UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO
DEPARTAMENTO DE BIOQUÍMICA E IMUNOLOGIA

Mateus Silveira Freitas

Avaliação do potencial imunomodulador e protetor de vesículas extracelulares produzidas por *Aspergillus fumigatus*

Ribeirão Preto
2023

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MATEUS SILVEIRA FREITAS

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Tese apresentada à Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo para obtenção do título de Doutor em Ciências.

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Resumo

FREITAS, M. S. **Avaliação do potencial imunomodulador e protetor de vesículas extracelulares produzidas por *Aspergillus fumigatus*.** 2023 Tese (Doutorado) em Imunologia Básica e Aplicada – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2023.

Aspergillus fumigatus é o principal agente causador da aspergilose invasiva, doença que apresenta alta taxa de mortalidade, e acomete principalmente indivíduos imunocomprometidos. *A. fumigatus* apresenta distribuição mundial e grande produção de conídios, fazendo com que o trato respiratório humano seja continuamente exposto aos conídios fúngicos. Normalmente, a destruição de conídios e o controle da infecção, é realizado por células da imunidade inata. Essas são capazes de identificar o fungo e seus produtos respondendo de forma adequada. Nesse sentido, temos que as vesículas extracelulares (EVs) produzidas pelo fungo podem interagir com células do sistema imune modulando sua resposta. As EVs são estruturas esféricas formadas por uma bicamada lipídica que possuem várias ações descritas. Podem atuar na comunicação celular, no desenvolvimento da parede celular, na aquisição de resistência a antifúngicos e na modulação da resposta imune do hospedeiro. Importantes estudos têm descrito a interação de EVs fúngicas com células do sistema imune inato, demonstrando potencial imunomodulador dessas EVs. Dessa forma, nos propomos a verificar a capacidade imunomoduladora de EVs de *A. fumigatus*, principalmente sobre células da imunidade inata. Nesse estudo, estimulamos células da imunidade inata e células mononucleares do sangue periférico (PBMCs, do inglês *peripheral blood mononuclear cell*) com EVs de *A. fumigatus* e avaliamos a proteção conferida pelas EVs em modelo *in vivo* de *Galleria mellonella*. Adicionalmente, verificamos que macrófagos RAW 264.7 e AMJ2-C11 foram capazes de produzir TNF-α quando estimulados com EVs, mas não NO, IL-6, IL1-β e superóxido. Entretanto, macrófagos RAW 264.7 aumentaram a expressão de iNOS e Arginase-1, enquanto macrófagos AMJ2-C11 aumentaram somente Arginase-1. O estímulo com EVs produzidas por *A. fumigatus* foi capaz de induzir alteração conformacional em células RAW 264.7, mas não em AMJ2-C11, como verificado por microscopia de luz e pela maior transcrição de C11b e CD18. Ainda, em neutrófilos estimulados com as EVs de *A. fumigatus*, não houve a produção de NETs (do inglês *neutrophil extracelular trap*) e tão pouco de TNF-α ou IL-10, enquanto PMBCs foram capazes de produzir IL-6, mas

não TNF-α e IL-10. Apesar da relativamente baixa resposta imune gerada, a administração de EVs em *G. Mellonella* foi capaz de proteger a larva frente ao desafio fúngico. Nossos resultados sugerem que EVs de *A. fumigatus* possuem limitada ação inflamatória em células da imunidade inata, porém exercem um papel na proteção em modelo experimental de infeção fúngica.

Palavras-chave: *Aspergillus fumigatus*. Vesículas extracelulares. Inflamação. Leucócitos inatos. Proteção.

Abstract

FREITAS, M. S. **Evaluation of the immunomodulatory and protective potential of extracellular vesicles produced by *Aspergillus fumigatus*.** 2023 Tese (Doutorado) em Imunologia Básica e Aplicada – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2023.

Aspergillus fumigatus is the main causative agent of invasive aspergillosis, a disease that has a high mortality rate and may affects immunocompromised patients. *A. fumigatus* has worldwide distribution and large conidia production, causing the human tract to be continuously exposed to conidia. Usually, conidia destruction and infection control are carried out by innate immune cells. They can identify the fungus and their products and respond appropriately. In this sense, we have the extracellular vesicles (EVs) produced by the fungus could interact with immune cells modulating its response. EVs are spherical structures formed by a lipid bilayer that have several described functions. They may act in cell communication, cell wall development, acquisition of resistance to antifungal agents, and modulation the host immune response. Important studies have described the interaction of fungal EVs with cells of the innate immune system, demonstrating the immunomodulatory potential of these EVs. This way we proposed to verify the immunomodulatory capabilities of *A. fumigatus* EVs, primarily over innate immunity cells. In this study, we stimulated innate immune cells and peripheral blood mononuclear cells (PBMCs) with *A. fumigatus* EVs and evaluated the conferred protection by EVs through *in vivo* model of *Galleria mellonella*. Furthermore, we verified that RAW 264.7 and AMJ2-C11 macrophages were able to produce TNF-α when stimulated with EVs, but not NO, IL-6, IL1-β, and superoxide. However, RAW 264.7 macrophages increased the expression of iNOS and Arginase-1, while AMJ2-C11 macrophages only increased Arginase-1. Stimulation with *A. fumigatus* EVs was able to induce conformational change in RAW 264.7 cells, but not in AMJ2-C11, as verified by light microscopy and increased transcription from C11b and CD18. Furthermore, neutrophils stimulated with *A. fumigatus* EVs, were not able to produce neutrophil extracellular traps (NETs) neither TNF-α and IL-10, while PMBCs were able to produce IL-6, but not TNF-α and IL-10. Despite the relatively low immune response produced, the administration of *A. fumigatus* EVs in *G. mellonella* was able to protect the larva against the fungal challenge. Our results suggest that *A. fumigatus*

EVs possess a limited inflammatory action over innate immune cells, however, exert a role in the protection against the fungal infection.

Keywords: *Aspergillus fumigatus*. Extracellular vesicles. Inflammation. Innate leukocytes. Protection.

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Lista de abreviaturas e siglas

×g	Rotações em *g*
µg	Microgramas
µm	Micrômetro
µM	Micromolar
A. flavus	*Aspergillus flavus*
A. fumigatus	*Aspergillus fumigatus*
A. nidulans	*Aspergillus nidulans*
ABPA	Aspergilose broncopulmonar alérgica
AIDS	Síndrome da imunodeficiência adquirida
BOD	Demanda Bioquímica de Oxigênio
C. albicans	*Candida albicans*
C. auris	*Candida auris*
C. neoformans	*Cryptococcus neoformans*
C1q	Complexo C1q
CCL	*C-C Motif Chemokine Ligand*
CD	*Cluster of differentiation*
cDNA	DNA complementar
CFU	Unidades formadoras de colônias
CGD	Doença granulomatosa crônica
CLR	Receptor de lectina tipo C
CO_2	Dióxido de carbono
CPA	Aspergilose pulmonar crônica
CR	Receptor de complemento
CXCL	C-X-C *motif chemokine ligand*
DCs	Células dendríticas
DMEM	*Dulbecco's Modified Eagle Medium*
DNA	Ácido desoxirribonucleico
EDTA	Ácido etilenodiamino tetra-acético
EGTA	Ácido egtazic
EVs	Vesículas extracelulares
FACS	Aparelho de citometria de fluxo
FCγR	Receptor da porção FC de IgG
FleA	*A. fumigatus lectin*
G. mellonella	*Galleria mellonella*
Gapdh	Gliceraldeído-3-fosfato desidrogenase
G-CSF	Fator estimulador de colônia de granulócitos
GM-CSF	Fator estimulador de colônia de granulócitos e macrófagos
GXM	Glucuronoxilomanana
h	Hora
H_3PO_4	Ácido fosfórico
HLA	*Human leukocyte antigen*
HOCl	Ácido hipocloroso
HSCT	Células-tronco hematopoéticas
IFN	Interferon
Ig	Imunoglobulina
IL	Interleucina
iNOS	Óxido nítrico sintase induzível
IPA	Aspergilose pulmonar invasiva
KH_2PO_4	Fosfato monopotássico
LPS	Lipopolissacarídeo
Mac-1	Antígeno de macrófago - 1
MHC-II	Complexo principal de histocompatibilidade - II
min	Minutos
MIP	Proteína inflamatória de macrófagos
mL	Mililitro
mRNA	RNA mensageiro
MyD88	*Myeloid differentiation primary response 88*
NADPH	Fosfato de dinucleótido de nicotinamida e adenina
NETs	Armadilhas extracelulares de neutrófilos
NF-κB	Factor nuclear kappa B
NK	Células natural killer
nm	Nanômetro
NO	Óxido Nítrico
NTA	*Nanoparticle-Tracking Analysis*
O2-	Superóxido
ºC	Graus celcius
P. brasiliensis	*Paracoccidioides brasiliensis*
Pam3CSK4	Pam3CysSerLys4
PAMPs	Padrões moleculares associados a patógenos
PBMCs	Células mononucleares de sangue periférico

PBS	Tampão Salina-Fosfato
pH	Potencial hidrogeniônico
PMA	*Phorbol myristate acetate*
PMNs	*Peritoneal polymorphonuclear neutrophils*
PRRs	Receptores de reconhecimento padrão
PTX3	Pentraxina 3
qPCR	PCR quantitativa em tempo real
RLU	*Relative light unit*
RNA	Ácido robonucleico
ROS	Espécies reativas de oxigênio
rpm	Rotações por minuto
SP-D	Proteína surfactante D
T. interdigitale	*Trichophyton interdigitale*
Th	Linfócito T helper
TLR	Receptor do tipo toll
TNF-α	Fator de necrose tumoral - alfa
Tγδ	Células T gama delta
WT	*Wild type*

Sumário

Capítulo I – Considerações gerais

1. Introdução

1.1. *Aspergillus fumigatus* e aspergilose

Os fungos pertencentes ao gênero *Aspergillus* são organismos saprófitos, ou seja, são capazes de utilizar matéria orgânica em decomposição pela liberação de enzimas específicas no meio extracelular (TEKAIA; LATGE, 2005). Por serem saprófitos, são encontrados em matéria orgânica em decomposição, além do solo e do ar, de modo que estão distribuídos de forma global (VAN DE VEERDONK; GRESNIGT; ROMANI; NETEA *et al.*, 2017). Como forma de reprodução assexuada, os fungos do gênero *Aspergillus*, são capazes de produzir grandes quantidades de conídios, por meio de uma estrutura chamada conidióforo (MCCORMICK; LOEFFLER; EBEL, 2010; VAN DE VEERDONK; GRESNIGT; ROMANI; NETEA *et al.*, 2017). Os conídios liberados dessa forma são capazes de colonizar outras áreas com material orgânico ou podem ser inalados por indivíduos, que podem vir a desenvolver a aspergilose (SEGAL, 2009).

Aspergillus fumigatus é a espécie desse gênero que mais desperta o interesse médico, pois é a principal causadora da aspergilose, uma infecção fúngica que pode ser fatal (ABAD; FERNANDEZ-MOLINA; BIKANDI; RAMIREZ *et al.*, 2010; BRAKHAGE; LANGFELDER, 2002; MORGAN; WANNEMUEHLER; MARR; HADLEY *et al.*, 2005). Algumas características de *A. fumigatus* o fazem o maior causador da aspergilose, incluem: liberação de grandes quantidades de conídios e sua efetiva dispersão pelo ar, termotolerância, capacidade de adquirir nutrientes no ambiente pulmonar e presença de hidrofobinas e melanina no conídio, que ajuda na proteção frente a ação do sistema imune (AKOUMIANAKI; KYRMIZI; VALSECCHI; GRESNIGT *et al.*, 2016; FANG; LATGE, 2018; FONTAINE; DELANGLE; SIMENEL; CODDEVILLE *et al.*, 2011; LATGE, 2001).

De fato, os conídios liberados por *A. fumigatus* apresentam maior capacidade de dispersão no ar, devido a sua característica hidrofóbica, esse dado é confirmado pela maior quantidade de conídios de *A. fumigatus* no ar, quando comparado com outras espécies de *Aspergillus* (ABDEL HAMEED; YASSER; KHODER, 2004; KWON-CHUNG; SUGUI, 2013; TAHA; POLLARD; SARKAR; LONGHURST, 2005). Essa característica hidrofóbica dos conídios é derivada de uma camada externa de proteínas hidrofóbicas, as hidrofobinas, sendo RodA a principal hidrofobina presente nos conídios de *A. fumigatus* (VALSECCHI; DUPRES; STEPHEN-VICTOR;

GUIJARRO *et al.*, 2017). Um experimento interessante demonstrou que uma cultura de 7 dias de *A. fumigatus* era capaz de disseminar seus esporos para outras 8 placas colocadas ao redor da primeira, quando na ausência de fluxo de ar, porém em um mesmo experimento, utilizando *A. nidulans*, foi verificado que as placas adjacentes não apresentaram nenhum crescimento fúngico (KWON-CHUNG; SUGUI, 2013). Além disso, os conídios de *A. fumigatus* são recobertos por uma camada de melanina que os protege da radiação UV (BRAKHAGE; LIEBMANN, 2005).

Outra característica de *A. fumigatus* que o faz ser capaz de causar doença em humanos é sua capacidade de se estabelecer em diferentes ambientes, com grandes variações de temperatura e pH. A temperatura ótima de crescimento de hifas *A. fumigatus* é de 37ºC, porém há relatos que descrevem que ele pode suportar temperaturas entre 12ºC e 75ºC (ABAD; FERNANDEZ-MOLINA; BIKANDI; RAMIREZ *et al.*, 2010; KOZAKIEWICZ; SMITH, 1994). Sua capacidade de reciclar matéria orgânica em decomposição, confere ao *A. fumigatus* uma capacidade de se adequar a vários tipos de condições ambientais, sendo capaz de produzir uma vasta gama de proteinases extracelulares e enzimas capazes de clivar açúcares complexos (ABAD; FERNANDEZ-MOLINA; BIKANDI; RAMIREZ *et al.*, 2010; FANG; LATGE, 2018). De fato, sua capacidade de termotolerância aliada com a capacidade de degradar proteínas e carboidratos do meio extracelular para adquirir energia para seu metabolismo pode estar relacionada com a sua capacidade de se estabelecer e prosperar nos pulmões de indivíduos imunocomprometidos acometidos (VAN DE VEERDONK; GRESNIGT; ROMANI; NETEA *et al.*, 2017). Por fim, a composição da parede celular fúngica também influencia na capacidade infectiva do fungo. Sua composição é variável, a depender do seu estágio de maturação (LEE; SHEPPARD, 2016), bem como do perfil imunológico do hospedeiro, como verificado em ensaios de infecção *in vivo* (LATGE; BEAUVAIS; CHAMILOS, 2017). A camada de hidrofobinas e melanina presentes nos conídios formam uma camada de proteção, uma vez que interagem fracamente com receptores de células imunes inatas, inibindo a resposta imune frente a conídios dormentes (GARCIA-RUBIO; DE OLIVEIRA; RIVERA; TREVIJANO-CONTADOR, 2019). Foi demonstrado que conídios provenientes de cepas mutantes de *A. fumigatus*, que apresentavam falha na produção de melanina, eram capazes de ativarem células dendríticas (DCs), visto pelo aumento de moléculas coestimuladoras como CD86, CD80 e CD40, bem como

de citocinas inflamatórias como TNF-α, IL-1β e IL-6 (BAYRY; BEAUSSART; DUFRENE; SHARMA *et al.*, 2014). Nesse caso, a presença de melanina nas cepas WT atuavam de forma a inibir a ativação de DCs, assim, esses conídios se comportavam como o controle negativo (BAYRY; BEAUSSART; DUFRENE; SHARMA *et al.*, 2014). Além disso, a camada de melanina ainda é capaz de proteger os conídios frente a acidificação de fagolisossomos, o que garante sua maior sobrevida quando fagocitadas por células epiteliais quando comparadas com cepas não melanizadas (AMIN; THYWISSEN; HEINEKAMP; SALUZ *et al.*, 2014).

Como destacado anteriormente, os conídios de *A. fumigatus* têm funções reprodutivas e são muito pequenos (2-3mm de diâmetro) o que ajuda no início da infecção (KANJ; ABDALLAH; SOUBANI, 2018; KWON-CHUNG; SUGUI, 2013). A inalação de conídios ocorre naturalmente, cerca de 100 conídios/dia (BRAKHAGE; LANGFELDER, 2002). Em indivíduos imunocompetentes, eles são eliminados devido a atividade mucociliar do trato respiratório acompanhado de eficiente resposta imune inata (KANJ; ABDALLAH; SOUBANI, 2018). Já é descrito na literatura grande prevalência de *A. fumigatus* em amostras clínicas de pacientes com fibrose cística (BAKARE; RICKERTS; BARGON; JUST-NUBLING, 2003; MORTENSEN; JENSEN; JOHANSEN; SKOV *et al.*, 2011). A fibrose cística é uma doença que acomete as vias aéreas, caracterizada pela inflamação crônica infecciosa, e clinicamente pode ser caracterizada pela acumulação anormal de muco (COWLEY; THORNTON; DENNING; HORSLEY, 2017). Por conta do defeito na atividade mucociliar e de produção de muco em pacientes com fibrose cística, é postulado que tais indivíduos possuam um fator de risco para o desenvolvimento de aspergilose (COWLEY; THORNTON; DENNING; HORSLEY, 2017).

Apesar de haverem fatores de virulência característicos presentes em *A. fumigatus*, o estabelecimento da infecção é, principalmente, dependente do estado imunológico do indivíduo (TEKAIA; LATGE, 2005). Dessa forma indivíduos mais suscetíveis ao desenvolvimento da aspergilose são aqueles que apresentam algum tipo de dano pulmonar preexistente (asma ou tuberculose, por exemplo) ou aqueles com algum grau de imunossupressão (leucemia, transplantados, sob tratamento com corticoides, infectados com o vírus da imunodeficiência humana ou que apresentam neutropenia) (KOUSHA; TADI; SOUBANI, 2011). Nesses casos as células da imunidade inata, principalmente macrófagos e neutrófilos, podem estar em número

reduzido ou apresentar ineficiência na destruição dos conídios, esses são capazes de germinarem em hifas e estabelecer a infecção (DAGENAIS; KELLER, 2009; WARRIS, 2014).

A manifestação clínica da doença é ampla e pode ser limitada a uma reação alérgica, podendo ocorrer até a infecção sistêmica. As principais manifestações clínicas são: aspergilose pulmonar invasiva (IPA); aspergilose pulmonar crônica (CPA); e aspergilose broncopulmonar alérgica (ABPA). A Figura 1 demonstra graficamente essas manifestações clínicas.

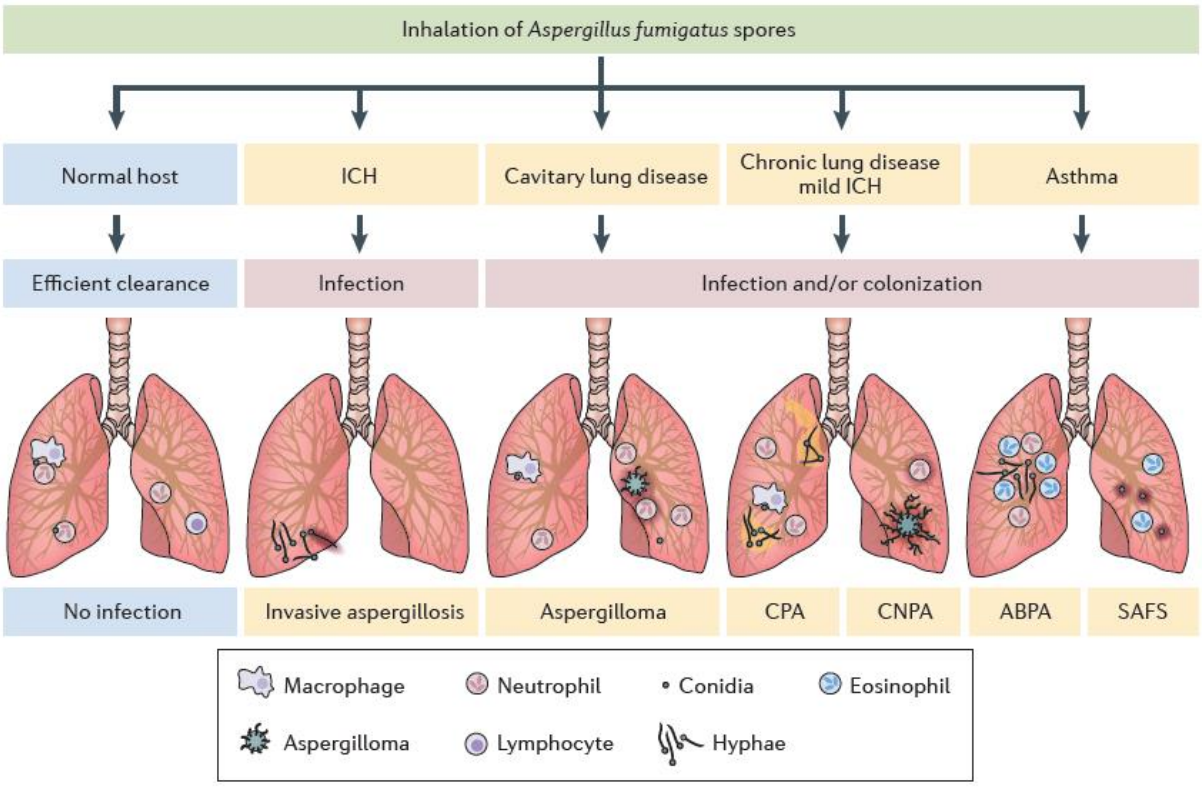
A IPA é a mais severa forma de aspergilose, apresentando uma alta taxa de mortalidade (30-95%) (CHABI; GORACCI; ROCHE; PAUGAM *et al.*, 2015; TACCONE; VAN DEN ABEELE; BULPA; MISSET *et al.*, 2015). Pacientes internados em unidade de terapia intensiva (UTI) com aspergilose apresentavam maior mortalidade e maior média de tempo de internação, quando comparados com aqueles pacientes internados na UTI, porém sem aspergilose (VANDEWOUDE; BLOT; BENOIT; COLARDYN *et al.*, 2004). Esse aumento do tempo de internação é um dos fatores que aumenta os custos diretos e indiretos com o tratamento do paciente (KIM; NICOLAU; KUTI, 2011). A população em risco é aquela que apresenta algum estado de imunossupressão grave, geralmente causado por transplante de células-tronco hematopoéticas (HSCT), transplante de órgãos sólidos, pacientes que fazem uso prolongado com corticosteroides, com síndrome da imunodeficiência adquirida (AIDS) ou aqueles com doença granulomatosa crônica (GAO; SOUBANI, 2019; PATTERSON; THOMPSON; DENNING; FISHMAN *et al.*, 2016). Além dos casos citados, a neutropenia também é um importante fator no desenvolvimento da IPA, dado a importância dessa célula na defesa inicial frente conídios de *A. fumigatus* (GERSON; TALBOT; HURWITZ; STROM *et al.*, 1984; KOUSHA; TADI; SOUBANI, 2011). Em todos os casos, o fator comum é a deficiência na resposta imune, causada pela ação de medicamentos, para inibir a rejeição do transplante, ou pela ação de microrganismos, que atacam células imunes inibindo a resposta imune, o que permite a rápida disseminação do fungo. A IPA é caracterizada pela invasão fúngica de arteríolas pulmonares e parênquima pulmonar, nesse cenário o rápido crescimento da hifa fúngica é capaz de causar necrose isquêmica, trombose intravascular e infarto hemorrágico (DAGENAIS; KELLER, 2009; GAO; SOUBANI, 2019). Uma vez na corrente sanguínea, o fungo é capaz de migrar para órgãos distantes do sítio inicial

da infecção, como rim, fígado, baço e os sistema nervoso central (LATGE; CHAMILOS, 2019). São estimados que ocorram mais de 200 mil casos de aspergilose invasiva no mundo por ano, mas esse número pode ser subestimado devido à falta de diagnóstico preciso (BROWN; DENNING; GOW; LEVITZ *et al.*, 2012).

A CPA é composta de um conjunto de doenças não invasivas causadas pela infecção, principalmente de *A. fumigatus* (ZARIF; THOMAS; VAYRO, 2021). Na maioria das vezes, a CPA se desenvolve em indivíduos sadios, mas que possuem algum dano pulmonar preexistente, principalmente aqueles com tuberculose prévia (SMITH; DENNING, 2011). O aspergilloma, por exemplo, é uma forma de CPA composto por uma massa formada de hifas fúngicas, fibrina, muco, células inflamatórias, elementos derivados do sangue e de células epiteliais, que cresce dentro de uma cavidade pulmonar preexistente, na maioria dos casos derivado de dano anterior por tuberculose (GAO; SOUBANI, 2019; ZARIF; THOMAS; VAYRO, 2021). Em 2011 foi estimado que a prevalência da CPA era de 3 milhões de casos mundialmente distribuídos, porém esse número pode ser altamente variável quando comparado países de regiões diferentes (DENNING; PLEUVRY; COLE, 2011). Variando de menos que 1 caso por 100.000 habitantes nos Estados Unidos e Europa ocidental a 42,9 casos por 100.000 habitantes na República democrática do Congo e Nigéria (DENNING; PLEUVRY; COLE, 2011).

A ABPA é uma condição inflamatória pulmonar causada pela hipersensibilidade a alérgenos derivados da colonização por *A. fumigatus*. Na maioria dos casos, encontram-se indivíduos com asma e fibrose cística (SHAH; PANJABI, 2016). Na ABPA, o fungo não eliminado persiste e se multiplica, liberando proteases e outros componentes que permitem sua instalação nas vias aéreas. É proposto que quimiocinas específicas são capazes de recrutar linfócitos Th2 reativos a epítopos fúngicos (KNUTSEN, 2003), além disso uma resposta imune enviesada para o perfil Th2, seja pelo perfil de quimiocinas produzidos ou pelo padrão de expressão de HLA em células apresentadoras de antígenos (APCs) induzem o surgimento da ABPA (CHAUHAN; SANTIAGO; HUTCHESON; SCHWARTZ *et al.*, 2000). Pacientes acometidos apresentam ainda eosinofilia local e sistêmica, altas concentrações séricas de IgG, IgA and IgE anti- *A. fumigatus*, tosse sistêmica e sibilo (AGARWAL, 2009; MOSS, 2005).

Figura 1: Apresentações clínicas da aspergilose.



ICH, immunocompromised host.

Fonte: (VAN DE VEERDONK; GRESNIGT; ROMANI; NETEA *et al.*, 2017)

1.2. Resposta imune frente a infecção por *A. fumigatus*

Para que ocorra o estabelecimento da infecção, os conídios inalados devem ultrapassar algumas barreiras inespecíficas da imunidade inata, como a produção de muco e a atividade mucociliar, sendo a maior parte dos conídios eliminados por essas barreiras (BALLOY; CHIGNARD, 2009; VAN DE VEERDONK; GRESNIGT; ROMANI; NETEA *et al.*, 2017). A Figura 2 demonstra as barreiras que o conídio invasor deve superar para estabelecer a infecção. A depuração mucociliar conta com um conjunto de células multiciliadas dispostas por todo epitélio respiratório, essas células ativamente transportam uma fina camada de muco, que retém partículas e microrganismos inalados para fora dos pulmões (NAWROTH; VAN DER DOES; RYAN FIRTH; KANSO, 2020). Além da atividade do transporte de muco, as células epiteliais brônquicas são capazes de interagir diretamente com os conídios de *A. fumigatus* por meio da interação de padrões moleculares associados a patógenos (PAMPs) fúngicos com receptores de reconhecimento padrão (PRRs) nas células epiteliais (BIGOT; GUILLOT; GUITARD; RUFFIN *et al.*, 2020). A partir dessa interação as células epiteliais podem fagocitar o conídio, produzirem citocinas, quimiocinas e

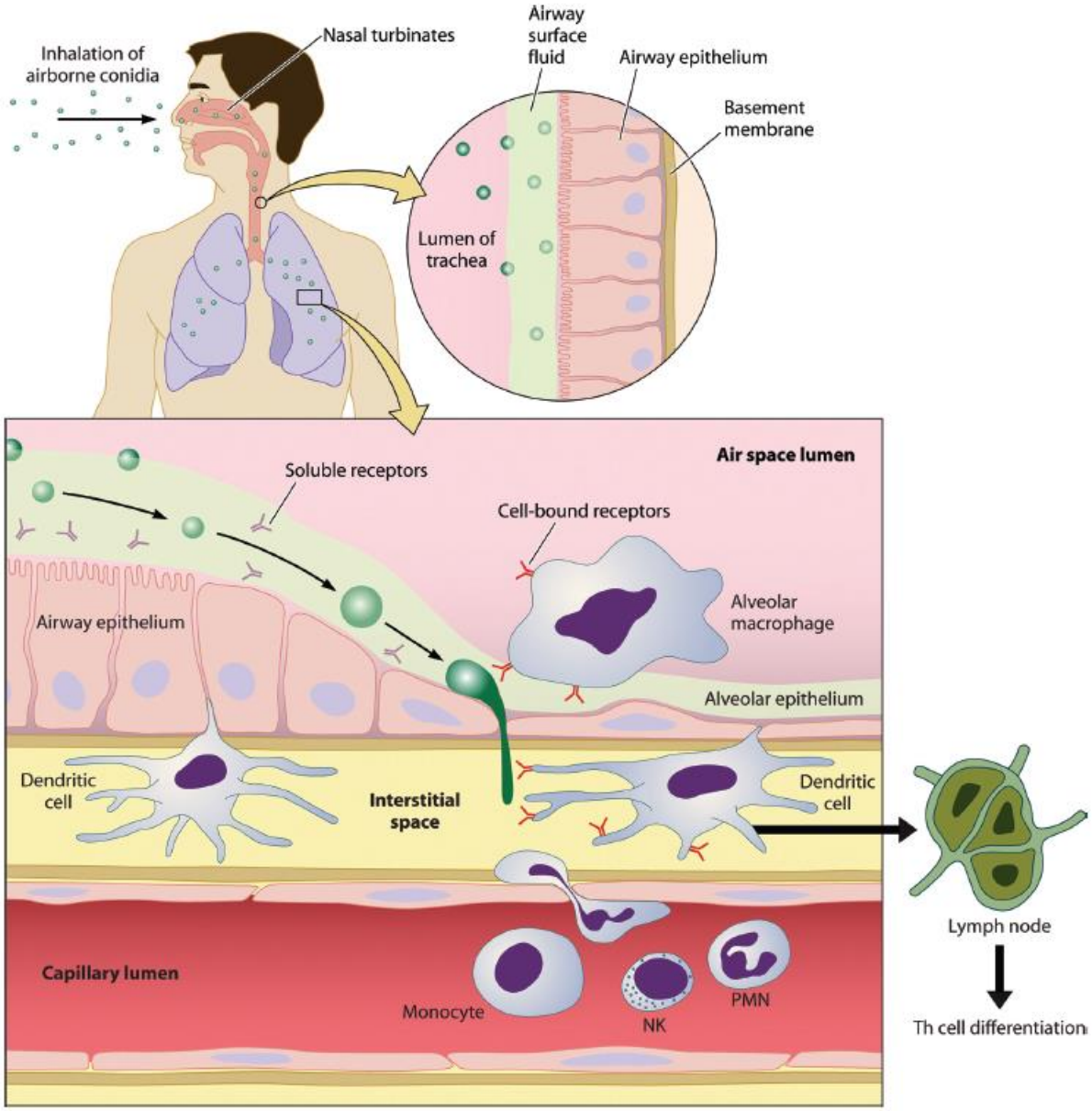
outros produtos microbicidas (BIGOT; GUILLOT; GUITARD; RUFFIN *et al.*, 2020). De forma interessante, células epiteliais brônquicas são ainda capazes de reconhecer um componente fúngico e inibir seu crescimento. FleA é uma lectina expressa por *A. fumigatus* que é capaz de se ligar avidamente com glicoproteínas presente no muco (KERR; FISCHER; SINHA; MCCABE *et al.*, 2016), além disso, a ligação de FleA, presente no fungo, com células epiteliais brônquicas, induz atividade fungistática mediada pelas células epiteliais (RICHARD; MARTI; VARROT; GUILLOT *et al.*, 2018).

Os conídios que vencem os obstáculos iniciais se alojam nos alvéolos e são protegidos do reconhecimento por células da imunidade inata devido a uma barreira composta de microfibrilas protéicas, compostas de hidrofobinas. Essa camada atua impedindo o acesso físico a principal fonte de antígenos para o sistema imune, a parede celular do conídio, onde estão contidos PAMPs que são passíveis de reconhecimento pela imunidade inata (AIMANIANDA; BAYRY; BOZZA; KNIEMEYER *et al.*, 2009; AIMANIANDA; LATGE, 2010). Os conídios ainda possuem uma camada de melanina que também atua na proteção do conídio, escondendo os PAMPs (CHAI; NETEA; SUGUI; VONK *et al.*, 2010). Apesar desses mecanismos de evasão, para que ocorra o início da infecção deve haver a germinação do conídio em hifa, esse processo degrada a camada de hidrofobina e a de melanina, culminando na exposição dos PAMPs fúngicos contidos na parede celular (PARIS; DEBEAUPUIS; CRAMERI; CAREY *et al.*, 2003; TRONCHIN; BOUCHARA; FERRON; LARCHER *et al.*, 1995). De fato, RodA, a principal hidrofobina, é responsável pela inibição da fagocitose de conídios de *A. fumigatus* por macrófagos murinos (DAGENAIS; GILES; AIMANIANDA; LATGE *et al.*, 2010). A remoção dessa camada de RodA, seja pela utilização de cepas mutantes, pela remoção química ou pela germinação do conídio restitui ao sistema imune a capacidade de reconhecimento do fungo, como verificado pela ativação de DCs e macrófagos humanos (AIMANIANDA; BAYRY; BOZZA; KNIEMEYER *et al.*, 2009). De forma interessante, a infecção *in vivo* por cepas deficientes na produção de RodA foi capaz de induzir maior recrutamento de neutrófilos e produção de citocinas pró-inflamatórias quando comparadas com a cepa WT (AIMANIANDA; BAYRY; BOZZA; KNIEMEYER *et al.*, 2009). Abaixo da camada de hidrofobinas, existe uma camada de melanina que possui várias atividades imunomoduladoras, que de forma geral impede o reconhecimento do conídio, ou promove sua sobrevida quando fagocitado (PEREZ-CUESTA; APARICIO-

FERNANDEZ; GURUCEAGA; MARTIN-SOUTO *et al.*, 2020). Foi demonstrado *in vitro* que a falta de melanina em cepas mutantes era a responsável pela ativação de DCs humanas (BAYRY; BEAUSSART; DUFRENE; SHARMA *et al.*, 2014). Uma vez que o conídio é fagocitado, a melanina é capaz de inibir a acidificação do fagolisossomo, etapa essencial na destruição microbiana, mas não interfere na fusão do fagossomo com o lisossomo (THYWISSEN; HEINEKAMP; DAHSE; SCHMALER-RIPCKE *et al.*, 2011). Esse fato foi verificado em macrófagos de linhagem, neutrófilos humanos recém isolados e linhagem de células epiteliais pulmonares (AMIN; THYWISSEN; HEINEKAMP; SALUZ *et al.*, 2014; THYWISSEN; HEINEKAMP; DAHSE; SCHMALER-RIPCKE *et al.*, 2011). No caso das células epiteliais pulmonares a presença de melanina induziu, de forma inesperada, maior internalização de conídios (AMIN; THYWISSEN; HEINEKAMP; SALUZ *et al.*, 2014), esse fato pode estar relacionado com outros relatos que propõe a melanina como um alvo para fagocitose. É postulado que uma proteína hidrofílica surfactante produzida por células epiteliais alveolares, a SP-D, é capaz de se ligar a melanina de conídios de *A. fumigatus*. A partir dessa ligação, o conídio é opsonizado, o que facilita o processo de fagocitose por macrófagos humanos derivados de monócitos (WONG; RANI; DODAGATTA-MARRI; IBRAHIM-GRANET *et al.*, 2018). Além disso, conídios opsonizados por SP-D foram capazes induzir uma forte resposta inflamatória por meio de macrófagos humanos derivados de monócitos, como verificado pela produção de TNF-α, IL-1β, IL-8, IL-6, IL-12 e IL-10, quando comparado com conídios não opsonizados (WONG; RANI; DODAGATTA-MARRI; IBRAHIM-GRANET *et al.*, 2018). Dessa forma, a melanina que recobre o conídio possui várias ações sobre o sistema, sendo capaz de proteger sua destruição intracelular, mas também atuando como um PAMP passível de reconhecimento.

Uma vez que o conídio encontra um local adequado para a germinação, ocorre a dissolução das camadas de hidrofobinas e de melanina, revelando vários PAMPs. Dentre os principais PAMPs presentes na parede celular de *A. fumigatus*, podemos destacar a presença de diferentes polissacarídeos, como β-1,3-glucanas, mananas, quitina, galactomananas e galactosaminagalactana (LATGE; BEAUVAIS, 2014; LATGE; MOUYNA; TEKAIA; BEAUVAIS *et al.*, 2005).

Figura 2: Representação das barreiras imunes as quais os conídios inalados devem superar a fim de iniciar a infecção.



Fonte: (PARK; MEHRAD, 2009)

Para que ocorra o reconhecimento de PAMPs fúngicos o sistema imune faz uso de receptores específicos que são capazes de reconhecer esses padrões, tais PRRs atuam na interação inicial do sistema imune frente a qualquer organismo invasor. Vários são os PRRs relacionados ao reconhecimento de *A. fumigatus* (GRESNIGT; NETEA; VAN DE VEERDONK, 2012), alguns deles são discutidos a seguir. Um dos principais PRR no reconhecimento de *A. fumigatus* é a dectina-1, que faz parte da família de receptores de lectinas do tipo C (CLR), a qual é expressa em DCs, macrófagos e neutrófilos, sendo capaz de reconhecer β-1,3-glucanas (SALAZAR; BROWN, 2018). A interação de dectina-1 com seu ligante ocorre somente

após a germinação do conídio, e pode acontecer no fagossomo ou na membrana (FARO-TRINDADE; WILLMENT; KERRIGAN; REDELINGHUYS *et al.*, 2012; STEELE; RAPAKA; METZ; POP *et al.*, 2005). A ativação desse receptor induz a produção de quimiocinas e citocinas, incluindo TNF-α, IL-6, IL-1α, IL-1β, G-CSF, GM-CSF, MIP-1α e MIP-1β (MARGALIT; KAVANAGH, 2015). De fato, a importância de dectina-1 na infecção por *A. fumigatus* fica evidente pela demonstração que camundongos knockout para esse receptor produzem quantidade diminuída de mediadores inflamatórios, o que resulta em diminuição de recrutamento de neutrófilos e consequentemente menor atividade fúngicida (WERNER; METZ; HORN; SCHOEB *et al.*, 2009). Outro CLR importante é a dectina-2, que atua no reconhecimento de hifas de *A. fumigatus* por células dendríticas plasmocitóides, induzindo a produção de TNF-α, IFN-α e maior atividade antifúngica (LOURES; ROHM; LEE; SANTOS *et al.*, 2015). Em macrófagos, dectina-2 é capaz de interagir com PAMPs de *A. fumigatus* durante sua germinação e na forma de hifa para induzir a produção de espécies reativas de oxigênio (ROS) e citocinas pró- e anti-inflamatórias como IL-1β, IL-23, TNF-α e IL-10 (SUN; XU; TIAN; SHAO *et al.*, 2014).

Os *toll like receptors* (TLR) são os receptores mais estudados e mais bem caracterizados dentre os PRR, e estão envolvidos no reconhecimento de *A. fumigatus*. Em modelos experimentais de aspergilose invasiva, ficou demonstrada a importância de TLR2, TLR4 e sua molécula adaptadora MyD88. Na falta desses, a mortalidade dos camundongos infectados com conídios de *A. fumigatus* se apresenta aumentada em relação ao grupo controle (BALLOY; SI-TAHAR; TAKEUCHI; PHILIPPE *et al.*, 2005; BELLOCCHIO; MONTAGNOLI; BOZZA; GAZIANO *et al.*, 2004). A importância desses receptores também é demonstrada *in vitro*. Macrófagos peritoneais sem TLR4 funcional não são capazes de ativar o fator de transcrição NF-κB, que é responsável pela expressão de genes que codificam moléculas inflamatórias. A falta de TLR2 e TLR4 pode afetar a produção de TNF-α e óxido nítrico (NO), além disso, camundongos que não possuíam esses receptores funcionais apresentavam menor migração de neutrófilos para o sítio da infecção (MEIER; KIRSCHNING; NIKOLAUS; WAGNER *et al.*, 2003).

Podemos citar também a pentraxina 3 (PTX3) como um receptor solúvel para *A. fumigatus*. PTX3 é produzida e secretada por uma gama de células, incluindo fagócitos, DCs, fibroblastos e células endoteliais, a partir da estimulação de TLRs

(PARK; MEHRAD, 2009). A principal atuação de PTX3 está no fato desse receptor ser uma ótima opsonina para conídios de *A. fumigatus*, PTX3 se liga a galactomanana e induz a ativação da via clássica do complemento, dependente de C1q, mas também é capaz de ativar a via alternativa, dessa forma contribui para a fagocitose mediada por macrófagos alveolares e DCs (BOTTAZZI; VOURET-CRAVIARI; BASTONE; DE GIOIA *et al.*, 1997; GARLANDA; HIRSCH; BOZZA; SALUSTRI *et al.*, 2002). O reconhecimento e fagocitose de conídios opsonizados por PTX3 é dependente do receptor de complemento 3 (CR3) e do receptor de IgG (FCγR) presente nas células fagocíticas (MOALLI; DONI; DEBAN; ZELANTE *et al.*, 2010). A importância de PTX3 é evidenciada por dados experimentais que mostram que camundongos PTX3-/- apresentam uma média de sobrevivência de apenas 3 dias após infecção por *A. fumigatus*, entretanto, esse quadro pode ser revertido pela administração de PTX3 exógena, aumentando a sobrevida média do grupo tratado para mais de 60 dias (MOALLI; DONI; DEBAN; ZELANTE *et al.*, 2010).

Após a interação de PAMPs fúngicos com macrófagos alveolares e neutrófilos em indivíduos imunocompetentes, ocorre a fagocitose do conídio e eliminação do patógeno. Macrófagos alveolares são células residentes no tecido e por isso são umas das primeiras a entrarem em contato com os conídios inalados (LEOPOLD WAGER; WORMLEY, 2014). Essas células controlam e limitam a infecção inicial e recrutam outras células imunes (XU; SHINOHARA, 2017). Uma das moléculas de superfície que atua nessa etapa da fagocitose é a integrina Mac-1, formada pelas cadeias CD11b/CD18 (BROWN, 2003; SCHITTENHELM; HILKENS; MORRISON, 2017). Ensaios *in vitro*, utilizando células epiteliais alveolares humanas e de camundongo, demonstraram que Mac-1 é responsável pela internalização de conídios de *A. fumigatus* (HAN; SU; LI; LIU *et al.*, 2021). Essas células matam conídios e hifas de *A. fumigatus* pela atividade microbicida de ROS ou pela ação de enzimas lisossomais (GAZENDAM; VAN HAMME; TOOL; HOOGENBOEZEM *et al.*, 2016; PHILIPPE; IBRAHIM-GRANET; PREVOST; GOUGEROT-POCIDALO *et al.*, 2003). A geração de ROS por ambos os tipos celulares é dependente da maquinaria de NADPH oxidase e mieloperoxidase, responsáveis por produzir ácido hipocloroso (HOCl) e radical superóxido (O_2^-), respectivamente (BALLOY; CHIGNARD, 2009; HOHL, 2017). Tais compostos são essenciais na eliminação de *A. fumigatus* (ARATANI; KURA; WATANABE; AKAGAWA *et al.*, 2002). A importância da função de

NADPH oxidase para a produção de ROS e eliminação de *A. fumigatus* é demonstrada em pacientes com doença granulomatosa crônica (CGD), esses indivíduos possuem uma mutação que afeta a geração de ROS pela NADPH, causando extrema suscetibilidade a aspergilose invasiva (GRIMM; VETHANAYAGAM; ALMYROUDIS; LEWANDOWSKI *et al.*, 2011). Em neutrófilos foi demonstrada que a ativação de Mac-1 está relacionada com a morte intracelular de hifas de *A. fumigatus*, fato esse dependente da ativação de NADPH oxidase e mieloperoxidases (GAZENDAM; VAN HAMME; TOOL; HOOGENBOEZEM *et al.*, 2016). Além disso, os macrófagos ainda respondem a interação fúngica pela produção de mediadores inflamatórios com perfis, mais ou menos definidos. Dessa forma os macrófagos ativados podem se polarizar em dois tipos distintos: M1, ou classicamente ativados, e M2, ou alternativamente ativados. Após a ativação de TLRs e CLRs por PAMPs fúngicos, macrófagos alveolares vão secretar mediadores inflamatórios, como citocinas (IL-12, TNF-α e IL-6) e quimiocinas (CXCL9, CXCL10 e CXCL11) e aumentam a expressão de enzimas relacionadas com a produção de espécies reativas de oxigênio como a óxido nítrico sintase induzível (iNOS) (LEOPOLD WAGER; WORMLEY, 2014). Entretanto, a atividade inflamatória de macrófagos M1 pode ser nociva ao hospedeiro, já que na busca pela eliminação do patógeno ocorre dano de tecido sadio, dessa forma os macrófagos M2 vão atuar principalmente no reparo tecidual pós eliminação do patógeno (YUNNA; MENGRU; LEI; WEIDONG, 2020). Macrófagos M2 promovem a geração de resposta Th2, reparo tecidual, angiogênese e diminuem a inflamação favorecendo o reparo tecidual. Essas células são capazes de secretar citocinas como IL-4, IL-10 e quimiocinas CCL24 e CCL17 (WANG; SMITH; HAO; HE *et al.*, 2019). Na infecção por *A. fumigatus*, o desenvolvimento de macrófagos M1 garante uma resposta adequada para garantir a defesa do hospedeiro (XIE; ZHOU; ZHANG; YU *et al.*, 2022). De fato, macrófagos humanos cultivados *in vitro* com conídios de *A. fumigatus* apresentaram aumento de mRNA para iNOS, TNFα, CD80 e CD86, bem como aumento na secreção de TNF-α (ZHANG; HE; GAO; WEI *et al.*, 2019). Apesar desse fato, um estudo demonstrou que camundongos infectados por *A. fumigatus* apresentavam uma rápida, após 48 h de infecção, e potente ativação alternativa de macrófagos alveolares, como demonstrado pela alta expressão de mRNA de Arginase-1 em macrófagos $CD11c^{+}$ (BHATIA; FEI; YARLAGADDA; QI *et al.*, 2011).

Além da fagocitose, neutrófilos ainda tem a sua disposição outro mecanismo para inibir o crescimento fúngico. Hifas que são grandes demais para serem fagocitadas podem sofrer a ação de armadilhas extracelulares de neutrófilos (NETs), que consistem em uma rede de DNA associada a outras proteínas citoplasmáticas que envolve o microrganismo (PAPAYANNOPOULOS, 2018). As NETs podem ser induzidas pela interação de conídios de *A. fumigatus* com Mac-1 presente na membrana do neutrófilo (SILVA; RODRIGUES; THOMPSON-SOUZA; MUNIZ *et al.*, 2020). A formação de NETs não é suficiente para inativar conídios ou inibir sua germinação, mas as hifas têm seu crescimento prejudicado na presença dessas estruturas (GAZENDAM; VAN HAMME; TOOL; HOOGENBOEZEM *et al.*, 2016; MCCORMICK; HEESEMANN; WAGENER; MARCOS *et al.*, 2010). A importância de neutrófilos no controle da infecção por *A. fumigatus* é bem estabelecida, já que é sabido que a neutropenia é um fator de risco para o desenvolvimento da aspergilose pulmonar (CHABI; GORACCI; ROCHE; PAUGAM *et al.*, 2015; KOUSHA; TADI; SOUBANI, 2011; STEPHENS-ROMERO; MEDNICK; FELDMESSER, 2005). Essas células são recrutadas para o local da infecção por mediadores solúveis e são capazes de matar conídios e hifas de forma mais eficiente quando comparados aos macrófagos (PARK; MEHRAD, 2009).

As células natural killer (NK) são linfócitos circulantes que são capazes de promover a morte de células cancerígenas e células infectadas por vírus através da secreção de perforinas e granzimas ou pela ação de receptores específicos. Essas células são capazes de produzir IFN-γ, TNF-α e GM-CSF que atuam aumentando a atividade fagocítica de macrófagos e neutrófilos, dessa forma, contribuem para o controle da infecção por *A. fumigatus* (LEHRNBECHER; SCHMIDT, 2019; SCHMIDT; CONDORELLI; KOLTZE; LEHRNBECHER, 2017). Células NK humanas são capazes de induzir a morte de hifas de *A. fumigatus*, mas não de conídios, *in vitro*. Esse efeito está relacionado com a produção de perforina, já que mesmo quando separadas por uma membrana a atuação das células NK frente as hifas é mantida. Além disso, a inibição de perforina diminui a morte dependente das NK (SCHMIDT; TRAMSEN; HANISCH; LATGE *et al.*, 2011). Em modelos animais de aspergilose invasiva foi demonstrado que o recrutamento defeituoso de células NK para o sítio de infecção, em camundongos tratados com inibidores de MCP-1/CCL2, resultou em menor sobrevida e aumento da carga fúngica nos pulmões quando comparado aos animais

controles (MORRISON; PARK; MOONEY; MEHRAD, 2003). As células NK ainda podem ser a fonte principal de IFN-γ em animais neutropênicos, na ausência dessas células animais apresentam baixa produção de IFN-γ e maior carga fúngica. Essa observação pode estar relacionada a baixa ativação de macrófagos dependente de IFN-γ (PARK; HUGHES; BURDICK; STRIETER *et al.*, 2009). Outro tipo de linfócito da imunidade inata que pode ter relação a resposta antifúngica é o linfócito Tγδ. Foi demonstrado em modelos experimentais que conídios de cepas de *A. fumigatus* que possuem menor quantidade de β-1,3-glucanas em sua parede celular são capazes de recrutar uma maior quantidade de eosinófilos para o sítio de infecção. O recrutamento dessas células se traduz em piora do quadro clínico do animal, com maior carga fúngica. Ficou demonstrado que o recrutamento aumentado de eosinófilos era dependente de linfócitos Tγδ, demonstrado pelo fato de que camundongos que não tinham essas células não apresentaram recrutamento aumentado de eosinófilos quando infectados por conídios de *A. fumigatus* expressando menor quantidade de β-1,3-glucanas (AMARSAIKHAN; O'DEA; TSOGGEREL; TEMPLETON, 2017; AMARSAIKHAN; SANDS; SHAH; ABDOLRASOULI *et al.*, 2017).

1.3. Vesículas extracelulares fúngicas e resposta imune

A capacidade de comunicação intercelular é uma característica de organismos multicelulares, que era determinado pela secreção de mediadores solúveis ou pela comunicação célula-célula, mas um terceiro mecanismo foi proposto, a produção de vesículas extracelulares (EVs), que mediariam a comunicação entre células distantes (RAPOSO; STOORVOGEL, 2013). EVs são conservadas desde bactérias até humanos, são estruturas esféricas compostas por uma bicamada lipídica e são bastante heterogêneas em seu conteúdo, podendo conter ácidos nucleicos, proteínas, lipídeos e variadas biomoléculas (COLOMBO; RAPOSO; THERY, 2014; DEATHERAGE; COOKSON, 2012). As EVs podem ser categorizadas em exossomos, microvesículas e corpos apoptóticos a depender da sua biogênese, conteúdo, tamanho e função (YANEZ-MO; SILJANDER; ANDREU; ZAVEC *et al.*, 2015). Os exossomos são EVs derivadas do sistema endocítico e geralmente são as menores EVs, possuindo tamanho de 30 a 150 nm de diâmetro (VAN NIEL; D'ANGELO; RAPOSO, 2018). Os exossomos são derivados da invaginação da membrana de endossomos tardios, que dão origem aos corpos multivesiculares

(MVBs) (ZHANG; YIN; LAI; LIM, 2014). Os MVBs podem ser direcionados para degradação, pela fusão com lisossomos, ou serem fundidos com a membrana plasmática da célula para que ocorra a liberação dos exossomos para o meio extracelular, de forma interessante, devido a biogênese dos exossomos sua membrana possui a mesma orientação da membrana celular de origem (BEBELMAN; SMIT; PEGTEL; BAGLIO, 2018; ZHANG; YIN; LAI; LIM, 2014). As microvesículas são EVs derivadas do brotamento da membrana plasmática celular e possuem tamanho mais heterogêneo, variando entre 50-1000 nm de diâmetro (VAN NIEL; D'ANGELO; RAPOSO, 2018). A formação das microvesículas provavelmente requer o recrutamento do citoesqueleto celular e ativação de actina e microtúbulos (DOYLE; WANG, 2019). Já os corpos apoptóticos são derivados de células em processo de morte celular. São as maiores EVs, possuindo entre 50 e 5000 nm de diâmetro, sendo que a grande maioria tendem a estar mais próximas a 5000 nm (DOYLE; WANG, 2019).

A primeira observação de que fungos poderiam produzir tais estruturas foi realizada, em 1973, por Takeo e colaboradores, utilizando *Cryptococcus neoformans* e técnicas de microscopia eletrônica (TAKEO; UESAKA; UEHIRA; NISHIURA, 1973). Em 1990, Anderson e colaboradores descreveram a presença de brotamentos de vesículas em “espinhas” na parede celular de *Candida albicans* (ANDERSON; MIHALIK; SOLL, 1990). Entretanto, somente em 2007 foi demonstrado de forma sistemática e inequívoca que *C. neoformans* era capaz de secretar vesículas, através da parede celular, que continham seu principal componente capsular a glucuronoxilomanana (GXM) (RODRIGUES; NIMRICHTER; OLIVEIRA; FRASES *et al.*, 2007). Desde então a caracterização de EVs fúngicas já foi observada para várias espécies, sendo elas patogênicas ou não (LIEBANA-JORDAN; BROTONS; FALCON-PEREZ; GONZALEZ, 2021; RIZZO; RODRIGUES; JANBON, 2020).

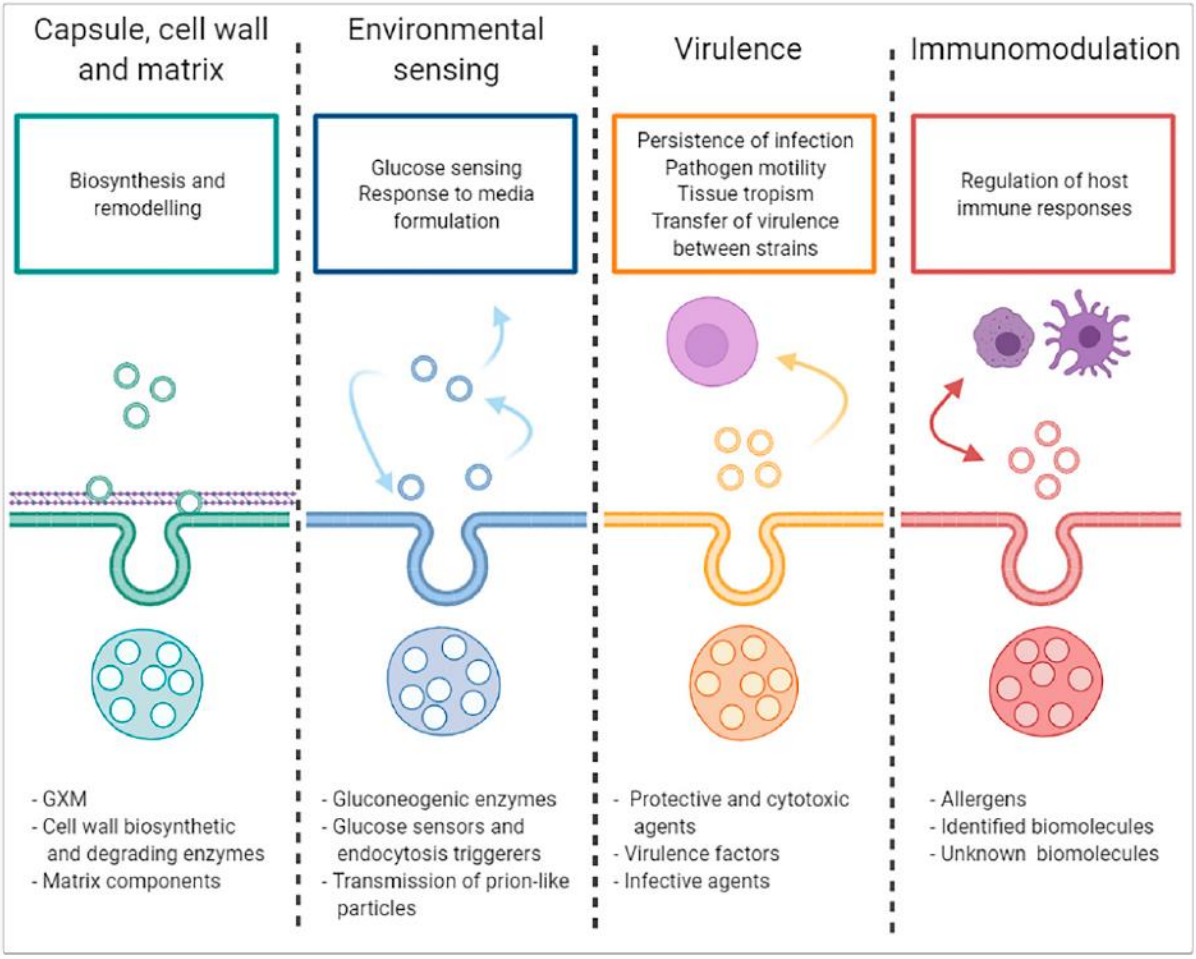
Dado o seu complexo conteúdo e origem celular, as EVs fúngicas podem realizar várias ações, seja mediando a comunicação entre células fúngicas, bem como na modulação da resposta imune do hospedeiro (RODRIGUES; CASADEVALL, 2018; ZAMITH-MIRANDA; NIMRICHTER; RODRIGUES; NOSANCHUK, 2018). Algumas dessas ações são demonstradas na Figura 3. Em *C. albicans*, as EVs produzidas são capazes de estimular a formação de biofilmes e induzir resistência a antifúngicos, duas características que contribuem para a

colonização no hospedeiro humano (ZARNOWSKI; NOLL; CHEVRETTE; SANCHEZ *et al.*, 2021). Em *C. auris*, foi demonstrado que o tratamento do fungo com caspofungina foi capaz de causar alterações celulares e nas vesículas secretadas por esse fungo, demonstrando a habilidade do fungo modular sua produção de EVs a depender de estímulos externos (AMATUZZI; ZAMITH-MIRANDA; MUNHOZ DA ROCHA; LUCENA *et al.*, 2022). Em *A. fumigatus*, a produção de EVs por protoplastos pode estar relacionada com a síntese da parede celular (RIZZO; CHAZE; MIRANDA; ROBERSON *et al.*, 2020). Um estudo do nosso grupo demonstrou que tanto *C. albicans*, *A. fumigatus*, e *Paracoccidioides brasiliensis* eram capazes de internalizar EVs obtidas de outro cultivo da mesma espécie. A partir dessa internalização foi verificado a alteração na expressão de genes específicos a cada espécie, induzido pelas EVs (BITENCOURT; HATANAKA; PESSONI; FREITAS *et al.*, 2022). Desse modo foi possível demonstrar que EVs podem atuar como estruturas mensageiras, alterando o perfil transcricional do fungo alvo. Para além dos exemplos descritos aqui há várias outras funções das EVs fúngicas que já foram descritas na literatura (RODRIGUES; NIMRICHTER, 2022).

Outra função importante das EVs fúngicas é a modulação do sistema imune do hospedeiro, estimulando a secreção de várias citocinas que podem influenciar o resultado da infecção (FREITAS; BONATO; PESSONI; RODRIGUES *et al.*, 2019). EVs originárias de *C. neoformans* são fagocitadas, *in vitro*, por macrófagos e induzem a produção de TNF-α, NO, TGF-β e IL-10, demonstrando uma dualidade entre mediadores pró- e anti-inflamatórios. Além disso, os macrófagos estimulados por EVs são capazes de fagocitar e matar leveduras de *C. neoformans* de forma mais eficiente (OLIVEIRA; FREIRE-DE-LIMA; NOSANCHUK; CASADEVALL *et al.*, 2010). Entretanto, em um modelo experimental de invertebrado, utilizando a larva de *Galleria mellonella*, a administração prévia de EVs de *C. neoformans* induziu a morte precoce das larvas desafiadas com o fungo, em relação ao controle negativo (COLOMBO; RELLA; NORMILE; JOFFE *et al.*, 2019). O uso de modelos invertebrados para o estudo de infecções fúngicas é amplamente aceito, ele apresenta algumas vantagens como baixo custo, facilidade de produção e maior facilidade de manipulação, além disso, a resposta imune de *G. mellonella* apresenta similaridades a resposta inata de mamíferos (BORMAN, 2018; SMITH; CASADEVALL, 2021). EVs derivadas de *C. albicans* também podem ser internalizadas por macrófagos e DCs derivados de

medula, após essa interação há a produção de citocinas inflamatórias como TNF-α, IL-12 e anti-inflamatórias como TGF-β e IL-10, bem como aumento de marcação para CD86 e MHC-II (VARGAS; ROCHA; OLIVEIRA; ALBUQUERQUE *et al.*, 2015). Além disso, as EVs eram capazes de proteger a larva de *G. mellonella* frente ao desafio com leveduras de *C. albicans* (VARGAS; ROCHA; OLIVEIRA; ALBUQUERQUE *et al.*, 2015). Em modelo vacinal utilizando camundongos, EVs de *C. albicans* foram capazes de proteger os animais frente ao desafio letal com *C. albicans* (VARGAS; HONORATO; GUIMARAES; RODRIGUES *et al.*, 2020). O número de unidades formadoras de colônias (CFU) nos animais tratados era menor em vários órgãos estudados, quando comparados a animais não vacinados (VARGAS; HONORATO; GUIMARAES; RODRIGUES *et al.*, 2020). *A. flavus*, *P. brasiliensis* e *Trichophyton interdigitale* apresentam uma certa similaridade na forma em que suas EVs estimulam macrófagos. As EVs destes fungos foram capazes de estimular a produção de mediadores inflamatórios como TNF-α, IL-6, IL-1β e NO. Essa forte resposta inflamatória se refletia na polarização dos macrófagos, que apresentavam maior expressão de iNOS, caracterizando sua polarização clássica (BITENCOURT; REZENDE; QUARESEMIN; MORENO *et al.*, 2018; BRAUER; PESSONI; BITENCOURT; DE PAULA *et al.*, 2020; DA SILVA; ROQUE-BARREIRA; CASADEVALL; ALMEIDA, 2016). Além disso, EVs de *A. flavus*, também são capazes de conferir proteção as larvas de *G. mellonella* tratadas, frente ao desafio fúngico (BRAUER; PESSONI; BITENCOURT; DE PAULA *et al.*, 2020). Ainda, EVs de *A. fumigatus* também são bioativas e induzem resposta imune *in vivo* e *in vitro*. Utilizando a cepa A1163, Souza e colaboradores demonstraram que EVs de *A. fumigatus* induziam a secreção de TNF-α e CCL2 em macrófagos, e ainda aumentavam a fagocitose e morte celular de conídios, por macrófagos e neutrófilos estimulados com EVs (SOUZA; BALTAZAR; CARREGAL; GOUVEIA-EUFRASIO *et al.*, 2019). Ademais, o mesmo grupo de pesquisa ainda demonstrou que a administração vacinal de EVs de *A. fumigatus* em camundongos foi capaz de reduzir a inflamação causada pelo desafio fúngico, porém os animais sucumbiam rapidamente frente a infecção, assim como animais não imunizados (SOUZA; GURGEL; MALACCO; MARTINS *et al.*, 2022). Além dos relatos aqui descritos, já foi observado efeito imunomodulador de outras EVs fúngicas, utilizando ensaios *in vivo* e *in vitro* (FREITAS; PESSONI; COELHO; BONATO *et al.*, 2021).

Figura 3: Algumas das funções descritas para EVs fúngicas.



Fonte: (LIEBANA-JORDAN; BROTONS; FALCON-PEREZ; GONZALEZ, 2021).

2. Objetivos

2.1. Objetivo geral

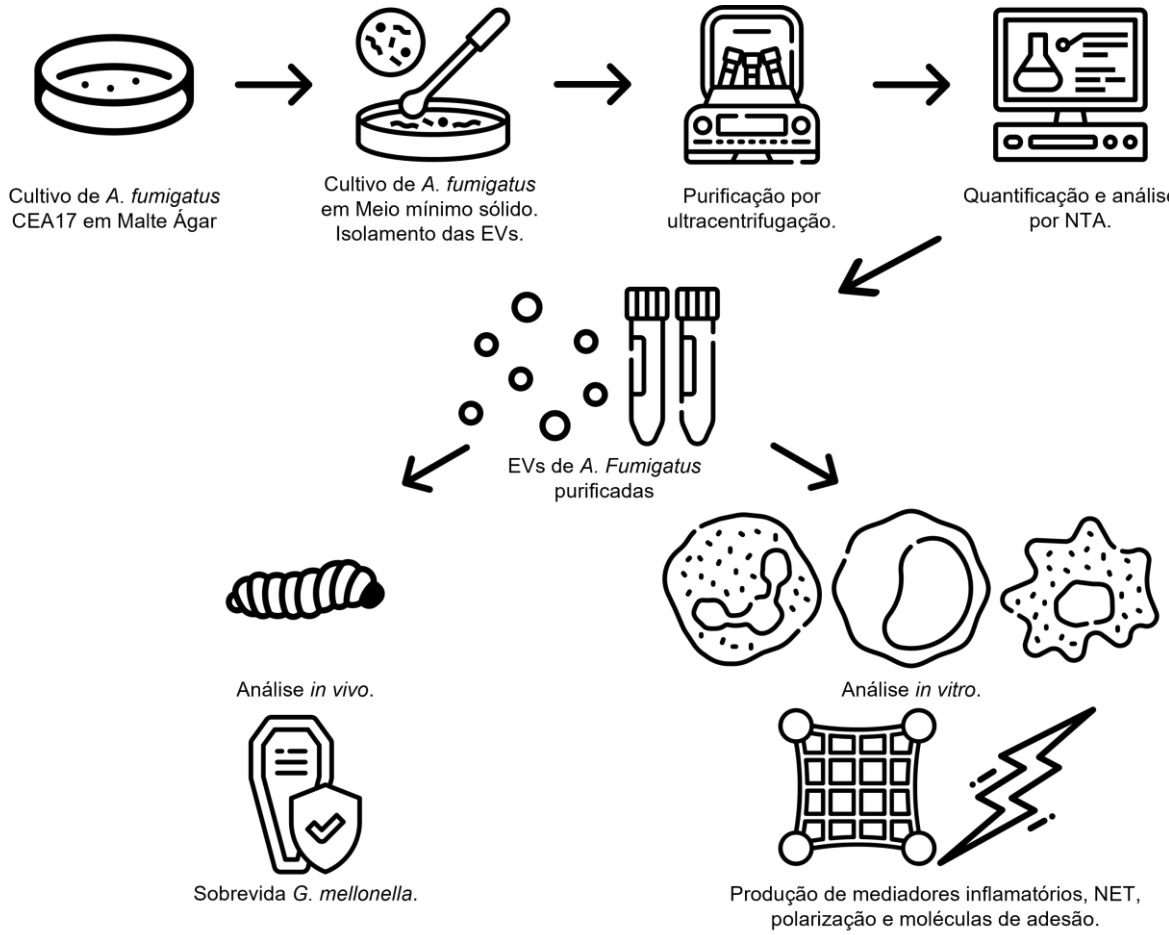
Isolar e purificar EVs de *A. fumigatus* CEA17 e avaliar sua ação em células imunes, verificando a produção de mediadores inflamatórios e polarização celular, bem como avaliar a atividade das EVs em modelo invertebrado utilizando larvas de *G. mellonella.*

2.2. Objetivos específicos

- Avaliar a produção dos seguintes mediadores inflamatórios por macrófagos RAW 264.7 e AMJ2-C11 tratados com EVs:
    - IL-6, TNF e IL-1β;
    - NO e superóxido;
- Avaliar a polarização de macrófagos RAW 264.7 e AMJ2-C11 tratados com EVs;
- Avaliar a expressão de RNA mensageiro para CD11b e CD18 em macrófagos RAW 264.7 e AMJ2-C11 tratados com EVs;
- Verificar a indução de NETs em neutrófilos humanos tratados com EVs;
- Verificar a produção de TNF-α, IL-6 e IL-10 em PBMCs humanos tratados com EVs;
- Verificar a proteção induzida pelo tratamento de EVs em *G. mellonella*.

3. Delineamento Experimental

Figura 4: Delineamento Experimental



Fonte: De autoria própria.

Capítulo II

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Aspergillus fumigatus Extracellular Vesicles Display Increased *Galleria mellonella* Survival but Partial Pro-Inflammatory Response by Macrophages

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Abstract: Fungal extracellular vesicles (EVs) mediate intra- and interspecies communication and are critical in host–fungus interaction, modulating inflammation and immune responses. In this study, we evaluated the *in vitro* pro- and anti-inflammatory properties of *Aspergillus fumigatus* EVs over innate leukocytes. *A. fumigatus* EVs induced a partial proinflammatory response by macrophages, characterized by increased tumor necrosis factor-alpha production, and increased gene expression of induced nitric oxide synthase and adhesion molecules. EVs induce neither NETosis in human neutrophils nor cytokine secretion by peripheral mononuclear cells. However, prior inoculation of *A. fumigatus* EVs in *Galleria mellonella* larvae resulted in increased survival after the fungal challenge. Taken together, these findings show that *A. fumigatus* EVs play a role in protection against fungal infection, although they induce a partial pro-inflammatory response.

Keywords: *Aspergillus fumigatus*; extracellular vesicles; inflammation; innate leukocytes; protection

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1. Introduction

Aspergillus fumigatus is the main causative agent of aspergillosis, which can be a fatal disease [1]. *Aspergillus* are saprophytic organisms that release many airborne conidia [2,3]. The first line of defense against inhaled *A. fumigatus* conidia are resident macrophages, which interact with pathogen-associated molecular patterns from swollen conidia and promote cytokine release, phagocytosis, and intracellular killing [4]. Soluble factors, such as CXCL1 and CXCL2, released by resident macrophages, and other immune cells, recruit neutrophils to the site of infection [5]. Neutrophils are pivotal in conidia elimination and host protection [6].

Extracellular vesicles (EVs) are released by virtually all living organisms. The spherical structures formed of a lipid bilayer membrane transport several biomolecules [7,8]. Several species of fungi produce EVs [9–14] that have important biological functions [15]. Although their role in the infective process is still not clear, fungal EVs have been reported to have an effect on host immune responses and could also mediate intraspecies fungal communication [16,17]. EVs from *A. flavus*, *Paracoccidioides brasiliensis*, and *Trichophyton interdigitale* have strong proinflammatory activity for macrophages, with the increased release of proinflammatory mediators such as nitric oxide (NO), IL-12p40, IL-12p70, IL-6, TNF-α, IL-1α, and IL-1β, augmented phagocytosis and fungal killing, and activation toward inflammatory or M1 macrophages [13,18,19]. On the other hand, EVs from *Histoplasma capsulatum* display anti-inflammatory activity for macrophages and might reduce phagocytosis, intracellular killing, and the production of reactive oxygen species (ROS) [20]. These studies

have demonstrated that fungal EVs have different actions on innate leukocytes, priming these cells to better control fungal infection or foster fungal growth.

Since EVs play an up- or down-modulatory role, in this study, we investigated the role of *A. fumigatus* EVs in the activation status of macrophages, neutrophils, and monocytes. These cells are pivotal in immune responses against *A. fumigatus*. Our data suggest that *A. fumigatus* EVs play a role in protection against fungal infection, inducing a partial pro-inflammatory response. Thus, our results suggest that *A. fumigatus* EVs could be exploited in antifungal therapies.

2. Materials and Methods

2.1. Ethics Statement

The protocols involving human cells used in this study were approved by the Research Ethics Committee of the University Hospital, Ribeirao Preto Medical School at the University of Sao Paulo (protocol 4.277.966), and informed written consent from all participants was obtained.

2.2. A. fumigatus Strain and Growth Conditions

A. fumigatus strain CEA17 was cultivated as described previously [16], with slight modifications. Freshly thawed vials containing conidia were seeded in a complete agar malt medium for 7 days at 37 °C. These plates were washed with PBS followed by centrifugation and filtration through sterile Miracloth (Millipore, Billerica, MA, USA) to obtain conidia suspensions. Conidia concentration was determined using a Neubauer chamber. Approximately 1×10^7 conidia were seeded in agar minimal medium [19]. After 2 days of incubation in 37 °C, the plates were washed with sterile PBS. EVs were then isolated and purified.

2.3. Isolation and Purification of EVs

Plates containing 2-day-old *A. fumigatus* cultures were washed twice with 5 mL of sterile PBS with the aid of inoculation loops. The recovered solution was filtered through sterile Miracloth (Millipore) and centrifuged at 5000× *g* for 15 min at 4 °C. The supernatant was recovered and centrifuged again at 15,000× *g* for 15 min at 4 °C. Supernatant concentration was performed in an Amicon system. Another centrifugation at 15,000× *g* for 15 min at 4 °C was performed, and the supernatant was filtered through a 0.45 µm filter. An ultracentrifugation was performed at 60,000 rpm for 1 h at 4 °C in a TLA-120.2 rotor (Beckman Coulter). The pellet was resuspended in sterile PBS. The due rotor fixed angle of rotation was 30 degrees; 60,000 rpm are equivalent of 98,800× *g* in its minimum radius. The obtained EVs were subjected to nanoparticle tracking analysis using a NanoSight NS300 system (Malvern Instruments, Malvern, UK) to verify their size, distribution, and concentration.

