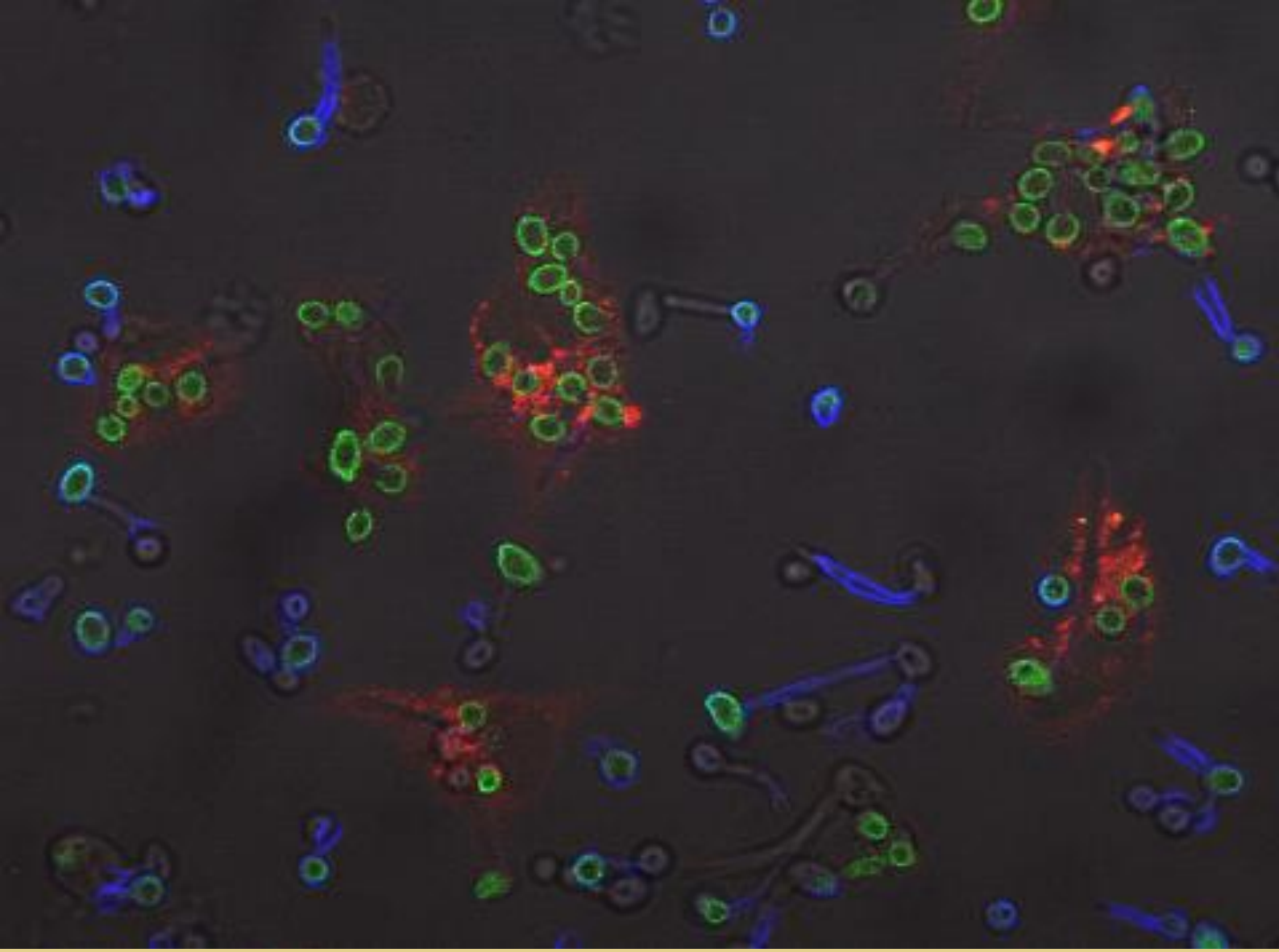

Lisa Catarina Oliveira Rodrigues

Candida-host interaction: Role of purines and adenosine A_{2A} receptors

Tese de Doutoramento do Programa de Doutoramento em Ciências da Saúde, ramo de Ciências Biomédicas, orientada pela Professora Doutora Teresa Gonçalves e pelo Professor Doutor Rodrigo Cunha e apresentada à Faculdade de Medicina da Universidade de Coimbra

Fevereiro 2015

· U C ·

UNIVERSIDADE DE COIMBRA

CANDIDA-HOST INTERACTION:

ROLE OF PURINES AND ADENOSINE A_{2A} RECEPTORS

INTERACÇÃO *CANDIDA*-HOSPEDEIRO:

FUNÇÃO DAS PURINAS E DOS RECEPTORES A_{2A} PARA A ADENOSINA

LISA CATARINA OLIVEIRA RODRIGUES

UNIVERSIDADE DE COIMBRA

2015

Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra, para prestação de provas de doutoramento do Programa de Doutoramento em Ciências da Saúde, ramo de Ciências Biomédicas, sob orientação científica da Professora Doutora Teresa Gonçalves e do Professor Doutor Rodrigo Cunha.

Trabalho financiado pela Fundação para a Ciência e Tecnologia, IP (SFRH/BD/74181/2010; PTDC/SAU-MIC/115598/200; PEst-C/SAU/LA0001/2011; UID/NEU/04539/2013); FEDER – COMPETE; DARPA (W911NF-10-1-0059); Gabinete de Apoio à Investigação (GAI) da Faculdade de Medicina da Universidade de Coimbra & Banco Santander Totta.

À minha Mãe

AGRADECIMENTOS

"Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive."
(Ricardo Reis)

As dúvidas que nos assolam, as opções que vamos tomando, o caminho que escolhemos percorrer…, tudo nos vai transformando constantemente e, acima de tudo, somos forçados a colocar de lado a nossa 'pequenez' e a crescer um pouquinho mais a cada dia que passa. "Ser inteiro" pode, efectivamente, revelar-se uma tarefa árdua, mas, de uma coisa, tenho a certeza: a cada desafio, a cada questão que coloco e que me é colocada, a cada incerteza, vai-se multiplicando também a vontade de me superar, de voar mais alto, de ser cada vez maior. E, como é óbvio, todo esse percurso nunca se faz sem companhia…

À Professora Teresa Gonçalves, que, sem me conhecer, me acolheu no seu laboratório; por me ter aberto portas (no sentido literal, mas não só) e por ter ajudado, ao longo destes anos, a fomentar em mim esta admiração pelo que é "muito pequenino e não se vê". Muito me honra poder partilhar o seu entusiasmo e aprender consigo: muito obrigada pela sua orientação, pela sua disponibilidade, pela sua amizade, pelo seu exemplo!

Ao Professor Rodrigo Cunha, co-orientador deste trabalho, por todo o apoio, pela constante disponibilidade e simpatia e por ter contribuído para que muitas dessas portas se abrissem definitivamente.

À Professora Catarina de Oliveira, por ter aceite ser minha tutora e pela constante preocupação e incentivo.

À Faculdade de Medicina, na pessoa do Professor Meliço-Silvestre, responsável pelo serviço de Microbiologia, e ao CNC, onde desenvolvi o meu trabalho.

Ao Programa Inter-Universitário de Doutoramento em Envelhecimento e Degenerescência dos Sistemas Biológicos Complexos e a todos os colegas e docentes, por terem tido um papel fulcral na minha decisão de 'mudar de rumo'.

Ao Professor José Roberto Meyer-Fernandes, à Michele e a todo o grupo de trabalho, por terem tornado a minha estadia no Rio de Janeiro numa das melhores experiências pessoais e profissionais que já vivi.

Ao Professor Acácio Gonçalves Rodrigues e à Professora Cidália Pina-Vaz, pelos seus importantes contributos e por terem aceite colaborar neste trabalho; a todo o grupo de investigação, por toda a ajuda, disponibilidade e simpatia.

À Isabel Miranda, pelo seu exemplo, pela sua incansável disponibilidade e ajuda, pela sua amizade e pelas constantes palavras de incentivo.

À Professora Maria de Fátima Carneiro, do Serviço de Anatomia Patológica do Hospital de São João e em particular ao Rui M. Correia, pela colaboração no estudo histológico.

À Geanne Matos, por ter aceite colaborar connosco e pela sua importante contribuição para o desfecho final de um dos últimos 'capítulos' deste trabalho.

A todos os colegas e às técnicas do CNC, por toda a simpatia e constante disponibilidade, em particular à Luísa Cortes, sem a qual parte deste trabalho não teria sido possível, pelos importantes conselhos e incentivos.

Ao LEF, pelas importantes colaborações e por todo o auxílio disponibilizado, nomeadamente, ao Francisco Queiroz e à Paula Canas; à Catarina Gomes, por tudo isto e também pelas suas palavras e pelo seu entusiasmo.

Ao MMYRG e a todos os elementos com quem cruzei nos últimos anos: trabalhar em espírito de equipa, num ambiente salutar e de amizade, inspira-nos a chegar mais longe. A todos os alunos e colegas que por cá têm passado e que muito contribuíram para o meu enriquecimento; à Filipa Curado, Carolina e Vítor, por terem dado os primeiros passos no que viria a ser o "meu" trabalho; à Filipa Moreira pela sua recente contribuição; à Alexandra, pela preciosa ajuda e gestão e por agilizar os mais pequenos detalhes; aos meus 'venenos' e companheiros de viagem, Rui, Marta, Cindy e

Branca, pelos pequenos e grandes momentos, pela amizade e por tornarem cada dia numa ‘nova e preciosa aventura’.

A todos os colegas e amigos que me têm acompanhado nesta jornada. Aos amigos de ‘sempre’, por, perto ou longe, estarem ‘sempre lá’, em especial à Patro (e à Clarinha!), à Jo, à Tatá e ao Ti. Ao Nuno que, mesmo sem se aperceber, me tem feito questionar, ‘desfocar’ e crescer para além do que eu própria me julgava capaz. À ‘minha’ Tunna e à ‘minha’ OTR. Aos meus ‘velhos amigos típicos’, em especial à Sandra, Paulo (e Pedro!), Xurro e Lara; ao Cousin, Chapitô, Aba, Raquel, Morangos, Magda, Fifi e tantos outros, por continuarem presentes, por todas as gargalhadas e puxões de orelhas, por partilharem, muitas vezes, das minhas preocupações, e pelas constantes palavras de apoio e incentivo. Ao CSIC e ao meu “gang”, Ana Marta, Isabel, Rita, Sofia e Pedro, por serem uma lufada de ar fresco e por, com a sua amizade, tornarem os meus dias mais leves.

A toda a minha família, por me ter ajudado a crescer como cresci.

Ao meu Pai e à minha Mãe, por me terem feito chegar “aqui”; por saber que, aconteça o que acontecer, estarão sempre lá, por me acompanharem e não me deixarem caminhar sozinha. (…à minha Mamã, por tudo o que me atura, pelo seu coração gigante e porque a sua mão nunca larga a minha! Porque, ainda que muito me esforce, não conseguirei ser mais que apenas uma pequena sombra da enorme Mulher que ela é e sempre foi!)

A todos os que têm cruzado o meu caminho, o meu mais sincero obrigada!

RESUMO

As leveduras do género *Candida* spp. são elementos da flora normal do organismo humano, mas também importantes agentes de infecção fúngica oportunista, particularmente em indivíduos com alterações do sistema imunitário ou muito debilitados. Também a imunossenescência natural, associada ao envelhecimento humano, leva a uma diminuição da capacidade de controlar as infecções, nomeadamente as fúngicas. O reconhecimento e erradicação de uma infecção fúngica pelo hospedeiro depende de células fagocíticas, tais como os macrófagos, sendo um processo complexo que envolve vários sistemas de reconhecimento e de resposta imune-inflamatória. Um importante sistema de homeostasia na imunidade e inflamação é operado pelas purinas, nomeadamente o ATP e a adenosina extracelulares. Em particular, o receptor A_{2A} de adenosina ($A_{2A}R$) contribui para este delicado controlo das respostas inflamatórias e imunológicas, eliminando as ameaças de forma eficiente e minimizando os danos infligidos.

Esta dissertação visa explorar a hipótese de que a adenosina e os seus receptores podem constituir um dos sistemas explorados por *Candida* spp., em particular por *Candida albicans*, o principal agente de infecção fúngica humana, para modular a resposta de macrófagos, reforçando o seu sucesso como agente patogénico. Este estudo incidiu sobre o papel dos receptores de adenosina, os $A_{2A}R$, na eficiência do fagolisossoma para eliminar leveduras. Utilizaram-se várias abordagens para estudar a interacção *in vitro* entre *C. albicans*, *Candida glabrata*, *Candida parapsilosis* ou *Saccharomyces cerevisiae* e uma linha celular de macrófagos. Observou-se que, após a infecção com essas leveduras, não ocorria aumento da expressão do gene Adora2a, que codifica para os $A_{2A}R$, em contraste com o aumento de 11 vezes registado para a

expressão do gene Adora2a em macrófagos tratados com lipopolissacarídeo (LPS) de bactérias Gram negativas. Paralelamente, observou-se que a exposição a fungos forçou os $A_{2A}R$ a localizarem-se em torno da membrana dos fagossomas dos macrófagos que continham células de leveduras. A utilização de uma abordagem farmacológica, com agonistas e antagonistas dos $A_{2A}R$, assim como o uso de macrófagos peritoneais de ratinhos com delecção genética (*knockout*) dos $A_{2A}R$, permitiu demonstrar que os $A_{2A}R$ controlavam a eficiência fagocítica dos macrófagos na internalização de *C. albicans* e na produção de moléculas sinalizadoras. Ademais, durante a infeção com *C. albicans*, a levedura estudada à qual está associada maior virulência, a quantidade de ATP extracelular, um sinal de perigo que estimula a inflamação conducente à eliminação dos agentes patogénicos, não aumenta, levando, assim, a uma menor eficiência dos macrófagos. Já durante a infecção por *C. glabrata* e *S. cerevisiae*, menos virulentos, há um aumento significativo do ATP extracelular. Para além disto, no decurso da infecção por *C. albicans*, a atividade total ecto-fosfatásica, diminuiu de cerca de 20 para 17 nmol Pi h^{-1} 10^{-6} células ($p<0.01$), enquanto que a atividade total ecto-5'-nucleotidásica se manteve constante. Para compreender a possível contribuição das ecto-nucleotidases ou ecto-fosfatases de *C. albicans* para o seu mecanismo de patogenicidade, foi realizado um estudo para esclarecer as propriedades dessas enzimas em *C. albicans*, tendo-se determinado que, nesta levedura, algumas particularidades destas enzimas poderão estar relacionadas com a sua capacidade de sobreviver ao ambiente do fagolisossoma, como o facto da atividade enzimática máxima se registar a um pH ácido de 4.0. Finalmente, foi construído um modelo gastrointestinal de infecção por *C. albicans* com o objetivo de decifrar o envolvimento dos $A_{2A}R$ na susceptibilidade à infecção e na resposta inflamatória dos tecidos gastrointestinais de ratinhos jovens, adultos e idosos, o que

permitiu estabelecer uma correlação entre infecção, inflamação, envelhecimento e localização dos $A_{2A}R$ nos tecidos do trato gastrointestinal.

Assim, o trabalho aqui apresentado demonstra que, após interação com os macrófagos, *Candida*, em particular *C. albicans* é capaz de modular o ATP extracelular, impedindo o seu aumento e afectando desta forma o seu efeito pro-inflamatório; por outro lado, força a re-localização dos $A_{2A}R$ para a membrana do fagossoma, o que contribuirá para o silenciamento da resposta dos macrófagos à infeção, contribuindo não só para o seu sucesso como agente patogénico, mas também para a manutenção dos reservatórios endógenos de agentes de infecção oportunista.

ABSTRACT

Yeasts of the genus *Candida* spp. are members of the human body normal flora that can turn into aggressive agents of opportunistic infection, namely in individuals with immune diseases or in debilitated conditions. This is prominent in elderly individuals, where the natural immunosenescence upon ageing leads to a decreased ability to control infections, including those caused by fungi. Yeast recognition and clearance by host phagocytic cells, such as macrophages, seems to be a complex procedure involving multiple recognition systems and inflammatory responses. Purines, namely extracellular ATP and adenosine, operate an important role in immunity and inflammation homeostasis. In particular, the adenosine A_{2A} receptor ($A_{2A}R$) critically contributes to the fine-tuning of inflammatory and immune responses, prompting an efficient elimination of threats while minimizing tissue damage.

This dissertation aims to explore the hypothesis that adenosine and its sensing devices may constitute one of the systems exploited by *Candida* spp., in particular by *Candida albicans*, to modulate the response of macrophages, bolstering its pathogenic success. This study focused on the role of $A_{2A}R$ in the efficiency of the phagolysosome to clear yeasts. Several approaches were used to study the *in vitro* interaction between *C. albicans, Candida glabrata, Candida parapsilosis* or *Saccharomyces cerevisiae* with a macrophage cell line. It was observed that upon infection with those yeasts, there was no increase of $A_{2A}R$ gene expression in contrast to the 11-fold increase of $A_{2A}R$ gene expression upon exposure of macrophages to lipopolysaccharide (LPS) from Gram negative bacteria. Furthermore, upon yeast infection, $A_{2A}R$ localize around phagosomes containing yeasts cells. The use of pharmacological approaches, with both $A_{2A}R$ agonists and antagonists, as well as peritoneal macrophages from $A_{2A}R$ knockout mice,

allowed concluding that $A_{2A}R$ control the phagocytic efficiency of macrophage to internalize *C. albicans* cells and the production of signaling molecules. Moreover, during the course of infection with *C. albicans*, the most virulent yeast studied, the extracellular levels of ATP, a danger signal contributing to stimulate inflammation leading to clearance of pathogens, did not increase, thus forcing a lower efficiency of macrophages. Nonetheless, during the course of infection with the less virulent yeasts *C. glabrata a*nd *S. cerevisiae* a significant increase in extracellular ATP levels was found. Adding to this, with *C. albicans* infection, total ectophosphatase activity decreased from about 20 to 17 nmol Pi h^{-1} 10^{-6} cells ($p<0.01$), while ecto-5'nucleotidase activity did not change. To tackle the contribution of *C. albicans* ecto-nucleotidases or ectophosphatases to its pathogenesis, an enzymatic study revealed some special properties of the enzymes relevant for the yeast survival inside the phagolysosome, such as the finding that their maximal enzyme activity was recorded under an acidic pH of 4. Finally, an *in vivo* model of sustained *C. albicans*-gastrointestinal infection was constructed with the objective of defining the involvement of $A_{2A}R$ on the susceptibility to yeast infection and on the inflammatory response of gastrointestinal tissues from young, adult and aged mice; this allowed to establish a correlation between infection, inflammation, ageing and $A_{2A}R$ localization in the gastrointestinal tract.

Overall, the work presented here shows that upon interaction with macrophages, *Candida*, in particular *C. albicans*, is able to modulate the extracellular ATP, preventing its increase and thus affecting its pro-inflammatory effect; on the other hand, forcing a re-localization of $A_{2A}R$ to the phagosome membrane, favors the silencing of the macrophages responses, contributing to the success of *Candida* spp. as pathogens but also for the persistence of endogenous reservoirs of opportunistic agents of infection.

CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER I

INTRODUCTION

INTRODUCTION

Among the great diversity of fungi, yeasts are ubiquitous eukaryotes that can interact with plants, animals and humans, establishing symbiotic, commensal, latent or pathogenic relationships. Many of those fungal species, such as *Pneumocystis jiroveci* or *Candida albicans*, have been actually co-evolving with mammalian hosts over millions of years, with consequent adaptations strategies from both sides (Romani, 2011). In the last decades, fungi have emerged as major cause of human disease, thus standing as an important public health problem (Romani, 2011). The increased number of opportunistic fungal diseases, namely due to *Candida* spp., are particularly relevant in individuals with debilitated physiological conditions and with local or systemic immune malfunction (Pfaller and Diekema, 2007) and numerous factors are contributing to this situation. Due to the natural progresses of modern medicine, organs transplantation, use of cytotoxic chemotherapies and broad-spectrum antibiotics or AIDS are major factors contributing to this increased pathogenicity (Pfaller and Diekema, 2007). As a result of natural immunosenescence, age is also a critical factor: in developed societies, elderly persons represent a rapidly growing portion of the population and worldwide it is expected that the age-group above 65 years will duplicate between 2000 and 2025 (Pfister and Savino, 2008). In all those situations, the outcome of any infection will thus obviously depend on the physiological robustness of the fungal pathogen, in one hand, but also, in the other hand, on the efficiency of host defenses against it (Brown *et al*., 2014). Consequently, deeper understanding of the relations and interactions established between both sides of this dilemma is, therefore, crucial to the effective management of any infection situation.

EPIDEMIOLOGY OF *CANDIDA* INFECTIONS

At any given time, *Candida* species colonize asymptomatically about 30 to 50% of the human population, normally existing as harmless commensal organisms in the microflora of skin, oral cavity, gastrointestinal and urogenital tracts of most healthy individuals (Calderone, 2002; Calderone and Clancy, 2012; Cheng *et al*., 2012; Brown *et al*., 2014). However, anytime the host defenses of the individuals are weakened, they can cause both mucosal and systemic infections, in a wide range of different niches within the host (Cheng *et al*., 2012; Brown *et al*., 2014), and *C. albicans* is particularly relevant, since it represents a major human fungal pathogen (Sobel, 2007; Revankar and Sobel, 2012).

Epidemiological results, although with slightly differences between studies, have shown that fungi cause about 15% of nosocomial infections and that *Candida* accounts for 70–90% of all invasive fungal infections. In the USA, *C. albicans* is presented as the 3th or 4th most commonly isolated microorganism in blood cultures, while in Europe it appears between the 6th and 10th positions of blood isolates (ranging from 45% to <60%). This is particularly important in Intensive Care Units, where invasive *Candida* infections are associated with high morbidity and mortality rates, ranging from 40% to 60% (Costa-de-Oliveira *et al*., 2008; Falagas *et al*., 2010; Delaloye and Calandra, 2014; Paramythiotou *et al*., 2014). Nonetheless, in the last decades, a change in the epidemiology of *Candida* infections worldwide has been observed, with progressive alterations from prevalent *C. albicans* toward predominance of non-*albicans Candida* species. As an example, *Candida glabrata* and *Candida parapsilosis* have been gradually emerging, accounting for up to 50% of infections in several countries, from USA to South America, Asia and Europe (Méan *et al*., 2008; Falagas *et al*., 2010;

Delaloye and Calandra, 2014). A recent analysis of the worldwide distribution of these species indicated that *C. glabrata* remains the most common non-*albicans Candida* spp, but *C. parapsilosis*, *Candida tropicalis* and *Candida krusei* are also frequently isolated (Papon *et al*., 2013). *C. parapsilosis* infections are shown to be particularly incident among newborns, while *C. glabrata* is a more common infectious agent among older patients (Zakikhany *et al*., 2008; Chow *et al*., 2012; Paramythiotou *et al*., 2014; Yapar, 2014).

Geography and patient's age are therefore main factors impacting on this *Candida* species distribution, but there is growing evidence suggesting a role of widespread use of antifungal agents, especially azoles, in this epidemiological alteration (Méan *et al*., 2008; Zakikhany *et al*., 2008; Delaloye and Calandra, 2014). As mentioned, incidence of infections is higher among infants younger than 1 year old and adults older than 70 years old and different incidence rates change depending on geographic region, although the reasons for those differences are not completely known (Yapar, 2014). In European countries, species distribution of *Candida* isolates differs as well from one country to another. In a Portuguese prospective study, it was shown that half the episodes of yeast infections are occurring in people over 65 years old attending a hospital in the centre of the country (Paulo *et al*., 2009). Another study, also published by a Portuguese group, aimed to observe fungaemia episodes in hospitalized people, over a year, showed as well predominance of cases in people over 65 (Costa-de-Oliveira *et al*., 2008). In line with the described to other countries, in both studies the most frequently isolated yeasts were *C. albicans*, followed by *C. parapsilosis* or *C. glabrata*.

Taking all this information into account, *Candida* opportunistic, disseminated infections are thus also associated to high healthcare costs, representing a major socioeconomic challenge for communities all over the world (Moyes and Naglik, 2011).

RISK FACTORS FOR *CANDIDA* INFECTIONS

Since *C. albicans* is an opportunistic pathogen obligately associated with warm-blooded animals, it will not cause infection unless the normal microbiota or the host antifungal defense responses has been perturbed (Brown *et al*., 2014; Delaloye and Calandra, 2014). Due to its natural localization on these niches, the skin and the urogenital and gastrointestinal tracts are thus main portals of entry for *Candida* infections. Accordingly, it is well established that colonization of skin and mucous membranes with *Candida* and alteration of natural host barriers due, for instance, to wounds, total parenteral nutrition, haemodialysis or chemotherapy, surgery (especially of the abdominal cavity) or insertion of indwelling intravascular and urinary catheters predispose to infections (Méan *et al*., 2008; Cheng *et al*., 2012; Delaloye and Calandra, 2014). While the application of central venous catheters, upon which *C. albicans* can form elaborate biofilms, allows direct access of the fungus to the bloodstream (Mayer *et al*., 2013; Brown *et al*., 2014), prolonged broad-spectrum antibiotic therapy normally used under this situations, enables fungal overgrowth (Méan *et al*., 2008; Mayer *et al*., 2013; Delaloye and Calandra, 2014). During the last three decades it was also identified that genetic variation plays an important role in host susceptibility to *Candida* infections (Smeekens *et al*., 2013). However, *C. albicans* is commonly isolated from individuals with compromised immune defenses, including diabetic and HIV/AIDS patients and premature babies (Mallick and Bennett, 2013; Brown *et al*., 2014), and is also associated to a set of different conditions, from prolonged hospitalization stays to elderly patients (Delaloye and Calandra, 2014). In fact, ageing progressively leads to profound and irreversible alterations in the human body structure and functional capacities. Besides the more perceptible features of this natural event, such as skin dryness and wrinkles, the immune system is obviously also affected. This gradual

impairment of immune functions, affecting both innate and adaptive immune responses in all cells and organs, is called immunosenescence. Among this aged-related alterations, progressive thymus involution (with changes in naïve, memory and effector T cells), comorbidities and chronic infections accumulated during life, will collaborate in the occurrence of a permanent pro-inflammatory state (inflamm-aging (Franceschi *et al*., 2000)), with chronic activation of nonspecific immune responses and reduction of immune system effectiveness. Therefore, anytime an infection occurs, elderly will have a reduced ability to eliminate pathogens, suffering from more severe symptoms, prolonged duration of disease and poorer prognosis. This decreased ability to control infections associated to a decreased response to vaccination are evidently main issues of concern (Aw *et al*., 2007; Pfister and Savino, 2008).

MANAGEMENT OF *CANDIDA* INFECTIONS

To decrease morbidity and mortality rates associated to *Candida* infections, various strategies must be adopted, in an attempt to save lives, time and money. As mentioned before, unless timely diagnostic and treatments are applied against it, invasive candidiasis can become, in most of the times, a fatal condition, particularly in immunocompromised patients. The ideal strategy should be the prevention of disease for example through vaccines administration (Cassone, 2008). Several approaches are being investigated in this field with animal models (Ibrahim *et al*., 2006; Lou *et al*., 2010; Fidel and Cutler, 2011), but until an effective approval and application in humans is achieved, treatment of *Candida* infections as quickly as possible should be mandatory. To accomplish this objective, the correct and rapid identification of the

Candida species and strains involved are determinant in the outcome of any infection and, the conventional methods used, should be complemented with advanced technologies (real-time PCR, DNA microarrays), allowing appropriate antifungal drugs prescription (Kabir and Ahmad, 2013). Four classes of antifungal drugs are presently available for the treatment of invasive fungal diseases, namely polyenes, azoles, echinocandins and pyrimidine analogues. Polyenes, like amphotericin B or nystatin are broad spectrum fungicidal drugs, binding to plasma membrane ergosterol. These drugs barely bind to host mammalian cholesterol, thereby affecting fungal cell membrane integrity and permeability, leading to pathogen death. However, since amphotericin B is often poorly tolerated and it has considerably toxic effects on human cells (*e.g.*, nephrotoxicity), new lipid formulations (liposome, lipid complexes and colloidal dispersions) have been developed to obtain toxicity reduction (Méan *et al*., 2008; Kabir and Ahmad, 2013; Paramythiotou *et al*., 2014). Azole drugs and it derivatives are fungistatic, having broad spectrum against yeast and filamentous fungi; these azoles, such as fluconazole, itraconazole or voriconazole, target ergosterol biosynthetic pathway, decreasing membrane ergosterol and inhibiting fungal growth. This alteration on plasma membrane also leads to inactivation of vacuolar ATPases or to hyphal development inhibition. Fluconazole is still one of the most commonly antifungal used, but non-*albicans Candida* spp. (like *C. krusei* or *C. glabrata*) natural resistance has been described worldwide, reinforcing the need to accurate species identification and antifungal susceptibility tests (Méan *et al*., 2008; Kabir and Ahmad, 2013; Paramythiotou *et al*., 2014). The echinocandins compounds, like caspofungin, are lipoproteins with fungicidal activity against *Candida*, inhibiting the synthesis of 1,3-β-glucan in the fungal cell wall. This class of antifungal agents is efficacious and safe, with a prominent role in the treatment of invasive candidiasis (Méan *et al*., 2008; Kabir

and Ahmad, 2013; Paramythiotou *et al*., 2014). Lastly, flucytosine is the main representative of pyrimidine analogues and it is its metabolite, 5-fluorouracil, that present toxicity for the fungal cell (Kabir and Ahmad, 2013; Paramythiotou *et al*., 2014).

Even if all this different antifungals are available to treat *Candida* infections, drug resistance, as pointed before, is becoming a reason of serious concern, both from the patients or health care institution view. High resistance levels against azoles are well known for *C. krusei*, for example, while *C. parapsilosis* or *Candida guilliermondii* stand out due to their decreased echinocandins susceptibility (Papon *et al*., 2013). In addition, it was recently also described the *in vivo* acquisition of *C. krusei* resistance against voriconazole, from isolates of leukemia patients with long term voriconazole therapies (Ricardo *et al*., 2014). Yeasts efflux pumps, draining drugs out from fungal cells, or genetic mutations of drugs targets are known to have primordial roles in the establishment of resistance, and, to counteract this phenomena, drug combination therapy has started to be tested in mice models and patients (Kabir and Ahmad, 2013; Delaloye and Calandra, 2014).

IMMUNITY RESPONSE TO *CANDIDA*

The mechanisms involved in host-*Candida* interactions, particularly those involved in fungal detection by cells of the host innate immune system, are important features remaining to be completely understood. Therefore, several host cells, with different characteristics and functions, have been intensely studied and are involved in host innate responses against *Candida* infections.

The mucosal epithelial cells are the first line of defense against *Candida* species, functioning as a physical barrier containing yeasts invasion of deeper tissues. Since epithelial cells express most of the TLRs (Toll-like receptors), they can recognize invading *Candida* spp. cells and trigger immune responses, recruiting other immune cells through cytokines and chemokines production.

Phagocytic cells are, in turn, the most efficient cells controlling and effectively clearing *Candida* infection. Professional phagocytes, like monocytes circulating in blood and macrophages in tissues, are responsible for the surveillance and killing invading pathogens; once these microorganisms are phagocyted, microbe-containing phagosomes fuse with lysosomes into phagolysosomes, where engulfed cells are killed by hydrolytic enzymes, antimicrobial peptides and reactive oxygen species (ROS). Lately, in addition to this direct killing, neutrophils were also shown to inhibit *Candida* growth by releasing neutrophil extracellular traps (NETs), containing the antifungal peptide calprotectin (Urban *et al*., 2006). Amongst phagocytes, polymorphonuclear neutrophils (PMNs) were, as well, shown to kill *C. albicans* and be able to inhibit germ tube formation, and dendritic cells (DCs) are able to ingest *Candida* cells once tissues are invaded. These professional antigen-presenting cells are able to distinguish yeast from hyphal forms, inducing T helper cell differentiation and linking innate and adaptive antifungal responses (Cheng *et al*., 2012).

Although the immune system has the ability to efficiently detect and recognize *Candida*, a fine balance between pro- and anti-inflammatory responses is absolutely required to maintain the equilibrium between host and fungi, avoiding pathologic consequences and maintaining homeostasis (Romani, 2011). Nevertheless, *C. albicans* can resist these defense mechanisms and remain viable inside these phagocytic cells.

CANDIDA HOST INVASION

✓ *CANDIDA* POLYMORPHISM

In the last decades, several efforts were being made in an attempt to elucidate the complex host-pathogen interactions. Due to its frequency and importance, most of the research and knowledge achieved in this field is related to *Candida albicans*. Clearly, *C. albicans* has therefore emerged as a model yeast, making of this organism one of the best studied pathogens.

C. albicans is a polymorphic organism that can be found in several forms, from yeasts (blastospores) to pseudohyphae, true hyphae or chlamydospores (Gow, 2013; Modrzewska *et al*., 2013). Therefore, this fungus can, for instance, grow and switch between unicellular budding yeast cells which are round, ovoid-shaped; pseudohyphae, which are chains of elongated ellipsoid cells with constrictions at the septa of adjacent cells; true hyphae, consisting of long tubes without constrictions or chlamydospores, which are thick-walled spore-like structures (Huang, 2012; Mayer *et al*., 2013). *C. albicans* can also undergo an understudied phenotypic switching system, mentioned as white-opaque transition. This switch from the white to the opaque state is a mating prerequisite in *C. albicans*. Both white and opaque forms are budding cells, but opaque cells, with unique pimples on its surface, share some common features with hyphae and are the only mating competent form (Huang, 2012; Mallick and Bennett, 2013). Recently, it was also described that *C. albicans* can enter another specific state designated by GUT (gastrointestinally induced transition), with cells resembling opaque ones, but missing surface pimples and not responding to mating factors. This phenotype allows the fungus to survive during long periods of time in the large intestine of a

healthy host: virulence genes are downregulated, allowing *C. albicans* to remain there in a regulated commensal state (Gow, 2013; Pande *et al*., 2013). Moreover, it was also been described that, in addition to this transition phenotypes, *C. albicans* CUG mistranslation (due to serine/leucine exchange) alter cell surface, increase adherence to host substrates and change β-glucans exposure, reducing consequent phagocytosis by macrophages. CUG mistranslation can therefore be pointed as another mechanism involved in yeasts-host interactions (Miranda *et al*., 2013).

Although with distinct functions, while yeast and true hyphae are regularly observed during infection, the *in vivo* biological significance of pseudohyphae, chlamydospores or white-opaque transition remains to be completely elucidated (Huang, 2012; Mayer, 2013). However, the genes or pathways involved in the described phenotypic transitions are often also required for virulence, indicating an important link between morpho- and pathogenesis in *C. albicans* and allowing it to rapidly adapt to host different environments (Huang, 2012).

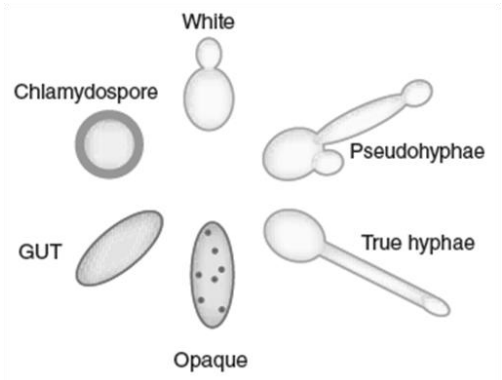

Figure 1. *Candida albicans* morphologies.

Pseudohyphae and true hyphae are important to tissue penetration and invasion, while white-phase yeast cells have important roles in establishing and disseminating *Candida* infections. Elongated opaque-phase cells are competent for mating, whereas an *in vivo* role for chlamydospores remains unknown. GUT cells resemble opaque-phase cells and are adapted for commensal growth. (From Gow, 2013.)

Among all this forms of growth, the ability to switch between yeast and filamentous forms is one of the most important characteristic in virulence and infection conditions, although both are indispensable for it pathogenic features (Huang, 2012; Modrzewska *et al*., 2013). Filamentous forms are more invasive and better at tissue penetration, while yeast forms are easier to disseminate in the bloodstream, but both forms are found in infected tissues (Huang, 2012).

