory response in the host appears as one of the possible ways that *C. albicans* survive within macrophages.

Adenosine plays an important role in inflammation. It binds to the receptors at the cell membrane, such as the A_{2A} receptor ($A_{2A}R$) promoting the development of anti-inflammatory responses, important for tissue protection when a deleterious inflammatory process occurs. Before, the research group described that in response to yeast infection $A_{2A}R$ at the cell membrane moves to the phagosomal membrane, inside the cell. According to this, the main objective of this study was to determine to what extent dipyridamole, an inhibitor of nucleoside transporters, influences the development of infection in the presence of adenosine, with the goal of emphasizing the presence of $A_{2A}R$ inside the cell.

The most important conclusion to be drawn from this work is the reduction of *C. albicans* viability within macrophages in the presence of adenosine and dipyridamole. In this same condition, a decreased viability of macrophages indicates that the macrophage is more activated and efficient in the clearance of yeasts. Moreover, the profile of expression of the genes coding for IL-1β and TNF-α production in the presence of adenosine and dipyridamole also enhance the results obtained previously.

This work can thus help the understanding of how *C. albicans* persists silently inside macrophages and to create new avenues of studying other strategies to effectively eliminate infections by this fungus.

Keywords: *Candida albicans*, adenosine, dipyridamole, and macrophages

Resumo

Com o desenvolvimento na sociedade atual, o aparecimento de novas metodologias mais drásticas para o tratamento de doenças, como o cancro, debilita o sistema imunitário, deixando-o mais suscetível a infeções oportunistas. *C.* albicans é um bom exemplo de um microrganismo que se aproveita do sistema imunitário comprometido. Como comensal é inofensivo, estando presente na microbiota de quase todos os humanos. No entanto, alterações no organismo do hospedeiro estimulam a passagem deste fungo a agente patogénico oportunista. Muitos fatores influenciam a sobrevivência deste microrganismo mesmo em ambientes desfavoráveis. Mecanismos de fuga ao sistema imunitário do hospedeiro é uma das estratégias de *C. albicans* para fugir à eliminação. A modulação da resposta inflamatória no hospedeiro surge como uma das possíveis vias pelas quais *C. albicans* sobrevive no interior de macrófagos.

A adenosina desempenha um importante papel na inflamação. A sua ligação a recetores da membrana citoplasmática, como os recetores A_{2A} ($A_{2A}R$) promove o desenvolvimento da resposta anti-inflamatória, importante para a proteção de tecidos quando ocorre uma resposta inflamatória exacerbada. Antes, o grupo de investigação descreveu que no decurso de uma infeção por *C. albicans* os recetores $A_{2A}R$ encontram-se na membrana do fagossoma que encerra células de levedura. O principal objetivo deste trabalho foi verificar em que medida o dipiridamole, um inibidor dos transportadores de nucleósidos, influencia o desenvolvimento da infeção na presença de adenosina, com a finalidade de enfatizar a importância da localização intracelular dos $A_{2A}R$.

A conclusão mais importante deste trabalho é a diminuição do número de *C. albicans* no interior dos macrófagos na presença de adenosina e de dipiridamole. Por outro lado, ocorre uma diminuição da viabilidade dos macrófagos nas mesmas condições o que pode constituir um indício de que os macrófagos estão mais ativados e são mais eficientes na eliminação. Por outro lado, o perfil de expressão dos genes que codificam para IL-1β e TNF-α, na presença de adenosina e dipiridamole reforça os resultados previamente obtidos.

Este trabalho poderá assim ajudar à compreensão deste mecanismo de proteção da *C. albicans* e futuramente ajudar à criação de novas vias estratégias para a eliminação eficaz deste fungo.

Palavras-Chave: *Candida albicans*, adenosina, dipiridamole, macrófagos

CHAPTER 1

INTRODUCTION

In today's society, infections caused by pathogens have received special attention. These infections caused by microorganisms such as bacteria, viruses, parasites and fungi can be transmitted directly or indirectly to the individual, representing a public health problem. With the advancement of science, new therapies have emerged. At times, these therapies interfere with the individual's immune system, making it more susceptible to the action of these pathogens and the development of infections. The comprehension of the mechanisms of action and protection of these same pathogens becomes essential.

The species of the genus Candida are one of the main pathogens that take advantage of this weakening of the immune system, leading sometimes to the death of the host. In this study, we looked to *Candida albicans*, one of the major infectious agents of this genus and the modulation of anti-inflammatory adenosine, during macrophage infection.

1.1 *Candida albicans*

Candida albicans is a diploid, polymorphic fungus that belongs to the genus *Candida*. They grow as budding cells and can achieve the mycelial state when the conditions are favorable (Dekte, 1998; Motaung, et al, 2015). A commensal pathogen that can be found on the microbial flora of the oral cavity, gut or vaginal tract. However, *C. albicans* only lives in 70% of the human population. This means that 30% may not be carriers of this pathogen or their colonization is not continuous (Niimi, Cannon & Monk, 1999; Nadeem, et al, 2013).

The interaction with the host is essential for the survival of this fungus. That is why it is usually isolated from human clinical samples and rarely isolated from the environment (Motaung, et al., 2015). As a commensal, the interaction with the host must always be balanced to fulfill the fungi needs, enabling the extraction of the resources necessary for its replication, without causing major damage to the host. The multiplication of *C.albicans* is maintained by host factors, such as competition with endogenous microbiota and with host defense mechanisms (Niimi, Cannon & Monk, 1999; Gow, 2013).

The transition from harmless commensal to pathogen is seen as a response to changes in the host environment. Individuals with compromised immune system due to HIV infection, cancer or undergoing organ transplantation or other types of major surgery, are some of the groups at higher risk (Niimi, Cannon & Monk, 1999; Nadeem, et al., 2013).

With the debilitation of the protective barriers of the host, colonization of this fungus occurs leading to an increased susceptibility to the development of infections. *C. albicans* is responsible for about 400,000 severe infections and a higher number of mucosal infections per year (Gow, 2013).

Mucosal infections affect different mucous membranes of various body surfaces, such as oropharyngeal and vaginal mucous membranes. Most of these infections, which includes vaginal and oral candidiasis, are benign. It may however be associated with high morbidity in cases of chronic mucocutaneous candidiasis and recurrent vaginal candidiasis. Systemic infections involve the dissemination of this pathogen in the bloodstream and subsequently to the other organs. In systemic infections, the mortality rate can reach 80% (Nadeem, et al., 2013; Whibley & Gaffen, 2015; Gow, 2013).

The capacity of *C. albicans* to cause infections is attributed to several virulence factors. Virulence is a complex and dynamic phenomenon involving both host and microbial features. The rapid ability of *C. albicans* to adapt to different environmental conditions in the host, such as different temperatures, pH, carbon dioxide (CO_2) levels, *stress* and available energy sources, can influence their prevalence (Motaung, et al., 2015; Mayer, et al., 2013). Using mutants of *C. albicans* it was revealed that aspects such as secretion of hydrolytic enzymes and cell wall architecture also influence this adaptation (Fernández-Arenas, et al., 2009; Chaffin, et al., 1998).

The resistance of *C. albicans* to antifungal drugs, detected in all *Candida* species with clinical relevance, presents different patterns, leading to unsuccessful therapies. The ability of this fungus to switch between unicellular yeast and different multicellular filamentous forms is also considered one of the major virulence factors of this organism (Motaung, et al., 2015).

1.1.1 Cell Morphology and its role in infection

Unicellular fungi, generally called yeasts species in general can grow with different morphologies, as yeast cells, pseudohyphae and hyphae (Fig. 1). Yeast cells are single oval-shaped cells with patterns of budding. The pseudohyphae and hyphae, also known as filamentous morphologies, grow in a polarized manner, elongated and connected together end to end. The pseudohyphae are ellipsoidal, wider in the center and narrower at the ends with several constrictions in the septal joints. On the other hand, the hyphae have two parallel sides, uniform in their width, with a true septum without constrictions. They also have pores on their septum allowing cell-to-cell communication. Despite the physical similarities between pseudohyphae and hyphae, the first are more similar to yeast cells. Besides, when in the filamentous form, some cells of these fungi can differentiate into large and round with a thick wall, the chlamydospores. These cells are formed at the tip of the hypha filaments, or intercolated, as a response to unfavorable conditions for the cell, such as lack of nutrients (Thompson, Carlisle & Kadosh, 2011; Nadeem et al., 2013).

Figure 1 **- Schematic representation of the principal *C. albicans* morphologies** (adapted from Thompson, Carlisle & Kadosh, 2011).

In *C. albicans* the morphological transition is one of the most important virulence factors, as referred above. Yeast cells and hyphae were observed during infection with distinct functions. However, the role of pseudohyphae is not well clarified and chlamydospores have not been observed in patient samples (Gow, et al., 2011).

The yeast form has been identified as a virulence factor in some pathogens like *Blastomyces dermatitidis* and *Penicillium marneffei.* This morphological form is not required for virulence of *C. albicans*, but is important to the processes of colonization

and dissemination in the host tissues, adherence to cell surfaces and biofilm formation (Thompson, Carlisle & Kadosh, 2011; Mayer, et al., 2013).

Changes in the immune status of the host can lead to the morphological transition and formation of hyphae. This transition promotes the virulent action of this pathogen by several mechanisms since the invasion of epithelial cells layers, which will be damaged and broken, to the lysis of macrophages and neutrophils after phagocytosis, due to the growth of the hyphae. Environmental factors can also influence this change. Hyphae may be induced from yeast unbudded cells by serum addition, temperature superior to 35°C, neutral pH and CO_2 (Sudbery, Gow & Berman, 2004). The importance of hyphal growth in infection has been inferred from the observation of double mutants of Enhanced Filamentous Growth (*EFG1*) and in *Candida* Pseudohyphal (*CPH1*) genes which codify proteins that enhance the filamentous growth. These mutants were unable to form hyphae in various environments. They also present defects in the transcription program that accompanies hyphal growth and are important for the fungus recognition and its invasion of the host cells, which includes the expression of cell wall proteins (CWP), adhesins and secreted aspartyl proteinases (SAPs) (Sudbery, 2011).

1.1.2 Invasion of the cells by *C. albicans*

The mechanism by which the pathogens invade the cells have gain a lot of attention throughout the years. *C. albicans* in particular is one pathogen studied in great detail. The invasion of epithelial and endothelial cells by this fungi, which can result in the development of infections, illustrates the importance to clarify those pathways. The production of lytic enzymes, such as SAPs during hyphal formation, are responsible for the digestion of elements of cell membrane, providing a way in to the microorganism (Mogensen, 2009). Another mechanism is the induction of endocytoses in cells, with the stimuli of pseudopods formation that surrounds and consequently leads to the internalization of the fungus (Filler & Shepard, 2006). Both yeast and hyphae can induce endocytosis. However, in a study by Park et al. (2005) they proved that hyphae are more efficient in stimulating this process, using double efg1Δ/efg1Δ mutant cells who could not germinate in oral epithelial cells. Their capacity to induce

endocytosis was significantly reduced. But live and killed cells can stimulate this process. A model was designed by scientists in which agglutinin like sequences 3 (ALS3), produced in hyphal state can bind to cadherins and other proteins on the cell surface, leading to the activation of clathrin dependent endocytoses and the internalization of *C. albicans* (Liu & Filler, 2011; Phan, et al., 2007).

1.1.3 Mechanisms of *C. albicans* escape from the host immune system

Besides of compromised immune system, the occurrence of invasive fungal infection is also dependent on the fungus ability to evade the host defense mechanisms. A great number of strategies employed by fungi have been described. For example the modulation of cytokines production helps *C. albicans* to control the host response. The activation of Toll Like Receptors 2 (TLR2) versus Toll Like Receptors 4 (TLR4) differs in terms of pro- and anti-inflammatory cytokine effect (Fig. 2B). It was demonstrated that *C. albicans* evades the host defense through TLR2-derived signals. TLR2-deleted macrophages have enhanced anti-candida capabilities. Hyphal forms of *C. albicans* tend to evade TLR4 in favor of a predominant TLR2 activation, which leads to a more pronounced anti-inflammatory Th2 (T helper cells) response (Chai, et al., 2009).

After its uptake, *C. albicans* can still escape the killing process in the phagocyte. The transition to hyphae can lead to the piercing and killing of the macrophages (Fig. 2A). It was also reported that *C. albicans* modulates the intracellular membrane trafficking by inhibiting the formation of phagolysosomes (Fig. 2C). The fusion of the phagosome containing the microorganism with the lysosome is a necessary step to kill the pathogen. Another mechanism of the phagocyte in the process of killing the pathogen is the production of reactive oxygen species (ROS). *C. albicans* counteracts this respiratory burst producing catalase and superoxide dismutase (SOD) (Cheng, et al., 2012; Erwig & Gow, 2016).