2.4. Culture of Macrophages

AMJ2-C11 and RAW 264.7 macrophages were maintained in Dulbecco's modified Eagle's medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 37 °C in a 5% CO_2 atmosphere. Cells were cultivated until confluence and detached from the flask bottom with Trypsin-EDTA solution (Sigma–Aldrich). The cells were counted in a Neubauer chamber and plated in 96- or 48-well culture plates (depending on the assay performed) and in a fetal bovine serum-free medium.

2.5. Isolation of PBMCs

Blood withdrawn under authorized supervision was collected using Vacutainer heparin collection tubes. PBMC separation was performed using Histopaque-1077 (Sigma–Aldrich). Briefly, collection tubes were centrifuged at 500× *g* for 20 min at 20 °C. The separated plasma was discarded. The remaining samples were diluted in PBS up to 15 mL.

The solution was carefully layered over 6 mL of Histopaque-1077 solution and centrifuged at 500× *g* for 30 min at 20 °C. The rings formed with PBMCs were harvested and washed with PBS and centrifuged at 500× *g* for 10 min at 20 °C. The cells were resuspended in RPMI 1640 medium (Sigma–Aldrich) with 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific Inc.) lacking fetal bovine serum.

2.6. Cytokine Measurement

AMJ2-C11 or RAW 264.7 macrophages were plated in 96-well plates (3×10^4 cells/well) at 37 °C in a 5% CO_2 atmosphere and rested overnight. Cells were stimulated with EVs (10^7 to 10^{10} EVs/mL), LPS (1 μg/mL) or medium alone for 24 h at 37 °C in a 5% CO_2 atmosphere. After incubation, the supernatant was tested for the presence of TNF-α, IL-6, and IL-1β via enzyme-linked immunosorbent assay using an OptEIA kit (Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions. For the PBMCs, the cells were plated in 96-well plates (2×10^5 cells/well) at 37 °C in a 5% CO_2 atmosphere, and soon after they were stimulated with EVs (10^9 to 10^{10} EVs/mL), LPS (1 μg/mL) or medium alone for 24 h at 37 °C in a 5% CO_2 atmosphere. The presence of human cytokines (TNF-α, IL-6 and IL-10) was assessed using an OptEIA kit as described above. Absorbance was read at 450 nm in the Power Wave-X microplate scanning spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

2.7. Nitric Oxide (NO) Measurement

NO was measured in the supernatant of AMJ2-C11 cells plated (3×10^4 cells/well) in 96-well plates at 37 °C in a 5% CO_2 atmosphere stimulated for 24 h with EVs (10^9 and 10^{10} EVs/mL), Pam3CSK4 (1 μg/mL), or medium alone. NO was quantified using the Griess method [21]. Briefly, 50 μL of cell supernatant was mixed with the same volume of Griess reagent (1.0% sulfanilamide, 0.1% naphthalenediamine dihydrochloride, and 2.5% H_3PO_4) at room temperature for 10 min in 96-well plates. Absorbance at 550 nm was read using a Power Wave-X microplate reader (BioTek Instruments, Inc.). The absorbance was converted to micromolar (μM) NO based on a standard curve generated using known concentrations of $NaNO_2$.

2.8. Detection of $O_2^{\bullet -}$ by Lucigenin Enhanced Chemiluminescence

Superoxide levels were measured in AMJ2-C11 and RAW 264.7 culture supernatants using the lucigenin-derived chemiluminescence assay. Both cells were plated (3×10^4 cells/well) in 96-well plates at 37 °C in a 5% CO_2 atmosphere and stimulated for 24 h with EVs (10^9 and 10^{10} EVs/mL), LPS (1 μg/mL), or medium alone. The reaction was carried out by the addition of 50 μL of the sample in the assay buffer, containing 50 mM KH_2PO_4, 1 mM EGTA, 150 mM sucrose, (pH 7.4) 5 μM lucigenin in 250 μL total volume. The addition of 0.1 mM of NAD(P)H started the reaction. Luminescence was measured with an Orion II luminometer (Berthold Technologies, Pforzheim, Germany). Blank was subtracted from each sample reading. The production of superoxide was expressed as relative light unit (RLU)/3×10^4 cells.

2.9. qPCR

AMJ2-C11 and RAW 264.7 macrophages were plated in 48-well plates (4×10^5 cells/well) at 37 °C in a 5% CO_2 atmosphere and rested overnight. The cells were stimulated with EVs (1×10^{10} EVs/mL), LPS (1 μg/mL), LPS + Pam3CSK4 (500 ng/mL each), or medium alone for 6 and 24 h at 37 °C in a 5% CO_2 atmosphere. The supernatant was discarded, and the cells were used for RNA extraction using the Illustra RNAspin Mini RNA isolation kit (GE Healthcare, Chicago, IL, USA) following the manufacturer's instructions. RNA concentration and quality were measured using a nanophotometer (Implen, Westlake Village, CA, USA). Before complementary DNA (cDNA) synthesis, RNA was treated with DNase (Sigma–Aldrich). cDNA synthesis was performed using a high-capacity cDNA reverse-transcription kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR Green

(Applied Biosystems) in the Step One Plus platform. The relative expression of transcripts was quantified using the ΔΔCt method and normalized relative to Gapdh expression. The primers used are listed in Table 1.

Table 1. Primers used in qPCR.

Gene	Sequence 5′–3′	Concentration (nM)	Efficiency (%)	Ref.
iNOS2	FWD: CCGAAGCAAACATCACATTCA REV: GGTCTAAAGGCTCCGGGCT	70	110.11	[18]
arginase-1	FWD: GTTCCCAGATGTACCAGGATTC REV: CGATGTCTTTGGCAGATATGC	100	99.33	[18]
CD11b	FWD: TACTTCGGGCAGTCTCTGAGTG REV: ATGGTTGCCTCCAGTCTCAGCA	300	94	[22]
CD18	FWD: CTTTCCGAGAGCAACATCCAGC REV: GTTGCTGGAGTCGTCAGACAGT	300	105.5	[23]
Gapdh	FWD: GGTGCTGAGTATGTCGTGGA REV: CGGAGATGATGACCCTTTTG	300	108.76	[24]

The term efficiency (%) is related to the efficiency of the qPCR reaction, considering the kinetics of the reaction, assuming a twofold increase in amplicon in each cycle for 100% efficiency. In general, PCR efficiency should be 90–110% for target and normalizing genes, which ensures accurate quantification. To verify this information, a standard curve was performed using two-fold serial dilutions; the reaction efficiency for each pair of primers is provided within the table.

2.10. Macrophage Adhesion Assay

High binding plates were coated with fibrinogen (20 µg/mL; Sigma–Aldrich) overnight at 4 °C. The plate was washed with sterile PBS, and RAW 264.7 macrophages were seeded at 6×10^4 cells/well. Immediately after seeding, the cells were treated with LPS (1 µg/mL) or EVs (1×10^{10} EVs/mL). The plate was kept in a humidified incubator at 37 °C in a 5% CO_2 atmosphere for 24 h. Each well was washed three times with 200 µL of PBS to remove non-adherent cells. After the last wash, the cells were incubated with a solution of 100 µM Resazurin (Sigma–Aldrich) in 150 µL of incomplete DMEM. After 4 and 24 h of incubation at 37 °C in a 5% CO_2 atmosphere, the plate was read in a plate reader (BioTek Instruments, Inc.) at 570 and 600 nm. The percentage of the reduced form of Resazurin was calculated following the manufacturer's instructions. Increased levels of reduced Resazurin were an indication of more cells in each well.

2.11. Isolation and Culture of Neutrophils

Healthy volunteers provided consent to serve as blood donors. Blood was withdrawn under authorized supervision. The blood was collected in tubes containing EDTA, and neutrophils were isolated by a Percoll gradient. Briefly, the gradient was layered by adding Percoll concentrations of 72, 64, 54, and 45%. Blood sample was added above the 45% layer. The gradient was centrifuged at $650\times g$ for 32 min. The layer of polymorphonuclear cells was collected, centrifuged at $400\times g$ for 10 min, and plated (5×10^5 cells/well). The cells were stimulated with EVs (10^9 or 10^{10} EVs/mL) overnight. As the positive control, cells were stimulated with 10 µM phorbol myristate acetate (PMA) for 2 to 4 h. PMA is a known inducer of NETosis [25].

2.12. Detection of NETs by Flow Cytometry

Cultured neutrophils were labeled with FVS780 viability stain (BD Biosciences, San Diego, CA, USA) and Sytox Green (Thermo Fisher Scientific). Fluorescence was detected using a FACS Melody device (BD Biosciences, San Jose, CA, USA). Analysis was performed using the FlowJo software (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

2.13. G. mellonella Survival Assay

G. mellonella larvae were kept in sterile glass flasks with modified lids, with a hole in the center covered with an ultra-fine stainless steel wire mesh for better ventilation. They

were maintained in a BOD SL-200 incubator (Solab cientifica) in the dark with a controlled temperature of 28 °C. They were fed with an in-house-produced mixture of bee wax, beer yeast, soy, honey, powder milk, and corn meal. *G. mellonella* survival was assayed as previously described [19]. Ten healthy *G. mellonella* larvae of similar weight (approximately 240 to 300 mg) were used in each group. Each larva was inoculated with 57 µL of 10^{10}, 10^{9}, or 10^{8} EVs, or with PBS as the control, into the last left proleg, directly on the hemocoel. After 48 h, all larvae were infected with 10 µL of a solution containing 100 conidia/larvae (1×10^{4} conidia/mL) of *A. fumigatus* at the same site of injection. Preliminary experiments using 10^{3} and 10^{6} conidia/larvae showed the early death of the larvae (2 days) after conidia inoculum. With the above-mentioned concentration of 100 conidia/larvae, the larvae were able to survive longer, and we could perform the survival analysis clearly. The larvae were kept at 37 °C in the dark. Mortality was monitored daily; a lack of movement after physical stimuli was indicative of larva death. We performed a test to verify that neither the inoculum volume nor the site of injection was the cause of larva death. The results showed that despite the inoculum volume (57 µL) and the site of the second injection (the same proleg) there was no increase in larvae mortality.

2.14. Statistical Analysis

The results are expressed as the mean ± SEM of the three independent experiments. The statistical analysis was carried out using the Graph Pad Prism software version 8.4.3 (GraphPad Software, San Diego, CA, USA). Homogeneous variance was analyzed, and the difference between the means of the groups was calculated by analysis of variance (one-way) and Dunnett's test thereafter. In addition, a two-tailed unpaired *t* test was used for the parametric data. For comparisons of survival curves, log rank (Mantel–Cox) tests were used. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. A. fumigatus EVs Induce TNF-α by Macrophages

Fungal EVs transport several molecules that up- or down-modulate inflammation and immune responses. To clarify the *in vitro* inflammatory properties of *A. fumigatus* EVs, we analyzed the AMJ2-C11 and RAW 264.7 macrophage lines stimulated with different concentrations of *A. fumigatus* EVs (10^{7} to 10^{10} EVs/mL) for 24 h. In parallel, those cells were stimulated with LPS, as a positive control, or were left unstimulated (medium). AMJ2-C11 macrophages showed significant and increased production of TNF-α at the highest concentration of EVs (10^{10} EVs/mL) compared to the unstimulated cells (medium), which were not statistically different from the LPS-stimulated cells (Figure 1A). The production of IL-6 was not altered (Figure S1A). Similar results were observed with RAW 264.7 cells; TNF-α production was increased upon stimulation with 10^{9} and 10^{10} EVs/mL (Figure 1D), but no alterations in IL-6 and IL-1β production were observed (Figure S1B,C).

Nitric oxide (NO) and superoxide ($O_2^{\bullet -}$) play important roles in killing invading organisms, acting in phagolysosomes inside phagocytes. $O_2^{\bullet -}$ is involved in host defense against *A. fumigatus* infection *in vivo* [26]. After 24 h of stimulation with *A. fumigatus* EVs at the highest concentration (10^{10} EVs/mL), AMJ2-C11 macrophages exhibited increased NO production (Figure 1C) and no $O_2^{\bullet -}$ production (Figure 1B). RAW 264.7 macrophages showed reduced $O_2^{\bullet -}$ production after stimulation with 10^{10} EVs/mL (Figure 1E).

3.2. A. fumigatus EVs Induce Lower Arginase-1 and Higher iNOS Transcription in RAW 264.7 Macrophages

Activated macrophages or M1 macrophages, which express the iNOS enzyme and produce proinflammatory mediators, are effective for killing *A. fumigatus* [27,28]; anti-inflammatory or M2 macrophages that express arginase 1 (Arg1) also have a phagocytic profile in an *in vivo* infection, but their killing capabilities have not been addressed [29]. To address the polarization status of macrophages in response to *A. fumigatus* EVs, we analyzed their transcriptional gene profile. AMJ2C-11 and RAW 264.7 cells were stimulated

for 24 h with 10^{10} *A. fumigatus* EVs/mL. Both mRNA levels of iNOS and arginase-1 were increased in RAW 264.7 cells after incubation with *A. fumigatus* EVs (Figure 2A,B). Only arginase-1 had increased gene expression in AMJ2-C11 cells (Figure 2C).

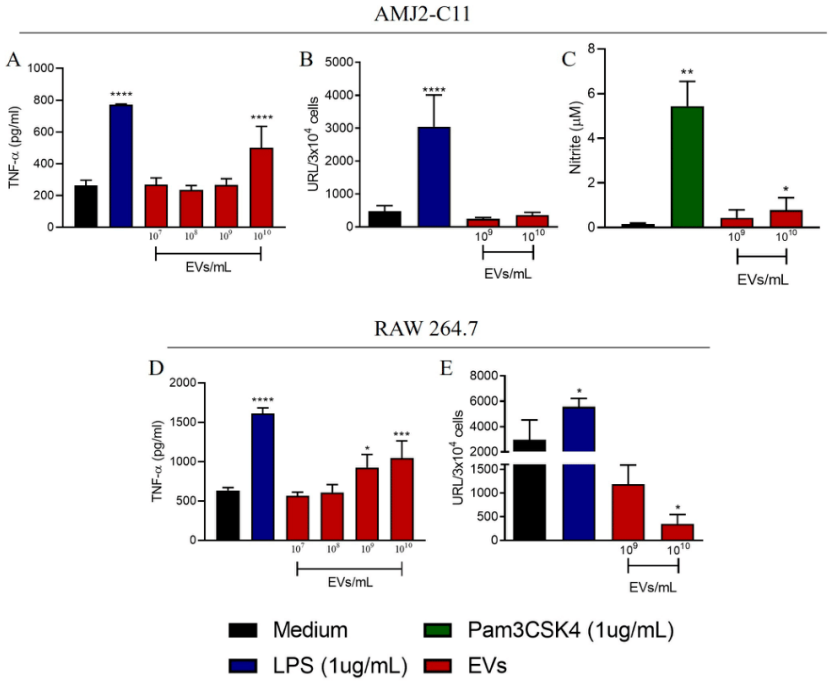

Figure 1. Production of inflammatory mediators by macrophages stimulated with EVs. AMJ2-C11 and RAW 264.7 macrophages were stimulated with EVs (10^7 to 10^{10} EVs/mL) for 24 h. For AMJ2-C11 macrophages, TNF-α (**A**), $O_2^{\bullet -}$ (**B**), and NO (**C**) were measured in the supernatant. For RAW 264.7 macrophages, TNF-α (**D**) and $O_2^{\bullet -}$ (**E**) were measured. The results are expressed as mean ± SEM and were compared to medium through one-way analysis of variance followed by Dunnett's test. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

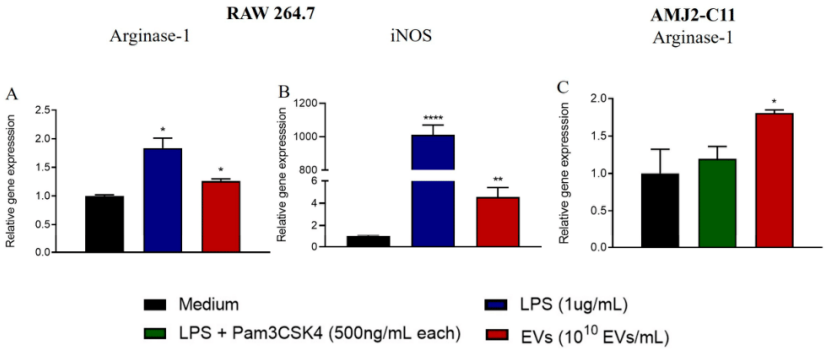

Figure 2. RAW 264.7 macrophages, but not AMJ2-C11, display polarization toward an M1 profile after stimulation with EVs. After 24 h of stimulation with 10^{10} EVs/mL, mRNA was extracted from AMJ2-C11 and RAW 264.7 macrophages. qPCR was performed to assess the expression of polarization signature genes. Panels A and B show the relative expression of arginase-1 (**A**) and iNOS (**B**) of RAW 264.7 macrophages. Panel (**C**) shows the relative expression of arginase-1 in AMJ2-C11 macrophages after 24 h of treatment. The results are expressed as mean ± SEM and were compared to the medium through an unpaired two-tailed *t* test. T test. **** $p < 0.0001$, ** $p < 0.01$ and * $p < 0.05$.

3.3. *A. fumigatus* EVs Augment Adhesion Molecule Gene Expression in RAW 264.7 Macrophages

Optical microscopy revealed the altered morphology of RAW 264.7 cells stimulated with *A. fumigatus* EVs for 24 h (Figure S2). The cells displayed more dendritic-like protrusions and adhered more avidly to the flask bottom, suggesting increased production of adhesion molecules. Treatment with EVs for 6 h had no influence on CD11b or CD18 transcript levels (Figure 3A,C), while increased CD11b and CD18 expression levels were detected after 24 h stimulation (Figure 3B,D). CD11b and CD18 form the alpha and beta chain of the Macrophage-1 antigen (Mac-1), respectively, which mediates cellular adhesion and other responses [30]. The optical microscopy results were consistent with qPCR results. Regarding AMJ2-C11 cells stimulated with *A. fumigatus*, EVs did not display morphological changes, and CD11b and CD18 transcript levels were not altered at any time point (Figure 3E–H). Using an adhesion assay with plates coated with fibrinogen, the ligand for Mac-1 [30], we showed that RAW 264.7 macrophages stimulated with *A. fumigatus* EVs showed no difference between treatment with EVs and unstimulated cells (medium); both showed similar percentages of reduced Resazurin (Figure S3). LPS induced increased adhesion of RAW 264.7 cells, as demonstrated by the higher percentage of reduced Resazurin, which correlated with the attachment of more cells in the wells (Figure S3).

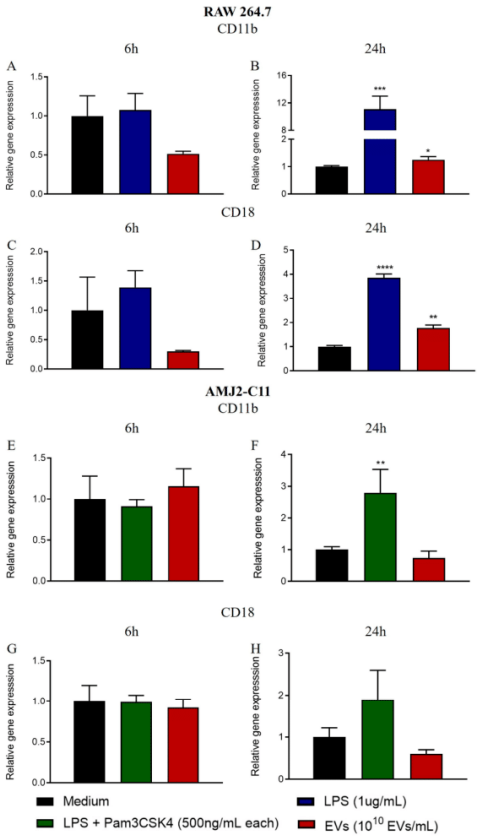

Figure 3. RAW 264.7 macrophages, but not AMJ2-C11, show increased mRNA levels in adhesion molecules after stimulation with EVs. AMJ2-C11 and RAW 264.7 cells were treated with 10^{10} EVs/mL for 6 and 24 h. The relative expression of CD11b and CD18 was assessed by qPCR. The relative expression of CD11b and CD18 after 6 and 24h of treatment in RAW 264.7 cells is shown in panels (**A**–**D**). The relative expression of CD11b and CD18 after 6 and 24 h of treatment in AMJ2-C11 cells is shown in panels (**E**–**H**). The results are expressed as mean ± SEM and were compared to the medium through an unpaired two-tailed *t* test. T test. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

3.4. *A. fumigatus* EVs Fail to Induce NETs Release and Cytokine Production by Neutrophils

To evaluate the effect of *A. fumigatus* EVs on the release of neutrophil extracellular traps (NETs), neutrophils isolated from the blood of healthy donors were stimulated with *A. fumigatus* EVs. Flow cytometry analysis confirmed that stimulation with (PMA) as the positive control induced the release of NETs and promoted neutrophil death, a mechanism known as NETosis. Increased NETosis or the release of NETs was not evident when neutrophils were stimulated with 10^9 or 10^{10} *A. fumigatus* EVs/mL was compared with the negative control (Figure 4A,B). Moreover, *A. fumigatus* EVs did not induce IL-6 and IL-1β production by human neutrophils (Supplementary Material). The results suggest that *A. fumigatus* EVs neither induce NET release nor alter cytokine production by human neutrophils. We also assessed the production of IL-6, TNF-α, and IL-10 by human PBMCs treated with *A. fumigatus* EVs for 24 h. IL-6 secretion was increased due to 10^{10} EVs/mL (Figure 5A); however, EVs did not induce TNF-α or IL-10 production (Figure 5B,C).

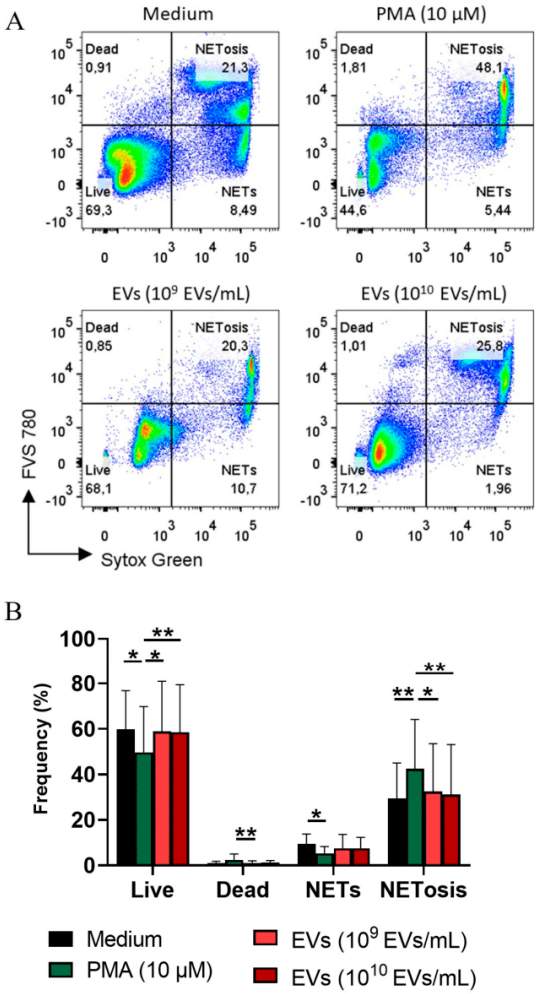

Figure 4. EVs do not induce NETosis in human neutrophils. Human neutrophils were stimulated with EVs or phorbol myristate acetate (PMA). Representative dot plot of neutrophils stimulated with PMA (positive control) or 10^9 or 10^{10} EVs/mL (**A**). Percentage of live cells (FVS780−Sytox−), dead cells (FVS780+Sytox−), NETs (FVS780−Sytox+), and NETosis (FVS780+Sytox+) (**B**). Data are representative of three experiments (n = 9/group) and are expressed as mean ± SD. * $p < 0.05$; ** $p < 0.001$.

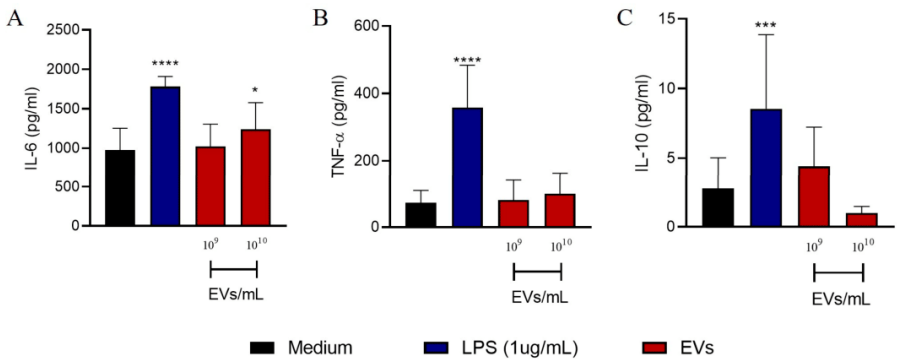

Figure 5. EVs induce the release of IL-6 by human PBMCs. PBMCs (2×10^5 cells/well) were stimulated with 10^9 or 10^{10} EVs/mL for 24 h. The production of IL-6 (**A**), TNF-α (**B**), and IL-10 (**C**) was measured in the supernatant. The results are expressed as mean ± SEM and were compared to the medium through one-way analysis of variance followed by Dunnett's test. **** $p < 0.0001$, *** $p < 0.001$, and * $p < 0.05$.

3.5. EVs Induce Increased Survival in Galleria Mellonella Model of A. fumigatus Infection

Since *A. fumigatus* EVs were unable to induce a robust proinflammatory response from innate leukocytes and human PBMCs, we assessed whether *A. fumigatus* EVs could influence *G. mellonella* survival in an *in vivo* model of infection. *G. mellonella* has an immune system that, in some respects, shows similarities to the mammal innate immune system which can be used to explore the interaction of fungal pathogens and the host [31]. *G. mellonella* larvae were treated with 10^8 to 10^{10} *A. fumigatus* EVs/larvae for 48 h and were then infected with *A. fumigatus* conidia. In the control group (PBS-treated), all larvae were dead by day 11. In contrast, survival was greater among the treated larvae (Figure 6). Treatment with 10^8 or 10^9 EVs significantly increased the survival of *G. mellonella*, leading to death only by days 15 and 16; this indicates the protective effect of *A. fumigatus* EVs *in vivo*.

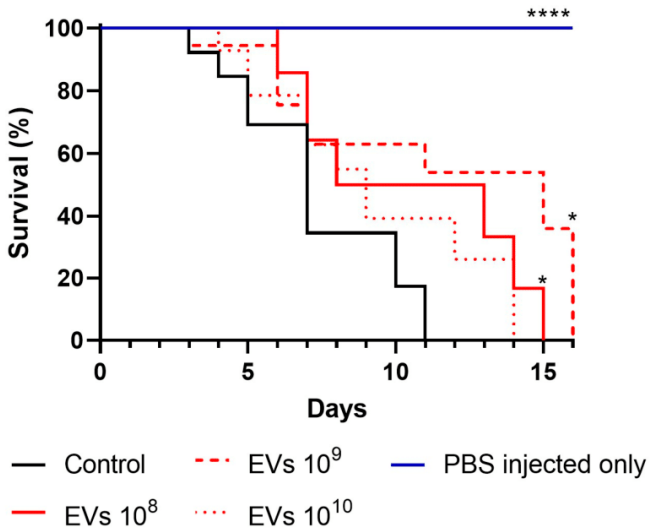

Figure 6. Treatment of *G. mellonella* with EVs increases larvae survival after fungal challenge. *G. mellonella* larvae were treated with 10^8, 10^9, or 10^{10} EVs. After 48 h, each larva was infected with 100 conidia of *A. fumigatus*. Larva survival was assessed daily until all larvae were dead. Log rank (Mantel–Cox) test for survival curve analysis. **** $p < 0.0001$, * $p < 0.05$. The PBS-injected-only group were injected with 57 µL of PBS in the last left proleg; after 48 h, 10 µL of PBS was injected at the same site, and mortality was recorded.

4. Discussion

Several biomolecules transported by EVs are related to fungal survival, virulence, and immune evasion [32]. Herein we investigated the *in vitro* pro- and anti-inflammatory properties of *A. fumigatus* EVs. A previous study by our group showed that treatment with *A. flavus* EVs induced the production of TNF-α, IL-6, IL-1β, and NO by bone marrow-derived macrophages (BMDMs) [19]. *A. flavus* EV-treated BMDMs showed an increased phagocytic index and intracellular killing abilities, which were related to an M1 macrophage polarization [19].

In this study, we used AMJ2-C11 and RAW 264.7 macrophages. AMJ2-C11 is an alveolar macrophage line. Thus, AMJ2-C11 cells would mimic the first phagocytes to interact with EVs *in vivo*, while RAW 264.7 macrophages would mimic the contact of EVs in a disseminated infection. Our results show a lower pro-inflammatory profile for *A. fumigatus* EVs compared to *A. flavus* EVs. Both macrophage cell lines displayed increased TNF-α production only when stimulated with the highest concentrations of EVs (10^9 and 10^{10} EVs/mL). NO production was only increased in AMJ2-C11 macrophages stimulated with the highest concentration of EVs (10^{10} EVs/mL). Superoxide was not induced by EVs in AMJ2-C11 cells, and a decrease was noted in RAW 264.7 cells, even when stimulated with the highest concentration of EVs compared to the control. The results suggest that *A. fumigatus* EVs may act differentially in both macrophage lines tested. In AMJ2-C11 alveolar macrophages derived from C57BL6J mice, EVs induced an increased expression of arginase-1 mRNA with no increased expression of iNOS mRNA. RAW 264.7 macrophages derived from the ascites of BALB/c mice displayed increased expression of arginase-1 and iNOS mRNA. Souza et al., 2019 also studied *A. fumigatus* EVs. They used the *A. fumigatus* A1163 strain grown in a YG liquid medium (yeast extract powder, glucose, and trace elements), while we have used the *A. fumigatus* CEA17 strain grown in a solid minimal medium. The nutrition status of the medium influences the fungal EVs' profile. Fungal species can produce different EVs with altered size and cargo depending on growth media [33]. EVs from *Histoplasma capsulatum* cultured in a rich medium (supplemented Ham's F-12) have double protein content, 18 times more sterol content, and altered protein expression when compared to a less nutritional medium such as RPMI-1640 [33]. Despite the differences in growth conditions and strain, Souza et al., 2019 showed increases in both the phagocytosis and fungal killing activities of RAW 264.7 macrophages, as well the production of TNF-α and chemokine CCL2, but not IFN-γ or IL-10 [34]. A similar response was noted when bone marrow-derived neutrophils (BMDNs) were tested, increasing phagocytosis and fungal killing but not prompting the production of TNF-α, IL-1β or IFN-γ [34]. Thus, these findings suggest that albeit the difference in the strain and culture conditions, EVs from *A. fumigatus* promote low pro-inflammatory cytokine production *in vitro* by some innate leukocytes. The production of inflammatory mediators after the interaction of *A. fumigatus* conidia with alveolar macrophages is necessary to convey the message to leukocytes indicating a microbe invasion. TNF-α secreted by alveolar macrophages is critical for fungal clearance [35], while NO and superoxide production within phagolysosomes are essential for fungal killing [26,27]. Secreted EVs may act as an immunomodulatory component of the invading *A. fumigatus* conidia. In this sense, it was demonstrated that immunization with *A. fumigatus* EVs could partially protect mice from fungal infection by evoking a less pronounced inflammation [36]. After the *A. fumigatus* conidia challenge, mice immunized with EVs displayed an immune regulatory profile in the lungs, evident as a reduced influx of inflammatory cells and primarily neutrophils and reduced vascular permeability. Furthermore, immunized animals produced fewer inflammatory cytokines in the lungs 48 h after infection, with increasing conidia phagocytosis and killing by neutrophils compared to non-immunized mice [36]. However, despite the less inflammatory profile of the EV-immunized mice, no difference in survival was observed when compared to the non-immunized ones [36]. Differing from this study, the above-mentioned study was performed using EVs from *A. fumigatus* strain A1163 cultivated in a liquid YG medium. Similar to the macrophage response, EVs failed to induce the production of IL-6 or IL1-β by human

neutrophils. The production of TNF-α and IL-1β by BMDNs was reportedly achieved only when the cells were treated with EVs and infected by *A. fumigatus in vitro* [34]. The findings demonstrate a priming role for *A. fumigatus* EVs. Likewise, when human PBMCs were treated with EVs, only IL-6 production was induced at the highest concentration of EVs, but no increased production of TNF-α or IL-10 was noted.

Fungal EVs can modulate innate cells [15]. *Candida albicans* EVs can induce the production of inflammatory mediators such as IL-12p40, TNF-α, IL-6, and NO by BMDCs, BMDMs, and RAW 264.7 cells [37,38]. *C. auris* EVs increased the production of IL-6 by BMDC [39]. *C. neoformans* EVs increased the production of TNF-α, TGF-β, IL-6, and NO by RAW 264.7 macrophages [40]. *P. brasiliensis* EVs induce a strong proinflammatory response by murine peritoneal macrophages with the production of IL-12p40, IL-12p70, IL-6, TNF-α, IL-1α, IL-1β, and NO, which was related to an M1 macrophage polarization [18]. Similarly, *T. interdigitale* EVs can induce inflammatory mediators by BMDMs, which include TNF-α, IL-6, IL-1β, and NO, also displaying M1 macrophage polarization. *H. capsulatum* EVs impair the phagocytosis and fungal killing of stimulated BMDMs [20]. Thus, fungal EVs play different roles in activating or modulating innate responses. Our group has summarized these properties elsewhere [15].

RAW 264.7, but not AMJ2-C11 cells, stimulated with *A. fumigatus* EVs displayed an altered morphology with the formation of elongated protrusions, suggesting augmented expression of adhesion molecules. Indeed, we demonstrated increased mRNA levels of CD11b and CD18 integrins in EV-stimulated RAW 264.7 cells. In contrast, an adhesion assay showed no difference comparing the negative control to EV-treated cells. Due to the nature of the assay, the sensitivity may be not high enough to capture subtle changes in the expression of adhesion molecules. Therefore, further studies are necessary to definitively understand whether *A. fumigatus* EVs influence the expression and function of adhesion molecules.

Integrins are involved in several steps in phagocyte function. They modulate adhesion to extracellular matrix, phagocytosis, migration, and spreading [41]. An *in vitro* assay demonstrated that peritoneal polymorphonuclear neutrophils (PMNs) use Mac-1 or CD11b ($\alpha_M\beta_2$), while peritoneal activated macrophages use Mac-1 and complement receptor 4 (CR4) ($\alpha_X\beta_2$) to engulf and kill germinated *C. albicans* cells [42]. *In vivo* experiments with *C. albicans* infection demonstrated that Mac-1 is required for the elimination of invading fungi by PMNs, while CR4 is necessary for macrophage fungal killing [42]. Additionally, Mac-1 is important for the adhesion and internalization of *A. fumigatus* conidia into alveolar epithelial cells [43] and plays a pivotal role in NET formation by human neutrophils [44,45].

Conidia from *A. fumigatus* induce NET formation by human neutrophils [44,45]. In the present study, EVs failed to induce NET formation by human neutrophils. NETs released by neutrophils do not directly kill *A. fumigatus* [45]. However, NETs are a powerful tool to promote the control of the hyphal form of *A. fumigatus* by virtue of their fungistatic activity [46,47].

G. mellonella is an established invertebrate model of fungal infection, as its immune system shares some similarities with mammal innate immunity [31,48]. The *G. mellonella* hemolymph, which is analogous to human blood, contains hemocytes. Hemocytes are immune cells capable of many functions, including phagocytosis, nodulation, and encapsulation [49]. Accordingly, *G. mellonella* larvae are used to study many fungal infections [50–55]. Here we demonstrate that treatment with EVs prior to the *A. fumigatus* conidia infection of *G. mellonella* protected the larvae from early death. *G. mellonella* lack adaptive immunity [31]. Thus, the observed outcome must reflect the activation of hemocytes, which in turn partially protect larvae. As discussed above, Souza et al., 2022 demonstrated the EVs' vaccinal properties from *A. fumigatus* in a pulmonary aspergillosis mice model, probably by engaging the adaptive arm of immunity. Our work shows that the activation of *G. mellonella* immune cells by *A. fumigatus* EVs resulted in increased larva survival, suggesting that *A. fumigatus* EVs could promote an immune cell activation that protects the larvae. Other fungal EVs have been used in a similar manner to study the protection conferred by these vesicles.

Our group showed that treatment with *A. flavus* EVs resulted in a reduced CFU count and increased survival of *G. mellonella* larvae after *A. flavus* infection [19]. We observed a similar survival curve in PBS-treated larvae after *A. fumigatus* or a *A. flavus* infection, with larvae infected with *A. fumigatus* conidia showing slightly earlier death. Previous reports showed the opposite: in one case, *A. flavus* showed an increased pathogenicity in *G. mellonella* larvae when compared to *A. fumigatus* or *A. nidulans* [56]. Although the same number of *A. flavus* or *A. fumigatus* conidia per inoculum was used in both studies (100 conidia per larvae), in this study a much higher concentration of EVs was capable of conferring protection for the larvae after fungal infection. *C. albicans* EVs did not affect larva survival but significantly decreased the fungal burden of *G. mellonella*-infected larvae [37]. Interestingly, *G. mellonella* treatment with *C. neoformans* EVs resulted in the early death of treated larvae [57].

In summary, this work shows that *A. fumigatus* EVs induce a partial pro-inflammatory response by mouse macrophage lines, human neutrophils, and PBMCs. Also, *A. fumigatus* EVs act differently in macrophage lines, probably because those macrophages express a differential density of pattern recognition receptors. Finally, EVs protect *G. mellonella* larvae in an infection model, demonstrating its effects over innate cells responsible for the larvae protection. This work contributes to elucidating the inflammatory properties of EVs, which may account for the modulation of immune responses and possibly the control of infection. The understanding of initial steps in the crosstalk between *A. fumigatus* EVs and innate leukocytes might drive the identification of critical targets for the development of new anti-fungal therapies and adjuvants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jof9050541/s1, Figure S1: EVs fail to induce the production of IL-6 and IL-1β by macrophages.; Figure S2: EVs induce membrane extended processes in RAW 264.7 macrophages.; Figure S3: EVs fail to increase macrophage adhesion.

Author Contributions: M.S.F. and F.A. conceived the study. M.S.F., F.A., T.A.B. and C.P.R. performed the experimental design. M.S.F., T.A.B., C.P.R., N.S.M. and T.d.M.H.D. performed the laboratory experiments. M.S.F. and F.A. wrote the manuscript. M.S.F., T.A.B., C.P.R., N.S.M., T.d.M.H.D., C.R.T. and F.A. contributed to the editing and preparation of the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the University Hospital, Ribeirao Preto Medical School at the University of Sao Paulo (protocol 4.277.966 approval in 8 July 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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References

1. Abad, A.; Fernandez-Molina, J.V.; Bikandi, J.; Ramirez, A.; Margareto, J.; Sendino, J.; Hernando, F.L.; Ponton, J.; Garaizar, J.; Rementeria, A. What makes *Aspergillus fumigatus* a successful pathogen? Genes and molecules involved in invasive aspergillosis. *Rev. Iberoam. Micol.* **2010**, *27*, 155–182. [CrossRef]
2. van de Veerdonk, F.L.; Gresnigt, M.S.; Romani, L.; Netea, M.G.; Latge, J.P. *Aspergillus fumigatus* morphology and dynamic host interactions. *Nat. Rev. Microbiol.* **2017**, *15*, 661–674. [CrossRef]
3. McCormick, A.; Loeffler, J.; Ebel, F. *Aspergillus fumigatus*: Contours of an opportunistic human pathogen. *Cell. Microbiol.* **2010**, *12*, 1535–1543. [CrossRef]

4. Xu, S.; Shinohara, M.L. Tissue-Resident Macrophages in Fungal Infections. *Front. Immunol.* **2017**, *8*, 1798. [CrossRef] [PubMed]
5. Chotirmall, S.H.; Al-Alawi, M.; Mirkovic, B.; Lavelle, G.; Logan, P.M.; Greene, C.M.; McElvaney, N.G. *Aspergillus*-associated airway disease, inflammation, and the innate immune response. *BioMed Res. Int.* **2013**, *2013*, 723129. [CrossRef]
6. Latge, J.P.; Chamilos, G. *Aspergillus fumigatus* and Aspergillosis in 2019. *Clin. Microbiol. Rev.* **2019**, *33*, e00140-18. [CrossRef]
7. Deatherage, B.L.; Cookson, B.T. Membrane vesicle release in bacteria, eukaryotes, and archaea: A conserved yet underappreciated aspect of microbial life. *Infect. Immun.* **2012**, *80*, 1948–1957. [CrossRef]
8. Yanez-Mo, M.; Siljander, P.R.; Andreu, Z.; Zavec, A.B.; Borras, F.E.; Buzas, E.I.; Buzas, K.; Casal, E.; Cappello, F.; Carvalho, J.; et al. Biological properties of extracellular vesicles and their physiological functions. *J. Extracell. Vesicles* **2015**, *4*, 27066. [CrossRef]
9. Albuquerque, P.C.; Nakayasu, E.S.; Rodrigues, M.L.; Frases, S.; Casadevall, A.; Zancope-Oliveira, R.M.; Almeida, I.C.; Nosanchuk, J.D. Vesicular transport in *Histoplasma capsulatum*: An effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. *Cell. Microbiol.* **2008**, *10*, 1695–1710. [CrossRef] [PubMed]
10. Gehrmann, U.; Qazi, K.R.; Johansson, C.; Hultenby, K.; Karlsson, M.; Lundeberg, L.; Gabrielsson, S.; Scheynius, A. Nanovesicles from *Malassezia sympodialis* and host exosomes induce cytokine responses—Novel mechanisms for host-microbe interactions in atopic eczema. *PLoS ONE* **2011**, *6*, e21480. [CrossRef] [PubMed]
11. Vallejo, M.C.; Matsuo, A.L.; Ganiko, L.; Medeiros, L.C.; Miranda, K.; Silva, L.S.; Freymuller-Haapalainen, E.; Sinigaglia-Coimbra, R.; Almeida, I.C.; Puccia, R. The pathogenic fungus *Paracoccidioides brasiliensis* exports extracellular vesicles containing highly immunogenic alpha-Galactosyl epitopes. *Eukaryot. Cell* **2011**, *10*, 343–351. [CrossRef]
12. Silva, B.M.; Prados-Rosales, R.; Espadas-Moreno, J.; Wolf, J.M.; Luque-Garcia, J.L.; Goncalves, T.; Casadevall, A. Characterization of *Alternaria infectoria* extracellular vesicles. *Med. Mycol.* **2014**, *52*, 202–210. [CrossRef] [PubMed]
13. Bitencourt, T.A.; Rezende, C.P.; Quaresemin, N.R.; Moreno, P.; Hatanaka, O.; Rossi, A.; Martinez-Rossi, N.M.; Almeida, F. Extracellular Vesicles from the Dermatophyte *Trichophyton interdigitale* Modulate Macrophage and Keratinocyte Functions. *Front. Immunol.* **2018**, *9*, 2343. [CrossRef] [PubMed]
14. Ikeda, M.A.K.; de Almeida, J.R.F.; Jannuzzi, G.P.; Cronemberger-Andrade, A.; Torrecilhas, A.C.T.; Moretti, N.S.; da Cunha, J.P.C.; de Almeida, S.R.; Ferreira, K.S. Extracellular Vesicles from *Sporothrix brasiliensis* Are an Important Virulence Factor That Induce an Increase in Fungal Burden in Experimental Sporotrichosis. *Front. Microbiol.* **2018**, *9*, 2286. [CrossRef] [PubMed]
15. Freitas, M.S.; Pessoni, A.M.; Coelho, C.; Bonato, V.L.D.; Rodrigues, M.L.; Casadevall, A.; Almeida, F. Interactions of Extracellular Vesicles from Pathogenic Fungi with Innate Leukocytes. *Curr. Top. Microbiol. Immunol.* **2021**, *432*, 89–120. [CrossRef]
16. Bitencourt, T.A.; Hatanaka, O.; Pessoni, A.M.; Freitas, M.S.; Trentin, G.; Santos, P.; Rossi, A.; Martinez-Rossi, N.M.; Alves, L.L.; Casadevall, A.; et al. Fungal Extracellular Vesicles Are Involved in Intraspecies Intracellular Communication. *mBio* **2022**, *13*, e03272-21. [CrossRef]
17. Bielska, E.; May, R.C. Extracellular vesicles of human pathogenic fungi. *Curr. Opin. Microbiol.* **2019**, *52*, 90–99. [CrossRef]
18. Freitas, M.S.; Oliveira, A.F.; da Silva, T.A.; Fernandes, F.F.; Goncales, R.A.; Almeida, F.; Roque-Barreira, M.C. Paracoccin Induces M1 Polarization of Macrophages via Interaction with TLR4. *Front. Microbiol.* **2016**, *7*, 1003. [CrossRef]
19. Brauer, V.S.; Pessoni, A.M.; Bitencourt, T.A.; de Paula, R.G.; de Oliveira Rocha, L.; Goldman, G.H.; Almeida, F. Extracellular Vesicles from *Aspergillus flavus* Induce M1 Polarization In Vitro. *mSphere* **2020**, *5*, e00190-20. [CrossRef]
20. Baltazar, L.M.; Zamith-Miranda, D.; Burnet, M.C.; Choi, H.; Nimrichter, L.; Nakayasu, E.S.; Nosanchuk, J.D. Concentration-dependent protein loading of extracellular vesicles released by *Histoplasma capsulatum* after antibody treatment and its modulatory action upon macrophages. *Sci. Rep.* **2018**, *8*, 8065. [CrossRef]
21. Green, L.C.; Wagner, D.A.; Glogowski, J.; Skipper, P.L.; Wishnok, J.S.; Tannenbaum, S.R. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal. Biochem.* **1982**, *126*, 131–138. [CrossRef]
22. Wang, X.Z.; Zhang, S.Y.; Xu, Y.; Zhang, L.Y.; Jiang, Z.Z. The role of neutrophils in triptolide-induced liver injury. *Chin. J. Nat. Med.* **2018**, *16*, 653–664. [CrossRef]
23. Huang, J.; Zhou, Q. CD8+T Cell-Related Gene Biomarkers in Macular Edema of Diabetic Retinopathy. *Front. Endocrinol.* **2022**, *13*, 907396. [CrossRef]
24. Ip, W.K.; Medzhitov, R. Macrophages monitor tissue osmolarity and induce inflammatory response through NLRP3 and NLRC4 inflammasome activation. *Nat. Commun.* **2015**, *6*, 6931. [CrossRef]
25. Pruchniak, M.P.; Demkow, U. Potent NETosis inducers do not show synergistic effects in vitro. *Cent. Eur. J. Immunol.* **2019**, *44*, 51–58. [CrossRef] [PubMed]
26. Aratani, Y.; Kura, F.; Watanabe, H.; Akagawa, H.; Takano, Y.; Suzuki, K.; Dinauer, M.C.; Maeda, N.; Koyama, H. Relative contributions of myeloperoxidase and NADPH-oxidase to the early host defense against pulmonary infections with *Candida albicans* and *Aspergillus fumigatus*. *Med. Mycol.* **2002**, *40*, 557–563. [CrossRef] [PubMed]
27. Ibrahim-Granet, O.; Philippe, B.; Boleti, H.; Boisvieux-Ulrich, E.; Grenet, D.; Stern, M.; Latge, J.P. Phagocytosis and intracellular fate of *Aspergillus fumigatus* conidia in alveolar macrophages. *Infect. Immun.* **2003**, *71*, 891–903. [CrossRef] [PubMed]
28. Philippe, B.; Ibrahim-Granet, O.; Prevost, M.C.; Gougerot-Pocidalo, M.A.; Sanchez Perez, M.; Van der Meeren, A.; Latge, J.P. Killing of *Aspergillus fumigatus* by alveolar macrophages is mediated by reactive oxidant intermediates. *Infect. Immun.* **2003**, *71*, 3034–3042. [CrossRef]
29. Bhatia, S.; Fei, M.; Yarlagadda, M.; Qi, Z.; Akira, S.; Saijo, S.; Iwakura, Y.; van Rooijen, N.; Gibson, G.A.; St Croix, C.M.; et al. Rapid host defense against *Aspergillus fumigatus* involves alveolar macrophages with a predominance of alternatively activated phenotype. *PLoS ONE* **2011**, *6*, e15943. [CrossRef]

30. Schittenhelm, L.; Hilkens, C.M.; Morrison, V.L. beta2 Integrins As Regulators of Dendritic Cell, Monocyte, and Macrophage Function. *Front. Immunol.* **2017**, *8*, 1866. [CrossRef]
31. Smith, D.F.Q.; Casadevall, A. Fungal immunity and pathogenesis in mammals versus the invertebrate model organism *Galleria mellonella*. *Pathog. Dis.* **2021**, *79*, ftab013. [CrossRef] [PubMed]
32. Freitas, M.S.; Bonato, V.L.D.; Pessoni, A.M.; Rodrigues, M.L.; Casadevall, A.; Almeida, F. Fungal Extracellular Vesicles as Potential Targets for Immune Interventions. *mSphere* **2019**, *4*, e00747-19. [CrossRef] [PubMed]
33. Cleare, L.G.; Zamith, D.; Heyman, H.M.; Couvillion, S.P.; Nimrichter, L.; Rodrigues, M.L.; Nakayasu, E.S.; Nosanchuk, J.D. Media matters! Alterations in the loading and release of *Histoplasma capsulatum* extracellular vesicles in response to different nutritional milieus. *Cell. Microbiol.* **2020**, *22*, e13217. [CrossRef] [PubMed]
34. Souza, J.A.M.; Baltazar, L.M.; Carregal, V.M.; Gouveia-Eufrasio, L.; de Oliveira, A.G.; Dias, W.G.; Campos Rocha, M.; Rocha de Miranda, K.; Malavazi, I.; Santos, D.A.; et al. Characterization of *Aspergillus fumigatus* Extracellular Vesicles and Their Effects on Macrophages and Neutrophils Functions. *Front. Microbiol.* **2019**, *10*, 2008. [CrossRef]
35. Phadke, A.P.; Mehrad, B. Cytokines in host defense against *Aspergillus*: Recent advances. *Med. Mycol.* **2005**, *43* (Suppl. S1), S173–S176. [CrossRef]
36. Souza, J.A.M.; Gurgel, I.; Malacco, N.; Martins, F.R.B.; Queiroz-Junior, C.M.; Teixeira, M.M.; Soriani, F.M. Pre-Exposure with Extracellular Vesicles from *Aspergillus fumigatus* Attenuates Inflammatory Response and Enhances Fungal Clearance in a Murine Model Pulmonary Aspergillosis. *Front. Cell. Infect. Microbiol.* **2022**, *12*, 898619. [CrossRef]
37. Vargas, G.; Rocha, J.D.; Oliveira, D.L.; Albuquerque, P.C.; Frases, S.; Santos, S.S.; Nosanchuk, J.D.; Gomes, A.M.; Medeiros, L.C.; Miranda, K.; et al. Compositional and immunobiological analyses of extracellular vesicles released by *Candida albicans*. *Cell. Microbiol.* **2015**, *17*, 389–407. [CrossRef]
38. Vargas, G.; Honorato, L.; Guimaraes, A.J.; Rodrigues, M.L.; Reis, F.C.G.; Vale, A.M.; Ray, A.; Nosanchuk, J.D.; Nimrichter, L. Protective effect of fungal extracellular vesicles against murine candidiasis. *Cell. Microbiol.* **2020**, *22*, e13238. [CrossRef] [PubMed]
39. Zamith-Miranda, D.; Heyman, H.M.; Couvillion, S.P.; Cordero, R.J.B.; Rodrigues, M.L.; Nimrichter, L.; Casadevall, A.; Amatuzzi, R.F.; Alves, L.R.; Nakayasu, E.S.; et al. Comparative Molecular and Immunoregulatory Analysis of Extracellular Vesicles from *Candida albicans* and *Candida auris*. *mSystems* **2021**, *6*, e00822-21. [CrossRef]
40. Oliveira, D.L.; Freire-de-Lima, C.G.; Nosanchuk, J.D.; Casadevall, A.; Rodrigues, M.L.; Nimrichter, L. Extracellular vesicles from *Cryptococcus neoformans* modulate macrophage functions. *Infect. Immun.* **2010**, *78*, 1601–1609. [CrossRef]
41. Brown, E.J. Integrins of Macrophages and Macrophage-Like Cells. In *The Macrophage as Therapeutic Target*; Gordon, S., Ed.; Springer: Berlin/Heidelberg, Germany, 2003; pp. 111–130.
42. Jawhara, S.; Pluskota, E.; Cao, W.; Plow, E.F.; Soloviev, D.A. Distinct Effects of Integrins $\alpha_X\beta_2$ and $\alpha_M\beta_2$ on Leukocyte Subpopulations during Inflammation and Antimicrobial Responses. *Infect. Immun.* **2017**, *85*, e00644-16. [CrossRef] [PubMed]
43. Han, X.; Su, X.; Li, Z.; Liu, Y.; Wang, S.; Zhu, M.; Zhang, C.; Yang, F.; Zhao, J.; Li, X.; et al. Complement receptor 3 mediates *Aspergillus fumigatus* internalization into alveolar epithelial cells with the increase of intracellular phosphatidic acid by activating FAK. *Virulence* **2021**, *12*, 1980–1996. [CrossRef]
44. Silva, J.C.; Rodrigues, N.C.; Thompson-Souza, G.A.; Muniz, V.S.; Neves, J.S.; Figueiredo, R.T. Mac-1 triggers neutrophil DNA extracellular trap formation to *Aspergillus fumigatus* independently of PAD4 histone citrullination. *J. Leukoc. Biol.* **2020**, *107*, 69–83. [CrossRef] [PubMed]
45. Gazendam, R.P.; van Hamme, J.L.; Tool, A.T.; Hoogenboezem, M.; van den Berg, J.M.; Prins, J.M.; Vitkov, L.; van de Veerdonk, F.L.; van den Berg, T.K.; Roos, D.; et al. Human Neutrophils Use Different Mechanisms to Kill *Aspergillus fumigatus* Conidia and Hyphae: Evidence from Phagocyte Defects. *J. Immunol.* **2016**, *196*, 1272–1283. [CrossRef] [PubMed]
46. Urban, C.F.; Nett, J.E. Neutrophil extracellular traps in fungal infection. *Semin. Cell Dev. Biol.* **2019**, *89*, 47–57. [CrossRef] [PubMed]
47. McCormick, A.; Heesemann, L.; Wagener, J.; Marcos, V.; Hartl, D.; Loeffler, J.; Heesemann, J.; Ebel, F. NETs formed by human neutrophils inhibit growth of the pathogenic mold *Aspergillus fumigatus*. *Microbes Infect.* **2010**, *12*, 928–936. [CrossRef]
48. Borman, A.M. Of mice and men and larvae: *Galleria mellonella* to model the early host-pathogen interactions after fungal infection. *Virulence* **2018**, *9*, 9–12. [CrossRef]
49. Pereira, T.C.; de Barros, P.P.; Fugisaki, L.R.O.; Rossoni, R.D.; Ribeiro, F.C.; de Menezes, R.T.; Junqueira, J.C.; Scorzoni, L. Recent Advances in the Use of *Galleria mellonella* Model to Study Immune Responses against Human Pathogens. *J. Fungi* **2018**, *4*, 128. [CrossRef]
50. Slater, J.L.; Gregson, L.; Denning, D.W.; Warn, P.A. Pathogenicity of *Aspergillus fumigatus* mutants assessed in *Galleria mellonella* matches that in mice. *Med. Mycol.* **2011**, *49* (Suppl. S1), S107–S113. [CrossRef] [PubMed]
51. Thomaz, L.; Garcia-Rodas, R.; Guimaraes, A.J.; Taborda, C.P.; Zaragoza, O.; Nosanchuk, J.D. *Galleria mellonella* as a model host to study *Paracoccidioides lutzii* and *Histoplasma capsulatum*. *Virulence* **2013**, *4*, 139–146. [CrossRef] [PubMed]
52. Scorzoni, L.; de Paula e Silva, A.C.; Singulani Jde, L.; Leite, F.S.; de Oliveira, H.C.; da Silva, R.A.; Fusco-Almeida, A.M.; Mendes-Giannini, M.J. Comparison of virulence between *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* using *Galleria mellonella* as a host model. *Virulence* **2015**, *6*, 766–776. [CrossRef]
53. Coleman, J.J.; Muhammed, M.; Kasperkovitz, P.V.; Vyas, J.M.; Mylonakis, E. *Fusarium* pathogenesis investigated using *Galleria mellonella* as a heterologous host. *Fungal Biol.* **2011**, *115*, 1279–1289. [CrossRef]
54. Firacative, C.; Duan, S.; Meyer, W. *Galleria mellonella* model identifies highly virulent strains among all major molecular types of *Cryptococcus gattii*. *PLoS ONE* **2014**, *9*, e105076. [CrossRef] [PubMed]

55. Bouklas, T.; Diago-Navarro, E.; Wang, X.; Fenster, M.; Fries, B.C. Characterization of the virulence of *Cryptococcus neoformans* strains in an insect model. *Virulence* **2015**, *6*, 809–813. [CrossRef] [PubMed]
56. St Leger, R.J.; Screen, S.E.; Shams-Pirzadeh, B. Lack of host specialization in *Aspergillus flavus*. *Appl. Environ. Microbiol.* **2000**, *66*, 320–324. [CrossRef] [PubMed]
57. Colombo, A.C.; Rella, A.; Normile, T.; Joffe, L.S.; Tavares, P.M.; de S. Araújo, G.R.; Frases, S.; Orner, E.P.; Farnoud, A.M.; Fries, B.C.; et al. Cryptococcus neoformans Glucuronoxylomannan and Sterylglucoside Are Required for Host Protection in an Animal Vaccination Model. *mBio* **2019**, *10*, e02909-18. [CrossRef]

Capítulo III – Considerações finais

Conclusões

Concluímos que EVs de *A. fumigatus* CEA17 induz atividade inflamatória limitada de macrófagos, neutrófilos humanos e PBMCs, como verificado pela produção de citocinas, NO, superóxido e NETs. Além disso, as EVs atuam de forma diferencial em linhagens de macrófagos, provavelmente devido a expressão diferencial de receptores. As EVs ainda são capazes de proteger larvas de *G. mellonella* frente o desafio com conídios de *A. fumigatus*, demonstrando sua ação benéfica em modelo experimental de infecção fúngica. Desse modo o trabalho adiciona conhecimentos acerca da resposta inicial elicitada por EVs de *A. fumigatus* e sua modulação de células da imunidade inata que combatem a infecção.

Referências bibliográficas

ABAD, A.; FERNANDEZ-MOLINA, J. V.; BIKANDI, J.; RAMIREZ, A. *et al.* What makes Aspergillus fumigatus a successful pathogen? Genes and molecules involved in invasive aspergillosis. **Rev Iberoam Micol**, 27, n. 4, p. 155-182, Oct-Dec 2010.

ABDEL HAMEED, A. A.; YASSER, I. H.; KHODER, I. M. Indoor air quality during renovation actions: a case study. **J Environ Monit**, 6, n. 9, p. 740-744, Sep 2004.

AGARWAL, R. Allergic bronchopulmonary aspergillosis. **Chest**, 135, n. 3, p. 805-826, Mar 2009.

AIMANIANDA, V.; BAYRY, J.; BOZZA, S.; KNIEMEYER, O. *et al.* Surface hydrophobin prevents immune recognition of airborne fungal spores. **Nature**, 460, n. 7259, p. 1117-1121, Aug 27 2009.

AIMANIANDA, V.; LATGE, J. P. Fungal hydrophobins form a sheath preventing immune recognition of airborne conidia. **Virulence**, 1, n. 3, p. 185-187, May-Jun 2010.

AKOUMIANAKI, T.; KYRMIZI, I.; VALSECCHI, I.; GRESNIGT, M. S. *et al.* Aspergillus Cell Wall Melanin Blocks LC3-Associated Phagocytosis to Promote Pathogenicity. **Cell Host Microbe**, 19, n. 1, p. 79-90, Jan 13 2016.

AMARSAIKHAN, N.; O'DEA, E. M.; TSOGGEREL, A.; TEMPLETON, S. P. Lung eosinophil recruitment in response to Aspergillus fumigatus is correlated with fungal cell wall composition and requires gammadelta T cells. **Microbes Infect**, 19, n. 7-8, p. 422-431, Jul-Aug 2017.

AMARSAIKHAN, N.; SANDS, E. M.; SHAH, A.; ABDOLRASOULI, A. *et al.* Caspofungin Increases Fungal Chitin and Eosinophil and gammadelta T Cell-Dependent Pathology in Invasive Aspergillosis. **J Immunol**, 199, n. 2, p. 624-632, Jul 15 2017.

AMATUZZI, R. F.; ZAMITH-MIRANDA, D.; MUNHOZ DA ROCHA, I. F.; LUCENA, A. C. R. *et al.* Caspofungin Affects Extracellular Vesicle Production and Cargo in Candida auris. **J Fungi (Basel)**, 8, n. 10, Sep 21 2022.

AMIN, S.; THYWISSEN, A.; HEINEKAMP, T.; SALUZ, H. P. *et al.* Melanin dependent survival of Apergillus fumigatus conidia in lung epithelial cells. **Int J Med Microbiol**, 304, n. 5-6, p. 626-636, Jul 2014.

ANDERSON, J.; MIHALIK, R.; SOLL, D. R. Ultrastructure and antigenicity of the unique cell wall pimple of the Candida opaque phenotype. **J Bacteriol**, 172, n. 1, p. 224-235, Jan 1990.

ARATANI, Y.; KURA, F.; WATANABE, H.; AKAGAWA, H. *et al.* Relative contributions of myeloperoxidase and NADPH-oxidase to the early host defense against pulmonary infections with Candida albicans and Aspergillus fumigatus. **Med Mycol**, 40, n. 6, p. 557-563, Dec 2002.

BAKARE, N.; RICKERTS, V.; BARGON, J.; JUST-NUBLING, G. Prevalence of Aspergillus fumigatus and other fungal species in the sputum of adult patients with cystic fibrosis. **Mycoses**, 46, n. 1-2, p. 19-23, Feb 2003.

BALLOY, V.; CHIGNARD, M. The innate immune response to Aspergillus fumigatus. **Microbes Infect**, 11, n. 12, p. 919-927, Oct 2009.

BALLOY, V.; SI-TAHAR, M.; TAKEUCHI, O.; PHILIPPE, B. *et al.* Involvement of toll-like receptor 2 in experimental invasive pulmonary aspergillosis. **Infect Immun**, 73, n. 9, p. 5420-5425, Sep 2005.

BAYRY, J.; BEAUSSART, A.; DUFRENE, Y. F.; SHARMA, M. *et al.* Surface structure characterization of Aspergillus fumigatus conidia mutated in the melanin synthesis pathway and their human cellular immune response. **Infect Immun**, 82, n. 8, p. 3141-3153, Aug 2014.

BEBELMAN, M. P.; SMIT, M. J.; PEGTEL, D. M.; BAGLIO, S. R. Biogenesis and function of extracellular vesicles in cancer. **Pharmacol Ther**, 188, p. 1-11, Aug 2018.

BELLOCCHIO, S.; MONTAGNOLI, C.; BOZZA, S.; GAZIANO, R. *et al.* The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. **J Immunol**, 172, n. 5, p. 3059-3069, Mar 1 2004.

BHATIA, S.; FEI, M.; YARLAGADDA, M.; QI, Z. *et al.* Rapid host defense against Aspergillus fumigatus involves alveolar macrophages with a predominance of alternatively activated phenotype. **PLoS One**, 6, n. 1, p. e15943, Jan 5 2011.

BIGOT, J.; GUILLOT, L.; GUITARD, J.; RUFFIN, M. *et al.* Bronchial Epithelial Cells on the Front Line to Fight Lung Infection-Causing Aspergillus fumigatus. **Front Immunol**, 11, p. 1041, 2020.

BITENCOURT, T. A.; HATANAKA, O.; PESSONI, A. M.; FREITAS, M. S. *et al.* Fungal Extracellular Vesicles Are Involved in Intraspecies Intracellular Communication. **mBio**, 13, n. 1, p. e0327221, Feb 22 2022.

BITENCOURT, T. A.; REZENDE, C. P.; QUARESEMIN, N. R.; MORENO, P. *et al.* Extracellular Vesicles From the Dermatophyte Trichophyton interdigitale Modulate Macrophage and Keratinocyte Functions. **Front Immunol**, 9, p. 2343, 2018.

BORMAN, A. M. Of mice and men and larvae: Galleria mellonella to model the early host-pathogen interactions after fungal infection. **Virulence**, 9, n. 1, p. 9-12, Jan 1 2018.

BOTTAZZI, B.; VOURET-CRAVIARI, V.; BASTONE, A.; DE GIOIA, L. *et al.* Multimer formation and ligand recognition by the long pentraxin PTX3. Similarities and differences with the short pentraxins C-reactive protein and serum amyloid P component. **J Biol Chem**, 272, n. 52, p. 32817-32823, Dec 26 1997.

BRAKHAGE, A. A.; LANGFELDER, K. Menacing mold: the molecular biology of Aspergillus fumigatus. **Annu Rev Microbiol**, 56, p. 433-455, 2002.

BRAKHAGE, A. A.; LIEBMANN, B. Aspergillus fumigatus conidial pigment and cAMP signal transduction: significance for virulence. **Med Mycol**, 43 Suppl 1, p. S75-82, May 2005.

BRAUER, V. S.; PESSONI, A. M.; BITENCOURT, T. A.; DE PAULA, R. G. *et al.* Extracellular Vesicles from Aspergillus flavus Induce M1 Polarization In Vitro. **mSphere**, 5, n. 3, May 6 2020.

BROWN, E. J. Integrins of Macrophages and Macrophage-Like Cells. *In*: GORDON, S. (Ed.). **The Macrophage as Therapeutic Target**. Berlin, Heidelberg: Springer Berlin Heidelberg, 2003. p. 111-130.

BROWN, G. D.; DENNING, D. W.; GOW, N. A.; LEVITZ, S. M. *et al.* Hidden killers: human fungal infections. **Sci Transl Med**, 4, n. 165, p. 165rv113, Dec 19 2012.

CHABI, M. L.; GORACCI, A.; ROCHE, N.; PAUGAM, A. *et al.* Pulmonary aspergillosis. **Diagn Interv Imaging**, 96, n. 5, p. 435-442, May 2015.

CHAI, L. Y.; NETEA, M. G.; SUGUI, J.; VONK, A. G. *et al.* Aspergillus fumigatus conidial melanin modulates host cytokine response. **Immunobiology**, 215, n. 11, p. 915-920, Nov 2010.

CHAUHAN, B.; SANTIAGO, L.; HUTCHESON, P. S.; SCHWARTZ, H. J. *et al.* Evidence for the involvement of two different MHC class II regions in susceptibility or protection in allergic bronchopulmonary aspergillosis. **J Allergy Clin Immunol**, 106, n. 4, p. 723-729, Oct 2000.

COLOMBO, A. C.; RELLA, A.; NORMILE, T.; JOFFE, L. S. *et al.* Cryptococcus neoformans Glucuronoxylomannan and Sterylglucoside Are Required for Host Protection in an Animal Vaccination Model. **mBio**, 10, n. 2, Apr 2 2019.

COLOMBO, M.; RAPOSO, G.; THERY, C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. **Annu Rev Cell Dev Biol**, 30, p. 255-289, 2014.

COWLEY, A. C.; THORNTON, D. J.; DENNING, D. W.; HORSLEY, A. Aspergillosis and the role of mucins in cystic fibrosis. **Pediatr Pulmonol**, 52, n. 4, p. 548-555, Apr 2017.

DA SILVA, T. A.; ROQUE-BARREIRA, M. C.; CASADEVALL, A.; ALMEIDA, F. Extracellular vesicles from Paracoccidioides brasiliensis induced M1 polarization in vitro. **Sci Rep**, 6, p. 35867, Oct 24 2016.

DAGENAIS, T. R.; GILES, S. S.; AIMANIANDA, V.; LATGE, J. P. *et al.* Aspergillus fumigatus LaeA-mediated phagocytosis is associated with a decreased hydrophobin layer. **Infect Immun**, 78, n. 2, p. 823-829, Feb 2010.

DAGENAIS, T. R.; KELLER, N. P. Pathogenesis of Aspergillus fumigatus in Invasive Aspergillosis. **Clin Microbiol Rev**, 22, n. 3, p. 447-465, Jul 2009.

DEATHERAGE, B. L.; COOKSON, B. T. Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. **Infect Immun**, 80, n. 6, p. 1948-1957, Jun 2012.

DENNING, D. W.; PLEUVRY, A.; COLE, D. C. Global burden of chronic pulmonary aspergillosis as a sequel to pulmonary tuberculosis. **Bull World Health Organ**, 89, n. 12, p. 864-872, Dec 1 2011.

DOYLE, L. M.; WANG, M. Z. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. **Cells**, 8, n. 7, Jul 15 2019.

FANG, W.; LATGE, J. P. Microbe Profile: Aspergillus fumigatus: a saprotrophic and opportunistic fungal pathogen. **Microbiology (Reading)**, 164, n. 8, p. 1009-1011, Aug 2018.

FARO-TRINDADE, I.; WILLMENT, J. A.; KERRIGAN, A. M.; REDELINGHUYS, P. *et al.* Characterisation of innate fungal recognition in the lung. **PLoS One**, 7, n. 4, p. e35675, 2012.

FONTAINE, T.; DELANGLE, A.; SIMENEL, C.; CODDEVILLE, B. *et al.* Galactosaminogalactan, a new immunosuppressive polysaccharide of Aspergillus fumigatus. **PLoS Pathog**, 7, n. 11, p. e1002372, Nov 2011.

FREITAS, M. S.; BONATO, V. L. D.; PESSONI, A. M.; RODRIGUES, M. L. *et al.* Fungal Extracellular Vesicles as Potential Targets for Immune Interventions. **mSphere**, 4, n. 6, Nov 6 2019.

FREITAS, M. S.; PESSONI, A. M.; COELHO, C.; BONATO, V. L. D. *et al.* Interactions of Extracellular Vesicles from Pathogenic Fungi with Innate Leukocytes. **Curr Top Microbiol Immunol**, 432, p. 89-120, 2021.

GAO, Y.; SOUBANI, A. Advances in the diagnosis and management of pulmonary aspergillosis. **Adv Respir Med**, 87, n. 6, p. 231-243, 2019.

GARCIA-RUBIO, R.; DE OLIVEIRA, H. C.; RIVERA, J.; TREVIJANO-CONTADOR, N. The Fungal Cell Wall: Candida, Cryptococcus, and Aspergillus Species. **Front Microbiol**, 10, p. 2993, 2019.

GARLANDA, C.; HIRSCH, E.; BOZZA, S.; SALUSTRI, A. *et al.* Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. **Nature**, 420, n. 6912, p. 182-186, Nov 14 2002.

GAZENDAM, R. P.; VAN HAMME, J. L.; TOOL, A. T.; HOOGENBOEZEM, M. *et al.* Human Neutrophils Use Different Mechanisms To Kill Aspergillus fumigatus Conidia and Hyphae: Evidence from Phagocyte Defects. **J Immunol**, 196, n. 3, p. 1272-1283, Feb 1 2016.

GERSON, S. L.; TALBOT, G. H.; HURWITZ, S.; STROM, B. L. *et al.* Prolonged granulocytopenia: the major risk factor for invasive pulmonary aspergillosis in patients with acute leukemia. **Ann Intern Med**, 100, n. 3, p. 345-351, Mar 1984.

GRESNIGT, M. S.; NETEA, M. G.; VAN DE VEERDONK, F. L. Pattern recognition receptors and their role in invasive aspergillosis. **Ann N Y Acad Sci**, 1273, p. 60-67, Dec 2012.

GRIMM, M. J.; VETHANAYAGAM, R. R.; ALMYROUDIS, N. G.; LEWANDOWSKI, D. *et al.* Role of NADPH oxidase in host defense against aspergillosis. **Med Mycol**, 49 Suppl 1, n. Suppl 1, p. S144-149, Apr 2011.

HAN, X.; SU, X.; LI, Z.; LIU, Y. *et al.* Complement receptor 3 mediates Aspergillus fumigatus internalization into alveolar epithelial cells with the increase of intracellular phosphatidic acid by activating FAK. **Virulence**, 12, n. 1, p. 1980-1996, Dec 2021.

HOHL, T. M. Immune responses to invasive aspergillosis: new understanding and therapeutic opportunities. **Curr Opin Infect Dis**, 30, n. 4, p. 364-371, Aug 2017.

KANJ, A.; ABDALLAH, N.; SOUBANI, A. O. The spectrum of pulmonary aspergillosis. **Respir Med**, 141, p. 121-131, Aug 2018.

KERR, S. C.; FISCHER, G. J.; SINHA, M.; MCCABE, O. *et al.* FleA Expression in Aspergillus fumigatus Is Recognized by Fucosylated Structures on Mucins and Macrophages to Prevent Lung Infection. **PLoS Pathog**, 12, n. 4, p. e1005555, Apr 2016.

KIM, A.; NICOLAU, D. P.; KUTI, J. L. Hospital costs and outcomes among intravenous antifungal therapies for patients with invasive aspergillosis in the United States. **Mycoses**, 54, n. 5, p. e301-312, Sep 2011.

KNUTSEN, A. P. Lymphocytes in allergic bronchopulmonary aspergillosis. **Front Biosci**, 8, p. d589-602, May 1 2003.

KOUSHA, M.; TADI, R.; SOUBANI, A. O. Pulmonary aspergillosis: a clinical review. **Eur Respir Rev**, 20, n. 121, p. 156-174, Sep 1 2011.

KOZAKIEWICZ, Z.; SMITH, D. Physiology of Aspergillus. *In*: SMITH, J. E. (Ed.). **Aspergillus**. Boston, MA: Springer US, 1994. p. 23-40.

