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Role of secreted aspartyl proteases in *Candida albicans* virulence, host immune response and immunoprotection in murine disseminated candidiasis

Tese de Doutoramento em Ciências
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Trabalho realizado sob a orientação da
Professora Doutora Célia do Sacramento Santos Pais
e do
Professor Doutor Manuel João Rua Vilanova

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Abstract

The polymorphic yeast *Candida albicans* is an important opportunistic human pathogen and the most common causative agent of fungal invasive infections. Host physical barriers and immune system integrity are crucial factors in controlling the establishment of *Candida* infections. However, the high adaptability of *C. albicans* to different host niches is also a determinant factor. The host-fungus interplay is dynamic and the balance between fungal elimination and tissue damage depends on both the host response and how the pathogen reacts to immune effector molecules and cells.

The murine model of hematogenously disseminated *C. albicans* infection is widely used to ascertain strain virulence and host-fungus interactions. However, distinct *C. albicans* strains may present distinct virulence phenotypes and elicit quantitatively and qualitatively different immune responses. Hence, results obtained with one strain cannot be taken as representative of the whole species. Moreover, most studies focused on *C. albicans* internalization and killing by phagocytes, rather than on the overall immune response. Here, a comparative analysis of the early immune response, host survival and kidney fungal burden was done in mice infected intravenously with three *C. albicans* strains with different attributed virulence, SC5314, ATCC 90028 and ATCC 32354. Strain SC5314 was the most virulent and elicited a more marked inflammatory response, with higher neutrophil recruitment. In contrast, ATCC 32354 presented the lowest virulence and stimulated less markedly the innate immune response than the other strains. These results provide additional evidence on the association between *C. albicans* virulence and the early immune response, which may be useful in delineating vaccination or immunotherapeutic strategies against disseminated candidiasis.

Secretion of *C. albicans* hydrolytic enzymes during infection is a virulence attribute that aids adhesion to and invasion of host tissues, and immune evasion. Among these enzymes, secreted aspartyl proteases (Sap) encoded by a 10-member gene family (*SAP1 to SAP10*) have been particularly studied. Several members of the Sap family were claimed to play a significant role in the progression of candidiasis established by the hematogenous or intraperitoneal routes. This assumption was based on the

observed attenuated virulence of *sap*-null mutant strains. However, the exclusive contribution of *SAP* genes to their attenuated phenotype was not unequivocally confirmed, as the Ura status of several mutant strains could also have contributed to the attenuation. In this study, the importance of *SAP1* to *SAP6* in murine models of hematogenously disseminated candidiasis and *C. albicans* peritonitis was reassessed by using *sap*-null mutant strains not affected in their URA3 gene expression. In systemic candidiasis established by intravenous infection, *SAT1*-flipping constructed Δ*sap123* and Δ*sap456* mutants did not present a significant reduction in virulence contrasting the attenuated virulence found in equivalent Ura-blaster mutants. Using the newly assessed mutant strains, the median survival time of BALB/c mice infected with the Δ*sap123* strain was similar to that of wild-type (WT) SC5314-infected mice, while those infected with mutant strains lacking *SAP5* showed slightly extended survival times. Nevertheless, WT and Δ*sap456* strains were equally able to invade mice kidneys. Likewise, *SAP4* to *SAP6* deficiency had no noticeable impact on the immune response elicited in the spleen and kidneys of *C. albicans*-infected mice. These results suggest that Sap1 to Sap6 do not play a significant role in *C. albicans* virulence in the murine model of hematogenously disseminated candidiasis. Contrastingly, in the murine model of *C. albicans* peritonitis, Δ*sap456*-infected mice presented lower kidney fungal burden than WT- or Δ*sap123*-infected mice. WT-infected mice presented higher proportions of T regulatory cells ($Foxp3^+$) in the spleen and mesenteric lymph nodes than non-infected or *sap*-null mutant-infected counterparts. In addition, $CD4^+CD25^+$ T cells of WT-infected mice were the most effective in suppressing the proliferative response of $CD4^+CD25^-$ T cells whereas those of Δ*sap456*-infected mice were the least suppressive. Furthermore, $CD4^+$ T cells of WT-infected mice were the ones producing the highest levels of IL-10. Interestingly, Δ*sap456*-infected mice presented less $Foxp3^+$ cells in kidney lesions than the other fungal challenged mice. Altogether, these results implicate Sap4 to Sap6 in the host immune response to *C. albicans* peritonitis, providing additional evidence for the role of these enzymes in this infection model.

To circumvent host immune defenses, *C. albicans* developed multiple evasion mechanisms. Among these, production of Saps has been particularly highlighted due to

their ability to degrade and/or inactivate diverse host immune effector molecules. The mammalian pattern recognition receptor galectin-3 (Gal-3) was shown to specifically bind β-1,2 mannosides on *C. albicans* cell wall. This interaction directly induces fungal cell death and also promotes pro-inflammatory cytokine TNF-α production by host cells. In this study, we assessed whether this lectin could be degraded by native Sap2 or recombinant Sap1 to Sap3 isoenzymes. All *C. albicans* Saps tested degraded and inactivated the host receptor Gal-3. This may constitute a fungal strategy to control and evade host immune mechanisms dependent on Gal-3. Degradation of this protein might thus generate a protective microenvironment of reduced Gal-3 activity, which may facilitate *C. albicans* survival in the host.

Previous reports have shown that mice immunized with Sap2 were protected against mucosal or peritoneal *C. albicans* infection. Here, we extended these studies and evaluated the suitability of *C. albicans* recombinant Sap2 (rSap2) as protective immunogen for vaccination against hematogenously disseminated candidiasis. Four different immunogenic preparations were tested, respectively using Alum, Imiquimod, Freund's or CpG plus Alum as adjuvants. Moreover, as the hypha-associated isoenzyme Sap5 is preferentially expressed during systemic candidiasis, it was also evaluated as target antigen for *Candida* vaccination, together with Alum or Imiquimod. All these approaches failed in protecting the immunized mice from fungal infection. This suggests that the *C. albicans* enzymes Sap2 and Sap5, despite their potential role in virulence, do not appear to be suitable target proteins in immunopreventive strategies against hematogenously disseminated candidiasis.

In summary, results presented in this thesis provide additional evidence for the differential involvement of Saps in distinct *C. albicans* infection models. Moreover, these results, by showing that host immune response to *C. albicans* is affected by lack of *SAP* expression, are in support of a previously hypothesized immunomodulatory role for Saps. Finally, the lack of host immune protection against hematogenously disseminated candidiasis by means of Sap immunization reinforces the limited role of these proteins in this type of infection.

Resumo

A levedura polimórfica *Candida albicans* é um importante patógeno oportunista em humanos, sendo o mais frequente agente causador de infeções fúngicas invasivas. A integridade das barreiras físicas do hospedeiro, bem como do seu sistema imunitário, são fatores cruciais no controlo de infeções por leveduras do género *Candida*. Todavia, a grande adaptabilidade de *C. albicans* a diferentes nichos do hospedeiro é também um fator determinante. As interações fungo-hospedeiro são dinâmicas e o equilíbrio entre a eliminação do fungo e o dano tecidular depende tanto da resposta do hospedeiro, como da forma como o patógeno reage às células e moléculas imunitárias. O modelo de candidíase sistémica originada pela via endovenosa em murganhos é amplamente utilizado para avaliar a virulência das estirpes e as interações entre fungo e hospedeiro. No entanto, estirpes diferentes de *C. albicans* podem apresentar fenótipos de virulência distintos e desencadear respostas imunitárias, quer qualitativa, quer quantitativamente, diferentes. Deste modo, os resultados obtidos com uma estirpe não poderão ser considerados representativos da espécie. Além disso, a maioria dos estudos incidiram na internalização e morte de *C. albicans* por células fagocíticas, em detrimento da resposta imunitária global. Nesta tese foi feita uma análise comparativa da resposta imunitária precoce, da carga fúngica renal e da sobrevivência de murganhos infetados pela via endovenosa com três estirpes de *C. albicans*, com reconhecidas diferenças de virulência, SC5314, ATCC 90028 e ATCC 32354. A estirpe SC5314 foi a mais virulenta e desencadeou uma resposta inflamatória mais exuberante, com um maior recrutamento de neutrófilos. Pelo contrário, a estirpe ATCC 32354 foi a menos virulenta e estimulou menos a resposta imunitária do que as outras estirpes. Estes resultados reforçam a associação entre a virulência de *C. albicans* e a resposta imunitária precoce, o que poderá ser útil no planeamento de estratégias de vacinação ou imunoterapêuticas contra a candidíase disseminada.

A secreção de enzimas hidrolíticas por *C. albicans* durante a infeção é considerada um fator de virulência que auxilia a adesão e invasão de tecidos do hospedeiro e a evasão à resposta imunitária. Entre estas enzimas, as proteases aspárticas secretadas (Sap), codificadas pela família de genes *SAP1* a *SAP10* foram estudadas mais aprofundadamente. Foi atribuído um papel importante a vários membros desta família

na progressão da candidíase estabelecida pelas vias endovenosa e intra-peritoneal. Para tal contribuíram a virulência atenuada observada em mutantes deficientes nestas proteases. Contudo, o contributo exclusivo dos genes *SAP* para o fenótipo observado não foi inequivocamente demonstrado, uma vez que a inserção ectópica do gene *URA3* em vários mutantes poderá ter contribuído para a diminuição da virulência. Neste estudo reavaliou-se a importância dos genes *SAP1* a *SAP6* na infeção disseminada por via endovenosa e na peritonite causada por *C. albicans*, usando mutantes deficientes nestes genes sem expressão de *URA3* afetada. Na candidíase sistémica estabelecida pela via endovenosa, os mutantes Δ*sap123* e Δ*sap456*, construídos pelo método "*SAT1*-flipping" não apresentaram uma redução significativa na virulência, contrariamente ao observado nos mutantes correspondentes, construídos pelo método "Ura-blaster". Utilizando os mutantes novos, o tempo médio de sobrevida de murganhos BALB/c infetados com o mutante Δ*sap123* foi muito semelhante ao observado em murganhos infetados com a estirpe selvagem SC5314, enquanto os murganhos infetados com mutantes deficientes na expressão do gene *SAP5* tiveram tempos de sobrevida ligeiramente mais alargados. No entanto, tanto a estirpe selvagem, como o mutante Δ*sap456,* foram igualmente capazes de invadir os rins de murganhos infetados. Da mesma forma, a deficiência na expressão de *SAP4* a *SAP6* não afetou significativamente a resposta imunitária desencadeada, quer no baço, quer nos rins, de murganhos infetados com a levedura. Estes resultados sugerem que as Sap1 a Sap6 não desempenham um papel essencial na virulência de *C. albicans* no modelo de candidíase sistémica estabelecido pela via endovenosa. Pelo contrário, no modelo de peritonite causado por *C. albicans*, os murganhos infetados com a estirpe Δ*sap456* apresentaram carga fúngica renal menor do que a dos murganhos infetados com as estirpes selvagem ou Δ*sap123*. Os murganhos infetados com a estirpe selvagem apresentaram frequências de células T reguladoras ($Foxp3^+$) superiores às observadas nos murganhos infetados com as estirpes mutantes, tanto no baço como nos gânglios linfáticos mesentéricos. Além disso, as células T $CD4^+CD25^+$ de murganhos infetados com a estirpe selvagem suprimiram de modo mais marcado a proliferação de células T $CD4^+CD25^-$ do que as células correspondentes de murganhos infetados com as estirpes mutantes, enquanto as células de murganhos infetados com a estirpe Δ*sap456* apresentaram o fenótipo menos supressor. Adicionalmente, as células T $CD4^+$

de murganhos infetados com a estirpe selvagem foram as que produziram níveis mais elevados de IL-10. É particularmente interessante a observação de uma menor presença de células Foxp3^{+} nas lesões renais de murganhos infetados com o mutante Δ*sap456* em comparação com o observado nos murganhos infetados com as outras estirpes. O conjunto dos resultados obtidos implica o envolvimento das enzimas Sap4 a Sap6 na resposta imunitária à peritonite causada por *C. albicans*, acrescentando informação adicional sobre o papel destas proteases neste modelo de infeção.

C. albicans desenvolveu diversos mecanismos para ultrapassar a defesa imunitária do hospedeiro. Entre eles, destaca-se a produção de Saps pela capacidade destas enzimas em degradar e/ou inativar diversas moléculas da resposta imunitária do hospedeiro. O recetor de padrões microbianos galectina-3 (Gal-3) liga especificamente oligomanosídeos β-1,2 presentes na parede celular de *C. albicans*. Esta interação induz a morte da levedura e promove a produção da citoquina pro-inflamatória TNF-α pelas células do hospedeiro. Neste estudo foi avaliada a degradação da Gal-3 pelas Sap2 nativa e Sap1, Sap2 e Sap3 recombinantes. Todas as Saps testadas foram capazes de degradar e inativar a Gal-3. Esta poderá constituir uma estratégia para controlar e evadir os mecanismos imunitários dependentes de Gal-3. A degradação deste recetor poderá originar um microambiente de atividade Gal-3 reduzida, o que poderá facilitar a sobrevivência do patogéneo no hospedeiro.

Estudos prévios mostraram que murganhos imunizados com Sap2 ficaram protegidos contra a candidíase mucocutânea e contra a peritonite causada por *C. albicans.* Aqui, alargamos estes estudos e avaliamos a adequação do uso das Sap2 recombinante (rSap2) como imunogénio para vacinação contra a candidíase sistémica estabelecida por via endovenosa. Foram testadas quatro formulações imunogénicas diferentes, utilizando como adjuvantes Alum, Imiquimod, Freund e uma mistura de CpG e Alum. Adicionalmente, como a isoenzyma Sap5 é expressa preferencialmente durante a candidíase sistémica, foi igualmente avaliado o seu uso como antigénio alvo para vacinação contra a doença, utilizando Alum ou Imiquimod como adjuvantes. Nenhuma das preparações testadas resultou na proteção dos murganhos imunizados contra a infeção fúngica. Estes resultados sugerem que as enzimas Sap2 e Sap5, apesar do seu potencial papel na virulência de *C. albicans*, poderão não ser proteínas-alvo adequadas

para utilização em estratégias imuno-preventivas contra a candidíase sistémica estabelecida pela via endovenosa.

Em resumo, os resultados apresentados nesta tese fornecem informações adicionais sobre o envolvimento diferencial das Saps em modelos de infeção distintos. Além disso, estes resultados, ao mostrarem que a resposta imunitária do hospedeiro é afetada pela falta de expressão de genes *SAP*, suportam um papel imuno-modulador das Saps que havia sido previamente sugerido. Finalmente, a falta de proteção imunitária do hospedeiro contra a candidíase sistémica estabelecida pela via endovenosa reforça o papel limitado destas proteases neste tipo de infeção.

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List of abbreviations

AIDS	Acquired Immune Deficiency Syndrome
Alum	Aluminium hydroxide gel
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATCC	American Type Culture Collection
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
CLR	C-type lectin receptor
CWP	Cell Wall protein
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercelular adhesion molecule 3-grabbing non-integrin
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting; flow citometry
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPI	Glycosylphosphatidylinositol-anchored
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KDa	Kilodalton
LIP	Lipase
LPS	Lipopolysaccharide
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MR	Mannose receptor
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor
NBT	Nitro-blue tetrazolium
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide

PLB	Phospholipase B
PPR	Pattern recognition receptor
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SAP	Secreted aspartyl protease
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamid gel electrophoresis
TAA	Tumour-associated antigen
TEA	Triethylamine
Th	T helper
THP-1	Human acute monocytic leukemia cell line
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
UK	United Kingdom
USA	United States of America
YCB	Yeast carbon base
YEPD	Yeast extract, Peptone and Dextrose
WO	White-opaque
WT	Wild-type

CHAPTER 1

General Introduction

1. General introduction

The genus *Candida* is composed of a very heterogenous group of more than 150 yeast species (1), yet only a few are recognized human pathogens. The species most commonly encountered in medical practices are *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei* (2, 3). Besides the yeast form, the majority of the members of the genus can also display a filamentous type of growth, as pseudohyphae. In addition, *C. albicans* and *C. dubliniensis* also form true hyphae, in addition to pseudohyphae (2).

Even though the prevalence of infections caused by non-*albicans Candida* species is rising, the majority of candidiasis cases, regardless of clinical setting and geographical location, are still caused by *C. albicans* (4).

C. albicans commonly colonizes the human gastrointestinal, respiratory and reproductive tracts, and the skin (5), generating no obvious pathology. However, under certain conditions, *C. albicans* is capable of causing a wide spectrum of infections, from superficial thrush to life-threatening systemic candidiasis, making it the most prevalent fungal pathogen in humans (6, 7).

Transition from colonization to mucosal invasion and/or systemic dissemination depends upon host and fungal factors (8). Infection of a host starts with the adherence of fungi to the epithelial surface layers and further dissemination to different host sites (9). The immune status of the host is important for the transition from commensal to pathogen (10). However, the adaptation of *C. albicans* to new niches within the host is also critical for this transition (2). Fungi respond to change in a specific environment by inducing transcriptional and translational changes that promote survival and fitness under the new environmental conditions (11-13).

2. *Candida* infections

Clinical disease can be divided into two broad categories, mucocutaneous and systemic infection, each with its own risk factors.

2.1. Mucocutaneous candidiasis

Infections of the skin and mucous membranes due to *Candida* spp. are growing in incidence and can occur either in immunocompromised or in immunocompetent patients (14-16).

Oropharyngeal candidiasis

Oropharyngeal candidiasis (OPC) is very common in HIV-positive individuals, affecting nearly 90% of subjects at some stage during the course of HIV disease progression (17-20). *C. albicans* is always the most prevalent species in this type of infection, followed by *C. glabrata*, *C. dubliniensis* and *C. tropicalis* (17).

In non-HIV-infected individuals, the epidemiological data of OPC are not as intensively studied as in HIV-infected individuals. Predisposing factors in these patients include concomitant treatments with corticosteroids, antibiotics or immunosuppressive and anticancer drugs (21), diabetes, elderly and infancy (22). The most important predisposing factor for OPC other than T-cell immunodeficiency is the wearing of removable dentures (23).

***Candida* esophagitis**

Candida esophagitis occurs in patients with chronic diseases, most of whom have been previously treated with antibiotics or steroids, but the disease most frequently occurs in those with advanced HIV infection and affects 10% of patients with AIDS (24, 25). Some of these patients have concomitant OPC (26).

Vulvovaginal candidiasis

Vulvovaginal candidiasis (VVC) is a mucosal infection caused by several *Candida* spp. with *C. albicans* as the most common yeast obtained from vaginal fungal cultures (27-30). Approximately 70% to 75% of women experience at least one episode of VVC in their lifetime, and 20% suffer from recurrence (28, 31), usually caused by the same single strain through sequential infections (32, 33). Subjects at higher risks for VVC are pregnant HIV-infected women and, to a lesser extent, HIV-negative pregnant women (34). HIV-infected women have higher frequencies of *Candida* spp. colonization than HIV-negative women (34-36), yet the influence of $CD4^+$ T cell numbers in the

occurrence of VVC is not consensual. Other risk factors are diabetes, and antibacterial vaginal or systemic therapy (5).

Chronic mucocutaneous candidiasis

Chronic mucocutaneous candidiasis (CMC) is a set of syndromes in which patients have chronic and/or recurrent infections of the skin, nails, and mucous membranes due to *Candida* spp. (5), without a known underlying cause (37).

2.2. Disseminated candidiasis

Candida species have emerged as an important cause of bloodstream and deep tissue infections. Bloodstream infections caused by these organisms are also designated as candidemia, which often lead to *Candida* spread to internal organs, a condition known as disseminated or systemic candidiasis. Most cases occur in hospitalized patients and up to half are associated with intensive care units (38-41).

Nosocomial *Candida* infections

The incidence of nosocomial fungal infections (defined as invasive fungal infections acquired in a health care-associated setting) has dramatically increased. As the population of immunosuppressed individuals has increased (secondarily to the increased prevalence of cancer, chemotherapy, organ transplantation, and autoimmune diseases), so has the incidence of *Candida* invasive infections (42, 43).

Frequency

Candida species are by far the most common fungi causing invasive disease in humans (44-46).

Data from the most recent studies in the US indicate that *Candida* spp. are now the third most frequent nosocomial bloodstream isolates (47-51). However, in Europe, *Candida* species are considered to be between the fifth and tenth most common causative pathogen of bloodstream infections (41). The true incidence of disseminated candidiasis may nevertheless be markedly underrepresented in studies focusing on blood cultures, because of the difficult diagnostic in these samples (52, 53) and thus,

studies which reflect clinical diagnoses of disseminated candidiasis, rather than relying upon blood cultures, have indicated a predicted value of 24 cases per 100,000 population in the US (54). Hence an estimated number of 60-70,000 cases of disseminated candidiasis occur per year in the US alone, with a health care cost associated of $2-4 billion/year (54, 55).

Through the late 1980s, the predominant species causing invasive *Candida* infections was *C. albicans*. Indeed, *C. albicans* is by far the most virulent species of *Candida* in animal models (56-61). However, since the 1990s there has been a steady increase in the relative frequencies of non-*albicans* species of *Candida* causing disseminated candidiasis. This epidemiological trend has profound consequences for selection of empiric antifungal therapy. *C. albicans* has been responsible for approximately 50% of invasive *Candida* infections, with *C. glabrata* generally as the second most common cause of infection in the US and much of Europe, causing 15-25% of cases (44, 47, 50, 62-70). In contrast, in Latin America, Portugal and Spain, *C. parapsilosis* is the second most common cause of invasive candidiasis (71-74). *C. tropicalis* was acknowledged as causative of 10-20% of cases in most studies. The frequency of other species remains low, except in major cancer centres where widespread azole prophylaxis is used. In such centres, *C. krusei* may cause 10% of cases of invasive *Candida* infections (71, 75-78).

Origin

The origin of the infecting strain in the establishment of systemic infections is still controversial. Although the GI tract of most people is colonized by *Candida*, it is not clear whether the strains that colonize healthy hosts are responsible for causing subsequent invasive disease when those hosts acquire the appropriate risk factors, or if infections are caused by acquisition of more virulent strains from environmental sources in the nosocomial setting. Available data suggest that in most cases, the source of an infecting *C. albicans* strain is indeed endogenous flora (79-83), but that in certain circumstances exogenous transmission may occur in the nosocomial setting (84, 85).

Risk factors

The majority of patients who develop disseminated candidiasis are not immunosuppressed in the classical sense (such as neutropenic, corticosteroid-treated, infected with HIV, etc.) (39, 41, 62, 63, 67, 70, 86, 87). Rather, the predominant risk factors for disseminated candidiasis are common iatrogenic and/or nosocomial conditions. In particular, 65-90% of patients with disseminated candidiasis harboured a central venous catheter (39, 62, 88-90). This and other medical devices are easily colonized by candidal cells from mucosal surfaces and provide the opportunity for these cells to form biofilms, which are more resistant to drugs and capable of greater tissue invasion, enabling fungal spread from one tissue site to another (91). Hospitalization in the intensive care unit (ICU) provides the opportunity for transmission of *Candida* among patients and has been shown to be an additional independent risk factor. Another important independent risk factor for development of disseminated candidiasis is colonization by *Candida* spp. (82, 92-96). Patients with higher colonization burdens and more sites colonized have a proportional higher risk of developing hematogenously disseminated disease (97, 98) (and treatments that lower colonization burden simultaneously decrease the risk of candidemia (99). Broad-spectrum antibiotic therapy may alter the growth of normal bacterial flora, resulting in increased *Candida* colonization burden (100-102) and, consequently, increasing the risk of disseminated candidiasis (103-105). Additionally, disruption of normal skin barriers, for example by burn injury or percutaneous catheter placement (104, 106-111), and disruption of gut mucosal barriers by abdominal surgery, instrumentation, induction of mucositis (63, 111, 112) or parenteral nutrition (113), are major risk factors for invasive *Candida* infections. Direct translocation of *Candida* across the GI tract of animals and humans has been well documented (8, 113, 114), and GI surgery is a well-described clinical risk factor for development of disseminated candidiasis (115-118). More recently, cardiac surgery has also been described as a major risk for disseminated candidiasis (119-121). Neutropenia or abnormalities in neutrophil function dramatically increases the risk of developing disseminated candidiasis and the expected mortality rate (67, 70, 92, 122-126). Concordant with their well-characterized suppression of phagocyte function, glucocorticoids also increase the risk of

disseminated candidiasis (123, 127-129). Similarly, diabetes markedly increases the incidence of both mucocutaneous and disseminated candidiasis (130, 131).

Candidemia in cancer patients is also thought to develop from initial GI colonization with subsequent translocation into the bloodstream after administration of chemotherapy. In a murine model of GI candidiasis, systemic chemotherapy with cyclophosphamide, which causes simultaneously neutropenia and GI mucosal damage, led to disseminated fungal infection and 100% mortality ensued in mice previously colonized with *C. albicans* (114). In this study, neutropenia alone or combined with macrophage depletion did not result in *Candida* translocation and mice death. Likewise, GI mucosal damage alone was not sufficient for the development of candidemia. Instead, both neutropenia and GI mucosal damage were crucial for *C. albicans* dissemination from the GI tract.

Patients with late stage HIV disease have an extremely high incidence of developing mucocutaneous candidiasis (130, 132). However, HIV infection is not an independent risk factor for disseminated candidiasis. The occurrence of disseminated candidiasis in patients infected with HIV is attributable to the increased incidence of the usual risk factors for candidemia, including indwelling catheters, broad-spectrum antibiotics, hospitalization in an ICU, parenteral nutrition, and neutropenia (106, 110). Patients infected with HIV who do not have additional risk factors for disseminated candidiasis are not at increased risk of developing the disease.

Therapeutic strategies

Not only are invasive *Candida* infections extremely difficult to diagnose, they are also difficult to treat. Even with first-line antifungal therapy, disseminated candidiasis has an attributable mortality of up to 40% (87, 133, 134) , or even over 50% attributable mortality in patients that undergone myeloablative chemotherapy (93, 105, 123) . Unfortunately, newer therapies, such as voriconazole, lipid-based amphotericin formulations, and echinocandins, have not considerably improved survival of patients with candidemia, compared to the classical amphotericin B deoxycholate (135-137). Data from several studies supported that delayed initiation of therapy for candidemia is associated with significantly higher mortality (138-140).

Because of the difficulties in confirming the diagnosis with laboratory studies, empiric therapy administration must often be based on a clinical diagnosis of disseminated candidiasis. A clinical diagnosis of disseminated candidiasis is typically made in a patient with signs, symptoms, and laboratory features consistent with infection, who does not respond to broad-spectrum antibacterials, and who has risk factors for disseminated candidiasis. In such patients, early empiric therapy is appropriately administered. If a clinical response is seen, a clinical diagnosis of disseminated candidiasis can be made retrospectively. Consensus guidelines on the empiric treatment of disseminated candidiasis are available (141, 142). In the guidelines of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), it is clear that all three echinocandins (anidulafungin, caspofungin and micafungin) are likely equivalently efficacious for the treatment of disseminated candidiasis in ICU patients. The use of fluconazole has only marginal support and voriconazole and liposomal amphotericin B have moderate support. Combination therapy is not recommended, either for these patients or for hemato-oncological patients. For the last group of patients, caspofungin and micafungin are the most appropriate antifungal drugs for treatment of invasive candidiasis and candidemia in neutropenic patients or hematopoietic stem cell transplant recipients. For empirical treatment in neutropenic patients, the use of caspofungin and liposomal amphotericin B have a strong recommendation, while there is moderate support for voriconazole and micafungin (142).

From these guidelines it is also clear that catheter removal is not mandatory if echinocandin is being used (142, 143).

3. *Candida* virulence factors

Candida pathogenicity is a complex and highly regulated multifactorial process(144). The expression of several *C. albicans* genes or proteins associated with infection has been extensively studied. The role of a determined putative virulence factor can be assessed by comparing the biological response of the fungus with and without the factor. The most convincing evidence for a factor to be considered as a virulence determinant is the simultaneous loss of the factor and of virulence, and the regaining

of virulence when the factor is restored (144). Virulence factors must help the pathogen to grow at elevated temperatures, facilitate adherence, penetration and dissemination, or assist in resistance against or evasion from innate immune defenses.
It has become clear that a complex combination of properties rather than a single putative virulence factor are usually required for a fungus to successfully adapt to the different host niches met during the infectious process (144, 145) .

3.1. Morphogenesis

One of the most unique characteristics of *C. albicans* is its ability to reversibly change between the unicellular yeast and filamentous (hyphae and pseudohyphae) growth modes (146). Polymorphism of *C. albicans* is known to be one of the most important virulence factors of the fungus (145, 147, 148) and has been associated with tissue destruction and invasion (149-153), although both *C. albicans* yeast and hyphal cells are found in infected host tissues (147). In fact, the nature of association between fungal morphogenesis and host invasion is a highly debated aspect of fungal virulence (154, 155).
The switch from yeast to hyphal growth is controlled both by the environment and by numerous other stimuli. The standard trigger of hyphal growth is nutrient poor media and a rise in temperature, together with N-acetyl-glucosamine (GlcNAc) or serum (146). External pH is another signal regulating morphogenesis (156). In the host, *C. albicans* switches to the hyphal form to adhere to and penetrate through tissues, and this fungal plasticity has been demonstrated in several studies to be strongly required for *C. albicans* pathogenesis (157-159).
The yeast-hypha transition process is regulated by different interconnected signal transduction pathways (160). Efg1 (161, 162), and Cph1 (161) have been identified as key transcriptional regulators for the cAMP-protein kinase (PKA) and the mitogen-activated protein kinase (MAPK) pathways, respectively. The role of these factors in morphogenesis and their relevance for *Candida* infections was demonstrated by using strains which lacked either functional Efg1, Cph1 or both factors (147, 161-170) . Disruption of *CPH1* caused attenuated *C. albicans* hyphal formation on solid media, while mutants lacking *EFG1* failed to produce hyphal cells under most conditions investigated. Moreover, mutants lacking *EFG1* had significantly attenuated virulence in

murine models of hematogenously disseminated infection (164, 171) , in a murine model of *Candida* peritonitis (172), in the interaction with macrophages (164), in the ability to colonize successfully on polyurethane central venous catheters (165), and also in the ability to invade or damage endothelial (166), epidermal (163, 173, 174) or intestinal cells (163).

The transcription factor Rim101 regulates pH-dependent transition and mutant strains lacking *RIM101* also have a defective virulence phenotype (175). The TEA/ATTS transcription factor Tec1 is essential for serum-induced filamentation (176) and the Δ*tuc1* mutant was found to be locked in the filamentous form, with negative consequences in its ability to cause disease. Furthermore, there are a number of other transcription factors (Czf1, Flo8, Hap5 Efh1, Ace2 Mcm1, Ash1 and Cph2) involved in filamentation under specific growth conditions (146). The attenuated virulence, or even avirulence, of the mutant strains lacking any of these genes seemed to be caused not only by the interference in the filamentation process, but also by a reduction or loss of expression of virulence-associated genes under their regulation (164, 177, 178) .

3.2. Phenotypic switching

Phenotypic switching in *C. albicans* affects several phenotypic and metabolic parameters, including recognized virulence traits. Colonies of *C. albicans* show reversible morphological variation that occurs spontaneously in stress, resulting in changes in cell surface, colony appearance, metabolic, biochemical and molecular attributes, with impact on fungal virulence (15, 179). Interestingly, strains isolated from vaginitis or systemically infected patients showed higher frequencies of switching, indicating a strong role for the switching phenomenon in establishing disease (180). Although many phenotype switching phenotypes have been described in *C. albicans*, the white-opaque system in strain WO-1 is the most studied (181, 182). The ability to switch to the opaque form (mating-competent form) depends on whether the mating type locus (MTL) is homozygous. Most *C. albicans* cells are unable to switch since they are heterozygous for the MTL (MTLa/MTLα) and express the heterodimeric α1/α2 repressor (182). Although transition occurs at very low levels, white-opaque switching can be induced by environmental signals. One of the most relevant is the induction of switching to the opaque phase by anaerobic conditions,

such as those encountered by passage of white cells through the mammalian intestine (183).

Like the yeast-hyphae transition, white-opaque phase transition also influences fungal virulence (180, 184). Macrophages seem to preferentially phagocytose white cells (185) and that has also been recently observed with neutrophils. Neutrophils phagocytosed more efficiently white cells that were beginning filamentation, indicating that suppression of filamentation observed in opaque cells avoided neutrophil recognition and may represent a strategy for immune evasion (186). In contrast, dendritic cells phagocytosed white and opaque phase cells equally (186). However, white cells are known to be more resistant to candidacidal activity of PMN (187). Indeed, the viability of opaque cells is reduced when compared to white cells under many growth conditions (188, 189). However, even though opaque cells are generally considered as less virulent than white cells in several murine models (184, 190), they were found to be better able than white cells to infect skin (180).

3.3. Adhesins

Adherence of *C. albicans* cells to host tissues is a complex multifactorial phenomenon employing several types of adhesins expressed on morphogenetically changing cell surfaces.

The *C. albicans* agglutinin-like sequence (ALS) gene family is composed of at least eight genes encoding cell-surface GPI-anchored proteins that have long been associated with adhesion of *C. albicans* cells to host tissues and even to abiotic surfaces (191, 192). High allelic variability has been shown in *ALS* genes, often within the same *C. albicans* strain, leading to strikingly different adherence profiles (191, 193). Of the ALS family members, the *ALS1* and *ALS3* genes encode adhesins with the broadest array of substrate affinity (193). The structural and functional diversity within the Als family most certainly provides *C. albicans* with an array of cell wall proteins capable of recognizing and interacting with a wide range of host constituents during infection (193). The expression of some ALS genes has also been involved in biofilm formation (194, 195) and in other growth-related functions (191). *ALS* gene family members were differentially expressed in response to specific stimuli *in vitro* and have been associated with hyphal morphogenesis (196-198). The expression of Als proteins was

detected by immunohistochemistry in *C. albicans* cells infecting the kidneys, spleen, heart, liver and lungs of mice (196). *ALS* genes might be differentially regulated in a niche-specific fashion. *ALS6* and *ALS7* were expressed at relatively low levels, and *ALS4* appeared to be downregulated in vaginal samples. Interestingly, *ALS* expression patterns determined by RT-PCR in clinical samples were similar to those observed in the corresponding animal models of oral, vaginal and systemic candidiasis, and in reconstituted human epithelial models (199-201). Als3, and to a lesser extent Als1 and Als5, were found to be important for the epithelial and endothelial invasion process (193). Als3 also binds cadherins and induces fungal endocytosis by host cells (202). Moreover, Als3 binds ferritin and allows *C. albicans* to use iron in oral cavities (203).

HWP1 (hyphal wall protein) encodes a cell-surface adhesin expressed during hyphal development (204, 205) that promotes strong interactions between *C. albicans* and host cells (206). Production of Hwp1 has been confirmed in the mouse model of systemic infection by immunohistochemistry (204). Studies with a *hwp1*-deficient *C. albicans* mutant have reported its reduced adherence and mortality in murine models (206, 207), and *HWP1* expression has been analysed in samples from human oral and vaginal infections (208, 209). Nevertheless, Hwp1 appears to be important not only for invasion but also for benign interactions of *C. albicans* with the host (208, 209).

3.4. Biofilm formation

Biofilms are surface-associated microbial communities with significant environmental and medical impact. *C. albicans* is able to form biofilms on catheters, endotracheal tubes, pacemakers and other prosthetic devices and has been involved in the establishment of device-associated nosocomial infections (91, 210). Moreover, biofilms have reduced susceptibility to several components of the host immune response and to antifungal drug therapy (211). *C. albicans* biofilms are heterogenous three-dimensional structures containing hyphae form cells, with altered phenotype, growth rate, and gene expression compared to planktonic cells, enclosed in an extracellular polymer matrix consisting of polysaccharides and proteins (212). Heterogeneity of these biofilms depends on the substrate composition, environmental conditions, and strains involved. Biofilm formation was found to be linked to dimorphism and phenotypic switching, well-known virulence traits for candidal cells

(210, 211, 213) and a variety of genes, such as the *ALS* gene family and *HWP1* are upregulated during biofilm formation (197, 199, 211, 214) , in particular, *ALS1* and *ALS3* expression was reported as necessary for biofilm formation (194, 215).

ALS1, *ALS3*, and *HWP1* are regulated by the transcription factor Bcr1 (biofilm and cell wall regulator), which is under the control of transcription factor Tec1 (214-217) . Nobile et al (194) suggested that Hwp1 and Als proteins might function as complementary adhesions in biofilm formation. The hypha-associated *EFG1* gene is also required for normal biofilm growth, and hypha-deficient mutants had defective biofilms that also adhered poorly to the substrate (165, 218). Besides, biofilms are under tight regulation of gene expression that is controlled through quorum sensing molecules (219).

3.5. Hydrolytic enzymes

Three types of secreted enzymes have been described extensively: phospholipases, lipases and secreted aspartyl proteases.

3.5.1. Phospholipases B

Phospholipases B (PLB) are enzymes that hydrolyze ester linkages of glycophospholipids and also possess lysophospholipase transacylase activity. Plb activity was the major phospholipase activity found in *C. albicans* culture supernatants (220). They play a central role in cellular processes such as signal transduction and inflammation through their effect on the metabolism of phospholipids and lysophospholipids. *C. albicans* extracellular Plb are encoded by five putative genes. *PLB1* (221), *PLB2* (222) and *PBL5* (223) have already been cloned and described. *PLB* expression has been detected in mucosal, gastrointestinal and systemic infection models. Differential expression of *PLB1* and *PLB2* was demonstrated, by using RT-PCR, in samples from human oral and vaginal infections (224, 225). Several studies investigated the role of Plb in *C. albicans* virulence. A clinical isolate producing high amounts of phospholipases was invasive in a murine model of disseminated candidiasis whereas a low-producing strain was not (226). In this infection model, phospholipase activity highly correlated with the severity of kidney burden (227) and phospholipase-activity was the only putative virulence factor tested that predicted

mortality (226). Leidich et al (228) and Mukherjee et al (229) showed that mutant strains lacking *PLB1*, which have 1% of the extracellular Plb activity of the wild-type strain, had significantly attenuated virulence in a model of disseminated infection in mice. In a similar model of infection, a mutant strain deficient in *PLB5* caused reduced liver and kidney fungal burdens (223). Noteworthy, the ability of Δplb1 null mutant to penetrate host cells was dramatically reduced (190, 230). However, adherence of the yeast cells to human endothelial or epithelial cells was not affected in a Δ*plb1* null mutant strain, suggesting that Plb most likely contribute to the pathogenicity of *C. albicans* by damaging host cell membranes and aiding the fungus to invade host tissues (231, 232). To this hypothesis certainly contribute the findings that Plb secretion is mainly concentrated at the growing tips of mature and developing hyphae (229, 233).

3.5.2. Lipases

At least ten members constitute *C. albicans* Lipase (*LIP*) gene family, and there is plenty evidence that this gene family is differentially expressed *in vivo* (234). Expression of *LIP5*, *LIP6*, *LIP8*, and *LIP9*, but not the other members was detected in a mouse model of *C. albicans* peritonitis (234). *LIP1*, *LIP3* and *LIP9* were found in infected gastric tissues, but undetectable in oral mucosa (235). Another study indicated that *LIP4* preferentially plays a role in superficial infections (236).

It is likely that these enzymes, like phospholipases, are involved in virulence of *C. albicans*, but their roles and functions during infection remain to be elucidated (234, 237).

3.5.3. Secreted aspartyl proteases

Microbial extracellular proteases are mostly secreted for the producing microorganisms to obtain readily available nutrients from complex materials or to compete with other environmental microbes (238). However, pathogenic microorganisms may also secrete proteases to accomplish other functions during the infective process, such as hydrolysing proteins of host cell membranes to facilitate adhesion and tissue invasion or damaging cells and molecules of the host defence system to evade or resist the host immune response (239-245). Classically, proteases

are ordered in four classes: serine, cysteine, metallo and aspartyl proteases. Aspartyl proteases are ubiquitous in nature and are involved in numerous biochemical processes (246). All secreted *C. albicans* proteases belong to the same class of enzymes – the aspartyl proteases. *C. albicans* secreted aspartyl proteases (saps) are essential for cell growth when proteins are the sole nitrogen source (247). Sap production is also involved in *C. albicans* pathogenicity and has been associated with several *C. albicans* virulence attributes, including hyphal formation, adhesion and phenotypic switching (241).

So far, 10 *SAP* genes have been identified with open reading frames ranging from 1173 to 1764 base pairs in length, and located on five different chromosomes.

Processing, activation, and regulation of *C. albicans* secreted aspartyl proteases

All 10 *SAP* genes of *C. albicans* encode preproprotein forms, which are processed along the secretory pathway. The prepropeptide, which includes a signal peptide and a propeptide, is approximately 60-200 amino acids longer than the mature enzyme and is essential for correct folding and secretion of the protein. The pathway of protease synthesis starts in the nucleus, from where the newly synthesized mRNA is transferred to the cytoplasm and translated into the preproenzyme on the rough endoplasmic reticulum (rER) where the N-terminal signal peptide is removed by the signal peptidase complex (248). The 'pro' region probably acts as an intramolecular chaperone that is required for proper folding in the rER, since Sap1 lacking its propeptide is retained in the rER (249). The 'pro' region is released and degraded by an exogenous protease leaving the folded enzyme in the active state (250). Later in the Trans Golgi Network (TGN), the propeptide is removed by a protease after conserved Lysine-Arginine sequences (251-253). The mature enzymes contain sequence motifs typical for all aspartyl proteases, including two highly conserved regions with reactive aspartyl residues of the active site and a third conserved region at the C-terminus of the protein (254, 255). Moreover, four cysteine residues, conserved in all aspartyl proteases, may form two disulphide bridges and are probably responsible for the maintenance of the three-dimensional structure (256). Once activated, mature Saps, with molecular weights between 35 and 50 kDa, are packaged into secretory vesicles and transported to the plasma membrane. There, Saps are either released into the

extracellular space (Sap1–8) or remain attached to the cell membrane or cell wall via a GPI anchor (Sap9 and Sap10, also called yapsin-like proteases) (254, 257).