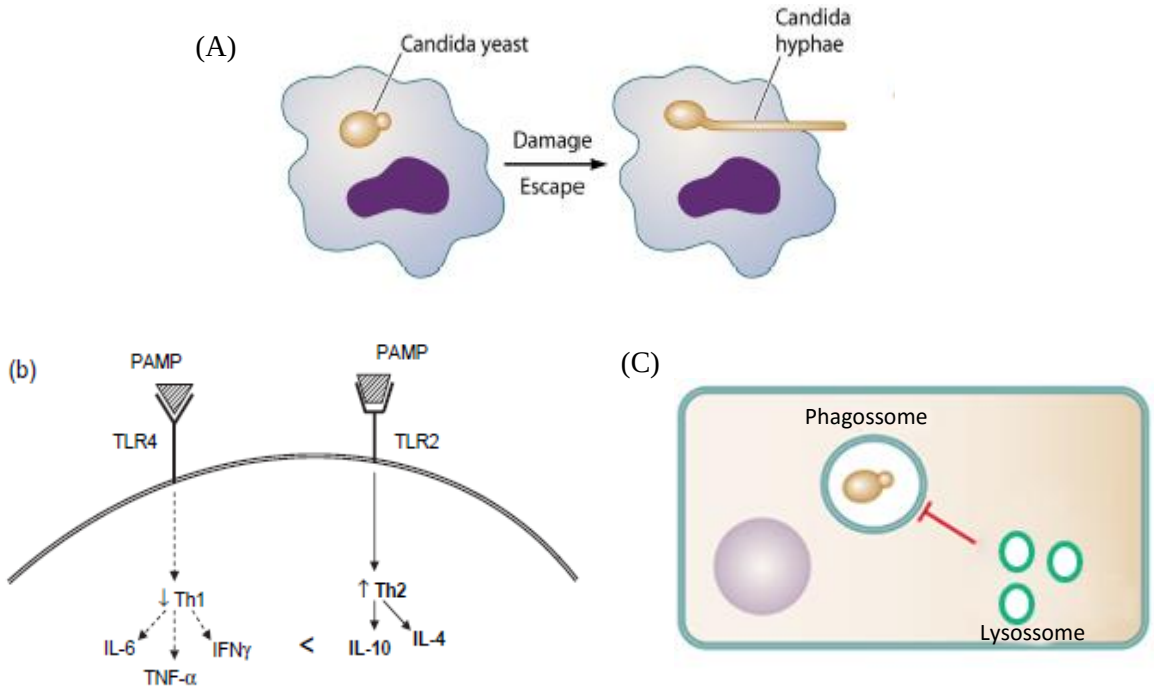

***Figure 2* – Examples of some escape strategies by *C.albicans*. (A)** Yeast- to hyphal-phase transition. **(B)** Preferential activation of TLR2 over TLR4 by certain fungal morphotypic PAMP stimulates a Th2 humoral response over a Th1-dependent antifungal proinflammatory cytokine production. **(C)** Inhibition of phagolysosome formation. (adapted from Cheng, et al., 2012; Chai et al., 2009).

1.2 Innate Immune Response

The innate immune system constitutes the first line of host defense. Based on physical and chemical barriers, as well as on different cell types, it plays a crucial role in the early recognition followed by the triggering of a proinflammatory response to the invasive pathogens (Mogensen, 2009).

One of the main cells involved in the killing of fungi are macrophages. These phagocytic cells, derived from monocytes circulating in the blood, continually patrol tissues and non-sterile interfaces on the surface of the epithelium. The phagocytic clearance of pathogens involves the accumulation thereof at the site where fungal cells are located. In response to *C. albicans*, macrophages trigger the production of Interleukin-1β (IL-1β), in particular during hyphae formation (Duhring, et al., 2015; Erwig & Gow, 2016).

This ability of phagocytic cells to recognize fungal cells is due to the existence of carbohydrates and cell wall proteins (CWP) specific to this type of cells and absent on the host. These features on the cell surface of fungi termed pathogen-associated

molecular patterns (PAMPs) interact with pattern recognition receptors (PRRs) present on the surface of phagocytic cells (Erwig & Gow, 2016; Gow, et al., 2011).

After the recognition of the danger by the host immune system, cells release damage-associated molecular pattern molecules (DAMPs) as endogenous danger signals in order to alert, in this case, the innate immune system to microbial invasion (Tang, et al., 2012).

1.2.1 Pathogen-associated molecular patterns (PAMPs)

Fungal cell wall is the major difference between fungi and most cells, in particular animal cells. For this reason its components are the first to be accessed by the host immune system. The principal PAMPs recognized are the glucose-containing macromolecules such as mannoproteins, β-glucans and chitin (Sorrell & Chen, 2009).

In *C. albicans*, two main layers in the cell wall can be distinguished. The outer layer is mainly composed of O- and N- linked mannose polymers (mannans) that are covalently associated with proteins, forming glycoproteins. On the other hand, the inner wall is majorly composed of chitin and β-glucans. However, due to the organization and dynamic composition of the cell wall during growth, the interaction of PAMPs with PRRs is dependent on their distribution and exhibition in the cell wall surface (Erwig & Gow, 2016; Gow, et al., 2011).

Even though the components of the membranes in the various forms of *C. albicans* are similar, some differences have been described such as the exposure of β-1,3-glucan. The yeast cells of *C. albicans* have in its inner cell wall chitin and β-1,3-glucan, which are exposed to cell surfaces on bud scars. This distribution makes it easier for the recognition of the pathogen by the immune system. β-1,3-glucan, in particular, is responsible for triggering phagocytosis and the production of ROS after recognition. In hyphae, fibrils mannans are smaller and less abundant in comparison with yeast cells. For this reason, β-1,3-glucan is not as exposed as in yeast cells. However, it still is recognized by the host cells (Erwig & Gow, 2016; Gow, et al., 2011).

1.2.2 Pattern recognition receptors (PRRs)

As previously referred, during infection PAMP-PRR binding triggers the response of the innate immune system. Several PRRs have been associated with these process in which we can highlight: Toll-like receptors (TLR), the C-type lectin receptors (CLRs) and NOD-like receptors (NLRs) (Mogensen, 2009; Gow, et al., 2011).

The TLR family represent the paradigmatic example of a PRR. Of the ten functional human TLRs the most associated with *C. albicans* recognition are TLR2 and TLR4. Both are responsible for the recognition of PAMPs situated on the microbial cell wall. TLR2 recognizes phospholipomannan, while the O-linked mannan have been shown to be recognized by TLR4. These two receptors are also related to the induction of proinflammatory cytokines (Gow, et al., 2011; Ebel & Heesemann, 2008).

Another family of receptors involved in the recognition process are CLRs. These receptors can recognize polysaccharide structures of the microorganism. In this family, the Dectin-1 plays an important role. This receptor recognizes β-1,3-glucan and mediates ligand uptake and phagocytosis. It is also involved in proinflammatory cytokine production and cooperates with TLR2 that leads to a synergetic proinflammatory response. Meanwhile, Dectin-2 recognizes mannose-rich structures in both yeast cells and hyphae, inducing the production of Tumor Necrosis Factor α (TNF-α) with the interaction with the Fc gamma receptor (Fcγ). Another receptor in this family is the macrophage mannose receptor that recognizes the N-linked mannan on the outer cell wall (Gow, et al., 2011; Cheng, et al., 2012).

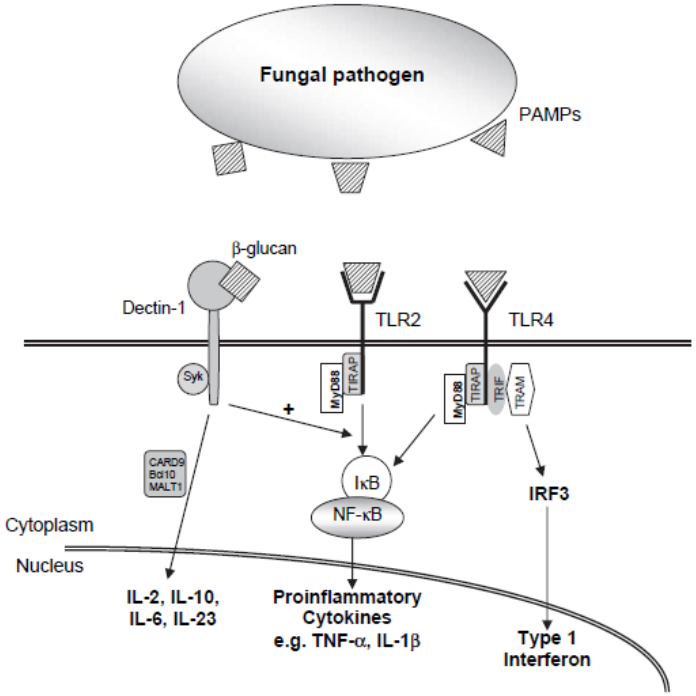

***Figure 3* -Pattern recognition of fungal pathogen and representative signaling pathways.** Specific pathogen-associated molecular patterns (PAMPs) on the surface of fungal cell wall engage their respective pattern-recognition receptor (PRR) like TLR2, TLR4 and dectin-1 receptor on immune cells. Complex signaling pathways are activated involving adaptors and protein kinases resulting in the release of transcription factors to the nucleus to regulate expression of inflammatory cytokines Adaptors: myeloid differentiation primary response gene 88 (MyD88), Toll/interleukin-1 receptor (TIR) adaptor protein (TIRAP),TIR-containing adaptor-inducing interferon β (TRIF), TRIF-related adaptor molecule (TRAM), caspase recruitment domain 9 CARD9)-B-cell chronic lymphocytic leukemia/lymphoma 10 (BCl10)-mucosal-associated lymphoid tissue translocation gene 1 (MALT1) complex Protein Kinase: spleen tyrosine kinase (Syk) IRF, Interferon regulatory factor NF-kB, nuclear factor-kappa B IkB, inhibitor or NF-kB (adapted from Chai, et al., 2009).

At last, NLRs recognize intracellular PAMPs. These receptors, who belong to the second line of recognition, are located in the cytoplasm and are mainly responsible for the activation of a protein complex called inflammasome, as a response to the recognition of the microbial PAMP or an endogenous signal such as ATP. Among the NLRs, the NLRP3 have an important role in *C. albicans* induced inflammation (Gow, et al., 2011; Erwig & Gow, 2016).

1.2.3 Damage-associated molecular pattern molecules (DAMPs)

In some circumstances host inflammatory responses can cause host cell death leading to tissue injury, and the release of host cellular components to the extracellular environment. These cellular components could be considered “messengers” for danger; known as DAMPs. These signals can include lipids, sugars,

metabolites and nucleic acids. The recognition of DAMPs by host PRRs can also initiate and perpetuate an immune response (Tang, et al., 2012; Jounai, et al., 2013).

Adenosine triphosphate (ATP) is almost known to everyone by its involvement in the energetic processes. However, extracellular ATP released from injured cells is a danger signal through the activation of the NLRP3 and caspase-1, which results in the stimulation of proinflammatory cytokines. Lipopolysaccharides (LPS) present in the cell wall of bacteria induce the stimulation of macrophages, accompanied by the release of ATP. It was verified that the binding of these extracellular ATP to the P_2X_7 receptor can mediate caspase-1 activation following IL-1β maturation, a well-known proinflammatory cytokine. The excess of extracellular ATP can also stimulate the anti-inflammatory response when converted to adenosine (Jounai, et al., 2013; Ferrari, et al., 2006).

1.3 Adenosine

Adenosine is a ubiquitous molecule present in several metabolic processes. Distributed in various tissues and organisms (Haskó, et al, 2008; Li, et al, 2012), the importance of this molecule in signaling processes, have been reported in *Drosophilla*, protozoa and mammals (Haskó, et al., 2009).

The concept of adenosine, as a signaling molecule, was first introduced in 1929 by Drury & Szent-Györgyi through studies in cardiac muscle extracts. These authors found that after injecting those extracts in animals, occurred a decreased in the heart rate and an increase on blood flow. The discovery of the applicability of adenosine in the heart aroused interest on this endogenous molecule. This purine nucleoside, consisting of an adenine attached to a ribose, is a precursor of ATP and a modulator of inflammatory responses in the body (Antonioli, et al, 2014; Borea, et al, 2016; Sachdeva & Gupta, 2013).