KWON-CHUNG, K. J.; SUGUI, J. A. Aspergillus fumigatus--what makes the species a ubiquitous human fungal pathogen? **PLoS Pathog**, 9, n. 12, p. e1003743, 2013.

LATGE, J. P. The pathobiology of Aspergillus fumigatus. **Trends Microbiol**, 9, n. 8, p. 382-389, Aug 2001.

LATGE, J. P.; BEAUVAIS, A. Functional duality of the cell wall. **Curr Opin Microbiol**, 20, p. 111-117, Aug 2014.

LATGE, J. P.; BEAUVAIS, A.; CHAMILOS, G. The Cell Wall of the Human Fungal Pathogen Aspergillus fumigatus: Biosynthesis, Organization, Immune Response, and Virulence. **Annu Rev Microbiol**, 71, p. 99-116, Sep 8 2017.

LATGE, J. P.; CHAMILOS, G. Aspergillus fumigatus and Aspergillosis in 2019. **Clin Microbiol Rev**, 33, n. 1, Dec 18 2019.

LATGE, J. P.; MOUYNA, I.; TEKAIA, F.; BEAUVAIS, A. *et al.* Specific molecular features in the organization and biosynthesis of the cell wall of Aspergillus fumigatus. **Med Mycol**, 43 Suppl 1, p. S15-22, May 2005.

LEE, M. J.; SHEPPARD, D. C. Recent advances in the understanding of the Aspergillus fumigatus cell wall. **J Microbiol**, 54, n. 3, p. 232-242, Mar 2016.

LEHRNBECHER, T.; SCHMIDT, S. Why are natural killer cells important for defense against Aspergillus? **Med Mycol**, 57, n. Supplement_2, p. S206-S210, Apr 1 2019.

LEOPOLD WAGER, C. M.; WORMLEY, F. L., Jr. Classical versus alternative macrophage activation: the Ying and the Yang in host defense against pulmonary fungal infections. **Mucosal Immunol**, 7, n. 5, p. 1023-1035, Sep 2014.

LIEBANA-JORDAN, M.; BROTONS, B.; FALCON-PEREZ, J. M.; GONZALEZ, E. Extracellular Vesicles in the Fungi Kingdom. **Int J Mol Sci**, 22, n. 13, Jul 5 2021.

LOURES, F. V.; ROHM, M.; LEE, C. K.; SANTOS, E. *et al.* Recognition of Aspergillus fumigatus hyphae by human plasmacytoid dendritic cells is mediated by dectin-2 and results in formation of extracellular traps. **PLoS Pathog**, 11, n. 2, p. e1004643, Feb 2015.

MARGALIT, A.; KAVANAGH, K. The innate immune response to Aspergillus fumigatus at the alveolar surface. **FEMS Microbiol Rev**, 39, n. 5, p. 670-687, Sep 2015.

MCCORMICK, A.; HEESEMANN, L.; WAGENER, J.; MARCOS, V. *et al.* NETs formed by human neutrophils inhibit growth of the pathogenic mold Aspergillus fumigatus. **Microbes Infect**, 12, n. 12-13, p. 928-936, Nov 2010.

MCCORMICK, A.; LOEFFLER, J.; EBEL, F. Aspergillus fumigatus: contours of an opportunistic human pathogen. **Cell Microbiol**, 12, n. 11, p. 1535-1543, Nov 2010.

MEIER, A.; KIRSCHNING, C. J.; NIKOLAUS, T.; WAGNER, H. *et al.* Toll-like receptor (TLR) 2 and TLR4 are essential for Aspergillus-induced activation of murine macrophages. **Cell Microbiol**, 5, n. 8, p. 561-570, Aug 2003.

MOALLI, F.; DONI, A.; DEBAN, L.; ZELANTE, T. *et al.* Role of complement and Fcgamma receptors in the protective activity of the long pentraxin PTX3 against Aspergillus fumigatus. **Blood**, 116, n. 24, p. 5170-5180, Dec 9 2010.

MORGAN, J.; WANNEMUEHLER, K. A.; MARR, K. A.; HADLEY, S. *et al.* Incidence of invasive aspergillosis following hematopoietic stem cell and solid organ transplantation: interim results of a prospective multicenter surveillance program. **Med Mycol**, 43 Suppl 1, p. S49-58, May 2005.

MORRISON, B. E.; PARK, S. J.; MOONEY, J. M.; MEHRAD, B. Chemokine-mediated recruitment of NK cells is a critical host defense mechanism in invasive aspergillosis. **J Clin Invest**, 112, n. 12, p. 1862-1870, Dec 2003.

MORTENSEN, K. L.; JENSEN, R. H.; JOHANSEN, H. K.; SKOV, M. *et al.* Aspergillus species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on Aspergillus fumigatus azole resistance. **J Clin Microbiol**, 49, n. 6, p. 2243-2251, Jun 2011.

MOSS, R. B. Pathophysiology and immunology of allergic bronchopulmonary aspergillosis. **Med Mycol**, 43 Suppl 1, p. S203-206, May 2005.

NAWROTH, J. C.; VAN DER DOES, A. M.; RYAN FIRTH, A.; KANSO, E. Multiscale mechanics of mucociliary clearance in the lung. **Philos Trans R Soc Lond B Biol Sci**, 375, n. 1792, p. 20190160, Feb 17 2020.

OLIVEIRA, D. L.; FREIRE-DE-LIMA, C. G.; NOSANCHUK, J. D.; CASADEVALL, A. *et al.* Extracellular vesicles from Cryptococcus neoformans modulate macrophage functions. **Infect Immun**, 78, n. 4, p. 1601-1609, Apr 2010.

PAPAYANNOPOULOS, V. Neutrophil extracellular traps in immunity and disease. **Nat Rev Immunol**, 18, n. 2, p. 134-147, Feb 2018.

PARIS, S.; DEBEAUPUIS, J. P.; CRAMERI, R.; CAREY, M. *et al.* Conidial hydrophobins of Aspergillus fumigatus. **Appl Environ Microbiol**, 69, n. 3, p. 1581-1588, Mar 2003.

PARK, S. J.; HUGHES, M. A.; BURDICK, M.; STRIETER, R. M. *et al.* Early NK cell-derived IFN-gamma is essential to host defense in neutropenic invasive aspergillosis. **J Immunol**, 182, n. 7, p. 4306-4312, Apr 1 2009.

PARK, S. J.; MEHRAD, B. Innate immunity to Aspergillus species. **Clin Microbiol Rev**, 22, n. 4, p. 535-551, Oct 2009.

PATTERSON, T. F.; THOMPSON, G. R., 3rd; DENNING, D. W.; FISHMAN, J. A. *et al.* Practice Guidelines for the Diagnosis and Management of Aspergillosis: 2016 Update by the Infectious Diseases Society of America. **Clin Infect Dis**, 63, n. 4, p. e1-e60, Aug 15 2016.

PEREZ-CUESTA, U.; APARICIO-FERNANDEZ, L.; GURUCEAGA, X.; MARTIN-SOUTO, L. *et al.* Melanin and pyomelanin in Aspergillus fumigatus: from its genetics to host interaction. **Int Microbiol**, 23, n. 1, p. 55-63, Jan 2020.

PHILIPPE, B.; IBRAHIM-GRANET, O.; PREVOST, M. C.; GOUGEROT-POCIDALO, M. A. *et al.* Killing of Aspergillus fumigatus by alveolar macrophages is mediated by reactive oxidant intermediates. **Infect Immun**, 71, n. 6, p. 3034-3042, Jun 2003.

RAPOSO, G.; STOORVOGEL, W. Extracellular vesicles: exosomes, microvesicles, and friends. **J Cell Biol**, 200, n. 4, p. 373-383, Feb 18 2013.

RICHARD, N.; MARTI, L.; VARROT, A.; GUILLOT, L. *et al.* Human Bronchial Epithelial Cells Inhibit Aspergillus fumigatus Germination of Extracellular Conidia via FleA Recognition. **Sci Rep**, 8, n. 1, p. 15699, Oct 24 2018.

RIZZO, J.; CHAZE, T.; MIRANDA, K.; ROBERSON, R. W. *et al.* Characterization of Extracellular Vesicles Produced by Aspergillus fumigatus Protoplasts. **mSphere**, 5, n. 4, Aug 12 2020.

RIZZO, J.; RODRIGUES, M. L.; JANBON, G. Extracellular Vesicles in Fungi: Past, Present, and Future Perspectives. **Front Cell Infect Microbiol**, 10, p. 346, 2020.

RODRIGUES, M. L.; CASADEVALL, A. A two-way road: novel roles for fungal extracellular vesicles. **Mol Microbiol**, 110, n. 1, p. 11-15, Oct 2018.

RODRIGUES, M. L.; NIMRICHTER, L. From fundamental biology to the search for innovation: The story of fungal extracellular vesicles. **Eur J Cell Biol**, 101, n. 2, p. 151205, Apr 2022.

RODRIGUES, M. L.; NIMRICHTER, L.; OLIVEIRA, D. L.; FRASES, S. *et al.* Vesicular polysaccharide export in Cryptococcus neoformans is a eukaryotic solution to the problem of fungal trans-cell wall transport. **Eukaryot Cell**, 6, n. 1, p. 48-59, Jan 2007.

SALAZAR, F.; BROWN, G. D. Antifungal Innate Immunity: A Perspective from the Last 10 Years. **J Innate Immun**, 10, n. 5-6, p. 373-397, 2018.

SCHITTENHELM, L.; HILKENS, C. M.; MORRISON, V. L. beta(2) Integrins As Regulators of Dendritic Cell, Monocyte, and Macrophage Function. **Front Immunol**, 8, p. 1866, 2017.

SCHMIDT, S.; CONDORELLI, A.; KOLTZE, A.; LEHRNBECHER, T. NK Cells and Their Role in Invasive Mold Infection. **J Fungi (Basel)**, 3, n. 2, May 19 2017.

SCHMIDT, S.; TRAMSEN, L.; HANISCH, M.; LATGE, J. P. *et al.* Human natural killer cells exhibit direct activity against Aspergillus fumigatus hyphae, but not against resting conidia. **J Infect Dis**, 203, n. 3, p. 430-435, Feb 1 2011.

SEGAL, B. H. Aspergillosis. **N Engl J Med**, 360, n. 18, p. 1870-1884, Apr 30 2009.

SHAH, A.; PANJABI, C. Allergic Bronchopulmonary Aspergillosis: A Perplexing Clinical Entity. **Allergy Asthma Immunol Res**, 8, n. 4, p. 282-297, Jul 2016.

SILVA, J. C.; RODRIGUES, N. C.; THOMPSON-SOUZA, G. A.; MUNIZ, V. S. *et al.* Mac-1 triggers neutrophil DNA extracellular trap formation to Aspergillus fumigatus independently of PAD4 histone citrullination. **J Leukoc Biol**, 107, n. 1, p. 69-83, Jan 2020.

SMITH, D. F. Q.; CASADEVALL, A. Fungal immunity and pathogenesis in mammals versus the invertebrate model organism Galleria mellonella. **Pathog Dis**, 79, n. 3, Mar 20 2021.

SMITH, N. L.; DENNING, D. W. Underlying conditions in chronic pulmonary aspergillosis including simple aspergilloma. **Eur Respir J**, 37, n. 4, p. 865-872, Apr 2011.

SOUZA, J. A. M.; BALTAZAR, L. M.; CARREGAL, V. M.; GOUVEIA-EUFRASIO, L. *et al.* Characterization of Aspergillus fumigatus Extracellular Vesicles and Their Effects on Macrophages and Neutrophils Functions. **Front Microbiol**, 10, p. 2008, 2019.

SOUZA, J. A. M.; GURGEL, I.; MALACCO, N.; MARTINS, F. R. B. *et al.* Pre-Exposure With Extracellular Vesicles From Aspergillus fumigatus Attenuates Inflammatory Response and Enhances Fungal Clearance in a Murine Model Pulmonary Aspergillosis. **Front Cell Infect Microbiol**, 12, p. 898619, 2022.

STEELE, C.; RAPAKA, R. R.; METZ, A.; POP, S. M. *et al.* The beta-glucan receptor dectin-1 recognizes specific morphologies of Aspergillus fumigatus. **PLoS Pathog**, 1, n. 4, p. e42, Dec 2005.

STEPHENS-ROMERO, S. D.; MEDNICK, A. J.; FELDMESSER, M. The pathogenesis of fatal outcome in murine pulmonary aspergillosis depends on the neutrophil depletion strategy. **Infect Immun**, 73, n. 1, p. 114-125, Jan 2005.

SUN, H.; XU, X. Y.; TIAN, X. L.; SHAO, H. T. *et al.* Activation of NF-kappaB and respiratory burst following Aspergillus fumigatus stimulation of macrophages. **Immunobiology**, 219, n. 1, p. 25-36, Jan 2014.

TACCONE, F. S.; VAN DEN ABEELE, A. M.; BULPA, P.; MISSET, B. *et al.* Epidemiology of invasive aspergillosis in critically ill patients: clinical presentation, underlying conditions, and outcomes. **Crit Care**, 19, n. 1, p. 7, Jan 12 2015.

TAHA, M. P.; POLLARD, S. J.; SARKAR, U.; LONGHURST, P. Estimating fugitive bioaerosol releases from static compost windrows: feasibility of a portable wind tunnel approach. **Waste Manag**, 25, n. 4, p. 445-450, 2005.

TAKEO, K.; UESAKA, I.; UEHIRA, K.; NISHIURA, M. Fine structure of Cryptococcus neoformans grown in vivo as observed by freeze-etching. **J Bacteriol**, 113, n. 3, p. 1449-1454, Mar 1973.

TEKAIA, F.; LATGE, J. P. Aspergillus fumigatus: saprophyte or pathogen? **Curr Opin Microbiol**, 8, n. 4, p. 385-392, Aug 2005.

THYWISSEN, A.; HEINEKAMP, T.; DAHSE, H. M.; SCHMALER-RIPCKE, J. *et al.* Conidial Dihydroxynaphthalene Melanin of the Human Pathogenic Fungus Aspergillus fumigatus Interferes with the Host Endocytosis Pathway. **Front Microbiol**, 2, p. 96, 2011.

TRONCHIN, G.; BOUCHARA, J. P.; FERRON, M.; LARCHER, G. *et al.* Cell surface properties of Aspergillus fumigatus conidia: correlation between adherence, agglutination, and rearrangements of the cell wall. **Can J Microbiol**, 41, n. 8, p. 714-721, Aug 1995.

VALSECCHI, I.; DUPRES, V.; STEPHEN-VICTOR, E.; GUIJARRO, J. I. *et al.* Role of Hydrophobins in Aspergillus fumigatus. **J Fungi (Basel)**, 4, n. 1, Dec 24 2017.

VAN DE VEERDONK, F. L.; GRESNIGT, M. S.; ROMANI, L.; NETEA, M. G. *et al.* Aspergillus fumigatus morphology and dynamic host interactions. **Nat Rev Microbiol**, 15, n. 11, p. 661-674, Nov 2017.

VAN NIEL, G.; D'ANGELO, G.; RAPOSO, G. Shedding light on the cell biology of extracellular vesicles. **Nat Rev Mol Cell Biol**, 19, n. 4, p. 213-228, Apr 2018.

VANDEWOUDE, K. H.; BLOT, S. I.; BENOIT, D.; COLARDYN, F. *et al.* Invasive aspergillosis in critically ill patients: attributable mortality and excesses in length of ICU stay and ventilator dependence. **J Hosp Infect**, 56, n. 4, p. 269-276, Apr 2004.

VARGAS, G.; HONORATO, L.; GUIMARAES, A. J.; RODRIGUES, M. L. *et al.* Protective effect of fungal extracellular vesicles against murine candidiasis. **Cell Microbiol**, 22, n. 10, p. e13238, Oct 2020.

VARGAS, G.; ROCHA, J. D.; OLIVEIRA, D. L.; ALBUQUERQUE, P. C. *et al.* Compositional and immunobiological analyses of extracellular vesicles released by Candida albicans. **Cell Microbiol**, 17, n. 3, p. 389-407, Mar 2015.

WANG, Y.; SMITH, W.; HAO, D.; HE, B. *et al.* M1 and M2 macrophage polarization and potentially therapeutic naturally occurring compounds. **Int Immunopharmacol**, 70, p. 459-466, May 2019.

WARRIS, A. The biology of pulmonary aspergillus infections. **J Infect**, 69 Suppl 1, p. S36-41, Nov 2014.

WERNER, J. L.; METZ, A. E.; HORN, D.; SCHOEB, T. R. *et al.* Requisite role for the dectin-1 beta-glucan receptor in pulmonary defense against Aspergillus fumigatus. **J Immunol**, 182, n. 8, p. 4938-4946, Apr 15 2009.

WONG, S. S. W.; RANI, M.; DODAGATTA-MARRI, E.; IBRAHIM-GRANET, O. *et al.* Fungal melanin stimulates surfactant protein D-mediated opsonization of and host immune response to Aspergillus fumigatus spores. **J Biol Chem**, 293, n. 13, p. 4901-4912, Mar 30 2018.

XIE, Y.; ZHOU, X.; ZHANG, J.; YU, H. *et al.* Immunomodulatory responses of differentially polarized macrophages to fungal infections. **Int Immunopharmacol**, 111, p. 109089, Oct 2022.

XU, S.; SHINOHARA, M. L. Tissue-Resident Macrophages in Fungal Infections. **Front Immunol**, 8, p. 1798, 2017.

YANEZ-MO, M.; SILJANDER, P. R.; ANDREU, Z.; ZAVEC, A. B. *et al.* Biological properties of extracellular vesicles and their physiological functions. **J Extracell Vesicles**, 4, p. 27066, 2015.

YUNNA, C.; MENGRU, H.; LEI, W.; WEIDONG, C. Macrophage M1/M2 polarization. **Eur J Pharmacol**, 877, p. 173090, Jun 15 2020.

ZAMITH-MIRANDA, D.; NIMRICHTER, L.; RODRIGUES, M. L.; NOSANCHUK, J. D. Fungal extracellular vesicles: modulating host-pathogen interactions by both the fungus and the host. **Microbes Infect**, 20, n. 9-10, p. 501-504, Oct-Nov 2018.

ZARIF, A.; THOMAS, A.; VAYRO, A. Chronic Pulmonary Aspergillosis: A Brief Review. **Yale J Biol Med**, 94, n. 4, p. 673-679, Dec 2021.

ZARNOWSKI, R.; NOLL, A.; CHEVRETTE, M. G.; SANCHEZ, H. *et al.* Coordination of fungal biofilm development by extracellular vesicle cargo. **Nat Commun**, 12, n. 1, p. 6235, Oct 29 2021.

ZHANG, B.; YIN, Y.; LAI, R. C.; LIM, S. K. Immunotherapeutic potential of extracellular vesicles. **Front Immunol**, 5, p. 518, 2014.

ZHANG, X.; HE, D.; GAO, S.; WEI, Y. *et al.* Aspergillus fumigatus enhances human NK cell activity by regulating M1 macrophage polarization. **Mol Med Rep**, 20, n. 2, p. 1241-1249, Aug 2019.

Anexos

Anexo I

Manuscrito - FREITAS, M. S.; BONATO, V. L. D.; PESSONI, A. M.; RODRIGUES, M. L.; CASADEVALL, A.; ALMEIDA, F. Fungal Extracellular Vesicles as Potential Targets for Immune Interventions. **mSphere**, 4, n. 6, Nov 6 2019.

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MINIREVIEW
Host-Microbe Biology

Fungal Extracellular Vesicles as Potential Targets for Immune Interventions

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ABSTRACT The release of extracellular vesicles (EVs) by fungi is a fundamental cellular process. EVs carry several biomolecules, including pigments, proteins, enzymes, lipids, nucleic acids, and carbohydrates, and are involved in physiological and pathological processes. EVs may play a pivotal role in the establishment of fungal infections, as they can interact with the host immune system to elicit multiple outcomes. It has been observed that, depending on the fungal pathogen, EVs can exacerbate or attenuate fungal infections. The study of the interaction between fungal EVs and the host immune system and understanding of the mechanisms that regulate those interactions might be useful for the development of new adjuvants as well as the improvement of protective immune responses against infectious or noninfectious diseases. In this review, we describe the immunomodulatory properties of EVs produced by pathogenic fungi and discuss their potential as adjuvants for prophylactic or therapeutic strategies.

KEYWORDS drug targets, extracellular vesicles, fungal infections, immune response

Extracellular vesicles (EVs) are spherical structures delimited by a bilayered membrane that are produced by prokaryotic and eukaryotic cells (1, 2). EVs transport various biomolecules, such as proteins, lipids, nucleic acids, and carbohydrates, and they are involved in several aspects of physiology and pathogenicity (2–4). EVs are classified as apoptotic bodies, ectosomes, or exosomes, depending upon their cellular origin and size (5). Apoptotic bodies are the largest (50 to 5,000 nm in size) and are derived from apoptotic cells. Ectosomes, also called microvesicles, are generated by outward budding from the plasma membrane, followed by pinching off and release to the extracellular space, resulting in EVs ranging from 100 to 1,000 nm in size. Exosomes are the smallest EVs (30 to 150 nm), and these structures originate from endosomal compartments (5–7). In this review, we adopt EVs as a general term for all of the above-mentioned vesicles.

In fungi, the first visual evidence of the production of EVs was produced in 1972 in *Aspergillus nidulans* (8). Microscopic evidence of fungal EVs was reproduced in 1973 in *Cryptococcus neoformans* (9), 1990 in *Candida albicans* (10), and 1998 in *Saccharomyces cerevisiae* (11), but the first characterization of extracellular membranous structures as fungal EVs dates to 2007 in the *C. neoformans* model (12). So far, the production of EVs has been observed in a number of fungal species (13–19). The composition of fungal EVs can vary, depending on the availability of nutrition and the immunological activity of host cells, and they typically contain proteins, RNA, lipids, complex carbohydrates, and pigments (20, 21). Due to the heterogeneity in their content, fungal EVs are able

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to participate in a number of physiological processes, including biofilm formation, the transport of virulence factors, and modulation of the host immune response (22, 23).

Deep mycoses, such as cryptococcosis, candidiasis, and aspergillosis, are responsible for approximately 1,270,000 annual global cases, and the mortality rates from these mycoses are comparable to those from malaria (24, 25). The drugs currently approved for treating human mycoses usually have low efficacy and high toxicity, and the widespread use of these medications is selecting for resistant strains (26–29). Given the high incidence of fungal diseases worldwide and their therapeutic limitations, it is important to study the biology of pathogenic fungi in an attempt to develop new immune interventions (30). In this review, we discuss the immunomodulatory potential of fungal EVs. Additionally, we highlight strategies where fungal EVs could be used as therapeutic targets and/or as components of therapeutic and prophylactic strategies.

THE INTERACTION OF FUNGAL EVs WITH THE IMMUNE SYSTEM

Most of the data resulting from the immunomodulatory effects of EVs are derived from studies involving Gram-negative bacteria (31). Macrophages that internalize *Neisseria gonorrhoeae* EVs undergo apoptosis due to the presence of the porin PorB within the vesicles, resulting in altered mitochondrial permeability and cytochrome *c* release (32). EVs derived from *Escherichia coli* can also cause apoptosis in human intestinal epithelial cells due to interleukin-8 (IL-8) production, possibly mediated by the intracellular receptor NOD-1 (33, 34). Similar effects were observed in Gram-positive bacteria, where the listeriolysin O within EVs produced by *Listeria monocytogenes* decreased the viability of J774 macrophages (35). *Streptococcus pneumoniae* EVs also induce inflamatory cytokine production and cell maturation (36). Additionally, EVs derived from *Streptococcus pneumoniae* interact with complement components that cannot directly interact with the bacteria, thus avoiding phagocytosis (36). Fungal EVs also possess immunogenic properties (37). The proteins, RNA, lipids, carbohydrates, and pigments in fungal EVs are recognized by pattern recognition receptors (PRRs) expressed on leukocytes and activate immune responses (38). These collective findings show that EVs of fungi might positively or negatively modulate the activation of innate immunity.

Cryptococcus. *C. neoformans* is the principal causative agent of cryptococcosis, a disease distributed worldwide. After inhalation of fungal cells, immunosuppressed individuals, such as those infected with HIV, can develop the invasive form of this disease (39, 40). EVs derived from *C. neoformans* carry many virulence factors, including its major capsular antigen, glucuronoxylomannan (GXM), and laccase, the enzyme responsible for melanin production (12, 41). GXM exerts an immunosuppressive action over macrophages, monocytes, neutrophils, and T lymphocytes (42). This polysaccharide enhances IL-10 production by monocytes, subsequently impairing IL-12 production and intracellular killing (43). The lack of IL-12 may be due to the low levels of production of gamma interferon (IFN-γ) by peripheral blood mononuclear cells (PBMC), which in turn hampers the development of the Th1 protective response (43). GXM also exerts a direct *in vivo* and *in vitro* cytotoxic effect on macrophages due to activation of the Fas/FasL pathway (44, 45). Indeed, it has been demonstrated that macrophages stimulated *in vitro* with EVs derived from *C. neoformans* produce anti-inflammatory cytokines, such as transforming growth factor β (TGF-β) and IL-10 (46). Interestingly, the production of both TNF-α and nitric oxide (NO), as well as an increased ability to phagocytize and kill fungal cells, suggests that several molecules present in EVs derived from *C. neoformans* play dual roles: positive and negative stimulation of macrophages (46). These findings reinforce the suggestion that the effect of *C. neoformans* EVs is more protective than deleterious for the host. The presence of specific antibodies against EV proteins in the sera of patients diagnosed with cryptococcosis confirms the activation of the humoral immune response by these EVs *in vivo* (41). The degradation of cryptococcal EVs in human sera might be highly efficient, since the vesicles are disrupted in the presence of albumin and galectin-3 (47, 48).

EVs derived from a virulent strain of *C. gattii* also modulate macrophage responses, ultimately facilitating the intracellular multiplication of less virulent strains without interfering with the phagocytosis rate (49). Therefore, the presence of fungal virulence factors might be critical for the protective or deleterious effect of EVs. Heat inactivation of EVs abolished this effect, suggesting that, besides the virulence factors, a plethora of compounds derived from EVs (lipids, RNA, and proteins) could play a pivotal role in the modulation of macrophage functions (49).

***Candida*.** *C. albicans* is the most common human commensal fungus. This fungus primarily colonizes the gastrointestinal tract and the oral and vaginal mucosa of healthy individuals. In immunosuppressed individuals, *C. albicans* can become an opportunistic pathogen, ultimately causing a range of diseases from mucosal infections to candidemia and disseminated candidiasis (50, 51). Collected findings show that *C. albicans* EVs exhibit immunomodulatory effects that benefit the activation of the innate response. When stimulated with EVs, RAW 264.7 macrophages produce NO, IL-12p40, and lower concentrations of IL-10 and TGF-β, while bone marrow-derived macrophages (BMDM) produce NO, IL-12p40, tumor necrosis factor alpha (TNF-α), and IL-10. Bone marrow-derived dendritic cells (BMDC) produce IL-12p40, TNF-α, and TGF-β and undergo cell maturation that coincides with a higher level of expression of major histocompatibility complex class II and CD86. The hallmark of dendritic cell (DC) activation by *C. albicans* EVs suggests that vesicle constituents might be important for the immune adaptive response against the pathogen (52). The phospholipids contained in *C. albicans* EVs may also play a role in immune cell activation, as EVs derived from phosphatidylserine synthase (CHO1)-knockout fungi were unable to activate NF-κB in BMDM and J774.14 macrophages (53). EVs produced by *C. albicans* may be more stable in human sera than other fungal vesicles due to the presence of the glucose transmembrane transporter Hgt1p, which binds to complement factor H to allow for evasion of complement alternative pathway activation and, ultimately, opsonization (54). Indeed, blockage of Hgt1p in fungal cells results in an increase in neutrophil phagocytosis and intracellular killing (54). The EVs produced by *C. albicans* also played a protective effect in a *Galleria mellonella* model of fungal infection. Inoculation of EVs into the larval form of this invertebrate 2 days prior to lethal challenge with live *C. albicans* fungal cells resulted in a lower fungal burden and an increased life span in the EV-treated larvae (52).

***Paracoccidioides*.** Paracoccidioidomycosis is a fungal infection endemic in Latin America that is caused by the thermally dimorphic fungi *Paracoccidioides brasiliensis* and *P. lutzii*. The infection initiates in the lungs but can spread to other organs (55). The activation of a Th1 immune response is an indicator of a good prognosis, while the activation of the Th2 and Th9 immune responses causes an uncontrolled, deleterious inflammatory process (56). It is well-known that classically activated macrophages (M1) play a protective role against *P. brasiliensis* (55, 57). Interestingly, it has been demonstrated that *in vitro* stimulation with *P. brasiliensis* EVs can induce the differentiation of M1 macrophages (58). The EV-induced stimulus leads to the production of proinflammatory cytokines and to the increased relative expression of the inducible nitric oxide synthase (iNOS) gene. Additionally, macrophage phagocytosis and intracellular killing were comparable to those that were observed after IFN-γ stimulation (58). Mammalian lectin microarray assays demonstrated that the interaction between EVs and DCs was dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) dependent, with no involvement of Dectin-1 and -2 (59). Sera obtained from patients diagnosed with paracoccidioidomycosis reacted with EVs through a process that required fungal α-galactosyl epitopes (15). Despite this, it remains unclear if the α-galactosyl epitopes in EVs participate in the generation of immune responses, as observed in *Trypanosoma cruzi* (15). As previously reported for *C. neoformans*, galectin-3 disrupts *P. brasiliensis* EVs, ultimately limiting the functionality of those vesicles (60).

***Histoplasma*.** *Histoplasma capsulatum* is a thermally dimorphic fungus that causes histoplasmosis. *H. capsulatum* is distributed worldwide and is highly endemic in North

and South America (61). In immunocompetent hosts, a Th1/Th17 immune response limits the progression of the infection (62). However, a severe form of histoplasmosis might occur in immunocompromised individuals (63, 64). Similar to the findings for other fungal EVs, vesicular proteins of *H. capsulatum* were recognized by sera from patients with histoplasmosis, with a dominant serological reactivity of heat shock protein 60 (Hsp60) and histone 2B (13). The *in vivo* generation of anti-Hsp60 antibodies might be of significance, as it has been demonstrated that *in vitro* incubation of EVs with anti-Hsp60 antibodies alters the morphology, protein cargo, and enzymatic activities (65). Although the interplay of *H. capsulatum* EVs with host leukocytes remains to be better investigated, those EVs reduced the phagocytic rates and intracellular fungal killing by BMDM (66), suggesting that they may be more suppressive than protective in the infection.

***Malassezia*.** *Malassezia* spp. are dimorphic fungi that colonize the human skin as commensal organisms and are typically harmless; however, these fungi can cause skin-related disorders (67). *Malassezia sympodialis* and *M. furfur* are two of the many *Malassezia* species associated with atopic eczema (AE) (68). *M. sympodialis* produces EVs that carry allergens recognized by IgE from AE patients, and those EVs might induce the production of IL-4 and TNF-α by peripheral blood mononuclear cells (PBMC) obtained from AE patients or from healthy individuals (14). *M. sympodialis* EVs also exert an indirect immune effect, as DCs are able to phagocytize these fungal structures and produce their own EVs containing *M. sympodialis* antigens. DC-derived vesicles also induce the production of IL-4 and TNF-α by PBMCs from AE patients (14). In addition to DCs, monocytes and keratinocytes could actively internalize *M. sympodialis* EVs (69). *M. furfur* EVs may also play a role in immune modulation and AE development. Collected evidence from *in vitro* and *in vivo* experiments showed that *M. furfur* EVs stimulated NF-κB activation and increased IL-1β gene expression and IL-6 production by keratinocytes (70).

***Trichophyton*.** Dermatophytes are associated with infections of keratin-rich tissues that represent the most common superficial mycoses in humans (24, 71). Dermatophyte infections are also called ringworms or tinea. *Trichophyton interdigitale* and *T. rubrum* are established as the most important species that cause skin and nail infections (71). *T. interdigitale* EVs induce a strong inflammatory response in BMDM and keratinocytes. Both types of cells produce NO, TNF-α, IL-6, and IL-1β in response to EVs. An increase in iNOS relative expression suggests that EV-treated macrophages differentiate into M1 macrophages, which exhibit a higher phagocytic and intracellular killing index than other macrophages when incubated with fungal conidia. Additionally, Toll-like receptor 2 activation was found to be required for EV-induced macrophage activation (18). These data suggest that *T. interdigitale* EVs positively modulate inflammatory and microbicidal innate responses.

***Sporothrix*.** Sporotrichosis, caused by *Sporothrix* spp., is a mycosis endemic in tropical and subtropical areas (72). In Brazil, *Sporothrix brasiliensis* is the main etiological agent of sporotrichosis, but *Sporothrix* spp. may be distributed worldwide (73, 74). After contact with *Sporothrix* species, both immunocompetent and immunosuppressed individuals can develop sporotrichosis, which varies from cutaneous manifestations to colonization of multiple organs (63, 72). EVs derived from *S. brasiliensis* exhibited immunoreactivity with sera obtained from mice with experimental sporotrichosis, revealing a humoral response against vesicle proteins (19). *S. brasiliensis* EVs played no role in cytokine production by BMDC. However, pretreatment of BMDC with EVs and subsequent exposure to *S. brasiliensis* yeast cells resulted in the differential production of IL-12 p40, TNF-α, and IFN-γ compared with that by nontreated BMDC and higher levels of phagocytosis but not intracellular killing of *S. brasiliensis* yeast cells by pretreated BMDC than by nontreated BMDC. The *in vivo* administration of EVs impaired the initial immune response, as initial skin lesions were larger and contained higher levels of fungal colonization (19). These results suggest that *S. brasiliensis* EVs may be more suppressive than protective in the infection.

FUNGAL EVs AS TARGETS FOR IMMUNE INTERVENTIONS

The urgent need to develop new strategies to prevent and combat fungal infections is efficiently illustrated by the global number of deaths (1.5 million annually) caused by invasive mycoses. There are no licensed antifungal vaccines, and only two vaccine candidates targeting vulvovaginal candidiasis are currently under development (75, 76).

Several groups have demonstrated that fungal EVs act as immunomodulators, as they carry a number of immunogenic molecules that elicit inflammatory and microbicidal responses, suggesting the possibility of their use as candidates for adjuvants, vaccines against invasive fungal infections, or immunotherapies. In this sense, liposomes resembling EVs could be promising structures for the development of vaccines. Liposomes are laboratory-generated round vesicles of various sizes that are formed by one or more lipid bilayers. They can be engineered to carry selected antigens in association with the lipid bilayer or within the vesicle core. Addition of pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMP) in liposomes promotes adjuvant-like properties (77, 78). In this context, EVs would play a role of natural liposomes, delivering fungal PAMP for leukocytes of the innate system and, possibly, driving the differentiation of $CD4^+$ subsets. P10 is an epitope derived from *P. brasiliensis* glycoprotein 43 (gp43) that exerts a protective effect against experimental paracoccidioidomycosis. gp43, which is present in EVs, is the most well studied antigen from *P. brasiliensis* (79). The immunization of healthy or immunosuppressed mice with P10 decreased *P. brasiliensis* fungal loads and increased the survival of mice lethally challenged with *P. brasiliensis* by a mechanism dependent on the Th1 immune response (80–82). This protection is achieved through the establishment of a Th1 response (80). Additionally, concomitant treatment of mice with P10 and antifungal drugs efficiently diminished the fungal burden in infected mice (81). Two facts—that P10 is a component of EVs and that EVs induce the differentiation of M1 macrophages—suggest that the protective potential of *P. brasiliensis* EVs as a candidate vaccine could be assessed.

The *C. albicans* protein 1,3-β-glucosyltransferase (Bgl2) is involved in cell wall biosynthesis and virulence. Bgl2 proteins are present in *C. albicans* EVs and elicit humoral immune responses, as concluded from their reactivity with sera from patients suffering from systemic candidiasis (83). Additionally, treatment of mice with Bgl2 in a vaccine model led to the increased survival of mice challenged with *C. albicans* (84). *C. albicans* EVs used as immunogens in *G. mellonella* reduced the fungal burden and increased larva survival after challenge with yeast cells (52). In this sense, *G. mellonella* provides a useful experimental model, as its innate immune response resembles the human innate immune response. Unlike humans, however, no adaptive immune response is present in this invertebrate. *G. mellonella* exhibits a short period of immune boost after an immune challenge. This period is called "primary immunization" or "primitive immunity" and is characterized by higher counts of hemocytes and the increased production of antimicrobial peptides (85). Thus, the protective effect of *C. albicans* EVs may be due to primary immunization rather than adaptive immunization.

In *C. neoformans*, fungal protein extracts or the recombinant proteins were able to induce protection in mice challenged with *C. neoformans* or *C. gattii* (86, 87). Similarly, the vaccination of mice with protein extracts derived from the cell wall or cytoplasm of *C. gattii* increased the survival of mice and decreased the fungal burden after challenge with *C. gattii* (88). The recruitment of lymphocytes to the infection sites was also reported (88). The association of these proteins with EVs has not been demonstrated, but on the basis of the complexity of the protein composition of EVs, results similar to those described above for protein extracts from *C. gattii* would not be surprising. Indeed, EVs produced by *C. neoformans* containing GXM and sterylglucosides induced protection in *G. mellonella* (89). Similar to what has been proposed for *C. albicans*, this effect could be related to a primary immunization mechanism.

EVs carry several virulence factors, suggesting that detrimental activity against the host cannot be ruled out. EVs transport pathogen antigens that can interact with the

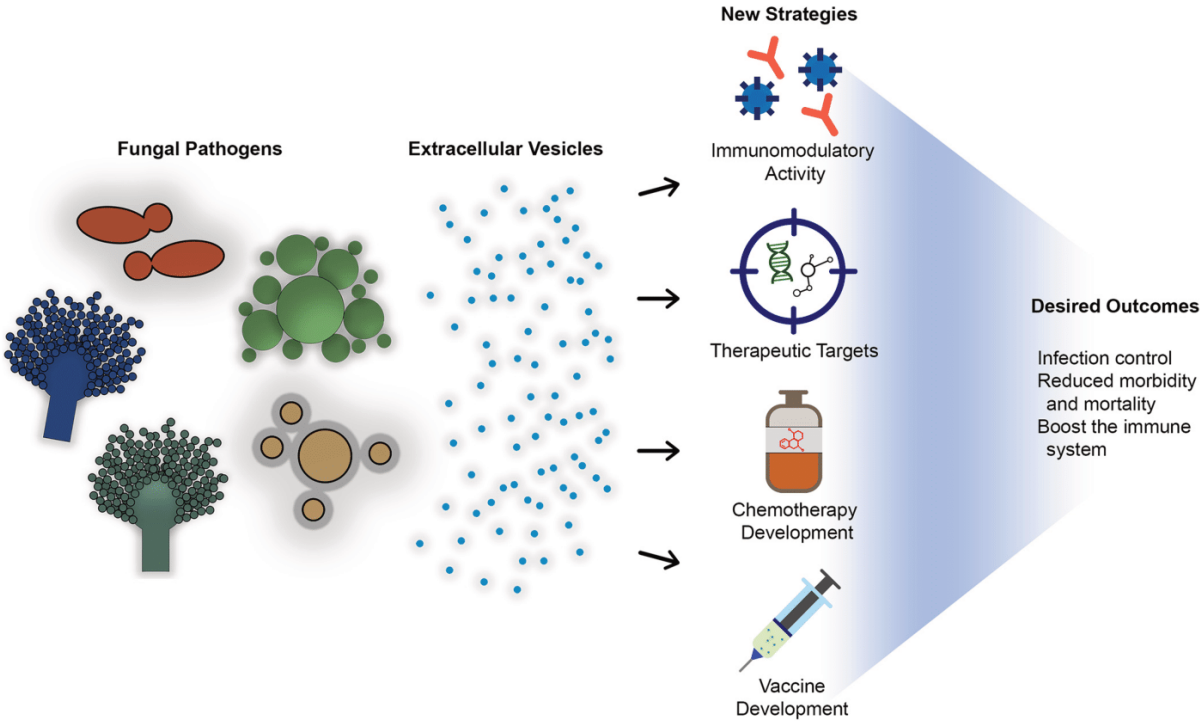

FIG 1 Fungal EVs may play a pivotal role in the establishment of fungal infections and can alter the infection process. These EVs are potential targets for new antifungal agents, as well as potential candidates for chemotherapy and vaccine development.

host immune system, ultimately resulting in immune memory. The controlled administration of fungal EVs in combination with an appropriate three-dimensional culture or animal model could be exploited to provide an adjuvant, a vaccine platform, or an immunotherapy platform. However, there is an equivoque with two commercially available vaccines derived from extracts of outer membrane vesicles from *Neisseria meningitidis* processed into a liposomal form (Bexsero and VA-Mengoc-BC); although these vaccines exhibit satisfactory efficacy (90–93), these materials cannot be considered EVs since these vaccines do not derive from secretions produced by bacterial cells (94). However, these previous successful approaches support the search for vaccines derived from fungal EVs.

The field of fungal EVs is still in its infancy, which agrees with the existence of many gaps in knowledge on how these structures can impact disease. Dissection of the physiological and pathogenic roles of fungal EVs will enhance our understanding of their potential applicability in models of vaccination. In this sense, we propose strategies to explore fungal EVs as potential prophylactic or therapeutic targets (Fig. 1).

We described above the interactions between various pathogenic fungal EVs and leukocytes of the innate system. The diversity of molecules present within these structures results in a diverse immune response. In some cases, the interactions between EVs and the immune system are beneficial to the host, and in other situations, the vesicles support disease development. It is clear that not all EV interactions are well established. Indeed, fungal EVs are important factors in immune response modulation, and they act at different levels, depending on the pathogen and the EV cargo. More studies are necessary to further elucidate this relationship and the possible use of such vesicles in host protection (23). The complexity of fungal EVs is enormous, but their potential to impact the interaction of fungi with host cells suggests that they have great potential in the development of new strategies to combat fungal infections.

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REFERENCES

1. Deatherage BL, Cookson BT. 2012. Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. Infect Immun 80:1948–1957. https://doi.org/10.1128/IAI.06014-11.
2. Yáñez-Mó M, Siljander PR-M, Andreu Z, Zavec AB, Borràs FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, Colás E, Cordeiro-da Silva A, Fais S, Falcon-Perez JM, Ghobrial IM, Giebel B, Gimona M, Graner M, Gursel I, Gursel M, Heegaard NHH, Hendrix A, Kierulf P, Kokubun K, Kosanovic M, Kralj-Iglic V, Krämer-Albers E-M, Laitinen S, Lässer C, Lener T, Ligeti E, Linē A, Lipps G, Llorente A, Lötvall J, Manček-Keber M, Marcilla A, Mittelbrunn M, Nazarenko I, Nolte-'t Hoen ENM, Nyman TA, O'Driscoll L, Olivan M, Oliveira C, Pállinger É, Del Portillo HA, Reventós J, Rigau M, Rohde E, Sammar M, et al. 2015. Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles 4:27066. https://doi.org/10.3402/jev.v4.27066.
3. van Niel G, D'Angelo G, Raposo G. 2018. Shedding light on the cell biology of extracellular vesicles. Nat Rev Mol Cell Biol 19:213–228. https://doi.org/10.1038/nrm.2017.125.
4. Brock CK, Wallin ST, Ruiz OE, Samms KM, Mandal A, Sumner EA, Eisenhoffer GT. 2019. Stem cell proliferation is induced by apoptotic bodies from dying cells during epithelial tissue maintenance. Nat Commun 10:1044. https://doi.org/10.1038/s41467-019-09010-6.
5. Malloci M, Perdomo L, Veerasamy M, Andriantsitohaina R, Simard G, Martinez MC. 2019. Extracellular vesicles: mechanisms in human health and disease. Antioxid Redox Signal 30:813–856. https://doi.org/10.1089/ars.2017.7265.
6. Raposo G, Stoorvogel W. 2013. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 200:373–383. https://doi.org/10.1083/jcb.201211138.
7. Maas SLN, Breakefield XO, Weaver AM. 2017. Extracellular vesicles: unique intercellular delivery vehicles. Trends Cell Biol 27:172–188. https://doi.org/10.1016/j.tcb.2016.11.003.
8. Gibson RK, Peberdy JF. 1972. Fine structure of protoplasts of Aspergillus nidulans. J Gen Microbiol 72:529–538. https://doi.org/10.1099/00221287-72-3-529.
9. Takeo K, Uesaka I, Uehira K, Nishiura M. 1973. Fine structure of Cryptococcus neoformans grown in vivo as observed by freeze-etching. J Bacteriol 113:1449–1454.
10. Anderson J, Mihalik R, Soll DR. 1990. Ultrastructure and antigenicity of the unique cell wall pimple of the Candida opaque phenotype. J Bacteriol 172:224–235. https://doi.org/10.1128/jb.172.1.224-235.1990.
11. Osumi M. 1998. The ultrastructure of yeast: cell wall structure and formation. Micron 29:207–233. https://doi.org/10.1016/S0968-4328(97)00072-3.
12. Rodrigues ML, Nimrichter L, Oliveira DL, Frases S, Miranda K, Zaragoza O, Alvarez M, Nakouzi A, Feldmesser M, Casadevall A. 2007. Vesicular polysaccharide export in Cryptococcus neoformans is a eukaryotic solution to the problem of fungal trans-cell wall transport. Eukaryot Cell 6:48–59. https://doi.org/10.1128/EC.00318-06.
13. Albuquerque PC, Nakayasu ES, Rodrigues ML, Frases S, Casadevall A, Zancope-Oliveira RM, Almeida IC, Nosanchuk JD. 2008. Vesicular transport in Histoplasma capsulatum: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. Cell Microbiol 10:1695–1710. https://doi.org/10.1111/j.1462-5822.2008.01160.x.
14. Gehrmann U, Qazi KR, Johansson C, Hultenby K, Karlsson M, Lundeberg L, Gabrielsson S, Scheynius A. 2011. Nanovesicles from Malassezia sympodialis and host exosomes induce cytokine responses—novel mechanisms for host-microbe interactions in atopic eczema. PLoS One 6:e21480. https://doi.org/10.1371/journal.pone.0021480.
15. Vallejo MC, Matsuo AL, Ganiko L, Medeiros LC, Miranda K, Silva LS, Freymuller-Haapalainen E, Sinigaglia-Coimbra R, Almeida IC, Puccia R. 2011. The pathogenic fungus Paracoccidioides brasiliensis exports extracellular vesicles containing highly immunogenic alpha-galactosyl epitopes. Eukaryot Cell 10:343–351. https://doi.org/10.1128/EC.00227-10.
16. Silva BMA, Prados-Rosales R, Espadas-Moreno J, Wolf JM, Luque-Garcia JL, Gonçalves T, Casadevall A. 2014. Characterization of Alternaria infectoria extracellular vesicles. Med Mycol 52:202–210. https://doi.org/10.1093/mmy/myt003.
17. Leone F, Bellani L, Muccifora S, Giorgetti L, Bongioanni P, Simili M, Maserti B, Del Carratore R. 2018. Analysis of extracellular vesicles produced in the biofilm by the dimorphic yeast Pichia fermentans. J Cell Physiol 233:2759–2767. https://doi.org/10.1002/jcp.25885.
18. Bitencourt TA, Rezende CP, Quaresemin NR, Moreno P, Hatanaka O, Rossi A, Martinez-Rossi NM, Almeida F. 2018. Extracellular vesicles from the dermatophyte Trichophyton interdigitale modulate macrophage and keratinocyte functions. Front Immunol 9:2343. https://doi.org/10.3389/fimmu.2018.02343.
19. Ikeda MAK, de Almeida JRF, Jannuzzi GP, Cronemberger-Andrade A, Torrecilhas ACT, Moretti NS, da Cunha JPC, de Almeida SR, Ferreira KS. 2018. Extracellular vesicles from Sporothrix brasiliensis are an important virulence factor that induce an increase in fungal burden in experimental sporotrichosis. Front Microbiol 9:2286. https://doi.org/10.3389/fmicb.2018.02286.
20. de Toledo Martins S, Szwarc P, Goldenberg S, Alves LR. 2019. Extracellular vesicles in fungi: composition and functions. Curr Top Microbiol Immunol 422:45–59. https://doi.org/10.1007/82_2018_141.
21. Bleackley MR, Dawson CS, Anderson MA. 2019. Fungal extracellular vesicles with a focus on proteomic analysis. Proteomics 19:e1800232. https://doi.org/10.1002/pmic.201800232.
22. Rella A, Farnoud AM, Del Poeta M. 2016. Plasma membrane lipids and their role in fungal virulence. Prog Lipid Res 61:63–72. https://doi.org/10.1016/j.plipres.2015.11.003.
23. Zamith-Miranda D, Nimrichter L, Rodrigues ML, Nosanchuk JD. 2018. Fungal extracellular vesicles: modulating host-pathogen interactions by both the fungus and the host. Microbes Infect 20:501–504. https://doi.org/10.1016/j.micinf.2018.01.011.
24. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. 2012. Hidden killers: human fungal infections. Sci Transl Med 4:165rv113. https://doi.org/10.1126/scitranslmed.3004404.
25. Bongomin F, Gago S, Oladele RO, Denning DW. 2017. Global and multinational prevalence of fungal diseases—estimate precision. J Fungi (Basel) 3:E57. https://doi.org/10.3390/jof3040057.
26. Farmakiotis D, Kontoyiannis DP. 2017. Epidemiology of antifungal resistance in human pathogenic yeasts: current viewpoint and practical recommendations for management. Int J Antimicrob Agents 50:318–324. https://doi.org/10.1016/j.ijantimicag.2017.05.019.
27. Robbins N, Caplan T, Cowen LE. 2017. Molecular evolution of antifungal drug resistance. Annu Rev Microbiol 71:753–775. https://doi.org/10.1146/annurev-micro-030117-020345.
28. Gao J, Wang H, Li Z, Wong AH, Wang YZ, Guo Y, Lin X, Zeng G, Liu H, Wang Y, Wang J. 2018. Candida albicans gains azole resistance by altering sphingolipid composition. Nat Commun 9:4495. https://doi.org/10.1038/s41467-018-06944-1.
29. Rybak JM, Ge W, Wiederhold NP, Parker JE, Kelly SL, Rogers PD, Fortwendel JR. 2019. Mutations in hmg1, challenging the paradigm of clinical triazole resistance in Aspergillus fumigatus. mBio 10:e00437-19. https://doi.org/10.1128/mBio.00437-19.
30. Almeida F, Rodrigues ML, Coelho C. 2019. The still underestimated problem of fungal diseases worldwide. Front Microbiol 10:214. https://doi.org/10.3389/fmicb.2019.00214.
31. Kuipers ME, Hokke CH, Smits HH, Nolte-'t Hoen ENM. 2018. Pathogen-derived extracellular vesicle-associated molecules that affect the host immune system: an overview. Front Microbiol 9:2182. https://doi.org/10.3389/fmicb.2018.02182.

32. Deo P, Chow SH, Hay ID, Kleifeld O, Costin A, Elgass KD, Jiang JH, Ramm G, Gabriel K, Dougan G, Lithgow T, Heinz E, Naderer T. 2018. Outer membrane vesicles from Neisseria gonorrhoeae target PorB to mitochondria and induce apoptosis. PLoS Pathog 14:e1006945. https://doi.org/10.1371/journal.ppat.1006945.
33. Kunsmann L, Ruter C, Bauwens A, Greune L, Gluder M, Kemper B, Fruth A, Wai SN, He X, Lloubes R, Schmidt MA, Dobrindt U, Mellmann A, Karch H, Bielaszewska M. 2015. Virulence from vesicles: novel mechanisms of host cell injury by Escherichia coli O104:H4 outbreak strain. Sci Rep 5:13252. https://doi.org/10.1038/srep13252.
34. Canas MA, Fabrega MJ, Gimenez R, Badia J, Baldoma L. 2018. Outer membrane vesicles from probiotic and commensal Escherichia coli activate NOD1-mediated immune responses in intestinal epithelial cells. Front Microbiol 9:498. https://doi.org/10.3389/fmicb.2018.00498.
35. Coelho C, Brown L, Maryam M, Vij R, Smith DFQ, Burnet MC, Kyle JE, Heyman HM, Ramirez J, Prados-Rosales R, Lauvau G, Nakayasu ES, Brady NR, Hamacher-Brady A, Coppens I, Casadevall A. 2019. Listeria monocytogenes virulence factors, including listeriolysin O, are secreted in biologically active extracellular vesicles. J Biol Chem 294:1202–1217. https://doi.org/10.1074/jbc.RA118.006472.
36. Codemo M, Muschiol S, Iovino F, Nannapaneni P, Plant L, Wai SN, Henriques-Normark B. 2018. Immunomodulatory effects of pneumococcal extracellular vesicles on cellular and humoral host defenses. mBio 9:e00559-18. https://doi.org/10.1128/mBio.00559-18.
37. Joffe LS, Nimrichter L, Rodrigues ML, Del Poeta M. 2016. Potential roles of fungal extracellular vesicles during infection. mSphere 1:e00099-16. https://doi.org/10.1128/mSphere.00099-16.
38. Patin EC, Thompson A, Orr SJ. 2019. Pattern recognition receptors in fungal immunity. Semin Cell Dev Biol 89:24–33. https://doi.org/10.1016/j.semcdb.2018.03.003.
39. May RC, Stone NR, Wiesner DL, Bicanic T, Nielsen K. 2016. Cryptococcus: from environmental saprophyte to global pathogen. Nat Rev Microbiol 14:106–117. https://doi.org/10.1038/nrmicro.2015.6.
40. Boulware DR. 2011. Cryptococcus: from human pathogen to model yeast. Lancet Infect Dis 11:434. https://doi.org/10.1016/S1473-3099(11)70140-2.
41. Rodrigues ML, Nakayasu ES, Oliveira DL, Nimrichter L, Nosanchuk JD, Almeida IC, Casadevall A. 2008. Extracellular vesicles produced by Cryptococcus neoformans contain protein components associated with virulence. Eukaryot Cell 7:58–67. https://doi.org/10.1128/EC.00370-07.
42. Monari C, Bistoni F, Vecchiarelli A. 2006. Glucuronoxylomannan exhibits potent immunosuppressive properties. FEMS Yeast Res 6:537–542. https://doi.org/10.1111/j.1567-1364.2006.00072.x.
43. Retini C, Kozel TR, Pietrella D, Monari C, Bistoni F, Vecchiarelli A. 2001. Interdependency of interleukin-10 and interleukin-12 in regulation of T-cell differentiation and effector function of monocytes in response to stimulation with Cryptococcus neoformans. Infect Immun 69:6064–6073. https://doi.org/10.1128/IAI.69.10.6064-6073.2001.
44. Villena SN, Pinheiro RO, Pinheiro CS, Nunes MP, Takiya CM, DosReis GA, Previato JO, Mendonca-Previato L, Freire-de-Lima CG. 2008. Capsular polysaccharides galactoxylomannan and glucuronoxylomannan from Cryptococcus neoformans induce macrophage apoptosis mediated by Fas ligand. Cell Microbiol 10:1274–1285. https://doi.org/10.1111/j.1462-5822.2008.01125.x.
45. Monari C, Pericolini E, Bistoni G, Casadevall A, Kozel TR, Vecchiarelli A. 2005. Cryptococcus neoformans capsular glucuronoxylomannan induces expression of Fas ligand in macrophages. J Immunol 174:3461–3468. https://doi.org/10.4049/jimmunol.174.6.3461.
46. Oliveira DL, Freire-de-Lima CG, Nosanchuk JD, Casadevall A, Rodrigues ML, Nimrichter L. 2010. Extracellular vesicles from Cryptococcus neoformans modulate macrophage functions. Infect Immun 78:1601–1609. https://doi.org/10.1128/IAI.01171-09.
47. Wolf JM, Rivera J, Casadevall A. 2012. Serum albumin disrupts Cryptococcus neoformans and Bacillus anthracis extracellular vesicles. Cell Microbiol 14:762–773. https://doi.org/10.1111/j.1462-5822.2012.01757.x.
48. Almeida F, Wolf JM, da Silva TA, DeLeon-Rodriguez CM, Rezende CP, Pessoni AM, Fernandes FF, Silva-Rocha R, Martinez R, Rodrigues ML, Roque-Barreira MC, Casadevall A. 2017. Galectin-3 impacts Cryptococcus neoformans infection through direct antifungal effects. Nat Commun 8:1968. https://doi.org/10.1038/s41467-017-02126-7.
49. Bielska E, Sisquella MA, Aldeieg M, Birch C, O'Donoghue EJ, May RC. 2018. Pathogen-derived extracellular vesicles mediate virulence in the fatal human pathogen Cryptococcus gattii. Nat Commun 9:1556. https://doi.org/10.1038/s41467-018-03991-6.
50. Nobile CJ, Johnson AD. 2015. Candida albicans biofilms and human disease. Annu Rev Microbiol 69:71–92. https://doi.org/10.1146/annurev-micro-091014-104330.
51. Kim J, Sudbery P. 2011. Candida albicans, a major human fungal pathogen. J Microbiol 49:171–177. https://doi.org/10.1007/s12275-011-1064-7.
52. Vargas G, Rocha JD, Oliveira DL, Albuquerque PC, Frases S, Santos SS, Nosanchuk JD, Gomes AM, Medeiros LC, Miranda K, Sobreira TJ, Nakayasu ES, Arigi EA, Casadevall A, Guimaraes AJ, Rodrigues ML, Freire-de-Lima CG, Almeida IC, Nimrichter L. 2015. Compositional and immunobiological analyses of extracellular vesicles released by Candida albicans. Cell Microbiol 17:389–407. https://doi.org/10.1111/cmi.12374.
53. Wolf JM, Espadas J, Luque-Garcia J, Reynolds T, Casadevall A. 2015. Lipid biosynthetic genes affect Candida albicans extracellular vesicle morphology, cargo, and immunostimulatory properties. Eukaryot Cell 14:745–754. https://doi.org/10.1128/EC.00054-15.
54. Kenno S, Speth C, Rambach G, Binder U, Chatterjee S, Caramalho R, Haas H, Lass-Flörl C, Shaughnessy J, Ram S, Gow NAR, Orth-Höller D, Würzner R. 2018. Candida albicans factor H binding molecule Hgt1p—a low glucose-induced transmembrane protein is trafficked to the cell wall and impairs phagocytosis and killing by human neutrophils. Front Microbiol 9:3319. https://doi.org/10.3389/fmicb.2018.03319.
55. Mendes RP, Cavalcante RS, Marques SA, Marques MEA, Venturini J, Sylvestre TF, Paniago AMM, Pereira AC, da Silva JF, Fabro AT, Bosco SMG, Bagagli E, Hahn RC, Levorato AD. 2017. Paracoccidioidomycosis: current perspectives from Brazil. Open Microbiol J 11:224–282. https://doi.org/10.2174/1874285801711010224.
56. Rodríguez Molina LM, Tobón Orozco AM. 2018. Paracoccidioidomycosis: global vision of a forgotten endemic mycosis. Curr Trop Med Rep 5:138–143. https://doi.org/10.1007/s40475-018-0148-4.
57. Leopold Wager CM, Wormley FL, Jr. 2014. Classical versus alternative macrophage activation: the Ying and the Yang in host defense against pulmonary fungal infections. Mucosal Immunol 7:1023–1035. https://doi.org/10.1038/mi.2014.65.
58. da Silva TA, Roque-Barreira MC, Casadevall A, Almeida F. 2016. Extracellular vesicles from Paracoccidioides brasiliensis induced M1 polarization in vitro. Sci Rep 6:35867. https://doi.org/10.1038/srep35867.
59. Peres da Silva R, Heiss C, Black I, Azadi P, Gerlach JQ, Travassos LR, Joshi L, Kilcoyne M, Puccia R. 2015. Extracellular vesicles from Paracoccidioides pathogenic species transport polysaccharide and expose ligands for DC-SIGN receptors. Sci Rep 5:14213. https://doi.org/10.1038/srep14213.
60. Hatanaka O, Rezende CP, Moreno P, Freitas Fernandes F, Oliveira Brito PKM, Martinez R, Coelho C, Roque-Barreira MC, Casadevall A, Almeida F. 2019. Galectin-3 inhibits Paracoccidioides brasiliensis growth and impacts paracoccidioidomycosis through multiple mechanisms. mSphere 4:e00209-19. https://doi.org/10.1128/mSphere.00209-19.
61. Woods JP. 2016. Revisiting old friends: developments in understanding Histoplasma capsulatum pathogenesis. J Microbiol 54:265–276. https://doi.org/10.1007/s12275-016-6044-5.
62. Kroetz DN, Deepe GS. 2012. The role of cytokines and chemokines in Histoplasma capsulatum infection. Cytokine 58:112–117. https://doi.org/10.1016/j.cyto.2011.07.430.
63. Queiroz-Telles F, Fahal AH, Falci DR, Caceres DH, Chiller T, Pasqualotto AC. 2017. Neglected endemic mycoses. Lancet Infect Dis 17:e367–e377. https://doi.org/10.1016/S1473-3099(17)30306-7.
64. Limper AH, Adenis A, Le T, Harrison TS. 2017. Fungal infections in HIV/AIDS. Lancet Infect Dis 17:e334–e343. https://doi.org/10.1016/S1473-3099(17)30303-1.
65. Matos Baltazar L, Nakayasu ES, Sobreira TJ, Choi H, Casadevall A, Nimrichter L, Nosanchuk JD. 2016. Antibody binding alters the characteristics and contents of extracellular vesicles released by Histoplasma capsulatum. mSphere 1:e00085-15. https://doi.org/10.1128/mSphere.00085-15.
66. Baltazar LM, Zamith-Miranda D, Burnet MC, Choi H, Nimrichter L, Nakayasu ES, Nosanchuk JD. 2018. Concentration-dependent protein loading of extracellular vesicles released by Histoplasma capsulatum after antibody treatment and its modulatory action upon macrophages. Sci Rep 8:8065. https://doi.org/10.1038/s41598-018-25665-5.
67. Harada K, Saito M, Sugita T, Tsuboi R. 2015. Malassezia species and their associated skin diseases. J Dermatol 42:250–257. https://doi.org/10.1111/1346-8138.12700.
68. Nowicka D, Nawrot U. 2019. Contribution of Malassezia spp. to the development of atopic dermatitis. Mycoses 62:588–596. https://doi.org/10.1111/myc.12913.
69. Johansson HJ, Vallhov H, Holm T, Gehrmann U, Andersson A, Johansson C, Blom H, Carroni M, Lehtio J, Scheynius A. 2018. Extracellular nanovesicles released from the commensal yeast Malassezia sympodialis are

enriched in allergens and interact with cells in human skin. Sci Rep 8:9182. https://doi.org/10.1038/s41598-018-27451-9.
70. Zhang YJ, Han Y, Sun YZ, Jiang HH, Liu M, Qi RQ, Gao XH. 2019. Extracellular vesicles derived from Malassezia furfur stimulate IL-6 production in keratinocytes as demonstrated in in vitro and in vivo models. J Dermatol Sci 93:168–175. https://doi.org/10.1016/j.jdermsci.2019.03.001.
71. Zhan P, Liu W. 2017. The changing face of dermatophytic infections worldwide. Mycopathologia 182:77–86. https://doi.org/10.1007/s11046-016-0082-8.
72. Chakrabarti A, Bonifaz A, Gutierrez-Galhardo MC, Mochizuki T, Li S. 2015. Global epidemiology of sporotrichosis. Med Mycol 53:3–14. https://doi.org/10.1093/mmy/myu062.
73. de Beer ZW, Duong TA, Wingfield MJ. 2016. The divorce of Sporothrix and Ophiostoma: solution to a problematic relationship. Stud Mycol 83:165–191. https://doi.org/10.1016/j.simyco.2016.07.001.
74. Gremiao ID, Miranda LH, Reis EG, Rodrigues AM, Pereira SA. 2017. Zoonotic epidemic of sporotrichosis: cat to human transmission. PLoS Pathog 13:e1006077. https://doi.org/10.1371/journal.ppat.1006077.
75. Nicola AM, Albuquerque P, Paes HC, Fernandes L, Costa FF, Kioshima ES, Abadio AKR, Bocca AL, Felipe MS. 2019. Antifungal drugs: new insights in research & development. Pharmacol Ther 195:21–38. https://doi.org/10.1016/j.pharmthera.2018.10.008.
76. Nami S, Mohammadi R, Vakili M, Khezripour K, Mirzaei H, Morovati H. 2019. Fungal vaccines, mechanism of actions and immunology: a comprehensive review. Biomed Pharmacother 109:333–344. https://doi.org/10.1016/j.biopha.2018.10.075.
77. De Serrano LO, Burkhart DJ. 2017. Liposomal vaccine formulations as prophylactic agents: design considerations for modern vaccines. J Nanobiotechnology 15:83. https://doi.org/10.1186/s12951-017-0319-9.
78. Wang N, Chen M, Wang T. 2019. Liposomes used as a vaccine adjuvant-delivery system: from basics to clinical immunization. J Control Release 303:130–150. https://doi.org/10.1016/j.jconrel.2019.04.025.
79. Vallejo MC, Nakayasu ES, Matsuo AL, Sobreira TJ, Longo LV, Ganiko L, Almeida IC, Puccia R. 2012. Vesicle and vesicle-free extracellular proteome of Paracoccidioides brasiliensis: comparative analysis with other pathogenic fungi. J Proteome Res 11:1676–1685. https://doi.org/10.1021/pr200872s.
80. Taborda CP, Juliano MA, Puccia R, Franco M, Travassos LR. 1998. Mapping of the T-cell epitope in the major 43-kilodalton glycoprotein of Paracoccidioides brasiliensis which induces a Th-1 response protective against fungal infection in BALB/c mice. Infect Immun 66:786–793.
81. Marques AF, da Silva MB, Juliano MA, Munhoz JE, Travassos LR, Taborda CP. 2008. Additive effect of P10 immunization and chemotherapy in anergic mice challenged intratracheally with virulent yeasts of Paracoccidioides brasiliensis. Microbes Infect 10:1251–1258. https://doi.org/10.1016/j.micinf.2008.07.027.
82. Munoz JE, Luft VD, Amorim J, Magalhaes A, Thomaz L, Nosanchuk JD, Travassos LR, Taborda CP. 2014. Immunization with P10 peptide increases specific immunity and protects immunosuppressed BALB/c mice infected with virulent yeasts of Paracoccidioides brasiliensis. Mycopathologia 178:177–188. https://doi.org/10.1007/s11046-014-9801-1.
83. Pitarch A, Jimenez A, Nombela C, Gil C. 2006. Decoding serological response to Candida cell wall immunome into novel diagnostic, prognostic, and therapeutic candidates for systemic candidiasis by proteomic and bioinformatic analyses. Mol Cell Proteomics 5:79–96. https://doi.org/10.1074/mcp.M500243-MCP200.
84. Gil-Bona A, Llama-Palacios A, Parra CM, Vivanco F, Nombela C, Monteoliva L, Gil C. 2015. Proteomics unravels extracellular vesicles as carriers of classical cytoplasmic proteins in Candida albicans. J Proteome Res 14:142–153. https://doi.org/10.1021/pr5007944.
85. Pereira TC, de Barros PP, Fugisaki LRO, Rossoni RD, Ribeiro FC, de Menezes RT, Junqueira JC, Scorzoni L. 2018. Recent advances in the use of Galleria mellonella model to study immune responses against human pathogens. J Fungi (Basel) 4:E128. https://doi.org/10.3390/jof4040128.
86. Specht CA, Lee CK, Huang H, Tipper DJ, Shen ZT, Lodge JK, Leszyk J, Ostroff GR, Levitz SM. 2015. Protection against experimental cryptococcosis following vaccination with glucan particles containing Cryptococcus alkaline extracts. mBio 6:e01905-15. https://doi.org/10.1128/mBio.01905-15.
87. Specht CA, Lee CK, Huang H, Hester MM, Liu J, Luckie BA, Torres Santana MA, Mirza Z, Khoshkenar P, Abraham A, Shen ZT, Lodge JK, Akalin A, Homan J, Ostroff GR, Levitz SM. 2017. Vaccination with recombinant Cryptococcus proteins in glucan particles protects mice against cryptococcosis in a manner dependent upon mouse strain and cryptococcal species. mBio 8:e01872-17. https://doi.org/10.1128/mBio.01872-17.
88. Chaturvedi AK, Hameed RS, Wozniak KL, Hole CR, Leopold Wager CM, Weintraub ST, Lopez-Ribot JL, Wormley FL, Jr. 2014. Vaccine-mediated immune responses to experimental pulmonary Cryptococcus gattii infection in mice. PLoS One 9:e104316. https://doi.org/10.1371/journal.pone.0104316.
89. Colombo AC, Rella A, Normile T, Joffe LS, Tavares PM, Araujo GRDS, Frases S, Orner EP, Farnoud AM, Fries BC, Sheridan B, Nimrichter L, Rodrigues ML, Del Poeta M. 2019. Cryptococcus neoformans glucuronoxylomannan and sterylglucoside are required for host protection in an animal vaccination model. mBio 10:e02909-28. https://doi.org/10.1128/mBio.02909-18.
90. Campos JH, Soares RP, Ribeiro K, Andrade AC, Batista WL, Torrecilhas AC. 2015. Extracellular vesicles: role in inflammatory responses and potential uses in vaccination in cancer and infectious diseases. J Immunol Res 2015:832057. https://doi.org/10.1155/2015/832057.
91. Ochoa-Azze RF. 2018. Cross-protection induced by VA-MENGOC-BC(R) vaccine. Hum Vaccin Immunother 14:1064–1068. https://doi.org/10.1080/21645515.2018.1438028.
92. Holst J, Oster P, Arnold R, Tatley MV, Næss LM, Aaberge IS, Galloway Y, McNicholas A, O'Hallahan J, Rosenqvist E, Black S. 2013. Vaccines against meningococcal serogroup B disease containing outer membrane vesicles (OMV): lessons from past programs and implications for the future. Hum Vaccin Immunother 9:1241–1253. https://doi.org/10.4161/hv.24129.
93. Petousis-Harris H. 2018. Impact of meningococcal group B OMV vaccines, beyond their brief. Hum Vaccin Immunother 14:1058–1063. https://doi.org/10.1080/21645515.2017.1381810.
94. Coelho C, Casadevall A. 2019. Answers to naysayers regarding microbial extracellular vesicles. Biochem Soc Trans 47:1005–1012. https://doi.org/10.1042/BST20180252.

Anexo II

Manuscrito - FREITAS, M. S.; PESSONI, A. M.; COELHO, C.; BONATO, V. L. D.; RODRIGUES, M. L.; CASADEVALL, A.; ALMEIDA, F. Interactions of Extracellular Vesicles from Pathogenic Fungi with Innate Leukocytes. **Curr Top Microbiol Immunol**, 432, p. 89-120, 2021.

Interactions of Extracellular Vesicles from Pathogenic Fungi with Innate Leukocytes

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Contents

Abstract Several studies have shown the immunomodulatory effects of extracellular vesicles (EVs) released by pathogenic fungi. Herein, we discuss the data regarding the immunomodulatory properties of fungal EVs, but also of EVs produced by infected leukocytes. This characterizes a two-way path, in which both host and

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fungal EVs could coexist and play crucial roles in disease progression or protection in fungal infections. We suggest that EVs can dictate the progress of fungal diseases, and their potential as therapeutic targets.

1 Important Virulence Factors Are Secreted in EVs

Fungal pathogens produce a plethora of molecules to promote successful infection in their hosts. Besides the molecules recognized by host cell surface receptors, virulence factors might also directly target the host and mediate adhesion and invasion, host damage, and, finally, counteract immune attack or allow immune evasion (Mayer et al. 2013; Hofs et al. 2016; Esher et al. 2018; Mihu and Nosanchuk 2012; Camacho and Nino-Vega 2017; Santos et al. 2020; Boral et al. 2018). Virulence factors modulate also fungal morphological changes which generate more invasive forms and biofilm formation (Brunke et al. 2016). Many of those virulence factors are exported in a concentrated form in extracellular vesicles (EVs), as "virulence bags", which can interact with host cells to promote the spread of infection (Rodrigues et al. 2014; Rodrigues and Casadevall 2018; Bielska and May 2019; Joffe et al. 2016; Freitas et al. 2019). Likewise, the host may have evolved mechanisms to counteract the microbial/fungal virulence bags.

Candida albicans produce and secrete aspartic proteinases (Sap) and agglutinin-like sequence protein (Als), both well-defined virulence factor families (Mayer et al. 2013; Hofs et al. 2016; Schaller et al. 2005). *C. albicans* possess a 10-member Sap family (Sap1–10), tasked with active penetration of host cell and nutrient acquisition from the extracellular space (Mayer et al. 2013; Naglik et al. 2003; Moyes et al. 2015). Saps also play an immune evasion function. Sap1–3 cleave C3b, C4b, and C5 complement factors and inhibit membrane attack complex formation; additionally, Saps can digest to degrade a myriad of proteins related to immunologic defense (Naglik et al. 2003; Singh et al. 2020; Gropp et al. 2009; Monika et al. 2017). In contrast with its immunomodulatory properties, recombinant Saps can also induce the production and secretion of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, by human monocytes, in a manner independent from enzymatic activity (Pietrella et al. 2010). In *C. albicans,* Sap2, Sap3, and Sap7–10 have been found in proteomics analysis of EVs (Konecna et al. 2019; Vargas et al. 2015). The Als family is composed of 8 members (Als1–7 and Als9) (Liu and Filler 2011), and of these proteins, Als2–4 are found in *C. albicans* EVs (Konecna et al. 2019; Vargas et al. 2015). Als3 is a key factor for *C. albicans* infection, as this protein is associated with adherence to host cells, biofilm formation, invasion, iron acquisition, and transition between yeast and hyphal form, the latter possessing a great invasive potential (Mayer et al. 2013; Hofs et al. 2016; Liu and Filler 2011). Given its many effector roles and high expression in *C. albicans* surface, Als3 is also a potential candidate for a vaccine. Indeed, in vivo experiments showed a protective response in

mice immunized with recombinant Als3 (Spellberg et al. 2006). A vaccine based on Als3 proteins is currently undergoing clinical trials (Cassone and Casadevall 2012).

