

Defining the calcineurin-dependent innate immune response of human
alveolar macrophages to *Aspergillus fumigatus*

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Declaration of originality

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ABSTRACT

Pulmonary aspergillosis is a lethal mould infection in the immunocompromised host. Understanding initial control of infection, and how this is altered in the immunocompromised host, is a key goal for understanding the pathogenesis of pulmonary aspergillosis. My thesis aims to characterise the outcome of human macrophage infection with *Aspergillus fumigatus*, and understand how this is altered in transplant recipients on calcineurin inhibitor immunosuppressants.

I initially quantified the impact of calcineurin inhibitors on fungal killing following *in vitro* human alveolar macrophage infection with *A. fumigatus*. A human monocyte-derived macrophage model was then used to systematically analyse calcineurin-dependent effects on phagocytosis, phagosomal maturation, reactive oxygen species and cytokine production. Calcineurin-dependent mechanisms of fungal killing were then further characterised by a combination of single cell fluorescence imaging, proteomics, and *in vivo* studies.

Human alveolar macrophage killing of *A. fumigatus* is calcineurin-dependent. Macrophage phagocytosis of *A. fumigatus* enabled control of 90% of fungal germination. However fungal germination in the late phagosome led to macrophage necrosis. During programmed necrosis, I observed frequent cell-cell transfer of *A. fumigatus* between macrophages which assists subsequent control of germination in recipient macrophages. Lateral transfer occurred through actin-dependent exocytosis of the late endosome in a vasodilator-stimulated phosphoprotein (VASP) envelope. Its relevance to the control of fungal germination was also shown by direct visualisation in a zebrafish aspergillosis model *in vivo*. The calcineurin inhibitor FK506/tacrolimus reduced cell death and lateral transfer *in vitro* by 50%. This resulted in uncontrolled fungal germination in macrophages and hyphal escape.

This thesis identifies programmed necrosis-dependent lateral transfer of *A. fumigatus* between macrophages as an important host strategy for controlling fungal germination. This process is

critically dependent on calcineurin. This provides fundamental insights into the pathogenesis of pulmonary aspergillosis in the immunocompromised host.

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Nomenclature

AM	Alveolar macrophages
AP-1	Activator protein-1
BAL	Bronchoalveolar lavage
CLR	C-type lectin receptor
CnA	Calcineurin A
CnB	Calcineurin B
CRAC	Calcium-release activated channel
CsA	Cyclosporine A
DAMP	Damage-associated molecular pattern
DRP1	Dynamin-related protein-1
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FK506	Tacrolimus
FMO	Fluorescence minus one
GFP	Green-fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
IFN- γ	Interferon-gamma
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
LAMP-1	Lysosomal-associated membrane protein 1
MDM	Monocyte-derived macrophages
NFAT	Nuclear Factor of Activating T cells
NF κ B	Nuclear factor kappa light-chain-enhancing of activated B cells
NHR	NFAT homology regions
NLR	NOD-like receptor
NOD-1	Nucleotide-binding oligomerization domain-containing protein 1
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PI	Propidium iodide
PLC- γ	Phospholipase C-gamma
PRR	Pattern recognition receptor
RCAN-1	Regulator of calcineurin
ROS	Reactive oxygen species
Syk	Spleen-like tyrosine kinase
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor-alpha
VASP	Vasodilator-stimulated phosphoproteins

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1 Introduction

1.1 Immunosuppression regimens in solid organ transplantation

1.1.1 Solid organ transplantation:

Humanity has long dreamed of hybrid creatures with the description of the '*chimera*' in Homer's Iliad coining the term *chimerism* to represent coexistence of donor and host cells. Descriptions of organ transplantation date back to ancient Indian and Chinese medical texts circa 1000BC with tissue flap autografts used to repair nose injuries by the Indian surgeon Samhita (Nagral, 1995, Hauben, 1984). The modern transplant techniques were developed in a series of animal experiments in the early 20th century by the French surgeon Alexis Carrel (Aida, 2014). Dr Carrel noted that the allogeneic organ failed due to what he termed '*biologic incompatibility*', one of the first descriptions of the concept of an immunological cause of graft failure.

The first solid organ transplant was performed around 60 years ago following the transplantation of a human kidney between identical twins (Merrill et al., 1984). Prior to this, experiments using animal models and work with skin allografts had significantly enhanced understanding of immunological host tolerance (Doyle et al., 2004). Using this knowledge, transplant physicians realised that for allogeneic transplantation to be achievable for a larger patient population, tools were necessary to prevent immune-mediated organ rejection. This was initially achieved using lymphoid irradiation which was seen to prevent renal allograft rejection in animals, and subsequently the newly available corticosteroid 'cortisone' (Murray et al., 1960, Dealy et al., 1960). An early insight into the complications of immunosuppression was seen with the majority of patients receiving lymphoid irradiation dying due to overwhelming infection (Dealy et al., 1960). Further breakthroughs eventually occurred with the identification of azathioprine as an immunosuppressant in solid-organ transplantation following its original synthesis for use in oncology (Murray et al., 1963). Azathioprine

and corticosteroids formed the basis of clinical immunosuppression until the early 1980's when calcineurin inhibitors were introduced into clinical practice (Kapturczak et al., 2004).

The significantly improved outcome associated with the introduction of calcineurin inhibitors when administered to primate experimental heart-lung transplants at Stanford University, enabled the first clinical trial of human heart-lung transplantation (Baumgartner et al., 2009). This occurred in 1981 with Mary Gohlke, a lady with primary pulmonary hypertension, which had no meaningful treatment at the time, receiving a heart-lung transplant (Reitz, 2011). The operative success and absence of post-operative acute allograft rejection opened an era of therapeutic lung transplantation (Reitz et al., 1982).

Since then, with an aging population surviving longer with end-stage organ failure, solid organ transplantation as a treatment modality has continued to expand. Technical improvements in organ retrieval alongside a greater awareness of organ donation in the general public have contributed to increased solid organ transplants (Watson and Dark, 2012). This has led to around 4000 solid organ transplants being performed in the UK every year with just under 200 lung transplants (Johnson et al., 2014). Nevertheless, there remains a disparity between organ supply and demand with a shortage of suitable organs available to transplant (Salomon et al., 2015). With improvement in the efficiency of the consent process and the utility of novel immunosuppressive strategies to transplant across ABO barriers, the cohort of patients with solid organ transplants is likely to increase in the future (Razdan et al., 2015, Han et al., 2014).

Throughout these advances, despite the development of novel immunosuppressant drugs such as mycophenolate mofetil, inhibitors of the mammalian target of rapamycin (e.g. Sirolimus) and induction therapy using monoclonal antibodies (e.g. Basiliximab), calcineurin inhibitors and corticosteroids still form the cornerstone of immunosuppression in solid organ transplantation (Calne, 2004).

1.1.2 Calcineurin inhibitors

The introduction of cyclosporine in the early 1980's constituted one of the major breakthroughs in modern medicine with significant improvements in organ transplantation outcome (Kapturczak et al., 2004, Kolata, 1983). Cyclosporin A (CsA) was initially isolated in 1971 from cultures of the fungus *Tolypocladium inflatum* by Borel in the Sandoz laboratory in Basel during an antibiotic screening process (Calne, 2004). It was soon discovered that a fungal extract containing CsA showed considerable immunosuppressive activity without significant cytotoxic properties (Borel et al., 1994). An initial description of its immunosuppressive activity *in vitro* alongside experiments prolonging skin graft survival initiated its use in animal solid organ allograft models and subsequently in human cadaveric renal allografts with considerable success (Kolata, 1983, Watson and Dark, 2012). Ten years later, in 1984, scientists at Fujisawa Pharmaceuticals discovered a bacterium named *Streptomyces tsukubaensis* within a fungal broth which produced a potent immunosuppressant named FK506, and later 'Tacrolimus' (Goto et al., 1987). *In vitro* tests were published in 1987 and following trials in animal transplantation, it was approved for the prevention of organ rejection in liver transplantation in 1994 (1994).

CsA is a cyclic endecapeptide (molecular mass of 1203 kDa) with *N*-methylated amino acids that make the molecule resistant to the gastrointestinal tract and usable as an oral drug (Kapturczak et al., 2004). FK506 is a macrolide antibiotic (molecular mass of 804 kDa) with high lipid solubility (Plosker and Foster, 2000). Both drugs bind intracellular proteins called immunophilins with CsA binding cyclophilins, and FK506 binding FK-binding proteins (Kapturczak et al., 2004). In 1992, calcineurin (also known as phosphatase 2B) was identified as the target of the CsA and FK506 immunophilin complex, with binding leading to an inhibition of its activity (Cardenas et al., 1994). This was subsequently shown to effectively block T-cell proliferation by inhibiting interleukin-2 (IL-2) production (Ferrini et al., 1986). Forty years after their discovery, immunosuppression in solid organ transplantation is still reliant on calcineurin inhibitors with FK506 more commonly used, given recent

trials showing a lower risk of acute rejection compared to CsA in renal transplantation (Vincenti et al., 2002, Guethoff et al., 2013).

1.2 The calcineurin/NFAT signalling pathway

1.2.1 Calcineurin

Calcineurin is a protein phosphatase first identified in 1976 by Wang and Desai (Wang and Desai, 1976). Reversible protein phosphorylation is an essential component of intracellular signalling and a mechanism utilised in the regulation of all living organisms (Rao, 2009). In mammalian cells, phosphorylation mainly occurs on three hydroxyl-containing amino acids; serine, threonine and tyrosine. Calcineurin (or PP2B) forms part of the phosphoprotein serine/threonine phosphatase (PSP) family and is unique in that it is regulated by calcium (Ca^{2+}) and calmodulin (Shi, 2009). It is ubiquitously expressed and regulates many biological processes including immune system responses and neuronal and muscle development. Selective gene knockout of the most common isoform of CnA causes defective peripheral T-cell activation (Rusnak and Mertz, 2000).

The structure of calcineurin consists of a catalytic subunit (calcineurin A or CnA) and a regulatory subunit (calcineurin B or CnB). CnA contains a phosphatase domain, a CnB-binding helical domain, a calcium (Ca^{2+})-calmodulin-binding region and an autoinhibitory element (Rusnak and Mertz, 2000). Calcineurin is inactive alone and only gains phosphatase activity upon association with Ca^{2+} -calmodulin. Binding to Ca^{2+} stabilises its heterodimeric structure and results in partial stimulation of calcineurin activity. Crucially, it additionally allows calmodulin binding which induces a conformational change and displacement of the autoinhibitory element from the active site to allow full activation (Shen et al., 2008).

The molecular mechanisms that allow calcineurin to recognise and dephosphorylate specific protein substrates are not well understood. Two short calcineurin-binding amino-acid motifs in substrates have been identified. 'PxIxIT' (where x denotes any amino acid) enables binding to CnA at a groove

distal from the catalytic centre and more recently 'LxVP' which enables binding at the CnA-CnB interface to encourage efficient dephosphorylation (Grigoriu et al., 2013, Roy and Cyert, 2009).

Both the calcineurin immunosuppressant-immunophilin complexes (FKBP-FK506 and cyclophilin-CsA) associate with the CnB-binding helical domain of CnA and CnB around 25 amino acids away from the catalytic active site and inhibit phosphatase activity by sterically blocking access of substrates to the catalytic site rather than direct inhibition of enzymatic activity (Ke and Huai, 2003).

1.2.2 NFAT family of transcription factors

The nuclear factor of activating T cells (NFAT) family of transcription factors consists of 5 proteins (NFATc1 (or NFAT2), NFATc2 (or NFAT1), NFATc3 (or NFAT4), NFATc4 (or NFAT3) and NFAT5) evolutionarily related to the Rel/NF κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) family (Macian, 2005). NFATc1-4 are regulated by calcium-calcineurin-dependent dephosphorylation with immune cells expressing NFATc1-3 (Macian, 2005). NFATc1-4 also consist of an overall conserved NFAT homology region (NHR) that includes a trans-activation domain, nuclear localisation signal, calcineurin-binding sites (PxIxIT) and multiple phosphorylation sites (Takeuchi et al., 2007). Within cells in their resting state, cytosolic NFAT proteins are highly phosphorylated on multiple serine residues located in their NHR domains (Hogan et al., 2003b). Dephosphorylation by activated calcineurin results in a conformational change that exposes a nuclear import sequence leading to nuclear transport and induction of NFAT-mediated gene transcription (Sharma et al., 2011). NFAT is able to function as a Rel/NF- κ B-like dimeric transcription factor on certain types of NFAT-binding DNA elements and also bind co-operatively with other transcriptional partners (notably activator protein 1 (AP-1)) (Hogan et al., 2003b, Chen et al., 1998). For the majority of NFAT-mediated gene transcription (including IL-1, GM-CSF and IL-4 but not TNF- α) a dependence on NFAT-AP-1 co-operation has been established with differential T-cell activation when NFAT is activated alone or in combination with AP-1 (Macian et al., 2001). Due to the co-operative transcriptional activity of NFAT and AP-1, there is thought to be considerable cross-talk between the

two signalling pathways. This is supported by T cells from JNK^{-/-} mice showing increased nuclear localisation of NFATc1 and additionally by JNK activation in T and B cells being calcium-calcineurin dependent (Dolmetsch et al., 1997, Healy et al., 1997, Dong et al., 1998).

Activated NFAT is negatively regulated by re-phosphorylation due to various NFAT nuclear export kinases (including members of the mitogen-activated protein kinases (p38 and JNK)) and cytosolic maintenance kinases (e.g. glycogen-synthase kinase 3 and casein kinase 1) with sustained transcriptional activity reliant on a prolonged calcium influx (Wu et al., 2003b, Ortega-Perez et al., 2005). A level of redundancy within NFAT proteins is suggested by gene knockout mice lacking certain individual NFAT proteins showing only mild alterations in immune function (Ranger et al., 1998). However, cell-specific differences in protein expression and complex regulatory networks suggest a highly developed transcriptional role. NFATc1 knockout is embryonically lethal, with conditional NFATc1 and NFATc2 deficiency resulting in abnormal Th2 cell responses and double-knockout in fetal liver chimeras causing grossly impaired T cell effector functions and B-cell hyperactivity. (Yoshida et al., 1998, Xanthoudakis et al., 1996, Peng et al., 2001) NFATc3 deficient mice show impairment of CD4 and CD8 single-positive cells with increased apoptosis of double-positive thymocytes (Crabtree and Olson, 2002).

1.2.3 Calcineurin activation in T- and B-cells

A critical role for calcineurin in the activation of lymphocytes was suggested by the early discovery that the calcineurin inhibitor immunosuppressants Cyclosporin A and FK506 affected lymphocyte activation alongside the high lymphocyte cellular expression of calcineurin (Rusnak and Mertz, 2000, Ferrini et al., 1986). Calcineurin A α ^{-/-} lymph node cells were subsequently found to generate defective antigen-specific T cell responses following *in vivo* stimulation with an OVA peptide (Zhang et al., 1996). Identification of the central role of the NFAT transcription factor family in lymphocyte activation has further underlined the importance of the calcineurin pathway (Macian, 2005).

T and B cell receptor engagement leads to activation of receptor-associated tyrosine kinases that induce activation of phospholipase C- γ (PLC- γ) (Wange, 2000). Activated PLC- γ cleaves phospholipid phosphatidylinositol-4,5-bisphosphate (PIP₂) into phosphatidylinositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to its receptors in the endoplasmic reticulum and induces a release of intracellular calcium stores (Rao, 2009). This transient increase triggers the STIM1 and Orai1-mediated opening of calcium-release-activated calcium (CRAC) channels in the plasma membrane, which leads to a sustained increase in intracellular calcium levels (Park et al., 2009). Calcium binds calmodulin and activates calcineurin. Activated calcineurin dephosphorylates NFAT proteins and induces nuclear translocation and subsequent gene transcription following interaction with transcriptional partners (Hogan et al., 2003a, Macian et al., 2001). NFAT-driven gene expression is highly dependent on sustained Ca²⁺ influx and activation by calcineurin with rapid export and cessation of transcription with decreased Ca²⁺ influx or inhibition of calcineurin activity (Loh et al., 1996).

NFAT has been shown to play a role in the transcription of numerous T cell cytokine genes including IL-2, IL-3, GM-CSF, IL-4, IL-5, IL-13 expressed by Th2 cells, IFN- γ expressed by Th1 cells and TNF- α amongst others (Rao et al., 1997). NFAT has additionally has been shown to play a key role in T cell tolerance induction (Macian et al., 2002, Macian, 2005). In response to tolerizing stimuli with the absence of co-stimulation, unchecked Ca²⁺ signalling leads to the formation of NFAT homodimers which induce the expression of genes encoding proteins that dampen signalling from the T cell receptor and inhibit IL-2 expression (Soto-Nieves et al., 2009). T regulatory cell development and function is also thought to be NFAT dependent through positive feedback pathways and regulation of *Foxp3* expression (Hu et al., 2007, Oh-hora and Rao, 2009, Bandukwala et al., 2011). In B cells, calcineurin regulates B cell proliferation through cyclin D2 with decreased B1 cells that are involved in the humoral immune response and involvement of NFAT in the induction and maintenance of B cell anergy (Barrington et al., 2006, Winslow et al., 2006).

1.2.4 Calcineurin activation in innate immune cells

Although the calcineurin/NFAT pathway regulates multiple adaptive T cell functions, there has been recent evidence to show it has a significant role in innate immunity and myeloid differentiation (Fric et al., 2012b, Wang et al., 2015, Kiani et al., 2007, Kiani et al., 2004). A number of innate immune cells including macrophages, neutrophils, NK cells and dendritic cells respond to microbial stimuli via recognition through innate pattern recognition receptors and subsequently influence adaptive immunity.

Early evidence of the importance of the calcineurin/NFAT pathway was seen with NFAT nuclear translocation after CD14 activation from lipopolysaccharide (LPS) stimulation in dendritic cells (Zanoni et al., 2009a). Subsequently, calcineurin has been shown to regulate Toll-like receptor activation pathways in murine macrophages following LPS stimulation, with an inhibitory peptide (VIVIT) that blocks the calcineurin-NFAT docking site impairing TNF- α and IL-12 cytokine secretion (Kang et al., 2007, Elloumi et al., 2012). Stimulation with zymosan, a particulate preparation from the cell wall of *Saccharomyces cerevisiae*, also activates the calcineurin/NFAT pathway in macrophages and dendritic cells through the C-type lectin pattern recognition receptor Dectin-1 (Goodridge et al., 2007).

Further evidence is seen in murine models of uropathogenic *Escherichia Coli* infection, where calcineurin inhibitors impair resistance due to decreased neutrophil migration and phagocytosis, epithelial chemokine secretion and decreased neutrophil and macrophage expression of nucleotide-binding oligomerization domain 1 (NOD1) pattern recognition receptor (Tourneur et al., 2013). In neutrophils, conditional deletion of calcineurin B has additionally been shown to impair resistance to invasive candidiasis in murine models, with defective *ex vivo* killing. The role of the calcineurin/NFAT pathway in the innate immune response is highly evolutionarily conserved with evidence seen in *Drosophila* and *Caenorhabditis elegans* (Dijkers and O'Farrell, 2007, Febus et al., 2014). More recent studies have also implicated a critical role of the calcineurin/NFAT pathway in myeloid

differentiation with calcineurin negatively regulating dendritic cell differentiation, and TNF α dependent NFAT activation in human macrophages regulating osteoclast differentiation (Fric et al., 2012a, Wang et al., 2015, Yarilina et al., 2011).

1.3 Opportunistic infections in solid organ transplantation

1.3.1 Invasive aspergillosis

Human *Aspergillus* infections were first reported in 1842 with an aspergilloma complicating tuberculosis (Warris, 2014). Invasive aspergillosis was subsequently reported in chronic granulomatous disease and neutropenic cancer patients in the early 1950s (Cawley, 1947, Rankin, 1953). This led to a realisation that although exposure to *Aspergillus* conidia through inhalation is common, only a minority of those exposed with a defective immune response will develop disease (Mansour et al., 2012). The clinical features, course and prognosis of *Aspergillus* infections in patients depends on the interaction between the pathogen and the immune response of the host. *Aspergillus*-related disease can range from an allergic bronchopulmonary aspergillosis or allergy due to a hyper-reactive immune response, chronic aspergillosis where there is pre-existing structural lung disease (e.g. previous cavitary tuberculosis) to invasive aspergillosis (Kosmidis and Denning, 2015, Segal, 2009). Of the 185 recognised species of *Aspergillus*, *A. fumigatus* accounts for around 65% of all invasive infections and is the most frequent in pulmonary infection (Baddley et al., 2009).

Patients groups at risk for invasive aspergillosis include those with prolonged neutropenia, recipients of haematological stem-cell transplants or solid-organ transplants and patients with advanced acquired immunodeficiency syndrome or chronic granulomatous disease (Segal, 2009). Due to medical advances and increasing numbers of patients receiving stem-cell and solid-organ transplants, the population at risk for invasive aspergillosis has increased. Additionally, due to the use of intensive care support in life-threatening diseases, there is an increasing spectrum of patients without traditional risk factors (e.g. chronic obstructive lung disease) who develop invasive

aspergillosis (Baddley et al., 2013, Garnacho-Montero et al., 2013, Ader et al., 2005). This has led to a steadily increasing frequency of invasive aspergillosis over the past 2 decades (Baddley et al., 2001, Marr et al., 2002, Pfaller et al., 2006).

Although *Aspergillus* can infect any organ in the body, the majority occurs in the sino-pulmonary region due to the inhalational route of infection (Segal, 2009). In a neutropenic host, angioinvasion, vascular thrombosis and tissue infarction are characteristic pathological findings with minimal inflammatory reaction (Stergiopoulou et al., 2007). This is in contrast to non-neutropenic hosts such as patients with solid-organ transplantation where cases are usually indolent and present over weeks rather than days with a lack of angioinvasion on imaging (Singh and Paterson, 2005).

In a large consortium of 23 US transplant centres, the overall 12 month cumulative incidence of invasive fungal infections in a cohort of almost 17,000 solid-organ transplant patients was 3.1%. The greatest probability of invasive fungal infections was associated with small bowel transplantation (11.6%) followed by lung and heart-lung transplants (8.6%). Invasive candidiasis was the most common invasive fungal infection overall followed by aspergillosis with *A. fumigatus* the most common species isolated (Pappas et al., 2010).

In lung transplantation, invasive aspergillosis was however the most common invasive fungal infection. The majority of cases of invasive aspergillosis occurred >90 days after transplantation, were limited to the lungs (78%), and was associated with a 12-month mortality of 41% (Pappas et al., 2010). A high incidence of *Aspergillus* tracheobronchitis is also seen in lung transplantation (a rare presentation outside of solid organ transplantation) (Mehrad et al., 2001). Studies have described colonisation frequencies of up to 40% in lung transplant recipients, with subsequent increased susceptibility to invasive disease (Iversen et al., 2007). Patients with *Aspergillus* colonisation additionally have significant late mortality with high 5 year mortality around 50% (Iversen et al., 2007). This high mortality in lung transplantation may relate to the association seen between *Aspergillus* colonisation with bronchiolitis obliterans syndrome (BOS) which is the pathological

presentation of chronic rejection in lung transplantation although a causal link has yet to be proven (Weigt et al., 2013, Garantziotis and Palmer, 2009, Weigt et al., 2009).

1.4 *Aspergillus fumigatus*

1.4.1 *A. fumigatus* life cycle

A. fumigatus is a ubiquitous saprophytic fungus that plays a significant role in global carbon and nitrogen recycling to which humans are exposed to on a daily basis (Pitt, 1994). It forms part of the genus *Aspergillus* to which there are almost 200 species (Samson et al., 2014). In susceptible individuals with an impaired immune system however, *A. fumigatus* is the major cause of invasive aspergillosis followed by *A. flavus*, *A. terreus* and *A. niger* (Pfaller et al., 2006). There are a number of factors which are thought to account for the success of *A. fumigatus* as a human and environmental pathogen. Although *A. fumigatus* grows optimally at 37°C and a pH ranging from 3.7-7.6, it can survive a broad range of environmental conditions (Ward et al., 2006, Smith et al., 1977). *A. fumigatus* predominantly grows on decaying vegetation and soil with self-heating compost a major environmental source of *A. fumigatus* (Clark et al., 1983).

The life-cycle of *Aspergillus* begins with the production of conidia or asexual spores which are easily dispersed into the air (Latge, 2001). *A. fumigatus* in comparison to other pathogens like *A. flavus* and *A. niger* are relatively small with an average size of 2-3µm (Latge, 1999). This enables inhalation, bypassing of mucociliary clearance and deposition deep into the bronchioles and alveoli (Dagenais and Keller, 2009). Each *A. fumigatus* conidial head additionally produces thousands of conidia with efficient dispersal from environmental currents due their conidial hydrophobicity conferred by a surface rodlet layer encoded by the *rodA* gene (Carrion Sde et al., 2013, Taha et al., 2005). The production of a number of secondary metabolites by *A. fumigatus* and enzymes such as catalases and superoxide dismutases which counter reactive oxygen species killing also enable survival and propagation in hostile conditions (Kwon-Chung and Sugui, 2009, Kwon-Chung and Sugui, 2013).

Resting *A. fumigatus* conidia that are released into the air are in a metabolically inactive state and are immunologically inert due to masking of their underlying cell wall by the complex hydrophobic outer layer (Girardin et al., 1999, Carrion Sde et al., 2013). Production of melanin by resting conidia which is responsible for the grey-green pigmentation additionally protects against reactive oxygen species and inhibits programmed cell death and acidification in phagocytes enabling increased survival (Bayry et al., 2014, Heinekamp et al., 2012). Activation leads to an isotrophic growth that bursts open the rigid surface layer and exposes the carbohydrates of the cell wall. These consist of an inner layer of branched $\beta(1,3);(1,6)$ -glucans linked to chitin and covalently bound to an outer layer of galactomannan which consists of linear α -mannan chains linked to $\beta(1,5)$ -galactofuranose residues (Gastebois et al., 2009, Dubey et al., 2014).

This germination readily occurs at the mammalian body temperature of 37°C with conidia shedding its hydrophobic layer and swelling in 4 hours to form swollen conidia. By 6-8 hours they have switched to a polar growth and formed short hyphae (germlings). These then extend to form hyphae (mycelia) which are filamentous, multi-nucleated structures and can lead to tissue invasion and symptomatic disease (Latge, 1999, Latge, 2001). The *A. fumigatus* cell wall composition additionally differs between swollen conidia and hyphae, with reduced melanin and $\beta(1,3)$ glucan in hyphae (Gastebois et al., 2009).

During germination *A. fumigatus* activates a highly co-ordinated gene expression profile that enables adaptation to nutrient depletion and hypoxia and activation of secondary metabolite expression including gliotoxin which is immunosuppressive and cytotoxic (Kwon-Chung and Sugui, 2009). Interestingly, calcineurin is conserved in *A. fumigatus* and has been shown to localise to hyphal tips and septa with deletion of calcineurin A leading to defective invasion and impaired virulence (Juvvadi et al., 2014, Steinbach et al., 2006). Previous work within our group however has shown that although calcineurin inhibitors have *in vitro* activity against *A. fumigatus*, its

immunosuppressive effects at clinical relevant dosages *in vivo* outweighs its antifungal activity (Herbst et al., 2013).

1.5 The human macrophage

1.5.1 Human alveolar macrophage

The innate immune system includes a number of specialised hemopoietic cell types such as phagocytes which include macrophages, neutrophils and monocytes, dendritic cells, innate lymphocytes (including natural killer (NK) cells), mast cells, basophils and eosinophils (Uthaisangsook et al., 2002). Differentiated tissue macrophages throughout the body arise from circulating monocytes recruited from the blood (Gordon and Taylor, 2005). Once differentiated, macrophages become long-lived cells and develop specialised functions according to the tissues in which the cells reside, resulting in considerable heterogeneity (Gordon and Taylor, 2005). Immune stimuli increase tissue recruitment of monocytes where macrophage differentiation occurs, thus contributing to host defence, tissue remodelling and homeostasis (Shi and Pamer, 2011).

Macrophages are essential for pulmonary host defence with pulmonary macrophages present in the airways, alveolar spaces (alveolar macrophages (AM)), and resident lung tissue. AM consist of over 90% of the pulmonary macrophage population (van oud Alblas and van Furth, 1979). In the absence of an inflammatory insult, AM are remarkably long-lived with ~40% turn-over in 1 year and have significant self-renewal properties with the local population being primarily self-sustained (Hashimoto et al., 2013). AM differentiation is highly dependent on local produced cytokines and environmental triggers with AM having a distinct phenotype compared to tissue macrophages and other pulmonary macrophages suggesting further specialisation within the lung (Guth et al., 2009). AM differentiation has been shown to be granulocyte/macrophage colony-stimulating factor dependent with subsequent priming leading to various activation states (Suzuki et al., 2014, Guth et al., 2009, Mosser and Edwards, 2008).

Historically these activation states have simplistically been classified as either classically activated M1 macrophages or alternatively activated M2 macrophages depending on whether the macrophages were deemed to be 'pro-inflammatory' or 'anti-inflammatory' (Mosser and Edwards, 2008). This was in part based upon *in vitro* transcriptional analysis of human AM polarised with IFN γ showing gene upregulation of Toll-like receptor 2 and 4, CXC-chemokine ligand 9, 10, 11 (M1 macrophage), and IL-4 and IL-13 polarisation associated with upregulation of genes for mannose receptor (CD206) and matrix metalloproteinases 2 (MMP2), 7 and 9 (M2 macrophage) (Gordon, 2003). Recent transcriptional and epigenetic data, however, has shown that this paradigm is outdated and macrophage activation is complex and tissue-specific (Xue et al., 2014a). Macrophages have been shown to be in a permanent 'activated' state based on environmental signals sensed by surface receptors leading to distinct tissue and signal specific transcriptional and epigenetic signatures (Lavin et al., 2014).

It is clear from studies in human patients, that AM display a range of phenotypes, in particular encompassing cells with dramatic differences in biochemistry and physiology including regulatory macrophages important in resolution of inflammation (Gordon and Martinez, 2010). The phenotype of healthy-state human AM is in addition not clear (Hussell and Bell, 2014). AM also have a considerable degree of plasticity and are able to change their phenotypes dramatically in response to cues from the microenvironment (Hussell and Bell, 2014). In the lung, resident AM continuously encounter inhaled substances due to the exposure nature of their location (Lambrecht, 2006). To avoid inappropriate inflammation and damage, AM are kept in a quiescent state, suppressing innate and adaptive immune responses. This is demonstrated by depletion of AM using clodronate-filled liposomes leading to inappropriate inflammatory reactions to otherwise harmless particulate and soluble antigens (Thepen et al., 1989). Infectious agents however, trigger pattern and non-pattern recognition receptors on the AM cell surface causing activation and release of pro-inflammatory cytokines, enhanced phagocytosis and recruitment of CCR2⁺ inflammatory monocytes (Naessens et al., 2012, Steinmuller et al., 2000, Warmington et al., 1999, Islam et al., 2013). It is thought that

macrophages are likely to differ in their reactivity towards tissue or stress-induced signals depending on their origin and ontogeny (Ginhoux and Jung, 2014). In the context of an acute stress insult in the lung, it is as yet unclear how the response of resident AM differs to that of recruited monocyte-derived macrophages (MDM).

Human AM function is thought to be impaired in a number of pathological conditions. In chronic obstructive pulmonary disease, initial transcriptome profiling showed expression of wound-healing markers alongside a deficiency in resolution of inflammation and a lack of plasticity leading to impaired bacterial phagocytosis and efferocytosis of apoptotic cells (Day et al., 2014, Marti-Llitas et al., 2009, Hodge et al., 2007, Shaykhiev et al., 2009). Recent transcriptome analysis in smokers has also revealed significant changes characterised by loss of antigen processing, inflammatory response and immune response regulation (Xue et al., 2014a). There is a paucity of information regarding the impact of solid-organ transplantation on human AM function. Studies have highlighted the important role of macrophages in not only promoting allograft injury and vascular disease but also in tissue repair and microvascular repair in transplant related injury (Broichhausen et al., 2012). In lung transplantation, there are low numbers of donor AM in the allografted lung with increased donor macrophages correlating with allograft rejection (Bittmann et al., 2001, Schmidt et al., 2007). Expression of a number of AM surface markers associated with a range of host defence functions (CD11a, CD11b, CD11c, HLA-DR, CD14) were also found to be lower in transplant patients with bronchiolitis obliterans (Ward et al., 2001). Following on from these findings, there is now considerable interest in utilising *ex vivo* expanded regulatory macrophages as a promising therapeutic modality with early animal studies showing a good safety profile and longevity of treatment (Suzuki et al., 2014).

1.6 The immune response to *A. fumigatus*

1.6.1 The innate immune response to *A. fumigatus*

In immunocompetent individuals, inhaled conidia are swiftly killed by cells of the pulmonary innate immune response (Latge, 1999). After inhalation, by virtue of their size, *A. fumigatus* resting conidia can pass through the bronchial tree to reach the bronchoalveolar space (Park and Mehrad, 2009). The role of lung epithelium in fungal innate immune defence is important with a highly ciliated nature and increased secretory cells producing antimicrobial proteins and mucins (e.g. β -defensins, lysozyme, lactoferrin, surfactant protein A and D) (Oshero, 2012). Inhaled conidia can get trapped in mucus and expelled towards the oropharynx via ciliary beating. *A. fumigatus* however secrete proteases to inhibit the action of the mucociliary elevator and prevent expulsion (Amitani et al., 1995). As well as interacting with macrophages, lung epithelial cells (including work in A549 models of alveolar epithelium) have also been shown to phagocytose *A. fumigatus* conidia with trafficking into late endosomal-lysosomal compartments but their killing ability is poor (Wasylanka and Moore, 2003). Therefore control of fungal infection is thought to depend on professional phagocytic innate immune cells. This importance is demonstrated by the fact that loss of any of the phagocytic innate cells (neutrophils, monocytes, macrophages and dendritic cells) or defects in their anti-fungal effector functions results in increased susceptibility to infection (Bhatia et al., 2011, Philippe et al., 2003, Mircescu et al., 2009, Hohl et al., 2009, Ramirez-Ortiz et al., 2011).

The innate recognition of fungi by the innate immune system occurs largely through sensing of cell wall constituents or fungal DNA (Brown, 2011, Latge, 2010). Therefore *A. fumigatus* is primarily only 'recognised' by the innate immune system in a swollen conidial state following release of the hydrophobic rodlet outer layer which conceals its carbohydrate-rich cell wall in the resting state (Carrion Sde et al., 2013). This host-pathogen interaction occurs through host recognition of conserved molecular structures known as pathogen-associated molecular patterns (PAMPs) essential

for the life-cycle of the pathogen by evolutionary conserved pattern recognition receptors (PRRs) present on innate immune cells (Mansour et al., 2012). Sensing of PAMPs through PRRs leads to rapid induction of host immune inflammatory responses via activation of complex signalling pathways with an aim to facilitate pathogen eradication (Park and Mehrad, 2009, Reid et al., 2009).

1.6.2 Innate immune recognition of *A. fumigatus*

There are a number of membrane-bound and soluble PRRs involved in the recognition and killing of *A. fumigatus* with secreted soluble PRRs which opsonise the pathogen enabling indirect recognition by membrane-bound PRRs (Brown, 2011). All of the major fungal cell wall carbohydrates can be recognised by the innate immune system, with specific receptors for distinct organisms and discrete subsets of receptors on different phagocytic cells enabling recognition for the same organism (Latge, 2010). Membrane-bound PRRs can be categorised into the C-type lectin family, Toll-like receptors (TLRs), scavenger receptors, and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Mogensen, 2009, Park and Mehrad, 2009).

C-type lectin family

Recent studies have identified the C-type lectin receptors (CLRs) as having a crucial role in antifungal innate immunity (Dennehy and Brown, 2007, Hardison and Brown, 2012). CLRs are a large family of receptors which bind carbohydrates through one or more carbohydrate recognition domains (CRDs), or which possess structurally similar C-type lectin-like domains (CTLDs) which do not necessarily recognise carbohydrate ligands (Geijtenbeek and Gringhuis, 2009). Within myeloid cells, CLRs can be broadly grouped independently of structure 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 (Osorio and Sousa, 2011).

Dectin-1 is a surface type 2 membrane protein and CLR that recognises β -1,3-glucan which couples Syk and is highly expressed on phagocytes (Brown, 2006). Due to recognising β -1,3-glucan, Dectin-1 recognises swollen and germinating *A. fumigatus* conidia either at the cell surface or intracellularly but not resting conidia (Hohl et al., 2005). Dectin-1 has previously been shown to be important for fungal immunity with Dectin-1 $-/-$ mice having enhanced susceptibility to *C. albicans* and *A. fumigatus* due to impaired inflammatory responses and fungal killing (Dennehy and Brown, 2007). In addition, haematological stem cell transplant patients homozygous for a polymorphism in human Dectin-1 (Y238X) resulting in reduced cell surface expression are more susceptible to invasive aspergillosis (Cunha et al., 2010). Dectin-1 contains an ITAM-like motif in its intracellular tail, which is responsible for Syk engagement. This subsequently leads to canonical NF- κ B signalling via recruitment of CARD9 and activation of I κ B kinase (IKK) complex through an activated CARD9/Bcl10/Malt1 module (Strasser et al., 2012). Dectin-1 can also activate non-canonical NF- κ B pathway (RelB) in an CARD9-independent fashion, and signal via the serine-threonine kinase Raf-1 in a Syk-independent fashion resulting in acetylation of NF- κ B p65 subunit which can become transcriptionally active in combination with p50 (Gringhuis et al., 2009). In response to *Candida albicans* and Zymosan which is a fungal particle derived from the cell wall of *Saccharomyces cerevisiae*, Dectin-1 has also been shown to signal through the calcineurin-NFAT pathway in macrophages and dendritic cells (Goodridge et al., 2007).

Dectin-2 (CLEC6A) is another member of the CLR family and is expressed on macrophages, monocytes and some dendritic cell subtypes (Sun et al., 2013, Loures et al., 2015). It binds α -mannans in the fungal cell wall exposed in swollen and germinating *A. fumigatus* conidia with high affinity and its expression is upregulated in AM after *A. fumigatus* infection in mouse models (Sun et al., 2013). Unlike Dectin-1, Dectin-2 does not have a clear intracellular signalling motif and associates with the ITAM-containing FcR γ chain to activate Src and Syk-dependent NF κ B signalling (Robinson et al., 2009). Dectin-2 has been shown to be important for macrophage cytokine secretion, reactive-oxygen species production and fungal killing *in vitro* after infection with *A. fumigatus* (Sun et al.,

2014). Dectin-2 is also important for the development of adaptive immunity with Dectin-2^{-/-} mice models of invasive candidiasis showing impaired Th17 responses (Robinson et al., 2009).

Mannose receptor (CD206) is highly expressed on AM and has 8 CRDs allowing recognition of high mannose, fucose, N-acetylglucosamine sulphated glycan structures on many microorganisms including the fungal cell wall of *A. fumigatus* (Stephenson and Shepherd, 1987). It is an ITAM/ITIM independent CLR with a tyrosine-based motif in its intracellular tail that promotes the delivery of mannosylated ligands to early endosomes (Osorio and Sousa, 2011). Its signalling capacity has not been determined but is inferred as CD206 deficient macrophages have impaired cytokine production following lipopolysaccharide (LPS) stimulation (Chavele et al., 2010). Although CD206 has been shown to mediate uptake of *Pneumocystis* in human AM, when expressed in non-professional phagocytes it is unable to mediate uptake of particulate ligands suggesting it may instead facilitate uptake by other receptors (Le Cabec et al., 2005, Ezekowitz et al., 1991).

Other CLRs expressed by macrophages which have been shown to be important for fungal immunity include DC-SIGN and Mincle (Brown, 2011). Mincle is another FcRγ-coupled receptor with Mincle^{-/-} mice showing increased susceptibility to *C. albicans* (Wells et al., 2008). DC-SIGN is predominantly expressed by human dendritic cells and at low levels by alveolar macrophages (Geijtenbeek et al., 2002). It has a single CRD, which binds to mannose branched structures and enables multimerization of the receptor, and a cytoplasmic tail containing internalization motifs. It is able to activate noncanonical NF-κB signalling via Raf-1 and is also thought to play a role in particulate uptake and endocytosis (Gringhuis and Geijtenbeek, 2010).

Toll-like receptor family

It was the discovery of the Toll-like receptor (TLR) family in the mid 1990's that developed understanding of the role of PRRs in the innate immune response (O'Neill et al., 2013). Currently, 11 human TLRs have been identified which are expressed in distinct cell subsets and tissues. They

recognise a diverse range of microbial PAMPs from nucleic acids to complex proteins. Ligation of TLRs to their respective PAMP triggers MyD88 dependent and independent signalling pathways that activates NF κ B, and eventually induces release of proinflammatory cytokines or secondary signals (Akira and Takeda, 2004). Fungal PAMPs are recognised by a number of TLRs including TLR1/2, TLR3, TLR4, TLR2/6, TLR7 and TLR9 (Bourgeois and Kuchler, 2012). The importance is clear with mice deficient in the adaptor protein Myd88 being highly susceptible to *C. albicans* and *A. fumigatus* infections (Bretz et al., 2008, Villamon et al., 2004). As the TLRs tend to act in collaboration the role of individual TLRs is less clear with single TLR receptor knockout mice not showing obvious fungal susceptibility (Mambula et al., 2002). In humans, however, several polymorphisms have been linked to increased fungal susceptibility. These include TLR4 polymorphism (Asp229Gly) leading to increased risk of invasive and chronic pulmonary aspergillosis, TLR9 polymorphism (T-1237C) associated with an increased risk of allergic bronchopulmonary aspergillosis and TLR1 polymorphism (Arg80Thr) and a combination of TLR1 (Asn248Ser) and TLR6 polymorphism (Ser249 Pro) associated with invasive aspergillosis in allogeneic stem-cell transplant patients (Pamer, 2008).

Due to collaboration between receptors, identifying specific TLR *A. fumigatus* PAMPs has been difficult. However, it has been shown that TLR2 recognises fungal β -glucan alongside as-yet unidentified ligands on *A. fumigatus* conidia and hyphae (Chai et al., 2011). *A. fumigatus* conidia activate TLR2/1 heterodimers and TLR4, with recruitment of TLR9 to fungal containing phagosomes and recognition of *A. fumigatus* gDNA at unmethylated CpG motifs (Kasperkovitz et al., 2010, Meier et al., 2003a). TLR3 is additionally activated by dsRNA from *A. fumigatus* conidia in lung epithelial cells (Beisswenger et al., 2012). The TLR expression profile of human AM differs from that of systemic monocytes suggesting a contribution to compartmentalised immune responses in the lung (Juarez et al., 2010).

The role of the calcineurin-NFAT pathway in TLR signalling after fungal infection is unclear. In a non-infection setting studies have suggested that calcineurin negatively regulates TLR-mediated

activation pathways by inhibiting Myd88 and TRIF adaptor proteins leading to increased activation of NF κ B and MAPK pathways (Kang et al., 2007). Further studies using NFAT peptide inhibitors and NFATc4^{-/-} mice have suggested that nuclear NFAT is directly required for TLR-activated innate immune response to LPS in macrophages (Minematsu et al., 2011).

CD14 receptor exists in a membrane and soluble form and has recently been shown to act as a co-receptor with TLR4 following LPS stimulation. Membrane localised CD14 has been shown to be required for microbe-induced endocytosis of TLR4 through tyrosine kinase Syk and phospholipase C- γ 2 (PLC) (Zanoni et al., 2011). In murine dendritic cells, stimulation with LPS has been shown to induce Src kinase and PLC γ 2 activation leading to Ca²⁺ mobilisation and calcineurin-dependent NFAT translocation independently of TLR4 (Zanoni et al., 2009b). Further studies suggest that CD14 and TLR4 signalling may additionally be involved in signalling in human monocytes stimulated with *A. fumigatus* hyphae suggesting a possible role in the calcineurin-dependent fungal innate immune response (Wang et al., 2001).

NOD-like receptors

Nucleotide oligomerization domain (NOD)-like receptors (NLRs) are a specialised group of intracellular proteins that play a critical role in the regulation of the host innate immune system (Chen et al., 2009). Due to the lack of signal peptides and transmembrane domains, NLRs are thought to remain localised in the cytosol where they act as scaffolding proteins that assemble signalling platforms to trigger NF- κ B and MAPK signalling pathways and control the activation of inflammatory caspases (Kufer and Sansonetti, 2011). There are 23 NLR family members in humans, with certain NLRs like NOD1 and NOD2 having high expression in human AM with ligation by microbial PAMPs resulting in pro-inflammatory cytokine expression and activation of autophagy (Kufer and Sansonetti, 2011, Juarez et al., 2012, Juarez et al., 2014). A role for NOD signalling in fungal innate immunity is suggested by up-regulation of NOD2 in mice infected with *A. fumigatus* with studies in mice macrophage cell-lines also implicating NOD2 in the innate immune response to

A. fumigatus (Li et al., 2012b, Zhang et al., 2008). Increased expression of NOD1 is also seen in human corneal epithelial cells infected with *A. fumigatus* with knockdown decreasing NFκB signalling and pro-inflammatory cytokine secretion (Zhang et al., 2014). Recent work has also identified NOD2 as an essential fungal chitin-recognition receptor with ligation critical in innate immune homeostasis and regulation via Il-10 production (Wagener et al., 2014). The calcineurin-NFAT pathway is implicated in NOD signalling, with calcineurin inhibition and NFATc1 knockdown in experimental murine models of bacterial acute pyelonephritis resulting in increased susceptibility, impaired phagocytosis, neutrophil migration and reduced NOD1 macrophage expression (Tourneur et al., 2013).

Soluble receptors

There are a variety of soluble *A. fumigatus* receptors that are thought to play a significant role in the innate immune response (Underhill and Lliev, 2014). Pulmonary collectins are a family of C-type lectins that include lung surfactant proteins A and D and mannan-binding lectin (MBL). They act as opsonins and are able to bind *A. fumigatus* through carbohydrate recognition domains in a calcium dependent manner (Madan et al., 1997a, van Asbeck et al., 2008). Binding of *A. fumigatus* to lung surfactant proteins A and D has been shown to enhance binding, phagocytosis, activation and killing by human AM and circulating neutrophils *in vitro* (Madan et al., 1997b). In addition, mice deficient in surfactant proteins A and D have increased susceptibility to *A. fumigatus* allergen induced pulmonary hypersensitivity suggesting a role in co-ordinating adaptive immune responses (Madan et al., 2005). MBLs have been shown to play a protective role in murine models of invasive aspergillosis treated with recombinant human MBL through increased oxidative neutrophils bursts and activation of the lectin complement pathway (Kaur et al., 2007).

Pentraxin-3 (PTX3) is another soluble receptor, and forms part of the family of long pentraxins and has been shown to be rapidly produced by mononuclear phagocytes and dendritic cells following infection with *A. fumigatus* (Mantovani et al., 2008, Salvatori and Campo, 2012). Mice deficient in

PTX3 have been shown to have defective phagocytosis of *A. fumigatus* conidia and increased susceptibility to invasive pulmonary aspergillosis with a protective effect of treatment with recombinant PTX3 (Garlanda et al., 2002, Lo Giudice et al., 2010). Its role in part is thought to be due to facilitation of pathogen recognition enabling increased phagocytosis, cytokine production and killing and additionally direct activation and modulation of the classical complement pathway cascade (Moalli et al., 2010b, Salvatori and Campo, 2012).

1.6.3 Intracellular fate of *A. fumigatus*

Following recognition of *A. fumigatus* conidia by PRRs, human macrophages are able to uptake conidia through an actin, dynamin and phosphoinositol-3 and tyrosine kinase dependent process with formation of pseudopodia and filopodia surrounding the conidia (Luther et al., 2008, Ibrahim-Granet et al., 2003, Kasperkovitz et al., 2010). Further characterisation by electron microscopy has shown strong local actin re-organisation without significant microtubular re-organisation and the presence of large ruffle-like structures resembling macropinocytosis (Luther et al., 2008).

Following internalisation, *A. fumigatus* conidia are contained within specialised phagosomal compartment that is tightly associated with the conidium surface (Ibrahim-Granet et al., 2003). The phagosome rapidly acquire specific markers of the endocytic pathway with recruitment of the Rab GTPases; initially Rab5 at the early endosomal stage and then Rab7 in the late endosome. There is rapid maturation into lysosomal-like compartments with Lamp-1 recruitment. Acidification of the phagosome during the maturation process occurs with accumulation of vacuolar ATPases and is necessary for effective fungal killing (Slesiona et al., 2012, Ibrahim-Granet et al., 2003). *A. fumigatus* conidia however are able to inhibit phagolysosomal acidification through production of the conidial pigment DHN-melanin (Thywissen et al., 2011).

Once within the phagosome, a number of oxidative and non-oxidative mechanisms are utilised to kill fungi (Brown, 2011). Fungal killing by human macrophages is dependent on production of reactive

oxygen intermediates (known as the respiratory burst), with AM from *p47phox*^{-/-} mice showing defective killing (Philippe et al., 2003). This is consistent with the increased susceptibility to invasive aspergillosis in chronic granulomatous disease where there is an inherited disorder of the NADPH oxidase complex with granulocytes defective in generating superoxide anion and its metabolites (Segal and Romani, 2009). The role of reactive nitrogen intermediates in fungal killing by human macrophages has not been fully established with conflicting evidence in studies to date (Fang, 2004). Non-oxidative mechanisms of killing include production of serine proteases such as Cathepsins which have been shown to be important for fungal immunity (Mittal et al., 2013). Nutrient depletion via iron and zinc sequestration by lactoferrin and calprotectin has additionally been shown to be an effective antifungal killing mechanism (Seifert et al., 2008, Zarembek et al., 2007, Vicente-Franqueira et al., 2015).

1.6.4 Cross-talk with the adaptive immune response to *A. fumigatus*

There is increasing evidence to support the role of early innate responses in the subsequent development of critical adaptive T-cell responses with development of T helper type 1 (Th1) immunity protective to subsequent lethal challenge in murine models (Bozza et al., 2003). This is corroborated by the efficacy of exogenous interferon-gamma (IFN γ) immunotherapy in patients with invasive fungal infection and over-expression of IFN γ in murine models of invasive aspergillosis (Armstrong-James et al., 2010, Shao et al., 2005). Immunocompetent mice challenged with *A. fumigatus* show a rapid CD4⁺ T cell recruitment with subsequent proliferation in mediastinal lymph nodes and trafficking to the airways (Rivera et al., 2005). Recognition of PAMPs by PRRs on innate antigen-presenting cells has been shown to instruct subsequent adaptive immune responses (Iwasaki and Medzhitov, 2015). In murine studies, infection with *A. fumigatus* induces concurrent Th1 and Th17 responses in a TLR/Myd88 and Dectin-1 dependent fashion. Dectin-1 signalling was found to promote a Th17 response by inhibiting Th1 CD4⁺ T cell differentiation thereby impairing antifungal immune resistance (Rivera et al., 2011, Zelante et al., 2009).

Although antigen presentation by the innate immune system has been thought to be primarily due to dendritic cell activation, recent evidence has implicated activation of alveolar and lung tissue-resident macrophages. Due to their considerable plasticity, they are thought to also be a key determinant in subsequent adaptive immune responses (Balhara and Gounni, 2012, Soroosh et al., 2013).

1.6.5 Calcineurin is required for an effective innate immune response to *A. fumigatus* infection in a mouse model of solid organ transplant invasive aspergillosis

To study the calcineurin-dependent effects on fungal killing in solid organ transplantation, our group has previously developed a murine model of solid organ transplant invasive aspergillosis (Herbst et al., 2013). Briefly, this involved infecting BALB/c mice with 5×10^6 *A. fumigatus* CEA10 conidia after immunosuppression with hydrocortisone and FK506. Fungal killing and clearance was shown to be calcineurin dependent with incremental mortality in mice treated with FK506 and hydrocortisone compared to hydrocortisone alone. Replication in Rag2^{-/-} mice mapped the calcineurin-dependent effect to the innate immune response. *In vivo*, calcineurin inhibition led to impaired cytokine and chemokine responses, delayed neutrophil recruitment and impaired fungal killing at 48h post infection. Despite early macrophage recruitment and almost exclusive *A. fumigatus* uptake by macrophages *in vivo*, no demonstrable defect in fungal killing could be found in bone marrow-derived murine macrophages *in vitro*. However, *A. fumigatus* was found to strongly activate NFAT in murine macrophages *in vitro* in a novel TLR9-BTK dependent fashion, with chemokine production the predominant calcineurin-dependent innate immune response (Herbst et al., 2013, Herbst et al., 2015).

On the basis of this murine work alongside the finding that AM were the primary immune cell interacting with conidia at early time points after infection, this thesis analyses the effects of FK506 on human AM inflammatory responses to *A. fumigatus in vitro*.

1.7 Aims and Hypothesis

1.7.1 Aim

The aim of this thesis is to define the calcineurin-dependent innate immune response of human AM to *A. fumigatus*. This will be achieved by performing a systematic analysis of the innate immune response (including phagocytosis, phagosomal maturation, cytokine release and fungal killing) of human macrophages to *A. fumigatus* in vitro. Further mechanistic work will subsequently be performed on calcineurin-dependent innate immune responses.

1.7.2 Hypothesis

The hypothesis is that the use of calcineurin inhibitors results in increased susceptibility to invasive aspergillosis in solid-organ transplantation due to impairment of calcineurin-dependent fungal killing by AM.

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 alveolar macrophages

A total of 240 ml saline bronchoalveolar lavage (BAL) was performed by flexible bronchoscopy and return transferred on ice for processing. BAL fluid was passed through a 100 µm cell strainer (BD Bioscience, UK) and centrifuged at 400 g for 10 min to pellet cells which were subsequently washed in cold PBS x2 and resuspended in pre-warmed RPMI 1640 (Invitrogen). Red cells if present were lysed using a red cell lysis buffer (Sigma, UK). AM were plated at the desired concentration and adhered for 1 h. Non-adherent cells were removed by washing x2 with warm RPMI. These purified AM were then further rested for 3 days in RPMI 1640 with 10% fetal calf serum (Sigma, UK) and 1% penicillin-streptomycin (Invitrogen) to ensure any residual calcineurin inhibitor/drug effect was removed.