Besides its role as a model pathogen, several differences can be found between *C. albicans* and other related yeasts. For example, unlike diploid *C. albicans*, *C. glabrata* is strictly haploid and usually only grows in the yeast form, with virulence independent of morphology (Brunke and Hube, 2013). In a similar manner, *C. parapsilosis* is also not able to grow true hyphae and exists in either yeast or pseudohyphal forms (Trofa *et al*., 2008). Naturally, this distinction will be reflected in species-specific pathogenic behaviors and host responses.

- ✓ ***CANDIDA* RECOGNITION**

Once in contact with host cells, invading pathogens stimulate host innate immunity to distinguish self from nonself, initiating a complex cascade of extracellular and intracellular responses. At the end, it is expected that innate antimicrobial and inflammatory responses and adaptive immunity will be able to control or eliminate infection.

Host pattern recognition receptors (PRRs) recognize relatively constant microbial structures referred as pathogen associated molecular patterns (PAMPs), which are common to related groups of microorganisms. Therefore, together with the recognition of specific antigens by T-cells in the adaptive immune response, PAMPs

recognition by PRRs are part of the innate defenses against fungal infections (Hajishengallis and Cambris, 2011; Moyes and Naglik, 2011; Cheng *et al*., 2012). At the same time, molecules implicated in host organelle or cellular damages, like purines and nucleic acids, are recognized by immune cells through danger associated molecular receptors (DAMPs). The concertized monitorization of both PAMPs and DAMPs, allows the immune cells to balance the responses against excessive host damages and pathogen control (Romani, 2011).

Different PRRs, expressed in different locations and cell types, usually recognize distinct PAMPs and this specificity, together with the ability of PRRs cooperation forming multi-receptor complexes, should theoretically allow host to detect nearly any type of infection, discriminating between different pathogens. Those PRRs are included in four main families, namely, Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-like receptors (RLRs) (Hajishengallis and Cambris, 2011; Moyes and Naglik, 2011). The *Candida* cell wall structure is composed of chitin, β-glucans and mannoproteins and these polysaccharides are mainly recognized by two classes of membrane bound PRRs: TLRs and CLRs. As an example, among TLRs, TLR2 and TLR4 respectively recognize phospholipomannan and O-linked mannan, while β-glucans and N-linked mannan are correspondently recognized by CLRs like dectin-1 and macrophage mannose receptor. In addition to PAMPs recognition by membrane bound receptors, other PRRs were shown to recognize *Candida* intracellularly, with for instance TLR9, recruited to phagosomes, recognizing *C. albicans* DNA. Another example of these intracellular PAMPs recognition is the NLRs receptors like NLRP3 (NLR family pyrin domain containing 3), which are able to activate inflammasome caspase-1, with an important anti-*Candida* role (Hajishengallis and Cambris, 2011; Cheng *et al*., 2012; Williams *et al*., 2013).

Moreover, an important example of a multi-receptor PRR complex is the cooperation between TLR2 and dectin-1, stimulating anti-fungal immunity. Each receptor can act independently, with TLR2 activation inducing cytokine production, while dectin-1 induces phagocytosis, but together they produce a powerful synergistic response (Dennehy *et al*., 2008; Ferweda *et al*., 2008; Hajishengallis and Cambris, 2011; Moyes and Naglik, 2011). In addition to these main receptors described, dectin-2, mincle, DC-SIGN, and galectin-3 have also been identified, although the role of these receptors is currently not fully established (Netea *et al*., 2006; Moyes and Naglik, 2011).

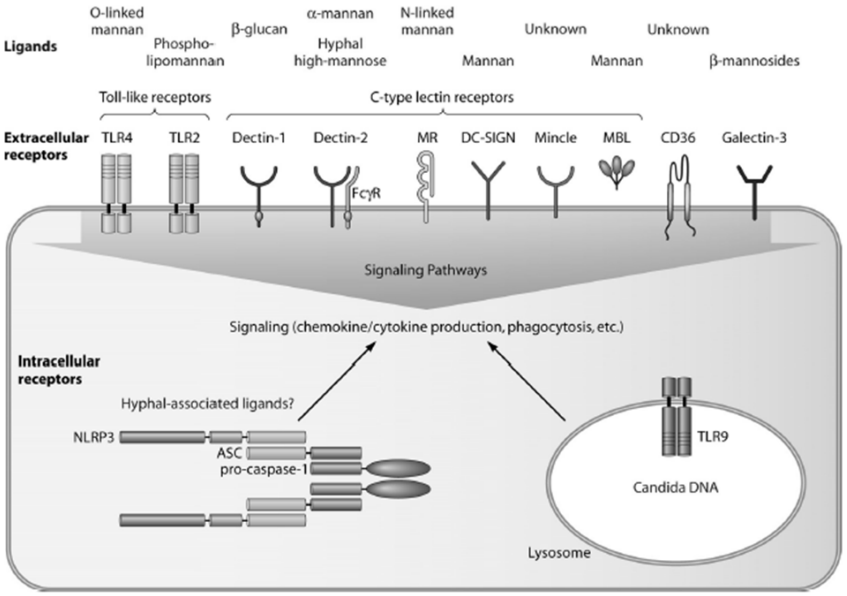

Figure 2. Pattern recognition receptors (PRRs) and their corresponding *Candida* PAMPs.

Candida cell wall components are mainly recognized extracellularly by Toll-like and C-type lectin receptors on host cell surface and lead to different downstream signaling, such as chemokine/cytokine production and phagocytosis. Once *Candida* is internalized/phagocytosed, the fungal PAMPs can further activate TLR9 or NLRP3 inflammasome activation. (From Cheng *et al*., 2012.)

✓ COLONIZATION *VERSUS* INVASION

Exactly how the host discriminates between commensal and pathogenic states of opportunistic yeasts is still not fully understood and deeper knowledge on these mechanisms will make possible the manipulation of host immunity to control infections.

Through β-glucans exposure, hyphal formation was identified as the main event triggering the activation of inflammasome and secretion of IL-1β in murine macrophages. Because IL-1β is a requisite for protective Th17 differentiation and response, this hyphae recognition might be essential in macrophages discrimination between *Candida* colonization and invasion (Joly *et al*., 2009; Cheng *et al*., 2012). Besides Th17, inflammasome activation is also considered determinant for the switch between colonization and invasion at mucosal surfaces (Gow *et al*., 2011). As previously described, *C. albicans* yeast to hyphae transition was demonstrated to be essential for host tissue invasion and phagocytosis evasion. Nevertheless, since this switch fuel an all set of host defense responses, it also leave *Candida* in a risky position to be eminently recognized by the host. Even if, in the past decades, much has been learned about the *in vitro* and *in vivo* mechanisms of host-*Candida* interplay, this interaction is, in no doubt, a lot more complex in its real host context, where several important questions remain to be answered (Cheng *et al*., 2012).

FROM COMMENSAL TO PATHOGEN: *CANDIDA* EVASION FROM HOST DEFENSES

The *C. albicans* ability to present as a commensal and as a life-threatening pathogen, infecting various host niches, is mainly due to its capability to sense and react

to the environment and is sustained by a wide range of virulence factors and fitness attributes (d'Enfert, 2009; Huang, 2012; Mallick and Bennett, 2013; Mayer *et al*., 2013). In response to the emergence of non-*albicans Candida* spp. as agents of human infection, several researches were initiated to investigate differences between host adaptation of *Candida* species. It is of utmost importance to keep in mind that since each of those yeasts exhibits specific traits (from ploidy to morphology, *e.g.*), survival in their specific hosts and natural niches will be in, some cases, markedly different between different species (Papon *et al*., 2013).

The morphological transition between yeast and hyphal forms is considered one of the most important *C. albicans* virulence factors. Some *in vivo* studies have revealed that both hypha- and yeast-locked mutants are avirulent or less virulent than wild-type strains, meaning that the switch between both forms accounts for *Candida* full virulence. While hyphae might be the invasive form, perforating phagocytes and invading the epithelium barrier, the yeast form is also needed for free dissemination in systemic infections (Cheng *et al*., 2012). Serum is taken as one of the most powerful inducers of this filamentous growth. In fact, it was discovered that bacterial peptidoglycans (from the commensal bacteria in the gastrointestinal tract (GI tract)) in the host serum are the major compounds triggering this switch. In addition to peptidoglycans, N-acetylglucosamine (a component of the GI tract mucus and bacterial cell wall) and CO_2 (a product of cellular respiration, with much higher levels in blood than in the atmosphere) are other factors strongly contributing to the yeast-hyphae transition. Host temperature (37°C), neutral pH, nutrient limitation/ starvation and low O_2 levels are also among the factors involved in *C. albicans* morphological changes. Yeasts can colonize distinct host niches where, for example, different pH levels are found: at low pH (< 6, like in vaginal tract) yeast forms of *C. albicans* are

predominantly found, while at a high pH (aprox. ≥ 7, like in some GI tract portions) hyphal growth is induced (Huang, 2012; Mayer *et al*., 2013). In addition to this ability to sense and adapt to the environmental pH, *C. albicans* is also able to modulate extracellular pH, alkalinizing its surroundings and thus autoinducing hypha formation (Mayer *et al*., 2013).

The yeasts cells ability to generate biofilms on host surfaces is another key feature to promote infection. Briefly, a biofilm formation includes adherence of yeast cells to the substrate, cell proliferation with formation of hyphae at the upper biofilm portion, accumulation of extracellular matrix material and, at last, dispersion of these cells into the host. Biofilms are thus microbial communities frequently attached to a living or non-living solid surface (intravascular and urinary catheters, *e.g.*), with cells generating and embedding in extracellular polymeric substances (EPS). These EPS helps both limiting the diffusion and access of several substances, and restricting phagocytotic cells into the biofilm, conferring a protective environment to yeasts degradation and penetration of host tissue structures. Hence, mature biofilms were shown to be more resistant to antimicrobial agents and host immune defenses (Mayer *et al*., 2013; Williams *et al*., 2013).

The first steps for this *Candida* persistence within the host are consequently determined for its adherence abilities, mainly mediated by adhesins. This specialized set of proteins, like agglutinin-like sequence Als3, interact and mediate adherence to other yeasts cells, microorganisms, abiotic surfaces and host cells (Mayer *et al*., 2013; Williams *et al*., 2013). *C. parapsilosis,* for instance, can also promote adhesion to host cells through ectophosphatase activity on its surface, suggesting that these enzymes may also contribute to fungal pathogenesis (Kiffer-Moreira *et al*., 2007). Once adherence has been established, degradation and penetration of host structures are decisive to yeasts

cells colonization and growth within the host. In order to get this, *C. albicans* can, for instance, produce hydrolytic enzymes, namely secreted aspartyl proteinases, with wide substrate activity, to damage and invade host cell structures (Williams *et al*., 2013). Evidently, through all this process, host is persistently trying to fight back, urging *Candida* to develop several mechanisms to "fool" those host defenses. In order to escape from phagocytosis, for example, *Candida* developed the ability to shield its surface, using outer cell wall components to protect PAMPs (like β-glucan) from recognition by PRRs (like dectin-1) (Cheng *et al*., 2012). One of the most important cell wall components with a strong immune homeostasis function is chitin (Wagener *et al*., 2014). However, even if yeasts like *C. albicans* have been actually engulfed by phagocytes, they are still capable of preventing phagolysosomes formation and maturation, modulating intracellular membrane trafficking, and escaping to host clearance. In addition, once inside a phagocytic cell, *Candida* species (such as *C. albicans* or *C. glabrata*) can inhibit ROS production, using catalase and superoxide dismutase to counteract the respiratory burst (Tavanti *et al*., 2006; Seider *et al*., 2010; Cheng *et al*., 2012).

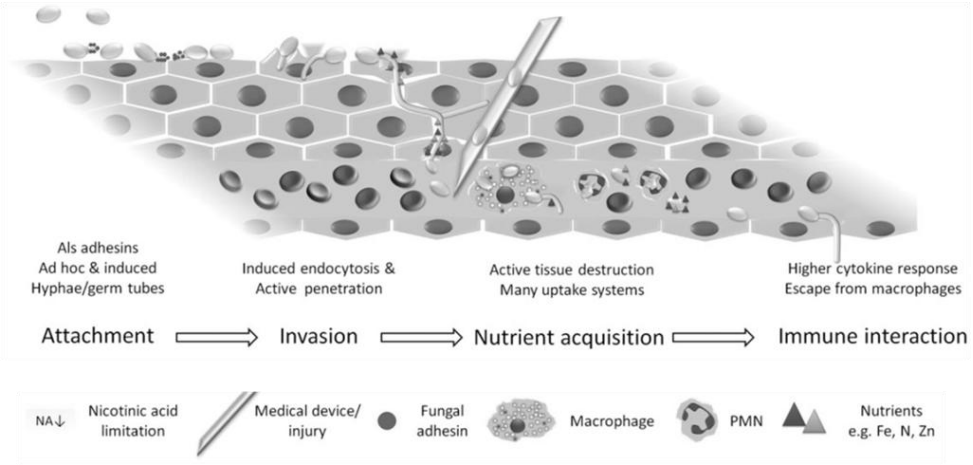

Figure 3. Infection strategies of *C. albicans.*

Under some circumstances, *C. albicans* actively invades epithelia, forms hyphae and aggressively destroys tissue, eliciting a strong immune response. Macro- and micronutrients from damaged host tissue are taken up by a broad

range of acquisition systems and macrophages may be killed by hyphae formation. (Adapted from Brunke and Hube, 2013.)

C. albicans is able to colonize several niches in the human body, from skin to GI tract, with consequent predictable interaction with other microorganisms. The contact between yeasts and the normal host microbiota can impact survival and pathogenesis of both communities: by physically blocking the available niches and through the production of several signaling molecules, *Lactobacillus* sp., *Enterococcus faecalis* and other GI tract bacteria can control *C. albicans* colonization (Garsin and Lorenz, 2013; Zaborin *et al*., 2014). Taking all this into account, it is not unexpected that the use of broad-spectrum antibiotics is many times associated to *C. albicans* infections and to the use of probiotics, in an attempt to repopulate the lost normal flora (Mallick and Bennett, 2013).

ROLE OF ADENOSINE RECEPTORS IN INFLAMMATION DUE TO INFECTION

The immune cells normally recruited to destroy pathogens, might also cause collateral damages to normal tissues, inducing exacerbate pro-inflammatory states. It has been shown that sepsis can initiate a sequence of inflammatory events that can actually activate the immune system and injure host tissues. Microbial factors, such as Gram negative bacteria LPS, origin host release of chemokines, pro- (e.g., tumor necrosis factor TNF–α, IL-1β) and anti-inflammatory cytokines (e.g., IL-10), reactive oxygen species and nitric oxide (Hogan *et al*., 2001; Sullivan *et al*., 2004; Németh *et al*., 2006). However, low incidence of post-inflammatory complications are many times observed, indicating that a ‘nonimmune danger-sensing’ mechanism might be

contributing to an effective protection of healthy tissues and cells. In line with this, the adenosine purine nucleoside is produced in conditions of metabolic stress and cell damage, with elevated extracellular levels associated to ischemia, hypoxia, trauma and inflammation (Haskó, 2008; Haskó and Pacher, 2008). In fact, the purinergic signalling system employs extracellular pyrimidines and purines (namely ATP and adenosine), released from living and dying cells, as signalling molecules of cell injury (Sitkovsky, 2004; Sitkovsky and Ohta, 2005; Di Virgilio, 2007; Fredholm, 2007; Burnstock and Verkhrasky, 2009; Kumar and Sharma, 2009). High extracellular adenosine levels are obtained through release of both intracellular adenosine and precursor adenine nucleotides from cells (mostly ATP). This ATP and other nucleotides are rapidly degraded to adenosine by a cascade of enzymes, called ectonucleotidases, including CD39 (nucleoside triphosphate diphosphorylase) and CD73 (ecto-5'-nucleotidase) (Robson *et al*., 2006; Haskó, 2008). Adenosine can induce several physiological responses by binding and activating four cell surface transmembrane adenosine receptors, designated as A_1, A_{2A}, A_{2B} and A_3. These receptors are usually coupled to intracellular GTP-binding proteins (G proteins): A_1 and A_3 receptors couple to Gi proteins, with an inhibitory effect, whereas A_{2A} and A_{2B} couple to Gs proteins and stimulate adenylyl cyclase and cyclic AMP (cAMP) production (Ohta and Sitkovsky, 2001; Haskó, 2008; Haskó and Pacher, 2008; Burnstock and Verkhratsky, 2009; Hajishengallis and Cambris, 2011). Nevertheless, only A_{2A} and A_{2B} receptors have been actually described as having a role in infection. A_{2B} receptors ($A_{2B}R$) may stand as a new therapeutic approach to control bacterial diseases. It was shown that $A_{2B}R$ activation can be useful to decrease and regulate sepsis inflammatory responses (Csóka *et al*., 2010) or to protect against helminth infections (Patel *et al*., 2014), whereas its inhibition might be valuable, for instance, preventing *Chlamydia trachomatis* (Pettengill

et al., 2009) or *Clostridium difficile* intoxication and infection (Warren *et al*., 2012). In turn, research on selective adenosine receptor ligands and mice without the A_{2A} receptors ($A_{2A}R$) genes (Adora2a) have shown that $A_{2A}R$ activation protect tissues, through an anti-inflammatory action . Adenosine activation of $A_{2A}R$ alters host release of cytokines and inhibits LPS-induced inflammation on many different cell types. As a consequence, interest has been deposit in the anti-inflammatory effect of $A_{2A}R$ agonists, since it can be particularly important in the treatment of bacterial diseases. As previously mentioned, antibiotics used in those situations kill large numbers of bacteria and can increase inflammation through rupture and dissemination of bacterial cell wall products into the host (Sullivan *et al*., 2004; Köröskényi *et al*., 2011).

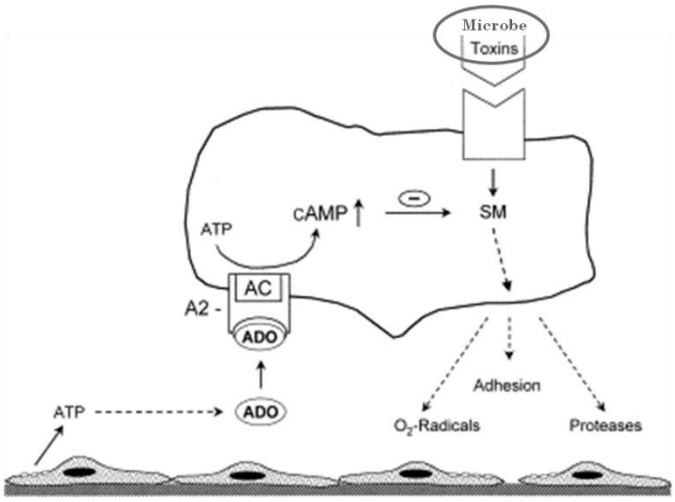

Figure 4. Model of adenosine cell protection.

Following phagocytes activation by pathogens, oxygen radical- and protease-mediated disruption of cell membrane results in adenine nucleotides release and released nucleotides (ATP, ADP, AMP) are degraded to adenosine by ectonucleotidases. Locally produced adenosine may neutralize additional cell injury, interacting with A_{2A} receptors and inhibiting exacerbated inflammatory reactions of these cells. [AC, adenylyl cyclase; SM, second messengers] (Adapted from Thiel *et al*., 2003.)

Interestingly, it has already been shown that *Staphylococcus aureus* can utilize adenosine anti-inflammatory effects, escaping phagocytes clearance, via cell wall

expression of adenosine synthase A (AdsA), which converts adenosine monophosphate into adenosine (Hajishengallis and Cambris, 2011). Additionally, another study showed that inhibition of adenosine deaminase displays an important role in the reduction of tissue injury, neutrophil infiltration and pro-inflammatory cytokine levels in a mice model of *C. difficile*-associated disease (Junqueira *et al*., 2011).

REFERENCES

Aw D, Silva AB and Palmer DB. (2007) Immunosenescence: emerging challenges for an ageing population. Immunology 120:435-446.

Brown AJP, Brown GD, Netea MG and Gow NAR. (2014) Metabolism impacts upon *Candida* immunogenicity and pathogenicity at multiple levels. Trends Immunol. 22(11): 614-622.

Brown AJP, Budge S, Kaloriti D, Tillmann A, Jacobsen MD, Yin Z, Ene IV, Bohovych I, Sandai D, Kastora S, Potrykus J, Ballou ER, Childers DS, Shahana S and Leach MD. (2014) Stress adaptation in a pathogenic fungus. J Exp Biol. 217: 144-155.

Brunke S and Hube B. (2013) Two unlike cousins: *Candida albicans* and *C. glabrata* infection strategies. Cell Microbiol. 15(5): 701–708.

Burnstock G and Verkhratsky A. (2009) Evolutionary origins of the purinergic signalling system. Acta Physiol. 195:415–447.

Calderone RA Ed., *Candida* and Candidiasis: ASM Press, Washington, DC, 2002.

Calderone RA, Clancy CJ. *Candida* and Candidiasis: ASM Press, Washington, DC, 2012.

Cassone A. (2008) Fungal vaccines: real progress from real challenges. Lancet Infect Dis. 8(2): 114-124.

Cheng SC, Joosten LAB, Kullberg BJ and Netea MG. (2012) Interplay between *Candida albicans* and the mammalian innate host defense. Infect Immun. 80(4): 1304-1313.

Chow BDW, Linden JR and Bliss JM. (2012) *Candida parapsilosis* and the neonate: epidemiology, virulence and host defense in a unique patient setting. Expert Rev Anti Infect Ther. 10(8): 935–946.

Costa-de-Oliveira S, Pina-Vaz C, Mendonça D and Gonçalves-Rodrigues A (2008) A first Portuguese epidemiological survey of fungaemia in a university hospital. Eur J Clin Microbiol Infect Dis. 24:23-30.

d'Enfert, C. (2009) Hidden killers: persistence of opportunistic fungal pathogens in the human host. Curr. Opin. Microbiol. *12*, 358–364.

Delaloye J and Calandra T. (2014) Invasive candidiasis as a cause of sepsis in the critically ill patient. Virulence. 5(1):161–169.

Dennehy KM, Ferwerda G, Faro-Trindade I, Pyz E, Willment JA, Taylor PR, Kerrigan A, Tsoni1 SV, Gordon S, Meyer-Wentrup F, Adema GJ, Kullberg BJ, Schweighoffer E, Tybulewicz V, Mora-Montes HM, Gow NAR, WilliamsDL, Netea MG and Brown GD. (2008) Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. Eur. J. Immunol. 38: 500–506.

Di Virgilio, F. (2007). Purinergic signalling in the immune system. A brief update. Purinergic Signal. *3*,1–3.

Falagas ME, Roussos N and Vardakas KZ. (2010) Relative frequency of albicans and the various non-albicans *Candida* spp among candidemia isolates from inpatients in various parts of the world: a systematic review. Int J Infect Dis. 14:e954–e966.

Ferwerda G, Meyer-Wentrup F, Kullberg BJ, Netea MG and Adema GJ. (2008) Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. Cell Microbiol. 10(10):2058-66.

Fidel PL Jr. and Cutler JE. (2011). Prospects for development of a vaccine to prevent and control vaginal candidiasis. Curr Infect Dis Rep. 13(1): 102-107.

Franceschi C, Bonafe M, Valensin S, Olivieri F, De Luca M, Ottaviani E and De Benedictis G. (2000) Inflammaging. An evolutionary perspective on immunosenescence. Ann N Y Acad Sci. 908:244–54.

Fredholm BB. (2007) Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ. *14*, 1315–1323.

Garsin DA and Lorenz MC (2013) *Candida albicans* and *Enterococcus faecalis* in the gut. Infect Immun. 81:189-200.

Gow N. (2013) A developmental program for *Candida* commensalism. Nat Genet. 45(9): 967-968.

Gow NAR, van de Veerdonk FL, Brown AJP and Netea MG. (2011) *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. Nat Rev Microbiol. 10(2):112-122.

Hajishengallis G and Lambris JD. (2011) Microbial manipulation of receptor crosstalk in innate immunity. Nat Rev Immunol. 11(3): 187–200.

Haskó G and Pacher P. (2008) A2A receptors in inflammation and injury: lessons learned from transgenic animals. J Leukoc Biol. 83: 447-455.

Haskó G, Linden J, Cronstein B and Pacher P. (2008). Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat. Rev. Drug Discov. *7*, 759-770.

Hogan CJ, Fang GD, Scheld WM, Linden J and Diduch DR. (2001) Inhibiting the inflammatory response in joint sepsis. Arthroscopy. 17:311–5.

Huang G. (2012) Regulation of phenotypic transitions in the fungal pathogen *Candida albicans*. Virulence 3(3):251–261.

Ibrahim AS, Spellberg BJ, et al. (2006). The anti-*Candida* vaccine based on the recombinant N-terminal domain of Als1p is broadly active against disseminated candidiasis. Infect Immun. 74(5): 3039-3041.

Joly S, et al. (2009) Cutting edge: *Candida albicans* hyphae formation triggers activation of the Nlrp3 inflammasome. J. Immunol. 183:3578–3581.

Junqueira AFTA, Dias AAM, Vale ML, Spilborghs GMGT, Bossa AS, Lima BB, Carvalho AF, Guerrant RL, Ribeiro RA and Brito GA. (2011) Adenosine

deaminase inhibition prevents Clostridium difficile toxin A-induced enteritis in mice. Infect Immun. 79(2):653-662.

Kabir MA and Ahmad Z. (2013) *Candida* infections and their prevention. ISRN Prev Med. doi: 10.5402/2013/763628.

Kiffer-Moreira T, de Sa Pinheiro AA, Alviano WS, Barbosa FM, Souto-Padron T, , Nimrichter L, Rodrigues ML, Alviano CS and Meyer-Fernandes JR. (2007) An ectophosphatase activity in *Candida parapsilosis* influences the interaction of fungi with epithelial cells. FEMS Yeast Res. **7**: 621-628.

Köröskényi K, Duró E, Pallai A, Sarang Z, Kloor D, Ucker DS, Beceiro S, Castrillo A, Chawla A, Ledent CA, Fésüs L and Szondy Z. (2011) Involvement of adenosine A2A receptors in engulfment-dependent apoptotic cell suppression of inflammation. J Immunol. 186:7144-7155.

Kumar V and Sharma A. (2009). Adenosine: an endogenous modulator of innate immune system with therapeutic potential. Eur. J. Pharmacol. *616*, 7-15.

Lou G, Ibrahim AS, et al. (2010). *Candida albicans* Hyr1p confers resistance to neutrophil killing and is a potential vaccine target. J Infect Dis. 201(11): 1718-1728.

Mallick EM and Bennett RJ. (2013) Sensing of the microbial neighborhood by *Candida albicans*. PLoS Pathog. 9(10): e1003661.

Mayer FL, Wilson D and Hube B. (2013) *Candida albicans* pathogenicity mechanisms. Virulence 4(2):119–128.

Méan M, Marchetti O and Calandra T. (2008) Bench-to-bedside review: *Candida* infections in the intensive care unit. Critical Care. 12:204 doi:10.1186/cc6212.

Miranda I, Silva-Dias A, Rocha R, Teixeira-Santos R, Coelho C, Gonçalves T, Santos MAS, Pina-Vaz C, Solis NV, Filler SG and Rodrigues AG. (2013) *Candida albicans* CUG mistranslation is a mechanism to create cell surface variation. mBio. 4(4):e00285-13.

Moyes DL and Naglik JR. (2011) Mucosal immunity and *Candida albicans* infection. Clin Dev Immunol. doi:10.1155/2011/346307.

Németh ZH, Csóka B, Wilmanski J, Xu D, Lu Q, Ledent C, Deitch EA, Pacher P, Spolarics Z and Haskó G. (2006) Adenosine A_{2A} receptor inactivation increases survival in polymicrobial sepsis. J. Immunol. *176,* 5616-5626.

Netea MG et al. (2006) Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. J. Clin. Invest. 116:1642–1650.

Ohta A and Sitkovsky M. (2001) Role of G-protein–coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. Nature. 414:916–20.

Pande K et al. (2013) Passage through the mammalian gut triggers a phenotypic switch that promotes *Candida albicans* commensalism. Nat Genet. 45:1088-1091.

Papon N, Courdavault V, Clastre M and Bennett RJ (2013) Emerging and emerged pathogenic *Candida* species: Beyond the *Candida albicans* paradigm. PLoS Pathog. 9(9): e1003550.

Paramythiotou E, Frantzeskaki , Flevari A, Armaganidis A and Dimopoulos G. (2014) Invasive fungal infections in the ICU: How to approach, how to treat. Molecules. 19:1085-1119.

Patel N, Wu W, Mishra PK, Chen F, Millman A, Csóka B, Koscsó B, Eltzschig HK, Haskó G and Gause WC. (2014) A2B adenosine receptor induces protective antihelminth type 2 immune responses. Cell Host Microbe. 15(3):339-50.

Paulo C, Mourão C, Veiga PM, Marques JM, Rocha G, Alves AF, Querol A, Meliço-Silvestre AA, Gonçalves I, Flores, Clemente C and Gonçalves T (2009) Retrospective analysis of clinical yeast isolates in a hospital in the centre of Portugal: spectrum and revision of the identification procedures. Med Micol. 47(8):836-844.

Pfaller MA, and Diekema DJ. (2007). Epidemiology of invasive candidiasis: a persistent public health problem. Clinic. Microbiol. Rev. *20,* 133–163.

Pfister and Savino (2008) Can the immune system still be efficient in the elderly? An immunological and immunoendocrine therapeutic perspective. Neuroimmunomodulation. 15:351-364.

Ricardo E, Miranda IM, Faria-Ramos I, Silva RM, Rodriges AG and Pina-Vaz C. (2014) *In vivo* and *in vitro* acquisition of resistance to voriconazole by *Candida krusei*. Antimicrob Agents Chemother. 58(8):4604-4611.

Robson SC, Sévigny J and Zimmermann H. (2006) The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal. 2(2):409-30.

Romani L. (2011) Immunity to fungal infections. Nat. Rev. Immunol. 11, 275-288.

Seider K, Heyken A, Lüttich A, Miramón P and Hube B. (2010) Interaction of pathogenic yeasts with phagocytes: survival, persistence and escape. Curr Opin Microbiol. 13, 392-400.

Sitkovsky M, Lukashev D, Apasov S, Kojima H, Koshiba M, Caldwell C, Ohta A and Thiel M. (2004) Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors. Annu. Rev. Immunol. 22, 657–682.

Sitkovsky MV and Ohta A. (2005) The 'danger' sensors that STOP the immune response: the A2 adenosine receptors? Trends Immunol. 26: 299-304.

Smeekens SP, van de Veerdonk FL, Kullberg BJ and Netea MG. (2013) Genetic susceptibility to *Candida* infections. EMBO Mol Med. 5(6):805-813.

Sullivan GW, Fang G, Linden J and Scheld WM. (2004) A_{2A} adenosine receptor activation improves survival in mouse models of endotoxemia and sepsis. J. Infect. Dis. 189:1897-1904.

Tavanti A, Campa D, Bertozzi A, Pardini G, Naglik JR, Barale R and Senesi S. (2006) *Candida albicans* isolates with different genomic backgrounds display a differential response to macrophage infection. Microbes Infect. 8:791e800.

Thiel M, Caldwell CC and Sitkovsky MV (2003) The critical role of adenosine A2A receptors in downregulation of inflammation and immunity in the pathogenesis of infectious diseases. Microbes Infect. 5:515-526.

Trofa D, Gacser A and Nosanchuk JD (2008) Candida parapsilosis, an emerging fungal pathogen. Clin. Microbiol.Rev. 21: 606–625.

Urban CF, Reichard U, Brinkmannand V and Zychlinsky A. (2006) Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. Cell Microbiol. 8(4): 668–676.

Wagener J, Malireddi RK, Lenardon MD, Köberle M, Vautier S, MacCallum DM, Biedermann T, Schaller M, Netea MG, Kanneganti TD, Brown GD,Brown AJ and Gow NA. (2014) Fungal chitin dampens inflammation through IL-10 induction mediated by NOD2 and TLR9 activation. PLoS Pathog. 10(4):e1004050.

Warren CA, Li Y, Calabrese GM, Freire RS, Zaja-Milatovic S, van Opstal E, Figler RA, Linden J and Guerranta RL. (2012) Contribution of Adenosine A2B Receptors in *Clostridium difficile* intoxication and infection. Infect Immun. 80(12): 4463–4473.

Williams DW, Jordan RPC, Wei XQ, Alves CT, Wise MP, Wilson MJ and Lewis MAO (2013) Interactions of *Candida albicans* with host epithelial surfaces. J Oral Microbiol. 5: 22434.

Yapar N (2014) Epidemiology and risk factors for invasive candidiasis. Ther Clin Risk Manag. 10:95–105.

Zaborin A, Smith D, Garfield K, Quensen J, Shakhsheer B, Kade M, Tirrell M, Tiedje J, Gilbert JA, Zaborina O and Alverdy JC. (2014) Membership and behavior of ultra-low diversity pathogen communities present in the gut of humans during prolonged critical illness. mBio 5(5):e01361-14.

Zakikhany K, Thewes S, Wilson D, Martin R, Albrecht A and Hube B. (2008) From attachment to invasion: Infection associated genes of *Candida albicans*. J Med Mycol. 49:245-251.

CHAPTER II

AIMS AND OUTLINE OF THE THESIS

AIMS OF THE STUDY

Candida spp., in particular *C. albicans*, can persist in the host with minimal damages, in a state of equilibrium between the yeast growth, its virulence phenotype and the immune surveillance mechanisms of the host. However, this also constitutes the natural reservoirs of the agent and, when the homeostasis is disturbed, namely during the aging process of immunosenescence, *C. albicans* passive colonization can turn into an aggressive infection. The switch between colonization and infection is characterized by a series of changes in the yeast biology that ultimately contributes for its ability to evade the defenses mechanisms of the host. On the other hand, since $A_{2A}R$ activation and consequent anti-inflammatory actions can indeed be protective and reduce tissue destruction, modulation of the purinergic system will be an excellent target to efficiently overcome an infection condition and it can be questioned and further investigated if adenosine and its sensing devices can actually be involved in the passive persistence and in the infection by *Candida* spp.

Therefore, the main objective of this dissertation was to determine, *in vitro* and *in vivo*, using a mouse model, if adenosine and adenosine A_{2A} receptors impact in the ability of *C. albicans* to be a facultative intracellular organism, resisting the noxious environment of the phagosome, and in its potential to opportunistically infect and cause disease in the more susceptible aged animal host.

In order to elucidate this possibility, the following specific aims were thus explored:

- determination of *C. albicans* phagocytosis rate, internalization and macrophage activation, under adenosine $A_{2A}R$ blockade or activation;

- quantification of extracellular ATP levels, a danger signal, associated with pro-inflammatory processes, and the correspondent ectonucleotidase activity upon yeast infection;

- assessement of the relative expression of the gene coding for adenosine A_{2A} receptors, *Adora2a* gene, in macrophages infected with yeasts;

- sub-cellular localization of adenosine A_{2A} receptors during macrophage infection by *Candida* spp.;

- relative gastrointestinal tract over-colonization/infection in aged, adult and young mice by *C. albicans*: gastrointestinal A_{2A}R mapping and modification upon *C. albicans* infection.

OUTLINE OF THE THESIS

The present dissertation is divided in eight chapters and results are shown as data compiled in different submitted or under preparation manuscripts.

CHAPTER I give respect to the introductory aspects of the thesis, reviewing the most important and relevant achievements on the field and allowing a global comprehension on the origins of the work presented.

CHAPTER II corresponds to the description of the main objectives of the work and to the description of the thesis structure, in an attempt to facilitate reading and understanding of the sequence of data presented.

CHAPTER III describes for the first time the undeniable $A_{2A}R$ involvement in yeast-macrophage interaction. We report a novel property of *C. albicans* to manipulate the macrophage response through purinergic signalling, damping the increase in extracellular ATP, a danger signal, downloading macrophage activation; not up-regulating adenosine A_{2A} receptors ($A_{2A}R$) and re-locating $A_{2A}R$ to the intracellular phagosomal membrane, allowing the silent internalization of yeasts without affecting their viability. This work is under submission to mBio Journal.

CHAPTER IV scrutinizes *C. albicans* ectophosphatase and ectonucleotidase activities, in order to elucidate mechanisms or pathways by which *C. albicans* could modulate the extracellular ATP clearance (a pro-inflammatory signal) and the adenosine availability (a stop signal of inflammation). This chapter is published in the journal FEMS Microbiology Letters.

CHAPTER V explores the same features described in the 3th chapter, comparing *C. albicans* with non-*albicans Candida* species and *Saccharomyces cerevisiae* in what

regards the purinergic response. The same $A_{2A}R$ profile was found under those conditions, but non-*albicans* yeasts modulate differently the extracellular ATP.

