

The utility of sphingolipid pathway inhibitors in the treatment of aspergillosis

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Declaration of Originality

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Abstract

A. fumigatus is a ubiquitous fungus that causes invasive pulmonary aspergillosis (IPA). IPA is one of the most life-threatening infections in immune compromised patients. Corticosteroids are commonly prescribed as immunosuppressive agents for transplant patients, and IPA mostly occurs in the setting of potent transplant maintaining immune suppressive therapy. Corticosteroid immune suppression represents a major risk factor for IPA, as it inhibits the killing capacity of alveolar macrophages and results in fungal growth that recruits non-sterilizing PMNs to the site of infection that leads to tissue damage. This PhD aimed to characterize effects of the sphingosine-1-phosphate (S1P) pathway inhibitors SKI-II and FTY720 for their utility in attenuating the characteristics of IPA immunopathology, such as inflammation and fungal development. Firstly, it was demonstrated that *A. fumigatus*-mediated cytokines and chemokines were down regulated *in vitro* in the presence of inhibitors, and direct sphingosine kinase (SphK) inhibition resulted in a phagocytic defect but not S1P₁ receptor antagonism. Inflammatory responses and fungal burden were then evaluated in immunocompetent mice with FTY720, and it was shown that cytokine and chemokine responses were inhibited without compromising fungal clearance. Further to this, FTY720 was evaluated for its efficacy in the hydrocortisone model of aspergillosis. Both pro-inflammatory mediators and fungal burden were reduced, and increased survival was observed. Secondly, the antifungal properties of both SKI-II and FTY720 were investigated. SKI-II was shown to have limited activity with germination kinetics but inhibited hyphal extension, whereas FTY720 was demonstrated to inhibit fungal germination and hyphal growth significantly. Genetic deletions in *A. fumigatus* of *SphK* and *gprD* putative genes were generated, corresponding to mammalian sphingosine kinase 1 and S1P₁. Phenotypic examination showed that mutants were sensitive to cell wall stress and had altered germination kinetics, and $\Delta gprD$ was less immunogenic. Overall these findings in this thesis indicate the S1P₁ inhibitor FTY720 has antifungal activity *in vitro* against *A. fumigatus* with therapeutic effects *in vivo* in the HC model of IPA and could be developed as an antifungal therapeutic.

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Glossary of Acronyms

AMs	Alveolar macrophages
ANOVA	Analysis of variance
APC	Antigen presenting cell
BLAST	Basic local alignment search tool
CF	Cystic fibrosis
CFU	Colony forming units
CPA	Chronic pulmonary aspergillosis
CTLs	C-type lectin-like domains
CTLR	C-type lectin-like receptor
DC-SIGN	Dendritic cell-specific intracellular adhesion molecule-grabbing non-integrin
dH ₂ O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunoabsorbent assay
ERKs	Extracellular signal-regulated kinases
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FTY720	2-Amino-2-[2-(4-octyl-phenyl)-ethyl]-propane-1,3-diol hydrochloride
FTY720-p	FTY720 phosphate
GM	Geometric mean
GVHD	Graft versus host disease
HIV	Human immunodeficiency virus
HSCT	Hematopoietic stem cell transplantation
IPA	Invasive pulmonary aspergillosis
IFN- γ	Interferon-gamma
IL	Interleukin
IPA	Invasive pulmonary aspergillosis
KC	Keratinocyte-derived chemokine
LPS	Lipopolysaccharide
MBL	Mannose-binding lectin
MCP	Monocyte chemoattractant protein

μl	Microlitre
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-1β	Macrophage inflammatory protein 1 beta
MIP-2	Macrophage inflammatory protein 2
MIP-2α	Macrophage inflammatory protein 2 alpha
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NCBI	National center for biotechnology information
NETs	Neutrophil extracellular traps
NLRs	Nucleotide-oligomerisation domain-like receptors
NK cell	Natural killer cell
NOD	Nucleotide-oligomerisation domain
PAMPs	Pattern-associated molecular patterns
PBS	Phosphate buffered saline
PTX3	Pattern recognition receptor pentraxin 3
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute culture medium
SC	Swollen conidia
SKI-II	2-(p-hydroxyanilo)-4-(p-chlorophenyl)thiazole
SphKs	Sphingosine kinases
SphK1	Sphingosine kinase 1
SphK2	Sphingosine kinase 2
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
S1PR ₁₋₅	Sphingosine-1-phosphate receptor family
TLRs	Toll-like receptors
TNF-α	Tumour-necrosis factor alpha

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1. Introduction

1.1. Mammalian lipids

1.1.1 Mammalian lipids

Lipids are generally accepted to be small hydrophobic or amphipathic molecules that are insoluble in water and soluble in organic non-polar solvents. Biological lipids are broadly categorized as fatty acids, waxes, eicosanoids, monoglycerides, diglycerides, triglycerides, phospholipids, sphingolipids, sterols, terpenes, prenols, and fat soluble vitamins such as vitamins A, D, E and K (1). They have a variety of known functions, such as providing energy storage, membrane structural components and cell messengers (1). Previously thought to be only structural components of the plasma membrane, lipids are now known to function as potent signalling molecules, and it is increasingly difficult to find an area of cell biology where lipids do not have important roles (2). The field of bioactive lipids is exemplified by sphingolipids, which have key roles from embryonic vascular development to cell death (3).

1.1.2 Sphingolipids

Sphingolipids are a diverse class of lipids with a common sphingosine backbone and are synthesized *de novo* in the endoplasmic reticulum (ER). It is the modifications to this characteristic backbone that give rise to the plethora of sphingolipids existing in the cell (4). *De novo* synthesis is initiated by the condensation of palmitoyl-CoA with serine. This reaction occurs on the cytoplasmic facing membrane of the ER (5), and is followed by the synthesis of dihydrosphingosine and sphingosine and related ceramides, the precursors of more complex sphingolipids (1,6).

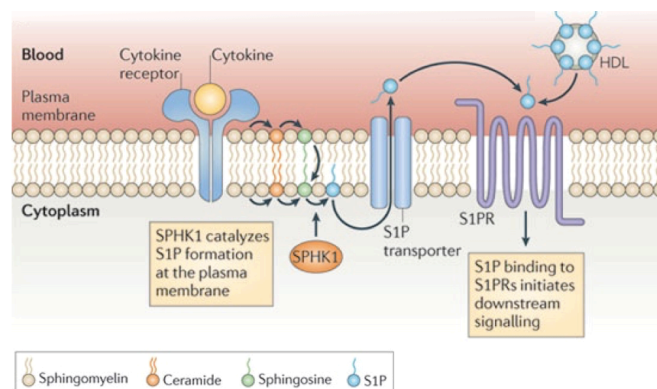


Figure 1.1: Sphingolipids on the cell membrane. Published by Spiegel and Milstein in 2001 (7). Permission was granted for use in this thesis by Nature Publishing Group (see 10.1). HDL, high density lipoproteins.

Sphingolipid metabolism involves numerous enzymes that generate bioactive sphingolipids. However, sphingosine-1-phosphate (S1P) has been the subject of the majority of study owing to its pleiotropic nature (7). As per Fig 1.1, S1P is generated by the phosphorylation of sphingosine by sphingosine kinases and is exported out of the cell by the S1P transporter SPNS2 (8,9). S1P is a well-established ligand of a family of five S1P-specific G protein coupled receptors, and has a number of intracellular targets. Bioactive sphingolipids have a diverse range of important roles in cell survival, proliferation, differentiation, migration, and immune function (10). There is now considerable evidence to support the role of SphKs and S1P in health and disease (249).

1.1.2.2 Sphingosine kinases

Two distinct isoforms of sphingosine kinase, 1 and SphK2, catalyse the ATP-dependent phosphorylation of sphingosine (Sph) to S1P (10). In humans, both enzymes are expressed as multiple splice variants that differ in the length of the N-terminal sequence (10–12). The SphK genes (SphK1 and SphK2) give rise to three SphK1 splice variants (SK1a, SK1b, SK1c) and two SphK2 variants (SK2a, SK2b). The functional significance for multiple variants of each isoform is yet to be established (12). Both SphK1 and SphK2 consist of five conserved domains (C1–C5). The catalytic domain is formed within C1–C3 and the ATP binding site is located in C2 (13). Owing to their conserved catalytic domains, SphKs are part of the diacylglycerol kinase catalytic domain (DAGK_cat) family of enzymes (10). SphKs are regulated in a spatial and temporal manner by post-translational modification (PTMs) and interact with intracellular proteins and lipids (10,14,15).

1.1.2.3 Regulation, cellular localization and activation of SphKs

Sphingolipids and associated enzymes are not only found in ER but also the plasma membrane, endosomes, Golgi and nucleus (16), see Fig 1.2.

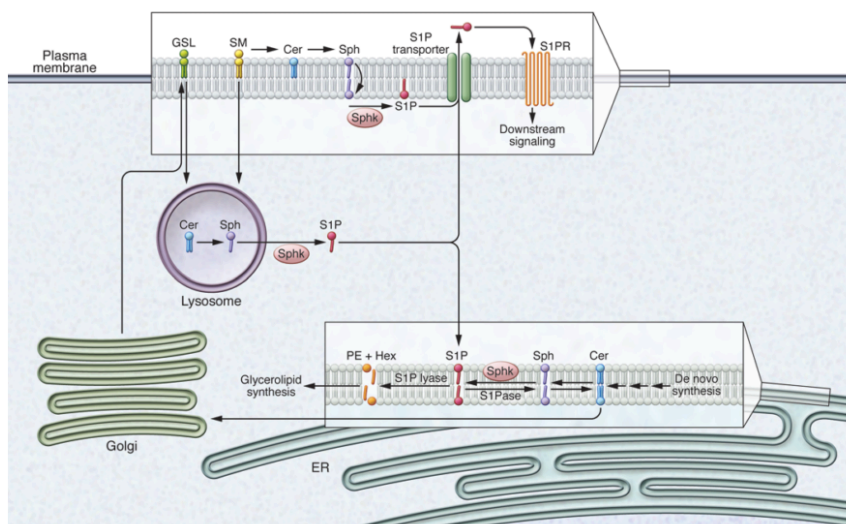


Figure 1.2: S1P location in the cell during synthesis, metabolism, and export.

Ceramide (Cer) is degraded to sphingosine (Sph), a process also occurring on lysosomes. Sphingosine kinase (SphK) is converted to S1P via phosphorylation by SphK and transported out of the cells by SPNS2 to act on GPCRs S1P₁₋₅ in an auto- or paracrine manner. S1P is degraded by S1P lyase into hexadecenal (Hex) and phosphoethanolamine (PE), and transferred to the glycerolipid biosynthetic pathway. Alternatively S1P is dephosphorylated by S1P phosphatase, and recycled back into sphingolipid metabolism. Permission for use in this thesis has been sought (see 10.2) Published by Proia and Hla in 2015 (5).

Levels of sphingolipids in the cell are controlled by multifactorial and bidirectional pathways that are both spatially and temporally regulated. Sphingolipid metabolism is complex, but SphKs have emerged as central regulators. Research is on-going to understand the molecular mechanisms that regulate the activity and subcellular localization of enzymes in this system. Sphingolipid based therapeutics could potentially be involved in the treatment of a range of inflammatory conditions including as sepsis (17), inflammatory bowel disease (18), and cancer (19–22).

SphKs have distinctive steady-state localizations and stimulus-dependent movement that underpins their biochemical roles in the cell (22–24). SphK1 is mostly cytosolic in unstimulated cells accounting for 60-80% of its activity (25), and some association with intracellular organelles. However, the membrane associated SphK1 has been reported as having a tenuous association that is easily disrupted by experimental washing during cell

fractionation to quantify lipids (25). Interestingly, murine SK1b, a short lived splice variant isoform that has been shown to have constitutive membrane association (25,26).

Both SphKs can be activated by extracellular signal-regulated kinase 1/2 (ERK1/2) mediated phosphorylation in response to agonists (27,28), but SphK1 activation is more pronounced. Upon activation SphK1 is translocated to the plasma membrane in a calcium and integrin-binding protein 1 (CIB1)-dependent manner (29), and is available for export (27,30–33). Other stimulatory agonists have been identified such as phorbol myristate acetate (PMA), a phorbol ester that generates an inflammatory response by binding to protein kinase C triggering NF- κ B (32,34), and both phospholipids phosphatidylserine (PS) (35,36) and phosphatidic acid (PA) (10).

Despite highly conserved domains in SphKs they have structural and functional differences (23), and their genes are located on different chromosomes, 17 and 19 for SphK1 and SphK2 respectively. In humans SphK2 is larger and includes an extended N-terminal that contains nuclear localization and export sequences that are lacking in SphK1 (37). SphK2 has been shown to be activated upon protein kinase D mediated phosphorylation (38), and shuttles between the nucleus and the cytoplasm with demonstrated localization to the ER and mitochondria (39). Additionally, it has been proposed that S1P produced in the nucleus has the potential to regulate gene expression (12). Furthermore, the SphKs exhibit partial redundancy since double knockout SphK1^{-/-}SphK2^{-/-} is embryonically lethal *in vivo* due to neurological and vascular defects, whereas single knockout mice are phenotypically normal (40).

1.1.2.4 Sphingosine-1-phosphate

Sphingosine 1-phosphate (S1P) is an endogenous pleiotropic lipid that has a wide variety of physiological and pathophysiological roles (7,41–43), including modulation of the vascular barrier, angiogenesis, and trafficking of immune cells (particularly lymphocytes) (44).

After transport out of the cell, S1P elicits cellular responses by binding to and activating a family of G protein coupled receptors S1P₁₋₅, with low nM affinity (45–47), and by interaction with intracellular target proteins (12). Generally, the effects of S1P (survival, differentiation, proliferation and migration) are largely opposed to those of ceramide (apoptosis, growth arrest and senescence) and thus, the ‘sphingolipid rheostat’ was proposed, whereby

interconversion of ceramide to sphingosine to S1P controls cellular fate (48). However, since this model was proposed it has become clear that sphingolipids are far more complex, and a new more nuanced model is required that incorporates both the receptor-mediated and intracellular effects of S1P (2,11).

1.1.2.5 Extracellular targets of S1P: S1P receptors

1.1.2.6 The S1P GPCR family

The five high-affinity GPCRs known as S1P₁₋₅, bind S1P with nanomolar dissociation constants (49,50). This family of receptors can generate multiple downstream signals to achieve specific effects that are regulated by distinct expression patterns in cells and tissues (51). The regulatory roles attributed to the action of S1P are owing to the variety, prevalence and variation of expression of the S1P receptor (44,52). However, S1P₁ has been the focus of much research and, knowledge of the roles of the other S1PRs are comparatively limited (52).

1.1.2.7 S1P receptor cellular expression

The S1P receptors S1P₁, S1P₂, and S1P₃ are broadly expressed in tissues (5), whereas S1P₄ and S1P₅ expression are tightly restricted to distinct cell types (52,53). However, S1P₁ is the most highly expressed S1P receptor in the S1PR family (53–55).

Endothelial cells (ECs) exhibit high expression of S1P₁ that increases with their maturation, which partially explains the widespread effects of S1P (5,52). The pro-inflammatory receptor S1P₂ expressed on ECs is implicated in a paracrine feedback loop involving EC-derived TNF- α that induces S1P₂ expression. S1P₂ expression leads to activation of NF- κ B and increases intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) (56), signature adhesion molecules that facilitate neutrophil migration from circulation into tissues.

Leukocyte S1PR expression is complex and is postulated to be in accordance with cell subtype- and situation-specificity (see Fig 1.3). Generally, S1P₂ repels macrophages from S1P, whereas S1P₁ and S1P₃ appear to induce migration towards S1P (57,58). Interestingly, S1P₂ has also been implicated in phagocytosis of fungal pathogens as alveolar macrophages of S1P₂ deficient mice had reduced phagocytosis of *Cryptococcus neoformans* (59). Neutrophils express all S1PRs but their expression and subsequent activity is thought to be

dependent on their activation status (60). S1P₂ has a role in circulating neutrophil, as mice lacking S1P lyase (an enzyme that degrades S1P) experience neutrophilia (61). Also, S1P₁ antagonism blocks neutrophil infiltration (52).

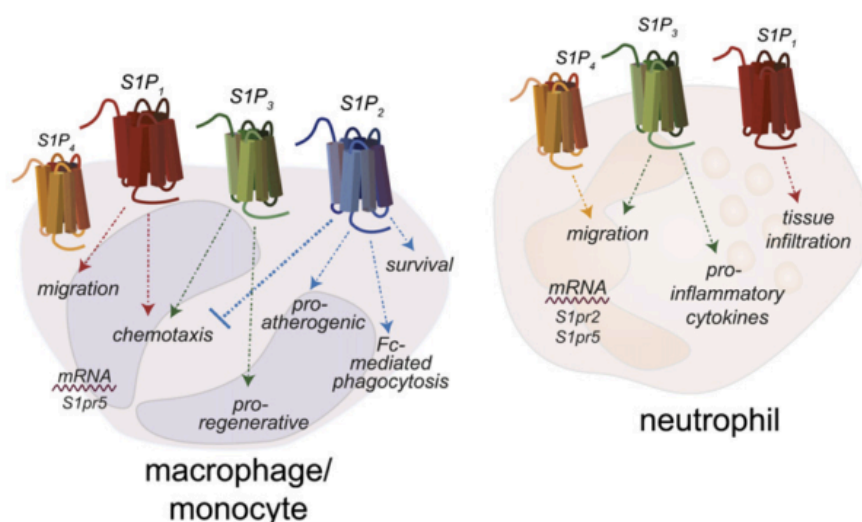


Figure 1.3: The expression of S1PRs on leukocytes. Permission was granted for use in this thesis by the American Society for Biochemistry and Molecular Biology (see 10.3). Published by Blaho and Hla in 2014 (52).

DCs express all S1PRs and exhibit varied responses to S1P stimulation (62–64). In sepsis models S1P₃ is required for IL-1 β production (65) and S1P₄ has been shown to play a role in their migration from lymph nodes (66).

NK cells in mice notably express S1P₅, and low mRNA transcript levels for S1P₁, S1P₂ and S1P₄ (67,68). Also, S1P₅ has been implicated in their egress from lymph nodes (68,69).

T cells predominantly express S1P₁ and S1P₄, but the transcription of mRNA for S1P_{2-3,5} indicate that they are also present (52). S1P₁ induces lymphocyte migration from secondary lymphoid tissues into the blood and lymph (70). In blood and lymph levels of S1P are high (nanomolar-micromolar), whereas in tissues S1P is maintained at low levels, creating an S1P gradient that encourages egress from lymph nodes to circulation (71).

1.1.2.8 Intracellular targets of S1P

In addition to membrane receptor signalling, numerous intracellular protein targets of S1P have come to light (72,73). These are thought to be differentially regulated by SphK1 and SphK2, most likely due to their different subcellular localisations (12).

In TNF- α receptor signalling, TRAF2 ubiquitination is crucial for NF- κ B activation (74,75). SphK1 interacts directly with TRAF2 (76), and its S1P is proximal to act as a cofactor for TRAF2 E3 ubiquitin ligase activity (76,77), as well as the next step in the signalling pathway, ubiquitination of RIP1 (78). Therefore, S1P acts to positively promote TRAF2-mediated NF- κ B activation (76). However, neither SphK1^{-/-} (79) or SphK2^{-/-} knockout mice (80) effect TNF- α -mediated responses (12), indicating that there are other factors at play in TNF- α /S1P signalling.

S1P is also reported to enhance the expression of nuclear hormone peroxisome proliferator-activated receptor γ (PPAR γ) target genes (81). PPAR γ is a nuclear receptor that is expressed mainly in macrophages, intestinal epithelial cells and adipose tissue (81–83). In neutrophils PPAR γ signalling targets gelatinase-associated lipocalin (NGAL), that is proposed to have anti-inflammatory effects (84), suggesting a role for S1P in inflammatory regulation (85).

Other known intracellular targets are with SphK2-derived S1P and occur in the nucleus.

In the nucleus SphK2 is in a repressor complex with histone H3 and histone deacetylase 1 and 2 (HDAC 1/2), which is found at the promoters of genes. Here complexed S1P sustains histone acetylation enhancing transcription of p21 (cyclin-dependent kinase inhibitor) and c-fos (transcriptional regulator) (72). SphK2-derived S1P also acts to regulate mitochondrial assembly and function (86,87); plays a role in stabilisation of telomerase (enhancing cell proliferation); and has been demonstrated to be upregulated in the brains of Alzheimer's patients thereby becoming a potential therapeutic target (12).

1.1.3 Sphingolipid pathway inhibitors

The sphingolipid pathway inhibitors used in this study, SKI-II and FTY720 (Fig 1.4) are detailed below.

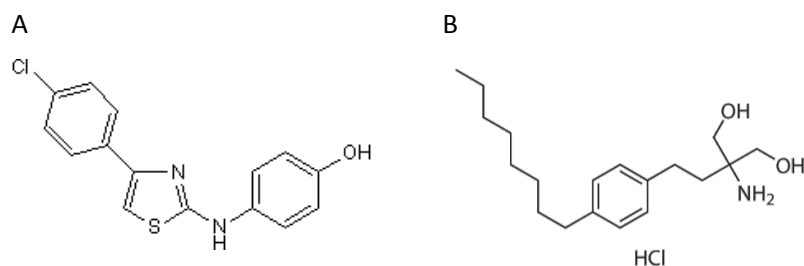


Figure 1.4: Sphingolipid pathway inhibitors. A) SKI-II and B) FTY720

SKI-II [2-(p-hydroxyanilo)-4-(p-chlorophenyl)thiazole], is a potent non-lipid inhibitor of sphingosine kinases, and is showed to induce SphK inhibitory effect at sub micromolar to micromolar concentrations (88). SKI-II reportedly causes irreversible inhibition of SphK1 by inducing its proteasomal and/or lysosomal degradation (89), and treatment of cells *in vitro* resulted in reduction of SphK1 activity and intracellular S1P levels (90,91). This compound has been extensively used to study the involvement of SphK1 and S1P in different cellular processes (92–94), but has also been shown to inhibit SphK2 (95). SKI-II exhibits no inhibition against other kinases such as ERK2, PI3-kinase, or PKC α at concentrations up to 60 μ M (88).

FTY720 (or Fingolimod, formulated as Gilenya™) is licenced as the first oral treatment for relapsing and remitting multiple sclerosis (12). This pro-drug, a structural analogue of sphingosine (96), is phosphorylated by SphK2 to yield active FTY720-p (96–98). FTY720-p is released from cells to act as an agonist for four of the five known S1P receptors, S1P₁, S1P₄ and S1P₅ (EC values of 0.3-0.6 nM) and S1P₃ (EC values of 3 nM) (97). Furthermore, FTY720-p is also a functional antagonist of S1P₁, promoting its internalization and proteasomal degradation (99). In addition, it has been reported that FTY720-p has SphK1, but not SphK2 inhibiting properties (100). The immunomodulatory properties of chronic FTY720 exposure are down-regulation of S1P₁, which results in sequestration of self-reactive lymphocytes in secondary lymphoid organs. This specifically, limits S1P₁-mediated inflammatory T cell invasion of the CNS (101), ameliorating the symptoms of multiple sclerosis with a favourable adverse-effect profile. In addition it reduces astrogliosis and supports nerve remyelination (12,102).

1.2. *Aspergillus fumigatus*

Aspergillus is a diverse genus of fungi with that includes species with high economic and social impact, and *Aspergillus fumigatus* is one of 339 species in the genus of *Aspergillus* (103,104). *A. fumigatus* is a ubiquitous fungus that plays an important role in the recycling of environmental carbon and nitrogen (105,106). Consequently, it is typically isolated from terrestrial soil habitats where it acts as a saprophyte of decomposing organic matter.

Aspergillus species have positive and negative implications for human life. They play important roles in industry and agriculture and have clinical importance. *Aspergillus nidulans* has proven an important model in genetic and cell biology studies (107). *Aspergillus niger* is utilized as a biocatalyst in the production of fine chemicals, its secreted proteases are procured for detergents and it is widely used in the food industry (108). Several species of *Aspergillus* are used in the making of beverages and sauces, such as *Aspergillus sojae* and *Aspergillus oryzae* that are utilized for their saccharification abilities in the production of soya sauce (109) and Sake (110), and *Aspergillus kawachii* in the brewing of the spirit shochu (111).

In contrast to their utility as biotechnological workhorses, *Aspergillus spp.* also have a significant negative impact on the food industry, ecosystems and human health. *Aspergillus flavus* and *Aspergillus parasiticus* are primarily responsible for the aflatoxin contamination of crops resulting in major economic losses. Aflatoxins are associated with severe toxicological effects to human and animals and as such are a global concern (112,113). Although a number of species of *Aspergillus* are known to cause infections, *A. fumigatus* is frequently reported as a pathogenic agent. *A. fumigatus* is the causative species clinically isolated from approximately 90% of invasive aspergillosis cases (114). Other pathogenic species include *A. flavus*, *A. niger* and *A. nidulans* and *A. terreus* (115–119).

All research to date suggests that the pathogenicity of *A. fumigatus* is multifactorial (120–123). Its multifactorial nature is due to a combination of its biological characteristics and the immune status of the host. Some reported characteristics contributing to its virulence as a human pathogen include conidial (spore) size, thermotolerance, resistance to oxidative stress, rapid hyphal growth rate, nutritional versatility and cell wall properties (120,123).

A. fumigatus proliferates from uninucleated conidia and it is estimated that any given individual can inhale on average up to 200 conidia per day (121,123). Due to their small size (2 to 3 μm in diameter), they are able to reach deep within the lung into the alveoli space, the primary site of infection (123,124).

Varying temperature and pH can apply a significant stress on biological systems. *A. fumigatus* grows optimally at the host conditions of 37°C between pH 3.7-7.6, and in the varying environment of the soil and exothermic environment of decaying matter, where it is known to tolerate between 12-65°C and a pH range of 2.1-8.8 (125). Its survival in these conditions suggests it has developed unique mechanisms of temperature and pH tolerance (126). In addition, its germination rate increases between 20-41°C, unlike its related genus counterparts (*A. flavus* and *A. niger*), which exhibit a decline in germination rate at these temperatures (127). The thermotolerance of *A. fumigatus* is linked to ribosome synthesis, which is particularly thermo-sensitive. In regards to this CgrA (a nuclear protein) was identified as being important to thermotolerance, and subsequently subject to many gene deletion studies (126,128,129). One such study showed the ΔcgrA mutant was less virulent compared to parental or reconstituted strains in an immune suppressed murine model (128).

1.2.1 *A. fumigatus* life cycle and cell wall composition

A. fumigatus dormant (resting) conidia are so ubiquitous in the environment that they are considered part of the natural airborne flora, and could be regarded as the most important pathogenic airborne fungi (121,123,130). Readily aerosolized due to their small size and hydrophobicity (121), they are passively transported great distances in normal atmospheric conditions (131). Resting conidia are covered in an outer layer of hydrophobic interwoven rodlet fascicles (132), a characteristic that aids air dispersal, and bestows protection from chemicals and enzymes (133).

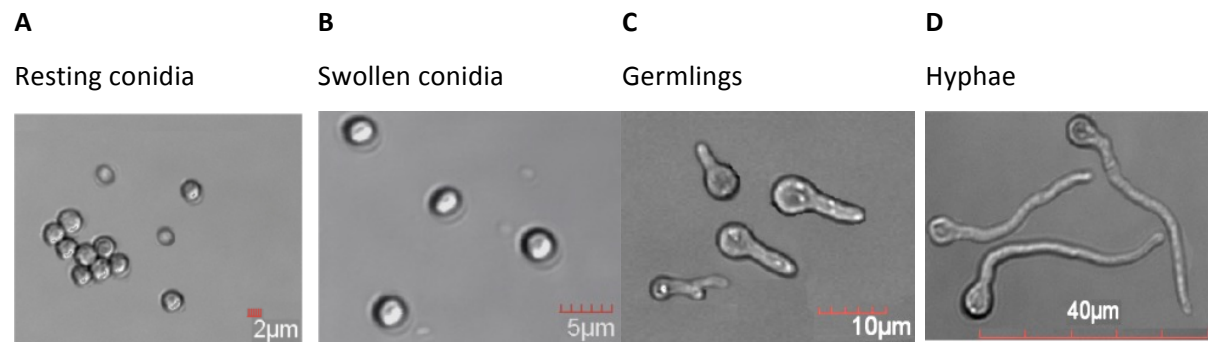


Figure 1.5: Life Cycle: resting, swollen, germinated and hyphal morphology.

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Microscopy images (134).

A significant factor contributing to the virulence of *A. fumigatus* is the dynamic nature of the cell wall during its life cycle. This has negative implications for the host as it allows for immune evasion upon first contact (124,135). The germination of *A. fumigatus* conidia into hyphal filaments follows a series of ordered morphological events (see Fig 1.5). Resting conidia are considered metabolically inactive. During the process of development from cellular to hyphal morphology, resting conidia swell, increasing in size from approximately 2 µM to 4 µM (136). During this maturation process the outermost cell-wall layer is shed to reveal fungal PAMPS recognizable by host immune cells, such as chitin, galactomannan and β1,3-glucans (132). Thus, the host immune system does not initiate an inflammatory responses to resting conidia (or recognise it as a pathogen), but once the cell wall composition is transformed during isotrophic swelling, innate cell recognition and activation ensues in earnest (124).

In addition to a hydrophobic coat, conidia are surrounded by a dense outer layer of 1,8-dihydroxynaphthalene melanin in their cell wall (125,132). Melanin is a pigment that gives conidia their distinctive grey-green colouration (137,138). It also protects from DNA damaging ultraviolet irradiation (125) and from intracellular reactions in phagocytes, with i.e. non-pigmented conidia 10-fold being more sensitive to ROS (132). In immune compromised hosts melanin prevents phagolysosome acidification, which enables *A. fumigatus* to germinate in macrophages and escape by hyphal extension (139). Melanin is therefore considered to contribute to the virulence of *A. fumigatus* (140).

The cell wall of *A. fumigatus* is composed of glucans, mannans and chitin. More specifically Latgé et al (2005) reported the cell wall as being composed of branched β 1,3-glucan that are covalently bound to chitin, β 1,3-, β 1,4-glucans, and galactomannan, that are embedded in α 1,3-glucan, galactomannan and polygalactosamin (141). With the central core of the chitin linked branched β 1,3-, and β 1,6-glucans account for about 30% of the total cell wall in *A. fumigatus* (141). Covalently associated cell wall proteins are typically enzymes related to its synthesis (142).

Following the break from dormancy, the next step in the life cycle for resting conidia is isotropic swelling (Fig 1.5 B), followed by polarization and emergence of a germ tube (Fig 1.5 C) and germ tube elongation (Fig 1.5 D). Once resting conidia adhere to a substrate containing water and a carbon source, nutrient acquisition and biosynthetic pathways are upregulated (143–145). Isotropic growth of conidia is as a result of water uptake (146), during which time conidia can double in size. Throughout this process the cell wall undergoes significant remodelling losing hydrophobin and melanin and exposing underlying cell wall components.

During the course of conidial swelling, polysaccharide hydrolysis by β 1,3-glucanase and chitinase activity softens and thins the original cell wall to allow for expansion and the resultant hyphae have a single cell wall mono-layer (141,147). In *A. fumigatus* the first mitosis preceded the isotropic polar switch (to hyphal formation) in 22% of conidia, a process which occurs in *A. nidulans* only after the first mitosis and in which initial nuclear duplication took 25% more time (148).

Before the switch to polarized filamentous growth, conidia dedicate a cell wall location where a germ tube will emerge (149). This site specification step is referred to as establishment of polarity, and results in the swollen conidia becoming pear shaped, coinciding with the first round of mitosis (148). In preparation for apical growth there is redirection of cellular machinery for plasma membrane and cell wall synthesis and extension, where new material is added exclusively to the apex, known as polarity maintenance (149,150). Following the shift to polarized growth a complex of branched hyphal filaments develops, known as mycelia. Hyphae are multi-nucleated structures and after a period of apical growth the first septum is reported to occur after the fourth mitosis.

Auxiliary branches to main hyphae are to establish sub-apical axes of polarity (149). In addition, second germ tube emergence is reported to occur at the third mitotic event (148).

The cell wall of hyphae differs from that of swollen conidia in that chitin and β 1,3-glucan can be detected on the surface of swollen conidia and recently formed germ tubes, but not on mature hyphal surfaces (147,151). During maturation from swollen conidia to hyphae galactosaminogalactan mediates adhesion to epithelial cells and aids host evasion by binding to and masking β -glucans and possibly other PAMPS also (similar to Rodlet A on resting conidia)(152). Through this mechanism *A. fumigatus* reduces detection by decreasing recognition by the host and thereby suppresses host inflammatory responses (153).

The life cycle for *A. fumigatus* completes with sexual or asexual reproduction, resulting in the production of conidia (conidiation) that occurs after hyphal formation. *A. fumigatus* develops specialised structures called conidiophores atop of aerial hyphae, which produce and disperse hundreds of hydrophobic conidia (143,154). Despite *Aspergillus* being first described 152 years ago, the sexual reproductive mechanism in *A. fumigatus* remained elusive. It was only in 2009 that a sexual cycle was reported in *A. fumigatus* otherwise known to reproduce only by asexual means (154). Sexual reproduction occurs when the opposite mating genotypes, MAT1-1 and MAT1-2, are in close proximity to each other, after which light-yellow spherical fruiting bodies develop known as Cleistothecia (154). The teleomorphic or sexual stage of *A. fumigatus* is known as *Neosartorya fumigata* (103,154,155). A sexual reproduction cycle of *A. fumigatus* provides a clear advantage, as genetically diverse offspring have the potential for increased virulence and or antifungal resistance (154).

1.2.2 Secondary metabolites

Aspergillus species produce a diversity of biologically active small organic molecules, known as secondary metabolites (SMs) (156,157). The antibiotic penicillin, the cholesterol-reducing drug lovastatin, and potent mycotoxins are just some of the secondary metabolites of the vast and unknown pharmacopoeia encoded in the *Aspergillus* genome (156).

SMs are thought to play important roles in colonizing the respiratory tract and although not all mechanisms and effects are clear, gliotoxin, verruculogen, and helvolic acid are mycotoxins frequently implicated in *A. fumigatus* pathogenesis (140,157,158). In *Aspergillus*,

genes controlling SMs are typically physically linked or organized in clusters on a chromosome, which can be highly variable even within species (156,159). These evolutionarily diverse regions are not only virulence attributes but are also postulated to be as a result of niche adaptation, and *A. fumigatus* contains 26 such gene clusters (140,159). LeaA is a global regulator of secondary metabolism in *Aspergillus*. In *A. fumigatus* LeaA is a transcriptional regulator upregulating the expression of 13 of 22 secondary metabolite gene clusters (140,160,161). It is required for the expression of the biosynthesis of mycotoxins like gliotoxin but also mycelial pigments, lovastatin, penicillin and sterigmatocystin (a carcinogen related to aflatoxin) (161). It is therefore accountable for much of the chemical diversity in SMs of *A. fumigatus* (140).

Gliotoxin is one of the most studied mycotoxins and has long been implicated as a major virulence factor for *A. fumigatus* (140,162). As an alkaloid derivative mycotoxin is demonstrated to have an immunosuppressive effect (162,163). Gliotoxin inhibits NADPH oxidase and can trigger apoptosis in macrophages through ROS (162,164), has a negative influence on phagocytosis by neutrophils (162,165), and interferes with leukocyte cytokine release and T-lymphocyte mediated cytotoxicity (166–169). In addition it causes cilliositis reducing mucociliary clearance and causes marked epithelial cell disruption *in vitro* (157,158). Gliotoxin is known to be produced during exponential stages of growth of *A. fumigatus* at host temperature of 37°C (170), and therefore has a minimal role in colonization but is implicated to be crucial in the later stages of infection, and has been detected in patients suffering from aspergillosis (157). Verruculogen is also implicated in the development of aspergillosis and has been demonstrated to alter pulmonary clearance through cilliositis and epithelial disruption *in vitro*, thereby increasing the colonization potential of *A. fumigatus* (157,158). It is therefore implicated in the initial stages of infection during conidial settlement (157). Lastly, helvolic acid is demonstrated to inhibit the oxidative burst in macrophages in addition to causing cilliositis and epithelial cell disruption (157,158,171,172).

1.3. Molecular recognition of *A. fumigatus*

1.3.1 Soluble proteins

Soluble proteins important in the immune response against *A. fumigatus*, include complement proteins, collectins, pentraxins and ficolins, and belong to the humoral arm of the innate immune system (173).

1.3.1.1 Complement

Complement is part of innate immunity and consists of more than 35 soluble proteins and membrane factors, which act in a sequential manner to eliminate pathogens directly or indirectly (174,175). Complement proteins are produced principally by the liver but AMs and ECs independently produce a number of factors (C2, C3, C4, C5 and factor B) (175). Products of the complement pathway are important to fungal immunity because they enhance phagocytosis by opsonizing pathogens and act as chemoattractants for migrating immune cells such as neutrophils (174). The classic, alternative and lectin pathways of complement converge on a common pathway in which C3 is cleaved by C3 convertase, and *Aspergillus* activates all three pathways in a morphologically dependent manner (176). Resting conidia activate the alternative pathway, and fungal development positively correlates with increasing classical pathway involvement (176).

When C3b interacts with C3 convertase it forms C5 convertase which then cleaves C5 to C5a and C5b (177). C5 is important in fungal immunity because it is a potent chemoattractant for PMNs, and C5 knockout mice are rendered more susceptible to IPA. Furthermore, treatment of knockout mice with serum containing complement factors increased neutrophil influx and survival, indicating the importance of complement at the early stages of infection (178). In the later stages of fungal infection, the classical pathway is largely driven by the interaction of the complement pattern recognition molecule C1q, and surface-bound fungal targeting IgG and IgM (176,177). The classical pathway can also be activated by interaction of C1q with pentraxins and c-reactive protein (179,180), the importance of this was demonstrated by C1q knockout mice that were rendered more susceptible to IPA (181,182).

1.3.1.2 Collectins

Collectins are soluble C-type lectin receptors that recognize and bind to carbohydrate structures in the fungal cell wall (175,183).

Pulmonary surfactant proteins SP-A and SP-D are important factors in the recognition of *A. fumigatus* (175). Secreted by ECs and Clara cells (175,184), these collectins bind to the surface of *A. fumigatus* conidia promoting their aggregation and efficient phagocytosis by macrophages *in vitro* (185). SP-D has facilitative roles in the clearance of conidia, and recognition with subsequent inflammatory response is mediated through PRRs (186,187). In addition, pulmonary surfactant proteins act as potent neutrophil chemoattractants (188).

and increase the fungicidal effects of AMs and neutrophils (175). They are also implicated in the later stages of infection as SP-D has been demonstrated to bind to hyphae (189), and *in vivo* treatment of immunocompromised mice with SP-D conferred a 60% survival rate with lethal doses of inoculum (190).

Mannose-binding lectin (MBL) activates the lectin pathway (191). MBL activates the MBL-associated serine proteases (MASPs) that results in conidial opsonisation (174)(192). MBL has been demonstrated to enhance conidial phagocytosis and anti-fungal activity of alveolar macrophages and neutrophils (193). Furthermore, the importance of MBL *in vivo* was demonstrated when mice treated with recombinant human MBL after *A. fumigatus* inoculation had reduced lung fungal burden, and enhanced and reduced inflammatory and anti-inflammatory cytokines respectively, that resulted in increased survival (193).

Pentraxin-3 (PTX3) is a soluble pattern recognition receptor (PRR), secreted by mononuclear cells, neutrophils, dendritic cells and pulmonary epithelial cells in response to conidia and inflammatory cytokines (175,181,194). PTX3 opsonizes conidia (181), and is demonstrated to not only improve macrophage but also neutrophil recognition and conidial uptake by neutrophils in an Fcγ receptor II dependent manner (182). Its critical role in response to *A. fumigatus* clearance was demonstrated *in vivo* with immunocompetent PTX3^{-/-} mice which were rendered highly susceptible to IPA was partly attributed to defects in fungal recognition (181).

Ficolins are also lectins and similarly to MBL activate the lectin pathway by forming complexes with MASPs (195). In a study using A549 epithelial cells, it was suggested that ficolin-a may play an important role in the opsonization of conidia and hyphae that resulted in enhanced adherence, and they were demonstrated to modulate the inflammatory response to conidia by increasing IL-8 production (196). Ficolins are co-operative with other soluble factors such as with PTX3, where Ficolin-2 enhances the binding of PTX3 to *A. fumigatus* conidia and vice versa (197).

1.3.2 Membrane-bound receptor recognition

The fungal cell wall is a highly complex mesh of carbohydrate polymers representing a major source of its pathogen-associated molecular patterns (PAMPs) (198). These fungal PAMPs mediate host recognition through the pattern recognition receptors (PRRs) of immune cells

(199). PRRs known to be important in detecting fungi are Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and nucleotide-oligomerisation domain (NOD)-like receptors (NLRs) (200,201).

1.3.2.1 Toll-like receptors

TLRs are membrane receptors that signal through the universal adaptor MyD88 (186,202), that activates NF- κ B-mediated expression of pro- and anti-inflammatory responses (186,203). TLR signalling is often characterised as either MyD88-dependent or -independent. The importance in MyD88 signalling in early responses to *A. fumigatus* was demonstrated in immunocompetent MyD88 knockout mice that exhibited a dysregulated initial immune response that resulted in increased fungal persistence that normalised by day 3 post inoculum suggesting compensatory alternative pathways (204).

TLR2 and TLR4 have been postulated to be the most important TLRs for fungal immunity against *A. fumigatus* (134), recognising fungal O-linked mannans. It is well established that TLR2 and TLR4 mediate an inflammatory response through the activation of NF- κ B (205–207). Interestingly, it has been demonstrated that TLR2 and TLR4 on macrophages discriminate between *A. fumigatus* morphologies by binding to swollen conidia and hyphae for the former and only conidia for the latter (151,208). Both TLRs trigger inflammatory responses, but the phenotypic switch of swollen conidia to hyphal filaments induces the expression of anti-inflammatory cytokine IL-10 in a TLR2-dependent manner. Due to this it has been suggested that immune modulation from pro- to anti-inflammatory cytokines is a potent virulence factor of *A. fumigatus* (208,209). *In vivo* studies with TLR2^{-/-} and TLR2^{-/-}/TLR4^{-/-} have demonstrated higher fungal burden and both receptors are required for optimal PMN recruitment (205,210).

TLR3 is expressed by dendritic cells in endosomal compartments detecting fungal RNA (211). TLR3 mediates an immune response to *A. fumigatus* through adapter protein TRIF (212,213), and an adaptive response after its migration to the lymph nodes where it primes T-cells. *In vivo* TLR3 knockout mice had a dysregulated inflammation response (214), were unable to activate a CD8⁺ T-cells (215), and had increased susceptibility to infection (215).

TLR9 is expressed by human plasmacytoid DCs (pDCs) and B cells in humans, and expressed in bronchial and vascular endothelium, and alveolar macrophages in both mice and humans

(216,217). TLR9 detects unmethylated CpG DNA release by *A. fumigatus* in the phagolysosome (216,218), and its recruitment to the phagosome is not morphology dependent, suggesting it is important throughout a *A. fumigatus* infection (218). A recent study showed that in response to *A. fumigatus*, phagosomal TLR9- and Bruton's tyrosine kinase-dependent signalling activated inflammatory signaling that is independent of MyD88 (219). Studies *in vivo* have suggested it is required for initiation of a robust proinflammatory response against *A. fumigatus* and plays an immunoregulatory role in preventing an excessive immune response that results in pulmonary immune pathology (220).

1.3.2.2 C-type lectin-like receptors

C-type lectin-like receptors (CLRs) are a family of receptors recognizing a wide range of carbohydrate structures (221).

Dectin-1 is highly expressed on macrophages, neutrophils and dendritic cells and plays a major role in fungal immunity (222). Dectin-1 recognises β -glucans that are exposed on the surface of hyphae and swollen conidia (223), and can occur on the cell membrane or intracellularly in phagolysosomes (135,151,224). Dectin-1 recognition mediates a signalling response through both Syk kinase and CARD9 resulting in NF- κ B-dependent expression of cytokines and chemokines. Pro-inflammatory cytokines included include TNF- α , IL-6, IL-1 α , IL-1 β , G-CSF, GM-CSF, and chemokines MIP-1 α and MIP-1 β (151,223,224). Additionally, it induces production of the signature anti-inflammatory cytokine IL-10, suggesting an immunoregulatory role (151). Furthermore, it is well established that Dectin-1 signalling synergizes with the MyD88 pathway to induce an appropriate inflammatory response to swollen conidia (135). In this respect, Dectin-1 and TLR2 act collaboratively in facilitating phagocytosis of *A. fumigatus* conidia (225,226). More specifically, while TLR2 is dispensable for the initial binding to the conidial surface, it does promote Dectin-1-mediated phagocytosis in MyD88-dependent manner (136).

Dectin-2 recognises α -mannan moieties (199), and has recently been implicated in the fungal immune response by a study that demonstrated it was highly expressed on alveolar macrophages during *A. fumigatus* interaction (227). Similar to Dectin-1, Dectin-2 interacts with swollen conidia, not resting, and mediates an NF- κ B-mediated pro-inflammatory response that is via Syk kinase-dependent (227,228). Silencing this pathway in macrophages *in vitro* resulted in impaired respiratory burst and consequentially less fungal killing (228).

1.3.2.3 NOD-like receptors

NOD-like receptors are a cytosolic class of PRRs primarily expressed by leukocytes, dendritic cells and epithelial cells (175,229).

Nucleotide-binding oligomerization domain-2 (NOD2) is implicated in *A. fumigatus* conidial recognition and NF- κ B activation, demonstrating potential for host defense signalling against *A. fumigatus* (229). Additionally, NOD2 may have a regulatory role as implicated in the induction of IL-10 in response to the fungal cell wall component chitin (230).

NLRPs are a subfamily of NOD-like receptors. NLRPs play a pivotal role in activation of acute phase inflammatory mediators IL-1 β and IL-18 in response to *A. fumigatus* hyphae but not conidia (231). Upon activation these receptors oligomerize into multi-protein signalling complexes termed inflammasomes. Inflammasome assembly leads to caspase-1 activation and pro-cytokine cleavage (232). The NLRP3 inflammasome activates an IL-1 β -mediated inflammatory response against *Aspergillus* swollen conidia and hyphae, suggesting importance throughout infection (231,233).

1.4. The immune response to *A. fumigatus*

1.4.1 Innate Immune response to *Aspergillus fumigatus*

Macrophages and neutrophils of the innate system provide the first line of defense against invading microbes (234). Innate immune cells respond by recognition, phagocytosis and intracellular killing of conidia, and co-ordination of an appropriate host inflammatory response (235–237).

Epithelial cells provide an inflammatory response that involves the expression of adhesion molecules to facilitate PMN recruitment, in addition to mucociliary clearance, this results in the effective removal of conidia from the lungs (238). However, innate cells cannot always recognise and eliminate pathogens (234). As aforementioned, *A. fumigatus* resting conidia maintain a hydrophobic proteinaceous coat that enables immune evasion (124). These innate responses are followed by an adaptive immune response, which is initiated by dendritic cells (DCs). DCs phagocytose fungal conidia, and migrate to the lymphoid organs to present antigens to T-cells (239). Lymphocytes of the adaptive immune system provide a pathogen specific defense that provides increased protection from reinvasion by the same organism (234). Innate immune cells play an important role in initiating and directing the

subsequent adaptive response, as well as working with lymphocytes to eliminate and clear dead pathogens (234). Furthermore, innate immunity has the critical role of controlling invading pathogens during the 4-7 days before the adaptive immune response comes into effect (234).

1.4.1.1 Alveolar macrophages

Macrophages are sentinels of the innate immune system, and patrol the tissues to eliminate pathogens, particulates and apoptotic cells. Alveolar macrophages (AMs) are pulmonary specific in that they develop from fetal monocytes that colonized the lung in the first few days after birth. The AM population is maintained by these self-renewing cells and only in the case of absolute depletion are they replenished by hematopoietic stem cells (240,241). Murine studies have demonstrated the importance of macrophages in an AM depleted model that exhibited increased fungal burden (242).

Since AMs reside on the luminal side of the lung surface they are the first innate immune cell to encounter conidia, and play a critical role in initiating and shaping innate and adaptive immune responses. AMs are primarily responsible for phagocytosis and intracellular killing of conidia, with concomitant initiation of an appropriate immune response. Phagocytosis of *A. fumigatus* conidia follows a series of complex and coordinated cellular mechanisms (188,206,219,243). Upon interaction with fungal cell wall PAMPS with host PRRs Dectin-1, TLR2 and TLR4, transcription factor NF- κ B is activated and initiates an inflammatory response resulting in production of cytokines and chemokines that stimulate leukocyte recruitment and ensuing antigen-specific immunity (226,244,245). Although macrophages competently and regularly clear inoculum from the lungs, conidia in large numbers can necessitate the involvement of neutrophils to achieve sterility (246). AMs work co-operatively with neutrophils to clear larger inocula and or persistent fungal burden and they secrete key mediators of neutrophil recruitment such as TNF- α , macrophage inflammatory protein-2 (MIP-2) and keratinocyte-derived chemokine (KC) (242). Therefore, for an immune competent host with routine clearance of *A. fumigatus* conidia, it is important to mount an appropriate response to this opportunistic pathogen.

As for antifungal effector functions, after phagocytosis AMs use oxidative and non-oxidative methods of intracellular conidial killing. Oxidative methods include the generation of ROS

like the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) and non-oxidative mechanisms of phagosomal acidification (246) (243).