2.3 Isolation of human monocytes and differentiation into monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from 60 ml of 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) and centrifuging at 450 g for 45 min. PBMCs were washed at 200 g in cold PBS x2 to remove platelets and monocytes subsequently purified by negative magnetic bead selection using a pan-monocyte isolation kit (Miltenyi Biotech, Auburn, CA). To obtain monocyte-derived macrophages (MDM), freshly isolated monocytes were cultured with RPMI 1640 medium supplemented with 10% human serum (Sigma, UK) and 5 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) (Peprotech, UK) for 7 days at 37°C.

2.4 *A. fumigatus* strains and culture

A. fumigatus AFCEA10 (FGSC A1163) was obtained from the Fungal Genetics Stock Center and was used for Western blot, Luminex, fungal burden, Imagestream, electron microscopy, phosphoproteomic and RNA sequencing experiments (McCluskey et al., 2010). An eGFP-expressing strain (ATCC46645-eGFP, a gift from Frank Ebel) was used for all microscopy experiments (Meier et al., 2003b). All strains were cultured on Sabouraud dextrose agar (Oxoid) for 3-5 d at 30°C. Conidia were harvested in 0.1% Tween/H₂O and filtered through MIRACLOTH (Calbiochem, UK). Conidial suspensions were washed twice in phosphate-buffered saline (PBS), centrifuged at 5000 g and resuspended in RPMI (Sigma) at concentrations shown. To generate swollen conidia (SC), resting conidia (RC) were suspended in RPMI at 1×10^6 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 0.1 M ammonium chloride for 10 min followed by further washes in PBS x4 with centrifugation at 5000 g.

2.5 Flow cytometry

2.5.1 Cell viability assay – (Zombie-Aqua staining)

Macrophages were harvested from tissue-culture plates by incubating in ice-cold PBS (Ca²⁺/Mg²⁺ free)/5 mM EDTA for 10 minutes following by repeat pipetting and 250 g centrifugation for 5 min.

Cells were re-suspended at 1×10^6 / ml in PBS containing a 1 in 1000 dilution of Zombie Aqua Fixable Viability kit (Biolegend, UK) and incubated at room temperature in the dark for 30 min. Cells were subsequently washed in PBS / 10% fetal calf-serum (FCS) (with centrifugation at 250 g for 5 min) and fixed in 0.2% paraformaldehyde (PFA) (if no *A. fumigatus* present) or 2% PFA (if *A. fumigatus* conidia present) for 10 min following by washing with PBS x1 and acquired on a Fortessa flow cytometer. Results were analysed using Flow Jo (Oregon, US).

2.5.2 Macrophage purity, viability and phenotyping

AM were harvested at D3 after isolation and MDM at D7 after differentiation as above and stained with Zombie-aqua cell viability stain as per above. Prior to fixation, cells were blocked on ice for 20 min with human FcR block (1 in 100 dilution in PBS / 0.1% BSA, BD Biosciences) and subsequently stained with antibodies against CD11b, CD11c, CD206, CD86, HLA-DR, TLR4, TLR2 and Dectin-1 for 30 min at RT in the dark (see table below). All antibodies were from Biolegend, UK. Cells were washed with PBS / 0.1% BSA x1, fixed as above and then acquired on a Fortessa flow cytometer. Results were analysed using Flow Jo (Oregon, US).

Antibody description	Manufacturer	Clone	Staining concentration µg/ml
anti-CD11b-PerCP/Cy5.5	Biolegend	ICRF44	4
anti-CD11c-PE/Cy7	Biolegend	3.9	4
anti-CD206-AF647	Biolegend	15-2	3
anti-CD86-Brilliant violet 605	Biolegend	IT2.2	0.7
anti-HLA-DR-APC/Cy7	Biolegend	L234	2
anti-Dectin-1-PE	Biolegend	15E.2	10
anti-TLR2-FITC	Biolegend	TL2.1	25
anti-TLR4-Brilliant violet 421	Biolegend	HTA125	10
Anti- CD45-APC-eFluor 780	eBioscience	2D1	5

Anti- CD209 (DC-SIGN)- PerCP/Cy5.5	Biolegend	9E9A8	2
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Human alveolar macrophage gating strategy is shown in Figure 2. Fluorescence minus one (FMO) controls were used to determine gating strategy.

For analysis of macrophage cell death, MDM were harvested at D7 either pre-infection or post infection with CEA10 *A. fumigatus* swollen conidia (MOI=1) and stained with Annexin V BV421 (Biolegend) according to manufacturer's instruction and with 1.25 µg/ml Propidium iodide (Invitrogen), and acquired on a Fortessa flow cytometer. Results were analysed using Flow Jo (Oregon, US). Gating was based on fluorescence minus one controls and a positive control using Triton x 100 (0.001%).

2.5.3 Quantification of macrophage mitochondrial reactive oxygen species production

MDM were plated at 5×10^5 / well in 24-well plates and infected with live *A. fumigatus* SC (MOI=1) following pre-loading with Mitotracker Orange CM-H2TMRos (Invitrogen) according to the manufacturer's instructions and pre-treatment with FK506 (10 ng/ml), or vehicle, for 1 h. Cells were harvested at the required time-points using ice-cold PBS/5 mM EDTA and fluorescence quantified by flow cytometry (Fortessa). Results were analysed using Flow Jo (Oregon, US).

2.6 Enzyme-linked immunoabsorbent assay (ELISA) and Luminex

MDM and AM were plated at 1×10^5 per well in a 96-well plate and pre-treated with FK506 (10 ng/ml, Calbiochem), VIVIT (1 µM), SC-514 (10 µM), human Dectin-1 blocking antibody (3 µg/ml, R&D Systems), Piceatannol (20 µM, Cayman Chemical) or vehicle for 1 h. AM were infected with live AF SC (MOI=0.1) for 24hrs and MDM with live *A. fumigatus* SC (MOI=1) for 6h. TNF-α production was assayed using a human TNF-α ELISA kit (R&D Systems) according to manufacturer's instructions. Luminex analysis of culture supernatants from MDM infection experiments was performed using the

Milliplex human cytokine/chemokine magnetic bead panel kit from Merck Millipore. Supernatants were assayed for TNF- α , GM-CSF, MCP-1, MIP-1 α and MIP-1 β .

2.7 Determination of in vitro fungal burden using quantitative real-time PCR

AM and human MDM were stimulated with live *A. fumigatus* SC (MOI=1) with FK506 or vehicle pre-treatment for 1 h. A total of 5×10^5 macrophages were lysed in Tri Reagent (Sigma-Aldrich) 1 and 6 h after stimulation. Chloroform was added at 2/5 of the starting volume and the sample vortexed and centrifuged at 8000 g for 10 min at 4°C. The aqueous phase was transferred to a fresh tube and an equal volume of isopropanol (VWR) was added. After mixing, the sample was centrifuged at 8000 g for 15 min at 4°C. The pellet was washed twice in ice-cold 70% ethanol and resuspended in RNase-free H₂O (Ambion). RNA was reverse-transcribed using the QuantiTect kit according to the manufacturer's instruction (Qiagen). Fungal burden was analysed by qPCR analysis for 18S ribosomal RNA genes of *A. fumigatus*. Primers RV: CAGGCTGGCCGCATTG FW: GCACGTGAAATTGTTGAAAGG bind to a highly conserved region of the 18S rRNA gene. A biotinylated oligonucleotide probe [6FAM]AGCCAGCGGCCCGCAAATG[TAM] was chosen that binds specifically to *A. fumigatus*. Primers were used at a final concentration of 250 nM and reverse transcription carried out with JumpStart Taq Polymerase (Sigma-Aldrich). Known concentration of purified genomic DNA from *A. fumigatus* was used to generate a standard curve. The reaction parameters were as below. The amount of *A. fumigatus* DNA was determined comparing the amplification threshold cycle of the unknown sample with the threshold cycle values of the standard.

Activation	95°C	15 min	50 cycles
Denaturing	95°C	5 sec	
Annealing/Extension/Acquisition	60°C	30 sec	

2.8 Quantification of *A. fumigatus* phagocytosis using Imagestream

5×10^5 MDM pre-treated with FK506 (10 ng/ml) and/or Cytochalasin D (5 μ M, Sigma) for 1 h were stimulated with live *A. fumigatus* SC (MOI=1) pre-stained with Calcofluor White (Sigma). Infections were synchronised by incubating cells at 4°C for 30 min pre and post-infection before starting the experiment by incubation at 37°C. At specified time-points, macrophages were washed twice with warm PBS to remove non-internalised *A. fumigatus* conidia. Cells were harvested by incubation with ice-cold PBS and repeat pipetting and centrifugation at 400 g for 10 min. Cells were stained with nuclear stain DRAQ5 (Cell signalling) and fixed with 2% PFA. Fixed cells were run on ImageStreamX (Amnis) and data analysed using IDEAS software. Single cells with in-focus nuclei were chosen for analysis and percentage of macrophages with internalised conidial quantified according to Calcofluor White fluorescence within the cell. (*Imagestream analysis performed in collaboration with Shichina Kannambath, postdoc in Darius Armstrong-James laboratory*).

2.9 Western blotting and preparation of tissue culture cell extracts

For whole-cell lysates, cells were lysed in 150 mM NaCl, 50 mM Tris (pH 8), 1% Triton X-100 containing proteinase/phosphatase inhibitor cocktail (Cell Signalling). Cytoplasmic and nuclear extracts were prepared using the NE-PER extraction reagents (Thermo Scientific) according to the manufacturer's instructions. Protein concentration was determined using a BCA assay kit (Thermo Scientific). Protein samples were denatured by the addition of x5 Protein loading buffer (National Diagnostics) and heating to 95°C for 5 min. 2-5 μ g of protein was loaded per lane and separated by 4–15% Bis-Tris gels (BioRad), and proteins transferred to PVDF membranes activated with methanol. Membranes were blocked in 5% milk power/TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 in H₂O) for 1 h at room temperature. Membranes were probed with primary antibodies overnight at 4°C followed by x3 washes in TBS-T, before the addition of a horse-radish peroxidase-linked secondary antibody for 1 h at room temperature. The membrane was washed a further three

times and proteins were detected using LumiGLO substrate (Cell Signalling). Primary antibodies used are listed below. Staining concentrations are provided or dilution of stock if concentration not available from supplier. Antibodies were diluted in 5% milk powder in TBS-T apart from analysis of phosphoproteins where 5% bovine serum albumen in TBS-TSA was substituted for all blocking and incubation steps.

Primary antibodies:

Antibody	Clone and manufacturer	Staining concentration or dilution of stock concentration
anti-NFATc2	sc-7296 , Santacruz	400 ng/ml
anti-NF- κ B p65	C22B4, Cell Signalling	1:1000
anti-HDAC1	10E2, Cell Signalling	1:1000
anti-Histone H3	D1H2, Cell Signalling	1:1000
anti- β -actin	8H10D10, Cell Signalling	1:4000
anti-DSCR1	D6694, Sigma	1.5 μ g/ml
anti-VASP	9A2, Cell Signalling	1:1000
anti-phospho-VASP (Ser239)	3114, Cell Signalling	1:1000
anti-SAPK/JNK	9252, Cell Signalling	1:1000
anti-phospho-SAPK/JNK (Thr183/Tyr185)	81E11, Cell Signalling	1:1000
anti-p38 MAPK	D13E1, Cell Signalling	1:1000
anti-phospho-p38 MAPK (Thr180/Tyr182)	D3F9, Cell Signalling	1:1000
anti-DRP1	D6C7, Cell Signalling	1:1000
anti-phospho-DRP1 (Ser637)	GTX5091, Genetex	1 μ g/ml

2.10 Confocal microscopy

For confocal 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 NH_4Cl 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 permeabilisation buffer (see below). Staining concentration are provided where possible or dilution of stock. After washing with PBS three times, cells were incubated with an anti-rabbit AF555 (Life Technologies), anti-rabbit Cy5 or anti-mouse Cy5 antibody with or without Phalloidin AF555 or AF643 (Life Technologies) for 45 min at room temperature in the dark and mounted with Vectashield mounting medium containing DAPI (Vector laboratories) and sealed with nail varnish to prevent evaporation. For imaging of Clathrin and Dynamin cells were fixed with 100% methanol at -20C for 5min followed by x3 washes in PBS instead of PFA. Immunostaining was carried out subsequently as above.

Images were captured on a Zeiss LSM-510 inverted confocal microscope. Image analysis was performed using Fiji Life-line version, 2015 (*NIH*) with 3D reconstructions performed using Volocity 6.3 (*PerkinElmer*) (Schindelin et al., 2012). Nuclear co-localisation analysis was performed using JACoP (Just Another Co-localization Plugin) with Manders' overlap coefficient used to quantify nuclear co-localisation (Bolte and Cordelières, 2006). The coefficient varies from 0 to 1, with 1 reflecting 100% co-localisation (Manders et al., 1992). To quantify fluorescence association with *A. fumigatus* phagosomes, phagosomes were selected based on *eGFP*-expressing *A. fumigatus* fluorescence. This region of interest was expanded x1 using the 'dilate' tool in Fiji. Fluorescent association was then quantified by using the command "Analyse → Measure". Parameters for measurement set were "Area" and "Integrated Density" as described previously (Pei et al., 2014).

Primary antibodies:

Antibody	Clone and manufacturer	Staining concentration or dilution of stock
anti-clathrin heavy chain	ab21679, Abcam	10 µg/ml
anti-dynamin 2	ab3457, Abcam	10 µg/ml
anti-LAMP-1	H4A3, Biolegend	10 µg/ml
anti-NFATc1	7A6, BD Biosciences	10 µg/ml
anti-NFκB p65	C20, Santa Cruz	2 µg/ml

anti-Rab5	C8BA, Cell Signalling	1:50
anti-Rab7	D95F2, Cell Signalling	1:50
anti-VASP	9A2, Cell Signalling	1:50

2.11 Phagosomal maturation assays

2.11.1 LysoTracker Red phagosomal acidification assay

MDM were plated at 1×10^5 cells / well in 8-well μ -slides (Ibidi) and pre-loaded with 1 μ M LysoTracker Red DND-99 for 2 h and either FK506 (10 ng/ml, Calbiochem) or vehicle for 1 h. LysoTracker Red DND-99 was then replaced by Live Cell Imaging Solution (Invitrogen), including FK506 or vehicle as above and cells infected with live e-GFP expressing AF SC Af (MOI=1). Infections were synchronised by cooling to 4°C for 20 min before and after infection with the experiment commencing by transferring to a humidified imaging incubation chamber at 37°C. Images were acquired at 30 min intervals to avoid photobleaching using a Leica SP5 inverted confocal microscope. Colocalisation of phagosomal acidification was assessed by quantifying the integrated density around phagosomes containing single conidia using ImageJ as explained above.

2.11.2 Magic Red® activated Cathepsin B assay

Imaging of activated Cathepsin B was performed on live cells using a Magic Red Cathepsin B staining kit (Immunochemistry Technologies) as per manufacturer's instructions. Briefly human MDM plated at 1×10^5 cells/well in 8-well μ -slides (Ibidi) were infected with live e-GFP expressing Af SC (MOI=1), following pre-treatment for 1 h with FK506 (10 ng/ml, Calbiochem) or vehicle. Following infection, Magic Red® Cathepsin B substrate was added to cells and nuclei stained with Hoescht 33342 cell permeable nuclear stain. Infections were synchronised by cooling to 4°C for 20 min before and after infection with the experiment commencing by transferring to a humidified imaging incubation chamber at 37°C. Images were acquired at 30 min intervals to avoid photobleaching using a Leica SP5 inverted confocal microscope. Colocalisation of activated Cathepsin B was assessed by

quantifying the integrated density around phagosomes containing single conidia using ImageJ as explained above.

2.12 Time-lapse live video microscopy

Live time-lapse imaging of macrophage - *A. fumigatus* interaction was performed by plating human MDM at 1×10^5 cells / well in 8-well μ -slides (Ibidi) and stimulating with live e-GFP expressing *A. fumigatus* SC, RC or fixed conidia (MOI=1). Cells were pre-treated for 1 h with FK506 (10 ng/ml, Calbiochem), Bafilomycin (100 nM, Sigma), Z-VAD-FMK (50 μ M, Promega), Necrostatin-1 (10 μ M, Calbiochem), Necrosulphonamide (5 μ M, Calbiochem) or vehicle. Following infection, propidium iodide was added to the media (1.25 μ g/ml, Invitrogen) and lateral transfer events visualised by confocal time-lapse imaging using a Leica SP5 inverted confocal microscope with motorised stage and full incubation chamber. To avoid effects on Af uptake, media was replaced to contain Cytochalasin D (5 μ M, Sigma) and Dynasore (100 μ M, Sigma) 1 h p.i. with un-internalised conidia removed by washing with warm RPMI x3. Cells were maintained at 37°C with 5% CO₂ during imaging and images analysed using ImageJ. A 20x 0.70 numerical aperture air objective was used with cells imaged using brightfield imaging, eGFP-expressing *A. fumigatus* using a Argon 488 laser and PI using a Diode 633 laser. Images were captured at 10 min intervals.

2.13 Phosphoprotein array

The Phospho Explorer Antibody Microarray was conducted by Full Moon BioSystems Inc (California, USA). Human MDMs were differentiated at 1×10^7 cells in 75mm² tissue culture flasks. At day 7, cells were stimulated with live CEA10 *A. fumigatus* SC (MOI=1) following pre-treatment for 1 h with FK506 (10 ng/ml, Calbiochem) or vehicle. At 1 h post stimulation, cells were washed (x5) with 10 ml ice-cold PBS with protease inhibitor (Cell signalling), and collected by gentle scraping and centrifugation (250 g for 10 min at 4°C). Cells were frozen at -80°C and transferred to Full Moon Biosystems on dry ice. The array consists of 1,318 phospho-specific antibodies. In brief, proteins

were labelled with biotin and placed on pre-blocked microarray slides. After washing, detection of total and phosphorylated proteins was conducted using Cy3-conjugated streptavidin. Expression of phosphorylated proteins was normalized to corresponding total protein expression. Fold change was calculated as follows: phosphorylation of FK506-treated cells/phosphorylation of untreated cells.

2.14 Electron microscopy

Human MDMs in 24-well plates were infected with live CEA10 *A. fumigatus* swollen conidia (MOI=1). Un-internalised conidia were removed by washing with warm RPMI x3 at 1 h p.i., and cultures were subsequently fixed in 2.5% glutaraldehyde in 0.05M cacodylate buffer at 15 min intervals from 105 min to 180 min p.i.. Samples were post-fixed in 1.0% osmium tetroxide, dehydrated through a methanol series (70-100%) and embedded in epoxy resin. Regions of interest were selected from 1.0 µm thick toluidine blue-stained resin survey sections. Ultrathin sections (70–80 nm) were contrast-stained with uranyl acetate and lead citrate then examined by transmission electron microscope (JEOL 1400+, Jeol Ltd, UK). Digital images were captured using an AMT 16X camera (Deben, UK). *(Electron microscopy preparation and imaging was performed with the assistance of Amelia Shoemark and Andrew Rogers.)*

2.15 Zebrafish model of invasive aspergillosis

2.15.1 Husbandry

All zebrafish experiments were approved by the United Kingdom Home Office and performed in accordance with the project license PPL 70/7446. Wild-type adult breeders were purchased from the Zebrafish International Resource Center (Eugene, OR). The Tg(UAS-E1b:Eco.NfsB.m Cherry)c24 (referred to as mpeg:mCherry) zebrafish lines have been described elsewhere (Mostowy et al., 2013). Embryos were raised in Petri dishes containing 0.5 × E2 medium supplemented with 0.3 µg/ml of methylene blue. For imaging studies, from 24 h post-fertilization (hpf), 0.003% 1-phenyl-2-

thiourea (Sigma-Aldrich) was added to the medium to prevent melanin synthesis. Embryos were reared at 30°C, and larvae were anaesthetized with 200 lg/ml tricaine (Sigma-Aldrich) during the injection and imaging procedures.

2.15.2 *A. fumigatus* infection and imaging

At 2 days post-fertilization (dpf), zebrafish larvae were transferred into 0.5 × E2. For zebrafish infection studies, 1×10^8 resting e-GFP *A. fumigatus* conidia were stained overnight at 4°C with Alexa-flour 488 5-SDP ester (Life Technologies) at 400 µg/ml in 250µl of 0.1M NaHCO₃ pH 8.3 buffer, washed twice with 0.1%Tween/H₂O followed by PBS (centrifugation at 5000 g) and re-suspended in PBS containing 1% polyvinylpyrrolidone (Sigma, UK). At Day 3, up to 10 µl of pre-stained *A. fumigatus* resting conidia from a stock concentration of 5×10^7 /ml was injected into the hindbrain resulting in an inoculum of 10 to 50 conidia per embryo. Infected larvae were embedded in 1% agarose in 35 mm live-imaging µ-dish (Ibidi) and covered with 0.5 × E2 media. Macrophage recruitment and *A. fumigatus* lateral transfer was imaged with a 20x 0.70 numerical aperture water immersion media objective with live time-lapse imaging at 5 min intervals in a humidified incubation chamber at 30°C using a Leica SP5 resonant inverted confocal microscope. Brightfield imaging was captured, with eGFP-expressing *A. fumigatus* excited using a 488nm Argon laser and mCherry-expressing macrophages with a Krypton 594nm laser. Image analysis was performed using Fiji Life-line version, 2015 (NIH) with 3D reconstructions performed using Volocity 6.3 (PerkinElmer) (Schindelin et al., 2012).

2.16 Statistics

Data are presented as mean ± SEM and were analysed using GraphPad Prism software (version 6.0; GraphPad). Paired non-parametric group data was analysed using a Wilcoxon matched pairs test, unpaired non-parametric data with a Mann-Whitney U test. Multiple comparison tests of non-parametric data was analysed using Kruskal-Wallis or Friedman test for repeated measures with

levels of significance calculated by Dunnett's multiple comparisons post-test. Where mean differences were analysed in groups split on two independent variables a 2-way ANOVA was used with Bonferroni post-test calculation. A $P < 0.05$ was considered statistically significant. (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.)

3 FK506 impairs fungal killing of *A. fumigatus* in human alveolar macrophages

3.1 Isolation and phenotype of human alveolar macrophages

To determine the calcineurin-dependent effects on the AM innate immune response to *A. fumigatus* infection in solid-organ transplantation, initial experiments utilised AM isolated from lung transplant patients at the Royal Brompton and Harefield NHS Foundation Trust. These were patients undergoing bronchoscopy for clinical purpose, usually for the exclusion of lung transplant rejection or infection. AM were isolated from bronchoalveolar lavage and rested for 3 days to remove any residual drug effect and as previously reported enable response to reactivation (Tomlinson et al., 2012).

The purity of AM isolation was assessed by flow cytometry. To accomplish this, AM after isolation by adherence, were removed and stained for surface markers CD45 and CD11c alongside a live-dead stain (Zombie Aqua Fixable Viability kit (Biolegend, UK)). CD45 antigen are proteins belonging to the protein tyrosine phosphatase family which are present on all differentiated haematopoietic cells except erythrocytes and plasma cells (Altin and Sloan, 1997). AM are known to highly express CD11c, a member of the leukointegrin family which has been shown to combine with CD18 to form an integrin that mediates phagocytosis of inactive complement-opsonized particles (Malhotra et al., 1986, Nham, 1999). CD11c is also expressed on dendritic cells and occasionally in activated lymphocytes (Vinay and Kwon, 2010). Cells were gated initially by their forward and size scatter characteristics followed by CD45 and CD11c positivity. Autofluorescence in the FITC channel was subsequently used to differentiate AM from dendritic cells and other lymphocytes (Figure 1). AM isolation was shown to be consistently >95% pure by flow cytometry.

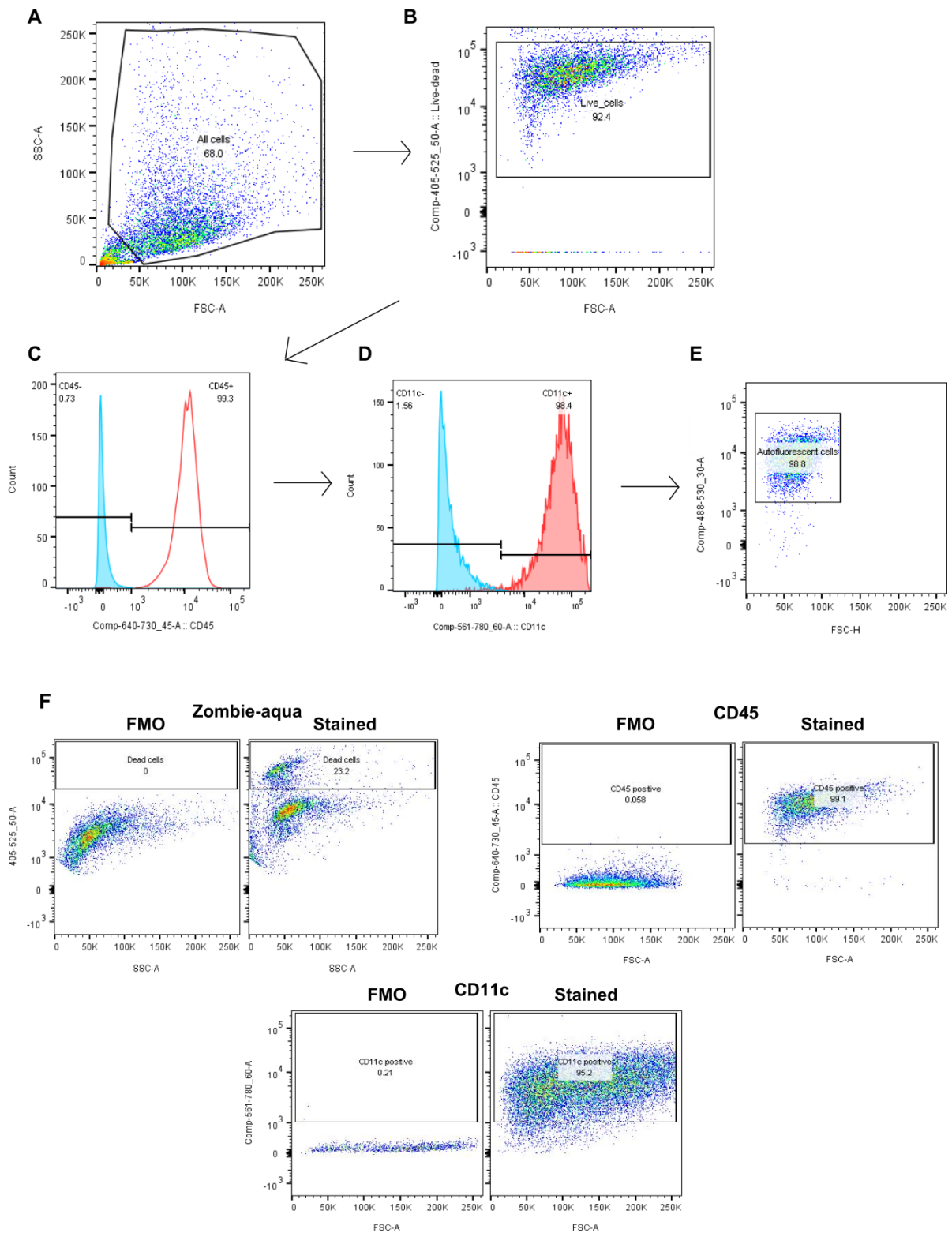


Figure 1: Representative flow cytometry gating strategy for analysis of alveolar macrophages.

A: All cells were gated by flow cytometry based on forward and size scatter. **B:** Live cells were selected for analysis with gating based on a positive control of cells treated with 0.01% Triton x100. Cells had >90% viability. **C:** Cells were subsequently gated on CD45 staining. Unstained cells are shown in blue with cells stained for CD45 in red. >99% of live cells were CD45 +ve. **D:** Cells were

further gated on CD11c staining. Unstained cells are again shown in blue with CD11c stained cells in red. >98% of live CD45 +ve cells were additionally CD11c +ve. **E:** Lastly cells were gated based on autofluorescence in the FITC channel. >98% of live CD45 +ve/CD11c +ve cells were autofluorescent. Cells were found to be consistently >95% purity based on CD45 +ve/CD11c +ve/autofluorescent +ve gating. **F:** Gating strategy based on fluorescence minus one controls shown for Zombie-aqua, CD45 and CD11c stains.

The phenotype of AM was subsequently assessed by flow cytometry. AM were selected by forward and side scatter initially, followed by gating for a CD11c+ve/autofluorescent +ve population as above and further stained for CD11b, CD206, HLA-DR and CD86. CD11b is an integrin which pairs with CD18 to form a heterodimeric transmembrane receptor known as Mac-1/complement receptor 3 (CR3) and contributes to LPS signalling (Ling et al., 2014). It is not expressed on AM but is expressed on interstitial macrophages and inflammatory monocytes which have been shown to be recruited to the lung during acute invasive fungal infection in murine models (Hussell and Bell, 2014, Espinosa et al., 2014). CD206 (mannose receptor) is a carbohydrate-binding C-type lectin receptor which is up-regulated following interleukin (IL)-4 stimulation (Stein et al., 1992, Gordon, 2003). Studies to date have shown varied levels of CD206 expression in healthy AM with up-regulation following inflammation (Kaku et al., 2014, Melgert et al., 2011). Macrophages were further assessed by CD86 expression which is a protein expressed on antigen-presenting cells that provides co-stimulatory signals necessary for T cell activation and survival (Mosser and Edwards, 2008). Surface expression of major histocompatibility complex (MHC) class II molecule HLA-DR, a MHC class II cell surface receptor that regulates peptide antigen presentation by AM was additionally analysed as a marker of macrophage activation (Choi et al., 2015, Rossi et al., 1986). HLA-DR surface expression has previously been shown to be up-regulated in pulmonary diseases (e.g. sarcoidosis, infection, fibrosis), and down-regulated in smokers compared to healthy controls (Popp et al., 1989, Pankow et al., 1995).

Phenotyping of lung transplant AM showed that cells exhibited high CD206 expression alongside high HLA-DR/MHC2 surface expression suggesting an activated state with significant antigen-presentation capacity. CD86 expression was variable but of a lower magnitude. This variability is likely to relate to the inflammatory state of the patient at the time of sampling. CD11b expression was very low, suggesting an absence of macrophage recruitment from inflammatory monocytes or the lung interstitium to the airway (Figure 2).

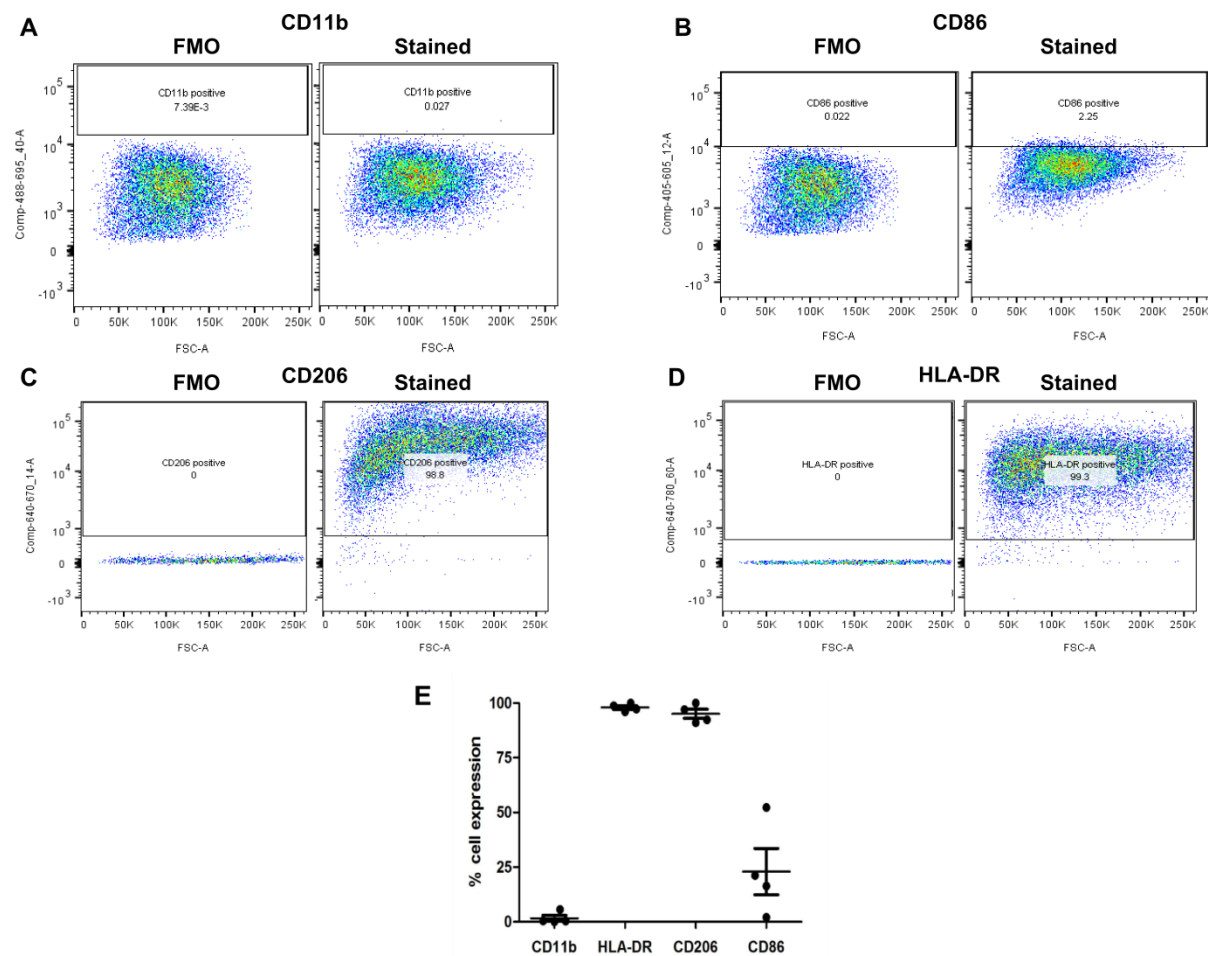


Figure 2: Lung transplant alveolar macrophages have an activated state with high CD206 and MHC2 surface expression.

AM were isolated by adherence from bronchoalveolar lavage samples from lung transplant recipients, rested for 3 days and following harvesting analysed by flow cytometry. AM were selected by forward and size scatter initially followed by gating for live/CD11c+ve/autofluorescent cells (as shown in Figure 1). Cells were further stained for CD11b, CD86, CD206 and HLA-DR. All gates were based on fluorescence minus one (FMO) controls (as shown). Representative images are shown from

flow cytometry analysis. **A:** Low CD11b expression is seen suggesting an absence of inflammatory monocyte recruitment at the time of bronchoscopy. **B:** Low levels of CD86 surface expression were seen in this patient. **C:** High surface expression of CD206 is seen with low CD86 surface expression. **D:** In contrast, however, high HLA-DR/MHC-2 expression is seen suggesting an activated state with high antigen-presentation capacity. **E:** Graph showing cell expression of CD11b, HLA-DR, CD206 and CD86 in AM isolated from 4 lung transplant patients. As seen, surface expression of CD206 and HLA-DR expression is high suggesting an activated state with high antigen-presentation capacity. CD86 expression is variable which is likely related to the inflammatory status of the patient. The absence of CD11b in transplant patients makes the presence of macrophages derived from recruited inflammatory monocytes unlikely; however this may again depend on the status of the patient.

3.2 FK506 inhibits activation of the calcineurin-NFAT pathway in the human alveolar macrophage immune response to swollen *Aspergillus fumigatus* conidia

To determine the calcineurin-dependent innate immune responses in human AM, we initially analysed TNF- α production in human AM infected with resting and swollen *A. fumigatus* conidia as a marker of innate immune activation. Human AM isolated from bronchoalveolar lavage samples in lung transplant patients were plated in 96-well plates and rested for 3 days to remove any residual drug effects and enable response to re-stimulation. Macrophages were subsequently infected overnight with resting and swollen CEA10 *A. fumigatus* conidia (MOI=0.1) and TNF- α production measured in supernatant at 18h by ELISA (Figure 3A). This showed that AM have greater innate immune activation to swollen *A. fumigatus* conidia than resting conidia likely due to the hydrophobic rodlet outer coating in resting conidia. Activation of the calcineurin-NFAT pathway was subsequently analysed by nuclear translocation of NFATc2 in western blot nuclear extracts from human lung transplant AM infected with swollen CEA10 conidia (MOI=1) (Figure 3B). NFATc1, NFATc2 and NFATc3 are calcium-responsive isomers known to be expressed in immune cells (e.g. macrophages). Previous work within our group analysing mRNA NFAT isomer expression revealed predominant expression of NFATc1 and NFATc2 isomers in human AM (work by Shichina Kannambath, postdoc in

Dr Armstrong-James lab, data not shown). Hence, western blot analysis of NFATc2 isomer was performed.

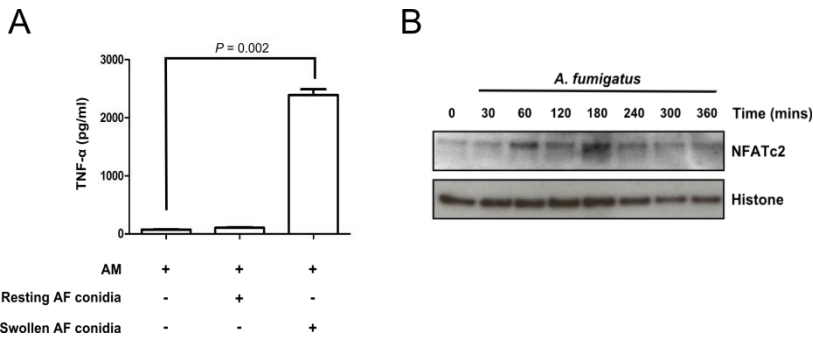


Figure 3: Lung transplant alveolar macrophages activate the calcineurin-NFAT pathway in response to live swollen *A. fumigatus* conidia.

AM were isolated by adherence from lung transplant BAL samples and rested for 3 days. **A:** TNF-α production measured by ELISA in human AM infected at day 4 post isolation with resting and swollen *A. fumigatus* conidia (MOI = 0.1) for 18 h. Significant TNF-α production was seen in response to swollen *A. fumigatus* conidia, in contrast to resting conidia where no TNF-α production was seen. n=5 (n = biological replicates). Bars represent mean + SEM and samples were analysed by Wilcoxon matched pairs test. **B:** Calcineurin-NFAT pathway activation was analysed using a western blot NFAT translocation assay to detect NFATc2 presence in nuclear extracts of human lung transplant AM infected with live *A. fumigatus* swollen conidia (MOI = 1). A representative western blot is shown. Resting AM have little nuclear NFATc2 presence, with translocation in response to live swollen *A. fumigatus* conidia with an early peak at 1 h post infection. Anti-Histone served as a loading control.

The effect of FK506 on lung transplant AM innate immune responses and specifically calcineurin-NFAT activation was further assessed. Cytotoxicity of FK506 was determined by incubating AM with increasing doses of FK506 and determining cytotoxicity using an amine-reactive live-dead flow cytometry assay (Zombie-aqua, Biolegend). A clinically-relevant 'trough-level' dose of 10 ng/ml was not cytotoxic and was subsequently used in further experiments (Figure 4A-C). The effect of FK506 on calcineurin-NFAT transcriptional activity in human lung transplant AM was assessed by analysing mRNA expression of RCAN1.4 (Regulator of Calcineurin 1, Isoform 4), a negative regulator of the calcineurin-NFAT pathway and a NFAT-dependent transcriptional target, by rt-PCR. RCAN1.4 mRNA expression was significantly up-regulated in response to *A. fumigatus* swollen conidia supporting data from the previous NFAT translocation assay by western blot. This effect was almost completely

abolished in the presence of FK506 suggesting effective blockade of the calcineurin-NFAT dependent innate immune response in AM infected with *A. fumigatus* (Figure 4D).

The effect of FK506 on the lung transplant AM innate immune response was further characterised by measuring TNF- α production in culture supernatants of macrophages infected for 18 h with swollen *A. fumigatus* conidia (MOI = 0.1) with 1 h pre-treatment with either FK506 (10 ng/ml) or vehicle. The NFAT and NF κ B-dependent AM innate immune response to *A. fumigatus* swollen conidia was analysed using NFAT and NF κ B specific peptide inhibitors (VIVIT and SC514 respectively) (Figure 4E). Analysing AM from 13 different lung transplant recipients showed that FK506 significantly down-regulated TNF- α production from lung transplant AM infected with swollen *A. fumigatus* conidia. TNF- α production is dependent on NFAT and NF- κ B signalling with down-regulation with VIVIT and SC514 respectively.

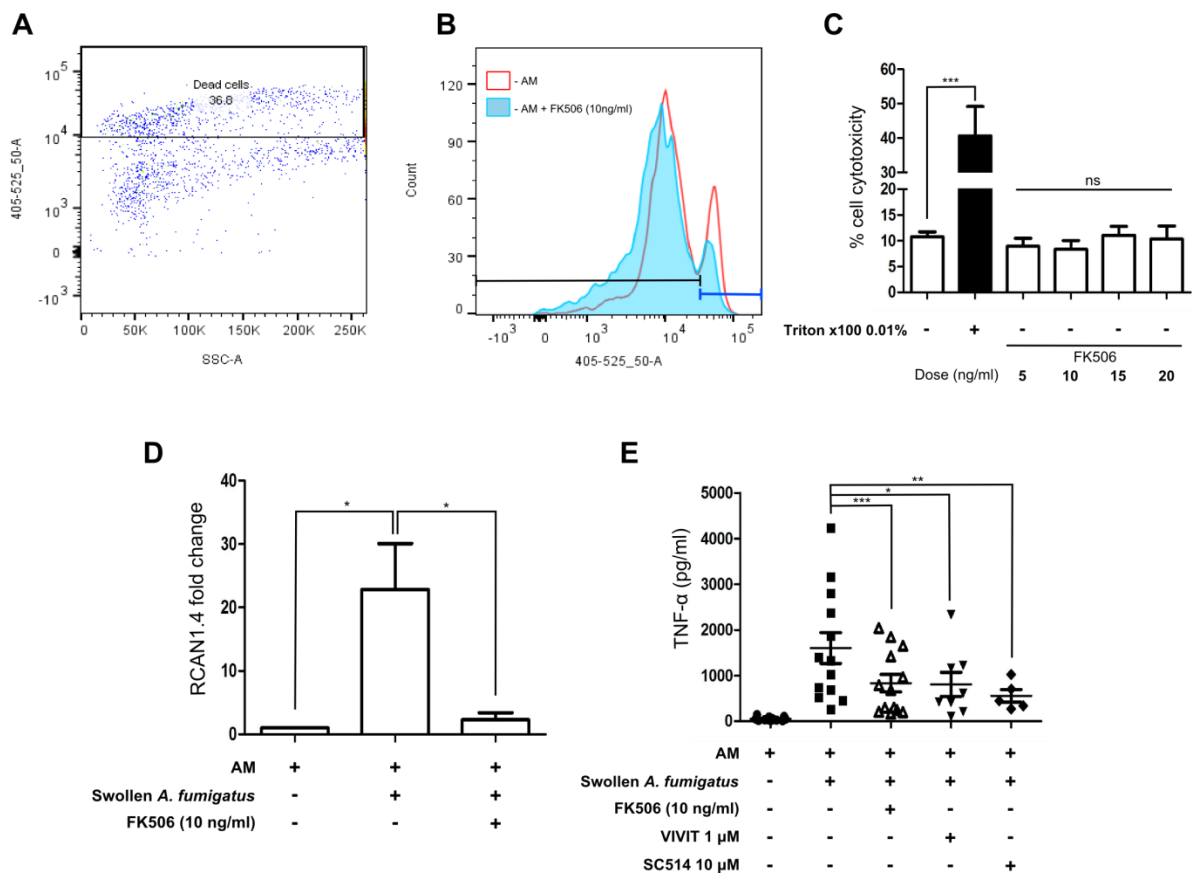


Figure 4: The lung transplant alveolar macrophage innate immune response to *A. fumigatus* is calcineurin-dependent.

Lung transplant AM isolated from BAL and plated in 24-well plates were exposed to increasing concentrations of FK506 for 6 h before harvesting and analysing cytotoxicity using an amine-reactive fixable stain (Zombie-aqua, Biolegend). **A:** Gating for cytotoxicity was based on unstained cells and one positive control (Triton x100 0.01%). A representative flow cytometry analysis of AM treated with Triton x100 is shown. A distinct subset of dead cells can be clearly seen. **B:** Representative histograms of AM treated with FK506 (10 ng/ml) or vehicle is shown. FK506 treated cells are shown in blue with untreated cells outlined in red. Gating for dead cells is shown in blue. There is no appreciable difference in cytotoxicity with FK506 treatment. **C:** Graph representing results of lung transplant AM cytotoxicity with FK506. No significant toxicity was detected at concentrations of 5, 10, 15 and 20 ng/ml FK506 for 6 h compared to untreated cells. (n = 5; n = biological replicates) **D:** Lung transplant AM plated in 24-well plates were infected with live *A. fumigatus* swollen conidia (MOI = 1) following 1 h pre-treatment with FK506 (10 ng/ml) or vehicle. Total RNA was extracted at 6 h p.i. and RCAN1.4 mRNA levels quantified by rt-PCR. Fold change was calculated using β -actin as a house-keeping gene. RCAN1.4 mRNA was significantly up-regulated following infection with swollen *A. fumigatus* conidia implying NFAT transcriptional activity. This was almost completely inhibited in the presence of FK506 suggesting effective blockade of calcineurin-NFAT signalling following *A. fumigatus* infection (n = 5; n = biological replicates). **E:** Lung transplant AM plated in 96-well plates were infected at day 3 with live swollen *A. fumigatus* conidia (MOI = 0.1) following pre-treatment with FK506 (10 ng/ml) (n=13), VIVIT (1 μ M) (n=8), SC514 (10 μ M) (n=5) or vehicle (n = biological replicates). TNF- α production was assayed in culture supernatants at 18 h p.i.. FK506 significantly impaired TNF- α production in lung transplant AM infected with live swollen *A. fumigatus* conidia. TNF- α production was dependent on both NFAT and NF κ B signalling with significant impairment with VIVIT and SC514 respectively. Bars represent mean + SEM and samples were analysed by Wilcoxon matched pairs test; ns = not significant; *p<0.05, **p<0.01, ***p<0.001.

The phenotype of AM has considerable plasticity and is dependent on the local environment (Hussell and Bell, 2014). To ensure that the calcineurin-dependent innate immune responses are applicable in AM from non-lung transplant patients, we compared macrophage phenotype and analysed calcineurin-dependent innate immune responses to *A. fumigatus* in AM from non-lung transplant patients. AM were obtained from 4 patients (1 idiopathic pulmonary fibrosis, 1 sarcoidosis, 1 hypersensitivity pneumonitis and 1 drug-induced interstitial lung disease) and phenotype by flow cytometry was compared. As well as analysing CD11b, CD206, HLA-DR and CD86, surface expression

of innate pattern recognition receptors (PRRs) thought to be important in *A. fumigatus* recognition were also analysed (Dectin-1, DC-SIGN, TLR4 and TLR2) (Figure 5).

Analysis revealed that the phenotype of AM in lung transplantation broadly resembles that from other chronic respiratory diseases with high CD206 expression alongside HLA-DR/MHC2 expression suggestive of an activated status with significant antigen-presentation capacity (Figure 5). As in lung transplantation, CD86 expression was highly variable presumably depending on the inflammatory state of the patient. Population shifts were seen with Dectin-1 and TLR2 staining compared to fluorescence minus one controls. This is likely to represent the varied levels of PRR surface receptor expression seen in this population however isotope controls would be required to completely exclude non-specific staining. Gates were placed to capture high (hi) positive Dectin-1 and TLR2 positive cells. Nevertheless, no significant differences in expression of surface PRRs was seen with highly variable cell surface expression levels in lung transplant recipients and other respiratory diseases (Figure 5). This may reflect patient heterogeneity or again the macrophage activation state. Further larger scale studies would be required however to determine disease related differences in PRR expression.

The effect of calcineurin inhibition in the innate immune response of non-transplant AM was determined by measuring TNF- α production following infection with *A. fumigatus* swollen conidia. In keeping with AM from lung transplant recipients, TNF- α production in response to swollen *A. fumigatus* conidia was calcineurin dependent. This was additionally the case when FK506 was combined with hydrocortisone treatment, replicating the scenario most often encountered in clinical practise (Figure 5F).

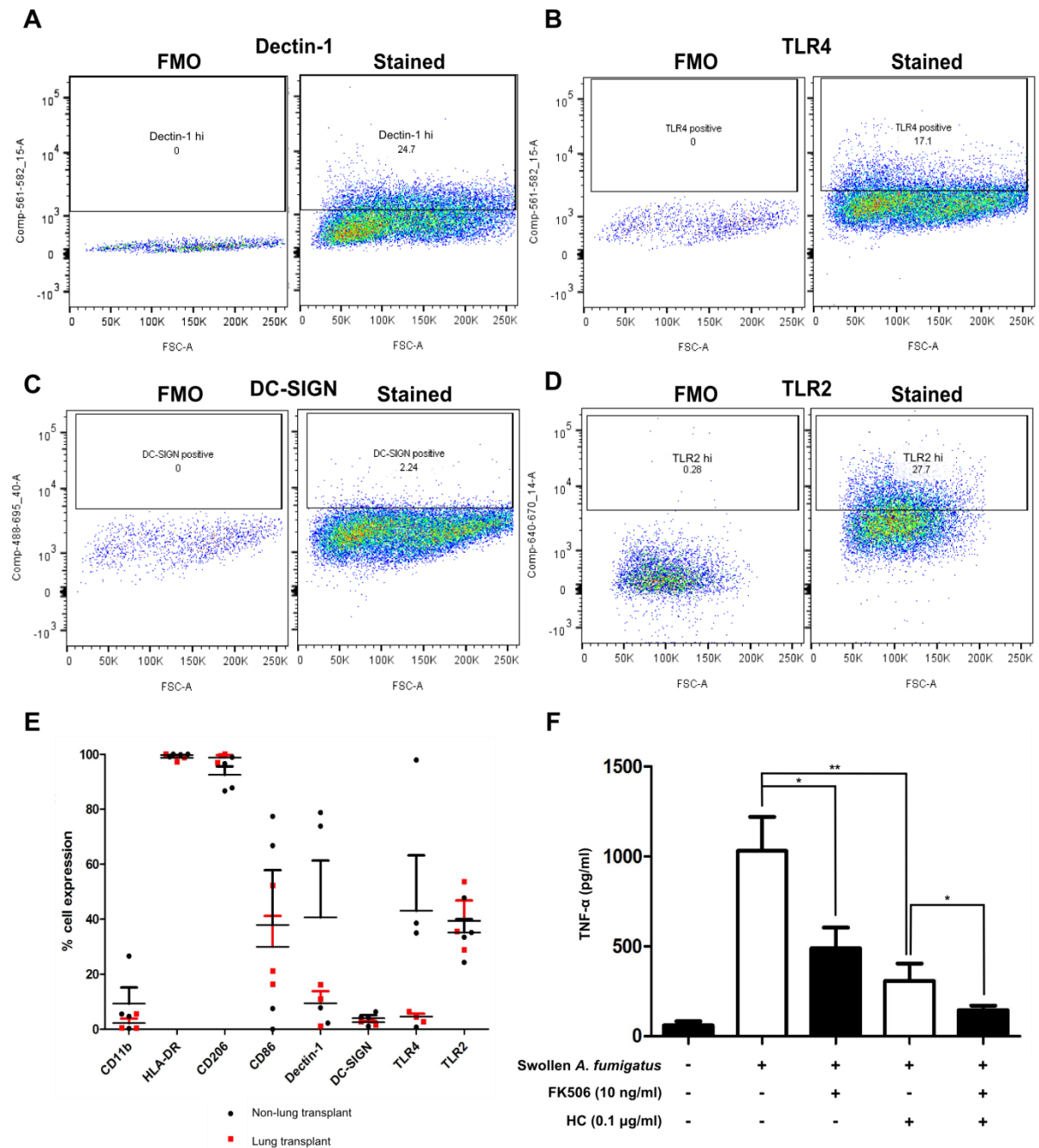


Figure 5: The phenotype and calcineurin-dependent innate immune response to *A. fumigatus* in alveolar macrophages in lung transplantation is similar to chronic respiratory disease.

AM isolated from BAL samples from lung transplant and non-transplant respiratory diseases were stained and analysed by flow cytometry. AM were selected by gating by forward and size scatter initially followed by CD11c +ve/autofluorescence +ve cells as shown in Figure 1. All gating was based on fluorescence minus one control samples as shown. **A:** Representative flow cytometry plot showing Dectin-1 staining. There was a population shift with Dectin-1 staining compared to FMO controls. Gating was based to capture high (hi) Dectin-1 positive cells. **B:** Representative flow cytometry plot showing positive TLR4 staining. **C:** Representative flow cytometry plot showing

positive DC-SIGN staining. **D:** Representative flow cytometry plot showing TLR2 staining. As with Dectin-1 staining there was again a population shift with TLR2 staining. Gating was used to capture high (hi) TLR2 positive cells. **E:** Graph showing flow cytometry phenotype and PRR expression in lung transplant (n=3) and non-transplant (n=4; 1 idiopathic pulmonary fibrosis, 1 sarcoidosis, 1 hypersensitivity pneumonitis, 1 drug-induced pulmonary fibrosis) alveolar macrophages. No significant differences in cell surface expression were seen with a broadly similar phenotype of low CD11b, high CD206 and HLA-DR and variable CD86 expression. PRR expression was additionally highly variable. **F:** AM from non-lung transplant patients were isolated from BAL, plated in 96-well plates and infected at day 4 with live swollen *A. fumigatus* conidia (MOI=0.1) following 1 h pre-treatment with FK506 (10 ng/ml), hydrocortisone (0.1 µg/ml), both or vehicle. TNF-α was subsequently measured in culture supernatant 18 h p.i. by ELISA. FK506 treatment both in isolation and in combination with hydrocortisone significantly impaired TNF-α production in response to live swollen *A. fumigatus* conidia. n=6 (n = biological replicates). Bars represent mean + SEM and samples were analysed by Wilcoxon matched pairs test; *p<0.05, **p<0.01.

