"Targeting the *Aspergillus fumigatus* Cell Wall with Polysaccharide-Specific Proteins"

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August 2018

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Abstract

Aspergillus fumigatus is a ubiquitous saprophytic mould that can cause life-threatening necrotizing pneumonia in immunocompromised patients. A primary component contributing to the virulence of *A. fumigatus* is its complex cell wall and ability to form robust biofilms. The cell wall of *A. fumigatus* is composed almost exclusively of polysaccharides, each with their own unique function. One outer cell wall polysaccharide important for virulence is galactosaminogalactan (GAG), which is essential in mediating adherence and biofilm formation, as well as masking inner cell wall components that have potent immune-reactivity. However, purified GAG has direct effects on the immune system, suggesting that mammalian systems have the capacity to recognize and respond to this molecule.

We hypothesize that the recognition of GAG by mammalian protein receptors plays an important role in the immune response towards an *A. fumigatus* infection. Furthermore, given the central role GAG plays in the virulence of *A. fumigatus*, we hypothesize that enzymatic degradation of this polysaccharide could form the basis of a novel treatment strategy.

The mammalian cytosolic lectin galectin-3 is a member of a family of lectins characterized by their ability to bind to galactoside-containing carbohydrates that has been implicated in a broad range of infectious diseases and other disorders. Because of this observation, we postulated that it could potentially bind to *A. fumigatus* GAG, and may be involved in the immune response against this pathogen. Galectin-3 was upregulated in the serum and bronchoalveolar lavage fluid of humans and mice with pulmonary aspergillosis, respectively, indicating that galectin-3 is released during an *Aspergillus* infection. Galectin-3 bound to both purified GAG, as well as native GAG on the surface of the hyphae. Despite binding to the surface of *A. fumigatus*, galectin-3 did not inhibit fungal growth, unlike previous observations with other fungi. However, galectin-3 deficient mice were more susceptible to *Aspergillus* infection, revealing that galectin-3 still played a beneficial role to the host. Galectin-3 was found to aid in the effective migration of neutrophils out of the vasculature and to the site of infection. Neutrophil adoptive transfer experiments revealed that while extrinsic galectin-3 was required for optimal neutrophil egress from the pulmonary vasculature, neutrophil intrinsic galectin-3 was dispensable for this process.

In addition to investigating the capacity of mammalian proteins to interact with GAG, we also studied the proteins involved in the biosynthesis of the polysaccharide. One protein, Sph3,

contained a glycoside hydrolase domain capable of cleaving the GAG polysaccharide chain. The hydrolase domain of Sph3, $Sph3_h$, was expressed and purified, and evaluated for its ability to disrupt *A. fumigatus* biofilms. Treatment of *A. fumigatus* biofilms with nanomolar concentrations of $Sph3_h$ markedly reduced the amount of detectable GAG on the surface of hyphae, as well as the adherence of the fungus. Hydrolysis of GAG increased the susceptibility of *A. fumigatus* to a variety of classes of antifungal compounds, and prevented the fungus from damaging human airway epithelial cells *in vitro*. A single dose of $Sph3_h$ was well tolerated by mice, and attenuated the virulence of *A. fumigatus* in a mouse model of invasive aspergillosis. Additionally, a similar glycoside hydrolase produced by the Gram-negative bacteria *Pseudomonas aeruginosa* was also able to disrupt *A. fumigatus* biofilms, suggesting that this therapeutic strategy could have cross-kingdom activity. This study has laid the foundation for the development of a new therapeutic strategy in combating recalcitrant biofilm infections.

Collectively, the findings of this thesis illustrate the diversity with which both mammalian and microbial proteins interact with the polysaccharides of the fungal cell wall. We have identified a new role for galectin-3 in the context of fungal immunology, and a novel avenue of therapeutic development through degradation of microbial biofilms with glycoside hydrolases.

Résumé

Aspergillus fumigatus est un champignon saprophytique ubiquitaire pouvant provoquer des pneumonies nécrosantes potentiellement mortelle chez les patients immunocompromis. Une première composante contribuant à la virulence d'*A. fumigatus* réside dans la complexité de sa paroi cellulaire et sa capacité à former de robuste biofilms. Ces parois et biofilms sont composés presque exclusivement de polysaccharides possédant chacun des fonctions uniques. L'un des polysaccharides les plus exposés et jouant un rôle clé dans la virulence est le galactosaminogalactane (GAG). Cette molécule est essentielle à l'adhérence et la formation de biofilm, ainsi qu'au masquage d'autres polysaccharides pariétaux fortement immunogènes. Cependant, malgré le rôle de masquage du GAG, celui-ci lorsque purifié présente lui aussi des effets directs sur le système immunitaire suggérant que les mammifères ont la capacité de le reconnaître et d'enclencher une réponse immunitaire subséquente.

Nous avons émis l'hypothèse que la reconnaissance du GAG par les récepteurs de mammifères joue un rôle important dans la réponse immunitaire contre les infections provoquées par *A. fumigatus*. De plus, considérant le rôle central de GAG dans la virulence, nous avons également postulé que la dégradation enzymatique de ce polysaccharide pourrait constituer une nouvelle stratégie de thérapie.

La lectine cytosolique de mammifère galectine-3 est un membre d'une famille de lectine caractérisée par leur capacité de liaison aux polysaccharides contenant du galactose, et connue pour leur implication dans une multitude de maladies infectieuses et autres pathologies. En conséquence, nous avons postulé que la galectine-3 pourrait potentiellement lier le GAG et être impliqué dans la réponse immunitaire provoquée par *A. fumigatus*. La galectine-3 a été retrouvé en plus grande abondance dans le sérum d'humains et le fluide bronchoalvéolaire de souris souffrant d'aspergillose indiquant une sécrétion de cette molécule lors d'une infection. La galectine-3 a aussi montré des capacités de liaison au GAG et ceux qu'il soit purifié ou natif à la surface des hyphes fongiques. Contrairement à ce qu'il a été précédemment observé avec d'autres espèces fongiques, la liaison de la galectine-3 aux hyphes d'*A. fumigatus* ne semble pas inhiber la croissance du champignon. Néanmoins, les souris déficientes en galectine-3 sont plus sensibles à *A. fumigatus*, révélant que la galectine-3 joue tout de même un rôle bénéfique dans la réponse de l'hôte. Cette lectine a de plus démontré améliorer la migration des neutrophiles hors des vaisseaux sanguins vers le site d'infection. Des expériences de transfert adoptif de

neutrophile ont montré que même si l'apport extrinsèque de galectine-3 est requis pour la sortie des neutrophiles à partir du système vasculaire, la galectine-3 intrinsèque aux neutrophiles est dispensable à ce procédé de migration.

Après avoir étudié la capacité de récepteurs de mammifère à interagir avec GAG, nous avons également étudié les protéines impliquées dans la voie de biosynthèse de ce polysaccharide. L'une des protéines, Sph3, présente un domaine glycoside hydrolase capable de cliver GAG. Ce domaine, $Sph3_h$, a été exprimé en système hétérologue, purifié et sa capacité de disruption de biofilm d'*A. fumigatus* évaluée. Le traitement des biofilms avec quelques nanomolaires de $Sph3_h$ a diminué considérablement la quantité de GAG détectable à la surface des hyphes, ainsi que l'adhérence du biofilm. L'hydrolyse du polymère a aussi permis de rendre *A. fumigatus* plus sensible à différentes classes d'antifongiques et de prévenir *in vitro* les dommages occasionnés aux cellules épithéliales alvéolaires humaines par le champignon. *In vivo*, une dose unique de $Sph3_h$ est tolérée par les souris, et a atténué la virulence d'*A. fumigatus* dans un modèle d'aspergillose invasive. De plus, une glycoside hydrolase similaire produite par la bactérie Gram négative *Pseudomonas aeruginosa* a aussi été en mesure de dégrader le biofilm formé par *A. fumigatus*, suggérant que cette stratégie thérapeutique pourrait avoir des activités traversant les règnes du vivant. Cette étude a mis en place les fondations du développement d'une nouvelle stratégie de thérapie en combattant des infections résistantes dues à la formation de biofilm.

Ensemble, les découvertes de cette étude illustrent la diversité avec laquelle les protéines mammifères et microbiennes interagissent avec les polysaccharides de la paroi fongique. Nous avons aussi identifié un nouveau rôle de la galectine-3 dans le contexte de l'immunologie fongique, et identifier une nouvelle avenue de thérapie basée sur la dégradation de biofilm microbien utilisant des glycoside hydrolases.

Acknowledgements

While my path to completing my degree has been a long one, it has been an incredibly fulfilling and rewarding experience. Throughout my time in Montréal I have had the great fortune to be surrounded by an exception group of people, both professionally and personally. Due to a lack of space I cannot thank everyone individually in this acknowledgment, however I want everyone with whom I have ever had an inspiring conversation, a stimulating discussion, a round of beers, a helping hand, or simply a kind word, to know that I am sincerely grateful for our time together.

It turns out that I really had no idea what I was in for when I accepted Dr. Donald Sheppard's offer to join his lab, but I would not change a minute of my time here. Don, you are a true force of nature, and it has been an absolute privilege to work with you for these past years. You have given me exceptional guidance and helped me develop and mature into a scientific investigator. Not just in the area of scientific research, but also in the other hats that a scientist has to wear, such as skills in management and "politics". I look forward to our continued discussions and interactions within the academic sphere in the future!

An important reason why I have cherished my time here is that the Sheppard Lab has been a truly delightful place to work. I would like to thank everyone in the lab, past and present, for always being there to bounce ideas back-and-forth, joke around, and lend a helping hand. Specifically I would like to thank Mélanie Lehoux, without whom I would still be trying to finish my mouse experiments; Josée Chabot, for keeping the lab running smoothly against all odds; François LeMauff for translating my abstract; and especially Mark Lee and James Stewart, for being great friends both at and outside of work. I also had the opportunity to mentor some amazing trainees: Yukiko Sato, Tianli Xiao, Victoria Bentley, and my cubicle from the 2013 class of MIMM212: it has been an honour to be a part of your training and watch many of you flourish into successful scientists.

I was very fortunate to have two exceptional Advisory Committee members that were not only great mentors, but also developed into close collaborations. Dr. Dao Nguyen, thank you for expanding my knowledge of *Pseudomonas* and bacterial biofilms, and the different techniques available to study them. And of course thank you Dr. Sachiko Sato, without whom this thesis would not have been possible. Thank you for our many long scientific conversations and debates about our data, and where to take it next. I have also had the privilege of working with some great collaborators. I would like to thank Dr. Lynne Howell and her lab for our stimulating quarterly inter-lab meetings, and being a great group of people to meet up with at conferences. And Drs. Maziar Divangahi, Irah King, and Jörg Fritz: thank you for opening my eyes to new techniques and ways of thinking about science.

Thank you to my friends that have helped me along the way, both here in Montréal and back home in BC. Thank you to the original Duff crew, the camping trips, intramural sports, and pub nights kept me sane all these years. Thank you Mark and Joanna Bazett and Marek Kobylarz, for always being a visit or quick text away for moral support. And thank you to the IDIGH community for an exciting time these last few years!

Finally, most importantly thank you to my wonderful parents, Jeff and Leslie Snarr, for your unconditional love, and shaping me into the man I am today. I know it has not been easy having your only child on the other side of the country all these years, and so I am incredibly grateful for you always supporting me, even if you only had a vague idea of what I was doing. I love you always!

Table of Contents

List of Tables

CHAPTER 3:

List of Figures

CHAPTER 1:

CHAPTER 2:

CHAPTER 3:

List of Abbreviations

ABPA	allergic bronchopulmonary aspergillosis
ACK	ammonium-chloride-potassium
AIDS	acquired immunodeficiency syndrome
AIF	alkali-insoluble fraction
AmB	amphotericin B
AMM	*Aspergillus* minimal media
ANOVA	analysis of variance
APC	allophycocyanin
BAL	bronchoalveolar lavage
BDP-PCZ	BODIPY-conjugated posaconazole
BMDC	bone marrow-derived dendritic cell
BMDM	bone marrow-derived macrophage
BODIPY	boron-dipyrromethene
BSA	bovine serum albumin
CARD	caspase-associated recruitment domain
CCL	C-C motif chemokine ligand
CD	cluster of differentiation
CF	cystic fibrosis
CFTR	cystic fibrosis trans membrane conductance regulator
CGD	chronic granulomatous disease
COPD	chronic obstructive pulmonary disorder
CPA	chronic pulmonary aspergillosis
CR	complement receptor
CRD	carbohydrate recognition domain
CXCL	chemokine (C-X-C motif) ligand
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
EC_{50}	effective concentration for 50% activity
ECM	extracellular matrix
eDNA	extracellular DNA
ELISA	enzyme-linked immunosorbent assay
FBS	foetal bovine serum
FcγR	Fcγ receptor
FE-SEM	field-emission scanning electron microscopy
FITC	fluorescein isothiocyanate
GAG	Galactosaminogalactan
GalNAc	*N*-acetyl-D-galactosamine

GalXM	galactoxylomannan
GH	glycoside hydrolase
GlcNAc	*N*-acetyl-D-glucosamine
GM	galactomannan
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	glycosylphosphatidylinositol
GRO	growth-related gene product
GXM	glucuronoxylomannan
HIV	human immunodeficiency virus
IA	invasive aspergillosis
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-1Ra	IL-1 receptor antagonist
iNOS	inducible nitric oxide synthase
IPA	invasive pulmonary aspergillosis
ITAM	immunoreceptor tyrosine-based activation motif
JAM	junctional adhesion molecule
KC	keratinocyte-derived chemokine
LB	Luria-Bertani
LDH	lactose dehydrogenase
LFA	lymphocyte function-associated antigen
LPS	lipopolysaccharide
Ly6	lymphocyte antigen 6
MAC	macrophage antigen
MASP	MBL-associated serine protease
MBL	Mannose-binding lectin
MCP	monocyte chemoattractant protein
MelLec	Melanin-sensing C-type lectin receptor
MHC	major histocompatibility complex
MIC	minimum inhibitory concentration
MIC-A	MHC class I chain-related molecule A
MIP	macrophage inflammatory protein
Mo-DC	monocyte-derived dendritic cell
MOI	multiplicity of infection
MOPS	3-(N-morpholino) propane-sulfonic acid
MR	mannose receptor
NADPH	nicotinamide adenine dinucleotide phosphate
ND	not detected

NET	neutrophil extracellular trap
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NOD	nucleotide-binding oligomerization domain-containing protein
NS	not significant
PAMP	pathogen-associated molecular pattern
PAS	periodic acid Schiff
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PBS-T	PBS-Tween-20
PCR	polymerase chain reaction
PE	phycoerythrin
PECAM	platelet/endothelial-cell adhesion molecule
PEG	polyethylene glycol
$PelA_h$	hydrolase domain of PelA
PFA	paraformaldehyde
PGSL	P-selectin glycoprotein ligand
PI3K	phosphoinositol-3 kinase
PMN	polymorphonuclear cell
PNAG	poly-β-1,6-*N*-acetyl-D-glucosamine
PRR	pattern recognition receptor
RANTES	regulated upon activation normal T cell expressed and secreted
RFP	red fluorescent protein
rGal-3	recombinant galectin-3
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
SD	standard deviation
SE	standard error
SEM	standard error of mean
SI	supplementary information
$Sph3_h$	hydrolase domain of Sph3
TGF	tumour growth factor
T_H	T helper
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNF	tumour necrosis factor
v/v	volume/volume
w/v	weight/volume

XTT 3’-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate

YPD yeast extract peptone dextrose

Contributions to Original Scientific Knowledge

The following key results are presented in this thesis:

1) Galectin-3 interacts with galactosaminogalactan of *Aspergillus fumigatus*:
    a) Galectin-3 binds to purified galactosaminogalactan in a dose-dependent manner.
    b) Galectin-3 binds to galactosaminogalactan via its carbohydrate recognition domain, as the binding can be inhibited with a canonical galectin-3 ligand.
2) Galectin-3 plays an important role during pulmonary *A. fumigatus* infection:
    a) Galectin-3 is upregulated in the serum of patients with pulmonary aspergillosis.
    b) Galectin-3 is upregulated in the lungs of mice during acute pulmonary *A. fumigatus* infection.
    c) Galectin-3 deficient mice are more susceptible to pulmonary *A. fumigatus* infection.
    d) Galectin-3 deficient mice are unable to efficiently recruit neutrophils to the airways to effectively clear an *A. fumigatus* infection.
    e) Extracellular galectin-3 is required for neutrophils to efficiently emigrate from the pulmonary vasculature to the site of the *A. fumigatus* infection, independent of neutrophil-derived galectin-3.
3) Enzymatic digestion of galactosaminogalactan disrupts *A. fumigatus* biofilm integrity:
    a) Glycoside hydrolase treatment reduces detectable galactosaminogalactan on the surface of *A. fumigatus* hyphae.
    b) Glycoside hydrolase treatment reduces adherence of *A. fumigatus* biofilms to abiotic surfaces.
    c) Glycoside hydrolase treatment renders *A. fumigatus* more susceptible to multiple classes of antifungal compounds.
    d) Glycoside hydrolase treatment reduces ability of *A. fumigatus* to damage airway epithelial cells in cell culture.
4) Glycoside hydrolases exhibit cross-kingdom activity:
    a) Use of glycoside hydrolase from *Pseudomonas aeruginosa* yields similar results for point 3) against *A. fumigatus*, indicating cross-kingdom activity.
5) Glycoside hydrolase treatment shows potential as a novel therapeutic:
    a) Glycoside hydrolases are non-toxic to mammalian cells in cell culture.

b) Glycoside hydrolases are non-toxic to immunocompetent mice receiving intratracheal treatment.
c) Glycoside hydrolase treatment at the time of infection reduces fungal burden in a mouse model of invasive pulmonary aspergillosis.

Contributions of Authors

This doctoral thesis was prepared in accordance with the guidelines stated in the McGill University "Guidelines for Thesis Preparations". The work of this thesis is presented in the "Manuscript-based thesis" format. All the studies have been performed under the sole supervision of Dr. Donald C. Sheppard. The detailed contributions of each authors are listed below. Authors are designated by their initials.

Chapter 1: General Introduction

Parts of the introduction were adapted from: **Snarr BD, Qureshi ST, Sheppard DC. (2017). Immune Recognition of Fungal Polysaccharides.** *J. Fungi* 2017 Aug 28;3(3);47.

BDS wrote the literature review. BDS, STQ and DCS edited the manuscript.

Chapter 2: Snarr BD, St. Pierre G, Ralph BAW, Lehoux M, Sato Y, Baistrocchi SR, Corsini R, Divangahi M, King IL, Takazono T, Satoh M, Sato S*, Sheppard DC*. (2018). Galectin-3 aids in neutrophil extravasation into the airways during pulmonary infection by *Aspergillus fumigatus.* *PLoS Pathog* (in preparation).

*Co-corresponding authors.

BDS performed 80% of experiments: BDS performed or was involved in all animal infections, tissue processing, and downstream analysis. BDS performed galectin staining microscopy experiments, galectin direct fungal killing assays, *in vitro* mouse neutrophil migration and fungal killing assays. GSP produced all recombinant galectin-3 and anti-galectin-3 antibody, measured galectin-3 in human and all mouse samples, performed all galectin-3 binding assays to purified GAG, and performed all *in vitro* human neutrophil migration assays. MS performed and analyzed live cell imaging. BAWR assisted with animal infections. ML was involved in animal infections and tissue processing. YS assisted in galectin staining microscopy experiments. SRB and RC assisted in animal tissue processing. TT provided human samples and measured their galectin-3 concentrations. MD & ILK provided invaluable advice and helpful discussions. BDS assembled all figures and wrote the paper. BDS, SS & DCS edited the manuscript.

Chapter 3: Snarr BD*, Baker P*, Bamford NC*, Sato Y, Liu H, Lehoux M, Gravelat FN, Ostapska H, Baistrocchi SR, Cerone RP, Filler EE, Parsek MR, Filler SG, Howell PL[‡],

Sheppard DC[‡]. (2017). Microbial glycoside hydrolases as antibiofilm agents with cross-kingdom activity. *Proc Natl Acad Sci USA* 2017 Jul 3;114(27);7124-7129.

*These authors contributed equally.

[‡]Co-corresponding authors.

BDS performed 70% of experiments. BDS performed and analyzed all fungal adherence assays, all lectin staining microscopy and quantification, all culture supernatant binding assays, all minimum inhibitory concentration assays, as well as analyzed epithelial cell fungal damage assay data and histology. PB produced glycoside hydrolases and analyzed hydrolase cellular toxicity data. NCB produced glycoside hydrolases, performed bacterial adherence assays and galactosaminogalactan hydrolysis assay. YS assisted with fungal adherence assays, lectin staining microscopy, and minimum inhibitory concentration assays. HL performed and analyzed all epithelial cell fungal damage assays. ML performed all *in vivo* experiments and performed and analyzed all downstream processing of samples and data collected. FNG generated fungal strains and performed all scanning electron microscopy imaging. HO assisted with minimum inhibitory concentration assays. SRB performed and analyzed posaconazole uptake assays and assisted with minimum inhibitory concentration assays. RPC assisted with minimum inhibitory concentration assays. EEL assisted with *in vivo* experiments. MRP provided bacterial strains. BDS assembled all figures and wrote the paper. BDS, PB, NCB, SGF, PLH & DCS edited the manuscript.

<u>Chapter 4:</u> General Discussion and Conclusions

Parts of this chapter were adapted from: **Snarr BD, Howell PL, Sheppard DC. (2018). Hoisted by their own petard: do microbial enzymes hold the solution to treating and preventing biofilm infections?** *Future Microbiology 2018 Feb 14;13(4);395-398.*

BDS wrote the editorial. BDS, PLH and DCS edited the manuscript.

CHAPTER 1: General Introduction

1.1 *Aspergillus* biology

The *Aspergillus* genus encompasses over 200 species of saprophytic moulds that are ubiquitous in nature. They are commonly found in soils, and play an important role in degrading and decomposing dead organic matter [1]. *Aspergillus* species thrive in this niche by utilizing two distinct cellular morphologies that are specialized for different roles: vegetative hyphae for degrading and digesting organic matter [1], and hydrophobic spores (or conidia) for dispersion to new environments [2].

1.1.1 Lifecycle and cellular morphologies

The lifecycle of *Aspergillus* begins with dormant asexual conidia, which can remain viable for extended periods of time. Conidia have also evolved to resist a variety of environmental stressors, including desiccation, ultra-violet radiation, and extreme temperatures [2]. Conidia are single-cell structures encased in a cell wall composed of a highly-organized proteinaceous outer layer and a loosely-organized inner layer, both of which are rich in polysaccharides [3]. In addition to these polysaccharides, the outer cell wall contains two other molecules, melanin and rodlets, that are critical for conidia viability and dispersion, respectively. The melanin coat provides the conidia resistance to ultra-violet radiation, allowing them to survive prolonged exposure to natural light, as well as oxidative stress [4]. The rodlet proteins form the outer-most layer of the conidial cell wall, and act to make the conidia strongly hydrophobic [5]. This hydrophobicity enhances conidia dispersion in the environment [2], and adhesion to organic surfaces [5]. The roles of both melanin and rodlets in virulence are discussed further in sections 1.4.2 and 1.4.1, respectively.

When an airborne conidium lands in an environment with the appropriate temperature and levels of water and nutrients, it will germinate, and transition out of its dormant state. The first morphological change that can be observed is an increase in conidia size and shedding of the rodlet layer of the cell wall [3]. This morphological state is colloquially referred to as “swollen conidia”, and represents the first step towards vegetative growth. With the rodlet layer removed, swollen conidia are no longer hydrophobic, as the deeper layers of the cell wall, primarily composed of polysaccharides, are now exposed [6].

As the rate of cellular metabolism and growth increases, conidia swelling halts and conidia begin to grow and extend with a polar directionality [7]. This is the beginning of the development of the hyphae, the morphology primarily associated with vegetative growth.

Aspergillus hyphae are filaments of elongated cells segmented by septa to yield cellular heterogeneity within the colony of hyphae (termed a mycelium or mycelial mat) and maintain structural integrity [8]. Hyphae grow with a tropism towards nutrients sources [9], and are capable of penetrating and invading dense strata in order to achieve this goal [10]. In addition to hyphal filament extension, hyphal branching can also occur, allowing for more rapid expansion in their environment [11]. The mature hyphae will thus form a mycelial mat that can mature into a biofilm [3], enhancing the extraction of nutrients and persistence within their environment.

As the mycelial mat depletes the nutrients within its environment, the fungus must disperse to colonize new environments. Upon detecting signals that its environment has become unfavourable, such as starvation, desiccation, or exposure to light, *Aspergillus* undergoes asexual reproduction through the formation of aerial specialized hyphae known as conidiophores [12]. The conidiophore is composed of an aerial stalk that grows perpendicular to the hyphal mat, terminating with a bulbous vesicle. These vesicles produce numerous elongated specialized cells known as phialides, from which conidia are generated [13]. As the conidia are strongly hydrophobic, they have evolved to be carried by air currents, and indeed conidiophores are structured in such a way to encourage this method of dispersion. The structures of the conidiophores vary slightly between the different *Aspergillus* species, and this difference can be used for species identification [14]. Conidia are then carried on air currents to new environments, completing the asexual life cycle of *Aspergillus*. Each mycelial mat will ultimately produce thousands of conidiophores, while each conidiophore is capable of producing and releasing more than 10,000 conidia into the air [15], resulting in the ubiquity of *Aspergillus* in nature.

1.1.2 Ecological niche

Aspergillus species play an important role in the environment as nutrient recyclers. They recycle nutrients by degrading the organic matter of dead and dying organisms [16]. *Aspergillus* species break down complex biomolecules into smaller units, returning nutrients and minerals sequestered in large biomolecules to their elementary building blocks. This conversion makes these nutrients accessible to the beginning of the food chain, continuing the circle of life in the ecosystem [17]. Vegetative hyphae are responsible for this degradation of decaying matter. The hyphae obtain nutrients through the secretion of digestive enzymes into their environment that break down complex biomolecules into their elementary components. These enzymes include proteases [18], nucleases [19] and lipases [20] to degrade proteins into amino acids, DNA and

RNA into nucleotides, and lipids into glycerides and fatty acids, respectively. As the bulk of the carbon found in plant biomatter is trapped in the form of complex polysaccharides such as cellulose, hemicellulose and pectin, *Aspergillus* species also produce a variety of glycoside hydrolase enzymes to digest these molecules into smaller units [17]. Following their degradation, the biomolecular components are then imported into the fungal cell to be utilized during cellular metabolism.

1.2 *Aspergillus* cell wall and biofilm

Both the conidia and hyphae will ultimately be exposed to a variety of environmental stressors during their respective stages of the *Aspergillus* life cycle. The fungus thus requires a means of protection from these stresses, and this is accomplished by the cell wall. The cell walls of the conidia and hyphae vary dramatically, as these two cellular morphologies differ greatly in both structure and function [3]. The cell wall therefore needs to be a dynamic structure to accommodate these various changes in cellular morphology and function, as well as the unique environments that these cell types occupy.

For both conidia and hyphae, the cell wall is composed of an inner and outer cell wall, each with their own unique composition and specific set of functions. The cell wall is composed of a variety of biomolecules, such as proteins, lipids and melanin [21]. However, polysaccharides are the most abundant molecule within the *Aspergillus* cell wall.

1.2.1 Inner cell wall

The primary role of the inner cell wall in both conidia and hyphae is to maintain the fungal cell shape and structural integrity. The inner cell wall of both hyphae and conidia is composed largely of chitin and β-1,3-glucan, which play important roles in maintaining structural integrity [21,22]. Chitin, a β-1,4-linked polymer of *N*-acetylglucosamine, appears to be an essential polysaccharide to the fungus, as no viable chitin-deficient *Aspergillus* mutants have been described to date. Mutants deficient in β-1,3-glucan are viable, however they have fragile and leaky cell walls, resulting in a markedly reduced growth rate and altered cellular morphology [23].

The inner cell wall of conidia also contains α-1,3-glucans, which are involved in the structuring of the conidial cell wall and the agglutination of conidia once they begin to swell and shed their outer rodlet layer [6,24]. The hyphal inner cell wall also contains α-glucan during

growth in liquid conditions, however under biofilm-forming conditions α-glucan is located at the interface between the outer cell wall and the extracellular matrix [3,25]. The function of α-glucan in the inner cell wall of hyphae is poorly understood, however it is likely involved in maintaining cell wall structure and organization.

Galactomannan, composed of an α-(1,2)(1,6)-linked mannose backbone with β-(1,5)-oligogalactofuranose branches connected by β-(1,3) and β-(1,6) linkages, is also present in the conidial inner cell wall where, in contrast to α-glucans, it plays a role in the separation of the conidia during sporulation [26]. The inner cell wall of hyphae also contains galactomannan, however it is synthesized via a different set of biosynthetic enzymes than those required for the galactomannan found within the inner cell wall of the conidia [26]. Hyphal galactomannan is found covalently linked to glycosylphosphatidylinositol, and proteins within the membrane, as well as secreted into the cell wall [27].

1.2.2 Outer cell wall

In contrast to the inner cell wall, the composition of the outer cell wall varies significantly between conidia and hyphae. The outer cell wall of conidia is composed primarily of proteins, as the resting conidia are coated in the hydrophobic rodlet protein layer [5]. A layer of melanin lies immediately below the rodlets, conferring resistance to the conidia from free radical damage caused by ultraviolet irradiation and oxidative stress [4].

In contrast, the outer cell wall of hyphae is primarily composed of an array of loosely organized polysaccharides that extends into a polysaccharide-rich extracellular matrix, which contributes to biofilm formation. While α-glucan localizes to the inner cell wall of hyphae grown in liquid cultures, it is found primarily at the interface between the outer cell wall and the extracellular matrix in aerial hyphae growing as a biofilm, where it is thought to be involved in the agglutination between neighbouring hyphae, and between the hyphal cell wall and other polysaccharides within the extracellular matrix [25].

The outer cell wall of hyphae also contain galactomannan, as the secreted galactomannan can reach the outer cell wall layer and be shed into the environment as a component of the extracellular matrix [3].

One of the outermost cell wall polysaccharides that is unique to the outer cell wall and the extracellular matrix of hyphae is galactosaminogalactan (GAG). GAG is a linear

heteropolysaccharide composed of galactose and *N*-acetylgalactosamine residues linked in an α-1,4 orientation [28]. Following synthesis and secretion to the extracellular space, the *N*-acetylgalactosamine residues are partially deacetylated to produce galactosamine, which undergoes protonation to produce a positively charged polymer [29]. The positive charge helps mediate adherence of the hyphae to biological surfaces in its environment, as well as to other hyphae [29]. Adhesion of the organism provides resistance to physical removal from its environment and also increases the structural integrity of biofilms. Additionally, the positive charge of GAG confers resistance towards cationic antimicrobial peptides [30], a common defence mechanism of plants and animals [31]. The role of GAG in virulence is discussed further in section 1.4.4.

1.3 Pathogenesis

Due to the ubiquity of *Aspergillus* conidia in the atmosphere, it is estimated that humans will inhale between 100 to 1000 conidia each day [2]. Because of their relatively small size and hydrophobicity, the conidia have the capacity to reach the lower portions of the respiratory tract [2]. As the natural role of *Aspergillus* in the environment is to degrade organic matter, fungal hyphae have the potential to cause disease in the lungs of the mammalian host, although this is largely dependent on both the species of *Aspergillus*, as well as the host immune status.

1.3.1 Disease-causing *Aspergillus* species

While there are over 200 *Aspergillus* species known, only approximately 40 are reported to cause disease in humans [32]. The most frequent *Aspergillus* species to cause human disease is *Aspergillus fumigatus* [32], however as described below, *Aspergillus nidulans* [33], *A. flavus,* and *A. terreus* can also cause human infections [34,35].

1.3.2 *Aspergillus* exposure in the "normal" host

When a healthy, immunocompetent human inhales *Aspergillus* conidia, disease is rare. The majority of the conidia are removed from the lungs through the action of the mucociliary escalator [36]. The conidia become embedded in the mucus layer that lines the upper respiratory tract, and through the beating action of the cilia of the epithelial cells, are swept back up the trachea to be swallowed or expectorated. Swallowed conidia are easily killed by the acids and enzymes of the digestive system.

Conidia that reach the terminal airways, such as the alveoli, are beyond the action of the mucociliary escalator. Sentinel alveolar macrophages phagocytose and kill conidia [37], thereby preventing conidial germination and the initiation of disease.

1.3.3 *Aspergillus* exposure in the immunocompromised host

Inhalation of conidia by an immunocompromised host can lead to the development of a progressive necrotizing pneumonia termed invasive aspergillosis (IA). Patient cohorts at the highest risk for IA include patients with acute leukemia, chronic lymphoproliferative disorders, allogeneic haematopoietic stem cell transplantation recipients, and recipients of solid-organ transplants [34]. While less common, IA can also be observed in patients with Acquired Immunodeficiency Syndrome (AIDS) or solid tumours, patients in the intensive care unit, as well as patients having received autologous haematopoietic stem cell transplantation or immunosuppressive treatments, such as corticosteroids or tumour necrosis factor (TNF) antagonists [34].

As is the case with an immunocompetent host, conidia that evade the mucociliary escalator in an immunocompromised host reach the terminal airways. However, due to the host's defective immune system, these conidia are not efficiently cleared from the airways by the alveolar macrophages, and instead germinate into filamentous hyphae. The hyphae can then penetrate the airway epithelium and invade the pulmonary tissue [10]. As in its natural environment, *A. fumigatus* hyphae then secrete an array of hydrolytic enzymes that digest and degrade pulmonary tissues, producing a liquefied zone of necrosis surrounding the fungal lesion [38]. Hyphae exhibit tropism towards blood vessels, where they invade the endothelial cell layer [10]. Following angioinvasion, hyphal fragments can travel through the circulatory system to disseminate to other parts of the body [10]. Common sites of secondary infection include the kidneys and liver, and to a lesser extent the skin [39] and heart [40].

Patients with chronic granulomatous disease (CGD), a disease caused by mutations in the NADPH oxidase complex resulting in the inability of neutrophils to produce neutrophil extracellular traps (NETs), are also at risk of developing IA [30]. The normally non-pathogenic *Aspergillus nidulans* is uniquely able to cause invasive disease in CGD patients, since unlike *A. fumigatus* it is normally susceptible to NETs. This susceptibility is due to low levels of production of GAG within the *A. nidulans* cell wall leading to an inability to repel the cationic antimicrobial peptides released with NET formation [30].

The total global incident rate of invasive aspergillosis is estimated to be more than 300,000 cases annually, from the over 10 million individuals who are at risk [41]. However the incident rate differs among specific at-risk patient groups, such as in acute myeloid leukemia patients, where the incidence rate is as high as 5 to 10% [42,43]. Importantly, invasive aspergillosis has an observed mortality rate of up to 90%, despite the use of currently available antifungal therapies, highlighting the need for new preventative and therapeutic approaches to managing this infection [32].

1.3.4 *Aspergillus* exposure in the context of pre-existing lung disease

While the presence of an intact systemic immune system greatly reduces the risk of invasive aspergillosis, *Aspergillus* can also cause pulmonary infections in patients with pre-existing chronic lung diseases who are not immunocompromised. Chronic lung disease commonly results in damaged pulmonary airways and impaired function of the mucociliary escalator, impairing the mechanical removal of conidia [44]. Uncleared conidia can then germinate in the airways, forming filamentous hyphae. Though hyphae are largely contained to the airways due to the intact immune system, they can induce a chronic inflammatory airway infection associated with lung injury due to the digestive enzymes and other biological products that hyphae secrete into their environment. In some patients, hyphae can even slowly invade damaged tissue to cause a chronic low grade invasive infection known as chronic pulmonary aspergillosis (CPA) [44]. The chronic inflammatory state of CPA results in increased morbidity of patients, as well as a further decline in lung function [44]. While the incidence rate of CPA is not known, it is estimated to affect approximately 3 million people worldwide [41].

One of the more common pre-existing lung diseases leading to CPA is cystic fibrosis (CF), a genetic disease caused by a mutation in the gene encoding the cystic fibrosis trans membrane conductance regulator (CFTR). The CFTR is a chloride ion channel that plays a critical role in regulating the transportation of salts and water across epithelial layers [45]. A mutation of the CFTR gene can lead to a build-up of thick, sticky mucus in the lungs, impairing the mucociliary escalator and permitting *Aspergillus* and other microbes to persist within the airways. This thick mucus environment also provides the microbes protection from killing by the host's immune system [46].

Similarly, *Aspergillus* is able to colonize the lungs of chronic obstructive pulmonary disorder (COPD) patients [47]. Excessive and long-term inhalation of tobacco smoke can lead to

the development of COPD, which is characterized as increased inflammation in the lower airways and surrounding parenchyma, leading to a loss of alveolar tissue elasticity (emphysema) and a blocking of the lower airways [48]. These characteristics can again result in an impaired ability of the lungs to effectively clear inhaled conidia, producing an environment where they can germinate and grow within airways.

Additionally, a subset of patients with airway colonization can develop an allergic sensitization response towards fungal antigens produced during chronic airway infection. This condition is known as allergic bronchopulmonary aspergillosis (ABPA) and is associated with elevated serum levels of immunoglobulin (Ig) E, pulmonary fibrosis, the development of chronic asthma and decline in lung function [49]. ABPA is almost exclusively caused by exposure to *A. fumigatus*, due to its heightened virulence [49]. The global incidence of ABPA is estimated to be approximately 4.5 million people [41]. ABPA is primarily observed in asthmatic patients (~1.4 million globally) and to lesser extent patients with CF (~6700).

1.4 *Aspergillus fumigatus* virulence factors

Despite representing only a small proportion of the total *Aspergillus* conidia in the air that humans breathe, *A. fumigatus* is responsible for approximately 60-90% of all IA cases [32,50]. While approximately 40 other *Aspergillus* species have been reported as pathogenic, with *A. flavus, nidulans, niger* and *terreus* also being isolated from invasive aspergillosis patients, these species are all less common then *A. fumigatus* [50]. The high frequency of *A. fumigatus* being isolated from patients suggests that *A. fumigatus* exhibits a higher fitness during infection. Indeed, the success of *A. fumigatus* as a pathogen has been attributed to its array of virulence factors [32].

Once *A. fumigatus* enters the lungs of the host, many of the characteristics of *A. fumigatus* that aid in surviving and recycling organic material in the environment can be repurposed towards making it a successful pathogen of humans. Factors protecting *A. fumigatus* from environmental stresses such as desiccation and ultraviolet irradiation also provide the fungus with resistance to host detection and immunological attack, respectively [32]. Additionally, the strategies employed by *A. fumigatus* to adhere to and degrade dead organic matter in the environment are also critical for fungal invasion and necrosis of host tissues during human infection. Our current understanding of these fungal virulence factors is detailed below.

1.4.1 Physical characteristics of conidia

An important aspect of *A. fumigatus* virulence is the ability of its spores to reach the terminal airways. This is due in large part to the relatively small size (2-3μm in diameter) and strong hydrophobicity [32] of its conidia. While these characteristics likely evolved to enhance conidial dispersion and resistance to desiccation, they also allow inhaled conidia to reach deep within the pulmonary system and evade physical removal from the lungs. Additionally, the hydrophobin rodlet coat also serves to conceal conidia from the immune system by masking the immunoreactive pathogen-associated molecular patterns (PAMPs) located deeper in the conidial cell wall, such as β-glucan. As a result resting conidia are relatively immune-inert. Studies of rodlet-deficient mutants have demonstrated that conidia from these strains are phagocytosed by macrophages at a significantly higher rate, and display reduced virulence in a mouse model of *A. fumigatus* corneal infection [51].