Conidia are primarily phagocytosed by AMs into a phagosome that matures by fusing with a lysosome to form a phagolysosome (185,243). In the phagolysosome conidia swell and shed their hydrophobic coats (of rodlet A proteins) over approximately 3 hours to expose the cell wall (124,185,247), after which fungal PAMPS activate lysosomal Dectin-1 and TLR9 (218,224). This intracellular incubation and sensing mechanism is central to innate recognition of and response to *A. fumigatus* (224). Non-oxidative acidification and hydrolytic enzymes also contribute to conidial killing. The phagolysosome undergoes acidification through a vacuolar ATPase- dependent mechanism, and in consort activation of hydrolytic enzymes such as chitinases and cathepsin-D contribute to fungal cell wall degradation (243).

After conidial maturation in the phagolysosome swollen conidia-mediated ROS generation is through activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or NOX (248–250). NADPH oxidases are a family of ROS generating enzymes. Seven of which are expressed in mammals, namely, NOX1, NOX2, NOX3, NOX4, NOX5, dual oxidase 1 (DUOX1) and DUOX2 (249). As a family they share the capacity to transport electrons across the plasma membrane from cytoplasmic NADPH to extracellular or phagosomal oxygen (248,249). This movement generates superoxide (O_2^-) from molecular oxygen (O_2) that is further reduced into hydrogen peroxide (H_2O_2) by addition of another electron (249,251). Thus, the biological function of NOX enzymes is the generation of ROS (249). Formation of active NADPH oxidase requires multiple proteins to act sequentially. Firstly, small G-proteins of the rac family Rac1/Rac2, and the cytoplasmic protein p47^{phox}, p67^{phox} and p40^{phox} are recruited to the cell membrane where they bind to NOX2/gp91^{phox} and gp22^{phox} to form active NADPH oxidase (248,252). For a long time only NADPH oxidase 2 (NOX2) was known, before the above mentioned homologous enzymes were identified (249). NOX2/gp91^{phox} is predominantly expressed in professional phagocytes, like macrophages and neutrophils (249), as well as eosinophils, dendritic and B cells (248). The production of ROS positively correlates with increased levels of fungal killing (253). *In vivo*, p47^{phox-/-} mice with inhibited ROS production have suppressed conidial killing (246), and their AMs are unable to prevent the growth of internalised conidia *in vitro* (253).

Moreover, in addition to phagosomal conidial killing, AMs also initiate downstream immune

responses by secreting cytokines and chemokines. These inflammatory mediators recruit leukocytes and lymphocytes to the site of infection such as neutrophils and NK cells. Interestingly, a link was made between ROS and leukocyte chemotaxis in a study that demonstrated CXCL2 and CCL3 genes involved in PMN recruitment were significantly upregulated after inoculation with *A. fumigatus* in WT mice compared to gp91^{phox-/-}. This suggests a role for ROS early in infection in neutrophil recruitment (254).

In summary, macrophages are therefore key regulators of pro-inflammatory cytokine responses, augmenting or attenuating cellular immunity to *A. fumigatus* as well as its phagocytosis and intracellular killing (185,246,255).

1.4.1.2 Neutrophils

Neutrophils are an essential part of the innate immune response against *A. fumigatus*. Neutropenia is a key risk factor for developing IPA in humans (123), and their importance has been demonstrated by numerous neutrophil depletion studies that resulted in the establishment of IPA with high mortality (256–258). Neutrophils are not resident in the lungs like macrophages, but they do mature from a common myeloid progenitor (234). They develop in the bone marrow and are released into the peripheral circulation fully matured. Interestingly, neutrophils account for approximately 50-70% of circulating leukocytes in humans (259) and only 30% in mice. Similarly, they have a short circulating half life of 6–10 h in humans and 11.4 h in mice (260,261).

Neutrophils migrate from the peripheral circulation in response to inflammatory stimuli. It has been demonstrated that this occurs mostly due to the subset CXC family of chemokines secreted by alveolar macrophages (262). Neutrophils arrive at the site of infection fully activated and within half an hour (263,264). Activation of circulating neutrophils is initiated with the detection of adhesion molecules expressed by ECs (234). The endothelium is activated by inflammatory mediators secreted by macrophages upon interaction with the pathogen, in particular TNF- α and IL-1 β (234,265). Adhesion molecules known as selectins are then quickly expressed on the surface of ECs following TNF- α stimulation (234). On leaving the circulation and entering the target tissue, neutrophils follow a chemotactic gradient to the site of infection (260,266). At the site of infection they work in concert with macrophages to mount an effective immune response against a fungal insult. Macrophages routinely and effectively clear inhaled conidia from the lungs. Nevertheless, in the case of a

large inoculum that cannot be promptly cleared or conidia that escape macrophage killing and germinate, neutrophils are recruited by secreted proinflammatory mediators such as IL-8 in humans and KC in mice (134,237). In this sense, neutrophils are key for early control of *A. fumigatus* infections (267).

Neutrophils both recognise and kill conidial and hyphal forms of *A. fumigatus*. Neutrophils bind, phagocytose and kill swollen conidia, and, similarly to macrophages, they employ oxidative and non-oxidative mechanisms. They release antimicrobial proteases through the process of de-granulation, microbicidal proteins (such as lactoferrin) and NOX-mediated ROS, and generate neutrophil extracellular traps (NETs) that have been demonstrated to damage and limit hyphal growth respectively (268–271).

The primary mechanism responsible for intracellular killing of *A. fumigatus* is non-oxidative. After phagocytosis, primary granules containing hydrolytic enzymes merge with the phagosome (272,273). An other means of non-oxidative fungicidal activity is through lactoferrin-mediated iron depletion (271,274). Iron is an essential micronutrient for virulence of *A. fumigatus in vivo* (275) as it is a key component to several enzymes required in cellular processes (276,277).

Oxidative mechanisms include the generation of ROS through NOX. Similarly to macrophages, neutrophils phagocytose and kill conidia via fusion of antimicrobial granules with the phagosome and the generation of ROS via the NADPH oxidase complex. In addition to direct killing by ROS, its release into the phagosome causes an increase the ionic strength within the vacuole that leads to the release of aforementioned granule proteins such as elastase and cathepsin G (278).

Neutrophils are classically considered to act primarily on hyphae (269) by attaching to the cell wall and spreading over its surface before degranulating, and in response to resting conidia they weakly stimulate ROS with minimal degranulation (279–281).

In addition to oxidative methods, activated neutrophils release chromatin and granule proteins to create fibres that form neutrophil extracellular traps (NETs). NETs immobilize microbes thereby preventing dissemination and allowing for a more targeted assault by the immune system, and are thus an essential part of the antifungal arsenal. Since their

description in 2004 (263), NETs have been implicated in host defence against *A. fumigatus* (274,282). The major structural component of NETs is decondensed DNA that is in complex with histones and granule proteins (283). This decondensed nuclear chromatin not only forms the network of extracellular fibres but also binds proteins from primary (neutrophil elastase, cathepsin G, and myeloperoxidase), secondary (lactoferrin) and tertiary granules (gelatinase). In addition, NETs are also fast developing and can form as quickly as 10 min after activation (263). NET formation (NETosis) is induced by proinflammatory mediators such as IL-8 and NADPH oxidase (284). NETosis is directly stimulated by *A. fumigatus* but is morphologically discriminatory. They can be directly stimulated by *A. fumigatus* hyphae and swollen conidia (282), which suggest activation via fungal PAMPS is involved. This is further suggested by NETosis activation by resting conidia of Δ rod mutants (with no hydrophobic layer) but not WT resting conidia (274,285). NETs released on swollen conidia do not prevent germination but can limit growth of their hyphae (282). NETs therefore are of particular use with pathogens too large to phagocytose such as fungal hyphae and form important defences against *A. fumigatus* in the later stages of infection (263,286). Recently it has been reported that *A. fumigatus* mediates resistance to NETs via a cell wall exopolysaccharide galactosaminogalactan (GAG), that mediates adherence and was demonstrated to be required for virulence in a murine model (287).

Neutrophils also contribute to shaping the unfolding inflammatory response by producing a variety of cytokines and chemokines (236). Neutrophils express a range of PRRs that sense *A. fumigatus* associated PAMPS that drive secretion of proinflammatory mediators (288). Similarly to macrophages they recognise and phagocytose conidia via TLR2 and TLR4, and Dectin-1 (162).

Once activated, neutrophils undergo a transcriptional burst that results in *de novo* synthesis of signalling molecules (289,290). Neutrophils have been reported to secrete TNF- α , IL-12, GM-CSF, IFN- γ , IL-6, IL-8, IL-10 and IL-1 β in response to *A. fumigatus* (291), but the primary cytokine is IL-8 (murine KC), which serves to recruit other neutrophils (206,236). In addition, neutrophil-derived IL-1 β and TNF- α stimulate other cells to produce neutrophil chemoattractants (291,292). Although neutrophils are considered weak producers of cytokines compared to macrophages, they are typically in large numbers at the site of infection and this increases their total cytokine contribution to the inflammatory environment (266,293).

In addition to production of proinflammatory mediators, neutrophil-derived serine proteases have an alternative function in to both activating and degrading cytokines, demonstrating proinflammatory or regulatory effects on other immune cells (269,294). Furthermore, some granule proteins that neutrophils release are chemotactic not only for other leukocytes (neutrophils, monocytes, and dendritic cells) but also T cells, highlighting their assistive role in co-ordination of the adaptive response (236,269,295).

In summary, tight regulation of neutrophils is critical for the host, not only to combat infection but because they have significant histotoxic potential and are extensively implicated in inflammatory related tissue injury (260).

1.4.1.3 Epithelial cells and Eosinophils

The first cells in contact with inhaled conidia are the airway-lining epithelial cells (ECs). The role of ECs extends beyond an anatomical barrier, to phagocytosis and proinflammatory responses.

Phagocytosis of *A. fumigatus* conidia was first reported in ECs in 1997 (296), and it was later defined as a TLR2 and TLR4 independent process (297). *Aspergillus* hyphae and secreted proteases promote the production of proinflammatory IL-6 and TNF- α , and chemokine IL-8 (murine KC) *in vitro* but irradiated (dead) conidia do not (297–300). Further, IL-6 produced during host-fungal interaction indirectly promotes chemokine release by lung ECs (301). After phagocytosis, ECs have also been demonstrated to upregulate adhesion molecules E-selectin and VCAM-1 playing a supportive role in leukocyte migration to the site of infection (134,257). Lastly, ECs were also found to produce high levels of β -defensins when exposed to resting or swollen conidia, and hyphae (302). Together these responses suggests ECs tailor responses according to the severity of the pathogen and as such could be important in the early stages of infection.

Eosinophils have been implicated in the immune response against *A. fumigatus* (303). Their granular content is hypothesised to contribute to their function against *A. fumigatus in vivo* (303), which they discharge into the phagosome and extracellular fluid upon activation (303,304). A study reported that decreased pulmonary clearance of *A. fumigatus* was associated with impairment of eosinophilic-derived cytokines and chemokines compared to WT (303).

1.4.1.4 NK cells

The nature of natural killer (NK) cell involvement in response to filamentous fungi is yet to be fully elucidated, but they have been implicated in the host response against *A. fumigatus* (305). However, their contribution to host antifungal activity appears to be multifactorial and mediated through soluble factors (305–309).

NK cells are large granular lymphocytes that are sentinels of the innate immune system. They represent approximately 10-15% of all peripheral blood lymphocytes, and are stimulated by IL-2 produced by dendritic and activated T cells (307,310). NK cells migrate to the site of infection (similarly to other leukocytes) by sensing chemoattractants, and their early recruitment to the lungs has been shown *in vivo* to be critical for early host defence against IPA in a neutropenic mouse model (309).

Both stimulated and unstimulated NK cells selectively affect *A. fumigatus* hyphae, with stimulated cells imposing significantly more damage. Thus, bestowing on IL-2-producing cells an important role in the promotion of NK cell anti-fungal potential. Morphological discrimination by NK cells was further confirmed when no NK cell-mediated damage was observed in resting conidia of WT or $\Delta RodA$ mutants (306). Moreover, NK-cell-hyphal contact has been shown to be crucial in inducing hyphal injury (305). This selectivity for actively growing *A. fumigatus* filaments suggests a role in the later stages of infection or for fungus that has escaped the first line of host defence by macrophages.

NK cells influence macrophages as a major source of IFN- γ which enhances their antimicrobial affects *in vitro*, thus assisting in proinflammatory responses during early infection (308). Hyphal morphologies are highly immunogenic and result in high levels of NK-cell derived IFN- γ and GM-CSF, which has been shown to be reduced during incubation with *A. fumigatus*, demonstrating that NK cells are also subject to its immunosuppressive mechanisms (306). Moreover, in NK cell depleted mice reduced IFN- γ was correlated with increased fungal burden and the transfer of activated NK cells from WT mice increased fungal clearance (308).

Furthermore, akin to fellow granulocytes, NK cells produce potent natural cytotoxic proteins, namely perforin, granzymes and granulysin. Although whether this activity against *A. fumigatus* is direct or indirect is yet to be entirely validated (306).

1.4.1.5 Dendritic cells

DCs are leukocytes derived from the common myeloid progenitor. Immature DCs enter tissues from circulation to function as innate immune sentinels. Pulmonary DCs are made up of a complex network of cells from various origins, whose categories include conventional DCs (cDCs), plasmacytoid DCs (pDCs) and monocyte-derived DCs (moDCs). Each category of DCs represents an independent developmental lineage, and each has been described as distinct and overlapping in function (311).

Immature DCs (iDCs) recognise and phagocytose *A. fumigatus* conidia and hyphae via Dectin-1 and DC-SIGN *in vitro*, triggering the maturation process (312–314). iDCs stimulated with *A. fumigatus* germlings produce a raft of proinflammatory cytokines that include CCL20, CXCL10, IL-6, IL-8, IL-10, IL-12, and TNF- α (314). DC-derived cytokines are morphologically discriminatory with IL-12 produced in response to conidia, TNF- α in response to conidia and hyphae, IL-4 and IL-10 in response to hyphae only (239), and chemoattractant IL-8 during the onset of infection (315). A study demonstrated that *A. fumigatus*-stimulated DCs produce CCL20 (a recruiter of effector memory Th1 cells to the infection site) and that monocyte-derived DCs (moDCs) initiate the Th1 response after exposure to hyphae (315,316). This study also postulated that DCs released CCL19 once in the lymph nodes to encourage DC-T cell interaction (315). Lastly, plasmacytoid DCs (pDCs) bind to and inhibit *A. fumigatus* growth (317), and during cell-hyphal contact pDCs form pDC extracellular traps or pETs similar to that of neutrophils (318). The role of pDCs in IPA was established in a depleted murine model which exhibited hyper-susceptibility (317).

1.4.2 Adaptive Immune response to *A. fumigatus*

After phagocytosis of *A. fumigatus*, DCs mature and present fungal antigens on their surface. This process is followed by migration to the lymph nodes in order to activate T lymphocytes, bridging the innate and adaptive responses (234).

It is well established that an adaptive response increases protection from re-infection by *A. fumigatus* (319). Yet despite not having protective vaccination, nu/nu mice lacking the adaptive arm of the immune system were not significantly more susceptible to aspergillosis via intratracheal, intravenous or intraperitoneal routes of infection (319). More recently, in a hydrocortisone immune suppressed model of pulmonary aspergillosis, RAG^{-/-} mice (lacking

competent adaptive immunity) were not more susceptible than WT C57BL/6 mice (319). Equally, SCID mice lacking B cell function also did not exhibit increased susceptibility (320).

T cell responses are classified in accordance with the main categories of cytokines produced, and are referred to as Th1, Th2 and Th17 responses (321,322). It is widely recognized that Th1 CD4⁺ T cells mediate protection against IPA by producing the macrophage-stimulatory cytokine IFN- γ (201,323). In contrast, Th2 responses are considered to be unfavorable to the host against *A. fumigatus* (324). Th2 mediated IL-4 secretion has also been reported to contribute to the allergic condition of ABPA (325). Furthermore, T-cells have been shown to tailor responses according to the pathogenic status of *A. fumigatus* inoculum. Mice infected with conidia had increased levels of IFN- γ (associated with Th1), compared to heat killed conidia which also expressed increased IL-4, a cytokine that plays a central role in Th2 differentiation of CD4⁺ T cells (326). However, the role of Th17 cells against *A. fumigatus* infection remains unclear. There is conflicting data in mice as to the utility of the Th17 cells in IPA, and further to this its utility in human cells is also debated, as human mononuclear cells are a weak inducer of IL-17 the Th17 signature cytokine. Subsequently, Th-17 response inhibition has been proposed as an *A. fumigatus* fungal virulence factor (327).

B cell-mediated antibody responses to *A. fumigatus* can occur due to a variety of exposures, environmental (routine pulmonary inhalation), in response to an aspergilloma (saprophytic colonization), hypersensitivity conditions (allergy) and invasive infections (IPA) (320). In patients without aspergillosis IgG and IgM serum antibodies against *A. fumigatus* components are broadly observed (328), and in hypersensitivity conditions over 80 proteins can bind IgE from sensitized patients demonstrating the potency of *A. fumigatus* in allergy (329). Hyphal filaments are potent B cell antigens (330–332), but there is conflicting evidence of B cell utility in IPA. A study in B-cell deficient neutropenic mice suggested antibodies were not protective (333,334), while immune serum administered to B cell-deficient mice and monoclonal antibodies for *A. fumigatus* cell wall component (β -1,3-glucan) both prolonged survival (335,336). Therefore further study on the function and usefulness of B cell responses is required under conditions of IPA.

1.5. Aspergillosis

Aspergillus species are opportunistic pathogens that are the causative agents of a range of infections from superficial to invasive and disseminated disease. The first recorded infection

attributed to *Aspergillus* was described in 1789 in Paris in a 23 year old soldier of the French revolution, as an infection of the maxillary sinus (337). However, due to conidia being small in size and airborne, for most patients the main portal of entry is the respiratory tract with other sites being comparatively uncommon (121,338). The ubiquitous nature of *Aspergillus spp.* conidia in the atmosphere coupled with their potential for pathogenicity in immunocompromised hosts has enabled it to become one of the most prevalent clinical fungal pathogens (120,339,340). *A. fumigatus* in particular therefore may be regarded as one of the most important airborne pathogenic fungi (130) .

In healthy individuals inhaled conidia are routinely cleared from the lung but in immune compromised individuals *A. fumigatus* conidia are allowed to persist and can cause a number of pulmonary diseases. Pulmonary diseases of *A. fumigatus* can be classified as per their location in the respiratory tract and extent of colonization and invasion, typically dependent on the immunological status of the host (121,341,342). Thus as an opportunistic human pathogen causing life-threatening invasive pulmonary aspergillosis (IPA) in vulnerable patient populations *A. fumigatus* is the focus of much study (340). Despite this it remains one of the most dreaded infections in susceptible patient groups due to its high mortality.

Susceptible patient groups include those with pre-existing lung conditions such as cystic fibrosis or chronic obstructive pulmonary disease (COPD) and those undergoing immunosuppressive treatment for prior illnesses such as transplant patients receiving corticosteroids and those receiving cyclophosphamide for the treatment of various cancers.

1.5.1 The spectrum of *Aspergillus spp.* disease

Aspergillus spp. are the causative agents of a spectrum of diseases in a variety of host environments from hyperactivity to immune dysfunction (see Fig 1.6). These conditions are regarded as allergic, saprophytic and invasive respectively and their severity and progression are dependent on the underlying host immunological status and are not mutually exclusive.

Pulmonary disorders such as asthma, emphysema, cystic fibrosis and tuberculosis infection can damage the lung, leaving cavities (bronchiectasis) and impaired clearance of secretions that potentiates colonization by fungi. Allergic diseases occur due to recurring exposure to *Aspergillus* antigens without colonization and removal of the environmental source usually

results in clinical improvement (121). However, contrary to this, the conditions of allergic bronchopulmonary aspergillosis (ABPA), growth of a fungal ball (known as an aspergilloma), and IPA require therapeutic intervention (121).

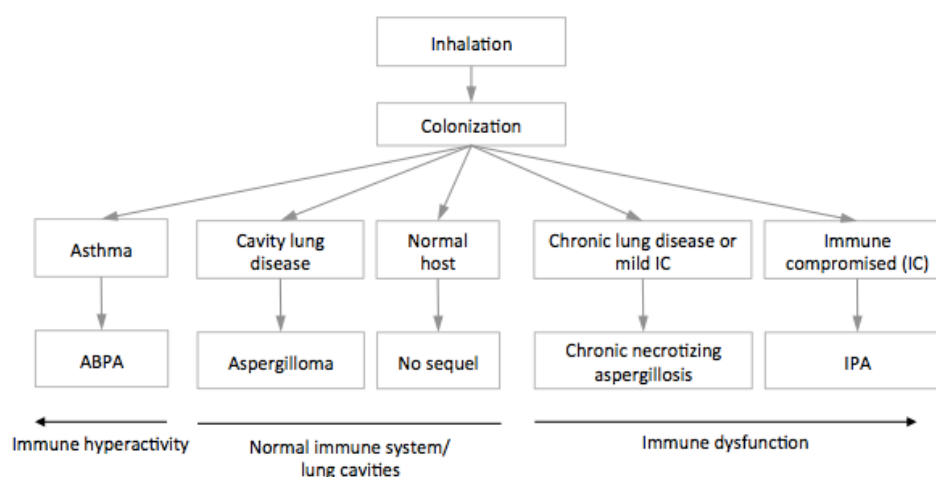


Figure 1.6: Spectrum of *Aspergillus* infection. Adapted from (156,343)

1.5.1.1 Allergic aspergillosis

ABPA is an allergic *Aspergillus* colonizing disease that affects more than 4 million people worldwide (344). It is the most severe allergic complication cause by *Aspergillus spp.* predominantly affecting those with the underlying conditions of asthma and CF, in which it affects 1-2.5% and 3-35% respectively (121,344–347). It is estimated that 2.5% of adults with asthma also have ABPA, which is approximately 4.8 million people worldwide (344). Patients with underlying conditions such as cystic fibrosis are also at risk of developing ABPA. *A. fumigatus* is isolated from 5.9-58.3% of CF patients, where its colonization can cause life-threatening declines in respiratory function (348–352). ABPA remains difficult to diagnose and in CF patient’s mucosal damage can occur without meeting diagnostic criteria. Without therapeutic treatment ABPA progresses to central bronchiectasis, pulmonary fibrosis and respiratory failure (121).

1.5.1.2 Saprophytic colonization

Aspergillomas or 'fungal balls' can form saprophytically in pulmonary cavities in the lungs of patients that are immunologically competent as well as those with active cavitating lung diseases such as tuberculosis and sarcoidosis (121,353). Despite being non-invasive, aspergillomas can cause haemoptysis, as a result of damage to the bronchial artery or proximal blood vessels, and can become extensive and even fatal (342,354–356). Additionally, aspergillomas can produce conidia that provoke hypersensitivity disorders such as ABPA, severe asthma and sinusitis (353). Lastly, chronic cavitating or necrotizing pulmonary aspergillosis (CNPA) is a semi-invasive condition seen commonly seen in patients with structural lung disease and HIV/AIDS. CNPA has been reported to damage the lung parenchyma without tissue invasion (357).

1.5.1.3 Invasive pulmonary aspergillosis (IPA)

IPA is the most common form of invasive aspergillosis (357) and the recent increase in the incidence of IPA is largely attributed to the rise in widespread use of immunosuppressive agents for solid organ and stem cell transplant recipients and cancer, in addition to immune suppressing infections such as the global HIV/AIDS pandemic (343,358,359).

1.5.2 Epidemiology

Aspergillus is one of the most common species of invasive mycoses (360). The Leading International Fungal Website currently reports that currently 'Over 30 million people are at risk of invasive aspergillosis each year' (www.life-worldwide.org/fungal-diseases/invasive-aspergillosis). Mortality of invasive fungal infections is so high there are as many or more annual deaths from the top 10 fungal infections as from TB or malaria (358).

Recent estimates of the epidemiology of IPA in France and Greece published in 2016 indicate that the incidence is between 1.8 and 10.4 cases respectively per 100,000 people (361,362). In the UK, an NHS commissioned annual report published in 2016 by The National Aspergillosis Service reported an increase of 27.3% in new aspergillosis patient referrals (457 referrals) in 2015/16 in England and Scotland compared to the previous 2014/15 year (359 referrals) (363,364).

However fungal infections are reported to be often undiagnosed or misdiagnosed (358), and as a consequence it is thought that 50% of invasive aspergillosis cases go undetected (344).

Therefore, with the combination of misdiagnosis and the lack of reporting the mortality is estimated to be much higher than is currently recorded. Confounding this, despite epidemiological data demonstrating that fungal infections are a growing problem, there is very little consistent global mycological surveillance from health agencies, with the exception of the USA CDC, but for which the only reportable fungal disease is Valley Fever (coccidioidomycosis) (358).

1.5.3 Risk factors

In an immunocompetent host, fungal conidia are readily cleared by AMs and recruited neutrophils (268). Therefore, invasive fungal infections are rare in immune competent individuals but do still occur (365). It is also worth noting that IPA can occur in the absence of classical risk factors, such as in critically ill patients without malignancies in intensive care units (ICUs), in whom it is now considered an emerging and devastating disease (343,366).

It is well established that down regulation of the immune response facilitates colonization and invasion by *A. fumigatus* in animals models and human patients (114,367). In particular, neutropenia and corticosteroid treatment are recognized as the predominant risk factors for development of IPA. Both patient groups present with impaired innate immunity, with the former notably affecting neutrophils and the latter macrophages. Consequently, the immunopathology of each condition differs greatly (114). Immune suppressive treatment and medical interventions notably include patients receiving transplant-maintaining drugs, chemotherapy for cancer (resulting in neutropenia) and those receiving high-dose and or long term corticosteroids for inflammatory and autoimmune conditions (123).

1.5.3.1 Neutropenia

Neutropenia is a reduction in numbers of circulating neutrophils and occurs in individuals undergoing cancer chemotherapy by drugs such as cyclophosphamide (76,77). Lack of neutrophils results in little inflammation at the site of infection despite TNF- α production, where AMs remain without the aid of infiltrating neutrophils, and are unable to clear conidia or contain infection, resulting in angioinvasion and dissemination (123). Invasive aspergillosis as a consequence of neutropenia has become a lesser problem since the effective application of anti-fungal prophylaxis.

1.5.3.2 Corticosteroid treatment

An important risk factor for IPA in patients in the early post-transplant period is neutropenia, for allogeneic hematopoietic stem-cell transplantation (HSCT) recipients in particular. However, IPA mostly occurs after neutrophil levels have recovered and in the setting of potent transplant maintaining immune suppressive therapy (368). Glucocorticoids such as prednisone and hydrocortisone have been routinely prescribed as immunosuppressive agents in transplant patients since 1963 to protect against graft versus host disease (GVHD) (369,370). The treatment of GVHD with corticosteroids and other potent immune suppressive agents increases the risk of IPA (371–375).

Corticosteroids represent a major risk factor for IPA and provide significant advantages to the pathogen in disease progression, and invasive fungal infections post transplantation are an increasing issue. Patients receiving transplant maintaining immunosuppressive treatment, develop persistent infections that are characterized by minimal fungal invasion but chronic inflammation causing pulmonary tissue destruction (78).

1.5.3.3 Epidemiology and mortality of IPA in neutropenic and corticosteroid treated patients

The mortality rate of *A. fumigatus* IPA exceeds 50% in neutropenic patients, and up to 90% in HSCT recipients receiving drugs such as corticosteroids (343,376). A review of 960 reported IPA cases by Steinbach et al. (2012) revealed 73.8% received corticosteroids (within 30 days of diagnosis) and 33.8% were neutropenic (377). With regards to post transplant prognosis, a large prospective study found that one-year survival rates for IPA patients who had undergone organ transplantation was 59%, and 25% for stem cell transplant recipients (378). Due to the severity of IPA in these groups, most studies of the clinical impact of IPA are conducted in haematological and transplant patients (379,380).

1.5.2.4 Effects of corticosteroids on cellular responses

Corticosteroids such as mineralocorticoids and glucocorticoids are naturally secreted by the zona fasciculata in the adrenal cortex (234,381). Natural glucocorticoids such as cortisol in humans and corticosterone in rodents, are classically released in response to stress (382) and inflammatory mediators such as cytokines or prostaglandins (381). Synthetic glucocorticoids like prednisone, dexamethasone and hydrocortisone, are powerful anti-inflammatory compounds that resemble natural glucocorticoids. They are widely used as

drugs to treat various chronic inflammatory conditions related to allergy, autoimmune diseases (asthma, IBS, eczema, MS and rheumatoid arthritis) and lymphoid malignancies (301,381–385).

Consistent with the pleiotropic effects of glucocorticoids, the glucocorticoid receptor (GR) are expressed in nearly every cell of the body including immune cells (386). Owing to their lipophilic nature, glucocorticoids can diffuse freely across cell membranes (383). Both natural and synthetic glucocorticoids, act by binding to the GR (367,381,387). Following ligand binding, GR undergoes a conformational change that results in its nuclear translocation. The GR dimer exerts its actions by binding to DNA target sequences called glucocorticoid-responsive elements (GREs) (387), and causing transcriptional repression or induction of its target genes, consisting of up to 10-20% of the human genome (381,388,389).

However, cellular response to glucocorticoids is remarkably diverse owing to different GREs and multiple GR isoforms (381), exhibiting profound variability in specificity and sensitivity (390,391). In addition to affecting the transcription of pro-inflammatory cytokines and prostaglandins (381,392), GRs can act further upstream by directly binding to transcription factors in the absence of GR elements such as AP-1 and NF- κ B (393). At pharmacological concentrations corticosteroids have significant deleterious effects on the function of innate immune cells, with a primary role in impairment of anti-conidial capabilities of macrophages (394).

In macrophages under glucocorticoid treatment, phagocytosis is unaffected but generation of ROS fails to inhibit conidial germination (395) and results in hyphal growth. Contributing to this effector function defect, it has also been postulated from electron microscopy that glucocorticoids stabilize lysosomal membranes to prevent phagolysosomal fusion (396,397). Furthermore, cellular migration and production of cytokines and chemokines is also impaired by glucocorticoids (123). Interestingly, the GR has been demonstrated to enhance macrophage phagocytosis of neutrophils, which acts to reduce inflammation (398). In contrast, glucocorticoids have been shown to cooperate with TNF- α to increase TLR-2 expression on epithelial cells which stimulates the secretion of proinflammatory IL-6 and chemoattractant IL-8 (399,400).

Neutrophils infiltrate the lung to the site of infection by interaction with adhesion molecules and follow chemoattractants released by innate and ECs. Glucocorticoids suppress neutrophil migration by repressing the expression of adhesion molecules (383). However, despite this effect, neutrophils are still recruited to the lung and in this instance persist to fashion an exacerbated inflammatory pulmonary environment, secreting cytokines, ROS and fungal proteases, that result in tissue damage (293,401). Furthermore, glucocorticoids are proposed to suppress neutrophil apoptosis via up-regulation of anti-apoptotic proteins such as Mcl-1 and XIAP (381,402).

In summary, the effects of glucocorticoids could be considered as both pro- and anti-inflammatory. *A. fumigatus* conidia require fast and effective actions of innate cells to prevent the development of hyphae which require neutrophil assistance to eliminate, but as they suppress many of the initial events in an inflammatory response they play an important role in allowing *A. fumigatus* to evade the most crucial arm of the immune system dedicated to its clearance. Therefore corticosteroids impair fungal killing, but may also further exacerbate disease by promoting non-sterilizing inflammation leading to tissue destruction.

1.5.2.5 The hydrocortisone model of aspergillosis

Identification of the contributing factors of fungal and host pathogenicity in IPA requires the use of *in vivo* models. For the cortisone model of murine aspergillosis mice are typically given intraperitoneal injections of hydrocortisone acetate 3 days prior to infection with *A. fumigatus* conidia followed by further injections at 2-3 day intervals over the course of infection to maintain immune suppression. Some small differences in immune responses are reported in the literature owing to variances in mouse strains, corticosteroid regimens and fungal inocula (403), but a lack of fungal clearance and immune impairment are universally observed.

After inoculation, resident macrophages in the lung are unable to clear conidia due to the action of hydrocortisone acetate which inhibits the killing capacity of AMs and results in the germination of conidia outside or inside macrophages even within one day of infection (404,405).

The corticosteroid model of IPA is characterised by an early and extensive PMN recruitment to the lung that is maintained until the death of the animals (403,406), and has been

demonstrated to be as high as 3 times that of immunocompetent mice. Despite high levels of pulmonary PMN infiltration, granular content release activity has been demonstrated to be relatively low early in infection (0-48 h), with a rapid and significant increase late in infection (72-96 h) by a factor of 4 compared to immune competent mice (403).

Fungal burden is described as non-invasive despite rapid germination and consequent hyphal development being permissible under hydrocortisone conditions (368,403,406). It has been reported that one day after *A. fumigatus* inoculation germinating conidia and short hyphae were observed in alveolar spaces and after three days mature septated hyphae in bronchiolar and alveolar spaces were seen (368).

Correspondingly, multifocal inflammatory lesions were observed around alveoli at day one, and after three days multifocal lesions on bronchi and bronchioles that extended to alveoli and small vasculature were present. Lesions were infiltrated mostly with neutrophils that were surrounded by activated macrophages (368). In addition, haemoglobin levels as a measure of tissue injury, and respiratory distress, have been shown to steadily increase over the course of *in vivo* IPA until the death of the animals (403).

Confounding fungal persistence in the lung, the production of IL-8 (murine KC) has been shown to be unaffected by hydrocortisone (407,408), despite its ability to inhibit the activity of pro-inflammatory transcription factors, such as NF- κ B (384). Therefore, neutrophils continue to be recruited to the lung due to lack of fungal clearance, maintain a non-sterilizing inflammatory environment that results in tissue damage. Additionally, NF- κ B inhibition during inflammation resolution lengthens the inflammatory response and inhibits apoptosis (409), and could also account for the persistence of PMNs in the lung (403).

In summary, due to the effects of hydrocortisone on macrophages, fungal clearance is inhibited, resulting in a persistence of *A. fumigatus* in the lung. The persistence a fungal PRR stimulus invariably leads to chronic inflammation by extensive infiltrating PMNs and although a fungal-mediated inflammatory response can prevent invasion and dissemination, the hydrocortisone model is associated with destructive and excessive inflammation (385). While this persistent inflammation may kill some fungal cells (368), the growth of *A. fumigatus* results in irreversible tissue injury leading to respiratory distress and ensuing death (403). However, this complex multifactorial immune suppression by hydrocortisone

and its alterations in the immune response to *A. fumigatus* continues to be characterised in the literature.

1.6. Antifungal compounds

The cell wall of *A. fumigatus* serves as a major defensive barrier against the hostile environment of a host (410). It prevents the cell from lysing and is thicker in resting conidia than hyphae. In addition to being protective it is composed of many fungal specific sugars, proteins and lipids that are absent in mammalian systems (132,133,150,151,208,410,411). Due to this and its essentiality, the cell wall has been the direct or indirect target of many antifungal drugs and the source of biomarkers used in diagnosis.

Due to the aggressive nature of IPA under immune suppressive regimes like hydrocortisone a timely diagnosis is key and the difficulties in diagnosis are due to varied and unspecific clinical signs. Current diagnostic tools developed over the past few decades targeting β -glucan, fungal DNA and galactomannan, show improved performance over the more conventional culture and microscopic examination. Therefore, early, rapid and specific diagnosis is required for prompt treatment to potentiate a positive outcome (412).

1.6.1 Antifungal compounds in current use

Currently, there are three major classes of antifungal compounds available to treat systemic invasive fungal infections, they are polyenes, azoles, and echinocandins (413). Polyenes and azoles were introduced into the clinics by 1980 (414), and since the licensing of echinocandins in 2001 there have been no new classes of approved antifungals.

1.6.1.1 Polyenes

Polyenes are the oldest class of antifungals, of which Amphotericin B (AMB) was the sole drug available for severe fungal infections for nearly 30 years (414,415). Ergosterol is a fungal membrane sterol that acts as a bioregulator of membrane stability and integrity in the fungal cell wall (416). The mode of action of AMB is membrane pore formation created by a ring of eight AMB molecules (417). Membrane pores depolarize the cell membrane causing leakage of cytoplasmic components that results in cell death (418,419). The action of AMB is fungicidal and it has the most of the broad-spectrum antifungal effects of current compounds. However, AMB is known to cause significant nephrotoxicity (415), due to polyenes binding to the mammalian membrane sterol cholesterol (418). Although efforts

have been made to reduce this using inhaled nebulized liposomal-AMB remains expensive and not widely available (414,418).

1.6.1.2 Azoles

Azoles were first discovered by Wooley in 1944 but the most widely used azoles in current use, fluconazole, itraconazole, voriconazole and posaconazole were introduced in 1990, 1992, 2002 and 2006 respectively (418). Azoles are the most widely used class of antifungals (420), and the most recent formulations of fluconazole, itraconazole, and voriconazole share a mode of action (415). This mode of action is to inhibit ergosterol synthesis (415) by inhibition of cytochrome P-450-dependent C14 α -sterol demethylase (421), a fungal enzyme involved in converting lanosterol to ergosterol (418). However, their ability to inhibit cytochrome P450 has predisposed them to interfere with the metabolism of a number of other drugs, but despite this complication they are well tolerated (414). In general, azoles (itraconazole, voriconazole, and posaconazole) are fungistatic (420), and while fluconazole has no clinically relevant activity against molds, voriconazole is fungicidal toward *A. fumigatus* (422). Currently voriconazole is the treatment of choice for *A. fumigatus* due to its superiority to AMB in a head-to-head clinical trial (414,423,424).

The newest azole isavuconazole, was licensed in its intravenous and oral formulations, and approved for the treatment of IPA in 2015 (425). Its MOA is similar to other azoles in targeting cytochrome P450, but its side arm allows for greater fungal binding. It is effective against yeasts, molds, and dimorphic fungi, with potent *in vitro* activity against *A. fumigatus*. In patients it is also demonstrated to be well tolerated, and its efficacy was reported to be non-inferior to that of voriconazole, and similar to that of AMB and posaconazole (426).

1.6.1.3 Echinocandins

Caspofungin was approved by the FDA in 2001, followed by micafungin and anidulafungin in 2005 and 2006 respectively (427). The fungal cell wall is mostly comprised of polymers and glucans form the most abundant polymers, making them the key components. The mode of action of echinocandins is to inhibit β 1,3-glucan synthesis by inhibition of the glucan polymerizing β 1,3-glucan synthase. This inhibition leads to increased cell wall instability and resultant cell lysis (414,418). The action of echinocandins is broadly fungistatic towards *Aspergillus spp.*, although caspofungin has been demonstrated to kill growing *A. fumigatus* (414,428).

1.6.2 Emerging azole resistance

The discovery and licencing of azoles dramatically improved the treatment of IPA and quickly led to their widespread use (418). However, a consequence has been the development of azole resistance (418). Azole resistance in *A. fumigatus* has now emerged as a global health problem (429), with reports in Europe, the Middle East, Asia, Africa, Australia and the Americas (430,431). Azole resistance was first reported in 1998 in the Netherlands with increasing incidence until the present day (429). In addition, due to prevalent world travel and trade, and *A. fumigatus* being an aerosolized pathogen, migration between countries can be rapid.

Azole-resistant aspergillosis is on the rise (up to 20% in CF or CPA patients), resistance mechanisms continue to emerge and in so doing, threaten the widespread use of these drugs in the management of IPA. Resistance to azoles, as with any drug, is either innate or acquired. It is therefore important to consider that azole prophylaxis and or monotherapy can provide selective pressure to create azole-resistant strains (432). Considering that azole resistance can develop in any *Aspergillus* infection, it is interesting that acquired resistance has only been reported in patients with a cavity or aspergilloma under long term use of antifungal therapy (433–435), and surprisingly, the majority of azole-resistance strains originate from the environment (429). Azole fungicides are used globally in plant and crop protection, and some have been found to have activity against *A. fumigatus* (436,437). It has been hypothesised that azole resistance in the environment developed due to the similarity of azole fungicides with medical triazoles (437), and although fungi are eukaryotes and do not exchange genes with the ease of bacteria in lateral transfer, *A. fumigatus* has a parasexual and sexual cycle. Fungicide-mediated cell stress coupled with the potential for *A. fumigatus* sexual reproduction in the environment, most likely increased the likelihood for genetic recombination conferring resistance (429). Resistant strains have been shown to have point mutations in the Cyp51A gene (or promoter region), the target of azoles, but in many isolates the Cyp51A gene is intact, suggesting alternative resistance mechanisms (429).

Due to the limited treatment options for IPA, patients with azole resistant *A. fumigatus* infections have poor treatment outcomes, with mortality between 50% and 100% (432,438–441). *In vitro* testing has demonstrated some of the originally reported isolates to have between 82.7-97.88% resistance across itraconazole, voriconazole and posaconazole (429),

illustrating the utility of the currently prescribed azoles to be very limited against resistant strains.

A global effort and cooperation will be required to identify and understand resistance mechanisms and implement measures that may impede the development of further resistance. However, evidence to date suggest that pan-azole resistance is on the rise, and the retention of azoles as treatment for IPA is now under threat (414). Therefore, the development of new classes of anti-fungal drugs is now urgently needed.

1.7 Hypothesis

S1P signalling inhibition has therapeutic potential in the treatment of aspergillosis.

1.8 Project Aims

1. To determine the effect of FTY720 and SKI-II, on *A. fumigatus* mediated TNF- α production in macrophages
2. To investigate the utility of FTY720 in the treatment of aspergillosis
3. To characterize the effects of sphingolipid pathway inhibition and gene deletion in *A. fumigatus*

2. Materials and Methods

2.1 *Aspergillus fumigatus* strains

Aspergillus fumigatus strains used in this study were CEA10 (FGSC A1163), a clinical isolate sequenced by Fedorova *et al.*, (2008), Af293 a clinical isolate sequenced by Nierman *et al.*, (2005), ATCC46645, a wildtype strain (443), and pyrG $\Delta ku80$ a derivative of CEA10 that has a non-homologous end joining deficiency (444).

2.2 *Aspergillus fumigatus* culture

2.2.1 Culture, harvest and storage of *A. fumigatus*

Strains were cultured on Sabouraud (SAB) (Oxoid Ltd, UK) agar or *Aspergillus* minimal medium (phenotypic testing) 90 mm plates or T25cm² flasks at 37°C for 3-5 days. Conidia were harvested in sterile 0.1% v/v Tween20 by agitation with a sterile spreader or shaking, plates and flasks respectively. Conidial suspensions were filtered to isolate conidia from hyphal fragments and conidiophores using Miracloth (Calbiochem) and centrifuged for 5 mins at 4000 rpm. Conidia were washed twice and re-suspended in dH₂O, prior to enumeration using a Nikon Eclipse TS100 microscope and a haemocytometer. Conidial counts were adjusted to experimental requirements with dH₂O or assay medium.

Aspergillus minimal media (AMM), was comprised of 1% glucose, 5 mM ammonium tartrate, 10 mg/ml biotin and 20 ml/l *Aspergillus* salt solution, supplemented with 1.5% No.1 agar (Oxoid Ltd, UK). *Aspergillus* salt solution is comprised of 26 g/l KCl, 26 g/l MgSO₄.7H₂O, 76 g/l KH₂PO₄ and 50 ml Trace element solution (40 mg/L Na₂B₄O₇.10H₂O, 400 mg/L CuSO₄.5H₂O, 800 mg/L FePO₄. 2H₂O, 800 mg/L MnSO₄.2H₂O, 800 mg/L Na₂MoO₄.2H₂O and 8 g/L ZnSO₄. 7H₂O). Strains were either stored on agar at 4°C for up to 4 weeks or for long-term storage in 20% (w/v) glycerol at -20°C.

2.2.2 Preparation of swollen conidia

For swollen conidia (SC), conidia were suspended in 50 ml of RPMI (Gibco) at a concentration of 1.5×10^6 conidia/ml, and incubated for 4 hours at 37°C in a 145 mm petri dish (Greiner bio-one). SC were harvested using a cell scraper (Corning Incorporated) and centrifuged at 4000 rpm for 10 mins. SC were resuspended in dH₂O or assay medium prior to enumeration using a Nikon Eclipse TS100 microscope and a haemocytometer. Conidial

counts were adjusted to experimental requirements with dH₂O or assay medium.

2.3 Bacterial strains and culture

Escherichia coli used for propagating plasmids pYES2 and pUC-hph are commercially available. Strains were grown on solid Luria Bertani (LB) medium at 30°C, and a single colony was used to grow a 10 ml overnight culture.

Competent *E. coli* strain DH5- α (NEB) were used in transformation and cultured on LB agar. Solid LB was prepared with addition of 2% (w/v) agar prior to autoclaving, then cooled to 50°C and supplemented with 100 μ g/ml ampicillin for transformant selection.

2.4 Plasmids

Full details of plasmids used and generated in this study are given in the respective chapters.

2.5 General molecular techniques

2.5.1 Polymerase chain reaction (PCR)

PCR reactions were performed as per manufacturer's instructions and using one of three enzymes: Q5TM High Fidelity DNA polymerase (New England BioLabs), Phusion[®] High-Fidelity DNA Polymerase (New England BioLabs), or Phusion Flash (Thermo Scientific). dNTPs used in all reactions were from Vivantis.

2.5.2 Separation of DNA fragments by electrophoresis

DNA was visualised by electrophoresis separation on 1% agarose gel (w/v). To facilitate DNA loading and display running front DNA was mixed with a purple 6x Gel Loading Dye (New England BioLabs). The gel was prepared by adding 1% agarose (Flowgen Bioscience) to 1x TBE or Tris-borate-EDTA buffer (Cold Spring Harbour Protocols; Tris base 0.1 M, Boric Acid 0.1 M, EDTA disodium salt 2 mM) and microwaved to dissolve. 50 ml of liquid gel was supplemented with 5 μ l of SYBR Safe DNA gel stain (Invitrogen), poured into a tank and allowed to set. Gels were run with 1 kb and 2-Log DNA ladders (New England BioLabs) to define the size of DNA fragments, at 70-90 V for 45 mins to 1 h using 1x TBE buffer. DNA was visualised on a UV transilluminator for imaging (ChemiDocTM MP Imaging Systems, Bio-Rad).

For purification of specific DNA fragments after electrophoretic separation, DNA was visualised using a blue light illuminator, Safe Imager (Invitrogen), and excised using a clean scalpel. DNA was isolated using a GenElute™ Gel Extraction Kit (Sigma) according to manufacturer's instruction.

2.5.3 Ligation (Gibson Assembly)

Vector fragment ligation were performed using Gibson Assembly® Master Mix (New England Biolabs), to join multiple overlapping DNA fragments in a single-tube isothermal reaction. This is achieved by an exonuclease which creates single-stranded 3' overhangs that facilitate the annealing of fragments that share complementarity, a DNA polymerase fills in the gaps within each annealed fragment and a DNA ligase seals nicks along the assembled DNA. Briefly, fragment DNA concentration was determined by nanodrop and between 0.02-0.1 pmols in 10 µl was combined with 10 µl of Gibson Assembly® Master Mix and incubated at 50°C for 60 minutes. Ligations reactions were stored on ice until subsequent transformation on the same day.

2.5.4 Bacterial transformation

Knockout plasmids were directly transformed into chemically competent bacteria (NEB® 5-alpha Competent DH5-α *E. coli* (High Efficiency), New England BioLabs) according to manufacturers instructions. Briefly, 100 ng of construct plasmid DNA was added to 50 µl of competent cells on ice. After 30 minutes cells were heat shocked at 42°C in a water bath for 30 s with subsequent incubation on ice for 5 mins. Cells were recovered with addition of pre-warmed (37°C) 950 µl SOC medium (New England BioLabs) and incubated at 37°C for 1 h with vigorous shaking at 250 rpm. Cells were plated in a serial dilution on pre-warmed 90 mm LB agar plates containing 100 µg/ml ampicillin, and incubated at 37°C overnight. The next day, individual colonies were transferred to 10 ml of LB broth supplemented 100 µg/ml ampicillin and grown overnight at 37°C, shaking at 180 rpm.

2.5.5 Plasmid DNA extraction

Isolation of recombinant plasmids from 10 ml overnight cultures of *E. coli* was performed using PureYield™ Plasmid Miniprep System (Promega) specifically designed to extract plasmid DNA, according to manufacturers instructions. Plasmid DNA was eluted in elution buffer.

2.5.6 Nucleic acid quantification

Plasmid DNA purity and concentration was quantified by Nano-drop using a ND-1000 Spectrophotometer (Nano-Drop®).

2.5.7 Restriction enzyme digests

DNA was isolated and subjected to restriction enzyme analysis with either Hind III, XhoI or NcoI according to the manufacturers specifications (New England BioLabs), yielding DNA fragments that were analysed for size on a 1% agarose gel.

2.5.8 DNA Sequencing

All plasmids were sequenced by Sanger's method at The Genomics Laboratory at Imperial College (MRC Clinical Sciences Centre) using a Sanger ABI 3730xl, performing automated fluorescent DNA sequencing. Each reaction mix was comprised of 500 ng of plasmid DNA, and 2 µl of the primers (20 µM) for a final volume of 10 µl.

2.6 *Aspergillus fumigatus* techniques

2.6.1 *A. fumigatus* genomic DNA extraction from conidia

DNA was extracted from *A. fumigatus* conidia at Manchester University using Dr Elaine Bignell's Lab protocol, as per the following: *A. fumigatus* strains were cultured on AMM agar at 37°C for 3-5 days. To break the conidia, a small section of conidia were lifted from the plate using a sterile toothpick and mixed with 200 µl of breaking buffer (2% Triton X-100, 1% SDS, 20 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). 300 mg of sterile acid washed glass beads, 0.4-0.6 mm in diameter (Sartorius UK) were added and samples were incubated for 30 mins at 70°C (in a heat block), vortexing every 10 minutes for 30 s. 200 µl of phenol:chloroform:isoamyl alcohol 25:24:1 was added and samples vortexed at a moderate speed for 5 minutes, and subsequently centrifuged for 8 min at 13,000 rpm at RT to remove conidial debris. The aqueous phase was transferred to a fresh tube and 1 ml of isopropanol was added and the tubes incubated for 30 mins at -20°C. DNA was pelleted by centrifugation at 13,000 rpm for 10 mins, and the pellet was washed twice with 70% ethanol, air dried, then dissolved in 20 µl of DNase free water. 1 µl of DNA was added to 2 µl of 6x loading buffer and analysed by 1% agarose gel electrophoresis.