3.3 FK506 impairs fungal killing in human alveolar macrophages

Our work to date in murine models has shown that FK506 increased mortality in murine solid-organ transplant models of invasive aspergillosis due to defects in chemokine secretion and neutrophil migration. *In vitro* analysis of bone marrow derived murine macrophages and murine macrophage cell lines have not identified defects in fungal killing. To determine whether the calcineurin-dependent innate immune responses in human AM are required for effective fungal killing and control of germination, fungal burden was analysed by 18S ribosomal RNA rt-PCR in lung transplant AM infected with swollen *A. fumigatus* conidia for 1 and 6 h and pre-treated for 1 h with FK506 (10 ng/ml) or vehicle.

This revealed significantly increased fungal burden in human AM pre-treated with FK506 (10 ng/ml) at 1 and 6 h post infection with live swollen *A. fumigatus* (MOI = 1) suggestive of impaired fungal killing (Figure 6).

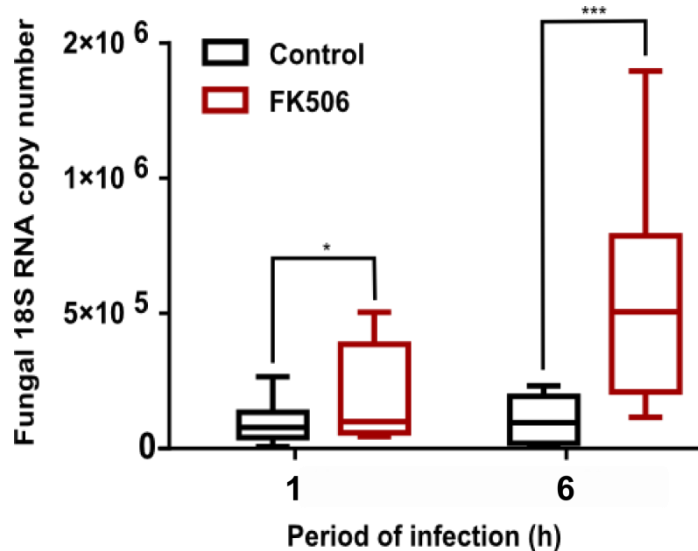


Figure 6: Human alveolar macrophage killing of *A. fumigatus* is calcineurin dependent.

Lung transplant AM were isolated from BAL and infected at day 4 with live swollen *A. fumigatus* conidia (MOI = 1) following pre-treatment for 1 h with FK506 (10 ng/ml) or vehicle. Total RNA was extracted at 1 h and 6 h p.i. and fungal burden quantified by 18S ribosomal RNA rt-PCR. FK506 pre-treatment significantly impaired human AM fungal killing with increased fungal burden 1 h and 6 h p.i..

n=5 (n = biological replicates). Bars represent mean + SEM and samples were analysed by Wilcoxon matched pairs test; *p<0.05, ***p<0.001.

3.4 Discussion

Invasive fungal infections are a major cause of morbidity and mortality in chronic respiratory disease and solid-organ transplantation (Pfaller et al., 2006, Taccone et al., 2015). Neutropenia has traditionally been thought to be the primary innate immunodeficiency leading to risk of invasive fungal infections (Baddley et al., 2001). However, recent epidemiological studies have highlighted a significant population of non-neutropenic patients (such as solid-organ transplant patients) with invasive fungal infections (Baddley et al., 2013, Taccone et al., 2015).

The AM represents the sentinel immune cell required for innate recognition and defence against invasive aspergillosis given the inhalation route of infection (Bhatia et al., 2011). AM have been shown to be critical for murine and human host defence against invasive fungal infections with

increased incidence with murine macrophage depletion, in GM-CSF deficient mice and additionally in alveolar proteinosis patients with increased GM-CSF auto-antibodies (Punatar et al., 2012, Takahashi et al., 2013, Bhatia et al., 2011, Chen et al., 2016). In murine invasive aspergillosis models, recruited inflammatory monocytes also appear to be critical for host defence (Espinosa et al., 2014). My results show that the human AM innate immune response and control of fungal germination is calcineurin dependent.

Initial studies revealed that macrophages isolated from lung transplant BAL were predominantly CD11c⁺/CD11b⁻. This is suggestive of a predominance of AM as opposed to interstitial macrophages or inflammatory monocytes. Further phenotyping suggest high CD206 surface expression which has been shown to be important for innate fungal immunity from murine studies (Bhatia et al., 2011). This was additionally seen in AM from patients with chronic respiratory disease, suggesting a disease and drug-independent phenotype. An over-exuberant inflammatory response to infectious pathogens in the lung is associated with acute lung injury and increased mortality (Johnston et al., 2012). High CD206 surface expression has been associated with inflammation resolution (Herold et al., 2011). Therefore, this phenotype of AM may present an evolutionary conserved state to preserve lung function in response to repetitive inhaled environmental and infectious stimuli. CD86⁺ activation state was variable, however, given the heterogeneous disease state of the patients, this is perhaps unsurprising. Given the plasticity of human AM however, further studies are required to confirm the phenotype of AM during fungal infection.

There have been few studies analysing innate PRR expression in human AM (Juarez et al., 2010). Studies suggest expression is highly variable both due to plasticity depending on local tissue environmental cues and cytokine stimuli, and also genetic heterogeneity and polymorphisms (Hussell and Bell, 2014, Georgel et al., 2009). Analysis of PRR expression on lung transplant and chronic respiratory disease human AM was additionally extremely variable. Further larger scale

studies are required to ascertain whether reduced TLR (e.g. TLR-4) or C-type lectin receptor (e.g. Dectin-1) expression correlates with increased susceptibility to infection.

When infected with *A. fumigatus* conidia in the resting state, human AM had almost no pro-inflammatory cytokine response. Further data additionally revealed an absence of transcription pathway activation (data from *Shichina Kannambath*, postdoc in Armstrong-James lab). The *A. fumigatus* conidia have a hydrophobic rodlet outer layer in the resting state which conceals the carbohydrate-rich cell wall that is recognised by the innate host immune system. Although resting conidia are phagocytosed by AM and killed efficiently, the receptor-mechanism of uptake and the killing mechanisms involved are as yet undefined. Given the ubiquitous distribution of *A. fumigatus* in the environment, the immunologically silent processing of resting conidia may well present an evolutionarily conserved pathway to prevent over-exuberant inflammatory responses, neutrophil influx and lung damage. Infection of human AM with germinated (swollen) *A. fumigatus* conidia, however, revealed a strong pro-inflammatory stimulus and calcineurin-NFAT pathway activation. Assays performed were 18 h p.i.. In most cases, human AM were able to contain fungal germination following infection with live swollen *A. fumigatus* so responses are related to swollen conidia rather than germling or hyphal *A. fumigatus* forms. Human macrophage NFAT pathway activation has previously been shown to be TNF α dependent, which may explain the absence of NFAT activation in response in resting *A. fumigatus* conidial infection (Yarilina et al., 2011). Limitations persist in that NFAT pathway activation was only shown with a single NFAT isomer (NFATc2) given activation has previously been shown to be isomer dependent (Crabtree and Olson, 2002). Further work is necessary to confirm whether this pattern of activation is consistent with all calcium and non-calcium responsive NFAT isomers expressed in human macrophages. Additionally, in this thesis I have focussed on the human macrophage response to resting and swollen *A. fumigatus* conidia as this is the initial inhalational route of exposure and infection. Initial work in our group had shown that human AM are unable to phagocytose germling or hyphal *A. fumigatus* with infection leading to cell

lysis (*data not shown*). Further work is required to ascertain and characterise in detail the human AM response to *A. fumigatus* germlings and hyphae.

Contrary to the previous literature suggesting a negative regulation of NFAT on NF- κ B-dependent TNF α production following LPS stimulation, lung transplant AM TNF- α production in response to swollen *A. fumigatus* conidial infection was also calcineurin-NFAT pathway dependent (Kang et al., 2007). This was additionally seen in AM from chronic respiratory disease patients where a further reduction in TNF- α in response to swollen *A. fumigatus* conidia was seen in the presence of FK506 and hydrocortisone compared to hydrocortisone alone. Similar effects have been shown in a murine macrophage model of experimental colitis, with VIVIT (a selective peptide inhibitor of the calcineurin-NFAT interaction) treatment resulting in impaired TNF- α production (Elloumi et al., 2012). This suggests that the effects of calcineurin-NFAT pathway activation on TNF- α production are complex and are likely to be stimulus and pathogen dependent.

As yet, the role of the calcineurin-NFAT pathway in patients on long-term corticosteroids is unclear. As corticosteroids are potent inhibitors of the NF κ B pathway, it is possible that calcineurin-NFAT activation represents a salvage pathway for innate immune activation. In contrast however, NFAT transcriptional activity is in large part dependent on its transcriptional partner AP-1 which is in itself inhibited by corticosteroids (Macian et al., 2001, Barnes, 2006). Independent NFAT activation due to AP-1 inhibition is likely to modify cellular responses. Given the large numbers of patients on long-term corticosteroid treatment, further studies are required to understand the effects of corticosteroids on calcineurin-NFAT pathway activation.

Although our previous studies showed calcineurin-dependent innate immune activation in murine macrophages, there was no calcineurin-dependent defect in fungal killing (Herbst et al., 2015). In contrast, fungal killing by human AM was shown to be calcineurin dependent. The contrast between murine and human macrophages has been well described (Mestas and Hughes, 2004). Differences in macrophage plasticity, phenotype and nitric oxide synthase production pathways suggest murine

macrophage mechanistic pathways may not be conserved in human macrophages (Spiller et al., 2015). A role for the calcineurin-NFAT pathway in neutrophil fungicidal activity against *C. albicans* has previously been shown, however the calcineurin-dependent killing mechanism remains unknown (Greenblatt et al., 2010). Further NOD-1 and calcineurin-NFAT dependent neutrophil bactericidal activity against uropathogenic *Escherichia coli* (UPEC) has also been shown (Vandewalle et al., 2014). Given the variable activation state of human AM, to identify the calcineurin dependent mechanism of fungal killing a healthy human monocyte-derived macrophage (MDM) model of fungal infection was utilised.

4 Utilising a human monocyte-derived macrophage model of the alveolar macrophage-*A. fumigatus* interaction to determine the calcineurin-dependent mechanism of fungal killing

4.1 Introduction – Macrophage differentiation in the human lung

For a long time, homeostasis of AM and indeed all tissue-resident macrophages was thought to be dependent on circulating bone-marrow derived monocytes (van Furth and Cohn, 1968). This notion was following monocyte transfer experiments in AM depletion murine experiments utilising intratracheal administration of diphtheria toxin (DT) to CD11c-DT receptor mice.(Landsman et al., 2007) This evidence was contradicted however, by genetic fate mapping experiments showing AM were either only partly or not at all of adult haematopoietic or monocytic origin (Schulz et al., 2012, Yona et al., 2013). More recent experiments have shown that colonisation of the airways with AM occurs in the first few days of life, deriving from fetal monocytes that seed the lung before birth and develop into the long-lived CD11c expressing AM shortly after birth. This differentiation process starts around the same time as the alveoli develop, is completed over the course of 1 week and is dependent on granulocyte-macrophage colony-stimulating factor (GM-CSF) (Guilliams et al., 2013). Macrophages from the airways of GM-CSF^{-/-} mice are not fully differentiated AM and are functionally limited, with reduced responsiveness to LPS and impaired phagocytosis (Shibata et al., 2001). This is additionally true in patients with pulmonary alveolar proteinosis where there is a functional deficiency of GM-CSF resulting in defective AM with inappropriate accumulation of surfactant, poor antigen presentation and pathogen clearance (Suzuki et al., 2015).

AM have been shown to have a marked capacity of self-renewal and during depletion following influenza infection are able to replenish their population (Ghoneim et al., 2013, Hashimoto et al., 2013). During a significant infection, such as pneumococcal pneumonia however, a distinct population of CD11b^{High} AM which are lacking monocyte markers are recruited to the airways. This recruitment has been shown to be dependent on CD11b and increased GM-CSF expression in the

lung environment in acute infection (Kirby et al., 2006). It is unclear whether these cells represent circulating monocytes or lung tissue resident macrophages that have differentiated within lung tissue to acquire an AM phenotype. A possible explanation is suggested from previous work showing that circulating monocytes initially differentiate into parenchymal lung macrophages which subsequently migrate into the alveolar space (Landsman and Jung, 2007).

Human blood monocytes are often used to generate, following differentiation, MDM in vitro as a model for tissue macrophages (Erbel et al., 2013). Given the considerable plasticity of tissue macrophages, depending on presence in vitro of different cytokines and differentiating agents, MDM are able to exhibit different phenotypes (Lacey et al., 2012). Unsurprisingly, given the GM-CSF dependency of AM differentiation and the high levels present in the lung, monocytes differentiated in the presence of GM-CSF appear to have a more AM-like morphology, phenotype and function, compared to other differentiation agents such as macrophage-CSF (M-CSF) (Komuro et al., 2001). Whole genome expression profiling has shown that there is a similar transcriptional signature of AM with GM-CSF differentiated MDM (Tomlinson et al., 2012). Nevertheless, recent work has highlighted the considerable plasticity of AM and MDM in response to diverse activation signals (Xue et al., 2014b). The patient-specific local lung environment is therefore likely to be a key determinant of AM function (Hussell and Bell, 2014).

Given the heterogeneity of AM isolated from lung transplant and non-transplant patients undergoing bronchoscopy for clinical necessity, coupled with the difficulty in obtaining AM from healthy volunteers, a model system is necessary to systematically dissect the calcineurin-dependent mechanism of fungal killing in human AM. Whilst murine models have been used to date, previous work in our group has shown no appreciable calcineurin-dependent effect on fungal killing in murine bone-marrow derived macrophages and murine macrophage cell lines (Herbst et al., 2015). This is in stark contrast to findings in human AM. Significant differences between the murine and human innate immune response to *A. fumigatus* have previously been reported (Jurgens et al., 2010).

Hence, to model the AM-A. *fumigatus* interaction and define the calcineurin-dependent mechanism of fungal killing, a GM-CSF differentiated human MDM model was used.

4.2 Phenotype of human monocyte-derived macrophages vs alveolar macrophages

To determine whether GM-CSF differentiated MDM displayed a similar phenotype to that found in lung transplant AM, monocytes from healthy volunteers were differentiated for 7 days in 10% human serum and GM-CSF (5 ng/ml) and analysed by flow cytometry. Macrophages were gated by forward and size scatter and an amine-reactive fixable dye used to select live macrophages (Zombie-aqua, Biolegend) as previously shown. Cells were stained for CD11c, CD11b, CD206, HLA-DR, CD86 and Dectin-1 and gating based on fluorescence minus one controls as shown in Figures 1 and 2.

MDM differentiated with GM-CSF and human serum for 7 days had high CD11c, HLA-DR and CD206 expression similar to that seen in human AM. This suggests an activated state with high antigen-presentation capability. Unsurprisingly given their origin, MDM had high CD11b expression compared to AM where this is absent. Interestingly, MDM additionally had significantly higher CD86 expression suggesting differences in activation state. There was no significant difference in Dectin-1 surface expression levels in MDM compared to AM.

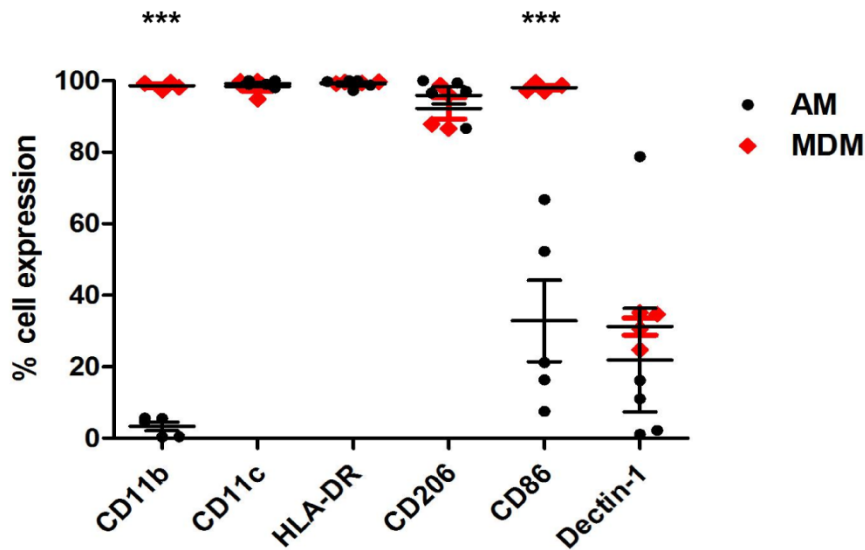


Figure 7: GM-CSF differentiated monocyte-derived macrophages have a broadly similar phenotype to human alveolar macrophages with differences in CD11b and CD86 expression.

Lung transplant AM (n=5) and MDM (n=4) were analysed by flow cytometry to assess surface phenotype. Cells were gated by forward and size scatter and live cells selected using a fixable amine-reactive stain (Zombie-aqua, Biolegend) and surface expression of CD11b, CD11c, HLA-DR, CD206, CD86 and Dectin-1 quantified. Similar to AM, MDM differentiated with GM-CSF and human serum also had high CD11c, HLA-DR and CD206 expression. In contrast MDM had high CD11b expression and high CD86 expression. Dectin-1 expression was variable in AM and MDM with no significant difference seen. Bars represent mean + SEM and samples were analysed by 2-way ANOVA with Bonferroni post-test calculation; ***p<0.001. (n = biological replicates).

4.3 FK506 impairs calcineurin-dependent innate responses in human monocyte-derived macrophages infected with *A. fumigatus*

To validate a MDM model of the AM - *A. fumigatus* interaction, calcineurin-NFAT activation in healthy volunteer MDM was analysed after infection with *A. fumigatus* swollen conidia (MOI = 1). As in human AM, initial analysis by rt-PCR within our group had shown predominant expression of NFATc1 and NFATc2 within human MDM (*data not shown*). Calcineurin-NFAT activation was assessed using two NFAT translocation assays (probing nuclear extracts for NFATc2 by Western blot and analysing NFATc1 nuclear translocation by confocal microscopy) and by quantifying RCAN 1.4 expression in whole cell lysate as a NFAT-specific transcriptional target.

A western blot NFATc2 and confocal NFATc1 nuclear translocation assay revealed that MDM infected with live *A. fumigatus* swollen conidia stimulated both NFATc1 and NFATc2 nuclear translocation at 30 - 60 min p.i. comparable to human AM (Figure 8A-E). NFκB p65 nuclear translocation was additionally seen at 30 min p.i. by western blot and confocal microscopy (Figure 8B and G). Pre-treatment with FK506 strongly inhibited NFATc1 and NFATc2 nuclear translocation with no significant effect on NFκB p65 nuclear translocation (Figure 8B, E and G). To confirm calcineurin-NFAT transcriptional activity, expression of RCAN1.4 was additionally up-regulated after infection of MDM with *A. fumigatus* swollen conidia with pre-treatment with FK506 resulting in almost complete inhibition in expression (Figure 8F).

To determine whether activation of the calcineurin-NFAT signalling pathway in human MDM is downstream of the Dectin-1-Syk receptor, NFATc1 nuclear translocation and TNF-α production was quantified by confocal microscopy and ELISA respectively following pre-treatment with either a blocking human Dectin-1 receptor antibody (R&D systems) or Piceatannol (Syk inhibitor, Sigma). Although ligation of the Dectin-1 receptor and activation of Syk was shown to be critical for TNF-α production, activation of calcineurin-NFAT signalling in human MDM infected with *A. fumigatus* swollen conidia is not Dectin-1-Syk dependent (Figure 9).

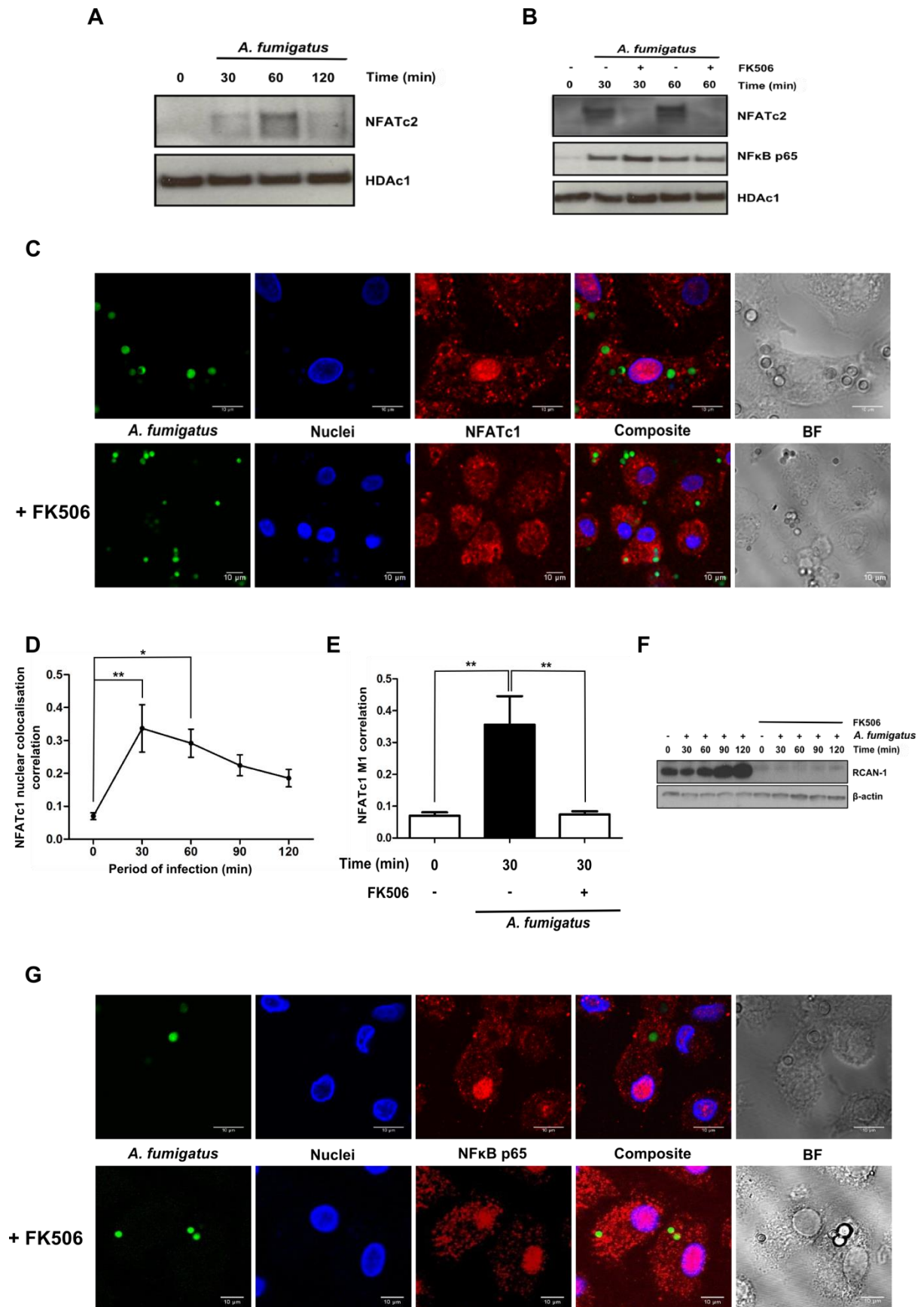


Figure 8: Human healthy monocyte-derived macrophage activation of the calcineurin-NFAT pathway in response to *A. fumigatus* infection mirrors that of alveolar macrophages.

Monocytes were extracted from healthy volunteer human blood and differentiated with GM-CSF and human serum into MDM. Nuclear extracts of MDM infected with live *A. fumigatus* swollen conidia (MOI = 1) were probed by Western blot for NFATc2 and NFκB. HDAC1 was used as a loading control. **A)** Representative western blot showing MDM translocate NFATc2 into the nucleus 30 mins p.i. with sustained activation at 1 h p.i.. **B)** Representative western blot showing that pre-treatment with FK506 blocks NFATc2 nuclear translocation but has no effect on NFκB nuclear translocation which also occurs at 30 min p.i. and is sustained for 1 h p.i.. **C)** Representative confocal microscopy of MDM infected with live eGFP expressing *A. fumigatus* (green) (MOI = 1) at 60 min p.i. stained for NFATc1 (red) and nuclei (blue). Top panel shows NFATc1 nuclear translocation in response to live *A. fumigatus* swollen conidia, which is inhibited in the presence of FK506 (10 ng/ml) (bottom panel). **D-E)** Quantification of NFATc1 nuclear translocation (using Manders' (M1) overlap nuclear correlation coefficient) in human MDM infected with live *A. fumigatus* swollen conidia (MOI = 1) by confocal microscopy. Significant nuclear translocation is seen at 30 min p.i. and is maintained till 60 min p.i.. FK506 pre-treatment results in almost complete inhibition of NFATc1 nuclear translocation in response to *A. fumigatus* infection. n=4 (n = biological replicates) **F)** Representative western blot of RCAN1.4 expression in whole cell lysates of healthy volunteer MDM infected with live *A. fumigatus* (MOI = 1) with 1 h FK506 (10 ng/ml) or vehicle pre-treatment. RCAN1.4 expression is up-regulated after MDM are infected with *A. fumigatus* with almost complete inhibition of expression in presence of FK506. **G)** Representative confocal microscopy image of MDM infected with live eGFP expressing *A. fumigatus* swollen conidia (green) (MOI = 1) for 60 min p.i. and stained for NFκB p65 (red) and nuclei (blue). NFκB nuclear translocation is clearly seen in the macrophage with internalised *A. fumigatus* conidia with no calcineurin-dependent effect on nuclear translocation. Bars represent mean + SEM and samples were analysed by Friedman test with Dunn's post-test calculation (D) and Wilcoxon matched pairs test (E); *p<0.05, **p<0.01. All scale bars shown represent 10µm.

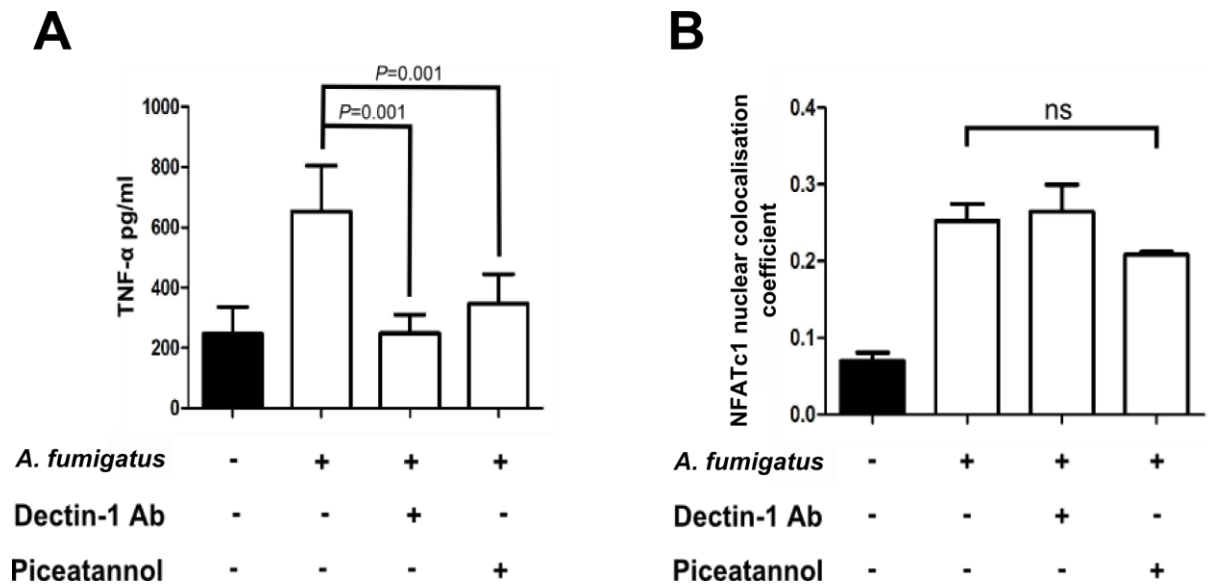


Figure 9: Calcineurin-NFAT signalling in healthy human monocyte-derived macrophages infected with live swollen *A. fumigatus* conidia is not Dectin-1-Syk receptor dependent.

MDM were differentiated from healthy human volunteer blood and infected at day 7 with live *eGFP*-expressing swollen *A. fumigatus* conidia (MOI = 1) with pre-treatment with a blocking human Dectin-1 receptor (R&D systems), Piceatannol (Sigma) or vehicle. Macrophages were fixed and stained for NFATc1 and nuclei, nuclear translocation assessed by confocal microscopy and quantified using Fiji. TNF-α production at 6 h p.i. was analysed by ELISA. **A)** TNF-α production at 6 h p.i. was significantly inhibited in the presence of a blocking dectin-1 receptor or a Syk inhibitor (Piceatannol). n=5 (n = biological replicates). **B)** NFATc1 nuclear translocation however, did not appear to be dependent on the Dectin-1 receptor or SyK activation with no inhibition of translocation seen with blockade of Dectin-1 receptor or inhibition of Syk. n=5 (n = biological replicates) . Bars represent mean + SEM and samples were analysed by Wilcoxon matched pairs test.

4.4 Fungal killing in healthy human monocyte-derived macrophages is calcineurin-dependent

4.41 Fungal burden

To confirm whether the calcineurin-dependent fungal killing in human lung transplant AM is also seen in healthy human MDM, fungal burden was quantified by 18S ribosomal RNA rt-PCR in human MDM infected with swollen *A. fumigatus* conidia at 1 and 6 h p.i.. Similar to the effect of calcineurin inhibition on fungal killing in human lung transplant AM, fungal killing in healthy human MDM was also calcineurin-dependent (Figure 10). This result in combination with the calcineurin-NFAT

pathway activation in response to *A. fumigatus* infection and similar surface phenotype validates the use of a healthy human MDM model of *A. fumigatus* infection to study the calcineurin-dependent mechanism of fungal killing in human AM.

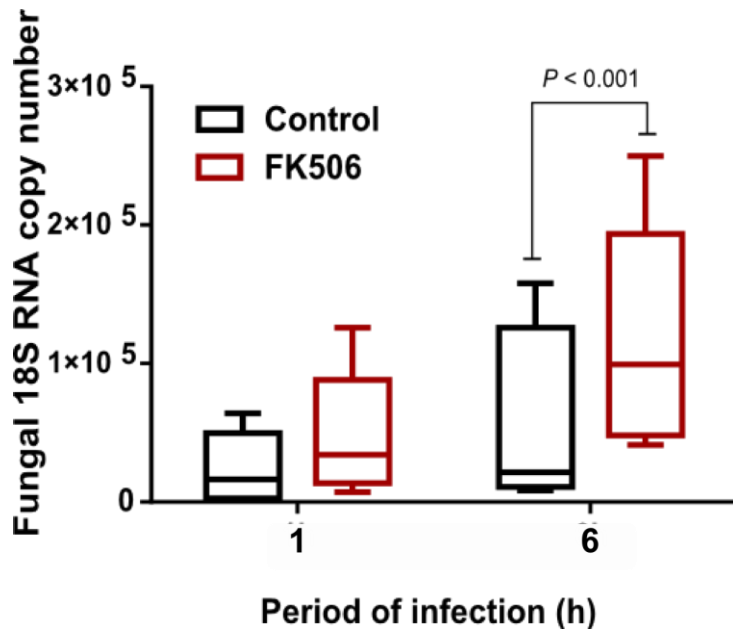


Figure 10:

Fungal killing in healthy human monocyte-derived macrophages is calcineurin-dependent.

MDM differentiated from healthy human volunteer blood was infected with live swollen CEA10 *A. fumigatus* conidia (MOI = 1) with 1 h FK506 (10 ng/ml) pre-treatment or vehicle. RNA was extracted at 1 h and 6 h p.i. and fungal burden was quantified using an 18S ribosomal RNA rt-PCR assay.

Fungal burden was significantly increased at 6 h p.i. in the presence of FK506. n=5 (n = biological replicates). Bars represent mean + SEM and samples were analysed by Wilcoxon matched pairs test

4.42 Phagocytosis and phagosomal maturation

There are various stages of the intracellular fate of *A. fumigatus* conidia upon interaction with macrophages. Initially the *A. fumigatus* conidium is internalised through an actin-dependent mechanism. The phagosome then matures with the acquisition of early and late endosomal Rab GTPase proteins (e.g. Rab5 and 7), acidifies and fuses with lysosomes to form mature phagolysosomes expressing LAMP-1. Through this process intracellular killing is achieved in part through the production of reactive oxygen species (ROS) and activation of lysosomal cysteine proteases (e.g. Cathepsins). To determine the calcineurin-dependent effect on human AM fungal killing, a systematic analysis of the effects of calcineurin inhibition on a MDM model of *A. fumigatus* infection was performed.

MDM differentiated from healthy volunteers were infected with live CEA10 swollen *A. fumigatus* conidia stained with calcofluor white (Sigma) (MOI = 1) following pre-treatment for 1 h with FK506 (10 ng/ml) or vehicle. Pre-treatment with the actin polymerisation inhibitor Cytochalasin D (5 μ M, Sigma) for 30 min was used as a negative control. Conidial uptake was analysed at 30 min intervals p.i. by Imagestream, with DRAQ5 (eBioscience) used to stain macrophage nuclei.

Calcineurin inhibition significantly delayed fungal uptake at early time-points post infection (30 min and 1 h) but not beyond 2 h (Figure 11). Further work performed by *Shichina Kannambath*, a post-doc in our lab has confirmed however that there is no calcineurin-dependent defect in uptake of resting *A. fumigatus* conidia or inert beads.

Further analysis of endosomal and phagolysosomal maturation was performed by quantifying Rab7 and LAMP-1 co-localisation to the *A. fumigatus* containing phagosome alongside phagosomal acidification (Figure 12). MDM differentiated from healthy volunteers were infected with eGFP-expressing *A. fumigatus* swollen conidia (MOI = 1) following pre-treatment for 1 h with FK506 (10 ng/ml) or vehicle. To assess phagosomal acidification, cells were additionally pre-loaded with LysoTracker red (1 μ M) and acidification assessed by imaging live cells with time-lapse confocal microscopy. Rab7 and LAMP-1 co-localisation was assessed by fixation at 30 min time-points p.i., and imaging after antibody staining by confocal microscopy.

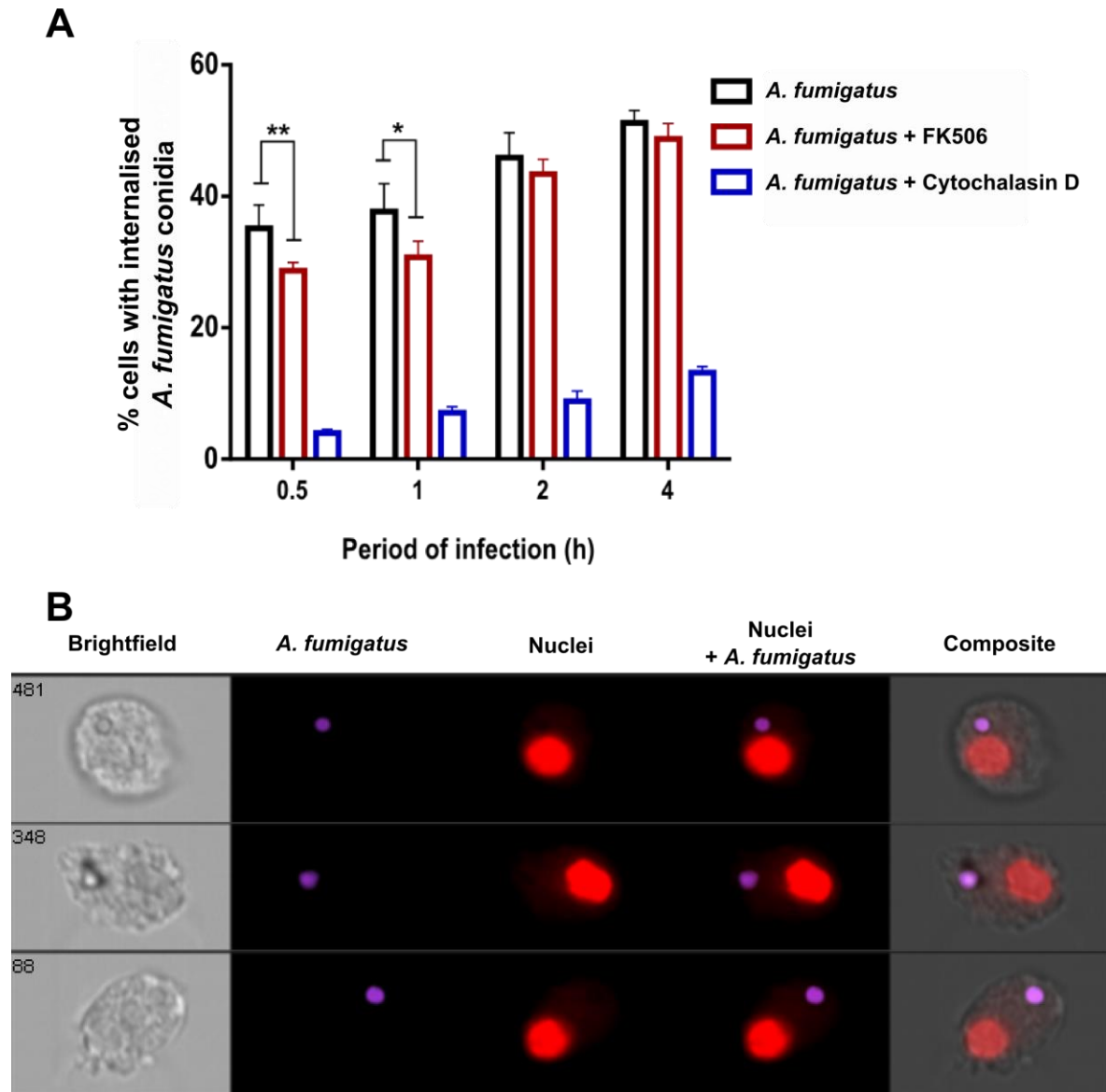


Figure 11: Human monocyte-derived macrophage phagocytosis of live swollen *A. fumigatus* conidia is calcineurin-dependent.

MDM differentiated from healthy human volunteer blood were infected with live swollen CEA10 *A. fumigatus* conidia stained with calcofluor white (MOI = 1) following pre-treatment with either FK506 (10 ng/ml), Cytochalasin D (5 μ M) or vehicle. Cells were harvested in ice-cold PBS/EDTA, stained with DRAQ5 nuclear stain, fixed with 2% PFA and uptake analysed by Imagestream. **A)** Calcineurin inhibition resulted in delayed uptake of live swollen *A. fumigatus* conidia at early time points (30 min and 1 h) post infection but not beyond 2 h. $n=5$ (n = biological replicates) Bars represent mean + SEM and samples were analysed by 2-way ANOVA with Bonferroni post-test calculation. * $p<0.05$, ** $p<0.01$. **B)** Representative images from Imagestream analysis of MDM *A. fumigatus* uptake (control cells shown). (Imagestream analysis performed in collaboration with Shichina Kannambath).

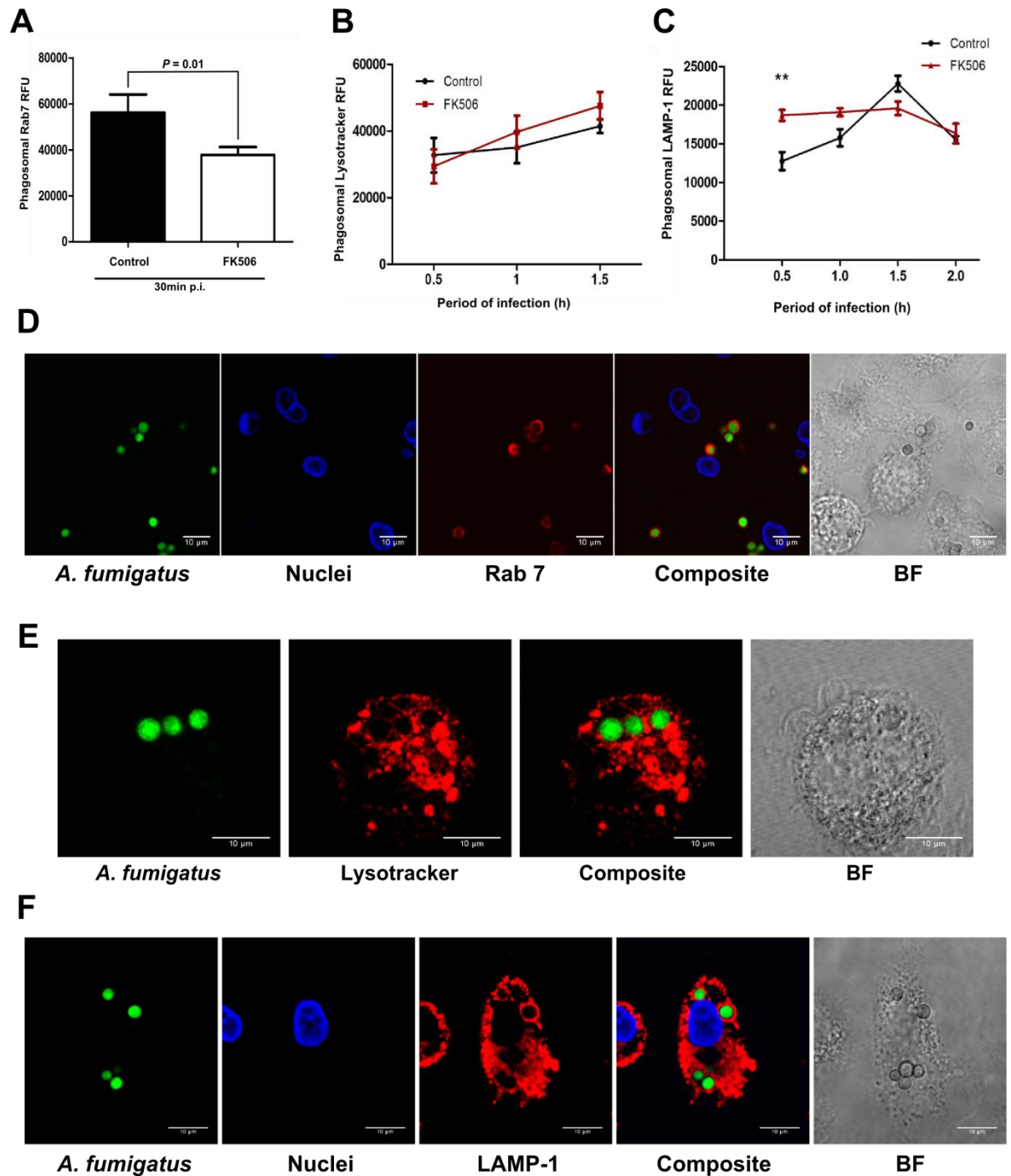


Figure 12: Human monocyte-derived macrophage phagosomal maturation after *A. fumigatus* infection is altered by calcineurin inhibition.

MDM differentiated from healthy human volunteer blood were infected with live *eGFP*-expressing swollen CEA10 *A. fumigatus* conidia (MOI = 1) following pre-treatment with either FK506 (10 ng/ml) or vehicle. **A)** Rab7 phagosomal co-localisation was quantified at 30 min p.i.. Calcineurin inhibition results in impaired Rab7 phagosomal colocalisation at 30 min p.i.. n=4, (n = biological replicates) **B)** Phagosomal acidification in human MDM infected with live *eGFP*-expressing *A. fumigatus* conidia is

not calcineurin-dependent. n=4 (n = biological replicates) **C)** LAMP-1 phagosomal co-localisation is significantly increased at 30 min p.i. after FK506 treatment with no difference from 1 h p.i.. n=4, (n = biological replicates). **D-F)** Representative images showing Rab7 phagosomal co-localisation (control cells shown) (D), phagosomal acidification (control cells shown) (E) and LAMP-1 phagosomal co-localisation in human MDMs infected with live *eGFP*-expressing *A. fumigatus* conidia (control cells shown). *A. fumigatus* conidia shown in green, Nuclei in blue, RAB7 (D), acidified vesicles (E), and LAMP-1 (F) shown in red. All scale bars shown represent 10µm. Bars represent mean + SEM and samples were analysed by Wilcoxon matched pairs test (**A**) and by 2-way ANOVA with Bonferroni post-test calculation (**B-C**), **p<0.01.

With FK506 pre-treatment human MDM infected with *A. fumigatus* have significantly increased LAMP-1 phagosomal co-localisation, but reduced Rab7 co-localisation at 30 min p.i. with no difference in phagosomal acidification or LAMP-1 phagosomal recruitment after 30 min p.i. (Figure 12). This suggests that although phagosomal maturation in human macrophages infected with *A. fumigatus* is not calcineurin-dependent, the kinetics of maturation after infection and internalisation does appear to be altered with calcineurin inhibition.

4.43 **Reactive oxygen-species production and Cathepsin B activation**

To further understand the calcineurin-dependent mechanism of fungal killing, production of activated Cathepsin B was analysed by live time-lapse confocal microscopy (Figure 13). Reactive oxygen species production in response to swollen *A. fumigatus* conidia was simultaneously analysed by a postdoc within our group (*Shichina Kannambath*). Results are shown with her permission.

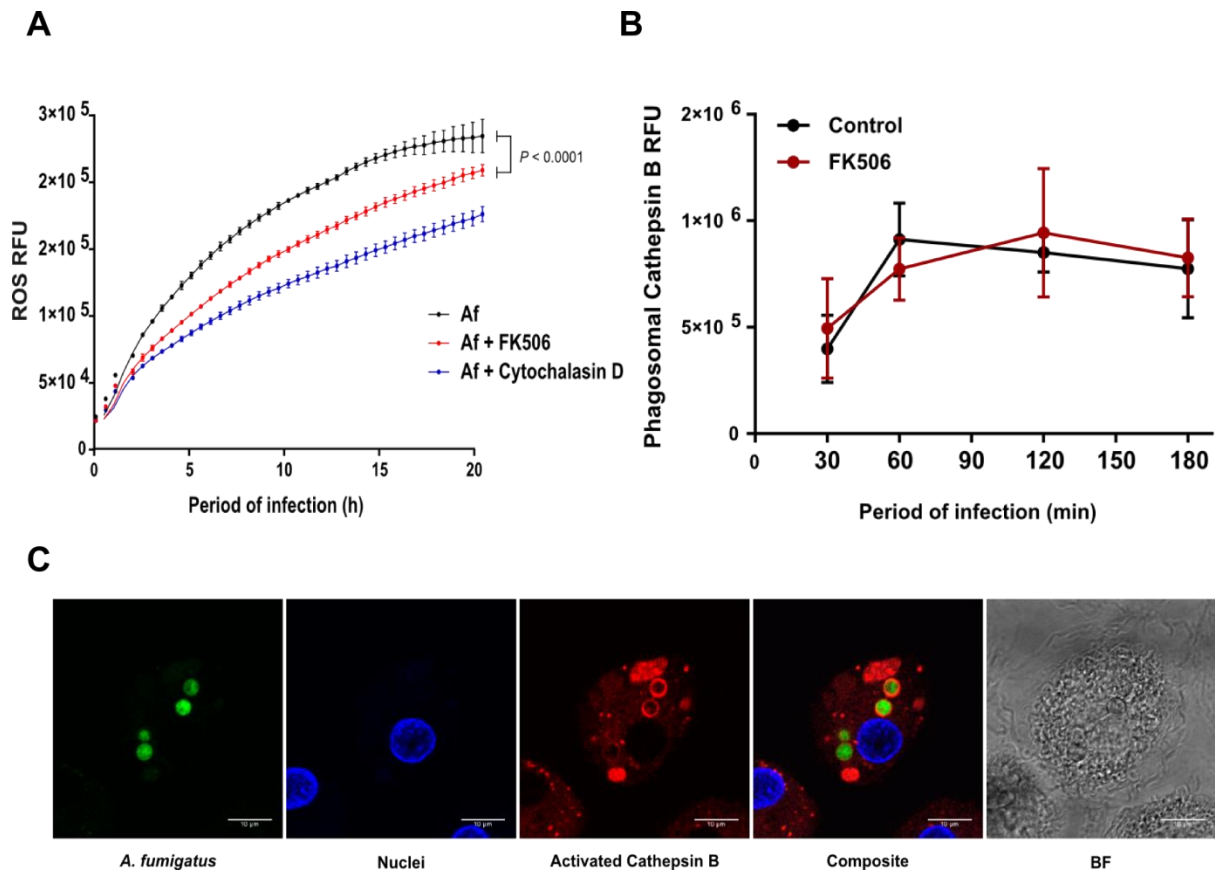


Figure 13: Human monocyte-derived macrophage reactive oxygen species production after *A. fumigatus* infection is calcineurin dependent.

MDM differentiated from healthy human volunteer blood were infected with either live CEA10 or *eGFP*-expressing swollen *A. fumigatus* conidia (MOI = 1), following pre-loading with dihydrorhodamine 123 (Life technologies) or Magic red activated Cathepsin B assay (Immunochemistry technologies). Reactive oxygen species production was quantified using a fluorescent plate reader (Tecan), and activated Cathepsin B production by image analysis of time-lapse confocal microscopy. **(A)** Reactive-species production by human MDM infected with live swollen *A. fumigatus* conidia is calcineurin-dependent. (data produced and shown with permission from Shichina Kannambath). $n=5$, (n =biological replicates) **(B)** Activated Cathepsin B production by human MDM infected with live *A. fumigatus* is not calcineurin-dependent. $n=4$, (n = biological replicates) **(C)** Representative images showing activated Cathepsin B in human MDM infected with live *eGFP*-expressing *A.fumigatus* conidia (MOI = 1) (control cells shown). *A. fumigatus* conidia are shown in green, nuclei in blue and activated Cathepsin B in red. All scale bars shown represent 10 μ m. Bars represent mean + SEM and samples were analysed by 2-way ANOVA with Bonferroni post-test calculation **(A-B)**.

With FK506 pre-treatment, human MDM infected with live swollen *A. fumigatus* conidia have significantly impaired reactive oxygen species production suggesting a calcineurin-dependent process (Figure 13A). In contrast, although significant activated cathepsin B colocalisation was seen to live swollen *A. fumigatus* containing phagosomes this was not found to be calcineurin-dependent (Figure 13B-C).

To ascertain whether the calcineurin-dependent effects on reactive oxygen species production are due to effects on mitochondria, mitochondrial reactive oxygen species production after infection with *A. fumigatus* was quantified by pre-loading cells with Mitotracker Orange CM-H2TMRos (Invitrogen) and analyzing fluorescence by flow cytometry (Figure 14). There was no calcineurin-dependent difference in mitochondrial reactive oxygen species production in human MDM infected with *A. fumigatus*.

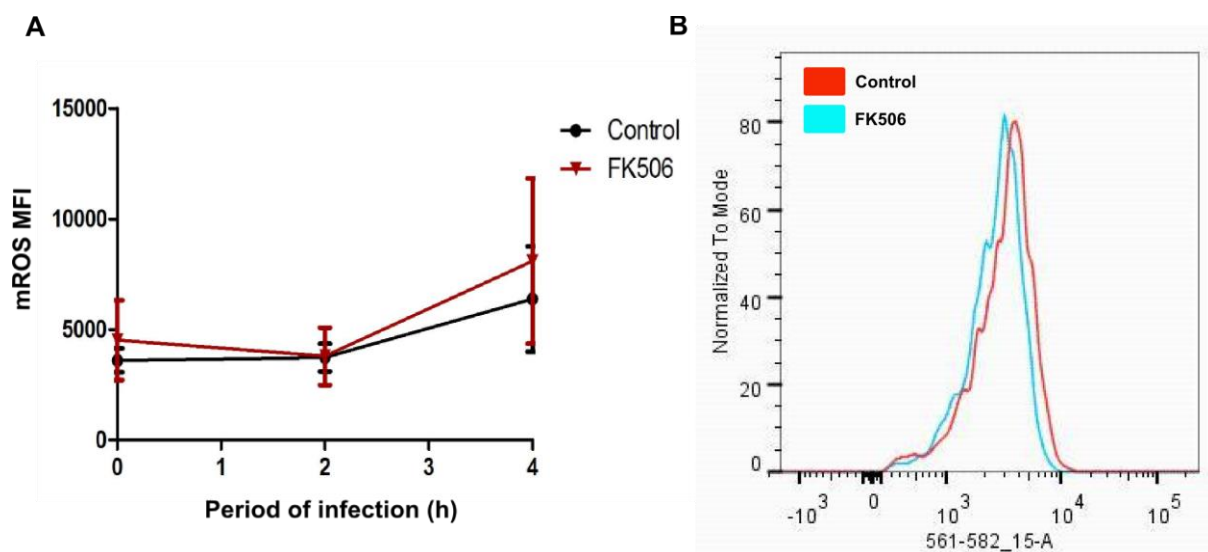


Figure 14: Human monocyte-derived macrophage mitochondrial reactive oxygen species production after *A. fumigatus* infection is not calcineurin dependent.

MDM differentiated from healthy volunteers were infected with live swollen *A. fumigatus* conidia (MOI = 1) following pre-loading with Mitotracker Orange CM-H2TMRos (Invitrogen) and pre-incubation with FK506 (10 ng/ml) or vehicle. Fluorescence was quantified by flow cytometry at 0,2 and 4 h p.i.. **(A)** There was no calcineurin-dependent difference in mitochondrial reactive oxygen species production in human MDM infected with *A. fumigatus*. **(B)** Representative histogram of mitochondrial reactive oxygen species fluorescence 4 h p.i.. n=3, (n = biological replicates). Bars

represent mean + SEM and samples were analysed by 2-way ANOVA with Bonferroni post-test calculation.

4.44 Pro-inflammatory cytokine production

To further understand the mechanism of calcineurin-dependent fungal killing, the effects of FK506 on cytokine production in human MDM infected with swollen *A. fumigatus* conidia was analysed by Luminex assay. Previous work has shown that an initial macrophage cytokine innate immune response is critical to anti-*Aspergillus* host defense. This includes TNF- α production by alveolar macrophages with immunoneutralisation resulting in impaired fungal clearance and increased mortality in murine in vivo studies (Mehrad et al., 1999b, Mehrad et al., 1999a). This is associated with impaired chemokine production including IL-8, MIP-1 α , MIP-1 β and MCP-1 (Brieland et al., 2001). Neutrophils are critical for the host antifungal response with neutropenia being a significant risk factor for the development of invasive aspergillosis in humans (Baddley et al., 2010). Neutrophil influx to the lung is dependent on chemokine production with immunoneutralisation of chemokines in murine invasive aspergillosis in vivo studies leading to impairment of neutrophil influx and significantly increased mortality (Mehrad et al., 1999a).

