

Defining the impact of Ibrutinib therapy and CFTR dysfunction on
macrophage function in the innate immune response to
Aspergillus fumigatus

Amelia Bercusson

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National Heart and Lung Institute
Imperial College London

Declaration of originality

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Abstract

Over recent years, the role of fungal pathogens within the respiratory microbiome has been increasingly recognised. *Aspergillus fumigatus* is the most commonly isolated organism and gives rise to a wide spectrum of disease, the nature of which depends on the outcome of the host-pathogen interaction. While the majority of people who inhale *Aspergillus* spores on a daily basis clear them without consequence, certain subgroups of the population appear to be pre-disposed to develop clinical disease. Patients with specific forms of immunocompromise are known to be susceptible to invasive infection but in others who develop *Aspergillus* lung disease, the nature of the immune dysfunction present is not clear. This PhD aimed to clarify the mechanisms of immune dysfunction that underlay two different clinical groups' predisposition to *Aspergillus* lung disease, namely patients treated with Ibrutinib and patients with Cystic Fibrosis. I established that Ibrutinib inhibits the activation of signalling pathways crucial to normal macrophage function, thus compromising the innate immune response to *Aspergillus fumigatus*. Ibrutinib was also shown to suppress macrophage programmed necrotic cell death. Following on from this finding, I was able to demonstrate that necroptosis is a significant mode of programmed cell death in *Aspergillus*-infected macrophages and is regulated by signalling along a TLR9-BTK mediated pathway. The clinical phenotype of fungal disease in Cystic Fibrosis is different, being characterised by chronic infection and allergy. In an in-vitro model, I showed that dysfunctional signalling within CFTR-deficient macrophages gives rise to a hyper-inflammatory response to *Aspergillus fumigatus* characterised by increased cytokine release, excess macrophage cell death and impaired fungal killing. In an in-vivo model, this exaggerated inflammatory response was ineffective in clearing *Aspergillus* from the airways and gave rise to a phenotype of chronic neutrophilic inflammation consistent with CF clinical disease.

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List of Abbreviations

ABPA	Allergic bronchopulmonary aspergillosis
AGM	Aorta, gonads, and mesonephros
AIM2	Absent in melanoma 2
AM	Alveolar macrophage
ASC	Adaptor Protein Apoptosis-Associated Speck-Like Protein Containing CARD
BAL	Bronchoalveolar lavage
BCR	B cell receptor
BMDM	Bone-marrow derived macrophages
BMX	Bone marrow expressed kinase
BTK	Bruton's Tyrosine Kinase
CCPA	Chronic cavitary pulmonary aspergillosis
CF	Cystic Fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFPA	Chronic fibrosing pulmonary aspergillosis
CFU	Colony Forming Unit
CGD	Chronic granulomatous disease
CLL	Chronic lymphocytic leukaemia
CLR	C-type lectin receptors
COPD	Chronic obstructive pulmonary disease
CPA	Chronic pulmonary aspergillosis
DAG	Diacylglycerol
DAI	DNA-dependent activator of IFN- regulatory factors
DAPI	4',6-diamidino-2-phenylindole

DMSO Dimethyl Sulfoxide

ELISA Enzyme-linked immunoabsorbent assay

FADD FAS-Associated via Death Domain

GFP Green fluorescent protein

GM-CSF Granulocyte-macrophage colony-stimulating factor

GTP Guanosine triphosphate

HDAC1 Histone deacetylase 1

HMGB1 High mobility group box 1 protein,

Ig Immunoglobulin

IL Interleukin

IPA Invasive pulmonary aspergillosis

IP-10 Interferon gamma-induced protein 10

IP₃ 1, 4, 5-triphosphate

ITAM Immunoreceptor tyrosine-based activation motif

ITIM Immunoreceptor tyrosine-based inhibitory motif

ITK Interleukin-2-inducible T cell kinase

LDH Lactate dehydrogenase

MAPK MAP kinase

M-CSF Macrophage colony-stimulating factor

MCL Mantle cell lymphoma

MCP-1 Monocyte chemoattractant protein 1

MIP Macrophage inflammatory protein

MDM Monocyte-derived macrophages

MLKL Mixed lineage kinase domain-like

NADPH Nicotinamide adenine dinucleotide phosphate (reduced form)

NET Neutrophil extracellular trap

NFAT Nuclear factor of activated T-cells

NF- κ B Nuclear factor kappa-light-chain-enhancer of activated B cells

NLRP3 Nod-like receptor pyrin-containing domain-3

NOD Nucleotide-binding oligomerisation domain

PAMP pathogen associated molecular patterns

PBMC Peripheral blood mononuclear cells

PFA Paraformaldehyde

PH Pleckstrin homology

PI Propidium iodide

PI3K Phosphoinositide 3-kinase

PIP2 Phosphatidylinositol 4,5-bisphosphate

PIP3 Phosphatidylinositol (3,4,5)-trisphosphate

PLC - γ Phospholipase- γ

PMA Phorbol 12-myristate 13-acetate

PRR pattern recognition receptors

PTX Pentraxin

RHIM RIP homotypic interaction motif

RIPK1 Receptor-interacting protein kinase 1

RIPK3 Receptor-interacting protein kinase 3

RFU Relative fluorescence units

RLK Resting lymphocyte kinase

ROI Region of interest

ROS Reactive oxygen species

SAFS Severe asthma with fungal sensitisation

SH1 *Src* homology 1

SH2 *Src* homology 2

SiRNA Small interfering RNA

SOCE Store operated calcium entry

Syk Spleen tyrosine kinase

TEC Tyrosine kinase expressed in hepatocellular carcinoma

TGF- β Transforming growth factor beta

TH Tec homology

Th1 Type 1 T helper cells

Th2 Type 2 T helper cells

Th17 Type 17 T helper cells

TIR Toll/IL-1 receptor

TLR Toll-like receptors

TNF- α Tumour necrosis factor-alpha

TRADD Tumour necrosis factor receptor type 1-associated DEATH domain

Treg regulatory T cells

TRIF TIR domain-containing adaptor protein inducing IFN- β

TRPC6 Transient Receptor Potential Cation Channel Subfamily C Member 6

WT Wildtype

XLA X-linked agammaglobulinemia

1 Chapter 1: Introduction

1.1 *Aspergillus spp.* lung disease

Aspergillus spp. are understood to give rise to a range of pulmonary diseases. These can be classified into three broad categories: *Aspergillus* airways disease, chronic pulmonary aspergillosis (CPA) and invasive pulmonary aspergillosis (IPA). The phenotype that manifests in any one individual is determined by the immunological status of the host and the interaction between host and pathogen. As these are not static factors, clinical presentations can evolve over time and overlap between these categories is not uncommon ¹.

1.1.1 Invasive pulmonary aspergillosis

Invasive pulmonary aspergillosis is the most life-threatening presentation of *Aspergillus* lung disease and is characterised by the invasion of lung tissue by fungal hyphae. In some cases fungus also invades the pulmonary vasculature causing intravascular thrombosis and haemorrhagic pulmonary infarction ^{2,3}. Once vascular penetration has occurred, lung disease can be complicated by dissemination, most frequently to the central nervous system, liver, skin, heart and kidneys ⁴. The mortality associated with IPA remains high. In the TRANSNET study, 12 month mortality among lung transplant recipients was 40% ⁵. Among critically ill patients on ICU, mortality was even higher at 79% ⁶.

A. fumigatus accounts for the vast majority of IPA cases ⁵ with the exception of chronic granulomatous disease (CGD) patients where *A. nidulans* is identified in 1/3rd of invasive aspergillosis cases ⁷. Groups at risk of IPA include: those with prolonged

neutropenia or neutrophil dysfunction, recipients of solid organ or haematopoietic stem cell transplants, patients with haematological malignancies or advanced acquired immunodeficiency syndrome and those receiving long-term glucocorticoid and immunosuppressive therapy ⁸. In addition to these established at risk groups, the use of intensive care support in critical illness has exposed a range of patients without traditional risk factors who also develop invasive disease, for example COPD patients ^{9,10}. The common factor among all these groups is some form of immunocompromise.

1.1.2 Chronic pulmonary aspergillosis

Chronic pulmonary aspergillosis encompasses a spectrum of disorders with similar risk factors and clinical presentations. Almost all cases of CPA occur in individuals with underlying lung disease, most commonly: previous TB, bronchiectasis, non-tuberculous mycobacterium infection, COPD, sarcoidosis and previously treated lung cancer ¹¹. The most clinically significant form of CPA is chronic cavitary pulmonary aspergillosis (CCPA), which if left untreated can progress to chronic fibrosing pulmonary aspergillosis (CFPA). Simple aspergillomas and nodules are usually less severe forms of CPA. Subacute invasive aspergillosis is a locally destructive form of *Aspergillus* infection, which occurs in mildly immunocompromised patients and develops over a period of weeks to a few months. It is commonly categorised as a form of CPA but is treated similarly to IPA ¹².

Treatment with anti-fungal therapy is indicated in symptomatic patients with CCPA, CFPA and semi-invasive disease. Surgical excision is recommended for low surgical risk patients with symptomatic aspergillomas, particularly recurrent large-volume haemoptysis ¹³. Treatment is not indicated for asymptomatic aspergillomas and

nodules. Morbidity and mortality in CPA are variable depending on the form of disease present. In a retrospective analysis of a cohort of 387 CPA patients referred to the UK National Aspergillosis Centre between 1992 and 2012, the authors report 5- and 10-year survival rates of 62% and 47% respectively ¹⁴. These numbers are in line with previously published data ^{15,16}.

The immune status profile of individuals susceptible to CPA is not so clear cut. Many patients with chronic lung disease have a history of long-term low-dose corticosteroid input which could impart a degree of immunocompromise. However, this by no means universal and more work is needed to clarify what immunological risk factors patients with CPA may carry.

1.1.3 *Aspergillus* airways disease

Aspergillus airways disease has classically been considered to consist of the hypersensitivity disorders allergic bronchopulmonary aspergillosis (ABPA) and severe asthma with fungal sensitisation (SAFS). ABPA occurs almost exclusively in individuals with Cystic fibrosis (CF) or asthma, two conditions with a high prevalence of atopy ^{17,18}. Sensitization to *Aspergillus* in the atopic host leads to release of Th2 cytokines and activation of Th2 lymphocytes, which play a key role in recruiting eosinophils. A predisposition to mount a Th2 response to *Aspergillus* thus seems likely to predispose individuals to ABPA.

Not all individuals who become colonised with *Aspergillus* trigger a hypersensitivity reaction, however. Over the past few years, the term *Aspergillus* bronchitis has been proposed to describe patients with pulmonary symptoms and *Aspergillus spp.* growth

who do not show evidence of an atopic response to the fungus. Diagnostic criteria to distinguish *Aspergillus* bronchitis from asymptomatic colonization have been proposed, however validation of these criteria as predictors of long-term outcomes in large cohorts remains to be performed ^{19,20}. CF patients are the main group which have been identified with *Aspergillus* bronchitis. Clinically they grow *Aspergillus* regularly in their sputum, have evidence of declining lung function in the absence of other untreated causes and develop raised *Aspergillus* specific IgG with normal total and *Aspergillus* specific IgE.

Although this clinical presentation is familiar to most CF physicians, clearly demonstrating an association between this clinical entity and lung function decline has been difficult. One group found an association between persistent *Aspergillus* infection and lower lung function, however it was not reported whether there was a difference in rate of decline of lung function ²¹. Others have not found an association with either lower lung function or decline over a 5 year period ²². One limiting factor in all these studies is that the study and control groups have been differentiated by the presence of *Aspergillus* colonisation alone. A subgroup of patients with fungal colonisation and a non-atopic immunological response, eg raised IgG, has never been separated out from those who are simply colonised with no evidence of immunological reactivity.

1.2 *Aspergillus fumigatus*

Aspergillus fumigatus is a member of the *Aspergillus* genus of saprophytic fungi. Aspergilli are ubiquitous in the environment and major contributors to global carbon and nitrogen recycling. They are used in industry for the production of enzymes, pharmaceuticals and food stuffs. For example, *A. niger* is used in the bread and beer

making industries ^{23,24} while statins, used in the treatment of hypercholesterolaemia, are a secondary metabolite of *A. terreus* ²⁵. In humans, however, *Aspergilli*, predominantly *A. fumigatus*, can be pathogenic giving rise to a spectrum of disorders in susceptible individuals.

1.2.1 *A. fumigatus* virulence factors

There are a number of factors which are thought to contribute to making *A. fumigatus* a successful human and environmental pathogen. Thermotolerance is one such factor. *A. fumigatus* can survive in a wide range of temperatures, up to 60°C, allowing it to survive in the environments in which it reproduces such as self-heating compost. It grows particularly well, however, at the human body temperature of 37°C, more so than other *Aspergillus* species. Compared to *A. niger* and *A. flavus*, *A. fumigatus* demonstrates an increased germination rate at this temperature ²⁶ and growth and germination rates at 37°C have been linked to pathogenic potential in a murine model of *Aspergillus* infection ²⁷.

A second factor is the continuous change in cell surface components during the *Aspergillus* life cycle, which interferes with the host's ability to recognise the pathogen ²⁸. Nutritional versatility is also thought to contribute to *Aspergillus* virulence ²⁹. *A. fumigatus* conidial spores, the most easily dispersible form of the fungus, are smaller than those of other *Aspergillus* species at 2-3 µm in diameter and thus easily inhaled, bypassing mucociliary clearance and reaching deep into the small airways and alveoli ³⁰. These resting spores are covered by the pigment melanin which protects the fungus against reactive oxygen species (ROS) and inhibits host programmed cell death and phagosomal acidification ^{31,32}. Finally, *A. fumigatus* produces a number of secondary

metabolites and enzymes such as catalases and superoxide dismutases that enable survival and propagation in hostile conditions ³³.

1.2.2 *A. fumigatus* life cycle

Although *A. fumigatus* is able to reproduce sexually ³⁴, it predominantly does so asexually. At the beginning of its life-cycle, *A. fumigatus* produces vast numbers of spores or conidia which are dispersed into the air. These conidia are released in a resting state where they are metabolically inactive and the cell wall is hidden behind a layer of RodA hydrophobins ³⁵. This renders resting conidia immunologically inert and allows healthy airway phagocytes to clear inhaled conidia without triggering a significant inflammatory response.

Once resting conidia gain access to water and a carbon source they start to germinate ³⁶. Water uptake results in swelling of the conidium during which the fungal cell wall undergoes extensive remodelling. Swelling conidia lose their outer hydrophobin and melanin layers and expose their underlying cell wall components. These consist of an inner layer of branched β (1,3);(1,6)-glucans linked to chitin via β (1,4)-linkages. This core is covalently bound to an outer layer of galactomannans made up of linear α -mannan chains linked to β (1,5)-galactofuranose residues ^{37,38}. About 6-8 hours after germination starts, the fungus switches to polar growth and conidia start to hyphenate. It is these hyphal structures that can penetrate through host cell layers and lead to tissue invasion ³⁹. Hyphae gradually extend to form filamentous, multi-nucleated structures called mycelia. The *A. fumigatus* life cycle completes with the formation of aerial hyphae and specialised conidiophores which produce and disperse hundreds of fresh conidia to restart the cycle.

1.3 The immune response to *A. fumigatus*

The human immune system is a complex network of structural, cellular and humoral components whose purpose is to protect the body from damage inflicted by invading pathogens. It can be broadly categorized into two main components, the innate and adaptive immune systems. The purpose of the former is to rapidly recognise what is “foreign” to the body and either expel or contain it, while the more delayed adaptive system mounts a more targeted response characterised by specificity and memory.

1.3.1 The innate immune response

A. fumigatus most commonly enters the body by inhalation into the airways. The first site of contact is the ciliated airway surface epithelium with its overlying mucoid layer of airway surface liquid. Conidia become trapped in this layer and are moved upwards and out of the airways by ciliary beating. *Aspergillus* conidia oppose this by secreting proteases that inhibit ciliary action ⁴⁰. However, the mucus layer contains anti-microbial peptides that have activity against *A. fumigatus* and its secreted products, including lactoferrin, chitinase and β -defensins ⁴¹.

Conidia that are not successfully removed by the mucociliary escalator will next encounter airway epithelial cells and alveolar macrophages, both of which cell types have phagocytic capacity. *Aspergillus* conidia have been shown to adhere to and be internalised by lung epithelial cells. However, the fungicidal activity of these cells is markedly reduced compared to that of macrophages ⁴². Effective control of *Aspergillus* infection depends primarily on the activity of professional phagocytes such as alveolar macrophages, neutrophils, inflammatory monocytes and dendritic cells. Loss of any these cells results in increased susceptibility to infection ^{43–45}.

1.3.2 Alveolar macrophages

Alveolar macrophages are the dominant cell type found within the alveolar space, making up over 90% of the cellular population ⁴⁶. Alongside interstitial macrophages they contribute to a heterogeneous group of lung macrophages whose members are distinguished by ontogeny, phenotype and function ⁴⁷.

Whereas interstitial macrophages derive predominantly from circulating bone marrow-derived monocytes, with a small perivascular population of F4/80+ yolk sac-derived “primitive” interstitial macrophages, alveolar macrophages derive from monocytes generated in the foetal liver ⁴⁷. From embryonic age 10.5 onward, haematopoietic progenitors from the yolk sac and the aorta, gonads, and mesonephros (AGM) region colonise the embryonic liver, establishing it as a major haematopoietic organ ⁴⁸. Foetal liver monocyte cells seed the lung before birth and develop into CD11c⁺/Siglec F⁺ alveolar macrophages shortly after birth under the influence of GM-CSF ⁴⁹ and TGF- β ⁵⁰. Under homeostatic conditions they are unusually long-lived cells with an annual turnover of 40% and the population is self-propagating, even during periods of acute and chronic inflammation ^{51,52}. If the population undergoes complete depletion, however, it can be re-populated by differentiation of circulating blood monocytes ⁵³.

Alveolar macrophages play a critical role in *Aspergillus* infection both by directly killing conidia and by recruiting other phagocytes to the airways through the release of inflammatory cytokines. Conidia are taken up into macrophages by phagocytosis primarily, though it has been shown that they can also be internalized through a process with features of micropinocytosis ⁵⁴. Once internalised, conidia are contained within an endosomal compartment that is tightly associated with the conidial surface ⁵⁵. The

endosome then enters a maturation pathway proceeding from early to late endosomal maturation and on to lysosomal fusion. This process is necessary as newly formed endosomes are unable to kill pathogens and must instead mature stepwise into phagolysosomes. Maturation of the endosome is signalled by recruitment of the early- and late-phase GTPases RAB5 and RAB7⁵⁶, regulatory enzymes that modulate the vesicular membrane's phospholipids, common docking sites for effector proteins ⁵⁷. Phago-lysosomal fusion is heralded by the accumulation of Lamp-1. Once matured, microbial killing is mediated through acidification, NADPH oxidase-dependent reactive oxygen species (ROS) production, and lysosomal hydrolases ⁵⁸.

The role of macrophages in controlling the early events after *Aspergillus* infection has been demonstrated in-vitro and in-vivo. In alveolar macrophage depleted mice, although mortality from aspergillosis is not increased, fungal clearance from the airways is delayed. This results in greater influx of neutrophils and increased production of inflammatory cytokines at later time points of infection ⁵⁹.

1.3.3 Neutrophils

Neutrophils originate from the same myeloid progenitor as macrophages. They differentiate in the bone marrow and are released into the circulation once fully matured. They become activated in response to the expression of adhesion molecules on endothelial cells. Expression of these adhesion signals is in turn promoted by chemotactic cytokines such as TNF- α and IL-6 ⁶⁰. On entering infected tissues, neutrophils follow a gradient of chemokines to the source ⁶¹. On reaching the site of infection, neutrophils deploy a range of anti-microbial mechanisms including degranulation, ROS production and neutrophil extracellular trap (NET) formation. Of

the four main categories of neutrophil granules, primary and secondary granules have the most important anti-microbial function. Primary granules contain myeloperoxidase, defensins, lysozymes and proteases whereas secondary granules contain lactoferrin; either granules can fuse with phagosomes inside the neutrophil or release their contents into the extracellular space ^{62,63}. Myeloperoxidase is involved in *Aspergillus* hyphal killing whereas Lactoferrin has been shown to mediate iron depletion and thus restrict fungal growth ^{64,65}.

NADPH-dependent ROS release contributes to fungal killing in *Aspergillus* infections. p47(phox)^{-/-} mice lack functional NADPH oxidase and are not able to kill *A. fumigatus* conidia in vitro ⁶⁶ or in vivo ⁶⁷. CGD patients are at significantly increased risk of invasive *Aspergillus* infections ⁷. Also dependent on NADPH activity is NET release. NETs are networks of extracellular fibres composed of decondensed nuclear chromatin that bind histones and antimicrobial granular proteins. Their release is particularly important for defence against pathogens too large to be phagocytosed, hence their anti-hyphal activity. NET release by neutrophils against *Aspergillus* has been demonstrated in-vivo ⁶⁸. Although they are not directly involved in fungal killing, they help restrict hyphal growth ⁶⁹.

1.3.4 Adaptive immune response to *A. fumigatus*

It has been well-established that activation of innate antigen presenting cells by *A. fumigatus*-derived antigens induces T cell responses in both murine and human experimental models ^{70 71}. Dendritic cells are the primary antigen-presenting cells in human lungs but there is evidence that alveolar macrophages, due to their marked plasticity, also contribute to T cell activation ^{72,73}. As well as in in-vitro experimental

models, *Aspergillus*-specific T cells have been detected in human peripheral blood samples from both healthy subjects ⁷⁴ and patients with a spectrum of *Aspergillus*-related disease. A dominant Th1 response is necessary for effective protection against invasive *Aspergillus* infection ⁷⁵. T-cells that express the Th1 markers CD154 and IFN- γ were isolated from patients recovering from invasive aspergillosis but not from those with progressive infection ⁷⁶. The protection mediated by the Th1 subset correlates with the production of the cytokine IFN- γ ⁷⁷, which potentiates the fungicidal activity of innate immune cells ⁷⁸.

In healthy subjects the Foxp3(+) regulatory T (T reg) cell population is expanded, consistent with the development of tolerance to a commonly encountered environmental organism ⁷⁹. Imbalance between pro-inflammatory Th17 and modulatory T reg cells has been linked to the pathogenesis of *Aspergillus* lung disease in CGD ⁸⁰ and cystic fibrosis ⁸¹. In subjects with *Aspergillus* allergies, instead, it is the Th2 population which dominates ⁷⁹.

1.3.5 Innate immune recognition of *A. fumigatus*

The exact nature of the cellular response to *A. fumigatus* is dependent on the degree to which incoming conidia are recognised as foreign pathogens. In their resting state, conidia can be taken up by phagocytes but will not provoke a significant immunological response as recognition of the pathogen associated molecular patterns (PAMPs) present in the fungal cell wall is blocked by the outer layer of RodA hydrophobins. As conidia swell, isotropic growth cracks open this layer and reveals the underlying PAMPs to host pattern recognition receptors (PRRs). Numerous membrane-bound and soluble receptors have been identified as having a role in fungal recognition. They include both

membrane-bound opsonic receptors, C-type lectins, nucleotide-binding oligomerisation domain (NOD)- like receptors and Toll-like receptors (TLR) and soluble opsonins.

Pentraxin-3 is a soluble PRR that opsonizes conidia for detection by phagocytes. In in-vitro studies, alveolar macrophages from PTX-/- mice showed impaired uptake and killing of conidia compared to wild type ⁸². All three complement pathways are activated by *A. fumigatus* with resting conidia triggering the alternative pathway and progressive involvement of the classical pathway as conidia mature. Depletion of Complement factor 3 from human serum in neutrophil cell media compromised phagocytosis of conidia ⁸³. Evidence exists from in-vitro and in-vivo studies for a role for opsonins in the immune response to *Aspergillus*. However, it has also been shown that opsonisation is not essential and non-opsonised conidia can also be internalised by phagocytes ⁸⁴.

C-type lectin receptors (CLRs) can be broadly grouped into CLRs that signal via spleen tyrosine kinase (Syk) coupling, CLRs that signal via Syk through association with an immunoreceptor tyrosine-based activation motif (ITAM)-bearing adaptor such as FcR γ chain, CLRs with immunoreceptor tyrosine-based inhibitory motif (ITIM) domains, and CLRs without ITAM or ITIM domains. Dectin-1 is the best characterised of the CLRs. Activation of Dectin-1 by β -1,3-glucan on the surface of swollen and germinating *Aspergillus* conidia triggers numerous Syk- dependent and independent signalling cascades that result in phagocytosis, ROS production, endocytosis and cytokine release ⁸⁵⁻⁸⁷. Dectin-1-/- mice show impaired cytokine release and neutrophil recruitment to the lungs as well as an overall increased mortality compared to wild type after *Aspergillus* infection ⁸⁸.

Dectin-2 recognises α -mannans in the fungal cell wall and is involved in macrophage cytokine secretion, ROS production and fungal killing in-vitro after infection with *A. fumigatus* ⁸⁹. Dectin-2 is also important for the development of adaptive immunity with Dectin-2-/- mice models of invasive candidiasis showing impaired Th17 responses ⁹⁰. MelLec is a recently discovered CLR that recognises melanin in conidial spores rather than a fungal wall carbohydrate. In a murine model, MelLec was required for protection against disseminated infection with *A. fumigatus* while a MelLec polymorphism increases susceptibility of stem-cell transplant recipients to disseminated *Aspergillus* infections ⁹¹.

TLRs are membrane bound PRRs that can be found both at the cell surface and internalised to the phagosome. Several of the TLRs have been found to contribute to *Aspergillus* immunity. Studies using bone-marrow derived macrophages (BMDM) from TLR-knockout mice, transfection of TLR plasmids into cell lines and in-vivo models have demonstrated TLR2-, TLR4- and TLR9-dependent cytokine production ⁹²⁻⁹⁴. Clinical studies have identified TLR4 and TLR1 and -6 (TLR2 co-receptors) and TLR9 polymorphisms that are associated with an increased susceptibility to invasive aspergillosis ^{95,96}. However, single TLR receptor knockout mice are not more susceptible to aspergillosis than wild type ^{97,98}.

TLRs are characterised by the presence of an extra-cellular leucine-rich recognition domain and an intracellular Toll/IL-1 receptor (TIR) domain that is responsible for signalling via adaptor proteins. MyD88 was the first of these to be described and is universal to all TLRs bar TLR3, which signals via a second adaptor protein TIR domain-containing adaptor protein inducing IFN- β (TRIF). MyD88 signalling contributes to

Aspergillus immunity but it is not indispensable. MyD88 knockout in a cyclophosphamide-treated murine host, but not in an immunocompetent host, gives rise to increased susceptibility to *Aspergillus* infection ^{93,99}. An example of MyD88-independent signalling that contributes to anti-fungal immunity is the TLR9-BTK pathway which has been recently described in murine macrophages ⁹⁴.

1.4 Bruton's tyrosine kinase

In 1993, two separate research groups identified the gene responsible for the human immunodeficiency disorder X-linked agammaglobulinemia (XLA) ^{100,101}. The gene identified codes for a member of the *Src* family of cytoplasmic tyrosine kinases, Bruton's tyrosine kinase (BTK), so named after the American paediatrician Ogden Bruton who reported the first case of agammaglobulinemia in 1952 ¹⁰². XLA is characterised by the absence of peripheral B lymphocytes and serum immunoglobulins. This clinical phenotype has helped delineate BTK's central role in B cell development and function ^{103,104}. Since then further work has identified critical roles in numerous other non-lymphoid cells including: monocytes and macrophages ^{105,106}, neutrophils ¹⁰⁷, dendritic cells ¹⁰⁸ and mast cells ¹⁰⁹.

1.4.1 BTK structure

BTK is one of five members of the Tec family of non-receptor tyrosine kinases. The other members are: tyrosine kinase expressed in hepatocellular carcinoma (TEC), interleukin-2-inducible T cell kinase (ITK), resting lymphocyte kinase (RLK) and bone marrow expressed kinase (BMX) ¹¹⁰. Similarly, to its sibling kinases TEC and ITK, BTK is made up of five protein-interaction domains. Starting from the N-terminus: a pleckstrin homology (PH) domain, a proline-rich Tec homology (TH) domain, *Src* homology 2 and

3 (SH2 and SH3) domains and a catalytically active kinase domain, the SH1 domain ¹¹¹.

The multiplicity of interaction partners allowed for by this multi-domain structure, localises BTK into numerous signalling pathways within cells, the most clearly delineated of which are those originating at the B cell receptor (BCR).

1.4.2 BTK's role in B cells

Since BTK's discovery, evidence has built up to support a critical role for this kinase in B cell development, differentiation, proliferation, survival and apoptosis ¹¹²⁻¹¹⁴. BTK's interaction with the BCR is the model upon which our understanding of how BTK is activated is based.

Upon antigen engagement of the BCR, tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAM), located in the Ig α /Ig β heterodimer cytoplasmic tails, are phosphorylated by *Src* family kinases such as LYN. This phosphorylation step creates docking sites for the spleen tyrosine kinase (SYK) ¹¹⁵ and allows LYN and SYK to phosphorylate the cytoplasmic tail of the B-cell co-receptor CD19 and/or the adaptor protein B-cell PI3K (BCAP), which facilitates recruitment and activation of phosphoinositide 3-kinase (PI3K) and the guanine nucleotide exchange factor VAV ^{116,117}. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which by binding to the PH domain of BTK encourages its recruitment to the cell membrane ¹¹⁸. It is this step, the generation of PIP3, which most likely mediates receptor-driven BTK activation in both lymphoid and myeloid cells ¹¹⁹.

Once translocated to the cell membrane, *Src* family kinases phosphorylate BTK at tyrosine residue Y551, which sits in the activation loop of the kinase domain ¹²⁰. Phosphorylation of BTK at Y551 promotes its catalytic activity and drives autophosphorylation at residue T223 in the SH3 domain ¹²¹. This second phosphorylation event is believed to stabilise BTK into a “kinase active” conformation ¹²². Activated BTK is then able to interact with numerous downstream proteins and activate signalling pathways essential for B cell survival and function.

1.4.3 BTK’s role in myeloid cells

As mentioned above, BTK expression in myeloid cells as well as in B cells has been established. Its functional role in these cells is still being revealed, however. BTK has been implicated in pathogen recognition via Toll-like receptors (TLR) in human and murine macrophages. Specific-ligand binding experiments have demonstrated TLR-mediated BTK activation for TLR2 ¹²³; TLR3 ¹²⁴; TLR4 ^{125,126}; TLR7/8 ^{127,128} and TLR9 ¹²⁹. BTK-dependent recognition and triggering of anti-microbial responses has been shown for whole pathogens also, including *Listeria monocytogenes* ¹³⁰, *Staphylococcus aureus* ¹³¹, dengue virus ¹²⁴ and *Aspergillus fumigatus* ⁹⁴.

BTK has also been identified as playing a key role in Nod-like receptor pyrin-containing domain-3 (NLRP3) inflammasome activation in macrophages. BTK interacts directly with NLRP3 and its adaptor protein Adaptor Protein Apoptosis-Associated Speck-Like Protein Containing CARD (ASC) resulting in the induction of ASC oligomerization and caspase-1 activation in a kinase-dependent manner ¹³². Monocytes from XLA patients demonstrate impaired inflammasome activity, suggesting that a genetic inflammasome deficiency may contribute to the immunocompromised XLA phenotype ¹³³ (Figure 1).

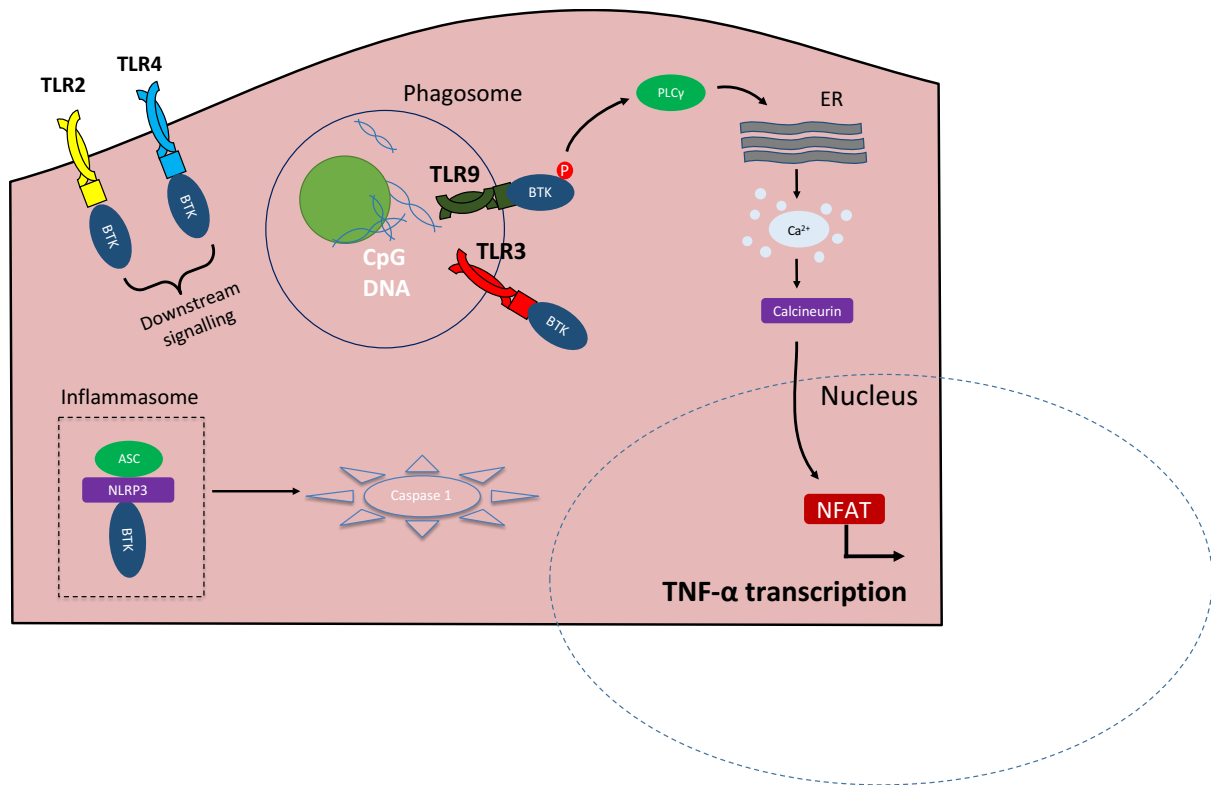


Figure 1. BTK is involved in multiple signalling pathways within myeloid cells

As well as its role in pathogen recognition, BTK is involved in myeloid cell maturation and survival. In BTK-deficient mice, absence of BTK correlated with reduced macrophage numbers due both to its role in haematopoiesis ¹³⁴ and in M-CSF-induced signalling pathways that regulate macrophage survival ¹³⁵. Neutrophils, though found to be increased in number in BTK-deficient mice, were immature and functionally impaired ¹³⁶. XLA patients are often found to be neutropenic, with neutrophils more susceptible to apoptosis ^{137,138}. In dendritic cells, the data on BTK's role is less consistent. In murine dendritic cells, BTK has been found to have a negative regulatory effect on cell maturation and LPS-induced T cell stimulation, mediated by reduced secretion of IL-10 and subsequent impaired activation of Stat3, a transcription factor critical for immune tolerance ¹³⁹. In human dendritic cells instead, BTK mediates TLR9-

induced cell activation with an impaired response in XLA patients and cell exposed to the BTK inhibitor Ibrutinib ¹²⁹.