Cryptococcus neoformans and other members of this pathogenic species complex are particularly known for producing a polysaccharide capsule and the protective phenolic pigment melanin (Kwon-Chung et al. 2017). Both of these act as virulence factors, protecting the fungi against host attack and dampening the host immune response (Zaragoza 2019). The predominant components of *C. neoformans* polysaccharide capsule are glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal) (Casadevall et al. 2019; O'Meara and Alspaugh 2012). GXM are recognized by innate receptors such as, Toll-like receptor 2 (TLR2), TLR4, CD14, and Dectin-3 (Barbosa et al. 2007; Levitz 2002; Huang et al. 2018). GXM polysaccharides can also be secreted into the extracellular space. Capsular and secreted GXM have numerous well-described immunosuppressive effects on macrophages, monocytes, neutrophils, and T-lymphocytes (Monari et al. 2006; Decote-Ricardo et al. 2019). These polysaccharides trigger enhanced IL-10 production by monocytes and induce an in vivo and in vitro Fas/FasL dependent cell death pathway over mammalian macrophages (Retini et al. 2001; Villena et al. 2008; Monari et al. 2005). Melanin is present in *C. neoformans* cell wall, as well as secreted and confers protection against degradative action by phagosomes by neutralizing reactive oxygen species (ROS) (Esher et al. 2018). Production of melanin is dependent on laccase enzymes, which use phenolic precursors, such as L-3,4-dihydroxyphenylalanine (L-DOPA), to synthesize this pigment (Esher et al. 2018). Both GXM and laccase are detected by immunostaining in *C. neoformans* EVs (Rodrigues et al. 2008, 2007; Eisenman et al. 2009) and proteomics analysis revealed the presence of other components (Rodrigues et al. 2008).

Replication within phagocytes is a remarkable feature of *Histoplasma capsulatum* infection (Shen and Rappleye 2017). Among other molecules, heat shock protein 60 (Hsp60) and catalase B, each contribute to *H. capsulatum* survival in the adverse cell environment following ingestion (Mihu and Nosanchuk 2012; Beyhan and Sil 2019). Hsp60 is an important chaperone associated with protein folding that is also relevant in the recognition and phagocytosis of the yeast by macrophages (Habich et al. 2006; Long et al. 2003). Phagocytosis is mediated by the interaction of Hsp60 with the complement receptor 3 (CR3) on human macrophages (Mihu and Nosanchuk 2012; Long et al. 2003). The CR3-based phagocytosis can be exploited by *H. capsulatum*. Sole activation of CR3 does not induce oxidative burst, it is hypothesized that the activation of others pattern-recognition receptors or cytokine signaling are required for cellular activation (Ehlers 2000). Fungal survival and replication inside phagosomes is associated with catalase activity that inhibits ROS action (Shen and Rappleye 2017; Beyhan and Sil 2019). The presence of Hsp60 and catalase activity was reported in *H. capsulatum* EVs, as well as other proteins (Albuquerque et al. 2008; Matos Baltazar et al. 2016).

The glycoprotein 43 (gp43) from *Paracoccidioides brasiliensis* is the most immunodominant molecule in this fungus, being recognized by 100% of paracoccidioidomycosis patient sera (Santos et al. 2020; Puccia and Travassos 1991). Gp43 is found intracellularly, in the cell wall and as secreted by the fungus,

both by conventional secretory pathways and within EVs (Straus et al. 1996; Vallejo et al. 2012; Puccia et al. 2017). Its primary function is to promote fungal adhesion to components of the extracellular matrix, mainly laminin and fibronectin (Vicentini et al. 1994; Mendes-Giannini et al. 2006), and appropriate adhesion to host cell, both essential processes for successful colonization and dissemination of the infection (Camacho and Nino-Vega 2017; Mendes-Giannini et al. 2000). Indeed, reduced expression of gp43 is associated with lower pathogenicity observed in vivo (Torres et al. 2013). This is accompanied by increased intracellular killing by activated macrophages resulting in low fungal burden in the lungs of infected mice (Torres et al. 2013). In addition to its adhesin functions, gp43 is a known immunomodulator. Gp43 inhibits macrophage phagocytosis, nitric oxide (NO) production, and intracellular killing of *P brasiliensis* yeast (Flavia Popi et al. 2002), maybe due to the action of P4 and P23 (gp43 epitopes) (Konno et al. 2012). Gp43 is recognized by TLR2 and TLR4 in human polymorphonuclear neutrophils inducing the production of IL-17A and prostaglandin E2 (PGE2), respectively (Gardizani et al. 2019). In human monocytes, gp43 interacts with TLR2, TLR4, and mannose receptors (MR), for the production of IL-10, while TNF-α production was restricted to TLR4 and MR activation (Nakaira-Takahagi et al. 2011). Importantly, due to its high expression in fungal cells and its immunodominant characteristics, vaccines using P10 (gp43 epitope) are being tested (Munoz et al. 2014; Marques et al. 2008; Braga et al. 2009).

We have noted some of the known and most studied virulence factors of some fungi, which are often found associated with EVs. In addition, many other molecules are present within EVs, such as proteins, lipids, nucleic acids, and carbohydrates (Yanez-Mo et al. 2015; van Niel et al. 2018). The highly variable constitution of the EVs suggests that this fungal component can interact with various host cells, generating diverse events. Fungal EVs are important to cell-to-cell communication with other fungal cells or host cells. They are capable of transferring virulence-associated molecules or sending a message for a morphological change; they can also interact with host immune cells, and depending on the fungus, aid in the spread or containing of infection (Rizzo et al. 2020a). Below we discuss the state of the art regarding EVs in human pathogenic fungi and their relationship with the host immune system, particularly innate immunity.

2 Interactions of EVS with Innate Immunity

2.1 Aspergillus *spp.*

Aspergillus spp. are ubiquitous saprophytic mold (Kanj et al. 2018). Naturally inhaled *Aspergillus* conidia are cleared by the innate system, in cooperation of epithelial barrier and phagocytes, mainly alveolar macrophages and neutrophils (Latge and Chamilos 2019). Although immunocompetent individuals rarely develop invasive aspergillosis, immunocompromised, and patients with underlying lung pathology develop a spectrum of diseases, from allergy to invasive infection (Kanj

et al. 2018; Muldoon et al. 2017). *Aspergillus fumigatus* and *A. flavus* are the most common causative agent of aspergillosis (Almeida et al. 2019; Pasqualotto 2009; Sugui et al. 2014). Due to the importance of macrophages and neutrophils in defense against *Aspergillus*, the response of these cells to EVs produced by *A. fumigatus* and *A. flavus* was studied. Bone marrow-derived macrophages (BMDM) stimulated with *A. flavus* EVs secreted high amounts of NO, TNF-α, IL-6, and IL-1β in a dose-dependent manner (Brauer et al. 2020). The phagocytic and fungicidal activity of EVs-stimulated macrophages was augmented since EV-treated BMDM showed similar results as the IFN-γ stimulated macrophages. The induction of a pro-inflammatory profile after EVs treatment followed a classical macrophage activation (M1). Indeed, upon *A. flavus* EVs stimulation, BMDM displayed a 62-fold increase in iNOS mRNA, compared to unstimulated cells (Brauer et al. 2020). The mRNA levels of alternatively activated macrophages (M2) polarization markers remained unchanged. Additionally, *A. flavus* EVs conferred protection against the infectious challenge in an invertebrate model of infection. *Galleria mellonella* larvae treated with *A. flavus* EVs exhibit a dose-dependent reduction in fungal burden, as shown by reduction in colony forming units (CFU), and enhanced survival (Brauer et al. 2020). The protection observed is attributed to "primary immunization" or "primitive immunity": a short period of an immune boost after an immune challenge. This boost is characterized by higher counts of hemocytes and increased production of antimicrobial peptides (Pereira et al. 2018), which may have helped to lower *A. flavus* burden in treated larvae. Thus, far EV-dependent immune boost has not been tested in mammalian models. Similar to *A. flavus* EVs, *A. fumigatus* EVs induce a strong inflammatory response in phagocytes. In vitro stimulation of RAW 264.7 macrophages with *A. fumigatus* EVs lead to increased production of pro-inflammatory mediators such as TNF-α and CCL2, however, no difference in INF-γ or IL-10 was detected (Souza et al. 2019). Pretreatment of RAW 264.7 macrophages with EVs and latter fungal challenge, induce the same cytokine and chemokine response. Moreover, a higher phagocytic index and higher intracellular killing of *A. fumigatus* conidia were observed in EVs treated macrophages compared to non-treated cells. It is interesting to note that low amounts of *A. fumigatus* EVs (0,1 or 0,2μg of total EVs protein) protected the conidia against intracellular killing, suggesting that through some unknown mechanism that low quantities of EVs may inhibit fungal clearance. Bone marrow-derived neutrophils (BMDN) stimulated with EVs elicited also increased phagocytosis and intracellular killing of *A. fumigatus* conidia (Souza et al. 2019). Antigenic proteins from *A. fumigatus* EVs are recognized by sera obtained from infected mice (Souza et al. 2019). Both studies have shown a host protective role for *A. fumigatus* and *A. flavus* EVs. A strong inflammatory response is elicited either by macrophages or neutrophils stimulated with these fungal EVs, with increased phagocytic and intracellular killing capabilities. A robust response induced by these innate leukocytes, with rapid conidia elimination, is key for fungal clearance and homeostasis maintenance (Hohl 2017).

2.2 Candida *spp.*

C. albicans is the most common human commensal fungi (Witherden et al. 2017). Colonization occurs primarily in the gastrointestinal tract and the oral and vaginal mucosa of healthy individuals (Schulze and Sonnenborn 2009). Immunosuppressed individuals are at high risk of invasive candidiasis, which can manifest as diverse conditions ranging from mucosal infections to disseminated candidiasis (Nobile and Johnson 2015; Kim and Sudbery 2011). As a result of the presence of major virulence factors in *C. albicans* EVs (Konecna et al. 2019; Vargas et al. 2015; Wolf et al. 2015; Gil-Bona et al. 2015), the immunomodulatory effects of those EVs over innate cells were striking. Upon *C. albicans* EVs stimulation, BMDM produced NO, IL-12p40, TNF-α, and IL-10; minor production of TGF-β was also detected (Vargas et al. 2015). RAW 264.7 macrophages secreted high amounts of IL-12p40 while a minor increase, although significant, of TGF-β and IL-10 was also detected (Vargas et al. 2015). For BMDC, EVs treatment caused an increase in the expression of IL-6, IL-12p40, TNF-α, IL-10, and TGF-β (Vargas et al. 2015, 2020; Zamith-Miranda et al. 2020). EVs from *C. auris* were able to stimulate IL-6 secretion from BMDC while reducing the production of TGF-β when compared to untreated cells (Zamith-Miranda et al. 2020). Cytokine production coincides with phagocytic cell activation and maturation illustrated by a higher expression of MHC-II and co-stimulatory molecules, such as CD80 and CD86, in BMDC upon stimulation by *C. albicans* or *C. auris* EVs (Vargas et al. 2015; Zamith-Miranda et al. 2020). Although clear phagocyte activation by EVs from *C. albicans* or *C. auris* was observed, there is no difference in phagocytosis of EVs treated or not macrophages (Zamith-Miranda et al. 2020). In contrast, the intracellular killing of *C. albicans* was augmented when macrophages were pretreated with EVs from the same fungi, while *C. auris* EVs treated macrophages show a trend in killing inhibition (Zamith-Miranda et al. 2020). Nevertheless, BMDC activation showed that these EVs could not only activate the innate arm of the immune system but also, potentiate the link with the adaptive immune system. Indeed, antibodies in the sera of infected mice or patients with candidiasis recognized proteins from EVs (Vargas et al. 2015; Gil-Bona et al. 2015). The property of *C. albicans* EVs to induce an adaptive immune response was demonstrated in a vaccination model, in which *C. albicans* EVs immunization showed a protective effect against *C. albicans* infection in immunosuppressed mice. Following an immunization protocol, the inoculation of *C. albicans* EVs alone leads to an increase in total IgG after 21 days, with the presence of reactive antibodies against *C. albicans* protein extract and EVs (Vargas et al. 2020). CFU numbers in tested organs (kidney, spleen, and liver), were reduced in *C. albicans* EVs immunized animals, but the immunization with EVs plus adjuvant induced a greater reduction. Both immunization protocols (single *C. albicans* EVs or EVs plus adjuvant) induced protection against disseminated candidiasis. Treated mice survived until the end of the experiment, while mice immunized with PBS or adjuvant alone died 15 days after infection. Reduction in CFU counts and increased survival rates in *C. albicans* EVs immunized mice was

accompanied by increased production of IL-12p70 and TNF-α; surprisingly increased production of anti-inflammatory cytokines such as IL-10, TGF-β, and IL-4 was also observed in these mice (Vargas et al. 2020). In a similar manner, a protective effect against a lethal dose of *C. albicans* was induced in *G. mellonella* larvae by pretreatment with *C. albicans* EVs (Vargas et al. 2015, 2020).

Some degree of cellular activation mediated by *C. albicans* EVs may be related to their lipid content. The lack of phosphatidylserine synthase (CHO1) gene, which is responsible for de novo phosphatidylserine (PS) synthesis in EVs from *C. albicans,* abolished its capacity to evoke NF-κB activation in BMDM or J774.14 macrophages (Wolf et al. 2015). Critically, although this gene deletion predicted that only the lipid production would be affected in *C. albicans*, the protein cargo of secreted EVs was different. Some of the virulence factors present in WT EVs were lacking in CHO1-deleted strain's EV, which could partially explain the inability for NF-κB activation (Wolf et al. 2015). It remains unknown how alterations in lipid composition affect the loading and cargo of EVs.

The presence of transmembrane protein high affinity glucose transporter 1 (Hgt1p), involved in glucose metabolism, in the membrane of *C. albicans* EVs is a mechanism by which the fungi may evade the complement activation (Kenno et al. 2018). Hgt1p binds to factor H (FH), which is a modulator for C3b/iC3b deposition, preventing both the fungi and its EVs were destroyed by complement activation (Kopp et al. 2012). Indeed, treatment of fungal cells with anti-Hgt1p antibodies inhibited FH deposition, leading to normal C3b/iC3b deposition and consequently, phagocytosis and killing by neutrophils (Kenno et al. 2018).

2.3 Cryptococcus *Pathogenic Species Complex*

Several species of *Cryptococcus* spp. can cause deadly disease, in what is now called the *Cryptococcus* species complex. *C. neoformans* and *C. gattii* species are the principal causative agents of cryptococcosis, a life-threatening disease occurring worldwide (Maziarz and Perfect 2016). After inhalation of fungal cells, immuno-competent patients usually resolve and control the infection. However, immunosuppressed individuals, such as those suffering from AIDS, can develop the invasive form of the disease, which accounts for 15% of AIDS-associated deaths (Fisher et al. 2016; Chang et al. 2015). The dissemination pattern of these fungi within the human body and animal models shows a strong tropism for the central nervous system (CNS) (May et al. 2016; Srikanta et al. 2014). In fact, *C. neoformans* EVs have a priming function for invading fungal cells by helping them transverse the blood-brain barrier (BBB) (Huang et al. 2012). The current hypothesis is *C. neoformans* can reach the brain through either a Trojan horse mechanism in macrophages or directly cross the BBB. In the latter mechanism, EVs could interact with human brain microvascular endothelial cells (HBMECs), the first constituent of BBB, leading to membrane reorganization that promotes fungal internalization and breaching of BBB, ultimately resulting in a greater fungal burden in the brain of

infected mice (Huang et al. 2012). In a similar manner, treatment of *G. mellonella* larvae with *C. neoformans* EVs leads to uncontrolled infection, after fungal challenge, as EVs-treated larvae died sooner than the PBS control (Colombo et al. 2019).

Studies with murine macrophages have revealed some of the molecular mechanisms underpinning the biological activities of *C. neoformans* EVs. The RAW264.7 macrophage internalized EVs with 30 min of exposure, which was followed by NO, TNF-α, TGF-β, and IL-10 production (Oliveira et al. 2010). It is interesting to note that EVs from acapsular strains induced a greater production of NO and TNF-α, as its EVs lack GXM and GXMGal, potent immunosuppressive molecules (Monari et al. 2006, 2005; Decote-Ricardo et al. 2019; Retini et al. 2001; Villena et al. 2008). As predicted, the addition of exogenous GXM to the RAW264.7 macrophages diminished NO production by acapsular EVs (Oliveira et al. 2010). No phagocytic index difference was detected in RAW 264.7 macrophages pretreated with an encapsulated strain of *C. neoformans* EVs. In contrast, the pretreatment with acapsular EVs resulted in an augmented phagocytosis index by RAW 264.7 macrophages (Oliveira et al. 2010). The microbicidal activity of macrophages treated with EVs was also distinct. While macrophages treated with acapsular EVs were able to reduce fungal loads after 5 h of infection, the encapsulated EVs treated macrophages took 8 h post-infection to reach similar levels of intracellular killing (Oliveira et al. 2010). Those results show that the cargo of *C. neoformans* EVs can affect macrophage activation, which can drastically affect the elicited innate response.

Proteins extracted from EVs of *C. neoformans* were recognized by antibodies obtained from human sera during *C. neoformans* infection (Rodrigues et al. 2008). This shows that besides generating an innate response, proteins from EVs are also antigenic. The capability of eliciting an immune response is particularly interesting in vaccine development. As *C. neoformans* EVs are coated with various immunogenic proteins, their protective properties were assessed (Rizzo et al. 2020b). Immunization with EVs from both WT and *cap59Δ* strain of *C. neoformans* induced a great antibody response, measured by increased levels of IgG, against EVs proteins compared to nonimmunized mice. Despite pronounced protection observed in *cap59Δ* EVs immunization, both vaccinal strategies (*cap59Δ* or WT EVs) rendered a great survival of mice challenge with WT strain of *C. neoformans*, when compared to the control group (Rizzo et al. 2020b). These findings reinforce the potential vaccinal use of fungal EVs.

Curiously, the capability of bovine albumin to disrupt EVs which causes the release of their cargo in the medium shows that the stability of EVs in the bloodstream may be very short (<1 min) (Wolf et al. 2012). Another molecule described as capable of lysing *C. neoformans* EVs is Galectin-3 (Gal-3), which lyses EVs after 1.5 min exposure in a manner dependent on its CRD region (Almeida et al. 2017). Serum albumin is the most abundant plasma protein in humans and based on the high structural conservation of albumin between species (Majorek et al. 2012), we hypothesize that human albumin could also disrupt *C. neoformans* EVs. The presence of Gal-3 in human sera could also limit the range of action of *C. neoformans* EVs through the body. The action of these vesicle-disrupting proteins could ensure that EV cargo would be quickly released into host tissues.

EVs from more virulent strains of *C. neoformans* are able to modulate the response of less virulent strains and enhance its pathogenic characteristics. *C. neoformans* virulent VNIa-5 lineage can deliver virulence factors to a low virulence lineage, inducing a greater virulence phenotype (Hai et al. 2020). When *G. mellonella* was infected with low virulent *C. neoformans* VNIa-5 lineage cultured with EVs from a virulent lineage, an early death of the larvae was noted. This effect was shown to originate from the EVs treatment, as vesical disruption abolished the transferred virulence. Additionally, this phenomenon is mediated by the presence of proteins in EVs, as proteinase treatment or EVs boiling abrogate the observed effect (Hai et al. 2020). Consistent with the aforementioned results, experiments using EVs from an outbreak strain of *C. gattii* (R265) presented a "division of labor" model (Bielska et al. 2018), where EVs from R265, a virulent strain associated with the British Columbia outbreak, could increase the virulence of ICB180, a less-virulent strain of *C. gattii*. When *C. gattii* ICB180-infected macrophages were exposed to EVs from *C. gattii* R265 strain, a rise in fungal intracellular proliferation rate (IPR) was observed, without augmented phagocytosis. Confocal imaging and immunostaining assays demonstrated the internalization of EVs from the outbreak strain and colocalization of EVs and cryptococci within the host phagosome (Bielska et al. 2018). This interaction EV-fungi within the phagosome is likely responsible for increased yeast survival. This phenomenon was restricted to EVs from the R265 outbreak strain, as the EVs from a non-outbreak strain had no impact in IPR in macrophages infected with R265 *C. gattii* (Bielska et al. 2018). This effect is due to a thermolabile component in EVs, as heat-treated EVs lost the capacity to increase IPR. It remains to be identified which component of R265 EVs can influence macrophages in such a striking manner. In a certain way, those vesicles function as messenger's effector components delivering signals that can induced phenotype changes in plastic strains of *Cryptococcus*.

2.4 Histoplasma capsulatum

H. capsulatum is a thermally dimorphic fungus that can cause histoplasmosis. This fungus is worldwide distributed and is highly endemic in North and South America (Woods 2016). Immunocompetent hosts generate a Th1/Th17 immune response that limits the progression of the infection (Kroetz and Deepe 2012). However, a severe form of histoplasmosis might occur in immunocompromised individuals (Queiroz-Telles et al. 2017; Limper et al. 2017). An interesting study showed data that support the idea that the interaction of fungal cells with soluble mediators of the immune system can alter the characteristics and contents of released EVs from *H. capsulatum* (Matos Baltazar et al. 2016). The pretreatment of *H. capsulatum* with two monoclonal antibodies (6B7 and 7B6 mAb) (Guimaraes et al. 2009) specific to Hsp60 showed that secreted EVs were larger, with increase in total protein cargo and significantly reduced phosphatase and laccase activity compared to EVs from non-treated fungi (Matos Baltazar et al. 2016). It is worth noting that proteome

analysis identified a series of differentially expressed proteins in EVs originated from *H. capsulatum* versus those originated from *H. capsulatum* treated with 6B7 or 7B6 mAb (Matos Baltazar et al. 2016; Baltazar et al. 2018). Similarly, a discrepancy in BMDM activation was noted when antibody-exposed EVs were used. Non-treated *H. capsulatum* EVs inhibited *H. capsulatum* phagocytosis by BMDM, and EVs derived from *H. capsulatum* treated with 6B7 or 7B6 mAb induced an even strong inhibition of phagocytosis and ROS production (Baltazar et al. 2018). EVs from non-treated *H. capsulatum* or EVs from *H. capsulatum* treated with 6B7 mAb leads to a similar inhibition of intracellular killing of fungal cells by BMDM. By contrast, the use of *H. capsulatum* EVs treated with 7B6 mAb lead to greater inhibition of intracellular killing (Baltazar et al. 2018). When sera from patients with histoplasmosis were tested against proteins present in *H. capsulatum* EV extracts (Albuquerque et al. 2008), Hsp60 was recognized by human antibodies. This suggests that Hsp60 in the human response to infection could interfere with *H. capsulatum* EVs and that in turn, could differentially activate macrophage function. Further work should investigate how the in vivo generation of anti-Hsp60 antibodies might affect EV production and how it regulates the outcome of infection.

In addition to soluble mediators of an immune response, changes as the media and nutrition availability chances affect also *H. capsulatum* EV cargo (Cleare et al. 2020). Protein, lipid, and metabolic cargo were different between EVs extracted from different media, which can correlate to the vast tissue that can be colonized by the fungus. Changes in EV content could potentially induce a distinct immune response, which in turn could favor fungal growth or elimination (Cleare et al. 2020).

2.5 Malassezia *spp.*

Malassezia spp. are dimorphic fungi that colonize the human skin as commensal organisms and are typically harmless. However, these fungi can cause skin-related disorders, such as dandruff, in a large percentage of the population, (Harada et al. 2015; Findley et al. 2013). *Malassezia sympodialis* and *M. furfur* are two of the many *Malassezia* species associated with atopic eczema (AE) and dermatitis (AD) (Nowicka and Nawrot 2019; Saunders et al. 2012). The development of AE and AD are related to host skin dysregulation as well as sensitization to *Malassezia* allergens (Nowicka and Nawrot 2019). Despite the layer of keratin in the outer layers of skin, keratinocytes interact with and internalize *M. furfur* EVs, which was demonstrated in vivo and in vitro. Human keratinocytes (HaCaT cells) internalized *M. furfur* EVs (Zhang et al. 2019). After vesicular exposure, HaCaT cells upregulated IL-1β and IL-6 mRNAs, confirmed by increased secretion of IL-6 (Zhang et al. 2019). IL-6 production was also detected after in vivo treatment with *M. furfur* EVs in intact or damaged mice skin, by immunohistochemical staining, but only in its highest EVs concentration (500 ng sterol). This may be important as deregulation of IL-6 production is involved in many cutaneous disorders, like

psoriasis (Hanel et al. 2013). IL-6 production by HaCaT cells was dependent on NF-kB activation, as measured by phosphorylation of p65, an active subunit of NF-kB, or via the use of an NF-kB specific inhibitor, helenalin. Strikingly, the penetration of EVs was demonstrated in vivo, in skin injury models. A lesion in the dorsal skin of mice was created using the tape stripping method, and after 8 h of exposure to *M. furfur* EVs, penetration below the basement membrane was detectable (Zhang et al. 2019). These observations describe potential roles from *M. furfur* EVs in eliciting or exacerbating cutaneous disorders in susceptible individuals.

M. sympodialis EVs, which were named MalaEX, are coated with allergens, since IgE from AE patients reacted with antigens in MalaEX (Gehrmann et al. 2011). MalaEX may also be engulfed by human keratinocytes and monocytes (Johansson et al. 2018), and perhaps, in a manner similar to *M. furfur,* initiate or exacerbate skin disorders (Di Meglio et al. 2011). After MalaEX stimulation, keratinocytes from healthy individuals showed a sixfold increase in ICAM-1 expression when compared to controls (Vallhov et al. 2020). ICAM-1 is an integrin-binding protein expressed in different cells, such as epithelial cells, which is induced by a plethora of factors. ICAM-1 binds to lymphocyte function-associated antigen (LFA-1) β2-integrin in T cells and macrophage-1 antigen (Mac-1 or CD11b) in macrophages and neutrophils, mediating cell migration to the site of infection (Lyck and Enzmann 2015; Walling and Kim 2018). So, the observed capacity of MalaEX to enhance ICAM-1 expression by keratinocytes implies a role in the cutaneous defense against *M. sympodialis* (Vallhov et al. 2020). These EVs possess cargo, which can stimulate an inflammatory response by leukocytes of the innate immunity. Stimulation of human peripheral blood mononuclear cells (PBMC) from AE patients with MalaEX stimulated increased IL-4 and TNF-α production, while PBMC from healthy individuals showed increased levels of TNF-α (Gehrmann et al. 2011). Interestingly, EVs from monocyte-derived dendritic cells (MDDC) co-cultured with *M. sympodialis* (DCexo Mala) are coated with both fungi allergens and HLA-DR, evidencing that infected cells could produce EVs with fungal components. Additionally, DCexo Mala, induced higher production of IL-4 and TNF-α in PBMC from AE patients, and TNF-α in PBMC from healthy individuals (Gehrmann et al. 2011).

2.6 Paracoccidioides *spp.*

Paracoccidioidomycosis is an endemic disease from Latin America that is caused by the thermally dimorphic fungi *P. brasiliensis* and *P. lutzii* (Martinez 2015). The infection initiates in the lungs but can spread to other organs (Mendes et al. 2017). The generation of a Th1 immune response is an indicator of a good prognosis, while the activation of Th2 and Th9 immune responses lead to an uncontrolled inflammatory process (Rodríguez Molina and Tobón Orozco 2018). It is well established that M1 macrophages have a protective role in paracoccidioidomycosis progression, being responsible for fungal clearance and inflammatory cytokine production (Mendes et al. 2017; Leopold Wager and Wormley 2014; Benard 2008).

Interestingly, EVs from *P. brasiliensis* have been demonstrated to induce phenotype and functional M1 macrophage in peritoneal macrophages (da Silva et al. 2016). When stimulated with *P. brasiliensis* EVs, peritoneal macrophages from C57BL/6 mice showed a dose-dependent increased release of NO, TNF-α, IL-6, IL-12p40, IL-12p70, IL-1α, and IL-1β compared to non-treated cells (da Silva et al. 2016). A similar pattern of response was obtained with J774 macrophage-like cells stimulated with *P. brasiliensis* EVs, as TNF-α, IL-6, and IL-12p40 levels were significantly increased compared to non-treated cells. The development of M1 macrophages was confirmed at the transcript level: *iNOS* was increased 5800-fold, while the expression of genes associated with M2 macrophages, such as *Arg-1*, *Ym-1*, and *Fizz-1*, was unchanged (da Silva et al. 2016). Furthermore, M2 macrophages could switch to M1 phenotype upon EV stimulation. *P. brasiliensis* EV-stimulated macrophages also showed enhanced fungicidal activity, using *P. brasiliensis* yeast assay (da Silva et al. 2016). In vivo generation of specific antibodies against compounds of *P. brasiliensis* EV was confirmed using sera from paracoccidioidomycosis patients tested with EV antigens (Vallejo et al. 2011). All these data depict the interaction between the immune system and *P. brasiliensis* EVs, showing that EVs can prime macrophages to combat the fungal infection as well as be recognized by specific antibodies.

2.7 Sporothrix

Sporotrichosis, caused by *Sporothrix spp.*, is an endemic mycosis of tropical and subtropical areas (Chakrabarti et al. 2015). *Sporothrix brasiliensis* is the main etiologic agent of sporotrichosis in Brazil, but *Sporothrix* spp. is distributed worldwide (de Beer et al. 2016; Gremiao et al. 2017). Contact of susceptible individuals with the fungus, primarily immunocompromised ones, may result in sporotrichosis, which like the other fungal diseases discussed here may generate different clinical manifestations from local and cutaneous to the colonization of multiple organs (Chakrabarti et al. 2015; Moreira et al. 2015). Unlike EVs from other fungal pathogens, those from *S. brasiliensis* were not very immunomodulatory and did not induce a direct response by BMDC, as shown by the lack of cytokine production (IL-1β, IL-4, IL-6, IL-10, IL-12 p70, IL-17, IL-12p40, TNF-α or IFN-γ). Only when EVs treated BMDC were exposed to *S. brasiliensis* yeast production of IL-12p40, TNF-α, and IFN-γ production was noted (Ikeda et al. 2018). Moreover, after *S. brasiliensis* EV stimulation, BMDC showed an increased phagocytic index followed by a rise in fungal burden within the cells. In vivo assay revealed news clues on the non-protective role of *S. brasiliensis* EVs. Mice that received *S. brasiliensis* EVs before *S. brasiliensis* infection, displayed a larger average lesion diameter after 21 days of infection that began to regress by the 28th day and was fully resolved by the 42nd day of infection. It is important to note that observed lesions in EVs-treated groups showed higher size in all time points of the experiment and this was accompanied by higher burden of fungal recovered at 21st and 35th day

after infection in mice (Ikeda et al. 2018). Although the production of IL-1β and TNF-α was detected in skin lesions, no statistical difference was noted between groups administered, or not, with *S. brasiliensis* EVs, and therefore, few clues exist on the causes of the increased lesion size when mice are preexposed to EVs. Additionally, specific antibodies against *S. brasiliensis* EVs are produced during fungal infection, as indicated by the positive reaction with sera from infected mice and vesicle antigens (Ikeda et al. 2018). The present data suggest that *S. brasiliensis* EVs have a role in *S. brasiliensis* pathogenesis, favoring the establishment of the infection.

2.8 Trichophyton *spp. and Other Dermathophytes*

Dermatophytes are associated with infections of keratin-rich tissues that represent the most common superficial mycoses in humans (Brown et al. 2012; Zhan and Liu 2017). Infection by dermatophytes causes dermatophytosis or tineas, also known as athlete's foot, onychomycosis, ringworm, and jock itch (Nenoff et al. 2014). *Trichophyton interdigitale* and *T. rubrum* are established as the most important species that cause skin and nail infections (Zhan and Liu 2017). Being able to populate the skin of a human, and cause diseases, the immune and biological activities of *T. interdigitale* EVs was evaluated. When stimulated with *T. interdigitale* EVs, both BMDM and HaCaT cells show a predominant proinflammatory profile (Bitencourt et al. 2018). *T. interdigitale* EVs appear to induce M1 macrophages, characterized by the higher relative expression of iNOS mRNA compared to non-treated cells. EVs-treated macrophages also showed an increase in phagocytosis and killing of *T. interdigitale* conidia. Moreover, pro-inflammatory mediators were produced, as EVs stimulated BMDM and HaCaT cells produced increased levels of NO, TNF-α, IL-6, and IL-1β; HaCaT cells also exhibited an increased production in IL-8 (Bitencourt et al. 2018). Those pro-inflammatory effects of *T. interdigitale* EVs were modulated by TLR2 activation in BMDM. In knockout TLR2 BMDM, cytokine production and iNOS mRNA expression were abolished, lack of TLR4 lead to a similar response as WT BMDM (Bitencourt et al. 2018). Likewise described for *P. brasiliensis* EVs, *T. interdigitale* possess EVs with a great proinflammatory nature that could play in favor of the host in a real infection.

3 Recognition of EVS by the Host Immune System

There is still little knowledge regarding the recognition of EVs by Pattern Recognition Receptors (PRRs). PRRs at the surface of leukocytes can recognize fungal compounds of EVs, for example, EVs from *P. brasiliensis* and *P. lutzii* EVs. Lectin microarray assays have shown that EVs carry carbohydrates that are recognized by

DC-SIGN (Peres da Silva et al. 2015), a C-type receptor mainly expressed by DCs, but not Dectin-1 or -2, classical receptors for *P. brasiliensis* (Goyal et al. 2018; de Quaglia et al. 2019). Gal-3, a lectin which is both secreted and membrane-bound, can also disrupt EVs from *C. neoformans (*Almeida et al. 2017) and *P. brasiliensis* EVs, limiting the area of diffusion and reach of the pathogenic EVs (Hatanaka et al. 2019). Interestingly, Gal-3 at the membrane surface may mediate uptake as wild-type peritoneal macrophages were three times more effective in EVs disruption than Gal-$3^{-/-}$ macrophages, and lack of Gal-3 even hampers the uptake of *P. brasiliensis* EVs by peritoneal macrophages (Hatanaka et al. 2019).

The type required EV, receptor ratio and the exact distribution of PRR recognizing EVs remain open scientific questions. Given the small size of EVs, it is also possible that they are internalized intact by host cells, including immune cells, via endocytosis, without triggering surface PRRs. It may be that PRRs are only triggered upon the action of digestive enzymes in the lumen of the lysosomal system or when EVs bypass surface PRRs, their compounds might be recognized by intracellular PRRs, in the phagosome or cytoplasm. Moreover, lysis of EVs extracellularly will release and expose cargo to the action of the immune system and subsequent immune processing to generate antibodies.

4 Fungal Components Detected in Host Immune EVs

As shown by the studies mentioned above, fungal EVs possess a wide range of invasive, immunomodulatory, and immunogenic properties. While fungal EVs have a direct effect on immune cells, those immune cells can produce and export their own EVs. Notably, EVs derived from host cells are critically involved in antigen presentation. Antigen-presenting cells (APCs) may acquire MHC and antigenic peptides from neighboring cells through EVs (Zeng and Morelli 2018), and EVs are also implicated at the immunological synapse between APCs and T lymphocytes (Choudhuri et al. 2014). These evidences show that not only pathogen EVs affect the course of a given disease, but also EVs released from host cells play a role in this process, by modulating the response in uninfected immune cells (Wang et al. 2018) and underscoring the prime role of EV in intercellular communication.

Most of the available data regarding EV and the pathogen-host interaction focus has come from bacterial studies, particularly from mycobacteria (Rodrigues et al. 2018). THP-1 macrophage-like cells infected with *Mycobacterium tuberculosis* or *M. bovis* release EVs containing various mycobacterial components such as LAM and the 19-kDa lipoprotein (Giri et al. 2010). These EVs have an effect in noninfected macrophages, inducing TNF-α production and iNOS expression (Bhatnagar et al. 2007). The action of EVs in THP-1 macrophage-like cells requires TLR2 and TLR4 recognition followed by MyD88 activation (Bhatnagar et al. 2007). Similarly, EVs derived from *M. tuberculosis* infected neutrophils trigger immune responses over macrophages, inducing higher expression of co-stimulatory molecules such as CD80 and CD86 and production of TNF-α and IL-6 (Alvarez-Jimenez

et al. 2018). Intranasal administration of EVs from THP-1 cells infected with *M. tuberculosis* or *M. bovis* to mice induced the production of IL-12p40, TNF-α, and recruitment of macrophages and neutrophils to the lungs (Bhatnagar et al. 2007; Singh et al. 2012). Likewise, BMDM treated with EVs from *M. bovis*-infected J774 cells could recruit unstimulated BMDM in in vitro-transwell systems (Singh et al. 2012). Furthermore, EVs from *M. bovis*-infected J774 cells can activate both $CD4^+$ and $CD8^+$ T cells in vivo and in vitro, as well as induction of DC maturation (Giri and Schorey 2008). It is hypothesized that EVs derived from infected macrophages could promote both innate and adaptive immune responses. Corroborating this hypothesis, it was shown that vaccination of mice with EVs derived from infected macrophages, and which contain *M. tuberculosis* proteins, induced comparable protection as the commercial vaccine BCG (Cheng and Schorey 2013). Analysis of EVs from *M. avium* infected J774 macrophages show a similar proinflammatory profile as EVs from *M. tuberculosis* or *M. bovis* infected macrophages. In that case, TNF-α and RANTES were produced, in a TLR2, TLR4, and MyD88 dependent manner, when macrophages were stimulated with EVs obtained from *M. avium* infected J774 macrophages (Bhatnagar and Schorey 2007).

To demonstrate the role of host EV in the immune communication, THP-1 macrophage like-cells and human monocytes were infected with *C. albicans* and the release of EVs was addressed. Both cells showed increased secretion of EVs. These EVs increased total protein content when compared to EVs from uninfected cells. EVs derived from THP-1 cells showed a greater size distribution while EVs derived from human monocytes had a more uniform size distribution (Reales-Calderón et al. 2017; Halder et al. 2020). EVs derived from infected macrophages showed the presence of *C. albicans* proteins. Despite the observed physical size difference between EVs, there were no detectable changes in the immunostimulatory properties of EVs obtained from *C. albicans* infected or uninfected THP-1 macrophage like cells. THP-1 cells and monocytes stimulated with both EVs showed increased TNF-α, IL-12p40, and IL-8 production with phosphorylation of ERK2 and p38 kinases (Reales-Calderón et al. 2017). Also, the fungicidal activity of both tested cells was enhanced by EV treatment. Only the TNF-α production by monocytes stimulated with EVs from infected THP-1 cells was significantly augmented. It is tempting to speculate these immunostimulatory differences will manifest in antigen-presentation assays.

C. albicans infected human monocytes secreted EVs containing CD14, indicating its monocytic origin. Interestingly, TGF-β1-transporting EVs were only produced by infected cells. The identification of EVs carrying TGF-β1 was observed in vitro, ex vivo, and in vivo (Halder et al. 2020). Interaction between *C. albicans*-surface bound β-glucans and CR3 in monocytes are required to induce the production of EVs containing TGF-β1. EVs transporting TGF-β1 binds to TGF-βRII on neighboring macrophages, monocytes, and endothelial cells and triggers a series of anti-inflammatory responses, such as inhibition of IL-6 production and activation of SMAD7 pathway, which in turn leads to impaired *IL1β* transcription. Thus, TGF-β1-transporting vesicles are favorable to the pathogens such as *C. albicans*, by promoting the infection (Halder et al. 2020; Andriantsitohaina and Papon 2020). Loading of

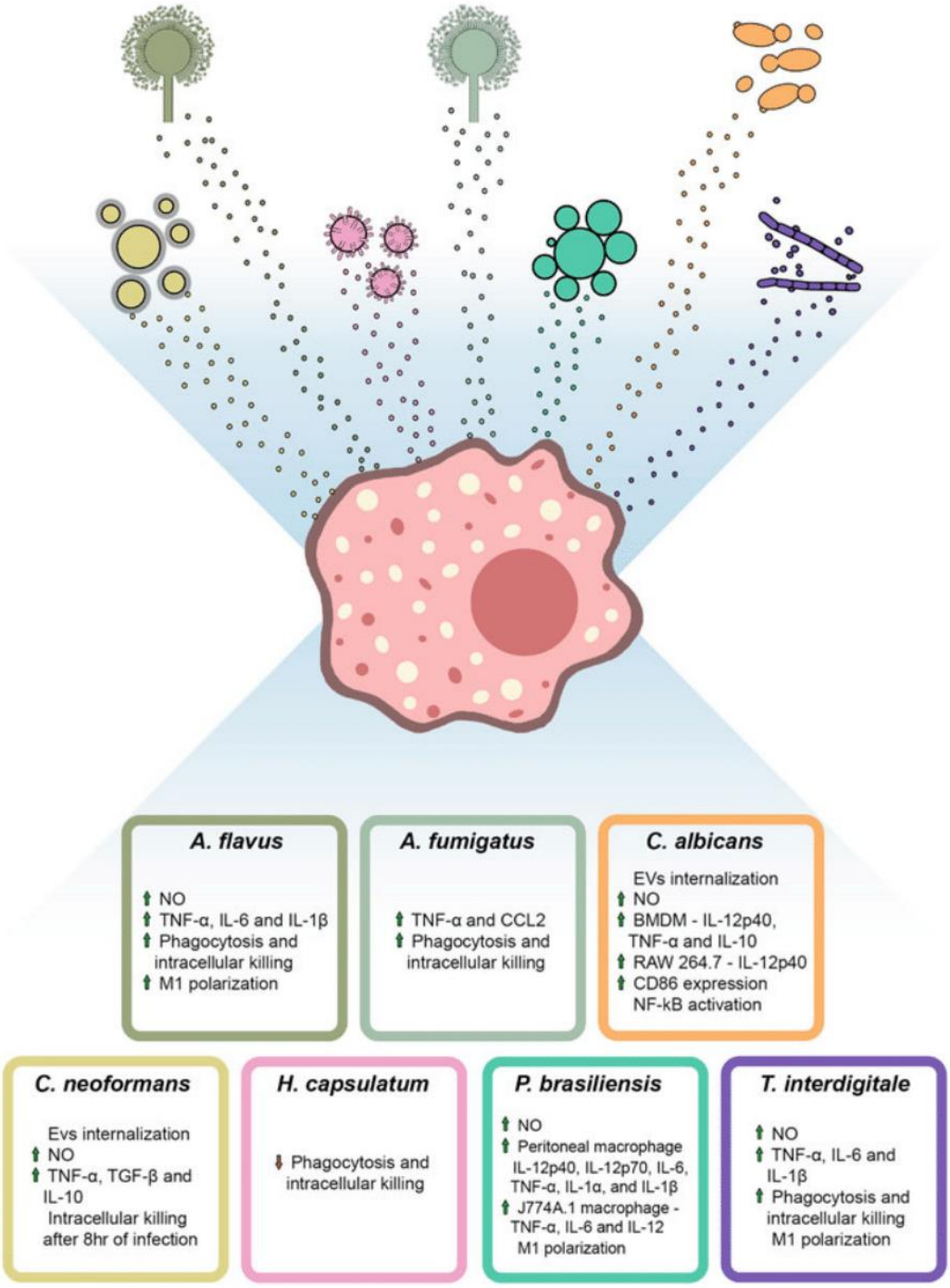

Fig. 1 Modulation of macrophage response upon fungal EVs stimuli. Fungal EVs interact with innate immune cells and promote a specific immune response that could either support the infection or benefit host defense. Refer to Table 1 to more detailed information

fungal antigens into macrophages has also been shown for J774.16 macrophages infected with *C. neoformans*. Despite no conclusive presence of GXM in EVs produced by *C. neoformans* in infected J774.16 macrophage like cells, these cells

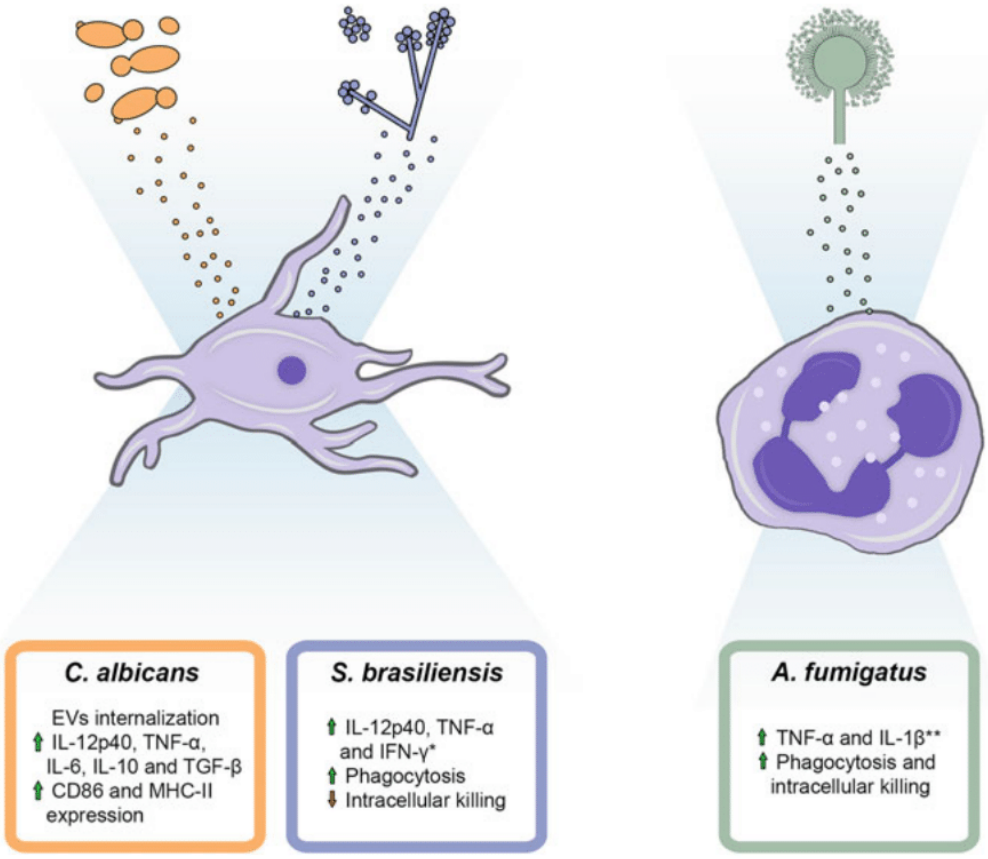

Fig. 2 Modulation of DCs (left) and Neutrophils (right) response after fungal EVs stimuli. Although less data is available when compared to macrophages, EVs can elicit an immune response from different types of immune cells. *Only when stimulated bone marrow-derived macrophage (BMDC) was co-cultured with *S. brasiliensis* yeast. **Only when stimulated bone marrow-derived neutrophil (BMDN) was co-cultured with *A. fumigatus* conidia. Refer to Table 1 to more detailed information

contain large amounts of polysaccharides (Rodrigues et al. 2007). Dendritic cells infected with *M. sympodialis* could also produce EVs containing *M. sympodialis* antigens (Gehrmann et al. 2011). EVs derived from infected DCs could significantly raise IL-4 and TNF-α production in autologous CD14, CD34 depleted PBMC from AE patients, but not in the same cell type from healthy individuals. Intriguingly EVs produced by fungal-infected leukocytes can also interfere with fungal growth, suggesting a direct antifungal effect and a role as "antifungal packages". EVs derived from *A. fumigatus*-infected neutrophils are produced in a larger quantity and are populated with antimicrobial proteins such as cathepsin G and azurocidin, as revealed by proteomics analysis (Shopova et al. 2020). EVs from infected neutrophils can physically interact with *A. fumigatus* conidia or hyphae inhibiting fungal growth by constraining hyphal extension, and this effect is, somehow, related to the presence of antimicrobial proteins in EVs (Shopova et al. 2020).

Table 1 Effects of fungal EVs over immune cells

Fungi EVs (strain)	Cell tested	Experiment performed	Observed outcome	Ref.
A. flavus (NRRL 6513)	BMDM	Cytokine production	Dose-dependent production of TNF-α, IL-6 and IL-1β	Brauer et al. (2020)
A. flavus (NRRL 6513)	BMDM	NO production	Dose-dependent production of NO	Brauer et al. (2020)
A. flavus (NRRL 6513)	BMDM	Phagocytosis and intracellular killing	Increased phagocytosis and intracellular killing of *A. flavus* conidia	Brauer et al. (2020)
A. flavus (NRRL 6513)	BMDM	Macrophage polarization	Increase in relative expression of iNOS mRNA (M1 polarization)	Brauer et al. (2020)
A. fumigatus (A1163)	RAW 264.7 macrophage and BMDN	Cytokine production	RAW 264.7 macrophages—TNF-α and CCL2 BMDN—production of TNF-α and IL-1β, only when stimulated cells were co-cultured with *A. fumigatus* conidia	Souza et al. (2019)
A. fumigatus (A1163)	RAW 264.7 macrophage and BMDN	Phagocytosis and intracellular killing	Increased phagocytosis and intracellular killing of *A. fumigatus* conidia for both cells	Souza et al. (2019)
C. albicans (11)	BMDM and BMDC	EVs internalization	Full internalization after 15 min of exposure	Vargas et al. (2015)
C. albicans (11)	BMDM	NO production	Increased production	Vargas et al. (2015)
C. albicans (11)	BMDM, BMDC and RAW 264.7 macrophage	Cytokine production	BMDM—IL-12p40, TNF-α and IL-10. Minor production of TGF-β. BMDC—IL-12p40, TNF-α, IL-6, IL-10 and TGF-β. RAW 264.7—IL-12p40. Minor production of TGF-β and IL-10	Vargas et al. (2015, 2020)

(continued)

Table 1 (continued)

Fungi EVs (strain)	Cell tested	Experiment performed	Observed outcome	Ref.
C. albicans (ATCC #90028)	RAW 264.7 and BMDM macrophage	Phagocytosis and intracellular killing	No difference in phagocytosis. Fungicidal activity in low doses of EVs	Zamith-Miranda et al. (2020)
C. albicans (11 and ATCC #90028)	BMDM and BMDC	Expression of co-stimulatory molecules	BMDM—slight increase in CD86 expression BMDC—high increase in CD80, CD86 and MHC-II expression	Vargas et al. (2015), Zamith-Miranda et al. (2020)
C. albicans (sc5314)	BMDM and J774.14 macrophage	NF-κB activation	Nuclear staining of NF-κB P65	Wolf et al. (2015)
C. albicans CHO1 KO (YLC337)	BMDM and J774.14 macrophage	NF-κB activation	Failed NF-κB activation	Wolf et al. (2015)
C. auris (MMC1 and MMC2)	RAW 264.7 and BMDM macrophage	Phagocytosis and intracellular killing	No difference in phagocytosis. Impaired killing in MMC2 EVs treated BMDM	Zamith-Miranda et al. (2020)
C. auris (MMC1 and MMC2)	BMDC	Cytokine production	IL-6. Decreased basal production of TGF-β in MMC1 EVs treated BMDC	Zamith-Miranda et al. (2020)
C. auris (MMC1 and MMC2)	BMDC	Expression of co-stimulatory molecules	Increased expression of CD80, CD86, and MHC-II	Zamith-Miranda et al. (2020)
C. neoformans	RAW 264.7 macrophage	EVs internalization	Internalization after 30 min of exposure	Oliveira et al. (2010)
C. neoformans (HEC3393 and B3501)	RAW 264.7 macrophage	NO production	Dose-dependent production	Oliveira et al. (2010)
C. neoformans (Cap 67)	RAW 264.7 macrophage	NO production	Dose-dependent production, higher than WT EVs	Oliveira et al. (2010)
C. neoformans (HEC3393)	RAW 264.7 macrophage	Cytokine production	TNF-α, TGF-β, and IL-10	Oliveira et al. (2010)
C. neoformans (Cap 67)	RAW 264.7 macrophage	Cytokine production	TNF-α, lower production of TGF-β and IL-10, when compared to WT EVs	Oliveira et al. (2010)

(continued)

Table 1 (continued)

Fungi EVs (strain)	Cell tested	Experiment performed	Observed outcome	Ref.
C. neoformans (HEC3393)	RAW 264.7 macrophage	Phagocytosis and intracellular killing	No difference in phagocytosis. Fungicidal activity after 8 h of infection	Oliveira et al. (2010)
C. neoformans (Cap 67)	RAW 264.7 macrophage	Phagocytosis and intracellular killing	Increased phagocytic index. Fungicidal activity after 5 h of infection	Oliveira et al. (2010)
H. capsulatum (G217B)	BMDM	Phagocytosis and intracellular killing	Reduced phagocytosis and intracellular killing of *H. capsulatum*	Baltazar et al. (2018)
H. capsulatum (G217B) + 6B7 mAb or 7B6 mAb	BMDM	Phagocytosis and intracellular killing	Greater inhibition of phagocytosis and intracellular killing of *H. capsulatum*, when compared with control EVs	Baltazar et al. (2018)
H. Capsulatum (G217B) + 6B7 mAb	BMDM	ROS production	Slight reduction when compared to control EVs	Baltazar et al. (2018)
H. Capsulatum (G217B) + 7B6 mAb	BMDM	ROS production	Greater reduction when compared to control EVs	Baltazar et al. (2018)
M. Furfur (14521)	HaCaT	Cytokine production	IL-6 production, NF-kB dependent	Zhang et al. (2019)
M. Furfur (14521)	Mice epidermal keratinocytes	Immunohistochemistry	IL-6 production, in intact or damaged skin	Zhang et al. (2019)
M. Sympodialis (42132)	Human keratinocytes and monocytes	EVs internalization	Active internalization after 16 h for keratinocytes and 2 h for monocytes	Johansson et al. (2018)
M. Sympodialis (42132)	PBMC from HC donors	Cytokine production	Dose-dependent production of TNF-α, mild production of IL-4 when compared to AE PBMC	Gehrmann et al. (2011)
M. Sympodialis (42132)	PBMC from AE patients	Cytokine production	Dose-dependent production of TNF-α and IL-4	Gehrmann et al. (2011)
M. Sympodialis (42132)	Human keratinocytes	ICAM-1 expression	6-fold increase in ICAM-1 expression	Vallhov et al. (2020)

(continued)

Table 1 (continued)

Fungi EVs (strain)	Cell tested	Experiment performed	Observed outcome	Ref.
P. brasiliensis (Pb18)	J774A.1 and murine peritoneal macrophage	Cytokine production	J774A.1—dose-dependent production of TNF-α, IL-6, and IL-12. Peritoneal macrophage—dose-dependent production of IL-12p40, IL-12p70, IL-6, TNF-α, IL-1α, and IL-1β	da Silva et al. (2016)
P. brasiliensis (Pb18)	Murine peritoneal macrophage	NO production	Dose-dependent production	da Silva et al. (2016)
P. brasiliensis (Pb18)	Murine peritoneal macrophage	Macrophage polarization	Increase in the relative expression of iNOS mRNA (M1 polarization)	da Silva et al. (2016)
P. brasiliensis (Pb18)	Murine peritoneal macrophage	Macrophage repolarization	Induction of classical M1 markers in previously M2 macrophages	da Silva et al. (2016)
S. brasiliensis (5110)	BMDC	Cytokine production	Production of IL-12p40, TNF-α and IFN-γ, only when stimulated BMDC were co-cultured with *S. brasiliensis* yeast	Ikeda et al. (2018)
S. brasiliensis (5110)	BMDC	Phagocytosis and intracellular killing	Increased phagocytosis and diminished intracellular killing of *S. brasiliensis* yeast	Ikeda et al. (2018)
T. Interdigitale (MYA3108)	BMDM and HaCaT	Cytokine production	BMDM—TNF-α, IL-6, and IL-1β. HaCaT cells—TNF-α, IL-6, IL-1β, and IL-8	Bitencourt et al. (2018)
T. Interdigitale (MYA3108)	BMDM and HaCaT	NO production	Dose-dependent production for both cells	Bitencourt et al. (2018)
T. Interdigitale (MYA3108)	BMDM	Macrophage polarization	Increase in the relative expression of iNOS mRNA (M1 polarization)	Bitencourt et al. (2018)
T. Interdigitale (MYA3108)	BMDM	Phagocytosis and intracellular killing	Significant rise in phagocytosis and	

(continued)

Table 1 (continued)

Fungi EVs (strain)	Cell tested	Experiment performed	Observed outcome	Ref.
			intracellular killing of *T. interdigitale* conidia	Bitencourt et al. (2018)

BMDM bone marrow derived macrophages, *NO* nitric oxide, *BMDN* bone marrow derived neutrophils, *BMDC* bone marrow derived dendritic cells, *NF-κB* nuclear factor kappa B, *CHO1 KO* phosphatidylserine synthase knockout strain of *C. albicans*, *6B7* IgG1 Protective antibody against H. capsulatum heat shock protein 60, *7B6* IgG2b nonprotective antibody against H. capsulatum heat shock protein 60, *ROS* reactive oxygen species, *HaCaT* human keratinocyte cell line, *PBMC* peripheral blood mononuclear cell, *HC* healthy controls; *AE* atopic eczema

Table 2 Recognition of fungi EVs by immune sera

Fungi EVs (strain)	Serum tested	Identified molecules	Ref.
A. fumigatus (A1163)	Infected mice	37–150 kDa proteins	Souza et al. (2019)
C. albicans (SC5314)	Patients with candidiasis	High molecular mass proteins	Gil-Bona et al. (2015)
C. albicans (11)	Infected mice	27 and 37 kDa proteins	Vargas et al. (2015)
C. neoformans (H99)	Patients with cryptococcosis	Seven major bands corresponding to 19, 27, 38, 48, 67, 101 and 131 kDa	Rodrigues et al. (2008)
H. capsulatum (G217B)	Patients with histoplasmosis	Various positive proteins. Positive reaction for histone 2B and Hsp60	Albuquerque et al. (2008)
M. Sympodialis (42132)	Patients with atopic eczema	70 kDa allergen[a]	Gehrmann et al. (2011)
P. brasiliensis (Pb18)	Patients with paracoccidioidomycosis	49, 64 and 75 kDa proteins. Faints bands observed in ~82 kDa range	Vallejo et al. (2011)
P. brasiliensis (Pb3)	Patients with paracoccidioidomycosis	47, 49, 64 and 75 kDa proteins and high molecular mass band	Vallejo et al. (2011)
S. brasiliensis (5110)	Infected mice	45, 50 and 100 kDa proteins	Ikeda et al. (2018)

[a]Rabbit anti-human IgE was used as secondary antibody

Table 3 *Galleria mellonella* protection model

Fungi EVs (strain)	Observed outcome	Ref.
A. flavus (NRRL 6513)	Dose-dependent reduction in CFU and enhanced survival of the larva	Brauer et al. (2020)
C. albicans (11)	Low fungal burden in EVs treated larvae, no difference in survival	Vargas et al. (2015)
C. neoformans (H99)	Early death of treated larvae after challenge	Colombo et al. (2019)

CFU colony forming units

Table 4 Other reported immune interactions of fungi EVs

Fungi EVs (strain)	Cell tested	Type of experiment	Observed outcome	Ref.
C. albicans (11)	Mice immunization	Antibody production	Increased total IgG concentration, production of specific IgG against fungal proteins and EVs	Vargas et al. (2020)
C. albicans (11)	Mice immunization	Fungal burden	Decreased CFU in kidney, spleen, and liver of EVs immunized mice	Vargas et al. (2020)
C. albicans (11)	Mice immunization	Cytokine profile	Increased production of IL-12p70, TNF-α, IL-10, TGF-β, and IL-4 in the spleen of EVs immunized mice	Vargas et al. (2020)
C. albicans (11)	Mice immunization	Mice survival	Full protection of EVs immunized mice after lethal infection with *C. albicans*	Vargas et al. (2020)
C. albicans (SN152)	EVs	Presence of Hgt1p	Presence of Hgt1p in EVs membrane, that binds to FH, which could protect EVs destruction by complement alternative pathway	Kenno et al. (2018)
C. neoformans (H99)	EVs	Vesicle lysis	Lysis in the presence of 300 mM BSA or mouse serum	Wolf et al. (2012)
C. neoformans (H99)	EVs	Vesicle lysis	Lysis in the presence of 0,1;1;10μg/ml Gal-3	Almeida et al. (2017)
C. neoformans (KN99α and *cap59Δ*)	Mice immunization	Antibody production	Production of specific IgG antibodies against EVs components	Rizzo et al. (2020b)
C. neoformans (KN99α and *cap59Δ*)	Mice immunization	Mice survival	Increased mice survival with both EVs immunization, pronounced survival in *cap59Δ* EVs immunized mice	Rizzo et al. (2020b)
H. capsulatum (G217B)	Fungus	Treatment with monoclonal antibodies (6B7 or 7B6)	Increased vesicle size and protein load. Reduced EVs phosphatase, laccase, and catalase activities. Differentially expressed protein profile	Matos Baltazar et al. (2016)
P. brasiliensis (Pb18 and Pb3)	EVs	Lectin microarray	Detection of surface carbohydrate ligand of DC-SIGN	Peres da Silva et al. (2015)
P. brasiliensis (Pb18)	EVs	Vesicle lysis	Lysis in the presence of 1 and 10μg/ml Gal-3	Hatanaka et al. (2019)

(continued)

Table 4 (continued)

Fungi EVs (strain)	Cell tested	Type of experiment	Observed outcome	Ref.
P. lutzii (Pb01)	EVs	Lectin microarray	Detection of surface carbohydrate ligand of DC-SIGN	Peres da Silva et al. (2015)
S. brasiliensis (5110)	Mice skin	Fungal load	Increased fungal load in EVs treated mice with *S. brasiliensis* infection	Ikeda et al. (2018)
S. brasiliensis (5110)	Mice skin	Skin lesion	Increased skin lesion in EVs treated mice with *S. brasiliensis* infection	Ikeda et al. (2018)
S. brasiliensis (5110)	Mice skin	Cytokine production	IL-1β and TNF-α production, no difference between EVs treated, or not, groups in mice with *S. brasiliensis* infection	Ikeda et al. (2018)

CFU colony forming units, *Hgt1p* high affinity glucose transporter 1, *FH* factor H, *BSA* bovine serum albumin, *Gal-3* galectin-3, *6B7* IgG1 protective antibody against H. capsulatum heat shock protein 60, *7B6* IgG2b nonprotective antibody against H. capsulatum heat shock protein 60, *DC-SIGN* dendritic cell-specific ICAM-grabbing non-integrin

5 Conclusion

Taken together, the data regarding the immunomodulatory properties of fungal EVs and data from EVs derived from infected leukocytes, show a two-way path, in which both EVs could coexist and play an important role in fungal infections and disease progression or protection. Overall, EVs produced by fungi can be recognized by host cells triggering activation of immune responses (Figs. 1 and 2). This response is both fungal-dependent and EV-composition-dependent. The information provided by current studies is compiled in Tables 1, 2, 3, and 4. We aim here to synthesize and provide a reference for the studies performed on this important topic. Table 1 resumes the effects of the interaction between various fungal EVs with innate leukocytes. Table 2 depicts that immune sera recognize EV antigens. Table 3 shows the protection conferred to *G. mellonella* treated with EVs. Table 4 shows other interactions between fungi EV and components of the immune system.

Overall, there is already sufficient data to support a detailed investigation of the vaccine potential from EV preparations, particularly if EVs contain the virulence cargo. Albeit several studies have tested the protective potential of EVs, the protection conferred to animals was dependent on the fungal species. Given the heterogeneity and variability in EV composition, these studies merely underscore the need to understand which EVs and which cargo may mediate protection, as well as to the need to understand the mechanisms of cargo loading and biogenesis of EVs.