CHAPTER VI involves the *in vivo* study of *C. albicans* infection and $A_{2A}R$ distribution on gastrointestinal tract of mice with different ages. The physiopathology of over-colonization of the gastrointestinal tract of aged mice differs from the one observed in younger mice, indicating a higher susceptibility of elderly. Concomitantly, these animals have lower number of A_{2A} receptors in the mucosal cells. This work is submitted to the International Journal of Medical Microbiology.

CHAPTER VII refers to an overall discussion of the results presented in the four previous chapters and to the global conclusions accomplished with the present work.

CHAPTER VIII points out future work directions, based on the achievements, to further understand the unanswered questions of this field.

CHAPTER III

CANDIDA ALBICANS EARLY INTERACTION WITH MACROPHAGES PREVENTS EXTRACELLULAR ATP INCREASE AND ADENOSINE A_{2A} RECEPTOR ACTIVATION

From:

Rodrigues L, Curado F, Coelho C, Cabral V, Figueiredo AB, Cortes L, Meyer-Fernandes JR, Cunha RA and Gonçalves T (2015) *Candida albicans* early interaction with macrophages prevents extracellular ATP increase and adenosine A_{2A} receptor activation. Submitted to mBio.

ABSTRACT

Purines (ATP and adenosine) are signaling molecules fine-tuning immune-inflammatory responses. Here we investigated if the adenosine sensing device, adenosine A_{2A} receptor ($A_{2A}R$), is exploited by *Candida albicans* to escape the macrophage, contributing to its pathogenic success. We report that co-culturing *C. albicans* with RAW264.7 macrophages did not change the extracellular ATP (eATP) levels and ectophosphatase and ectonucleotidase activities, while the less virulent strains *Candida glabrata* and *Saccharomyces cerevisiae* increase eATP and have lower enzymatic activities. Furthermore, *C. albicans* did not increase macrophage $A_{2A}R$ gene expression, in contrast to the 12-fold increase triggered by *E.coli* LPS. Instead, *C. albicans* forced a re-localization of $A_{2A}R$ from the plasma membrane to the phagosomal membrane enclosing yeast cells, both in macrophages and in primary dendritic cells. Notably, yeast cells were internalized more efficiently by peritoneal macrophages of $A_{2A}R$ knockout compared to wild type mice. However, once internalized, neither $A_{2A}R$ agonist (NECA) nor antagonist (ZM) altered *C. albicans* viability, nor did they alter TNF-α and Il-1β expression, corroborating the intracellular arrest of $A_{2A}R$, out of reach of $A_{2A}R$ agonist and antagonist. Overall, these results indicate that *C. albicans* exploits the purinergic system to infect and strive in macrophages, by preventing increased eATP and the up-regulation of macrophage $A_{2A}R$, to lower the activation of macrophages; this allows the internalization of *C. albicans* cells, surrounded by the phagosomal membrane retaining $A_{2A}R$, ultimately contributing to the "silent" persistence of *C. albicans* inside macrophages.

INTRODUCTION

Over the past decades, the number of opportunistic fungal diseases has increased and *Candida* species, namely *Candida albicans*, are the fourth leading cause of nosocomial bloodstream infections (Pfaller and Diekema, 2007) with an associated mortality rate of 30-55% (Armstrong-James and Harrison, 2012). These fungi take advantage of deteriorated physiological conditions or of local or systemic immune malfunction to strive; accordingly, organ transplants, cytotoxic drugs, diabetes or AIDS are major factors contributing to this increased fungal pathogenicity (Pfaller and Diekema, 2007). Therefore, due to the increased number of patients with fragile immune conditions and the failure of available therapies against severe *C. albicans* infections (Pfaller and Diekema, 2007; Sardi *et al*., 2013), it is of major importance to bolster our understanding of the mechanisms of yeast infection to devise novel therapeutic strategies.

As a result of the different manifestations of *C. albicans* infection, from minor local infections to severe situations of systemic spreading, and of the different morphologies of this yeast species, the resulting host immune response is a complex process involving adapting strategies both from the fungus and the host (Plantinga *et al*., 2012; Seider *et al*., 2010; d'Enfert, 2009). Macrophages have a primordial role in the host immune response to candidial invasion (Vásquez-Torres and Balish, 1997), being responsible for an early recognition and consequent orchestration of a pro-inflammatory response against these pathogens (Martinez-Solano *et al*., 2006; Romani, 2011). Nevertheless, *C. albicans* manipulates the phagocytic pathway and survives macrophage attack through several mechanisms (Chinen *et al*., 1999; Marcil *et al*., 2002; Lorenz *et al*., 2004; Frohner *et al*., 2009; Barelle *et al*., 2006; Netea *et al*., 2006; Heinsbroek *et al*., 2008; Fernandez-Arenas *et al*., 2008), some still unclear.

Purines are important endogenous signalling molecules in immunity and inflammation, contributing for the efficient elimination of pathogens by the immune-inflammatory system while minimizing the ensuing damage to healthy tissues (Haskó *et al*., 2008; Kumar and Sharma, 2009; Junger, 2011). This is achieved by a coordinated action of ATP and its metabolite, adenosine; thus, ATP is released as a danger signal to bolster the immune-inflammatory response (Bours *et al*., 2006; di Virgilio, 2007), whereas adenosine is mainly released as a consequence of cellular stress by the injured tissue to curtail excessive activation of the immune-inflammatory system, restraining damage to healthy tissue (Fredholm, 2007). Indeed, the activation of the immune-inflammatory system leads to an up-regulation of adenosine A_{2A} receptors ($A_{2A}R$), which are a main STOP signal of this system (Sitkovsky *et al*., 2004; Sitkovsky and Ohta, 2005). In particular, it is well documented that $A_{2A}R$ are robustly up-regulated in macrophages upon bacterial infection (Khoa *et al*., 2001; Murphee *et al*. 2005; Thiel *et al*., 2003). In accordance with this opposite role of these two purine signals (ATP and adenosine), ecto-nucleotidases that convert extracellular ATP into adenosine are a determinant of the efficiency of infection of microorganisms (Kas-Deelena *et al*., 2001; Romio *et al*., 2011; Mahamed *et al*., 2012) and the up-regulation of ecto-phosphatases seems to be a correlate of their pathogenicity (de Jesus *et al*., 2002; Bisaggio *et al*., 2003; Pinheiro *et al*., 2006; Samson *et al*., 2007; Figueiredo *et al*., 2012).

Since it is currently unknown if fungal infections also affect or are controlled by the adenosine modulation system, the present study investigated if adenosine and its sensing device ($A_{2A}R$) may constitute one of the systems exploited by *C. albicans* to "silence" macrophage response, thus explaining its success as a pathogen. By exposing a macrophage cell line to *C. albicans*, we observed that $A_{2A}R$ agonists decreased the internalization of yeast cells and, accordingly, peritoneal macrophages from $A_{2A}R$

knockout mice have a higher phagocytosis rate than wild type mice peritoneal macrophages. Remarkably, *C. albicans* avoids the over-expression of $A_{2A}R$ characteristic of bacterial infections, decreases ATP-derived adenosine formation by lowering ATP release and the activity of ectonuclotidases, and promotes an intracellular trapping of $A_{2A}R$ around the phagosomes containing yeast cells.

MATERIALS AND METHODS

Strains, media and cell culture conditions

Candida albicans YP0037 and *Candida glabrata* YP0852 were isolated from haemocultures while *Saccharomyces cerevisiae* YP0907 was isolated from urine, all belonging to the Microbiology Pathogenic Yeast Collection, University of Coimbra. Yeast cells were grown on YPD agar (0.5 % yeast extract, 1 % bacto-peptone, 2 % agar and 2 % glucose) plates, at 30 ºC, overnight, and then washed 3 times with PBS (Phosphate-Buffered Saline) and counted with a haemocytometer before use. Whenever needed, washed *C. albicans* cells were killed at 65°C for 30 min (Bain *et al.*, 2014) and the cell suspension was plated on YPD to confirm nonviability. RAW 264.7 murine macrophages were obtained from the European Collection of Cell Cultures and maintained in DMEM (Sigma-Aldrich) with 10 % non-inactivated FCS (Fetal Calf Serum), 10 mM HEPES, 12 mM sodium bicarbonate and 11 mg/mL sodium pyruvate at 37 ºC in a humidified atmosphere with 5 % CO_2. The medium was changed every 2 days, until ~70% confluence was reached. RAW 264.7 cells were resuspended in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% inactivated FBS, 23.8 mM sodium bicarbonate and 50 mM glucose for the experimental assays.

Bone-marrow derived dendritic cells

Bone-marrow-derived dendritic cells were obtained from C57BL/6 bone marrow as previously described (Lutz *et al.*, 1999). Briefly, bone marrow cells were isolated from the femur and tibia and cells were cultured in RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine and 50 μM β-mercaptoethanol, pH 7.2. Cells were plated in Petri dishes at a concentration of 3 x 10^5 cells/mL, and incubated at 37°C / 5% CO_2. GM-CSF (R&D Systems) was added on the days 0, 3 and 6, at a concentration of 3 ng/mL (1050 U/mL), and non-adherent cells were collected on the ninth day of culture.

Infection assays

Macrophages-yeasts interaction assays were carried out essentially as previously described (Fernández-Arenas *et al.*, 2007). Briefly, RAW 264.7 macrophage cells were platted in 12-well multiwell plates (with or without 16 mm glass cover-slips, depending on the type of measurement required) for 18 h, at 37 ºC under a 5% CO_2 atmosphere. When the confluence reached around 70%, yeast cells were added to the macrophages at a MOI (Multiplicity of Infection) of 1:1. Similarly, dendritic cells-yeasts interaction assays were carried out in 12-well multiwell plates (with cover-slips), at a MOI of 1:1, for 1.5 h at 37 ºC under a 5% CO_2 atmosphere.

ATP quantification

For ATP quantification, supernatants were collected from macrophages-yeasts co-cultures at 1.5 h, 3 h and 6 h and stored at -80 ºC. ATP quantification was made as previously described (George *et al.*, 2015) using the Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma), to record luminescence with a VICTOR^3 Multilabel Plate Reader (PerkinElmer).

Measurement of the activities of ecto-5´-nucleotidase and ecto-phosphatase

RAW 264.7 and yeasts cells maintained and co-cultured as previously described were incubated for 1 hour at 37 ºC and 5% CO_2 in 1 mL of reaction mixture containing 116 mM NaCl, 5.4 mM glucose, 50 mM HEPES-MES-Tris buffer (pH 7.2) and 5 mM 5´-AMP (Sigma) as substrate for ecto-5´-nucleotidase. Cell supernatants were collected to quantify inorganic phosphate (Pi) (Guilherme *et al.*, 1991). Briefly, the enzymatic reaction was stopped by the addition of 2 mL of 25% charcoal in 0.1 M HCl to the supernatants, followed by a centrifugation at 1500 *g* for 15 min at 4 ºC. Supernatants (100 μL) were added to 100 μL of Fiske-Subbarow mixture and the absorbance was measured at 650 nm (SpectraMax Plus 384 spectrophotometer, Molecular Devices). Ecto-5´-nucleotidase activity was calculated by subtracting the non-specific 5´-AMP hydrolysis measured in the absence of cells from the total Pi released, both determined by extrapolation of a concentration curve of standard Pi.

Differences in total ecto-phosphatase activity were determined as previously described (Fernandes *et al.*, 1997), using the same conditions as for ecto-5´-nucleotidase, but replacing 5'-AMP by 5 mM *p*-nitrophenyl phosphate (from VWR) as substrate. To determine the concentration of *p*-nitrophenol formed by ecto-phosphatase activity, the reaction was stopped by the addition of 2 mL of NaOH (1 M), followed by spectrophotometric analysis (SpectraMax Plus 384 spectrophotometer, Molecular Devices) at 425 nm to interpolate a *p*-nitrophenol standard curve (Fernandes *et al.*, 1997).

***Adora2a* relative gene expression**

In order to quantify the relative expression of the $A_{2A}R$ gene (*Adora2a*) in macrophages interacting with *C. albicans* or in macrophages exposed to 100 ng/ml

lipopolysaccharide (LPS, from *E. coli* serotype 026:B6; L-8274, Sigma-Aldrich), we used a real-time PCR approach, selecting the 18S rRNA gene as the reference gene. After carrying out the infection assay as described above, the multiwell plates were iced, scraped and samples transferred to RNAse free tubes at 0 h, 1.5 h, 3 h and 6 h. After centrifugation (10,000 rpm; Minifuge) for 5 min at 4 ºC, RNA was extracted using the Magma Pure Compact RNA Isolation Kit (Roche). The concentration and purity of the extracted RNA was assessed, followed by reverse transcription using a Transcriptor First Strand cDNA Synthesis Kit (Roche). The reverse transcription reaction proceeded in a GeneAmp PCR System 2400 (Perkin Elmer), following the manufacturer's guidelines. The obtained cDNA was used for quantitative RT-PCR on a LightCycler II Carousel-Based System (Roche, software LightCycler 2.0) using A_{2A} primers (Streitová *et al.*, 2010), forward CCGAATTCCACTCCGGTACA and reverse CAGTTGTTCCAGCCCAGCAT and, as reference, 18S gene primers, forward AGGGGAGAGCGGGTAAGAGA and reverse GGACAGGACTAGGCGGAACA. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was applied to quantify the relative gene expression in macrophages under the different activation conditions.

TNF-α and IL-1β relative gene expression

Supra-maximal but selective concentrations of an $A_{2A}R$ antagonist ({4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a]-[1,3,5]triazin-5-yl amino]ethyl)phenol} - ZM241385, 50 nM) or an agonist (5'-N-ethylcarboxamido adenosine - NECA, 50 nM and adenosine – ADO, 10 mM) were added to cells during the interaction assays (Csóka *et al.*, 2012; Cunha *et al*., 1997). The quantification of the relative expression of TNF-α and IL-1β genes was made by RT-qPCR as described above, using the following primers (Ghosh *et al.*, 2010):

5'-CATGATCCGCGACGTGGAACTG-3'/5'-AGAGGGAGGCCATTTGGGAACT-3' (195 bp) and 5'-AACTGTTCCTGAACTCAACTGT-3'/5'-GAGATTTGAAGCTGGATGCTCT-3' (150 bp). 18S rRNA gene was used as reference gene.

Immunofluorescence and microscopy analysis

Macrophages grown in coverslips were infected with Oregon Green-labelled *C. albicans* (described below). At the end of each infection period, coverslips were washed twice with ice-cold PBS and fixed with 4 % paraformaldehyde in PBS for 15 min at room temperature. Macrophages were permeabilized with a PBS solution containing 0.2 % Triton X-100 (10 min at room temperature), followed by treatment with a blocking solution of 3 % bovine serum albumin in PBS for 1 h at room temperature. Washing with PBS was made between these incubation periods. Cells were then incubated 1 h at room temperature with the primary goat polyclonal antibody against $A_{2A}R$ (sc7504-R18; Santa Cruz Biotechnology), diluted (1:400) in blocking solution. Coverslips were washed three times and incubated for 1 h at room temperature with the secondary donkey anti-goat IgG antibody labelled with AlexaFluor 594 (A11058, Invitrogen). Dendritic cells were also infected with Oregon Green-labelled *C. albicans* and labelled with primary guinea-pig polyclonal antibody against $A_{2A}R$ (A2A-GP-Af1000, Frontier Institute Co.) and secondary goat anti-guinea-pig IgG antibody conjugated with AlexaFluor 594 (A11076, Invitrogen), both diluted 1:400. Finally, cells were incubated with DAPI 0.02% for 10 min at room temperature. Cells were subsequently washed three times and the coverslips were mounted with DAKO mounting medium and kept at -20ºC until visualized with confocal or fluorescence microscopy. Digital images were captured using a Zeiss LSM 510 Meta Confocal Microscope with PlanApoChromat

63x/1.40 immersion objective and viewed on Zeiss LSM Image Browser, using ImageJ software to analyse the images. Dendritic cells images were captured using a Zeiss Imager Z2 Microscope with a Plan-Apochromat 63x/1.40 oil objective and viewed on AxioVision LE64 software.

Isolation and maintenance of murine peritoneal macrophages

Wild type and $A_{2A}R$ KO C57Bl/6 male mice were obtained from our local breeding colonies (Centre for Neuroscience and Cell Biology, University of Coimbra, Portugal). All procedures were according to EU guidelines (2010/63) and approved by the Animal Care Committee of the Center for Neurosciences and Cell Biology of the University of Coimbra. Animals were kept under 12h:12h light-dark cycle, controlled temperature (21-24ºC) and humidity conditions (50-60%) and with free access to water and food. On the day of macrophage isolation, mice were killed by cervical displacement and sprayed with ethanol. Briefly, 4-5 mL of ice-cold PBS (with 3% FCS) were injected into the mice peritoneal cavity, with care to avoid puncturing any organs. After injection, the peritoneum was gently massaged, dislodging any attached cells into the PBS solution, and the fluid was collected with needle and syringe. The collected cell suspension was kept in tubes on ice, until spinning at 1500 rpm for 8 minutes. Supernatants were discarded and cells resuspended at the desired concentration in culture media. Cells were allowed to adhere to the substrate by culturing them for 1 to 2 h at 37°C and non-adherent cells were removed by gently washing with warm PBS. Subsequent phagocytosis assays were done as described below.

Macrophage phagocytosis assay

Prior to infection, *C. albicans* cells were labelled with 1 μM Oregon Green 488 (Invitrogen), for 1 h at 30 ºC with continuous gentle shaking in the dark, and then washed twice with PBS containing 100 mM glycine and re-suspended at the desired density. Before infection with yeast cells, peritoneal macrophages (previously seeded on cover-slips) were also labelled with 100 mM Lysotracker Red DND-99 (Invitrogen) to follow phagolysosome maturation as previously described (Marcil *et al.*, 2002), and the infection assay carried out as described above. During the assay, Lysotracker Red DND-99 was kept in solution at a concentration of 50 nM. After the period of infection, coverslips were washed with PBS and fixed in 4% paraformaldehyde in PBS, washed with PBS and stained with 12.5 nM Calcofluor White (Sigma-Aldrich). After washing, the coverslips were mounted with specific mounting medium (DAKO) and stored at -20 ºC until observation.

Yeast viability assays

The yeast cell viability upon phagocytosis was assessed by a colony forming unit (CFU) assay. After co-culturing, peritoneal macrophages were lysed with 0.5% Triton X-100 and sterile distilled water and then plated over YPD agar plates at a 1:100 dilution, representing the number of yeast cells internalised. Plates were incubated at 30 ºC and the number of colonies was counted after 3 days of incubation.

Statistics

Data are presented as means ± SEM. Statistical differences were determined, whenever applied, with either a Student's *t* test or a one or two-way ANOVA respectively

followed by a Newman-Keuls or Bonferroni post hoc test. Data were considered statistically significant at $p<0.05$.

RESULTS

Extracellular ATP and its metabolism did not change during the course of infection

Due to its recognized importance as an endogenous trigger of the immuno-inflammatory system, we first determined if RAW264.7 macrophages infection with *C. albicans* altered the extracellular levels of ATP and the ability of macrophages to clear this extracellular ATP (eATP). Clearly, *C. albicans* infection, at least during the 6 h-infection period, did not trigger a significant increase in eATP. In contrast, macrophage infection with the less pathogenic non-*albicans* yeast species, *C. glabrata* and *S. cerevisiae*, caused a marked increase of eATP (Figure 1A).

The extracellular catabolism of ATP is important to eliminate this pro-inflammatory signaling molecule, but it is also responsible for the generation of another signaling molecule, adenosine, which is a negative modulator of the immune-inflammatory process. The ectophosphatase activity, responsible for converting ATP into ADP and AMP, and the ectonucleotidase activity, that converts AMP into adenosine, were measured in the intact co-cultured cells during macrophage-yeast cells infection assays. Therefore, the measured ecto-enzymatic activity results from the activity of ecto-enzymes present either in RAW264.7 macrophages and/or in yeast cells. In the first 60 minutes of infection, the ectophosphatase activity during *C. glabrata*-macrophages interaction was lower comparing to *C. albicans* (240 ± 46 *vs* 835 ± 163 nmol Pi h^{-1} 10^{-6}

cells; p<0.001; Fig. 1B); *S. cerevisiae* also tended to lower ectophosphatase activity (609 ± 36 nmol Pi h^{-1} 10^{-6} cells; Figure 1B). In what regards ecto-5'-nucleotidase activity, macrophage-*C. glabrata* and macrophage-*C. albicans* co-cultures displayed similar activities (183 ± 30 *vs* 247 ± 57 nmol Pi h^{-1} 10^{-6} cells), which was lower (108 ± 7.2 nmol Pi h^{-1} 10^{-6} cells) in macrophage-*S. cerevisiae* co-cultures (Figure 1B). We also observed that the ectophosphatase activity during *C. albicans*-macrophage interaction decreases to 77 % of control, while the ectonucleotidase activity does not significantly change when a macrophage cell culture was infected with *C. albicans* cells during the first 1.5 h (Fig. 1 - insert).

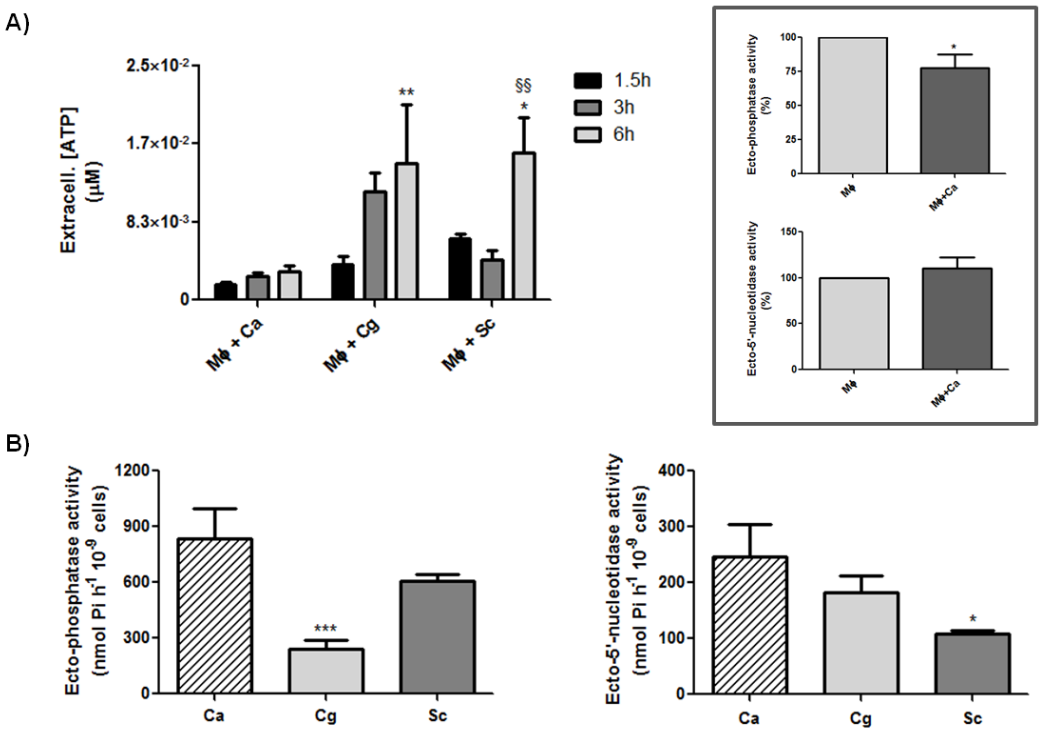

Figure 1. Extracellular ATP is kept low during *C. albicans* infection.

A) Measurement of ATP in the culture supernatant of macrophages infected with yeasts (*C. albicans* – Ca; *C. glabrata* – Cg; *S. cerevisiae* – Sc) after different periods (1.5 h, 3 h and 6h) of incubation. The measurement was carried out by luminescence using the Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma). * p<0.05, ** p<0.01, 1.5h *vs* 6h; §§ p<0.01, 3h *vs* 6h. B) Measurement of the activities of ecto-phosphatase (left) and

ecto-5´-nucleotidase (right) in RAW 264.7 macrophages co-cultured with *C. albicans*, *C. glabrata* or *S. cerevisiae*. [Insert: Percentage of ecto-phosphatase (top) and ecto-5´-nucleotidase (bottom) activities of RAW 264.7 macrophages *versus* co-cultured with *C. albicans*.] The substrate for ecto-phosphatase was *p*-nitrophenyl phosphate (5 mM) while the substrate for ecto-5´-nucleotidase was 5´-AMP (5 mM). The absorbance obtained for each reaction was analysed spectrophotometrically. *p<0.05; ***p<0.001. Data were derived from at least three independent experiments (mean ±SEM), each performed in experiment triplicates.

Adenosine increases cytokine production during *C. albicans* infection

Since our main purpose was to study the involvement of adenosine, a STOP signal of inflammation, and its sensing device $A_{2A}R$, we began by testing if $A_{2A}R$ activation or blockade would lead to a differential expression of two main pro-inflammatory cytokines, TNF-α and Il-1β. RT-qPCR showed that after 1.5 h of *C. albicans* infection of RAW264.7 macrophages, neither the activation (with 50 nM NECA, an $A_{2A}R$ agonist) nor the blockade (with 50 nM ZM241385, an $A_{2A}R$ antagonist) of $A_{2A}R$ affected the expression of the genes coding for TNF-α or for Il-1β (Fig. 2A,B). Furthermore, the exogenous addition of adenosine (10 mM) during *C. albicans* infection did not increase the expression of TNF-α gene (Fig. 2A); however, it caused a 80-fold increase in Il-1β expression (Fig. 2B). Of note is the fact that the incubation of *C. albicans* heat-inactivated (HK-Ca) by macrophages in the presence of adenosine 10 mM significantly increased TNF-α gene expression by 2-fold, while Il-1β gene expression was increased by 75-fold, similar to the value obtained by addition of adenosine during live *C. albicans* infection (Fig. 2C,D). As expected, exposure of macrophages to LPS caused a 600-fold increase in Il-1β expression and a 20-fold increase in TNF-α expression (Fig. 2A, B).

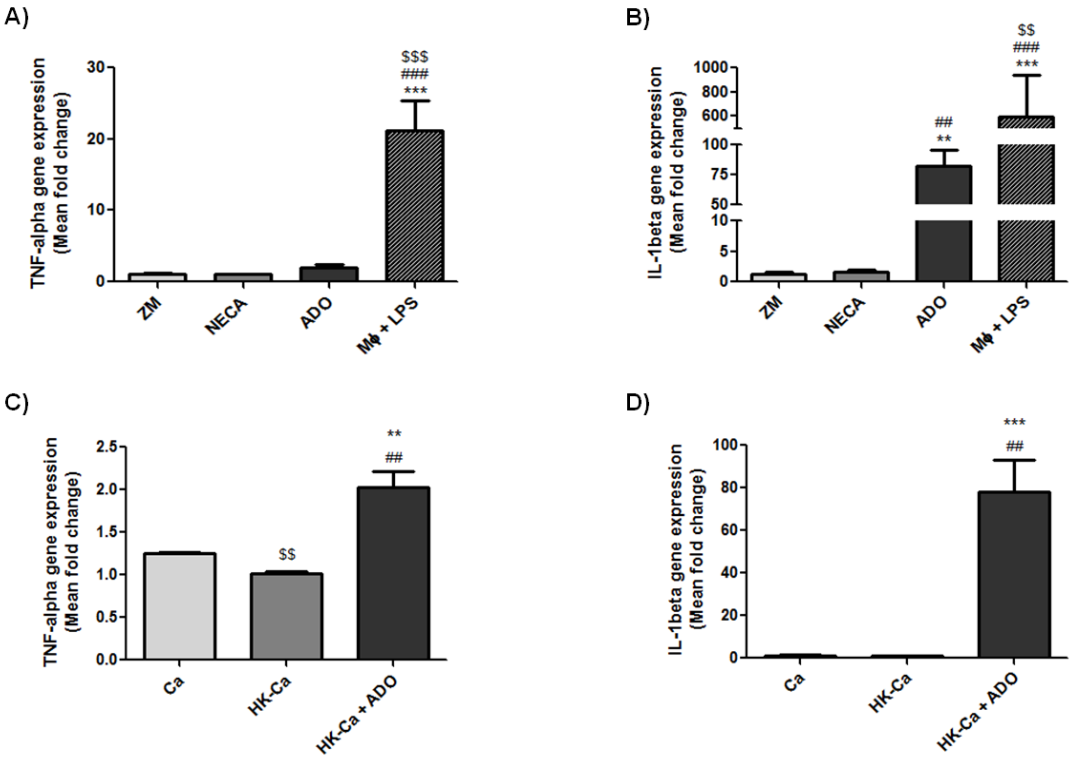

Figure 2. $A_{2A}R$ modulation impacts in cytokine production during infection.

Relative expression of TNF-α and IL-1β genes in macrophages infected with yeasts (1.5h), under different experimental conditions: A, B) Co-cultures of RAW 264.7 cells and *C. albicans* treated with an $A_{2A}R$ antagonist (50 nM ZM 241385, ZM), with an $A_{2A}R$ agonist (50 nM NECA) or adenosine (10 mM, ADO) *versus* RAW 264.7 macrophages treated with LPS. Data are mean fold elevation compared to RAW 264.7 cells infected with *C. albicans*. **p<0.01, ***p<0.001, ZM *vs* others; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001, NECA *vs* others; $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001, ADO *vs* others. C, D) RAW264.7 macrophages infected with *C. albicans* – Ca; heat-killed *C. albicans* – HK-Ca or heat-killed *C. albicans* plus 10 mM adenosine – HK-Ca + ADO. Data are mean fold elevation compared to control untreated RAW 264.7 cells. **p<0.01, ***p<0.001, HK-Ca +ADO *vs* Ca; $^{\#\#}$p<0.01, HK-Ca + ADO *vs* HK-Ca; $^{\$\$}$p<0.01, HK-Ca *vs* Ca. Data were derived from three independent experiments (±SEM), each performed in triplicates.

C. albicans does not induces an up-regulation of *Adora2a* expression in macrophages

The lack of eATP increase during macrophage interaction with *C. albicans* and the lack of a clear change in the external ATP metabolism activity indicated that the formation

of ATP-derived adenosine would be scarce. Moreover, the pharmacological manipulation of $A_{2A}R$ did not change the production of pro-inflammatory cytokines, except with a concentration of adenosine far exceeding that found in pathophysiological conditions. So, we tested if *C. albicans* infection altered the expression of the gene that codes for the main receptor associated with the adenosine-mediated control of immune-inflammation, *i.e.* $A_{2A}R$. As shown in Fig. 3, the co-culture for 1.5, 3 and 6 h of RAW 264.7 macrophages with *C. albicans* failed to induce significant modifications of the expression of the $A_{2A}R$ gene (*Adora2a*). This clearly contrasted with the robust 12-fold increase of $A_{2A}R$ gene expression in macrophages exposed to *E. coli* LPS (100 nM) during 1.5 h, decreasing to a 2-fold induction of $A_{2A}R$ mRNA at 3 and 6 h of LPS exposure. These results show that macrophages do not respond to yeasts with an increase in *Adora2a* expression, as observed in response to LPS.

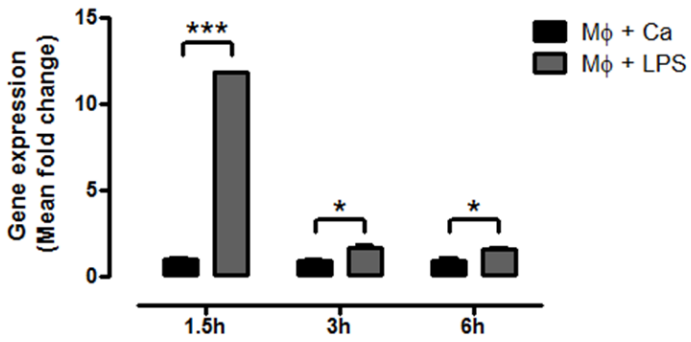

Figure 3. *Adora2a* expression is not increased during yeast infection.

Relative expression of adenosine A_{2A} receptor *Adora2a* gene in macrophages following the interaction with *C. albicans* or exposure to *E. coli* LPS (100 ng/ml) for 1.5 h, 3 h and 6 h. Data are mean fold elevation (± SEM) over control untreated RAW264.7 cells (n=4); * $p<0.05$; *** $p<0.001$.

Phagocytosis of yeasts induces the re-localization of $A_{2A}R$ to the phagosomal membrane

We next investigated if yeasts could affect $A_{2A}R$ signaling by impacting on the distribution of $A_{2A}R$ within the macrophage. Surprisingly, the confocal microscopy immunolabeling study of $A_{2A}R$ localization in RAW264.7 macrophages infected with *C. albicans* revealed an higher density of $A_{2A}R$ staining in the phagosome membrane enclosing the yeast cells in clear contrast with the profile of $A_{2A}R$ distribution in macrophages exposed to LPS, where $A_{2A}R$ immunoreactivity was disperse along the plasma membrane (Fig. 4A). We excluded the presence of $A_{2A}R$ in *C. albicans* using either immunocytochemical or receptor binding assays with the selective $A_{2A}R$ ligand ^{3}H-SCH58261 (data not shown), and concluded that *C. albicans* infection indeed triggers a redistribution of macrophage $A_{2A}R$ into the membranes enclosing the yeast cells. A more detailed analysis of the immunoreactivity of $A_{2A}R$ in *C. albicans*-infected macrophages failed to reveal a co-localization of $A_{2A}R$ staining among macrophages and yeasts cells, which confirmed that $A_{2A}R$ staining was located in the phagosomal membrane of macrophages (Fig. 4B). Of notice is the fact that $A_{2A}R$ is mainly located in the phagosomal membranes surrounding the hyphae of internalized *C. albicans* (Fig. 4C).

This unexpected redistribution of $A_{2A}R$ in macrophages leads us to test whether this was exclusively found in macrophages infected with yeast cells. So, we prepared primary cultures of bone marrow-derived dendritic cells from C57Bl6 mice. We observed that, similarly to macrophages, *C. albicans* infection of dendritic cells also leads to a redistribution of the $A_{2A}R$ into the membrane of phagosomes enclosing *C. albicans* cells (Fig. 4D).

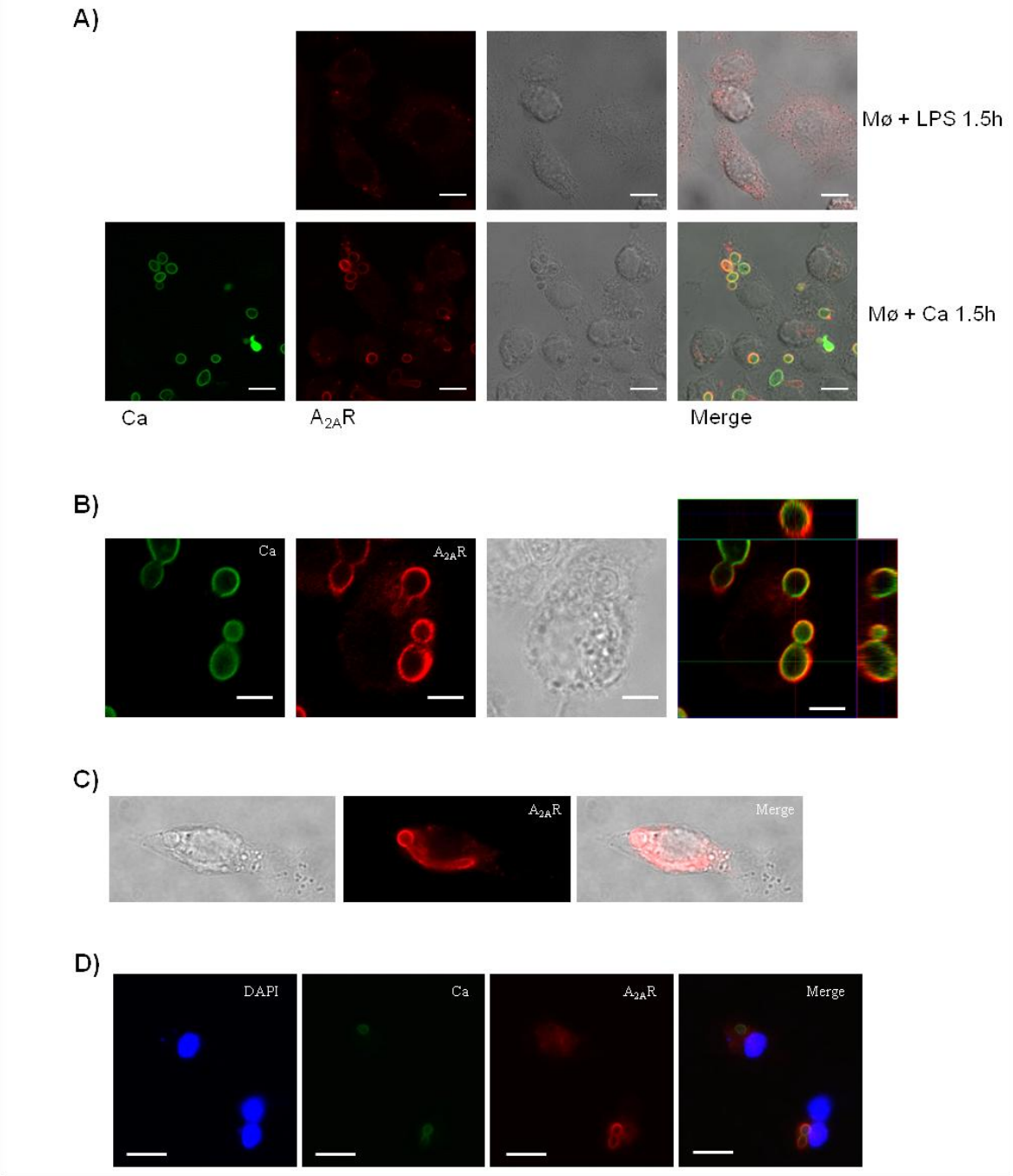

Figure 4. Re-localization of $A_{2A}R$ in the phagosome membrane enclosing yeast cells.