1.5 Ibrutinib

Given the crucial role of BTK in BCR signalling it is not surprising that abnormal expression and activity of this kinase is a feature of several B cell malignancies. Primary mantle cell lymphoma (MCL) cells show strong expression of Y223-phosphorylated BTK ¹⁴⁰ and BTK is needed for retention of MCL cells in lymphoid tissues ¹⁴¹ Chronic lymphocytic leukaemia (CLL) cells show constitutive activation of BTK ¹⁴². Ibrutinib is an irreversible inhibitor of BTK that was first licenced by the US FDA as a treatment for relapsed and refractory mantle cell lymphoma (MCL) in November 2013 and then for relapsed and refractory CLL in February 2014 ¹⁴³. Its use has since expanded so that it is now licenced for Waldenstrom's macroglobulinaemia and, in the USA, for marginal zone lymphoma and graft-versus-host disease. Chemically, Ibrutinib is a small molecule piperidine. It binds covalently and irreversibly to the non-catalytic cysteine residue 481 (Cys481) in the extended hinge region near the BTK active site and blocks BTK's full activation by inhibiting its autophosphorylation at tyrosine residue 223.

As the clinical use of Ibrutinib has expanded, an association between Ibrutinib therapy and invasive *Aspergillus* infections has become apparent. Initial case reports ^{144,145} were followed by a phase-II study of Ibrutinib in primary central nervous system lymphoma, which reported a 39% incidence of invasive aspergillosis in patients concurrently treated with corticosteroids, in the absence of neutropenia ¹⁴⁶. The mechanisms behind this increased susceptibility have not been fully established, however. There is evidence that BTK is involved in macrophage responses to *Aspergillus* in murine cells ⁹⁴ and

macrophages play a key role in early control of *Aspergillus* infections. It may be thus that it is Ibrutinib's influence on myeloid cell activity that is responsible for this susceptibility.

Just as Ibrutinib-treated patients appear to have a susceptibility to invasive *Aspergillus* infections, CF patients also have a disproportionately high incidence of *Aspergillus* lung disease. It is not invasive disease that affects this latter group but rather airways disease, both atopic and not. It is known that the immune response to bacterial infections is altered in CF but data on the impact of CFTR dysfunction on anti-fungal immunity is not so conclusive.

1.6 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive inherited condition caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. It is the most common inherited life-limiting disease among Caucasians, affecting 1 in 2500 newborns, and the major indication for lung transplantation in the UK. Chronic suppurative lung disease leading to progressive respiratory failure is the principal cause of morbidity and mortality. Median age of survival has improved over the past 50 years and is now more than 40 years in western countries ¹⁴⁷.

1.6.1 CFTR

The CFTR gene was identified by Lap-Chee Tsui's research group in 1989 and published in a series of three papers ¹⁴⁸⁻¹⁵⁰. Since then more than 2000 mutations have been identified ¹⁵¹, though Phe508del is by far the most common. The CFTR protein is a cAMP-activated ion channel that conducts Cl⁻ and HCO₃⁻ ions across the cell membrane

¹⁵². CFTR also drives HCO₃⁻ flow indirectly by the coordinated activity of CFTR and SLC26 transporters (Cl⁻/HCO₃⁻ exchangers) ¹⁵³. Loss of functional CFTR interferes with anion transport out of the cell and, through its regulatory effect on the ENaC sodium channel, sodium reabsorption. The classical hypothesis in CF pathophysiology is that this disruption of ion movements leads to abnormal hydration of the airway surface liquid and impaired mucociliary clearance ^{154,155}. More recent studies in newborn CF pigs and ferrets, however, did not find any evidence of sodium hyper-reabsorption ^{156,157}. The hydration model might thus not fully explain the abnormalities seen in muco-ciliary clearance in CF. Bicarbonate deficiency related ^{158,159} changes in pH, mucous tethering and function are also thought to contribute ^{152,160,161}.

1.6.2 The immune response in CF

CF airways are characterised by the presence of chronic microbial contamination and inflammation. While there is no doubt that epithelial cell dysfunction contributes to this phenotype, there is increasing evidence that other components of the innate and adaptive immune responses are also involved the pathogenesis of CF airways disease.

1.6.3 CF macrophages

CFTR expression has been demonstrated in human monocyte-derived macrophages ¹⁶² and murine and human alveolar macrophages ^{158,159}. These authors have reported impaired macrophage killing of *P. aeruginosa* and have proposed impaired phagosomal acidification as the mechanism underlying this defect. Others, however, have challenged this hypothesis ¹⁶³. Impaired autophagy has also been reported in CF macrophages and may contribute to the killing defect towards *B. cenocepacia* observed in this study ¹⁶⁴. In

a 2018 paper, correction of an anti-Pseudomonal killing defect by treatment of CF macrophages with the CFTR corrector Lumacaftor (VX-809) was reported ¹⁶⁵.

As well as failing to kill pathogens effectively, CF macrophages have also been observed to generate hyper-inflammatory responses to infectious stimuli. *B. cenocepacia* induced greater inflammatory cytokine release and inflammatory cell death in monocyte-derived macrophages from CF patients versus healthy controls ^{166,167}. Inflammatory cytokine release by CF murine and human macrophages stimulated with LPS was increased versus wild type and healthy controls ¹⁶⁸. In a murine CF model of *A. fumigatus* infection, ¹⁶⁹ activation of the NLRP3 inflammasome was increased in CF lung macrophages versus wild type controls.

The mechanisms that link CFTR dysfunction to altered macrophage behaviour are less clear. NF- κ B activation is exaggerated in CF epithelial and macrophage cells ^{170,171}. This could be secondary to altered calcium homeostasis, which has been observed with increased store operated calcium entry (SOCE) and calcium release from the endoplasmic reticulum demonstrated in Δ F508 epithelial cells ^{172,173}. Given the central role of Ca²⁺ as a universal second messenger, dysregulated calcium flux could play an important aetiological role beside altered chloride flux in CF pathophysiology.

1.6.4 CF neutrophils

Neutrophilic inflammation is the key feature of CF airways. The presence of chronic microbial infection acts as a persistent draw, swelling airway neutrophil numbers. However, it is not just that neutrophils are inappropriately drawn to the airways, but their activity is also abnormal and contributes to the chronic inflammation seen. CF

neutrophils show reduced rates of apoptosis and so persist longer in the airways than non-CF neutrophils ¹⁷⁴. These long-living neutrophils then undergo increased rates of NET-osis in response to *Pseudomonas* infection, resulting in the release of toxic intracellular contents into the airway ¹⁷⁵. NET release becomes an ineffective anti-microbial measure in chronic infection, leaving the NET strands to act as an inflammatory stimulus to surrounding macrophages ¹⁷⁶.

In-vivo, CF murine and human neutrophils produce larger amounts of the pro-inflammatory cytokine IL-17A and express more IL-17 receptor than wild type or healthy human neutrophils in response to *Pseudomonas* infection. Interestingly, sputum IL-17A levels correlated inversely with lung function ^{177,178} in CF. Finally, although neutrophil-mediated killing of *A. fumigatus* was not impaired, ROS production and release was increased. Again this increased ROS production by peripheral blood neutrophils was associated with an increased number of clinical exacerbations in the previous year and reduced lung function ¹⁷⁹.

1.7 Hypothesis

The hypothesis of this thesis is that inhibition of BTK and loss of normal CFTR function both result in an impaired innate immune response to *Aspergillus fumigatus* leading to airway infection and clinical lung disease. This effect is mediated by BTK and CFTR's regulation of key intra-cellular signalling pathways, namely the calcineurin-NFAT and NFκB activation pathways.

Chapter 2

Materials and Methods

2.1 Ethics statement

Collection and study of human macrophages was approved by the Biomedical Research Unit (Advanced lung disease) Biobank research project (NRES reference 10/H0504/9), Royal Brompton and Harefield NHS Trust (AS1). All experiments conformed to the principles set out in the WMA declaration of Helsinki and the Department of Health and Human Services Belmont Report. Informed consent was obtained from lung transplant recipients from the Royal Brompton and Harefield NHS Trust undergoing flexible bronchoscopy.

2.2 Isolation of human peripheral blood monocytes and differentiation into monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteer blood or from leukocyte cones purchased from the National blood service, Colindale, UK by mixing 3:1 in warm RPMI and layering over Ficoll-Paque PLUS (GE healthcare). Samples were centrifuged at 450g for 45 minutes and the PBMC layer removed. PBMCs were incubated with red cell lysing buffer Hybri-Max (Sigma) for 10 minutes and then washed at 200g in warm PBS x 2 to remove platelets. Monocytes were subsequently purified by negative magnetic bead selection using a pan-monocyte isolation kit (Miltenyi Biotech, Auburn, CA). To obtain monocyte-derived macrophages (MDMs), freshly isolated monocytes were cultured with RPMI 1640 medium supplemented with 10% human serum (Sigma, UK), 200 IU/ml Penicillin-Streptomycin and 5 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) (Peprotech, UK) for 7 days at 37°C.

2.3 Isolation of human alveolar macrophages

Bronchoalveolar lavage (BAL) fluid was passed through a 100µm cell strainer (BD Bioscience, UK) and centrifuged at 400g for 10 min to pellet cells, which were re-suspended in red cell lysing buffer. Cells were subsequently washed in warm PBS x 2 and re-suspended in RPMI 1640 prior to plating at the desired concentration. Alveolar macrophages (AM) were allowed to adhere for 1 hour after which non-adherent cells were removed by washing x 2 with warm RPMI. Residual purified AMs were then further rested for 3 days at 37°C to ensure any residual drug effect was removed.

2.4 Culture of THP1 macrophages

THP1 cells are a human macrophage cell line derived from a 1-year-old male patient with acute monocytic leukaemia. Cells were propagated in T75 cell culture flasks in RPMI 1640 supplemented with 10% FCS and 50mM β-mercaptoethanol (referred to as complete media or cRPMI) and sub-cultured when cell concentration reached 8×10^5 cells/ml. For differentiation, cells were plated in media supplemented with 100 ng/ml PMA for 72 hrs. The media was then changed to remove the PMA and cells rested for 24 hours prior to experimentation. For long-term storage, cells were re-suspended in cRPMI containing 10% Dimethyl Sulfoxide (DMSO), frozen over night at -80°C and transferred into the vapour phase of liquid nitrogen.

2.5 Generation and culture of bone-marrow derived macrophages (BMDMs)

WT C57BL/6 or knock-out mice (aged 8 to 10 weeks) were culled by overdose of phenobarbitone and the femur and tibia removed aseptically. Excess muscle was removed from the legs and the leg bones carefully cut open adjacent to each joint. A 28-G

needle was inserted into the bone marrow cavity and the marrow flushed into a 50 ml Falcon tube using cold PBS without calcium or magnesium (PBS_{Ca-/Mg-}) supplemented with 2mM EDTA (Gibco by Life Technologies) until the bone appeared white. The cells were centrifuged for 10 min at 500g at room temperature and re-suspended in Red Blood cell lysis buffer (Sigma-Aldrich). After 10 min incubation, an equal amount of PBS was added, and the cells were spun down for 5 min at 500g. The remaining cell pellet was re-suspended in differentiation media consisting of RPMI 1640 supplemented with 10% FCS, 40 ng/ml recombinant murine M-CSF (eBioscience) and 200 IU/ml Penicillin/Streptomycin and counted by haemocytometer. 5×10^6 cells were plated per 15 mm Petri dish in 8 ml of media and incubated at 37 °C. After 3 days of culture, 4 ml of fresh differentiation media was added to each petri dish. After 7 days of culture, differentiated macrophages were washed once with PBS and harvested in PBS_{Ca-/Mg-} supplemented with 2mM EDTA. For long-term storage, cells were re-suspended in RPMI 1640 supplemented with 10% FCS and 5% DMSO, frozen at -80 °C overnight and stored in the vapour phase of liquid nitrogen. For culture after differentiation, macrophages were thawed and re-suspended in culture media consisting of RPMI containing 10% FCS, 20 ng/ml recombinant murine M-CSF and 200 IU/ml Penicillin/Streptomycin, seeded and rested for 2 days before being used for experimentation. On the day before experimentation, the medium was replaced with culture media supplemented by 200 IU/ml IFN- γ for 24 hours. On the day of experimentation, the IFN- γ was removed.

2.6 *Aspergillus fumigatus* strains and culture

Aspergillus fumigatus AF CEA10 (FGSC A1163) was obtained from the Fungal Genetics Stock Centre and was used for Western blot, ELISA, Luminex, fungal killing, LDH release

and plate reader microscopy experiments. An eGFP-expressing strain (ATCC46645-eGFP, a gift from Frank Ebel) was used for confocal and widefield microscopy, flow cytometry and in-vivo animal infections. All strains were cultured on Sabouraud dextrose agar (Oxoid). Conidia were harvested in 0.1% Tween/H₂O and filtered through MIRACLOTH (Calbiochem, UK). Conidial suspensions were washed twice in sterile water and resuspended in RPMI 1640 at concentrations shown. To generate swollen conidia (SC), resting conidia (RC) were suspended in RPMI 1640 at 1.5×10^6 conidia/ml and swollen at 37°C for 4 h. For fixed conidia, resting or swollen conidia were fixed in 4% paraformaldehyde in PBS for 10 minutes, washed in PBS and then quenched in 0.1M ammonium chloride for 10 minutes followed by further washes in PBS x4.

2.7 Tissue culture conditions

For all experiments using murine macrophages, cells were seeded and activated overnight in cRPMI containing 20 ng/ml M-CSF and 200 U/ml IFN- γ (Peprotech). The next day the medium was replaced with fresh cRPMI with M-CSF only. Human macrophages did not require IFN- γ activation. Cells were pre-treated with inhibitors for one hour prior to experimentation and the inhibitors remained in the culture medium throughout the period of stimulation.

2.8 Western blotting and lysate preparation

Materials

- RIPA buffer: 50 mM Tris HCl (pH 8), 150 mM NaCl, 1% Triton X100, 0.5% sodium deoxycholate, 0.1% SDS.
- Hypotonic buffer: 20 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl.

- Nuclear extraction buffer: 100 mM Tris (pH 7.4), 100 mM NaCl, 1% Triton X100, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate.

All lysis buffers were supplemented with Proteinase/phosphatase inhibitor cocktail (Cell signalling). Phospho-protein cell lysates were prepared with buffers also supplemented with 1 mM PMSF and 1 mM sodium orthovanadate.

Methods

For whole cell lysates, cells were lysed in RIPA buffer. Samples were then agitated for 30 minutes at 4°C before being centrifuged for 20 minutes at 12000 rpm. Supernatants were frozen at -80° C. For phospho-BTK western blots, cell cultures were incubated with sodium orthovanadate for 1 hr at 4° C prior to cell lysis. Nuclear extracts were prepared by sequential lysis of the plasma and nuclear membranes. Cells were detached from the tissue culture plate by incubation with ice-cold PBS (Ca-/Mg-) supplemented with 2 mM EDTA for 10 mins at 4°C and then pelleted by centrifugation at 2000 rpm for 5 minutes. The cells were then first swollen in hypotonic buffer for 15 minutes followed by disruption of the outer cell membrane by the addition of Triton X100 to a final concentration of 0.5%. The samples were vortexed and spun down at 14000 rpm for 10 mins. The supernatant was frozen at -80° C.

Protein concentration of lysates was determined using the BSA assay kit from Thermo - Scientific. Protein samples were denatured by addition of Protein loading buffer (National Diagnostics) and heating to 95° C for 5 mins. 5-20 µg of protein was loaded per lane and separated in gels with an acrylamide percentage of 10-15% (see table x for recipe). The proteins were transferred onto nitrocellulose or PVDF membranes, followed

by blocking of the membrane in 5% milk powder or 5% BSA in TBS-T for 1 hour at room temperature. Membranes were probed with primary antibodies over night at 4° C. The next morning, blots were washed three times in TBS-T, before being probed with horse radish peroxidase-linked secondary antibodies for 1 hour at room temperature. Blots were then washed twice in TBS-T and once in TBS. Proteins were detected using Clarity™ ECL blotting substrate (Bio-Rad). Primary antibodies used were:

Antibody	Clone and manufacturer	Staining concentration
Anti-NFATc1	7A6, Santa-Cruz	1:100
Anti- NF-κB p65	C22B4, CST	1:1000
Anti-HDAC1	10E2, CST	1:1000
Anti-Histone H3	D1H2	1:1000
Anti-BTK	D3H5, CST	1:1000
Anti-phospho-BTK	Tyr223, CST	1:1000

2.9 Enzyme-linked immunoabsorbent assay (ELISA) and Luminex

For the quantification of cytokines in tissue culture and BAL supernatants, ELISAs were performed according to the manufacturer's instruction using the TNF- α , KC and IL-1 β DuoSet kits from R& D systems. In addition, cytokines in supernatants were measured

with a Meso Scale Discovery (MSD) multiplex assay kit Containing antibodies for the following analytes: IL-1 β , IL-2, IL-4, IL-5, IL-6, KC-GRO, IL-10, IL-12p70, TNF- α , IFN- γ , IL-9, MCP-1, IL-33, IL-27p28, IL-15, IL-17, MIP-1 α , IP-10 and MIP-2.

2.10 Confocal and widefield microscopy

For confocal and widefield microscopy, cells were seeded on glass coverslips in 24-well plates and treated as indicated. Before staining, cells were washed in PBS x1 and fixed in 2% PFA for 15 minutes followed by quenching in 50mM NH₄Cl for 10 min. Cells were blocked and permeabilised in PBS containing 10% goat serum (Sigma-Aldrich) and 0.1% Saponin (Sigma-Aldrich) for 2h at room temperature and incubated overnight at 4°C with a primary antibody diluted in the blocking and permeabilization buffer (see table). After washing with PBS three times, cells were incubated with anti-rabbit Cy5 or anti-mouse Cy5 antibodies (Life Technologies) for 45min at room temperature in the dark, mounted with Vectashield mounting medium containing DAPI (Vector laboratories) and sealed with nail varnish to prevent evaporation. Image analysis was performed using Image J software. NFAT and NF- κ B translocation studies were conducted with a Zeiss LSM-510 widefield microscope. Nuclear translocation was quantified by determining the percentage overlap of the NFAT/ NF- κ B channel with the DAPI channel (the Mander's coefficient) using a macro written for the project (thanks to Stephen Rothery, Imperial Facility for Imaging by Light Microscopy). Recruitment of BTK to phagosomes containing *Aspergillus* conidia was imaged with a Zeiss LSM-510 confocal microscope and quantified by creating a region of interest (ROI) around the conidium and quantifying the average pixel intensities of the marker of interest in the resulting ROI.

Antibody	Clone and manufacturer	Staining concentration
Anti-NFATc1	7A6, Santa-Cruz	1:100
Anti-NFKB p65	Clone F6, Santa-Cruz	1:100
Anti-BTK	7F12H4, Novus Biologicals	1:100
Anti-phospho-BTK	Tyr223, CST	1:100

2.11 Plate reader fluorescence microscopy

Cells were seeded in in black-walled clear-bottomed 96-well microplates (Corning). On day of experiment, *A. fumigatus* conidia were added to appropriate wells and plates were centrifuged at 900 rpm for 3 min. Plates were incubated at 37° C for 1 hour. RPMI containing Propidium iodide (PI) and Ac-DEVD-AMC (Enzo Life Sciences) was added to each well and PI-positive cell death and AMC cleavage by Caspase 3 (an analogue measure of apoptosis) were measured over 16 hrs by time-lapse fluorescence microscopy using a TECAN Infinite PRO microplate reader.

2.12 Time lapse video microscopy

Live time-lapse imaging of macrophage-*A. fumigatus* interactions was performed by plating cells in white-walled, clear-bottomed 96-well microplates and stimulating with live e-GFP expressing *A. fumigatus* conidia at a MOI of 1. Following infection, propidium iodide was added to the media to a final concentration of 1.25µg/ml and cell death events

and e-GFP *A. fumigatus* growth was visualised over 16 hours by widefield time-lapse imaging using a Zeiss LSM-510 Widefield microscope. Cells were maintained at 37°C with 5% CO₂ during imaging. Images were analysed using ImageJ software and a macro written for the project (thanks to Stephen Rothery, Imperial FILM).

2.13 siRNA knockdown

After initial dose-titration experiments, SMARTpool siGENOME BTK siRNA (Dharmacon) and Scramble control siRNA (Ambion) were used at a concentration of 75nM. For siRNA knockdown, primary monocyte cells were differentiated for 7 days. On day 4 siRNA was transfected using Viromer Blue (Lipocalyx) according to the manufacturer's instructions.

2.14 Flow cytometry

2.14.1 Phagocytosis

Cells were seeded in 24-well plates and on the day of experimentation infected with biotinylated e-GFP *A. fumigatus* conidia at a MOI of 1 for 2 hours. Unattached conidia were washed off and cells fixed in 2% PFA for 15 minutes followed by quenching in 50mM NH₄Cl for 10 min. Cells were counterstained with Cy3 biotin antibody and acquired on a Fortessa flow cytometer. Data was analysed using FlowJo software (Oregon, USA).

2.14.2 Analysis of cellular infiltrates by flow cytometry

10 µl of each BAL sample was removed and a haemocytometer was used to count the average cell concentration per sample. The BAL fluid was spun down at 2000 rpm for 10 min and the cell pellet resuspended in PBS containing an Fc receptor blocking antibody

(anti-CD16/CD32, clone 93, eBio- science, diluted 1:100). The cells were incubated on ice for 20 min. A pre-mixed surface stain marker cocktail was directly added to the cell suspension. Samples were stained for 20 min in the dark at room temperature. After 1 wash in PBS, cells were fixed in 2% PFA and NH₄Cl and acquired on a Fortessa flow cytometer. Data was analysed using FlowJo software. Absolute cell counts were calculated by using proportions derived from flow cytometry and cell concentrations for each sample counted by haemocytometer.

2.15 Fungal killing

2.15.1 Colony Forming Unit (CFU) counts

For in-vitro killing assays, cells were infected with *A. fumigatus* swollen conidia (MOI=1) for 6 hrs. Cells were then washed to remove extra-cellular conidia and lysed in 0.1% Tween 20/dH₂O. Serial dilutions were plated onto Sabouraud agar plates. Plates were incubated at 37° C overnight and CFUs were counted the next day. For fungal burden in BAL samples, serial dilution of BAL fluid was performed in 0.1% Tween 20/dH₂O and plated onto Sabouraud agar plates. Plates were incubated at 37° C overnight and CFUs were counted the next day.

2.15.2 Galactomannan release

Galactomannan levels in tissue culture and BAL supernatants were measured according to the manufacturer's instructions using the Platelia™ *Aspergillus* Ag kit (Bio-rad).

2.16 Lactate dehydrogenase (LDH) release

LDH levels in tissue culture supernatants were measured by colorimetric formazan product formation using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's instructions.

2.17 Animals

All mouse experiments were approved by the United Kingdom Home Office and the Imperial College animal ethics committee, performed in accordance with the project license PPL 70/7941. C57BL/6 male mice (18-22g) were ordered from Charles River, UK. WT and litter-mate homozygote knockout *Cftr*^{tm1Unc}-Tg(FABPCFTR)1Jaw/J mice were bred by Uta Griesenbach's lab and were a kind gift from her. All mice were housed in individually vented cages with free access to autoclaved food and water. WT and CFTR^{-/-} mice were co-housed with separation by gender and age cohort rather than genotype.

2.18 Infections

Mice were infected with e-GFP *A. fumigatus* resting conidia administered intra-nasally under isoflurane anaesthesia in 75 µl of PBS.

2.19 Bronchoalveolar lavage

Mice were culled prior to bronchoalveolar lavage by intra-peritoneal overdose of phenobarbitone. Bronchoalveolar lavage (BAL) was performed by tracheal cut-down and intubation with a self-designed intubation catheter. Lungs were lavaged by installing 3 x 800 µl of PBS/2mM EDTA. The lavage fluid was stored on ice until further processing.

110 μ l was removed for fungal burden analysis. Cells and supernatant were separated by spinning at 1500 rpm for 8 min. Cell pellets were processed immediately for flow cytometry while supernatants were stored at -80 C for further analysis.

Chapter 3

BTK mediates endosomal calcineurin-NFAT and NF- κ B activation in response to *A. fumigatus* infection in human macrophages

3.1 Introduction

BTK has recently been identified as a key kinase involved in mediating calcineurin activation in murine macrophages in response to recognition of *A. fumigatus* by the CpG DNA receptor TLR9 ⁹⁴. *In vitro*, the BTK inhibitor LFM-A13 blocked NFATc2 activation and TNF- α release. This inhibitory effect was similar to that seen in TLR9-/- BMDM versus wild-type BMDM. Dual inhibition of TLR9 and BTK by pre-treatment of TLR9-/- BMDM with LFM-A13 did not show any additive effect, suggesting that TLR9 and BTK operate in the same calcineurin activation pathway. *In vivo*, BTK-/- mice showed increased mortality in a murine model of aspergillosis with increased peri-bronchial mononuclear cell and neutrophil inflammation, focal interstitial pneumonia and large airway mucous plugs ¹⁴⁶, changes that mirror the findings in macrophage-depleted models rather than neutropenic models of pulmonary aspergillosis.

Ibrutinib is a small molecule inhibitor of BTK licenced for the treatment of chronic lymphocytic leukaemia, Waldenström's macroglobulinemia and as a second-line treatment for mantle cell lymphoma, marginal zone lymphoma, and chronic graft vs. host disease ¹⁸⁰. Recent clinical trials have reported an association with invasive pulmonary and CNS *Aspergillus* infections in patients receiving Ibrutinib ^{145, 146}.

Using the murine work mentioned above as a starting point, I commenced my research by examining the role of BTK in human macrophage responses to *A. fumigatus* with the

aim of gaining a mechanistic understanding of what might underlie the observed infection risk in patients receiving Ibrutinib.

Experiments were performed in differentiated THP-1 cells, a human monocytic cell line, and primary human blood monocyte-derived macrophages. Given the large number of cells required to generate a detectable phosphorylation signal by western blot, THP-1 cells were used to establish initial findings, which were then confirmed in primary cells. THP-1 cells have been used extensively to model both monocyte and macrophage function in-vitro. They can be differentiated into a macrophage-like phenotype by incubation with PMA, whereupon they acquire a flattened adherent morphology with an increased concentration of lysosomes and mitochondria, all changes consistent with maturation to a macrophage state. Flow cytometry has shown that differentiated THP-1 cells show increased surface expression of mature macrophage markers including CD11b, CD11c and CD68 and reduced expression of monocytic markers such as CD14 181.

Monocyte-derived macrophages are able to exhibit different phenotypes depending on the presence in-vitro of different cytokines and differentiating agents 182. As the respiratory tract is most common site of infection for *A. fumigatus*, my particular interest is in the response of alveolar macrophages. Obtaining alveolar macrophages from healthy volunteers is difficult however, making an alternative model system useful. I used GM-CSF - differentiated blood monocytes as a model as they appear to have a more alveolar macrophage like morphology, phenotype and function, compared to other differentiation agents such as macrophage-CSF (M-CSF) 183.

3.2 BTK is recruited to endosomes containing swollen *A. fumigatus* conidia

BTK activation is understood to be a two-step process that entails first membrane localisation of the kinase and secondly sequential phosphorylation of tyrosine residues in the kinase and SH3 domains ¹⁸⁴. Subcellular localisation of BTK in human monocyte-derived macrophages infected with *A. fumigatus* was examined by confocal microscopy. I found that a fluorescently labelled BTK antibody co-localised with swollen eGFP *Aspergillus* conidia within monocyte-derived macrophages. As *Aspergillus* conidia are known to be taken up into endosomes on entry into the cell, this finding provides indirect evidence that BTK is recruited to endosomes containing swollen *Aspergillus* conidia. Of note, this recruitment was dependent on the morphological state of the fungus. BTK was recruited to endosomes containing swollen but not resting *Aspergillus* conidia (Figure 2). This is consistent with findings in murine and human macrophages that resting conidia do not induce a significant inflammatory response ¹⁸⁵.

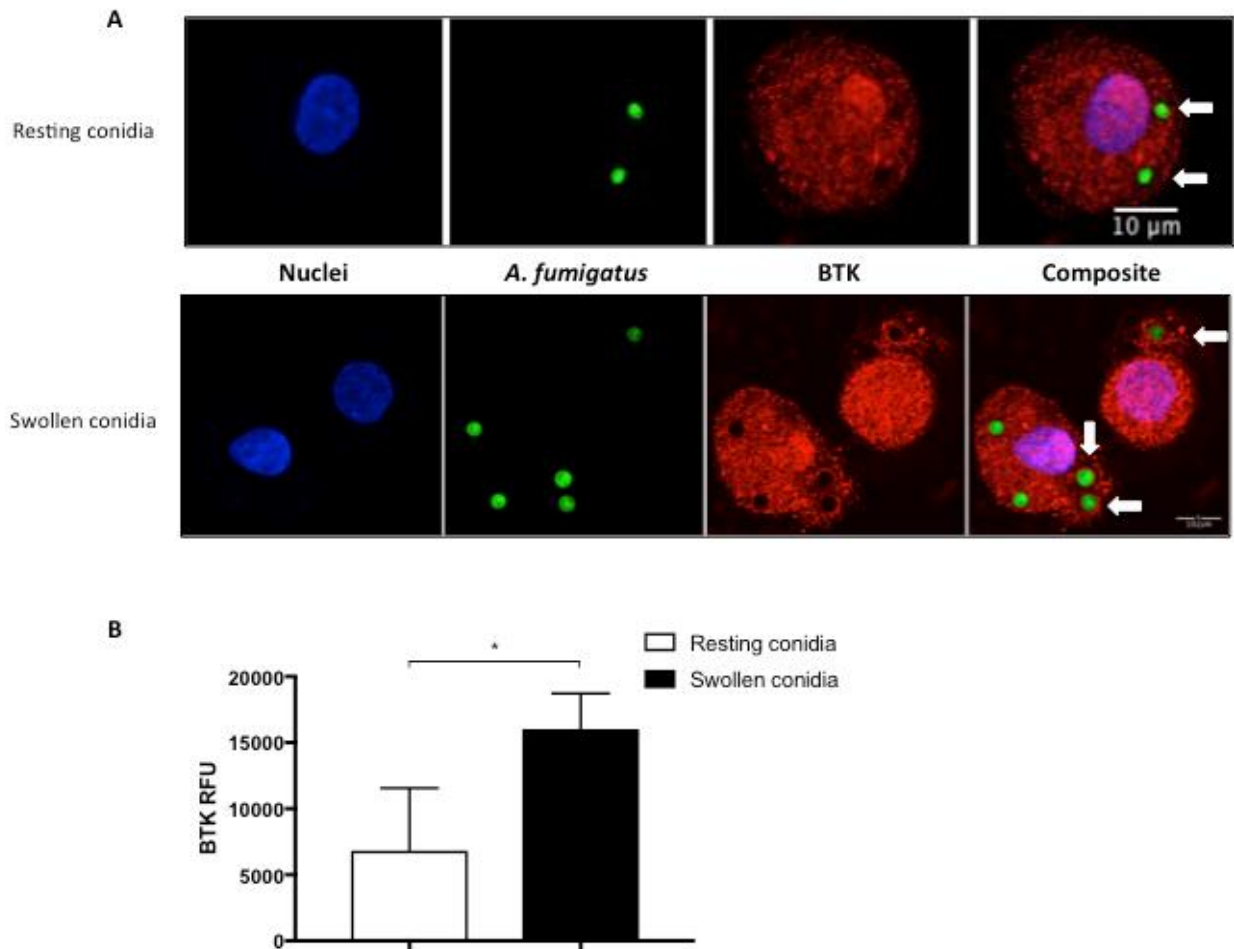


Figure 2. BTK is recruited to endosomes containing swollen but not resting *A.*

***fumigatus* conidia.** Human monocyte-derived macrophages were infected with either resting or swollen eGFP expressing *A. fumigatus* (green) (MOI=1) for 45min. Cells were then fixed and permeabilised and stained for BTK (red) and nuclei (blue). (A) Representative confocal microscopy images showing co-localisation of BTK and swollen *A. fumigatus* conidia (B) BTK/conidial co-localisation was quantified by measuring the average pixel intensity around single conidia using Image J software. N=3. RFU = relative fluorescence units.

3.3 *A. fumigatus* induces auto-phosphorylation of BTK at Tyr 223 in human macrophages

Following membrane localisation, BTK must undergo phosphorylation to become enzymatically active. Tyrosine residue 551 in the activation loop is trans-phosphorylated first, usually by a Src family kinase ¹²⁰. This phosphorylation step switches on BTK's intrinsic kinase activity and one of its' first targets is Tyrosine residue 223 in the SH3 domain. Auto-phosphorylation of Tyr 223 is thus an indicator of BTK activation ¹²¹.

Ibrutinib is an irreversible inhibitor of BTK that works by blocking the phosphorylation of BTK at Tyrosine 223. To assess whether BTK is involved in *A. fumigatus* induced signalling, THP1 and then monocyte-derived macrophages were pre-treated with Ibrutinib or vehicle control and then infected with *A. fumigatus*.

To confirm that any inhibitory effects seen in subsequent experimentation with Ibrutinib were not due to cell toxicity, I performed a dose titration on THP-1 cells and monocyte-derived macrophages (Figure 3A). Concentrations of 1 μ M and below were not associated with significant toxicity. I then performed a dose titration against TNF- α production to assess drug efficacy on macrophages and determine a suitable working concentration (Figure 3B). TNF- α release was significantly inhibited at 1 μ M. All further experiments were performed with Ibrutinib 1 μ M. On my review of the literature I could not find reference to tissue concentrations achieved with standard oral dosing of Ibrutinib. Pharmacokinetic and pharmacodynamic data derived from a human trial suggest a plasma concentration of ~ 0.5 μ M with a standard daily dose of 560 mg ¹⁸⁶.

