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Beyond Toll-Like Receptor 9: Interactions Between Plasmacytoid Dendritic Cells and Aspergillus Fumigatus: A Dissertation

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BEYOND TOLL-LIKE RECEPTOR 9: INTERACTIONS BETWEEN PLASMACYTOID DENDRITIC CELLS AND *ASPERGILLUS FUMIGATUS*

A Dissertation Presented

By

Zaida Gisela Ramirez-Ortiz

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"It is always what I have already said: always the wish that you may find patience enough in yourself to endure, and simplicity enough to believe; that you may acquire more and more confidence in that which is difficult, and in your solitude among others. And for the rest, let life happen to you. Believe me: life is right, in any case."

— ***Rainer Maria Rilke***

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Abstract

The opportunistic fungus, *Aspergillus fumigatus*, is a leading cause of morbidity and mortality among the immunocompromised population. Experimental and clinical findings have established that phagocytic defenses are critical in the recognition and clearance of *A. fumigatus*. Previous studies found that Toll-like receptors (TLRs), specifically TLR2 and TLR4, were essential in the detection of the mold. Furthermore, one study found that mice deficient in TLR9 lived longer than their wild-type counterparts following challenge with *A. fumigatus*. We sought to determine the role of TLR9 during *A. fumigatus* infection. Our results show that *A. fumigatus* contains unmethylated CpG DNA, the natural ligand of TLR9. Furthermore, *A. fumigatus* DNA stimulates a potent pro-inflammatory response in mouse bone marrow derived dendritic cells (BMDCs) and human plasmacytoid dendritic cells (pDCs). A genome wide analysis showed that *A. fumigatus* DNA contains 87 human and 23 mouse putative immunostimulatory motifs. The response to *A. fumigatus* DNA is TLR9-dependent, as BMDCs from $TLR9^{-/-}$ mice were unresponsive to the fungal DNA. In addition, HEK293 cells cotransfected with human TLR9 and NFκB driven Luciferase conferred responsiveness to *A. fumigatus* CpG-rich sequences found in the fungal DNA. Our results show that TLR9 detects *A. fumigatus* DNA, resulting in the secretion of proinflammatory cytokines.

While pDCs secrete IFNα in response to *A. fumigatus* DNA, these cells have been mainly described to play critical roles in the antiviral responses. The role of pDCs during

fungal infections remains to be elucidated. Our data show that $CD304^{+}$ peripheral blood pDCs challenged with *A. fumigatus* hyphae secrete large concentrations of IFNα and TNFα in response to infection. Furthermore, the response appears to be TLR9-independent. However, pDCs spread over the hyphae and inhibit fungal growth. Furthermore, pDCs undergo cell lysis upon incubation with *A. fumigatus.* The antifungal activity of the pDCs was retained in the cell lysates, suggesting that this response was mediated by an intracellular factor. Addition of exogenous Zn^{2+}, but not Fe^{3+}, partially restores hyphal growth. In addition, western blot of pDC lysates show that these cells have the Zn^{2+}-binding protein calprotectin.

Over 60% cell death is observed in the pDC population following a 2 hour incubation with *A. fumigatus*. The observed pDC cell death can be partially attributed to gliotoxin, as pDCs challenged with *A. fumigatus* stains deficient in production of the mycotoxin result in decreased pDC cytotoxicity. Furthermore, pDC cell death occurs independent of contact with the mold, confirming that pDC cell death is mediated by a secreted fungal factor. In addition, our results show that pDCs are required for the host response against *A. fumigatus*. Mice depleted of their pDCs are more susceptible to *A. fumigatus* infection than the control counterparts, suggesting that pDCs play a role in the antifungal response. Also, we observe a 5-fold increase in the pDC population in the lungs of infected mice. Therefore, the possibility of these cells playing a role in recruiting and communicating with other immune cells cannot be eliminated.

Upon maturation, pDCs acquire characteristics of conventional DCs (cDCs) such as upregulation of major histocompatability complex (MHC) and becoming more phagocytic. Whether mature pDCs are involved in the detection of and responses against fungal pathogens remains to be determined. Here we show that mature pDC secrete IFNα and TNFα in response to *A. fumigatus* conidia as early as 6 hours post-challenge. While cytokine secretion of mature pDCs against *A. fumigatus* does not require opsonization, it requires for *A. fumigatus* being alive and growing. Furthermore, supernatants from conidial growth induced cytokine secretion by the mature pDCs.

The work presented in this thesis establishes that the nucleic acids in *A. fumigatus* serve as a pathogen associated molecular pattern (PAMP) that can induce a TLR9-dependent response. Furthermore, I show that pDCs secrete cytokines and induce an antifungal response against *A. fumigatus* conidia and hyphae. While the pDC population in the blood appears to be small, our work shows that these cells could be intimately involved in the antifungal responses against *A. fumigatus*.

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List of Abbreviations:

Absent in Melanoma 2	AIM2
Allergic bronchopulmonary aspergillosis	ABPA
Antigen presenting cells	APCs
Blood Dendritic Cell Antigen	BDCA
Bone marrow-derrived Dendritic cells	BMDCs
Chronic Granulomatous Disease	CGD
Complement Alternative Pathway	AP
Complement Classical Pathway	CP
Conventional Dendritic cells	cDCs
C-type Lectin Domains	CTLD
C-type Lectin Receptors	CLRs
dendritic cell specific ICAM-3-grabbing non-integrin	DC-SIGN
Dendritic cells	DCs
Deoxyribonucleic acid	DNA
DNA-dependent activator of IFN-regulatory factors	DAI
Double Stranded DNA	dsDNA
Enzyme-linked Immunosorbent Assay	ELISA
Epipolythiodioxopiperazine	ETP
Fetal Bovine Serum	FBS
Glycophosphatidylinositol	GPI

Granulocyte macrophage colony stimulating factor	GM-CSF
Hematopoietic stem cell transplant	HSCT
Human Immunodeficiency Virus	HIV
Interferon	IFN
Interferon Regulatory Factors	IRF
Interferon α and β Receptor knockout mice	IFNα/β$^{-/-}$
Invasive Aspergillosis	IA
Major histocompatability complex	MHC
Mannose Binding Lectin	MBL
Mannose Receptor	MR
Melanoma Differentiation Associated Gene 5	MDA-5
Myeloid Dendritic Cells	mDCs
NACHT, LRR and PYD domains containing protein 3	NALP3
Neutrophil Extracellular Traps	NETs
NOD-Like Receptors	NLRs
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κB
Nucleotide-binding Oligomerization Domain	NOD
Oligodinucleotides	ODN
Pathogen Associated Molecular Patterns	PAMPs
Pattern Recognition Receptors	PRR
Peripheral Blood Mononuclear Cells	PBMC

Phosphate Buffered Saline	PBS
Plasmacytoid Dendritic cells	pDCs
Polymorphonuclear Neutrophils	PMN
Resting Conidia	RC
Retinoid acid Inducible Gene	RIG-I
Ribonucleic acid	RNA
RIG-I Like Receptors	RLR
Single Nucleotide Polymorphism	SNP
Single Stranded DNA	ssDNA
Swollen Conidia	SC
Terminal deoxynucleotidyl transferase dUTP nick end labeling	TUNEL
T-helper	Th
Toll/IL-1R	TIR
Toll-Like Receptors	TLRs
Tumor Necrosis Factor α	TNFα
Wild-type	WT

Chapter I

Introduction

Aspergillus

Overview of fungal pathogens

The kingdom Mycota includes between 100,000 to 1,000,000 species that habituate in a variety of environments (Levitz 2004) . These eukaryotic disease causing organisms in humans are saprophytic organisms that can adapt to changes between environmental temperature and body temperatures (Garcia-Solache and Casadevall 2010; Murphy 1991). Due to increased numbers of immunocompromised hosts, the clinical relevance of fungal infections has increased significantly over the last three decades (Romani 2004). Mycotic disease rarely occurs in "normal" hosts; however, these opportunistic pathogens infect hosts that have a variety of immunodeficiencies. Furthermore, pathogenic fungi can cause a range of infections; from allergy to systemic infections. Among the most studied fungal pathogens are *Cryptococcus neoformans*, *Candida albicans*, *Histoplasma capsulatum* and *Aspergillus fumigatus* (Guarro et al. 1999).

Fungi are usually non-invasive organisms. Fungal pathogens cause infection by taking advantage of weaknesses in the host immune system, evading the immune response or overwhelming the host response mechanisms (Murphy 1991; Romani 2004). For example, *C. albicans* is part of the natural flora of the genital tract. Under normal conditions this poses no threats; however, the pathogenicity of *C. albicans* is greatly dependent on host immune status and disease presentation (Achkar and Fries 2010). *C. albicans* infections of the mucosal surfaces are more prevalent among

immunocompromised hosts, but vulvovaginal candidiasis is a common disease among immunocompetent woman (Achkar and Fries 2010). Other fungal pathogens, like *C. neoformans*, *H. capsulatum* and *A. fumigatus*, are found in the environment and infection normally occurs via inhalation of the organism (Karkowska-Kuleta et al. 2009).

The frequency of invasive fungal infections has greatly increased as a result of progress in transplantology, increased HIV infections and increased cancer treatment (Person et al. 2010). Although anti-fungal medicine is available; some fungal pathogens have evolved to become resistant to drug treatment. Furthermore, scientists are just starting to understand the mechanisms of pathogenesis of these infectious agents (Levitz 2004; Mansour et al. 2004; Romani 2004). The work presented in this thesis concentrates on the immune responses against *A. fumigatus.*

The Genus *Aspergillus*

The genus *Aspergillus* belongs to the ascomycete group and contains approximately 200 species, but only a few are considered human pathogens (Dagenais and Keller 2009; Hohl and Feldmesser 2007a). These saprophytic molds are ubiquitous to the environment and play essential roles in nitrogen and carbon recycling (Latge 1999). Organisms in this genus were first characterized in 1729 by Pier Antonio Micheli, a priest biologist, who noticed the resemblance between the spore bearing structure of the fungi and the *aspergillum* (holy water sprinkler) (Bennett 2010). Three centuries later, we now use the word aspergillum to describe the asexual spores used by members of the *Aspergillus* genus. However, it is important to note that at least one third of the

Aspergillus species can also reproduce sexually (Dyer and Paoletti 2005; Kwon-Chung and Sugui 2009a).

Members of the *Aspergillus* genus are widely distributed in nature, growing in almost all environments. *Aspergillus* spores are common components of aerosols, and because of their small size, they can easily disperse (Bennett 2010; Latge 1999). Similar to other genuses in the fungal kingdom, *Aspergilli* are characterized by their particular eating mechanism known as digestion then ingestion. These organisms degrade matter by enzyme secretion into the environment, where they obtain the necessary nutrients, and then they ingest the polymers (Bennett 2010; Latge 1999).

Aspergilli are of great importance for humans. Some species play roles in industrial processes including enzymes, commodity chemicals and food preparation (Dagenais and Keller 2009). *Aspergillus* spp are used in research to understand the parasexual cell cycle, metabolic pathways and hyphal polarity. Other fungi from this genus are plant pathogens; affecting crops with toxic metabolites that can cause disease in animals, including humans. Finally, a few species are known to cause invasive disease in humans (Bennett 2010; Dagenais and Keller 2009). The most studied of these species is *A. fumigatus*.

Aspergillus fumigatus

Aspergillus fumigatus belongs to the genus *Aspergillus*, subgenus *Fumigati* division *fumigati*. *A. fumigatus* was first described in 1863 by Johann Baptist Gerog Wolfgang Fresenius (Latge 1999). Like other members of the *Aspergillus* spp, *A.*

fumigatus plays a role in the aerobic decomposition of organic materials and recycling environmental carbon and nitrogen (Dagenais and Keller 2009; Latge 1999). Although the natural environment of *A. fumigatus* is the soil, this mold can be found in almost every environment in the world.

A. fumigatus and other *Aspergillus* species, produces small hydrophobic spores known as conidia during the asexual reproduction cycle. The small size of the conidia and the properties of the cell wall allow it to be easily distributed in the water or air. Once in the air, the small conidia (2-3 μm) can travel great distances and colonize new niches. The primary ecological niche for this fungus is decaying vegetation (Latge 1999). Upon colonization, *A. fumigatus* releases enzymes that breakdown and degrade the organic material that is subsequently taken up by the fungus (Tekaia and Latge 2005).

A. fumigatus conidia not only colonize organic matter, the aerosolized conidia are readily inhaled by humans and upon the right conditions disease is established. *A. fumigatus* is the most common species of *Aspergillus* spp. to cause human disease in immunocompromised hosts (Levitz 2004; Montagnoli et al. 2006; Netea et al. 2006). The most common route of infection is inhalation of the conidia, where it can reach deep pockets in the alveoli. If the host conditions are appropriate, life-threatening invasive and disseminated disease may develop (Askew 2008).

***A. fumigatus* Growth and Culture**

The small and dormant conidia (2-3 μm) are round, uniform size with nuclei that are arrested in interphase. In the presence of nutrient sources, morphological changes can be observed as early as 1 hour post addition of nutrient rich media or nutrients found in the environment. In the earliest stages of germination, nuclear decondensation and adhesion to the substrate are observed (Bennett 2009). The next step in conidial germination is rehydration and cell wall growth, a process known as conidial swelling. Conidial swelling takes places within 4 hours at 37°C from the time dormancy is broken. The swollen conidia polarize and the first germ tube is observed within 6 hours at 37°C. Nuclear decondensation prepares *A. fumigatus* conidia for DNA replication, a process that is usually observed within a couple of hours, followed by mitosis. Germ tubes continue polar growth to become hyphae. Hyphae continue polar growth (Latge 1999; Latge 2007). Within 2-4 hours from germ tube germination, septa form and the first branches emerge from the apical side of the septa. Hyphae continue to grow, extend and branch; giving rise to visible colonies within 24 hours of awakening. Conidiophores, the asexual reproductive structures, can be observed by 36 hours (Fontaine et al. 2010) allowing the cycle to begin again (Figure 1.1).

The life cycle and growth of the filamentous fungus *A. fumigatus* can be considered an essential pathogenicity factor. *A. fumigatus* exhibits multiple morphology changes during its life cycle (Pihet et al. 2009; Tronchin et al. 2008). The asexual conidia are the primary means of environmental dispersal, due to the small size and the

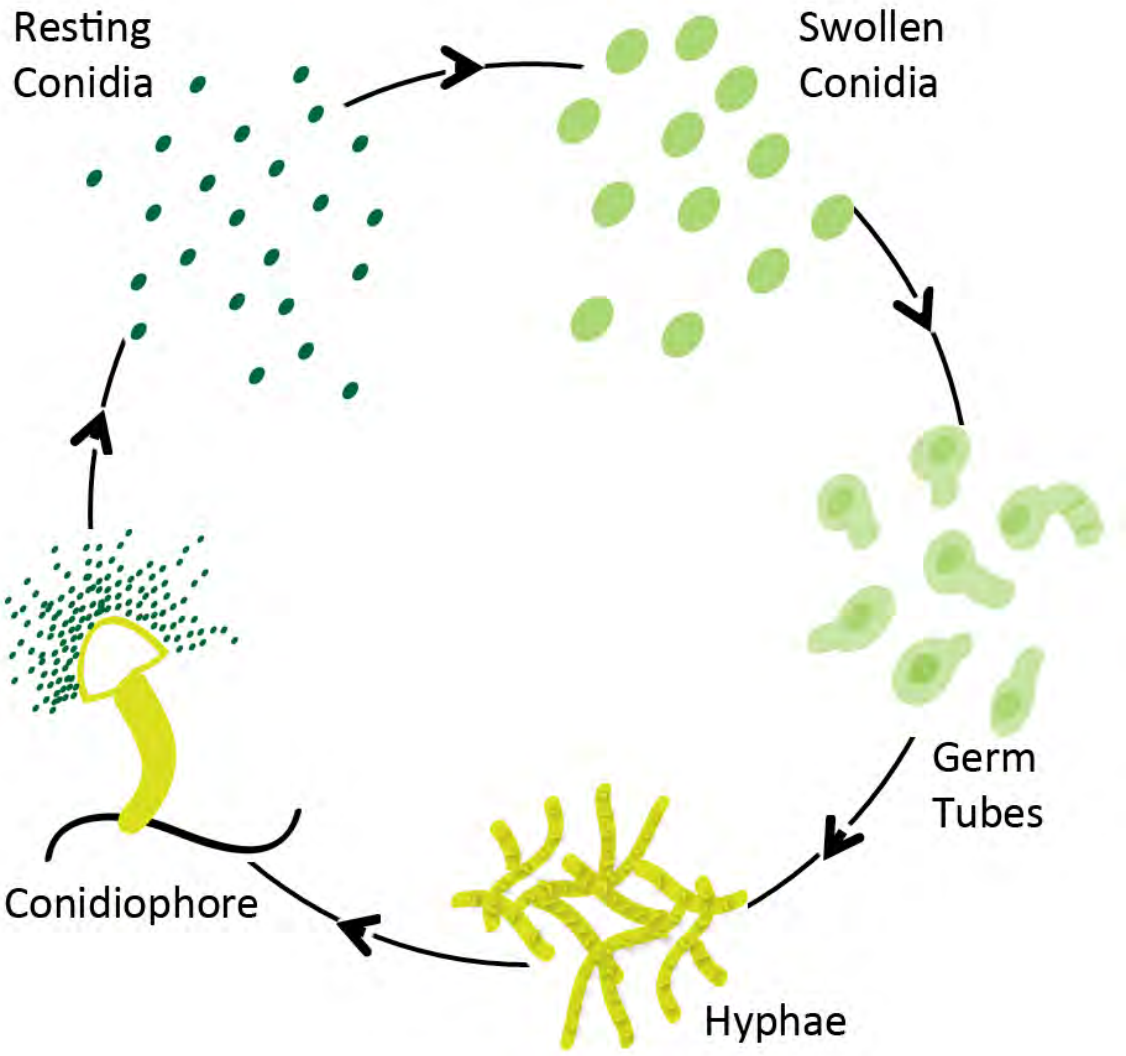
Resting
Conidia
Swollen
Conidia
Germ
Tubes
Hyphae
Conidiophore

Figure 1.1. Biological cycle of *A. fumigatus*

The natural niche of *A. fumigatus* is decaying matter and organic material. Colonies of the mold produced from conidiophores generate thousands of green-grey spores, also known as resting conidia. These small conidia (2-3μm) become readily aerosolized. Upon encountering a nutrient-rich environment, the resting conidia shed the hydrophobic outer layer and begin to grow. During conidial swelling, *A. fumigatus* rehydrates and changes in the cell wall expose carbohydrate moieties that were protected by the outer layer. Swollen conidia polarizes, giving rise to germ tubes. Additional changes in the cell wall are responsible for the plasticity of the germ tubes. These germ tubes become septated and continue their polar growth to become hyphae, the invasive form of the mold.

resistance to severe environmental conditions. Upon encountering the appropriate environmental conditions, which may be after years of latency as a conidium, *A. fumigatus* activates a series of physiological and biochemical pathways that allows conidial germination. *A. fumigatus* is unable to germinate in anaerobic condition or in the presence of respiratory chain inhibitors; proper growth conditions require the presence of water, carbon, sources of phosphate and nitrate, and oxygen (Krappmann and Braus 2005; Rhodes 2006).

The fungal cell wall

During *A. fumigatus* germination, growth and polarization, the fungal cell wall undergoes dramatic changes (Latge 1999). The *A. fumigatus* cell wall is primarily composed of a polysaccharide matrix with some proteins required for cell wall synthesis and nutrient uptake anchored to the "cytoskeleton". In the *A. fumigatus* resting conidia, there are two layers comprising the cell wall. The most outer layer is hydrophobic and mainly comprised of melanin, small cystein-rich proteins that form a hydrophobic layer known as hydrophobins and rodlet proteins (Fontaine et al. 2010; Pihet et al. 2009). The second electron translucent layer is comprised of polysaccharides (Fontaine et al. 2010).

During conidial germination and swelling, the cell wall undergoes dramatic changes. The first change observed during conidial swelling is the loss of the most outer layer, resulting in the exposure of different sugars to the environment. Although there are six main glycans that comprise the cell wall of *A. fumigatus*: β-1,3 glucan, chitin, galactomannan, α-1,3 glucan, β-1,3/1,4 glucan and a polymer of galatosaminogalactan;

conidial swelling is characterized by the emergence of β-1,3 glucans to the cell surface (Latge 2007; Latge et al. 2005).

In addition to the polysaccharide structure, the cell wall is coated with proteins. The major group of cell wall proteins is the glycophosphatidylinositol(GPI)-linked proteins, which is connected to the polysaccharide membrane (Levdansky et al. 2010). Other proteins required for cell wall biosynthesis and nutrient uptake have also been found in association with the *A. fumigatus* cell wall.

The morphological changes of *A. fumigatus* and the plasticity of the cell wall can be considered important virulence factors of the filamentous mold. During fungal growth and morphological changes, some of the cell wall components like galactomannan are released into the environment where they can elicit an immune response (Latge 1999; Latge 2001; Latge 2007; Latge 2010). Furthermore, the fungal cell wall contains many transient proteins and enzymes which have been found to be antigenic (Gastebois et al. 2009).

Aspergillosis

A. fumigatus is the primary causative species of human aspergillosis (Dagenais and Keller 2009). Infection occurs via the respiratory tract, upon encountering the proper host *A. fumigatus* germinates resulting in disease. The clinical manifestations of this multifaceted disease are determined by the host immune response. Aspergillosis can present itself as allergic, saprophytic (germination of the mold on the mucous crust of sinonasal cavities) or invasive forms (Latge 1999; Levitz 2004).

The allergic presentations of aspergillosis occur following repeated exposure to the conidia and antigens of Aspergillus; however, there is no colonization of the lungs. Some forms of allergic disease include asthma, allergic sinusitis and alveolitis. Most severe forms of the disease involve mycelial growth of *A. fumigatus* in the lungs, and therapeutic intervention is usually required. Allergic bronchopulmonary aspergillosis (ABPA) is one of the most severe complications caused by *Aspergillus* spp and is mainly found in patients suffering from asthma and cystic fibrosis (Dagenais and Keller 2009; Latge 1999; Levitz 2004; Pettigrew et al. 2010). Aspergilloma or fungus ball is usually found in preexisting pulmonary cavities caused by other lung disorders and in chronically obstructed paranasal sinuses. Aspergillomas consist of a spheroid mass of hyphae with sporulating structures at the periphery; the most common symptom of aspergillomas is hemoptosis.

Invasive aspergillosis (IA) is the most severe form of disease, and has emerged as an infectious disease with high morbidity and mortality among immunocompromised

patients. The higher risk groups involve acute leukemia patients, bone marrow and solid organ transplant patients, cancer patients and HIV-infected hosts. IA is also found as a common infectious complication of chronic granulomatous disease; however, this disease is rarely found in immunocompetent hosts. In neutropenic patients, IA is characterized by thrombosis and hemorrhage from rapid and extensive hyphal growth (Dagenais and Keller 2009). However, the observed outcome of infection with *A. fumigatus* as determined by the host immune status and response to the fungal infection.

The pathogen *A. fumigatus*

***A. fumigatus* infectious cycle**

The saprophyte *A. fumigatus* can be readily found in the environment, as its natural niche is dead or decaying matter. During the asexual reproduction cycle, conidiophores produce and releases conidia, the infectious propagules of the mold. The secretion and propagation of the small spores is the first step in the infectious cycle. The small size of the conidia allows them to be easily dispersed into the air, where they can be found in both indoor and outdoor environments. Human infection begins with inhalation of the airborne conidia. The microscopic size of the conidia allows the deposition of these spores in the bronchioles or alveoli. In healthy individuals, this is the end of the infectious cycle. Epithelial cells and alveolar macrophages, the primary resident phagocytes of the lungs, recognize and clear the infection without sign of disease.

In immunocompromised host, conidia are able to bypass mucociliary clearance and establish infection in the alveoli. *A. fumigatus* conidia that escape the host's first line

of defense undergo conidial swelling. The isotropic growth of the conidia marks the emergence of *A. fumigatus* from dormancy. Establishment of infection requires a warm and nutrient rich environment, conditions shared between the usual environmental niche of *A. fumigatus* in compost and the pulmonary alveoli. Upon germination in the alveoli polar growth results in generation of hyphae, the invasive form of *A. fumigatus*. Therefore, the morphological switch from unicellular conidia to multicellular hyphae allows *A. fumigatus* to invade host tissue.

Cells of the immune system are key regulators of *A. fumigatus* infection. Phagocytes are the first immune cell type to encounter the pathogenic mold and clear the infection, in most cases without adverse effects. Conidia are phagocytosed by alveolar macrophages, while hyphae are killed extracellularly by neutrophils. In addition to cell mediated clearance of *A. fumigatus*, several host factors have been described to be involved in the development of disease.

A. fumigatus **Virulence Factors**

The virulence of *A. fumigatus* is mediated by several factors such as proteins involved in fungal growth and conidial germination, as well as secreted factors from the cell wall (Latge 2001). In addition to secreted factors, the virulence *A. fumigatus* is dependent on escaping recognition of the conidia by the host immune system, allowing deposition in the lung alveoli and the establishment of infection.

Environmental conidia are covered with a hydrophobin layer formed by the RodA protein that limits their recognition by the immune system; hiding the β-1,3-glucans from

recognition by alveolar macrophages (Aimanianda et al. 2009; Brakhage et al. 2010a). Furthermore, the green pigment that confers conidia its unique color, DHN-melanin, an important component of the cell wall, was also identified as virulence factor for *A. fumigatus* (Pihet et al. 2009). The DHN-melanin layer is lost upon conidial swelling, allowing the exposure of highly immunogenic β-glucans. One study suggested a role for *A. fumigatus* melanin in the modulation of host response, as conidia lacking the melanin layer stimulated human PBMC defective in dectin-1 less potently than cells isolated from healthy donors (Chai et al. 2009).

A. fumigatus produces and secretes various hydrolytic enzymes that facilitate lung tissue colonization. Three major proteases have been shown to be secreted during infection: an alkaline serine protease, Alp; a metalloprotease, Mep; and an aspartic protease, Pep. These enzymes are known to play a role in the recycling of organic material in the environment; however, the penetration into the lung requires the fungi to use extracellular enzymes to penetrate the pulmonary epithelium (Rementeria et al. 2005).

***A. fumigatus* Secondary Metabolites**

Secondary metabolites also play a role in *A. fumigatus* virulence. These secreted small molecules have been shown to be detrimental to the host cells by exerting immunosuppressive and proapoptotic effects on host cells. The *A. fumigatus* Af293 genome has revealed numerous genes that encode for non-ribosomal peptides and polyketide synthetases, which are responsible for secondary metabolite production.

Secondary metabolites play roles in conidial germination, hyphal growth, and cell wall structural components. However, some of these secondary metabolites have also been shown to be implicated in *A. fumigatus* pathogenicity.

Gliotoxin, fumagilin, fumagatin and helvoic acid are some of the *A. fumigatus* toxins (Bok and Keller 2004). Regulation of these toxins is mediated by LaeA, a nuclear protein required for the expression of secondary metabolites. Studies with LaeA deletion strain of *A. fumigatus* (*ΔLaeA)* show reduced virulence in a mouse infection model. Furthermore, the decreased virulence of *ΔLaeA* correlated with the lack of secondary metabolite production (Bok et al. 2005; Bok and Keller 2004; Kale et al. 2008). In addition, *ΔLaeA A, fumigatus* did not show any changes in the morphology of the conidia, cell wall composition or fungal growth (Sugui et al. 2007a). Recent data suggest that the difference in fungal recognition by macrophages between *A. fumigatus ΔLaeA* and the wild type counterpart is due to decreased hydrophobin (RodAp) layer in the deletion strain. In addition to a deficiency in the production of secondary metabolites, wild type *A. fumigatus* was shown to have 60% more RodAp than the LaeA deletant (Dagenais et al. 2010).

The best studied secondary metabolite is the epipolythiodioxopiperazine (ETP) gliotoxin (Kwon-Chung and Sugui 2009b; Sugui et al. 2007b). Gliotoxin was first identified in 2004 as the product of a 12-gene cluster in the *A. fumigatus* genome that resembles the ETP sirodesmin (Gardiner and Howlett 2005). Mutations in different genes of the cluster resulted in loss of gliotoxin production, confirming the initial

findings (Bok et al. 2006; Kwon-Chung and Sugui 2009b; Spikes et al. 2008; Sugui et al. 2007b). Additional studies have showed that gliotoxin can be recovered from sera during IA (Lewis et al. 2005). Deletion studies in *GliZ*, a Zn_2Cys_6 binuclear transcription factor, resulted in loss of detectable gliotoxin. Expression of multiple copies of *GliZ* in the *A. fumigatus* genome resulted in increased gliotoxin production, suggesting that *GliZ* is essential for gliotoxin production. In a mouse pulmonary infection model, Bok *et al.* showed decreased virulence in *A. fumigatus* strains lacking *LaeA* when compared to the wild-type and complemented strains (Bok et al. 2005; Bok et al. 2006; Bok and Keller 2004; Kale et al. 2008). Although decreased survival was observed in mice infected with *ΔGliZ* and *ΔGliP*, the difference was not significant between mice infected with wild-type *A. fumigatus* and the deletion strains (Bok et al. 2006; Sugui et al. 2007b). Gliotoxin has also been shown to inhibit the nuclear translocation of the transcription factor NF-κB, to inhibit reactive oxygen species and inhibit phagocytosis. Recent studies showed that *A. fumigatus* gliotoxin can induce apoptosis but not necrosis in human monocytes and neutrophils, serving as an evasion mechanism for the fungus (Orciuolo et al. 2007; Stanzani et al. 2005).

Host factors involved in disease

Qualitative and quantitative disorders on phagocyte function are among the most important host factors predisposing patients to invasive infection with *A. fumigatus* (Shoham and Levitz 2005). The major risk factor for development of IA is neutropenia, the duration and severity of the immunodeficiency has been found to be proportional to

the development of disease (Maschmeyer et al. 2007). In hematopoietic stem cell transplant patients (HSCT), T cell suppression induced by total body irradiation increases the risks of developing IA. Furthermore, high-dose corticosteroids and T cell suppressive drugs given pre-transplantation have also been identified as risk factors (Maschmeyer et al. 2007; Sherif and Segal 2010).

Recently, patient specific risk factors were described. Polymorphisms in toll-like receptors (TLRs) were found to correlate with increased susceptibility to *A. fumigatus* infection (Bochud et al. 2008; Carvalho et al. 2008a; Kesh et al. 2005; Pamer 2008). In HSCT patients, polymorphisms in TLR1 and TLR6 were correlated to increased susceptibility to IA (Kesh et al. 2005); while polymorphisms in the IL-10 gene may either play a protective role or increase the risk of IA (Sainz et al. 2007; Seo et al. 2005).

Overview of the Immune System

The observation that an individual infected with a certain pathogen could gain protection against this pathogen gave rise to the field of immunology. Our bodies have evolved to fight infections, and protect us from subsequent infections with the same organisms. This protection is based on the initial recognition of pathogens, differentiating the pathogenic cells from self and generating memory against invading pathogens. Therefore, host defenses can be divided into two categories: innate and adaptive (or acquired) immune responses.

The adaptive arm of the immune system is responsible for the elimination of the pathogens in the late phase of infection (Loiarro et al. 2010). Unlike the innate arm of the immune system, the adaptive immune system recognizes infectious agents based on clonal selection. A pre-existing repertoire of memory B cells and T cells, generated by genetic recombination of antigen receptor genes after exposure, are capable of recognizing and selectively eliminating foreign antigens. This antigenic specificity allows the adaptive immune system to distinguish extremely subtle differences among antigens. In addition, there is ample diversity among the recognition molecules that allows recognition of billions of unique structures on foreign antigens. The specificity of acquired immune system can differentiate between two organisms with minor genetic variations. One important aspect of acquired immunity is the ability to develop immunogenic memory and generate a specific reaction the second time the same antigen is encountered. This allows for life-long immunity to an array of infectious agents after initial exposure.

The innate immune system constitutes the first line of defense against infection. The evolutionarily conserved system provides protection by means of physical, chemical and cellular barriers mediated by germline-encoded receptors that can detect a limited repertoire of microbial structures associated with infection (Kawai and Akira 2010).

The primitive innate immune system is capable of discriminating self antigens from exogenous antigens. The detection of exogenous antigens is facilitated by the recognition of pathogen associated molecular patterns (PAMPs), a process which usually

leads to an inflammatory response. These PAMPs include nucleic acids, viral proteins, components of the bacterial and fungal cell wall such as LPS and β-1,3-glucans that are conserved among a wide range of pathogens making them the perfect target for host recognition. The host response is mediated by pattern recognition receptors (PRRs), germ line encoded receptors responsible for recognizing PAMPs and initiating a signaling cascade that leads to the activation of cytokines and chemokines. By the initiation of these signaling cascades, the innate immune system communicates with other components for the activation of the adaptive immune response.

The mammalian immune system depends on the fine interplay between innate and adaptive immunity for the effective clearance of invading pathogens (Akira 2009; Kawai and Akira 2009). Phagocytes, like macrophages and dendritic cells (DCs), mediate these "non-specific" responses by recognizing and triggering proinflammatory response against invading pathogens (Mogensen 2009).

Pattern Recognition Receptors

The innate immune system relies on evolutionary conserved structures on PAMPs through a limited number PRRs. The innate immune response is primarily mediated by phagocytes and antigen presenting cells (APCs) like macrophages and dendritic cells, which express different PRR. To date, several groups of PRRs have been described and characterized into four main families: the TLRs, the retinoid acid-inducible gene I (RIG-I) like receptor (RLR), the C-type lectin receptors (CLRs) and the nucleotide-binding

oligomerization domain (NOD) like receptors (NLRs) (Akira 2009; Kawai and Akira 2009) (Figure 1.2).

PRRs are expressed on the cell surface or in intracellular compartments and their activation leads to the secretion of proinflammatory cytokines. Phagocytic cells, such as dendritic cells (DCs), neutrophils and macrophages, are primarily responsible for mediating this evolutionarily ancient form of defense. Each phagocyte bears a subset of PRRs that mediates recognition of the pathogen by either direct recognition (by expression in the cell surface or intracellular vesicles) or indirect via soluble PRRs in bodily fluids. Recognition of PAMPs results in an array of cellular responses triggered by a variety of intracellular signaling cascades. Activation of these cascades results in microbial uptake by phagocytosis, induction of microbial killing machinery such as the respiratory burst and production of cytokines and chemokines.

The TLRs comprise the best studied family of PRR. The TLR family is composed of thirteen (TLR1-TLR13) members, all responsible for the detection and signaling in response to different PAMPs. TLRs recognize non-self antigens either on the cell wall or the lumen of intracellular vesicles, such as the phagolysosome. TLRs can detect a wide range of PAMPs including lipids, lipoproteins, proteins, glycans and nucleic acids (Kumagai and Akira 2010; Takaoka and Shinohara 2008).

The RLRs and NLRs were characterized by their ability to detect nucleic acids on the cytosol of host cells (Figure 1.2). In addition to DNA, the NLRs can detect components of the bacterial cell wall, viruses and parasites (Kumagai and Akira 2010).

The RLRs RIG-I, melanoma differentiation associated gene 5 (MDA-5) and the negative regulator LGP2 were identified as RNA helicases involved in antiviral responses. These RLRs recognize viral RNA in the cytosol, during viral replication, and are capable of inducing the activation of IFNα. Recently a cytoplasmic DNA receptor was identified as a member of this family of PRR, DNA-dependent activator of (interferon regulatory factor) IRF (DAI). DAI can recognize Z-form DNA in the cytosol and induce the production of type I IFNs (Takeuchi and Akira 2010; Vilaysane and Muruve 2009).

On the other hand, the NLRs are cytoplasmic PRRs composed of a central nucleotide binding domain and C-terminal leucine-rich repeats. NLRs contain either a pyrin domain or a baculovirus inhibitor of apoptosis protein repeat domain and a CARD domain; therefore, they are not are not involved in transcriptional regulation of inflammation. Rather, NLRs are components of the inflammasome and are responsible for the regulation of caspase 1 activation. In conjunction with TLRs, the NOD receptors are capable of inducing type 1 IFN (Takaoka and Shinohara 2008).

The CLRs are membrane bound receptors characterized by the presence of carbohydrate binding domain. These CLRs are responsible for detecting sugars on bacteria and fungi (Figure 1.2). Upon their activation they can stimulate the production and subsequent secretion of proinflammatory cytokines.

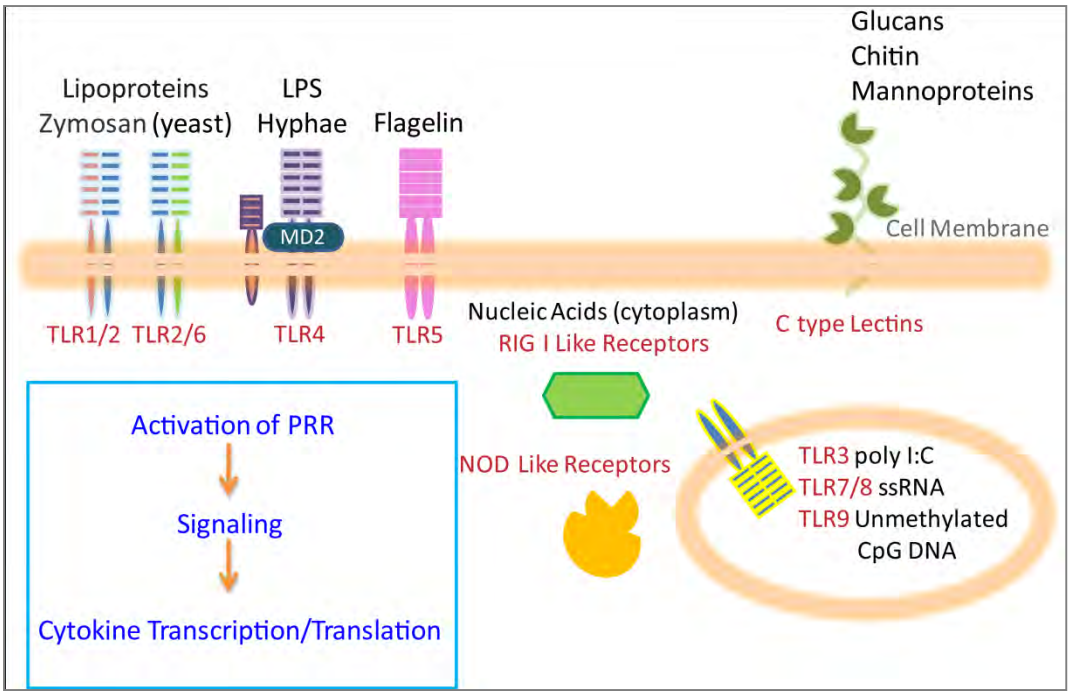
Glucans
Chitin
Mannoproteins
Lipoproteins
Zymosan (yeast)
LPS
Hyphae
Flagelin
MD2
Cell Membrane
TLR1/2
TLR2/6
TLR4
TLR5
Nucleic Acids (cytoplasm)
RIG I Like Receptors
C type Lectins
Activation of PRR
Signaling
Cytokine Transcription/Translation
NOD Like Receptors
TLR3 poly I:C
TLR7/8 ssRNA
TLR9 Unmethylated
CpG DNA

Figure 1.2. Overview of Pattern Recognition Receptors and their ligands

Overview of TLRs (TLRs and signaling)

TLRs are a conserved family of Type I transmembrane proteins, originally identified by their homology with *Drosophila melanogaster* Toll. Toll was first identified by its role in dorso-ventral development and essential anti-*Aspergillus* response in the fly (Lemaitre et al. 1996). TLRs are a family of glycoproteins composed of an extracellular ligand binding domain, a series of leucine-rich repeats and a cytoplasmic Toll/IL-1R (TIR) domain. Ten TLRs have been identified in humans and twelve have been identified in mice. These TLRs are expressed on a number of cell types, like DCs, macrophages, T cells, B cells and epithelial cells. In addition, TLRs can be found in cell surface or in intracellular compartments; where they act as PRRs.