GM-CSF and IFN γ production have been linked to effective phagocytic function of macrophages and neutrophil effector functions and have been shown to potentially useful as immunotherapeutic agents in invasive fungal infections (Brummer et al., 2002, Brummer et al., 2001, Abu Jawdeh et al., 2000, Schelenz et al., 1999, Nagai et al., 1995, Warris et al., 2005). Both cytokines have additional likely complex immunomodulatory actions at transcriptional level (Roilides et al., 1993, Warris et al., 2005). Lung damage and host mortality is also dependent on the ability to regulate the innate immune response to prevent excess damage (Moretti et al., 2014). IL-10 and IL-12p40 are cytokines secreted by macrophages that have been shown to have an immunoregulatory role following *A. fumigatus* infection with IL-12p40 knockout mice having increased susceptibility to invasive

aspergillosis following neutrophil depletion (Cenci et al., 1999). In contrast, immunocompetent Il-10-deficient mice had increased resistance to invasive fungal infection (Clemons et al., 2000). Lastly, *A. fumigatus* infection induces a strong inflammasome response in human macrophages characterised by Il-1 β secretion and Il-1 α -dependent leukocyte recruitment (Karki et al., 2015a, Said-Sadier et al., 2010b). Il-1Ra production has been shown to be critical in regulation of the inflammasome response in human macrophages stimulated with *A. fumigatus* (Iannitti et al., 2016). On this basis, human MDM were isolated from healthy human volunteers and infected with swollen CEA10 *A. fumigatus* conidia (MOI = 1) and culture supernatants tested for TNF- α , GM-CSF, MCP-1, MIP-1 α , MIP-1 β , Il-8, Il-10, Il-12p40, Il-1 α and Il-1 β and Il-1ra by Luminex assay 6 h p.i. (Figure 15). This revealed that TNF- α , GM-CSF, MCP-1, MIP-1 α , MIP-1 β and Il-1ra production by human MDM infected with *A. fumigatus* is calcineurin-dependent. Unfortunately, no Il-1 β was detected in culture supernatants within this assay.

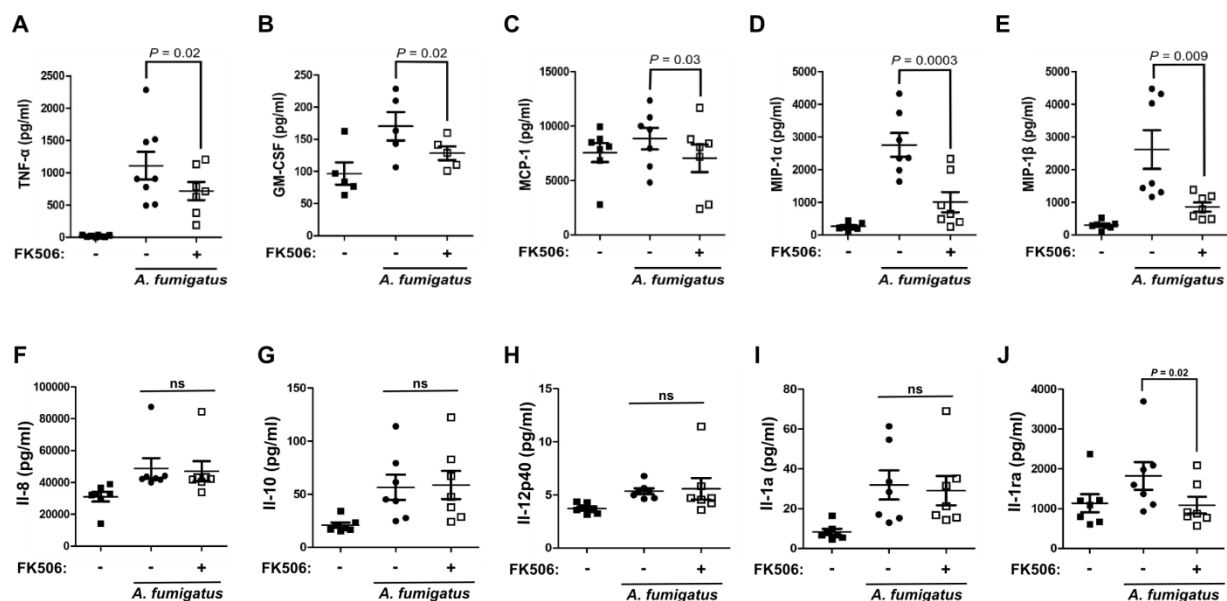


Figure 15: The innate immune response of human monocyte-derived macrophages infected with *A. fumigatus* is calcineurin-dependent.

MDM differentiated from healthy volunteers were infected with live swollen *A. fumigatus* conidia (MOI = 1) following pre-treatment with FK506 (10 ng/ml) or vehicle. Culture supernatants were tested for TNF- α , GM-CSF, MCP-1, MIP-1 α , MIP-1 β , Il-8, Il-10, Il-12p40, Il-1 α and Il-1ra by Luminex assay 6 h p.i. (A-E) TNF- α , GM-CSF, MCP-1, MIP-1 α , MIP-1 β production by human MDM infected

with live swollen *A. fumigatus* conidia is calcineurin-dependent. **(F-I)** There was no calcineurin-dependent difference in IL-8, IL-10, IL-12p40, IL-1a production by human MDM infected with *A. fumigatus*. **(J)** IL-1ra production by human macrophages infected with *A. fumigatus* is calcineurin-dependent. n=7, (n = biological replicates). Bars represent mean + SEM and samples were analysed by Wilcoxon matched pairs test.

4.5 Discussion

This chapter indicates that healthy human MDM differentiated in human serum and GM-CSF show a broadly similar phenotype to lung transplant AM with high CD11c, HLA-DR and CD206 expression. This is suggestive of high antigen-presenting capability with high CD206 expression consistent with an activated phenotype potentially important for wound repair (Tomlinson et al., 2012). As with any model system, there remains differences in phenotype however. Human MDM differentiated in human serum and GM-CSF had comparatively high CD86 surface expression suggestive of a highly activated state. Unsurprisingly, given the heterogeneous clinical state of the lung transplant patients from whom AM were isolated, CD86 activation was variable. Additionally, in contrast to AM, MDM have high CD11b surface expression given their lineage.

There is increasing evidence of the role of integrins such as CD11b in the innate immune response (Means and Luster, 2010). CD11b/CD18 has been previously shown to be a key integrin in *C. albicans* interaction with human leukocytes as well as being involved in antifungal eosinophilic activity, with *C. albicans* infection additionally modulating CD11b expression in polymorphonuclear neutrophils (PMNs) in certain circumstances (Forsyth et al., 1998, Salvenmoser et al., 2010, Yoon et al., 2008). The role of CD11b in *A. fumigatus* infection is suggested by increased Pentraxin-3 dependent CD11b activation and localisation at the phagocytic cup in PMNs following infection (Moalli et al., 2010a). It is unclear at present whether the increased CD11b expression seen in human MDM compared to AM significantly alters the innate immune response to *A. fumigatus*. Therefore, although human MDM

share a similar phenotype to human AM, limitations persist and any results ultimately require verification in AM.

Consistent with findings in lung transplant AM however, healthy MDM also showed early activation of the calcineurin-NFAT pathway following infection with swollen *A. fumigatus* conidia which was effectively inhibited in the presence of clinically-relevant doses of FK506. NFAT nuclear translocation was seen to peak around 30-60 min post exposure and subsequently decline. Our previous work has indicated an endosomal-dependent NFAT activation pathway which may explain the timing of activation (Herbst et al., 2015). Calcineurin-NFAT pathway regulation has been shown to be highly complex and depends on a combination of autoregulation alongside kinase activity controlling nuclear export, sumoylation and endogenous calcineurin inhibitors (Macian, 2005). Further studies in mast cells with GFP-tagged NFAT have additionally revealed isoform-dependent highly rapid nuclear localisation dynamics in response to cellular stimulation (Yissachar et al., 2013). Although NFAT nuclear localisation in response to live swollen *A. fumigatus* has been shown in human MDM with NFATc1 and NFATc2, further work is required to determine whether this is consistent across all calcium and non-calcium dependent isomers and detail NFAT isomer dynamics with activation. A regulatory response to calcineurin-NFAT activation was seen with increasing RCAN-1 expression. Recent studies have suggested a dose-dependent regulatory role of RCAN-1, which acts as a pathway inhibitor at low-levels but a facilitator at higher levels (Shin et al., 2011). Consistent with our murine studies, calcineurin-NFAT pathway activation in human MDM was Dectin-1 and Syk-independent (Herbst et al., 2015). This is again in contrast to macrophage NFAT activation following *Candida albicans* infection which has been shown to Dectin-1 and Syk dependent with NFAT dependent regulation of IL-2, IL-10 and IL-12 p70 (Goodridge et al., 2007). Whether activation following human macrophage *A. fumigatus* infection is TLR9-BTK dependent as seen in murine macrophages remains to be determined. Nevertheless, this represents the first evidence of calcineurin-NFAT pathway activation following *A. fumigatus* infection in human AM and further

studies are required to detail calcineurin-NFAT dynamics and pathway activation during human macrophage *A. fumigatus* infection.

As with AM, fungal killing by human MDM was calcineurin dependent. Further dissection of killing mechanisms, revealed calcineurin-dependent defects in conidial phagocytosis and reactive oxygen species production but no difference in lysosomal cysteine protease (Cathepsin B) production. Calcineurin-NFAT activation has been shown downstream of various particulate phagocytic receptors including Fcγ, Dectin-1, NOD1 and MARCO (Fric et al., 2014). Thus, calcineurin-dependent down-regulation of fungal phagocytic receptors may present one mechanism for the delayed phagocytosis following human macrophage *A. fumigatus* infection. Consistent with this, recent literature has shown decreased NOD1 expression in murine macrophages treated with Cyclosporine A and following NFATc1 silencing. Decreased neutrophil NOD1 expression was additionally seen in human transplant patients on calcineurin inhibitors (Tourneur et al., 2013).

However, further next-generation RNA sequencing performed within our group analysing the calcineurin-dependent transcriptome of healthy human MDM infected with swollen *A. fumigatus* conidia at 1 h and 6 h post infection did not identify any transcription-level down-regulation of innate PRRs (*data not shown, but performed by Shichina Kannambath, postdoc in Dr Armstrong-James lab*). Recent studies have also shown a role for calcineurin in actin cytoskeletal remodelling and this potentially represents another mechanism for delayed actin-dependent fungal phagocytosis (Wang et al., 2005, Li et al., 2015).

Intraphagosomal reactive oxygen species (ROS) production secondary to NADPH oxidase activity has been shown to be critical for macrophage *A. fumigatus* conidial killing with increased susceptibility in chronic granulomatous disease patients with defective NADPH oxidase function (Grimm et al., 2011). AM have been shown to be able to kill *A. fumigatus* in anaerobic conditions as well however, suggesting the presence of alternative killing pathways (Schaffner et al., 1983). In addition to phagosomal ROS production, mitochondria are thought to be the largest producers of intracellular

reactive oxygen species (mROS). Although calcineurin has been implicated in mitochondrial fission and mROS production, we did not see a calcineurin-dependent difference in mROS production following MDM *A. fumigatus* infection (Zaja et al., 2014). This intimates that calcineurin-dependent macrophage fungal killing may be in part due to phagosomal ROS production, however studies utilising sensitive phagosomal-only ROS assays would be required for definitive confirmation (Balce and Yates, 2013).

To date, there have been few studies analysing the role of calcineurin in reactive oxygen species production. In mammals, NADPH oxidases are divided into three subfamilies: NOX1–NOX4 (NADPH oxidase 1–4), NOX5 and DUOX (dual oxidase) (Panday et al., 2015). Calcium-dependent kinases, such as protein kinase C α and/or Ca²⁺/calmodulin-dependent protein kinase II, are known to phosphorylate and activate NOX5 and DUOX, suggesting a likely role for calcium-activated phosphatases in ROS regulation (Dubiella et al., 2013, Pandey et al., 2011, Rigutto et al., 2009). This is strengthened by a recent study implicating calcineurin A β in the regulation of NOX2 and NOX4 in an NFAT-dependent manner in renal cells (Williams and Gooch, 2014). Together with our results, this suggests a possible role for the calcineurin-NFAT pathway in the regulation of pathogen-dependent phagosomal ROS production. Further studies are however required to determine the precise interaction between calcineurin and NADPH oxidase regulation.

Phagosomal maturation is key to pathogen clearance following phagocytosis. This is achieved by multiple fusion and fission events by the phagosomal membrane with early and late endosomes and lysosomes finally resulting in an inhospitable environment resulting in pathogen degradation (Smith and May, 2013). Within fungal infection, this has been well characterised in macrophage *C. albicans* infection with intracellular host trafficking of Rab GTPases critical to the formation of mature phagosome and fungal killing (Okai et al., 2015). The role of calcineurin in macrophage phagosomal maturation following fungal infection has not been fully explored. In this chapter, I show that calcineurin inhibition results in increased LAMP-1 compared to Rab7 recruitment to the phagosome

at early time-points (30 min) following infection suggesting an accelerated phagosomal maturation process. Given no difference in phagolysosomal acidification post infection was noted, the significance of this is not clear. Studies in *Mycobacterium tuberculosis* have suggested that calcineurin activation results in cessation of phagosomal maturation. Co-opting of the actin-binding protein coronin-1 by mycobacteria to activate calcineurin results in prevention of phagosomal maturation (Jayachandran et al., 2007). Calcineurin inhibitors therefore may result in an accelerated phagosomal maturation process. *Cryptococcus neoformans* has been shown to manipulate phagosomal maturation by causing premature removal of Rab 5 and Rab 11 resulting in hindering of acidification and protease activity (Smith et al., 2015). Further work is required to establish whether the calcineurin-dependent accelerated phagosomal maturation seen during fungal infection of human macrophages is directly detrimental to host fungal killing.

Effective fungal killing in vivo has been shown to be dependent on TNF- α and GM-CSF production and chemokine dependent neutrophil, monocyte and NK cell influx (Mehrad et al., 1999b, Brummer et al., 2001, Kasahara et al., 2016, Phadke and Mehrad, 2005, Mehrad et al., 2000). My results show that TNF- α , GM-CSF and chemokine (MCP-1, MIP1- α and MIP-1 β) production by human MDM is calcineurin-dependent. This is consistent with our groups previous work utilising murine and zebrafish in vivo models of invasive aspergillosis (Herbst et al., 2015). A critical role for the inflammasome has additionally been shown in the innate immune response to fungal infection and susceptibility to invasive fungal infections post solid-organ transplantation (Wojtowicz et al., 2013, Tavares et al., 2015, van de Veerdonk et al., 2015, Karki et al., 2015b). Although no calcineurin-dependent difference was seen in IL-1 α production, there was a significant reduction in IL-1 receptor antagonist (IL-1Ra) levels. The failure to detect significant IL-1 β in culture supernatants may represent a failure of the assay and hence requires repetition for validation. IL-1Ra has been shown to ameliorate inflammasome-dependent inflammation, with defective secretion leading to an over-exuberant inflammatory response and host damage in human cystic fibrosis (Iannitti et al., 2016). Further studies are required to determine the calcineurin-dependent regulation of inflammasome

production in invasive aspergillosis, but defective IL-1Ra production may present a mechanism of increased inflammasome-dependent inflammation and host damage in solid-organ transplantation.

In summary, in this chapter I show that human MDM infected with germinated *A. fumigatus* conidia activate the calcineurin-NFAT pathway and kill *A. fumigatus* conidia through calcineurin-dependent phagocytosis, phagosomal reactive oxygen species and cytokine production.

5.1 Actin-dependent lateral transfer of live *A. fumigatus* swollen conidia between human macrophages

5.1.1 Introduction - Host-pathogen interaction – microbial escape

5.1.1.1 Actin-based bacterial escape mechanisms

Intracellular pathogens utilise host cells to facilitate replication and evade the immune system (Thi et al., 2012). Ultimately however, pathogen exit is of fundamental importance to transmission and dissemination of disease. Within bacterial infection, microbial exit has been shown to be a strictly co-ordinated process mediated by both bacterial and cellular factors (Traven and Naderer, 2014b). A wide-range of pathogens (including bacterial and viral) have evolved to exploit actin-based motility as a means of spreading into neighbouring host cells or escaping into the cytosol (Traven and Naderer, 2014b). The actin cytoskeleton is a highly dynamic three-dimensional framework within eukaryotic cells and provides force to enable directional movement and shape formation in response to stimuli (Abd-el-Basset and Fedoroff, 1994, Castellano et al., 2001a). Actin exists in two forms; monomeric (G)-actin which is cytosol soluble and filamentous (F)-actin which are thin flexible helically arranged actin polymers (Gaspar and Tapon, 2014). Assembly of actin is asymmetric and utilises ATP-bound monomers which polymerise on to the ‘barbed’ end of actin filaments, capping proteins to block filament assembly and severing proteins to break into short filaments (Callan-Jones and Voituriez, 2016). Trimers of actin (actin nuclei) form the nucleating structures for spontaneous polymerisation of monomeric actin into filaments. This dynamic scaffold is exploited by numerous pathogens such as *Listeria monocytogenes*, *Shigella flexneri* and *Vaccinia* virus which manipulate the activity of the highly conserved eukaryotic Arp (‘actin-related protein’)2/3 complex for de novo actin nucleation (Ally et al., 2004, Welch et al., 1997, Komano et al., 2004). The Arp2/3 forms a complex with other cellular proteins such as members of the Wiskott-Aldrich syndrome protein (WASP)

family to stimulate the nucleation of actin filaments that grow from actin filament barbed ends (Millard et al., 2004, Higgs and Pollard, 2001).

In *L. monocytogenes*, following internalisation, a phagocytic vacuole is formed and then rapidly lysed (possibly through exploitation of cellular ion homeostasis and host ion-channel function), releasing the bacterium into the cytosol (Radtke et al., 2011, Haschka et al., 2015). The *L. monocytogenes* protein ActA mediates actin assembly by binding to the Arp2/3 complex to form actin-tails and enable spreading from one cell into adjacent cells by pushing against cell membranes to form a finger-like protrusion that contains the bacterium at its tip. This is then taken up by the adjacent cell by an endocytic process. The pore-forming toxin Listeriolysin O (LLO) is required for *L. monocytogenes* cell-to-cell spread in macrophages due to its ability to cause damage to the plasma membrane and subsequent hijacking of efferocytosis (a process by which dying or dead cells are removed by phagocytosis) (Czuczman et al., 2014). A similar Arp2/3 dependent process occurs in *Shigella flexneri* and *Vaccinia virus*. More recently, *Burkholderia pseudomallei* and *B. mallei*, which are bacterial pathogens that use the trimeric autotransporter BimA to facilitate actin-based motility, have been shown to mimic different host actin-polymerising proteins. *B. pseudomallei* and *B. mallei* BimA mimic host Formin and/or Enabled Vasodilator associated phosphoprotein (Ena/VASP) family proteins in their ability to nucleate, elongate and bundle actin filaments (Benanti et al., 2015). This ability of pathogens to imitate the full spectrum of host actin-polymerising pathways to spread between cells is thought to be critical in the pathogen's ability to disseminate and evade the host immune system (Czuczman et al., 2014, Traven and Naderer, 2014b).

5.1.1.2 Fungal pathogen escape mechanisms

Fungal pathogens have also evolved to develop mechanisms of macrophage escape. This was initially shown with the encapsulated yeast *Cryptococcus neoformans* (Ma et al., 2006, Alvarez and Casadevall, 2006). *C. neoformans* has been shown to undergo a process of non-lytic expulsion as well as direct cell-cell spread. Macrophage phagosomes containing intracellular *C. neoformans* undergo

repeated cycles of actin polymerisation creating a highly dynamic actin cage dependent with WASP-Arp2/3 complex mediated actin filament nucleation. This acts to prevent pathogen expulsion with failure resulting in fusion of the phagosomal and plasma membrane, phagosomal permeabilization and ultimately non-lytic expulsion and pathogen dissemination (Johnston and May, 2010). In addition, *C. neoformans* can also rarely escape macrophages through a process of host cell-to-cell transfer which is actin-dependent (Alvarez and Casadevall, 2007).

Non-lytic expulsion and cell-to-cell spread has additionally been described for *C. albicans* and *C. krusei* (Bain et al., 2012, Garcia-Rodas et al., 2011). *C. albicans* has additionally recently been shown to hijack the host programmed cell death defence pathway, achieving macrophage escape through triggering of pyroptosis, a caspase-1 dependent pro-inflammatory form of cell death during hyphal transition (Uwamahoro et al., 2014). *A. fumigatus*, in contrast, has been shown to primarily achieve macrophage escape following phagocytosis by inhibition of phagolysosomal acidification and causing macrophage lysis through fungal germination (Slesiona et al., 2012). To date, non-lytic expulsion or cell-to-cell spread for *Aspergillus spp.* has not been shown.

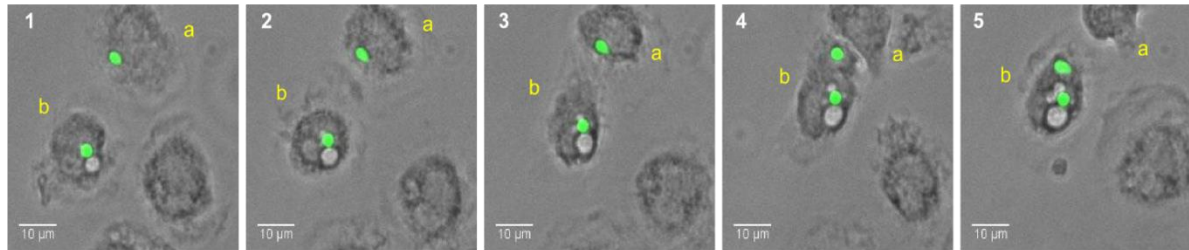
5.1.2 Human macrophages infected with *A. fumigatus* conidia undergo calcineurin-dependent lateral transfer to neighbouring macrophages.

To further characterise the effects of calcineurin inhibition on macrophage control of *A. fumigatus* infection, live time-lapse widefield imaging was performed. Human MDM derived from healthy volunteers were infected with live eGFP-expressing *A. fumigatus* swollen conidia (MOI = 1) following pre-treatment with FK506 (10 ng/ml) or vehicle for 1 h. Images were taken at 10 min intervals for 12 h p.i..

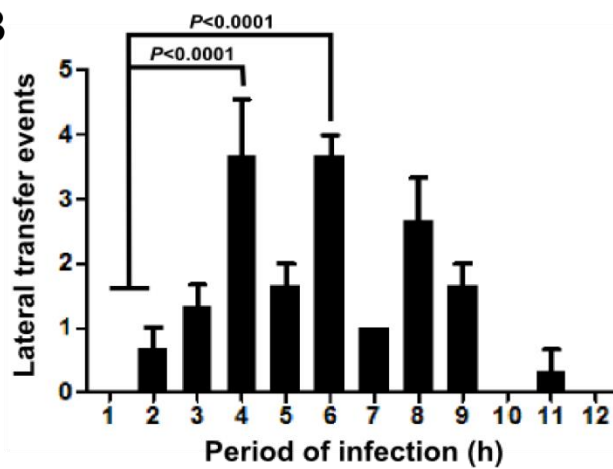
We observed frequent lateral transfer events of *A. fumigatus* swollen conidia between human MDM. Lateral transfer events occurred in $3.0 \pm 0.4\%$ of cells infected with live *A. fumigatus* swollen conidia,

peaking at 4 to 6 h p.i.. Calcineurin inhibition with FK506 resulted in reduced lateral transfer (53.9 $\pm$ 10.3%) suggesting a calcineurin-dependent mechanism (Figure 16).

A



B



C

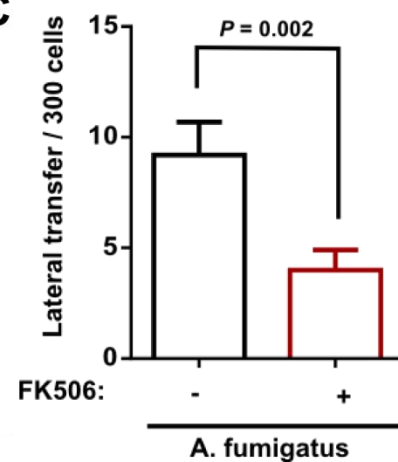


Figure 16: Human macrophages infected with *A. fumigatus* conidia undergo calcineurin-dependent lateral transfer to neighbouring macrophages.

MDM differentiated from healthy volunteers were infected with live *eGFP*-expressing swollen *A. fumigatus* conidia (MOI = 1) following pre-treatment with FK506 (10 ng/ml) or vehicle and visualized with time-lapse widefield microscopy at 10 min intervals for 12 h p.i.. Lateral transfer events were visualized and innumerated manually. **(A)** Representative time-lapse widefield microscopy images of *A. fumigatus* lateral transfer in human MDM infected with live *A. fumigatus* swollen conidia (shown in green). Transferring macrophage labelled 'a' and receiving macrophage labelled 'b'. Images taken at 10 min intervals. n=3, (n = biological replicates). Scale bars represent 10 μ m. **(B)** Timing of lateral transfer events in macrophages infected with live *A. fumigatus* swollen conidia is shown. Lateral transfer of conidia peaks at late time-points (4 and 6 h p.i.) compared to phagocytosis n=3, (n = biological replicates). **(C)** Human MDM pre-treated with FK506 (10 ng/ml) or vehicle were stimulated with live *A. fumigatus* swollen conidia and lateral transfer events quantified by time-lapse widefield imaging at 10 min intervals for 12 h p.i.. Lateral transfer of live *A. fumigatus* swollen conidia between macrophages was calcineurin-dependent. n=5, (n = biological replicates). Bars represent mean + SEM and samples were analysed by Kruskal-Wallis test with Dunn's post-test calculation **(B)** and

Wilcoxon matched pairs test (C). Videos are available to view at <http://www.atsjournals.org/doi/suppl/10.1164/rccm.201601-0070OC>

5.1.3 Macrophage transfer endosomes containing live germinating *A. fumigatus* to neighbouring cells through an actin-dependent process.

We further assessed the fungal contribution to lateral transfer of *A. fumigatus* by time-lapse microscopy of metabolically inactive resting conidia or dead fixed swollen conidia (MOI = 1) infected human MDM from healthy volunteers. Lateral transfer of *A. fumigatus* between human macrophages was dependent on both live and swollen *A. fumigatus* conidia (Figure 17A). Human macrophages infected with swollen *A. fumigatus* conidia (MOI = 1) were fixed and stained for actin, dynamin and clathrin at 15 min intervals between 2-3 h p.i. following phagocytosis and washing of external conidia 45 min p.i.. An actin, dynamin and clathrin-dependent process was suggested by co-localisation to conidia undergoing lateral transfer (Figure 17B-D). This was confirmed by time-lapse widefield microscopy after treating human MDM with actin polymerisation and dynamin inhibitors (Cytochalasin D and Dynasore) following phagocytosis (Figure 17A). The live swollen *A. fumigatus*-containing cargo transferred between human macrophages was further defined by staining for endo-lysosomal markers (Rab 5, Rab7 and LAMP-1). This showed that *A. fumigatus* transferred between human MDM are in predominantly Rab7-positive and Rab5 and LAMP-1-negative compartments, suggesting late endosomal trafficking (Figure 17D-E). Lastly, characterisation by transmission electron microscopy was performed by fixing human MDM infected with live swollen *A. fumigatus* conidia at 15 min intervals from 2-3 h p.i. following phagocytosis and washing of external conidia 45 min p.i.. This demonstrated transfer of *A. fumigatus*-containing cargo between human MDM in membrane-bound structures (Figure 17F).

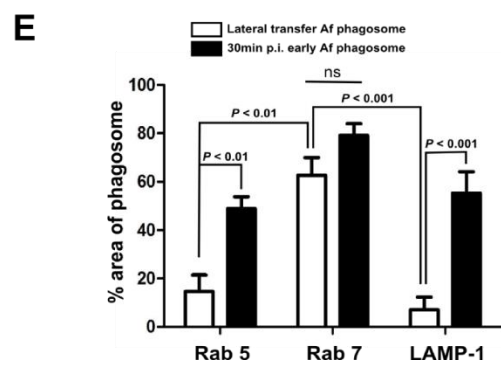
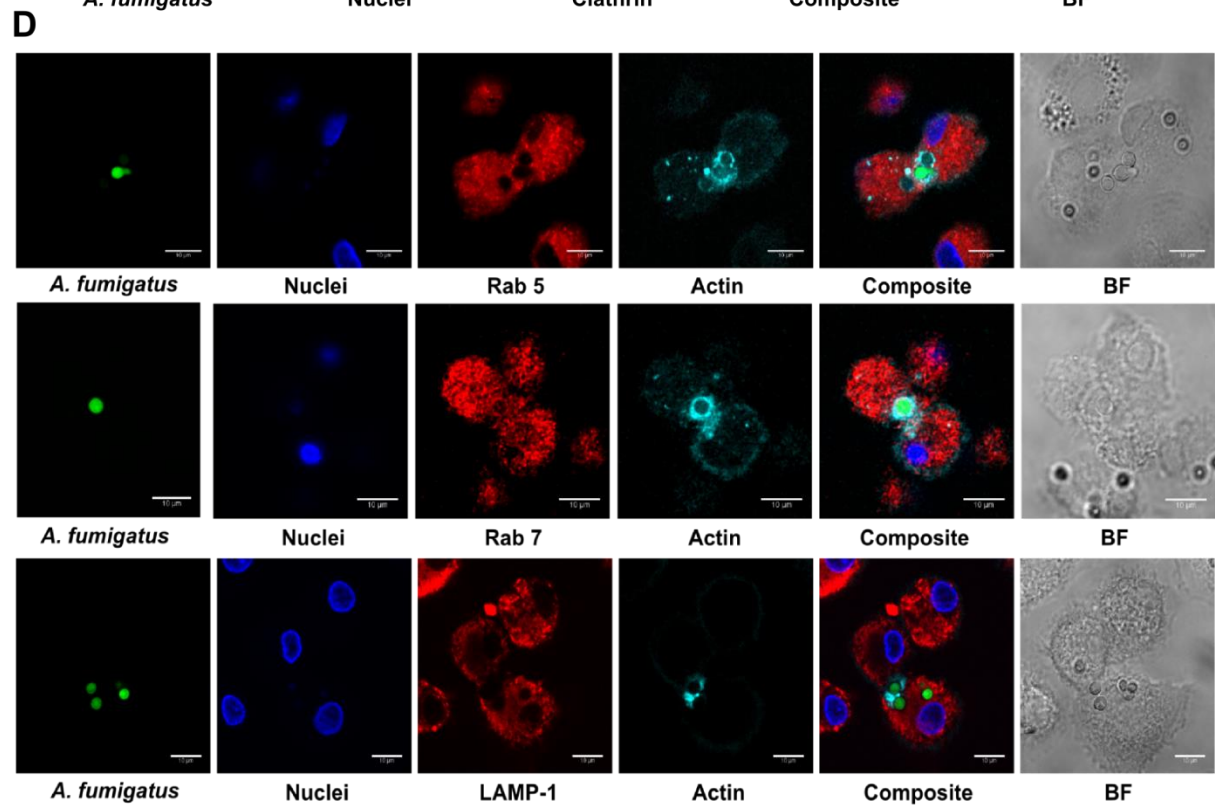
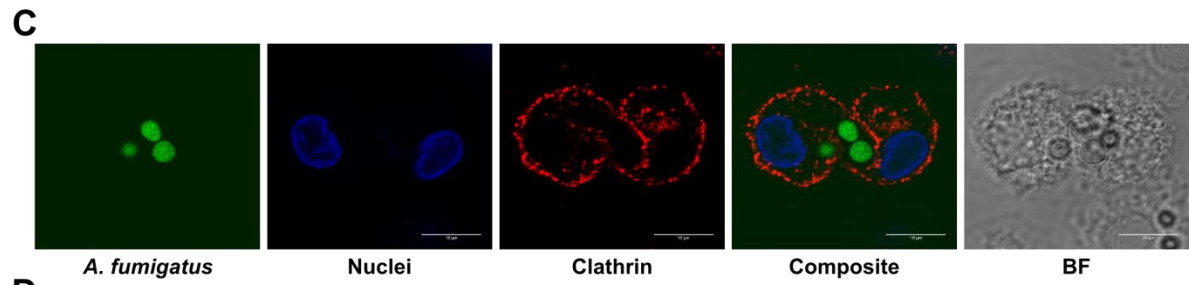
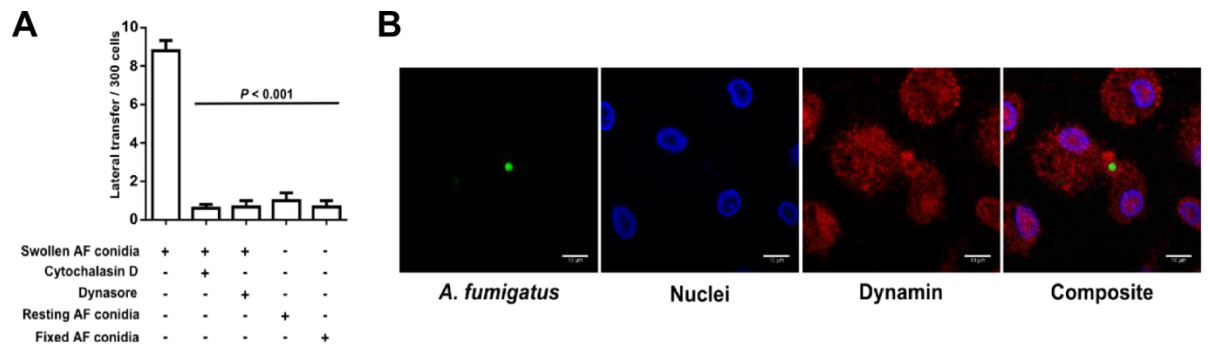


Figure 17: Macrophage transfer endosomes containing live germinating *A. fumigatus* to neighbouring cells through an actin-dependent process.

(A) MDM differentiated from healthy volunteers were infected with *eGFP*-expressing resting, live and fixed swollen *A. fumigatus* conidia (MOI = 1). Following phagocytosis, unbound conidia were washed away at 45 min p.i. and Cytochalasin (5 nM), Dynasore (100 μ M) or vehicle added at 90 min p.i.. Time-lapse widefield microscopy was then performed and lateral transfer events quantified. Lateral transfer of *A. fumigatus* between human macrophages was dependent on live germinating conidia and was actin and dynamin dependent. $n=4$, (n = biological replicates) **(B-D)** MDM were infected with live swollen *A. fumigatus* conidia and unbound conidia washed away post-phagocytosis (45 min p.i.). Macrophages were then fixed at 15 min intervals 2-3 h p.i., stained for dynamin **(B)**, clathrin **(C)**, actin and endolysosomal markers (Rab5, Rab 7 and LAMP-1) **(D)** and imaged by confocal microscopy. **(B)** Representative images showing significant co-localisation of dynamin to *A. fumigatus* conidia being transferred between human macrophages. **(C)** *A. fumigatus* conidia being transferred also appeared to be within clathrin-coated compartments suggesting clathrin-mediated endosomal trafficking. **(D)** Representative images showing endolysosomal and actin staining during human macrophage *A. fumigatus* lateral transfer. Significant actin co-localisation to *A. fumigatus* conidia being transferred between human macrophages is seen suggesting an actin-dependent process of transfer. Images show positive Rab7 localisation to *A. fumigatus* containing endosomes during lateral transfer with little Rab5 localisation and no LAMP-1 localisation. **(E)** Phagosomal intensity of maturation markers (Rab5, Rab7 and LAMP-1) assayed by confocal microscopy in MDMs infected with live *A. fumigatus* swollen conidia undergoing lateral transfer and 30 mins p.i. was quantified by *ImageJ*. Lateral transfer of *A. fumigatus* between macrophages occurs in phagosomes with significant Rab7 localisation compared to Rab5 and LAMP-1. This is in contrast to early phagocytosis where significantly increased Rab5 and LAMP-1 phagosomal localisation is present. This suggests lateral transfer of *A. fumigatus* occurs within those late endosomal compartments that have not fully matured. $n=3$ (n = biological replicates, Each biological replicate includes 5 *A. fumigatus* containing phagosomes). **(F)** Representative transmission electron microscopy image of *A. fumigatus* lateral transfer between human macrophages (conidia highlighted with red arrows). *A. fumigatus* conidia are seen being transferred within membrane-bound structures into the receiving macrophage. Bars represent mean + SEM and samples were analysed by Kruskal-Wallis test with Dunn's post-test calculation. Scale bars represent 10 μ m **(B-D)** and 2 μ m **(F)**.

5.1.4 Alveolar macrophages isolated from lung transplant recipients and infected with *A. fumigatus* conidia undergo lateral transfer to neighbouring macrophages.

Although human MDM differentiated with human serum and GM-CSF are broadly similar in phenotype to AM there remains some significant differences (see Figure 7). To identify whether lateral transfer is a macrophage conserved process that is relevant to human AM, time-lapse confocal microscopy was performed using human AM isolated from lung transplant recipients and infected with live *eGFP*-expressing swollen *A. fumigatus* conidia. Similar lateral transfer of swollen *A. fumigatus* conidia was seen to neighbouring macrophages at late time-points after infection (see Figure 18).

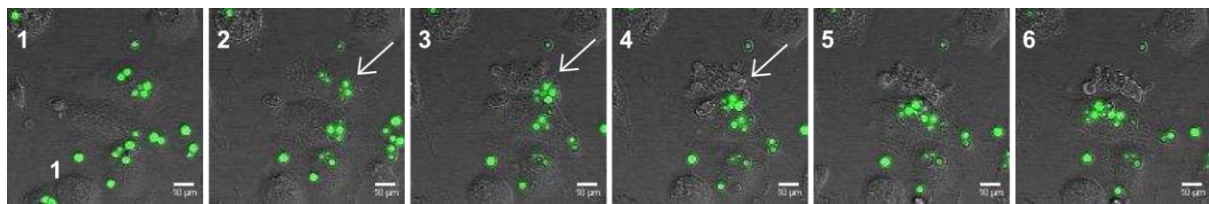


Figure 18: Human alveolar macrophages from lung transplant recipients infected with swollen *A. fumigatus* conidia undergo lateral transfer to neighbouring macrophages.

Human AM were isolated from lung transplant recipients, rested for 3 days and then infected with live *eGFP*-expressing swollen *A. fumigatus* conidia (MOI = 1). Time-lapse confocal microscopy was then performed and lateral transfer events identified. The representative images above show lateral transfer from human AM infected with *eGFP*-expressing swollen *A. fumigatus* conidia to neighbouring macrophages. The white arrow highlights the conidia during the transfer process. Images are at 10 min intervals, and occur 4 h post-infection. Videos are available online to view at <http://www.atsjournals.org/doi/suppl/10.1164/rccm.201601-0070OC>.

5.1.5 In vivo macrophage lateral transfer of *A. fumigatus* conidia: Macrophages in zebrafish embryos infected with *A. fumigatus* conidia undergo lateral transfer to neighbouring macrophages.

To evaluate the in vivo relevance of macrophage lateral transfer of *A. fumigatus* conidia, we utilised our zebrafish model of invasive aspergillosis. Mpeg-mCherry zebrafish embryos were infected with *eGFP*-expressing *A. fumigatus* conidia further labelled with Alexa-fluor 488 and high-resolution time-lapse confocal microscopy performed to observe the macrophage-*A. fumigatus* interaction over 12 h post-infection. Confirming its in vivo relevance and an evolutionary conserved role, macrophage lateral transfer of *A. fumigatus* conidia to neighbouring cells was seen (Figure 19).

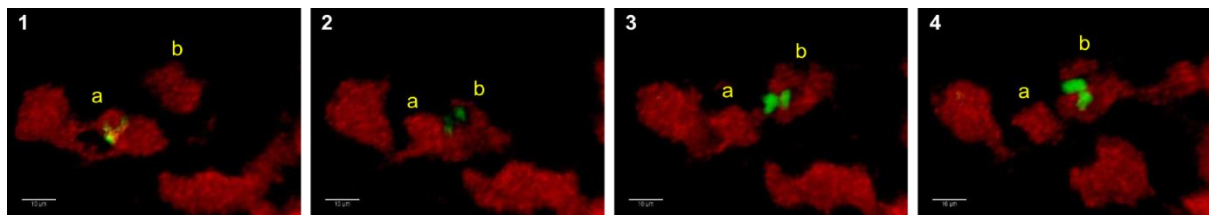


Figure 19: Zebrafish macrophages infected with swollen *A. fumigatus* conidia undergo lateral transfer to neighbouring cells.

MPEG:mCherry Zebrafish larvae were infected with ~50 live *eGFP*-expressing conidia of *A. fumigatus* and time-lapse confocal microscopy performed. Representative images show *A. fumigatus* lateral transfer between macrophages. The transferring macrophage is labelled 'a' and the receiving macrophage labelled 'b'. Images shown are at 7 min intervals. Images representative of 3 lateral transfer events over 7 fish. Videos are available online to view at <http://www.atsjournals.org/doi/suppl/10.1164/rccm.201601-0070OC>.

5.2 Calcineurin-dependent lateral transfer of *A. fumigatus* within actin-VASP enveloped endosomes in human macrophages undergoing programmed necrosis controls fungal germination.

5.2.1 Introduction: Actin-based cytoskeletal remodelling.

5.2.1.1 Role of calcineurin in actin-cytoskeletal remodelling

A major role of macrophages is their ability to migrate and internalise particles larger than 0.5 μm by a process known as 'phagocytosis'. This is dependent on changes in cell morphology due to actin polymerisation (Flannagan et al., 2012). The actin cytoskeleton is a three-dimensional filamentous protein network that provides a structural framework to the cell and governs cell migration, adhesion, phagocytosis and vesicular trafficking (Gardel et al., 2010, Guerra et al., 2014, Croise et al., 2014). It also provides a platform for signal transduction and enables modulation of signalling pathways to affect cellular function (Nusco et al., 2006, Coppolino et al., 2001). Actin polymerisation is dependent on the Arp2/3 complex which is in itself reliant on nucleation-promotion factors (NPFs) to initiate new filament formation (Beltzner and Pollard, 2008). Specifically for Fc γ R, the most widely studied phagocytic receptor, binding to opsonized particles results in receptor clustering and small Rho-family GTPase recruitment which via NPFs (e.g. Wiscott-Aldrich syndrome protein (WASP) family proteins) activate the Arp2/3 complex (Tollis et al., 2010). This promotes actin filament branching resulting in an increase in the number of uncapped ends and isotropic growth of the actin network (Flannagan et al., 2012).

The importance of calcium signalling, calcineurin and NFAT within actin cytoskeletal remodelling and signalling has been suggested by studies showing a role for the actin cytoskeleton in the regulation of calcium dynamics and NFAT activation during T cell activation (Rivas et al., 2004). Further studies have indicated that increases in cytosolic calcium concentration may play a role in phagocytosis,

although its exact role is unclear (Nunes and Demaurex, 2010, Stossel, 1973). Studies have additionally shown reactive-oxygen species production during phagocytosis and phagosomal maturation to additionally be a calcium-dependent process (Gordeeva et al., 2003, Malik et al., 2000).

As a calcium-dependent phosphatase, calcineurin has been thought to play a role in cytoskeletal remodelling (Halpain et al., 1998, Liu and Berg, 1999). Calcineurin was first implicated in neuronal cytoskeletal remodelling, with effects on microtubular assembly and disassembly, and regulation of endocytosis (Goto et al., 1985, Wu et al., 2014, Lai et al., 1999). Further roles have been identified in actin-associated cytoskeletal remodelling. Cofilin is a potent regulator of actin filament dynamics and its activity is determined by its phosphorylation state at a unique site (Ser3) (Bravo-Cordero et al., 2013). Cofilin mediates its rapid turnover by severing the actin filaments and stimulating disassembly near the leading edge. Its activity is inhibited by phosphorylation at Ser-3 by LIM kinases and TES kinases (Bierne et al., 2001). Recent studies have shown that calcium-induced calcineurin activation results in dephosphorylation of protein phosphatase Slingshot-1L (SSH1L) and subsequent increased SSH1L-mediated cofilin dephosphorylation and activation (Wang et al., 2005).

Further studies have implicated a role for calcineurin in regulation of WAVE1 proteins which are part of the WASP family of proteins (Ceglia et al., 2010). Members of the WASP family have a conserved C-terminus containing one or two WASP homology (WH2) domains responsible for Arp2/3 complex binding and activation, a proline-rich region and an N-terminal region which contains domains thought to be important for interaction with regulatory proteins (Pollitt and Insall, 2009). The phosphorylation state of WASP proteins are thought to be critical in regulating their function (Kim et al., 2006, Ceglia et al., 2010, Pollitt and Insall, 2009). Serine-threonine phosphorylation of WAVE1 inhibits its ability to activate the Arp2/3 complex in vitro and in vivo (Kim et al., 2006). Calcineurin has been shown to be a critical serine-threonine phosphatase involved in WAVE1 activation (Ceglia

et al., 2010, Li et al., 2015). The role and importance of calcineurin in host actin cytoskeletal remodelling during fungal infection, however, has yet to be characterised.

5.2.1.2 Vasodilator stimulated phosphoprotein

Actin cytoskeletal function is determined by specific actin-binding proteins which enable nucleation, elongating, capping, bundling/crosslinking and severing (Stricker et al., 2010, dos Remedios et al., 2003). Cell motility and phagocytosis are driven by lamellipodia, which are protruding structures at the leading edge of the cell composed of short-branched actin filaments (Flannagan et al., 2012, Hansen and Mullins, 2015). The Ena/Vasodilator stimulated phosphoproteins (VASP) are a family of proteins important in regulating actin dynamics (Krause et al., 2003). They localise to sites of actin assembly in focal adhesions, actin-based protrusions at cell-cell contacts, and at lamellipodia, where they gather and rapidly elongate actin-barbed ends by antagonising inhibition by capping proteins and reduce filament branching (Edwards et al., 2014, Castellano et al., 2001b).

VASP are large multi-domain proteins with conserved domains. The N-terminal Ena-VASP homology 1 (EVH1) domain binds FP4 motif proteins (such as vinculin, lamellipodin and ActA) for localisation and recruitment (Hansen and Mullins, 2015, Coppolino et al., 2001, Huttelmaier et al., 1998, Machner et al., 2001). The central proline rich region contains binding sites for SH3 domains and the G-actin binding protein profilin which are required for multimerisation and for F-actin binding (Lambrechts et al., 2000, Winkelmann et al., 2014). The C-terminal coiled-coil facilitates tetramerisation and is necessary for processive actin filament elongation and in vivo function (Krause et al., 2003). It has been recently shown that VASP reinforces Arp2/3 complex-based actin assembly when recruited by WAVE interaction (Chen et al., 2014).

VASP was initially shown to be recruited to the surface of *Listeria monocytogenes* and required for optimal bacterial motility in vivo and in vitro (Laurent et al., 1999). Further studies have since

revealed VASP co-localisation with Arp2/3 in the phagocytic cups of macrophages and recruitment to FcγR-mediated phagosomes (Coppolino et al., 2001). Recruitment of VASP to FcγR phagosomes was required for phagocytosis-induced actin cytoskeletal remodelling, pseudopodial extension and efficient particle internalisation (Coppolino et al., 2001).

VASP function is tightly regulated by differential phosphorylation (Geese et al., 2002, Thomson et al., 2011, Gomez and Robles, 2004). Human VASP has three phosphorylation sites: serine 157 (S157), serine 239 (S239), and threonine 278 (T278) (Thomson et al., 2011). Phosphorylation is dependent on the cAMP and cGMP-dependent serine and threonine kinases, PKA and PKG (Walter et al., 1993, Becker et al., 2000, Li et al., 2003). Phosphorylation at S157 has been shown to influence VASP localisation but has minor impact on F-actin assembly (Lambrechts et al., 2000, Benz et al., 2008). S239 and T278 phosphorylation however, has been shown to significantly impair actin-filament formation (Benz et al., 2009, Doppler and Storz, 2013). The serine-threonine phosphatases involved in VASP regulation have not been well characterised.

Additionally, VASP has been shown to be a critical protein involved in bacterial escape mechanisms (Lee et al., 2015b, Bierne et al., 2005). Recruitment of VASP and Arp2/3 via the bacterial ActA protein in *Listeria monocytogenes* to the bacterial surface disguises the bacteria from autophagic recognition and enables bacterial intracellular motility and escape (Yoshikawa et al., 2009). As previously mentioned, recent studies have additionally shown *Burkholderia* spp utilise VASP mimic proteins (BimA) to exploit actin assembly pathways and enable cell-to-cell spread and dissemination (Benanti et al., 2015).

5.2.2 Introduction: Programmed cell death

5.2.2.1 Programmed cell death in the innate immune response to fungi

The initial response to infection is the stimulation of an inflammatory cascade, production of enzymes and molecules designed to kill the offending pathogen and recruitment of neighbouring inflammatory cells (Brown, 2011). However, should the above mechanisms fail, the ability of infected cells to die or undergo 'programmed cell death' to try and protect the remaining cells remains an important intrinsic part of the host defence (Ashida et al., 2011).

Electron microscopy study of cell ultrastructure during programmed cell death reveals different morphologies suggestive of different pathways (Burattini and Falcieri, 2013). Classically, this was divided into apoptotic and necrotic cell death (Duprez et al., 2009). Apoptosis is characterised morphologically by cytoplasmic shrinkage, nuclear condensation and the retention of membrane and organelle integrity (Elmore, 2007). For a long-time necrosis, characterised morphologically by cellular swelling and rupture was thought to be an uncontrolled 'non-programmed' event without signalling pathway activation (Zong and Thompson, 2006).

Since then, further research has significantly enhanced our understanding of programmed cell death including highlighting the importance of programmed necrotic cell death (Chan et al., 2015). Currently, programmed cell death is broadly divided into 4 subtypes; Apoptosis, Necrosis, Pyroptosis and Autophagic cell death (Duprez et al., 2009).

Apoptosis forms a key cellular defence response during pathogen infection with mitochondria playing a key role (Ashida et al., 2011, Behar et al., 2011, Desagher and Martinou, 2000). Two main protein families are involved in apoptosis; the Bcl-2 family of proteins which control mitochondrial integrity and the cysteinyl-aspartate specific proteases or caspases which are involved in the execution process (Czabotar et al., 2014, Kumar, 2007). Furthermore, apoptosis can be triggered by either an intrinsic or extrinsic pathway (Elmore, 2007). The extrinsic pathway is activated by

transmembrane death receptors such as FasL, TNF-R1, TRAIL-R and Apo2/Apo-3 which transmit apoptotic signals resulting in activation of caspase-3 and caspase-7 (Czabotar et al., 2014, Guicciardi and Gores, 2009). The intrinsic pathway is activated by the release of mitochondrial signalling factors which cause activation of Bcl2 homology 3 (BH3-only) proteins (Shamas-Din et al., 2011). This overcomes the inhibitory effect of Bcl-2 proteins, promoting oligomerisation of pro-apoptotic proteins in the mitochondrial outer membrane resulting in cytoplasmic release of cytochrome C. Cytochrome C helps form the apoptosome (a multimeric protein complex) which acts as a scaffold for caspase activation and ultimately apoptosis (Jiang and Wang, 2004).

Apoptosis has been shown to be beneficial in the host bacterial response, with induction of intrinsic apoptosis beneficial in dendritic cell clearance of *Legionella pneumophila* and defective apoptosis resulting in enhanced disease in murine models of pneumococcal infection (Nogueira et al., 2009, Dockrell et al., 2003). Given the importance of apoptosis in the host immune response, pathogens have gained the ability to modulate the host apoptotic response. For example, *Staphylococcus aureus* and *Listeria monocytogenes* have been shown to be able to activate cell apoptosis to prevent an appropriate inflammatory response (Esen et al., 2001, Carrero and Unanue, 2012). The role of programmed cell death in the host innate immune defence to invasive aspergillosis is poorly defined. *Aspergillus fumigatus* has been shown to produce gliotoxin, which induces apoptosis in a number of mammalian cells through a signalling pathway involving phosphorylation of the BH3-only protein Bim (Geissler et al., 2013). It can also however, inhibit AM apoptosis after infection in a melanin-dependent manner (Volling et al., 2007, Volling et al., 2011).

Recent evidence has identified multiple pathways that share the morphological characteristics of programmed 'necrotic' cell death (Vanden Berghe et al., 2014). TNF- α mediated cell death was initially identified as a form of regulated or programmed cell necrosis, with the subsequent discovery of receptor interacting protein kinase-1 and -3 (RIPK1 and RIPK3) dependent cell death being termed 'necroptosis' (Pasparakis and Vandenabeele, 2015). Ligation of TNF- α activates the TNF-receptor1

(TNFR1) and in turn recruits RIPK1 via the adaptor protein TNF receptor-associated death domain (TRADD) to the membrane (Sosna et al., 2014, Pasparakis and Vandenabeele, 2015). This is then recruited to form a complex containing ubiquitinated RIPK1, NF-κB essential modulator (NEMO) and inhibitor of NF-κB (IκB) kinase (IKK) complex resulting in NF-κB activation (Sawai, 2014, Han et al., 2011). Binding of death receptors also recruits RIPK1 to the cytosol for assembly of the death inducing signalling complex (DISC) which is comprised of adaptor protein FAS-associated death domain (FADD), caspase 8 and cellular FLICE-like inhibitor protein (cFLIP) (Tsuchiya et al., 2015). In the absence of active caspase 8, cytosolic RIPK1 can interact with RIPK3 kinase to generate the RIPK1/RIPK3 containing complex known as the 'necrosome' (Li et al., 2012a). The auto- and trans-phosphorylation of RIPK1 and RIPK3 and the recruitment of mixed lineage kinase domain-like (MLKL) then ultimately leads to the induction of necroptosis via translocation to mitochondria-associated membranes, reactive oxygen species production and JUN N-terminal kinase (JNK) activation (Moriwaki and Chan, 2013, Zhang et al., 2015, Christofferson and Yuan, 2010, Marshall and Baines, 2014). The necrosome interaction and phosphorylation of MLKL by RIPK3 has been shown to be a critical determinant of necroptosis downstream of RIPK3 with MLKL^{-/-} mice unable to undergo necroptosis (Cai et al., 2014, Wu et al., 2013).