1.4.2 Melanin

Another important virulence factor found in the conidial cell wall is melanin. *A. fumigatus* produces a melanin known as melanin-1,8-dihydroxynaphthalene (DHN-melanin), that is distinct from the melanin produced by mammalian cells. The melanin serves to protect conidial DNA from degradation by shielding it from environmental stresses such as ultraviolet irradiation and oxidation. In the setting of an infection, melanin helps to protect the conidia from attack by the host's immune system. Pigmentless conidia lacking melanin exhibit a smooth surface when observed under scanning electron microscopy, in contrast to the echinulated surface of wild-type and melanin-complemented strains [52,53]. This alteration in surface morphology correlated with increased binding of complement to the conidial surface, and increased phagocytosis by human neutrophils [52]. However, it is unclear whether melanin is involved in masking PAMPs along with the rodlet layer, or if melanin deficiency leads to global alterations of the conidial cell wall resulting in exposure of immunoreactive molecules.

In addition to reducing the recognition and phagocytosis of conidia by the immune system, melanin also protects conidia by partially quenching reactive oxygen species (ROS) produced by phagocytes. Wild-type conidia survived exposure to significantly higher concentrations of ROS hydrogen peroxide or sodium hypochlorite than melanin-deficient strains [54]. These effects by melanin clearly provide the fungus with an advantage, as melanin-deficient mutants are less virulent in an intravenous mouse model of aspergillosis [52,53].

Recently it has been discovered that the melanin layer is detectable by the mammalian melanin-sensing C-type lectin receptor (MelLec) [55]. MelLec is absent from most epithelial cells, however it is expressed on endothelial cells and pulmonary epithelial cells [55]. While MelLec is absent from myeloid cells in mice, it is expressed by human myeloid cells. The MelLec-mediated response to an intratracheal *A. fumigatus* infection in mice involves the rapid release of the pro-inflammatory molecules KC and GM-CSF, and the subsequent influx of neutrophils to the lungs by 4 hours post-infection [55]. However, by 24 hours post-infection neutrophil recruitment is indistinguishable between the MelLec-deficient mice and the parental strain. Consequently, immunocompetent MelLec-deficient mice are not susceptible to pulmonary challenge with *A. fumigatus*. Conversely, MelLec is essential to mounting a successful immune response against disseminated aspergillosis, as MelLec-deficient mice exhibit greater mortality in a model of intravenous *A. fumigatus* infection [55].

1.4.3 Thermotolerance

A key difference between *A. fumigatus* and many non-pathogenic *Aspergillus* species is its ability to grow at physiological temperatures [32]. *A. fumigatus* grows well at temperatures between 30 and 45°C, to a maximum temperature of 52°C [56,57], and conidia can remain viable at temperatures as high as 75°C [32]. It is hypothesized that *A. fumigatus* evolved this thermotolerance through growth in compost heaps, in which similarly high temperatures can be reached [56,57].

Several classes of genes have been implicated in conferring thermotolerance in *A. fumigatus*. These include an array of genes encoding heat-shock proteins, which act as molecular chaperones, that are upregulated following a temperature shift from 30 to 48°C [58]. However, the apparent redundancy of the heat shock proteins makes it difficult to determine their necessity *in vivo*. Synthesis of trehalose, an α,α,1,1-linked glucose disaccharide, allows for hyphal growth at 50°C and resistance to oxidative shock. Paradoxically, however, an *A. fumigatus* strain unable to synthesize trehalose had heightened virulence in a mouse model of invasive aspergillosis [59]. The genes *afpmt1* and *afmnt1* encode proven and putative mannosyltransferases, respectively, that are involved in maintaining cell wall integrity at elevated temperatures [32]. Lastly, the gene *cgrA,* encoding a protein involved in the synthesis of the 60S ribosomal subunit, is required for optimal ribosome biogenesis at 37°C [57]. Both *afmnt1* and *cgrA* are necessary for full virulence in a mouse model of invasive aspergillosis [32,57].

1.4.4 Galactosaminogalactan

A number of virulence factors are specific to the hyphal morphology. An important trait of *A. fumigatus* hyphae is their ability to adhere to abiotic and biological surfaces and form biofilms. The exopolysaccharide galactosaminogalactan is essential for this process, as *A. fumigatus* mutants deficient in GAG are unable to adhere to multiple surfaces [60]. This ability to adhere and form biofilms is important for the organism's virulence, as GAG-deficient mutants are less virulent in multiple immunocompromised mouse models of invasive aspergillosis [60]. Further support for the role of *A. fumigatus* GAG in virulence comes from the study of *A. nidulans*, a less virulent relative of *A. fumigatus* that produces poorly-adherent GAG and consequently a weak biofilm. Genetic manipulation of *A. nidulans* to force its production of an *A. fumigatus*-like GAG results in an increase in its adherence *in vitro,* as well as its invasiveness and virulence *in vivo* [30].

In addition to its effects on adherence, GAG also plays an important role in defending the fungus from the host immune system. Similar to the rodlet and melanin layers of conidia, GAG masks the detection of β-glucans located in the inner cell wall of the hyphae [60]. GAG-deficient hyphae have increased β-glucan exposure, as determined by fluorescently-labelled dectin-1 binding, and induce a stronger pro-inflammatory cytokine response from bone marrow-derived dendritic cells *in vitro* compared to wild-type fungus [60]. Similarly, the positive charge of GAG also acts to protect the hyphae from cationic antimicrobial peptides [30]. An *A. nidulans* mutant that expresses *A. fumigatus*-like GAG is more resistant to cationic peptides found in neutrophil extracellular traps *in vitro*, and exhibits greater virulence in mice only in a non-leukopenic model of infection, when compared to wild-type *A. nidulans* [30]. In addition to these more "passive" properties of GAG to protect *A. fumigatus*, secreted GAG directly affects the immune system in several ways. GAG induces the production of the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra) *in vivo*, thereby limiting the pro-inflammatory response against the pathogen [61]. GAG is also able to induce neutrophil apoptosis through a process involving natural killer cells, thus limiting neutrophil-mediated immune defences against the pathogen (as detailed in section 1.6.2.4) [28,61].

1.4.5 Nutrient acquisition

One generic antimicrobial strategy of the host is to limit nutrient availability to the pathogen. As such, a successful pathogen must be able to acquire sufficient nutrients for growth during

infection. *A. fumigatus* is particularly adept at growing in nutrient-poor environments, given the large array of hydrolytic enzymes it secretes [32]. *A. fumigatus* has the capacity to produce at least 99 suspected or confirmed proteolytic enzymes [32], allowing it to acquire essential amino acids from host proteins.

In addition to their role in acquiring nutrients, *A. fumigatus* proteases are able to degrade extracellular matrix proteins such as collagen [62], elastin [63], laminin and fibrinogen, enhancing the invasiveness of the fungus [32]. Some *A. fumigatus* proteases also have the capacity to bind to and hydrolyze soluble host factors, such as human growth hormone releasing factor, potentially disrupting extracellular signalling [64]. *A. fumigatus* also secretes several phospholipases that cleave the phospholipids of cell membranes, potentially destabilizing host cell membranes, leading to cell lysis [32]. However, it should be noted that due to the redundancy of these degradative enzymes, it has been challenging to elucidate the contributions of the individual enzymes to the overall virulence of *A. fumigatus*.

A. fumigatus also secretes a variety of proteins involved in the acquisition of vital minerals, such as iron and zinc [32]. Iron is sequestered by the host as a strategy to limit infection, and thus pathogens require specialized molecules, known as siderophores, to scavenge iron from their environments. While an *A. fumigatus* strain deficient in the gene *sidA* required for siderophore biosynthesis grows normally in rich media, it is unable to grow in iron-poor media, and is avirulent in a mouse model of invasive aspergillosis [65]. For efficient zinc acquisition, *A. fumigatus* expresses several zinc-transporter proteins, ZrfA, B, and C [66]. In contrast to ZrfA and B, which have homologues in the avirulent fungus *Saccharomyces cerevisiae* and are expressed in acidic environments, ZrfC is expressed at neutral and alkali pH, similar to the conditions found within the airways. ZrfC also contains an extended N-terminal domain with 4 zinc-binding motifs, hypothesized to enhance its ability to scavenge zinc from the environment [66]. Consistent with these observations, of these transporters, only ZrfC was required for virulence in a mouse model of invasive aspergillosis [67].

1.4.6 Mycotoxins

Aspergillus fumigatus has the capacity to produce an array of toxic secondary metabolites, which have a variety of effects on mammalian cells [32]. Of these toxins, gliotoxin is the best studied. Gliotoxin is produced abundantly during infection and exerts many effects on mammalian cells [32]. The structure of gliotoxin contains a disulphide bridge that is essential for

its toxicity, enabling the toxin to cross-link proteins through their cysteine residues [68]. Gliotoxin suppresses mast cell degranulation, macrophage phagocytosis, neutrophil phagocytosis and ROS production [32,68]. Gliotoxin can also inhibit NF-κB signalling to suppress cytokine production and inflammatory responses, as well as induce apoptosis in a variety of immune cells [68]. *A. fumigatus* mutants deficient in gliotoxin are less virulent *in vivo*, but only in non-neutropenic mouse models of invasive aspergillosis [68], underscoring the importance of neutrophils as target cells for the effect of gliotoxin.

Aspergillus fumigatus also produces the ribotoxin restrictocin, which inhibits protein synthesis by specifically cleaving the sarcin/ricin loop domain of the 18S ribosomal RNA [69]. While restrictocin is a potent toxin able to cause cell death at nanomolar concentrations, restrictocin-deficient *A. fumigatus* strains are not hypovirulent, suggesting that this toxin does not play a major role in the pathogenesis of this fungus [32,70].

Fumagillin is a metabolite of *A. fumigatus* associated with neurotoxicity, and hematotoxicity such as granulocytopenia and thrombocytopenia [71,72]. Fumagillin is able to affect several neutrophil functions, such as phagocytosis, degranulation and respiratory burst [73]. While the role of fumagillin during an *A. fumigatus* infection has not been evaluated [32], *in vitro* this toxin is approximately 100 times less potent than gliotoxin at inhibiting mammalian cell growth [74]. Interestingly, fumagillin is under active investigation for use as a therapeutic agent for a number of conditions. Obese rodents that were administered fumagillin experienced sustained weight loss, suggesting its possible use to treat obesity [75]. Fumagillin inhibits angiogenesis, leading to the study of synthetic fumagillin analogues as agents to treat prostate cancer and melanoma [71]. Lastly, the antibiotic activity of fumagillin against microsporidia has led to its successful use against this microbe in both humans [72] and honeybees [76].

Together, this diverse assortment of secondary metabolites produced by *A. fumigatus* has the potential to affect the host and their response to the infection through a variety of mechanisms.

1.4.7 Growth under hypoxic conditions

During invasive aspergillosis infection, tissue damage and thrombosis lead to the development of an oxygen-limiting hypoxic microenvironment. This is then amplified in the context of inflammation, as the aerobic metabolic activity of both immune and fungal cells rapidly depletes the available oxygen [77]. *A. fumigatus* is able to grow under hypoxic conditions, a trait likely acquired through the growth in hypoxic compost heaps [77]. This ability

to grow under hypoxic conditions is likely advantageous to the fungus in an infection setting, as a screen of clinical isolates revealed a correlation between hypoxic growth and virulence in an immunosuppressed mouse model [78]. This correlation was supported when serial passage of an *A. fumigatus* strain under hypoxic conditions was found not only to increase the ability of the resulting strain to grow under hypoxic conditions, but also its virulence in an immunosuppressed mouse model [78]. The deletion of *A. fumigatus srbA*, a transcription factor responsible for regulating growth in hypoxic conditions, results in a strain that is unable to grow under hypoxia and is avirulent in multiple immunocompromised mouse models of invasive aspergillosis [79].

1.5 Treatment options

Antifungal options for the treatment of *Aspergillus* infection are relatively limited. The three primary classes of antifungal drugs used for both treatment and prophylaxis of *Aspergillus* infections are the polyenes, the azoles, and the echinocandins [80]. These drugs all target unique components of the fungal cell wall or plasma membrane not present in mammalian cells and exhibit excellent antifungal activity *in vitro*. However, real world activity of these agents in patients remains suboptimal, with mortality rates for invasive aspergillosis in acute myeloid leukemia patients remaining between 50 and 90% [43].

1.5.1 Polyenes

The polyenes are a class of drug that bind to ergosterol, a sterol present in the lipid membranes of fungi and other microorganisms that is similar in structure and function to mammalian cholesterol. At high enough concentrations, polyene drugs form pores in the fungal membrane, leading to leakage of intracellular contents and death of the fungal cell [81]. The only member of the polyene drug class used in the treatment of aspergillosis is amphotericin B, which is also effective against other fungal infections, as well as leishmaniasis [81].

Although amphotericin B has efficacy against most *Aspergillus* species (a notable exception being *A. terreus*, which is inherently resistant via an unknown mechanism), use of this agent is associated with a high incidence of renal and other toxicities due to binding of amphotericin B to cholesterol in mammalian cells [81]. Newer formulations in which amphotericin B is encapsulated in liposomes or other lipid carrier molecules are much better tolerated, and have largely replaced conventional amphotoricin B [81]. Randomized clinical trials have also demonstrated that it is inferior to the azole voriconazole for primary treatment of invasive

aspergillosis [82]. Currently amphotericin B is most commonly used as a second line therapy in azole-refractory disease, or in patients intolerant of voriconazole.

1.5.2 Azoles

The azole class of antifungal compounds act by binding to and inhibiting the fungal enzyme cytochrome P450 (cyp450), which is involved in an intermediate step of ergosterol biosynthesis. Inhibition of cyp450 results in the cellular accumulation of toxic intermediate compounds, and the death of the fungus. Three different azoles are primarily used against *Aspergillus*: voriconazole, posaconazole, and isavuconazole [80]. These azoles each have different hydrophobicity profiles, resulting in different pharmacokinetic and pharmacodynamic properties.

Voriconazole is the smallest and most polar of the three azoles, and is the preferred first-line azole treatment [80]. Voriconazole remains the most effective therapeutic option available against aspergilloses of the central nervous system [80,83], likely due to its ability to penetrate the blood-brain barrier. Voriconazole toxicities, which include visual disturbances, hepatotoxicity and photosensitivity, often limit use of this agent [84].

In contrast to voriconazole, posaconazole is a highly lipophilic molecule that accumulates in the lipid membrane of host cells, and is transferred to fungal membranes upon contact [85]. Posaconazole accumulates in the membranes of both structural [86] and immune [38] cells, thus increasing its bioavailability. Due to its accumulation to high concentrations in tissues, posaconazole is the preferred drug for prophylactic treatment of haematological patients [80]. Posaconazole is much better tolerated than voriconazole but has only been studied as a prophylactic agent to prevent *Aspergillus* infections [87].

Isavuconazole is the newest azole approved as a treatment for aspergillosis diseases, having hydrophobic properties intermediate between voriconazole and posaconazole. One benefit of isavuconazole is that it can be administered as a water-soluble pro-drug, which is then cleaved into its more hydrophobic, active moiety by plasma esterases [88]. Unlike posaconazole however, the active moiety predominantly associates with serum albumin rather than cells, resulting in good bioavailability in the central nervous system [89]. A recent clinical trial suggests that isavuconazole is non-inferior to voriconazole as primary therapy for invasive aspergillosis and is associated with less toxicity [90].

Over the past decade, the incidence rate of azole resistance in *Aspergillus* species has been rising [91]. Mutations in efflux transporters contributing to azole resistance were first described

in patients with chronic *Aspergillus* infections receiving long-term azole therapy [91]. More recently however, resistance has been reported in azole-naïve patients and has been linked to the development of resistance in *Aspergillus* isolates in the environment exposed to agricultural azoles [91]. Resistance in these strains is predominantly the result of mutations in the gene encoding the target enzyme, cyp450 and its promoter region that lead to both reduced azole binding and overexpression, respectively [91].

1.5.3 Echinocandins

The echinocandin class of antifungal compounds work by inhibiting the plasma membrane β-glucan synthase enzyme necessary for the synthesis of β-glucan [92]. As β-glucan is an essential cell wall component of *Aspergillus*, inhibiting synthesis of this glycan leads to a fragile cell wall and impaired fungal growth. Of the three echinocandins that are currently approved for therapeutic use (anidulafungin, caspofungin, and micafungin), caspofungin is most commonly used to treat aspergilloses [92]. As a monotherapy, caspofungin has relatively poor efficacy and is considered inferior to azoles under most conditions [80]. However, due to its targeting of a unique fungal pathway, caspofungin is generally favoured over amphotericin B and has shown promise in combination therapy with either polyenes or azoles [92].

1.6 Immune response to *Aspergillus fumigatus*

As previously mentioned, the majority of inhaled conidia are physically removed from the lungs via the action of the mucociliary escalator. However, conidia that escape physical removal require clearance by sentinel cells, such as pulmonary epithelial cells, alveolar macrophages and neutrophils. This second line of defense is dependent on the immune system's capacity to recognize and respond to *Aspergillus*.

1.6.1 Initial response

The conidia that evade physical removal from the lungs and reach the alveolar space will come in contact with two important resident cell types: alveolar epithelial cells and alveolar macrophages. Both cell types are able to phagocytose conidia and kill them through acidification of the phagolysosome [93,94]. In *in vitro* assays using the A549 alveolar epithelial cell line, phagocytosis of conidia only occurs after swelling, and requires an interaction between alveolar epithelial cell dectin-1 and conidial β-glucan that is exposed following the shedding of the rodlet

layer. Internalization is inefficient, and only approximately one third of adherent conidia are internalized by these cells [95]. A small percentage (approximately 3%) of internalized conidia can survive the phagosome to germinate and escape the epithelial cell [95]. In contrast, alveolar macrophages can phagocytose resting conidia, internalizing up to 90% of conidia that they are exposed to in *ex vivo* assays [93,96]. Under *in vitro* conditions, alveolar macrophages kill only approximately 50% of the conidia over a 6-hour period, however *in vivo* studies suggest that in the lung environment they are more effective and can kill up to 90% of conidia by 36 hours post infection [96].

In addition to directly killing the conidia, both alveolar epithelial cells and alveolar macrophages produce cytokines and chemokines such as IL-6, TNF-α, GM-CSF, and MCP1 in response to *A. fumigatus* swollen or germinating conidia [93,94]. This response attracts additional immune cells to the alveoli, most notably circulating neutrophils. Neutrophils account for the majority of the body's circulating phagocytes, with the pulmonary capillaries acting as a reservoir for approximately 40% of these cells [97]. Neutrophils migrate to the alveoli (described in detail in section 1.6.4), phagocytose and kill conidia and hyphae, and are necessary for the early control of an *A. fumigatus* infection [98]. Chemical depletion experiments suggest that alveolar macrophages are dispensable for this neutrophil recruitment and that alveolar epithelial cells alone are able to efficiently recruit neutrophils [98]. All three of these cell types require NADPH oxidase to efficiently kill *A. fumigatus*, underscoring its importance in the antifungal response and the resulting susceptibility of CGD patients.

1.6.2 Innate immune response towards *A. fumigatus*

If the initial response by alveolar epithelial cells, alveolar macrophages, and circulating neutrophils fails to clear the conidia, either due to a prohibitively high inoculation or immunosuppression, the conidia will then germinate into hyphae. These hyphae present a different set of PAMPs to the host immune system, and secrete both digestive enzymes and toxins, which in turn can modulate the immune response. These interactions lead to the further release of pro-inflammatory cytokines such as GM-CSF, MIP-1α, MIP-2, MCP-1, KC, IL-1β, IL-8, IL-18, IL-12, IL-6, IL-10 and IFN-γ [97] and the recruitment of additional immune cells which are critical in controlling early infection. The majority of these interactions involve an array of mammalian pathogen recognition receptors (PRRs) that bind to the carbohydrates

located on the hyphal cell wall and are essential for efficient fungal recognition and initiation of the innate immune response (these interactions are reviewed in detail in section 1.7).

1.6.2.1 Neutrophils

An effective neutrophil response is essential for successfully clearing an *A. fumigatus* infection. Mice rendered neutropenic [98], and patients receiving allogeneic haematopoietic stem transplantation, or with acute myeloid leukemia undergoing intensive chemotherapy [43] are highly susceptible to invasive aspergillosis.

Neutrophils are recruited to the lungs during a pulmonary aspergillosis infection through the release of IL-8, KC, and MIP-2 by alveolar epithelial cells and alveolar macrophages in response to swollen or germinating conidia [93,94,97]. The mechanisms behind neutrophil recruitment and extravasation are discussed in detail in section 1.6.4. Once in contact with *A. fumigatus*, the mechanisms of neutrophil-mediated fungal killing are dependent on the morphology of the fungus. Human neutrophils phagocytose *A. fumigatus* conidia *in vitro* in a dectin-1-independent, complement receptor 3 (CR3, also known as MAC-1 or CD11b/CD18 integrin) dependent manner. Mice with haematopoietic deficiencies in either dectin-1 or CD18 do not have increased susceptibility to *A. fumigatus* conidia, suggesting there is functional redundancy in this system [99,100]. Killing of phagocytosed conidia is primarily dependent on non-oxidative granule-mediated killing, however NADPH oxidase-dependent ROS production can contribute to this process [99,101].

Neutrophils are uniquely adept at killing *A. fumigatus* hyphae. Hyphae are too large to be phagocytosed, and as a result neutrophils release various antifungal mediators into the extracellular space to kill the fungus. Neutrophils possess preformed cytosolic granules containing cytotoxic proteins such as the proteases elastin and cathepsin G, the ROS-producing enzyme myeloperoxidase (MPO), the siderophore lactoferrin, opsonins such as pentraxin-3, and a variety of antimicrobial peptides [101]. Neutrophil recognition of *A. fumigatus* hyphae is not well understood, but induces the release the contents of their granules to the extracellular space. Neutrophils generate ROS superoxide and hydrogen peroxide through the NADPH complex, while secreted MPO converts hydrogen peroxide into the longer-lived ROS hypochlorous acid [101].

In addition to granule-release, neutrophils can also release neutrophil extracellular traps (NETs) in response to hyphae. The exact mechanisms underlying NET formation are unclear,

however it appears to involve NADPH ROS production [101], and in some cases IL-8 [102]. NET formation involves chromatin decondensation and translocation of granules to the nucleus [101]. Neutrophils then release their nuclear contents into the extracellular space, trapping pathogens in DNA embedded with granular contents such as MPO, proteases and antimicrobial peptides. It is believed that NETs enhance the antimicrobial activity of neutrophils by concentrating their antimicrobial peptides and proteins in close proximity to the target pathogen. However, while NETs have been observed to minimally inhibit the growth of *A. fumigatus* [102], they are not fungicidal, as degradation of NETs with DNAse *in vitro* resulted in no reduction of fungal killing [101]. Studies of non-*fumigatus Aspergillus* species have suggested that hyphal resistance to NETs is mediated, at least in part, by the production of cationic GAG [30]. The GAG-deficient species *Aspergillus nidulans* is more susceptible to killing by NETs [30]. Increasing GAG expression in this strain by heterologous expression of GAG-synthetic genes renders this species resistant to NET-mediated killing [30].

1.6.2.2 Eosinophils

While eosinophils have a well-documented detrimental allergic role in ABPA through their interactions with elevated serum IgE [49], they play an important role in mediating effective fungal clearance in the early stages of a pulmonary *A. fumigatus* invasive infection [103]. In an immunocompetent pulmonary aspergillosis model, eosinophil-deficient mice exhibit higher fungal burden up to 48 hours post-infection, as well as lower concentrations of the pro-inflammatory cytokines IL-1β, IL-6, IL-17A, G-CSF, GM-CSF and KC [103]. Eosinophils are also effective at killing *A. fumigatus in vitro* through the release of antimicrobial granules, suggesting that they contribute to the antifungal response through direct killing, as well as immune cell recruitment [103].

1.6.2.3 Mast cells

Similar to eosinophils, mast cells are detrimental to the host during ABPA, as they are recruited to the airways in an IgE-dependent manner and contribute to airway inflammation and hyper-responsiveness in mouse models of allergic disease [104]. However, *in vitro* studies have also revealed that mast cells degranulate in response to mature *A. fumigatus* hyphae in an IgE-independent manner, suggesting that they are also involved in the innate response towards this fungus [105]. Multiple *Aspergillus* mutants deficient in GAG production were found to induce

less mast cell degranulation, suggesting GAG may be the fungal molecule responsible for inducing mast cell degranulation [105]. However, as the contribution of mast cells to the response against *A. fumigatus* remains to be studied *in vivo*, it is currently unknown whether they are beneficial or detrimental to the host in this context.

1.6.2.4 Natural Killer cells

Natural Killer (NK) cells are a class of innate lymphocytes with potent cytotoxic activity [106]. NK cells are attracted to the site of *A. fumigatus* infection in a neutropenic mouse model through the release of MCP-1 [107]. Neutralization of MCP-1 specifically blocked NK cell recruitment, and depletion of either MCP-1 or NK cells resulted in significantly greater mortality in neutropenic mice, suggesting that NK cells provide an important "second-line" of defence against *A. fumigatus* in immunocompromised hosts [107]. Interestingly, an increase in MCP-1 is not observed in non-neutropenic mice infected with *A. fumigatus* [107], and *in vitro* studies revealed that co-culturing human NK cells with neutrophils inhibited the activation and antifungal effects of the NK cells [108], suggesting that the activity of NK cells against *A. fumigatus* is suppressed in the presence of neutrophils.

Aspergillus may also play an important role in modulating the interactions between NK cells and neutrophils as it has been reported that *A. fumigatus* GAG induces neutrophil apoptosis through a process involving NK cells [109]. When human cells were co-incubated with GAG, GAG induced ROS production by the neutrophils, subsequently resulting in an increased expression of MHC-I chain-related molecule A (MICA) on the neutrophil surface. MICA expression allowed for the interaction between the neutrophil and NK cell via NKG2D, resulting in NK cell activation and induction of neutrophil apoptosis via the Fas/FasL pathway [109]. However, these *in vitro* co-culture observations have not been validated *in vivo* or with live fungal organisms, and therefore their significance during *A. fumigatus* infection remains unknown.

1.6.2.5 Monocytes

Monocytes are myeloid-derived phagocytic cells that circulate in the blood stream with the capacity to respond to inflammatory cues, and can differentiate into inflammatory or tissue-resident macrophages and dendritic cells [110]. Mice depleted of inflammatory monocytes and monocyte-derived dendritic cells (Mo-DCs) through targeting of the CCR2 surface receptor have

normal pulmonary neutrophil recruitment but exhibit similar levels of susceptibility to invasive aspergillosis as is seen in neutropenic mice [111]. Monocytes can directly phagocytose and kill *A. fumigatus* conidia as well as enhance neutrophil activity through the release of inflammatory mediators such as TNFα and IL-12 [111]. The observation that specific depletion of either neutrophils or monocytes renders mice highly susceptible to *A. fumigatus* infection underscores the importance of monocyte-mediated neutrophil activation in the immune response towards this pathogen. Additionally, co-culture of human monocytes with *A. fumigatus* conidia revealed the conidial killing to be dependent on dectin-1 recognition of β-glucan, and thus is dependent on conidia swelling and shedding their rodlet layer for efficient killing [112].

Inflammatory monocytes and their derivatives are important antigen-presenting cells (APCs) that represent a key link between the innate and adaptive immune systems [113]. In a murine model of pulmonary aspergillosis, monocytes and Mo-DCs are recruited to the lungs, where Mo-DCs phagocytose *A. fumigatus* conidia and traverse to the draining lymph nodes [113]. It is unclear whether these monocytes differentiate into Mo-DCs prior to, or following conidial uptake. Following *A. fumigatus* infection, CCR2-depleted mice fail to present conidial antigens to draining lymph nodes and mount an effective CD4 T-cell response [113], highlighting the importance of inflammatory monocytes and Mo-DCs in the transition from an innate to an adaptive immune response. Interestingly, this difference in T-cell response is abrogated in a systemic model of *A. fumigatus* infection, suggesting that antigen presentation by Mo-DCs is tissue-specific [113].

1.6.3 Adaptive immune response towards *A. fumigatus*

A range of adaptive immune responses to *A. fumigatus* has been reported. These responses [114,115] can be beneficial to the host resulting in fungal clearance, or contribute to disease progression through heightened immunopathology, depending on the type of antigen presented and the host conditions in which it is presented. The overall outcome of infection depends not only on the type of adaptive response elicited, but also the timing of the response, as different T-cell responses are required at different stages of disease.

1.6.3.1 T_H2/T_H9 response

Allergic bronchopulmonary aspergillosis (ABPA, discussed previously) is an allergic response to chronic fungal antigen exposure during airway infection. ABPA is characterized by

an exuberant T_H2 response with elevated IL-4, -5, -10, and -13, pulmonary recruitment of eosinophils and mast cells, high IgE titres, goblet cell hyperplasia and airway hyperreactivity [116]. T_H9 cells are also recruited through elevated IL-4, and contribute to the airway hyperreactivity [117]. The detrimental effects of a T_H2/T_H9 response against *A. fumigatus* have been observed in mouse models of allergy and infection. Neutralizing IL-4, IL-5 or IL-13 reduced morbidity in animal models of ABPA [116], and mice deficient in IL-4 are protected against invasive aspergillosis [115,118]. Similarly, neutralization of the T_H9 negative regulator programmed cell death ligand 2 (PD-L2) resulted in increased morbidity in ABPA mice [117]. *A. fumigatus*-susceptible CD2F1 mice administered a sublethal intravenous dose of conidia produce less IL-4 and instead more IFNγ by their splenocytes than mice administered a lethal infection, and are protected from a subsequent lethal infection [119]. Conversely, blocking the effects of IL-4 through treatment with soluble IL-4 receptor increases the survival of mice challenged with a lethal dose of conidia [119].

1.6.3.2 T_H1 response

In contrast to the detrimental T_H2/T_H9 immune responses, the T_H1 response is thought to be beneficial to the host during an *A. fumigatus* infection. The induction of a T_H1 response results in the production of interferon-γ (IFNγ), which acts on numerous innate immune cells including neutrophils, macrophages, and monocytes [120], increasing their antifungal activity [121]. IFNγ-deficiency results in increased susceptibility to *Aspergillus* challenge [118] and transient over-expression of IFNγ using an adenovirus vector prior to fungal challenge increases the survival of BALB/c mice in an immunosuppressed model of invasive pulmonary aspergillosis [122]. This increased resistance to infection is associated with increased IL-12, enhanced fungicidal activity of pulmonary macrophages and leukocytes, and reduced fungal burden [122]. While NK cells have been shown to be an important source of IFNγ during invasive aspergillosis [123], the importance of T_H1 cell-derived IFNγ is illustrated through the observation that adoptive transfer of *Aspergillus*-specific T_H1 cells to naïve mice confers protection against invasive aspergillosis [124].

Further evidence for the importance of a T_H1 response in protection against *Aspergillus* infection comes from comparative studies of C57BL/6 and BALB/c mice. Following the intravenous administration of a sublethal dose of *A. fumigatus* conidia, T_H1-skewed C57BL/6 mice clear the infection from their pulmonary tissue at a faster rate than T_H2-skewed BALB/c

mice [125]. This enhanced fungal clearance is associated with greater induction of IFNγ in the lungs, as well as greater recruitment of neutrophils, macrophages and monocytes to the lung tissue by the C57BL/6 mice [125]. In contrast, the delayed fungal clearance in the BALB/c mice is associated with greater induction of pulmonary IL-4 and IL-17 [125].

Observations suggesting a beneficial role for the T_H1-associated response in protection against *Aspergillus* infection have also been made in humans, where polymorphisms in the promoter of the gene for IFNγ are associated with greater production of IFNγ, higher antifungal capacity of macrophages, and a reduced risk of developing invasive aspergillosis [126]. IFNγ-producing *Aspergillus*-specific T-cells were isolated from patients recovering from invasive aspergillosis, but were absent in patients with progressive disease [127]. IFNγ therapy has been associated with clinical improvement in chronic pulmonary aspergillosis patients that were recalcitrant to antifungal therapy [128], and has been observed to enhance the capacity of leukocytes in patients with invasive fungal infections to produce pro-inflammatory cytokines [129], suggesting IFNγ therapy as a possible adjunctive approach to treating aspergilloses.

Taken together, these observations in both mice and humans demonstrate the overall beneficial effects of the T_H1 response against aspergillosis.

1.6.3.3 T_H17 response

The hallmark of the T_H17 response, classically associated with mucosal immunity, is the production of IL-17 [121]. IL-17 plays multiple roles in the inflammatory response to fungal infection including recruitment of neutrophils to the site of infection as well as the production of additional pro-inflammatory cytokines, such as IL-1β, IL-6, IL-22, G-CSF and TNFα, production of ROS, proteases and antimicrobial peptides by neutrophils, and the production of antimicrobial peptides by epithelial cells [121]. However, there is conflicting evidence as to whether the T_H17 response is effective at resolving an *A. fumigatus* infection, or exacerbates the disease through immunopathology.

The importance of the T_H17 response in fungal infections is illustrated by experiments in which administration of neutralizing anti-IL-17 antibodies to wild-type mice increased susceptibility to pulmonary *A. fumigatus* infection [130]. Dectin-1-deficient mice, which are highly susceptible to *A. fumigatus* infection, are unable to efficiently produce T_H17-associated cytokines as compared to wild-type mice [130]. Additionally, COPD patients without active *Aspergillus* infection have a higher percentage of *Aspergillus*-specific T_H17 cells than T_H1 cells

in lung explant tissue, despite having more *Aspergillus*-specific T_H1 cells in their peripheral blood [131], suggesting that T_H17 cells serve a protective role at the site of infection.

The T_H17 response has also been associated with immunopathology during *A. fumigatus* infection. Unregulated or prolonged activation of a T_H17 response can result in chronic inflammation, and tissue damage which can paradoxically increase susceptibility to fungal infection [121]. Production of the T_H17-inducing cytokine IL-23 by dendritic cells during *A. fumigatus* pulmonary infection inhibits the production of IL-12 due to the two cytokines sharing a common subunit, preventing the induction of a beneficial T_H1 response and resulting in uncontrolled inflammation and fungal growth [132]. Mouse neutrophils exposed to IL-23 and IL-17 are significantly more resistant to apoptosis than neutrophils exposed to IFNγ, suggesting a possible mechanism of how skewing towards a T_H17 response and away from a T_H1 response contributes an uncontrolled chronic inflammatory state [132]. Mice lacking the T_H17 negative regulator Toll IL-1R8 (Tir8) are more susceptible to *A. fumigatus* infection, exhibiting reduced survival and heightened fungal burden, despite higher levels of T_H1 and T_H17-associated cytokines [133]. The cytokine IL-17 directly promotes the germination of *A. fumigatus* conidia [134], possibly enhancing its pathogenicity under these settings. In CF patients, while not directly linked to *Aspergillus* infection, higher percentages of T_H17 cells in peripheral blood were associated with poor lung function [135], suggesting that an exacerbated T_H17 response contributes to CF pathology.

Taken together these data show that while a T_H17 response likely plays an important role in the clearance of *A. fumigatus* during infection, it must be tightly regulated to avoid a detrimental hyper-inflammatory state. Furthermore, neutrophils have been observed to produce IL-17 in an autocrine manner *in vivo*, enhancing antifungal killing in a mouse keratitis model [136]. It is currently unknown whether T_H17- and neutrophil or other cell-derived IL-17 play cooperating or conflicting roles in antifungal immunity.

1.6.3.4 Antibody response

A. fumigatus produces a host of antigenic proteins to which humans make antibodies. However, *A. fumigatus* antibodies do not seem to play an important protective role. Many of the antigens secreted by *A. fumigatus* during infection, such as galactomannan and proteases, lead to the generation of IgE antibodies and a non-protective T_H2 response [137-139]. While the severity of ABPA has long been correlated to elevated levels of *Aspergillus*-specific IgE [140,141], the

exact role that IgE plays in disease remains poorly understood. ABPA patients receiving azole therapy exhibited reductions of *A. fumigatus*-specific IgE and asthma severity [142], while treating ABPA CF patients with anti-IgE-blocking antibodies provided no clinical benefit [143].

In a model of ABPA, IgE-deficient 129/SVEV mice develop similar levels of airway hyperreactivity and lung eosinophilia as compared to wild-type mice [144]. However, IgE-deficient BALB/c mice exhibit reduced pulmonary eosinophil numbers [104], suggesting that the phenotype may be strain specific. In addition to reduced numbers of pulmonary eosinophils, IgE-deficient BALB/c mice do not experience an expansion of mast cells, nor an increase in mast cell activity that is observed in the wild-type mice [104]. However, airway hyperreactivity and overall disease progression was not evaluated in the IgE-deficient BALB/c mice [104], and as such the contribution of mast cell activation to disease progression remains poorly understood.

BALB/c mice deficient in B cells, and thus lacking the capacity to produce antibodies, exhibit similar levels of airway hyperresponsiveness, overall inflammation and collagen deposition as compared to wild-type mice in a model of ABPA [145]. However, the sensitized B cell-deficient mice produce lower levels of the T_H1/T_H2 cytokines IL-12, IL-4 and IL-13 in the BAL fluid, and consequently exhibit reduced goblet cell hyperplasia and mucus production. These mice produce higher levels of the T_H17 cytokines IL-6 and IL-17, accompanied by greater levels of eosinophilia and neutrophilia in the BAL fluid as compared to the wild-type mice [145].

These studies suggest that while the natural antibody response, in particular IgE, does not affect the overall severity or outcome of ABPA, it does have some influence on cellular recruitment and activation.

Adoptive transfer of serum from mice pre-exposed to a sub-lethal intravenous infection of *A. fumigatus* was unable to protect mice from a lethal intravenous challenge, despite the presence of *A. fumigatus*-specific antibody [146]. Instead, adoptive transfer of splenic macrophages from pre-exposed mice offered significant protection to a lethal intravenous *A. fumigatus* infection [146], suggesting that pre-exposure to *A. fumigatus* results predominately in cell-mediated, and not antibody-mediated, protection from infection.

While antibodies naturally produced against *A. fumigatus* antigens during infection provide little protection to the host, recent vaccination studies focussing on fungal cell wall components have yielded promising results. Monoclonal mouse IgG antibodies generated through immunization with synthetic analogues of the oligogalactofuranose of galactomannan recognize

the surface of *A. fumigatus* hyphae, however their ability to confer protection was not evaluated *in vivo* [147]. Similarly, a monoclonal mouse IgM antibody generated by immunization with proteins secreted from cultures of *A. fumigatus* bound secreted proteins, and antigens on the conidial and hyphal surfaces [148]. While this antibody was not evaluated for its ability to protect against *A. fumigatus* infection, it was able to prevent the adherence of the fungus to a variety of surfaces [148]. As detailed in section 1.7, an IgG antibody raised against β-glucan partially protected mice from intravenous *A. fumigatus* infection [149]. Additionally, an IgM monoclonal antibody specific for the oligosaccharide sialyl-lacto-*N*-tetraose of group B streptococci bound to *A. fumigatus* conidia and hyphae, and protected mice against both intravenous and pulmonary *A. fumigatus* challenge [150].

Taken together, these observations suggest that while *A. fumigatus* infection typically does not naturally generate protective antibodies, a careful selection of antigen could lead to the development of an effective vaccine against this fungus.

1.6.4 Neutrophil recruitment to the site of infection

As described previously, the recruitment of neutrophils to the site of infection is a critical component of an effective immune response against *A. fumigatus.* This is exemplified in the susceptibility of neutropenic mice and humans to invasive pulmonary aspergillosis [151,152]. Given the central role neutrophils play in combating an *Aspergillus* infection, it is important to understand the molecular mechanisms by which they migrate to the site of infection.

Neutrophils are attracted to the site of infection through the detection of various chemokines, a family of low molecular weight cytokines with chemotactic properties [153]. Some of the best-characterized neutrophil chemokines are CXCL1 (growth-related gene product-α GRO-α in humans or keratinocyte-derived chemokine KC in mice), CXCL2 (GRO-β in humans or macrophage inflammation protein-2 MIP-2 in mice), CXCL8 (IL-8 in humans), CCL2 (monocyte chemoattractant protein-1 MCP-1), CCL3 (macrophage inflammatory protein-1α MIP-1α), and CCL4 (MIP-1β). Neutrophils will detect concentration gradients of these molecules through G protein-coupled receptors and migrate towards higher concentrations, towards their source [153].