TLRs play an extremely important role in the control of most pathogens by means of recognizing specific PAMPs (See table 1.1). In general, TLRs can be subdivided into groups, according to the PAMP they recognize: TLR1, TLR2, TLR4 and TLR6 recognize lipids, while TLR3, TLR7, TLR8 and TLR9 recognize nucleic acids (Figure 1.4). The natural ligands of TLRs are indispensable components of the pathogen, and because of this the pathogen will not mutate to lack the essential building blocks recognized by TLRs. However, due to the high rate of mutations in nucleic acids, TLRs detect pathogenic DNA or RNA due to small differences between the host and the pathogenic nucleic acids (Haas et al. 2008; Hacker et al. 2000; Kawai and Akira 2008).

Signaling for all TLRs, except TLR3 and partially TLR4, are dependent on the adaptor molecule MyD88. Interactions between the TLRs and the adaptor molecule takes

place via TIR domain interactions, leading to activation of signaling cascade which results in the production of proinflammatory cytokines and chemokines. However, MyD88 is not the only adaptor protein. TLR4 is able to signal via a MyD88-dependent and MyD88-independent mechanisms. TLR3 and MyD88-independent TLR4 signal via the TIR-domain-containing adapter-inducing interferon β (TRIF). Signaling via TRIF results in the activation of interferon regulatory factor 3 (IRF3) and IFNβ production.

TLRs	Ligands	Target Microbes
TLR1	Triacyl lipopeptides	*Mycobacterium*
TLR2	Peptidoglycans	Gram Positive Bacteria
	GPI-linked proteins	Trypanosomes
	Lipoproteins	*Mycobacterium*
	Zymosan	Yeast and other fungi
	Conidia and Hyphae	*Aspergillus* spp
	Phospolipomannan	*Candida albicans*
TLR3	Double Stranded RNA (dsRNA)	Viruses
TLR4	LPS	Gram Negative Bacteria
	F-protein	Respiratory Syncytial Virus
	Conidia	*Aspergillus fumigatus*
	Mannan and Glucoronoxylomannan	*Cryptococcus neoformans*
	O-linked mannose	*Candida albicans*
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides	*Mycobacterium*
	Zymosan	Fungi
TLR7	Single Stranded RNA (ssRNA)	Viruses
TLR8 (human)	ssRNA	Viruses
TLR9	Unmethylated CpG Dinucleotides	Bacteria
	DNA-containing immune complexes	Autoimmunity

	Viral infection	Viruses
	Unmethylated DNA	*Aspergillus fumigatus* *Candida albicans* *Cryptococcus neoformans*
TLR10	Unknown	Unknown
TLR11 (mouse)	Uropathogenic Bacteria	Bacteria
	Profilin-like molecule	*Toxoplasma gondii*
TLR12	Unknown	Unknown
TLR13	Unknown	Unknown

Table 1.1 Toll-like Receptors and their natural ligands

Toll-like Receptor 9

Endosomal TLRs play an important role in the recognition of nucleic acids such as single-stranded RNA (ssRNA), double-stranded RNA (dsRNA) and unmethylated CpG DNA. TLR9 is responsible for the detection of single-stranded DNA (ssDNA) rich in unmethylated CpG motifs (Figure 1.3A). From a historical perspective, the observation that bacterial DNA from *Mycobacterium bovis* was capable of promoting immunostimulatory and antitumor effects was a landmark (Bauer et al. 2008; Tokunaga et al. 1984). This was the first piece of evidence that DNA from pathogens could stimulate an inflammatory response in non-B and non-T cells, and that the response could be reproduced by stimulation with short single stranded oligonucleotides (ssODN) (Ishii and Akira 2006). The immunostimulatory ability of microbial DNA was found to be mediated by the presence of palindromic unmethylated CpG dinucleotides (Krieg 1995; Krieg et al. 1995). In contrast to microbial DNA, vertebrate DNA was shown to have reduced stimulatory activity due to methylation of the CpG DNA and the relatively low frequency of these motifs in the genome (Figure 1.3B). However, during certain autoimmune diseases humans are capable of reacting to their own DNA (Bauer et al. 2008).

TLR9 signaling requires translocation of the receptor from the endoplasmic reticulum into the endosome. Upon binding DNA, activation of TLR9 requires endosomal acidification and dimerization in order to initiate a response (Figure 1.3A). Upon recognition of CpG DNA, TLR9 undergoes conformational changes that results in

the interaction with MyD88. Subsequent activation of a signaling cascade results in the translocation of interferon regulatory factors 7 (IRF7), IRF3 and NF-κB (Latz et al. 2007a; Latz et al. 2004b). These transcription factors translocate to the nucleus were they are responsible for initiating the transcription of cytokines. To date, three classes of CpG have been identified: the A-class ODNs are potent inducers of IFNα in plasmacytoid DCs (pDCs) and poor inducers of B cell activation, the B- class ODNs are strong B-cell stimulation, and C-class ODNs are capable of inducing B cell activation and IFNα secretion. Expression of TLR9 differs between human and mouse cells. This difference in expression is most evident in DCs, which are responsible for linking innate and adaptive immune responses. Whereas TLR9 is expressed exclusively on pDCs in humans, TLR9 is expressed in both conventional DCs and pDCs (Ishii and Akira 2006).

The best studied role of TLR9 is during recognition of unmethylated viral ssDNA. During viral replication, ssDNA can be found in the cytoplasm and endosome of infected cells, where it can induce a TLR9-dependent response (Gilliet et al. 2008). Furthermore, bacterial unmethylated ssDNA is also capable of engaging TLR9 and inducing a response (Figure 1.3A). The immunostimulatory response of each bacterial species analyzed correlated with the number of CpG motifs found in the genome (Dalpke et al. 2006). Parroche *et al* showed that DNA from *Plasmodium falciparum* was capable of initiating a TLR9-dependent response; and that the response was enhanced when the DNA was associated with hemozoin (Parroche et al. 2007). In addition to the microbial DNA recognition, TLR9 has been shown to play a role in autoimmunity. TLR9 recognizes

unmethylated self DNA in Lupus patients. (Lamphier et al. 2006; Means et al. 2005; Tian et al. 2007).

While umethylated CpG DNA was immunostimulatory, TLR9 signaling can be inhibited by methylation of the DNA, inverted CG sequence or cleavage at the CG sequences (Duramad et al. 2005) (Figure 1.3B). The initial observation was made by Krieg *et al*; he observed that Adenovirus serotype 2 was non-stimulatory and was capable of inhibiting activation by bacterial DNA (Krieg 2006; Krieg et al. 1998). Furthermore, methylated bacterial CpG DNA motifs were shown to be inhibitory for TLR9 stimulation (Pisetsky and Reich 2000). Similar results were observed for human telomeric DNA repeats (Gursel et al. 2003).

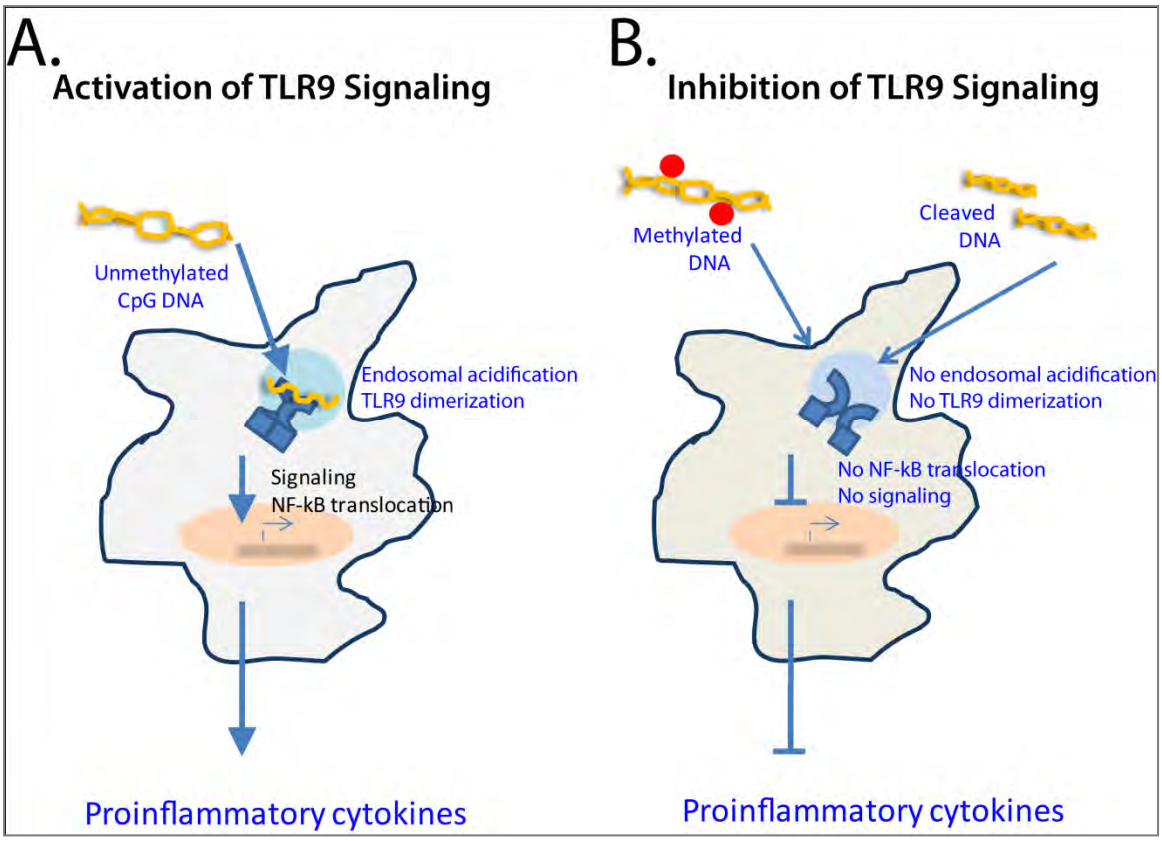
A.
Activation of TLR9 Signaling
Unmethylated
CpG DNA
Endosomal acidification
TLR9 dimerization
Signaling
NF-kB translocation
Proinflammatory cytokines
B.
Inhibition of TLR9 Signaling
Methylated
DNA
Cleaved
DNA
No endosomal acidification
No TLR9 dimerization
No NF-kB translocation
No signaling
Proinflammatory cytokines

Figure 1.3. Overview of TLR9 signaling

A. Unmethylated CpG DNA is taken into endosomal $TLR9^{+}$ compartment. Once in the endo-lysosome, the DNA binds to TLR9 which induces conformational chanes and dimerization of the receptor. Signaling cascade is subsequently activated, leading the nuclear translocation of NF-κB and transcription of IRF3/7. As a result, proinflammatory cytokines are secreted; including type I IFN and TNFα.

B. Upon methylation of the DNA, inversion of CG sequences or cleavage of CG, the DNA enters the $TLR9^{+}$ compartments. While TLR9 still binds the DNA, there are no comformational changes and the receptor will not dimerize. As a result, the signaling cascade is blocked or inhibited. Alternatively, inhibition of endo-lysosomal acidification will block dimerization of the receptor and activation of downstream signaling.

Host Immune Responses against *A. fumigatus*

Host responses to *A. fumigatus* begin with the physical barriers of the respiratory tract. Upon inhalation, the conidia are deposited in airway surfaces where they can be removed by the ciliary action of the respiratory epithelium. The respiratory epithelium works by trapping the conidia, recognizing the pathogen via soluble pattern recognition molecules and secreting microbicidal peptides responsible for killing the pathogen. As the size of the conidia is ideal for alveolar deposition, the inhaled spores can bypass these physical barriers and reach the alveoli (Figure 1.4)

Phagocytes are essential in the clearance of *A. fumigatus*. Once the conidia reach the broncheo-alveolar spaces, alveolar macrophages recognize and phagocytose *A. fumigatus*, resulting in killing of the spores. With a variety of soluble and surface bound PRR, alveolar macrophages secrete proinflammatory cytokines and restrict the initial spread of the conidia. Killing of the conidia by alveolar macrophages has been shown to occur via non-oxidative mechanisms.

In addition to alveolar macrophages, neutrophils and conventional DCs are also responsible for phagocytosing *A. fumigatus* conidia. Neutrophils have been shown to eliminate swollen conidia in the lungs after internalization by respiratory burst and degranulation. The host immune system has evolved to recognize the different morphologies of *A. fumigatus* (Park and Mehrad 2009). The conidia that are not eliminated during the host's first round of defense can germinate to become hyphae. Neutrophils are the primary cell type responsible for killing of the hyphae. Hyphal

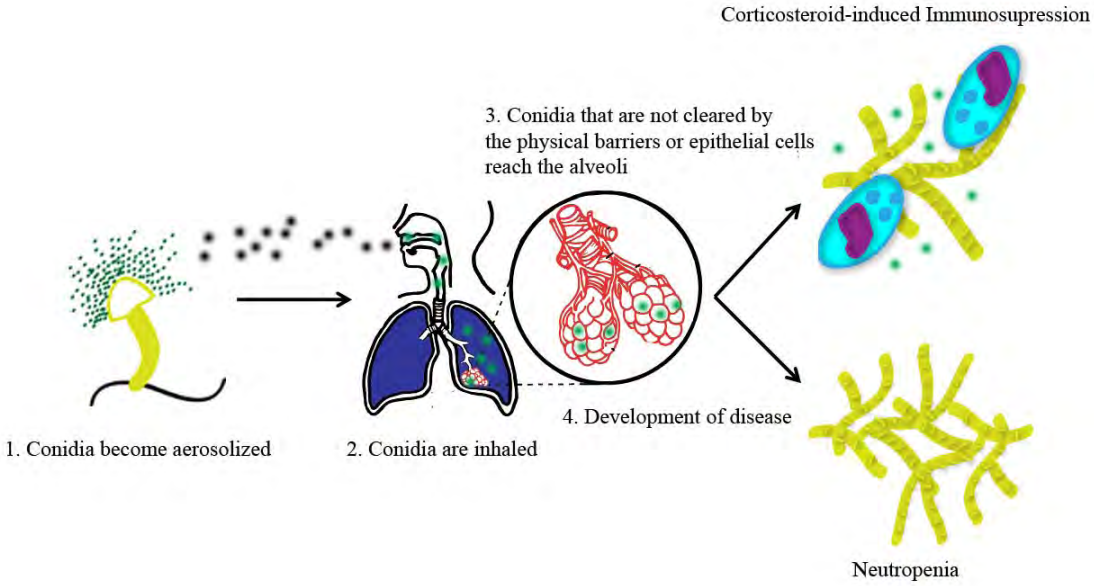
Corticosteroid-induced Immunosupression
3. Conidia that are not cleared by the physical barriers or epithelial cells reach the alveoli
1. Conidia become aerosolized
2. Conidia are inhaled
4. Development of disease
Neutropenia

Figure 1.4. Infectious Cycle of *A. fumigatus*.

Infectious cycle of *A. fumigatus* starts with the inhalation of aerosolized conidia by a susceptible host. Due to the perfect size of the conidia, these spores can reach bronchoalveolar spaces in the lungs. In an immunocompentent host, the infection is usually eliminated by professional phagocytes such as blood alveolar macrophages. In the immunocompromised host, conidia can reach the alveoli. In these hosts, due to the lack of immune recognition, *A. fumigatus* germinates into hyphae- the invasive form of the mold. Development of disease is highly dependent on the status on the immune system of the host. During neutropenia, the lack of PMNs allows for excessive hyphal growth and dissemination of the mold. Corticosteroid induced immunosuppression, on the other hand, allows for PMN recruitment and the exacerbated response causes tissue damage.

killing by neutrophils occurs via both oxidative and non-oxidative mechanisms (Figure 1.5). The importance of the oxidative killing mechanism by neutrophils has been well characterized in the context of chronic granulomous disease (CGD). This inherited disease results from mutations in genes encoding for components of the NADP (NADPH) oxidase complex, resulting in an impaired oxidative response and higher susceptibility to IA (Grimm et al. 2010). Although mechanisms of oxidative stress are important in the killing of *A. fumigatus* during infection, neutrophils are capable of eliminating hyphae by both oxidative and non-oxidative mechanisms (Park and Mehrad 2009).

DCs are the link between innate and adaptive immunity (Figure 1.5). Previous data showed that DCs could phagocytose conidia and transport them to the lymph nodes (Bozza et al. 2002). The recognition and phagocytosis of *A. fumigatus* by DCs occurs via distinct mechanisms, dependent on whether the DCs encounter conidia or hyphae. In addition, priming of T-lymphocytes at the lymph nodes was dependent on the morphology encountered; as confirmed by cytokine secretion (Bozza et al. 2002). Signaling of DCs, via cytokines, stimulates disparate Th1/Th2 T-cell responses that could be either beneficial or detrimental to the host (Bozza et al. 2009). Furthermore, activation of Th17 cells has also been suggested to play a role in the outcome of *A. fumigatus* infection.

Recognition of *A. fumigatus*, in all its morphologies, occurs via both soluble and cell associated receptors that recognize microbial structures. For example, binding and phagocytosis of resting conidia induce scant inflammatory responses. In a pulmonary

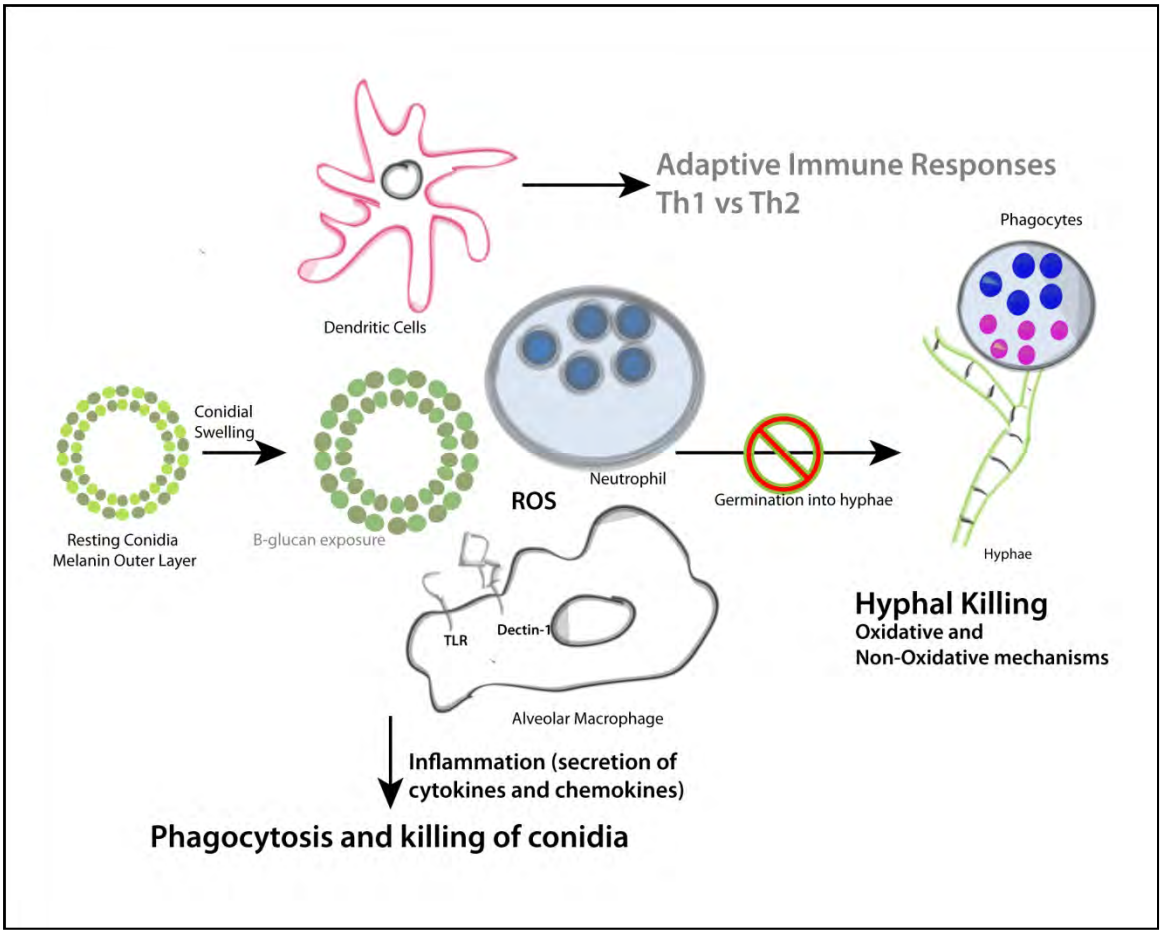
Adaptive Immune Responses
Th1 vs Th2
Dendritic Cells
Phagocytes
Conidial
Swelling
Resting Conidia
Melanin Outer Layer
B-glucan exposure
Neutrophil
ROS
Germination into hyphae
Hyphae
Hyphal Killing
Oxidative and
Non-Oxidative mechanisms
TLR
Dectin-1
Alveolar Macrophage
Inflammation (secretion of
cytokines and chemokines)
Phagocytosis and killing of conidia

Figure 1.5. Host Responses against *A. fumigatus*.

Inhaled resting conidia in the lungs are covered with a melanin outer layer which is secreted into the environment upon conidial swelling. Exposure of β-glucans in swollen conidia allows alveolar macrophages to detect the conidia by TLR2 and by Dectin-1. Upon detection by alveolar macrophages, an inflammatory response is initiated resulting in the phagocytosis and killing of the conidia in phagolysosomal compartments. Neutrophils are also recruited into the site of infection where the conidia are killed via oxidative and non-oxidative mechanisms. In most cases if the infection is controlled, the conidia will not germinate into hyphae. During disease, some of the conidia escape killing by professional phagocytes and germinate into hyphae. PMNs recognize and kill the hyphae via oxidative and non-oxidative mechanisms, as well as the generation of neutrophil extracellular traps (NETs) which inhibit fungal growth. In addition, DCs recognizes components of the mold and initiate an adaptive immune response. A dysregulation of the Th1/Th2 response will determine the outcome of disease. While a Th1 response is favorable for the outcome of the disease, a Th2 response will benefit the pathogen and may contribute to an unfavorable outcome of the disease. Activation of Th17 pathway, acting by negatively regulating the Th1 mediated immune resistance to *A. fumigatus*, resulting in an uncontrolled Th1 inflammatory response. In addition, Tregs have been shown to play a role in the early phase of infection by mediating an anti-inflammatory response.

infection model, mice were challenged with live and heat killed resting conidia. Results showed optimal $CD4^+$ T cells to live conidia but not heat-killed resting conidia. Exposure of β-glucan, particularly in the tips of *A. fumigatus* hyphae, is associated with increased secretion of tumor necrosis factor (TNFα) and CXCL2 by macrophages (Balloy et al. 2005; Meier et al. 2003; Steele et al. 2005). In addition, increased hyphal damage is mediated by neutrophils, resulting in an inflammatory response and host tissue damage.

Antifungal responses mediated by TLRs

TLRs play an important role in the control of most pathogens; therefore, it was expected that they played a role in the recognition and antifungal responses to *A. fumigatus*. Over the last decade the evidence that TLRs play a role in the antifungal response has increased significantly, particularly the role of TLR2 and TLR4. Studies with *A. fumigatus* and TLRs have yielded conflicting results, depending on the strain and infection model used.

Initial studies with human blood stimulated with *A. fumigatus* hyphae suggested that secretion of TNFα, IL-1β and IL-6 occur via a TLR4 dependent mechanism (Wang et al. 2001). Subsequent studies showed a reduction in TNFα secretion when mouse macrophages deficient in TLR2 and MyD88 were stimulated with different morphologies of *A. fumigatus* (Mambula et al. 2002). The same study showed that CD14 played a role in the antifungal response in human cells, but not in the mouse system (Mambula et al. 2002). Later studies (Meier et al. 2003; Netea et al. 2003) showed that both receptors, TLR2 and TLR4, were involved in the anti-*A. fumigatus* response. In one study,

peritoneal macrophages from TLR4$^{-/-}$ ScCr mice showed decreased TNFα, IL1α and IL1β secretion when stimulated with conidia but not hyphae, as compared to the wild-type controls. In addition, a reduction in cytokine secretion was observed upon stimulation of TLR2$^{-/-}$ macrophages, with either conidia or hyphae (Netea et al. 2003). Meier *et al* showed TLR2 and TLR4 mediated NF-κB translocation upon stimulation with *A. fumigatus* (Meier et al. 2003). Consistent with the previous findings, *A. fumigatus* antigens induce secretion of IL-12 via TLR2 and IL-6 via TLR4 in mouse BMDCs (Braedel et al. 2004). Stimulation of human monocyte-derived dendritic cells with *A. fumigatus* results in CD86 expression and upregulation of MHC and co-stimulatory proteins (Braedel et al. 2004). Furthermore, PMN mediated antifungal activity occurs via two distinct oxidative pathways that involve TLR2 or TLR4 (Bellocchio et al. 2004b).

The role of TLRs has also been shown *in vivo*. Studies with mice deficient in TLR2, TLR4, TLR9, MyD88 and IL1-R1 and wild type mice, showed decreased survival for MyD88$^{-/-}$ and TLR4$^{-/-}$ but increased survival was observed for the remaining groups (Bellocchio et al. 2004a). Studies with immunocompromised TLR2$^{-/-}$ mice challenged with *A. fumigatus* showed decreased survival, as well as cytokine secretion, when compared to the TLR2$^{+/+}$ counterparts (Balloy et al. 2005).

In addition to TLR 2 and TLR4, other TLRs have been shown to also play a role in the detection of *A. fumigatus* PAMPs. One study showed that TLR9$^{-/-}$ mice infected with *A. fumigatus* conidia were more susceptible to the infection than the wild type or MyD88 deficient counterparts. Furthermore, the fungal burden in TLR9 deficient mice was

higher than the fungal burden in the wild type mice (Bellocchio et al. 2004a; Bellocchio et al. 2004b). In addition, studies in mouse infection models showed delayed mortality when $TLR9^{-/-}$ mice, depleted of neutrophils, were challenged with *A. fumigatus* when compared to $TLR9^{+/+}$ immune deficient counterparts (Ramaprakash et al. 2009).

The importance of TLRs has also been shown during *A. fumigatus* disease. Polymorphisms in different TLRs have been shown to increase susceptibility to infection in an array of immunodeficiencies (Pamer 2008). Studies performed with a cohort of 336 hematopoietic-cell transplant patients showed that polymorphisms in TLR4 can increase the risk of IA (Bochud et al. 2008). Furthermore, Bochud *et al* showed that the haplotype was present in two single nucleotide polymorphisms (SNPs) that resulted in a strong linkage disequilibrium that affected TLR4 function. These result were confirmed by comparing 103 IA patients to control patients (Bochud et al. 2008). Another study examined the association between SNP in TLR1, TLR4 and TLR6 genes and development of IA from 22 patients with IA and 105 unaffected hematopoietic stem cell transplant patients (Kesh et al. 2005). Kesh *et al* found that the mutations in TLR1 239G>C (Arg80>Thr) and TLR6 745C>T (Ser249>Pro) were associated with IA (Kesh et al. 2005). The role of TLRs during infection was also assessed for susceptibility to non-invasive forms of pulmonary aspergillosis (Carvalho et al. 2008a). Carvalho *et al* found an association between the TLR4 mutation Asp299Gly and chronic cavitary pulmonary aspergillosis. This group also found that ABPA was associated with a mutation on TLR9 (T-1237C) (Carvalho et al. 2008a).

Other Aspects of Innate Immune System involved in anti-*A. fumigatus* responses

In addition to the TLR responses to *A. fumigatus*, other PRRs have also been shown to be involved in the recognition and antifungal responses to this opportunistic pathogen. Recognition of *A. fumigatus* cell wall components exposed at different growth stages has become an important topic of research, particularly in the search for vaccines.

Other PRR (C-type Lectins, Cytosolic PRR)

PRR are widely distributed, and can be found bound to cell membranes or soluble (Figure 1.6). In addition to TLRs, other cytosolic PRRs have been identified. Among these non-TLR s receptors are the RLR and the NLR. The RLRs belong to the family of RNA helicases that can detect viral RNA in the cytoplasm of infected cells and induce a type I IFN response. To date, the RLRs have only been shown to play a role in the antiviral response; however, it is possible that pathogenic DNA and RNA from other pathogens could induce an RLR-dependent response.

The NLR constitute a large family of cytosolic PRR characterized by the presence of N-terminal protein interaction domain, a central nucleotide-biding domain and a C-terminal LRR. The NLRs and the inflammasomes are responsible for the activation of inflammatory processes including pyroptosis (Fernandes-Alnemri et al. 2007; Suzuki and Nunez 2008). *A. fumigatus* was recently shown to activate the NLRP3 inflammasome (Said-Sadier et al. 2010) (Figure 1.6). Although no IL-1β could be detected upon conidia challenge, a human monocyte cell line secreted the cytokine in response to hyphal

fragments. The ability of *A. fumigatus* hyphae to induce the NLRP3 inflammasome is dependent on production of reactive oxygen species (ROS) and pro-IL-1β cleavage was shown to be dependent on the activation of Syk tyrosine kinase, which has been identified to be downstream from dectin-1 (Said-Sadier et al. 2010).

Another group of non-TLR PRRs are the CLRs. This superfamily of proteins is characterized by the presence of one or more C-type Lectin-like domains (CTDLs). Most proteins in this family are type II transmembrane proteins, with two exceptions- Mannose Receptor and DEC-205. C-type lectins play essential roles in cell-cell adhesion, detection and recognition of pathogens, and apoptosis.

Several CLRs play roles in the clearance of fungal pathogens. As with TLRs, the distribution of these receptors is varied among different cell types. During the antifungal response, the CLRs bind exposed carbohydrates on the fungal cell wall (Figure 1.6). Among the CLRs, Mannose Receptor (MR), dectin-1, dectin-2, dendritic cell specific ICAM-3-grabbing non-integrin (DC-SIGN) and the collectins have been shown to be involved in the antifungal response.

One of the best characterized CLRs in the anti-*Aspergillus* response is Dectin-1. This CLR is a glycosylated type II transmembrane receptor, that contains a carbohydrate binding domain and a ITAM-like motif (Reid et al. 2009). Dectin-1 is the major receptor for recognition of β-1,3-glucans, a major component of the *A. fumigatus* cell wall. Dectin-1 is localized in the cell membrane of various cell types, such as: neutrophils, monocytes and dendritic cells. Upon engagement of the receptor, Dectin-1 can induce

the secretion of proinflammatory cytokines such as TNFα, IL-2, IL-10, IL-12 and MIP2; induce ligand phagocytosis; and induce a respiratory burst. Although signaling via Dectin-1 can induce the secretion of cytokines independently of the TLRs; however, they may cooperate to induce a specific response to the pathogen (Brown 2006; Goodridge et al. 2009).

Exposure of β-1,3-glucans during *A. fumigatus* conidial germination leads to activation of dectin-1 (Hohl et al. 2005). Hohl *et al* showed that dectin-1 is internalized into the phagolysosome of alveolar macrophages that have ingested conidia where it can stimulate the secretion of TNFα and MIP2 upon β-glucan exposure. The response was shown to be specific to dectin-1, as blocking the receptor with antibody resulted in decreased TNFα/MIP2 (Hohl et al. 2005). Subsequently, it was shown that dectin-1 is responsible for recognizing swollen conidia and small hyphae ; with a responses that correlated with the level of exposed β-glucan (Gersuk et al. 2006; Steele et al. 2005). Further studies have shown that dectin-1 and TLR2 can cooperate to augment the anti-*Aspergillus* response (Gersuk et al. 2006; Luther et al. 2007).

Other CLRs have also been shown to play a role in the responses to *A. fumigatus*. DC-SIGN is one of these CLRs and is primarily expressed on DCs (van Kooyk and Geijtenbeek 2003). DC-SIGN was shown to bind conidia using stably transfected monocytes. In addition, the binding and internalization of *A. fumigatus* conidia by DC-SIGN was confirmed by blocking the receptor with anti-DC-SIGN antibody (Serrano-Gomez et al. 2004). Another member of the CLR family, MR, is expressed primarily on

macrophages and DCs (Willment and Brown 2008). Recent data suggests a role for MR in the recognition of *A. fumigatus* galactomannans, which is exposed after the melanin outer layer on conidia is shed (Chai et al. 2009). Expression of other CLRs, such as dectin-2 is more limited to DCs (Willment and Brown 2008). Dectin-2 signals by association with FcRγ, and subsequent phosphorylation that leads to activation of NFκB, production of TNFα and phagocytosis (Hollmig et al. 2009). Dectin-2 recognizes high mannose structures, similar to those found in hyphal forms of fungi. Recently it was shown that Dectin-2 plays a role in the recognition of *A. fumigatus* (Barrett et al. 2009).

Role of complement and antibody in responses against *A. fumigatus*

Inhaled conidia quickly encounter the host complement system in addition to phagocytic cells (Figure 1.6). Complement activation can take place via three pathways: the alternate pathway, the lectin pathway (MBL) and the classical pathway (CP). Even though target discrimination is different among these pathways, their activation leads to C3b binding to invading cell for opsonization and phagocyte killing. Activation of the alternative pathway is mediated by reverse recognition; it recognizes markers on host cells and activates on cells lacking these markers. For example, Factor H is the first host pattern recognition molecule, capable of recognizing host-associated molecular patterns such as polyanions present on host cells but not on pathogens. (Pangburn et al. 2008).

Activation of the alternative complement pathway begins with the cleavage of C3. Subsequently, progression of the cascade leads to generation of C5 convertase and the formation of complement complexes. The formation of these complexes forms pores on the target surfaces, leading to pathogen killing. These complexes have been shown to play a role in bacterial clearance; however, due to protection impaired by the melanin outer layer of the conidia these complexes play a minor role in the antifungal response(Sturtevant and Latge 1992a; Sturtevant and Latge 1992b).

A. fumigatus, like other pathogenic fungi, can induce cleavage of C3, deplete hemolytic complement activity and conidia can bind C3b (Figure 1.6). Initiation of the alternative complement pathway by *Aspergillus* spp is dependent on the morphology encountered. Activation of the pathway is slow with resting conidia, which correlates with the low immunogenicity of the outer layer (Kozel 1996). Deposition on C3b on conidial cell wall has been shown to induce phagocytosis in macrophages (Bouchara et al. 1994). One study with 29 strains of *A. fumigatus* showed that conidia can only activate the alternative complement pathway and that activation of MBL by the conidia can lead to cleavage of C3 bypassing C2 (Dumestre-Perard et al. 2008). Furthermore, it was shown that Factor H, a central component of the alternative pathway, binds to conidia but not hyphae (Behnsen et al. 2010).