Further research has since identified numerous other regulated necrosis pathways including ferroptosis, ETosis and parthanatos (Vanden Berghe et al., 2014, Dixon et al., 2012, Wartha and Henriques-Normark, 2008, David et al., 2009). These all share the morphological definition of regulated necrosis but are reported to occur independently of RIPK1 or RIPK3. Recent work has shown that plasmacytoid dendritic cells (pDC) infected with *A. fumigatus* conidia stimulate the formation of pDC extracellular traps containing DNA and citrullinated histone H3 in a dectin-2 and type-1 interferon dependent process which closely resembles neutrophil extracellular traps (NETs) (Loures et al., 2015). Macrophage extracellular traps (METs), a newly described process, have been identified following infection of murine J774A.1 and peritoneal macrophages with *Candida albicans*,

but have been shown to have limited microbicidal activity in vitro (Liu et al., 2014). As yet, there have been no studies identifying MET formation following *A. fumigatus* infection.

It is thought programmed necrosis may be an important mechanism to clear damaged cells during infection via recruitment of inflammatory cells (Christofferson and Yuan, 2010). Toll-like receptor and C-type lectin receptor recognition of damage-associated molecular patterns (DAMPs) alongside pathogen-associated molecular patterns (PAMPs) have been shown to regulate necrosome formation and cell death (Sancho and Reis e Sousa, 2013, Kaczmarek et al., 2013). The importance of programmed necrosis to the host immune response to infection is however as yet unclear. As with apoptosis, studies have revealed that a number of viral and bacterial pathogens modulate the cell programmed necrosis response. Diverse bacterial pathogens that produce a pore-forming toxin (PFT) (e.g. *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Listeria monocytogenes* and uropathogenic *Escherichia coli* (UPEC)) induce necroptosis of macrophages with RIPK1 inhibitors reducing severity of *S. marcescens* pneumonia in a murine model (Gonzalez-Juarbe et al., 2015). *Salmonella typhimurium* additionally has been shown to induce type-1 interferon associated macrophage necroptosis causing increased pathogenicity in murine models (Robinson et al., 2012). As yet, the relevance and importance of programmed necrosis in invasive fungal infections is undetermined.

Pyroptosis has been termed to describe a caspase-1 dependent form of cell death initially seen with *Salmonella enterica* infection which results in the formation of pores in the cell membrane and release of cytosolic contents in particular IL-1 β and IL-18 causing inflammation and fever (Fink and Cookson, 2007). Caspase-1 activation is dependent on Nod-like receptor (NOD) receptor formed inflammasomes with pyroptosis shown to be a potent innate immune effector mechanism to clear intracellular pathogens within in vivo models of *Francisella novicida* infection (Miao et al., 2011, Mariathasan et al., 2005). Recent data has shown that bone marrow derived macrophages (BMDMs) infected with *C. albicans* die in part through a pyroptotic mechanism with BMDMs from Caspase-1^{-/-}

mice showing resistance to cell death (Uwamahoro et al., 2014). Given, that susceptibility to invasive aspergillosis in murine in vivo studies has been shown to be Nod-like receptor pyrin-containing domain-3 (NLRP3), (a critical component of the inflammasome), and Caspase-1 dependent with a strong inflammasome response seen in vitro, pyroptosis is likely to be important in the host immune response to *A. fumigatus* (Karki et al., 2015b, Said-Sadier et al., 2010b). To date however, there have been no studies identifying pyroptosis induction following macrophage infection with *A. fumigatus*.

A mechanism of intracellular pathogen survival after phagocytosis is escape into the cytoplasm (Traven and Naderer, 2014a). Autophagy is a lysosomal degradation pathway that mediates clearance of intracellular pathogens (e.g. *M.tuberculosis*) by engulfment into autophagosomes after their escape to the cytosol (Campoy and Colombo, 2009, Gutierrez et al., 2004). The autophagosomes, which are double membrane cytoplasmic vesicles, then fuse with lysosomes to form autolysosomes leading to digestion of their cargo (Deretic et al., 2013). Autophagic cell death is a morphological observation of cell death associated with autophagosome/autolysosome formation (Tsujimoto and Shimizu, 2005). As with other programmed cell death mechanisms, several pathogens (e.g. *Legionella pneumophila*, *Coxiella burnetti* and *Brucella abortus*) have been shown to modulate autophagy machinery to use autophagosomes as a means to escape detection and systemic immunity and allow replication (Gutierrez et al., 2004). Recent work has highlighted the importance of autophagy machinery for fungal clearance in human monocytes infected with *A. fumigatus* (Kyrmizi et al., 2013).

5.2.2.1 Role of calcineurin in programmed cell death

Calcium-regulated cell death has been well established since studies analysing myocardial cell death post-infarction (Persoon-Rotherth et al., 1994). Calcium-mediated mitochondrial permeability was subsequently shown to play a key role in apoptosis regulation (Lemasters et al., 2009). The activation of calcium-dependent kinases and phosphatases are a method of transcriptional regulation of programmed cell death (Orrenius et al., 2003). A role for calcineurin was suggested when

cyclosporine A was shown to prevent calcium-dependent apoptosis in lymphoid cells (Yazdanbakhsh et al., 1995). Further studies, however showed a protective role for calcineurin against oxidant-induced stress in cardiac myocytes (De Windt et al., 2000). Thus, although cell death regulation may be calcineurin-dependent its exact function may be stimulus and cell type dependent (Orrenius et al., 2003, Sun and Wang, 2012).

Studies have since established a pro-apoptotic mechanism for calcineurin by dephosphorylation of the Bcl-2 family member Bad (Bcl-2 associated death promoter) resulting in translocation from the cytoplasm to the mitochondria and stimulation of cytochrome c release and subsequent caspase activation (Wang et al., 1999, Li et al., 2013). The Bcl-2 family proteins, known to be critical regulators of apoptosis have also been shown to be highly important regulators of TNF- α mediated and Z-VAD-FMK induced necroptosis (Jiang et al., 2013, Hitomi et al., 2008, Tischner et al., 2012). A recent genome-wide siRNA screen revealed Bcl-2 modifying factor (Bmf), (a BH3-only Bcl-2 family member) to be a critical regulator of necroptosis (Hitomi et al., 2008). Thus, effects of calcineurin on Bcl-2 family protein activation are likely to have regulatory effects on apoptosis and necroptosis.

Although there is some controversy, mitochondrial fission and permeability appear to have a role in programmed necrosis as well as apoptosis (Elgass et al., 2013, Guo et al., 2014, Moujalled et al., 2014). Mitochondrial fission requires translocation of dynamin related protein-1 (Drp-1) to the mitochondria (Cereghetti et al., 2010). This process is dephosphorylation and calcineurin dependent, and has been shown to be required for programmed necrosis in human lymphocytes (Cereghetti et al., 2006, Bras et al., 2007). This may potentially represent another calcineurin-regulatory pathway of programmed cell death.

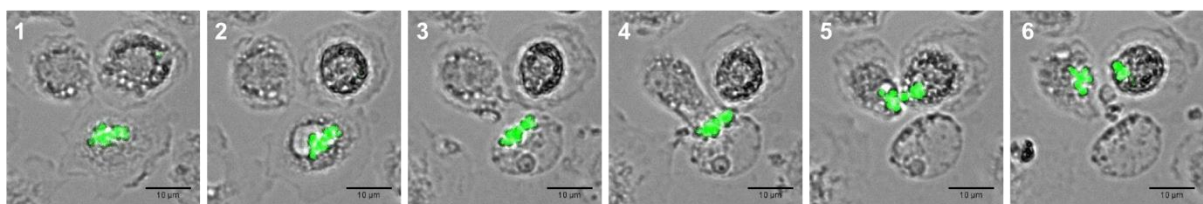
Lastly, numerous studies have shown the p-38 MAPK and JNK pathways to be important regulators of programmed cell death (Deacon et al., 2003, Kim and Lee, 2005, Liu and Lin, 2005, Xu et al., 2014). As regulators of the calcineurin-NFAT pathway through p38-MAPK and JNK mediated phosphorylation of NFAT, and intrinsically linked through negative feedback loops, any effect on

MAPK/JNK pathways secondary to calcineurin-NFAT inhibition is likely to have effects on programmed cell death regulation (Gonzalez-Guerrero et al., 2013, Liang et al., 2003, Wu et al., 2003a, Lee et al., 2012). As with calcineurin, the effects of JNK on programmed cell death are likely to be cell type and stimulus dependent (Liu and Lin, 2005).

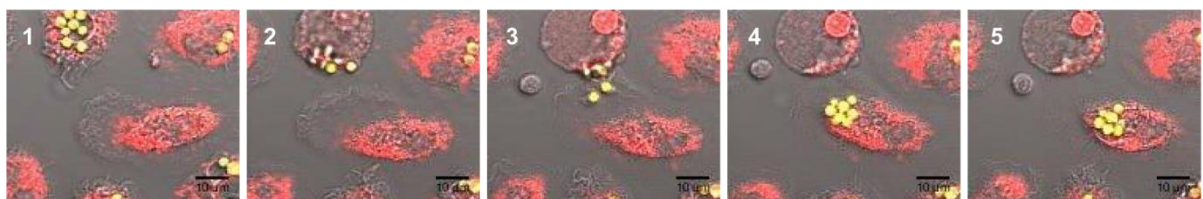
5.2.3 Human macrophages transfer *A. fumigatus* conidia during programmed necrosis to prevent fungal germination

During time-lapse widefield and confocal imaging of lateral transfer of *A. fumigatus* conidia between infected human MDM and AM to neighbouring macrophages, transferring macrophages had morphological appearances consistent with a programmed cell death response (Figure 20A-B). Transferring macrophages had increasingly translucent cytoplasm, organelle swelling and increased cell volume followed by cell lysis immediately following lateral transfer of conidia to neighbouring recipient macrophages (Figure 20A-B). Cell death in the transferring macrophage was confirmed by the addition of propidium iodide to the media to identify loss of membrane integrity (Figure 20C).

A



B



C

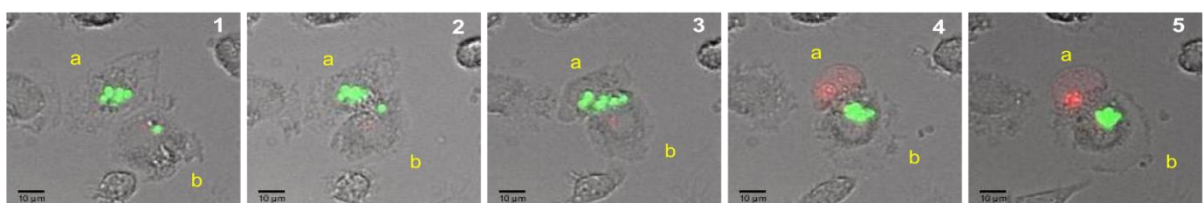


Figure 20: Human macrophages infected with *A. fumigatus* undergo lateral transfer during a programmed cell death response.

Human MDM were infected with live *eGFP*-expressing swollen *A. fumigatus* conidia (MOI = 1) and time-lapse confocal microscopy performed. **(A)** Representative images of human MDMs undergoing lateral transfer of *A. fumigatus* conidia (green) with the transferring macrophage showing features of programmed cell death (increasingly translucent cytoplasm, organelle swelling and increased cell volume followed by cell lysis). Images are 210 mins post infection at 10 min intervals. **(B)** Representative images of human MDM pre-loaded with Mitotracker Red and infected with live *eGFP*-expressing *A. fumigatus* conidia (MOI = 1). Again during lateral transfer the transferring macrophage has features consistent with programmed cell death with no mitochondrial activity demonstrated in the cytoplasm after lateral transfer. Images are 190 mins post infection at 10 min intervals. **(C)** Representative images of human MDM infected with *eGFP*-expressing *A. fumigatus* conidia (MOI = 1) with propidium iodide (PI) pre-added to the media. Programmed cell death in the transferring macrophage (labelled 'a') is confirmed with loss of membrane integrity and increased fluorescence following transfer. The receiving macrophage (labelled 'b') membrane is still alive following uptake of *A. fumigatus* conidia with intact membranes and no PI fluorescence. Images are 240 min post infection at 10 min intervals. All scale bars shown represent 10 μ m. Videos are available online to view at <http://www.atsjournals.org/doi/suppl/10.1164/rccm.201601-0070OC>.

Another feature consistently seen during time-lapse microscopy of human macrophages infected with *A. fumigatus* conidia was large vacuolar formation at later time-points post phagocytosis (Figure 21). Vacuole formation has been previously shown to be a feature of programmed cell necrosis. Transmission electron microscopy was used to further define the vacuole formation seen during *A. fumigatus* infection of human macrophages. This showed *A. fumigatus* conidia uptake within intact phagosomes at early timepoints post phagocytosis (Figure 21B). At later time-points post phagocytosis however, *A. fumigatus* conidia were seen to be residing within large cytoplasmic vacuoles (Figure 21C-D). Vacuole formation was inhibited by pre-treatment with the vacuolar H⁺-ATPase inhibitor Bafilomycin, with a trend towards decreased transfer (Figure 21E).

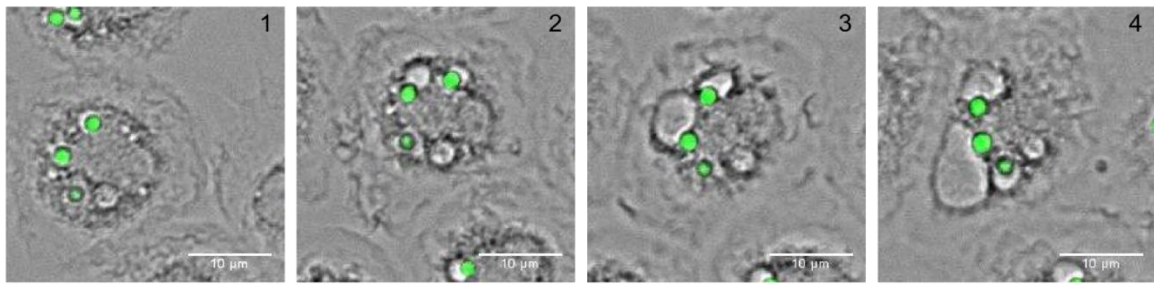
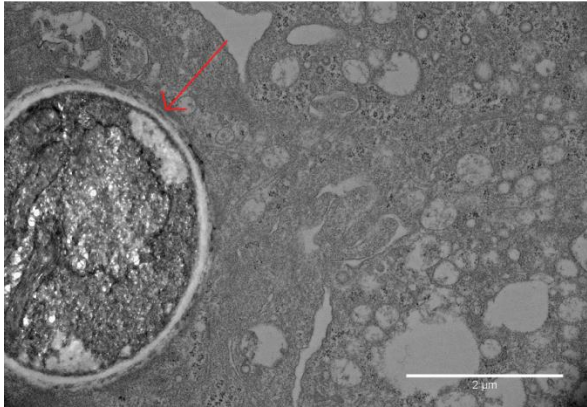
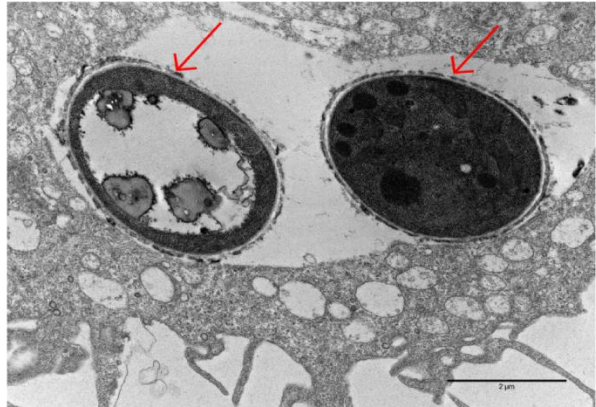
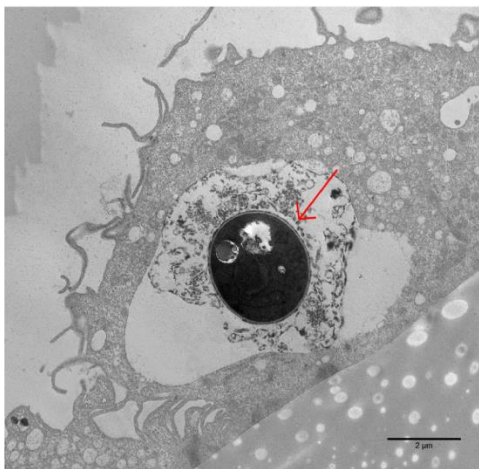
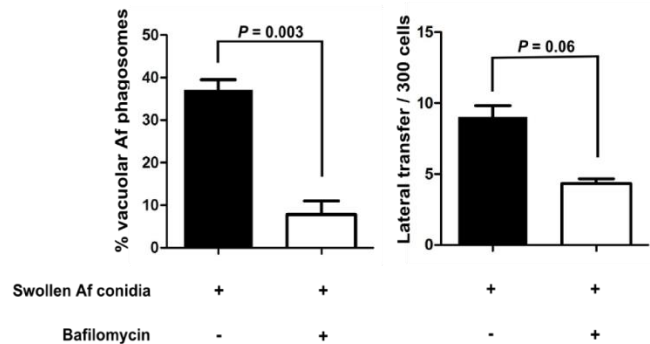
A**B****C****D****E**

Figure 21: Human macrophages infected with *A. fumigatus* form large vacuoles at late time-points post phagocytosis.

Human MDM were infected with live *eGFP*-expressing swollen *A. fumigatus* conidia (MOI = 1) and time-lapse widefield microscopy performed. **(A)** Representative widefield microscopy images showing vacuolar formation in human MDM infected with live *eGFP*-expressing *A. fumigatus* conidia at late time-points following infection. Multiple vacuole formation can be seen adjacent to internalised *A. fumigatus* conidia. Images are 120 min post infection at 20 min intervals. Scale bars represent 10 μ m. **(B)** Representative transmission electron microscopy image showing uptake of *A. fumigatus* conidia (labelled with a red arrow) 30 min post infection within intact phagosomes. **(C–D)**

Representative transmission electron microscopy images showing *A. fumigatus* conidia residing within large cytoplasmic vacuoles at late time-points following infection (180 mins post infection). Scale bars shown in **(B-D)** represent 2 μ m. **(E)** Quantification of vacuolar development and lateral transfer in human MDM pre-treated with Bafilomycin (100 nM) and infected with live swollen *eGFP*-expressing *A. fumigatus* conidia. (MOI = 1). Vacuolar development was dependent on endosomal acidification, with an additional trend towards decreased *A. fumigatus* lateral transfer in Bafilomycin-treated cells. n=3, (n = biological replicates). Bars represent mean + SEM and samples were analysed by Wilcoxon matched pairs test. (*Transmission electron microscopy performed with the assistance of Amelia Shoemark and Andrew Rogers.*) Videos are available online to view at <http://www.atsjournals.org/doi/suppl/10.1164/rccm.201601-0070OC>.

To attempt to define the programmed cell death response of human macrophages infected with live swollen *A. fumigatus* conidia, flow cytometry analysis was performed of human MDM pre and 6 h p.i.. Macrophages were stained with propidium iodide (PI) to analyse membrane integrity and Annexin V to stain membrane phosphatidylserine exposure as a marker of early apoptosis. Although PI staining will also identify late apoptotic events where membrane integrity is lost, this method has widely been used to differentiate apoptotic or necrotic programmed cell death pathways. Apoptotic cells are defined as Annexin V +ve/PI negative and necrotic cells as PI positive. Post-infection, although there were mild increases in apoptotic cell death, the majority of macrophage cell death was PI positive suggestive of a programmed necrotic cell death response (Figure 22). At 6 h post infection, ~15% of cells underwent necrotic cell death, suggesting that human MDM have a moderately low level of cell death at this time point following infection with live swollen *A. fumigatus* conidia at a MOI of 1.

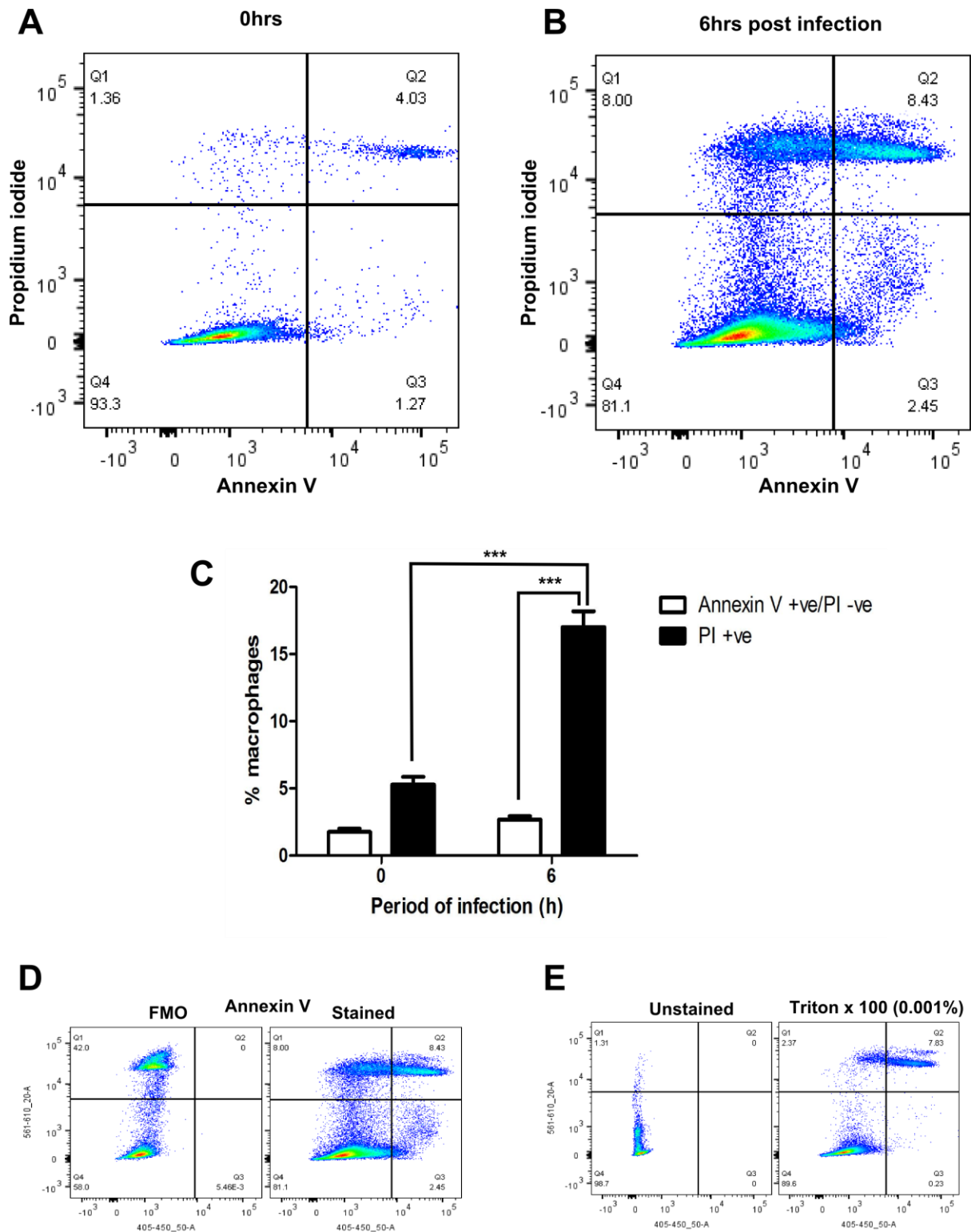


Figure 22: Human macrophages infected with *A. fumigatus* undergo necrotic cell death.

Human monocyte derived macrophages were infected with CEA10 swollen *A. fumigatus* conidia (MOI = 1). Pre-infection and 6 h post-infection, cells were harvested and stained with propidium iodide and Annexin V and analysed by flow cytometry. Macrophages were gated based on forward and size scatter as shown in Figure 1 and Annexin V and propidium iodide gating based on

fluorescence minus one controls and a positive cytotoxic control (0.001% Triton x100). **(A)** Representative flow cytometry gate of MDM pre-infection. Minimal cell death (PI or Annexin V +ve) is seen. **(B)** Representative flow cytometry gate 6 h post infection. Increased PI positive cells are seen (either single or double positive with Annexin V as well) representing necrotic cell death. Although an increase in Annexin V +ve/PI –ve cells is also seen, this is modest compared to PI. **(C)** Graph showing Annexin V +ve/PI –ve cells and PI +ve cells pre and 6 h post *A. fumigatus* infection. There is significantly more PI positive cells 6 h post-infection compared to pre-infection and also compared to Annexin V +ve/PI –ve cells. This would suggest human macrophages primarily undergo a necrotic cell death following *A. fumigatus* infection. n=3, (n = biological replicates) **(D)** Representative image showing gating strategy based on Annexin V fluorescence minus one staining at 6 h post infection. **(E)** Representative image of gating strategy based on unstained cells and positive control with Triton x100 (0.001%). Bars represent mean + SEM and samples were analysed by 2-way ANOVA with Bonferroni post-test calculation. *** - p<0.001.

To define the relationship between cell death and transfer, induction of necroptosis with the pan-caspase inhibitor Z-VAD-FMK (an inhibitor of caspase-dependent apoptosis which increases necroptosis due to de-regulation of caspase-8 inhibition) was performed (Wu et al., 2011, Kim and Li, 2013). This significantly increased programmed necrosis (as seen by increased PI fluorescence) and lateral transfer (Figure 23A). Addition of Necrostatin-1, an inhibitor of RIPK1 kinase-dependent necroptosis, to Z-VAD-FMK, inhibited both cell death and lateral transfer induced by Z-VAD-FMK alone (Figure 23A). However, induction of necroptosis with Z-VAD-FMK led to increased fungal burden due to recipient macrophage death and uncontrolled cell death (Figure 23B). Necrostatin-1 alone or Necrosulphonamide (a small molecule inhibitor which selectively targets the mixed lineage kinase domain-like protein (MLKL) to block necrosome formation) could not inhibit macrophage programmed cell death or lateral transfer (Figure 23A). As pyroptosis has recently been shown following macrophage infection with *C. albicans*, we additionally specifically blocked pyroptosis with an irreversible cell permeable caspase-1 inhibitor, (Z-YVAD-FMK), which did not affect cell death or lateral transfer (Figure 23A) (Krysan et al., 2014). These observations alongside increased vacuole formation post infection, are consistent with transfer of *A. fumigatus* between human macrophages

during a programmed necrotic cell death process. To summaries, although lateral transfer could be elicited by inducing necroptosis using artificial stimulation with pan-caspase inhibition (i.e. Z-VAD-FMK), inhibition of RIPK1 and MLKL-mediated necrosome formation was unable to inhibit lateral transfer and *A. fumigatus* induced macrophage necrosis.

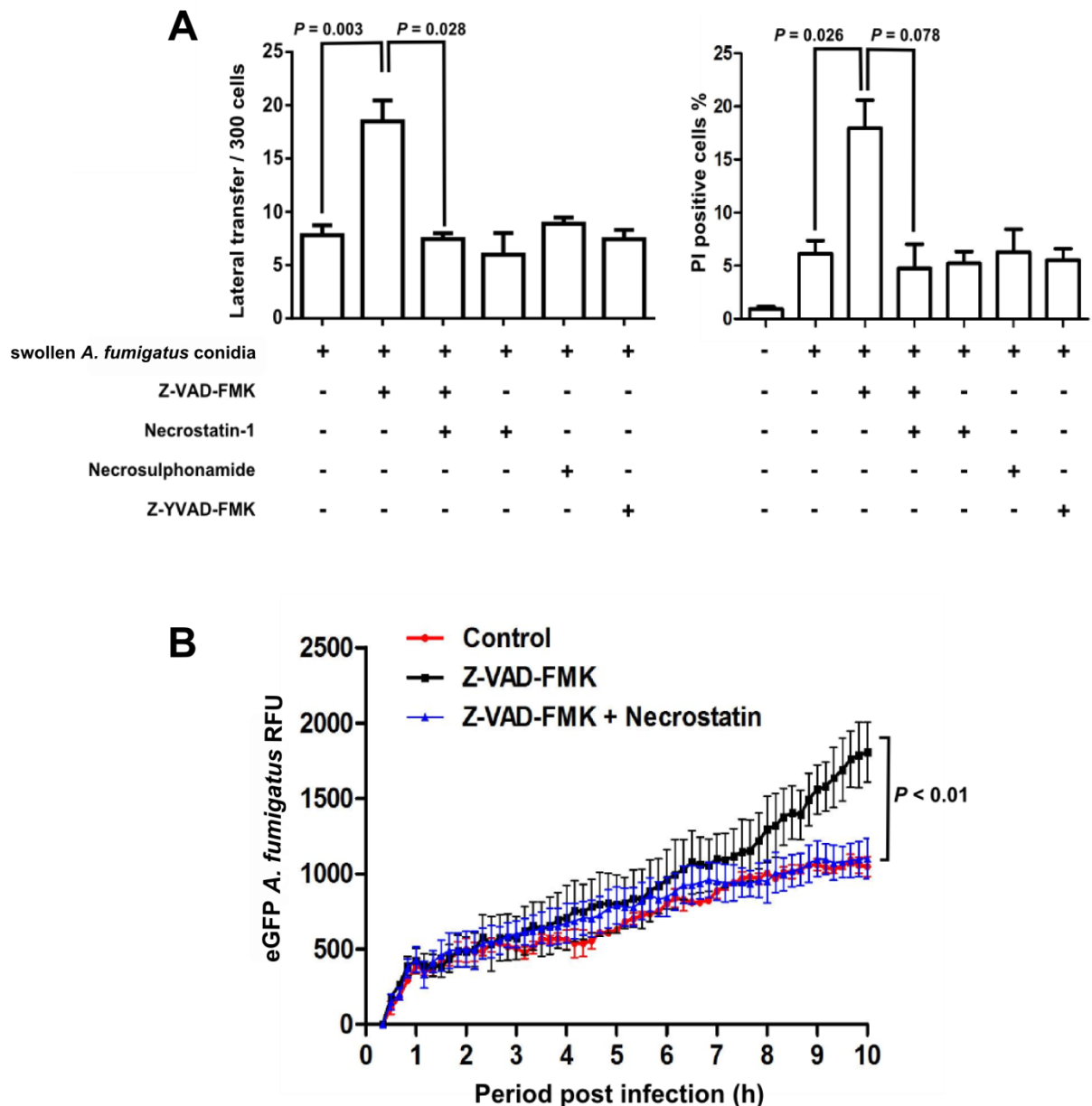


Figure 23: Human macrophages infected with *A. fumigatus* undergo lateral transfer to neighbouring macrophages during programmed necrosis.

Human MDM were infected with live *eGFP*-expressing swollen *A. fumigatus* conidia (MOI = 1) and time-lapse confocal microscopy performed. **(A)** Human MDM pre-treated with a pan-caspase

inhibitor (Z-VAD-FMK 50 μ M), RIPK-1 inhibitor (Necrostatin-1 10 μ M), caspase-1 inhibitor (Z-YVAD-FMK 50 μ M), Necrosulphonamide (10 μ M) or vehicle were stimulated with live *eGFP*-expressing *A. fumigatus* SC (MOI = 1) and lateral transfer events and cell death quantified by propidium iodide fluorescence. Treatment with Z-VAD-FMK induced necrotic cell death coupled to increased *A. fumigatus* lateral transfer, which was inhibited by the addition of a RIPK-1 inhibitor (Necrostatin-1). Inhibition of RIPK1 and MLKL-mediated necrosome formation using Necrostatin-1 or Necrosulphonamide was however unable to inhibit programmed cell necrosis and lateral transfer during *A. fumigatus* infection. n=3, (n = biological replicates) **(B)** MDM pre-treated with a pan-caspase inhibitor (Z-VAD-FMK 50 μ M), RIPK-1 inhibitor (Necrostatin-1 10 μ M), both or vehicle were stimulated with *eGFP*-expressing live *A. fumigatus* SC (MOI = 1) and fungal burden was quantified by analysis of eGFP fluorescence over time. Induction of necroptosis by Z-VAD-FMK treatment significantly increased fungal burden which was prevented by the addition of Necrostatin-1. n=3, (n = biological replicates). Bars represent mean + SEM and samples were analysed by Wilcoxon matched pairs test **(A)** and 2-way ANOVA with Bonferroni post-test calculation **(B)**.

To determine the relevance and importance of macrophage programmed necrosis and *A. fumigatus* lateral transfer to host control of fungal germination, further analysis was performed. Quantification of fungal burden using eGFP fluorescence in macrophages treated with a dynamin inhibitor (Dynasore) post-phagocytosis to inhibit *A. fumigatus* lateral transfer showed significantly increased fungal burden in dynamin treated macrophages. This suggests lateral transfer of *A. fumigatus* conidia enables recipient macrophage control of hyphal germination (Figure 24A). Further time-lapse confocal imaging of human macrophages in media loaded with PI and infected with live *eGFP*-expressing *A. fumigatus* was used to determine fungal germination rates in alive and dying macrophages. Germination of *A. fumigatus* conidia was well controlled in living macrophages compared to macrophages undergoing programmed necrosis (Figure 24B). Transfer of *A. fumigatus* conidia from dying human MDM enhanced control of germination, compared to dying MDM where conidia were not transferred (Figure 24B). Macrophages infected with *A. fumigatus* and undergoing programmed necrosis contained conidia with significantly increased conidial area compared to living macrophages (Figure 24C). There was no difference in conidial area in macrophages undergoing lateral transfer during programmed necrosis compared to those that did not (Figure 24C). Put

together, these results suggest that human macrophage *A. fumigatus* lateral transfer to neighbouring cells is a host-beneficial process occurring to contain fungal germination and prevent fungal escape with phagosomal fungal germination a possible checkpoint for programmed necrosis and subsequent lateral transfer.

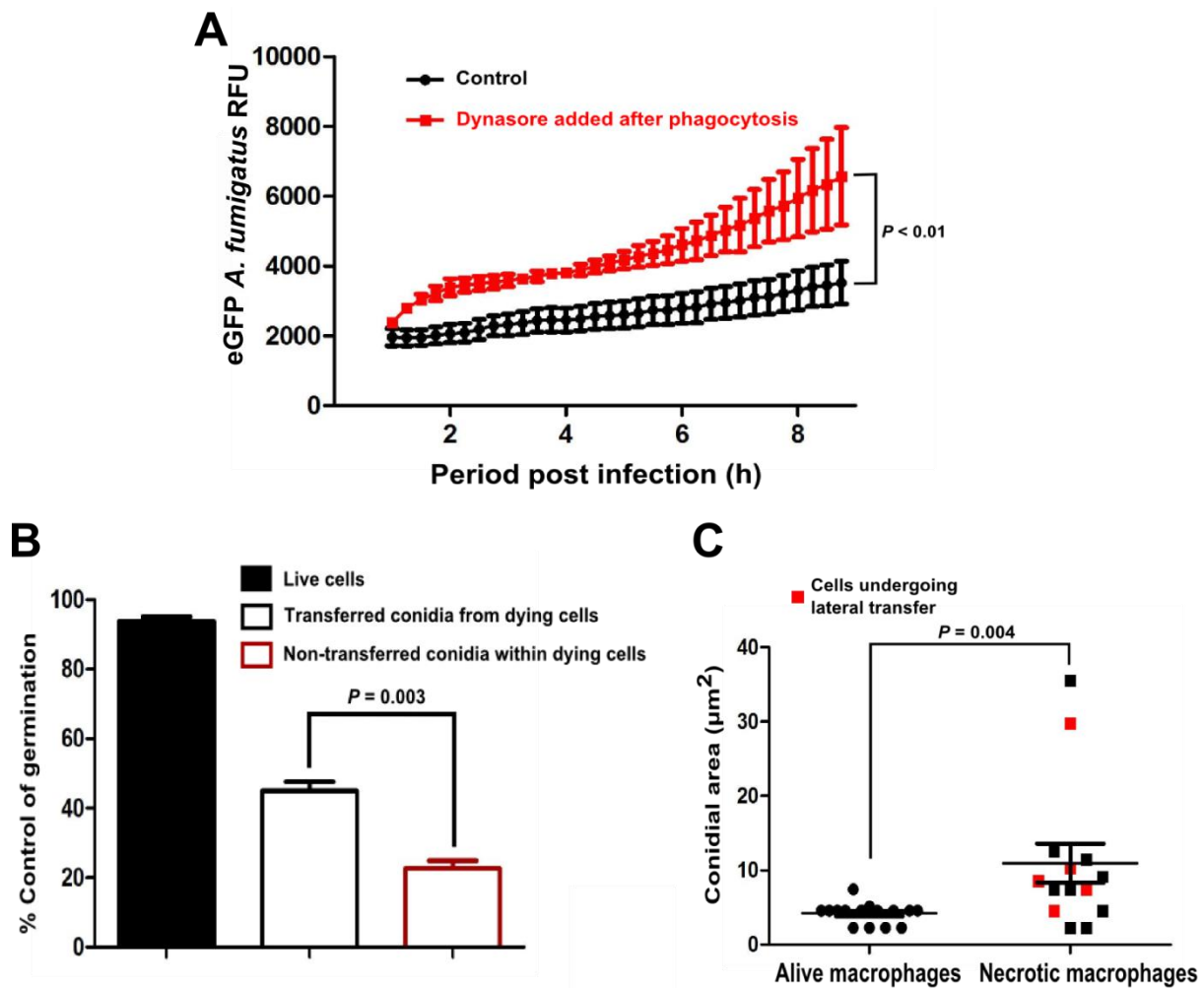


Figure 24: Human macrophage lateral transfer of *A. fumigatus* during infection enables control of fungal germination.

Human MDM pre-loaded with PI were infected with live *eGFP*-expressing swollen *A. fumigatus* conidia (MOI=1) and time-lapse confocal microscopy performed. **(A)** Human MDM were stimulated with *eGFP*-expressing live *A. fumigatus* SC (MOI = 1) and treated with Dynasore (100 μM) or vehicle following phagocytosis. Fungal burden was quantified by analysis of *eGFP* fluorescence over time. Inhibition of lateral transfer by the addition of Dynasore resulted in significantly increased fungal burden over time. $n=3$, (n = biological replicates) **(B)** Time-lapse confocal microscopy was used to quantify the fate of transferred vs non-transferred conidia from dying human MDM (PI +ve) and also

of conidia within live macrophages (PI –ve). Germination was assessed over a 12-hour period. Control of fungal germination was significantly increased in dying human MDM that transferred conidia to neighbouring cells compared to those that did not. n=4, (n = biological replicates) **(C)** The conidial area (μm^2) as a marker of fungal germination was quantified within cells undergoing programmed necrosis (PI +ve) and surrounding live cells (PI -ve). Conidia that undergo lateral transfer are highlighted in red. Fungal germination was significantly increased within cells undergoing programmed necrosis. n=14, (n = cells). Bars represent mean + SEM and samples were analysed by 2-way ANOVA with Bonferroni post-test calculation **(A)** and Wilcoxon matched pairs test **(B-C)**.

5.2.4 Calcineurin-dependent programmed macrophage necrosis and subsequent *A. fumigatus* lateral transfer enhances control of fungal germination

As calcineurin regulates cell death, we assessed its role in macrophage necrosis following fungal infection (Erin et al., 2003). Calcineurin inhibition significantly reduced macrophage programmed necrosis by 57% +/- 13.7 (Figure 25A). Lateral transfer occurred in $37 \pm 5\%$ of dying macrophages, suggesting its importance as a host-defence mechanism. Our results previously indicate intraphagosomal fungal germination to be a possible checkpoint for programmed cell necrosis. At late time-points following infection, human macrophages treated with FK506 had increased intracellular conidial area, with subsequent accelerated fungal germination in FK506-treated macrophages that fail to undergo necrosis-dependent transfer (Figure 25B-C). This is consistent with a calcineurin-dependent checkpoint for programmed necrosis during *A. fumigatus* germination. Together, these results indicate that human macrophages infected with live *A. fumigatus* undergo calcineurin-dependent cell death with features of programmed necrosis and transfer *A. fumigatus*-containing endosomes to neighbouring cells to facilitate control of fungal germination.

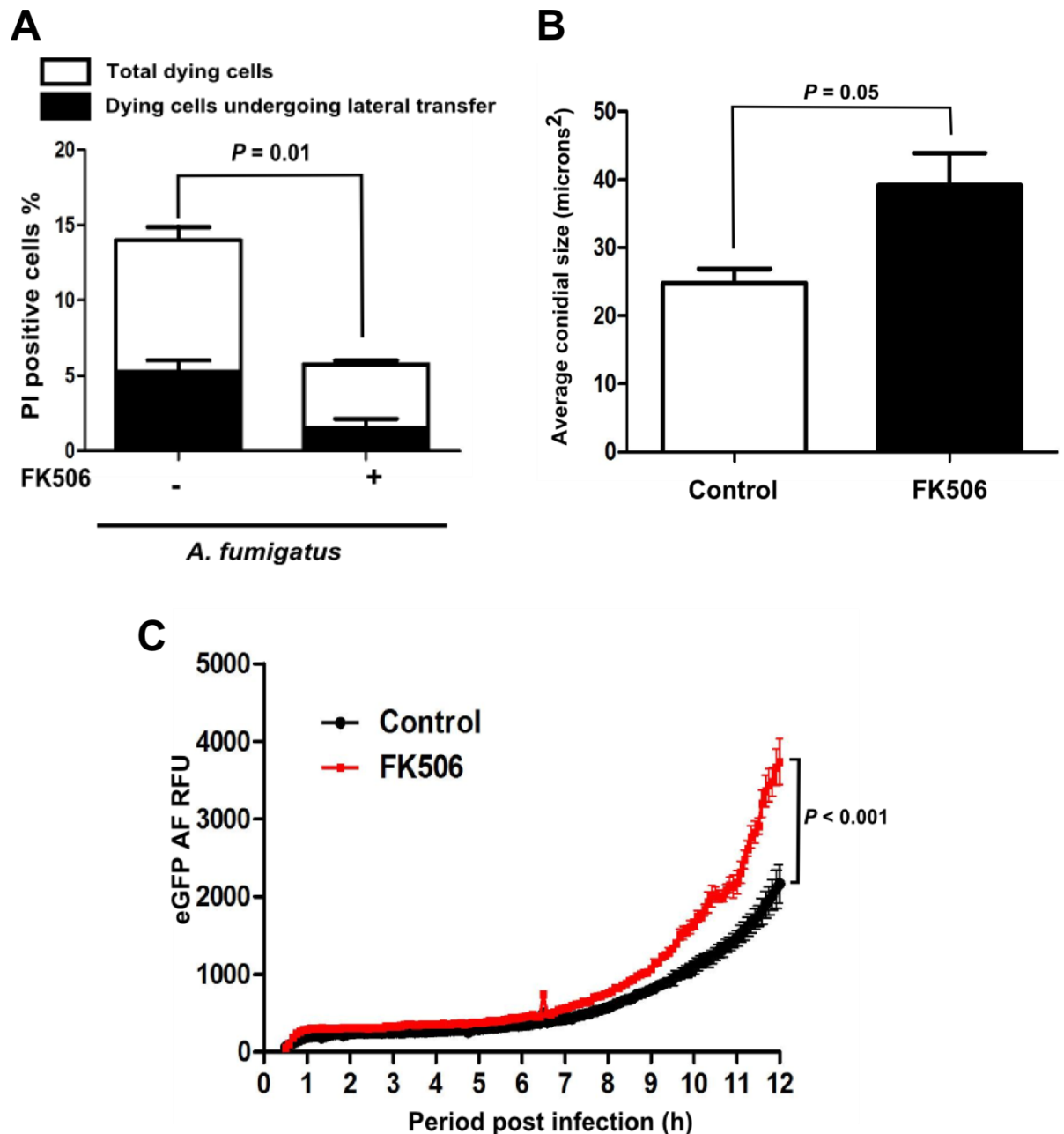


Figure 25: Human macrophages infected with *A. fumigatus* undergo calcineurin-dependent programmed necrosis and lateral transfer to enable control of fungal germination.

Human MDM pre-loaded with PI and pre-treated with FK506 (10 ng/ml) or vehicle were infected with live *eGFP*-expressing swollen *A. fumigatus* conidia (MOI = 1) and time-lapse confocal microscopy performed. **(A)** Cell death was assayed by propidium iodide fluorescence over a 6 h period. The programmed macrophage cell death response to live *A. fumigatus* swollen conidia was calcineurin-dependent. The graph also shows the proportion of lateral transfer events in dying cells (~35% of dying cells). $n=4$, (n = biological replicates). **(B-C)** Fungal germination was quantified by average conidial size 6 h p.i **(B)** and fungal burden by *eGFP* fluorescence quantification over time **(C)**. Treatment with FK506 resulted in increased fungal burden at late time-points after infection. This

was associated with increased conidial size in FK506 treated cells suggesting increased fungal germination. n=4, (n = biological replicates). Bars represent mean + SEM and samples were analysed by Wilcoxon matched pairs test **(A-B)** and 2-way ANOVA with Bonferroni post-test calculation **(C)**.

Studies have suggested a likely role for the MAPK-AP-1 pathway, and dynamin-related protein 1 (Drp1) mediated mitochondrial fission in programmed cell necrosis pathways including necroptosis (Wu et al., 2011, Kanamaru et al., 2012). As mitochondrial translocation of Drp1, and MAPK-AP-1 pathway regulation are both thought to be calcineurin-dependent, this was explored in *A. fumigatus* infected human MDM (Cereghetti et al., 2008, Huang et al., 2008, Wu et al., 2003b). Significant modulation of MAPK-AP-1 pathway and Ser637 P-Drp1 phosphorylation was observed (Figure 26). Thus, protein-level analyses suggest that calcineurin pathway inhibition in the macrophages infected with *A. fumigatus* results in effects on the MAPK-AP1 pathway and Drp1 phosphorylation which may have an effect on programmed cell death pathways. Further studies analysing the effect of MAPK-AP1 inhibition (e.g. small peptide inhibitors or siRNA) on programmed macrophage cell death following *A. fumigatus* infection is required to confirm this hypothesis. Likewise, analysis of DRP1 mitochondrial colocalisation and DRP1 phosphomimetic mutants is required to ascertain the importance of DRP1 dephosphorylation within macrophage programmed cell necrosis following *A. fumigatus* infection.

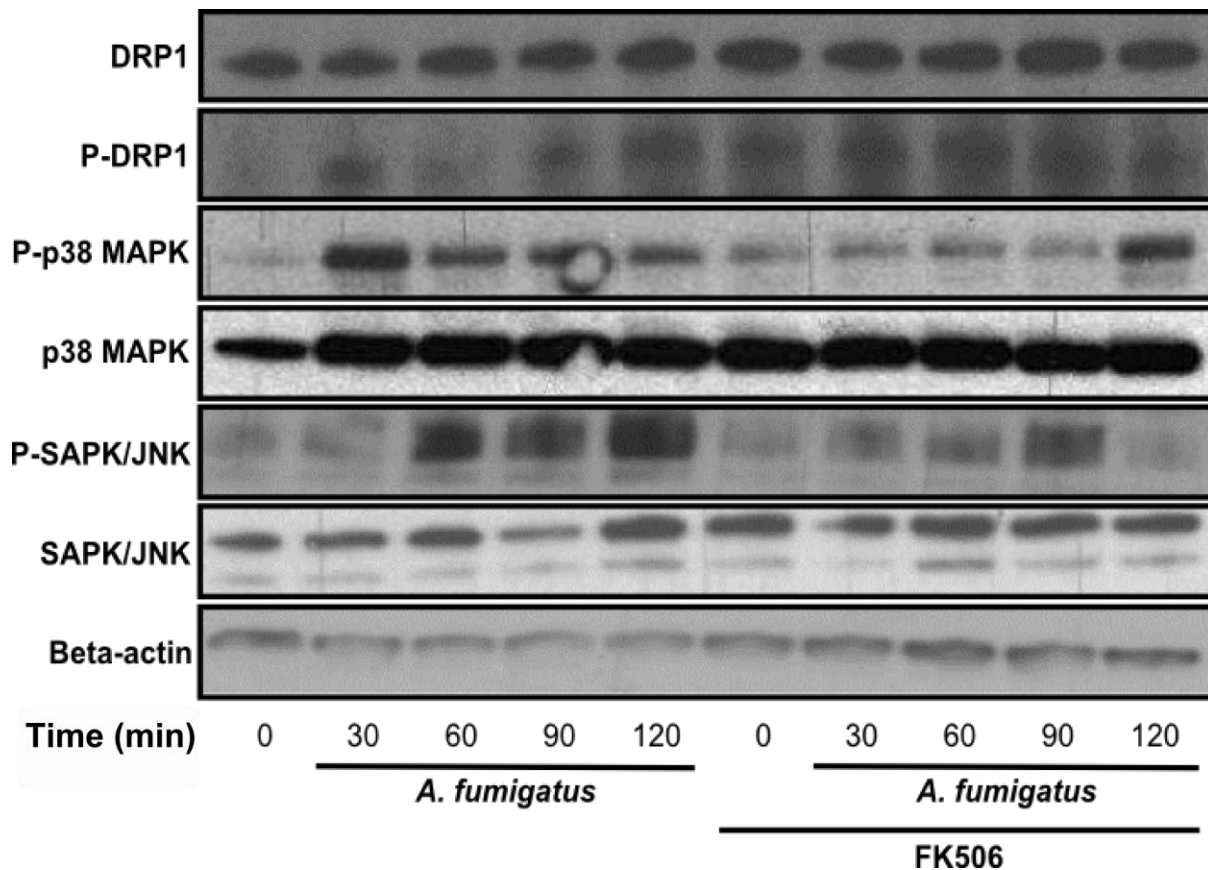


Figure 26: MAPK-JNK activation and Drp-1 phosphorylation in human macrophages infected with *A. fumigatus* is regulated by calcineurin.

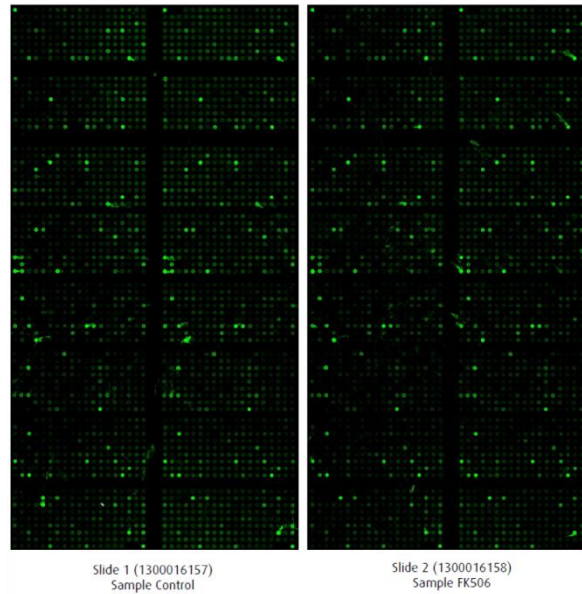
MDM pre-treated with FK506 (10 ng/ml) or vehicle were stimulated with live *A. fumigatus* swollen conidia (MOI = 1) and whole cell lysates probed by western blot for Drp-1 phosphorylation and MAPK pathway activation. Representative western blots of n=3 (n = biological replicates) are shown. Ser637 DRP-1 dephosphorylation in macrophages infected with live *A. fumigatus* swollen conidia was calcineurin-dependent. Additionally significant cross-control between the calcineurin-NFAT pathway and the MAPK pathway with dysregulated p38 MAPK and JNK phosphorylation seen in macrophages treated with FK506 and infected with live *A. fumigatus* swollen conidia.

5.2.5 Calcineurin-dependent *A. fumigatus* lateral transfer between human macrophages occurs within actin-VASP enveloped endosomes.

Our results have suggested multiple calcineurin-dependent effects on actin cytoskeletal components of cellular function such as phagocytosis alongside actin-dependent lateral transfer of *A. fumigatus*. Furthermore, calcineurin dependent effects are seen on programmed cell death pathways.

Calcineurin has been shown to have multiple NFAT-independent effects on cytoskeletal reorganisation and regulation of MAPK pathways with numerous described and undescribed phosphatase targets (Zhao et al., 2012, Huang et al., 2008, Escolano et al., 2014).

To identify calcineurin phosphatase targets in human macrophages infected with *A. fumigatus*, we performed a comparative phosphoproteomic array on FK506 vs. vehicle pre-treated human MDM infected with *A. fumigatus* swollen conidia (Figure 27A). Full array findings are shown in Appendix 8.1. Validating the effects of FK506 in our in vitro system, differential phosphorylation of calcineurin pathway regulatory proteins were seen such as Calmodulin and AKT1. In addition, consistent with our previous findings there were calcineurin-dependent effects on the phosphorylation of proteins within the MAPK pathway including c-Jun, MEK1 and P90RSK and cell cycle regulatory proteins such as CDC25A, p27Kip1 and HDAC1 (Figure 27B). As above, further work is necessary to evaluate the importance of these proteins with calcineurin-dependent macrophage programmed cell death.

A**B**

	Phosphoprotein	Fold change in phosphoprotein to non-phosphoprotein ratio (FK506 treated vs Control)
Calcineurin/NFAT pathway	AKT1 (Phospho-Ser124)	0.45
	Calmodulin (Phospho-Thr79/Ser81)	0.13
Actin cytoskeleton	VASP (Phospho-Ser238)	26.70
Cell-cycle regulation	CDC25A (Phospho-Ser75)	3.82
	p27Kip1 (Phospho-Ser10)	0.44
	SMC1 (Phospho-Ser957)	0.49
	HDAC1 (Phospho-Ser421)	0.49
MAPK pathway	c-Jun (Phospho-Tyr170)	2.20
	MEK1 (Phospho-Ser298)	2.69
	P90RSK (Phospho-Thr359/Ser363)	0.44
Angiogenesis	VEGFR2 (Phospho-Tyr1175)	0.47

Figure 27: Phosphoproteomic array showing calcineurin-dependent cell cycle and MAPK pathway regulation in human macrophages infected with *A. fumigatus*.