Upon stimulation by inflammatory cytokines and chemokines, neutrophils leave the bone marrow to enter the bloodstream and transit to the site of infection. Endothelial cells within blood vessels proximal to the site of infection are activated in response to mediators such as

complement, thrombin and histamine, and rapidly translocate the lectin P-Selectin from intracellular Weibel-Palade bodies to their luminal surface, initiating the multistep process of neutrophil transendothelial migration [154]. In a mouse model of cerebral aspergillosis, increased P-selectin staining is observed in cerebral capillaries proximal to immune cell infiltrates [155]. P-Selectin interacts with PGSL-1 on the surface of neutrophils to "tether" the neutrophils to the endothelial wall, and begin the process of "rolling". The neutrophils, in turn, express L-selectin on their surface, which interacts with PGSL-1 on the endothelial surface, strengthening these interactions [156].

In response to TNF-α, activated endothelial cells also express E-selectin *de novo* [157], which interacts with the neutrophils to participate in the "slow rolling" step of transendothelial migration [158]. In response to *A. fumigatus* hyphae *in vitro,* endothelial cells express E-selectin and vascular cell adhesion molecule-1 (VCAM-1), as well as release TNF-α and IL-8 [159]. Increased expression of E-selectin, VCAM-1, TNF-α and KC is also observed in mouse models of pulmonary aspergillosis [159]. Neutrophil integrins in their low-affinity conformation are also involved in the slow rolling process [158,160]. Slow rolling gives the neutrophils an opportunity to sample the cytokines in their surrounding milieu, and in the presence of IL-8, neutrophil LFA-1 (a heteromolecule composed of CD11a and CD18) and other integrins convert to their high-affinity conformation [158]. In parallel, nearby endothelial cells activate and express several integrin ligands, such as intercellular adhesion molecule (ICAM)-1 and ICAM-2, in response to TNF-α and IL-1β [161]. In the cerebral aspergillosis model, increased ICAM-1 staining is also observed in the cerebral capillaries [155]. Co-cultures of *A. fumigatus* with human pulmonary microvasculature endothelial cells induced the expression of E-selectin and ICAM-1, as well as IL-1β and IL-6 [162]. The interaction of ICAM-1 and 2 with neutrophil integrins mediates neutrophil "arrest" and "adherence" [157]. The neutrophils then begin "crawling", probing the endothelial cell layer with their pseudopods, searching for a suitable transmigration site. Crawling requires the interactions between the leukocyte integrin MAC-1, a heteromolecule composed of CD11b and CD18, and ICAM-1 on the endothelial cell surface [157].

Neutrophils exhibit crawling behaviour until they find a suitable site for diapedesis. Such a site is typically the intercellular junction between two endothelial cells for paracellular transmigration, however in rare cases neutrophils can penetrate the endothelial cell for transcellular transmigration [157,158]. In the case of paracellular transmigration, neutrophils

migrate across the endothelial-cell junction through interactions with many of the endothelial cell junctional proteins. Leukocyte integrin LFA-1 can interact with junctional adhesion molecule (JAM)-A and ICAM-2, while MAC-1 can interact with JAM-C and ICAM-2 [157]. Neutrophils can also express adhesion molecules found in the endothelial-cell junction that are involved in homophilic interactions, such as platelet/endothelial-cell adhesion molecule 1 (PECAM1), JAM-A, and CD99 [157]. The requirement of different junctional proteins for neutrophil transmigration is stimulus-specific, as PECAM1, JAM-A and ICAM2 are required for migration in response to IL-1β, but not TNFα [157]. Additionally, cell signalling through junctional proteins, such as JAM-A and PECAM1, can weaken the interactions between endothelial-cell cadherins, promoting paracellular transmigration of the neutrophils out of the vascular lumen [157]. The combination of weakened inter-endothelial cell interactions and direct binding of neutrophils to the junctional proteins allows these cells to navigate across the endothelial cell junction.

Beyond the endothelial layer the neutrophils must traverse other barriers, including the basement membrane and vasculature-associated cells called pericytes [157]. The basement membrane is a loosely organized collection of extracellular molecules which include the laminins -8 and -10, and collagen IV [157]. Neutrophils navigate through the basement membrane and pericyte layers through penetration of regions of lower matrix density, as well as the use of elastases and other proteases which partially degrade the matrix [163]. Gaps in the pericyte layer have been observed to correspond to low-density regions of the basement membrane, thus facilitating neutrophil passage.

The process of neutrophil transendothelial migration in the lung is somewhat atypical, given the unique structure of the organ [161,163]. The cross-sectional area of the alveolar capillaries is smaller than that of a neutrophil, thus neutrophils must deform in order to pass through the capillaries. Possibly as a result of this low flow state, tethering and rolling are not observed during pulmonary neutrophil recruitment [161]. However, the molecular interactions involving crawling and transendothelial migration are conserved in neutrophil recruitment in the lungs. Additionally, given the close proximity of the alveolar endothelium with the alveolar epithelium to allow for efficient gas exchange, the two layers have a thin common basement membrane, produced by fibroblasts, thus reducing the barrier to neutrophil transmigration [161].

Preface to Chapter 1, Section 7

Aspergillus fumigatus is the cause of a spectrum of diseases, which are associated with exceedingly high rates of morbidity and mortality, and for which current antifungal therapies remain limited. *A. fumigatus* predominately infects patients with underlying impaired systemic or local pulmonary immunity, and under normal physiological conditions the host is able to eradicate the invading fungus before the establishment of infection. A complex array of host receptors exists that recognize the unique polysaccharide structures found on the surface of *A. fumigatus,* and are critical to initiate and maintain a protective immune response to fungal challenge while differentiating potential fungal pathogens from commensal organisms. The role of host receptors in the immune response to fungal glycans is reviewed in the following section, originally published in the Journal of Fungi in August 2017.

Review

1.7 Immune Recognition of Fungal Polysaccharides

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Adapted from: *J. Fungi* 2017 Aug 28;3(3);47

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1.7.1 Abstract

The incidence of fungal infections has dramatically increased in recent years, in large part due to increased use of immunosuppressive medications as well as aggressive medical and surgical interventions that compromise natural skin and mucosal barriers. There are relatively few currently licensed antifungal drugs, and rising resistance to these agents has led to interest in the development of novel preventative and therapeutic strategies targeting these devastating infections. One approach to combat fungal infections is to augment the host immune response towards these organisms. The polysaccharide-rich cell wall is the initial point of contact between fungi and the host immune system, and therefore represents an important target for immunotherapeutic approaches. This Review highlights the advances made in our understanding of the mechanisms by which the immune system recognizes and interacts with exopolysaccharides produced by four of the most common fungal pathogens: *Aspergillus*

fumigatus, Candida albicans, Cryptococcus neoformans, and *Histoplasma capsulatum*. Work to date suggests that inner cell wall polysaccharides that play an important structural role are the most conserved across diverse members of the fungal kingdom, and elicit the strongest innate immune responses. The immune system senses these carbohydrates through receptors such as lectins and complement proteins. In contrast, a greater diversity of polysaccharides is found within the outer cell walls of pathogenic fungi. These glycans play an important role in immune evasion, and can even induce anti-inflammatory host responses. Further study of the complex interactions between the host immune system and the fungal polysaccharides will be necessary to develop more effective therapeutic strategies, as well as to explore the use of immunosuppressive polysaccharides as therapeutic agents to modulate inflammation.

Keywords: polysaccharide; immune receptor; fungal cell wall; *Aspergillus fumigatus*; *Candida albicans*; *Cryptococcus neoformans*; *Histoplasma capsulatum*

1.7.2 Introduction

Over the past several decades, there has been a marked increase in the use of immunosuppressive therapy for the treatment of haematologic malignancies, stem cell and solid organ transplantation, and rheumatologic disorders. In parallel, there has also been an increased use of novel surgical techniques, indwelling central venous catheters, and other prosthetic devices in hospitalized patients. These changes in health care, combined with the HIV epidemic, have resulted in a rapid expansion in the number of patients with acquired defects in innate, acquired, and mucosal immunity. This patient population is at increased risk for the acquisition of a wide range of fungal infections, leading to a resurgence of interest in the development of novel antifungal therapeutics.

One approach to combating fungal infections is to augment host recognition and immune response to these organisms. Fungal cell wall glycans and exopolysaccharides play a critical role in these fungal–host interactions. The cell wall is the first point of physical contact between the pathogen and host cells, and fungal polysaccharides have been identified both as ligands for innate immune receptors, and antigens that can stimulate adaptive immune responses. This review will summarize our current understanding of the immune response to fungal exopolysaccharides, and the molecular mechanisms underlying the recognition of these glycans.

We have focussed our attention on four of the most common medically relevant fungi: *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Histoplasma capsulatum* (**Figure 1**). Studies of immune interactions with the polysaccharides produced by these organisms reveal a common theme. Polysaccharides that are common to multiple fungi, and play a major role in cell wall structure, are associated with the strongest host immune responses through interactions with soluble and cell-associated pattern recognition receptors. However, medically relevant fungi have also developed unique exopolysaccharides that often serve to mask the more conserved glycans from detection by the host, and can even directly downregulate inflammatory responses. Gaps in our current understanding of these host–glycan interactions, and potential areas for future research, will be highlighted.

1.7.3 *Candida albicans*

Candida albicans is a commensal yeast commonly found in the gastrointestinal tract of healthy individuals. However, *C. albicans* is also an opportunistic pathogen that is the most common cause of invasive fungal infection in hospitalized patients [164]. Risk factors for invasive candidiasis include the use of broad-spectrum antibiotics that lead to *C. albicans* overgrowth, surgical and medical interventions that breach normal mucosal barriers to *Candida* invasion, and immunosuppressive illnesses or therapies that impair host immune response [165]. Additionally, *Candida* can form biofilms on biomedical devices, including urinary and vascular catheters [166]. During infection, *C. albicans* can switch morphologies between yeast cells, pseudohyphae and hyphae [167]. This ability to undergo morphogenesis is a critical virulence factor of *C. albicans*, and allows it to exploit a variety of environmental niches within the host [167]. The interactions of the host immune system with *C. albicans* glycans are the best studied among the medically relevant fungi, and have greatly advanced our understanding of innate immune recognition of these organisms.

1.7.3.1 Beta-Glucan

β-glucans are key structural polysaccharides found largely within the *C. albicans* inner cell wall. β-(1,3)-linked glucans are the most abundant of these glucans, with shorter chains of β-(1,6)-glucan that serve to cross-link the inner and outer cell wall [168]. β-(1,3)-glucans are pathogen associated molecular pattern (PAMP) ligands that are recognized by the pattern

recognition receptor (PRR) dectin-1 (also known as CLEC7A) [169]. This interaction is the best studied of all fungal–innate immune interactions, and is common to most pathogenic fungi.

Dectin-1 is a transmembrane C-type lectin that is primarily expressed on the surface of immune cells, such as dendritic cells, alveolar macrophages, and neutrophils. The extracellular portion of dectin-1 consists of a carbohydrate recognition domain (CRD) atop a stalk region, while the intracellular portion contains an immunoreceptor tyrosine-based activation motif (ITAM) involved in signalling [170]. The CRD of dectin-1 recognizes β-(1,3)-glucan fragments that are a minimum of seven glucose residues long [171]. Dectin-1 activation is proportional to β-glucan polymer length, and it has been suggested that dectin-1 activation requires receptor clustering as part of the phagocytic synapse [171,172]. Dectin-1 signalling induces phosphorylation of Syk and IκB, and translocation of NF-κB to the nucleus [173,174], as well as Syk-independent signalling through Raf1 activation [175]. Dectin-1 activation controls a wide range of immune responses, including regulation of leukocyte phagocytosis, recruitment of Toll-like receptor (TLR) 9 to phagolysosomes, phagolysosome maturation, activation of autophagy, production of reactive oxygen species (ROS), activation of the inflammasome, and induction of pro- and anti-inflammatory cytokine secretion ([176,177], and reviewed in [178]).

Dectin-1 signalling in response to β-glucan can be modulated by interaction with other host proteins. The soluble galactose-specific lectin galectin-3 (previously known as Mac-2, εBP, or CBP30/35) physically interacts with dectin-1, likely through binding of glycosylated domains of the receptor to form multivalent oligomers that enhance clustering and activation of dectin-1 [179]. Galectin-3 exists in both a cytoplasmic and secreted form, and it remains to be determined if interaction of this lectin with dectin-1 occurs in the intracellular or extracellular space. Galectin-3 knockdown and overexpression studies in macrophages suggest that galectin-3 interaction with dectin-1 augments TNF production in response to *C. albicans* β-glucan [180]. However, the significance of this specific interaction is unclear, since, as detailed below, galectin-3 also interacts with *C. albicans* mannans [181], and there are conflicting reports as to the role of galectin-3 in host defence *in vivo*. A recent study reported that galectin-3 deficiency in mice increased resistance to *C. albicans* infection, and that intracellular galectin-3 suppressed Syk signalling within neutrophils to decrease ROS production [182]. These results contrast with an earlier report in which galectin-3 deficiency was associated with increased susceptibility of mice to *C. albicans* infection [183]. The molecular mechanisms underlying these conflicting

studies are unknown, but may reflect different roles of intracellular and extracellular galectin-3 in the modulation of immune responses.

Other host receptors have been found to participate in the recognition and response to β-glucan. Complement receptor 3 (CR3, also known as Mac-1 and integrin $\alpha_M\beta_2$) enhances fungal responses through recognition of β-(1,6)-glucan [184]. In mouse bone marrow-derived neutrophils, CR3 expression is upregulated following dectin-1 recognition of β-glucan particles, resulting in phagocytosis of the particles and ROS production [185]. While CR3 is not required for phagocytosis of β-glucan particles by mouse peritoneal macrophages [185], IL-1β release by mouse bone marrow-derived macrophages (BMDMs) and bone marrow derived dendritic cells (BMDCs), in response to purified β-glucan and heat-killed *C. albicans*, is dependent on CR3 [186]. Consistent with these findings, CR3-deficent mice exhibit higher mortality and fungal burden compared to wild-type animals, when challenged with *C. albicans*, highlighting the importance of this receptor in host defence [185].

It is likely that other host receptors participate in the recognition of β-glucan. For example, β-glucan on the surface of *C. albicans* hyphae was reported to induce the production of IL-1 receptor antagonist (IL-1Ra) by peripheral blood mononuclear cells (PBMCs) via a dectin-1 and CR3-independent pathway [187]. While the mechanism of IL-1Ra induction is currently not well understood, inhibition of Akt and PI3K significantly reduced IL-1Ra production [187]. The identification and characterization of novel β-glucan recognition receptors is an area of great interest for future studies.

There is also evidence to suggest that some *C. albicans* strains are recognized by the host by dectin-1/β-glucan independent mechanisms. Support for this hypothesis comes from a screen of 14 *C. albicans* strains from a range of clades, which were analyzed for their ability to stimulate cytokine release by human peripheral blood mononuclear cells (PBMCs) [188]. One strain induced PBMC IL-1β production that was inhibited by blocking of the mannose receptor (see below), but was unaffected by dectin-1 blockade [188]. Interestingly, this strain was hypovirulent in an intravenous mouse infection model [189], and failed to stimulate KC and MIP-2 production by M-1 murine renal epithelial cells, or mediate cellular damage *in vitro* [190]. Taken together, these findings suggest that some strains of *C. albicans* have developed adaptations to evade dectin-1 detection, but these changes are associated with a loss of virulence.

In addition to natural recognition of β-glucan by the innate immune system, efforts have been made to use these glycans to augment adaptive immune responses against fungi. A β-glucan vaccine was generated by conjugating laminarin, composed of a β-(1,3)-glucan backbone with β-(1,6)-glucan branches isolated from the alga *Laminaria digitata*, to the diptheria toxoid CRM197 [191]. Immunization with this antigen increased mouse survival from 10 to 70%, as compared to the adjuvant alone, in a model of systemic candidiasis [191]. Protection was antibody-mediated, as treating naïve mice with either serum from vaccinated mice, or a monoclonal IgG antibody raised against the β-glucan antigen, was effective at limiting fungal burden and improving survival. These antibodies reduced *C. albicans* adherence to human epithelial cells, as well as inhibited fungal growth *in vitro* [149,191]. The β-glucan vaccine provided only modest protection in a model of vaginal candidiasis, likely due to poor penetration of β-glucan-specific IgG antibodies to the vaginal mucosa [192]. However, vaccinated mice exhibited increase resistance to intravenous challenge with *Aspergillus fumigatus* [191] and intravenous challenge with *Cryptococcus neoformans* [193], highlighting the potential of β-glucan immunization to protect against a broad range of fungal pathogens.

1.7.3.2 Mannan

The outer layer of the *C. albicans* cell wall is composed of an array of heavily mannosylated proteins that are glycosylphosphatidylinositol (GPI)-modified and cross-linked to β-(1,6)-glucans [194,195]. Gas chromatography and nuclear magnetic resonance studies have suggested that *N*-linked mannans are large branched structures that consist primarily of an α-(1,6)-mannose backbone with α-(1,2)-oligomannose sidechains that are commonly capped with β-(1,2)-linked mono-, di-, tri, or tetramannans [196]. Genetic studies have suggested that phospholipomannans comprised of β-(1,2)-oligomannans can also be attached to the *N*-linked mannans via β-(1,2)-mannosyltransferases [197]. In contrast, *O*-linked mannans of *C. albicans* are primarily linear α-(1,2)-oligomannans [196]. The relative abundance and composition of the mannans differs between *C. albicans* yeast and hyphae, with reduced phosphodiesterification of the β-(1,2)-oligomannans and decreased branching of the α-(1,6) backbone in hyphae [196].

Multiple studies have demonstrated that the outer mannan layer plays an important role in concealing β-glucans from host immune detection. Treatment of mouse BMDCs with heat-killed yeast producing *N*-linked mannans deficient in β-(1,2)-mannan, resulted in the release of higher levels of pro-inflammatory cytokines, such as IL-6, IL-12p40, IL-23, and TNFα, as compared

with wild-type *C. albicans* [198]. Similarly, live yeast cells deficient in mannan branching induced greater levels of IL-1β, IL-10, and TNFα secretion by human PBMCs, largely due to enhanced β-glucan exposure [199]. Deletion of α-(1,6)-mannosyltransferase in *C. albicans* produced a strain with severely-truncated *N*-linked mannans and increased β-glucan exposure, which resulted in higher IL-6 and TNFα production by mouse peritoneal macrophages [200]. Similarly, a *C. albicans* strain deficient in *O*-linked mannans exhibited increased β-glucan exposure, and was unable to prevent phagolysosome maturation in RAW and J774 macrophage cell lines, as well as mouse peritoneal macrophages, resulting in a reduced ability of *C. albicans* to lyse and escape from these cells [201]. Finally, chemical removal of the mannan layer of the cell wall resulted in enhanced activation of the alternative pathway of the complement cascade, as determined by the ability of human neutrophils to phagocytize yeast cells in the presence of human serum [202].

Although mannans play an important role in immune evasion, a number of host receptors can directly recognize mannans and augment host defences. The C-type lectin, dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN, also known as CD209), was found to bind to *N*-linked mannans [203]. DC-SIGN is comprised of an extracellular stalk consisting of multiple CRDs, and an intracellular domain containing motifs that participate in internalization [204]. The CRD of DC-SIGN shows specificity for mannose-containing oligosaccharides [205]. These multiple CRDs are thought to aid in receptor multimerization and clustering [206]. Consistent with the internalization motifs on the cytosolic domain, DC-SIGN is thought to function largely as an endocytic receptor, and is primarily expressed on professional antigen-presenting cells, such as macrophages and dendritic cells [204]. Phagocytosis of *C. albicans* yeast cells by human monocyte-derived dendritic cells, and release of IL-6, is dependent on DC-SIGN and *N*-linked mannans [203,207].

N-linked mannans are also recognized by a number of other receptors. The mannose receptor (MR, also known as CD206), a transmembrane lectin found predominantly in macrophages [208], can recognize terminal mannose structures [209]. While the MR is primarily expressed on the cell surface, a soluble form can also be released through proteolytic cleavage [209]. TLR4, on both human mononuclear cells and murine macrophages, recognizes *O*-linked mannans on the surface of *C. albicans* yeast cells [208]. Optimal release of TNFα, IL-6, IL-10, and interferon (IFN)γ by these cells required recognition of both *N*- and *O*-linked mannans by MR and TLR4,

respectively [208]. Human mononuclear cells incubated with *C. albicans* deficient in either *N*- or *O*-linked mannans produced lower levels of cytokines, which could be further reduced with blocking antibodies targeting the receptor for the other mannan structure [208]. Finally, dectin-2 (also known as CLEC6A or CLECSF10), a C-type lectin commonly expressed by tissue macrophages, dendritic cells, and PBMCs, has also been implicated in the recognition of *C. albicans* mannans [210]. The CRD of dectin-2 is specific for α-(1,2)-mannan structures, which are significantly masked by the β-linked mannan caps [211]. Mouse peritoneal macrophages exhibited increased IL-6 and KC release *in vitro*, in response to *C. albicans* lacking β-mannans, in a dectin-2-dependent manner [211]. Importantly, dectin-2 lacks a cytoplasmic signalling motif, and therefore, must associate with other receptors to transduce signals. Dectin-2 forms heterodimers with dectin-3 (also known as CLEC4D or CLECSF8), another C-type lectin that recognizes α-mannans, to activate intracellular signalling cascades [212]. This heterodimeric complex is thought to recruit the Fcγ receptor (FcγR) to further activate signalling cascades [212-214]. RAW264.7 macrophages, stably expressing both dectin-2 and dectin-3, produced greater amounts of TNF-α in response to purified α-mannans, than did cells expressing only one of the receptors [212]. Dectin-2-deficient mice are hypersusceptible to intravenous challenge with both *C. albicans* and *Candida glabrata*, exhibiting decreased survival, greater kidney fungal burden, and reduced production of T helper (T_H) 1- and T_H17-derived cytokines by splenocytes, as compared to the wild type control mice [211,215]. Dectin-3-deficient mice were also hypersusceptible to *C. albicans* intravenous infection, with higher mortality and kidney fungal burden as compared with wild type mice [212], illustrating the importance of the dectin-2/dectin-3 heterodimer in recognizing α-mannans and mounting a successful anti-fungal immune response.

Soluble receptors can also interact with *Candida* mannans. The β-(1,2)-mannan sidechains of the *N*-linked mannans on *C. albicans* yeast cells were specifically recognized by galectin-3 [181]. While phagocytosis by J774 macrophages was not found to be dependent on galectin-3, the interaction between galectin-3 and β-(1,2)-mannan was required for optimal TNFα release by both J774 cells and mouse peritoneal macrophages [181]. This signalling occurred via TLR2 activation in macrophages, and may provide a mechanism for the host to discriminate between pathogenic *C. albicans* and the commensal *Saccharomyces cerevisiae*, which lacks these β-(1,2)-mannan sidechains [181]. Intriguingly, it has been reported that binding of human recombinant

galectin-3 to β-(1,2)-mannan can directly kill *C. albicans* yeast cells in the absence of any other immune effectors [216], although the mechanisms underlying this observation remain unknown.

Mannose-binding lectin (MBL, also known as MBL2) is likely another important host receptor for *C. albicans* mannans. MBL is a soluble circulating collectin-class lectin of hepatic origin that forms helical quaternary structures that increase its avidity for carbohydrates [217]. Upon binding to carbohydrates, MBL induces complement activation via complex formation with MBL-associated serine proteases (MASPs) [218]. While the precise carbohydrate ligand remains unclear, human recombinant MBL bound strongly to budding and young yeast cells, as well as hyphae [219]. This binding was temperature-specific, as MBL bound strongly to mature yeast grown at 37 °C, but not to yeast grown at 23 °C [219]. Mannans purified from cultures grown at 23 °C were recognized by MBL, suggesting that failure to detect MBL ligand at this temperature is due to masking by another polymer [219]. As with galectin-3, MBL binds poorly to *S. cerevisiae*, suggesting a role in discriminating between commensal and pathogenic fungi [219]. Human MBL initiates the agglutination of *C. albicans* hyphae [220], and can recruit additional host factors to aid in immune recognition of these fungal structures. Binding of MBL to the cell wall resulted in enhanced deposition of complement proteins C4 and C3b from normal human serum, and augmented phagocytosis of yeast by human blood polymorphonuclear cells (PMN) [221], but not human monocyte-derived DCs. These actions are likely due to heterocomplexes formed between MBL and other serum proteins, as complexes of MBL and either pentraxin-3 or serum amyloid P, result in C4 and C3b-mediated human PMN phagocytosis of *C. albicans* yeast [222]. As with galectin-3, MBL binding to *C. albicans* also directly inhibits growth of the fungus, suggesting that they may bind similar glycans on the fungal cell wall [220]. The MBL-pentraxin-3 heterocomplex activates complement-mediated killing through deposition of complement protein C1q [222]. Taken together, these findings suggest that MBL mediates a complex anti-*C. albicans* response through both complement-mediated killing and opsonisation. Consistent with these observations, prophylactic treatment of mice with MBL improves survival following intravenous *C. albicans* challenge [219]. Furthermore, genome-wide association studies have identified polymorphisms in the human *MBL* gene, with heightened susceptibility to vulvovaginal candidiasis and increased rates of recurrence of this condition [223], suggesting a role for *MBL* in mucosal immunity against *C. albicans*.

Several studies have evaluated the virulence of mannan-deficient strains of *C. albican*s [200,208,224]. Strains deficient in *O*-linked mannan had slower growth rates and greater antifungal susceptibility, suggesting that *O*-linked mannosylation may also be required for normal mannoprotein function. Consistent with these observations, *O*-linked mannan-deficient strains exhibited attenuated virulence in a mouse model of systemic infection [224]. Mice infected with *N*-linked mannan-deficient *C. albicans* exhibited higher survival and lower kidney fungal burden, as well as reduced levels of kidney IL-6 and TNFα, as compared to those infected with wild-type *C. albicans* [208]. Similarly, despite no observed defect in growth rate, an α-(1,6)-mannosyltransferase-deficient mutant that produces a severely-truncated *N*-linked mannan backbone, also exhibited attenuated virulence in a mouse model of systemic candidiasis, in association with increased T_H1 and T_H17 responses and increased kidney levels of IFN-γ, IL-6, and IL-17 [200]. Thus, despite the presence of a range of host receptors that can mediate recognition of *Candida* mannans, these animal studies suggest that the virulence promoting characteristics of these polysaccharides predominate during invasive infection.

1.7.3.3 Chitin

Chitin is a ubiquitous exopolysaccharide composed of β-(1,4)-*N*-acetylglucosamine that is produced by a wide array of arthropods, parasites, and fungi. Chitin is found within the innermost layer of the *C. albicans* cell wall [195], where it plays an important role in maintaining cell rigidity and resistance to physical stress. While it is one of the least well-studied cell wall components of *C. albicans*, several recent studies using purified *C. albicans* chitin have begun to shed light onto the immunomodulatory effects of this glycan. Pure chitin is a strong activator of the complement cascade, and can induce cleavage of complement protein C3 via the alternative complement pathway to produce C3a, a potent anaphylatoxin [225]. Human PBMCs pre-treated with chitin produced lower levels of the pro-inflammatory cytokines TNFα, IL-6, and IL-1β in response to *C. albicans* yeast exposure [226]. Treatment of *C. albicans* with sub-therapeutic concentrations of caspofungin, which increased the surface exposure of chitin, also resulted in a reduced pro-inflammatory cytokine response [226]. While the authors reported no difference in β-glucan content in caspofungin-treated and untreated *C. albicans* [226], it is difficult to exclude the possibility that inhibitory effects of this agent on β-glucan synthesis may also have contributed to these observations [227]. Treatment of mice with intranasal chitin induced release of IL-25 and IL-33 by airway epithelial cells, resulting in type-2 innate lymphoid cell production

of IL-5, and pulmonary recruitment of eosinophils and M2 macrophages [228]. Intraperitoneal administration of chitin induced eosinophilia and suppressed the TNFα response to LPS challenge in mice [229]. Similarly, purified *C. albicans* chitin directly enhanced the release of the anti-inflammatory cytokine IL-10 by human PBMCs [229]. IL-10 production in these studies was dependent on the MR, and involved TLR9 and NOD2 activation [229]. Chitin-mediated anti-inflammatory responses have been postulated as a mechanism of resolving inflammation when non-viable chitin "ghosts" remain following successful killing of the *C. albicans* yeast cells [229].

1.7.3.4 *Candida albicans* Biofilms

During infection, *C. albicans* commonly grows in biofilms formed on prosthetic devices or mucosal surfaces [230,231]. While the majority of studies examining the host response to *C. albicans* have been performed using planktonic cells, there have been recent efforts to examine the immune response to biofilm-grown organisms (reviewed in [232]). Cytokine production by PBMCs has been reported to differ between biofilm and planktonic cells with high levels of IL-1β, IL-10, and MCP-1, and lower IL-6 and MIP1β production, in response to biofilm-grown organisms [233]. Impaired phagocytosis and killing of biofilm-associated organisms by PBMCs [233], monocytes [234], and neutrophils [234-236] have all been reported. Impaired activation of neutrophils has been linked to β-glucans [236], as well as the GPI-anchored cell wall protein Hyr1 [237]. *Candida* biofilms formed on mucosal surfaces are characterized by the infiltration of abundant neutrophils [238-242], a process that has been linked to the production of chemotactic factors, such as alarmins, by epithelial cells in response to fungal colonization [239,243-245].

1.7.3.5 Non-*albicans Candida* Species

While *C. albicans* are the most common species isolated from *Candida* infections, rates of infections with other *Candida* species are increasing [246]. Among these species, *C. glabrata* and *C. parapsilosis* are the most frequently implicated in nosocomial infections [246]. Differences in host–pathogen interactions between these strains have been reported in a number of studies.

C. glabrata is more closely genetically related to the non-pathogenic yeast *Saccharomyces cerevisiae*, and produces surface mannans more closely related to this organism [247,248]. In contrast to *C. albicans*, disruption of mannosyltransferases that mediate synthesis of *N*-linked

mannans enhanced the virulence of these strains [249], suggesting that recognition of these glycans by host PRRs is important in innate host defence. J774 macrophages phagocytosed *C. glabrata* more avidly than *C. albicans* [250], and *C. glabrata* survival within the phagosomes of macrophages has been linked to decreased chitin exposure [251]. Whether this chitin masking is a consequence of cell wall mannans remains to be determined.

Although less is known about the cell wall composition of *C. parapsilosis*, lectin staining suggests differences in chitin exposure as compared with *C. albicans* [252]. Deletion of the α1,6-mannosyltransferase, responsible for initiating *N*-linked mannan production, resulted in a strain that induced higher levels of pro-inflammatory cytokine production by PBMCs [253]. Increased cytokine production was associated with increased exposure of β-glucans, and was reduced by laminarin-mediated blocking of dectin-1 or with antibodies to TLR4 [253]. As with *C. albicans*, loss of *N*-linked mannans was associated with attenuated virulence [253]. In contrast, β-elimination trimming of *O*-linked mannans increased production of IL-10 by PBMCs stimulated with live wild type cells, and reduced pro-inflammatory cytokine induction by *N*-linked mannan-deficient organisms [253]. Collectively, these observations suggest a pro-inflammatory role for *O*-linked mannans of *C. parapsilosis*, although further studies are required to validate these observations *in vivo*.

1.7.4 *Aspergillus fumigatus*

Aspergillus fumigatus is an ubiquitous mould that produces abundant airborne conidia. Every day, humans inhale up to one hundred of these conidia, which are largely eliminated by the airway cell mucociliary action or killed by pulmonary macrophages, before they undergo germination [70]. Dormant conidia are coated in a layer of hydrophobic rodlet proteins that are largely immunoinert and conceal cell wall polysaccharides from immune detection [21]. If conidia evade these initial immune defences, they begin to swell and shed this layer of hydrophobins, exposing deeper cell components. Swollen conidia then undergo germination to produce filamentous hyphae, which can invade host tissues and blood vessels [10]. The cell wall composition of each of these fungal growth stages differs [21], and as a result, the host exhibits stage-specific immune responses to *A. fumigatus*. Despite current antifungal therapies, the mortality of invasive aspergillosis remains between 50% and 90%, highlighting the need for new treatment options for this infection [97]. Strategies targeting cell wall polysaccharide synthesis, and the immune response to these glycans, are two promising therapeutic approaches.

1.7.4.1 Beta-Glucan

Mutants devoid of β-glucan are viable, but produce leaky, fragile cell walls, and are markedly impaired in growth and development [23]. In resting conidia, β-glucan is concealed by a layer of hydrophobic proteins, termed rodlets [21]. During germination, conidia shed this rodlet layer to display high levels of surface exposed β-glucan [254], leading to an increased production of dectin-1-dependent CXCL1, CXCL2, and TNFα by BMDMs [51]. Alveolar macrophages isolated from dectin-1-deficient mice were impaired in their ability to produce proinflammatory cytokines, such as IL-1α, IL-1β, TNFα, MIP-1α, MIP-1β, and KC in response to live *A. fumigatus* conidia after 24 h of growth [130], a finding that has been validated *in vivo* in an *Aspergillus* keratitis model [173]. Similarly, human monocyte-derived dendritic cells incubated with young hyphae exhibited significantly reduced expression of IL-12 and TNFα when treated with either dectin-1 blocking antibodies or transfected with dectin-1 silencing RNA [255]. Additionally, thioglycolate-elicited neutrophils from dectin-1-deficient mice produced lower levels of ROS when challenged with swollen *A. fumigatus* conidia, and exhibited impaired killing of the fungus *in vitro* [130]. As hyphae mature, β-glucan is again masked by the production of the exopolysaccharide galactosaminogalactan (discussed further below) [60].

Dectin-1 is required for normal production of IL-23 by dendritic cells in response to *Aspergillus* [256]. Production of IL-23 plays an important role in defence against fungal infection through stimulating neutrophil IL-17 production. Mice deficient in dectin-1 produced lower levels of IL-17A, exhibited reduced neutrophil recruitment to the site of infection, and had increased mortality following pulmonary challenge with *A. fumigatus* [130]. Dectin-1-dependent IL-23 secretion was also required for optimal IL-22 responses in a mouse model of pulmonary aspergillosis [130]. IL-22 induction was necessary for optimal IL-1α, IL-12 (both p40 and p70), CCL3, CCL4, and TNFα release, leading to control of fungal infection [257]. These findings have been corroborated with a model of fungal keratitis, where dectin-1 was required for optimum IL-1β and KC production, and control of fungal growth [173]. These dectin-1-mediated responses are most important against germinating conidia and young hyphae, as β-glucans are cloaked by the exopolysaccharide galactosaminogalactan produced by growing hyphae (discussed further below) [60,254].

Dectin-1 also plays a role in facilitating the adaptive immune response to *A. fumigatus*. Dectin-1-deficiency resulted in alterations in *A. fumigatus*-specific T cell maturation following

adoptive transfer and pulmonary challenge with *A. fumigatus* [258]. Analysis of bronchoalveolar lavage (BAL) fluid from these mice revealed a greater abundance of IL-17-producing T cells in wild-type mice than was found in dectin-1 deficient animals in which IFN-γ-positive T cells were most abundant [258]. In a mouse model of *A. fumigatus*-induced allergy, production of IL-17, IL-4 and IL-13 by T cells was dectin-1-dependent, leading to increased airway resistance and allergic pathology [259]. These dectin-1-dependent effects were mediated by IL-22 production, illustrating that while this cytokine is beneficial in the context of acute *A. fumigatus* infection, it can be detrimental in allergic disease.

As with *C. albicans*, other host molecules are thought to participate in the detection and response to β-glucans during *A. fumigatus* infection. M-ficolin, also known as ficolin-1, is a member of the ficolin family of opsonins that mediate recognition of pathogens *Escherichia coli* and *Staphylococcus aureus*, and activation of the complement pathway [260]. Recombinant human M-ficolin binds to conidia and young hyphae of *A. fumigatus*, to β-(1,3)-glucan-containing *A. fumigatus* alkali-insoluble hyphal cell wall fraction (AIF) [261], and to purified β-(1,3)-glucan [262]. M-ficolin binding to AIF and purified β-(1,3)-glucan activates the lectin-dependent complement pathway *in vitro* [262], and enhances IL-8 secretion by A594 airway epithelial cells, when incubated *in vitro* with AIF [262]. A synergistic interaction between M-ficolin and the soluble pattern recognition receptor pentraxin-3 has also been observed *in vitro*, resulting in greater M-ficolin binding to the β-glucan of *A. fumigatus*, and C4 deposition and activation of the complement cascade [263]. While M-ficolin has been detected in granulocytes and monocytes at the periphery of pulmonary aspergillomas in humans [262], the role of M-ficolin during experimental *A. fumigatus* infection has not been studied.

1.7.4.2 Galactomannan

A. fumigatus galactomannan (GM), is composed of an α-(1,2)(1,6)-mannopyranose backbone with short branches of β-(1,5)-oligogalactofuranose connected by β-(1,3) and β-(1,6) linkages [264]. GM is found in the hyphal cell wall, conjugated to both proteins [265] and glucans [26], as well as in a soluble form that is shed into the environment. Additionally, a second species of GM, produced by the action of a unique set of mannosyltransferases, is present exclusively in the conidia, where it appears to be involved in conidial separation during sporulation [26]. A mutant lacking these mannosyltransferases produced conidia with altered cell wall organization and reduced viability [26]. The solubility of GM, as well as its relative

specificity for *Aspergillus* species, makes it a useful diagnostic marker of *Aspergillus* infection [266,267].

The mannan and galactofuranose components of GM are differentially recognized by the host. The mannan core closely resembles cell wall mannans of other fungi, and as a consequence, interacts with many of the host mannose receptors described above. The best described receptor for the mannan core is DC-SIGN [268]. Antibodies to DC-SIGN dramatically reduce binding and phagocytosis of conidia by human monocyte-derived dendritic cells [269]. While the ligand interacting with DC-SIGN was not defined in this study, purified *A. fumigatus* GM was found to block binding of conidia to dendritic cells [269,270]. Surprisingly, DC-SIGN played no role in the cytokine response of human monocyte-derived dendritic cells to *A. fumigatus* young hyphae, as DC-SIGN knockdown experiments revealed no change in *TNFA* or *IL12* gene expression, as compared with vector controls [255]. There are several possible interpretations for these results. It is possible that *Aspergillus* mannan is not a potent inducer of cytokine responses, or that other mannan receptors can compensate for the loss of DC-SIGN. An alternate, intriguing hypothesis is that DC-SIGN is more specific for the recognition of the unique species of soluble GM found in conidia, which were not tested in this study [26].

Dectin-2 also plays an important role in the detection of the α-mannan backbone of GM. Binding of swollen conidia and hyphae by THP-1 macrophages is dectin-2-dependent, leading to Syk-dependent signalling and NF-κB-specific activation [271]. These NF-κB-dependent responses include release of IL-1β, IL-10, IL-23, and TNFα, as well as the generation of ROS [271]. These responses were not observed in response to resting conidia, likely due to the rodlet layer of the conidia masking cell wall GM [51]. Consistent with this hypothesis, rodlet-deficient mutants induced higher levels of CXCL2 and TNFα production by BMDMs in a dectin-2-dependent manner [51]. Recognition of *A. fumigatus* hyphae by human plasmacytoid dendritic cells was also found to be dectin-2 dependent, leading to release of TNFα and IFN-α [272], and production of extracellular traps by these cells [272]. In mouse bone marrow neutrophils, dectin-2 surface expression was induced in response to IL-6 and IL-23, where it augmented IL-17 release, leading to increased killing of *A. fumigatus* hyphae *in vitro* [136]. Finally, human data support a role for dectin-2 in the pathogenesis of invasive aspergillosis, as increased expression of dectin-2, largely restricted to macrophages, was observed during pulmonary infection [273].