Representative confocal images of cell cultures immunostained with an antibody against A_{2A} receptors ($A_{2A}R$) (red). A) RAW 264.7 macrophages upon interaction with LPS (upper panels) or with Oregon green-labeled *C. albicans* (Ca, bottom panels) during 1.5 h of infection; scale bars are 10 µm; B) Confocal 3D reconstruction: orthogonal images of *x-z* (top) and *y-z* (right) plane showing a lack of co-localization between $A_{2A}R$ immunoreactivity and yeast cells; scale bars are 5 µm. C) Detail of $A_{2A}R$ staining of hyphal tips in *C. albicans* engulfed by macrophages. The confocal images were obtained with a Zeiss LSM 510 Meta Confocal Microscope, using Plan-ApoChromat 63x/1.4 oil objective. D) Dendritic cells upon interaction with Oregon green-labeled *C. albicans* during 1.5 h of infection; scale

bars are 10 μm; images were obtained with a Zeiss Imager Z2 Microscope, using Plan-Apochromat 63x/1.40 oil objective.

C. albicans phagocytosis by macrophages is under the control of $A_{2A}R$

The role of $A_{2A}R$ in *C. albicans* phagocytosis by macrophages was tested by comparing the ability of peritoneal macrophages extracted from wild type and from *Adora2a* knockout C57Bl6 mice ($A_{2A}R$ KO) to phagocyte yeasts. As shown in Figure 5A, peritoneal macrophages from $A_{2A}R$ KO mice were more competent to internalize *C. albicans* cells than macrophages from wild type mice, but the viability of yeast cells was not compromised (Fig. 5B). A differential microscopic fluorescence probing methodology that allows the distinction between total yeast cells (green), non-internalized (blue) and yeast cells inside activated phagolysosomes (yellowish to reddish), allowed us to conclude that there were less yeast cells outside the macrophages from $A_{2A}R$ KO than wild type mice (Fig. 5C). It is worth noticing that $A_{2A}R$ KO mice macrophages are more yellowish/red due to Lysotracker red fluorescence than wild type mice macrophages, indicating more acidic phagosomes, supposedly, a sign of phagolysosome maturation and consequently, of macrophage activation. However, at 1.5 h post-phagocytosis these differences in Lysotracker red fluorescence were not reflected in different number of viable cells (Fig 5B).

Using a CFU assay we also tested whether the presence of either ZM248385 ($A_{2A}R$ antagonist) or NECA ($A_{2A}R$ agonist) would change the efficiency of RAW 264.7 cells to kill internalized *C. albicans* cells. The yeast cells were allowed to be phagocyted during 1 h; then, the non-internalized yeast cells were discarded and the macrophage cultures infected with *C. albicans* were exposed to ZM248385 (50 nM) or to NECA (50 nM) for an additional 1.5 h or 3 h period. Neither the activation nor the inhibition of

macrophage $A_{2A}R$, using the $A_{2A}R$ agonist or antagonist, respectively, modified the viability of yeasts cells once they were inside the phagosomal compartment of macrophages (data not shown), in accordance with the predominant intracellular localization of $A_{2A}R$ upon *C. albicans* infection.

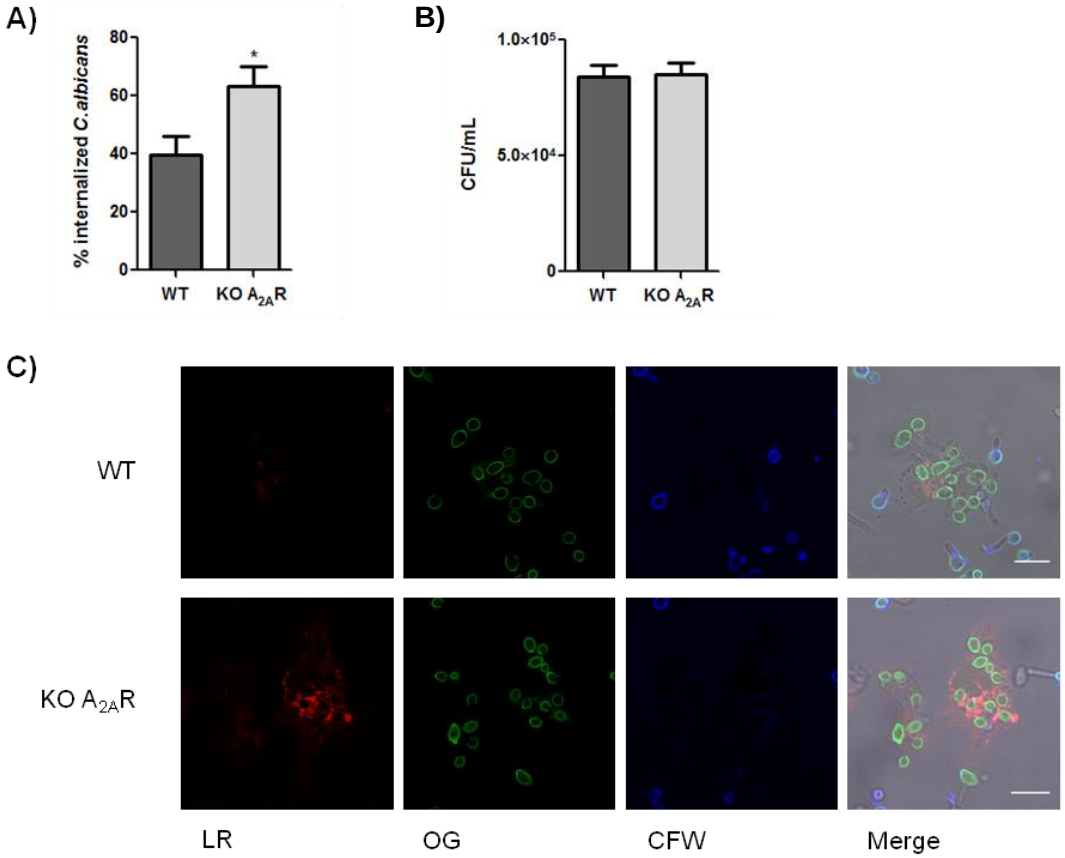

Figure 5. Peritoneal macrophages from $A_{2A}R$ knockout mice phagocyte more efficiently *C. albicans* cells.

A) *C. albicans* internalization by peritoneal macrophages from wild type (WT) and from Adora2a knockout mice ($A_{2A}R$ KO), during 1.5 h. Percentage of internalization was calculated from cell counting (number of internalized yeasts *vs* total cell number) of differential fluorescence staining microscopic images. *p<0.05. B) *C. albicans* viability after 1.5 h with peritoneal macrophages. C) Representative images of WT and $A_{2A}R$ KO peritoneal macrophages and *C. albicans* after 1.5 h of interaction; scale bar represents 10 µm. Cells are distinguished through their different fluorescence staining with LysoTracker Red (LR - macrophages red phagolysosomes), Oregon Green (OG - green yeast cells) and CalcoFluor White (CFW - non-internalized blue yeasts). Images were obtained with a Zeiss Axioskop 2 plus Microscope, using a PlanNeofluar 40x/0.75 objective.

DISCUSSION

The early interaction of *C. albicans* with the host innate immune system, in particular with macrophages is a crucial step in the pathogenesis of yeasts and in the genesis of an infection. The results obtained in the present study provide new insights on the role of purines and of adenosine A_{2A} receptors ($A_{2A}R$) in the control of infection, specifically in the interaction between macrophages and *C. albicans*. It was concluded that, during *C. albicans* infection, the extracellular levels of ATP are manipulated to avoid the overt activation of macrophages. Simultaneously, *C. albicans* force a unique adaptation of $A_{2A}R$ in macrophages, evading its up-regulation and imposing a novel and unexpected subcellular redistribution of $A_{2A}R$ into the phagosomal membrane.

The first major conclusion of this study is the ability of *C. albicans*, during the early interaction with macrophages, to keep low extracellular levels of ATP, which is a well recognized danger signal participating in the activation of macrophages. It has previously been described that yeasts have the ability to constitutively release ATP during an infection process (Koshlukova *et al*., 2000; Vylkova *et al*., 2007). It was also reported that, *in vitro*, *C. albicans* releases adenosine although with no effect on neutrophil killing (Smail *et al*., 1992). Now we report that, in contrast to *C. glabrata* and *S. cerevisiae*, the interaction of *C. albicans* with macrophages does not increase the extracellular levels of ATP. This dampens several macrophage ATP-dependent functions resulting from the activation of different P2 (ATP) receptors controlling chemotaxis (Isfort *et al*., 2011; Kronlage *et al*., 2010; Elliot *et al*., 2009), phagocytosis (Elliot *et al*., 2009; Marques-da-Silva *et al*., 2011) and the production of pro-inflammatory cytokines (Ferrari, 1997; Haskó, 2000a; Netea, 2010) and of radical species by macrophages (Sperlágh *et al*., 1998; Pfeiffer *et al*., 2007), all of which may

provide a biological advantage to yeast infection. This prompt us to post the working hypothesis that the ability of *C. albicans* to prevent the increase of eATP during the initial steps of infection might be a strategy to decrease the activation of macrophages and the release of macrophage-derived cytotoxic mediators, thus increasing yeast survival.

The extracellular metabolism of ATP, through the activity of ectonucleotidases (Dwyer *et al*., 2007) also defines the formation of extracellular adenosine, a fine regulator of inflammation and of tissue damage (Fredholm, 2007; Sitkovsky *et al*., 2004; Sitkovsky and Ohta, 2005) that acts as a STOP signal of inflammatory cells (Sitkovsky *et al*., 2004). The presence of two different types of cells lead us to measure, for the first time, the overall ectophosphatase activity (responsible for the conversion of ATP into ADP and AMP), and the overall ectonucleotidase (responsible for converting AMP into adenosine), resulting from yeast cells and macrophage cells enzymatic activities. This allowed us to re-enforce our contention that *C. albicans* orchestrates a decreased accumulation of eATP, since the ecto-nucleotidase and ecto-phosphatease activities during the infection with *C. albicans* were higher than during the infection with *C. glabrata* and with *S. cerevisiae*, considered less virulent than *C. albicans*. Moreover, despite the decreased ecto-phosphatase activity during infection with *C. albicans*, lack of an increased ecto-nucleotidase and of a bolstered release of ATP results in decreased formation of extracellular adenosine, necessary to activate $A_{2A}R$ to control macrophage reactivity (Haskó *et al*., 2000; Mayne *et al*., 2001; Day *et al*., 2005; Csóka *et al*., 2007; Köröskényi *et al*., 2011), as shown during bacterial infections (Sullivan, 2004; Nemeth *et al*., 2006; Belikoff, 2011). Strikingly, *C. albicans* did not trigger an up-regulation of $A_{2A}R$ gene expression, which seems to be a general hallmark of the activation of immune-inflammation (reviewed by Hásko *et al*., 2004; Junger,

2011; Sitkovsky *et al*., 2004), and in particular of macrophages (Murphee *et al*., 2005; Grinberg *et al*., 2009; Streitová *et al*., 2010). Indeed, whereas LPS triggered a robust increase of the expression of the *Adora2a* gene in macrophages, as previously described (Khoa *et al*., 2001, Streitová *et al*., 2010), we observed no significant up-regulation of *Adora2a* expression in macrophages exposed to *C. albicans*, showing that the infection of macrophages with *C. albicans* fails to bolster this crucial system (Csóka *et al*., 2012; Grinberg *et al*., 2009; Pinhal-Enfield *et al*., 2003). Notably, the absence of functional $A_{2A}R$ increased the number of yeast cells ingested by peritoneal macrophages from $A_{2A}R$ KO mice. This indicates that the engagement of $A_{2A}R$ decreased the ability of macrophages to ingest *C. albicans*, providing a novel link between $A_{2A}R$ and the phagocytosis of *C. albicans*. Given that the progression of *C. albicans* infection requires the entry of *C. albicans* cells into phagocytic cells (counteracted by $A_{2A}R$ activation), while avoiding its destruction by activated macrophages (promoted by ATP), the registered dual ability to simultaneously disable the increase of extracellular ATP and evading the up-regulation of $A_{2A}R$ provides an ideal manipulation of the purinergic system to bolster the success of *C. albicans* infection by promoting its intracellular life and persistence inside the macrophage as a "silent" agent.

Instead of up-regulating the expression of $A_{2A}R$, *C. albicans* triggered an unexpected and novel re-distribution of $A_{2A}R$ within macrophages. In fact, upon *C. albicans* infection, $A_{2A}R$ were mostly present in the phagosomal membrane surrounding internalized *C. albicans*, either in the yeast form or in the hyphal form. This re-localization of $A_{2A}R$ is not restricted to macrophages since it was also observed when *C. albicans* infected dendritic cells, another cell type of the mononuclear phagocyte system. In accordance with the unexpected localization of $A_{2A}R$ in *C. albicans*-infected macrophage membranes and its absence from the cytoplasmic membrane, the blockade

or the activation of $A_{2A}R$ with drugs described as $A_{2A}R$ antagonist and agonist (ZM or NECA, respectively) did not change phagocytosis or the expression of genes coding for TNF-α or IL-1β. Otherwise, extracellular addition of adenosine resulted in the activation of IL-1β gene expression while TNF-α gene expression does not change. According to recent publications, the activation of macrophages by ATP and LPS leads to a rapid increase in IL-1β, and the $A_{2A}R$ antagonist, ZM241385, decreases IL-1β while the agonist NECA increases this IL-1β response (Ouyang *et al*., 2013). So, the lack of IL-1β in the early response of macrophage perception of *C. albicans*, as observed by us and by others (Joly *et al*., 2013), can be attributed to the absence of strong stimuli such as ATP and the lack of $A_{2A}R$ activation, due to its removal from the plasma membrane, a process equivalent to the antagonism of this adenosine receptor. This strongly indicates a tolerogenic state leading to the sustainability of inflammasome activation, as described by Ouyiang and co-authors (Ouyang *et al*., 2013). Taking both observations together, it seems this *C. albicans*-induced re-distribution of $A_{2A}R$ in macrophages is designed to assist the entrance of yeast cells into the macrophage, while simultaneously controlling the outcome of yeast cells within the phagosome, as testified by the observation that, after yeasts internalization, exogenously added $A_{2A}R$ agonists or antagonists have no effect on the viability of the phagocyted yeast cells. Otherwise, when the agonist (adenosine) is taken up by the cell, through nucleoside transporters (Cabrita *et al*., 2013), one can observe cell activation and increase in Il-1β.

Although the mechanisms underlying this yeast-induced re-distribution of $A_{2A}R$ in macrophages is currently unknown, it is of interest to speculate, based on the similarity of cytokine gene expression response with infection by live and heat-killed *C. albicans* cells, that it is possible that the $A_{2A}R$ localization/migration to the membrane of *C. albicans*-enclosing phagosomes is dependent on yeast pathogen-associated molecular

patterns (PAMPs). Moreover, this re-localization is absent during *Escherichia coli* and *Leishmania amazonensis* dendritic cells infection (AB Figueiredo, unpublished work). In fact, $A_{2A}R$ can become associated with some TLRs during bacterial infection, to promote phagosome closure and internalization (Pinhal-Enfield *et al*., 2003). As for the therapeutic potential of manipulating $A_{2A}R$ to control the infection by *C. albicans*, we now observed that neither the external activation nor the external blockade of $A_{2A}R$ controlled the progress of *C. albicans* infection, except when exposed to adenosine, that can be transported to the cytoplasm by nucleoside transporters. In fact it was described that adenosine uptake is required for ATP extracellular toxicity to human cervical cancer cells (Mello *et al*., 2003) and that adenosine uptake inhibition increases anti-inflammatory responses during sepsis (Ramakers *et al*., 2011) and increases chemotaxis and phagocytosis of neutrophils (Alsharif *et al*., 2015). A final and crucial aspect that remains to be defined is the status of yeast cells in the un-maturated phagosome surrounded by $A_{2A}R$. It is tempting to speculate that *C. albicans* may persist inside the un-maturated phagolysosome in a latent stage, as postulated by the "*Trojan horse model*" (d'Enfert, 2009; Fernandez-Arenas *et al*., 2008). It is worth noting that the observed control by $A_{2A}R$ of the maturation of yeast-containing phagosomes suggests that the control of $A_{2A}R$ function might be a window of opportunity to test a novel and provocative hypothesis to explain how *C. albicans* may actually behave as an intracellular organism, persisting in intracellular reservoirs and that can turn into an aggressive pathogen. Recently, we described that *C. albicans* ectonucleotidase maximal activity is obtained with very low (4 - 5) pH conditions similar to the phagosome millieu (Chapter IV - Rodrigues *et al*., 2015). This could lead to the synthesis of adenosine inside the phagosome, leading to activation of $A_{2A}R$ in the phagosome

membrane, resulting in the downregulation of pro-inflammatory signals. The novelty of our findings is that all this would happen inside the phagosome and not extracellularly.

In conclusion, the present results indicate that both the control of the extracellular levels of ATP as well as the re-distribution of $A_{2A}R$ seem to play a role in the infection of macrophages by yeasts in particular by *C. albicans*. On one hand, the interaction of *C. albicans* with macrophages does not promote the pro-inflammatory boost of extracellular ATP, thus disabling or lowering macrophage reactivity. On the other hand, *C. albicans* avoids the up-regulation of $A_{2A}R$, which would change macrophage phenotype and decrease of yeast internalization, while it forces a re-distribution of the existing $A_{2A}R$ into the phagosomal membrane supposedly to control the maturation of the phagolysosome. All together, these new results provide new avenues to exploit the impact of the purinergic system in the control of the infectious process of *C. albicans*.

REFERENCES

Alsharif, K.F., Thomas, M.R., Judge, H.M., Khan, H., Prince, L.R., Sabroe, I., Ridger, V.C. and R.F. Storey. (2015) Ticagrelor potentiates adenosine-induced stimulation of neutrophil chemotaxis and phagocytosis. Vascul Pharmacol. 71:201-7.

Armstrong-James D and Harrison TS. (2012). Immunotherapy for fungal infections. Curr Opin Microbiol. 15, 434-439.

Bain, J.M., Louw, J., Lewis, L.E., Okai, B., Walls, C.A., Ballou, E.R., Walker, L.A., Reid. D., Munro, C.A., Brown, A.J., Brown, G.D., Gow, N.A. and L.P. Erwig. (2014) *Candida albicans* hypha formation and mannan masking of β-glucan inhibit macrophage phagosome maturation. MBio. 5(6):e01874.

Barelle CJ, Priest CL, MacCallum DM, Gow NA, Odds FC and Brown AJ. (2006). Niche-specific regulation of central metabolic pathways in a fungal pathogen. Cell Microbiol. 8, 961-971.

Belikoff BG, Hatfield S, Georgiev P, Ohta A, Lukashev D, Buras JA, Remick DG and Sitkovsky M. (2011). A_{2B} adenosine receptor blockade enhances macrophage-mediated bacterial phagocytosis and improves polymicrobial sepsis survival in mice. J Immunol.186: 2444-2453.

Bisaggio DF, Peres-Sampaio CE, Meyer-Fernandes JR and Souto-Padrón T. (2003). Ecto-ATPase activity on the surface of *Trypanosoma cruzi* and its possible role in the parasite-host cell interaction. Parasitol Res. 91: 273-282.

Bours MJL, Swennen ELR, Di Virgilio F, Cronstein BN and Dagnelie PC. (2006). Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol. Ther. 112: 358–404.

Cabrita, M.A., Baldwin, S.A., Young, J.D. and C.E. Cass. (2002) Molecular biology and regulation of nucleoside and nucleobase transporter proteins in eukaryotes and prokaryotes. Biochem Cell Biol. 80(5):623-38.

Chinen T, Qureshi MH, Koguchi Y and Kawakami K. (1999). *Candida albicans* suppresses nitric oxide (NO) production by interferon-gamma (IFN-gamma) and lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages. Clin. Exp. Immunol. 115, 491-497.

Csóka B, Németh ZH, Virág L, Gergely P, Leibovich SJ, Pacher P, Sun CX, Blackburn MR, Vizi ES, Deitch EA and Haskó G. (2007). A_{2A} adenosine receptors and C/EBPbeta are crucially required for IL-10 production by macrophages exposed to *Escherichia coli*. Blood 110, 2685-2695.

Csóka B, Selmeczy Z, Koscsó B, Németh ZH, Pacher P, Murray PJ, Kepka-Lenhart D, Morris SM Jr, Gause WC, Leibovich SJ et al. (2012). Adenosine promotes alternative macrophage activation via A_{2A} and A_{2B} receptors. FASEB J. 26, 376-386.

Cunha RA, Almeida T and Ribeiro JA. (2001). Parallel modification of adenosine extracellular metabolism and modulatory action in the hippocampus of aged rats. J. Neurochem. 76, 372-382.

Cunha RA, Constantino MD and Ribeiro JA. (1997). ZM241385 is an antagonist of the facilitatory responses produced by the A_{2A} adenosine receptor agonists CGS21680 and HENECA in the rat hippocampus. Br. J. Pharmacol. 122, 1279-1284.

d'Enfert C. (2009). Hidden killers: persistence of opportunistic fungal pathogens in the human host. Curr. Opin. Microbiol. 12, 358–364.

Day YJ, Huang L, Ye H, Linden J and Okusa MD. (2005). Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages. Am. J. Physiol. 288, F722-F731.

de Jesus JB, de Sá Pinheiro AA, Lopes AH and Meyer-Fernandes JR. (2002). An ectonucleotide ATP-diphosphohydrolase activity in Trichomonas vaginalis stimulated by galactose and its possible role in virulence. Z Naturforsch C. 57, 890-896.

Di Virgilio F. (2007). Purinergic signalling in the immune system. A brief update. Purinergic Signal. 3,1–3.

Dwyer KM, Deaglio S, Gao W, Friedman D, Strom TB and Robson SC. (2007). CD39 and control of cellular immune responses. Purinergic Signal. 3, 171-180.

Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, Park D, Woodson RI, Ostankovich M, Sharma P, et al. (2009). Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature 461, 282-286.

Fernandes EC, Meyer-Fernandes JR, Silva-Neto MA and Vercesi AE. (1997). *Trypanosoma brucei*: ecto-phosphatase activity present on the surface of intact procyclic forms. Z Naturforsch C. 52, 351-358.

Fernandez-Arenas E, Bleck CK, Nombela C, Gil C, Griffiths G and Diez-Orejas R. (2008). *Candida albicans* actively modulates intracellular membrane trafficking in mouse macrophage phagosomes. Cell. Microbiol. 11, 560-589.

Fernández-Arenas E, Cabezón V, Bermejo C, Arroyo J, Nombela C, Diez-Orejas R and Gil C. (2007). Integrated proteomics and genomics strategies bring new insight into *Candida albicans* response upon macrophage interaction. Mol. Cell Proteomics 6, 460-478.

Ferrari D, Chiozzi P, Falzoni S, Dal Susino M, Melchiorri L, Baricordi OR and di Virgilio F. (1997). Extracellular ATP triggers IL-1 beta release by activating the purinergic P2Z receptor of human macrophages. J. Immunol. 159, 1451-1458.

Figueiredo AB, Serafim TD, Marques-da-Silva EA, Meyer-Fernandes JR and Afonso LC. (2012). *Leishmania amazonensis* impairs DC function by inhibiting CD40 expression via A_{2B} adenosine receptor activation. Eur. J. Immunol. 42, 1203-1215.

Fredholm BB. (2007). Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ. 14, 1315–1323.

Frohner IE, Bourgeois C, Yatsyk K, Majer O and Kuchler K. (2008). *Candida albicans* cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance. Mol. Microbiol. 71, 240-252.

George, J., Gonçalves, F.Q., Cristóvão, G., Rodrigues, L., Meyer-Fernandes, J.R., Gonçalves, T., Cunha, R.A. and C.A. Gomes. (2015) Different danger signals differently impact on microglial proliferation through alterations of ATP release and extracellular metabolism. Glia. 63(9):1636-45.

Ghosh, S., Howe, N., Volk, K., Tati, S., Nickerson, K.W. and 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 Med Microbiol. 60(1):63-73.

Grinberg S, Haskó G, Wu D and Leibovich SJ. (2009). Suppression of PLCbeta2 by endotoxin plays a role in the adenosine A(2A) receptor-mediated switch of macrophages from an inflammatory to an angiogenic phenotype. Am. J. Pathol. 175, 2439-2453.

Guilherme A, Meyer-Fernandes JR and Vieyra A. (1991) Reversible inhibition by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid of the plasma membrane (Ca(2+)+Mg2+)ATPase from kidney proximal tubules. Biochemistry 30, 5700-5706.

Haskó G, Kuhel DG, Chen JF, Schwarzschild MA, Deitch EA, Mabley JG, Marton A and Szabó C. (2000). Adenosine inhibits IL-12 and TNF-[alpha] production via adenosine A_{2a} receptor-dependent and independent mechanisms. FASEB J. 14, 2065-2074.

Haskó G, Kuhel DG, Salzman AL and Szabó C. (2000). ATP suppression of interleukin-12 and tumour necrosis factor-alpha release from macrophages. Br. J. Pharmacol. 129, 909-914.

Haskó G, Linden J, Cronstein B and Pacher P. (2008). Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat. Rev. Drug Discov. 7, 759-770.

Heinsbroek SE, Taylor PR, Martinez FO, Martinez-Pomares L, Brown GD and Gordon S. (2008). Stage-specific sampling by pattern recognition receptors during *Candida albicans* phagocytosis. PLoS Pathog. 4:e1000218.

Isfort K, Ebert F, Bornhorst J, Sargin S, Kardakaris R, Pasparakis M, Bähler M, Schwerdtle T, Schwab A and Hanley PJ. (2011). Real-time imaging reveals that P2Y2 and P2Y12 receptor agonists are not chemoattractants and macrophage chemotaxis to complement C5a is phosphatidylinositol 3-kinase (PI3K)- and p38 mitogen-activated protein kinase (MAPK)-independent. J Biol Chem. 286, 44776-44787.

Joly S, Ma N, Sadler JJ, Soll DR, Cassel SL and FS Sutterwala. (2009) Cutting edge: *Candida albicans* hyphae formation triggers activation of the Nlrp3 inflammasome. J Immunol. 183(6):3578-81.

Junger WG. (2011). Immune cell regulation by autocrine purinergic signalling. Nat Rev Immunol. 11, 201-212.

Kas-Deelena AM, Bakker WW, Olinga P, Visser J, de Maar EF, van Son WJ, The TH and Harmsen MC. (2001). Cytomegalovirus infection increases the expression and activity of ecto-ATPase (CD39) and ecto-5Pnucleotidase (CD73) on endothelial cells. FEBS Lett. 491, 21-25.

Khoa ND, Montesinos MC, Reiss AB, Delano D, Awadallah N and Cronstein BN. (2001). Inflammatory cytokines regulate function and expression of adenosine A(2A) receptors in human monocytic THP-1 cells. J. Immunol. 167, 4026-4032.

Kiffer-Moreira T, de Sá Pinheiro AA, Alviano WS, Barbosa FM, Souto-Padrón T, Nimrichter L, Rodrigues ML, Alviano CS and Meyer-Fernandes JR. (2007). An ectophosphatase activity in *Candida parapsilosis* influences the interaction of fungi with epithelial cells. FEMS Yeast Res. 7, 621-628.

Köröskényi K, Duró E, Pallai A, Sarang Z, Kloor D, Ucker DS, Beceiro S, Castrillo A, Chawla A, Ledent CA, et al. (2011). Involvement of adenosine A_{2A} receptors in engulfment-dependent apoptotic cell suppression of inflammation. J. Immunol. 186: 7144-7155.

Koshlukova SE, Araujo MW, Baev D and Edgerton M. (2000). Released ATP is an extracellular cytotoxic mediator in salivary histatin 5-induced killing of *Candida albicans*. Infect. Immun. 68, 6848-6856.

Kronlage M, Song J, Sorokin L, Isfort K, Schwerdtle T, Leipziger J, Robaye B, Conley PB, Kim HC, Sargin S, et al. (2010). Autocrine purinergic receptor signaling is essential for macrophage chemotaxis. Sci. Signal. 3:ra55.

Kumar V and Sharma A. (2009). Adenosine: an endogenous modulator of innate immune system with therapeutic potential. Eur. J. Pharmacol. 616, 7-15.

Livak KJ and Schmittgen TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

Lorenz MC, Bender JA and Fink GR. (2004). Transcriptional response of *Candida albicans* upon internalization by macrophages. Eukaryot. Cell 3, 1076-1087.

Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N and G Schuler. (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223: 77-92.

Mahamed DA, Mills JH, Egan CE, Denkers EY and Bynoe MS. (2012). CD73-generated adenosine facilitates Toxoplasma gondii differentiation to long-lived tissue cysts in the central nervous system. Proc. Natl. Acad. Sci. U S A 109,16312–16317.

Marcil A, Harcus D, Thomas DY and Whiteway M. (2002). *Candida albicans* killing by RAW 264.7 mouse macrophage cells: effects of Candida genotype, infection ratios, and gamma interferon treatment. Infect. Immun. *70*, 6319-6329.

Marques-da-Silva C, Rodrigues JC, Corte-Real S, Coutinho-Silva R, and Persechini PM. (2011) Differential modulation of ATP-induced P2X7-associated permeabilities to cations and anions of macrophages by infection with *Leishmania amazonensis.* PLoS One. 6, e25356.

Martinez-Solano L, Nombela C, Molero G and Gil C. (2006). Differential protein expression of murine macrophages upon interaction with *Candida albicans*. Proteomics. 6, S133-144.

Mayne M, Fotheringham J, Yan HJ, Power C, Del Bigio MR, Peeling J and Geiger JD. (2001). Adenosine A_{2A} receptor activation reduces proinflammatory events and decreases cell death following intracerebral hemorrhage. Ann. Neurol. 49, 727-735.

Mello PA, Filippi-Chiela EC, Nascimento J, Beckenkamp A, Santana DB, Kipper F, Casali EA, Nejar Bruno A, Paccez JD, Zerbini LF, Wink MR, Lenz G and A Buffon. (2014) Adenosine uptake is the major effector of extracellular ATP toxicity in human cervical cancer cells. Mol Biol Cell. 25(19):2905-18.

Murphree LJ, Sullivan GW, Marshall MA and Linden J. (2005). Lipopolysaccharide rapidly modifies adenosine receptor transcripts in murine and human macrophages: role of NF-κB in A2A adenosine receptor induction. Biochem. J. 391, 575–580.

Németh ZH, Csóka B, Wilmanski J, Xu D, Lu Q, Ledent C, Deitch EA, Pacher P, Spolarics Z and Haskó G. (2006). Adenosine A_{2A} receptor inactivation increases survival in polymicrobial sepsis. J. Immunol. 176, 5616-5626.

Netea MG, Gow NAR, Munro CA, Bates S, Collins, Ferwerda G, Hobson RP, Bertram G, Hughes HB, Jansen T, et al. (2006). Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. J. Clin. Invest. 116, 1642–1650.

Netea MG, Simon A, van de Veerdonk F, Kullberg BJ, Van der Meer JW and Joosten LA. (2010). IL-1beta processing in host defense: beyond the inflammasomes. PLoS Pathog. 6, e1000661.

Ouyang X, Ghani A, Malik A, Wilder T, Colegio OR, Flavell RA, Cronstein BN and Mehal WZ. (2013). Adenosine is required for sustained inflammasome activation via the A_{2A} receptor and the HIF-1α pathway. Nat. Commun. 4, 2909. doi: 10.1038/ncomms3909.

Pfaller MA and Diekema DJ. (2007). Epidemiology of invasive candidiasis: a persistent public health problem. Clinic. Microbiol. Rev. 20, 133–163.

Pfeiffer ZA, Guerra AN, Hill LM, Gavala ML, Prabhu U, Aga M, Hall DJ and Bertics V. (2007). Nucleotide receptor signaling in murine macrophages is linked to reactive oxygen species generation. Free Radic. Biol. Med. 42, 1506-1516.

Pinhal-Enfield G, Ramanathan M, Haskó G, Vogel SN, Salzman AL, Boons GJ and Leibovich SJ. (2003). An angiogenic switch in macrophages involving synergy between Toll-like receptors 2, 4, 7, and 9 and adenosine A_{2A} receptors. Am. J. Pathol. 163, 711-721.

Pinheiro CM, Martins-Duarte ES, Ferraro RB, Fonseca de Souza AL, Gomes MT, Lopes AH, Vannier-Santos MA, Santos AL and Meyer-Fernandes MR. (2006). *Leishmania amazonensis:* Biological and biochemical characterization of ecto-nucleoside triphosphate diphosphohydrolase activities. Exp. Parasitol. 114, 16-25.

Plantinga TS, Johnson MD, Scott WK, Joosten LA, van der Meer JW, Perfect JR, Kullberg BJ and Netea MG. (2012). Human genetic susceptibility to *Candida* infections. Med. Mycol. 50,785-794.

Ramakers BP, Riksen NP, Stal TH, Heemskerk S, van den Broek P, Peters WH, van der Hoeven JG, Smits P and P Pickkers. (2011) Dipyridamole augments the antiinflammatory response during human endotoxemia. Crit Care. 15(6):R289.

Rodrigues L, Russo-Abrahão T, Cunha RA, Gonçalves T and JR Meyer-Fernandes. (2015) Characterization of extracellular nucleotide metabolism in *Candida albicans*. FEMS Microbiol Let. DOI:10.1093/femsle/fnv212.

Romani L. (2011). Immunity to fungal infections. Nat. Rev. Immunol. 11, 275-288.

Romio M, Reinbeck B, Bongardt S, Hüls S, Burghoff S and Schrader J. (2011). Extracellular purine metabolism and signaling of CD73-derived adenosine in murine Treg and Teff cells. Am. J. Physiol. Cell Physiol. 301, C530–C539.

Sansom FM, Newton HJ, Crikis S, Cianciotto NP, Cowan PJ, d'Apice AJ and Hartland EL. (2007). A bacterial ecto-triphosphate diphosphohydrolase similar to human CD39 is essential for intracellular multiplication of *Legionella pneumophila*. Cell Microbiol. 9, 1922-1935.

Sardi JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM and Mendes Giannini MJS. (2013). Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J. Med. Microbiol. 62, 10–24.

Seider K, Heyken A, Lüttich A, Miramón P and Hube B. (2010). Interaction of pathogenic yeasts with phagocytes: survival, persistence and escape. Curr Opin Microbiol. 13, 392-400.

Sitkovsky M, Lukashev D, Apasov S, Kojima H, Koshiba M, Caldwell C, Ohta A and Thiel M. (2004). Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors. Annu. Rev. Immunol. 22, 657–682.

Sitkovsky MV and Ohta A. (2005). The 'danger' sensors that STOP the immune response: the A2 adenosine receptors? Trends Immunol. 26, 299-304.