1.3.1 Adenosine and the anti-inflammatory response

Adenosine has been identified as an endogenous ligand of the receptor P1 family, also referred to as adenosine receptors (Matsumoto, et al, 2012; Ravelic & Burnstock, 1998).

These receptors are distinguished, by biochemical and pharmacological differences, being subdivided into A_1, A_{2A}, A_{2B} and A_3 (Ravelic & Burnstock, 1998). The adenosine receptors are composed of seven transmembrane units coupled to intracellular G proteins. Its C-terminus is found in the intracellular space and the N-terminus on the extracellular space, where the ligands bind to them on the superior portion of a pore, formed due to the three-dimensional arrangement of the transmembrane subunits. Despite the structural similarities, small differences in their composition lead to different roles. For example, the A_{2A} receptor, coupled to the G_s protein appear to have an important role in inflammatory processes. The activation of A_{2A} receptor leads to an increase of cAMP levels and activation of protein kinase A (PKA). PKA phosphorylates a transcription factor (CREB) which causes an increase in the transcription of the CEBPβ gene. The CEBPβ protein binds the promoter of the Interleukin 10 (IL-10) gene, which triggers IL-10 transcript and therefore leads to the release of this well-known anti-inflammatory cytokine (Haskó, et al., 2008; Lapa,.et al., 2014).

1.3.2 Main pathways to obtain adenosine

At the intracellular level (Fig. 4), adenosine is obtained from adenosine mono-phosphate (AMP), due to the action of the enzyme 5'nucleotidase. The intracellular adenosine then follows two main metabolic pathways: can be metabolized to inosine by adenosine deaminase or into hypoxanthine and uric acid by xanthine oxidase (Sheth, et al., 2014). The concentration of this molecule is still regulated by adenosine kinase, which in the presence of phosphate, mediates the conversion of adenosine to AMP, and may subsequently form ATP. The balance between the ATP and adenosine in cells is dependent on the energy levels of the cell (Ham & Evans, 2012).

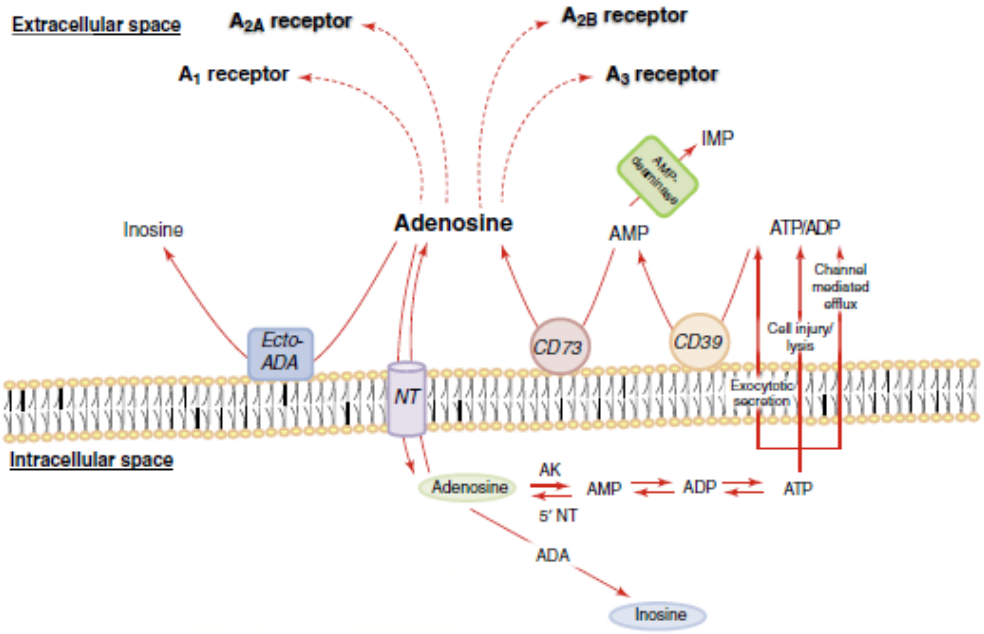

***Figure 4*- Schematic diagram showing the adenosine formation.** Once released into the extracellular environment, through channels, cell injury and/or lysis or other extrusion systems, ATP is degraded by ecto-ATPase (CD39) and ecto-5′-nucleotidase (CD73) into adenosine, which selectively interacts with A_1, A_{2A}, A_{2B} and A_3 receptors. Nucleoside transporters (NT) and ecto adenosine deaminase (ecto-ADA), which operate the uptake or deamination of extracellular adenosine, respectively. After intracellular uptake, adenosine undergoes a rapid phosphorylation to AMP by adenosine kinase (AK), or deamination to inosine by adenosine deaminase (ADA). Abbreviations: IMP, inosine monophosphate (adapted from Antonioli, et al., 2014).

On an extracellular level (Fig. 4), adenosine is a product of ATP dephosphorylation, which is the principal mechanism for its production (Sheth et al., 2014). Under normal conditions, this molecule can be found at low concentrations in the extracellular space (Milne & Palmer, 2011). However, in situations of stress to the cell, such as ischemia, hypoxia or infection, ATP is released into the extracellular space. With the increase in extracellular ATP levels, there is an increase in phosphatase activity, as ectophosphatase CD39 (nucleoside triphosphate diphosphohydrolase) responsible for the degradation of ATP to adenosine diphosphate (ADP) and AMP, as well as the increase in ectonuclease activity CD73 (5'-ribonucleotide phosphohydrolase), which transforms the previously formed AMP to adenosine and leads to its accumulation in the extracellular space. Adenosine present in the extracellular space is also subjected to regulation processes. Contrary to what happens intracellularly, the extracellular adenosine is mainly regulated by adenosine

deaminase, which turns it into inosine. The concentration of adenosine present in the extracellular medium can be an indicator of tissue damage, which may trigger a protective response by the body in the tissue (Ham & Evans, 2012).

1.3.3 Nucleoside Transporters

Besides the mechanisms of adenosine synthesis, its concentration may be regulated by membrane transporters, denominated nucleoside transporters (NT). These carriers, which regulate the homeostasis of nucleosides, are essential for the regulation of various metabolic processes in which these molecules are key (Sheth, et al, 2014; Young, et al, 2013). One of the processes most affected by these carriers are signaling processes triggered by adenosine (Johnson et al., 2012).

In mammals are currently known two distinct families of NTs: equilibrative nucleosides transporters (ENT) and concentrative nucleosides transporters (CNT) (King, et al., 2006).

CNTs transport unidirectionally nucleosides against a concentration gradient, with energy consumption (Molina-Arcas, et al, 2009; Johnson, et al., 2014). The known isoforms of this family are more selective showing preference for a certain type of substrate, with the exception of uridine which is common to all (King, et al., 2006). They are mostly present in specialized epithelial tissue. Its contribution to the transport of these nucleosides in the tissues is minimal in comparison with ENTs (Molina-Arcas, et al., 2009). There is still no known inhibitor with a high affinity for this type of carriers (Parkinson, et al., 2011).

The ENTS, consisting of 4 subtypes, are widely distributed among eukaryotes (King, et al, 2006; Young, et al, 2013.).

These proteins catalyze passive diffusion processes, with bidirectional transport mediating the influx and efflux of substrates. Within this family of transporters, ENT1 and ENT2 are those who are best characterized, being present in most cells and tissues (Molina-Arcas, et al, 2009; Parkinson, et al, 2011). Such carriers have a wide variety of substrates, accepting the majority of purines and pyrimidines. On the other hand, they are more sensitive to inhibition by coronary vasodilators such as dipyridamole (DIP) (Molina-Arcas, et al., 2009; King, et al., 2006). Dipyridamole is an anti-platelet agent

with marked vasodilator, anti-oxidant, and anti-inflammatory activity. It inhibits adenosine uptake and improves tissue perfusion through adenosine-mediated vasodilator effects, by blocking the ENTs. Dipyridamole will increase the extracellular endogenous adenosine concentration, mainly in situations of increased extracellular formation of adenosine, such as occurs during hypoxia or inflammation (Puri, et al., 2016; Ramakers, et al., 2011).

1.4 Aims

The main goal of this work was to verify the effect of dipyridamole on the modulation of intracellular adenosine on cells of the immune system, during infection. For this purpose macrophages and its response to *C. albicans* in the presence of these drugs was studied.

CHAPTER 2

MATERIALS AND METHODS

2.1 Microorganism Culture

2.1.1 Yeast Strain

During this work the *C. albicans* strain used (YP0037) was obtained in the Microbiology Pathogenic Yeast Collection of the University of Coimbra. Yeast cells were grown at 30°C in Yeast Extract Peptone Dextrose (YPD) agar medium plates with the following composition: 0.5% yeast extract (Panreac); 1% Bacto-peptone (Panreac); 2% glucose (Sigma-Aldrich); 2% agar (Cultimed). The medium was autoclaved at 121ºC for 20 min at a pressure of 1.2 atm and distributed into sterile Petri dishes.

2.1.2 Yeast Harvesting

Prior to the assay, a 24 h growth culture was harvested and suspended in 500 µL of cold Phosphate-buffered saline (PBS: 10 mM Na_2PO_4; 1.8 mM KH_2PO_4; 137 mM NaCl; 2.7 mM KCl at pH 7.4). The suspension was centrifuged at 16,060 *g* for 2 min and resuspended in cold PBS. This procedure was performed twice. The suspension was then diluted 100x and the cell number was counted, using a hemocytometer for this purpose.

2.1.3 Viability of yeast cells in the presence of adenosine and dipyridamole

The effect of adenosine (ADO) 10 mM (Sigma-Aldrich) and dipyridamole (DIP) 10 µM (Tocris) on *C. albicans* was assessed by incubating the yeast cells with these drugs in cell culture medium and counting the colony forming units (CFUs).

A culture of *Candida albicans*, grown overnight was prepared to a density of $2.5x10^5$ cells/mL as described in 2.1.2. The yeast cells were then incubated in 96 well plates (Costar), with the respective drugs for periods of 1.5 h and 3 h, at 37 ^oC in a humidified atmosphere with 5% CO_2.

Finished the incubation time, the multiwells were placed on ice and the wells were scraped. Next, serial dilutions were performed with 50 µL of suspension plated on YPD agar and incubated for 3 days at 30 °C. Afterwards CFUs were counted.

2.2 Infection Assays

2.2.1 Cell Culture

RAW 264.7 macrophage cells were purchased from the European Collection of Cell Cultures. These were maintained in Dulbecco's Modified Eagle Medium (D5648, Sigma-Aldrich) supplemented with 10% FBS (Fetal Bovine Serum) non-inactivated, 10 mM HEPES, 12 mM sodium bicarbonate and 11 mg/mL pyruvate sodium. The cells were cultured in 75 cm^2 flasks (Corning) at 37 ^oC in a humidified atmosphere with 5% of CO_2.

For their maintenance, the cells were plated with ~10% of confluence until reaching approximately 70%. All the procedures described herein, were performed with cells not older than the 15^{th} generation.

For the experimental assays, the RAW264.7 cells were resuspended in RPMI 1640 medium (R1383, Sigma-Aldrich) supplemented with 10% FBS inactivated, 23.8 mM sodium bicarbonate and 50 mM glucose.

2.2.2 Cells Harvesting

Cells were mechanically removed through scrapping and were then counted in a hemocytometer. Only viable cells were counted. For this, cells were diluted with Trypan Blue 4% (Sigma-Aldrich). Cells then were plated in multiwells with the desired number in each well and supplemented with RPMI 1640 medium, up to the work volume. They were then incubated in a humidified atmosphere with 5% CO_2 at 37°C, 15-18h prior to the realization of the assay, in order to obtain approximately 60% of confluency.

2.2.3 Infection of macrophages with *C. albicans*

In all infection assays, macrophages were plated according to the described on 2.2.2. On the day of the assay, the medium from cells was discarded and the cells were washed once with sterile PBS heated at 37°C. Fresh medium with yeast cells, prepared as described previously, were added to the macrophage cell cultures. The plates were

incubated for 1 h at 37 ^{o}C and 5% CO_2. The multiplicity of infection (MOI) in all these experiments was 1:1.

After the incubation period, the medium was removed. The cells were again washed with sterile PBS heated at 37 °C. New medium with 10 µM DIP was added and the cells were again incubated at 37^{o}C and 5% of CO_2. After 30 min, ADO 10 mM was added and the cells were incubated again in the same conditions for 1.5 h and 3 h.

2.2.4 *C. albicans* viability assay

The viability of *C. albicans* in the presence of these drugs was evaluated again, in this case on an infection context. This also allows to evaluate the phagocytic efficiency of the macrophages. Cells were plated in 96-wells plates at a density of $1.25x10^5$ cells/mL according to the procedure 2.2.2. The infection assay occurred as previously described in 2.2.3. Cells were incubated for periods of 1.5 h and 3 h.