Although MBL binding to GM has not been specifically demonstrated, purified MBL binds to the surface of *A. fumigatus* resting conidia [274], an interaction that could be inhibited with mannose, N-acetylglucosamine, and EDTA [275]. Human corneal epithelial cells up-regulate and secrete MBL in response to *A. fumigatus* antigens [276], and MBL enhances phagocytosis of conidia, and killing of *A. fumigatus* hyphae by human PMNs in the presence of serum [277]. As with *C. albicans*, interactions between MBL and *A. fumigatus* activate the complement cascade, however, there are conflicting reports in the literature as to whether this occurs via C4 deposition [277], or the C2 bypass mechanism [274].

Mouse models have suggested site and condition-specific roles for MBL in the pathogenesis of *Aspergillus* disease. In a model of invasive pulmonary aspergillosis, a single dose of 0.05 mg/kg of recombinant human MBL increased mouse survival from 0 to 80% [277]. MBL-mediated protection was associated with increased splenocyte production of TNFα and IL-1β, and decreased IL-10 production [277]. In contrast, during intravenous *A. fumigatus* infection, MBL-deficient mice were more resistant to fungal challenge [278]. In a model of *A. fumigatus*-induced asthma, MBL-deficient mice exhibited significantly lower production of type-2 cytokines and reduced airway hyperresponsiveness at 4 days post challenge, suggesting that MBL contributes to the allergic response towards *Aspergillus* [279]. However, by 28 days post challenge, minimal differences were observed between wild type and MBL-deficient mice [279], suggesting that MBL is not involved in the airway remodeling seen in chronic fungal asthma. In humans, polymorphisms resulting in reduced MBL expression have been associated with chronic necrotizing pulmonary aspergillosis [280]. Taken together, these findings suggest that MBL-mediated recognition of *A. fumigatus* mannans is likely important during early pulmonary host–fungal interactions.

While the mannan core of GM is not recognized by sera from aspergillosis patients [264], the oligogalactofuranose side chains of GM are antigenic in experimental animals [281]. An anti-galactofuranose monoclonal antibody forms the basis for the non-culture based *Aspergillus* antigen EIA, which has revolutionized the early diagnosis of invasive aspergillosis in immunocompromised patients [282,283]. No host receptors specific for *A. fumigatus* oligogalactofuranose have been described to date.

1.7.4.3 Alpha-Glucan

The α-(1,3)-glucan of *A. fumigatus* is found within the outer cell wall during growth *in vitro*, where it is involved in cell wall stability and agglutination of germinating conidia and hyphae [3,6,284]. However, the role of α-glucan in mediating aggregation may vary by morphology and environment, as electron microscopy studies of hyphae during pulmonary infection localized α-glucan largely within the inner cell wall of hyphae [285].

No host receptor for α-glucan has yet been identified, however, this polysaccharide is thought to play both direct and indirect roles in the immune response against *A. fumigatus*. Purified α-glucan inhibits both TLR2 and TLR4-mediated IL-6 production by PBMCs, although the molecular mechanisms underlying this observation remain unclear [286]. *A. fumigatus* mutants deficient in α-glucan produce conidia in which the normally-inert rodlet layer is covered by an amorphous layer of glycoproteins [24]. During germination, these conidia display increased amounts of surface exposed β-glucan and chitin [24], are more readily phagocytosed and killed by mouse alveolar macrophages, and induce higher levels of TNFα secretion by these cells *in vitro* [24]. Mutants lacking α-glucan are hypovirulent in mouse models of invasive aspergillosis, where conidia fail to germinate into hyphae [24], likely as a consequence of the dramatic alterations in cell wall structure. Collectively, these findings suggest that α-glucan plays an important role in masking cell wall PAMPs from immune recognition during early germination.

Although natural antibodies to α-glucan have not been described, a synthetic α-(1,3)-glucan pentasaccharide has been used successfully to generate anti-α-glucan antibodies [287]. These antibodies recognized native α-glucan on the surface of germinating *A. fumigatus* conidia, however, their potential as diagnostic tools and the ability of vaccination with this pentasaccharide or administration of anti-α-glucan antibodies to protect against *A. fumigatus* infection have yet to be evaluated.

1.7.4.4 Chitin

Chitin is located within the inner cell wall of *A. fumigatus* and plays an important role in structural integrity of the fungal cell. No host cell receptor for *A. fumigatus* chitin has been identified to date, however, the interaction of this glycan with a number of soluble factors has been implicated in the modulation of inflammation.

M-ficolin interacts with chitin on the surface of *A. fumigatus* young hyphae, and results in cleavage of the complement protein C4 by the protease MASP-2 [262]. Incubating A549 airway

epithelial cells with M-ficolin and *A. fumigatus* extract resulted in elevated IL-8 production by these cells [262], suggesting that recognition of chitin by M-ficolin may alter inflammatory responses. Other members of the ficolin family H-ficolin (also known as ficolin-3), L-ficolin (also known as ficolin-2), and its murine ortholog A-ficolin have also been reported to recognize *A. fumigatus* conidia [288-290]. While the specific fungal ligands bound by these ficolins have not been defined, *N*-acetylglucosamine inhibits the binding of these soluble factors to *A. fumigatus*, suggesting that they may recognize chitin [288-290]. As with M-ficolin, treatment with H- and L-ficolin increased *A. fumigatus* conidia binding to A549 airway epithelial cells, and enhanced the release of IL-8 [290,291]. Intriguingly, both A- and L-ficolin treatment decreased the amount of IL-1β, IL-6, IL-8, and TNFα released by human monocyte-derived macrophages and neutrophils in response to conidia, despite increased levels of fungal uptake and killing [291]. H-ficolin enhanced activation of the lectin-dependent complement cascade *in vitro*, leading to increased deposition of C3 onto the conidial surface [290]. Studies using transgenic mice deficient in specific ficolins would greatly improve our understanding of the role of these proteins in the pathogenesis of *A. fumigatus* infection.

Purified *A. fumigatus* chitin has been reported to induce anti-inflammatory effects via induction of IL-1Ra production by human PBMCs [292]. IL-1Ra production was mediated by anti-chitin IgG antibodies found in normal human serum interacting with FcγRII, leading to phagocytosis of chitin particles [292]. Interestingly, in the presence of TLR-2, -4, or NOD2 ligands, this response could be re-programmed to augment release of pro-inflammatory IL-1β [292]. Thus, the immune consequences of host-chitin interactions are likely context specific, and may vary during different stages of infection.

In animal models of fungal allergy, chitin exacerbates detrimental type-2 responses. Repeated exposure of mice to *A. fumigatus* conidia, or commercial crab shell chitin alone, was unable to induce a significant adaptive T_H2 cell response, however, a combination of the two resulted in increased type-2 cytokines, such as IL-4, IL-5, and IL-13, eosinophilia, and high IgE antibody titers [225]. This phenomenon was initiated by C3 protein cleavage to generate C3a via the alternative pathway of the complement cascade, leading to a suppression of regulatory dendritic and T cells, and induction of allergy-promoting T_H2 cells [225]. Similar findings were reported using purified crab shell chitin as an adjuvant when administering *A. fumigatus* culture filtrate intraperitoneally to mice [293]. Although priming with chitin prior to challenge reduced

the release of IL-4, -5, and -13 in response to culture filtrate challenge, chitin priming still enhanced both eosinophil recruitment and the secretion of IgE antibodies [293]. Similarly, mice receiving repeated intranasal inoculation of conidia of an *A. fumigatus* strain with increased levels of exposed chitin resulted in enhanced eosinophil recruitment to the lungs, as compared to those exposed to the wild type strain Af293 [294]. The resulting T-helper cells were skewed to a type-2 phenotype, producing less IFN-γ, and more IL-4 [294]. This heightened type-2 response was detrimental to the host, as eosinophil-deficient, sensitized mice had significantly greater survival in a neutropenic model of *A. fumigatus* infection, as compared with wild type sensitized mice [294]. Taken together, these studies suggest that chitin elicits a predominantly type-2 immune response, although the host receptors involved in mediating this response remain undefined.

1.7.4.5 Galactosaminogalactan

Galactosaminogalactan (GAG) is a linear heteropolysaccharide composed of α-(1,4)-linked galactose and *N*-acetylgalactosamine (GalNAc) [28,295] that is found in the outer cell wall and extracellular matrix of hyphae [3]. Partial *N*-deacetylation of GalNAc residues renders the polymer cationic, and allows GAG to mediate adherence to the hyphal cell wall, as well as other anionic surfaces, such as human cells, plastic, and glass [60,296].

GAG has been described to play a number of passive and active roles in counteracting host immune responses. GAG conceals more immunoreactive cell wall components, such as β-glucan from host detection [60]. The cationic nature of GAG also protects the hyphae from neutrophil-mediated killing by repelling the cationic peptides found in neutrophil extracellular traps [30]. GAG also plays an active role in altering immune responses to *A. fumigatus*. A purified fraction of GAG was found to induce apoptosis of human neutrophils in whole blood samples [28], a process mediated by natural killer (NK) cells [109]. Soluble GAG induced neutrophil ROS production through an unknown mechanism, which in turn increased expression of MHC class I chain-related molecule A (MIC-A) on the surface of neutrophils [109]. MIC-A binding to NKG2D on the surface of the NK cells was then linked to Fas-dependent apoptosis via the caspase-8 pathway [109]. Purified GAG can also stimulate IL-1Ra secretion by human PBMCs, resulting in a suppression of T_H1 and T_H17 responses [61]. Finally, GAG has been reported to bind and activate human platelets, resulting in degranulation and exposure of CD62P on their

surface [297]. The mechanism of these GAG-dependent direct effects on immune cells and the host receptors involved in GAG recognition are largely unknown.

As with other exopolysaccharides, GAG is antigenic in humans. Anti-GAG antibodies are present in up to 40% of human sera samples, even in the absence of prior history of *Aspergillus* disease [28]. Importantly, however, many of these antibodies also reacted with glycoproteins of *Campylobacter jejuni*, suggesting that the antibodies may have developed in response to other microbial glycans with structural similarity to GAG. This hypothesis, that GAG shares similarities with bacterial exopolysaccharides, is supported by a recent study demonstrating cross-species activity of bacterial glycoside hydrolases from *Pseudomonas aeruginosa* against *A. fumigatus* GAG [298].

1.7.4.6 Non-*fumigatus Aspergillus* Species

While *A. fumigatus* represents roughly 80% of all *Aspergillus*-related infections, it is not the most abundant species isolated from environmental sampling, suggesting that it expresses unique virulence factors to enable it to cause human infection. *Aspergillus nidulans*, while commonly isolated from the environment, is rarely associated with infections, except in patients with NADPH oxidase deficiency (chronic granulomatous disease, CGD). Although the reasons underlying this observation are not fully understood, it has been suggested that differences in cell wall GAG production may contribute to the pathogenicity of *A. nidulans* in patients with CGD. *A. nidulans* produces low levels of cell wall GAG due to reduced expression of the glucose 4-epimerase UgeB that synthesizes *N*-acetylgalactosamine, and as a result, is more sensitive to killing by neutrophil extracellular traps (NETs) [30]. Patients with CGD are unable to form NETs, and are thus lacking a key element of host defence against this pathogen. Low levels of cell wall associated GAG in *A. nidulans* have also been linked to increased production of pro-inflammatory cytokines by CGD PBMCs [299].

Aspergillus terreus is another uncommon cause of invasive aspergillosis [300]. Conidia of *A. terreus* display higher levels of β-glucan and galactomannan then do those of *A. fumigatus* [301]. Conidia of *A. terreus* were more rapidly phagocytosed by murine alveolar macrophages than those of *A. fumigatus*, and this was dependent on dectin-1 and mannose receptors [301]. Interestingly, unlike *A. fumigatus*, conidia of *A. terreus* failed to germinate within the phagolysosome, and persisted in a dormant but viable state, without inducing macrophage injury [301]. Another unique feature of *A. terreus* is its capacity to produce accessory conidia, in

addition to those formed by phialides, both *in vitro* as well as *in vivo*. These accessory conidia are physically distinct, and exhibit higher levels of exposed β-glucan, resulting in increased dectin-1 dependent production of pro-inflammatory cytokines by murine alveolar macrophages, *in vitro* and *in vivo* [302].

1.7.5 *Cryptococcus neoformans*

Infection with the yeast *Cryptococcus neoformans* is acquired by inhalation of dessicated yeast cells or basidiospores from fungi that are ubiquitous in the environment [303]. This exposure usually results in limited asymptomatic pulmonary infection; however, immunocompromised patients are at risk of developing pneumonia, disseminated disease, and meningitis. During infection, *C. neoformans* produces a large, mucoid capsule that surrounds and protects the yeast cells and is shed in large amounts during growth. The capsule is composed of three major components: glucuronoxylomannan, galactoxylomannan, and mannoproteins [304] that play key roles in the pathogenesis of cryptococcosis, by interfering with host recognition of β-glucans and mannoproteins within the cell wall [305], and cell phagocytosis, as well as by facilitating intracellular survival, replication, and extrusion through complex immunosuppressive and immunomodulatory mechanisms [306-308].

1.7.5.1 Glucuronoxylomannan

Glucuronoxylomannan (GXM) is the outermost and most abundant component of the capsule, forming > 90% of its mass. GXM is composed of a poly-α-(1,3)-mannose backbone that can be 6-*O* acetylated, and substituted with β-(1,2)-linked glucuronic acid sidechains, and β-(1,2)- or β-(1,4)-linked xylose sidechains, depending on the serotype [303]. While the full repertoire of host receptors for this glycan remain poorly defined, several studies have suggested that GXM plays an important role in host–fungal interactions (reviewed in [309]). GXM is recognized by CD14, CD18, TLR2, and TLR4 *in vitro*; however, none of these pattern recognition receptors were absolutely required for serum clearance or hepatosplenic polysaccharide accumulation *in vivo* [310]. Knockout mouse models have shown a modest role for TLR2 or CD14, but not TLR4, on survival after cryptococcal infection [311]. Notably, deletion of the intracellular protein MyD88 had a much more significant effect on survival, fungal burden, and GXM levels in the lungs and sera after intranasal infection, suggesting that additional innate immune receptors that signal via this adaptor mediate the host response to

GXM [311]. GXM can also directly interact with FcγRIIB, which has been implicated in *C. neoformans* uptake by phagocytic cells [312,313]; however, this interaction produces inhibitory signals that contribute to immune unresponsiveness [314].

Invasion and lysis of A549 airway epithelial cells by *C. neoformans* can be inhibited by anti-GXM antibodies [315]. Studies of leukocyte interactions with purified GXM *in vitro* have also reported a variety of host responses to this glycan. These include the production of TNFα, IL-6, IL-10, and RANTES by murine peritoneal macrophages [316], and induction of TGFβ, iNOS, and nitric oxide, leading to autophagy and ultimately apoptosis in RAW 264.7 macrophages [317]. Rat peritoneal macrophages also produce iNOS and undergo nitric oxide-dependent apoptosis in response to GXM [318]. Macrophage apoptosis was also dependent on CD18, FcγRII, and protein kinase C activation, but was associated with down-regulation of caspase-3 activity, suggesting that GXM-mediated apoptosis was mediated through a caspase-independent pathway [318]. GXM was reported to bind to CD18 on human neutrophils, raising the possibility that GXM may activate the β2-integrin apoptosis pathway, although apoptosis was not directly studied in this report [318].

In vitro studies also suggest that GXM can influence the adaptive immune response through inhibition of CD4 T cell activation [319]. Internalization of GXM by mouse BMDCs reduced their ability to induce antigen-specific T cell proliferation and IL-2 release [320]. Although this phenomenon was independent of cell death, a second study reported that GXM internalization by human monocyte-derived macrophages led to the Fas-mediated apoptosis of T cells [321]. GXM treatment also directly reduced T cell proliferation in response to PMA/ionomycin and anti-CD3 antibodies [322].

In addition to these direct effects on the immune response, GXM may also conceal ligands deeper in the capsule and cell wall from immune detection [305]. *C. neoformans* mutants lacking GXM induced higher levels of pro-inflammatory cytokines IL-12p40 and TNFα production by dendritic cells, than did wild type fungi [320].

C. neoformans strains deficient in GXM xylosylation are severely attenuated in virulence in a murine intravenous infection model [323], while strains deficient in GXM *O*-acetylation exhibit heightened virulence [324]; however, neither of these studies reported on the immune response mounted against these strains. Studies reporting the effects of purified GXM on immune responses *in vivo* have validated some of the *in vitro* observations discussed above.

Intraperitoneal injection of purified GXM resulted in uptake by peritoneal macrophages and nitric oxide production in rats [318], as well as increased Fas/FasL-dependent peritoneal macrophage apoptosis in mice [317]. Intrapulmonary administration of GXM led to upregulation of pulmonary iNOS in rats [318], and induced IL-10 and TNFα secretion in mice [316]. Co-administration of GXM with chitin elicited higher levels of IL-10, IL-17, and TNFα release, than either glycan alone [325]. Thus, it is possible that this synergistic response to multiple glycans enhances the specificity of the immune response to fungal pathogens, while avoiding deleterious responses to environmental glycans.

1.7.5.2 Galactoxylomannan

A second important polysaccharide found within the *C. neoformans* capsule is galactoxylomannan (GalXM), composed of an α-(1,6)-galactose backbone with trisaccharide branches of mannose-α-(1,3)-mannose-α-(1,4)-galactose-β-(1,3) [303]. These branches may be further xylosylated through β-(1,2) and β-(1,3) linkages [303]. GalXM is located deep within the capsule, adjacent to the cell wall, and forms 5–10% of its mass [304].

As with GXM, the effects of purified GalXM on leukocyte responses have also been studied. GalXM binds CD18 on human neutrophils [326], and induces TGFβ, TNFα and iNOS production by RAW macrophages, leading to autophagy and apoptosis mediated by Fas/FasL interactions [317]. GalXM has a more marked effect on the cells of the adaptive immune system, as compared to GXM. Purified GalXM induces the release of IFN-γ and IL-10 [327] by human PBMCs, suppresses purified human T lymphocyte proliferation, and directly induces Fas/FasL-dependent T cell apoptosis. GalXM-induced apoptosis of human T cells is dependent on interactions with CD7 and CD43 (also known as leukosialin or sialophorin) that activate both extrinsic and intrinsic apoptosis pathways through caspase-8 cleavage [328,329]. As with GXM, peritoneal injection of GalXM also increases Fas/FasL-dependent apoptosis of resident macrophages [317]. Administration of GalXM, in this model, was associated with reduced inflammatory cytokine expression by splenocytes, and caspase- and Fas-dependent apoptosis of antigen-specific B cells, resulting in a state of immune paralysis [330]. While virulence studies using GalXM-deficient *C. neoformans* have revealed these strains to be hypovirulent *in vivo* [331], detailed studies of the GalXM-specific immune response have yet to be performed.

1.7.5.3 Mannoproteins

Mannoproteins comprise a small fraction (<1%) of the *C. neoformans* capsule. Mannosylation of these proteins is extensive, such that mannose residues comprise 80–90% of the weight of these molecules [332], and is required for mannoprotein-mediated T cell stimulation [333]. Like GalXM, mannoproteins are situated deep within the capsule, which conceals them from immune recognition [304,334].

Unlike GalXM and GXM that have complex immunosuppressive and immunomodulatory effects, mannoproteins appear to induce a predominately pro-inflammatory immune response [335]. In studies using purified capsule components, mannoproteins were found to be the strongest inducers of human PBMC proliferation [336] and IL-6 production [337]. Proliferation of PBMCs in response to mannoproteins could be inhibited by antibodies against ICAM-1, LFA-1, and MHC class II, suggesting that this response is dependent on antigen recognition [336]. Human dendritic cells have been found to internalize and process mannoproteins via the mannose receptor, leading to the maturation and activation of these cells [338]. Mannoprotein-activated dendritic cells produced IL-12 and TNFα, and were efficient at stimulating CD8 and CD4 T cell proliferation, and T cell differentiation towards a T_H1 phenotype [339]. Mice lacking the mannose receptor died significantly faster than wild type mice, with higher lung fungal burdens at 4 weeks after infection, and they displayed impaired $CD4^+$ T cell responses to mannoprotein [340]. Mannoprotein-dependent T_H1 responses also provided cross-fungal immunity against lethal *C. albicans* challenge [341]. Thus, although mannoproteins are highly effective at inducing protective antifungal immune responses, their abundance and location deep within the capsule likely limits their role as activators of protective antifungal immune responses.

1.7.5.4 *Cryptococcus gattii*

While *C. neoformans* typically only causes disease in immunosuppressed individuals, such as AIDS patients, a recently emerged strain of *Cryptococcus gattii* isolated from the Pacific Northwest has been reported to infect and cause disease in immunocompetent hosts [342]. Studies of the differences in virulence, that could contribute to this difference in host requirement, found that this strain of *C. gattii* induced a much lower inflammatory response, as compared with *C. neoformans* [343-345]. Immunocompetent mice infected with *C. gattii* exhibited limited cellular recruitment to the site of infection, and depletion of CD4-positive cells had no effect on survival, while mice infected with *C. neoformans* displayed a robust cellular immune response, and CD4 cell depletion significantly reduced survival time [345].

Coincubation of the two *Cryptococcus* strains with dendritic cells, *in vitro*, found that while *C. neoformans* stimulated a strong IL-6 response, *C. gattii* failed to induce IL-6 production by these cells [345]. This difference in cytokine response was related to partial deacetylation of *C. gattii* GXM, which was absent in *C. neoformans*. Chemical deacetylation of GXM from both species abolished their recognition by dendritic cells [345]. The mechanism underlying this deacetylation-dependent difference in host response is unknown, though it is interesting to hypothesize that there are similarities between this process and the role of partially deacetylated *A. fumigatus* GAG in immune evasion. Complement protein C3 also binds more strongly to GXM of *C. neoformans* than *C. gattii*, although this difference in C3 binding was attributed to differences in polysaccharide branching, rather than deacetylation [346].

1.7.6 *Histoplasma capsulatum*

Histoplasma capsulatum is a thermally dimorphic fungus that is a primary human pathogen [347]. *H. capsulatum* grows in a filamentous form in the environment, where it produces conidia that can be disseminated, particularly during excavation or other physical disruption [348]. Following inhalation, and exposure to higher body temperatures, conidia develop into their yeast form, causing pulmonary and disseminated infection [347]. Studies of immune interactions with *H. capsulatum* have been largely limited to elucidating the strategies by which this organism conceals β-glucans.

1.7.6.1 Alpha-(1,3)-Glucan

As with *A. fumigatus*, α-(1,3)-glucan is found in the outer cell wall of *H. capsulatum*, where it can mask β-glucans from detection by dectin-1. Strains deficient in α-glucan were significantly attenuated in their ability to kill murine P388D1 macrophage-like cells in co-culture [349], and were rapidly phagocytosed by these cells, leading to increased dectin-1-dependent TNFα secretion [350]. Interestingly, α-glucan-deficient strains of *H. capsulatum* and wild type *C. albicans* induced similar levels of TNFα production [350], suggesting that the efficiency of β-glucan masking by *H. capsulatum* may contribute to the success of this organisms as a primary pathogen. Masking of β-glucans by α-glucan is not universal among strains of *H. capsulatum*, as α-glucan-deficient strains (chemotype I), have been reported [351]. Although these yeast bind dectin-1 during log phase growth, dectin-1 binding is lost during stationary phase [351]. While the effects of deleting α-glucan synthase in chemotype I strains on stationary phase masking of

β-glucans has not been studied, inhibition of α-glucan synthase function via RNA interference resulted in no decrease in virulence, either *in vitro* or *in vivo* [351]. These findings suggest that β-glucan-masking in chemotype I strains is α-glucan independent [351,352]. Despite the absence of α-glucan, chemotype I yeast retain their ability to be internalized by, and kill, mouse macrophages [351], although less rapidly than α-glucan-sufficient strains [353].

In addition to α-glucan masking of β-glucans from immune recognition, *H. capsulatum* also secretes β-glucanase enzymes to further limit surface exposure of this glycan [352,354]. Strains from both chemotypes that are deficient in β-glucanase exhibit increased recognition by dectin-1, resulting in greater amounts of TNFα and IL-6 release by murine peritoneal macrophages during infection [352,354]. In α-glucan sufficient (chemotype II) strains, deletion of α-glucan synthase resulted in a greater increase in dectin-1 binding than did deletion of β-glucanase, although the effects were additive [352]. These observations suggest that while the masking of β-glucan with the α-glucan layer is the dominant mechanism of immune evasion, the two strategies are complementary. It has been suggested that the role of the secreted β-glucanase is to "trim" off any exterior β-glucan that remains exposed beyond the α-glucan coat surrounding the yeast cell [352].

Studies in mouse models have mirrored these *in vitro* findings. Chemotype II *H. capsulatum* strains deficient in α-glucan were significantly attenuated in virulence and exhibited reduced ability to disseminate beyond the lung in a mouse model of pulmonary infection [351]. In contrast, naturally α-glucan-deficient chemotype I strains remained virulent in mouse models of pulmonary histoplasmosis, and deletion of α-glucan synthase in these strains had no effects on overall virulence [351]. Although both chemotypes of *H. capsulatum* are capable of causing lethal diseases, studies using a sub-lethal pulmonary infection mouse model revealed differences in the immune response to these two strain types. Infection with α-glucan-deficient chemotype I *H. capsulatum* was associated with greater pulmonary levels of IFN-γ, IL-1β, IL-12, and TNFα, in association with increased weight loss, more severe pathology in lung histology, and higher pulmonary fungal burden later in the infection [353]. These differences in the kinetics of infection further support the hypothesis that chemotype I strains utilize unique virulence factors to support infection. Studies in mice have also confirmed the importance of the β-glucanase in virulence [352]. Mice infected intranasally with β-glucanase-deficient *H. capsulatum* strains had significantly lower pulmonary fungal burden than mice infected with the wild type parent strain,

regardless of chemotype [352]. This difference in fungal burden was not observed in dectin-1-deficient mice, confirming that these alterations in virulence were due to differences in β-glucan recognition [352].

1.7.7 Thoughts and Perspectives

Significant progress has been made in recent years regarding the study of fungal exopolysaccharides and the effects they have on the host immune system. These findings have led to a range of new therapeutic strategies targeting fungal polysaccharides [355]. In addition to the currently licensed echinocandins, inhibitors of glycosyl phosphatidylinositol synthesis that prevent incorporation of mannoproteins into the fungal cell wall, are currently in clinical trials [356]. Other efforts include generating antibodies against *C. albicans* mannans [357], cell wall glycoproteins [358], and *A. fumigatus* α-glucan [287], to test the vaccine potential of these cell wall components. Finally, we have recently reported the use of microbial glycoside hydrolases to degrade *Aspergillus* GAG, increase β-glucan exposure, and reduce virulence in a mouse model of invasive aspergillosis [298,359]. While many of these approaches remain in the early experimental phase, the therapeutic potential of the fungal cell wall is enormous.

Another promising avenue of research is to exploit immunomodulatory properties of fungal exopolysaccharides as treatments for inflammatory and autoimmune diseases. For example, *A. fumigatus* GAG has been proposed as a treatment for colitis through the induction of IL-1Ra [61]. Similarly, *C. neoformans* GalXM has been investigated as a treatment for rheumatoid arthritis, due to its ability to induce T cell apoptosis and inhibit IL-17 production [360].

Despite the tremendous advances in our understanding of the host immune response to fungal polysaccharides, significant challenges remain. Purification and characterization of cell wall polysaccharides remains in its infancy, and it is highly likely that variations in polymer length and post-synthetic modifications have a major impact on the host recognition and response to these molecules. Further, as evidenced by the results of studies using chitin and GXM [325], the immune response to combinations of polysaccharides may differ from those observed with isolated single polysaccharides. The study of fungal mutants that are deficient in specific polysaccharides is helpful, however, compensatory changes in cell wall composition through activation of the cell wall integrity and other pathways can lead to misleading results. Further, strain-, species-, and growth condition-dependent differences in cell wall composition may limit the generalizability of observations from *in vitro* and *in vivo* studies. Lastly, our ability

to study the dynamics of cell wall polysaccharide synthesis and modification during infection remains in its infancy. A combination of experimental approaches, and the development of new tools to assay, manipulate, and quantify polysaccharide production *in vitro* and *in vivo* are required to move the field forward and maximize the therapeutic potential of these microbial molecules.

1.8 Galectins

As detailed in section 1.7.4, proteins that bind *A. fumigatus* play an essential role in coordination of the immune response to infection. One class of such proteins that have been found to bind fungi and play an important role in the outcome of infection are the members of the galectin family [361,362]. The galectins are a family of soluble, non-glycosylated, cytosolic lectins that bind to β-galactoside-containing oligosaccharides [363]. While relatively few glycosylated ligands are found within the cytosol, galectins can be released into the extracellular space via cell damage or the non-classical "leaderless" secretion pathway, where they interact with glycosylated proteins on the surface of cells as well as the extracellular matrix [361]. Consistent with their presence in these two distinct cellular compartments, and the wide range of glycosylated proteins that can serve as binding partners, galectins have been implicated in a diverse array of biological processes (reviewed in [363]).

The galectin family is divided into 3 distinct groups, based on their structures [362,364]. The "prototypic" group consists of galectins including galectins 1, 2, 7 and 10, which are expressed as a single carbohydrate recognition domain (CRD) that exists either as a monomer or dimer. The "tandem-repeat" group contains galectins 4, 8, 9, and 12, and are expressed as two CRDs separated by a short linker region [362,364]. Galectin-3 is the sole member of the "chimeric" group, consisting of a CRD and a non-lectin tail.

1.8.1 Galectin-3

Galectin-3 is one of the most intensely studied members of the galectin family, having been implicated in the involvement of diseases including cancer, autoimmunity, infectious diseases, and heart failure [365]. Almost all mammalian cell types express galectin-3 [365], but some of the highest expression levels are found in immune cells, such as macrophages, monocytes, mast cells, and eosinophils [365].

1.8.2 Galectin-3 structure

The structure of galectin-3 is composed of a single C-terminal CRD, and an N-terminal domain. The N-terminal domain is approximately 260 amino acids long (ranging from 243 to 286 depending on the species) containing a tandem repeat rich in proline, glycine and tyrosine residues [366]. This domain gives galectin-3 the unique ability to multimerize following binding to a carbohydrate ligand. It is hypothesized that multimerization is suppressed in the absence of

carbohydrate binding via interactions between the CRD and the N-terminal domain. Galectin-3 multimerization is a dynamic process, forming up to pentameric structures [366]. Galectin-3 oligomers have their CRDs exposed, resulting in a multivalent structure capable of cross-linking multiple CRD ligands.

The CRD of galectin-3 is roughly 135 amino acid residues in length, and can bind to a variety of carbohydrate and protein ligands. While primarily known for binding β-galactoside-containing carbohydrates such as polylactosamine-containing polysaccharides [367], galectin-3 is also able to bind other non-galactose-containing carbohydrates, such as mannans [216,368]. In the cytosol, the galectin-3 CRD interacts with a variety of proteins, such as the pro-apoptotic protein Bax, forming a heterodimer and resulting in resistance to apoptosis [369].

1.8.3 Galectin-3 function

Given the breadth of diseases in which galectin-3 has been implicated, the scope of background information in this literature review will be restricted to its role in infectious diseases. Galectin-3 has been observed to exert an effect on the immune response against pathogens via multiple mechanisms, including direct inhibition of microbial growth, aiding in immune recognition by cross-linking receptors, and by indirectly modulating the immune system.

Galectin-3 interactions play an important role in the pathogenesis of a number of bacterial infections. When co-incubated in the presence of galectin-3, the bacteria *Helicobacter pylori* form clumps and exhibit a loss of viability [370]. Galectin-3-deficient macrophages exhibit a reduced ability to kill the bacteria, and galectin-3-deficient mice are unable to control an *H. pylori* infection [370], confirming that galectin-3 is important in the host response against this pathogen. Similarly, when grown in the presence of galectin-3, the Gram positive bacteria *Streptococcus pneumoniae* exhibits reduced colony formation units [371], however both the bacterial ligand and the mechanism of inhibition remain unknown. In addition to directly inhibiting the growth of *S. pneumoniae*, galectin-3 plays an important role in coordinating the immune response against this pathogen. Mice deficient in galectin-3 exhibit reduced neutrophil recruitment to the site of infection, due to a deficiency in the ability of the neutrophils to emigrate from the vasculature [371-373]. Interactions between the outer core region of the *Pseudomonas aeruginosa* lipopolysaccharide and galectin-3 were found to enhance binding of the bacteria to mouse cornea [374], suggesting that galectin-3 could play a role in the immune

recognition of this opportunistic pathogen. Finally, galectin-3 also recognizes *Mycobacterium* through binding to both phosphotidylinositol mannosides [375], as well as mycolic acids [376]. Galectin-3-deficient mice infected intravenously with *M. bovis* exhibit reduced clearance of the pathogen [375], suggesting that galectin-3 plays an important role in the immune response, either as an opsonin, or in modulating the immune system.

Galectin-3 also plays an important role in antifungal immunity. Galectin-3 binds to the capsule of the yeast *Cryptococcus neoforman,* and inhibits fungal growth [377]. Galectin-3 is thought to bind to mannans found on the surface of extracellular vesicles within the capsule, causing vesicle lysis and disrupting the capsule architecture. This binding likely contributes to host defence, as galectin-3-deficient mice are more susceptible to pulmonary infection with *C. neoformans* [377]. Galectin-3 can also bind to the surface of the dimorphic fungus *Candida albicans in vitro* [368], via β-1,2-oligomannans found on its cell wall. This binding directly induces the death of *C. albicans* [216], however the mechanism by which this occurs remains unclear. In addition to directly killing *C. albicans*, galectin-3 also aids in the differentiation of *C. albicans* from non-pathogenic *Saccharomyces cerevisiae* by the immune system. *S. cerevisiae* lacks β-1,2-oligomannans, and interactions of galectin-3 with the mammalian receptors TLR2 [181] or dectin-1 [179] enhance the immune system's ability to discern between *C. albicans* and *S. cerevisiae*. Galectin-3 has also been observed to be detrimental in the response towards *C. albicans,* as neutrophil-intrinsic galectin-3 attenuates the ability of neutrophils to generate ROS and kill phagocytosed fungi [182]. Collectively these data suggest that galectin-3 plays multiple roles during fungal infection, and can have both beneficial and detrimental effects on host immunity during these infections.

There is also conflicting evidence as to whether galectin-3 is beneficial or detrimental to the host during an intravenous *C. albicans* infection. Two studies involving similar candidemia models using the same strains of *C. albicans* and mice observed opposing results. One study administered 10^5 yeast cells via the tail vein and observed significantly greater mortality in the galectin-3-deficient mice than the wild-type mice, with median survival times of 3 and 15 days, respectively [183]. In contrast, another study administered 5×10^5 yeast cells via the tail vein and found the galectin-3-deficent mice to be protected from infection, exhibiting only 30% mortality after 10 days post infection, while the wild-type mice experienced a median survival time of 5

days [182]. It is therefore likely that other factors such as the host microbiome or environmental differences modulate the role of galectin-3 in *Candida* infection.

As with bacteria and fungi, galectin-3 mediates host defence against parasitic infection. Galectin-3 binds to galactose-containing moieties of the lipophosphoglycan of the protozoan parasite *Leishmania major* [378], although it does not exert direct anti-parasitic effects. Instead, it is involved in the recruitment of neutrophils to the site of infection, a process dependent on the presence of galectin-3 epitopes on the parasite's surface [379,380]. Interestingly, *L. major* expresses a metalloprotease that cleaves the N-terminal domain of galectin-3, separating it from the CRD and disabling its ability to multimerize [378]. It has been speculated that this cleavage of galectin-3 by *L. major* is a mechanism of evasion from the immune system. Similarly, galectin-3 also binds to the surface of the protozoan parasite *Trichomonas vaginalis* via lipophosphoglycan [381]. Unlike *L. major* however, binding of galectin-3 to *T. vaginalis* depletes the amount of free lectin available in the extracellular space, thus contributing to immune evasion by the parasite.

Thus, galectin-3 participates in the immune response against infectious agents through a variety of mechanisms including mediating direct antimicrobial effects, opsonisation, and immune modulation. To date, the role that galectin-3 plays in the context of an *A. fumigatus* infection remain unknown.

1.9 Hypothesis and objective of the research

A. fumigatus is distinguished from other *Aspergillus* species by the production of high levels of the exopolysaccharide galactosaminogalactan (GAG), allowing it to form robust biofilms [30,60]. While to date no mammalian receptor for GAG has been described, mammalian cells [28], mice and humans [61] directly respond to purified GAG, suggesting that they have the capacity to recognize the polysaccharide. The overarching hypothesis of this thesis is that the interactions of proteins with galactosaminogalactan represent a promising avenue for therapeutic development. The main goal of this doctoral work was to investigate proteins that interact with GAG and characterize the subsequent response, as well as evaluate the potential to exploit these proteins for a therapeutic advantage.

The first objective of this thesis was to identify and define a mammalian GAG receptor. The soluble lectin galectin-3 was found to bind to both purified and native GAG on the surface of the fungi. Unlike previous observations with other fungi, galectin-3 binding to the surface of *A.*

fumigatus did not inhibit fungal growth. Rather, galectin-3 was required for optimal cell recruitment to the site of infection, aiding in neutrophil emigration from the vasculature.

The second objective of this thesis was to investigate the utility of fungal enzymes involved in GAG catabolism as potential therapeutics. A glycoside hydrolase, Sph3, was identified in the GAG biosynthetic pathway that was capable of cleaving the mature GAG polysaccharide [359]. Treatment with a recombinant hydrolase domain from this protein disrupted pre-formed *A. fumigatus* biofilms, reducing their ability to mediate adherence, increasing their susceptibility to antifungal agents, and attenuating *Aspergillus* virulence in a mouse model of infection. Excitingly, a similar enzyme from the Gram-negative bacteria *Pseudomonas aeruginosa* was effective against both *A. fumigatus* and *P. aeruginosa* biofilms, suggesting that hydrolases can exhibit cross-kingdom activity and have potential therapeutic use.