References

Albuquerque PC, Nakayasu ES, Rodrigues ML, Frases S, Casadevall A, Zancope-Oliveira RM et al (2008) Vesicular transport in Histoplasma capsulatum: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. Cell Microbiol 10(8):1695–1710

Almeida F, Wolf JM, da Silva TA, DeLeon-Rodriguez CM, Rezende CP, Pessoni AM et al (2017) Galectin-3 impacts Cryptococcus neoformans infection through direct antifungal effects. Nat Commun 8(1):1968

Almeida F, Rodrigues ML, Coelho C (2019) The still underestimated problem of fungal diseases worldwide. Front Microbiol 10:214

Alvarez-Jimenez VD, Leyva-Paredes K, Garcia-Martinez M, Vazquez-Flores L, Garcia-Paredes VG, Campillo-Navarro M et al (2018) Extracellular vesicles released from Mycobacterium tuberculosis-infected neutrophils promote macrophage autophagy and decrease intracellular mycobacterial survival. Front Immunol 9:272

Andriantsitohaina R, Papon N (2020) Extracellular vesicles: new bullets to fight fungal infections. Trends Cell Biol 30(8):589–590

Baltazar LM, Zamith-Miranda D, Burnet MC, Choi H, Nimrichter L, Nakayasu ES et al (2018) Concentration-dependent protein loading of extracellular vesicles released by Histoplasma capsulatum after antibody treatment and its modulatory action upon macrophages. Sci Rep 8 (1):8065

Barbosa FM, Fonseca FL, Figueiredo RT, Bozza MT, Casadevall A, Nimrichter L et al (2007) Binding of glucuronoxylomannan to the CD14 receptor in human A549 alveolar cells induces interleukin-8 production. Clin Vaccine Immunol 14(1):94–98

Benard G (2008) An overview of the immunopathology of human paracoccidioidomycosis. Mycopathologia 165(4–5):209–221

Beyhan S, Sil A (2019) Sensing the heat and the host: virulence determinants of Histoplasma capsulatum. Virulence 10(1):793–800

Bhatnagar S, Schorey JS (2007) Exosomes released from infected macrophages contain Mycobacterium avium glycopeptidolipids and are proinflammatory. J Biol Chem 282(35):25779–25789

Bhatnagar S, Shinagawa K, Castellino FJ, Schorey JS (2007) Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo. Blood 110(9):3234–3244

Bielska E, May RC (2019) Extracellular vesicles of human pathogenic fungi. Curr Opin Microbiol 52:90–99

Bielska E, Sisquella MA, Aldeieg M, Birch C, O'Donoghue EJ, May RC (2018) Pathogen-derived extracellular vesicles mediate virulence in the fatal human pathogen Cryptococcus gattii. Nat Commun 9(1):1556

Bitencourt TA, Rezende CP, Quaresemin NR, Moreno P, Hatanaka O, Rossi A et al (2018) Extracellular vesicles from the dermatophyte trichophyton interdigitale modulate macrophage and keratinocyte functions. Front Immunol 9:2343

Boral H, Metin B, Dogen A, Seyedmousavi S, Ilkit M (2018) Overview of selected virulence attributes in aspergillus fumigatus, Candida albicans, Cryptococcus neoformans, Trichophyton rubrum, and Exophiala dermatitidis. Fungal Genet Biol 111:92–107

Braga CJ, Rittner GM, Munoz Henao JE, Teixeira AF, Massis LM, Sbrogio-Almeida ME et al (2009) Paracoccidioides brasiliensis vaccine formulations based on the gp43-derived P10 sequence and the Salmonella enterica FliC flagellin. Infect Immun 77(4):1700–1707

Brauer VS, Pessoni AM, Bitencourt TA, de Paula RG, de Oliveira Rocha L, Goldman GH et al (2020) Extracellular vesicles from aspergillus flavus induce M1 polarization in vitro. mSphere 5 (3):e00190-20

Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC (2012) Hidden killers: human fungal infections. Sci Transl Med 4(165):165rv13

Brunke S, Mogavero S, Kasper L, Hube B (2016) Virulence factors in fungal pathogens of man. Curr Opin Microbiol 32:89–95

Camacho E, Nino-Vega GA (2017) Paracoccidioides spp.: virulence factors and immune-evasion strategies. Mediat Inflamm 2017:5313691

Casadevall A, Coelho C, Cordero RJB, Dragotakes Q, Jung E, Vij R et al (2019) The capsule of Cryptococcus neoformans. Virulence 10(1):822–831

Cassone A, Casadevall A (2012) Recent progress in vaccines against fungal diseases. Curr Opin Microbiol 15(4):427–433

Chakrabarti A, Bonifaz A, Gutierrez-Galhardo MC, Mochizuki T, Li S (2015) Global epidemiology of sporotrichosis. Med Mycol 53(1):3–14

Chang CC, Sorrell TC, Chen SC (2015) Pulmonary cryptococcosis. Semin Respir Crit Care Med 36 (5):681–691

Cheng Y, Schorey JS (2013) Exosomes carrying mycobacterial antigens can protect mice against Mycobacterium tuberculosis infection. Eur J Immunol 43(12):3279–3290

Choudhuri K, Llodra J, Roth EW, Tsai J, Gordo S, Wucherpfennig KW et al (2014) Polarized release of T-cell-receptor-enriched microvesicles at the immunological synapse. Nature 507 (7490):118–123

Cleare LG, Zamith D, Heyman HM, Couvillion SP, Nimrichter L, Rodrigues ML et al (2020) Media matters! Alterations in the loading and release of Histoplasma capsulatum extracellular vesicles in response to different nutritional milieus. Cell Microbiol 22(9):e13217

Colombo AC, Rella A, Normile T, Joffe LS, Tavares PM, de SAGR et al (2019) Cryptococcus neoformans glucuronoxylomannan and sterylglucoside are required for host protection in an animal vaccination model. MBio 10(2):e02909-18

da Silva TA, Roque-Barreira MC, Casadevall A, Almeida F (2016) Extracellular vesicles from Paracoccidioides brasiliensis induced M1 polarization in vitro. Sci Rep 6:35867

de Beer ZW, Duong TA, Wingfield MJ (2016) The divorce of Sporothrix and Ophiostoma: solution to a problematic relationship. Stud Mycol 83:165–191

de Quaglia ESJC, Della Coletta AM, Gardizani TP, Romagnoli GG, Kaneno R, Dias-Melicio LA (2019) Involvement of the dectin-1 receptor upon the effector mechanisms of human phagocytic cells against Paracoccidioides brasiliensis. J Immunol Res 2019:1529189

Decote-Ricardo D, LaRocque-de-Freitas IF, Rocha JDB, Nascimento DO, Nunes MP, Morrot A et al (2019) Immunomodulatory role of capsular polysaccharides constituents of Cryptococcus neoformans. Front Med (Lausanne) 6:129

Di Meglio P, Perera GK, Nestle FO (2011) The multitasking organ: recent insights into skin immune function. Immunity 35(6):857–869

Ehlers MR (2000) CR3: a general purpose adhesion-recognition receptor essential for innate immunity. Microbes Infect 2(3):289–294

Eisenman HC, Frases S, Nicola AM, Rodrigues ML, Casadevall A (2009) Vesicle-associated melanization in Cryptococcus neoformans. Microbiology 155(Pt 12):3860–3867

Esher SK, Zaragoza O, Alspaugh JA (2018) Cryptococcal pathogenic mechanisms: a dangerous trip from the environment to the brain. Mem Inst Oswaldo Cruz 113(7):e180057

Findley K, Oh J, Yang J, Conlan S, Deming C, Meyer JA et al (2013) Topographic diversity of fungal and bacterial communities in human skin. Nature 498(7454):367–370

Fisher JF, Valencia-Rey PA, Davis WB (2016) Pulmonary Cryptococcosis in the immunocompetent patient-many questions, some answers. Open Forum Infect Dis 3(3):ofw167

Flavia Popi AF, Lopes JD, Mariano M (2002) GP43 from Paracoccidioides brasiliensis inhibits macrophage functions. An evasion mechanism of the fungus. Cell Immunol 218(1–2):87–94

Freitas MS, Bonato VLD, Pessoni AM, Rodrigues ML, Casadevall A, Almeida F (2019) Fungal extracellular vesicles as potential targets for immune interventions. mSphere 4(6):e00747-19

Gardizani TP, Della Coletta AM, Romagnoli GG, Puccia R, Serezani APM, de Campos Soares AMV et al (2019) 43 kDa glycoprotein (gp43) from Paracoccidioides brasiliensis induced IL-17A and PGE2 production by human polymorphonuclear neutrophils: involvement of TLR2 and TLR4. J Immunol Res 2019:1790908

Gehrmann U, Qazi KR, Johansson C, Hultenby K, Karlsson M, Lundeberg L et al (2011) Nanovesicles from Malassezia sympodialis and host exosomes induce cytokine responses--novel mechanisms for host-microbe interactions in atopic eczema. PLoS One 6(7):e21480

Gil-Bona A, Llama-Palacios A, Parra CM, Vivanco F, Nombela C, Monteoliva L et al (2015) Proteomics unravels extracellular vesicles as carriers of classical cytoplasmic proteins in Candida albicans. J Proteome Res 14(1):142–153

Giri PK, Schorey JS (2008) Exosomes derived from M. Bovis BCG infected macrophages activate antigen-specific CD4+ and CD8+ T cells in vitro and in vivo. PLoS One 3(6):e2461

Giri PK, Kruh NA, Dobos KM, Schorey JS (2010) Proteomic analysis identifies highly antigenic proteins in exosomes from M. tuberculosis-infected and culture filtrate protein-treated macrophages. Proteomics 10(17):3190–3202

Goyal S, Castrillon-Betancur JC, Klaile E, Slevogt H (2018) The interaction of human pathogenic fungi with C-type lectin receptors. Front Immunol 9:1261

Gremiao ID, Miranda LH, Reis EG, Rodrigues AM, Pereira SA (2017) Zoonotic epidemic of sporotrichosis: cat to human transmission. PLoS Pathog 13(1):e1006077

Gropp K, Schild L, Schindler S, Hube B, Zipfel PF, Skerka C (2009) The yeast Candida albicans evades human complement attack by secretion of aspartic proteases. Mol Immunol 47 (2–3):465–475

Guimaraes AJ, Frases S, Gomez FJ, Zancope-Oliveira RM, Nosanchuk JD (2009) Monoclonal antibodies to heat shock protein 60 alter the pathogenesis of Histoplasma capsulatum. Infect Immun 77(4):1357–1367

Habich C, Kempe K, Gomez FJ, Lillicrap M, Gaston H, van der Zee R et al (2006) Heat shock protein 60: identification of specific epitopes for binding to primary macrophages. FEBS Lett 580(1):115–120

Hai TP, Tuan TL, Anh DV, Mai TN, Huong LNP, Thwaites GE et al (2020) The virulence of the Cryptococcus neoformans VNIa-5 lineage is highly plastic and associated with isolate background. bioRxiv

Halder LD, Jo EAH, Hasan MZ, Ferreira-Gomes M, Kruger T, Westermann M et al (2020) Immune modulation by complement receptor 3-dependent human monocyte TGF-beta1-transporting vesicles. Nat Commun 11(1):2331

Hanel KH, Cornelissen C, Luscher B, Baron JM (2013) Cytokines and the skin barrier. Int J Mol Sci 14(4):6720–6745

Harada K, Saito M, Sugita T, Tsuboi R (2015) Malassezia species and their associated skin diseases. J Dermatol 42(3):250–257

Hatanaka O, Rezende CP, Moreno P, Freitas Fernandes F, Oliveira Brito PKM, Martinez R et al (2019) Galectin-3 inhibits Paracoccidioides brasiliensis growth and impacts paracoccidioidomycosis through multiple mechanisms. mSphere 4(2)

Hofs S, Mogavero S, Hube B (2016) Interaction of Candida albicans with host cells: virulence factors, host defense, escape strategies, and the microbiota. J Microbiol 54(3):149–169

Hohl TM (2017) Immune responses to invasive aspergillosis: new understanding and therapeutic opportunities. Curr Opin Infect Dis 30(4):364–371

Huang SH, Wu CH, Chang YC, Kwon-Chung KJ, Brown RJ, Jong A (2012) Cryptococcus neoformans-derived microvesicles enhance the pathogenesis of fungal brain infection. PLoS One 7(11):e48570

Huang HR, Li F, Han H, Xu X, Li N, Wang S et al (2018) Dectin-3 recognizes glucuronoxylomannan of Cryptococcus neoformans Serotype AD and Cryptococcus gattii serotype B to initiate host defense against Cryptococcosis. Front Immunol 9:1781

Ikeda MAK, de Almeida JRF, Jannuzzi GP, Cronemberger-Andrade A, Torrecilhas ACT, Moretti NS et al (2018) Extracellular vesicles from Sporothrix brasiliensis are an important virulence factor that induce an increase in fungal burden in experimental sporotrichosis. Front Microbiol 9:2286

Joffe LS, Nimrichter L, Rodrigues ML, Del Poeta M (2016) Potential roles of fungal extracellular vesicles during infection. mSphere 1(4)

Johansson HJ, Vallhov H, Holm T, Gehrmann U, Andersson A, Johansson C et al (2018) Extracellular nanovesicles released from the commensal yeast Malassezia sympodialis are enriched in allergens and interact with cells in human skin. Sci Rep 8(1):9182

Kanj A, Abdallah N, Soubani AO (2018) The spectrum of pulmonary aspergillosis. Respir Med 141:121–131

Kenno S, Speth C, Rambach G, Binder U, Chatterjee S, Caramalho R et al (2018) Candida albicans factor H binding molecule Hgt1p - a low glucose-induced transmembrane protein is trafficked to the cell wall and impairs phagocytosis and killing by human neutrophils. Front Microbiol 9:3319

Kim J, Sudbery P (2011) Candida albicans, a major human fungal pathogen. J Microbiol 49 (2):171–177

Konecna K, Klimentova J, Benada O, Nemeckova I, Jandourek O, Jilek P et al (2019) A comparative analysis of protein virulence factors released via extracellular vesicles in two Candida albicans strains cultivated in a nutrient-limited medium. Microb Pathog 136:103666

Konno FT, Maricato J, Konno AY, Guereschi MG, Vivanco BC, Feitosa Ldos S et al (2012) Paracoccidioides brasiliensis GP43-derived peptides are potent modulators of local and systemic inflammatory response. Microbes Infect 14(6):517–527

Kopp A, Hebecker M, Svobodova E, Jozsi M (2012) Factor h: a complement regulator in health and disease, and a mediator of cellular interactions. Biomol Ther 2(1):46–75

Kroetz DN, Deepe GS (2012) The role of cytokines and chemokines in Histoplasma capsulatum infection. Cytokine 58(1):112–117

Kwon-Chung KJ, Bennett JE, Wickes BL, Meyer W, Cuomo CA, Wollenburg KR et al (2017) The case for adopting the "species complex" nomenclature for the etiologic agents of cryptococcosis. mSphere 2(1)

Latge JP, Chamilos G (2019) Aspergillus fumigatus and Aspergillosis in 2019. Clin Microbiol Rev 33(1):e00140-18

Leopold Wager CM, Wormley FL Jr (2014) Classical versus alternative macrophage activation: the Ying and the Yang in host defense against pulmonary fungal infections. Mucosal Immunol 7 (5):1023–1035

Levitz SM (2002) Receptor-mediated recognition of Cryptococcus neoformans. Nippon Ishinkin Gakkai Zasshi 43(3):133–136

Limper AH, Adenis A, Le T, Harrison TS (2017) Fungal infections in HIV/AIDS. Lancet Infect Dis 17(11):e334–ee43

Liu Y, Filler SG (2011) Candida albicans Als3, a multifunctional adhesin and invasin. Eukaryot Cell 10(2):168–173

Long KH, Gomez FJ, Morris RE, Newman SL (2003) Identification of heat shock protein 60 as the ligand on Histoplasma capsulatum that mediates binding to CD18 receptors on human macrophages. J Immunol 170(1):487–494

Lyck R, Enzmann G (2015) The physiological roles of ICAM-1 and ICAM-2 in neutrophil migration into tissues. Curr Opin Hematol 22(1):53–59

Majorek KA, Porebski PJ, Dayal A, Zimmerman MD, Jablonska K, Stewart AJ et al (2012) Structural and immunologic characterization of bovine, horse, and rabbit serum albumins. Mol Immunol 52(3–4):174–182

Marques AF, da Silva MB, Juliano MA, Munhoz JE, Travassos LR, Taborda CP (2008) Additive effect of P10 immunization and chemotherapy in anergic mice challenged intratracheally with virulent yeasts of Paracoccidioides brasiliensis. Microbes Infect 10(12–13):1251–1258

Martinez R (2015) Epidemiology of paracoccidioidomycosis. Rev Inst Med Trop Sao Paulo 57 (Suppl 19):11–20

Matos Baltazar L, Nakayasu ES, Sobreira TJ, Choi H, Casadevall A, Nimrichter L et al (2016) Antibody binding alters the characteristics and contents of extracellular vesicles released by Histoplasma capsulatum. mSphere 1(2)

May RC, Stone NR, Wiesner DL, Bicanic T, Nielsen K (2016) Cryptococcus: from environmental saprophyte to global pathogen. Nat Rev Microbiol 14(2):106–117

Mayer FL, Wilson D, Hube B (2013) Candida albicans pathogenicity mechanisms. Virulence 4 (2):119–128
Maziarz EK, Perfect JR (2016) Cryptococcosis. Infect Dis Clin N Am 30(1):179–206
Mendes RP, Cavalcante RS, Marques SA, Marques MEA, Venturini J, Sylvestre TF et al (2017) Paracoccidioidomycosis: current perspectives from Brazil. Open Microbiol J 11:224–282
Mendes-Giannini MJ, Taylor ML, Bouchara JB, Burger E, Calich VL, Escalante ED et al (2000) Pathogenesis II: fungal responses to host responses: interaction of host cells with fungi. Med Mycol 38(Suppl 1):113–123
Mendes-Giannini MJ, Andreotti PF, Vincenzi LR, da Silva JL, Lenzi HL, Benard G et al (2006) Binding of extracellular matrix proteins to Paracoccidioides brasiliensis. Microbes Infect 8 (6):1550–1559
Mihu MR, Nosanchuk JD (2012) Histoplasma virulence and host responses. Int J Microbiol 2012:268123
Monari C, Pericolini E, Bistoni G, Casadevall A, Kozel TR, Vecchiarelli A (2005) Cryptococcus neoformans capsular glucuronoxylomannan induces expression of fas ligand in macrophages. J Immunol 174(6):3461–3468
Monari C, Bistoni F, Vecchiarelli A (2006) Glucuronoxylomannan exhibits potent immunosuppressive properties. FEMS Yeast Res 6(4):537–542
Monika S, Malgorzata B, Zbigniew O (2017) Contribution of aspartic proteases in Candida virulence. Protease inhibitors against Candida infections. Curr Protein Pept Sci 18 (10):1050–1062
Moreira JA, Freitas DF, Lamas CC (2015) The impact of sporotrichosis in HIV-infected patients: a systematic review. Infection 43(3):267–276
Moyes DL, Richardson JP, Naglik JR (2015) Candida albicans-epithelial interactions and pathogenicity mechanisms: scratching the surface. Virulence 6(4):338–346
Muldoon EG, Strek ME, Patterson KC (2017) Allergic and noninvasive infectious pulmonary aspergillosis syndromes. Clin Chest Med 38(3):521–534
Munoz JE, Luft VD, Amorim J, Magalhaes A, Thomaz L, Nosanchuk JD et al (2014) Immunization with P10 peptide increases specific immunity and protects immunosuppressed BALB/c mice infected with virulent yeasts of Paracoccidioides brasiliensis. Mycopathologia 178 (3–4):177–188
Naglik JR, Challacombe SJ, Hube B (2003) Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev 67(3):400–428
Nakaira-Takahagi E, Golim MA, Bannwart CF, Puccia R, Peracoli MT (2011) Interactions between TLR2, TLR4, and mannose receptors with gp43 from Paracoccidioides brasiliensis induce cytokine production by human monocytes. Med Mycol 49(7):694–703
Nenoff P, Kruger C, Ginter-Hanselmayer G, Tietz HJ (2014) Mycology - an update. Part 1: dermatomycoses: causative agents, epidemiology and pathogenesis. J Dtsch Dermatol Ges 12 (3):188–209. quiz 10, 188–211; quiz 2
Nobile CJ, Johnson AD (2015) Candida albicans biofilms and human disease. Annu Rev Microbiol 69:71–92
Nowicka D, Nawrot U (2019) Contribution of Malassezia spp. to the development of atopic dermatitis. Mycoses 62(7):588–596
Oliveira DL, Freire-de-Lima CG, Nosanchuk JD, Casadevall A, Rodrigues ML, Nimrichter L (2010) Extracellular vesicles from Cryptococcus neoformans modulate macrophage functions. Infect Immun 78(4):1601–1609
O'Meara TR, Alspaugh JA (2012) The Cryptococcus neoformans capsule: a sword and a shield. Clin Microbiol Rev 25(3):387–408
Pasqualotto AC (2009) Differences in pathogenicity and clinical syndromes due to aspergillus fumigatus and aspergillus flavus. Med Mycol 47(Suppl 1):S261–S270
Pereira TC, de Barros PP, Fugisaki LRO, Rossoni RD, Ribeiro FC, de Menezes RT et al (2018) Recent advances in the use of Galleria mellonella model to study immune responses against human pathogens. J Fungi (Basel) 4(4):128

Peres da Silva R, Heiss C, Black I, Azadi P, Gerlach JQ, Travassos LR et al (2015) Extracellular vesicles from Paracoccidioides pathogenic species transport polysaccharide and expose ligands for DC-SIGN receptors. Sci Rep 5:14213

Pietrella D, Rachini A, Pandey N, Schild L, Netea M, Bistoni F et al (2010) The inflammatory response induced by aspartic proteases of Candida albicans is independent of proteolytic activity. Infect Immun 78(11):4754–4762

Puccia R, Travassos LR (1991) 43-kilodalton glycoprotein from Paracoccidioides brasiliensis: immunochemical reactions with sera from patients with paracoccidioidomycosis, histoplasmosis, or Jorge Lobo's disease. J Clin Microbiol 29(8):1610–1615

Puccia R, Vallejo MC, Longo LVG (2017) The cell wall-associated proteins in the dimorphic pathogenic species of Paracoccidioides. Curr Protein Pept Sci 18(11):1074–1089

Queiroz-Telles F, Fahal AH, Falci DR, Caceres DH, Chiller T, Pasqualotto AC (2017) Neglected endemic mycoses. Lancet Infect Dis 17(11):e367–e377

Reales-Calderón JA, Vaz C, Monteoliva L, Molero G, Gil C (2017) Candida albicans modifies the protein composition and size distribution of THP-1 macrophage-derived extracellular vesicles. J Proteome Res 16(1):87–105

Retini C, Kozel TR, Pietrella D, Monari C, Bistoni F, Vecchiarelli A (2001) Interdependency of interleukin-10 and interleukin-12 in regulation of T-cell differentiation and effector function of monocytes in response to stimulation with Cryptococcus neoformans. Infect Immun 69 (10):6064–6073

Rizzo J, Rodrigues ML, Janbon G (2020a) Extracellular vesicles in fungi: past, present, and future perspectives. Front Cell Infect Microbiol 10:346

Rizzo J, Wong SSW, Gazi AD, Moyrand F, Chaze T, Commere PH et al (2020b) New insights into Cryptococcus extracellular vesicles suggest a new structural model and an antifungal vaccine strategy. bioRxiv

Rodrigues ML, Casadevall A (2018) A two-way road: novel roles for fungal extracellular vesicles. Mol Microbiol 110(1):11–15

Rodrigues ML, Nimrichter L, Oliveira DL, Frases S, Miranda K, Zaragoza O et al (2007) Vesicular polysaccharide export in Cryptococcus neoformans is a eukaryotic solution to the problem of fungal trans-cell wall transport. Eukaryot Cell 6(1):48–59

Rodrigues ML, Nakayasu ES, Oliveira DL, Nimrichter L, Nosanchuk JD, Almeida IC et al (2008) Extracellular vesicles produced by Cryptococcus neoformans contain protein components associated with virulence. Eukaryot Cell 7(1):58–67

Rodrigues ML, Nakayasu ES, Almeida IC, Nimrichter L (2014) The impact of proteomics on the understanding of functions and biogenesis of fungal extracellular vesicles. J Proteome 97:177–186

Rodrigues M, Fan J, Lyon C, Wan M, Hu Y (2018) Role of extracellular vesicles in viral and bacterial infections: pathogenesis, diagnostics, and therapeutics. Theranostics 8(10):2709–2721

Rodríguez Molina LM, Tobón Orozco AM (2018) Paracoccidioidomycosis: global vision of a forgotten endemic mycosis. Curr Trop Med Rep 5(3):138–143

Santos LA, Grisolia JC, Burger E, de Araujo Paula FB, Dias ALT, Malaquias LCC (2020) Virulence factors of Paracoccidioides brasiliensis as therapeutic targets: a review. Antonie Van Leeuwenhoek 113(5):593–604

Saunders CW, Scheynius A, Heitman J (2012) Malassezia fungi are specialized to live on skin and associated with dandruff, eczema, and other skin diseases. PLoS Pathog 8(6):e1002701

Schaller M, Borelli C, Korting HC, Hube B (2005) Hydrolytic enzymes as virulence factors of Candida albicans. Mycoses 48(6):365–377

Schulze J, Sonnenborn U (2009) Yeasts in the gut: from commensals to infectious agents. Dtsch Arztebl Int 106(51–52):837–842

Shen Q, Rappleye CA (2017) Differentiation of the fungus Histoplasma capsulatum into a pathogen of phagocytes. Curr Opin Microbiol 40:1–7

Shopova IA, Belyaev I, Dasari P, Jahreis S, Stroe MC, Cseresnyés Z et al (2020) Human neutrophils produce antifungal extracellular vesicles against Aspergillus fumigatus. Am Soc Microbiol 11 (2):e00596-20

Singh PP, Smith VL, Karakousis PC, Schorey JS (2012) Exosomes isolated from mycobacteria-infected mice or cultured macrophages can recruit and activate immune cells in vitro and in vivo. J Immunol 189(2):777–785

Singh DK, Toth R, Gacser A (2020) Mechanisms of pathogenic Candida species to evade the host complement attack. Front Cell Infect Microbiol 10:94

Souza JAM, Baltazar LM, Carregal VM, Gouveia-Eufrasio L, de Oliveira AG, Dias WG et al (2019) Characterization of Aspergillus fumigatus extracellular vesicles and their effects on macrophages and neutrophils functions. Front Microbiol 10:2008

Spellberg BJ, Ibrahim AS, Avanesian V, Fu Y, Myers C, Phan QT et al (2006) Efficacy of the anti-Candida rAls3p-N or rAls1p-N vaccines against disseminated and mucosal candidiasis. J Infect Dis 194(2):256–260

Srikanta D, Santiago-Tirado FH, Doering TL (2014) Cryptococcus neoformans: historical curiosity to modern pathogen. Yeast 31(2):47–60

Straus AH, Freymuller E, Travassos LR, Takahashi HK (1996) Immunochemical and subcellular localization of the 43 kDa glycoprotein antigen of Paracoccidioides brasiliensis with monoclonal antibodies. J Med Vet Mycol 34(3):181–186

Sugui JA, Kwon-Chung KJ, Juvvadi PR, Latge JP, Steinbach WJ (2014) Aspergillus fumigatus and related species. Cold Spring Harb Perspect Med 5(2):a019786

Torres I, Hernandez O, Tamayo D, Munoz JF, Leitao NP Jr, Garcia AM et al (2013) Inhibition of PbGP43 expression may suggest that gp43 is a virulence factor in Paracoccidioides brasiliensis. PLoS One 8(7):e68434

Vallejo MC, Matsuo AL, Ganiko L, Medeiros LC, Miranda K, Silva LS et al (2011) The pathogenic fungus Paracoccidioides brasiliensis exports extracellular vesicles containing highly immunogenic alpha-Galactosyl epitopes. Eukaryot Cell 10(3):343–351

Vallejo MC, Nakayasu ES, Matsuo AL, Sobreira TJ, Longo LV, Ganiko L et al (2012) Vesicle and vesicle-free extracellular proteome of Paracoccidioides brasiliensis: comparative analysis with other pathogenic fungi. J Proteome Res 11(3):1676–1685

Vallhov H, Johansson C, Veerman RE, Scheynius A (2020) Extracellular vesicles released from the skin commensal yeast Malassezia sympodialis activate human primary keratinocytes. Front Cell Infect Microbiol 10:6

van Niel G, D'Angelo G, Raposo G (2018) Shedding light on the cell biology of extracellular vesicles. Nat Rev Mol Cell Biol 19(4):213–228

Vargas G, Rocha JD, Oliveira DL, Albuquerque PC, Frases S, Santos SS et al (2015) Compositional and immunobiological analyses of extracellular vesicles released by Candida albicans. Cell Microbiol 17(3):389–407

Vargas G, Honorato L, Guimaraes AJ, Rodrigues ML, Reis FCG, Vale AM et al (2020) Protective effect of fungal extracellular vesicles against murine candidiasis. Cell Microbiol:e13238

Vicentini AP, Gesztesi JL, Franco MF, de Souza W, de Moraes JZ, Travassos LR et al (1994) Binding of Paracoccidioides brasiliensis to laminin through surface glycoprotein gp43 leads to enhancement of fungal pathogenesis. Infect Immun 62(4):1465–1469

Villena SN, Pinheiro RO, Pinheiro CS, Nunes MP, Takiya CM, DosReis GA et al (2008) Capsular polysaccharides galactoxylomannan and glucuronoxylomannan from Cryptococcus neoformans induce macrophage apoptosis mediated by Fas ligand. Cell Microbiol 10(6):1274–1285

Walling BL, Kim M (2018) LFA-1 in T cell migration and differentiation. Front Immunol 9:952

Wang J, Yao Y, Chen X, Wu J, Gu T, Tang X (2018) Host derived exosomes-pathogens interactions: potential functions of exosomes in pathogen infection. Biomed Pharmacother 108:1451–1459

Witherden EA, Shoaie S, Hall RA, Moyes DL (2017) The human mucosal mycobiome and fungal community interactions. J Fungi (Basel) 3(4)

Wolf JM, Rivera J, Casadevall A (2012) Serum albumin disrupts Cryptococcus neoformans and Bacillus anthracis extracellular vesicles. Cell Microbiol 14(5):762–773

Wolf JM, Espadas J, Luque-Garcia J, Reynolds T, Casadevall A (2015) Lipid biosynthetic genes affect Candida albicans extracellular vesicle morphology, cargo, and immunostimulatory properties. Eukaryot Cell 14(8):745–754

Woods JP (2016) Revisiting old friends: developments in understanding Histoplasma capsulatum pathogenesis. J Microbiol 54(3):265–276

Yanez-Mo M, Siljander PR, Andreu Z, Zavec AB, Borras FE, Buzas EI et al (2015) Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicl 4:27066

Zamith-Miranda D, Heyman HM, Couvillion SP, Cordero RJB, Rodrigues ML, Nimrichter L et al (2020) Comparative molecular and immunoregulatory analysis of extracellular vesicles from Candida albicans and Candida auris. bioRxiv

Zaragoza O (2019) Basic principles of the virulence of Cryptococcus. Virulence 10(1):490–501

Zeng F, Morelli AE (2018) Extracellular vesicle-mediated MHC cross-dressing in immune homeostasis, transplantation, infectious diseases, and cancer. Semin Immunopathol 40(5):477–490

Zhan P, Liu W (2017) The changing face of dermatophytic infections worldwide. Mycopathologia 182(1–2):77–86

Zhang YJ, Han Y, Sun YZ, Jiang HH, Liu M, Qi RQ et al (2019) Extracellular vesicles derived from Malassezia furfur stimulate IL-6 production in keratinocytes as demonstrated in in vitro and in vivo models. J Dermatol Sci 93(3):168–175

Anexo III

Manuscrito - BITENCOURT, T. A.; HATANAKA, O.; PESSONI, A. M.; FREITAS, M. S.; TRENTIN, G.; SANTOS, P.; ROSSI, A.; MARTINEZ-ROSSI, N. M.; ALVES, L. L.; CASADEVALL, A.; RODRIGUES, M. L.; ALMEIDA, F. Fungal Extracellular Vesicles Are Involved in Intraspecies Intracellular Communication. **mBio**, 13, n. 1, p. e0327221, Feb 22 2022.

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RESEARCH ARTICLE

Fungal Extracellular Vesicles Are Involved in Intraspecies Intracellular Communication

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ABSTRACT Fungal infections are associated with high mortality rates in humans. The risk of fungal diseases creates the urgent need to broaden the knowledge base regarding their pathophysiology. In this sense, the role of extracellular vesicles (EVs) has been described to convey biological information and participate in the fungus-host interaction process. We hypothesized that fungal EVs work as an additional element in the communication routes regulating fungal responses in intraspecies interaction systems. In this respect, the aim of this study was to address the gene regulation profiles prompted by fungal EVs in intraspecies recipient cells. Our data demonstrated the intraspecies uptake of EVs in pathogenic fungi, such as *Candida albicans*, *Aspergillus fumigatus*, and *Paracoccidioides brasiliensis*, and the effects triggered by EVs in fungal cells. In *C. albicans*, we evaluated the involvement of EVs in the yeast-to-hypha transition, while in *P. brasiliensis* and *A. fumigatus* the function of EVs as stress transducers was investigated. *P. brasiliensis* and *A. fumigatus* were exposed to an inhibitor of glycosylation or UV light, respectively. The results demonstrated the role of EVs in regulating the expression of target genes and triggering phenotypic changes. The EVs treatment induced cellular proliferation and boosted the yeast to hyphal transition in *C. albicans*, while they enhanced stress responsiveness in *A. fumigatus* and *P. brasiliensis*, establishing a role for EVs in fungal intraspecies communication. Thus, EVs regulate fungal behavior, acting as potent message effectors, and understanding their effects and mechanism(s) of action could be exploited in antifungal therapies.

IMPORTANCE Here, we report a study about extracellular vesicles (EVs) as communication mediators in fungi. Our results demonstrated the role of EVs from *Candida albicans*, *Aspergillus fumigatus*, and *Paracoccidioides brasiliensis* regulating the expression of target genes and phenotypic features. We asked whether fungal EVs play a role as message effectors. We show that fungal EVs are involved in fungal interaction systems as potent message effectors, and understanding their effects and mechanisms of action could be exploited in antifungal therapies.

KEYWORDS fungal infections, extracellular vesicles, fungal biology, *Aspergillus fumigatus*, *Candida albicans*, cellular communication, *Paracoccidioides brasiliensis*

Fungal infections are responsible for over 1.6 million deaths per year. It is estimated that more than a billion cases of severe fungal diseases affect the world population. Despite these numbers it is likely that they represent an underestimate of the fungal diseases that ail humans (1, 2). The diseases caused by *Aspergillus* spp., *Candida* spp.,

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and the agents of mycoses such as *Paracoccidioides* species are among the deadliest mycoses (1, 2).

In recent years, extracellular vesicles (EVs) have been studied in cell-walled microorganisms. In fungi, they were first described in 2007 in *Cryptococcus neoformans* (3). So far, EVs have been characterized in approximately 20 fungal species, including yeasts forms of *H. capsulatum*, *Sporothrix schenckii*, *C. parapsilosis*, *Saccharomyces cerevisiae*, *Malassezia sympodialis*, *P. brasiliensis*, *C. albicans*, *Pichia fermentans*, *C. gattii*, *S. brasiliensis*, *P. lutzii*, and *Exophiala dermatitidis* (4–13) and filamentous fungi such as *Alternaria infectoria*, *Trichophyton interdigitale*, *Rhizopus delemar*, *Fusarium oxysporum* f. sp. *vasinfectum*, *Trichoderma reesei*, *Aspergillus fumigatus*, and *Aspergillus flavus* (14–20).

EVs function as vehicles carrying complex cargoes with diverse biological functions, including proteins, carbohydrates, pigments, nucleic acids, and lipids. EVs can contribute to fungal infection outcomes (21). EVs likely have roles in bidirectional communication, raising the possibility of communication between fungal cells (22). Previous reports have demonstrated the participation of fungal EVs in biofilm formation (10), stimulation of cytokine production (6, 9, 11, 15, 20, 23), and favoring pathogen infection (4, 24). Bidirectional communication mediated by fungal EVs has been demonstrated in the interaction of fungal cells with plants (25, 26) and in communication with mammalian cells (27, 28).

The possibility of EV-mediated virulence transfer and/or antifungal resistance between strains has gathered attention. In the *Cryptococcus* model, previous studies demonstrated that the Vancouver Island outbreak strain of *C. deuterogatti*, namely, R265, can transfer its ability to proliferate within the host macrophages to an avirulent strain, which was attributed to an EV-regulated process (11, 29). Another report showed that the supernatant from a highly virulent strain of *C. neoformans* culture stimulated the pathogenic potential of a less virulent isolate, an effect also attributed to EVs (30). Furthermore, EVs isolated from biofilms of wild-type (WT) *C. albicans* restored the biofilm production and fluconazole tolerance in mutant strains with a negative background in orthologs of endosomal sorting complexes required for transport (ESCRT) subunits (31). Recently, a study advocated the role of EVs from *C. albicans* as messaging compartments involved in growth, morphogenesis, and biofilm production (32). Altogether, these studies suggested a role for fungal EVs in intraspecies communication; however, at the molecular level, many aspects related to the mechanisms switched on by EVs remain to be investigated. Here, we sought to analyze the fungal cellular communication mediated by EVs using the fungal pathogens *P. brasiliensis*, *A. fumigatus*, and *C. albicans* using multiple approaches. Our data demonstrate that fungal EVs mediate cellular communication by regulating the expression of target genes and by controlling cellular proliferation.

RESULTS

Intercellular transfer of fungal EVs. Because EVs can transport multiple molecules that play essential roles in fungal biology (22, 28, 33), we examined whether fungal EVs would be transferred intercellularly from cells of the same species. Using [1-^{14}C] palmitic acid metabolic labeling, radiolabeled fungal EVs were produced as previously described (34, 35). Radioactive assays confirmed that fungal EVs produced by *P. brasiliensis* (Fig. 1A), *A. fumigatus* (Fig. 1B), and *C. albicans* (Fig. 1C) could be transferred from cell to cell within the same species. We tracked the radioactivity added to the fungal cell cultures during the time course after 0 h (control), 1 h, 6 h, 12 h, and 24 h from the addition of radiolabeled EVs to the cultures (Fig. 1). The fungal cells were pelleted and washed with phosphate-buffered saline (PBS), and pulse-chase measurements were performed. We verified that purified radiolabeled EVs were taken up by fungal cells or at least associated with the cell surface as the radioactive signal increased over

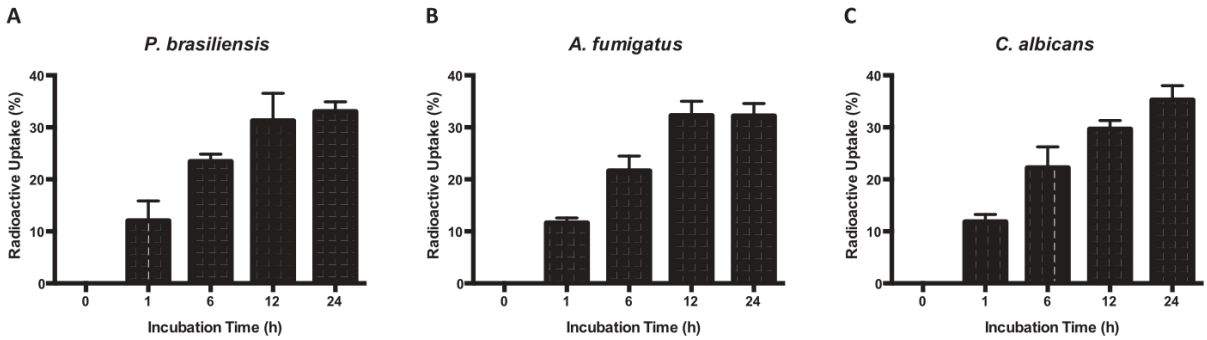

FIG 1 Evaluation of extracellular vesicles (EVs) uptake in different fungal species. The absorption of radioactive VEs was evaluated after 0, 1, 6, 12, and 24 h in the following yeast cells: *P. brasiliensis* (A), *A. fumigatus* (B), and *C. albicans* (C).

time. In this respect, we posit that EVs are taken up and internalized, or at least associated, with the fungal cell surface.

***P. brasiliensis* EVs as cellular communicator during ER stress.** We previously demonstrated that the genes *HACA* and *IRE1* showed increased expression during tunicamycin (TM) treatment (36), suggesting that they are involved in the endoplasmic reticulum (ER) stress response of *P. brasiliensis*. Thus, we purified EVs from *P. brasiliensis* treated with TM (TM EVs) and added these EVs to *P. brasiliensis* yeast cells that did not receive TM treatment (Fig. 2A). We observed that TM EVs increased *HACA* and *IRE1* expression significantly (Fig. 2B and C, red bars). On the other hand, fungal EVs obtained from untreated *P. brasiliensis* yeast cells (CONTROL EVs) were added to *P. brasiliensis* yeast cells, which did not alter *HACA* and *IRE1* expression (Fig. 2B and C, blue bars). PBS (NO EVs) was used as a negative control (white bar), while TM was the positive-control condition (black bars). These results strongly suggested that *P. brasiliensis* EVs could participate in intercellular communication during ER stress, possibly promoting fungal adaptive responses.

EVs from *A. fumigatus* act as stress message effectors. To verify whether the observations made with *P. brasiliensis* applied to other fungi, we isolated EVs from UV-irradiated cultures and from nonirradiated *A. fumigatus* cells (control). The EVs obtained from UV-treated cells were named UV EVs, whereas the EVs obtained from regular cultures from *A. fumigatus* were named CONTROL EVs. Our data showed an overall population size in the range of 100 to 200 nm and a minor population of EVs with sizes varying from 320 to 394 nm in regular cultures or a range of 240 to 614 nm in cultures that underwent UV irradiation (Fig. S1).

EVs obtained from both *A. fumigatus* cultures after UV irradiation (UV EVs) or without UV irradiation (CONTROL EVs) (Fig. 3A) caused a prominent decrease in colony formation in *A. fumigatus* (Fig. 3B). Approximately 40% of colony reduction was achieved after EVs uptake (Fig. 3B). Thereafter, the gene expression analysis reinforced a possible role of these EVs as stress message effectors, showing the upregulation of the *mpkC* gene under both EVs uptake conditions. The *mpkC* gene, which encodes the mitogen-activated protein kinase (MAPK) ortholog, known as Hog1p, plays a role in adaptive responses to different stress agents, such as osmotic stress, oxidative stress, heat shock, and cell wall damage and also acts in cellular division regulation, as demonstrated in *A. fumigatus* (37, 38). The cultures that received UV EVs showed higher induction of the *mpkC* gene (Fig. 3C). In addition, UV EVs also caused a significant increase in the *akuA* transcript levels (Fig. 3D), supporting a role of EVs in cellular communication events. The *akuA* gene encodes the Ku70 component involved in DNA repair (39). In addition, the presence of EVs caused a subtle downregulation of the *nimA* gene, the encoding gene of cell cycle-regulated protein kinase, which may reflect another level of cell cycle regulation (Fig. 3E). To prove that the communication process relies only on intact EVs, we also tested heated EVs. In such cases, we evaluated the effects triggered by heated

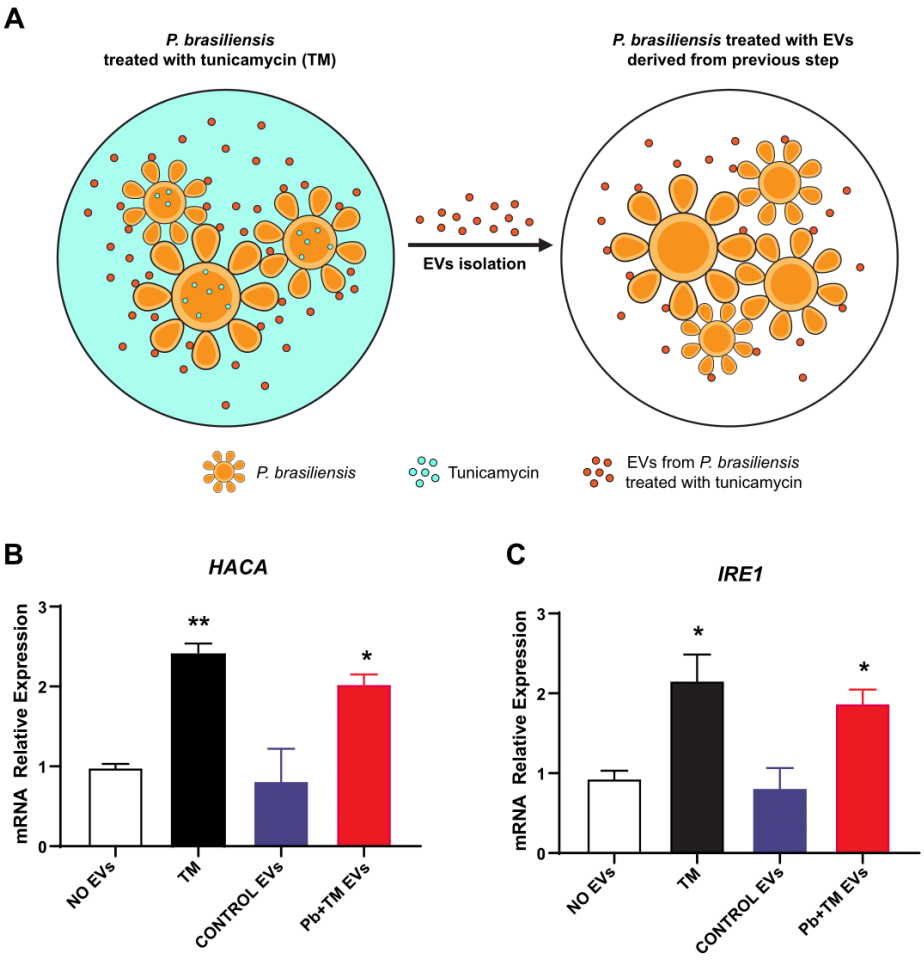

FIG 2 EVs from *P. brasiliensis* as cellular communicator during ER stress. (A) Schematic representation of EVs obtained after tunicamycin exposure. The relative expression of UPR genes *HACA* (B) and *IRE1* (C) was determined after EVs uptake (4×10^9/ml EVs in 10^5/ml recipient cells). Significantly different values are indicated by asterisks as determined using ANOVA followed by Tukey's *post hoc* test ($P < 0.05$). The calibrator was the NO EVs condition, and the positive control was tunicamycin (TM).

EVs from both conditions (CONTROL EVs and UV EVs) on colony formation and *akuA* modulation (Fig. S2). Similar profiles of colony formation were observed when recipient cells were treated with heated EVs or non-heated EVs (about 40 to 45% reduction). Conversely, the uptake of heated UV EVs did not impact *akuA* modulation. The condition control EVs (heated) were associated with a decrease in *akuA* transcript levels.

EVs induce hypha formation and regulate the cell cycle in *C. albicans*. Next, we investigated whether EVs obtained from yeast culture and a yeast-to-hypha culture in the *C. albicans* model would mediate communication, affecting its morphological transition. Therefore, we isolated EVs from *C. albicans* from hypha-inducing cultures and from yeast cultures. We analyzed the size and distribution of EVs in both cultures (see Fig. S1 in the supplemental material). EVs obtained after *C. albicans* grown on cultures in YPD at pH 6.3 at 30°C were named CONTROL EVs, whereas EVs obtained from *C. albicans* grown on cultures in YPD at pH 7.4 at 37°C were named TRANS EVs. Our results showed a heterogeneous EV distribution profile within these conditions, and the majority of EVs obtained from yeast-to-hypha transition cultures (TRANS EVs) had a size

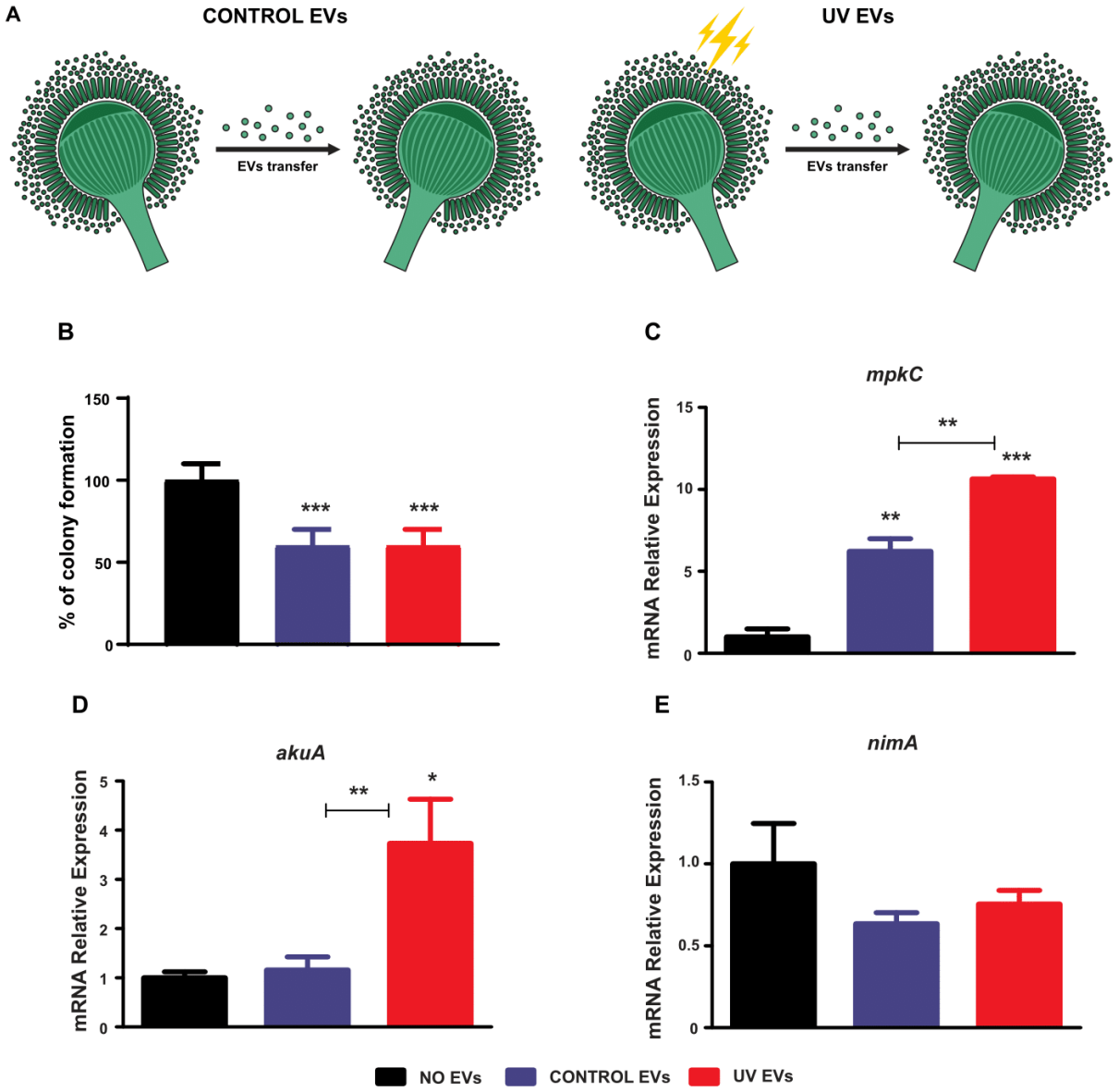

FIG 3 EVs from *A. fumigatus* as stress message effectors. (A) Schematic representation of EVs obtained from regular cultures, without UV exposure (CONTROL EVs), and EVs obtained from UV-exposed cultures (UV EVs). The thunder diagram represents UV light exposure. The EVs uptake was performed with 5×10^8/ml of EVs in 10^4/ml recipient cells. (B) The colony formation was counted and a percentage value was determined after EVs uptake. (C to E) RT-qPCR for *A. fumigatus* genes *mpkC* (C), *akuA* (D), and *nimA* (E). Relative expression was assessed using the NO EVs condition as a reference sample and 18S and *βtub* as a reference for normalization. Significantly different values are indicated by asterisks as determined using ANOVA followed by Tukey's *post hoc* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

range from 99 to 182 nm and minor populations with sizes varying from 23 to 85 nm and 232 to 440 nm. For EVs isolated from *C. albicans* yeast cultures (CONTROL EVs), the majority of EVs had a size range from 107 to 154 nm and a minor population in the range of 211 to 440 nm. Previous studies have demonstrated the heterogeneity of EVs from *Candida* spp., showing a distribution profile with population sizes ranging from 60 to 280 nm, and in *C. albicans*, the sizes varied from 100 nm to 600 nm (5, 40, 41).

We also evaluated the gene expression profiles of selected genes (*HWP1*, *SAP5*, *CHT2*, and *SEC24*) during *C. albicans* growth in yeast-to-hypha transition cultures

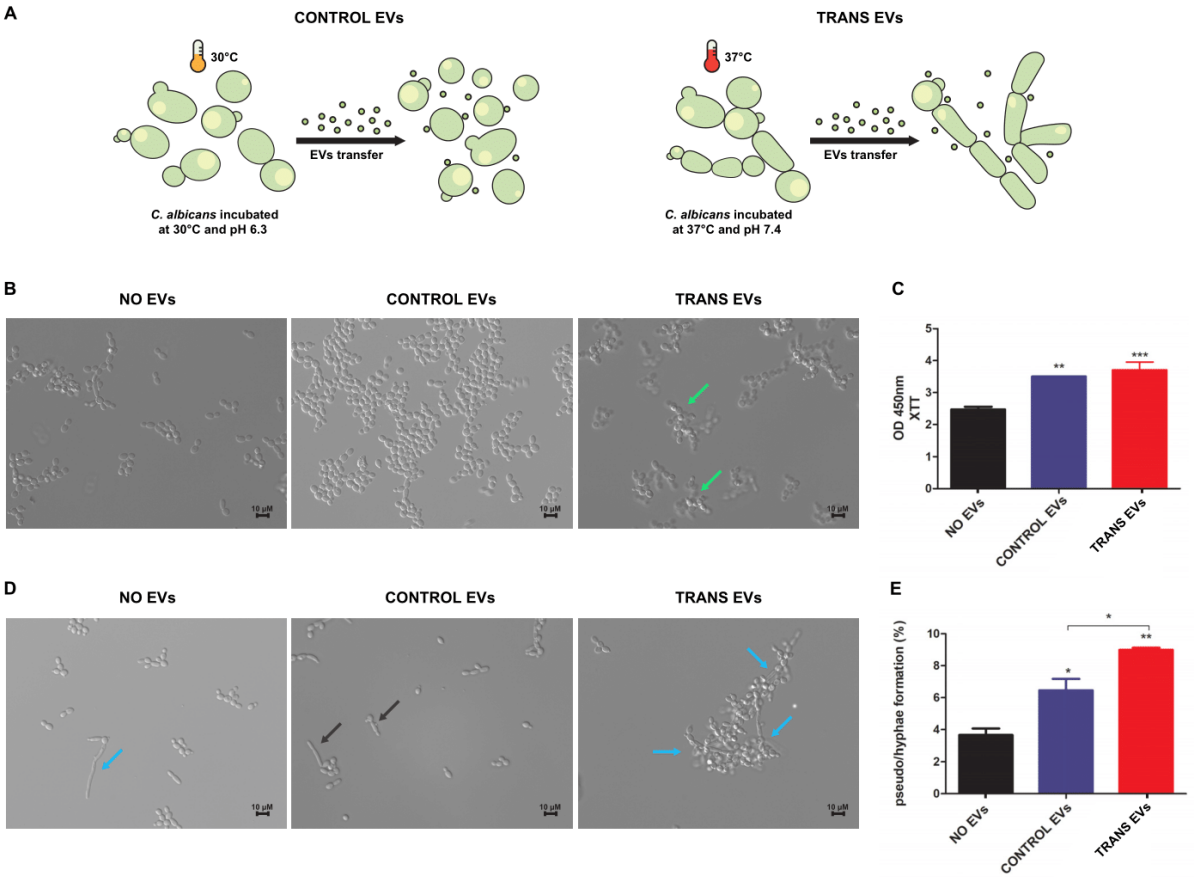

FIG 4 EVs heighten the cellular proliferation and pseudohypha formation in *C. albicans*. (A) Schematic representation of EVs obtained from yeast cultures (CONTROL EVs) and yeast-to-hypha cultures (TRANS EVs). (B) Morphological appearance was investigated by microscope images of *C. albicans* cells grown on YPD, pH 7.4, at 37°C after EVs uptake for 2 h. Green arrows depict clump structures observed in cultures that underwent TRANS EVs uptake. (C) XTT reduction assay to assess cellular proliferation after EVs uptake during 2 h of growth on transition condition. Identification of hypha and pseudohypha morphologies in *C. albicans* grown on transition condition for 4 h is shown. (D) Black arrows depict pseudohyphae and blue arrows depict hypha structures. (E) Estimation of morphotype abundance after 4 h of *C. albicans* growth on transition condition.

compared to yeast cultures after different incubation times, such as 0.5, 1, 2, and 4 h (Fig. S3). These genes were previously modulated in microarray data of *C. albicans* grown as hyphae with serum at 37°C, and *SAP5* (secreted aspartyl protease) and *HWP1* (cell wall protein hyphal wall protein 1) were highly upregulated (42). We identified a time-dependent modulation for the genes *CHT2* (endochitinase) and *SEC24* (ER to Golgi transport). A prominent upregulation was verified for the *HWP1* gene at all time points evaluated. The *SAP5* gene was upregulated at all times analyzed. Taking advantage of this information, we compared gene expression profiles after EVs uptake in *C. albicans*. Thus, the *C. albicans* cells were incubated with TRANS EVs or CONTROL EVs (Fig. 4A). Thereafter, the cells were recovered through centrifugation and resuspended in a yeast-to-hypha medium. By analyzing the morphological features of *C. albicans* under yeast-to-hypha conditions assessed throughout the incubation time points, we identified the occurrence of the three main morphologies presented by *C. albicans*, yeast, pseudohyphae, and hyphae. Moreover, the cultures that underwent TRANS EVs uptake appeared to present changes in yeast development in which a particular cellular clumping was detected at earlier time points (Fig. 4B). Our results also showed a possible increase in cellular proliferation after EVs uptake compared to NO EVs uptake cultures. The 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[carbonyl (phenylamino)]-2H-tetrazolium hydroxide (XTT) assay reinforced the involvement of EVs in cell cycle

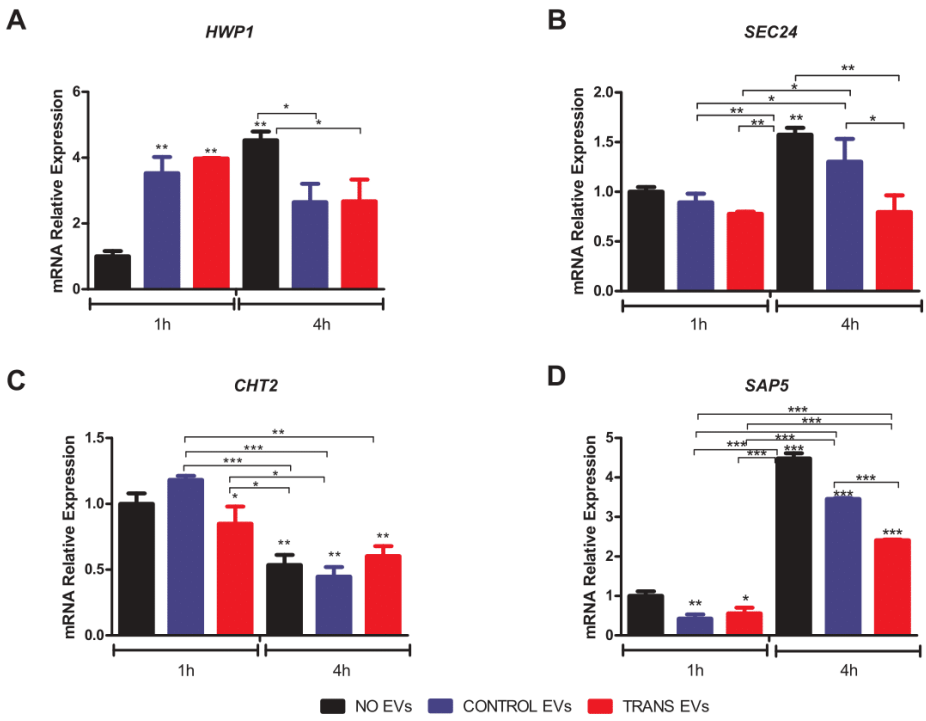

FIG 5 EVs prompted a boost in yeast-to-hypha transition gene expression response. The EVs uptake was carried out with EVs (5×10^8/ml) and *C. albicans* in cellular density adjusted to an OD_{600} of 0.100 to 0.130. RT-qPCR evaluated a set of *C. albicans* genes after EVs uptake during 1 h and 4 h of growth on transition cultures. *HWP1* (A), *SEC24* (B), *CHT2* (C), and SAP*5* (D). The relative expression was assessed using NO EVs condition at the 1-h time point as the reference sample after normalization with the *RPP2B* and *TDH3* genes. Significantly different values are indicated by asterisks, determined using ANOVA followed by Tukey's *post hoc* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

regulation (Fig. 4C). Furthermore, during the course of growth, the cultures that received TRANS EVs displayed more developed hyphae than the other conditions, NO EVs and CONTROL EVs (Fig. 4D). The estimation of morphotype abundance also indicated that *C. albicans* cultures receiving EVs presented an enhancement of pseudohypha and/or hypha formation, which was mostly predominant after TRANS EVs treatment (Fig. 4E). In parallel, the effect of heated EVs on the regulation of morphotypes was analyzed. Although *C. albicans* treated with heated EVs displayed heightened pseudohypha and/or hypha formation (about 7%), regardless of the type of EV treatment, CONTROL EVs (heated) or TRANS EVs (heated) (Fig. S4), it was lower than uptake of intact TRANS EVs (about 9%).

EVs uptake caused a rapid response to hypha-inducing stimuli, with a more significant upregulation of the *HWP1* gene at an earlier incubation time point (1 h) followed by a decay in its transcript levels later (4 h), which was observed for both uptake conditions, CONTROL EVs and TRANS EVs (Fig. 5A). The transition panel (Fig. S3) suggested a fluctuation in *HWP1* modulation, with a higher increase in the transcript levels after 2 h, showing an approximately 50-fold difference. In addition, the EVs from control and transition cultures promoted a repression of the *SEC24* gene after 4 h, which was more pronounced by TRANS EVs treatment (Fig. 5B). The transition panel demonstrated its downregulation, particularly in delayed incubation time points, in which hyphae were more commonly found. In addition, the *CHT2* gene was repressed after EVs uptake from transition cultures after 1 h, and no change in its transcription levels was observed after 4 h compared with the NO EVs condition at the same time point (Fig. 5C). A previous study showed that the downregulation of this *CHT2* gene is a

remarkable feature in hyphae (43). Therefore, our data suggest a boost in hypha induction after EVs stimulation. Curiously, *SAP5*, which is mainly upregulated in cells with hyphal morphology, presented a decrease in transcription levels under EVs uptake conditions (Fig. 5D). We also investigated the effects of heated EVs on the regulation of the *HWP1* gene and observed a subtle induction triggered by CONTROL EVs (heated), with an increase in transcript levels of about 1.34-fold. No differences in gene modulation were induced by TRANS EVs (heated) compared with NO EVs (Fig. S4).

DISCUSSION

Intercellular communication occurs through contact with cells or by the secretion of molecules such as quorum-sensing (QS) effectors. A recent study demonstrated that EVs enriched with Fks1 and Chs3 from *Saccharomyces cerevisiae* rescued the yeast cells from cell wall disturbance (44), which suggested the possibility that EVs function in fungal intraspecies communication.

Here, we evaluated whether EVs are capable of mediating intraspecies communication in fungi by applying three approaches in three different fungal pathogens. We used uptake times of 1 and 2 h, which were sufficient to allow the incorporation of radioactive EVs or at least to promote their aggregation with fungal cells, as mentioned above (Fig. 1). It is expected that subtle differences in EVs uptake could be responsible for altering and regulating gene expression, because fungi have the refined ability to sense any change in the environment and adjust their cellular machinery in response to these changes.

Our study demonstrated a role for EVs as stress message effectors in the fungal agents *P. brasiliensis* and *A. fumigatus*. In the *P. brasiliensis* model, the genes belonging to the UPR pathway were studied after cultures underwent EVs uptake (EVs were obtained from previous tunicamycin-exposed cultures) compared to cells directly exposed to tunicamycin. The UPR pathway is switched on to reestablish endoplasmic reticulum homeostasis by heightening the folding capability and controlling misfolded protein disposal (45). In fungi, this pathway comprises the ER-transmembrane sensor Ire1/IreA (Ser/Thr kinase) and transcription factor Hac1/HacA (46). Our experiments demonstrated the upregulation of both genes after TM EVs uptake, suggesting that EVs function in fungal communication.

In the *A. fumigatus* model, EVs obtained from a regular or an irradiated culture triggered a stress response in fresh *A. fumigatus* cultures, as evidenced by colony reduction formation and upregulation of a stress response gene, the *mpkC* gene. We hypothesize that fungitoxic compounds in EVs from *A. fumigatus* are responsible for these effects. Previously, the production of antifungal compounds by filamentous fungi such as *A. fumigatus* was reported (47). However, the characterization and understanding of the processes mediated by EVs in *A. fumigatus* have been poorly explored. Recently, a study showed the production and cargo description of EVs from *A. fumigatus* in which approximately 60 proteins were identified with potential roles in immunomodulation and pathogenicity (19).

In this study, the uptake of EVs from cultures exposed to UV light prompted the most pronounced upregulation of the *mpkC* gene. It also promoted a remarkable increase in transcript levels of the *akuA* gene. *akuA* is involved in DNA repair (39). UV exposure causes DNA damage through mutation and pyrimidine dimers and induces oxidative stress status (48, 49). A previous study demonstrated the involvement of *akuA* in cellular protection against UV radiation exposure, showing that *akuA* deletion was responsible for a significant reduction in survival rates of the mutant strain compared to the *A. fumigatus* wild type (50). Moreover, we revealed that the cultures that underwent EVs uptake from *A. fumigatus* showed a trend of downregulating an encoding gene of cyclin-dependent kinase, NimA, involved in cell cycle transition (51).

In *C. albicans*, we assessed the potential of EVs to favor hyphal formation. It is widely accepted that dimorphism in *C. albicans* is important for virulence (52). The yeast-to-hypha transition is a tightly regulated process of sensing and responding to environmental cues (53). Hypha formation is mainly associated with invasion and

adaptive responses during fungus-host interactions (54). Recently, single-cell transcriptome sequencing data showed the dynamic regulation of transition genes during macrophage infection with *C. albicans* and also highlighted the occurrence of bimodal modulation in genes related to hypha formation and cell wall remodeling concomitantly with differences in immunomodulation responses (55).

In our data, we detected a new response characterized by cell clumping after TRANS EVs uptake in which the EVs were obtained from *C. albicans* hypha-inducing cultures. Cell clumping effects probably reflect changes in surface hydrophobicity. This feature may result from cell surface changes activated in the regulation of hyphal expansion (56, 57). Furthermore, the gene modulation profile assessed after EVs uptake also showed a boost in yeast-to-hypha transition response, mainly prompted after TRANS EVs uptake, which is reinforced by the presence of long hyphae in this treatment (Fig. 4D). Hyphal growth is associated with a hypha-specific transcriptional program that paves the way toward the expression of genes related to virulence traits such as adhesion (ALS3 and HWP1), invasion (ALS3), oxidative stress response (SOD5), proteolytic activity (SAPs), and others (54). In a previous high-throughput study, a set of genes showed differential modulation after transition stimuli, including the genes *SAP5*, *HWP1*, *SEC24*, and *CHT2* (42). Furthermore, hyphal development relies on a complex network of transduction signals that respond to environmental cues and activate the molecular processes involved in driving the high-polarization growth of hyphal forms (58, 59).

Aspects of cell cycle organization are also unique in hyphae compared to pseudohyphae and yeasts. Hyphal forms showed different branching patterns, regulation of the cell cycle, and polarization (59). In addition, a phenomenon known as QS governs responses related to hyphal formation and control of cell density (60, 61). Many QS molecules might also be found within fungal EVs; however, it is also possible to speculate a cross-regulation between small RNA content in EVs and QS phenomena in fungi. A recent study showed the involvement of QS molecules such as farnesol derivatives and medium-chain fatty acids in EVs obtained from yeast culture as potential regulators of cellular proliferation and yeast-to-hypha development (32). The EVs from *C. albicans* reduced the hypha and biofilm formation and the impact of *C. albicans* EVs on invasive hypha development and virulence, as shown in a *Galleria mellonella* model (32). Moreover, that study was conducted exploring EVs obtained from yeast cultures, and it is reasonable that the cargo and the message within these EVs contributing to cells remain in this form or just evolve toward pseudohyphal morphology, as inferred from our data.

Current knowledge regarding the differences in cell cycle regulation in hyphal development associated with our data prompted the investigation of cellular proliferation after EVs uptake. We demonstrated that the conditions that underwent EV uptake presented an increase in mitochondrial activity, suggesting a function of EVs in heightening cellular metabolism and/or proliferation. EVs were previously shown to enhance cellular proliferation in *C. albicans* (32) as well as in *C. neoformans* within macrophages. The high activation of the *C. neoformans* proliferation rate was named the division of labor mechanism (11). It was ascribed to an important virulence trait transferred from an outbreak strain to a nonoutbreak strain (11).

Collectively, our data pave new avenues for EV functions in fungal intraspecies communication, supporting the role of EVs as potent message effectors regulating pathophysiological mechanisms. Although we recognize the need to develop our knowledge about the complex cellular communication circuit switched on by EVs, and regardless of how it will be addressed in the future, this study sheds new light on the role of fungal EVs in intraspecies cellular communication.

MATERIALS AND METHODS

Fungal strains and growth conditions. *Paracoccidioides brasiliensis* strain 18 (Pb18) was cultivated as previously described (62). The Pb18 yeast form was maintained in Fava Netto semisolid medium and incubated at 36°C. Yeast growth was determined by inoculating cultures in liquid YPD medium (2%

peptone, 1% yeast extract, and 2% glucose) at 36°C on a rotary shaker at 100 rpm for 72 h. Tunicamycin (TM) was used to induce ER stress. TM (15 μg/ml) was prepared in 20 mM NaOH, and it was added to yeast cultures in liquid YPD for 5 days at 36°C on a rotary shaker at 100 rpm to obtain EVs derived from ER-stressed fungi. The same fungal growth was seen without TM to obtain Pb18 CONTROL EVs.

The *Aspergillus fumigatus* strain CEA17 used in this study was grown in a complete agar malt medium (YAG medium supplemented with malt extract: 2% [wt/vol] glucose, 0.2% [wt/vol] yeast extract, 2% [wt/vol] malt extract, 2% [wt/vol] agar, and 0.1% [vol/vol] trace elements) for 7 days at 37°C. Conidial suspensions were obtained from 7-day-old plates with sterilized PBS, recovered by centrifugation, and filtered through sterile Miracloth (Millipore, Billerica, MA, USA). Conidial concentration was determined using a Neubauer chamber. Approximately 1×10^3 conidia were directly inoculated into complete YAG medium or the conidial suspension was first exposed to UV light for 60 s and then inoculated into a complete YAG medium. The cultures were incubated for 2 days at 37°C. These plates were used for the following experiments: EV isolation and communication assay.

Candida albicans strain ATTC-64548 was grown at 30°C in Sabouraud medium (Oxoid, Basingstoke, United Kingdom) for 72 h. One fresh colony was inoculated into 10 ml of yeast extract-peptone-dextrose medium (YPD pH 6.3) and cultured overnight at 30°C with shaking (150 rpm). The overnight cultures of *C. albicans* then were diluted to an optical density at 600 nm (OD_{600}) range of 0.100 to 0.130 with YPD medium (pH 6.3) or YPD medium (pH 7.4) and grown at 30°C or 37°C, respectively. The cultures grown on YPD at pH 6.3 represent the control cultures in which no stimuli for hypha differentiation were offered, whereas cultures grown on YPD at pH 7.4 represent the transition cultures in which a stimulus conferred by pH (7.4) and temperature (37°C) were offered to prompt hypha differentiation. After that, the cultures were used to perform the EVs isolation experiment, the gene expression profile of yeast to hyphae, and the communication assay.

EVs isolation. EVs from Pb18 yeast cultivated in the presence or absence of TM were isolated as previously described (63). Yeast cultures of Pb18 growth in YPD medium were depleted from cell pellets by serial centrifugation at 5,000 $\times$ *g* for 15 min and 15,000 $\times$ *g* for 30 min at 4°C. The supernatants, containing EVs, were concentrated and filtered using Amicon systems (Millipore, Billerica, MA, USA) with a 100-kDa-cutoff membrane. The concentrated material was centrifuged again at 15,000 $\times$ *g* for 30 min at 4°C, and the supernatants were centrifuged at 100,000 $\times$ *g* for 1 h at 4°C to collect vesicles. The EVs pellets were resuspended in PBS for nanoparticle-tracking analysis (NTA) and experiments. EVs obtained from TM-treated Pb18 were termed TM EVs, whereas EVs obtained from nontreated cultures were termed Pb EVs.

Regarding the isolation of *A. fumigatus* EVs, the procedures were performed as previously described (19, 41), with slight modifications. Two-day-old plates obtained for a regular culture or a UV-exposed culture were used to isolate EVs. UV light exposure cultures were obtained after conidial irradiation. Approximately 10^4 conidia/ml were irradiated with a UV germicidal light (G1578 UV lamp) at 16-cm distance for 60 s with constant shaking, and then 100 μl of this irradiated suspension was plated on YAG medium, yielding about 10% of the CFU compared to nonirradiated cultures. The cells were washed twice with 3 ml of sterile PBS, recovered from the dish plates with inoculation loops, and then transferred to centrifuge tubes. Thereafter, the cell suspension was filtered through sterile Miracloth (Millipore, Billerica, MA, USA), and then a sequential centrifugation and supernatant concentration in the Amicon system was performed. The EVs obtained from UV light exposure were named UV EVs, whereas the EVs obtained from regular cultures were called control EVs.

Isolation of *C. albicans* EVs was performed as described previously, with slight modifications (9, 44). The concentration of the *C. albicans* cultures was adjusted as mentioned above, and the preinocula were added into 300 ml of YPD (pH 6.3) or YPD (pH 7.4) and incubated for 4 h at 30°C or 37°C, with shaking (100 rpm). For EVs isolation, the cells and debris were removed by sequential centrifugation at 4,000 $\times$ *g* for 15 min and 15,000 $\times$ *g* for 15 min. Supernatants were concentrated using an Amicon ultraconcentration system (cutoff, 100 kDa; Millipore, Billerica, MA, USA). The resulting concentrated supernatant was ultracentrifuged at 100,000 $\times$ *g* for 1 h at 4°C. Pellets (EV-containing) were collected and resuspended in ultrapure water (Sigma-Aldrich, St. Louis, MO, USA) supplemented with protease inhibitor cocktail (10×; Sigma) (0.2%, vol/vol) to prevent cargo degradation, and then the EVs were stored at −80°C. Taking into account the culture conditions used to promote yeast-to-hypha transition (YPD medium at pH 7.4, incubated at 37°C), an additional step was included in which the cultures were first filtered using sterile Miracloth (Millipore, Billerica, MA, USA) and then subjected to differential centrifugation. EVs obtained after *C. albicans* grown on YPD at pH 6.3 at 30°C were named CONTROL EVs, while EVs obtained from *C. albicans* grown on YPD at pH 7.4 and incubated at 37°C were named TRANS EVs.

NTA. To determine the size distribution and quantification of EVs isolated from *C. albicans* cultures in two different stages, a yeast culture condition (CONTROL EVs) and a yeast-to-hypha culture condition (TRANS EVs), NTAs were performed. We also performed the NTA to measure and characterize the size distribution of EVs isolated from regular and UV-exposed *A. fumigatus* cultures. We obtained the profiles of *P. brasiliensis* EVs as previously described (35). NTA was performed using a Nanosight NS300 appliance (Malvern Instruments, Malvern, UK) with NTA 3.0. The parameters were set as recommended by the manufacturer's manual. The camera level was increased to a level of >14, in which all particles were distinctly visible, and the threshold was determined to capture as many particles as possible, within an ideal range of 20 to 100 particles per frame. The NTA is an optical dispersion technique employed to measure the size distribution of particles in a solution at the nanometer scale (64).

Determination of EVs uptake by fungal cells. To obtain radiolabeled EVs, each one of the fungal species (*P. brasiliensis*, *A. fumigatus*, and *C. albicans*) was pulsed with [1-^{14}C] palmitic acid for 72 h before EVs isolation (65). The culture medium used for each species and EVs isolation process were described above. Radiolabeled EVs from the same species (10^9/ml) were added to the corresponding yeast-phase

fungi (10^7/ml) and incubated for 0, 1, 6, 12, and 24 h at 37°C in 9.5% CO_2. After incubation, culture supernatants containing EVs that were not taken up were removed, fungal cells were washed thrice with PBS and lysed in 200 μl of 25 mM deoxycholate, and the resultant material was collected for scintillation counting.

Gene expression profile of *C. albicans* in yeast-to-hypha transition stage. The *C. albicans* inoculum was prepared, and its concentration was adjusted as previously described. It was added to 5 ml of YPD (pH 6.3) and incubated for 0.5, 2, and 4 h at 30°C (yeast culture condition). Thereafter, the cultures were centrifuged at 4,000 $\times$ *g* for 10 min at 4°C. The resulting pellet was stored at −80°C until RNA extraction. The *C. albicans* inoculum was also added to 5 ml of YPD (pH 7.4) and incubated for 0.5, 2, and 4 h at 37°C (hypha-yeast culture condition). The pellet obtained from these cultures was stored at −80°C until RNA extraction. We used the resulting material to create a panel that reflects the expression profiles of *HWP1*, *SEC24*, *SAP5*, and *CHT2* genes during the *C. albicans* transition stage.

Communication assay mediated by EVs. (i) Effect of EVs on *P. brasiliensis* endoplasmic reticulum stress. The *P. brasiliensis* yeast cells were treated with TM as previously described (36). The EVs from TM-treated and untreated yeast cultures were isolated, and the uptake of EVs (4×10^9/ml) with *P. brasiliensis* yeast cells (10^5/ml) was performed for 2 h at 37°C in brain heart infusion medium (Sigma). RNA isolation, cDNA synthesis, and quantitative PCR (qPCR) assays were performed as described previously (36).

(ii) Effects of EVs on *A. fumigatus* UV stress. The assay was performed with 5 ml of *A. fumigatus* conidial suspension (10^4 conidia/ml). The conidial suspension was incubated with 5×10^8 EVs/ml obtained from *A. fumigatus* regular cultures or UV light-exposed cultures. Additionally, the EVs from both cultures were incubated at 90°C for 15 min, and these heated EVs were also added with fungal conidia. Uptake was performed for 1 h at 37°C in PBS with shaking (100 rpm). Therefore, the fungal cells were recovered by centrifugation at 4,000 $\times$ *g* for 10 min at room temperature, and the cultures were resuspended in 5 ml of PBS. Subsequently, approximately 100 μl of conidia was plated on the YAG medium. After incubation at 37°C in the dark for 2 days, the CFU were counted. CFU from conidia without EVs uptake were counted as controls. The colonies were stored at −80°C until RNA extraction.

(iii) Effects of EVs on *C. albicans* dimorphism. About 5×10^8 EVs obtained from *C. albicans* yeast cultures (CONTROL EVs) or *C. albicans* yeast-to-hypha transition cultures (TRANS EVs) were added into a fresh culture of *C. albicans* with the cell density adjusted to an OD_{600} of 0.10 to 0.130, as previously mentioned, in 5 ml of YPD (pH 6.3 for 1 h at 30°C), with shaking (100 rpm). Additionally, the EVs obtained from both *C. albicans* cultures were incubated at 90°C for 15 min, and these heated EVs were also added into the fresh yeast culture, as mentioned above. The yeast cells were then recovered by centrifugation. The pellets were resuspended in 5 ml of YPD (pH 7.4) and incubated for 1 h and 4 h at 37°C under shaking (100 rpm). After incubation, the cultures were centrifuged at 4,000 $\times$ *g* for 10 min at 4°C, and the pellet was stored at −80°C until RNA extraction. Similar conditions were employed to analyze cellular proliferation through the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[carbonyl (phenylamino)]-2H-tetrazolium hydroxide (XTT) reduction assay. The XTT assay was performed as previously described (66), with slight modifications. After the uptake of EVs in *C. albicans* cultures, the yeast pellets were recovered through centrifugation at 4,000 $\times$ *g* for 10 min at room temperature, and the pellets were resuspended in YPD (pH 7.4) and incubated for 2 h at 37°C. The medium then was removed, and the pellet was resuspended in XTT solution (1 mg/ml in PBS) and menadione (1 mM in acetone) and incubated for 3 h with gentle shaking. The activity of the yeast mitochondrial dehydrogenase reduces the tetrazolium salt XTT to formazan salts, which results in a colorimetric change that might correlate with cell viability. Colorimetric changes were measured using an enzyme-linked immunosorbent assay microplate reader (Multiskan FC; Thermo Scientific) at 450 nm. Additionally, we monitored the changes in yeast-to-hypha transition in *C. albicans* cultures after EVs uptake by microscopy analysis. Images were obtained using a Zeiss Observer Z.1 microscope with AxioVision SE64 software after 1-h, 2-h, and 4-h time points. As controls, cultures that had not undergone the EVs uptake process were analyzed.

RNA extraction and RT-qPCR. The *C. albicans* cells were treated with lysis solution (20 mg/ml lysozyme, 0.7 M KCl, and 1 M $MgSO_4$, pH 6.8) for 1 h with shaking (100 rpm). Next, the supernatant was removed by centrifugation at 1,000 $\times$ *g* for 10 min, and total RNA was extracted using the Illustra RNAspin Mini RNA isolation kit (GE Healthcare) by following the manufacturer's instructions. The *A. fumigatus* mycelia were lysed by mechanical pulverization with a pestle and mortar in liquid nitrogen, and total RNA extraction was performed using an Illustra RNAspin mini RNA isolation kit (GE Healthcare). *P. brasiliensis* cells were also lysed with the aid of a small mortar and pestle in liquid nitrogen before total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), as described previously (36). RNA concentration and quality were estimated using a nanophotometer (Implem). The RNA was pretreated with DNase (Sigma). cDNA synthesis was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems) by following the manufacturer's instructions. Real-time qPCR (RT-qPCR) was conducted as described previously (67). The qPCR experiments were performed with SYBR green master mix (Applied Biosystems) in the Step One Plus platform. Primer sequences were retrieved from the IDT DNA primer quest tool (www.idtdna.com/primerquest/Home/Index), and the oligonucleotide sequences are listed in Table 1. The algorithm used for gene expression analysis was the relative quantification $2^{-\Delta\Delta CT}$ method (68), and graphs were generated using GraphPad Prism v.5 software (GraphPad).

Statistical analyses. The results are presented as mean values from independent experiments ± standard deviations. Significant differences were determined by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* tests or unpaired *t* test, using GraphPad Prism 5 software.