Since the *SAP* gene family encode preproenzymes, the regulation of protease expression can be controlled either at mRNA or protein levels. Comparisons of Sap protein and mRNA levels at identical time points (258) and kinetic studies of protease secretion by pulse chase (259) suggested that protease synthesis and secretion were tightly coupled, strongly implying that regulation of Sap activity occurred predominantly at the mRNA level. Most Sap proteins contain putative N-glycosylation sites, and treatment with endoglycosidase EndoH has revealed that Sap4, 6, 7, 8, 9 and 10 were N-glycosylated (260), confirming previous observations of Albrecht et al (261) that the yapsin-like Saps were highly glycosylated.

Purification, activity and biochemical properties of the *C. albicans* secreted proteases

C. albicans proteolytic activity was first described by Staib (262) based on cell growth in media containing BSA as the sole source of protein, later attributed to an extracellular protease (263). Numerous studies of the biochemical properties of Sap isoenzymes have been performed afterwards, by using purified culture supernatants (264-266)or recombinant proteins obtained by recombinant expression in *Pichia pastoris* (260, 267) or *Escherichia coli* (268). Sap2 is the most abundantly secreted protein *in vitro* when *C. albicans* is grown in protease inducing media (247, 258). Thus, data from studies using culture supernatants largely correspond to Sap2 proteolytic activity. Structural studies of the *C. albicans* protease family have also concentrated on Sap2 (269, 270). The structure of Sap2 corresponds to the classical aspartyl protease pepsin.

Studies on the biochemical properties of recombinant Saps revealed similarities and differences in their structures and active sites, pH optima and substrate specificities. The majority of the Sap isozymes (Sap1-6) showed highest proteolytic activity at acidic pH 3.0-6.0 (260, 264, 266-268, 271) , which is typical for aspartyl proteases. Sap1 to Sap3 have highest activity at lower pH values and Sap4 to Sap6 have highest activity at higher pH values, (260, 267, 268). Sap8 has the lowest optimum pH (2.5) (260). In contrast, Sap7 showed optimal activity at neutral pH, which is an unusual pH for

aspartyl protease activity (260). In addition to different pH optima, cleavage site specificity of *Candida* Sap proteins has been suggested (265, 272).

Sap versatility may contribute to the success of *C. albicans* as an opportunistic pathogen by providing *C. albicans* with a biological advantage and enhancing the pathogenic ability of the fungus *in vivo* (241). The production of several Sap isoenzymes with distinct pH optima may promote *C. albicans* colonization and infection of different tissues and host niches. A family of proteases with different substrate specificities would allow *C. albicans* to make use of an array of host proteins at several host sites to provide nitrogen for the yeast. However, *C. albicans* produce Saps with highly similar substrate specificities and similar optimum pH ranges simultaneously, which makes it possible that they have similar target proteins and overlapped functions. Nevertheless, it is still not clear whether the digestion of substrates by Saps *in vivo* is similar to that shown *in vitro*. Although the specific host targets of Saps *in vivo* are not accurately known, some conclusions can be inferred from the combination of Sap *in vitro* proteolytic properties and results from several models of infection in which protease inhibitors or *sap*-deficient mutant strains were used.

The great majority of studies focusing Sap substrate specificity were done in protease-inducing growth medium, known to favour *SAP2* expression (247, 258). Thus, the potential targets of the Sap family *in vivo* determined therein have been mainly based on Sap2 activity *in vitro*. One of the most remarkable properties of Sap2 is the diversity of proteins it can cleave. Sap2 is able to degrade many human proteins, like the ones that protect mucosal surfaces and cell-surface proteins, such as keratin, collagen, vimentin, fibronectin, laminin and mucins (273-278). The secretory immunoglobulin A (IgA) is also degraded (272, 279), what is worth mentioning since it is considerably more resistant to proteolysis than are monomeric or serum immunoglobulins. Wu and Samaranayake (280) suggested that *Candida* Sap proteins could degrade salivary proteins in the oral cavity because a reduction of total salivary protein concentration correlated with the degree of Sap expression, and Meiller et al (281) proved that histatin-5 was degraded by aspartyl proteases. *C. albicans* proteases may also evade host defenses by directly degrading molecules such as salivary lactoferrin, lactoperoxidase, cathepsin D (an intracellular lysosomal enzyme of leukocytes), and

complement (274, 282-284). In addition, Sap2 can degrade β2-macroglobulin, a natural protease inhibitor in human plasma (285), and cystatin A, a cysteine protease inhibitor found in human epidermal tissues and fluids (286). Sap can cleave pro-interleukin-1β into the proinflammatory cytokine interleukin-1β (287) and can also activate Hageman factor, a serine protease of the kallikrein-kinin system, which may cause increased vascular permeability *in vivo* (288). Similarly, Sap2 may also act on the blood clotting system by activating coagulation cofactor X (289), clotting factor XII, or prothrombin, which may result in the generation of thrombin and consequently blood clotting (290). These substrates have been mainly tested with Sap2, but it is likely that other Saps also target part of them, which may result in overlapping functional roles *in vivo* between members of this gene family. Despite Sap2 ability to degrade such a huge set of proteins, these can nevertheless be at environments in which pH does not favour *SAP2* expression and Sap2 proteolytic activity. Indeed, Sap2 is almost inactive at neutral pH, the pH found in the milieu of most of these proteins.

Sap9 and Sap10 are regulatory proteases that may play a role in the cell surface integrity and shedding of cell-surface proteins (261). Target proteins may be proteins of the cell membrane, of the cell wall, or secreted proteins, including other Saps. However, Sap2 processing is independent of Sap9 and Sap10, since total proteolytic activity of Δ*sap9* and Δ*sap10* mutants was not reduced when comparing with the wild-type strain (261). Processing of Sap9 and Sap10 target proteins is not essential for normal growth though it is necessary for normal separation of cells after budding (261).

Differential expression and transcriptional regulation of *SAP* genes

The existence of 10 *SAP* genes suggested that different proteases might target distinct host cells and tissues during *C. albicans* infections and might play key roles in the pathogenesis of *C. albicans* infections. In that sense, the different *SAP* genes would be differentially regulated and expressed under a variety of laboratory growth conditions, during the experimental *C. albicans* infections, and also *in vivo*.

In vitro SAP expression and regulation

The initial *SAP* expression studies were performed *in vitro* to comprehend the regulation of *SAP* gene expression induced by changes in growth environment, morphological transition from yeast to hypha and phenotypic switching (241, 256, 291). Under the protease-inducing conditions generally used, the major protease gene expressed in the yeast form of *C. albicans* was *SAP2*, which is regulated by exogenous protein or peptides. The accumulation of proteolytic products of Sap2 and peptides of eight or more amino acid residues, resulting from proteolysis of high-molecular weight proteins, led to the induction of *SAP2* gene expression via a positive-feedback mechanism (247, 292). The widely used SC5314 strain possesses two *SAP2* alleles, which are differentially regulated, either *in vitro* or *in vivo*. Under appropriate conditions, the *SAP2-2* allele may serve as a signal sensor and amplifier to enhance its own expression, as well as to induce the *SAP2-1* allele to achieve maximal proteolytic activity (293). Two other *SAP* genes, *SAP1* and *SAP3*, are known to be differentially expressed *in vitro* when *C. albicans* undergoes phenotypic switching (264, 294). Both isoenzymes are expressed in the opaque but not in the white form of strain WO-1 (264, 294).

Curiously, *SAP4–6* were almost exclusively expressed during hyphal formation at neutral pH, even in defined protein-free media, albeit most aspartyl proteases are usually only active under acidic conditions (247, 258). The *SAP4–6* subfamily was usually assessed as a group during these early studies, and thus conclusions are to be applied to the subfamily as a whole. *SAP8* expression is more strongly induced at 25ºC, suggesting that its expression is temperature regulated *in vitro* and, possibly *in vivo* (254). More recently, *SAP9* and to a lesser extent *SAP10* were found to be expressed in YPD preculture growth, and appeared to be independent of environmental conditions and morphology (271). It should also be noted that under all the laboratory growth conditions tested in these experiments *SAP7* expression was never detected. After treatment with subinhibitory concentrations of antifungal agents, increased levels of mRNA transcripts were reported for several *SAP* genes. *SAP2* and *SAP9* expression was induced upon exposure to fluconazole and caspofungin in protease-inducing medium (295), while in non-inducing conditions, only *SAP5* expression was found increased when treated with caspofungin. Barelle et al (296) also reported increased expression

of *SAP4*, *SAP5* and *SAP6* after exposure to subinhibitory concentrations of azole antifungal agents in hyphal-inducing growth conditions.

Protein expression studies have confirmed that the secretion of Sap5 in *C. albicans* was induced during hyphae formation, followed by secretion of Sap4 and Sap6 under acidic conditions, which occurred *in vitro* in parallel to the degradation of BSA (297).

***In vivo SAP* expression and regulation**

The *in vitro* studies confirmed the differential expression of *SAP* gene family, indicating that it was a highly regulated process, and suggested that different members of the *SAP* family might also be differentially expressed *in vivo*, and might play distinct roles during different types or stages of infection and during antifungal treatment. This has been confirmed, in both mucosal and systemic *C. albicans* infections, by several studies using technologies such as reverse transcription polymerase chain reaction (RT-PCR) and *in vivo* expression technology (IVET) to assess *SAP* expression in human samples or in *in vitro* and *in vivo* experimental models. Using *C. albicans*-infected oral and vaginal reconstituted human epithelium (RHE), Naglik et al (173) reported that within 24h of infection, *SAP9* was highly and constitutively expressed while *SAP10* was also constitutively expressed though at lower levels. *SAP5* was the only *SAP* gene that was clearly up-regulated during the infection period. The unique up-regulation of *SAP5* during vaginal RHE infection was also reported by Lermann and Morschhaeuser (174). These results contrast the previous reported expression of *SAP1–3* subfamily in the initial stages of epithelial colonization (298, 299).

Expression of *SAP* gene family has been also assessed upon interaction with cells of the innate immune system. *C. albicans SAP4–SAP6* expression was demonstrated during interaction with murine macrophages (267) and in cells incubated with human plasma, but not in cells which were incubated with whole blood or phagocytosed by polymorphonuclear neutrophils (300).

To determine whether the *in vitro* models were representative of the *in vivo* situation, *SAP* expression was analysed in numerous animal infection models and in human samples. In a murine model of oropharyngeal candidiasis, *SAP9* transcripts were detected continuously throughout the course of infection being *SAP5* and *SAP9* the most highly expressed (301). Staib et al (302) have also reported strong activation of

SAP5 and *SAP6* during invasion of oesophageal mucosa by *C. albicans* hyphae. In contrast, in a mouse model of gastrointestinal infection, *SAP4* and *SAP6* were constitutively expressed, with *SAP2*, *SAP3* and *SAP5* mRNA only occasionally being detected (303). *SAP* gene expression was compared during gastric candidiasis in immunocompetent and immunodeficient mice by RT-PCR (304). Gene expression patterns were similar in both, suggesting that the immune status of the host has only a minor or no influence on the expression of *SAP* genes.

The *SAP* expression profile of *C. albicans* was analysed in the course of murine vaginal infection using an *in vivo* expression technology (IVET) and green fluorescent protein-expressing *C. albicans* reporter strains (302). Of the six *SAP* genes that were analysed (*SAP1–SAP6*) only *SAP4* and *SAP5* were detectable during infection. This contrasts the results of De Bernardis et al (305), in a rat vaginitis model, which reported the preferential expression of *SAP2* though *SAP1* and *SAP3* expression was also found in this model. Discrepancies between these results may be explained by the fact that physiological mouse vaginal pH ranges between 6.2 and 6.5 (306), a pH interval which favours hyphal growth and thus expression of the hyphal-associated genes *SAP4–6*, while at the acidic rat pH (4.5) the expression of *SAP1* to *SAP3* might be preferential (307).

Naglik *et al*. analysed *SAP* expression in subjects with oral and vaginal *C. albicans* infection or asymptomatic carriage (308, 309). In these studies, *SAP1*, *SAP3*, *SAP4*, *SAP7* and *SAP8* mRNA transcripts were predominantly expressed in infected patients as opposed to asymptomatic *Candida* carriers. However, more recently, the same group has analysed other groups of patients with oral and vaginal candidiasis and could not confirm such an association between *SAP* expression and infection/carriage. *SAP9* and *SAP5* were the most highly expressed genes in both types of infection and *SAP2, SAP5*, *SAP9* and *SAP10* were the most commonly expressed genes. These authors have also confirmed previous findings (308, 309) that *SAP1*, *SAP3*, and *SAP8* expression were more commonly found in vaginal rather than in oral disease.

The differential expression of *SAP* genes was also observed during systemic infections. *SAP5* was the first *SAP* gene induced after intraperitoneal (i.p.) infection or haematogenous dissemination (302). *SAP5* expression at this stage of the infection did not correlate with the presence of germ tubes or hyphae, and *SAP6* gene activation

was detected only when *C. albicans* hyphae were observed in the infected tissue. These authors also reported *SAP2* induction in the late stages of infection (302). Similar conclusions can be taken from a similar model of i.p. infection in which expression of *SAP2*, *SAP4–6* and *SAP9* was reported for cells invading parenchymal organs (172).

SAP genes are also regulated by biofilm formation, and higher levels of *SAP5*, *SAP6* and *SAP9* mRNA transcripts were detected in biofilm rather than during planktonic growth (310).

The expression of *SAP1–SAP6* was confirmed at the protein level by using immunoelectron microscopy and two different antibodies directed against Sap1–3 and Sap4–6 (172, 311) and the results largely agree with the studies reported above. Sap1 to Sap3 antigens were found on yeast and hyphal cells, while Sap4 to Sap6 antigens were predominantly found on hyphal cells in close contact with host cells, in particular, eosinophilic leukocytes (172). Sap4 to Sap6 were also detected on hyphal cells that have been phagocytosed by murine macrophages (267).

In conclusion, all these studies indicated that *SAP* gene expression and regulation, and consequently protein production, highly depend on the type and stage of *C. albicans* infection and on the pH conditions and substrate availability in the local environment.

Global transcriptional regulators and *SAP* expression

Key transcriptional regulators are known to manage *C. albicans* morphogenesis and the expression of several hyphal associated genes (2). The two transcriptional activators Cph1 and Efg1, which are controlled via the MAP kinase or cAMP pathways, respectively, regulate not only hypha formation (148), but also the expression of hyphal-associated genes, such as *SAP4–6*. Mutants lacking *CPH1* clearly had reduced expression levels of *SAP4-6*, even though filamentous growth in infected tissue was not visibly impaired (177). Although *efg1* and *cph1*/*efg1* mutants did not produce germ tubes during infection, expression of the hyphal associated genes *SAP4-6* was reduced but not completely abolished, demonstrating that in contrast to *in vitro* conditions, *SAP4-6* activation during infection does not strictly depend on growth of *C. albicans* in the hyphal form (172, 177, 312). Nevertheless, these data suggest that the two signal transduction pathways are important for *SAP4-6* expression *in vivo* (177).

Another transcription factor, Tec1, a member of the TEA/ATTS family of transcription factors, is predominantly expressed in the hyphal form of *C. albicans*. The promoters of the hyphal-associated *SAP4–6* subfamily contain repetitive TEA/ATTS consensus sequence motifs, and expression of *SAP4–6* was clearly affected in a *tec1* null mutant (176). An additional transcriptional factor, Nrg1, represses a subset of genes regulated by the general repressor Tup1. Inactivation of Nrg1 results in filamentous and invasive growth and derepresses hypha-specific genes, such as *SAP5* (313). At last, the transcription factor Mig1, like Nrg1, targets the Tup1 repressor to specific subsets of genes, including *SAP9*. However, Tup1 can act Mig1- and Nrg1-independently and repress the expression of *SAP6* and *SAP7* (314). In conclusion, it seems that synchronized regulation of germ-tube formation and protease production is essential for optimal invasive growth, because mutants lacking either the ability to produce hyphal cells or the secretion of hyphal associated proteases Sap4–6 have defects in parenchymal organ invasion (172).

Sap production and *C. albicans* virulence

The role of Saps as *C. albicans* virulence factors has been intensively investigated during the last decades. Proteolytic activity has been found *in vitro* in most isolates of pathogenic *Candida* species, including *C. dubliniensis* (315), C. *tropicalis* (255, 316, 317) and *C. parapsilosis* (318, 319). Sap activity is regarded as a virulence factor for *C. albicans* (256) and *C. tropicalis* (320), and its role as a virulence factor for *C. parapsilosis* has been recently highlighted (321). The proteolytic activity of other non-pathogenic *Candida* species is generally lower, suggesting that virulence is correlated with the level of Sap production (322).

Secreted aspartyl proteases also seem to be important for the establishment of mucosal *C. dubliniensis* infections. Like *C. albicans*, *C. dubliniensis* possesses several *SAP* genes (315, 323), but interestingly, *C. dubliniensis* does not have the orthologue of *SAP5* and *SAP6*, genes involved in systemic *C. albicans* infections, which may account for the almost absent involvement of this species in systemic infections (323, 324).

C. albicans virulence modulation by aspartyl protease inhibitors

The contribution of aspartyl proteases to *C. albicans* infectious process was first confirmed by using the classical aspartyl inhibitor pepstatin A. Colina et al. (273) showed that digestion of mucin could be inhibited by pepstatin, indicating that *Candida* Sap proteins may degrade mucosal barrier proteins. This may allow *C. albicans* to gain access to the oral and GI mucosa and may consequently indicate a role for *Candida* proteases in dissemination from these colonized sites. The addition of pepstatin inhibited the *in vitro* digestion of soluble and immobilized extracellular matrix proteins produced by a human endothelial cell line (277), what suggests that *Candida* Sap proteins contribute to cell damage and invasion of the subendothelial extracellular matrix, which in turn could facilitate dissemination via the circulatory system. Likewise, adherence of *C. albicans* to human mucosa (325), human buccal epithelial cells (Watts et al 1998), epidermal corneocytes (326), and epidermal keratinocytes (327) has been shown to be inhibited by pepstatin A, indicating that proteolytic activity of aspartyl proteases is necessary for the adhesion properties of *C. albicans.* In RHE models or epidermis of oral (173, 311), cutaneous (328) and vaginal candidiasis (173, 329), pepstatin reduced the tissue damage caused by Saps. But even in the presence of pepstatin, epithelial tissue damage was observed in late stages of RHE infections (173). This suggested that certain members of the Sap family might not be inhibited effectively by pepstatin or other hydrolytic enzymes of *C. albicans,* such as phospholipases and lipases that might also contribute to the development of mucosal lesions. On the contrary, Lermann and Morschhaeuser (174) stated that no inhibition in RHE damage could be observed upon pepstatin usage, indicating that dependence on Sap activity during the *C. albicans* pathogenesis process is highly variable. Besides, the authors also gave the possibility that pepstatin A did not fully inhibit Sap activity under the conditions used in the RHE infection experiments (174). In another *in vitro* model of oral epithelium, tissue invasion by *C. albicans* was reduced in the presence of pepstatin. The tissue invasion mechanism proposed was the degradation of E-cadherin by Saps, in particular by Sap5, since overexpression of *SAP5* rescued the attenuated invasive phenotype of a Δ*rim101* null mutant, in which *SAP4* to *SAP6* expression is diminished (330).

Protective effects of pepstatin were also demonstrated in a rat vaginitis model, where administration of pepstatin after infection greatly accelerated the clearance of *C. albicans* from the rat vagina (331, 332). In a study of murine peritonitis, the addition of pepstatin significantly reduced liver and pancreas damage, as determined by decreased levels of alanine aminotransferase (ALT) and α-amylase enzyme activities, respectively (333). These findings indicated that pepstatin could attenuate *C. albicans* virulence in a systemic murine intraperitoneal model, probably by inhibiting tissue damage and invasion by Sap4 to Sap6, since a *sap4-6* null mutant strain caused decreased tissue damage that could be no further reduced by the addition of pepstatin (333).

While the evidence supporting a protective role for pepstatin in mucosal animal models is convincing, its potential use in acute systemic candidiasis remains unpersuasive. No real protective effect was observed after intravenous challenge with *C. albicans* by using pepstatin *in vivo* (334-337). Fallon et al. (334) showed that pretreatment of neutropenic mice with pepstatin A afforded strong dose-dependent protection against a subsequent lethal intranasal dose of *C. albicans*. Inhibition of Sap proteins by pepstatin prevented the initial penetration of *C. albicans* through mucosal surfaces, but not the dissemination of *C. albicans* once the cells had already reached the blood vessels, since no protection by pepstatin A was observed in mice challenged intravenously, and protection was markedly attenuated in mice given pepstatin A after intranasal challenge.

Thus, one conclusion may be that pepstatin can prevent disseminated infections by inhibiting *Candida* penetration through the mucosal route, but cannot inhibit systemic infections when the *Candida* is administered via the intravenous route. However, pepstatin is not selective and is slightly toxic in animals, probably due to its inhibitory action on host aspartyl proteases, including cathepsin D and renin (335). Moreover, pepstatin accumulates in the liver and not the kidneys, which is a major target organ of *C. albicans* in systemic infections, and is thus likely to be rapidly cleared from the blood *in vivo*. Therefore, while pepstatin is a potent inhibitor of the *C. albicans* aspartyl proteases *in vitro*, its suitability as an antifungal agent *in vivo* is not convincing.

The interest in discovering new compounds that inhibit Sap activity has grown considerably as a result of the evolving HIV epidemic. In the last two decades, the

treatment of HIV-positive patients with highly active antiretroviral therapy (HAART), which includes a cocktail of HIV reverse transcriptase and HIV protease inhibitors, has proved successful in delaying the onset of AIDS. Administration of HAART resulted in a significant improvement in the immune status of the HIV-positive individual reflected by an increase in the $CD4^+$ T cell population. These patients also had a dramatically reduced incidence of oropharyngeal candidiasis (338-341). Given that *Candida* proteases and the HIV protease are members of the same aspartyl protease family, these findings indicated that HIV protease inhibitors could also inhibit *Candida* aspartyl proteases *in vivo* and consequently prevent or reduce *Candida* infections (342-344). In fact, several HIV protease inhibitors were able to inhibit Sap activity (343, 345-348), and to reduce the adherence of *C. albicans* to epithelial cells, further giving evidence that protease activity is involved in the attachment of *Candida* cells to host surfaces (303, 342, 344), what could explain the reduction of *Candida* infections in HIV patients treated with HAART. Regardless the strong inhibition of Sap expression in the oral cavity, these HIV protease inhibitors had a limited effect on *C. albicans* viability, thus having a limited potential as therapeutic agents in the treatment of *C. albicans* infections (349).

HIV protease inhibitors did not influence the phagocytosis by polymorphonuclear neutrophils (348). Sap inhibition with pepstatin did not affect *C. albicans* cell viability in a *Candida*-neutrophils interaction study, confirming the view that Saps are not associated with survival in neutrophils (300).

Role of Saps in *C. albicans* virulence using *sap*-deficient mutant strains

Genetic manipulation of *C. albicans* has always been complicated by the diploid nature of the fungus and the fact that it has an unproven naturally functional sexual stage. As a result, for a long time it was very difficult to create mutant strains for the analysis of virulence properties. Before the development of modern molecular biology tools, many of the early studies investigated the role of proteases in *C. albicans* virulence using less proteolytic or nonproteolytic *C. albicans* mutants that were induced by chemical or UV mutagenesis. Macdonald and Odds (350) and Ross et al. (351) showed that proteolysis-deficient mutant strains of *C. albicans* were less pathogenic in mice than was the parental strain. This attenuated virulence for one of the mutants was

confirmed for the rat vaginitis model by De Bernardis et al. (352). Also, Kwon-Chung et al. (353) demonstrated similar reduced virulence with another proteolysis deficient mutant and showed that a spontaneous revertant, that regained half of its original proteolytic activity, was almost as virulent as the parental strain. However, all these studies have used protease-deficient mutants obtained by chemical or UV mutagenesis and, most certainly, these mutants also contained nonspecific mutations at other gene loci that may have affected the growth and/or virulence of *C. albicans.* Thus, the results had to be interpreted with extreme caution.

Targeted gene disruption and the use of expression vectors have enabled the construction of null mutants lacking specific *SAP* genes or strains overexpressing determined *SAP* genes (174, 261, 354-356) . These *sap* null mutants have highlighted the relevance of distinct Saps in different models of *Candida* infection. Actually, the most definitive data regarding the contribution of the *SAP* family to *Candida* pathogenicity have been obtained, most probably, from the behaviour of the various selectively *SAP*-disrupted strains.

Sap production and mucosal infections

The role played by *C. albicans* proteases in mucosal infections has been frequently assessed by the ability of mutant strains deficient in one or multiple *SAP* genes to cause tissue damage in RHE models of oral and vaginal candidiasis. The results from the first studies have indicated that Sap1, Sap2, and Sap3 enzymes were important in both the oral (311, 357) and vaginal (299) models, since mutant strains lacking these genes caused less tissue damage than did the parental strain. These results were in accordance with previous observations that Sap1 and Sap3 were associated with mucosal tissue damage (298). However, more recently, two different groups, using independently constructed sap null mutant strains, have shown that even mutants lacking all of the *SAP1–SAP3* or the *SAP4–SAP6* genes displayed the same capacity to invade and damage both oral and vaginal RHE as their wild-type parental strain (173, 174). They have stated that hypha formation was the predominant cause of tissue damage in these models, but Albrecht et al (261)proved that Sap9 and Sap10 were essential for maximal pathogenicity during interaction with oral epithelial tissue, since mutant strains deficient in *SAP9* and/or *SAP10* genes had altered adhesion properties

to oral epithelial cells and induced considerably smaller tissue damage, which was restored by reintroducing the genes(261).

Schaller et al. reported that *SAP1–3*, but not *SAP4–6*, contributed to *C. albicans* infections on cutaneous epidermis (328). De Bernardis et al. (358) also showed that *sap1*, *sap2*, and *sap3* mutants, but not the *sap4* to *sap6* mutant, were less virulent in a rat vaginitis model, comparatively to wild-type strain SC5314, in particular the *sap2* mutant. Reintroduction of the *SAP2* gene recovered the ability of the *sap2* mutant to efficiently infect vaginal tissues, showing that Sap2 may play a pathogenic role in rat vaginitis. In contrast, although *SAP7* is induced after murine vaginal infection, a *sap7* mutant strain was not affected in its virulence in this infection model, highlighting that *SAP7* expression does not correlate with *C. albicans* virulence in vaginal infection in mice (356).

In another model of mucosal candidiasis, the murine model of gastrointestinal infection, no demonstrable differences between *sap1*- to *sap6*-deficient strains and the parental strain could be observed in the ability to invade the stomach or to disseminate to the brain or in the number of fungi persisting in the faeces (359). It is possible that, contrarily to oral or vaginal infections, the infectious process of *C. albicans* gastrointestinal infection does not depend on Sap activity. Similar conclusions were taken from the results obtained in the infection model using chick chorioallantoic membrane, which has been described as an alternative to evaluate *C. albicans* virulence. In this model, virulence of *SAP*-disrupted mutants was not attenuated (360).

It has become clear that the importance of Saps in general, and of individual Sap isoenzymes, for the virulence of *C. albicans* varies strongly, depending on the infection model, with even minor differences in the experimental setup having a significant impact on the dependence on protease activity for successful invasion and establishment in various host niches.

Sap production and systemic infections

The use of *SAP*-deficient strains has also implicated the Sap family in systemic *C. albicans* infections. During invasion of host cell tissue and during escape of host cells that have internalized the fungus, hypha-associated factors such as secreted hydrolases may be crucial. *SAP4–6* were shown to be almost exclusively expressed

during hyphal formation at near neutral pH values *in vitro* (247, 258) and may thus be important for the invasive properties of *C. albicans* hyphal cells.

The first real evidence that Sap4–6 may target different tissue compartments and substrates *in vivo* came from animal experiments performed using *SAP*-disrupted mutants, which indicated that the Sap4–6 family contributed to systemic infections (355). In guinea pig and murine models of disseminated candidiasis, upon intravenous infection with the *sap1*, *sap2*, *sap3*, and *sap4* to *sap6* mutants, all animals had increased survival rates compared with those infected by the parental strain (354, 355). The *sap4* to *sap6* mutant displayed the greatest attenuation not only in terms of lethality but also in terms of fungal burden in host organs, such as the kidneys and liver. However, the authors concluded that although Sap4 to Sap6 appeared to contribute more to systemic infections in both animal models than did Sap1 to Sap3, none of the *C. albicans* proteases was a single dominant factor during disseminated infections. In murine models of acute systemic candidiasis, *sap7* mutant strain had partial attenuated virulence (356), and *sap9* and *sap10* mutant strains exhibited virulence phenotypes similar to the parental strain (261).

In a model of murine peritonitis, the invasive properties of *sap1*, *sap2*, or *sap3* mutants were indistinguishable from those of wild-type cells, but the *sap4* to *sap6* triple mutant showed strongly reduced invasiveness (172) and induced a significantly reduced activity of ALT and α-amylase (markers of liver and pancreas damage, respectively) in comparison to the parental strain, despite still producing hyphal cells (333). When the tissue damage of liver and pancreas caused by single *sap4*, *sap5*, and *sap6* and double *sap4* and *sap6*, *sap5* and *sap6*, and *sap4* and *sap5* double mutants was compared to the damage caused by wild-type cells, all mutants which lacked functional *SAP6* showed significantly reduced tissue damage (172).

These data indicated that the Sap4 to Sap6 subfamily contribute to tissue damage and invasion in systemic infections, probably by aiding penetration of tissue and survival of the fungus in phagocytes.

Differential control and coordination of *SAP* expression appears to be essential for pathogenesis as overexpression of *C. albicans* Sap2 even reduced virulence. Furthermore, heterologous expression of *SAP2* did not enhance virulence in the non-

pathogenic yeast *Saccharomyces cerevisiae* (361), indicating that *SAP* expression *per se* does not necessarily correlate with virulence.

It is clear that the use of *sap*-deficient strains has significantly advanced our understanding of the possible roles and functions of the proteases during *Candida* infections. However, almost the totality of these studies were based on the use of Ura-blaster *sap* null mutants, which have not been complemented and thus, the nature of these mutants remain to be confirmed. The great majority of these mutant strains were generated from auxotrophic laboratory strain CAI4 with the most common method used for disrupting genes in *C. albicans*, the Ura blaster technique (362). The use of the *URA3* marker for mutant construction in *C. albicans* can lead to a misinterpretation of the results in mutant virulence studies (363-365) . Although this can now be overcome by the integration of *URA3* at the *ENO1* (366) or *RPS10* (364) locus, the mutant strains used in earlier studies did not share a site of *URA3* integration. Therefore, it is conceivable that the Ura status could have influenced the results, and thus, the attenuated nature of these mutants during acute systemic candidiasis remains to be confirmed unequivocally (367). More recently, an alternative method for constructing null mutants from the prototrophic wild-type strain SC5314, the *SAT1*-flipping strategy, has been published (368), and a new set of single, double and triple *sap* null mutants has been constructed by using this strategy (174), avoiding the recognized effect of Ura influence.

Still, results obtained with disrupted strains should be interpreted with some caution, since the disruption of one *SAP* gene may be compensated for by the up-regulation or down-regulation of other *SAP* genes, or even other unrelated virulence-associated genes, which may account for some of the reported observations. These forced adaptations that *C. albicans* may need to undergo as a result of *SAP* gene disruption may introduce undesirable changes that could lead to misinterpretation of the obtained results. An example is the loss of heterozigosity for the chromosome R and loss of *SAP2-2* allele in the Ura-blaster triple mutant *Δsap4-6*, which has resulted in an impaired ability of this mutant strain to utilize proteins as the sole nitrogen source, and that may have resulted in other misidentified phenotypic characteristics (369). In addition, only a single *C. albicans* strain has usually been used for all *SAP* disruption

studies and thus it is not known whether this strain is representative of all other *C. albicans* strains that cause clinical infections.

In summary, the majority of the *in vitro*, animal model and *in vivo* studies support the view that distinct proteases of the Sap isoenzyme family have distinct functions during the different types and stages of infection (172, 311, 333, 355, 358, 359) yet, some of the roles and functions of Sap enzymes during *C. albicans* infections may need confirmation.

Sap production and evasion of host immune responses

C. albicans possesses several mechanisms of evasion of the host immune response and Saps have also been implicated in some of those processes. In macrophage killing assays, a *sap4-6* triple mutant was killed more efficiently after phagocytosis by murine macrophages than was the parent strain (267). As a result of macrophage ingestion, *C. albicans* may germinate inside the phagolysosome and secrete Sap4 to Sap6, which are optimally active at the same pH (4.7 to 4.8) as that found in the phagolysosome (267). The production of functional Sap4 to Sap6 proteases within the phagolysosome may result in the digestion of enzymes associated with *Candida* killing and might render *C. albicans* more resistant to macrophage attack. The macrophage proteins targeted by Sap4 to Sap6 are not yet known, but Sap4 to Sap6 may act either directly on phagolysosomal enzymes involved in microbial killing (cathepsin D and/or antifungal peptides) or on key enzymes of macrophage metabolism that are essential for optimal microbial killing (239). Interestingly, Sap4–6 do not appear to be involved in evasion of phagocytosis by human blood polymorphic nuclear (PMN) neutrophils, because disruption of the whole subfamily has no effect on *C. albicans* survival or escape and none of the individual members were found to be up-regulated after phagocytosis (300, 370). These findings strongly indicated that Sap4 to Sap6 might facilitate *C. albicans* evasion from host innate defences by resisting macrophage attack, but not neutrophil control. Interestingly, Sap9 has a major impact on recognition of *C. albicans* by PMN cells (371). Yet, the expression of *SAP9* does not constitute an evasion mechanism, since killing of *sap9* null mutants by PMNs was reduced in comparison to wild-type *C. albicans* and the induced PMN chemotaxis towards Δ*sap9* filaments, and

consequent effective PMN activation, was also reduced (371). In contrast, *SAP9* deletion had no impact on *C. albicans* phagocytosis and killing by macrophages (271).

The production of secreted proteases has also been shown to interfere and inactivate host innate immune effector components, such as complement proteins and antimicrobial peptides. Saps in the culture supernatant of *C. albicans* cells and also recombinant Sap1, Sap2 and Sap3 degrade host complement components C3b, C4b and C5 besides inhibiting terminal complement complex formation. A triple knock out *C. albicans* strain Δ*sap1–3* and also the non-pathogenic yeast *S. cerevisiae* lack such degrading activities. In brief, the secretion of complement degrading proteases mediates complement escape and generates a protective microenvironment of reduced host complement activity (282).

Additionally, Saps may impair antimicrobial peptide function. Sap9 was responsible for degradation and deactivation of histatin-5, a host antimicrobial peptide involved in the protection of the oral mucosa against *C. albicans*, resulting in loss of its anti-candidal potency (281).

At last, Saps may contribute to evasion of the host humoral immune response by degrading immunoglobulins (372), including IgA, which is known to neutralize many toxins and enzymes (373) and to inhibit *C. albicans* attachment to buccal epithelial cells (374).

These findings strongly indicate a functional role for Saps in the evasion and possible manipulation of mucosal and systemic immune responses.

Sap production and host immune response

Since Saps play a role in *C. albicans* virulence, it is thus expected that the host would respond to Sap production by counteracting Saps to prevent local infection and damage. In fact, high titres of anti-Sap antibodies have been observed in sera of candidiasis patients, indicating the presence of Sap antigens during human systemic infections (322, 325). Besides, Sap antigens have been detected in biopsies of oral epithelial lesions collected from HIV-infected patients (311) and in almost all autopsied organs of immunocompromised patients who had died of systemic *C. albicans* infections (375). In all cases, antigens were found within the cell wall of yeast and hyphal cells. By using immunofluorescence with an anti-Sap antibody, amorphous

deposits of protease antigen formed a ring surrounding *C. albicans* micro-abscesses in the kidney, which may correspond to pH-mediated denaturation of secreted proteases (375). The observation that most Sap antigens were present within the *Candida* cell wall was later confirmed (172, 298, 311, 328, 359, 376). The location of Sap antigen during infections, assessed by using specific antibodies against the different Sap proteins, showed that Sap1–3 antigens were present in both yeast and hyphal cells, while Sap4–6 antigens were mainly found in hyphal cells (172, 267). Expression of different isoforms of Saps was also demonstrated by using immunoelectron microscopy in samples from patients suffering from oral and cutaneous candidiasis, showing abundant Sap1-3 antigen levels and few Sap4-6 antigen levels (311, 377).

While Sap antibodies were useful in demonstrating the expression and localization of the enzymes, the protective effect of Sap antibodies produced by infected hosts is more uncertain. Saps are known to be immunogenic and induce potentially protective host defences in mucosal and systemic animal models (331, 378-381). However, Ghadjari et al (382) attempted to protect mice from lethal *C. albicans* intravenous infection after passive antibody transfer with no success. The antibodies used were human recombinant antibodies against two Sap2 B-cell epitopes from a patient who had recovered from disseminated candidiasis. It is not yet known whether a strong immunological response against individual or multiple members of the Sap family can neutralize, inhibit or prevent extracellular protease activity resulting in effective protection against *C. albicans* infections *in vivo*.

Finally, although an immunomodulatory role has already been suggested for some of these proteases (267, 329, 381, 383), the specific interactions between *Candida* aspartyl proteases and the host immune response remain to be investigated.

3.6. *C. albicans* pathogenicity beyond virulence factors

The majority of the studies focusing putative virulence factors support the view that secreted hydrolases (SAPs, PLBs and LIPs) contribute to nutrient acquisition and promote fungal penetration of host barriers (224), whereas the Als proteins and Hwp1 promote adhesion to host tissue (196, 366, 384).

However, the new genome-wide expression profiling studies examine the global transcriptional response of *C. albicans* to the host using *ex vivo* and *in vivo* infection

models. These studies have revealed that most infection-related changes in *C. albicans* gene expression reflect environmental adaptation and that the initial contacts with the host, and also disease progression, are highly associated with metabolic and stress adaptation. *C. albicans* nitrogen metabolism change following exposure to host immune defenses or growth in biofilms (300, 310, 385, 386) . Also, amino acid biosynthetic genes are induced following neutrophil attack, but not during tissue invasion (387). This is consistent with the observation that the inactivation of the amino acid starvation response or specific amino acid biosynthetic pathways does not attenuate the virulence of *C. albicans* (364, 388). Therefore, tissues appear to be amino acid replete and thus, *SAP* expression is probably induced in these infection models for other purposes other than nitrogen availability (144). Other genes that are up-regulated during the development of *C. albicans* tissue invasion are genes involved in iron and phosphate assimilation (387). Also the expression of pH sensing functions changes during exposure to human blood and during tissue invasion (300, 370, 387). These transcript profiling data are in agreement with previous mutant studies indicating that iron assimilation and pH sensing are required for the overall virulence of *C. albicans* (307, 389). Data from these global transcriptional studies further supported that stress adaptation is essential for the virulence of *C. albicans*. These global transcript profiling studies support previous observations that several stress genes, involved in the detoxification of reactive oxygen and nitrogen species, are induced when C. *albicans* cells are exposed to macrophages, neutrophils, blood or epithelial cells, or during oral infections (300, 370, 385, 386, 390) . Genes involved in protective functions, such as heat shock proteins, have also been found to be up-regulated in infected kidneys and liver (387, 391). Stress adaptation is known to be essential for overall virulence, since inactivation of catalase, superoxide dismutase, or a flavohemoglobin involved in NO detoxification (300, 392-394) attenuates *C. albicans* virulence. Similar results are obtained by disrupting the stress-activated protein kinase, Hog1 (395), which is required for oxidative stress resistance (395, 396).

Therefore, *C. albicans* appears to activate adaptive stress responses in a niche-specific fashion during disease establishment and progression. Genomic studies show that diverse changes in *C. albicans* gene expression occur during infection and that,

although virulence factors are important, environmental adaptation appears to be the main key to pathogenicity.

4. *C. albicans* cell wall

The yeast cell wall is a crucial extracellular organelle that maintains the viability of fungal cells by protecting the cell from lysis during environmental stress and morphogenesis (257). It corresponds to the primary way in which the organism interacts with its host and contributes to pathogenesis by mediating interactions with host-cells and eliciting host immune responses (397). Fungal cell walls combine skeletal and matrix components. The skeletal component of the *C. albicans* cell wall is based on a core structure of β-(1,3)-glucan covalently linked to β-(1,6)-glucan and chitin (poly-β-(1,4)-*N*-acetylglucosamine (GlcNAc), which are located towards the inside of the cell wall. The outer layer is enriched with cell wall proteins that are attached to this skeleton. Despite being buried beneath the mannoprotein outer layer, β-glucans and chitin can nevertheless become exposed at cell surface in the bud scars (398). *C. albicans* cell wall contains a matrix mainly composed of glycosylated proteins that represent 30-40% of the cell wall dry weight (399). Lipids are minor components of the cell wall, but still have important functions (400). In fact, phospholipidomannan (PLM), is an important glycolipid of *C. albicans*, with linear β-1,2-oligomannose chains as the major component. β-1,2-oligomannosides of PLM have a role in virulence and immunomodulation (401) and are thought to be strong immunogens (402). Curiously, β-1,2-oligomannosides are expressed only in *C. albicans* and *C. tropicalis*, which are the most pathogenic *Candida* species.