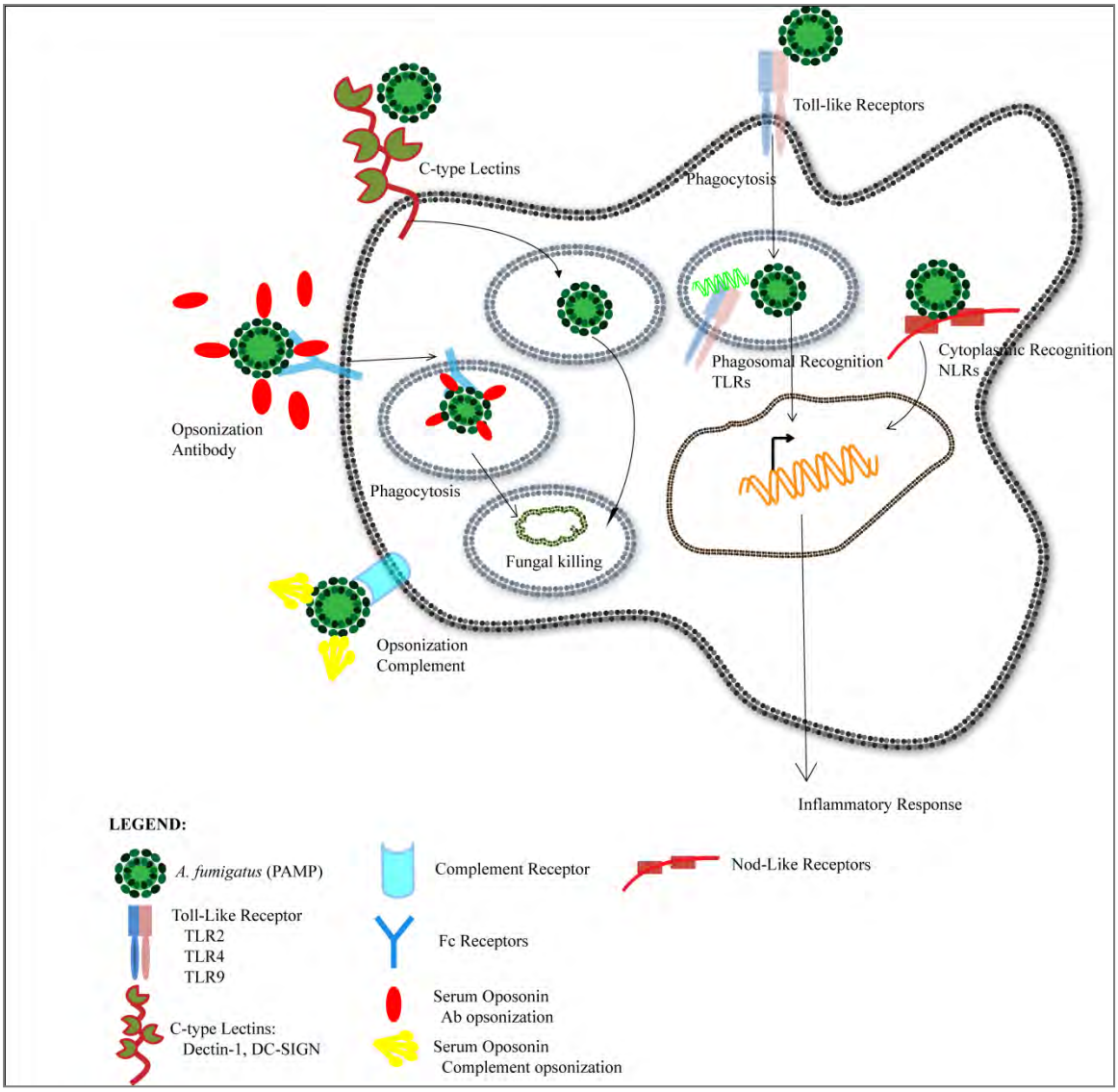
Toll-like Receptors
C-type Lectins
Phagocytosis
Phagosomal Recognition
TLRs
Cytoplasmic Recognition
NLRs
Opsonization
Antibody
Phagocytosis
Fungal killing
Opsonization
Complement
Inflammatory Response
LEGEND:
A. fumigatus (PAMP)
Toll-Like Receptor
TLR2
TLR4
TLR9
C-type Lectins:
Dectin-1, DC-SIGN
Complement Receptor
Fc Receptors
Serum Oposonin
Ab opsonization
Serum Oposonin
Complement opsonization
Nod-Like Receptors

Figure 1.6. Innate Immune Recognition of *A. fumigatus*

Thesis overview

Phagocytic defenses have been shown to be critical for effective host defenses against *Aspergillus fumigatus*. One study (Bellocchio et al. 2004a) found that mice deficient in TLR9 challenged with *A. fumigatus* lived longer than wild type mice. Furthermore, polymorphisms in the TLR9 have been suggested to play a role in the development of aspergillosis (Carvalho et al. 2008b; Lanciotti et al. 2007). However, whether *A. fumigatus* DNA could induce aTLR9-dependent immune response remains to be elucidated.

Our work shows that human plasmacytoid dendritic cells secrete pro-inflammatory cytokines in response to fungal DNA (Ramirez-Ortiz et al. 2008). In addition, it has been suggested (Romani et al. 2004; Romani et al. 2006; Romani et al. 2009) that *A. fumigatus* conidia can stimulate an IDO-dependent TLR9 response in both human and mouse pDCs. Most of the previous works on fungal infections have focused on the responses by conventional dendritic cells (Ibrahim-Granet et al. 2003; Latge 1999). Furthermore, pDCs are high producers of type I interferon and can be found circulating in the blood, where they constitute less than 1% of the total mononuclear cells. Therefore, we wanted to determine whether pDCs play a role in the antifungal response to *Aspergillus fumigatus*.

It has been shown that the type of immune response to fungal pathogens is dependent on the fungal morphotype encountered (Bozza et al. 2002). Previous data (Bozza et al. 2002; Ibrahim-Granet et al. 2003; Paris et al. 1997) have shown that *A.*

fumigatus conidia are phagocytosed by macrophages and neutrophils. Furthermore, one study suggested that murine pulmonary DCs internalize both conidia and hyphae through distinct phagocytic mechanisms and receptors. Our published data show that immature pDCs secrete cytokines in response to fungal DNA suggesting that these cells may be involved in the immune response to *A. fumigatus* (Ramirez-Ortiz et al. 2008) in both the mouse and human system. In the mouse, TLR9 is expressed in cDCs and pDCs, as well as macrophages. In the human, macrophages and cDCs lack TLR9 and it is mainly expressed in pDCs and B cells. These cells are a subset of DCs characterized by the lack of TLR4 and the presence of TLR7/9 (Kadowaki et al. 2001; Kool et al. 2009). Furthermore, data from clinical studies (Carvalho et al. 2008a) on immunocompromised patients suffering from invasive aspergillosis show that polymorphisms in the TRL9 gene increases susceptibility to infection with the mold (Carvalho et al. 2009; Carvalho et al. 2008a). Human and mouse pDCs have been shown to be important during the antiviral response (Takeuchi and Akira 2009); however, whether human pDCs play a role in antifungal host defenses remains to be elucidated.

During the germination process, morphological changes in the dimorphic fungi, *A. fumigatus*, result in the exposure of different sugars and proteins in the cell wall (Bernard and Latge 2001; Gastebois et al. 2009). During an infection, fungal conidia germinate into hyphae. In a healthy individual, the infection is cleared before germination of the conidia by alveolar macrophages and neutrophils. However, in an immunocompromised host, the conidia may germinate to hyphae and immune cells are exposed to the stimuli for a longer period of time. Very little is known about the role of

human dendritic cells in the antifungal response to *A. fumigatus*. Furthermore, whether mature pDCs are involved in the process remains to be elucidated. Since mature pDCs acquire characteristics of conventional DCs, and they become phagocytic (Drenou et al. 2005) it is possible that they may be involved in the clearance of *Aspergillus* resting or swollen conidia.

Aim 1: To determine whether fungal DNA contains unmethylated CpG motifs capable of stimulating TLR9; thereby potentially influencing the host response to fungal challenge

Aim 2: To determine whether *Aspergillus fumigatus* hyphae interact with human plasmacytoid dendritic cells; thereby, potentially contributing to the antifungal immune response

Aim 3: To determine whether mature pDCs recognize and induce a response against the different morphologies of *A. fumigatus*; thereby contributing to the anti-*A. fumigatus* response.

Preface to Chapter II

This Chapter has been published in Infection and Immunity

Zaida G. Ramirez-Ortiz, Charles A. Specht, Jennifer P. Wang, Chrono K. Lee, Daniella C. Bartholomeu, Ricardo T. Gazzinelli, and Stuart M. Levitz. *Toll-Like Receptor 9-Dependent Immune Activation by Unmethylated CpG Motifs in Aspergillus fumigatus DNA*. (2008)

Chrono K. Lee performed BMDC isolations

Ricardo T. Gazzinelli and Daniella C. Bartolomeu performed Genome Wide Scanning for CpG motifs

Jennifer P. Wang helped with pDC isolations and experimental design

Zaida G. Ramirez-Ortiz performed remaining experiments

Zaida G. Ramirez-Ortiz, Charles A. Specht and Stuart M. Levitz designed the experiments and wrote the manuscript

Chapter II

Toll-Like Receptor 9-Dependent Immune Activation by Unmethylated CpG Motifs in *Aspergillus fumigatus* DNA

Abstract

Phagocytic defenses are critical for effective host defenses against the opportunistic fungal pathogen *Aspergillus fumigatus*. Previous studies found that following challenge with *A. fumigatus*, Toll-Like Receptor 9 (TLR9) knockout mice survived longer than wild type (WT) mice. However, the mechanism responsible was not defined. Here we demonstrate that *A. fumigatus* contains unmethylated CpG sequences, the natural ligand for TLR9. *A. fumigatus* DNA and synthetic CpG-rich oligodeoxynucleotides (ODNs) containing sequences found in the *A. fumigatus* genome potently stimulated the production of pro-inflammatory cytokines in mouse bone marrow-derived dendritic cells (BMDCs) and human plasmacytoid dendritic cells. The response was decreased when the fungal DNA was treated with a CpG methylase or CpG-specific endonucleases. A role for TLR9 was demonstrated as cytokine production was abolished in BMDCs from TLR9-deficient mice. Moreover, transfection of HEK293 cells with human TLR9 conferred responsiveness to synthetic CpG-rich ODNs containing sequences found in *A. fumigatus* DNA. Taken together, these data demonstrate that TLR9 detects *A. fumigatus* DNA resulting in the secretion of pro-inflammatory cytokines, which may contribute to the immune response to the pathogen.

Introduction

Aspergillus fumigatus is a widely distributed opportunistic fungal pathogen. Exposure generally occurs when airborne spores (conidia) are inhaled into the lungs or sinuses (Shoham and Levitz 2005). While inhalation of conidia rarely causes disease in the normal host, immunocompromised persons are prone to develop invasive aspergillosis (Hohl and Feldmesser 2007b). Despite new additions to the antifungal armamentarium, mortality rates in those with established disease exceed 50 percent (Hohl and Feldmesser 2007b). Clinical and experimental studies have demonstrated that the innate immune responses of phagocytes are essential for effective host defenses against *A. fumigatus* (Shoham and Levitz 2005; Walsh et al. 2005).

A critical component of innate defenses is the ability of phagocytes to recognize structurally unrelated and evolutionarily conserved microbial constituents referred to as "pathogen- (or pattern-) associated molecular patterns" (PAMPs) (Akira et al. 2006). Among the receptors that recognize PAMPs are the mammalian toll-like receptors (TLRs), a family of at least 12 members that initiate signaling pathways through a series of adaptor proteins, including MyD88, TIRAP/MAL, TRIF, and TRAM. This results in activation of downstream signaling pathways and the subsequent production of inflammatory cytokines and the initiation of adaptive immune responses. Some TLRs, including TLR1, TLR2, TLR4, TLR5 and TLR6, are primarily expressed on the plasma membrane where they recognize specific molecules on the surface of microbes, including fungi (Akira et al. 2006; Levitz 2004). Others, including TLR3, TLR7, TLR8 and TLR9,

are found in intracellular compartments and signaling responses require ligand internalization (Akira et al. 2006; Latz et al. 2004a).

TLR9 is activated by DNA rich in unmethylated CpG motifs, whereas DNA lacking such sequences is either inert or inhibitory (Hemmi 2000; Latz et al. 2007b). Studies using CpG-containing oligodeoxynucleotides (ODN) have defined sequences that stimulate immune responses(Krieg 2006). The optimal CpG motif appears to be GACGTT for mice and GTCGTT for humans. The number of CpG motifs and the spacing of the motifs also influence immunostimulatory capacity. A-class CpG-containing ODNs strongly stimulate plasmacytoid DC (pDC) IFNα responses but poorly induce B cell proliferation(Krieg 2006). In contrast, ODNs of the B-class are weak inducers of pDC IFNα but strong inducers of B cell proliferation. C-class ODNs have intermediate effects.

In the human, TLR9 is found mainly on pDC and B cells. In contrast, the cellular distribution of TLR9 in the mouse is much broader and includes myeloid DC, pDC, monocytes, macrophages, and B cells. Clinical trials of CpG-DNA, used as adjuvants in vaccines and as part of treatment for infectious diseases and neoplasms, are ongoing (Krieg 2007). Originally, TLR9 was thought to be specific for bacterial and viral DNA (both of which are rich in unmethylated CpG motifs). However, recent studies have suggested a role for TLR9 in the recognition of eukaryotic DNA. Mammalian DNA, when complexed with anti-DNA antibodies, is a potent self-antigen for TLR9 and may play a role in promoting systemic lupus erythematosus and other autoimmune diseases

(Barrat et al. 2005). Moreover, TLR9-dependent stimulation of murine DC by malarial DNA has been demonstrated (Parroche et al. 2007).

A possible role for TLR9 in host defenses against fungal infections was suggested by Bellocchio et al. (Bellocchio et al. 2004a; Bellocchio et al. 2004b). TLR9$^{-/-}$ mice paradoxically survived longer than wild-type mice following challenge with *Candida albicans* hyphae and *A. fumigatus* conidia. Compared with wild-type mice, the TLR9$^{-/-}$ mice had an organ fungal burden that was significantly lower and a shift in Th1/Th2 reactivity in favor of Th2 cells. Moreover, the TLR9$^{-/-}$ mice infected with *A. fumigatus* had a markedly reduced inflammatory response in the lungs. These data led us to hypothesize that fungal DNA contains unmethylated CpG motifs capable of stimulating TLR9 and thereby influencing the host response to fungal challenge. Here we demonstrate that DNA obtained from *A. fumigatus* stimulates TLR9-dependent responses in human and murine cells.

Results

A. fumigatus contains unmethylated CpG DNA.

We sought to determine if genomic DNA extracted from *A. fumigatus* contained unmethylated CpG motifs. DNA was treated with *HpaII* and *MspI*. These two endonucleases cleave DNA containing the sequence 5'…CCGG…3'. However, they differ in that *HpaII* only cleaves unmethylated CpG sequences whereas *MspI* cleaves both methylated and unmethylated CpG sequences. Both *HpaII* and *MspI* cleaved *A. fumigatus* DNA (Figure 2.1). To demonstrate the specificity of the cleavage reaction, the DNA was treated with the CpG methyltransferase, *M.SssI*, prior to restriction enzyme digest. Methylation abolished the capacity of *HpaII* but not *MspI* to cleave the DNA. These results demonstrate that *A. fumigatus* DNA contains unmethylated CpG sequences.

A. fumigatus induces TNFα and IL-12p70 production in mouse BMDCs.

In order to determine if *A. fumigatus* DNA was immunostimulatory, we measured TNFα and IL-12p70 release following incubation of the DNA with BMDCs. Cells treated with the fungal DNA potently stimulated production of both cytokines, with concentrations observed similar to that seen following stimulation with *E. coli* DNA and the synthetic ODN, CpG 1826 (Figure 2.2). However, when the cells were treated with enzymatically methylated *A. fumigatus* DNA, the cytokine response was nearly completely abolished. Similarly, treatment of the DNA with the CpG-specific endonucleases, *HpaII* and *MspI*, potently reduced cytokine production.

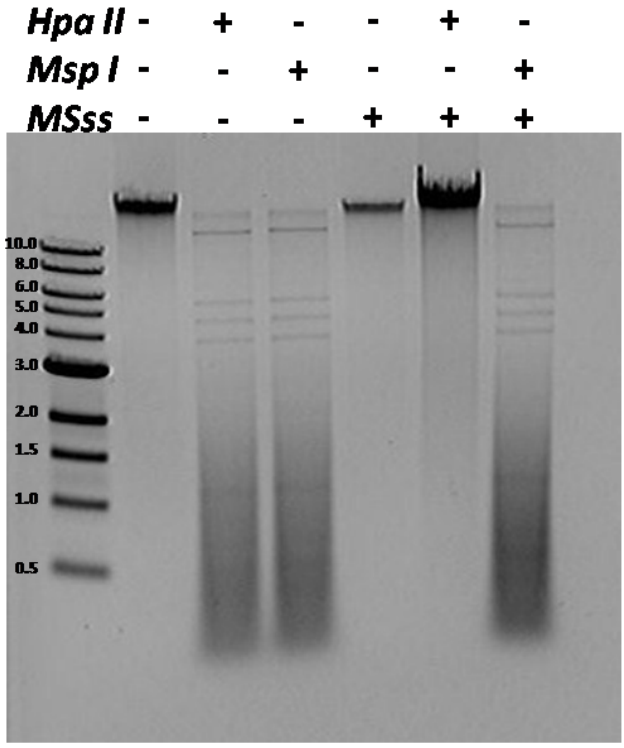
Hpa II - + - - + -
Msp I - - + - - +
MSss - - - + + +
10.0
8.0
6.0
5.0
4.0
3.0
2.0
1.5
1.0
0.5

Figure 2.1: *A. fumigatus* DNA contains unmethylated CpG sequences.

Genomic DNA was isolated from *A. fumigatus* and left untreated or treated with the indicated enzymes. "+" indicates treatment, "-" indicates absence of treatment. The digest was then analyzed by 1% Agarose gel electrophoresis with ethidium bromide-staining. Left lane shows a DNA ladder with numbers indicating the size of the DNA, in kB. Figure is representative of 12 individual experiments, each with similar results

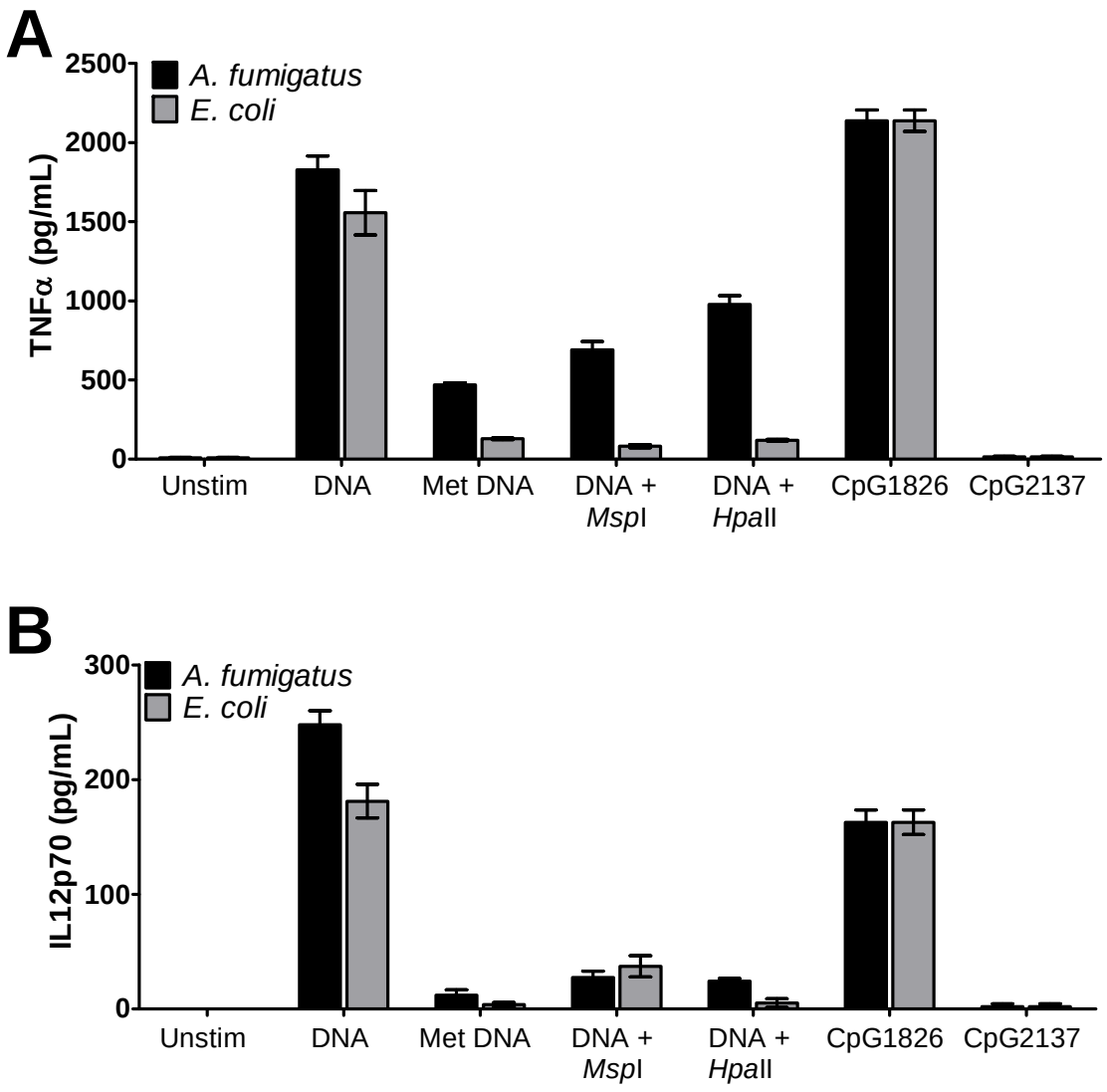
A
2500
2000
1500
1000
500
0
TNFα (pg/mL)
A. fumigatus
E. coli
Unstim
DNA
Met DNA
DNA + MspI
DNA + HpaII
CpG1826
CpG2137
B
300
200
100
0
IL12p70 (pg/mL)
A. fumigatus
E. coli
Unstim
DNA
Met DNA
DNA + MspI
DNA + HpaII
CpG1826
CpG2137

Figure 2.2: Stimulation of murine BMDC by *A. fumigatus* DNA.

BMDCs were stimulated by transfecting 2.5 μg/mL of *A. fumigatus* DNA or *E. coli* DNA that was left untreated (DNA), or treated with the CpG methyltrasferase, *M.SssI* (Meth DNA), *MspI* or *HpaII*. CpG 1826 at 3 μg/mL served as a positive control while unstimulated cells and GpC 2137 were negative controls. After 24 hours, supernatants were collected and TNFα (A) and IL-12p70 (B) concentrations were determined by ELISA. Data are means ± SE of 5 individual experiments. $p<0.001$ by one-way ANOVA with Bonferroni post test comparing TNFα and IL-12p70 stimulated by untreated *A. fumigatus* or *E. coli* DNA compared with any treatment.

A. fumigatus DNA stimulates TNFα secretion in a TLR9-dependent manner.

Given the role of TLR9 as an intracellular sensor of unmethylated CpG-rich DNA, we next compared cytokine responses of BMDC derived from WT and TLR9$^{-/-}$ mice following stimulation with *A. fumigatus* DNA. In the BMDC from WT mice, native DNA stimulated TNFα release in a dose-dependent manner (Figure 2.3). Methylated DNA also induced TNFα production, although lower levels were detected compared with untreated DNA. However, neither untreated nor methylated *A. fumigatus* stimulated TNFα release from TLR9$^{-/-}$ BMDC.

DNA from _A. fumigatus_ stimulates the production of Type I IFN in human pDC. Immunostimulatory CpG-rich motifs differ between mice and humans (Hochrein and Wagner 2004). Therefore, we next determined whether *A. fumigatus* DNA would also stimulate human pDC, a cell type which highly expresses TLR9 (Uematsu and Akira 2007). *A. fumigatus* DNA stimulated IFNα production in human pDC obtained from 7 different donors (Figure 2.4a) in a dose-dependent fashion (Figure 2.4b). As was observed with murine DC, cytokine production was significantly decreased if the DNA was methylated.

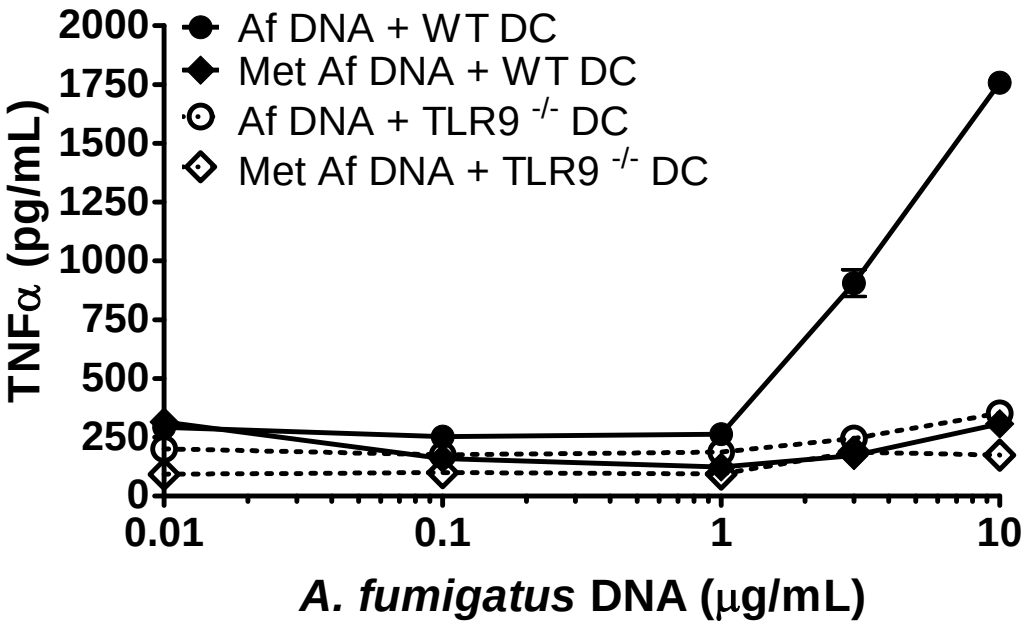
2000
1750
1500
1250
1000
750
500
250
0
TNFα (pg/mL)
Af DNA + WT DC
Met Af DNA + WT DC
Af DNA + TLR9 -/- DC
Met Af DNA + TLR9 -/- DC
0.01
0.1
1
10
A. fumigatus DNA (μg/mL)

Figure 2.3: *A. fumigatus* DNA stimulates TNFα secretion in a TLR9-dependent manner.

BMDCs from WT and TLR9$^{-/-}$ mice were transfected with the indicated concentrations of *A. fumigatus* DNA that was left untreated (Af DNA), or treated with the CpG methyltrasferase, *M.SssI* (met Af DNA) and incubated at 37° C. After 24 hours, supernatants were collected and TNFα concentrations were determined by ELISA. Concentrations of TNFα released from unstimulated WT and TLR9$^{-/-}$ BMDC were 225 pg/mL ± 40 and 134 pg/mL ±18, respectively. Data are means ± SE of a representative (out of four) experiment performed in triplicate. $p<0.001$ by one-way ANOVA with Bonferroni post-test comparing TNFα stimulated by Af DNA in WT BMDC with any other group at DNA concentrations of 3 and 10 μg/mL.

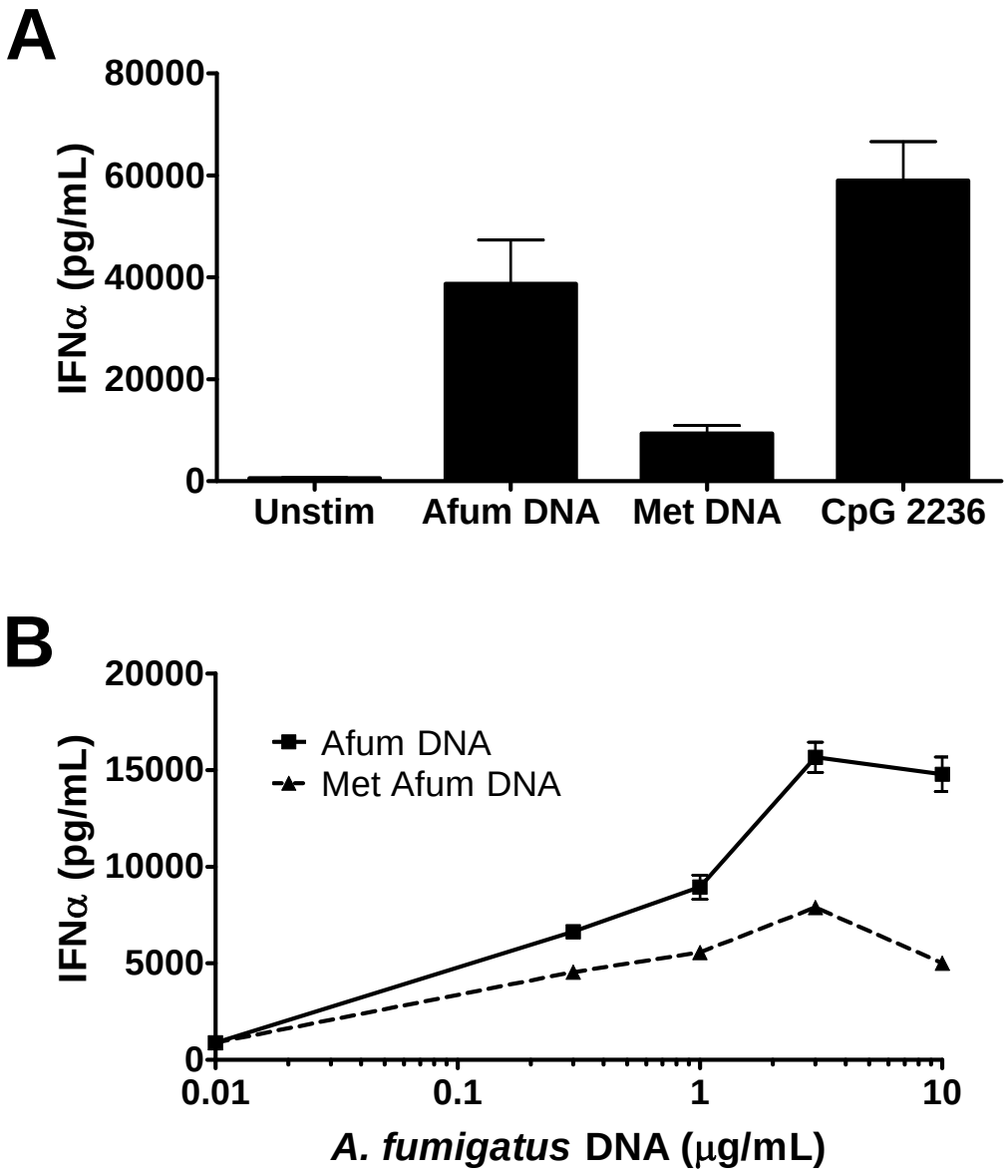
A
80000
60000
40000
20000
0
IFNα (pg/mL)
Unstim
Afum DNA
Met DNA
CpG 2236
B
20000
15000
10000
5000
0
IFNα (pg/mL)
Afum DNA
Met Afum DNA
0.01
0.1
1
10
A. fumigatus DNA (μg/mL)

Figure 2.4: Stimulation of human pDC by *A. fumigatus* DNA.

Human pDCs were isolated from PBMC by positive selection using CD304+ magnetic beads. (A) pDCs were stimulated with 10 μg of *A. fumigatus* DNA which was either left untreated or methylated with the CpG methyltrasferase, *M.SssI*. Unstimulated and CpG 2236 (10 μg/ml)-stimulated pDC served as negative and positive controls, respectively. After 18-24 hours, supernatants were collected and analyzed by ELISA for IFNα. Results are shown as means ± SE of seven individual donors. $p<0.001$ comparing untreated and methylated DNA by one-way ANOVA with Bonferroni post-test. (B) As in (A), except a dose response curve was performed comparing untreated and methylated *A. fumigatus* DNA. Data are means ± SE of a representative (out of three) experiment performed in triplicate. $p<0.001$ comparing untreated and methylated DNA at concentrations 1, 3 and 10 μg/mL by two way ANOVA with Bonferroni post test.

ODNs containing CpG-rich motifs present in the *A. fumigatus* genome stimulate signaling and cytokine responses in a TLR9-dependent fashion.

Next, we took advantage of the nearly complete (98.0%) deciphering of the *A. fumigatus* genome to determine the GC content of the genome and to search for putative immunostimulatory CpG-rich motifs in *A. fumigatus* DNA. The GC content of the sequenced portion of the *A. fumigatus* genome was determined to be 49.8%. Moreover, the percentage of CpG dinucleotides sequences was 5.35%. Using the conservative search criteria described in the Methods section, 23 and 87 potential murine and human immunostimulatory motifs were identified, respectively (Table 2.1). A sample of these ODNs was then synthesized on a phosphothiorate background and tested for their capacity to stimulate murine BMDC and human TLR9-transfected HEK293 cells.

The six synthesized ODNs containing murine-like motifs stimulated BMDC derived from wild-type mice to secrete TNFα, although there was considerable variation in their stimulatory capacities (figure 2.5a). The most potent of the ODNs, mAF2 and mAF5, stimulated TNFα concentrations that were nearly as high as that seen with CpG 1826. In contrast, cytokine levels stimulated by mAF3 and mAF6 were barely above background. None of the ODNs stimulated cytokine release from $TLR9^{-/-}$ BMDC above background levels (Figure 2.5b).

Human-Like Motifs			Mouse-Like Motifs		
ID	**CpG Motif**	**Number of Hits**	**ID**	**CpG Motif**	**Number of Hits**
hAF1	TCGTCGTTGTCGTCGTC	9	mAF1	TCGACGTCGACGTT	2
hAF2	TCGTCGTTGTCGTC	8	mAF2	TTGACGTTTCCGACGTT	1
hAF3	TCGTCGTCGTCGTT	8	mAF3	TTGACGTTGGGGACGTT	1
hAF4	TCGTCGTCGTCGTCGTT	4	mAF4	CTGACGTTCATGGACGTT	1
hAF5	TTGTCGTTGTTGTCGTT	2	mAF5	CCGACGTTCCTGACGTT	1
hAF6	CTGTCGTTCGAGGTCGTT	1	mAF6	TTGACGTTGACTGACGTA	1
hAF7	TCGTCGTTCTCGTCGTC	1		TTGACGTTCGACGTG	1
hAF8	TCGTCGTCGTCCGTCGTT	1		TTGACGTCTGTGACGTT	1
hAF9	CCGTCGTGACTGGTCGTT	1		TCGACGTTTGAGACGTA	1
hAF10	CTGTCGTTCCGTCGTC	1		TCGACGTTTAGGACGTA	1
	TTGTCGTCGTCGTT	6		TCGACGTTCCGACGTC	1
	TCGTCGTTGTCGTT	4		TCGACGTTCCCCGACGTC	1
	TTGTCGTTGTCGTT	3		CTGACGTTTGTGACGTG	1
	TCGTCGTTATCGTCGTC	2		CTGACGTTTCGACGTG	1
	TCGTCGTCGTTGTCGTT	2		CTGACGTTGGGAGACGTA	1
	TCGTCGTTTCGTCGTT	1		CTGACGTCCAGGACGTT	1
	TCGTCGTTGGCGTCGTT	1		CTGACGTATTGACGTT	1
	TCGTCGTTGCGTCGTT	1		CCGACGTTTGACGTG	1
	TCGTCGTTGAAGTCGTT	1		CCGACGTTGGTGACGTC	1
	TCGTCGTTCTCGTCGTT	1		CCGACGTTGCCGACGTA	1
	CTGTCGTTTCTGTCGTT	1		CCGACGTCGAGACGTT	1
	CTGTCGTTGTGTCGTT	1		CCGACGTCGACGTT	1
	CTGTCGTTATAGTCGTT	1			
	CTGTCGTCGTCGTT	1			
	CCGTCGTTCACAGTCGTT	1			
	TTGTCGTTGTTGTCGTA	1			
	TTGTCGTTGTGGTCGTA	1			
	TTGTCGTTGTCGTCGTC	1			
	TTGTCGTTGTCGTC	1			
	TTGTCGTTGTCGTA	1			
	TTGTCGTTGGTCGTG	1			
	TTGTCGTTAGTCGTC	1			
	TTGTCGTCACAGTCGTT	1			
	TCGTCGTTGTTGTCGTC	1			
	TCGTCGTTGTCGTCGTG	1			
	TCGTCGTTGCTGTCGTC	1			
	TCGTCGTTCTTGTCGTA	1			
	TCGTCGTTAGTCGTCGTC	1			

	TCGTCGTGGTCGTT	1
	TCGTCGTGACGTCGTT	1
	TCGTCGTCTCCGTCGTT	1
	TCGTCGTCCGTCGTT	1
	TCGTCGTCAACGTCGTT	1
	CTGTCGTGTCGTCGTT	1
	CTGTCGTCGGTGTCGTT	1
	CCGTCGTTTTGTCGTC	1
	CCGTCGTTGTCGTC	1
	CCGTCGTGTCGTCGTT	1
	CCGTCGTGCGTCGTT	1

Table 2.1. Putative Immuno-stimulatory sequences found in *A. fumigatus* genome

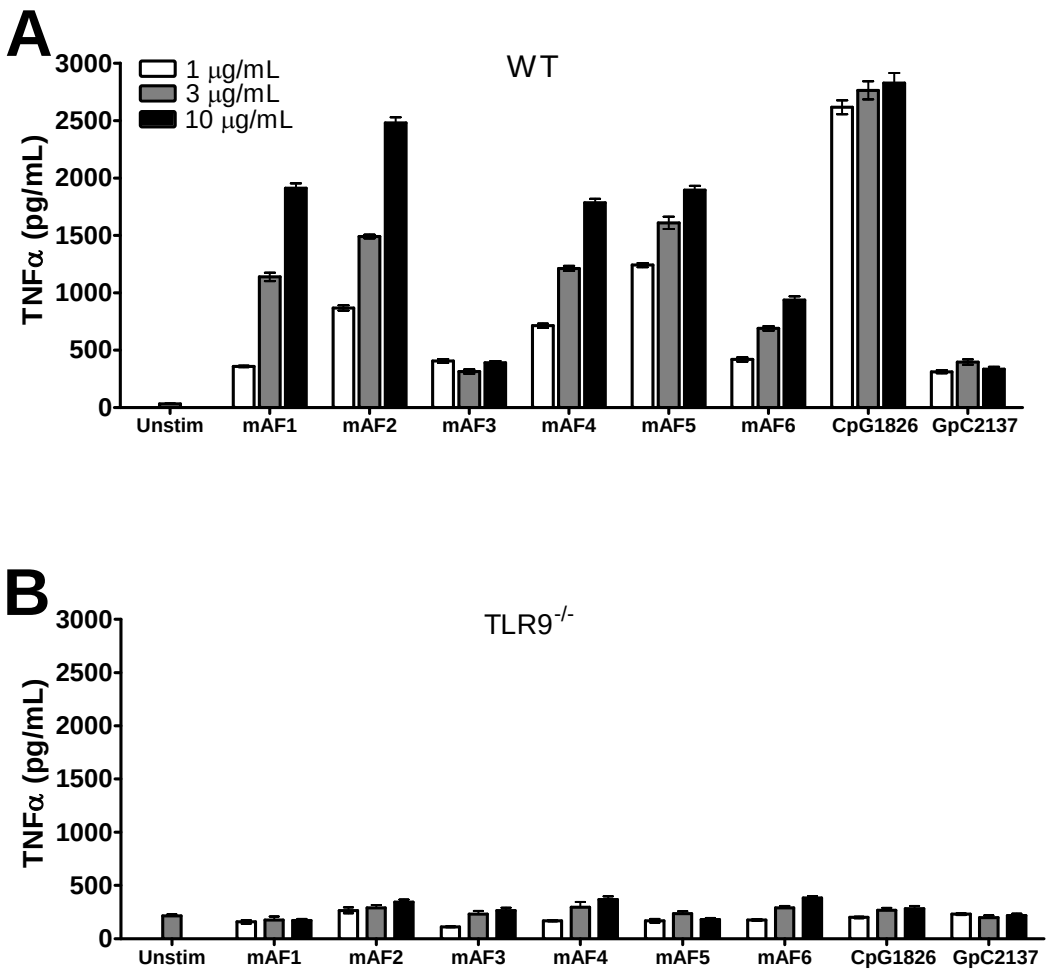
A
WT
1 μg/mL
3 μg/mL
10 μg/mL
TNFα (pg/mL)
3000
2500
2000
1500
1000
500
0
Unstim
mAF1
mAF2
mAF3
mAF4
mAF5
mAF6
CpG1826
GpC2137
B
TLR9-/-
TNFα (pg/mL)
3000
2500
2000
1500
1000
500
0
Unstim
mAF1
mAF2
mAF3
mAF4
mAF5
mAF6
CpG1826
GpC2137

Figure 2.5: Stimulation of murine BMDC by ODNs containing CpG-rich motifs present in the *A. fumigatus* genome.