TABLE 1 Primers used in RT-qPCR

Gene ID	Sequence (5′–3′)	Concn (nM)	Efficiency (%)
RPP2B	FWD, TGGTGTTGAAGCCGAAGAA; REV, CGGATGGGACAGAAGCTAAT	100	95.18
TDH3	FWD, CACGGTAGATACAAGGGTGAAG; REV, GGAATGTTAGCTGGGTCTCTTT	150	108.6
HWP1	FWD, GCCTGATGACAATCCTCCTATT; REV, GAGTAGTAGCTGGAGTTGTTGG	400	97.75
CHT2	FWD, TGGTGGTGTTGGTGACTATG; REV, CAGCGTCATCAAATGGTCTTTC	300	92.49
SAP5	FWD, GCGAAGCTACCGAGTTTGAT; REV, GCTTCAGCAGAGTTAAGGTAGAG	500	100.53
SEC24	FWD, GAGCTGGATATGGCGGATATG; REV, GTGGAAGGTCCCTTGATAAGTC	300	99.62
18S	FWD, AAGTGCGCGGCAATAACA; REV, CTCGGCCAAGGTGATGTACT	100	89.34
Btub[a]	FWD, TTCCCAACAACATCCAGACC; REV, CGACGGAACATAGCAGTGAA	70	119
akuA	FWD, GCTCCTGTGTACCTGAAAGATG; REV, GGGACCGACCGAGAATTTATG	70	100.74
mpkC	FWD, TTCCGAGGTCCTTGACTATCT; REV, GTTCAAGAGCACTCGGATCAA	100	105.13
nimA	FWD, TCAGCGGCAAGCAAGAATA; REV, TGAGGGAAGATCGGGTATATCA	100	92.65
HACA	FWD, GATTCACCCACTCTTGTCCC; REV, GAATCTGTGAGGTCCAAGTCC	100	97.38
IRE1	FWD, CACAATTTACAGGAGCTTGCG; REV, GAACCCTTGTCTCGTCTAACTC	100	95.43
A-tubulin	FWD, CGGCTAATGGAAAATACATGGC; REV, GTCTTGGCCTTGAGAGATGCAA	100	93.12

[a]See reference 69.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, EPS file, 1.6 MB.

FIG S2, EPS file, 0.9 MB.

FIG S3, EPS file, 1.6 MB.

FIG S4, EPS file, 0.8 MB.

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F.A., O.H., A.C., M.R., and T.B. conceived the study. F.A., O.H., and T.B. performed the experimental design and laboratory experiments. A.P. and M.S.F. contributed to *A. fumigatus* assays. A.P. and P.S. performed the illustrative designs. G.T. assisted in the *C. albicans* EV isolation assay. T.B., N.M., A.R., A.C., L.A., M.R., and F.A. wrote the manuscript.

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REFERENCES

1. Almeida F, Rodrigues ML, Coelho C. 2019. The still underestimated problem of fungal diseases worldwide. Front Microbiol 10:214. https://doi.org/10.3389/fmicb.2019.00214.
2. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. 2012. Hidden killers: human fungal infections. Sci Transl Med 4:165rv13.
3. Rodrigues ML, Nimrichter L, Oliveira DL, Frases S, Miranda K, Zaragoza O, Alvarez M, Nakouzi A, Feldmesser M, Casadevall A. 2007. Vesicular polysaccharide export in Cryptococcus neoformans is a eukaryotic solution to the problem of fungal trans-cell wall transport. Eukaryot Cell 6:48–59. https://doi.org/10.1128/EC.00318-06.
4. Ikeda MAK, de Almeida JRF, Jannuzzi GP, Cronemberger-Andrade A, Torrecilhas ACT, Moretti NS, da Cunha JPC, de Almeida SR, Ferreira KS. 2018. Extracellular vesicles From Sporothrix brasiliensis are an important virulence factor that induce an increase in fungal burden in experimental sporotrichosis. Front Microbiol 9:2286. https://doi.org/10.3389/fmicb.2018.02286.
5. Vargas G, Rocha JDB, Oliveira DL, Albuquerque PC, Frases S, Santos SS, Nosanchuk JD, Gomes AMO, Medeiros LCAS, Miranda K, Sobreira TJP, Nakayasu ES, Arigi EA, Casadevall A, Guimaraes AJ, Rodrigues ML, Freire-de-Lima CG, Almeida IC, Nimrichter L. 2015. Compositional and immunobiological analyses of extracellular vesicles released by Candida albicans. Cell Microbiol 17:389–407. https://doi.org/10.1111/cmi.12374.
6. Gehrmann U, Qazi KR, Johansson C, Hultenby K, Karlsson M, Lundeberg L, Gabrielsson S, Scheynius A. 2011. Nanovesicles from Malassezia sympodialis and host exosomes induce cytokine responses–novel mechanisms for host-microbe interactions in atopic eczema. PLoS One 6:e21480. https://doi.org/10.1371/journal.pone.0021480.
7. Albuquerque PC, Nakayasu ES, Rodrigues ML, Frases S, Casadevall A, Zancope-Oliveira RM, Almeida IC, Nosanchuk JD. 2008. Vesicular transport in Histoplasma capsulatum: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. Cell Microbiol 10:1695–1710. https://doi.org/10.1111/j.1462-5822.2008.01160.x.
8. Oliveira DL, Nakayasu ES, Joffe LS, Guimarães AJ, Sobreira TJP, Nosanchuk JD, Cordero RJB, Frases S, Casadevall A, Almeida IC, Nimrichter L, Rodrigues ML. 2010. Characterization of yeast extracellular vesicles: evidence for the participation of different pathways of cellular traffic in vesicle biogenesis. PLoS One 5:e11113. https://doi.org/10.1371/journal.pone.0011113.
9. Vallejo MC, Matsuo AL, Ganiko L, Medeiros LCS, Miranda K, Silva LS, Freymüller-Haapalainen E, Sinigaglia-Coimbra R, Almeida IC, Puccia R. 2011. The pathogenic fungus Paracoccidioides brasiliensis exports extracellular vesicles containing highly immunogenic alpha-galactosyl epitopes. Eukaryot Cell 10:343–351. https://doi.org/10.1128/EC.00227-10.
10. Leone F, Bellani L, Muccifora S, Giorgetti L, Bongioanni P, Simili M, Maserti B, Del Carratore R. 2018. Analysis of extracellular vesicles produced in the

biofilm by the dimorphic yeast Pichia fermentans. J Cell Physiol 233:2759–2767. https://doi.org/10.1002/jcp.25885.
11. Bielska E, Sisquella MA, Aldeieg M, Birch C, O'Donoghue EJ, May RC. 2018. Pathogen-derived extracellular vesicles mediate virulence in the fatal human pathogen Cryptococcus gattii. Nat Commun 9:1556. https://doi.org/10.1038/s41467-018-03991-6.
12. Peres da Silva R, Longo LGV, Cunha JPC, Sobreira TJP, Rodrigues ML, Faoro H, Goldenberg S, Alves LR, Puccia R. 2019. Comparison of the RNA content of extracellular vesicles derived from Paracoccidioides brasiliensis and Paracoccidioides lutzii. Cells 8:765. https://doi.org/10.3390/cells8070765.
13. Lavrin T, Konte T, Kostanjšek R, Sitar S, Sepčič K, Prpar Mihevc S, Žagar E, Župunski V, Lenassi M, Rogelj B, Gunde Cimerman N. 2020. The neurotropic black yeast Exophiala dermatitidis induces neurocytotoxicity in neuroblastoma cells and progressive cell death. Cells 9:963. https://doi.org/10.3390/cells9040963.
14. Silva BMA, Prados-Rosales R, Espadas-Moreno J, Wolf JM, Luque-Garcia JL, Gonçalves T, Casadevall A. 2014. Characterization of Alternaria infectoria extracellular vesicles. Med Mycol 52:202–210. https://doi.org/10.1093/mmy/myt003.
15. Bitencourt TA, Rezende CP, Quaresemin NR, Moreno P, Hatanaka O, Rossi A, Martinez-Rossi NM, Almeida F. 2018. Extracellular vesicles from the dermatophyte Trichophyton interdigitale modulate macrophage and keratinocyte functions. Front Immunol 9:2343. https://doi.org/10.3389/fimmu.2018.02343.
16. Liu M, Bruni GO, Taylor CM, Zhang Z, Wang P. 2018. Comparative genome-wide analysis of extracellular small RNAs from the mucormycosis pathogen Rhizopus delemar. Sci Rep 8:5243. https://doi.org/10.1038/s41598-018-23611-z.
17. Bleackley MR, Samuel M, Garcia-Ceron D, McKenna JA, Lowe RGT, Pathan M, Zhao K, Ang C-S, Mathivanan S, Anderson MA. 2019. Extracellular vesicles from the cotton pathogen Fusarium oxysporum f. sp. vasinfectum induce a phytotoxic response in plants. Front Plant Sci 10:1610. https://doi.org/10.3389/fpls.2019.01610.
18. de Paula RG, Antoniêto ACC, Nogueira KMV, Ribeiro LFC, Rocha MC, Malavazi I, Almeida F, Silva RN. 2019. Extracellular vesicles carry cellulases in the industrial fungus Trichoderma reesei. Biotechnol Biofuels 12:146. https://doi.org/10.1186/s13068-019-1487-7.
19. Souza JAM, Baltazar LM, Carregal VM, Gouveia-Eufrasio L, de Oliveira AG, Dias WG, Campos Rocha M, Rocha de Miranda K, Malavazi I, Santos DA, Frézard FJG, de Souza DG, Teixeira MM, Soriani FM. 2019. Characterization of Aspergillus fumigatus extracellular vesicles and their effects on macrophages and neutrophils functions. Front Microbiol 10:2008. https://doi.org/10.3389/fmicb.2019.02008.
20. Brauer VS, Pessoni AM, Bitencourt TA, de Paula RG, de Oliveira Rocha L, Goldman GH, Almeida F. 2020. Extracellular vesicles from Aspergillus flavus induce M1 polarization in vitro. mSphere 5:e00190-20. https://doi.org/10.1128/mSphere.00190-20.
21. de Toledo Martins S, Szwarc P, Goldenberg S, Alves LR. 2019. Extracellular vesicles in fungi: composition and functions. Curr Top Microbiol Immunol 422:45–59. https://doi.org/10.1007/82_2018_141.
22. Rizzo J, Rodrigues ML, Janbon G. 2020. Extracellular vesicles in fungi: past, present, and future perspectives. Front Cell Infect Microbiol 10:346. https://doi.org/10.3389/fcimb.2020.00346.
23. Oliveira DL, Freire-de-Lima CG, Nosanchuk JD, Casadevall A, Rodrigues ML, Nimrichter L. 2010. Extracellular vesicles from Cryptococcus neoformans modulate macrophage functions. Infect Immun 78:1601–1609. https://doi.org/10.1128/IAI.01171-09.
24. Huang SH, Wu CH, Chang YC, Kwon-Chung KJ, Brown RJ, Jong A. 2012. Cryptococcus neoformans-derived microvesicles enhance the pathogenesis of fungal brain infection. PLoS One 7:e48570. https://doi.org/10.1371/journal.pone.0048570.
25. Regente M, Pinedo M, San Clemente H, Balliau T, Jamet E, de la Canal L. 2017. Plant extracellular vesicles are incorporated by a fungal pathogen and inhibit its growth. J Exp Bot 68:5485–5495. https://doi.org/10.1093/jxb/erx355.
26. Cai Q, Qiao L, Wang M, He B, Lin F-M, Palmquist J, Huang S-D, Jin H. 2018. Plants send small RNAs in extracellular vesicles to fungal pathogen to silence virulence genes. Science 360:1126–1129. https://doi.org/10.1126/science.aar4142.
27. Silva VKA, Rodrigues ML, May RC. 2019. Deciphering fungal extracellular vesicles: from cell biology to pathogenesis. Curr Clin Micro Rep 6:89–97. https://doi.org/10.1007/s40588-019-00128-1.
28. Freitas MS, Bonato VLD, Pessoni AM, Rodrigues ML, Casadevall A, Almeida F. 2019. Fungal extracellular vesicles as potential targets for immune interventions. mSphere 4:e00747-19. https://doi.org/10.1128/mSphere.00747-19.
29. Voelz K, Johnston SA, Smith LM, Hall RA, Idnurm A, May RC. 2014. "Division of labour" in response to host oxidative burst drives a fatal Cryptococcus gattii outbreak. Nat Commun 5:5194. https://doi.org/10.1038/ncomms6194.
30. Hai TP, Tuan TL, Anh DV, Mai TN, Huong LNP, Thwaites GE, et al. 2020. The expression of virulence by the Cryptoccocus neoformans VNIa-5 lineage is plastic and associated with host immune background. bioRxiv https://doi.org/10.1101/2020.02.24.962134.
31. Zarnowski R, Sanchez H, Covelli AS, Dominguez E, Jaromin A, Bernhardt J, Mitchell KF, Heiss C, Azadi P, Mitchell A, Andes DR. 2018. Candida albicans biofilm-induced vesicles confer drug resistance through matrix biogenesis. PLoS Biol 16:e2006872. https://doi.org/10.1371/journal.pbio.2006872.
32. Honorato L, Demetrio JF, Ellis CC, Piffer A, Pereira Y, Frases S, et al. 2021. Extracellular vesicles regulate yeast growth, biofilm formation, and yeast-to-hypha differentiation in Candida albicans. bioRxiv https://doi.org/10.1101/2021.01.21.427696.
33. Brown L, Wolf JM, Prados-Rosales R, Casadevall A. 2015. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. Nat Rev Microbiol 13:620–630. https://doi.org/10.1038/nrmicro3480.
34. Wolf JM, Rivera J, Casadevall A. 2012. Serum albumin disrupts Cryptococcus neoformans and Bacillus anthracis extracellular vesicles. Cell Microbiol 14:762–773. https://doi.org/10.1111/j.1462-5822.2012.01757.x.
35. Hatanaka O, Rezende CP, Moreno P, Freitas Fernandes F, Oliveira Brito PKM, Martinez R, Coelho C, Roque-Barreira MC, Casadevall A, Almeida F. 2019. Galectin-3 inhibits Paracoccidioides brasiliensis growth and impacts paracoccidioidomycosis through multiple mechanisms. mSphere 4:e00209-19. https://doi.org/10.1128/mSphere.00209-19.
36. Almeida F, Antoniêto ACC, Pessoni AM, Monteiro VN, Alegre-Maller ACP, Pigosso LL, Pereira M, Soares CMA, Roque-Barreira MC. 2016. Influence of N-glycans on expression of cell wall remodeling related genes in Paracoccidioides brasiliensis yeast cells. Curr Genomics 17:112–118. https://doi.org/10.2174/1389202917666151116212705.
37. Bruder Nascimento ACMO, Dos Reis TF, de Castro PA, Hori JI, Bom VLP, de Assis LJ, Ramalho LNZ, Rocha MC, Malavazi I, Brown NA, Valiante V, Brakhage AA, Hagiwara D, Goldman GH. 2016. Mitogen activated protein kinases SakA(HOG1) and MpkC collaborate for Aspergillus fumigatus virulence. Mol Microbiol 100:841–859. https://doi.org/10.1111/mmi.13354.
38. Pereira Silva L, Alves de Castro P, Dos Reis TF, Paziani MH, Von Zeska Kress MR, Riano-Pachon DM, et al. 2016. Genome-wide transcriptome analysis of Aspergillus fumigatus exposed to osmotic stress reveals regulators of osmotic and cell wall stresses that are SakA(HOG1) and MpkC dependent. Cell Microbiol https://doi.org/10.1111/cmi.12681.
39. Pannunzio NR, Watanabe G, Lieber MR. 2018. Nonhomologous DNA end-joining for repair of DNA double-strand breaks. J Biol Chem 293:10512–10523. https://doi.org/10.1074/jbc.TM117.000374.
40. Karkowska-Kuleta J, Kulig K, Karnas E, Zuba-Surma E, Woznicka O, Pyza E, Kuleta P, Osyczka A, Rapala-Kozik M, Kozik A. 2020. Characteristics of extracellular vesicles released by the pathogenic yeast-like fungi Candida glabrata, Candida parapsilosis and Candida tropicalis. Cells 9:1722. https://doi.org/10.3390/cells9071722.
41. Reis FCG, Borges BS, Jozefowicz LJ, Sena BAG, Garcia AWA, Medeiros LC, Martins ST, Honorato L, Schrank A, Vainstein MH, Kmetzsch L, Nimrichter L, Alves LR, Staats CC, Rodrigues ML. 2019. A novel protocol for the isolation of fungal extracellular vesicles reveals the participation of a putative scramblase in polysaccharide export and capsule construction in Cryptococcus gattii. mSphere 4:e00080-19. https://doi.org/10.1128/mSphere.00080-19.
42. Nantel A, Dignard D, Bachewich C, Harcus D, Marcil A, Bouin A-P, Sensen CW, Hogues H, van Het Hoog M, Gordon P, Rigby T, Benoit F, Tessier DC, Thomas DY, Whiteway M. 2002. Transcription profiling of Candida albicans cells undergoing the yeast-to-hyphal transition. Mol Biol Cell 13:3452–3465. https://doi.org/10.1091/mbc.e02-05-0272.
43. McCreath KJ, Specht CA, Robbins PW. 1995. Molecular cloning and characterization of chitinase genes from Candida albicans. Proc Natl Acad Sci U S A 92:2544–2548. https://doi.org/10.1073/pnas.92.7.2544.
44. Zhao K, Bleackley M, Chisanga D, Gangoda L, Fonseka P, Liem M, Kalra H, Al Saffar H, Keerthikumar S, Ang C-S, Adda CG, Jiang L, Yap K, Poon IK, Lock P, Bulone V, Anderson M, Mathivanan S. 2019. Extracellular vesicles secreted by Saccharomyces cerevisiae are involved in cell wall remodelling. Commun Biol 2:305. https://doi.org/10.1038/s42003-019-0538-8.
45. Ron D, Walter P. 2007. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 8:519–529. https://doi.org/10.1038/nrm2199.

46. Moore KA, Hollien J. 2012. The unfolded protein response in secretory cell function. Annu Rev Genet 46:165–183. https://doi.org/10.1146/annurev-genet-110711-155644.
47. Bladt TT, Frisvad JC, Knudsen PB, Larsen TO. 2013. Anticancer and antifungal compounds from Aspergillus, Penicillium and other filamentous fungi. Molecules 18:11338–11376. https://doi.org/10.3390/molecules180911338.
48. Ikehata H, Ono T. 2011. The mechanisms of UV mutagenesis. J Radiat Res 52:115–125. https://doi.org/10.1269/jrr.10175.
49. Silva-Junior AC, Asad LM, Felzenszwalb I, Asad NR. 2011. Mutagenicity induced by UVC in Escherichia coli cells: reactive oxygen species involvement. Redox Rep 16:187–192. https://doi.org/10.1179/1351000211Y.0000000010.
50. Blachowicz A, Raffa N, Bok JW, Choera T, Knox B, Lim FY, Huttenlocher A, Wang CCC, Venkateswaran K, Keller NP. 2020. Contributions of spore secondary metabolites to UV-C protection and virulence vary in different Aspergillus fumigatus strains. mBio 11:e03415-19. https://doi.org/10.1128/mBio.03415-19.
51. Osmani SA, Ye XS. 1996. Cell cycle regulation in Aspergillus by two protein kinases. Biochem J 317:633–641. https://doi.org/10.1042/bj3170633.
52. Mayer FL, Wilson D, Hube B. 2013. Candida albicans pathogenicity mechanisms. Virulence 4:119–128. https://doi.org/10.4161/viru.22913.
53. Biswas S, Van Dijck P, Datta A. 2007. Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of Candida albicans. Microbiol Mol Biol Rev 71:348–376. https://doi.org/10.1128/MMBR.00009-06.
54. Jacobsen ID, Wilson D, Wachtler B, Brunke S, Naglik JR, Hube B. 2012. Candida albicans dimorphism as a therapeutic target. Expert Rev Anti Infect Ther 10:85–93. https://doi.org/10.1586/eri.11.152.
55. Muñoz JF, Delorey T, Ford CB, Li BY, Thompson DA, Rao RP, Cuomo CA. 2019. Coordinated host-pathogen transcriptional dynamics revealed using sorted subpopulations and single macrophages infected with Candida albicans. Nat Commun 10:1607. https://doi.org/10.1038/s41467-019-09599-8.
56. Whiteway M, Bachewich C. 2007. Morphogenesis in Candida albicans. Annu Rev Microbiol 61:529–553. https://doi.org/10.1146/annurev.micro.61.080706.093341.
57. Kumamoto CA. 2008. Molecular mechanisms of mechanosensing and their roles in fungal contact sensing. Nat Rev Microbiol 6:667–673. https://doi.org/10.1038/nrmicro1960.
58. Sudbery P, Gow N, Berman J. 2004. The distinct morphogenic states of Candida albicans. Trends Microbiol 12:317–324. https://doi.org/10.1016/j.tim.2004.05.008.
59. Sudbery PE. 2011. Growth of Candida albicans hyphae. Nat Rev Microbiol 9:737–748. https://doi.org/10.1038/nrmicro2636.
60. Finkel JS, Mitchell AP. 2011. Genetic control of Candida albicans biofilm development. Nat Rev Microbiol 9:109–118. https://doi.org/10.1038/nrmicro2475.
61. Albuquerque P, Casadevall A. 2012. Quorum sensing in fungi–a review. Med Mycol 50:337–345. https://doi.org/10.3109/13693786.2011.652201.
62. Dos Reis Almeida FB, Carvalho FC, Mariano VS, Alegre ACP, Silva RN, Hanna ES, Roque-Barreira MC. 2011. Influence of N-glycosylation on the morphogenesis and growth of Paracoccidioides brasiliensis and on the biological activities of yeast proteins. PLoS One 6:e29216. https://doi.org/10.1371/journal.pone.0029216.
63. da Silva TA, Roque-Barreira MC, Casadevall A, Almeida F. 2016. Extracellular vesicles from Paracoccidioides brasiliensis induced M1 polarization in vitro. Sci Rep 6:35867. https://doi.org/10.1038/srep35867.
64. Rupert DLM, Claudio V, Lasser C, Bally M. 2017. Methods for the physical characterization and quantification of extracellular vesicles in biological samples. Biochim Biophys Acta 1861:3164–3179. https://doi.org/10.1016/j.bbagen.2016.07.028.
65. Almeida F, Wolf JM, da Silva TA, DeLeon-Rodriguez CM, Rezende CP, Pessoni AM, Fernandes FF, Silva-Rocha R, Martinez R, Rodrigues ML, Roque-Barreira MC, Casadevall A. 2017. Galectin-3 impacts Cryptococcus neoformans infection through direct antifungal effects. Nat Commun 8:1968. https://doi.org/10.1038/s41467-017-02126-7.
66. Costa-Orlandi CB, Sardi JC, Santos CT, Fusco-Almeida AM, Mendes-Giannini MJ. 2014. In vitro characterization of Trichophyton rubrum and T. mentagrophytes biofilms. Biofouling 30:719–727. https://doi.org/10.1080/08927014.2014.919282.
67. Bitencourt TA, Oliveira FB, Sanches PR, Rossi A, Martinez-Rossi NM. 2019. The prp4 kinase gene and related spliceosome factor genes in Trichophyton rubrum respond to nutrients and antifungals. J Med Microbiol 68:591–599. https://doi.org/10.1099/jmm.0.000967.
68. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262.
69. Campos Rocha M, Fabri JHTM, de Godoy KF, de Castro PA, Hori JI, da Cunha AF, Arentshorst M, Ram AFJ, van den Hondel CAMJJ, Goldman GH, Malavazi I. 2016. Aspergillus fumigatus MADS-box transcription factor rlmA is required for regulation of the cell wall integrity and virulence. G3 Genes Genomes Genetics 6:2983–3002. https://doi.org/10.1534/g3.116.031112.

Anexo IV

Manuscrito - BRAUER, V. S.; PESSONI, A. M.; FREITAS, M. S.; CAVALCANTI-NETO, M. P.; RIES, L. N. A.; ALMEIDA, F. Chitin Biosynthesis in Aspergillus Species. **J Fungi (Basel)**, 9, n. 1, Jan 6 2023.

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Review

Chitin Biosynthesis in *Aspergillus* Species

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Abstract: The fungal cell wall (FCW) is a dynamic structure responsible for the maintenance of cellular homeostasis, and is essential for modulating the interaction of the fungus with its environment. It is composed of proteins, lipids, pigments and polysaccharides, including chitin. Chitin synthesis is catalyzed by chitin synthases (CS), and up to eight CS-encoding genes can be found in *Aspergillus* species. This review discusses in detail the chitin synthesis and regulation in *Aspergillus* species, and how manipulation of chitin synthesis pathways can modulate fungal growth, enzyme production, virulence and susceptibility to antifungal agents. More specifically, the metabolic steps involved in chitin biosynthesis are described with an emphasis on how the initiation of chitin biosynthesis remains unknown. A description of the classification, localization and transport of CS was also made. Chitin biosynthesis is shown to underlie a complex regulatory network, with extensive cross-talks existing between the different signaling pathways. Furthermore, pathways and recently identified regulators of chitin biosynthesis during the caspofungin paradoxical effect (CPE) are described. The effect of a chitin on the mammalian immune system is also discussed. Lastly, interference with chitin biosynthesis may also be beneficial for biotechnological applications. Even after more than 30 years of research, chitin biosynthesis remains a topic of current interest in mycology.

Keywords: *Aspergillus* spp.; chitin; chitin synthase; fungal cell wall; caspofungin paradoxical effect; calcineurin; protein kinase C; cell wall integrity pathway

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1. Introduction

The fungal cell wall (FCW) is a vital and dynamic structure [1], which accommodates changes in morphology, confers plasticity and protects against potentially harmful environmental factors such as osmotic pressure and temperature [1,2]. The FCW is therefore crucial in mediating the interaction of the fungus with the environment and contains a broad number of hydrolytic molecules, which play important roles in specific niche colonization [3]. The FCW composition changes depending on the fungal species. In filamentous fungi, the main CW components are polysaccharides such as glucans and chitin that represent 50–60% and 10–20%, respectively, of the FCW dry weight; as well as glycoproteins, that constitute 20–30% of the FCW dry weight [4]. These components are cross-linked, resulting in a complex network that constitutes the core and structural basis of the FCW [2]. Glucans confer tensile strength to the cell wall, and together with chitins and glycoproteins, they are responsible for the maintenance of the FCW shape [5]. In addition, chitin also confers rigidity to the cell wall, whereas glycoproteins can function in signal transduction, adhesion, absorption of molecules, protection against unfamiliar substances and synthesis and remodeling of the FCW [2,6].

Preceded only by cellulose, chitin is the second most abundant natural polysaccharide in nature [7] and the major component of crustacean shells, insect exoskeletons and FCWs,

including the fungal genus *Aspergillus* [7,8]. Chitin was first described in 1811 by Henri Braconnot from raw materials isolated from mushroom species such as *Agaricus volvaceus, A. acris, A. cantarellus, A. piperatus, Hydnum repandum, H. hybridum* and *Boletus viscidus* [8,9]. Chitin is a white and rigid homopolymer, composed of linear β-(1,4)-N-acetylglucosamine (GlcNAc) chains with approximately 6–7% of nitrogen [8]. There are 3 forms of chitin, called α, β and γ, which anneal to each other in different conformations during chitin synthesis. α-chitin, the most abundant form, is when the GlcNAc chains are ordered in an anti-parallel form, whereas parallel stacking of the chains gives rise to β-chitin. In contrast, γ-chitin consists of two or three polysaccharide chains in parallel orientation, whereas the third chain is in the anti-parallel order. γ-chitin is therefore considered a combination of α- and β-chitin [10,11]. The orientation of chitin chains results in an intermolecular hydrogen bond formation, creating fibers that are highly hydrophobic and have a strong tensile force [7,11,12], contributing to the FCW integrity [1].

The interaction between chitin and α-1,3-glucan results in the formation of a highly hydrophobic core in the FCW, which could accommodate pigment molecules, such as melanin [13]. This inner part of the FCW confers rigidity to the structure and is embedded in a hydrated and mobile matrix rich in β-1,3-, β-1,4- and β-1,6-glucans [13]. The structural role of FCW components was also confirmed by Chakraborty et al., 2021 [14], who used different mutant strains lacking chitin, α-1,3-glucan, galactosaminogalactan (GAG) or galactomannan (GM) linked to the β-1,3-glucan-chitin core. A thick cell wall is observed in mutants lacking GAG, GM and α-1,3-glucan. The production of the β-1,3-glucan is increased in the inner part of the FCW in response to the lack of the α-1,3-glucan. In addition, chitin fibril deposition is altered in this mutant lacking the α-1,3-glucan (Δ*ags1* Δ*ags2* Δ*ags3* triple mutant), resulting in a strain with increased rigidity in the FCW. The lack of chitin, as a result of the simultaneous deletion of *csmA*, *csmB*, *chsF* and *chsG* chitin synthase (CS) genes, results in a strain deficient in growth, with the inner domain of the FCW formed basically by α-1,3-glucan and β-1,3-glucan, with no chitin-β-glucan-GAG core formation. The lack of GM results in the absence of proteins and the compensatory increase in GAG and chitin, resulting in a highly hydrophobic and rigid cell wall. The GAG deficient strain does not present so many changes in the inner core of the FCW when compared to the WT strain, but the lack of GAG causes difficulties in water retention, which reflects on the rigidity of the cell wall [14]. This study illustrates the importance of the plasticity and rigidity of the FCW for fungal growth and development.

The genus *Aspergillus* is composed of filamentous saprophytic fungi that are ubiquitous in the soil and air [15]. Several species from this genus are relevant for different industries, including the pharmaceutical and medical sectors [16]. For example, *A. oryzae* and *A. sojae* are used for the production of oriental foods such as soy sauce or sake [17]; whereas *A. niger* is the main producer of citric acid, an organic acid that is used as an acidifier, a food flavoring and a chelating agent [18]. In contrast, *A. terreus* is a prolific producer of statin, a secondary metabolite and hypolipidemic agent, used to treat hypercholesterolemia [17,18]. *A. flavus* and *A. parasiticus* are important food spoilage organisms for a variety of edible crops. Their pathogenicity is characterized by aflatoxin production, a secondary metabolite with hepatotoxicity, teratogenicity and immunotoxicity actions that present a health hazard to humans and cattle [19]. Lastly, *A. fumigatus* is globally recognized as the most important opportunistic human filamentous fungal pathogen. It causes a wide range of diseases in patients with different types of immunodeficiencies, and some of these diseases are accompanied by mortality rates as high as 90% [20].

In this review, we will provide an overview of chitin biosynthesis in *Aspergillus* species while highlighting the need for additional studies to elucidate different aspects (mentioned hereafter) of chitin biosynthesis. We will discuss how chitin is biosynthesized before focusing on the classification and cellular roles of chitin synthases (CS) in different *Aspergillus* species. We will emphasize the importance of CS for fungal morphology, which is intrinsically linked to antifungal drug resistance and interactions with mammalian immune cells. Furthermore, we provide an in-depth discussion of stress- and morphology-

related signaling pathways that regulate chitin biosynthesis. Lastly, we will also discuss how CS gene manipulation can be beneficial for biotechnological applications that currently use *Aspergillus* species.

2. Chitin Biosynthesis

2.1. Metabolic Steps Leading to Chitin Biosynthesis

Chitin biosynthesis occurs through a process that is conserved in all organisms capable of synthesizing it. In general, the process consists of converting sugars such as glucose (or storage compounds such as glycogen) or the disaccharide trehalose into linear chitin chains [21,22]. These structures are subsequently secreted into the extracellular space, where they aggregate into microfibrils and are organized in the extracellular matrix, allowing the formation of structures such as fungal cell walls [23]. The chitin biosynthesis process is illustrated in Figure 1.

In fungi, the conversion of glycogen into glucose-1-phosphate by the action of the enzyme glycogen phosphorylase represents an important initial step in this process. Glucose-1-phosphate can (1) be converted into glycolysis intermediates or (2) serve as a precursor for trehalose biosynthesis [24]. Glucose-1-phosphate is converted to glucose-6-phosphate by phosphoglucomutase, generating a central glycolysis molecule, which has multiple destinations in cell metabolism [25,26]. In addition, the enzyme Uridine Triphosphate (UTP)-glucose-1-phosphate uridyltransferase catalyzes the production of Uridine Diphosphate (UDP)-glucose and pyrophosphate from glucose-1-phosphate and UDP, while releasing glucosyl residues [27]. Subsequently, the glucosyl residues are transferred to glucose-6-phosphate by trehalose-6-phosphate synthase, resulting in the production of trehalose-6-phosphate, which, when dephosphorylated, results in trehalose production [24]. The trehalose, in turn, can be hydrolyzed by the enzyme trehalase, generating glucose, which will feed into the glycolytic pathway, where it is phosphorylated by hexokinase and converted into glucose-6-phosphate. Subsequently, the glucose-6-phosphate isomerase converts glucose-6-phosphate into fructose-6-phosphate, a molecule that, in association with glutamine, forms the main precursors of UDP-GlcNAc [25] and represents the intersection between glycolysis and chitin biosynthesis.

The first and limiting step in chitin biosynthesis is the formation of glucosamine 6-phosphate from fructose 6-phosphate and glutamine, a reaction catalyzed by the enzyme glutamine-fructose-6-phosphate amidotransferase (GFAT). The importance of GFAT in the synthesis of chitin has been shown by GFAT-encoding gene (*gfa1*) expression analyses in different microorganisms and higher eukaryotes. In *S. cerevisiae*, genetically or chemically induced cell wall stress strongly induced the expression of *gfa1* [28]. Similarly, calcofluor white (CFW) and caspofungin (CP)-induced CW stress increased *gfaA* mRNA levels in *A. niger*, *Penicillium chrysogenum* and *Fusarium oxysporum*. An increase in chitin levels in the *A. niger* CW after incubation with CFW was also observed. These results support the induction of chitin biosynthesis as a general response mechanism to cell wall stress [29].

The second step includes the formation of N-acetylglucosamine (GlcNAc)-6-phosphate, through the donation of the acetyl group derived from coenzyme-A to Glycosamine-6-phosphate, by the action of the enzyme glucosamine-6-phosphate acetyltransferase (GNA1). Acetylation is characteristic of N-acetylglucosamine (GlcNAc) polymers, and it is one of the main differences in the structure and physicochemical properties when comparing chitin and cellulose molecules [30]. Computational studies have revealed the role of acetylation in the formation of hydrogen bonds, which is important for the crystalline structure of chitin, which confers a strong resistance to physical damage [31]. In vitro studies with *A. fumigatus* Δ*gna1* revealed that this strain was unable to grow in culture in the absence of exogenously added GlcNAc, suggesting that GNA1 is crucial for *A. fumigatus* survival, representing a potential target for the development of antifungal agents [32].

Subsequently, phosphate is transferred from C6 to C1 by the action of the enzyme phosphoacetylglucosamine mutase (termed Agm1 in *Aspergillus* spp.), resulting in the formation of N-acetylglucosamine (GlcNAc)-1-phosphate [25,33]. Agm1 was shown to be

an essential enzyme in *A. fumigatus* through the construction of a conditional mutant [34]. In this strain, the *agm1* open reading frame (ORF) was placed under the control of the *alcA* (alcohol dehydrogenase) promoter. In the presence of glucose (the repressive condition), *agm1* is repressed, whereas the removal of glucose and the addition of other carbon sources (the inductive condition), including ethanol, results in *agm1* transcription. The lack of *agm1* expression resulted in a thinner cell wall in both conidia and hyphae, with the conidia being unable to retain the melanin layer. Indeed, analysis of the cell wall in the *agm1* conditional mutants showed increased levels of α-glucan and β-glucan and lower levels of glycoproteins and chitin when compared to the wild-type strain. Interestingly, crystallography analyses revealed several amino acid changes near the substrate binding site in *A. fumigatus* AGM1 when compared to the human homologue, making this enzyme a potential target for the design of fungus-specific selective inhibitors [34].

The next step in chitin biosynthesis is catalyzed by UDP-GlcNAc pyrophosphorylase (UAP), a nucleotidyltransferase characteristic of the metabolism of aminosugars, which catalyzes the formation of UDP-GlcNAc through the uridylation of GlcNAc-1-phosphate. During this reaction, pyrophosphate is released, and UDP-N-acetylglucosamine, a substrate for CS, is generated [35,36]. In *A. fumigatus*, the *uap1* gene is essential, and a conditional mutant was constructed for additional characterization [34]. Upon *uap1* gene repression, a considerable reduction in cell growth, accompanied by structural changes in conidia and hyphae, including cell wall thinning and reduction of the content of α-glucan, chitin and GlcNAc in the mycelial cell wall was observed when compared to the WT strain. In addition, the presence of cell wall-interfering compounds such as Congo red, CFW, SDS and hygromycin B increased sensitivity to these reagents when *uap1* expression was repressed, suggesting that the reduction of *uap1* expression results in impaired cell wall integrity. Indeed, the mycelial α-glucan and chitin levels, as well as, GlcNAc were reduced by 36%, 11% and 23%, respectively, in the conditional mutant strain when compared to the WT strain, suggesting that Uap1 is crucial for the cell wall structure and integrity [34].

All aforementioned enzymatic steps occur in the cytoplasm [25], whereas all subsequent steps involving chitin polymerization occur in specialized microdomains of the membrane on the growing buds of yeast cells [37] and at hyphal tips [38]. The generated UDP-N-acetylglucosamine (GlcNAc) molecules serve as a substrate for CS, which will catalyze the transfer of the sugar moiety of UDP-GlcNAc and promote the formation of growing chitin chains. CS is considered the only enzyme specifically committed to chitin biosynthesis and, for this reason, has been described as the key enzyme in this process [25,39]. CSs are integral membrane-bound glycosyltransferases, that mediate the transfer of GlcNAc from the nucleotide uridine diphosphate (UDP)-GlcNAc to a chitin chain in linear expansion, subsequently releasing UDP as a by-product, which requires a divalent ion for its activity [2,25,40–42]. CSs are located in the Golgi complex and plasmatic membranes, as well as in chitosomes, which are intracellular vesicles responsible for transporting these enzymes from the endoplasmic reticulum to the cell surface [41,43–45]. They are crucial for fungal growth and developmental processes, being intrinsically associated with the cell wall, mechanisms of morphogenesis, growth and hyphal differentiation and conidia formation [41,46].

2.2. Initiation of Chitin Biosynthesis: An Unknown Mechanism

The molecular mechanisms involved in chitin synthesis initiation and deposition still require further investigation due to the lack of structural data as well as knowledge about post-catalytic events [47]. The biochemistry and reaction mechanisms that underlie chitin biosynthesis were mainly obtained from CS and related glycosyltransferase studies [25]. The currently proposed CS enzymatic mechanism involves the transfer of GlcNAc from the donor sugar nucleotide UDP-GlcNAc to the non-reducing end of an extended glycan chain. The process takes place through an SN2-like displacement reaction. The C4 hydroxyl group at the non-reducing end of the acceptor glycan attacks the anomeric C1 of the sugar bound to the donor UDP, releasing UDP. The polymerization involves a reaction mechanism

where the nucleophilic attack by the acceptor hydroxyl group results in an inversion of the anomeric carbon of the donor substrate [48–50].

How polymerization starts during chitin synthesis has not been fully elucidated. Hypotheses suggesting the requirement for soluble or covalently bonded primers for polymerization initiation remain unproven [51]. Chitin polymerization may be mediated by CS itself or by an associated protein when using glycogen as a primer [45,52]. Possible initiation mechanisms involve the generation of a UDP-linked disaccharide, resulting from a reaction between two UDP-linked saccharides, or monosaccharide transfer from a UDP sugar to some low molecular weight initiator molecule. However, given that the UDP-linked disaccharide represents an initiation product characteristic of a reducing-end extension mechanism, this hypothesis conflicts with the framework-based model for elongation of the non-reducing end chain [53]. Gyore et al., 2014 used different strains of *Saccharomyces cerevisiae*, which expressed a single chitin synthase, Chs2, to demonstrate that the formation of chitin oligosaccharide (CO) chains by these enzymes can be stimulated by the addition of GlcNAc and 2-acylamido analogues, and that the latter molecules act as acceptors for the transfer of GlcNAc from UDP-GlcNAc. In addition, the study revealed that 2-acylamido analogues can stimulate the synthesis of insoluble chitin. The authors conclude that CS uses GlcNAc analogues as primers and CO formation occurs by transferring GlcNAc units at a time. The study strongly suggests that GlcNAc itself can initiate the formation of CO and chitin in vitro [54]. Similar studies have not yet been conducted in *Aspergillus* spp.

Once the cell wall space has been reached, hydrogen bonds occur between chitin chains, and microfibril structures are formed, allowing subsequent crystallization [2] that associates with other extracellular components, resulting in the formation of the FCW [55]. Before the chitin chains are organized into microfibrils and deposited on the cell surface, they must first be translocated across the plasma membrane [56]. The biochemical and ultrastructural data from the fungal systems revealed that the catalytic machinery involved in chitin chain translocation is composed of an assembly of tightly packed membrane-bound polymerizing enzymes with cytoplasmic exposure of the catalytic site [57,58] and, as a consequence, the translocation of chains of chitin can occur from the intracellular domain outwards. However, the mechanism of translocation across plasma membranes has not been fully elucidated, and some hypothetical models have been proposed [55,59] (Figure 1).

One of these hypothetical models is based on the structural organization and topographical location of cellulose synthase, an enzyme analogous to CS. Transmission microscopy analysis of freeze-fractured membranes revealed that cellulose synthase has a hexagonal configuration composed of six particles, called "Rosetta" structures [60–62]. These structures show transmembrane segments constituting the cellulose synthesis machinery. In addition, it is observed that these protein complexes are organized in the shape of a pore through which, supposedly, the cellulose chains are translocated. Data obtained from the analysis of the enzyme hyaluronan synthase, a glycosyltransferase that catalyzes the transfer of repeated disaccharide units of hyaluronic acid and GlcNAc, and subsequent formation of the biopolymer hyaluronan, also suggest that the transmembrane domains present in these structures present a pore-like arrangement [63]. These studies lend theoretical support and corroborate the hypothesis of the formation of a pore-shaped complex in studies that show the presence of CS transmembrane domains integrated into the cell membrane of different organisms; although the actual structure of the complex catalyzing chitin chain translocation remains to be defined [55].

Recently, structural and functional data based on Chs1 from *Phytophthora soybean*, a pathogenic oomycete that causes root and stem rot in soybean, were found to contribute to the mechanistic understanding of chitin biosynthesis at the atomic level [64]. The analysis of *Ps*Chs1 by cryo-electron microscopy (cryo-EM) identified five structures that reflected different states of the enzyme: apo, linked to the substrate (UDP-GlcNAc), catalytic state, linked to the nascent chitin oligomer, and the product release state. In the apo state, the channel that connects the extracellular side of the membrane with the intracellular reaction

chamber of the CS is blocked by a loop that controls chitin access to the reaction chamber. Chitin synthesis initiates when UDP-GlcNAc binds to the substrate-binding site, followed by its hydrolysis, releasing GlcNAc, or when the substrate is exogenously added to the system. This first GlcNAc molecule remains trapped inside the chitin-translocating channel, whereas the second added GlcNAc molecule is at the entrance and extends outside of the channel. As a result, the "loop gate" that blocked the chitin-translocation channel opens. In addition, these changes in loop conformation also prevent the donor substrate from leaving before it can bind to a growing chitin oligomer, and direct the new chitin chain towards the extracellular side of the plasma membrane. Finally, after many reactions, the enzyme changes its conformation to the "post-released state", where the chitin chain is released from the CS. Subsequently, the loop gate blocks the chitin-translocated channel again, until a new substrate comes, re-starting the cycle of chitin chain biosynthesis [64].

An alternative hypothesis for chitin chain export involves the participation of microvesicular organelles called chitosomes. They are vesicles with a diameter of approximately 40–70 nm, supposedly originating from the endoplasmic reticulum and Golgi complex. It is suggested that chitosomes contain multi-units of the CS polymerizing enzyme [44] and for this reason, they would have an important role in the processes of trafficking individual or packaged CS units [55] as well as in the synthesis of chitin polymers [65,66]. However, the idea that the chitosomal compartment could function as a vehicle for CS clusters and assist in the translocation of chitin chains does not represent a plausible strategy, since the fusion of the chitosomal vesicle with plasma membranes does not provide a solution as to how chitin chains are translocated across the plasma membrane. Among the limitations of the hypothesis is the size of the vesicles, which are too small to store chitin chains. In addition, the entry of substrate molecules into the vesicles would require the existence of specific transport mechanisms in the membranes of chitosomes [55,67].

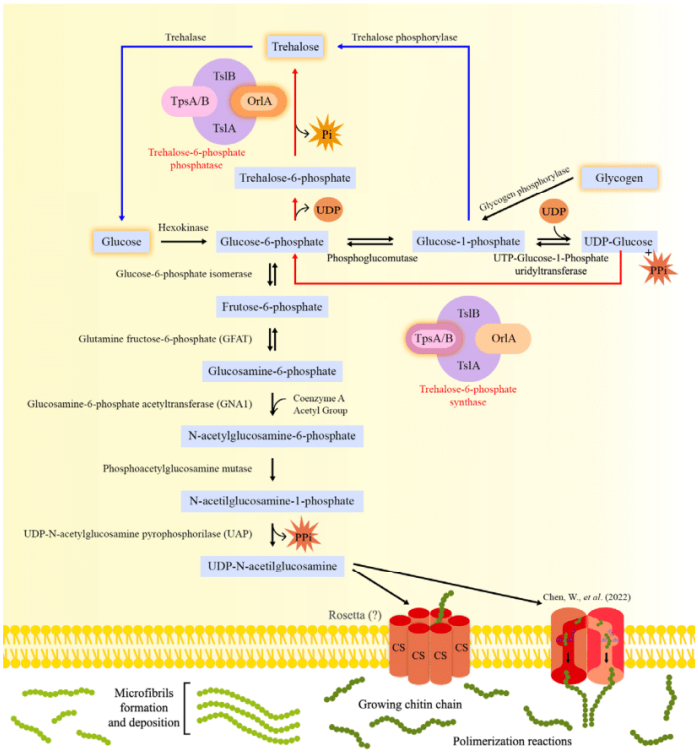

Figure 1. The chitin biosynthesis pathway in *Aspergillus.* Glucose-6-phosphate (G6P) is the precursor for UDP-N-acetylglucosamine (UDP-N-GlcNAc), the main building block of fungal chitin. Glucose is

first converted to G6P by hexokinase and then to fructose-6-phosphate (F6P) by G6P isomerase, before an additional 4 enzymatic steps result in the biosynthesis of UDP-N-GlcNAc. The first two steps in UDP-N-GlcNAc biosynthesis are the same as in the glycolytic pathway. The generation of F6P represents the intersection between the glycolytic pathway and chitin synthesis. F6P is converted to glucosamine-6-phosphate by the enzyme glutamine-fructose-6-phosphate (GFAT), marking the beginning of the first and limiting stage of chitin biosynthesis. The enzyme Glucosamine-6-phosphate acetyltransferase (GNA1) catalyzes the addition of an acetyl group, derived from acetyl-CoA, to glucosamine-6-phosphate generating N-acetylglucosamine-6-phosphate, which is then converted into N-acetylglucosamine-1-phosphate by phosphoacetylglucosamine mutase (AGM-1). The next step involves the enzyme UDP-GlcNAc pyrophosphorylase (UAP), which catalyzes the uridylation of GlcNAc-1-phosphate and the formation of UDP-N-GlcNAc, releasing pyrophosphate as a by-product. Chitin synthase (CS), an enzyme found in specialized microdomains of the plasma membrane, uses UDP-N-GlcNAc as a substrate in order to transfer GlcNAc (N-acetylglucosamine) from UDP-GlcNAc to the emerging chitin chains. The growing chitin chains must be translocated across the plasma membrane from the intracellular domain to the outside, a mechanism that remains to be fully elucidated but may involve the participation of molecular complexes termed "Rosettas". Chen et al., 2022 [64] recently suggested a different mechanism for chitin chain translocation, whereby the CSs present five different conformations: apo, linked to the substrate (UDP-GlcNAc), catalytic, linked to the nascent chitin oligomer, and the product release state. CSs were shown to form a loop that functions as a gate and that prevents the donor substrate from leaving the reaction site without being bound to a growing chitin oligomer. Furthermore, these CSs also direct the new chitin chain to the extracellular space. After reaching the cell wall space, hydrogen bonds form between chitin chains, causing them to be organized into microfibrils. The microfibrils subsequently crystallize in association with other extracellular components, resulting in the formation of the fungal cell wall. In addition, the intracellular carbon storage compounds trehalose and glycogen can also be converted to glucose or G6P, respectively, through a series of enzymatic reactions. The trehalase converts trehalose to glucose, whereas the glycogen is first converted to glucose-1-phosphate (G1P) and then to G6P by the enzymes glycogen phosphorylase and phosphoglucomutase. Furthermore, G1P and G6P derived from glucose or glycogen, respectively, function as precursors for trehalose. G1P is directly converted to trehalose by trehalose phosphorylase, whereas G6P is converted to trehalose by two enzymatic steps involving the large enzymatic complex Trehalose-6-phosphate phosphatase. Red lines: represent the pathway of trehalose production through the transference of glucosyl residues to G6P by the enzyme trehalose-6-phosphate synthase (described in red letters on the figure), releasing trehalose-6-phosphate, that it will be dephosphorylated by trehalose-6-phosphate phosphatase (described in red letters on the figure), forming trehalose. Blue lines: represent the trehalose production through G1P.

3. Chitin Synthases in *Aspergillus* Species

Reverse genetic assays, including CS gene disruptions, deletions, complementation and interference RNA (RNAi) studies [68–73], showed that different CS genes are expressed during different fungal cell cycle stages, at different sites of fungal structures during vegetative growth, and can be induced by different environmental conditions, indicating distinct functionality between them [74,75].

Figure 2 compares the protein sequences of *Aspergillus* spp. orthologous CS-encoding genes. Orthologs perform similar functions between species, although the reverse is not always true [76], and we will see in the next topic that similar functions can be attributed to CS gene orthologs.

3.1. Classification of Chitin Synthases (CS)-Encoding Genes

In *S. cerevisae*, the three CS genes *chs1, chs2 and chs3* are well described [41], where they encode enzymes with distinct functions during cell wall expansion, budding and septum formation [77]. CS genes from *S. cerevisiae* are expressed in a regulated manner during the different phases of the cell cycle [78], with increased expression of *chs1* in the M/G1

phase, *chs2* in the M phase and *chs3* right before cell division [79]. During early growth and cell budding, chitin synthesis is required at the bud tip; after the budding process and during isotropic growth, chitin is distributed all across the nascent fungal cell [80]. Following nuclear cell division, chitin biosynthesis is directed to the mother-bud neck, at the site of the bud scars [81]. Yeast CS gene expression is required on the bud site in order to restart the yeast growth/division cycle [82]. The situation is more complex in filamentous fungi, where chitin synthesis is governed by a higher number of CSs, since chitin content in filamentous fungi is significantly higher (10–20%) when compared to *S. cerevisiae* (1–2%) and *Candida albicans* (2–5%) [4,80]. Filamentous fungi undergo a more complex vegetative growth and exhibit elaborate sexual cell cycles, requiring a different demand for chitin synthesis, which is reflected in a differential expression of CS genes at distinct fungal sites, such as the apical tip or during septum formation [25,79,83].

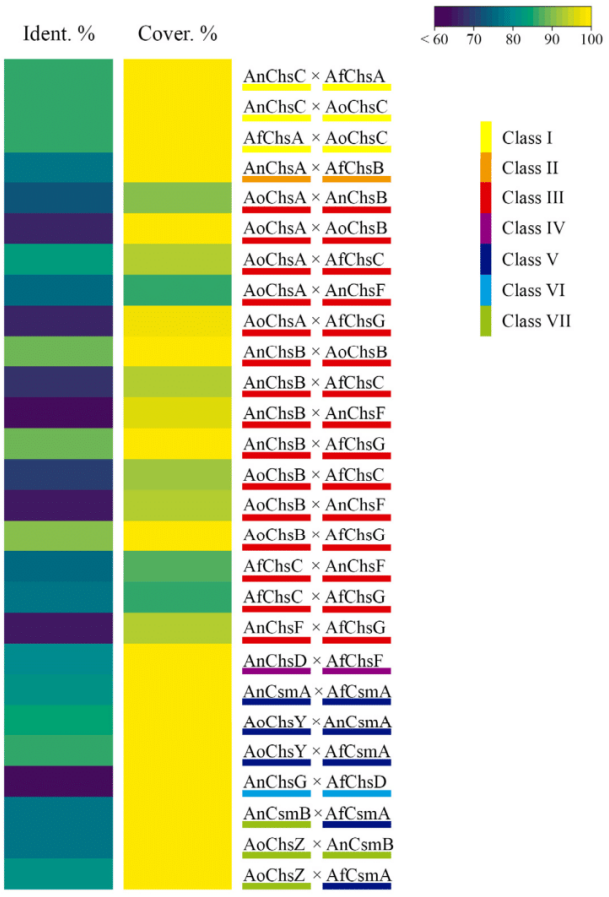

Figure 2. Chitin synthases (CS) of different classes are highly conserved amongst *Aspergillus* species. A heat map depicting the percentage (%) of identity and coverage between protein sequences of CS from the same classes of different *Aspergillus* species. The protein sequences were retrieved from the FungiDB database before pairs were submitted to BlastP for alignment analysis (BLAST Tool NCBI). An, *Aspergillus nidulans*; Af, *Aspergillus fumigatus*; Ao, *Aspergillus oryzae*. On the right, different coloured bars underlining the *Aspergillus* CSs are compared with each other, indicating the class of CS. On the left, heat maps depicting the percentage of identity and coverage between two CS protein sequences from different *Aspergillus* species.

CS can be classified into divisions and classes (Table 1) based on their protein sequence, phylogenetic position and similarities in catalytic domains [84,85]. Several studies have

been published about CS classification, and as a consequence, the nomenclature became heterogenous, with differences in the number of divisions and classes [3,41,86–89]. In an attempt to standardize and update CS classification, Gonçalves and colleagues (2016) performed a multi-species comparative analysis (eukaryotes, bacteria and viruses) and showed that fungal CSs are grouped into two divisions (1 and 2), supporting the study carried out by Niño-Vega et al., 2004 [85,88]. CSs from division 1, which include classes I, II and III, have a PF01644 domain, a type 1 CS catalytic domain (CS1) and a PF08407 CS N-terminal domain (CS1N). The members of division 2, which include classes IV, V and VII, comprise enzymes with a catalytic domain preceded by the PF00173 domain, which is a cytochrome b5-like binding domain with a predicted function of binding to lipid ligands [83,85]. Furthermore, they also possess a type 2 CS domain 2 (PF03142) and a DEK C-terminal domain (PF08766) with unknown functions. Classes V and VII of CSs further have an N-terminal myosin-motor such as the PF00063 domain [90], which is associated with the intracellular trafficking of the CS and the site of chitin secretion [91]. CSs of class VI are the only enzymes to have a PF03142 domain [84,90], and they are clustered separately from the other CS classes. Ninõ-Vega et al., 2004 classified the class VI CS into Division 2, but Gonçalves et al., 2016 suggested that a recombination event between two family 2 glycosyltransferases might be the origin of this class of CS. Class VI CS protein sequences have a duplication of the QXXEY motif, a C-terminal region similar to other CSs, and an N-terminal region similar to the one found in hyaluronan synthase [85]. Alternatively, the ancestor of this class of CS underwent accelerated evolution, a process that blurred the phylogenetic signal [85]. Due to the two aforementioned hypotheses and the fact that no CS activity has been shown for this class of CS, these CSs are termed "recCS" (recombined CS) [85].

Table 1. Chitin Synthase genes on *Aspergillus* species classified into divisions and classes.

Aspergillus Specie	Chitin Synthase Gene	ID Gene [#]	Alias	Division	Class
A. nidulans	*chsA*	AN7032	-	1	II [68]
	chsB	AN2523	-	1	III [68]
	chsC	AN4566	-	1	I [69]
	chsD	AN1555	*chsE*	2	IV [43,92]
	chsF	AN4367	-	1	III [93]
	chsG	AN1046	-	2	VI [79]
	csmA	AN6318	*chsD*	2	V [94]
	csmB	AN6317	-	2	VII [95]
A. fumigatus	*chsA*	Afu2g01870	-	1	I [70]
	chsB	Afu4g04180	-	1	II [70]
	chsC	Afu5g00760	-	1	III [70]
	chsD	Afu1g12600	-	2	VI [96]
	chsF	Afu8g05630	-	2	IV [70,93]
	chsG	Afu3g14420	-	1	III [97]
	csmA	Afu2g13440	*chsE*	2	V [71,98,99]
	csmB	Afu2g13430	-	2	VII [71]
A. oryzae	*chsA*	AO090012000084	-	1	III [100]
	chsB	AO090701000589	-	1	III [72]
	chsC	AO090011000449	-	1	I [72]
	chsY	AO090026000323	-	2	V [100]
	chsZ	AO090026000321	-	2	VII [100]
A. niger	*chsC*	An8g04350	-	1	III [73]
	chsF	An12g10380	-	1	III [101,102]

[#] The gene IDs were extracted from the database FungiDB and were adopted during the review as CS gene designations.

The number of CS genes varies depending on the fungal species, with eight CS genes being present in the genome of *A. nidulans*, *A. fumigatus* and *A. niger*, and five CS genes encoded in the genome of *A. oryzae*. Table 1 lists each gene from these species, together with

gene IDs according to the FungiDB database, whereas division and classes are according to Niño-Vega et al., 2004. We subsequently adopted this nomenclature in this review.

3.2. Aspergillus Mutants of CS Genes from Division 1: Morphological Features

In *A. nidulans*, deletion of the class I CS *AnchsC* resulted in a strain with no defects in growth, fungal morphology and conidiation [69]. This is similar to the *A. fumigatus* class I *chsA* (Afu2g01870) deletion strain [103]. In *A. nidulans* and *A. fumigatus*, deletion of the class II CSs Δ*AnchsA* and Δ*AfchsB* did not result in any growth defects, although the Δ*AnchsA* strain has defects in conidiation and a significantly decreased hyphal chitin content [68,103,104]. The double Δ*AnchsA* Δ*AnchsC* mutant presented growth and morphological defects when compared to the respective single deletion strains [105]. In addition, this strain was highly sensitive to SDS (sodium dodecyl sulphate), chitin-binding dyes and chitin synthase inhibitors [105]. Furthermore, the Δ*AnchsA* Δ*AnchsC* strain presented a heterogeneous and dense lateral cell wall, electron-transparent regions situated immediately outside the membrane, denser septa and large septal spores when compared to the WT and single deletion strains, suggesting abnormalities in the hyphal cell wall [106]. Together, these results suggest that the *An*ChsA and *An*ChsC CSs have overlapping functions during the conidiation process [93] and cell wall development as well as having redundant functions in septum formation and development [105,106].

Deletion of the class III CS *An*ChsB (AN2523) resulted in a strain unable to form colonies and conidia and to sustain hyphal growth in the presence of an osmotic stabilizer [68,107]. A similar phenotype was also observed when the promoter region of *AnchsB* was substituted by the *alcA* promoter (*alcA(p)*::*AnchsB*). Under repressing conditions, the *alcA(p)*::*AnchsB* strain presented growth defects, hyphal morphological abnormalities and abnormal asexual development [108], suggesting that this CS is important for all developmental stages of the fungal life cycle. A similar functional activity of *An*chsB orthologs during hyphal development, growth and conidiation was also observed in *A. fumigatus* [Afu3g14420 (ChsG] and *A. oryzae* [AO090701000589 (ChsB)]. *Af*ChsG and *Ao*ChsB are approximately 89% identical to *An*ChsB at the protein level (Figure 2). Deletion of the respective genes resulted in strains exhibiting compact colonies, hyperbranching hyphae, and aberrant conidia formation [72,97,103,109]. In addition, Δ*AfchsG* conidia did not mature and presented a disorganized cell wall with a poorly adherent layer of melanin and showed decreased levels in GlcNAc. Moreover, significantly decreased CS activity was observed for this deletion strain when compared to the WT strain, suggesting that *Af*ChsG is the major contributor of CS activity in *A. fumigatus* [97,103,109]. Deletion of *AfchsC* (Afu5g00760), encoding an additional class III CS in *A. fumigatus* and with a 76.94% similarity to *AfchsG*, did not cause obvious morphological and growth abnormalities, although a reduction of the GlcNAc content in the conidia cell wall was observed [97,103]. The simultaneous deletion of *AfchsG* and *AfchsC* led to decreased colony and conidiophore length, increased hyphal density, and decreased CS activity when compared to the WT strain [97], suggesting that, *Af*ChsG is also important for *A. fumigatus* growth and conidiation.

The genetic relationship between *AnchsA* and *AnchsB*, and *AnchsC* and *AnchsB*, using the *alcA(p)*::*AnchsB* strain [110] was also analyzed with regards to hyphal morphology, growth rate and chitin content [111]. The *alcA(p)*::*AnchsB* Δ*AnchsA* strain presented similar growth when compared to the *alcA(p)*::*AnchsB* strain, but the colony color was dark brown and fewer aerial hyphae were produced. In addition, the *alcA(p)*::*AnchsB* Δ*AnchsA* strain presented increased branching when compared to the parental strains. These results suggest that both *A. nidulans* ChsB (class III) and ChsA (class II) have distinct roles and do not have redundant functions during growth and development. The *alcA(p)*::*AnchsB* Δ*AnchsC* strain also presented similar growth when compared to the *alcA(p)*::*AnchsB* strain, although the hyphal mass was decreased in the periphery of the colony. In addition, the hyphae showed a disorganized pattern, and the sparse growth at the periphery of the colony was abolished by the addition of CFW and Congo red, resulting in a decrease in colony diameter when compared to the parental strains. These studies suggest that the *A. nidulans* CSs ChsB (Class

III) and ChsC (class I) have distinct and redundant (deletion of *chsC* alone does not affect growth and morphology) functions during fungal growth and hyphal morphogenesis. Supporting these results is the observation that chitin content was significantly increased in both the *alcA(p)*::*AnchsB* Δ*AnchsA* and *alcA(p)*::*AnchsB* Δ*AnchsC* strains, suggesting that the simultaneous absence of two CSs resulted in a different cell wall composition. Subsequently, when *AnchsA* was expressed ectopically under the control of the *AnchsB* promoter, before growth rates, colony and hyphal morphologies were determined and found to be similar to the parental strain, suggesting that *An*ChsA and *An*ChsB have non-redundant and non-overlapping functions [111].

With the aim to clarify the function of each *A. fumigatus* CS from division 1, Muszkieta and colleagues (2014) constructed single and multiple CS deletion strains. The Δ*AfchsG* (Class III) and the Δ*AfchsA* (Class I) Δ*AfchsC* (Class III) Δ*AfchsB* (Class II) Δ*AfchsG* (Class III) strains had reduced growth when compared to the WT strain, and together with the Δ*AfchsA* Δ*AfchsC* and Δ*AfchsA* Δ*AfchsC* Δ*AfchsB* strains also presented increased hyphal branching. Furthermore, the Δ*AfchsA* Δ*AfchsC* Δ*AfchsB* Δ*AfchsG* strain produced abnormal conidiophores and significantly fewer conidia, even in the presence of KCl, when compared to the single deletion and WT strains. Conidia from the Δ*AfchsG* and Δ*AfchsA* Δ*AfchsC* Δ*AfchsB* Δ*AfchsG* strains were swollen, with thin, disorganized cell walls and a melanin layer that was loosely attached to the adjacent cell wall. In addition, the Δ*AfchsG* and Δ*AfchsA* Δ*AfchsC* Δ*AfchsB* Δ*AfchsG* strains presented significantly reduced CS activity when compared to the WT strain. No significant reduction in GlcNac was observed for all mutant strains, but a reorganization of the mycelial cell wall is predicted to occur, as levels of β-1,3-glucan decreased and α-1,3-glucan concentrations increased in all single deletion strains and the Δ*AfchsA* Δ*AfchsC* Δ*AfchsB* Δ*AfchsG* strains. In contrast, GlcNac concentrations were significantly reduced in the cell wall of conidia in all single deletion strains and in the Δ*AfchsA* Δ*AfchsC* Δ*AfchsB* Δ*AfchsG* strain. Together, these results suggest that (i) the *Af*ChsG CS accounts for a large proportion of total CS activity and chitin biosynthesis and (ii) that the other division 1 CSs have less important and/or redundant roles in chitin biosynthesis during asexual development in *A. fumigatus* [103]. This is in contrast with *A. nidulans*, where CS have both distinct and redundant roles; although it is difficult to draw this comparison as a strain deleted for all division 1 CSs has not been generated in *A. nidulans*. Figure 3 summarizes CS gene functions during the fungal development.

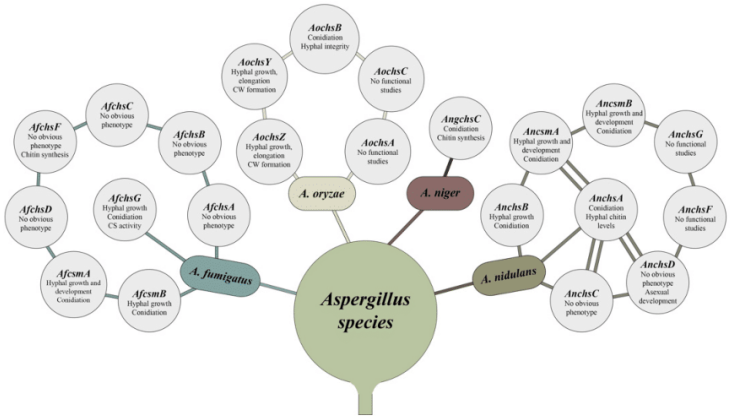

Figure 3. An overview of the functions of chitin synthases (CS) in different *Aspergillus* species. The functional description is based on studies using the respective gene deletion strain. The diagram also depicts CS whose gene deletion has not been carried out and, thus, where no functional studies were performed. Our understanding of the role of CS in *Aspergillus* species is mainly derived from *A. nidulans* and *A. fumigatus*. Overlapping functions between *AnchsA* and *AnchsC* in hyphal growth and conidiation, between *AnchsA* and *AnchsD* in conidiation and between *AnchsA* and *AncsmA* in hyphal integrity under low osmotic conditions in *A. nidulans* are depicted with a double line.

3.3. Aspergillus Mutants of CS Genes from Division 2: Morphological Features

Deletion of class IV CS did not result in obvious phenotypes. The Δ*AnchsD* (AN1555) strain presented no defect in the production of conidia, sexual structures such as ascospores and cleistothecia, cell growth and morphology [43,92]. The Δ*AnchsD* strain had a 35% reduction in chitin content when compared to the WT strain, irrespective of whether an osmotic stabilizer was added or not [43]. A similar phenotype was also observed for the *A. fumigatus AfchsF* (Afu8g05630) deletion strain, coding for a CS with 79.62% of identity to *An*ChsD. No defects in conidiation, growth and hyphal morphology were observed, although a reduction in mycelial chitin content was observed for this mutant strain [103]. These results suggest that *Aspergillus* can accommodate a certain loss of cell wall chitin or perhaps compensate for the loss of chitin by increasing the concentration of other cell wall polysaccharides as is the case during the caspofungin paradoxical effect (CPE, see below).

The simultaneous deletion of genes encoding *An*ChsD and the class I CS *An*ChsA, resulted in a strain with reduced conidia formation, suggesting that *An*ChsA and *An*ChsD have redundant functions during conidia formation in *A. nidulans* [68,92,104]. Furthermore, fewer conidiophores were also observed under repressing conditions when the promoter region of *AnchsB* was replaced with the *alcA* promoter in the Δ*AnchsD* background strain [110]. Reduced growth rates and abnormal hyphae were also observed, suggesting that the lack of *An*ChsB increases the functional importance of *An*ChsD during growth (mainly in high osmolarity conditions) and during asexual reproduction [110].

3.3.1. Chitin Synthases with Myosin-Motor Like (MMD) and Chitin Synthase Domains (CSD)

CSs from class V (*An*CsmA, *Af*CsmA, *Ao*ChsY) and class VII (*An*CsmB, *Af*CsmB, *Ao*ChsY) contain an N-terminal myosin-motor like domain (MMD), in addition to the C-terminal chitin synthase domain (CSD).

The role of the *An*CsmA MMD in cell wall assembly and integrity was investigated through the construction of several different mutants, including a deletion mutant, the N630 strain (deletion of the 1220 C-terminal amino acids, expressing only the 630 N-terminal amino acids); the CH5 and CH9 strains, where the CS domain was put under the regulatory control of the *alcA* promoter; the CS3 and CS5 strains, where the entire protein was under the regulatory control of the *alcA* promoter; as well as the Δ*AnchsD* (Class IV) Δ*AncsmA* (DM-3) strain [112]. The Δ*AncsmA* strain did not form cleistothecia during self-mating, aerial mycelia, conidiophores and conidia, independent of the presence of an osmotic stabilizer [43]. In addition, the Δ*AncsmA* strain presented aberrant growth, abnormal hyphal morphology with balloon-like structures and intrahyphal hyphae features, and abnormal septum formation. The hyphal balloon shape and intrahyphal hyphae were also seen when the CH5 strain was cultivated in *AncsmA* inducible conditions, suggesting that the *An*CsmA CS is important for the maintenance of the hyphal cell wall and polarized cell wall synthesis, but that the MMD is dispensable for the formation of normal hyphae. No phenotypical differences were observed between the Δ*AncsmA* and Δ*AnchsD* Δ*AncsmA* strains, with the latter also presenting hyphae with morphological abnormalities; thus, suggesting an overlapping function between these CSs. In agreement with the function of *An*CsmA in cell wall integrity, Yamada et al., 2005 showed that *An*CsmA and *An*ChsA have overlapping functions in maintaining hyphal cell wall integrity mainly in the presence of low osmotic conditions. In addition, the growth of the triple deletion mutant Δ*AnchsA* Δ*AnchsC* Δ*AncsmA* was extremely slow, accompanied by abnormal hyphal morphology, abnormal chitin deposition and irregular septa distribution in low osmotic conditions. Furthermore, an increase in mRNA levels of *AncsmA* in high osmotic conditions was observed for the Δ*AnchsA* Δ*AnchsC* strain when compared to the WT, Δ*AnchsA* and Δ*AnchsC* strains. Altogether, these data suggest that *An*CsmA is important for polarized growth and also may have a role in cell wall repair and maintenance, as mRNA levels of *csmA* are expressed in the presence of osmotic stress [113].

With 82.13% of identity, the *A. fumigatus Af*CsmA (Afu2g13440) CS is orthologous to *An*CsmA. Deletion of *AfcsmA* resulted in a strain with reduced growth when compared to the parental strain [103]. In addition, the Δ*AfcsmA* strain exhibited 70–80% of intrahyphal growth, a decreased sporulation process, conidia with permeable cell walls, less chitin content and a reduced number of conidiophores with altered morphology. Medium supplemented with osmotic stabilizer agents (such as KCl or sucrose), partially restored conidiation of this strain [71]. Alsteens and colleagues (2013) subsequently analyzed architecture, hydrophobicity and polysaccharide concentrations of cell walls from conidia of this mutant strain. In contrast to *A. fumigatus* WT conidia, conidia from the Δ*AfcsmA* strain had low amounts of homogenous rodlets and presented an amorphous and granular surface, with tip-induced alterations, suggesting that Δ*AfcsmA* conidia are more fragile [108].

Some of the growth and developmental abnormalities observed for the Δ*AfcsmA* strain were similar to the Δ*AfchsG* strain, deleted for a class III CS-encoding gene. The double deletion of Δ*AfcsmA* and Δ*AfchsG* strains presented decreased radial growth when compared to the WT and Δ*AfcsmA* strains but grew similarly than with the Δ*AfchsG* strain. In addition, colonies from the double deletion strain were more compact, with increased hyphal branching and severely decreased sporulation in the absence of the osmotic stabilizers KCl or sorbitol. Conidia of the Δ*AfcsmA* and Δ*AfchsG* strains were spherical and pear-shaped, respectively, when compared to conidia of the WT strain, and germination rates were significantly increased. Furthermore, chitin synthase activity decreased, and chitin content was also severely reduced in the double deletion strain when compared to the parental strain. The reduction in chitin resulted in an increase in other cell wall polysaccharides, such as α-glucan. It is possible that the genetic reduction in chitin destabilized the cell wall glucan content and structures, highlighting the importance of CsmA and ChsG for *A. fumigatus* hyphal growth, development and sporulation [109].

A. oryzae also contains a CS-encoding gene, *chsY* (AO090026000323), that has high similarity at the protein level with *An*CsmA (83.97%) and with *Af*CsmA (86.23%). This gene codes for class V CS, and is predicted to be important for hyphal growth, elongation and cell formation. Expression of *AochsY* occurred mainly in liquid cultures with complete medium, whereas transcription of this gene was reduced in solid and minimal medium [100]. To date, no studies investigating the role of *AochsY* in fungal growth and development have been carried out.

The CS from class VII, are encoded by the genes *AncsmB* (AN6317) in *A. nidulans*, *AfcsmB* (Afu2g13430) in *A. fumigatus*, and *AochsZ* (AO09002600321) in *A. oryzae*. The CSD is highly conserved between the species, when compared to the MMD. The CSD from *An*CsmB has 55% similarity with the class V CSD from *An*CsmA [114]. Similarly, in *A. fumigatus*, the *Af*CsmB CSD has 60% similarity with the class V *Af*CsmA CSD [71]. In contrast, MMD significantly differs between class VII CS, with MMD from *An*CsmB and *An*CsmA having 21% similarity with each other [114], and 11% similarity between MMD from *Af*CsmA and *Af*CsmB [71]. The MMD domain from class VII CS is smaller than the MMD from class V CS, and the ATP-binding motifs are not conserved [100,114]; hence the classification of *An*CsmB, *Af*CsmB and *Ao*ChsZ as class VII of CS.

Intrigued by the similarities and differences of the MMD and CSD from classes V and VII CS, Tsuizaki and colleagues, 2013, constructed strains that produce chimeric proteins in order to further investigate the function of these domains. They fused the MMD from *An*CsmA to the CSD from *An*CsmB in a strain termed MACB, and fused the MMD from *An*CsmB to the CSD from *An*CsmA in a strain named MBCA. Constructions were placed under the control of the *An*CsmA or *An*CsmB promoter regions in strains that were deleted for the open reading frame (ORF) of either *An*CsmA (strains were termed MACBΔA1 and MBCAΔA1) or *An*CsmB (strains were named MACBΔB1 and MBCAΔB1). The MACBΔA1 strain had increased chitin content, balloon-shaped hyphae with increased lysis at subapical regions, and presented growth rates that were significantly reduced when compared to the WT. In contrast, no differences in growth rates between the MBCAΔA1 and WT strains were observed, although the mutant strain presented some hyphal abnormalities and

reduced conidiation. These results suggest that the *An*CsmB CSD cannot substitute for the *An*CsmA CSD, but that the MMDs have similar functions in both CSs. The MACBΔB1 strain presented no differences in growth rate, chitin content or hyphal structures when compared to the WT strain. Similar to the *An*CsmB deletion strain, the MBCAΔB1 strain manifested severely reduced growth, and exhibited balloon-shaped hyphae, brown clumps and lysis at hyphal subapical regions. Again, these results show that the MMDs fulfill similar roles in both fungi, but that, interestingly, the CSDs have different, non-overlapping functions in both CS proteins. Classes V and VII of CS, significantly differ, despite similar amino acid sequences, in the function of their CSD [115].