Smail EH, Cronstein BN, Meshulam T, Esposito AL, Ruggeri RW and Diamond RD. (1992). *In vitro*, *Candida albicans* releases the immune modulator adenosine and a second, high-molecular weight agent that blocks neutrophil killing. J. Immunol. 148, 3588-3595.

Sperlágh B, Haskó G, Németh Z and Vizi ES. (1998). ATP released by LPS increases nitric oxide production in raw 264.7 macrophage cell line via P2Z/P2X7 receptors. Neurochem. Int. 33, 209-215.

Streitová D, Hofer M, Holá J, Vacek A and Pospísil M. (2010). Adenosine A_1, A_{2a}, A_{2b}, and A_3 receptors in hematopoiesis. 2. Expression of receptor mRNA in resting and lipopolysaccharide-activated mouse RAW 264.7 macrophages. Physiol. Res. 59, 139-144.

Sullivan GW, Fang G, Linden J and Scheld WM. (2004). A_{2A} adenosine receptor activation improves survival in mouse models of endotoxemia and sepsis. J. Infect. Dis. 189, 1897-1904.

Thiel M, Caldwell CC and Sitkovsky MV. (2003). The critical role of adenosine A2A receptors in downregulation of inflammation and immunity in the pathogenesis of infectious diseases. Microbes Infect. 5, 515-526.

Vásquez-Torres A and Balish E. (1997). Macrophages in resistance to candidiasis. Microbiol. Mol. Biol. Rev. 61, 170–192.

Vylkova S, Sun JN and Edgerton M. (2007). The role of released ATP in killing *Candida albicans* and other extracellular microbial pathogens by cationic peptides. Purinergic Signal. 3, 91-97.

CHAPTER IV

CHARACTERIZATION OF EXTRACELLULAR NUCLEOTIDE METABOLISM IN *CANDIDA ALBICANS*

From:

Rodrigues L, Russo-Abrahão T, Cunha RA, Gonçalves T and JR Meyer-Fernandes. (2015) Characterization of extracellular nucleotide metabolism in *Candida albicans*. FEMS Microbiol Let. DOI:10.1093/femsle/fnv212.

ABSTRACT

Candida albicans is the most frequent agent of human disseminated fungal infection. Ectophosphatase and ectonucleotidase activities are known as influencing the infectious potential of several microbes, including other non-*albicans Candida* species. With the present work we aim to characterize this ecto-enzymatic activities in *C. albicans*. We found that *C. albicans* does not have a classical ecto-5'-nucleotidase enzyme and 5'AMP is cleaved by a phosphatase instead of exclusively by a nucleotidase, that also can use 3'AMP as a substrate. Moreover, these enzymatic activities are not dependent of secreted soluble enzymes and changed when the yeast cells are under infection conditions, including low pH, higher temperature and CO_2 content.

INTRODUCTION

Among the great diversity of fungi, only a small subset can be considered pathogenic to humans, since different species are found to be ubiquitous in the environment and part of human body normal flora (Brown, 2012). However, among those, some have emerged as important human agents of infection. In the last decades, *Candida albicans* is being considered the most important opportunistic agent of fungal infections, with an associated mortality of 30-55%, especially concerning individuals with immune diseases or with weakened immune system (Pfaller and Diekema, 2007; Grubb *et al*., 2008; Rodloff *et al*., 2011; Czaika *et al*., 2013).

The biological properties of *C. albicans* and its ability to successfully adapt to different environments with, for instance, the yeast-to-hypha switching, adaptation of

metabolism and expression of several proteins that contribute to adherence and invasion, hampers the understanding of *C. albicans* pathogenic mechanisms (Grubb *et al*., 2008; Bain *et al*., 2012; Cheng *et al*., 2012; Brunke and Hube, 2013; Gow *et al*., 2013; Mayer *et al*., 2013). Undoubtfully, ectoenzymes attached to the cell membrane or secreted to extracellular milieu, represent important virulence factors, contributing to adhesion, invasion and residence inside phagosomes (*e.g.*, Schaller *et al*., 2005; Frohner *et al*., 2009; Bhat *et al*., 2011). In this group of factors, secreted hydrolases, lipases, phospholipases and proteases facilitate penetration of host cells by *C. albicans* and contribute to extracellular nutrient recruitment (reviewed by Mayer *et al*., 2013). Also, the importance of ectonucleotidases, hydrolyzing extracellular nucleotides to its respective nucleosides, and of ectophosphatases, dephosphorylating specific protein residues, is undeniable (Robson *et al*., 2006; Gomes *et al*., 2011; Paletta-Silva and Meyer-Fernandes, 2012). Indeed, several studies have been revealing that the conversion of extracellular ATP into adenosine (ADO) is determinant in the efficiency of microorganisms infection (Kas-Deelena *et al*., 2001; Romio *et al*., 2011; Mahamed *et al*., 2012), affecting pathogen interaction (Kiffer-Moreira *et al*., 2007; Russo-Abrahão *et al*., 2011) and adhesion to different host cells (Kiffer-Moreira *et al*., 2010; Portela *et al*., 2010; Kneipp *et al*., 2012; Cosentino-Gomes *et al*., 2013) and being a correlate of their pathogenicity (de Jesus *et al*., 2002; Bisaggio *et al*., 2003; Pinheiro *et al*., 2006; Sansom *et al*., 2007; Figueiredo *et al*., 2012). In that context, various proteins involved in the complex machinery of ATP-ADP-AMP-ADO cascade, with active sites facing external medium, have been highlighted in host-pathogen interactions. In *Streptococcus agalactiae* it was recently described that the absence of ecto-5'nucleotidase leads to decreased viability of bacteria in blood and that this effect is mediated by adenosine (Firon *et al*., 2014). In fact, in recent years and due to its anti-inflammatory and

immunosuppressive effects, adenosine and its sensing devices, namely adenosine 2A receptors, have emerged as important targets in the context of infection (Thiel *et al*., 2003; Németh *et al*., 2006). Thammavongsa and colleagues (2009) have shown that some pathogens, like *Staphylococcus aureus* and *Bacillus anthracis*, can use adenosine to overcome host immune responses. Thus, due to the arising interest in the purinergic system, mechanisms leading to the recognition and generation of specific external and internal signals have been widely described in several microorganisms. This is not the case of *C. albicans*, where the role of these enzymes is far from being fully understood and defined in the infection context. So, the present work was aimed to characterize the properties of *C. albicans* ectophosphatase and ecto-5'-nucleotidase activities.

MATERIALS AND METHODS

Strain, media and cell culture conditions

Candida albicans YP0037 strain (Microbiology Pathogenic Yeast Collection, University of Coimbra) was isolated from a hemoculture. Yeast cells were maintained and grown on YPD (Yeast Peptone Dextrose) agar (0.5 % yeast extract, 1 % bacto-peptone, 2 % agar and 2 % glucose) plates at 28 ºC. For the different enzymatic activity measurements, yeasts were grown in those condition for, at least, 48 h. Cells were harvested and obtained by centrifugation after washed twice in a solution containing 116 mM NaCl, 5.4 mM glucose, 50 mM HEPES buffer (pH 7.2). Cell growth was assessed by counting the yeast cells in a Neubauer chamber.

Ecto-5'-nucleotidase activity measurements

Ecto-5'-nucleotidase activity were determined by the rate of inorganic phosphate (Pi) released. Intact cells (3 x 10^9 cells) were incubated for 1 h at RT in 0.5 mL of reaction mixture containing, unless otherwise specified, 116 mM NaCl, 5.4 mM glucose, 50 mM HEPES-MES-Tris buffer (pH 4) and 5 mM 5'AMP as substrate. The reaction was stopped by the addition of 1 mL of 25% charcoal in 0.1 M HCl (Guilherme *et al*., 1991). Controls in which cells were added after reaction stop were considered as blanks. After the reaction, tubes were centrifuged at 1500 g for 15 min at 4^{o}C, and 0.1 mL of the supernatant was added to 0.1 mL of Fiske Subbarow reactive mixture (Fiske and Subbarow, 1925). The absorbance of the released Pi was measured spectrophotometrically at 650 nm. The ecto-5'-nucleotidase activity was calculated by subtracting the nonspecific 5'AMP hydrolysis in blanks; the concentration of Pi released in the reaction was determined using a standard curve of Pi. Several variables were tested, including, cell number, pH, and incubation conditions (temperature, CO_2 content, incubation time), as well as diverse substrates (mono-, di- and trinucleotides, under the same conditions).

Ectophosphatase activity measurements

Differences in phosphatase activity were determined under the same conditions used for quantification of ecto-5'-nucleotidase activity, with a reaction mixture containing 5 mM *p*-nitrophenyl phosphate (*p*-NPP) as substrate. To determine the concentration of *p*-nitrophenol formed due to phosphatase activity, the reaction was stopped with the addition of 1 mL of 1 M NaOH. The quantification procedure was similar to the described above, with mixture measured spectrophotometrically at 425 nm and using *p*-nitrophenol as standard (Fernandes *et al*., 1997).

Competition assay

Ectophosphatase activity of living *C. albicans* was also quantified under the cumulative presence of *p*-NPP and 5'AMP. The assay conditions described above were kept during this assay, but 5 mM 5'AMP was added to the normal reaction mixture.

Inhibition assay

C. albicans ecto-5'-nucleotidase and ecto-phosphatase activities during 5'AMP and *p*-NPP cleavage, were tested in the presence of different concentrations (0.01, 0.02, 0.05, 0.10, 0.20, 0.50 and 1 mM) of three phosphatase inhibitors: sodium orthovanadate, sodium fluoride and zinc chloride. When testing for ATP hydrolysis, 1 mM of sodium orthovanadate was also assessed.

Statistics

Data are presented as means ± SEM. Statistical differences were determined with Student's *t* test and one and two-way ANOVA. Results with $p<0.05$ were considered statistically significant.

RESULTS

The presence of ectonucleotidase activity in *Candida albicans* was confirmed during this work by the fact that in different assay conditions intact yeast cells were able to hydrolyse 5'AMP. Actually, it seems that, in this cellular model, ecto-5'-nucleotidase activity is dependent of several variables, as seen with the different responses obtained. As expected, ecto-5'-nucleotidase activity seems to be proportional to the cell number

used in the study (Figure 1A), reason why, in order to achieve confident results, a high cell number (3 x 10^9 cells) was selected and used in all the subsequent assays. A range of different pH values was then tested and, as seen with cell number, the enzymatic activity varies with pH (Figure 1B). The yeast cells ecto-5'-nucleotidase activity was higher under acidic pH levels and decreases as pH increases to higher alkalinity. We also measured ecto-5'-nucleotidase activity under conditions closely related to the ones yeast cells found during host infection, i.e. temperature around 37 ºC and higher CO_2 content, and a decreased enzymatic activity was found (Figure 1C). Besides, when (accidentally) testing for different oxygenation conditions, we found lower enzymatic activity under low oxygenation, even though, yeast viability and ecto-5'-nucleotidase activity remained unaltered under the different incubation periods (data not shown). To verify if 5'AMP cleavage was or not dependent of secreted soluble enzymes and to confirm the putative ecto-localization of those enzymes, we designed an assay aimed to compare intact cells and supernatants. Supernatants were obtained after centrifugation of a normal reaction mixture with cells incubated in the absence of 5'AMP and tested for nucleotidase activity. As seen in Figure 1D, supernatants failed to hydrolyze 5'AMP substrate, while intact cells retained the efficiency of 5'AMP hydrolyses.

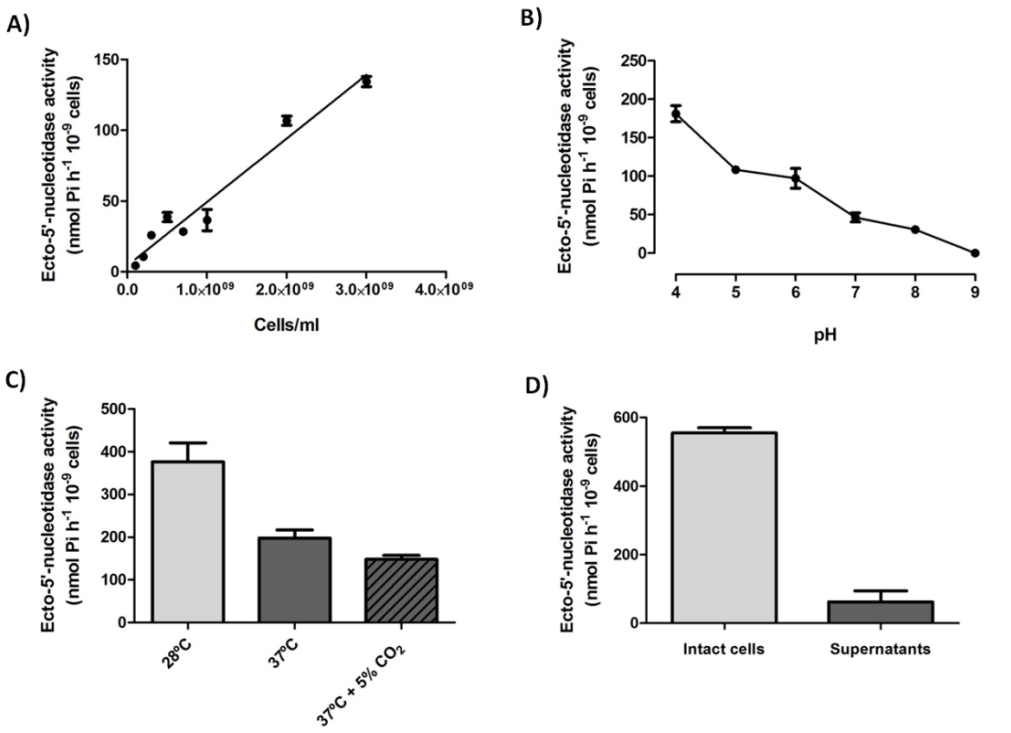

Figure 1. *C. albicans* ecto-5'-nucleotidase activity in diferent conditions.

Enzymatic activity seems to be dependent of: A) cell number, as enzymatic activity increases with the cell number; B) pH, with maximal Pi cleavage at low pH values and; C) temperature, when comparing the usual temperature for yeast growth with temperature and CO_2 concentration close to the ones found in an infection condition. All this enzymatic activity was quantified in intact cells and seems to be independent of eventual secreted enzymes (D). Data are shown as means ± SEM: A and B are representative of a set of experiments; C and D represent the means of at least three independent experiments.

Besides the ecto-5'-nucleotidase activity, ecto-phosphatase enzymatic activity was also assessed in this yeast (Figure 2). Under the same conditions, *p*-NPP hydrolysis (a phosphatase substrate cleaved into *p*-nitrophenol, detected by a colorimeric reaction) seems to be about two-fold higher when compared with 5'AMP cleavage. These results, somehow lead to question about the specificity of AMP hydrolysis.

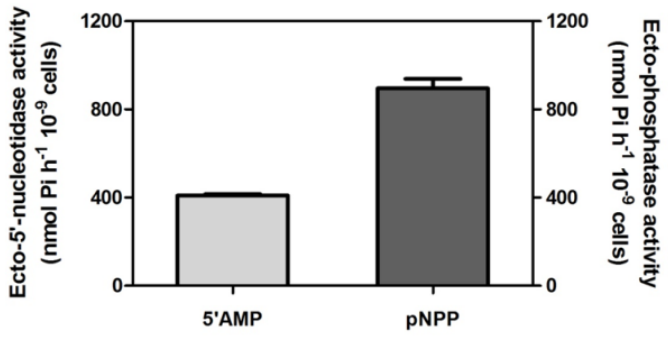

Figure 2. *C. albicans* ecto-5'-nucleotidase and ecto-phosphatase enzymatic activity.

Intact cells were incubated for 1h at RT in the reaction medium described in Materials and methods, using 5'AMP or pNPP as substrates. Data are shown as means ± SEM of at least three independent experiments.

When intact cells were incubated with *p*-NPP and 5'AMP, together, as substrates, no relevant differences on *p*-nitrophenol formation was detected (Figure 3A). Moreover, the putative *C. albicans* ecto-5'-nucleotidase enzyme was affected by several phosphatase inhibitors, reinforcing the doubts about this enzyme specificity. In fact, nucleotidase activity was inhibited by potent phosphatase inhibitors such as sodium orthovanadate, sodium fluoride and zinc chloride (Figure 3B).

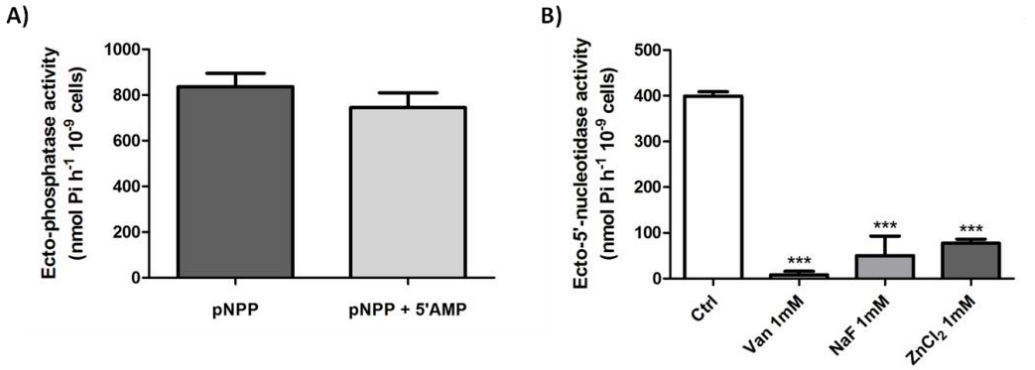

Figure 3. *C. albicans* ecto-phosphatase and ecto-5'-nucleotidase enzymatic activity modulation by competition between substrates and phosphatase inhibitors.

Intact cells were incubated for 1h at room temperature in the reaction medium described in Materials and methods. A) Putative competition between 5'AMP and pNPP substrates assessed using both compounds with ecto-phosphatase

activity methodology. B) 5'AMP cleavage was measured through ecto-5'-nucleotidase enzymatic activity technique in the presence of sodium orthovanadate, sodium fluoride and zinc chloride (1mM). Data are shown as means ± SEM of at least three independent experiments; *** - $p<0.001$.

The specificity of substrates of yeasts ecto-5'-nucleotidase was further investigated. Intact *C. albicans* cells were able to hydrolyze all monophosphate substrates tested (AMP, CMP, GMP, IMP and UMP), with slight differences, not statistically significant among, as well as the correspondent di- and triphosphate substrates, as indicated in Figure 4A, B and C. Remarkably, it should be pointed that *C. albicans* cells were able of hydrolyze 3'AMP (Figure 4A). Aligned with the findings that phosphatase inhibitors affect the *C. albicans* ecto-5'-nucleotidase, we also observed that sodium orthovanadate, under two different concentrations (0.1 and 1 mM), was able to inhibit nucleotidase activity in about 60% and 80%, respectively (Figure 4D).

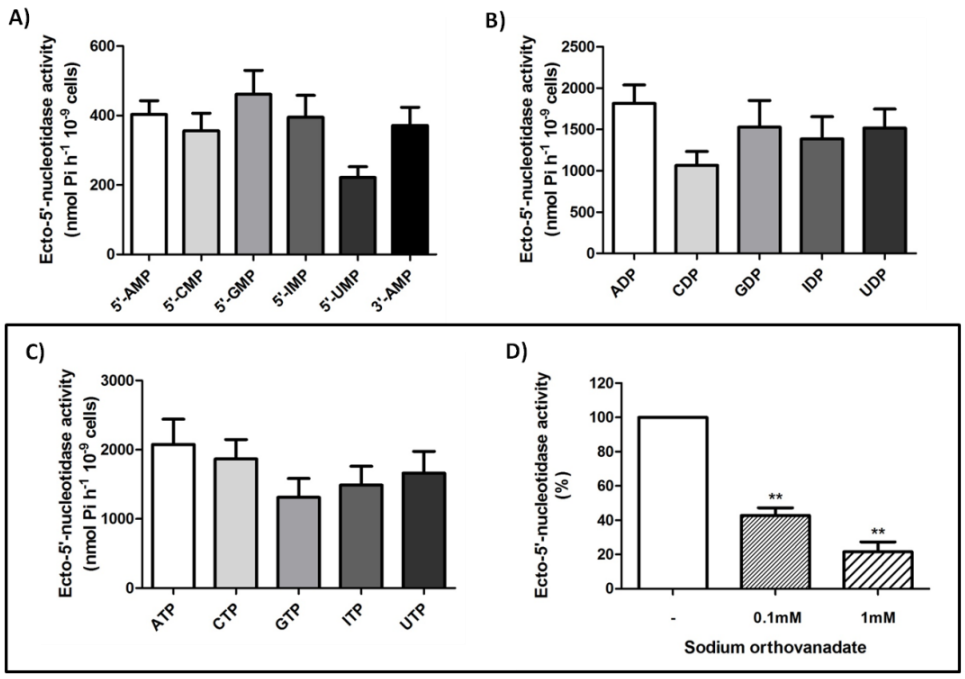

Figure 4. *C. albicans* ectonucleotidase activity substrate specificity.

Intact cells were incubated for 1h at RT in the reaction medium described in Materials and methods, with 5 mM of each indicated **A)** mononucleotide, **B)** dinucleotide and **C)** trinucleotide substrate. **D)** Effect of sodium orthovanadate on ectonucleotidase activity: ATP (5 mM) was used as substrate with 0.1 or 1 mM of sodium orthovanadate. Data are

shown as means ± SEM of at least three independent experiments; ** - $p<0.01$, with 2076.82 ± 363.3 nmol Pi h^{-1} 10^{-9} cells considered as absolute values.

DISCUSSION

The extracellular metabolism of ATP has been identified as controlling several homeostasis processes from thermogenesis and homeothermy, along with hormonal regulation (Silva, 2006), to multiple mechanisms of cellular energetic supplies, of undoubtedly importance, for instance, to skeletal and cardiac muscles sustainment (Hochachka *et al*., 1997; Dzeja and Terzic, 2003). In microbes, the understanding was that ectophosphate activity was important as a mean of obtaining nutrients from the environment. Lately it emerged the importance of this metabolism in human pathogens. *Leishmania donovani, Cryptococcus neoformans, Candida parapsilosis* or *Staphylococcus aureus* were described as having ectophosphatases and ectonucleotidases that are considered as determinants in its virulence (Gomes *et al*., 2011; Russo-Abrahão *et al*., Freitas-Mesquita *et al*., 2014). As an example, while ecto-phosphatases were described to be present in *C. neoformans*, ecto-5'-nucleotidase was found to be important in *Saccharomyces cerevisiae* (Collopy-Junior *et al*., 2006; Itoh, 1994). However, in *Leishmania* spp. or *C. parapsilosis* (with ecto-ATPase, ecto-ADPase) both types of enzymes can be found (Paletta-Silva and Meyer-Fernandes, 2012; Kiffer-Moreira *et al*., 2007 and 2010; Russo-Abrahão *et al*., 2011). Taking this information into account, we now characterised *C. albicans* in what regards both ectophosphatase and ecto-5'-nucleotidase activities.

The ectophosphatases usually described in fungi are considered acid ectophosphatases with pH range inferior to 6. In *C. parapsilosis*, for instance, it was

described ecto-phosphatase activity in optimum pH levels of 4.5 (Russo-Abrahão *et al*., 2011), while in *C. neoformans* this activity was found higher at 5.5 (Collopy-Junior *et al*., 2006). Now, in *C. albicans*, we observed a maximal ectonucleotidase activity at pH 4. In fact this yeast is mainly associated with animal hosts, especially with human compartments. In particular, *C. albicans* is usually described as being able to cope with the low pH of the phagolysosome of innate immune cell (Geisow *et al*., 1981; Vylkova and Lorenz, 2014). So, we believe that this maximal activity at a pH lower than in other pathogenic yeasts might be relevant to *C. albicans* cells survival/escape to phagolysosome when infecting host cells, a key feature contributing to its success as a facultative intracellular pathogen. On the other hand, ecto-5'-nucleotidase activity decreases with a temperature of 37 ºC and with CO_2 5%, the ambient conditions with what *C. albicans* copes when colonizing the host. Moreover, yeast cells grown in the absence of oxygen and left to grow until late stationary (batch culture, no oxygenation, 48h), have lower activity (results not shown). So, taken together, all these results prompt us to wonder if during infection, when the yeast cells are inside the host cells, in particular inside acidic compartments, yeasts are more prone to hydrolyze these substrates than during extracellular life (either under non-infectious conditions or infecting the host but extracellularly, as observed when yeast cells are colonizing mucosal surfaces).

As described previously, it was reported that *C. parapsilosis* possesses both ecto-phosphatases and ecto-5'-nucleotidase enzymes, with specific properties associated to each type of enzyme (Kiffer-Moreira *et al*., 2007; Russo-Abrahão *et al*., 2011). Nevertheless, it seems that *C. albicans* does not have a classical ecto-5'-nucleotidase enzyme, as described in other organisms. *C. albicans* ectonucleotidases are able to use 5'AMP as substrate, but contrarily to what was observed in *C. parapsilosis*, this enzyme

is inhibited by several phosphatase inhibitors, revealing an unlike enzymatic profile. In addition to this, *C. albicans* ectonucleotidases are also able to use numerous other substrates, with particular relevance on 3'AMP. In fact, a 3'nucleotidase/nuclease activity was observed in *Leishmania* spp., related to different aspects of parasite virulence, from nutrition to infection and escape to neutrophil extracellular traps (NETs) (Palleta-Silva and Meyer-Fernandes, 2012; Guimarães-Costa *et al*., 2014). This activity was not described in other yeasts until now, but since it is known that this substrate together with cAMP (3'-5'-cyclic adenosine monophosphate) are involved and determinant to the yeast-to-hypha transition of *C. albicans* (Sabie and Gadd, 1992; Sudbery, 2011), we can speculate on the importance of this enzymatic activity in yeast survival and persistence either in the epithelial surfaces and/or once internalized by phagocytic cells. Although the exact nature and specificity of *C. albicans* ectonucleotidases are not completely established, data presented here highlights the importance of those enzymes in the context of infection, helping yeasts to overcome host defences.

REFERENCES

Bain JM, Lewis LE, Okai B, Quinn J, Gow NAR and Erwig LP. (2012) Non-lytic expulsion/exocytosis of *Candida albicans* from macrophages. Fungal Genet Biol. 49: 677–678.

Bisaggio DF, Peres-Sampaio CE, Meyer-Fernandes JR and Souto-Padrón T. (2003) Ecto-ATPase activity on the surface of *Trypanosoma cruzi* and its possible role in the parasite-host cell interaction. Parasitol Res. 91, 273-282.

Brown GD. (2012) Actin' dangerously. Nature 485: 589-590.

Brunke S and Hube B (2013) Two unlike cousins: *Candida albicans* and *C. glabrata* infection strategies. Cel. Microbiol. 15(5), 701–708.

Cheng SC, Joosten LAB, Kullberg BJ and Netea MJ. (2012) Interplay between *Candida albicans* and the Mammalian Innate Host Defense. Infect Immun. doi:10.1128/IAI.06146-11.

Collopy-Junior I, Esteves FF, Nimrichter L, Rodrigues ML, Alviano CS and Meyer-Fernandes JR. (2006) An ectophosphatase activity in *Cryptococcus neoformans*. FEMS Yeast Res. 6(7):1010-7.

Czaika V, Nenoff P, Glöckner A, Fegeler W, Becker K and Schmalreck AF. (2013) Epidemiology and Changes in Patient-Related Factors from 1997 to 2009 in Clinical Yeast Isolates Related to Dermatology, Gynaecology, and Paediatrics. Inter J Microbiol. doi.org/10.1155/2013/703905.

de Jesus JB, de Sá Pinheiro AA, Lopes AH and Meyer-Fernandes JR. (2002) An ectonucleotide ATP-diphosphohydrolase activity in *Trichomonas vaginalis* stimulated by galactose and its possible role in virulence. Z Naturforsch C. 57, 890-896.

Dzeja PP. and Terzic A. (2003) Phosphotransfer networks and cellular energetic. J Exp Biol. 206: 2039-2047.

Fernandes EC, Meyer-Fernandes JR, Silva-Neto MA and Vercesi AE (1997) *Trypanosoma brucei*: ecto-phosphatase activity present on the surface of intact procyclic forms. Z Naturforsch C. 52(5-6):351-8.

Figueiredo AB, Serafim TD, Marques-da-Silva EA, Meyer-Fernandes JR and Afonso LC (2012) *Leishmania amazonensis* impairs DC function by inhibiting CD40 expression via A_{2B} adenosine receptor activation. Eur. J. Immunol. 42, 1203-1215.

Fiske CH. and Subbarow Y. (1925) The colorimetric determination of phosphorus. J. Biol. Chem. 66:375-400.

Freitas-Mesquita, Leocadio A and Meyer-Fernandes JR. (2014) Biochemical properties and possible roles of ectophosphatase activities in fungi. Int. J. Mol. Sci. 15: 2289-2304.

Frohner IE, Bourgeois C, Yatsyk K, Majer O and Kuchler K. (2009) *Candida albicans* cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance. Mol Microbiol. 71: 240–252.

Geisow MJ, D'Arcy Hart P and Young MR. (1981) Temporal changes of lysosome and phagosome pH during phagolysosome formation in macrophages: studies by fluorescence spectroscopy. J Cell Biol. 89(3):645-52.

Gomes MT, Lopes AH and Meyer-Fernandes JR. (2011) Possible roles of ectophosphatases in host-parasite interactions. J Parasitol Res. doi:10.1155/2011/479146.

Gow NAR, van de Veerdonk F, Brown AJP and Netea MG. (2013) *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. Nat Rev Microbiol. 10(2): 112–122.

Grubb SEW, Murdoch, Sudbery PE, Saville SP, Lopez-Ribot JL and Thornhill MH. (2008) *Candida albicans*-endothelial cell interactions: a key step in the pathogenesis of systemic candidiasis. Infect Immun. 76, 10: 4370–4377.

Guilherme A, Meyer-Fernandes JR and Vieyra A. (1991) Reversible inhibition by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid of the plasma membrane (Ca(2+)+Mg2+)ATPase from kidney proximal tubules. Biochemistry 30(23):5700-6.

Guimarães-Costa AB, DeSouza-Vieira TS, Paletta-Silva R, Freitas-Mesquita AL, Meyer-Fernandes JR and Saraiva EM. (2014) 3'-nucleotidase/nucleasse activity. Allows *Leishmania* parasites to escape killing by neutrophil extracellular traps. Infect Immun. 82(4):1732-40

Hochachka PW and McClelland GB. (1997) Cellular metabolic homeostasis during large-scale change in ATP turnover rates in muscles. J Exp Biol. 200: 381–386.

Itoh R. (1994) Purification and some properties of an IMP-specific 5'-nucleotidase from yeast. Biochem J. 298 Pt 3:593-8.

Kas-Deelena AM, Bakkerb WW, Olingad P, Visserd J, de Maarc EF, et al. (2001) Cytomegalovirus infection increases the expression and activity of ecto-ATPase (CD39) and ecto-5Pnucleotidase (CD73) on endothelial cells. FEBS Letters 491, 21-25.

Kiffer-Moreira T, de Sa Pinheiro AA, Alviano WS, Barbosa FM, Souto-Padron T, et al. (2007) An ectophosphatase activity in *Candida parapsilosis* influences the interaction of fungi with epithelial cells. FEMS Yeast Res. 7, 621-628.

Kiffer-Moreira T, Sampaio MEF, Alviano DS, Axelband F, Cesar GV, Cosentino-Gomes D, Rodrigues ML, Nimrichter L, Vieyra A, Alviano CS and Meyer-

Fernandes JR. (2010) Biochemical characterization of an ecto-ATPdiphosphohydrolase activity in *Candida parapsilosis* and its possible role in adenosine acquisition and pathogenesis. FEMS Yeast Res 10: 735–746.

Mahamed DA, Mills JH, Egan CE, Denkers EY and Bynoe MS. (2012) CD73-generated adenosine facilitates *Toxoplasma gondii* differentiation to long-lived tissue cysts in the central nervous system. PNAS 109, 16312–16317.

Mayer FL, Wilson D and Hube B (2013) *Candida albicans* pathogenicity mechanisms. Virulence 4:2, 119–128.

Nemeth ZH, Csoka B, Wilmanski J, Xu D, Lu Q., et al. (2006) Adenosine A_{2A} receptor inactivation increases survival in polymicrobial sepsis. J. Immunol. 176, 5616-5626.

Paletta-Silva R and Meyer-Fernandes JR. (2012) Adenosine and immune imbalance in visceral leishmaniasis: The possible role of ectonucleotidases. J Trop Med. doi:10.1155/2012/650874.

Pfaller MA and Diekema DJ. (2007) Epidemiology of invasive candidiasis: a persistent public health problem. Clinic. Microbiol. Rev. 20, 133–163.

Pinheiro CM, Martins-Duarte ES, Ferraro RB, Fonseca de Souza AL, Gomes MT, et al. (2006) *Leishmania amazonensis*: Biological and biochemical characterization of ecto-nucleoside triphosphate diphosphohydrolase activities. Exp. Parasitol. 114, 16-25.

Rodloff AC, Koch D and Schaumann R (2011) Epidemiology and antifungal resistance in invasive candidiasis. Eur J Med Res. 16: 187-195.

Romio M, Reinbeck B, Bongardt S, Hüls S, Burghoff S and Schrader J. (2011) Extracellular purine metabolism and signaling of CD73-derived adenosine in murine Treg and Teff cells. Am J Physiol Cell Physiol 301: C530–C539.

Russo-Abrahão T, Cosentino-Gomes D, Daflon-Yunes N and Meyer-Fernandes JR. (2011) *Giardia duodenalis*: Biochemical characterization of an ecto-5'-nucleotidase activity. Expl Parasitol. 127: 66–71.

Russo-Abrahão T, Cosentino-Gomes D, Gomes MT, Alviano DS, Alviano CS, Lopes AH and Meyer-Fernandes JR. (2011) Biochemical properties of *Candida*

parapsilosis ecto-5'-nucleotidase and the possible role of adenosine in macrophage interaction. FEMS Microbiol Lett. 317(1):34-42.

Sabie FT and Gadd GM. (1992) Effect of nucleosides and nucleotides and the relationship between cellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) and germ tube formation in *Candida albicans*. Mycopathologia. 119(3):147-56.

Sansom FM, Newton HJ, Crikis S, Cianciotto NP, Cowan PJ, et al. (2007) A bacterial ecto-triphosphate diphosphohydrolase similar to human CD39 is essential for intracellular multiplication of *Legionella pneumophila*. Cell Microbiol. 9, 1922-1935.

Schaller M, Borelli C, Korting HC and Hube B. (2005) Hydrolytic enzymes as virulence factors of *Candida albicans*. Mycoses, 48: 365–377.

Silva JE. (2006) Thermogenic mechanisms and their hormonal regulation. Physiol Rev 86: 435–464.

Sudbery PE. (2011) Growth of *Candida albicans* hyphae. Nat Rev Microbiol. 9(10):737-48.

Thammavongsa V, Kern JW, Missiakas DM and Schneewind O. (2009) *Staphylococcus aureus* synthesizes adenosine to escape host immune responses. J. Exp. Med. 206, 11: 2417-2427.

Thiel M, Caldwell CC and Sitkovsky MV. (2003) The critical role of adenosine A_{2A} receptors in downregulation of inflammation and immunity in the pathogenesis of infectious diseases. Microbes Infect. 5: 515–526.

Vinaya Bhat, Sharma SM, Shetty V, Shastry CS and Rao V. (2011) Extracellular enzymes of *Candida albicans* and their role in development of denture stomatitis. JIADS 2(1):26-30.

Vylkova S and Lorenz MC. (2014) Modulation of phagosomal pH by *Candida albicans* promotes hyphal morphogenesis and requires Stp2p, a regulator of amino acid transport. PLoS Pathog. 10(3):e1003995.

CHAPTER V

ATP, ADENOSINE AND ADENOSINE A_{2A} RECEPTORS IN MACROPHAGE - NON-*ALBICANS* INTERACTION

Part of these results are included in the MS:

Rodrigues L, Curado F, Coelho C, Cabral V, Figueiredo AB, Cortes L, Meyer-Fernandes JR, Cunha RA and Gonçalves T (2015) *Candida albicans* early interaction with macrophages prevents extracellular ATP increase and adenosine A_{2A} receptor activation. Submitted to mBio.