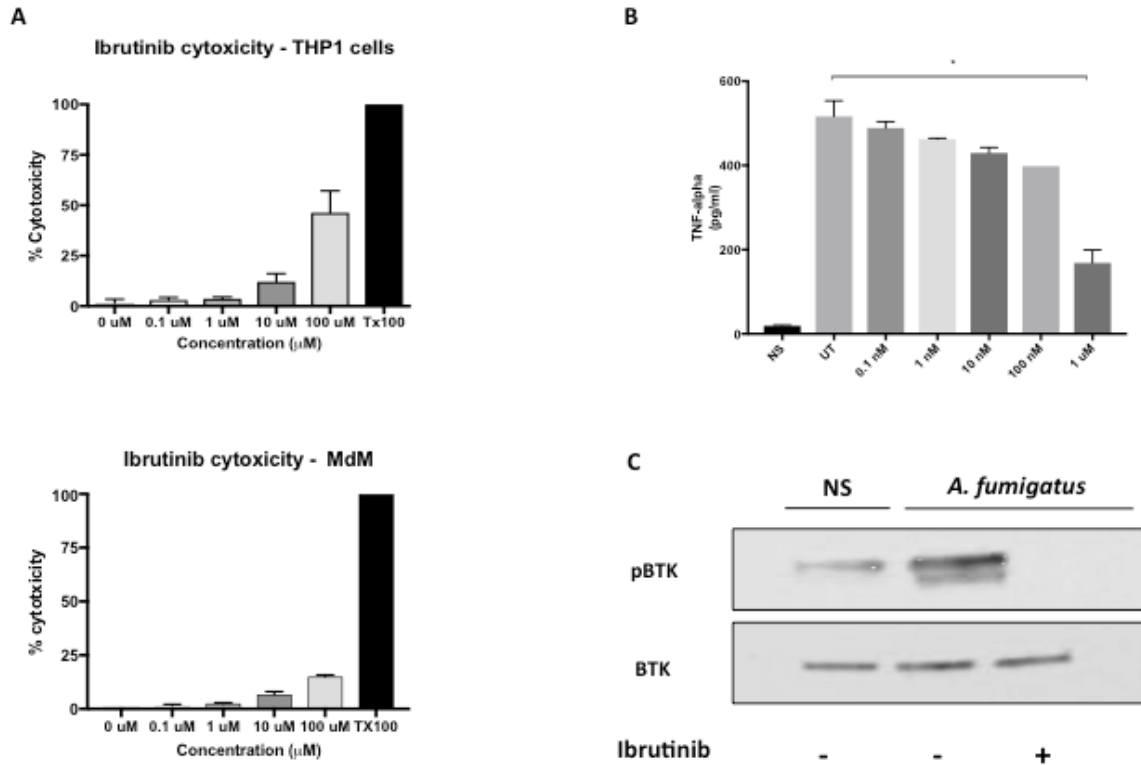


Figure 3. *A. fumigatus* induces auto-phosphorylation of BTK at Tyr 223 in human macrophages and this is inhibited by Ibrutinib. (A) THP1 and monocyte-derived macrophages were treated with rising concentrations of Ibrutinib for 18 hrs. LDH release in supernatants was measured and % cytotoxicity calculated compared to Triton X-100 treated cells. (B) Monocyte-derived macrophages were pre-treated with rising concentrations of Ibrutinib for 1 hour and then stimulated with *A. fumigatus* swollen conidia (MOI=1) for 6 hrs. TNF- α levels were measured in tissue culture supernatants by ELISA. (C) THP1 macrophages were pre-treated with Ibrutinib (1 μ M) or vehicle control before infection with *A. fumigatus* swollen conidia (MOI=5) for 2 hrs. Whole cell lysates were separated by SDS-PAGE, followed by Western Blotting. Membranes were probed with anti-Tyr 223 pBTK and anti-BTK antibodies. Bars represent mean + SD. p values calculated by one-way ANOVA with Dunnett's correction for multiple comparisons; p < 0.05. (A) N = 2, (B) N = 3 (C) image representative of 2 biological replicates.

Western blot analysis of whole cell lysates from macrophage infection experiments revealed that BTK Tyrosine 223 is auto-phosphorylated upon infection with *Aspergillus* and that Ibrutinib inhibits this step (Figures 3C and 4B).

3.4 *A. fumigatus* induces auto-phosphorylation of BTK along an endosomal TLR9-mediated pathway

It has been shown that TLR9 recognises *Aspergillus* DNA in murine and human myeloid cells triggering pro-inflammatory cytokine release¹⁸⁷. TLR9 recruitment to phagosomes containing *Aspergillus* conidia has also been demonstrated in murine macrophages¹⁸⁸. Others meanwhile have demonstrated TLR9-BTK interaction in THP-1 cells in response to CpG stimulation¹⁸⁹. To determine if BTK auto-phosphorylation is dependent on TLR9 recognition of *A. fumigatus*, macrophage cells were pre-treated with the TLR9 inhibitory oligonucleotide ODN 2088 prior to infection. ODN 2088 also antagonises TLR7 and 8 so a negative control for ODN 2088, ODN control, which inhibits TLR7 and 8 but not TLR9, was also tested. Western blot analysis of cell lysates showed that BTK auto-phosphorylation is TLR9 - dependent (Figure 4B). TLR9 is known to be an endosomal TLR, so to confirm that BTK is part of an endosomal signalling pathway, BTK auto-phosphorylation was assessed in the presence and absence of Cytochalasin D, an inhibitor of actin polymerisation. Blocking phagocytosis reduced BTK auto-phosphorylation almost back to baseline (Figure 4A).

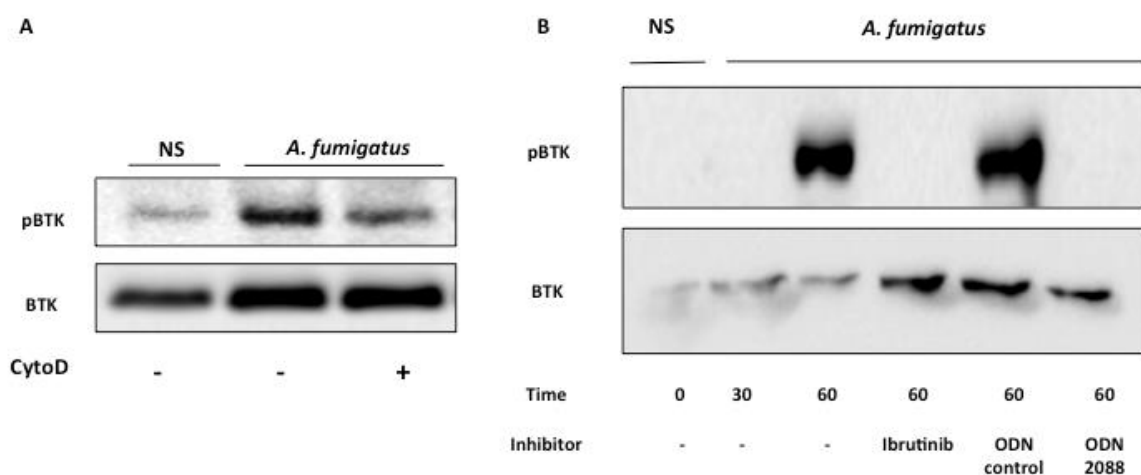


Figure 4. *A. fumigatus* induces auto-phosphorylation of BTK along an endosomal TLR9- mediated pathway. (A + B) Monocyte-derived macrophages were pre-treated with Cytochalasin D Ibrutinib (1 μ M), ODN 2088 (10 μ M), ODN control (10 μ M) or vehicle control for 1 hr. Cells were then stimulated with *A. fumigatus* swollen conidia (MOI=1) for 1 hr. Whole cell lysates were separated by SDS-PAGE, followed by Western Blotting. Membranes were probed with anti-pBTK and BTK antibodies. (A + B) Images representative of 2 biological replicates.

These results suggest that *A. fumigatus* activates an endosomal TLR9 - BTK signalling pathway in human macrophages as has been demonstrated in murine macrophages.

3.5 *A. fumigatus*-dependent calcineurin activation occurs downstream of an endosomal TLR9-BTK pathway

Activation of the calcineurin-NFAT pathway is required for TNF- α production by macrophages and subsequent neutrophil chemotaxis to the airways during pulmonary aspergillosis ⁹⁴. In murine macrophages this pathway is initiated by TLR9 mediated recognition of phagocytosed *A. fumigatus*⁹⁴ and auto-phosphorylation of BTK. Activated BTK phosphorylates PLC- γ via its PH domain and allows its catalysation of PIP2 to the

second messengers DAG and IP3. IP3 binding to IP3 receptors triggers calcium release into the cytoplasm. The resultant rise in cytoplasmic calcium concentration allows the calcium sensor Calmodulin to activate calcineurin. Calcineurin dephosphorylates NFAT, exposing nuclear localisation signals that allow NFAT to move into the nucleus and, in association with AP1, initiate transcription ¹⁹⁰.

To establish if a similar relationship exists in human macrophages, monocyte derived macrophages were pre-treated with the TLR9 antagonist ODN 2088, the non-binding ODN control, Ibrutinib or vehicle control prior to infection with *A. fumigatus*. Cells were lysed 1hour post-infection and nuclear extracts were analysed by western blot. NFATc2 nuclear translocation was found to be dependent on both TLR9 engagement and BTK auto-phosphorylation (Figure 5).

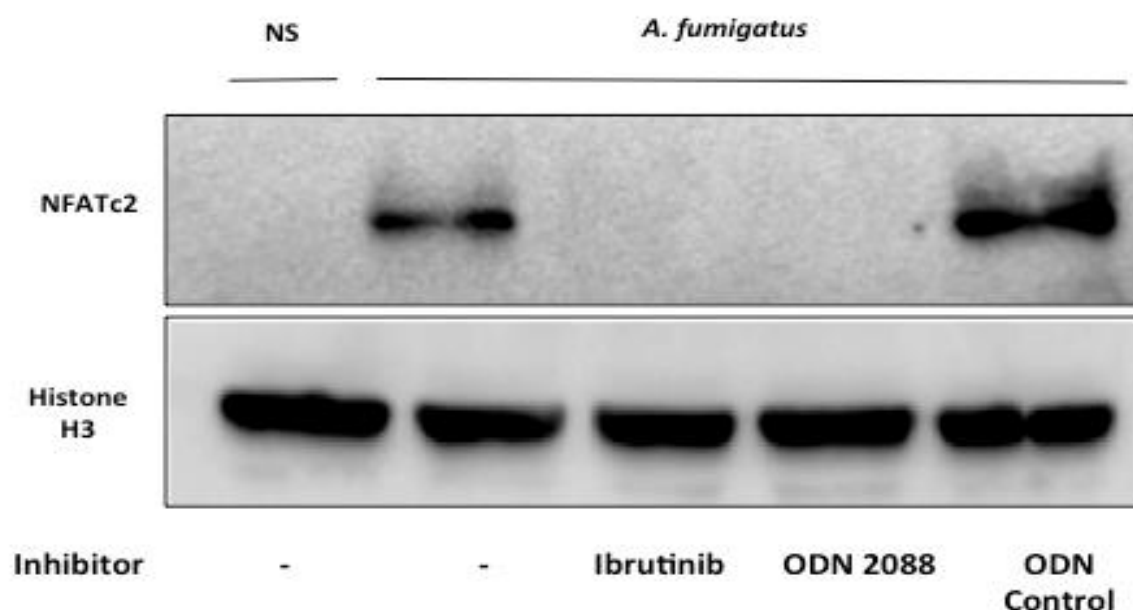


Figure 5. TLR9 engagement and BTK phosphorylation are required for NFAT activation in response to *A. fumigatus* in human monocyte-derived macrophages.

Human monocyte-derived macrophages were pre-treated with Ibrutinib (1 μ M), ODN 2088 (10 μ M), ODN control (10 μ M) or vehicle control for 1 hr. Cells were then stimulated with *A. fumigatus* swollen conidia (MOI=1) for 1 hr. Nuclear extracts were separated by SDS-PAGE, followed by Western Blotting. Membranes were probed with anti-NFATc2 and Histone H3 antibodies.

This relationship was confirmed and quantified using a second widefield microscopy method whereby transcription factor nuclear translocation was quantified by calculating the percentage overlap of the nuclear DAPI and transcription factor-linked fluorophore channels. Both techniques are able to detect the movement of transcription factors into the nucleus and so are corroborative. Microscopy, however, has the advantage for quantitative assessment of requiring fewer cells to detect a signal than western blotting, which is relevant when working with primary cells.

In a proof of principle experiment for this technique, macrophage cells were pre-treated with the calcineurin inhibitor FK506 and then stimulated with the calcium ionophore Ionomycin. Nuclear translocation was measured by western blotting of nuclear extracts (Figure 6A) and by quantification of the % overlap of the nuclear DAPI and transcription factor-linked fluorophore channels (Figure 6C), the Mander's co-efficient, using a macro written for the project. An FK506 blocked the nuclear translocation of NFAT as measured by western blotting (Figure 6A and B) and by widefield microscopy (Figure 6C and D).

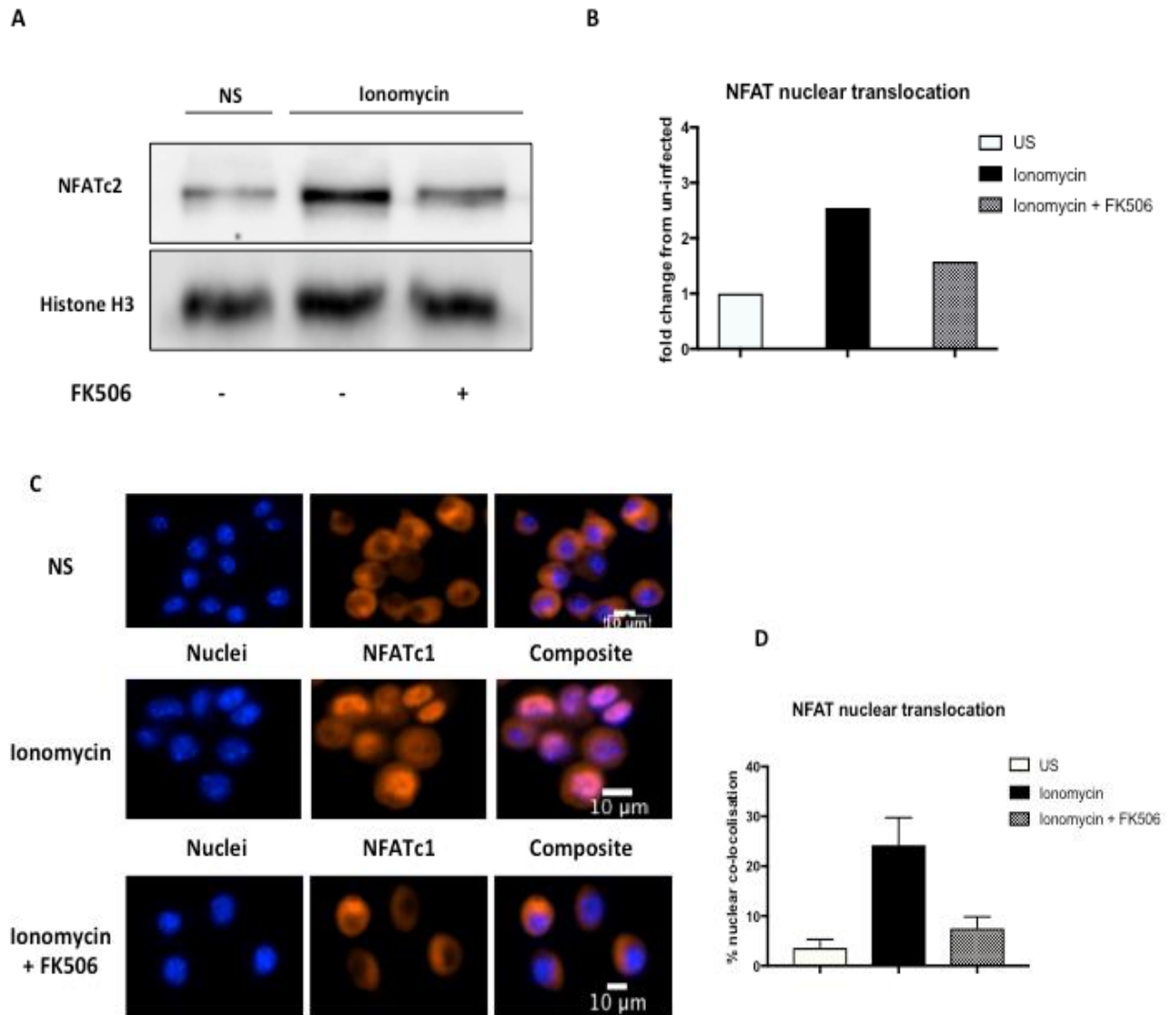
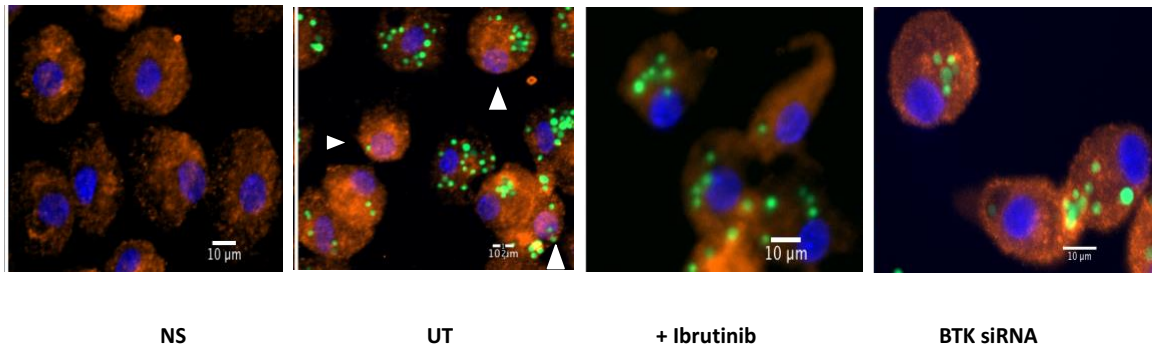


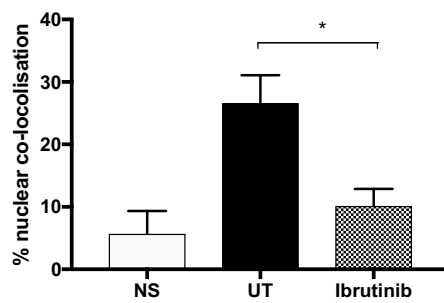
Figure 6. Nuclear translocation of transcription factors can be quantified using a widefield microscopy technique. (A-D) Human monocyte derived macrophages were pre-treated with FK506 (12.5 ng/ml) or vehicle control before stimulation with Ionomycin (2 μ g/ml) for 1 hr. (A) Nuclear extracts were separated by SDS-PAGE, followed by Western Blotting. Membranes were probed with anti-NFATc2 and Histone H3 antibodies. (B) Fold change in NFATc2 band intensity relative to un-stimulated (C) NFATc1 translocation was measured by widefield microscopy. (D) Nuclear translocation was quantified by calculating the % overlap of the nuclear DAPI and transcription factor-linked fluorophore channel. Data was calculated from 7 fields of view taken at random.

NFAT translocation as measured by widefield microscopy was significantly reduced by Ibrutinib inhibition of BTK auto-phosphorylation (Figure 7A and B). To rule out non-specific effects of Ibrutinib on other Tec family kinases, NFAT translocation was assessed in monocyte-derived macrophages treated with Scramble and BTK specific siRNA. A knockdown of 70% was confirmed by Western blotting (Figure 7D). Consistent with the results obtained with Ibrutinib, siRNA knockdown of BTK inhibited NFAT translocation in response to *A. fumigatus* (Figure 7A and C).

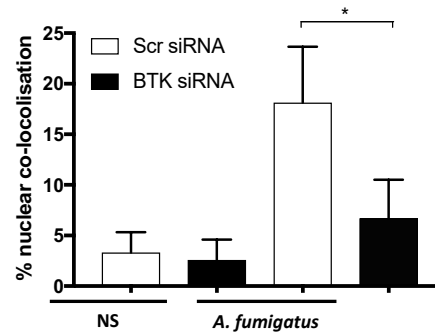
A



B



C

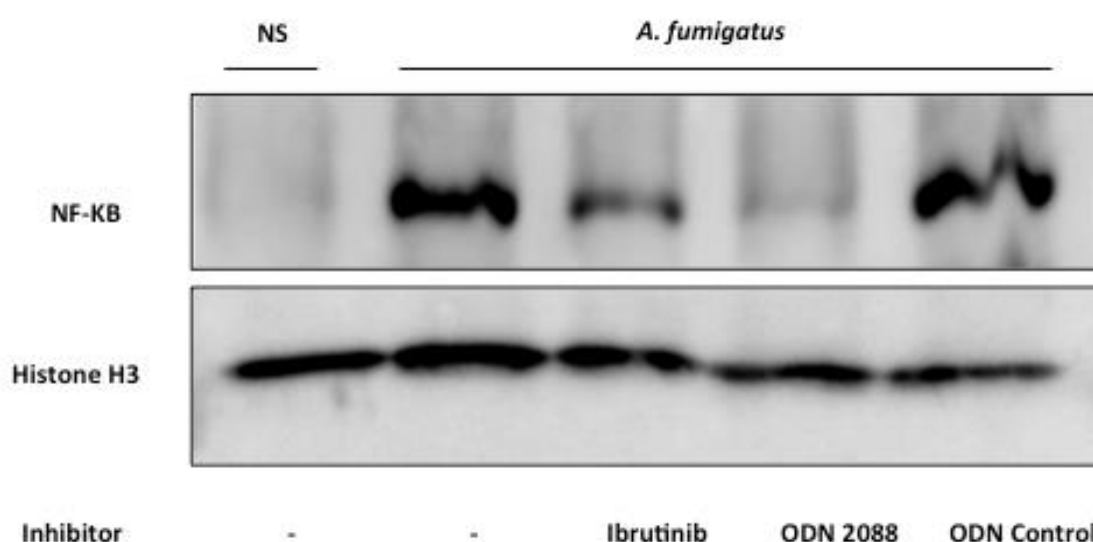


D



Figure 7. *A. fumigatus*-induced NFAT activation is BTK dependent. (A-D) Monocyte-derived macrophages were pre-treated with Ibrutinib (1 μ M) or vehicle control for 1 hour or Scramble or BTK-targeting siRNA (100 nM) for 72 hrs. (A-C) Cells were then stimulated with eGFP *A. fumigatus* swollen conidia (MOI=1) for 1 hr and NFATc1 translocation was quantified by widefield microscopy. (A) Representative widefield microscopy of monocyte-derived macrophages infected with eGFP expressing *A. fumigatus* (green) (MOI=1) at 60min p.i., stained for NFATc1 (orange) and nuclei (blue). (B-C) Nuclear translocation was quantified by calculating the % overlap of the nuclear DAPI and transcription factor-linked fluorophore channels. Data was calculated from 7 fields of view taken at random per biological repeat. Mean and SD of 4 biological repeats are represented. Statistical analysis was performed using one-way ANOVA: ns, not significant; *p<0.05. NS, non-stimulated, UT, un-treated. (D) Whole cell lysates were separated by SDS-PAGE, followed by Western Blotting. Membranes were probed with anti-BTK and B-actin antibodies.

3.6 *A. fumigatus* induces NF- κ B activation along an endosomal TLR9-BTK pathway



BTK siRNA knockdown re-affirmed the presence of a BTK-dependent pathway for NF- κ B activation (Figure 9A and B). To confirm the endosomal localisation of this newly described Aspergillus-dependent TLR9 – BTK – NF- κ B activation pathway, siRNA treated cells were pre-incubated with the actin inhibitor Cytochalasin D or vehicle control prior to stimulation with *A. fumigatus*. When phagocytosis was blocked, the inhibitory effect on NF- κ B activation of BTK knockdown was lost (Figure 9C). This demonstrates that Aspergillus is able to activate NF- κ B both via established cell surface initiated non-BTK dependent pathways and also via a novel endosomal BTK dependent pathway.

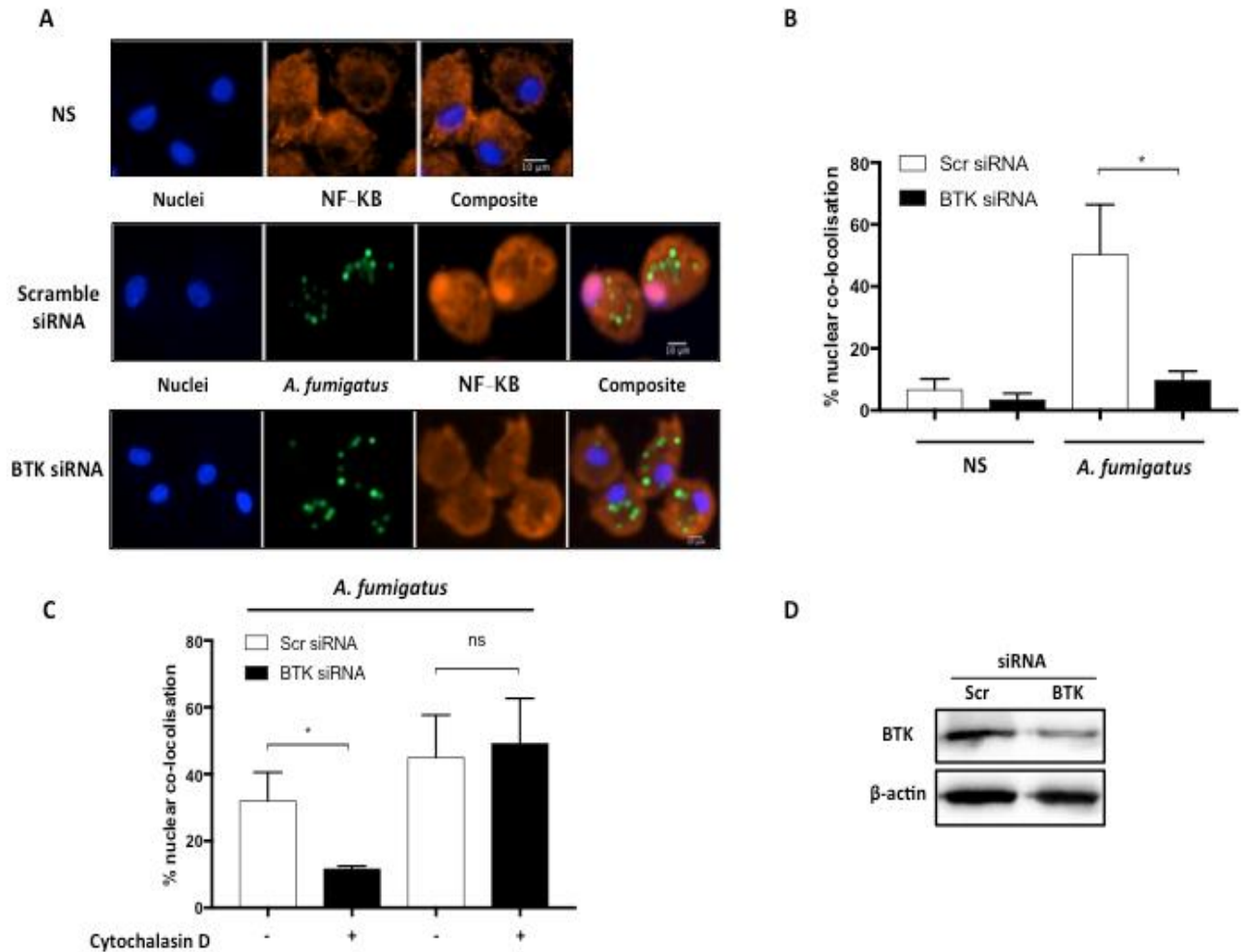


Figure 9. *A. fumigatus* induces NF-κB activation along an endosomal TLR9-BTK dependent pathway. Monocyte-derived macrophages were pre-treated with Scramble or BTK-targeting siRNA (100 nM) for 72 hrs and (C) Cytochalasin D (4μM) or vehicle control for 1 hour prior to infection. Cells were then stimulated with eGFP *A. fumigatus* swollen conidia (MOI=1) for 1 hr and NF-κB translocation was quantified by widefield microscopy. (A) Representative widefield microscopy of monocyte-derived macrophages infected with eGFP expressing *A. fumigatus* (green) (MOI=1) at 60min p.i. stained for NF-κB (orange) and nuclei (blue). (C) Nuclear translocation was quantified by calculating the % overlap of the nuclear DAPI and transcription factor-linked fluorophore channels. Data was calculated from 7 fields of view taken at random per biological repeat. Mean and SD of 3 biological repeats are represented. Statistical analysis was performed using one-way ANOVA: ns, not significant; *p<0.05. NS, non-stimulated, UT, un-treated.

3.7 TNF- α production is BTK dependent in human macrophages

TNF- α is a transcriptional target of both NFAT and NF- κ B. As would be expected, inhibition of BTK phosphorylation with Ibrutinib and BTK siRNA knockdown blocked *Aspergillus*-dependent TNF- α release by monocyte-derived macrophages (Figure 10A and C). This BTK dependency was seen in alveolar macrophages infected with *Aspergillus* as well (Figure 10B) suggesting that the effects of Ibrutinib seen in our monocyte-derived macrophage model may well reflect the impact of this drug on alveolar macrophage function in patients taking it as part of chemotherapeutic regimens.

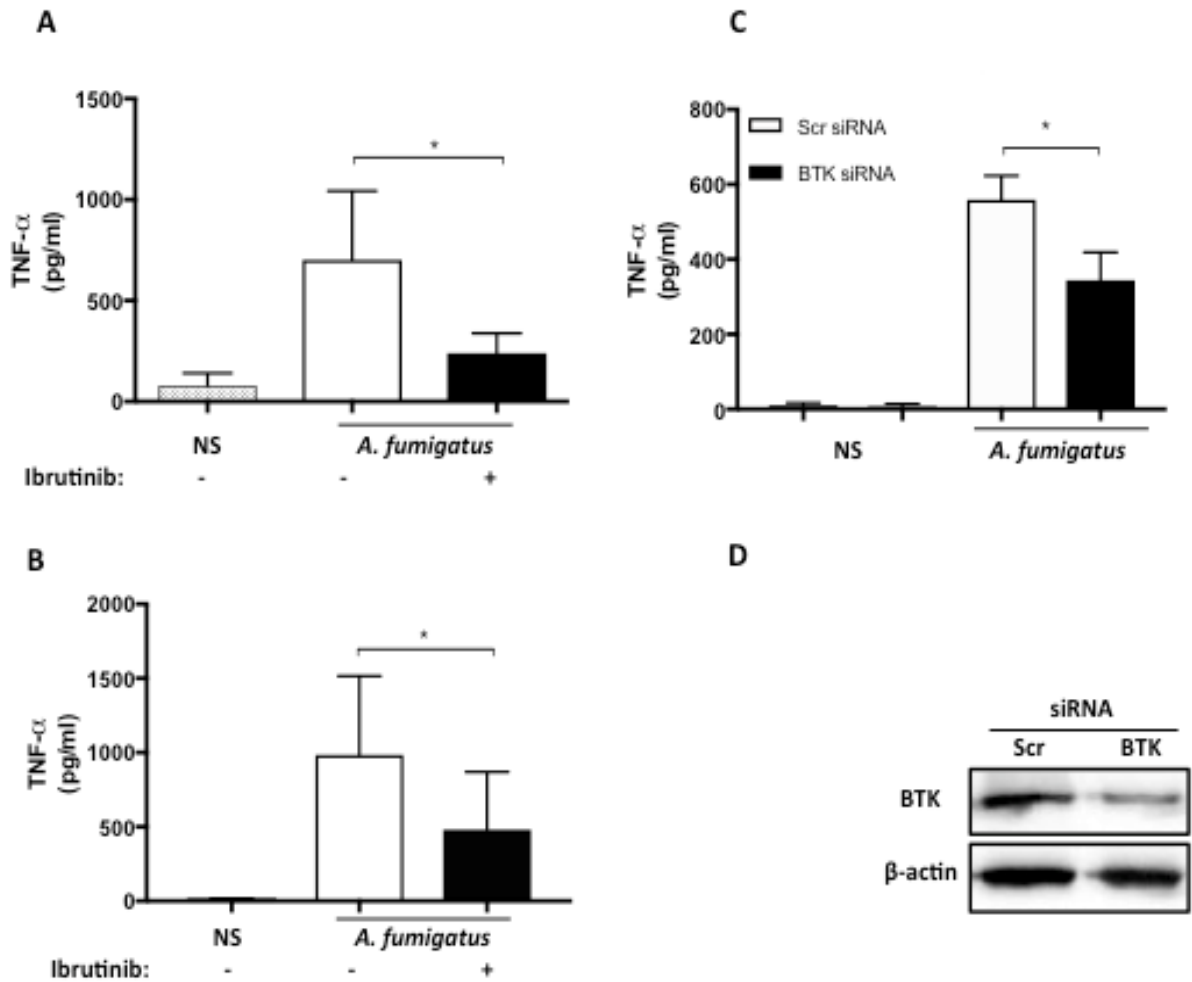
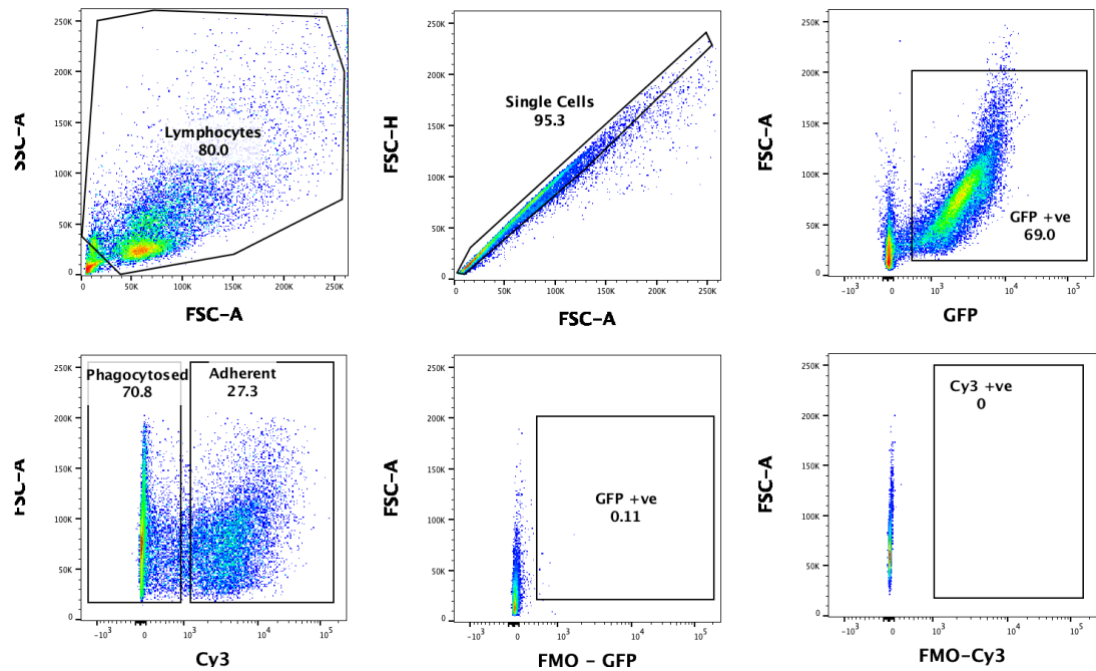


Figure 10. TNF- α release by human macrophages in response to *A. fumigatus* is BTK dependent. (A, C + D) Monocyte-derived macrophages and (B) alveolar macrophages were pretreated with (A + B) Ibrutinib (1 μ M) or vehicle control for 1 hr or (C + D) Scramble or BTK-targeting siRNA (100 nM) for 72 hrs. (A-C) Cells were stimulated with *A. fumigatus* swollen conidia (MOI=1) for 6 hrs. TNF- α levels in the tissue culture supernatants were measured by ELISA. (D) Whole cell lysates were separated by SDS-PAGE, followed by Western Blotting. Membranes were probed with anti-BTK and anti- β -actin antibodies. (A-C) Mean and SD of 3 biological repeats are represented. Statistical analysis was performed using paired Student's t-tests. * p <0.05. NS, non-stimulated.