2.6.2 Construction of *A. fumigatus* mutant strains

Transformation of *A. fumigatus* was performed at Manchester University according to Dr Elaine Bignell's Lab protocol, as per the following: After PCR of the knockout cassettes from the correctly assembled vectors, the PCR mixture was used directly in *A. fumigatus* transfection reactions. Transformation of *A. fumigatus* was performed using the standard method. *A. fumigatus* strains were streaked and grown on ACM at 37°C for 3 days in T25 cm² cap filtered flasks. Conidia were harvested by addition of 15 ml dH₂O and shaking. Conidial suspensions were filtered through sterile miracloth to isolate conidia and centrifuged at 3500 rpm for 10 mins. Pellets were washed in 15 ml dH₂O and spun down at 3500 rpm for 10 mins, and were transferred to a 1.5 ml tube in 1 ml dH₂O, and kept at 4°C. Conidia were enumerated at a 1:200 dilution in dH₂O using a haemocytometer. 40 ml of ACM liquid medium was inoculated with 2x10⁸ total conidia and transferred to a 250 ml conical flask. Conidial suspensions were incubated at 30°C for overnight (16 h) shaking at 150 rpm. The next day hyphae were filtered through sterile Miracloth into a 50 ml tube. All fungal matter was transferred in 10 ml liquid ACM to a fresh 250 ml conical flask containing 10 ml of 2x protoplasting solution (1.28 g VinoFlow, Novo Nordisk A/S). Protoplasting suspension was incubated at 37°C for 4 h shaking at 180 rpm. Protoplasts were separated from hyphal fragments using a 40 µM cell strainer into a 50 ml tube, the suspension was made up to 50 ml with 1 M KCl and centrifuged at 1800 rpm for 10 mins. Supernatant was gently decanted, and the protoplast pellet was washed with 50 ml KCl, centrifuged at 1800 rpm for 10 mins and then transferred to a 1.5 ml tube in 1 ml KCl. Protoplasts were washed twice with 1 ml KCL, intermittently centrifuged at 5300 rpm for 3 mins, resuspended gently to avoid damaging cells, and finally resuspended in 1 ml KCl.

For the transformation reaction 10 µl of recombinant vector DNA was added to 100 µl of protoplasts and vortexed 8 times successively for 1 s. 50 µl of 0% PEG was added and reaction was vortexed 4 times successively for 1 s followed by incubation on ice for 20 mins. 1 ml of PEG was added and gently mixed with a pipette prior to incubation at RT for 30 mins. Whole 1 ml transformation reactions were added to 50 ml pre-warmed (50-60°C) 0.4% agarose overlay agar medium supplemented with 180 µg/ml hygromycin B (Roche), and mixed by inversion. 10 ml of overlay containing protoplasts was poured onto ACM agar plates, which were left over night at RT (agar side down). The next day plates were parafilmed to prevent cross contamination, then inverted and transferred to a 37°C incubator for 4 days. Plates were observed for evidence of clearly defined hygromycin B

resistant colonies. Purification of strains was performed by sub-culturing 3 times, via conidial transfer from a mature AMM plate to a fresh one, using an inoculation loop.

2.6.3 Antifungal susceptibility testing

2.6.3.1 EUCAST broth microdilution method

Susceptibilities were determined by European Committee on Antimicrobial Susceptibility Testing (EUCAST) method (445). Culture medium was prepared as a double-strength solution and sterilised by filtration with final concentrations in RPMI-1640 (Sigma-Aldrich) with L-glutamine and a pH indicator (without bicarbonate) of 0.165 M 3-(N-morpholino) propanesulfonic acid (MOPS), and 2% w/v glucose. Flat-bottom 96-well microdilution plates (Corning Costar) containing 100 µl of serial dilutions of antifungal drugs in double-strength EUCAST defined medium were inoculated with 100 µl of inoculum containing 1.25×10^5 CFU/ml in sterile water. Drug-free medium wells were included for growth and sterility controls. Inoculum was harvested as previously described. If conidia were self-adherent, suspensions were resuspended in 0.1% v/v Tween20 and vortexed for 15 seconds followed by centrifugation and resuspension in water. These steps were repeated until single cell suspensions were achieved. Inoculum was enumerated with a haemocytometer and adjusted to 2.5×10^6 CFU/ml in sterile water. Suspensions were then diluted 1:10 with sterile water to obtain a final working inoculum of 2.5×10^5 CFU/ml, which resulted in a final concentration of 1.25×10^5 CFU/ml or 2.5×10^4 CFU/well. Plates were incubated at 37°C and MIC readings were taken after 24 and 48 h. A no-growth end point was determined by eye and corresponded to the lowest drug concentration that showed a clear well without growth against a black background seen using a viewing mirror.

2.6.3.2 Multi anti-mycotic susceptibility testing

Multiple antimycotic susceptibility assays were performed using commercially available MICRONAUT-AM MHK 2 test plates (Merlin, Germany), with 9 antimycotics (Anidulafungin, Amphotericin B, Caspofungin, Fluconazole, 5-Fluorocytosine, Itraconazole, Micafungin, Posaconazole and Voriconazole) in up to 11 concentrations. Commercially available MICRONAUT culture 1x medium was RPMI-1640 (with L-glutamine and a pH indicator but without bicarbonate or phenol red) supplemented with glucose to a final concentration of 2% w/v glucose, and 3-(N-morpholino) propanesulfonic acid (MOPS) at a final concentration of 0.165 M (pH 7.0) as a buffer). Flat-bottomed 96-well microdilution plates containing lyophilised anti-mycotics were filled with in 50 µl of medium with or without FT720 or SKI-

II, and inoculated with 50 µl of inoculum containing 1.25×10^5 CFU/ml in culture medium. Drug-free medium wells were included in the plate for growth and sterility controls. Inoculum was harvested as previously described. If conidia were self-adherent, suspensions were resuspended in 0.1% v/v Tween20 and vortexed for 15 seconds, followed by centrifugation and resuspension in water. These steps were repeated until single cell suspensions were achieved. Plates were incubated at 37°C and MIC readings were taken after 24 and 48 h. A no-growth end point was determined by eye and corresponded to the lowest drug concentration that showed a clear well without growth against a black background seen using a viewing mirror.

2.7 Tissue Culture

2.7.1 Cell line

J774A.1 cells obtained from HPACC (UK) were cultured in T75cm² flasks at 37°C with 5% CO₂ and humidity in 0.22µM filtered media consisting of RPMI supplemented with 10% heat inactivated foetal bovine serum (FBS) (Gibco) and 50 mM β-mercaptoethanol (Gibco). Media is referred to as complete RPMI or cRPMI. Cells were cultured to 70% confluency and harvested by scraping. Cells were seeded in cRPMI with 200 U/ml recombinant murine IFN-γ (R&D Systems) and incubated overnight for experimentation the next day. The following day medium was replaced with fresh cRPMI with or without FTY720 (Selleck) or SKI-II (Calbiochem) at the concentration stipulated for 1 hour prior to addition of stimuli.

2.7.2 Isolation and differentiation of human peripheral blood monocytes to macrophages

Monocytes were isolated from peripheral blood using Ficoll-Paque gradient and human Pan Monocyte Isolation Kit (MACS, Miltenyi Biotec) as per manufacturers instructions. Briefly, 12 ml of room temperature RPMI was added to 20 ml of blood, gently layered on 13 ml of Ficoll-Paque, and centrifuged at 1800 rpm for 40 minutes. PBMCs were transferred to a fresh tube using a sterile Pasteur pipette and washed with 45 ml of RPMI. Cells were centrifuged at 1800 rpm for 10 minutes and re-suspended in 10 ml of Red blood cell lysis buffer (Sigma) for 5 mins. Followed by a wash with 40 ml PBS and re-suspension in RPMI. Cells were enumerated, centrifuged and re-suspended in MACS buffer before being isolated by negative selection column.

2.7.3 Phagocytosis

J774.1 murine macrophages were seeded the day before at 2×10^5 cells per well in 24-well plate onto 13mm sterile glass slips with activated with 200 U/ml recombinant murine IFN- γ (R&D). *Aspergillus fumigatus* conidia were harvested as per Chapter 2 and enumerated using a haemocytometer. Conidia were biotinylated and labeled with concanavalin A conjugated FITC, then kept over night in water at 4°C. The following day, macrophages were co-cultured with resting conidia at an MOI of 3:1 and spun down at 1000 rpm for 1 min to bring conidia into contact with the bottom of the wells, considered time 0 h. The co-culture was incubated at 37°C with 5% CO₂ and humidity. After 15, 30 and 60 minute intervals macrophages were washed with PBS twice to remove superfluous conidia and all cells were fixed with 4% PFA. PFA was removed and remaining molecules quenched with 50 mM ammonium chloride before being washed twice with PBS. Cells were then incubated with streptavidin conjugated Cy3 followed by a wash with PBS. Cells were mounted on glass slides using solid medium and sealed with nail varnish. The FITC signal was used to determine conidia inside macrophages (phagocytosed) and Cy3 conidia that resided outside macrophages (not phagocytosed). Using widefield microscopy z-stack images of cells were captured and used to accurately quantify phagocytosed conidia by analyzing each slice. Below is an example montage displaying the number of slices used in quantification and the height difference on the sequential z-stack images (Fig 2.1).

2.8 Stimuli preparations

Stimulants such as ultrapure LPS (Invitrogen) or swollen *A. fumigatus* conidia suspended in cRPMI were added in 10-20 μ l and were present throughout the stated stimulatory period. Conidia were commonly used at an MOI of 1:1 or 3:1 and ultrapure LPS 8 or 25 ng/ml (for murine and human cells respectively/6-16 h). For ELISA sample preparations, cells were seeded in a 96-well microtiter plate in triplicate and after experimental incubation period supernatants were transfer to fresh plates for storage at -80°C.

For mRNA extraction, cells were seeded in triplicate in 24- well microtiter plates, and after experimental incubation period supernatants were removed and cells were washed once with warm PBS then lysed with TriReagent (Sigma) and stored at -80°C until processed.

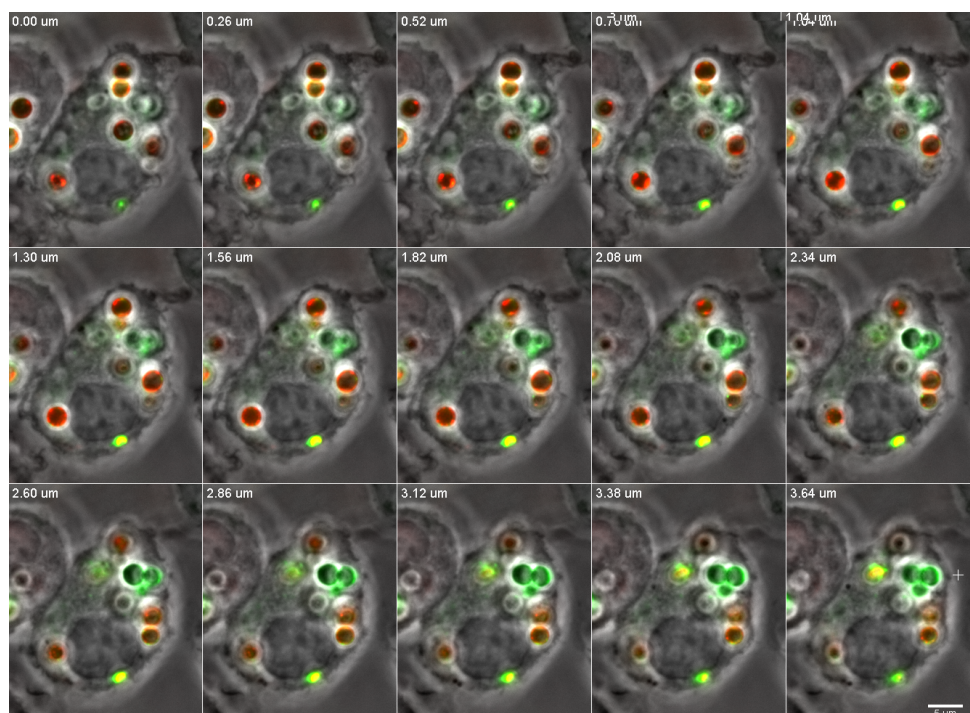


Figure 2.1: Z-stack of murine macrophages identifying phagocytosed and external *A. fumigatus* conidia. Phagocytosed conidia were quantified using z-stack images of J774A.1 cells incubated with biotinylated *A. fumigatus* conidia at an MOI of 3:1 at intervals of 15, 30 and 60 mins of co-culture. Green are phagocytosed conidia labelled with concavalin A conjugated FITC antibody and red conidia are extracellular labelled with streptavidin conjugated Cy3 antibody. Z stack images were captured by widefield microscopy and sharpened using Huygens Analysis deconvolution software.

2.9 Drug cytotoxicity

FTY720 and SKI-II cytotoxicity against J774A.1 cells and human MDMs in culture was investigated using MTT colorimetric assay and Flow cytometry.

2.9.1. MTT

Cell viability was measured using MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) dye reduction assay (Sigma). Cells were seeded in cRPMI in triplicate in 96-well microtiter plates and activated overnight with 200 U/ml recombinant murine IFN- γ (R&D Systems). The following day cells were incubated with FTY720 or SKI-II for 6 h or 16 h, after which 50 μ l of 5 mg/ml MTT sterile solution in phosphate-buffered saline was added to each well. Plates were further incubated at 37°C for 1 h, after which plates were centrifuged at 1000 rpm for 5 mins and assay medium was carefully removed. 100 μ l of dimethyl

sulfoxide was added to each well to solubilize MTT formazan crystals and absorbance of each well was measured at 540 nm spectrophotometrically by a microtiter plate reader.

2.9.2 Analysis of cellular death by flowcytometry

Cells were seeded in cRPMI in duplicate in 24-well microtiter plates and activated overnight with 200 U/ml recombinant murine IFN- γ (R&D Systems). The following day cells were incubated with FTY720 or SKI-II for 6 h or 16 h, after which supernatants were removed and cells were wash once with warm PBS. Cell detachment was performed by incubating cells on ice for 5 mins in the presence of sterile citric saline, and subsequent gentle repetitive pipetting.

2.10 Animals

All murine experiments were approved by the United Kingdom Home Office and conducted under and in accordance with the project licence PPL 70/7324. Groups of five C57BL/6 male mice were (18-22 g; Harlan Ortech, UK) were housed in individually vented cages with free access to autoclaved food and water in a dedicated facility at Imperial College London. Drinking water sterile dH₂O supplemented with 1 g/L tetracycline hydrochloride and 64 mg/L ciprofloxacin (both from Sigma) as prophylaxis against bacterial infection.

2.10.1 Murine Immunosuppression

Mice were immunosuppressed with 125 mg/kg hydrocortisone (Hydrocortistab, Sovereign Medical) or 250 mg/kg hydrocortisone acetate (Sigma) suspended in saline (autoclaved and 0.22 μ M filtered 0.9% NaCl) by sonication and vortexing. A change was made from premade suspension to lab made suspension due to the unavailability of the former. After initial dose titration, C57BL/6 mice received 250 mg/kg hydrocortisone acetate (HC), subcutaneously on day -3 and -1 before infection, and thereafter every 3 days. FTY720 was administered daily from day -2 via intraperitoneal injection at 3 mg/kg.

2.10.2 Murine infection with *A. fumigatus*

A. fumigatus was grown on SAB agar for 5 days prior to infection. Conidia were freshly harvested with sterile saline and filtered through Miracloth (Calbiochem) to remove hyphal fractions. Conidial suspension was centrifuged for 10 mins at 4500 rpm, washed twice and re-suspended with saline. Conidia were enumerated using a haemocytometer and re-

suspended in saline to desired concentration (2.5×10^7 conidia/ml for an inoculum of 1×10^6 per mouse). Inoculation suspensions were made and kept in glass vials. Mice were anaesthetised by isoflurane inhalation and typically received 1×10^7 and 1×10^6 resting conidia via the intranasal route in 40 μ l, for competent and immunocompromised animals respectively. To verify viable CFUs administered in inoculum, a serial dilution was performed on SAB agar with saline and incubated at 37°C for 1-2 days. Mice were weighed every day, from day -3 and inspected 2-3 times a day. For survival studies the end of the experiment corresponded to 20% loss in body weight from day 0 (day of inoculation), at such time mice were culled.

2.10.3 Histopathology

Whole lungs were removed immediately after mice were culled and fixed in 10% (w/v) formaldehyde for 24 h before transfer to 70% ethanol until processing. Histological processing was performed by Lorraine Lorence at the National Heart and Lung Institute (NHLI) at Imperial College London. Lungs were embedded in paraffin prior to sectioning and stained with Periodic acid-Schiff (PAS), Hematoxylin and eosin (H & E) and light green and Grocott's methenamine silver (GMS) staining. Histological analysis was performed using ImageJ, reporting inflammation as a percentage of dense tissue of whole lung area using a threshold tool.

2.10.4 Bronchoalveolar lavage (BAL)

BAL was collected as per (219). Briefly, after mice were culled by cervical dislocation, the trachea cut downwards and intubated with 20G x 1 $\frac{1}{4}$ " cannula (Vasofix®). Lungs were lavaged with 1 ml of sterile saline and collected lavage fluid was stored on ice until processing. BAL was centrifuged at 1200 rpm for 10 mins at 4°C to isolate cells from liquid fraction. Supernatants were stored at -80°C until processing and cells were re-suspended for processed by FACS for identification of cell type.

2.10.5 Analysis of cellular infiltrates into alveolar space by fluorescence activated cell sorting (FACS)

BAL fluid was centrifuged at 1200 rpm for 10 mins at 4°C, and cells were re-suspended in PBS containing an Fc receptor blocking antibody (anti-CD16/CD32, clone 93, eBioscience, diluted 1:100), and incubated on ice for 20 mins. Cells were then further incubated for 30 mins in the dark after the addition of 2.5 μ l anti-F4/80- APC/Cy7 (clone BM8, Biolegend), 2 μ l

anti- CD45-PE/Cy7 (clone 30-F11, eBioscience) and 2.5 µl anti-Ly-6G-BV421 (clone 1A8, Biolegend). Cell suspension then underwent red blood cell lysis in 1 ml BD FACS Lysing Solution for 10 mins in the dark. Cells were then washed with 2 ml PBS and fixed with 300 µl 2% formaldehyde. Cellular analysis was performed using a Fortessa flow cytometer.

2.11 Determination of cytokines by qPCR

Cytokine activity was measured using samples from *in vitro* and *in vivo* experimentation. For murine studies whole lungs were homogenised using a hand held homogenizer in 1 ml of PBS on ice and 100 µl of homogenate was added to 1ml of TriReagent (Sigma-Aldrich). For *in vitro* studies with J774A.1 cells and human monocyte derived macrophages (MDMs) in microtiter plates, TriReagent was added directly into wells. Total RNA was isolated using the phenol/chloroform method. TriReagent was added to samples as per above, followed by chloroform at a ratio of 2:5. Samples were spun down for 5 mins at 12000 rpm at 4°C, and aqueous phase was transferred to a fresh tube. Isopropanol was added at an equal volume to aqueous phase, and spun down for 15 mins at 12000 rpm at 4°C. Nucleic acid pellet was washed with 70% ethanol and spun down for 15 mins at 12000 rpm at 4°C. Finally pellets were air dried for 5 mins at room temperature resuspended in RNase-free water (Ambion). Nucleic acid was quantified spectrophotometrically by NanoDrop and reverse transcription was carried out with the QuantiTect kit (Qiagen) according to the manufacturers instructions. mRNA copy number was determined by SYBR Green Jumpstart™ Taq Ready Mix (Sigma). All primers were used at a concentration of 0.25 mM and targets were amplified as per below cycling profile: The reaction was run in a RotorGene RG-3000 (Corbett Research) and transcriptional analysis was performed using RotorGene software (Rotogene software version 5-6).

2.12 Determination of fungal burden by qPCR and CFU

Fungal burden was determined in J774A.1 cells, human MDM's and in the lungs of mice (at times indicated) post inoculation by quantitative PCR and CFU counts.

Fungal burden was estimated by quantification of *A. fumigatus* 18s rRNA levels (GenBank accession number AB008401) as a measurement of conidial/mycelial mass, as previously described by Werner et al., (2009). In Taq Ready Mix (Sigma), *A. fumigatus* 18s rRNA levels were quantified via fluorescence of a 5' nuclease probe during amplification. Primers were

used at a concentration of 0.25 mM and targets were amplified as per below cycling profile and reaction parameters.

For colony forming units (CFU) quantification, serial dilutions were performed in 0.05% Tween20 (in dH₂O) on 90 mm Sabouraud agar plates. Plates were incubated at 37°C for 1-2 days and CFUs were counted manually.

2.13 Enzyme-linked immunosorbent assay (ELISA)

Cytokine activity was measured quantitatively using supernatants from *in vitro* and *in vivo* experimentation, for detection of proteins in supernatants of tissue culture, and BAL or whole lung homogenates respectively. ELISAs were performed using commercial kits from R&D Systems (UK) according to manufacturer instructions, for TNF- α DuoSet kit (DY410 and DY210 for mouse and human respectively) and Mouse CXCL1/KC DuoSet (DY453), all from R&D Systems, UK.

2.14 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. For statistically significant difference between the two groups and multiple comparison analysis, the students t-test and one-way analysis of variance (ANOVA) was performed respectively.

For murine survival experiments, animals that exhibited signs of vestibular infection were culled and excluded from analysis. Animals were randomly distributed in groups of 5 animals per cage and each cage was subject to one condition to avoid interference between treatments via coprophagia.

3. The effects of sphingolipid pathway inhibitors on *A. fumigatus* mediated Inflammatory responses of macrophages *in vitro*

3.1 Introduction

The innate immune system is central to the defence against *A. fumigatus* infection. Macrophages and neutrophils play a central role in recognition and killing of fungal pathogens in the lung (134,251). It is the resident alveolar macrophages that prevent infection by routinely clearing inhaled conidia, and as such are predominately the first innate cell to come into contact with this pathogen. However, despite their importance the host pathogen interaction between macrophages and *A. fumigatus* still not well understood.

Alveolar macrophages are responsible for pulmonary immune surveillance, and as the main phagocytic cell of the innate immune system they not only recognise and internalize foreign particles (like fungal conidia), but also host material such as apoptotic cells and cellular debris. Clearance of *A. fumigatus* conidia involves dectin-1 dependent phagocytosis followed by intracellular killing by acidification in a maturing phagolysosome (247). Recognition of *A. fumigatus* as a pathogen is through membrane located fungal specific moieties called pathogen associated molecular patterns (PAMPS) by innate cell pathogen recognition receptors (PRRs). PRRs are prevalent on innate leukocytes like macrophages and neutrophils and include Toll like receptors (TLRs) and C-type lectin receptors (CLRs). In consort with Dectin-1 dependent phagocytosis, recognition leads to the activation of TLRs and CLRs that result in the secretion of a variety of cytokines that mediate an inflammatory response. Important inflammatory mediators include tissue necrosis factor α (TNF- α) and macrophage inflammatory protein 2 (MIP-2).

TNF- α is a pleiotropic cytokine produced by many different cell types. It is one of the major initiators of inflammation. Its secretion is stimulated by a wide variety of pathogens through PRR activation along side the transcription of other inflammatory mediators that orchestrate an inflammatory response. Macrophages are the main producers of TNF- α and are also very responsive to it. MIP-2 is one of the major neutrophil chemoattractant molecules along with keratinocyte-derived chemokine (KC), secreted by macrophages in response to TLR signaling and dectin-1 mediated phagocytosis (446,447). MIP-2 facilitates infiltrating neutrophils, which are critical for resolving an *A. fumigatus* infection (448).

In summary, despite having key phagocytic effector functions and an essential role in the initiation of inflammation, macrophage involvement in the removing of *A. fumigatus* from the lung remains

contended in the literature. Mircescu et al. (2009) reported that alveolar macrophages when depleted by dichloromethylenebisphosphonate (clodronate) liposomes in C57B/L6 mice were dispensable for clearance of *A. fumigatus*, while in contrast Bhatia et al. (2011) demonstrated that BALB/c mice treated with clodronate liposomes had increased fungal burden and elevated levels of infiltrating neutrophils but failed to clear the infection.

Additionally, while neutrophils are demonstrated to be critical in an infection setting, they can also be the cause of immunopathology in response to *A. fumigatus*. Balloy et al. (2005) characterised the hydrocortisone (HC) model of *Aspergillus* as having high levels of infiltrating neutrophils associated with destruction of bronchi and alveoli but minimally invasive *A. fumigatus*. In this disease model the hyper-inflammatory response exacerbated by neutrophil recruitment resulted in the immunopathology that lead to death.

Moreover, clinically, IPA now largely presents in the setting of chronic respiratory diseases. In humans high dose corticosteroid treatment is a classical risk factor for developing IPA, especially in liver and kidney transplant recipients (413). It is estimated that over 30 million people are at risk of IPA each year due to the use of corticosteroids and other therapies (life-worldwide.org).

Taken together this evidence suggests macrophages play a supporting role to neutrophils in resolving an *A. fumigatus* infection *in vivo*, and a key role in routine clearance of conidia followed by initiating an inflammatory response if required.

Sphingolipids are a family of lipids defined by their sphingoid backbone (6). They are located throughout the cell in the golgi apparatus, endoplasmic reticulum, nucleus, and cell membrane (450). Sphingolipids play important roles in cellular adhesion, endocytosis and migration, and are also known to be involved in inflammatory processes (17,451).

The potent bioactive sphingolipid S1P is produced by phosphorylation of sphingosine by isoenzymes sphingosine kinase 1 (SphK1) and sphingosine kinase 2 (SphK2). Upon export from the cell by transporters such as spinster homolog 2 (Spns2) (8), S1P is recognised by a family of five G protein coupled receptors namely S1P₁₋₅ activating downstream effectors such as PI-3-kinase, protein kinase C, or intracellular calcium (452). The coupling of receptors to different downstream G-proteins and the differential tissue and cell type distribution of these receptors gives them the ability to effect numerous cellular processes including inflammation (452).

A study by Daniel et al. (2007) demonstrated that FTY720 could reduce secreted TNF- α in a murine model of colitis, characterised by severe colonic inflammation. Moreover, S1P acts as an intracellular secondary messenger interacting with signalling proteins of the Toll pathway (Fig 3.1). S1P directly regulates Toll signalling through interaction with TLR adapter protein TNF- α receptor-associated factor 6 (TRAF6) as a co-factor (78).

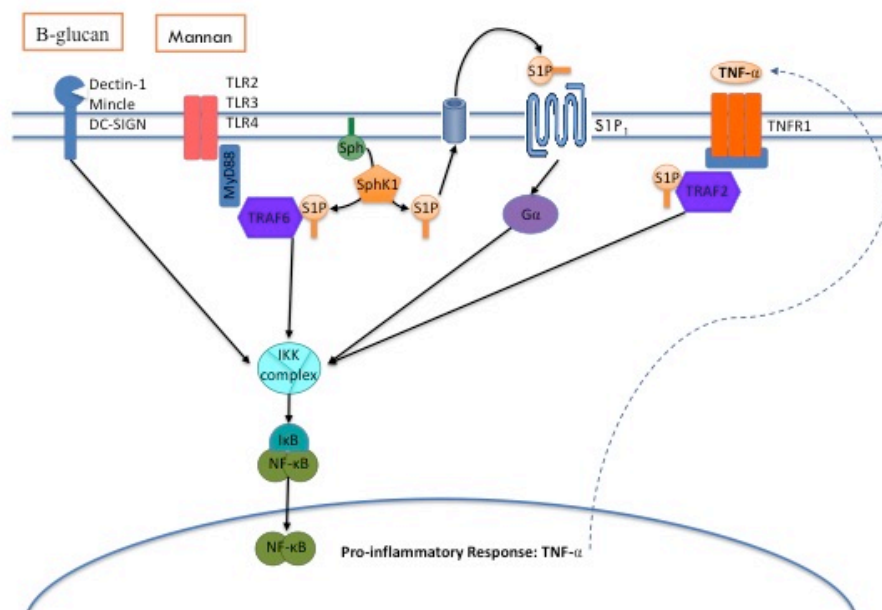


Figure 3.1: Sphingolipid involvement in fungal signalling

It was therefore hypothesised that sphingolipids could be utilized to reduce inflammatory mediators produced by macrophages in response to *A. fumigatus*. These anti-inflammatory effects could be helpful in attenuating the hyper-inflammatory phenotype seen in the HC model of aspergillosis that is immunopathological. To demonstrate the utility of sphingosine kinase inhibition in attenuation of *in vivo* inflammation, Puneet et al. (2010) showed that inhibition of SphK1 using 5c (a SphK1 specific inhibitor) and siRNA provided protection from polymicrobial sepsis, a life threatening condition characterized by excessive inflammation (455,456).

In this study sphingolipid pathway inhibitors SKI-II and FTY720 were investigated for their anti-inflammatory activity in macrophages co-cultured with *A. fumigatus* and their effects on phagocytosis. SKI-II comprehensively inhibits S1P production, thereby directly regulating pathogen recognition signaling. FTY720 (a structural homologue of the substrate sphingosine), is a high-affinity

antagonist of S1P₁, that inhibits downstream GPCR signalling (3,457). It is also a licensed drug for relapsing remitting multiple sclerosis. It is utilized for its ability to prevent circulation of self-reactive lymphocytes by inhibiting their chemotaxis from the secondary lymphoid organs (458). FTY720 can also reduce circulating monocytes (459).

In summary, with respect to mediating the hyper-inflammatory response associated with IPA, S1P signalling is a prime target to disrupt, as it is downstream of fungal recognition and upstream of TNF- α expression. Therefore, SphK enzyme inhibition is upstream of NF- κ B and has the potential to broadly attenuate inflammatory mediators during non-sterilizing and hyper-inflammatory phases of aspergillosis. Additionally, S1P receptor signaling inhibition could have effects on inflammation via its family of GPCRs S1P₁₋₅, which have been demonstrated to be involved in the inflammatory process.

3.2 Cytotoxicity of sphingolipid pathway inhibitors

Firstly, drug toxicity was tested in the murine macrophage cell line J774A.1 to establish the highest concentration of SKI-II and FTY720 that did not have a deleterious effects on cell viability. Metabolic activity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay to quantify cell metabolism and flow cytometry to assess cell viability using FACS Zombie Aqua™ (an amine group binding antibody) as secondary confirmation.

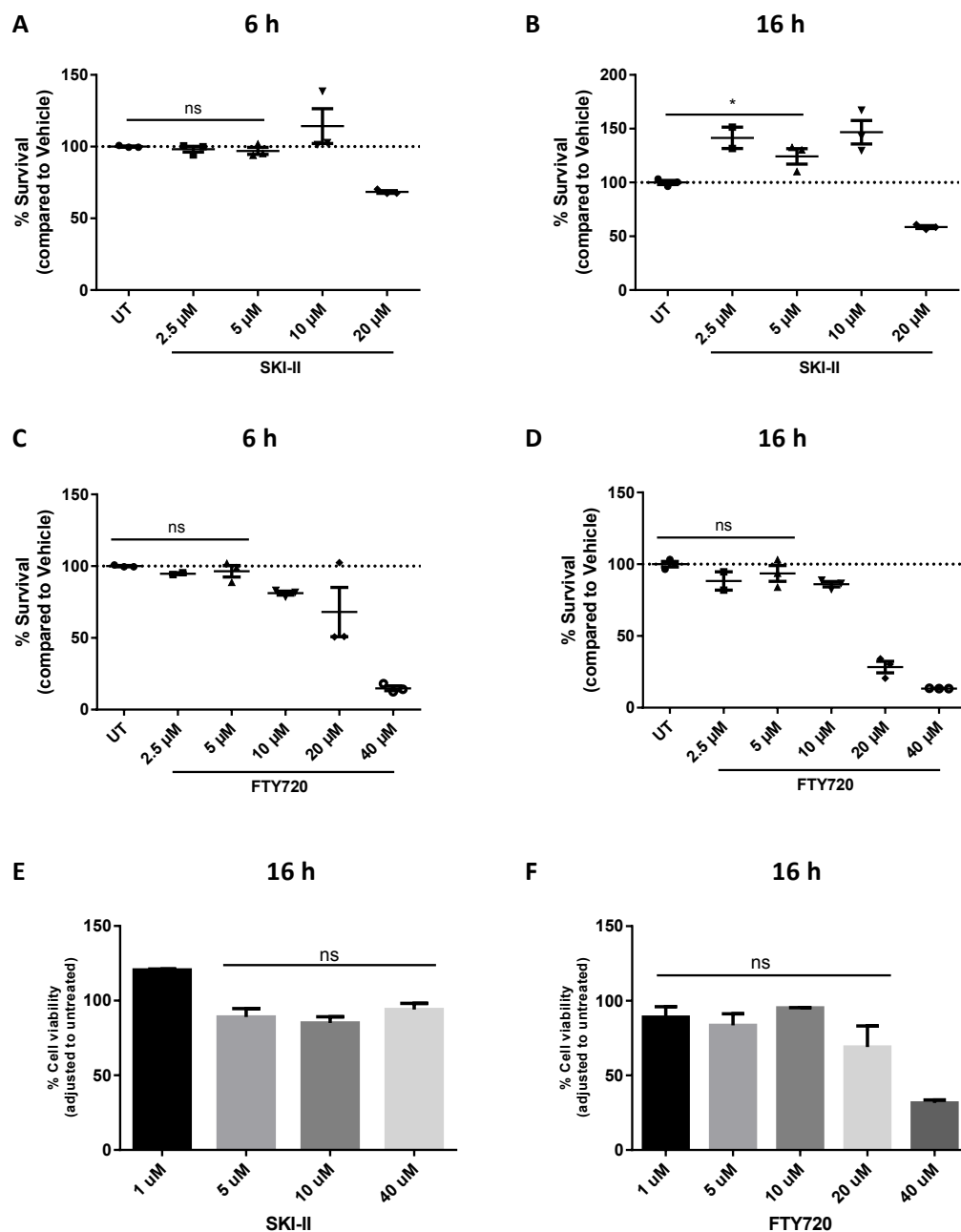


Figure 3.2: Effect of SKI-II and FTY720 on cellular viability of J774A.1 murine macrophages. Dose response curves for cell metabolism measured by MTT assay for 6 h A) and 16 h B) hours exposure to SKI-II C) and FTY720 D) titrations (2.5-40 μ M) (n=3). Cell viability was also determined by flow cytometry at 16 h using Zombie AquaTM (1:100 dilution) and without cell fixation for E) SKI-II and F) FTY720 titrations (1-40 μ M) (n=2). Data are expressed as $\pm$ SEM. Statistical significance was calculated by ANOVA. MTT for SKI-II at 6 h and 16 h (A and B: ns. $p=0.5488$ and * $p=0.0168$) and FTY720 6 h and 16 h (C and D: ns. $p=0.4496$ and $p=0.3136$), and flow cytometry for SKI-II (E: ns. $p=0.3073$) and FTY720 (F: ns. $p=0.1473$).

MTT data showed decreasing macrophage viability in a dose- and time-dependent manner to increasing drug concentrations after 6 and 16 h incubation times. Loss of viability occurred in cells treated with upwards of 10 μ M SKI-II as measured by the MTT assay (Fig 3.2 A, C), though no toxicity was indicated by flow cytometry (Fig 3.2 E). Cytotoxic effects of FTY720 were apparent at 10 μ M, which significantly increased towards 20 μ M (Fig 3.2 C, D). Flow cytometry for FTY720 indicated that 10 μ M had little effect on cell viability, with signs of toxicity being exhibited from 20 μ M (Fig 3.2 F).

In light of these data it was decided that 5 μ M was a suitable non-toxic concentration of SKI-II and FTY720 for *in vitro* experimentation with J774A.1 murine macrophages.

3.3 *A. fumigatus*-mediated TNF- α is attenuated by sphingolipid pathway inhibitors

Given the potential involvement of sphingolipids in *A. fumigatus*-mediated inflammatory responses as per their reported importance in Toll signaling (208), the effects of SKI-II and FTY720 on cytokine responses were investigated.

Murine macrophages were activated with IFN- γ overnight and pre-treated with SKI-II or FTY720 for 1 h prior to the addition of swollen *A. fumigatus* conidia at an MOI of 1:1 and 3:1. Resting conidia, as opposed to swollen conidia, are known to be immunologically inert, initiating no inflammatory response with NF- κ B (223).

After 6, 16 and 24 h of co-culture the supernatants were removed and analysed by ELISA for secreted TNF- α protein. The MOI for the 6 h incubation was higher at 3:1 in order to provoke a robust inflammatory response.

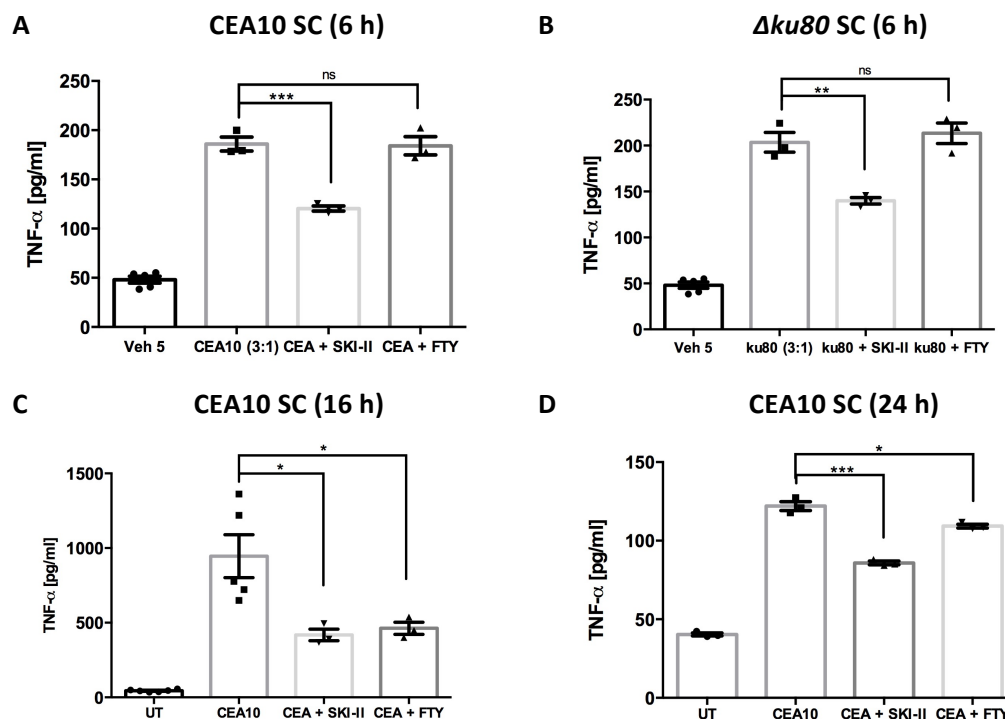


Figure 3.3: *A. fumigatus*-stimulated TNF- α secreted by murine macrophages is reduced by pre-treatment with SKI-II or FTY720. J774A.1 cells pre-treated with 5 μ M SKI-II or FTY720 for 1 h prior to co-incubation with swollen *A. fumigatus* conidia at an MOI of 3:1

for 6 h with strains A) CEA10 and B) *Δku80*, and at an MOI of 1:1 with CEA10 after C) 16 h and D) 24 h incubations. Supernatants were analysed for TNF- α protein. Data are representative of three independent experiments (n=3), each with triplicate cultures. Data are expressed as $\pm$ SEM. Statistical significance was calculated by t-test (A: *** p=0.0009 and ns p=0.8824, B: ** p=0.0050 and ns p=0.5579, C: * p=0.0345 and * p=0.0473, and D: *** p=0.0003 and * p=0.0136).

Data from two strains show that *Aspergillus* dependent TNF- α is significantly reduced by SKI-II but not FTY720 after 6 h at an MOI of 3:1 (Fig 3.3 A and B), but after 16 h and 24 h incubation with an MOI of 1:1 both SKI-II and FTY720 both had significant inhibitory effects (Fig 3.3 C-D).

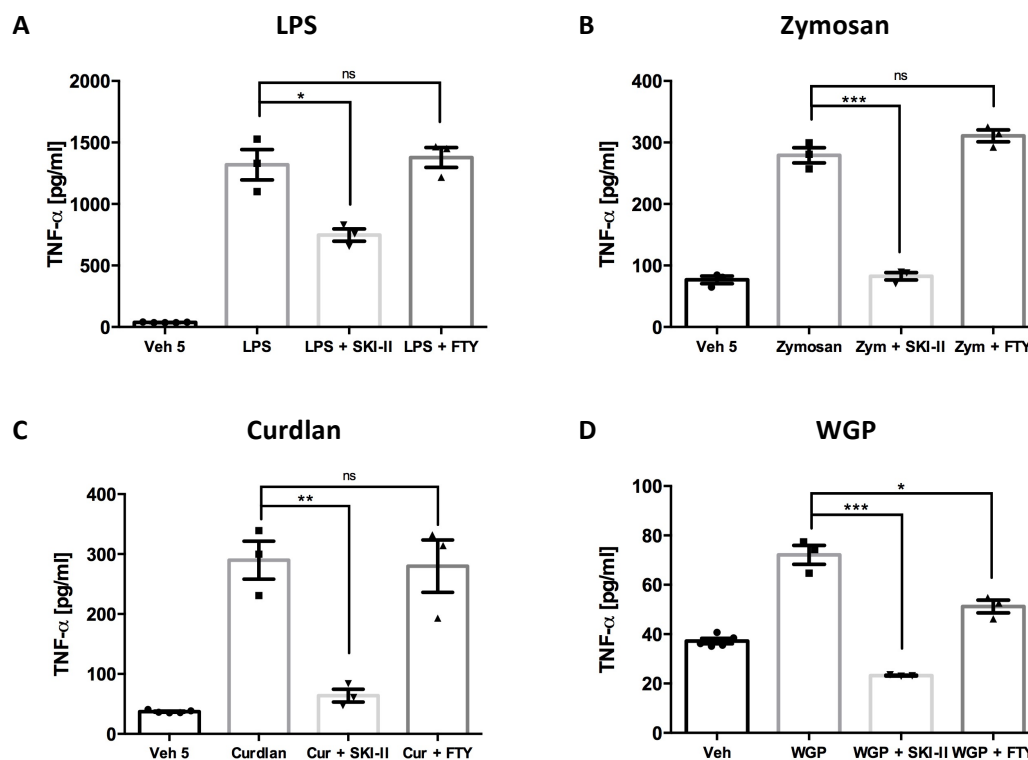


Figure 3.4: TLR- and CLR-mediated TNF- α is inhibited in murine macrophages by pre-treatment by SKI-II or FTY720. J774A.1 cells pre-treated with 5 μ M SKI-II or FTY720 for 1 h and stimulation for 6 h by addition of A) 20 ng/ml LPS, B) 1 μ g/ml Zymosan, C) 10 μ g/ml Curdlan, and D) 100 μ g/ml dispersible WGP. Data are representative of three independent experiments (n=3), each with triplicate cultures. Data are expressed as $\pm$ SEM. Statistical significance was calculated by t-test (A: LPS ns p=0.7106 and * p=0.0127, B: Zymosan ns p=0.1117 and *** p=0.0001, C: Curdlan ns p=0.8607 and ** p=0.0025, and D: WGP * p=0.0104 and *** p=0.0002).

To investigate the effects of SKI-II and FTY720 on macrophages stimulated by different ligands, responses to lipopolysaccharide (LPS), zymosan, curdlan and whole glucan particles (WGP) were determined at 6 h (Fig 3.4).

TNF- α stimulated by LPS (ultrapure) a TLR4 agonist, was attenuated by SKI-II but not FTY720. Zymosan, a TLR2, TLR6 and Dectin-1 agonist also exhibited the same responses with only SKI-II being effective in reducing TNF- α . Both selective Dectin-1 selective agonists Curdlan and WGP, had inhibition of TNF- α by SKI-II but only WGP stimulated TNF- α was reduced by FTY720. These data demonstrate that both sphingolipid pathway inhibitors had anti-inflammatory effects in response to fungal mediated TNF- α after 6 h of exposure.

The effects of swollen *A. fumigatus* conidia on murine macrophages was then investigated on a transcriptional level to gain further insights into the initial host pathogen interaction. Macrophages were co-cultured with swollen conidia at an MOI of 3:1 and after 6 hours macrophages were harvested and mRNA was extracted and analysed for RNA copy number.

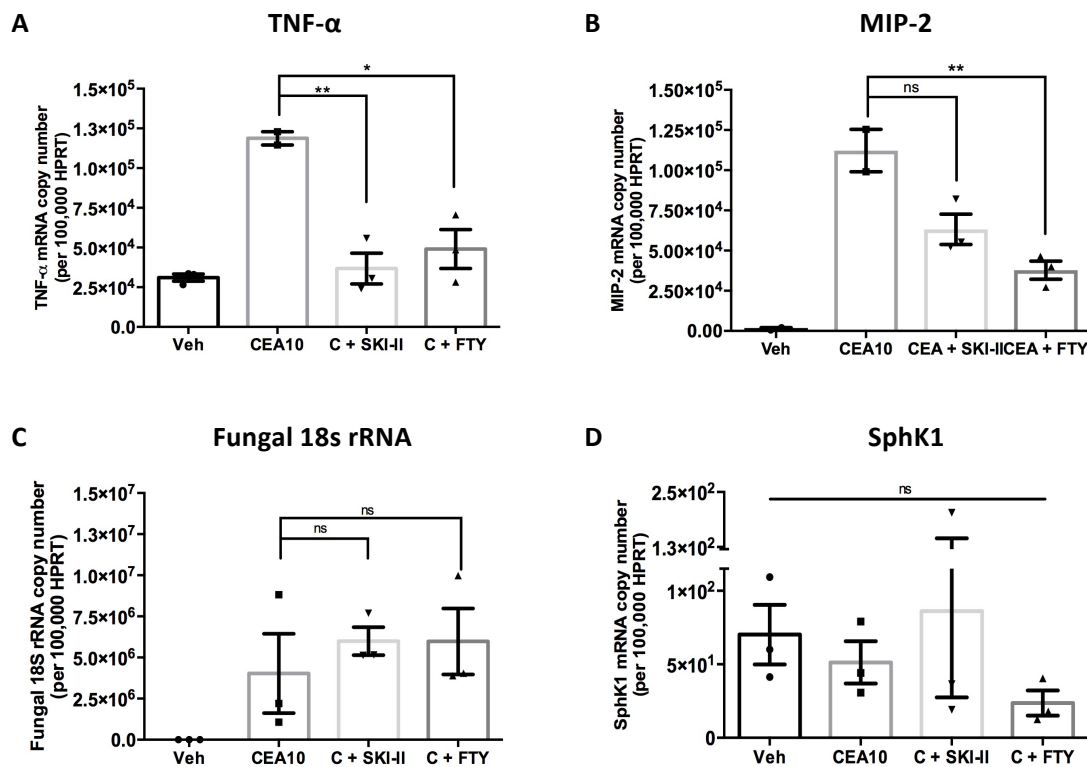


Figure 3.5: *A. fumigatus* stimulated cytokines, sphingosine kinase 1 and fungal burden in murine macrophages after pre-treatment with SKI-II or FTY720. J774A.1 cells pre-treated with 5 μ M SKI-II or FTY720 for 1 h followed by co-culture with CEA10 *A. fumigatus* conidia at an MOI of 3:1 for 6 h. mRNA was harvested from cell using Trizol-chloroform-isopropanol method and reverse transcribed. qPCR was performed using Sybr Green (Sigma) and RotorGene hardware. RNA copy numbers for the following were quantified and adjusted to 1×10^5 HPRT copies A) TNF- α , B) MIP-2, C) Fungal 18S rRNA, D) SphK1. Data are representative of three independent experiments (n=3), each with triplicate cultures. Data are expressed as $\pm$ SEM. Statistical significance was calculated by t-test for all, except for SphK1 by ANOVA (A: TNF- α ** p=0.0079 and * p=0.0227, B: MIP-2 α ns p= 0.0090, **p=0.0523, C: Fungal 18S rRNA ns p=0.4870 and ns p=0.5696, and D: SphK1 ns p=0.5773,).

Similar to secreted protein in the supernatant, mRNA copy number of TNF- α transcripts in response to SKI-II was significantly reduced (Fig 3.5 A). Contrary to TNF- α protein data, FTY720 treatment resulted in a significant reduction in TNF- α mRNA copy number also. Macrophage inflammatory protein 2-alpha (MIP-2), a chemoattractant for PMNs was reduced by SKI-II and significantly so by FTY720 (Fig 3.5 B). Fungal burden measured by 18S ribosomal RNA was not statistically affected by SKI-II or FTY720 (Fig 3.5 D). Lastly, SphK1 mRNA showed no differences between drug treatment and vehicle (Fig 3.5 E).

3.4 Immunomodulatory effects of sphingolipid pathway inhibitors are independent of potential antifungal effects

Rittershaus *et al.* (2006) demonstrated that inositol-phosphoryl ceramide (IPC) synthase, an enzyme in the sphingolipid pathway, was essential for the virulence of *C. neoformans*. They showed that melanin production was compromised and its ability to grow inside macrophages was also inhibited, thereby highlighting the potential for sphingolipid inhibitors to possess antifungal properties beyond the scope of their effects on mammalian cells. Therefore, to investigate the effects of SKI-II and FTY720 in the absence of their potential antifungal activity dead swollen conidia were co-cultured with macrophages to induce an inflammatory response.