ABSTRACT

Although *Candida albicans* is still the leading cause of opportunistic fungal infections, non-*albicans* species like *Candida glabrata* and *Candida parapsilosis* have emerged as important pathogens. Yeasts recognition and elimination by host phagocytic cells, such as macrophages, is a complex process involving multiple recognition systems. It is known that ATP and adenosine are important endogenous signalling molecules in immunity and inflammation, contributing to the fine-tuning of these responses and eliminating the danger to the host with minimal damage to healthy tissues. We described here the involvement of ATP and it metabolism through ectoenzymes, adenosine and adenosine A_{2A} receptors in the macrophage response to the yeasts presence. As previously seen with *C. albicans*, upon non-*albicans* species phagocytosis by macrophages, A_{2A} receptors are also relocated into the phagosomal membrane, without altering the relative expression of the Adora2a gene. Even if slightly differences among the different yeasts were observed (probably regarding its physiology and intrinsic behaviour characteristics), it can be concluded that all these opportunistic pathogens, might be modulating the purinergic responses to overcome the immune responses used to clear infections.

INTRODUCTION

Candida species represent the fourth cause of worldwide nosocomial bloodstream infections, with high morbidity and mortality rates associated (Pfaller and Diekema, 2007). *Candida albicans* is responsible for the majority of this opportunistic

infections, but the number of reports due to non-*C. albicans* species has been increasing in the last decades (Nguyen *et al*., 1996; Rodloff *et al*., 2011; Silva *et al*.; 2012). *Candida glabrata* (Pfaller and Diekema, 2007; Seider *et al*., 2011) and *Candida parapsilosis* (Trofa *et al*., 2008; Tóth *et al*., 2014), for instance, are both being described, in recent years, as the second most frequent causes of fungal infections. In addition, several yeasts species, commonly isolated from foods (Loureiro and Querol, 2000; Loureiro and Malfeito-Ferreira, 2003), are as well being considered as important infection agents in debilitated, more susceptible hosts. Among those species, *C. glabrata* and *C. parapsilosis* (Arabatzis *et al*., 2004; Paulo *et al*., 2009; Costa-de-Oliveira *et al*., 2008), together with *Saccharomyces cerevisiae* (Perapoch *et al*., 2000), are frequently isolated from severe yeast infections. Some *in vivo* studies showed that, due to immune system impairments, these pathogens can translocate and disseminate into different organs (Takahashi *et al*., 2003), but it is not completely clear if fungal organisms isolated from foods have actually the ability to cause disease. The importance of macrophages in host immune responses against *Candida* spp. it is nonetheless well established (Vásquez-Torres *et al*., 1997; Martinez-Solano *et al*., 2006; Romani, 2011). Additionally, the involvement of the purinergic system, namely adenosine and adenosine A_{2A} receptors ($A_{2A}R$), arosed as important in the context of infections (Haskó *et al*., 2008; Kumar and Sharma, 2009; Junger, 2011). Robust upregulation of $A_{2A}R$ in macrophages upon bacterial infection was previously described (Khoa *et al*., 2001; Murphee *et al*., 2005; Thiel *et al*., 2003) and *C. albicans* also has the ability to modulate $A_{2A}R$, through it recruitment in macrophages (Chapter III).

However, even though *C. albicans*, *C. glabrata* and *C. parapsilosis* are pathogens, the strategies to invade host cells and to evade the protective response by the host are different among them (Seider *et al*., 2014; Tóth *et al*., 2014). However, while

C. albicans has been widely studied, much remain to be elucidated concerning non-*albicans* species such as *C. glabrata* or *C. parapsilosis* infection (Nucci and Marr, 2005; Seider *et al*., 2011; Tóth *et al*., 2014). Therefore, we explore here the putative involvement of $A_{2A}R$ in the outcome of macrophage infection with non-*albicans* species. In fact and in essence, we show that, regarding *C. glabrata*, *C. parapsilosis* and *S. cerevisiae*, as observed in *C. albicans* infection model, macrophage redistribution of $A_{2A}R$ was also found, despite no changes in the correspondent gene expression was perceived. However, some alterations in ATP levels, ecto-5'-nucleotidase and ecto-phosphatase activities seems to evidence some natural interspecies differences, concerning macrophages and yeasts responses to infection. Although much remain to be elucidated, the present work hence add a new feature to non-*albicans*-host interaction knowledge, opening other possibilities to overcome fungal infections.

MATERIALS AND METHODS

Strains, media and cell culture conditions

Candida albicans YP0037, *Candida glabrata* YP0852, *Candida parapsilosis* YP0515 were isolated from hemoculture while *Saccharomyces cerevisiae* YP0907 was isolated from urine, all belonging to the Microbiology Pathogenic Yeast Collection, University of Coimbra. Yeast cells were grown on YPD agar (0.5 % yeast extract, 1 % bacto-peptone, 2 % agar and 2 % glucose) plates, at 30 ºC, overnight. RAW 264.7 murine macrophages were obtained from the European Collection of Cell Cultures and maintained in DMEM (Sigma-Aldrich) with 10 % non-inactivated FCS (Fetal Calf Serum), 10 mM HEPES, 12 mM sodium bicarbonate and 11 mg/mL sodium pyruvate at

37 ºC, 5 % CO_2 in a humidified atmosphere. Medium was changed every 2 days, until ~70% confluence was reached. RAW 264.7 cells were resuspended in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% inactivated FBS, 23.8 mM sodium bicarbonate and 50 mM glucose for the experimental assays.

Infection assays

Macrophages-yeasts interaction assays were carried out essentially as described by Fernandez-Arenas and co-authors (2008). Briefly, RAW 264.7 macrophage cells were platted in 12-well multiwell plates (with or without 16 mm glass cover-slips, depending on the type of measurement required) for 18 h, at 37 ºC under a 5% CO_2 atmosphere. When the confluence reached around 70%, fungal cells were then added to the macrophages at a MOI (Multiplicity of Infection) of 1:1.

ATP quantification

For ATP quantification, supernatants were collected either from macrophages alone or from macrophages-yeasts co-cultures at 1.5 h, 3 h and 6 h and stored at -80 ºC. ATP quantification was made using the Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma), accordingly with manufactures intructions. The obtained luminescence was measured in a VICTOR^3 Multilabel Plate Reader (PerkinElmer).

Measurement of the activities of ecto-5´-nucleotidase and ecto-phosphatase

RAW 264.7 and yeast cells maintained and co-cultured as previously described were incubated for 1 hour at 37 ºC and 5% CO_2 in 1 mL of reaction mixture containing 116 mM NaCl, 5.4 mM glucose, 50 mM HEPES-MES-Tris buffer (pH 7.2) and 5 mM 5´-AMP (Sigma) as substrate for ecto-5´-nucleotidase. Cell supernatants were collected to

quantify inorganic phosphate (Pi) (Guilherme *et al*., 1991). Briefly, the enzymatic reaction was stopped by the addition of 2 mL of 25% charcoal in 0.1 M HCl to the supernatants, followed by a centrifugation at 1500 *g* for 15 min at 4 ºC. Supernatants (100 μL) were added to 100 μL of Fiske-Subbarow mixture and the absorbance was measured at 650 nm (Spectra Plus spectrophotometer, Molecular Devices). Ecto-5´-nucleotidase activity was calculated by subtracting the non-specific 5´-AMP hydrolysis measured in the absence of cells from the total Pi released, both determined by extrapolation of a concentration curve of standard Pi.

Differences in total ecto-phosphatase activity were determined as previously described (Fernandes *et al*., 1997), using the same conditions as for ecto-5´-nucleotidase, but replacing 5'-AMP by 5 mM *p*-nitrophenyl phosphate (from VWR) as substrate. To determine the concentration of *p*-nitrophenol formed by ecto-phosphatase activity, the reaction was stopped by the addition of 2 mL of NaOH (1 M), followed by spectrophotometric analysis (Spectra Plus spectrophotometer, Molecular Devices) at 425 nm to interpolate a *p*-nitrophenol standard curve (Fernandes *et al*., 1997).

Adora2a relative gene expression

In order to quantify the relative expression of the $A_{2A}R$ gene (Adora2a) in macrophages interacting with *C. albicans*, *C. glabrata*, *C. parapsilosis* and *S. cerevisiae* or in macrophages exposed to 100 ng/ml lipopolysaccharide (LPS, from *Escherichia coli* serotype 026:B6; L-8274, Sigma-Aldrich), we used a real-time PCR approach, selecting the 18S rRNA gene as the reference gene. After carrying out the infection assay as described above, the multiwell plates were iced, scraped and samples transferred to RNAse free tubes at 0 h, 1.5 h, 3 h and 6 h. After centrifugation (10,000 rpm; Minifuge) for 5 min at 4ºC, RNA was extracted using the Magma Pure Compact RNA Isolation

Kit (Roche). The concentration and purity of the extracted RNA was assessed, followed by reverse transcription using a Transcriptor First Strand cDNA Synthesis Kit (Roche). The reverse transcription reaction proceeded in a GeneAmp PCR System 2400 (Perkin Elmer), following the manufacturer's guidelines. The obtained cDNA was used for quantitative RT-PCR on a LightCycler II Carousel-Based System (Roche, software LightCycler 2.0) using A_{2A} primers (63), forward CCGAATTCCACTCCGGTACA and reverse CAGTTGTTCCAGCCCAGCAT and, as reference, 18S gene primers, forward AGGGGAGAGCGGGTAAGAGA and reverse GGACAGGACTAGGCGGAACA. The $2^{-\Delta\Delta CT}$ method (Livak, 2001) was applied to quantify the relative gene expression in macrophages under the different activation conditions.

Immunofluorescence and confocal microscopy

Macrophages grown in coverslips were infected with Oregon Green-labelled yeasts. At the end of each infection period, coverslips were washed twice with ice-cold PBS and fixed with 4 % paraformaldehyde in PBS for 15 min at room temperature. Macrophages were permeabilized with a PBS solution containing 0.2 % Triton X-100 (10 min at room temperature), followed by treatment with a blocking solution of 3 % bovine serum albumin in PBS for 1 h at room temperature. Washing with PBS was made between these incubation periods. Cells were then incubated 1 h at room temperature with the primary goat polyclonal antibody against $A_{2A}R$ (sc7504-R18; Santa Cruz Biotechnology), diluted (1:400) in blocking solution. Coverslips were washed three times and incubated for 1 h at room temperature with the secondary donkey anti-goat IgG antibody labelled with AlexaFluor 594 (A11058, Invitrogen). Cells were subsequently washed three times and the coverslips were mounted with DAKO mounting medium and kept at -20ºC until visualized with fluorescence and/or confocal

microscopy. Digital images were captured using a Zeiss Axioskop2 Plus Microscope or with a Zeiss LSM 510 Meta Confocal Microscope, using Zeiss Zen Blue and ImageJ software to analyse the images.

Cytokines quantification

For TNF-alfa and IL-1beta quantification by ELISA technique, supernatants were collected as described for ATP quantification. Cytokines quantification were respectively made using the Mouse TNF alpha ELISA Ready-SET-Go! and Mouse IL-1 beta ELISA Ready-SET-Go! Kits (eBioscience), according to manufacturer's instructions. The obtained luminescence was then measured at 450 nm in a Spectra Plus spectrophotometer (Molecular Devices).

Statistics

Data are presented as means ± SEM. Statistical differences were determined, whenever applied, with Student's *t* test and one or two-way ANOVA respectively followed by a Newman-Keuls or Bonferroni post hoc test. Data were considered statistically significant at $p<0.05$.

RESULTS

Extracellular levels of ATP during the course of infection

Since ATP represents an important immune-inflammatory signal, we first look at possible alterations in its extracellular levels during *C. albicans*, *C. glabrata*, *C. parapsilosis* and *S. cerevisiae* infection of a macrophage culture. As we previously

reported (Chapter III), because our model includes ATP release and metabolism by two different cell types, we only have find small changes in extracellular levels of ATP, with some variability among them. Macrophage infection with *C. glabrata*, *C. parapsilosis* and *S. cerevisiae* revealed to induced slightly higher ATP levels than in *C. albicans* co-cultures. Whenever macrophages cultures are infected with yeasts, the levels of extracellular ATP (eATP) are higher than in non-infected cell cultures (Figure 1A). Although extracellular ATP levels were similar in most of the different time points, we found significantly higher levels in supernatants of macrophage - *C. glabrata* and - *S. cerevisiae* co-cultures collected at 6 h (respectively, $p<0.01$, 1.5h *vs* 6h and $p<0.05$, 1.5h *vs* 6h; $p<0.01$, 3h *vs* 6h; Figure 1A).

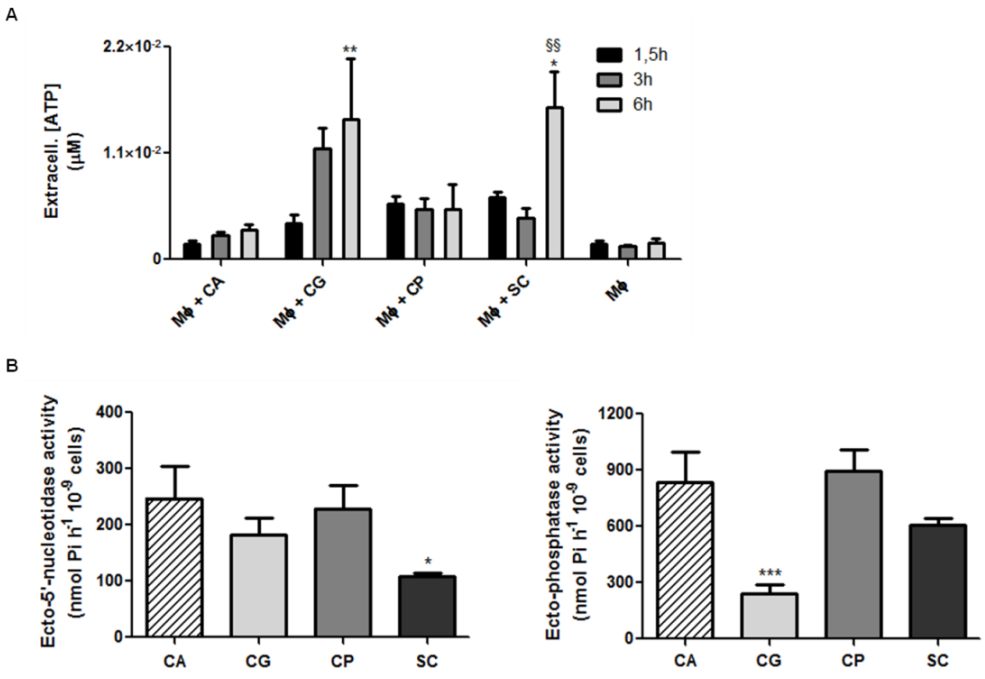

Figure 1. Extracellular levels and metabolism of ATP during the course of infection.

A) Measurement of ATP in the supernatant of macrophages alone and with yeast (CA- *C. albicans*; CG- *C. glabrata*; CP- *C. parapsilosis*; SC- *S. cerevisiae*), after different periods (1.5h, 3h and 6h) of phagocytosis. The measurement was obtained using an ATP Bioluminescent Assay Kit (Sigma); * $p<0.05$, ** $p<0.01$, 1.5h *vs* 6h; §§ $p<0.01$, 3h *vs* 6h. B) Measurement of the enzymatic activities of ecto-5´-nucleotidase and ecto-phosphatase in RAW 264.7 cells co-

cultured during 1 hour with *C. albicans*, *C. glabrata* , *C. parapsilosis* or *S. cerevisiae*. 5´-AMP was used as substrate for ecto-5´-nucleotidase and 5 mM *p*-nitrophenyl phosphate as substrate for ecto-phosphatase. The absorbance obtained for each reaction was spectrophotometrically analysed (Spectra Plus spectrophotometer, Molecular Devices); * $p<0.05$, *** $p<0.001$, *vs* CA. Results shown are derived from at least three independent experiments (±SEM); in each experiment triplicate measurements were performed.

Ecto-5'-nucleotidase and ecto-phosphatase activity in yeast cells exposed to macrophages

ATP levels are also dependent on it extracellular metabolism, so we questioned how enzymatic activities responsible for its catabolism into adenosine, negative modulator of the immune-inflammatory process, will be. The ecto-phosphatase activity, responsible for converting ATP into ADP and AMP, and the ecto-5'-nucleotidase activity, converting AMP into adenosine, were measured in intact cells during macrophage-yeast interaction assays and resulted from ecto-enzymes activity either of macrophages and/or yeast cells. We observed that the ecto-5'-nucleotidase activity of *C. glabrata* and *C. parapsilosis* are similar to the ones found in *C. albicans* (ranging from ~180 to ~250 nmol Pi h^{-1} 10^{-6} cells), while *S. cerevisiae* had significantly lower levels (~100nmol Pi h^{-1} 10^{-6} cells, $p<0.05$; Figure 1B). Conversely, *C. glabrata* ecto-phosphatase activity is, in this case, the only one to have different levels comparing with *C. albicans* (~240 vs 835 nmol Pi h^{-1} 10^{-6} cells; $p<0.001$); despite *S. cerevisiae* seem to present a certain tendency to have lower enzymatic activity levels (~610 nmol Pi h^{-1} 10^{-6} cells), both *S. cerevisiae* and *C. parapsilosis* (900 nmol Pi h^{-1} 10^{-6} cells) are no statistically different from *C. albicans* (Figure 1B).

Macrophage gene expression of $A_{2A}R$ upon yeasts infection

Since we have previously described that, despite decreased concentration of extracellular ATP and ectophosphatase activity, no modifications of the expression of the $A_{2A}R$ gene (Adora2a) was found during macrophage interaction with *C. albicans*, we looked at the same gene expression of macrophages during *C. glabrata, C. parapsilosis* and *S. cerevisiae* interaction. As shown in Figure 2, no modifications in Adora2a expression were found at 1.5, 3 or 6 h co-cultures, similarly to the findings with *C. albicans*. Thus, these results show that macrophages do not respond as well against *C. glabrata, C. parapsilosis* and *S. cerevisiae*, resembling what we have found with *C. albicans.*

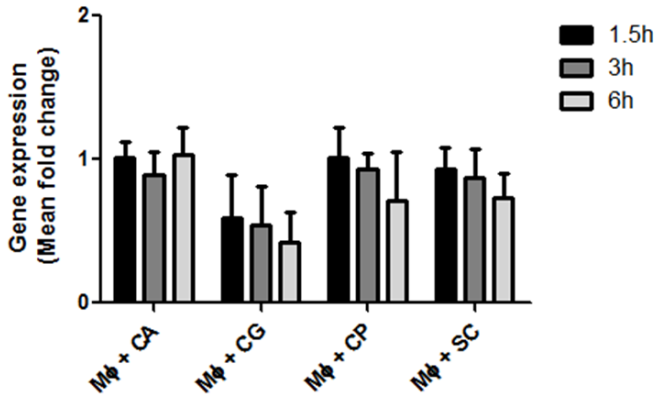

Figure 2. Adora2a relative expression.

Relative expression of adenosine A_{2A} receptor Adora2a gene in macrophages following the interaction with *C. albicans*, *C. glabrata* , *C. parapsilosis* or *S. cerevisiae* for 1.5h, 3h and 6h. Data are expressed as mean fold elevation compared to control untreated RAW 264.7 cells (means ± SEM, n=3).

Cellular distribution of $A_{2A}R$ in macrophages after yeast phagocytosis

Given that $A_{2A}R$ gene (Adora2a) expression profile in *C. glabrata*, *C. parapsilosis* and *S. cerevisiae* seem to be very similar to what we had described with *C. albicans*

infection, we also looked at $A_{2A}R$ distribution within macrophages. Immunofluoresence microscopy analysis revealed that the $A_{2A}R$ distribution in macrophages infected with *C. glabrata*, *C. parapsilosis* and *S. cerevisiae* is similar to the pattern observed in *C. albicans*, with an higher density of $A_{2A}R$ staining in the phagosome membrane enclosing the yeast cells (Figure 3). It can be thus concluded that different yeast species can actually trigger a redistribution of macrophage $A_{2A}R$ into the membranes enclosing the yeast cells.

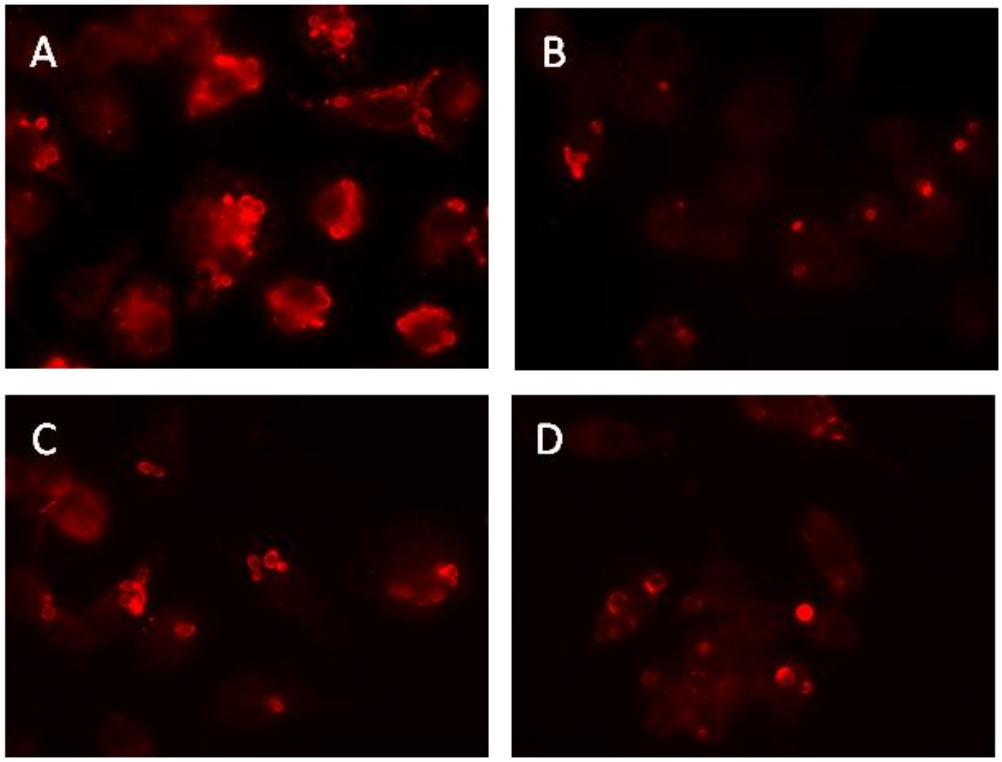

Figure 3. Macrophages adenosine A_{2A} receptors localization.

Representative confocal images of RAW 264.7 cell culture immunostained with an antibody against $A_{2A}R$ (red). Macrophages upon interaction with A) *C. albicans*, B) *C. glabrata*, C) *C. parapsilosis* and D) *S. cerevisiae* during 1.5h. The confocal images were obtained with a Zeiss LSM 510 Meta Confocal Microscope, using Plan-ApoChromat 63x/1,4 oil objective.

Cytokine levels in response to yeast cells

Taking into account adenosine ability to decrease inflammation of host damaged tissues, we quantified TNF-α and Il-1β cytokines, involved in inflammation and damage. Exposure of macrophages to *C. albicans*, *C. glabrata*, *C. parapsilosis* or *S. cerevisiae* during 1.5, 3 or 6 h did not significantly modify the extracellular levels of either TNF-α or Il-1β (n=3, p>0.05), in contrast with what we found with LPS stimulation (Figure 4). However, the TNF-α values seem to have a slight increase with time, for all species studied. The Il-1β levels did not show any pattern of changing during the 6-hour infection period, except at 3h of exposure to LPS (Figure 4).

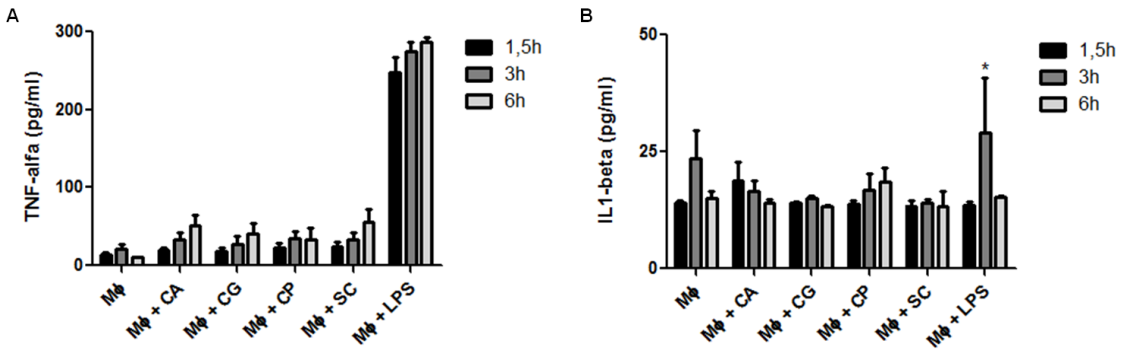

Figure 4. Cytokines quantification upon yeasts macrophage infection.

A, B) TNF-alpha and IL-1beta quantification by ELISA technique, under different experimental conditions: with macrophages alone - Mϕ; with macrophage infection by *C. albicans* – CA, *C. glabrata*- CG, *C. parapsilosis*- CP, *S. cerevisiae*- SC or LPS after a 1.5h, 3h and 6h period of interaction. Results shown are derived from three independent experiments (±SEM); in each experiment, duplicate measurements were performed; * p<0.05.

DISCUSSION

In this study it is provided evidences showing the purinergic system involvement on macrophages and non-*albicans* yeast species interaction. The results here presented adding to the increasing evidences of the involvement of $A_{2A}R$ in bacterial and *C. albicans* infections (Chapter III; Nemeth *et al*., 2006; Csoka *et al.*, 2007; Hasko *et al*., 2008), highlight novel inter-specific properties of the non-albicans *Candida* interaction with macrophages, in what regards the purinergic system.

The roles of ATP and adenosine in inflammation were extensively reviewed (see for instance Sitkovsky *et al*., 2004 and 2005). Thus, whenever a macrophage-yeast interaction occurs, any ATP produced by either of the cell types, would be relevant in the pro-inflammatory signalization and could also be available as a source of adenosine (Fredholm, 2007; Sitkovsky *et al*., 2004 and 2005). The data collected during this study showed that in co-cultures of macrophages with either *C. albicans, C. glabrata, C. parapsilosis* or *S. cerevisiae* different ATP concentration levels were registered, with higher amounts in *C. glabrata* and *S. cerevisiae* samples, indicating that during the infection of these two species a higher pro-inflammatory state is registered. On the other hand, the higher ecto-phosphatase activities were registered in the presence of *C. albicans* and *C. parapsilosis*. Previously, it was described that yeast strains exhibiting higher pathogenicity, namely of the species *C. parapsilosis,* had higher ectophosphatase activity (Kiffer-Moreira *et al*., 2007; MacCallum *et al*., 2009). It could also be expected that some macrophage ATP-dependent function like pro-inflammatory cytokines production will be affected (Ferrari *et al*., 1997; Haskó *et al*., 2000; Netea *et al*., 2010). These variations in ATP and associated metabolism regarding different yeast species, could also predict different outcomes in infection signalling: lower ATP concentrations

observed during the infections assays could thus be linked to higher ecto-phophatase/ecto-5'-nucleotidase activities, conferring advantages into effective *C. albicans* and *C. parapsilosis* macrophage infection. On the contrary, high ATP associated to decreased metabolism and adenosine levels, will render macrophages more efficient in the elimination/digestion of *C. glabrata* and *S. cerevisiae* cells. Before it was described that *C. glabrata* does not have some key virulence factors found in *C. albicans* cells, as hyphal formation or tissues inflammation upon colonization, but, as it closely related non-pathogenic *S. cerevisiae*, it can survive and replicate within the macrophages (Seider *et al*., 2011). *C. glabrata* is thus able to inhibit phagolysosome formation and normal phagosome acidification, as seen with *C. albicans*, through alkalinization of phagosome environment (Seider *et al*., 2011; Kasper *et al*., 2014). Under our experimental conditions, using RAW264.7 macrophages infected with yeasts during 6 h, the different yeast species used during this study failed to alter both TNF-α and IL-1β citokynes production. The lack of TNF-α and IL-1β responses in *C. glabrata* were already reported (Seider *et al*., 2011), but it would be expectable to register detectable levels of these factors for the other yeast species, particularly to *C. albicans* and *C. parapsilosis*. Gosh *et al*. (2010) proved that this macrophage cell line is able to increase cytokines levels but using a gene expression assay. Adenosine $A_{2A}R$ revealed to be one of the most promising purinergic receptors involved in the immune-inflammatory system regulation (Hasko *et al*., 2008; Belikoff *et al*., 2011). As we formerly described for *C. albicans* infection (Chapter III) the stimulation of the Adora2a gene expression with non-*albicans* species does not occur. This clearly shows that similarly to *C. albicans*, *C. glabrata*, *C. parapsilosis* and *S. cerevisiae* did not enhance a stimulation of one of the macrophages effector systems, as observed during a bacterial infection (Khoa *et al*., 2001; Streitova *et al*., 2009). However, when looking at confocal

images obtained from the different groups of yeasts, $A_{2A}R$ staining in phagosomes enclosing *C. glabrata*, *C. parapsilosis* or *S. cerevisiae* clearly stands out and resembles *C. albicans* findings. Why and actually how is this happen still raises many questions to be solved and, nonetheless, the effective activation of $A_{2A}R$ and it possible role in phagocytosis/ingestion of non-*albicans* cells by macrophages remain to be elucidated. However, this clearly supports the hypothesis that $A_{2A}R$ recruitment to the phagosomal membrane is a key feature in the ability of yeasts, even those with lower virulence, to behave as an efficient intracellular organism.

In conclusion, among the complexity of macrophage-yeast interactions, our results highlight that a decrease in ATP concentration with correspondent higher ecto-phophatase/ecto-5'-nucleotidase activities together with $A_{2A}R$ located in phagosomes enclosing yeasts, compromises inflammatory signaling and death rate, in clear benefit of fungal survival.

REFERENCES

Arabatzis M, Kollia K, Menounos M, Logotheti M and Velegraki A (2004) Delineation of *Clavispora lousitaniae* clinical isolates by polymerase chain reaction-single strand conformation polymorphism analysis of the ITS1 region: a retrospective study comparing five typing methods. Med Mycol 42: 27-34.

Bader O. (2014) Looking into the virulence of *Candida parapsilosis:* A diagnosticperspective. Virulence. 5:4, 457–459.

Belikoff B, Hatfield S, Sitkovsky M and Remick DG. (2011) Adenosine negative feedback on A2A adenosine receptors mediates hyporesponsiveness in chronically septic mice. Shock. 35(4):382-7.

Belikoff BG, Hatfield S, Georgiev P, Ohta A, Lukashev D, Buras JA, Remick DG and Sitkovsky M. (2011). A_{2B} adenosine receptor blockade enhances macrophage-mediated bacterial phagocytosis and improves polymicrobial sepsis survival in mice. J Immunol.186: 2444-2453.

Boyle et al (2006) Probiotic use in clinical practice: what are the risks. Am J Clin Nut 83:1256-64.

Cassone et al (2003) Outbreak of *Saccharomyces cerevisiae* subtype boulardii fungemia in patients neighboring those treated with a probiotic preparation of the organism. J Clin Microbiol 41: 5340-5343.

Costa-de-Oliveira S, Pina-Vaz C, Mendonça D and Gonçalves-Rodrigues A (2008) A first Portuguese epidemiological survey of fungaemia in a university hospital. Eur J Clin Microbiol Infect Dis 24:23-30.

Csóka B, Németh ZH, Virág L, Gergely P, Leibovich SJ, Pacher P, Sun CX, Blackburn MR, Vizi ES, Deitch EA and Haskó G. (2007). A_{2A} adenosine receptors and C/EBPbeta are crucially required for IL-10 production by macrophages exposed to *Escherichia coli*. Blood 110, 2685-2695.

de Llanos et al (2006) Food and probiotic strains from the Saccharomyces cerevisiae species as a possible origin of human systemic infections. Int J Food Microbiol.

Fernandes EC, Meyer-Fernandes JR, Silva-Neto MA and AE Vercesi. (1997) *Trypanosoma brucei*: ecto-phosphatase activity present on the surface of intact procyclic forms. Z Naturforsch C. 52:351-358.

Fernandez-Arenas E, Bleck CK, Nombela C, Gil C, Griffiths G and Diez-Orejas R. (2008). *Candida albicans* actively modulates intracellular membrane trafficking in mouse macrophage phagosomes. Cell. Microbiol. *11*, 560-589.

Ferrari D, Chiozzi P, Falzoni S, Dal Susino M, Melchiorri L, Baricordi OR and di Virgilio F. (1997). Extracellular ATP triggers IL-1 beta release by activating the purinergic P2Z receptor of human macrophages. J. Immunol. 159, 1451-1458.

Guilherme A, Meyer-Fernandes JR and A Vieyra. (1991) Reversible inhibition by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid of the plasma membrane (Ca(2+)+Mg2+)ATPase from kidney proximal tubules. Biochemistry.30:5700-5706.

Haskó G, Kuhel DG, Chen JF, Schwarzschild MA, Deitch EA, Mabley JG, Marton A and Szabó C. (2000). Adenosine inhibits IL-12 and TNF-[alpha] production via adenosine A_{2a} receptor-dependent and independent mechanisms. FASEB J. 14, 2065-2074.

Haskó G, Linden J, Cronstein B and Pacher P. (2008). Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat. Rev. Drug Discov. 7, 759-770.

Junger WG. (2011). Immune cell regulation by autocrine purinergic signalling. Nat Rev Immunol. 11, 201-212.

Kasper L, Seider K, Gerwien F, Allert S, Brunke S, Schwarzmüller T, Ames L, Zubiria-Barrera C, Mansour MK, Becken U, Barz D, Vyas JM, Reiling N, Haas A, Haynes K, Kuchler K and Hube B. (2014) Identification of *Candida glabrata* genes involved in pH modulation and modification of the phagosomal environment in macrophages. PLoS One. 9(5):e96015.

Khoa ND, Montesinos MC, Reiss AB, Delano D, Awadallah N and Cronstein BN. (2001) Inflammatory cytokines regulate function and expression of adenosine A(2A) receptors in human monocytic THP-1 cells. J Immunol. 167(7):4026-32.

Kiffer-Moreira T, de Sa Pinheiro AA, Alviano WS, Barbosa FM, Souto-Padron T, et al. (2007) An ectophosphatase activity in *Candida parapsilosis* influences the interaction of fungi with epithelial cells. FEMS Yeast Res. 7, 621-628.

Kumar V and Sharma A. (2009). Adenosine: an endogenous modulator of innate immune system with therapeutic potential. Eur. J. Pharmacol. 616, 7-15.

Livak KJ and TD Schmittgen. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Loureiro and Querol (2000) The prevalence and control of spoilage yeasts in foods and beverages. Trends in Food Science & Technology 10, 11: 356-365.

Loureiro V and Malfeito-Ferreira M (2003) Yeasts in spoilage. Encyclopedia of Food Science and Nutrition (2nd ed.) Caballero B, Trugo L & Finglas P (eds.) pp5530-5536. Academic Press, London.

Németh ZH, Csóka B, Wilmanski J, Xu D, Lu Q, Ledent C, Deitch EA, Pacher P, Spolarics Z and Haskó G. (2006). Adenosine A_{2A} receptor inactivation increases survival in polymicrobial sepsis. J. Immunol. 176, 5616-5626.

Netea MG, Simon A, van de Veerdonk F, Kullberg BJ, Van der Meer JW and Joosten LA. (2010). IL-1beta processing in host defense: beyond the inflammasomes. PLoS Pathog. 6, e1000661.

Nucci M and Marr KA. (2005) Emerging fungal diseases. Clin Infect Dis. 41(4):521-6.

Paulo C, Mourão C, Veiga PM, Marques JM, Rocha G, Alves AF, Querol A, Meliço-Silvestre AA, Gonçalves I, Flores, Clemente C and Gonçalves T (2009) Retrospective analysis of clinical yeast isolates in a hospital in the centre of Portugal: Spectrum and revision of the identification procedures. Med Mycol 47(8): 836-844.

Perapoch J, Planes AM, Querol A, Lopéz V, Martinez-Bendayán I, Tormo R, Fernández F, Peguero G and Salcedo S (2000) Fungemia with *Saccharomyces cerevisiae* in two newborns, only one of whom had been treated with Ultra-Levura. Eur J Clin Microbiol Infect Dis 19: 468-470.

Pfaller MA and Diekema DJ (2007) Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol. Rev. 20:133-163.