After each incubation time, plates were placed on ice. To each well was added sterile MilliQ water and Triton X-100 0.5% (BDH), in order to collect the macrophages and internalized yeasts adhered to the well, by scrapping it with the tip of a 200 µL pipette. Afterwards they were plated on YPD medium and incubated at 30°C for 3 days. After this time, the CFUs were counted, thereby determining the viability of the yeast.

2.2.5 Interaction between *C. albicans* and macrophages

The interaction between macrophages and *C. albicans* in the presence of ADO and DIP was observed by a differential fluorescence protocol. This allows the distinction between yeast cells (green) and acidic organelles in macrophages (yellowish to red).

Macrophages were collected according to 2.2.2 and a density of $1.25x10^5$ cells/mL of viable macrophages were plated on Multiwells 24 (Costar) with 12 mm coverslips.

On the day of the assay, yeast cells were harvested as described 2.1.2 and labeled with Oregon Green® 488 2 µM (Life Technologies) in the dark for 1 h, at 30ºC

and 180 rpm. After this period, the yeast cells were again centrifuged for 5 min, at 7,412 *g* and 4° C. They were resuspended in PBS with 100 mM glycine (Sigma-Aldrich).

At the same time, macrophages were labeled with Lysotracker Red® DND-99 (Invitrogen Molecular Probes) for 1 h at 37 ^{o}C and 5% CO_2. After this time the macrophages were infected with *C. albicans* and treated with the drugs the same way as in in 2.2.3.

At the end of the incubation period, the cells were washed with sterile PBS and fixed with paraformaldehyde (PFA) 4% (Sigma-Aldrich) for 15 min at room temperature. Then they were again washed with sterile PBS. The coverslips were mounted with Dako (Dako) mounting medium and left overnight in the dark. Images were acquired in a Zeiss Axio Observer Z1 Microscope with Plan-ApoChromat 63x/1.40 immersion objective and visualized in Zen Blue 2012 software.

2.2.6 Macrophage viability

2.2.6.1 MTT Assay

Cells were prepared in accordance with the described in 2.2.2. For this assay, cells were plated at a density of 2.5x105 cells/mL in Multiwells 12 (Costar) in RPMI medium without Phenol Red (R8755, Sigma-Aldrich) supplemented with 10% FBS inactivated, 23.8 mM sodium bicarbonate and 38.9 mM glucose.

On the day of the assay, yeast cells were prepared as described above in point 2.1.2. The infection and subsequent addition of the drugs proceeded in the same manner as in 2.2.3.

After the incubation times, Thiazolyl Blue Tetrazolium Bromide (MTT reagent) (Sigma-Aldrich) was added to each well. The plates were incubated again. After 2 hours, DMSO (Merck) was added to each well to form formazan crystals. The crystals absorbance was measured at 570 nm on a plate reader (Spectra Max Plus 384).

2.2.6.2 Trypan Blue Assay

Trypan blue 4% (Sigma-Aldrich) was also used to verify the viability of macrophages since it only stains dead cells.

Again, cells were prepared in accordance with 2.2.2. In this assay cells were plated at a density of 1.25x105 cells/mL in 96- wells plates (Costar). Preparation of yeast cells and infection assay occurred as described in 2.1.2 and 2.2.3, respectively.

After each incubation period, the cell medium was discarded, cells were washed with PBS and fresh medium was added. The wells were scraped with the tip of 200 µL pipette and the contents of the wells were transferred to microtubes. This step was repeated twice. Then 2x dilutions were made with 10 µL of the suspension and 10 µL Trypan Blue 4%. Live and dead cells were counted in hemocytometer in a phase contrast microscope (Nikon LWD 0.52).

2.2.7 Relative quantification of TNF-α and IL-1β gene expression in macrophages

With the purpose of quantifying the relative expression of the genes coding for the inflammatory cytokines IL-1β and TNF-α in RAW 264.7 cells during the course of infection, in the presence of ADO and DIP, a Real-Time RT-PCR approach was applied. The 18S rRNA gene was used as reference gene.

The cells for this assay were prepared in accordance with point 2.2.2. The day before the assay, cells were plated at a density of $2.5x10^5$ cells/mL in 12-wells plates. On the day of the assay, the yeast cells were prepared according to 2.1.2. The infection and the addiction of the drugs took place as described in 2.2.3. After the incubation time of 1.5 h plates were placed on ice and in the wells were scrapped and transferred to ice cold microtubes. Afterwards they were centrifuged for 5 min, at 12,354 g at 4 °C. The pellet obtained was resuspended in ice cold PBS and centrifuged at 12,354 g and 4 °C, for 8 min. The obtained pellet was again resuspended in 100 µL of ice cold PBS and stored at -80 ^{o}C, until additional experiments.

RNA extraction was performed using the RNA-Cell protocol of the Magna Pure Compact RNA Isolation Kit (04802993001, Roche) according to the manufacturer's instructions. Briefly, 100 µL of lysis buffer was added to the cell pellets and gently mixed, at room temperature. The late mixture was transferred to sample tubes and placed in the Magna Pure Compact Equipment (Roche). The RNA concentration and purity of the extracted samples was assessed, using NanoDrop2000 (Thermoscientific). Reverse Transcription was performed, using the Transcriptor First Strand cDNA

Synthesis Kit (04896866001, Roche) according to the manufacturer's instructions. Firstly, 4 µL of Transcriptor Reverse Transcriptase Reaction Buffer, 2 µL of Deoxynucleotide Mix, 0.5 µL of Protector RNase Inhibitor and 0.5 µL were added sterile microtubes. A volume of 2 µL of Random Hexamer Primer was added to which microtube alongside the respect volume of which sample. RNAse-free water was added to which microtube, in order to make up a total volume of 20 µL. The reverse transcription reaction was performed in a GeneAmp PCR System 2400 (PerkinElmerTM), with the followed program: 25 ^{o}C for 10 min, 50 ^{o}C for 60 min; 85 ^{o}C for 5 min, and lastly, completion at 4 ^{o}C.

Quantitative Real Time PCR was then performed. First, it was prepared a mixture comprising 10 µL of Eva Green (BioRad), 0.5 µL of primer forward and 0.5 µL of primer reverse (represented on the table below), 4 µL RNAse-free water and 5 µL of the previously synthesized cDNA. This was added to the LightCycler 2.0 (Roche). The final concentration of primer used was 0.5 µM.

***Table I* – Primers used in Real Time PCR.**

Gene	Sequence	Amplicon	Reference
TNF-α	5'-CATGATCCGCGACGTGGAACTG-3' / 5'-AGAGGGAGGCCATTTGGGAACT-3'	195 bp	Ghosh, et al., 2010
IL-1β	5'-AACTGTTCCTGAACTCAACTGT-3' / 5' GAGATTTGAAGCTGGATGCTCT-3'	150 bp	
18S	5'-CGGCTACCACATCCAAGGAA-3' / 5'-GCTGGAATTACCGCGGCT-3'	241 bp	Rodrigues, 2016

2.3 Statistic analysis

Statistical differences among several sample types were analyzed by the Student's two tailed t-test (unpaired test), in order to compare two groups. Significance values were indicated as $p<0.05$, $p<0.01$ and $p<0.001$. At least three samples were used for three independent experiments. All results are presented as mean ± SD with at least n=3.

CHAPTER 3

RESULTS

3.1 Effects of adenosine and dipyridamole on *C. albicans* viability

As referred in Chapter 1, adenosine is a nucleoside that mediates anti-inflammatory response and dipyridamole is a well-known nucleoside transporter inhibitor.

Before studying their effect on the macrophage *C.albicans* interaction, it was studied its effect directly in *C. albicans*. For that, yeast cells were incubated with which one of drugs and the combination of the two for 1.5 h and 3 h, at 37 ^{o}C and 5 % of CO_2. A CFU assay was then performed to study the cell viability.

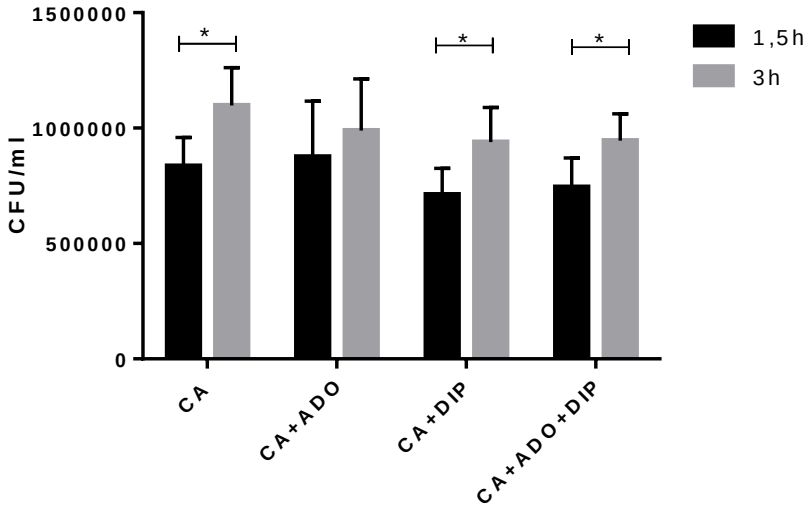

***Figure 5* - Effect of ADO and DIP in *C. albicans* viability.** *C. albicans* (CA) viability after 1.5 h and 3 h period treated with dipyridamole (DIP) and Adenosine (ADO) through a CFU assay. Results shown are derived from at least three independent experiments (means ± SD); *- $p<0.05$.

The results (Fig. 5) showed that in all the conditions tested a slight increase of CFU number at 3 h, when compared with 1.5 h. Those results indicate that these drugs do not affect *C. albicans* viability, so that the cells keep dividing with time. However, when compared to non-treated yeasts, the presence of adenosine and dipyridamole seems to slightly decrease *C. albicans* viability. Nevertheless, no statistical significance was found between untreated and treated cells.

3.2 Internalization of *C. albicans* by RAW 264.7 cells

The ability of internalized *C. albicans* to duplicate inside the macrophages, in the presence of both drugs, was tested by a CFU assay. In this assay, *C. albicans* was left to be internalized by macrophages during one hour, prior to the administration of the treatment. Then, the non-internalized yeast cells were washed, corresponding to time zero. In this type of study only the internalized yeasts were taken in consideration.

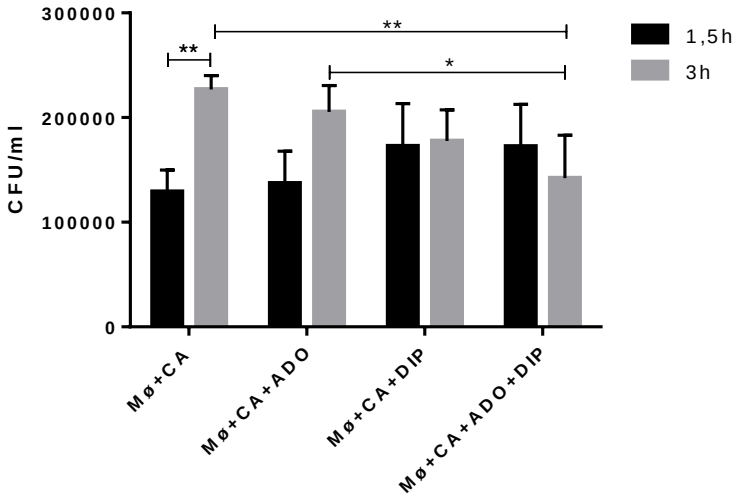

***Figure 6* - Viability of *C. albicans* inside macrophages.** *C. albicans* (CA) viability after 1.5 h and 3 h period with Macrophages (MΦ) and treated with dipyridamole 10 μM (DIP) and adenosine 10 mM (ADO) through a CFU assay. Results shown are derived from at least three independent experiments (means ± SD); *- p<0.05; **- p<0.01

An increase of the number of *C. albicans* cells was observed after 3 h on the non-treated cells and when only adenosine was present (Fig. 6). However, in this last case no statistical significance was found. Otherwise, when dipyridamole is present, it appears to decrease the number of yeast cells inside the macrophages. When the two drugs were applied, a decreased in *C. albicans* viability was visible at 3 h. Since this decrease was not observed in the control, i. e., when *C. albicans* alone was treated with dipyridamole and adenosine, the administration of these two drugs conditions the *C. albicans*-macrophage interaction.

DIP also seems to prevent the division of *C. albicans* inside the macrophages and when DIP + ADO are present it begins dying.