Cell wall glucans

β-glucans are the most abundant polysaccharides of the fungal cell wall and represent approximately 60% of the cell wall components (399). Contrarily to other fungal species, *C. albicans* does not contain α-glucans. β-glucans occur as β-(1-3)-linked glucose polymers with β-(1-6)-linked side chains of varying length, tertiary structure and distribution, characteristics that have been shown to influence host immunity modulation (403). In general, large β-glucans lead directly to leukocyte activation, triggering phagocytosis, the production of cytokines, chemokines and other

inflammatory mediators (404). Intermediate-sized β-glucans (glucan phosphate) do not elicit leukocyte response *in vitro* yet being active *in vivo*. Small and low molecular weight β-glucans, such as the algae β-glucan laminarin, are recognised by glucan receptors but do not stimulate downstream signals (404). The ability of β-glucans to modulate immune recognition, and consequently the immune response, has caught pharmaceutical interest (405). Glucans and mannans are released by *C. albicans* in synthetic medium, as well as in the blood of infected patients, and these molecules can induce anaphylactic shock and coronary arthritis in murine models (406). On the other hand, treatment with β-glucans reduced fungal burdens and increased survival of infected animals. β-glucans have been shown to inhibit tumour growth and increase survival times, but the success of this treatment is dependent on a number of factors including the type of tumour (407, 408). The use of β-glucans as immune boosters has been assessed in clinical trials with promising results, though the mechanism anti-infective is not completely understood (404, 409). The anti-tumour activity is the best-examined property of β-glucans. In general, β-glucans are considered as safe but they have been implicated in triggering autoimmune diseases such as arthritis and are thought to be involved in respiratory burst disorders. Also, intravenous injection of particulate β-glucan caused the formation of granulomas, though it can be overcome by using active soluble glucans (410).

Chitin

Chitin is a β-(1,4)-linked homopolymer of GlcNAc that forms antiparallel hydrogen-bonded chains called microfibrils (411). Three genes encoding chitin synthases are described in *C. albicans*. *CHS2* is preferentially expressed in the hyphal state although its lack does not have any effect on chitin levels, the yeast to hyphal transition or virulence in a mouse model (412). Chs1 is involved in septum formation and essential for cell integrity and virulence (413). Strains defective in Chs3 are less virulent in a mouse model than the parental strain. Exposure of *C. albicans* to cell wall stresses such as $CaCl_2$ or Calcofluor White can increase the chitin synthase activity (414). Additionally, caspofungin treatment, which targets the β-(1,3)-glucan synthesis increases Chs3 levels in the cell (415). A fourth Chs was identified by in silico analysis: Chs8, which is similar to Chs2 and is responsible for 25% of the chitin synthase activity

but not essential for growth (416). The chitin synthase can be used as a target to control fungal infections because of the importance of chitin in the structure of the cell wall and its absence in the host. So far, two important inhibitors of chitin synthase have been described: polyoxins and nikkomycins. Nevertheless, they show high antifungal *activity in vitro* but they do not present effective activities in *in vivo* studies (417).

Cell wall proteins

Cell wall proteins can fall into two classes. Class I proteins are not covalently linked to the cell wall and are extractable with detergents or chaotropic agents. Class 2 proteins can only be solubilized after the destruction of structural polysaccharides or by breaking the specific bonds which link them to the polysaccharides (417). Within this group is the major class of cell wall proteins, the glycosylphosphatidylinositol (GPI)-anchored cell wall proteins, attached through a GPI remnant to β-(1,3)-glucan or chitin by a highly branched β-(1,6)-glucan linker (418, 419). These proteins are usually highly glycosylated with mannose-containing polysaccharides, and carbohydrates can account for up to 90% of their molecular mass. Mannoproteins are bound to the β-glucan/chitin inner layer through lateral chains of β-(1,6)-glucan or to β-(1,3)-glucan via alkali-sensitive linkages. The mannan structures are very important for the discrimination between fungal species and serotypes and are known to be present in *C. albicans* but absent in *S. cerevisiae.* Three different types of β-1,2-mannose have been found in distinct *Candida* species (420-422). In *C. albicans,* phospholipomannan reacts with antibodies specific to β-1,2-mannose (423). For phospholipomannan, it has been suggested that it may have relevance in adhesion, protection and signaling (424, 425). Similar to β-glucans, both mannans and mannoproteins from *C. albicans* cell wall have important immunostimulatory activities (426-430) .

The number of in silico predicted GPI proteins identified in *C. albicans* is almost twice as high as of that identified in *S. cerevisiae*, though the function for many of them is yet to be discovered (431). GPI proteins are located at the cell surface and are thus expected to interact with the host cells. In addition, *C. albicans* is highly adapted to its environment compared to other opportunistic fungi suggesting the evolutionary development of numerous mechanisms to colonize its host.

5. Immunity to *C. albicans* infections

The host immune response to fungal infections comprises diverse mechanisms and involves both the innate and the adaptive immune system. A complex and continuous balance between pro- and anti-inflammatory signals is required to maintain a stable host-fungus relationship, the disruption of which can have pathological consequences. The relative importance of specific innate and adaptive defence mechanisms in *C. albicans* infections differs, depending on the anatomical site of infection and on organism characteristics, such as morphotype (yeast, pseudohyphae and hyphae).

5.1. Recognition of *C. albicans* by the innate immune system

Pattern recognition receptors (PRRs) expressed on host innate immune cells recognise pathogen-associated molecular patterns (PAMPs) in fungi (432). The receptors that are involved in the recognition of *C. albicans* by leukocytes and the respective fungal PAMPs are depicted in Figure 1. PRRs on phagocytes initiate downstream intracellular events that promote the activation of the immune system and the clearance of fungi, being the specific immune response generated dependent on the PRRs triggered. However, PRR-mediated cell activation might as well promote some infections and cause tissue damage. Fungi have developed several mechanisms that exploit PRRs in order to manipulate host immune responses and survive, or even replicate, within the host.

5.1.1. PAMPs

The fungal cell wall varies in composition depending on the morphotype, growth stage and environment of the fungal species, and is the main source of PAMPs that are recognised by PRRs on mammalian cells. During the course of *C. albicans* infection, multiple host PRRs are likely to be stimulated by the fungal PAMPs depending on the *C. albicans* morphotype and on the host cell types involved. Therefore, the final immune response will depend not only on the relative degree of stimulation of the individual receptors, but also on receptors synergy or antagonism and on their cellular localization.

5.1.2. Pattern recognition receptors and their targets

Toll-like receptors (TLRs). The functional role of TLRs in antifungal host defence was first reported by Lemaitre et al. (433), who showed that drosophila flies deficient of toll were highly susceptible to infection with *Aspergillus fumigatus*. Of the 13 TLRs described in human and mice to date, only TLR2, TLR4, TLR6 and TLR9 have been reported to be involved in the defence against *C. albicans*.
Fungicidal pathways appear to be dependent on signalling via MyD88, the signalling adaptor that is a major component of TLR signalling and an essential component of IL-1R signalling (434).

TLR2. Although inhibition or deletion of TLR2 resulted in decreased production of pro-inflammatory cytokines and neutrophil recruitment after stimulation with *C. albicans* (435, 436), TLR2-deficient mice had increased resistance to disseminated candidiasis associated with increased production of IL-12 and INF-γ and decreased production of the anti-inflammatory cytokine IL-10 (434, 437). Furthermore, TLR2-deficient macrophages were able to clear *C. albicans* infections better than wild type counterparts (438). Such an anti-inflammatory role for TLR2 in host defence is supported by the demonstration that zymosan can induce immunological tolerance through a TLR2-mediated pathway (439). Even though the TLR2 ligand from *C. albicans* remains unidentified, there is evidence that phospholipomannan and β-glucans might be recognised by TLR2 and TLR6 (424, 440).

TLR4. There are controversial studies about the role of TLR4 during *C. albicans* disseminated infection. Netea et al (435) have reported that it is important for chemokine production, neutrophil recruitment and kidney fungal burden control, even if no changes in cytokine production could be found. In contrast, Gil and Gozalbo (441) and Murciano (442) stated that TLR4 was not relevant for the survival of mice infected with *C. albicans*. TLR4 is the receptor for bacterial lipopolysaccharide (LPS) (443, 444), but the nature of fungal PAMPs recognised by TLR4 is still poorly known. *C. albicans* and *S. cerevisiae* mannan, especially shorter O-linked mannan (445, 446) might be recognised by this receptor.

TLR6. The role of these receptors in response to *C. albicans* is less well studied. TLR2/TLR6 heterodimers are involved in zymosan recognition, but cytokine production is only moderately reduced in TLR6-deficient macrophages. Also, TLR6 does not seem to play a role in disseminated *C. albicans* infections (440).

TLR9. Blocking TLR9 in human monocytes reduces production of the anti-inflammatory cytokine IL-10 after stimulation with *C. albicans*. This effect is also observed in murine TLR9-deficient macrophages (van de Veerdonk et al, 2008). The natural ligands for TLR9 are unmethylated CpG sequences. A recent study indicates that TLR9 recognises *C. albicans* DNA driving to IL-12 production by myeloid DCs (447). However, no increased susceptibility of TLR9-deficient mice to disseminated candidiasis has been observed (434, 447).

C-type lectin receptors (CLRs)

The CLRs proved to recognise fungal PAMPs are Dectin-1, Dectin-2, macrophage mannose receptor (MR), galectin-3, dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN) and mincle.

Dectin-1 is the most extensively studied receptor implicated in fungal recognition. Dectin-1 recognises β-1,3 glucans via its extracellular C-type lectin-like domain (CTLD) (403). Dectin-1 stimulation with curdlan, a linear β-(1,3)-glucan, stimulates IL-2 and IL-10 production by DCs. ROS production upon zymosan and *C. albicans* stimulation requires Dectin-1 (398, 448, 449). However, the role of this receptor in *C. albicans* ROS signalling is still unclear because ROS production is not altered in macrophages from dectin-1-deficient mice when challenged with *C. albicans* (450). In addition, Dectin-1 is required for phagocytosis (398, 451, 452). Dectin-1-deficient mice are more susceptible to infection with *C. albicans*, resulting in increased fungal burden and lower survival (448). However, another study using a different mouse strain of dectin-1-deficient mice found increased susceptibility to *Pneumocystis* but not to *C. albicans* (450). Dectin-1 can also cooperate with TLRs to induce pro-inflammatory responses. In macrophages, cooperative signalling through Dectin-1 and TLR2 heterodimers is

required for the induction of TNF-α in response to *C. albicans* and zymosan (404, 449). Remarkably, Dectin-1 amplifies TLR4-dependent pathways (453). Furthermore, Dectin-1 can couple with other TLRs, resulting in the synergistic induction of TNF-α and IL-10 (454). In murine macrophages, a collaboration of Dectin-1 and DC-SIGNR1 for fungal binding exists (455), and in human DCs, a co-stimulation of DC-SIGN and Dectin-1 induces arachidonic acid signalling (456).

Dectin-2 has specificity for high mannose structures (457, 458). The receptor preferentially recognises hyphal forms of *C. albicans*. However, the receptor can also weakly recognise yeast forms (457, 459). The cytoplasmic tail of Dectin-2 appears to associate with the FcγR chain (459, 460), a signalling adaptor associated with several other transmembrane receptors. This induces TNF-α and IL-1ra in response to hyphal forms of *C. albicans* (459, 461). Blocking of Dectin-2 in a *C. albicans* infection model did not affect innate immune resistance but abrogated Th17 response (462), and when combined with Dectin-1 loss, the Th1 response decreased (461).

The human **dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN)** is primarily expressed on immature DCs, but has also been found in macrophage populations (463, 464). DC-SIGN recognises high mannose structures in a calcium dependent way (465). Eight orthologues of DC-SIGN exist in mice, although these molecules appear to have different expression profiles and several structural differences (466). The role of this receptor in response to fungi has not been studied extensively, but DC-SIGN has been proposed to mediate fungal uptake (467). Among the murine homologues, only SIGNR1 (also termed murine DC-SIGN) and SIGNR3 recognise fungal PAMPs (455, 468). DC-SIGN can induce intracellular signalling, modulating TLR-mediated responses (469).

Mannose Receptor (MR) recognises oligosaccharides that terminate in mannose, fucose and GlcNAc through several carbohydrate-recognition domains. MR was shown to preferentially recognise branched N-linked mannans (445). A recent study indicated that IL-17 production was induced by the MR, and that Dectin-1/TLR2 amplified the IL-17 production (470).

Mincle appears to be involved in the recognition of *C. albicans* by macrophages, although it is not essential for phagocytosis. Mincle-deficient mice are susceptible to *C. albicans*, and blocking of Mincle in macrophages leads to reduced TNF-α (471, 472). Like other CLRs, Mincle induces inflammatory cytokines and chemokines via the association with the Fc gamma (Fcγ) chain (473).

Complement receptor 3 (CR3). The CR3 integrin mediates adhesion, chemotaxis and phagocytosis in complement-dependent but also complement-independent ways (474-477) and recognises β-glucans of unopsonised yeast (477). The recognition of β-glucans by CR3 promoted phagocytosis but did not trigger protective host responses, such as the respiratory burst, and could suppress pro-inflammatory signals (478). In agreement with these immunosuppressive effects *in vitro*, CR3-deficient mice are more resistant to disseminated candidiasis. Curiously, a recent study using human neutrophils suggested that CR3 but not Dectin-1, was the major receptor for β-glucan bearing particles (479).

Galectin-3 is a receptor mainly expressed by macrophages crucial for the recognition of β-1,2 linked oligomannosides (480). Galectin-3 on the surface of murine macrophages can discriminate between pathogenic *C. albicans*, that bears β-1,2 linked oligomannosides, and non-pathogenic *S. cerevisiae*, which lack these residues, in collaboration with TLR2 (481). Binding of recombinant Galectin-3 to the specific β-1,2 linked mannosides of *C. albicans* directly induces death of *C. albicans* cells (482).

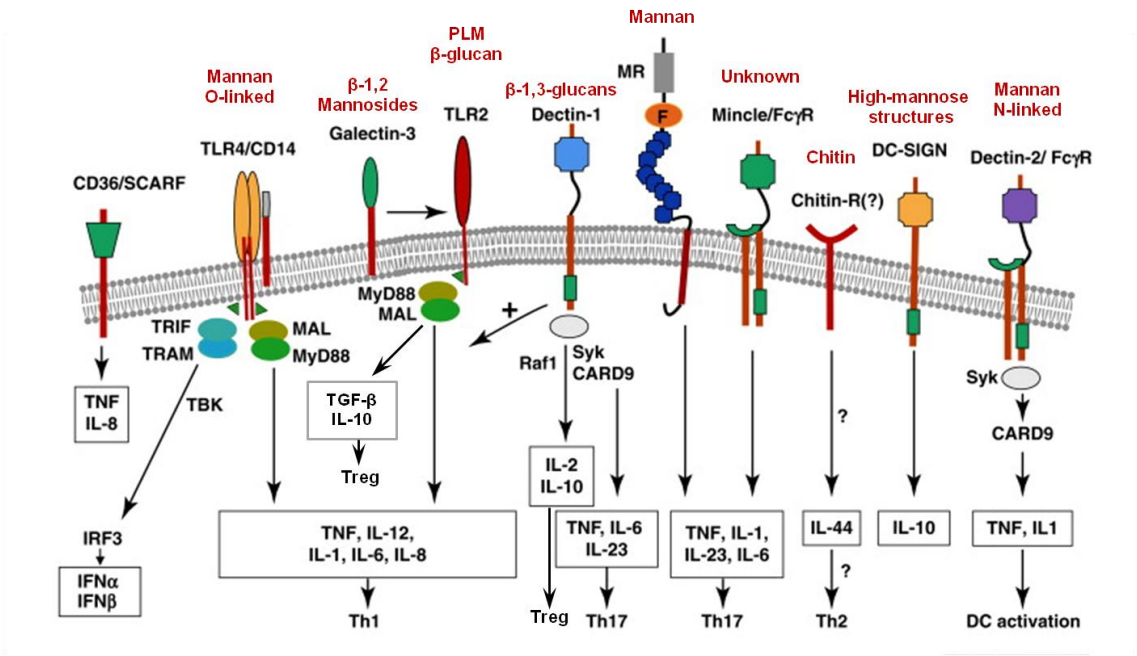

Figure 1. Cell membrane pattern recognition receptors sensing *C. albicans*. Recognition is mediated by TLRs and CLRs. Both TLR4 and TLR2 can induce proinflammatory signals in monocytes, macrophages and DCs through the MyD88 and Mal-mediated pathways, as well as the TRIF pathway to initiate Th1 responses. A second downstream effect of TLR4 signalling involves TRIF and TRAM adapter proteins that activate TBK1 and the IRF3 transcription factor required for the expression of type I interferon genes. TLR2 binding stimulates strong IL-10 and TGF-β and induces proliferation of regulatory T cells (Treg) and immunosuppression, but is also able to induce proinflammatory cytokines such as TNF-α or IL-6. However, through an Erk and c-Fos-dependent pathway, TLR2 is also able to inhibit IL-12 synthesis and Th1 responses. The proinflammatory effects of TLR2 can be amplified by dectin-1 and galectin-3. In addition to the amplification of TLR2 effects, the lectin-like receptor dectin-1 induces IL-2, IL-10 and Th17 responses through a Syk/CARD9 cascade, independent of its interaction with TLR2. The MR induces proinflammatory effects in monocytes and macrophages, whereas chitin-dependent stimulation induces mainly Th2 responses, although the identity of its receptor is unknown. Other less well characterized pathways include stimulation of inflammatory cytokine release by dectin-2, Mincle, and CD36/SCARF lectin receptors, and of the immunosuppressive cytokine IL-10 by DC-SIGN in DCs. Adapted from Netea and Maródi 2011 (483)

MAL, MyD88 adapter-like; IRAK, interleukin-1 receptor-associated kinase; TRAF, TNF receptor-associated factor; TAK, tumour growth factor (TGF) β-activated kinase; TRIF, Toll–interleukin-1 receptor domain-containing adapter inducing interferon b; TRAM, TRIF-related adapter molecule; TBK, TRAF family member-associated NF-kB activator-binding kinase; IRF, interferon response factor; Syk, T cell lineage-specific tyrosine kinase; MR, mannose receptor; DC-SIGN, DC-specific intracellular adhesion molecule-grabbing non-integrin.

5.2. Innate Immunity

The so-called innate immunity comprises constitutive mechanisms that are used by the host to respond to fungal pathogens in a rapid and conserved manner. Innate immune mechanisms are present at sites of continuous interaction with fungi and include the barrier function of the skin and mucosal epithelial cell surfaces of the respiratory, GI and genitourinary tracts. It is the first line of defence and is a crucial instructor of the subsequent adaptive immune response (484, 485). Microbial antagonism, defensins, collectins and the complement system also provide constitutive defence mechanisms (484). *C. albicans* activates complement by the classical and alternative pathways with deposition of C3 on the cell fungal surface. Complement activation facilitates the recruitment of phagocytes to infected tissues and enhances their anti-*Candida*l activity. Mice deficient in C5 production have an increased propensity to develop disseminated infection (486-488).

One of the functions of the innate immune system during fungal infections is the destruction of the pathogen through phagocytosis or secretion of microbicidal compounds against fungal elements that cannot be ingested. Phagocytosis and direct pathogen killing is mainly effected by monocytes, macrophages and neutrophils (485).

Protection against disseminated candidiasis relies mainly on components of innate immunity. The role of phagocytic cells such as macrophages, monocytes and, in particular, neutrophils is paramount, since quantitative and qualitative abnormalities of these cells are often associated with a lethal outcome (489). Namely, inborn errors of reactive oxygen radical production, leading to defective intracellular killing, as is the case in patients with chronic granulomatous disease make these hosts more prone to *Candida* infections (37).

Apart from the phagocytic cells residing in target organs at the time of infection, other effector cells, like neutrophils and monocytes, are recruited to sites of infection guided by inflammatory mediators such as chemokines and complement fragments.

Neutrophils and monocytes recognise and engulf opsonised and non-opsonized yeast cells via cell-surface PRRs. Binding to individual TLRs and IL-1 receptor (IL-1R) activates specialized antifungal effector functions on neutrophils and other phagocytes (434). TLR signalling on monocytes/macrophages and DCs also play an important role in the

orchestration of neutrophilic inflammation by the indirect regulation of neutrophil recruitment, activation and survival (490, 491).

Killing occurs by oxidative mechanisms, including generation of reactive oxygen and nitrogen intermediates, and by non-oxidative mechanisms (485, 492)). Phagocytosis and killing are augmented by opsonization and pro-inflammatory cytokines. Larger structures of *Candida* hyphae and pseudohyphae may prevent phagocytosis and in those cases, several phagocytes collaborate to effect extracellular killing. Although defective neutrophil function can result in disseminated infection, inappropriate or overwhelming neutrophil activation can also be a major cause of disease.

The interaction of macrophages with fungi is complex and the exact mechanism and efficiency of fungal killing are likely to depend on numerous factors, including macrophage source and environment, activation state, and fungal pathogenicity and morphology (493). Human macrophages, for example, are more efficient in killing C. *albicans*, than mouse macrophages, and *C. albicans* is able to escape from the latter by growing hyphae that break out the cell, releasing fungal cells into the extracellular environment.

Besides their role as professional phagocytes, innate immune cells can initiate the adaptive immune response via secretion of pro-inflammatory cytokines and chemokines and presentation of antigens.

Dendritic cells (DCs)

Fungal recognition by DCs is crucial for antigen-processing and presentation to T-cells (397, 494, 495) . DCs capture and process antigens, express cell-surface lymphocyte co-stimulatory molecules, migrate to lymphoid organs and secrete cytokines to initiate immune responses (496-499)(. In this way, DCs play an important role in linking innate with adaptive immunity (497). The type of response depends in part upon the morphotype of *Candida* encountered and the antigens displayed by the fungus (500). DCs can phagocytose both *Candida* yeast and hyphae, through receptors distinct for each morphotype, which lead to DC maturation and activation (500, 501). DCs that ingest the yeast form induce differentiation of $CD4^+$ T cells toward a T helper type-1 (Th1) pathway, whereas CDs that ingest hyphae induce T helper type-1 (Th2) responses by inhibiting IL-12 production and inducing IL-4 (500, 502). The multiple, functionally

distinct, receptor signalling pathways in DCs, by affecting the balance between $CD4^+$ effector T cells and $T_{reg,}$ are likely to be exploited by fungi to facilitate host commensalism or invasion (484).

5.3. Adaptive immunity

Adaptive immune responses comprise two major types, the so-called cell mediated and humoral immunity. They are mediated by different machineries of the immune system and have distinct roles during infection.

T cell-mediated responses

The Th1 and Th2 cell subsets were initially shown to participate and determine the outcome of *C. albicans* infections (503) . However, other T cell subsets such as Th17 or Treg cells were subsequently shown to be also involved in the host immune response to this pathogen (484, 504, 505). Development of a Th1-type response was initially shown to be determinant in the host's ability to control fungal infections and correlated with protection. Development of Th1 responses is influenced by the concerted action of several cytokines, prominently IL-12, in the relative absence of Th2 cytokines, such as IL-4 (505). The immunosuppressive cytokine IL-10 may also contribute to impair Th1 protective responses (505, 506). Progression of infection is associated with predominance of a Th2-type response (484) and, depending on the type of infection, of Treg (437).

Activation of Th17 cells may also occur in fungal infections, and be involved in what was classically defined as Th1- and Th2-type responses. Indeed, a role for Th17 cells in supporting Th1 cell responses has been shown in murine models of mucosal candidiasis (507, 508). The ability of IL-17A, produced by Th17 cells, to mobilize neutrophils and induce the production of defensins greatly contributes to efficient control of an infection at different body sites. However, the role of Th17 cells in the course of *Candida* infections remains unequivocally determined, as it may depend on the stage and site of infection, and is probably influenced by environmental stimuli (484). This cell subset may also be involved in the immunopathogenesis of fungal diseases (509). Nevertheless, this subject, or its precise mechanisms remain a matter of debate.

Although T cells have an acknowledged role in host defence against candidiasis, the importance of these cells appears to be rather limited during disseminated *Candida* infections (489, 510).

$CD8^+$ T cells are believed to exert direct anti-*Candida* activity, lysing yeast cells as well as yeast-containing phagocytes (505). However, in a model of acute systemic candidiasis, $CD8^+$ T cells were not necessary for host protection but had a role in limiting host's pathology (511). In contrast, these cells were shown to be important for resistance to gastric and disseminated candidiasis of endogenous origin (512).

Regulatory T cells (Treg)

During a fungal infection, an optimal immune response must eliminate the fungus while limiting collateral damage to tissues and restoring a homeostatic environment. Secretion of IL-10 represents one of the mechanisms by which Treg mediate suppression and control inflammatory responses (513). Several clinical observations indicated that an inverse relationship between IFN-γ and IL-10 production might occur in patients with fungal infections (514, 515). High levels of IL-10, which negatively affect IFN-γ production, are detected in chronic *Candida* infections, and thus have been linked to increased susceptibility to fungal infections (437, 484, 516, 517). TGF-β production has also been associated with disease progression (518). Moreover, during *C. albicans* infection the absence of IL-10 is beneficial for the host response (506). However, taking into account its major role in the resolution of inflammation, IL-10 production may be a consequence, rather than a cause of infection. To be so, in the case of chronic fungal infections, which are characterized by persistent inflammation, IL-10 may act as a homeostatic response aimed at keeping inflammation under control. However, as $T_{reg\ activity}$ may impair the efficacy of protective immunity, fungal persistence may be a host detrimental consequence. Thus, T_{reg} may be responsible for different infection outcomes, ranging from protective tolerance to increased fungal burden (437, 519, 520). Additionally, Treg participate in the development of protective memory after yeast priming (520).

B cell-mediated responses

The role of B cells in protection against *Candida* is not as extensively studied as the one played by T cells. Clinically, B cell deficiency is not associated with increased susceptibility to infection. Moreover, mice that lack functional T and B cells develop ongoing mucosal infection (most certainly due to the lack of T cells) without progression to disseminated disease (521). In contrast, susceptibility to acute systemic candidiasis was increased in mice deficient in B cells (522).

Antibodies

The role of antibodies during *Candida* infections is less clear as earlier studies suggested that *Candida*-specific antibodies do not have a protective role (5, 523). In fact, high titre anti-*Candida* antibodies are regularly found in patients that cannot clear *Candida* (524). Recently there has been renewed interest in antibody-mediated protection against fungal infections, with a focus on different *Candida* antigen targets, such as mannans, glucans, heat-shock proteins, Saps, and adhesins, with the aim of producing monoclonal or recombinant antibodies as prospective novel treatments (525, 526). The understanding of the role played by antibodies in the host response to *Candida* is currently growing. However, in murine models, protective and non-protective antibodies have been described and monoclonal anti-*Candida* antibodies are currently undergoing clinical trials in humans (527). It has been observed that the protective potential of antibodies with enhanced phagocytosis and killing of the fungus is dependent upon epitope specificity, serum titre, and ability to rapidly and efficiently fix complement to the fungal surface (528).

5.4. Mechanisms of immune evasion in *C. albicans*

Candida has developed numerous sophisticated mechanisms aimed at to elude and overcome host defences (3, 529, 530) . *C. albicans* may avoid inflammation, and this contributes to fungal adaptation and opportunism (531). This fungus can block recognition by masking β-1,3-glucans under the mannose/mannoprotein layer. These glucans are exposed in the bud scar of *C. albicans* yeasts but are masked on hyphae, thus favouring fungal escape from recognition by dectin-1 (532, 533).

C. albicans may also inhibit opsonisation/complement function by binding negative regulators of the complement cascade (534-536). Once detected by the immune system, blocking phagocytosis is another potential survival strategy. Cell size is an effective restriction to ingestion, and hyphal forms of *C. albicans* are not efficiently internalized (537). Another mechanism employed by *C. albicans* to avoid phagocyte attack is the contact-independent inhibition of NO production (538, 539). Even after internalization, *C. albicans* may affect phagocyte function, by escaping from the phagocyte after elongation of the hyphae and causing disruption of the phagocyte. Interestingly, this only occurs in certain monocyte/macrophage populations, whereas neutrophils can inhibit hyphae formation (371). Alterations of the intracellular fate have also been described. Trafficking of phagocytosed *C. albicans* was aberrant in murine macrophages, and a significant fraction of fungal cells ended up in a membrane-bound compartment associated with the ER, often at near neutral pH (540), although the underlying mechanism is unknown. As reported above, *C. albicans* Saps are able to degrade several proteins involved in the host immune response such as histatin-5 and proteins of the complement system (281, 282). In addition to all these mechanisms, a soluble factor, yet to be characterized, released by live *C. albicans* shifts tryptophan metabolism and inhibits host Th17 responses (504).

6. Vaccination

Because of the rising incidence of life-threatening candidiasis, high treatment failure rates, and huge health care costs, more effective prophylactic and therapeutic strategies are needed (44, 45). Recently, a renewed interest in the development of vaccination strategies against both mucosal and disseminated *Candida* infections has arisen, including induction of cell-mediated immunity by active immunization and also passive vaccination with antibodies (541, 542).

6.1. Vaccination against disseminated candidiasis

For many years the development of fungal vaccines did not receive much attention from the pharmaceutical companies. One reason was the general conviction that most patients who develop life-threatening fungal infections have profound defects in

immunity. One major concern about vaccinating those patients against invasive fungal infections was the belief that the immune systems of such patients were unlikely to respond protectively to vaccination and might suffer from aggravation of the immunological disorder following the immunostimulation by vaccine antigens and adjuvants. However, only 10-20% of patients who develop *Candida* bloodstream infection are seriously immunocompromised. Vaccination of high-risk groups of patients is a particularly promising strategy to prevent disseminated fungal infections because risk factors are clearly identified and correspond to common iatrogenic and/or nosocomial conditions that result in a substantial increase in the colonization burden of *Candida* spp. or in the disruption of protective anatomical barriers rather than to severe immunosuppression (541, 543, 544). Furthermore, development of these risk factors precedes infection, affording a window of opportunity to vaccinate highly at-risk patients before the establishment of infection. Such patients - including thosecandidate to transplants, patients that will undergo gastric or cardiac surgery and those affected by tumours - could benefit of advance active immunization against *Candida*, since the therapy or medical proceedings predispose to fungal infection (193). Since many of these risk factors are of relatively short duration, normally 4 to 6 weeks, an immunization approach would need to protect patients just for the short period of time during their increased susceptibility. In theory, antibodies can be induced by vaccination in at-risk subjects before predisposing therapy or immunosuppression (541). Because of the relative longevity of IgG (from weeks to months, depending on the IgG isotype), its persistence at a good protective level in the circulation even during a relatively prolonged immunosuppression or hospitalization period is to be expected (544). Vaccines merely eliciting antifungal CMI will not be appropriate for this purpose.

6.2. Proposed *Candida* vaccines

Protection against disease may be actively or passively acquired through vaccination and the transfer of preformed antibodies. The nature of immunizing antigen and its immunodominant epitopes, interaction with APCs (usually DCs), antigen processing through MHC class II or MHC class I pathways, and type of adjuvant, all determine the nature of elicited immunity and its outcome in terms of protection.

Protection against disseminated candidiasis has been reported following both active vaccination and passive transfer of antibodies (Table 1) and it is clear that antigens targeted for vaccination need not be restricted to virulence factors, which markedly increases the antigen repertoire available for testing.

Table 1. Proposed *C. albicans* vaccines

Antigen	Protection[a] Mucosal; Disseminated	Nature of protection[b]	Reference
Mannan, Mannoproteins	+;ND	Abs, B cells, CMI – Th1	(331, 379, 545-547)
1,3-β-glucan (Laminarin)	+;+	Abs	(548-552)
Secreted aspartyl protease 2	+;+[c]	Abs	(331, 378-381)
Agglutin-like sequences 1 and 3	+; +	CMI – Th1,Th17	(547, 553-556)
Phosphoglicerate Kinase	+;ND	Abs	(557)
Enolase	ND;+	Abs, CMI – Th1	(558)
Low virulent strains	-; +	CMI	(559)
Candida membrane antigen	ND; +[d]	Abs, CMI	(560, 561)
Heat shock proteins	+;+		(562, 563)
Heat killed cells	ND;+	Unknown	(564, 565)
Hyr-1	ND;+	Abs	(534, 566)
β-1,2-Mannotriose and mannobiose	ND;+	Abs	(567, 568)
***C. albicans* ds DNA**	+[e];ND	CMI – Th1	(569)
DCs transfected with fungal RNA	ND;+	CMI – Th1	(570, 571)

[a]+: protective; -: not protective, may enhance virulence; ND: not determined;
[b]Abs- Antibodies; CMI- Cell mediated immunity; [c] *C. albicans* peritonitis ; [d]Disseminated of endogenous origin in newborns; [e] gastrointestinal

The protective mechanism for most active vaccines against disseminated or mucosal candidiasis studied to date, relies on the induction of cell-mediated, pro-inflammatory, Th1 or Th17 responses, which improve phagocytic killing of the fungus (541, 544) Cutler et al 2007,. However, antibody participation in antifungal protection cannot be ruled out when CMI responses are elicited by vaccination. In fact, a type-1 cytokine response could be necessary for the formation of some protective antibodies against *C. albicans* proteins and most polysaccharide antigens, which, in murine models, are of the IgG2a isotype (572).

The best protective effects observed to date have been obtained by immunization with viable cells from virulent or avirulent *C. albicans* strains. DNA vaccines are now

believed to be the strongest immunization approach for CD8 cytotoxic effector generation, due to preferential antigen processing through MHC class I pathway (569). Whole cell or DNA vaccines maintain a persistent activation of CMI effectors. While positively controlling the infectious agent, they may nonetheless be also inducing strong inflammation with potential undesirable effects (544). Moreover, *C. albicans* is a member of the normal microbiota and vaccines against this opportunistic pathogen might result in unwanted inflammation, such as allergic manifestations (543, 573).

Antibodies have long been considered irrelevant in host defense against disseminated candidiasis. The existence of inhibitory antibodies, rather than the absence of protective ones, has mostly contributed to this assumption. The clinical evidence that antibodies are protective against fungal infections is limited to few cases (527, 574, 575) , but over the last two decades, a number of antibodies directed against *C. albicans* cell wall polysaccharides and glycopeptides, proteins and peptide epitopes, have been shown to confer protection in experimental models (Table 1). The demonstration of protective anti-*Candida* antibodies makes possible their use in immunoprophylaxy or immunotherapy against disseminated *Candida* infections through passive vaccination, an intervention that would have some advantages. There are several examples of recombinant antibodies against fungal infection (576, 577). Some protective antibodies can be generated in a human format devoid of Fc component, suggesting that they can work without the cooperation of the immune system, and thus could be appropriate for use in severely immunocompromised patients. Other monoclonal antibodies against *Candida* do need the Fc component and complement activation and deposition on cell surface for protection (572, 573).

Great efforts have been made in the characterization of the protective immune mechanisms against *Candida* infections and in the vaccination field but still, no therapeutic vaccine successfully used to fight *Candida* infections have been so far provided. Nevertheless, the increasing evidence, from clinical observations and animal models of candidiasis, that some *Candida*-specific antibodies can be immunoprotective during infection, points to the viability of an immunotherapeutic approach for the treatment and management of candidiasis, particularly in severely immunosuppressed patients, in combination with antifungal therapy (544, 572) .

7. Objectives and outline of the thesis

The work presented in this dissertation was developed at Centre of Molecular and Environmental Biology of Minho University (CBMA-UM) and Instituto de Ciências Biomédicas Abel Salazar of Porto University (ICBAS-UP).

Secreted aspartyl proteases have long been implicated in *Candida albicans* pathogenesis, mostly taking into account the observed attenuated virulence of Δ*sap* null mutant strains. However, the exclusive contribution of *SAP* genes for their attenuated phenotype remains to be unequivocally confirmed since the Ura-status of these mutant strains could have also contributed for the attenuation. Although Sap2 has been successfully used in several vaccination assays against mucosal and peritoneal candidiasis, the efficacy of Sap2 formulations in immunoprotection against hematogenously disseminated candidiasis is yet to be attempted. Moreover, the usage of Sap4-6 as antigen targets for vaccination against this type of *C. albicans* infection might be promising.

Thus, considering the contribution of Saps to *C. albicans* disseminated candidiasis, and the potential usage of these virulence determinants as antigen targets in vaccination assays to protect against this infection, the general objectives of this work were: i) to characterize the host immune response to systemic candidiasis elicited by different *C. albicans* strains; ii) to evaluate the role and importance of Saps in the course of murine disseminated candidiasis, established either by the hematogenous or the intraperitoneal routes; iii) to evaluate Sap1-3 contribution to host response evasion by degrading and inactivating host galectin-3; iv) to establish immunoprotective protocols against hematogenously disseminated candidiasis by using Sap2 and Sap5 as target antigens.

This dissertation is organized in seven chapters:

Chapter 1 consists of a general introduction presenting a review of the current knowledge on *Candida* infections, with a focus on systemic candidiasis, including data on incidence, independent risk factors and groups at risk. *Candida albicans* virulence factors are also addressed here, with a particular emphasis on Saps. Moreover, a

description of *C. albicans* cell wall components and their recognition by host Pattern Recognition Receptors is presented. Innate and adaptive immunity to *C. albicans* infections is also reviewed in this chapter. Finally, a review of the usefulness of immune-based prevention and therapy of *C. albicans* infections and a list of published proposed vaccines is included.

Chapter 2 presents data concerning the relative virulence of three *C. abicans* reference strains in a murine model of hematogenously disseminated candidiasis and the elicited host immune response. Different studies published on vaccination against *Candida* infections use distinct *C. albicans* strains. However, this usage appears to be based on laboratory strain availability rather than on an appropriate selection. Moreover, comparative studies that addressed *C. albicans* virulence barely focused on the overall elicited immune response. The main objective of this chapter is thus to comparatively evaluate virulence of three reference *C. albicans* strains, often used in virulence and antifungal drug testing, and characterize several features of the innate and adaptive immune response to infection established through the hematogenous challenge with each strain.

Chapter 3 re-addresses the importance of the *SAP1-SAP6* genes in a murine model of hematogenously disseminated candidiasis. This work aimed at confirming the importance of Saps in this infection model, by using single or triple mutant strains deficient in these virulence-associated genes, and also at characterizing the elicited host immune response.

Chaper 4 focuses on the role of *SAP1-SAP6* genes in a murine model of *C. albicans* peritonitis. Here, the analysis performed in the previous chapter is extended to the murine model of *Candida* peritonitis, with the objective of re-evaluating virulence of Δ*sap* null mutant strains in this model as well as the elicited host immune response.

In **Chapter 5** the ability of Sap1-3 to contribute for *C. albicans* evasion of host immunity, by degrading the host PRR galectin-3 is explored. Galectin-3 recognizes *C. albicans* and induces fungal death. Thus, impairing recognition through galectin-3 would favour pathogen survival. This work aimed at addressing if galectin-3 is a

substrate for Sap1, Sap2 or Sap3 and whether degradation of this PRR occurs *in vivo* in *C. albicans* infected tissues.

Chapter 6 presents data concerning the development of immunoprotective protocols against hematogenously disseminated candidiasis by using Sap2 and Sap5 enzymes as target antigens. Several approaches using commercially available adjuvants are tested in order to fulfil the main objective which consisted in establishing a vaccination strategy that could confer protection to mice against disseminated candidiasis.

Chapter 7 presents the concluding remarks consisting of an integrated discussion of the results achieved in the Chapters 2, 3, 4, 5, and 6 in the context of the initially proposed objectives. Future perspectives are also suggested at the end of the chapter.

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CHAPTER 2

Differential virulence and elicited immune response in mice infected by the hematogenous route with *Candida albicans* strains SC5314, ATCC 90028 or ATCC 32354

Differential virulence and elicited immune response in mice infected by the hematogenous route with *Candida albicans* strains SC5314, ATCC 90028 or ATCC 32354

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ABSTRACT

The murine model of hematogenously disseminated *Candida albicans* infection is widely used to ascertain strain virulence and host-fungus interaction. Here, a comparative study assessed survival and kidney fungal burden in mice infected intravenously with three different *C. albicans* strains with different attributed virulence, SC5314, ATCC 90028 and ATCC 32354. A comprehensive analysis of the early immune response in the spleen of yeast-challenged mice was also done. SC5314 was the most virulent strain and elicited a more marked inflammatory response, with higher neutrophil recruitment. In contrast, ATCC 32354 presented the lowest virulence and stimulated less markedly the innate response than the other strains. These results provide additional evidence on the association between *C. albicans* virulence and the host early innate immune response.

INTRODUCTION

The opportunistic human pathogen *Candida albicans* can cause both mucocutaneous and disseminated infections, particularly in debilitated or immuno-compromised hosts, being the most common cause of fungal invasive infections (1, 2). The murine model of hematogenously disseminated candidiasis closely resembles infection introduced into patients directly through catheters, is highly reproducible, and its clinical course is similar to untreated clinical disseminated candidiasis (3-5). Therefore, it has been widely used for investigating *C. albicans* virulence, host-fungus interactions, and efficacy of antifungal agents (6). Sepsis has been recognized as the major cause of death in the murine model of disseminated candidiasis (4), and the kidney is the main target organ for infection in this model (3, 5). However, evaluation of virulence only in terms of fungal kidney burdens and survival times gives little information of the fungus-host interactions and elicited host immune responses.

The innate immune system is the first line of host defence upon intravenous *C. albicans* challenge (7, 8). This arm of the immune system is mainly comprised of phagocytic cells that must recognize and respond to fungi, opposing fungal invasion and eliminating fungi from infected tissues and organs (9-11). The nature of the fungus is determinant in fungal recognition and drives the production of chemokines and directive cytokines, expression of co-stimulatory molecules and presentation of fungal antigens to T lymphocytes, affecting polarization of the T helper type of response (8, 12). The interplay between the host and the fungus is dynamic, and the balance between elimination of the fungus and tissue damage at the site of infection will depend not only on the host response, but also on the fungus ability to react to host effector molecules and cells (10, 12). It is well documented that properties of different yeast isolates, such as cell wall composition and morphogenesis, may modify fungal recognition and the ensuing immune response (12-16).