(A) A phosphoprotein array analysis of human MDM pre-treated with FK506 and stimulated with live *A. fumigatus* swollen conidia (MOI = 1) for 1 h p.i.. **(B)** Proteins with significant fold change differences in the phosphoprotein to non-phosphoprotein ratio between FK506 and control macrophages stimulated with live *A. fumigatus* swollen conidia are shown. Calcineurin/NFAT regulatory proteins AKT1 and Calmodulin were differentially phosphorylated. FK506 pre-treatment significantly increased the Ser238 VASP phospho to non-phosphoprotein ratio at 1 h p.i. as shown in red. Differential phosphorylation was also seen in the cell cycle regulatory proteins (e.g. CDC25A) and MAPK pathway proteins (e.g. c-Jun, MEK1 and P90RSK) in the presence of FK506 pre-treatment.

The phosphoproteins array was performed with 2 biological replicates each with 2 technical replicates.

To determine whether Ser238 VASP is a direct target of calcineurin, further analysis of VASP Ser238 phosphorylation was performed by Western blot. Human MDM Ser238 VASP phosphorylation was stimulated by the addition of a cell permeable cGMP analogue (8-pCPT-cGMP) (Pilz et al., 1995). As calcineurin is the only calcium-activated serine-threonine phosphatase, calcineurin activation was stimulated by the addition of ionomycin (a calcium ionophore) in the presence or absence of FK506 (Vasauskas et al., 2012). Protein lysate was probed by Western blot to analyse P-Ser238 VASP levels compared to total VASP. This showed a significant increase in P-Ser238 VASP/VASP levels in the presence of FK506 suggesting P-Ser238 VASP is a direct calcineurin target (Figure 28).

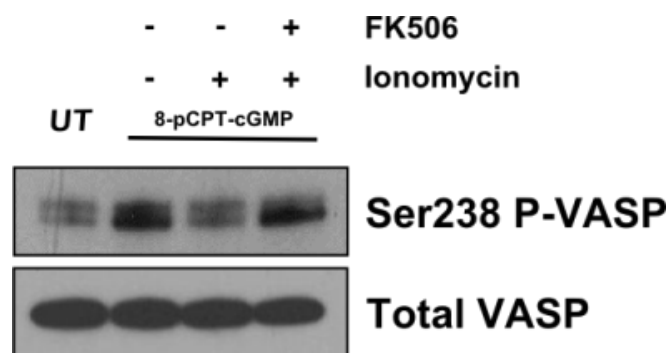


Figure 28: P-Ser238 VASP is a direct target of calcineurin in human macrophages.

Human MDM were pre-treated with a cell-permeable cGMP analogue (8-pCPT-cGMP 25 mM) to induce Ser238 VASP phosphorylation and FK506 (10 ng/ml) or vehicle. They were then stimulated with a calcium ionophore (Ionomycin 2 µg/ml) to activate calcineurin. Macrophage whole cell extracts were probed by western blot for Ser238 VASP and total VASP. Ionomycin caused Ser238 VASP dephosphorylation which was inhibited by FK506 pre-treatment implying calcineurin dependent dephosphorylation of Ser238 VASP.

As VASP is an important actin cytoskeletal regulator, and calcineurin is required for optimal phagocytosis and lateral transfer of *A. fumigatus*, it was hypothesized that VASP is involved in actin-dependent phagocytosis and lateral transfer of *A. fumigatus*. High-resolution confocal microscopy was performed to analyse VASP localisation during human macrophage infection with *A. fumigatus*.

VASP was seen to be constitutively expressed, highly co-localised to actin with expression and localisation not calcineurin dependent (Figure 29A-B). Co-localisation of VASP was further seen to *A. fumigatus* at phagocytic cups, which disappeared following internalisation (Figures 29C-D). Co-localisation of VASP to *A. fumigatus* was again not calcineurin-dependent (*data not shown*). During *A. fumigatus* lateral transfer at later time-points, high intensity co-localisation of VASP and actin staining was observed to *A. fumigatus*-containing endosomes. High-resolution 3D reconstruction revealed that *A. fumigatus* containing endosomes trafficked between macrophages are enveloped within actin-VASP rich cargo (Figure 29C-G).

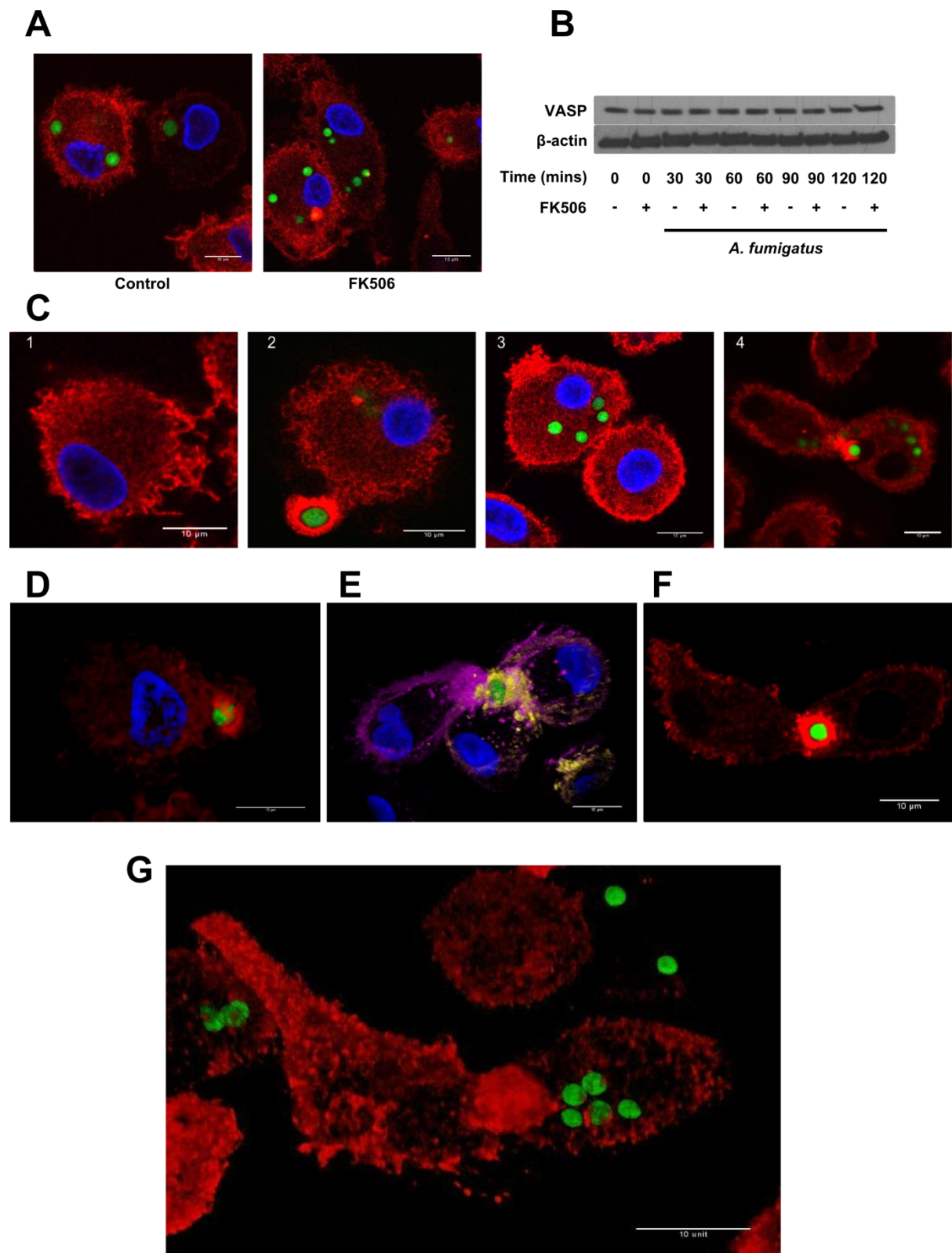


Figure 29: Lateral transfer of *A. fumigatus* between human macrophages occurs within actin-VASP enveloped endosomes.

(A) Representative confocal microscopy images of human MDM infected with *A. fumigatus* swollen conidia (MOI = 1) 1 h p.i.. No calcineurin-dependent difference is seen in VASP localisation or expression. VASP shown in red, nuclei in blue and *A. fumigatus* in green. **(B)** Representative western blot of VASP expression in lysate of human MDM infected with *A. fumigatus* swollen conidia (MOI = 1). Results confirm constitutive VASP expression in human macrophages with no calcineurin-dependent difference in expression at baseline and post infection. **(C)** Human MDM phagocytosis and lateral transfer of live *A. fumigatus* swollen conidia (MOI = 1). VASP shown in red, nuclei in blue and *A. fumigatus* in green. Strong VASP co-localisation is shown to *A. fumigatus* conidia being phagocytosed (shown in 2) compared to uninfected human MDM (shown in 1). VASP co-localisation then disappears at later time-points following phagocytosis (shown in 3) with further co-localisation at lateral transfer points (shown in 4). **(D)** 3D reconstruction of confocal microscopy image showing *A. fumigatus* phagocytosis at 30 min p.i. with VASP shown in red, *A. fumigatus* in green and nuclei in blue. VASP is seen co-localising to the early *A. fumigatus* phagosome during uptake. **(E)** 3D reconstruction of confocal microscopy image of *A. fumigatus* lateral transfer with VASP shown in yellow, actin in magenta, *A. fumigatus* in green and nuclei in blue. VASP co-localises strongly to actin at areas of cell-cell contact during *A. fumigatus* lateral transfer with *A. fumigatus* containing endosomes transferred as VASP-actin enveloped cargo. **(F-G)** Representative z-slice confocal microscopy image **(F)** and 3D reconstruction **(G)** of *A. fumigatus* lateral transfer. VASP shown in red, *A. fumigatus* in green. VASP co-localises strongly at lateral transfer points. All scale bars shown represent 10 μm . Videos are available online to view at <http://www.atsjournals.org/doi/suppl/10.1164/rccm.201601-0070OC>.

To determine the role of VASP in macrophage *A. fumigatus* phagocytosis, siRNA knockdown of VASP was performed in human macrophages (THP-1), followed by analysis of uptake of calcofluor white-stained live *A. fumigatus* swollen conidia compared to control. This showed that phagocytosis of live *A. fumigatus* swollen conidia at early time-points is VASP-dependent (Figure 30), consistent with the defect previously seen for FK506-based calcineurin inhibition (Figure 11). Taken together, these results indicate that calcineurin has a key role in macrophage actin cytoskeleton re-organisation during fungal infection, by direct de-phosphorylation and activation of the actin polymerase VASP. This enables optimal actin-dependent *A. fumigatus* phagocytosis and lateral transfer of *A. fumigatus*

containing endosomes during programmed necrosis, which are critical to achieve control of germinating conidia.

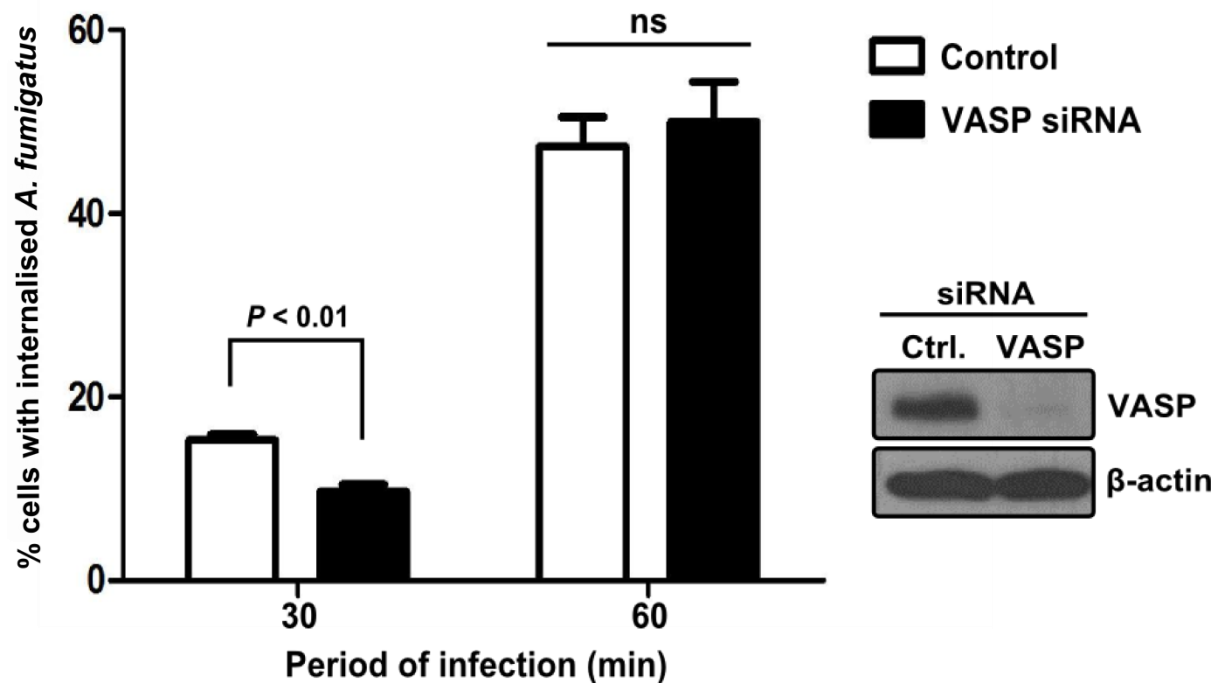


Figure 30: Human macrophage early phagocytosis of *A. fumigatus* is VASP-dependent.

THP-1 macrophages treated with either control or VASP siRNA (50 μ M) were infected with live *A. fumigatus* swollen conidia and stained with calcofluor-white (Sigma) (MOI = 1). Phagocytosis was quantified by flow cytometry. Early phagocytosis of *A. fumigatus* swollen conidia at 30 min p.i. was VASP dependent with no effect seen at 60 min p.i.. $n=3$ (n = technical replicates). Bars represent mean + SEM and samples were analysed by Wilcoxon matched pairs test.

5.3 Discussion

Invasive pulmonary aspergillosis has emerged as an increasingly common serious infectious complication of a range of immunocompromised states and chronic respiratory diseases (Taccone et al., 2015). Overt infection is typically characterised histologically by hyper-inflammatory tissue destruction and necrosis in invasive aspergillosis with more complex relationships involving airway inflammation, fibrosis and fungal colonisation in semi-invasive or chronic presentations (Tochigi et al., 2013).

Programmed necrosis has been shown to be an integral part of the innate immune response to virus and bacterial infection, with mitophagy-dependent necroptosis additionally contributing to the development of chronic airways disease (Mocarski et al., 2014, Chan et al., 2015, Mizumura et al., 2014). In this chapter, human macrophage programme necrosis is shown to be a primary outcome following *A. fumigatus* infection. Necrotic cell death was described based on morphological changes during light and electron microscopy and by propidium iodide and Annexin V fluorescence by a combination of flow cytometry and live time-lapse microscopy. For confirmation of necrotic cell death, analysis of LDH cellular release and analysis of caspase activation by use of fluorogenic caspase substrate or western blot is required.

At a MOI of 1, during infection of human macrophages with live swollen *A. fumigatus* conidia, however, programmed necrosis was only seen in 10-15% of cells. This is comparatively low compared to that seen in fungal toxin mediated epithelial necrosis (e.g. due to 'Candidalysin') (Moyes et al., 2016). This suggests that human macrophages are highly efficient at controlling fungal infection. Fungal growth is hence dependent on macrophage escape with ~90% of cells controlling germination. This chapter suggests that uncontrolled fungal germination is a trigger for programmed cell necrosis with increased conidial diameter in cells undergoing programmed necrosis. Studies with *C. neoformans* have shown the ability of fungi to modulate phagolysosomal pH and Rab-GTPase interaction to promote replication and ultimately cause phagolysosomal membrane leakage and cell

lysis (Tucker and Casadevall, 2002). *A. fumigatus* has additionally been shown to inhibit phagolysosomal maturation and acidification and whether phagolysosomal leakage represents a checkpoint and trigger for programmed necrosis remains to be confirmed (Thywissen et al., 2011).

Remarkably, during programmed necrosis I observed actin-dependent lateral transfer of *A. fumigatus* conidia to neighbouring macrophages. This occurred in ~30% of dying macrophages and was additionally seen in vivo within a zebrafish model of invasive aspergillosis, suggesting an evolutionarily-conserved clinically-relevant process. Although, lateral transfer has previously been rarely shown following macrophage fungal infection with *C. neoformans*, *C. albicans* and *C. krusei*, this represents the first evidence of a lateral transfer process involving *A. fumigatus*. Lateral transfer of *A. fumigatus* conidia was shown within Rab7 +ve, Rab5 and Lamp-1 –ve endosomes suggesting the late endosome as a potential checkpoint for programmed necrosis. Whether the actin-dependent endosomal transfer of *A. fumigatus* has similarities to previously described Rab11 and Rab27 dependent pathogen exocytosis remains to be determined (Szumowski et al., 2014, Song et al., 2009). Further studies to create a model-based system with a greater necrosis percentage and enable reporter cell lines for Rab-GTPase live imaging and siRNA silencing are required to study this in greater detail.

Pan-caspase inhibition has been shown to stimulate necroptosis through degulation of caspase-8 dependent inhibition. Although, induction of lateral transfer and programmed cell death was achieved using a pan-caspase inhibitor (Z-VAD-FMK), it is unclear how well this relates to the biological programmed cell death response in human MDM infected with *A. fumigatus*. RIPK1 inhibition (necrostatin-1) was able to prevent the increased programmed necrosis and lateral transfer seen during necroptosis induction (Z-VAD-FMK). However, programmed necrosis and lateral transfer following macrophage fungal infection were still present despite inhibition of necrosome formation. This suggests that although programmed necrosis and lateral transfer of *A. fumigatus* can occur through exogenous necroptosis induction, this may not represent the underlying macrophage

programme cell death response to *A. fumigatus* infection. Necrosome formation is dependent on MLKL phosphorylation by RIPK3. Further work in our lab has since shown evidence of phospho-MLKL colocalisation with programmed macrophage necrosis following *A. fumigatus* infection suggesting necroptosis may present one pathway of necrosis in this setting. The creation of a BMDM model of the macrophage-*A. fumigatus* programmed cell death response will allow the utilisation of MLKL^{-/-} and RIPK1^{-/-} and RIPK3^{-/-} derived BMDMs to characterise and dissect the programmed macrophage necrosis response to *A. fumigatus* in greater detail. Our understanding of programmed necrosis is rapidly expanding with several recently discovered novel pathways (Vanden Berghe et al., 2014). Further research is now required to fully dissect the programmed necrosis response involved in human macrophage *A. fumigatus* infection.

Previous descriptions of lateral transfer involving *C. neoformans* have been presumed to be pathogen-dependent. Given a number of bacterial and viral pathogens (e.g. *Listeria monocytogenes*, *Shigella flexneri* and *Vaccinia virus*) that have been shown to exploit actin-based motility to spread into neighbouring cells or escape in to the cytosol following infection this is perhaps unsurprising (Benanti et al., 2015, Lee et al., 2015a, Czuczman et al., 2014, Frischknecht et al., 1999). Strikingly this chapter suggests that human macrophage lateral transfer of *A. fumigatus* is a host-beneficial process to limit fungal germination. Inhibition of *A. fumigatus* lateral transfer (utilising dynasore) led to increased fungal burden. In addition, transfer of *A. fumigatus* conidia to neighbouring macrophages during programmed necrosis enhanced control of fungal germination. This represents the first description of a host-beneficial pathogen transfer process. Comparative to bacterial and viral infection, control of *A. fumigatus* infection is primarily dependent on prevention of fungal germination (Waldorf, 1989). Once hyphal transition has occurred, host defense is reliant on neutrophil influx and extracellular release of microbial peptides, enzymes, oxygen radicals and DNA with inevitable host damage (Gazendam et al., 2016). Hence, transfer of *A. fumigatus* conidia to neighbouring macrophages during programmed necrosis represents another opportunity for phagolysosomal containment and fungal killing. A limitation of this work is the use of only one strain

of *A. fumigatus*. Whether *A. fumigatus* has the ability to modulate the host actin-cytoskeletal network as seen with bacteria, and whether there is virulence-dependent variability in host programmed necrosis and subsequent lateral transfer remains to be determined (Traven and Naderer, 2014b).

Notably, this chapter suggests that both macrophage programmed necrosis and lateral transfer following *A. fumigatus* infection are calcineurin-dependent, indicating that regulated necrotic control of infection is likely to be impaired in organ transplantation. Calcineurin has previously been shown to have a role in programmed cell death through dephosphorylation of Bcl-2 family proteins (Springer et al., 2000). Further analysis of the calcineurin-dependent transcriptome following macrophage *A. fumigatus* infection confirmed transcriptional level regulation of a number of Bcl-2 family proteins (*analysis performed by Shichina Kannambath, postdoc in Armstrong-James lab*). Determining the calcineurin-dependent phosphoproteome in human macrophages following *A. fumigatus* infection additionally revealed differential phosphorylation of a number of cell cycle proteins (e.g. CDC25A), suggesting widespread calcineurin-dependent effects on programmed cell death.

As transcriptional partners (NFAT and AP-1), the calcineurin-NFAT and MAPK pathways are closely linked with multiple levels of differential pathway regulatory control (Escolano et al., 2014, Huang et al., 2008, Lee et al., 2012, Macian et al., 2001). Hence, unsurprisingly, inhibition of the calcineurin-NFAT pathway during macrophage *A. fumigatus* infection resulted in differential MAPK pathway phosphorylation. Given p-38 MAPK and JNK activation are known to regulate programmed cell death, this potentially represents another mechanism for calcineurin-dependent programmed cell death (Liu and Lin, 2005, Wu et al., 2011). The direct effects of individual MAPK pathway activation on programmed cell death and lateral transfer during human macrophage *A. fumigatus* infection are however as yet unknown and further pathway dissection utilising small peptide inhibitors or siRNA is required to confirm the significance of these findings. Additionally, the significance of calcineurin-

dependent DRP-1 dephosphorylation to programmed macrophage necrosis given the absence of calcineurin-dependent differences in mitochondrial ROS production is additionally unclear. Analysis of DRP-1 mitochondrial colocalisation and utilisation of phosphomimetic mutants would be required to explore this in more detail.

Calcineurin has recently been shown to have a role in actin-cytoskeletal regulation (Wang et al., 2005, Li et al., 2015). Although, impaired programmed necrosis is a likely mechanism for the reduced *A. fumigatus* transfer seen following *A. fumigatus* infection in macrophages treated with calcineurin inhibitors, phosphoproteomic analysis additionally revealed direct calcineurin-dependent dephosphorylation of the actin polymerase VASP. Co-localisation and transcriptional silencing revealed the importance of VASP in macrophage *A. fumigatus* phagocytosis with co-localisation suggesting lateral transfer of *A. fumigatus* conidia within actin-VASP enveloped endosomes. Due to the ability of VASP to rapidly elongate actin-barbed ends at lamellipodia by antagonising inhibition by capping proteins and reducing filament branching, VASP has previously been shown to be important for directional motility and macrophage phagocytosis (Chen et al., 2014, Edwards et al., 2014, Geese et al., 2002, Winkelman et al., 2014).

Although highly co-localised, the VASP-dependence of *A. fumigatus* lateral transfer is currently unknown. The recent development of a cell-permeable VASP peptide inhibitor, however, enables a means of investigation in the future (Opitz et al., 2015). Analysis of the human VASP amino-acid structure potentially identifies a conserved calcineurin-binding motif with a PxxxIx motif (as seen with the calcineurin substrate phosphatidylinositol 4,5-bisphosphate-binding protein SIm2) similar to that seen in NFAT family members (PxIxxT) (see Appendix 8.2) (Li et al., 2011, Bultynck et al., 2006). Further research to confirm the calcineurin-VASP docking site and use of specific calcineurin-binding motif inhibitors may allow delineation of calcineurin-dependent VASP dephosphorylation effects in the future. A number of bacterial pathogens have additionally been shown to be able to either imitate or manipulate host Ena-VASP proteins through actin-assembly (ActA) inducing proteins to

enable cell-cell spread or escape (Lee et al., 2015a, Laurent et al., 1999, Lee et al., 2015b, Benanti et al., 2015). Whether *A. fumigatus* is able to manipulate host actin polymerases including VASP is unknown and further research utilising fungal actin-specific gene targeting is required.

To summarise, this chapter, shows that human macrophages undergo a calcineurin-dependent lateral transfer of *A. fumigatus* conidia within actin-VASP enveloped endosomes to neighbouring macrophages during programmed necrosis to prevent fungal germination.

6 Concluding remarks

There is an increasing incidence of invasive aspergillosis worldwide, with high associated mortality and morbidity (Taccone et al., 2015, Pfaller et al., 2006, Baddley et al., 2013). Historically, neutropenia was the most prevalent risk-factor for the development of invasive aspergillosis (Baddley, 2011). Recent epidemiological data, however, suggests that amongst patients with invasive fungal infections admitted to intensive care units, non-neutropenic patients are increasingly common (Taccone et al., 2015). This cohort largely consists of patients with chronic respiratory disease (e.g. COPD) and solid organ transplantation, in particular lung transplantation, which is a growing population of patients with a high annual incidence of invasive fungal infection (Garnacho-Montero et al., 2013, Baddley et al., 2013, Pappas et al., 2010). The increased risk of invasive aspergillosis seen in non-neutropenic patients has focussed attention onto the role of the human AM in the host innate defense to invasive aspergillosis (Segal, 2007). Recent studies from murine models of invasive aspergillosis, including that from within our group has highlighted the importance of A in early control of infection (Philippe et al., 2003, Brummer et al., 2001, Bhatia et al., 2011).

Calcineurin inhibitors, alongside corticosteroids form the mainstay of immunosuppression in solid organ transplantation and is additionally used as first-line therapy for graft-vs-host disease following bone marrow transplantation (Calne, 2004, Wolff et al., 2009). Given the increased risk of invasive fungal infections seen in both these cohorts, there has been increased interest in the effects of calcineurin inhibitors on the host immune response to invasive aspergillosis (Greenblatt et al., 2010). The calcium-dependent calcineurin-NFAT pathway is specific to vertebrate species and is known to play a significant role in adaptive immunity and T cell activation (Rusnak and Mertz, 2000, Takeuchi et al., 2007). The weak-DNA binding ability of NFAT, however, requires the presence of additional transcription factors (e.g. AP-1) for effective transcription, hence enabling a diversification of function through combination with more ancient signalling pathways (e.g. MAPK-AP-1) (Rao, 2009, Macian et al., 2001). Further development of NFAT binding regions within older signalling pathways

(e.g. NFκB) has led to the development of a complex pathway with multiple functions and regulatory levels (Serfling et al., 2004, Boise et al., 1993). Recent work from within our group has shown increased mortality with the addition of calcineurin inhibitors to a hydrocortisone Rag2^{-/-} murine model of invasive aspergillosis (Herbst et al., 2015). This confirms a significant role for the calcineurin-NFAT pathway in the host innate immune response to invasive aspergillosis. Further murine data shows almost exclusive phagocytosis by macrophages at early time-points following *A. fumigatus* infection with neutrophil influx occurring at later time-points, suggesting a profound effect on the AM response (Herbst et al., 2015).

This thesis provides evidence of a significant role for calcineurin in the human AM immune response to invasive aspergillosis. Activation of the calcineurin-NFAT pathway is shown following infection with germinating *A. fumigatus* conidia. Fungal killing by human macrophages is shown to be calcineurin-dependent with activation of the calcineurin pathway critical for *A. fumigatus* phagocytosis and reactive oxygen species production. Remarkably, actin-dependent endosomal lateral transfer of *A. fumigatus* associated with human macrophage programmed necrosis is seen to prevent hyphal transition as a primary outcome following intraphagosomal germination of *A. fumigatus*. Detailed biochemical, high-resolution microscopy and proteomic analysis show that programmed necrosis and lateral transfer of *A. fumigatus* following infection are calcineurin-dependent with effects on programmed cell death associated signalling pathways and regulation of actin cytoskeletal function through differential phosphorylation of the actin polymerase VASP. As visually represented below, this suggests a model whereby following intraphagosomal germination of *A. fumigatus* conidia, calcineurin orchestrates actin-dependent lateral transfer of *A. fumigatus* containing endosomes to neighbouring macrophages during programmed necrosis to prevent hyphal transition and pathogen escape.

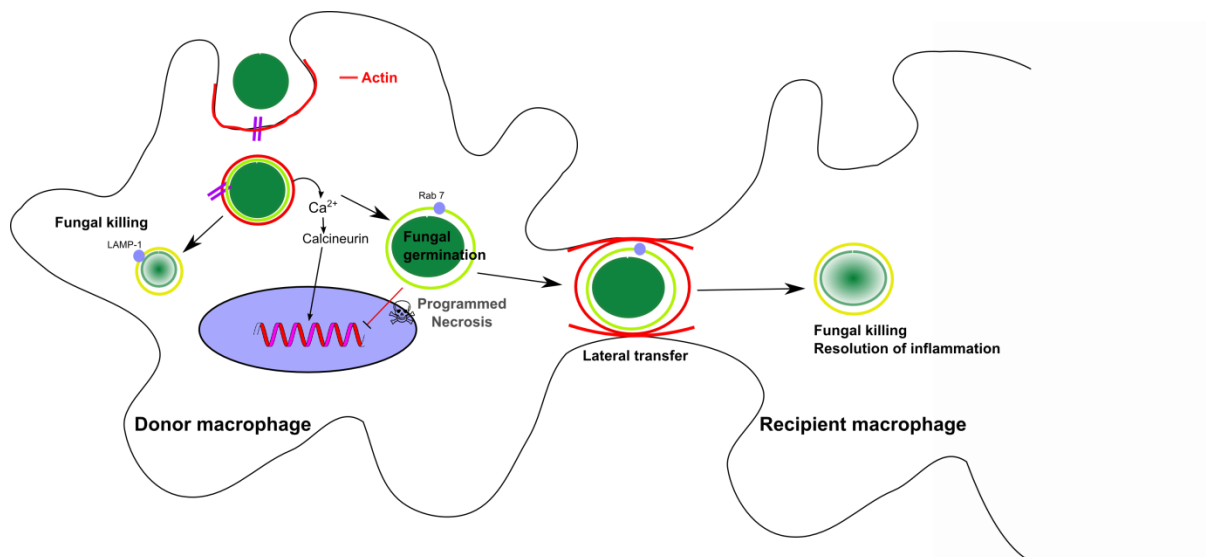


Figure 31: Model diagram illustrating calcineurin-dependent human macrophage lateral transfer of *A. fumigatus* endosomes during programmed necroptosis to enable containment of fungal germination.

A. fumigatus conidia are phagocytosed by an actin-dependent process resulting in calcineurin pathway activation. This either results in fungal killing and resolution of inflammation or persistent fungal germination. Phagosomal inability to contain fungal germination triggers programmed necrosis and calcineurin-actin-dependent lateral transfer of *A. fumigatus* containing endosomes to neighbouring macrophages. This then enables further phagolysosomal containment, fungal killing and resolution of inflammation.

Historically, transplant rejection and tolerance has been attributed to the adaptive immune system, with research showing the necessity of anti-graft effector T cells for organ rejection and the presence of regulatory T cells for long-term graft tolerance (Dong et al., 1999). Hence, the success of calcineurin inhibitors in preventing graft rejection was unsurprisingly attributed to effects on the T cell and adaptive immunity (Calne, 2004). Recent literature however, has shown an increasing role for the innate immune in transplant rejection and tolerance (Hanidziar et al., 2010, Land, 2012, Murphy et al., 2011). This thesis shows that the calcineurin-NFAT pathway has a significant role in the innate immune response. To date, however, it is unclear whether the innate immune effects of calcineurin inhibitors are contributing to transplant tolerance. Hence, future work is required to

explore the possibility of uncoupling the beneficial effects on innate immunity obtained by calcineurin inhibition from deleterious effects on the innate immune response to microbial pathogens.

Innate signalling through the calcineurin-NFAT pathway in human macrophages has not been fully characterised (Fric et al., 2012b). In murine macrophages, our group recently described calcineurin activation through a novel TLR9-BTK endosomal signalling pathway (Herbst et al., 2015). It is unclear as yet whether this is preserved in human macrophages. The involvement of NOD-like receptors in the calcineurin-NFAT activation described in murine models has additionally not been confirmed in human macrophages (Tourneur et al., 2013, Vandewalle et al., 2014). Obtaining a better understanding of the innate fungal pattern recognition receptors that signal through the calcineurin-NFAT pathway, alongside an understanding of salvage pathways and their receptors that are activated following fungal infection in solid-organ transplantation may enable better patient risk stratification for invasive fungal infections and optimal targeted prophylaxis post-transplant.

At present, the majority of patients with solid-organ transplantation are also on corticosteroid treatment (Doyle et al., 2004). Corticosteroids have been shown to impair NF- κ B and AP-1 signalling, thus likely having indirect effects on the calcineurin-NFAT pathway (Barnes, 2006). In non-transplant patients on corticosteroid treatment, it is intriguing to suggest whether the calcineurin-NFAT pathway represents a salvage pathway for innate activation. This is suggested by my results which show NFAT activation only following infection with germinating *A. fumigatus* conidia with little inflammatory response to resting conidia. Contrary to previous work, data within our murine models does not suggest a glucocorticoid receptor potentiating effect from calcineurin inhibition, however this needs confirming in human macrophages (Herbst et al., 2015, Herbst et al., 2013, Jaaskelainen et al., 2011, Takeuchi et al., 2015). Further research is therefore required to analyse calcineurin-NFAT pathway activation during corticosteroid treatment and the effects of further calcineurin inhibition on the host innate immune response.

This thesis also highlights programmed necrosis as a primary outcome following *A. fumigatus* macrophage infection and uncontrolled fungal germination in the phagosome. Our understanding of programmed necrosis is evolving with greater mechanistic understanding of pyroptotic and necroptotic signalling pathways and regulation (Pasparakis and Vandenabeele, 2015, Vanden Berghe et al., 2014, Inoue and Tani, 2014). There is additionally increasing interest in the role of regulated or programmed necrosis in the host immune response (Ashida et al., 2011). Although necrotic cell death and cell leakage is highly inflammatory, recent work has shown that it can enable elimination of intracellular pathogens that are unable to be killed through apoptosis due to pathogen-encoded caspase inhibitors (Mocarski et al., 2014). The role of programmed cell death in human *A. fumigatus* infection however is unclear. Although studies have suggested that *A. fumigatus* may be able to inhibit AM apoptosis in a melanin-dependent manner, very little is known regarding the role of programmed necroptosis, pyroptosis and autophagy in the host immune response to *A. fumigatus* (Volling et al., 2011, Kanayama and Shinohara, 2016, Said-Sadier et al., 2010a). This thesis suggests that although programmed necrosis is a primary outcome in human macrophages unable to kill intraphagosomal germinating *A. fumigatus* conidia, uncontrolled necrosis leads to increased fungal burden. Lateral transfer of *A. fumigatus* endosomes to neighbouring cells during regulated necrosis therefore appears to be a salvage mechanism to prevent escape and fungal germination. Intraphagosomal fungal germination and hyphal transition appears to be a critical step and perhaps a checkpoint for macrophage programmed necrosis. The role of hyphal growth in fungal virulence is well established within *A. fumigatus*, *C. albicans* and other fungal pathogens (Brand, 2012). The primary function is to invasive the substrate they are adhered to and acquire nutrients. The effect of *A. fumigatus* mutant strains with abherent germination and hyphal transition on programmed macrophage necrosis and lateral transfer would enable confirmation (Fortwendel et al., 2005).

At present, the programmed cell necrosis pathway during macrophage *A. fumigatus* infection and lateral transfer is unclear. Exogenous induction of necroptosis using pan-caspase inhibition stimulates increased lateral transfer of *A. fumigatus*, however inhibition of necrosome formation

was unable to inhibit programmed cell death and lateral transfer. Further research is clearly required to dissect the signalling pathways critical to programmed necrosis following human macrophage *A. fumigatus* infection, and additionally better characterise the type of regulated necrosis. The subsequent development of a murine BMDM model of this interaction in our group may allow the utilisation of knockout strains to examine the MLKL and RIPK3 dependence of programmed macrophage necrosis during *A. fumigatus* infection. As a primary marker of uncontrolled fungal infection, however, biomarker assays of necrosis may have diagnostic potential and help determine treatment response.

Additionally, this thesis shows an intrinsic role for calcineurin in regulating programmed cell death during macrophage fungal infection and necrosis-dependent *A. fumigatus* lateral transfer. Given studies suggesting an important role for calcineurin in the innate immune response to bacterial (e.g. uropathogenic *E. coli*) and *C. albicans* infections, further research is required to determine the calcineurin-dependent effect on the programmed cell death response to a broader pathogen profile (Goodridge et al., 2007, Tourneur et al., 2013). Further characterisation of the calcineurin-dependent pathways involved in the macrophage programmed necrosis response to *A. fumigatus* infection may allow improved drug development to negate off-target effects not necessary in achieving organ tolerance. Better pathway characterisation may also potentially identify targets for modulatory therapy. Programmed cell death inhibitors or inducers are now currently in clinical and trial use for a number of oncological disorders (Homet Moreno and Ribas, 2015, Lemke et al., 2014). A better understanding of the host programmed cell death response to intracellular pathogens including *A. fumigatus* may eventually allow the development of similar complimentary therapies in invasive fungal infections to improve outcome.

Numerous studies have highlighted how bacterial pathogens subvert the host cell cytoskeletal system to promote their own survival, replication and dissemination (Traven and Naderer, 2014b, Thi et al., 2012). This work suggests that lateral transfer of *A. fumigatus* within actin-VASP enveloped

endosomes to neighbouring cells during programmed necrosis is a host beneficial process. The role of *A. fumigatus* in triggering host necrosis and whether there is any pathogen-mediated subversion of the host cytoskeleton or endosomal trafficking is unclear. Analysis of the importance of the pH-responsive PacC transcription factor of *A. fumigatus* utilising a $\Delta pacC$ mutant might enable further insights (Bertuzzi et al., 2014). Recent studies have suggested *Cryptococcus neoformans* has the ability to subvert the host macrophage immune response with modification of phagolysosomal maturation, permeabilization and fusion with the plasma membrane to enable escape (Johnston and May, 2010). Whether there is differing virulence, macrophage necrosis and lateral transfer between clinical strains of *A. fumigatus* remains to be seen. A significant limitation of this work is the utilisation of only 1 wild-type clinical strain of *A. fumigatus*. Further work within our group is now analysing the effects of several fully sequenced strains of *A. fumigatus* on macrophage programmed necrosis and lateral transfer to elucidate significant pathogen-specific virulence factors. Studies have previously suggested that gliotoxin production from *A. fumigatus* may have the ability to modulate the host actin cytoskeleton (Comera et al., 2007). At present, the actin-dependent uptake mechanisms during *A. fumigatus* phagocytosis at the various germination stages have been poorly characterised (Luther et al., 2008). The specific roles and importance of actin polymerases, such as VASP which appears to be calcineurin-regulated, during *A. fumigatus* phagocytosis is again poorly understood. Fundamental dissection of the host actin-cytoskeletal organisation and signalling pathways during *A. fumigatus* uptake will allow a deeper understanding of the effects of immunosuppression on host actin-dependent processes.

Overall, this thesis suggests a critical role for calcineurin in the innate immune response of human AM infected with *A. fumigatus*. Calcineurin appears to orchestrate lateral transfer of *A. fumigatus* during programmed macrophage necrosis to prevent fungal germination and pathogen escape. Thus, the increased incidence of invasive fungal infections seen within solid-organ transplantation likely relates to unintended consequences of immunosuppression on the host innate immune

response. Further research is required to separate beneficial calcineurin-inhibitor effects on organ tolerance from deleterious consequences on host defence.

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8 Appendix

8.1 Phosphoarray of human monocyte-derived macrophages infected with *A. fumigatus* swollen conidia at 1h p.i.: FK506 vs Control.

A phosphoprotein array analysis (*Full Moon Biosystems*) of human MDM pre-treated with FK506 and stimulated with live *A. fumigatus* swollen conidia (MOI = 1) for 1 h p.i. is shown. Fold change differences in the phosphoprotein to non-phosphoprotein ratio between FK506 and control macrophages stimulated with live *A. fumigatus* swollen conidia are shown. A colour scale is used to represent decreased phosphoprotein to non-phosphoprotein ratio (red) to increased phosphoprotein to non-phosphoprotein ratio (blue).

Phospho Protein	Non Phospho Protein	Signal Ratio of Phospho Protein to Non-Phospho Protein		Fold Changes in Ratios
		Control	FK506	FK506 vs Control
14-3-3 theta/tau (Phospho-Ser232)	14-3-3 theta/tau (Ab-232)	1.52	1.80	1.18
14-3-3 zeta (Phospho-Ser58)	14-3-3 zeta (Ab-58)	1.78	1.57	0.88
14-3-3 zeta/delta (Phospho-Thr232)	14-3-3 zeta/delta (Ab-232)	0.23	0.15	0.66
4E-BP1 (Phospho-Ser65)	4E-BP1 (Ab-65)	0.11	0.06	0.54
4E-BP1 (Phospho-Thr36)	4E-BP1 (Ab-36)	1.67	1.95	1.16
4E-BP1 (Phospho-Thr45)	4E-BP1 (Ab-45)	0.81	0.80	1.00
4E-BP1 (Phospho-Thr70)	4E-BP1 (Ab-70)	1.25	0.99	0.79
Abl1 (Phospho-Tyr204)	Abl1 (Ab-204)	0.04	0.04	1.27
Abl1 (Phospho-Thr754/735)	Abl1 (Ab-754/735)	1.55	1.50	0.97
ACC1 (Phospho-Ser79)	ACC1 (Ab-79)	1.69	0.95	0.56
ACC1 (Phospho-Ser80)	ACC1 (Ab-80)	0.20	0.14	0.74
ACTIN Pan(a/b/g) (Phospho-Tyr55/53)	ACTIN Pan(a/b/g) (Ab-55/53)	0.17	0.18	1.06
AFX/FOXO4 (Phospho-Ser197)	AFX/FOXO4 (Ab-197)	0.32	0.34	1.09

AKT (Phospho-Thr308)	AKT (Ab-308)	0.41	0.41	1.00
AKT (Phospho-Tyr326)	AKT (Ab-326)	0.56	0.89	1.58
AKT1 (Phospho-Ser124)	AKT1 (Ab-124)	0.82	0.37	0.45
AKT1 (Phospho-Ser246)	AKT1 (Ab-246)	0.69	0.67	0.98
AKT1 (Phospho-Thr450)	AKT1 (Ab-450)	2.32	1.85	0.80
AKT1 (Phospho-Tyr474)	AKT1 (Ab-474)	0.80	1.00	1.24
AKT1 (Phospho-Thr72)	AKT1 (Ab-72)	0.32	0.32	0.98
AKT1S1 (Phospho-Thr246)	AKT1S1 (Ab-246)	0.22	0.14	0.65
AKT2 (Phospho-Ser474)	AKT2 (Ab-474)	0.92	1.06	1.15
ALK (Phospho-Tyr1507)	ALK (Ab-1507)	1.56	2.13	1.37
ALK (Phospho-Tyr1604)	ALK (Ab-1604)	1.34	0.89	0.66
AMPK1 (Phospho-Thr174)	AMPK1 (Ab-174)	0.84	0.91	1.08
AMPK1/AMPK2 (Phospho-Ser485/491)	AMPK1/AMPK2 (Ab-485/491)	0.96	0.95	0.98
AMPKbeta1 (Phospho-Ser182)	AMPKbeta1 (Ab-182)	0.74	0.44	0.60
Amyloid beta A4 (Phospho-Thr743/668)	Amyloid beta A4 (Ab-743/668)	0.72	0.66	0.92
Androgen Receptor (Phospho-Ser213)	Androgen Receptor (Ab-213)	0.47	0.55	1.18
Androgen Receptor (Phospho-Ser650)	Androgen Receptor (Ab-650)	0.43	0.44	1.03
A-RAF (Phospho-Tyr301/302)	A-RAF (Ab-301/302)	0.78	0.51	0.65
Arrestin-1 (Phospho-Ser412)	Arrestin-1 (Ab-412)	1.35	0.82	0.61
ASK1 (Phospho-Ser83)	ASK1 (Ab-83)	1.14	1.22	1.07
ASK1 (Phospho-Ser966)	ASK1 (Ab-966)	0.66	0.62	0.95
ATF-1 (Phospho-Ser63)	ATF-1 (Ab-63)	0.05	0.10	1.89
ATF2 (Phospho-Ser112/94)	ATF2 (Ab-112/94)	0.64	0.60	0.94
ATF2 (Phospho-Ser62/44)	ATF2 (Ab-62/44)	0.91	0.90	0.99
ATF2 (Phospho-Thr69/51)	ATF2 (Ab-69/51)	1.06	0.90	0.84
ATF2 (Phospho-Thr71/53)	ATF2 (Ab-71/53)	1.80	2.09	1.16
ATF2 (Phospho-Thr73/55)	ATF2 (Ab-73/55)	1.01	1.00	0.98
ATF4 (Phospho-Ser245)	ATF4 (Ab-245)	0.76	0.84	1.11
ATP1A1/Na+K+ ATPase1 (Phospho-Ser23)	ATP1A1/Na+K+ ATPase1 (Ab-23)	1.63	2.35	1.44
ATPase (Phospho-Ser16)	ATPase (Ab-16)	0.29	0.39	1.36
ATP-Citrate Lyase (Phospho-Ser454)	ATP-Citrate Lyase (Ab-454)	0.10	0.13	1.31
ATRIP (Phospho-Ser68/72)	ATRIP (Ab-68/72)	0.06	0.05	0.85
AurB (Phospho-Thr232)	AurB (Ab-232)	2.97	2.83	0.95

AurB (Phospho-Tyr12)	AurB (Ab-12)	2.48	2.36	0.95
AURORA KINASE (Phospho-Thr288)	AURORA KINASE (Ab-288)	1.55	1.76	1.13
BAD (Phospho-Ser112)	BAD (Ab-112)	0.59	0.61	1.03
BAD (Phospho-Ser134)	BAD (Ab-134)	0.89	0.98	1.11
BAD (Phospho-Ser136)	BAD (Ab-136)	1.09	0.85	0.78
BAD (Phospho-Ser155)	BAD (Ab-155)	0.61	0.70	1.15
BAD (Phospho-Ser91/128)	BAD (Ab-91/128)	0.95	0.85	0.90
BCL-2 (Phospho-Ser70)	BCL-2 (Ab-70)	3.94	3.16	1.07
BCL-2 (Phospho-Thr56)	BCL-2 (Ab-56)	1.62	1.43	0.88
BCL-2 (Phospho-Thr69)	BCL-2 (Ab-69)	2.69	1.95	0.73
BCL-XL (Phospho-Ser62)	BCL-XL (Ab-62)	0.08	0.09	1.14
BCL-XL (Phospho-Thr47)	BCL-XL (Ab-47)	0.10	0.07	0.72
BCR (Phospho-Tyr177)	BCR (Ab-177)	1.09	1.03	0.94
BCR (Phospho-Tyr360)	BCR (Ab-360)	1.91	1.97	1.03
BID (Phospho-Ser78)	BID (Ab-78)	0.65	0.55	0.84
BIM (Phospho-Ser69/65)	BIM (Ab-69/65)	0.86	0.92	1.06
BLNK (Phospho-Tyr84)	BLNK (Ab-96)	0.35	0.68	1.92
BLNK (Phospho-Tyr96)	BLNK (Ab-96)	0.26	0.48	1.88
B-RAF (Phospho-Ser446)	B-RAF (Ab-446)	0.57	0.60	1.05
B-RAF (Phospho-Ser601)	B-RAF (Ab-601)	0.81	1.14	1.42
B-RAF (Phospho-Thr598)	B-RAF (Ab-598)	0.14	0.11	0.80
BRCA1 (Phospho-Ser1423)	BRCA1 (Ab-1423)	0.28	0.23	0.83
BRCA1 (Phospho-Ser1457)	BRCA1 (Ab-1457)	0.45	0.89	1.99
BRCA1 (Phospho-Ser1524)	BRCA1 (Ab-1524)	0.30	0.32	1.05
BTK (Phospho-Tyr223)	BTK (Ab-223)	0.90	0.62	0.69
BTK (Phospho-Tyr550)	BTK (Ab-223)	1.62	1.00	0.62
c-Abl (Phospho-Tyr245)	c-Abl (Ab-412)	0.53	0.50	0.94
c-Abl (Phospho-Tyr412)	c-Abl (Ab-412)	0.45	0.47	1.05
Calmodulin (Phospho-Thr79/Ser81)	Calmodulin (Ab-79/81)	0.42	0.05	0.13
Calsenilin/KCNIP3 (Phospho-Ser63)	Calsenilin/KCNIP3 (Ab-63)	3.60	2.78	0.77
CaMK1-a (Phospho-Thr177)	CaMK1-a (Ab-177)	0.29	0.32	1.12
CaMK2 (Phospho-Thr305)	CaMK2-beta/gamma/delta (Ab-287)	0.07	0.06	0.93
CaMK2-beta/gamma/delta (Phospho-Thr287)	CaMK2-beta/gamma/delta (Ab-287)	0.10	0.10	1.00
CaMK4 (Phospho-Thr196/200)	CaMK4 (Ab-196/200)	0.80	0.88	1.11

CaMKII (Phospho-Thr286)	CaMKII (Ab-286)	0.24	0.29	1.20
CASP1 (Phospho-Ser376)	CASP1 (Ab-376)	1.24	2.34	1.88
CASP2 (Phospho-Ser157)	CASP2 (Ab-157)	1.22	1.23	1.01
CASP6 (Phospho-Ser257)	CASP6 (Ab-257)	0.60	0.41	0.69
CASP8 (Phospho-Ser347)	CASP8 (Ab-347)	0.47	0.46	0.98
CASP9 (Phospho-Thr125)	CASP9 (Ab-125)	1.16	0.67	0.58
Caspase 9 (Phospho-Ser144)	Caspase 9 (Ab-144)	0.64	0.76	1.18
Caspase 9 (Phospho-Tyr153)	Caspase 9 (Ab-153)	0.99	0.51	0.52
Caspase 9 (Phospho-Ser196)	Caspase 9 (Ab-196)	0.29	0.23	0.78
Caspase-3 (Phospho-Ser150)	Caspase-3 (Ab-150)	5.26	3.33	0.63
Catalase (Phospho-Tyr385)	Catalase (Ab-385)	0.26	0.19	0.72
Catenin beta (CTNNB) (Phospho-Tyr489)	Catenin beta (CTNNB) (Ab-489)	0.89	1.63	1.83
Catenin beta (Phospho-Ser33)	Catenin beta (CTNNB) (Ab-33)	0.39	0.47	1.21
Catenin beta (Phospho-Ser37)	Catenin beta (Ab-37)	0.16	0.10	0.60
Catenin beta (Phospho-Thr41/Ser45)	Catenin beta (Ab-41/45)	0.67	0.72	1.08
Catenin beta (Phospho-Tyr654)	Catenin beta (Ab-654)	0.74	0.73	0.98
Catenin delta-1 (Phospho-Tyr228)	Catenin delta-1 (Ab-228)	2.41	1.63	0.68
Caveolin-1 (Phospho-Tyr14)	Caveolin-1 (Ab-14)	0.96	0.89	0.94
CD19 (Phospho-Tyr531)	CD19 (Ab-531)	0.88	1.21	1.37
CD227/mucin 1 (Phospho-Tyr1243)	CD227/mucin 1 (Ab-1243)	2.72	2.48	0.91
CD3Z (Phospho-Tyr142)	CD3Z (Ab-142)	1.61	1.28	0.80
CD4 (Phospho-Ser433)	CD4 (Ab-433)	0.13	0.15	1.13
CD5 (Phospho-Tyr453)	CD5 (Ab-453)	0.39	0.35	0.90
CDC2 (Phospho-Tyr15)	CDC2 (Ab-15)	1.23	1.05	0.85
CDC25A (Phospho-Ser124)	CDC25A (Ab-124)	10.52	8.14	0.77
CDC25A (Phospho-Ser75)	CDC25A (Ab-75)	0.37	1.40	3.82
CDC25B (Phospho-Ser323)	CDC25B (Ab-323)	0.54	0.46	0.85
CDC25B (Phospho-Ser353)	CDC25B (Ab-353)	5.17	3.24	0.63
CDC25C (Phospho-Ser216)	CDC25C (Ab-216)	0.04	0.04	0.92
CDC25C (Phospho-Thr48)	CDC25C (Ab-216)	0.05	0.03	0.59
CDK1/CDC2 (Phospho-Thr14)	CDK1/CDC2 (Ab-14)	2.06	2.29	1.11
CDK2 (Phospho-Thr160)	CDK2 (Ab-160)	0.41	0.36	0.88
CDK5 (Phospho-Tyr15)	CDK5 (Ab-15)	0.23	0.22	0.96
CDK7 (Phospho-Thr170)	CDK7 (Ab-170)	1.21	0.79	0.65