1.10 Figure

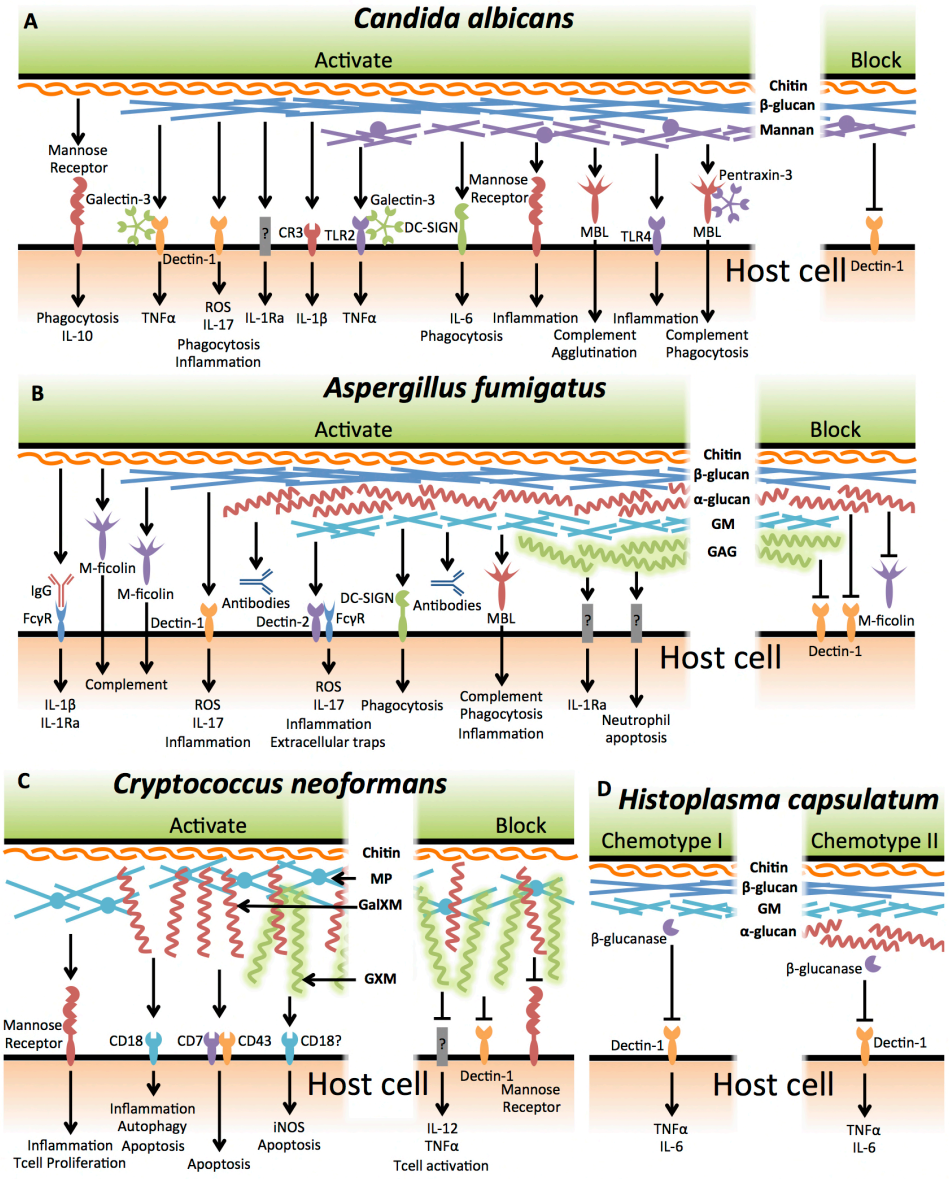

Figure 1. Graphical overview of interactions between fungal polysaccharides and host elements. (**A**) *Candida albicans,* (**B**) *Aspergillus fumigatus,* (**C**) *Cryptococcus neoformans,* and (**D**) *Histoplasma capsulatum*. Abbreviations used: CR3, complement receptor 3; MBL, mannose-binding lectin; GM, galactomannan; GAG, galactosaminogalactan; MP, mannoprotein; GalXM, galactoxylomannan; GXM, glucuronoxylomannan. Green indicates fungal cell, and tan, the host cell.

Preface to Chapter 2

Galactosaminogalactan is an important virulence factor for *Aspergillus fumigatus*, enabling the fungus to adhere to abiotic and biological substrates, form robust biofilms, and resist detection and attack by the host's immune system. Mammalian systems directly respond to galactosaminogalactan, with human mononuclear cells secreting interleukin-1 receptor antagonist, and neutrophils undergoing apoptosis via a mechanism involving natural killer cells. These responses by host cells suggest that a mammalian receptor exists that can recognize galactosaminogalactan, although no such receptor has been characterized to date. Here we demonstrate that the soluble, cytosolic lectin galectin-3 specifically recognizes galactosaminogalactan by its carbohydrate recognition domain. However, this recognition was secondary to its primary role during an *A. fumigatus* infection, which is to enhance neutrophil egress from the vasculature to the site of infection.

CHAPTER 2: Galectin-3 aids in neutrophil extravasation into the airways during pulmonary infection by *Aspergillus fumigatus*

Galectin-3 aids in neutrophil extravasation into the airways during pulmonary infection by *Aspergillus fumigatus*

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2.1 Abstract

The mould *Aspergillus fumigatus* is an opportunistic pathogen that infects immunocompromised patients, as well as patients with chronic lung diseases. The factors governing the host response to *A. fumigatus* remain poorly defined. Galectin-3 is a mammalian β-galactose-binding lectin that has been implicated in the defense against multiple organisms including pathogenic yeasts and dimorphic fungi. The role that galectin-3 plays in the defense against moulds has not been studied. We hypothesized that, as with other fungi, galectin-3 plays an important role in mediating protection against *A. fumigatus* infection.

Galectin-3 expression was markedly up-regulated in mice and humans infected with *A. fumigatus*. Galectin-3 bound to galactosaminogalactan on the surface of *A. fumigatus* hyphae, but did not affect fungal viability or growth. Galectin-3 deficient mice displayed increased susceptibility to *A. fumigatus* infection with higher mortality and pulmonary fungal burden, suggesting that galectin-3 is important for an effective immune response to *A. fumigatus*. Following *A. fumigatus* infection, the bronchoalveolar lavage fluid of galectin-3 deficient mice was found to contain higher levels of chemokines but fewer neutrophils than wild-type mice. Galectin-3 deficient mice exhibited normal numbers of neutrophils within whole lung digests, suggesting that galectin-3 is required for neutrophil entry into the airspaces. Neutrophil adoptive transfer experiments demonstrated that extrinsic rather than neutrophil-derived galectin-3 is required for efficient neutrophil migration to the airspaces. Exogenous galectin-3 increased human neutrophil movement and migration *in vitro*, suggesting that galectin-3 is an important mediator of neutrophil activation.

Taken together, these data demonstrate that galectin-3 facilitates effective recruitment of neutrophils to the site of *A. fumigatus* infection, and reveals a novel role for galectin-3 in the context of fungal infection.

2.2 Author Summary

The innate immune response against the mould *Aspergillus fumigatus* involves a complex array of factors leading to a neutrophil-mediated killing of the fungus. Many of these factors remain poorly defined. Here we provide evidence that the mammalian lectin galectin-3 plays an important role in the innate immune response to *A. fumigatus*. Galectin-3 levels were elevated in the blood and airways of *A. fumigatus* infected humans and mice, respectively. In contrast to

other fungal pathogens, galectin-3 had no direct effects on *A. fumigatus* growth, despite binding to its surface. Mice lacking galectin-3 were more susceptible to *A. fumigatus* infection, with both higher fungal burden and greater pulmonary damage. Galectin-3 deficient mice released greater amounts of chemokines and exhibited reduced neutrophil recruitment to the airways during *A. fumigatus* infection. Intravascular staining and adoptive transfer experiments demonstrated that extrinsic galectin-3 was required for optimal neutrophil recruitment during *A. fumigatus* infection. This study furthers our insight into the complex network of factors involved in the immune response against *A. fumigatus*.

2.3 Introduction

The mould *Aspergillus fumigatus* is an opportunistic pathogen that commonly infects the respiratory tract of immunocompromised patients, and patients with chronic lung diseases such as cystic fibrosis [41,43,45]. Pulmonary infection with *A. fumigatus* manifests as a necrotizing pneumonia, which can disseminate via the bloodstream to distal organs such as the brain. The mortality rate of invasive aspergillosis is between 30 and 95% [382], despite the use of current antifungal therapies. As such there is an urgent need for novel approaches to treat *A. fumigatus* invasive infections. One such approach is to develop strategies to enhance innate immune responses to this filamentous fungus.

While many of the factors underlying the innate immune response to *A. fumigatus* remain unknown, recognition of the carbohydrate-rich fungal cell wall by mammalian lectin receptors plays a critical role in initiating protective immune responses (reviewed in [22,383]). Dectin-1 has been identified as a key antifungal receptor that recognizes the cell wall component β-1,3-glucan [169]. Both mice and humans lacking functional dectin-1 exhibit increased susceptibility to *A. fumigatus* infections [384,385]. The opsonin M-ficolin also binds to β-glucan, resulting in the activation of the complement cascade [262]. Several mammalian receptors are able to recognize the mannan core of the *A. fumigatus* polysaccharide galactomannan, including "dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin" (DC-SIGN), dectin-2 and mannose-binding lectin, activating antifungal cellular responses [269,271,275]. However, it is highly likely that other mammalian lectin receptors play an important role in *A. fumigatus* immunity.

Galectin-3 is a mammalian lectin that has been implicated in the immune response against diverse microbial infections, including bacteria [370,372], parasites [378,380,386], and a number

of fungal pathogens. Galectin-3 is a soluble intracellular protein expressed in both immune and stromal cells, and can be released into the extracellular space by both the non-classical "leaderless" secretion pathway [387], and cellular injury [364].

Galectin-3 is composed of a single C-terminal carbohydrate recognition domain (CRD), and an N-terminal domain. While the canonical ligands of the CRD are β-galactoside-containing carbohydrates such as polylactosamine-containing polysaccharides [367], it has also been reported to bind other non-galactose-containing carbohydrates, such as mannans [216,368]. The N-terminal domain is approximately 260 amino acids long and is an intrinsically disordered tandem-repeat region rich in proline, glycine and tyrosine [388]. Once the CRD has engaged a carbohydrate ligand, this domain can interact with the N-terminal domain of other galectin-3 monomers, allowing for multimerization [366]. It is hypothesized that interactions between the CRD and the N-terminal domain suppress multimerization in the absence of carbohydrate binding. Galectin-3 multimerization is a dynamic process, resulting in the formation of up to pentameric structures [366]. The orientation of the galectin-3 oligomers results in the exposure of CRDs, allowing them to bind and cross-link ligands. Given the promiscuity of CRD binding, galectin-3 is then able to cross-link a wide variety of carbohydrates and glycosylated proteins, resulting in a diverse range of outcomes depending on the ligands and cell types involved (reviewed in [180]). From the point of view of infection, galectin-3 has been reported to mediate host defence against microbes through multiple mechanisms including binding to and directly killing pathogens, mediating pathogen detection through cross-linking canonical immune receptors, and/or enhancing immune cell recruitment [179,216,379].

The role of galectin-3 in host defence against fungal infections varies by species. Galectin-3 deficient mice exhibit increased sensitivity to infection with the pathogenic basidiomycete *Cryptococcus neoformans* [377], but are more resistant to infection with the dimorphic pathogen *Histoplasma caspulatum* [389]. Two studies examining the susceptibility of galectin-3 deficient mice to infection with the pathogenic yeast *Candida albicans* have reported conflicting results, with these mice exhibiting reduced survival following intravenous fungal challenge in one study, and increased survival in the second [182,183]. The role of galectin-3 in the pathogenesis of mould infections has not been studied.

In light of these reports, we hypothesized that galectin-3 may play a role in the innate immune response to *A. fumigatus* infection. High levels of extracellular galectin-3 were found in

bronchoalveolar fluid of wild-type mice infected with *A. fumigatus*, and in the serum of human patients with pulmonary aspergillosis. Galectin-3 bound to the surface of the hyphae, but this binding did not affect fungal growth or viability. Galectin-3 deficient mice were more susceptible to *A. fumigatus* infection, and exhibited reduced neutrophil egress from blood vessels and recruitment to the site of infection within the airspaces following fungal challenge. Adoptive transfer experiments revealed that extrinsic, rather than neutrophil-derived galectin-3 was required for effective neutrophil recruitment to the airways. Recombinant galectin-3 enhanced the motility and migration of human neutrophils *in vitro*, suggesting that extracellular galectin-3 plays a direct role in modulating neutrophil responses.

2.4 Results

Galectin-3 is released in response to *A. fumigatus in vivo*. To determine if galectin-3 is involved in the immune response against *A. fumigatus*, both wild-type and galectin-3 deficient mice were infected intratracheally with *A. fumigatus* Af293 conidia and lungs were harvested at 36 hours post infection for histopathology. Anti-galectin-3 staining of uninfected wild-type lungs revealed that galectin-3 staining was primarily restricted to solitary cells in the airspaces (**Figure 1A**). In contrast, infected wild-type mice exhibited intense galectin-3 staining of leukocytes and the pulmonary epithelium (**Figure 1A**). No galectin-3 was detected in lung samples from infected, galectin-3 deficient mice (**Figure 1B**), confirming the specificity of the anti-galectin-3 antibody. To confirm these findings, galectin-3 levels in bronchoalveolar lavage (BAL) fluid and serum from infected and uninfected wild-type mice were measured by ELISA. Increased levels of galectin-3 were detected in the BAL fluid of infected mice as compared to uninfected animals (**Figure 1C**). No differences in serum galectin-3 levels were observed between infected and uninfected animals (**Figure 1C**). Collectively these data suggest that galectin-3 is released into mouse airways in response to *A. fumigatus* infection.

To determine if galectin-3 expression is also induced in humans during pulmonary *Aspergillus* infection, BAL fluid and serum samples from patients with invasive pulmonary aspergillosis, chronic pulmonary aspergillosis, and controls without fungal infection were analyzed for galectin-3 content. Serum galectin-3 levels were significantly elevated in the patients with either form of pulmonary aspergillosis as compared with controls (**Figure 1D**). A trend towards increased galectin-3 in the BAL fluid of patients with either form of pulmonary aspergillosis was observed, however this was not statistically significant (**Figure 1D**). Taken as a

whole, these data suggest that galectin-3 expression and secretion is induced during *A. fumigatus* infection in both mice and humans.

Galectin-3 binds to galactosaminogalactan on the surface of *A. fumigatus* hyphae, but is not directly fungicidal. Galectin-3 has been reported to bind to *C. albicans* and *C. neoformans* and directly inhibit fungal growth [216,377]. We therefore tested the ability of purified recombinant galectin-3 (rGal-3) to bind to, and kill *A. fumigatus*. Immunofluorescent confocal microscopy demonstrated that rGal-3 bound to the surface of *A. fumigatus* hyphae (**Figure 2A**). Hyphae of the galactosaminogalactan (GAG)-deficient *A. fumigatus* Δ*uge3* mutant [60] exhibited markedly reduced rGal3 staining, suggesting that galectin-3 binds to hyphal-associated GAG. rGal-3 also exhibited dose-dependent binding of purified immobilized GAG by ELISA (**Figure 2B**). rGal3 binding was inhibited by lactose (**Figure 2C**), a disaccharide which binds galectin-3 via its carbohydrate recognition domain [367], suggesting that galectin-3 recognizes GAG specifically through its lectin domain. Galectin-3 did not mediate direct fungicidal activity against *A. fumigatus,* as exposure of hyphae to rGal-3 concentrations as high as 850 μg/ml had no effect on fungal growth or viability (**Figure 2D**), as determined by calcofluor white staining of the resulting biomass.

Galectin-3 deficient mice are more susceptible to *A. fumigatus* pulmonary infection. To assess the role of galectin-3 in the pathogenesis of *A. fumigatus* pulmonary infection, wild-type and galectin-3 deficient mice were infected intratracheally with two different strains of *A. fumigatus* conidia. When compared to wild-type mice, galectin-3 deficient mice exhibited reduced survival when infected with the highly virulent CEA10 strain (**Figure 3A**) [78], or the less-virulent Af293 strain (**Figure 3B**). Given the rapid mortality during infection with strain CEA10, strain Af293 was selected for further studies to allow for complete sampling of experimental groups. To determine if the lower survival of galectin-3 deficient mice was a reflection of poorer control of fungal infection, pulmonary fungal burden was measured. The pulmonary fungal burden of galectin-3 deficient mice was significantly higher than was observed in wild-type mice (**Figure 3C**). Galectin-3 deficient mice also exhibited increased total protein concentrations in the BAL fluid following *A. fumigatus* infection as compared to wild-type animals, suggesting that galectin-3 deficiency was associated with increased pulmonary injury

[371] (**Figure 3D**). No sex-specific differences in survival and fungal burden were observed in the mice during infection with *A. fumigatus* (**Supplementary Figures S1A-C**). Periodic acid-Schiff staining of pulmonary tissue sections revealed that fungal lesions in galectin-3 deficient animals were surrounded by reduced levels of inflammation and leukocyte recruitment as compared with wild-type mice. (**Figure 3E**). Collectively these results suggest that galectin-3 may mediate defence against *A. fumigatus* infection by enhancing the innate immune response.

Galectin-3 is required for early neutrophil migration to the airways. To characterize the role of galectin-3 in inflammation, the levels of cytokines in BAL fluid were compared between wild-type and galectin-3 deficient mice infected with *A. fumigatus*. Levels of the pro-inflammatory cytokines granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1α, IL-1β, IL-6, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein-2 (MIP-2) were increased in the BAL fluid of infected galectin-3 deficient mice compared to infected wild-type mice (**Figure 4A**). In contrast, levels of IL-1 receptor antagonist (IL-1Ra), IL-12, IL-17, monocyte chemoattractant protein 1 (MCP-1) and "regulated upon activation normal T cell expressed and secreted" (RANTES) were decreased in the BAL fluid of infected galectin-3 deficient mice compared to wild-type controls (**Figure 4B**). Levels of macrophage inflammatory protein-1α (MIP-1α) and tumour necrosis factor α (TNFα) were increased to the same degree in both strains of mice (**Supplementary Figure S2A**), and levels of interferon-γ, IL-4, IL-5, IL-10 and IL-13 were not significantly induced by 36 hours during an *A. fumigatus* pulmonary infection in either mouse strain (**Supplementary Figure S2B**). Collectively these data indicate that levels of neutrophil chemoattractant cytokines are elevated in the BAL fluid of galectin-3 deficient mice in response to *A. fumigatus* infection. Furthermore, as many of the cytokines found to be expressed at lower levels in the BAL fluid of galectin-3 deficient mice are produced by neutrophils, these cytokine data are consistent with the histopathology findings of reduced inflammatory cell recruitment to the alveoli in these mice during *A. fumigatus* infection.

To test the hypothesis that galectin-3 plays an important role in neutrophil recruitment to the airways during *A. fumigatus* infection, the immune cell populations in the whole lung digest and BAL fluid of wild-type and galectin-3 deficient mice were analyzed. No difference was observed in the number of neutrophils present in total lung samples between wild-type and galectin-3

deficient mice, however significantly fewer neutrophils were recovered from the BAL fluid of infected galectin-3 deficient animals compared to their infected wild-type controls (**Figure 5A**). This difference between strains was also significant when the fraction of total pulmonary neutrophils present within the airways of each strain was calculated (**Figure 5B**). The total number of macrophages, inflammatory monocytes, and conventional dendritic cells in the BAL fluid and the airway fraction of these cells within the lung were also reduced in galectin-3 deficient mice as compared to the infected wild-type animals (**Supplementary Figures S3A&B**). No significant differences were observed in eosinophils or alveolar macrophages populations between mouse strains during infection (**Supplementary Figure S3A**).

To confirm that there was a defect in migration of neutrophils and other leukocytes from the vascular compartment into the airways, vascular staining was used to quantify the pulmonary neutrophil population that remained within the vasculature. Consistent with the results of leukocyte quantification in BAL fluid, a higher proportion of neutrophils were found within the pulmonary vasculature of galectin-3 deficient mice compared to wild-type mice during *A. fumigatus* infection (**Figure 5C**). Greater than 99% of the neutrophils collected from blood and less than 1% of the neutrophils collected in the BAL fluid were positive for vascular staining, confirming the specificity of this approach (**Figure 5C**). Both alveolar macrophages and inflammatory monocytes within the total lung cell population also exhibited increased vascular staining in the lungs of infected galectin-3 deficient mice as compared to wild-type animals, while no difference was seen with eosinophils (**Supplementary Figure S3C**). Other cell types were not analyzed. These data are strongly suggestive that galectin-3 plays an important role in enhancing neutrophil migration from the pulmonary vasculature to the site of infection within the pulmonary airspaces.

Neutropenia ablates the phenotypic differences between wild-type and galectin-3 deficient mice. Although numbers of macrophages, inflammatory monocytes, conventional dendritic cells and neutrophils were all reduced in galectin-3 deficient mice during *A. fumigatus* infection, this effect was greatest with neutrophils. As neutrophils play a critical role in the innate defence against *A. fumigatus* infection, we hypothesized that galectin-3-mediated neutrophil recruitment to the site of infection is the primary mechanism by which this lectin contributes to immunity against this organism. To test this hypothesis, the effects of neutrophil

depletion using an antibody specific for Ly6G were tested [390]. Less than 200 neutrophils were detected in the BAL fluid of any mouse following anti-Ly6G treatment, confirming the successful depletion of neutrophils with this treatment (**Figure S4**). Neutrophil depletion abrogated the difference in susceptibility to *A. fumigatus* between wild-type and galectin-3 deficient mice. No statistical differences were observed in survival (**Figure 6A**), pulmonary fungal burden (**Figure 6B**) or pulmonary damage (**Figure 6C**) between neutrophil-depleted wild-type and galectin-3 deficient mice. These data suggest that the increased susceptibility of immunocompetent galectin-3 deficient mice to *A. fumigatus* pulmonary infection is primarily due to differences in neutrophil recruitment to the airways between the mouse strains.

Adoptive transfer of neutrophils reveals an extrinsic galectin-3-dependent migration defect. To determine if the differences observed in neutrophil migration to the airways between the wild-type and galectin-3 deficient mice were due to extrinsic galectin-3 in the lung environment, or required neutrophil intrinsic galectin-3, an adoptive transfer approach was taken.

To determine the effects of extrinsic galectin-3, neutrophils were purified from the bone marrow of wild-type B6-CD45.1 mice and transfused into both infected wild-type and galectin-3 deficient mice (both of which express the CD45.2 alloantigen of CD45) via the tail vein at 24 hours post infection with *A. fumigatus*. At 12 hours post adoptive transfer, a significantly greater proportion of the pulmonary B6-CD45.1 neutrophils were found in the airways of recipient wild-type mice compared to the galectin-3 deficient animals (**Figure 7A**), suggesting that extrinsic galectin-3 is necessary for effective neutrophil recruitment to the airways.

To confirm these findings, the inverse experiment was performed in which neutrophils were purified from both wild-type and galectin-3 deficient mice, and administered to B6-CD45.1 mice infected with *A. fumigatus* (**Figure 7B**). No differences in the fraction of neutrophils recruited to the airways were observed between wild-type and galectin-3 deficient donor neutrophils, suggesting that endogenous galectin-3 is dispensable in neutrophils for efficient recruitment to the airways.

Galectin-3 deficient neutrophils are not impaired in their ability to kill *A. fumigatus.* The ability of galectin-3 deficient neutrophils to migrate normally into the lungs of galectin-3 sufficient mice infected with *A. fumigatus* suggests that these neutrophils are not intrinsically

dysfunctional. To determine if galectin-3 deficient neutrophils exhibit normal antifungal activity, the ability of neutrophils from both wild-type and galectin-3 deficient mice to kill *A. fumigatus* were evaluated *in vitro*. Galectin-3 deficient neutrophils purified from bone marrow were able to kill young *A. fumigatus* hyphae to the same extent as wild-type neutrophils (**Figure 8**), suggesting that galectin-3 is not required for normal neutrophil antifungal activity.

Exogenous galectin-3 enhances neutrophil motility and migration *in vitro*. The results of our mouse studies suggest that galectin-3 enhances neutrophil migration during pulmonary *Aspergillus* infection. The elevated levels of galectin-3 in the sera of patients with pulmonary aspergillosis suggest that galectin-3 may play a similar role in humans. To explore this hypothesis, the effects of rGal-3 on human neutrophil motility was examined using live cell imaging. Human peripheral blood neutrophils were treated with rGal-3 and their motility was quantified using single cell tracking analysis. Even in the absence of a microbial stimulus, rGal-3 exposure significantly enhanced neutrophil motility (**Figure 9A**). Neutrophil migration paths were significantly longer in rGal3 treated samples, although in the absence of a specific chemotactic signal, no specific directionality of migration was observed. To determine if galectin-3 enhances directional movement, the effects of rGal-3 on neutrophil migration to the chemoattractant IL-8 was measured using a transwell-system. The addition of rGal-3 was found to significantly enhance the ability of the neutrophils to migrate towards the neutrophil chemoattractant IL-8 (**Figure 9B**), confirming that galectin-3 also enhances neutrophil migration.

2.5 Discussion

Our findings suggest that galectin-3 plays an important role in the immune response against an *A. fumigatus* pulmonary infection. Galectin-3 is significantly up-regulated in the context of *A. fumigatus* infection, in both pulmonary aspergillosis patients and infected mice. While not directly anti-fungal, galectin-3 mediated the efficient recruitment of neutrophils to the airways of infected mice. Neutrophil adoptive transfer experiments revealed that extrinsic, rather than neutrophil intrinsic galectin-3 is required for neutrophil recruitment to the airways. Galectin-3 treatment of human neutrophils *in vitro* increased motility and augmented migration, suggesting that extracellular galectin-3 directly enhances neutrophil motility. These studies reveal a novel mechanism of action for this protein in pulmonary fungal infection.

The findings of this study extend our understanding of the multiple roles that galectin-3 can play in fungal innate immunity. Galectin-3 directly inhibits growth of *C. neoformans* through binding to extracellular vesicles within the capsule, presumably via β-mannosides, causing vesicle lysis and disrupting capsule architecture [377]. Similarly, galectin-3 binds to *C. albicans* β-1,2-oligomannans within the cell wall [368], directly killing the fungus [216] via an unknown mechanism. We observed that while galectin-3 bound to GAG on the surface of *A. fumigatus*, it did not directly affect the growth or viability of the fungus, suggesting that galectin-3 is not inherently antifungal against *A. fumigatus*. Conversely, it has also been reported that cytosolic galectin-3 suppresses the ability of neutrophils to kill *C. albicans* yeast through attenuating ROS production [182]. We found that galectin-3 deficient neutrophils displayed normal activity against *A. fumigatus in vitro*, suggesting that galectin-3 is dispensable for neutrophil recognition and killing of *A. fumigatus*. ROS has been observed to play only a partial role in killing phagocytosed *A. fumigatus* [100,391], possibly explaining the differences between these observations.

Galectin-3 bound β-1,2-oligomannans of the cell wall of *C. albicans* [368], and has been hypothesized to bind to a similar structure of the capsule of *C. neoformans* [377]. These observations suggest that the traditional view of galectin-3 as a β-galactoside-specific lectin requires reassessment. Our finding that galectin-3 binds to *A. fumigatus* GAG, a heteropolysaccharide of α-1,4-linked galactose and partially deacetylated GalNAc, expands the repertoire of carbohydrates recognized by galectin-3. While it remains to be determined which of these sugar(s) is bound by galectin-3, multiple lines of evidence suggest that this α-linked polysaccharide is bound specifically by galectin-3. Galectin-3 bound to the surface of GAG-producing *A. fumigatus* hyphae, but not those of the GAG-deficient Δ*uge3* mutant. Galectin-3 bound to purified GAG in a dose-dependent manner, and this binding could be inhibited by the galectin-3 ligand, lactose. Galectin-3 did not recognize α-mannans *in vitro,* suggesting that galectin-3 was not able to interact with the mannan core of galactomannan, the only known mannose-containing polysaccharide within the *A. fumigatus* cell wall.

Interaction between galectin-3 and β-1,2-oligomannans on the surface of *C. albicans* induced death of the organism, however the exact mechanism of killing is not clear [216]. On the surface of *C. albicans,* galectin-3 preferentially binds to the β-1,2-oligomannans of phospholipomannan, a glycosylated phosphoceramide synthesized in the Golgi complex [392].

While phospholipomannan is shed into the outer cell wall and released into the environment [392,393], it is primarily concentrated within the inner layer of the cell wall [394,395]. It is thus possible that galectin-3 binding to phospholipomannan in the inner *Candida* cell wall leads to a destabilization of the inner cell wall architecture and death of the fungus, similar to the proposed mechanism of galectin-3 inhibition of *C. neoformans* growth [377]. As GAG is localized to the outer cell wall and extracellular matrix of *A. fumigatus*, galectin-3-induced cell wall changes would be confined to the outer cell wall, which plays a much less important structural role in *A. fumigatus*, thus explaining the species-specific differences in antifungal activity of this lectin.

Through our immunophenotyping and neutrophil adoptive transfer studies we found that extrinsic galectin-3 is required for effective neutrophil egress from the blood vessels and into the site of the infection within the airways. Similar observations have been reported with models of bacterial [371-373] and parasitic infection [379], where extracellular galectin-3 is required for effective neutrophil recruitment to the site of infection [372]. However, the mechanisms underlying the roles of galectin-3 in neutrophil extravasation remain unclear as galectin-3 does not function as a neutrophil chemoattractant [372]. Galectin-3 deficient endothelial cells are observed to express lower levels of intercellular adhesion molecule-1 (ICAM1), a membrane protein involved in leukocyte crawling prior to extravasation [396]. There is evidence that extracellular galectin-3 can crosslink and aid in the association of receptors found on the surfaces of neutrophils and endothelial cells, promoting diapedesis [180,373,397,398]. Galectin-3 has been reported to associate with CD11b [399], a subunit of the β2-integrin Mac-1 (CD11b/CD18). However, several studies suggest that integrin-dependent and galectin-3-dependent neutrophil migration are independent processes. Antibody-mediated CD18-blockade significantly reduces airway neutrophil recruitment during a mouse model of pulmonary *Escherichia coli* infection [400], while galectin-3 deficiency enhances the recruitment of neutrophils to the airways [372]. Similarly, neutrophil recruitment to the airways is significantly reduced in galectin-3 deficient mice in a model of streptococcal pneumonia [372], but is unaffected by CD18 deficiency [401]. While CD18 deficiency abrogates neutrophil recruitment to the cornea during experimental *A. fumigatus* keratitis [402], the effects of CD18 deficiency during pulmonary *A. fumigatus* infection are not known. The pulmonary capillary bed is unique in that neutrophils are larger than the cross-sectional diameter of the alveolar capillaries, requiring neutrophils to deform themselves to pass through the capillaries [161]. Possibly as a result of being in contact with the

endothelium and the low flow rate, the early steps of neutrophil diapedesis that are observed in other organs do not occur in the pulmonary capillaries [161]. It is therefore possible that neutrophils are recruited to the cornea and the airways via different mechanisms during *A. fumigatus* infection.

The results of these studies establish a role for galectin-3 in the innate immune response to *A. fumigatus* infection. In contrast to *C. albicans* and *C. neoformans*, galectin-3 did not exhibit direct antifungal effects against *A. fumigatus*, but rather was required for efficient immune cell recruitment to the site of infection. These findings highlight the diversity of mechanisms by which galectin-3 aids in the response to infection, and identifies a novel factor in the host response against pathogenic moulds. Taken together this study furthers our understanding of the complex interplay between the host and *A. fumigatus.*

2.6 Materials and Methods

Strains and reagents. *Aspergillus fumigatus* strain Af293 was generously provided by Dr. P. Magee, and CEA10 strain was a generous gift by Dr. J.P. Latgé. Frozen Af293 conidial stocks were cultivated on Yeast-Peptone-Dextrose (YPD) agar plates for 6 days at 37°C, while CEA10 was cultivated on *Aspergillus* Minimal Media (AMM) agar plates for 3 days at 37°C, at which point the conidia were harvested by gentle washing of the mycelial mat with phosphate-buffered saline (PBS) + 0.05% (v/v) Tween-20 (PBS-T). Galectin-3 deficient (B6.Cg-*Lgals3*^{tm1Poi}/J) mice were purchased from The Jackson Laboratory and bred at our facility, and wild-type controls (C57BL/6) were purchased from The Jackson Laboratory and Charles River Laboratories. The anti-galectin-3 antibody was purified from the M3/38 hybridoma cell line using GE Healthcare HiTrap Protein G HP column as previously published [378]. Murine bone marrow, blood, lung digestate, and bronchoalveolar lavage (BAL) fluid were stained for flow cytometry using the following fluorescently-labeled antibodies (all purchased from BD Biosciences): CD11c-allophycocyanin (clone HL3), CD11b-phycoerythrin (PE)-CF594 (clone M1/70), CD11b-fluorescein isothiocyanate (FITC) (clone M1/70), CD45-allophycocyanin (APC)-Cy7 (clone 30-F11), CD45-PE-Texas Red® (clone 30-F11), CD45.1-FITC (clone A20), CD45.2-APC-Cy7 (clone 104), SiglecF-brilliant violet 421 (clone E50-2440), Ly6G-PE (clone 1A8), Ly6C-Alexa Fluor®700 (clone AL-21), and F4/80-Alexa Fluor®488(clone 6F12). Anti-Ly6G antibody (clone 1A8) to induce neutropenia was purchased from BioXcell. Recombinant galectin-3 was produced

in *Escherichia coli* as previously described [378]. Purified galectin-3 was then passed through an ActiClean Etox endotoxin-removing column (Sterogene) for *in vivo* and *in vitro* experiments.

Ethics Statements. All experiments involving mice were approved by the Animal Care Committees of the McGill University Health Centre. Clinical studies using patient samples were approved by the Nagasaki University School of Medicine Research Ethics Committee (Approval number: 17091125-2). Peripheral blood was collected from healthy volunteers under a license from "Comité d'éthique de la recherche du CHU de Québec-Université Laval".

Endotracheal conidia infection. Mice 8 - 10 weeks of age were anaesthetized with isoflurane and endotracheally infected with 5 x 10^7 Af293 or CEA10 conidia in 50 µl PBS-T. Mice were monitored daily for signs of distress and moribund animals were euthanized by CO_2 overdose. To induce neutropenia, mice received an intraperitoneal injection of 200 µg of anti-Ly6G antibody the day prior to infection, and every 48 hours until the end of the experiment.

Histopathological preparation and staining. At 36 hours post infection, mice were euthanized, their lungs removed, inflated with 10% PBS-buffered formalin, and fixed in formalin. Fixed lungs were embedded in paraffin, and 4 µm-thick sections were stained with either periodic acid Schiff (PAS) stain, or immunohistochemical staining using the anti-galectin-3 antibody and visualized by staining with donkey-anti-rat antibody conjugated to horseradish peroxidase and developed using 3,3'-diaminobenzidine.

Quantification of galectin-3 in serum and BAL fluid. Following euthanasia, cardiac puncture was performed on the mice and blood collected in Microvette® 500 Z-Gel tubes and centrifuged to yield serum.

Mice were then tracheotomised and a blunt 18-gauge needle was inserted into the trachea. Lungs were then lavaged with 1 ml PBS, and the resulting bronchoalveolar lavage (BAL) fluid was centrifuged at 100 *xg* for 10 mins and the supernatant collected for galectin-3 or cytokine analysis.

Human samples (BAL fluid and serum) were collected from patients who were diagnosed with pulmonary aspergillosis in Nagasaki University Hospital, Japan. Control samples were obtained from patients undergoing bronchoalveolar lavage for pulmonary sarcoidosis who had no signs of fungal infection and were not receiving corticosteroids. BAL was performed with 20 to 40 ml of normal saline in the area of the affected pulmonary lesion.

All samples were stored at -80°C until use. Galectin-3 levels were measured in the human samples by the Human Galectin-3 Quantikine ELISA kit (R&D systems), as per manufacturer's instructions. Galectin-3 was quantified in mouse serum samples using the R&D DY1197 Mouse Galectin-3 DuoSet ELISA according to manufacturer's instructions. Galectin-3 was detected in mouse BAL samples using an in-house ELISA. Briefly, ELISA plates were coated overnight with R&D MAB11541 anti-galectin-3 antibody at 2 μg/ml in PBS. After blocking with PBS 5% BSA, BAL samples where added and incubated 2 hours at room temperature. Mac-2 anti-galectin-3 antibody was then added at 1 μg/ml, followed by horseradish peroxidase conjugated anti-rat antibody. Finally, TMB substrate solution was added to produce a colorimetric signal and the reaction was stopped using 0.18 M H_2SO_4. Absorbance was read at 450 nm with Perkin Elmer Wallac Victor2 Microplate Reader.

Pulmonary fungal burden determination. Excised lungs were resuspended in PBS and homogenized, and the resulting homogenate was diluted and plated onto YPD agar containing 1% (v/v) penicillin/streptomycin via sterile glass beads. Colonies were counted following an overnight incubation at 37°C.

Measurement of proteins released into the BAL fluid. Total protein content of the BAL fluid was determined using the bicinchoninic acid (BCA) assay (Thermo Scientific), as per manufacturer's instructions.

Cytokine analysis of the BAL fluid was performed using a custom 16-plex mouse cytokine multiplex array (GM-CSF, IFNγ, IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17, KC, MCP-1, MIP-1α, RANTES, and TNFα; Quansys Biosciences), as per manufacturer's instructions. Individual ELISAs were also used to quantify IL-1α (eBioscience), KC, MIP-2, and IL-1Ra (all from R&D Biosystems), as per manufacturer's instructions.

Immunofluorescent hyphal staining by rGal-3. *A. fumigatus* Af293 expressing the red fluorescent protein (RFP) mRFP1 [86] conidia were grown in Dulbecco's Modified Eagle Medium (DMEM). Samples were washed with PBS and blocked with PBS containing 3% (w/v) 1X crystallized bovine serum albumin (blocking buffer). Samples were washed and incubated with blocking buffer containing 75 μg/ml biotinylated recombinant galectin-3. Samples were washed and incubated with blocking buffer containing 2.5 μg/ml streptavidin conjugated to Alexa Fluor® 488 (Jackson Immunoresearch), and fixed with 4% (w/v) PFA in PBS. Coverslips were then mounted in SlowFade Diamond Antifade mounting medium (Thermo) and sealed.

Samples were acquired on an Olympus confocal fluorescent microscope using a 60x/1.42 Oil objective lens at a resolution of 640X640 with a 1.7X digital zoom, using 488 and 543 nm lasers to excite Alexa Fluor® 488 and RFP, respectively. The channels were acquired separately, with Alexa Fluor® 488 being detected at 520 nm, and RFP at 581 nm. Z-stacks were acquired with a spacing of 0.2 μm. Stacks were combined using the "Maximum Intensity Projection" algorithm of the ImageJ software. A 20 μm scale bar was then added using ImageJ before being exported as a .png file format.

Galectin-3 polysaccharide binding assay. ELISA plate wells were coated with GAG (purified as previously described [30]), asialofetuin, or α-mannan at 0.5mg/ml in PBS overnight. After blocking with 10 mg/ml BSA, biotinylated galectin-3 was added in the absence or presence of lactose (an antagonist of galectin-3) and incubated for 2 hours. Following washing, galectin-3 bound to GAG was detected using streptavidin-horseradish peroxidase and TMB substrate solution. The reaction was stopped using 0.18 M H_2SO_4. Absorbance was read at 450nm with a Perkin Elmer Wallac Victor2 Microplate Reader.

Direct fungal killing by rGal-3. *A. fumigatus* Af293 conidia were grown for 24 hours in DMEM supplemented with 850 μg/ml recombinant galectin-3 or 8 μg/ml amphotericin B. The resulting biomass was then measured by staining with 1 mg/ml solution of calcofluor white, and measuring the fluorescence with an excitation of 340 nm and an emission at 440 nm.

Cellular recruitment analysis by flow cytometry. Lungs were lavaged twice with 1 ml PBS, and the resulting BAL fluid was centrifuged at 100 *xg* for 10 mins. The resulting cell pellets recovered after centrifugation were combined. Lungs were minced with scalpel blades and digested by 150 units/ml collagenase type IV (Sigma) in Roswell Park Memorial Institute Medium (RPMI) supplemented with 5% (v/v) Fetal Bovine Serum (FBS). Samples were drawn through an 18G needle to further disrupt large pieces, and strained through a 70 μm cell strainer.

Erythrocytes were lysed by incubating the samples with ACK buffer, and the remaining cells were stained with 0.1% (v/v) fixable viability dye (eBioscience). Samples were washed in PBS + 2% (v/v) FBS (staining buffer), and incubated with unlabelled anti-CD16/32 antibodies (FcBlock; BD Pharmingen) to block the Fc receptors. Samples were stained with the fluorescently-labelled antibodies listed in the Reagents section above. Cell were washed with staining buffer and fixed with 2% (w/v) PFA in PBS, and subsequently washed and resuspended in PBS prior to data acquisition. Samples were spiked with BD CountBright absolute counting

beads and data was acquired on an LSR Fortessa flow cytometer using FACSDiva software (BD Biosciences). Following acquisition, data were analyzed by using FlowJo software version 10 (FlowJo, LLC), with immune cell subsets being defined as follows: neutrophils, $CD45^+$ $Ly6G^+$ $CD11c^-$ $CD11b^+$; alveolar macrophages, $CD45^+$ $CD11c^+$ $siglecF^+$ $CD11b^{neg/low}$; eosinophils, $CD45^+$ $CD11b^+$ $CD11c^-$ $siglecF^+$; inflammatory monocytes, $CD45^+$ $Ly6C^+$ $Ly6G^-$ $CD11b^+$; macrophages, $CD45^+$ $Ly6C^-$ $Ly6G^-$ $CD11b^+$ $CD11c^-$ $F4/80^+$; conventional dendritic cells, $CD45^+$ $Ly6C^-$ $Ly6G^-$ $CD11b^+$ $CD11c^+$ $F4/80^-$.