Dead inoculum was prepared by fixing swollen *A. fumigatus* conidia (prepared as per previous assays) followed by fixing with 2% paraformaldehyde. Murine macrophages were stimulated with fixed swollen *A. fumigatus* conidia over night and TNF- α protein secreted into the supernatant was quantified by ELISA.

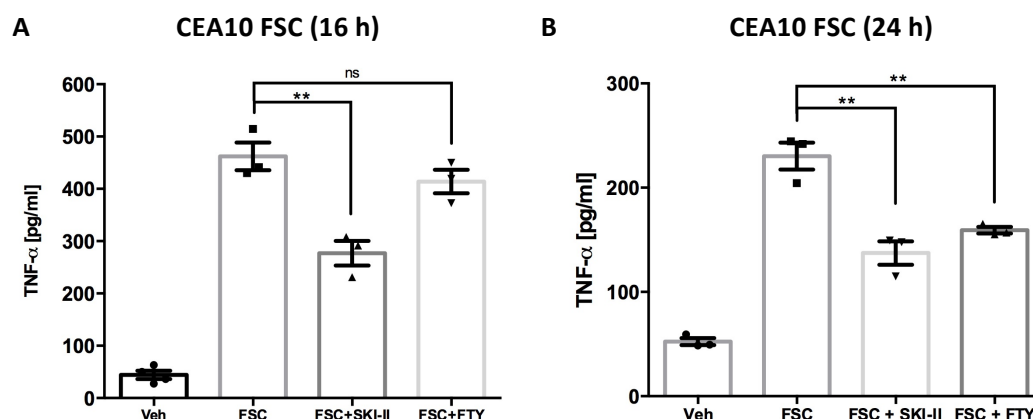


Figure 3.6: FSC-mediated TNF- α expression is inhibited by SKI-II or FTY720 in murine macrophages J774A.1 cells were pre-treated with 5 μ M SKI-II or FTY720 for 1 h followed by co-culture with CEA10 *A. fumigatus* fixed swollen conidia (FSC) at an MOI of A) 2:1 for 16 h and B) 1:1 and 24 h. Supernatants were analysed by ELISA for TNF- α protein. Data are representative of three independent experiments for 16 h and one for 24 h (n=1-3), each with triplicate cultures. Data are expressed as $\pm$ SEM. Statistical significance was calculated by t-test (A: ns p=0.2366 and ** p=0.0063, B: ** p=0.0056, ** p=0.0059).

Data show that SKI-II and FTY720 reduce fixed swollen conidia (FSC) mediated TNF- α production, thereby verifying the capacity of sphingolipid inhibition to down regulate inflammatory signaling (Fig

3.6). Confirming that the observed reduction in TNF- α seen with live conidia is due to the immune modulating effects of SKI-II and FTY720, and not their antifungal activity.

3.5 Conidial phagocytosis by murine macrophages is inhibited by SKI-II but not by FTY720

Alveolar macrophages line the lung epithelium, clearing cellular debris and inhaled pathogens. Phagocytosis is a key effector function of macrophages, allowing for intracellular phagosomal degradation of particles and killing of pathogens. This process is crucial to the clearance of *A. fumigatus* conidia (246).

To examine the effects of sphingolipid inhibition on phagocytic activity, murine macrophages were co-cultured with *A. fumigatus* conidia at an MOI of 3:1 over a time course of 1 hour. Conidia were biotinylated and labelled with concanavalin A conjugated FITC before co-culture. At each time point cells were fixed with 2% PFA and external conidia were labeled with streptavidin labelled Cy3. This allowed for the identification of internal and external conidia by FITC and Cy3 fluorescent signals respectively. Cells were imaged using widefield microscopy z-stacks and fluorescence polarized using Huygens Analysis deconvolution software.

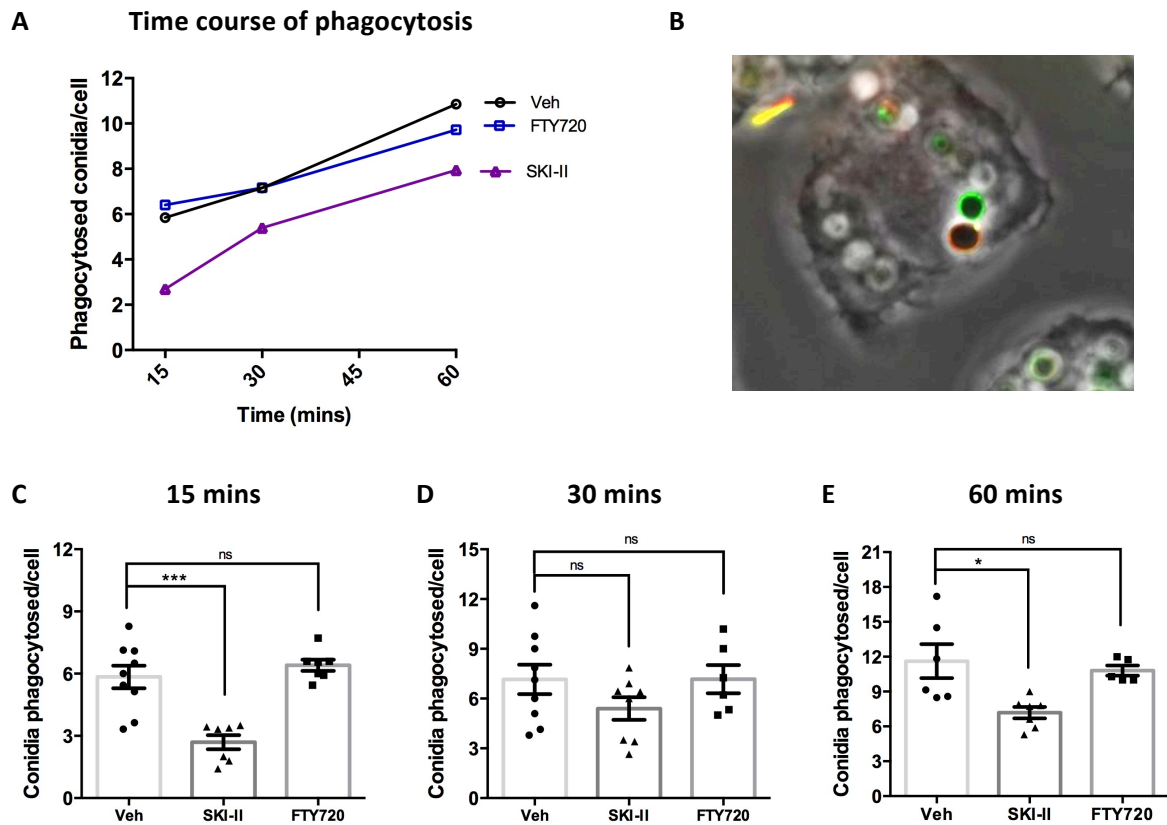


Figure 3.7: Inhibition of sphingosine kinases but not S1P₁ inhibits phagocytosis of *A. fumigatus* conidia.

J774A.1 cells were pre-treated with 5 μ M SKI-II or FTY720 for 1 h followed by co-culture with FITC labelled CEA10 *A. fumigatus* conidia at an MOI of 3:1 for 15, 30 and 60 mins. Cells and conidia were the fixed with 2% PFA and those attached to macrophages but not phagocytosed were labelled with Cy3. Z stack images of fields of view were sharpened by Huygens Analysis deconvolution software, and A) the average number of phagocytosed conidia were enumerated per cell per image using z-stacks, using B) widefield images where green and red conidia are internal and external respectively. Individual time point data for C) 15, D) 30 and E) 60 mins. Data are expressed as $\pm$ SEM of triplicate cultures. Statistical significance was calculated by t-test (C: 15 mins ns $p=0.4134$ and *** $p=0.0005$, D: 30 mins ns $p=0.9919$ and ns $p=0.1433$, and E: 60 mins ns $p=0.6389$ and * $p=0.0108$).

Macrophages treated with FTY720 showed no difference in phagocytosis of swollen conidia compared to vehicle across the time course. In contrast, treatment of macrophages with SKI-II significantly inhibited conidial uptake (Fig 3.7).

3.6 Discussion

This study investigated the ability of sphingolipid inhibitors to attenuate fungal-mediated inflammatory responses of murine macrophages. Sphingolipid biosynthesis was inhibited using SKI-II to impede S1P genesis and FTY720 to inhibit S1P₁ receptor signalling. Both drugs resulted in attenuated responses of macrophages. Cellular responses were examined by quantification of secreted cytokines, RNA transcription and phagocytosis of swollen conidia.

Firstly, cellular toxicity of SKI-II and FTY720 against murine macrophages was established to be 10 μ M and above for both drugs and 5 μ M was determined to be a safe dose for *in vitro* experimentation. It is interesting to note that after 16 h incubation, SKI-II had increased the cellular metabolism of murine macrophages above that of UT to a statistically significant level between 2.5 and 10 μ M. A reason for this might be that SKI-II, in inhibiting both SphKs induces a stress response that results in an up-regulation of metabolic processes.

Production of TNF- α by murine macrophages after *A. fumigatus* co-culture was significant at 6, 16 and 24 h, with 16 h having the highest secreted protein levels. Data show that a longer incubation time of 16 h is necessary for FTY720 to significantly reduce TNF- α , but a reduction in TNF- α mRNA transcripts is seen at 6 h. These data suggest that the inflammatory inhibiting mechanism of FTY720 has slower kinetics than SKI-II, which significantly inhibited TNF- α from 6 h. It may be that as FTY720 is initially an agonist for S1P₁ the effects of its internalization and degradation cause a delay in anti-inflammatory effects. This same pattern of inhibition is seen in co-culture with FSC, which are less immunogenic. At 16 h SKI-II significantly reduces TNF- α and at 24 h both drugs achieve significant inhibition. Incubation with FSC also confirmed that the reduction in live *A. fumigatus* mediated TNF- α was not due to the potential anti-fungal effects SKI-II and FTY720 might possess, but due to immunomodulatory effects.

Recognition of fungal mannan structures by PRRs is mediated through TLRs. Both TLR2 and TLR4 have been implicated in the recognition of *Aspergillus* conidia and hyphae. Both TLRs signal through adapter protein Myd88 with subsequent translocation of NF- κ B, resulting in production of cytokines such as TNF- α and MIP-2 (186,461). TLR4 and TLR2 mediated TNF- α protein production is significantly reduced by SKI-II but not FTY720. However, TNF- α is reduced by both SKI-II and FTY720 when stimulated by WGP. Demonstrating that PAMP recognition signaling via TLRs is sphingolipid dependent, and although it is less clear why, downstream inhibition by FTY720 can have inhibiting effects on TNF- α through Dectin-1 signalling. Similarly, TNF- α mRNA is also significantly inhibited by

both SKI-II and FTY720. The reduction of TNF- α mRNA copy number of FTY720 and not protein indicate that while inhibition of TNF- α may not be as clear as the upstream inhibitor SKI-II, on a transcription level it is just as effective. An explanation for the effectiveness of SKI-II could be that with the inhibition of both sphingosine kinases, SKI-II is able to comprehensively inhibit the synthesis of S1P, thus preventing its actions as a co-factor for TLR signaling in addition to the subsequent autocrine S1P receptor signaling. Similarly for zymosan, an agonist of TLR2 (and Dectin-1), SKI-II effectively inhibited TNF- α production.

The effects of sphingolipid pathway inhibition on Dectin-1 mediated TNF- α however are diverse. While SKI-II inhibited TNF- α in response to curdlan, FTY720 was ineffective, yet both SKI-II and FTY720 had significant down regulation of TNF- α mediated by WGP. The reason for this disparity is likely due to the differences in complexity of these two ligands. Curdlan is β 1,3-glucan residues from bacteria where as WGP is hollow *S. cerevisiae* 'ghosts' that consists predominantly of long β 1,3-glucan polymers. Thus WGP is a more complex stimulant with greater potential to activate fungal PRRs, increasing the potential for sphingolipid involvement.

Furthermore, significant transcriptional inhibition of MIP-2 (a leukocyte chemoattractant) by FTY720, suggests its utility in reducing cellular recruitment to the lung in response to *A. fumigatus*. This is an important finding because the hydrocortisone murine model of IPA is characterised by immunopathology resulting from high levels of cellular infiltrates (Balloy et al. 2005).

It is somewhat surprising then that SphK1 mRNA levels had no statistically significant differences between treatments, although SKI-II data could indicate the start of SphK1 transcript up-regulation in response to its inhibition. An explanation could be that if SphK1 was recognised as inactive, the cell may initiate *de novo* synthesis to replace it. This would also explain why FTY720 doesn't increase SphK1 transcripts as its effects are on downstream inflammatory signalling and not on aberration of S1P production by SphK inhibition. These data are to be interpreted with caution because mRNA is not a quantification of active enzyme or enzymatic activity, but it does demonstrate that SKI-II could potentially result in increased SphK1 production.

A study by Philippe and Pre, et al. (2003) reported that after 6 hours macrophages killed 6.6% of ingested swollen conidia when incubated at an MOI of 1:1. In this study an MOI of 3:1 was used to increase effects of fungal interaction with regards to signalling and phagocytosis but this may have lowered the cells ability to kill the conidia in the time frame of this assay. Taking this into

consideration, it is unsurprising that no differences were observed in fungal burden when comparing drug to vehicle treated cells. This inference is taken with the assumption that treatment by drugs had no effect on fungal 18s rRNA, i.e. no antifungal effects after 6 h.

A key effector function of pulmonary macrophages is phagocytosis of pathogens entering the lungs. Tafesse et al. (2015) demonstrated that inhibiting *de novo* sphingolipid biosynthesis and more specifically ceramide synthesis (the precursor of sphingosine), inhibited the phagocytic uptake of *C. albicans*. They go on to suggest that inhibition of sphingolipid biosynthesis results in defective phagocytic cup formation where they speculate sphingolipids aid the formation of the actin-rich pseudopods that are generated during membrane curvature. In addition, phagocytosis is known to be triggered by ligation of PAMPS and PRRs. PRRs such as Dectin-1 and TLR2 that recognise fungal ligands (β -glucan and mannan respectively) and are known to be associated with lipid rafts which are characterized by dynamic assemblies of cholesterol and sphingolipids (225,463). Lipid rafts exercise protein selectivity and this compartmentalization allows for increased receptor localization at the pathogen host interface (464). Data in this study is concurrent with the idea that sphingosine biosynthesis plays an important role in the phagocytosis of fungi. Data show that inhibition of S1P biosynthesis by SKI-II results in a phagocytosis defect, whereas FTY720 that does not inhibit sphingolipid biosynthesis but inhibits S1P₁ receptor binding, conferring no difference in phagocytic activity compared to vehicle.

In summary, although SKI-II effectively inhibited secretion of TNF- α in response to swollen *A. fumigatus* conidia, it significantly impaired conidial phagocytosis by murine macrophages. FTY720 on the other hand had inhibitory effects on TNF- α and MIP-2, but resulted in no phagocytic defect. Together these data demonstrate that sphingolipids play an important role in phagocytosis, and fungal mediated TNF- α is partially an S1P-dependent process that can be attenuated by sphingolipid pathways inhibitors.

4. The effects of sphingolipid pathway inhibitors in the murine model of aspergillosis

4.1 Introduction

In an immunocompetent host, pulmonary defenses against *A. fumigatus* are initially mediated by alveolar macrophages (AMs). Resident AMs are the first line of phagocytic defense. Through recognition of non-self motifs or PAMPS (on resting and swollen conidia respectively) conidia are phagocytosed. In the event of a high fungal insult or germinated conidia that evade phagocytosis and or killing by macrophages, AMs coordinate the early PMN infiltration to the site of infection via antigen-specific secretion of cytokines and chemokines (114,403). Neutrophils make up 90% of the infiltrating cells and are responsible for killing of *A. fumigatus* hyphae by degranulation into the extracellular environment (175). Leukocyte responses in an immunocompetent host are characterized by an acute inflammatory response that is entirely resolved within 72 h of inoculation (403). Initial responses are mediated by the innate immune system and are thought to be responsible for resolving infection. Although adaptive responses provide assistance in pulmonary sterilization through T and B cells, it has demonstrated that mice without adaptive immune cells (Rag2^{-/-} mice) have normal neutrophil and monocyte recruitment to the lung and do not develop invasive disease after a high (8×10^7) *A. fumigatus* insult (465).

Prolonged high dose corticosteroid therapy is a classical risk factor for developing aspergillosis. Corticosteroids are typically prescribed for a patients receiving solid organ or stem cell transplantation for the prevention or treatment of allogeneic rejection (343,466,467). The HC murine model is characterized by major pulmonary PMN recruitment, concomitant with production of inflammatory cytokines. Mortality is observed to be due to pulmonary lesions caused by an excessive and chronic host response (403). In the AMs of corticosteroid treated mice, intracellular killing of conidia is inhibited by impairment of the production of reactive oxidant intermediates, as well as phagosomal acidification and NADPH oxidase activity (468). As a result, conidia that fail to be killed germinate and grow causing disruption of the phagosomal membrane, followed by cellular outgrowth and host cell death (246). This hyphal outgrowth from macrophages is followed by overwhelming PMN recruitment due to fungal persistence.

FTY720 was selected for use in the HC model of aspergillosis and not SKI-II because of the anti-inflammatory effects observed in Chapter 3. Data suggests that FTY720 reduces *A. fumigatus*-mediated TNF- α production, and most notably inhibited MIP-2 mRNA transcript numbers more significantly than SKI-II. Lastly, SKI-II inhibited phagocytosis of resting conidia, whereas FTY720

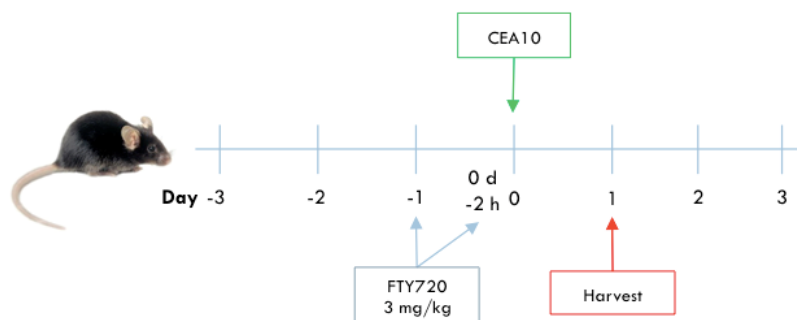
treated cells exhibited no difference compared to vehicle. Therefore, due to its demonstrated anti-inflammatory effects on TNF- α and MIP-2, it was hypothesised that FTY720 could be beneficial in a HC model of aspergillosis where mortality was mediated by inflammation as opposed to uncontrolled fungal growth.

4.2 FTY720 inhibits *A. fumigatus*-mediated cytokines in immunocompetent mice

Firstly, it was determined if the anti-inflammatory effects of FTY720 on *A. fumigatus*-mediated cytokines was detrimental or advantageous to immunocompetent mice. Murine lungs were harvested at 48 h after inoculation and mechanically homogenized in 1 ml saline on ice. Homogenates were analysed for TNF- α by ELISA and fungal burden determined by fungal 18s rRNA.

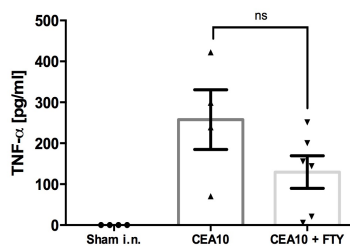
Mice underwent two regimens of FTY720 treatment, a low dose regime with two injections before inoculation (at day -1 and day 0) and a daily dose regime (from day -2 until the end of the experiment). It was unclear which dosing schedule would be effective and since both regimes were reported in the literature the low dose regime was employed in initial experiments investigating single time points (24-48 h). Further to data from initial experiments, it was decided to adjust to a daily dose schedule to more distinctly define the effects of FTY720 by increasing the dosage.

A



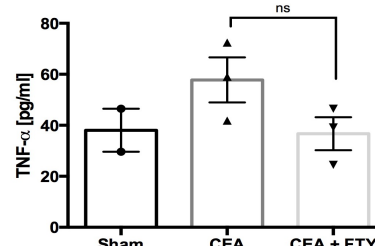
B

TNF- α



C

TNF- α



D

Fungal 18s rRNA

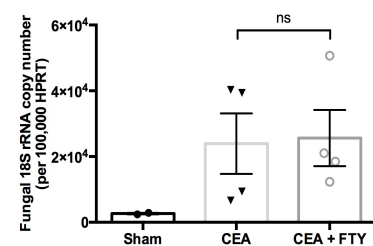


Figure 4.1: FTY720 affects TNF- α in response to *A. fumigatus*. Mice were treated with i.p. FTY720 on day -1 and day 0, 2 h before inoculation with *A. fumigatus* conidia suspended in 40 μ l of saline and sham given saline only. Mouse strains BALB/c and C57BL/6 were given B) 2.5×10^6 and C) 1×10^7 conidia respectively. Lung homogenates for both strains were tested for TNF- α protein by ELISA and only C57BL/6 for fungal 18s rRNA by qPCR D). Data are expressed as $\pm$ SEM. Statistical significance was calculated by t-test (B: ns p=0.5347, C: p=0.1267, D: p=0.8968).

Two strains of mice, namely BALB/c and C57BL/6 were treated with FTY720 one day prior to and on the day of inoculation (Fig 4.1 A). Both immunocompetent mouse strains appeared to have marginally lower TNF- α in whole lung homogenates at 24 h (Fig 4.1 C, D). Fungal burden was unaffected at 24 h by treatment with FTY720 (Fig 4.1 E).

To further assess the effect of FTY720 on fungal development and inflammatory responses to *A. fumigatus*, cytokines and fungal burden in mice were monitored over a time course from 6 to 72 h after inoculation.

Immune competent C57BL/6 mice were treated daily with FTY720 prophylactically from day -2 before inoculation until the end of the experiment (Fig 4.2 A). BAL fluid was harvested in place of whole lung homogenates to increase concentration of secreted proteins and because immune competent mice do not develop invasive aspergillosis therefore fungal burden can also be determined without lung homogenization.

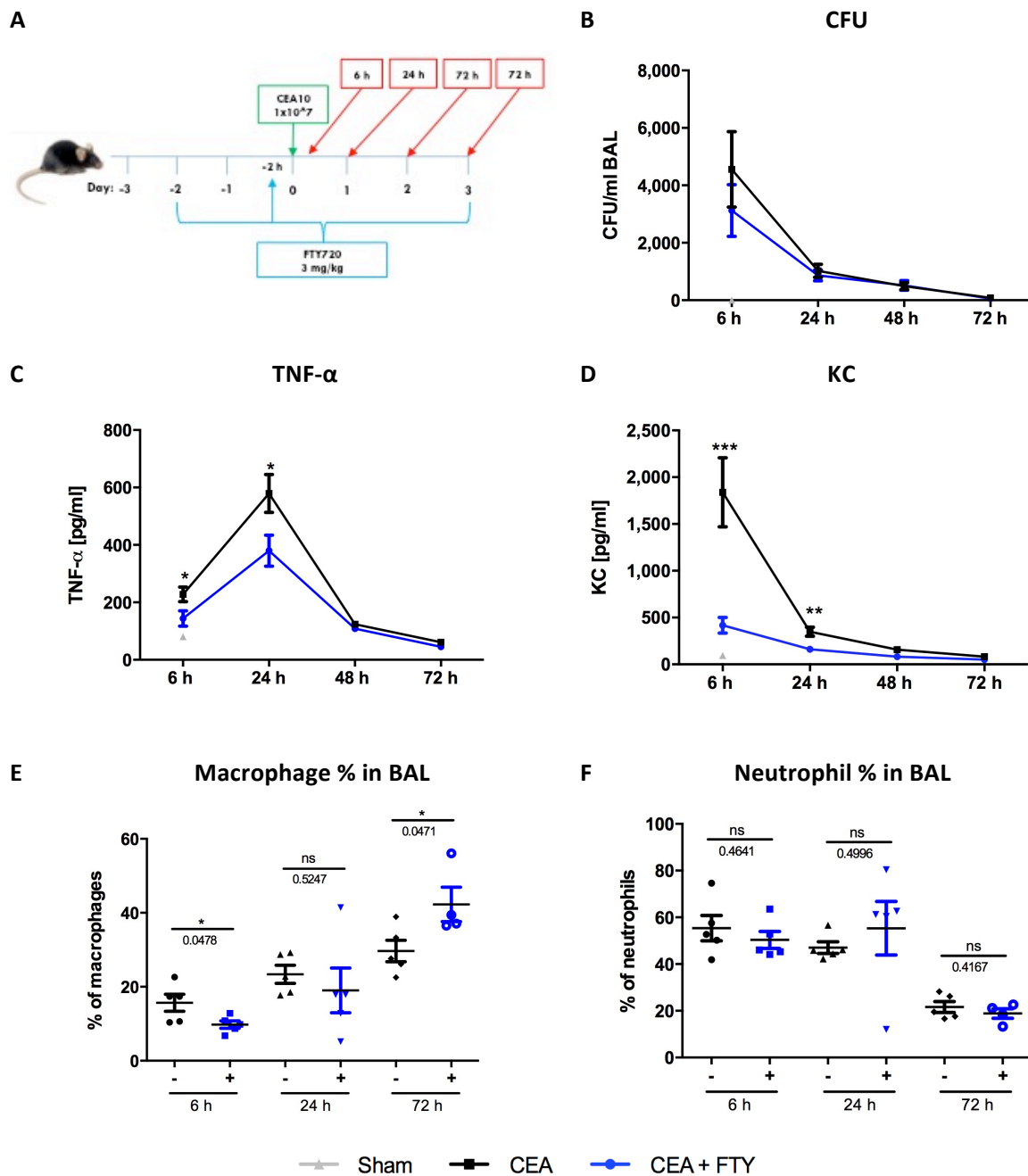


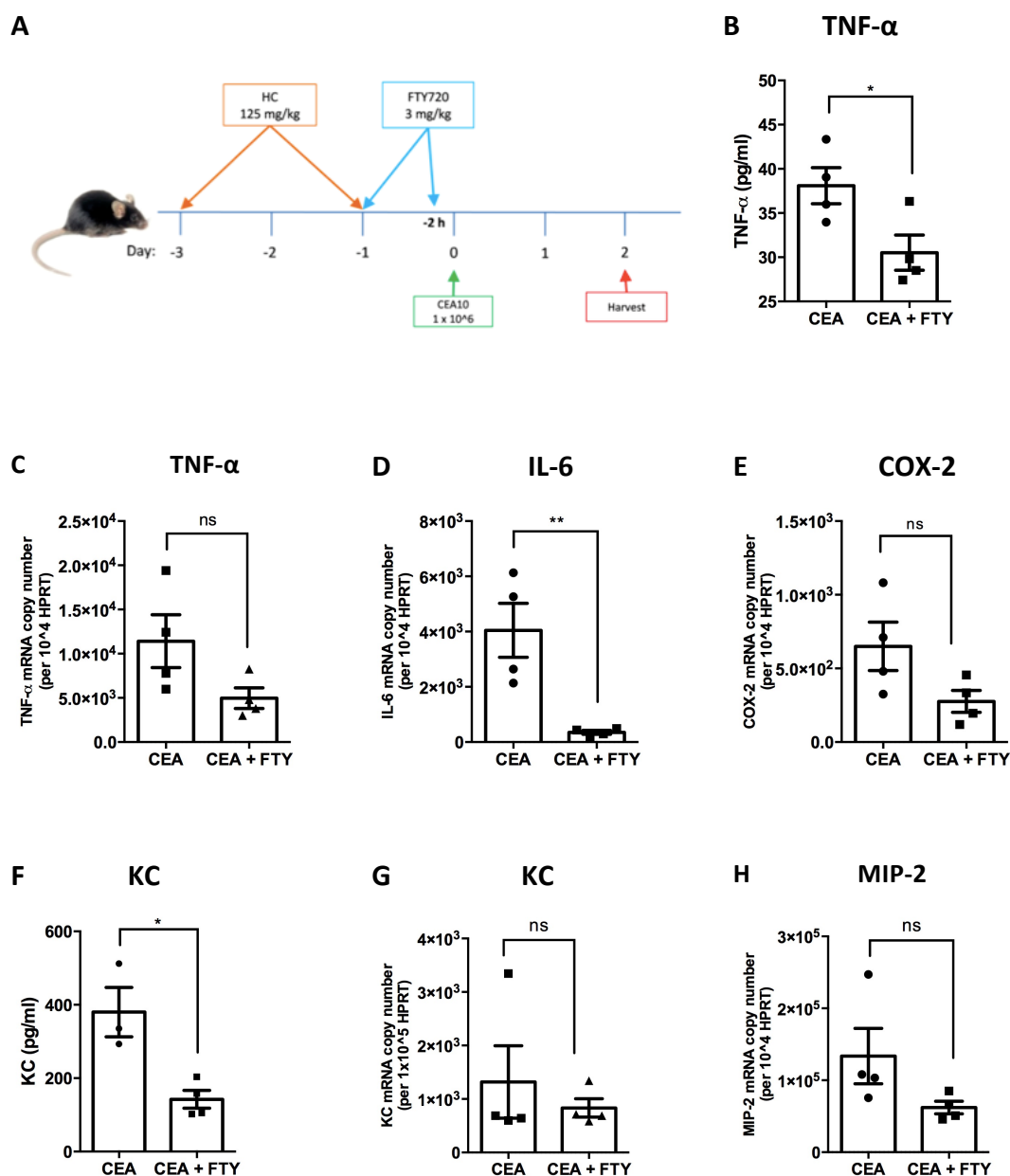
Figure 4.2: FTY720 affects TNF- α , KC and pulmonary infiltrate population in response to *A. fumigatus*. A) Mice were treated with FTY720 i.p. daily or given vehicle (saline) i.p. two days before inoculation with 1×10^7 conidia. Conidia were suspended in 40μ l saline and sham given saline only. Mice were anaesthesia by isoflurane inhalation and prior to intranasal inoculation. After cervical dislocation and femoral artery transection, lungs of mice were lavaged with 1 ml of saline and recovered fluid was analysed for B) TNF- α and C) KC by ELISA, and leukocytes by FACS. Data are expressed as $\pm$ SEM of 2-3 independent experiments where mice/n=5. Statistical significance was calculated by t-test (C: * $p=0.0304$ and * $p=0.0266$, D: *** $p=0.0007$ and ** $p=0.0030$, E: * $p=0.0478$, ns $p=0.5247$ and * $p=0.0471$, and F: ns $p=0.4641$, ns $p=0.4996$ and ns $p=0.4167$).

Similar to Fig 4.1 D, mice showed no significant differences in fungal burden, although the average CFU at 6 h was lower for FTY720 treated mice (Fig 4.2 B). The TNF- α expression of FTY720 treated mice followed the same expression pattern as vehicle but was significantly reduced at 6 and 24 h (Fig 4.2 C). Correspondingly, KC in FTY720 treated mice followed a similar expression pattern to that of vehicle but was extensively inhibited at the early time point of 6 h and marginally at 24 h (Fig 4.2 D). Leukocytes analysed by flow cytometry indicate that of the total cells recovered in BAL, the % of macrophages was initially significantly lower at 6 h but became significantly higher at 72 h in FTY720 treated mice, but neutrophil % were unaffected (Fig 4.2 E, F).

4.3 FTY720 inhibits inflammatory cytokines and fungal burden in HC model of aspergillosis

Next, the effects of FTY720 on the inhibited and dysregulated immune responses of immune compromised mice to *A. fumigatus* were investigated. Mice were immune suppressed with 125 mg/kg hydrocortisone acetate (HC) at days -3 and -1 prior to inoculation (Fig 4.3 A).

Corticosteroid-induced immunosuppression is a major risk factor for the development of invasive aspergillosis (114). To ensure invasive and non-invasive fungus was quantified, whole lungs were harvested at 48 h and homogenized in 1 ml of saline. Whole lung homogenates were analysed for cytokine expression and fungal burden in response to FTY720 prophylactic treatment.



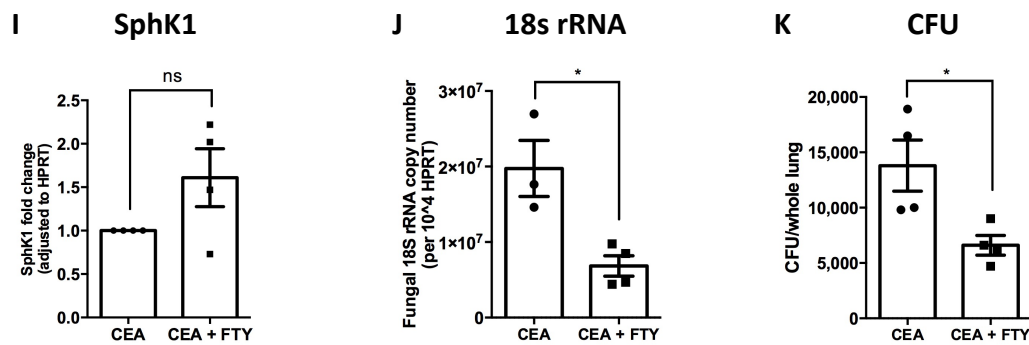


Figure 4.3: Inflammatory mediators are reduced by FTY720. A) Mice were immune suppressed with 125 mg/kg hydrocortisone acetate at days -3 and -1, and treated with FTY720 i.p. or given vehicle (saline) i.p. at day -1 and on the of inoculation at -2 h. Inoculum of 1x10⁶ conidia was given in 40 µl saline. Mice were anesthetised with isoflurane inhalation prior to intranasal inoculation. Lungs of mice were harvested and homogenized in 1 ml of saline. Whole lung homogenates were analysed for B) TNF-α and F) KC by ELISA, and for mRNA transcription of C) TNF-α, D) IL-6, E) COX2, G) KC, H) MIP-2, I) SphK1 and J) fungal 18s rRNA. CFU were calculated per whole lung from dilutions plated on SAB. Data are expressed as ± SEM (mice/n=4). Statistical significance was calculated by t-test (B: * p=0.0379, C: ns p=0.0915, D: ** p=0.0093, E: ns p=0.0840, F: * p=0.0130, G: ns p=0.5129, H: ns p=0.1204, I: ns p=0.1171, J: * p=0.0141, and K: * p=0.0269).

Hydrocortisone immune suppressed mice treated with FTY720 had significant down regulation of TNF-α and KC protein production, and IL-6 at the transcriptional level at 48 h after inoculation (Fig 4.3 B, F, D). Of note, fungal burden as determined by 18s rRNA and CFU was significantly reduced in FTY720 treated mice (Fig 4.3 J,K).

Immune suppression by corticosteroids is associated with high levels of PMN recruitment and their persistence in the lung causing tissue damage (114). To corroborate, the finding that FTY720 inhibits *A. fumigatus*-mediated chemoattractant KC, with inflammation in an immune suppressed model (Fig 4.3 F), murine lungs were harvested at 48 h after inoculation and examined by histological analysis using a periodic acid-Schiff (PAS) stain.

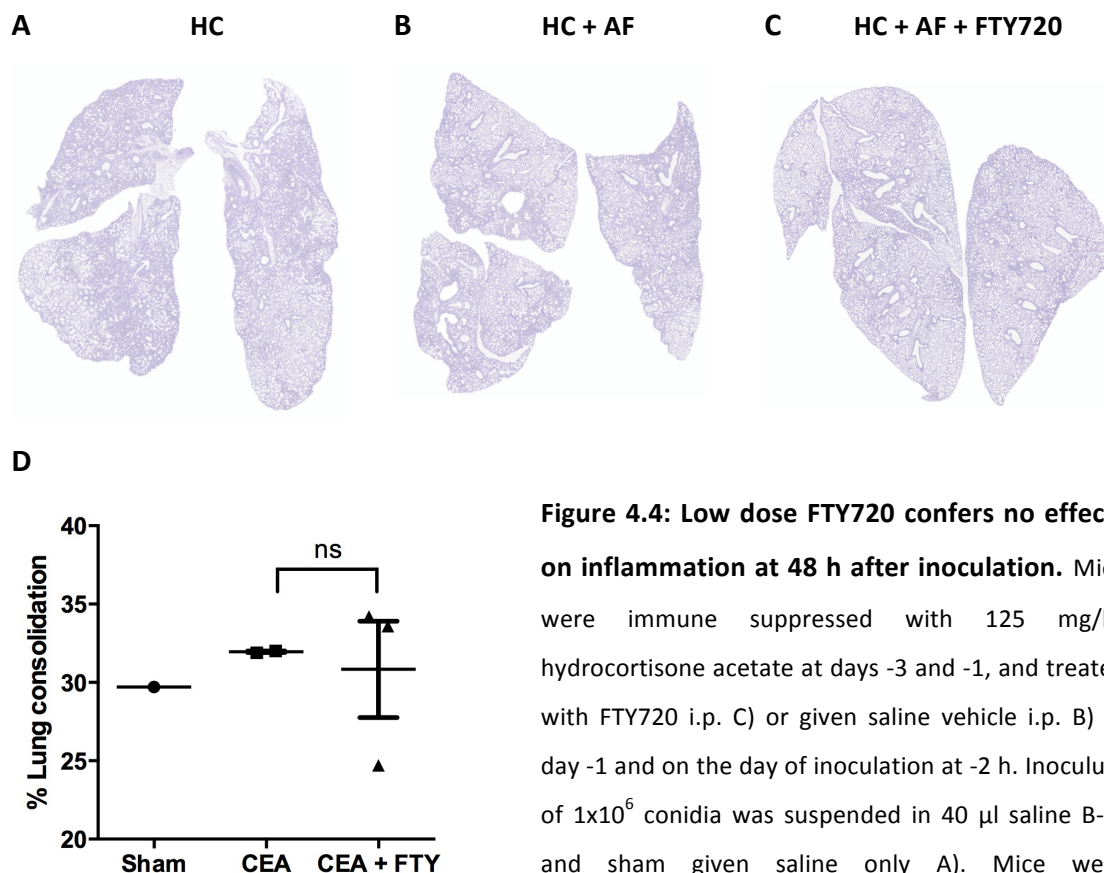


Figure 4.4: Low dose FTY720 confers no effects

on inflammation at 48 h after inoculation.

Mice were immune suppressed with 125 mg/kg hydrocortisone acetate at days -3 and -1, and treated with FTY720 i.p. C) or given saline vehicle i.p. B) at day -1 and on the day of inoculation at -2 h. Inoculum of 1×10^6 conidia was suspended in 40 μ l saline B-C) and sham given saline only A). Mice were anaesthetised with isoflurane inhalation prior to

intranasal inoculation. Lungs of mice were harvested at 48 h and fixed in a PBS buffered 10% formaldehyde for 24 h at RT. 3 μ m lung sections were treated with periodic acid-Schiff (PAS) stain. Images were captured by widefield microscopy and analysed blindly for consolidation ImageJ analysis (by mixing the set of images). Data are expressed as $\pm$ SEM (mice/n=2-3 for vehicle and FTY720 treated mice respectively). Statistical significance was calculated by t-test (ns p=0.1843).

Whole lung images were captured by widefield microscopy and analysed by ImageJ for inflammation. Pulmonary inflammation was quantified by lung consolidation and calculated as a percentage of tissue density across the whole lung area using a threshold tool.

Histological sections indicate similar levels of inflammation in both low dose FTY720 and vehicle treated mice at 48 h after inoculation, although data are from a small study and are interpreted with caution. Therefore to determine the utility of FTY720 in attenuating the immunopathological affects of a developing *A. fumigatus* infection under hydrocortisone suppression, a survival study was performed.

4.4 FTY720 improved survival in the murine HC model of aspergillosis

To determine if the anti-inflammatory effect of daily FTY720 would prolong survival, mice were immuno-suppressed with 125 mg/kg HC at days -3 and -1 prior to inoculation in combination with daily FTY720 treatment from day -2 (Fig 4.5 A).

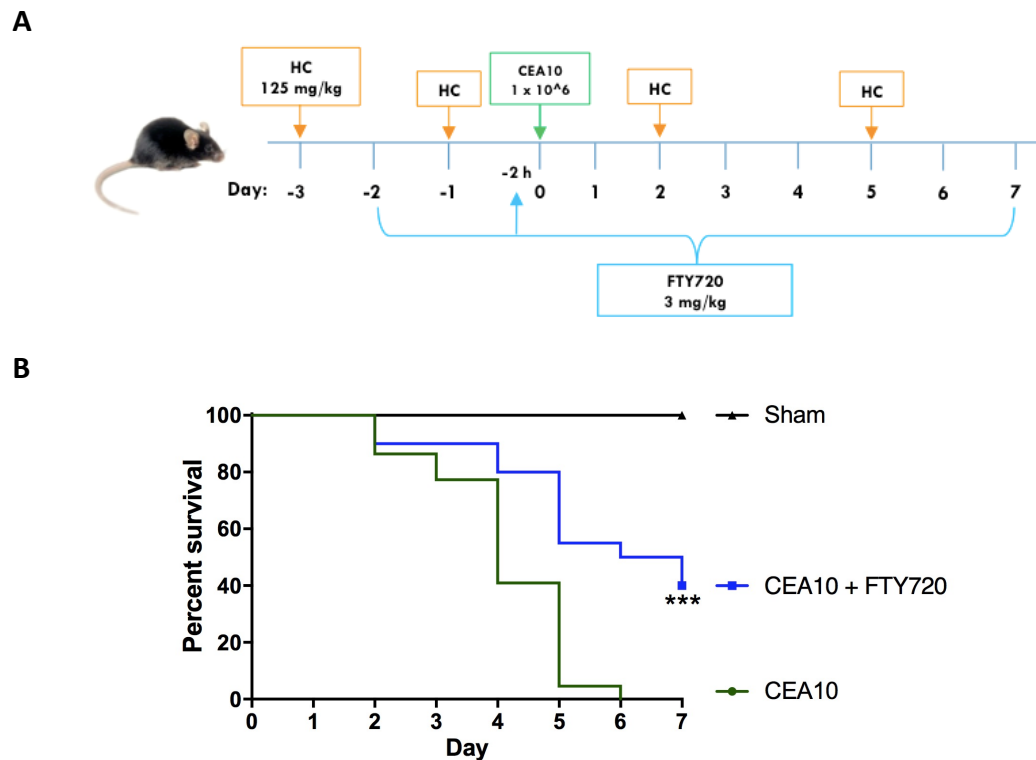


Figure 4.5: FTY720 improved survival in HC model of aspergillosis. Mice were immune suppressed with 125 mg/kg HC at days -3 and -1, and treated with daily 3 mg/kg FTY720 i.p. from day -2 (blue) or given saline vehicle i.p. (green and black). Inoculum of 1×10^6 conidia was suspended in 40 μ l saline (blue and green) and sham given saline only (black). Mice were anaesthetised with isoflurane inhalation prior to intranasal inoculation. Mice were monitored daily for weight and behaviour, and culled when either weight was less than $\leq 80\%$ of that at day 0 or if severity level was determined to be moderate. Data are expressed as $\pm$ SEM of 2 independent experiments (mice/n=8). Statistical significance was performed using Log-rank (Mantel-Cox) test as *** p=0.0001. Median survival for FTY720 treated and vehicle treated were 6.5 and 4 days respectively.

Data demonstrate that prophylactic treatment with daily FTY720 significantly prolonged survival, although did not completely protect mice from *A. fumigatus* infection (Fig 4.5 B). FTY720 and vehicle treated mice succumbed to *A. fumigatus* infection with a median survival of 6.5 and 4 days after inoculation respectively.

4.4 FTY720 reduces lung inflammation in the murine HC model of aspergillosis

Further to chemoattractant data suggesting the potential of FTY720 in reducing pulmonary inflammation (Fig 4.2 C, D and Fig 4.3 B, F) and survival curve data showing attenuation of disease (Fig 4.5), histological analysis was performed on lungs of mice at day 5 after inoculation. Day 5 is of particular interest because HC treated mice at this time are at the terminal stage of disease. FTY720 treated mice had begun to stabilize at day 5 in terms of survival (Fig 4.5).

Mouse lungs were harvested and pulmonary inflammation was examined by histological analysis of with periodic acid-Schiff (PAS) stain using ImageJ lung consolidation.

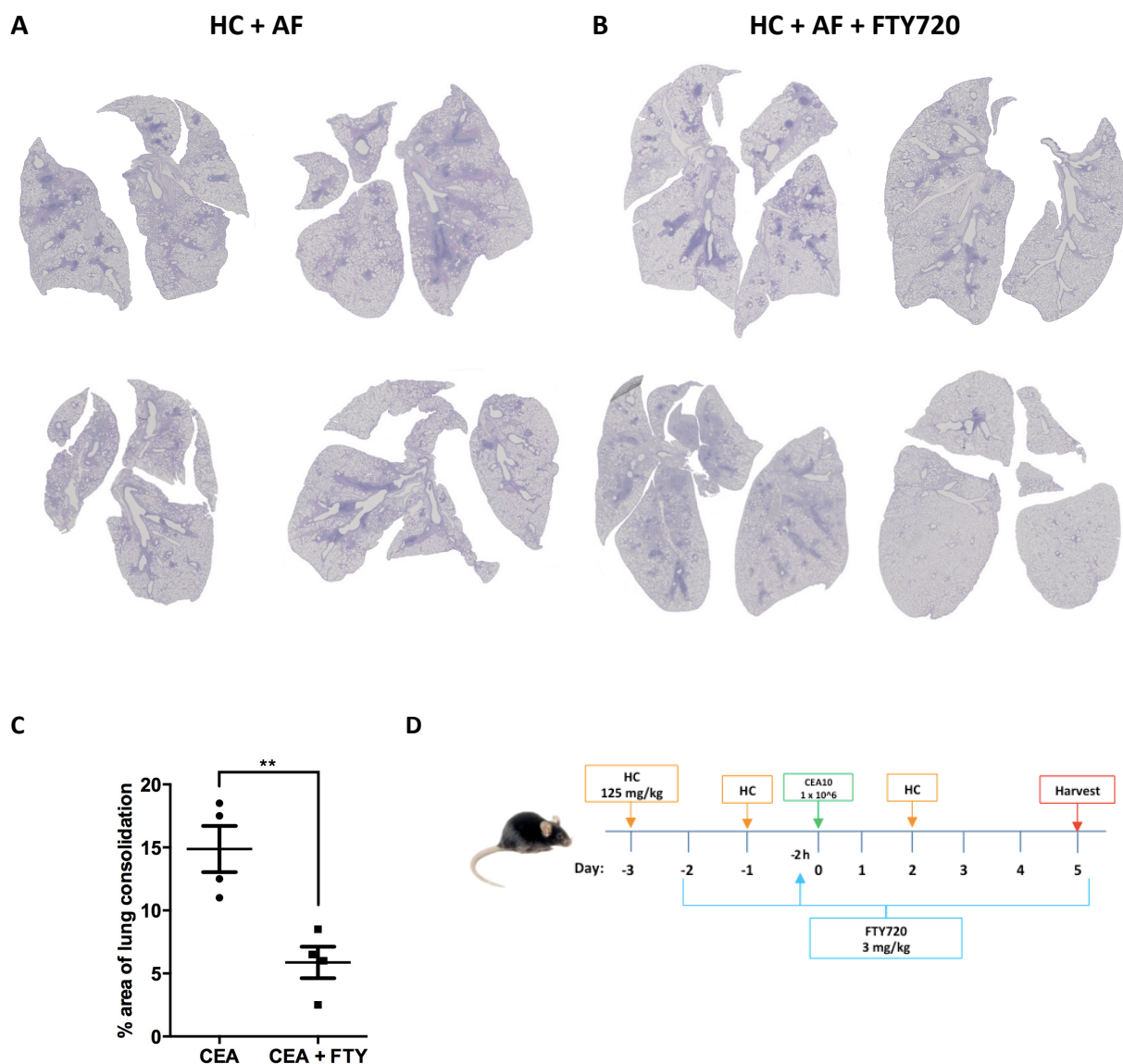


Figure 4.6: FTY720 reduces pulmonary inflammation at 5 days after inoculation. Mice were immune suppressed with 125 mg/kg hydrocortisone acetate at days -3 and -1, and treated daily with 3 mg/kg FTY720 i.p. B) or given saline vehicle i.p. A) daily from day 2. Inoculum of 1×10^6 conidia was suspended in 40 μ l saline and sham given saline only. Mice were anaesthetised with isoflurane inhalation prior to intranasal inoculation D). Lungs of mice were harvested 5 days after inoculation and fixed in a PBS buffered 10% formaldehyde for 24 h at RT. 3 μ m lung sections were stained with periodic acid-Schiff (PAS) stain by Imperial College histological laboratory. Images by widefield microscopy. Data are expressed as $\pm$ SEM (mice/n=4). Statistical significance was calculated by t-test (C: ** p=0.0068).

Histological sections (Fig 4.6 A-B) indicate a significantly lower level of lung consolidation in FTY720 treated mice (p=0.0068) compared to vehicle at 5 days after inoculation (Fig 4.6 C).

4.5 Discussion

The data in this study demonstrates that FTY720 attenuates innate pulmonary responses against *A. fumigatus* infection and is associated with a reduction in pulmonary inflammation and fungal burden.

The immune response of immunocompetent mice is characterized by an early moderate response to an *A. fumigatus* insult that is resolved by 48 h after inoculation. The features of this inflammatory response are an increased TNF- α at 24 h that returns to normal levels by 48 h and a rapid production of KC at 6 h that falls to almost normal levels by 24 h. These data are consistent with other studies demonstrating a rapid, moderate transient inflammation that results in fungal clearance (403). In this immune competent model FTY720 not only significantly reduced inflammatory cytokine TNF- α and the central chemokine KC, but did so not only without increasing fungal burden but appeared to result in marginally less CFUs albeit with no statistical significance. Therefore these data suggest that TNF- α and KC are somewhat dispensable in the immunocompetent mice for clearance and that FTY720 has utility in reducing fungal burden *in vivo*. Neutrophil percentage in pulmonary infiltrates was demonstrated to be unaffected between groups despite a reduction in KC, although this is not a measure of total number of cells. Macrophages were also shown to have increased percentage in infiltrating cells in FTY720 treated mice at the later time point of 72 h which has been associated with a successful immune response (403). Together these data suggests that FTY720 has utility in reducing TLR-mediated inflammation and chemokines that are responsible for the high level of pulmonary infiltrating cells.