3.3 Macrophage viability

In order to investigate if the decrease of *C. albicans* internalized by RAW 264.7 cells is somehow related to the decrease of host cell viability, the same was tested. For this purpose two different experiments were made. The MTT assay is a colorimetric method, which determines cell viability due to the conversion of the yellow MTT reagent to purple formazan crystals. The other method used was the Trypan Blue method, which distinguishes live and death cells due to the coloring of the last ones. During this assays, the same conditions were tested.

3.3.1 MTT Assay

In the MTT assay, its reagent is metabolized by the mitochondria of viable cells, forming purple formazan. Cells were infected and treated in the same way as in the previous assay. After the incubation times of 1.5 h and 3 h, MTT reagent was added and the cells were again incubated for additional 2h. Afterward, DMSO was added to which well in order to form formazan crystals. The absorbance was measured at 570 nm. On the analyses of the results, untreated macrophages were considered our value of reference (100% of viability).

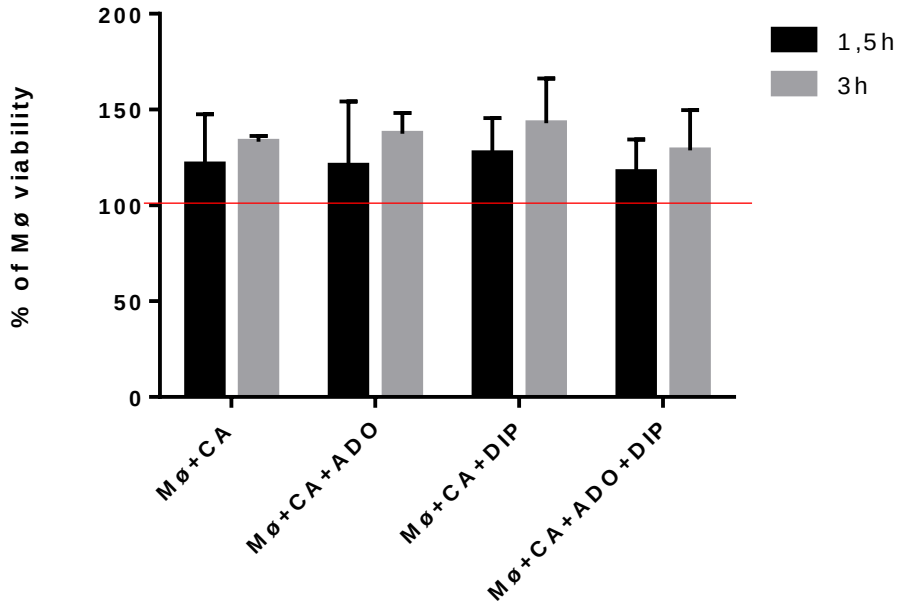

***Figure 7* - Percentage of Viable Cells.** Macrophages (MΦ) were infected with *C. albicans* (CA) and treated with dipyridamole (DIP) and Adenosine (ADO). Cell viability was evaluated at 1.5 h and 3 h by the MTT assay. Red line corresponds to the untreated cells. Results shown are derived from three independent experiments (means ± SD).

With this methodology the macrophages viability is above 100% of the control (which corresponds to the non-infected macrophages) in all of the conditions tested (Figure 7). These results indicate that not only viable cells are contributing to the formation of the formazan crystals, but also the yeast cells. Since this method is dependent of functional mitochondria of eukaryotes, who are also present in the yeast cells, interfering with the final result.

The result above described, does not give insight on the viability of macrophages cells during the infection assay. For this reason, another assay to test the RAW 264.7 viability was performed.

3.3.2 Trypan Blue assay

In this method, Trypan Blue was used as a way to distinguish live and death RAW 264.7 cells. This dye can penetrate the damaged membrane of the cells, dying it blue. It also allows distinguishing the RAW 264.7 cells from the yeast cells, because the assessment is made under the light microscope and, this way, it is possible to distinguish between the two types of cells. After the infection assay, and fulfilled the incubation

periods, dilutions of 2x were prepared (10 µL of the suspension + 10 µL of Trypan Blue 4%). Live and dead macrophage cells were counted in a hemocytometer.

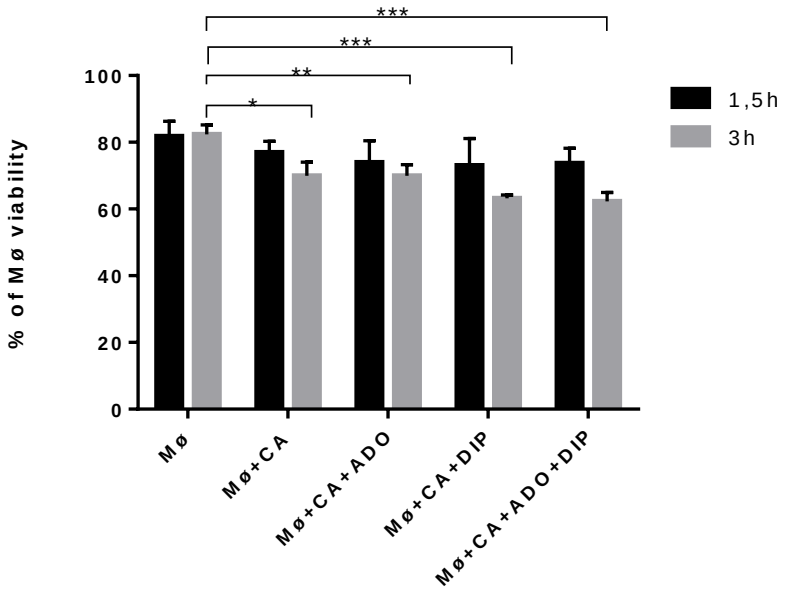

Figure 8 **- Percentage of Viable Host Cells with Trypan blue assay**. Macrophages (MΦ) were infected with *C. albicans* (CA) and treated with dipyridamole (DIP) and Adenosine (ADO). Cell viability was evaluated at 1.5 h and 3 h. Results shown are derived from three independent experiments (means ± SD);*- p<0.05; **- p<0.01; ***- p<0.001.

In the graph represented on Figure 8, it is possible to see a decrease in cell viability in cells that were infected compared to uninfected cells. When macrophages weren't subjected to infection, there is practically no difference between the viability during the 1.5 h period of the study. However, in the remaining conditions, a significant decrease of macrophage viability is visible at 3 h, in comparison with the un-infected cells. It is also important to note that in both conditions in which dipyridamole was present the cells were more compromised, at 3 h. These results seem to indicate that there is in fact a decrease of viability in macrophages, which can affect their phagocytic capability. At 1.5 h, even though there is a slight decrease in cells viability when compared to the control group, no statistical significance was found.

3.4 Interaction between *C. albicans* and macrophages

The internalization of *C. albicans* by RAW 264.7 cells were visualized by a differential fluorescence assay. In this experiments, yeast cells were dyed with Oregon

Green® 488, for 1 h prior to the infection, while RAW 264.7 cells were dyed with Lysotracker Red® DND 99. Oregon green® is a pH insensitive dye which allows monitoring *C. albicans* development. On the other hand, Lysotracker Red® is a red dye that labels acidic compartments of live cells. The use of this dye allows the labeling of phagolysosome, and consequently, besides distinguishing the yeast cells from the RAW 264.7, and to observe relative acidification between treatments. In figures 9 and 10 are illustrated the different conditions in study, at the 1.5 h and 3 h, respectively.

On figure 9 are represented the interactions between *C. albicans* and RAW264.7 cells at 1.5 h. It is visible that in the conditions were treatment is present (Fig. 9C, D), the number of RAW 264.7 cells present in the image is lower. In the presence of adenosine (Fig.9C), hypha appear to develop faster than in the untreated condition (Fig. 9B). The presence of dipyridamole together with adenosine (Fig. 9D) also stimulated hypha formation when compared to untreated cells (Fig. 9B), but not as much as when adenosine was present alone (Fig. 9C). Dipyridamole also seems to interfere with fluorescence, which did not allowed a clear observation using the green filter. Due to this fact, only the red filter, corresponding to Lysotracker Red® DND 99 probing, alongside the brightfield microscopy are shown (Fig. 9D).

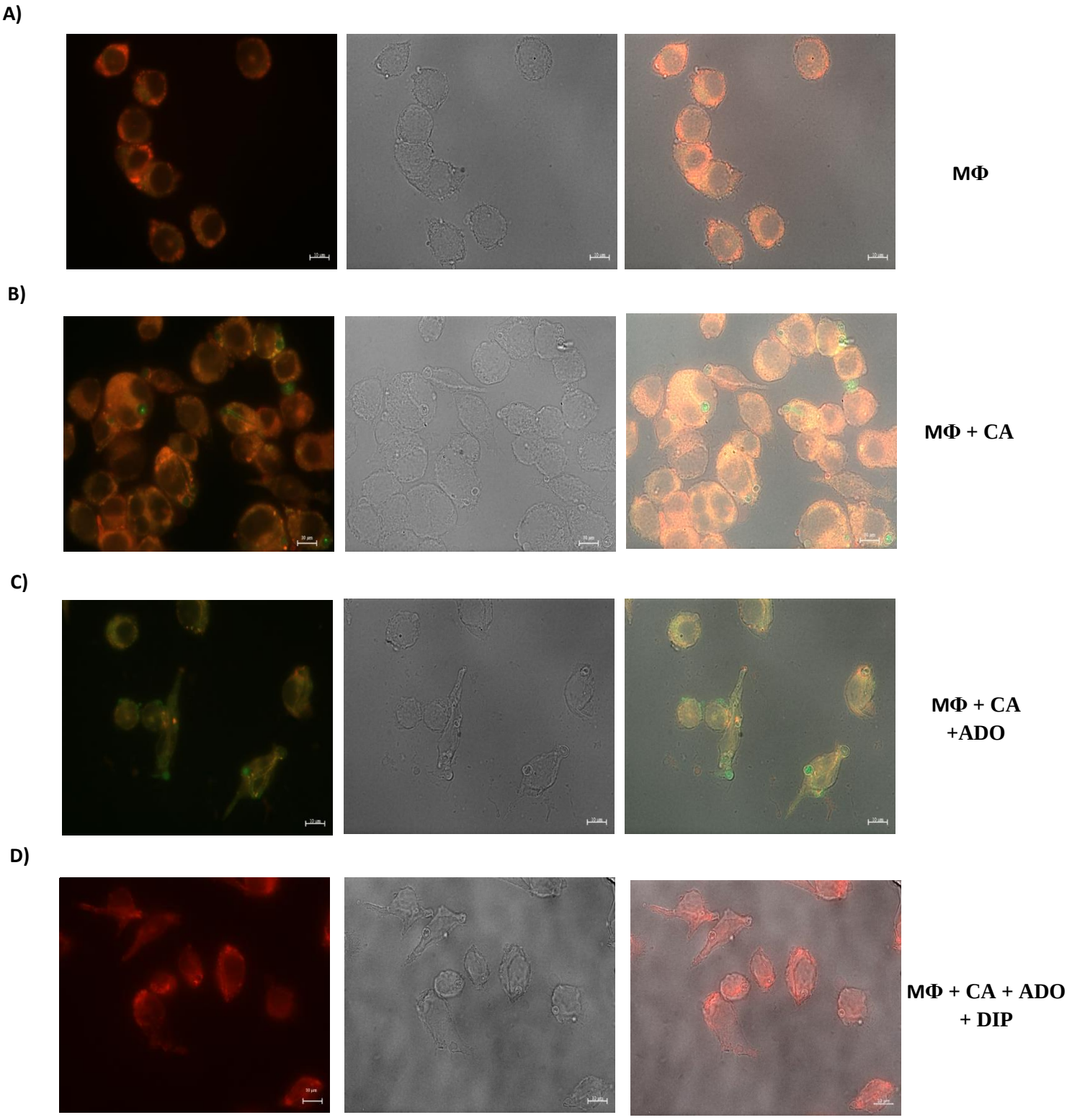

Figure 9 **- Macrophages interaction with *C. albicans* at 1.5 h. A)** Macrophages (MΦ); **B)** Macrophages and *C. albicans* (CA); **C)** Macrophages and *C. albicans* treated with adenosine 10 mM (ADO); **D)** Macrophages and *C. albicans* treated with adenosine 10 mM and dipyridamole 10 µM (DIP). MOI 1:1. Scale bar indicates 10µm. Cells marked with Lysotracker Red and *C. albicans* marked with Oregon Green. Images were acquired in Zeiss Axio Observer Z1 Microscope (Carl Zeiss) with Plan-ApoChromat 63x/1.40 immersion objective.