Within *C. albicans*, clinical isolates were found to differ considerably in virulence for mice, as revealed by differences in survival times and organ fungal burden after systemic infection (17-20). Nevertheless, the effects on the host response of the virulence properties of different *Candida* strains have been poorly evaluated, and focused on *C. albicans* internalization and killing by phagocytes, rather than on the

overall immune response (11, 21-23). In this respect, the study presented here aimed at evaluating the virulence of three different isolates of *C. albicans*, SC5314, ATCC 90028, and ATCC 32354, in a murine model of hematogenously disseminated infection, and comparing both innate and acquired immunity against the intravenous challenge with the different strains.

MATERIALS AND METHODS

Mice

Male BALB/c mice, 8-10 weeks old, were purchased from Charles River (Barcelona, Spain) and kept under specific pathogen-free conditions at the Animal Facility of Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal. All procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123), 86/609/EEC Directive and Portuguese rules (DL 129/92). Authorization to perform the experiments was issued by the competent national board authority (Direção Geral de Veterinária), document number 0420/000/000/2010.

Candida albicans strains and culture conditions

The *C. albicans* strains used in this study were the wild-type strains SC5314, ATCC 90028 and ATCC 32354. All strains were maintained as frozen stocks in 30% glycerol at -80ºC. To prepare the inocula for infection, strains were grown in a shaking incubator for 14 to 16 hours at 30ºC in Winge medium (0,2% glucose, 0,3% yeast extract). Yeast cells were harvested, washed twice with sterile, nonpyrogenic phosphate-buffered saline, counted in a haemocytometer and resuspended at the appropriate concentrations. Inocula were confirmed by Colony Forming Unit (CFU) counts on Sabouraud dextrose agar (Difco, Detroit, MI, USA) for up to 48 h at 37ºC.

Candida albicans hematogenously disseminated infections

Mice were injected intravenously (i.v.), in the lateral tail vein, with 1×10^5 *C. albicans* yeast cells in 0.2 ml PBS. To evaluate the progress of hematogenously disseminated candidiasis, mice were weighed and monitored twice daily, for a maximum of 70 days. Moribund mice were humanely terminated, and their deaths recorded as occurring the following day.

Alternatively, mice were sacrificed 18 and 72 h post-infection to determine organ fungal burden and/or immunological parameters. Control mice were injected i.v. with PBS. Kidneys were aseptically removed, homogenized, and quantitatively cultured on

Sabouraud dextrose agar (Difco) at 37ºC. Values were expressed as log CFU per gram of tissue.

Flow cytometric analysis

The assessment of cell surface and cytoplasmic lineage or activation markers on different splenic leukocyte populations was performed by flow cytometric analysis (FACS). Spleens were aseptically removed and homogenized to single cell suspensions in Hank's Balanced Salt Solution (HBSS) (Sigma Aldrich, St Louis, MO, USA). A number of $1x10^6$ leucocytes were stained per sample.

The following monoclonal antibodies (mAbs), along with the respective isotype control were used (at previously determined optimal dilutions) for immunofluorescence cytometric analysis in FACScan (Becton-Dickinson, San Jose, CA, USA) using CELLQUEST software (Becton-Dickinson):

Phycoerythrin (PE) conjugated rat anti-mouse CD40 (1C10) (Southern Biotechnology Associates, Birmingham, ALA, USA); Fluorescein isothiocyanate (FITC) conjugated anti-mouse/rat Foxp3 (FJK-16s), Phycoerythrin-Cyanin 5 (PE-Cy5) rat anti-mouse CD4 (L3T4) (RM4-5), and PE anti-mouse F4/80 antigen (BM8) (eBioscience, San Diego, CA, USA); Biotin conjugated anti-mouse PDCA-1(JF05-1C2.4.1) (Miltenyi Biotech, Inc., Auburn, CA, USA); FITC hamster anti-mouse CD11c (HL3), FITC anti-mouse Ly-6G and Ly-6C (Gr-1) (RB6-8C5), PE anti-mouse CD25 (PC61), PerCP-Cy5 anti-mouse CD19 (RA3-6B2), PE anti-mouse CD80 (B7-1) (16-10A1), PE anti-mouse CD86 (B7-2) (GL1), PE rat anti-mouse I-Ad/I-Ed (2G9), PE conjugated rat anti-mouse IL-4 (BVD4-1D11), FITC anti-mouse IFN-γ (XMG1.2), PE rat anti-mouse IL-17A (TC11-18H10), and PE rat anti-mouse IL-10 (JES5-2A5) (all from BD Pharmingen, San Diego, CA). Biotin conjugated mAbs were revealed with Streptavidin-PE-Cy5 (BD Pharmingen). Cells were pre-incubated for 15 minutes with anti-FcγR (a kind gift of Dr Jocelyne Demengeot, Gulbenkian Institute of Science, Oeiras, Portugal) before CD11c and Foxp3 staining. The Foxp3 Staining Buffer Set (eBioscience) was used for fixation and permeabilization of splenocytes surface stained with CD4 and CD25 mAbs.

The intracellular expression of the cytokines IFN-γ, IL-4, IL-17A and IL-10 was detected in splenic $CD4^+$ T lymphocytes. Splenocytes were obtained as described above. Red blood cell lysis was performed by incubation with 0,15M ammonium chloride. Cells

were washed and resuspended in complete RPMI medium (Sigma) (RPMI 1640 supplemented with 50U of penicillin/ml, 50 µg of streptomycin/ml, 1% HEPES buffer (Sigma), 10% FCS (Invitrogen, Carlsbad, CA, USA), and 5 µM 2-mercaptoethanol). $1x10^6$ cells were transferred to 96-well tissue culture plates (Nunc, Roskilde, Denmark) and stimulated for 4,5 hours with 20 ng/ml phorbol myristate acetate (Sigma) and 200 ng/ml ionomycin (Sigma) in the presence of 10 µg/ml of brefeldin A (Roche, Penzberg, Germany).

Staining of cell surface markers CD4 was performed as described above, after a pre-incubation step of 15 minutes with anti-FcγR, followed by fixation with 2% formaldehyde. Cells were permeabilized with 0,5% saponin in flow cytometric buffer (PBS containing 1% BSA and 0,01M sodium azide) and, subsequently, cells were incubated for 15 minutes with anti-FcγR and stained for 30 minutes at room temperature with the appropriate antibody. Intracellular staining with the isotypic control was performed to confirm the specificity of antibody binding.

The IFN-γ^+/IL-4^+ $CD4^+$ T cell ratios were calculated to determine the polarization of the immune response towards a Th1- or Th2-type. The immune response in non-infected mice was defined as unpolarized.

Statistical Analysis

Unless otherwise stated, results shown are from one experiment, representative of two independent experiments. Statistical significance of results was determined by one-way ANOVA and *post-hoc* Bonferroni's multiple comparison test, and survival data were analysed with the log-rank test, by using the GraphPad Prism 4 Software (GraphPad Software, Inc., La Jolla, CA, USA). Results were considered statistically significant with *P* values of less than 0.05.

RESULTS

Virulence of *C. albicans* strains SC5314, ATCC 90028 and ATCC 32354 in intravenously infected BALB/c mice

The widely used *Candida albicans* strain SC5314 and strains ATCC 90028 and ATCC 32354 with different reported virulence abilities (17) were compared for their virulence in a model of hematogenously disseminated candidiasis. Striking differences in survival time were observed after i.v. infection of BALB/c mice with SC5314 and ATCC 90028 or ATCC 32354 (P= 0.0013 and P= 0.0066, respectively) (Figure1A).

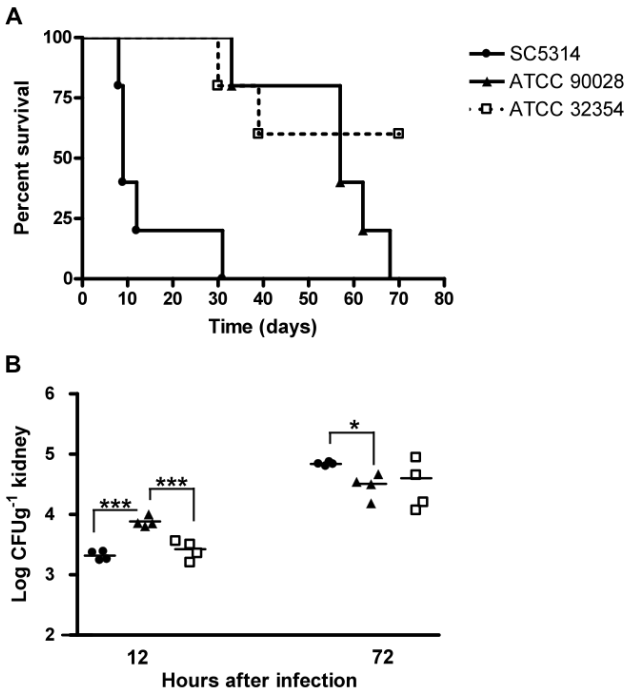

Figure 1. *C. albicans* virulence in the murine model of hematogenously disseminated candidiasis. (A) Survival curves of BALB/c mice injected i.v. with 1×10^5 cells yeast cells of *C. albicans* WT strains SC5314, ATCC 90028 and ATCC 32354. P=0.0013 SC5314 *vs* ATCC90028 and P=0.0066 SC5314 vs ATCC 32354, as determined by log-rank test (n=5); (B) Kidney fungal burden of BALB/c mice 12 and 72 hours after i.v. infection with 1×10^5 SC5314 (●), ATCC 90028 (▲) or ATCC 32454 (□) *C. albicans* cells, as indicated. Data are from one experiment representative of two independent experiments. Each symbol represents an individual mouse, horizontal bars are means of CFU numbers in each group (n=4), * P<0.05; *** P<0.001, as determined by one-way ANOVA and *post-hoc* Bonferroni's multiple comparison test.

The median survival time of SC5314- and ATCC 90028-infected infected mice was 9 and 57 days, respectively. All mice injected with either SC5314 or ATCC 90028 strains succumbed to candidal infection. In contrast, mice infected with ATCC 32354 had an extended overall survival time, compared to mice infected with the other *C. albicans* strains. At the end of the experiment (70 days), 60% of the mice infected with ATCC 32354 survived the infection (Figure 1A). 12 h after i.v. challenge, SC5314-infected mice presented significantly lower CFU counts than ATCC 90028-infected mice, and similar kidney counts to ATCC 32354-infected mice (Figure 1B), though mice infected with the former strain presented a greatly diminished survival time. Nevertheless, as depicted in Figure 1B, 72 h after challenge, kidneys from mice infected with strain SC5314 presented higher fungal burdens than those of ATCC 90028- and ATCC 32354-infected mice, which had no different kidney CFU numbers, despite the differences observed in survival. No later time points were assessed, since mice inoculated with 1×10^5 SC5314 cells were frequently moribund by 6 to 7 days after infection.

Early host immune response in *C. albicans*-challenged mice

Given the marked differences found in mice survival times, we evaluated the early host immune response to an i.v. challenge with the different tested strains. At the earliest time point analysed, mice infected with strain ATCC 90028, which presented the highest kidney fungal burden, showed the highest proportions and numbers of splenic inflammatory monocytes ($F4/80^+Gr\text{-}1^+$) and reduced frequency and numbers of macrophages ($F4/80^{hi}Gr\text{-}1^-$) whereas neutrophils ($F4/80^-Gr\text{-}1^+$) did not vary from controls or the other infected groups (Figure 2). This scenario changed completely by 72 h after infection. SC5314-infected mice, which presented the highest kidney CFU counts at this time point, had a significant increase in the proportions and numbers of neutrophils, as compared with any of the other groups (Figure 2). Inflammatory monocytes were present in higher proportions and numbers in the spleen of mice infected with the most virulent strains, SC5312 and ATCC 90028. Curiously, no relevant changes were observed in the assessed myeloid spleen cell populations in mice infected with ATCC 32354.

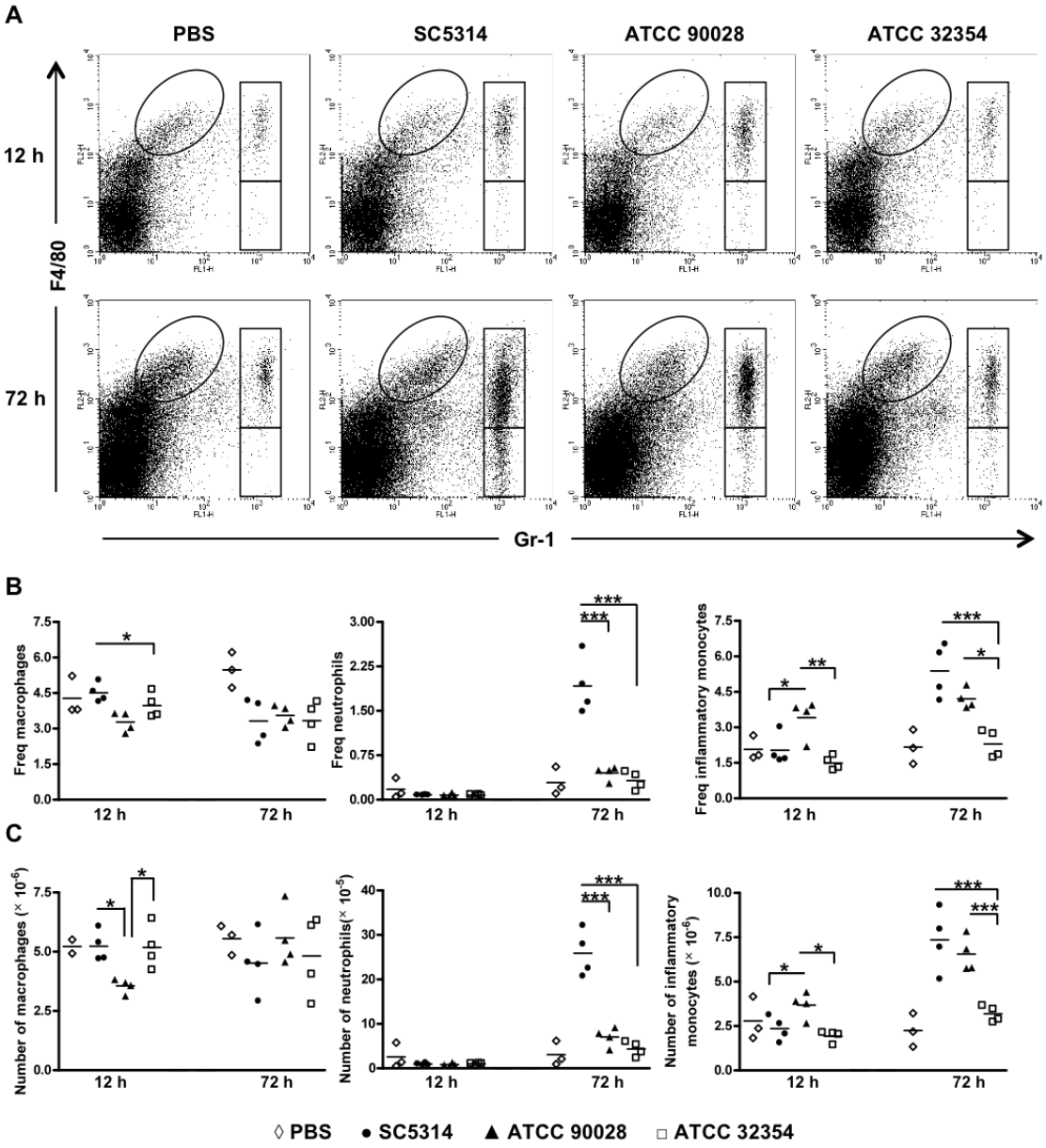

Figure 2 (A) Representative examples of flow cytometric analysis of F4/80 and Gr-1 expression on the surface of mice splenic cells 12 and 72h after i.v. injection with PBS or 1×10^5 *C. albicans* SC5314, ATCC 90028 or ATCC32354 cells, as indicated. Gated populations correspond to macrophages ($F4/80^{high}Gr\text{-}1^{neg}$), neutrophils ($F4/80^{neg/low}Gr\text{-}1^{high}$) and inflammatory monocytes ($F4/80^{high}Gr\text{-}1^{high}$). (B) Scatter plots of the frequencies of macrophages, neutrophils and inflammatory monocytes ; (C) Scatter plots of the total numbers of macrophages, neutrophils and inflammatory monocytes, as indicated. Each symbol represents an individual mouse. (n=3 for controls and n=4 for infected mice). **P*<0.05; ***P*<0.01; ****P*<0.001. Statistical comparison with non-infected controls was omitted for simplicity.

In general, expression of MHC class II and co-stimulatory molecules CD80, CD86, CD40 on the surface of spleen conventional and plasmacytoid dendritic cells (cDCs and pDCs, respectively) did not markedly change upon infection. Some alterations could however

be observed. Mice infected with SC5314 had increased expression of the co-stimulatory molecule CD86 on the surface of both cDCs and pDCs 72 h after infection, indicating a higher activation of these cells as compared to the other infected groups (Figure 3).

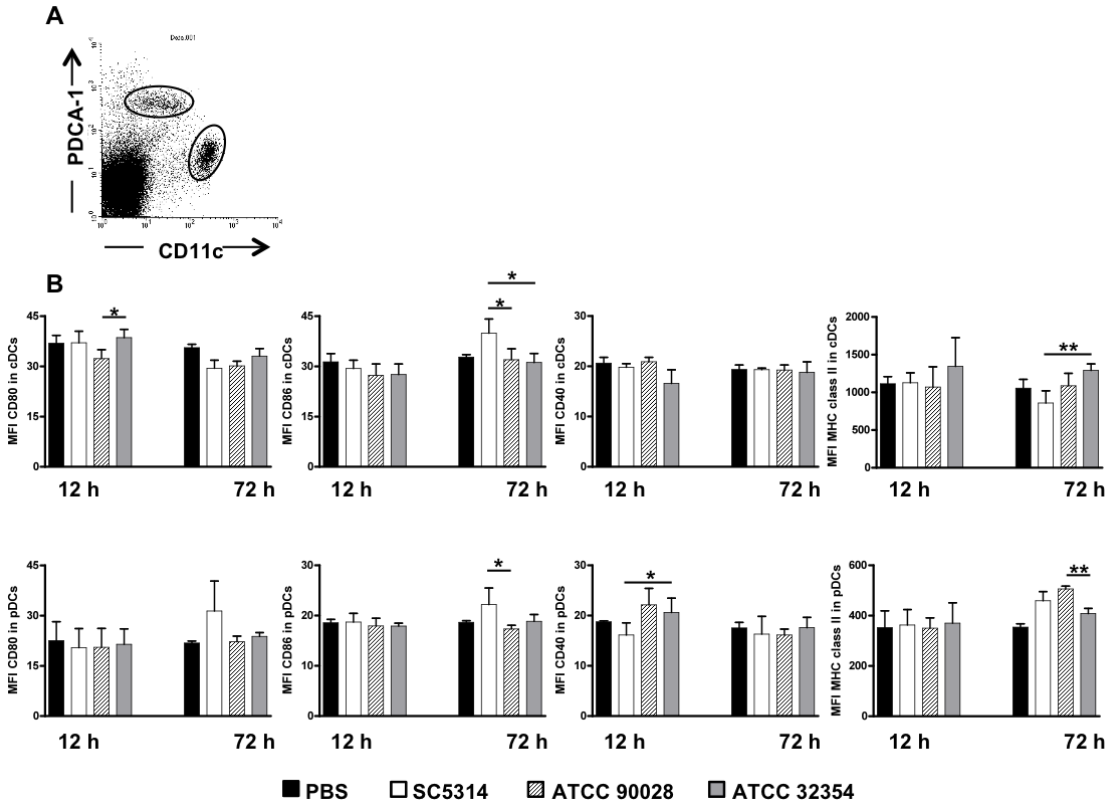

Figure 3 Representative flow cytometric analysis of CD11c and PDCA-1 on splenic BALB/c mice cells. (A) Conventional dendritic cells (cDC) were defined as $CD11c^{high}$ and plasmacytoid dendritic cells (pDC) as $CD11c^{low}$ $PDCA\text{-}1^{+}$, as shown. (B) CD80, CD86, CD40 or MHC class II expression on the surface of splenic cDC or pDC, as indicated, 12 and 72 h after *C. albicans* i.v. Infection (n=3 for controls and n=4 for infected mice). * $P<0.05$; ** $P<0.01$.

An elevated expression of CD86 on the surface of B cells was also observed in SC5314-infected mice (data not shown). In contrast, SC5314-infected mice presented a diminished expression of CD40 on the surface of pDCs at the earliest time point and of MHC class II molecules on cDCs 72 h upon challenge. Mice infected with strain ATCC 90028 had diminished CD80 and elevated MHC class II expression on cDCs at 12 h and 72 h after infection, respectively (Figure 3).

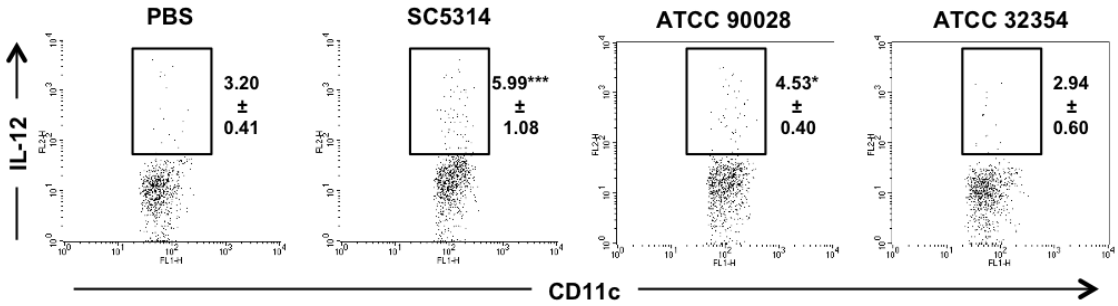

Figure 4 Representative examples of flow cytometric analysis of intracellular IL-12 expression in CD11c$^+$-gated spleen cells, 72 h after i.v. injection with PBS or 1×10^5 *C. albicans* SC5314, ATCC 90028 or ATCC32354 cells. Numbers inside dot-plots correspond to mean percentage ± SD of IL-12$^+$ cells (n=3 for controls and n=4 for infected mice). **P*<0.05; ****P*<0.001 when comparing with either PBS or ATCC 32354 groups. No other significant differences were observed.

Mice infected with the most virulent strains had the highest frequencies of spleen cDCs expressing the pro-inflammatory molecule IL-12 (Figure 4), yet the frequencies of CD4$^+$ T cells expressing IFN-γ were not significantly different from the one found in ATCC 32354-infected mice (Figure 5A).

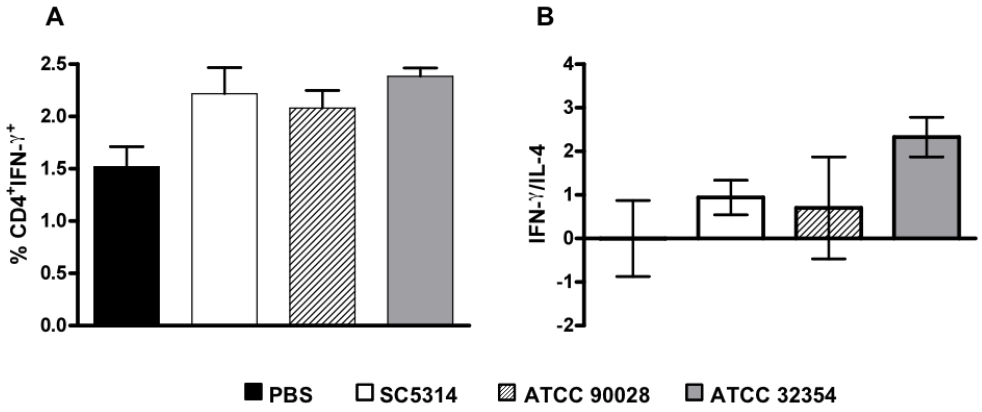

Figure 5 (A) Expression of intracellular IFN-γ in gated spleen CD4$^+$ T cells, 72 h after *C. albicans* i.v. Infection. (B) IFN-γ/IL-4 ratio normalized by the control mean ratio (n=3 for controls and n=4 for infected mice).

Interestingly, the IFN-γ$^+$/IL-4$^+$ cell ratio in these mice was the highest, suggesting a Th1 bias in the immune response. No significant differences were observed in IL-10 and IL-17A expression by CD4$^+$ T cells. Expression of the regulatory T cell marker Foxp3 within CD4$^+$CD25$^+$ T cells was reduced in SC5314- and ATCC 90028-infected mice groups, but not in mice infected with the less virulent ATCC 32354 strain (data not shown).

Altogether, these results confirm virulence differences among the tested strains, in this infection model, and suggest an association between *C. albicans* virulence and the elicited inflammatory response.

DISCUSSION

Distinct *C. albicans* isolates may show very distinct virulence and infection patterns in mice (20, 21, 24, 25) and elicit both qualitatively and quantitatively different host responses (18, 21). The virulence of three different *C. albicans* strains, SC5314, ATCC 90028 and ATCC 32354 was compared here by determining survival and fungal burden in the kidneys of BALB/c mice after intravenous challenge. The evaluation of the ability to colonize and invade the kidneys has been usually used to measure virulence of *C. albicans* strains (3, 26). A near-linear relationship has been demonstrated between mouse median survival times and *C. albicans* kidney CFU counts, determined 12 h after challenge (3, 27). However, we could not find such an association between kidney CFU counts and mice survival time at this early time point. In the infection model used, SC5314 was the most virulent *C. albicans* strain tested, causing lethal infection in a relatively short time. This *C. albicans* strain has already been classified as highly virulent in the murine model of hematogenously disseminated candidiasis and causing severe infection in the kidney (3, 18, 19). Not surprisingly, strain ATCC 32354 presented lower virulence than the two other strains tested, since in a previous report it has been shown to have intermediate virulence in murine hematogenously disseminated candidiasis (17). Accordingly, neutrophilic infiltration and edematous corneal stroma were prominent in corneas infected with SC5314, but less severe with strain ATCC 32354 (28). Unexpectedly, mice infected with ATCC 90028, which was considered a highly virulent strain in the i.v. infection model (17), presented a markedly higher survival time than SC5314-infected counterparts.

Several studies support a direct relationship between colony count in the infected kidney and mortality (3, 27, 29). However, although renal failure was long considered to be the cause of mice death in the murine model of disseminated candidiasis (29-32), the major cause of death was later proved to be progressive sepsis (4). Here, such a relationship between kidney fungal burden and mortality was not obvious as mice infected with SC5314 or ATCC 32354, which greatly differed in survival times, presented similar *C. albicans* kidney load. This might indicate that survival may be instead determined by the host immune response. This hypothesis may be in agreement with a previous report showing that the early innate immune response,

assessed through cytokine and chemokine measurement in the spleen and kidneys, determines and predicts the progression of *C. albicans* infection (21).

It is noteworthy that the highest virulent strain, SC5314, induced a more marked inflammatory response, as assessed by the high recruitment of neutrophils and inflammatory monocytes into the spleen. Also, DCs from mice infected with this strain produced the highest levels of the pro-inflammatory cytokine IL-12 and presented the highest up-regulation of co-stimulatory molecule CD86 on their surface. The generated inflammation may be worsening organ pathology rather than protecting the host, as suggested before (12, 21). That would explain the more precocious death of SC5314-infected mice. Contrastingly, in mice infected with the other strains, a lower splenic neutrophil influx could reflect a milder inflammatory response, translated in extended survival times. It is intriguing that SC5314 strain, which display a high virulence in the model used here, in which immune protection mainly depends on polymorphonuclear neutrophils was reported to be much less efficient at infecting the mucosa (18, 33), for which cell mediated immunity is essential for host protection (7).

The ATCC 32354 strain barely elicited a detectable inflammatory response, as assessed by leukocyte recruitment or activation in the spleen. Nevertheless, mice infected with this strain, were the ones presenting the highest IFN-γ/IL-4 ratio. This could indicate a bias towards a Th1-type immune response, presumably protective (7, 12), without aggressive inflammatory pathology. In this regard, it must be noted that mice infected with ATCC 32354 strain, contrasting with what was observed in the mice infected with the most virulent strains, did not present a decrease in splenic T regulatory cell proportions. It is conceivable that this may help in inflammation control as these cells have been implicated in limiting inflammatory pathology in the murine model of hematogenously disseminated candidiasis (34).

The vast majority of gene disruption studies have been carried out in a single strain, SC5314, background. It is already known that different strains may present distinct virulence phenotypes and elicit dissimilar immune responses. In consequence, results obtained with one strain cannot be taken as representative of the whole species. This must be taken into account when delineating vaccination or immunotherapeutic strategies to prevent or manage systemic *C. albicans* infections.

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CHAPTER 3

Limited role of secreted aspartyl proteases Sap1-6 in *Candida albicans* virulence and host immune response in murine hematogenously disseminated candidiasis

Limited role of secreted aspartyl proteases Sap1-6 in *Candida albicans* virulence and host immune response in murine hematogenously disseminated candidiasis

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ABSTRACT

Candida albicans secreted aspartyl proteinases (Sap) are considered virulence-associated factors. Several members of the Sap family have been claimed to play a significant role in the progression of candidiasis established by the hematogenous route. This assumption was based on the observed attenuated virulence of *sap* null mutant strains. However, the exclusive contribution of *SAP* genes for their attenuated phenotype was not unequivocally confirmed as the Ura-status of these mutant strains could also have contributed to the attenuation. In this study, we have re-assessed the importance of *SAP1-SAP6* in a murine model of hematogenously disseminated candidiasis, using *sap* null mutant strains not affected in their *URA3* gene expression and compared their virulence phenotype with Ura-blaster *sap* mutants. The median survival time of BALB/c mice intravenously infected with a mutant strain lacking *SAP1-3* was equivalent to that of the wild-type SC5314, while those infected with mutant strains lacking *SAP5* showed slightly extended survival. Nevertheless, no differences could be observed between the wild-type and a *Δsap456* mutant in their ability to invade and colonize mice kidneys. Likewise, deficiency in *SAP4-6* had no noticeable impact on the immune response elicited in the spleens and kidneys of *C. albicans* infected mice. These results contrast with the behaviour of equivalent Ura-blaster mutants, which presented a significant reduction in virulence. Our results suggest that Sap1-6 do not play a significant role in *C. albicans* virulence in a murine model of hematogenously disseminated candidiasis and that, in this model, Sap1-3 are not necessary for successful *C. albicans* infection.

INTRODUCTION

The polymorphic yeast *Candida albicans* is an important opportunistic human pathogen, causing infections that range from superficial mucosal lesions to life-threatening systemic disease. It is by far the most common cause of fungal invasive infections, which could be attributed to the little immunosuppression required to predispose to invasive *Candida* infections (39). Host physical barriers and immune system integrity are crucial factors in controlling the establishment of infection. However, the high adaptability of *C. albicans* to different host niches, by the expression of appropriate sets of virulence-related genes, is also determinant (18, 51). Several of these virulence attributes may participate in and influence the infective process, depending on the site and stage of invasion and on the nature of the host response (36). The secretion of hydrolytic enzymes during infection is required as a virulence attribute to aid adhesion, invasion, and destruction of host immune factors, in addition to nutrient acquisition (20). Within these, secreted aspartyl proteases (Sap), encoded by a ten-member gene family (*SAP1-SAP10)* have been the most extensively studied (34). The 10 *SAP* genes that compose this family can be divided into subfamilies based on amino acid sequence homology alignments (*SAP1-3*, *SAP4-6*, *SAP9-10*). These genes exhibit differential expression profiles at different stages and sites of infection (34, 32, 46, 49) and have been linked with the virulence of the fungus since their discovery (10, 26, 48).

The contribution to virulence of *SAP1-3*, *SAP4-6*, *SAP7* and *SAP9-10* genes in different models of infection has been studied by using *sap* null mutant strains (1; 13; 15; 19; 22; 24, 25, 28, 33, 43, 54). The subfamily of genes *SAP4-6*, in particular, were shown to contribute significantly to *C. albicans* virulence in models of acute systemic candidiasis, murine peritonitis and *Candida* gastrointestinal infection (15, 24, 43). These genes are mainly expressed during hypha formation (21, 33) and *SAP5*, in particular, was found to be up-regulated at all time-points after either intravenous or intraperitoneal mice infection (44, 50, 57).

Hube *et al*. (19) reported that *Δsap1*, *Δsap2* and *Δsap3* null mutants displayed attenuated virulence in models of acute systemic candidiasis. The triple deletion of *SAP4-6* resulted in a more marked impact on *C. albicans* virulence, in similar

experimental settings, suggesting an important role for these hypha-related genes in the establishment of disseminated candidiasis (43). These mutant strains were generated from the auxotrophic laboratory strain CAI4 with the most common method used for disrupting genes in *C. albicans*, the Ura-blaster technique (16). The use of the *URA3* marker for mutant construction in *C. albicans* can lead to misinterpretation of the results in mutant virulence studies (5, 8, 11, 27). Though this can now be overcome by integration of *URA3* at the *ENO1* (52) or *RPS10* loci (8), the mutant strains used in the referred studies did not share a common site of *URA3* integration. Therefore, it is conceivable that the Ura-status could have influenced the results and thus, the attenuated nature of these mutants during acute systemic candidiasis remains to be confirmed unequivocally (37). Therefore, in this study, we have used a set of *Δsap* null mutants constructed by Lermann and Morschhäuser (28) from the prototrophic wild-type strain SC5314 using the *SAT1*-flipping stategy (41) to re-address the importance of the *SAP1-SAP6* genes in a murine model of hematogenously disseminated candidiasis. In addition, we analyzed the histopathology of several organs and aspects of the immune response elicited in the spleens and kidneys of BALB/c mice infected with the wild-type strain and a *sap* null mutant lacking *SAP4* to *SAP6*.

MATERIALS AND METHODS

Mice

Male BALB/c mice, 8-10 weeks old, were purchased from Charles River (Barcelona, Spain) and kept under specific pathogen-free conditions at the Animal Facility of Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal. All procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123), 86/609/EEC Directive and Portuguese rules (DL 129/92).

Candida albicans and culture conditions

The *C. albicans* strains used in this study are listed in TABLE 1.

TABLE 1. *Candida albicans* strains used in this study

Strain	Genotype	Reference
SC5314	Prototrophic wild-type strain	16
SAP123MS4C and -D	*sap1*Δ::*FRT*/*sap1*Δ::*FRT* *sap2*Δ::*FRT*/*sap2*Δ::*FRT* *sap3*Δ::*FRT*/*sap3*Δ::*FRT*	27
SAP456MS4A and -B	*sap4*Δ::*FRT*/*sap4*Δ::*FRT* *sap5*Δ::*FRT*/*sap5*Δ::*FRT* *sap6*Δ::*FRT*/*sap6*Δ::*FRT*	27
SAP4MS4A	*sap4*Δ::*FRT*/*sap4*Δ::*FRT*	27
SAP5MS4A	*sap5*Δ::*FRT*/*sap5*Δ::*FRT*	27
SAP6MS4A	*sap6*Δ::*FRT*/*sap6*Δ::*FRT*	27
CAF2-1	*URA3/ura3Δ::imm434*	15
M119	*sap1Δ::hisG/sap1Δ::hisG* *sap2Δ::hisG/sap2Δ::hisG* *sap3Δ::hisG/sap3Δ::hisG::URA3::hisG*	23
DSY459	*sap6Δ::hisG/sap6Δ::hisG* *sap4Δ::hisG/sap4Δ::hisG* *sap5Δ::hisG/sap5Δ::hisG::URA3::hisG*	42

All strains were maintained as frozen stocks in 30% glycerol at -80ºC. Yeast growth was tested in synthetic glucose minimal (2% glucose, 0.67% Bacto-yeast nitrogen base without amino acids) and complex YPD (2% glucose, 1% yeast extract, 2% Bacto-peptone) liquid media at 30 and 37ºC, in a shaking incubator for 24 h. Growth was measured at 60 minute-intervals and the generation times were calculated. Hyphae formation was induced by the addition of calf serum (Invitrogen, Carlsbad, CA, USA) (10%) or N-acetyl-glucosamine (2.5 mM) (Sigma, St Louis, USA) at 37ºC. To prepare the inocula for infection, *C. albicans* strains were grown in a shaking incubator for 14 h at 30ºC in Winge medium (0.2% glucose, 0.3% yeast extract). Yeast cells were harvested, washed twice with sterile, nonpyrogenic phosphate-buffered saline (PBS), counted in a haemocytometer and resuspended at the appropriate concentrations. Inocula were confirmed by Colony Forming Unit (CFU) counts on Sabouraud dextrose agar (Difco, Detroit, MI, USA) for up to 48 h at 37ºC.

***C. albicans* hematogenously disseminated infections**

Mice were injected intravenously (i.v.), in the lateral tail vein, with $1x10^5$ or $5x10^5$ *C. albicans* yeast cells in 0.2 ml PBS. To evaluate the progress of hematogenously-disseminated candidiasis, mice were weighed and monitored twice daily. Moribund mice were humanely terminated, and their deaths recorded as occurring the following day.

Other groups of mice were infected with $5x10^4$ yeast cells and sacrificed 3 and 7 days post-infection to determine organ fungal burden and/or immunological parameters. Control mice were injected i.v. with PBS. Kidneys were aseptically removed, weighted, homogenized, and quantitatively cultured on Sabouraud dextrose agar (Difco) at 37ºC. Values were expressed as log CFU per gram of tissue. Alternatively, kidneys, liver, lungs and brain were fixed in 10% phosphate-buffered formaldehyde, followed by periodic acid-Schiff reagent staining and counterstaining with haematoxylin of the paraffin-embedded tissues, in order to evaluate both fungal morphology and composition and distribution of inflammatory infiltrates.

Quantitative real-time PCR (qRT-PCR)

BALB/c mice were i.v. infected with $1x10^5$ *C. albicans* SC5314, SAP123MS4C and SAP456MS4A yeast cells. Total RNA was isolated from the kidneys of three mice per group, 3 and 7 days after infection. Briefly, the kidneys were removed, homogenized in PBS and centrifuged at 1500 g, 4ºC for 10 min. Pellets were washed twice with ice cold RNAse-free water and frozen in liquid nitrogen until RNA extraction which was performed by using the hot acidic phenol method (4) Total RNA was incubated with Deoxyribonuclease I (DNAse I), Amplification Grade (Invitrogen) for 15 min at room temperature to eliminate genomic DNA contamination. DNAse I was inactivated according to the manufacturer's instructions.

The Superscript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green was used to generate first-strand cDNA from each DNAse I-treated RNA sample. The RT conditions were 10 min at 65°C, 60 min at 37°C, and 10 min at 65°C. Quantitative PCR was performed with Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). Primers were added to the reaction mix at a final concentration of 200 nM. Three microliters of each cDNA sample were added to a 25 µl PCR mixture containing 12.5 µl of Platinum® SYBR® Green qPCR SuperMix-UDG, 0.5 µl of 10µM specific forward and reverse primers (TABLE 2) and 8.5 µl of RNase free water (Invitrogen). Each reaction was performed in a Corbett Rotor-Gene 6000 (Quiagen). Thermocycling conditions for *SAP* and *ACT1* quantification were 2 min at 50ºC (UDG incubation), 5 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. The specificity of each primer pair was verified by the presence of a single melting temperature peak. Calibration and efficiency of *SAP* and *ACT1* primers were assessed in titration experiments using *C. albicans* SC5314 genomic DNA (500 ng to 5 pg) in serial dilutions. Negative (water) control and a four point curve of SC5314 genomic DNA were included in each run. DNAse I-treated RNA was used to exclude genomic DNA contamination. *SAP1-10* gene expression was normalized to the housekeeping gene *ACT1* and analysed by using both the standard curve and the comparative C_t ($\Delta\Delta C_t$) methods. Data are presented as fold difference in expression relative to the WT gene expression from infected mice. Each experimental condition was performed in triplicate and reactions were done in duplicate in different days for reproducibility purposes.

TABLE 2. *SAP* and *ACT1* primers used in this study and expected fragment length

Gene	Primer 5'-3'		Amplicon (bp)
	Forward	Reverse	
ACT1	TGCTGAACGTATGCAAAAGG	TGAACAATGGATGGACCAGA	186
SAP1	TGAGGCTGCTGGTGATTATG	TGCCAACAGCTTTGAGAGAA	224
SAP2	ATCAGCTGGTTTCGTTGCTT	GGGACAGCTTGTCTTTTGGA	105
SAP3	TGTTACTGGTCCCCAAGGTGAA	CTTGTCCTTGACCAGCTTGACAT	209
SAP4	AATGATGTGGGCAAAAGAGG	ACGGCATTTGAATCTGGAAC	155
SAP5	ATTTCCCGTCGATGAGACTG	ACCACGCCATTTTGGAATAC	205
SAP6	GTCAACGCTGGTGTCCTCTT	GCAGGAACGGAGATCTTGAG	197
SAP7	TTCTCGTGATGCTGTCCAAG	CCAGCAGGAAGACCATAAGC	183
SAP8	TTTGGTGGGGTTGATAATGC	GGCAGCAGCCAATTTATCAG	198
SAP9	ACCGGGTCTTCAGATTTGTG	TTCCTCGTCGGTTTCTATGG	180
SAP10	AACGGAAATGTTGCTTCTGG	TGAATCGCCTATCGAAAACC	192

Flow cytometric analysis

The assessment of cell surface and cytoplasmic lineage or activation markers on different splenic leukocyte populations was performed by flow cytometric analysis (FACS). Spleens were aseptically removed and homogenized to single cell suspensions in Hank's Balanced Salt Solution (HBSS) (Sigma). A number of $1x10^6$ leucocytes were stained per sample.