Initial inflammatory responses by phagocytic cells to an *A. fumigatus* challenge are key in mounting an effective antifungal immune response. Initiation of an immune response by alveolar macrophages typically results in TNF- α and KC production. An *in vivo* study by De Filippo et al. (2008) in C57BL/6 mice showed that KC is one of the major chemoattractants produced by alveolar macrophages (262). In the HC model of aspergillosis, mortality is as a result of chronic inflammation (not fungal growth), therefore reducing inflammatory signals such as cytokines and especially chemokines is critical for attenuating disease progression. This study demonstrated that FTY720 reduces TNF- α , KC, and IL-6 in a HC model of aspergillosis. This inhibition in pro-inflammatory mediators, specifically the chemoattractant KC could be the cause of the significant reduction in pulmonary inflammation at day 5. Furthermore, this attenuation in inflammation is the likely cause of decreased mortality in the murine survival curves. Together these data suggest that FTY720 is able to dampen the *A. fumigatus*-

mediated pathological immune response that is associated with aberrant tissue damage during persistent fungal colonization in the HC model of aspergillosis.

In summary, in an immunocompetent model of infection, FTY720 demonstrated utility in reducing inflammatory cytokines in response to *A. fumigatus* with potential for assisting fungal clearance. In the HC model, FTY720 reduced inflammatory mediators associated with immunopathology, reduced fungal burden and pulmonary inflammation and increased murine survival. Therefore, FTY720 has been shown to attenuate the pathological characteristic of the HC model of aspergillosis.

5. An investigation into the antifungal effects of sphingolipid pathway inhibitors against *A. fumigatus*

5.1 Introduction

The rising prevalence of fungal infections has highlighted the need for more efficacious antifungal drugs. Fungal infections have increased significantly over the last few decades most notably due to prevalence of the immune deficient population, with an estimated 1.5 million people dying every year from invasive mycoses (469). To confound the issue, only a few classes of antifungal drugs are available and these agents display limited fungal killing capability *in vivo*. High toxicity of antifungals is attributed to biological and biochemical similarities between mammals and fungi. No new classes of antifungal drugs have become available since the licensing of echinocandins in 2002 (470) and in light of the rise of azole resistance (429), effective new antifungals are urgently required.

Sphingolipid pathways are highly conserved between fungi and mammalia (471), though despite a number of conserved steps in sphingolipid biosynthesis and degradation (Fig 5.1-2), there are defined differences in molecular structures. Many studies have shown that inhibition and deletion of complex sphingolipids have resulted in growth defects in fungi and their attenuated virulence *in vivo* (472). In this context, sphingolipid inhibition has emerged as a new potential area for antifungal drug development (473).

Many studies focus on disruption of novel branches and molecules of the fungal sphingolipid pathway containing inositol-phosphoryl ceramide (IPC) or glucosylceramide (GlcCer) (460,469,474,475). This focus on complex sphingolipids leaves a vacuum in the literature on other components of sphingolipid biosynthesis, such as sphingosine kinases, S1P and S1PRs. Also, most research has been performed in *S. cerevisiae* with few studies focused on pathogenic fungi.

SKI-II and FTY720 are known to inhibit sphingolipid biosynthesis via inhibition of sphingosine kinase enzymes (SphK1 and SphK2) and functional antagonism of S1P₁ respectively. These drugs are designed for, and validated with, mammalian cells and systems, and so it was therefore unclear if they would possess cross reactivity against their fungal counterparts.

Current antifungals typically have high toxicity to the human host, and there are also examples of sphingolipid inhibitors exhibiting toxicity against mammalian cells. For example Fumonisin B1, an inhibitor of fungal ceramide synthase has cytotoxicity in J774A.1 macrophages and is linked to cancer (476). On the other hand, Aureobasidin A, an inhibitor of fungal inositol phosphorylceramide

synthase (469), has low toxicity with cultured cells (477,478), but little activity against *A. fumigatus* (479). However, FTY720 is a sphingolipid receptor inhibitor (already licensed for the treatment of MS) that is demonstrated to have low toxicity in the human host (480) and is well tolerated (101,481–484). Furthermore, it is reported to be 10-100 times more potent gram for gram than the immune suppressant cyclosporine A (485), and this is reflected in its working dose of 0.5 mg/day but it has been shown that 5 mg/day is also well tolerated (483). FTY720 is based on the fungal metabolite myriocin. Myriocin is an active metabolite isolated from *Myriococcum albomyces*, *Isaria sinclairi*, and *Mycelia sterilia* (486), a traditional ingredient in Eastern medicine (485), and inhibits the initial step in sphingolipid biosynthesis (487). Myriocin has been shown to affect both mammalian cells (485) and the growth of *Aspergillus nidulans* (488).

It was hypothesized that SKI-II might have inhibiting effects on fungal sphingosine kinase due to a homologue being present in fungi and no investigations into this had been reported in the literature. Similarly, it was hypothesized that FTY720 may have activity because GPCRs have been identified in *A. fumigatus*, although much research is required to understand their structure, function and interactive molecules.

Therefore, it is possible for sphingolipid inhibitors to possess cross species reactivity (between fungi and mammalia), giving potential to mammalian sphingolipids inhibitors to have antifungal properties. Nonetheless, the same similarities that allow for cross species reactivity have presented with mammalian toxicity, or have only permitted low activity against *A. fumigatus*. However, both SKI-II and FTY720 have low toxicity at immunologically efficacious concentrations *in vitro* with murine macrophages, and in light of this they were tested for antifungal activity.

5.2 Anti-fungal activity of sphingolipid pathway inhibitors

SKI-II and FTY720 were investigated for antifungal activity using standard testing techniques; minimum inhibitory concentration (MIC) assays and radial growth on supplemented agar, as well as hyphal extension quantification by turbidity, germination by widefield microscopy and drug-drug interaction with clinically available antifungal agents.

5.2.1 EUCAST minimum inhibitory concentration testing

EUCAST MICs were performed to investigate the anti-fungal properties of SKI-II and FTY720 against *A. fumigatus* resting conidia.

Table 5.1: Minimal inhibitory concentration of FTY720 against *A. fumigatus* strains in accordance with new European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology

Strain(s)	MIC (mg/L)		MIC (μM)	
	GM	Range	GM	Range
CEA10	17	20, 20, 10	50	60, 60, 30
<i>Δku80</i>	17	20, 20, 10	50	60, 60, 30

MIC: minimum inhibitory concentration. GM: geometric mean.

To encompass a broad range of concentrations both drugs were initially titrated using a log₁₀ serial dilution, Table 5.1. SKI-II had no MIC and no observable effect on germination of resting conidia or subsequent hyphal extension and is therefore not listed in data table. FTY720 exhibited a clear MIC between 10-20 mg/L, with a geometric mean of 17 mg/L in both CEA10 and *Δku80* strains.

Table 5.2: Minimal inhibitory concentration of FTY720 against *A. fumigatus* strains in accordance with EUCAST methodology.

Strain(s)	MIC (mg/L)		MIC (μM)	
	GM	Range	GM	Range
CEA10	13	13, 15, 11	38	38, 44, 32
<i>Δku80</i>	13	14, 13, 12	38	41, 38, 35
ATCC466	12	13, 14, 10	36	38, 41, 29

Subsequent MICs were performed using a 1 mg/L step serial dilution to more precisely determine the MIC value for FTY720, Table 5.2. FTY720 exhibited an MIC between 10-15 mg/L, with a geometric mean of 12-13 mg/L across CEA10, *Δku80* and ATCC46645 strains.

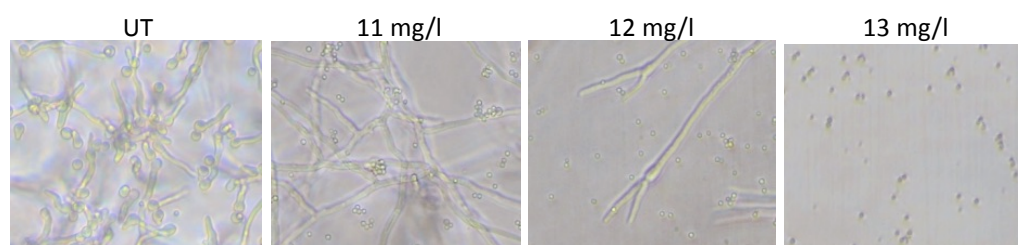


Figure 5.3: Microscopic *Aspergillus* morphology. FTY720 inhibits germination of *A. fumigatus* resting conidia at 13 mg/L in EUCAST MIC medium after 48 h incubation at 37°C. Magnification is x40 using Nikon Eclipse TS100 microscope camera. Representative images.

To determine fungal growth under experimental concentrations microscopic images were captured (Fig 5.3). These images confirm that the MIC of 13 mg/L FTY720 comprehensively inhibits germination of *A. fumigatus* conidia, whereas 11 and 12 mg/L show a gradual increase in germination and hyphal extension when compared to control.

5.2.2 Effects of SKI-II and FTY720 on radial growth

Arendrup *et al.*, (2010) reported that agar methods such as Etest have been more reproducible and with less errors than MIC microdilutions for echinocandins. The effects of SKI-II and FTY720 on fungal growth on solid media were therefore investigated further, to look for similarities and differences to MIC data. Colony size was determined using colony diameter.

Unexpectedly SKI-II and FTY720 had an affect on the surface tension of the water suspended inoculum, causing it to disperse differentially on drug-supplemented agar. Most notably, FTY720 caused the inoculum to disperse over a larger area than vehicle controls. Therefore, to correct for differential seeding areas, radial growth was adjusted to the discrete colony diameter apparent at 24 h after inoculation.

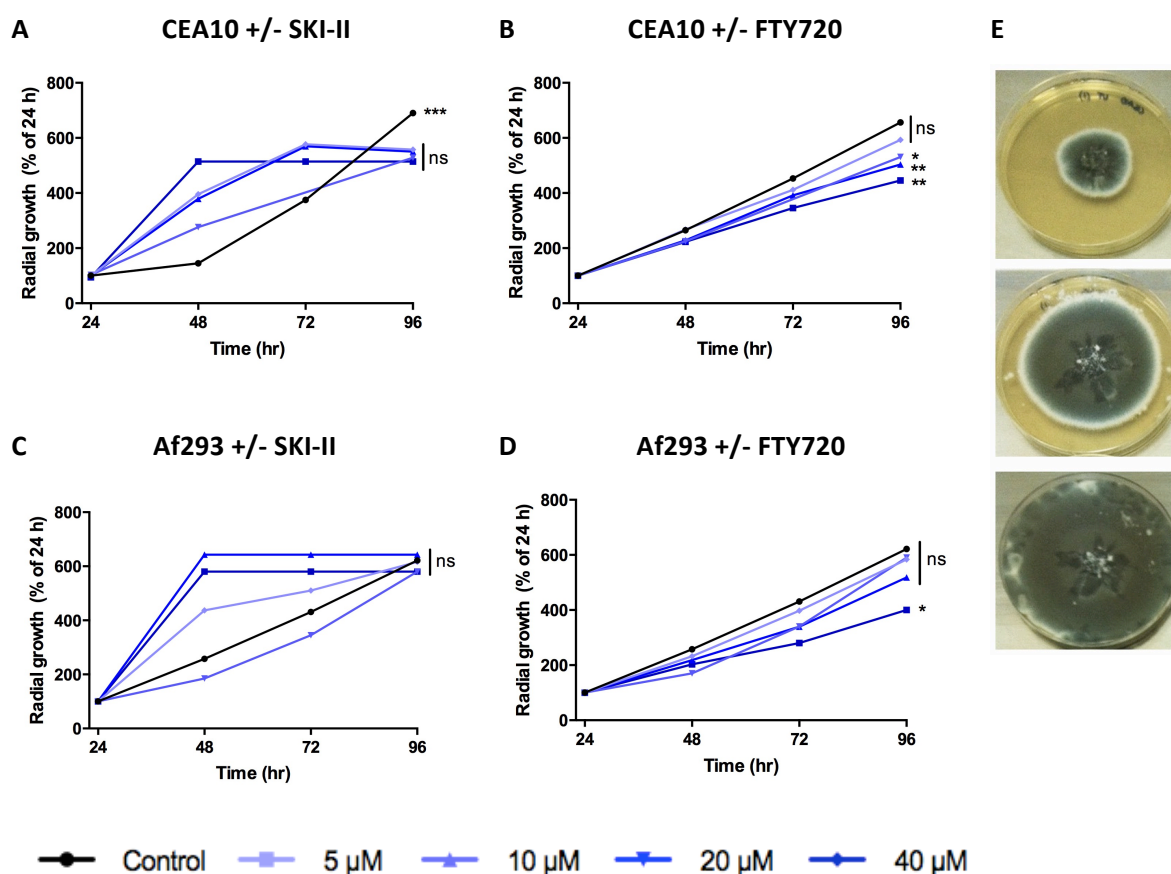


Figure 5.4: Effect of SKI-II or FTY720 on *A. fumigatus* radial growth on Sabouraud (SAB) agar. *A. fumigatus* resting conidia from strains CEA10 (A-B) and Af293 (C-D) were plated on 90 mm SAB agar plates containing increasing drug concentrations of SKI-II (A and C), FTY720 (B and D). Images of CEA10 at 48, 72 and 96 h (E). SAB medium was supplemented with 40 μ M (dark

blue), 20 μ M (blue), 10 μ M (light blue), 5 μ M (lightest blue) and no drug in control plates (black). SAB agar plates were inoculated with 1×10^3 resting conidia in 10 μ l of dH₂O, incubated at 37°C, radial filamentous growth was measured in mm daily using a ruler, and adjusted to percentage of 24 h colony size. Vehicle SAB contained the same concentration of DMSO as drug-supplemented agar. Data are representative of three independent experiments (n=3). Data are expressed as $\pm$ SEM. Statistical significance calculated by t-test and ANOVA (A: *** p=0.0002 and ns p=0.1211, B: * p=0.0429, ** p=0.0019, ***p=0.0037 and ns = 0.0879, C: ns p=0.9479, and D: * p=0.9816 and ns p=0.9784).

SKI-II initially elevated growth in almost all drug concentrations across the strains. Despite this initial promotion in hyphal extension, SKI-II treated *A. fumigatus* had significantly inhibited growth at 96 h compared to vehicle control in CEA10 but not Af293. FTY720 treated *A. fumigatus* was consistently inhibited throughout the time course in a dose dependent manner in both strains (Fig 5.4 B, D).

Radial growth under the influence of SKI-II and FTY720 was recapitulated in *Aspergillus* minimal medium (AMM) with the hypothesis that restricted nutrition could accentuate differences in growth inhibition. In addition, colony size was determined by area using ImageJ to increase accuracy, and measurements were adjusted to colony size at 48 h, when colonies exhibited clearly defined edges.

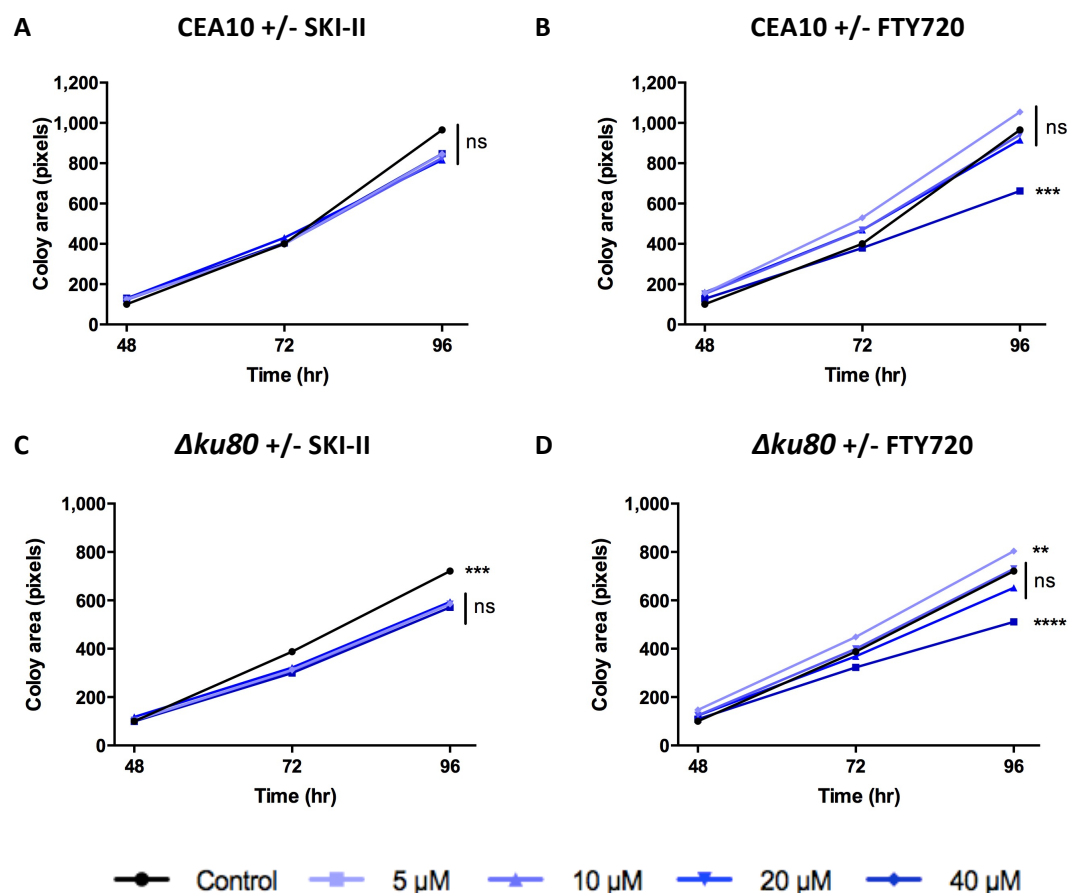


Figure 5.5: Effect of SKI-II or FTY720 on *A. fumigatus* radial growth on *Aspergillus* minimal medium (AMM) agar. *A. fumigatus* resting conidia from strains CEA10 (A-B) and $\Delta ku80$ (C-D) were plated on 90 mm AMM agar plates containing indicated drug concentrations of SKI-II (A and C), FTY720 (B and D). AMM agar plates were inoculated with 1×10^3 resting conidia in 5 μ l of dH₂O, incubated at 37°C, and radial filamentous growth was measured in mm daily using a ruler, and adjusted to percentage of 48 h colony size. Untreated SAB contained vehicle DMSO. Data are representative of three independent experiments (n=3). Data are expressed as $\pm$ SEM. Statistical significance calculated by t-test and ANOVA (A: ns p=0.2738, B: ns p=0.6653 and *** p=0.0002, C: *** p=0.0002 and ns p=0.6653, D: ** p=0.0059, ns p=0.0604 and *** p< 0.0001)

In contrast to data from SAB agar, SKI-II treated *A. fumigatus* exhibited on AMM consistent growth inhibition that was statistically significant in $\Delta ku80$ strain (Fig 5.5 A, C). FTY720 also showed an opposing pattern to that of SAB, in that it induced elevated growth 10 μ M (Fig 5.5 B, D), and permitted a differential growth response in FTY720 that surprisingly was not dose dependent. However both CEA10 and $\Delta ku80$ exhibited significant growth inhibition with 40 μ M, its approximate MIC value in EUCAST liquid media. These data show that AMM allowed for a more robust growth inhibition by SKI-II and a more significant reduction in growth with 40 μ M FTY720.

5.2.3 Effects of SKI-II and FTY720 on hyphal extension

A microbroth turbidity assay was developed by Meletiadis, Meis, and Mouton (2001) to examine filamentous fungal growth in liquid medium using 405 nm absorbance. Resting conidia from CEA10 and $\Delta ku80$ strains were inoculated in RPMI liquid medium supplemented with SKI-II or FTY720 from a 5 μ M (nontoxic cell culture concentration) to the approximate MIC of 40 μ M. Plates were incubated overnight at 37°C, shaken at 180 rpm, and 405 nm readings taken at 30 min intervals.

Due a slight variation in inoculums between replicates all data was adjusted to percentage of initial 405 nm absorbance reading for each condition at 0 h.

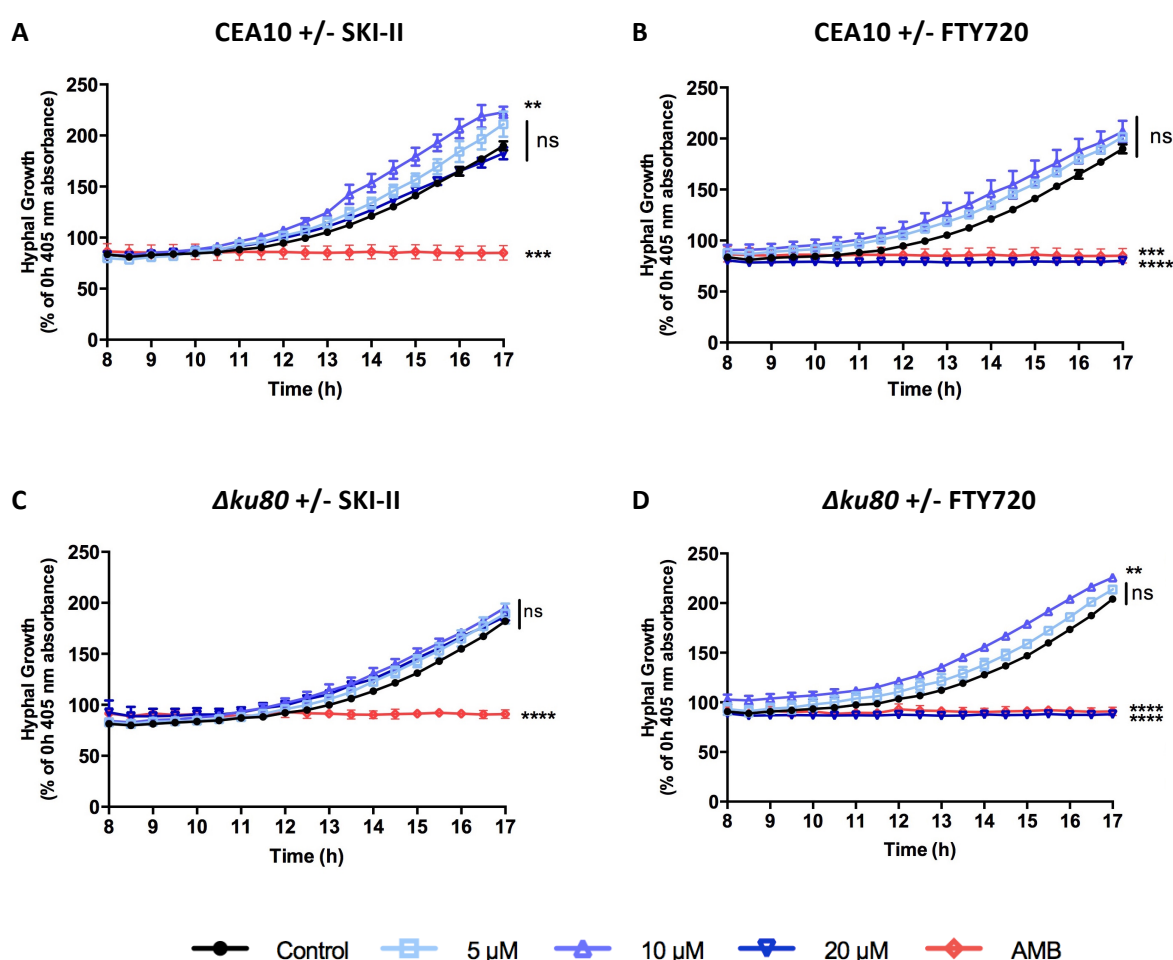


Figure 5.6: Effect of SKI-II or FTY720 on filamentous growth of *A. fumigatus* in RPMI liquid medium. *A. fumigatus* resting conidia from strains CEA10 (A-B) and $\Delta ku80$ (C-D) were added to 96-well plates containing indicated drug concentrations of 5 μ M to 40 μ M SKI-II (A and C), FTY720 (B and D) respectively. Microtiter plates were inoculated with 2.5×10^3 resting conidia per well, and incubated at 37°C overnight with 405 nm absorbance readings taken by spectrophotometer at 30 min intervals.

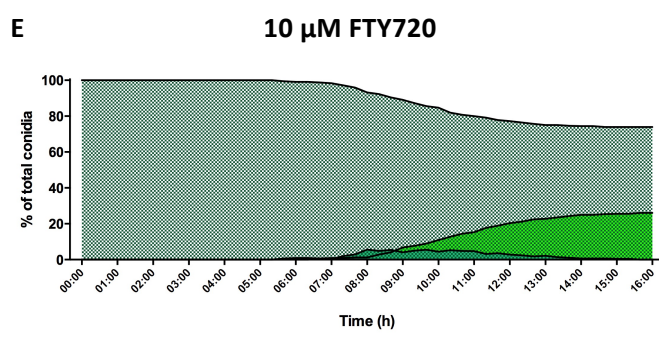
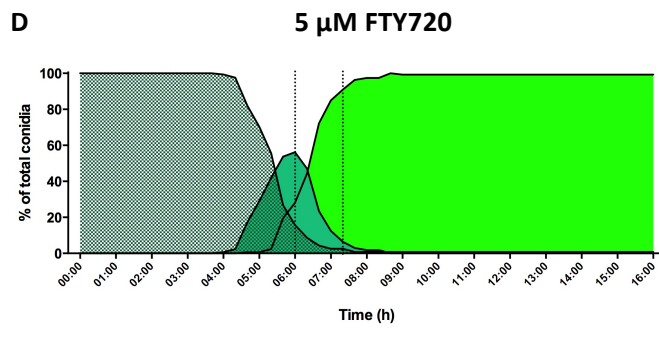
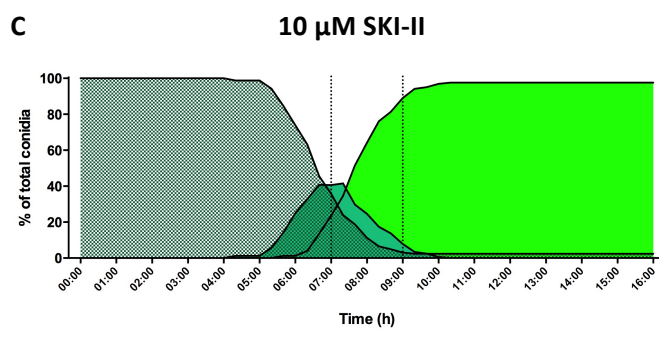
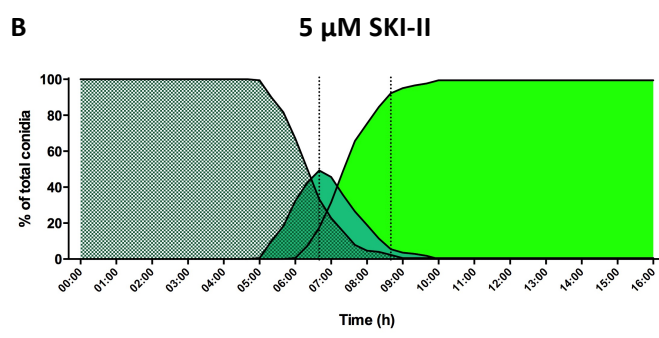
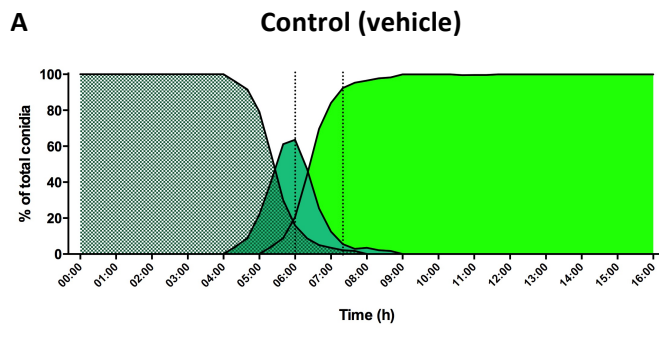
Data are representative of three independent experiments (n=3) with triplicate cultures. Data are expressed as $\pm$ SEM. Statistical significance calculated by t-test and ANOVA (A: ** p=0.0092, ns p=0.1127 and *** p=0.0002, B: ns p=0.2787, *** p=0.0002 and **** p=< 0.0001, C: ns p=0.4410 and **** p=<0.0001, D: ** p=0.0013, ns p=0.0873, **** p=<0.0001, **** p=<0.0001).

Absorbance values indicated that most drug concentrations resulted in a slight elevation of hyphal growth above vehicle, with 10 μ M FTY720 and 10 μ M SKI-II having statistical significance (Fig 5.6 A, D). Although, this elevated absorbance may have been caused by the drugs absorbance at 405 nm, which was not accounted for in this assay. Despite this, growth curves for 40 μ M FTY720 indicate a highly significant growth inhibition in both strains, equivalent to the growth inhibiting activity of 2 μ M AMB (a positive antifungal control). AMB has a known potent antifungal activity at 0.5-2.0 μ M, so it can be concluded that 40 μ M FTY720 has substantial antifungal activity at this concentration.

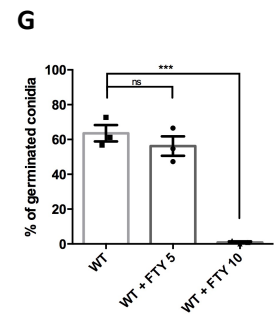
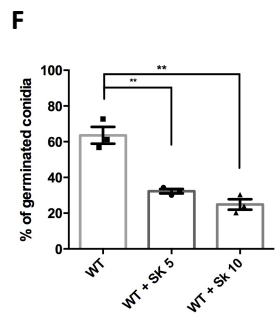
5.2.4 Effects of SKI-II and FTY720 on germination kinetics

The transition from resting conidia to hyphal filaments is an important developmental transition in *A. fumigatus* pathogenesis. Resting conidia are immunologically inert due to the presence of hydrophobic proteins and are easily cleared by macrophages, but swollen conidia with exposed fungal PAMPS induce a host inflammatory response. Once a germ tube has emerged it is increasingly difficult for phagocytosis by macrophages to occur. Additionally, swollen conidia can germinate from within cells and lyse them, at which point inflammatory mediators recruit neutrophils to the site of the infection. Thus, clearance at the earlier resting stage, before *A. fumigatus* can become fully metabolically active is preferential for killing.

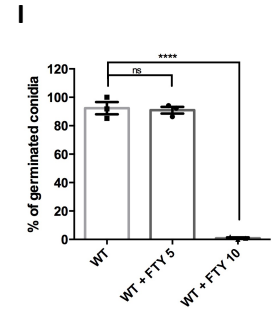
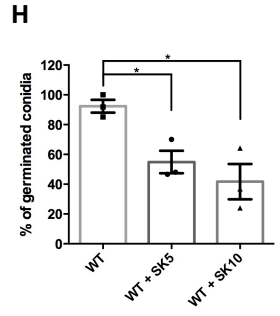
Microscopic analysis of germinating CEA10 conidia was performed using widefield microscopy. Liquid AMM was used as a medium as slower growth of *A. fumigatus* was observed in solid AMM, with the prospect therefore that the stages of germination would be more detectable. Images were captured at 20 min intervals and analysed for germination kinetics (Fig 5.7) as well as germinated morphotypes (Fig 5.9), and at the end of the assay 405 nm absorbance measurements were taken by spectrophotometer to determine final fungal biomass (Fig 5.10).



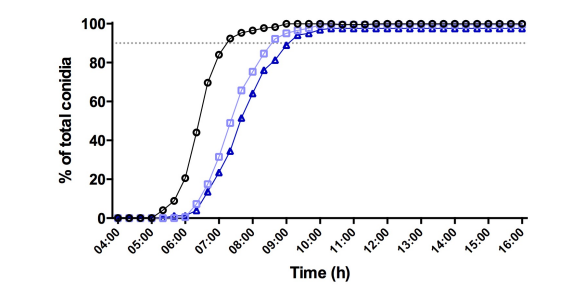
% of swollen conidia at 06:00



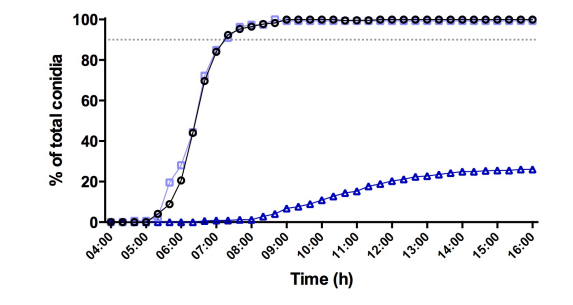
% of germinated conidia at 07:20



SKI-II (% of germinated conidia)



FTY720 (% of germinated conidia)



Legends:

- A-E RC SC Germlings
- J-K Control 5 μ M 10 μ M

Figure 5.7: Germination kinetics of SKI-II or FTY720 treated conidia. CEA10 strain *A. fumigatus* resting conidia were added to 96-well microtiter plate and images of germinating conidia were taken at 20 min intervals by widefield microscopy. Growth kinetics were assessed and categorized by swelling, germination and germ tube formation. Germination kinetics of in A) AMM (control) B) 5 μ M SKI-II, C) 10 μ M SKI-II, D) 5 μ M FTY720 and E) 10 μ M FTY720. Dark green, green and light green represent resting, swollen and germinated conidial populations respectively. Percentage of swollen conidia at 06:00 for when untreated conidia are $\geq 90\%$ germination for F) SKI-II and G) FTY720, and % of germinated conidia at 07:20 for H) SKI-II and I) FTY720. Comparison of germination rates for J) SKI-II and K) FTY720 (where dotted line $\geq 90\%$ germinated). Data are representative of two independent experiments (n=2) with triplicate cultures. Data are expressed as $\pm$ SEM (150 conidia observed in total per condition). Statistical analysis by t-test (F: ** p=0.0031 and ** p=0.0022, G: ns p=0.3711 and *** p=0.0002, H: * p=0.0126 and * p=0.0161, and I: ns p=0.7895 and **** p=<0.0001)

Both swelling of conidia and rate of germination were quantified, as these are the most fundamental stages of germination. SKI-II delayed the onset of swelling by approximately 1 hour in both 5 and 10 μ M from 04:00 to 06:00, therefore when compared with vehicle at the peak of swollen conidia (SC), both drug treatments had significantly less (Fig 5.7 F). The transition from a resting state to germination was delayed by SKI-II in a dose dependent manner, conidia reaching $\geq 90\%$ at 08:40 and 09:00 for SKI-II 5 μ M and 10 μ M respectively, compared to vehicle at 07:20 (Fig 5.7 A-C, H).

Conidia treated with 5 μ M FTY720 exhibited no differences in swelling (Fig 5.7 D, G) or germination rate (Fig D, I) compared to vehicle. Most surprisingly, 10 μ M FTY720 resulted in very significant differences in both swelling and germination rate (Fig 5.7 E, G, I). The onset of swelling was delayed by 3 h (from 04:00 in vehicle to 07:00), attainment of $\geq 90\%$ germination was not achieved, and those conidia that did swell did so slowly with only 26% producing hyphae (Fig 5.7 I, K).

5.2.5 Effects of SKI-II and FTY720 on morphology of germinated conidia

During analysis of germination kinetics it was observed that SKI-II and FTY720 induced distinct changes in the occurrence of germinated morphotypes. Morphotypes in this assay are designated as unipolar, dipolar and tripolar+ for germings that have one, two and three or more hyphal filaments extending from the conidium (Fig 5.8).

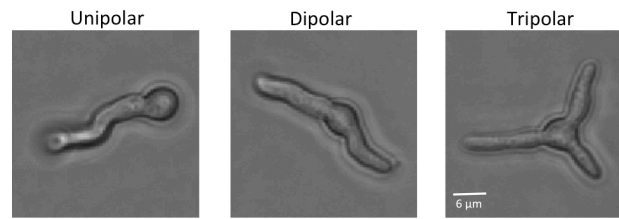


Figure 5.8: Morphotypes of germinated conidia. Categorization of germinated conidial morphotypes (unipolar, dipolar and tripolar+ germings)

It was hypothesised that these germling morphotypes could correlate to differences in pathogenesis, as more filaments could pose a greater challenge to phagocytosis but it was uncertain if one or many hyphal branches would render advantage as resources could be more concentrated or divided.

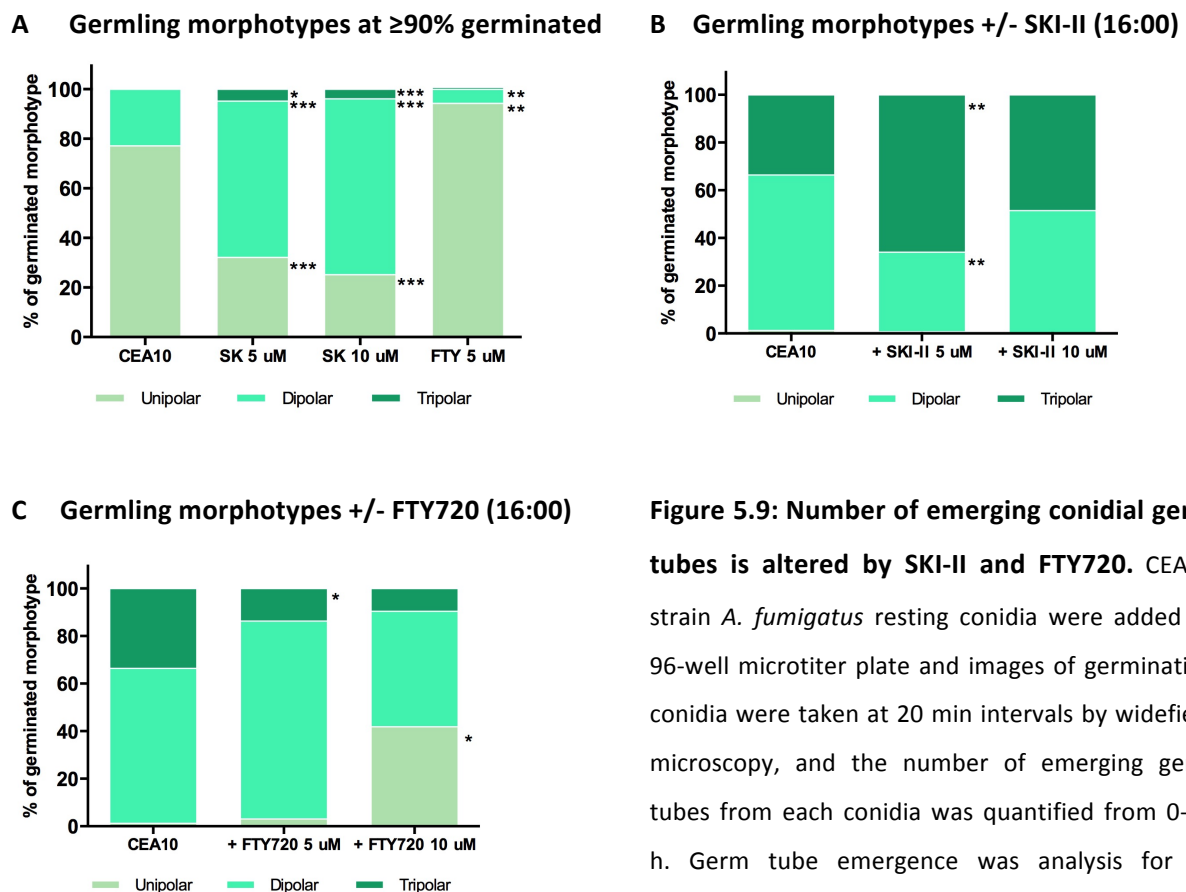


Figure 5.9: Number of emerging conidial germ tubes is altered by SKI-II and FTY720. CEA10 strain *A. fumigatus* resting conidia were added to 96-well microtiter plate and images of germinating conidia were taken at 20 min intervals by widefield microscopy, and the number of emerging germ tubes from each conidia was quantified from 0-16 h. Germ tube emergence was analysis for A) percentage of germling morphotypes at ≥90% germinated conidia for all conditions (UT 07:20, 5

µM SKI-II 08:40, 10 µM SKI-II 09:00 and 5 µM FTY720 07:20) and germinated morphotypes at 16 h for C) SKI-II and D) FTY720. Data are representative of two independent experiments (n=2) with triplicate cultures and 150 conidia analysed per strain. Data are expressed as ± SEM (150 conidia observed in total per condition). Statistical analysis by t-test (A: 5 µM SKI-II * p=0.0456, *** 0.0002, *** p=0.0003; 10 µM

SKI-II *** $p=0.0002$, *** $p=0.0003$, *** $p=0.0003$; 5 μ M FTY720 ** $p=0.0097$ ** $p=0.0097$, B: ** $p=0.0037$ and ** $p=0.0030$, and C: * $p=0.0104$ and $p=0.0105$).

Both SKI-II and FTY720 delayed or inhibited the onset of germination (Fig 5.6 H, I), and further to this it was hypothesised that sphingolipid inhibition may cause morphological differences of those germinated conidia. Therefore, to compare the morphology of germinated conidia between drugs and concentrations, the number of emerging germ tubes were quantified at the time of exponential growth ($\geq 90\%$ germination). Exponential growth in 5 μ M FTY720 and 5 and 10 μ M SKI-II occurred at 07:20, 08:40 and 07:20 respectively (Fig 5.7 B-D). However, conidia incubated with 10 μ M FTY720 underwent sufficient growth inhibition as to not allow for a clear exponential phase within the time frame of this assay (Fig 5.7 E) and as such were not included in Fig 5.9 A.

At $\geq 90\%$ germination, both 5 and 10 μ M SKI-II had mostly dipolar germlings with some unipolar and a small population of tripolarity. FTY720 at 5 μ M was dominated by unipolar germlings with a small population of dipolarity.

However, at 16 h SKI-II treated conidia had significantly more tripolar+ conidia compared to vehicle correlating with its earlier emergence of tripolarity (Fig 5.9 B), and FTY720 had decreasing polarity that correlated with drug concentration (Fig 5.9 C).

5.2.6 SKI-II and FTY720 reduce mycelial biomass

At the end of the germination kinetic assay at 16 h, the optical density of fungal cultures was determined by 405 nm absorbance to determine final fungal biomass for each condition (Fig 5.10).

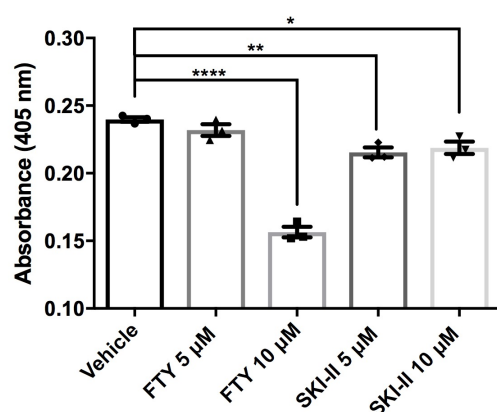


Figure 5.10: Effect of SKI-II or FTY720 on mycelial biomass of parental versus mutant strains. Optical density of cultures was measured by 405 nm absorbance by spectrophotometer to quantify fungal biomass. Biomass is compared in presence of FTY720 and SKI-II for *A. fumigatus* CEA10 strain. Data are representative of three independent experiments (n=3) with triplicate cultures. Data are expressed as $\pm$ SEM. Statistical analysis by t-test (**** $p < 0.0001$, ** $p = 0.0037$ and * $p = 0.0123$).

Fungal biomass was significantly reduced in 5 and 10 μ M SKI-II and although 5 μ M FTY720 exhibited no growth inhibition, 10 μ M had a significant impact.

5.3 Investigating the susceptibility of *A. fumigatus* to antifungal drug combinations

Antimycotics are routinely administered to animals and humans both prophylactically and therapeutically against fungal infection. MICRONAUT-AM MHK 2 (Merlin) are commercially available microtiter plates containing nine lyophilised antimycotics in up to eleven concentrations for MIC testing. MICRONAUT-AM MHK 2 plates were utilized to determine drug activity between SKI-II or FTY720 with currently available antimycotic agents.

Conidial suspensions consistent with the standard EUCAST MIC inoculations were added in provided RPMI based medium with or without SKI-II or FTY720 at concentrations of 2.5 μ M or 5 μ M (in parallel with cell culture assays).

5.3.1 Combination testing of antifungal agents and sphingolipid pathway inhibitors

Firstly SKI-II and FTY720 were tested for their independent MIC values in the supplied MICRONAUT assay medium.

Table 5.3: Minimal inhibitory concentration of FTY720 on *A. fumigatus* strains CEA10 and $\Delta ku80$

Strain(s)	MIC (mg/L)		MIC (μ M)	
	GM	Range	GM	Range
CEA10	7	10, 5, 5	20	29, 15, 15
$\Delta ku80$	7	10, 5, 5	20	29, 15, 15

MIC: minimum inhibitory concentration. GM: geometric mean.

FTY720 had an average MIC value of 20 μ M (7 mg/L) in MICRONAUT medium and SKI-II had no MIC value in accordance with previous data.

Further to this MICRONAUT plates were inoculated with conidial suspensions in combination with 2.5 and 5 μ M SKI-II and FTY720.

Table 5.4: Minimal inhibitory concentration of Amphotericin B with FTY720 on *A. fumigatus* strains CEA10 and $\Delta ku80$

Drug(s)	MIC (mg/L)			
	CEA10		$\Delta ku80$	
	GM	Range	GM	Range
Amphotericin B	2	2, 2, 2, 2, 2	2	2, 0.5, 2, 2
Amphotericin B + 2.5 μ M FTY720	1.63	2, 0.5, 2, 2	1.5	2, 0.5, 2
Amphotericin B + 5 μ M FTY720	1.2	2, 0.5, 1, 2	1.125	1, 0.5, 1, 2

Table 5.5: Minimal Inhibitory Concentration of itraconazole with FTY720 on *A. fumigatus* strains CEA10 and $\Delta ku80$

Drug(s)	MIC (mg/L)			
	CEA10		$\Delta ku80$	
	GM	Range	GM	Range
Itraconazole	3.5	4, 2, 4, 4	1.8	2, 2, 2, 2, 1
Itraconazole + 2.5 μ M FTY720	1	1, 1, 1	1	1, 1, 1
Itraconazole + 5 μ M FTY720	1.05	0.25, 1, 1, 2	1	0.5, 1, 1, 1

Table 5.6: Minimal Inhibitory Concentration of voriconazole with FTY720 on *A. fumigatus* strains CEA10 and $\Delta ku80$

Drug(s)	MIC (mg/L)			
	CEA10		$\Delta ku80$	
	GM	Range	GM	Range
Voriconazole	0.38	0.5, 0.25, 0.5, 0.25, 0.5	0.35	0.5, 0.5, 0.25, 0.25
Voriconazole + 2.5 μ M FTY720	0.38	0.25, 0.25, 0.25, 0.5	0.417	0.5, 0.5, 0.25
Voriconazole + 5 μ M FTY720	0.23	0.125, 0.25, 0.25, 0.25	0.425	0.025, 0.5, 0.125, 0.25

Table 5.7: Minimal Inhibitory Concentration of posaconazole with FTY720 on *A. fumigatus* strains CEA10 and $\Delta ku80$

Drug(s)	MIC (mg/L)			
	CEA10		$\Delta ku80$	
	GM	Range	GM	Range
Posaconazole	0.175	0.125, 0.25, 0.25, 0.125, 0.125	0.15	0.125, 0.25, 0.125, 0.125
Posaconazole + 2.5 μ M FTY720	0.219	0.125, 0.5, 0.125, 0.125	0.167	0.125, 0.25, 0.125
Posaconazole + 5 μ M FTY720	0.131	0.031, 0.0125, 0.0125, 0.25, 0.125	0.109	0.0125, 0.0125, 0.062, 0.125

Previous data showed that SKI-II had no MIC, and similarly caused no differences in MIC values in combination with antifungal agents with significance (Appendix 9.1).

FTY720 had no interaction with voriconazole and posaconazole at concentrations tested (Table 5.6-7) but did have effects on the MICs of AMB and Itraconazole. However the MIC of AMB was reduced from 2 mg/L to 1.63 mg/L and 1.2 mg/L, with 2.5 μ M and 5 μ M FTY720 respectively (Table 5.4). Itraconazole showed a reduction in MIC from 3.5 mg/L to 1 mg/L and 1.05 mg/L, with 2.5 μ M and 5 μ M FTY720 respectively (Table 5.5) (Appendix 9.1).

5.3.2 Analysis of drug interactions by fractional inhibitory concentration analysis

To assess the *in vitro* pharmacodynamic interaction of FTY720 and AMB against CEA10 and $\Delta ku80$ strains, microdilution broth checkerboards were performed.

Both AMB and FTY720 were titrated across a 96-well plate which was inoculated with an identical inoculum to EUCAST MIC and MICRONAUT assays to investigate the potential for synergy, additive and antagonistic effects.

Data were analysed to determine the fractional inhibitory concentration (FIC) index or Σ FIC, calculated using the sum of the FICs for each drug tested. The FIC is determined for each drug by dividing the value of the drugs in combination in all the wells corresponding to a MIC, with the MIC of drugs alone (491). This model is based on the assumption that drugs alone cannot self-interact and can only be additive resulting in a FIC index of 1. Cut off values of 0.5 and 2 have been suggested for confirmation of synergism and antagonism respectively (492).

Table 5.8: Fractional inhibitory concentration of FTY720 and AMB in combination

<i>Aspergillus</i>		Σ FIC	
strain	Mean	Median	Range
CEA10	0.91	0.99	0.66, 0.99, 1.10
$\Delta ku80$	0.98	0.98	0.79, 0.98, 1.18

Data analysis revealed a mean Σ FIC of 0.91 and 0.98 for CEA10 and $\Delta ku80$ strains respectively, indicating that the interaction of FTY720 with AMB is additive (Table 5.8).