Chk1 (Phospho-Ser280)	Chk1 (Ab-280)	2.35	3.19	1.35
Chk1 (Phospho-Ser286)	Chk1 (Ab-286)	0.71	0.70	0.99
Chk1 (Phospho-Ser317)	Chk1 (Ab-317)	0.81	0.81	0.99
Chk1 (Phospho-Ser345)	Chk1 (Ab-345)	0.43	0.42	0.98
Chk2 (Phospho-Thr383)	Chk2 (Ab-383)	0.26	0.26	0.97
Chk2 (Phospho-Thr387)	Chk2 (Ab-387)	0.29	0.33	1.14
Chk2 (Phospho-Ser516)	Chk2 (Ab-516)	0.58	0.71	1.23
Chk2 (Phospho-Thr68)	Chk2 (Ab-68)	0.10	0.08	0.83
c-Jun (Phospho-Tyr170)	c-Jun (Ab-170)	0.54	1.19	2.20
c-Jun (Phospho-Thr239)	c-Jun (Ab-239)	0.95	0.95	1.00
c-Jun (Phospho-Ser243)	c-Jun (Ab-243)	0.39	0.37	0.97
c-Jun (Phospho-Ser63)	c-Jun (Ab-63)	1.78	1.29	0.72
c-Jun (Phospho-Ser73)	c-Jun (Ab-73)	0.04	0.04	0.83
c-Jun (Phospho-Thr91)	c-Jun (Ab-91)	2.58	2.33	0.90
c-Jun (Phospho-Thr93)	c-Jun (Ab-93)	1.37	2.38	1.73
CK1-A (Phospho-Thr321)	CK1-A (Ab-321)	2.80	3.20	1.14
CK2-b (Phospho-Ser209)	CK2-b (Ab-209)	1.12	0.87	0.78
c-Kit (Phospho-Tyr721)	c-Kit (Ab-721)	1.46	1.84	1.26
claudin 3 (Phospho-Tyr219)	claudin 3 (Ab-219)	1.35	1.60	1.19
claudin 7 (Phospho-Tyr210)	claudin 7 (Ab-210)	2.31	3.16	1.37
c-met (Phospho-Tyr1003)	c-met (Ab-1003)	1.47	1.45	0.98
cofilin (Phospho-Ser3)	cofilin (Ab-3)	0.45	0.51	1.14
Connexin 43 (Phospho-Ser367)	Connexin 43 (Ab-367)	0.89	0.54	0.61
Cortactin (Phospho-Tyr421)	Cortactin (Ab-421)	5.90	6.12	1.04
Cortactin (Phospho-Tyr466)	Cortactin (Ab-466)	0.51	0.56	1.10
COT (Phospho-Thr290)	COT (Ab-290)	0.80	0.77	0.95
CPI17 α (Phospho-Thr38)	CPI17 α (Ab-38)	0.93	0.94	1.01
c-PLA2 (Phospho-Ser505)	c-PLA2 (Ab-505)	0.70	0.69	0.99
c-Raf (Phospho-Ser296)	c-Raf (Ab-296)	0.72	0.64	0.89
c-Raf (Phospho-Ser43)	c-Raf (Ab-43)	0.34	0.30	0.90
CREB (Phospho-Thr100)	CREB (Ab-100)	1.46	1.73	1.19
CREB (Phospho-Ser121)	CREB (Ab-121)	1.03	1.17	1.13
CREB (Phospho-Ser129)	CREB (Ab-129)	0.70	0.83	1.19
CREB (Phospho-Ser133)	CREB (Ab-133)	0.86	0.71	0.82

CREB (Phospho-Ser142)	CREB (Ab-142)	0.80	0.65	0.81
CrklI (Phospho-Tyr221)	CrklI (Ab-221)	0.60	0.56	0.92
CSFR (Phospho-Tyr561)	CSFR (Ab-561)	1.95	2.75	1.41
Cyclin B1 (Phospho-Ser126)	Cyclin B1 (Ab-126)	0.91	0.67	0.73
Cyclin B1 (Phospho-Ser147)	Cyclin B1 (Ab-147)	0.92	0.84	0.91
Cyclin D1 (Phospho-Thr286)	Cyclin D1 (Ab-286)	0.77	0.63	0.82
Cyclin D1 (Phospho-Thr286)	Cyclin D1 (Ab-90)	1.68	1.48	0.88
Cyclin D3 (Phospho-Thr283)	Cyclin D3 (Ab-283)	1.01	0.65	0.64
Cyclin E1 (Phospho-Thr395)	Cyclin E1 (Ab-395)	0.89	0.89	1.00
Cyclin E1 (Phospho-Thr77)	Cyclin E1 (Ab-77)	0.16	0.15	0.89
Cytokeratin 18 (Phospho-Ser52)	Cytokeratin 18 (Ab-52)	2.65	2.93	1.11
Cytokeratin 8 (Phospho-Ser431)	Cytokeratin 8 (Ab-431)	1.53	1.24	0.81
DAB1 (Phospho-Tyr220)	DAB1 (Ab-220)	0.62	0.72	1.17
DAB1 (Phospho-Tyr232)	DAB1 (Ab-232)	0.45	0.57	1.26
DAPP1 (Phospho-Tyr139)	DAPP1 (Ab-139)	1.81	1.35	0.74
DARPP-32 (Phospho-Thr34)	DARPP-32 (Ab-34)	0.22	0.23	1.05
DARPP-32 (Phospho-Thr75)	DARPP-32 (Ab-75)	1.51	0.94	0.62
DAXX (Phospho-Ser668)	DAXX (Ab-668)	0.08	0.06	0.83
DDX5/DEAD-box protein 5 (Phospho-Tyr593)	DDX5/DEAD-box protein 5 (Ab-593)	0.70	0.62	0.90
DNA-PK (Phospho-Thr2638)	DNA-PK (Ab-2638)	0.88	0.72	0.82
DNA-PK (Phospho-Thr2647)	DNA-PK (Ab-2647)	1.84	1.73	0.94
Dok-1 (Phospho-Tyr362)	Dok-1 (Ab-362)	0.60	0.50	0.84
Dok-1 (Phospho-Tyr398)	Dok-1 (Ab-398)	0.74	0.78	1.06
Dok-2 (Phospho-Tyr299)	Dok-2 (Ab-299)	0.65	0.55	0.84
DYN1 (Phospho-Ser774)	DYN1 (Ab-774)	0.06	0.08	1.32
E2F1 (Phospho-Thr433)	E2F1 (Ab-433)	0.47	0.31	0.67
EEF2 (Phospho-Thr56)	EEF2 (Ab-56)	0.66	0.59	0.89
eEF2K (Phospho-Ser366)	eEF2K (Ab-366)	0.61	0.61	1.01
EGFR (Phospho-Tyr1016)	EGFR (Ab-1016)	0.67	0.67	1.00
EGFR (Phospho-Tyr1069)	EGFR (Ab-1069)	2.34	3.07	1.31
EGFR (Phospho-Ser1070)	EGFR (Ab-1070)	0.06	0.07	1.07
EGFR (Phospho-Tyr1092)	EGFR (Ab-1092)	0.19	0.22	1.14
EGFR (Phospho-Tyr1110)	EGFR (Ab-1110)	1.20	1.58	1.31
EGFR (Phospho-Tyr1172)	EGFR (Ab-1172)	0.45	0.41	0.90

EGFR (Phospho-Tyr1197)	EGFR (Ab-1197)	0.62	0.59	0.94
EGFR (Phospho-Thr678)	EGFR (Ab-678)	1.59	1.52	0.96
EGFR (Phospho-Thr693)	EGFR (Ab-693)	0.33	0.31	0.95
EGFR (Phospho-Tyr869)	EGFR (Ab-869)	0.15	0.10	0.63
eIF2A (Phospho-Ser51)	eIF2A (Ab-51)	0.49	0.36	0.74
eIF4G (Phospho-Ser1108)	eIF4G (Ab-1108)	0.27	0.23	0.85
Elk-1 (Phospho-Ser383)	Elk-1 (Ab-383)	1.44	1.81	1.25
Elk1 (Phospho-Ser389)	Elk1 (Ab-389)	0.71	0.70	0.98
Elk1 (Phospho-Thr417)	Elk1 (Ab-417)	1.15	1.66	1.45
eNOS (Phospho-Ser1177)	eNOS (Ab-1177)	0.69	0.89	1.30
eNOS (Phospho-Thr495)	eNOS (Ab-495)	1.16	1.47	1.27
eNOS (Phospho-Ser615)	eNOS (Ab-615)	1.77	1.67	0.94
EPB41 (Phospho-Tyr418/660)	EPB41 (Ab-418/660)	2.18	1.83	0.84
EPHA2/3/4 (Phospho-Tyr588/596)	EPHA2/3/4 (Ab-588/596)	0.62	0.65	1.05
EPHB1/2 (Phospho-Tyr594/604)	EPHB1/2 (Ab-594/604)	7.76	5.04	0.65
Ephrin B (Phospho-Tyr330)	Ephrin B (Ab-330)	0.18	0.14	0.77
Ephrin-B1 (Phospho-Tyr317)	Ephrin-B1 (Ab-317)	1.03	0.96	0.93
Epo-R (Phospho-Tyr368)	Epo-R (Ab-368)	0.92	0.90	0.98
ERK3 (Phospho-Ser189)	ERK3 (Ab-189)	1.34	1.60	1.20
Estrogen Receptor- α (Phospho-Ser104)	Estrogen Receptor- α (Ab-104)	0.42	0.44	1.05
Estrogen Receptor- α (Phospho-Ser106)	Estrogen Receptor- α (Ab-106)	0.06	0.05	0.79
Estrogen Receptor- α (Phospho-Ser118)	Estrogen Receptor- α (Ab-118)	2.46	3.14	1.28
Estrogen Receptor- α (Phospho-Ser167)	Estrogen Receptor- α (Ab-167)	0.58	0.57	0.98
ETK (Phospho-Tyr40)	ETK (Ab-40)	1.25	1.37	1.10
ETK (Phospho-Tyr566)	ETK (Ab-566)	10.35	9.44	0.91
Ezrin (Phospho-Tyr353)	Ezrin (Ab-353)	0.79	0.83	1.04
Ezrin (Phospho-Tyr478)	Ezrin (Ab-478)	1.02	0.74	0.73
Ezrin (Phospho-Thr566)	Ezrin (Ab-566)	0.10	0.09	0.91
FADD (Phospho-Ser194)	FADD (Ab-194)	0.15	0.16	1.05
FAK (Phospho-Tyr397)	FAK (Ab-397)	0.55	0.47	0.85
FAK (Phospho-Tyr407)	FAK (Ab-407)	0.91	1.00	1.10
FAK (Phospho-Tyr576)	FAK (Ab-576)	2.09	1.52	0.73
FAK (Phospho-Tyr861)	FAK (Ab-861)	0.93	0.91	0.98
FAK (Phospho-Ser910)	FAK (Ab-910)	0.15	0.16	1.02

FAK (Phospho-Tyr925)	FAK (Ab-925)	0.43	0.53	1.23
FER (Phospho-Tyr402)	FER (Ab-402)	2.76	2.98	1.08
FGFR1 (Phospho-Tyr154)	FGFR1 (Ab-154)	1.03	1.27	1.23
FGFR1 (Phospho-Tyr654)	FGFR1 (Ab-654)	0.04	0.04	1.09
FGFR1 (Phospho-Tyr766)	FGFR1 (Ab-766)	0.47	0.43	0.92
Filamin A (Phospho-Ser2152)	Filamin A (Ab-2152)	0.59	0.66	1.11
FKHR (Phospho-Ser256)	FKHR (Ab-256)	0.74	0.68	0.92
FKHR (Phospho-Ser319)	FKHR (Ab-319)	0.66	0.73	1.11
FKHRL1/FOXO3A (Phospho-Ser253)	FKHRL1/FOXO3A (Ab-253)	0.42	0.40	0.95
FLT3 (Phospho-Tyr842)	FLT3 (Ab-599)	1.27	1.23	0.97
FLT3 (Phospho-Tyr969)	FLT3 (Ab-599)	1.51	1.64	1.09
Fos (Phospho-Thr232)	Fos (Ab-232)	0.51	0.87	1.70
FosB (Phospho-Ser27)	FosB (Ab-27)	0.97	0.85	0.87
FOXO1/3/4-PAN (Phospho-Thr24/32)	FOXO1/3/4-PAN (Ab-24/32)	0.58	0.50	0.87
FOXO1A (Phospho-Ser329)	FOXO1A (Ab-329)	0.70	0.75	1.07
G3BP-1 (Phospho-Ser232)	G3BP-1 (Ab-232)	0.57	0.80	1.40
GAB1 (Phospho-Tyr627)	GAB1 (Ab-627)	0.94	0.87	0.93
GAB1 (Phospho-Tyr659)	GAB1 (Ab-659)	0.50	0.52	1.04
GAB2 (Phospho-Tyr643)	GAB2 (Ab-623)	3.81	3.74	0.98
GABA-RB (Phospho-Ser434)	GABA-RB (Ab-434)	0.90	1.00	1.12
GAP43 (Phospho-Ser41)	GAP43 (Ab-41)	0.45	0.37	0.83
GATA1 (Phospho-Ser142)	GATA1 (Ab-142)	0.87	0.90	1.04
GATA1 (Phospho-Ser310)	GATA1 (Ab-310)	1.27	1.24	0.97
GluR1 (Phospho-Ser849)	GluR1 (Ab-849)	0.07	0.07	1.02
GluR1 (Phospho-Ser863)	GluR1 (Ab-863)	1.07	1.06	0.99
GluR2 (Phospho-Ser880)	GluR2 (Ab-880)	0.05	0.05	1.00
GRB10/Growth factor receptor-bound protein 10 (Phospho-Tyr67)	GRB10/Growth factor receptor-bound protein 10 (Ab-67)	1.14	1.37	1.20
GRB2 (Phospho-Ser159)	GRB2 (Ab-159)	1.27	1.30	1.03
GRK1 (Phospho-Ser21)	GRK1 (Ab-21)	1.92	1.63	0.85
GRK2 (Phospho-Ser29)	GRK2 (Ab-29)	0.34	0.28	0.83
GSK3a-b (Phospho-Tyr216/279)	GSK3a-b (Ab-216/279)	1.94	2.14	1.11
GSK3α (Phospho-Ser21)	GSK3α (Ab-21)	2.16	1.87	0.87
GSK3β (Phospho-Ser9)	GSK3β (Ab-9)	3.61	2.75	0.76

GTPase activating protein (Phospho-Ser387)	GTPase activating protein (Ab-387)	0.32	0.32	1.00
HCK (Phospho-Tyr410)	HCK (Ab-410)	0.03	0.02	0.93
HDAC1 (Phospho-Ser421)	HDAC1 (Ab-421)	0.09	0.05	0.49
HDAC2 (Phospho-Ser394)	HDAC2 (Ab-394)	0.92	0.89	0.97
HDAC3 (Phospho-Ser424)	HDAC3 (Ab-424)	0.29	0.27	0.92
HDAC4 (Phospho-Ser632)	HDAC4 (Ab-632)	1.37	1.93	1.41
HDAC5 (Phospho-Ser259)	HDAC5 (Ab-259)	1.15	1.08	0.94
HDAC5 (Phospho-Ser498)	HDAC5 (Ab-498)	1.23	1.43	1.16
HDAC6 (Phospho-Ser22)	HDAC6 (Ab-22)	0.97	1.23	1.27
HDAC8 (Phospho-Ser39)	HDAC8 (Ab-39)	0.82	0.76	0.92
HER2 (Phospho-Tyr1221/Tyr1222)	HER2 (Ab-1221/1222)	0.40	0.86	2.14
HER2 (Phospho-Tyr1248)	HER2 (Ab-1248)	0.52	0.60	1.17
HER2 (Phospho-Tyr877)	HER2 (Ab-877)	1.17	1.19	1.02
HER3/ErbB3 (Phospho-Tyr1222)	HER3/ErbB3 (Ab-1222)	1.28	1.09	0.86
HER3/ErbB3 (Phospho-Tyr1289)	HER3/ErbB3 (Ab-1289)	2.34	1.83	0.78
HER4/ErbB4 (Phospho-Tyr1284)	HER4/ErbB4 (Ab-1284)	0.58	0.47	0.81
Histone H2A.X (Phospho-Ser139)	Histone H2A.X (Ab-139)	0.38	0.37	0.96
Histone H3.1 (Phospho-Ser10)	Histone H3.1 (Ab-10)	1.06	0.85	0.81
HNF4α (Phospho-Ser313)	HNF4α (Ab-313)	0.96	1.01	1.05
HRS (Phospho-Tyr334)	HRS (Ab-334)	1.25	1.29	1.03
HSF1 (Phospho-Ser303)	HSF1 (Ab-303)	1.02	0.90	0.88
HSL (Phospho-Ser552/563)	HSL (Ab-552/563)	0.34	0.59	1.71
HSL (Phospho-Ser554)	HSL (Ab-554)	0.62	0.71	1.15
HSP 90-beta (Phospho-Ser226)	HSP 90-beta (Ab-226)	2.97	3.70	1.24
HSP27 (Phospho-Ser15)	HSP27 (Ab-15)	3.52	3.31	0.94
HSP27 (Phospho-Ser78)	HSP27 (Ab-78)	2.76	1.96	0.71
HSP27 (Phospho-Ser82)	HSP27 (Ab-82)	0.80	0.79	0.98
Hsp90 co-chaperone Cdc37 (Phospho-Ser13)	Hsp90 co-chaperone Cdc37 (Ab-13)	1.12	0.93	0.83
HSP90B (Phospho-Ser254)	HSP90B (Ab-254)	1.29	1.55	1.20
ICAM-1 (Phospho-Tyr512)	ICAM-1 (Ab-512)	1.45	1.54	1.06
IGF-1R (Phospho-Tyr1161)	IGF-1R (Ab-1161)	2.27	2.34	1.03
IGF-1R (Phospho-Tyr1165/1166)	IGF-1R (Ab-1165/1166)	1.24	1.50	1.20
IGF2R (Phospho-Ser2409)	IGF2R (Ab-2409)	0.73	0.70	0.96
IκB-alpha (Phospho-Ser32/36)	IκB-alpha (Ab-32/36)	0.67	0.52	0.77

IkB-alpha (Phospho-Tyr42)	IkB-alpha (Ab-42)	0.35	0.32	0.93
IkB-beta (Phospho-Thr19)	IkB-beta (Ab-19)	1.28	0.92	0.71
IkB-beta (Phospho-Ser23)	IkB-beta (Ab-19)	0.46	0.46	1.00
IkB-epsilon (Phospho-Ser22)	IkB-epsilon (Ab-22)	0.14	0.15	1.07
IKK gamma (Phospho-Ser85)	IKK gamma (Ab-85)	0.53	0.57	1.07
IKK-a/b (Phospho-Ser180/181)	IKK-a/b (Ab-180/181)	1.26	0.69	0.55
IKK-alpha (Phospho-Thr23)	IKK-alpha (Ab-23)	0.36	0.32	0.89
IKK-beta (Phospho-Tyr188)	IKK-beta (Ab-188)	0.25	0.16	0.65
IKK-beta (Phospho-Tyr199)	IKK-beta (Ab-199)	0.17	0.17	1.04
IKK-GAMMA (Phospho-Ser31)	IKK-GAMMA (Ab-31)	0.14	0.11	0.84
IL-10R-A (Phospho-Tyr496)	IL-10R-A (Ab-496)	0.61	0.78	1.27
IL-13R/CD213a1 (Phospho-Tyr405)	IL-13R/CD213a1 (Ab-405)	2.31	2.15	0.93
IL-2RA/CD25 (Phospho-Ser268)	IL-2RA/CD25 (Ab-268)	0.83	0.90	1.09
IL3R (Phospho-Tyr593)	IL3R (Ab-593)	1.55	1.33	0.86
IL-4R/CD124 (Phospho-Tyr497)	IL-4R/CD124 (Ab-497)	1.09	1.09	1.00
Integrin beta-1 (Phospho-Thr788)	Integrin beta-1 (Ab-788)	0.60	0.45	0.75
Integrin beta-1 (Phospho-Thr788)	Integrin beta-1 (Ab-789)	0.34	0.27	0.80
Integrin beta-3 (Phospho-Tyr773)	Integrin beta-3 (Ab-773)	0.81	0.77	0.96
Integrin beta-3 (Phospho-Tyr785)	Integrin beta-3 (Ab-785)	0.85	0.90	1.06
Interferon-gamma receptor alpha chain precursor (Phospho-Tyr457)	Interferon-gamma receptor alpha chain precursor (Ab-457)	3.23	2.78	0.86
IR (Phospho-Tyr1355)	IR (Ab-1361)	0.77	0.66	0.86
IR (Phospho-Tyr1361)	IR (Ab-1361)	0.95	0.76	0.80
IRS-1 (Phospho-Ser307)	IRS-1 (Ab-307)	0.59	0.75	1.27
IRS-1 (Phospho-Ser312)	IRS-1 (Ab-312)	1.01	0.64	0.63
IRS-1 (Phospho-Ser323)	IRS-1 (Ab-323)	0.48	0.45	0.94
IRS-1 (Phospho-Ser636)	IRS-1 (Ab-636)	0.60	0.62	1.03
IRS-1 (Phospho-Ser639)	IRS-1 (Ab-639)	0.09	0.10	1.09
IRS-1 (Phospho-Ser794)	IRS-1 (Ab-794)	1.49	1.53	1.03
ITGB4 (Phospho-Tyr1510)	ITGB4 (Ab-1510)	0.86	0.91	1.06
JAK1 (Phospho-Tyr1022)	JAK1 (Ab-1022)	0.78	0.73	0.93
JAK2 (Phospho-Tyr1007)	JAK2 (Ab-1007)	0.42	0.41	0.98
JAK2 (Phospho-Tyr221)	JAK2 (Ab-221)	0.92	0.97	1.05
JNK1/2/3 (Phospho-Thr183/Tyr185)	JNK1/2/3 (Ab-183/185)	1.60	1.50	0.93

JunB (Phospho-Ser259)	JunB (Ab-259)	0.89	0.82	0.92
JunB (Phospho-Ser79)	JunB (Ab-79)	1.00	0.75	0.75
JunD (Phospho-Ser255)	JunD (Ab-255)	1.13	1.13	1.01
Keratin 18 (Phospho-Ser33)	Keratin 18 (Ab-33)	0.62	0.50	0.80
Keratin 8 (Phospho-Ser73)	Keratin 8 (Ab-73)	0.36	0.46	1.28
KIT (Phospho-Tyr703)	KIT (Ab-936)	1.36	1.38	1.01
KIT (Phospho-Tyr936)	KIT (Ab-936)	2.02	1.95	0.96
KSR (Phospho-Ser392)	KSR (Ab-392)	0.54	0.67	1.23
Kv1.3/KCNA3 (Phospho-Tyr135)	Kv1.3/KCNA3 (Ab-135)	1.15	1.43	1.24
Lamin A (Phospho-Ser22)	Lamin A (Ab-22)	9.19	11.19	1.22
Lamin A/B(lamin A/C) (Phospho-Ser392)	Lamin A/B(lamin A/C) (Ab-392)	0.02	0.03	1.21
LAT (Phospho-Tyr171)	LAT (Ab-171)	0.39	0.32	0.81
LAT (Phospho-Tyr191)	LAT (Ab-191)	0.53	0.48	0.91
LCK (Phospho-Ser59)	LCK (Ab-59)	0.56	0.61	1.08
LCK (Phospho-Tyr192)	LCK (Ab-192)	1.35	1.03	0.76
Lck (Phospho-Tyr393)	LCK (Ab-393)	0.19	0.24	1.28
LCK (Phospho-Tyr504)	LCK (Ab-504)	1.37	1.00	0.73
LIMK1 (Phospho-Thr508)	LIMK1 (Ab-508)	0.64	0.71	1.11
LKB1 (Phospho-Thr189)	LKB1 (Ab-189)	0.41	0.70	1.71
LKB1 (Phospho-Ser428)	LKB1 (Ab-428)	1.46	1.54	1.05
LYN (Phospho-Tyr507)	LYN (Ab-507)	0.04	0.03	0.76
MAPKAPK2 (Phospho-Ser272)	MAPKAPK2 (Ab-272)	1.05	1.05	1.01
MAPKAPK2 (Phospho-Thr334)	MAPKAPK2 (Ab-334)	0.10	0.05	0.51
MARCKS (Phospho-Ser158)	MARCKS (Ab-158)	0.45	0.45	1.01
MARCKS (Phospho-Ser163)	MARCKS (Ab-163)	0.70	0.51	0.73
M-CSF Receptor (Phospho-Tyr809)	M-CSF Receptor (Ab-809)	2.69	3.12	1.16
MDM2 (Phospho-Ser166)	MDM2 (Ab-166)	0.05	0.04	0.70
MEF2A (Phospho-Thr312)	MEF2A (Ab-312)	0.46	0.43	0.95
MEF2A (Phospho-Thr319)	MEF2A (Ab-319)	0.90	0.93	1.04
MEF2A (Phospho-Ser408)	MEF2A (Ab-408)	0.41	0.36	0.87
MEF2C (Phospho-Ser396)	MEF2C (Ab-396)	0.65	0.82	1.26
MEK1 (Phospho-Ser217)	MEK1 (Ab-217)	0.63	0.66	1.05
MEK1 (Phospho-Ser221)	MEK1 (Ab-221)	1.86	2.84	1.53
MEK1 (Phospho-Thr286)	MEK1 (Ab286)	0.40	0.40	0.98

MEK1 (Phospho-Thr291)	MEK1 (Ab-291)	0.37	0.36	0.98
MEK1 (Phospho-Ser298)	MEK1 (Ab-298)	0.05	0.14	2.69
MEK-2 (Phospho-Thr394)	MEK-2 (Ab-394)	1.68	2.40	1.43
Merlin (Phospho-Ser10)	Merlin (Ab-10)	1.78	1.27	0.72
Merlin (Phospho-Ser518)	Merlin (Ab-518)	1.11	1.39	1.25
Met (Phospho-Tyr1234)	Met (Ab-1234)	1.24	1.32	1.06
Met (Phospho-Tyr1349)	Met (Ab-1349)	0.53	0.68	1.27
MITF (Phospho-Ser73)	MITF (Ab-73)	0.66	0.74	1.12
MKK3 (Phospho-Ser189)	MKK3 (Ab-189)	0.76	0.73	0.95
MKK3/MAP2K3 (Phospho-Thr222)	MKK3/MAP2K3 (Ab-222)	0.61	0.60	0.99
MKK6 (Phospho-Ser207)	MKK6 (Ab-207)	0.41	0.39	0.97
MKK7/MAP2K7 (Phospho-Ser271)	MKK7/MAP2K7 (Ab-271)	0.90	0.75	0.83
MKK7/MAP2K7 (Phospho-Thr275)	MKK7/MAP2K7 (Ab-271)	1.14	1.15	1.01
MKP-1 (Phospho-Ser359)	MKP-1 (Ab-359)	1.22	1.16	0.95
MKP-1/2 (Phospho-Ser296/318)	MKP-1/2 (Ab-296/318)	0.36	0.26	0.70
Mnk1 (Phospho-Thr385)	Mnk1 (Ab-385)	1.90	1.85	0.98
MSK1 (Phospho-Ser360)	MSK1 (Ab-360)	0.20	0.19	0.94
MSK1 (Phospho-Ser376)	MSK1 (Ab-376)	1.77	1.46	0.83
MSK1 (Phospho-Thr581)	MSK1 (Ab-581)	0.36	0.37	1.03
Mst1/Mst2 (Phospho-Thr183)	Mst1/Mst2 (Ab-183)	1.94	1.72	0.89
mTOR (Phospho-Thr2446)	mTOR (Ab-2446)	2.67	2.54	0.95
mTOR (Phospho-Ser2448)	mTOR (Ab-2448)	1.53	1.63	1.06
mTOR (Phospho-Ser2481)	mTOR (Ab-2481)	0.51	0.59	1.16
Myc (Phospho-Thr358)	Myc (Ab-358)	0.17	0.12	0.75
Myc (Phospho-Ser373)	Myc (Ab-373)	0.48	0.46	0.96
Myc (Phospho-Thr58)	Myc (Ab-58)	0.76	1.02	1.33
Myc (Phospho-Ser62)	Myc (Ab-62)	0.39	0.46	1.18
Myosin regulatory light chain 2 (Phospho-Ser18)	Myosin regulatory light chain 2 (Ab-18)	0.71	0.69	0.97
NFAT4 (Phospho-Ser165)	NFAT4 (Ab-165)	1.95	1.86	0.95
NFkB-p100/p52 (Phospho-Ser865)	NFkB-p100/p52 (Ab-865)	0.20	0.16	0.83
NFkB-p100/p52 (Phospho-Ser869)	NFkB-p100/p52 (Ab-869)	0.71	0.70	0.99
NFkB-p105 (Phospho-Ser927)	NFkB-p105 (Ab-927)	0.11	0.09	0.87
NFkB-p105/p50 (Phospho-Ser337)	NFkB-p105/p50 (Ab-337)	5.10	3.32	0.65

NFkB-p105/p50 (Phospho-Ser893)	NFkB-p105/p50 (Ab-893)	0.36	0.50	1.39
NFkB-p105/p50 (Phospho-Ser907)	NFkB-p105/p50 (Ab-907)	0.93	0.87	0.93
NFkB-p105/p50 (Phospho-Ser932)	NFkB-p105/p50 (Ab-932)	0.57	0.78	1.38
NFkB-p65 (Phospho-Thr254)	NFkB-p65 (Ab-254)	0.85	1.62	1.91
NFkB-p65 (Phospho-Ser276)	NFkB-p65 (Ab-276)	1.41	1.04	0.74
NFkB-p65 (Phospho-Ser311)	NFkB-p65 (Ab-311)	1.70	2.06	1.22
NFkB-p65 (Phospho-Thr435)	NFkB-p65 (Ab-435)	0.83	0.82	0.98
NFkB-p65 (Phospho-Ser468)	NFkB-p65 (Ab-468)	1.66	2.21	1.33
NFkB-p65 (Phospho-Ser529)	NFkB-p65 (Ab-529)	0.55	0.56	1.02
NFkB-p65 (Phospho-Ser536)	NFkB-p65 (Ab-536)	2.60	2.00	0.77
NMDAR1 (Phospho-Ser897)	NMDAR1 (Ab-897)	0.63	0.75	1.20
NMDAR2B (Phospho-Tyr1472)	NMDAR2B (Ab-1472)	0.11	0.09	0.80
Opioid Receptor (Phospho-Ser375)	Opioid Receptor (Ab-375)	3.82	5.06	1.32
p130Cas (Phospho-Tyr165)	p130Cas (Ab-165)	0.81	0.69	0.85
p130Cas (Phospho-Tyr410)	p130Cas (Ab-410)	0.65	0.86	1.33
p21Cip1 (Phospho-Thr145)	p21Cip1 (Ab-145)	1.93	1.67	0.87
p27Kip1 (Phospho-Ser10)	p27Kip1 (Ab-10)	6.85	3.02	0.44
p27Kip1 (Phospho-Thr187)	p27Kip1 (Ab-187)	2.34	3.64	1.56
P38 MAPK (Phospho-Thr180)	P38 MAPK (Ab-180)	1.81	1.91	1.05
P38 MAPK (Phospho-Tyr182)	P38 MAPK (Ab-182)	0.09	0.09	1.01
P38 MAPK (Phospho-Tyr322)	P38 MAPK (Ab-322)	0.39	0.57	1.45
p44/42 MAP Kinase (Phospho-Thr202)	p44/42 MAP Kinase (Ab-202)	2.29	2.63	1.15
p44/42 MAP Kinase (Phospho-Tyr204)	p44/42 MAP Kinase (Ab-204)	1.76	2.61	1.48
p53 (Phospho-Ser15)	p53 (Ab-15)	1.47	1.88	1.28
p53 (Phospho-Thr18)	p53 (Ab-18)	0.58	0.45	0.78
p53 (Phospho-Ser20)	p53 (Ab-20)	0.26	0.23	0.86
p53 (Phospho-Ser315)	p53 (Ab-315)	0.14	0.14	0.95
p53 (Phospho-Ser33)	p53 (Ab-33)	0.33	0.40	1.21
p53 (Phospho-Ser37)	p53 (Ab-37)	0.44	0.55	1.24
p53 (Phospho-Ser378)	p53 (Ab-378)	1.23	1.95	1.59
p53 (Phospho-Ser392)	p53 (Ab-392)	0.55	0.49	0.88
p53 (Phospho-Ser46)	p53 (Ab-46)	0.67	0.68	1.02
p53 (Phospho-Ser6)	p53 (Ab-6)	1.20	1.04	0.87
p53 (Phospho-Ser9)	p53 (Ab-9)	0.48	0.43	0.91

P70S6K (Phospho-Thr229)	P70S6K (Ab-229)	1.08	0.78	0.73
P70S6K (Phospho-Ser371)	P70S6K (Ab-371)	1.01	0.77	0.76
P70S6K (Phospho-Ser411)	P70S6k (Ab-411)	0.63	0.59	0.93
P70S6K (Phospho-Ser418)	P70S6K (Ab-418)	0.60	0.64	1.06
P70S6k (Phospho-Thr421)	P70S6k (Ab-421)	0.52	0.49	0.94
P70S6K (Phospho-Ser424)	P70S6K (Ab-424)	1.47	1.67	1.14
P70S6k-beta (Phospho-Ser423)	P70S6k-beta (Ab-423)	0.60	0.60	1.00
P73 (Phospho-Tyr99)	P73 (Ab-99)	1.13	1.52	1.35
P90RSK (Phospho-Thr359/Ser363)	P90RSK (Ab-359/363)	0.24	0.11	0.44
P90RSK (Phospho-Ser380)	P90RSK (Ab-380)	0.93	0.80	0.86
P90RSK (Phospho-Thr573)	P90RSK (Ab-573)	0.55	0.76	1.39
P95/NBS1 (Phospho-Ser343)	P95/NBS1 (Ab-343)	1.32	1.28	0.97
PAK1 (Phospho-Ser204)	PAK1 (Ab-204)	0.77	1.23	1.60
PAK1 (Phospho-Thr212)	PAK1 (Ab-212)	1.28	0.76	0.59
PAK1/2 (Phospho-Ser199)	PAK1/2 (Ab-199)	1.12	0.88	0.78
PAK1/2/3 (Phospho-Ser141)	PAK1/2/3 (Ab-141)	0.81	0.74	0.92
PAK1/2/3 (Phospho-Thr423/402/421)	PAK1/2/3 (Ab-423/402/421)	0.07	0.04	0.68
PAK2 (Phospho-Ser192)	PAK2 (Ab-192)	0.99	1.60	1.61
PAK3 (Phospho-Ser154)	PAK3 (Ab-154)	0.93	1.13	1.21
Paxillin (Phospho-Tyr118)	Paxillin (Ab-118)	1.16	1.84	1.59
Paxillin (Phospho-Tyr31)	Paxillin (Ab-31)	1.40	0.98	0.70
PDGF R alpha (Phospho-Tyr849)	PDGF R alpha (Ab-849)	1.76	1.11	0.63
PDGF R beta (Phospho-Tyr1021)	PDGF R beta (Ab-1021)	2.02	2.74	1.36
PDGF R beta (Phospho-Tyr740)	PDGF R beta (Ab-740)	3.82	3.83	1.00
PDGF R beta (Phospho-Tyr751)	PDGF R beta (Ab-751)	0.50	0.37	0.74
PDK1 (Phospho-Ser241)	PDK1 (Ab-241)	0.36	0.30	0.85
PEA-15 (Phospho-Ser104)	PEA-15 (Ab-116)	0.93	1.08	1.15
PEA-15 (Phospho-Ser116)	PEA-15 (Ab-116)	0.56	0.63	1.12
PECAM-1 (Phospho-Tyr713)	PECAM-1 (Ab-713)	0.05	0.04	0.79
PI3-kinase p85-subunit alpha/gamma (Phospho-Tyr467/Tyr199)	PI3-kinase p85-subunit alpha/gamma (Ab-467/199)	1.07	0.68	0.63
Pim-1 (Phospho-Tyr309)	Pim-1 (Ab-309)	1.20	1.19	0.99
PKA CAT (Phospho-Thr197)	PKA CAT (Ab-197)	0.44	0.47	1.07
PKC alpha (Phospho-Tyr657)	PKC alpha (Ab-657)	0.87	0.91	1.05

PKC alpha/beta II (Phospho-Thr638)	PKC alpha/beta II (Ab-638)	0.35	0.42	1.20
PKC beta/PKCB (Phospho-Ser661)	PKC beta/PKCB (Ab-661)	0.55	0.72	1.32
PKC delta (Phospho-Thr505)	PKC delta (Ab-505)	1.09	1.29	1.18
PKC delta (Phospho-Ser645)	PKC delta (Ab-645)	0.80	0.43	0.54
PKC epsilon (Phospho-Ser729)	PKC epsilon (Ab-729)	0.61	0.66	1.09
PKC pan activation site (Phospho)	PKC pan activation site	0.43	0.30	0.70
PKC theta (Phospho-Thr538)	PKC theta (Ab-538)	0.01	0.01	0.79
PKC theta (Phospho-Ser676)	PKC theta (Ab-676)	0.61	0.97	1.57
PKC zeta (Phospho-Thr410)	PKC zeta (Ab-410)	0.80	0.77	0.96
PKC zeta (Phospho-Thr560)	PKC zeta (Ab-560)	0.50	0.59	1.19
PKD1/PKC mu (Phospho-Ser205)	PKD1/PKC mu (Ab-205)	1.04	1.04	0.99
PKD1/PKC mu (Phospho-Tyr463)	PKD1/PKC mu (Ab-463)	0.66	0.66	1.00
PKD1/PKCmu (Phospho-Ser910)	PKD1/PKCmu (Ab-910)	0.35	0.42	1.20
PKD2 (Phospho-Ser876)	PKD2 (Ab-876)	3.85	3.19	0.83
PKR (Phospho-Thr446)	PKR (Ab-446)	1.06	0.95	0.90
PKR (Phospho-Thr451)	PKR (Ab-451)	0.72	0.71	0.98
PLC beta (Phospho-Ser1105)	PLC beta (Ab-1105)	0.37	0.45	1.19
PLC beta3 (Phospho-Ser537)	PLC beta3 (Ab-537)	0.57	0.79	1.38
PLCG1 (Phospho-Tyr771)	PLCG1 (Ab-771)	0.35	0.56	1.62
PLCG1 (Phospho-Tyr783)	PLCG1 (Ab-783)	0.44	0.45	1.02
PLCG2 (Phospho-Tyr1217)	PLCG2 (Ab-1217)	0.30	0.50	1.67
PLCG2 (Phospho-Tyr753)	PLCG2 (Ab-753)	0.24	0.13	0.55
PLD1 (Phospho-Ser561)	PLD1 (Ab-561)	0.78	0.71	0.92
PP1-B485alpha (Phospho-Thr320)	PP1-alpha (Ab-320)	2.44	1.92	0.79
PP2A-alpha (Phospho-Tyr307)	PP2A-alpha (Ab-307)	0.17	0.17	1.00
PPAR-beta (Phospho-Thr1457)	PPAR-beta (Ab-1457)	0.58	0.71	1.22
PPAR-gamma (Phospho-Ser112)	PPAR-gamma (Ab-112)	0.00	0.01	1.14
Progesterone Receptor (Phospho-Ser190)	Progesterone Receptor (Ab-190)	1.44	1.67	1.16
PTEN (Phospho-Ser370)	PTEN (Ab-370)	0.19	0.19	1.01
PTEN (Phospho-Ser380)	PTEN (Ab-380)	0.42	0.36	0.87
PTEN (Phospho-Ser380/Thr382/Thr383)	PTEN (Ab-380/382/383)	0.76	0.73	0.95
Pyk2 (Phospho-Tyr402)	Pyk2 (Ab-402)	0.76	0.93	1.23
Pyk2 (Phospho-Tyr580)	Pyk2 (Ab-580)	0.22	0.24	1.13
Pyk2 (Phospho-Tyr881)	Pyk2 (Ab-881)	0.29	0.16	0.55

Rac1/cdc42 (Phospho-Ser71)	Rac1/cdc42 (Ab-71)	0.60	0.60	1.01
RAD51 (Phospho-Tyr315)	RAD51 (Ab-309)	1.07	0.98	0.92
RAD52 (Phospho-Tyr104)	RAD52 (Ab-104)	0.01	0.01	0.81
Raf1 (Phospho-Ser259)	Raf1 (Ab-259)	1.78	2.94	1.65
Raf1 (Phospho-Ser338)	Raf1 (Ab-338)	0.54	0.51	0.95
Raf1 (Phospho-Tyr341)	Raf1 (Ab-341)	0.94	0.75	0.79
Raf1(Phospho-Ser621)	Raf1 (Ab-621)	0.56	0.55	0.99
Ras-GRF1 (Phospho-Ser916)	Ras-GRF1 (Ab-916)	0.48	0.47	0.98
Rb (Phospho-Ser608)	Rb (Ab-608)	1.28	1.31	1.02
Rb (Phospho-Ser780)	Rb (Ab-780)	0.18	0.15	0.86
Rb (Phospho-Ser795)	Rb (Ab-795)	0.48	0.45	0.92
Rb (Phospho-Ser807)	Rb (Ab-807)	0.27	0.18	0.66
Rb (Phospho-Ser811)	Rb (Ab-811)	0.37	0.37	1.01
Rel (Phospho-Ser503)	Rel (Ab-503)	0.46	0.48	1.05
RelB (Phospho-Ser552)	RelB (Ab-552)	0.72	0.90	1.25
Ret (Phospho-Tyr905)	Ret (Ab-905)	1.67	1.50	0.90
Rho/Rac guanine nucleotide exchange factor 2 (Phospho-Ser885)	Rho/Rac guanine nucleotide exchange factor 2 (Ab-885)	1.06	1.40	1.32
RSK1/2/3/4 (Phospho-Ser221/227/218/232)	RSK1/2/3/4 (Ab-221/227/218/232)	0.51	0.50	0.99
RyR2 (Phospho-Ser2808)	RyR2 (Ab-2808)	1.17	1.21	1.03
S6 Ribosomal Protein (Phospho-Ser235)	S6 Ribosomal Protein (Ab-235)	0.55	0.73	1.33
SAPK/JNK (Phospho-Thr183)	SAPK/JNK (Ab-183)	1.00	1.16	1.16
SAPK/JNK (Phospho-Tyr185)	SAPK/JNK (Ab-185)	0.82	1.03	1.27
SEK1/MKK4 (Phospho-Thr261)	SEK1/MKK4 (Ab-261)	0.17	0.18	1.02
SEK1/MKK4 (Phospho-Ser80)	SEK1/MKK4 (Ab-80)	0.08	0.07	0.92
SEK1/MKK4/JNKK1 (Phospho-Ser257)	SEK1/MKK4/JNKK1 (Ab-257)	1.27	1.49	1.18
Shc (Phospho-Tyr349)	Shc (Ab-349)	0.08	0.11	1.29
Shc (Phospho-Tyr427)	Shc (Ab-427)	0.23	0.20	0.87
SHP-2 (Phospho-Tyr542)	SHP-2 (Ab-542)	1.72	1.27	0.74
SHP-2 (Phospho-Tyr580)	SHP-2 (Ab-580)	0.57	0.65	1.15
SLP-76 (Phospho-Tyr128)	SLP-76 (Ab-128)	0.91	0.87	0.96
Smad1 (Phospho-Ser187)	Smad1 (Ab-187)	2.28	1.82	0.80
Smad1 (Phospho-Ser465)	Smad1 (Ab-465)	1.43	1.24	0.87
Smad2 (Phospho-Thr220)	Smad2 (Ab-220)	0.89	0.41	0.47

Smad2 (Phospho-Ser250)	Smad2 (Ab-250)	2.05	1.69	0.83
Smad2 (Phospho-Ser467)	Smad2 (Ab-467)	1.15	0.69	0.60
Smad2/3 (Phospho-Thr8)	Smad2/3 (Ab-8)	1.35	1.16	0.86
Smad3 (Phospho-Thr179)	Smad3 (Ab-179)	0.71	0.49	0.69
Smad3 (Phospho-Ser204)	Smad3 (Ab-204)	3.38	1.94	0.57
Smad3 (Phospho-Ser213)	Smad3 (Ab-213)	2.79	2.95	1.06
Smad3 (Phospho-Ser425)	Smad3 (Ab-425)	0.36	0.33	0.94
SMC1 (Phospho-Ser957)	SMC1 (Ab-957)	1.46	0.72	0.49
SP1 (Phospho-Thr739)	SP1 (Ab-739)	1.19	1.19	1.00
Src (Phospho-Tyr418)	Src (Ab-418)	0.04	0.04	0.96
Src (Phospho-Tyr529)	Src (Ab-529)	0.32	0.31	0.99
Src (Phospho-Ser75)	Src (Ab-75)	0.68	0.50	0.74
SREBP-1 (Phospho-Ser439)	SREBP-1 (Ab-439)	0.21	0.25	1.19
SRF (Phospho-Ser77)	SRF (Ab-77)	0.95	0.73	0.76
SRF (Phospho-Ser99)	SRF (Ab-99)	0.26	0.28	1.08
STAM2 (Phospho-Tyr192)	STAM2 (Ab-192)	3.56	4.24	1.19
STAT1 (Phospho-Tyr701)	STAT1 (Ab-701)	2.98	3.90	1.31
STAT1 (Phospho-Ser727)	STAT1 (Ab-727)	0.38	0.52	1.38
STAT2 (Phospho-Tyr690)	STAT2 (Ab-690)	1.76	1.58	0.90
STAT3 (Phospho-Tyr705)	STAT3 (Ab-705)	1.46	1.26	0.86
STAT3 (Phospho-Ser727)	STAT3 (Ab-727)	1.27	1.09	0.86
STAT4 (Phospho-Tyr693)	STAT4 (Ab-693)	0.96	1.21	1.26
STAT5A (Phospho-Tyr694)	STAT5A (Ab-694)	3.06	2.28	0.75
STAT5A (Phospho-Ser780)	STAT5A (Ab-780)	0.44	0.75	1.70
STAT5B (Phospho-Ser731)	STAT5B (Ab-731)	0.63	0.53	0.85
STAT6 (Phospho-Tyr641)	STAT6 (Ab-641)	0.73	0.95	1.30
STAT6 (Phospho-Thr645)	STAT6 (Ab-645)	0.48	0.48	1.00
Stathmin 1(Phospho-Ser15)	Stathmin 1(Ab-15)	0.34	0.47	1.36
Stathmin 1(Phospho-Ser24)	Stathmin 1(Ab-24)	0.51	0.40	0.78
Stathmin 1(Phospho-Ser37)	Stathmin 1(Ab-37)	0.55	0.69	1.25
Survivin (Phospho-Thr117)	Survivin (Ab-117)	0.74	0.95	1.29
SYK (Phospho-Tyr348)	SYK (Ab-348)	0.31	0.32	1.04
SYK (Phospho-Tyr525)	SYK (Ab-525)	0.15	0.16	1.09
SYN1-Synapsin1 (Phospho-Ser62)	SYN1-Synapsin1 (Ab-62)	0.73	0.62	0.85

Synapsin (Phospho-Ser9)	Synapsin (Ab-9)	0.97	0.77	0.80
Synaptotagmin (Phospho-Thr202)	Synaptotagmin (Ab-202)	1.17	1.08	0.92
Synaptotagmin (Phospho-Ser309)	Synaptotagmin (Ab-309)	0.69	0.56	0.81
Synuclein alpha (Phospho-Tyr125)	Synuclein alpha (Ab-125)	0.29	0.47	1.62
Synuclein alpha (Phospho-Tyr133)	Synuclein alpha (Ab-133)	0.60	0.56	0.93
TAK1 (Phospho-Thr184)	TAK1 (Ab-184)	0.57	0.64	1.11
Tau (Phospho-Thr181)	Tau (Ab-181)	1.01	0.83	0.82
Tau (Phospho-Thr205)	Tau (Ab-205)	0.37	0.37	1.01
Tau (Phospho-Thr212)	Tau (Ab-212)	0.12	0.12	0.94
Tau (Phospho-Ser214)	Tau (Ab-214)	0.17	0.15	0.90
Tau (Phospho-Thr231)	Tau (Ab-231)	0.54	0.65	1.19
Tau (Phospho-Ser235)	Tau (Ab-235)	0.53	0.50	0.95
Tau (Phospho-Ser262)	Tau (Ab-262)	0.51	0.50	0.97
Tau (Phospho-Ser356)	Tau (Ab-356)	0.04	0.05	1.36
Tau (Phospho-Ser396)	Tau (Ab-396)	0.96	1.16	1.20
Tau (Phospho-Ser404)	Tau (Ab-404)	0.92	0.78	0.85
Tau (Phospho-Ser422)	Tau (Ab-422)	0.85	0.96	1.13
TIF-IA (Phospho-Ser649)	TIF-IA (Ab-649)	1.60	2.16	1.35
TOP2A/DNA topoisomerase II (Phospho-Ser1106)	TOP2A/DNA topoisomerase II (Ab-1106)	0.82	0.83	1.01
Trk A (Phospho-Tyr680/681)	Trk A (Ab-496)	0.30	0.30	1.00
Trk A (Phospho-Tyr701)	Trk A (Ab-496)	1.08	1.29	1.19
Trk A (Phospho-Tyr791)	Trk A (Ab-496)	0.38	0.40	1.07
Trk B (Phospho-Tyr515)	Trk B (Ab-515)	0.64	0.59	0.92
Trk B (Phospho-Tyr705)	Trk B (Ab-515)	0.57	0.42	0.74
Tuberin/TSC2 (Phospho-Thr1462)	Tuberin/TSC2 (Ab-1462)	0.39	0.57	1.46
Tuberin/TSC2 (Phospho-Ser939)	Tuberin/TSC2 (Ab-939)	0.25	0.21	0.87
TYK2 (Phospho-Tyr1054)	TYK2 (Ab-1054)	1.25	1.11	0.89
Tyrosine Hydroxylase (Phospho-Ser19)	Tyrosine Hydroxylase (Ab-19)	0.53	0.44	0.84
Tyrosine Hydroxylase (Phospho-Ser31)	Tyrosine Hydroxylase (Ab-31)	0.66	0.47	0.72
Tyrosine Hydroxylase (Phospho-Ser40)	Tyrosine Hydroxylase (Ab-40)	0.49	0.60	1.23
Tyrosine Hydroxylase(TH) (Phospho-Ser8)	Tyrosine Hydroxylase(TH) (Ab-8)	0.38	0.47	1.24
VASP (Phospho-Ser157)	VASP (Ab-157)	0.26	0.22	0.85
VASP (Phospho-Ser238)	VASP (Ab-238)	0.03	0.72	26.70

VAV1 (Phospho-Tyr174)	VAV1 (Ab-160)	0.67	0.69	1.02
VAV1 (Phospho-Tyr174)	VAV1 (Ab-174)	2.32	1.87	0.80
VAV2 (Phospho-Tyr142)	VAV2 (Ab-142)	2.51	2.13	0.85
VEGFR1 (Phospho-Tyr1333)	VEGFR1 (Ab-1333)	0.40	0.49	1.22
VEGFR2 (Phospho-Tyr1054)	VEGFR2 (Ab-1054)	0.46	0.69	1.50
VEGFR2 (Phospho-Tyr1059)	VEGFR2 (Ab-1059)	0.36	0.70	1.98
VEGFR2 (Phospho-Tyr1175)	VEGFR2 (Ab-1175)	0.80	0.38	0.47
VEGFR2 (Phospho-Tyr1214)	VEGFR2 (Ab-1214)	0.03	0.03	0.97
VEGFR2 (Phospho-Tyr951)	VEGFR2 (Ab-951)	0.07	0.06	0.88
Vinculin (Phospho-Tyr821)	Vinculin (Ab-821)	1.18	2.12	1.80
WASP (Phospho-Tyr290)	WASP (Ab-290)	0.48	0.52	1.09
WAVE1 (Phospho-Tyr125)	WAVE1 (Ab-125)	0.84	1.02	1.21
WEE1 (Phospho-Ser642)	WEE1 (Ab-53)	1.03	1.00	0.97
XIAP (Phospho-Ser87)	XIAP (Ab-87)	0.20	0.22	1.12
Zap-70 (Phospho-Tyr292)	Zap-70 (Ab-292)	0.55	0.71	1.28
Zap-70 (Phospho-Tyr319)	Zap-70 (Ab-319)	0.42	0.44	1.05
Zap-70 (Phospho-Tyr493)	Zap-70 (Ab-493)	0.62	0.83	1.33

Appendix Table 1 - A phosphoprotein array analysis (*Full Moon Biosystems*) of human MDM pre-treated with FK506 and stimulated with live *A. fumigatus* swollen conidia (MOI = 1) for 1 h p.i. is shown. Fold change differences in the phosphoprotein to non-phosphoprotein ratio between FK506 and control macrophages stimulated with live *A. fumigatus* swollen conidia are shown. A colour scale is used to represent decreased phosphoprotein to non-phosphoprotein ratio (red) to increased phosphoprotein to non-phosphoprotein ratio (blue).

8.2 Human vasodilator-stimulated phosphoproteins (VASP) protein sequence.

```
1   msetvicssr atvmlyddgn krwlpagtgp qafsrvqiyh nptansfrvv grkmqpdqqv
61  vincaivrgv kynqatpnfh qwrdaqvwg lnfgskedaa qfaagmasal ealegggppp
121 ppalptwsvp ngpspeeveq qkrqqg

pse



hi

errvs nag gppappaggp ppppgppppp
181 gpppppglpp sgvpaaahga gggppppappl paaqgpgggg agapglaaa aiagaklrkvsk
241 qeeasggpta pkaesgrsgg gglmeemnam larrrrkatqv gektpkdesa nqeepearvp
301 aqsesvrrpw eknsttlprm kssssvttse tqpctpsssd ysdqlrvkqe lleevkkelq
361 kvkeeieaf vqelrkrqsp
```

Protein sequence highlighted in yellow showing a possible conserved calcineurin-binding motif (Pxxlx) with similarities to the PxIxxT seen in NFAT family members.

8.3 Paper in American Journal of Respiratory and Critical Care Medicine, 2016 – Calcineurin orchestrates lateral transfer of *Aspergillus Fumigatus* during macrophage cell death

QA1 Calcineurin Orchestrates Lateral Transfer of *Aspergillus fumigatus* during Macrophage Cell Death

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is order correct?

Abstract

Rationale: Pulmonary aspergillosis is a lethal mold infection in the immunocompromised host. Understanding initial control of infection and how this is altered in the immunocompromised host are key goals for comprehension of the pathogenesis of pulmonary aspergillosis.

Objectives: To characterize the outcome of human macrophage infection with *Aspergillus fumigatus* and how this is altered in transplant recipients on calcineurin inhibitor immunosuppressants.

Methods: We defined the outcome of human macrophage infection with *A. fumigatus*, as well as the impact of calcineurin inhibitors, through a combination of single-cell fluorescence imaging, transcriptomics, proteomics, and *in vivo* studies.

Measurements and Main Results: Macrophage phagocytosis of *A. fumigatus* enabled control of 90% of fungal germination. However, fungal germination in the late phagosome led to macrophage necrosis. During programmed necroptosis, we observed frequent

cell–cell transfer of *A. fumigatus* between macrophages, which assists subsequent control of germination in recipient macrophages. Lateral transfer occurred through actin-dependent exocytosis of the late endosome in a vasodilator-stimulated phosphoprotein envelope. Its relevance to the control of fungal germination was also shown by direct visualization in our zebrafish aspergillosis model *in vivo*. The calcineurin inhibitor FK506 (tacrolimus) reduced cell death and lateral transfer *in vitro* by 50%. This resulted in uncontrolled fungal germination in macrophages and also resulted in hyphal escape.