The following monoclonal antibodies (mAbs), along with the respective isotype controls were used (at previously determined optimal dilutions) for immunofluorescence cytometric analysis in FACScan (Becton-Dickinson, San Jose, CA, USA) using CELLQUEST software (Becton-Dickinson):

Phycoerythrin (PE) rat anti-mouse CD40 (1C10), biotin rat anti-mouse Major Histocompatibility Complex (MHC) class II (NIMR-4) (Southern Biotechnology Associates, Birmingham, ALA, USA); fluorescein isothiocyanate (FITC) anti-mouse/rat Foxp3 (FJK-16s), PE-Cy5 rat anti-mouse CD4 (L3T4) (RM4-5), and PE anti-mouse F4/80 antigen (BM8) (eBioscience, San Diego, CA, USA); FITC hamster anti-mouse CD11c (HL3), FITC anti-mouse Ly-6G and Ly-6C (Gr-1) (RB6-8C5), PE anti-mouse CD25 (PC61), FITC anti-mouse CD45R/B220 (RA3-6B2), PE anti-mouse CD80 (B7-1) (16-10A1), PE anti-mouse CD86 (B7-2) (GL1), PE rat anti-mouse IL-4 (BVD4-1D11), FITC anti-mouse IFN-□ (XMG1.2), and PE rat anti-mouse IL-10 (JES5-2A5) (all from BD Pharmingen, San Diego, CA). Biotin conjugated mAbs were revealed with Streptavidin-PE-Cy5 (BD Pharmingen). Cells were preincubated for 15 min with anti-Fc□R (a kind gift of Dr Jocelyne Demengeot, Gulbenkian Institute of Science, Oeiras, Portugal) before CD11c

and Foxp3 staining. The Foxp3 Staining Buffer Set (eBioscience) was used for fixation and permeabilization of splenocytes previously surface stained with CD4 and CD25 mAbs.

The intracellular expression of the cytokines IFN-γ, IL-4 and IL-10 was detected in splenic $CD4^+$ T lymphocytes. Intracellular expression of the cytokines IFN-γ and IL-4 was also detected in renal $CD4^+$ T lymphocytes. Splenocytes were obtained as described above. Red blood cell lysis was performed by incubation with 0.15M ammonium chloride. Cells were washed and resuspended in complete RPMI medium (Sigma) (RPMI 1640 supplemented with 50U of penicillin/ml, 50 µg of streptomycin/ml, 1% HEPES buffer (Sigma), 10% FCS (Invitrogen), and 5 µM 2-mercaptoethanol). The kidneys were minced with a razor blade and incubated for 30 min at 37ºC in RPMI 1640 complete medium containing collagenase (Sigma-Aldrich) at 2 µg/ml. Cells were homogenized to single cell suspensions, washed and resuspended in RPMI 1640 complete medium. Mononuclear cells were separated from the above suspensions by layering 5 ml onto 2.5 ml of a polysucrose-sodium ditrizoate solution (Histopaque 1083®, Sigma) and centrifuged at 800 g for 20 min at room temperature. Mononuclear cells collected from the medium-Histopaque interface were washed and resuspended in RPMI 1640 complete medium. Spleen and renal $1x10^6$ cells were transferred to 96-well tissue culture plates (Nunc, Roskilde, Denmark) and stimulated for 4.5 h with 20 ng/ml phorbol myristate acetate (Sigma) and 200 ng/ml ionomycin (Sigma) in the presence of 10 µg/ml of brefeldin A (Roche, Penzberg, Germany).

Staining of cell surface marker CD4 was performed as described above, after a preincubation step of 15 min with anti-FcγR, followed by fixation with 2% formaldehyde. Cells were permeabilized with 0.5% saponin in flow cytometric buffer (PBS containing 1% BSA and 10mM sodium azide) and, subsequently, cells were incubated for 15 min with anti-FcγR and stained for 30 min at room temperature with the appropriate antibody. Intracellular staining with the isotypic controls was performed to confirm the specificity of antibody binding.

Th1 cells were defined as $CD4^+IFN\text{-}\gamma^+Il\text{-}4^-$ and Th2 cells were defined as $CD4^+IFN\text{-}\gamma^-Il\text{-}4^+$. Ratios of Th1/Th2 were generated to determine the presence of a polarized immune response. The immune response in non-infected mice was defined as unpolarized.

Serum IFN-γ, IL-4 and IL-10 measurements

The concentration of IL-4 in the sera of *C. albicans* infected mice and non-infected controls was quantified with the Quantikine® M Murine IL-4 ELISA kit and serum IFN-γ and IL-10 were quantified with the Duo-Set ELISA kits (all from R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

Statistical Analysis

Unless otherwise stated, results shown are from one experiment, representative of three independent experiments. Statistical significance of results was determined by unpaired Student t-test and survival data were analyzed with the log-rank test, using the GraphPad Prism 4 Software (GraphPad Software, Inc., La Jolla, CA, USA). Results were considered statistically significant with *P* values of less than 0.05.

RESULTS

Virulence of *C. albicans* in a murine model of hematogenously disseminated infection

Prior to the virulence studies, we have determined the generation time for each strain in synthetic defined and complex media at 30º and 37ºC, and the ability to form hyphae in serum- or N-acetylglucosamine-containing media. No yeast growth defects were observed under the conditions tested (similar generation times) and filamentous growth was similar to that of the wild-type (WT) strain SC5314 under the hypha-inducing conditions analyzed (data not shown).

To explore the role of aspartyl proteases Sap1-6 as virulence factors in the course of hematogenously disseminated candidiasis, BALB/c mice were initially infected i.v. with $5x10^5$ and $1x10^5$ cells of *C. albicans* WT strain SC5314 and two independent series of homozygous deletion triple mutant strains lacking *SAP1-3* (SAP123MS4C and SAP123MS4D) or lacking *SAP4-6* genes *(*SAP456MS4A and SAP456MS4B) (28). The independent mutant strains behaved indistinctively (data not shown) and therefore results presented below correspond to those obtained with strains SAP123MS4C and SAP456MS4A.

Mice infected with the highest inoculum showed 100% mortality for every group by day 6 after infection and no differences could be observed (data not shown).

No differences in survival time were observed after i.v. infection of BALB/c mice with $1x10^5$ CFU of the WT or *Δsap123* mutant SAP123MS4C strains (*P*= 0.5698 by log rank test) (Figure 1A). Mice infected with the *Δsap456* mutant strain SAP456MS4A had an extended overall survival time, compared to the WT infected counterparts (Fig. 1). The median survival time of SAP456MS4A-infected mice were 13 and 16 versus 10 and 12 days of WT-infected mice, as determined on the independent experiments shown in Figure 1 A, and B, respectively. All mice injected with either the WT or the triple mutant strain SAP123MS4C succumbed to candidal infection within 17 days. In contrast, at the end of the experimental period (30 days), 15 to 20% of the mice infected with the *Δsap456* mutant survived infection. Nevertheless, the kidneys of the surviving mice had *Candida* microabscesses and granulomas as revealed by histopathological analysis (data not shown). Despite these differences, statistical comparisons of the survival curves revealed that survival of mice infected with the WT

strain did not differ significantly from those of mice infected with the *Δsap456* mutant (*P*=0.1405 and *P*=0.1331, Figure 1 A and B, respectively).

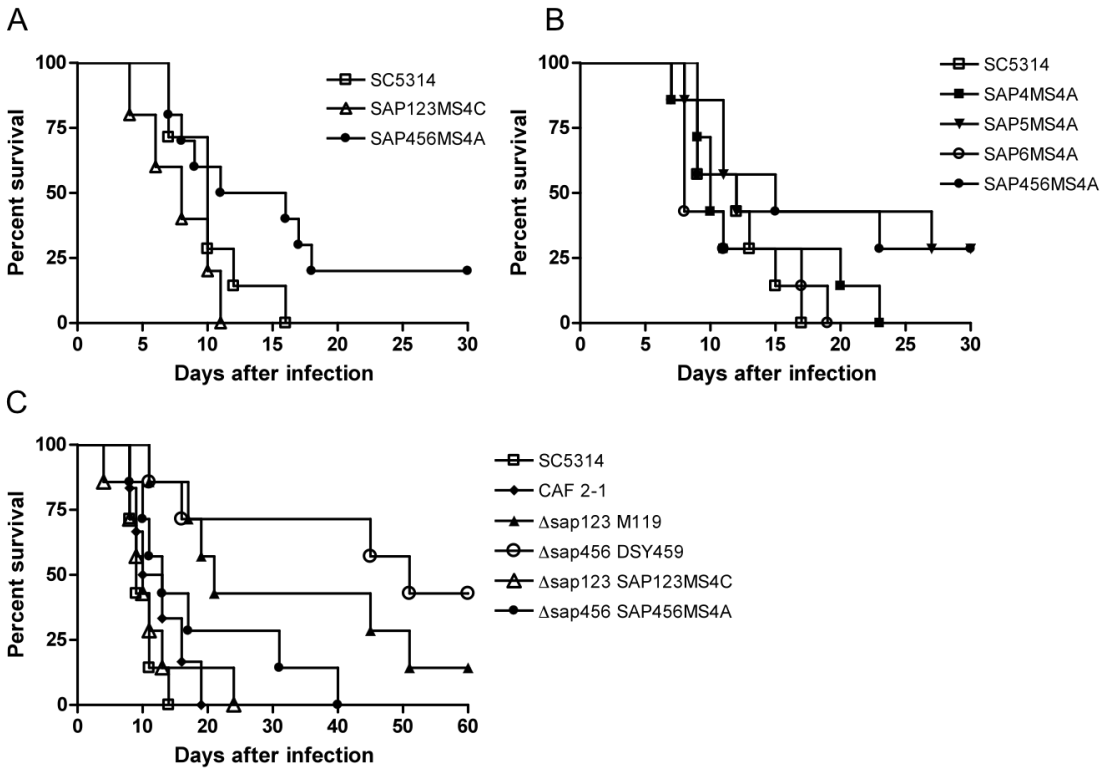

Figure 1 Influence of *SAP1-6* on *C. albicans* virulence in a murine model of hematogenously disseminated candidiasis. Male BALB/c mice were i.v. injected with $1x10^5$ cells of (A) *C. albicans* WT strain SC5314 and the mutant strains SAP123MS4C and SAP456MS4A; (B) *C. albicans* WT strain SC5314 and the mutant strains SAP4MS4A, SAP5MS4A, SAP6MS4A and SAP456MS4A; (C) *C. albicans* WT strain SC5314 and CAF2-1, and the mutant strains SAP123MS4C, M119, SAP456MS4A, and DSY459. Each strain was injected into 7 mice per group, per experiment, and survival was monitored twice daily for 30 days (A and B) or for 60 days (C). Results are representative of two to three independent experiments.

To ascertain the contribution of each of the deleted genes of the triple *Δsap456* mutant to the slightly extended survival observed, *Δsap4*, *Δsap5* and *Δsap6* single mutant strains were used. As shown in Figure 1B, survival time of mice infected with SAP4MS4A, SAP5MS4A and SAP6MS4A strains was statistically equivalent to that of WT-infected counterparts (*P*= 0.6265, *P*= 0.2121 and *P*= 0.9465, respectively). The observed median survival time was, however, consistently longer in mice infected with mutants lacking *SAP5*, such as with strains SAP5MS4A and SAP456MS4A. The survival curves of mice injected with these two strains were similar (*P*= 0.8621).

These results are in apparent contrast with previous studies, in a similar model of murine hematogenously disseminated candidiasis, using single mutant strains deficient in *SAP1*, *SAP2* or *SAP3* (19) and a triple mutant lacking the *SAP4* to *SAP6* genes (43), constructed with the Ura-blaster technique (16). In those studies, mutant strains deficient in each of the *SAP1* to *SAP3* genes and the *Δsap456* triple mutant strain DSY459, were reported to survive longer than WT-infected mice, and the latter, to have significantly decreased mean CFU in the kidneys (43). Therefore, parallel experiments with the *SAT1*-flipping mutants and Ura-blaster triple mutants *Δsap123* M119 (23) and *Δsap456* DSY459 (43) were performed using the same batch of BALB/c mice and the same experimental conditions. Strain CAF2-1 (16) was also included. As shown in Figure 1C, significant differences in survival time were observed between mice infected with strains *Δsap123* M119 and *Δsap456* DSY459, and the respective control strain CAF2-1 (P=0.0089 and P=0.0065, respectively). Mice infected with the Ura-blaster mutants survived significantly longer than mice infected with the equivalent *SAT1*-flipping mutants. The medium survival time of mice infected with mutants lacking *SAP1-3* was 21 days for the Ura-blaster constructed *Δsap123* M119, and 10 days for *Δsap123* mutant SAP123MS4C (P=0.0127). Similar results were found when comparing the triple mutants lacking *SAP4-6* genes. Medium survival time was 51 days for mice infected with the Ura-blaster mutant and 13 days with the *SAT1*-flipping mutant (P=0.0096). Survival of mice infected with the WT strain SC5314 and with the *URA3* heterozygous strain CAF2-1 was similar (P=0.7005)

The evaluation of fungal ability to invade the kidneys has been frequently used to measure virulence of *C. albicans* strains (29, 37). The number of *C. albicans* CFU in the kidneys of mice infected, either with the *SAT1*-flipping or the Ura-blaster mutants, as well as with the respective control strains was similar, except for mice infected with the *Δsap456* DSY459 strain (Figure 2). Seven days after infection, CFU numbers were significantly reduced in mice infected with the latter mutant (P=0.0149, CAF2-1 *vs* *Δsap456* DSY459).

The two sets of mutants tested behaved distinctly in the same experimental model, suggesting that the observed differences could be due to the effect of ectopic *URA3* insertion, and not caused by disruption of *SAP* genes. However, the limited impact of *SAP* gene deletion in *C. albicans* virulence could be due to a compensatory expression

of the non-deleted *SAP* genes, as reported for the Ura-blaster mutants (33; 45). Expression of *SAP1-10* in SC5314 and in *SAT1*-flipping triple mutants was evaluated by qRT-PCR in kidney samples, 3 and 7 days after infection. Only the results of the latter time-point analyzed are presented, since after 3 days the fungal burden was often insufficient to obtain reproducible results. The expression levels were always inferior or similar to *ACT1* expression In the WT strain, except for *SAP7*.

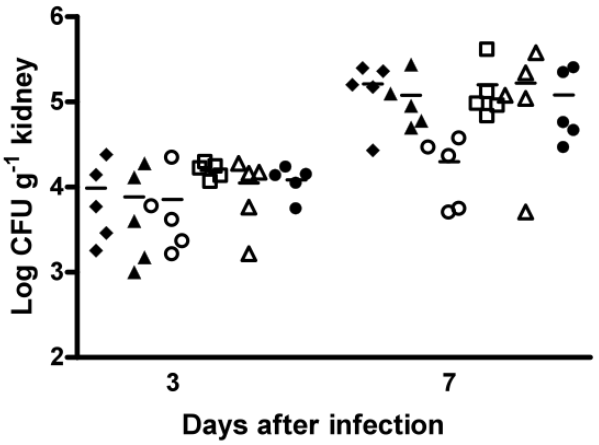

Figure 2 Kidney fungal burden of BALB/c mice 3 and 7 days after i.v. infection with $5x10^4$ CAF2-1 (◆), M119 (▲), DSY459 (○) SC5314 (□), SAP123MS4C (△), and SAP456MS4A (●) *C. albicans* cells. Data are representative of two independent experiments. Each symbol represent an individual mouse, horizontal bars are means of CFU numbers in each group.

No significant differences in *SAP1* to *SAP10* expression were observed in either mutant compared with SC5314. However, mRNA levels of *SAP4* were higher in the *Δsap123* mutant and the expression of *SAP1*, *SAP2* and *SAP3* trended higher in the *Δsap456* mutant (Figure 3). Thus, the virulence phenotypes observed do not seem to be due to significant compensatory upregulation.

Although not significant, a reduction in virulence was consistently seen in the mutant *Δsap456.* Therefore, the impact of *SAP4* to *SAP6* deficiency on *C. albicans* virulence was further evaluated.

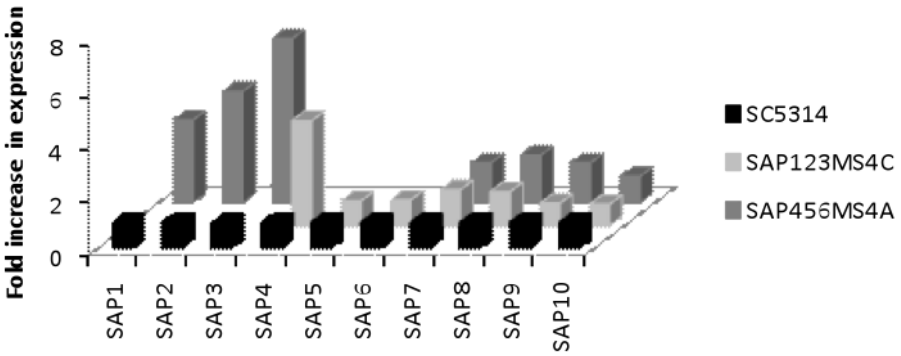

Figure 3 Compensatory upregulation of *SAP1-10* in *sap* triple mutants SAP123MS4C, and SAP456MS4A from kidney homogenates, 7 days after i.v. infection with $1x10^5$ cells. Results are presented as fold difference in expression relative to the WT SC5314 from infected mice.

The ability of WT and *Δsap456* triple mutant strains to infect and injure several organs was assessed by histopathological analysis of the kidneys, liver, lungs and brain, 3 and 7 days after infection. As shown in Figure 4, similar *C. albicans* cell morphology and invasive abilities by the WT and mutant strains were observed. The kidneys of both mice groups showed moderate multifocal renal medullary interstitial neutrophylic infiltration, with small areas of ductular necrosis. Intralesional PAS-positive organisms both in yeast and septated, branched hyphal morphology, were detected 3 days after *C. albicans* i.v. infection with strains SC5314 (Figure 4A) and SAP456MS4A (Figure 4B). At the later time-point tested, 7 days after infection, analysis of the kidneys of WT (Fig. 4C) and mutant-infected (Figure 4D) mice showed moderate to severe, focally extensive to coalescing, renal medullary interstitial neutrophylic infiltration surrounding numerous PAS-positive organisms. These were present mainly as septated, branched hyphal structures, which largely effaced the medulla and invaded the urothelium. Invasion of liver, lungs and brain was not consistently seen (data not shown). Altogether, these results suggest that *SAP4* to *SAP6* genes are not essential for invasion of the kidneys during hematogenously disseminated candidiasis.

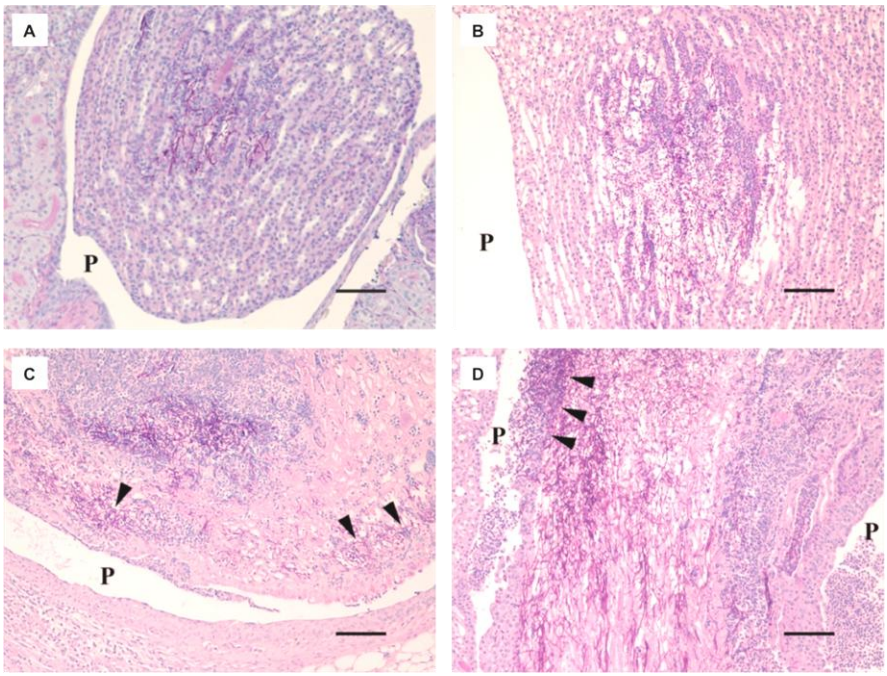

Figure 4. Representative photomicrographs of histological sections of kidneys from BALB/c mice infected with $5x10^4$ SC5314 (A and C) and SAP456MS4A (B and D) *C. albicans* cells. Three days post i.v. infection, PAS-positive hyphae and yeast-like organisms were present in the renal medulla, partially effacing the renal tubuli and eliciting moderate neutrophylic infiltration both in WT- (A) and mutant-infected mice (B). 7 days post i.v. infection, numerous PAS-positive hyphae and yeast-like organisms were present in the renal medulla, extensively effacing the renal tubuli, eliciting intense neutrophylic infiltration and invading the urothelium (arrows) with no clear differences between WT (C) and mutant (D) strains. P-renal pelvis. Bar=100 µm.

Host immune response to hematogenously disseminated candidiasis

To determine the effect of *SAP4* to *SAP6* disruption on the immune response elicited by *C. albicans* systemic infection, BALB/c mice were infected i.v. with 5×10^4 *C. albicans* yeast cells of WT and *Δsap456* mutant strains. At days 3 and 7 upon infection, absolute numbers and phenotype of different splenic leukocyte populations were quantitated by flow cytometric analysis. Macrophages and neutrophils, represent the first line of host immune defence when *C. albicans* cells infect the bloodstream or the endothelia (47, 58). Macrophages typically express the F4/80 cell surface marker, whereas neutrophils have a Gr-1^{high} surface phenotype. Murine splenic cells expressing both antigens with either inflammatory or immunosuppressive function have also been described in the context of *C. albicans* infections (30, 56). According to the

expression of these two surface markers, three cell populations were analyzed in this study: F4/80^{high}Gr-1^{neg}, F4/80^{high}Gr-1^{high} and F4/80$^{neg/low}$Gr-1^{high}.

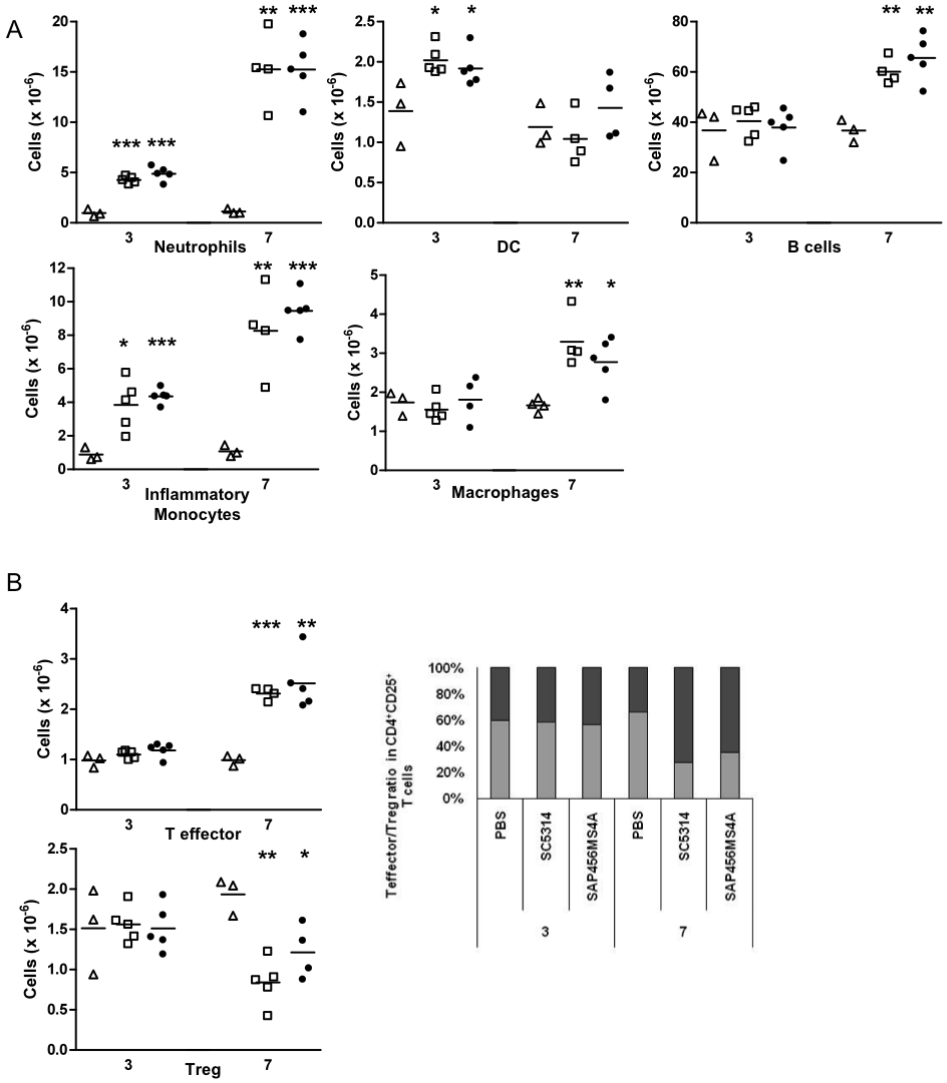

Figure 5 (A) Scatter plots of the total numbers of neutrophils (F4/80$^{neg/low}$Gr-1^{high}), inflammatory monocytes (F4/80^{high}Gr-1^{high}), macrophages (F4/80^{high}Gr-1^{neg}), cDC (CD11c^{high}), B cells (B220^{+}), Treg (CD4^{+}CD25^{+}Foxp3^{+}), and Teffector (CD4^{+}CD25^{+}Foxp3^{-}) as indicated, observed 3 and 7 days after infection in the spleens of non-infected control mice (open triangles) and mice challenged i.v. with 5x10^{4} WT (open squares) and SAP456MS4A (filled circles) *C. albicans* cells. Data are representative of three independent experiments. Each symbol represents an individual mouse, horizontal bars are means of cell numbers in each group (n=3 for control and n=4 to 5 for infected mice groups). Statistically significant differences between controls and *C. albicans* infected mice are indicated *P<0.05; ** P<0.01; *** P<0.001. (B) Relative proportions of splenic Teffector (black bars) and Treg (white bars) in the spleens of non-infected controls (PBS) and SC5314 or SAP456MS4A i.v. infected mice, 3 and 7 days after challenge.

These were respectively designated as macrophages, inflammatory monocytes and neutrophils (55). An extensive recruitment of neutrophils and inflammatory monocytes into the spleen could be observed in infected mice compared to non-infected controls, 3 and, more markedly, 7 days after infection. Higher numbers of spleen macrophages were also detected in the infected mice 7 days upon infection. The total numbers of these myeloid cell populations in WT-infected mice were equivalent to the ones in mice infected with the *Δsap456* triple mutant (Figure 5A).

Dendritic cells were previously shown to play a major role in the induction of the cell-mediated immune response to *C. albicans* infection (9, 38) and to directly influence the infection outcome (6). Therefore, the numbers and surface maturation markers of splenic conventional dendritic cells (cDC), defined as $CD11c^{high}$ cells, were assessed upon *C. albicans* infection. Higher numbers of splenic cDC, comparatively to non-infected controls, were observed at the earlier time-point analyzed. The cDC surface expression of the co-stimulatory molecules CD40 and CD80, remained practically unchanged after *C. albicans* i.v. infection, as evaluated by flow cytometry and recorded as Mean Fluorescence Intensities (MFI). In contrast, the co-stimulatory molecule CD86 was upregulated on the surface of splenic cDC from *C. albicans*-infected mice, 3 and 7 days after infection. The cDC surface expression of MHC class II molecules was slightly down-regulated in infected mice 3 days after infection, and upregulated at day 7 post-infection, as compared to that of non-infected controls. No significant differences on cDC numbers (Figure5A) and surface expression of any of the assessed co-stimulatory and antigen-presenting molecules was detected between WT and SAP456MS4A-infected mice at the two tested time-points (Figure 6).

B cells have been shown to mediate host resistance to i.v. established *C. albicans* systemic infection (60). B cell numbers were significantly increased 7 days after infection in either WT or SAP456MS4A challenged mice (Figure 5A). As observed on splenic cDC, an up-regulation of the co-stimulatory molecule CD86 was observed 3 and 7 days post-infection on the surface of B-cells of the infected mice, compared to non-infected controls. The expression of CD80 and MHC class II molecules on the surface of B cells was observed up-regulated only at day 7 after challenge.

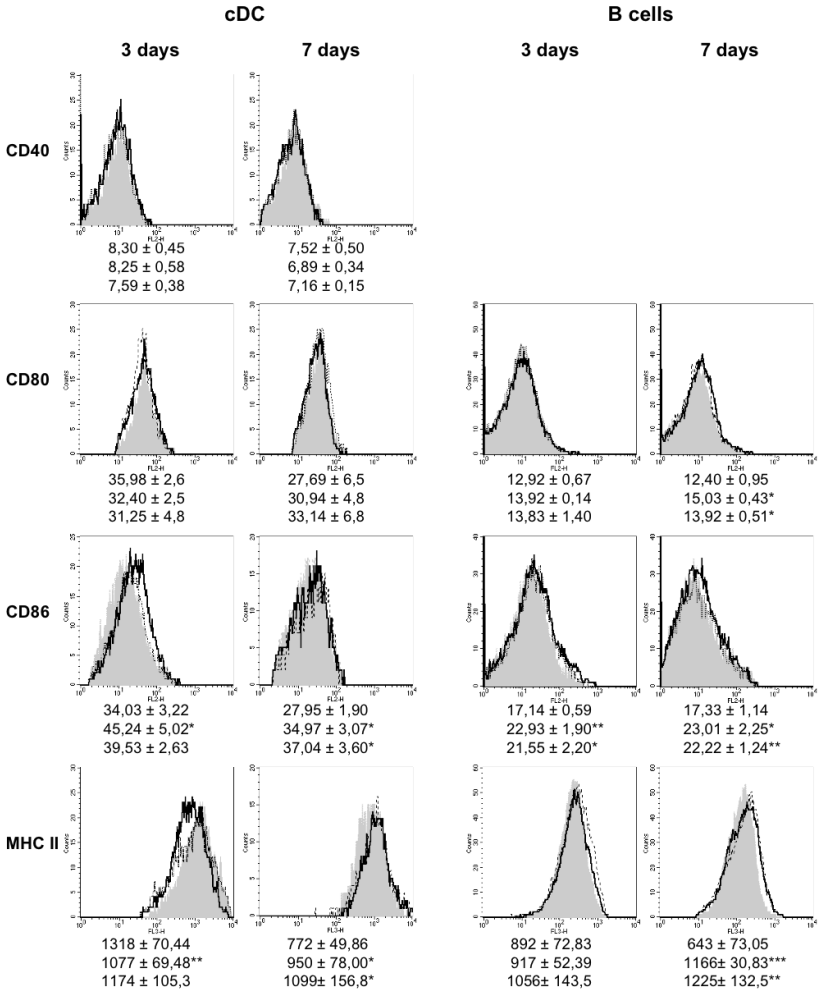

Figure 6 Expression of CD40, CD80, CD86, and MHC II molecules on the surface of spleen conventional dendritic cells (cDC) and B cells of BALB/c mice 3 and 7 days after i.v. injection with PBS (controls, grey histograms) or i.v. infection with 5×10^4 SC5314 (solid line) and SAP456MS4A (dashed line) *C. albicans* yeast cells. Staining with respective isotypic controls was omitted for simplicity. Numbers below histograms represent means ± one standard deviation of the mean fluorescence intensities of antibody stainings for non-infected controls (up) and SC5314 (middle) or SAP456MS4A (down) infected mice. (n=3 for control and n=5 for infected mice groups). Statistically significant differences between controls and *C. albicans* infected mice are indicated (* P<0.05; ** P<0.01). Results shown are from one experiment, representative of three independent experiments.

Challenge with the *SAP4* to *SAP6* deficient mutant did not result in any differences to the WT strain, regarding either B cell numbers or co-stimulatory molecules expression (Figures 5A and 6).

Although $CD4^+$ T cells have been reported to have little influence on survival and on fungal burden during acute systemic candidiasis (3, 23), the $CD4^+$ T cell subset of

naturally occurring regulatory T cells (Treg) has been shown to promote host susceptibility to *C. albicans* (35, 53), to limit tissue damage and/or enhance healing, but not to directly augment clearance of the organism from infected tissues (31). To ascertain the impact of *SAP4* to *SAP6* deficiency on the immune response mediated by $CD4^+$ T lymphocyte cell populations, the numbers of $CD4^+$, $CD4^+CD25^+$, $CD4^+CD25^+Foxp3^+$ (Treg) cells and also of $CD4^+CD25^+Foxp3^-$ (Teffector) cells were assessed in the spleens of the infected BALB/c mice. The numbers of splenic $CD4^+$ and $CD4^+CD25^+$ T cells of infected mice were not significantly different from the ones of non-infected controls (data not shown). However, as assessed by Foxp3 expression within the $CD4^+CD25^+$ T cell population, reduced percentages and numbers of Treg were observed 7 days after *C. albicans* infection. This correlated with higher splenic percentage and numbers of Teffector cells in these mice (Figure 5A), resulting in higher Teffector/Treg ratios compared to non-infected controls (Figure 5B). The percentage and numbers of both Teffector and Treg cells were not significantly different between the two *C. albicans*-infected groups.

To better elucidate the effector function of the $CD4^+$ T cells from WT and SAP456MS4A-infected mice, the proportion of splenic $CD4^+$ T cells producing IFN-γ, IL-4 and IL-10 was determined by intracytoplasmatic cytokine staining analysis. An increased frequency of $CD4^+$ T cells expressing IFN-γ or IL-4 was observed in *C. albicans*-infected mice, comparatively to the non-infected controls. Although the frequency of cells producing either cytokine increased upon infection in the spleens of *C. albicans*-challenged mice, a bias towards a Th1 type response was observed (high IFN-γ/IL-4 ratio). The frequency of $CD4^+$ T cells expressing IL-10 was also increased in the spleens of infected mice (Figure 7). The intracellular expression of IFN-γ and IL-4 was also evaluated in kidneys $CD4^+$ T cells as it associates with the outcome of infection (48). As observed in the spleen, the frequency of $CD4^+$ T cells expressing IFN-γ and IL-4 cytokines increased in infected mice. Although a trend for extended survival was observed in mice infected with the mutant strain SAP456MS4A, the percentage of $CD4^+$ T cells producing the cytokines IFN-γ or IL-4 in the kidneys of either group of infected mice were similar, resulting in equivalent Th1/Th2 cell ratios (Figure 7). No serum IFN-γ, IL-4 and IL-10 were detected by ELISA, 3 and 7 days after infection, either in infected or uninfected mice (data not shown).

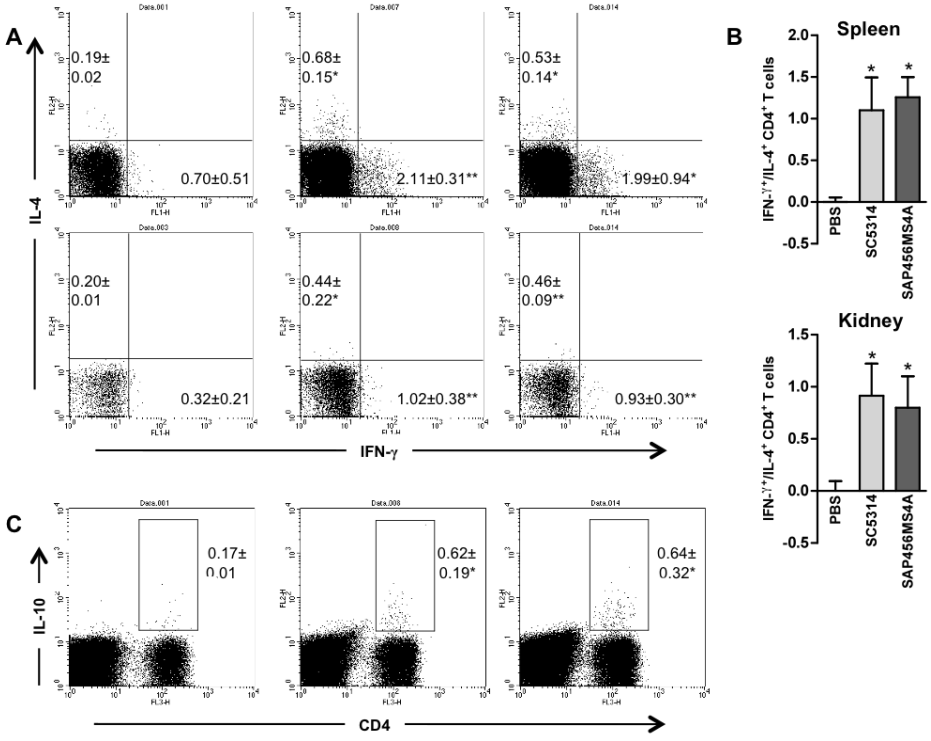

Figure 7 Cytokine production in the spleen and kidneys of BALB/c mice infected i.v. with 5×10^4 SC5314 and SAP456MS4A *C. albicans* cells. (A) Representative examples of flow cytometric analysis of intracellular IFN-γ and IL-4 expression on gated splenic and renal $CD4^+$ T cells, as indicated. Numbers inside dot plot regions represent means ± one standard deviation of the frequency of IFN-γ^+ or IL-4^+ $CD4^+$ T cells. (B) IFN-γ^+/IL-4^+ $CD4^+$ T cell ratio, normalized considering the ratio of non-infected controls as basal (zero value). (C) Dot plots showing the percentage of splenic $CD4^+$ T cells producing IL-10. Numbers inside dot plots represent means ± one standard deviation of the frequency of IL-10^+ $CD4^+$ T cells. Data are representative of two independent experiments (n=4 for non-infected control and n=7 for infected mice groups). Statistically significant differences between controls and *C. albicans* infected mice are indicated *P<0.05; ** P<0.01.

Overall, these results indicate that *SAP4* to *SAP6* deficiency do not have a significant impact on the immune response elicited in the spleen and kidneys of BALB/c mice hematogenously challenged with *C. albicans*.

DISCUSSION

The secretion of aspartyl proteases has long been recognized as a virulence-associated trait of *Candida albicans* (10, 26, 49). The importance of specific Sap isoenzymes for the pathogenicity of this fungus has been investigated in different infection models by comparing the virulence of mutants deficient in individual or multiple *SAP* genes with that of a WT control strain. In this study, the importance of *SAP1* to *SAP6* gene expression for *C. albicans* virulence was evaluated by using *sap* null mutants derived from the WT strain SC5314. Virulence of mutant strains lacking *SAP1* to *SAP3* was indistinguishable from that of the WT SC5314 strain, while *SAP4* to *SAP6* deletion caused a slight attenuation in virulence. Previous reports have shown that deficiency in *SAP4* to *SAP6* attenuated virulence to a higher extent than deficiency in *SAP1*, *SAP2* or *SAP3* (19, 43). Here, an increased median survival time was consistently observed in mice infected with mutants lacking the *SAP*5 gene, such as the single SAP5MS4A or the triple SAP456MS4A deletion mutant strains, comparatively to that of animals infected with the WT SC5314 strain. However, the differences found were narrow and not always significant. Moreover, histopathology analysis did not indicate a reduced ability of the SAP456MS4A mutant to invade the kidneys, though *sap* null mutant strains, lacking *SAP6* gene, were previously shown to have reduced invasiveness in a model of experimental peritonitis (15). Deletion of *SAP4* to *SAP6* did not result in clear differences in hypha formation and similar morphotypes were observed, both *in vitro* and *in vivo,* for WT and mutant strains. This is not unexpected, as *SAP4* to *SAP6* expression is associated with, but not required for hyphal morphology (15). Additionally, expression levels of *SAP4* to *SAP6* may not be directly linked to organ invasion, since a *C. albicans* strain expressing high levels of *SAP4* to *SAP6* was non-invasive (57).

The results obtained with *SAT1*-flipping mutants contrast with the ones obtained when using the Ura-blaster *sap* null mutants, which survived much longer. When analyzing another parameter associated with *C. albicans* virulence, such as kidneys CFU, no differences were observed among mice groups, except for the ones infected with strain DSY459, which presented a lower fungal burden. Discrepancy between different

methods of evaluating virulence has already been reported in mice intravenously infected with *C. albicans* mutant strains, including *sap* null mutants, where differences in mice survival were not associated with differences in organ fungal burden (19, 54, 62). The differences found between the two sets of mutants are most likely due to the ectopic insertion of *URA3,* which must have contributed to the reduced virulence of the Ura-blaster constructed mutant strains. It is widely known that the Ura-status of *C. albicans* strains influences adherence (5) and virulence (27, 52). Although this can be overcome by integration of *URA3* at the *ENO1* (52) or *RPS10* loci (8), the strains used in this study and in previous reported studies (19, 43) did not share a common site of *URA3* integration.

The disruption of *SAP1-3* and *SAP4-6* led to an increased expression of *SAP4* and *SAP1-3*, respectively, suggesting that *C. albicans* attempts to compensate the functional loss of these subfamilies by upregulating alternative *SAP* genes during hematogenously disseminated candidiasis. Therefore, the compensatory upregulation observed could be, in some extent, contributing to the lack of phenotype seen in these mutants. However, the equivalent Ura-blaster mutants, despite the compensatory upregulation reported (33, 45) showed a markedly reduced virulence in this model

Recently, Lermann and Morschhäuser (28) and Naglik *et al*. (33) have reevaluated the role of *SAP1-6* in a model of reconstituted human epithelia (RHE) and reported that *SAP1-6* were not essential for successful *C. albicans* RHE infection, in contrast to earlier reports (45, 46). The present study thus reports an additional model in which the *SAP* gene subfamilies *SAP1-3* and *SAP4-6* seem to have little influence on the outcome of infection.

As mice infected with the *Δsap456* triple mutant displayed a slightly extended survival time, it could be expected that it might result from a more effective host immune response. This would be in agreement with a previous report suggesting an immunomodulatory role of Sap4-6 upon macrophage phagocytosis (7). However, the analysis of diverse features of the innate and acquired immune response elicited in BALB/c mice upon infection with either the WT or the *Δsap456* triple mutant did not show any significant differences between these two yeast strains. The similar ability of both strains to recruit inflammatory cells is in accordance with their similar observed

virulence, taking into account the prominent role of innate immunity and of neutrophils in particular, in host protection against disseminated candidiasis (2, 60).