BMDCs from WT (top panel) and TLR9$^{-/-}$ (bottom panel) mice were stimulated with the indicated mouse-like CpG motifs (see supplemental table). CpG 1826 served as a positive control while unstimulated (Unstim) cells and GpC 2137 were negative controls. After 24 hours, supernatants were collected and TNFα concentrations were determined by ELISA. Data are means ± SE of a representative (out of three) experiment performed in triplicate.

Next, ODNs representing human-like CpG motifs were assessed for their capacity to stimulate HEK293 cells stably co-transfected with human TLR9 and an NF-κB-driven luciferase reporter gene. All ODNs tested induced luciferase activity, with the level of activity generally increasing in a dose-dependent manner (figure 2.6). As was observed with the murine-like motifs, the stimulatory capacity of the individual ODNs was quite variable. hAF1, which occurs nine times in the *A. fumigatus* genome, was at least as potent as the positive control, CpG 2007. The control ODN, GpC 2137, did not induce the activation of the TLR9-transfected cells. The ODNs tested in figure 6 did not induce significant luciferase activity in HEK293 cells stably transfected with human TLR7 and an NF-κB-driven luciferase reporter gene (data not shown).

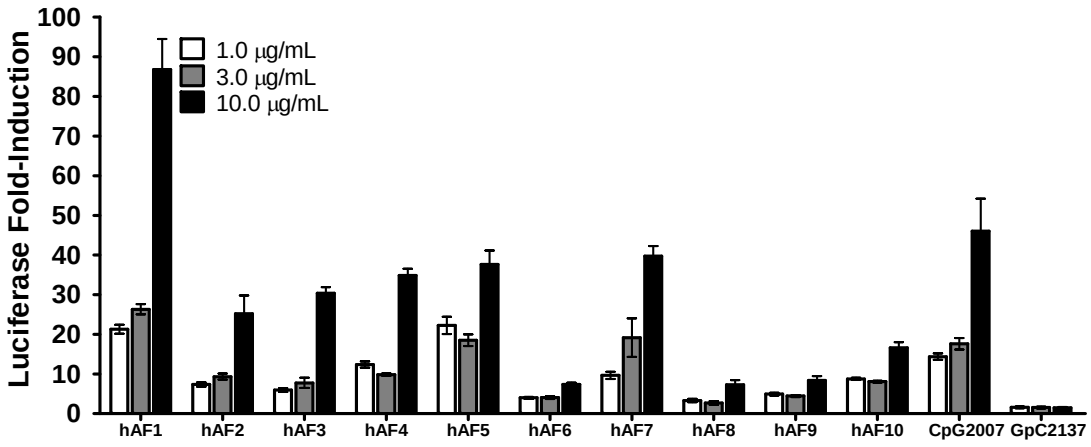
Luciferase Fold-Induction
100
90
80
70
60
50
40
30
20
10
0
1.0 μg/mL
3.0 μg/mL
10.0 μg/mL
hAF1
hAF2
hAF3
hAF4
hAF5
hAF6
hAF7
hAF8
hAF9
hAF10
CpG2007
GpC2137

Figure 2.6: Stimulation of human TLR9-transfected HEK293 cells by ODNs containing CpG-rich motifs present in the *A. fumigatus* genome.

HEK293 cells stably co-expressing human TLR9 and the NF-κB-driven luciferase reporter construct were stimulated with the indicated human-like CpG motifs (see supplemental table). CpG 2007 served as a positive control while GpC 2137 was a negative control. After 18 hours, luciferase activity was determined in the cell lysates. Results are expressed as fold induction over unstimulated cells. Data are means ± SE of a representative (out of four) experiment performed in triplicate.

Discussion

Innate immunity plays a critical role in microbial defenses by allowing the host to rapidly respond to a challenge and by providing a bridge to adaptive immunity. The strong association of invasive aspergillosis with qualitative and quantitative disorders of phagocyte function suggests innate recognition of *Aspergillus* is of particular importance in defending against this ubiquitous fungus. Previous studies by our laboratory and others have demonstrated that following incubation of phagocytes with *A. fumigatus* conidia and hyphae, TLR2- and TLR4-dependent signaling cascades are stimulated, leading to proinflammatory cytokine production (Chignard et al. 2007; Luther et al. 2007; Mambula et al. 2002; Roeder et al. 2004a; Roeder et al. 2004b). Here, we demonstrate a putative contribution of another TLR, TLR9, to defenses against aspergillosis.

Whereas the ligands for TLR2 and TLR4 on *A. fumigatus* remain undefined, several lines of evidence establish unmethylated CpG-rich fungal DNA as the ligand for TLR9. First, using restriction endonucleases and DNA methylases, the presence of unmethylated CpG sequences (the natural ligand for TLR9) in *A. fumigatus* DNA was demonstrated. Second, stimulation of murine DC from $TLR9^{-/-}$ mice with *A. fumigatus* DNA resulted in greatly impaired cytokine production compared with that seen with wild-type DC. Nevertheless, the possibility that some stimulation induced by the *A. fumigatus* DNA was due to mechanisms independent of TLR9 cannot be excluded. DC from $TLR9^{-/-}$ mice did secrete some residual TNFα after treatment with *A. fumigatus* DNA. Similarly, some residual cytokine secretion was observed after stimulation of

wild-type DC with methylated DNA. This residual response could be due to other intracellular DNA sensors, including DAI (DNA-dependent activator of IFN-regulatory factors) (Takaoka et al. 2007) and an as yet unidentified sensor dependent on TANK-binding kinase 1 (Ishii et al. 2008).

In a survey of 15 bacterial species, the immunostimulatory capacity of bacterial DNA samples directly correlated with the frequency of CpG dinucleotides (Dalpke et al. 2006). In that study, the CpG frequency ranged from 1.44% to 12.21%. Our analysis of the *A. fumigatus* genome determined the frequency of CpG dinucleotides sequences to be 5.35%, similar to that of bacterial DNA. Mammalian DNA is thought to be less immunostimulatory compared with bacterial DNA because the frequency of the CpG motif is suppressed. In addition, mammalian, but not bacterial, DNA is highly methylated (Akira et al. 2006). Although mammals and fungi share membership in the eukaryotic kingdom, we found that CpG motifs in *A. fumigatus* DNA had a low level of methylation. Thus, the pattern of degradation of *A. fumigatus* DNA following treatment with *HpaII*, an endonuclease specific for unmethylated CCGG sequences, was similar to the pattern seen after treatment with *MspI*, an endonuclease which cleaves both methylated and unmethylated CCGG sequences. Consistent with these findings, *A. fumigatus* DNA stimulated DC cytokine responses comparable to that by *E. coli* DNA, which has a CpG frequency of 7.27%.

An *in silico* genome-wide analysis of the fungal DNA demonstrated the abundant presence of CpG-rich motifs of the type predicted to be stimulatory for mouse and human

TLR9. The analysis was conservative and additional stimulatory motifs likely exist within the genome. Synthetic ODNs containing CpG-rich motifs found in *A. fumigatus* DNA stimulated mouse and human cells. Interestingly, of the synthetic ODNs tested, the one that was most stimulatory had a sequence which appeared the most often in the *A. fumigatus* genome. The potential for this ODN to be used as an immunostimulant in people should be considered given that it stimulated human cells as well as a CpG-rich ODN undergoing clinical trials.

The relative contribution of *A. fumigatus* DNA to the immunology of human aspergillosis remains to be determined. Future studies will examine the conditions under which DNA is released from the fungal cell, an event that presumably would be a prerequisite for an interaction with TLR9 to occur. In addition, in its tissue-invasive hyphal phase, *A. fumigatus* is mostly an extracellular pathogen and TLR9 is located intracellularly in the endosomal compartments. Nevertheless, disparities between wild-type and $TLR9^{-/-}$ mice with regards to their susceptibility to aspergillosis (Bellocchio et al. 2004a) suggest that the interaction of *A. fumigatus* DNA and TLR9 occurs in vivo. In addition, a recent study examined variants in TLR genes in a population of Italian children with hematological malignancies (Lanciotti et al. 2007). The frequency of the C allele of the TLR9 T-1486C polymorphism was significantly higher in patients with invasive mold infections compared with patients without invasive fungal infections. Furthermore, a recent study (Carvalho et al. 2008b) found an association of the TLR9 T-1237C polymorphism with allergic bronchopulmonary aspergillosis, a form of aspergillosis mainly found in patients that suffer from asthma and cystic fibrosis.

An inflammatory response is beneficial to the host if it enables it to contain or eliminate the pathogen. However, the immune response can be detrimental if it results in damage to host tissues. The finding that TLR9$^{-/-}$ mice survived longer than wild-type mice following challenge with *A. fumigatus* suggests that, at least in some circumstances, the response to *A. fumigatus* DNA favors the pathogen rather than host. The nature of the immune response might be important too. In addition to direct inflammatory effects resulting from stimulation of proinflammatory cytokines, including type I interferons, CpG-rich DNA biases towards Th1-type responses (Krieg 2002). Finally, recent work by Miyazato *et al.* showed that BM-DCs are capable of stimulating a TLR9-dependent response following challenge with *C. albicans* DNA (Miyazato et al. 2009). While TLR9 has been suggested to play a role in recognition of fungal DNA, the role of type I IFNs during these infections remains to be elucidated.

Materials and Methods

Reagents: Reagents, unless otherwise stated, were obtained from Sigma-Aldrich (St Louis, Missouri). Dulbecco's Minimal Essential Media (DMEM) and RPMI-1640 Media were obtained from GIBCO (Invitrogen; Carlsbad, California). Low-endotoxin fetal bovine serum (FBS) was obtained from Tissue Culture Biologicals (Tulare, California). R10 Media consisted of RPMI-1640 supplemented with 100U/mL Penicillin, 100U/mL Streptomycin, 2mM L-glutamine, 50 μM β-2-Mercaptoethanol, and 10% heat inactivated FBS. *E. coli* K-12 DNA containing undetectable levels of endotoxin was purchased from Invivogen (San Diego, California). ODNs (Supplemental table) based upon the sequences found in the *A. fumigatus* genome were synthesized on a phosphothiorate backbone by Alpha DNA (Canada). Stimulatory CpG 1826, CpG 2007, and CpG 2236 ODNs, and control GpC 2137, GpC 2243 ODNs, with phosphothiorate linkages were purchased from Coley Pharmaceuticals (Wellesley, Massachusetts).

Genomic DNA preparation: DNA was obtained from two strains of *A. fumigatus*. Strain Af293 was obtained from the Fungal Genetic Stock Center (University of Missouri, Kansas City). The genome of this strain has been sequenced (Nierman et al. 2005a). The other strain of *A. fumigatus* used was a clinical isolate (Mambula et al. 2002). The data shown are with DNA from strain Af293, except for figure 2B which utilized DNA from the clinical isolate. In preliminary studies, the two sources of DNA stimulated similar quantities of cytokines (data not shown). *A. fumigatus* was grown on minimal liquid media consisting of yeast extract supplemented with 2.5% Glucose, 3.4

G/L Yeast Nitrogen Base supplemented with ammonium sulfate and amino acids at 37^{o} for 40-48 hours. Fungi were harvested through a nylon mesh filter and washed with Tris-Sodium-EDTA (TSE; 0.05M Tris-HCl pH 8.0, 0.150M NaCl, 0.1M EDTA pH8)(Specht et al. 1982). Subsequently, hyphae were freeze-dried and ground to a powder, and the DNA was extracted with chloroform/isopropanol. The DNA was treated with 20 µg/mL of RNAse B and 20 µg/mL of Proteinase K, and further extracted with ethanol. Extracted fungal DNA was purified over a cesium-chloride gradient by centrifugation at 30K RPM (Beckman Ultracentrifuge L8-80M) for 40 hours at 20^{o}C. Purified DNA collected from the gradient was extracted with saturated N-butanol and further extracted by ethanol extraction. DNA was then tested for the presence of glucans and endotoxin using the *Limulus* Amebocyte Lysate Test (Cape Cod Associates). Endotoxin levels were found to be ≤0.03 endotoxin units/µg of DNA.

Enzyme Treatment of DNA: Isolated genomic *A. fumigatus* DNA was subjected to CpG methylation using CpG methyltransferase, *M.SssI* (NEB). The methylation reaction was performed following the manufacturer's instructions. In addition, DNA that was either methylated or untreated was subjected to cleavage at CpG sequences by restriction enzyme digest with the isoschizomers *MspI* or *HpaII*. Restriction digest was confirmed by 1% agarose gel electrophoresis of the treated and untreated samples. Sizes of the resulting digested DNA fragments were compared to a DNA ladder (NEB).

Mice: All mice were specific pathogen-free and housed in the University of Massachusetts Medical Center animal facility. C57Bl/6J mice were purchased from

Jackson Laboratories (Bar Harbor, Maine). The TLR9-deficient mice were obtained from Robert Finberg (UMass Medical School) who obtained the mice from Shizuo Akira (Osaka, Japan). The knockout mice were backcrossed 12 times to a C57Bl/6 background.

Generation and stimulation of BMDCs: BMDCs were obtained according to the protocol of Lutz et.al (Lutz et al. 1999), and as in our previous studies (Kelly et al. 2005; Mansour et al. 2006; Wozniak et al. 2006). Briefly, mice were euthanized and bone marrow cells were harvested from the femurs and tibiae. After treatment with RBC lysis buffer, the remaining cells were suspended at a final density of 1 X 10^6 cells per mL in R10 media supplemented with 10% supernatant from J558L cells (as a source of GM-CSF). Cells were seeded in non-tissue cultured treated Petri dishes and incubated at 37^{o}C in air supplemented with 5% CO_2. Media was changed every 3 days. On day 8 or 9, cells were harvested and the BMDCs were positively selected on a magnetic column using CD11c-coated magnetic beads, as per the manufacturer's instructions (Miltenyi Biotech).

BMDCs (2 X 10^5 cells per well) were added to 48-well plates containing a final volume of 500 uL media (R10 with 10% J558L supernatant). BMDC were transfected with DNA using 30 μg/mL of N-[1-(2,3-Dioloyloxy)propyl-N,N,N-trimethylammonium methylsulfate (DOTAP; Roche) according to the manufacturer's instructions. Briefly, DNA and DOTAP were mixed together in HEPES-buffered saline and incubated for 15 minutes at 25˚C. The DNA-DOTAP complexes were then added to BMDC and further mixed by pipetting. Treated cells were incubated for 24 hours at 37^{o}C in air supplemented with 5% CO_2. Supernatants were collected and cytokine production was

analyzed for TNFα and IL-12p70 by ELISA according to the manufacturer's instructions (eBioscience; San Diego, California).

Isolation and stimulation of human pDC: pDC were isolated as described (Wang et al. 2006). Briefly, peripheral blood was obtained by venipuncture following informed consent from healthy volunteers using a protocol approved by the University of Massachusetts Medical Center Institutional Review Board. Blood was anticoagulated with heparin, diluted 1:1 with HBSS (BioWhittaker; Walkersville, MD) and the peripheral blood mononuclear cells (PBMC) collected following Ficoll-Hypaque (Lymphoprep^{TM}; Westbury, NY) density gradient centrifugation . Human pDCs were positively selected from the PBMC using CD304 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA).

pDCs ($5x10^4$) were stimulated with DNA-DOTAP complexes in 96-well flat-bottom plates containing a final volume of 200 µl RPMI 1640 supplemented with 10% FCS and 10 ng/ml rIL-3 (R&D Systems, Minneapolis, MN). After 20 hours culture in a 37°C/5% CO2 incubator, supernatants were collected and human IFNα levels were measured using a commercially available ELISA kit (Bender MedSystems module set, Burlingame, CA). Samples were assayed in duplicate at dilutions that fell within the range of the standard curves.

Luciferase Assay: HEK293 cells stably transfected with hTLR9 and a NF-kB-driven luciferase construct (HEK/hTLR9/NF-kB), and HEK293 cells stably transfected with hTLR7and a NF-kB luciferase construct (HEK/hTLR7/NF-kB) were a gift from the Eisai Research Institute (Andover, MA). Cells were maintained in tissue culture flasks

containing DMEM supplemented with 10% FBS. Cells were treated with the ODNs at the specified concentrations at a final volume of 150 µl/well, and incubated at 37°C in air supplemented with 5% CO_2 for 18 hours. Luciferase activity in cell lysates was measured using the Steady Glo Luciferase Assay System (Promega, Madison, WI) on a luminometer (Envision, Perkin Elmer).

Genome-wide scanning for GC content, CpG dinucleotides, and TLR9 stimulatory sequences: Both mouse- and human-like motifs were searched for in both DNA strands of the assembled contigs, release date 05/12/2007 (AF.contigs.031704), downloaded from the Sanger fpt site (ftp://ftp.sanger.ac.uk/pub/pathogens/A_fumigatus). Sequences matching the following patterns were selected: human-like, [CT][CT]GTCGTTN(0,4)GTCGTT, and mouse-like, [CT][CT]GACGTTN(0,4)GACGTT. Residues between square parentheses indicate ambiguities acceptable in the specified positions, N indicates any nucleotide and the numbers between parentheses indicate the number of times residues can appear in that position. Mismatches were allowed only at the eighth or the last position. GC content was calculated from the number of G's and C's found for one strand divided by the number of base pairs sequenced. CpG dinucleotides were found searching both strands, and the frequency calculated using two times the number of base pairs sequenced. The "fuzznuc" algorithm (EMBOSS program: http://bioweb.pasteur.fr/seqanal/EMBOSS) was used for each of the searches.

Statistical Analysis: Means ± standard errors (SE) were analyzed by one-way or two-way ANOVA using a statistical software package (GraphPad Prism 4.02, San Diego, CA). Pairs of group means were then compared by Bonferroni's multiple comparison test. Statistical significance was defined as $p<0.05$.

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Preface to Chapter III

The data presented in this chapter has not been published.

Zaida G. Ramirez-Ortiz, Chrono Lee, Charles A. Specht and Stuart M. Levitz

"Interactions of plasmacytoid dendritic cells with *Aspergillus fumigatus*"

Zaida G. Ramirez-Ortiz performed all the human pDC work

Zaida G. Ramirez-Ortiz and Chrono Lee performed mouse survival experiments and pDC depletion assays

Chapter III

Interactions of plasmacytoid dendritic cells with *Aspergillus fumigatus*

Abstract:

While plasmacytoid dendritic cells (pDCs) are regarded as critical components of the innate immune responses to viruses, their role in host defenses against fungal infections has received scant study. We examined the interactions of human peripheral blood pDCs, purified by positive selection for CD304, with *Aspergillus fumigatus* hyphae. pDCs spread over hyphae and inhibited their growth, as determined by assays for fungal metabolic activity and by direct measurement of hyphal length via microscopy. pDC-mediated antifungal activity was retained in cell lysates, did not require direct contact with fungi, and was partially reversed by zinc, suggesting a contribution of calprotectin. After a 2 hour incubation with hyphae, >60% of the pDC died and stained positive for fragmented DNA by TUNEL assay. pDC cytotoxicity could be partly attributed to fungal gliotoxin secretion, as determined by studies with gliotoxin-deficient *A. fumigatus* mutants and with clinically relevant concentrations of purified gliotoxin. Furthermore, following hyphal stimulation, pDCs released the proinflammatory cytokines, TNFα and IFNα, via mechanism independent of TLR7 and TLR9. Finally, mice depleted of pDCs were more susceptible to *A. fumigatus* infection than their control counterparts. These data demonstrate two broad mechanisms by which pDCs may contribute to antifungal defenses. First, pDCs directly inhibit fungal growth via a mechanism that involves *A. fumigatus*-induced pDC death and the release of antifungal mediators. Second, when stimulated with *A. fumigatus* hyphae, pDCs release cytokines known to activate and recruit antifungal effector cells.

Introduction:

Aspergillus fumigatus has emerged as the most common cause of invasive mold infections. Mortality rates for persons with invasive aspergillosis are very high due to the severely immunocompromised status of most inflicted individuals and the relatively weak fungicidal activity of the available therapeutic options (Hohl and Feldmesser 2007a; Walsh et al. 2008). Morbidity and mortality can also result from allergic and saprophytic forms of aspergillosis. Exposure to this ubiquitous fungus is frequent and typically occurs by inhalation of airborne conidia. In the suitable host, the inhaled conidia swell and germinate into hyphae, the invasive form of the fungus. Clinical and experimental studies have strongly implicated both innate and adaptive immune responses as being critical for protection against aspergillosis. While neutrophils appear to be of paramount importance, vital contributions of monocytes, macrophages, conventional dendritic cells (DC) and T cells have been demonstrated (Bozza et al. 2002; Dagenais and Keller 2009; Diamond et al. 1983; Diamond et al. 1978; Feldmesser 2006). While conidia are efficiently ingested by phagocytes, the large size of the hyphal morphotype generally precludes phagocytosis. However, phagocytes can spread over the hyphae surface and inhibit and kill the fungus via oxidative and non-oxidative mechanisms (Bruns et al. 2010; Diamond and Clark 1982; Diamond et al. 1983; Levitz et al. 1986; Philippe et al. 2003). Moreover, co-incubation of neutrophils and *A. fumigatus* results in the formation of neutrophil extracellular traps (NETs) with direct antifungal activity (Bruns et al. 2010).

Plasmacytoid DCs (pDCs), also known as natural type I IFN producing cells, rapidly produce copious amounts of type I interferons upon stimulation with viruses (Colonna et al. 2004). In humans, pDCs comprise 0.2 – 0.8% of the total peripheral blood mononuclear cells (PBMCs) and express the endosomal Toll-like receptors (TLRs) 7 and 9, but not any of the other known TLRs. Upon viral exposure, pDCs initiate protective antiviral responses by secreting up to 1000-fold more type I interferon (IFNs) than other cell types, predominantly via mechanisms dependent on sensing viral nucleic acids via TLR7 and TLR9 (Blasius and Beutler 2010; Lande and Gilliet 2010; Lui et al. 2009; Masten et al. 2006; Yang et al. 2005; Yu et al. 2010). Activated pDCs link innate to adaptive immunity by secreting cytokines such as IFNα and tumor necrosis factor (TNFα) and by differentiating into mature pDCs with upregulated MHC and co-stimulatory molecules capable of priming naïve T cells (Lande and Gilliet 2010; Stary et al. 2009; Yu et al. 2010).

pDCs have also been implicated in the pathogenesis of autoimmune diseases and in maintenance of the immunosuppressive environments in neoplasms (Lande and Gilliet 2010; Lande et al. 2007). Recently, certain bacteria were shown to stimulate pDCs via TLR9 dependent and independent mechanisms (Ang et al. 2010; Krieg 2002; Parcina et al. 2008; Petzke et al. 2009; Pietras et al. 2006; Veckman and Julkunen 2008). However, whether pDCs play a role in the detection and responses to fungal pathogens has not been well studied. We have shown that unmethylated CpG motifs in *A. fumigatus* DNA stimulate human pDCs to produce IFNα (Ramirez-Ortiz et al. 2008) while Perruccio et al.

demonstrated that *A. fumigatus* and *Candida albicans* RNA can stimulate a cytokine response in murine and human pDCs (Perruccio et al. 2004). However, it is uncertain whether quantities of fungal DNA and RNA released during the course of a mycotic infection are sufficient to stimulate pDCs. Therefore, in the present investigation, we sought to determine whether human pDCs sense live *A. fumigatus* hyphae and, if so, what are the consequences of the interaction. We found that pDCs directly inhibit fungal growth via a mechanism that involves *A. fumigatus*-induced pDC death and the release of antifungal mediators. Moreover, following stimulation with *A. fumigatus* hyphae, pDCs release IFNα and TNFα via a TLR-independent mechanism.

Results:

Antimicrobial activity of pDCs against *A. fumigatus*. Initial experiments focused on determining whether human pDCs, purified from peripheral blood, had direct antimicrobial activity against the invasive hyphal morphotypes of *A. fumigatus*. pDCs were incubated with *A. fumigatus* hyphae for 2 hours at 37°C. Antifungal activity was then measured by the XTT assay. We found that pDCs have potent antifungal activity against *A. fumigatus*, with nearly 80% antifungal activity observed at the highest ratio (50:1) of pDCs to hyphae tested (Figure 3.2A). Surprisingly, even at a ratio of 1 pDC to 20 hyphae, over 40% antifungal activity was seen. To confirm the XTT data using an independent assay, we directly measured the hyphal length of *A. fumigatus* following 2 and 4 hour incubations with pDCs at a 1:2 pDCs to hyphae ratio (Figure 3.2B). Consistent with the XTT data, hyphal growth was significantly inhibited in the presence of pDCs. Nevertheless, it should be noted that the hyphae did show modest growth suggesting that the pDCs were predominantly fungistatic, rather than fungicidal.

Confocal microscopy of pDCs incubated with *A. fumigatus*. Following a 2 hours co-incubation of pDCs and *A. fumigatus*, the cells were fixed and observed via confocal microscopy (Figure 3.2C). Nearly all pDCs were found to be closely associated with fungi. The pDCs spread over hyphae that were too large to be ingested and phagocytosed *A. fumigatus* swollen conidia.

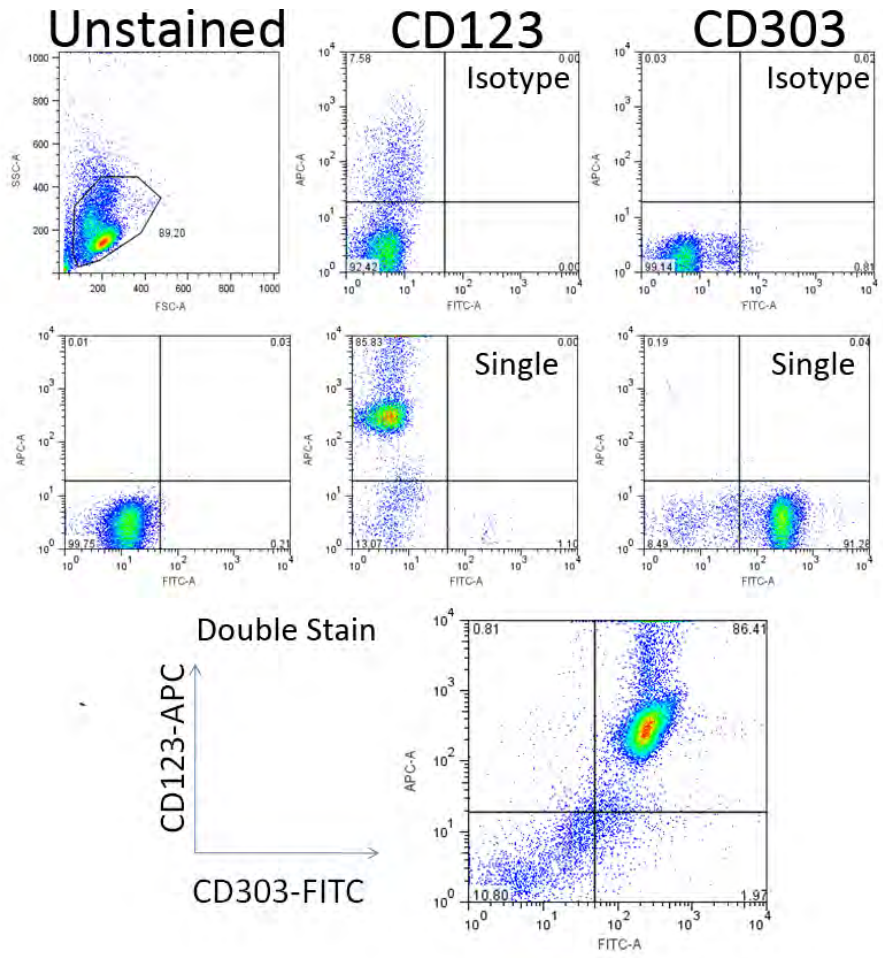
Unstained
CD123
CD303
Isotype
Isotype
Single
Single
Double Stain
CD123-APC
CD303-FITC
SSC-A
FSC-A
APC-A
FITC-A
89.20
7.58
0.00
92.42
0.03
99.14
0.01
99.75
85.83
13.07
1.10
0.19
8.49
91.28
0.81
86.41
10.80
1.97

Figure 3.1. Purity of pDC population following CD304^{+} enrichment.

pDCs were isolated from whole blood by CD304^{+} selection. The purity of the pDCs was measured by staining 100,000 cells with αCD123-APC antibody and αCD303-FITC antibody. Cells were fixed and analyzed by flow cytometry. Double positive stain represents pDC population. Representative experiment of 3 individual experiments with similar results.

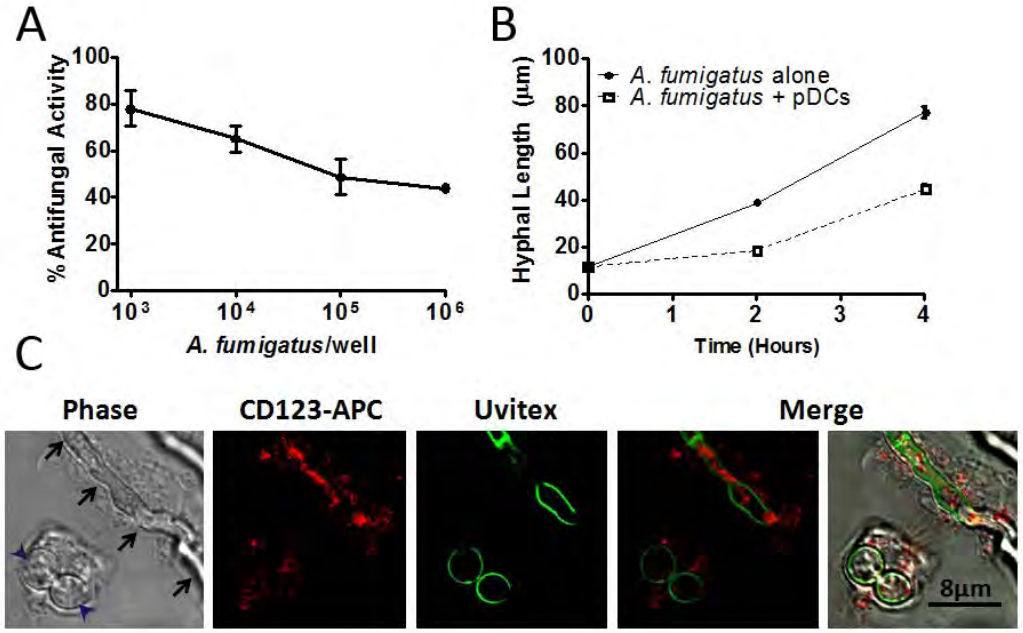
A
% Antifungal Activity
100
80
60
40
20
0
10 3
10 4
10 5
10 6
A. fumigatus/well
B
Hyphal Length (µm)
A. fumigatus alone
A. fumigatus + pDCs
Time (Hours)
C
Phase
CD123-APC
Uvitex
Merge
8µm

Figure 3.2. Antimicrobial activity of pDCs against *A. fumigatus*.

(A) pDCs ($5x10^4$) were incubated with the indicated number of *A. fumigatus* hyphae for 2 hours. Antifungal activity of pDCs was then measured by the XTT assay. Data represent means ± SE from two donors, each tested in duplicate. (B) *A. fumigatus* hyphae were incubated for the specified times with or without pDCs and hyphal length determined. Data represent means ± SE from two experiments. For each variable, at least 75 hyphal measurements were recorded. $p<0.0001$ by Student t-test, when comparing hyphal lengths in the presence or absence of pDCs at the 2 and 4 hour time points. (C) pDCs ($1x10^5$) were incubated with *A. fumigatus* ($1x10^5$) at 37°C for 2 hours. After fixation, pDCs were stained with anti-CD123-efluor650 (red) and *A. fumigatus* hyphae were stained with Uvitex (green). Samples were analyzed by confocal microscopy. Arrowheads point to two swollen conidia phagocytosed by a pDC. Arrows point to a hypha which is covered by a pDC. The photomicrographs depicted are representative of three independent experiments with similar results.

Effect of *A. fumigatus* on viability of pDCs. While observing pDCs by microscopy, we noticed that many of the pDCs incubated with *A. fumigatus* did not appear healthy, as judged by poor adherence to tissue culture wells and swollen morphology. Thus, we examined pDC death, as measured by LDH release, following a 2 hour incubation with or without *A. fumigatus* hyphae (Figure 3.3A). Remarkably, there was a large increase in death of the pDCs in the presence of *A. fumigatus.*

Antifungal activity of pDC lysates. The finding that pDCs died following incubation with hyphae raised the question of whether dying pDC had antifungal activity. To examine whether this was indeed the case, we lysed pDCs and compared the activity of pDC lysates with intact pDCs against *A. fumigatus* hyphae (Figure 3.3B). We found that the lysates and the live pDCs had comparable antifungal activity.

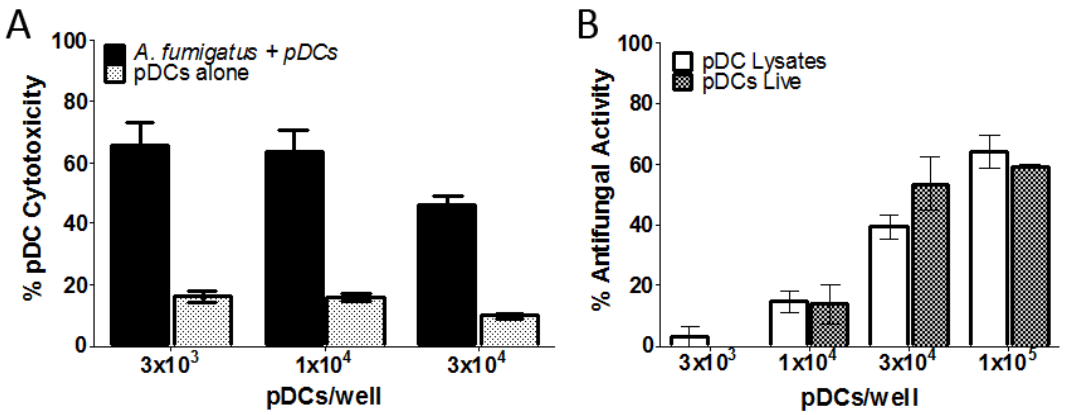
A
A. fumigatus + pDCs
pDCs alone
% pDC Cytotoxicity
100
80
60
40
20
0
3x10^3
1x10^4
3x10^4
pDCs/well
B
pDC Lysates
pDCs Live
% Antifungal Activity
100
80
60
40
20
0
3x10^3
1x10^4
3x10^4
1x10^5
pDCs/well

Figure 3.3. Effect of *A. fumigatus* on viability of pDCs.

(A) The indicated number of pDCs was incubated with or without $3x10^4$ *A. fumigatus* hyphae for 2 hours. Supernatants were then collected and cytotoxicity of pDC was assessed by LDH release. Data represent means ± SE of three donors, each studied in triplicate. $p< 0.0001$ by Student t-test when comparing cytotoxicity with and without hyphae for each concentration of pDCs. (B) *A. fumigatus* hyphae were incubated with live pDCs or lysates obtained from the indicated number of pDCs. Antifungal activity was measured by the XTT assay. Data represent means ± SE of three donors, each studied in triplicate. There were no significant differences comparing pDC lysates with live pDCs at any of the cell concentrations studied.

The contribution of Zn^{++} and Fe^{+++} deprivation to the antifungal activity of pDCs. The zinc-binding protein calprotectin and iron-binding proteins, such as lactoferrin, are constituents of some phagocytic populations and can exert broad antifungal activity by chelating divalent cations essential for fungal growth (Mambula et al. 2000; Okutomi et al. 1998; Urban et al. 2009). To determine whether nutritional deprivation of zinc or iron contributes to the antifungal activity of the pDCs, we examined whether $ZnCl_2$ or $FeCl_3$ supplementation rescued pDC-mediated growth inhibition of *A. fumigatus* hyphae (Figure 3.4A). These experiments were performed by direct microscopic measurement of fungal growth as high ferric concentrations can interfere with the XTT assay (Knight and Dancis 2006). We found that while addition of exogenous $FeCl_3$ had no effect, 10μM $ZnCl_2$ partially, but significantly, reversed pDC-mediated growth inhibition. While these data suggest a role for calprotectin, the presence of calprotectin in pDCs has not, to our knowledge, been previously documented. Therefore, Western blots probing for calprotectin were performed on pDC lysates. A band of the expected size was found, suggesting that pDCs contain calprotectin (Figure 3.4B).