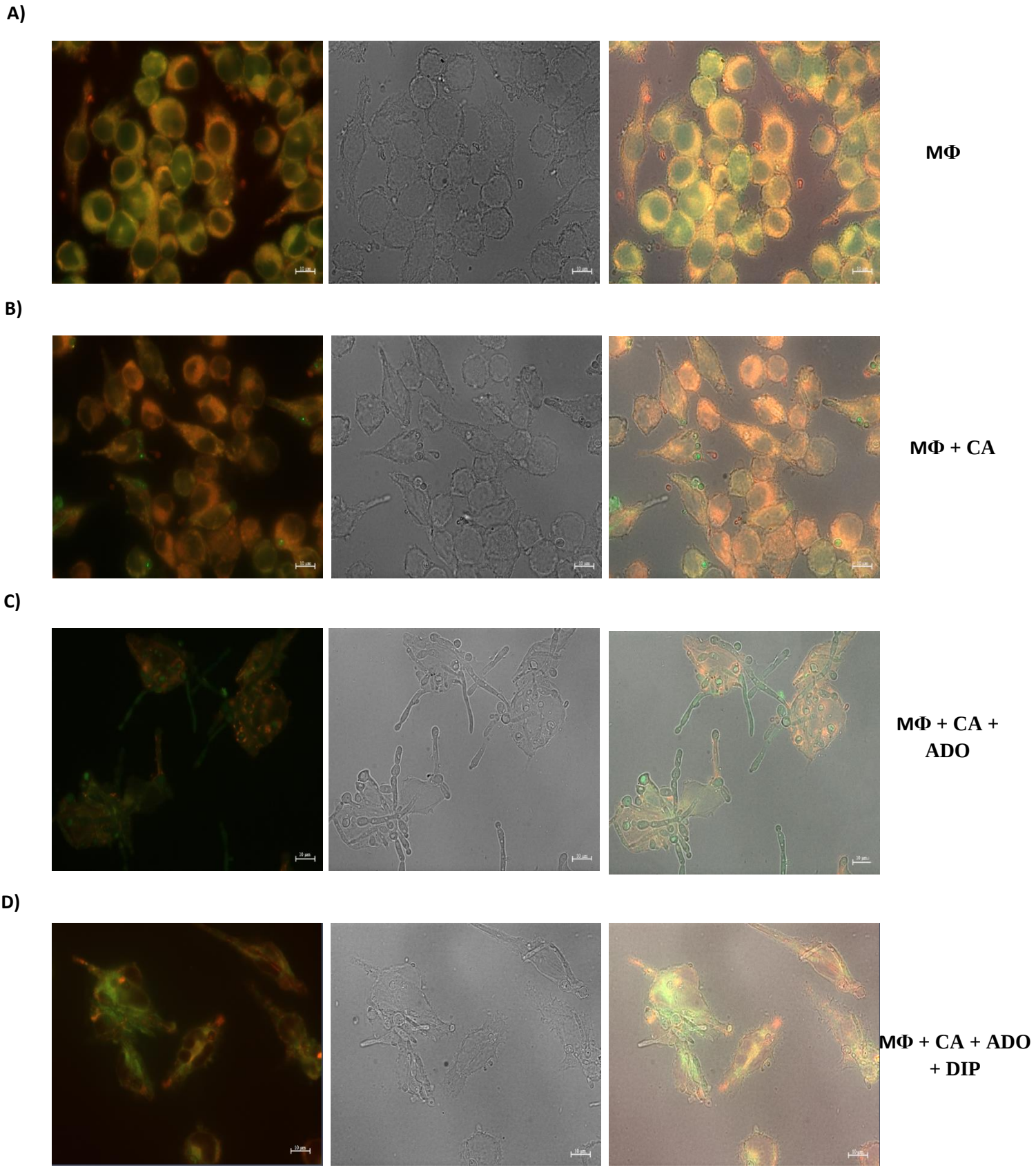

Figure 10 **- Macrophages interaction with *C. albicans* at 3 h. A)** Macrophages (MΦ); **B)** Macrophages and *C. albicans* (CA)*;* **C)** Macrophages and *C. albicans* treated with adenosine 10 mM (ADO); **D)** Macrophages and *C. albicans* treated with adenosine 10 mM and dipyridamole 10 µM (DIP). MOI 1:1. Scale bar indicates 10µm. Cells marked with Lysotracker Red and *C. albicans* marked with Oregon Green. Images were acquired in Zeiss Axio Observer Z1 Microscope (Carl Zeiss) with Plan-ApoChromat 63x/1.40 immersion objective.

The same conditions were studied after an incubation period of 3 h, as illustrated in Figure 10. Notorious differences can be seen in this panel, especially when drugs were present during the infection assay (Fig. 10C, D), when compared to the untreated co-cultures (Fig.10B). It is possible to observe that at 3 h there was an increase of the number of *C. albicans* cells inside the macrophages. However, in the Figures 10C and 10D, which corresponds to the treatment with adenosine and adenosine with dipyridamole, the hyphal growth seems to be over-stimulated when compared with untreated co-cultures (Fig. 11A). Moreover, the interaction of macrophages and *C. albicans* in the presence of these drugs, leads to the formation of chlamydospores (Fig. 11B; red arrows), corresponding to the differentiation of cells when *C. albicans* is in the filamentous form, that are visible only in conditions of *stress* to the pathogen. Besides, it is also possible that the hyphae growth damages the host cells membranes (Fig.11B), which can allow the yeast to escape to the extracellular environment avoiding the killing process. Contrary to what was described at 1.5 h, dipyridamole did not interfere extensively with the fluorescence at 3 h.

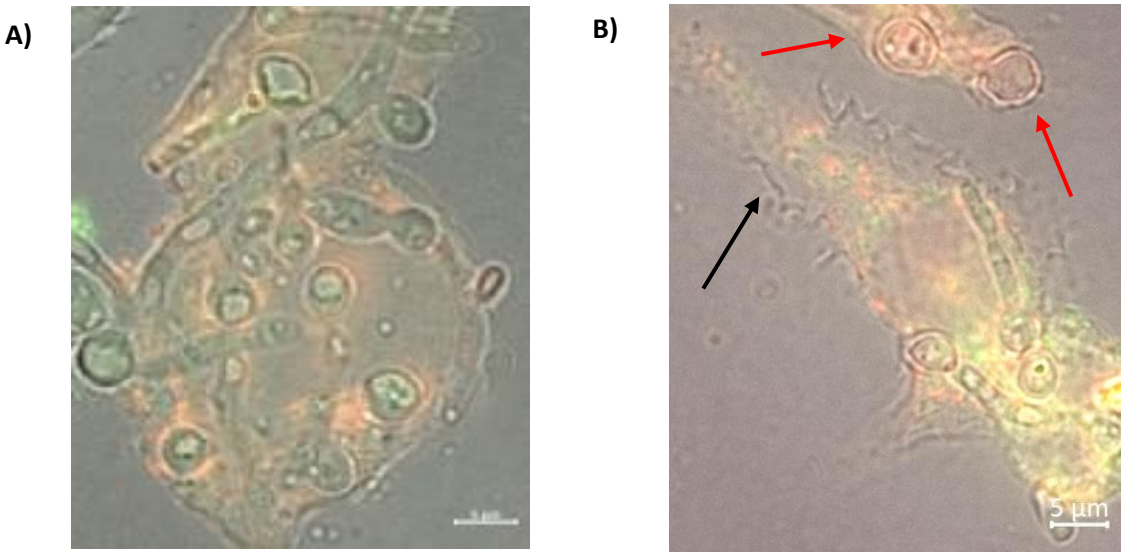

***Figure 11 - Morphological detail of the interaction of* macrophages with *C. albicans* at 3 h. A)** Detail of Macrophages (MΦ) infected with *C. albicans*, treated with Adenosine 10 mM; **B)** Death of a cell when Macrophages and *C. albicans* were treated with adenosine 10 mM and dipyridamole 10 µM (DIP) (black arrows); Chlamydospores are also visible (red arrows indicate this formations). MOI 1:1. Scale bar indicates 5 µM. Cells marked with Lysotracker Red and *C. albicans* marked with Oregon Green. Images were acquired in Zeiss Axio Observer Z1 Microscope (Carl Zeiss) with Plan-ApoChromat 63x/1.40 immersion objective.

3.5 Relative TNF-α and IL-1β gene expression

The host cells have the capability to recognize specific molecules on the cell wall of pathogens, as discussed in Chapter 1. This recognition will trigger the response of the host to microorganisms, which can include the release of proinflammatory cytokines, including the major inflammatory cytokines Interleukin-1β (IL-1β) and the Tumor Necrosis Factor-α (TNF-α). Upon recognition of the PAMPs present on the cell wall of *C. albicans* by its respective PRRs, these two cytokines are two of the first to be released. Besides promoting inflammation, they also stimulate the recruitment of more phagocytic cells to the local of infection. For this reason, the expression of the genes coding for these cytokines was quantified by real time RT-PCR. The profile obtained for the expression of these two cytokines genes (Figure 12) at 1.5 h, revealed that in the presence of adenosine and dipyridamole, there is a two-fold decrease in IL-1β gene expression. Meanwhile, an approximately 6-fold increase in TNF-α gene expression is visible.

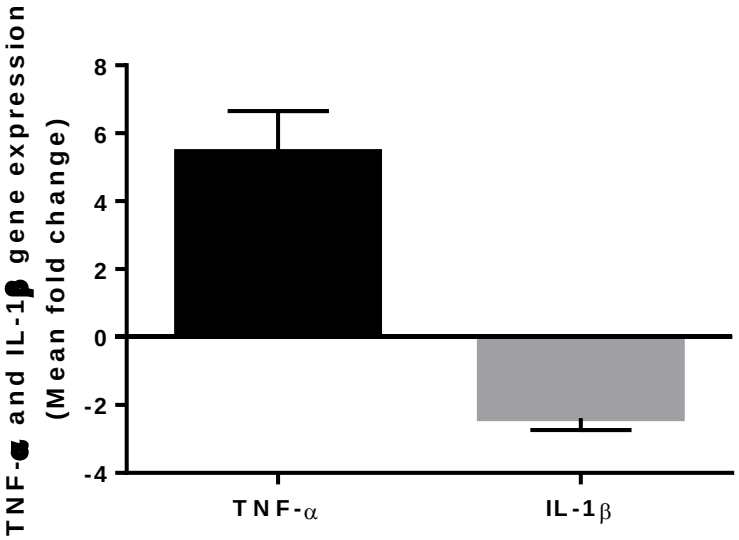

Figure 12 **- Relative expression of TNF-α and IL-1β genes.** Macrophages infected with *C. albicans* and subjected to treatment with dipyridamole (10 μM) and adenosine (10 mM). Data are mean fold elevation compared to RAW 264.7 cells infected with *C. albicans*. Data were derived from two independent experiments (±SEM).

CHAPTER 4

DISCUSSION

Over the years the occurrence of fungal infections have significantly increased due to the advances in today societies, which can be associated with the increase of lifespan in population and the subsequent loss of immune-competence with age. The technologic advances in the medical field, the development of more intensive treatments, which can compromised the defenses of the patient, are also associated with increased opportunistic infections such as fungal infections. Also the administration of antibacterial treatments with a large spectrum of action, allows patients with infections to survive longer, without overcoming the underlying disease, but keeping them more susceptible to these infections (Rodloff, Koch & Schaumann, 2011).

Infections can be mild and superficial or life-threatening illnesses. The major causes of infection are the inhalation of spores, ingestion of a toxin in contaminated food and penetration into the mucosa by commensal organisms. A good example of the last case is *C. albicans*. In healthy individuals, *C. albicans* normally belongs to the microbial flora of the individual, living as a commensal. In this state this pathogen is harmless. However, with disruptions in the balance of the normal microflora and/or the immune system compromised, the pathogen can develop freely causing symptoms of disease (Badiee & Hashemizadeh, 2014; Mavor, Thewes & Hubes, 2005).

As previously described in Chapter 1 this pathogen gained escape mechanisms that empower avoiding the overall killing process by the host. The modulation of the inflammatory signs can be one of them (Cheng, et al., 2012).

One of the main keys in the inflammation process is the release of ATP into the extracellular space, when in presence of danger stimuli. Binding of ATP to specific receptors will stimulate the production of pro-inflammatory factors such as cytokines. But also, ATP in the extracellular millieu can be converted in adenosine, a molecule known for its anti-inflammatory activity. With the binding of adenosine to A_{2A} receptors, an increase of anti-inflammatory cytokines, such as IL-10 occurs. These increase in anti-inflammatory activity helps to prevent an exacerbated response by the host immune system, preventing damages to the tissues. However, some pathogens can take advantage of this signal, benefiting themselves (Russo-Abrahão, et al., 2011; Antonioli, et al., 2013). In a recent study by Rodrigues and colleagues (2016), they verified that with the internalization of *C. albicans*, the A_{2A} receptors previously

located on the membrane of the macrophage, were also internalized and relocated to the phagolysosome membrane. This monopolization of the anti-inflammatory system is believed to protect *C. albicans* in the interior of the macrophage, inside the phagosome, in order to protect it from elimination.