Rodloff AC, Koch D and Schaumann R. (2011) Epidemiology and antifungal resistance in invasive candidiasis. Eur J Med Res.16: 187-195.

Seider K, Brunke S, Schild L, Jablonowski N, Wilson D, Majer O, Barz D, Haas A, Kuchler K, Schaller M and Hube B. (2011) The facultative intracellular pathogen *Candida glabrata* subverts macrophage cytokine production and phagolysosome maturation. J Immunol. 187(6):3072-86.

Seider K, Gerwien F, Kasper L, Allert S, Brunke S, Jablonowski N, Schwarzmüller T, Barz D, Rupp S, Kuchler K and Hube B. (2014) Immune evasion, stress Resistance, and efficient nutrient acquisition are crucial for intracellular survival of *Candida glabrata* within macrophages. Eukaryot Cell. 13(1):170-83.

Silva S, Negri M, Henriques M, Oliveira R, Williams DW and Azeredo J. (2012) *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology,

epidemiology, pathogenicityand antifungal resistance. FEMS Microbiol Rev. 36:288–305.

Sitkovsky M, Lukashev D, Apasov S, Kojima H, Koshiba M, Caldwell C, Ohta A and Thiel M. (2004). Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A_{2A} receptors. Annu. Rev. Immunol. 22, 657–682.

Sitkovsky MV and Ohta A. (2005). The 'danger' sensors that STOP the immune response: the A_{2A} adenosine receptors? Trends Immunol. 26, 299-304.

Streitová D, Hofer M, Holá J, Vacek A and Pospísil M. (2010). Adenosine A_1, A_{2a}, A_{2b}, and A_3 receptors in hematopoiesis. 2. Expression of receptor mRNA in resting and lipopolysaccharide-activated mouse RAW 264.7 macrophages. Physiol. Res. 59, 139-144.

Takahashi et al (2003) Translocation model of Candida albicans in DBA-2J mice with protein calorie malnutrition mimics hematogeneous candidiasis in humans. Microbiol Pathogenesis 35:179-187.

Tóth R, Tóth A, Papp C and Jankovics F. (2014) Kinetic studies of *Candida parapsilosis* phagocytosis by macrophages and detection of intracellular survival mechanisms. Front Microbiol. 20;5:633.

Trofa D,Gacser A and Nosanchuk JD.(2008). *Candida parapsilosis*, an emerging fungal pathogen. Clin. Microbiol.Rev. 21, 606–625.

CHAPTER VI

INCREASED INFECTION OF THE GASTROINTESTINAL TRACT BY *CANDIDA ALBICANS* IN ELDERLY: INVOLVEMENT OF ADENOSINE A_{2A} RECEPTORS

From:

Rodrigues L, Miranda IM, Andrade GM, Mota M, Cortes L, Gonçalves-Rodrigues A, Cunha RA and Gonçalves T (2015) Increased infection of the gastrointestinal tract by *Candida albicans* in elderly: involvement of adenosine A_{2A} receptors. Manuscript submitted to Internacional Journal of Medical Microbiology.

ABSTRACT

With the aging of the Western population, immunosenescence of the elderly is becoming a public health issue, since it is associated with increased morbidity and mortality rates associated with infections and inflammatory gut diseases in the elderly. We now compared, in aged, adult and young mice, the relative intestinal over-colonization by ingested *Candida albicans* and their translocation to other organs. Moreover, we studied, in the three groups of mice, the distribution of adenosine A_{2A} receptors, a stop signal of severe inflammation, in the gastrointestinal tract and the correlation between the density of A_{2A} receptors and tissue damage due to yeast infection. We show that elderly mice are more prone to over-colonization by *C. albicans*, than adults and young mice. This fungal over-colonization in the aged animals seems to be related with higher growth rate in the intestinal lumen, independent of invasion of gut tissues, but resulting in higher inflammation of the GI tract. Also, we observed a particularly high colonization in the stomach, with high rate of yeast-to-hypha transition in the elderly. On the other hand, when compared with young and adults, aged mice have a lower density of adenosine A_{2A} receptor in gut tissues and *C. albicans* infection failed to increase the density of A_{2A} receptor. In conclusion, the present study shows that aged mice have a lower ability to cope with inflammation due to *C. albicans* over-colonization, which is likely related to the inability to adaptively adjust the density of adenosine A_{2A} receptors. This paves the way to exploit the impact of the purinergic system, in particular the adenosine A_{2A} receptor, in the control of the gut over colonization/infectious process of *C. albicans* in the elderly.

INTRODUCTION

Over the past decades, the number of opportunistic fungal diseases has increased, taking advantage of debilitated physiological conditions or of local or systemic immune malfunction (Falagas *et al*., 2010; Pfaller and Diekema, 2007). In fact, fungal infections have a particular incidence upon aging since individuals above 65 years display more frequently signs of immunosenescence, with reduced effectiveness of the immune system (Aw *et al*., 2007). Furthermore, inflammation increases with age (Biagi *et al*., 2010) and can shift the balance between commensalism and infection towards the latter (Round and Mazmanian, 2009), making aged individuals more susceptible to opportunistic infections. This represents a complex and challenging problem worldwide since the number of elderly individuals is expected to duplicate between 2000 and 2025 (Pfister and Savino, 2008).

Candida albicans is often described as commensal, being part of the human microbiome. However, when the immune status and/or the host microbiota are altered, commensalism evolves toward extensive colonization of mucosal surfaces and disease. In these cases, gut is a main gateway for *C. albicans* colonization (d'Enfert, 2009). The balance between pro- and anti-inflammatory signals that keeps the host-fungus equilibrium, can also cause severe host damage, namely during chronic inflammation, that will render fungal cells more aggressive (Romani, 2011). Another factor that might be involved in the control of gut inflammation is adenosine through the recruitment of adenosine receptors, in particular A_{2A} receptors ($A_{2A}R$) (Estrela and Abraham, 2011; Hasko *et al*., 2008). This major controller of the reactivity of the immune-inflammatory system (Sitkovsky *et al*., 2004) undergoes a decreased capacity to control inflammation

in some organs upon aging (Fredholm *et al*., 2003), but it is unknown if $A_{2A}R$ density is modified in the gut of aged individuals.

We now aimed to understand using a murine model, the relation between host age and susceptibility to over-colonization or infection by *C. albicans*, and its impact on the inflammation of the gut, and explored the localization and density of adenosine $A_{2A}R$. The *in vivo* results indicate that aging favors the over-colonization of the intestinal lumen and that this bolsters inflammatory signs. Moreover, aged mice display a lower $A_{2A}R$ density, which remains unchanged upon *C. albicans* infection, in contrast to young or adult mice.

MATERIALS AND METHODS

Strain, media and growth conditions

Candida albicans YP0037 strain (Microbiology Pathogenic Yeast Collection, University of Coimbra) was isolated from a hemoculture. Yeast cells were grown overnight at 30ºC on YPD agar (0.5% yeast extract, 1% bacto-peptone, 2% agar and 2% glucose) plates, harvested by centrifugation and resuspended in PBS. Cell density was determined using Neubauer chamber cell counting.

Mice and gastrointestinal model

Young, adult and aged (respectively, 2-, 9- and 18-months-old) C57Bl/6JRj male mice were obtained from Janvier Labs and maintained under specific pathogen-free conditions at the animal facilities of the Faculty of Medicine, University of Oporto. All procedures were according to EU guidelines (2010/63) and approved by the Directorate General of Food and Veterinary Medicine of the European Union (authorization no.

6411). Animals were individually housed in sterilized cages, supplied with sterile bedding, sterile water and sterile mouse chow and kept under 12h:12h light-dark cycle, under controlled temperature (21-24ºC) and humidity (50-60%). The gastrointestinal infection model was carried out as previously described (Koh *et al*., 2008) with slight modifications (Figure 1). Briefly, to reduce commensal bacterial and fungal flora, mice were treated for 3 days with sterile drinking water containing 2 mg/mL streptomycin (Reig Jofré, Spain), 2000 U/mL penicillin G (Atral, Portugal) and 0.25 mg/mL fluconazole (Pfizer, Germany) and then switched for a further day to water containing the same concentrations of streptomycin and penicillin G. Animals were then provided, during 5 days, with sterile drinking water with *C. albicans* ($1x10^7$ colony forming units, CFU/mL) and the same concentrations of streptomycin and penicillin G. After this period of yeasts ingestion, mice were maintained with sterile water containing 2 mg/mL streptomycin, 2000 U/mL penicillin G and 0.2 mg/mL gentamicin (Labesfal, Portugal) until the end of the experiment. Two additional groups of mice were maintained without *C. albicans* administration, one of the groups being kept under the same antibiotherapeutic protocol and the other group was never exposed to any antibiotics. To confirm the depletion of the endogenous microflora, stools were collected before beginning *C. albicans* administration.

Thereafter, in order to monitor *C. albicans* colonization, the stools were collected from individual mice every two days. Stools were homogenized in 1 mL PBS and serial dilutions were made in order to obtain 30 to 50 CFU in each YPD agar plate. Samples were plated in triplicate. Plates were then incubated for 2 days at 30 ºC and the yeast load was quantified by counting the number of colonies corresponding to viable yeast cells and expressed as CFU per g of stools. Mice were sacrificed 19 days after *C. albicans* exposure and kidneys, livers, stomachs, small intestines and large intestines

were harvested and weighted. Sets of equivalent tissues were then divided into three groups, each processed as follows: 1) fixed in formalin solution for subsequent histology analysis; 2) immediately snap-frozen in isopentane and kept at -80 ºC, for further immunohistochemistry; 3) homogenized in PBS + 0.05% Triton X-100, diluted and plated on YPD as described above, to determine the tissue content of *C. albicans*.

Histology

Formalized tissues were processed using standardized paraffin embedding protocols and stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) methods. Sections of the different organs were then microscopically analyzed (Zeiss Axio Imager Z2 Microscope, with EC Plan-Neofluar, 5x/0.16 and 40x/0.75, objectives) in order to evaluate the presence of yeasts and/or inflammation. Stomach and small intestine sections were evaluated according to the criteria of Kelly *et al*. (1994). Briefly, a 1-cm segment of each histological section was assessed on a scale of 0 (absence of alterations) and 1 (mild) to 3 (severe) for disruption of the villi, presence of red blood cells, and neutrophil infiltration. For large intestine, the presence and intensity of histological changes were evaluated with the use of a score index, according to Vilaseca *et al*. (1990) including the following criteria: presence of epithelial cell loss, presence and intensity of inflammatory infiltration and presence of blood cells.

Immunohistochemistry

Snap-frozen samples were analysed using an immunohistochemistry standard protocol against $A_{2A}R$. Briefly, OCT (Tissue-Tek, Sakura) ultrathin (10 μm) sections (Cryostat CM3050S, Leica Biosystem) were obtained from stomach, small and large intestine of the different mice. Tissue sections were fixed with pre-cooled acetone (-20°C) for 10

min, then washed with PBS and permeabilized with PBS and 0.25% Triton X-100 for 30 min. After PBS washing, 10% albumin bovine serum (BSA) in PBS was used as blocking buffer and sections slides were incubated in a humidified chamber at room temperature for 1 h. The rabbit anti-$A_{2A}R$ primary antibody (1:300; sc-13937 from Santa Cruz Biotechnology) was diluted in PBS with 1% BSA and incubated with the sections overnight at 4°C in a humidified chamber. Sections were then washed with PBS, and treated with the Alexa Fluor 488-labeled donkey anti-rabbit secondary antibody (1:2000; A21206 from Molecular Probes) diluted in PBS with 1% BSA. Sections were again incubated in a humidified chamber at room temperature for 2 h. After washing with PBS, nuclei were counterstained with DAPI (Sigma), during 5 min at room temperature, protected from light, and again rinsed with PBS. Coverslips were mounted with Vectashield HardSet mounting medium and kept at 4 ºC until visualized with fluorescence and/or confocal microscopy. Digital images were captured using a Zeiss Axio Imager Z2 Microscope (with ApoTome2 structured illumination acquisition system and a Plan-Apochromat 20x/0.8 objective), and using Zeiss ZEN 2012 (blue edition) and ImageJ software was used to analyse the images.

Statistics

Data are means ± SEM. Statistical differences were determined with one or two-way ANOVA respectively followed by a Newman-Keuls or Bonferroni post hoc test. Histological damage scores were analysed with Kruskall Wallis and Mann-Whitney tests. Statistical significance was considered at $p<0.05$.

RESULTS

C. albicans load in mice stools and target organs

Since mice are not normally colonized by *C. albicans*, we made use of an established protocol for gastrointestinal (GI) colonization (Cole *et al*., 1996; Koh *et al*., 2008; Figure 1), which requires an initial elimination of the existent microflora with antibiotics followed by infection of the mice with an oral suspension of yeasts, applied through the drinking water. Using this GI infection model, none of the mice from the different groups suffered significant weight alterations (data not shown) and no mice succumbed during the experiments, in line with previous studies (Vautier *et al*., 2012).

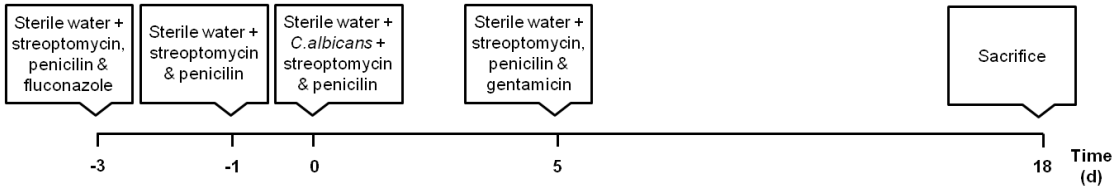

Figure 1. Schematic timeline of the gastrointestinal murine model of infection.

Young, adult and aged mice were first maintained with antibiotics in the drinking water to eliminate the gut microbiota (days -3 to 0); this allows an oral infection with *C. albicans* (days 0 to 5) to trigger an effective colonization of the GI tract (days 5-18). Mice were sacrificed at day 18 for collection of tissues for analysis.

To check GI tract colonization, the fungal load of stools was monitored every two days; as expected, at day 0 fungal cells were absent from the mice commensal flora (Figure 2A). Along the experiment, following the infection with *C. albicans*, the levels of yeasts elimination in adult mice remained constant over time, beginning to be detected at day 1 with values ranging between $3x10^6$ and $7x10^6$ CFU/g stool (Figure 2A). During the initial period of *C. albicans* infection *per os*, the stool of young and aged mice displayed

similar values of fungal load, although with some point differences. In fact, in the last day of *C. albicans* administration (day 5), the excretion levels of yeasts in young and aged mice were significantly higher than in adult animals (~$1x10^7$ CFU/g stool, $p<0.001$ and $p<0.05$, respectively, *vs*. adult mice; Figure 2A). After this period, between the days 7 and 9, the number of viable yeast cells eliminated in the stools of young and aged mice became similar to the profile found in the adults group until the end of the study. However, the aged group showed a significant increase in yeast elimination in the last time point, at day 11, when compared with the other age groups (~$2x10^7$ CFU/g stool, $p<0.001$; Figure 2A).

At the end of the study, the animals were sacrificed and the fungal content was investigated in kidneys, livers, stomachs, small and large intestines. No yeast colonization was found in either kidneys or livers from any group (Figure 2B). *C. albicans* cells were found in all the gastrointestinal tract tissues, stomach, small intestine and large intestine from infected mice of the three age groups. Although we observed a great variability of results (values ranging from ~$8x10^4$ to ~$1x10^6$ CFU/g tissue), the samples taken from the large intestine of young, adult and aged mice displayed larger fungal loads, when compared with the other GI tissues (~$1x10^6$ CFU/g tissue; Figure 2B). However, when comparing between the three age groups, aged mice tend to present a lower colonization level in the stomach and intestine, although not statistical significant compared to adult mice (Figure 2B). The histological analysis revealed that, at day 11, the colonization of the stomach mucosa was more evident than in the intestine (small and large). Interestingly, it was possible to observe *C. albicans* yeast-to-hyphae transition in the stomachs, which was more exuberant in aged mice than in young and adult mice (Figure 2C).

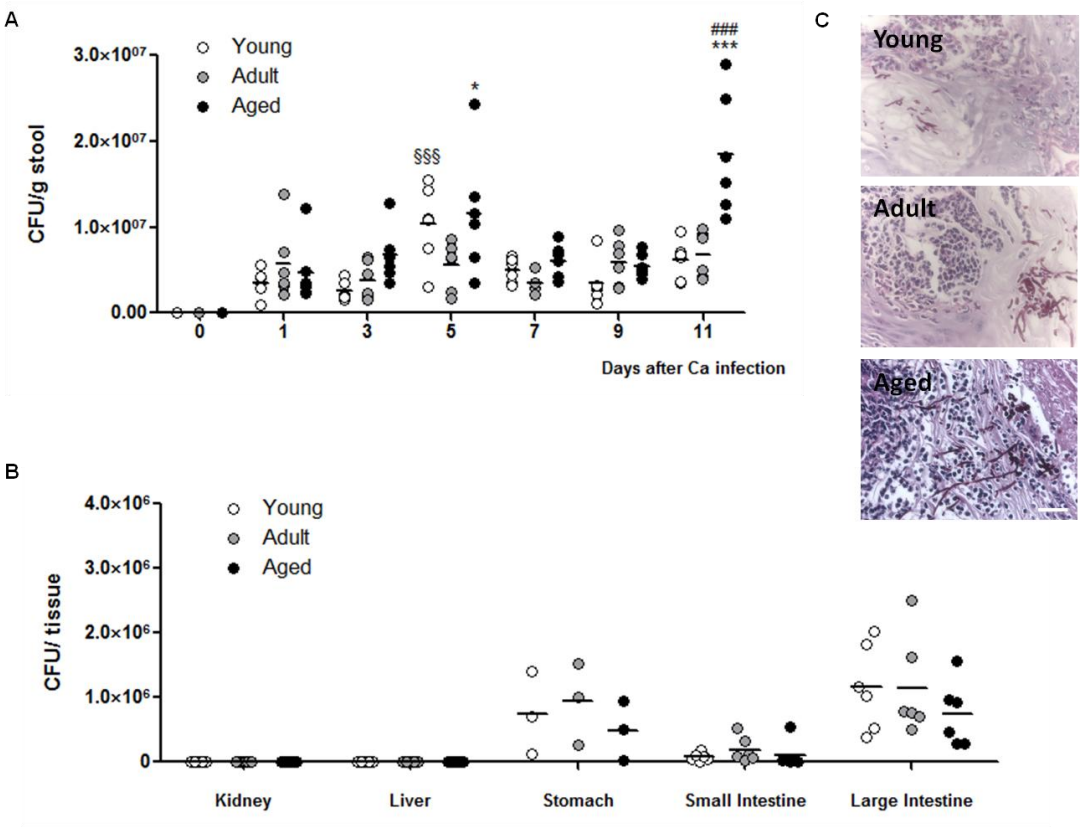

Figure 2. Yeast load in mice stools and target organs.

A) Stool fungal burdens of young, adult and aged mice before (day 0) and in consecutive days after *C. albicans* oral infection. Stools were collected every two days until the end of the experiment, from mice housed individually. B) Viable *C. albicans* counts in different tissues of young, adult and aged mice at the end of the experimental procedure (day 18). Dots represent data from individual mice and horizontal lines represent the mean of each group of mice; * $p<0.05$, *** $p<0.001$, aged *vs.* adult mice; $^{###}$ $p<0.001$, aged *vs.* young mice and §§§ $p<0.001$, adult *vs.* young mice. C) *C. albicans* hyphal forms were found to be present in stomachs from young, adult and aged mice 18 days after infection with *C. albicans*. Scale bar: 5 μm.

Inflammation processes in the GI tract

The analysis of the histological sections of the different GI tract tissues from control mice of the three age groups did not reveal any significant age-dependent alterations (Figures 3 and 4). According to the inflammatory criteria used in the present study to calculate a score index, we found a score of 0 (Table 1), with the absence of alterations in the tissues sections from control mice. By contrast, *Candida* infection induced a severe degree of disruption of the villi, haemorrhagic damage, and increase of inflammatory cells infiltration on the gastric mucosa (Figures 3; "Infected" panels). Particularly, infected aged animals had large inflammatory infiltrates (Figure 3A), and infected adult animals displayed the presence of lymphoid tissue (Figure 3B). In the small intestine, there was a mild disruption of villi and inflammatory infiltrate in the aged infected group, which did not reach significance compared to control mice (Figure 3 and Table 1). The large intestine from aged infected mice displayed mild epithelial cell loss and moderate haemorrhage (Figure 3 and Table 1). However, slight alterations were found in small and large intestines of infected mice from the different age groups (Figures 3; Table 1; score from 0.5 to 2). When looking at stomach sections of young, adult and aged mice, the presence of *C. albicans* filamentous forms seemed to induce an altered state of the tissue, with more severe alterations, regarding villi and haemorrhage and inflammatory infiltrates (Table 1, score 2 and 3), confirmed by the inflammatory processes illustrated in Figures 3. We did not observe differences between the groups with respect to the presence of glycogen or mucopolysaccharides in foveolar (stomach) and goblet cells (intestine) or presence of mucus when tissues were stained with PAS (Figure 4). Thus, both ageing and infection seems to be affecting more prominently GI tissues of the mice, with some alterations in the tissues structures being suggestive of a higher inflammatory damage (Figures 3 and Table 1).

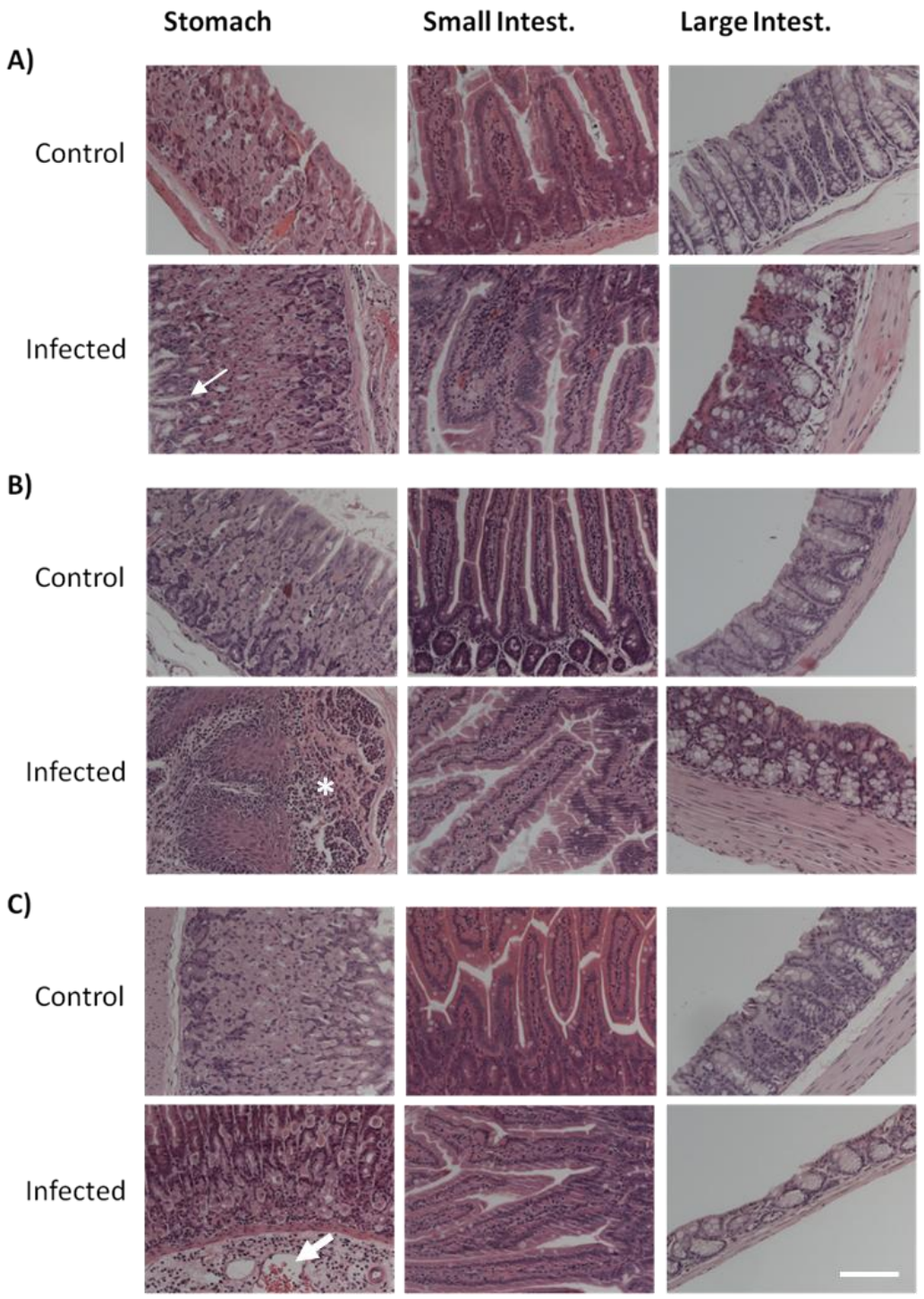

Figure 3. Histological analysis of Haematoxylin-Eosin (HE) stained tissues.

Stomach, small and large intestine sections from both control and *C. albicans* infected mice from A) aged, B) adult and C) young groups. Images from infected mice illustrate *C. albicans* infection capacity to induce severe degree of villi disruption, hemorrhagic damage and inflammatory cells infiltration on gastric mucosa, particularly evident in aged animals. Photographs point to the yeast-induced epithelial cell loss (thin arrow), inflammatory infiltrate (asterisk), and hemorrhage (large arrow). Images were taken using a Zeiss Axio Imager Z2 Microscope; scale bar: 50 μm.

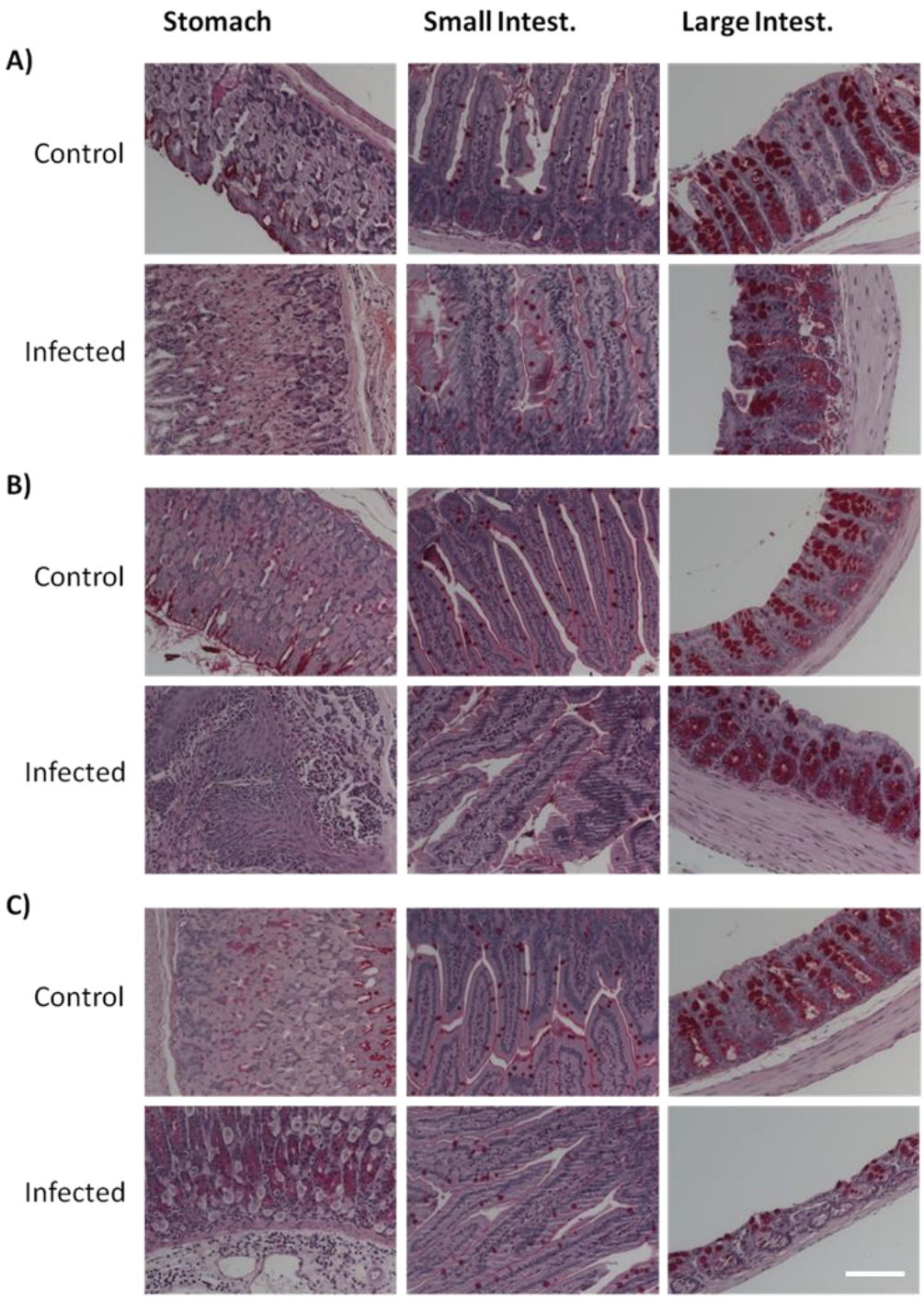

Figure 4. Histological analysis of Periodic Acid Schiff (PAS) stained tissues.

Stomach, small and large intestine sections from both control and *C. albicans* infected mice from A) aged, B) adult and C) young groups. No differences in glycogen and mucopolysaccharides presence were observed between groups. Images were taken using a Zeiss Axio Imager Z2 Microscope; scale bar: 50 μm.

Table 1. Histological evaluation scores from mice GUT tissues.

Histological sections of stomachs, small and large intestines of control and infected mice groups (aged, adult and young) were analyzed regarding villi (vilos.), haemorrhage (hem.) and inflammatory infiltrates (infilt.).

Stomach	Control		Infected	
	Vilos.	Hem., Infilt.	Vilos.	Hem., Infilt.
Aged	0 (0-0)	0 (0-0)	2	3
Adult	0 (0-0)	0 (0-0)	3	3
Young	0 (0-0)	0 (0-0)	3	3

Small Intestine	Control		Infected	
	Vilos.	Hem., Infilt.	Vilos.	Hem., Infilt.
Aged	0 (0-0)	0 (0-0)	1 (0-1)	1.5 (1-2)
Adult	0 (0-0)	0 (0-0)	0.5 (0-1)	1 (0-1)
Young	0 (0-0)	0 (0-0)	0.5 (0-1)	0.5 (0-1)

Large Intestine	Control		Infected	
	Vilos.	Hem., Infilt.	Vilos.	Hem., Infilt.
Aged	0 (0-0)	0 (0-0)	1 (0-1)	2 (0-2)*
Adult	0 (0-0)	0 (0-0)	0 (0-1)	1 (1-2)
Young	0 (0-0)	0 (0-0)	1 (0-1)	0 (0-0)$^{\#}$

Scores represent absence (0) to mild (1) and severe (3) alterations. *, $p< 0.05$ - adult infected *vs* aged infected;

$^{\#}$, $p<0.05$ – aged infected *vs* young infected.

Mapping of A_{2A} receptors ($A_{2A}R$) in the gut of infected mice

Since infection situations trigger an up-regulation of $A_{2A}R$, which feedback to curtail inflammation (Sitkovsky *et al*., 2004), we investigated if there was a correlation between $A_{2A}R$ density, inflammation, tissue damage and age. As expected, adult and young animals displayed a more marked $A_{2A}R$ signal in GI tissues sections upon infection with *C. albicans* compared to controls (Figure 5B, C). This $A_{2A}R$ staining was particularly evident in the gastric epithelium layers of both adult and young mice, and also appeared with high intensity in the *muscularis mucosa* of stomach and small and large intestines from adult and young infected mice (Figure 5B, C, D). However, notorious differences were observed when analyzing GI tissues from aged mice: Figure

5 shows that $A_{2A}R$ immunofluorescence was lower in GI tissues from aged mice compared to young and adult mice. Furthermore, the dynamic plasticity of $A_{2A}R$ density upon infection was lost in aged mice: thus, *C. albicans* infection failed to modify $A_{2A}R$ staining in aged rats compared to non-infected aged rats, in contrast to what was observed in young and adult mice (Figure 5A).

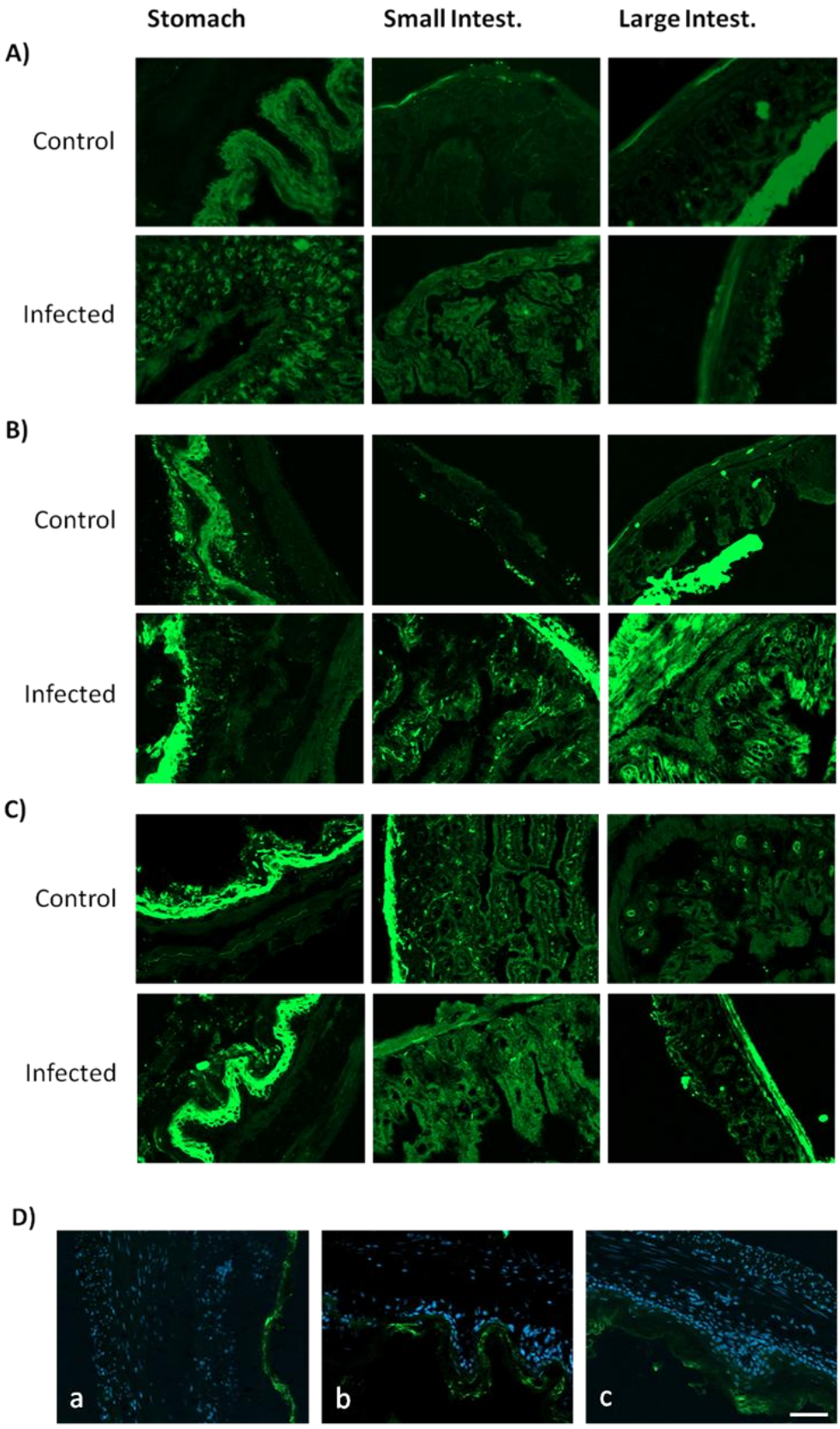

Figure 5. Adenosine A_{2A} receptors localization on mice GI tract tissues.