3.8 Fungal killing but not phagocytosis is BTK dependent in human macrophages

BTK has been implicated in the regulation of Fcγ receptor mediated phagocytosis in murine and human macrophages^{194 195}. However, particularly in human cells, the data is mixed with some studies finding BTK to be redundant in phagocytosis¹⁹⁶. To investigate the role of BTK in the phagocytosis of *Aspergillus* conidia, monocyte-derived macrophages were pre-treated with Ibrutinib or vehicle control prior to infection with biotinylated GFP-expressing *Aspergillus* conidia. Cells were then counterstained with Cy3 biotin antibody and phagocytosis measured by flow cytometry. Lymphocytes were selected by forward and side scatter followed by gating of a GFP positive population. GFP positive cells were then divided into cells with adherent conidia and cells with internalised conidia based on Cy3 positivity (Figure 11A). Phagocytosis was not BTK dependent (Figure 11B).

A



B

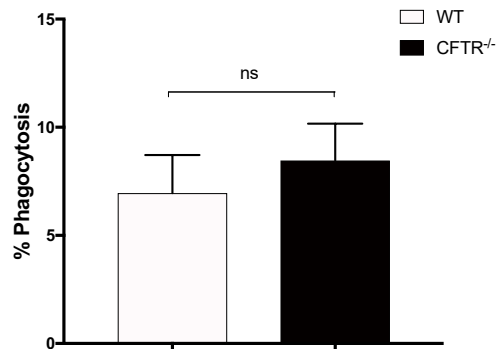


Figure 11. Phagocytosis of *A. fumigatus* is not BTK dependent in human macrophages. Human monocyte-derived macrophages were pre-treated with Ibrutinib (1 μ M) for 1 hr. Cells were infected with biotinylated GFP *A. fumigatus* swollen conidia (MOI=1) for 2 hrs. Cells were then counterstained with Cy3 biotin antibody and phagocytosis was measured by flow cytometry. (A) Gating strategy for measurement of conidial phagocytosis. (B) Percentage of cells with phagocytosed conidia. Mean and SD of 3 biological repeats are represented. Statistical analysis by paired t-test, ns = non-significant.

These results confirm that the BTK dependency of *Aspergillus*-induced NFAT and NF- κ B activation observed so far is due to BTK's role in endosomal signalling rather than reduced cellular uptake.

Fungal killing of *A. fumigatus* has been found to be calcineurin-dependent in human monocyte-derived macrophages and alveolar macrophages¹⁸⁵. It would not be unexpected therefore to find that fungal killing is also BTK dependent given BTK is hypothesized to lie upstream of calcineurin activation. Fungal killing was assessed by CFU counts at 6 hours post macrophage infection and by measurement of galactomannan, an *Aspergillus* cell wall antigen, in cell culture supernatants at 6 hrs. Fungal killing was reduced as evaluated by both measures (Figure 12A and B).

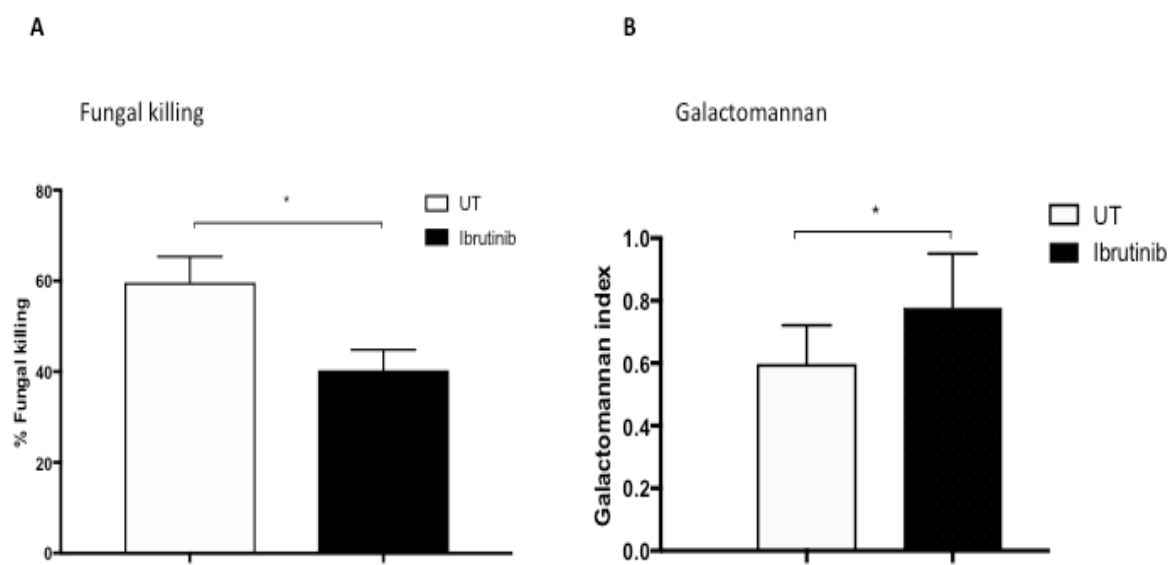


Figure 12. Fungal killing is BTK dependent. Monocyte-derived macrophages were pre-treated with Ibrutinib (1 μ M) for 1 hr. Cells were stimulated with *A. fumigatus* swollen conidia (MOI=1) for 6 hrs. (A) Cells were washed to remove extra-cellular conidia and then lysed in 0.1% Tween 20/dH₂O and serial dilutions plated onto Sabouraud agar plates. Plates were incubated at 37 °C overnight and CFUs were counted

the next day. (B) Galactomannan levels in the tissue culture supernatants were measured with a galactomannan detection kit. (A +B) Mean and SD of 3 biological repeats are represented. Statistical analysis by paired Student's t-test. *p<0.05.

3.9 Discussion

A. fumigatus most commonly enters the body by inhalation into the lungs where it is rapidly internalized by airway macrophages. Uptake of *Aspergillus* conidia into cells occurs primarily via phagocytosis, though there is evidence that an endocytic process with features of macro-pinocytosis also contributes to uptake ⁵⁴. Subject to the morphotype and germination state of the fungus at the time of uptake, phagocytosis is dependent on the coordinated activity of C-type lectin receptors ¹⁹⁷, Toll-like receptors ⁵⁴ and opsonin receptors ¹⁹⁸.

Once internalized, conidia enter an endocytic pathway that is broadly unidirectional, proceeding from early to late endosomes to lysosomes. Early and late endosomes are defined by the recruitment of the small GTPases RAB5 (early) and RAB7 (late) ⁵⁶. Early endosomes are generally unable to kill microorganisms and must undergo a sequential maturation process in order to form phagolysosomes. Once matured, microbial killing is mediated through acidification, NADPH oxidase-dependent reactive oxygen species production and lysosomal hydrolases ⁵⁸.

As well as facilitating fungal killing, the endosomal system also allows for the activation of multiple signalling pathways responsible for regulating macrophage effector functions. In order to initiate these pathways and indeed decide which pathways to activate, the cell must first recognise what it has internalised as self or non-self and

second determine its pathogenicity. Several endosomal receptors have been identified as playing a role in instigating immune responses to fungi including the TLRs: TLR 3, 7, 8 and 9 and the CLRs Dectin-1 and Mannose receptor. Endosomal TLRs recognise nucleic acids released by pathogen degradation during endosomal maturation. Distinction between self and non-self nucleic acids is thought to rely on nucleic acid sequence-specific motifs being distinct between species and distinguishable by TLRs, endosomal segregation of nucleic-acid sensing TLRs and concentration of nucleic acids above an immuno-stimulatory level within endosomes ¹⁹⁹.

It has been shown in murine macrophages that TLR9 is recruited to endosomes containing *Aspergillus* conidia¹⁸⁸ guided in part by fungal DNA's methylation status. Fungal DNA is less widely methylated than mammalian DNA and TLR9 preferentially recognises unmethylated CpG sequences ²⁰⁰. It had previously been thought that TLR9 expression was limited to B cells and dendritic cells in humans. However, more recent studies have provided evidence for TLR9 activity in human macrophages as well ^{201 202}. TLR9 recognises *Aspergillus* unmethylated CpG DNA in both murine and human cells as the first step in the potential triggering of anterograde signalling pathways ¹⁸⁷. Like other receptors, TLR9 must recruit adaptor molecules in order to transfer signals. TLR9 has been shown to interact with BTK in THP-1 cells in response to CpG stimulation ¹⁸⁹. I found that BTK is recruited to endosomes containing *Aspergillus* conidia and that this recruitment is dependent on conidial swelling. When cells were infected with resting conidia, there was no co-localisation of BTK with fungal conidia. This is consistent with our findings in murine and human macrophages that resting conidia do not induce a significant inflammatory response ¹⁸⁵.

In contrast, TLR9 recruitment has been found to be independent of fungal morphological state ²⁰³. This discrepancy suggests that while TLR9 may be found within the fungal endosome, it does not always recruit BTK. The reason for this is not yet clear. One hypothesis is that swollen conidia, lacking the rigid layer of hydrophobins surrounding the cell wall present in resting conidia, are more easily degraded exposing stimulatory CpG DNA. Alternatively, conidial swelling may allow for an as yet unspecified co-stimulatory interaction in the endosome that triggers BTK engagement. Irrespective of how exactly TLR9 recruits BTK, it is clear that BTK engagement is an important checkpoint in the activation of inflammatory pathways in response to *A. fumigatus* infection. Such a role for BTK in relation to TLR9 signalling is not unique. BTK has been found to be involved in signalling downstream of other TLR receptors. In human PBMCs from XLA patients and healthy controls, BTK was required for TLR2 and TLR4-induced TNF- α production ¹²³. In TLR7/8 signalling in human monocytes, BTK acts upstream of NF- κ B activation, regulating the serine phosphorylation of p65RelA and thereby its nuclear localisation ²⁰⁴.

Upon localisation to the fungal endosome, I found that BTK is activated in a TLR9-dependent manner, as indicated by auto-phosphorylation of tyrosine residue 223. BTK activation in turn triggers PLC- γ induced calcium influx into the cytoplasm, which enables NFAT translocation into the nucleus. Although, it is well established that NF- κ B is activated along canonical and non-canonical pathways in response to cell surface Dectin-1 binding ²⁰⁵, I found that NF- κ B translocation also lies downstream of this endosomal TLR9-BTK mediated pathway (Figure 13). The exact mechanism by which this occurs is not yet clear. BTK has been implicated in TLR4-mediated NF- κ B activation in response to LPS where it is involved in the pathway leading to phosphorylation of

p65 on serine 536 but is not involved in I κ B α degradation ²⁰⁶. In B cells BTK activates Protein kinase C (PKC) via PLC- γ activity. PKC in turn phosphorylates I κ B kinase (IKK), which degrades I κ B α allowing NF- κ B to move into the nucleus ²⁰⁷. Further work is needed to establish the remaining components of the pathway linking TLR9-BTK and NF- κ B activation in *Aspergillus*-infected macrophages.

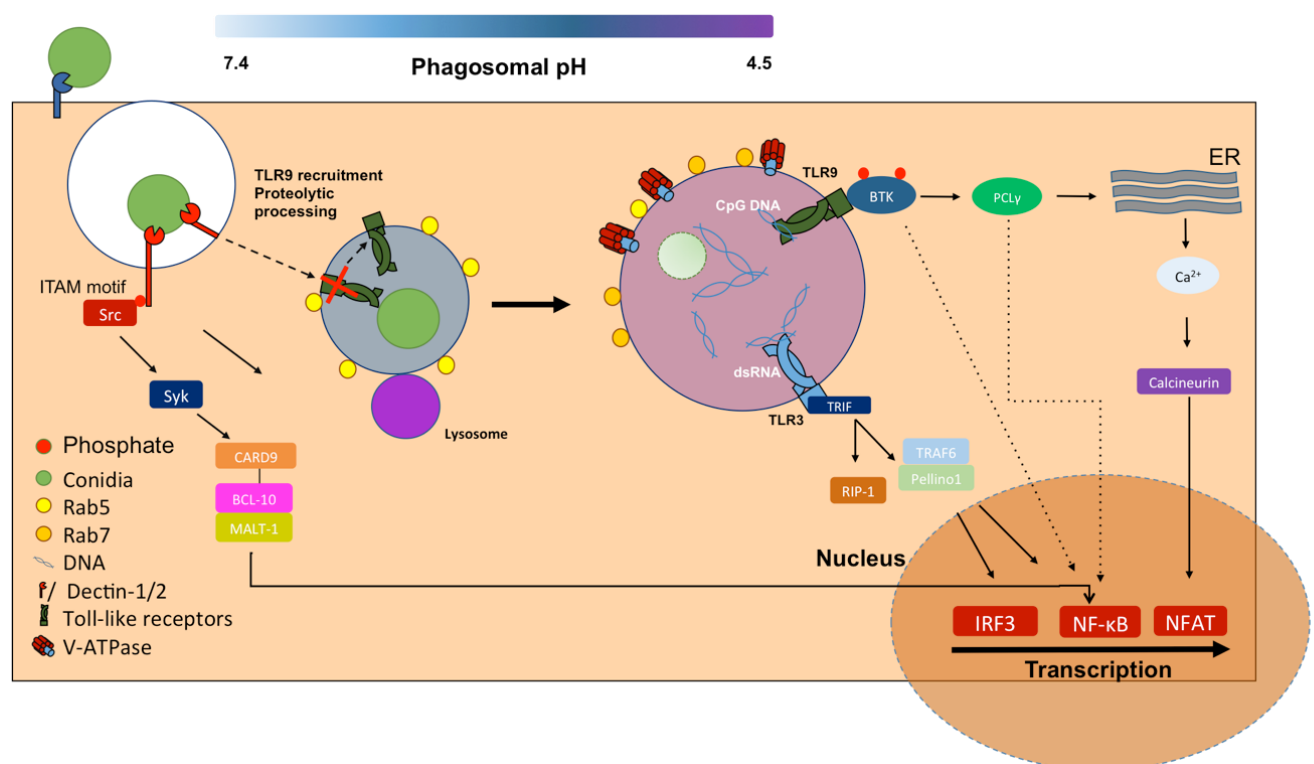


Figure 13. Working model for BTK-dependent signalling in macrophages infected with *A. fumigatus*. *A. fumigatus* conidia are phagocytosed by macrophages in the lung. Phagosomes mature and recruit pattern recognition receptors such as TLR9 and TLR3. If the conidia are swollen, TLR9 attracts BTK which undergoes activation before in turn phosphorylating phospholipase C- γ (PLC- γ). The resultant rise in cytoplasmic Calcium drives NFAT into the nucleus to initiate transcription. NF- κ B is activated from the cell-surface via canonical and non-canonical pathways. NF- κ B can also be activated from the endosome via a TLR9-BTK mediated pathway.

BTK is one of a family of non-receptor tyrosine kinases, the Tec kinases. BMX and TEC are expressed in monocytes and macrophages alongside BTK ²⁰⁸ and there is some evidence for their involvement in the host response to pathogens. BMX is required for TLR4-dependent IL-6 production in LPS-stimulated macrophages via a stabilising effect on IL-6 mRNA, although the exact mechanism is not known ²⁰⁹. In a murine model, TEC contributed to the assembly of the non-canonical caspase 8 inflammasome in response to *Candida albicans* infection ²¹⁰. ITK is expressed in T cells and acts downstream of the T-cell receptor to activate PLC γ and thus affect signalling via the calcineurin-NFAT, NF- κ B and MAPK pathways ²¹¹, similar to the role of BTK in myeloid cells. It is possible that there is an element of functional redundancy between the Tec family kinases, which might explain why invasive fungal disease is not common among XLA patients. This is in contrast to the observed association between Ibrutinib therapy and cases of invasive aspergillosis. Ibrutinib is recognized to have an off-target inhibitory effect on the wider Tec family kinases ²¹², which might account for the discrepancy between the incidence of invasive fungal disease between these two clinical groups.

As regards my experimental findings, off-target inhibition of non-BTK Tec kinases may have contributed to the observed impact of Ibrutinib on macrophage function, but a significant role for BTK in macrophage immunity was confirmed by the use of BTK-specific siRNA. This is supported by the literature where macrophages derived from XLA patients demonstrate impaired secretion of pro-inflammatory cytokines in response to TLR8 and 9 stimulation compared to healthy controls ^{127,189}.

Alveolar macrophages play a key role in controlling pulmonary fungal infection in-vivo both through direct killing of phagocytosed pathogens ⁵⁵ and via the recruitment of

neutrophils, monocytes and NK cells to the site of infection ²¹³. Fungal growth control was impaired in macrophages treated with Ibrutinib suggesting a role for BTK in intracellular fungal killing. TNF- α production by macrophages infected with *Aspergillus* was also BTK-dependent and inhibited by Ibrutinib. These results suggest that BTK is important for the control of *Aspergillus* infections in-vivo. Recent clinical trials have found that patient's taking Ibrutinib are at increased risk of *Aspergillus* infections^{146 145}. My results demonstrate the central role BTK has in endosomal signalling pathways in human macrophages and the impact of inhibition of this kinase on vital macrophage effector functions. These findings may help explain the link that has been observed clinically between Ibrutinib and invasive *Aspergillus* infections.

Chapter 4

Aspergillus-induced programmed macrophage cell death is regulated by a TLR9-BTK dependent pathway

4.1 Introduction

Programmed cell death has emerged over recent years as an important component of the host response to infection. Host cell death can be unplanned, occurring secondary to damage caused by trauma or infection, or it can be programmed and executed in response to activation of as yet incompletely delineated signalling pathways within the cell. Whether host cell death is beneficial to the host or the pathogen is a complex question. It is evident that the death of host cells can be advantageous for certain pathogens, allowing cellular egress and dissemination ²¹⁴. However, it is also clear that for other pathogens, host cell death is detrimental as it removes a protective intracellular niche ²¹⁵. Those pathogens that seek an intracellular niche have evolved strategies to manipulate and inhibit host cell death to their advantage ^{216,217}. This suggests that the death of infected host cells can be a protective end-point for the host as a whole.

The term “programmed cell death” describes not a single homogenous process but rather covers a range of modes of cell death. These can be classified into lytic and non-lytic forms of cell death. Lytic cell death is inflammatory with the potential to cause damage to the host through the release of toxic intracellular contents. Lytic forms of programmed cell death include necroptosis, pyroptosis, parthanatos, ferroptosis,

mitochondrial permeability transition (MPT)-dependent necrosis, pyro-necrosis, and ET-osis ²¹⁸. Non-lytic programmed cell death includes apoptosis and is typically more immunologically silent.

Most of the work demonstrating the role of programmed cell death in host defence has been conducted using bacteria or viruses as the infecting pathogens. There is evidence, however, that programmed cell death is also triggered in response to fungal infections. Macrophages infected with *Candida* undergo pyroptosis ^{219,220} as well as apoptosis ²²¹ and the fungus can trigger ET-osis in neutrophils ^{222,223}. Dendritic cells release IL-1 β in a caspase 1 dependent manner and activate the AIM2 and NLRP3 inflammasomes ²²⁴ when infected with *Aspergillus fumigatus*. While pyroptosis is generally considered to be a Caspase 1 or 11 dependent process, *Aspergillus* induced lytic cell death has not yet been demonstrated to be dependent on either of these caspases. *Aspergillus* manipulates programmed cell death pathways by inhibiting apoptosis in macrophages in its conidial form ²²⁵ and inducing it in its hyphal form ²²⁶.

In work conducted by my group, *Aspergillus* has been shown to induce morphological changes suggestive of necroptotic cell death in human macrophages unable to control fungal germination ¹⁸⁵. In about a third of cells undergoing this necrotic cell death, the infecting conidia were transferred to neighbouring cells allowing control of hyphal escape. Both host cell death and lateral transfer were calcineurin-dependent. In the work presented in my first chapter I demonstrated how calcineurin-NFAT activation occurs in response to *Aspergillus* triggering of a TLR9-BTK dependent signalling pathway in human macrophages and others in our group have shown this relationship in murine macrophages ⁹⁴. I continued my research by seeking to clarify how

Aspergillus infection activates programmed cell death in macrophages and what pathways are involved in its regulation.

4.2 *A. fumigatus* induces RIPK3-dependent necroptosis in macrophages

Once Aspergillus conidia are phagocytosed and taken up into the endosome, macrophage effector mechanisms aimed at killing the infecting pathogen are activated. If the cell fails to kill the conidia, then the fungus will germinate leading to cellular escape through hyphal piercing of the cell membrane ²⁸. Others in my group have shown that the onset of fungal germination can also be a trigger for the activation of programmed cell death and lateral transfer of conidia to neighbouring cells. Infected macrophages develop an increasingly translucent cytoplasm, organelle swelling and increased cell volume followed by cell lysis, all changes suggestive of a necrotic form of cell death ¹⁸⁵.

Necroptosis is a form of programmed necrosis. The serine/threonine protein kinase receptor-interacting protein kinase 3 (RIPK3) must be activated to trigger its auto-phosphorylation, oligomerization and onward phosphorylation of the necroptosis effector mixed lineage kinase domain-like (MLKL). Following phosphorylation, MLKL undergoes a conformational change that allows for interaction with the inner leaflet of the plasma membrane where MLKL oligomerizes to form the necroptotic pore. After pore formation osmotic pressure from ion and water influx results in rupture of the plasma membrane ²²⁷. To confirm that the necrotic death observed in macrophages infected with *A. fumigatus* is RIPK3 dependent necroptosis, J774A1 cells were pre-treated with the RIPK3 inhibitor GSK'872 for 1 hour prior to infection with *A. fumigatus*

conidia. The DNA stain propidium iodide (PI) was added to the culture media as a fluorogenic marker of all cause cell death whereas the Caspase 3 substrate Ac-DEVD-AMC was added as a specific marker of apoptosis. Lytic cell death was assessed by measurement of LDH release. Unexpectedly, PI+ve macrophage cell death was increased when RIPK3 was inhibited (Figure 14A) whereas LDH release was reduced (Figure 14C). These discrepant results suggest both that macrophages undergo a RIPK3-dependent form of lytic cell death in response to *A. fumigatus* infection consistent with necroptosis and that inhibition of RIPK3 with GSK'872 leads to an increase in a non-lytic form of cell death. This hypothesis was confirmed by the observation of a significant increase in apoptosis in GSK'872 treated cells (Figure 14B).

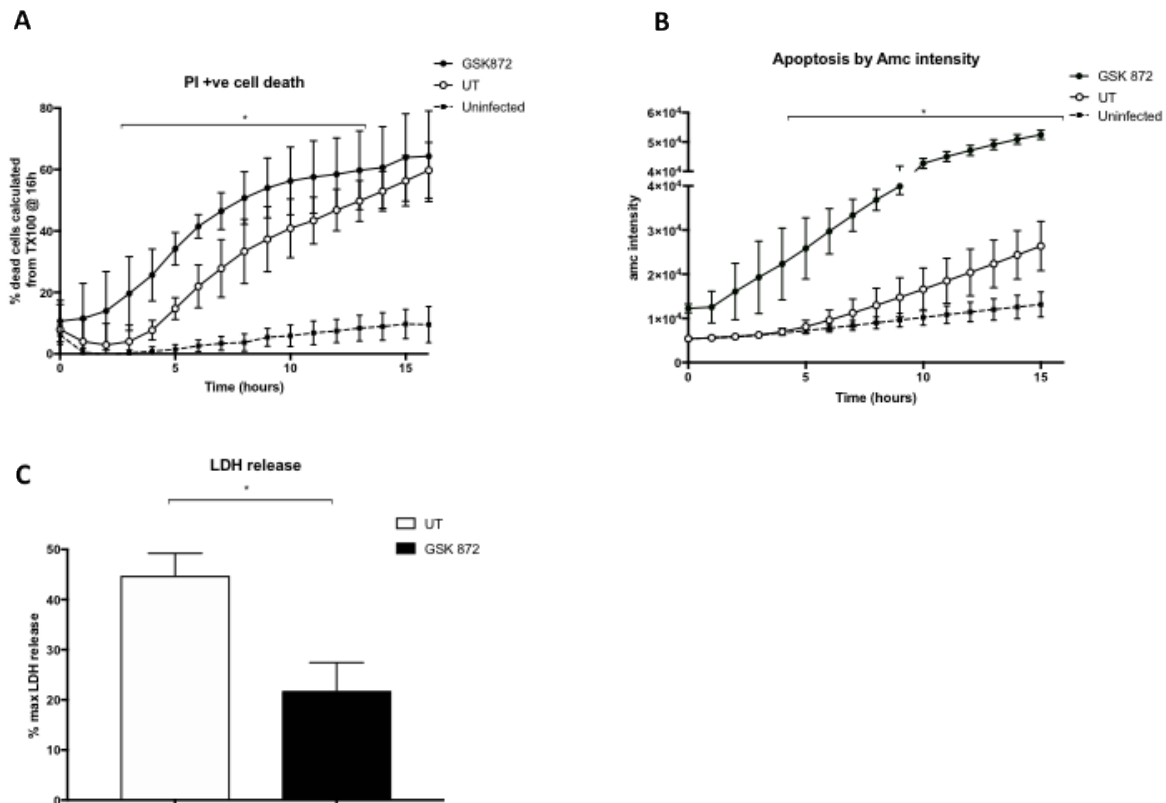


Figure 14. RIPK3 can act as a switch balancing inflammatory necroptotic cell death and non-lytic apoptotic cell death. (A + B) J774A1 cells were pre-treated with 3 μ M GSK'872 for 1 hour prior to infection with CEA *A. fumigatus* swollen conidia (MOI=2). Following infection, propidium iodide and Ac-DEVD-AMC Caspase-3 Fluorogenic Substrate were added to the media. Live time-lapse imaging was performed overnight using a fluorescent plate reader (Tecan). (A) PI +ve cell death was expressed as % of Triton x100 treated cells (B) Apoptosis was measured by monitoring amc intensity. Mean and SD from 3 independent experiments are represented. P value calculated by two-way ANOVA * $p < 0.05$. (C) LDH levels in the tissue culture supernatants were measured by colorimetric formazan product formation assay. P value calculated by paired Student's t-test: * $p < 0.05$.

These results are consistent with previous findings that GSK'872 can induce apoptosis in a RIPK3-dependent manner. This is an on-target effect which is not related to RIPK3's kinase activity but rather due to the conformation-dependent capacity of RIPK3 to

recruit RIPK1 via RHIM binding and trigger Caspase 8-dependent apoptosis ²²⁸. GSK'872 has a high specificity for RIPK3. Unexpected experimental results are more likely to be related to RIPK3's varied role within cell death and inflammatory pathways rather than off-target effects.

4.3 RIPK1 is a necessary mediator of *A. fumigatus* induced necroptosis

Classical necroptosis is understood to occur in response to the binding of TNF- α to the TNF receptor which leads eventually to the interaction of RIPK1 and RIPK3 through their RHIM domains to form the necrosome complex ^{229,230}. Other proteins including TLR3, TLR4, TRIF and DAI also have RHIM domains and are able to form so called “non-classical” necrosomes directly with RIPK3 without the involvement of RIPK1 ²³¹. The role of RIPK1 in TLR and TRIF mediated necroptosis is not clear. Murine cytomegalovirus (MCMV)-induced necroptosis is RIPK1 independent, occurring instead following the direct interaction of DAI and RIPK3 ²³². However, others have found that TLR3/TLR4 induced necrosis is blocked by the RIPK1 inhibitor Necrostatin-1 ^{233,234}.

To determine the role of RIPK1 in *Aspergillus*-induced necroptosis, BMDM from C57BL/6 mice were pre-treated with Necrostatin-1 for 1 hour prior to infection with either eGFP, for the live widefield microscopy, or CEA, for the plate-reader microscopy, *A. fumigatus* conidia. Both PI +ve cell death and LDH release were reduced by inhibition of RIPK1 kinase activity (Figure 15 A-C). Although PI is not a specific necroptotic cell death marker, this result provides indirect evidence that *Aspergillus* induces a RIPK1-dependent form of lytic cell death, that is necroptosis. Of note apoptosis was also reduced by RIPK1 inhibition (Figure 15D). RIPK1 forms part of cytoplasmic Complex 2,

alongside TRADD, Caspase 8 and FADD, which lies upstream of the apoptosis effectors caspases 3 and 7 in both intrinsic and extrinsic activation pathways ²³⁵.

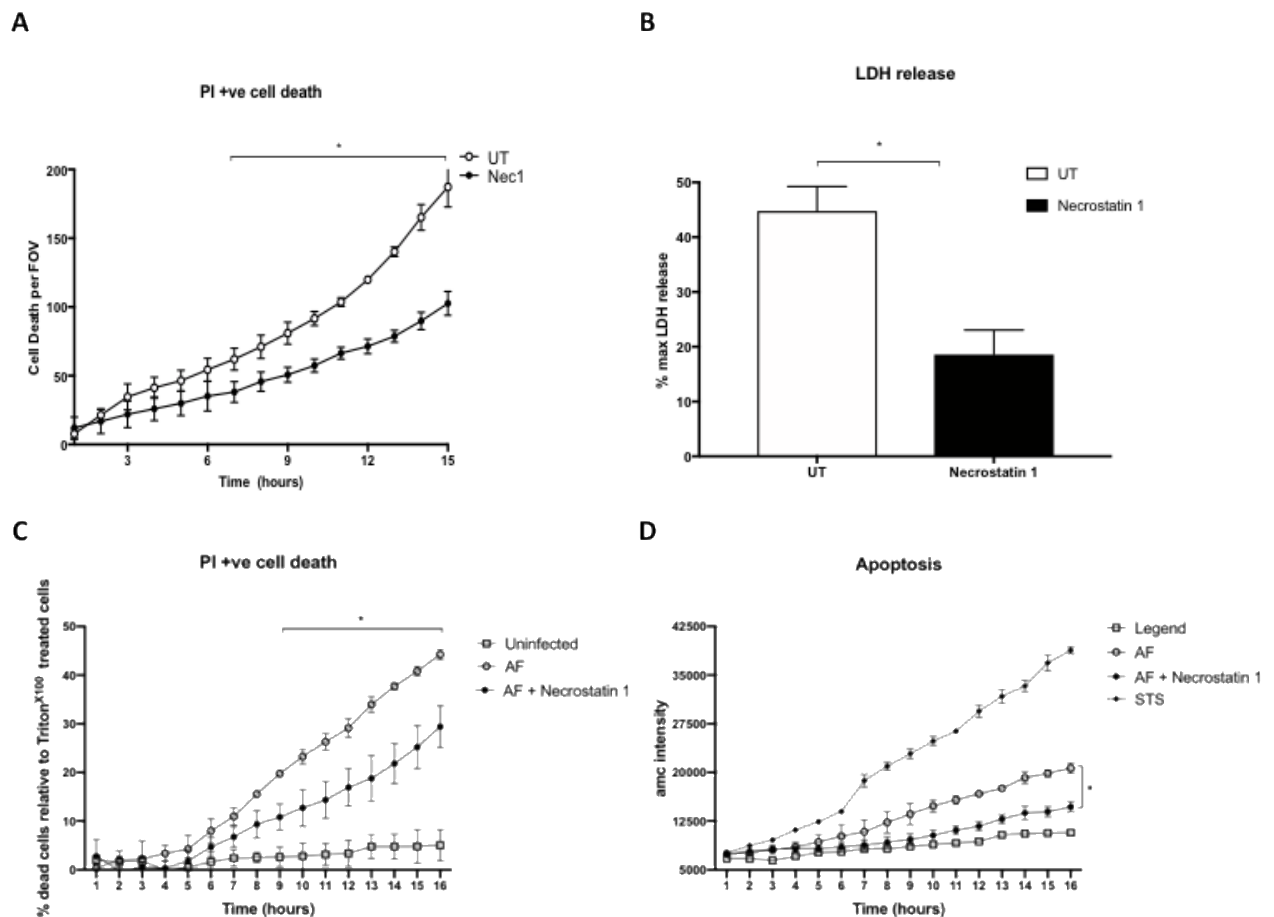


Figure 15. RIPK1 is required for both necroptotic and apoptotic cell death in response to *A. fumigatus* infection. (A-D) BMDM from C57BL/6 mice were pre-treated with 20 μ M Necrostatin-1 for 1 hour prior to infection with (A + B) eGFP *A. fumigatus* or (C+D) CEA *A. fumigatus* swollen conidia (MOI=2) or treatment with STS. (A) Following infection, propidium iodide was added to the media. Live time-lapse imaging was performed overnight to monitor PI +ve cell death events. Mean and SD from 3 independent experiments are represented. P values calculated by two-way ANOVA * $p < 0.05$. (B) LDH levels in the tissue culture supernatants were measured by colorimetric formazan product formation assay. P value calculated by paired Student's t-test: * $p < 0.05$. (C + D) Following infection, propidium iodide and Ac-DEVD-AMC

Caspase-3 Fluorogenic Substrate were added to the media. Live time-lapse imaging was performed overnight using a fluorescent plate reader (Tecan). (C) PI +ve cell death was expressed as % of Triton x100 treated cells. (D) Apoptosis was measured by monitoring amc intensity. Mean and SD from 3 independent experiments are represented. P value calculated by two-way ANOVA *p<0.05

4.4 *A. fumigatus*-induced macrophage lytic cell death is not dependent on Caspase 1 or 11 activity

Pyroptosis is a Caspase-dependent mode of programmed lytic cell death. Both *Candida albicans* and *Cryptococcus neoformans* have been identified as inducing pyroptotic cell death in myeloid cells. Live *C. albicans*-induced pyroptosis has been found to be exclusively Caspase-1-dependent ²³⁶, whereas Caspase-8 also contributes to IL-1 β release. Interestingly, Caspase 8 appears to contribute in a beta-glucan-dependent manner to cell death induced by heat-killed *C. albicans* ²³⁷. Live *C. neoformans*-induced pyroptosis and IL-1 β release, instead, are not entirely Caspase-1-dependent as Caspase-8 takes over this role in the absence of Caspase-1 via activation of the non-canonical inflammasome ²³⁸.

A. fumigatus-induced IL-1 β release has been shown to require both Caspase-1 and Caspase-8 ²²⁴. The authors did not assess cell death in their paper. To investigate if Caspase-1-dependent pyroptosis contributes significantly to *A. fumigatus*-induced macrophage cell death, BMDM from WT and Caspase 1/11-/- C57BL/6 mice were infected with CEA *A. fumigatus* conidia and PI +ve cell death was monitored. There was no change in macrophage cell death between Caspase 1/11-/- BMDM versus WT (Figure 16) suggesting that Caspase 1/11-dependent Pyroptosis is not an important mode of

cell death in macrophages infected with *A. fumigatus* (data from Thomas Colley, post-doc in Armstrong-James Lab).