Staining of vasculature-associated cells. To determine the proportion of cells in the lung digest that was in the vascular system, intravascular staining with anti-CD45 antibody was performed [403]. Three minutes prior to euthanasia, mice were injected intravenously with 300 µl PBS containing 3 µg anti-CD45-PE-Texas Red® (clone 30-F11). Following euthanasia, blood was obtained by cardiac puncture and collected in heparinized tubes. BAL fluid and lungs were collected and processed as above. Samples were stained *ex vivo* as above with the following antibodies: CD45-APC-Cy7, CD11b-FITC, CD11c-APC, Ly6G-PE, Ly6C-Alexa Fluor®700, and SiglecF-Brilliant Violet 421. Samples were analyzed by flow cytometry, with neutrophils, eosinophils, alveolar macrophages, and inflammatory monocytes populations as defined as above. Cells associated with the vasculature were defined as positive for both CD45 markers.

Mouse bone marrow neutrophil isolation. Bone marrow was collected from the femurs and tibias of mice, 8 - 10 weeks of age. Neutrophils were isolated using the mouse MACS Neutrophil Isolation Kit and LS columns (Miltenyi Biotec), as per manufacturer's instructions.

Adoptive transfer of neutrophils. Isolated neutrophils were counted and their concentration adjusted to 5 x 10^6 cells / ml in PBS. Neutrophils were administered to mice at 24 hours post infection via the lateral tail vein at a volume of 200 µl (10^6 live neutrophils / mouse). At 36 hours post infection (12 hours post neutrophil administration), mice were euthanized and their blood, lungs and BAL fluid were collected and processed as above. Samples were stained as above with the following antibodies: CD45.1-FITC, CD45.2-APC-Cy7, CD11b-PE-Texas Red®, CD11c-APC, Ly6G-PE, Ly6C-Alexa Fluor®700, and SiglecF-Brilliant Violet 421. Samples were analyzed by flow cytometry. Neutrophils were defined as $Ly6G^+$ $Ly6C^{mid}$ $CD11c^-$ $CD11b^+$ before being gated as either $CD45.1^+$ $CD45.2^-$ or $CD45.1^-$ $CD45.2^+$.

In vitro neutrophil-mediated fungal killing assay. *A. fumigatus* Af293 was grown for 6 hours in DMEM + 10% (v/v) heat-inactivated FBS + 1% (v/v) penicillin/streptomycin at a

concentration of 3 x 10^3 conidia / well. Isolated mouse bone marrow neutrophils were co-incubated with hyphae for 16 hours at a concentration of 2 x 10^5 cells / well (MOI 1:67). The resulting hyphal growth was then measured by staining with a 1 mg/ml solution of calcofluor white, and measuring the fluorescence with an excitation of 340 nm and an emission at 440 nm.

Human neutrophil isolation. Neutrophils were purified from the blood of healthy volunteers, as previously described [404].

Neutrophil live cell imaging and migration assays. Neutrophil motility assays were performed as previously described [405,406]. Briefly, purified human neutrophils were added to collagen-coated wells and allowed to adhere for 1 hour. PBS or rGal-3 was added to a final concentration of 1 μM, and cells were imaged every 3 mins overnight. Single cells were tracked and their movements plotted. Neutrophil migration assays were performed as previously described [372], with minor modifications. Following loading the neutrophils with calcein AM, neutrophils were added to 3-μm pore transwell inserts, with or without 2 μM rGal-3 and incubated for 1 hour at 37°C and 5% CO_2.

Statistical analysis. All graphs were generated and statistical analyses were performed using Prism v6.0 (GraphPad Software). Statistical significance calculated as indicated. All data presented as the mean ± standard error of mean (SEM), unless otherwise noted.

2.7 Acknowledgements

Research described in this paper is supported by Canadian Institutes of Health Research (CIHR) grants 81361 and 123306, and GlycoNet (Networks of Centres of Excellence of Canada-Canadian Glycomics Network) grant AM-7. B.D.S. has been supported by graduate scholarships from CFC and CIHR. D.C.S. is supported by a Chercheur-Boursier Award from the Fonds de Recherche Quebec Santé.

2.8 Figures

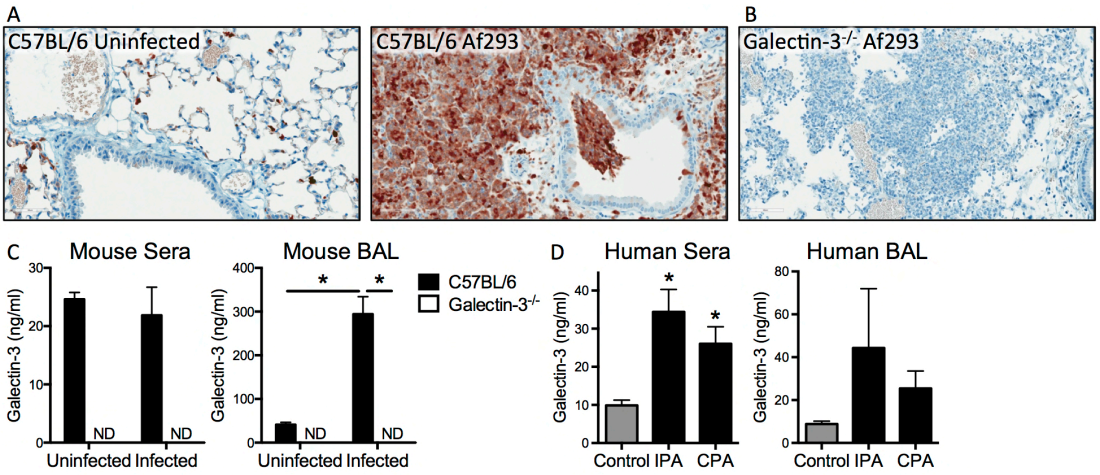


Figure 1. ***Aspergillus fumigatus*** **induces galectin-3 release** ***in vivo.*** (**A**) Pulmonary tissue sections from wild-type C57BL/6 mice infected or not with Af293 conidia stained for galectin-3 (brown) 36 hours post-infection. (**B**) Pulmonary tissue sections from galectin-3 deficient mice stained for galectin-3 as in (A). (**C**) Galectin-3 quantification by ELISA in sera and BAL fluid from the indicated infected and uninfected mouse strains. At least 6 wild-type and 4 galectin-3 deficient mice were tested per group. ND: not-detected. (**D**) Galectin-3 quantification by ELISA in the sera and BAL of human patients with invasive pulmonary aspergillosis (IPA), chronic pulmonary aspergillosis (CPA), and without fungal infection (control). $n = 5$ for IPA patients, 20 for CPA patients, and 10 for patients without fungal infection. *: $p < 0.05$; 2-way ANOVA for (C), or 1-way ANOVA for (D), both with Sidak's multiple comparison test.

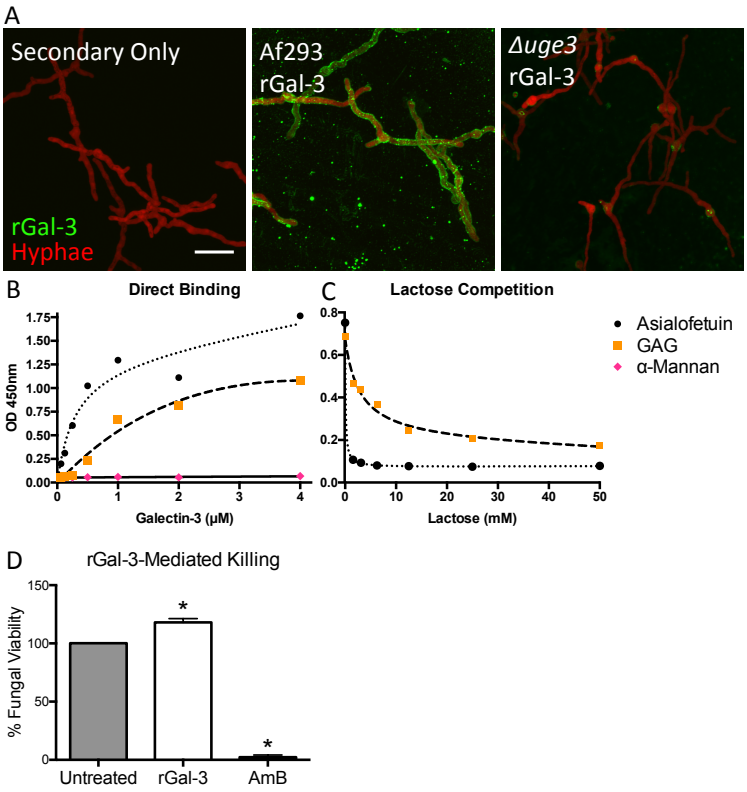


Figure 2. Galectin-3 binds to galactosaminogalactan (GAG) on the surface of *A. fumigatus* hyphae, but does not affect fungal growth or viability. (**A**) Wild-type Af293 and GAG-deficient Δ*uge3* hyphae (red) stained with recombinant galectin-3 (rGal-3, green). Scale bar = 20 μm. (**B**) Binding of the indicated concentrations of rGal-3 to immobilized GAG, the known galectin-3 ligand asialofetuin or α-mannan (non-galectin-3 ligand control) by ELISA. (**C**) Effects of the indicated concentration of lactose on the binding of 4 μM rGal3 to immobilized GAG. Non-linear fit via least-squares. (**D**) The viability of Af293 grown for 24 hours in the presence of 850 μg/ml rGal-3 or 8 μg/ml amphotericin B (AmB). Mean of 3 independent experiments. *: $p < 0.05$; 1-way ANOVA with Sidak's multiple comparison test.

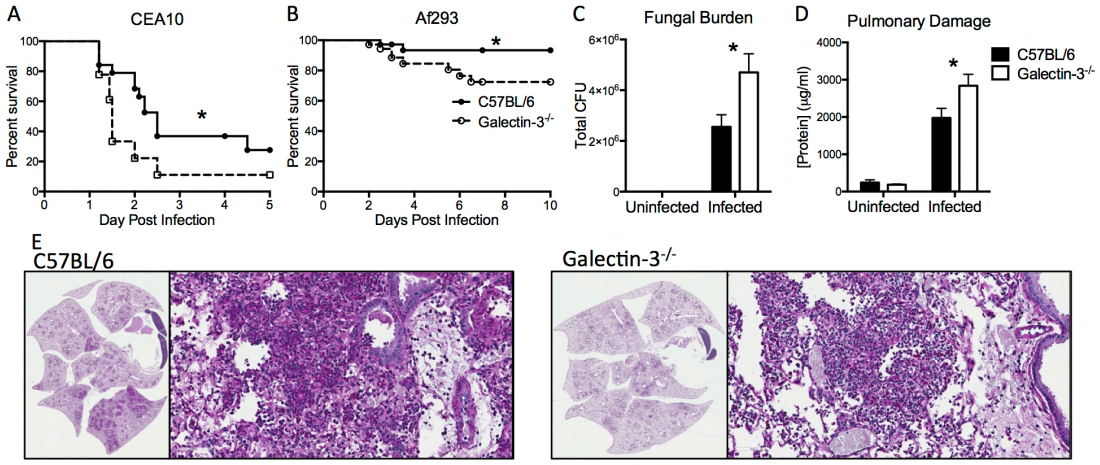


Figure 3. Galectin-3 deficient mice are more susceptible to pulmonary *A. fumigatus* infection. Survival of wild-type and galectin-3 deficient mice infected with conidia of *A. fumigatus* strain (**A**) CEA10 or (**B**) Af293. *n* = at least 18 mice per group from 2 independent experiments for (A), and at least 35 mice per group from 4 independent experiments for (B). *: $p < 0.05$; Mantel-Cox log rank test. (**C**) Pulmonary fungal burden and (**D**) total protein content in the BAL fluid of the indicated mouse strain at 36 hours post-infection with Af293 conidia. At least 20 infected and 7 uninfected mice per group from 3 independent experiments for (C) and (D). *: $p < 0.05$; 2-way ANOVA with Sidak's multiple comparison test. (**E**) Periodic acid-Schiff staining of lungs sections from wild-type and galectin-3 deficient mice 36 hours after infection with Af293 conidia.

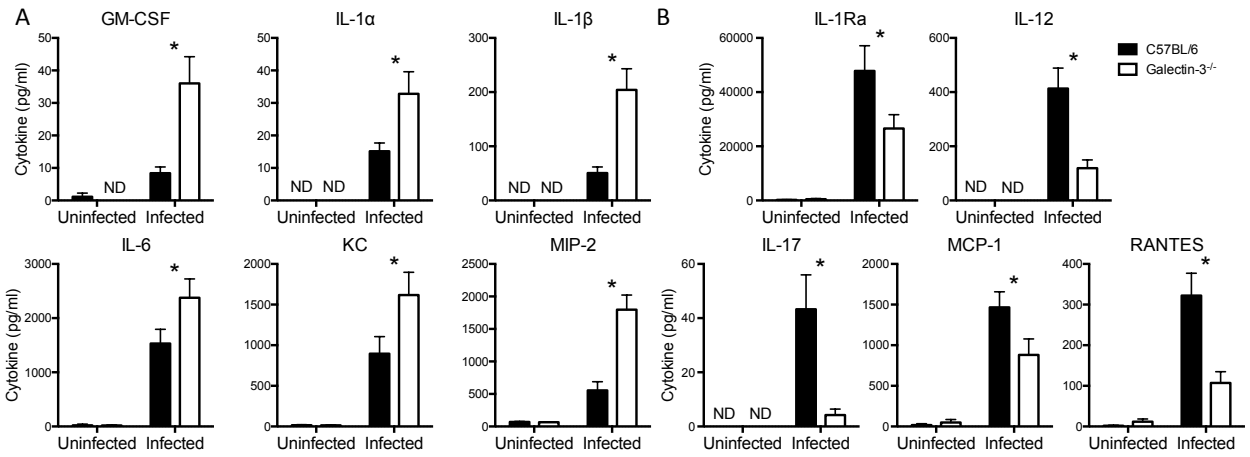


Figure 4. Galectin-3 deficient mice have altered cytokine responses during pulmonary *A. fumigatus* infection. Cytokine concentrations were measured in the BAL fluid of mice 36 hours post infection. (**A**) Cytokines up-regulated in infected galectin-3 deficient mice. (**B**) Cytokines down-regulated in infected galectin-3 deficient mice. At least 13 infected and 7 uninfected mice per group from 2 independent experiments. ND: not-detected. *: $p < 0.05$; 2-way ANOVA with Sidak's multiple comparison test.

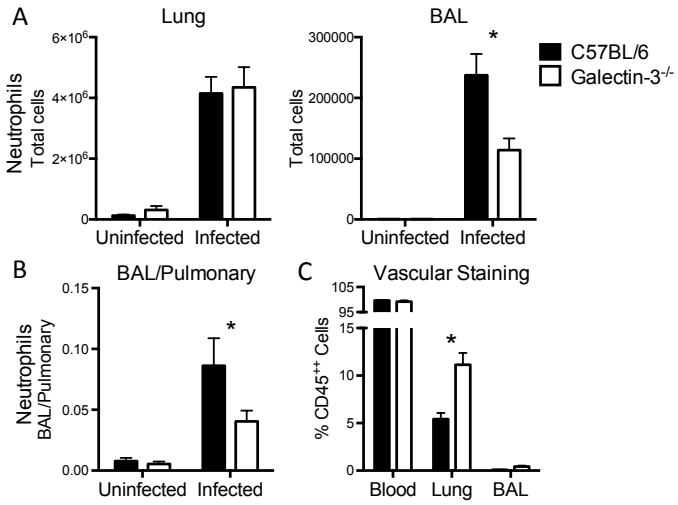


Figure 5. Galectin-3 deficient mice have impaired neutrophil recruitment to the airways during pulmonary *A. fumigatus* infection. (**A**) Quantification of the neutrophils in the whole lung digest and BAL fluid of the indicated mouse strains 36 hours post-infection. At least 18 infected and 11 uninfected mice per group from at least 3 independent experiments. (**B**) The proportion of total pulmonary neutrophils located within in the airways in (A). (**C**) The proportion of vasculature-associated neutrophils within the lung of the indicated mouse strains 36 hours post-infection with *A. fumigatus* conidia. At least 4 mice per group from 1 experiment. *: $p < 0.05$; 2-way ANOVA with Sidak's multiple comparison test.

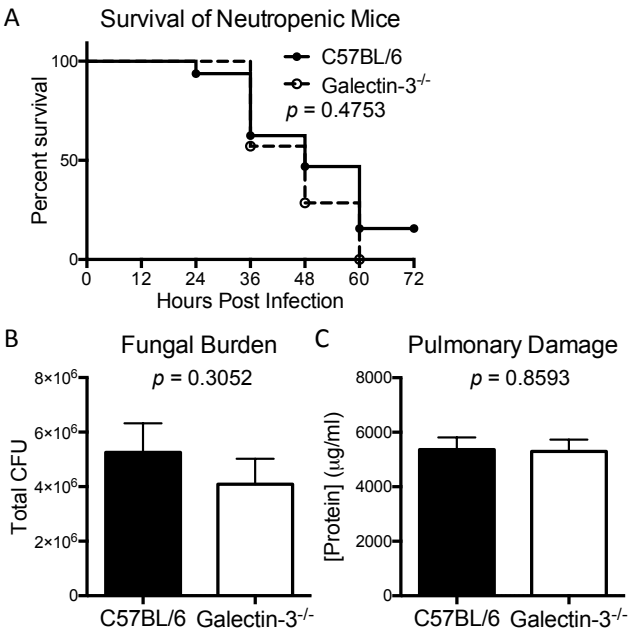


Figure 6. Wild-type and galectin-3 deficient neutropenic mice are equally susceptible to *A. fumigatus* in a pulmonary infection model. (**A**) Survival of neutropenic wild-type and galectin-3 deficient mice infected with Af293 conidia. Groups consist of at least 14 mice. Mantel-Cox log rank test. (**B**) Pulmonary fungal burden and (**C**) total protein content in the BAL fluid at 36 hours post-infection. $n = 6$ mice per group for (B) and (C). Mann Whitney test.

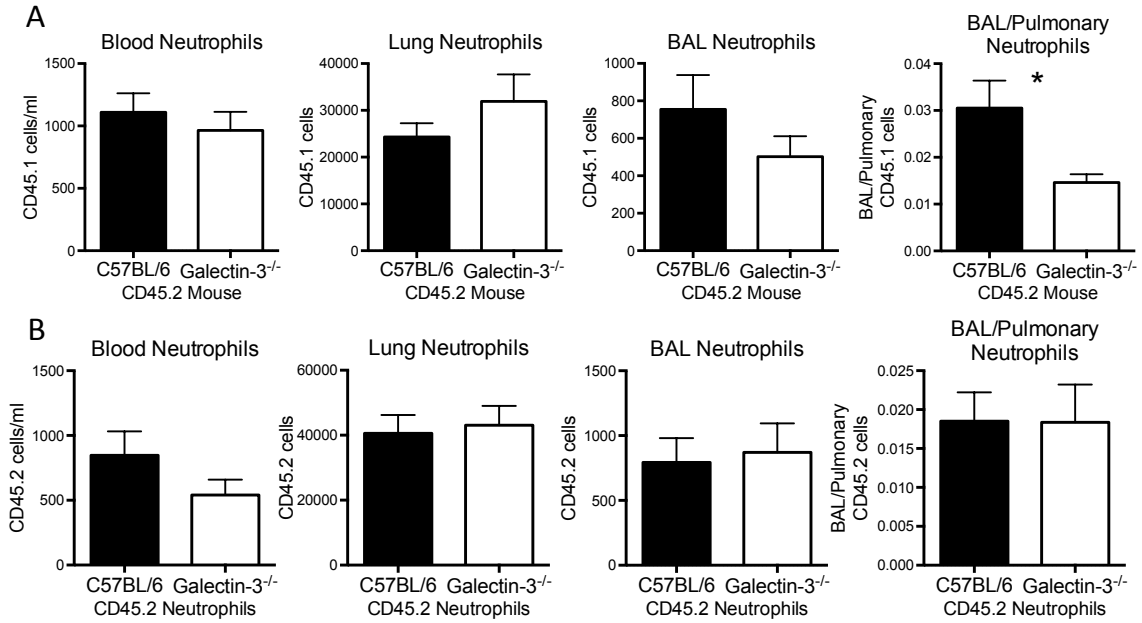


Figure 7. Neutrophil migration requires extrinsic galectin-3 during pulmonary *A. fumigatus* infection. (**A**) Neutrophils were isolated from the bone marrow of B6-CD45.1 mice, and injected intravenously into infected C57BL/6 and galectin-3 deficient mice. $n = 17$ mice per group from 4 independent experiments. (**B**) Neutrophils were isolated from the bone marrow of C57BL/6 and galectin-3 deficient mice, and injected intravenously into infected B6-CD45.1 mice. $n = 12$ mice per group from 2 independent experiments. Donor neutrophils in the indicated compartments were quantified by flow cytometry in both (A) and (B). *: $p < 0.05$; unpaired t-test.

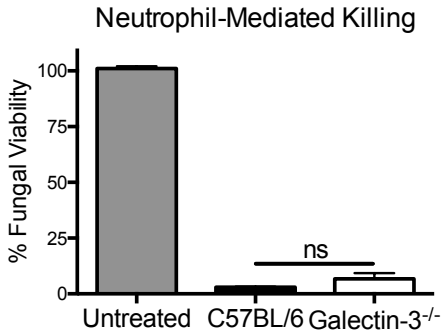


Figure 8. Endogenous galectin-3 is not required for neutrophil antifungal activity against *A. fumigatus*. The viability of Af293 hyphae incubated in the presence of murine bone marrow-isolated neutrophils (MOI 1:67) *in vitro* as determined by calcofluor white staining. Mean of 3 independent experiments. ns: not significant; 1-way ANOVA with Sidak's multiple comparison test.

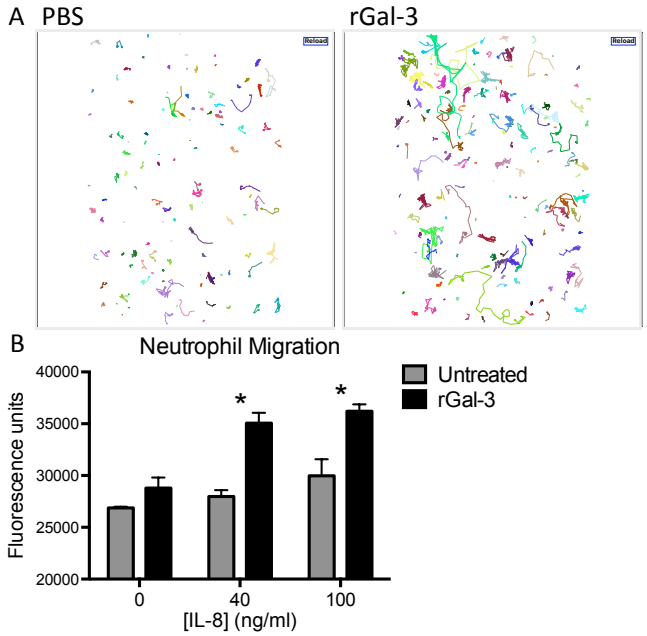
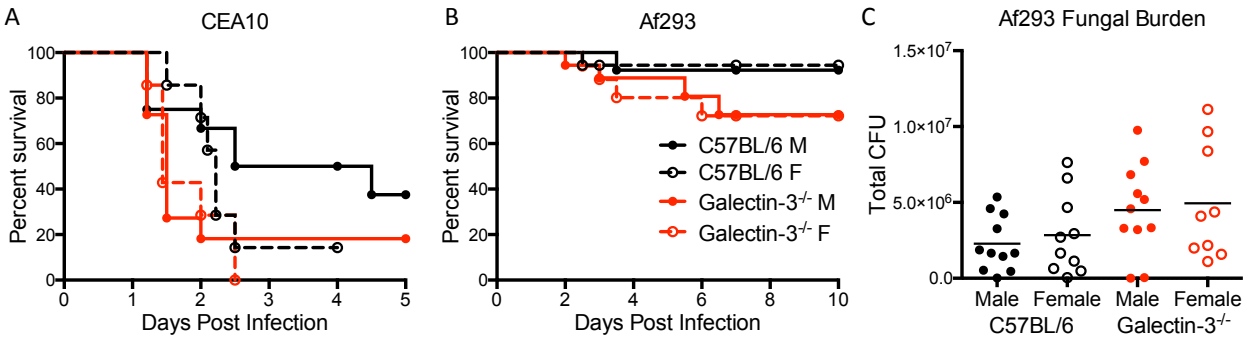
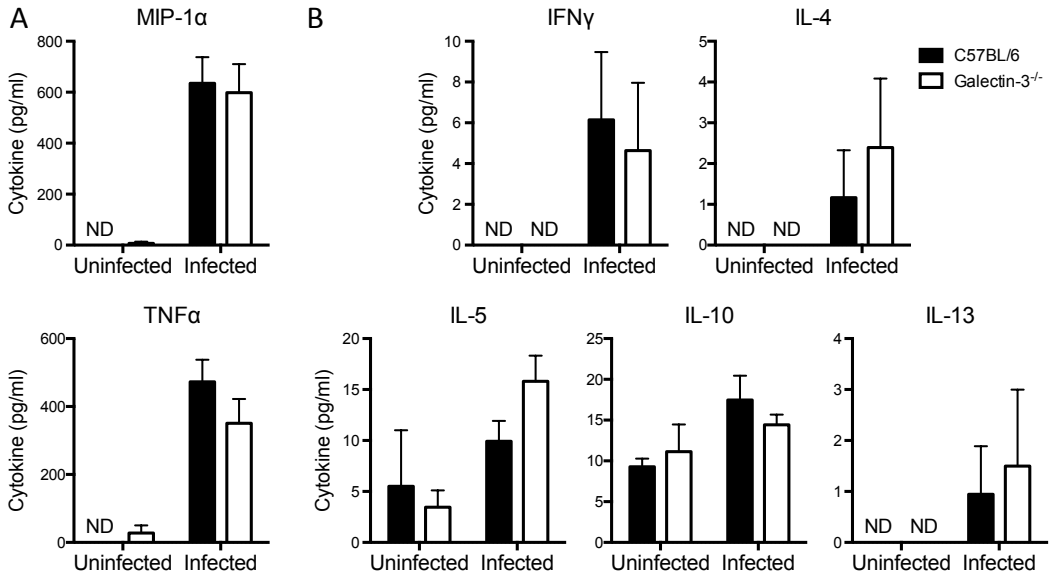


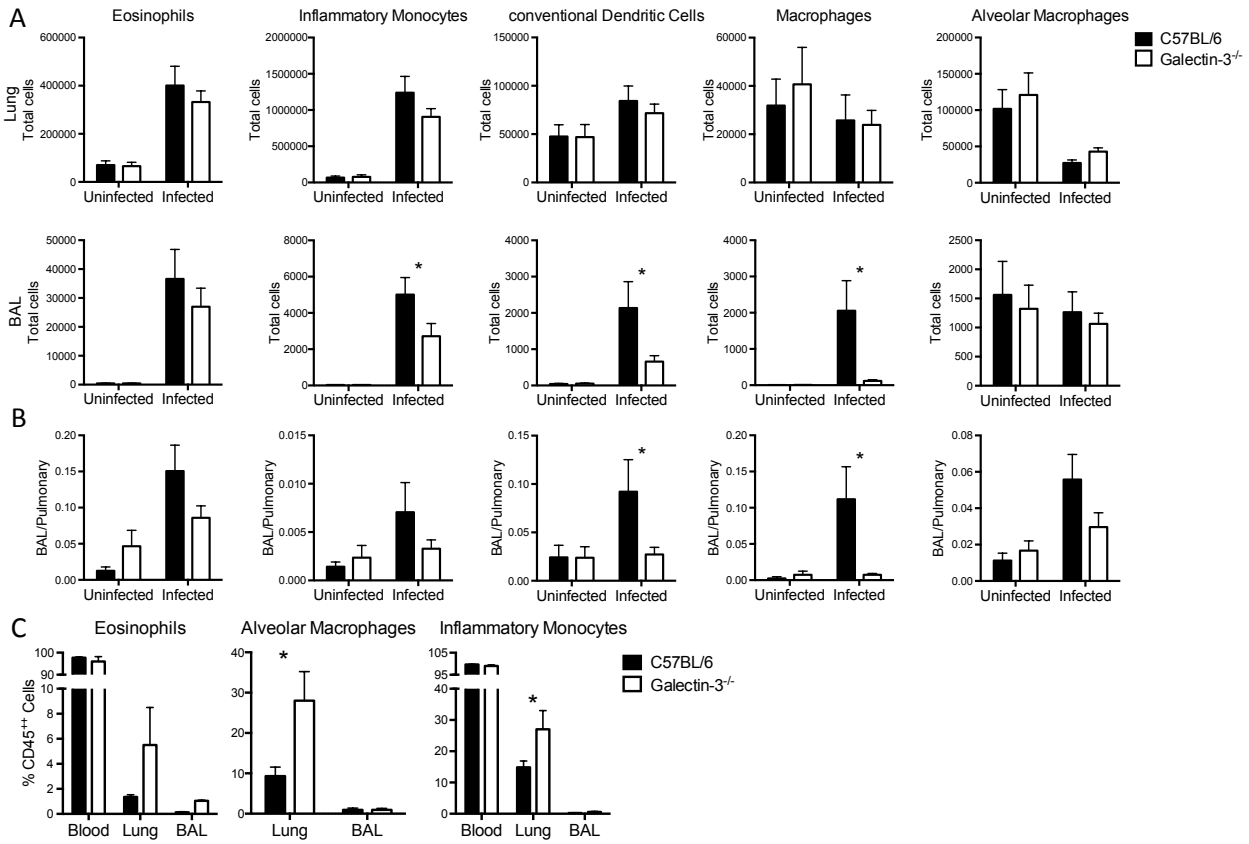
Figure 9. Exogenous galectin-3 enhances neutrophil motility and migration *in vitro.* (**A**) The motility of human neutrophils isolated from the peripheral blood in the presence or absence of rGal-3. Cells were incubated in the presence or absence of 1 µM rGal-3 and imaged every 3 min overnight. The resulting movements of each cell were then tracked using in-house software. Data are a representative example of 3 independent experiments. (**B**) The ability of calcein-labeled human neutrophils to migrate across a transwell membrane towards human IL-8 in the presence or absence of 2 µM rGal-3. Neutrophil migration was quantified by calcein fluorescence determination in the bottom well after 1 hour incubation. Mean ± SD from 1 experiment. *: $p < 0.05$; 2-way ANOVA with Sidak's multiple comparison test.



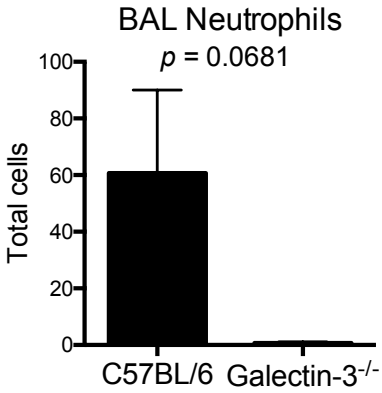
Supplementary Figure S1. Galectin-3 deficient mice exhibit no sex-dependent susceptibility to pulmonary *A. fumigatus* infection. (**A - C**) Stratification by sex of Figures 2A - C, respectively. *n* = at least 7 mice per group from 2 independent experiments for (A), at least 17 mice per group from 4 independent experiments for (B), and at least 9 mice per group from 3 independent experiments for (C). Mantel-Cox log rank test (A & B), 2-way ANOVA with Sidak's multiple comparison test (C).



Supplementary Figure S2. Expression levels of cytokines whose levels were not differentially expressed in galectin-3 deficient mice during pulmonary *A. fumigatus* infection. Cytokine concentrations were measured in the BAL fluid of mice 36 hours post infection. (**A**) Cytokines up-regulated equally in infected wild-type and galectin-3 deficient mice. (**B**) Cytokines not induced during pulmonary *A. fumigatus* infection. At least 13 infected and 7 uninfected mice per group from 2 independent experiments. ND: not-detected. 2-way ANOVA with Sidak's multiple comparison test.



Supplementary Figure S3. Pulmonary leukocyte populations during pulmonary *A. fumigatus* infection. (**A**) Quantification of eosinophils, inflammatory monocytes, conventional dendritic cells, macrophages, and alveolar macrophages in the whole lung digest and BAL fluid of the indicated mouse strains 36 hours post-infection. (**B**) The proportion of the total pulmonary cells in (A) located within in the airways. At least 12 infected and 9 uninfected mice per group from at least 2 independent experiments. (**C**) The proportion of vasculature-associated eosinophils, inflammatory monocytes, and alveolar macrophages of the indicated mouse strains 36 hours post-infection. At least 4 mice per group from 1 experiment. *: $p < 0.05$; 2-way ANOVA with Sidak's multiple comparison test.



Supplementary Figure S4. Ly6G antibody treatment depletes pulmonary neutrophils during *A. fumigatus* infection. Total neutrophil counts in the BAL fluid at 36 hours post-infection. $n = 6$ mice per group. Unpaired t-test.

Preface to Chapter 3

During glycan synthesis, GAG is bound by a number of proteins as it is modified and transported to the outer cell wall. We hypothesized that one or more of these glycan-binding proteins could be repurposed to bind and interfere with GAG localization or assembly within fungal biofilms. Within the biosynthetic cluster for galactosaminogalactan, we identified a membrane-bound protein capable of cleaving the polysaccharide, denoted Sph3. When the glycoside hydrolase domain of Sph3 was recombinantly expressed as a soluble protein ($Sph3_h$), it was able to degrade mature galactosaminogalactan on the surface of *A. fumigatus* hyphae, rendering it non-adherent and more susceptible to antifungal therapy. When administered to mice at the time of an *A. fumigatus* infection, $Sph3_h$ treatment was well tolerated and reduced pulmonary fungal burden to the level of the galactosaminogalactan-deficient strain. Excitingly, a hydrolase domain involved in the synthesis of a similar polysaccharide produced by the Gram-negative bacteria *Pseudomonas aeruginosa* ($PelA_h$) was also effective against *A. fumigatus* biofilms. This cross-kingdom activity suggests that a hydrolase-based antibiofilm therapy could exhibit broad-spectrum applicability. This work originally appeared in The Proceedings of the National Academy of Science in July 2017.

CHAPTER 3: Microbial glycoside hydrolases as antibiofilm agents with cross-kingdom activity

Microbial glycoside hydrolases as antibiofilm agents with cross-kingdom activity

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Adapted from: *Proc Natl Acad Sci USA* 2017 Jul 3;114(27);7124-7129.

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3.1 Abstract

Galactosaminogalactan and Pel are cationic heteropolysaccharides produced by the opportunistic pathogens, *Aspergillus fumigatus* and *Pseudomonas aeruginosa,* respectively. These exopolysaccharides both contain 1,4-linked *N*-acetyl-D-galactosamine and play an important role in biofilm formation by these organisms. Proteins containing glycoside hydrolase domains have recently been identified within the biosynthetic pathway of each exopolysaccharide. Recombinant hydrolase domains from these proteins ($Sph3_h$ from *A. fumigatus* and $PelA_h$ from *P. aeruginosa*) were found to degrade their respective polysaccharides *in vitro*. We therefore hypothesized that these glycoside hydrolases could exhibit anti-biofilm activity, and further, given the chemical similarity between galactosaminogalactan and Pel, that they might display cross-species activity. Treatment of *A. fumigatus* with $Sph3_h$ disrupted *A. fumigatus* biofilms with an EC_{50} of 0.4 nM. $PelA_h$ treatment also disrupted pre-formed *A. fumigatus* biofilms with EC_{50} values similar to those obtained for $Sph3_h$. In contrast, $Sph3_h$ was unable to disrupt *P. aeruginosa* Pel-based biofilms, despite being able to bind to the exopolysaccharide. Treatment of *A. fumigatus* hyphae with either $Sph3_h$ or $PelA_h$ significantly enhanced the activity of the antifungals posaconazole, amphotericin B and caspofungin, likely through increasing antifungal penetration of hyphae. Both enzymes were non-cytotoxic and protected A549 pulmonary epithelial cells from *A. fumigatus*-induced cell damage for up to 24 hours. Intratracheal administration of $Sph3_h$ was well tolerated, and reduced pulmonary fungal burden in a neutropenic mouse model of invasive aspergillosis. These findings suggest that glycoside hydrolases can exhibit activity against diverse microorganisms and may be useful as therapeutic agents by degrading biofilms and attenuating virulence.

3.2 Significance

The production of biofilms is an important strategy used by both bacteria and fungi to colonize surfaces and to enhance resistance to killing by immune cells and antimicrobial agents. We demonstrate that glycoside hydrolases derived from the opportunistic fungus *Aspergillus fumigatus* and Gram-negative bacterium *Pseudomonas aeruginosa* can be exploited to disrupt pre-formed fungal biofilms and reduce virulence. Additionally, these glycoside hydrolases can be utilized to potentiate antifungal drugs by increasing their hyphal penetration, to protect human cells from fungal-induced injury and to attenuate virulence of *A. fumigatus* in a mouse model of invasive aspergillosis. The findings of this study identify recombinant microbial glycoside

hydrolases as promising therapeutics with the potential for anti-biofilm activity against pathogens across different taxonomic kingdoms.

3.3 Introduction

The mould *Aspergillus fumigatus* and the Gram-negative bacterium *Pseudomonas aeruginosa* are opportunistic pathogens that cause pulmonary infection in immunocompromised patients and individuals who suffer from chronic lung diseases such as cystic fibrosis and bronchiectasis. *A. fumigatus* is the second most common nosocomial fungal infection [407] and ~10% of all nosocomial bacterial infections are caused by *P. aeruginosa* [408]. Mortality associated with *P. aeruginosa* infections is high [409], and has increased with the emergence of multi- and even pan-resistance to antibiotics [409,410]. Similarly, invasive aspergillosis is associated with mortality rates of up to 50% [382], and increasing rates of antifungal resistance have been reported worldwide [411]. These factors underscore the urgent need for new effective therapies for these infections.

Although *A. fumigatus* and *P. aeruginosa* are members of different taxonomic kingdoms, both produce biofilms that constitute a protective lifestyle for the organism. Biofilms are complex communities of microorganisms that grow embedded in an extracellular matrix composed of DNA, protein, and exopolysaccharide [412]. Biofilm formation provides a significant advantage to these organisms as the matrix mediates adherence to host cells [296,413] and aids in the resistance to both antimicrobial agents [414,415] and host immune defences [30,416]. *A. fumigatus* biofilm formation is dependent on the cationic polysaccharide galactosaminogalactan (GAG), a heteroglycan composed of α1,4-linked galactose and *N*-acetyl-D-galactosamine (GalNAc) that is partially deacetylated [28,29]. In comparison, *P. aeruginosa* has the genetic capacity to produce three biofilm exopolysaccharides; alginate, Psl and Pel [417]. GAG shares several similarities with Pel, which has been identified as a cationic heteroglycan composed of 1,4-linked GalNAc and *N*-acetyl-D-glucosamine (GlcNAc) [418]. Like GAG, the cationic nature of Pel results from partial deacetylation of the polymer [418]. Most clinical and environmental isolates of *P. aeruginosa* utilize Pel and Psl during biofilm formation [419]. Alginate is dispensable for biofilm formation and is only observed in chronic pulmonary infection when strains switch to a mucoid phenotype [419,420].