The proportion of splenic Teffector and Treg cells was highly similar in the spleens of mice infected with either the WT or the Δ*sap456* mutant. Likewise, the frequencies of $CD4^+$ T cells expressing IFN-γ, IL-4 and IL-10, cytokines previously shown to be relevant for resistance or susceptibility to systemic candididasis (48), were similar in the two infected mice groups. *In vivo* models indicate that regulatory T cells attenuate Th1-type antifungal responses and induce tolerance to the fungus (31, 35). As higher IFN-γ/IL-4 ratios were observed in splenic and renal $CD4^+$ T cells of infected mice than in non-infected controls, it can be assumed that even though the kidneys of infected mice presented high fungal burden 7 days after challenge, a protective Th1-type response of equivalent magnitude might be occurring both in WT and *Δsap456* mutant infected mice.

Although our results suggest that B cells may have a role in the activation of T cells during experimental disseminated candidiasis, in accordance with the increased susceptibility observed in B cell-deficient mice (61), they also indicate that *SAP4* to *SAP6* deficiency does not affect such a role of B cells.

Differences in *C. albicans* morphology have been frequently shown to influence both type and magnitude of the host immune response in the course of candidiasis. Dendritic cells, and also neutrophils, modulate adaptative responses to the fungus depending on the *Candida* morphotype encountered (38, 42, 47). As indistinguishable morphotypes were found for WT and *Δsap456* mutant both *in vitro* and *in vivo*, this is also in agreement with the lack of significant differences observed in the immune response elicited by these strains.

A relative independence on aspartyl protease activity for the establishment of hematogenously disseminated candidiasis was previously reported (14), as treatment with pepstatin A, a potent protease inhibitor, did not protect mice against intravenous infection with *C. albicans*. As previously suggested, an explanation for these observations may be the requirement for Sap only where anatomical barriers had to be crossed prior to dissemination (14, 25). When *C. albicans* cells are delivered directly into the bloodstream, low molecular weight peptides are available and yeast growth may be protease independent.

The relative importance of specific *SAP* genes for *C. albicans* pathogenicity is highly determined by the type of infection and its dependence on protease activity for successful invasion and colonization of various host niches. Treatment with pepstatin A resulted in reduced virulence in intranasal (14) and intraperitoneal (25) models, but had no protective effect in the intravenous model. The subfamily of *SAP* genes *SAP1* to *SAP3*, *SAP2* in particular, was proved important in a model of rat vaginal infection, while *SAP4* to *SAP6* had little impact on this infection model (12). On the opposite, only *Δsap456* mutants, *Δsap6* in particular, showed reduced virulence in a murine model of *Candida* peritonitis and keratitis, while deletion of *SAP1*, *SAP2* or *SAP3* genes had no significant effect on these infection models (15, 22). Moreover, immunological neutralization of Sap2 was shown to have a protective effect in *C. albicans* infected hosts during vaginal and oral infection (12, 40), and also in experimental peritonitis (59).

Though individual processes resulting from the action of a single or small group of genes may be important in specific stages of infection, cooperative gene functions are essential for the multiple processes of *C. albicans* infection (37). Thus, although the protease family as a whole may contribute to *C. albicans* virulence in the course of acute systemic candidiasis, other factors must be the major contributors to invasion and cell damage in this model.

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CHAPTER 4

T cell function is affected by *Candida albicans* secreted aspartyl protease expression in murine peritonitis

T cell function is affected by *Candida albicans* secreted aspartyl protease expression in murine peritonitis

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ABSTRACT

Candida albicans secreted aspartyl proteases (Saps) have been considered virulence-associated factors. Here, we assessed the importance of *SAP1* to *SAP6* expression in the immune response induced in mice intraperitoneally infected with the wild-type (WT) strain SC5314 and *SAT1*-flipping mutants Δ*sap123* and Δ*sap456*. WT-infected mice presented higher proportions of T regulatory cells ($Foxp3^+$) in the spleen and mesenteric lymph nodes than control and *sap*-null mutant-infected counterparts. In addition, $CD4^+CD25^+$ T cells of WT-infected mice were the most effective in suppressing the proliferative response of $CD4^+CD25^-$ T cells whereas those of Δ*sap456*-infected mice were the least suppressive. Moreover, $CD4^+$ T cells of WT-infected mice were the ones producing the highest levels of IL-10. Interestingly, Δ*sap456*-infected mice presented less $Foxp3^+$ cells in kidney lesions and lower kidney *C. albicans* CFU than the other infected mice. Altogether, these results implicate Sap expression in the modulation of the host immune response to *C. albicans*.

INTRODUCTION

The opportunistic yeast *Candida albicans* is an important human pathogen causative of superficial to deep-seated infections. It represents the most frequently isolated species in *Candida* peritonitis, an infection with current increasing incidence and high associated mortality rates (1, 2).

Different T helper (Th) cell subsets, which include Th1, Th2, Th17, and T regulatory cells (Treg) have been shown to influence the outcome of *C. albicans* infections (3-7). Treg play an important role in controlling local and systemic immune responses during fungal infections (4, 8, 9). These cells, classically defined as $CD4^+CD25^+Foxp3^+$ T cells (10), can respond to self-antigens and control autoimmunity (11). Yet, they may also recognize antigens expressed by different pathogens (12-16), playing either a host protective or detrimental role in the immune response to infectious agents (16). This differential role depends on the pathogen and on the nature of the infection (16). A host protective role of Treg was shown in murine gastrointestinal (4) and oral (7) models of candidiasis. Conversely, a host deleterious role of Treg was shown in murine hematogenously disseminated candidiasis, in which depletion of this T cell subset resulted in extended survival and lower fungal burden (5).

C. albicans displays a number of virulence factors, among which the ten-member family of secreted aspartyl proteases (Saps) has been included (17, 18). Sap isoenzymes are differentially expressed during infection (19, 20) and may affect its outcome (21-24). The hypha-associated Sap4-6 were considered important for effective organ invasion and damage in experimental *Candida* peritonitis, while Sap1-3 did not seem to have such a determinant role (21, 23). Several functions have been attributed to Saps, from adhesion to nutrient acquisition, tissue degradation and immune evasion (25). Although not yet demonstrated, an immunomodulatory role has also been suggested for some of these proteases (26-29). In this study, the effect of *SAP1* to *SAP6* expression on the host immune response to experimental *C. albicans* peritonitis, was examined after intraperitoneal infection with wild-type (WT) strain SC5314 or with *SAT-1* flipping constructed mutant strains lacking *SAP1 to SAP3* or *SAP4 to SAP6* (30).

MATERIALS AND METHODS

Mice

Male BALB/c mice, 8-10 weeks old (Charles River, Barcelona, Spain) were kept under specific pathogen-free conditions at the animal facilities of Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal. Procedures were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123), 86/609/EEC Directive and Portuguese law (DL 129/92). Authorization to perform the experiments was issued by the competent national board authority, Direcção Geral de Veterinária (0420/000/000/2010).

Candida albicans and culture conditions

The *C. albicans* strains used in this study were the WT strain SC5314 (31) and its *SAT1*-flipping-derived triple mutants lacking *SAP1-3* genes, SAP123MS4C and SAP123MS4D (sap1 Δ : : FRT/sap1 Δ : : FRT; sap2-1 Δ : : FRT/sap2-2 Δ : : FRT;sap3 Δ : : FRT/sap3 Δ : : FRT) or lacking *SAP4-6* genes, SAP456MS4A and SAP456MS4B (sap4-1Δ : : FRT/sap4-2 Δ : : FRT; sap5-1 Δ : : FRT/sap5-2 Δ : : FRT; sap6-1 Δ : : FRT/sap6-2 Δ : : FRT) (30). Inocula were prepared at the appropriate concentrations as previously described (32).

C. albicans infections

Mice were injected intraperitoneally (i.p.) with 1×10^7 *C. albicans* yeast cells and sacrificed 2, 6, 12 and 72 h or 7 days after infection. Peritoneal exudates were collected upon injection of 2 or 5 mL of PBS for chemokine or inflammatory cell counts, respectively. Control mice were injected with PBS alone. Kidneys and liver were aseptically removed, weighted, homogenized, and quantitatively cultured on Sabouraud dextrose agar at 37ºC. Values were expressed as log colony-forming units (cfu) per gram of tissue. Alternatively, histopathology of kidneys, liver, lungs, heart and brain was evaluated as described (32).

Immunohistochemistry

Immunohistochemistry was used to assess the number and frequency of $Foxp3^+$ cells in kidneys and liver formalin-fixed, paraffin-embedded 4 µm sections mounted on amino-

propyl-tri-ethoxy-silane (Sigma-Aldrich) coated slides. Immunostaining was performed as previously described (33) with modifications. Namely, antigen retrieval was done by incubating the slides in 10mM citrate buffer (pH 6) for 3 min in a pressure cooker before endogenous peroxidase blocking. Normal rabbit serum (Dako) (1:5), unlabeled rat anti mouse/rat Foxp3 mAb (FJK-16s, eBioscience) (1:100), and biotin-labelled rabbit anti-rat antibody (Dako) (1:200) were used. Slides were evaluated under light microscopy. Numbers of $Foxp3^+$ T cells were registered and the frequency was calculated relatively to the respective lesion area.

Flow cytometric analysis

Spleens and mesenteric lymph nodes (MLN) were aseptically removed and homogenized in Hank's Balanced Salt Solution (Sigma). Anti-mouse mAb of the indicated specificities and fluorophore conjugates and clones (within brackets), or respective isotype controls were used for immunofluorescence cytometric analysis in a FACScan (Becton-Dickinson) using CELLQUEST software (Becton-Dickinson): CD40 (PE; 1C10), Major Histocompatibility Complex (MHC) class II (Biotin; NIMR-4) (Southern Biotechnology Associates); Foxp3 (FITC; FJK-16s), CD4 (PE-Cy5; RM4-5), and F4/80 (PE; BM8) (eBioscience); CD11c (FITC; HL3), Ly-6G and Ly-6C (Gr-1) (FITC; RB6-8C5), CD25 (PE; PC61), CD45R/B220 (FITC; RA3-6B2), CD80 (PE; 16-10A1), CD86 (PE; GL1), IL-4 (PE; BVD4-1D11), IFN-γ (FITC; XMG1.2), IL-17A (PE; TC11-18H10) and IL-10 (PE; JES5-2A5). Biotin conjugated mAb were revealed with Streptavidin-PE-Cy5 (all from BD Pharmingen). Staining for CD11c, Foxp3 or cytokines IFN-γ, IL-4, IL-17A and IL-10 was performed as described elsewhere (32).

Cell cultures and suppression assays

Mononuclear spleen cells to be used as antigen presenting cells (APC) were prepared from naïve mice as previously described (32). Collected cells were irradiated at 3000 rad in a Gammacell 1000 Elite irradiator (Nordion International). $CD4^+$, $CD4^+CD25^-$ and $CD4^+CD25^+$ cells from control and 3-day-infected mice were isolated from pooled spleen cells of four mice per group, by using a magnetic cell sorting $CD4^+CD25^+$ T-cell isolation kit (Miltenyi Biotech) following manufacturer's instructions. Sorted cells were

plated at 2.5 × 10^4/well in U-shape 96-well plates together with 10^5 APC without stimulus or stimulated with 1 µg/mL anti-CD3 mAb (145.2C11, BD Pharmingen). Cultures were set in sextuplicates and maintained for 72 h at 37ºC and 5% CO_2. The CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes, Invitrogen) was used for naive $CD4^+CD25^-$ T cell (responder cells) labelling, at a final concentration of 5 µM CFSE (5-(and-6)-carboxyfluorescein diacetate succinimidyl ester), according to manufacturer's instructions. Responder cells were plated at 2.5 × 10^4/well in U-shape 96-well plates together with 10^5 APC and 1µg/mL anti-CD3 mAb. To evaluate Treg suppressive function, $CD4^+CD25^+$ T cells from the different groups were added at different $CD4^+CD25^+$:responder T cell ratios (1:1, 0.5:1, 0.25:1 and 0.1:1). Responder cells without anti-CD3 stimulus were used as negative controls. Stimulated responder cells with no suppressor populations added were used as positive controls. Unlabelled stimulated responder cells were used to define cell auto-fluorescence. Stimulated responder cells co-cultured with variable ratios of $CD4^+CD25^-$ T cells were used to exclude suppression due to cell number/well. Cultures were set in sextuplicates/condition and maintained for 72h at 37ºC and 5% CO_2. Proliferation/suppression was determined based on CFSE fluorescence by flow cytometric analysis.

Cytokine measurements

The concentration of IL-4 in culture supernatants of $CD4^+$, $CD4^+CD25^-$ and $CD4^+CD25^+$ T lymphocytes from infected mice and controls was quantified with the Quantikine® M Murine IL-4 ELISA kit (R&D Systems) and IFN-γ, IL-10 and IL-17A were quantified with the Mouse IFN-γ, IL-10 and IL-17A (homodimer) ELISA Ready-Set-Go!® kits (all from eBioscience), according to manufacturer's instructions. The concentration of IL-10 in liver and kidney homogenates was similarly determined. Liver and kidneys were homogenized in 4 mL and 2 mL of PBS, respectively, and the homogenates were centrifuged and 0.45 mm pore-size filtered before use. The concentration of KC, MIP-2 and MCP-1 chemokines in peritoneal cavity lavage fluids was evaluated by using the respective ELISA Duo-Set® kits (R&D Systems), according to manufacturer's instructions.

Statistical Analysis

Statistical significance of results was determined by one-way ANOVA and *post-hoc* Bonferroni's multiple comparison test, by using the GraphPad Prism 4 Software (GraphPad Software). Results were considered statistically significant with *P* values of less than 0.05.

RESULTS

Limited effect of *SAP* expression on the inflammatory response to *C. albicans* i.p. infection

Expression of *SAP4-6*, has been implicated in the success of *C. albicans* experimental peritonitis (21, 23). However, how these genes affect the host immune response in this model is not known. Recruitment of the different leukocyte populations into the peritoneal cavity was assessed in BALB/c mice challenged i.p. with *C. albicans* WT or mutant strains lacking *SAP1* to *SAP3*, or *SAP4* to SAP6. Two independently generated mutant strains for each *SAP* subset were used to exclude unspecific effects resultant from gene disruption. The independent mutant strains behaved indistinctively (data not shown) and therefore, results presented below correspond to those obtained with Δ*sap123*, and Δ*sap456* strains SAP123MS4C and SAP456MS4A, respectively. Among all infected mice groups, similar frequencies and numbers of neutrophils, eosinophils, macrophages and inflammatory monocytes, respectively defined by the Gr-1^{hi}, F4/80^{+}Gr-1^{int}SSC^{hi}, F4/80^{hi} and Gr-1^{+}F4/80^{+} phenotypic characteristics (34), as well as of dendritic cells (CD11c^{hi}), were observed 6 h after fungal challenge (Supplementary Table 1). In agreement, among infected mice groups, no significant differences were observed in KC, MIP-2 and MCP-1 chemokine levels in the peritoneal lavage fluids collected 2 h upon infection that remained not different 12 h upon challenge (Supplementary Table 2). In contrast, 72 h upon infection, strains lacking *SAP4* to *SAP6* induced a significantly higher recruitment of inflammatory monocytes (Figure 1). No other significant differences were observed among the other analysed cell populations at this time point (Supplementary Table 1). These results, by showing that lack of *SAP4* to *SAP6* expression allows a higher recruitment of inflammatory monocytes, suggest that Sap4-6 impair the early inflammatory response to *C. albicans*.

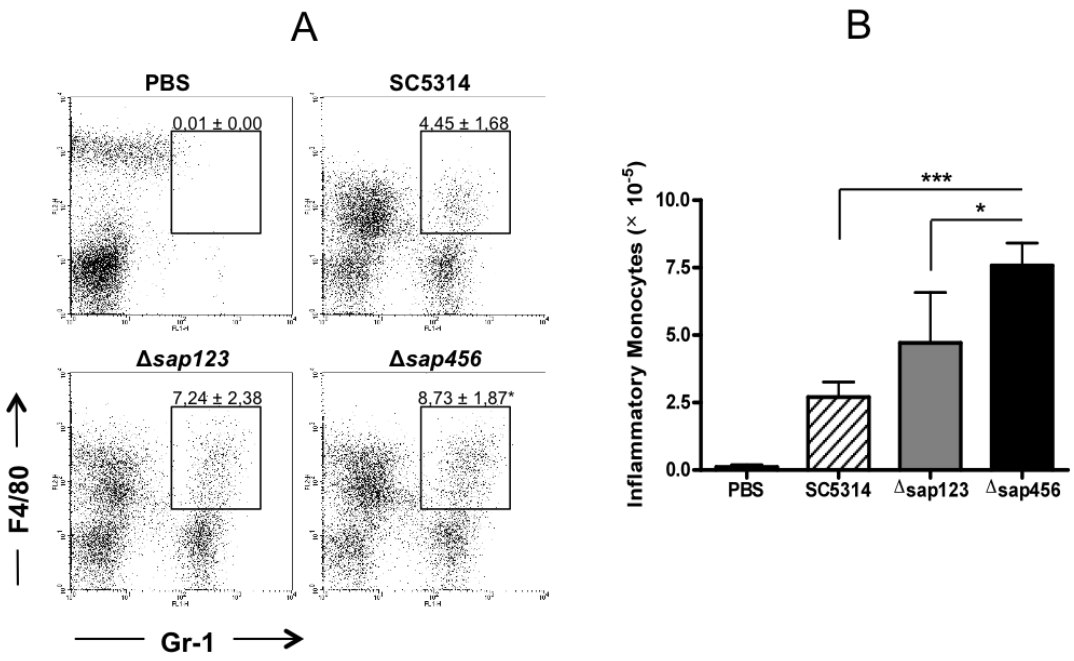

Figure 1 (A) Flow cytometry analysis of F4/80 and GR-1 expression on the surface of BALB/c mice peritoneal exsudate cells 72h upon i.p. challenge with PBS or WT, Δ*sap123* or Δ*sap456 C. albicans* strains, as indicated. Numbers within dot plots correspond to the mean ± one standard deviation of the frequency of the gated population, corresponding to inflammatory monocytes. Statistically significant difference between WT and Δ*sap456* infected mice is indicated (* P<0.05) (B) Number of inflammatory monocytes recruited into the peritoneal cavity 72 h post infection with WT, Δ*sap123* or Δ*sap456* or treatment with PBS alone, as indicated (n=4). Statistically significant differences among *C. albicans* infected mice are indicated (* P<0.05; *** P<0.001). Results shown are from one experiment representative of three independent experiments.

Higher frequency and numbers of effector T cells in Δ*sap456*-infected mice

Given the reported importance of $CD4^+$ T cells in host defense against candidiasis, splenic and MLN $CD4^+$ T cells were analysed in mice infected i.p. with the WT or mutant strains. Interestingly, Treg ($Foxp3^+$) frequency, in both $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets, was found reduced in the spleen and MLN of Δ*sap456*-infected mice, as compared with the WT-infected counterparts, 3 days after infection (Figure 2). Moreover, the mean fluorescence intensity (MFI) of Foxp3 staining in both cell subsets was also lower in Δ*sap456*- than in WT-infected mice (Figure 2). Similar frequencies of CD25-expressing $CD4^+$ T cells were found among the infected groups.

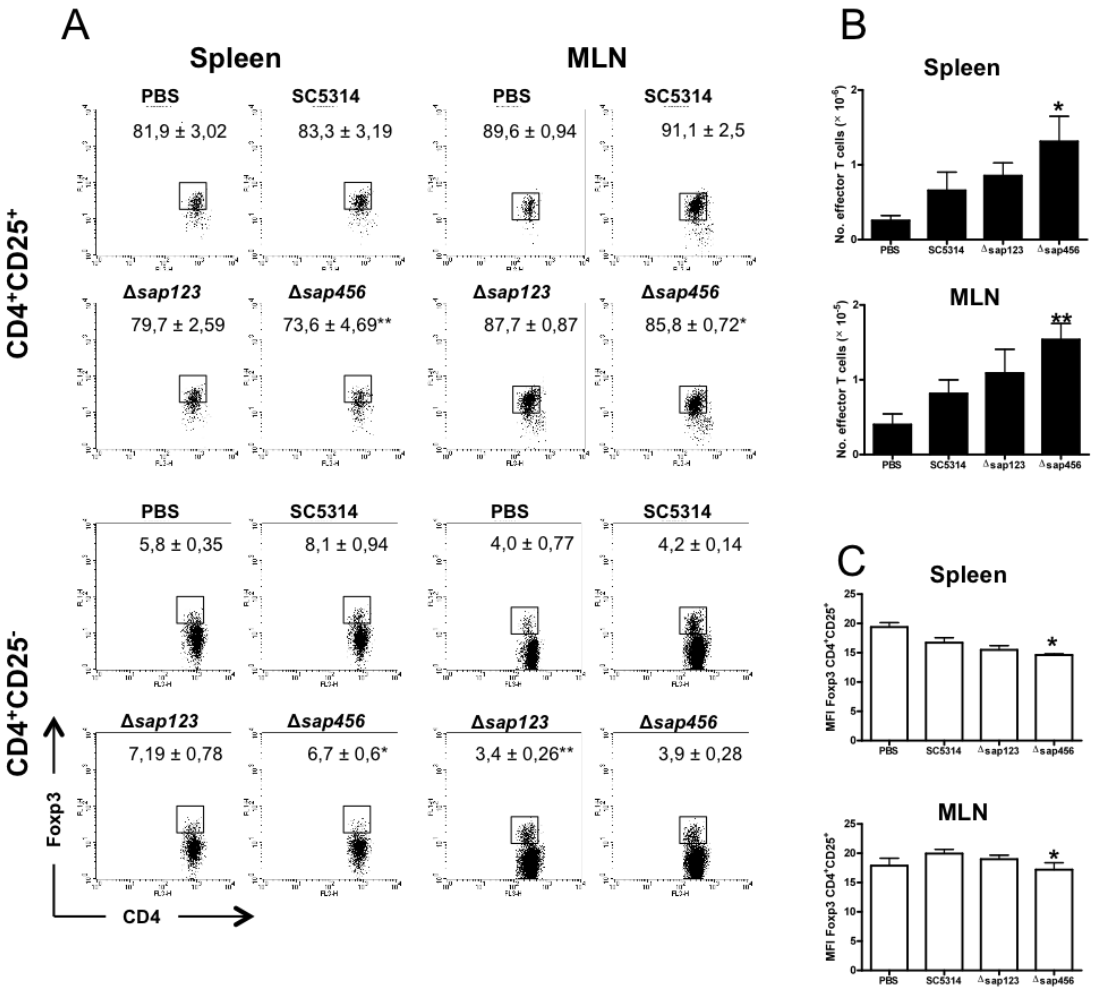

Figure 2 (A) Flow cytometry analysis of intracellular Foxp3 expression in splenic and MLN $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from BALB/c mice, 3 days after i.p. challenge with PBS or 1×10^7 *C. albicans* WT, Δ*sap123* and Δ *sap456* yeast cells, as indicated. Dot plots are a representative example of each group. (B) Number of T effector ($CD4^+CD25^+Foxp3^-$) cells observed in the spleens and MLN of i.p. injected mice, 3 days after challenge. (C) Mean fluorescence intensity of Foxp3 staining within splenic and MLN $CD4^+CD25^+$ T cells. Results are of a representative experiment out of five independent experiments (n=4 in each group). Statistically significant difference between WT and Δ *sap456* infected mice is indicated (**P*<0.05; ***P*<0.01).

The lower proportion of Treg within the $CD4^+CD25^+$ subset implied an increased frequency of T effector ($CD4^+CD25^+Foxp3^-$) cells (Teff) in the Δ*sap456*-infected mice, which was accompanied by an increase in total splenic and MLN Teff numbers. Conversely, no significant differences in Treg numbers were observed in these organs among infected groups (data not shown). No noticeable differences were detected 7 days after infection in the assessed $CD4^+$ T cell subsets frequency and numbers among the infected groups. Frequencies and numbers of B lymphocytes, myeloid cells and

DCs, as well as co-stimulatory and MHC class II molecules expression on B cells and DCs, also did not vary significantly among the different infected groups (data not shown). The above results indicate that *SAP4* to *SAP6* expression influences Treg/Teff ratio in the course of murine *C. albicans* peritonitis.

$CD4^+CD25^+$ T cells from WT-infected mice suppress more efficiently the proliferation of responder cells

Although Foxp3 is the best available marker to identify Treg, it does not allow their isolation for functional tests. Therefore, as CD25 is a good marker for murine Treg (35), the immunosuppressive ability of splenic $CD4^+CD25^+$ T cells isolated from non-infected or infected mice groups, was comparatively evaluated by measuring their ability to inhibit the *in vitro* proliferation of CFSE- labelled responder cells upon anti-CD3 mAb stimulation. $CD4^+CD25^+$ T cells sorted from WT- or Δ*sap456*-infected mice presented the highest and lowest suppressive abilities, respectively, whereas $CD4^+CD25^+$ T cells from Δ*sap123*-infected mice presented intermediate suppressive activity (Figure 3).

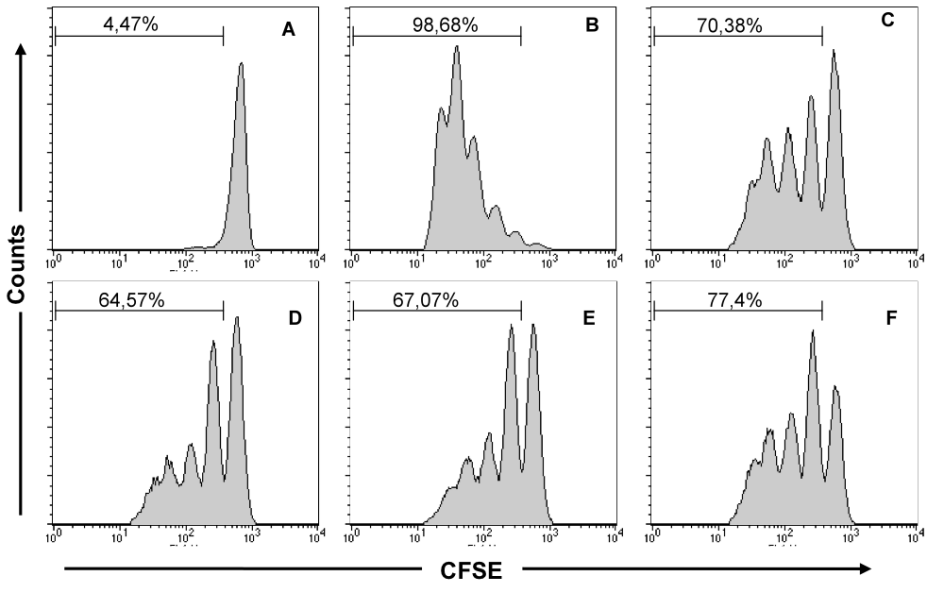

Figure 3 Flow cytometric evaluation of anti-CD3 mAb (1 μg/ml) induced proliferative response of 2,5 x 10^4 CFSE-labelled naive $CD4^+CD25^-$ T cells cultured for 3 days with 10^5 irradiated APC/well, in the absence (A- negative control, no mAb added; B-positive control) or presence of $CD4^+CD25^+$ T cells obtained from: C- control mice or mice infected with D- WT, E- Δ*sap123*, F- Δ*sap456* strains. Histograms correspond to the optimal determined ratio 0.5 $CD4^+CD25^+$:1

responder cells. Indicated percentages correspond to cells that divided at least once. The proliferation indexes for each condition were: A: 1.01; B: 9.06; C: 3.64; D: 2.05; E: 2,91; F: 5,16. $CD4^+CD25^+$ cells added in each condition were sorted from pooled splenic cells of 4 mice per group. Results are a representative example out of five independent experiments.

The proliferative response was not inhibited following co-culture with $CD4^+CD25^-$ T cells from the different groups, confirming that suppression was due to $CD4^+CD25^+$ T cell activity and not to cell density *per se* (proliferation index of 17.6, 17.1, 16.4, and 16.8 in co-cultures using cells from non-infected, WT-, Δ*sap123*-, and Δ*sap456*-infected mice, respectively). These results show that *C. albicans SAP* expression affects the suppressive activity of host Treg.

$CD4^+$ T cells from WT-infected mice produce higher levels of IL-10

Previous studies associated IFN-g and IL-17 with resistance to *C. albicans* infection, whereas IL-4 and IL-10 were associated with host susceptibility to this fungus (36). Therefore, cytokine expression was evaluated in splenic $CD4^+$ T cells through intracellular cytokine staining for IFN-γ, IL-4, IL-10 and IL-17A. No significant differences were detected in the frequencies of $CD4^+$ T cells expressing any of the analysed cytokines, among the different mice groups, 3 and 7 days upon infection (data not shown). Nevertheless, increased levels of IL-10 were detected in culture supernatants of splenic $CD4^+$, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells sorted from WT-infected mice, as compared with the ones from Δ*sap456*-infected counterparts (Figure 4). Conversely, the different sorted subsets of $CD4^+$ T cells from infected mice groups produced similar levels of IFN-γ, IL-4 and IL-17A (data not shown). Cytokine levels in control cultures were always the lowest (Figure 4 and data not shown) and those in unstimulated cultures were below or near detection threshold. IL-10 levels were also evaluated in parenchymal organs presenting *C. albicans* induced lesions, the liver and kidneys. Interestingly, liver homogenates from WT-infected mice had the highest IL-10 levels (Figure 4D). No such difference could be observed in the kidneys (data not shown). These results show that lack of *SAP* expression reduced the production of IL-10 elicited in the host upon *C. albicans* i.p. infection.

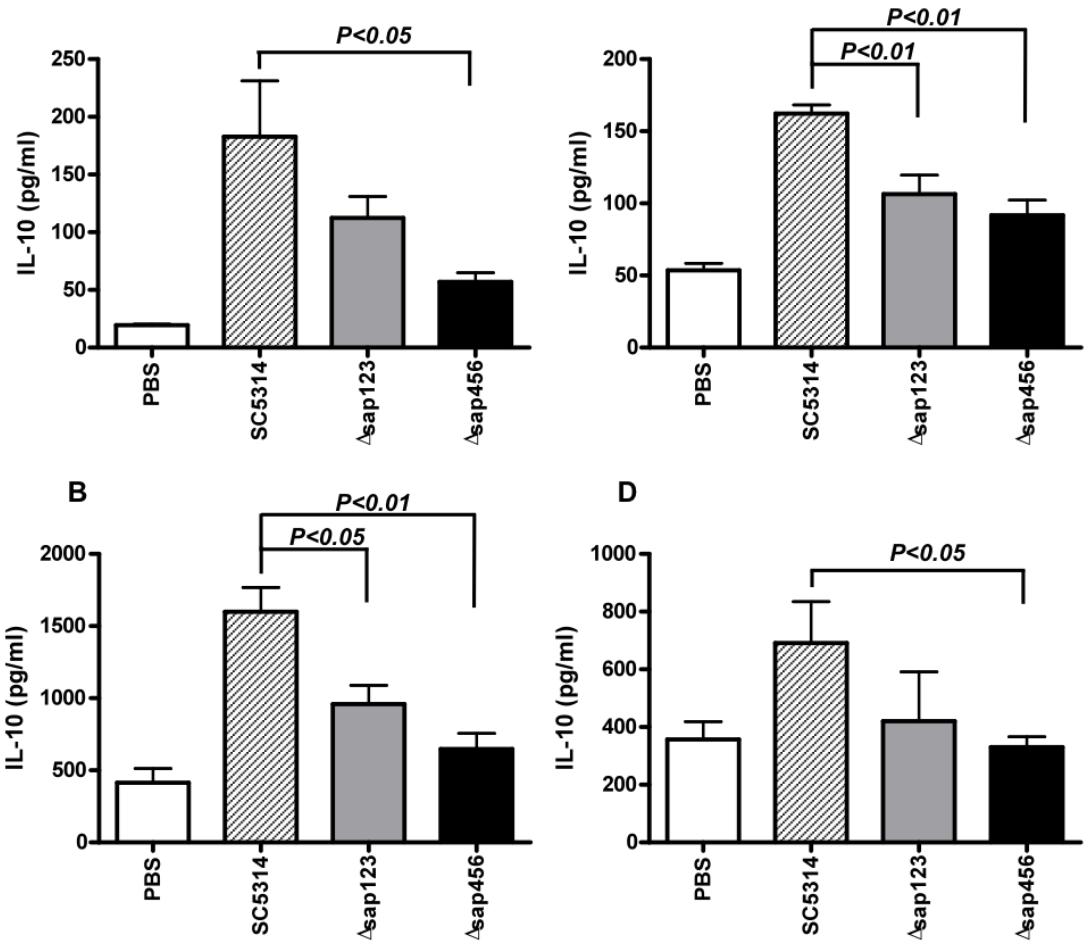

Figure 4 IL-10 cytokine concentration in the supernatants of anti-CD3 mAb-stimulated (1 µg/ml) 72 h cultures of 2,5 x 10^4 (A) $CD4^+$, (B) $CD4^+CD25^-$ and (C) $CD4^+CD25^+$ T cells from control or i.p.-infected mice, as indicated. Bars represent the mean + one SD of six wells per group. Results are a representative example out of five independent experiments (D) IL-10 concentration in liver homogenates of mice infected 72 h before with the indicated strains or sham –infected controls (PBS). Data represent means + one SD (n=4) and are representative examples of three independent experiments.

Treg cells accumulate in liver and kidneys lesions in *C. albicans* infected mice

The extent to which $Foxp3^+$ T cells were recruited into *C. albicans* induced lesions and their distribution therein was evaluated in infected mice. $Foxp3^+$ cells accumulated at low frequency at the periphery of liver and kidney microabscesses and no differences in their frequency or distribution were observed among the infected groups. In contrast, $Foxp3^+$ cells were more abundant and found within the infiltrate area in kidney lesions with smaller inflammatory infiltrates, like interstitial nephritis. Interestingly, $Foxp3^+$ cells were 2 to 3-fold less frequent within these lesions in

Δ*sap456*-infected mice, though no differences could be observed in cell distribution (Figure 5). No accumulation of these cells was observed in non-infected mice organs or in non-lesioned areas of infected organs.

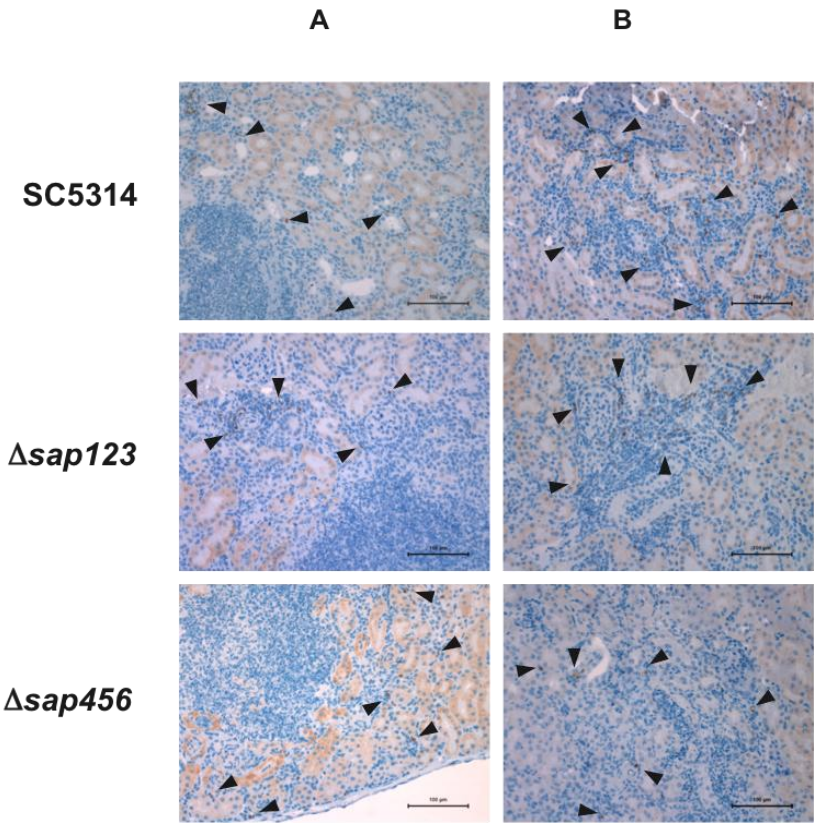

Figure 5 Detection of Foxp3$^+$ cells in *C. albicans* induced kidney lesions, 3 days after i.p. infection with WT, Δ*sap123* and Δ*sap456* strains, as indicated. Foxp3$^+$ cells (brown, denoted by arrows) accumulated in small number at the periphery of *C. albicans* microabscesses (A). In contrast, a larger number of Foxp3$^+$ cells were found within the infiltrates of interstitial nephritis (B). Recruitment of Foxp3$^+$ cells to interstitial nephritis lesions of mice infected with Δ*sap456* was smaller than the observed in WT and Δ*sap123* infected mice. Microscopic images of the slides viewed at 200× magnification. These results are representative of data from three independent experiments (n=4 per group, per experiment).

Lower fungal burden in the kidneys of Δ*sap456*-infected mice

As shown in Figure 6, higher cfu numbers were found in the kidneys of WT-infected mice, as compared with Δ*sap456*-infected mice, whereas in D*sap123*-infected mice renal cfu numbers were not different from the ones of the other infected groups.

Moreover, 58% and 51% of Δ*sap456*- and Δ*sap123*-infected mice, respectively, completely eliminated the fungus from the kidneys and liver by day 7 after infection, while this was observed in only 35% of WT-infected mice. Liver fungal burden was not different among the infected mice groups (Figure 6).

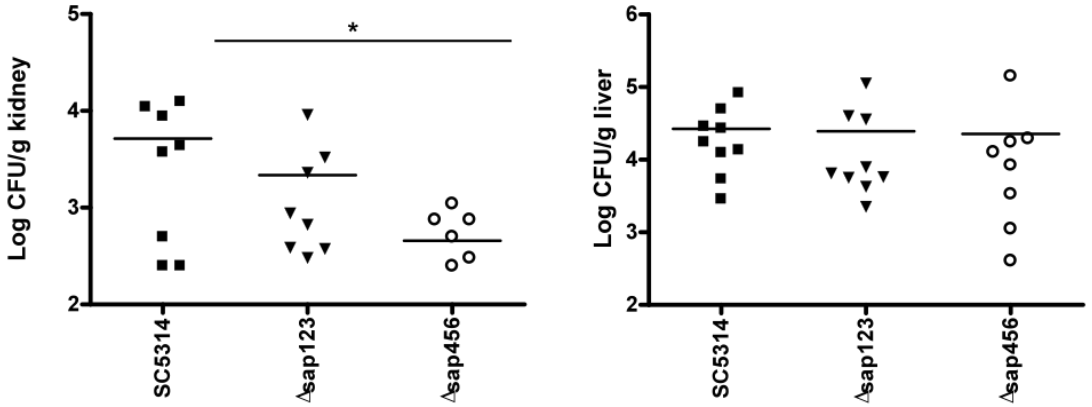

Figure 6 Kidney and liver fungal burden of BALB/c mice 3 days after i.p. infection with 1×10^7 WT (□), *sap123* (▼) or Δ*sap456* (○) *C. albicans* cells, as indicated. Data are representative examples of six independent experiments, (n=8 per group). Each symbol represents an individual mouse; horizontal bars correspond to medians of CFU numbers in each group.

The above data shows that, in this peritonitis model used here, lack of *SAP4* to *SAP6* expression impairs the ability of *C. albicans* to infect the kidneys, though no clear differences could be observed in *C. albicans*-induced lesions either in the kidneys or in the liver (Supplementary Figure 1).

DISCUSSION

Impaired organ colonization and tissue damage was reported for the Δ*sap4-6* mutant DSY459 in murine models of peritonitis (21, 37). Additionally, it was shown that *SAP5* was immediately induced after *C. albicans* i.p. inoculation (19) and *SAP4-6* were found upregulated during liver invasion (38). All these data implicate *SAP4-6* as virulence determinants in *C. albicans*-induced peritonitis. One explanation for the lower virulence of those mutants would be an impaired capacity to invade the host from the peritoneal cavity and ultimately infect parenchymal organs. Here, however, no clear differences were observed concerning organ invasion, among the used *C. albicans* strains, either in the morphology of the fungi or in the depth and extension of the lesions. Nevertheless, a higher fungal load was found in the kidneys of WT-infected mice comparatively to that of Δ*sap456*-infected mice. The observed variability in size and number of fungal-induced lesions, particularly in the kidneys, could result from SC5314 strain intrinsic characteristics. Indeed, a *C. albicans* clinical isolate previously used to induce peritonitis (27) infected more evenly the liver and kidneys of BALB/c mice and yielded higher cfu numbers than did SC5314 (our unpublished results). A reduced ability in colonizing mucosal surfaces has also been reported for this strain (39). By using the *SAT-1* flipping constructed Δ*sap456* mutants we did not observe an attenuated invasiveness, as reported for the DSY459 strain (37, 40). Nevertheless, the experimental conditions used here differ from those used in the referred studies and the disparity of results may not be directly attributable to the strains themselves.

An alternative explanation for the lower fungal burden found in Δ*sap456*-infected mice could be that, as already suggested (21, 26), Sap4-6 have an immunomodulatory role, either direct or indirect, which would be deleterious to the host. We hypothesize that lack of *SAP4-6* expression may allow the host to mount a more effective immune response against an i.p. challenge with *C. albicans*. The higher recruitment of inflammatory monocytes to the peritoneal cavity of Δ*sap456*-infected mice could represent a first step in this response, as these cells are considered important effectors in protective immunity to several pathogens (41-44). Interestingly, in a model of respiratory aspergillosis, inflammatory monocytes facilitated the adaptive $CD4^+$ T cell response (41). Assessing their role in *C. albicans*-infected mice would be worth to

investigate since Δ*sap456*-infected mice, which presented higher numbers of these cells in the peritoneal cavity, had a higher Teff/Treg ratio than WT-infected counterparts.