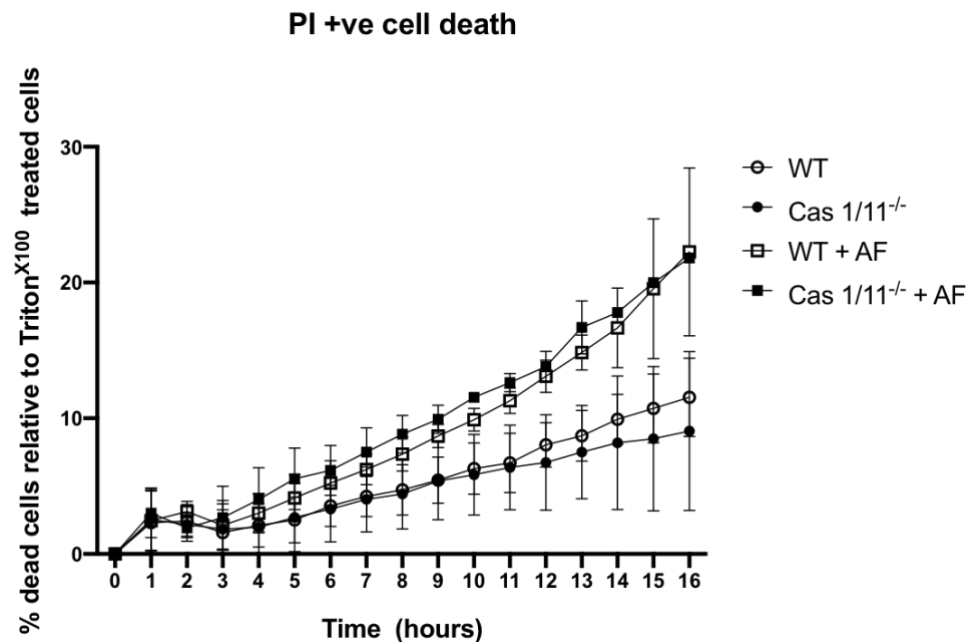


Figure 16. *A. fumigatus*-induced macrophage lytic cell death is not Caspase 1 or 11 dependent. BMDM from C57BL/6 WT and Cas 1/11^{-/-} mice were infected with CEA *A. fumigatus* swollen conidia (MOI=2). Following infection, propidium iodide was added to the media. Live time-lapse imaging was performed overnight using a fluorescent plate reader (Tecan). PI +ve cell death was expressed as % of Triton x100 treated cells.

4.5 *A. fumigatus*-induced necroptosis is TLR9-dependent

In vitro studies have shown that necroptosis can be triggered by ligation of the toll-like receptors 3 and 4 as well as the TNF receptor. For both TLR3 and TLR4, necroptosis occurs following interaction of these TLRs with RIPK3 via the adaptor protein TRIF rather than as a result of autocrine activation of the TNF receptor classical necroptosis

pathway ²³⁴. Other TLRs have also been shown to induce necroptosis in vitro, in the presence of caspase inhibition, when stimulated with synthetic ligands. However, in the case of TLR2, TLR5 and TLR9, cell death was TNF- α dependent ²³³. Correlation between studies performed between synthetic ligands and pathogens understood to bind the same receptors is not clear-cut. TLR3 recognises dsRNA viruses and although cells transfected with reovirus RNA underwent necroptosis in a TLR3-dependent manner, death of reovirus infected cells was TLR3 independent ²³⁹.

To ascertain if *Aspergillus*-induced necroptosis is TLR9-dependent, C57BL/6 BMDM were pre-treated with the competitive antagonist ODN 2088 for one hour prior to infection with either eGFP, for the live widefield microscopy, or CEA, for the plate-reader microscopy, *A. fumigatus* conidia. Both PI +ve cell death (Figure 17A) and LDH release were significantly reduced by blockage of TLR9 recognition of *A. fumigatus* (Figure 17B). This TLR9-dependency was confirmed by the observation that TLR9^{-/-} BMDM underwent significantly reduced macrophage PI +ve cell death in response to infection than WT BMDM (Figure 17C). Of note, *Aspergillus* induced apoptosis was also reduced in TLR9^{-/-} BMDM (Figure 17D).

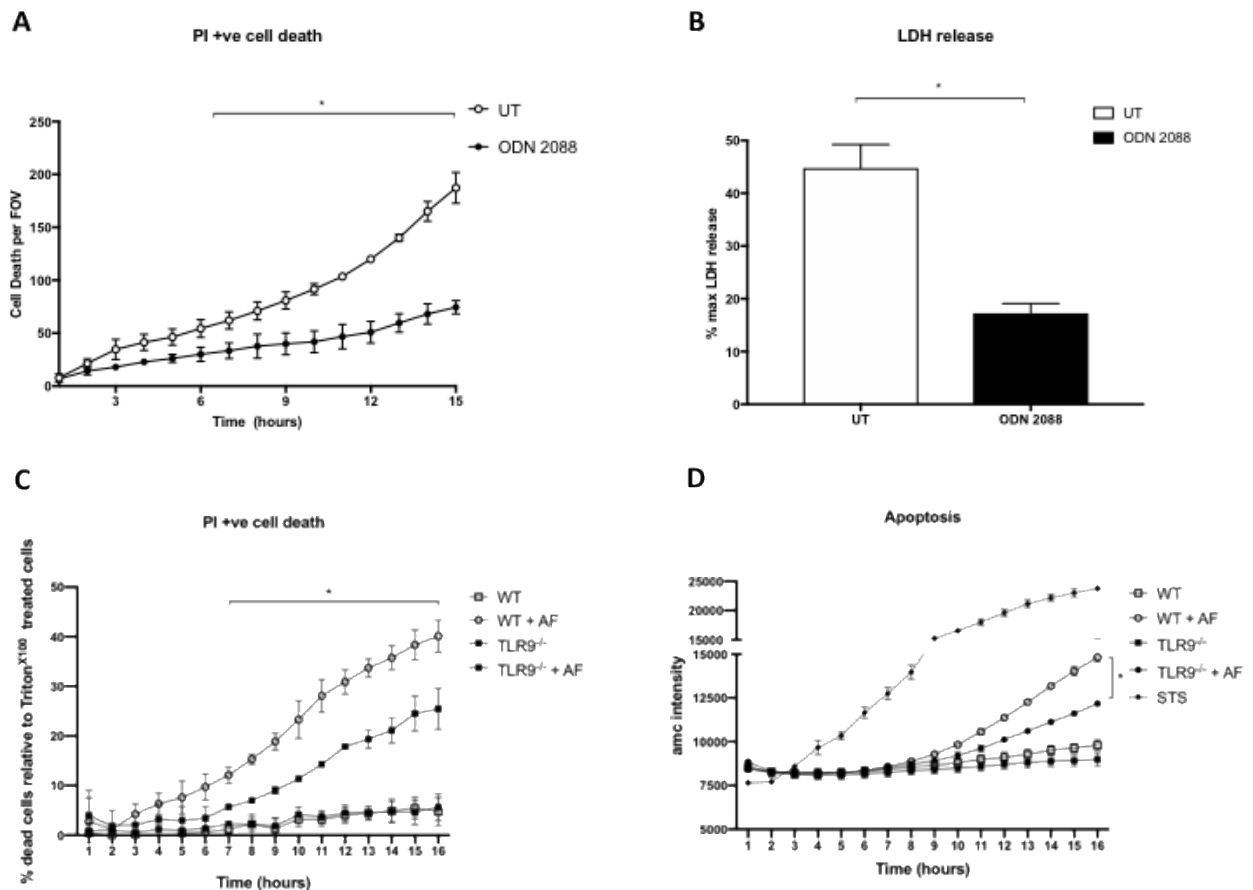


Figure 17. *A. fumigatus* induced necroptosis is TLR9-dependent.

(A -D) BMDM from WT and TLR9^{-/-} C57BL/6 mice were infected with eGFP or CEA *A. fumigatus* conidia (MOI=2). (A) WT BMDM were pre-incubated with 10 μ M ODN 2088 for 1 hr prior to infection. Propidium iodide was added to culture media and live time-lapse imaging was performed overnight to monitor PI +ve cell death events. Mean and SD from 3 independent experiments are represented. P values calculated by two-way ANOVA * $p < 0.05$. (B) LDH levels in the tissue culture supernatants were measured by colorimetric formazan product formation assay. P value calculated by paired Student's t-test: * $p < 0.05$. (C-D) Following infection, propidium iodide and Ac-DEVD-AMC Caspase-3 Fluorogenic Substrate were added to the media. (C) PI +ve cell death was monitored using a fluorescent plate reader (Tecan). Cell death expressed as % of Triton^x100 treated cells (B) Apoptosis was quantified by monitoring amc intensity using a fluorescent plate reader (Tecan). Mean and SD from 3 independent experiments are represented. P value calculated by two-way ANOVA * $p < 0.05$.

4.6 Ibrutinib inhibits *A. fumigatus* induced necroptosis

Our group has identified BTK as having a key role in propagating several key signalling pathways lying downstream of TLR9 recognition of *A. fumigatus*, including the calcineurin-NFAT and NF- κ B activation pathways^{94,240}. Our group has also observed that *Aspergillus*-induced macrophage cell death is calcineurin dependent in human monocyte-derived macrophages¹⁸⁵. Having established that macrophage necroptosis is TLR9-dependent my next step was to determine its dependency on BTK kinase activity.

C57BL/6 BMDM were pre-treated with Ibrutinib for one hour prior to infection with either eGFP, for the live widefield microscopy, or CEA, for the plate-reader microscopy, *A. fumigatus* conidia. Both PI +ve cell death (Figure 18A + C) and LDH release (Figure 18B) were reduced by inhibition of BTK kinase activity.

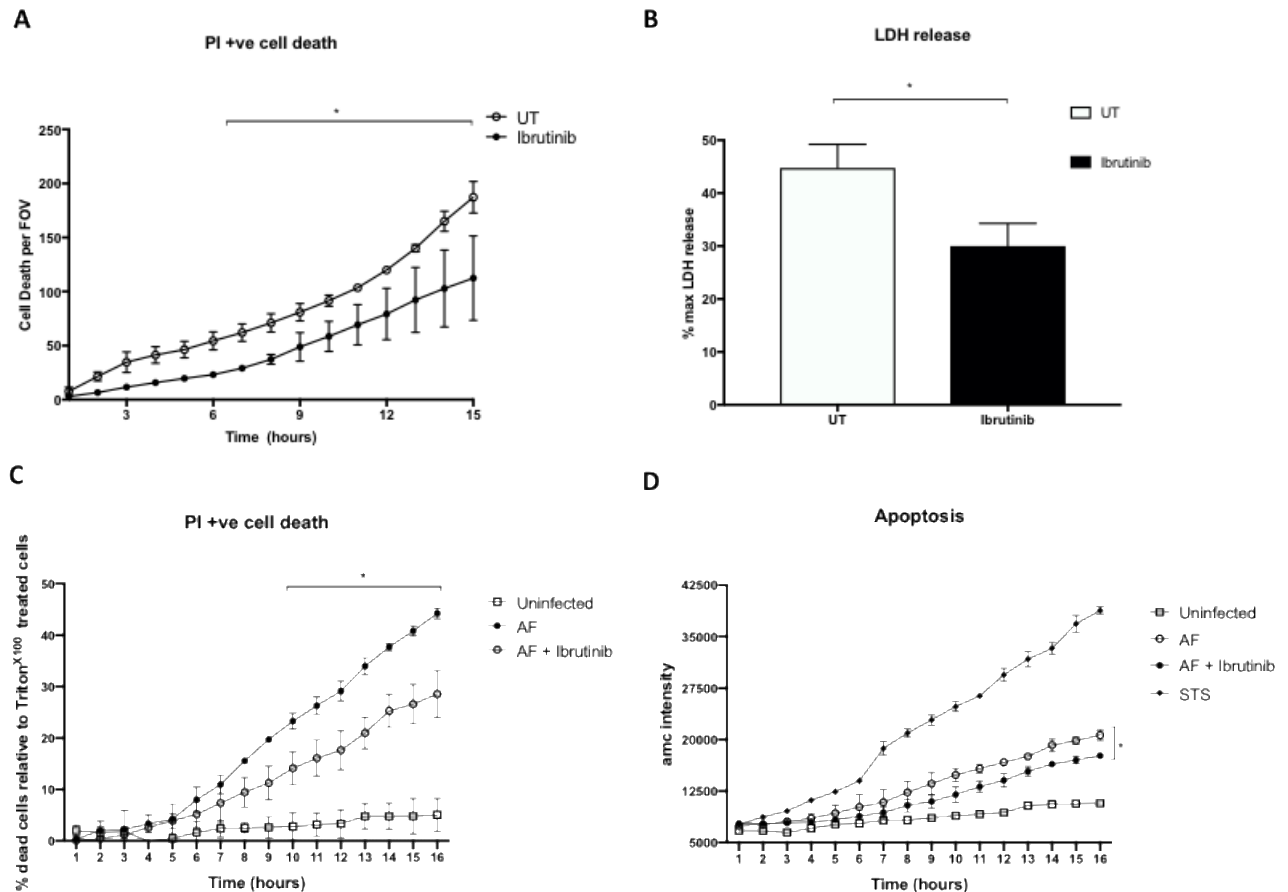


Figure 18. Ibrutinib inhibits *A. fumigatus* induced programmed cell death pathways. (A -D) BMDM from WT C57BL/6 mice were infected with eGFP or CEA *A. fumigatus* conidia (MOI=2). (A) WT BMDM were pre-incubated with 1 μ M ODN 2088 for 1 hr prior to infection. Propidium iodide was added to culture media and live time-lapse imaging was performed overnight to monitor PI +ve cell death events. Mean and SD from 3 independent experiments are represented. P values calculated by two-way ANOVA * $p < 0.05$. (B) LDH levels in the tissue culture supernatants were measured by colorimetric formazan product formation assay. P value calculated by paired Student's t-test: * $p < 0.05$. (C-D) Following infection, propidium iodide and Ac-DEVD-AMC Caspase-3 Fluorogenic Substrate were added to the media. (C) PI +ve cell death was monitored using a fluorescent plate reader (Tecan). Cell death expressed as % of Triton^x100 treated cells (D) Apoptosis was quantified by monitoring amc intensity using a fluorescent plate reader (Tecan). Mean and SD from 3 independent experiments are represented. P value calculated by two-way ANOVA * $p < 0.05$.

4.7 Discussion

Programmed cell death pathways, both lytic and non-lytic, play a key role in the development, homeostasis and host defence of multicellular organisms. Apoptosis is responsible for most of the cell death in development and homeostasis. Key to this role is the fact that it is an immunologically silent process, allowing cell populations to turn over without generating potentially destructive inflammation. As well as its role in development and homeostasis, the intrinsic pathway of apoptosis activation is thought to have originally evolved as a pathogen-sensing pathway ²⁴¹. As caspase-dependent death is believed to be the most ancient form of programmed cell death, it has been hypothesised that many pathogens evolved to encode caspase inhibitors that would allow them to evade the first form of programmed cell death they encountered ²⁴². It is in this context that necroptotic pathways, which become active once caspases are inhibited, may have evolved to allow infected host cells without functional caspase enzymes to die, thus removing a protective intracellular niche for pathogen replication ²⁴³.

Evidence for the role of necroptosis in host defence comes primarily from studies using viral and, to a lesser extent, bacterial pathogens. The Vaccinia virus has been shown to induce the formation of a pro-necrotic RIPK1/RIPK3 complex in-vitro and RIPK3^{-/-} mice succumb to Vaccinia viral infection in-vivo ²⁴⁴. The Influenza A virus is also able to trigger necroptosis in-vitro and in-vivo ²⁴⁵, though interestingly the virus has not yet been shown to encode a caspase 8 inhibitor, raising the possibility that there may be other mechanisms, apart from caspase inhibition, by which cells are sensitized to necroptosis. Further evidence for the role of necroptosis in anti-viral immunity comes

from the discovery that some viruses have evolved mechanisms to evade necroptotic death, just as they have with apoptosis. As well as 3 separate inhibitors of caspase activation, the murine Cytomegalovirus (MCMV) also encodes a viral inhibitor of RHIM activation (vIRA) that interferes with the RHIM-RHIM interaction of RIPK3 and DAI, the Z-DNA binding protein that mediates RIPK3 activation in response to herpesvirus infection ^{232,246}.

Unlike viruses, not all bacteria require a host cell for replication. Thus, the role of necroptotic cell death in anti-bacterial host defence is less clear cut. *Salmonella enterica* induces necroptosis in host cells as a means of cellular escape and dissemination ²⁴⁷ suggesting it might contribute to the bacterium's pathogenicity. In vivo, however, RIPK3^{-/-} mice do not show impaired survival or increased bacterial burden with *Salmonella* infection compared to wild-type. Injection of RIPK3^{-/-} macrophages into WT mice, interestingly, does increase bacterial burden ²⁴⁸. *Staphylococcus aureus* also activates RIPK3- and MLKL-dependent necroptosis. In response to in-vivo infection, MLKL^{-/-} mice demonstrate increased bacterial load, an inability to limit IL-1 β production and excessive inflammation compared to WT mice, whereas in RIPK3^{-/-} mice these findings were reversed ²⁴⁹. These results suggest not only that necroptotic cell death has a protective role in *S. aureus* infection but that RIPK3 has necroptosis-independent activity that contributes to the pathogenicity of bacterial infections.

There is even less data published on the role of necroptosis in anti-fungal host defence. *Cryptococcus* has been observed to induce necrotic changes in brain endothelial cells and cytoplasmic translocation of HMGB1, changes consistent with though not necessarily specific to necroptosis ²⁵⁰. In-vivo, MLKL^{-/-} mice did not show any reduction

in survival compared to WT mice following infection with *Cryptococcus neoformans* ²⁵¹.

No other results apart from effect on survival were presented, however. To my knowledge there is no published evidence on whether cells undergo necroptosis in response to *Candida* species.

I found that murine macrophages undergo RIPK3-dependent lytic cell death, that is necroptotic cell death, in response to *A. fumigatus* infection. These results support the work of others in my group who have shown that human monocyte-derived macrophages undergo morphological changes suggestive of necroptotic cell death in cells unable to control fungal germination ¹⁸⁵. It would be important to confirm these results in further work by comparing *Aspergillus*-dependent cell death in WT and RIPK3 and MLKL-knockout macrophages. Using MLKL-knockout cells as well as RIPK3-knockout cells would pick out any effects of RIPK3 on non-necroptotic cell death pathways. This is relevant given the pro-apoptotic effect of GSK'872. The overall significance of necroptosis in terms of host defence against *Aspergillus* infection is also not entirely clear. Lateral transfer of germinating *Aspergillus* conidia from cells undergoing programmed necrotic cell death to neighbouring healthy cells has been seen to occur in-vitro and this appears to assist in control of fungal germination ¹⁸⁵. However, in-vivo studies with RIPK3 and MLKL knockout mice are needed to better characterise the role of necroptosis in anti-fungal immunity.

As well as reducing lytic cell death, the RIPK3 inhibitor GSK'872 significantly increased apoptotic cell death. A pro-apoptotic effect associated with loss of normal RIPK3 activity has been noted before. Mice bearing the catalytically inactive D161N RIPK3 mutation are non-viable and die in-utero secondary to excess RIPK1- and Caspase-8-dependent

apoptosis of endothelial cells ²⁵². In addition, GSK'872 has been found to induce the RIPK3-dependent assembly of a RIPK1-FADD-cFLIP_L-Caspase 8 complex that drives apoptosis. Interestingly, this pro-apoptotic effect is thought to be due to conformational changes in RIPK3 that induce increased RHIM-mediated binding to RIPK1 and trigger formation of the above complex rather than due to loss of its kinase activity. This is supported by the finding that other kinase-inactivating RIPK3 mutations have been identified that inhibit necroptosis without inducing apoptosis ²²⁸.

As well as being RIPK3 dependent, I found that *Aspergillus* induced lytic cell death is RIPK1 dependent. This suggests that RIPK1 lies upstream of RIPK3. This RIPK1 dependency indicates that *Aspergillus* does not signal directly via its receptor to RIPK3 as in other non-classical necroptotic pathways. However, it also does not confirm that *Aspergillus* induces necroptosis via autocrine activation of the classical necroptosis pathway. TLR3/4-dependent necroptosis has been shown to be inhibited by Necrostatin-1, suggesting RIPK1 may mediate as yet undescribed TNF- α -independent necroptotic pathways. Further work could help answer this question by repeating these cell death assays in wild type and TNF- α knockout cells or in the presence of a TNF receptor antagonist.

In contrast to the RIPK1 and RIPK3 dependency identified, Caspase-1 and -11 were not required for *Aspergillus* induced lytic cell death. This suggests either that pyroptosis is not a significant contributor to overall *A. fumigatus* induced macrophage cell death or that pyroptosis does occur but that Caspase-1 and -11 are not essential for it. This result contrasts with data from other investigators who have found that *Aspergillus* activates the AIM2 and NLRP3 inflammasomes and provokes IL-1 β release in a Caspase 1- and 8-

dependent manner ²²⁴. Of note, the authors did not assess pyroptosis in their paper. It may be that pyroptosis is not an inevitable consequence of inflammasome activation, as is seen in *Salmonella typhimurium* infected neutrophils where NLRC4 driven IL-1 β release is not accompanied by pyroptosis ²⁵³. Alternatively, it may be that Caspase 8 can take over pyroptosis in the absence of Caspase 1 and 11 as occurs with *Cryptococcus*. Further work with Caspase 8 siRNA knockdown in Caspase 1/11^{-/-} cells would help clarify this question.

TLR9 and BTK were both found to have a regulatory effect on lytic cell death. Again, this could be due to the impact on TNF- α release by the cell or due to interference in a more direct pathway. Certain TLR receptors, specifically TLR3 and 4 have been found to drive necroptosis independently of TNF- α in response to binding by their specific ligands and in the presence of caspase inhibitors (Kaiser *et al.*, 2013). TLR9 induction of necroptosis by CpG was TNF- α dependent in this same paper, which would suggest a predominantly autocrine necroptotic pathway. However, synthetic ligand binding experiments do not always correlate with live infection studies. Whole *Aspergillus* conidia may activate additional pathways that simple ligands do not. Interestingly, BTK is also known to directly phosphorylate TLR3 leading to formation of a downstream RIPK1-TRIF-TBK1 complex in response to Poly (I:C) ¹²⁴. BTK may also mediate a direct interaction between TLR9 and RIPK1 in the context of *Aspergillus* infection. Immunoprecipitation studies in WT, TLR9 and BTK deficient cells would help identify if TLR9 interacts with any of the RHIM-containing proteins known to interact with RIPK1 and RIPK3.

In summary, I have identified that murine macrophages undergo RIPK3 and RPIK1 dependent necroptosis in response to *A. fumigatus* infection. This necroptotic cell death process is regulated by signalling along a TLR9-BTK pathway. What is not yet clear is whether necroptosis is activated along a direct pathway from the endosome or whether it is the result of autocrine activation of the classical TNF- α necroptosis pathway. Of course, it may be that both pathways are relevant and operate in parallel. Further work is needed to establish the TNF- α -dependency of *Aspergillus* induced necroptosis. Caspase 1/11-dependent pyroptosis is not a significant contributor to overall *Aspergillus*-induced cell death, though it may be that caspase 8 takes over in the absence of these caspases and pyroptosis does have a role in *Aspergillus* infection. Further in-vivo work with relevant knockout mice would help elucidate the role of different cell death mechanisms on host defence to *Aspergillus* infection.

Chapter 5

Loss of the CFTR ion channel is associated with a hyper-inflammatory macrophage response to *A. fumigatus*

5.1 Introduction

Although CF has traditionally been thought of as a disorder of epithelial cell function, there is an increasing body of evidence that immune cell activity is also affected ²⁵⁴. Whether this is because of an intrinsic defect in immune cell function or occurs secondary to the influence of the abnormal environment of the chronically infected CF airway, is still disputed ²⁵⁵. The CFTR protein has been localised to the surface membrane of human ¹⁶² and murine macrophages ¹⁷¹. Changes in macrophage cytokine production, microbicidal activity, autophagy, inflammasome activation, TLR4 expression and phagosomal acidification ^{159,162,169,171} have been identified, though the last of these findings has been disputed ¹⁶³. Almost all of these discoveries, however, have been made based on work examining the interaction between macrophages and bacterial or chemical stimulants. Apart from the work of Luigina Romani's group ^{81,169}, there is little data available on the response of CF immune cells to fungal stimuli, even though fungal species are significant pathogens within the CF microbiome.

Aspergillus fumigatus has been found to colonise the airways of a significant proportion of CF patients ²⁵⁶. The clinical consequences of colonisation vary; in some patients the fungus appears to have no pathogenic effect whereas in others allergic sensitization or ABPA develops and in still others a fungal bronchitis ²⁵⁷. Understanding how the

immune response to *Aspergillus* is altered in CF airways and how this relates to the type of CFTR mutation present, is key to understanding this clinical variability and may help us predict which patients are likely to be most affected by fungal colonisation.

A. fumigatus spores are normally cleared from the airway by the muco-ciliary escalator and the action of airway phagocytes, primarily macrophages. We know that muco-ciliary clearance is impaired in cystic fibrosis but whether macrophage handling of *Aspergillus* conidia is altered is less clear. I decided to start my research into this question by using bone marrow-derived macrophages (BMDM) from CFTR^{-/-} and WT litter-mate controls in an in-vitro model of macrophage-fungus interaction. Using macrophages from knockout mice instead of from CF patients for my initial mechanistic investigations had several advantages. Firstly, cells with a complete gene knockout would be more likely to display the effects of loss of function on phenotypic behaviour than cells carrying some residual gene function, as is present in some CF patients ²⁵⁸. Secondly, knock-out cells provide a uniform genotypic background on which to establish an experimental phenotype, whereas the degree of CFTR function present varies between CF individuals ²⁵⁹. Thirdly, findings based on experiments using BMDMs from un-infected mice, rather than primary cells from CF patients, can be more clearly attributed directly to the intrinsic effect of loss of CFTR rather than to the influence of a chronically infected pulmonary environment.

5.2 *A. fumigatus* induced NFAT activation is increased in CFTR ^{-/-} macrophages

Upon encountering macrophages within the airway, *Aspergillus* conidia are rapidly taken up into endosomes. As the endosome matures, several signalling pathways

responsible for coordinating the cell's response to the fungus are activated. The calcineurin-NFAT pathway has been shown to play a key role in regulating cytokine release, fungal killing, the kinetics of phagosomal maturation and programmed cell death pathways ^{94,185}. To investigate if activation of this signalling pathway was altered in CF, BMDMs from CFTR^{-/-} and WT littermate control mice were infected with eGFP *A. fumigatus* conidia (MOI=1) for 1 hour. Nuclear translocation of NFAT was then measured by widefield microscopy (Figure 19). For each sample the microscope was programmed to select 7-10 fields of view at random. The ImageJ macro written for the project then detected every DAPI positive cell within that field of view, on average 400-500 cells per field of view and thus up to 5000 cells per sample.

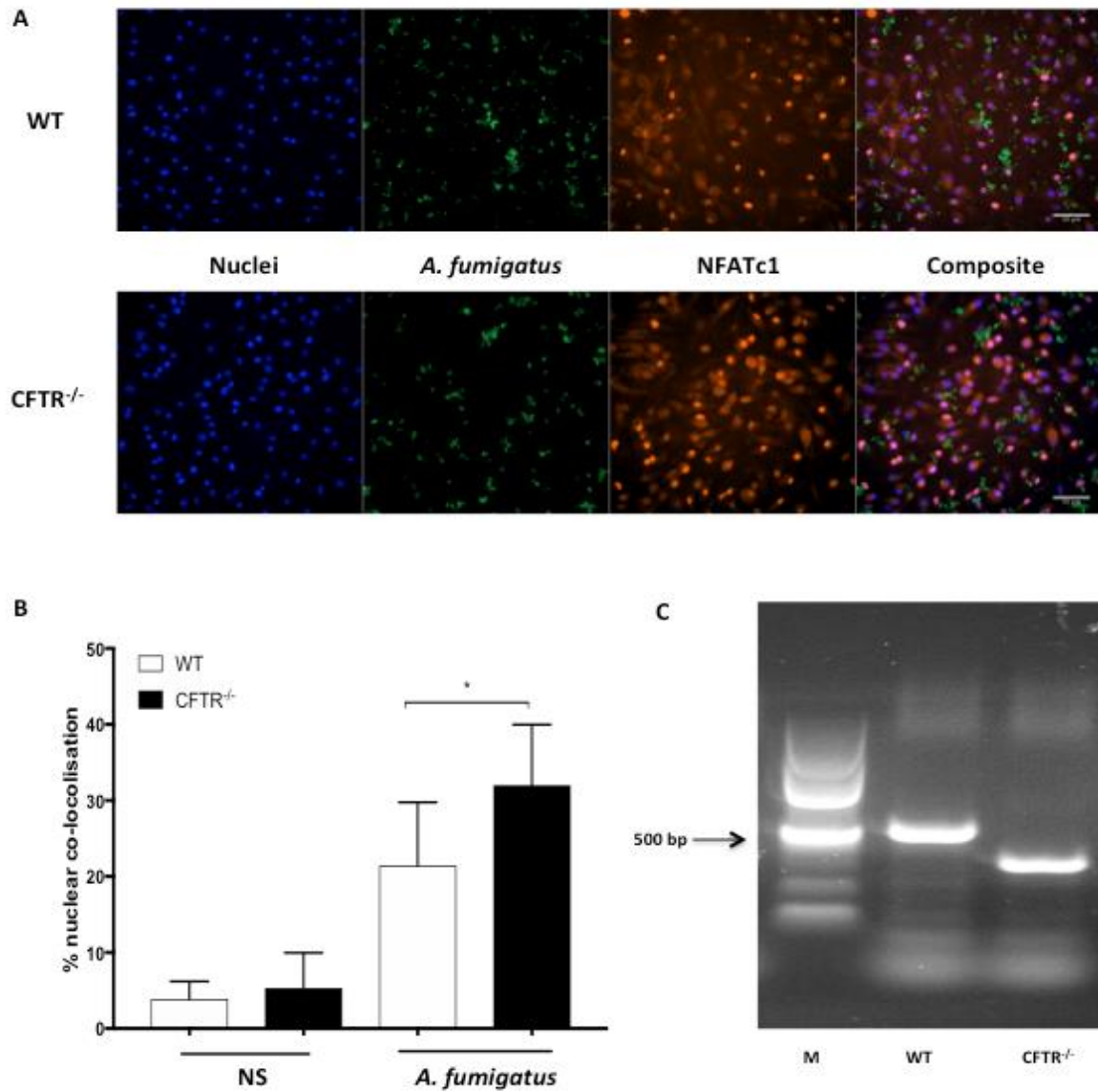


Figure 19. Nuclear translocation of NFAT in response to *A. fumigatus* infection is increased in CFTR^{-/-} macrophages. WT and CFTR^{-/-} bone-marrow derived macrophages (BMDM) were stimulated with eGFP *A. fumigatus* swollen conidia (MOI=1) for 1 hr and NFATc1 translocation was quantified by widefield microscopy. (A) Representative widefield microscopy of BMDM infected with *A. fumigatus* (green) for 60min and stained for NFATc1 (orange) and nuclei (blue). (B) Nuclear translocation was quantified by calculating the % overlap of the nuclear DAPI and transcription factor-linked fluorophore channels. Data was calculated from 7-10 fields of view taken at random. Mean and SD of 3 independent experiments are represented. P value

calculated by one-way ANOVA: * $p < 0.05$. NS, non-stimulated. (C) Gel electrophoresis confirming knockout of CFTR gene (PCR kindly performed by Cuixang Meng).

A. fumigatus induced NFAT activation was significantly increased in CFTR^{-/-} BMDM compared to WT cells (Figure 19). The calcineurin-NFAT pathway has not been previously studied in the context of CF, apart from one paper which observed increased NFAT activation in F508del CD4⁺ T cells stimulated with anti-CD3 and anti-CD28 antibodies ²⁶⁰. This is the first time that increased NFAT activation has been reported in CF macrophages in response to an infective stimulus.

5.3 *A. fumigatus*-induced NF- κ B activation is increased in CFTR^{-/-} macrophages

NF κ B is a key transcription factor that regulates the expression of cytokines and chemokines in fungal innate immunity ²⁶¹. Increased NF κ B activation has been reported previously in CF cells including CF macrophages, but always in response to bacterial stimuli ^{171,262,263}. To assess NF- κ B activity in response to *A. fumigatus* infection, BMDMs from CFTR^{-/-} and WT control mice were infected with eGFP *A. fumigatus* conidia (MOI=1) for 1 hour. Nuclear translocation of NF- κ B was then quantified by widefield microscopy, again with an average 400-500 cells per field of view and up to 5000 cells per sample. I found that *A. fumigatus*-induced NF κ B activation was significantly increased in CFTR^{-/-} versus wild-type macrophages (Figure 20).