In *A. nidulans*, deletion of the *An*CsmB MMD resulted in a strain with reduced growth and balloon-like hyphal structures [114]. The absence of the *An*CsmB MMD inhibited precipitation of *An*CsmB CS with actin filaments, suggesting that this domain is important for CS localization (as discussed below), chitin synthesis, and interaction of this CS with actin [114]. Deletion of the *An*CsmB ORF resulted in a strain with reduced growth and abnormalities in hyphal development, where balloon-like structures in the subapical regions of hyphae were observed when compared to the WT strain. Furthermore, intrahyphal hyphae, brownish clumps, a low rate of conidiation and the presence of abnormal conidiophores were also observed in the Δ*AncsmB* strain when compared to the WT strain. The addition of the osmotic stabilizer KCl reversed some of the aforementioned phenotypes, although conidia production remained low in all conditions. Simultaneous deletion of *AncsmA* and *AncsmB* (class V and class VII) was unsuccessful, suggesting that both CSs are essential for fungal growth and development. Furthermore, duplication of *AncsmA* in the *AncsmB* deletion background, resulted in an *An*CsmA CS overexpression strain. *An*CsmA overexpression was not able to restore growth phenotypes, suggesting that the *An*CsmB CS is crucial for growth and development and that both CSs have different functions.

In *A. fumigatus*, deletion of *AfcsmB* did not impair CS activity, cell wall chitin content or the expression of other CS-encoding genes. In contrast, the Δ*AfcsmB* strain presented decreased radial growth on solid media, with 10–20% of intrahyphal growth. Furthermore, the Δ*AfcsmB* strain produced a severely reduced number of conidia, even in the presence of an osmotic stabilizer, and conidiophores presented structural abnormalities [71]. These phenotypes were also observed for the double deletion strains Δ*AfcsmA* and Δ*AfcsmB*, with the exception that this strain presented 70–80% intrahyphal growth [71]. The cell wall of Δ*AfcsmB* conidia manifested both hydrophobic and hydrophilic features as well as exposed polyssacharides when compared to the WT strain; whereas Δ*AfcsmA* and Δ*AfcsmB* conidia had heterogenous morphological features similar to conidia from the Δ*AfcsmB* strain, with rodlets being easily damaged, suggesting a fragile and disorganized conidial structure [108].

3.3.2. Deletion of Other CSs from Division 2

To understand the role of CS from division 2 in *A. fumigatus* growth and development, Muszkieta and colleagues (2014), constructed single, double, triple and quadruple CS mutant strains (Δ*AfcsmA* Δ*AfcsmB* Δ*AfchsF* Δ*AfchsD* (Afu1g12600)) [103]. A reduction in radial growth that was proportional to the increase in the number of deleted genes encoding division 2 CS (e.g., Δ*AfcsmA* Δ*AfcsmB*, Δ*AfcsmA* Δ*AfcsmB* Δ*AfchsF*, Δ*AfcsmA* Δ*AfcsmB* Δ*AfchsF* Δ*AfchsD*) was observed. Hyphae of the Δ*AfcsmA*, Δ*AfcsmB*, Δ*AfcsmA* Δ*AfcsmB* Δ*AfchsF* and Δ*AfcsmA* Δ*AfcsmB* Δ*AfchsF* Δ*AfchsD* strains were balloon-shaped, and chitin distribution within the mycelial cell wall was also impaired. Division 2 CS gene deletion strains, with the exception of Δ*AfchsF* and Δ*AfchsD*, had significantly reduced conidiation, with conidiation defects being proportional to the number of deleted genes. The addition of KCl rescued the conidiation defect of the Δ*AfcsmA* Δ*AfcsmB* Δ*AfchsF* Δ*AfchsD* strain. In addition, conidia of the Δ*AfcsmA*, Δ*AfcsmB*, Δ*AfchsF*, Δ*AfchsD*, Δ*AfcsmA* Δ*AfcsmB*, Δ*AfcsmA* Δ*AfcmsB* Δ*AfchsF* and Δ*AfcsmA* Δ*AfcsmB* Δ*AfchsF* Δ*AfchsD* strains presented a loose cell wall with a layer of melanin that easily detached from the inner cell wall layer, resulting in conidia with increased hydrophilicity when compared to the parental strain. Germination

of the Δ*AfcsmA* Δ*AfcsmB* Δ*AfchsF* and Δ*AfcsmA* Δ*AfcsmB* Δ*AfchsF* Δ*AfchsD* strains resulted in hyphae with abnormal chitin deposits, suggesting fragile cell walls that were more susceptible to stress conditions. All *A. fumigatus* division 2 CS deletion strains, with the exception of the Δ*AfchsF* and Δ*AfchsD* strains, presented decreased CS activity, although no significant differences in cell wall chitin content were observed, with the exception of the Δ*AfchsD* strain, which contained 125% chitin when compared to the parental strain. Conidia from the Δ*AfcsmA,* Δ*AfcsmB* and Δ*AfchsF* strains presented significantly reduced content of cell wall chitin. Furthermore, short chitin microfibrils were observed for the Δ*AfcsmA*, Δ*AfcsmB* and Δ*AfcsmA* Δ*AfcsmB* Δ*AfchsF* Δ*AfchsD* strains, possibly explaining the observed morphological abnormalities during asexual development. Additional differences in cell wall polysaccharide concentrations were also observed for the division 2 CS deletion strains. Deletion of the class VI CS Δ*AfchsD* resulted in a strain with normal hyphal morphology, conidiation, germination and chitin synthase activity, but with significantly increased amounts of β-1,3-glucan and decreased concentrations of α-1,3-glucan. In contrast, deletion of class V CS Δ*AfcsmA* resulted in a strain with reduced β-1,3-glucan concentrations and increased concentrations of α-1,3-glucan [96,103].

As chitin content was significantly reduced in the deletion strain Δ*AfchsG* (division 1) and the division 2 Δ*AfcsmA,* Δ*AfcsmB* and Δ*AfchsF* single deletion strains, Muszkieta and colleagues (2014) constructed a strain deleted for the aforementioned four genes. The resulting strain presented significantly reduced growth, with hyphae that did not grow more than 1.3 cm after 3 weeks, in addition to having balloon-shaped hyphae and increased hyphal branching when compared to the parental strain. The same strain also produced conidiophores without conidia, whereas mycelial cell wall chitin and α-1,3-glucan concentrations were significantly reduced, and β-1,3-glucan concentrations were increased. These results suggest that CS from divisions 1 and 2 cooperate in the biosynthesis of the *A. fumigatus* cell wall chitin skeleton, a structure that is important for antifungal drug resistance, growth and virulence [103].

3.4. Localization of CSs in Aspergillus Spp.

In agreement with the changing requirements of chitin synthesis during the different fungal developmental stages [83], CS are located at different loci within the cell as well as expressed in a growth-dependent manner. *An*ChsA and *An*ChsC are present in the metulae, phialides and conidia of *A. nidulans* [105], with the simultaneous deletion of these genes resulting in severely decreased conidial production and abnormal conidiophores. Similarly, *An*chsD is expressed in asexual structures, such as vesicles, metulae, phialide and conidiophores in *A. nidulans* [116].

AnchsB, which encodes a CS with 4–7 transmembrane domains, is targeted to the membrane during septa formation, and is localized at hyphal tips, conidia [107] and the Spitzenkörper (SPK) [117]. The SPK is a structure present in the hyphal apices of actively growing filaments, that dictates direction and growth, and that supplies the hyphal tip with proteins required for growth and cell-wall maintenance [118]. *An*ChsB forms clusters at the hyphal apex in close proximity to the plasma membrane; and these clusters change shape from a globular to a crescent form, representing the changes in vesicle accumulation at the SPK site to fusion with the apical plasma membrane [119].

Vesicles containing biomolecules, cell wall and cell membrane components accumulate at hyphal apices, forming the SPK, which dictates fungal cell growth dynamics [120–122]. Through quantitative super-resolution photoactivation localization microscopy (PALM) analysis, Zhou et al., 2018, showed that vesicles accumulate at the SPK during hyphal slow growth, and then, the vesicles fuse with the plasma membrane to elicit fast growth [119]. The speed of the vesicles containing the *An*ChsB CS-protein at the anterograde movement (from the back to the tip) was either 2–4 μm s^{-1} or 7–10 μm s^{-1}. The retrograde transport (from tip to back) was less common and had a reduced speed (<~7 μm s^{-1}). Analyzing the transport of vesicles containing the early endosomal marker protein GFP-RabA and the secretory vesicle (SV) marker protein mEosFPthermo (monomeric variant of EosFP,

a fluorescent protein whose fluorescence changes from green to red at 390 nm)-TeaR, it was shown that early endosomes (EE) move at a slow speed (2.0 ± 0.5 μm s^{-1}, with identical values for anterograde and retrograde movement), and SV move at higher speeds (7.9 ± 3.6 μm s^{-1} for anterograde movements and 8.3 ± 3.8 μm s^{-1} for retrograde movements) [119]. This suggests that the *An*ChsB CS-protein can be carried either by the EE or via a SV [119]. Furthermore, MyoV (myosin-5) was also shown to be important for the transport of *An*ChsB-containing vesicles to the hyphal tip. Considering that some *A. nidulans* proteins are transported along microtubules and actin filaments to the hyphal tips [123,124], it was suggested that the CS *An*CsmA and *An*ChsB are shuttled to the hyphal tips by kinesins, small molecular motor proteins involved in intracellular transport [117,125]. Deletion of *myoV* resulted in the absence of an accumulation of vesicles at the hyphal tip, suggesting that myosin-5 carries the CS-laden vesicles to the site of exocytosis. The absence of the kinesin UncA also inhibited *An*ChsB CS-protein accumulation at the hyphal tip, resulting in random, fast movement of this CS within the hyphae and suggesting that the *An*ChsB CS-protein may also be transported through secretory vesicles [119]. Secretion dynamics of *An*ChsB were shown to occur at hyphal apical regions via indirect endocytic recycling, where the diffused protein is internalized by subapical actin patches (an endocytosis site) and reconducted from endosomes to the trans-Golgi network cisternae in a Sec7-, GARP-(Golgi-associated retrograde protein) and Rab6-dependent manner [126]. In addition, the adaptor protein (AP)-2 complex, which is involved in the endocytosis process [127], promotes *An*ChsB internalization from the subapical collars of the hyphal surface, and is also important for *An*ChsB localization at hyphal tips [128].

In addition to *An*ChsB, the absence of KinA, but not UncA, resulted in the subapical localization of GFP-*An*CsmA when compared to the WT and *uncA*-deleted strains. Both GFP-*An*CsmA and GFP-*An*ChsB are transported together, with their retrograde and anterograde transport—but not velocity of movement—being dependent on KinA and UncA. In addition, the transport of GFP-*An*CsmA and GFP-*An*ChsB was abolished in a mRFP-KinA^{rigor} strain, where a point mutation in the ATP-binding domain inhibited movement along microtubules, suggesting that *An*CsmA and *An*ChsB CSs are transported to hyphal tips by KinA [117]. Furthermore, the same study showed that the KinA-dependent transport of *An*CsmA is independent of the MMD [117]. Further evidence for the interaction between *An*ChsB and *An*CsmA with mRFP-KinA^{rigor} was obtained in experiments with strains expressing FLAG-*An*ChsB mRFP-KinA^{rigor} and HA-*An*CsmA mRFP-KinA^{rigor}. Through performing cellular fractionation experiments, FLAG-*An*ChsB and *An*CsmA-HA were detected in fractions that contain vesicles, Golgi structures and endosomal membranes. Furthermore, it was shown that although both *An*CsmA and *An*ChsB are transported by KinA to the growing hyphal tip, they are transported in different vesicles [117].

*An*CsmA is present at forming septa and as mobile spots in the cytoplasm and along the apical membrane [107,117]. The MMD in the *An*CsmA protein is predicted to be responsible for the localization of the CS domain, and therefore, for the correct localization of chitin synthesis [94]. Indeed, *An*CsmA-HA was shown to colocalize with actin at hyphal tips and at sites where septa are formed [129], and deletion of MMD resulted in *An*CsmA-HA localizing to large organelles and tubular structures [129]. Furthermore, *An*CsmA-HA was detected every 24 h by western blot when mycelia were grown in a complete medium for 5 days continuously, suggesting a patterned expression of this enzyme [130]. Cellular fractionation experiments showed that *An*CsmA-HA was present in low-speed pellets, suggesting that the *An*CsmA CS is an integral protein with several transmembrane domains. In agreement with the *An*CsmA transcriptional patterns, *An*CsmA-HA concentrations were higher in hyphae grown under low osmotic conditions than when compared to high osmotic conditions, further supporting a role for *An*CsmA in cell wall integrity during hyphal extension [113,130].

The class VII CS *An*CsmB localizes to hyphal tips and at forming septa, and similar to *An*CsmA and the *An*CsmB MMD, interacts with actin F. Once septa are mature, *An*CsmB disappears from the sites, suggesting that this protein, together with *An*CsmA, is essen-

tial for and has a compensatory effect on fungal cell wall integrity during growth and development [95].

3.5. Regulatory Pathways Controlling the Expression of CS-Encoding Genes

The fungal cell wall is the first point of contact and primary interaction site for the fungus with the extracellular environment. Subsequently, external cues, such as pH variation, heat shock, osmotic stress, oxidative stress, nutrient limitation and antifungal drugs, have an effect on the cell wall and activate and/or modulate intracellular signaling pathways [131] (Figure 4). In response to these cues, the fungal cell wall is constantly being remodeled in order to withstand and counteract adverse extracellular conditions, and to ensure growth and reproduction and to avoid cell death [132]. In addition, cell wall compensatory alterations can occur in a condition-specific manner, including increased chitin and glucan biosynthesis and/or a redistribution of chitin [78].

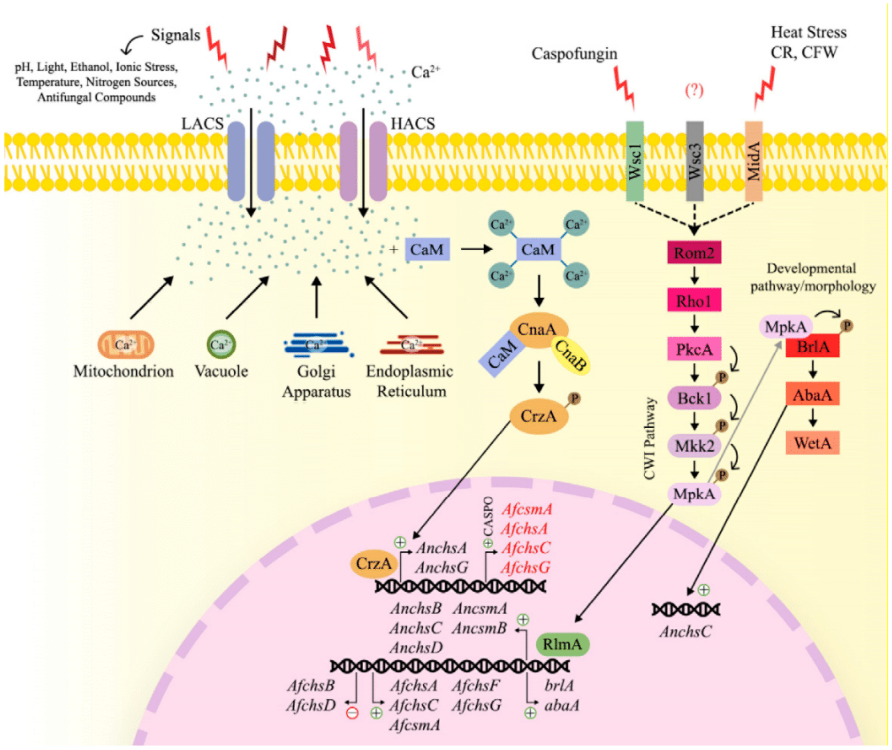

Figure 4. Regulation of chitin biosynthesis in response to extracellular signals and/or in the presence of stresses, including changes in pH, temperature, light, food sources and in the presence of antifungal compounds, an increase in intracellular Ca^{2+} concentrations occur. Calcium channels regulate the influx of calcium through the "High affinity Ca^{2+} influx system" (HACS), which is activated when Ca^{2+} availability is low, or the "Low affinity Ca^{2+} influx system" (LACS), when calcium availability is high. In addition, Ca^{2+} ions are also released from intracellular stores, such as mitochondria, vacuoles, the Golgi apparatus and the endoplasmic reticulum. Upon an increase in cytosolic Ca^{2+} concentrations, the protein Calmodulin (CaM) binds to 4 Ca^{2+} molecules, which triggers conformational changes in this enzyme. Subsequently, CaM is able to bind to calcineurin, a heterodimeric protein composed of catalytic (CnaA) and regulatory (CnaB) subunits, and activate it. Calcineurin, in its active form, phosphorylates the transcription factor CrzA, which subsequently translocates to the nucleus. CrzA binds to the promoter region of several *A. nidulans* chitin synthase-encoding genes, including *AnchsA* and *AnchsG*, initiating transcription of these genes. In *A. fumigatus*, the calcium-calmodulin-calcineuring signaling pathway is also activated during the caspofungin paradoxical effect (CPE), where CrzA binds to the promoter region of *AfcsmA*, *AfchsA*, *AfchsC* and *AfchsG*. In addition, the antifungal compound

caspofungin, can also be sensed by the transmembrane protein Wsc1. Furthermore, MidA, an additional transmembrane sensor, is involved in the resistance against heat stress and cell wall perturbing agents, such as calcofluor white (CFW) and Congo red (CR). Wsc1 and MidA activate the small GTPase Rho1 through the interaction with Rom2 (a guanine nucleotide exchange factor). Rho1 will activate Protein Kinase C (PkcA), which in turn activates the cell wall integrity (CWI) pathway. The CWI pathway is composed of the MAPK kinase kinase Bck1, the MAPK kinase Mkk2 and the MAPK MpkA, which phosphorylate one another. As a result, MpkA translocates to the nucleus, where it interacts with the transcription factor RlmA. RlmA is involved in the expression of chitin synthase-encoding genes in *A. nidulans* and *A. fumigatus*, including *AnchsB, AnchsC, AnchsD, AncsmA, AncsmB, AfchsA, AfchsC, AfchsF, AfchsG* and *AfcsmA*. RlmA also regulates the repression of the *AfchsB* and *AfchsD* genes in these conditions. RlmA binds to the promoter regions of the genes *brlA* and *abaA*, both of which are involved in fungal developmental and conidiation processes. Furthermore, MpkA physically interacts with BrlA and AbaA regulates the expression of *AnchsC*, further supporting the notion that the regulation of chitin biosynthesis in fungal cell walls is subject to multiple pathways and the crosstalk between these pathways. Dotted lines: represent the signal transduction in response of Wsc1, Wsc3 and MidA activation.

3.5.1. Intracellular Trehalose Levels

Trehalose biosynthesis is catalyzed by trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase [24]. In *S. cerevisiae*, trehalose-6-phosphate synthase (Tps1p) and trehalose-6-phosphate phosphatase (Tps2p) form a protein complex with the two regulatory subunits Tsl1p and Tps3p [133]. *A. fumigatus* has two paralogous genes for *tps1*, named *tpsA* and *tpsB* (*tpsA/B)*, one orthologous gene for *tps2*, termed *orlA*, and the two regulatory subunits TslA and TslB [133]. The trehalose regulatory subunit TslA was shown to be important for intracellular trehalose levels in mycelia and conidia, as well as for cell wall polysaccharide concentrations. The Δ*tslA* strain presented increased cell wall chitin content and CS activity, and reduced β-glucan exposure [134]. In addition, the CS *Af*CsmA physically interacts with TslA, the latter of which is important for the correct localization of this CS at hyphal tips and septa [134]. Furthermore, TslA was also shown to interact with the multifunctional RNA-binding protein SsdA. Deletion of *ssdA* resulted in a strain with significantly increased intracellular trehalose concentrations in both mycelia and conidia, whereas decreased trehalose levels were observed in a *ssdA* overexpression (OE::*ssdA*) strain [135]. Both strains had decreased growth rates. The Δ*ssdA* strain had increased resistance to the cell wall perturbing agents Congo red, calcofluor white and caspofugin; whereas the OE::*ssdA* strain was highly sensitive to these agents. SsdA was shown to be important for cell wall polysaccharide concentrations, with the Δ*ssdA* strain having decreased chitin levels and CS activity and the OE::*ssdA* strain having increased chitin levels and CS activity. Both the deletion and overexpression strains also presented lower levels of β-1,3-glucan in their cell wall. SsdA was also shown to be important for the correct cellular localization of CsmA, with CsmA being randomly dispersed within the hyphae in the OE::*ssdA* strain. In summary, these data suggest that intracellular levels of trehalose are important for *A. fumigatus* cell wall integrity, and that TslA functions as a chitin biosynthetic regulator in *A. fumigatus* [134,135]. This is perhaps not surprising as the type of extracellular nutrient source, in particular different carbon sources, have been shown to affect the composition and organization of the cell wall in a number of fungi.

3.5.2. The Calcium-Calcineurin Signaling Pathway

In addition to the trehalose metabolic pathway, the calcineurin signaling pathway was also shown to be crucial for fungal cell wall integrity, cation homeostasis, cell cycle progression, hyphal branching, sclerotial development and pathogenesis [136] (Figure 4). Calcineurin is a Ca^{2+}/calmodulin (CaM)-phosphatase-dependent heterodimeric protein that is composed of a catalytic (CnaA) and a regulatory (CnaB) subunit [137]. Extracellular signals, such as ionic stress, ethanol, light, temperature, pH, nitrogen sources

and antifungal compounds, activate the plasma membrane calcium system, which can either be the high-affinity Ca^{2+} influx system (HACS) when the availability of calcium is low, or the low-affinity Ca^{2+} influx system (LACS) when Ca^{2+} availability is high [138]. The release of calcium from intracellular storages, such as vacuoles, the endoplasmic reticulum, mitochondrion and Golgi apparatus, results in an increase in intracellular Ca^{2+} concentrations [139]. The increased intracellular calcium concentration is sensed by calmodulin, which binds 4 Ca^{2+} ions [136]. As a consequence, calmodulin undergoes a conformational change and binds to calcineurin. Calcineurin is now in its active form and dephosphorylates the transcription factor CrzA, which will translocate to the nucleus and bind to the target promoters of approximately 102 genes. These genes encode enzymes involved in transport, regulation of biological processes, response to stress, chemicals and developmental processes, and the transcriptional response modulates the cellular response to the extracellular cue [140,141]. The predicted CrzA DNA bindings consensus sequence— (G(T/G)GGC(T/A)G(T/G)G)—is present in the promoter regions of the CS genes *AnchsC* and *AnchsG* (AN1046), suggesting a possible transcriptional regulations of these two CS by CrzA in *A. nidulans* [142].

Further evidence for the involvement of calcium signaling in cell wall integrity was obtained from studies in *A. fumigatus*. Deletion of *cnaA* in this fungus, resulted in a strain with severe defects in growth, filamentation, conidia production and morphology [143]. Furthermore, the *A. fumigatus* Δ*cnaA* mutant strain presented a reduced β-glucan hyphal content, when compared to the WT strain, and this decrease is even greater when the Δ*cnaA* strain is treated with caspofungin, a β-1,3-glucan synthase inhibitor. In contrast, the addition of the CS inhibitor Nikkomycin Z leads to a compensatory increase in β-glucan levels in the Δ*cnaA* strain. Indeed, transcriptional expression of the CS-encoding genes *AfchsA*, *AfchsC*, *AfcsmA*, *AfchsF* and *AfchsG* is significantly reduced in the Δ*cnaA* strain [144]. Similar defects in growth, cell wall architecture and septa formation were also observed for the Δ*cnaB* and Δ*cnaA* Δ*cnaB* strains. In addition, a ~ 40% reduction in β-glucan content was observed in the Δ*cnaA*, Δ*cnaB* and Δ*cnaA* Δ*cnaB* strains when compared to WT strain. Furthermore, chitin content was increased by ~40% in the Δ*cnaA* Δ*cnaB* strain and by ~20% in the single deletion strains when compared to the WT strain, showing a compensatory effect. Despite the observed increase in chitin content in these strains, the expression of *AfchsA*, *AfchsB*, *AfchsC*, *AfchsD*, *AfcsmA*, *AfchsF*, *AfchsG* and *AfcsmB* was downregulated in the Δ*cnaB* and Δ*cnaA* Δ*cnaB* strains. These observations suggest that possibly an abnormal assembly of cell wall components in the calcineurin deficient strains is occurring, with a likely deficient incorporation of chitin into the cell wall, due to a decreased β-glucan content [145].

In agreement with the observed reduced levels of β-glucan in the Δ*cnaA* strain, Cramer and colleagues (2008) also showed a decreased expression of *fksA* (-2.1-fold expression change) in this strain [144]. The gene *fksA* codifies for the catalytic subunit of the β(1,3)-glucan synthase complex, that together with the regulatory subunit Rho1 are responsible for biosynthesising β-glucan. FksA is localized to the hyphal apex in proximity to Rho1, a GTP-ase that is highly expressed in the Δ*cnaA* (10,9-fold expression change) strain when compared to the WT strain [144,146]. Interestingly, Rho1 can elicit cell wall composition via activation of FksA, and also, this GTP-ase is a component of the cell wall integrity (CWI) pathway [147–149].

3.5.3. The Cell Wall Integrity (CWI) Pathway

The CWI pathway is an intracellular signaling cascade, which responds to various environmental stimuli that perturb the cell wall and membrane homeostasis; this signaling pathway modulates gene expression to keep the integrity of the fungal cell wall intact [131,150]. In *A. fumigatus*, cell wall abnormalities are detected by sensor transmembrane proteins, namely Wsc1, Wsc3 and MidA (Figure 4). Wsc1 is required for resistance against the antifungal agent caspofungin, whereas MidA is involved in resistance to heat stress and chitin binding agents, such as Congo red and CFW. All three proteins have

overlapping functions, as abnormalities in these sensor proteins result in defects in growth and conidiation [148]. Once cell wall stress activates a given transmembrane sensor, the small GTPase Rho1 is activated through interaction with the guanine nucleotide exchange factor (GEF) Rom2, which is localized between the sensor and Rho1 [149]. Subsequently, Rho1 activates the protein kinase C PkcA. When the *A. fumigatus* CEA17 WT strain was treated with the β-glucan-intercalating agent Congo red, the expression of genes *pkcA, mpkA, rlmA, fksA* was highly induced. Similarly, the CS-encoding genes *AfchsA*, *AfchsC*, *AfcsmA*, *AfchsF*, *AfchsG* had increased expression in response to Congo red. PkcA has been shown to be important for cell wall maintenance in the presence of Congo Red, as it regulates CS-encoding genes [151]. Using a strain that harbors a Gly579Arg substitution in the PkcA C1B domain, Rocha and colleagues, 2015, demonstrated a higher sensitivity of this mutant strain to cell wall perturbing agents (Congo red, CFW, anidulafungin, SDS and caffeine), nikkomycin Z and fluconazole [151]. The most prominent growth effects observed for the $pkcA^{G579R}$ mutant strain, were for Congo red and CFW. In the WT strain, a time-dependent increase in MpkA phosphorylation was observed in the presence of Congo red; this phenomenon was not observed for the $pkcA^{G579R}$ strain, where the presence of Congo red, resulted in reduced *mpkA* expression and MpkA phosphorylation. The expression of *rlmA,* coding for a transcription factor, was also decreased in the mutant strain in the presence of Congo red, suggesting the involvement of PkcA-MpkA in RlmA activation in the presence of Congo red. The authors also showed that Congo red increases the expression of CS genes (*AfchsA, AfchsC, AfchsF, AfchsG and AfcsmA*) in the WT strain, which was not seen in the $pkcA^{G579R}$ strain. After Congo red exposure, the expression of *AfchsA, AfchsC, AfchsF, AfchsG and AfcsmA* were reduced in the mutant strain. In contrast, this was not observed for the expression of *AfchsB, AfchsD* and *AfcsmB*. In the WT strain, the presence of Congo red resulted in the repression of *AfchsB* and *AfchsD*. In the $pkcA^{G579R}$ strain, before the addition of Congo red, the expression of *AfchsB* and *AfchsD* was higher than when compared to the WT; after incubation in the presence of Congo red, the expression levels of these CS were decreased, suggesting a possibly compensation mechanism for the absence of PkcA. Furthermore, the expression of *AfcsmB*, was also not modulated in the presence of Congo red in the WT and mutant strains [151]. Altogether, these data suggest the importance of PkcA for CS gene expression.

The deletion of MpkA and RlmA, in the presence of Congo red resulted in the transcriptional regulation of the genes *fksA, mpkA, rlmA, AfchsA*, *AfchsC*, *AfcsmA*, *AfchsF* and *AfchsG* by RlmA and MpkA. Furthermore, the same study showed that *pkcA* was negatively regulated by RlmA and MpkA, and *AfcsmB* was positively regulated by RlmA and negatively by MpkA. The CS-encoding genes *AfchsB* and *AfchsD* were both negatively regulated by RlmA, and *AfchsD* was positively regulated by MpkA in the presence of Congo red [152]. These studies suggest a complex interplay between PkcA, RlmA and MpkA in regulating cell wall maintenance in *A. fumigatus*.

Similar to *A. fumigatus*, PkcA was also involved in the regulation of CS-encoding genes in *A. nidulans*. Katayama et al., 2014, constructed a strain where PkcA was ectopically activated through the substitution of PkcA Arg429 to alanine and placed under the control of the *alcA* promoter (R429A-1 strain). When R429A-1 was transferred from repressing to inducing conditions, a significant increase in the mRNA levels of the CS-encoding genes *AnchsB*, *AnchsC*, *AnchsD*, *AncsmA* and *AncsmB* but not *AnchsG* was observed, suggesting that PkcA is involved in the regulation of these genes [142].

Protein kinase C activates the cell wall integrity (CWI) pathway, which consists of a mitogen-activated protein kinase (MAPKs) cascade. In the CWI pathway, this cascade is composed of the MAPK kinase kinase Bck1, the MAPK kinase Mkk2 and the MAPK MpkA, which phosphorylate one another [153]. Upon phosphorylation, MpkA translocates to the nucleus, where it activates the transcription factor RlmA, which subsequently transcriptionally upregulates cell wall biosynthesis genes and tolerance to oxidative stress [152]. To determine whether the expression of CS-encoding genes is directly regulated by PkcA or by downstream components, such as RlmA; Katayama et al., 2015, constructed the Δ*rlmA-1*

(*rlmA* deleted) and R429AΔ*rlmA-1* [*pkcA*(R429A) under the control of the *alcA* promoter in addition to a wild type *pkcA* copy, and also with a deleted *rlmA* gene strains. The expression of *AnchsB, AnchsC, AnchsD, AncsmA* and *AncsmB* is mainly regulated by RlmA, as the mRNA levels of these genes are similar between the Δ*rlmA-1* and R429AΔ*rlmA-1* strains when shifting from repressing to inducing conditions. The authors suggested that constitutively activated PkcA leads to the higher expression of some CS-encoding genes through the activation of the CWI pathway and the TF RlmA [142]. In contrast, the *AnchsG* mRNA levels were lower in the R429AΔ*rlmA-1* strain when compared to Δ*rlmA-1* strain in the same conditions, suggesting that the expression of *AnchsG* is independent of RlmA [142]. Similarly, the expression of *AnchsC* also occurs in a RlmA-independent manner, as *AnchsC* mRNA levels were higher in the R429AΔ*rlmA-1* strain, when compared to the Δ*rlmA-1* strain [142].

The *A. fumigatus* Δ*rlmA* strain has a decrease in conidia production, and exhibits undifferentiated aerial hyphae formation, suggesting that RlmA is important for conidiation [152]. A delay in conidial formation was also observed for the Δ*rlmA* strain, as well as for the $\Delta pkcA^{G579R}$ and Δ*mpkA* strains, which was accompanied by reduced conidial hydrophobicity. When analyzing chitin content in these mutants, it was observed that the $\Delta pkcA^{G579R}$ strain had similar chitin levels as the WT strain, whereas the Δ*mpkA* and Δ*rlmA* strains had lower and higher chitin levels, respectively, when compared to the parental strains. All these data highlight the relevance of the CWI pathway for the initial steps of conidiation [154].

3.5.4. The Developmental Signaling Pathway

In *Aspergillus* species, conidiation is regulated by a developmental signaling pathway. The central regulators of conidiation constitute of BrlA, AbaA and WetA [155] (Figure 4). BrlA is involved in the initiation of conidiation, as it is expressed at the early stages of conidia production. Additionally, BrlA is important in the initial steps of conidiophore formation, and this transcriptional regulator is necessary for the expression of other conidiation-related genes including *abaA, wetA, vosA and rodA* [156,157]. BrlA also transcriptionally induces *abaA*, whose function is related to the differentiation and functionality of phialide development during conidiophore maturation [156]. The *abaA* gene codifies for a protein with a DNA-binding motif, ATTS sequence/TEA and a leucine zipper [158]. In addition, AbaA binds to the consensus sequence 5′-CATTCY-3′, termed the "AbaA response element" (ARE), that is present in the promoter region of several genes, including *brlA, wetA, rodA* and *abaA* [155,159]. The gene *wetA* is activated by AbaA, and is responsible for the final steps of conidiation, including the synthesis of the C4 inner layer of the conidia cell wall, which is important for the maturation and impermeability of conidia [156,157]. In addition, WetA is important for conidia trehalose production, conidia germination and early steps of fungal growth [156,157].

An interesting crosstalk between the CWI pathway and the central regulators of asexual development in *A. fumigatus* were described by Rocha and colleagues, 2020 [154]. The expression of genes coding for central regulators of asexual development (*brlA, abaA* and *wetA*) was also increased in the WT strain during conidiation, whereas this expression was absent in the CWI pathway mutants ($pkcA^{G579R}$, Δ*mpkA*, Δ*rlmA*), corroborating the conidiation abnormalities observed in these mutant strains. Furthermore, RlmA was shown to bind in vivo to the promoter regions of *brlA* and *abaA*, and the authors also found that MpkA physically interacts with BrlA, suggesting an important role of the CWI pathway during conidiation in *A. fumigatus* [154].

In *A. nidulans*, Park and colleagues, 2003, demonstrated that AbaA was able to bind to, at least in vitro, to three putative AREs in the promoter region of the CS-encoding gene *AnchsC*, and in vivo assays demonstrated that AbaA directly activates transcription of *AnchsC* [160]. In agreement, expression of *AnchsC* is reduced in the *brlA* and *abaA* deletion strains, reinforcing the participation of AbaA in the regulation of chitin synthesis during conidiation [106,160]. Furthermore, expression of *brlA* and *abaA* was observed in

the WT, Δ*AnchsA* and Δ*AnchsC* strains during conidiophore development, whereas this transcriptional upregulation is lost in the Δ*AnchsA* Δ*AnchsC* double mutant strain [106], suggesting that *brlA* and *abaA* are important for *A. nidulans* conidiophore development.

In *A. nidulans*, extracellular cues, such as heat and osmotic stress, activate the aforementioned signaling and regulatory pathways, resulting in the transcriptional induction of CS-encoding genes in a cue-dependent manner. This further highlights the importance of cell wall dynamics and integrity for fungal survival and withstanding different stresses. The expression of *AnchsA* was increased during conidiophore development and the GFP-tagged *AnchsA* protein was observed in asexual structures. Osmotic stress and sodium acetate also increased the expression of this CS-encoding gene, whereas heat stress and different carbon sources did not influence the transcriptional expression of *AnchsA*. Expression of *AnchsB* was shown to be ubiquitous in the fungus and less dependent on the fungal developmental stage. Similar to *AnchsA, AnchsB* is transcriptionally induced in the presence of higher concentrations of sodium acetate, but not in the presence of osmotic or heat stress or in the presence of simple sugars. The expression of *AnchsC* was highly dependent on the hyphal developmental stage and transcriptional expression was observed in vegetative mycelia and during sexual differentiation. Furthermore, *AnchsC* was transcriptionally induced in the presence of osmotic stress and sodium acetate but not when the fungus was exposed to heat stress and simple sugars. Taken together, these data suggest that the expression of CS-encoding genes is dependent on the fungal developmental stage and also responds to different stresses as well as the presence of growth-stimulating carbon sources in a signal-specific manner [161]. To further investigate the effect of heat stress on CS gene expression in *A. nidulans*, Katayama and colleagues, 2014, constructed a temperature-sensitive *pkcA* mutant strain (*pkcA*-ts), where PkcA is inactivated at restrictive temperatures (42 °C). When strains were grown at the permissive temperature of 20 °C and then shifted to the restrictive temperature, the mRNA levels of *AnchsA*, *AnchsF*, *AncsmA* and *AncsmB* significantly increased in the WT strain, whereas expression of *AncsmA* and *AncsmB* did not occur in the *pkcA*-ts strain. In summary, these data suggest that, in the presence of heat stress, PkcA is involved in the regulation of several CS-encoding genes [142].

Together, the aforementioned studies highlight the complex regulatory network in which CS-encoding genes are embedded, and how the expression of these genes is highly variable and dependent on defined conditions, such as fungal developmental stage, temperature shifts and the presence of extracellular cell walls and cell membrane-damaging agents [79].

4. Chitin Biosynthesis as a Target for Antifungal Drugs

Opportunistic fungal infections, including those caused by *Aspergillus* spp. have become a major global health problem for both immunocompetent and immunocompromised patients. It is estimated that invasive fungal infections result in over 1.5 million deaths per year, which is higher than patients dying globally from breast cancer or malaria [162]. The fungal cell wall, including chitin synthesis, presents an interesting target for antifungal drug development, simply because humans do not have a cell wall [1]. Indeed, inhibition of chitin synthesis by these antifungal agents, negatively affects cell wall architecture and fungal morphology, subsequently resulting in reduced fungal viability and survival [2,162].

Nikkomycins and polyoxins are the most studied inhibitors of chitin synthesis. They are pyrimidine nucleosides linked to a di- or tri-peptide moiety, with a similar structure to the CS substrate UDP-GlcNAc. Thus, they function as competitive inhibitors, binding to the catalytic site of CS with more affinity than UDP-GlcNAc [163,164].

There are several types of nikkomycins: nikkomycin D, I, J, X and Z, that differ from each other mainly by the nucleoside moiety [164,165]. In vitro, nikkomycin X and Z were effective against the dimorphic fungi *Coccidioides immitis, Blastomyces dermatidis*, but were less effective against *C. albicans, C. tropicalis, Fusarium oxysporum* and ineffective against *A. fumigatus, A. flavus, A. niger, A. versicolor* and *A. nidulans* [166–168]. In in vivo murine models, nikkomycin Z was more effective than nikkomycin X, and was able to

almost eradicate coccidioidomycosis infection, leading to increased mouse survival during blastomycosis and histoplasmosis infection [166,169]. Nikkomycin Z went to phase II of clinical trials to be used as a commercial antifungal drug against coccidiomycosis, but no further results were published [170].

The synergism between nikkomycin Z with other antifungals agents in so-called combination therapies has been investigated intensely. Li and Rinaldi, 1999, analyzed the in vitro synergistic effect of nikkomycin Z and the ergosterol biosynthesis inhibitors itraconazole or fluconazole for 110 fungal species, and found increased antifungal efficacy against *C. albicans, C. parapsilosis, C. neoformans and C. immitis* when these antifungal compounds were used in combination than when compared to single drug use only. They also observed a synergism for the combination of nikkomycin Z and itraconazole against *A. fumigatus* and *A. flavus* [168]. In vitro synergism was also observed for nikkomycin Z in combination with the echinocandins micafungin and caspofungin against *A. fumigatus*. Echinocandins are antifungal agents that inhibit β-1,3-glucan synthase, which synthesizes cell wall β-glucan. Indeed, disruption of one cell wall polysaccharide can have profound effects on the structure and arrangement of other cell wall polysaccharides. Verwer and colleagues, 2012, observed that *A. fumigatus* treated with >0.5 μg/mL of nikkomycin Z presented a high β-glucan content and a decreased chitin content at nikkomycin concentrations of 16 μg/mL. Caspofungin at 4 μg/mL significantly reduced β-glucan levels, whereas higher concentrations of 32 μg/mL resulted in increased cell wall chitin content. *A. fumigatus* strains treated with the combination of two drugs (0.125 μg/mL of caspofungin + 2 μg/mL of nikkomycin Z) presented a significant decrease in β-glucan content, and increased chitin levels [171], suggesting that a compensatory effect occurs when the synthesis of chitin or β-glucan are blocked. Antifungal drug synergism against *A. fumigatus* was not observed for polyoxin D in combination with echinocandins or the cell membrane ergosterol biosynthesis targeting drugs polyenes and triazoles [171–173].

Plagiochin E (PLE), a macrocyclic *bis* (bibenzyls) compound, is isolated from the liverwort plant *Marchantia polymorpha* L., and presents antifungal properties. In *Candida albicans*, PLE causes alteration in the FCW, with decreased activity of CS in vitro, inhibition of chitin synthesis in situ, decreased expression of *chs1*, and increased expression of *chs2* and *chs3* [174]. In addition, PLE causes reactive oxygen species (ROS) accumulation in *C. albicans.* ROS function as mediators of apoptosis, as *C. albicans* treated with PLE undergoes apoptosis through the activation of metacaspases [175,176]. PLE shows synergism with fluconazole against a fluconazole-resistant *C. albicans* strain, and the mechanism of this synergism is through the inhibition of efflux pumps by PLE, resulting in increased intracellular concentrations of fluconazole in *C. albicans* [177]. Despite these promising properties, to date, there are no studies testing PLE on *Aspergillus* species. Fortunately, several antifungal compounds, including some with novel antifungal mechanisms, are currently in phases II and III of clinical trials with promise to be commercially available soon [178]. These compounds do not target chitin biosynthesis and will therefore not be reviewed here.

Furthermore, CS confer resistance to antifungal agents and cell wall targeting compounds. Mellado et al., 2003, showed that the *A. fumigatus* strain simultaneously deleted for *AfcsmA* and *AfchsG*, and the single *AfchsG* deletion strain, are more susceptible to the echinocandin LY303,336. These strains exhibited significantly reduced chitin synthase activity and chitin content when compared to the parental strain [109]. In contrast, deletion of *AfchsG*, and a quadruple deletion of genes encoding CS from division 1 (*AfchsA AfchsC AfchsB AfchsG*) resulted in strains that were resistant to Congo red, Calcofluor white and nikkomycin Z, with these strains containing less β-1,3-glucan and high α-glucan concentrations in their cell wall. A compensatory effect in cell wall glucan biosynthesis therefore occurred. Deletion of the division 2 CS *AfchsD* resulted in increased β-1,3-glucan levels and decreased concentrations of α-1,3-glucan. With the exception of the Δ*AfchsF* and Δ*AfchsD* strains, all division 2 CS deleted strains were resistant to itraconazole, voriconazole and nikkomycin Z, but sensitive to Congo red and Calcofluor white when compared to the WT strain, suggesting an impairment in cell wall permeability [103].

4.1. The Caspofungin Paradoxical Effect (CPE)

The CPE is probably the most studied cell wall polysaccharide compensatory effect. The CPE is an event observed in some strains of *Aspergillus* and *Candida* species, whereby increased concentrations of caspofungin, in contrast to lower concentrations, are not fungicidal/fungistatic, but rather result in improved fungal growth [179–181]. The occurrence of the paradoxical effect (PE) is a strain- and echinocandin-dependent trait, as five out of seven *A. fumigatus* clinical isolates presented paradoxical growth in the presence of caspofungin only, but not in the presence of micafungin and anidulafungin [182]. Similarly, the routinely used *A. fumigatus* wild-type strain Af293, presents a compensatory growth effect in the presence of caspofugin, but not when grown in the presence of the echinocandins micafungin and anidulafungin. In addition to *A. fumigatus*, the PE was also observed in *A. flavus* and *A. niger* in the presence of caspofungin and in *A. terreus* in the presence of caspofungin and micafungin [183–185]. One mechanism through which the CPE is predicted to occur, is through an increase in cell wall chitin content when β-1,3-glucan content is decreased by the echinocandin [171,186]. Indeed, in the presence of caspofungin (0.2 or 2 μg/mL), several genes involved in cell wall biosynthesis are induced, such as CS-encoding genes (*AfchsA* and *AfchsG*), α-glucan synthase-encoding genes (*ags1* and *ags3*), as well as genes coding for β-glucan synthase (*fks1*) and other enzymes in the *A. fumigatus* CEA17 and AF293 strains [187]. The Δ*AfchsA,* Δ*AfchsC* and Δ*AfchsA* Δ*AfchsC* strains showed increased cell wall chitin content similar to the WT strain in the presence of caspofungin, and compensatory increased transcriptional expression of other CS-encoding genes, such as *AfchsA, AfchsB, AfchsD, AfcsmA, AfchsF* and *AfchsG* was not observed. These results suggest that the increase in cell wall chitin concentrations is likely due to additional post-transcriptional downstream events [188] or that other mechanisms not related to the increased cell wall chitin exist. Furthermore, the class III CS AfChsG plays a critical role in the response of *A. fumigatus* to caspofungin. In contrast to strains deleted for CS from classes II and IV, the Δ*AfchsG* strain did not show compensatory chitin content production in response to the presence of caspofugin. In addition, the Δ*AfchsC* Δ*AfchsG* strain was highly susceptible to caspofungin, even in the presence of $CaCl_2$ and CFW, suggesting that AfChsG is the main CS that responds to the presence of caspofungin [189].

4.1.1. Signaling Pathways Involved in the CPE

Several signaling pathways, and the crosstalk between them, have been shown to be involved in establishing the CPE. The calcium-calcineurin signaling pathways is involved in the CPE, with the Δ*cnaA* strain not showing a compensatory increase in cell wall chitin upon the addition of high concentrations of caspofungin [190]. Indeed, increased expression of the CS-encoding genes *AfchsA* (CS of class I) and *AfchsC* (CS of class III) in the presence of caspofungin was not observed for the Δ*cnaA* strain in comparison to the WT strain [182]. The expression of other CS-encoding genes, including *AfchsD, AfcsmA, AfchsF* and *AfchsG* was also increased in the WT strain when compared to the Δ*cnaA* strain, although this increase was not statistically significant, suggesting that two CS-genes are under the regulatory control of the calcium-calcineurin signaling pathway [182].

In the presence of the CPE (4 μg/mL of caspofungin), an increase in cytoplasmic Ca^{2+} concentrations occurs in the *A. fumigatus* CEA10 strain, together with an increased expression of calmodulin (*cmdA*) and calcineurin A (*cnaA*) genes, suggesting an activation of the calcium-calmodulin pathway [191], resulting in the activation of the transcription factor CrzA. Ries et al., 2017 showed that CrzA binds to the promoter regions of *AfchsA, AfchsC, AfchsG* and *AfcsmA* in the presence of the CPE [192]. CrzA binds to the CG [GCC(A/T)C] motif in the promoter regions of approximately 600 genes, including *AfchsA* and the β-1,3-glucan synthase-encoding gene *fks1*, in the presence of 2 μg/mL caspofungin [187]. These results provide further evidence for the involvement of the calcineurin signaling pathway in the CPE. Indeed, deletion of *crzA* resulted in a strain with a significantly thicker cell wall and increased concentrations of cell wall GlcNAc, suggesting that CrzA does not only regulate the expression of CS-encoding genes during the CPE, but is also required for cell wall

organization in the absence of caspofungin. In addition, the transcription factor ZipD, which is upregulated in the presence of calcium and negatively regulated by calcineurin, was also shown to be important for the CPE. Deletion of *zipD* resulted in a strain with increased sensitivity to caspofungin, a decrease in the CPE and in the expression of CS-encoding genes. ZipD translocated to the nucleus in the presence of calcium or caspofungin in a calcineurin-dependent manner, suggesting that ZipD is involved in regulating the CPE via the calcium-calcineurin signaling pathway, although the mechanism underlying this regulation remains subject to future investigations [192]. Not surprisingly, ZipD was also associated with correct cell wall organization and composition in *A. fumigatus* [193]. The Δ*zipD* strain was sensitive to cell wall-perturbing compounds, such as Congo red and calcofluor white, had lower levels of cell wall β-1,3-glucan, higher chitin levels and a thicker cell wall when compared to the WT strain [193].

In addition to the calcineurin pathway, the CWI pathway was also shown to be important for the *A. fumigatus* CPE, with both pathways being subject to crosstalk [192]. Colabardini and colleagues, 2021, showed that the deletion of *crzA* in *A. fumigatus* (CEA17 [$\Delta crzA^{CEA17}$] and Af293 [$\Delta crzA^{Af293}$] strains) resulted in decreased MpkA phosphorylation in both *crzA* deletion background strains in the presence of most tested caspofungin concentrations. This study suggests that CrzA is important for MpkA activation, but not essential [187]. In the CEA17 WT strain, the presence of fungistatic concentrations of caspofungin resulted in increased phosphorylation of MpkA and, thus, the activation of the CWI pathway and establishment of the CPE. Increasing concentrations of caspofungin also caused transcriptional upregulation of the CS genes *AfchsA, AfchsC, AfchsG* and *AfcsmB*. Deletion of *mpkA* and *rlmA* resulted in the loss of the CPE and a differential expression pattern of CS-encoding genes. In the Δ*mpkA* strain, *AfchsA, AfchsB, AfchsC, AfchsD, AfcsmA, AfchsG* and *AfcsmB* expression was significantly increased in the presence of caspofungin, when compared to the WT strain. In the Δ*rlmA* strain, expression of *AfcsmA* and *AfchsG* was increased in response to caspofungin, whereas expression of *AfchsC* was decreased during the CPE [192].

4.1.2. Additional Factors Involved in the CPE

In addition to ZipD, Valero et al., 2020, showed that eleven additional transcription factors (TFs) are involved in the CPE. Strains deleted for the TFs *cbfA, nctA, nctB, nctC, fhdA, znfA, atfA, rlmA, zfpA,* AFUB_054000 and *zipD* were sensitive to caspofungin concentrations at which the CPE is usually observed in the WT strain. All these strains were also sensitive to lower concentrations (0.5 µg/mL) of caspofungin, with the exception of Δ*nctA* and Δ*rlmA*, suggesting that a correlation between reduced CPE and caspofungin resistance exists [194]. The NctA and NctB (negative factors two A and B, respectively) are transcription factors that are members of the C-repeat binding factor (CBF)/nuclear factor Y (NF-Y) family of regulators. They are part of the same transcription regulatory complex (NCT), that act as negative regulators of ergosterol biosynthesis, and are involved in the transcription of the ABC transporter CDR1B-encoding gene, which was shown to play a role in azole resistance. Deletion of *A. fumigatus nctA* and *nctB* resulted in a strain with increased resistance to various azoles (itraconazole, voriconazole, posaconazole), amphotericin B and terbinafine. In contrast, the same strain was highly sensitive to echinocandins (caspofungin and micafungin), CFW and Congo red [195]. These results highlight the importance of these TFs for mediating the *A. fumigatus* response to antifungal agents. NctC and CbfA are also members of the CBF/NF-Y TF family, proteins that are important for the mitochondrial electron transport chain [196]. NctC and CbfA were also important for fungal growth, secondary metabolite production, with the deletion of *nctC* resulting in an avirulent *A. fumigatus* strain, in a neutropenic mice model [194]. ZnfA is a TF with a zinc-finger associated domain and AtfA is a leucine zipper TF. Deletion of *atfA* in *A. fumigatus* resulted in a strain with conidia that were highly sensitive to heat and oxidative stress, contained less intracellular trehalose levels and were delayed in germination [197]. Furthermore, AtfA was shown to positively regulate genes important for oxidative stress resistance (e.g.,

catalases), and to negatively regulate genes encoding enzymes involved in germination (e.g., *calA* and *calB*), suggesting a role for AtfA in mediating conidial dormancy [198]. The expression of the TF ZfpA is regulated by CrzA and induced in the presence of voriconazole and calcium [140,199]. The role of the TF AFUB_05400 has not been described and does not have an identity with any described TF [194].

The *fdhA* gene encodes a TF containing a fork-head domain, which was shown to be important for cell wall stress tolerance, as the corresponding deletion strain was sensitive to Congo red, calcofluor white and high temperatures. Through performing transcriptional analyses of the Δ*fhdA* and WT strains in the presence of caspofungin, Valero et al., 2020, observed an induction of genes related to iron metabolism. The growth of the Δ*fhdA* strain in iron-limiting conditions resulted in increased sensitivity to caspofungin and a reduced CPE. This is in contrast to growth in the presence of iron-rich medium, where the CPE took place, suggesting that the homeostasis of iron is important for the resistance of *A. fumigatus* to caspofungin [194]. Indeed, Colabardini and colleagues, 2021, demonstrated that iron depletion is essential for the CPE in the *A. fumigatus* Af239 strain, as growth of Af239 in minimal media without iron (MM-Fe) and supplemented with 8 µg/mL of caspofungin was significantly reduced when compared to iron rich media and caspofungin. Furthermore, the presence of 2 µg/mL caspofungin induced the expression of the biosynthetic gene cluster (BGC) 9 in Af239. BGC9 contains genes that code for enzymes involved in the synthesis of the iron chelator hexadehydroastechrome (HAS). Overexpression of *hasA* completely abolished the CPE, suggesting that the iron homeostasis is important during the CPE in *A. fumigatus* [187].

Caspofungin inhibits β-1,3-glucan synthesis through noncompetitive binding to Fks1, a subunit of β-1,3-glucan synthase [200]. It was shown that the expression of *fks1* is also important for the establishment of the CPE [201]. To further investigate this, a conditional mutant $fks1_{tetOn}$ was constructed, where the *fks1* promoter region was replaced by the doxycycline-inducible Tet-On promoter. During repressing conditions (absence of doxycycline), the CPE in the presence of 8 µg/mL of caspofungin was not observed in this conditional mutant. In addition, prolonged exposure to caspofungin also did not induce paradoxical growth of this strain in the presence of caspofungin, suggesting the requirement of Fks1 for the establishment of the CPE. Furthermore, the expression of *fks1* was shown to correlate with fungal sensitivity to caspofugin, where low *fks1* expression led to higher resistance against caspofungin, and high *fks1* expression resulted in increased sensitivity to caspofungin [201].

5. Chitin and Host Immune System Interaction

The fungal cell wall is the interface and first point of contact between the fungal cell and the host immune cells. The fungal cell wall contains a myriad of pathogen associated molecular patterns (PAMPs), molecules containing conserved motifs that are associated with pathogen infection and that serve as ligands for immune cell receptors. Thus, PAMPs stimulate a host-specific immune response directed at eliminating the pathogen from the host. Chitin is a major fungal PAMP and the ability of chitin to induce an immune response relies on several factors, such as its source, size, and concentration [202–204]. Chitin can interact with pattern recognition receptors (PRRs) present on immune cells and is recognized by TLR2, Dectin-1 and mannose receptors (MR). This interaction can lead to fungal phagocytosis and cytokine secretion [12,203,204].

Studies using purified *A. fumigatus* chitin have shown that this type of chitin elicits an anti-inflammatory response in vitro (Figure 5A). Incubation of *A. fumigatus* chitin (strain 237) with human peripheral blood mononuclear cells (PBMCs) stimulated the secretion of IL-10, but not TNF- α, although no statistical difference was observed when compared to unstimulated cells [205]. In another study, purified *A. fumigatus* chitin (strain CEA17_Δ$akuB^{KU80}$) did not stimulate human PBMCs to produce proinflammatory cytokines (IL-1β, TNF-α or IL-6) or IL-10 [206]. In contrast, *A. fumigatus* chitin stimulated the secretion of IL-1 receptor antagonist (IL-1Ra), an anti-inflammatory cytokine. The

production of IL-1Ra by human PBMCs was dependent on the opsonization of chitin by serum IgG, which in turn interacted with membrane-bound Fc-γ receptors resulting in Syk kinase and phosphatidylinositol 3-kinase (PI3K) activation [206]. Interestingly, the observed effect was Dectin-1, TLR2, TLR4, MR and NOD2-independent.

Additional studies, which were carried out in a more realistic setting and included the presence of other cell walls associated with PAMPs that showed an increased in proinflammatory response. Chitin synergizes with muramyl dipeptide (MDP), Pam3ys and LPS to induce the production and secretion of proinflammatory mediators, such as IL-1β and TNF-α by human PBMCs [206]. Similarly, intranasal administration of *A. fumigatus* AIF (alkali-insoluble cell wall fragments), containing both chitin and β-glucan polysaccharides, as well as chitinase-treated AIF or glucanase-treated AIF in mice, induced lung inflammation and a great influx of immune cells; however, recruitment of neutrophils and eosinophils was only significantly increased in AIF treated mice [207]. Similarly, during in vivo and in vitro experiments with RAW 264.7 macrophages, stimulation with AIF, chitinase-treated AIF or glucanase-treated AIF resulted in increased TNF-α production in the presence of wholesome AIF, suggesting a synergistically interaction of chitin and β-glucan for immune activation [207]. AIF from *A. fumigatus* also had an in vitro inhibitory effect on PBMC cytokine production. Pretreatment of human PBMCs with *A. fumigatus* AIF, and later stimulation with the TLR4 agonist LPS, resulted in decreased secretion of IL-6, when compared to non-pretreated cells [208]. The presence of β-glucan and galactomannan, but not chitin, in AIF, was responsible for the inhibition of IL-6 secretion [208]. These results suggest that the mammalian immune response to *A. fumigatus* is elicited by different cell wall polysaccharides.

Furthermore, the immune response is also modulated by the amount of cell wall polysaccharides, including chitin, present in the cell wall of *A. fumigatus*. Mice that inhaled *A. fumigatus* conidia expressing high levels of chitin (strain Af5517) display reduced gene transcription of adiponectin, an anti-inflammatory mediator [209] in the lungs homogenates compared to mice that aspirated a low chitin expressing strain (Af293) [210]. When adiponectin knockout mice inhaled strain Af5517 conidia, an increase in neutrophil recruitment was observed, and the transcription levels of inflammatory mediators were increased, when compared to strain Af293 [210]. In contrast, WT mice infected with strain Af5517 had an increase in eosinophil recruitment to the lungs, when compared to mice infected with different low chitin-containing strains [211,212] (Figure 5B).

The activation and recruitment of immune cells, primarily eosinophils, mediated by *A. fumigatus* chitin may be γδ T cell-dependent. In the presence of the antifungal drug caspofungin, which induces *A. fumigatus* chitin levels in vitro and in vivo, an increase in lung eosinophil influx was observed in infected mice [211,213]. When γδ T cell-deficient mice were infected with *A. fumigatus* and treated with caspofungin, they displayed increased survival, low fungal burden and low eosinophil counts in the lungs, when compared to nontreated mice [213]. Furthermore, WT mice infected with the high-chitin strain Af5517, presented increased lung eosinophil recruitment when compared to mice infected with the normal chitin strain Af293. In γδ T cell-deficient mice, the eosinophil recruitment in the presence of strain Af5517 was abolished [211]. These studies highlight the importance of γδ T cells in eosinophil recruitment, a response that appears to be dependent on *A. fumigatus* chitin content. Moreover, the increase in eosinophil recruitment to the lungs of mice infected with *A. fumigatus* strain Af5517, is dependent on a Th2 immune response. Mice infected with strain Af5517 exhibited a reduction in CD4 T cells, which produce IFN-γ, and a transcriptional increase in Th2 chemokines and cytokines (CCL11 and CCL22) in the lungs, when compared to infection with strain Af293 [212].

Although the high-chitin strain Af5517 elicited an increased early inflammatory response in an infection model of aspirated conidia [210,211], the development and progression of disease in a mouse model of invasive aspergillosis (IA), is less detrimental than for strain Af293, as assessed by mouse survival, disease score and fungal burden in the murine lung [214]. Such difference may be related to a delay in the growth of strain

Af5517 [214], or the increased recognition and clearance by immune cells; indeed higher concentrations/differential exposure of chitin and β-glucans trigger recognition by Dectin-1 and phagocytosis [215,216]. Alternatively, in order to promote IA, mice had to be immunosuppressed, so the lack of neutrophils in this murine model may also be related to the observed disease outcome when infected with both high- and low-chitin *Aspergillus* isolates. O'Dea et al. (2014) showed that the observed reduction in disease severity, in a model of IA infection, was related to the presence of eosinophils. BALB/c mice that aspirated Af5517 conidia, but not Af293, displayed an increase in eosinophil numbers in BALF. Additionally, eosinophil-deficient mice that were repeatedly challenged with *A. fumigatus* strain Af5517, showed increased survival, low disease score and decreased lung fungal burden when compared to neutropenic mice infected with the same strain [212] (Figure 5B).

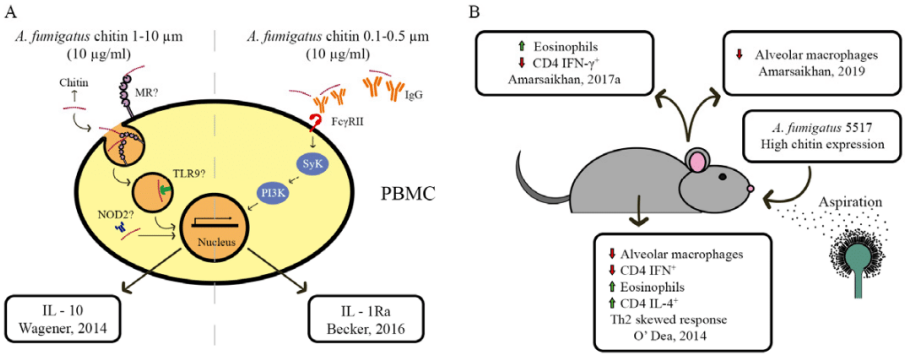

Figure 5. Chitin is important for the recognition of fungal pathogens by the mammalian immune system. (**A**) *A. fumigatus* purified chitin and its interaction with human PBMCs. Human PBMCs showed an increase in IL-10 secretion upon stimulation with *A. fumigatus* chitin, although this was not statistically significant. Furthermore, the receptor that mediates the increase in IL-10 upon stimulation with purified chitin, remains to be determined. Instead, when *A. fumigatus* chitin was opsonized with IgG, production of the anti-inflammatory cytokine IL-1Ra was observed. Subsequently, the *A. fumigatus* chitin-IgG complex activates the PBMC Fc-γ receptors, resulting in the activation of the Syk and phosphatidylinositol 3-kinase (PI3K) kinases and cytokine secretion. PBMC: peripheral blood mononuclear cells; IL-: Interleukin; MR: mannose receptor; TLR: toll-like receptor 9; NOD2: nucleotide-binding oligomerization domain containing 2. (**B**) Mice that aspired conidia of an *A. fumigatus* high chitin-containing strain (Af5517) displayed an increased Th2-mediated response in BALF. Indeed, inhaled Af5517 conidia led to increased eosinophils and CD4 IL-4+ T cells in BALF, while alveolar macrophages and CD4 IFN+ showed reduced numbers. BALF: bronchoalveolar lavage fluid [205,206,210–212].

These studies suggest that chitin present in the *A. fumigatus* cell wall induces the recruitment of eosinophils, which are the main cells related to allergic inflammatory responses. Intraperitoneal immunization of C57BL/6 mice with *A. fumigatus* allergen extract (1WCF) with crab shell chitin as adjuvant, led to an increase in total IgE and specific serum IgE and IgG1 [217]. These results indicate an allergic property of 1WCF that is enhanced in the presence of chitin. In agreement, the activity of eosinophil peroxidase (EPO) in BALF cell pellet lysate was increased during 1WCF immunization with chitin as adjuvant, supporting an increase in the number of eosinophils in the lung [217]. Strong et al. (2002) employed a different approach to unveil the allergic properties of chitin during *Aspergillus* infection. Mice were immunized with *A. fumigatus* allergen extract (Afu), using alum as an adjuvant, and then intranasally challenged with Afu and purified chitin (Sigma-Aldrich) or Afu and PBS. A reduction in total serum IgE, specifically in Afu IgG, as well as in peripheral blood eosinophils, was observed in mice challenged with Afu and chitin when compared to mice challenged with Afu and PBS. Furthermore, the levels of Th1 cytokines (IL-12, TNF-α and IFN-γ) were significantly increased in the spleen of mice challenged with Afu and

chitin, while IL-4 levels were decreased when compared to mice challenged with Afu and PBS [218]. In agreement, the respiratory capacity and lung histology of mice challenged with Afu and chitin were preserved when compared to those of mice challenged with Afu and PBS [218]. These results suggest a modulation of the allergic response elicited by *A. fumigatus* extracts that is somewhat dependent on the phase of chitin administration.

Differences in chitin source and size, as well as the experimental design could explain the differences observed in the immune modulation exerted by chitin in both studies that used only immunized mice and the same adjuvant. Similar to Dubey et al. (2015), Roy et al. (2013) showed that mice sensitized with a combination of *A. fumigatus* antigens and chitin purified from crab shells presented a strong allergic response. They observed an increase in eosinophilia in the lungs as well as expression of Th2 cytokines (IL-4, IL-5 and IL13), and increased serum IgE levels when compared to mice treated with either chitin or *A. fumigatus* antigens alone [219]. The immunological response in mice sensitized with *A. fumigatus* antigens and crab shell chitin was dependent on chitin complement opsonization and cleavage of C3, leading to the activation of the C3a receptor (C3aR). A similar opsonization-dependent response to *A. fumigatus* purified chitin was also observed in vitro in PBMC-treated cells [206]. Mice sensitized with *A. fumigatus* antigens and crab shell chitin also showed a less tolerogenic phenotype in lung plasmacytoid dendritic cells (pDCs) that were related to an increased inflammatory T cell response in the lungs, and which consisted of $CD4^+$ $IL\text{-}4^+$ and $CD4^+$ $IL\text{-}17^+$ T cells. This increased inflammatory response was associated with the presence of C3 and C3aR activation, as the corresponding C3KO mice presented a more tolerogenic response to *A. fumigatus* antigens and crab shell chitin, which was accompanied by an increase in $CD4^+$ $Foxp3^+$ T cells, and a reduction in $CD4^+$ $IL\text{-}4^+$ and $CD4^+$ $IL\text{-}17^+$ T cells [219].

Furthermore, the use of *A. fumigatus* CS deletion mutants highlighted the importance of chitin and cell wall organization for fungal interaction with the mammalian immune system. de Jesus Carrion and colleagues (2019) showed that the *A. fumigatus* Δ*AfchsA* Δ*AfchsC* Δ*AfchsB* Δ*AfchsG* (division 1) and Δ*AfcsmA* Δ*AfcsmB* Δ*AfchsD* Δ*AfchsF* (division 2) strains were more susceptible to human neutrophil killing, as assessed by the XTT viability of fungal cells, when compared to the parental strain. In a murine model of corneal infection, *A. fumigatus* strains deleted for division 1 or 2 of CS-encoding genes, caused a reduction in virulence as measured by a decrease in CFU in the eyes of mice and less corneal disease when compared to the parental strain [98]. Interestingly, neutrophil-mediated killing of fungal hyphae depended on the activity of acidic mammalian chitinase (AMCase), an enzyme that hydrolyzes chitin. Indeed, the fungicidal properties of neutrophils in the presence of chitinase inhibitors or when using neutrophils from $AMCase^{-/-}$ mice were impaired [98]. A disturbance in chitin synthesis due to the absence of CS-encoding genes causes restructuring/reorganization of the fungal cell wall. Indeed, the deletion of *AfcsmA*, *AfcsmB* or both of these genes resulted in the disruption or complete loss of the *A. fumigatus* conidial rodlet layer, which in turn exposed mannan and chitin, resulting in an increased immunological response [108]. Indeed, DCs cultivated with the single and double deletion strains of these CS-encoding genes had increased surface activation and displayed an increase in maturation markers, such as CD80, CD86, CD40, CD83 and in the expression of the major histocompatibility complex class II (HLA-DR) [108]. The *A. fumigatus* CS encoded by gene *AfchsG* from division 1 seems to play a major role in chitin biosynthesis in conidia, as deletion of the corresponding gene caused a 60% reduction in conidial chitin, whereas a small reduction in mycelial chitin levels were observed [103]. Strains Δ*AfchsB* and Δ*AfchsC*, also deleted for division 1 CS-encoding genes, display a subtle reduction in mycelial chitin, whereas the Δ*AfchsA*, Δ*AfchsB* and Δ*AfchsC* strains showed a small reduction in conidial chitin [103]. A strain deleted for all four CS-encoding genes from division 1 (Δ*AfchsA* Δ*AfchsC* Δ*AfchsB* Δ*AfchsG*) was as virulent (assessed through survival curves and histological analyses) as the parental strain in both immunosuppressed murine and insect (*Galleria mellonella*) models of infection [103]. This can be explained by the insignificant reduction in mycelial and conidial chitin content in the quadruple mutant

when compared to the WT strain. Deletion of the *A. fumigatus* CS-encoding gene Δ*AfchsF* from division 2 resulted in a strain with a 25% reduction in mycelial chitin, whereas strains Δ*AfchsF*, Δ*AfcsmB* and Δ*AfcsmA* (deleted for division 2 CS-encoding genes) displayed a reduction in cell wall chitin content in the conidia, with Δ*AfcsmA* mutant showing the greatest reduction (~80%) when compared to the parental strain. The quadruple mutant of division 2 CS-encoding genes (Δ*AfcsmA* Δ*AfcsmB* Δ*AfchsF* Δ*AfchsD*) showed comparable chitin content (in mycelia and conidia) than the parental strain, although chitin microfibrils showed significant structural differences, which could account for the observed hypovirulent phenotype of this strain in both immunosuppressed murine and insect models of aspergillosis. The structural differences were not observed for the class 1 quadruple deletion strain, suggesting that CS from division 2 is more important for chitin structure and cell wall integrity than CS from division 1 [103].

In addition to gene-encoding proteins involved in chitin metabolism, *A. fumigatus* gene encoding proteins involved in cell wall morphogenesis and biosynthesis also affect cell wall chitin content and interaction with mammalian immune cells. The *A. fumigatus Afcps1* gene was identified from a genome-wide random insertional mutagenesis screening as an important regulator of morphogenesis [220]. *Afcps1* encodes for capsular polysaccharide synthase 1 (Cps1), which is required for normal colony development. The absence of *Afcps1* resulted in reduced α-glucan, β-glucan, and chitin levels in *A. fumigatus* mycelia, as well as in a decreased transcription of CS-encoding genes [220]. Incubation of Δ*Afcps1* conidia with BMDMs revealed that the absence of *Afcps1* resulted in greatly induced BMDM cellular activation and upregulation of genes involved in killing, biological adhesion and the production of cytokines and chemokines (*IL1b*, *IL12b*, *IL10* and *Ccl22*, among others) [220]. Even though the Δ*Afcps1* strain presented reduced expression of cell wall α-glucan, β-glucan and chitin, the immune stimulatory properties of this strain were significantly enhanced when compared to the WT strain. Similarly, deletion of *ssdA*, encoding a multifunctional RNA-binding protein involved in conidial and mycelial trehalose metabolism in *A. fumigatus*, resulted in a strain with increased trehalose, decreased chitin and β-1,3-glucan levels, while *ssdA* overexpression (OE::*ssdA*) caused decreased trehalose, increased chitin and reduced β-1,3-glucan levels [135]. In a murine model of invasive aspergillosis, the OE::*ssdA* (high chitin content) strain had decreased virulence, while the Δ*ssda* (low chitin content) strain was as virulent as the WT strain. Increased mice survival, reduction in immune infiltrate in BALF and a reduction in lung fungal growth were observed for mice infected with the OE::*ssdA* strain [135]. Furthermore, the absence of the regulatory subunit TslA (Δ*tslA*) involved in trehalose biosynthesis in *A. fumigatus*, resulted in a strain with decreased trehalose and β-glucans levels, and increased chitin levels [21]. Indeed, the Δ*tslA* strain presented increased chitin synthase activity, due to negative regulation in the CS *AfcsmA* (Afu2g13440) by TslA [134]. In a murine model of invasive pulmonary aspergillosis, the *A. fumigatus* Δ*tslA* strain was as virulent as the WT strain. However, mice infected with the Δ*tslA* strain displayed an increased inflammatory state, with increased inflammatory lesions in the lungs, greater recruitment of neutrophils and macrophages to BALF, and increased secretion of inflammatory cytokines and chemokines (TNF-α, MIP-1α and CXCL1) [134]. This result contradicts a study that was carried out by the same group in 2019, where the authors observed that overexpression of SsdA resulted in increased fungal cell wall chitin and a reduction in virulence [135]. Similarly, the Δ*zipD* strain, which codes for a transcription factor involved in the CPE and the calcium-calcineurin pathway, also presented decreased β-1,3-glucan content, increased levels of chitin and a thicker cell wall in the presence of caspofungin when compared to the WT strain, suggesting that ZipD is important for cell wall maintenance and integrity. In a murine model of invasive pulmonary aspergillosis, the Δ*zipD* strain was avirulent, with conidia from this stain being more susceptible to BMDM-killing, which was accompanied a by higher production of the proinflammatory cytokines IL-12p40, Il-6, IL-1β and TNF-α. A similar inflammatory phenotype was observed in immunocompetent mice, where infection with Δ*zipD* caused a greater influx of innate and adaptive inflammatory cells to the lungs, accompanied by an

increased secretion of proinflammatory cytokines and a decrease in fungal burden. The authors of this study suggested that ZipD-dependent cell wall organization is important for *A. fumigatus* immune evasion, although additional mechanisms that are regulated by this transcription factor cannot be excluded [193]. Together, these studies suggest that the deletion and overexpression of different genes, causes additional alterations in fungal cell development and fitness, which can impact cell wall integrity and composition, and in turn promote different immune responses. It is also worth noting that the aforementioned studies show that a reduction in chitin did not affect virulence, whereas an increase in cell wall chitin caused a reduction in virulence. It would be interesting to measure cell wall thickness in all these deletion strains and determine whether a correlation with strain-specific virulence exists. Alternatively, each of the aforementioned gene deletions caused effects other than cell wall remodeling on the fungal cell, which could also account for the observed differences in virulence. For example, the Δ*Afcps1 A. fumigatus* strain showed compact and wrinkled colony shapes, reduced colony size, conidiation and delayed fungal growth when compared to the parental strain [220]. The Δ*ssdA* and OE::*ssdA* strains also displayed a decrease in mycelial radial growth and reduced biomass in liquid culture when compared to the parental strain [135]. Furthermore, Δ*ssdA* conidia germinate faster, and OE::*ssdA* conidia germinate slower when compared to the parental strain. The Δ*tslA* displayed delayed conidia germination when compared to the parental strain [134]. Together, the observed differences in immune responses to the deletion strains may be a combination of defects, including cell wall organization, growth and development.