In the WT-infected mice, the higher recruitment of Treg into the kidneys early after challenge might delay the arrival or activation of effector cells, therefore hindering fungal clearance. A similar early local recruitment of Treg was reported in invasive aspergillosis, having a detrimental effect in recruited neutrophils (45).

The effect of *SAP4-6* expression seems to occur mainly at the regulatory level of the immune response. Our results, by showing a much lower *in vitro* suppression of T cell proliferation by $CD4^+CD25^+$ T cells sorted from Δ*sap456*- than WT-infected mice, support this hypothesis. Moreover, the detection of lower levels of the immunosuppressive cytokine IL-10 in Δ*sap456*-infected mice liver homogenates and splenic $CD4^+$ T cell culture supernatants, than in WT counterparts, further agrees with a role for Sap4-6 in the impairment of the host immune response to *Candida* peritonitis. Several studies recognized the role of fungal secreted proteases on T cell stimulation and overall immune response (46-49). Different fungal proteases, including aspartyl proteases, were shown to impact on T cell function either directly or indirectly via DC modulation. However, as surface expression of maturation markers on splenic and MLN DCs, assessed 12 h upon infection, was not affected by *SAP* deficiency (our unpublished results), a DC-dependent mechanism seems unlikely.

Fungal aspartyl proteases have been successfully used in experimental vaccination assays that often resulted in the production of host protective cytokines, longer median survival rates, reduction of fungal burden, and amelioration of inflammatory pathology (27, 46, 47). Moreover, T cells from mice immunized with an aspartyl protease of *Coccidioides posadasii* produced IL-10 upon *in vitro* antigen restimulation (49). Interestingly, treatment of live *C. albicans* with the aspartyl protease inhibitor pepstatin significantly reduced IL-10 levels detected in peritoneal exudates of i.p.-infected mice (50).

Mice infected with strains lacking *SAP1* to *SAP3* frequently presented an intermediate effect between WT and Δ*sap456* mutants. Although we cannot exclude a compensatory effect by other *SAP* genes, which could have diluted the impact of the

missing genes in virulence, evidence from this and other studies referred herein support a relatively less important role of Sap1-3 in this infection model. The importance of Saps for the course of candidiasis is known to highly depend on the infection model. In fact, a role for Saps in a murine model of hematogenously disseminated candidiasis was not evident, since the outcome of infection and ensuing host immune response were not different in mice infected with the WT or the Δ*sap* null mutant strains used in the present study (32).

Globally, results presented herein implicate Sap4-6 in the modulation of the host immune response to *C. albicans* peritonitis, providing additional evidence for the role of these enzymes as virulence determinants in this infection model.

These enzymes may constitute one of the mechanisms used by the fungus to dampen host immune response and therefore, their usage as target antigens in *C. albicans* vaccination could be worth to investigate.

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Supplementary Table 1. Frequencies and counts of inflamammatory cells recruited into the peritoneal cavity of BALB/c mice 6 and 72h upon i.p. infection.

Cell Type	Marker	6h							
		PBS		SC5314		*Δsap123*		*Δsap456*	
		Freq. (%)	Cells (× 10^{-5})	Freq. (%)	Cells (× 10^{-5})	Freq. (%)	Cells (× 10^{-5})	Freq. (%)	Cells (× 10^{-5})
Resident macrophage	F4/80^{hi}	39,66 ± 9,14	16,52 ± 4,44	13,8 ± 1,66^{a}	9,58 ± 1,44	13,16 ± 0,31^{a}	11,54 ± 0,75	15,8 ± 1,09^{a}	11,11 ± 6,77
Neutrophils	Gr-1^{hi}	0,4 ± 0,09	0,17 ± 0,07	71,5 ± 2,32^{a}	49,72 ± 6,12^{a}	70,29 ± 3,74^{a}	61,51 ± 1,2^{a}	68,07 ± 0,51^{a}	48,50 ± 11,62^{a}
Inflammatory monocytes	F4/80^{+}Gr-1^{+}	0,01 ± 0,00	0,01 ± 0,00	0,12 ± 0,01^{b}	0,83 ± 0,19^{c}	0,15 ± 0,02^{a}	0,11 ± 0,02^{a}	0,13 ± 0,03^{b}	0,87 ± 0,01^{a}
Eosinophils	F4/80^{+}Gr-1^{int}SSC^{hi}	2,03 ± 0,20	0,87 ± 0,25	1,88 ± 0,41	1,28 ± 0,17	1,44 ± 0,22	1,26 ± 0,20	2,34 ± 0,48	1,62 ± 0,47
Dendritic cells	CD11c^{hi}	1,08 ± 0,11	0,46 ± 0,1	3,75 ± 1,79	2,52 ± 1,09^{c}	2,86 ± 0,36^{b}	2,51 ± 0,41^{c}	4,48 ± 1,3^{a}	2,95 ± 0,62^{c}
		72h							
Resident macrophages	F4/80^{hi}	36,49 ± 3,48	15,40 ± 3,4	41,31 ± 5,59	27,83 ± 10,66	39,99 ± 4,79	25,50 ± 4,73	39,74 ± 2,49	35,92 ± 10,07^{c}
Neutrophils	Gr1^{hi}	0,28 ± 0,08	0,11 ± 0,08	21,82 ± 7,44^{b}	14,67 ± 7,21^{c}	18,79 ± 3,55^{b}	11,89 ± 1,97^{b}	21,94 ± 6,77^{b}	19,48 ± 7,14^{b}
Inflammatory monocytes	F4/80^{+}Gr-1^{+}	0,01 ± 0,00	0,02 ± 0,00	4,45 ±1,68^{c}*	2,70 ± 0,56^{c}***	7,24 ± 2,38^{b}	4,71 ±1,86^{b}*	8,73 ± 1,87^{a}	7,58 ± 0,83^{a}
Eosinophils	F4/80^{+}Gr-1^{int}SSC^{hi}	1,91 ± 0,46	0,83 ± 0,34	6,89 ± 1,21	3,80 ± 0,97	6,54 ± 3,71	3,02 ± 1,63	6,89 ± 0,52	5,52 ± 1,27
Dendritic cells	CD11c^{hi}	1,03 ± 0,18	0,44 ± 0,14	1,88 ± 0,22^{c}	1,24 ± 0,36^{c}	1,70 ± 0,28	1,10 ± 0,30	1,63 ± 0,35	1,44 ± 0,29^{b}

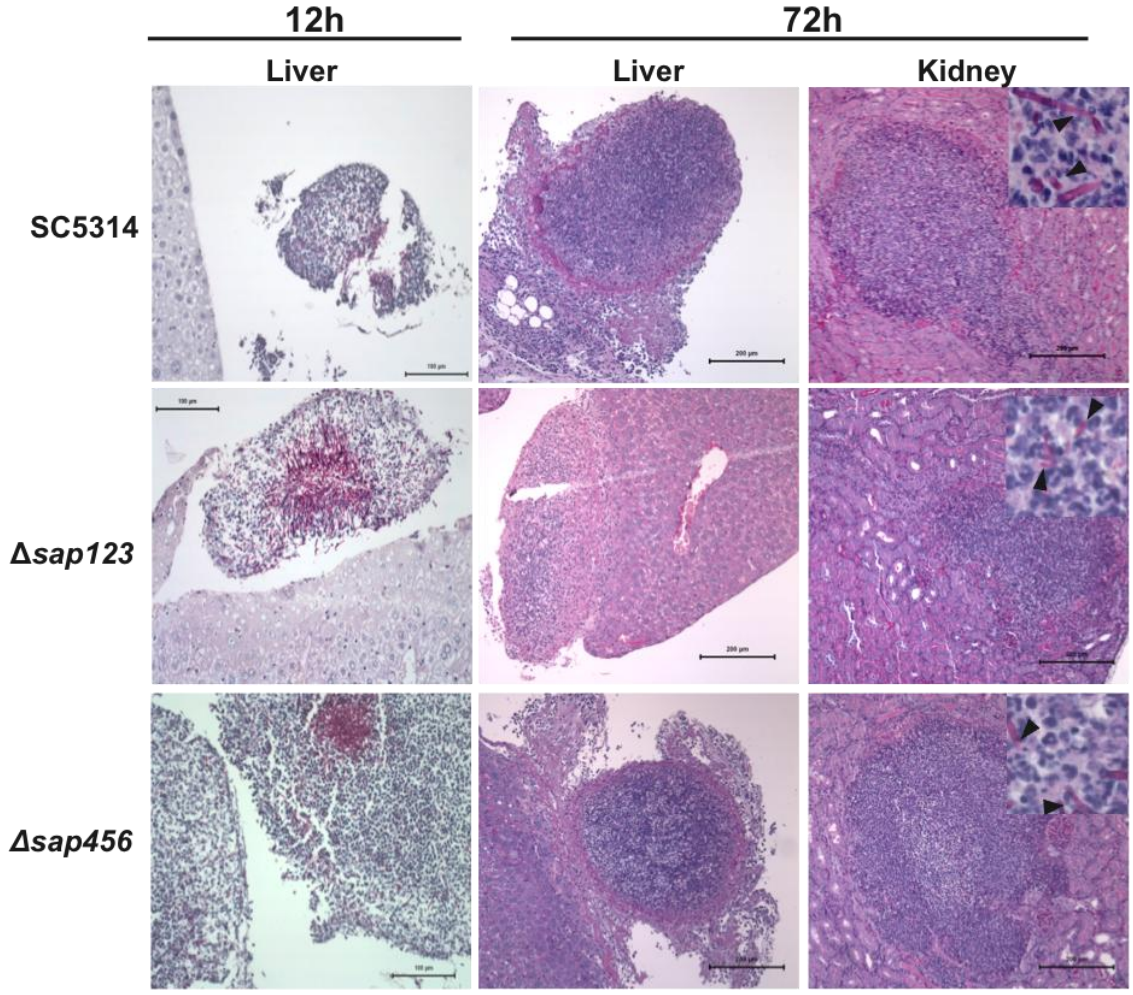
Different cell types were identified with markers and flow cytometry. Data represent means ± SD; n=4. Data are from one experiment representative of three independent experiments.

[a] P<0,001; [b] P<0,01; [c] P<0,05, when compared with PBS. * P<0,05; ***P<0,001, when compared with Δ*sap456*;

Supplementary Table 2. Chemokine levels in the peritoneal cavity lavage fluids of BALB/c mice 2 and 12h upon i.p. infection (pg/peritoneal cavity)

Chemokine	2h			
	PBS	**SC5314**	***Δsap123***	***Δsap456***
KC	79,31 ± 70,76	3727,30 ± 322,32[a]	3221,44 ± 88,44[a]	3719,39 ± 783,94[a]
MIP-2	ND	894,41 ± 351,81[c]	1196,63 ± 94,78[b]	1307,11 ± 351,88[a]
MCP-1	63,47 ± 67,21[a]	691,80 ± 202,05[a]	689,14 ± 146,87[a]	933,30 ± 386,03[a]
	12h			
KC	11,01 ± 11,46	631,55 ± 427,71	392,98 ± 453,64	180,85 ± 74,92
MIP-2	ND	ND	ND	ND
MCP-1	3,30 ± 1,46	810,33 ± 152,97[b]	499,43 ± 214,67	364,41 ± 245,64

ND – not detectable; [a] $P<0,001$; [b] $P<0,01$; [c] $P<0,05$, when compared with PBS.



Supplementary Figure 1 Lack of *SAP* expression did not significantly affect *C. albicans*-induced organ pathology. Representative photomicrographs of PAS-stained histological sections of liver and kidneys from BALB/c mice infected with *C. albicans* WT, D*sap123* and D*sap456*, at the indicated time points upon i.p. infection. Structures containing filamentous *C. albicans* and inflammatory cells whithin fibrin deposits could be observed in close contact with the liver serosa, without detectable organ invasion (12 h). Lesions in the liver comprised superficial microabscesses, often detached, showing healing by fibrosis (72 h). Microabscesses with moderate to intense neutrophylic infiltration, as well as interstitial nephritis, were observed mostly in the kidneys cortical area in addition to the organ surface, and interstitial nephritis was often observed (72 h). Most of the times, multiple lesions of variable size and type were present in each organ. Hyphal fungal elements were visible inside the larger lesions (arrows on magnified insets). Localization and size of the *C. albicans* induced lesions as well as composition of the inflammatory infiltrates was mostly similar in all infected groups. PAS-positive hyphae organisms within lesions are denoted by arrows. Seven days after infection.

CHAPTER 5

Pathogen recognition receptor Galectin-3 is cleaved by *Candida albicans* secreted aspartyl proteases

Pathogen recognition receptor Galectin-3 is cleaved by *Candida albicans* secreted aspartyl proteases

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ABSTRACT

The mammalian pattern recognition receptor galectin-3 was shown to specifically bind beta-1,2 mannosides on the cell wall of the pathogenic yeast *Candida albicans*. This interaction directly induces fungal cell death and also promotes pro-inflammatory cytokine TNF-alpha production by the host cells. To circumvent host immune defenses, *C. albicans* developed multiple evasion mechanisms. Among these, production of secreted aspartyl proteases (Saps) has been particularly highlighted due to their ability to degrade and/or inactivate diverse host immune effector molecules. Although *C. albicans* possesses a 10 member Sap family, Sap2, belonging to the Sap1-3 subfamily, is the most extensively characterized. In this study, we assessed the pattern of galectin-3 expression in the kidneys of mice i.v.-infected with *C. albicans* and investigated whether this lectin could be degraded by Sap1 to Sap3 isoenzymes.

INTRODUCTION

Galectins are a family of β-galactoside-binding animal lectins that are involved in several biological processes and are expressed in a wide variety of cell types, including macrophages, dendritic cells and epithelial cells (1, 2). Initially thought to bind only endogenous glycans and mediate development processes and regulation of immune homeostasis (1-3), it has now become clear that galectins can also bind glycans on the surface of several microorganisms and parasitic worms, and mediate recognition and effector functions in innate immunity (4). Within this family, galectin-3 (Gal-3) is the best characterized and has been linked to modulation of immune and inflammatory responses (5, 6). Gal-3 recognizes and binds β-1,2-linked oligomannosides on the cell wall of *Candida albicans* and, in consequence, has been considered to function as a macrophage receptor for this fungal pathogen (7). Gal-3 can differently sense *C. albicans* and the non-pathogenic yeast *Saccharomyces cerevisiae* through a mechanism involving TLR2 and Gal-3, leading to increased production of the inflammatory cytokine TNF-α upon *C. albicans* recognition (8). In a mouse model of experimental colitis, *C. albicans* colonization is promoted by pre-existing inflammatory conditions, which in turn augmented the inflammatory process through Gal-3 with up-regulation of TLR2 expression and TNF-α (9). Accordingly, synthetic analogues of β-1,2 oligomannosides administered orally prevented yeast colonization in the gut (10). This supports the roles of Gal-3 in both inflammation and the regulation of host immune responses to *C. albicans*. Interestingly, the selective binding of Gal-3 to the *C. albicans* cell wall glycans is fungicidal (11), an observation that gives emphasis to the direct effector functions of galectins in innate immunity.

C. albicans has developed efficient strategies to evade the host recognition and immune attack (12, 13). *C. albicans* secreted aspartyl protease (Sap) family comprises 10 different members (14, 15) which are differentially expressed (16-20) and that have been implicated in *C. albicans* pathogenesis (14, 21). Saps are able to degrade many human proteins, like the ones that protect mucosal surfaces and cell-surface proteins, such as keratin, collagen, vimentin, fibronectin, laminin, secretory immunoglobulin A (IgA), histatin-5 and mucins (22-30). Degradation of complement proteins and histatin-5 by Saps is included in the multiple immune evasion mechanisms explored by this

opportunistic pathogen (23, 24). Furthermore, these proteases damage host epithelial and endothelial barriers and thus facilitate tissue penetration by hyphae and infection of host cells (22, 27).

Given the central role of Saps for infection and pathogenicity of *C. albicans* and as Gal-3 specifically recognizes and directly kills bound yeast, besides augmenting pro-inflammatory response by the innate immune system, the ability of three secreted aspartyl proteases - Sap1, Sap2 and Sap3- to degrade Gal-3 is explored here.

MATERIAL AND METHODS

Secreted aspartyl proteases

Recombinant Sap1 (rSap1), rSap2 and rSap3 were produced in *Pichia pastoris* and were kindly provided by Dr Bernhard Hube (Jena University, Germany). All purified recombinant proteins exhibited protease activity *in vitro*, as assessed by using a standard fluorescence-based casein assay (Molecular Probes). Reactions were also performed in the presence of the general aspartic protease inhibitor pepstatin A (Sigma Aldrich). *C. albicans* SC5314 cells were grown in inducing medium (yeast carbon base (Difco) supplemented with 0.5% BSA (Sigma) ph 4.0) for 72 h at 30ºC. Culture supernatants containing native Sap2 (nSap2) were filtered and concentrated in Amicon Ultra-15 devices (Millipore Corporation, Billerica, USA).

Galectin-3

Recombinant human Gal-3 was obtained as previously described (Stowell et al 2008). Recombinant Gal-3 was purified by affinity chromatography on lactosyl-Sepharose, and bound lectin was eluted with 100 mm lactose in PBS, 14 mm βME. Prior to derivatisation, βME was removed from galectin samples by using a PD-10 gel filtration column (GE Healthcare), followed by the addition of lactose (100 mm final concentration) to help maintain the stability of each galectin and reduce the likelihood of adduct formation at or near the carbohydrate recognition domain.

Cleavage activity

The proteolytic activity of recombinant Sap1, Sap2, Sap3 (0.5 µg each) or of supernatants derived from wild type *C. albicans*, containing nSap2 was assayed by incubation for 24 h with purified Gal-3 protein in 0.1M citrate buffer at 37 ◦C (pH 5.2 and 6.8). Aliquots were taken at different time points, the reaction was stopped, and samples were frozen at -80º C until use. Reactions were also performed in presence of 15 µM pepstatin A (Sigma), the general aspartyl protease inhibitor.

Immunoblotting

The reactivity of anti-Gal-3 immunoglobulins was visualized by Western blot analysis. For that purpose, 1 µg of Gal-3 incubated with rSap1, rSap2 and rSap3, as well as with native Sap2, in the presence and absence of pepstatin A, were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide gels and then blotted overnight onto nitrocellulose membranes (Sigma). Nonspecific binding was prevented by blocking with 2% BSA in Tris buffered saline (TBS) for 1 h at room temperature. After washing, membranes were incubated for 2 h with polyclonal IgY anti-Gal-3 (1 µg/ml) diluted in TBST 0.1% BSA. Alternatively, membranes were incubated with supernatant from the rat hybridoma producing monoclonal anti-mouse/human Gal-3 (M3/38) (diluted 1:20 in TBST 0.1% BSA). Bound antibodies were detected with alkaline phosphatise (AP)-conjugate anti-chicken IgY (diluted at 1:10000 in TBST 0.1% BSA) or AP-conjugated goat anti-rat IgG (Southern Biotechnology Associates, Birmingham), and revealed by using NBT/BCIP solution (Roche, Penzberg, Germany) as substrate. The reaction was stopped in distilled water.

Mice

Male BALB/c mice, 8-10 weeks old, were purchased from Charles River (Barcelona, Spain) and kept under specific pathogen-free conditions at the Animal Facility of Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal. All procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123), 86/609/EEC Directive and Portuguese rules (DL 129/92). Authorization to perform the experiments was issued by the competent national board authority (Direcção Geral de Veterinária), document number 0420/000/000/2010.

***Candida albicans* and culture conditions**

C. albicans wild-type strain SC5314 (31) was used in this study. To prepare the inocula for infection, *C. albicans* was grown in a shaking incubator for 14 hours at 30ºC in Winge medium (0,2% glucose, 0,3% yeast extract). Yeast cells were harvested, washed twice with sterile, nonpyrogenic phosphate-buffered saline (PBS) (Gibco®, Grand Island,

NY, USA), counted in a haemocytometer and resuspended at the appropriate concentrations. The inoculum was confirmed by Colony Forming Unit (CFU) counts on Sabouraud dextrose agar (Difco, Detroit, MI, USA) for up to 48 h at 37ºC.

***C. albicans* hematogenously disseminated infections**

Mice were injected intravenously (i.v.), in the lateral tail vein, with $5x10^4$ *C. albicans* yeast cells in 0.2 ml PBS. Control mice were injected with PBS. Mice were sacrificed 7 days after infection and the kidneys were removed and fixed in 10% Phosphate-buffered formaldehyde, followed by dehydration and paraffin wax embedment. Sections of 4 µm were cut from each representative paraffin blocks for staining with haematoxilin-eosin and with periodic acid-Schiff reagent followed by counterstaining with haematoxylin, in order to evaluate both fungal morphology and composition and distribution of inflammatory infiltrates.

Immunohistochemistry

The presence of Gal-3 in kidney *C. albicans* induced lesions were assessed by immunohistochemistry in formalin-fixed, paraffin-embedded 4 µm sections of *C. albicans* i.v. infected mice mounted on amino-propyl-tri-ethoxy-silane (Sigma-Aldrich, St Louis, MO, USA) coated slides.

Immuno-staining was performed by using the modified avidin-biotin-peroxidase complex (ABC) method (32). Unlabeled monoclonal antibodies rat anti mouse/human Gal-3 (clone M3/38) (eBioscience, San Diego, CA, USA) was used.

Tissue sections were deparaffinised in xylene, rehydrated by graded washes of ethanol in water, ending in a final rinse in deionized water. Antigen retrieval was performed by incubating the slides in 10 mM citrate buffer (pH = 6) for 3 min in a pressure cooker. The slides were cooled and rinsed three times in Tris-buffered saline (TBS; 50 mM Tris, 150mM NaCl, pH=7,6) for 5 min. Endogenous peroxidase activity was blocked by immersing slides in methanol containing 3% hydrogen peroxide for 10 min, followed by TBS washing. To reduce non-specific antibody binding, slides were incubated with normal rabbit serum (Dako, Glostrup, Denmark) diluted at 1:5 in TBS containing 10% bovine serum albumin (BSA), in a humidified chamber for 20 min at room

temperature. Excess normal serum was removed and replaced by the hybridoma supernatant containing anti-Gal-3 mAb (diluted at 1:10). After overnight incubation at 4ºC, slides were washed with TBS and incubated for 30 min with a 1:200 dilution of biotin-labelled rabbit anti-rat secondary antibody (Dako). Slides were then washed with TBS and incubated for 30 min with the avidin-biotin complex (Dako) diluted at 1:100. Detection was performed for 3 to 5 min with 0.05% 3,3 diaminobenzidinetetrahydrochloride (DAB) freshly prepared in 0.05 M Tris/hydroxymethylaminomethane buffer, pH 7.6, containing 0.1% hydrogen peroxide (Dako). Finally, sections were lightly counterstained with Mayer's haematoxylin and mounted in Entellan® mounting medium (Merck, Darmstadt, Germany). Dilution of primary antibody, biotin-labelled secondary antibody, and avidin-biotin complex were made with TBS containing 5% BSA. Negative controls were performed by substitution of the primary antibody with irrelevant immunoglobulins of the same subclass and concentration as the monoclonal antibodies. Slides were evaluated under light microscopy.

RESULTS

C. albicans secreted aspartyl proteases cleave murine galectin-3

The ability of *C. albicans* secreted proteases to cleave galectin-3 was assessed by incubating different Sap proteins with this lectin. The proteolytic activity of *C. albicans* wild-type SC5314 culture supernatant grown in Sap inducing medium (containing mostly native Sap2) and the recombinant Sap proteins rSap1, rSap2 and rSap3 were incubated with galectin at 37ºC, pH 5.2 or 6.8, and aliquots were collected after 1, 3, 6, 12 and 24 h incubation. The reaction mixtures were separated by SDS-Page and Gal-3 cleavage was evaluated by Western blotting. Gal-3 cleavage could be detected already at the first assessed time point at pH 5.2, and was more evident along the other time points. The results, obtained with protein mixtures collected after 3 h incubation, are shown as representative examples (Figure 1A). Assessment of Gal-3 degradation was based on the disappearance of the protein bands of higher molecular weight as well as by the appearance of novel fragments of lower molecular weights. Native Sap2 and recombinant Sap2 and Sap3 were the most effective in cleaving Gal-3. This lectin was also cleaved, to a lesser extent, by rSap1.

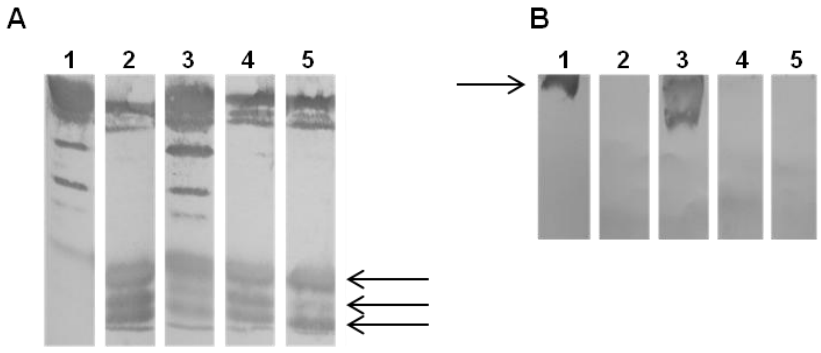

Figure 1 Culture supernatants derived from wild-type *C. albicans* containing nSap2 and rSap1, rSap2 and rSap3 have proteolytic activity and cleave Gal-3. Saps were added to Gal-3 and incubated for 3 h at 37ºC, pH5.2. The mixtures were then separated by SDS-Page, transferred to a nylon membrane. (A) Gal-3 and the resulting degradation fragments were identified by using a polyclonal anti-Gal-3 Ig-Y; cleavage products are indicated by the arrows; (B) Gal-3 integrity was evaluated by using a monoclonal anti-Gal-3 antibody (M3/38) that reacts with the N-terminal nonlectin domain of the protein. Arrow indicate full-length Gal-3. Gal-3 (lane 1); Gal-3 incubated with: nSap2 (lane 2), rSap1 (lane 3), rSap2 (lane 4), and rSap3 (lane 5). A representative example out of three independent assays is shown.

Gal-3 integrity was evaluated by using a monoclonal anti-Gal-3 antibody (M3/38) that reacts with the N-terminal nonlectin domain of the protein. The N-terminal non-lectin tandem repeated domain of Gal-3, necessary for its oligomerization and immunomodulatory activity, was removed during Sap-mediated cleavage (Figure 1B). Protein mixtures incubated at pH 6.8 did not exhibit detectable Gal-3 degradation (data not shown). Pepstatin A, an inhibitor of aspartyl proteases, largely blocked Gal-3 degradation (Figure 2), confirming that the proteolytic activity observed was specifically due to this class of proteases.

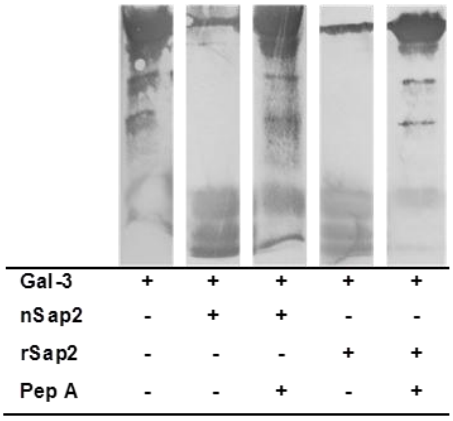

Figure 2 Inhibition of aspartyl protease activity. Gal-3 was incubated with nSap2 and rSap2 for 3 h in citrate buffer at pH 5.2 alone or in the presence of pepstatin-A. Degradation of Gal-3 was largely blocked by pepstatin A, an inhibitor of aspartyl proteases. The mixtures were separated by SDS-Page, transferred to a nylon membrane and Gal-3 and the resulting degradation fragments were identified by using a polyclonal anti-Gal-3 Ig-Y. A representative example out of three independent assays is shown.

Gal-3 accumulates in inflammatory infiltrates observed in the kidneys of *C. albicans* i.v.-infected mice

Gal-3, with intact N-terminal non-lectin tandem repeated domain, accumulated in the inflammatory infiltrates recruited into the kidneys of BALB/c mice upon i.v. infection with *C. albicans*. The presence of this lectin was however detected in lower amounts in lesions where *C. albicans*, mainly in the hyphal form, was present in higher numbers, suggesting that *C. albicans* was able to regulate and/or degrade Gal-3.

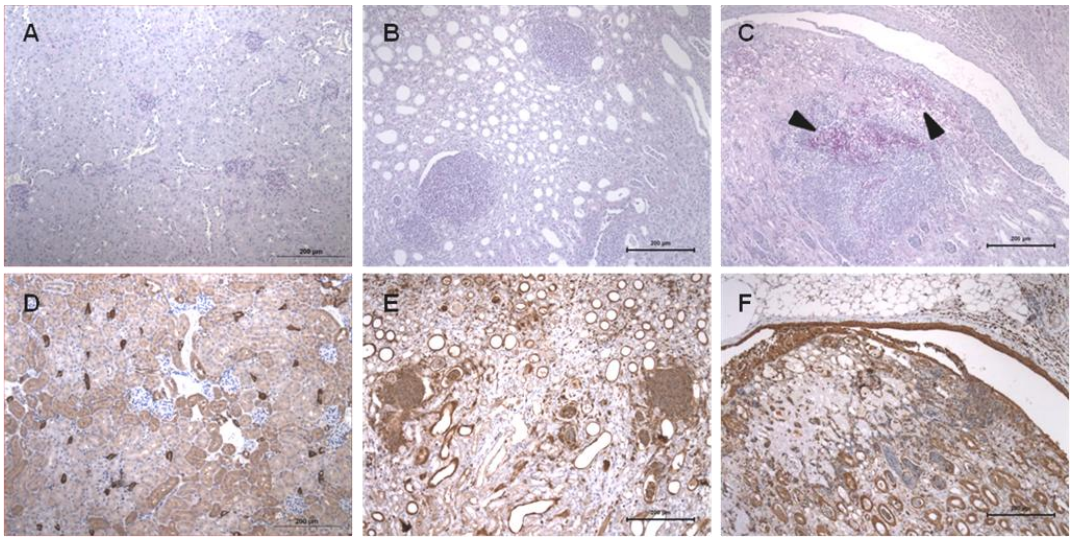

Figure 3 Representative photomicrographs of PAS-stained histological sections of kidneys from BALB/c control mice (A) or mice i.v.-infected with $5x10^4$ *C. albicans* SC5314 cells (B and C). At 7 days after i.v. infection, moderate to intense neutrophilic infiltration was observed, without visible fungal cells (B) or with numerous PAS-positive organisms with hyphal morphology (arrowheads), extensively effacing the renal tubuli and invading the urothelium (C); Immunohistochemistry of kidney sections from control (D) and infected mice (E and F), using a monoclonal antibody anti-N-terminal domain of Gal-3 (M3/38) and revealed with DAB, showing higher expression of Gal-3 in the inflammatory infiltrates with low fungal burden (E). Gal-3 is expressed at high levels in the collecting ducts of both control and infected mice.

DISCUSSION

Here we show that *C. albicans* secreted aspartyl proteases Sap1, Sap2 and Sap3 can degrade and inactivate the host receptor Gal-3. This activity might generate a microenvironment of impaired host innate immune function. Taking into account the concomitant decrease of intact Gal-3 and increase of Gal-3 cleavage products, the proteolytic activity of Sap2 and Sap3 seems stronger than that of Sap1. This result emphasizes that, even presenting high homology, different Saps may have a different enzymatic activity on specific substrates, (22, 33). In this regard, Sap1 and Sap2 have been reported to cleave proteins of the complement system more effectively than Sap3 (23). Here, Sap3 was as efficient as Sap2 in cleaving Gal-3, whereas Sap1 cleaved Gal-3 to a lesser extent. Each recombinant Saps and *C. albicans* culture supernatants showed comparable cleavage patterns suggesting that all Saps tested, native or recombinant, cleave Gal-3 at the same sites. Though Sap2 is the mainly secreted Sap when *C. albicans* is grown in media containing proteins as sole nitrogen sources, all three Saps tested here may be present in *C. albicans* supernatants (20). Sap proteins may participate in innate immune evasion by affecting diverse effectors with antifungal activity. These fungal proteins cleave the central host complement components and degrade other host innate immune components such as lactoferrin and histatin-5 of the saliva, the protease inhibitor α-macroglobulin, enzymes which mediate the respiratory burst of macrophages and several immunoglobulins, including secretory IgA, which normally is rather resistant to bacterial proteases (14, 34). Secretion of host Gal-3-degrading proteases, which could impair Gal-3-mediated *C. albicans* recognition and/or affect immune regulatory effects dependent on this lectin, may represent an alternative immune evasion mechanism. The cleavage of Gal-3 upon binding *Leishmania major* promastigotes, presumably by a membrane-bound zinc metalloprotease was previously shown (35).

Sap expression is induced in *C. albicans* cells upon infection (14) suggesting that these proteases may also contribute to *in vivo* Gal-3 inactivation and immune evasion. Here, an accumulation of Gal-3 was observed in *C. albicans*-induced kidney lesions. However, Gal-3 was detected in significantly lower amounts inside lesions containing numerous fungal cells. It would be interesting to evaluate whether this may result from Gal-3

cleavage by Saps. Interestingly, Gal-3 was previously shown to promote neutrophil recruitment into bacterial infection sites (36, 37). As neutrophils play a major role in controlling *C. albicans* infections, *C. albicans*-mediated Gal-3 inactivation could additionally result in reduced neutrophil recruitment into the kidneys, the preferential infected organ during invasive *Candida* infections (38).

In summary, *C. albicans* may use Gal-3 degradation as a strategy to control and evade host immune Gal-3-mediated recognition, and the ensuing inflammatory response, or even avoid Gal-3-mediated *C. albicans* killing. Here we show that the three Sap proteins Sap1, Sap2 and Sap3 degrade and inactivate the pattern recognition receptor Gal-3. This may compromise Gal-3 function, for which an intact N-terminal non-lectin domain was shown to be essential (39-41). Degradation of host Gal-3 might thus generate a protective microenvironment of reduced Gal-3 activity which may facilitate *C. albicans* survival in the host.

ACKNOWLEDGMENTS

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CHAPTER 6

Immunization with *Candida albicans* secreted aspartyl proteases does not protect BALB/c mice against hematogenously disseminated candidiasis

Immunization with *Candida albicans* secreted aspartyl proteases does not protect BALB/c mice against hematogenously disseminated candidiasis

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ABSTRACT

The usefulness of *Candida albicans* recombinant secreted aspartyl protease (rSap2) as an immunogen for vaccination against invasive candidiasis was evaluated in a murine model of hematogenously disseminated candidiasis. Four different active immunization protocols were assayed, with four different adjuvants tested: (i) Alum; (ii) topical Imiquimod; (iii) Freund's; (iv) CpG plus Alum. Moreover, as the hypha-associated isoenzyme Sap5 is preferentially expressed during systemic candidiasis, it was also evaluated as target antigen for *Candida* vaccination, together with Alum or Imiquimod. Results showed that all these approaches failed to have a relevant and statistically significant effect on the infection course, determined by survival curves and kidney fungal burden. This suggests that the *C. albicans* Sap2 and Sap5 isoenzymes, despite their potential role in virulence, do not appear to be suitable target proteins for the development of immunopreventive strategies against acute disseminated candidiasis.

INTRODUCTION

Invasive candidiasis ranks among the third or fourth most frequent nosocomial bloodstream infections both in the United States and in many European countries, and *Candida albicans* accounts for approximately 50% of the cases of candidemia (1-3). Disseminated candidiasis typically occurs after multiple weeks of hospitalization and is associated with a mortality of 40 to 50% and a high morbidity in those who survive, even after treatment with modern antifungal agents (4-6). Furthermore, development of *Candida* spp resistance to conventional antifungal therapies is of serious concern. Given the high morbidity and mortality rates, associated with high health care costs (7), the development of more effective prophylactic and therapeutic strategies is needed. The predominant risk factors for disseminated candidiasis are the disruption of protective anatomical barriers or situations leading to substantial increase in the colonization burden of *Candida* spp., such as indwelling catheters and parenteral nutrition, abdominal or cardiac surgery, prolonged hospital stay or stay in an intensive care unit, and receipt of broad-spectrum antibiotics (2). Because the risk factors for disseminated candidiasis precede the development of infection and are often easily identifiable, vaccination of at-risk patients to prevent the onset of disseminated candidiasis is promising. Furthermore, these patients are usually not profoundly immunosuppressed and would be expected to respond favorably to vaccination (8). Moreover, an immunization approach would need to protect patients just for the short period of time of increased susceptibility (9).

The protective role of antibodies against invasive candidiasis has been controversial. For some effective vaccines against invasive fungal infections, the key to protection has been reported to be the induction of cell-mediated, pro-inflammatory, Th1 or Th17 responses, which improve phagocytic killing of the fungus (10, 11). Nevertheless, over the last two decades, strong evidence has accumulated that some *Candida*-specific antibodies or their engineered derivatives can be immunoprotective against *C. albicans* infections, following both active vaccination and passive transfer of immune serum or antibodies (12-19).

In addition to the host status, the pathogenicity of *C. albicans* also depends on a complex set of fungal attributes that are considered as putative virulence factors for

their involvement in the infectious process. Often, these consist of secreted or membrane bound proteins, which are among the first targets encountered by host defense mechanisms (20), making them attractive vaccine candidates. The secreted aspartyl proteases (Sap) have long been considered putative virulence factors (21, 22). The isoenzyme preferentially expressed following induction *in vitro*, Sap2 (23), has already been used in vaccination strategies against oral, vaginal, and intraperitoneal *C. albicans* infections (15, 24-26). Intra-dermical (i.d.) immunization with native Sap2 plus Alum adjuvant conferred protection to BALB/c mice, against *C. albicans* peritonitis. The mechanism of protection was not completely elucidated, but antibodies by themselves were at least partially responsible for the protective effect (15). For the achievement of a candidate vaccine against acute systemic candidiasis, the availability of recombinant antigen would greatly overcome the well-known difficulties in producing, purifying and standardizing a native antigen.

The present studies were performed to evaluate the efficacy of alternative vaccine presentation strategies, using recombinant Sap2 (rSap2) in protecting immunocompetent BALB/c mice against hematogenously disseminated candidiasis. Moreover, as the hypha-associated isoenzyme Sap5 was preferentially expressed during systemic candidiasis (27), it was also evaluated as target antigen for *Candida* vaccination.

MATERIAL AND METHODS

Mice

Male BALB/c mice, 8-10 weeks old, were purchased from Charles River (Barcelona, Spain) and kept under specific pathogen-free conditions at the Animal Facility of Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal. All procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123), 86/609/EEC Directive and Portuguese rules (DL 129/92). Authorization to perform the experiments was issued by the competent national board authority (Direção Geral de Veterinária), document number 0420/000/000/2010.

Secreted aspartyl proteases

Recombinant Sap1 (rSap1), rSap2, rSap3 and rSap5 were produced in *Pichia pastoris* and were kindly provided by Dr Bernhard Hube (Jena University, Germany). All purified recombinant proteins exhibited protease activity *in vitro*, as assessed by using a standard fluorescence-based casein assay (Molecular Probes, Invitrogen, Eugene, OR, USA). Reactions were also performed in the presence of the general aspartyl protease inhibitor pepstatin A (Sigma Aldrich, St Louis, MO, USA). Native Sap2 (nSap2) was purified as previously described (15). Contaminant endotoxin was removed from all Sap samples by passage through a polymixin B column (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). Samples used were confirmed to be endotoxin-free, as assessed by the *limulus* test (E-toxate, Sigma).

In vitro splenocyte cell cultures and flow cytometric analysis

Spleen cells from BALB/c naive mice were obtained by gently teasing the organ in RPMI-1640 supplemented with penicillin (100 IU/mL), streptomycin (50 µg/mL), polymixin B (50 µg/mL), 2-mercaptoethanol (0.05 M) and 10% fetal bovine serum (all from Sigma). Cell suspensions were washed with RPMI, distributed in 96-well plates (1×10^6 cells/well) and cultured for 6 and 12 h at 37ºC in a humidified atmosphere containing 5% CO_2 in air. Plated cells were stimulated with medium alone or with different concentrations (6 to 100 µg per mL of culture medium) of recombinant or

native Sap2. rSap2 and nSap2 denatured by heat treatment for 2 min at 100ºC at the highest concentration were used to determine if Sap2-mediated biological effects were dependent on protein structure and function. Medium containing 2.5 µg/mL LPS or 2.5 µg/mL Concanavalin A were used as a positive controls. Polymixin B was added to each condition at a final concentration of 50 µg/mL to exclude contribution of endotoxin contamination to the measured Sap2-mediated biological effects. Each condition was set in triplicate. The cultured cells were washed and resuspended in PBS supplemented with 1% BSA and 10 mM sodium azide. The following monoclonal antibodies (mAbs) were used for immunofluorescence cytometric analysis in a FACScan (Becton-Dickinson, San Jose, CA, USA) using CELLQUEST software (Becton-Dickinson): FITC-conjugated rat anti-mouse B220 (clone), PE-conjugated hamster anti-mouse CD69 (clone), and FITC-conjugated rat anti-mouse CD4 (clone) (all from BD PharMingen, San Diego, CA, USA). Dead cells were excluded by propidium iodide (PI) incorporation.

***In vivo* treatments with Sap2**

Mice were i.p. injected with 250 µg of Sap2 in 0.5 mL of PBS. Control animals were injected with 0.5 mL of PBS alone.