Representative images of stomach, small and large intestine sections immunostained with an antibody against $A_{2A}R$ (green) of A) aged, B) adult and C) young mice. In adults and young mice, increased $A_{2A}R$ density during *C. albicans*

infection was observed. Aged mice, in contrast, have lower levels and slight alterations on $A_{2A}R$ immunolabeling between conditions. D) Infected stomachs of a) aged, b) adult and c) young mice showing increased $A_{2A}R$ density on epithelium and *muscularis mucosa* layers (cell nuclei stained blue). Images were obtained using a Zeiss Axio Imager Z2 Microscope with ApoTome2 structured illumination acquisition system; scale bar: 10 μm.

DISCUSSION

In this study, we tested the susceptibility of different age groups to *C. albicans* over-growth or over-colonization of the GI tract, upon ingestion of high loads of yeast cells. More, we aimed to study the influence of the adenosine A_{2A} receptors ($A_{2A}R$) in the gastrointestinal tissues of young, adults and aged mice upon an over-colonization by *C. albicans* and subsequent inflammation. *C. albicans* infection/over-colonization of elderly mice stomach lead to a more exuberant yeast-to-hypha transition with higher inflammation when compared to the other age groups. We also found that the overgrowth of *C. albicans* in the intestinal lumen of aged mice induced an inflammation of the mucosal tissues, which was independent on the invasion of intestinal tissues by yeast cells. The pattern of $A_{2A}R$ distribution in the GI tract showed that aged mice have less $A_{2A}R$ in the GI tract tissues, and that its density does not increases in response to *C. albicans* over-growth/infection, in contrast to the increased $A_{2A}R$ density observed in young and adult mice upon yeast infection.

Using a well established and sustained murine GI infection model (Koh *et al*., 2008; Vautier *et al*., 2012, 2014) based on the elimination of mice microbiota, an effective *C. albicans* GI colonization was achieved, without mice death owed to infection, in all age groups. Due to evidences of microbiota transference between different animals (Mason *et al*., 2012; Vautier *et al*., 2012), mice were housed individually to exclude this possibility. Additionally, we confirmed *C. albicans* identity

in stools and tissues using a molecular biology technique, by PCR and correspondent ITS-5.8S sequencing (data not shown), whereas in control animals (without *C. albicans* administration), no yeast cells were found in stools (data not shown).

The major conclusion of our study is that aged animals were more prone to yeast over-growth in the gut, which displayed a heighted inflammatory profile. First, we observed that *C. albicans* over-colonization of the gastric compartment (during the first 11 days of infection) in aged mice proceeds with a more exuberant morphological switch to the hyphal form, when compared with young and adult mice. Concomitantly, higher inflammation is observed in the gastric tissues of elderly mice. The importance of *C. albicans* morphologic switch for infection and dissemination in the gut has been lately updated (Gow *et al*., 2013; Vautier *et al*., 2014). GI tract colonization primarily favors the yeast form of *C. albicans* (Vautier *et al*., 2014) and a novel concept arose of the yeast GUT (gastrointestinally induced transition) a unicellular morphotype that promotes colonization (Gow *et al*., 2013; Pande *et al*., 2013). This morphological switch and over-colonization of the gastric compartment was higher than that observed in the intestinal segments, where the fungus was predominantly located in the intestinal lumen, as attested by the elimination of high levels of viable *C. albicans* cells in the stools and low levels of intestinal tissue colonization. This novel aspect of the gut infection/colonization by *C. albicans* was also observed by the group of Brown and co-workers (Vautier *et al*., 2012, 2014), and strongly suggests that the intestinal inflammation results from an overgrowth of yeast cells in the lumen rather than an invasion of the intestinal tissue. This is particularly evident in the aged mice. Whatsoever, inflammation can facilitate translocation phenomena (e.g., Andrutis *et al*., 2000). The colonization (or over-colonization) of the gut by yeasts has been associated with the translocation and hematogenous dissemination in immunosuppressed mice

(Koh *et al*., 2008; Takahashi *et al*., 2003). However, we did not observe a strong invasive infection of the intestinal tissues by *C. albicans*. This prompts the conclusion that the inflammation of the intestine is most probably triggered by the recognition of cell wall components of *C. albicans*, or to the production of soluble molecules that are known to stimulate host cells such as farnesol (Gosh *et al*., 2010) or small peptides originated by SAP2 external metabolism (Bras *et al*., 2012). In contrast with previous findings regarding systemic *C. albicans* infection (particularly relevant in aged mice as stated by Murciano *et al*., 2006, for instance), we have found low yeast levels in the kidneys and livers of all animals, using our infection model via drinking water. However, in those systemic studies, a substantial decrease in kidneys and livers colonization levels was found along the infection, being residual over the end of the experiment suggesting an efficient immunity response. Since we only harvested the tissues at the end of the experiment, instead of assessing organ colonization at different infection time points, the reduced fungal burden obtained probably corresponds to a post-eradication period. Nevertheless, the low colonization rates now observed was also previously reported by others (Kennedy and Volz, 1985; Samonis *et al*., 1990; Vautier *et al*., 2014).

The second major finding of our study is the tentative association of adenosine A_{2A} receptors ($A_{2A}R$) in the GI tissues with the over-colonization by *C. albicans* and subsequent inflammation. The involvement of purines and their receptors in the pathophysiology of inflammatory gut diseases is best heralded by combined observations that, while extracellular ATP mediates inflammation (Kurashima *et al*., 2015), CD73, an ecto-nucleotidase responsible for converting AMP in adenosine, plays an essential role in the recovery from severe colonic inflammation (Bynoe *et al*., 2012). In fact, adenosine is recognized as an anti-inflammatory agent, contributing to

ameliorate the damage induced by inflammation (Haskó and Pacher, 2007; Sitkovsky *et al*., 2004). Accordingly $A_{2A}R$ are involved in inhibitory mechanisms of pro-inflammatory cytokines secretion during colitis or enteritis, and the intestinal luminal adenosine levels are increased in the inflamed intestine due several pathological conditions such as inflammatory bowel disease (Ye and Rajendran, 2009). Our study provides the first qualitatively analysis of $A_{2A}R$ distribution in the GI tract of mice from different age groups. Notably, we observed that aged mice have a lower $A_{2A}R$ density than young and adult mice. Moreover, instead of increasing the density of $A_{2A}R$ during *C. albicans* infection, as observed in adults and young mice, $A_{2A}R$ immunolabelling showed that $A_{2A}R$ density is maintained or slightly decreases. Therefore, we conclude that the inability to increase $A_{2A}R$ density in the GI tract of aged mice, contributes to a higher inflammatory score, most probably with a lower ability to overcome the deleterious effects of *C. albicans* over-colonization, thus leading to a higher tissue damage. Overall, this supports the hypothesis that increased $A_{2A}R$ density at the surface of GI mucosa cells is a protective mechanisms against *C. albicans* overgrowth that is present throughout adulthood and decreases with age.

We also observed that in aged mice, yeast-to-hypha transition of *C. albicans* was more exuberant, especially in the stomach. We now propose that this may result from the lower density of $A_{2A}R$; in fact, adenosine and $A_{2A}R$ in the stomach contribute to the control of gastric secretion (Yip and Kwok, 2004), and the lower $A_{2A}R$ density in the stomach of aged mice is expected to dampen the ability of the gastric compartment of aged mice to control pH, leading to a stressful condition that triggers the morphological switch of this trimorphic fungus. In fact, it was described that, under acidic conditions, *C. albicans* can raise the pH from 4 to neutral or alkaline, resulting in yeast-to-hypha transition, considered one of its most important virulent traits (Vylkova *et al.*, 2011).

This age-related alteration of $A_{2A}R$ density, together with the ability of *C. albicans* to modulate the external pH (Vylkova *et al*., 2011), might also explain our finding of a low rate of yeast-to-hypha transition in young mice, increasing in adults, and displaying a maximal rate in aged mice. This novel working hypothesis is of particular interest in the context of gastrointestinal diseases, which have increased incidence in elderly patients (Holt, 2003), namely for elderly patients with gastric ulcers who are more colonized by *C. albicans* (Kumamoto, 2011).

In summary, the overgrowth of *C. albicans* in the intestinal lumen of aged mice induced inflammation of the mucosal tissues, which was independent on the invasion of intestinal tissues by yeast cells. The pattern of $A_{2A}R$ distribution in the GI tract showed that aged mice have less $A_{2A}R$ in the GI tract tissues, and that its density does not increases in response to *C. albicans* overgrowth/infection, in contrast to the increased $A_{2A}R$ density observed in young and adult mice upon yeast infection.. The impact of these findings is that the lack/reduced number of $A_{2A}R$, associated with beneficial effects under inflammatory conditions, likely contributes to increased inflammation and lower ability to control *C. albicans* infection and the deleterious effects of this opportunistic agent in the elderly.

REFERENCES

Andrutis KA, Riggle PJ, Kumamoto CA and Tzipori S. (2000) Intestinal lesions associated with disseminated candidiasis in an experimental animal model. J Clin Microbiol. 38(6):2317-23.

Aw D, Silva AB and Palmer DB. (2007) Immunosenescence: emerging challenges for an ageing population. Immunology 120:435-446.

Biagi E, Nylund L, Candela M, Ostan R, Bucci L, Pini E, Nikkïla J, Monti D, Satokari R, Franceschi C, Brigidi P and De Vos W. (2010) Through ageing, and beyond: Gut microbiota and inflammatory status in seniors and centenarians. PLoS ONE 5(5): e10667.

Bras G. et al. (2012) Extracellular aspartic protease SAP2 of *Candida albicans* yeast cleaves human kininogens and releases proinflammatory peptides, Met-Lys bradykinin and des-Arg 9 -Met-Lys-bradykinin. Biol Chem. 393:829-839.

Bynoe MS, Waickman AT, Mahamed DA, Mueller C, Mills JH and Czopik A. (2012) CD73 is critical for the resolution of murine colonic inflammation. J Biomed Biotechnol. 2012:260983.

Cole GT, Halawa AA and Anaissie EJ. (1996) The role of the gastrointestinal tract in hematogenous candidiasis: From the laboratory to the bedside. Clin Infect Dis. 22 (Suppl 2):873-88.

d'Enfert C. (2009) Hidden killers: perisitence of opportunistic fungal pathogens in the human host. Curr Opin Microbiol. 12: 358-364.

Estrela AB and Abraham WR. (2011) Adenosine in the inflamed gut: a Janus faced compound. Curr Med Chem . 18:2791-2815.

Falagas ME, Roussos N and Vardakas KZ. (2010) Relative frequency of albicans and the various non-albicans *Candida* spp among candidemia isolates from inpatients in various parts of the world: a systematic review. Int J Infect Dis. 14:e954–e966.

Fredholm BB, Chen JF, Cunha RA, Svenningsson P and Vaugeoisx JM. (2005) Adenosine and brain function. Int Rev Neurobiol. 63:191-270.

Garsin DA and Lorenz MC. (2013) *Candida albicans* and *Enterococcus faecalis* in the gut. Infect Immun. 81:189-200.

Ghosh S, Howe N, Volk K, Tati S, Nickerson KW and TM. (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 Med Microbiol. 60(1):63-73.

Gow NAR. (2013) A developmental program for *Candida* commensalism. Nature Genet. 45: 967–968.

Hasko G, Linden J, Cronstein B and Pacher P. (2008) Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat Rev Drug Discov. 7(9):759–770.

Haskó G and Pacher P. (2008) A_{2A} receptors in inflammation and injury: lessons learned from transgenic animals. J Leukoc Biol. 83: 447-455.

Holt PR. (2003) Gastrointestinal diseases in the elderly. Curr Opin Clin Nutr Metab Care. 6(1):41-48.

Kelly CP, Becker S, Linevsky JK, Joshi MA, O'Keane JC, Dickey BF, LaMont JT and Pothoulakis C. (1994) Neutrophil recruitment in *Clostridium difficile* toxin A enteritis in the rabbit. J Clin Invest. 93(3):1257-65.

Kennedy MJ and Volz PA. (1985) Effect of various antibiotics on gastrointestinal colonization and dissemination by *Candida albicans.* Sabouraudia 23:265-273.

Koh AY, Köhler JR, Coggshall KT, Van Rooijen N and Pier GB. (2008) Mucosal damage and neutropenia are required for *Candida albicans* dissemination. PLoS Pathog. 4(2):e35.

Kumamoto CA. (2011) Inflammation and gastrointestinal Candida colonization. Curr Opin Microbiol. 14(4): 386–391.

Kurashima Y, Kiyono H and Kunisawa J. (2015) Pathophysiological role of extracellular purinergic mediators in the control of intestinal inflammation. Mediators Inflamm. doi:10.1155/2015/427125.

Mason KL, Downward JRE, Mason KD, Falkowski NR, Eaton KA, Kao JY, Young VB and Huffnaglea GB. (2012) *Candida albicans* and bacterial microbiota interactions in the cecum during recolonization following broad-spectrum antibiotic therapy. Infect. Immun. 80(10):3371.

Murciano C, Villamón E, Yáñez A, O'Connor JE, Gozalbo D and Gil ML. (2006) Impaired immune response to *Candida albicans* in aged mice. J Med Microbiol. 55:1649-1656.

Pande K, Chen C and Noble SM. (2013) Passage through the mammalian gut triggers a phenotypic switch that promotes *Candida albicans* commensalism. Nat Genet. 45:1088-1091.

Pfaller MA and Diekema DJ. (2007) Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol. Rev. 20:133-163

Pfister and Savino. (2008) Can the immune system still be efficient in the elderly? An immunological and immunoendocrine therapeutic perspective. Neuroimmunomodulation 15:351-364

Plantinga TS, van Bergenhenegouwen J, Jacobs C, Joosten LAB, van't Land B, Garssen J and Netea MG. (2012) Modulation of Toll-like receptor ligands and *Candida albicans* induced cytokine responses by specific probiotics. Cytokine. 59:159–165.

Romani L. (2011) Immunity to fungal infections. Nat Rev Immunol. 11:276-288.

Round JL and Mazmanian SK. (2009) The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol. 9:313-323.

Samonis G, Anaissie EJ, Rosenbaum B and Bodey GP. (1990) A model of sustained gastrointestinal colonization by *Candida albicans* in healthy adult mice. Infect. Immun.58:1514-1517.

Sitkovsky M, Lukashev D, Apasov S, Kojima H, Koshiba M, Caldwell C, Ohta A and M Thiel. (2004) Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A_{2A} receptors. Annu. Rev. Immunol. 22:657–682.

Takahashi K, Kita E, Konishi M, Yoshimoto E, Mikasa K, Narita N and Kimura H. (2003) Translocation model of *Candida albicans* in DBA-2/J mice with protein calorie malnutrition mimics hematogenous candidiasis in humans. Microb Pathog. 35(5):179-87.

Vautier S, Drummond RA, Redelinghuys P, Murray GI, MacCallum DM and Brown GD. (2012) Dectin-1 is not required for controlling *Candida albicans* colonization of the gastrointestinal tract. Infect. Immun. 80(12):4216-4222.

Vautier S, Drummond RA, Chen K, Murray GI, Kadosh D, Brown AJP, Gow NAR, MacCallum DM, Kolls JK and Brown GD. (2014) *Candida albicans* colonization and dissemination from the murine gastrointestinal tract: the influence of morphology and Th17 immunity. Cell Microbiol. doi:10.1111/cmi.12388.

Vilaseca J, Salas A, Guarner F, Rodriguez R and Malagelada JR. (1990) Participation of thromboxane and other eicosanoid synthesis in the course of experimental inflammatory colitis. Gastroenterology. 98(2):269-77.

Vylkova S, Carman AJ, Danhof HA, Collette JR, Zhou H and Lorenz MC. (2011) The fungal pathogen *Candida albicans* autoinduces hyphal morphogenesis by raising extracellular pH. MBio. 2(3):e00055-11.

Ye JH and Rajendran V. (2015) Adenosine: an immune modulator of inflammatory bowel diseases. World J Gastroenterol 15:4491-4498.

Yip L and Kwok YN. (2004) Role of Adenosine A_{2A} Receptor in the regulation of gastric somatostatin release. J Pharmacol Exp Ther. 309(2):804-15.

CHAPTER VII

GLOBAL DISCUSSION AND CONCLUSIONS

GLOBAL DISCUSSION

In its daily routines, mankind is constantly challenged by a huge array of stresses and aggressions, and microorganisms are important contributors of those offenses. Moreover, most of the epithelial surfaces of our body are colonized by a number of microbial cells that exceeds that of our own cells, most of the times with no deleterious effects to the host. Yeasts are ubiquitous opportunistic fungi and, for instance, can either be found in the environment, as part of the human body normal flora or isolated from foods of animal origin, being thus always in close proximity with Man. However, in the last decades, genus *Candida*, particularly *C. albicans*, has been described as one of the most important agents of human infections, with enormous impact in health management (Romani, 2011). This is significantly relevant in individuals with immune diseases or weakened immune system, as we can found in the elderly growing portion of our societies (Pfaller and Diekema, 2007; Pfister and Savino, 2008). Ideally, it is expected that, anytime an infection occurs, the host immune system will be able to efficiently counteract the yeasts presence. To overcome an infection, different cells, namely the innate immune cells such as macrophages, due to it primordial phagocytosis competence, should recognize yeasts presence and engulf foreign cells, digesting it in phagolysosomes and thus clearing the invading pathogens (Cheng *et al*., 2012). Nevertheless, as mentioned above, infections happens and often lead to high morbidity and mortality rates. On one hand, a compromised immune system will be, *per si*, less competent to defeat invading yeasts, allowing them to grow and proliferate more easily, while, on the another hand, yeast cells have also developed several mechanisms to beat host defences. The host success over the presence of fungal cells will thus depend on a delicate equilibrium between host and pathogen (Brown *et al*., 2014). Anytime this

balance is disrupted, yeasts will overgrow, invading and infecting the host, causing disease, or hence being completely depleted or controlled and maintained as harmless commensals.

Most of the yeasts attributes to fight host defences are well established, but in many situations the complete responsible pathways leading to those mechanisms are yet not fully understood (Mayer *et al*., 2013; Williams *et al*., 2013). The results presented along this dissertation had established for the first time an unquestionable role of adenosine and $A_{2A}R$ in this yeasts-host interaction. It is well known that a potent immune response is necessary to eradicate an infection and that a consequent excessive inflammation will be also harmful to the host. Hence, ATP and its metabolite, adenosine, are described as important signalling molecules to achieve this tight regulation and both host and yeasts have the ability to release them (Sitkovsky and Ohta, 2005; Kumar and Sharma, 2009).

As described along this work, every time a macrophage-yeast interaction occurs, ATP produced by either of the intervenients, will be crucial in the pro-inflammatory responses and activating macrophages. However, the mentioned cleavage of ATP by the enzymatic cascade of ecto-phosphatases and ecto-5'-nucleotidases will function, together with an intrinsic cell release, as a source of adenosine, a potent STOP signal of inflammation.

In this work it is shown that the increased levels of ATP upon *C. glabrata* and *S. cerevisiae* infection of macrophages were associated to low activities of, respectively, ectophosphatase and ectonucleotidase, that concomitantly will contribute to keep the extracellular levels of ATP and to prevent the increase in extracellular adenosine. This might be in accordance with those yeasts infection profile: even if they can survive and replicate within the macrophages (surrounded by $A_{2A}R$, as characterized during this

work), *S. cerevisiae* is considered a non-pathogenic yeast, while *C. glabrata*, closely related to it, does not have key *C. albicans* virulence factors, as hyphal formation or tissues inflammation upon colonization, thus leaving this yeasts in a position to be more efficiently eliminated by the host (Seider *et al*., 2011). In line with this, contrary ATP levels and related enzymatic activities of *C. albicans* and *C. parapsilosis* are probably in accordance to its higher virulence and pathogenicity profiles, compromising host inflammatory defences and increasing yeast survival.

As reported before by several other research groups regarding bacterial infections and toxins, adenosine activation of $A_{2A}R$, can control inflammation and macrophages reactivity, with a robust trigger of macrophages Adora2a gene expression. Noticeably, instead of the this initially expected activation and increased gene expression, none of the yeast species used were able to induce any alteration in the Adora2a relative gene expression, compromising the responses associated to this crucial system. Although, in *C. albicans*-macrophage interaction, we have shown that regardless of the absence of up-regulation, modulation of $A_{2A}R$ proved to have an impact in the outcome of the infection. Hence, agonist activation of $A_{2A}R$ decreased the internalization of *C. albicans* cells. Moreover, the absence of functional $A_{2A}R$ in *C. albicans* infection of peritoneal macrophages of $A_{2A}R$ knockout mice, showed to increase the number of yeast cells ingested, when compared to wild type.

One of the most relevant novelties of this thesis is the first description of the functional relocalization of $A_{2A}R$ to intracellular compartments other than the intracellular trafficking associated with receptor inactivation. In fact, the interaction between macrophages and yeast cells leads to the intracellular localization of $A_{2A}R$, recruited to the membranes of macrophages enclosing the internalized yeasts cells.

Taking all these information together, this constitutes an unknown feature associated with the yeast's ability to efficiently infect and persist inside macrophages: the recruitment of anti-inflammatory adenosine receptors, $A_{2A}R$, functioning as a form of $A_{2A}R$ antagonism, a biological advantage that ultimately contributes to its success as pathogens. Once in contact with macrophages, yeast cells sequester, by an unknown mechanism, $A_{2A}R$ into the host intracellular compartments and modulate Adora2a gene expression, decreasing ATP and adenosine signaling levels, thus escaping phagolysosome efficient eradication and enabling persistence inside the host cells (Figure 1). These decreased levels of ATP and adenosine are probably achieved through modulation of ectonucleotidases activities, working as virulence factors, contributing to a modulation on the microenvironment concentrations of purines, in particular on phagolysosomes acidic compartments where it is expected to have higher enzymatic activities. Nonetheless, the involved specific key points and complete pathways are not fully addressed and differences among different yeast species and host cells, might have singular responses, namely in ectonucleotidases activities and consequent ATP and adenosine levels, depending on its phenotypic characteristics and need to be further investigated before extrapolated to a global vision. Still, it might be speculated that the described intracellular $A_{2A}R$ localization could possibly correspond to a conserved response among facultative intracellular microbes, functioning as an advantage protective feature of these organisms.

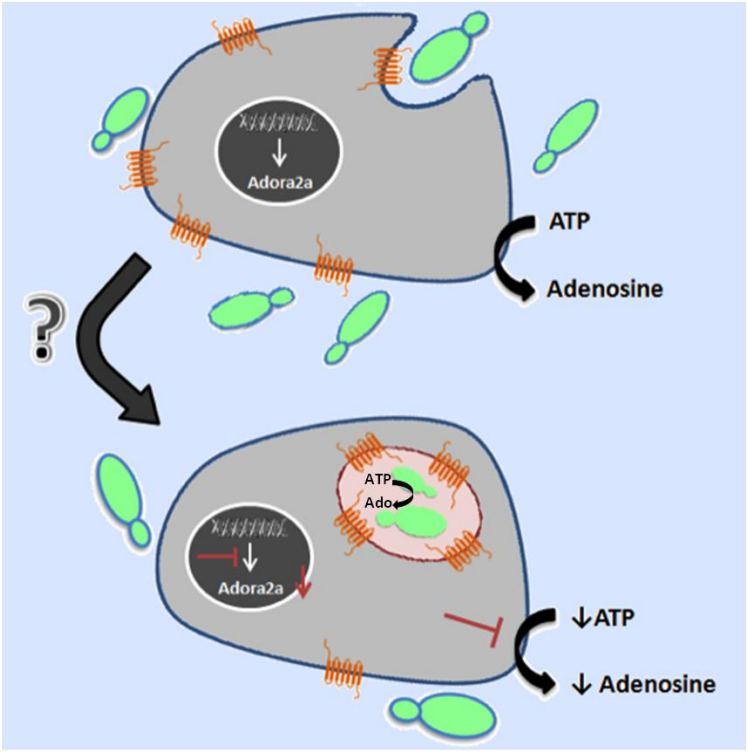

Figure 1. Schematic model of the $A_{2A}R$ involvement in yeast-macrophage interaction.

Upon macrophages (gray cells) phagocytosis of yeasts (green cells), $A_{2A}R$ (in orange) are recruited (migration from the cell membrane or *de novo* synthesis) into the phagosome membranes (bottom panel; pink compartment). This, together with a lack of increased $A_{2A}R$ gene expression (dark gray compartments) and compromised enzymatic and ATP and adenosine levels might be responsible in host cells yeast persistence upon infection. It is also predictable that, once inside the phagosome, when the phagosomal millieu is still characterised by low pH, yeasts increase the ectonucleotidase activity leading to the formation of adenosine. This adenosine is free to bind $A_{2A}R$, resulting in the downregulation of the intracellular pathway triggering macrophage activation and pathogen clearance. Moreover, activation of $A_{2A}R$ leads to the control of the phagosomal pH towards neutrality.

The observations at the cellular level, using phagocytic cells of the innate immune system, were complemented with an *in vivo* mice model of *C. albicans* infection. The purpose was to study the influence of $A_{2A}R$ in the gut infection of *C. albicans* and to relate that with age and inflammation of the gut tissues (Figure 2). Previously it was described that aged individuals were more susceptible to systemic *C. albicans* infection (Murciano *et al.*, 2006), clearly a serious life threatening pathology occurring in

situations of immunodepression or very debilitated conditions of the patients. The *in vivo* model studied here corresponds to a less dangerous condition, compatible with the normal development and aging process, and that, in what concerns the gut infection, corresponds to a autolimited gut infection or, as indicated by the inflammatory signs present in the gut tissues, a chronic inflammation due to *C. albicans* infection (in the absence of the bacterial microbiota usually associated with homeostasis). Overall, it could be concluded that during development and aging there is a progressive loss of the $A_{2A}R$ associated to gut tissues, *i.e.*, the $A_{2A}R$ gut mapping showed a lower density of this receptor in the gut of aged mice. This, as described by several groups of experts in adenosine receptors (*e.g.*, Haskó and Pacher, 2008; Estrela and Abraham, 2011), is immediately associated with a lower ability to control tissue damage and inflammation. When young, adults and aged mice were infected *per os*, in drinking water, with *C. albicans*, there was an increase in the immunodetection of $A_{2A}R$ in the gut tissues of young and adults, while in the aged mice this was absent. So, with this scenario regarding $A_{2A}R$ mapping before and after *C. albicans* sustained overgrowth/infection of the gut it is not surprising that the aged mice presented severe signs of inflammation such as haemorrhagic damages or inflammatory infiltrates. It is worth mention the observation of the virulent *C. albicans* yeast-to-hypha transition in the elderly mice stomach. This is probably related to the fact that $A_{2A}R$ is involved in the gastric pH homeostasis (Yip and Kwok, 2004).

One of the novelties of these *in vivo* results is that $A_{2A}R$ contributes to *C. albicans* persistence as a commensal, not inflicting damage in the young or adult host, while its absence in the aged host immediately triggers inflammation and yeast phenotypes associated with disease. This is probably an echo of the reported novel feature regarding

response to *C. albicans* at the cellular level, that also indicated that the recruitment of $A_{2A}R$ to the phagosome membrane leads to the silent persistance of *C. albicans.*

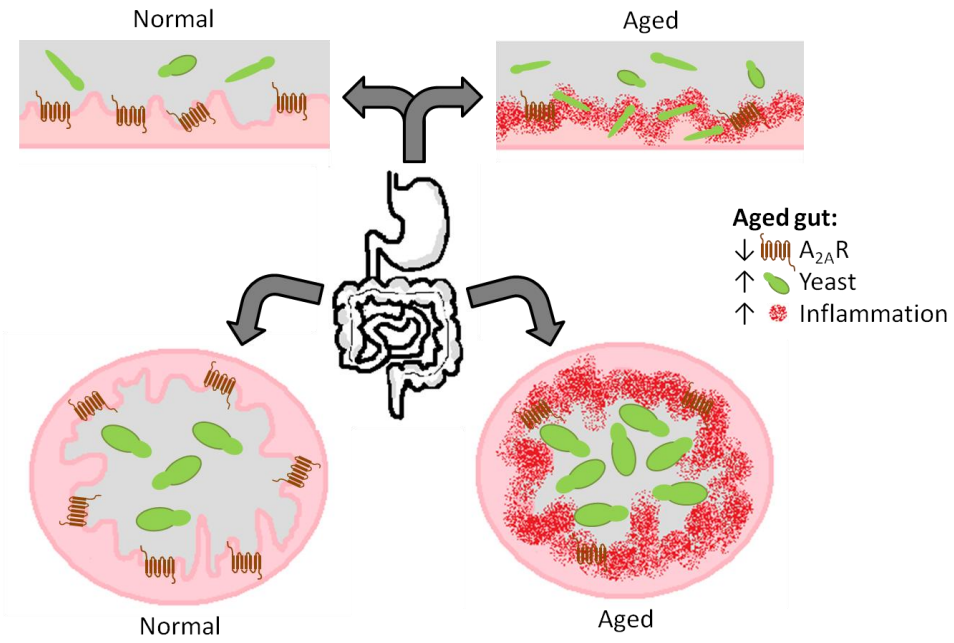

Figure 2. Schematic model of the $A_{2A}R$ involvement in yeast-gut interaction.

Yeasts (in green) administered to young, adult (Normal) and Aged mice, via drinking water, are found in gut lumen, from stomachs (top panels) to small and large intestines (bottom panels). The more exuberant infection and gut inflammation (red), particularly established in older animals, can be due to a possible concomitant role between a decreased density of gut $A_{2A}R$ (in brown) upon ageing and a yeast cells overgrowth in the lumen of those mice. Yeast-to-hyphae transition is also found higher in stomachs of aged mice (right top panel), thus compromising the equilibrium between host and the invading yeasts. Taking together, infection and ageing (with lower $A_{2A}R$ levels), are therefore expected to exacerbate the inflammatory processes and worsen the aged host damages.

This research has thus provided new insights into the complex field of yeasts-host interactions, establishing a new $A_{2A}R$ possible route on host *Candida* survival and persistence as a commensal, with an impact in maintenance of reservoirs that can lead to a severe infection if homeostasis is affected.

REFERENCES

Brown AJP, Budge S, Kaloriti D, Tillmann A, Jacobsen MD, Yin Z, Ene IV, Bohovych I, Sandai D, Kastora S, Potrykus J, Ballou ER, Childers DS, Shahana S and Leach MD. (2014) Stress adaptation in a pathogenic fungus. J Exp Biol. 217: 144-155.

Cheng SC, Joosten LAB, Kullberg BJ and Netea MG. (2012) Interplay between *Candida albicans* and the mammalian innate host defense. Infect Immun. 80(4): 1304-1313.

Estrela AB and Abraham WR. (2011) Adenosine in the inflamed gut: a Janus faced compound. Curr Med Chem . 18:2791-2815.

Haskó G and Pacher P. (2008) A_{2A} receptors in inflammation and injury: lessons learned from transgenic animals. J Leukoc Biol. 83: 447-455.

Kumar V and Sharma A. (2009). Adenosine: an endogenous modulator of innate immune system with therapeutic potential. Eur. J. Pharmacol. *616*, 7-15.

Mayer FL, Wilson D and Hube B. (2013) *Candida albicans* pathogenicity mechanisms. Virulence 4(2):119–128.

Murciano C, Villamón E, Yáñez A, O'Connor JE, Gozalbo D and Gil ML. (2006) Impaired immune response to *Candida albicans* in aged mice. J Med Microbiol. 55:1649-1656.

Pfaller MA, and Diekema DJ. (2007). Epidemiology of invasive candidiasis: a persistent public health problem. Clinic. Microbiol. Rev. *20*, 133–163.

Pfister and Savino (2008) Can the immune system still be efficient in the elderly? An immunological and immunoendocrine therapeutic perspective. Neuroimmunomodulation. 15:351-364.

Seider K, Brunke S, Schild L, Jablonowski N, Wilson D, Majer O, Barz D, Haas A, Kuchler K, Schaller M and Hube B. (2011) The facultative intracellular pathogen *Candida glabrata* subverts macrophage cytokine production and phagolysosome maturation. J Immunol. 187(6):3072-86.

Sitkovsky MV and Ohta A. (2005) The 'danger' sensors that STOP the immune response: the A_{2A} adenosine receptors? Trends Immunol. 26: 299-304.

Williams DW, Jordan RPC, Wei XQ, Alves CT, Wise MP, Wilson MJ and Lewis MAO (2013) Interactions of *Candida albicans* with host epithelial surfaces. J Oral Microbiol. 5: 22434.

Yip L and Kwok YN. (2004) Role of Adenosine A_{2A} Receptor in the regulation of gastric somatostatin release. J Pharmacol Exp Ther. 309(2):804-15.

CONCLUSIONS

The work presented in this dissertation leads to a main conclusion that yeasts modulate extracellular purines to decrease the pro-inflammatory extracellular ATP and, by sequestering the anti-inflammatory adenosine A_{2A} receptors, have the ability to persist inside the macrophages silencing the macrophage response to clear the invading agent. Moreover, a lower level of $A_{2A}R$ in the gut tissues of older individuals might be in the genesis of worsening host damage. The arguments that contributed to draw this conclusion include the following:

- *C. albicans*, *C. glabrata*, *C. parapsilosis* and *S. cerevisiae* while interacting with macrophages modulate ATP levels, together with overall ectophosphatase and ectonucleotidase activities; and only *C. glabrata* and *S. cerevisiae* were not able to decrease the extracellular ATP when infecting a macrophage cell line, thus contributing for the early inflammation of infected tissues.

- The different yeast species did not trigger an up-regulation of $A_{2A}R$ gene expression under macrophages infection but,
- *C. albicans*, *C. glabrata*, *C. parapsilosis* and *S. cerevisiae* force a novel macrophage redistribution of $A_{2A}R$ into the phagosomal membrane enclosing yeasts cells;
- Activation of $A_{2A}R$ by adenosine increases IL-1β gene expression upon *C. albicans* infection, although $A_{2A}R$ agonism or antagonism did not change phagocytosis or the expression of genes coding for the pro-inflammatory TNF-α or IL-1β cytokines;
- $A_{2A}R$ KO mice peritoneal macrophages internalize more efficiently *C. albicans* cells.

- Aged individuals are more prone to *C. albicans* gut overgrowth, and concomitantly present higher inflammation of the tissues;

- Aged individuals have less $A_{2A}R$ in the gastrointestinal tissues and *C. albicans* infection does not trigger increased number of this class of receptors in gut tissues, as observed in adult and young mice.

Re-distribution of $A_{2A}R$ in macrophages, together with the remaining alterations associated to the purinergic system, seems to support macrophage engulfment of yeasts, controlling phagosome maturation, compromising inflammatory responses and yeasts effective killing, thus contributing to yeasts persistence in the host and preventing clearance by phagocytic cells.

CHAPTER VIII

FUTURE PERSPECTIVES

FUTURE PERSPECTIVES

During the development of the present work several questions arose and much remain to be elucidated, although some of this doubts are presently being addressed.

As mentioned along the discussion, different microorganisms and different host cells might behave differently and interactions among them should be further investigated to deeper understanding of $A_{2A}R$ modulation under infections conditions. Special attention should be taken, for instance, on bacteria and killed yeasts infections assays, when looking at the host $A_{2A}R$ localization. This specific localization of $A_{2A}R$ should be confirmed as well as the intracellular trafficking. The effective concentrations of ATP and adenosine in the cells microenvironments are still unknown and should also be additionally studied, together with specific modulation of the several enzymes involved in this cleavage cascade. The complete adenosine and $A_{2A}R$ activation pathway should be modulated point by point in an attempt to understand which of the steps are actually involved in this system modulation. To confirm $A_{2A}R$ redistribution on host cells, transfection assays with a plasmidic $A_{2A}R$-GFP protein, *e.g.*, should also be optimized. This, together with further studies on $A_{2A}R$ KO cells and mice should provide important conclusions on effective $A_{2A}R$ contribution. Pharmacological modulation of $A_{2A}R$ responses should also be additionally studied, trying to confirm the different outcomes, for example, on infection upon non-*albicans* yeasts presence. The hypothetical contribution of other receptors, from $A_{2B}R$ to TLR-2 or dectin-1 should also be considered. All these treats should at the end be also confirmed in additional *in vivo* studies, corroborating the effective involvement of those receptors on real infection conditions. The use of caffeine as an inverse agonist and modulator of $A_{2A}R$ responses have been shown as a host protective in several conditions and should also be investigated in our cell and animal models. The *in vivo* results obtained here seem to

indicate that $A_{2A}R$ agonism might constitute a good therapeutic approach to gut chronic inflammation, so this could also be explored as a future perspective study to improve knowledge in this field.