Strains of *Aspergillus* and *P. aeruginosa* with impaired GAG, or Pel and Psl biosynthesis exhibit attenuated virulence [60,421], suggesting that targeting these exopolysaccharides may be

a useful therapeutic strategy. We previously demonstrated that recombinant glycoside hydrolases $PelA_h$ and $PslG_h$, encoded in the *pel* and *psl* operons of *P. aeruginosa*, respectively, target and selectively hydrolyze the Pel and Psl exopolysaccharide components of the *Pseudomonas* biofilm matrix [422]. Treatment with these enzymes rapidly disrupts established biofilms, increasing the susceptibility of *P. aeruginosa* to human neutrophil killing and potentiation of the antibiotic colistin [422].

Our recent work on *Aspergillus* has identified a cluster of five genes, which encode the proteins necessary for GAG biosynthesis [29]. As with *P. aeruginosa*, we found that the product of one of these genes contains a glycoside hydrolase domain, $Sph3_h$, that is capable of hydrolyzing purified and cell wall-associated GAG [359]. In the present study we assessed the therapeutic potential of $Sph3_h$ in disrupting fungal biofilms. We establish that the exogenous addition of $Sph3_h$ is capable of rapidly disrupting existing biofilms of this organism at nanomolar concentrations. Additionally, we demonstrate cross-kingdom activity, as the *P. aeruginosa* glycoside hydrolase, $PelA_h$, was able to disrupt *A. fumigatus* biofilms. While $Sph3_h$ was able to bind Pel, it was unable to disrupt pre-formed *P. aeruginosa* Pel-mediated biofilms. Treatment with $Sph3_h$ or $PelA_h$ increased the susceptibility of wild-type and azole-resistant *A. fumigatus* strains to lipophilic antifungal drugs. Kinetic studies with labeled posaconazole indicate that the increased susceptibility to antifungals is due to increased penetration of fungal cells by these agents. Both $Sph3_h$ and $PelA_h$ were non-toxic to mammalian cells and protected epithelial cells from *A. fumigatus*-induced damage for up to 24 hours. Intratracheal delivery of $Sph3_h$ was well tolerated by mice and significantly reduced the fungal burden of immunocompromised mice infected with *A. fumigatus*. Our results suggest that glycoside hydrolases have the potential to be effective anti-biofilm therapeutics that can mediate activity against evolutionarily diverse microorganisms.

3.4 Results

$Sph3_h$ disrupts preformed *A. fumigatus* biofilms. Our previous work demonstrated that $Sph3_h$ from *A. fumigatus* and *Aspergillus clavatus* can hydrolyze both purified and cell wall-bound GAG on young hyphae [359]. We therefore sought to determine if the degradation of GAG by $Sph3_h$ could disrupt established *A. fumigatus* biofilms. Treatment with $Sph3_h$ for one hour disrupted established *A. fumigatus* biofilms with an effective concentration for 50% activity

(EC_{50}) of 0.45 ± 1.31 nM (**Figure 1a**). Biofilm disruption was associated with a marked reduction in hyphae-associated GAG as detected by lectin staining (**Figure 1b and c**) and scanning electron microscopy (**Figure 1d**). A catalytic variant $Sph3_h$ D166A, which does not mediate GAG hydrolysis [359], displayed a greater than 500-fold reduction in anti-biofilm activity (**Figure 1a**) and failed to mediate degradation of biofilm-associated GAG (**Figure 1b and c**). Collectively, these data suggest that biofilm disruption is mediated through the enzymatic hydrolysis of GAG.

To validate that fungal biofilm disruption by $Sph3_h$ is not restricted to the *A. fumigatus* laboratory strain Af293, the activity of $Sph3_h$ was evaluated against four clinical *A. fumigatus* isolates. $Sph3_h$ disrupted biofilms of all isolates tested at EC_{50} values < 0.15 nM (**Supplementary Figure S1**). These results confirm the role of GAG in biofilm formation and indicate that $Sph3_h$ exhibits anti-biofilm activity across a wide range of *A. fumigatus* strains.

The bacterial hydrolase $PelA_h$ hydrolyses GAG and disrupts fungal biofilms. Given that GAG and Pel are both cationic exopolysaccharides containing 1,4-linked GalNAc [28,418], we hypothesized that $PelA_h$ might exhibit activity against GAG. Consistent with this hypothesis, an *in vitro* reducing sugar assay demonstrated that $PelA_h$ was capable of hydrolyzing purified GAG (**Supplementary Figure S2**). Furthermore, using the crystal violet biofilm assay, we found that $PelA_h$ disrupted *A. fumigatus* fungal biofilms with an EC_{50} value of 2.80 ± 1.14 nM (**Figure 2a**). The treatment of *A. fumigatus* hyphae with $PelA_h$ also resulted in a reduction in the amount of cell wall-associated GAG (**Figure 2b-d**) as was observed with $Sph3_h$ treatment. The $PelA_h$ catalytic variant, $PelA_h$ E218A, which is markedly impaired in Pel hydrolysis and is inactive against *P. aeruginosa* biofilms [422] did not significantly hydrolyze GAG at concentrations as high as 12 μM (**Supplementary Figure S2**). Consistent with this observation, $PelA_h$ E218A was also several hundred-fold less active against *A. fumigatus* biofilms and did not degrade hyphae-associated GAG (**Figure 2b and c**). These results suggest that $PelA_h$ disrupts *A. fumigatus* biofilms through the hydrolysis of biofilm-associated GAG.

$Sph3_h$ binds Pel but does not disrupt established *P. aeruginosa* biofilms. Given that $PelA_h$ can hydrolyze GAG and disrupt GAG-mediated biofilms, we hypothesized that $Sph3_h$ may exhibit activity against Pel and Pel-mediated biofilms. The inability to purify sufficient quantities

of Pel precluded us from utilizing it as a substrate. Therefore, to examine whether $Sph3_h$ was capable of hydrolyzing Pel, the enzyme was exogenously applied to biofilms produced by the Pel overproducing *P. aeruginosa* strain PAO1 Δ*wspF* Δ*psl* P_{BAD}*pel*. Treatment of these established biofilms with $Sph3_h$ did not affect levels of Pel within the biofilms as visualized by lectin staining (**Figure 3a and b**), nor did it reduce biofilm biomass, even at concentrations exceeding 10 μM (**Figure 3c**).

Since $Sph3_h$ did not hydrolyze Pel within *P. aeruginosa* biofilms, we tested whether the enzyme was capable of recognizing and binding this polysaccharide. Using an ELISA-based binding assay we observed dose-dependent binding of $Sph3_h$ to culture supernatants from the Pel over-producing *P. aeruginosa* strain, but not from supernatants of the Pel-deficient strain PAO1 Δ*wspF* Δ*pel* Δ*psl* (**Figure 3d**). These data suggest that the inability of $Sph3_h$ to disrupt Pel-mediated biofilms is likely a consequence of an inability to hydrolyze Pel rather than being unable to bind the polysaccharide. Dose-dependent binding of the inactive $Sph3_h$ D166A variant to GAG-containing culture supernatants was also observed (**Supplementary Figure S3**), suggesting that binding of hydrolases to exopolysaccharides is insufficient to disrupt established biofilms in the absence of enzymatic cleavage of the polymer.

$Sph3_h$ and $PelA_h$ potentiate antifungals by enhancing their intracellular penetration. The Pel polysaccharide enhances resistance to several antibiotics including aminoglycosides and colistin [422-424]. Since biofilm formation by *A. fumigatus* is associated with increased resistance to a number of antifungal agents [425-427], we hypothesized that GAG may have an analogous function to Pel in enhancing resistance to antifungal agents. To test this hypothesis, we investigated whether $Sph3_h$ or $PelA_h$ could potentiate the activity of commonly used antifungal drugs. Treatment of established fungal biofilms with either enzyme resulted in a significant reduction in the MIC_{50} of the azole posaconazole, the polyene amphotericin B, and the echinocandin caspofungin (**Figure 4a**). $Sph3_h$ or $PelA_h$ treatment produced a similar increase in sensitivity to posaconazole for both azole-sensitive and azole-resistant strains of *A. fumigatus* (**Supplementary Figure S4**). Susceptibility to voriconazole, a smaller and more polar azole, was unaffected by treatment with either glycoside hydrolase (**Figure 4a**). Since both posaconazole and voriconazole have the same intracellular target, these findings suggest that cationic GAG mediates antifungal resistance by hindering cellular uptake of large, nonpolar molecules such as

posaconazole. To investigate this hypothesis, the effect of $Sph3_h$ on intracellular penetration of posaconazole was examined using posaconazole conjugated to the fluorophore BODIPY (BDP-PCZ). Previous work has established that BDP-PCZ displays similar cellular and subcellular pharmacokinetics to unmodified posaconazole [86]. Fluorometric studies revealed that $Sph3_h$-treatment resulted in higher accumulation of BDP-PCZ within *A. fumigatus* hyphae (**Figure 4b**). This finding indicates that GAG protects *A. fumigatus* from the action of lipophilic antifungals by limiting their penetration into hyphae.

Recombinant $Sph3_h$ and $PelA_h$ protect epithelial cells from damage by *A. fumigatus*. *A. fumigatus* GAG-mediated adherence is required for *A. fumigatus* to damage A549 pulmonary epithelial cells *in vitro* [60]. We therefore tested whether treatment with either $Sph3_h$ or $PelA_h$ could protect epithelial cells from fungal-induced injury using an established chromium (^{51}Cr) release damage assay [428]. We first established that the enzymes were not cytotoxic and that the addition of $Sph3_h$ or $PelA_h$ to uninfected A549 cell monolayers did not cause detectable cellular damage (**Supplementary Figure S5a**), a finding verified with the IMR-90 human lung fibroblast cell line (**Supplementary Figure S5b**). These data are consistent with the lack of cytotoxicity previously reported for $PelA_h$ [422]. Next, we assessed whether $Sph3_h$ or $PelA_h$ were able to protect A549 cell monolayers from damage by *A. fumigatus*. $Sph3_h$ significantly reduced epithelial cell injury for up to 24 hours (**Figure 5a**). Treatment with $PelA_h$ also protected epithelial cells from *A. fumigatus*-induced damage (**Figure 5a**). The protective effect of $PelA_h$ was shorter than that observed with $Sph3_h$, and was lost before 24 hours of treatment. The addition of protease inhibitors extended $PelA_h$-mediated epithelial cell protection to 24 hours (**Supplementary Figure S5c**), suggesting that the decrease in $PelA_h$ mediated protection was likely due to proteolytic degradation of the recombinant protein. Epithelial cell protection was not observed with the catalytic variants, $PelA_h$ E218A or $Sph3_h$ D166A, suggesting that the hydrolytic activity of the enzymes is required for protection (**Figure 5a**).

Intratracheal $Sph3_h$ is well tolerated, and attenuates fungal virulence in an immunocompromised mouse model of pulmonary aspergillosis. Given the ability of $Sph3_h$ to protect epithelial cells for over 24 hours, this hydrolase was selected for evaluation *in vivo*. BALB/c mice treated intratracheally with doses up to 500 μg of $Sph3_h$ exhibited no signs of

stress, weight loss or change in body temperature post-treatment (**Supplementary Figure S6a and b**). Additionally, no significant increase in pulmonary injury or inflammation between treated and untreated mice were observed as measured by bronchoalveolar lavage lactate dehydrogenase activity and total pulmonary leukocyte populations (**Supplementary Figure 5b and Figure S6c**). Collectively these results suggest that a single intratracheal dose of $Sph3_h$ is well tolerated by mice.

To determine the ability of $Sph3_h$ to attenuate virulence of *A. fumigatus*, neutropenic BALB/c mice were infected intratracheally with *A. fumigatus* conidia with or without the co-administration of 500 μg of $Sph3_h$. Four days after infection, mice infected with *A. fumigatus* and treated with $Sph3_h$ had a significantly lower pulmonary fungal burden to untreated, infected mice as measured by both fungal DNA (**Figure 5c**) and pulmonary galactomannan content (**Supplementary Figure S7**). The fungal burden of the $Sph3_h$-treated mice was similar to that observed with mice infected with the GAG-deficient hypovirulent strain Δ*uge3* [60]. Consistent with the fungal burden data, histopathologic examination of lung sections revealed the presence of fungal lesions in untreated, infected mice, but no detectable lesions in the lungs of infected mice treated with $Sph3_h$, or those infected with conidia of the Δ*uge3* mutant (**Figure 5d**). These findings suggest that $Sph3_h$-mediated degradation of GAG can limit the growth of *A. fumigatus in vivo*, to the same degree as is observed with GAG-deficient organisms.

3.5 Discussion

In this study, we demonstrate that the fungal glycoside hydrolase $Sph3_h$ is able to degrade pre-formed *A. fumigatus* biofilms. This study is an example of the use of a glycoside hydrolase to disrupt a fungal biofilm. Further, we establish that the glycoside hydrolase $PelA_h$ displays activity against biofilms formed by organisms across different microbial kingdoms. Both glycoside hydrolases potentiated the penetration and activity of antifungal agents *in vitro*, exhibited no toxicity against mammalian cells and protected epithelial cells from *A. fumigatus*-induced damage. Pulmonary administration of $Sph3_h$ was well tolerated and limited fungal growth in an immunocompromised mouse model, suggesting that these enzymes are promising therapeutic agents for the treatment of fungal disease.

The mechanism by which the biofilm matrix enhances *A. fumigatus* resistance to antifungals is poorly understood. The effect of hydrolase treatment on the antifungal sensitivity of *A. fumigatus* provides some insight into this question and establishes a role for GAG in biofilm-

associated antifungal resistance. Multiple observations suggest that GAG enhances antifungal resistance by acting as a barrier to antifungal penetration of hyphae. First, glycoside hydrolase degradation of GAG enhanced the activity of multiple antifungals with different mechanisms of action. Second, the activity of posaconazole, but not voriconazole, was enhanced even though both azoles target the same enzyme, CYP51A. These hydrolases also display similar activity against azole-resistant and azole-sensitive strains. The cationic nature of GAG may explain the differential effects on voriconazole as compared with other antifungals. The GAG barrier would be predicted to be most effective against large, lipophilic or cationic antimicrobial agents, and thus therapeutic hydrolases may be most effective as adjuvants for lipophilic antifungals. Previous studies have reported that the enzymatic degradation of neutral α-glucans of *A. fumigatus* did not enhance susceptibility to antifungals [25], further supporting our hypothesis that exopolysaccharide charge plays a role in mediating antibiotic resistance. Similarly, hydrolysis of cationic Pel exopolysaccharide by $PelA_h$ enhances the activity of the polycationic antibacterial colistin [422]. Interestingly, degradation of biofilm-associated extracellular DNA (eDNA) has previously been reported to enhance *A. fumi*gatus susceptibility to caspofungin and amphotericin B, though the effects on posaconazole and voriconazole susceptibility were not reported in the study [425]. Recent work has suggested that Pel anchors eDNA within *P. aeruginosa* biofilms through charge-charge interactions [418]. Given the similarities between Pel and GAG, it is possible that GAG-mediated binding of eDNA may also contribute to enhancing antifungal resistance.

The results of these studies add to an emerging body of evidence that fungal biofilms share structural [429-432] and functional [425,433,434] similarity with those formed by pathogenic bacteria. The finding that glycoside hydrolases can display activity against the exopolysaccharides and biofilms of both fungi and bacteria provides evidence that these similarities could potentially be exploited for the development of therapeutics active against both organisms. Additionally, the similarity between the exopolysaccharides of *P. aeruginosa* and *A. fumigatus*, coupled with the interspecies activity of their glycoside hydrolases suggest the intriguing possibility that exopolysaccharide interactions may occur between organisms during multispecies biofilm formation. Co-colonization with *P. aeruginosa* and *A. fumigatus* is not uncommon in patients with chronic pulmonary disease such as cystic fibrosis [435]. Although studies of the formation of mixed fungal-bacterial biofilms during pulmonary infection are

limited, a recent study of patients with chronic lung disease reported that antibacterial therapy for *P. aeruginosa* was associated with a reduction in fungal colonization, suggesting the possibility of microbial cooperation [436]. Further studies examining the role of cross-species exopolysaccharide and exopolysaccharide-modifying enzyme interactions are required to establish a role for cooperative biofilm interactions in pulmonary disease.

While $PelA_h$ exhibited cross-species activity and disrupted pre-formed fungal biofilms, $Sph3_h$ bound Pel, but was unable to disrupt established Pel-mediated biofilms. This difference in activity may reflect differences in the composition or conformation of each polysaccharide since GAG is a heteropolymer of GalNAc and galactose while Pel is comprised of GalNAc and GlcNAc. It is likely that these differences influence the ability of $Sph3_h$ and $PelA_h$ to hydrolyze the polymer. The inability of $Sph3_h$ to degrade pre-formed *P. aeruginosa* biofilms may suggest that mature Pel adopts a configuration or undergoes post-synthetic modification that renders it incompatible with the catalytic active site of $Sph3_h$ and resistant to cleavage. Detailed studies of these enzymes to determine the mechanisms underlying their differential activity against Pel will require purified polysaccharide, which is currently not available.

Both $Sph3_h$ and $PelA_h$ were found to be non-cytotoxic, and a single dose of intratracheal $Sph3_h$ was well tolerated by BALB/c mice. Co-administration of $Sph3_h$ with wild-type conidia to neutropenic mice greatly reduced fungal outgrowth within the lungs of these mice. Together these results provide proof-of-concept that the glycoside hydrolases can be used to improve the outcome of fungal infection, with minimal side effects and toxicity. These findings will pave the way for future work to evaluate the utility of these agents as antifungal therapeutics including detailed pharmacokinetic and toxicity studies, as well as the evaluation of these enzymes for the treatment of established fungal infections alone and in combination therapy with lipophilic antifungal agents such as posaconazole or amphotericin B.

3.6 Methods

Strains and culture conditions. Strains used in this study are detailed in **Table S1** and detailed culture conditions are described in the Supplementary Information (SI).

Recombinant hydrolase expression and purification. Hydrolases were expressed and purified as described previously [359,422].

Treatment of *A. fumigatus* with glycoside hydrolases. To visualize the effects of hydrolases on cell wall-associated GAG, hyphae were treated with recombinant hydrolases and

stained with fluorescein-conjugated soybean agglutinin as previously described [359], with minor modifications. Hyphae were counterstained with a 1:1000 dilution of DRAQ5 (eBioscience) in phosphate buffered saline (PBS) for 5 min prior to paraformaldehyde (PFA) fixation. Complete image acquisition and processing methods can be found in the SI. To study the effects of hydrolases on biofilms, 10^4 conidia were grown in Brian media in polystyrene, 96-well plates for 19 h at 37 °C and 5% CO_2 and then treated with the indicated concentration of glycoside hydrolase in PBS for 1 h at room temperature. Biofilms were then gently washed, stained with 0.1% (w/v) crystal violet and de-stained with 100% ethanol for 10 min. The optical density of the de-stain fluid was measured at 600 nm (OD_{600}).

Scanning electron microscopy. Conidia were grown for 9 h in Dulbecco's Modified Eagle's Medium (DMEM) at 37 °C, 5% CO_2 on glass coverslips, washed once with Ham's F- 12K (Kaighn's) Medium, and incubated with 500 µL in F-12K Medium with or without 0.5 µM hydrolase for 3 h at 37°C, 5% CO_2. Coverslips were processed for scanning electron microscopy as previously described [60], and detailed in the SI.

Treatment of *P. aeruginosa* with glycoside hydrolases. For biofilm disruption, static *P. aeruginosa* cultures were grown for 22 h at 30 °C, at which point the planktonic cells were aspirated and LB + 0.5% arabinose + 0.5 µM glycoside hydrolase was added for an additional 3 h. For the detection of Pel, samples were incubated with 30 µg/ml of fluorescein-conjugated *Wisteria fluoribunda* lectin for 2 h at 4 °C, fixed with 8% (w/v) PFA for 20 min at 4° C and imaged as detailed in the SI. The ability of hydrolases to disrupt established biofilms were studied as previously described [422].

Culture supernatant production. *P. aeruginosa* cultures were grown at 30 °C for 24 h shaking at 200 rpm. Cultures were then centrifuged at 311 *x g* for 10 mins, and supernatants were filtered using 0.44 µm syringe filters. Culture supernatants were stored at -20 °C until use.

Hydrolase binding quantification. Undiluted culture supernatants were incubated on Immunolon® 2HB high-binding 96 well microtiter plates overnight at 4 °C. Wells were washed 3X with Wash Buffer (PBS + 0.05% (v/v) Tween-20) and blocked for 30 min at 4 °C in Blocking Buffer (1% (w/v) Bovine Serum Albumin in Wash Buffer). Wells were washed 1X with Wash Buffer and incubated with the indicated concentrations of $Sph3_h$ diluted in Blocking Buffer for 3 h at 4 °C. Wells were washed 3X with Wash Buffer and incubated with polyclonal rabbit anti-$Sph3_h$ (custom-produced by Cedarlane Laboratories) diluted 1/100 in Blocking Buffer

for 1.5 h at 4 °C. Wells were then washed 3X with Wash Buffer and incubated with donkey-anti-rabbit secondary antibody conjugated to horseradish peroxidase diluted 1/2000 in Blocking Buffer for 1 h at 4 °C. Wells were then washed 4X with Wash Buffer and incubated with TMB substrate (ThermoFisher®) for 15 mins at room temperature. The reaction was stopped with the addition of 2 N H_2SO_4 and absorbance read at 450 nm with a 570 nm correction.

Effects of glycoside hydrolases on antifungal susceptibility of *A. fumigatus*. Fungal biofilms were prepared in tissue culture treated 24 well plates in RPMI 1640 medium (Life Technology) buffered with MOPS (3-(N-Morpholino) Propane-Sulfonic Acid) (Fisher) (RPMI-MOPS) for 9 h at 37 °C, 5% CO_2. Serial dilutions of antifungal compounds with or without 0.5 μM of $Sph3_h$ or $PelA_h$ were added to wells and the plates incubated at 37 °C and 5% CO_2 for 15 h. Fungal viability was measured using the sodium 3′-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) metabolic assay as described previously [437]. The concentration of antifungal resulting in a 50% decrease in viability (MIC_{50}) was used as a measure of antifungal effect.

Fluorometric quantification of hyphal uptake of BDP-PCZ. $2.5x10^4$ conidia of red fluorescent protein (RFP)-expressing *A. fumigatus* were grown in a 96-well black, clear-bottom plate for 8 h at 37 °C, 5% CO_2. Hyphae were treated with 1 μM of $Sph3_h$ in PBS for 90 min at 37 °C, 5% CO_2 then exposed to 2 μg/mL BDP-PCZ for 10 mins. The plate was then read using an Infinite M1000 fluorescent plate reader with excitation wavelengths of 532 and 488 nm for RFP and BDP-PCZ, respectively. Background fluorescence was subtracted from both RFP and BDP-PCZ signals and the BDP-PCZ signal was then normalized to total RFP fluorescence for each well.

Effects of glycoside hydrolases on *A. fumigatus*-induced epithelial cell damage. A549 pulmonary epithelial cell damage by *A. fumigatus* was tested using the ^{51}Cr release assay as previously described [60,428]. Recombinant hydrolases were added to the A549 cultures at the time of infection at a final concentration of 0.5 μM.

Characterization of pulmonary damage by $Sph3_h$. All procedures involving mice were approved by the Animal Care Committees of the McGill University Health Centre. Female BALB/c mice 5-6 weeks of age were anaesthetized with isoflurane and administered a single endotracheal injection of 500 μg $Sph3_h$ in 50 μL PBS and monitored daily for 7 days. Mice were then euthanized by CO_2 overdose and their airways lavaged with 1 mL PBS that was

administered and collected through a needle inserted in the trachea. A total of 2 lavages were performed and pooled. The presence of LDH in the BAL fluid was used as an indicator of pulmonary damage; LDH activity was measured in the fluid using a commercial assay (Promega), as per manufacturer's instructions.

Effects of $Sph3_h$ in a severely immunocompromised mouse model of invasive pulmonary aspergillosis. Mice were immunosuppressed with cortisone acetate and cyclophosphamide as previously described [60,438]. Mice were infected with an endotracheal injection of $5x10^3$ *A. fumigatus* conidia, resuspended in either PBS alone, or in combination with 500 μg of $Sph3_h$. Mice were monitored daily and moribund animal were euthanized. At 4 days post infection mice were euthanized and their lungs were harvested. For fungal burden analysis, lungs were homogenized in 5 mL PBS containing protease inhibitor cocktail (Roche), and aliquots were stored at -80°C until use. Pulmonary fungal burden was determined as previously described [29], and detailed in the SI. For histological examination, lungs were inflated with 10% buffered formalin (Fisher Scientific) and immersed in formalin overnight to fix. Lungs were then embedded in paraffin and 4 μm thick sections were stained with periodic acid Schiff (PAS) stain.

3.7 Acknowledgements

Research described in this paper is supported by operating grants from the *Canadian Institutes of Health Research* (CIHR) (#81361 to P.L.H. and D.C.S; #123306 to D.C.S.; and #43998 to P.L.H) and Cystic Fibrosis Canada (CFC) (to D.C.S. and P.L.H). B.D.S has been supported by graduate scholarships from CFC and CIHR. N.C.B has been supported in part by graduate scholarships from the *Natural Sciences and Engineering Research Council of Canada*, Mary H. Beatty, and Dr. James A. and Connie P. Dickson Scholarships from the University of Toronto, CFC, and The Hospital for Sick Children. P.B. has been supported in part by a CFC postdoctoral fellowship and a Banting Fellowship from CIHR. P.L.H. is the recipient of a Canada Research Chair. D.C.S is supported by a Chercheur-Boursier Award from the *Fonds de Recherche Quebec Santé*.

3.8 Figures

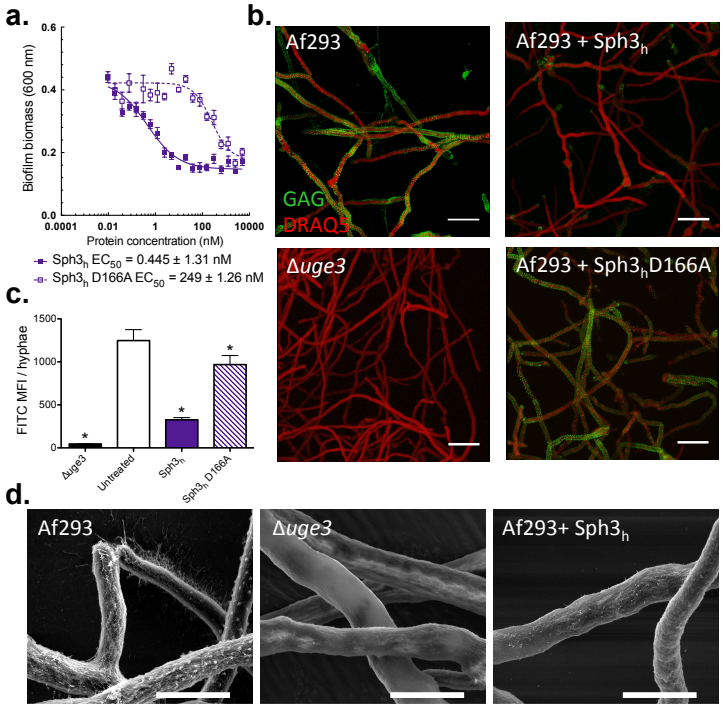


Figure 1. Treatment with $Sph3_h$ disrupts *A. fumigatus* biofilms and degrades GAG on the surface of hyphae. (**a**) Crystal violet staining of established *A. fumigatus* biofilms treated with the indicated concentration of each hydrolase. Each data point represents the mean of $n = 20$ with error bars indicating standard error (SE). EC_{50} indicates the 50% effective concentration ± SE. (**b**) Effects of the indicated hydrolases on cell wall associated GAG. Hyphae of the indicated strains grown in the absence of hydrolase treatment (left column) or following exposure to 0.5 μM of the indicated hydrolases (right column). Cell wall-associated GAG was visualized using FITC-conjugated lectin staining (green) with DRAQ5 as a counterstain (red). Scale bars = 20 μm. The GAG-deficient Δ*uge3* mutant was included as a negative control. (**c**) Quantification of lectin-staining from panel (b). Each data point represents the mean fluorescence intensity of at least 7 hyphae with error bars indicating SE. * indicates a significant difference ($p < 0.05$) relative to the untreated *A. fumigatus* as determined by one-way ANOVA with Dunnett's multiple comparison test. (**d**) Scanning electron micrographs of hyphae of the indicated strains grown in the absence of hydrolases (left & middle) and following exposure to 0.5 μM of $Sph3_h$ (right column). Scale bars = 5 μm.

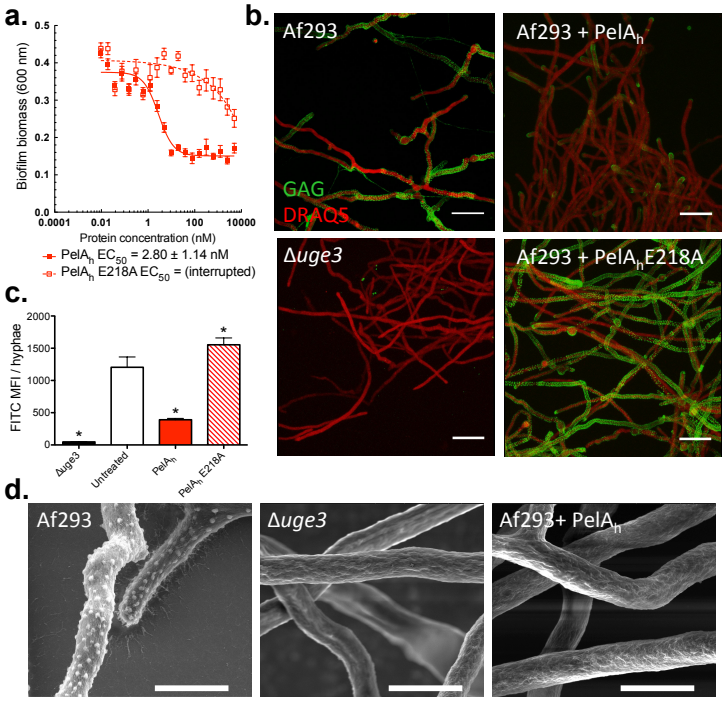


Figure 2. PelA$_h$ disrupts *A. fumigatus* biofilms and degrades GAG. (**a**) Effects of PelA$_h$ on *A. fumigatus* biofilms. Crystal violet staining of established *A. fumigatus* biofilms treated with the indicated concentration of PelA$_h$ or PelA$_h$ E218A. Each data point represents the mean of $n = 20$ with error bars indicating SE. EC$_{50}$ reported ± SE. (**b**) Effects of the indicated hydrolases on cell wall-associated GAG. Established hyphae of the indicated strains were untreated (left column) or exposed to 0.5 μM of the indicated hydrolases (right column). Cell wall-associated GAG was detected using FITC-conjugated lectin staining (green), with DRAQ5 as a counterstain (red). Scale bars = 20 μm. (**c**) Mean fluorescent intensity of lectin staining in panel (b). Each data point represents the mean of at least 7 hyphae with error bars indicating SE. The * indicates a significant difference ($p < 0.05$) relative to the untreated *A. fumigatus* as determined by one-way ANOVA with Dunnett's multiple comparison test. (**d**) Scanning electron micrographs of hyphae of the indicated strains grown in the absence of hydrolase treatment (left & middle) or following treatment with 0.5 μM of PelA$_h$ (right). Scale bars = 5 μm.

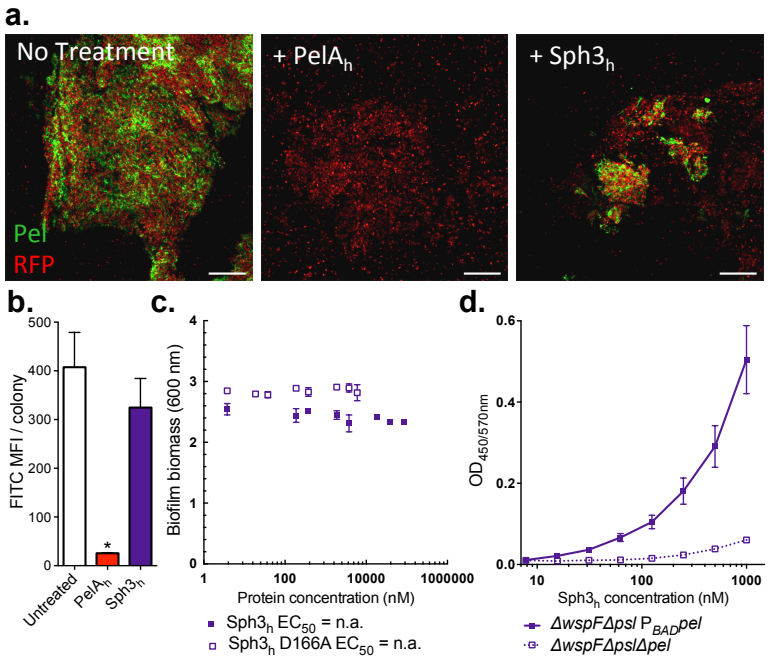


Figure 3. Sph3$_h$ binds Pel but is inactive against established *P. aeruginosa* biofilm. (**a**) Established biofilms of RFP-producing *P. aeruginosa* overexpressing the Pel operon (red) were untreated (left) or exposed to 0.5 μM of the indicated hydrolases (middle, right). Biofilm-associated Pel was detected using FITC-conjugated *Wisteria fluoribunda* lectin staining (green). Scale bars = 20 μm. (**b**) Mean fluorescent intensity of lectin staining in panel (a). Each data point represents the mean of at least 4 *P. aeruginosa* colonies with error bars indicating SE. * indicates a significant difference ($p < 0.001$) relative to the untreated *P. aeruginosa* as determined by 1-way ANOVA with Dunnett's multiple comparison test. (**c**) Effects of Sph3$_h$ on Pel-mediated *P. aeruginosa* biofilms. Crystal violet staining of established biofilms of *P. aeruginosa* overexpressing the Pel operon incubated with the indicated concentrations of Sph3$_h$ or Sph3$_h$ D166A. Each data point represents the mean of $n = 3$ with error bars indicating SE. EC$_{50}$ reported ± SE. (**d**) Sph3$_h$ binding to Pel polysaccharide. Microtiter plates were coated with culture supernatants of the indicated *P. aeruginosa* strains and the binding of Sph3$_h$ was determined using an anti-Sph3$_h$ antibody. Each data point represents the mean of 3 independent experiments with error bars indicating SE.

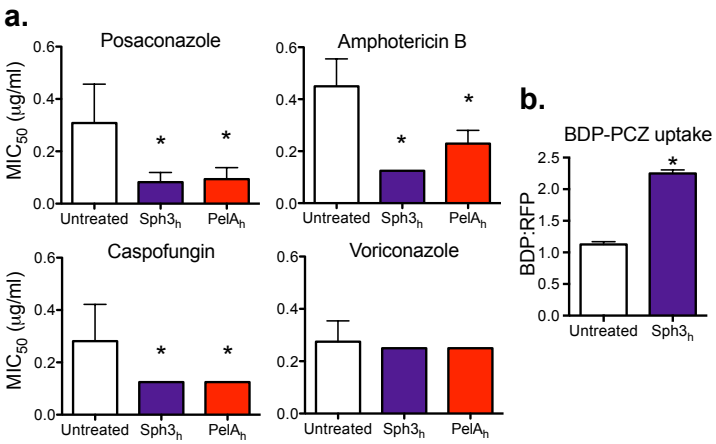


Figure 4. Glycoside hydrolases increase sensitivity of *A. fumigatus* to antifungal agents. (**a**) Established biofilms of wild-type *A. fumigatus* strain Af293 were treated with the indicated concentrations of antifungals with or without 0.5 μM of the indicated hydrolase and the viability of the resulting biofilms was then measured using the XTT metabolic assay. Susceptibility to antifungals was quantified by determining the antifungal concentration resulting in a 50% reduction in fungal metabolic activity (MIC_{50}) as compared to untreated controls. Bars represent the mean of at least $n = 4$ with error bars indicating SE. (**b**) Effects of hydrolase therapy on antifungal uptake. *A. fumigatus* hyphae were treated with 1 μM $Sph3_h$ then exposed to 2 μg/ml BDP-PCZ. Uptake of BDP-PCZ was quantified via fluorometry. Each bar represents the mean of 3 independent experiments with error bars indicating SE. The * indicates a significant difference ($p < 0.05$) relative to untreated control samples as determined by 1-way ANOVA with Dunnett's multiple comparison test in (a), or two-tailed student's T-test in (b).

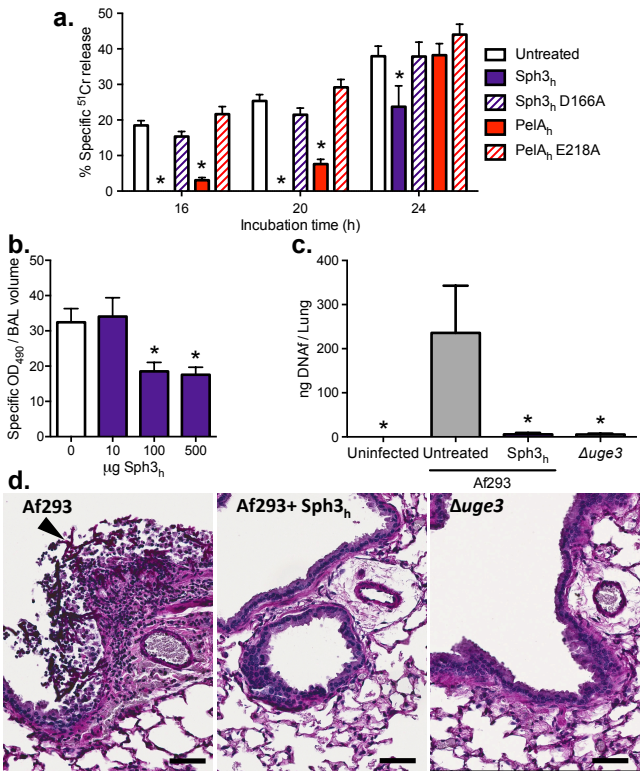


Figure 5. Effects of hydrolases on *A. fumigatus*-induced airway epithelial cell damage and *in vivo* pulmonary infection. (**a**) ^{51}Cr-loaded A549 pulmonary epithelial cells were incubated with conidia of wild-type *A. fumigatus* in the presence or absence of 0.5 µM concentrations of the indicated hydrolases. Epithelial cell damage was determined by measurement of the amount of ^{51}Cr released into supernatant at the indicated time points. Each bar represents the mean of at least 5 independent experiments performed in duplicate with error bars indicating SE. (**b**) Pulmonary injury as measured by lactose dehydrogenase activity of the bronchoalveolar lavage fluid from BALB/c mice treated intratracheally or not with the indicated quantities of $Sph3_h$ and sacrificed 7 days post treatment. Data represents the mean of at least n = 5, with error bars representing SE. (**c**) Fungal burden of neutropenic mice as determined by quantitative PCR following 4 days of infection with the indicated *A. fumigatus* strain with or without treatment with a single dose of 500 µg $Sph3_h$. Data represents the mean of at least n = 12, from two independent experiments with error bars indicating SE. The * indicate a significant difference $p < 0.01$ for (a) and (c), and < 0.05 for (b), relative to untreated controls using a two-way ANOVA for (a) and a one-way ANOVA for (b) and (c) with a Dunnett's multiple comparison test. (**d**) Histopathological analysis of lung sections of mice from (c) stained with Periodic acid Schiff reagent. Arrow indicates hyphal lesion. Scale bars = 20 µm.