ELISPOT assays

The numbers of splenic Ig-secreting cells were assessed by an ELISA-spot assay as described (28). Briefly, polystyrene microtitre plates (Nunc, Roskilde, Denmark) were coated overnight at 4ºC with 5 µg/mL goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, AL, USA) or with 10 µg/mL rSap2. The wells were then saturated for 90 min with 2% BSA in PBS at 37ºC. Appropriate serial suspensions of spleen cells in RPMI 1640 supplemented with 2% fetal calf serum (Invitrogen Life Technologies, Carlsbad, CA, USA) and 0.05% Tween 20 were incubated in the plates for 6 h at 37°C in a humidified atmosphere of 5% CO2 in air. The plates were then rinsed with 0.075% Tween 20 in water (Sigma) and washed four times with PBS containing 0.075% Tween 20. Ag-specific or total Ab-secreting cells were revealed by the addition of alkaline phosphatase-coupled monoclonal goat anti-mouse-Ig (Southern Biotechnology Associates) overnight at 4ºC. After washing, 5-bromo 4-chloro 3-indolyl

phosphate (Sigma) in 2-amino 2-methyl 1-propanol (Sigma) buffer was used as substrate for 2 h at 37ºC. After washing four times with distilled water, the number of spots was quantified in triplicate wells with a dissecting microscope.

Immunization protocols

Alum adjuvant. Mice were injected intra-dermically (i.d.) three times, with a 3-week intervening period, with 10 µg of rSap2 in 100µL of a 1:1 PBS/Alum suspension (Aluminium hydroxide gel; Brenntag, Frederikssund, Denmark, a kind gift of Dr Erik Lindblad, Biosector, Frederikssund, Denmark). The respective control animals received 100 µL of 1:1 PBS/Alum. Mice were also immunized by using 5 µg of rSap5 plus Alum adjuvant, twice at a 3-week interval.

Imiquimod adjuvant. Mice were injected subcutaneously (s.c.) four times, with a 1-week intervening period, with 20 µg of rSap2 in 100µL PBS, followed by topical application of Imiquimod (Aldara^{TM} cream, 5% Imiquimod, Laboratoires 3M Santé, Cergy Pontoise, France). The respective control animals received 100µL of PBS, followed by topical application of Imiquimod. The equivalent to 1.25 mg of Imiquimod was applied to each mouse in the site of injection. A similar immunogenic procedure was performed by using 5 µg of rSap5 as target antigen.

Freund's adjuvant. Mice were injected intra-dermically (i.d.) with 10 µg of rSap2 in a 1:1 PBS/Complete Freund suspension (Sigma). After a 3-week intervening period, mice were boosted with 10 µg of rSap2 in a 1:1 PBS/Incomplete Freund suspension (Sigma). The respective control animals received 100 µL of 1:1 PBS/Complete Freund followed by PBS/Incomplete Freund.

CpG adjuvant. Mice were immunized by an intramuscular administration of a single dose of a mixture containing 10 µg of rSap2, 10 µg of oligodeoxynucleotide (ODN) CpG (Invivogen, San Diego, CA, USA) and 0,3% Alum in a 30 µL final volume. Control mice were similarly immunized with PBS, CpG ODN and Alum. Mice immunized with 10 µg of rSap2,10 µg of non-CpG ODN (Invivogen) and Alum or PBS, non-CpG ODN and Alum were included as another control group.

Antibody detection

Specific anti-Sap2 immunoglobulin in mice sera, collected by submandibular bleeding, was quantified by enzyme-linked immunosorbent assay (ELISA). Briefly, polystyrene microtitre plates (Nunc) were coated with 5 µg/mL of rSap2, nSap2, rSap1, rSap3 or rSap5, and incubated overnight at 4ºC. Wells were then saturated for 1 h at room temperature with 2% BSA in 0.05% Tween 20-Tris buffered saline (TBST). Serial dilutions of the serum samples were then plated and incubated for 2 h at room temperature. After washing, alkaline phosphatase-conjugate goat anti-mouse IgG, IgG1 or IgG2a (all from Southern Biotechnology Associates) antibodies were added and incubation was kept for an additional 30 min at room temperature. After washing, the bound antibodies were detected by development with substrate solution containing p-nitrophenyl phosphate (Sigma) and the reaction was stopped by the addition of 0.1M EDTA, pH 8.0. The absorbance was measured at 405 nm. The ELISA antibody titres were expressed as the reciprocal of the highest dilution giving an absorvance of 0.1 above that of the control (no serum added).

Purification of serum IgG antibodies

Sera from mice inoculated i.d. with a 3-week intervening interval, with a 1:1 PBS/Alum suspension or 1:1 PBS/Alum suspensions containing 10 µg of rSap2, as described above in immunization protocol section, were collected and pooled 21 days after the second inoculation. Purification of IgG from these pooled sera was then performed as follows: pooled serum samples were equilibrated in binding buffer (20mM sodium phosphate, pH 7.0) by using Amicon Ultra-15 centrifugal devices (Millipore, Billerica, MA, USA). 3 ml of these preparations were applied to a Protein G HP affinity column (HiTrap; Amersham Biosciences, Bucks, UK) for each separation, according to manufacturer's instructions.

Immunoblotting

The reactivities of anti-Sap2 and anti-Sap5 immunoglobulins were visualized by Western blot analysis. For that purpose, 1 µg of rSap1, rSap2, rSap3, and rSap5, as well as native Sap2 were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels and then blotted overnight

onto nitrocellulose membranes (Sigma). Nonspecific binding was prevented by blocking with 3% BSA in Tris buffered saline (TBS) for 2 h at room temperature. After washing, membranes were incubated for 4 h with mouse polyclonal antibodies anti-rSap2 (5 μg/mL) or with nSap2 antiserum (1:200) diluted in TBST. Alternatively, membranes were incubated with rSap5 antiserum (1:200). Bound antibodies were detected with alkaline phosphatase-conjugate goat anti-mouse IgG (Southern Biotechnology Associates), using NBT/BCIP solution (Roche, Penzberg, Germany) as substrate. The reaction was stopped in distilled water.

Reactivity of the purified anti-rSap2 IgG were also tested by ELISA, as described above, in polystyrene microplates (Nunc) coated with 5 μg/mL of rSap1, rSap2, rSap3, rSap5, rSap6, and nSap2.

Candida albicans and culture conditions

The *C. albicans* wild-type strain SC5314 was used in this study (29) and maintained as frozen stocks in 30% glycerol at -80ºC. To prepare the inoculum for infection, *C. albicans* was grown in a shaking incubator for 14 hours at 30ºC in Winge medium (0,2% glucose, 0,3% yeast extract). Yeast cells were harvested, washed twice with sterile, nonpyrogenic phosphate-buffered saline (PBS) (Gibco®, Grand Island, NY, USA), counted in a haemocytometer and resuspended at the appropriate concentrations. Inocula were confirmed by Colony Forming Unit (CFU) counts on Sabouraud dextrose agar (Difco, Detroit, MI, USA) for up to 48 h at 37ºC.

C. albicans hematogenously disseminated infections

Mice were injected intravenously (i.v.), in the lateral tail vein with 2×10^5 *C. albicans* yeast cells in 0.2 mL PBS. To evaluate the progress of hematogenously disseminated candidiasis, mice were weighed and monitored twice daily, for a maximum of 30 days. Moribund mice were humanely terminated, and their deaths recorded as occurring the following day.

In some immunization protocols, a second group of similarly injected mice were infected with 1×10^5 and sacrificed 7 days post-infection to determine organ fungal burden. Kidneys were aseptically removed, homogenized, and quantitatively cultured

on Sabouraud dextrose agar (Difco) at 37ºC. Values were expressed as log CFU per gram of tissue.

Statistical Analysis

Unless otherwise stated, results shown are from one experiment, representative of at least three independent experiments. Statistical significance of results was determined by unpaired Student t-test, using the GraphPad Prism 4 Software (GraphPad Software, Inc., La Jolla, CA, USA). Results were considered statistically significant with *P* values of less than 0.05.

RESULTS

Sap2 has been shown to be an effective target antigen in immunoprotective protocols against systemic *C. albicans* infection established i.p. in BALB/c mice (15). Here, the same immunization protocol was extended to the murine model of hematogenously disseminated candidiasis by using rSap2 and rSap5, to evaluate their host protective effect in mice infected with *C. albicans* strain SC5314.

Immunomodulatory effects of rSap2

The ability of rSap2 to induce B- and T-lymphocyte activation was tested *in vitro*. Moreover, rSap2-mediated B-lymphocyte activation was also tested *in vivo*. rSap2 added to splenocyte cultures failed to induce the expression of the early activation marker CD69 on B and T lymphocytes (Figure 1).

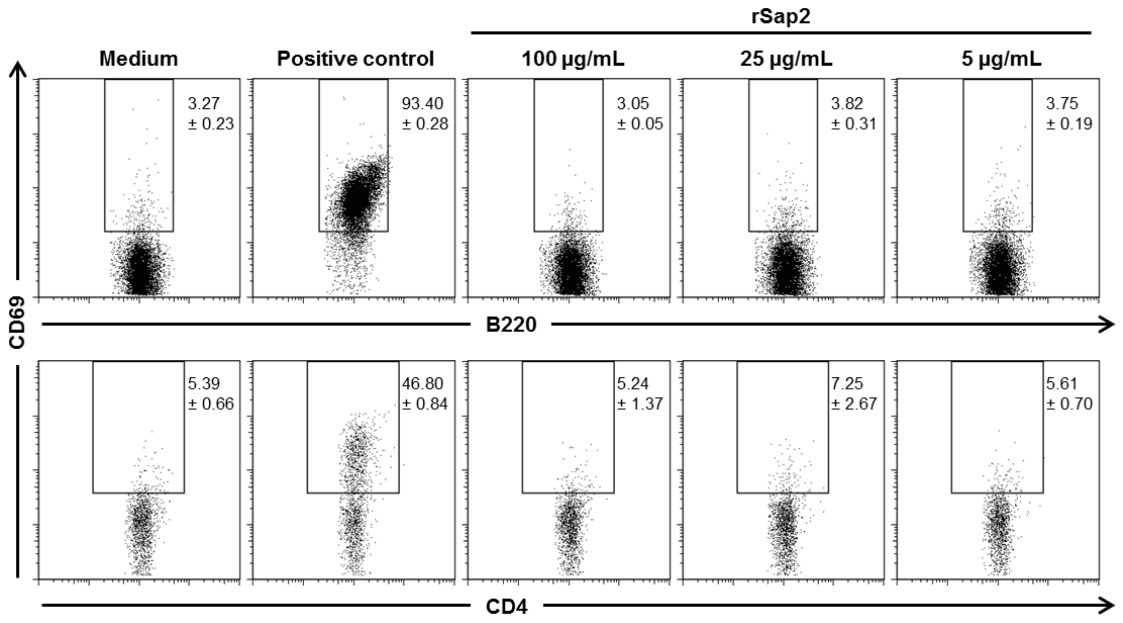

Figure 1 *In vitro* lymphocyte stimulatory effect of recombinant secreted aspartyl protease 2 (rSap2). Flow cytometric analysis of CD69 expression on the surface of BALB/c mice B ($B220^+$) and $CD4^+$ T cells in spleen mononuclear cell cultures after 12 hours of incubation with rSap2 or medium alone. LPS and ConA were used as positive controls for B and T cells, respectively. Numbers represent the mean values ± standard deviation (SD) of three samples per group. This is a representative result of four independent experiments.

Similarly, BALB/c mice that received 250 µg of rSap2 i.p. showed no significant increase in the number of splenic immunoglobulin-secreting cells when compared with control animals (Figure 2A). Moreover, rSap2 injection did not induce DC maturation, as no altered expression of the activation markers CD80, CD86 and MHC class II was observed on the surface of $CD11c^{hi}$ cells (Figure 2B). In summary, an immunomodulatory role of rSap2 could not be observed either *in vitro* or *in vivo*.

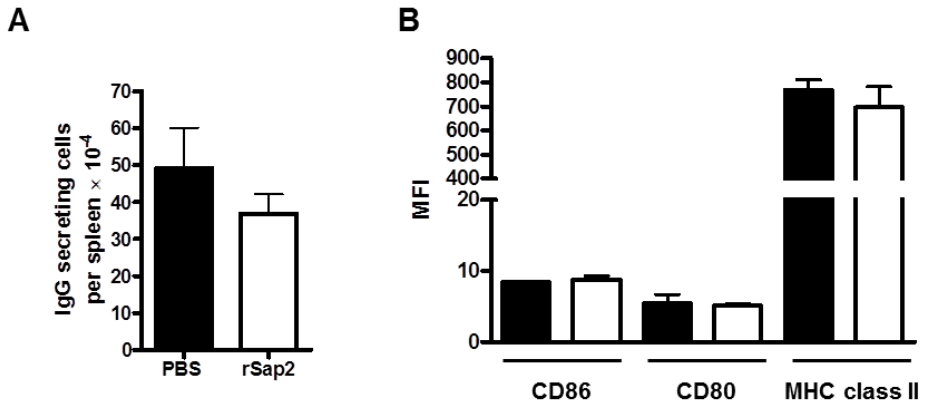

Figure 2 *In vivo* stimulatory effect of rSap2 in BALB/c mice, 5 days after i.p. treatment with phosphate-buffered saline (PBS) (black bars) or with 250 µg of rSap2 (white bars). (A) Numbers of spleen immunoglobulin-secreting cells. (B) Expression of CD80, CD86, and MHC class II molecules on the surface of spleen conventional dendritic cells, as assessed by the mean fluorescence intensities of antibody staining. The values represent the mean + 1 SD of four mice per group and are a representative result of two independent experiments.

Elevated anti-Sap2 and anti-Sap5 immmunoglobulins in the serum of immunized mice

To determine the immunogenic efficiency of the different rSap2 and rSap5 preparations, sera from mice immunized with different adjuvants were tested for the presence of antibodies specific for this protein. ELISAs were performed for the quantitative measurement of anti-Sap immunoglobulins in the sera collected before infection. The titres of anti-Sap2 and anti-Sap5 IgG were significantly higher in mice immunized with rSap2 and rSap5, respectively, than in the controls, regardless of the adjuvant or immunization protocol used. In mice immunized with rSap2 alone or rSap2 plus Alum, IgG1 was the main IgG isotype found in serum antibodies whereas IgG2a antibodies were mostly absent. In rSap5 plus Alum or imiquimod-immunized mice, only production of antigen-specific IgG1 was observed. Conversely, in mice immunized

with rSap2 using Imiquimod, Freund or CpG plus Alum as adjuvants, mixed IgG1 and IgG2a production was detected, although with IgG1 predominance (Figure 3).

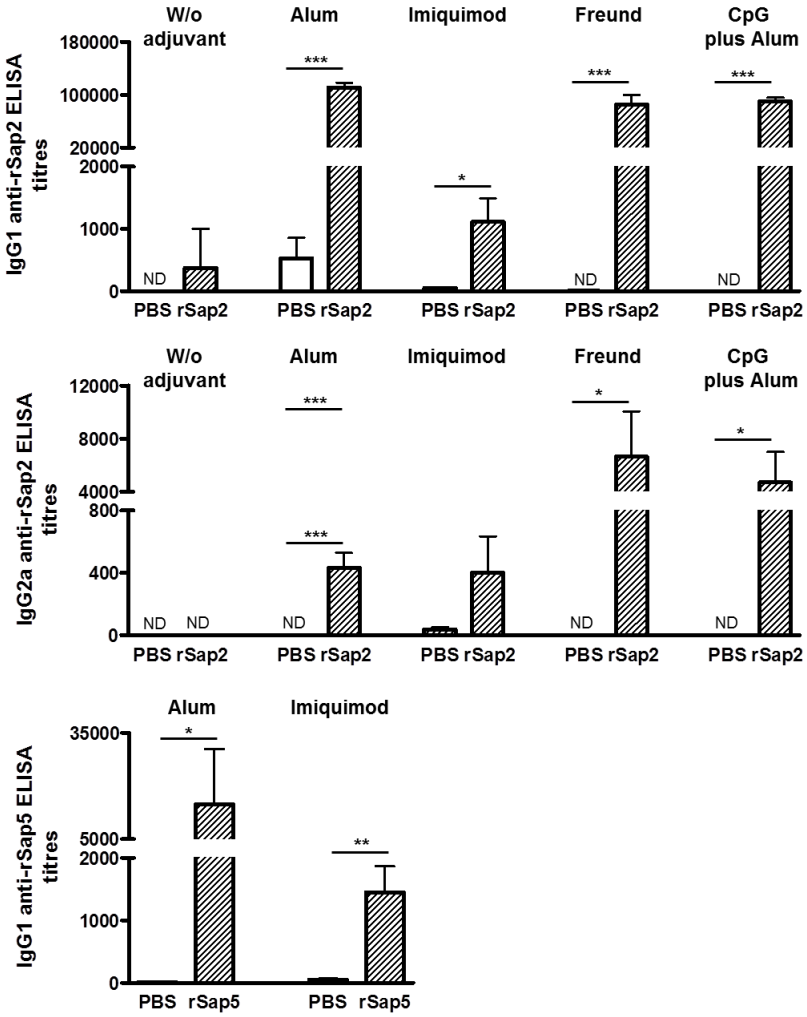

Figure 3 Serum IgG1- and IgG2a rSap2- or rSap5-specific antibody titres in mice sera, as indicated. Sera were collected 15 days after the last immunization for all groups except for the CpG group in which sera were collected 21 days after the single immunization. Bars represent mean antibody titres ± SD. n=4 to 8 mice per group. *$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

Western blot analysis was used to reveal purified IgG reactivity against blotted Sap isoenzymes. Specific reactivity was readily detectable in mice immunized with rSap2 against rSap2 and nSap2. Anti-rSap2 IgG also reacted with rSap1 and rSap3. In contrast, these immunoglobulins did not react with rSap5. The reaction was always stronger with sera from rSap2-immunized mice against rSap1, rSap2, rSap3 and nSap2 than the background anti-Sap2 reactivity detected in sera from control mice (Figure 4A). Sera from rSap5-immunized mice reacted only with rSap5 (Figure 4B). These

results were confirmed by ELISA. Unexpectedly, rSap2-specific antibodies recognized nSap2 with lower intensity than rSap2 (IgG titers of 77500 ± 26299 and 12750 ± 12473, specific for rSap2 and nSap2, respectively).

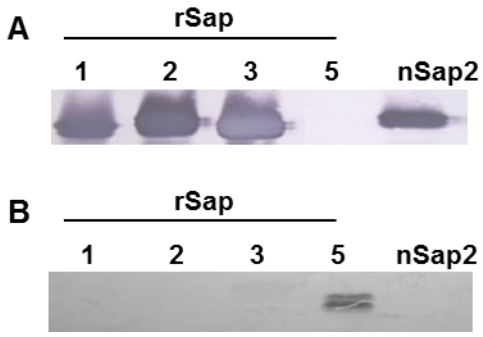

Figure 4 Western blot analysis of (A) anti-rSap2 IgG or (B) anti-rSap5 sera reactivities against the indicated antigens. Purified IgG antibodies and sera were obtained,15 days after the last immunization, from rSap2-immunized or rSap5-immunized mice, using Alum adjuvant.

Immunization with rSap2 did not confer protection against acute systemic candidiasis

Having ascertained that all immunization procedures assessed raised Sap-specific serum antibody levels, confirming the immunogenicity of the immunizing preparations, the protective effect of rSap2 immunization against hematogenously disseminated candidiasis was evaluated in the different groups of immunized mice.

Immunization with rSap2 in the absence of adjuvant

Mice immunized with rSap2 alone and sham-immunized controls had no detectable differences in the number of *C. albicans* CFU in the kidneys, on day 7 after inoculation i.v. with *C. albicans* (Figure 5A).

Immunization with Alum adjuvant

As observed by using rSap2 alone, no significantly different CFU counts were found in the kidneys of mice immunized with rSap2 plus Alum and controls, 7 days upon *C. albicans* infection (Figure 5B). In accordance, this immunization procedure did not protect mice against infection with a lethal *C. albicans* inoculum (Figure 5C). Both

sham-immunized and rSap2-immunized mice showed similar median survival times and all mice in these groups were dead by day 8 and day 10, respectively.

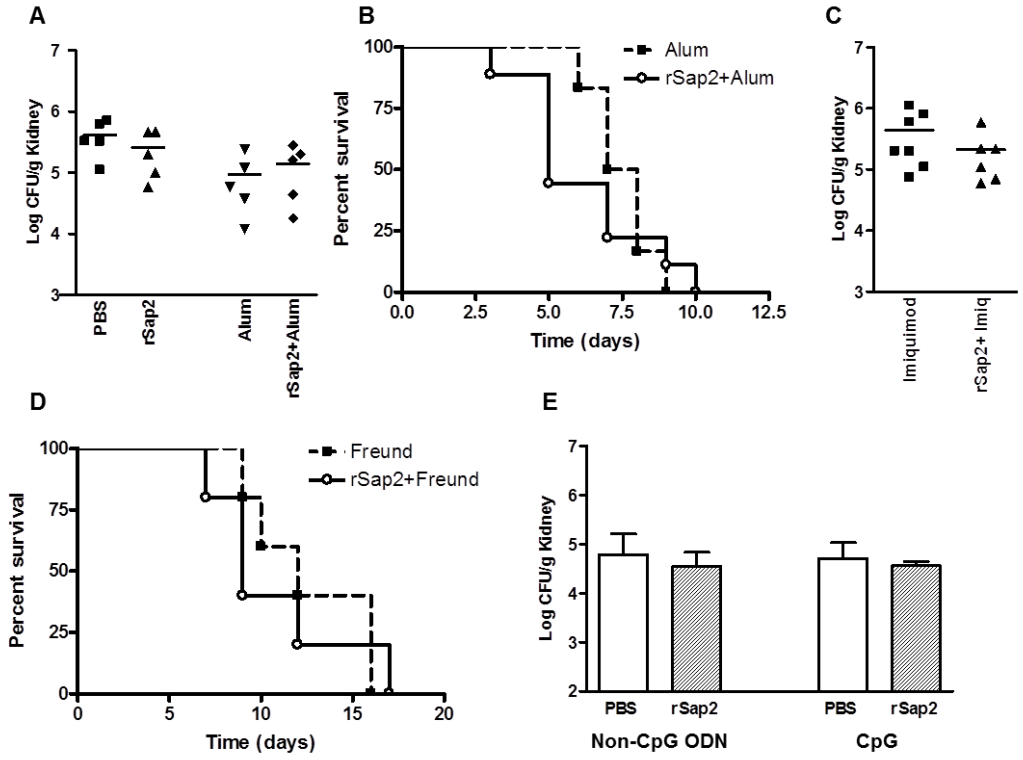

Figure 5 (A) CFU recovered from the kidneys of BALB/c mice immunized i.d. with rSap2 in PBS, rSap2 in alum adjuvant, or respective sham-immunized controls, and infected i.v. with 1×10^5 *C. albicans* SC5314 cells three weeks after the last i.d. immunization. Data are representative of two independent experiments. Each symbol represents an individual mouse, and horizontal bars are means of CFU numbers for each group; n=5. (B) Survival rates in mice immunized i.d. with rSap2 in alum adjuvant or sham-immunized controls and infected i.v. with 2×10^5 *C. albicans* cells, three weeks after the last i.d. immunization. The median survival rates of mice immunized with rSap2 plus alum and adjuvant inoculated mice, as determined using the Log rank test, were not different (*P*= 0.60); n=8; results are one representative experiment out of two independent experiments. (C) CFU recovered from the kidneys of BALB/c mice immunized s.c. with rSap2 plus topical Imiquimod application or sham-immunized controls and infected i.v. with 1×10^5 *C. albicans* cells 20 days after the last immunization. Each symbol represents an individual mouse, and horizontal bars are means of CFU numbers for each group; n=8; (D) Survival rates in mice immunized i.d. with rSap2 in Freund's adjuvant or sham-immunized controls and infected i.v. with 2×10^5 *C. albicans* cells, three weeks after the last i.d. immunization. The median survival rates of mice immunized with rSap2 plus Freund's and adjuvant inoculated mice, as determined using the Log rank test, were not different (*P*= 0.77); n=5; (E) CFU recovered from the kidneys of BALB/c mice immunized i.m. with rSap2 in CpG plus Alum adjuvant or sham-immunized controls and infected i.v. with1×10^5 *C. albicans* cells 30 days after the last immunization. Bars represent means ± SD; n=4.

Immunization with imiquimod adjuvant

Mice were immunized by subcutaneous (s.c.) injection with rSap2 followed by topical application of imiquimod. Sham-immunized animals were s.c. injected with PBS followed by similar imiquimod application. As shown in Figure 5D, no significant reduction in fungal kidney burden was observed in the immunized mice, comparatively to controls. Two out of eight immunized mice died before the experiment was terminated. Moreover, two other immunized mice were moribund by the time kidney CFU counts were assessed. Interestingly, these mice were the ones presenting the highest IgG levels (data not shown).

Immunization with Freund's adjuvant

As shown in Figure 5E, *C. albicans* infected mice immunized with rSap2 plus Freund's adjuvant showed survival rates equivalent to those of sham-immunized controls injected with PBS and adjuvant alone. Sham-immunized mice showed a median survival time of 12 days, while the median survival time of rSap2-immunized mice was 9 days, and all mice in these groups were dead by day 16 and day 17, respectively.

Immunization with CpG plus Alum adjuvant

Similarly to the other immunization approaches tested, immunization with rSap2 plus CpG/Alum adjuvant also failed to protect mice from an i.v. *C. albicans* challenge, as the number of kidney CFU was not significantly different between the immunized mice and controls (Figure 5F). A mouse of the rSap2 plus CpG/Alum-immunized group died prior to the termination of the experiment.

Altogether, these results showed that no protection against systemic *C. albicans* infection established by the hematogenous route was obtained by immunizing mice with rSap2, independently of the used adjuvant.

Immunization with rSap5 failed to protect mice against acute systemic candidiasis

As observed by using rSap2 as target antigen, immunization with rSap5 with Alum or imiquimod adjuvants did not protect mice from a hematogenous challenge with *C. albicans* cells. No significantly different CFU counts were found in the kidneys of mice

immunized with rSap5 plus either adjuvant and controls, 7 days upon *C. albicans* infection (Figure 6A). Also no extended survival was observed in rSap5 plus Alum-immunized mice, as compared to sham-immunized controls (Figure 6B).

Immunization with rSap5 using imiquimod as adjuvant, also failed to reduce kidney fungal burden, comparative to that of controls (Figure 6C).

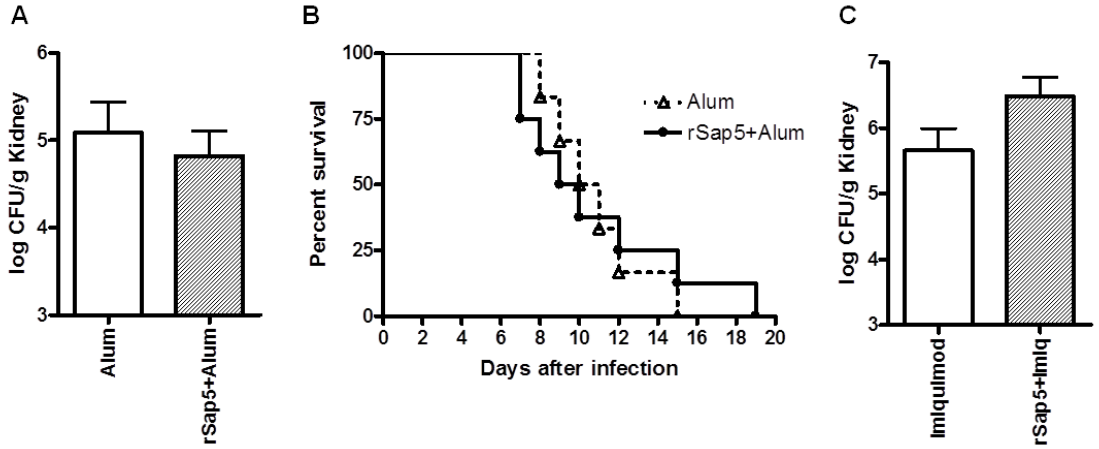

Figure 6 (A) Numbers of *C. albicans* CFU recovered from the kidneys of BALB/c mice immunized twice i.d. with rSap5 in Alum adjuvant, or respective sham-immunized controls, and infected i.v. with 1×10^5 *C. albicans* cells 3 weeks after the last i.d. immunization. Bars represent means ± SD; n=6. (B) Survival rates in mice immunized i.d. with rSap5 in Alum adjuvant or sham-immunized controls and infected i.v. with 2×10^5 *C. albicans* cells, three weeks after the last i.d. immunization. The median survival rates of mice immunized with rSap5 plus Alum and adjuvant inoculated mice, as determined using the Log rank test, were not different (*P=0.91*); n=8 for rSap5 plus Alum group and n=6 for sham-immunized group.

DISCUSSION

Previous reports have shown that mice immunized with Sap2 were protected against mucosal or peritoneal *C. albicans* infection (15, 24, 25, 30). Here immunization with Sap antigens was done, using different adjuvants and immunization schedules, to attempt protect mice against acute systemic candidiasis.

Alum is a licensed adjuvant used in human vaccine formulations (31). It was previously employed to successfully immunize mice against *C. albicans* peritonitis (Vilanova et al., 2004). Therefore, it was used here as a first choice adjuvant in order to assess the protective effect of rSap2 and rSap5 as target antigens in a murine model of hematogenously disseminated candidiasis. Nevertheless, as this adjuvant usually favors an antibody-mediated immune response, other adjuvants that promote a cellular-mediated Th1-type immune response, such as imiquimod, CpG or Freund's (32), were alternatively used for immunization with Sap proteins. Imiquimod was approved to be used for topical skin treatment in humans. It binds to TLR7 in mice and TLR7 and 8 in humans and stimulates the production of a characteristic cytokine profile, promoting a Th1 bias (33). The adjuvant effects of imidazoquinolines, including imiquimod, specifically increase IgG2a responses (34, 35). Although not yet licensed for human usage, the TLR 9 agonist CpG (oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs) has been safely used in human clinical trials (36, 37). It directly stimulates human B lymphocytes and plasmacytoid dendritic cells to produce pro-inflammatory cytokines, leading to the induction of Th1 responses and triggering the activation of APCs (38-40). Moreover, it was shown to be more effective than Alum and imidazoquinolines in augmenting both humoral and cell mediated immune responses (40, 41). Furthermore, when used in combination with Alum, it had greater potential to augment immune responses and provided higher antibody titres than Freund's complete adjuvant, with only minimal tissue damage (40).

Despite the powerful properties of the used adjuvants, the lack of observed immune protection in mice infected i.v. with *C. albicans* may nevertheless indicate that those adjuvants were inadequate for the generation of a protective immune response. Anti-sap2 IgG antibodies were previously shown to be host protective against *Candida* peritonitis (15), oral (24) and vaginal *Candida* infection (24, 25, 30). Thus, it could be

hypothesized that the magnitude of the immune response elicited by the immunization procedures assayed here was below effective levels. However, the anti-rSap2 titres were similar or even higher than the ones reported for mice immunized with native Sap2 and Alum (15). Nevertheless, mice presenting higher rSap2-specific antibody titres, upon immunization with rSap2 plus Imiquimod, became more susceptible to the i.v. infection. The immune response elicited by immunization could actually be deleterious to the host. Although with IgG1 preponderance, a mixed IgG1/IgG2a isotypic profile was observed in these mice, which presented antibody titres below those detected by using the other adjuvants. Other immune mechanisms than antibody production could have been elicited by the Imiquimod adjuvanted immunization, resulting in uncontrolled inflammation. Alternatively, the lack of protection in the immunized mice may indicate that rSap2 was an inadequate target antigen. We actually observed that sera collected from rSap2-immunized mice reacted more intensely with the recombinant protein than with nSap2. This differential reactivity was only observed in ELISA assays, in which no denaturation of the antigen was expected. This might indicate that structural differences may exist between recombinant and native Sap2 that might affect the protein epitopes recognized by the antibodies. The usage of rSap5 as target antigen was also ineffective in inducing protection. However, it must be noted that rSap5-specific antibody titres raised by immunization were lower than the ones reached by using rSap2. It could have been expected that rSap5 was more promising as a target antigen in systemic candidiasis than rSap2, as Sap5 expression has been associated with invasive candidiasis (27, 42). Nevertheless, *C. albicans* could be less dependent on Sap2 activity to successfully infect the host in the acute model of systemic candidiasis than in other infection models, in which vaccination with Sap2 was found to be protective. The reported observation that Sap inhibitor pepstatin A was ineffective in protecting mice against hematogenously disseminated candidiasis supports this hypothesis (43). Moreover, in this infection model, Sap1 to Sap3 proved to be unnecessary for *C. albicans* infection establishment, as mutant stains deficient in *SAP1* to *SAP3* were equivalent in virulence to the wild-type strain. Furthermore, mutant strains lacking *SAP4* to *SAP6* presented only a marginal virulence attenuation (44). It is widely recognized that the relative importance of specific *SAP* genes for *C. albicans* pathogenicity is greatly determined by

the type of infection and its dependence on protease activity for the successful invasion and colonization of various host niches (44-47). Thus, immunization strategies targeting Sap2 would be more effective in controlling *C. albicans* infections dependent on protease activity than in the murine model of acute systemic candidiasis which is relatively protease independent. In addition, in the present work we have used *C. albicans* SC5314 strain to infect mice. This strain was not used in any of the previous reports where immunization with Sap2 was assessed (15, 24, 25, 30). Thus, we cannot exclude that protection conferred by this immunization strategy might be strain dependent and that the *C. albicans* strain used here to infect the immunized mice could not strictly depend on Sap2 and Sap5 activities to successfully colonize the host.
Overall the results presented herein indicate that immunization with *C. albicans* Sap proteins may have no effect in protecting the host against candidiasis established by the hematogenous route. Nevertheless, they do not completely rule out the possibility that using Sap antigens combined with other adjuvants might result in host protection in this infectious condition.

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CHAPTER 7

Concluding Remarks

CONCLUDING REMARKS

The increasing incidence of invasive candidiasis, associated to a high treatment failure rate, fully justify the search for more effective prophylactic and therapeutic strategies. To fulfil this goal, a more comprehensive understanding is needed of *C. albicans* virulence mechanisms and of the host immune response to this fungus, in the different types of candidiasis. Moreover, several studies have underlined that distinct *C. albicans* isolates may show very distinct virulence phenotypes and elicit dissimilar immune responses, compromising the generalization of results obtained with a single isolate to the whole species. In addition, the majority of those studies gave little information of the fungus-host interactions and elicited host immune responses, since they focused on phagocytosis and intracellular killing assays. The studies presented here comparatively evaluating the virulence of three reference *C. albicans* strains in the murine model of hematogenously disseminated candidiasis show remarkable differences among them. Interestingly, the highest virulent strain studied here, SC5314, induced a more marked inflammatory response with a high recruitment of neutrophils and inflammatory monocytes into the spleen and kidney tissues. In general, the most virulent strains tested here generated higher inflammation, which may be worsening organ pathology rather than protecting the host. These results might explain why SC5314 strain, well known to be highly virulent in this infection model, in which immune protection mainly depends on neutrophils, is much less efficient at infecting the mucosa, where cell mediated immunity is essential for host protection. In future studies concerning *C. albicans* virulence, it would be advisable to assess pathology together with an analysis of local immune response in affected organs. Also, it would be interesting to compare the cell wall composition and cell surface associated proteins, such as Sap9 that modulates the interaction of *C. albicans* with human neutrophils.

The vast majority of gene disruption studies have been carried out in the SC5314 background. As this strain elicits an uncontrolled host inflammatory response that results in sepsis and host death soon after intravenous challenge, this may negatively affect mutant virulence assessment. Other factors affecting *C. albicans* mutants

virulence assessment are undesired effects that may result from their construction. The majority of *C. albicans* mutant strains were constructed by using the Ura-blaster technique, which has been shown to generate mutant strains with altered *URA3* expression, influencing *C. albicans* virulence. Although this can now be overcome by the directed integration of *URA3*, mutant strains used in earlier studies did not share a site of *URA3* integration and virulence phenotypes of all these mutants should thus be confirmed. Therefore, the importance of *SAP1* to *SAP6* was re-evaluated in a model of acute systemic candidiasis by using mutant strains constructed with the *SAT1*-fliping strategy. The obtained results show that Δ*sap123* and Δ*sap456* are as virulent as the WT strain contrasting the reduced virulence of equivalent Ura-blaster generated mutants, for which the Ura-status must have thus contributed to their attenuated virulence. Altogether, these results suggest that Sap1 to Sap6 do not play a significant role in *C. albicans* virulence in hematogenously disseminated candidiasis. Nevertheless, as shown in this thesis, Sap4 to Sap6 play a role in the infectious process of *C. albicans* peritonitis. In Δ*sap456*-infected mice, the lower recruitment of Treg into the kidneys early after challenge might facilitate the arrival or activation of effector cells, therefore enhancing fungal clearance. Also, $CD4^+CD25^+$ T cells sorted from these mice presented a much lower ability for *in vitro* suppression of T cell proliferation and production of the immunosuppressive cytokine IL-10 than WT-infected mice. These observations are in agreement with, and support, the hypothesis that Sap4 to Sap6 play a role in the impairment of the host immune response to *Candida* peritonitis. Further studies are needed to determine the mechanism by which Sap4-6 interfere with the host immune response. As no differences were observed between the used WT and *sap*-null strains in their ability to activate DCs, other cell targets, such as monocytes or macrophages should be studied in more detail. Indeed, inflammatory monocytes were differentially recruited into the peritoneal cavity of mice infected with the WT or Δ*sap456* strains. Another mechanism needing to be clarified concerns Treg function in WT or *sap*-null infected mice. It would be important to ascertain the role of IL-10 in the suppressive activity of these cells. Antibody-mediated neutralization of this cytokine or cell transfer experiments using IL-10-deficient mice as recipients may be useful in that regard. Altogether, the results obtained with the *sap*-null mutant strains, in two different infection models, further highlight that the importance of specific *SAP* genes for *C.*

albicans pathogenicity is greatly determined by the type of infection and its dependence on protease activity for the successful invasion and colonization of various host niches. These Saps may be necessary to degrade human proteins, enabling nutrient acquisition, host tissue invasion, and immune evasion. The pattern recognition receptor Gal-3 selectively binds to *C. albicans* β-1,2-oligomannosides leading to fungal death and increased production of the inflammatory cytokine TNF-α upon *C. albicans* recognition. In this study, *C. albicans* Sap1, Sap2 and Sap3 isoenzymes were shown to degrade and inactivate Gal-3 *in vitro*. These results suggest that host Gal-3 might be also cleaved *in vivo* by Saps. Hence, *C. albicans* may degradate Gal-3 to control and evade host immune Gal-3-mediated recognition, and the ensuing inflammatory response, or even avoid Gal-3-mediated *C. albicans* killing. In future studies, co-localization of Sap1-3 or Sap4-6 and Gal-3 in the infected tissues would provide a more compelling evidence of the Gal-3 degradation *in vivo*. It would be also important to use other infection models, such as experimental vaginal or oral candidiasis, where a prominent role of Sap2 in infection was already demonstrated, to determine the importance of Sap-mediated Gal-3 degradation in the establishment of candidiasis.

The above-mentioned results suggest that *C. albicans* Sap isoenzymes may be one of the mechanisms used by this fungus to dampen host immune response, making these proteins natural target antigen candidates in *C. albicans* vaccination. Previous reports have shown that mice immunized with Sap2 were protected against mucosal or peritoneal *C. albicans* infection. Here, a similar approach was attempted to protect mice against hematogenously disseminated candidiasis. Mice were immunized with rSap2 and rSap5 as target antigens, using different adjuvants and immunization schedules. All the tested approaches failed to protect against this type of candidiasis. In some occasions, the immune response elicited by immunization could actually have been deleterious to the host, resulting in uncontrolled inflammation. Although in this infection model Sap1 to Sap3 proved unnecessary for *C. albicans* infection establishment, as mutant stains deficient in *SAP1* to *SAP3* were equivalent in virulence to the WT strain, immunization with Sap2 could nevertheless result in host protection. It would be expected that rSap5 was more promising as a target antigen in systemic

candidiasis than rSap2, since Sap5 expression has been associated with invasive candidiasis. Nevertheless mutant strains lacking *SAP4* to *SAP6* presented only marginal virulence attenuation in this infection model. The results described here indicate that *C. albicans* is less dependent on Sap activity to successfully infect the host in hematogenously disseminated candidiasis than in *C. albicans* peritonitis, for which vaccination with Sap2 was protective. The observation reported by others that Sap inhibitor pepstatin A was ineffective in protecting mice against hematogenously disseminated candidiasis supports this hypothesis. Thus, immunization strategies targeting Sap2 would be more effective in controlling *C. albicans* infections more dependent on protease activity than in the murine model of hematogenously disseminated candidiasis, which is relatively protease independent. Nevertheless, it cannot be excluded that protection conferred by this immunization strategy might be strain dependent. In fact, it is shown here that strain SC5314 elicits a host immune response with a high inflammatory character, which might have contributed to the lack of protection. It would be interesting to determine whether vaccination with Saps may nevertheless be protective in this infection model, when using strains causing a less marked inflammatory response to establish infection. As the cell surface associated Sap9 was shown by others to have a major impact on recognition of *C. albicans* by neutrophils, promoting the activation and chemotaxis of these leukocytes, it would be interesting to use this antigen to attempt vaccination against yeast strains inducing an exacerbated neutrophilic inflammation, such as SC5314.

Overall, the results presented herein provide additional evidence for the differential involvement of Saps in distinct *C. albicans* infection models and, by showing that host immune response to *C. albicans* is affected by lack of *SAP* expression, support a previously hypothesized immunomodulatory role for Saps. They also indicate that immunization with *C. albicans* Sap proteins may have no effect in protecting the host against candidiasis established by the hematogenous route, reinforcing the limited role of these proteins in this type of infection. Nevertheless, they do not completely rule out the possibility that Sap antigens, the ones used here or other, with different adjuvant combinations, might result in host protection in this type of disseminated infection.