Although RPMI has been widely proposed as a standard medium by NCCLS for antifungal susceptibility testing and for EUCAST (supplemented with 2% glucose), it has been reported that RPMI can produce differential fractional inhibitory concentration data from checkerboards. For instance, the interactive activity of a fluoroquinolone with AMB against *A. fumigatus* was reported as antagonistic in RPMI but synergistic in yeast nitrogen based medium (493). A reason for this was suggested by Meletiadiis *et al.*, (2001), who inferred that the faster growth rates in alternative media to RPMI might allow for better intracellular drug penetration and concentrations at the site of its

activity thus increasing its affect. Therefore, synergy between FTY720 and AMB could be possible in alternative media.

5.2 Discussion

This study utilized various antifungal susceptibility assays including standard MICs and lab developed techniques for detection of subtle effects such as germination kinetics. Differences in antifungal properties were observed in SKI-II and FTY720 inhibitors, which are assumed to target sphingolipid biosynthesis and downstream signaling respectively, according to their targets in mammalian cells. FTY720 was found to have significant antifungal activity while SKI-II surprisingly had few detectable effects.

SKI-II had no MIC and as such was shown to not prevent conidial germination but interestingly did have effects on hyphal extension on solid medium. An unanticipated finding was that on SAB agar supplemented with SKI-II, radial growth was above that of vehicle control in almost all concentrations between 24-72 h. Possible explanations for this might be that between 24-72 h *A. fumigatus* may undergo either a stress response, a build up of growth stimulating sphingolipid precursors and or switching to alternative parts of the sphingolipid pathway. These potential responses could be restricted to threshold concentrations, which would account for the differential dose response. In contrast to this, in nutrient restrictive media (AMM) SKI-II resulted in a clustering of data points where all inhibition was alike irrespective of drug concentration. This could indicate a threshold growth retardation that corresponds with the level of fungal sphingosine kinases involvement. This hypothesis could account for the discreet and statistically significant growth reduction seen in CEA10 and $\Delta ku80$ respectively.

In addition, the substrate dependent differential responses exhibited by *A. fumigatus* to SKI-II could be explained by different medium formulas. SAB is rich in nutrients and glucose (4%) and could support changes in nutritional requirements caused by chemical perturbations thereby 'masking' their effects, whereas a restricted medium like AMM has limited resources to accommodate deviations from atypical growth requirements and as such can potentially exaggerate affects of such perturbations.

The MIC of FTY720 was 38 μM for both CEA10 and $\Delta ku80$ strains, demonstrating its ability to inhibit processes essential for germination. Consistent with this germination impairment, *A. fumigatus* grown on FTY720 supplemented SAB agar, exhibited a dose dependent growth retardation. This confirmed that down stream sphingolipid signaling is also important for hyphal growth and not just germination. However, despite maintaining a dose response pattern of inhibition on supplemented AMM agar, low dose FTY720 (5 μM) resulted in growth above that of untreated *A. fumigatus*. An

explanation for this increased growth could be that low doses, which have no detectable antifungal capabilities, trigger a stress response that results in increased growth. Whereas, increasing drug concentration counteracts potential 'stress-response induced growth', as concentrations above 5 μM have been demonstrated to have antifungal effects.

Also, it is interesting that while FTY720 significantly reduced growth on agar at 40 μM , it did not completely inhibit germination or hyphal extension, despite being above its liquid media MIC (38 μM). This reduction in sensitivity to FTY720 on solid media maybe due to a lack of drug exposure due its binding with elements in the agar. This is especially true in SAB where the agar grade is low (and contains impurities). It could also be due to a reduction in surface area exposure to the drug, which is highest in liquid medium.

In the lung pulmonary fluid covers the surface of the alveolar membrane. Antifungal assays reproduce elements of this environment by growing on a substrate (agar) and being in contact with liquid (liquid assay). It is important therefore to consider both assays when considering FTY720 is to its potential efficacy *in vivo*. Taken together, agar and liquid assay data suggest that FTY720 can inhibit the germination of conidia and slow hyphal extension, and could therefore aid immune cells in fungal clearance.

With regards to hyphal growth in liquid media, when *A. fumigatus* was grown in RPMI 1640 liquid medium containing 0.2% glucose (Fig 5.6), growth was completely inhibited with 20 μM (6.8 mg/L) FTY720, almost half of its 38 μM (13 mg/L) MIC value. This increase in susceptibility could be attributed to the differences between liquid medias of the EUCAST MIC assay and RPMI 1640 (a reduction in glucose or the presence of sodium bicarbonate). However, the presence of a buffer would more likely not effect growth in stabilizing pH and therefore the increased susceptibility to FTY720 is most likely due to a reduction in glucose, readily metabolised available carbon/energy source.

These data reconfirm that down-stream sphingolipid signalling is important for both germination and growth and that glucose availability could significantly impact on susceptibility to sphingolipid pathway inhibition. This result may be explained by the fact that synthesis of fatty acids like palmitoyl-CoA are derived from glucose (494). Palmitoyl-CoA is an essential molecule in the first step of *de novo* sphingolipid biosynthesis (Fig 5.1), therefore, reduced glucose availability would result in

a reduction in sphingolipid biosynthesis, and consequently their ability to fulfil their dynamic functional roles (e.g. membrane stability and protein transport) would be diminished.

Further to MICs the more subtle effects of low dose SKI-II and FTY720 on the germination kinetics of *A. fumigatus* were investigated in supplemented AMM liquid medium. SKI-II pushed back conidial swelling by 40 min and 1 h, and $\geq 90\%$ germination by 1 h 20 min and 1 h 40 min, for 5 μM and 10 μM respectively. Therefore, although SKI-II caused a delay in (the peak of) swollen conidia that resulted in a corresponding delay in conidial germination, once underway the rate of germination was not significantly different to that of vehicle conidia, suggesting that SphKs are involved in conidial swelling but not germination. FTY720 at 5 μM had almost identical kinetics to that of vehicle treated conidia, but in stark contrast 10 μM FTY720 significantly inhibited the swelling and germination processes. This dramatic onset of inhibition could be explained by a threshold value for FTY720, where FTY720 or FTY720-p concentration is above that of its competing fungal counterpart, thereby increasing its binding to and saturation of its target molecule.

In summary, SKI-II delayed the onset of swelling by approximately 1 hour and conidia took longer to transition through the swelling phase to germination, suggesting that sphingolipids could be involved in the environmental sensing prior to swelling or the process of expansion during isotropic swelling. In contrast, 10 μM FTY720 did not permit widespread germination and resulted in low levels of swollen conidia and germling polarity. This is important because phagocytic activity in the lung by macrophages and other phagocytic cells is a key effector function in clearance of *A. fumigatus*. Once germinated the conidia become far more difficult to phagocytose and kill, and require neutrophil involvement. Therefore, the period of time taken to transition from resting conidia to germling is critical in removing *A. fumigatus* conidia. If this time can be extended, in the way that SKI-II induced, it could potentially allow for more phagocytic activity, and in the case of 10 μM FTY720 where germination was significantly inhibited there is clear advantage for the host.

With relation to multi-polarity, increased hyphal tip branching has been demonstrated by Cheng *et al.*, (2001) to be an indicator of growth inhibition. Conidia in this assay were too close together to accurately determine hyphal branching over the time course but it was noticed that there were differences in the number of hyphal extensions coming from germinated conidia. To investigate this further, germinated conidial uni- and multi-polar morphotypes were quantified and compared.

It was hypothesized in this study, that if conidia develop with multi polarity points this may result in slower growth than that of unipolar forms for which all activity is focused on a single hyphae. In

addition, multiple polarity could limit the physical radial spread of fungal mass making it more dense and restricting it to a smaller area. SKI-II treated conidia exhibit more multi-polarity at the end of exponential growth and at 16 h, which is consistent with this hypothesis and the reduction in radial growth seen in agar. FTY720 on the other hand, had significantly less multi-polarity, which could be explained by its inhibitory properties in relation to germination and hyphal extension.

Therefore, it could be concluded that multi-polarity is a partially sphingolipid-dependent process.

In addition, SKI-II had a slight but significant reduction in fungal biomass, which corresponds with the delayed onset of germination and an increase in tripolarity compared to vehicle. This could be because tripolar germlings have shorter hyphae that give rise to more discrete and denser clumps of mycelia that result in an overall lower turbidity in the assay. The fungal biomass in 5 μ M FTY720 was not significantly different from vehicle treated conidia that corresponding to the lack of differences in germination kinetics. However, 5 μ M FTY720 had more dipolar and less tripolar+ germlings than vehicle suggesting a growth defect but not significantly increased turbidity readings per the above hypothesis, but this could be explained by the 405 readings approaching saturation. Lastly, as expected, 10 μ M FTY720 had a significantly lower biomass than vehicle in accordance with its germination dysregulation and low number of multipolar germlings.

The MICRONAUT multimycotic agent assay revealed a lower MIC for FTY720 than EUCAST methodology from 38 μ M (13 mg/L) to 20 μ M (7 mg/L). An explanation for this reduction in MIC could be due to differences in formulation. However, the MICRONAUT supplied assay medium contains RPMI with 2% glucose and L-glutamine, without added bicarbonate, which is identical to EUCAST medium and a personal correspondence from Merlin confirmed that the medium follows current EUCAST guidelines. Therefore if the medium can be considered equitable, then the methodology of having 100 μ l final volume in combination with a plate sealer (preventing gaseous exchange), in the MICRONAUT assay could explain the disparity between these data. This hypothesis is consistent with Warn *et al.*, (2004) who reported that a 2-4 fold reduction in *in vitro* MIC of AMB can occur in hypoxic conditions. The MIC of AMB in this assay was 2 mg/L, which is within the accepted susceptibility range, but was as high as 4 mg/L in EUCAST medium during some replicates (data not shown). Therefore, the potential for hypoxia could explain the increased sensitivity (of the same number of conidia) to FTY720 in this reduced assay medium volume in the absence of gaseous exchange.

With regards to the combination of FTY720 with antifungal agents, the data from the MICRONAUT assay revealed AMB and itraconazole to have a significantly reduced MIC value when combined with 2.5 μ M and 5 μ M FTY720. Consequently, FTY720 was titrated in a checkerboard with AMB and Σ FIC was determined. The Σ FIC values were 0.91 and 0.98 for CEA10 and $\Delta ku80$ respectively, suggesting the activity of FTY720 with AMB is additive, but as the value is less than one it could still imply that there is potential for synergistic activity. This is also because the mode of action of AMB is to destabilize the plasma membrane and sphingolipids are known to provide membrane stability. Thus, further research would include investigation into synergy between membrane perturbing agents (such as FTY720 and other agents) and investigation into the synergistic potential of itraconazole as per Tables 5.3, 5.5, and Appendix 9.1.

To summarize the findings of this chapter, the antifungal properties of SKI-II and FTY720 were investigated due to the potential for cross reactivity of these compounds with fungal cells. FTY720 showed consistent antifungal activity by preventing germination, reducing radial growth and hyphal mass, as well as having an additive effect with AMB against resting conidia. SKI-II had no effect on germination but did reduce radial growth on solid media and fungal biomass insignificantly in AMM. Therefore, the present study raises the possibility that inhibition of sphingolipid biosynthesis and down stream signalling could be effective antifungal drug strategies, with greater potential in the latter. In addition, these data also suggest that SKI-II and FTY720 could assist in clearance of conidia by delaying the onset of germination, providing support to innate immune cells and thereby attenuating the progression of aspergillosis infection.

6. *A. fumigatus* sphingolipid pathway mutants and phenotyping

6.1 Introduction

Fungal sphingolipids are known to play important roles in a variety of highly conserved cellular processes such as cell division in *S. cerevisiae* and apoptosis in *A. nidulans* (496,497). Virulence determinants such as pH tolerance and heat stress response have also been demonstrated to affect the growth of *C. neoformans* and *S. cerevisiae* (498,499). In addition, sphingolipids have been demonstrated to be involved in subcellular domain formation (such as lipid rafts), hyphal formation in *C. albicans*, and conidial germination in *Aspergillus spp.* (474,500,501). Although some sphingolipid-dependent processes in fungi have been demonstrated, the functions of the sphingolipid pathways in pathogenic fungi such as *A. fumigatus* are yet to be fully elucidated, especially in terms of virulence.

It is known that some areas of sphingolipid metabolism are conserved across species, with mammalia and fungi sharing similar pathways and sphingolipidomes (Fig 5.1-2). Hence, both sphingosine kinases and S1PRs contain conserved regions and notable amino acid residues related to active sites. To identify and analyse cross species homology the amino acid sequences were aligned and scrutinized.

A. fumigatus uptake of exogenous DNA occurs with a low efficiency relative to other fungal species. This, coupled with high rates of non-homologous recombination have slowed progress in genetic manipulations of this species. Recently isolates lacking one component of the ku70-ku80 dimer have been demonstrated as efficient vehicles for genetic manipulation of *A. fumigatus*. For this reason an *A. fumigatus* $\DeltaakuB^{KU80}$ isolate (444) was used as the progenitor of gene replacement mutants. DNA repair in *A. fumigatus* is mediated in part by the ku70-ku80 heterodimer, a DNA-dependent protein kinase catalytic subunit (502). The $\DeltaakuB^{KU80}\Delta ku80$ strain is constructed in a CEA10 genetic background and due to inactivation of the $\DeltaakuB^{KU80}$ gene exhibits a significantly increased rate of homologous recombination in preference to non-homologous end joining. This allows for a much higher efficiency of direct gene replacement. It has been demonstrated that $\DeltaakuB^{KU80}$ gene deletion has no measurable affect on *Aspergillus* infection progression in a low dose mouse model of invasive aspergillosis (444).

In this study, two *A. fumigatus* mutants, $\Delta SphK$ and $\Delta gprD$, were constructed in the *A. fumigatus* $\DeltaakuB^{KU80}$ genetic background and their phenotypes, virulence and immunogenicity were characterized.

6.2 Methods

6.2.1 *Aspergillus fumigatus* culture and strains

Strains were cultured on Sabouraud agar (Oxoid) or *Aspergillus* minimal medium (AMM) in 90 mm plates or in T25cm² flasks at 37°C for 3-5 days. Conidia were harvested by agitation with a sterile spreader on plates or manual shaking in flasks in sterile 0.1% Tween20 in dH₂O. Suspensions were filtered to isolate conidia from hyphal fragments and conidiophores using sterile Miracloth (Calbiochem). Conidial suspensions were centrifuged for 5 mins at 3500 rpm and washed twice with sterile dH₂O prior to enumeration by haemocytometer. Conidial counts were adjusted as per experimental requirements in dH₂O or assay medium. All strains used in this study are listed in Table 6.1.

Table 6.1: *A. fumigatus* strains used in this study

Genotype	Abbreviation	Reference
Clinical isolate (wild type) <i>MAT1-1</i>	CEA10	(442)
<i>ΔakuB (KU80);ΔpyrG1; MAT1-1</i>	<i>Δku80</i>	(503)
<i>ΔakuB (KU80);ΔpyrG1; ΔSphK MAT1-1</i>	<i>ΔSphK</i>	This study
<i>ΔakuB (KU80);ΔpyrG1 ΔgprD MAT1-1</i>	<i>ΔgprD</i>	This study

6.2.2 Construction of gene deletion plasmids, transformation and screening of *A. fumigatus* transformants

6.2.2.1 Gene replacement strategy

Plasmids utilized and generated in this study are listed in Table 6.2, using commercially available plasmids pYES2 and pUC-hph that were gifts from Dr Elaine Bignell's lab.

Table 6.2: Plasmids used in this study

Isolate	Selection in <i>Aspergillus</i>	Selection in <i>E. coli</i>	<i>Aspergillus</i> target locus	Reference
pUC19	N/A	100 µg/ml Ampicillin	N/A	(504)
pUC-hph	180 µg/ml Hygromycin B	N/A	N/A	(505)
pYES2	N/A	100 µg/ml Ampicillin	N/A	(506)
pYES2	180 µg/ml Hygromycin B	100 µg/ml Ampicillin	AFUB_010400, Sphingosine kinase (SphK), putative	(442)
pYES2gprD	180 µg/ml Hygromycin B	100 µg/ml Ampicillin	AFUB_028290, integral membrane protein	(442)

Recombinant DNA for direct replacement of the *SphK* or *gprD* genes were constructed via recombinational cloning, using pYes2 as the recipient vector, and pUC_hph as the source of a hygromycin resistance cassette (Fig 6.1). The resulting plasmids, pYES2SphK and pYES2gprD, were then used for *A. fumigatus* transformation.

In the first step of generating transformation plasmids (pYES2SphK and pYES2gprD KO vectors) pUC-hph and pYES2 plasmids were utilized (Fig 6.1). The pUC-hph plasmid contains a sequence encoding the promoter region of the constitutively expressed *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*), controlling the expression of the subsequent hygromycin phosphotransferase gene (*hph*). The *gpdA* promoter is constitutively active during mycelial growth (507).

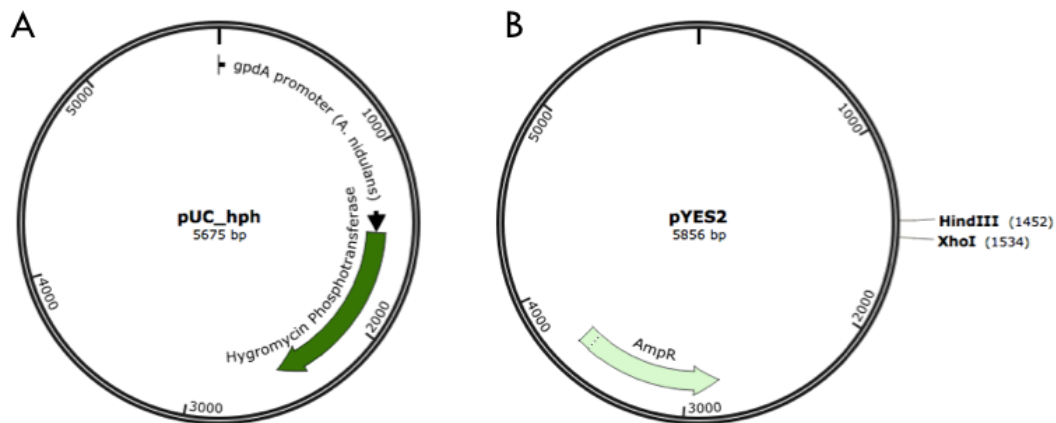


Figure 6.1: Schematic representation of pUC_hph and pYES2 plasmids.

In order to construct the gene replacement cassettes, a hygromycin resistance cassette, was fused to 5' and 3' flanking regions of the gene of interest into a pYES2 backbone using the commercially available Gibson assembly cloning kit (NEB). Primers consisted of 20 bp specific to the GOI flanking region and 30 bp homologous to the pYES2 vector backbone insertion sites (Fig 6.1). Primers used to amplify genes of interest were *SphK_yes_F*, *SphK-gpdA_R*, *SphK_hph_F* and *SphK_yes_R*, and *gprD_yes_F*, *gprD-gpdA_R*, *gprD_hph_F* and *gprD_yes_R*, for each GOI respectively. Primers are listed in Table 6.8 and Fig 6.2 shows primer scheme of primers.

The flanking regions for each of the genes of interest (GOI) of approximately 800 bp were amplified by PCR from *A. fumigatus* gDNA using long oligonucleotides, with additional sequences added to 5' and 3' ends for recombineering. Template *A. fumigatus* gDNA was isolated by a colleague and run on an agarose gel to confirm purity before use.

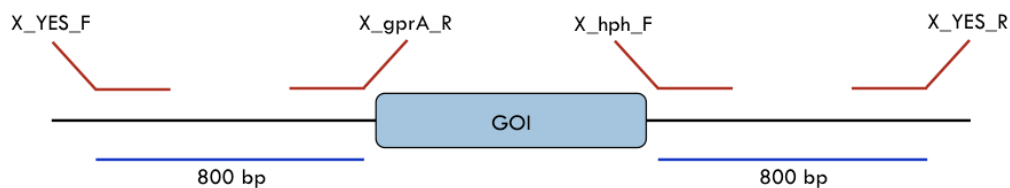


Figure 6.2: Schematic showing location of primers for amplification of flanking regions adjacent to gene of interest (with overhangs for recombineering)

The hygromycin resistance cassette was amplified by PCR from pUC_hph, using oligonucleotide primers gpdA_hph_F (sequence from 5' to 3' CTTGGTTGAATTAGAACGTGG) and gpdA_hph_R (sequence from 5' to 3' CTATTCCTTTGCCCTCGGACGAG). See Appendix 10.3 Fig 1 for 1% agarose electrophoresis gel. The backbone vector pYES2 was linearised by double digest using HindIII and XhoI (NEB) restriction enzymes, as per the manufacturer's instructions. The cutting restriction enzyme recognition sites can be seen in (Appendix 9.3) and the linearized pYES2 run on a 1% agarose gel in B). To assemble the gene knockout constructs purified DNA fragments were ligated using the exonuclease, DNA polymerase and DNA ligase activity of the Gibson Assembly Cloning Kit (NEB) according to manufacturer's instruction. The resulting construct comprised of a hygromycin B resistance cassette flanked by approximately 800 bp of the 5' and 3' regions of the putative *SphK* or *gprD* genes to direct homologous recombination at AFUB_010400 and AFUB_028290 ORFs respectively. Recombinant vectors were transformed into competent *E. coli* strain NEB 5- α and selected for using 100 μ g/ml ampicillin LB agar. Single colonies were picked and grown overnight in 10 ml of 100 μ g/ml ampicillin LB broth and isolated by DNA miniprep (Sigma).

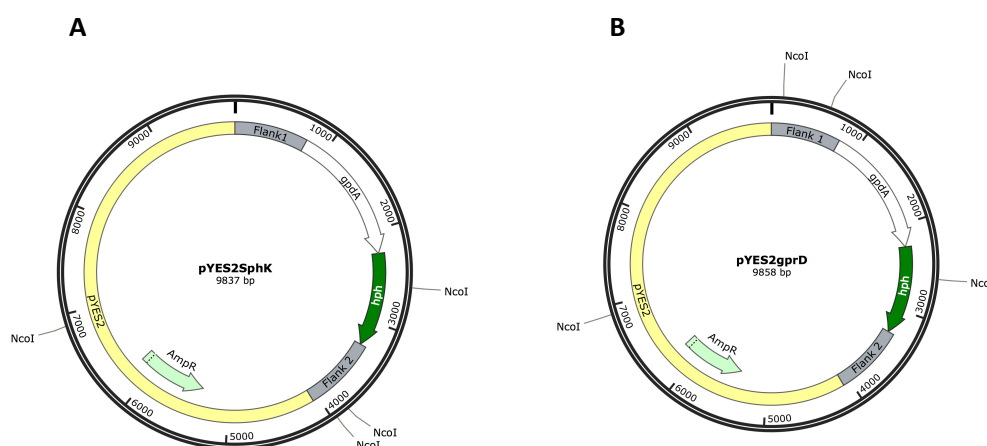


Figure 6.3: Schematic representation of recombinant vectors pYES2SphK and pYES2gprD A) pYES2SphK (9837 bp) and B) pYES2gprD (9858 bp), with restriction sites indicated for NcoI (NEB) restriction enzyme used in screening.

Screening of *E. coli* propagated plasmids for correctly assembled constructs was performed by restriction endonuclease enzyme digest and PCR. Plasmids were digested with NcoI (NEB) according to the manufactures instructions (Fig 6.2) and 10 µl of the linear DNA was run on a 1% agarose gel to assess fragments sizes (Appendix 10.3).

Screening by PCR was performed using primers pYES2_F and pYES2_R, with expected amplicons sizes of 4244 and 4223 bp for pYES2SphK or pYES2gprD respectively (Appendix 10.3). Oligonucleotides used to construct plasmids are listed in Table 6.3.

Table 6.3: Primers used in assembly of knockout constructs

Name	oligonucleotide sequence (5' to 3')	Use
gpdA_hph_F	CTTGTTGAATTTAGAACGTGG	This study
gpdA_hph_R	CTATTCCTTTGCCCTCGGACGAG	
SphK_yes_F	GCTGTAATACGACTCACTATAGGGAATATTacggcatgaagagagcaagt	
SphK_yes_R	CATAACTAATTACATGATGCGGCCCTCTAGtcgagtcatacatgaccatc	
gprD_yes_F	GCTGTAATACGACTCACTATAGGGAATATTgacttttgctcgtgctctt	
gprD_yes_R	CATAACTAATTACATGATGCGGCCCTCTAGtccagcttcagtcagagct	
SphK_hph_F	CCCAGCACTCGTCCGAGGGCAAAGGAATAGtaaagtcgatgccatcattg	
SphK-gpdA_R	CAATAGTGCCACGTTCTAAATTCAACCAAGtactacctgcaaaagcgagt	
gprD_hph_F	CCCAGCACTCGTCCGAGGGCAAAGGAATAGatcgtcctacttcgttcatt	
gprD-gpdA_R	CAATAGTGCCACGTTCTAAATTCAACCAAGgtttatcatgccccgttcaa	
pYES2_F	GGATCGGACTACTAGCAGCTG	
pYES2_R	CTTCAGGTTGTCTAACTCCTTCC	

6.2.2.3 Transformation plasmid sequencing

Both constructs (pYES2SphK and pYES2gprD) were sequenced by Sanger sequencing at The Genomics Laboratory (a dedicated Imperial College facility), by a MiSeq personal sequencer using oligonucleotide primers designed to hybridise to template DNA at 500 bp intervals.

A protein alignment of the expected sequence from the *hph* gene sequences using Clustal Omega (online alignment tool) revealed that they retained 100% identity.

6.2.2.4 Transformation of *A. fumigatus*

Plasmids used for transformations in the course of this work were the following: pYES2SphK and pYES2gpdA (as described above). In preparation for transformation of *A. fumigatus*, gene replacement cassettes were amplified by PCR using Phusion Flash PCR Master Mix (Thermo Scientific) using primers pYES2_F and pYES2_R (see Table 6.8). Resultant amplicons were analysed by agarose gel electrophoresis prior to use to estimate sizes (Appendix 6.5). The amplicons were purified using a Nucleospin Gel & PCR clean-up kit (Macherey-Nagel) before *A. fumigatus* transformation.

For the transformation, $\Delta ku80$ was grown on SAB agar and conidia were harvested with sterile distilled water. Conidia were separated from hyphal fragments using sterile Miracloth, then washed with water, re-suspended in water and enumerated using a haemocytometer. A total of 2×10^8 conidia were added to 250 ml of *Aspergillus* complete medium (ACM) in a conical flask and incubated overnight in an orbital shaking incubator at 30°C and 150 rpm. The next day Vinoflow (digesting enzyme mixture) was resuspended in KCL and filter sterilised. The mycelium was harvested using Miracloth and added to the Vinoflow solution. After 4 hours the protoplasts were harvested using a 40 μ M cell strainer, washed 5 times and re-suspended in KCL, before being resuspended in KCl/CaCl₂. Protoplasts, gene replacement cassettes and PEG were co-incubated for 30 mins at room temperature and then added to warm molten agarose overlay agar that was poured over 15 ml *Aspergillus* minimal media (AMM) plates containing 180 μ g/ml hygromycin B. Thus, transformants lacking the integration of the hygromycin resistance cassette should not proliferate, and *hph* positive transformants grew through the top agar to produce distinct colonies.

After 2-3 days of incubation at 37°C, conidia from transformant mycelial colonies growing on the surface agar were transferred with a sterile toothpick onto fresh individual AMM selection plates

containing 180 µg/ml hygromycin B. Transformants were purified by subculturing three times, with one transformant per plate.

6.2.2.5 gDNA extraction from *A. fumigatus* transformants for screening

After purification of *A. fumigatus* transformants genomic DNA was extracted from conidia for mutant screening by PCR. Extraction of gDNA was performed by harvesting conidia and underlying mycelia and combining with acid washed glass beads with breaking buffer in a 2 ml tube. The conidia were subject to constant vortexing for 30 mins followed by incubation at 65°C on a heat block for 30 mins, vortexing every 10 min for 30 s. Phenol:Chloroform:Isoamylalcohol was added and the mixture vortexed for 5 mins. After centrifugation at 13k rpm for 8 min the upper aqueous layer was transferred to a fresh tube and an equal volume of isopropanol added followed by centrifugation at 13k rpm for 10 min. The DNA pellet was washed with 70% ethanol and centrifuged at 13k rpm for 10 mins to re-pellet. Ethanol was removed and the residual ethanol was allowed to evaporate while tubes were placed face down on tissue paper. To check the gDNA for purity and size the isolated DNA was analysed by agarose gel electrophoresis. The appearance of the gDNA suggested that good quality gDNA had been extracted (Appendix 9.2).

6.2.2.6 Mutant identification by PCR screening

Gene replacement was confirmed by PCR and Southern blotting. *A. fumigatus* $\Delta SphK$ and $\Delta gprD$ mutants were identified by PCR on genomic (conidial) DNA from multiple hygromycin-resistant colonies. A set of four primers pairs were used in the screening (listed in Table 6.4) and their location indicated in Fig 6.4 below.



Figure 6.4: Schematic showing primers used in transformant screening by PCR

Table 6.4: Primers used for screening of *A. fumigatus* transformants

No.	Primer name	Oligonucleotide sequence (5' to 3')	Expected PCR amplicon size	Amplification target
P1	SK_SetA_F	CTGCGGATTACTATCAAACCTCG	833 bp	Gene of interest
P2	SK_SetA_R	GTGACAAGAAAGATAGGGTTCG		
P3	gD_SetA_F	GATTCATTTTCTGGCAGCGT	936 bp	
P4	gD_SetA_R	AGACTTGACCCGAGACTTCTTG		
P5	Hyg_SetB_F	CGAAGGAGAATGTGAAGCCA	1092 bp	Hygromycin B resistance cassette
P6	Hyg_SetB_R	CGCATATGAAATCACGCCAT		
P7	SK_SetC_F	CATCCTACAAGGAGTTCTTGCC	+ve: 2754 bp	Gene knockout cassette
P8	SK_SetC_R	AGCGAAGCCACATCACAACA	-ve: 2030 bp	
P9	gD_SetC_F	GGAACGAAAACCTCAAGACCATG	+ve: 2909 bp	
P10	gD_SetC_R	AGCACATGCAAGCCTTCAAA	-ve: 1775 bp	

6.2.2.7 Confirmation of single homologous gene replacement by Southern blotting

Gels were purinated in 250mM HCl for 15 mins, at room temperature with shaking (24.55ml of 33-35% HCl + H₂O to 1 litre). Followed by a wash for 10 mins with ddH₂O. Gels were denatured in denaturation solution twice for 15 mins, at room temp with shaking, followed by a rinse for 10 mins in ddH₂O. Gels were neutralized in neutralisation solution twice for 15 mins, at room temp with shaking, followed by a rinse with for 10 mins in ddH₂O. Gels were allowed to equilibrate for 10 mins in 20x SSC. Blots were transferred over night, after which membranes were placed DNA side down onto Whatman paper soaked in 2x SSC. Blots were UV cross-linked and allowed to dry. Blots were pre-hybridised for a minimum of 30 mins with gentle agitation. For hybridization, probe was added to 50 µl of ddH₂O, and added to boiling water for 5 mins to denature probe, and placed on ice. The denatured probe was added to 10 ml of pre warmed Prehyb solution to form hybridisation buffer. Plots were hybridized over night at the T_{hyb}. Blots were washed with low stringency buffer (2x SSC, 0.1% SDS), at room temp with shaking twice for 5 mins. Blots were then washed with 65°C pre-warmed high stringency wash buffer twice for 15 mins with gentle agitation. Membranes were washed with washing buffer (0.3% Tween in Maleic Acid buffer pH 7.5) for 2 mins at room temp with shaking. Membranes were blocked with blocking solution (1% blocking in Maleic acid buffer pH 7.5) for 30 mins at room temp with shaking. Membranes were incubated in antibody solution for 30 mins with gentle agitation, then washed with washing buffer twice for 15 mins at room temp with shaking. Then membranes were equilibrated in detection buffer (0.1M Tris-HCl, 0.1M NaCl pH 9.5) for 3 mins, room temp

with shaking. Finally, chemiluminescent substrate was added, and blot incubated at room temp for 5 mins prior to development of blot

6.2.2.8 Phenotypic testing of identified *A. fumigatus* mutants

A. fumigatus wild-type and mutant strains were streaked from glycerol stock onto on SAB agar and cultured for 3 days at 37°C in T25cm flasks. Cultures were gently shaken into suspension with 0.1% Tween20 in dH₂O, and conidia isolated by filtration through Miracloth and washed twice with PBS. Conidial suspensions were enumerated by haemocytometer and serially diluted 1:10 to achieve 2x10⁵/ml, 2x10⁴/ml and 2x10³/ml. Plates were spotted with 5 µl of each suspension resulting in inoculations of 1x10³, 1x10² and 1x10¹ conidia per spot. Plates were incubated at 37°C for 48-96 h. Colonies were imaged using a 1.5 megapixel camera secured at a fixed height.

Conditions employed for the purpose of screening transformants were acidic to basic pH, hyperosmolarity using NaCl, and cell membrane and cell wall stress by sodium dodecyl sulfate (SDS), Congo red (CR), or calcofluor white (CFW). AMM was cooled to 50°C prior to addition of the supplements advised in Table 6.5.

Table 6.5: Conditions for phenotypic analysis *A. fumigatus* transformants: Media and buffers used for *A. fumigatus* phenotypic screening

Media	Test condition	Comments
AMM pH 3	Acidic pH	Buffered with 100 mM Na ₂ HPO ₄ and 50 mM citric acid
AMM pH 4	Acidic pH	Buffered with 100 mM Na ₂ HPO ₄ and 50 mM citric acid
AMM pH 5	Acidic pH (phagolysosome)	Buffered with 100 mM Na ₂ HPO ₄ and 50 mM citric acid
AMM pH 6	Neutral pH	Buffered with 100 mM Na ₂ HPO ₄ and 50 mM citric acid
AMM pH 7.3	Physiological (host) pH	Buffered with 100 mM Na ₂ HPO ₄ and 50 mM citric acid
AMM pH 8	Alkaline pH	Buffered with 100 mM Na ₂ HPO ₄ and 50 mM citric acid
AMM 0.5 M NaCl	Hyperosmolarity	Sodium chloride
AMM 1.0 M NaCl	Hyperosmolarity	Sodium chloride
AMM 1.5 M NaCl	Hyperosmolarity	Sodium chloride
AMM 50 µg/ml SDS	Cell membrane stress	50 mg/ml SDS solution
AMM 50 µg/ml CFW	Cell wall stress	10 mg/ml CFW solution
AMM 100 µg/ml CFW	Cell wall stress	10 mg/ml CFW solution
AMM 200 µg/ml CFW	Cell wall stress	10 mg/ml CFW solution
AMM 300 µg/ml CFW	Cell wall stress	10 mg/ml CFW solution
AMM 400 µg/ml CFW	Cell wall stress	10 mg/ml CFW solution
AMM 500 µg/ml CFW	Cell wall stress	10 mg/ml CFW solution
AMM 20 µg/ml CR	Cell wall stress	1% (w/v) CR solution
AMM 40 µg/ml CR	Cell wall stress	1% (w/v) CR solution
AMM 80 µg/ml CR	Cell wall stress	1% (w/v) CR solution
AMM 160 µg/ml CR	Cell wall stress	1% (w/v) CR solution

AMM was prepared as described by Cove (1976) and buffered to appropriate pH (see table 6.10). NaCl was added to AMM and dissolved by stirring, and SDS, CFW and CR were added to media from a higher concentration stock solution. All plates were made the day before use and stored at 4°C. AMM plates containing CFW were wrapped in foil. After harvest and enumeration conidia were re-suspended at 1×10^4 in water and then serially diluted 1:10 in a 96-well plate for final concentrations. 5 µl of each conidial suspension were spotted onto media. Parental and mutant strains were seeded on each plate and two plates were seeded per experiment (2 technical replicates) and 3 experimental replicates were performed. Growth was monitored at 24 h intervals up to 96 h.

6.2.3 Growth Curve

After harvest and enumeration by haemocytometer conidia were re-suspended at 1.25×10^5 /ml in RPMI. Conidial suspensions were transferred to 96-well microtiter plates containing 200 µl per well and incubation at 37°C for 16 h. Absorbance readings at 405 nm were taken by a Tecan plate (200 pro) reader at 30 min intervals.

6.2.6 Analysis of germination by widefield microscopy

Germination experiments were carried out as per Chapter 5.

6.2.4 Cytokine quantification

TNF-α was quantified from cell culture supernatants by ELISA as per Chapter 2.

6.2.5 Galactomannan quantification

Galactomannan was quantified from cell culture supernatants using the Platelia *Aspergillus* EIA kit (Bio-RAD, #62796), an immunoenzymatic sandwich microplate assay. The kit was used as per the manufacturer's instructions. Briefly, neat or PBS diluted supernatants were added along with peroxidase-linked monoclonal antibodies to a 96-well plate containing lyophilized monoclonal antibodies for the detection of *Aspergillus* galactomannan antigen. A monoclonal antibody-galactomannan-monoclonal antibody/peroxidase complex is formed if galactomannan is present. Wells were washed to remove any unbound molecules and a chromogen TMB substrate solution added that turns blue in the presence of antibody and antigen complexes. The reaction was stopped by the addition of 1.5 N sulphuric acid, and optical density by absorbance at 405 nm was determined using a spectrophotometer. A positive, negative and cut-off control (for minimum assay detection limit) are provided with the kit.

6.3 Comparative analysis of sphingolipid pathway proteins in mammals and *A. fumigatus*

6.3.1 *A. fumigatus* sphingosine kinase homolog and sequence analysis

Human sphingosine kinase 1 (SPHK1) has 3 isoforms and a total of 4 transcript variants. An alignment of SPHK1 isoforms (Figure 6.5), demonstrates that all variants contain conserved diacylglycerol kinase (DAGK) regions and catalytic domains, and the sphingosine substrate-binding site. It also identifies isoform 2 as the longest sequence, which was consequently used for further alignments to *Aspergillus*.

```

SphingosineKinaseIsoform1
SphingosineKinaseIsoform2
SphingosineKinaseIsoform3
SphingosineKinaseIsoform3

-----MSAQVLGFLRSWTFPLAAPRGPAAGNDAGAPATAPGGEGEPHSRPCDARLGSTDKEL
-----

SphingosineKinaseIsoform1
SphingosineKinaseIsoform2
SphingosineKinaseIsoform3
SphingosineKinaseIsoform3

-----MDPVVG-CGRGLFGFVFSAGGPRGVLPKPCRVLVLLNPRGGKQKALQLF
KAGAAATGSAPTAPGTPWQREPRVEVMDPAGGPRGVLPKPCRVLVLLNPRGGKQKALQLF
-----MDPAGGPRGVLPKPCRVLVLLNPRGGKQKALQLF
-----MDPAGGPRGVLPKPCRVLVLLNPRGGKQKALQLF
: *****

SphingosineKinaseIsoform1
SphingosineKinaseIsoform2
SphingosineKinaseIsoform3
SphingosineKinaseIsoform3

RSHVQPLLAEEISFTMLTERRNHARELVSEELGRWDALVVMSGDGLMHEVVNGLMER
RSHVQPLLAEEISFTMLTERRNHARELVSEELGRWDALVVMSGDGLMHEVVNGLMER
RSHVQPLLAEEISFTMLTERRNHARELVSEELGRWDALVVMSGDGLMHEVVNGLMER
RSHVQPLLAEEISFTMLTERRNHARELVSEELGRWDALVVMSGDGLMHEVVNGLMER
*****

SphingosineKinaseIsoform1
SphingosineKinaseIsoform2
SphingosineKinaseIsoform3
SphingosineKinaseIsoform3

PDWETAIQKPLCSLPAGSGNALAASLNHYAGYEQVTNEDLLTNCTLLCRRLSPMNLLS
PDWETAIQKPLCSLPAGSGNALAASLNHYAGYEQVTNEDLLTNCTLLCRRLSPMNLLS
PDWETAIQKPLCSLPAGSGNALAASLNHYAGYEQVTNEDLLTNCTLLCRRLSPMNLLS
PDWETAIQKPLCSLPAGSGNALAASLNHYAGYEQVTNEDLLTNCTLLCRRLSPMNLLS
*****

SphingosineKinaseIsoform1
SphingosineKinaseIsoform2
SphingosineKinaseIsoform3
SphingosineKinaseIsoform3

LHTASGLRLFSVLSLAWGFIADVDLESEKYRRLGEMRFTLGTFLRLAALRTYRGLAYLP
LHTASGLRLFSVLSLAWGFIADVDLESEKYRRLGEMRFTLGTFLRLAALRTYRGLAYLP
LHTASGLRLFSVLSLAWGFIADVDLESEKYRRLGEMRFTLGTFLRLAALRTYRGLAYLP
LHTASGLRLFSVLSLAWGFIADVDLESEKYRRLGEMRFTLGTFLRLAALRTYRGLAYLP
*****

SphingosineKinaseIsoform1
SphingosineKinaseIsoform2
SphingosineKinaseIsoform3
SphingosineKinaseIsoform3

VGRVGSKTPASPVVVQGGPVDAHLVPLEEPVPSHWTVPDEDFVLVLALLHSHLGSEMFA
VGRVGSKTPASPVVVQGGPVDAHLVPLEEPVPSHWTVPDEDFVLVLALLHSHLGSEMFA
VGRVGSKTPASPVVVQGGPVDAHLVPLEEPVPSHWTVPDEDFVLVLALLHSHLGSEMFA
VGRVGSKTPASPVVVQGGPVDAHLVPLEEPVPSHWTVPDEDFVLVLALLHSHLGSEMFA
*****

SphingosineKinaseIsoform1
SphingosineKinaseIsoform2
SphingosineKinaseIsoform3
SphingosineKinaseIsoform3

APMGRCAAGVMHLFYVRAGVSRAMLLRLFLAMEKGRHMEYECPLYVYVPVAVRLEPKDG
APMGRCAAGVMHLFYVRAGVSRAMLLRLFLAMEKGRHMEYECPLYVYVPVAVRLEPKDG
APMGRCAAGVMHLFYVRAGVSRAMLLRLFLAMEKGRHMEYECPLYVYVPVAVRLEPKDG
APMGRCAAGVMHLFYVRAGVSRAMLLRLFLAMEKGRHMEYECPLYVYVPVAVRLEPKDG
*****

SphingosineKinaseIsoform1
SphingosineKinaseIsoform2
SphingosineKinaseIsoform3
SphingosineKinaseIsoform3

KGVFAVDGELMVSEAVQGGVHPNYFWMVSGCPEPPPSWKPPQMPPEEPL
KGVFAVDGELMVSEAVQGGVHPNYFWMVSGCPEPPPSWKPPQMPPEEPL
KGVFAVDGELMVSEAVQGGVHPNYFWMVSGCPEPPPSWKPPQMPPEEPL
KGVFAVDGELMVSEAVQGGVHPNYFWMVSGCPEPPPSWKPPQMPPEEPL
*****

```

Figure 6.5: Protein homology of human SPHK1 isoforms. In bold is the diacylglycerol kinase (DAGK) region, in blue the catalytic domain and in yellow the substrate binding site.

Sphingosine kinase (AFUB_010400) is annotated as an uncharacterized hypothetical protein in the CEA10 *A. fumigatus* genome as per the submitted genome sequence by Fedorova *et al* in 2008.

On comparing the DAG kinase catalytic domain of human SPHK1 with the designated 'putative sphingosine kinase' of *A. fumigatus*, a 39.5% homology was found in addition to an identical substrate binding site (Fig 6.6). This conserved DAGK domain feature suggests that *A. fumigatus* SphK could maintain the same functions as mammalian SPHK1.

```

SphKputative[CEA10]      KRYRRFKVLINPFGGKGIASRLYHQYAAPILAAAHCVVEVEETHGGHATEIAEQIDIDA
SphK1Isoform2[HomoSapien] ---RVLVLLNPRGGKGKALQLFRSHVQPLLAEEISFTLMLTERRNHARELVRSEELGR
                        *, **:* ***** :*:::.. *:* * . : * : ** *::.. ::

SphKputative[CEA10]      YDAIVCCSGDGLPYEVFNGLAKKPNAREALSKLAVAMIPGGSGNAMAWNLCGTGSVSVAA
SphK1Isoform2[HomoSapien] WDALVVMSSDGLMHEVVNGLMERPDWETAIQKP-LCSLPAGSGNALAASLNHYA-----
                        :*:* ***** :**.* ** :*: . *:* . :*.*****:* . * .

SphKputative[CEA10]      LAIVKGVRTPIDLVSVTQGK
SphK1Isoform2[HomoSapien] -----

```

Figure 6.6: Homology of DAG kinase catalytic domains of human SPHK1 was compared with that of *A. fumigatus* (CEA10) putative SphK. Substrate binding site is highlighted in yellow.

A protein BLAST using the CEA10 DAGc domain sequence revealed that sphingosine kinase is conserved across pathogenic and non-pathogenic species of *Aspergillus* with a high (80-100%) degree of homology (Table 6.6).

Table 6.6: Protein BLAST of the CEA10 DAGc domain against other *Aspergillus spp.*

Description	Max score	E value	Ident	Accession
Putative SphK, <i>A. fumigatus</i> Af293	724	990E-93	100%	Q4WT07
Putative SphK, <i>A. fischeri</i>	707	350E-90	96.4%	A1D2Y0
Sphingoid long chain base 4, <i>A. udagawae</i>	706	490E-90	96.4%	A0A0K8LMG4
<i>A. clavatus</i>	659	640E-84	89.2%	A1CQ16
Putative SphK, <i>A. calidoustus</i>	618	1.1E-75	83.5%	A0A0U5A2H7
SphK, <i>A. oryzae</i>	614	4.7E-75	81.3%	A0A064BB04
Putative SphK, <i>A. flavus</i>	614	4.7E-75	81.3%	B8NJP4
Putative SphK, <i>A. nomius</i>	609	23E-75	81.3%	A0A0L1J3X1
SphK, <i>A. niger</i>	609	28E-75	80.0%	A0A124BX85
SphK, <i>A. kawachii</i>	609	28E-75	80.0%	G7XRJ9

In correlation with the level of homology seen between human SPHK1 and CEA10 SphK, a protein BLAST of the human DAGc domain sequence against *Aspergillus spp.* showed that homology is maintained at between 40-42% (Table 6.7).

Table 6.7: Protein BLAST of the human SPHK1 DAGc domain against *Aspergillus spp.*

Description	Max score	Query cover	E value	Ident	Accession
SphK, <i>A. fumigatus</i> Af293	79.7	96%	1e-17	41%	XP_752463.1
Putative SphK, <i>A. fischeri</i>	79.0	93%	2e-17	41%	XP_001264670.1
Sphingoid long chain base 4, <i>A. udagawae</i>	81.3	96%	3e-18	41%	GAO88829.1
Putative SphK, <i>A. calidoustus</i>	84.7	96%	2e-19	41%	CEN62361.1
Putative SphK, <i>A. clavus</i>	84.3	96%	2e-19	42%	XP_001269163.1
SphK, <i>A. oryzae</i>	84.3	96%	2e-19	42%	XP_001825245.2
Putative SphK, <i>A. flavus</i>	84.3	96%	2e-19	42%	XP_002380413.1
Putative SphK, <i>A. nomius</i>	82.8	96%	8e-19	42%	XP_015407303.1
Putative SphK, <i>A. niger</i>	81.6	96%	2e-18	40%	GAQ41616.1
SphK, <i>A. kawachii</i>	81.6	96%	2e-18	40%	GAA89460.1

Lastly, phytosphingosine kinase was also investigated as a potential knockout as phytosphingosine has been identified as a main sphingoid base in fungi (500). Amino acid alignment showed overall homology to human SPHK1 of phytosphingosine is 17% whereas with *A. fumigatus* putative SphK it is 28% (Appendix 9.3). In addition, *A. fumigatus* SphK shares 100% homology with the identified substrate-binding site whereas phytosphingosine did not.

6.3.2 *A. fumigatus* S1P₁ receptor homolog and sequence identity analysis

An *in silico* study of the *Aspergillus* genome by Lafon *et al.*, (2006) indicated that there are 15 GPCRs (Table 6.8). Yet a BLAST of human S1P₁ (using the NCBI online tool) against *Aspergillus spp* yields a single result of a putative cytochrome P450 monooxygenase (CEL01510.1) from *A. calidoustus* with a query cover of 25% and 31% identity, although the E value of 8.4 suggests this could be an artifact of searching in a database this size. Due to the lack of a direct homologous protein being identified through a direct BLAST search, all known GPCRs of *A. fumigatus* were investigated for homology to S1P₁ on an individual basis.