Effect of cell contact on pDC cytotoxicity and antifungal activity: Given our observations that incubation with *A. fumigatus* results in both pDC cell death and fungal growth inhibition, we next sought to determine whether contact between the pathogen and the pDCs was required for these events to transpire. This was accomplished by growing hyphae on the bottom of tissue culture wells fitted with a permeable insert (Transwell) with a size exclusion of 0.4 μm. pDCs were then added either to the top of

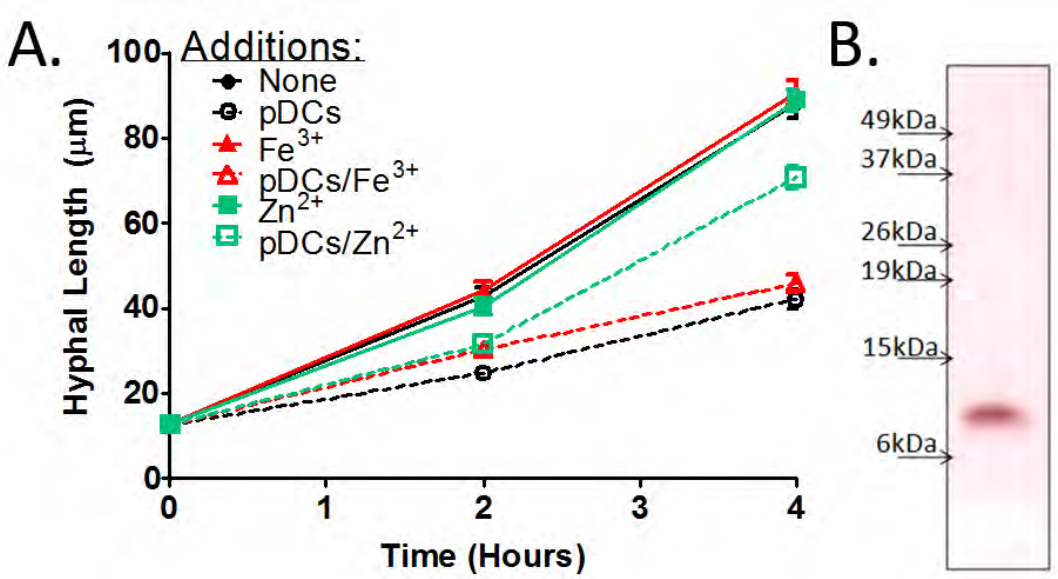
A.
Additions:
None
pDCs
Fe3+
pDCs/Fe3+
Zn2+
pDCs/Zn2+
Hyphal Length (μm)
Time (Hours)
0
20
40
60
80
100
0
1
2
3
4
B.
49kDa
37kDa
26kDa
19kDa
15kDa
6kDa

Figure 3.4. Contribution of Zn^{++} and Fe^{+++} deprivation to the antifungal activity of pDCs.

(A) *A. fumigatus* hyphae were incubated with or without pDCs ($5x10^4$) in the presence or absence of 10mM $ZnCl_2$ or 10mM $FeCl_3$. At the specified times, hyphal length was measured as described in *Methods*. Solid lines represent *A. fumigatus* alone whereas dotted lines represent *A. fumigatus* incubated with pDCs. Data represent means ± SE of two individual experiments. $p<0.001$ by two-way ANOVA, when comparing fungal growth in the presence or absence of pDCs. (B) pDCs were hypotonically lysed and 100 µg of total protein were analyzed by Western blot using a monoclonal antibody against human calprotectin. Blot is representative of 3 separate experiments. Arrows point to where the indicated molecular size standards ran on the gel.

the insert (so that no contact of intact pDCs with hyphae could occur) or underneath the insert (allowing contact to occur). We then measured pDC cytotoxicity and the antifungal activity of the pDCs (Figure 3.5). pDC cell death occurred regardless of whether the pDCs were in direct contact with hyphae, although cytotoxicity was greater when contact was allowed (Figure 3.5A). Similarly, while direct contact promoted antifungal activity, some antifungal activity was still observed in the absence of contact (Figure 3.5B).

Role of gliotoxin and other *A. fumigatus* secondary metabolites in the induction of pDC cell death: Our findings that pDC cell death occurs independently of contact with *A. fumigatus* suggest that secreted factor(s) are responsible for the observed effect. One such candidate is *A. fumigatus* gliotoxin, which is known to induce apoptosis in many cell types (Bok et al. 2006; Murayama et al. 1996; Stanzani et al. 2005). Therefore, we incubated pDCs with concentrations of purified gliotoxin within the range found in patients with invasive aspergillosis (Lewis et al. 2005; Spikes et al. 2008; Stanzani et al. 2005). We found that gliotoxin induced pDC cell death in a dose-dependent manner (Figure 3.6).

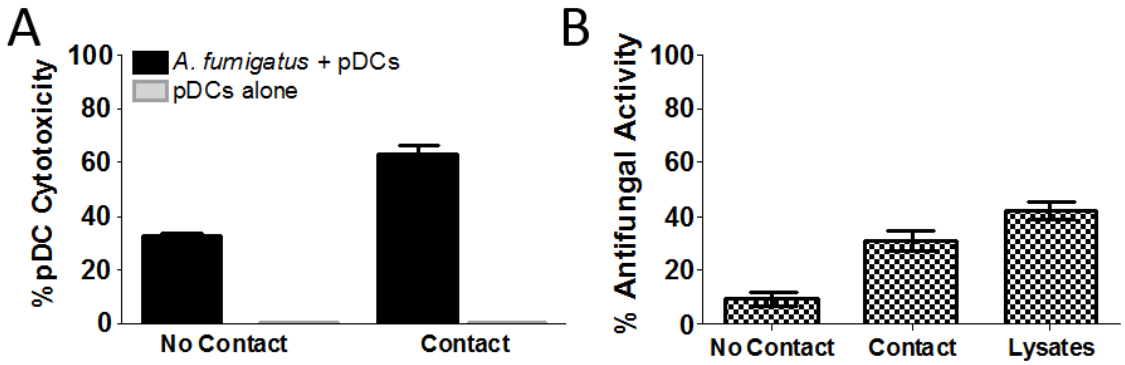
A
% pDC Cytotoxicity
100
80
60
40
20
0
A. fumigatus + pDCs
pDCs alone
No Contact
Contact
B
% Antifungal Activity
100
80
60
40
20
0
No Contact
Contact
Lysates

Figure 3.5. Influence of cell contact on pDC cytotoxicity and antifungal activity. (A) *A. fumigatus* hyphae ($5x10^4$) were grown on the bottom of wells containing a permeable insert (Transwell) with a size exclusion limit of 0.4 μm. pDCs ($5x10^4$) were then added either to the top of the insert (No Contact) or below the insert (Contact). After a 2 hour incubation, supernatants were collected and pDC cytotoxicity was measured by LDH assay. $p<0.0001$ by one-way ANOVA, when comparing pDCs in the presence or absence of *A. fumigatus* for both the "No Contact" and "Contact" groups. (B) As in part A, except antifungal activity was measured by the XTT assay. In addition, the antifungal activity of lysates from $5x10^4$ pDCs was assayed. $p< 0.0001$ by two-way ANOVA, when comparing any two groups except "Contact" and "Lysates". For both parts A and B, the data represent means ± SE from four different pDC donors, each tested in triplicate.

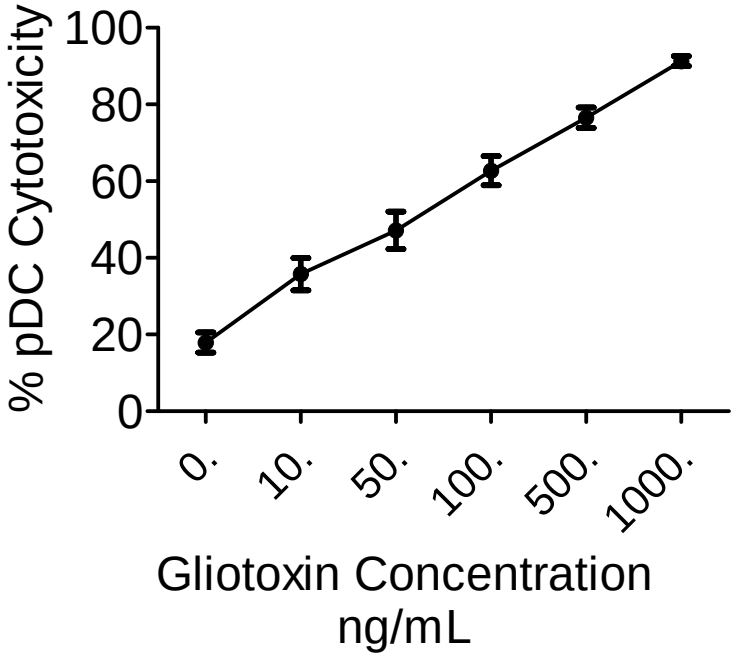
% pDC Cytotoxicity
100
80
60
40
20
0
0.
10.
50.
100.
500.
1000.
Gliotoxin Concentration
ng/mL

Figure 3.6. Gliotoxin induces pDC cell death.

pDCs ($5x10^4$) were treated with the indicated concentrations of gliotoxin. After a 2 hour incubation, supernatants were collected and cell mediated cytotoxicity was measured by LDH assay. Data represent means ± SE from four pDC donors, each tested in duplicate.

Next, we sought to determine whether hyphae-stimulated pDC cell death was due to gliotoxin secretion. Before performing our assay we wanted to determine whether fungal growth was comparable between wild-type and gliotoxin deficient and complemented strains; therefore we performed XTT assay for all strains (Figure 3.7). Our results show that all strains grow a similar rate. pDC cytotoxicity was compared following incubation with hyphae from wild-type, gliotoxin-deficient and complemented *A. fumigatus* strains (see Table 3.1). Our results suggest that pDC cell death is partially mediated by *A. fumigatus* gliotoxin, as strains mutated for gliotoxin production induced significantly less pDC cell death compared with wild type or complemented strains (Figure 3.8A). Similarly, we observed that the antifungal activity of pDCs against *A. fumigatus* strains deficient in gliotoxin production was reduced compared with wild-type or complemented strains (Figure 3.8B). In contrast, as expected, the antifungal activity of pDC lysates was similar against wild-type, gliotoxin-deficient and gliotoxin-complemented *A. fumigatus* strains (Figure 3.8C).

Mechanism of pDC cell death. In order to gain insights on the mechanism of pDC cell death, we performed TUNEL assays on pDCs incubated with *A. fumigatus* hyphae (Figure 3.9). We found DNA fragmentation in approximately 60% of the pDCs stimulated with hyphae (Figure 3.9), suggesting that the pDC cell death demonstrated in figure 2 is due to apoptosis or pyroptosis. A similar percentage of TUNEL positive cells were seen following incubation of pDCs with gliotoxin. In contrast, only about 10% of unstimulated pDCs had TUNEL-positive staining.

Strain name	Genotype	Phenotype	Mutation	Reference
Af293	Wild type	Wild type	None	(Nierman et al. 2005b; Ramirez-Ortiz et al. 2008)
Δ*GliZ*	*gliZ::pyrG pyrG1*	Deficient in gliotoxin production	Deletion of a putative Zn_2Cys_6 binuclear transcription factor	(Bok et al. 2005; Bok et al. 2006)
GliZ Comp	*gliZ hygB ,gliZ::pyrG pyrG1*	Complemented for gliotoxin	Complemented Δ*GliZ* strain	(Bok et al. 2005; Bok et al. 2006)
Δ*LaeA*	*laeA::pyrG pyrG1*	Deficient in production of gliotoxin and other secondary metabolites	Aberrant in toxin biosynthesis and spore development	(Bok et al. 2005; Bok et al. 2006)
LaeA Comp	*laeA hygB, laeA::pyrG pyrG*	Complemented for production of gliotoxin and other secondary metabolites	Complemented Δ*LaeA* strain	(Bok et al. 2005; Bok et al. 2006)

Table 3.1. Description of the *A. fumigatus* strains used in our studies.

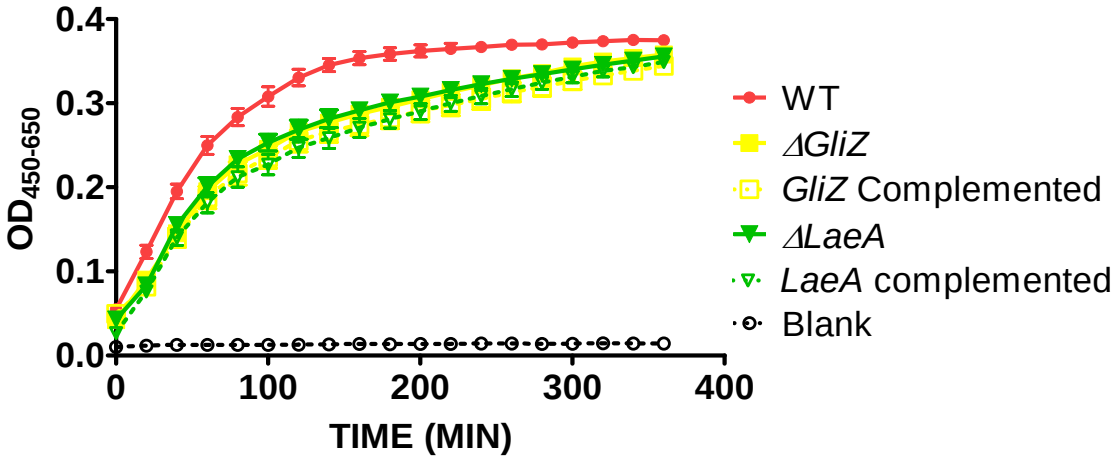
0.4
0.3
0.2
0.1
0.0
OD450-650
0
100
200
300
400
TIME (MIN)
WT
ΔGliZ
GliZ Complemented
ΔLaeA
LaeA complemented
Blank

Figure 3.7. Growth curves for *A. fumigatus* strains deficient in production of secondary metabolites.

Growth of *A. fumigatus* ($5x10^4$) from the indicated strains was determined by XTT assay. Graph representative of two individual experiments, both with similar results. No significant differences were found between mutant strains and WT *A. fumigatus*. Data represent means ± SE of triplicate samples.

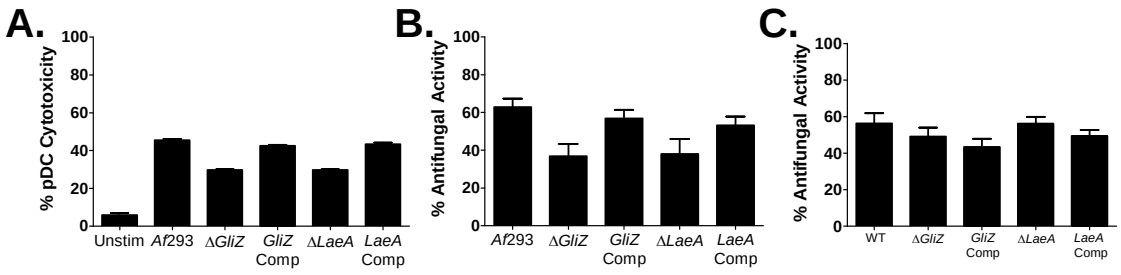
A.
% pDC Cytotoxicity
100
80
60
40
20
0
Unstim
Af293
ΔGliZ
GliZ Comp
ΔLaeA
LaeA Comp
B.
% Antifungal Activity
100
80
60
40
20
0
Af293
ΔGliZ
GliZ Comp
ΔLaeA
LaeA Comp
C.
% Antifungal Activity
100
80
60
40
20
0
WT
ΔGliZ
GliZ Comp
ΔLaeA
LaeA Comp

Figure 3.8. Role of gliotoxin and other *A. fumigatus* secondary metabolites in the induction of pDC cell death.

(A, B) pDCs ($5x10^4$) were left unstimulated (Unstim) or incubated for 2 hours with hyphae ($5x10^4$) from the indicated strain of *A. fumigatus* (see Table 3.1). pDC cytotoxicity and antifungal activity were analyzed by LDH and XTT assays, respectively. Data represent means ± SE of duplicate experiments from separate donors, each of which was performed in triplicate. $p<0.0001$ by one-way ANOVA, when comparing deletion strains with their complemented counterparts as well as wild-type *A. fumigatus* (Af293). (C) pDC lysates were generated by hypotonic lysis and the indicated strains of *A. fumigatus* hyphae were incubated for 2 hours at 37°C with volume of pDC lysate comparable to $5x10^4$ cells. The antifungal activity of the lysates against the hyphae was measured by XTT assay. No statistical differences were found among the different treatments.

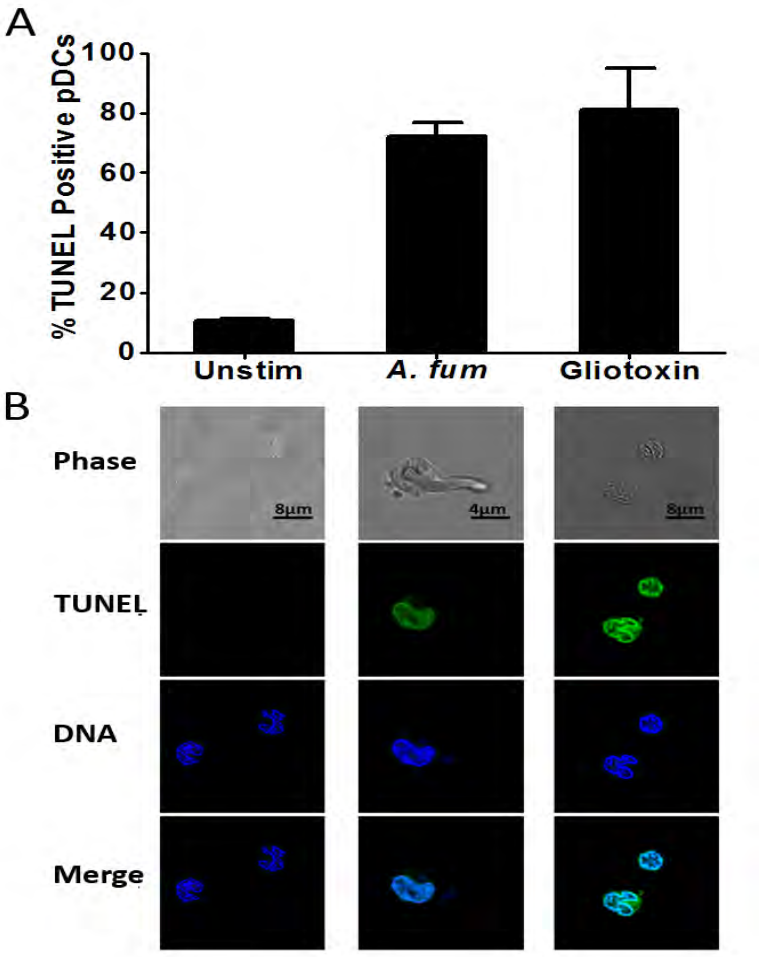
A
% TUNEL Positive pDCs
100
80
60
40
20
0
Unstim
A. fum
Gliotoxin
B
Phase
TUNEL
DNA
Merge
8µm
4µm
8µm

Figure 3.9. Mechanism of pDC cell death.

pDCs ($1x10^5$) were left unstimulated (Unstim) or stimulated for 2 hours with either *A. fumigatus* ($1x10^5$) hyphae or gliotoxin (20 ng/ml). Following incubation, samples were fixed and stained for DNA fragmentation by TUNEL (Alexa 594) and total DNA (Hoechst 33342). Cells were then examined by confocal microscopy. (A) TUNEL positivity was determined for at least 100 pDCs per group. Data represent means ± SE of two individual experiments performed in duplicate; $p<0.01$ by one-way ANOVA, when comparing unstimulated with *A. fumigatus* or gliotoxin. (B) Representative confocal microscopy images.

Cytokine release by pDCs stimulated with *A. fumigatus* hyphae. In the final set of experiments, we examined whether the interaction of pDCs with hyphae could lead to enhanced immune responses due to cytokine release. pDCs were stimulated with *A. fumigatus* hyphae for 6 hours following which concentrations of IFNα and TNFα were determined in the supernatants (Figure 3.10). For comparison, the "flow through" fraction, consisting of the PBMCs which did not adhere to the CD304-coated magnetic beads, was also studied. We found IFNα and TNFα were released from both the pDCs and flow through fractions following stimulation with *A. fumigatus* hyphae. Human pDCs are $TLR9^+$ but $TLR4^-$ (Gilliet et al. 2008; Lande and Gilliet 2010; Pietras et al. 2006; Schreibelt et al. 2010; Tel et al. 2010). Consistent with this observation, the TLR9 ligand, CpG, potently stimulated the pDCs whereas the TLR4 ligand, lipopolysaccharide (LPS), failed to stimulate these cells. As expected, LPS stimulated the flow through fraction (which contains LPS-responsive monocytes) to release IFNα and TNFα.

Role of TLR9 in hyphal stimulation of IFNα by pDCs. We previously demonstrated that purified *A. fumigatus* DNA stimulates TLR9-dependent cytokine release (Ramirez-Ortiz et al. 2008). Here we sought to determine whether stimulation of IFNα release by intact *A. fumigatus* hyphae was also mediated by TLR9. As endosomal acidification is essential for TLR9 signaling (Lewis and Cobb 2010; Rutz et al. 2004), we studied the effect of pDC pretreatment with the endosomal acidification inhibitors chloroquine and bafilomycin A on IFNα release stimulated by *A. fumigatus* hyphae (Figure 3.10C). Inhibiting endosomal acidification did not affect hyphae-induced IFNα release by pDCs,

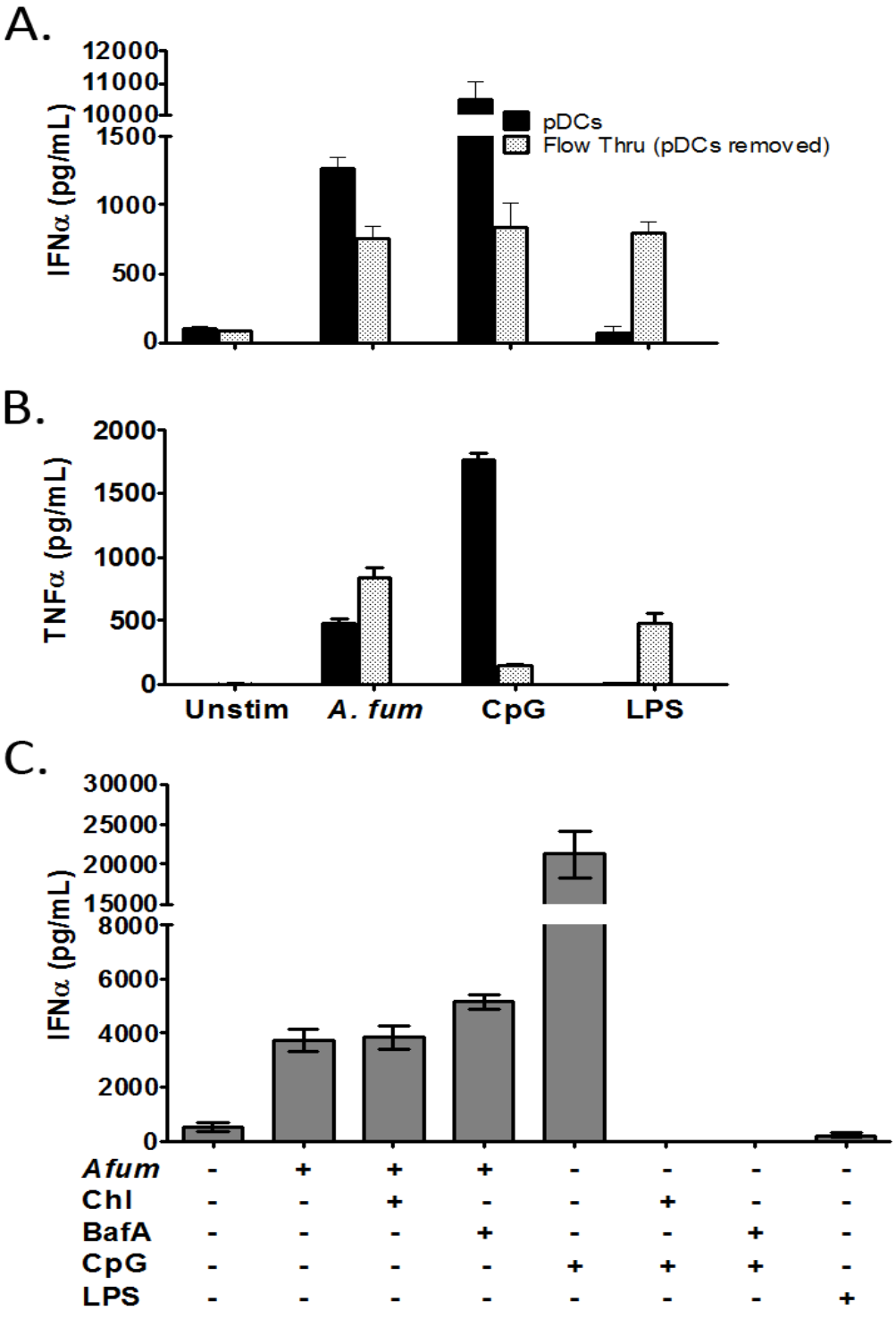
A.
IFNα (pg/mL)
12000
11000
10000
1500
1000
500
0
pDCs
Flow Thru (pDCs removed)
B.
TNFα (pg/mL)
2000
1500
1000
500
0
Unstim
A. fum
CpG
LPS
C.
IFNα (pg/mL)
30000
25000
20000
15000
8000
6000
4000
2000
0
Afum - + + + - - - -
Chl - - + - - + - -
BafA - - - + - - + -
CpG - - - - + + + -
LPS - - - - - - - +

Figure 3.10. Cytokine release by pDCs stimulated with *A. fumigatus* hyphae.

(A) and (B). PBMC were separated into pDC positive (pDCs) and negative (Flow through) fractions using CD304-coated magnetic beads. The pDCs and flow through cells ($5x10^4$/well) were then left unstimulated (Unstim) or stimulated for 6 hours with *A. fumigatus* hyphae ($5x10^4$), CpG (100 ng/ml) or LPS (10 ng/ml). Supernatants were collected and analyzed by ELISA for IFNα (A) and TNFα (B). Data represent means ± SE of cytokine concentrations from two donors, each analyzed in duplicate. $p<0.0001$ by two-way ANOVA when comparing cytokine secretion by unstimulated cells with any stimulus except for unstimulated pDCs compared with LPS-stimulated pDCs. (C) pDCs ($5x10^4$) were pretreated for 60 minutes with 10 µg/ml chloroquine (Chl), 10 µg/ml of bafilomycin A (BafA), or left untreated prior to 6 hour stimulation with *A. fumigatus* hyphae ($5x10^4$), CpG (100 ng/ml) or LPS (10 ng/ml). Supernatants were analyzed by ELISA for IFNα. Data represent means ± SE of cytokine concentrations from two donors, each analyzed in triplicate. $p<0.0001$ by two-way ANOVA, when comparing unstimulated pDCs to pDCs incubated with *A. fumigatus* or CpG. IFNα levels stimulated by *A. fumigatus* were not significantly affected by treatment with BafA or Chl.

suggesting a TLR9-independent mechanism of stimulation. As expected, chloroquine and bafilomycin A abrogated CpG-stimulated IFNα release.

pDCs are recruited into the lungs of *A. fumigatus*-infected mice. In order to determine whether pDCs were recruited into the lungs of *A. fumigatus*-infected mice, single cell suspensions obtained from lungs of infected mice were analyzed by flow cytometry 48 hours post infection (Figure 3.11). Our results show an approximately 5-fold increase in the number of pDCs, defined as $CD45^{+}$ $F4/80^{-}CD11c^{Int}$ $PDCA\text{-}1^{+}$ cell populations following challenge with *A. fumigatus*. In addition, we confirmed that administration of 120G8 results in profound depletion of pDCs in the lungs.

pDCs play a non-redundant role in host defenses against *A. fumigatus*. In order to determine whether pDCs play roles in vivo during *A. fumigatus* infection, mice were treated with either the pDC-depleting mAb 120G8 or a control mAb and then challenged with *A. fumigatus*. Our results show that mice depleted of pDCs were more susceptible to *A. fumigatus* infection, regardless of whether the challenge was via the pulmonary or intravenous route (Figure 3.12). As pDCs are the major producer of type I IFNs, Next, we examined the susceptibility of $IFN\alpha/\beta R^{-/-}$ mice to invasive aspergillosis. We found the KO mice were significantly more susceptible compared with their wild-type counterparts, although not as susceptible as mice which were depleted of pDCs.

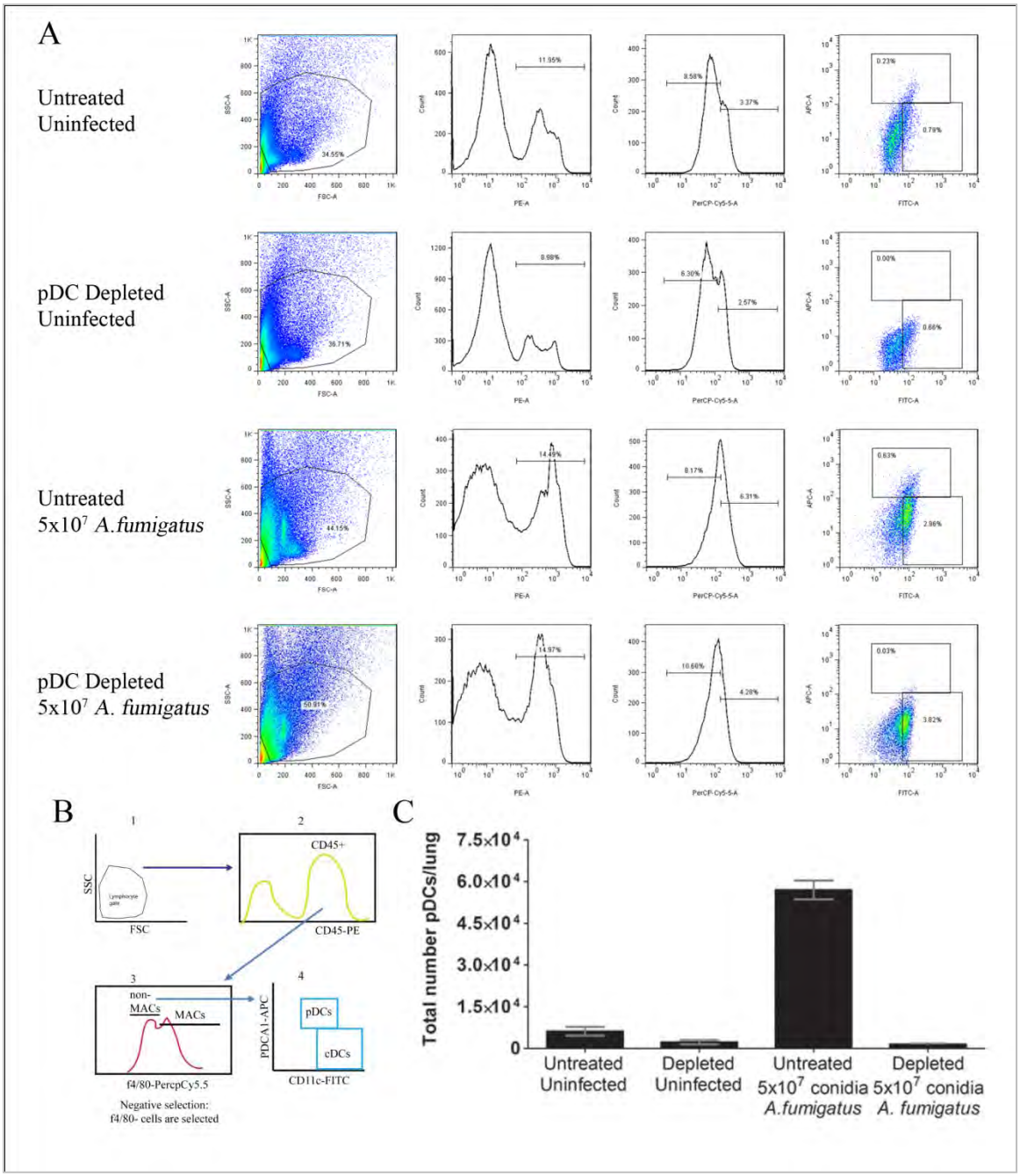
A
Untreated
Uninfected
pDC Depleted
Uninfected
Untreated
5x10^7 A.fumigatus
pDC Depleted
5x10^7 A. fumigatus
B
1
2
SSC
FSC
Lymphocyte gate
CD45+
CD45-PE
3
4
non-MACs
MACs
f4/80-PercpCy5.5
PDCA1-APC
pDCs
cDCs
CD11c-FITC
Negative selection:
f4/80- cells are selected
C
Total number pDCs/lung
7.5x10^4
6.0x10^4
4.5x10^4
3.0x10^4
1.5x10^4
0
Untreated Uninfected
Depleted Uninfected
Untreated 5x10^7 conidia A.fumigatus
Depleted 5x10^7 conidia A. fumigatus

Figure 3.11. pDCs are recruited into the lungs of *A. fumigatus* infected mice. Age and sex matched C57Bl/6J mice were either pretreated with 250 µg of 120G8 pDC depleting antibody via i.p. injection or left untreated. 24-hours later mice received a second dose of 150 µg of pDC depleting antibody. Following the second i.p. injection, mice were infected with $5x10^7$ *A. fumigatus* conidia via o.t. infection or mock infected with PBS/0.01% Tween 20. Mice were euthanized 48 hours post infection. Lungs were harvested and collagenase and DNAse digested for 1 hour at 37°C. Total number of lung cells was determined by counting on a hemocytometer. Then the lung cells were stained for the following cell surface markers: CD45-PE, F4/80-PercpCy5.5, CD11c-FITC and PDCA1- APC. Stained cells were analyzed by flow cytometry (A) following the diagram depicted above (B). pDC cell counts (C) were calculated using the following equation: (total number of cells*%pDC from total file)/% lymphocytes. Data represent means +/- SEM of one experiment performed with n=4 mice per group. $p<0.0001$ when comparing the total number of pDC of uninfected or depleted mice to the total number of pDCs of untreated/infected group.

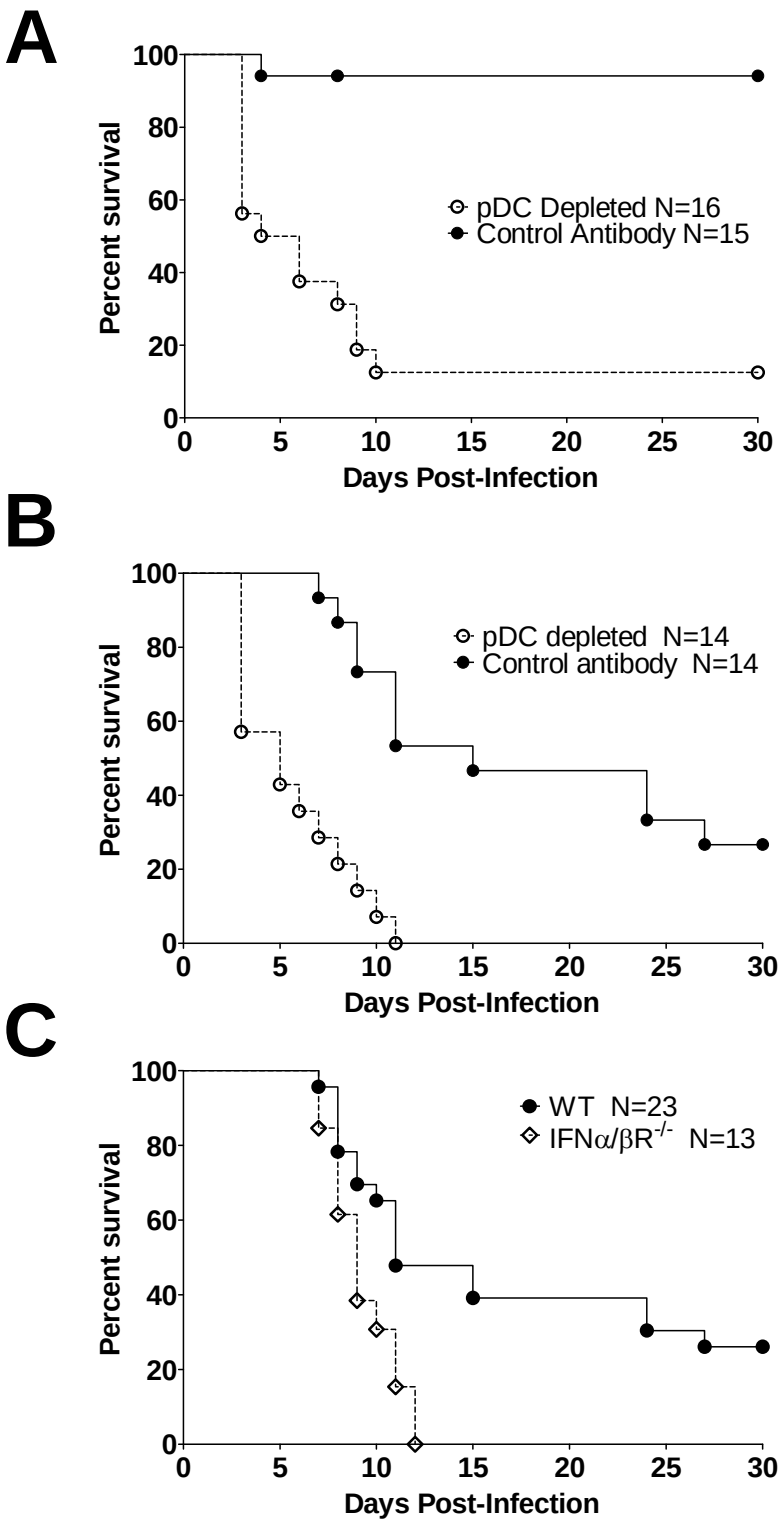
A
100
80
60
40
20
0
Percent survival
pDC Depleted N=16
Control Antibody N=15
0
5
10
15
20
25
30
Days Post-Infection
B
100
80
60
40
20
0
Percent survival
pDC depleted N=14
Control antibody N=14
0
5
10
15
20
25
30
Days Post-Infection
C
100
80
60
40
20
0
Percent survival
WT N=23
IFNα/βR-/- N=13
0
5
10
15
20
25
30
Days Post-Infection

Figure 3.12. pDCs are required *in-vivo* during *A. fumigatus* infection. C57Bl/6J mice were infected with $5x10^7$ *A. fumigatus* conidia via o.t. infection (A) or $1x10^6$ conidia via i.v. injection (B and C). (A and B) Mice were pretreated with 250 µg of 120G8 monoclonal pDC depleting antibody or control antibody via i.p. injection 24 hours prior to infection. On the day of the infection, mice were treated with 150µg of either antibody followed by infection with *A. fumigatus*. Mice were then injected with 150 µg of either depleting antibody or control antibody every other day for 14 days. (C) IFNα/βR$^{-/-}$ mice (which lack receptors for type I IFN) and WT mice were infected with $1x10^6$ *A. fumigatus* conidia via i.v. infection. All groups were followed for survival for up to 30 days. Data represent combined survival curves of 2 independent experiments, each with similar results. $p<0.0001$ when comparing survival of pDC depleted mice vs control antibody treated mice. $p<0.003$ when comparing survival of INFα/βR$^{-/-}$ mice to WT mice.