The majority of the adenosine produced by the host cells is derived from ATP degradation in the extracellular space (Antonioli et al., 2014). In order to reach the receptors present in the membrane of the phagolysosome, adenosine with a concentration of 10 mM was used to stimulate the infected cells throughout the assays. The dipyridamole, a well-known drug with vasodilator, anti-oxidant, and anti-inflammatory activity was tested. The application of this drug in the experiments was the same used by Mello et al. (2014). The main purpose of this work was to verify if the modulation of adenosine signal using a nucleoside transporter inhibitor, influenced of effect in the response of macrophages infected with *C. albicans*.

4.1 Effect of dipyridamole on *C. albicans* viability.

The interference of dipyridamole in inflammation has been already proven. On those studies this drug augments the anti-inflammatory response, partially due to the accumulation of adenosine on the extracellular space (Puri, et al., 2016; Ramakers, et al., 2011). Not many studies have been conduct with dipyridamole in infections caused by pathogens. However, is role in viral infections have received some attention. Studies have proven that dipyridamole can inhibit virus replication and reactivation (Fata-Hartley & Palmenberg, 2005; Tenser, Gaydos & Hay, 2001).

In the present work, when *C. albicans* was treated with dipyridamole and/or adenosine, there was no decreased viability of yeast cells. However, the administration of these two drugs, caused a decrease in the number of *C. albicans* cells inside the macrophages RAW264.7 when compared with infection assays without the drugs. This result suggests that since dipyridamole blocks the transport of adenosine, this molecule cannot reach and bind to the A_{2A} receptors that previously were described by Rodrigues (2016) as being re-located intracellularly at the phagosomal membrane surrounding internalized yeast cells. Hypothesizing that the $A_{2A}R$ inside the cells are not activated (due to absence of free adenosine) the cells do not develop an anti-

inflammatory response, meaning that the killing process by the macrophage is more effective. It can also be speculated that somehow the presence of both drugs interferes with *C. albicans* growth, which can lead to a premature death of the macrophages and the release of *C. albicans* to the extracellular space. The methodology used, that eliminates the fungal cells not inside the macrophages, would explain a lower number of yeast cells after three hours of infection assay.

4.2 Morphologic alterations are visible in *C. albicans* in the presence of adenosine and dipyridamole.

As it was possible to observe in the images on figures 9 and 10 in the previous chapter, significant differences were found when infected cells were subjected to treatment on the two times tested. The major differences, appear in the conditions were treatment was administrated. With both drugs, besides hyphal growth, it is possible to observe chlamydospores (Fig. 11B), another morphological form for dimorphic fungi such as *C. albicans* and that only appears in extreme conditions for the same. One of the plausible motives to the formation of this structures is the administration of drugs, in this case adenosine with a high concentration and dipyridamole. Also the alterations on the environment can promote the formation of cAMP by *C. albicans*, which in turn can stimulate the hyphal growth (Hall & Mühlschlegel, 2010). Even though, one of the main pathways activated by adenosine is the production of cAMP in macrophages (Antonioli et al., 2014), no correlation between this and hyphal growth in *C. albicans* have been described.

When in the presence of dipyridamole, hyphal growth is still visible but not in the same capacity as in the previous case. Although it is possible to observe an increase in the number of yeast cells inside macrophages at 3 h when compared to 1.5 h, it is also possible the observation that cells seem to be dying. Figure 11B, represents a detail of a macrophage that apparently bursted. This results strength the first theory presented above. For this reason the cells viability was also tested.

4.3 Presence of adenosine and dipyridamole reduces macrophage viability.

Macrophages are phagocytic cells that belong to the immune system. These cells are one of the first lines of defense in the organism, protecting it from microbial infections as well as maintaining healthy tissues by removing dead/dying cells and toxic materials from the body (Murray & Wynn, 2012). To test of viability of RAW264.7 cells two methods were used. First the MTT assay used proved to be inappropriate for the purpose of this work because the results obtained were generated both from the RAW264.7 viability but also from the *C. albicans* viability. The principle of the MTT method is very simple. Its reagent is metabolized to formazan by mitochondrial enzyme succinate dehydrogenase of viable cells (Stockert, et al., 2012). The problem was the interference of viable *C. albicans* who can also metabolize this reagent. So another method to determine cell viability was used. The Trypan Blue method, consist on the coloring of dying cells that allows distinguishing not only between live and death cells but also *C. albicans* from RAW264.7 cells (Strober, 2001). This happens due to the observation at a light microscope allows the distinction between the two types of cells. After fulfilling its mission, macrophages die (Kolaczkowska, et al., 2010). So a slight decrease in cell viability in the two times tested was expected in the infected cells. The highlight goes to the conditions in which dipyridamole is present, that register the higher decrease between the two times.

4.4 The administration of both dipyridamole and adenosine slight increase the production of TNF-α but decrease the production of IL-1β.

As previously mentioned, macrophages can produce cytokines to augment the immune response. Two of the main pro-inflammatory cytokines produced are TNF-α and IL-1β.

For this reason, the profile of the expression of these two genes were also verified. According to Rodrigues (2016), in the presence of adenosine the levels of TNF-α were maintained unaltered. In the results present in figure 12, where co-cultures of macrophages and *C.albicans* were treated with adenosine and dipyridamole, a tendency to an increase of TNF-α is seen. The difference between the profile obtained

and the results presented by the authors is in the IL-1β expression. These authors reported a significant increase in the IL-1β gene expression when adenosine was present. In the presence of dipyridamole, the tendency of IL-1β gene expression is to decrease. The difference between the two results can be explained because as proven by Ouyang and collaborators (2013), the activation of A_{2A} receptors is necessary to the production of this cytokine. With the use of dipyridamole, uptake of adenosine is blocked, and can't reach the receptors that by this time are already re-located to the membrane of the phagolysosome inside the cell.

CHAPTER 5

CONCLUSION

The understanding of *C. albicans* mechanisms to invade and escape the host cells is a point of interest to fight infections caused by this fungus. Infections caused by *C. albicans* affect more and more patients every year. It becomes necessary to find new ways to strike against this pathogen. Comprehending the ways that *C. albicans* modulates the anti-inflammatory signal can help understanding how this fungus can stay alive inside phagocytic cell.

The main objective of this work was to verify the modulation of the adenosine, an important molecule to the development of anti-inflammatory signal, on macrophages in the presence of dipyridamole, during infection with *C. albicans*. In this work the presence of dipyridamole does in fact affect the immune response by the macrophages, especially in combination with adenosine. The administration of both drugs seems to stimulate the entry of yeast cells in the RAW264.7 cells and promote the death of the same. The results presented seem to support the idea that blocking nucleoside entry increases the efficiency of macrophages clearing a yeast infection..

However more studies need to be conducted with these two drugs, in order to administrate them in case of infection. The verification of the viability of *C. albicans* in the supernatant should be verified. This assay can give an idea of the *C. albicans* present in the extracellular space to give more information of what happens after the 3 h time of infection.

Assays with longer times of infection should also be realized, in order to see the behavior of the cells and the drugs to longer periods of time.

Also the combination of adenosine and dipyridamole should be tested in other fungus besides *C. albicans* species. This would allow studying if the behavior seen in this work, also occurs in other fungal pathogens.

Besides these, the analyses of inflammatory cytokines should be continued. Due to technical problems, only two assays could be accounted. In the presence of adenosine and dipyridamole, analysis of anti-inflammatory cytokines gene expression could also be interesting, to see how the balance between inflammatory and anti-inflammatory response behaves in the presence of both drugs.

CHAPTER 6

REFERENCES

Antonioli, L., Blandizzi, C., Pacher, P., & Haskó, G. (2013). Immunity, inflammation and cancer: a leading role for adenosine. *Nature Reviews Cancer*, *13*(12), 842-857. doi: 10.1038/nrc3613.

Antonioli, L., Csóka, B., Fornai, M., Colucci, R., Kókai, E., Blandizzi, C., & Haskó, G. (2014). Adenosine and inflammation: what's new on the horizon? *Drug Discovery Today, 19*(8), 1051-1063. doi:10.1016/j.drudis.2014.02.010.

Badiee, P. & Hashemizadeh, Z. (2014). Opportunistic invasive fungal infections: diagnosis & clinical management. *Indian Journal of Medical Research, 139*(2), 195-204.

Borea, P. A., Gessi, S., Merighi, S., & Varani, K. (2016). Adenosine as a Multi-Signalling Guardian Angel in Human Diseases: When, Where and How Does it Exert its Protective Effects? *Trends in Pharmacological Sciences, 37*(6), 419-434. doi:10.1016/j.tips.2016.02.006.

Chaffin, W. L., López-Ribot, J. L., Casanova, M., Gozalbo, D., & Martínez, J. F. (1998). Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. *Microbiology and Molecular Biology Review, 62*(1), 130-180.

Chai, L., Netea, M. G., Vonk, A. G., & Kullberg, B. (2009). Fungal strategies for overcoming host innate immune response. *Medical Mycology, 47*(3), 227-236. doi:10.1080/13693780802209082.

Cheng, S. C., Joosten, L. A., Kulberg, B. J., & Netea, M. G. (2012). Interplay between *Candida albicans* and the mammalian innate host defense. *Infection and Immunity*, *80*(4) 1304-1313.doi: 10.1128/IAI.06146-11.

Detke, S. (1998). Cloning of the Candida albicans nucleoside transporter by complementation of nucleoside transport-deficient Saccharomyces. *Yeast, 14*(14), 1257-1265. doi:10.1002/(SICI)1097-0061(1998100)14:14<1257::AID-YEA326>3.0.CO;2-6.

Drury, A. N., & Szent-Gyorgyi, A. (1929). The Physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *The Journal of Physiology*, doi: 6I2.I74.OI9.9:547.857.7.

Duhring, S., Germerodt, S., Skerka, C., Zipfel, P. F., Dandekar, T., & Schuster, S. (2015). Host-pathogen interactions between the human innate immune system and *Candida albicans* - understanding and modeling defense and evasion strategies. *Frontiers in Microbiology, 6*(625). doi: 10.3389/fmicb.2015.00625.

Ebel, F., & Heesemann, J. (2008). Toll-Like Receptors and Fungal Recognition. In A. A. Brakhage & P. F. Zipfel (Eds.) *Human and Animal Relationships*, *6* (Chapter 12), 243-261. Springer Berlin Heidelberg.

Erwig, L. P., & Gow, N. A. (2016). Interactions of fungal pathogens with phagocytes. *Nature Reviews Microbiology, 14*(3), 163-176. doi:10.1038/nrmicro.2015.21.

Fata-Hartley, C. L., & Palmenberg, A. C. (2005). Dipyridamole Reversibly Inhibits Mengovirus RNA Replication. *Jounarl of Virology, 79*(17), 11062-11070. doi:10.1128/JVI.79.17.11062–11070.2005.

Fernández-Arenas, E., Bleck, C. K., Nombela, C., Gil, C., Griffiths, G., & Diez-Orejas, R. (2009). *Candida albicans* actively modulates intracellular membrane trafficking in mouse macrophage phagosomes. *Cellular Microbiology, 11*(4), 560-589. doi:10.1111/j.1462-5822.2008.01274.x.

Ferrari, D., Pizzirani, C., Adinolfi, E., Lemoli, R. M., Curti, A., Idzko, M., Panther, E., Di Virgilio, F. (2006). The P2X7 receptor: a key player in IL-1 processing and release. *The Journal of Immunology, 176*(7), 3877-3883. doi:10.4049/jimmunol.176.7.3877.

Filler, S. C., & Sheppard, D. C. (2006). Fungal invasion of Normally Non-Phagocytic Host Cells. *PLoS Pathogens, 2*(12), e129. doi: 10.1371/journal.ppat.0020129.

Ghosh, S., Howe, N., Volk, K., Tati, S., Nickerson, K. W., & Petro, T. M. (2010). *Candida albicans* cell wall components and farnesol stimulate the expression of both inflammatory and regulatory cytokines in the murine RAW264.7 macrophage cell line. *FEMS Immunol Medical Microbiology,60*, 63-73. doi: 10.1111/j.1574-695X.2010.00717.x.