3.9 Supplementary Material and Methods

Growth conditions: Unless specified, fungal strains were grown on yeast-extract peptone dextrose (YPD) agar (Fisher Scientific) at 37 °C for 6 days, and the conidia were harvested by gentle washing with phosphate buffered saline (PBS) + 0.01% (v/v) Tween-20. *P. aeruginosa* strains were grown in Luria-Bertani (LB) media to stationary phase overnight, at which point cultures were diluted to an OD_{600} of 0.05 in LB broth supplemented with 0.5 % (w/v) arabinose and grown at 30 °C, as indicated. *P. aeruginosa* strains were modified by transformation with a plasmid containing a constitutively expressing red-fluorescent protein (RFP).

Confocal image acquisition and modifications:

***A. fumigatus*:** Samples were acquired on a Zeiss LSM780 confocal fluorescent microscope using a Plan-Apochromat 63x/1.40 Oil DIC M27 objective lens at a resolution of 1024X1024, using 488 and 633 nm lasers to excite fluorescein and DRAQ5, respectively. The channels were acquired in tandem, with fluorescein being detected for 493-585 nm, and DRAQ5 for 661-759 nm. Z-stacks were acquired with a spacing of 0.74 μm. Stacks were composited into 3-dimensional renderings with the "3D Project" function of the ImageJ software using the "Brightest Point" Projection Method. A 20 μm scale bar was then added to each image before being exported as a .jpeg file format and assembled into the final figure in Microsoft PowerPoint.

***P. aeruginosa*:** Samples were acquired on a Zeiss LSM780 confocal fluorescent microscope using a Plan-Apochromat 63x/1.40 Oil DIC M27 objective lens at a resolution of 1024X1024, using 488 and 561 nm lasers to excite fluorescein and RFP, respectively. The channels were acquired separately, with fluorescein being detected for 490-577 nm, and RFP for 577-691 nm. Z-stacks were acquired with a spacing of 0.74 μm. Stacks were combined using the "Extended Depth of Focus" algorithm of Zeiss Zen software. A 20 μm scale bar was then added to each image using ImageJ before being exported as a .jpeg file format. The brightness was increased 54% for all images in Microsoft PowerPoint before being assembled into the final figure.

Introduction of constitutively expressed Red Fluorescent Proteins into *P. aeruginosa*: A plasmid containing the gene for the monomeric second-generation RFP protein mCherry under the control of a constitutive promoter [439] was transformed into *P. aeruginosa* via chemical transformation.

Scanning electron microscopy: Coverslips were washed twice with PBS, then fixed with 2.5% (v/v) gluteraldehyde in 0.1 M sodium cacodylate buffer at 4°C overnight, sequentially dehydrated in ethanol, and critical-point dried (Leica Microsystems EM CPD030 Critical Point Dryer Model). Samples were then sputter coated with Palladium (Leica Microsystems EM ACE600 High Resolution Coater), and imaged with a field-emission scanning electron microscope (FEI Inspect F-50 FE-SEM).

***A. fumigatus* culture supernatant production:** *A. fumigatus* conidia were inoculated into Brian media at 10^4 conidia/ml and grown at 37 °C for 72 h shaking at 200 rpm. Supernatants were collected by filtration through Miracloth. Culture supernatants were stored at -20 °C until use.

***In vitro* galactosaminogalactan hydrolysis assays:** The capacity of the glycoside hydrolases to degrade purified galactosaminogalactan was evaluated using a reducing sugar assay, as described previously [359].

Cell viability and morphology assays: Potential toxic and morphological effects of the glycoside hydrolases on IMR-90 fibroblasts were performed as described previously [422].

Daily monitoring of mice: Mice were monitored daily for signs of stress (ruffled fur, inactivity, hunched posture) and body weights and temperatures were taken. Body weight was measured using a top-loading balance, while surface body temperature was taken on the abdomen using a digital infrared thermometer.

Pulmonary leukocyte recruitment: Lungs were harvested and washed in PBS before being minced and digested for 60 mins at 37 °C in RPMI media containing 5% (v/v) fetal bovine serum (FBS) and 150 U/ml of collagenase (Sigma). The resulting suspension was passed through a 70 μm cell strainer to obtain a single cell suspension, at which point the erythrocytes were lysed using ACK buffer and the remaining cells were resuspended in PBS. Approximately 10^6 cells were resuspended in 1 ml PBS containing 1 μl fixable viability dye (eBioscience) and incubated for 30 mins at 4 °C. Cells were then washed in PBS + 2% FBS (staining buffer) and Fc receptors were blocked by incubating with unlabeled anti-CD16/32 antibodies (FcBlock; BD Pharmingen) for 15 mins at 4 °C. Cell surface components were stained for 30 mins at 4 °C using the following fluorescently-labeled antibodies (all purchased from BD Biosciences): CD3-fluorescein isothiocyanate (clone 17A2), CD11c-allophycocyanin (clone HL3), CD11b-phycoerythrin-CF594 (clone M1/70), CD45-allophycocyanin-Cy7 (clone 30-F11), CD19-

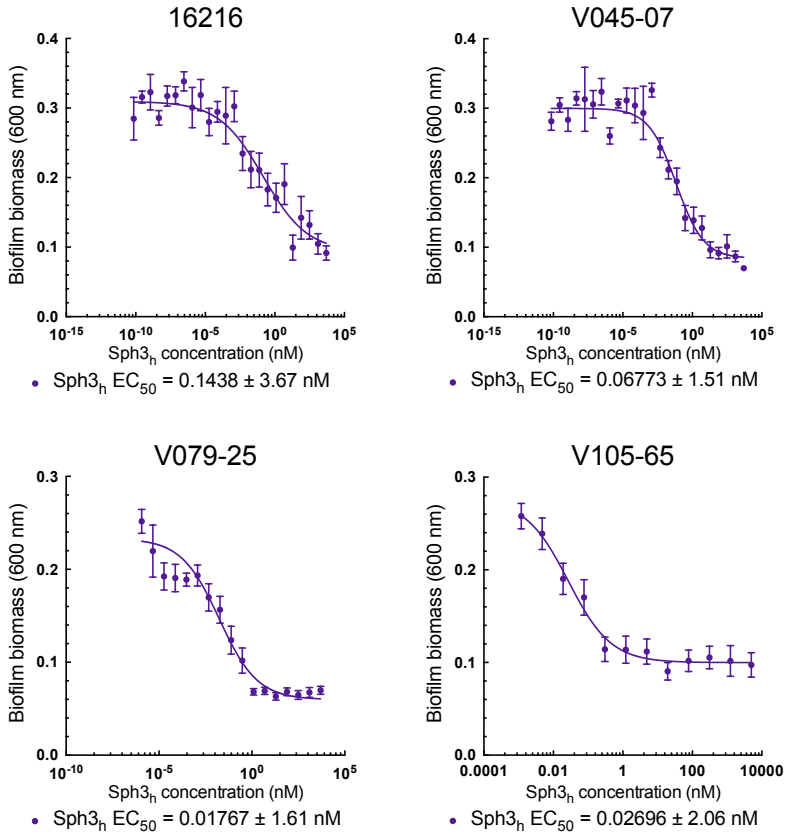
phycoerythrin-Cy7 (clone 1D3), SiglecF-brilliant violet 421 (clone E50-2440), and Ly6G-phycoerythrin (clone 1A8). Cells were washed with staining buffer and fixed with 2% (w/v) paraformaldehyde in PBS for 15 mins at 4 °C. Cells were washed and resuspended in PBS prior to data acquisition. Data was acquired on an LSR Fortessa flow cytometer using FACSDiva software (BD Biosciences). Data was further analyzed using FlowJo software version 10 (FlowJo, LLC). Immune cell subsets were defined as follows: neutrophils, $CD45^+$ $Ly6G^+$ $CD11c^-$ $CD11b^+$; alveolar macrophages, $CD45^+$ $CD11c^+$ $siglecF^+$ $CD11b^{neg/low}$; lymphocytes, $CD45^+$ $CD11c^-$ $Ly6G^-$ $CD3^+$ or $CD45^+$ $CD11c^-$ $Ly6G^-$ $CD19^+$; eosinophils, $CD45^+$ $CD11c^-$ $siglecF^+$. Total cell numbers of each population were calculated using the BD CountBright absolute counting beads during data acquisition.

Pulmonary fungal burden by galactomannan content: Pulmonary galactomannan content was determined using the Platelia *Aspergillus* immunoassay kit (Bio-Rad), according to manufacturer's instructions.

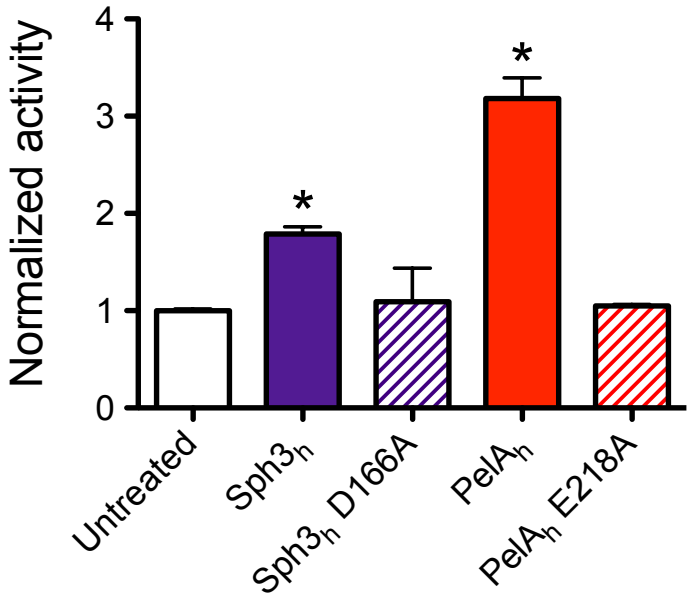
Pulmonary fungal DNA quantification: Total DNA was extracted from mouse lung homogenates using the High Pure PCR Template Preparation Kit (Roche Diagnostics), as per manufacturer's instructions. To quantify fungal DNA, real-time PCR was performed on 150 ng of total lung DNA using TaqMan Universal PCR Master Mix (Roche Diagnostics). Fungal DNA was quantified using primers targeting the *Aspergillus* 18S ribosomal RNA gene (forward: GGCCCTTAAATAGCCCGGT; reverse: TGAGCCGATAGTCCCCCTAA; labeled probe: 6-FAM-AGCCAGCGGCCCGCAAATG-MGB) by comparing to a standard curve of *A. fumigatus* DNA. Quantitative PCR was run as follows: 2 min at 50 °C; 10 min at 95 °C; and 50 cycles of 15 sec at 95 °C +1 min at 60°C.

Statistical analysis: A non-linear least-squares fitting to a dose-response model was used to calculate EC_{50} values. Data are presented and statistical significance calculated as indicated. All graphs were generated and statistical analyses performed using Prism v6.0 (GraphPad Software).

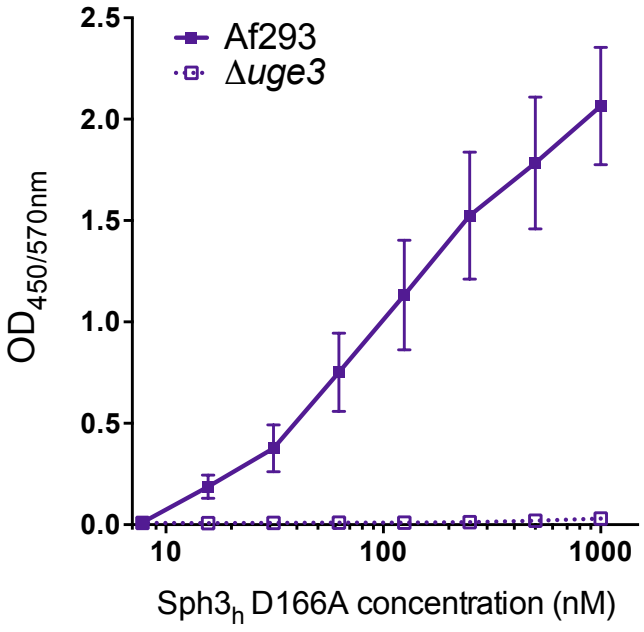
3.10 Supplementary Figures:



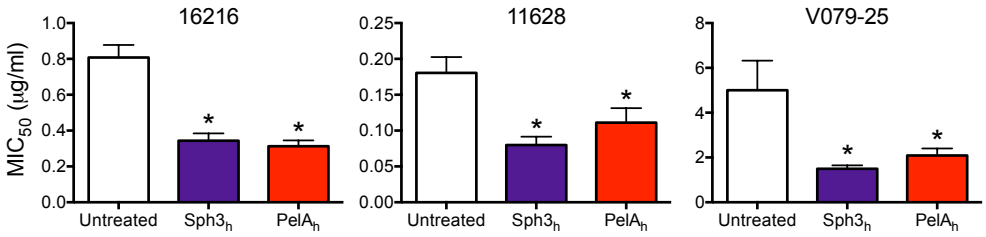
Supplementary Figure 1. Effects of $Sph3_h$ on biofilms formed by clinical isolates of *A. fumigatus*. Crystal violet staining of pre-grown *A. fumigatus* biofilms treated with the indicated concentration of $Sph3_h$. Data represents the mean of 3 independent experiments with error bars indicating SE. 50% effective concentration (EC_{50}) reported ± SE.



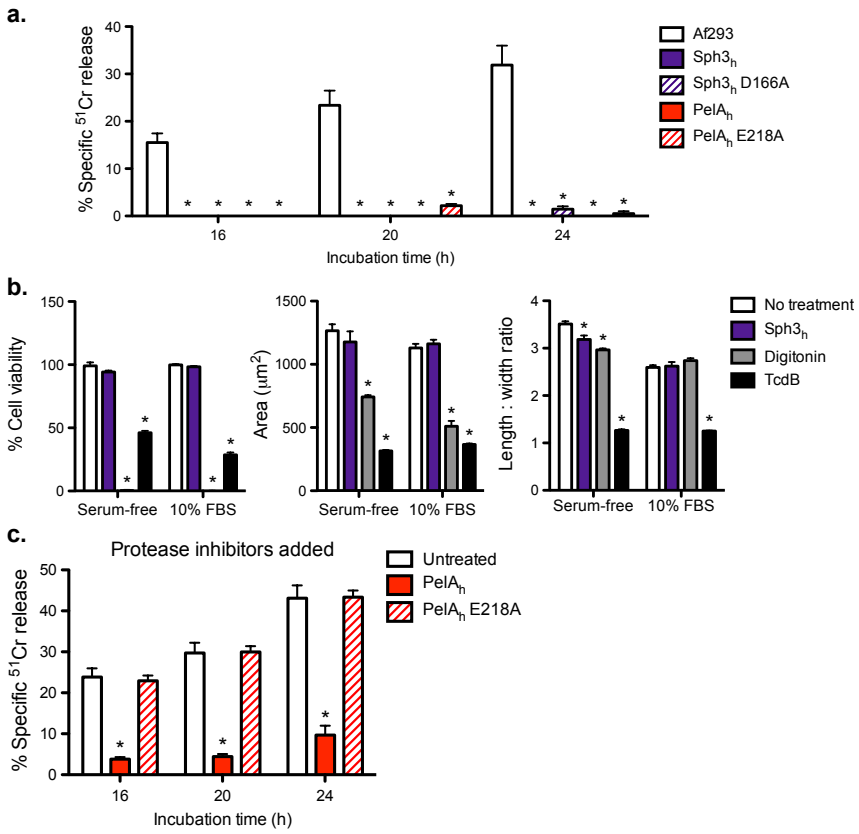
Supplementary Figure 2. $Sph3_h$ and $PelA_h$ can hydrolyze purified GAG. Purified GAG was incubated with 12 μM of the indicated hydrolase for 24 h. GAG hydrolysis was measured through quantification of the release of reducing sugars. The * indicates significant difference ($p < 0.01$) from the untreated samples by unpaired t-test. Each bar represents the mean of two independent experiments.



Supplementary Figure 3. $Sph3_h$ D166A binds to GAG. Microtiter plates were coated with culture supernatants of the indicated *A. fumigatus* strains and the binding of the indicated concentrations of $Sph3_h$ D166A was determined as above. Data represents the mean of 3 independent experiments with error bars indicating SE.

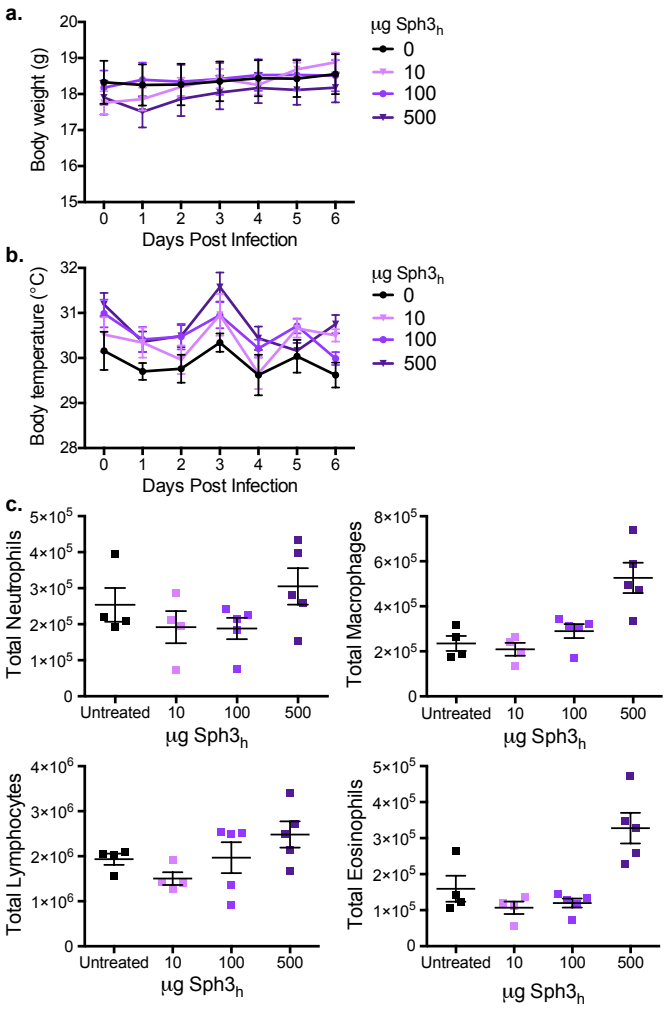


Supplementary Figure 4. Susceptibility of azole-resistant isolates of *A. fumigatus* to posaconazole when treated with $Sph3_h$ or $PelA_h$. Susceptibility to antifungals was quantified by determining the antifungal concentration resulting in a 50% reduction in fungal metabolic activity (MIC_{50}) as compared to untreated controls. The * indicates a significant difference ($p < 0.05$) from untreated control samples as determined by 1-way ANOVA with Dunnett's multiple comparison test. Each bar represents the mean of at least 3 independent experiments with error bars indicating SE.

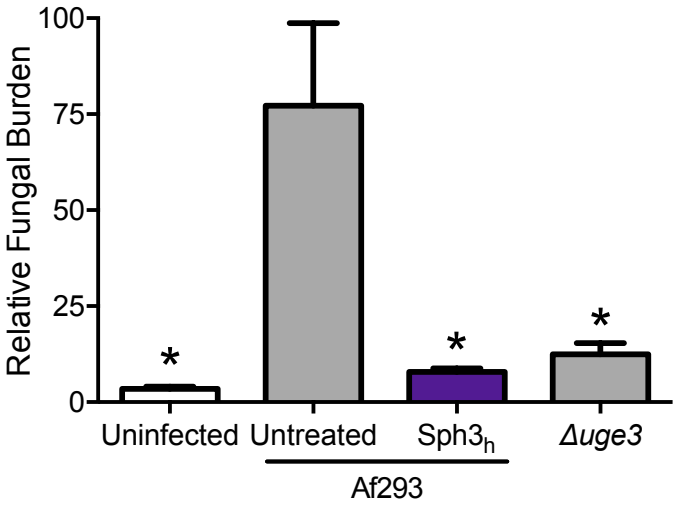


Supplementary Figure 5. $Sph3_h$ and $PelA_h$ are non-toxic to mammalian cell lines *in vitro*. (**a**) Chromium-loaded A549 pulmonary epithelial cells were incubated with conidia of wild-type *A. fumigatus* or 0.5 μM of the indicated hydrolases. Epithelial cell damage was determined by measurement of the amount of chromium released into supernatant at the indicated time points. Each bar represents the mean of at least $n = 3$ with error bars indicating SE. (**b**) Potential toxic and morphological effects of the $Sph3_h$ on IMR-90 fibroblasts were assayed as previously described. IMR-90 fibroblast cell viability assay using PrestoBlue® reagent (left). All data was normalized to a no treatment control (100%). IMR-90 cellomics assay to measure the area (centre) and length-to-width ratio (right) of the cells using CellTracker Orange CMRA. The *C. difficile* toxin TcdB was used as a positive control in cell morphology assays and the detergent digitonin was utilized as a negative control in cell viability assays. Each data point represents the mean from $n = 3$ from cellomic and PrestoBlue® measurements in microtiter plate well with error bars indicating SE. (**c**) Chromium-loaded A549 cells were incubated with conidia in the presence or absence of 0.5 μM of the indicated hydrolases and supplemented with 0.1% (v/v) protease inhibitor cocktail. Each bar represents the mean of 4 independent experiments

performed in triplicate with error bars indicating SE. The * indicates a significant difference ($p < 0.05$) compared with cells infected with *A. fumigatus* alone (a, c) or no treatment (b), as determined by 2-way ANOVA with Dunnett's multiple comparison test.



Supplementary Figure 6. Intratracheal administration of $Sph3_h$ is well tolerated in BALB/c mice. Body weight (**a**) and surface body temperature (**b**) of immunocompetent BALB/c mice administered an intratracheal injection of the indicated amount of $Sph3_h$ and monitored daily. Data represents the mean of at least $n = 4$, with error bars indicating SE. No significance compared to untreated controls, 2-way ANOVA with Sidak's multiple comparisons test. (**c**) Total pulmonary leukocyte populations of the mice after 7 days post-treatment, as determined by flow cytometry. No significance compared to untreated controls, Kruskal-Wallis test with Dunn's multiple comparisons test.



Supplementary Figure 7. $Sph3_h$ coadministration attenuates *A. fumigatus* invasive infection in BALB/c mice. Fungal burden of neutropenic mice as determined by galactomannan quantification following 4 days of infection with the indicated *A. fumigatus* strain with or without treatment with a single dose of 500 μg $Sph3_h$. Data represents the mean of at least n = 12, from two independent experiments with error bars indicating SE. $p < 0.001$ compared to untreated mice infected with wild-type *A. fumigatus* using a one-way ANOVA and Dunnett's multiple comparison test.

3.11 Supplementary Table

Supplementary Table 1. A list of fungal and bacterial strains used in this study.

Organism	Strain or genotype	Source or reference
Aspergillus fumigatus	Af293 (wild–type)	Paul T Magee
	Δ*uge3*	[60]
	Af293 pRFP	[86]
	16216 (wild-type)	William W Hope
	11628 (wild-type)	
	V045-07 (wild-type)	Paul E Verweij
	V079-25 (wild-type)	
	V107-65 (wild-type)	
Pseudomonas aeruginosa	PAO1 *ΔwspFΔpsl*P_{BAD}*pel*	[422]
	PAO1 *ΔwspFΔpsl*P_{BAD}*pel pRFP*	This study

CHAPTER 4: General Discussion and Conclusions

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Despite our currently available antifungal armamentarium, *Aspergillus fumigatus* pulmonary infections continue to be associated with a high degree of morbidity and mortality. New approaches to prevent or treat these infections are therefore desperately needed. The goal of this thesis was to examine the interactions of proteins with the *A. fumigatus* exopolysaccharide galactosaminogalactan (GAG) to explore new avenues of therapeutic intervention. We used a two-pronged approach, focussing not only on investigating mammalian proteins for their potential to bind GAG, but also to determine if proteins native to the fungus had the potential to be repurposed for use against the organism itself.

A. fumigatus rarely infects healthy individuals, and is limited to hosts that are severely immunocompromised or have pre-existing lung disease. This observation suggests that individuals with a functioning immune system are able to efficiently detect and eradicate the fungus, via innate immune responses involving specific mammalian protein receptors. As GAG is located on the outer cell wall of the fungus, it is exposed to the host cell receptors, and is a promising candidate to be recognized by host lectins. We explore the possibility of a novel mammalian receptor for *A. fumigatus* GAG in Chapter 2 of this thesis, where we report the binding of galectin-3 to GAG. However, while galectin-3 bound to GAG, this binding did not affect fungal growth or viability. This observation of galectin-3 differs from reports in other fungi in which binding of this lectin to fungal polysaccharides directly inhibits their growth. In the setting of an *A. fumigatus* infection the primary role for galectin-3 was found to be in mediating early neutrophil recruitment to the airways.

In Chapter 3 we turn our attention towards the fungus itself, to determine if any of the proteins involved in the biosynthesis of GAG can be repurposed to serve as anti-GAG therapeutics. We identified and expressed the glycoside hydrolase domain of the *Aspergillus* protein Sph3 and found it was able to both digest galactosaminogalactan and disrupt fungal biofilms *in vitro* and *in vivo*. This study has laid the foundation for the use of enzyme therapy in these fungal infections, and is being actively pursued in other infectious diseases.

4.1 Galectin-3 as a mammalian GAG receptor

While galactosaminogalactan functions effectively to cloak the inner cell wall components of *A. fumigatus*, such as β-glucan, from detection by the immune system [60], it is not immune-inert. Neutrophils and mononuclear cells actively respond to GAG by undergoing apoptosis or

through the production and release of IL-1 receptor antagonist, respectively [61,109], suggesting that GAG is engaging a mammalian receptor to induce these responses.

We found that the soluble mammalian lectin galectin-3 binds *A. fumigatus* GAG. Galectin-3 was identified through a knowledge-directed approach focussing on the galectin family of galactose-binding lectins, given that GAG is rich in both galactose and *N*-acetyl galactosamine [28,398]. Galectin-3 levels in the sera and BAL fluid were found to be elevated in humans and mice infected with *Aspergillus*, respectively, indicating that this lectin is released during an *Aspergillus* infection. Galectin-3 was observed to bind to both native GAG on the surface of the fungus, as well as GAG purified from culture supernatants. This binding was not due to non-specific interactions, as inhibition studies with the canonical galectin-3 ligand lactose revealed that the binding involved the lectin's carbohydrate recognition domain.

While galectin-3 was observed to bind GAG *in vitro*, this binding did not inhibit the growth of *A. fumigatus,* contrary to previous observations with other fungi. Instead, galectin-3 deficient mice failed to effectively recruit neutrophils to the airways during a pulmonary aspergillosis infection, and were thus more susceptible to infection. Detailed studies found elevated levels of neutrophil chemoattractants in the airways of infected galectin-3 deficient mice, indicating that the ability to recognize *A. fumigatus* conidia in this context and initiate the appropriate signals for neutrophil recruitment is galectin-3 independent. However, neutrophils in galectin-3 deficient mice were attenuated in their ability to exit the vasculature and enter the alveolar lumen. While similar observations have been reported in an animal model of streptococcal pneumonia [371,372], to our knowledge this is the first time this role for galectin-3 has been found during fungal infection.

Adoptive transfer experiments revealed that endogenous galectin-3 is not required for neutrophil recruitment to the airways. Additionally, endogenous galectin-3 is not required for neutrophil antifungal effects against *A. fumigatus*, as galectin-3 deficient neutrophils exhibited normal killing of hyphae in *in vitro* assays. These findings are in contrast to a report that intracellular galectin-3 attenuated neutrophil killing of the pathogenic yeast *C. albicans*, through a restriction of ROS production, resulting in galectin-3 deficient mice being more susceptible to experimental candidiasis [182]. However, as other studies of intravenous *C. albicans* infection found galectin-3 deficient mice to be more susceptible to infection [183], the importance of galectin-3 in *Candida* infections remains an unanswered question.

Adoptive transfer experiments also revealed that the lack of extrinsic galectin-3 attenuates neutrophil migration. Similarly, *in vitro* assays suggest that extracellular galectin-3 augments both the mobility and migration of human neutrophils, despite galectin-3 not functioning as a neutrophil chemoattractant itself [372]. While previous studies have observed enhanced neutrophil migration in the presence of extracellular galectin-3 in the context of *Streptococcus pneumoniae* and *Leishmania major* infections [372,379], this is the first report of exogenous galectin-3 directly enhancing the mobility of neutrophils, potentially representing a novel mechanism by which galectin-3 promotes leukocyte diapedesis in lung tissues.

To further expand our knowledge on the role of galectin-3 in the immune response to *A. fumigatus*, future studies will focus on confirming our *in vitro* mobility and migration findings, and will be expanded to include neutrophils isolated from wild-type and galectin-3 deficient mice. Administration of recombinant galectin-3 to galectin-3 deficient mice infected with *A. fumigatus* will be investigated to determine if it has an effect on neutrophil recruitment and can rescue the susceptibility of galectin-3 mice to *A. fumigatus* infection, as has been demonstrated in the streptococcal pneumonia model [371,372]. Beyond this, it would also be interesting study the galectin-3 deficient mice in a model of chronic pulmonary aspergillosis [440], to determine the long-term ramifications of galectin-3 in the immune response to *A. fumigatus*.

Preface to Chapter 4, Section 2

The past decade has seen a greater understanding of the importance of infections caused by biofilm-forming microbes. Up to 90% of all nosocomial bacterial and fungal infections are caused by microbes capable of forming biofilms. Biofilm infections are exceptionally difficult to treat, as the microbes have heightened resistance to physical removal, attack by the immune system, and antimicrobial therapies. As a result, great interest has been given to the study of enzymes and compounds that directly target the architecture of the biofilms. These investigations have uncovered a collection of enzymes, predominantly of microbial origin, that are active against a variety of microbial biofilms. The following section provides an overview of the avenues of research currently being pursued, and the potential implications that they may have on future therapeutic strategies. This section was originally published in Future Microbiology in February 2018.

4.2 Hoisted by their own petard: do microbial enzymes hold the solution to treating and preventing biofilm infections?

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Adapted from: *Future Microbiology* 2018 Feb 14;13(4);395-398.

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Biofilms are microbial communities that grow within a self-produced heterogeneous extracellular matrix (ECM). The production of ECM by pathogenic bacteria and fungi during biofilm growth confers a number of advantages during infection including mediating adherence to host tissues and biomedical devices as well as enhancing resistance to antimicrobial agents and host immune defences. Exopolysaccharides are a key component of the ECM and have been directly implicated in mediating adhesion, immune evasion and antimicrobial resistance. Recent studies have suggested that microbial enzymes can be used to degrade the biofilm exopolysaccharides of several pathogens and increase their susceptibility to antimicrobials *in vitro* and *in vivo*. We will review current progress in this area, and highlight areas for future research and development.

4.2.1 Current lines of research:

4.2.1.1 Bacterial biofilms

***Staphylococcus* species**. The polysaccharide poly-β-1,6-*N*-acetyl-D-glucosamine (PNAG) is an important biofilm component of many *Staphylococcus aureus* and *Staphylococcus epidermidis* strains, as well as some Gram negative pathogens such as *Acinetobacter baumannii, Klebsiella pneumonia, Yersinia pestis* and *Escherichia coli* [441]. A recombinant glycoside hydrolase (GH) known as DispersinB originating from *Actinobacillus actinomycetemcomitans* cleaves PNAG [442], and can disrupt PNAG-dependent biofilms of these organisms *in vitro*. DispersinB treatment enhanced the antimicrobial activity of cefamandole nafate against both *S. aureus* and *S. epidermidis* biofilms *in vitro* [443]. Animal model studies have also demonstrated that DispersinB enhanced the antimicrobial effects of silver-nanoparticles in a mouse methicillin-resistant *S. aureus* chronic wound model [444]. Systemic use of DispersinB in other models of staphylococcal infection have not been reported.

Yersinia species. NghA is a GH active against PNAG produced by *Yersinia pseudotuberculosis*. The related pathogen *Yersinia pestis* lacks a functional *nghA* gene and, as a result, is able to form PNAG-based biofilms within its flea vector [445]. Heterologous expression of *nghA* in *Y. pestis* prevented biofilm formation in the flea gut, and biofilm formation by *Y. pestis* and *S. epidermidis in vitro* was inhibited by recombinant NghA [445]. The effects of NghA on the susceptibility of *Yersinia sp*, or on virulence in mammalian infection models has yet to be reported.

Pseudomonas aeruginosa. *P. aeruginosa* produces three exopolysaccharides: alginate, Pel and Psl. The expression of these glycans varies by strain, and they are thought to play complementary roles in biofilm formation and antibiotic resistance.

Alginate is produced in abundance by mucoid *P. aeruginosa* strains commonly isolated from patients with chronic lung disease and increases resistance to antimicrobial agents and leukocyte phagocytosis. Alginate lyases are enzymes produced by a range of organisms, including *P. aeruginosa* itself, that catalyze the degradation of alginate. There are conflicting reports as to the ability of alginate lyases to inhibit or disrupt biofilms [446,447], possibly reflecting the differences in intrinsic activity of these proteins against *P. aeruginosa* alginate [448], as well as variability of substrate specificity [447]. Despite this, alginate lyase treatment has been reported to enhance the antimicrobial activity of both gentamicin and tobramycin *in vitro* [449]. In a rabbit model of mucoid *P. aeruginosa* endocarditis, intravenous administration of alginate lyase resulted in a reduction of *P. aeruginosa* to levels similar to that of a non-mucoid strain [450].

The Pel and Psl polysaccharides mediate adherence and biofilm structural integrity in *P. aeruginosa*. Glycoside hydrolase domain-containing proteins are encoded by genes within the biosynthetic operons of each of these glycans. Recombinant GH domains from these proteins, $PelA_h$ and $PslG_h$, are able to degrade their respective polysaccharides [422]. Nanomolar concentrations of $PelA_h$ and $PslG_h$ inhibit *P. aeruginosa* biofilm formation and degrade pre-formed biofilms [422]. $PslG_h$ treatment of Psl-dependent *P. aeruginosa* biofilms enhances susceptibility to colistin [422]. $PelA_h$ treatment also renders Pel-dependent *P. aeruginosa* biofilms more susceptible to both colistin and to killing by a neutrophil-like cell line [422]. Consistent with these *in vitro* studies, $PslG_h$ alone and in combination with tobramycin is also active against *P. aeruginosa* biofilms in a mouse peritoneal implant model [451].

4.2.1.2 Fungal biofilms

Candida albicans. *C. albicans* is the most common invasive fungal pathogen of humans, and forms biofilms on central venous catheters and on mucosal surfaces. *C. albicans* contain β-(1,3)-glucans which increase resistance to the antifungal fluconazole through binding and sequestering this agent [452]. In contrast to other studies of exopolysaccharide-degrading enzymes, treatment of *C. albicans* biofilms with β-glucanases from several microbial sources reduces fungal viability [452]. β-glucanase therapy also exhibits anti-biofilm activity and augments the antifungal effects of fluconazole in a rat intravenous catheter model [452].

Aspergillus fumigatus. Biofilm formation by the filamentous opportunistic fungal pathogen *A. fumigatus* is dependent on the heteropolysaccharide galactosaminogalactan (GAG) [60,295]. As with *P. aeruginosa*, one of the products of the gene cluster required for GAG biosynthesis is a glycoside hydrolase ($Sph3_h$) [359]. Nanomolar concentrations of recombinant $Sph3_h$ degrade GAG and disrupt pre-formed *A. fumigatus* biofilms. $Sph3_h$ treatment increases antifungal penetration into fungal biofilms and enhances their activity [298]. $Sph3_h$ treatment protects airway epithelial cells from *A. fumigatus*-induced damage *in vitro*, and intratracheal administration of $Sph3_h$ to neutropenic mice is well tolerated and attenuates the virulence of *A. fumigatus* in these mice [298]. $PelA_h$ is also active against *A. fumigatus* GAG, possibly reflecting similarities in the composition of these two glycans [29]. As with $Sph3_h$, $PelA_h$ treatment disrupts pre-formed *A. fumigatus* biofilms, enhances the activity of antifungals and protects airway epithelial cells from *A. fumigatus in vitro* [298]. The use of $PelA_h$ *in vivo* has not yet been reported.

4.2.2 Perspectives:

The use of microbial enzyme therapy to combat biofilms has several potential advantages over conventional small molecule therapeutics. Unlike many biofilm therapeutics, microbial enzymes are active against pre-formed biofilms *in vitro* and *in vivo*. Enzymes are by their nature processive, and therefore can exhibit disproportionate effects even at low concentrations. Through disrupting biofilm integrity, biofilm-degrading enzymes increase the sensitivity of microorganisms to a wide range of antimicrobials, and therefore have the capacity to "rescue" agents that are ineffective against microorganisms growing within biofilms. Loss of exopolysaccharides also renders many organisms more susceptible to host immune killing even in the absence of an additional antimicrobial agent. The cross-species activity of the bacterial GH $PelA_h$ against *A. fumigatus* suggests that broad-spectrum activity may be achievable with careful selection of enzymes or enzyme combinations. Finally, the extracellular location of the exopolysaccharide targets of microbial enzymes offers other advantages. Intracellular penetration of organisms is not required and efflux pump activity has no effect on these agents. Similarly, there is likely a high bar for the development of resistance, as these therapeutic enzymes are outside the organism itself.

While microbial enzyme therapy appears promising, there are potential challenges that need to be addressed. Data on the safety and efficacy of these agents in animal models remains limited. As foreign proteins, the potential for immunogenicity cannot be overlooked, although strategies such as chimeric protein production or chemical modification through PEGylation or other approaches may mitigate these problems. Off target effects of these enzymes against host glycans will need to be examined, particularly as they are used for infections at different body sites, which may express different glycans than those in lung or skin where these enzymes have been used to date. Finally, although degrading exopolysaccharides can render the organisms within biofilms more susceptible to immune and antimicrobial killing, there may be unintended consequences to this microbial unmasking. Exposure of molecular patterns within these organisms has the potential to trigger hyper-inflammatory responses with potential detrimental effects on the host. Similarly, degrading biofilms *in situ* could enhance dispersion of microorganisms and worsen outcomes in infection, although combining enzyme therapy with antimicrobials may prevent this from occurring. These effects are likely to vary by organism and

site of infection, and will require careful study in appropriate animal models before moving to human trials.

Despite the possible challenges associated with microbial enzyme therapy, the potential benefits of this approach strongly support pursuing their development. The rising rates of antimicrobial resistance have raised the spectre of a post-antibiotic era within our lifetime and highlighted the need for novel strategies to combat antimicrobial resistance. The use of microbial enzymes as anti-biofilm therapeutics is one such promising therapy. The enzymes discussed above are only a small sample of the wide range of microbial lytic enzymes produced by biofilm-forming organisms, highlighting the potential of this approach to combat antimicrobial resistance in a variety of infections [441,453,454]. The results of early proof of concept pre-clinical studies are promising, however significant work remains to complete the pre-clinical assessment of these agents and bring them to early clinical trials.

4.3 Conclusions

In conclusion, the work presented in this thesis has expanded the knowledge of the interactions between both host and fungal proteins with the cell wall of *Aspergillus fumigatus*. We characterized the first interaction between galactosaminogalactan and a mammalian protein, showing that galectin-3 specifically recognized the fungal polysaccharide. Ultimately however, this interaction was found to be incidental to the role galectin-3 plays in enhancing the recruitment of neutrophils to the site of infection. In focussing on the fungus, we found that *A. fumigatus* expresses a protein capable of hydrolyzing galactosaminogalactan, and that a recombinant version of this protein could disrupt *A. fumigatus* biofilms when applied to their surface. Hydrolase treatment rendered *A. fumigatus* more susceptible to antifungal therapy and inhibited its ability to damage mammalian cells in culture. A related protein from *Pseudomonas aeruginosa* had a similar effect, suggesting enzymatic activity across taxonomic kingdoms. We provide the first example of anti-biofilm monotherapy being effective against a fungal infection *in vivo*, opening the door for the development of microbial enzymes as a new class of anti-biofilm therapeutics.

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