Discussion:

Innate responses of phagocytes are thought to be paramount to host defenses against *A. fumigatus*. Neutrophils, monocytes, macrophages and conventional DCs have been shown to recognize and exert antifungal responses that promote clearance of this opportunistic fungus (Bozza et al. 2009; Diamond et al. 1983; Feldmesser 2006; Ibrahim-Granet et al. 2003; Latge 1999; Mezger et al. 2008; Serbina et al. 2009). Here we show that human pDCs inhibit the growth of *A. fumigatus* hyphae and produce cytokines capable of activating and recruiting other immune cells.

Hyphae, the tissue invasive form of *A. fumigatus*, rapidly grow to a size that precludes phagocytosis. However, upon incubation of pDCs with *A. fumigatus*, we found that within two hours, pDCs had spread over the hyphal surface. While the repertoire of surface receptors on pDCs associated with fungal recognition has not been well defined, human pDCs have been shown to express dectin-2, but not dectin-1, mannose receptor and DC-SIGN (Graham and Brown 2009; Meyer-Wentrup et al. 2008). Moreover, human pDCs express some complement and Fc receptors, although we found that hyphal recognition did not require opsonization. Future studies are needed to define receptor(s) and their cognate ligand(s) responsible for recognition of *A. fumigatus* hyphae by pDCs.

Other cell types, including neutrophils and monocytes, spread over hyphae and cause damage to *A. fumigatus* by oxidative and non-oxidative mechanisms (Diamond et al. 1983; Diamond et al. 1978). Using two independent assays, one that assesses

metabolic activity and the other that directly measures hyphal elongation; we demonstrated that human pDCs have antifungal activity against *A. fumigatus*. However, as opposed to the situation with neutrophils and monocytes, where both growth inhibition and killing of *A. fumigatus* have been demonstrated, we only found evidence for growth inhibition following incubation of hyphae with pDCs. Thus, hyphal growth proceeded, but at a significantly slower rate, in the presence of pDCs.

In the absence of activating signals, pDCs reportedly undergo spontaneous apoptosis (Grouard et al. 1997; Lepelletier et al. 2010). Interestingly though, after a 2 hours incubation of pDCs with *A. fumigatus* hyphae, over half of the pDCs died. Moreover, pDC cell lysates had antifungal activity against *A. fumigatus* and some antifungal activity was retained even if the pDCs were separated from the hyphae by a 0.4 μm permeable insert (Transwell). These observations strongly suggest that diffusible, preformed mediators were responsible for the antifungal activity. The essential role of Fe^{3+} and Zn^{2+} as fungal growth factors along with the known presence of chelators of these cations in other leukocyte populations (Mambula et al. 2000; Okutomi et al. 1998; Urban et al. 2009), led us to examine whether supplemental Fe^{3+} or Zn^{2+} would reverse the pDC-mediated growth inhibition. The observation that $ZnCl_2$ (but not $FeCl_3$) partially restored fungal growth suggests a role for the Zn^{2+} binding protein, calprotectin (Urban et al. 2009). Neutrophils contain large amounts of cytoplasmic calprotectin. Free and NET-associated calprotectin, released from dying neutrophils, inhibit the growth of *C. albicans* and other fungi by chelating zinc (Lulloff et al. 2004). Recently, NETs were

shown to have antifungal activity against *A. fumigatus* (Bruns et al. 2010). Although it is unknown whether a process similar to NETosis occurs with pDCs, we do demonstrate that human pDCs contain calprotectin. It is important to emphasize that Zn^{2+} supplementation only partially restored hyphal growth, suggesting that the antifungal activity of pDCs could be mediated by more than one pathway.

Two lines of evidence strongly suggest that the high rate of pDC cytotoxicity following incubation with *A. fumigatus* hyphae is at least partially due to secreted factors released by the fungi. First, pDC cytotoxicity was observed (albeit at a lower level) when the pDCs and hyphae were separated by a Transwell. Second, pDC cytotoxicity was significantly reduced following incubation with hyphae from *A. fumigatus* strains genetically engineered to be deficient in gliotoxin production. Moreover, purified gliotoxin at concentrations found in the lungs of patients with invasive pulmonary aspergillosis killed pDCs in a dose-dependent manner (Lewis et al. 2005; Stanzani et al. 2005).

Gliotoxin is a low molecular weight mycotoxin secreted by many fungal species including *A. fumigatus* (Bok et al. 2006; Kupfahl et al. 2008; Sutton et al. 1994; Yoshida et al. 2000). Induction of apoptosis by gliotoxin has been described in many cell types, including PMNs and monocytes (Stanzani et al. 2005). To dissect the mechanism of pDC cell death induced by *A. fumigatus* hyphae, we performed a TUNEL assay. The majority of pDCs incubated with hyphae or purified gliotoxin was TUNEL positive,

suggesting that the pDCs are dying by apoptosis. However, recent studies have shown that cells undergoing pyroptosis may also exhibit degradation of DNA and a positive TUNEL reaction (Fink et al. 2008; Fink and Cookson 2006). The finding that pDCs release cytokines when stimulated with hyphae points to their undergoing an inflammatory (rather than an apoptotic) cell death, although it is possible that the fraction of pDCs which remain viable is responsible for the cytokine secretion.

pDCs secrete large amounts of type I IFN in response to viral infections and certain DNA and RNA sequences (Cao and Liu 2007; Pietras et al. 2006). Recent reports suggest that type I IFNs may play a role during fungal infections (Biondo et al. 2008; Inglis et al. 2010b). Compared with wild-type mice, mice lacking the IFNα/β receptor died after challenge with *Cryptococcus neoformans* and failed to develop protective Th1 cytokine responses (Biondo et al. 2008). Moreover, *Histoplasma capsulatum* conidia were shown to induce type I IFN responsive genes in macrophages (Inglis et al. 2010b). While Romani et al. did not find INFα secretion by pDCs stimulated by *A. fumigatus* resting conidia (Romani et al. 2004), we found that the tissue invasive hyphal morphotype stimulates pDCs to release INFα. Furthermore, induction of type I IFN appears to be independent of TLR7 and TLR9 as inhibition of endosomal acidification had no effect on hyphae-stimulated IFNα production. This is perhaps not surprising given that hyphae are extracellular and TLR7 and TLR9 are endosomally localized. TLR-independent cytosolic viral sensors that lead to type I IFN responses have been identified, including RIG-1 (retinoic acid-inducible gene) and MDA-5 (melanoma

differentiation-associated gene 5) (Goutagny et al. 2010). In addition, surface receptors on pDCs that down-modulate the type I IFN response have been described (Lande and Gilliet 2010). However, the stimulatory receptors and downstream signaling pathways that enable pDCs to signal in response to *A. fumigatus* hyphae remain to be elucidated.

In an *in-vivo* infection model, we observe that mice lacking pDCs are more succeptible to *A. fumigatus* infection than their control counterparts suggesting that pDCs play a role in the antifungal response against the mold. Our data show that both pDCs and type I INF are important in the clearance of *A. fumigatus*. Furthermore, our data suggest that pDCs may be more important than type I IFN in the clearance of the mold, as mice depleted of their pDCs succumb to the infection faster that the IFNα/βR$^{-/-}$ mice. In addition, we observe an approximately 5-fold increase in the total number of pDCs in the lungs of infected mice demonstrating that these cells are recruited to the site of infection. Thus, our data shows in an in-vivo infection model that pDCs play a nonredundant role in host defenses against *A. fumigatus*. While the studies with IFNα/βR$^{-/-}$ implicate a contribution of pDC-derived type I IFNs, future studies are required to determine the exact mechanisms by which pDCs mediate protection. Moreover, pending pathology studies should help resolve whether the observed survival trends are a result of damage caused by the fungus or due to an overly exuberant host inflammatory response.

Thus, our data demonstrate a putative role for pDCs in host defenses against fungal infections. The host-pathogen interaction between pDCs and *A. fumigatus* has

novel, yet seemingly paradoxical, features. The significant antifungal activity of pDCs against *A. fumigatus* hyphae appears to be dependent, at least in part, on dying pDCs releasing antifungal effector molecules, such as zinc chelators. This process is enhanced by fungal release of cytotoxic molecules, including gliotoxin, which induce apoptosis or pyroptosis of the pDCs. Release of cytokines by fungal stimulated pDCs may serve to recruit and activate other immune cells, thereby boosting innate responses and helping to initiate adaptive immunity.

Materials and Methods:

Ethics statement. Informed consent was obtained from healthy volunteers using a protocol approved by the University of Massachusetts Medical School Institutional Review Board.

Reagents and cell culture. Reagents were obtained from Sigma-Aldrich (St Louis, Missouri), unless otherwise stated. DMEM and RPMI-1640 without phenol red were obtained from GIBCO (Invitrogen; Carlsbad, California). pDC media consisted of RPMI-1640 without phenol red supplemented with 100U/ml penicillin, 100U/ml streptomycin, 2mM L-glutamine, 0.5mM HEPES, 1mM sodium pyruvate and 10% autologous serum. All incubations were in a 37°C incubator containing humidified air supplemented with 5% CO_2, unless otherwise indicated. The stimulatory TLR9 ligand, CpG 2007 oligonucleotide (ODN) (Latz et al. 2007a), was synthesized with phosphothiorate linkages by Integrated DNA Technologies (Coralville, IA). Ultrapure LPS from *Escherichia coli* was generated as previously described (Huang et al. 2009). All antibodies were purchased from eBiosciences (San Jose, CA), unless otherwise specified. Monoclonal antibody against human calprotectin (also known as Calgranulin A/B; clone 2Q396C) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

***A. fumigatus* strains and culture.** Wild-type *A. fumigatus* strain 293 was obtained from the Fungal Genetic Stock Center, Kansas City, MO. Δ*GliZ*, *GliZ* Comp, Δ*LaeA*, and *LaeA* Comp (Bok et al. 2005; Bok and Keller 2004; Bouhired et al. 2007; Hohl et al.

2005) were generous gifts of Dr. Nancy Keller (University of Wisconsin, Madison). These genetically manipulated strains were all on the 293 background. Table 1 contains a description of the fungal strains used. There were no differences in growth rates among the different strains as determined using the XTT assay (data not shown). Except where otherwise noted, strain 293 was used in the studies.

Cultivation of *A. fumigatus*, harvesting of conidia and growth into hyphae was performed as in our previous studies with slight modifications (Mambula et al. 2002). Briefly, fungi were grown on Sabouraud Dextrose Agar slants at 37°C for 4 to 7 days. Conidia were harvested by adding PBS containing 0.01% Tween 20 followed by vortexing. The collected conidia were then filtered through a 40-μm nylon mesh to remove hyphae and conidiophores. The conidia in the filtrate were washed twice with phosphate buffered saline (PBS), counted and stored in water at 4°C for up to a week. To generate hyphae, conidia were incubated at 21^{o}C for 16 hours in pDC medium to swell the conidia, and then an additional 3 hours at 37°C to promote germination into hyphae.

Isolation of human pDCs and autologous sera. Human pDC were isolated as described (Ramirez-Ortiz et al. 2008; Wang et al. 2006). Peripheral blood was collected by venipuncture. A portion was clotted and the autologous serum collected following centrifugation. The remainder of the blood was anticoagulated with heparin, diluted 1:1 with Hank's buffered salt solution (HBSS) (BioWhittaker; Walkersville, MD) and the peripheral blood mononuclear cells (PBMCs) purified by Ficoll-Hypaque

(Lymphoprep™; Westbury, NY) density gradient centrifugation. Human pDCs were positively selected from the PBMC using CD304-coated magnetic beads, according to the manufacturer's instructions (Miltenyi Biotech, Auburn, CA). For some experiments, the negatively selected "flow through" cells, consisting of PBMCs depleted of $CD304^+$ cells, were collected too. pDC lysates were generated by hypotonic lysis of the pDCs. $CD304^+$ pDCs were washed 3 times with PBS, resuspended in sterile distilled water and then incubated at 37°C for 30 minutes. Lysis was verified by microscopy.

The high purity of the pDC population (Figure 3.1) was confirmed by analyzing the expression of the pDC markers CD123 and CD303 by flow cytometry (Colonna et al. 2004). Briefly, freshly isolated pDCs were incubated on ice with anti-CD123-APC and/or anti-CD303-FITC for 30 minutes. Non-specific binding was reduced by using Fc Block (Becton Dickinson). Cells were fixed with 2% paraformaldehyde and analyzed using a Becton Dickinson LSRII flow cytometer (BD Biosciences, San Jose CA). Isotype controls were included in all experiments.

XTT assay. XTT assay was performed as described (Meshulam et al. 1995). Briefly, *A. fumigatus* conidia were plated in 96-well half area plates and allowed to germinate in pDC media to hyphae of 10-20 µm average length. The hyphae were preopsonized by incubation in 10% autologous serum for 30 minutes followed by washing. Experiments comparing preopsonized and unopsonized hyphae yielded similar results (data not shown). pDCs were then added to the hyphae in a final volume of 100 µl pDC media.

For some experiments, where indicated, pDCs lysates were added in lieu of live pDCs or the experiments were conducted in Transwell chambers rather than half area wells. Following a 2 hour incubation, the pDCs were subjected to hypotonic lysis by three gentle washes and 20 minute incubation with sterile distilled water. Supernatants then were removed, taking care not to remove the hyphae. pDC media without serum containing 400 µg/ml of XTT and 50 µg/ml of Coenzyme Q were added and the wells were incubated for 2 hours at 37°C. The OD_{450} and OD_{650} were then measured and data expressed as percent antifungal activity according to the following formula:

$$\% \text{ Antifungal Activity} = \left[1 - \left(\frac{OD_{Af+pDCs} - OD_{pDCs}}{OD_{Af} - OD_{Blank}}\right)\right] \times 100$$

$OD_{Af + pDC}$ is $(OD_{450} - OD_{650})$ of wells containing *A. fumigatus* hyphae with pDCs. OD_{pDC} is $(OD_{450} - OD_{650})$ of wells containing pDCs. OD_{Af} is $(OD_{450} - OD_{650})$ of wells containing *A. fumigatus* hyphae alone. OD_{blank} is $(OD_{450} - OD_{650})$ of wells containing media alone.

Hyphae Growth Inhibition Assay. *A. fumigatus* hyphae were incubated in pDC media at 37°C in 8-well coverslip chambers (Nunc, Rochester NY) with or without pDCs. Where indicated, pDC media were supplemented with 10 mM $ZnCl_2$ or 10mM $FeCl_3$. At the specified times, pDCs were lysed with water and hyphae were fixed with 2% paraformaldehyde. Hyphal length was then measured by microscopy (Nikon Eclipse TE200) using software (SOFTMax Advace, SPOT Imaging Solutions) equipped with a

curved and calibrated cursor. A minimum of 10 visual fields containing at least 75 hyphae were scored per group.

Cytotoxicity Assay. The LDH release assay (Roche Applied Science, Indianapolis IN) was performed and analyzed according to the manufacturer's instructions. Briefly, pDCs were incubated with or without preopsonized *A. fumigatus* or gliotoxin for 2 hours in 150 µl pDC media. Following centrifugation, supernatants were collected, incubated with an equal volume of developing solution for 25 minutes at room temperature and the OD_{492} was measured. The maximum LDH released was determined by lysing pDCS with Triton X-100. All samples were measured in triplicate.

Confocal Microscopy. Preopsonized *A. fumigatus* hyphae in 35mm tissue culture slide dishes (Mattek Corporation, Andover, MA) were incubated with pDCs in pDC media for 2 hours at 37^{o}C. Cell surface staining was then performed by incubating with the pDC specific antibody CD123-efluor650 (eBiosciences, San Jose CA) for 30 minutes at 0^{o}C. Following three washes with PBS supplemented with 2% FBS, fungal cell walls were stained with the chitin-specific fluorescent dye, 1% Uvitex 2B (Polysciences, Warrington PA), by incubation for 20 minutes at 0^{o}C. Samples were fixed with 2% buffered paraformaldehyde and visualized by confocal microscopy using a Leica SP2 AOBS confocal microscope (Leica Microsystems, Bannockburn IL) with a 63X plan apochromatic objective (Zeiss).

Transwell Assay. Transwell 96 well plates containing 0.4 μm pore size polycarbonate membrane inserts were obtained from Corning Life Sciences (Lowell, MA). *A. fumigatus* conidia ($5x10^5$ in 100μl) were added to the surface of the wells and germinated to hyphae as described above. pDCs ($5x10^5$ in 100μl) were then added either below (allowing direct contact with hyphae) or above (allowing no contact with hyphae) the insert. After 2 hours incubation, pDC cytotoxicity and antifungal activity were assayed as described above.

TUNEL Assay. TUNEL assay was performed following the manufacturer's instructions (Molecular Probes, Eugene OR). Briefly, pDCs and *A. fumigatus* hyphae were incubated for 2 hours at 37°C in tissue culture chamber slides. Cells were fixed with 2% paraformaldehyde for 20 minutes at room temperature. pDCs were stained for the surface marker CD123 (CD123-efluor650) for 30 minutes at 0°C. After washing 3 times, cells were permeabilized and stained for fragmented DNA by the Click-it reaction (Alexafluor 594). After additional washes, total cellular DNA was stained using Hoechst 33342. All samples were visualized by confocal microscopy, as described above.

Mice. Wild-type (C57Bl/6J) mice were purchased from Jackson Laboratories (Bar Harbor, ME). IFNα/β$R^{-/-}$, which lack type I IFN receptors,were a kind gift from Jon Sprent and were backcrossed to the C57Bl/6J background for at least 12 generations (Kolumam et al. 2005; Thompson et al. 2006; Wang et al. 2010) . Mice were 6 to 8 weeks old at the start of the experiments and all groups were matched for sex and age.

Mice were maintained under microisolation conditions at the University of Massachusetts Medical School under a protocol approved by the Institutional Animal Use and Care Committee.

For oro-tracheal infections (OT), mice were anesthesized with Isofluorane and subsequently infected with *A. fumigatus* conidia suspended in 100 µL of sterile PBS supplemented with 0.01% Tween20 and administered in two doses of 50 µL each. For intravenous (i.v.) infections, mice were injected by the tail vein with 10^6 *A. fumigatus* conidia suspended in sterile PBS supplemented with 0.01% Tween 20 (Bellocchio et al. 2004a; Werner et al. 2009). For survival studies, infected mice were monitored at least daily for 30 days. Moribund mice were euthanized.

pDC Influx Assay. Mice were infected OT as described above. 48-hours post infection, mice were euthanized by CO_2 inhalation and their pulmonary arteries were perfused with PBS. Lungs were harvested, minced and incubated with 1 mg/mL of Collagenase type IV (Sigma Aldrich, MO) and 75 µg/mL bovine pancreatic DNAse (Sigma) supplemented with 5% FBS in PBS for 1 hour at 37°C (Wozniak et al. 2006). Following digestion, cells were passed through a 70µm filter in order to obtain single cell suspensions. Cells were counted and stained for flow cytometry using the following markers: PDCA-1, CD11c, CD45 and F4/80 (all antibodies were obtained from eBiosciences, San Diego CA) (Ang et al. 2010). In order to establish the specificity of the antibodies, isotype controls were performed and analyzed for each antibody used. Samples were analyzed

using BD LSRII (Becton Dickinson, San Jose, CA) and data were analyzed using Flojo 7.6 version for PC (Tree Star Inc, Ashland, OR).

pDC depletion. pDC depletion in mice was achieved by intraperitoneal injection (i.p.) with monoclonal rat anti-mouse IgG 120G8 pDC depleting antibody or monoclonal rat anti-mouse IgG control antibody GL113 againt *E.coli* β-galactosidase (Ang et al. 2010) (Gift of Dr. Louis Boon, Bioceros). Briefly, mice were injected with 250µg of either 120G8 or GL113 the day before infection (day -1). At day 0, mice received an additional dose of 150µg antibody. Following treatment mice were infected with *A. fumigatus* as described above. In order to maintain depletion, mice were treated with 150µg of antibody every 48 hours until day 14 post-infection.

Cytokine Secretion. The indicated concentrations of pDCs or flow through cells were incubated with *A. fumigatus* hyphae in 96-well flat-bottom plates containing a final volume of 200 µl pDC media supplemented with 10% autologous serum. After 6 hours, supernatants were collected and human IFNα and TNFα levels were measured using ELISA kits (Bender MedSystems module set, Burlingame, CA for IFNα and eBiosciences, San Diego, CA for TNFα) according to the manufacturers' instructions. Samples were assayed in duplicate at dilutions that fell within the range of the standard curve.

For the inhibition experiments, pDCs were left untreated or incubated for one hour with either 10 µg/ml of bafilomycin A or 10 µg/ml of chloroquine. pDCs were then incubated for 6 hours with preopsonized *A. fumigatus* hyphae, CpG or LPS in a final

volume of 200 µl of pDC media without serum. Samples were then assayed for IFNα by ELISA as described above.

Statistical Analysis. For comparisons of two groups, means ± standard errors (SE) were analyzed by the two-tailed unpaired Student *t*-test with the Bonferroni correction applied when making multiple comparisons. For comparisons of greater than two groups, significance was determining using the one- or two-way analysis of variance (ANOVA) with the Tukey multiple correction. Calculations were performed using a statistical software package (GraphPad Prism 4.02, San Diego, CA) and statistical significance was defined as $p<0.05$.

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Preface

Chapter IV

The data presented in this chapter has not been published

Zaida G. Ramirez-Ortiz, Charles A. Spetch and Stuart M. Levitz "Innate Immune Responses of Mature pDCs Against *Aspergillus fumigatus* conidia"

All the work presented in this chapter was performed by **Zaida G. Ramirez-Ortiz**

Chapter IV

Innate Immune Responses of Mature pDCs Against *Aspergillus fumigatus* conidia

Abstract

Upon maturation, pDCs acquire characteristics similar to cDCs. While we showed that freshly isolated pDCs are capable of inducing an antifungal response against *A. fumigatus*, the role of mature pDCs during infection remains unclear. Here, $CD304^{+}$ human pDCs, isolated from the blood of donors were matured by incubation with IL-3, CD40L and GM-CSF for seven days. Our results show that $CD304^{+}$ mature pDCs produced Type I IFN and TNFα in response to incubation with live *A. fumigatus*. As pDCs are characterized by being $TLR4^{-}$ and $TLR9^{+}$, cells were tested with ultrapure LPS and CpG DNA as purity controls. Cytokines were detected upon CpG DNA, but not LPS stimulation, suggesting that the cell population was not contaminated with macrophages or cDCs. Purity of the cell population was confirmed by flow cytometry. These data demonstrate that *A. fumigatus* is capable of stimulating cytokine secretion in mature human pDCs.

Introduction

Over the last three decades, *Aspergillus fumigatus* has emerged as the most common cause of invasive mold infections in the immunocompromised host (Sherif and Segal 2010). *A. fumigatus* can be found widely distributed in the environment. The small airborne spores or conidia are inhaled into the lungs, where in virtue of their small size, they can reach the alveoli. In the immunocompetent host, the conidia are readily cleared by resident immune cells of the pulmonary system. However, in the appropriate host, conidia can escape recognition by cells of the immune system and they can germinate to establish an infection (Hosseini-Moghaddam and Husain 2010).

Germination of *A. fumigatus* starts when resting conidia encounter an appropriate niche. Given nutrients and water, resting conidia begin to grow and germinate (Rhodes 2006). First, the resting conidia undergo a period of isotropic growth. The outermost layer of the resting conidia is characterized by the presence of hydrophobins and melanin, both of which protect *A. fumigatus* from environmental distress and enable the mold to form a more efficient aerosol (Paris et al. 2003; Pihet et al. 2009). One of the first steps of germination is the uncoating of this hydrophobic outer layer. Within a few hours, *A. fumigatus* conidia undergoing isotropic growth undergo changes in the cell wall composition. Swollen conidia exposes carbohydrate moieties such as β-(1-3)-glucans, β-(1,3-1,4)-glucans and chitin (Bernard and Latge 2001; Gastebois et al. 2009). Exposure of these carbohydrate moieties, upon conidial swelling, enables phagocytes to recognize and kill *A. fumigatus*. Once the conidia have undergone swelling, *A. fumigatus* switches

to polarized growth (Rhodes 2006). During this polarized growth, germ tubes increase their adhesion properties. The fast growing rate of germ tubes gives rise to hyphae, the invasive form of the mold.

Phagocytes are essential in the clearance of *A. fumigatus*. Upon inhalation, alveolar macrophages, dendritic cells (DCs) and neutrophils are the first line of defense against *A. fumigatus* conidia. These phagocytes are capable of phagocytosing and killing the spores in the phagolysosome. In some cases, conidia escape recognition by the alveolar macrophages and germinate into hyphae. Neutrophils are responsible of eliminating hyphae via oxidative and non-oxidative mechanisms. Immunocompromised hosts, particularly those that are neutropenic, are extremely susceptible to developing aspergillosis (Hohl and Feldmesser 2007a; Levitz 2004; Shoham and Levitz 2005).

Plasmacytoid dendritic cells (pDCs) are characterized by secreting large amounts of type I Interferon (IFN) (Cella et al. 1999). Initially pDCs were described as plasmacytoid T cells based on their plasma-cell-like morphology and the presence of CD4. pDCs have been extensively characterized over the last decade. Expression of the interleukin-3 receptor (IL3 or CD123), blood dendritic cell antigen-2 (BDCA-2 or CD303) and ILT7 distinguishes pDCs from other cells in the blood (Dzionek et al. 2001; Swiecki and Colonna 2010). In addition, pDCs express CD4, major histocompatability complex (MHC) class II, CD68 and CD2 (Colonna and Cella 2007). However, human pDCs lack the lineage markers CD3, CD19, CD14 and the DC marker CD11c. pDCs are generated in the bone marrow, where they are released into the blood in very small

number. These cells make less than 0.5% of the total peripheral blood mononuclear cells (PBMCs). pDCs can be found mainly in T-cell areas such as the spleen and lymph nodes, in mucosal associated tissues and thymus (Barchet et al. 2005; Kreisel et al. 2006).

Immature pDCs can readily be found in the blood. In their non-active state, pDCs are incapable of inducing T-cell proliferation. However, upon encountering an antigen they become activated. Upon activation, pDCs secrete large amounts of type I IFNs. In addition, mature pDCs acquire DC morphology, upregulate MHC and T-cell costimulatory molecules that enables pDCs to directly activate naïve T-cells (Lande and Gilliet 2010). When in culture, freshly isolated pDCs can be matured by incubation with IL3 or IL3 plus CD40L (Grouard et al. 1997). Maturation of pDCs in-vitro by CD40L or viral infection results in the formation of a functionally distinct type of DC also known as DC2 (Cella et al. 2000). These mature pDCs secrete bioactive IL12, and can induce Th2 responses by secretion of lL4. In vivo, pDCs migrate to the site of infection. Once pDCs encounter antigen, they acquire cDC-like characteristics such as antigen uptake, MHC upregulation and antigen presentation to T-cells in the lymph nodes. In vitro, maturation of pDCs does not affect their ability to generate and secrete cytokines; however, it has been suggested that in vivo, mature pDCs downregulate secretion of type I IFN and IL12 (Mittelbrunn et al. 2009).

pDCs secrete huge amounts of type I IFN, which have been shown to play an important role in the antiviral response (Gilliet et al. 2008). Secretion of type I IFN has been show to augment the expression of antiviral molecules and promote apoptosis of

infected cells. In addition, human pDCs are the only subset of DCs that express the endosomal TLRs, TLR7 and TLR9. These TLRs have been shown to recognize ssRNA and unmethylated CpG DNA, respectively (Ishii and Akira 2006; Vilaysane and Muruve 2009). Although a role for pDCs in the antifungal response has not been described to date, TLR9 has been shown to recognize and stimulate secretion of type I IFN in response to *A. fumigatus* DNA (Ramaprakash et al. 2009; Ramirez-Ortiz et al. 2008). However, whether pDCs are capable of inducing a cytokine response upon challenge with live *A. fumigatus* remains to be elucidated. Here we show that pDCs, both mature and immature, secrete cytokines in response to stimulation with *A. fumigatus* conidia.

Results

Mature pDCs spread over *A. fumigatus* hyphae

In order to determine whether mature pDCs recognized and interacted with *A. fumigatus*, we incubated matured pDCs with conidia for 18 hours and analyzed the interaction by light microscopy (Figure 4.1). While we incubated matured pDCs with *A. fumigatus* conidia, by the time we examined the cells, the conidia had already germinated into hyphae. Therefore, our results show that mature pDCs are capable of interacting and spreading over *A. fumigatus*.

Mature pDCs secrete proinflammatory cytokines in response to *A. fumigatus*

Since the microscopy suggested that there was a close interaction between *A. fumigatus* and the mature pDCs, we sought to determine whether mature pDCs were capable of stimulating a proinflammatory response against conidia. During infection, *A. fumigatus* can be encountered by the pDCs as either resting conidia or swollen conidia. First we sought to determine whether day 1, day 5 and day 7 mature pDCs induce an antifungal response against *A. fumigatus*; therefore, we analyzed cytokine secretion by pDCs challenged with either resting or swollen conidia. Our results show that *A. fumigatus* stimulates secretion of IFNα and TNFα in mature pDCs (Figure 4.2). Although day 1 mature pDCs secrete cytokines in response to *A. fumigatus*, optimal cytokine secretion for SC and RC was observed when pDCs were matured for 7 days in media

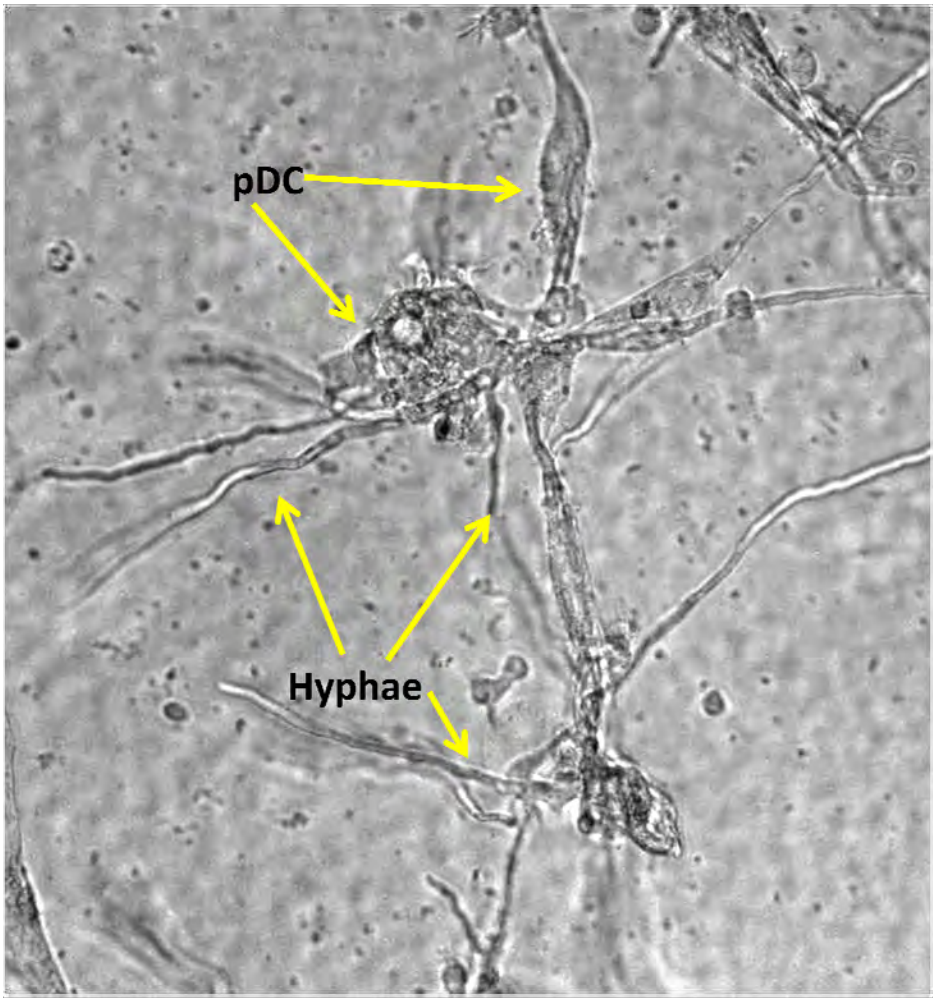
pDC
Hyphae

Figure 4.1: Mature pDCs Recognize and Interact with *Aspergillus fumigatus*. CD304+ pDCs ($5x10^4$ cells per well in 96-well plates) were matured for 7 days in RPMI supplemented with 10% FBS, 10ng/mL of recombinant IL-3, 10ng/mL CD40L and 150 ng/mL of GMCSF at 37°C. At day 7, cells were washed and the media was changed (RPMI supplemented with 10% autologous serum, 10ng/mL IL-3, 10ng/mL CD40L and 150 ng/mL GM-CSF). Consequently, pDCs were challenged with pre-opsonized *A. fumigatus* conidia for 18 hours at 37°C. Cell interactions were analyzed by microscopy. Photomicrograph represents 18 hours post-stimulation with swollen conidia. Sample visualized at 40X magnification.

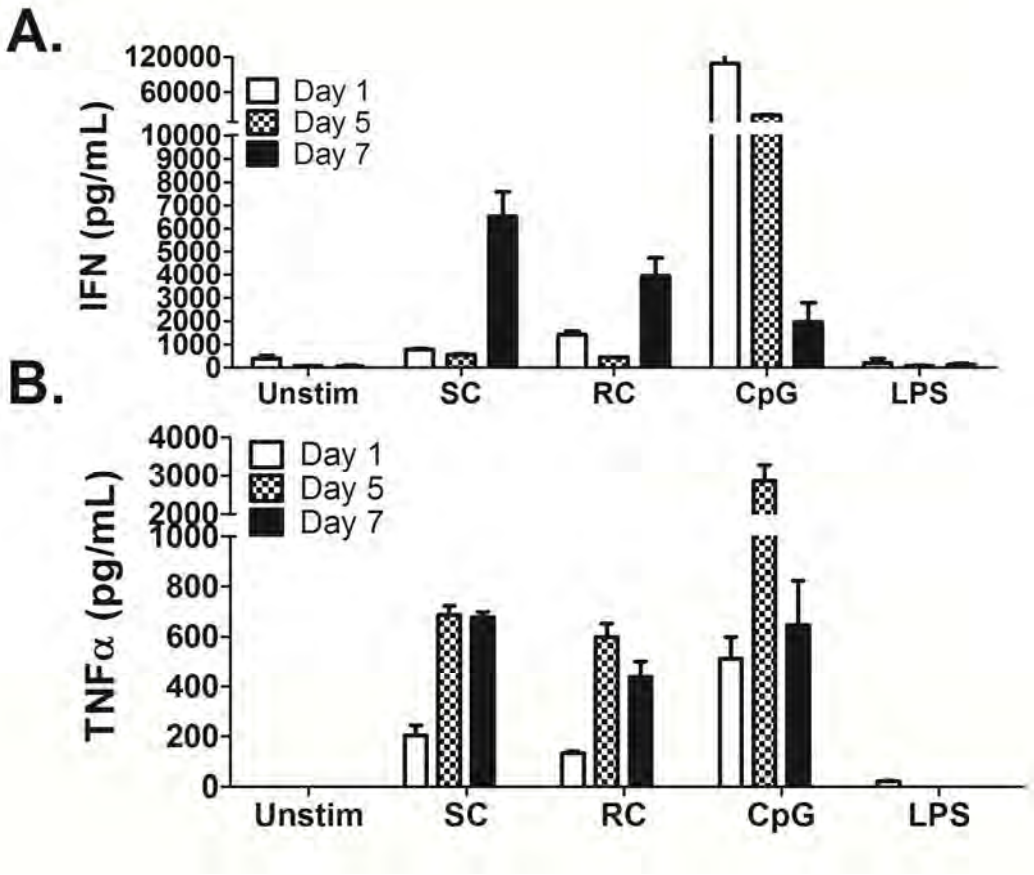
A.
IFN (pg/mL)
120000
60000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Day 1
Day 5
Day 7
Unstim
SC
RC
CpG
LPS
B.
TNFα (pg/mL)
4000
3000
2000
1000
800
600
400
200
0
Day 1
Day 5
Day 7
Unstim
SC
RC
CpG
LPS

Figure 4.2: Secretion of IFNα and TNFα by day 1, 5, 7 mature pDCs in response to *A. fumigatus*.

CD304+ pDCs (50,000/well in 96-well plates) were matured for 1, 5 or 7 days in RPMI supplemented with 10% FBS, 10 ng/mL of recombinant IL-3, 10 ng/mL CD40L and 150 ng/mL of GMCSF at 37°C. At the specified maturation day, pDCs were challenged with $5x10^4$ conidia per well (either swollen or resting) and incubated at 37°C for 6 hours. IFNα (A) and TNFα (B) secretion in supernatants were measured by ELISA. Data represent means ±SE of two independent experiments performed in duplicate.

SC- Swollen Conidia, RC- Resting Conidia

supplemented with IL-3, GM-CSF and CD40L. Based on our results, we decided to perform all the subsequent assays with day-7 mature pDCs.

Our results show that mature pDCs secrete IFNα, TNFα and IL-12p70 in response to both morphologies of *A. fumigatus* (Figure 4.3). Furthermore, our results show that mature pDCs secrete cytokines in response to *A. fumigatus* as early as 6 hours post-infection suggesting that mature pDCs recognize both conidial morphologies and induce a proinflammatory response against the mold. In addition, over the 6 hour and 18 hour incubation period, *A fumigatus* conidia undergoes morphological changes. Within 6 hours of incubation on a nutrient rich environment, the resting conidia will become swollen conidia and the swollen conidia will become hyphae. Within 18 hours both morphologies will germinate into hyphae. Therefore, the possibility of mature pDCs recognizing and secreting cytokines in response to hyphae cannot be eliminated.