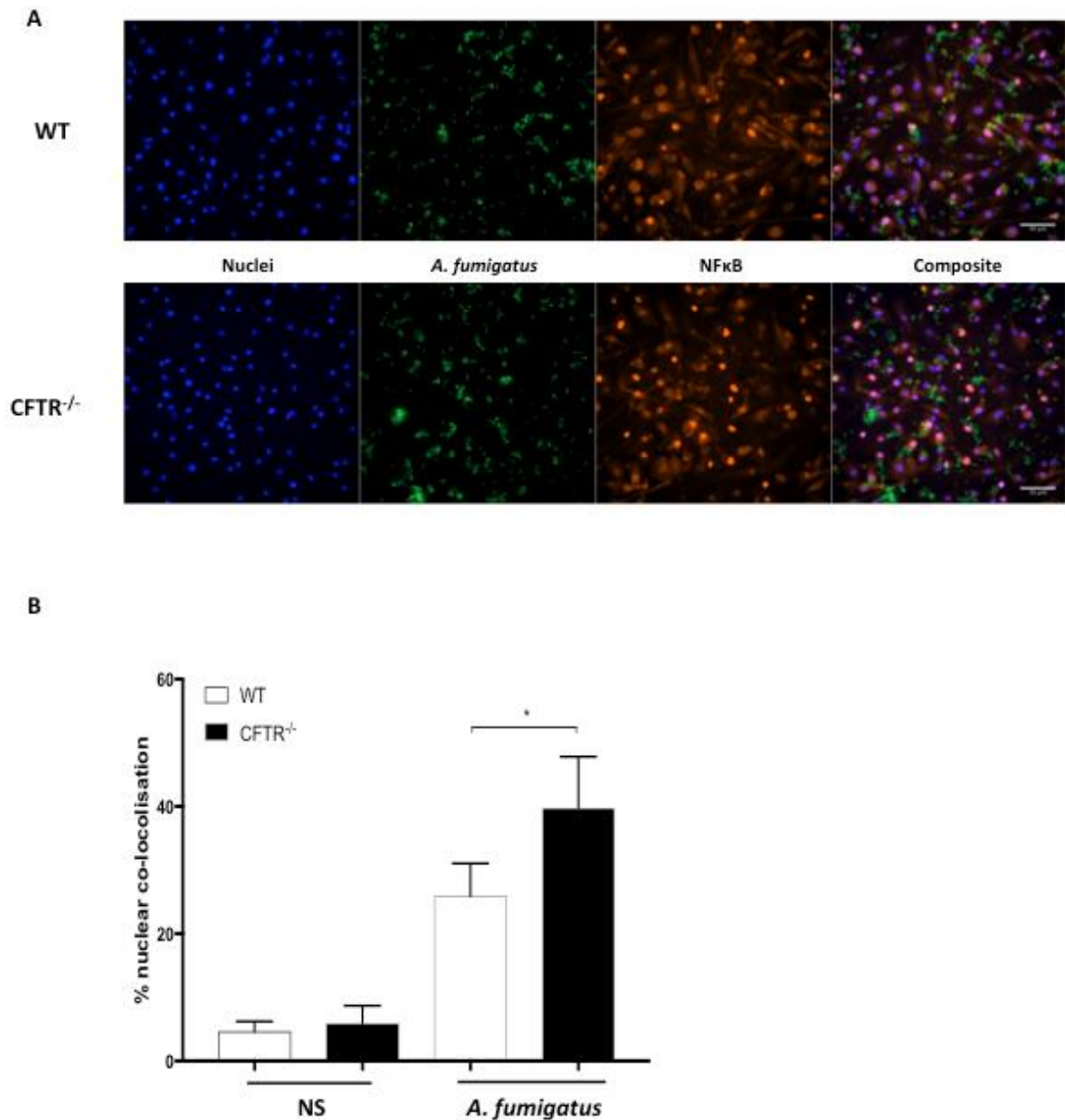


Figure 20. Nuclear translocation of NF-κB in response to *A. fumigatus* infection is increased in CFTR^{-/-} macrophages. WT and CFTR^{-/-} BMDM were stimulated with eGFP *A. fumigatus* swollen conidia (MOI=1) for 1 hr and NF-κB translocation was quantified by widefield microscopy. (A) Representative widefield microscopy of BMDM infected with *A. fumigatus* (green) at 60min p.i. stained for NFκB (orange) and nuclei (blue). (B) Nuclear translocation was quantified by calculating the % overlap of the nuclear DAPI and transcription factor-linked fluorophore channels. Data was calculated from 7-10 fields of view taken at random. Mean and SD of 3 independent experiments are represented. P value calculated by one-way ANOVA: *p<0.05. NS, non-stimulated.

Interestingly, I did not observe any increase in NFAT or NF- κ B activation in uninfected CFTR^{-/-} macrophages. There have been variable reports of baseline transcription factor activity in CF cells, with some papers demonstrating increased activity in resting cells 263 and others only showing a stimulant-induced signal increase 171,262.

5.4 *A. fumigatus* infection induces an exaggerated release of inflammatory cytokines in CFTR^{-/-} macrophages

Both NFAT and NF- κ B are involved in regulating cytokine and chemokine secretion by macrophages in response to *A. fumigatus*. As would be expected given the increased activation of these transcription factors in CFTR^{-/-} macrophages, *Aspergillus*-induced release of several inflammatory cytokines was increased in CFTR^{-/-} macrophages compared to wild-type (Figure 21).

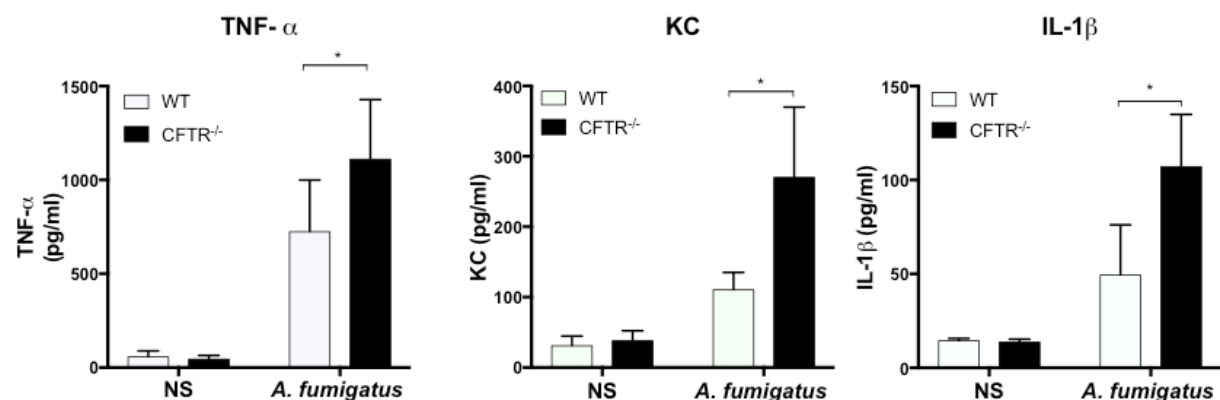


Figure 21. *A. fumigatus*-dependent cytokine release is augmented in CFTR^{-/-} macrophages. WT and CFTR^{-/-} BMDM were stimulated with *A. fumigatus* swollen conidia (MOI=1) for 16 hrs. Cytokine levels in tissue culture supernatants were measured by ELISA. P values were calculated by paired t-test. Mean and SD of 3 independent experiments are represented. *p<0.05. NS, non-stimulated.

TNF- α and KC are both pro-inflammatory cytokines that act as powerful chemo-attractants for neutrophils and monocytes. It is well established from human studies of sputum and bronchoalveolar lavage samples, that CF airways are characterised by chronic neutrophilic infiltration ^{264,265}. Airway macrophages are a key source of chemokines and these findings provide evidence to support the hypothesis that intrinsic hyper-activation of transcription factor activity in CFTR-deficient airway macrophages drives increased inflammatory cytokine release and promotes excess neutrophil recruitment.

I also found that CFTR-/- macrophages release more IL-1 β than WT cells when infected with *A. fumigatus*. Murine studies have found increased IL-1 β in whole lung homogenates of CFTR-/- mice infected with *A. fumigatus* and *P. aeruginosa* ¹⁶⁹. My in-vitro work suggests that some of this excess IL-1 β release derives from the exaggerated response of airway macrophages.

5.5 NFAT and NF- κ B activation are increased in human monocyte-derived macrophages in response to *A. fumigatus* infection

Having established a phenotype in murine macrophages, I went on to confirm these findings in human macrophages. Monocyte-derived macrophages from CF patients at the Royal Brompton and Harefield NHS Trust and healthy controls were infected with eGFP *A. fumigatus* conidia (MOI=1) for 1 hour. Nuclear translocation of NFAT and NF- κ B was then measured by widefield microscopy. Similar to my findings with murine

macrophages, I found that NFAT and NF- κ B activation were increased in macrophages derived from CF patients versus healthy controls (Figure 22).

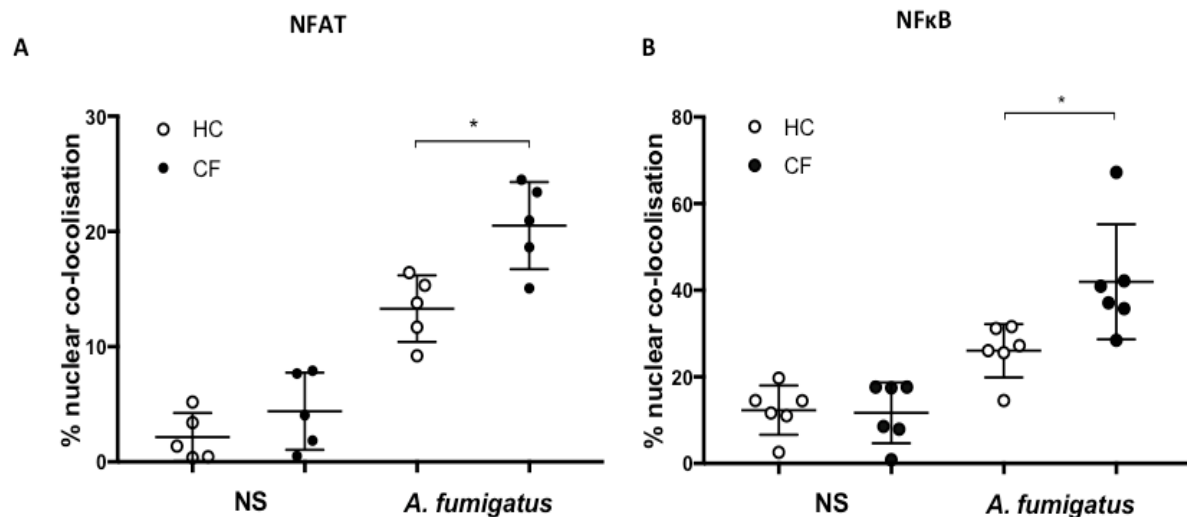


Figure 22. Nuclear translocation of NFAT and NF κ B is increased in monocyte-derived macrophages from CF patients versus healthy controls in response to *A. fumigatus* infection. Monocyte-derived macrophages from CF patients and healthy controls were stimulated with eGFP *A. fumigatus* swollen conidia (MOI=1) for 1 hr and transcription factor translocation was quantified by widefield microscopy. (A) NFAT (B) NF κ B. Nuclear translocation was quantified by calculating the % overlap of the nuclear DAPI and transcription factor-linked fluorophore channels. Data was calculated from 7 fields of view taken at random. Mean and SD of (A) 5 and (B) 6 independent experiments are represented. P value calculated by one-way ANOVA: * $p < 0.05$. NS, non-stimulated.

5.6 Impaired phagocytosis of *A. fumigatus* is not an intrinsic feature of CFTR^{-/-} macrophages

Aspergillus conidia are taken up into macrophages by phagocytosis. The data on phagocytosis in CF macrophages is variable. One group found that the phagocytic efficiency of murine macrophages to take up zymosan particles and *Pseudomonas* bacteria varied depending on the specific CFTR mutation ¹⁵⁸. In cells carrying the class II mutation, Phe508del, phagocytosis was impaired, whereas cells with a complete gene knock-out or the class III mutation, G551D, had preserved phagocytic function. Others have found impaired phagocytosis in macrophages but not neutrophils ²⁶⁶ and still others have attributed any phagocytic defect seen, to the damaging effect on cell-surface phagocytic receptors of excess levels of neutrophil elastase in CF airways ²⁶⁷. In order to examine if there was an intrinsic CFTR-related defect in phagocytosis, I incubated CFTR^{-/-} and wild-type BMDM with biotinylated eGFP *A. fumigatus* swollen conidia (MOI=1) for 2 hours and measured phagocytosis by flow cytometry. I did not find any significant difference in phagocytosis between the WT and CFTR^{-/-} BMDM (Figure 23).

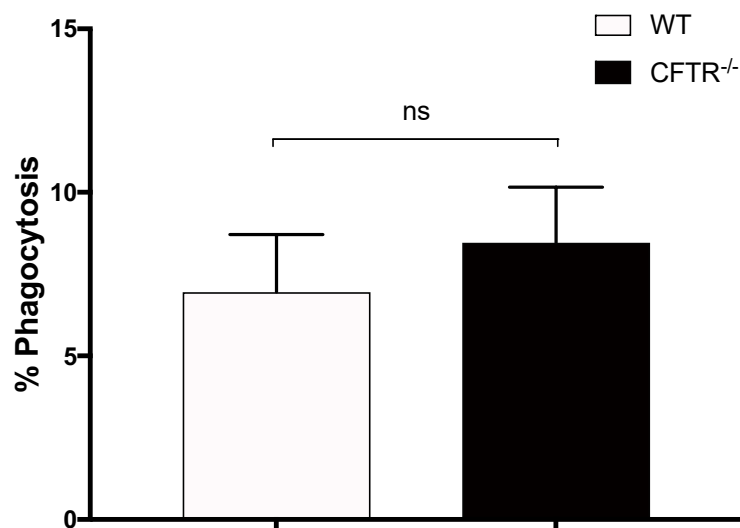


Figure 23. Macrophage phagocytosis of *A. fumigatus* is not CFTR dependent. WT and CFTR^{-/-} BMDM were infected with biotinylated GFP *A. fumigatus* swollen conidia (MOI=1) for 2 hrs. Cells were then counterstained with Cy3 biotin antibody and phagocytosis was measured by flow cytometry. Graph represents data from 3 independent experiments. Bars represent mean + SD and samples were analysed by paired Student's t-test, ns = non-significant.

5.7 Fungal killing is impaired in CFTR^{-/-} macrophages

Although uptake of fungal conidia was not impaired in CFTR^{-/-} macrophages, I found that fungal killing was. Cells were incubated with *Aspergillus* conidia for 6 hours and surviving spores were assessed by CFU counts. The number of viable conidia extracted from CFTR^{-/-} macrophages was increased compared to WT macrophages, suggesting a killing defect (Figure 24). Given one of the primary functions of airway macrophages in the innate immune response to fungal infection is to kill conidia and thus prevent conidial germination and cellular escape, this conidicidal defect may help explain fungal persistence within the CF airway.

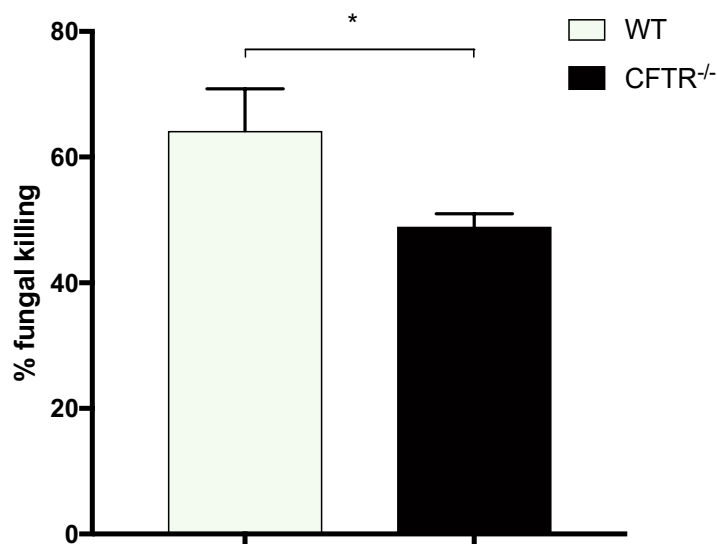


Figure 24. Fungal killing is impaired in CFTR-deficient macrophages. WT and CFTR^{-/-} BMDM were infected with eGFP *A. fumigatus* swollen conidia (MOI=1) for 6 hrs. Cells were washed to remove extra-cellular conidia and then lysed in 0.1% Tween 20/dH₂O and serial dilutions plated onto Sabouraud agar plates. Plates were incubated at 37°C overnight and CFUs were counted the next day. Data was analysed by paired Student's t-test. *p<0.05.

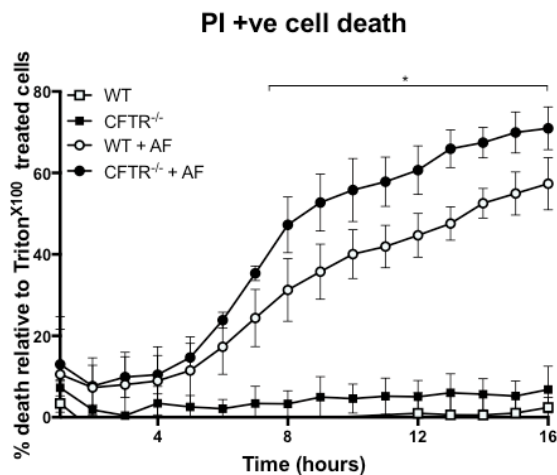
5.8 Inflammatory cell death is increased in CFTR^{-/-} macrophages

If a cell should fail to kill an invading pathogen, it can choose to undergo 'programmed cell death' in an attempt to prevent the pathogen escaping and damaging surrounding cells ²⁶⁸. Programmed cell death pathways can be broadly divided into those that are inflammatory and result in the release of toxic intra-cellular contents such as Necroptosis and Pyroptosis and those that are associated with cell shrinkage and containment of cell contents such as Apoptosis and Autophagic cell death ²⁶⁹.

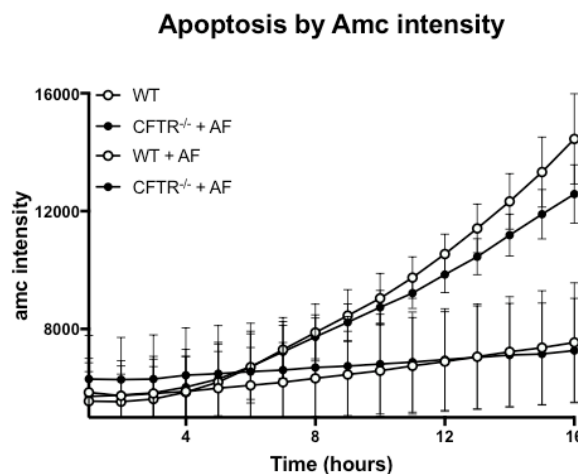
To investigate if cell death outcomes were altered by loss of CFTR, BMDM from CFTR^{-/-} and wild-type mice were infected with *A. fumigatus* conidia (MOI=1). Propidium iodide,

a nucleic acid stain, and Ac-DEVD-AMC, a fluorogenic substrate of Caspase-3 used to monitor apoptosis, were added to the culture media and PI +ve cell death and apoptosis were monitored by fluorescent microscopy over 16 hours. I found that overall PI +ve cell death was increased in CFTR^{-/-} macrophages compared to wild-type. (Figure 25A). This increase in cell death could not be accounted for by an increase in apoptosis (Figure 25B) however, instead represented an increase in inflammatory cell death (Figure 25C).

A



B



C

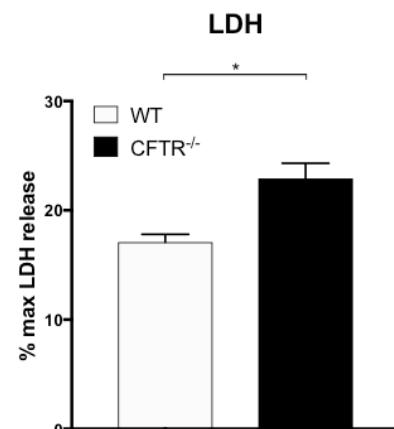


Figure 25. Macrophage inflammatory cell death is increased in cystic fibrosis.

(A + B) BMDM from WT and CFTR^{-/-} mice were infected with eGFP *A. fumigatus* swollen conidia (MOI=1) and following infection, propidium iodide and Ac-DEVD-AMC Caspase-3 Substrate were added to the media and plates were incubated overnight in a fluorescence plate reader (Tecan) (A) PI +ve cell death was expressed as % of Triton x100 treated cells (B) Apoptosis was quantified by monitoring Amc intensity. Mean and SD from 3 independent experiments are represented. P value calculated by two-way ANOVA. *p<0.05. (C) LDH levels in tissue culture supernatants were measured by colorimetric formazan product formation assay. P value calculated by paired Student's t-test: * p < 0.05.

5.9 Increased macrophage cell death is associated with impaired control of fungal growth

To assess the link between increased macrophage cell death and control of fungal growth, WT and CFTR^{-/-} BMDM were infected with eGFP *A. fumigatus* conidia (MOI=1) and PI +ve cell death and fungal growth were monitored over 16 hours by live time-lapse widefield microscopy. Increased cell death of CFTR^{-/-} macrophages. (Figure 26A) was accompanied by impaired control of fungal germination and increased fungal outgrowth (Figure 26B).

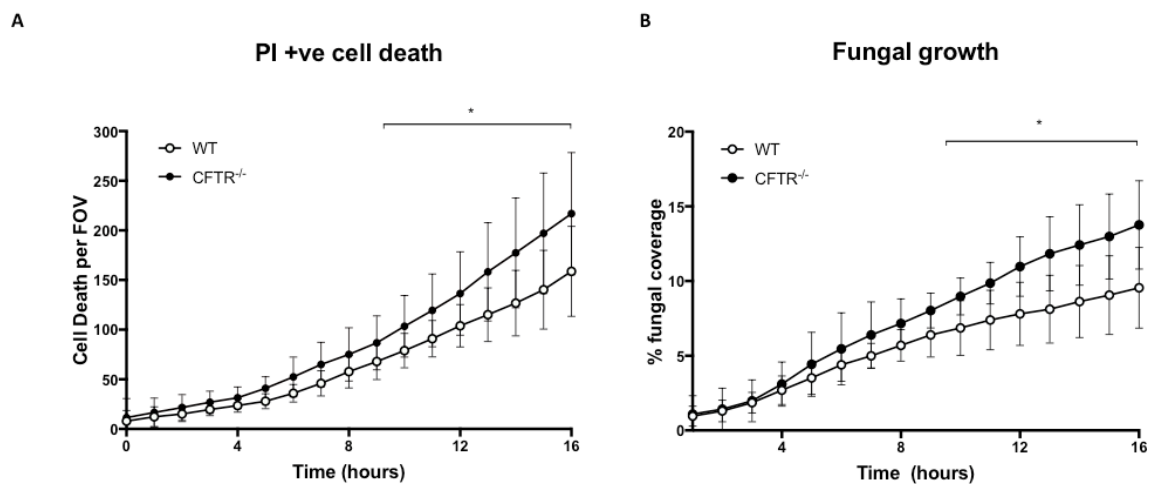


Figure 26. CFTR^{-/-} macrophages are less able to control outgrowth of *A. fumigatus*. (A + B) BMDM from WT and CFTR^{-/-} mice were infected with eGFP *A. fumigatus* swollen conidia (MOI=1) and following infection, propidium iodide was added to the media. (A) Live time-lapse imaging was performed overnight to monitor PI +ve cell death events. (B) The % coverage of the field of view by GFP expressing *A. fumigatus* was measured. Mean and SD from 3 independent experiments are represented. P values were calculated by two-way ANOVA. *p<0.05.

5.10 Discussion

The vast majority of patients with CF will die from respiratory failure caused by progressive lung disease. The pathology driving the damage to CF lungs is chronic inflammation generated by a failure to clear microorganisms from the airways paired with a dysregulated inflammatory response ²⁷⁰.

Effective removal of *Aspergillus* spores from the lungs depends on the presence of a correctly functioning innate immune response. Inhaled conidia are trapped within a mucous layer and propelled towards the oropharyngeal junction by ciliary beating whereupon they are either swallowed or expectorated ²⁷¹. If not removed from the airways by the muco-ciliary escalator, conidia encounter airway phagocytes, in particular alveolar macrophages. These cells play a key role in the early stages of fungal infection when the fungus first enters the lung in its conidial form. Spores are taken up into macrophages and killed. If the internalised conidia have already started to swell, an intermediate stage preceding germination, then signalling pathways are activated within the macrophage that will result in the release of chemotactic cytokines.

Neutrophils, needed to deal with the hyphenating form of the fungus, are recruited to the airways alongside circulating monocytes. Thus, the host immune response is a graduated one aimed at dealing effectively with the pathogen without causing excess collateral damage through an uncontrolled inflammatory response. In CF some of these controls are lost and the inflammatory response becomes excessive, while at the same time proving inefficient in killing the fungus.

I found that *A. fumigatus* triggered the activation of the key transcription factors NFAT and NF- κ B in a significantly greater proportion of infected CF macrophages than wild-type macrophages. These findings were replicated in human as well as murine macrophages. Although increased NF- κ B activity has been previously reported in CF epithelial cells ^{262,272}, and, in one paper, in CF macrophages ¹⁷¹, this has always been in response to bacterial stimuli. This is the first description of a fungal stimulant generating increased NF κ B activity in CF macrophages. This is also the first report of increased NFAT activation in CF macrophages in response to any infective stimulus. The calcineurin-NFAT pathway is not one that has been extensively studied in CF, with only one other paper examining its activity in T cells ²⁷³.

What the mechanistic link is between loss of the CFTR ion channel and increased transcription factor activation, has not yet been fully clarified. NFAT activation occurs in response to sustained calcium influx into the cytoplasm ²⁷⁴. Calcium signalling has been found to be dysregulated in CF epithelial cells with several mechanisms being identified as an explanation. Increased insertion of Orai1 calcium channels and consequent increased Store Operated Ca²⁺ Entry (SOCE) was observed in a F508 Δ epithelial cell line and in primary human CF epithelial cells ¹⁷². CFTR channels have been found to associate with TRPC6 (Transient Receptor Potential Cation Channel Subfamily C Member 6) calcium channels in lipid-raft based multi-protein complexes ²⁷⁵. Within these complexes, CFTR down-regulates OAG-dependent calcium influx and this in turn up-regulates CFTR-dependent Cl⁻ transport. Potentiation of normal CFTR ion channel activity by Ivacaftor reduced OAG-induced calcium influx into cells ²⁷⁶.

The data available on calcium flows in CF macrophages is more limited. In one study there was no difference in calcium influx in response to Ionomycin or PAF (platelet activating factor) between CF and non-CF macrophages but there was a significantly smaller signal in response to PMA in CF macrophages ¹⁶⁴. Further studies are needed to measure calcium ion movements in CFTR^{-/-} and wild-type macrophages in response to phagocytosis of *Aspergillus* conidia. If there is a larger or more sustained calcium influx, then this would account for the increased NFAT signal detected.

Regulation of NF- κ B activation is complex and influenced by several different signalling pathways. CFTR has been described as a negative regulator of NF κ B-mediated inflammatory signalling ²⁷⁷ with cell membrane localisation of a functional CFTR ion channel necessary to prevent hyper-activation of NF- κ B activation pathways ²⁶³. One of the ways wild-type CFTR influences NF- κ B is via promoting the degradation of TRADD, which forms part of a multi-protein complex that enables the phosphorylation of the inhibitory protein I κ B α ²⁷⁸.

The combination of increased NFAT and NF- κ B activation resulted in increased release of pro-inflammatory cytokines including TNF- α , KC, the murine equivalent of human IL-8, and IL-1 β . Both TNF- α and IL-8 are potent neutrophil chemokines and CF is a neutrophilic airways disease. IL-1 β is released following inflammasome activation and increased NLRP3 inflammasome activity has been noted in murine and human CF cells ¹⁶⁹. These in-vitro findings using infection-naïve macrophages provide evidence to support the hypothesis that intrinsic CFTR-related abnormalities in macrophage signalling pathways contribute to the generation of inflammation in CF airways.

As well as secreting cytokines, macrophages are responsible for directly killing conidia and preventing their germination and escape from the cell. I found that although uptake of conidia into the cell was not impaired in CF macrophages, fungal killing was. Killing defects have been identified previously in CF macrophages in response to bacterial pathogens ^{162,164}. This is, to my knowledge, the first demonstration of a defect in fungal killing capacity in CF macrophages.

The failure to kill *Aspergillus* at the conidial stage leads inexorably to fungal germination within the cell. One of the ways the cell can respond to this is by activation of programmed cell death pathways, in particular necroptosis. Fungal germination is thought to be a checkpoint in the activation of macrophage necroptosis. I found that *Aspergillus* infection induced more cell death in CF macrophages than wild-type and that the increase seen was in an inflammatory lytic mode of cell death. Necroptosis is regulated by the calcineurin-NFAT pathway with inhibition of this pathway by the calcineurin inhibitor FK506 blocking necroptotic cell death and cell-to-cell lateral transfer of fungus ¹⁸⁵. My results suggest that in CF macrophages, impaired killing of *Aspergillus* conidia allows fungal germination to occur within the cell. This triggers the cell to undergo necroptotic cell death, the threshold for activation of which may already have been lowered in CF cells due to increased activation of the calcineurin-NFAT pathway observed in these cells. Increased necroptotic death of macrophages is deleterious to the host not only because of the associated loss of control of fungal outgrowth but also as lytic cell death releases toxic intra-cellular contents into the cell's environment, which in the case of alveolar macrophages is the airway.

The results of my in-vitro studies show that loss of normal CFTR function increases activation of key intracellular pro-inflammatory pathways and consequently release of inflammatory cytokines. Loss of CFTR also interferes with effective fungal killing allowing conidia to germinate within the cell and drive inflammatory modes of programmed cell death.

Chapter 6

A. fumigatus induces a hyper-inflammatory airway response in CFTR^{-/-} mice

6.1 Introduction

My in-vitro experiments demonstrated that CFTR-deficient murine macrophages are impaired in their ability to kill *Aspergillus* conidia allowing increased intra-cellular fungal germination and macrophage cell death. Their response to fungal infection is hyper-inflammatory, with necrotic modes of cell death predominating and increased release of pro-inflammatory cytokines. The next step in my research was to see if this inflammatory phenotype would be replicated in an in-vivo CF animal model of *Aspergillus* infection.

The murine model is the most commonly used in-vivo model for *Aspergillus* infection. It is widely available, numerous gene-deficient and transgenic strains have been developed and the intra-nasal or intra-tracheal instillation of conidia mimics the natural route of infection in humans. Immunocompetent mice, like humans, do not develop invasive disease but can be manipulated by chemical or genetic means to mimic the various forms of immunocompromise in which invasive aspergillosis occurs, such as neutropenia, corticosteroid therapy or chronic granulomatous disease ²⁷⁹⁻²⁸¹. Non-invasive forms of aspergillus lung disease including allergic sensitization and ABPA have also been studied using immunocompetent mouse models ²⁸²⁻²⁸⁴.

The mouse has also been extensively used as an in-vivo model for CF ²⁸⁵. Early gene-knockout animals were limited in their usefulness by their reduced life expectancy with most animals dying of intestinal obstruction by day 40 post-partum. Animal survival was prolonged by the development of a “gut-corrected” model. In this model, human CFTR cDNA is expressed under the control of the rat fatty acid binding protein (FABP) promoter, thereby localising CFTR expression to the intestinal epithelium only and increasing the longevity of CF mice ²⁸⁶. Mice expressing specific CFTR mutations including the F508Δ ²⁸⁷ and G551D ²⁸⁸ mutations have also been created to investigate the impact of different modes of CFTR ion channel dysfunction.

The usefulness of the CF mouse as a model for human CF lung disease has been the subject of debate. CF mice do not develop the severe pathology characteristic of established human CF lung disease consisting of chronic respiratory infection, inflammation, mucus plugging, and progressive bronchiectasis ²⁸⁹

Aspergillus infection has been studied in CF mouse models before but with different foci of interest. In their 2012 paper, Chaudhary et al investigated epithelial cell responses to *Aspergillus* ²⁹⁰. Luigina Romani’s group has examined immune responses but at late time points, specifically 3, 7 and 14 days post-infection with a focus on the inflammasome and adaptive immune response ^{81,169,291}. My interest is in the early innate immune response in which macrophages play a key role; an area which has not yet been investigated in an in-vivo experimental CF model.

6.2 *A. fumigatus* infection induces non-fatal pathology in CFTR^{-/-} mice

CFTR^{-/-} and age-matched wild-type control mice (12-16 weeks old) were infected intra-nasally with 1×10^7 eGFP *A. fumigatus* resting conidia and monitored over a three-week period. There were no deaths in either group (Figure 27A). The CFTR^{-/-} mice did, however, lose significantly more weight than their wild type counterparts and were slower to return to their baseline weight, which suggests some pathological abnormality in the host response to infection (Figure 27B).

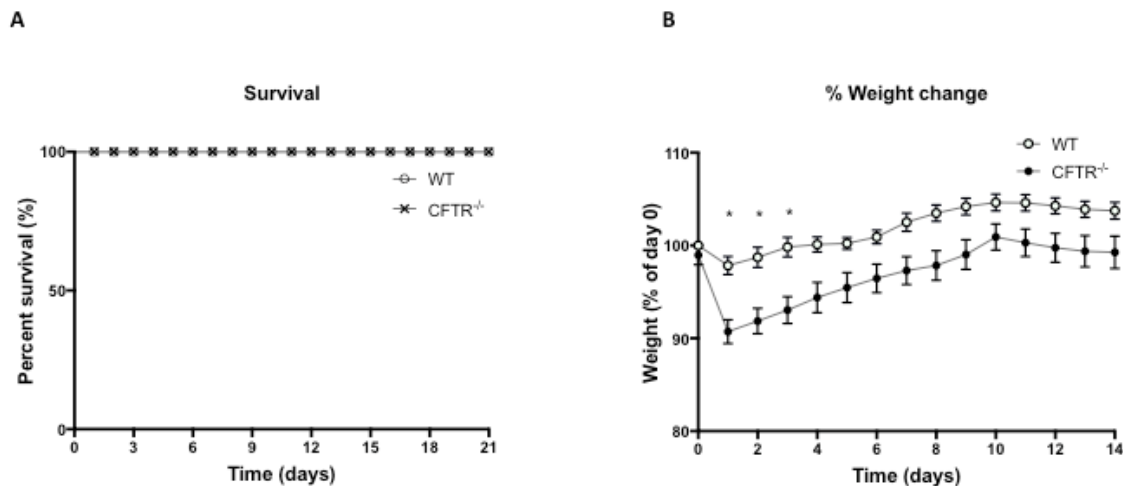


Figure 27. *A. fumigatus* infection is not fatal but does induce increased weight loss in CFTR^{-/-} mice. (A + B) Wild type and CFTR^{-/-} mice were infected intra-nasally with 1×10^7 eGFP *A. fumigatus* resting conidia and (A) survival and (B) weight were monitored daily for 3 weeks post. P-values calculated by two-way ANOVA, $p < 0.05$. $n=8$ in each group.

6.3 Uninfected CFTR^{-/-} murine airways are characterised by increased cellular infiltration

One of the unresolved questions in CF pathogenesis research is whether the inflammation seen in CF airways develops only following exposure to infection or if it starts before pathogen exposure, as a result of intrinsic immune dysfunction ²⁷⁰. To address this question, I performed bronchoalveolar lavages on wild type and CFTR^{-/-} mice that had undergone intra-nasal instillation of sterile PBS saline. Alveolar macrophages were defined as CD11c⁺/Siglec F⁺ positive cells whereas neutrophils were defined as CD11c⁻ and CD11b⁺/Ly6G⁺ (Figure 28A). I found that alveolar macrophage and neutrophil cell numbers in the airways were significantly higher in CFTR^{-/-} mice compared to wild type (Figure 28C).

The most marked difference was in alveolar macrophage numbers. Studies in murine and human subjects have found increased airway cellular, and in particular macrophage, infiltration in the absence of infection in CF ^{289,292}. Whether this is due to increased recruitment and differentiation of alveolar macrophages in foetal and early post-natal life or occurs secondary to reduced apoptosis and efferocytosis of aging cells is not clear. Also, more neutrophils were observed at baseline in the CFTR^{-/-} versus wild type mice (Figure 28C), although the cell number was very small in both cases representing less than 5% of CD45⁺ cells.