6. CS Gene Manipulation and Biotechnological Applications

Several species from the *Aspergillus* genus have important biotechnological and biomedical applications, due to their high metabolic diversity, and subsequent production and secretion of industrially relevant enzymes and secondary metabolites [221]. *Aspergillus* species, such as *A. niger, A. oryzae* and *A. terreus* are routinely used for citric acid production, oriental food production (such as soy sauce, sake brewing and soybean paste), and the production of statins, which are cholesterol-lowering drugs [222,223].

With the acquisition of more advanced genetic tools, the analysis of fungal transcriptional responses during industrial processes can be assessed. Yin and colleagues, 2016, demonstrated that during citric acid production, *A. niger* had an increase in the expression of cell wall related genes, including the CS-encoding gene *AngchsF* (An12g10380) [101]. This gene was also significantly expressed during glucoamylase synthesis [224], suggesting that chitin synthesis, perhaps not surprisingly, is also important for fungal morphology under industrial culture conditions.

Indeed, genetic engineering targeting CS-encoding genes, and the productivity of the resulting strains under industrial culture conditions were carried out. In *A. niger*, RNA interference (RNAi) was used to silence *AngchsC* (An8g04350) expression, resulting in a strain with decreased conidia production and chitin concentrations, highly compact mycelial pellets in submerged cultures and increased production of citric acid [73]. Needless to say, high citric acid-producing strains in bioreactors are highly desired for industrial citric acid production.

In *A. oryzae*, Müller and colleagues showed that the deletion of a CS-encoding gene can have positive effects on the rheological features of the culture [225,226]. In submerged cultures, *AochsB* (ΔAO090701000589) strain presented decreased hyphal growth, when compared to A1560 WT strain, and no differences on α-amylase yield were observed between the strain [226]. The *AochsB* deletion strain has more hyperbranched hyphae [72,225,226] and a ~88% reduction in extension rate when compared to the WT strain, thus, having severe defects in hyphal extension [225]. The fermentation broth of the *AochsB* deletion strain was significantly less viscous when compared to the one from the WT strain, a feature that is desired for improving submerged culture conditions and increasing enzyme yield [226].

The aforementioned studies further emphasize the close relationship between chitin synthesis and fungal morphology. Unfortunately, not many studies have investigated the

role of cell wall polysaccharide biosynthesis in optimizing bioreactor and culture conditions, although the aforementioned studies clearly show some promising results. Targeting cell wall chitin and other sugar biosynthesis is therefore, an interesting strategy for improving yields of fungal products as well as improving culture conditions, especially those that are dependent on fungal morphology.

7. Summary and Conclusions

Chitin is a structural component of the fungal cell wall, which, together with other polysaccharides, such as glucan, confers cell rigidity and shape, is essential for fungal survival and interaction with the extracellular space. Chitin biosynthesis is catalyzed by different classes of chitin synthases (CS) in *Aspergillus* species, and the expression of these enzymes is dependent on the stage of fungal growth and the presence of extracellular cues, such as cell wall stressors. Up to eight CS-encoding genes can be present in the genomes of *Aspergillus* species. CS are crucial for fungal growth and development, as deletion of the respective genes often results in strains with morphological defects in hyphal structure and growth as well as conidia formation. Deletion of some CS-encoding genes did not result in an obvious phenotype, and their specific function remains unknown. Figure 3 shows an overview of CS genes and their functions in *Aspergillus* species.

Although the metabolic steps of chitin biosynthesis are well described, processes involved in the initiation of chitin biosynthesis remain to be elucidated in all *Aspergillus* species (Figure 1). Similarly, how CS are localized to the extending hyphal tip or to hyphal branches as well as to conidiophores remains to be investigated in many *Aspergillus* species. The exception is *A. nidulans*, where the transport of some CS to the actively growing hyphal tip was shown to be dependent on kinesins and occurred along microtubules and actin filaments. Nevertheless, further investigation is required to determine the localization and transport of cell wall modifying enzymes to sites of growth and or conidiation in *Aspergillus* species.

Chitin biosynthesis is regulated by many different pathways, which ensure cell wall integrity, respond to extracellular stresses and provide the energy to support fungal growth and development (Figure 4). Crosstalk exists between these pathways, which accommodate different signals and allow the cell to adequately respond to these cues while continuing growth.

Considering the importance of chitin biosynthesis for fungal development and the complexity of regulation underlying chitin biosynthesis, it is perhaps not a surprise that these processes are intrinsically linked to antifungal drug resistance and interaction with mammalian immune cells. Indeed, many antifungal drugs or a combination thereof, target the synthesis of cell wall polysaccharides (CWP), including chitin, in order to disrupt fungal growth and cause cell death. CWP are interesting targets for the development of antifungal strategies, as these sugars are not present in mammalian cells. Unfortunately, inhibiting the synthesis of one CWP can lead to an increase in another CWP, thus, conferring fungal resistance to the drug. The most prominent example of this is the CPE, where inhibition of glucan biosynthesis leads to an increase in CW chitin levels and improved growth in the presence of the echinocandin caspofungin. The mechanism underlying the CPE has proven itself to be extremely complex, and recent studies have shown that many regulators are involved in sustaining the CPE in the presence of high concentrations of caspofungin. Our understanding of the CPE is far from complete, and additional studies are required to fully understand the fungal cellular mechanisms that support the CPE in order to counteract this phenomenon and maximize the potential of echinocandins in treating invasive fungal diseases.

Similarly, chitin, in conjunction with other CWP, is an important target that is recognized by mammalian immune cells and triggers an adaptive response. Deletion of genes coding for CS or regulators of chitin biosynthesis often results in strains with a significant reduction in virulence, although this may be due to the strain-specific morphological defects and a general reduction in fitness caused by the respective deletion. Nevertheless, chitin

and other CWP are crucial for triggering an immune response and keeping opportunistic fungal infections at bay, making it a possible therapeutic target for antifungal strategies, such as vaccines, although this has not been successful to date.

Manipulation of chitin biosynthesis is not only relevant for biomedical applications, but a few studies have shown a potential for biotechnological applications, where genetic interference of chitin biosynthesis resulted in increased secretion of a valuable product or improved culture conditions. Unfortunately, not many studies have addressed this, and manipulation of fungal cell wall properties certainly makes an interesting case for strain engineering in biotechnological applications.

Together, the mechanisms underlying chitin biosynthesis are crucial for fungal homeostasis and, thus, for antifungal drug resistance, interaction with mammalian immune responses and biotechnological applications. Fungal chitin metabolism, therefore, continues to be a highly interesting topic that is worth investigating even after more than 30 years of research in this area.

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References

1. Free, S.J. Fungal cell wall organization and biosynthesis. *Adv. Genet.* **2013**, *81*, 33–82. [CrossRef]
2. Bowman, S.M.; Free, S.J. The structure and synthesis of the fungal cell wall. *Bioessays* **2006**, *28*, 799–808. [CrossRef]
3. Latgé, J.P. The cell wall: A carbohydrate armour for the fungal cell. *Mol. Microbiol.* **2007**, *66*, 279–290. [CrossRef]
4. Garcia-Rubio, R.; de Oliveira, H.C.; Rivera, J.; Trevijano-Contador, N. The Fungal Cell Wall: Candida, Cryptococcus, and *Aspergillus* Species. *Front. Microbiol.* **2020**, *10*, 2993. [CrossRef]
5. Geoghegan, I.; Steinberg, G.; Gurr, S. The Role of the Fungal Cell Wall in the Infection of Plants. *Trends Microbiol.* **2017**, *25*, 957–967. [CrossRef]
6. Pontón, J. The fungal cell wall and the mechanism of action of anidulafungin. *Rev. Iberoam. Micol.* **2008**, *25*, 78–82. [CrossRef]
7. Singh, R.; Shitiz, K.; Singh, A. Chitin and chitosan: Biopolymers for wound management. *Int. Wound J.* **2017**, *14*, 1276–1289. [CrossRef]
8. Khoushab, F.; Yamabhai, M. Chitin research revisited. *Mar. Drugs* **2010**, *8*, 1988–2012. [CrossRef]
9. Muzzarelli, R.A.A.; Boudrant, J.; Meyer, D.; Manno, N.; DeMarchis, M.; Paoletti, M.G. Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. *Carbohydr. Polym.* **2012**, *87*, 995–1012. [CrossRef]
10. Liaqat, F.; Eltem, R. Chitooligosaccharides and their biological activities: A comprehensive review. *Carbohydr. Polym.* **2018**, *184*, 243–259. [CrossRef]
11. Khan, F.I.; Rahman, S.; Queen, A.; Ahamad, S.; Ali, S.; Kim, J.; Hassan, M.I. Implications of molecular diversity of chitin and its derivatives. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 3513–3536. [CrossRef]
12. Bueter, C.L.; Specht, C.A.; Levitz, S.M. Innate sensing of chitin and chitosan. *PLoS Pathog.* **2013**, *9*, e1003080. [CrossRef]
13. Kang, X.; Kirui, A.; Muszyński, A.; Widanage, M.C.D.; Chen, A.; Azadi, P.; Wang, P.; Mentink-Vigier, F.; Wang, T. Molecular architecture of fungal cell walls revealed by solid-state NMR. *Nat. Commun.* **2018**, *9*, 2747. [CrossRef]
14. Chakraborty, A.; Fernando, L.D.; Fang, W.; Dickwella Widanage, M.C.; Wei, P.; Jin, C.; Fontaine, T.; Latgé, J.-P.; Wang, T. A molecular vision of fungal cell wall organization by functional genomics and solid-state NMR. *Nat. Commun.* **2021**, *12*, 6346. [CrossRef]

15. Lass-Flörl, C.; Roilides, E.; Löffler, J.; Wilflingseder, D.; Romani, L. Minireview: Host defence in invasive aspergillosis. *Mycoses* **2013**, *56*, 403–413. [CrossRef]
16. Baker, S.E.; Bennett, J.W. An Overview of the Genus Aspergillus. In *The Aspergilli: Genomics, Medical Aspects, Biotechnology, and Research Methods*; Goldman, G.H., Osmani, S.A., Eds.; CRC Press Taylos & Francis Group: Boca Raton, FL, USA, 2008.
17. Bennett, J.W. Aspergillus: A primer for the novice. *Med. Mycol.* **2009**, *47*, S5–S12. [CrossRef]
18. Park, H.S.; Jun, S.C.; Han, K.H.; Hong, S.B.; Yu, J.H. Diversity, Application, and Synthetic Biology of Industrially Important *Aspergillus* Fungi. *Adv. Appl. Microbiol.* **2017**, *100*, 161–202. [CrossRef]
19. Kumar, P.; Mahato, D.K.; Kamle, M.; Mohanta, T.K.; Kang, S.G. Aflatoxins: A Global Concern for Food Safety, Human Health and Their Management. *Front. Microbiol.* **2017**, *7*, 2170. [CrossRef]
20. Sugui, J.A.; Kwon-Chung, K.J.; Juvvadi, P.R.; Latgé, J.-P.; Steinbach, W.J. Aspergillus fumigatus and related species. *Cold Spring Harb Perspect. Med.* **2014**, *5*, a019786. [CrossRef]
21. Becker, A.; Schlöder, P.; Steele, J.E.; Wegener, G. The regulation of trehalose metabolism in insects. *Experientia* **1996**, *52*, 433–439. [CrossRef]
22. François, J.; Parrou, J.L. Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **2001**, *25*, 125–145. [CrossRef]
23. Lesage, G.; Bussey, H. Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 317–343. [CrossRef]
24. Puttikamonkul, S.; Willger, S.D.; Grahl, N.; Perfect, J.R.; Movahed, N.; Bothner, B.; Park, S.; Paderu, P.; Perlin, D.S.; Cramer, R.A., Jr. Trehalose 6-phosphate phosphatase is required for cell wall integrity and fungal virulence but not trehalose biosynthesis in the human fungal pathogen *Aspergillus fumigatus*. *Mol. Microbiol.* **2010**, *77*, 891–911. [CrossRef]
25. Merzendorfer, H. The cellular basis of chitin synthesis in fungi and insects: Common principles and differences. *Eur. J. Cell Biol.* **2011**, *90*, 759–769. [CrossRef]
26. Masuda, C.A.; Xavier, M.A.; Mattos, K.A.; Galina, A.; Montero-Lomeli, M. Phosphoglucomutase is an in vivo lithium target in yeast. *J. Biol. Chem.* **2001**, *276*, 37794–37801. [CrossRef]
27. Thoden, J.B.; Holden, H.M. The molecular architecture of glucose-1-phosphate uridylyltransferase. *Protein Sci.* **2007**, *16*, 432–440. [CrossRef]
28. Lagorce, A.; Le Berre-Anton, V.; Aguilar-Uscanga, B.; Martin-Yken, H.; Dagkessamanskaia, A.; François, J. Involvement of GFA1, which encodes glutamine-fructose-6-phosphate amidotransferase, in the activation of the chitin synthesis pathway in response to cell-wall defects in *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **2002**, *269*, 1697–1707. [CrossRef]
29. Ram, A.F.; Arentshorst, M.; Damveld, R.A.; vanKuyk, P.A.; Klis, F.M.; van den Hondel, C.A. The cell wall stress response in *Aspergillus niger* involves increased expression of the glutamine: Fructose-6-phosphate amidotransferase-encoding gene (gfaA) and increased deposition of chitin in the cell wall. *Microbiol. (Read.)* **2004**, *150*, 3315–3326. [CrossRef]
30. Chawla, S.P.; Kanatt, S.R.; Sharma, A.K. Chitosan. In *Polysaccharides: Bioactivity and Biotechnology*; Ramawat, K.G., Mérillon, J.-M., Eds.; Springer International Publishing: Cham, Switzerland, 2015; pp. 219–246.
31. Cui, J.; Yu, Z.; Lau, D. Effect of Acetyl Group on Mechanical Properties of Chitin/Chitosan Nanocrystal: A Molecular Dynamics Study. *Int. J. Mol. Sci.* **2016**, *17*, 61. [CrossRef]
32. Lockhart, D.E.A.; Robinson, D.A.; Fang, W.; Ferenbach, A.T.; van Aalten, D.M.F. Validation of *Aspergillus fumigatus* glucosamine-6-phosphate N-acetyltransferase as a potential antifungal target. *Lancet* **2014**, *383*, S69. [CrossRef]
33. Dahiya, N. Chitin Metabolism In Fungi. In *Progress in Mycology*; Rai, M., Kövics, G., Eds.; Springer: Dordrecht, The Netherlands, 2010; pp. 409–423.
34. Fang, W.; Du, T.; Raimi, O.G.; Hurtado-Guerrero, R.; Urbaniak, M.D.; Ibrahim, A.F.M.; Ferguson, M.A.J.; Jin, C.; van Aalten, D.M.F. Genetic and structural validation of *Aspergillus fumigatus* UDP-N-acetylglucosamine pyrophosphorylase as an antifungal target. *Mol. Microbiol.* **2013**, *89*, 479–493. [CrossRef]
35. Mengin-Lecreulx, D.; van Heijenoort, J. Copurification of glucosamine-1-phosphate acetyltransferase and N-acetylglucosamine-1-phosphate uridyltransferase activities of Escherichia coli: Characterization of the glmU gene product as a bifunctional enzyme catalyzing two subsequent steps in the pathway for UDP-N-acetylglucosamine synthesis. *J. Bacteriol.* **1994**, *176*, 5788–5795. [CrossRef]
36. Strominger, J.L.; Smith, M.S. Uridine diphosphoacetylglucosamine pyrophosphorylase. *J. Biol. Chem.* **1959**, *234*, 1822–1827. [CrossRef]
37. Elorza, M.V.; Rico, H.; Sentandreu, R. Calcofluor white alters the assembly of chitin fibrils in *Saccharomyces cerevisiae* and Candida albicans cells. *J. Gen. Microbiol.* **1983**, *129*, 1577–1582. [CrossRef]
38. Watanabe, H.; Azuma, M.; Igarashi, K.; Ooshima, H. Analysis of Chitin at the Hyphal Tip of *Candida albicans* Using Calcofluor White. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 1798–1801. [CrossRef]
39. Merzendorfer, H.; Zimoch, L. Chitin metabolism in insects: Structure, function and regulation of chitin synthases and chitinases. *J. Exp. Biol.* **2003**, *206*, 4393–4412. [CrossRef]
40. Bulawa, C.E. Genetics and molecular biology of chitin synthesis in fungi. *Annu. Rev. Microbiol.* **1993**, *47*, 505–534. [CrossRef]
41. Roncero, C. The genetic complexity of chitin synthesis in fungi. *Curr. Genet.* **2002**, *41*, 367–378. [CrossRef]
42. Chaudhary, P.M.; Tupe, S.G.; Deshpande, M.V. Chitin synthase inhibitors as antifungal agents. *Mini Rev. Med. Chem.* **2013**, *13*, 222–236.

43. Specht, C.A.; Liu, Y.; Robbins, P.W.; Bulawa, C.E.; Iartchouk, N.; Winter, K.R.; Riggle, P.J.; Rhodes, J.C.; Dodge, C.L.; Culp, D.W.; et al. The chsD and chsE genes of *Aspergillus nidulans* and their roles in chitin synthesis. *Fungal Genet. Biol.* **1996**, *20*, 153–167. [CrossRef]
44. Ruiz-Herrera, J.; Lopez-Romero, E.; Bartnicki-Garcia, S. Properties of chitin synthetase in isolated chitosomes from yeast cells of Mucor rouxii. *J. Biol. Chem.* **1977**, *252*, 3338–3343. [CrossRef] [PubMed]
45. Bowen, A.R.; Chen-Wu, J.L.; Momany, M.; Young, R.; Szaniszlo, P.J.; Robbins, P.W. Classification of fungal chitin synthases. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 519–523. [CrossRef] [PubMed]
46. Cabib, E.; Shaw, J.A.; Mol, P.C.; Bowers, B.; Choi, W.J. Chitin Biosynthesis and Morphogenetic Processes. In *Biochemistry and Molecular Biology*; Brambl, R., Marzluf, G.A., Eds.; Springer: Berlin/Heidelberg, Germany, 1996; pp. 243–267.
47. Cohen, E. Chitin synthesis and inhibition: A revisit. *Pest Manag. Sci.* **2001**, *57*, 946–950. [CrossRef]
48. Lairson, L.L.; Withers, S.G. Mechanistic analogies amongst carbohydrate modifying enzymes. *Chem. Commun. (Camb.)* **2004**, *20*, 2243–2248. [CrossRef] [PubMed]
49. Lairson, L.L.; Henrissat, B.; Davies, G.J.; Withers, S.G. Glycosyltransferases: Structures, functions, and mechanisms. *Annu. Rev. Biochem.* **2008**, *77*, 521–555. [CrossRef]
50. McNamara, J.T.; Morgan, J.L.; Zimmer, J. A molecular description of cellulose biosynthesis. *Annu. Rev. Biochem.* **2015**, *84*, 895–921. [CrossRef]
51. Merz, R.A.; Horsch, M.; Nyhlén, L.E.; Rast, D.M. Biochemistry of chitin synthase. *Exs* **1999**, *87*, 9–37. [CrossRef]
52. Gibbons, B.J.; Roach, P.J.; Hurley, T.D. Crystal structure of the autocatalytic initiator of glycogen biosynthesis, glycogenin. *J. Mol. Biol.* **2002**, *319*, 463–477. [CrossRef]
53. Morgan, J.L.; Strumillo, J.; Zimmer, J. Crystallographic snapshot of cellulose synthesis and membrane translocation. *Nature* **2013**, *493*, 181–186. [CrossRef]
54. Gyore, J.; Parameswar, A.R.; Hebbard, C.F.F.; Oh, Y.; Bi, E.; Demchenko, A.V.; Price, N.P.; Orlean, P. 2-Acylamido analogues of N-acetylglucosamine prime formation of chitin oligosaccharides by yeast chitin synthase 2. *J. Biol. Chem.* **2014**, *289*, 12835–12841. [CrossRef]
55. Cohen, E. Chapter 2—Chitin Biochemistry: Synthesis, Hydrolysis and Inhibition. In *Advances in Insect Physiology*; Casas, J., Simpson, S.J., Eds.; Academic Press: Cambridge, MA, USA, 2010; Volume 38, pp. 5–74.
56. Merzendorfer, H. Chitin synthesis inhibitors: Old molecules and new developments. *Insect Sci.* **2013**, *20*, 121–138. [CrossRef] [PubMed]
57. Durán, A.; Bowers, B.; Cabib, E. Chitin synthetase zymogen is attached to the yeast plasma membrane. *Proc. Natl. Acad. Sci. USA* **1975**, *72*, 3952–3955. [CrossRef] [PubMed]
58. Sentandreu, R.; Martinez-Ramon, A.; Ruiz-Herrera, J. Localization of Chitin synthase in Mucor rouxii by an autoradiographic method. *J. Gen. Microbiol.* **1984**, *130*, 1193–1199. [CrossRef] [PubMed]
59. Merzendorfer, H. Insect chitin synthases: A review. *J. Comp. Physiol. B* **2006**, *176*, 1–15. [CrossRef]
60. Delmer, D.P. Cellulose Biosynthesis. *Annu. Rev. Plant Physiol.* **1987**, *38*, 259–290. [CrossRef]
61. Delmer, D.P. Cellulose Biosynthesis: Exciting Times for a Difficult Field of Study. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1999**, *50*, 245–276. [CrossRef]
62. Lindeboom, J.; Mulder, B.M.; Vos, J.W.; Ketelaar, T.; Emons, A.M. Cellulose microfibril deposition: Coordinated activity at the plant plasma membrane. *J. Microsc.* **2008**, *231*, 192–200. [CrossRef]
63. Heldermon, C.; DeAngelis, P.L.; Weigel, P.H. Topological organization of the hyaluronan synthase from Streptococcus pyogenes. *J. Biol. Chem.* **2001**, *276*, 2037–2046. [CrossRef]
64. Chen, W.; Cao, P.; Liu, Y.; Yu, A.; Wang, D.; Chen, L.; Sundarraj, R.; Yuchi, Z.; Gong, Y.; Merzendorfer, H.; et al. Structural basis for directional chitin biosynthesis. *Nature* **2022**, *610*, 402–408. [CrossRef]
65. Bracker, C.E.; Ruiz-Herrera, J.; Bartnicki-Garcia, S. Structure and transformation of chitin synthetase particles (chitosomes) during microfibril synthesis in vitro. *Proc. Natl. Acad. Sci. USA* **1976**, *73*, 4570–4574. [CrossRef]
66. Cohen, E. In vitro chitin synthesis in an insect: Formation and structure of microfibrils. *Eur. J. Cell Biol.* **1982**, *26*, 289–294. [PubMed]
67. Berninsone, P.M.; Hirschberg, C.B. Nucleotide sugar transporters of the Golgi apparatus. *Curr. Opin. Struct. Biol.* **2000**, *10*, 542–547. [CrossRef] [PubMed]
68. Yanai, K.; Kojima, N.; Takaya, N.; Horiuchi, H.; Ohta, A.; Takagi, M. Isolation and characterization of two chitin synthase genes from *Aspergillus nidulans*. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 1828–1835. [CrossRef] [PubMed]
69. Motoyama, T.; Kojima, N.; Horiuchi, H.; Ohta, A.; Takagi, M. Isolation of a chitin synthase gene (chsC) of *Aspergillus nidulans*. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 2254–2257. [CrossRef] [PubMed]
70. Mellado, E.; Aufauvre-Brown, A.; Specht, C.A.; Robbins, P.W.; Holden, D.W. A multigene family related to chitin synthase genes of yeast in the opportunistic pathogen *Aspergillus fumigatus*. *Mol. Gen. Genet.* **1995**, *246*, 353–359. [CrossRef]
71. Jiménez-Ortigosa, C.; Aimanianda, V.; Muszkieta, L.; Mouyna, I.; Alsteens, D.; Pire, S.; Beau, R.; Krappmann, S.; Beauvais, A.; Dufrêne, Y.F.; et al. Chitin synthases with a myosin motor-like domain control the resistance of *Aspergillus fumigatus* to echinocandins. *Antimicrob. Agents Chemother.* **2012**, *56*, 6121–6131. [CrossRef]
72. Müller, C.; Hjort, C.M.; Hansen, K.; Nielsen, J. Altering the expression of two chitin synthase genes differentially affects the growth and morphology of *Aspergillus oryzae*. *Microbiology* **2002**, *148*, 4025–4033. [CrossRef]

73. Sun, X.; Wu, H.; Zhao, G.; Li, Z.; Wu, X.; Liu, H.; Zheng, Z. Morphological regulation of *Aspergillus niger* to improve citric acid production by chsC gene silencing. *Bioprocess Biosyst. Eng.* **2018**, *41*, 1029–1038. [CrossRef]
74. Munro, C.A.; Gow, N.A. Chitin synthesis in human pathogenic fungi. *Med. Mycol.* **2001**, *39* (Suppl. S1), 41–53. [CrossRef]
75. Wang, Q.; Liu, H.; Szaniszlo, P.J. Compensatory expression of five chitin synthase genes, a response to stress stimuli, in Wangiella (Exophiala) dermatitidis, a melanized fungal pathogen of humans. *Microbiol. (Read.)* **2002**, *148*, 2811–2817. [CrossRef] [PubMed]
76. Koonin, E.V. Orthologs, paralogs, and evolutionary genomics. *Annu. Rev. Genet.* **2005**, *39*, 309–338. [CrossRef]
77. Kong, L.A.; Yang, J.; Li, G.T.; Qi, L.L.; Zhang, Y.J.; Wang, C.F.; Zhao, W.S.; Xu, J.R.; Peng, Y.L. Different chitin synthase genes are required for various developmental and plant infection processes in the rice blast fungus Magnaporthe oryzae. *PLoS Pathog.* **2012**, *8*, e1002526. [CrossRef] [PubMed]
78. Lenardon, M.D.; Munro, C.A.; Gow, N.A. Chitin synthesis and fungal pathogenesis. *Curr. Opin. Microbiol.* **2010**, *13*, 416–423. [CrossRef] [PubMed]
79. Rogg, L.E.; Fortwendel, J.R.; Juvvadi, P.R.; Steinbach, W.J. Regulation of expression, activity and localization of fungal chitin synthases. *Med. Mycol.* **2012**, *50*, 2–17. [CrossRef] [PubMed]
80. Brown, H.E.; Esher, S.K.; Alspaugh, J.A. Chitin: A "Hidden Figure" in the Fungal Cell Wall. In *The Fungal Cell Wall: An Armour and a Weapon for Human Fungal Pathogens*; Latgé, J.-P., Ed.; Springer International Publishing: Cham, Switzerland, 2020; pp. 83–111.
81. Sheu, Y.J.; Barral, Y.; Snyder, M. Polarized growth controls cell shape and bipolar bud site selection in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **2000**, *20*, 5235–5247. [CrossRef]
82. Fischer, R.; Zekert, N.; Takeshita, N. Polarized growth in fungi–interplay between the cytoskeleton, positional markers and membrane domains. *Mol. Microbiol.* **2008**, *68*, 813–826. [CrossRef]
83. Riquelme, M.; Bartnicki-García, S. Advances in understanding hyphal morphogenesis: Ontogeny, phylogeny and cellular localization of chitin synthases. *Fungal Biol. Rev.* **2008**, *22*, 56–70. [CrossRef]
84. Li, M.; Jiang, C.; Wang, Q.; Zhao, Z.; Jin, Q.; Xu, J.R.; Liu, H. Evolution and Functional Insights of Different Ancestral Orthologous Clades of Chitin Synthase Genes in the Fungal Tree of Life. *Front. Plant Sci.* **2016**, *7*, 37. [CrossRef]
85. Gonçalves, I.R.; Brouillet, S.; Soulié, M.C.; Gribaldo, S.; Sirven, C.; Charron, N.; Boccara, M.; Choquer, M. Genome-wide analyses of chitin synthases identify horizontal gene transfers towards bacteria and allow a robust and unifying classification into fungi. *BMC Evol. Biol.* **2016**, *16*, 252. [CrossRef]
86. Mandel, M.A.; Galgiani, J.N.; Kroken, S.; Orbach, M.J. Coccidioides posadasii contains single chitin synthase genes corresponding to classes I to VII. *Fungal Genet. Biol.* **2006**, *43*, 775–788. [CrossRef]
87. Choquer, M.; Boccara, M.; Gonçalves, I.R.; Soulié, M.C.; Vidal-Cros, A. Survey of the Botrytis cinerea chitin synthase multigenic family through the analysis of six euascomycetes genomes. *Eur. J. Biochem.* **2004**, *271*, 2153–2164. [CrossRef] [PubMed]
88. Nino-Vega, G.A.; Carrero, L.; San-Blas, G. Isolation of the CHS4 gene of Paracoccidioides brasiliensis and its accommodation in a new class of chitin synthases. *Med. Mycol.* **2004**, *42*, 51–57. [CrossRef] [PubMed]
89. Ruiz-Herrera, J.; Ortiz-Castellanos, L. Analysis of the phylogenetic relationships and evolution of the cell walls from yeasts and fungi. *FEMS Yeast Res.* **2010**, *10*, 225–243. [CrossRef]
90. Liu, R.; Xu, C.; Zhang, Q.; Wang, S.; Fang, W. Evolution of the chitin synthase gene family correlates with fungal morphogenesis and adaption to ecological niches. *Sci. Rep.* **2017**, *7*, 44527. [CrossRef] [PubMed]
91. Schuster, M.; Treitschke, S.; Kilaru, S.; Molloy, J.; Harmer, N.J.; Steinberg, G. Myosin-5, kinesin-1 and myosin-17 cooperate in secretion of fungal chitin synthase. *Embo J.* **2012**, *31*, 214–227. [CrossRef] [PubMed]
92. Motoyama, T.; Fujiwara, M.; Kojima, N.; Horiuchi, H.; Ohta, A.; Takagi, M. The *Aspergillus nidulans* genes chsA and chsD encode chitin synthases which have redundant functions in conidia formation [corrected and republished article originally appeared in *Mol. Gen. Genet.* **1996**, *251*, 442–450]. *Mol Gen Genet* **1997**, *253*, 520–528. [CrossRef]
93. Horiuchi, H. Functional diversity of chitin synthases of *Aspergillus nidulans* in hyphal growth, conidiophore development and septum formation. *Med. Mycol.* **2009**, *47* (Suppl. S1), S47–S52. [CrossRef] [PubMed]
94. Fujiwara, M.; Horiuchi, H.; Ohta, A.; Takagi, M. A novel fungal gene encoding chitin synthase with a myosin motor-like domain. *Biochem. Biophys. Res. Commun.* **1997**, *236*, 75–78. [CrossRef]
95. Takeshita, N.; Yamashita, S.; Ohta, A.; Horiuchi, H. Aspergillus nidulans class V and VI chitin synthases CsmA and CsmB, each with a myosin motor-like domain, perform compensatory functions that are essential for hyphal tip growth. *Mol. Microbiol.* **2006**, *59*, 1380–1394. [CrossRef]
96. Mellado, E.; Specht, C.A.; Robbins, P.W.; Holden, D.W. Cloning and characterization of chsD, a chitin synthase-like gene of *Aspergillus fumigatus*. *FEMS Microbiol. Lett.* **1996**, *143*, 69–76. [CrossRef]
97. Mellado, E.; Aufauvre-Brown, A.; Gow, N.A.; Holden, D.W. The *Aspergillus fumigatus* chsC and chsG genes encode class III chitin synthases with different functions. *Mol. Microbiol.* **1996**, *20*, 667–679. [CrossRef] [PubMed]
98. Carrion, S.J.; Abbondante, S.; Clark, H.L.; Marshall, M.E.; Mouyna, I.; Beauvais, A.; Sun, Y.; Taylor, P.R.; Leal, S.M.; Armstrong, B.; et al. Aspergillus fumigatus corneal infection is regulated by chitin synthases and by neutrophil-derived acidic mammalian chitinase. *Eur. J. Immunol.* **2019**, *49*, 918–927. [CrossRef] [PubMed]
99. Aufauvre-Brown, A.; Mellado, E.; Gow, N.A.; Holden, D.W. Aspergillus fumigatus chsE: A gene related to CHS3 of *Saccharomyces cerevisiae* and important for hyphal growth and conidiophore development but not pathogenicity. *Fungal Genet. Biol.* **1997**, *21*, 141–152. [CrossRef] [PubMed]

100. Chigira, Y.; Abe, K.; Gomi, K.; Nakajima, T. chsZ, a gene for a novel class of chitin synthase from *Aspergillus oryzae*. *Curr. Genet.* **2002**, *41*, 261–267. [CrossRef] [PubMed]
101. Yin, X.; Shin, H.-D.; Li, J.; Du, G.; Liu, L.; Chen, J. Comparative genomics and transcriptome analysis of *Aspergillus niger* and metabolic engineering for citrate production. *Sci. Rep.* **2017**, *7*, 41040. [CrossRef] [PubMed]
102. Baltussen, T.J.H.; Zoll, J.; Verweij, P.E.; Melchers, W.J.G. Molecular Mechanisms of Conidial Germination in *Aspergillus* spp. *Microbiol. Mol. Biol. Rev.* **2020**, *84*, 1–31. [CrossRef]
103. Muszkieta, L.; Aimanianda, V.; Mellado, E.; Gribaldo, S.; Alcàzar-Fuoli, L.; Szewczyk, E.; Prevost, M.C.; Latgé, J.P. Deciphering the role of the chitin synthase families 1 and 2 in the in vivo and in vitro growth of *Aspergillus fumigatus* by multiple gene targeting deletion. *Cell. Microbiol.* **2014**, *16*, 1784–1805. [CrossRef]
104. Culp, D.W.; Dodge, C.L.; Miao, Y.; Li, L.; Sag-Ozkal, D.; Borgia, P.T. The chsA gene from *Aspergillus nidulans* is necessary for maximal conidiation. *FEMS Microbiol. Lett.* **2000**, *182*, 349–353. [CrossRef]
105. Fujiwara, M.; Ichinomiya, M.; Motoyama, T.; Horiuchi, H.; Ohta, A.; Takagi, M. Evidence that the *Aspergillus nidulans* class I and class II chitin synthase genes, chsC and chsA, share critical roles in hyphal wall integrity and conidiophore development. *J. Biochem.* **2000**, *127*, 359–366. [CrossRef]
106. Ichinomiya, M.; Yamada, E.; Yamashita, S.; Ohta, A.; Horiuchi, H. Class I and class II chitin synthases are involved in septum formation in the filamentous fungus *Aspergillus nidulans*. *Eukaryot. Cell* **2005**, *4*, 1125–1136. [CrossRef]
107. Fukuda, K.; Yamada, K.; Deoka, K.; Yamashita, S.; Ohta, A.; Horiuchi, H. Class III chitin synthase ChsB of *Aspergillus nidulans* localizes at the sites of polarized cell wall synthesis and is required for conidial development. *Eukaryot. Cell* **2009**, *8*, 945–956. [CrossRef] [PubMed]
108. Alsteens, D.; Aimanianda, V.; Hegde, P.; Pire, S.; Beau, R.; Bayry, J.; Latgé, J.P.; Dufrêne, Y.F. Unraveling the nanoscale surface properties of chitin synthase mutants of *Aspergillus fumigatus* and their biological implications. *Biophys. J.* **2013**, *105*, 320–327. [CrossRef] [PubMed]
109. Mellado, E.; Dubreucq, G.; Mol, P.; Sarfati, J.; Paris, S.; Diaquin, M.; Holden, D.W.; Rodriguez-Tudela, J.L.; Latgé, J.P. Cell wall biogenesis in a double chitin synthase mutant (chsG-/chsE-) of *Aspergillus fumigatus*. *Fungal Genet. Biol.* **2003**, *38*, 98–109. [CrossRef]
110. Ichinomiya, M.; Motoyama, T.; Fujiwara, M.; Takagi, M.; Horiuchi, H.; Ohta, A. Repression of chsB expression reveals the functional importance of class IV chitin synthase gene chsD in hyphal growth and conidiation of *Aspergillus nidulans*. *Microbiology* **2002**, *148*, 1335–1347. [CrossRef] [PubMed]
111. Ichinomiya, M.; Horiuchi, H.; Ohta, A. Different functions of the class I and class II chitin synthase genes, chsC and chsA, are revealed by repression of chsB expression in *Aspergillus nidulans*. *Curr. Genet.* **2002**, *42*, 51–58. [CrossRef] [PubMed]
112. Horiuchi, H.; Fujiwara, M.; Yamashita, S.; Ohta, A.; Takagi, M. Proliferation of intrahyphal hyphae caused by disruption of csmA, which encodes a class V chitin synthase with a myosin motor-like domain in *Aspergillus nidulans*. *J. Bacteriol.* **1999**, *181*, 3721–3729. [CrossRef] [PubMed]
113. Yamada, E.; Ichinomiya, M.; Ohta, A.; Horiuchi, H. The class V chitin synthase gene csmA is crucial for the growth of the chsA chsC double mutant in *Aspergillus nidulans*. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 87–97. [CrossRef] [PubMed]
114. Tsuizaki, M.; Takeshita, N.; Ohta, A.; Horiuchi, H. Myosin motor-like domain of the class VI chitin synthase CsmB is essential to its functions in *Aspergillus nidulans*. *Biosci. Biotechnol. Biochem.* **2009**, *73*, 1163–1167. [CrossRef]
115. Tsuizaki, M.; Ohta, A.; Horiuchi, H. Myosin motor-like domain of class VI chitin synthase CsmB of *Aspergillus nidulans* is not functionally equivalent to that of class V chitin synthase CsmA. *Biosci. Biotechnol. Biochem.* **2013**, *77*, 369–374. [CrossRef]
116. Lee, J.I.; Choi, J.H.; Park, B.C.; Park, Y.H.; Lee, M.Y.; Park, H.M.; Maeng, P.J. Differential expression of the chitin synthase genes of *Aspergillus nidulans*, chsA, chsB, and chsC, in response to developmental status and environmental factors. *Fungal Genet. Biol.* **2004**, *41*, 635–646. [CrossRef]
117. Takeshita, N.; Wernet, V.; Tsuizaki, M.; Grun, N.; Hoshi, H.O.; Ohta, A.; Fischer, R.; Horiuchi, H. Transportation of *Aspergillus nidulans* Class III and V Chitin Synthases to the Hyphal Tips Depends on Conventional Kinesin. *PLoS ONE* **2015**, *10*, e0125937. [CrossRef] [PubMed]
118. Riquelme, M.; Sanchez-Leon, E. The Spitzenkorper: A choreographer of fungal growth and morphogenesis. *Curr. Opin. Microbiol.* **2014**, *20*, 27–33. [CrossRef] [PubMed]
119. Zhou, L.; Evangelinos, M.; Wernet, V.; Eckert, A.F.; Ishitsuka, Y.; Fischer, R.; Nienhaus, G.U.; Takeshita, N. Superresolution and pulse-chase imaging reveal the role of vesicle transport in polar growth of fungal cells. *Sci. Adv.* **2018**, *4*, e1701798. [CrossRef] [PubMed]
120. López-Franco, R.; Bartnicki-Garcia, S.; Bracker, C.E. Pulsed growth of fungal hyphal tips. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 12228. [CrossRef]
121. Ishitsuka, Y.; Savage, N.; Li, Y.; Bergs, A.; Grün, N.; Kohler, D.; Donnelly, R.; Nienhaus, G.U.; Fischer, R.; Takeshita, N. Superresolution microscopy reveals a dynamic picture of cell polarity maintenance during directional growth. *Sci. Adv.* **2015**, *1*, e1500947. [CrossRef]
122. Virag, A.; Harris, S.D. The Spitzenkörper: A molecular perspective. *Mycol. Res.* **2006**, *110*, 4–13. [CrossRef]
123. Abenza, J.F.; Pantazopoulou, A.; Rodriguez, J.M.; Galindo, A.; Penalva, M.A. Long-distance movement of *Aspergillus nidulans* early endosomes on microtubule tracks. *Traffic* **2009**, *10*, 57–75. [CrossRef]

124. Taheri-Talesh, N.; Horio, T.; Araujo-Bazán, L.; Dou, X.; Espeso, E.A.; Peñalva, M.A.; Osmani, S.A.; Oakley, B.R. The tip growth apparatus of *Aspergillus nidulans*. *Mol. Biol. Cell* **2008**, *19*, 1439–1449. [CrossRef]
125. Hirokawa, N.; Noda, Y.; Tanaka, Y.; Niwa, S. Kinesin superfamily motor proteins and intracellular transport. *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 682–696. [CrossRef]
126. Hernández-González, M.; Bravo-Plaza, I.; Pinar, M.; de Los Ríos, V.; Arst, H.N.; Peñalva, M.A. Endocytic recycling via the TGN underlies the polarized hyphal mode of life. *PLoS Genet.* **2018**, *14*, e1007291. [CrossRef]
127. Martzoukou, O.; Amillis, S.; Zervakou, A.; Christoforidis, S.; Diallinas, G. The AP-2 complex has a specialized clathrin-independent role in apical endocytosis and polar growth in fungi. *Elife* **2017**, *6*, e20083. [CrossRef] [PubMed]
128. Jin, J.; Iwama, R.; Takagi, K.; Horiuchi, H. AP-2 complex contributes to hyphal-tip-localization of a chitin synthase in the filamentous fungus *Aspergillus nidulans*. *Fungal Biol.* **2021**, *125*, 806–814. [CrossRef] [PubMed]
129. Takeshita, N.; Ohta, A.; Horiuchi, H. CsmA, a class V chitin synthase with a myosin motor-like domain, is localized through direct interaction with the actin cytoskeleton in *Aspergillus nidulans*. *Mol. Biol. Cell* **2005**, *16*, 1961–1970. [CrossRef] [PubMed]
130. Takeshita, N.; Ohta, A.; Horiuchi, H. csmA, a gene encoding a class V chitin synthase with a myosin motor-like domain of *Aspergillus nidulans*, is translated as a single polypeptide and regulated in response to osmotic conditions. *Biochem. Biophys. Res. Commun.* **2002**, *298*, 103–109. [CrossRef] [PubMed]
131. Dichtl, K.; Samantaray, S.; Wagener, J. Cell wall integrity signalling in human pathogenic fungi. *Cell. Microbiol.* **2016**, *18*, 1228–1238. [CrossRef]
132. Latgé, J.P.; Beauvais, A.; Chamilos, G. The Cell Wall of the Human Fungal Pathogen *Aspergillus fumigatus*: Biosynthesis, Organization, Immune Response, and Virulence. *Annu. Rev. Microbiol.* **2017**, *71*, 99–116. [CrossRef] [PubMed]
133. Thammahong, A.; Puttikamonkul, S.; Perfect, J.R.; Brennan, R.G.; Cramer, R.A. Central Role of the Trehalose Biosynthesis Pathway in the Pathogenesis of Human Fungal Infections: Opportunities and Challenges for Therapeutic Development. *Microbiol. Mol. Biol. Rev.* **2017**, *81*, e00053-16. [CrossRef]
134. Thammahong, A.; Caffrey-Card, A.K.; Dhingra, S.; Obar, J.J.; Cramer, R.A. Trehalose-Regulatory Subunit Homolog Moonlights To Mediate Cell Wall Homeostasis through Modulation of Chitin Synthase Activity. *mBio* **2017**, *8*, e02381-17. [CrossRef]
135. Thammahong, A.; Dhingra, S.; Bultman, K.M.; Kerkaert, J.D.; Cramer, R.A. An Ssd1 Homolog Impacts Trehalose and Chitin Biosynthesis and Contributes to Virulence in *Aspergillus fumigatus*. *mSphere* **2019**, *4*, 1–16. [CrossRef]
136. Juvvadi, P.R.; Lamoth, F.; Steinbach, W.J. Calcineurin-mediated regulation of hyphal growth, septation, and virulence in *Aspergillus fumigatus*. *Mycopathologia* **2014**, *178*, 341–348. [CrossRef]
137. Juvvadi, P.R.; Lee, S.C.; Heitman, J.; Steinbach, W.J. Calcineurin in fungal virulence and drug resistance: Prospects for harnessing targeted inhibition of calcineurin for an antifungal therapeutic approach. *Virulence* **2017**, *8*, 186–197. [CrossRef] [PubMed]
138. De Castro, P.A.; Chiaratto, J.; Winkelströter, L.K.; Bom, V.L.P.; Ramalho, L.N.Z.; Goldman, M.H.S.; Brown, N.A.; Goldman, G.H. The Involvement of the Mid1/Cch1/Yvc1 Calcium Channels in *Aspergillus fumigatus* Virulence. *PLoS ONE* **2014**, *9*, e103957. [CrossRef] [PubMed]
139. Liu, S.; Hou, Y.; Liu, W.; Lu, C.; Wang, W.; Sun, S. Components of the Calcium-Calcineurin Signaling Pathway in Fungal Cells and Their Potential as Antifungal Targets. *Eukaryot. Cell* **2015**, *14*, 324–334. [CrossRef] [PubMed]
140. De Castro, P.A.; Chen, C.; de Almeida, R.S.; Freitas, F.Z.; Bertolini, M.C.; Morais, E.R.; Brown, N.A.; Ramalho, L.N.; Hagiwara, D.; Mitchell, T.K.; et al. ChIP-seq reveals a role for CrzA in the *Aspergillus fumigatus* high-osmolarity glycerol response (HOG) signalling pathway. *Mol. Microbiol.* **2014**, *94*, 655–674. [CrossRef] [PubMed]
141. Thewes, S. Calcineurin-Crz1 signaling in lower eukaryotes. *Eukaryot. Cell* **2014**, *13*, 694–705. [CrossRef] [PubMed]
142. Katayama, T.; Ohta, A.; Horiuchi, H. Protein kinase C regulates the expression of cell wall-related genes in RlmA-dependent and independent manners in *Aspergillus nidulans*. *Biosci. Biotechnol. Biochem.* **2015**, *79*, 321–330. [CrossRef] [PubMed]
143. Steinbach, W.J.; Cramer, R.A.; Perfect, B.Z.; Asfaw, Y.G.; Sauer, T.C.; Najvar, L.K.; Kirkpatrick, W.R.; Patterson, T.F.; Benjamin, D.K.; Heitman, J.; et al. Calcineurin Controls Growth, Morphology, and Pathogenicity in *Aspergillus fumigatus*. *Eukaryot. Cell* **2006**, *5*, 1091. [CrossRef]
144. Cramer, R.A., Jr.; Perfect, B.Z.; Pinchai, N.; Park, S.; Perlin, D.S.; Asfaw, Y.G.; Heitman, J.; Perfect, J.R.; Steinbach, W.J. Calcineurin target CrzA regulates conidial germination, hyphal growth, and pathogenesis of *Aspergillus fumigatus*. *Eukaryot. Cell* **2008**, *7*, 1085–1097. [CrossRef]
145. Juvvadi, P.R.; Fortwendel, J.R.; Rogg, L.E.; Burns, K.A.; Randell, S.H.; Steinbach, W.J. Localization and activity of the calcineurin catalytic and regulatory subunit complex at the septum is essential for hyphal elongation and proper septation in *Aspergillus fumigatus*. *Mol. Microbiol.* **2011**, *82*, 1235–1259. [CrossRef]
146. Beauvais, A.; Bruneau, J.M.; Mol, P.C.; Buitrago, M.J.; Legrand, R.; Latgé, J.P. Glucan synthase complex of *Aspergillus fumigatus*. *J. Bacteriol.* **2001**, *183*, 2273–2279. [CrossRef]
147. Fuchs, B.B.; Mylonakis, E. Our paths might cross: The role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. *Eukaryot. Cell* **2009**, *8*, 1616–1625. [CrossRef] [PubMed]
148. Dichtl, K.; Helmschrott, C.; Dirr, F.; Wagener, J. Deciphering cell wall integrity signalling in *Aspergillus fumigatus*: Identification and functional characterization of cell wall stress sensors and relevant Rho GTPases. *Mol. Microbiol.* **2012**, *83*, 506–519. [CrossRef] [PubMed]
149. Samantaray, S.; Neubauer, M.; Helmschrott, C.; Wagener, J. Role of the guanine nucleotide exchange factor Rom2 in cell wall integrity maintenance of *Aspergillus fumigatus*. *Eukaryot. Cell* **2013**, *12*, 288–298. [CrossRef] [PubMed]

150. Yoshimi, A.; Miyazawa, K.; Abe, K. Cell wall structure and biogenesis in *Aspergillus* species. *Biosci. Biotechnol. Biochem.* **2016**, *80*, 1700–1711. [CrossRef] [PubMed]
151. Rocha, M.C.; Godoy, K.F.; de Castro, P.A.; Hori, J.I.; Bom, V.L.; Brown, N.A.; Cunha, A.F.; Goldman, G.H.; Malavazi, I. The *Aspergillus fumigatus* pkcA G579R Mutant Is Defective in the Activation of the Cell Wall Integrity Pathway but Is Dispensable for Virulence in a Neutropenic Mouse Infection Model. *PLoS ONE* **2015**, *10*, e0135195. [CrossRef] [PubMed]
152. Rocha, M.C.; Fabri, J.H.; Franco de Godoy, K.; Alves de Castro, P.; Hori, J.I.; Ferreira da Cunha, A.; Arentshorst, M.; Ram, A.F.; van den Hondel, C.A.; Goldman, G.H.; et al. Aspergillus fumigatus MADS-Box Transcription Factor rlmA Is Required for Regulation of the Cell Wall Integrity and Virulence. *G3 (Bethesda)* **2016**, *6*, 2983–3002. [CrossRef]
153. Valiante, V.; Macheleidt, J.; Föge, M.; Brakhage, A.A. The *Aspergillus fumigatus* cell wall integrity signaling pathway: Drug target, compensatory pathways, and virulence. *Front. Microbiol.* **2015**, *6*, 325. [CrossRef]
154. Rocha, M.C.; Fabri, J.; Simões, I.T.; Silva-Rocha, R.; Hagiwara, D.; da Cunha, A.F.; Goldman, G.H.; Cánovas, D.; Malavazi, I. The Cell Wall Integrity Pathway Contributes to the Early Stages of *Aspergillus fumigatus* Asexual Development. *Appl. Environ. Microbiol.* **2020**, *86*, e02347-19. [CrossRef]
155. Yu, J.-H. Regulation of Development in *Aspergillus nidulans* and *Aspergillus fumigatus*. *Mycobiology* **2010**, *38*, 229–237. [CrossRef]
156. Tao, L.; Yu, J.H. AbaA and WetA govern distinct stages of *Aspergillus fumigatus* development. *Microbiol. (Read.)* **2011**, *157*, 313–326. [CrossRef]
157. Park, H.S.; Yu, J.H. Developmental regulators in *Aspergillus fumigatus*. *J. Microbiol.* **2016**, *54*, 223–231. [CrossRef] [PubMed]
158. Sewall, T.C. Cellular effects of misscheduled brlA, abaA, and wetA expression in *Aspergillus nidulans*. *Can. J. Microbiol.* **1994**, *40*, 1035–1042. [CrossRef] [PubMed]
159. Andrianopoulos, A.; Timberlake, W.E. The *Aspergillus nidulans* abaA gene encodes a transcriptional activator that acts as a genetic switch to control development. *Mol. Cell. Biol.* **1994**, *14*, 2503–2515. [CrossRef] [PubMed]
160. Park, B.C.; Park, Y.H.; Park, H.M. Activation of chsC transcription by AbaA during asexual development of *Aspergillus nidulans*. *FEMS Microbiol. Lett.* **2003**, *220*, 241–246. [CrossRef] [PubMed]
161. Lee, J.I.; Yu, Y.M.; Rho, Y.M.; Park, B.C.; Choi, J.H.; Park, H.M.; Maeng, P.J. Differential expression of the chsE gene encoding a chitin synthase of *Aspergillus nidulans* in response to developmental status and growth conditions. *FEMS Microbiol. Lett.* **2005**, *249*, 121–129. [CrossRef]
162. Li, B.; Wang, K.; Zhang, R.; Shen, Y.; Ji, Q. Design, synthesis and biological evaluation of novel diazaspiro [4.5]decan-1-one derivatives as potential chitin synthase inhibitors and antifungal agents. *Eur. J. Med. Chem.* **2019**, *182*, 111669. [CrossRef]
163. Jackson, K.E.; Pogula, P.K.; Patterson, S.E. Polyoxin and nikkomycin analogs: Recent design and synthesis of novel peptidyl nucleosides. *Heterocycl. Commun.* **2013**, *19*, 375–386. [CrossRef]
164. Hasim, S.; Coleman, J.J. Targeting the fungal cell wall: Current therapies and implications for development of alternative antifungal agents. *Future Med. Chem.* **2019**, *11*, 869–883. [CrossRef]
165. Liao, G.; Li, J.; Li, L.; Yang, H.; Tian, Y.; Tan, H. Selectively improving nikkomycin Z production by blocking the imidazolone biosynthetic pathway of nikkomycin X and uracil feeding in Streptomyces ansochromogenes. *Microb. Cell Fact.* **2009**, *8*, 61. [CrossRef]
166. Hector, R.F.; Zimmer, B.L.; Pappagianis, D. Evaluation of nikkomycins X and Z in murine models of coccidioidomycosis, histoplasmosis, and blastomycosis. *Antimicrob. Agents Chemother.* **1990**, *34*, 587–593. [CrossRef]
167. Tariq, V.N.; Devlin, P.L. Sensitivity of fungi to nikkomycin Z. *Fungal Genet. Biol.* **1996**, *20*, 4–11. [CrossRef] [PubMed]
168. Li, R.K.; Rinaldi, M.G. In vitro antifungal activity of nikkomycin Z in combination with fluconazole or itraconazole. *Antimicrob. Agents Chemother.* **1999**, *43*, 1401–1405. [CrossRef]
169. Graybill, J.R.; Najvar, L.K.; Bocanegra, R.; Hector, R.F.; Luther, M.F. Efficacy of nikkomycin Z in the treatment of murine histoplasmosis. *Antimicrob. Agents Chemother.* **1998**, *42*, 2371–2374. [CrossRef] [PubMed]
170. NIH. Safety and PK of Nikkomycin Z for Coccidioides Pneumonia Treatment. Available online: https://clinicaltrials.gov/ct2/show/study/NCT00614666?id=NCT00614666&draw=2&rank=1&load=cart (accessed on 17 November 2021).
171. Verwer, P.E.; van Duijn, M.L.; Tavakol, M.; Bakker-Woudenberg, I.A.; van de Sande, W.W. Reshuffling of *Aspergillus fumigatus* cell wall components chitin and β-glucan under the influence of caspofungin or nikkomycin Z alone or in combination. *Antimicrob. Agents Chemother.* **2012**, *56*, 1595–1598. [CrossRef] [PubMed]
172. Ganesan, L.T.; Manavathu, E.K.; Cutright, J.L.; Alangaden, G.J.; Chandrasekar, P.H. In-vitro activity of nikkomycin Z alone and in combination with polyenes, triazoles or echinocandins against *Aspergillus fumigatus*. *Clin. Microbiol. Infect.* **2004**, *10*, 961–966. [CrossRef]
173. Chiou, C.C.; Mavrogiorgos, N.; Tillem, E.; Hector, R.; Walsh, T.J. Synergy, pharmacodynamics, and time-sequenced ultrastructural changes of the interaction between nikkomycin Z and the echinocandin FK463 against *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **2001**, *45*, 3310–3321. [CrossRef]
174. Wu, X.-Z.; Cheng, A.-X.; Sun, L.-M.; Lou, H.-X. Effect of plagiochin E, an antifungal macrocyclic bis(bibenzyl), on cell wall chitin synthesis in Candida albicans. *Acta Pharmacol. Sin.* **2008**, *29*, 1478–1485. [CrossRef]
175. Wu, X.-Z.; Cheng, A.-X.; Sun, L.-M.; Sun, S.-J.; Lou, H.-X. Plagiochin E, an antifungal bis(bibenzyl), exerts its antifungal activity through mitochondrial dysfunction-induced reactive oxygen species accumulation in Candida albicans. *Biochim. Biophys. Acta (BBA)—Gen. Subj.* **2009**, *1790*, 770–777. [CrossRef]

176. Wu, X.Z.; Chang, W.Q.; Cheng, A.X.; Sun, L.M.; Lou, H.X. Plagiochin E, an antifungal active macrocyclic bis(bibenzyl), induced apoptosis in Candida albicans through a metacaspase-dependent apoptotic pathway. *Biochim. Biophys. Acta* **2010**, *1800*, 439–447. [CrossRef]
177. Guo, X.L.; Leng, P.; Yang, Y.; Yu, L.G.; Lou, H.X. Plagiochin E, a botanic-derived phenolic compound, reverses fungal resistance to fluconazole relating to the efflux pump. *J. Appl. Microbiol.* **2008**, *104*, 831–838. [CrossRef]
178. Jacobs, S.E.; Zagaliotis, P.; Walsh, T.J. Novel antifungal agents in clinical trials. *F1000Research* **2021**, *10*, 507. [CrossRef] [PubMed]
179. Wiederhold, N.P. Attenuation of echinocandin activity at elevated concentrations: A review of the paradoxical effect. *Curr. Opin. Infect. Dis.* **2007**, *20*, 574–578. [CrossRef] [PubMed]
180. Wagener, J.; Loiko, V. Recent Insights into the Paradoxical Effect of Echinocandins. *J. Fungi.* **2017**, *4*, 5. [CrossRef] [PubMed]
181. Steinbach, W.J.; Lamoth, F.; Juvvadi, P.R. Potential Microbiological Effects of Higher Dosing of Echinocandins. *Clin. Infect. Dis.* **2015**, *61* (Suppl. S6), S669–S677. [CrossRef]
182. Fortwendel, J.R.; Juvvadi, P.R.; Perfect, B.Z.; Rogg, L.E.; Perfect, J.R.; Steinbach, W.J. Transcriptional regulation of chitin synthases by calcineurin controls paradoxical growth of *Aspergillus fumigatus* in response to caspofungin. *Antimicrob. Agents Chemother.* **2010**, *54*, 1555–1563. [CrossRef]
183. Antachopoulos, C.; Meletiadis, J.; Sein, T.; Roilides, E.; Walsh, T.J. Concentration-dependent effects of caspofungin on the metabolic activity of *Aspergillus* species. *Antimicrob. Agents Chemother.* **2007**, *51*, 881–887. [CrossRef]
184. Li, Y.; Wan, Z.; Liu, W.; Li, R. Identification and susceptibility of *Aspergillus* section nigri in china: Prevalence of species and paradoxical growth in response to echinocandins. *J. Clin. Microbiol.* **2015**, *53*, 702–705. [CrossRef]
185. Hadrich, I.; Neji, S.; Makni, F.; Ayadi, A.; Elloumi, M.; Ranque, S. Trailing or paradoxical growth of *Aspergillus* flavus exposed to caspofungin is independent of genotype. *J. Med. Microbiol.* **2014**, *63*, 1584–1589. [CrossRef]
186. Aruanno, M.; Glampedakis, E.; Lamoth, F. Echinocandins for the Treatment of Invasive Aspergillosis: From Laboratory to Bedside. *Antimicrob. Agents Chemother.* **2019**, *63*, e00399-19. [CrossRef]
187. Colabardini, A.C.; Wang, F.; Dong, Z.; Pardeshi, L.; Rocha, M.C.; Costa, J.H.; Dos Reis, T.F.; Brown, A.; Jaber, Q.Z.; Fridman, M.; et al. Heterogeneity in the transcriptional response of the human pathogen *Aspergillus fumigatus* to the antifungal agent caspofungin. *Genetics* **2021**, *220*, iyab183. [CrossRef]
188. Rogg, L.E.; Fortwendel, J.R.; Juvvadi, P.R.; Lilley, A.; Steinbach, W.J. The chitin synthase genes chsA and chsC are not required for cell wall stress responses in the human pathogen *Aspergillus fumigatus*. *Biochem. Biophys. Res. Commun.* **2011**, *411*, 549–554. [CrossRef] [PubMed]
189. Walker, L.A.; Lee, K.K.; Munro, C.A.; Gow, N.A. Caspofungin Treatment of *Aspergillus fumigatus* Results in ChsG-Dependent Upregulation of Chitin Synthesis and the Formation of Chitin-Rich Microcolonies. *Antimicrob. Agents Chemother.* **2015**, *59*, 5932–5941. [CrossRef] [PubMed]
190. Fortwendel, J.R.; Juvvadi, P.R.; Pinchai, N.; Perfect, B.Z.; Alspaugh, J.A.; Perfect, J.R.; Steinbach, W.J. Differential Effects of Inhibiting Chitin and 1,3-β-D-Glucan Synthesis in Ras and Calcineurin Mutants of *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **2009**, *53*, 476. [CrossRef]
191. Juvvadi, P.R.; Muñoz, A.; Lamoth, F.; Soderblom, E.J.; Moseley, M.A.; Read, N.D.; Steinbach, W.J. Calcium-Mediated Induction of Paradoxical Growth following Caspofungin Treatment Is Associated with Calcineurin Activation and Phosphorylation in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **2015**, *59*, 4946–4955. [CrossRef] [PubMed]
192. Ries, L.N.A.; Rocha, M.C.; de Castro, P.A.; Silva-Rocha, R.; Silva, R.N.; Freitas, F.Z.; de Assis, L.J.; Bertolini, M.C.; Malavazi, I.; Goldman, G.H. The *Aspergillus fumigatus* CrzA Transcription Factor Activates Chitin Synthase Gene Expression during the Caspofungin Paradoxical Effect. *mBio* **2017**, *8*, e00705-17. [CrossRef]
193. De Castro, P.A.; Colabardini, A.C.; Manfiolli, A.O.; Chiaratto, J.; Silva, L.P.; Mattos, E.C.; Palmisano, G.; Almeida, F.; Persinoti, G.F.; Ries, L.N.A.; et al. Aspergillus fumigatus calcium-responsive transcription factors regulate cell wall architecture promoting stress tolerance, virulence and caspofungin resistance. *PLoS Genet.* **2019**, *15*, e1008551. [CrossRef] [PubMed]
194. Valero, C.; Colabardini, A.C.; Chiaratto, J.; Pardeshi, L.; de Castro, P.A.; Ferreira Filho, J.A.; Silva, L.P.; Rocha, M.C.; Malavazi, I.; Costa, J.H.; et al. Aspergillus fumigatus Transcription Factors Involved in the Caspofungin Paradoxical Effect. *mBio* **2020**, *11*, 1–22. [CrossRef]
195. Furukawa, T.; van Rhijn, N.; Fraczek, M.; Gsaller, F.; Davies, E.; Carr, P.; Gago, S.; Fortune-Grant, R.; Rahman, S.; Gilsenan, J.M.; et al. The negative cofactor 2 complex is a key regulator of drug resistance in *Aspergillus fumigatus*. *Nat. Commun.* **2020**, *11*, 427. [CrossRef]
196. Kim, I.S.; Sinha, S.; de Crombrugghe, B.; Maity, S.N. Determination of functional domains in the C subunit of the CCAAT-binding factor (CBF) necessary for formation of a CBF-DNA complex: CBF-B interacts simultaneously with both the CBF-A and CBF-C subunits to form a heterotrimeric CBF molecule. *Mol. Cell. Biol.* **1996**, *16*, 4003–4013. [CrossRef]
197. Hagiwara, D.; Suzuki, S.; Kamei, K.; Gonoi, T.; Kawamoto, S. The role of AtfA and HOG MAPK pathway in stress tolerance in conidia of *Aspergillus fumigatus*. *Fungal Genet. Biol.* **2014**, *73*, 138–149. [CrossRef]
198. Hagiwara, D.; Takahashi, H.; Kusuya, Y.; Kawamoto, S.; Kamei, K.; Gonoi, T. Comparative transcriptome analysis revealing dormant conidia and germination associated genes in *Aspergillus* species: An essential role for AtfA in conidial dormancy. *BMC Genom.* **2016**, *17*, 358. [CrossRef] [PubMed]
199. Ferreira, M.E.D.S.; Malavazi, I.; Savoldi, M.; Brakhage, A.A.; Goldman, M.H.S.; Kim, H.S.; Nierman, W.C.; Goldman, G.H. Transcriptome analysis of *Aspergillus fumigatus* exposed to voriconazole. *Curr. Genet.* **2006**, *50*, 32–44. [CrossRef] [PubMed]

200. Walker, L.A.; Gow, N.A.R.; Munro, C.A. Fungal echinocandin resistance. *Fungal Genet. Biol.* **2010**, *47*, 117–126. [CrossRef] [PubMed]
201. Loiko, V.; Wagener, J. The Paradoxical Effect of Echinocandins in *Aspergillus fumigatus* Relies on Recovery of the β-1,3-Glucan Synthase Fks1. *Antimicrob. Agents Chemother.* **2017**, *61*, e01690-16. [CrossRef]
202. Da Silva, C.A.; Chalouni, C.; Williams, A.; Hartl, D.; Lee, C.G.; Elias, J.A. Chitin is a size-dependent regulator of macrophage TNF and IL-10 production. *J. Immunol.* **2009**, *182*, 3573–3582. [CrossRef]
203. Elieh Ali Komi, D.; Sharma, L.; Dela Cruz, C.S. Chitin and Its Effects on Inflammatory and Immune Responses. *Clin. Rev. Allergy Immunol.* **2018**, *54*, 213–223. [CrossRef]
204. García-Carnero, L.C.; Martínez-Álvarez, J.A.; Salazar-García, L.M.; Lozoya-Pérez, N.E.; González-Hernández, S.E.; Tamez-Castrellón, A.K. Recognition of Fungal Components by the Host Immune System. *Curr. Protein Pept. Sci.* **2020**, *21*, 245–264. [CrossRef]
205. Wagener, J.; Malireddi, R.K.; Lenardon, M.D.; Köberle, M.; Vautier, S.; MacCallum, D.M.; Biedermann, T.; Schaller, M.; Netea, M.G.; Kanneganti, T.D.; et al. Fungal chitin dampens inflammation through IL-10 induction mediated by NOD2 and TLR9 activation. *PLoS Pathog.* **2014**, *10*, e1004050. [CrossRef]
206. Becker, K.L.; Aimanianda, V.; Wang, X.; Gresnigt, M.S.; Ammerdorffer, A.; Jacobs, C.W.; Gazendam, R.P.; Joosten, L.A.B.; Netea, M.G.; Latgé, J.P.; et al. Aspergillus Cell Wall Chitin Induces Anti- and Proinflammatory Cytokines in Human PBMCs via the Fc-γ Receptor/Syk/PI3K Pathway. *mBio* **2016**, *7*, e01823-15. [CrossRef]
207. Dubey, L.K.; Moeller, J.B.; Schlosser, A.; Sorensen, G.L.; Holmskov, U. Induction of innate immunity by *Aspergillus fumigatus* cell wall polysaccharides is enhanced by the composite presentation of chitin and beta-glucan. *Immunobiology* **2014**, *219*, 179–188. [CrossRef]
208. Chai, L.Y.A.; Vonk, A.G.; Kullberg, B.J.; Verweij, P.E.; Verschueren, I.; van der Meer, J.W.M.; Joosten, L.A.B.; Latgé, J.-P.; Netea, M.G. Aspergillus fumigatus cell wall components differentially modulate host TLR2 and TLR4 responses. *Microbes Infect.* **2011**, *13*, 151–159. [CrossRef] [PubMed]
209. Luo, Y.; Liu, M. Adiponectin: A versatile player of innate immunity. *J. Mol. Cell Biol.* **2016**, *8*, 120–128. [CrossRef] [PubMed]
210. Amarsaikhan, N.; Stolz, D.J.; Wilcox, A.; Sands, E.M.; Tsoggerel, A.; Gravely, H.; Templeton, S.P. Reciprocal Inhibition of Adiponectin and Innate Lung Immune Responses to Chitin and *Aspergillus fumigatus*. *Front. Immunol.* **2019**, *10*, 1057. [CrossRef] [PubMed]
211. Amarsaikhan, N.; O'Dea, E.M.; Tsoggerel, A.; Templeton, S.P. Lung eosinophil recruitment in response to *Aspergillus fumigatus* is correlated with fungal cell wall composition and requires γδ T cells. *Microbes Infect.* **2017**, *19*, 422–431. [CrossRef] [PubMed]
212. O'Dea, E.M.; Amarsaikhan, N.; Li, H.; Downey, J.; Steele, E.; Van Dyken, S.J.; Locksley, R.M.; Templeton, S.P. Eosinophils are recruited in response to chitin exposure and enhance Th2-mediated immune pathology in *Aspergillus fumigatus* infection. *Infect Immun.* **2014**, *82*, 3199–3205. [CrossRef]
213. Amarsaikhan, N.; Sands, E.M.; Shah, A.; Abdolrasouli, A.; Reed, A.; Slaven, J.E.; Armstrong-James, D.; Templeton, S.P. Caspofungin Increases Fungal Chitin and Eosinophil and γδ T Cell-Dependent Pathology in Invasive Aspergillosis. *J. Immunol.* **2017**, *199*, 624–632. [CrossRef]
214. Amarsaikhan, N.; O'Dea, E.M.; Tsoggerel, A.; Owegi, H.; Gillenwater, J.; Templeton, S.P. Isolate-Dependent Growth, Virulence, and Cell Wall Composition in the Human Pathogen *Aspergillus fumigatus*. *PLoS ONE* **2014**, *9*, e100430. [CrossRef]
215. Brown, G.D.; Herre, J.; Williams, D.L.; Willment, J.A.; Marshall, A.S.; Gordon, S. Dectin-1 mediates the biological effects of beta-glucans. *J. Exp Med.* **2003**, *197*, 1119–1124. [CrossRef]
216. Herre, J.; Willment, J.A.; Gordon, S.; Brown, G.D. The role of Dectin-1 in antifungal immunity. *Crit. Rev. Immunol.* **2004**, *24*, 193–203. [CrossRef] [PubMed]
217. Dubey, L.K.; Moeller, J.B.; Schlosser, A.; Sorensen, G.L.; Holmskov, U. Chitin enhances serum IgE in *Aspergillus fumigatus* induced allergy in mice. *Immunobiology* **2015**, *220*, 714–721. [CrossRef]
218. Strong, P.; Clark, H.; Reid, K. Intranasal application of chitin microparticles down-regulates symptoms of allergic hypersensitivity to Dermatophagoides pteronyssinus and *Aspergillus fumigatus* in murine models of allergy. *Clin. Exp. Allergy* **2002**, *32*, 1794–1800. [CrossRef] [PubMed]
219. Roy, R.M.; Paes, H.C.; Nanjappa, S.G.; Sorkness, R.; Gasper, D.; Sterkel, A.; Wüthrich, M.; Klein, B.S. Complement component 3C3 and C3a receptor are required in chitin-dependent allergic sensitization to *Aspergillus fumigatus* but dispensable in chitin-induced innate allergic inflammation. *mBio* **2013**, *4*, e00162-13. [CrossRef] [PubMed]
220. Wang, S.; Yuan, A.; Zeng, L.; Hou, S.; Wang, M.; Li, L.; Cai, Z.; Zhong, G. The putative polysaccharide synthase AfCps1 regulates *Aspergillus fumigatus* morphogenesis and conidia immune response in mouse bone marrow-derived macrophages. *J. Microbiol.* **2021**, *59*, 64–75. [CrossRef]
221. Krull, R.; Wucherpfennig, T.; Esfandabadi, M.E.; Walisko, R.; Melzer, G.; Hempel, D.C.; Kampen, I.; Kwade, A.; Wittmann, C. Characterization and control of fungal morphology for improved production performance in biotechnology. *J Biotechnol.* **2013**, *163*, 112–123. [CrossRef] [PubMed]
222. Jin, F.-J.; Hu, S.; Wang, B.-T.; Jin, L. Advances in Genetic Engineering Technology and Its Application in the Industrial Fungus *Aspergillus oryzae*. *Front. Microbiol.* **2021**, *12*, 353. [CrossRef]
223. Cairns, T.C.; Nai, C.; Meyer, V. How a fungus shapes biotechnology: 100 years of *Aspergillus niger* research. *Fungal Biol. Biotechnol.* **2018**, *5*, 13. [CrossRef] [PubMed]

224. Kwon, M.J.; Jørgensen, T.R.; Nitsche, B.M.; Arentshorst, M.; Park, J.; Ram, A.F.J.; Meyer, V. The transcriptomic fingerprint of glucoamylase over-expression in *Aspergillus niger*. *BMC Genom.* **2012**, *13*, 701. [CrossRef]
225. Müller, C.; McIntyre, M.; Hansen, K.; Nielsen, J. Metabolic engineering of the morphology of *Aspergillus oryzae* by altering chitin synthesis. *Appl. Environ. Microbiol.* **2002**, *68*, 1827–1836. [CrossRef] [PubMed]
226. Muller, C.; Hansen, K.; Szabo, P.; Nielsen, J. Effect of deletion of chitin synthase genes on mycelial morphology and culture viscosity in *Aspergillus oryzae*. *Biotechnol. Bioeng.* **2003**, *81*, 525–534. [CrossRef]