A. fumigatus recognition by mature pDCs is independent of opsonization

The pattern of cytokine secretion by mature pDCs was similar for all three cytokines tested regardless of the *A. fumigatus* morphology encountered, suggesting that there may be another factor involved in the recognition of the conidia. In all our assays we have opsonized the conidia with autologous serum prior to addition to the mature pDCs. Since serum contains Fc receptors and antibodies that have been shown to coat *A. fumigatus*, we wondered whether opsonization of the conidia was responsible for the observed cytokine secretion pattern. Our results show that cytokine secretion by mature pDCs in

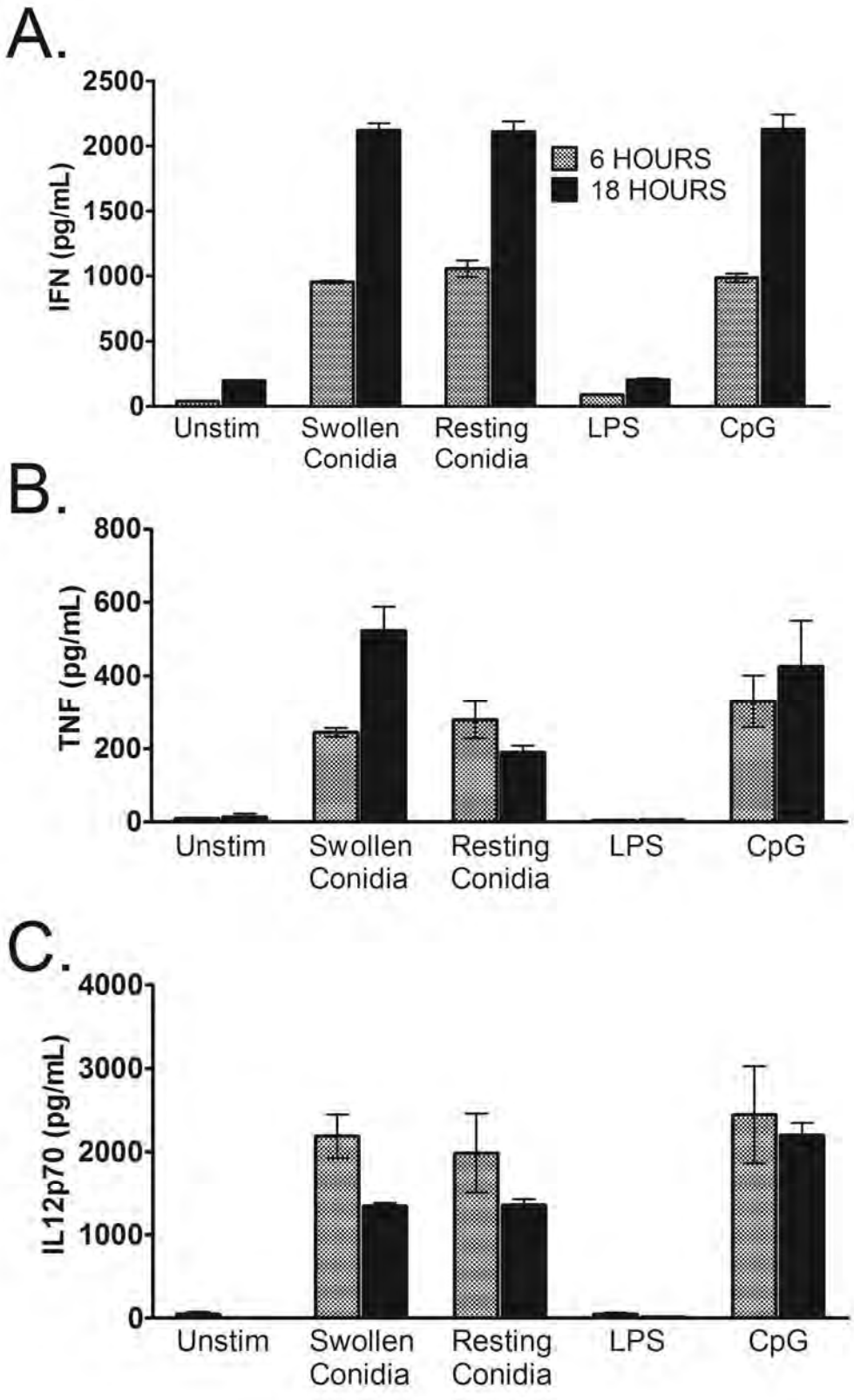
A.
IFN (pg/mL)
2500
2000
1500
1000
500
0
6 HOURS
18 HOURS
Unstim
Swollen Conidia
Resting Conidia
LPS
CpG
B.
TNF (pg/mL)
800
600
400
200
0
Unstim
Swollen Conidia
Resting Conidia
LPS
CpG
C.
IL12p70 (pg/mL)
4000
3000
2000
1000
0
Unstim
Swollen Conidia
Resting Conidia
LPS
CpG

Figure 4.3: Mature pDCs secrete cytokines within 6 hours of *A. fumigatus* challenge.

CD304+ pDCs (50,000/well in 96-well plates) were matured for 7 days in RPMI supplemented with 10% FBS, 10ng/mL of recombinant IL-3, 10ng/mL CD40L and 150 ng/mL of GMCSF at 37°C. At day 7, cells were washed and the media was changed (RPMI supplemented with 10% autologous serum, 10ng/mL IL-3 and 150 ng/mL GM-CSF). Consequently, pDCs were infected with opsonized swollen or resting *A. fumigatus* Af293 conidia and incubated at 37°C at 1:1 ratio. Supernatants were collected at 6 and 18 hours and analyzed by IFNα (A), TNFα (B) and IL12p70 (C) ELISA. Unstimulated cells and LPS (10 ng/mL) were used as negative and cell purity controls, respectively. CpG2007 (100 ng/mL) was used as a positive control. Data represent means ±SE of triplicate values. p value < 0.0001 when comparing 6 hours post- stimulation to 18 hours post-stimulation. Experiment was performed with 2 individual donors.

response to *A. fumigatus* conidia occurs independently of opsonization (Figure 4.4). Furthermore, our results show that secretion of IFNα and TNFα in response to un-opsonized *A. fumigatus* conidia was independent of the morphology encountered.

Only live *A. fumigatus* stimulates a cytokine response by mature pDCs

Since recognition and cytokine secretion by pDCs occurs via a mechanism independent of opsonization, we wanted to determine whether heat killed *A. fumigatus* was able to induce a response. Mature pDCs were capable of secreting IFNα only in response to live *A. fumigatus* (Figure 4.5). Our results show that mature pDCs challenged with low concentrations of heat killed *A. fumigatus* do not secrete cytokines; however, a response can be observed upon challenge with live conidia.

Mature pDC stimulation may be mediated by a fungal secreted factor

We noticed that there was no difference in the cytokine secretion profile of mature pDCs when incubated with *A. fumigatus* resting conidia or swollen conidia. As part of our method, the conidia were collected (resting) or swelled and stored at 4°C until ready to use, but not longer than two weeks. It was possible that the observed stimulation was due to a secreted fungal factor. In order to address whether there was a fungal factor secreted into the media capable of activating secretion of cytokines by mature pDCs, we incubated the mature pDCs with washed *A. fumigatus*, unwashed *A. fumigatus* or fungal supernatants. Our results show that fungal supernatants are capable of inducing a proinflammatory response in mature pDCs (Figure 4.6). Furthermore, *A. fumigatus* supernatants are capable of inducing a strong IL-6 response (Figure 4.6C). In addition,

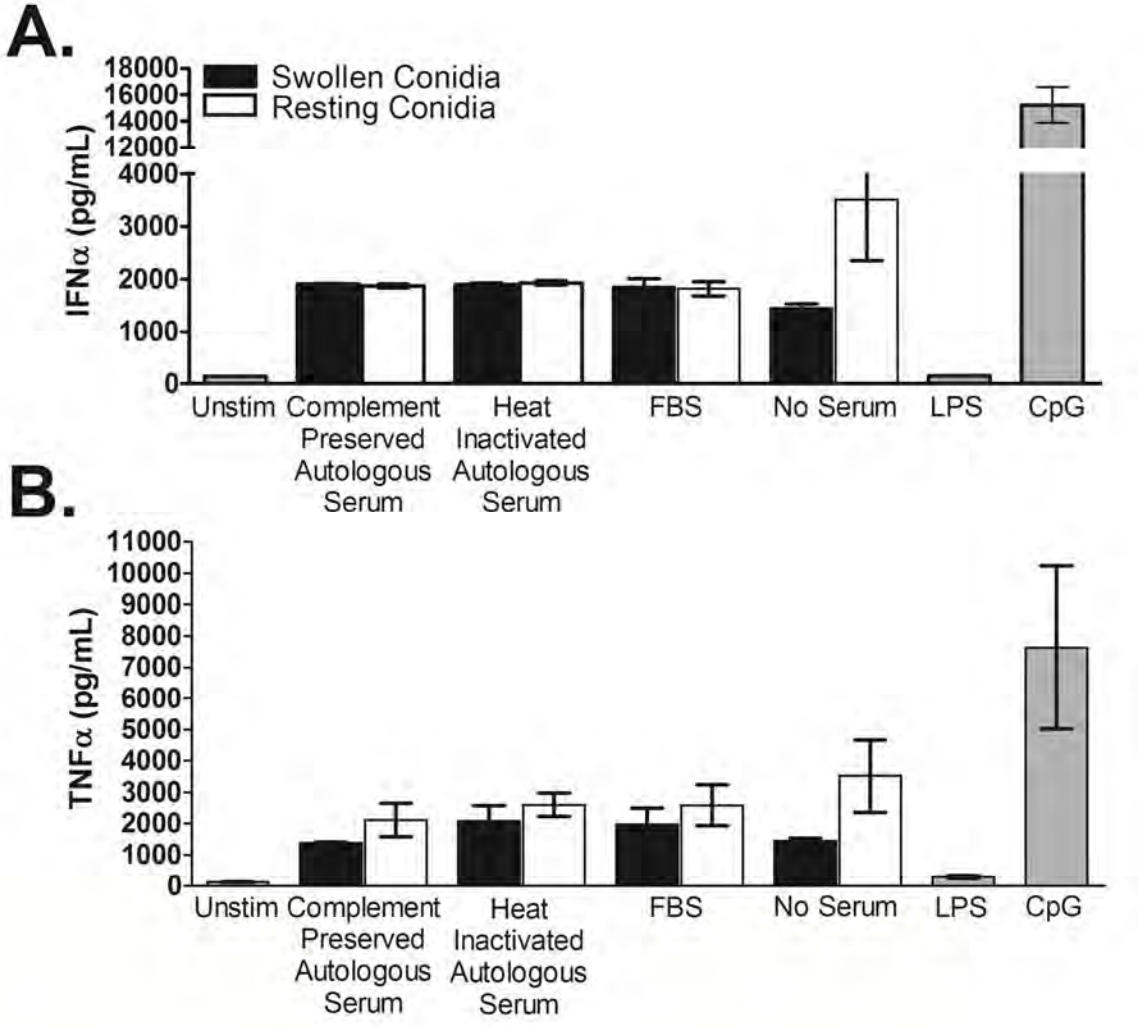
A.
Swollen Conidia
Resting Conidia
IFNα (pg/mL)
18000
16000
14000
12000
4000
3000
2000
1000
0
Unstim
Complement Preserved Autologous Serum
Heat Inactivated Autologous Serum
FBS
No Serum
LPS
CpG
B.
TNFα (pg/mL)
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Unstim
Complement Preserved Autologous Serum
Heat Inactivated Autologous Serum
FBS
No Serum
LPS
CpG

Figure 4.4: Stimulation of pDCs by *Aspergillus* conidia is independent of opsonization.

CD304+ pDCs (50,000/well in 96-well plates) were matured for 7 days in RPMI supplemented with 10% FBS, 10ng/mL of recombinant IL-3, 10ng/mL CD40L and 150 ng/mL of GMCSF at 37°C. pDCs were infected with either swollen or resting *A. fumigatus* Af293 conidia and incubated for 6 hours at 37°C at 1:1 ratio in the presence of the following treatments: 10% complement preserved autologous serum; 10% heat inactivated autologous serum, 10% heat-inactivate FBS or no serum. Supernatants were collected 6 hours post-incubation and analyzed by IFNα, TNFα and IL12p70 ELISA. Unstimulated cells and LPS (10 ng/mL) were used as negative and cell purity controls, respectively. CpG2007 (100 ng/mL) was used as a positive control. Data represent means ±SE of quadruplicate samples. The experiment was performed with 2 individual donors. No statistical differences were found when comparing challenge of pDCs by swollen or resting conidia.

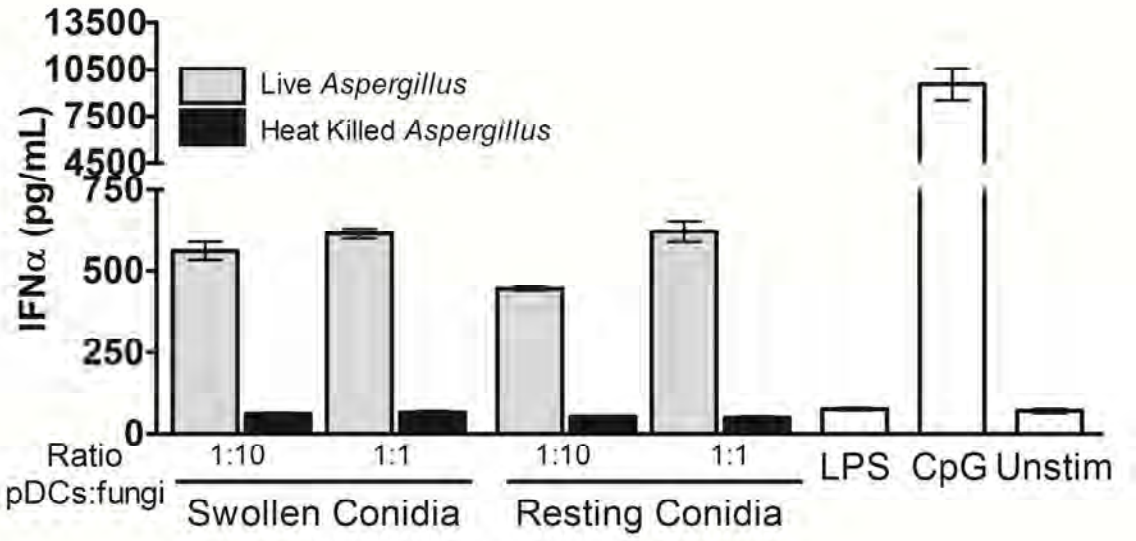
13500
10500
7500
4500
750
500
250
0
IFNα (pg/mL)
Live Aspergillus
Heat Killed Aspergillus
Ratio
pDCs:fungi
1:10
1:1
1:10
1:1
LPS
CpG
Unstim
Swollen Conidia
Resting Conidia

Figure 4.5: Live, but not heat-killed, *Aspergillus* stimulates pDCs to secrete cytokines.

CD304+ pDCs (50,000/well in 96-well plates) were matured for 7 days in RPMI supplemented with 10% FBS, 10ng/mL of recombinant IL-3, 10ng/mL CD40L and 150 ng/mL of GMCSF 37°C. At day 7, cells were washed and the media were changed (RPMI supplemented with 10% autologous serum, 10 ng/mL IL-3, 10 ng/mL CD40L and 150 ng/mL GM-CSF). Fungal conidia, swollen and resting, were heat-killed at 56°C for 30 min. Consequently, pDCs were infected with opsonized live or heat-killed *A. fumigatus* Af293 conidia (swollen or resting) and incubated at 37°C at the specified ratio. Supernatants were collected at 18 hours and analyzed by IFNα, TNFα and IL12p70 ELISA. Unstimulated cells and 10 ng/mL LPS were used as a negative control. CpG2007 (100 ng/mL) was used as a positive control. No statistical differences were found when comparing challenge with either swollen or resting conidia. Data are representative experiment of 3 performed in duplicate. Difference (p value ≤ 0.001) was observed when comparing challenge of pDCs with live vs. heat killed *A. fumigatus*.

our results show that unwashed *A. fumigatus* conidia stimulate higher secretion of IFNα when compared to conidia that underwent a washing step or to fungal supernatants (Figure 4.6A). However, there was no difference in the secretion of TNFα and IL-12p70 among the different treatments (Figure 4.6B and 4.6D).

Since our results show that *A. fumigatus* supernatants induce a potent proinflammatory response in mature pDCs, we sought to determine whether a fungal immunogenic factor was secreted into the media during conidial growth and germination. In order to determine whether contact was required for *A. fumigatus* to secrete the potential immunostimulatory factor and induce a response by mature pDC, a Transwell assay was performed. s We challenged pDCs with *A. fumigatus* with either swollen or resting conidia incubated in the same compartment (contact) or separated by a permeable membrane (Figure 4.7). Our results show that pDCs secrete IFNα independent of contact (Figure 4.7A). However, secretion of TNFα is reduced when pDCs and *A. fumigatus* are separated by the Transwell (Figure 4.7B). Our results suggest that *A. fumigatus* secretes an immunogenic factor into the environment during fungal growth.

A. fumigatus secretes proteins and carbohydrates during conidial germination

During condial germination, *A. fumigatus* secretes a large number of cell wall-associated proteins and carbohydrates. Some of these secreted factors have been shown to be immunostimulatory. In order to assess the nature of the fungal factor responsible for inducing a cytokine response in mature pDCs, we measured the protein and carbohydrate content secreted during *A. fumigatus* growth. We grew *A. fumigatus* to the different

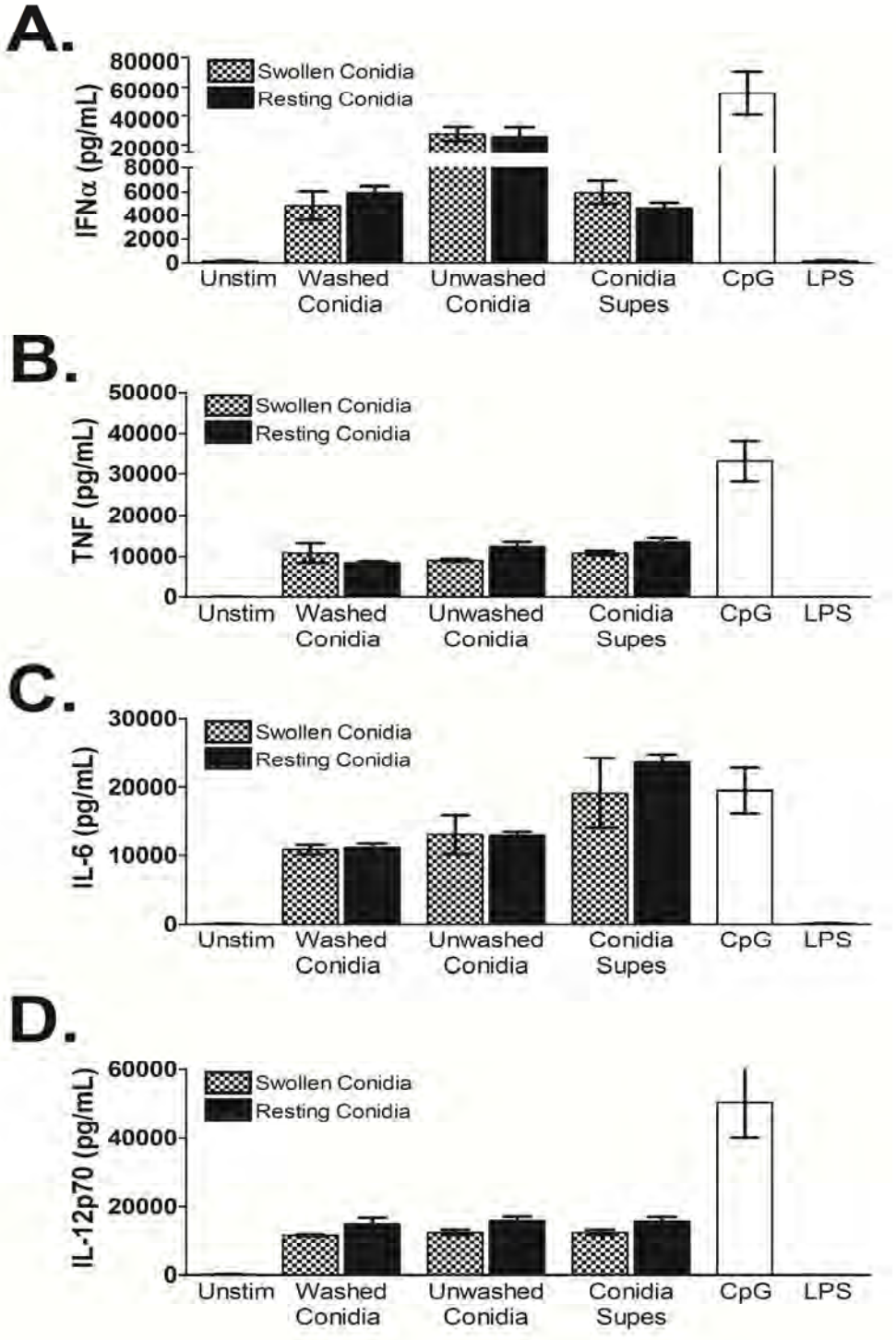
A.
IFNα (pg/mL)
80000
60000
40000
20000
8000
6000
4000
2000
0
Swollen Conidia
Resting Conidia
Unstim
Washed Conidia
Unwashed Conidia
Conidia Supes
CpG
LPS
B.
TNF (pg/mL)
50000
40000
30000
20000
10000
0
Swollen Conidia
Resting Conidia
Unstim
Washed Conidia
Unwashed Conidia
Conidia Supes
CpG
LPS
C.
IL-6 (pg/mL)
30000
20000
10000
0
Swollen Conidia
Resting Conidia
Unstim
Washed Conidia
Unwashed Conidia
Conidia Supes
CpG
LPS
D.
IL-12p70 (pg/mL)
60000
40000
20000
0
Swollen Conidia
Resting Conidia
Unstim
Washed Conidia
Unwashed Conidia
Conidia Supes
CpG
LPS

Figure 4.6: Stimulation of pDCs appears to be mediated by a fungal secreted factor. CD304+ pDCs (50,000/well in 96-well plates) were matured for 7 days in RPMI supplemented with 10% FBS, 10 ng/mL of recombinant IL-3, 10 ng/mL CD40L and 150 ng/mL of GMCSF at 37°C. At day 7, cells were washed and the media was changed (RPMI supplemented with 10% autologous serum, 10ng/mL IL-3, 10ng/mL CD40L and 150 ng/mL GM-CSF). Consequently, pDCs were infected with washed swollen or resting *A. fumigatus* Af293 conidia and incubated for 6 hours at 37°C at 0.1:1 (Fungi:pDC). pDCs were also treated with 10µL of supernatants collected from centrifuged *A. fumigatus* conidia (swollen and resting). Supernatants were collected after 6 hours incubation and analyzed by IFNα (A), TNFα (B), IL6 (C) and IL12p70 (D) ELISA. Unstimulated cells and 10 ng/mL LPS were used as negative and cell purity controls, respectively. CpG2007 (100 ng/mL) was used as a positive control. Data represent means ±SE of duplicate experiments. Comparison between results from swollen and resting conidia does not yield statistical differences by 2-way ANOVA.

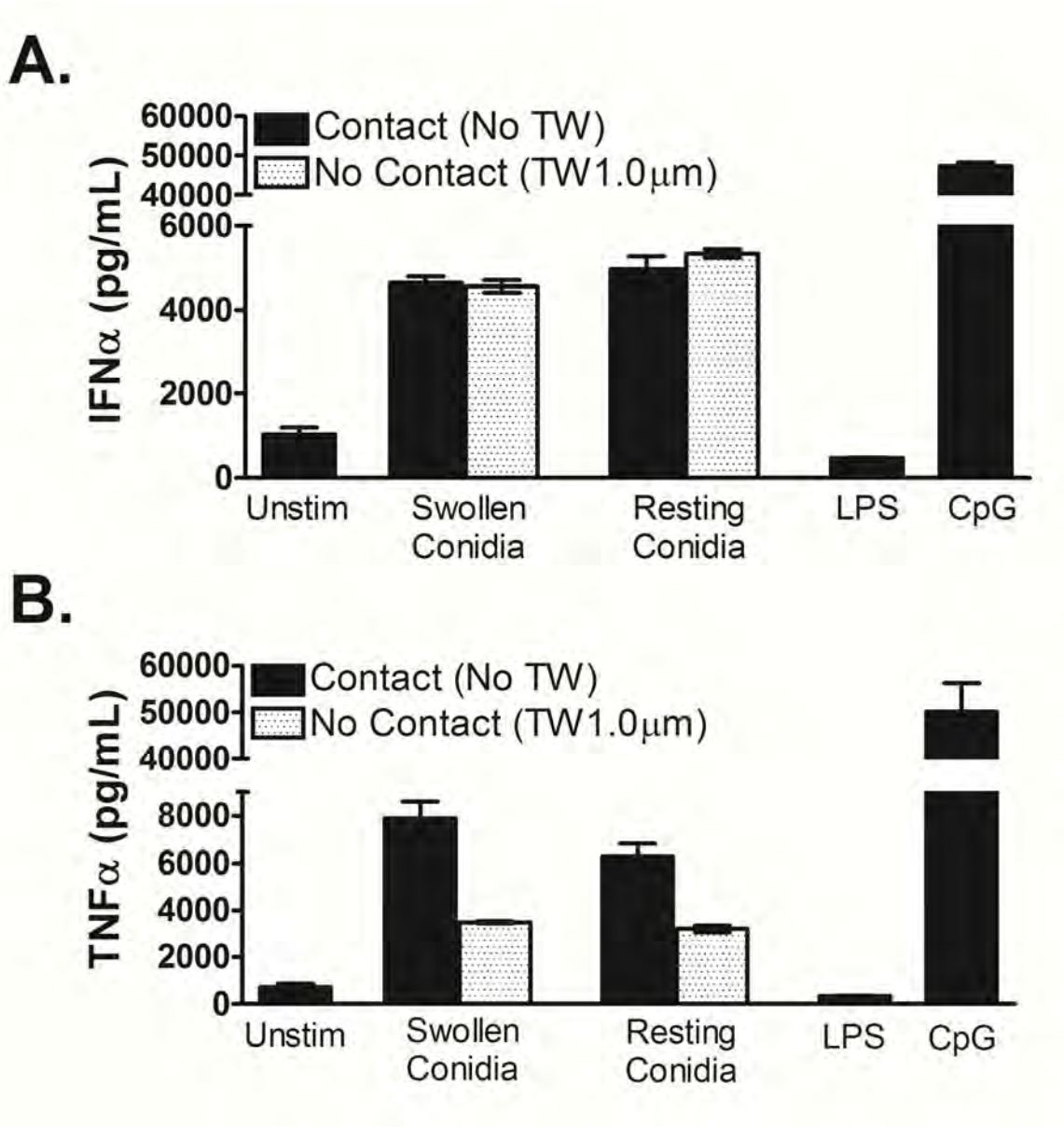
A.
60000
50000
40000
6000
4000
2000
0
IFNα (pg/mL)
Contact (No TW)
No Contact (TW1.0μm)
Unstim
Swollen Conidia
Resting Conidia
LPS
CpG
B.
60000
50000
40000
8000
6000
4000
2000
0
TNFα (pg/mL)
Contact (No TW)
No Contact (TW1.0μm)
Unstim
Swollen Conidia
Resting Conidia
LPS
CpG

Figure 4.7. *A. fumigatus* stimulates cytokine secretion in mature pDC via a secreted factor.

pDCs (50,000/well) were matured for 7 days on pDC media supplemented with 10 ng/mL recombinant IL3, 10 ng/mL CD40L and 150 ng/mL GM-CSF. On day 7, pDCs were challenged with 50,000 resting or swollen conidia either in contact with the pDCs or separated by a 1µm permeable insert (Transwell). pDCs and *A. fumigatus* were co-incubated for 6 hours at 37°C. Supernatants were collected and analyzed for cytokine secretion by IFNα and TNFα ELISA. Graphs represent means ±SE of duplicate experiments. No statistical difference was found in the secretion of IFNα for pDCs in contact with conidia when compared to cells separated by a Transwell, p value < 0.001 by Student's T-test for TNFα secretion when comparing these treatments.

conidial germination stages and collected supernatants from each of the growth stages (Figure 4.8). Our results show that *A. fumigatus* secretes between 600-1000 µg/mL of protein (Figure 4.8A). Furthermore, *A. fumigatus* secretes large amounts of sugars into the media with concentrations reaching approximately 20 mg/mL (Figure 4.8B). While our results show that *A. fumigatus* secretes proteins and carbohydrates into the environment, it is possible for other bioactive factors to be secreted during fungal growth. Therefore, future work is required in order to determine the nature of the immunostimulatory factor.

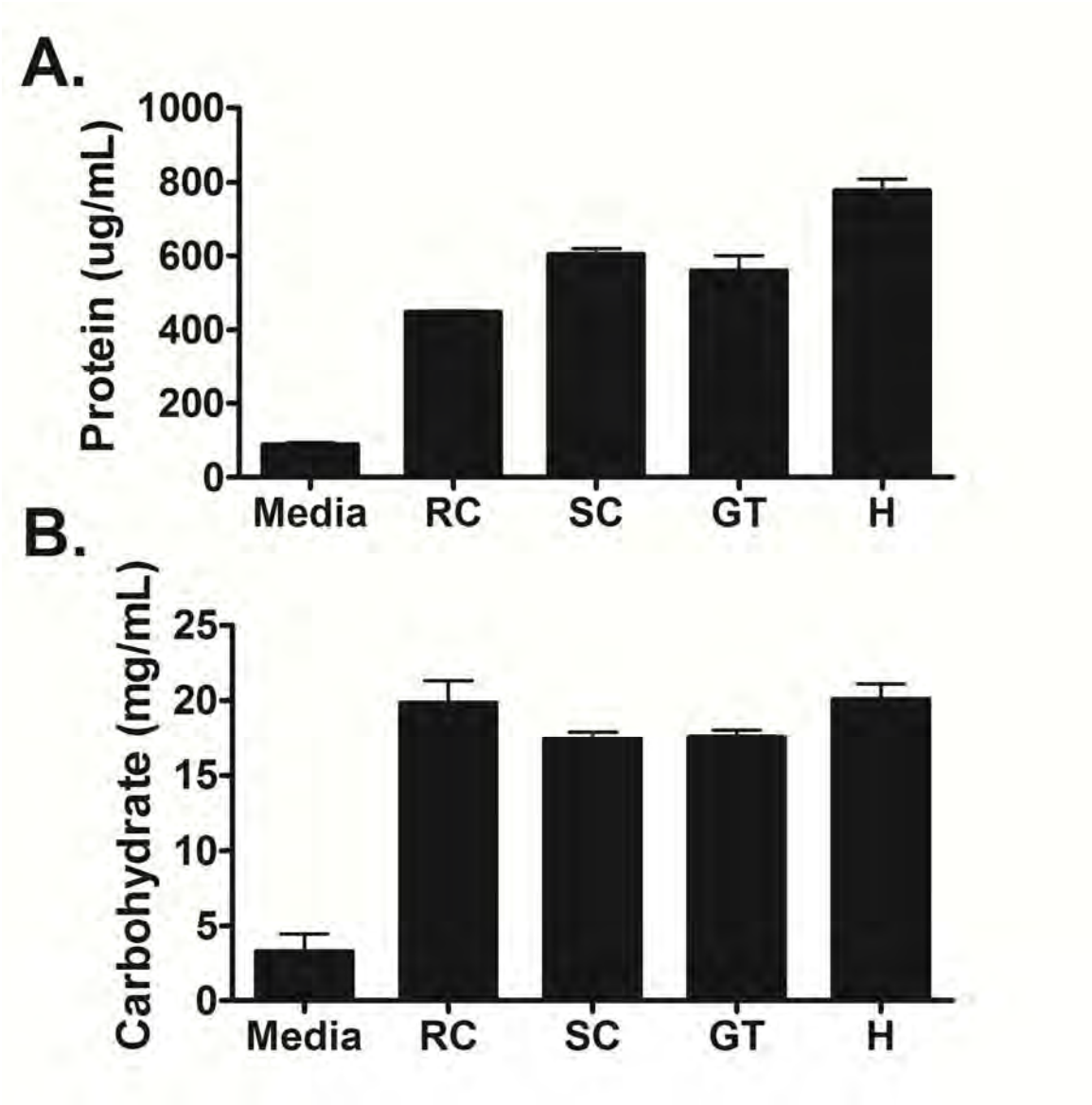
A.
Protein (ug/mL)
1000
800
600
400
200
0
Media
RC
SC
GT
H
B.
Carbohydrate (mg/mL)
25
20
15
10
5
0
Media
RC
SC
GT
H

Figure 4.8. *A. fumigatus* secretes proteins and carbohydrates into the environment during fungal growth.

A. fumigatus ($5x10^4$ conidia per well) were grown to swollen conidia, germ tubes or hyphae, or plated as resting conidia on 96-well plate at 37°C in pDC media. Once *A. fumigatus* reached the specified morphology (growth stage), 100 µL of supernatants were collected. The concentration of protein (A) during fungal growth was determined by BCA Assay. The concentration of carbohydrates (B) was determined by the Dubois Method (Dubois et al. 1951). Data represent means ±SE of two individual experiments. For protein assay, p value ≤0.0001 by one way ANOVA for different fungal growth stages. For carbohydrate assay, p value ≤ 0.0004 by one way ANOVA for different fungal growth stages.

RC- resting conidia, SC- swollen conidia, GT- germ tubes, H- hyphae

Discussion

Phagocytes have been shown to be essential in the antifungal response and clearance of *A. fumigatus* (Diamond 1988; Loeffler et al. 2009). Although TLR9 has been shown to play a role in the detection of fungal DNA, and our results (Chapter 3) show that immature pDCs play a role in the antifungal response, whether mature pDCs are involved in the overall immune response against *A. fumigatus* remains to be determined (Ramirez-Ortiz et al. 2008). Here we show that mature pDCs secrete cytokines in response to *A. fumigatus* conidia. Furthermore, mature pDCs are inducing a proinflammatory response independent of opsonization.

pDCs play a vital role in the antifungal response (Gilliet et al. 2008; Lande and Gilliet 2010). In addition to the role of pDCs in antiviral responses, these cells were shown to be involved in the antibacterial and antiparasite response (Bauer et al. 2001; Liese et al. 2008). pDCs were found to be rapidly recruited to the lungs of mice infected with *Legionella*. In addition, bacterial load was larger in mice depleted of their pDCs (Ang et al. 2010). However, pDCs have not been shown to be involved in the antifungal response.

As mature pDCs have been shown to acquire DC-like morphology, and their phagocytic ability increases with maturation, we sought to determine whether they were capable of interacting with *A. fumigatus* (Lande and Gilliet 2010; Tan and O'Neill 2005). Our results show that mature pDCs spread over the hyphae. This suggests that pDCs are capable of recognizing *A. fumigatus*, but does not tell us whether mature pDCs are capable of initiating a proinflammatory response.

Upon maturation, cytokine secretion by pDCs decreases and their ability to induce an adaptive immune response increases (Lande and Gilliet 2010; Swiecki and Colonna 2010). Incubation of pDCs with IL3 and CD40L is one way of inducing maturation of these cells (Grouard et al. 1997). Maximal response of the pDCs to *A. fumigatus* conidia was observed at day 7 post-isolation from blood. pDCs secrete type I IFN and TNFα in response to *A. fumigatus* conidia. The secretion of cytokines by mature pDCs is possibly a way of these cells to communicate with other cells and initiate the adaptive immune response. Mature pDCs have been shown to initiate T-helper responses, and the secretion of different subsets of cytokines can lead to a Th1 vs Th2 response (Bellocchio et al. 2005; Svirshchevskaya et al. 2001). In the case of IA, a Th2 response is thought to be unfavorable to the host (Bozza et al. 2009; Chai et al. 2010; Gafa et al. 2010). Therefore, future work will focus in determining whether challenge of pDCs with *A. fumigatus* leads to a Th1 or Th2 response.

Mature pDCs are capable of detecting *A. fumigatus* and inducing a response independent of serum opsonization. This suggests that pDCs have a receptor that can recognize PAMPs in the conidia or hyphae. It is also possible that as *A. fumigatus* grows, components of the cell wall or other molecules are secreted into the media. During conidial germination, the conidia cell wall undergoes changes that result in the release of carbohydrates and other components into the environment (Dague et al. 2008). One of those components is melanin. This electron dense outer layer, imparts a grey-green color upon *A. fumigatus* conidia and has been described as a virulence factor for the mold and other pathogenic fungi (Youngchim et al. 2004). It is possible that melanin released into

the PBS where we had been storing the conidia was responsible for the observed pDC stimulation. However, it is important to note that swollen conidia do not have a melanin outer layer. In addition, it is possible the combination of melanin with other secreted polysaccharides, such as galactosaminogalactan, can be highly immnunostimulatory. However, galactosaminogalactan is only secreted during hyphal growth. Other polyssacharides such as galactomannan are secreted in high concentrations during *A. fumigatus* growth (Pinel et al. 2003). While we did not assess the nature of the immunostimulatory factor, we observed that heat killed conidia did not stimulate an inflammatory response in pDCs. The cytokine response was only observed when pDCs were incubated with live *A. fumigatus,* suggesting that the factor responsible for the inflammatory response is exposed or released during fungal growth. Future work should concentrate on determining the nature of the secreted factor, as well as determining what receptors are involved in the detection of this immunostimulatory molecule. In addition, it is possible that the factor is released following germination; therefore, whether mature pDCs are capable of detecting *A. fumigatus* hyphae should be analyzed.

As we noted that there was no change in the response between swollen and resting conidia, we decided to look back at how we were generating the conidia. For our assays, we collected the conidia as previously described, counted it and saved it at 4°C until ready to use for a maximum of two weeks. The possibility that the stimulatory factor was released from the conidia as it germinated into other morphologies cannot be eliminated. Furthermore, supernatants from conidial growth stimulate cytokine secretion by pDCs. This suggests that the cytokine secretion was due to a released factor into the media,

rather than changes in the conidia cell wall or morphology. However, since in our method we saved the conidia on PBS for up to two weeks, it is possible that the RC was starting to become SC. Although we started our assays with either resting or swollen conidia, we were incubating the cells with live *A. fumigatus*. As we co-incubated the pDCs with *A. fumigatus* for 6 hours, it is possible that the observed response was in response to hyphae rather than the starting morphology. Furthermore, *A. fumigatus* grows slowly when stored at 4 degrees in media or PBS but not when stored in water (Osherov 2009); therefore, it is possible that for some of our assays the resting conidia had already started to swell.