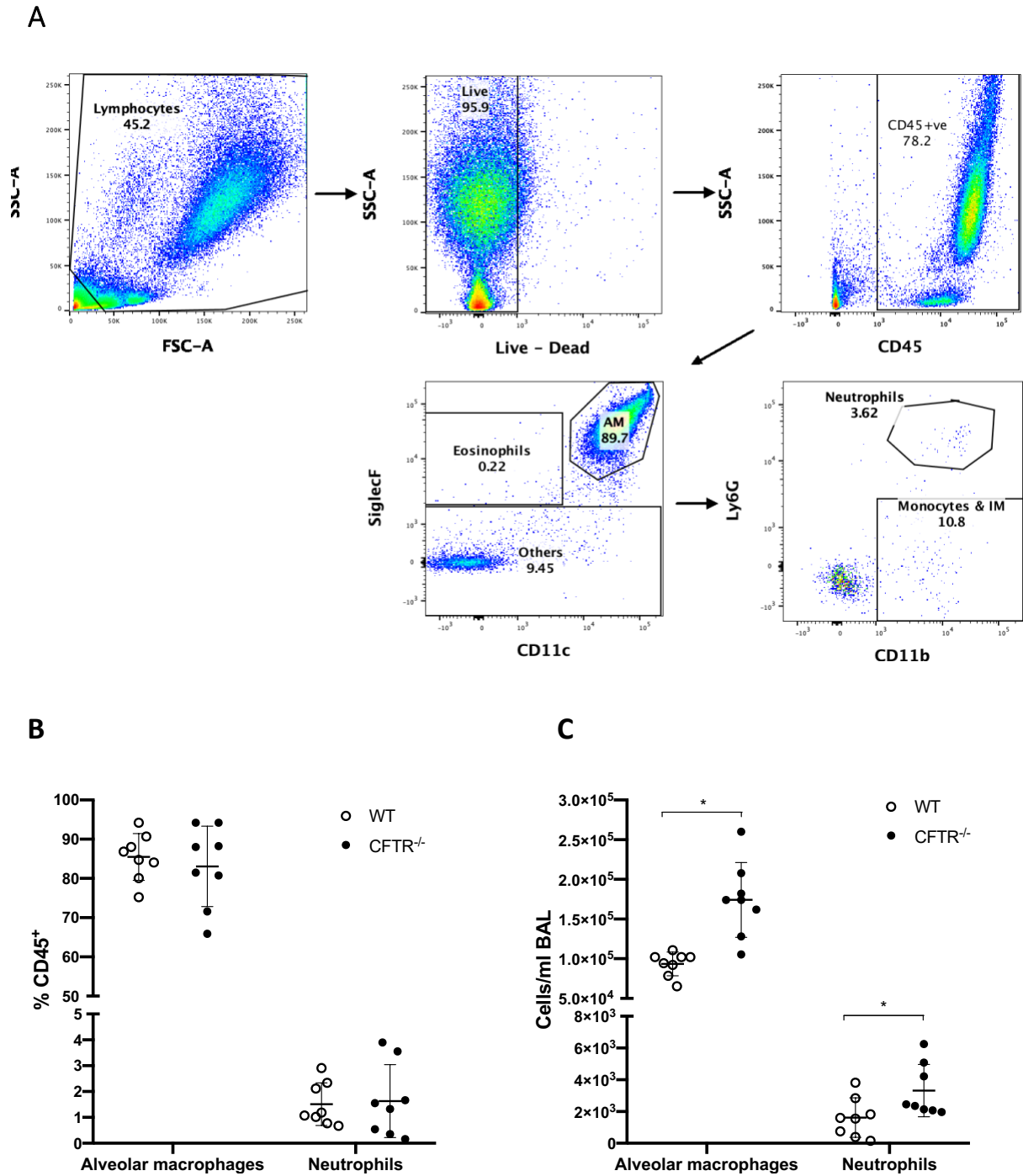


Figure 28. Un-infected CF airways are characterised by cellular infiltration.

WT and CFTR^{-/-} mice received sterile PBS via intra-nasal inhalation. Mice were culled at 18 hours and BAL performed to study cell influx by flow cytometry. (A) Gating strategy (B) Cell populations as % CD45+ve cells (C) Total cell numbers/ml BAL fluid. Each dot represents 1 animal and lines mean + SD, n=8 per group. Statistical analysis performed by Student's t-test, $p < 0.05$.

Alveolar macrophages play a crucial role in maintaining lung homeostasis. The presence of an increased number of these cells in CF airways does not necessarily imply that, in the absence of infection, they are the source of pathogenic inflammation. To assess the degree of airway inflammation present, cytokine levels in bronchoalveolar lavage supernatants were measured by MSD, a multiplex immuno-assay. I found no significant differences in pro-inflammatory cytokine levels between WT and CFTR^{-/-} mice (Figure 29), despite the presence of excess macrophages. This suggests that in the absence of an infectious stimulus there is no significant spontaneous activation of pro-inflammatory pathways in these cells. However, their presence in such numbers in the lung does suggest that the CF innate immune system is set-up to generate an augmented host response to incoming pathogens.

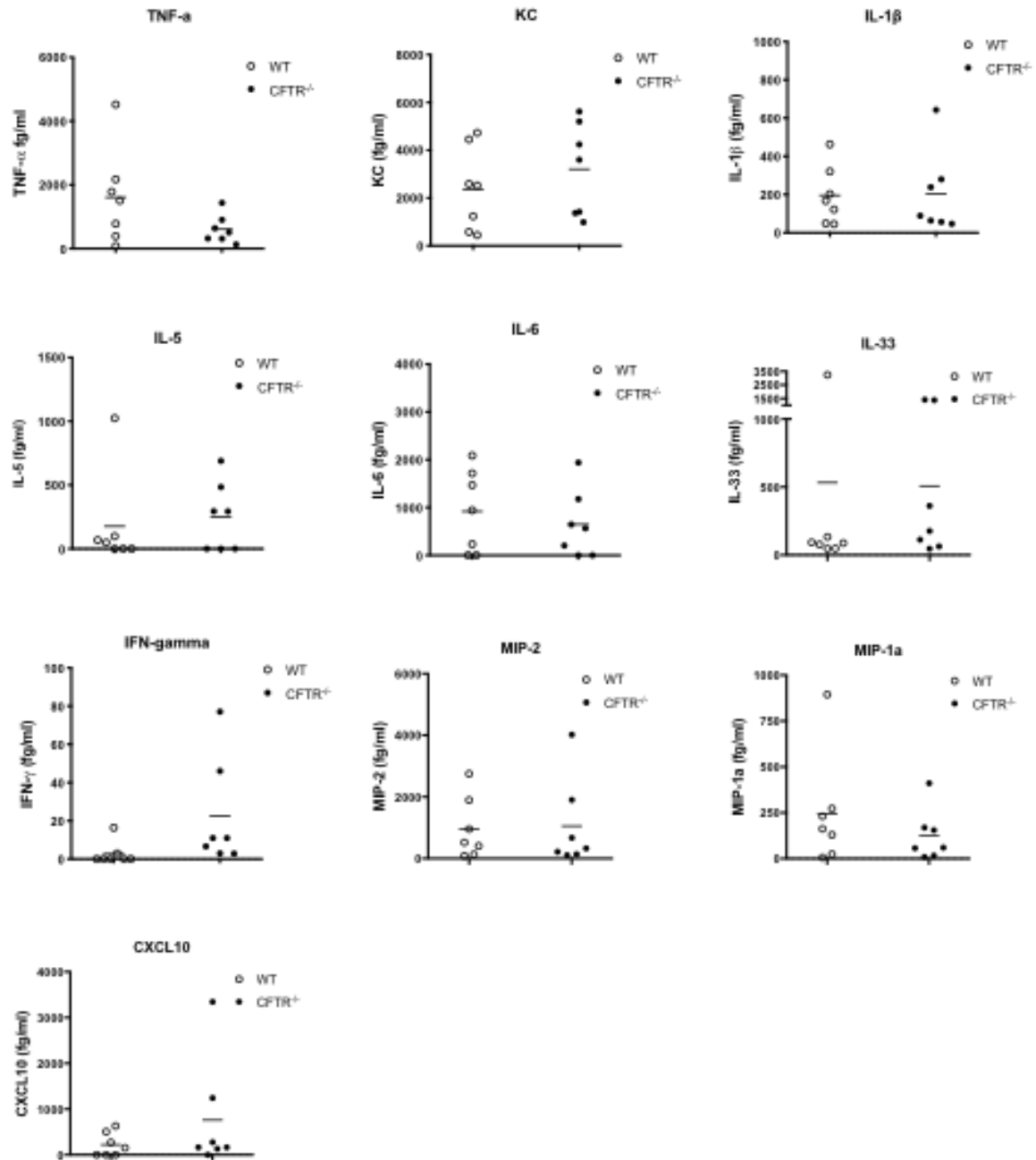


Figure 29. Airway cytokine levels are comparable between non-infected WT and CFTR^{-/-} mice. WT and CFTR^{-/-} mice underwent intra-nasal inhalation of sterile PBS. Bronchoalveolar lavage was performed at 18 hrs and cytokine levels in the BAL fluid were measured by MSD. Each dot represents 1 animal and lines mean + SD, n=8 per group. Statistical analysis performed by Student's t-test.

6.4 *A. fumigatus* infection induces significantly more macrophage cell death in CFTR^{-/-} than wild type mice

In my in-vitro studies I found that *A. fumigatus* induced more cell death in CFTR^{-/-} versus WT macrophages. To discover if this phenotype would hold true in an in-vivo model, wild-type and CFTR^{-/-} mice were infected intra-nasally with 1×10^7 resting *A. fumigatus* conidia. Mice were then culled at 6- and 18-hours post-infection and underwent bronchoalveolar lavage. Cell population analysis was performed by flow cytometry using the gating strategy depicted in Figure 26. Although alveolar macrophage numbers decreased in both genotypes post-infection, there was a significantly greater loss of cells in the CFTR^{-/-} mice compared to wild type; suggesting increased *Aspergillus*-induced macrophage cell death (Figure 30).

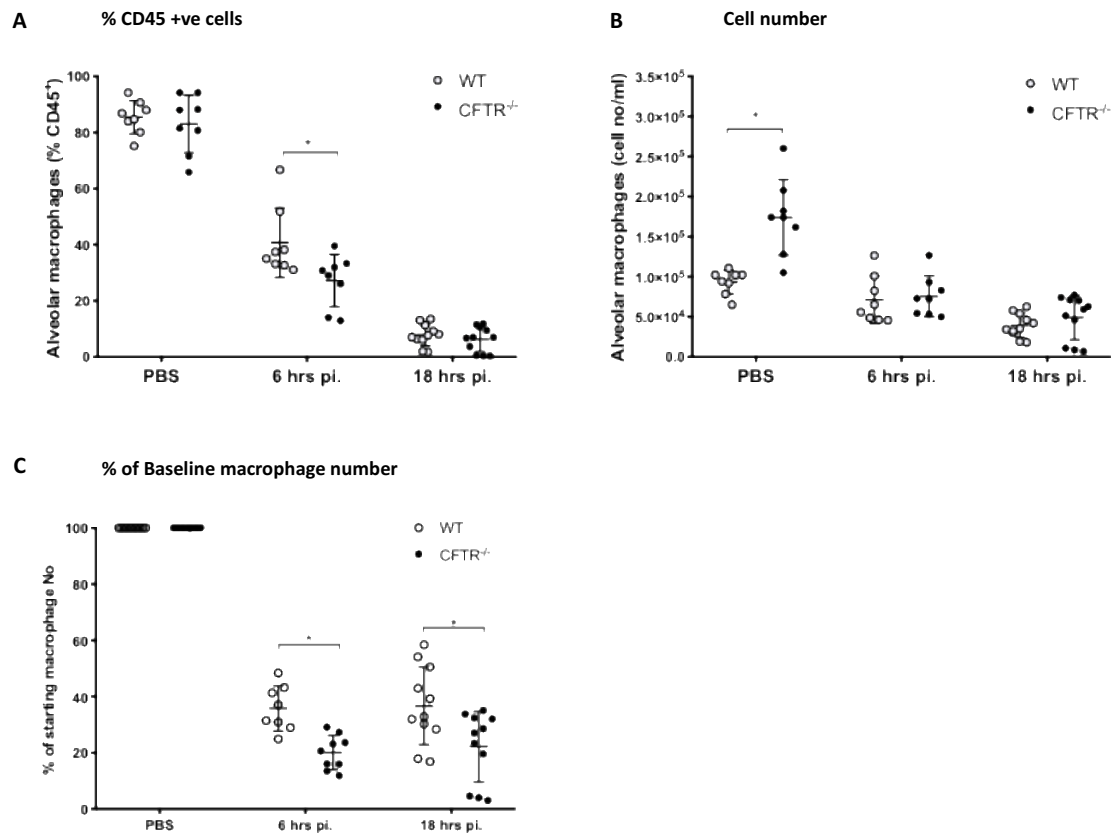


Figure 30. *A. fumigatus* induced macrophage cell death is increased in-vivo in CFTR^{-/-} mice. WT and CFTR^{-/-} mice were infected intranasally with 1×10^7 eGFP *A. fumigatus* conidia. Mice were culled at 6 and 18 hours and BAL performed to study cell influx by flow cytometry. (A) Macrophages as % of CD45+ve cells (B) Total macrophage number/ml BAL fluid. (C) % of average baseline macrophage numbers. Each dot represents 1 animal and lines mean + SD, n=8-11 per group. Statistical analysis performed by Student's t-test, $p < 0.05$.

6.5 *A. fumigatus* infection induces an exaggerated neutrophilia in CFTR^{-/-} mice

CF is recognised as a neutrophilic airways' disease. Consistent with this, I found that neutrophil recruitment to the airways was significantly increased in the CFTR^{-/-} mice compared to WT at both 6- and 18-hours post-infection (Figure 31).

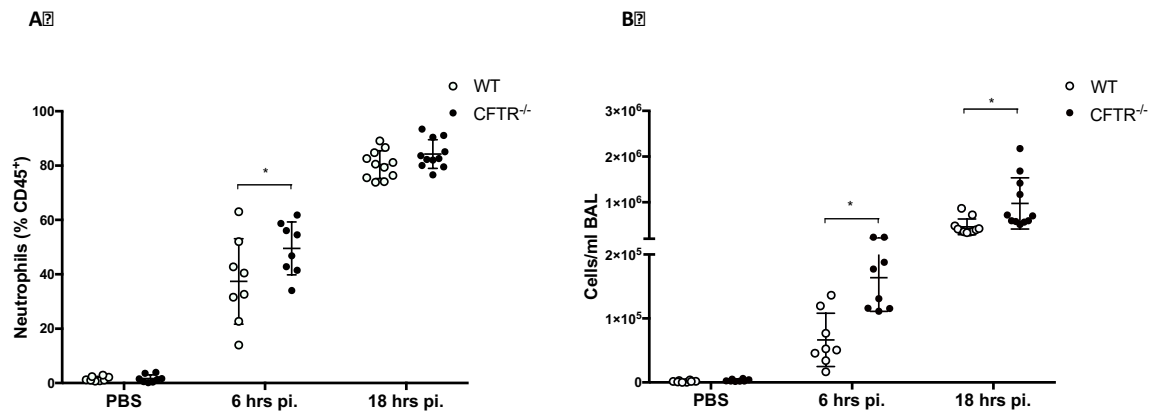


Figure 31. *A. fumigatus* infection induces an exaggerated neutrophilia in CF. WT and CFTR^{-/-} mice were infected intranasally with 1×10^7 eGFP *A. fumigatus* conidia. Mice were culled at 6 and 18 hours and BAL performed to study cell influx by flow cytometry. (A) Macrophages as % of CD45⁺ cells (B) Macrophage number/ml BAL fluid. Each dot represents 1 animal and lines mean + SD, n=8-11 per group. Statistical analysis performed by Student's t-test, $p < 0.05$.

Critical to the recruitment of neutrophils to the airways is the early release of chemotactic cytokines such as IL-8/KC, TNF- α and MIP-2. MSD multiplex analysis of bronchoalveolar lavage samples revealed increased KC release at 6 hours post-infection in CFTR^{-/-} versus WT mice (Figure 32). There was a trend towards increased MIP-2 but this did not reach significance. MCP-1 and CXCL10 levels were both significantly raised in CFTR^{-/-} samples. Both of these cytokines are classically known as monocyte chemokines, however there is evidence from murine infections studies that they also contribute to neutrophil chemotaxis and activity ^{293,294}.

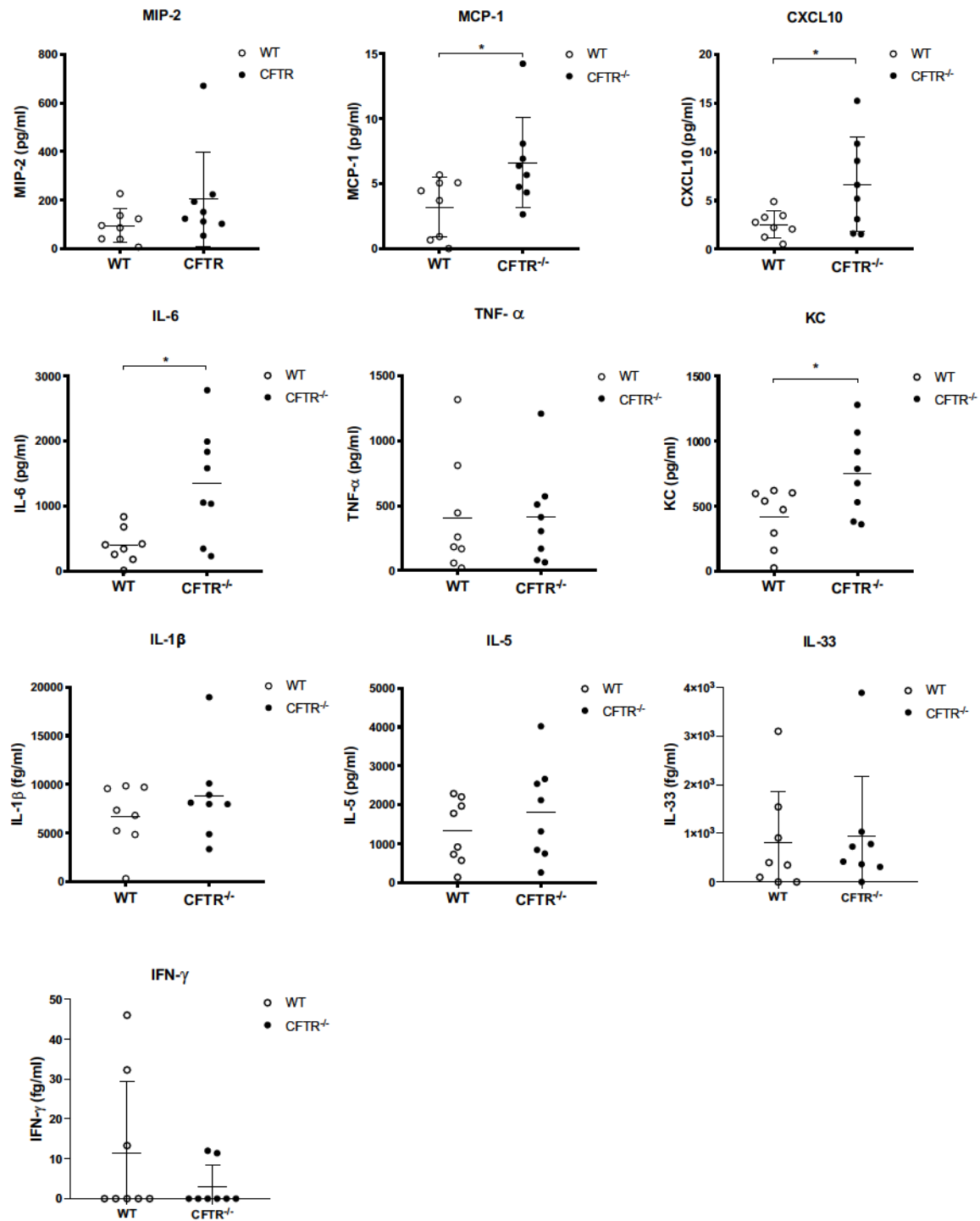


Figure 32. Early *A. fumigatus*-dependent neutrophil and monocyte chemokine release is increased in CFTR^{-/-} mice. WT and CFTR^{-/-} mice underwent intranasal inhalation of 1×10^7 *A. fumigatus* resting conidia. Bronchoalveolar lavage was performed at 18 hrs and cytokine levels in the BAL fluid were measured by MSD. Each dot represents 1 animal and lines mean + SD, n=8 per group. Statistical analysis performed by Student's t-test.

6.6 Airway clearance of *A. fumigatus* is impaired in CFTR^{-/-} mice

In my in-vitro experiments I had already identified a fungal killing defect in CFTR-deficient macrophages. To assess fungal clearance in-vivo, colony forming units (CFUs) in bronchoalveolar lavage fluid obtained 6- and 18- hours post-infection, were assessed. Fungal survival was significantly increased in CFTR^{-/-} mice at both time-points (Figure 33B). The geometric mean fluorescent intensity of the eGFP-*A. fumigatus* in alveolar macrophage cells was also significantly increased in CFTR^{-/-} mice versus wild-type, confirming an alveolar-specific killing defect in-vivo (Figure 33A).

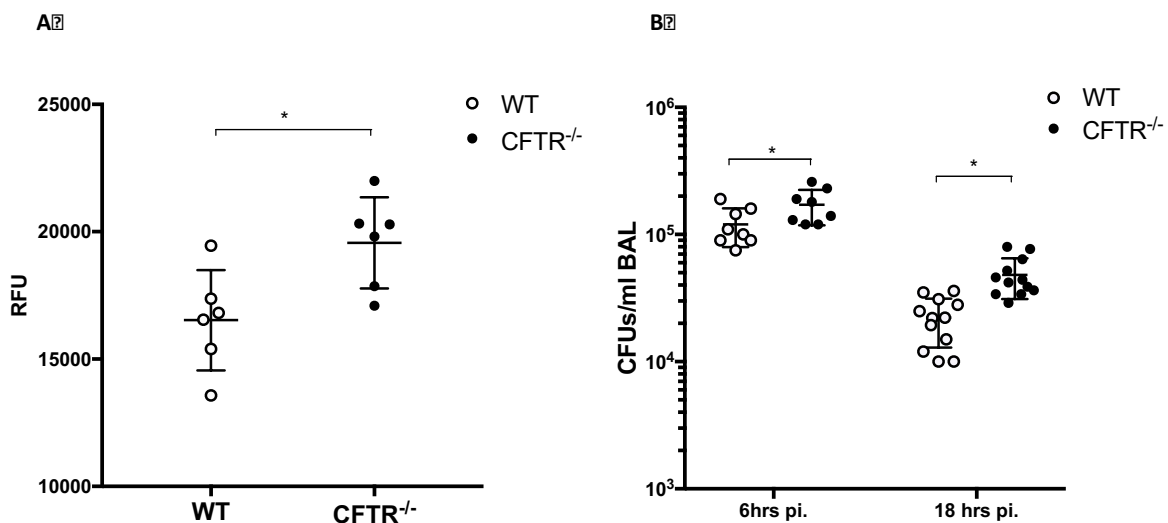


Figure 33. Fungal clearance is impaired in CFTR^{-/-} mice. WT and CFTR^{-/-} mice were infected intranasally with 1×10^7 eGFP *A. fumigatus* conidia and then culled and BAL performed at 6- and 18- hours. (A) GFP +ve alveolar macrophage population was identified by flow cytometry and geometric mean of GFP fluorescent intensity was calculated. Each dot represents 1 animal and lines mean + SD, n=6 per group. (C) Colony forming units (CFUs) were determined in the BAL fluid at the time points indicated. Each dot represents 1 animal and lines mean + SD, n=8-12 per group. Statistical analysis performed by Student's t-test, $p < 0.05$.

6.7 Late inflammatory cytokine release is increased in CFTR^{-/-} mice.

The impaired clearance of fungus from the airway in CFTR^{-/-} mice allows for ongoing immune stimulation. This is manifested in the increased release of inflammatory cytokines at 18 hours post-infection. MSD analysis revealed increased MIP-1a, MIP-2, MCP-1, TNF- α , IL-1 β , IL-6 and IL-33 in lavage samples from CFTR^{-/-} mice versus wild type (Figure 34).

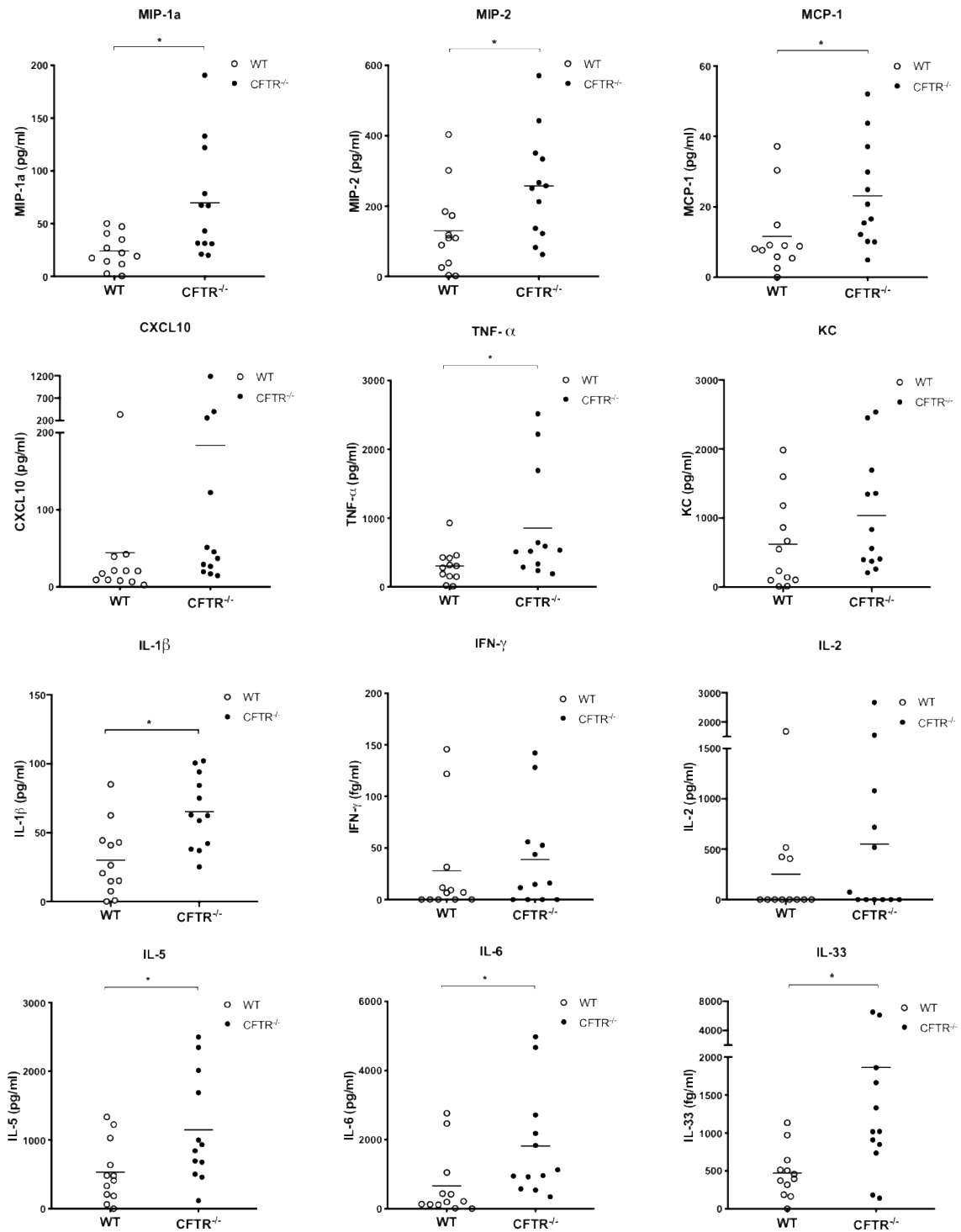


Figure 34. Late *A. fumigatus*-dependent inflammatory cytokine release is increased in CFTR^{-/-} mice. WT and CFTR^{-/-} mice underwent intra-nasal inhalation of 1×10^7 *A. fumigatus* resting conidia. Bronchoalveolar lavage was performed at 18 hrs and cytokine levels in the BAL fluid were measured by MSD. Each dot represents 1 animal and lines mean + SD, n=8 per group. Statistical analysis performed by Student's t-test.

6.8 Recovery of baseline airway cell population profile is delayed in CFTR^{-/-} mice.

An important feature of CF pathology is the chronicity of airway inflammation. Acute inflammatory changes induced by episodes of infection fail to resolve leading to chronic airway damage, recurrent infections and bronchiectasis. To assess whether resolution of the inflammatory changes induced by *Aspergillus* infection is delayed in CF, WT and CFTR^{-/-} mice were infected with 1×10^7 resting conidia and culled at 3 weeks post-infection. Flow cytometric analysis of bronchoalveolar lavage fluid showed that airway cell populations remained different at 3 weeks post-infection. Alveolar macrophages made up a smaller proportion of CD45⁺ve cells in CFTR^{-/-} mice compared to WT (Figure 34A) and were significantly delayed in returning to baseline numbers (Figure 35C). Neutrophils were still significantly increased in CFTR^{-/-} airways at three weeks whereas they returned to baseline levels in the WT mice (Figure 35 A + B).

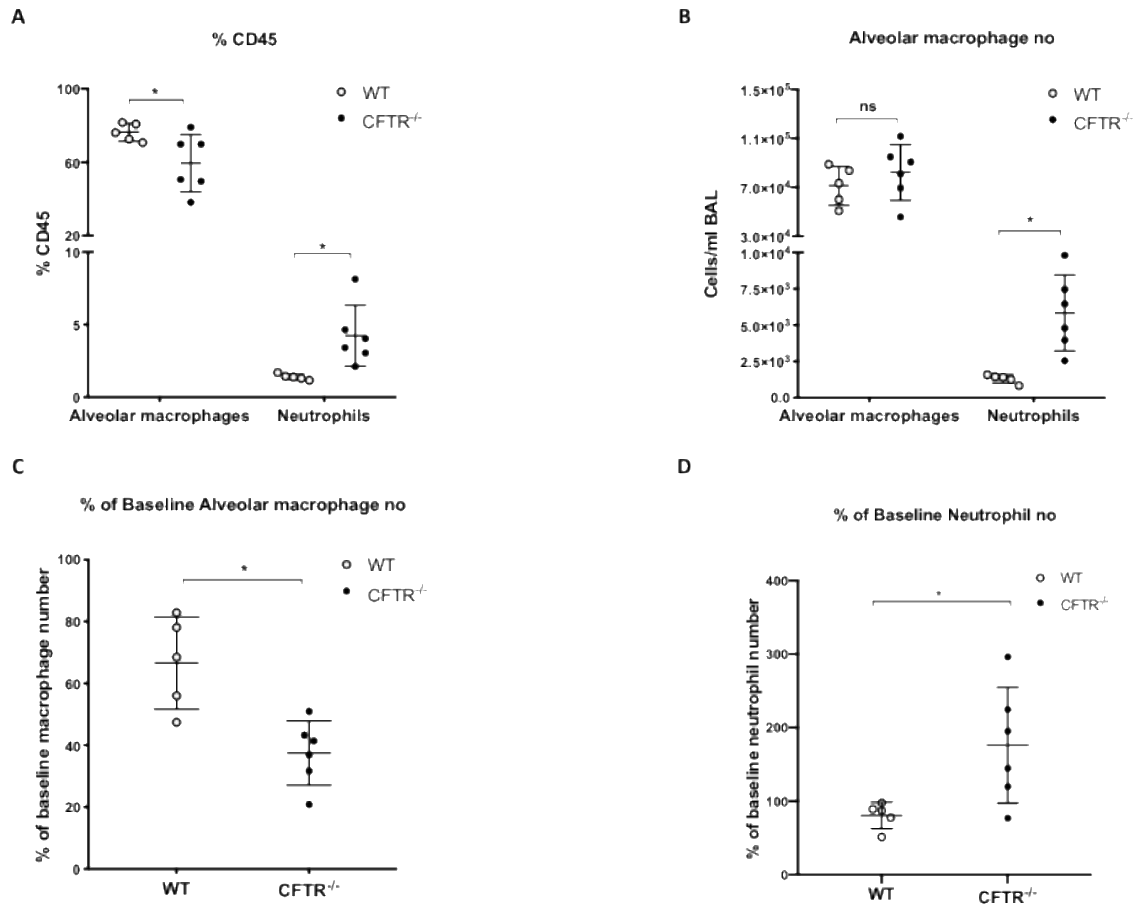


Figure 35. Resolution of airway inflammation is delayed in Cystic Fibrosis. WT and CFTR^{-/-} mice were infected intranasally with 1×10^7 eGFP *A. fumigatus* conidia. Mice were culled at 21 days post infection and BAL performed to study cell influx by flow cytometry. (A) Macrophages as % of CD45+ve cells (B) Total macrophage number/ml BAL fluid. (C) % of average baseline macrophage numbers. (D) % of average baseline neutrophil numbers. Each dot represents 1 animal and lines mean + SD, n = 6 per group. Statistical analysis performed by Student's t-test, $p < 0.05$.

6.9 Discussion

Aspergillus airway colonisation is an increasingly common finding in the CF population^{295,296}. In immunocompetent non-CF individuals inhaled conidia are cleared from the airways by the action of airway phagocytes and the mucociliary escalator. It is well established that the latter component of the host innate immune response is defective in CF but there is increasing evidence that the former is also compromised. I have shown in-vitro that CFTR-deficient macrophages have an intrinsic killing defect leading to increased cell death and that abnormal regulation of key intra-cellular signalling pathways leads to a hyper-inflammatory cell response to infection. In my murine studies my aim was to establish whether this phenotype would be present in an in-vivo model.

A. fumigatus infection did not result in death in either the WT or CFTR^{-/-} mice. The lack of deaths in the WT mice is consistent with our knowledge that immunocompetent mice, like immunocompetent humans, do not develop lethal aspergillosis when exposed to *Aspergillus* conidia²⁹⁷. That there were no deaths in the CFTR^{-/-} group is also not unexpected. CF patients do not develop angio-invasive *Aspergillus* disease unless they are otherwise immunosuppressed such as post-transplant. Of note, CF patients have a higher incidence of invasive *Aspergillus* disease post-lung transplant compared to non-CF organ recipients²⁹⁸, most likely due to higher rates of *Aspergillus* colonisation at the time of transplantation²⁹⁹. The lack of fatalities in the CFTR^{-/-} group suggests that loss of the CFTR ion channel does not give rise to the kind of profound immunocompromise associated with the development of acute invasive aspergillosis.

The CFTR^{-/-} mice did lose more weight than the WT mice, however, and were slower to return to their baseline weights. This suggests that infection with *A. fumigatus* induced a more potent and prolonged inflammatory response in the CFTR^{-/-} mice compared to wild type.

My initial experiments were performed in PBS saline-challenged mice to determine if there were features of airway inflammation present in the absence of infection. I found that there was increased cellular infiltration in the airways of CFTR^{-/-} mice compared to WT. The most marked difference was in CD11c⁺/Siglec F⁺ alveolar macrophages.