Conclusions: These observations identify programmed, necrosis-dependent lateral transfer of *A. fumigatus* between macrophages as an important host strategy for controlling fungal germination. This process is critically dependent on calcineurin. Our studies provide fundamental insights into the pathogenesis of pulmonary aspergillosis in the immunocompromised host.

Keywords: pulmonary fungal diseases; macrophage; necrosis; *Aspergillus*; calcineurin

Aspergillus fumigatus (*Af*) is a mold with small airborne conidia that are a ubiquitous component of the aerial mycobiota (1). Normally inhaled conidia are cleared by

alveolar macrophages to prevent fungal germination, tissue invasion, and destructive lung disease (2, 3). Our previous work with murine and zebrafish models

of invasive aspergillosis showed the importance of macrophages in control of *Af* early postinfection, with neutrophil influx as a later event (4, 5). Previous

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Author Contributions: D.P.A.-J., A.S., S.K., S.S., and S.M. designed the experiments; D.P.A.-J., A.S., S.K., S.H., and A. Rogers conducted the experiments; S.S., A. Reed, and M.C. obtained consent from the patients and undertook the bronchoscopies; and A.S. and S.K. wrote the first draft of the manuscript. All authors contributed to the data analysis and the writing of the manuscript.

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MRCP MSc PhD

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

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Shah, Kannambath, Herbst, *et al.*: Macrophage Necroptosis in Pulmonary Aspergillosis

1

At a Glance Commentary

Scientific Knowledge on the

Subject: Macrophages are the frontline defense against pulmonary infection with the mold pathogen *Aspergillus fumigatus*. Phagocytosis and generation of reactive oxygen species are known to be crucial for control of infection. However, the cellular outcome from progressive macrophage infection with *A. fumigatus* has not been studied systematically.

What This Study Adds to the

Field: We present a systematic analysis of human macrophage infection with *A. fumigatus*. We show that successful fungal germination in the phagosome leads to necrotic cell death and can result in cell–cell transfer of germinating *A. fumigatus* between macrophages. Ultimately, this process assists control of fungal germination and is orchestrated through the calcium-responsive serine-threonine phosphatase calcineurin. Our systematic analysis of the macrophage response to *A. fumigatus* defines necroptosis as a key early event in the pathogenesis of pulmonary aspergillosis.

studies have highlighted the importance of inflammatory monocytes in murine invasive aspergillosis (6).

A spectrum of immunocompromised states predisposes individuals to high-mortality invasive or chronic forms of pulmonary aspergillosis (PA) (7). Organ transplant recipients are at high risk of PA, with lung transplant recipients being particularly susceptible and having a mortality greater than 40% (8, 9). There are an estimated 3 million individuals with chronic PA and 4.5 million with allergic bronchopulmonary aspergillosis globally (10, 11). *Af* has also been implicated in the pathogenesis of asthma, chronic obstructive airways disease, and bronchiectasis (11–13). Treatment options remain limited, and high mortality rates persist (14). Better understanding of susceptibility to PA in the nonneutropenic host is a key clinical research priority.

Calcineurin–nuclear factor of activated T cells (NFAT) signaling is the target of the calcineurin inhibitor immunosuppressants used in organ transplantation (15). We have previously shown that calcineurin inhibitor immunosuppressants (FK506 [tacrolimus]) increase susceptibility to murine PA through inhibition of the macrophage Toll-like receptor 9–calcineurin–NFAT pathway (4, 5). This pathway is crucial for activation of inflammatory responses to *Af* and for recruitment of fungicidal neutrophils to the site of infection. Calcineurin–NFAT signaling has also been shown to be critical for the innate immune response to *Candida albicans* and *Escherichia coli* through endocytic mechanisms convergent on phospholipase C- γ -dependent calcium flux (16, 17). These observations underscore the importance of calcineurin–NFAT signaling for myeloid immunity, and they reveal a novel relationship between innate sensing and calcineurin–NFAT signaling in the lung that requires further clinical definition.

Here we show that host programmed necrosis is an important outcome of *Af* germination in the human macrophage. Cell death occurred in response to phagosomal germination, with necroptosis-associated lateral transfer of *Af* between macrophages. Cell death–dependent transfer was calcineurin dependent and enabled control of germination in recipient macrophages. Crucially, inhibition of either calcineurin-dependent cell death or lateral transfer enabled hyphal escape from the macrophage. In this study, we identify programmed necrosis as a key cellular response in PA and show that it is likely to be critically impaired in organ transplant recipients taking calcineurin inhibitors. Our studies reveal cell–cell transfer as a novel and important cell death–associated defense mechanism that enables control of fungal germination in the myeloid compartment.

Methods

Ethics Statement

The present study was approved by the Biomedical Research Unit (National Research Ethics Service reference 10/H0504/9), Royal Brompton and Harefield NHS Trust (AS1). All experiments conformed to the World Medical Association Declaration of Helsinki.

Written informed consent was obtained from all participants.

Zebrafish Care and Maintenance

Our experiments were approved by the U.K. Home Office in accordance with project license PPL 70/7446. Full details are given in the EXPERIMENTAL PROCEDURES section of the online supplement.

Fungal Strains and Culture

A. fumigatus strain CEA10 was used for Western blotting, Luminex (Luminex, Austin TX), fungal burden, ImageStream (EMD Millipore, Billerica, MA), phosphoproteomic, and RNA sequencing experiments. ATCC 46645 enhanced green fluorescent protein (American Type Culture Collection, Manassas, VA), a gift from Frank Ebel, was used for microscopy experiments. See EXPERIMENTAL PROCEDURES section in the online supplement for details.

Isolation of Human Macrophages

Human alveolar macrophages (hAMs) were isolated from bronchoalveolar lavage by adherence. Purified hAMs were rested for 3 days. Monocytes were isolated from peripheral blood mononuclear cells from healthy volunteers by Ficoll-Paque gradient centrifugation and negative magnetic bead isolation (Pan Monocyte Isolation Kit; Miltenyi Biotec, Auburn, CA). Human monocyte-derived macrophages (hMDMs) were differentiated using 5 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10% human serum for 7 days. See EXPERIMENTAL PROCEDURES section in the online supplement for details.

RNA-seq Analysis

Illumina RNA-seq libraries (Illumina, San Diego, CA) were prepared from hMDMs ($n = 6$ healthy donors) pretreated with 10 ng/ml FK506 for 1 hour or vehicle and stimulated with live *Af* swollen conidia (SC) (multiplicity of infection, 1) for 1 hour and 6 hours. Illumina paired-end reads were mapped to the human reference genome (hg19) using TopHat2 (18) (Gene Expression Omnibus database accession number GSE74924), and differential expression analyses were performed using Cufflinks v2.2.1 (19). Gene analyses were performed using DAVID v6.7 (20). Human protein–protein interaction network modules were obtained using MCODE (21) analysis in Cytoscape. See EXPERIMENTAL

PROCEDURES section in the online supplement for details.

Phosphoproteomic Analysis

Analysis with the Phospho Explorer Antibody Microarray was conducted by Full Moon BioSystems Inc. (Sunnyvale, CA).

hMDMs were differentiated at 1×10^7 cells in 75-mm² tissue culture flasks. At Day 7, cells were stimulated with live *Af* SC (multiplicity of infection, 1) following pretreatment for 1 hour with FK506 (10 ng/ml; Calbiochem, San Diego, CA) or vehicle (dimethyl sulfoxide diluted in RPMI

1640 medium). At 1 hour postinfection, cells were washed five times with 10 ml of ice-cold phosphate-buffered saline containing protease inhibitors (Cell Signaling Technology, Danvers, MA) and collected by centrifugation ($250 \times g$ for 10 min at 4°C). Cells were frozen at -80°C

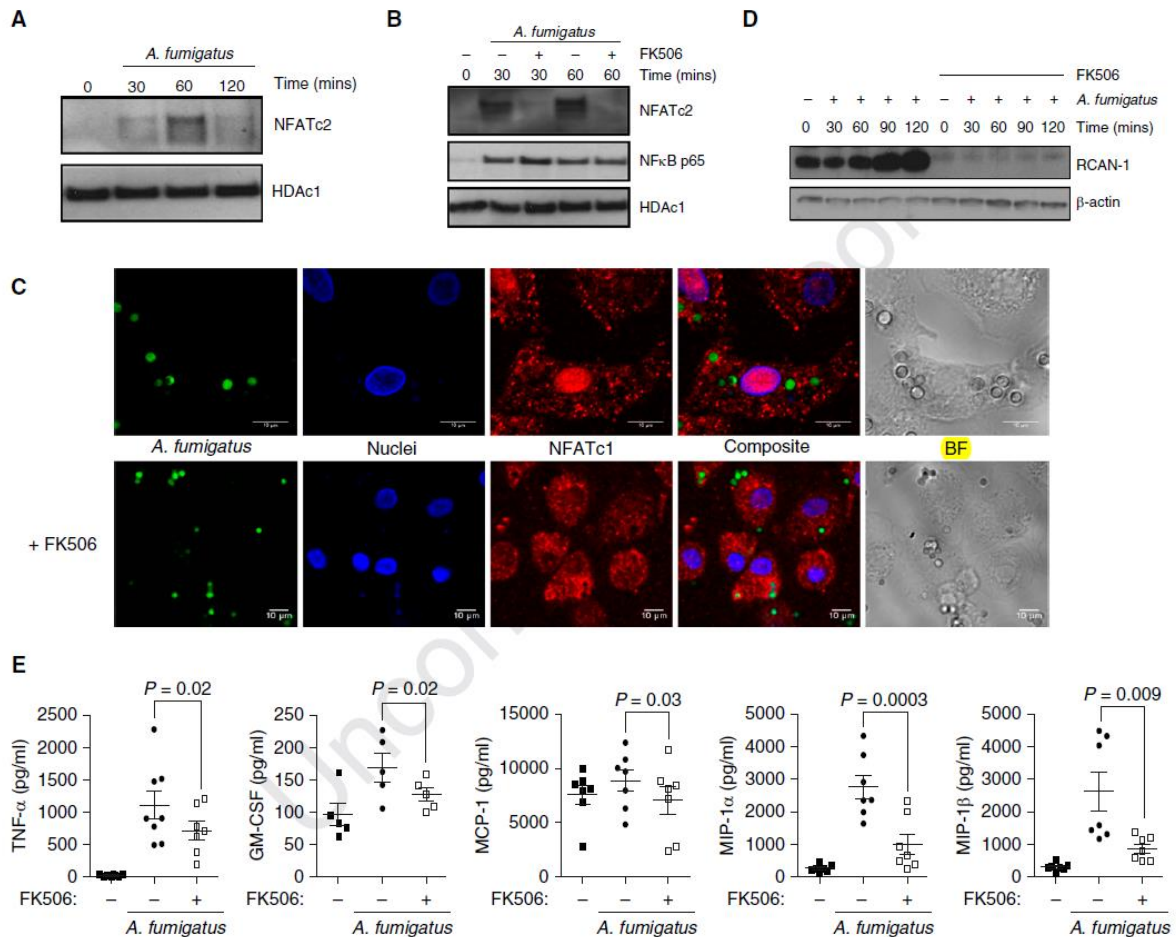


Figure 1. *Aspergillus fumigatus* (*Af*) activates calcineurin–nuclear factor of activated T cells (NFAT)–dependent inflammatory responses in human macrophages. (A) Human monocyte-derived macrophages (hMDMs) were stimulated with live *Af* swollen conidia (SC) (multiplicity of infection [MOI], 1). hMDM nuclear extracts were collected at 30, 60, and 120 minutes postinfection and probed by Western blotting for nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2 (NFATc2). The maximum translocation of NFATc2 to the nucleus was observed at 60 minutes postinfection. Western blots show representative data from four experiments. (B and C) hMDMs were pretreated with FK506 (10 ng/ml) and stimulated with live *Af* SC (MOI, 1). Nuclear extracts were probed by Western blotting for NFATc2 and whole-cell extracts for regulator of calcineurin 1 (RCAN-1). NFAT translocation was assessed by confocal microscopy. NFAT is shown in red, nuclei in blue, and *Af* conidia in green. Representative images of live conidia stimulating NFAT nuclear translocation are shown. Western blots show representative data from four experiments. (D) hMDMs were pretreated with FK506 (10 ng/ml) and stimulated with live *Af* SC (MOI, 1). FK506 completely inhibited RCAN-1 production. Western blot shows representative data from four experiments. (E) hMDMs were pretreated with FK506 (10 ng/ml) and stimulated with live *Af* SC (MOI, 1). Culture supernatant cytokines were measured at 6 hours postinfection by Luminex assay ($n = 7$). GM-CSF = granulocyte-macrophage colony-stimulating factor; HDAc1 = histone deacetylase 1; MCP-1 = monocyte chemoattractant protein 1; MIP-1 = macrophage inflammatory protein 1; NF-κB = nuclear factor-κB; TNF-α = tumor necrosis factor-α.

and transferred to Full Moon BioSystems on dry ice. The array consisted of 1,318 phospho-specific antibodies. Proteins were labeled with biotin and adhered to preblocked microarray slides. After a washing step, detection of total and phosphorylated proteins was conducted using cyanine 3-conjugated streptavidin. Expression of phosphorylated proteins was normalized to the corresponding total protein abundance. Fold change was calculated as the phosphoprotein-to-nonphosphoprotein ratio of FK506-treated cells divided by the phosphoprotein-to-nonphosphoprotein ratio of untreated cells.

Statistical Analysis

The results are presented as mean $\pm$ SEM and were analyzed using Prism software (version 6.0; GraphPad Software, La Jolla, CA). Significance was determined using Student's *t* test for unpaired observations. When three or more groups were

compared, we used one-way analysis of variance with the Bonferroni correction. Full details of the experimental procedures are available in the online supplement.

Results

A. *fumigatus* Activates Calcineurin-NFAT-Dependent Inflammatory Responses in Human Macrophages

Af phagocytosis activates NFAT nuclear translocation in murine macrophages (4). We therefore characterized the role of NFAT in hMDMs. hMDMs were infected with live *Af* SC or resting conidia (RC), and NFAT translocation was determined by Western blot analysis and confocal microscopy. SC phagocytosis led to sustained NFAT translocation after 30 minutes (Figures 1A and 1B; see also Figure E1A in the online supplement). RC induced a weak, transient NFAT activation

response (Figures E1B and E1C). NFAT translocation in response to SC was confirmed in hAMs from lung transplant recipients (Figure E1D). Maximum translocation of NFATc2 to the nucleus was seen in response to *Af* at 60 minutes postinfection. NFAT translocation was blocked by the calcineurin inhibitor FK506 (Figures 1B, 1C, and E1E), but nuclear factor- κ B Rel65 translocation was unaffected (Figure 1B).

NFAT transcriptional activity was defined by measuring expression of the NFAT-specific target regulator of calcineurin 1 (*RCAN1*). SC infection increased *RCAN1* expression, which was abrogated by FK506 (Figures 1D and E1F). To determine the role of the calcineurin-NFAT pathway in inflammatory responses, we quantified chemokine and cytokine production in hMDMs during SC infection. Expression of tumor necrosis factor- α (*TNF- α*), *GM-CSF*, monocyte

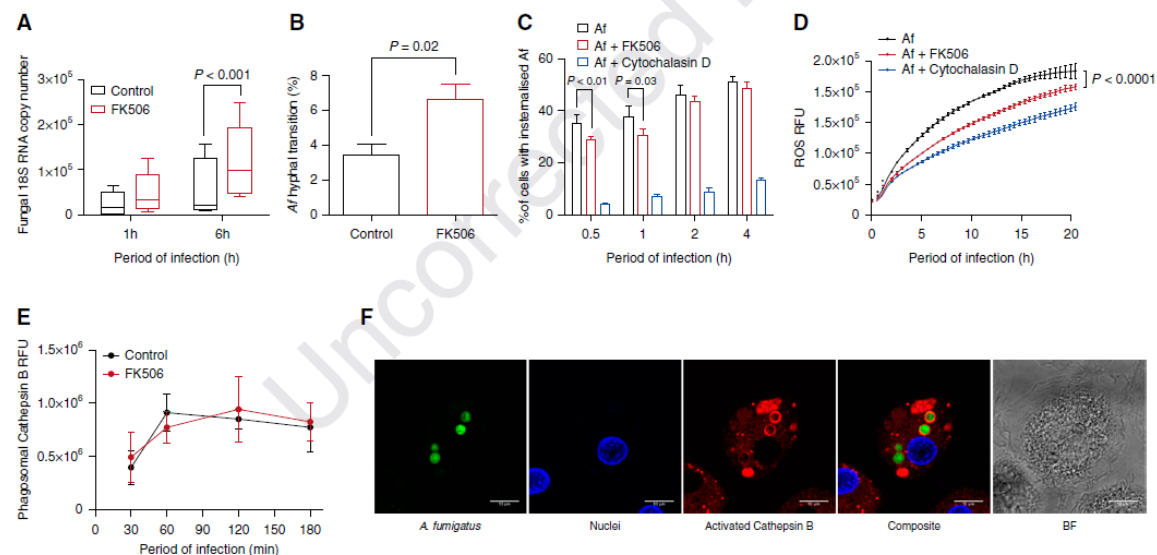


Figure 2. Human macrophage phagocytosis, reactive oxygen species (ROS) production, and control of *Aspergillus fumigatus* (*Af*) growth are calcineurin dependent. (A) Human monocyte-derived macrophages (hMDMs) pretreated with FK506 (10 ng/ml) or vehicle were stimulated with live *Af* swollen conidia (SC) (multiplicity of infection [MOI], 1), and total RNA was isolated at 1 and 6 hours postinfection. Fungal growth was assayed by reverse transcription-polymerase chain reaction of fungal RNA ($n = 5$). (B) hMDMs pretreated with FK506 (10 ng/ml) or vehicle were infected with live enhanced green fluorescent protein-expressing *Af* SC (MOI, 1), and hyphal transition was assessed using time-lapse video microscopy over a 10-hour period ($n = 4$). (C) hMDMs pretreated with FK506 (10 ng/ml) or cytochalasin D (5 nM) or vehicle were stimulated with live *Af* SC (MOI, 1) prestained with calcofluor white. Phagocytosis was quantified by using an ImageStream flow cytometer postinfection ($n = 4$). (D) hMDMs pretreated with FK506 (10 ng/ml) or vehicle were stimulated with live *Af* SC (MOI, 1), and ROS production was assayed by luminescence ($n = 4$). (E) hMDMs pretreated with FK506 (10 ng/ml) or vehicle were stimulated with live *Af* SC (MOI, 1), and cathepsin B activation was assayed by live confocal time-lapse microscopy. Phagosomal cathepsin B activation was quantified by using ImageJ software (National Institutes of Health, Bethesda, MD) ($n = 4$). (F) Representative confocal microscopic image showing cathepsin B activation in *Af* conidia-containing phagosomes in MDMs. Activated cathepsin B is shown in red, nuclei in blue, and *Af* in green. Data are presented as mean $\pm$ SEM.

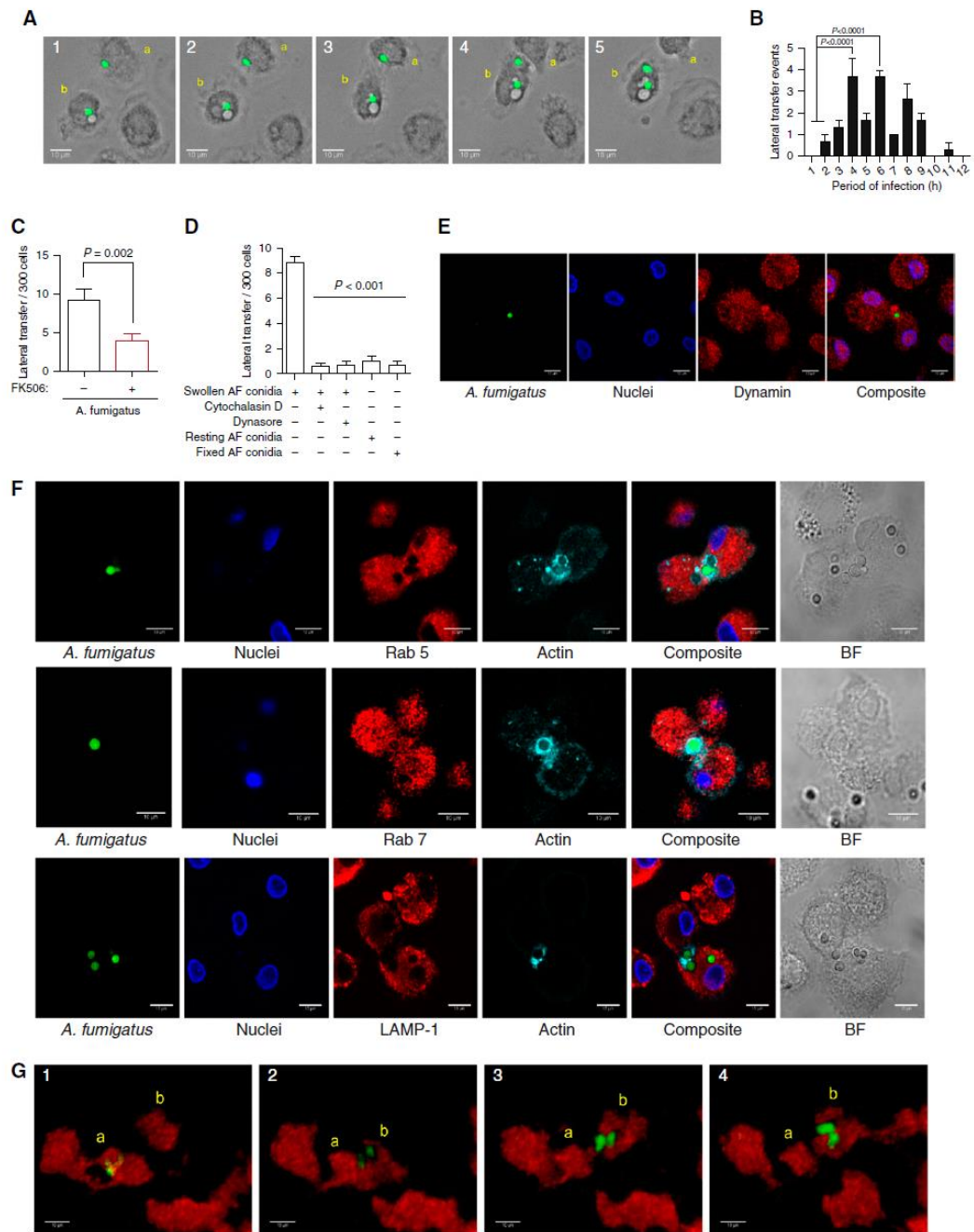


Figure 3. Macrophages infected with swollen *Aspergillus fumigatus* (Af) undergo calcineurin-dependent lateral transfer. (A) Representative time-lapse widefield microscopic images of Af lateral transfer in monocyte-derived macrophages (MDMs) infected with live Af swollen conidia (SC) (multiplicity of infection [MOI], 1), shown in green. Transferring macrophage is labeled a, and receiving macrophage is labeled b. Images were taken at 10-minute intervals. (B) hMDMs were stimulated with live enhanced green fluorescent protein (eGFP)-Af SC (MOI, 1), and time-lapse widefield imaging was performed

chemoattractant protein 1 (*MCP-1*), *MCP-1 α* , and *MCP-1 β* was attenuated by calcineurin inhibition ($P = 0.02$, $P = 0.02$, $P = 0.03$, $P = 0.0003$, and $P = 0.009$, respectively) (Figures 1E and 1G). Flow cytometric characterization of hMDMs and lung transplant hAMs revealed similar inflammatory activation status with CD11c⁺/major histocompatibility complex class II⁺/CD206⁺ surface expression. There were significant differences in surface CD11b ($P \leq 0.001$) and CD86 ($P \leq 0.001$) expression (Figure 1H). Blockade of the fungal C-type lectin receptor Dectin-1 ($P = 0.001$) or inhibition of downstream Syk signaling ($P = 0.001$) impaired TNF- α responses, but it had no effect on NFAT translocation (Figures 1I and 1J). These results indicate that the calcineurin-NFAT signaling pathway is critical for early macrophage inflammatory responses to *Af*, independent of Dectin-1 and Syk, in humans.

Human Macrophage Phagocytosis, Reactive Oxygen Species Production, and Killing of *A. fumigatus* Are Calcineurin Dependent

Next, we characterized the role of calcineurin-NFAT in killing of *Af*. hMDMs were infected with SC following treatment with FK506 or vehicle, and fungal growth was analyzed at 1 and 6 hours postinfection. Both hMDMs and hAMs pretreated with FK506 exhibited impaired control of fungal growth ($P < 0.001$ and $P < 0.0001$, respectively) (Figures 2A and 2A). Time-lapse video microscopy demonstrated increased *Af* hyphal transition in FK506-treated macrophages ($P = 0.02$) (Figure 2B). To identify the calcineurin-dependent mechanism of fungal growth inhibition, we assessed the efficiency of *Af* phagocytosis. Calcineurin

inhibition delayed phagocytosis of SC in hMDMs ($P < 0.01$) and hAMs ($P = 0.04$) early postinfection, but phagocytosis of RC was not affected (Figures 2C, 2B, and 2C). We measured reactive oxygen species (ROS) production and cathepsin B (a lysosomal cysteine protease) activation, which are critical for *Af* killing (22, 23). ROS production was partially calcineurin dependent ($P < 0.0001$) but had no effect on mitochondrial ROS (Figures 2D, 2D, and 2E) or activated cathepsin B production (Figures 2E and 2F). Calcineurin-dependent effects on phagosomal maturation were analyzed by phagosomal acidification and recruitment of the late phagosomal maturation marker lysosomal-associated membrane protein 1 (LAMP-1) to *Af*-containing phagosomes. Calcineurin inhibition increased LAMP-1 recruitment at early time points after infection ($P < 0.05$) (Figures 2F and 2G). Phagosomal acidification was not calcineurin dependent (Figures 2H and 2I). These results indicate that inhibition of the calcineurin-NFAT pathway impairs the ability of human macrophages to control fungal hyphal transition, delays fungal phagocytosis, and reduces ROS production.

Macrophages Traffic *Af*-containing Endosomes to Neighboring Cells through Calcineurin-Dependent Lateral Transfer

During live time-lapse confocal imaging, we observed lateral transfer of germinating *Af* between hMDMs (Figure 3A and Movie E1). This was confirmed in alveolar macrophages from lung transplant recipients (Movie E2). Lateral transfer events occurred in $3.0 \pm 0.4\%$ of cells infected with SC, peaking at 4–6 hours postinfection (Figure 3B). Calcineurin

inhibition markedly reduced lateral transfer ($53.9 \pm 10.3\%$) ($P = 0.002$) (Figure 3C).

We assessed the role of fungal germination in lateral transfer of *Af* by time-lapse microscopy of metabolically inactive RC or dead, fixed SC. Lateral transfer was dependent on both live and swollen *Af* ($P < 0.001$) (Figure 3D). We observed colocalization of actin and dynamin to conidia undergoing lateral transfer (Figures 3E and 3F), suggesting an actin- and dynamin-dependent process. This was confirmed by treating hMDMs with actin polymerization or dynamin inhibitors (cytochalasin D or dynasore, respectively) following phagocytosis ($P < 0.001$) (Figure 3D). Fungal burden was increased after inhibition of lateral transfer post-phagocytosis using dynasore, suggesting that transfer enables recipient macrophage control of hyphal germination ($P < 0.01$) (Figure 3A). Transmission electron microscopy showed that transfer of *Af*-containing cargo between hMDMs occurred in membrane-bound compartments (Figure 3B). The *Af*-containing cargo transferred between hMDMs was further characterized by staining for endolysosomal markers (Ras-related protein 5 [Rab 5], Rab 7, and LAMP-1). This showed that *Af* was transferred between hMDMs in Rab 7-positive and Rab 5- and LAMP-1-negative compartments, suggesting late endosomal trafficking (Figures 3F and 3C).

To determine whether this phenomenon occurred *in vivo*, we exploited our zebrafish invasive aspergillosis model (4). Using an mpeg:mCherry transgenic zebrafish line and enhanced green fluorescent protein-expressing *Af*, we examined the macrophage-*Af* interaction *in vivo* using time-lapse high-resolution

Figure 3. (Continued). at 10-minute intervals for 12 hours postinfection. The timing of lateral transfer events per hour in macrophages infected with live *Af* SC is shown ($n = 3$). (C) hMDMs pretreated with FK506 (10 ng/ml) or vehicle were stimulated with live eGFP-*Af* SC (MOI, 1), and lateral transfer events were quantified by time-lapse widefield imaging at 10-minute intervals for 12 hours postinfection ($n = 5$). Total lateral transfer events over the 12-hour period were quantified for each biological replicate. (D) hMDMs were stimulated with live or fixed *Af* SC or resting conidia (MOI, 1), and lateral transfer events were quantified by time-lapse widefield microscopy. Cytochalasin D (5 nM) and dynasore (100 μ M) were added at 90 minutes postinfection, and unbound conidia were washed away 45 minutes postinfection ($n = 4$). (E) Representative confocal microscopic image of dynamin-dependent lateral transfer in MDMs stimulated with live *Af* SC. Dynamin is shown in red, nuclei in blue, and *Af* in green. (F) MDMs were stimulated with live *Af* SC (MOI, 1), and nonphagocytosed conidia were washed away at 45 minutes postinfection. Lateral transfer events were captured by fixing cells every 10 minutes from 120 to 180 minutes postinfection. Characterization of *Af* lateral transfer was performed by staining for Ras-related protein 5 (Rab 5), Rab 7, and lysosomal-associated membrane protein 1 (LAMP-1) (shown in red) alongside actin (shown in cyan). Nuclei are shown in blue and live *Af* SC in green. Representative images show positive Rab 7 localization to *Af*-containing endosomes during lateral transfer, with little Rab 5 localization and no LAMP-1 localization. (G) mpeg:mCherry zebrafish larvae were infected with approximately 50 eGFP-expressing conidia of *Af*, and time-lapse confocal microscopy was performed. Representative images show *Af* lateral transfer between macrophages. The transferring macrophage is labeled a, and the receiving macrophage is labeled b. Images shown were taken at 7-minute intervals. Data are represented as mean $\pm$ SEM.

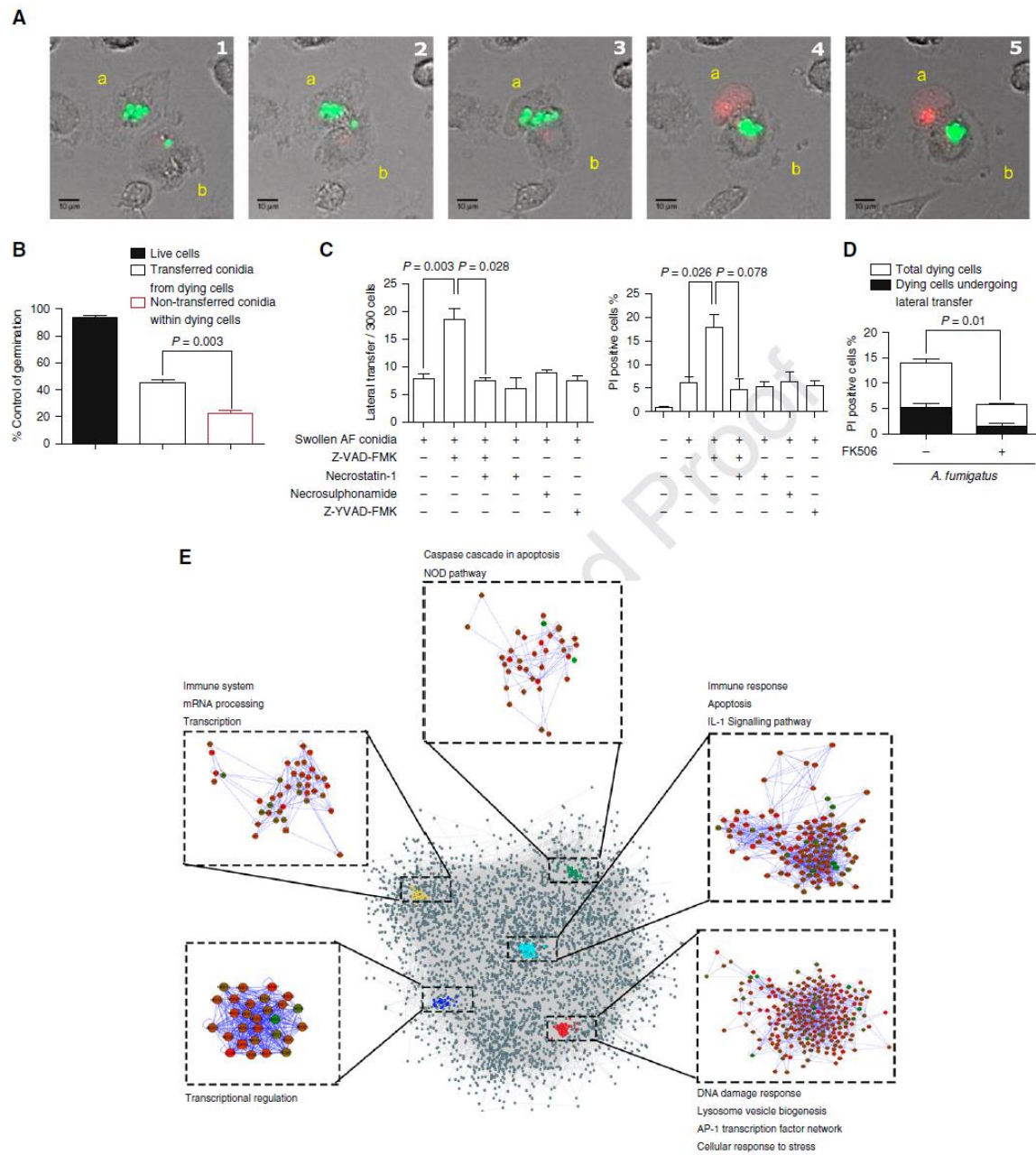


Figure 4. Macrophages undergoing programmed necrosis laterally transfer germinating *Aspergillus fumigatus* (Af) conidia in endosomes through an actin-dependent process. (A) Representative time-lapse confocal microscopic images of lateral transfer of live Af swollen conidia (SC) (shown in green) between monocyte-derived macrophages (MDMs). Propidium iodide (PI) staining (red) shows cell death of macrophage transferring Af conidia (labeled a) to neighboring macrophage (labeled b). Images were taken at 10-minute intervals. (B) Time-lapse confocal microscopy was used to quantify the fate of transferred versus nontransferred conidia from dying human MDMs and to conidia within live macrophages. Germination was assessed over a 12-hour period. Control of fungal germination was significantly increased in dying hMDMs that transferred conidia to neighboring cells compared with those that

confocal microscopy. We observed similar macrophage *Af* lateral transfer in zebrafish larvae, consistent with an evolutionarily conserved macrophage response to *Af* infection that occurs *in vivo* (Figure 3G and Movie E3). Taken together, these observations indicate that successful fungal germination in the late endosome triggers exocytic transfer of *Af* between macrophages.

Macrophages Undergoing Calcineurin-Dependent Programmed Necrosis Transfer *Af* Conidia to Neighboring Cells to Control Fungal Germination

hMDMs transferring *Af* had increasingly translucent cytoplasm, organelle swelling, and increased cell volume, suggesting programmed cell death during transfer. This was confirmed by confocal microscopy using propidium iodide (Figure 4A and Movie E4). Around 90% of macrophages successfully control fungal germination and do not die (Figure 4B). Propidium iodide staining revealed that, in dying macrophages ($13 \pm 2\%$), lateral transfer occurred in $37 \pm 5\%$ (Figure 4D). This represents approximately 3–4% of total macrophages, as previously described for those infected with *Af* SC. Transfer of *Af* conidia from dying hMDMs enhanced control of germination as compared with dying hMDMs, where conidia were not transferred ($P = 0.003$) (Figure 4B). Germination of *Af* in live hMDMs was well controlled compared with cells undergoing programmed necrosis (Figure E3D), further supporting a model where programmed cell death and lateral transfer occur in response to successful fungal germination in macrophages to enable containment of infection in recipient macrophages (Figure 4B).

We observed massive vacuole formation adjacent to the *Af*-containing compartment consistent with programmed

cell death (Figure E3E and Movie E5) (24). Transmission electron microscopy also showed conidia residing within large vacuoles (Figure E3F). Vacuole formation was inhibited by pretreatment with the vacuolar H^+ -ATPase inhibitor bafilomycin ($P = 0.003$), with a trend toward decreased transfer ($P = 0.06$) (Figure E3G).

To define the relationship between cell death and transfer, induction of necroptosis with the pan-caspase inhibitor Z-VAD-FMK (an inhibitor of caspase-dependent apoptosis that increases necroptosis) was performed (25, 26). This increased lateral transfer ($P = 0.003$) (Figure 4C). Addition of necrostatin-1, an inhibitor of receptor-interacting serine/threonine-protein kinase 1-dependent necroptosis, to Z-VAD-FMK inhibited both cell death ($P = 0.078$) and lateral transfer ($P = 0.028$) induced by Z-VAD-FMK alone (Figure 4C). However, induction of necroptosis with Z-VAD-FMK led to increased fungal burden due to recipient macrophage death ($P < 0.01$) (Figure E3H). Necrostatin-1 alone or necrosulfonamide could not inhibit macrophage programmed cell death or lateral transfer (Figure 4C). As pyroptosis following macrophage infection with *C. albicans* was recently shown, we blocked pyroptosis with an irreversible cell-permeable caspase 1 inhibitor (Z-YVAD-FMK), which did not affect cell death or lateral transfer (Figure 4C) (27). These observations are consistent with transfer of *Af* between macrophages during a programmed cell death process with hallmarks of necroptosis.

As calcineurin regulates cell death, we assessed its role in macrophage necrosis following infection (28). Calcineurin inhibition reduced macrophage programmed necrosis by $57 \pm 13.7\%$ ($P = 0.01$) (Figure 4D) and was associated with accelerated fungal germination at late time points in macrophages that failed to undergo necrosis-dependent transfer

($P < 0.001$) (Figures E3I and E3J). This is consistent with a calcineurin-dependent checkpoint for programmed necrosis during *Af* germination.

Together, these results indicate that hMDMs infected with live *Af* SC undergo calcineurin-dependent cell death with hallmarks of programmed necroptosis and transfer *Af*-containing endosomes to neighboring cells to facilitate control of fungal germination.

Calcineurin Regulates Cell Death and the Mitogen-activated Protein Kinase Pathway during *Af* Infection

To further define the macrophage response to *Af*, we undertook next-generation transcript profiling of hMDMs during phagocytosis. hMDMs pretreated with FK506 were infected with SC for 1 hour or 6 hours, and genes differentially expressed compared with baseline and untreated infection controls were identified (Figure E4A). The major gene sets regulated by calcineurin at 6 hours postinfection were associated with cell death, inflammatory responses, and transcriptional regulation (Figure E4B). Fifty-one genes associated with the cell death pathway were calcineurin dependent at 6 hours postinfection (Table E1).

In overrepresentation analysis using ConsensusPathDB, we identified calcineurin-dependent control of the programmed cell death, mitogen-activated protein kinase (MAPK) signaling, and cytokine–cytokine interaction pathways at 6 hours postinfection (Figure E4C). To assess the interaction between the pathways regulated by calcineurin, a protein–protein interaction network was created. By use of this method, we identified significant interactions and clustering of transcriptional regulation pathways, immune response pathways, apoptosis, and the activator protein 1 (AP-1) transcription factor network (Figure 4E). Other studies

Figure 4. (Continued). did not ($n = 4$). (C) hMDMs pretreated with a pan-caspase inhibitor ($50 \mu\text{M}$ Z-VAD-FMK), a receptor-interacting serine/threonine-protein kinase (RIPK-1) inhibitor ($10 \mu\text{M}$ necrostatin-1), a caspase 1 inhibitor ($50 \mu\text{M}$ Z-YVAD-FMK), 10 ng/ml FK506, or vehicle were stimulated with live *Af* SC (multiplicity of infection [MOI], 1), and lateral transfer events and cell death were quantified by propidium iodide fluorescence–based time-lapse confocal microscopy. Treatment with Z-VAD-FMK induced necroptotic cell death coupled to increased *Af* lateral transfer, which was inhibited by the addition of a RIPK-1 inhibitor (necrostatin-1) ($n = 3$). (D) hMDMs pretreated FK506 (10 ng/ml) or vehicle were stimulated with live *Af* SC (MOI, 1), and cell death was assayed by propidium iodide fluorescence and quantified by time-lapse confocal microscopy over a 6-hour period. The graph also shows the proportion of lateral transfer events in dying cells (approximately 30% of dying cells) ($n = 4$). (E) The network modules of the significantly expressed genes in FK506-pretreated macrophages stimulated with live *Af* SC (MOI, 1) for 6 hours. Significantly expressed genes were mapped onto a protein–protein interaction network, and network modules were identified using the MCODE plugin application of Cytoscape. Overrepresented pathways involving these network modules were identified using the Kyoto Encyclopedia of Genes and Genomes (“KEGG”) and WikiPathways databases ($n = 6$). Data are presented as mean $\pm$ SEM. AP-1 = activator protein 1; NOD = nucleotide-binding oligomerization domain.

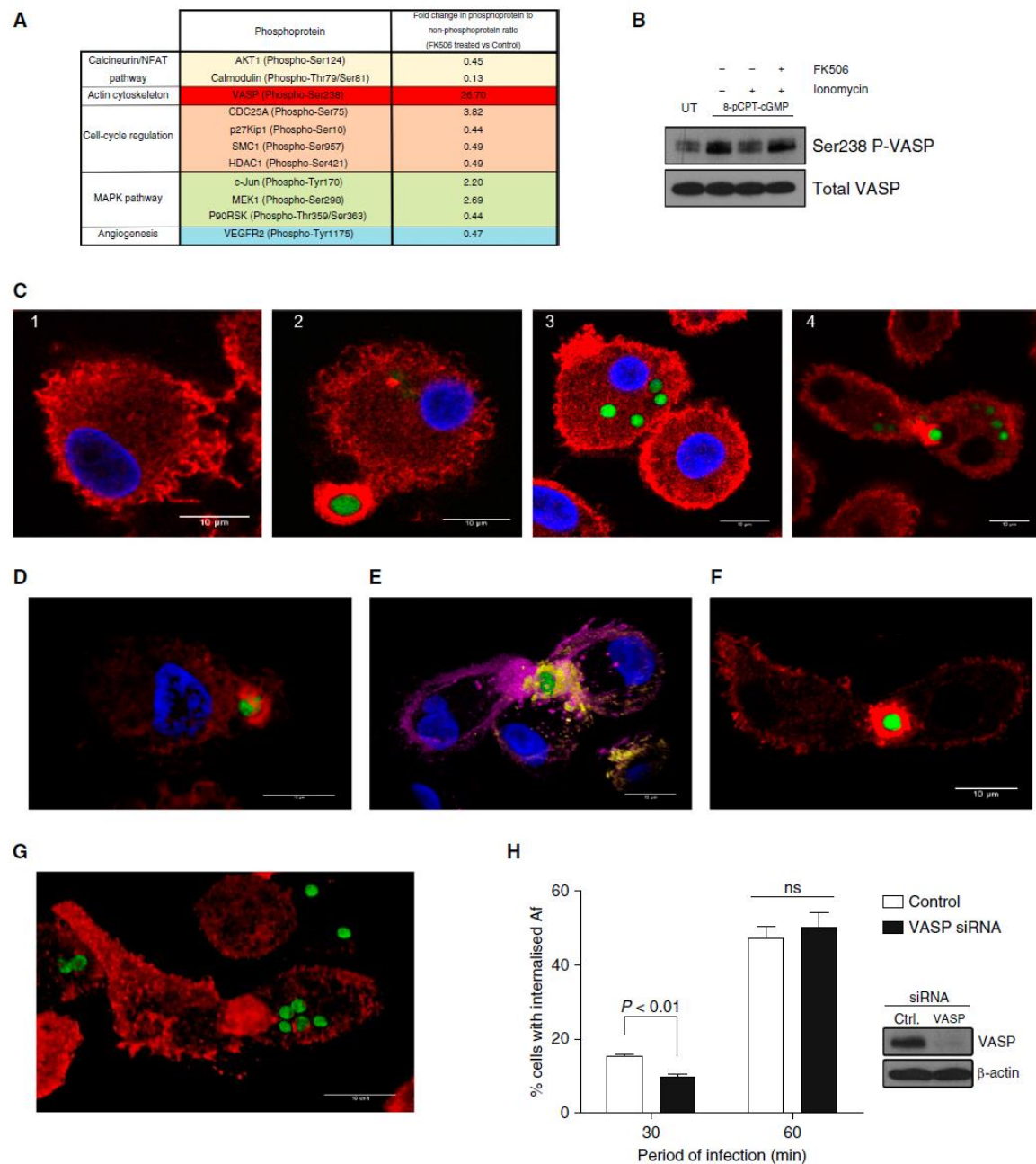


Figure 5. Lateral transfer of *Aspergillus fumigatus* (Af) occurs through vasodilator-stimulated phosphoprotein (VASP) tunnel-like structures. (A) A phosphoprotein array analysis was performed with human monocyte-derived macrophages (hMDMs) pretreated with FK506 and stimulated with live Af swollen conidia (SC) (multiplicity of infection [MOI], 1) for 1 hour postinfection. Proteins with significant fold change differences in the phosphoprotein-to-nonphosphoprotein ratio between FK506 and control macrophages stimulated with live Af SC are shown. (B) hMDMs were pretreated with a cell-permeable cyclic guanosine monophosphate analog (25 mM 8-[4-chlorophenylthio]-guanosine 3',5'-cyclic monophosphate [8-pCPT-cGMP]) to induce Ser238 VASP phosphorylation and with FK506 (10 ng/ml) or vehicle. They were then stimulated with a calcium ionophore (2 μg/ml ionomycin) to activate

have suggested a critical role for the MAPK-AP-1 pathway and dynamin-related protein 1 (Drp1)-mediated mitochondrial fission in necroptosis (25, 29). As mitochondrial translocation of Drp1 and MAPK-AP-1 pathway regulation are thought to be calcineurin dependent, this was explored in *Af*-infected hMDMs (30–32). Significant modulation of MAPK-AP-1 pathway activation and Ser637 P-Drp1 phosphorylation was observed (Figure E4D). Thus, RNA- and protein-level analyses further support a role for calcineurin in the macrophage programmed cell death response to *Af*.

***Af*-containing Endosomes Transferred from Macrophages to Macrophage Are Enveloped within Actin-Vasodilator-Stimulated Phosphoprotein-Rich Cargo**

Calcineurin phosphatase activity has multiple NFAT-independent effects on cytoskeletal reorganization and regulation of MAPK pathways (31, 33, 34). To identify calcineurin phosphatase targets, we performed a comparative phosphoproteomic array on FK506- versus vehicle-pretreated hMDMs infected with SC (Figure 5A). There were differences in phosphorylation of proteins within the MAPK pathway, including c-Jun and the cell-cycle regulatory protein M-phase inducer phosphatase 1. This is consistent with our transcriptomic findings of cross-control between calcineurin and MAPK pathways and cell death pathways.

The analysis revealed a 26-fold increase in Ser238 phosphorylated vasodilator-stimulated phosphoprotein (VASP) levels in FK506-treated hMDMs (Figure 5A). VASP is an actin polymerase that promotes assembly of actin networks, and it is important for directional motility and phagocytosis (35–37). Its function is tightly regulated, with Ser238 dephosphorylation

required for actin filament formation. We therefore postulated that VASP is a direct calcineurin target. As calcineurin is the only Ca^{2+} -activated serine-threonine phosphatase, we measured Ser238 P-VASP following the addition of the calcium ionophore ionomycin (to selectively activate calcineurin) to hMDMs with phosphorylated VASP (induced using a cell-permeable cyclic guanosine monophosphate analog) in the presence or absence of FK506 (Figure 5B). This confirmed that VASP is a major dephosphorylation target of calcineurin.

As VASP is an important actin cytoskeletal regulator and calcineurin is required for optimal phagocytosis and transfer of *Af*, we hypothesized that VASP is involved in actin-dependent phagocytosis and lateral transfer of *Af*. Using high-resolution confocal microscopy, we observed colocalization of VASP to *Af* at phagocytic cups, which disappeared following internalization (Figures 5C and 5D and Movie E6). Localization of VASP to *Af* was not calcineurin dependent. During *Af* lateral transfer at late time points, high-intensity colocalization of VASP and actin staining to *Af*-containing endosomes was observed (Figure 5E). High-resolution three-dimensional reconstruction revealed that *Af*-containing endosomes trafficked between macrophages are enveloped within actin-VASP-rich cargo (Figures 5C–5G and Movie E7).

To determine the role of VASP in macrophage *Af* phagocytosis, small interfering RNA knockdown of VASP was performed in human macrophages, followed by analysis of uptake of calcofluor white-stained live *Af* SC compared with control. This showed that phagocytosis of live *Af* SC at early time points is VASP dependent ($P < 0.01$) (Figure 5H),

consistent with the defect seen for calcineurin inhibition (Figure 2B). Taken together, these results indicate that calcineurin, by direct dephosphorylation and activation of the actin polymerase VASP, has a key role in macrophage actin cytoskeleton reorganization during fungal infection. This enables optimal actin-dependent *Af* phagocytosis and lateral transfer of *Af*-containing endosomes during programmed necrosis, which are critical to achieving control of germinating conidia.

Discussion

PA has emerged as a serious infectious complication of a range of immunocompromised states and chronic respiratory diseases. Overt infection is typically characterized by hyperinflammatory tissue destruction, with more complex relationships between airway colonization and progression of chronic respiratory diseases (38). Here we report human macrophage programmed necrosis as an important response to germination of *Af*. While necrosis has been recognized as a form of cell death since the mid-19th century, it has recently become clear that programmed necrosis may occur through receptor-interacting serine/threonine-protein kinase 3 necroptotic cell death (39). Necroptosis occurs as a firstline defense against intracellular pathogens and is central to the pathogenesis of a number of chronic inflammatory conditions, including emphysematous change (40, 41).

Remarkably, we observed frequent lateral transfer of *Af* during necroptosis, which ultimately enabled control of hyphal escape from the macrophage. Transfer occurred through a late endosomal compartment, suggesting that this process

Figure 5. (Continued). calcineurin. Macrophage whole-cell extracts were probed by Western blotting for Ser238 VASP and total VASP. (C) Representative confocal microscopic images of hMDM phagocytosis and lateral transfer of live *Af* SC (MOI, 1). VASP is shown in red, nuclei in blue, and *Af* in green. (1) Uninfected hMDMs. (2) Initial phagocytosis of *Af* by MDMs. (3) Late phagocytosis of *Af* by hMDMs. (4) Lateral transfer of *Af* between hMDMs. (D) Three-dimensional reconstruction of confocal microscopic images of phagocytosis and lateral transfer of live *Af* SC (MOI, 1) between hMDMs. *Af* phagocytosis at 30 minutes postinfection with VASP is shown in red, *Af* in green, and nuclei in blue. (E) Three-dimensional reconstruction of confocal microscopic image of *Af* lateral transfer with VASP shown in yellow, actin in magenta, *Af* in green, and nuclei in blue. (F and G) Representative z-slice confocal microscopic image (F) and three-dimensional reconstruction (G) of *Af* lateral transfer. VASP is shown in red, and *Af* is shown in green. (H) THP-1 macrophages treated with either control or VASP small interfering RNA (siRNA) (50 μM) were infected with live *Af* SC and stained with Calcofluor White (Sigma-Aldrich, St. Louis, MO) (MOI, 1). Phagocytosis was quantified by flow cytometry ($n = 3$). Data are presented as mean $\pm$ SEM. AKT1 = RAC- α serine/threonine protein kinase; CDC25A = M-phase inducer phosphatase 1; HDAC1 = histone deacetylase 1; MAPK = mitogen-activated protein kinase; MEK1 = mitogen-activated protein kinase 1; NFAT = nuclear factor of activated T cells; SMC1 = structural maintenance of chromosomes protein 1; VEGFR2 = vascular endothelial growth factor receptor 2.

may have similarities to exocytosis (42). While lateral transfer has previously been observed for *Cryptococcus neoformans* at low frequency, the molecular basis for transfer and its relationship to cell death or control of infection have not previously been defined (43). The pathogen escape and transfer processes described to date are pathogen mediated and enhance either tissue dissemination or evasion from immune attack (47). However, our observations are consistent with host-dependent lateral transfer of *Af* to limit hyphal escape during macrophage death. The observation of cell-cell transfer of *Af* supports a model whereby inhaled conidia may persist for prolonged periods in the myeloid compartment as spores. This has potentially important implications for individuals who subsequently undergo immunosuppression.

Notably, we observed that both fungal germination-driven programmed necrosis and lateral transfer were calcineurin dependent, indicating that necroptotic control of infection is likely to be impaired in organ transplant patients. Calcineurin has been postulated to have a role in the regulation of programmed cell death

through interaction with Bcl-2 family proteins (25, 26). Consistent with this, we found significant calcineurin-dependent expression of myeloid cell leukemia 1, a Bcl-2 protein that inhibits apoptosis, and Bcl2-modifying factor, a member of the prodeath BH3-only subgroup of Bcl-2 family proteins required for TNF- α -induced necroptosis (27). In transcriptomic and phosphoproteomic analysis, we defined calcineurin as a key phosphatase mediating cross-control of cell death, the MAPK-AP-1 innate pathway, and VASP-dependent cytoskeletal remodeling during macrophage infection. Calcineurin inhibition impaired these cellular processes, leading to enhanced fungal germination in the late phagosome, impaired lateral transfer, and ultimately hyphal escape. Interestingly, virulent *Burkholderia* spp. have recently been shown to mimic VASP actin polymerases to enable cell fusion and spread (44). Whether fungi are able to directly manipulate host actin polymerases remains to be determined.

In summary, we have identified macrophage programmed necrosis as an important response to progressive

germination of *Af* in the human macrophage, and we report a previously undescribed phenomenon of cell death-dependent lateral transfer as a cooperative macrophage behavior that assists in limiting hyphal escape. We show that calcineurin is a key orchestrator of this process, further defining the importance of this pathway in innate immunity. Our findings yield novel insights into host innate immunity to *Af* in the lung, extend current understanding of the pathogenesis of PA in organ transplantation, and have broader implications for transplant immunity and chronic lung disease. ■

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