Table 6.8: *A. fumigatus* GPCRs

Class	Name	A1163	Annotation/notes
1	GprA	AFUB_034900	Putative pheromone receptor
2	GprB	AFUB_055410	Putative pheromone receptor
3	GprC	AFUB_090350	Putative carbon sensor; Rhodopsin-like G-protein coupled receptor
3	GprD	AFUB_028290	Putative carbon sensor
4	GprF	AFUB_052610	Putative nitrogen sensor; PQ loop repeat
4	GprG	AFUB_011350	Putative nitrogen sensor; PQ loop repeat
4	GprJ	AFUB_007220	Putative nitrogen sensor; PQ loop repeat
5	GprH	AFUB_052650	Putative cAMP receptor; Secretin-like G-protein coupled receptor
5	GprI	AFUB_047630	Putative cAMP receptor; Secretin-like G-protein coupled receptor
5	GprL	AFUB_046660	Putative cAMP receptor (unique to <i>A. fumigatus</i>) Secretin-like G-protein coupled receptor
6	GprK	AFUB_101830	G-protein signalling (RGS) domain, unique to filamentous fungi
7	GprM	AFUB_090880	Homology to rat growth hormone-releasing factor receptors
8	GprO	AFUB_096360	Putative zinc regulator; Haemolysin III-related protein
8	GprP	AFUB_073100	Identity to bacterial opsins; Haemolysin III-related protein
9	NopA	AFUB_088000	Identity to bacterial opsins; Putative bacterial rhodopsin Family G-protein coupled receptor (GPCR)-like protein

An alignment of all known *A. fumigatus* GPCRs (Table 6.8) against human S1P₁ shows GprM, GprD and GprC as having the highest overall homology with 20% identity for the former and 19% for the latter two. However, when comparing the core 7TM sequence of human S1P₁ the sequence homology is 23% for GprD and GprC and 19% for GprM.


```

S1P1 [Homo]  FIGNLALSDLLAGVAYTANLLLSGATTYKLTTPAQWFLREGSMFVALSA-----
GprM [Af]    VCFTLGICFM--EIAFIIPLGVKPDQC-----YNQITPNDMH-SSLSCAFTGSLLLGGW
GprC [Af]    LIYNLVIADLQQSLAFLI-----C-----IKWIAEDKIEASSAACFLQGFWLQIGDP
GprD [Af]    LIYNLLLVLDIQQATAFLL-----C-----LHWFAFAGGVNYPAAACVLQGWIIQTADP
              .  . * : :      * :                . :                : : .

S1P1 [Homo]  -SV--FSLLAIA--IER-YITMLKMKLHNGSNNFRLFLLISACWVISLILGGLPIMGWNC
GprM [Af]    MVVVWSFLRTVAFHLQVCWEVILGPKFMWGALIFGWVPAVGLTVMLILT-----
GprC [Af]    ASG--LFVLAIA--IHTFLLVAMGHKLSYRIFVLG----VVGLWVFVAILVIIPLAAHGR
GprD [Af]    GSG--LFVLAIA--IHTGAVVLRGRQLPYTTFVWC----VVGLWAFIILLGLIAVGLYGS
              : : * : .      . : :                . : : :

S1P1 [Homo]  ISALS---SC-----STVLPLYHKHYILFCTTVFTLLLL--SIVILYC-RIYSL-VRT
GprM [Af]    -VSFRFGTVCHINIDGALQ-----DYWIPIISFAVAALILQLATMAYCIHVYVKS LFDT
GprC [Af]    FVFIPSGAWCWISEEYEAURLW-THYIWIFLAIEFGTVCL---YAVMWFQLRAQIK---Q
GprD [Af]    RTS-----VISEAGW-GHYVWIFLSEFGTILL---YGIMFFYLRRQMK---Q
              :      *      *      : : :

S1P1 [Homo]  RS----RRL-TFRKNISKASRSSEKSLALLKTVII-----VLSVFIACWAPLFIL
GprM [Af]    DSTNSSGLPSYSASV--RTVSARQAYRRIRRVLQLQWRGVTLVLIIIANVIFFSVTFIE
GprC [Af]    SA----IL-----GSNHTASLKRRLRVIG-----YMVIYPIAYIV
GprD [Af]    AA----AL-----RQNHQESLNRLNRVVV-----YMVIYPLVYVL
              :      *      . : : * :                : : : :

S1P1 [Homo]  LLLDVG-----CK-----VKTC DILFRAEYFLV-----LAVLNSGTNP II
GprM [Af]    LDSSLKPTAENMEKALPWVACLAATNGDREKCD--PEAAKFRPSEGLLLAVL----VLL
GprC [Af]    LSLPLA--AGRMATA-----RGNT--PSLAYFCV-AGALITSS-GLVDVLL
GprD [Af]    LSLPLA--EGRMSTA-----RHVV--PSKEYFAA-AGALMALS-GLVDVVV
              * :                .      *      : : : :

```

Figure 6.7: Amino acid sequence alignment for *Aspergillus* GPCRs against human S1P₁.

Active sites and residues of note are highlighted as follows: S1P binding site (yellow), phosphorylation site (blue), ligand binding and recognition (pink), hydrophobic binding pocket (green), and are amino acids that perform critical roles in ligand recognition, binding, and receptor activation (pink).

Further investigation into the protein sequence of the human S1P₁ receptor, revealed that although there is no direct homology between human S1P₁ and the *A. fumigatus* substrate binding region highlighted in yellow (NCBI), experimental evidence indicates several residues as the phosphorylation site, highlighted in blue (NCBI). GprM, GprC and GprD have direct homology for the leucine residue (L/Leu²³⁶) but none for serine (S/Ser³⁵¹) (Fig 6.7).

Furthermore, mutagenesis studies identified residues important for S1P₁ ligand binding and recognition, and receptor activation (pink highlight). Of note, three residues required for S1P recognition have been identified as R/Arg¹²⁰ (TM3), E/Glu¹²¹ (TM3) and R/Arg²⁹² (TM7) (510,511), for which GprD is the only receptor to share directly identity residue with R/Arg¹²⁰ (TM3).

With regards to the hydrophobic binding pocket interactions between ligand and the S1P₁ complimentary sites (green highlight except for E/Glu¹²¹ which is pink), E/Glu¹²¹ (TM3), F/Phe¹²⁵ (TM3) and W/Trp¹⁸² (TM4), have been reported as important (49,510). Although, there is

no direct homology between human S1P₁ and *Aspergillus spp.* GPCRs, GprD expresses a tyrosine (Y) in place of the S1P₁'s phenylalanine (F) at position 125, with both residues being structurally similar aromatic amino acids containing hydrophobic side chains. In addition, S1P₁ has tryptophan (W) at position 182, a tyrosine (Y) in GprD, which again has structural similarity, as they are both aromatic amino acids with hydrophobic side chains.

In addition, structural similarities can be seen between the Phyre2 (Protein Homology/analogy Recognition Engine V 2.0, <http://www.sbg.bio.ic.ac.uk/phyre2/>) predicted 3D structure of S1P₁ and GprD and GprC as per below in Fig 6.8 A-C (512).

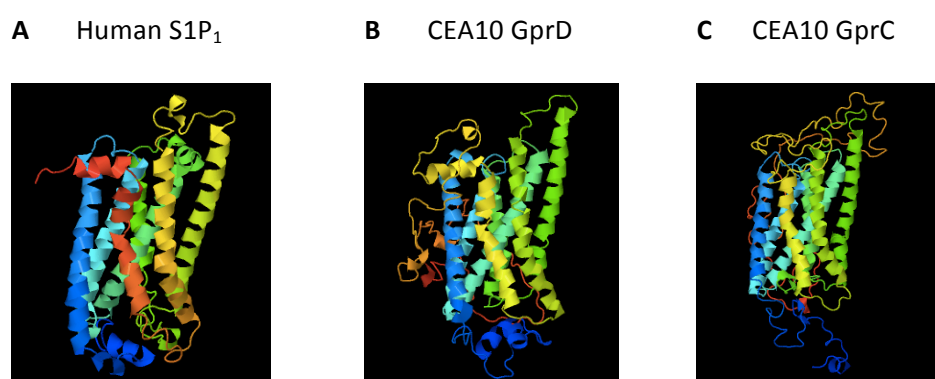


Figure 6.8: Tertiary structure prediction of human S1P₁ and *Aspergillus* GprD and GprC A) human S1P₁, B) *A. fumigatus* GprD and C) *A. fumigatus* GprC. Heat map showing high (red) to low (blue) confidence in 3D structure.

In light of the overall protein sequence homology of *Aspergillus* GPCRs against S1P₁ and degree of direct identity with respect to important ligand binding and receptor activation residues, GprD was selected for the gene deletion.

6.4 Construction of *A. fumigatus* knockout strains $\Delta SphK$ and $\Delta gprD$

Two *A. fumigatus* mutants were generated in this study using the $\Delta ku80$ strain as a progenitor isolate. Both mutants were validated by PCR amplification of gDNA and Southern blotting (the latter performed in Dr Elaine Bignell's laboratory at the University of Manchester in the UK).

Both mutants were initially tested for the integrity of the gene of interest (GOI) with primers designed to amplify an approximately 1 kb amplicon from the *SphK* and *gprD* coding regions (Fig 6.9, A), therefore for successful gene replacement no amplicon was expected. Thus, replacement of the genes of interest AFUB_010400 (for $\Delta SphK$) and AFUB_028290 (for $\Delta gprD$) was confirmed by primer Set A (SK_SetA_F and SK_SetA_R, and gD_SetA_F and gD_SetA_R for $\Delta SphK$ and $\Delta gprD$ respectively). $\Delta SphK$ mutant showed an absence of the GOI and PCR products resulting from $\Delta gprD$ DNA included some non-specific products. A robust positive signal of the correct size was detectable in the case of the $\Delta ku80$ progenitor strain (positive control) for both genes.

The presence of the hygromycin B resistance cassette was then confirmed with primers designed to amplify within the *gpdA* promoter and *hph* gene region (Fig 6.9, B). Primer Set B (Hyg_SetB_F and Hyg_SetB_R) was employed for both mutants, with an expected amplicon size of 1092 bp. Amplicons were detected for both mutants and construct plasmids with some non-specific amplification observed in the case of pYES2gprD.

The integration of the *hph* gene in place of the GOI was confirmed by primer Set C (SK_SetC_F and SK_SetC_R, and gD_SetC_F and gD_SetC_R for $\Delta SphK$ and $\Delta gprD$ mutants respectively). Amplification across the target region (from flanking regions), resulted in a variation of expected amplicon sizes to distinguish between native and transformant isolates. A successfully integrated hygromycin resistance cassette for $\Delta SphK$ would yield an amplicon of 2754 bp and a native DNA with no replacement of the GOI would yield an amplicon of 2030 bp, for $\Delta gprD$ the expected amplicon sizes for positive and negative transformants was 2909 bp and 1775 bp respectively. Appropriately amplified hygromycin resistance cassettes were seen for both mutants as well as for control plasmids, and the expected native amplicon size for the $\Delta ku80$ parental strain was also detectable (Fig 6.9, C).

To verify the correct *hph* gene replacement, two primer pairs P5/P8 or P5/P10 (Table 6.4) were utilized. Forward primer annealing within the *hph* gene and the reverse annealing from genomic DNA (in the flanking region used in the construct). The expected PCR product sizes were 1793 and

1840 bp for $\Delta SphK$ and $\Delta gprD$ respectively, if the transforming DNA had been integrated at the homologous site correctly. Appropriately sized amplicons were detectable for both mutants and plasmids (positive controls) with some unspecific amplification in the case of pYES2 vector and WT (Fig6. 9, D).

In addition to gDNA transformants were subjected to Southern blot analysis to confirm proper deletion of the *SphK* and *gprD* genes (Fig 6.10).

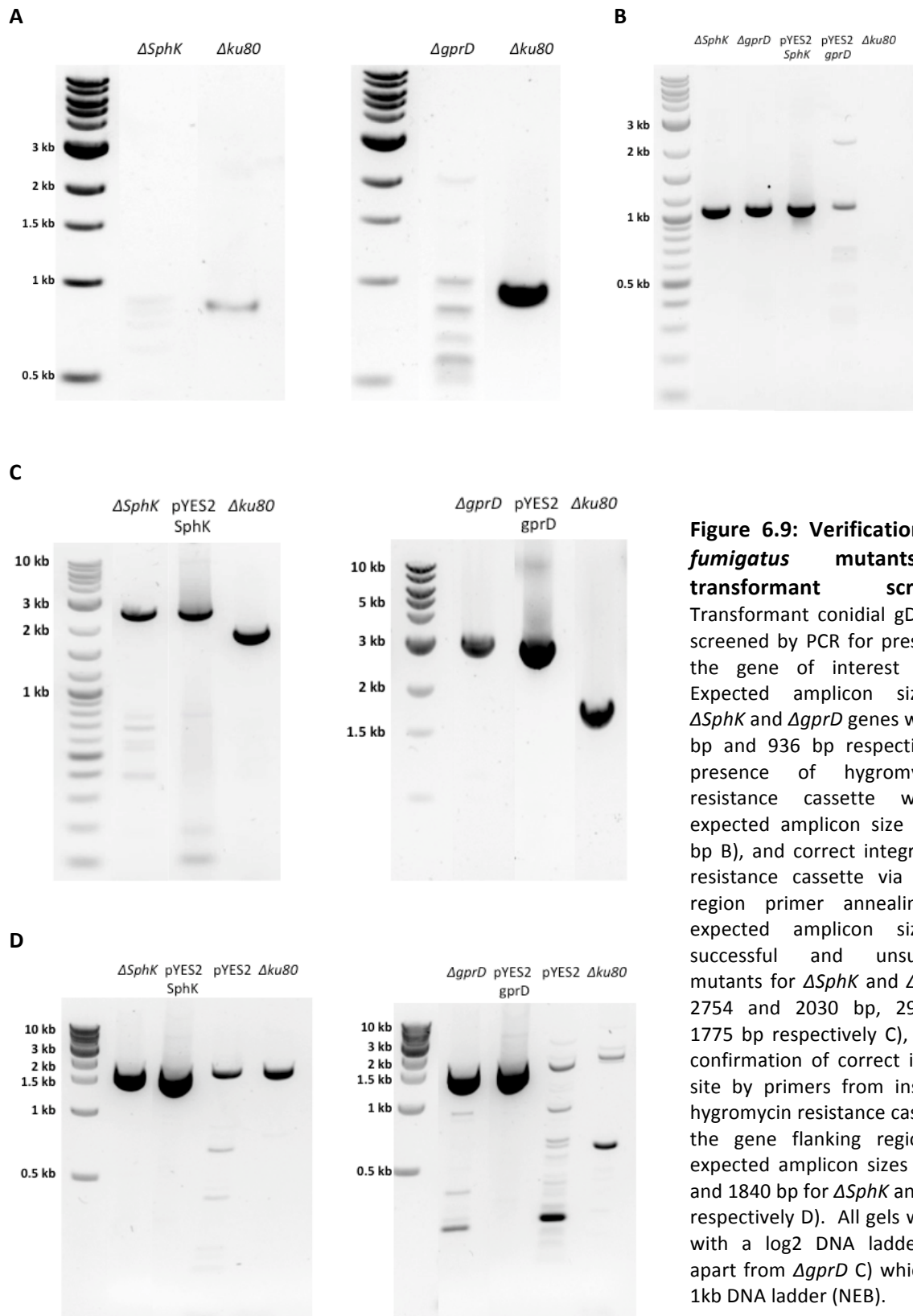


Figure 6.9: Verification of *A. fumigatus* mutants by transformant screening. Transformant conidial gDNA was screened by PCR for presence of the gene of interest (GOI). Expected amplicon sizes for $\Delta SphK$ and $\Delta gprD$ genes were 833 bp and 936 bp respectively A), presence of hygromycin B resistance cassette with an expected amplicon size of 1092 bp B), and correct integration of resistance cassette via flanking region primer annealing with expected amplicon sizes for successful and unsuccessful mutants for $\Delta SphK$ and $\Delta gprD$ at 2754 and 2030 bp, 2909 and 1775 bp respectively C), and the confirmation of correct insertion site by primers from inside the hygromycin resistance cassette to the gene flanking region with expected amplicon sizes of 1793 and 1840 bp for $\Delta SphK$ and $\Delta gprD$ respectively D). All gels were run with a log2 DNA ladder (NEB) apart from $\Delta gprD$ C) which has a 1kb DNA ladder (NEB).

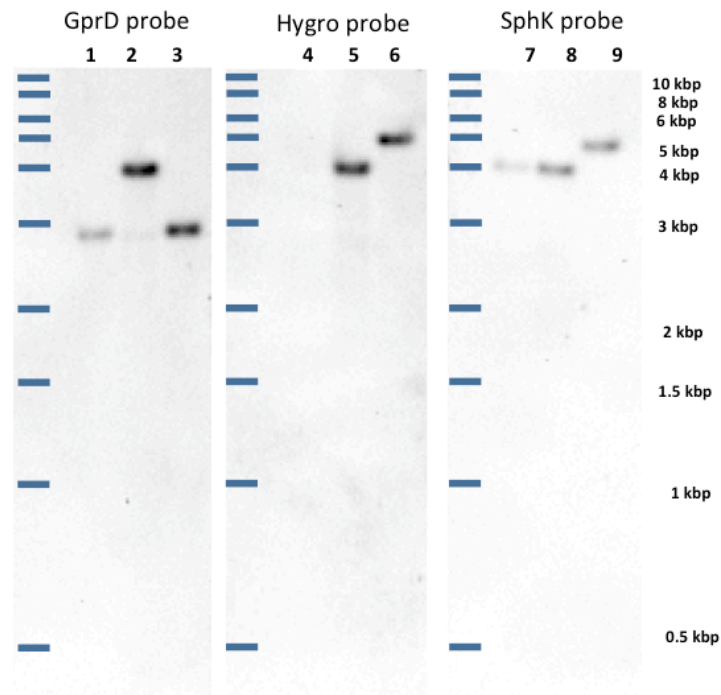


Figure 6.10: Southern blot analysis of *HindIII*-digested chromosomal DNA from *A. fumigatus* WT and mutant strains $\Delta SphK$ and $\Delta gprD$. Lanes 1-3 were probed for GprD. Lane 1, wild-type strain $\Delta ku80$ (expected size 2823 bp); lane 2, mutant strain $\Delta gprD$ (expected size 3941 bp); and lane 3, mutant strain $\Delta SphK$ (expected size 2823 bp). Lanes 4-6 were probed for *Hph*. Lane 4, wild-type strain $\Delta ku80$ (no band expected); lane 5, mutant strain $\Delta gprD$ (expected size 3941 bp); and lane 6, mutant strain $\Delta SphK$ (expected size 4827 bp). Lanes 7-9 were probed for SphK. Lane 7, wild-type strain $\Delta ku80$ (expected size 3793 bp); lane 8, mutant strain $\Delta SphK$ (expected size 3793 bp); and lane 9, mutant strain $\Delta gprD$ (expected size 4827 bp).

6.5 Phenotypic characterisation of *A. fumigatus* $\Delta SphK$ and $\Delta gprD$ mutants

The phenotype of CEA10 and $\Delta ku80$ (WT strains) and $\Delta SphK$ and $\Delta gprD$ mutants (constructed in the $\Delta ku80$ genetic background) were assessed and compared on *Aspergillus* minimal media (AMM) agar. Little is known about the sphingolipid pathway in *A. fumigatus* and as such a range of conditions were tested that had potential for sphingolipid involvement.

Standard AMM buffered to pH 3-8 was tested, including physiologically relevant pH 5 and pH 7.2 relating to the phagosome (513) and the physiological pH of murine lungs respectively (514). Conidia were seeded with 1×10^3 or 1×10^5 conidia per spot, and experiments performed in triplicate. Colonies were assessed for growth differences using ImageJ, as determined by area.

6.5.1 Agar phenotyping of transformants

Firstly, radial growth was assessed by spotting inoculum on AMM and incubating for 48 h at 37°C. Transformant colonies were purified by subculture and spotted at 1×10^3 in 5 μ l on selective AMM containing 180 μ g/ml hygromycin B to maintain selective pressure (Figs 6.11 A-B).

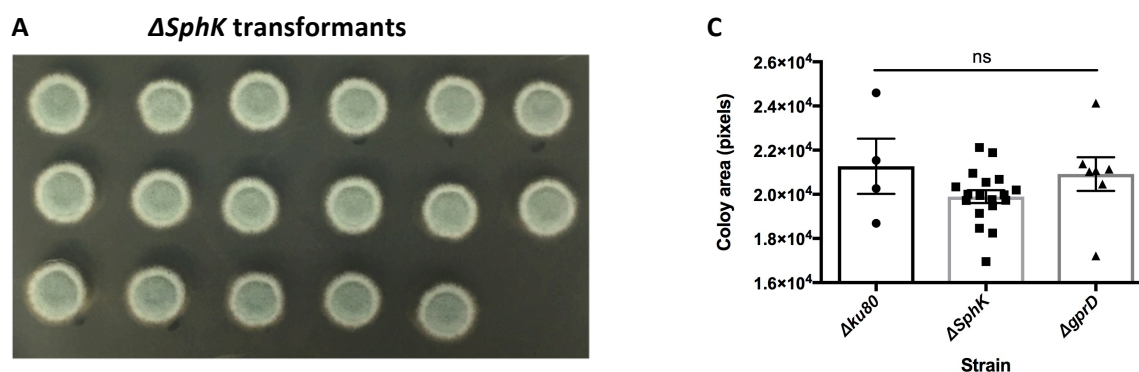


Figure 6.11: Growth of transformants on AMM. After purification 1×10^3 conidia of A) $\Delta SphK$ and B) $\Delta gprD$ transformants were spotted on AMM containing 180 μ g/ml Hygromycin B. Plates were incubated at 37°C and after 48 h colony sizes were examined. Colony sizes were quantified by area using ImageJ. Statistical significance was calculated by ANOVA and expressed as $\pm$ SEM (ns $p=0.1919$).

No significant differences were detectable amongst CEA10, $\Delta ku80$ and respective mutants for colony size (Fig 6.10 C). In addition, no differences were observed in colony undulation, margin, or

pigmentation. It was therefore concluded that SphK and GprD are dispensable for maintenance of normal radial growth rates on standard AMM agar at pH 6.5.

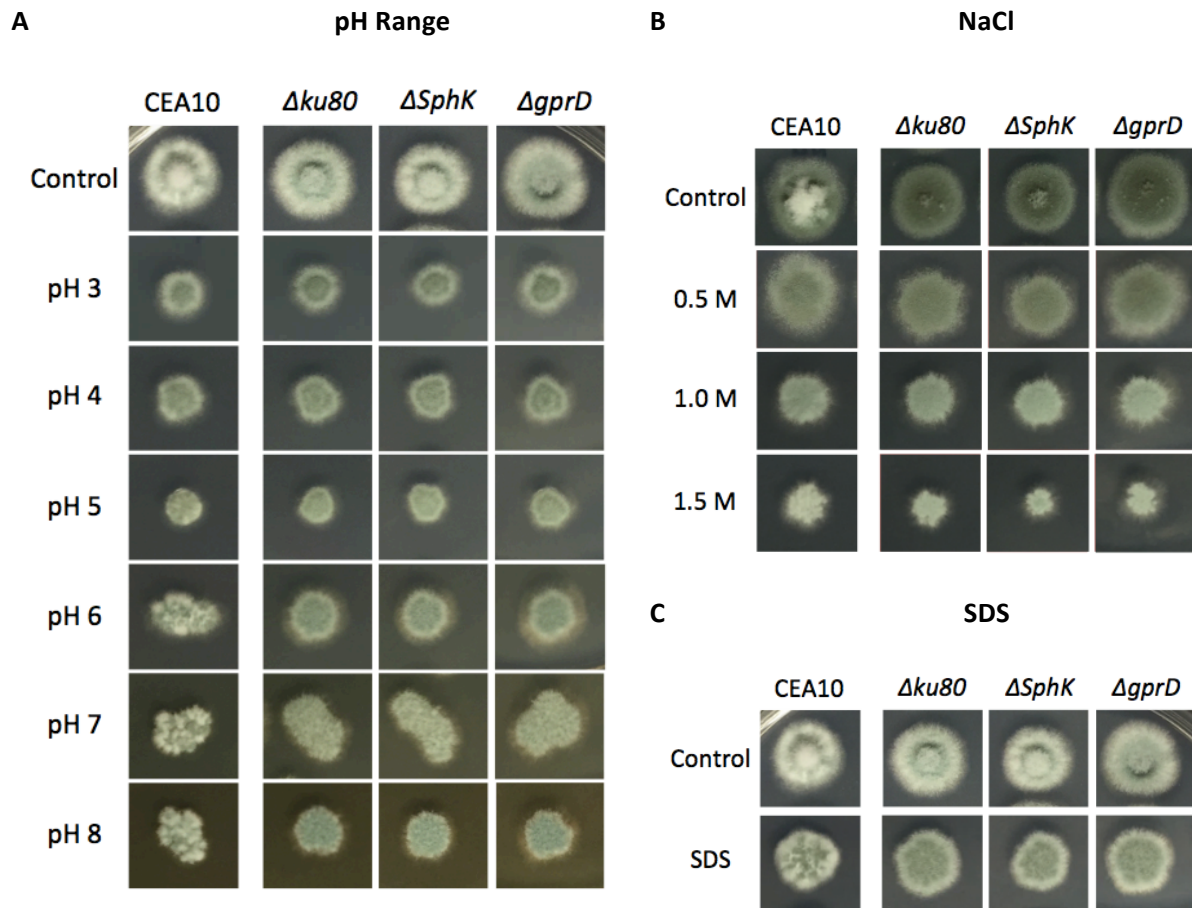


Figure 6.12: Phenotypic analysis of parental and mutant strains in conditions of varying pH, saline and cell wall stress. *A. fumigatus* CEA10 (clinical isolate and control strain), $\Delta ku80$ (parental strain) and respective mutants $\Delta SphK$ and $\Delta gprD$ were harvested and suspended in PBS. AMM was supplemented with A) to pH3-8, B) 0.5, 1.0 and 1.5 M NaCl and C) 50 $\mu\text{g/ml}$ SDS. Plates were inoculated with 1×10^2 per spot in 5 μl and incubated for 48 h at 37°C in a dry incubator. Images are representative of 3 experimental replicates.

Despite a reduction in radial growth in almost all conditions, no significant differences in colony size were observed between CEA10, $\Delta ku80$ or respective mutants during pH, hyperosmotic (by NaCl) or cell wall stress (by SDS) (Fig 6.12 A-C).

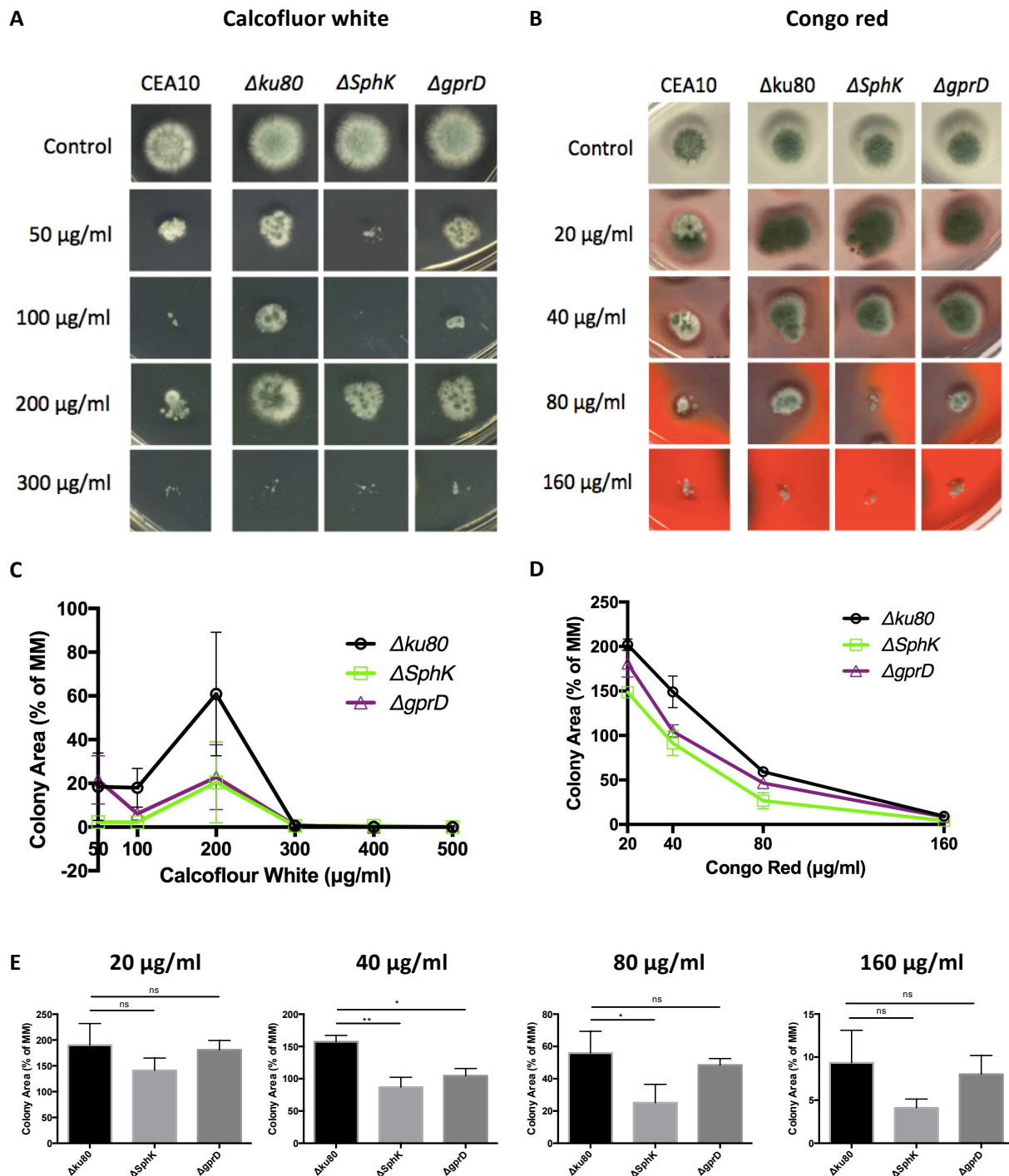


Figure 6.13: Phenotypic analysis of parental and mutant strains in conditions of cell wall stress. *A. fumigatus* CEA10 (clinical isolate and control strain), $\Delta ku80$ (parental strain) and respective mutants $\Delta SphK$ and $\Delta gprD$ were harvested and suspended in PBS. AMM was supplemented with A) calcofluor white at 50, 100, 200 and 300 $\mu\text{g/ml}$ and B) Congo red at 20, 40, 80 and 160 $\mu\text{g/ml}$. Plates were inoculated with 1×10^2 conidia per spot in 5 μl followed by incubation for 96 h at 37°C in a dry incubator. Percentage colony area was measured using ImageJ.

Data are representative of three independent experiments (n=3. Data are expressed as $\pm$ SEM. Statistical analysis by t-test (20 $\mu\text{g/ml}$: $\Delta SphK$ ns $p=0.1538$ and $\Delta gprD$ ns $p=0.7946$; 40 $\mu\text{g/ml}$: $\Delta SphK$ ** $p=0.0026$ and $\Delta gprD$ * $p=0.0115$; 80 $\mu\text{g/ml}$: $\Delta SphK$ * $p=0.0393$ and $\Delta gprD$ ns $p=0.4122$;; and 160 $\mu\text{g/ml}$: $\Delta SphK$ ns $p=0.0913$ and $\Delta gprD$ ns $p=0.6397$).

Figure 6.13 (A-B) shows the results of phenotypic tests for cell wall stress by calcofluor white and Congo red. Observation time was increased for CR and CFW supplemented AMM as colonies took longer to grow on these media. However, vehicle treated colonies over grew over into each other after 72 h and so colony sizes are compare to WT colony sizes at 48 h where they remained discrete.

Calcofluor white significantly reduced radial growth of both mutants at 100 $\mu\text{g/ml}$ and $\Delta SphK$ at 300 $\mu\text{g/ml}$. Although both mutants exhibit inhibition, $\Delta SphK$ shows consistent inhibition. Furthermore, in the presence of 200 $\mu\text{g/ml}$ CFW parental and mutant strains experience a growth resurgence, which was inhibited at the higher concentrations of 300 $\mu\text{g/ml}$ (Fig 6.13 C). Congo red inhibited the growth of both mutants in a dose dependent manner (Fig 6.13 B, D) with statistical significance between 40-80 $\mu\text{g/ml}$ (Fig 6.13 E).

6.5.2 Anti-fungal activity of sphingolipid pathway inhibitors on *A. fumigatus* mutant strains

6.5.2.1 EUCAST minimum inhibitory concentration testing

Minimum inhibitory concentration (MIC) assays were performed for SKI-II (Merck Chemicals) and FTY720 (Selleck) against *A. fumigatus* resting conidia as per EUCAST methodology (445). The drug concentration range studied was 0.125-128 mg/L, as this correlated with previous MICs, as no accepted MIC ranges had been established by EUCAST or CLSI for 100 µl of SKI-II or FTY720. Resting conidia were added to 96-microtiter plates in 100 µl of ddH₂O followed by SKI-II or FTY720 suspended in double strength assay medium, resulting in the desired concentrations specified below. MIC plates were incubated at 37°C for 48 h, at which point they were read for MIC values.

Table 6.9: Minimal inhibitory concentration of FTY720 on *A. fumigatus* parental and mutant strains in accordance EUCAST methodology.

Strain(s)	MIC (mg/L)		MIC (µM)	
	GM	Range	GM	Range
CEA10	10	16, 8, 8, 8	39	46.5, 23, 23, 23
$\Delta ku80$	14	16, 16, 8, 16	42	46.5, 46.5, 23, 46.51
$\Delta SphK$	12	16, 8, 8, 16	35	46.5, 23, 23, 46.5
$\Delta gprD$	16	16, 16, 16, 16	47	46.5, 46.5, 46.5, 46.5

Against *A. fumigatus* CEA10, the geometric mean of FTY720 MICs was 10 mg/L, which was lower than 14 mg/L for parental strain $\Delta ku80$ but still in line with previous MIC data. The MIC value for $\Delta SphK$ was 12 mg/L ($p=0.5370$) corresponding to 35 µM but $\Delta gprD$ had an increased MIC of 16 mg/ml ($p=0.3559$) that corresponds with 47 µM but without statistical significance (Table 6.9). As per previous MICs, SKI-II had no MIC value in any strain.

6.5.2.2 Investigating the susceptibility of *A. fumigatus* mutants to current antifungal agents

As per the previous chapter, MICRONAUT-AM MHK 2 plates were utilized to determine susceptibility to currently available antimycotic agents, against *A. fumigatus* WT and mutants. MICRONAUT microtiter plates contained lyophilised antimycotic drugs at concentrations ranging from 0.015625 - 128 $\mu\text{g/ml}$.

Conidial suspensions consistent with the standard EUCAST MIC inoculations were added in provided RPMI based medium. Data were analysed for evidence and statistical significance of drug resistance or susceptibility.

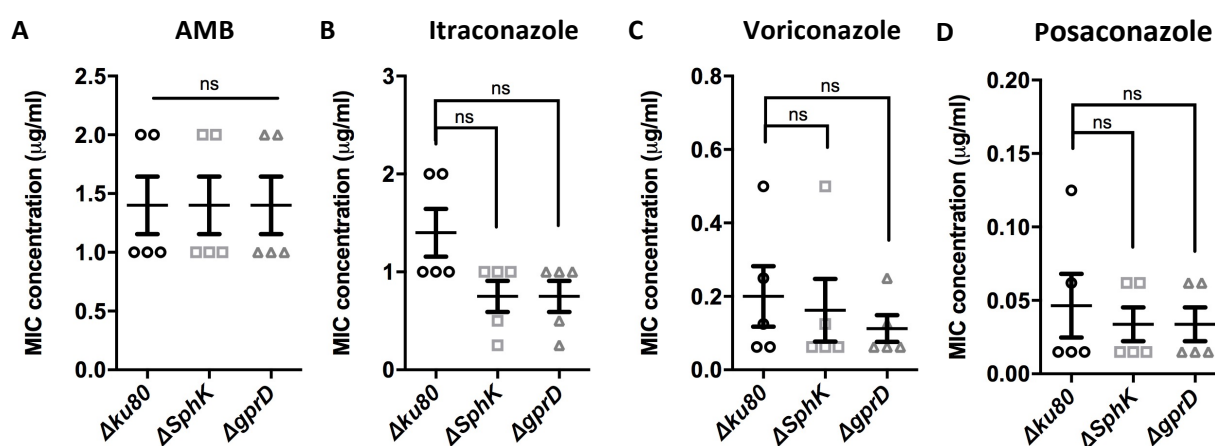


Figure 6.14: Minimal Inhibitory Concentrations (MICs) of anti-mycotic compounds against parental and mutant strains in liquid medium. *A. fumigatus* resting conidia from parental ($\Delta ku80$) and mutant strains ($\Delta sphK$ and $\Delta gprD$) were added to 96-well microtiter plates containing varying concentrations of anti-mycotic compounds. Plates were incubated at 37°C for 48 h and MICs were determined according to EUCAST methodology. Compounds that exhibited MICs against the strains were A) AMB, B) Itraconazole, C) Voriconazole and D) Posaconazole. Data are representative of four independent experiments (n=4). Statistical significance was calculated by t-test, except AMB which was calculated by ANOVA. Statistics are expressed as $\pm$ SEM (A: ns $p=0.3966$, B: $\Delta sphK$ ns $p=0.0563$ and $\Delta gprD$ ns $p=0.0563$, C: $\Delta sphK$ ns $p=0.7598$ and $\Delta gprD$ ns $p=0.3600$, and D: $\Delta sphK$ ns $p=0.6213$ and $\Delta gprD$ ns $p=0.6213$).

The susceptibility and resistance break points for AMB and Itraconazole are ≤ 1 mg/L and > 2 mg/L respectively. No statistical differences in MIC values between parental ($\Delta ku80$) and mutants ($\Delta sphK$ and $\Delta gprD$) strains were observed and all were susceptible. Voriconazole had varying results in the

WT and posaconazole was also varied in MIC across the strains, but despite this disparity data remained within susceptibility limits of ≤ 0.12 mg/L and ≤ 1 mg/ml respectively for susceptibility.

6.5.3 Growth curve analysis

To determine hyphal growth differences between parental ($\Delta ku80$) and mutant strains ($\Delta SphK$ and $\Delta gprD$) the hyphal growth turbidity assay developed by Meletiadis *et al.*, (2001) was performed. 96-well microtiter plates were inoculated with 2.5×10^3 resting conidia in liquid medium and sequential turbidity measurements were captured at 30 mins intervals.

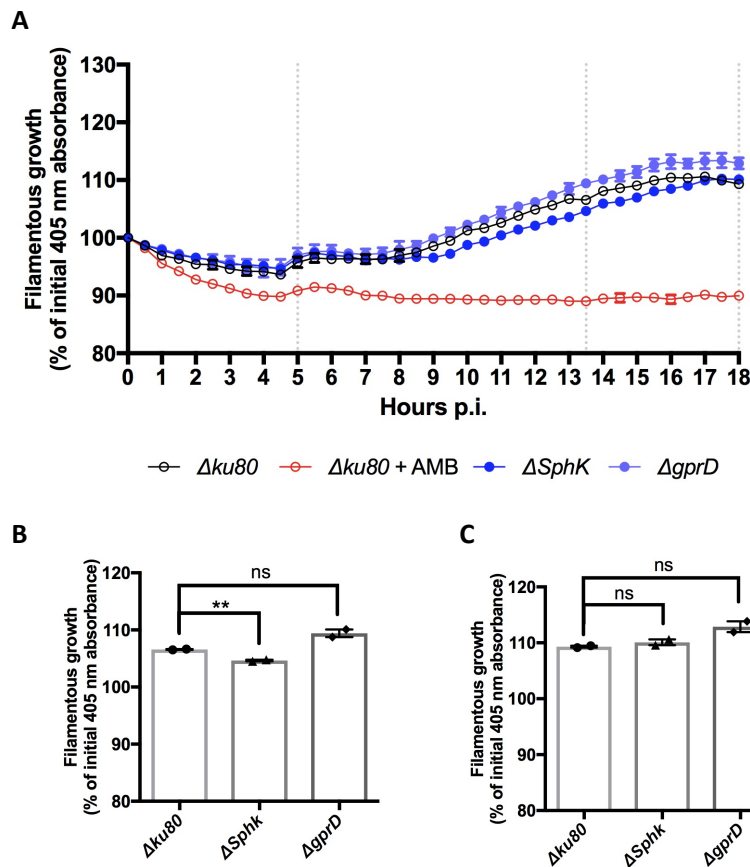


Figure 6.15: Filamentous growth of parental and mutant strains in liquid medium.

Resting conidia from parental ($\Delta ku80$), and mutant ($\Delta SphK$ and $\Delta gprD$) strains were added to 96-well microtiter plates in cRPMI. Microtiter plates were inoculated with 2.5×10^3 resting conidia per well and incubated at 37°C for 24 h with 405 nm absorbance readings were taken by spectrophotometer at 30 min intervals. Data are shown as A) filamentous growth curve over 0-16 h (Black, $\Delta ku80$; blue, $\Delta SphK$; light blue, $\Delta gprD$; red, AMB), B) filamentous growth at 13.6 h, and C) filamentous growth at 18 h. Data are triplicate cultures expressed as

$\pm$ SEM. Statistical analysis by t-test (B: $\Delta SphK$ ** $p=0.0061$ and $\Delta gprD$ ns $p=0.0525$, and C: $\Delta SphK$ ns $p=0.2915$ and $\Delta gprD$ ns $p=0.0681$).

The negative slope at the beginning of the assay is assumed to be due to conidia settling to the bottom of the well. Subsequently, after an initial lag phase the cultures began elevating in turbidity from 5 h and consistently from 8 h (Fig 6.15 A). During the succeeding period cultures remained in log phase increasing continuously until 17 h. At mid-log phase (13.5 h), $\Delta SphK$ cultures had

significantly reduced turbidity (Fig 6.15 B). After log phase at 18 h, no statistically significant differences in absorbance were observed comparing mutants to WT (Fig 6.15 C).

6.5.4 Germination analysis by widefield microscopy

6.5.4.1 Effects of *SphK* and *gprD* deletion on germination kinetics of *A. fumigatus*

As per the previous chapter, microscopic analysis of germinating conidia in liquid AMM of $\Delta ku80$ parental and $\Delta SphK$ and $\Delta gprD$ mutant strains was performed by widefield microscopy.

Images were captured at 20 min intervals, and analysed for germination stages (Fig 6.16 A-C) as well as germinated morphotypes (Fig 6.17 A-C). Subsequently, absorbance measurements of each condition were taken by spectrophotometer to determine fungal biomass (Fig 6.18 A-D).

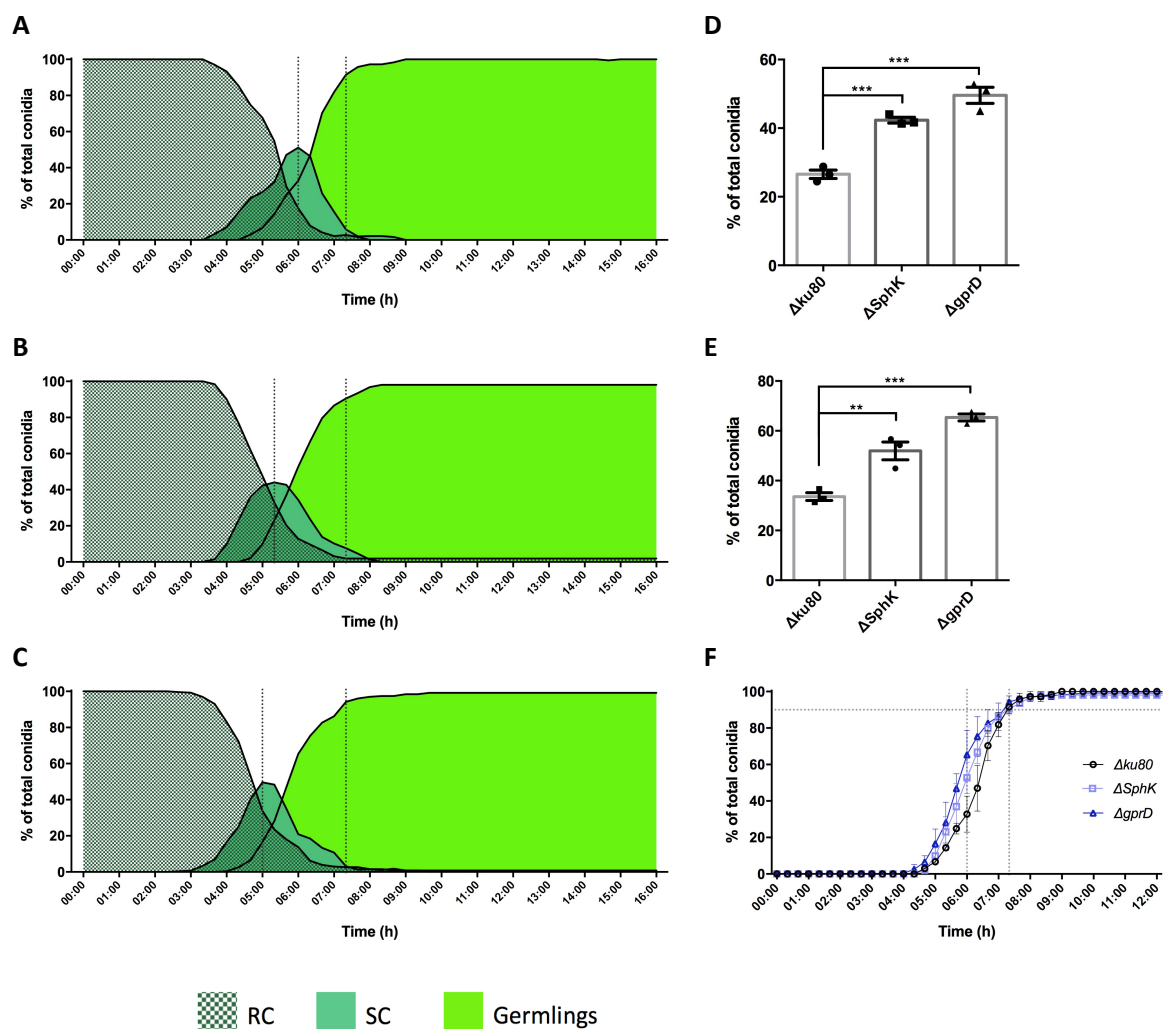


Figure 6.16: Germination kinetics of parental and mutant strains. *A. fumigatus* resting conidia from parental ($\Delta ku80$) and mutant strains ($\Delta SphK$ and $\Delta gprD$) were added to 96-well microtiter plates and images of germinating conidia were taken at 20 min intervals by Widefield

microscopy. Growth kinetics were assessed and categorized by swelling, germination and germ tube formation for A) $\Delta ku80$, B) $\Delta SphK$, and C) $\Delta gprD$. Dark green, green and light green represent resting, swollen and germinated conidial populations respectively. Percentage of D) swollen conidia at 5 h, E) germinated conidia at 6 h, and F) Comparison of germination rates between parental Vs. mutant strains (dotted line $\geq 90\%$ germinated). Data are expressed as $\pm$ SEM (150 conidia observed in total per condition). Statistical analysis by t-test (D: $\Delta SphK$ *** $p=0.0005$, $\Delta gprD$ *** $p=0.0010$, and E: $\Delta SphK$ ** $p=0.0095$, $\Delta gprD$ *** $p=0.0001$).

The time taken to transition from 100% resting conidia to a peak in the number of SC is greatest in $\Delta ku80$ (WT) occurring at 06:00 (Fig 6.16 A), whereas for $\Delta SphK$ and $\Delta gprD$ this occurred at 05:20 (Fig 6.16 B) and 05:00 (Fig 6.16 C) respectively. This transition period from resting to swollen (before log phase germination), is the most effective time for phagocytic clearance, and therefore if shortened could provide advantage for the pathogen. $\Delta SphK$ conidia begin swelling at the same time as $\Delta ku80$ but peak in number 05:20 (40 minutes earlier). Therefore, the time it takes this mutant to transition from a peak in SC to $\geq 90\%$ germinated is 40 min longer. However, $\Delta SphK$ begins conidial germination and reaches $\geq 90\%$ germinated at the same time as $\Delta ku80$.

$\Delta gprD$ conidia start swelling 1 h earlier than $\Delta ku80$ (Fig 6.16 A, C), and accordingly the mutant has a peak in SC also 1 h earlier. Fig 6.16 D shows that at 05:00 (when $\Delta gprD$ SC conidia peak in number), $\Delta ku80$ and $\Delta SphK$ are significantly delayed. In addition, the germination of $\Delta gprD$ initiates 40 mins earlier than $\Delta ku80$, but surprisingly reaches $\geq 90\%$ germination at the same time as the parental strain. Consequently, due to its shift in earlier swelling kinetics $\Delta gprD$ strain has the longest transition time from a peak in SC to being $\geq 90\%$ germinated at 2 h and 20 mins, 1 h longer than parental $\Delta ku80$ strain.

In addition, during mid log phase growth at 6 h $\Delta gprD$ and $\Delta SphK$ have significantly more conidia germinated than parental $\Delta ku80$ (Fig 6.16 E) but at 07:00 all strains simultaneously reach $\geq 90\%$ germination (Fig 6.16 F). These data suggests that $\Delta ku80$ has a steeper rate of germination towards the end of log phase growth.

6.5.4.2 Germling morphotypes

In the previous chapter it was observed that FTY720 and SKI-II induced changes in the occurrence of the germinated morphotypes designated as unipolar, dipolar and tripolar germings (Fig 5.7 D).

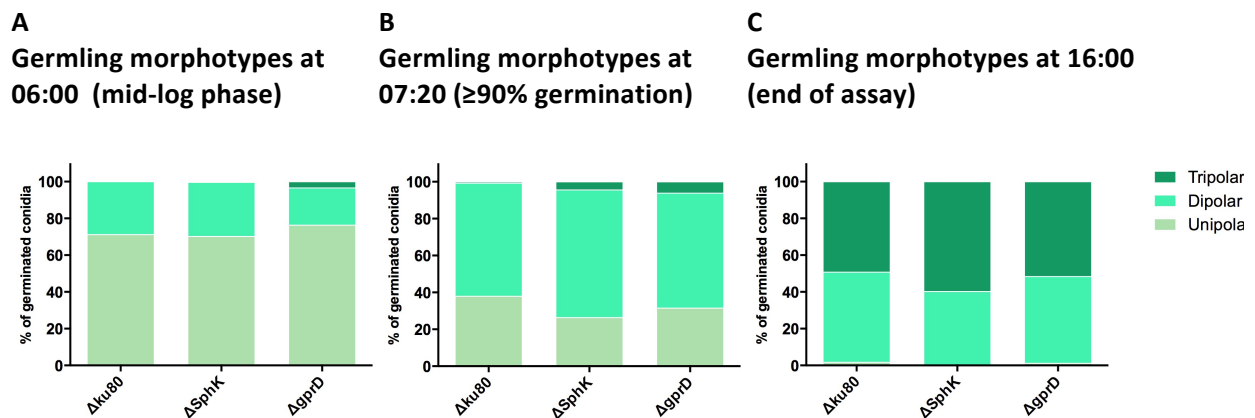


Figure 6.17: Number of emerging conidial germ tubes in parental and mutant strains. A. *Aspergillus fumigatus* resting conidia from parental ($\Delta ku80$) and mutant strains ($\Delta sphK$ and $\Delta gprD$) were added to 96-well microtiter plate and images of germinating conidia were taken at 20 min intervals by Widefield microscopy, and the number of emerging germ tubes from each conidia was quantified from 0-16 h. Germ tube emergence was analysis at A) 06:00 (mid-log phase), at B) 07:20 (when conidia were $\geq 90\%$ germinated) and at C) 16 h (stationary phase). Categorization of germinated conidial morphotypes is as unipolar, dipolar and tripolar+ germings. Data are from 2 independent experiments with 3 technical replicates (150 conidia each strain).