Alveolar macrophages are believed to derive from foetal monocytes that seed the lung before birth and develop into CD11c⁺ alveolar macrophages shortly after birth under the influence of GM-CSF⁴⁹ and TGF- β ⁵⁰. Under homeostatic conditions they are unusually long-lived with an annual turn-over of 40% and the population is mostly self-propagating⁵¹. Whether the increased number of alveolar macrophages seen in CF mice is due to increased recruitment of foetal monocytes during pre-natal life, an increased rate of self-propagation or reduced apoptosis and efferocytosis of aging cells is not clear.

In a study of CF and non-CF human foetuses, Hubeau et al. found that whereas macrophage numbers decreased during foetal development in non-CF foetuses, they increased in the CF lung such that at the late saccular/alveolar stage, macrophage numbers were significantly higher²⁹². To my knowledge, there is no published evidence on rates of self-propagation of CF and non-CF alveolar macrophages. Of note, cell population analysis at 3 weeks post-infection showed that alveolar macrophage numbers were similar in CFTR^{-/-} and WT mice. This is in contrast to pre-infection where

macrophage numbers were significantly increased in CF, suggesting that CF alveolar macrophages cannot replenish their numbers at an increased rate compared to WT.

The published data on apoptosis in CF cells is mixed. Most studies of epithelial cells have found increased rates of apoptosis in CF cells at baseline or in response to chemical stimuli ³⁰⁰⁻³⁰². Some studies have found reduced apoptosis in CF epithelial cells, but interestingly this was in response to an infectious stimulus ^{290,303}. CF neutrophils have been reported to display reduced apoptosis rates ^{174,304}. There is no published data on apoptosis rates in CF macrophages. In my in-vitro studies, I found that apoptosis was reduced in un-infected CFTR^{-/-} BMDM compared to WT. The mechanism connecting lack of CFTR to impaired apoptosis pathways is not yet known and requires further study. This finding might, however, help explain the higher airway cell numbers found in un-infected CFTR^{-/-} mice.

In contrast to the differences observed in airway cell populations, inflammatory cytokines were not elevated in CF bronchoalveolar lavage samples from un-infected mice. This is consistent with my in-vivo data where I did not find increased production of inflammatory cytokines from un-stimulated BMDM. Data on the presence of inflammation prior to infection onset in CF is mixed. Studies in CF ferrets have found that CF-associated alterations in inflammatory pathways and immune cell function exist in the sterile lungs of C-sectioned CF animals as well as after pathogen exposure at birth ³⁰⁵. Studies in CF pigs by contrast did not find evidence of increased inflammation in newborn animals ³⁰⁶. My results indicate that loss of CFTR alone is not sufficient to induce to a spontaneous inflammatory response from airway macrophages. However, the presence of excess macrophages at baseline suggests that the CF lung has the

infrastructure in place to generate a potentiated inflammatory response to any incoming pathogens.

This became evident post-infection where there was a significant increase in the release of pro-inflammatory cytokines in the CFTR^{-/-} mice. Neutrophil and monocyte chemokines were increased including KC, MCP-1 and CXCL10 at 6 hours and MIP-1a, MIP-2, MCP-1 and TNF- α at 18 hours. Release of these cytokines drives cell recruitment to the airways and consistent with this I found increased neutrophil recruitment to the airways in the CFTR^{-/-} mice from 6 hours post-infection. Interestingly, the Th1 cytokines IL-2 and IFN- γ were not significantly increased in samples from the CF mice whereas the Th2 cytokines IL-5, IL-6 and IL-33 were. IL-6 inhibits Th1 differentiation through the up-regulation of suppressor of cytokine signalling 1 (SOCS1)³⁰⁷ and promotes Th2 differentiation by the induction of NFAT³⁰⁸ and c-Maf³⁰⁹ during early CD4⁺ T cell activation. IL-33 is primarily produced by epithelial cells and enhances IL-5 and IL-13 expression in mouse Th2-polarized cells in vitro³¹⁰, activates murine bone marrow-derived myeloid DCs to prime naive T cells to produce IL-5 and IL-13³¹¹ and directly stimulates CD4⁺ cells to differentiate into Th2 cells producing IL-5 and IL-13³¹². IL-5 encourages the differentiation and proliferation of eosinophil progenitors³¹³.

The finding of increased production of IL-5, IL-6 and IL-33 suggests that *Aspergillus* induces the early release of cytokines that will promote a pro-allergic Th2-dominant adaptive immune response in cystic fibrosis. Although of benefit in fighting parasites, this is less useful for combating the more common pathogens encountered in CF airways such as bacteria and fungi. CF patients who become chronically colonised with *A. fumigatus* retain a chronic stimulus driving a Th2-predominant response in the

airways. This not only encourages the development of pathologies such as ABPA, which is more common in CF than in the general population ³¹⁴, but may compromise the development of an appropriate Th1-predominant response to other inhaled pathogens such as *P. aeruginosa*. Interestingly, a Th2-dominant immune response has been associated with chronic *P. aeruginosa* lung infection in CF patients ³¹⁵, whereas high levels of Th1-cytokines have been linked with fewer pulmonary exacerbations ³¹⁶. Future work could include assessing secretion of other Th2 polarising cytokines such as IL-25, TSLP and amphiregulin, a marker of tissue inflammation secreted by type 2 innate lymphoid cells that has been detected in sputum from CF patients ³¹⁷.

Post-infection with *A. fumigatus* there was a markedly greater drop in alveolar macrophage numbers in the CFTR^{-/-} mice compared to WT, indicating increased macrophage cell death consistent with my in-vivo results. It is likely that this is due to the killing defect identified within CF macrophages, which allows increased fungal germination and activation of necroptotic cell death pathways. Of note IL-33, which was increased in bronchoalveolar lavage samples from CFTR^{-/-} mice, is also an alarmin released by dying cells, primarily epithelial cells but also myeloid-lineage cells ³¹⁸. This increase in macrophage cell death is significant not only because of the loss of airway phagocytes but also due to the release of toxic intra-cellular contents into the airway lumen. This massive loss of macrophages is accompanied by a reduced ability to kill and clear fungus from the airway as indicated by increased live fungal burden at 18 hours post-infection.

In cystic fibrosis, airway inflammation becomes chronically established leading to the development of bronchiectatic damage. I found that at 3-week post-infection, whereas

live neutrophil numbers had returned to normal in the WT, they were still increased in the CFTR^{-/-} mice. Macrophage numbers were also delayed in recovering to pre-infection levels, both in terms of cell number and % CD45⁺ cells.

In summary, I have shown that *Aspergillus* induces a potent inflammatory response in CFTR^{-/-} mice characterised by increased macrophage cell death and release of pro-inflammatory cytokines. CFTR^{-/-} mice are impaired in their ability to clear fungus from the airways leading to persistent neutrophilic airway inflammation. There has been much debate as to the clinical significance of *Aspergillus* colonisation in CF. My findings lend evidence to support the argument that this common environmental fungus is not just a passive coloniser but in fact is able to induce a pathological host response which contributes to the pathogenesis of CF airways disease.

Chapter 7

Concluding Remarks

The physician J.H. Bennett was the first to report a case of pulmonary *Aspergillus* infection when in 1842 he described the finding of a fungus in the lungs of a post-mortem patient with tuberculosis, an apparent case of an aspergilloma ³¹⁹. Since then cases of invasive aspergillosis ^{320,321} and ABPA ³²² have been described for the first time. These historic cases all demonstrate the spectrum of disease that *Aspergillus spp.* can cause in hosts with different susceptibilities. It is relevant that immunocompetent individuals inhale hundreds of conidia on a daily basis and clear them without developing disease ³²³. Reflecting on the known risk factors for each of these disease phenotypes, it becomes apparent that it is the status of the host immune response and the nature of the interaction of that host response with the pathogen that determines the clinical features, course and prognosis of *Aspergillus* lung disease.

The use of Ibrutinib in the treatment of haematological malignancies has emerged over recent years as a risk factor for invasive *Aspergillus* infections. This thesis provides evidence of a mechanism that might explain this susceptibility. Using high-resolution microscopy, I have shown that upon uptake into the cell, BTK is recruited to endosomes containing swollen but not resting *Aspergillus* conidia, consistent with our understanding that resting conidia do not activate inflammatory pathways within the macrophage. Once recruited, BTK undergoes sequential trans- and then auto-phosphorylation at tyrosine residues 551 and 223. This second auto-phosphorylation

step is required to switch on BTK's intrinsic trans-kinase activity and it is this step that is irreversibly inhibited by Ibrutinib.

The impact of this inhibition is felt on several downstream signalling pathways critical for orchestrating macrophage responses to *A. fumigatus*. The calcineurin-NFAT pathway has been shown in in-vitro and in-vivo experiments to be important for regulating the kinetics of endosomal maturation, for cytokine release, ROS production and fungal killing^{94,185}. Calcineurin-NFAT is also thought to act as a checkpoint for initiating necroptotic cell death and lateral transfer of conidia when the cell fails to control germination. Ibrutinib prevented activation of this pathway and also of a previously undescribed endosomal BTK-NF- κ B activation pathway. Blocking both of these pathways had profound effects on downstream macrophage effector functions.

Ibrutinib is used clinically in haematological malignancies for its effect on lymphoid cells. Given the importance of macrophages in the innate immune response to *A. fumigatus* and the impact of Ibrutinib on macrophage function, the findings reported in this thesis suggest that it is the effect of Ibrutinib on the myeloid components of innate immunity that underlies the increased susceptibility to *Aspergillus* infections in patients taking this drug.

Ibrutinib was also found to inhibit *A. fumigatus* provoked macrophage necroptosis. Programmed necrosis has been shown to occur in response to uncontrolled fungal germination in human macrophages¹⁸⁵. However, they were unable to show that inhibition of necrosome formation significantly reduced cell death. In this thesis I have shown that murine macrophages undergo RIPK1 and RIPK-3 dependent lytic cell death

in response to *A. fumigatus* infection, that is necroptosis. Caspase-1/11-dependent cell death, contrastingly, was not a significant contributor to overall *Aspergillus*-induced lytic cell death. This suggests that Caspase-1/11-dependent pyroptosis is not a predominant form of cell death in macrophages infected with *Aspergillus*. This contrasts with what is known about *Candida* infection where Caspase-1-dependent pyroptosis does occur. It is also possible that pyroptosis does occur but that other caspases take over mediating it in the absence of Caspase-1, as is the case with *C. neoformans*. This seems less likely however as *Aspergillus*-dependent IL-1 β requires both Caspase-1 and -8. Overall, this suggests that macrophage necroptosis does occur in response to *Aspergillus* infection and that it is the predominant form of programmed necrosis induced.

Necroptosis was found to be both TLR9 and BTK dependent. Others have found that programmed necrosis and lateral transfer of germinating conidia is calcineurin-NFAT dependent. My results concur with this as calcineurin-NFAT signalling has been shown to be dependent on TLR9-BTK signalling. Lateral transfer of *A. fumigatus* endosomes to neighbouring cells during programmed necrosis has been shown to be a salvage mechanism to prevent fungal escape. Ibrutinib inhibition of programmed necroptosis could be another contributing factor to the increased susceptibility to *Aspergillus* infections seen in Ibrutinib-treated patients.

The findings outlined in Chapters 3 and 4 help demonstrate how suppression of protective inflammatory macrophage anti-fungal activity, particularly that which is dependent on calcineurin-NFAT and NF- κ B signalling, renders the host comparatively immunocompromised and thus vulnerable to invasive *Aspergillus* infection. In the

second half of my PhD, I was able to show how in Cystic Fibrosis an opposite hyperinflammatory response can also interfere with the host's ability to efficiently clear fungal infection.

My in-vitro macrophage studies identified CFTR-related excess activation of key *Aspergillus*-responsive signalling pathways, specifically the calcineurin-NFAT and NF- κ B activation pathways. Although increased NF- κ B signalling has been detected in CF macrophages before in response to bacterial stimuli, this is the first demonstration of augmented activation of this pathway by a fungal pathogen. The finding of increased calcineurin-NFAT activation in CF cells is another novel finding and the first time this has been described in relation to an infectious stimulus. Hyper-activation of these two pathways results in a hyper-inflammatory response to *Aspergillus* with increased release of TNF- α , KC and IL-1 β . Although fungal killing has been found to be impaired in the absence of effective calcineurin-NFAT activation in human and murine macrophages, fungal killing was also impaired in CFTR-/- macrophages. This suggests that non-calcineurin dependent killing mechanisms are impaired in cystic fibrosis macrophages.

The findings from the in-vivo *A. fumigatus* infection model were consistent with the in-vitro results. Infection triggered a hyper-inflammatory response with increased cytokine release and excess macrophage cell death. Fungal clearance from the airway was impaired and resolution of inflammation was significantly delayed. These findings are consistent with our understanding of CF as a clinical disease characterised by persistent microbial colonisation and chronic neutrophilic inflammation. The finding of such marked pathogen-induced macrophage cell death has not been previously

demonstrated and could be significant in understanding CF airway disease pathogenesis. Alveolar macrophages play a key role not only in clearing pathogens from the airway but also in controlling and modulating of the inflammatory response generate, which can of itself be pathogenic to the host. They help clear dying cells and other cellular debris from the airways by efferocytosis. This process is essential for successful resolution of inflammation and the return to lung homeostasis. Disruption of this process, such as through depletion of efferocytotic cells, leads to secondary necrosis of accumulating apoptotic cells, further release of cell debris and subsequent uncontrolled inflammatory activation of the innate immune system by the release of damage associated molecular patterns (DAMPS) ^{324,325}.

In summary this thesis delineates how abnormalities in the innate immune response in both the direction of immunocompromise and of hyperinflammation compromise the host's ability to successfully clear *Aspergillus* infection. Although loss of normal BTK and CFTR activity drives macrophages in opposing directions in terms of inhibiting and potentiating key signalling pathways, they are acting on the same key pathways, namely the calcineurin-NFAT and NF- κ B activation pathways. Both groups also demonstrate impaired fungal killing resulting in an inability on the part of the host to clear *A. fumigatus* from the airway. Despite these similarities, the clinical trajectory taken secondary to this shared fungal persistence is divergent with Ibrutinib-treated hosts more susceptible to invasive disease whereas CF hosts are prone to chronic airway infection and allergy.

Chapter 8

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9 List of Publications

1. Warris A, Bercusson A, Armstrong-James D. 2019 *Aspergillus* colonization and antifungal immunity in cystic fibrosis patients. *Med Mycol.* 57 (Supplement_2): S118-S126.
2. Bercusson A, Colley T, Shah A, Warris A, Armstrong-James D. 2018 Ibrutinib blocks Btk-dependent NF- κ B and NFAT responses in human macrophages during *Aspergillus fumigatus* phagocytosis. *Blood.* 132(18):1985-1988.
3. Abdolrasouli A, Bercusson AC, Rhodes JL, Hagen F, Buil JB, Tang AYY, de Boer LL, Shah A, Milburn AJ, Elborn JS, Jones AL, Meis JF, Fisher MC, Schelenz S, Simmonds NJ, Armstrong-James D. 2018 Airway persistence by the emerging multi-azole-resistant *Rasamsonia argillacea* complex in cystic fibrosis. *Mycoses.* 61(9):665-673.
4. Armstrong-James D, de Boer L, Bercusson A, Shah A. 2018 From phagocytosis to metaforosis: Calcineurin's deadly role in innate processing of fungi. *PLoS Pathog.* 14(1): e1006627.
5. Bercusson A, de Boer L, Armstrong-James D. 2017 Endosomal sensing of fungi: current understanding and emerging concepts. *Med Mycol.* 55(1):10-15.

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TO THE EDITOR:

Ibrutinib blocks Btk-dependent NF- κ B and NFAT responses in human macrophages during *Aspergillus fumigatus* phagocytosis

Amelia Bercusson,¹ Thomas Colley,¹ Anand Shah,¹ Adilia Warris,² and Darius Armstrong-James^{1,3,4}

¹National Heart and Lung Institute, Imperial College London, London, United Kingdom; ²Medical Research Council (MRC) Centre for Medical Mycology, University of Aberdeen, Aberdeen, United Kingdom; ³MRC Centre for Molecular Bacteriology and Infection, Imperial College London, London, United Kingdom; and ⁴MRC and Asthma UK Centre in Allergic Mechanisms of Asthma, London, United Kingdom

Ibrutinib is a small molecule Bruton tyrosine kinase (Btk) inhibitor approved by the Food and Drug Administration for clinical use in the treatment of chronic lymphocytic leukemia, Waldenström macroglobulinemia, and as a second-line treatment of lymphoma and chronic graft-versus-host disease.¹ An association with pulmonary aspergillosis was observed shortly after Ibrutinib was licensed for use.² A recent phase Ib study of Ibrutinib treatment of primary central nervous system lymphoma reported a 39% incidence of invasive aspergillosis, in patients concurrently treated with corticosteroids, in the absence of neutropenia.³ Studies of *Aspergillus fumigatus* infection in Btk^{-/-} mice revealed focal pneumonia and large airway mucous plugs, mirroring findings in macrophage-depleted models of pulmonary aspergillosis.³

We recently described a key role for Btk in macrophage immune responses during experimental pulmonary aspergillosis.⁴ Btk was critical for endosomal signaling responses during murine macrophage phagocytosis of *A fumigatus*. Btk activation led to calcineurin-NFAT signaling, which was crucial for orchestrating neutrophil recruitment during pulmonary aspergillosis and was dependent on the endosomal DNA receptor TLR9. These observations suggest that defects in macrophage Btk signaling contribute to susceptibility to pulmonary aspergillosis. Here we show that Ibrutinib is a potent inhibitor of both NFAT and nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) responses in human macrophages during infection with *A fumigatus*. We show that *A fumigatus* induces human macrophage Btk phosphorylation, and that Btk depletion impairs NFAT and NF- κ B responses in human macrophages. Our findings suggest Btk involvement in a TLR9-dependent endosomally driven pathway in accordance with previous findings in our murine model. In addition, our results show that Ibrutinib is a strong inhibitor of macrophage responses to *A fumigatus*, which may increase the susceptibility of patients on Ibrutinib to invasive aspergillosis.

Peripheral blood samples were collected from unscreened healthy donors. THP-1–derived macrophages, human monocyte–derived macrophages (hMDMs), and alveolar macrophages were isolated and differentiated as previously described.⁵ The study was approved by the Biomedical Research Unit (National Research Ethics Service reference 10/H0504/9[DAJ3]), Royal Brompton, and Harefield NHS Trust.

A fumigatus strain CEA10 (FGSC A1163) and *Candida albicans* ATCC 90028 were obtained from the Fungal Genetics Stock Center. ATCC 46645-eGFP was a kind gift from Frank Ebel (Germany). Strains were cultured as previously described.⁵

Macrophages were incubated with 1 μ M Ibrutinib (Selleck Chemicals), 10 μ M ODN2088 (TLR9-blocking nucleotide), 10 μ M ODN20958 (control nucleotide, Miltenyi Biotec), 50 μ g/mL zymosan, or vehicle. SMARTpool siGENOME BTK small interfering RNA (siRNA; Dharmacon) was used at a concentration of 75 nM. For siRNA knockdown, primary monocyte cells were differentiated for 7 days. On day 4, siRNA was transfected using V1romer Blue (Lipocalyx) according to the manufacturer's instructions. Scramble siRNA was used as a control for all experiments.

Confocal microscopy was performed as previously described.⁵ Cells were permeabilized in phosphate-buffered saline containing 10% goat serum and 0.1% Saponin (Sigma, UK) for 2 hours and then incubated overnight at 4°C with a primary antibody (anti-NFATc1, clone 7A6, BD Biosciences; anti-NF- κ B p65, clone F6, Santa-Cruz Biotech; anti-BTK, clone 7F12H4, Novus Biologicals) in blocking buffer. After washing with phosphate-buffered saline, cells were incubated with anti-rabbit Cy5 or anti-mouse Cy5 antibody (Life Technologies) for 45 minutes at room temperature and mounted with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

Tumor necrosis factor- α (TNF- α) release was quantified in culture supernatants using DuoSet ELISA Development kit (R&D Systems) following the manufacturer's instructions. Galactomannan was quantified in supernatants using Platelia *Aspergillus* Ag kit (Bio-Rad) following the manufacturer's instructions. Western blotting for nuclear and cytoplasmic fractions was performed as previously described.⁵ For Btk phosphorylation studies, macrophages were incubated in 100 μ M sodium pervanadate for 2 hours at 4°C prior to cell lysis. Membranes were probed with anti-NFATc1 (7A6; Santa-Cruz), anti-NF κ B p65 (C22B4), anti-HDAC1 (10E2), anti-histone H3 (D1H2), anti-phospho-BTK (Tyr 223), and anti-BTK (D3H5) antibodies, all from Cell Signaling.

To determine whether *A fumigatus* activates Btk macrophages, THP-1 macrophages were infected with swollen conidia and

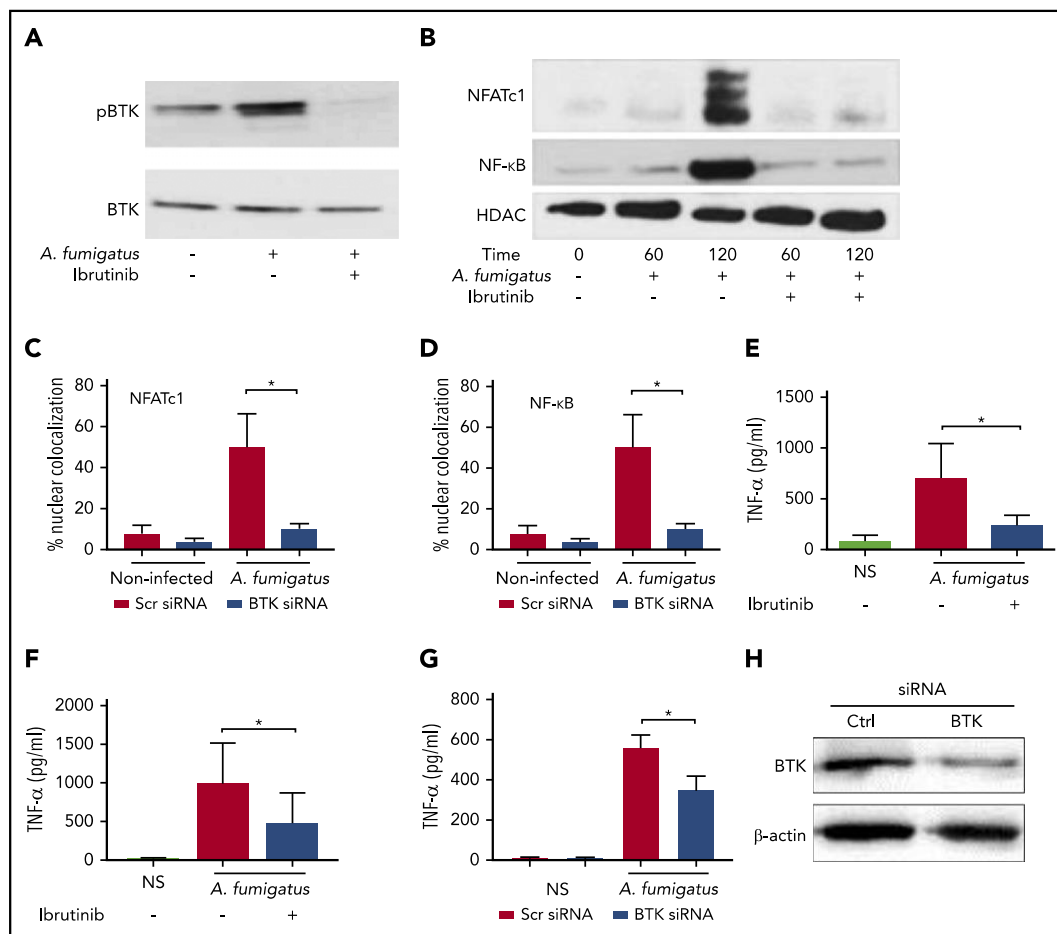


Figure 1. Ibrutinib blocks Btk-dependent activation of NFAT and NF-κB in human macrophages during *A. fumigatus* infection. (A) *A. fumigatus* induces autophosphorylation of Btk at Tyr 223, and this is inhibited by Ibrutinib. THP1 macrophages were pretreated with Ibrutinib (1 μM) for 1 hour. Cells were stimulated with *A. fumigatus* swollen conidia (multiplicity of infection [MOI] = 5) for up to 2 hours. Whole cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blotting. Membranes were probed with anti-pBTK and anti-BTK antibodies. (B) BTK phosphorylation is required for NFAT and NF-κB activation in response to *A. fumigatus* in THP1 macrophages. THP1 macrophages were pretreated with Ibrutinib (1 μM) for 1 hour. Cells were stimulated with *A. fumigatus* swollen conidia (MOI = 5) for up to 2 hours. Whole cell lysates were separated by SDS-PAGE, followed by western blotting. Membranes were probed with anti-NFATc1, NF-κB, and HDAC antibodies. (C-D) BTK mediates NFAT and NF-κB activation pathways in hMDMs (supplemental Figure 2). Monocyte-derived macrophages were pretreated with Scramble or BTK-targeting siRNA (75 nM) for 72 hours. Cells were stimulated with eGFP *A. fumigatus* swollen conidia (MOI = 1) for 1 hour, and NFATc1 and NF-κB translocation were measured by confocal microscopy. Nuclear translocation was quantified by calculating the percent overlap of the nuclear DAPI and transcription factor-linked fluorophore channels. Data were calculated from 7 fields of view taken at random per biological repeat. Mean and standard deviation of 3 biological repeats are represented. Statistical analysis was performed using paired Student t tests: ns, not significant; **P* < .05; NS, nonstimulated. N = 3. Af, *A. fumigatus*; Ca, *C. albicans*; Zym, zymosan. (E-H) TNF-α release by human macrophages in response to *A. fumigatus* is BTK dependent. Monocyte-derived macrophages (E, G-H) and alveolar macrophages (F) were pretreated with Ibrutinib (1 μM) for 1 hour or scramble or BTK-targeting siRNA (100 nM) for 72 hours. (E-G) Macrophages were stimulated with *A. fumigatus* swollen conidia (MOI = 1) for 16 hours. TNF-α levels in the tissue culture supernatants were measured by enzyme-linked immunosorbent assay. (H) Whole cell lysates were separated by SDS-PAGE, followed by western blotting. Membranes were probed with anti-BTK and anti-β-actin antibodies. Statistical analysis was performed using paired Student t tests. **P* < .05. NS, nonstimulated. N = 3 to 5.

phosphorylation of Btk at Tyr 223 determined by western blotting (Figure 1A). Infection induced phosphorylation of Btk, which was blocked by Ibrutinib. In addition, Ibrutinib inhibited *A. fumigatus*–dependent nuclear translocation of NFAT and NF-κB (Figure 1B). Zymosan, but not *C. albicans*, was able to induce BTK phosphorylation (supplemental Figure 1, available on the Blood Web site). The role of Btk in NFAT and NF-κB translocation was confirmed by Btk siRNA knockdown during *A. fumigatus* infection of hMDMs, by confocal microscopy (Figure 1C-D; supplemental

Figure 2). Accordingly, both Ibrutinib and Btk siRNA inhibited hMDM and alveolar macrophage TNF-α responses during *A. fumigatus* infection (Figure 1E-H). These observations indicate that Ibrutinib blocks inflammatory responses to *A. fumigatus* in human macrophages through a Btk-dependent pathway.

Our murine studies indicated that Btk-dependent macrophage responses to *A. fumigatus* are mediated through an endosomal TLR9 signaling pathway.⁴ Consistent with these observations,

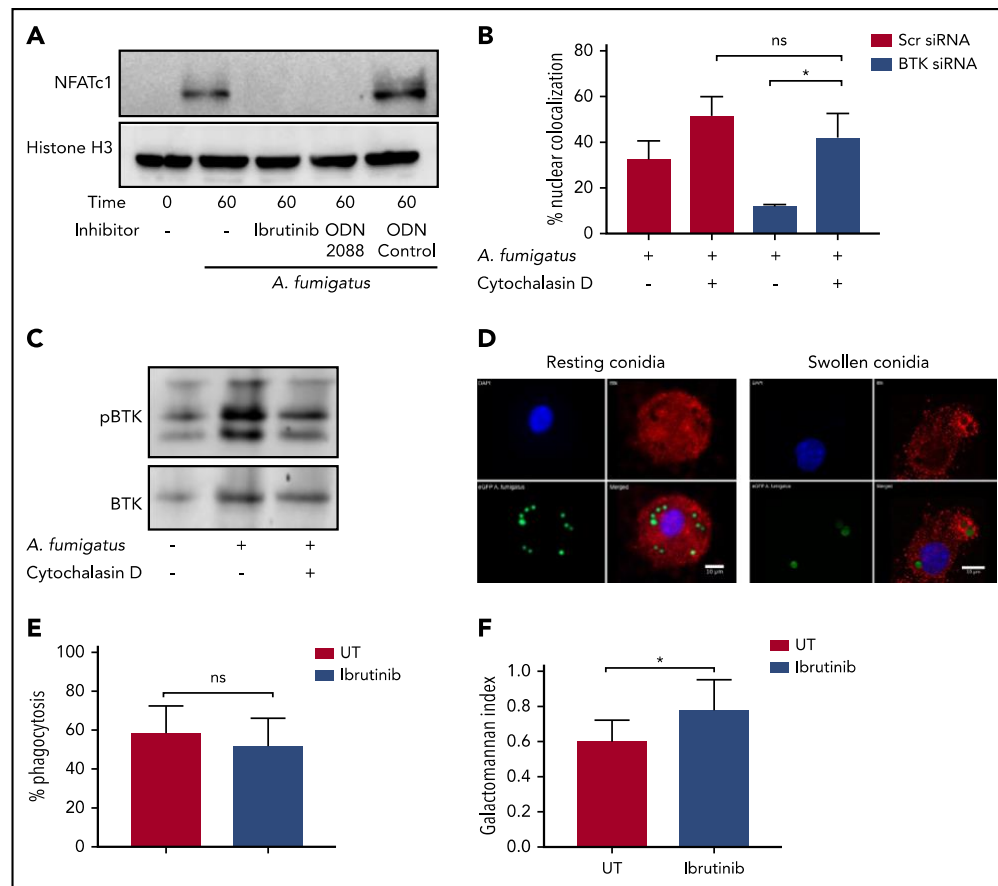


Figure 2. Endosomally driven Btk responses during human macrophage infection with *A. fumigatus* are required for optimal fungal growth control. (A) TLR9 engagement and BTK phosphorylation are required for NFAT activation in response to *A. fumigatus* in hMDMs. hMDMs were pretreated with Ibrutinib (1 μ M), the TLR9-blocking nucleotide ODN2088 (10 μ M), or ODN nucleotide control (10 μ M) for 1 hour. Macrophages were then infected with *A. fumigatus* swollen conidia (MOI = 1) for 1 hour. Whole cell lysates were separated by SDS-PAGE, followed by western blotting. Membranes were probed with anti-NFATc1 and Histone H3 antibodies. (B-C) BTK mediates an endosomal NF- κ B activation pathway in human macrophages. (B) Monocyte-derived macrophages were pretreated with Scramble or BTK-targeting siRNA (100 nM) for 72 hours and additionally with cytochalasin D (10 μ M) or vehicle. Macrophages were stimulated with eGFP *A. fumigatus* swollen conidia (MOI = 1) for 1 hour, and NFATc1 and NF- κ B translocation were measured by confocal microscopy. Nuclear translocation was quantified by calculating the percent overlap of the nuclear DAPI and transcription factor-linked fluorophore channels. Data were calculated from 7 fields of view taken at random per biological repeat. Mean and standard deviation of 3 biological repeats are represented. (C) Whole cell lysates were separated by SDS-PAGE, followed by western blotting. Membranes were probed with anti-BTK and anti- β -actin antibodies. Statistical analysis was performed using paired Student t tests: ns, not significant; * P < .05; NS, nonstimulated. (D) BTK is recruited to the membranes of endosomes containing swollen but not resting conidia in hMDMs. Representative confocal microscopy of monocyte-derived macrophages infected with eGFP expressing *A. fumigatus* (green) (MOI = 1) at 45 minutes propidium iodide stained for BTK (red) and nuclei (blue) (original magnification $\times 60$). (a) Nuclei; (b) BTK; (c) eGFP; (d) composite images. (E) Ibrutinib does not block *A. fumigatus* phagocytosis. Monocyte-derived macrophages were pretreated with Ibrutinib (1 μ M) for 1 hour. Macrophages were infected with biotinylated *A. fumigatus*-GFP (green fluorescent protein) swollen conidia (MOI = 1) for 2 hours. External conidia were then counterstained with Cy3 biotin antibody, and phagocytosis was measured by flow cytometry. (F) Ibrutinib impairs macrophage control of fungal growth in vitro. Monocyte-derived macrophages were pretreated with Ibrutinib (1 μ M) for 1 hour. Cells were stimulated with *A. fumigatus* swollen conidia (MOI = 1) for 6 hours. Galactomannan levels were measured in the tissue culture supernatants. UT, untreated.

A. fumigatus-dependent NFAT translocation in hMDMs was blocked by both Ibrutinib and the TLR9-blocking nucleotide ODN2088 (Figure 2A). Furthermore, inhibition of phagocytosis of *A. fumigatus* by hMDMs using cytochalasin D led to a loss of Btk dependency for NFAT-dependent signaling responses (Figure 2B). In accordance with this finding, cytochalasin D inhibited Btk phosphorylation in hMDMs during *A. fumigatus* infection (Figure 2C). Using confocal fluorescence microscopy, we conformed that Btk colocalizes with swollen, but not resting, *A. fumigatus* conidia during phagocytosis (Figure 2D). However, Ibrutinib had no inhibitory effect on phagocytosis (Figure 2E).

Ibrutinib impaired fungal growth control by macrophages (Figure 2F). These observations suggest that *A. fumigatus*-dependent macrophage Btk signaling is endosomally driven and dependent on TLR9. Btk has also been shown to regulate reactive oxygen species production, inflammasome activation, chemotaxis, and adhesion in myeloid cells.⁶⁻¹⁰ Further studies have shown that BTK and TEC kinase are also important for myeloid immunity to *C. albicans*.^{11,12} Furthermore, Ibrutinib is known to have off-target effects on other TEC kinases.^{13,14} Future studies should focus on defining the wider impact of Ibrutinib on TEC kinase-dependent innate immunity to fungi.¹⁵

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Authorship

Contribution: D.A.-J., A.W., and A.B. conceived the study; A.B., T.C., and A.S. performed the experiments; and A.B., T.C., A.S., A.W., and D.A.-J. wrote the manuscript.

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ORCID profile: D.A.-J., 0000-0002-1014-7343.

Correspondence: Darius Armstrong-James, Fungal Pathogens Laboratory, Sir Alexander Fleming Building, National Heart and Lung Institute, Imperial College London, SWZ 2AZ, London, United Kingdom; e-mail: d.armstrong@imperial.ac.uk.

Footnotes

The online version of this article contains a data supplement.

There is a *Blood* Commentary on this article in this issue.

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