Gow, N. A., van de Veerdonk, F., Brown, A., & Netea, M. (2011). *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. *Nature Reviews Microbiology, 10*(2), 112-122. doi: 10.1038/nrmicro2711.

Gow, N. R. (2013). A developmental program for *Candida* commensalism. *Nature genetics*, *50* (9), 967-968. doi: 10.1038/ng.2737.

Hall, R. A., & Mühlschlegel, F. A. (2010). A multi-protein complex controls cAMP signalling and filamentation in the fungal pathogen *Candida albicans*. *Molecular Microbiology, 75*(3), 534-537. doi:10.1111/j.1365-2958.2009.06979.x.

Ham, J., & Evans, B. A. (2012). An emerging role for adenosine and its receptors in bone homeostasis. *Frontiers in Endocrinology*, *3*(113), 1-8, doi: 10.3389/fendo.2012.00113.

Haskó, G., Linden, J., Cronstein, B., & Pacher, P. (2008). Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nature Review Drug Discovery*,*7*(9), 759-770. doi: 10.1038/nrd2638.

Haskó, G., Linden, J., Cronstein, B., & Pacher, P. (2009). A_{2B} adenosine receptors in immunity and inflammation. *Trends Immunology*, *30*(6), 263-270. doi:10.1016/j.it.2009.04.001.

Johnson, Z. L., Cheong, C.-G., & Lee, S.-Y. (2012). Crystal structure of a concentrative nucleoside transporter from Vibrio cholerae at 2.4 A. *Nature, 483*(7390), 489 - 493. doi:10.1038/nature10882.

Johnson, Z. L., Lee, J.-H., Lee, K., Lee, M., Kwon, D.-Y., Hong, J., & Lee, S.-Y. (2014). Structural basis of nucleoside and nucleoside drug selectivity by concentrative nucleoside transporters. *eLife*, e03604. doi:10.7554/eLife.03604.001

Jounai, N., Kobiyama, K., Takeshita, F., & Ishii, K. J. (2012). Recognition of damage-associated molecular patterns related to nucleic acids during inflammation and vaccination. *Frontiers in Cellular and Infection Microbiology, 2*(168), 1-13. doi:10.3389/fcimb.2012.00168

King, A. E., Ackley, M. A., Cass, C. E., Young, J. D., & Baldwin, S. A. (2006). Nucleoside transporters: from scavengers to novel therapeutic targets. *Trends in Pharmacological Sciences, 27*(8), 416-425. doi: 10.1016/j.tips.2006.06.004.

Kolaczkowska, E., Koziol, A., Plytycz, B., & Arnold, B. (2010). Inflammatory macrophages, and not only neutrophils, die by apoptosis during acute peritonitis. *Immunobiology, 215*(6), 492-504. doi:10.1016/j.imbio.2009.07.001.

Lapa, F. R., Macedo Jr, S. J., Cerutti, M. L. & Soares Santos, A. R. (2014). Pharmacology of Adenosine Receptors and Their Signaling Role in Immunity and Inflammation, InTech & S. Gowder (Ed.), *Pharmacology and Therapeutics*, Chapter 4, 85-130. doi: 10.5772/57206.

Li, Y., Figler, R. A., Kolling, G., Bracken, T. C., Rieger, J., Stevenson, R. W., Linden, J., Guerrant, R. L., Warren, C. A. (2012). Adenosine A_{2A} receptor activation reduces recurrence and mortality from *Clostridium difficile* infection in mice following vancomycin treatment. *BMC Infectious Diseases*, *12*(342), 1-12, doi:10.1186/1471-2334-12-342.

Liu, Y., & Filler, S. G. (2011). *Candida albicans* Als3, a Multifunctional Adhesin and Invasin. *Eukaryotic Cell, 10*(2), 168-173. doi:10.1128/EC.00279-10.

Matsumoto, T., Tostes, R. C., & Webb, R. C. (2012). Alterations in vasoconstrictor responses to the endothelium-derived contracting factor uridine adenosine tetraphosphate are region specific in DOCA-salt hypertensive rats. *Pharmacol. Res.*, *65*(1), 81-90. doi:10.1016/j.phrs.2011.09.005.

Mavor, A. L., Thewes, S., & Hube, B. (2005). Systemic fungal infections caused by Candida species: epidemiology, infection process and virulence attributes. *Current Drug Targets, 6*(8), 863-874. doi:10.2174/138945005774912735.

Mayer, F., Wilson, D., & Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. *Virulence, 4*(2), 119-128. doi:10.4161/viru.22913.

Mello, P. A., Filippi-Chiela, E. C., Nascimento, J., Beckenkamp, A., Santana, D. B., Kipper, F., Casali, E. A., Nejar Bruno, A., Paccez, J. D., Zerbini, L. F., Wink, M. R., Lenz, G., Buffon, A. (2014). Adenosine uptake is the major effector of extracellular ATP toxicity in human cervical cancer cells. *Molecular Biology of the Cell, 25*(19), 2905-2918. doi:10.1091/mbc.E14-01-0042.

Milne, G. R., & Palmer, T. M. (2011). Anti-Inflammatory and Immunosuppressive Effects of the A_{2A} Adenosine Receptor. *The Scientific World Journal*, *11*, 320-339. doi: 10.1100/tsw.2011.22.

Mogensen, T. H. (2009). Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. *Clinical Microbiology Reviews, 22*(2), 240-273. doi:10.1128/CMR.00046-08.

Molina-Arcas, M., Casado, F. J., & Pastor-Anglada, M. (2009). Nucleoside Transporter Protein. *Current Vascular Pharmacology, 7*(4), 426-434. doi:10.2174/157016109789043892#sthash.zkKF4usj.dpuf.

Motaung, T. E., Albertyn, J., Pohl, C. H., & Kohler, G. (2015). *Candida albicans* mutant construction and characterization of selected virulence determinants. *Journal of Microbiological Methods, 115*, 153-165. doi:10.1016/j.mimet.2015.06.004.

Murray, P. J., & Wynn, T. A. (2012). Protective and pathogenic functions of macrophage subsets. *Nature Reviews Immunology, 11*(11), 723-737. doi:10.1038/nri3073.

Nadeem, S. G., Shafiq, A., Hakim, S. T., Anjum, Y., & Kazm, S. U. (2013). Effect of Growth Media, pH and Temperature on Yeast to Hyphal Transition in *Candida albicans*. *Journal of Medical Microbiology, 3*, 185-192. doi:10.4236/ojmm.2013.33028.

Niimi, M., Cannon, R. D., & Monk, B. C. (1999). *Candida albicans* pathogenicity: a proteomic perspective. *Electrophoresis, 20*(11), 2299-2308. doi: 10.1002/(SICI)1522-2683(19990801)20:11<2299::AID-ELPS2299>3.0.CO;2-7.

Ouyang, X., Ghani, A., Malik, A., Wilder, T., Colegio, O. R., Flavell, R. A., Cronstein, B. N., Mehal, W. Z. (2013). Adenosine is required for sustained inflammasome activation via the A_{2A} receptor and the HIF-1α pathway. *Nature Communications, 4*(2909), 1-20. doi: 10.1038/ncomms3909.

Park, H., Myers, C. L., Sheppard, D. C., Phan, Q. T., Sanchez, A. A., Edwards Jr, J. E., & Filler, S. G. (2005). Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. *Cellular Microbiology, 7*(4), 499-510. doi:10.1111/j.1462-5822.2004.00476.x.

Parkinson, F. E., Damaraju, V. L., Graham, K., Yao, S. Y., Baldwin, S. A., Cass, C. E., & Young, J. D. (2011). Molecular Biology of Nucleoside Transporters and their Distributions and Functions in the Brain. *Current Topics in Medical Chemistry, 11*(8), 948-972.

Phan, Q. T., Myers, C. L., Sheppard, D. C., Yeanman, M. R., Welch, W. H., Ibrahim , A. S., Edwards, J. E. jr, Filler, S. G. (2007). Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLOS Biology, 5*(3), e64. doi:10.1371/journal.pbio.0050064.

Puri, N., Mohey, V., Singh, M., Kaur, T., Pathak, D., Buttar, H. S., & Singh, A. P. (2016). Dipyridamole attenuates ischemia reperfusion induced acute kidney injury through adenosinergic A_1 and A_{2A} receptor agonism in rats. *Naunyn-Schmiedeberg's Archives of Pharmacology, 389*(4), 361-368. doi:10.1007/s00210-015-1206-2.

Ramakers, B. P., Riksen, N. P., Stal, T. H., Heemskerk, S., van den Broek, P., Peters, W. H., van der Hoeven, J. G., Smits, P., Pickkers, P. (2011). Dipyridamole augments the antiinflammatory response during human endotoxemia. *Critical Care, 15*(6), R289. doi:10.1186/cc10576.

Ravelic, V., & Burnstock, G. (1998). Receptors for Purines and Pyrimidines. *Pharmacological Reviews*. *50*(3), 413 - 492.

Rodloff, A. C., Koch, D., & Schaumann, R. (2011). Epidemiology and antifungal resistance in invasive candidiasis. *European Journal of Medical Research, 16*(4), 187-195. doi:10.1186/2047-783X-16-4-187.

Rodrigues, L. (2016). Candida-host interaction: Role of purines and adenosine A_{2A} receptors. (PhD Thesis) Faculdade de Medicina, Universidade de Coimbra, Portugal.

Russo-Abrahão, T., Cosentino-Gomes, D., Gomes, M. T., Alviano, D. S., Alviano, C. S., Lopes, A. H., & Meyer-Fernandes, J. R. (2011). Biochemical properties of *Candida parapsilosis* ecto-5'-nucleotidase and the possible role of adenosine in macrophage interaction. *FEMS Microbiology Letters, 317*(1), 34-42. doi:10.1111/j.1574-6968.2011.02216.x.

Sachdeva, S., & Gupta, M. (2013). Adenosine and its receptors as therapeutic targets: An overview. *Saudi Pharmaceutical Journal*, *21*(3) 245-253. doi: 10.1016/j.jsps.2012.05.011.

Sheth, S., Brito, R., Mukherjea, D., Rybak, L. P., & Ramkumar, V. (2014). Adenosine Receptors: Expression, Function and Regulation. *International Journal of Molecular Sciences, 15*(2), 2024-2052. doi:10.3390/ijms15022024.

Sorrell T, Chen S (2009) Fungal-derived immune modulating molecules. In P. G. Fallon (Ed.) *Advances in Experimental Medicine and Biology*, *666* (Chapter 9), 108–120. New York. Springer Science.

Strober, W. (2001). Trypan blue exclusion test of cell viability. *Current protocols in immunology*, A3-B. doi:10.1002/0471142735.ima03bs21.

Stockert, J. C., Blázquez-Castro, A., Cañete, M., Horobin, R. W., & Villanueva, Á. (2012). MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta Histochemica, 114*(8), 785-796. doi:10.1016/j.acthis.2012.01.006.

Sudbery, P. (2011). Growth of *Candida albicans* hyphae. *Nature Reviews, 9*, 737-748. doi:10.1038/nrmicro2636.

Sudbery, P., Gow, N., & Berman, J. (2004). The distinct morphogenic states of *Candida albicans*. *Trends in Microbiology, 12*(7), 317-324. doi:10.1016/j.tim.2004.05.008.

Tang, D., Kang, R., Coyne, C. C., Zeh, H. J., & Lotze, M. T. (2012). PAMPs and DAMPs: signal 0s that spur autophagy and immunity. *Immunological Reviews, 249*(1), 158–175. doi:10.1111/j.1600-065X.2012.01146.x.

Tenser, R. B., Gaydos, A., & Hay, K. A. (2001). Inhibition of Herpers Simplex Virus Reativation by Dipyridamole. *Antimicrobial Agents and Chemotherapy, 45*(12), 3657-3659. doi:10.1128/AAC.45.12.3657–3659.2001.

Thompson, D. S., Carlisle, P. L., & Kadosh, D. (2011). Coevolution of Morphology and Virulence in *Candida* Species. *Eukaryot Cell, 10*(9), 1173-1182. doi:10.1128/EC.05085-11.

Whibley, N., & Gaffen, S. (2015). Beyond *Candida albicans*: Mechanisms of immunity to non-albicans *Candida* species. *Cytokine, 76*(1), 42-52. doi:10.1016/j.cyto.2015.07.025.

Young, J. D., Yao, S. Y., Baldwin, J. M., Cass, C. E., & Baldwin, S. A. (2013). The human concentrative and equilibrative nucleoside transporter families, SLC28 and SLC29. *Molecular Aspects of Medicine, 34*(2-3), 529-547. doi:10.1016/j.mam.2012.05.007.