During mid-log phase growth at 6 h (Fig 6.17 A), a higher presence of unipolar germings was observed for $\Delta gprD$. This could account for the increased rate of germination of $\Delta gprD$ (as the resources of the conidium are focused on a single hyphal projection) where conidia would otherwise have germinated with two hyphae simultaneously. However, it was observed that conidia also develop through the stages of unipolar, dipolar and tripolar over time.

The same pattern of germling morphotypes is followed by $\Delta sphK$ at the later time point of 7 h 20 min (Fig 6.17 B) where all strains (parental and mutants) were $\geq 90\%$ conidial germinated.

At 16 h (8 h 40 mins after) all strains are approximately uniform in their morphotype populations, suggesting that the increased level of tripolar germlings of *ΔgprD* strain earlier could be a result of faster germination kinetics.

6.5.4.3 Fungal Biomass

After a 16 h incubation in AMM for analysis of germination kinetics, the optical density of the fungal cultures was determined by 405 nm absorbance readings by spectrophotometer to determine fungal biomass.

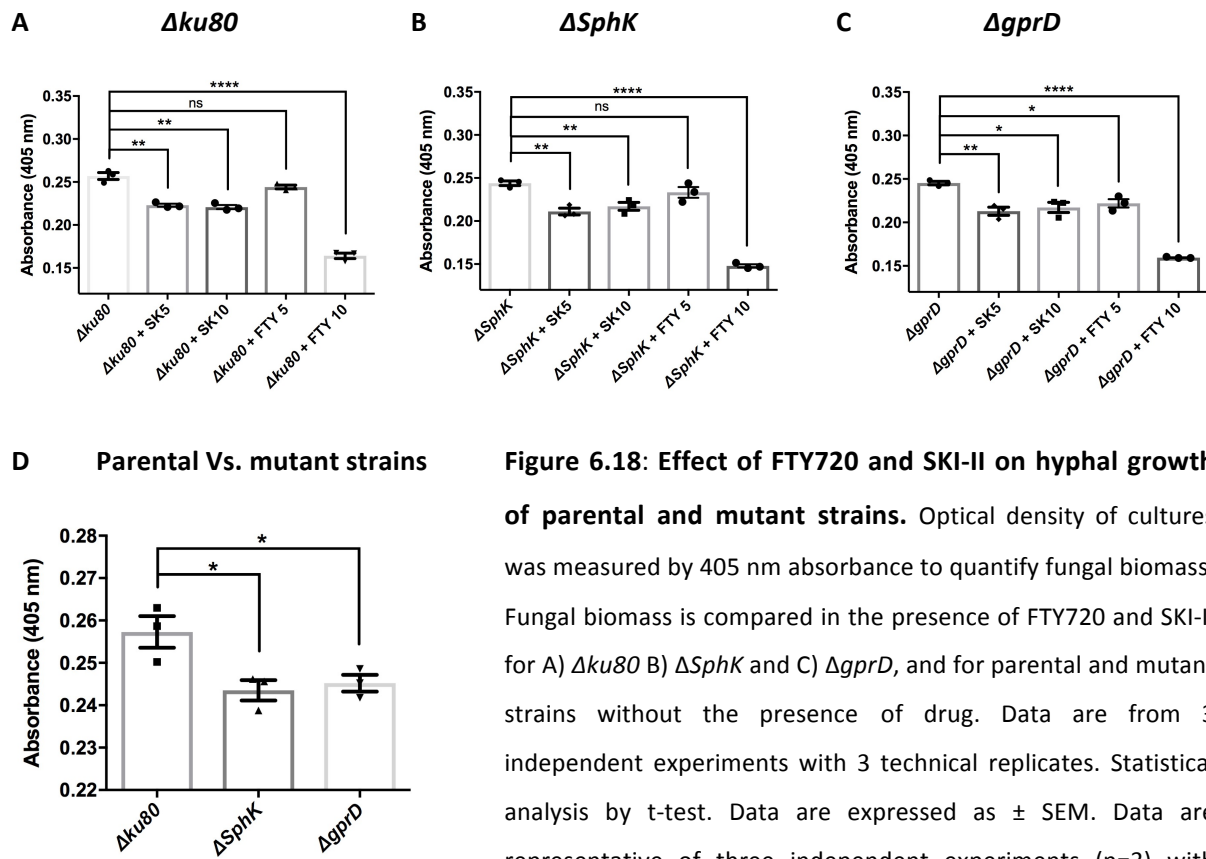


Figure 6.18: Effect of FTY720 and SKI-II on hyphal growth of parental and mutant strains. Optical density of cultures was measured by 405 nm absorbance to quantify fungal biomass.

Fungal biomass is compared in the presence of FTY720 and SKI-II for A) $\Delta ku80$ B) $\Delta SphK$ and C) $\Delta gprD$, and for parental and mutant strains without the presence of drug. Data are from 3 independent experiments with 3 technical replicates. Statistical analysis by t-test. Data are expressed as $\pm$ SEM. Data are representative of three independent experiments (n=3) with triplicate cultures. Data are expressed as $\pm$ SEM. Statistical

analysis by t-test A: ns $p=0.0517$, **** $p<0.0001$, ** $p=0.0016$ and ** $p=0.0016$, B: ns $p=0.1931$, **** $p<0.0001$, ** $p=0.0022$ and ** $p=0.0074$, C: * $p=0.0108$, **** $p<0.0001$, ** $p=0.0030$ and * $p=0.0107$, and D: $\Delta SphK$ * $p=0.0362$ and $\Delta gprD$ $p=0.0458$).

The parental strain $\Delta ku80$ showed some inhibition at 5 μM FTY720 and very significantly at 10 μM , while both 5 and 10 μM SKI-II had equally significant retardation on biomass (Fig 6.18, A). While both mutant strains $\Delta SphK$ and $\Delta gprD$, yielded the same pattern of response as parental strain, $\Delta gprD$ had significantly more inhibition in response to the lower FTY720 concentration of 5 μM (Fig 6.18, B-C).

In contrast to radial growth and hyphal extension in RPMI medium, *SphK* and *gprD* gene deletion resulted in growth inhibition. When parental and mutant strains are compared without the presence of FTY720 or SKI-II (Fig 6.18, D), a significant reduction in mycelial biomass was observed. These data

suggest that although germination rate is similar, the mycelial biomass density is lower. It was therefore hypothesised that gene *SphK* and *gprD* deletions may render mutants more susceptible to macrophage killing.

6.6 Immunogenicity and survival of $\Delta SphK$ and $\Delta gprD$ mutant strains during macrophage challenge

The phenotypic differences observed between parental and $\Delta SphK$ and $\Delta gprD$ mutants led to the investigation of their interaction with murine macrophages. Macrophages are the first line of defence against inhaled *Aspergillus* conidia, interacting in the airway as professional phagocytes and are crucial for rapid clearance (401).

Resting conidia are not immunogenic and do not induce TNF- α expression (data not shown). In contrast, swollen conidia have exposed PAMPS that interact with host PRRs initiating a host inflammatory response such as the release of cytokines.

To examine the inflammatory TNF- α response of macrophages to *A. fumigatus* mutants $\Delta SphK$ and $\Delta gprD$ *in vitro*, J774A.1 murine macrophages were challenged with swollen conidia at an MOI of 3:1 for 6 h. Supernatants were analysed by ELISA (R&D systems) to quantify secreted TNF- α , and analysed for the presence of *Aspergillus* galactomannan particles using Platelia™ *Aspergillus* Ag kit (BIO-RAD).

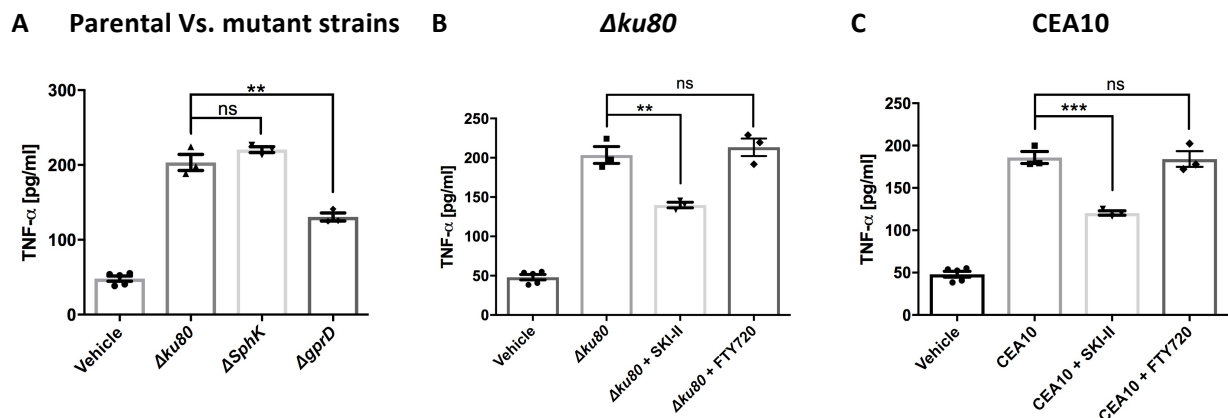


Figure 6.19: Murine macrophage inflammatory response to *A. fumigatus* parental and mutant strains. J774A.1 cells were co-cultured with *A. fumigatus* conidia at an MOI of 3:1 for 6 h with A) parental $\Delta ku80$ strain and mutants, and WT strains $\Delta ku80$ and CEA10 +/- 5 μ M FTY720 or SKI-II (vehicle was DMSO). Supernatants were analysed for TNF- α by ELISA. Data are representative of three independent experiments (n=3), each with triplicate cultures. Data are expressed as $\pm$ SEM (A: ns p=0.2092 and ** p=0.0037, B: **, p=0.0050 and ns p=0.5579, and C: ***, p=0.0009 and ns p=0.8824)

$\Delta SphK$ induced much the same TNF- α response as WT, unlike swollen conidia of the $\Delta gprD$ mutant that stimulated a significantly reduced TNF- α response (Fig 6.19 A). Whereas macrophages challenged with WT strains showed reduction in TNF- α with SphK inhibition (with SKI-II) but no effect with FTY720 (Fig 6.19 B-C) at 6 h.

To determine if a reduction in the inflammatory response of macrophages against *A. fumigatus* mutants ($\Delta SphK$ and $\Delta gprD$) was a result of increased or decreased killing by macrophages, supernatants were analysed for the presence of shed *Aspergillus* galactomannan antigens (Fig 6.20).

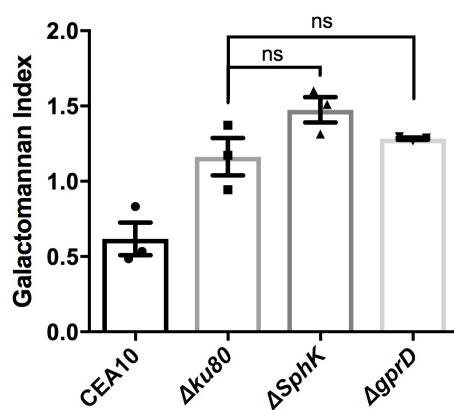


Figure 6.20: Galactomannan particles shed from parental and mutant strains in culture with murine macrophages. J774A.1 cells were co-cultured with *A. fumigatus* swollen conidia at an MOI of 3:1 for 6 h. Quantitative determination of galactomannan particles in supernatants was performed by Platelia ELISA. Data are representative of 3 independent experiments (n=3), with 3 technical replicates. Statistical analysis by t-test. Data are expressed as $\pm$ SEM (ns p=0.1059 and ns p=0.3879).

Across parental and mutant strains no statistical significance of galactomannan antigen was detected.

6.7 Discussion

The role of sphingolipids has been studied intensely in yeast and somewhat in *A. nidulans* but not in detail in pathogenic fungi such as *A. fumigatus*. The body of fungal sphingolipid research indicates that they have important roles in germination, growth, reproduction, and potentially for virulence (474,498–501). Initial experiments on AMM revealed no growth rate defects in either mutant with regards to colony size, suggesting that neither SphK or GprD expression is essential for normal rates of radial growth. This was unexpected as FTY720, the hypothetical ligand for GprD, exhibits an MIC against *A. fumigatus*. An explanation for this could be genetic redundancy of GprD, or a compensatory mechanism (preventing germination). Further to growth on AMM agar, all strains were then screened for growth defects under a range of environmental stresses to identify processes in which sphingolipids play a role, such as pH tolerance and the cell wall integrity pathway. Corresponding stress conditions were employed that include pH, hyper-osmolarity (by NaCl), and cell wall stress (by SDS, CR and SDS/CFW).

pH tolerance is an important virulence factor for *A. fumigatus*, as a natural saprophyte it can be found in decaying vegetation and soil where it is reported to exhibit optimal growth between pH 3.7-7.6. Fungi have a generally accepted range of growth between pH 3-8 (515)(515), but more importantly there are deleterious pH challenges in the host environment, namely exposure to acidification in macrophages. On first contact *A. fumigatus* conidia are exposed to the airway surface liquid in a healthy murine lung which is physiologically neutral with an average of pH 7.28 (514). A study by Hywiben *et al.*, (2011) indicated that the phagolysosome of murine macrophages containing *A. fumigatus* conidia was between pH 6-7, and furthermore, phagolysosomes in epithelial cells containing *Aspergillus* conidia have been reported to be as low as pH 4 *in vitro* (338). Additionally, at pH 4 inhibition of complex sphingolipids has been demonstrated to impede fungal growth in *C. neoformans* (498). The link between pH tolerance and *Aspergillus* virulence has been well investigated (517,518), and as such the growth of $\Delta SphK$ and $\Delta gprD$ mutant strains was observed across a wide pH range from alkaline pH 8, to phagosomal related pH 4-7, and down to acidic pH 3. However, despite links between complex sphingolipid and pH tolerance, no significant differences were observed over the pH range, and as such it is assumed that the physiological stress exerted by changes in pH upon *A. fumigatus* is not dependent on *SphK* or *gprD*.

Hyperosmotic stress in yeast activates the high-osmolarity glycerol response (HOG) mitogen-activated protein (MAP) kinase pathway in a saline environment as demonstrated by Tanigawa *et al.* (2012), who showed that depletion of sphingolipids caused the HOG pathway activation and

addition of sphingolipid pathway intermediates resulted in its inactivation. Thus, if sphingolipid depletion is a normal osmotic adaptive stress response, inhibition of down stream sphingolipid interacting proteins (such SphK and GprD) should have no affect on its ability to grow unless *A. fumigatus* transformation had off target affects on genes related to the regulation of hyperosmotic adaptive responses. However, in response to NaCl growth was reduced, but still permitted and no differences in growth were seen between parental and mutant strains, suggesting that intermediate fungal sphingolipids such as SphK and GprD are not required in response to the hyperosmotic stress.

Mutant strains proved to be more susceptible to cell wall stress. SDS is a denaturing detergent that dissolves cell membranes, typically used in the process of solubilizing and purifying membrane proteins. When used as a perturbing agent it can select for cells with cell wall defects (520), that allow it to penetrate through and act on the cell membrane (521). On account of sphingolipids being employed to stabilize cell membranes in response to heat and being components of lipid rafts, it was hypothesized that a genetic perturbation to sphingolipid signaling in combination with SDS could result in reduced growth. However, no differences in radial growth was observed, suggesting that either downstream sphingolipids are not essential in adaptive responses to cell wall/membrane perterbation when grown on solid media or that there are compensatry mechanism.

With regards to direct cell wall stress, it was hypothesised that if fungal cell wall biosynthesis, which is known to be affected by up stream sphingolipid inhibition (on a different branch) i.e. IPC, would it also be affected if the inhibition was mid or down stream of sphingolipid biosynthesis i.e. SphK and GprD. Thereby, probing whether both branches of sphingolipids are important for fungal growth. Cell wall stressors calcofluor white (CFW) and Congo red (CR) act on cell wall by interaction with beta-glucans and chitin, thus interfering with its construction (522). Both CFW and CR are known to interact with β 1,3-glucans *in vitro* (523), with CFW directly interacting with nascent chitin microfibrils (521,524) and to a lesser extent glucans (520). It is consequently postulated that these interactions inhibit the linking of β -glucans and chitin, thus interfering with cell wall assembly by the impeding linkage of these essential components (522). Sphingolipids have also been shown to be important for cell wall biosynthesis via inhibition of polarized hyphal extension. A study by Cheng *et al.*, (2001) in *A. nidulans* demonstrated that *de novo* sphingolipid biosynthesis is required for both establishment of polarity in germinating conidia and maintenance of cell polarity in already established germlings via control of the actin cytoskeleton. In addition, this perturbation promoted significant atypical hyphal tip branching. It was therefore expected that CFW and CR, in addition to

sphingolipid pathway protein deletions, would be additive in compounding growth inhibition and this is what was observed.

Similarly in *S. cerevisiae*, it was demonstrated that blocking the synthesis of IPC mimicked inositol starvation by activation of protein kinase C signaling which resulted in phosphorylation of Slt2p, the terminal MAPK in the CWI pathway (525). Furthermore, in *A. niger* a transcriptomic adaptation after inhibition of IPC synthase (by AbaA), had 236 differentially expressed genes, with genes relating to chitin, α - and β -1,3-glucan synthesis and actin polarization being significantly up-regulated. This also resulted in up regulation of diacylglycerol (DAG), a secondary messenger in both mammals and fungi, known to activate protein kinase C of the CWI pathway (526,527). Thus, although inhibition of sphingolipids can elicit differential gene expression they collectively activate the same CWI stress response pathway. Therefore, it is unsurprising that growth is slowed even further when cell wall stressors and sphingolipid inhibition is combined. In conclusion, the data from this study are in agreement with observations made in yeast and other *Aspergillus spp*, in suggesting that cell wall integrity and polar growth are not just dependent on inositol sphingolipids but also non-inositol sphingolipids such as those produced by sphingosine kinase, an otherwise uninvestigated enzyme in a different arm off of the sphingolipid biosynthesis pathway.

MIC assays were performed with resting conidia of *A. fumigatus* mutants in an attempt to validate the targets for SKI-II and FTY720. SKI-II exhibited no MIC against WT or mutant strains, which could suggest that SphK is the target of SKI-II (as there is no growth defect with mutant alone) and equally that SKI-II has little/no chemical interaction with fungal sphingosine kinases, or that SphK is dispensable for normal growth. An elevated MIC observed in $\Delta gprD$ against FTY720, could suggest that GprD is the target of FTY720, and that in its absence compensatory mechanisms/pathways are activated. The lack of growth inhibition could also indicate that there are no significant 'off target' effects by FTY720 in *A. fumigatus*.

With regards to multidrug testing, no synergistic or additive affects of SKI-II or FTY720 were seen on mutant strains compared to parental. MICs were observed for AMB and azoles. A lack of lowered MIC values with mutants suggests that neither SphK nor GprD are not significantly involved in signaling pathways in response cell wall stress caused by AMB or azoles.

Lastly, due to similar existing molecules to SphK and GprD in *A. fumigatus*, namely phytosphingosine kinase and other GPCRs, there is a potential for functional redundancy, whereby related proteins

that are mechanistically similar maintain cellular signaling requirements, resulting in a dampening or a lack of phenotype in response to gene deletion.

A growth curve analysis performed in cRPMI to assess the fitness of the mutant strains under cell culture conditions showed that despite an early growth retardation of $\Delta SphK$ (compared to parental strain) during mid-log phase, no statistically significant differences were observed between strains at 18 h. This suggests that hyphal extension rate at the end of log phase growth is unaffected by gene deletions possibly due to alternative signaling diversion or functional redundancy of SphK and GprD, although $\Delta gprD$ strain growth appears slightly elevated.

Germination kinetics data show that deletions in $\Delta SphK$ and $\Delta gprD$ permit earlier conidial swelling and subsequent potential for earlier germination. However, despite swifter rates of development initially, both parental and mutant strains achieve $\geq 90\%$ germination simultaneously at 7 h 20 min. in accordance to growth curve data, this suggests that while some processes are expedited during the breaking of dormancy, there are no significant differences in overall germination rate across parental and mutant strains at later time points. Similar to data in the previous chapter, WT and mutants follows the same pattern of germling morphotypes, with $\Delta SphK$ increasing tripolar emergence like SKI-II when $\geq 90\%$ of conidia were germinated and at 16 h, but $\Delta gprD$ showed similar levels to parental strain.

The ability of mutant strains to generate an inflammatory response from murine macrophages was investigated and its corresponding fungal biomass determined by shed galactomannan particles. $\Delta SphK$ -mediated TNF- α was similar to WT, and correspondingly no difference from WT in galactomannan particles were detected. However, $\Delta gprD$ -mediated TNF- α was significantly reduced and this was not due to a reduction in fungal burden as shed galactomannan particles were not significantly different from WT. Therefore, the reduction in immunogenicity could be attributed to a reduction in the constitution of cell wall PAMPS (perturbing normal recognition by the host), or that the cell wall is more fragile in the $\Delta gprD$ mutant and as such are more easily killed resulting in less TNF- α . This hypothesis is consistent with the literature in that membrane sphingolipids are known to act functionally in aiding transport and consolidation of membrane proteins (like receptors) to lipid rafts to aid pathogen recognition. Testing this hypothesis and understanding immunogenicity in term of GprD receptor deletion would require further study to correspond cell wall components and utility of GprD in cell wall construction and corresponding host signaling. This hypothesis is also consistent with $\Delta gprD$ exhibiting increased sensitivity to cell wall stress.

In summary, *SphK* and *gprD* genes were deleted in *A. fumigatus* to investigate their involvement in germination, hyphal growth and immunogenicity. It was found that mutant strains displayed similar growth rates to parental strain, in both solid and liquid medium, but differential germination kinetics and increased sensitivity to cell wall stressors. Thus, SphK and GprD could provide some tolerance to cell wall perturbations but despite this, sensitivity was observed in MICs with cell wall perturbing antifungals like polyenes (AMB) and azoles. Although, more subtle sensitivities to antifungals could potentially be detected by testing a smaller concentration range across the plate, as per the previous chapter.

In spectrophotometric data, when mutants were cultured without macrophages in liquid AMM there was a significant decrease in fungal biomass after 16 h in both strains, but when cultured in cRPMI no difference between mutants and parental strains was observed at 16 h, demonstrating that growth defects are only detectable in a nutrient depleted environment.

With regards to fungal virulence, when strains were cultured with macrophages similar levels of galactomannan by mutants and parental strains were detected, suggesting a similar level of fungal survival. However, in spite of its survival $\Delta gprD$ induced significantly less TNF- α , which could be due to subversion of the immune response, i.e. exposure of less immunogenic PAMPs or a more fragile cell wall that lead to easier killing. To further investigate these findings, sensitivities to H₂O₂ (as in macrophage phagosomes) could be investigated. This is particularly relevant as phagosomes are nutrient scarce environments and this has been demonstrated to reduce mycelial mass. Additionally, survival of immune suppressed mice infected with $\Delta gprD$ could be used to determine *in vivo* virulence.

Together these data suggest that the fungal enzyme SphK and GprD g-protein coupled receptor, are of somewhat minor importance for cell wall integrity and or have functional redundancy to other similar proteins *in vitro*. In addition, loss of function of the *SphK* and *gprD* genes may inhibit overall mycelial biomass but not germination rate, and as such its role in virulence is unclear.

7. Conclusions

S1P is a pleiotropic lipid signaling molecule that is involved in cell proliferation, migration, cytoskeletal rearrangement adhesion and inflammation in innate and adaptive immune cells (480). In this thesis, the inhibition of *A. fumigatus*-mediated inflammatory responses was investigated *in vitro* and *in vivo*. The antifungal effects of S1P signalling inhibitors were also assessed for potential antifungal properties against *A. fumigatus*.

Analysis of *A. fumigatus*-mediated responses in macrophages showed that both SphK inhibition and S1P₁ antagonism resulted in a down-regulation of inflammatory signalling *in vitro*, without affecting fungal killing. TNF- α is a pleiotropic cytokine that is important in initiating an inflammatory immune response (528,529). In this study TNF- α was markedly reduced in macrophages by SphK inhibition with SKI-II and FTY720. This is in accordance with reports that S1P plays important roles in TNF- α signalling through interaction with adapter protein TRAF2 (530) and potentially in PAMP signaling with TRAF6 (531). Moreover, S1P-mediated TNF- α signaling has been implicated in a variety of inflammatory conditions. For instance, TNF- α is crucial for acute systemic inflammatory conditions like sepsis (532) and chronic autoimmune disorders such as rheumatoid arthritis and atherosclerosis (533). TNF- α is a signature pro-inflammatory mediator in sepsis associated with severe tissue injury (534). SphK1 has been reported as an important regulator of acute inflammatory responses in sepsis, as mice with genetic deletion of *Sphk1* have significantly reduced lethality of LPS-induced sepsis (535). The inflammatory condition of rheumatoid arthritis (RA) is characterised by inflammation in moveable joints (41), and high levels of S1P have been detected in the synovial fluid of RA patients (536). In a murine model of TNF-induced chronic inflammatory arthritis with SphK genetic deletion, the synovial and periarticular inflammation was reduced (537). Also in this model fewer mature osteoclasts were found in the ankle joint with significantly less severe clinically evident swelling and deformity. Together these murine models demonstrated that abolition of SphK1-derived S1P attenuated acute inflammation and had utility in management of chronic inflammatory disorders. These reports and the data in this study suggested that disruption of S1P signalling *in vivo* could temper the pathological inflammation associated with IPA.

The phagocytic activity of macrophages was also assessed, and surprisingly, while both S1P pathway inhibitors showed impairment to macrophage–cytokine responses, SKI-II was detrimental to uptake of resting conidia. Interestingly, increased phagocytosis of *C. neoformans* by AMs of SphK1^{-/-} mice has been reported when the fungal cells had been prior opsonized with an IgG1 antibody (59). It has

also been observed that the addition of extracellular S1P increased antibody-mediated phagocytosis through S1P₂ and that S1P₂^{-/-} mice had decreased Fcγ receptor expression, thereby consequently ingesting fewer *C. neoformans* compared to WT (538). The fact that S1P is required for fungal uptake of *C. neoformans* could go some way to explaining the reason for SKI-II inhibiting conidial phagocytosis, in that it inhibits both SphKs. FTY720 however induced no reduction in phagocytic index of *A. fumigatus* and this could be explained by a study that showed that FTY720-p has significant S1P₂ receptor agonist activity (539).

Overall, these data suggest that while the potent inhibition of cytokines by SKI-II is ideal for acute inflammatory conditions that are TNF-α-dependent like sepsis, FTY720 also inhibits cytokines but without compromising phagocytosis, and therefore its application in IPA would be a better option.

The *in vivo* work in this thesis indicates that inhibition of signaling through S1P₁, a receptor targeted by the licensed drug FTY720, has anti-inflammatory effects in both immunocompetent and immune suppressed mice that could be protective in *A. fumigatus* infection. FTY720 inhibited production of TNF-α and KC in inoculated immunocompetent mice without changes to fungal clearance, suggesting that the conidiacidal functions of macrophages and neutrophils remained intact. Furthermore, FTY720 attenuated *A. fumigatus*-mediated inflammatory responses such as TNF-α, IL-6 and KC up-regulation that are associated with immunopathology in the HC murine model. This inflammatory inhibition was accompanied by a reduction in fungal burden that was partly attributed to the potential fungi-static or -cidal effects of FTY720 on *A. fumigatus* conidia. Additionally, FTY720 was demonstrated to increase survival and result in reduced lung inflammation in the later stages of infection *in vivo*.

Currently, the effects of FTY720 in *A. fumigatus* infection have not been reported in the literature, but FTY720 has been demonstrated to attenuate the pathological elements of the HC model in other inflammatory conditions. These characteristics include inhibiting neutrophil accumulation such as that in ischaemia–reperfusion injury (540), and inflammatory cytokines and chemokines such as that in atherosclerosis (533,541). In ischaemia–reperfusion injury, cytokines and chemokines promote activation of the innate immune system leading to a progressive interstitial fibrosis (542). S1P₁ expression has been demonstrated to be increased in kidney cells, and FTY720 treatment attenuated ischaemia–reperfusion injury by down-regulating neutrophil accumulation (540). This study concluded that the protective effects were mediated FTY720 through S1P₁ but the mechanism of protection might be due to reduced peripheral lymphocytes or by direct effects on S1P₁ expressing

kidney cells. Atherosclerosis is a chronic inflammatory disease characterized by inflammatory mediators, and lipid and innate cell accumulation in arteries that results in the formation of plaques (541). Along with high concentrations of cholesterol, sphingomyelin is a major lipid found in arterial plaques (533,543). Furthermore, in isolated aortic segments and cultured vascular smooth muscle cells, FTY720 inhibited the chemokine MCP-1 in an S1P₃ dependent manner, and a 41.8% reduction of macrophage content in lesions was observed *in vivo* (541). Additionally, atherosclerosis-prone, LDL-receptor-deficient mice, responded to low dose FTY720 treatment with markedly reduced plasma levels of pro-inflammatory cytokines such as IFN- γ , TNF- α and IL-6, and inhibition of atherosclerotic lesion development (533).

The key findings of the *in vitro* and *in vivo* studies in macrophages and immune suppressed mice along with the literature suggest that FTY720 has utility in not only reducing inflammatory mediators but also the associated recruitment of immune cells. These observations provide a therapeutic rationale for the use of S1P signaling blockade to abrogate the destructive immunopathology occurring in IPA, and that further investigation into the effects of FTY720 in this setting are warranted.

Much of the clinical interest in sphingolipids and their signalling in mammals was instigated by the discovery of the lymphopenia inducing drug FTY720 (544), and so far no studies reporting on the antifungal effects of FTY720 against *A. fumigatus* have been published. Moreover, at the time of investigation no sphingosine kinase or fungal GPCR inhibitors had been demonstrated as antifungals, but studies in mutants of fungal specific sphingolipids (not shared by mammalia) had exhibited growth retardation (469). So to investigate this gap in the literature, this thesis addressed the aim of characterizing the effects of sphingolipid inhibitors of conserved proteins with drugs and genetic deletions in *A. fumigatus*.

SKI-II delayed conidial swelling and subsequent germination and reduced fungal biomass. In contrast, FTY720 had a variety of inhibitory effects from the breaking of dormancy to mycelial formation. At high concentration, FTY720 was demonstrated to abrogate germination (in MIC testing) and inhibit filamentous growth in a dose dependent manner on solid medium. At low concentration, FTY720 caused a delay in the onset of conidial swelling and resulted in a very significant (74%) reduction in the number of germinated conidia. It also reduced fungal biomass of germinated conidia. Lastly, analysis of drug interactions by FTY720 indicated a potential for synergy

with AMB and Itraconazole, with fractional inhibitory concentration analysis suggesting FTY720 was additive to the action of AMB.

The inhibition by FTY720 on solid media was dose dependent but not as potent as that observed in liquid media, this is likely due to the increased level of exposure to the drug in liquid. In the lung *A. fumigatus* conidia are in contact with the epithelium in pulmonary fluid and due to their small size, are likely to have maximum exposure to the drug at the concentration present. The pulmonary tissue concentration of FTY720 relative to dose has not been reported as studies have focused on serum and brain concentrations in light of its utility in MS. Doses of 0.3 mg - 40 mg have been investigated for their pharmacokinetics in humans, and in a single dose study, 40 mg dose resulted in 26.9 ng/ml blood concentration (545) equivalent to 78 nM which is less than the concentrations used in this study. Nonetheless, FTY720 easily penetrates cells due to its amphipathic nature, has been reported to be efficiently absorbed with a bioavailability of >90% (546) and has extensive distribution in body tissues (547). Additionally, studies in rats have shown that with low dose (0.3 mg), FTY720 concentrations in the brain can be between 5 and 27 times higher than trough blood levels (548). Therefore, it is not clear whether the reduction in fungal burden *in vivo* is due to the antifungal effects of FTY720 or its effects on immune cells. However, it has been demonstrated that FTY720 significantly reduces germination at a concentration 4x less than its MIC, and could therefore have effects at low concentrations on resting conidia that are not detectable in growth or germination assays, but which reduce the virulence of conidia and aid clearance by immune cells.

Antifungals are known to have high toxicity due to cross reactivity with mammalian cells. Combination therapy is utilized in the hope of improving outcome over monotherapy, and although drug combinations can potentiate increased pharmacokinetic drug interactions, a reduction in dosage could also temper the undesirable side effects of one or either drug (549). Also broad drug target (coverage) can increase effectiveness against resistance mechanisms. Combination therapy for IPA using azoles and echinocandins is currently being used and a recent meta-analysis has indicated a potential benefit for voriconazole and anidulafungin over voriconazole alone in primary IPA. However, the utility of combination therapy in clinical settings remains in debate (550).

Analysis of drug interactions by FTY720 indicated potential for synergy with AMB and Itraconazole, with fractional inhibitory concentration analysis suggesting FTY720 was additive to the action of AMB. This suggests that fungal GPCRs could be a potential new target for additive and maybe even potentially synergistic interactions with antifungal agents.

Lastly, the effects of *SphK* and *gprD* gene deletions were investigated under a range of conditions to demonstrate sphingolipid involvement, and key findings for mutant phenotypes include increased cell wall sensitivity, a reduction in mycelial mass and perturbation of germination kinetics. Mutant strains $\Delta SphK$ and $\Delta gprD$ exhibited increased susceptibility to cell wall stressors, and, with regards to germination kinetics, while both $\Delta SphK$ and $\Delta gprD$ had earlier conidial swelling, they also had similar germination rates to that of the parental strain. This suggests that while SphK and GprD are both implicated in the isotropic swelling process, germination is not an SphK or GprD dependent process. Both mutants also exhibited a reduction in mycelial biomass after overnight growth compared to parental strain, suggesting that although germination is not inhibited, mycelial density is reduced. Also, the survival and immunogenicity of mutant strains was assessed *in vitro* by co-culture with murine macrophages and $\Delta gprD$ induced significantly less TNF- α , but, interestingly, the supernatant galactomannan levels indicated fungal burden was the same as WT. The reduction in immunogenicity of $\Delta gprD$ was attributed to a potential reduction in macrophage recognition, possibly due to an altered cell wall that resulted in macrophages not recognizing $\Delta gprD$ as much of a threat. Or, that $\Delta gprD$ was more susceptible to macrophage and this resulted in reduced TNF- α . These hypotheses are consistent with the observation that mutant strains had increased sensitivity to cell wall stress, which could be due to altered cell wall composition.

This thesis investigated the hypothesis that S1P signalling inhibition could be beneficial in the prophylactic treatment of aspergillosis. It was demonstrated that the S1P₁ inhibitor FTY720 has antifungal activity *in vitro* and therapeutic effects *in vivo* in the HC model of IPA. Although FTY720 administered to mice in this study was higher in respect of its currently prescribed dose, it is known to be well tolerated and given that current antifungals have a high degree of toxicity there is a rationale for development. Also, in recent years major advances in the understanding of the role of S1P and its receptor expression have been made, yet there has been no new class of drugs since the licensing of echinocandins almost 17 years ago. In summary, increase in the burden of IPA and the rise of antifungal resistance necessitates the development of new antifungal drugs, and the data in this study indicate that FTY720 could be developed as a novel antifungal agent.

Future Work

S1PRs are known to have varied expression in immune cells, but much research is required to determine expression associated with activation status and especially those expressed in IPA. To further understand the utility of S1PRs in fungal mediated signaling, S1PR antagonist or AMs from mice with genetic deletions could be used to investigate the contribution of each receptor to the inflammatory response to *A. fumigatus* *in vitro*.

In light of the reduction in fungal burden by FTY720 demonstrated in the HC model of IPA in this study, the antifungal properties of FTY720 and other S1PR antagonists/inhibitors could be investigated in a neutropenic model of IPA. This model could also be used to investigate *in vivo* drug combinations (that are additive and synergistic) in terms of drug toxicity and efficacy of fungal clearance. Also, the antifungal properties of FTY720 could be further investigated for effects on conidial survival in murine macrophages (without immune cell involvement) by pre-incubating conidia in FTY720 before by co-culture. This could indicate if low dose FTY720 affects conidial integrity and its resistance to phagosomal conditions.

In relation to other immune cells with significant effects in IPA, the inflammatory responses in neutrophils in culture with *A. fumigatus* could be investigated with focus on effector functions such as phagocytosis, degranulation and the production of NETs.

The role of S1P₁ is well established in the migration of T cells, but its effects on the trafficking of innate cells in response to fungi are still being defined. Further to the reduction in chemokines seen *in vitro* and *in vivo* by FTY720, future work could quantify pulmonary infiltrates during *A. fumigatus* infection, especially monocytes and neutrophils. Also, histological data across the time course of infection resolution would inform on the cell types involved, fungal burden and fungal morphology (conidial or hyphal forms). The use of S1PR knockout mice could also prove useful in this endeavor.

Furthermore, FTY720 was shown to be additive to the effects of AMB, which has significant nephrotoxicity (551) and is usually administered intravenously as a last resort. Therefore, if AMB could be administered with FTY720 in these circumstances, its associated toxicity to the host could be appreciably reduced. Moreover, providing an AMB/FTY720 combination by inhalation through a nebulizer could also attenuate the lymphocyte sequestering effects of FTY720. In addition, FTY720 could be tested for synergy with itraconazole as data in this suggested, as well as other known agents such as isavuconazole (the new azole), and in a variety of media as (RPMI has been shown to

produce differential data). Lastly, it would also be of interest to further investigate the interaction of FTY720 with other experimental and newly developing antifungal compounds, and high throughput MIC testing could quickly identify potential interactions.

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9. Appendices

Appendix 9.1: MIC values for combination of antifungal agents with SKI-II and FTY720

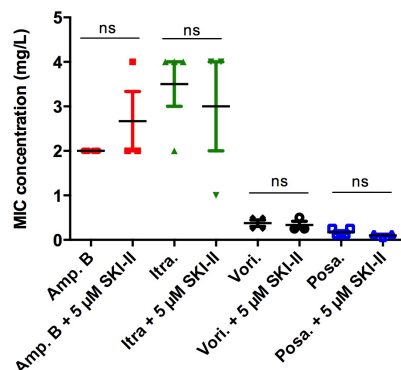


Figure 1: MIC values for combination of antifungal agents with SKI-II. *A. fumigatus* strain CEA10 resting conidia were added to 96-well MICRONAUT-AM MHK 2 microtiter plates containing 9 of the most commonly prescribed antimycotics currently available with or without SKI-II 5µM. MIC readings were taken after incubation for 48 h at 37°C. Data are from 3 experiments with statistical significance was calculated by unpaired t-test. Data are expressed as $\pm$ SEM.

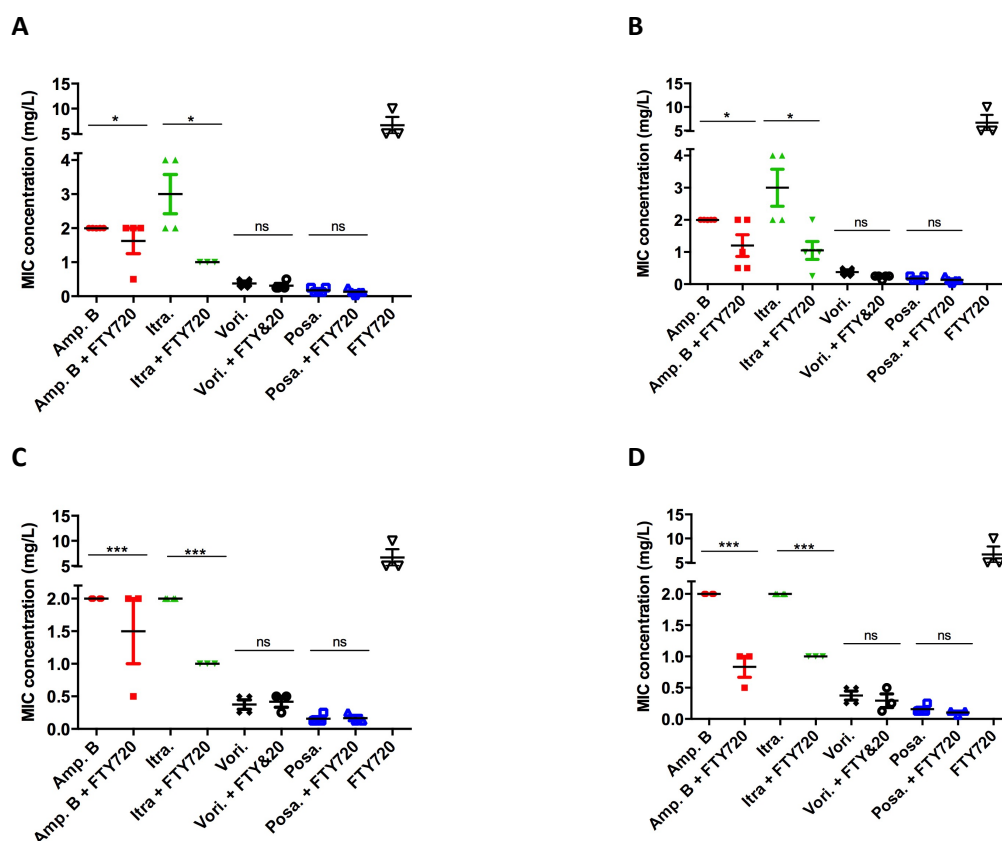


Figure 1: MIC values for combination of antifungal agents with FTY720. *A. fumigatus* strain CEA10 and $\Delta ku80$ resting conidia were added to 96-well MICRONAUT-AM MHK 2 microtiter plates containing 9 of the most commonly prescribed antimycotics currently available with or without FTY720 2.5 µM and 5 µM . MIC readings were taken after incubation for 48 h at 37°C. A-B) CEA10 and C-D) $\Delta ku80$. Data are from 3 experiments with statistical significance was calculated by unpaired t-test. Data are expressed as $\pm$ SEM.

Appendix 9.2: Agarose gels of DNA fragments for gene knockout constructs

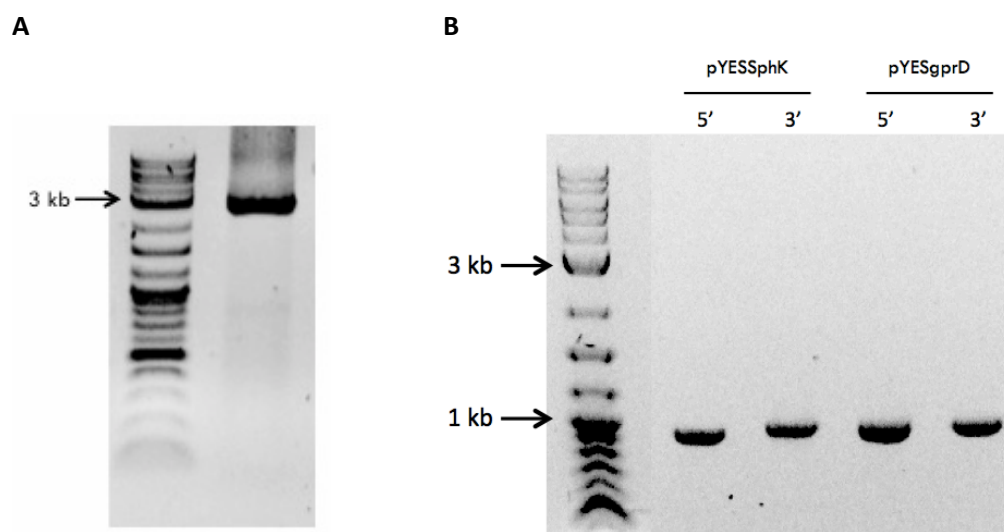


Figure 1: Amplification of knockout vector DNA components. 1% agarose electrophoresis gel of A) 5 μ l of the PCR product amplifying the hygromycin B resistance (gpdA-hph) cassette, and B) 5 μ l of PCR product amplifying flanking regions (of approximately 800 bp) adjacent to *A. fumigatus* genes of interest. Log2 DNA ladder (NEB).

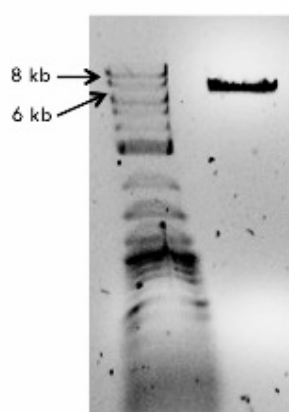


Figure 2: Linearization of pYES2 backbone vector. A) pYES2 vector map with restriction sites for linearization with HindIII and XhoI (NEB), and B) 1% agarose electrophoresis gel of 5 μ l of linearized pYES2 vector. Log2 DNA ladder (NEB).

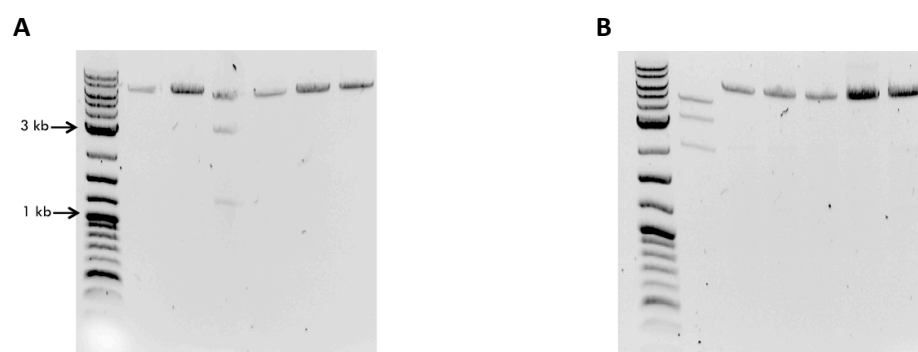


Figure 3: DNA fragments of pYES2SphK and pYES2gprD vectors after restriction enzyme digest. 1% agarose electrophoresis gel of 5 μ l of A) pYES2SphK or B) pYES2gprD vectors DNA fragments after restriction digest with NcoI. Log2 DNA ladder (NEB). For pYES2SphK lane 3 shows a positive result with three visible bands of expected size (1225, 2933 and 5554 bp), all other lanes are negative. For pYES2gprD lane 1 shows a positive result with three visible bands of expected size (2039, 3069 and 4294 bp), all other lanes are negative.

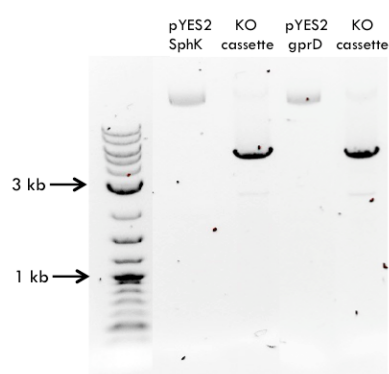


Figure 4: Gene knockout constructs and PCR amplicons. 1% agarose electrophoresis gel 1 μ l of pYES2SphK and pYES2gprD constructs followed by 10 μ l of PCR amplicons of gene knockout cassette vectors. Log2 DNA ladder (NEB).

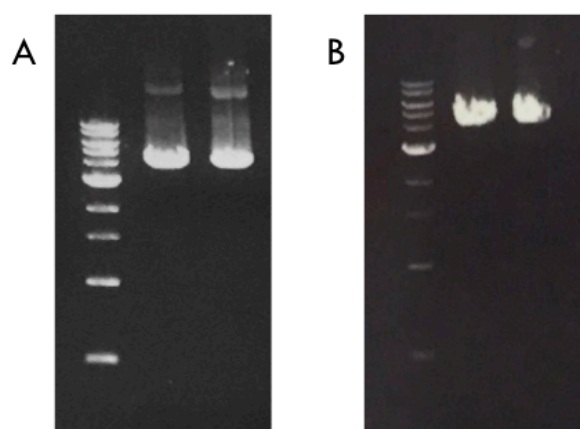


Figure 5: Gene knockout cassette DNA of pYES2SphK and pYES2gprD on 1% agarose electrophoresis gels of A) 2 μ l of the PCR product amplifying the gene knockout cassettes from pYES2SphK and pYES2gprD, and B) 5 μ l of purified gene knockout cassettes from pYES2SphK and pYES2gprD. 1kb DNA ladder (NEB).

Phytosphingosine kinase is annotated on *A. fumigatus* Af293 genome (159) as putative via its comparison to *S. cerevisiae* phytosphingosine kinase gene Sur2p. Sur2p gene in *A. fumigatus* (Afu1g16850) has a CEA10 homolog (AFUB_016240). When comparing the amino acid sequences, homology to human sphingosine kinase (Q9NYA1), for *A. fumigatus* putative sphingosine kinase (B0XQL7) is 28% and putative phytosphingosine kinase (B0XNZ8) 17%.

[illegible]

Percent Identity Matrix - created by Clustal2.1

Hu.SphK1	17.37	27.75	100.00
Af.SphK	17.60	100.00	27.75
Af.pSphK	100.00	17.60	17.37

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