Thus, our data demonstrate that mature pDCs secrete proinflammatory cytokines in response to *A. fumigatus* conidia as early as six hours post-challenge. While both fungal growth stages stimulated pDCs to secrete similar levels of cytokines, it is possible that the stimulating factor is secreted during conidial germination. In addition, pDCs only secrete cytokines in response to live *A. fumigatus*. Further evidence was obtained by separating pDCs and *A. fumigatus* by a permeable membrane. During conidial germination and growth, *A. fumigatus* secretes proteins and carbohydrate that may have immunostimulatory activity. Furthermore, recent reports showed that *A. fumigatus* secretes at least 35 immunoreactive fungal proteins during germination and growth (Singh et al. 2010a; Singh et al. 2010b). It is possible that mature pDCs recognize and secrete cytokine to a mixture of carbohydrates and proteins secreted into the environment during fungal growth. Mature pDCs may be contributing to the overall anti-*Aspergillus* response in multiple ways. First, since mature pDC acquire cDC-like characteristics it is

possible that they upregulate receptors such as Dectin-1 or the membrane bound TLR2 and TLR4. However, additional work is needed in order to determine whether mature pDCs express any of these receptors. In addition, by secreting cytokines, mature pDCs may be contributing to the anti-*Aspergillus* response by recruiting other immune cells and initiating adaptive immune responses to the mold.

Materials and Methods:

Reagents and cell culture. Reagents were obtained from Sigma-Aldrich (St Louis, Missouri), unless otherwise stated. RPMI-1640 was obtained from GIBCO (Invitrogen; Carlsbad, California). pDC media consisted of RPMI-1640 supplemented with 100U/ml penicillin, 100U/ml streptomycin, 2mM L-glutamine, 0.5mM HEPES, 1mM sodium pyruvate. Opsonization media consisted of pDC media supplemented with 10% autologous serum. All incubations were in a 37°C incubator containing humidified air supplemented with 5% CO_2, unless otherwise indicated. The stimulatory TLR9 ligand, CpG 2007 oligonucleotide (ODN) (Latz et al. 2007a), was synthesized with phosphothiorate linkages by Integrated DNA Technologies (Coralville, IA). Ultrapure LPS from *Escherichia coli* was generated as previously described (Huang et al. 2009). All antibodies were purchased from eBiosciences (San Jose, CA), unless otherwise specified.

Fungal Growth and Strains. *A. fumigatus* Af293 was obtained from Fungal Genetic Stock center (Kansas City, Mo). Cultivation of *A. fumigatus*, and harvesting of conidia was performed as in our previous studies with slight modifications (Mambula et al. 2002). Briefly, fungi were grown on Sabouraud Dextrose Agar slants at 37°C for 4 to 7 days. Conidia were harvested by adding PBS containing 0.01% Tween 20 followed by vortexing. The collected conidia were then filtered through a 40-µm nylon mesh to remove hyphae and conidiophores. The conidia in the filtrate were washed twice with

phosphate buffered saline (PBS), counted and stored in water at 4°C for up to two weeks in some cases.

pDC Enrichment and Maturation. Human pDC were isolated as described (Ramirez-Ortiz et al. 2008; Wang et al. 2006). Peripheral blood was collected by venipuncture. A portion was clotted and the autologous serum collected following centrifugation. The remainder of the blood was anticoagulated with heparin, diluted 1:1 with Hank's buffered salt solution (HBSS) (BioWhittaker; Walkersville, MD) and the peripheral blood mononuclear cells (PBMCs) purified by Ficoll-Hypaque (Lymphoprep^{TM}; Westbury, NY) density gradient centrifugation. Human pDCs were positively selected from the PBMC using CD304-coated magnetic beads, according to the manufacturer's instructions (Miltenyi Biotech, Auburn, CA).

After isolation, pDCs were incubated for 7 days at 37°C/5%CO_2 in pDC media supplemented with 10ng/mL of recombinant IL3, 150 ng/mL granulocyte macrophage colony stimulating factor (GMCSF) and 10ng/mL CD40L (all three cytokines from Prepotech; Rocky Hill, NJ) . At days 3 and 5, half of the media was removed and new media was added to the wells containing the pDCs. At day 7, pDCs were considered to be mature.

Flow Cytometry. The purity of the immature pDC population was confirmed by analyzing the expression of the pDC markers CD123 and CD303 by flow cytometry (Colonna et al. 2004). Briefly, freshly isolated pDCs were incubated on ice with anti-CD123-APC and/or anti-CD303-FITC for 30 minutes. All antibodies were obtained from

eBiosciences (San Jose CA). Isotype controls were included in all experiments (Figure 3.1).

Cytokine Secretion. The indicated concentrations of pDCs or flow through cells were incubated with *A. fumigatus* hyphae in 96-well flat-bottom plates containing a final volume of 200 µl pDC media supplemented with 10% autologous serum. After 6 or 18 hours, supernatants were collected and human IFNα (Bender Med Systems module set, Burlingame, CA), TNFα, IL12p70 and IL6 levels were measured using ELISA kits (eBiosciences, San Diego, CA for TNFα, IL12p70 and IL6) according to the manufacturers' instructions. Samples were assayed in duplicate at dilutions that fell within the range of the standard curve.

Transwell Assay. Transwell 96 well plates containing 1.0 µm pore size polycarbonate membrane inserts were obtained from Corning Life Sciences (Lowell, MA). Human pDCs ($5x10^4$ per well) were matured on the reservoir plate, as described above. *A. fumigatus* resting or swollen conidia ($5x10^4$ in 100µL) were added either to the Transwell (no contact) or to the reservoir plate (contact). Mature pDCs and *A. fumigatus* were co-incubated for 6 hours at 37°C/5%CO_2. Supernatants were collected and cytokine secretion was measured by ELISA.

Protein Assay. We wanted to determine whether *A. fumigatus* was secreting proteins into the storage media. For sample preparation, *A. fumigatus* was collected from slants and conidia were counted. Resting conidia were collected and stored at 4°C until ready to

use. In order to generate swollen conidia, we collected resting conidia and incubated it on pDC media at 37 °C for approximately 4 hours. Conidial swelling was determined by visualizing the conidia at different time points via microscopy. All conidia were plated to a final concentration of $5x10^4$ conidia per well in 100 µL pDC media. Germ tubes and hyphae were generated by plating $5x10^4$ conidia per well on 96 well plate at 37°C for 8-12 hours on pDC media (without FBS). For each one of the morphologies, the storage tubes or plates were centrifuged 800xg (Beckman TJ-6 Centrifuge) for 10 minutes and 100 µl of supernatants were transferred to a clean 96-well plate. BCA protein assay was performed and analyzed following manufacturer's instructions (Pierce Cat # 23225; Thermo Scientific, Rockford IL). Samples were assayed in triplicate dilutions that fell within the range of the standard curve.

Carbohydrate Assay. The phenol sulfuric acid method of Dubois was used to determine the polysaccharide concentration (Dubois et al. 1951) with the following changes: *A. fumigatus* supernatants from the different morphologies were diluted (1:10 and 1:100) to a final volume of 40 µL in LAL reagent water (LRW; Associates of Cape Cod, East Falmouth, MA) and added to 360 µl dH_20 in 13 x 100 millimeter (mm) borosilicate glass test tubes (Fisher). To each tube, 10 µl of buffer-saturated phenol (Invitrogen) was added, followed by quickly adding 1 ml of 95-98% sulfuric acid (Sigma). Using an Eppendorf repeater pipette, add 1 mL of ~98% Sulfuric acid (Sigma-Aldich, MO). Color was allowed time to develop and 100 µl samples were transferred to a 96-well flat-bottom plate (Costar). The concentration of carbohydrate is reflected by a colorimetric

change, and the plates were read at 490 nm on a plate reader (Molecular Devices, Sunnyvale, CA). The carbohydrate content of the samples was calculated by comparing with a standard curve of known amounts of glucose.

Statistical Analysis. For comparisons of two groups, means ± standard errors (SE) were analyzed by the two-tailed unpaired Student *t*-test with the Bonferroni correction applied when making multiple comparisons. For comparisons of greater than two groups, significance was determining using the one- or two-way analysis of variance (ANOVA) with the Tukey multiple correction. Calculations were performed using a statistical software package (GraphPad Prism 4.02, San Diego, CA) and statistical significance was defined as $p<0.05$.

Chapter V

Discussion

Overview:

The research goal of the work presented in this thesis was to understand the contribution of TLR9 and pDCs, TLR9-rich cells, in the immune responses to *A. fumigatus*. My work demonstrates that *A. fumigatus* DNA contains unmethylated CpG motifs that are capable of inducing a TLR9-dependent response. Furthermore, we showed that *A. fumigatus* DNA contains both, human-like and mouse-like immunostimulatory motifs. The observation that DNA isolated from *A. fumigatus* and transfected into pDCs results in the secretion of type I IFN, led us to investigate the role of human pDCs in the anti-*Aspergillus* response. My work illustrates that pDCs are capable of inducing a cytokine response upon challenge with *A. fumigatus* hyphae. pDCs spread over the hyphae, as the size of this fungal morphology is too big to induce phagocytosis. The observed interaction between *A. fumigatus* hyphae and pDCs is paradoxical. The interaction between *A. fumigatus* and the pDCs results in a fungistatic activity against the mold, possibly via the release of Zn^{2+} chelators such as calprotectin into the environment. At the same time, *A. fumigatus* is secreting gliotoxin and inducing pDC cell death. Thus, the immune evasion mechanism employed by *A. fumigatus* results in the initiation of a host response against the hyphae. I also illustrate that pDCs, mature and immature, are capable of secreting proinflammatory cytokines in response to *A. fumigatus* suggesting a role for pDCs in the recruitment of other immune cells. The work presented in this thesis describes a novel and unique host-pathogen interaction between pDCs and *A. fumigatus*, a major infectious agent among immunocompromised patients.

A. fumigatus DNA as a PAMP

Work performed previously in our laboratory and others had shown that TLRs are essential in the recognition and clearance of *A. fumigatus* conidia (Mambula et al. 2002; Wang et al. 2001). However, most of the previous work concentrated on the extracellular TLR2 and TLR4 (Chignard et al. 2007; Luther et al. 2007; Mambula et al. 2002). In 2004, Bellocchio *et al.* showed that mice deficient in MyD88 were extremely susceptible to *A. fumigatus* infection. However, mice deficient in TLR9 showed increased survival and decreased fungal burden when compared to wild type mice (Bellocchio et al. 2004a). The observation that TLR9 mice were relatively resistant to infection with *A. fumigatus* suggested to us that the involvement of the receptor may be detrimental to the host. As the natural ligand of TLR9 is unmethylated CpG DNA, we first wanted to determine whether *A. fumigatus* DNA was capable of mounting a TLR9-dependent immune response. By restriction enzyme digest, we showed that *A. fumigatus* DNA contained multiple CCGG sequences in its genome. In addition, we showed that *A. fumigatus* DNA can be enzymatically methylated.

A. fumigatus DNA shows all the characteristics needed to stimulate TLR9, suggesting that it could be acting as a PAMP during infection. Our results show that *A. fumigatus* DNA can stimulate the secretion of proinflammatory cytokines. Furthermore, enzymatic methylation of the DNA and cells from mice deficient in TLR9 show decreased stimulation. Our data suggests that *A. fumigatus* DNA can induce a response that is partially dependent on TLR9; therefore, other nucleic acid receptors may play a

role in the detection of *A. fumigatus* DNA. Cytoplasmic DNA receptors have been recently identified, including RIG-I, MDA-5, DAI (DNA-dependent activator or IFN-regulatory factors) and multiple components of the inflammasome (Fernandes-Alnemri et al. 2009; Hise et al. 2009; Hornung et al. 2009; Muruve et al. 2008; Rathinam et al. 2010; Roberts et al. 2009; Vilaysane and Muruve 2009). Further experimentation is needed in order to determine the contribution of other cytoplasmic receptors; however, our data suggest that they may be playing a role in the recognition of *A. fumigatus* DNA. Another explanation for the residual cytokine response would be that the DNA is not completely methylated. The later explanation does not take into consideration that residual TNFα was observed when stimulating BMDCs from TLR9$^{-/-}$ mice with *A. fumigatus* DNA. Therefore, it is highly possible for other nucleic acid sensors such as the newly identified cytoplasmic DNA receptors to contribute to the antifungal response; although, additional experiments are required to determine whether these receptors play a role in the detection of *A. fumigatus* DNA.

Previous data show that mammalian, but not microbial DNA, is highly methylated (Akira et al. 2006). Although fungi and mammals are members of the eukaryotic kingdom, we found that *A. fumigatus* DNA had more in common with bacterial DNA than with mammalian DNA. In our studies, *A. fumigatus* DNA shows low levels of CpG methylation. The observed pattern of degradation of *A. fumigatus* DNA after treatment with the restriction endonuclease *HpaII*, which is specific for unmethylated CCGG sequences, was similar to the one observed when treated with *MspI*, an endonuclease that cleaves CCGG regardless of the level of methylation. Consistent with the theory that

unmethylated CpG DNA is the natural ligand of TLR9 (Akira et al. 2006), we found that enzymatic methylation of *A. fumigatus* DNA results in decreased cytokine secretion. Furthermore, degradation of the *A. fumigatus* DNA also results in a decreased response.

The immunostimulatory activity of DNA directly correlates with the frequency of CpG dinucleotides, as shown in a survey of 15 bacterial species (Dalpke et al. 2006). Genomic analysis of the bacterial DNA revealed that the frequency of CG dinucleotides ranged from 1.44% to 12.21% among the different bacteria species analyzed. Our data show that *A. fumigatus* contains approximately 5.35% CG dinucleotides in its DNA. The frequency of CG dinucleotides found in the A. fumigatus genome is comparable to the frequency of CG in some bacterial species. For example, *E. coli* contains approximately 7.2% CG dinucleotides, *Yersenia enterocolitica* contains approximately 5.4% and *Neisseria meningitidis* contains approximately 7.9%. In the study, all three bacterial species were capable of inducing similar levels of TLR9 activation; as measure by secretion of IL-8 (Dalpke et al. 2006). Similarly, we observed that *A. fumigatus* and *E.coli* stimulate TNFα and IL12p70 to comparable levels in a TLR9-dependent manner.

Although the percentage of CpG dinucleotides is an important characteristic of the immunostimulatory activity of microbial DNA, the presence of specific sequences has also been shown to be important in the stimulation of human and mouse TLR9 (Putta et al. 2010; Struthers et al. 2010). Even though TLR9 is an evolutionarily conserved germ-line encoded receptor, the DNA binding-motif recognizes slightly different sequences in the human and the mouse. An *in silico* genome wide analysis of the *A. fumigatus* DNA

demonstrated the presence of immunostimulatory sequences specific for mouse and human TLR9. Our analysis was extremely conservative, and given the high percentage of CpG dinucleotides, it is possible that other stimulatory motifs exist within the fungal genome. In addition, we found that synthetic ODNs containing CpG motifs found in *A. fumigatus* DNA induced a TLR9-dependent response. The immunostimulatory activity of one of our ODNs was of particular interest, as it induces a TLR9 dependent response in human cells to levels comparable to a CpG ODN undergoing clinical trials. Given the high immunostimulatory activity of the *A. fumigatus* ODN, the possibility of using it as an adjuvant in a vaccine against *A. fumigatus* cannot be eliminated.

Possible Contribution of *A. fumigatus* DNA to Immunology of Aspergillosis

Even though our data show that *A. fumigatus* DNA is acting as a TLR9 agonist, the contribution of the fungal DNA to the overall host response during aspergillosis remains to be elucidated. It has been suggested that *A. fumigatus* DNA can be detected in the early stages of infection (Chamilos and Kontoyiannis 2006); however, future work is required to determine whether the DNA is capable of reaching endosomal compartments where TLR9 is present. In addition, three factors most be taken into consideration: 1) in its invasive hyphal form, *A. fumigatus* is an extracellular organism and TLR9 is found in the endosomal compartments, 2) neutropenia is a major risk factor in the development of invasive aspergillosis, 3) all PCR-based methods used in the diagnosis of aspergillosis require a cell lysis step. Considering these factors, the large size of the hyphae restricts it from endosomal compartments. In addition, neutrophils are the primary cell type

responsible for killing hyphae; therefore, further work should focus on determining whether *A. fumigatus* damage caused by neutrophils results in the release of fungal DNA into the environment. It is possible that during aspergillosis, other immune cells are trying to control hyphal growth and the damage made by those cell results in the secretion of DNA into the environment where it can be taken into endosomal compartments rich in TLR9. Nevertheless, the observed differences in survival between wild-type and $TLR9^{-/-}$ infected with *A. fumigatus* suggest a role for this receptor *in-vivo* (Bellocchio et al. 2004a).

The observed differences in survival between WT and $TLR9^{-/-}$ mice may be due to defects in the anti-*Aspergillus* response mediated by macrophages. *A. fumigatus* conidia are readily phagocytosed by macrophages (Brakhage et al. 2010b; Segal 2007). In the mouse, TLR9 can be found in the endosome of most immune cells. Once in the endosome of macrophages, conidia are killed by oxidative mechanisms, where DNA can be released into a $TLR9^{+}$ compartment (Ibrahim-Granet et al. 2003; Luther et al. 2006; Luther et al. 2008; Luther et al. 2007; Philippe et al. 2003). In this scenario, *A. fumigatus* DNA may be responsible for inducing a TLR9 dependent response. Although this is true in the mouse infection model, humans mainly express TLR9 in pDCs and B-cells (Gilliet et al. 2008). To date, neither one of these human cell populations has been shown to phagocytose *A. fumigatus* conidia. It is possible that monocytes phagocytose *A. fumigatus* conidia and kill it in the endosome, where it can induce activation of low levels of TLR9. A recent report (Fiola et al. 2010) suggested that monocytes express low levels of TLR9 and that these cells are responsive to TLR9 agonist. Furthermore, Fiola *et al.* showed that

TLR2 and TLR9 collaborate to induce a cytokine response during Epstein Bar Virus (EBV) infection (Fiola et al. 2010). It is possible for *A. fumigatus* to be inducing a response similar to EBV in monocytes, as TLR2 has been shown to be a key receptor in the detection of the mold. Future work should determine whether *A. fumigatus* conidia (and hyphae) induce a TLR2/TLR9 dependent response in monocytes and whether *A. fumigatus* conidia are killed in the endosomes of pDCs.

Polymorphisms in the *TLR9* gene have also been shown to play a role in the susceptibility to development ofaspergillosis. One study examined variants of the TLR genes in a population of Italian children with hematological malignancies (Lanciotti et al. 2008). Two variants in *TLR9* were analyzed: T-1237C and T-1486C. Lanciotti et al. showed that the frequency of the C allele in the TLR9 T-1486C polymorphism was significantly higher in patients with invasive mold infections than the healthy controls. An association between TLR9 T-1237C and patients suffering from allergic bronchopulmonary aspergillosis, a disease that affects mainly patients suffering from allergy or asthma, was also established (Carvalho et al. 2008a; Carvalho et al. 2008c).

TLR9-dependent Host Responses to *A. fumigatus*

An inflammatory response is only beneficial to the host if the infection can be contained or eliminated. In some cases the pathogen is not eliminated and host tissue damage results from the inflammatory response. The observation that $TLR9^{-/-}$ mice survived longer than the wild-type counterparts suggests that the immune response is favoring the pathogen rather than the host. In addition, using an asthma infection model,

Ramaprakash *et al.* showed that $TLR9^{-/-}$ mice infected with resting conidia have lower airway hyper-responsiveness than $TLR9^{+/+}$ counterparts (Ramaprakash et al. 2009). Furthermore, in their infection model, mice deficient in TLR9 appear to have higher fungal growth than the $TLR9^{+/+}$ counterparts (Ramaprakash et al. 2009).

The nature of the immune response to *A. fumigatus* is of great importance to the outcome of the infection. In addition to secretion of proinflammatory cytokines such as type I IFNs, activation of TLR9 also signals the activation of T helper (Th) cell responses. CpG-rich DNA is known to bias towards a Th1 response (Krieg 2002). While a Th1 bias will provide protective immunity to *A. fumigatus* (Brakhage et al. 2010b), the Th1/Th2 deregulation constitutes an important aspect of disease development (Bozza et al. 2009).

Role of pDCs in the anti-*Aspergillus* response

Controlling the immune responses against pathogens is essential in the prevention of host tissue damage due to prolonged inflammation, a hallmark of chronic diseases. Inflammatory responses to *A. fumigatus* often serve to limit the infection; however, the exacerbated host inflammatory response may contribute to the pathogenicity of the mold. The involvement of TLR9 in the host response against *A. fumigatus* may be beneficial to the pathogenicity of the fungus. Our initial studies were focused on the role of TLR9 in the detection of *A. fumigatus* DNA using an *in-vitro* system. While our results showed a contribution to the host response upon recognition of the fungal PAMP, additional studies are necessary to show the mechanism of *A. fumigatus* DNA release and entry into TLR9-

rich compartments in host cells.. During infection, host cells are most likely to encounter live *A. fumigatus*.

In humans, TLR9 is found in the endosomal compartments of pDCs and B cells. The finding that human pDCs secrete type I IFN in response to *A. fumigatus* DNA made us wonder whether pDCs mount an inflammatory response against live fungi. The role of pDCs in the immune response to pathogens has been extensively described during viral infections (Swiecki and Colonna 2010). However, whether pDCs play a role in the detection and antifungal responses to fungal pathogens has received scarce attention.

Recognition of *A. fumigatus* by pDCs

While neutrophils, macrophages, monocytes and conventional DCs have been shown to recognize and exert antifungal responses against *A. fumigatus* (Balloy and Chignard 2009; Dagenais and Keller 2009), a role for pDCs has been suggested but has not been established (Romani et al. 2006). Fungal recognition by monocytes, macrophages and neutrophils has been well characterized. Receptors such as TLR2, TLR4 and members of the C-type lectin receptor family have been shown to be involved in the recognition of *A. fumigatus* by these cell types (Gersuk et al. 2006; Luther et al. 2007; Reid et al. 2009). We show that pDCs are capable of interacting with *A. fumigatus* hyphae. While pDCs have been shown to phagocytose viruses and bacteria (Petzke et al. 2009; Swiecki and Colonna 2010), the size of the hyphae is too big to be phagocytosed. Instead, we observed that pDCs were capable of ingesting conidia and spreading over the

A. fumigatus hyphae within 2 hours. However, the repertoire of surface receptors on pDCs associated with fungal recognition has not been well defined.

Human pDCs have been shown to express dectin-2, but not dectin-1, mannose receptor and DC-SIGN (Graham and Brown 2009; Meyer-Wentrup et al. 2009). In addition, dectin-2 in mouse BMDCs has been shown to stimulate a rapid and robust stimulation of inflammatory lipid mediators against *A. fumigatus* (Barrett et al. 2009). Since dectin-2 has been shown to be expressed in pDCs (Seeds et al. 2009), it is possible for this receptor to be involved in the detection of *A. fumigatus* in our experimental system. Future studies should look at the receptor-ligand interactions between dectin-2 and *A. fumigatus* in human pDCs.

In addition to dectin-2, other receptors on pDCs may also be involved in the recognition of *A. fumigatus*. pDCs express some complement receptors, as well as some Fc receptors (Gilliet et al. 2008). We observed that pDCs were capable of interacting with *A. fumigatus* hyphae independent of opsonization, suggesting that the pDCs express a receptor capable of directly detecting the hyphae. Although we can speculate on which pDC receptors are responsible for the detection and interaction with *A. fumigatus*, further experimentation is required to define these interactions.

pDCs required for the anti-*Aspergillus* response in-vivo

While the role of pDCs in the antiviral and bacterial response has been extensively studied, the role of these cells during mycosis has received little attention. To date, there is no evidence that pDCs play a role in anti-*Aspergillus* responses in-vivo.

Here we show, for the first time, that mice lacking pDCs are more susceptible to *A. fumigatus* infection than control mice. These results are similar to the observation made by Ang et al where depletion of pDCs in mice increased their susceptibility to *Legionella* infection (Ang et al. 2010). In addition to the observation that mice depleted of their pDCs have lower survival rates than control mice, we observed an influx of pDCs in to the lungs of *A. fumigatus* infected mice 48 hours post-infection. However, there is one fundamental difference between the observations with *Legionella* and our studies. In our infection model, type I IFN appears to play a role in the anti-*Aspergillus* response while during *Legionella* infection type I IFN may be dispensable. While we observed that mice deficient in type I IFN receptors had lower survival rates than WT mice, we did not analyze the role of this cytokine during mycosis. One could speculate that type I IFNs may be involved in the activation of the adaptive immune response. A recent study showed that during viral infection, depletion of pDCs affects the expansion of NK-cells and the accumulation of cytotoxic T-lymphocytes (Swiecki et al. 2010). While it is possible for a similar mechanism to be involved in the anti-*Aspergillus* response, future work should analyze the number of T-lymphocytes and NK cells in mice depleted of pDCs following infection with the mold.

Fungistatic Activity of pDCs

Previous work concentrated on the role of neutrophils and monocytes against *A. fumigatus*. Both of these cell types spread over the hyphae and cause damage to *A. fumigatus* via oxidative and non-oxidative mechanisms (Bozza et al. 2002; Diamond et al. 1978). The interactions of these phagocytes with *A. fumigatus* are of prime

importance in the clearance of infection; however, during infection other cell types such as pDCs may be involved in the antifungal response. In our assays, we observe that human pDCs have antifungal activity against *A. fumigatus*. Using two independent assays, one that measures metabolic activity and one that measures hyphal enlongation, we were able to demonstrate that pDCs inhibit fungal growth. The antifungal activity of pDCs appears to be different than the activity observed in monocytes and neutrophils. These two cell types have been shown to both inhibit growth and kill *A. fumigatus*; however, we did not observed hyphal killing by the pDCs. Instead, hyphal growth proceeded at a significantly slower rate in the presence of pDCs.

The fungistatic activity of pDCs led us to further analyze the interactions between pDCs and *A. fumigatus* hyphae. Confocal microscopy results show that pDCs are capable of spreading over *A. fumigatus* hyphae, suggesting that pDCs are capable of detecting fungal components. In 1978, Diamond *et al.* showed that PMNs damaged *A. fumigatus* hyphae by spreading over the hyphae and undergoing degranulation (Diamond et al. 1978). It is possible that the fungistatic activity of pDCs is mediated by a mechanism similar to the one used by PMNs. We noted that pDCs interacting with *A. fumigatus* hyphae were looking sick. Since pDCs have been reported to undergo apoptosis in the absence of activating signals and in response to some viral infections we analyzed pDC cell viability (Grouard et al. 1997; Lepelletier et al. 2010). In our assay, we observed over 50% pDC cytotoxicity when the pDCs were stimulated with *A. fumigatus* hyphae. During viral and parasitic infections, pDCs have been shown to undergo apoptosis as a mechanism to prevent viral replication inside the cell via

activation of TNF-related apoptosis inducing ligand (TRAIL). DC apoptosis during these conditions results in a bystander effect of immunosuppression (Kushwah and Hu 2010). Interactions between pDCs and *A. fumigatus* are similar to those with viruses; however, further experiments are necessary to determine whether a receptor such as TRAIL is involved in pDC cell death. Our results show that pDCs have fungistatic activity against *A. fumigatus* while undergoing cell death. These two independent observations led us to analyze whether pDC cell lysates were playing a role in the anti-*Aspergillus* response. pDC cell death may result in the secretion of cytoplasmic factors that could be responsible for the fungistatic activity of pDCs. Our results show that pDC cell lysates induce an antifungal response against *A. fumigatus*, with up to 70% antifungal activity as measured by XTT assay. Furthermore, the response mediated by the pDC cell lysates was similar to the response mediated by live pDCs suggesting that the observed fungistatic activity may be mediated by secreted pDC factors resulting from cell death.

Iron and zinc are essential growth factors. These two transition metals play critical roles in a variety of biochemical processes in microbes and host cells. Therefore, it is not surprising that leukocytes and other professional phagocytes have proteins responsible for chelating these factors. Moreover, chelators of Fe^{3+} and Zn^{2+} in host cells have been shown to play a role in antifungal responses (Mambula et al. 2000; Urban et al. 2009). Since other immune cells contain chelators of these cations, we wondered whether supplemental Fe^{3+} and Zn^{2+} would revert the observed antifungal effect of pDCs. The observation that $ZnCl_2$, but not $FeCl_3$, partially restored growth of *A. fumigatus* hyphae suggests a role for Zn^{2+} binding protein. Induction of hypozincemia can be

induced by secretion of calprotectin by neutrophils, monocytes, epithelial and endothelial cells in response to infection (Striz and Trebichavsky 2004). Calprotectin, free and associated with NETs, has been shown to inhibit the growth of *C. albicans* and other pathogenic fungi (Lulloff et al. 2004; Urban et al. 2009). In addition, NETs were recently shown to play a role in host responses against *A. fumigatus* (McCormick et al. 2010). Similar to our findings, McCormick *et al.* found that *A. fumigatus* growth is inhibited and that fungal growth can be restored with the addition of exogenous Zn^{2+} (McCormick et al. 2010). Although additional work should be performed in order to determine whether pDCs are capable of inducing a NETosis-like antifungal response, we demonstrate that human pDCs contain calprotectin. In addition, given the high number of Zn^{2+}-binding proteins in the cell, future experiments should be performed to confirm that calprotectin is involved in the antifungal response against *A. fumigatus*. The observation that exogenous Zn^{2+} only partially restores *A. fumigatus* growth suggests that pDCs may have more than one pathway mediating the antifungal response.

A. fumigatus hyphae fights back

pDC cell death appears to be a mechanism employed by the cells to halt hyphal growth; however, our data strongly suggest that pDC cell death is partly induced by secreted fungal factors. Although at a lower level, we observed pDC cytotoxicity when the pDCs and the hyphae were separated by a Transwell suggesting that cell death was mediated by a secreted factor.

A. fumigatus produces a wide variety of secondary metabolites. The best characterized of these metabolites is gliotoxin, a low molecular weight mycotoxin shown to be immunosuppressive. *A. fumigatus* gliotoxin has been shown to induce cell death in multiple immune cells including neutrophils and monocytes (Murayama et al. 1996; Stanzani et al. 2005). Furthermore, *A. fumigatus* gliotoxin has been shown to play a role in immune evasion by inhibiting NF-κB and the oxidative response (Spikes et al. 2008). We found that *A. fumigatus* gliotoxin induces pDC cell death. However, whether gliotoxin is affecting signaling pathways that would lead to killing of *A. fumigatus* remains to be determined.

To dissect the mechanism of pDC cell death induced by *A. fumigatus* hyphae we performed TUNEL assay. We observed that pDCs incubated with *A. fumigatus* or purified gliotoxin resulted in TUNEL positive staining, suggesting apoptosis-mediated cell death. Even though TUNEL positive staining suggests that apoptosis is the mechanism of cell death, other mechanisms such as pyroptosis may be playing a role in pDC cell death. Recently, it was shown that cells undergoing pyroptosis exhibit DNA degradation and upregulation of pro-apoptotic proteins resulting in a positive TUNEL reaction (Fink et al. 2008; Fink and Cookson 2005; Fink and Cookson 2006). Furthermore, TUNEL staining confirmed our finding that more than fifty percent of the pDCs undergo cell death. Further experimentation, including staining for apoptosis-specific and pyroptosis-specific markers, is required in order to determine the mechanism of pDC cell death. One way of determining the mechanism of cell death would be to perform intracellular staining with Annexin 5 and IL1β. A pyroptosis positive stain

would show staining for both Annexin 5 and IL1β; while an apoptosis positive result should only show staining of Annexin 5. This staining assay can also be performed using TUNEL Assay, where cells undergoing pyroptosis should show TUNEL positive and $IL1\beta^{bright}$ staining.

It is possible that pDC cell death is a combination of *A. fumigatus* immune evasion via immunosuppression and a mechanism of pDCs to induce tolerance. During viral infections, DCs undergo apoptosis via death receptor pathways (extrinsic pathways), which results in a bystander effect of induced immunosuppression (Fugier-Vivier et al. 1997). In addition, it has been shown that transplant patients treated with corticosteroids have decreased levels of circulating pDCs (Kushwah and Hu 2010), and these patients are highly susceptible to *A. fumigatus* infection. Additional experiments are required in order to understand the relationship between pDC cell death and the antifungal response to *A. fumigatus* mediated by these cells.

Inflammatory cytokines

The antifungal response of pDCs against *A. fumigatus* goes beyond halting hyphal growth. Human pDCs have been shown to secrete huge amounts of type I IFN in response to viruses and certain RNA and DNA sequences; however, the role of IFNs in the antifungal response has received little attention. Recent reports have suggested a role for type I IFN during fungal infections (Biondo et al. 2008; Inglis et al. 2010a). In a mouse infection model, infection of mice deficient in IFNα/β receptor with *Cryptococcus neoformans* resulted in decreased survival when compared to the wild-type counterparts.

In addition, IFNα/β receptor knock-out mice infected with *C. neoformans* failed to generate a protective Th1 response (Biondo et al. 2008). Upregulation of type I IFN genes has also been observed in macrophages infected with *Histoplasma capsulatum* (Inglis et al. 2010a). Even though these publications suggest a role for type I IFN during fungal infections, the role of type I IFN during infection in humans remains to be elucidated. One report by Romani et al. showed that pDCs are capable of inducing a tolerogenic response to *A. fumigatus*; however, they observed low concentrations of IFNα secretion by pDCs infected with conidia (Romani et al. 2006). While Romani et al. did not see much of a IFNα response by pDCs following challenge with *A. fumigatus* conidia, our result show that pDCs are capable of secreting IFNα and TNFα in response to *A. fumigatus* hyphae. The differences observed may be due to a number of reasons, such as the method used to generate the pDCs by each group.

TNFα plays an important role in the clearance of *A. fumigatus* by enhancing leukocyte-mediated killing of the mold (Filler et al. 2005). We found that pDCs secrete TNFα upon stimulation with *A. fumigatus* hyphae. It is possible that TNFα secretion by pDCs is a communication mechanism employed by these cells to augment hyphal killing. One report showed that incubation of neutrophils with recombinant TNFα increases killing of the hyphae by these immune cells (Roilides et al. 1998). Furthermore, this group showed that alveolar macrophages phagocytose more *A. fumigatus* conidia in the presence of recombinant TNFα. Although further experimentation is required, it is possible for secretion of TNFα by pDCs to be involved in the observed antifungal response against *A. fumigatus* hyphae. TNFα may be playing multiple roles in the

antifungal response to *A. fumigatus*. The pDC-secreted TNFα could be acting as a chemotaxin, attracting neutrophils to the site of infection to kill the hyphae. In addition, TNFα may be playing a role in pDC cell death by activation of apoptotic pathways in pDCs. Future work should focus on determining the role of TNFα during *A. fumigatus* infection.

The cytokine response by pDCs is induced via a TLR7/9-independent mechanism. Pre-treatment of pDCs with either bafilomycin A or chloroquine, both V-ATPase inhibitors (Martinson et al. 2010; Nakamura et al. 2008) did not affect secretion of type I IFN in response to *A. fumigatus* hyphae. It is possible during infection for other host cells such as macrophages and neutrophils to kill *A. fumigatus*, resulting in release of fungal DNA into the environment. While our results show that pDCs secrete cytokines independent of activation of TLR7/9, we were looking at the responses of pDCs in isolation from other cell types. For example, neutrophils release NETs that cause fungal damage. These NETs contain host DNA and DNA binding proteins that can possibly bind fungal DNA and deliver the complexes into $TLR9^{+}$ compartments (Papayannopoulos and Zychlinsky 2009). In addition, whether A. fumigatus DNA can stimulate a response by cytoplasmic DNA receptors such as DAI or the inflammasome remains to be determined.

Mature pDCs and *A. fumigatus* conidia

Upon maturation, pDCs become more phagocytic and behave more like conventional DCs (Drenou et al. 2005; Gibson et al. 2002; Guiducci et al. 2006). While

pDC maturation in the blood is dependent on encountering antigen and communication with other cells via cytokines and chemokines, maturation of pDCs in culture requires supplementing the media with IL3 and CD40L (Schnurr et al. 2004). Mature pDCs have been shown to be involved in antigen presentation and modulation of adaptive immune responses (Cella et al. 2000; Mittelbrunn et al. 2009); however, these cells have not been shown to be involved in antimicrobial responses. While immature pDCs have fungistatic activity against *A. fumigatus* hyphae, mature pDCs do not appear to mediate an antifungal response. Similar to immature pDCs, seven-day mature pDCs stimulated with *A. fumigatus* recognize the fungi and spread over the hyphae.

"Mature" pDCs secrete an array of cytokines in response to *A. fumigatus* conidia. We observed that seven-day mature pDCs secrete IFNα, TNFα and IL12-p70 in response to stimulation with *A. fumigatus* conidia. This array of cytokines correlates with the induction of an inflammatory response against the mold; however, mature pDCs are not known to secrete large amounts of cytokines in response to infection (Schnurr et al. 2004). One report showed that maturation of pDCs is mediated by detection of an antigen, in this scenario the main role of mature pDCs is to uptake the antigen and present it to T-cells to initiate an adaptive immune response (Jahn et al. 2010; Jegalian et al. 2009; Tel et al. 2010). However, maturation of pDCs *in vitro* requires supplementing the media with CD40L and IL-3 (Schnurr et al. 2004). It is possible that at the time of performing our assays, the pDCs were still immature. Therefore, further experimentation, by flow cytometry, should analyze whether pDC maturation occurs in

vitro. Independent of whether we had mature or immature pDCs, we found that pDCs recognized and induced a response against *A. fumigatus*.

Detection of *A. fumigatus* conidia showed similar cytokine profiles for both resting and swollen conidia. It is possible that cytokine secretion by pDCs was mediated by detection of hyphae rather than conidia, as we stimulated pDCs for 18 hours. In addition, cytokine secretion by pDCs required *A. fumigatus* to be alive. These findings correlate with the observed responses of immature pDCs stimulated with hyphae. In the case of mature pDCs, during the maturation process these cells acquire characteristics that resemble cDCs (Sadaka et al. 2009). It is possible that mature pDCs express surface receptors such as dectin-1 or TLR2 that allow for recognition of *A. fumigatus*. However, whether there is a difference in the expression of surface receptors between mature and immature pDCs remains to be elucidated.

Stimulation of cytokine secretion by *A. fumigatus* conidia occur independent of contact. During conidial germination and hyphal growth, *A. fumigatus* secretes components of the cell wall such as melanin or galactomannan into the environment where it can have immunostimulatory activity. Further experiments should concentrate on determining the nature of the immunostimulatory factor secreted by *A. fumigatus*.

Future Directions:

Multiple lines of research will need to be pursued in order to further understand the role of human pDCs and nucleic acid sensors during *A. fumigatus* infection.

First, my research shows that *A. fumigatus* DNA can act as ligand for TLR9 and initiate a TLR9-dependent immune response. However, upon treatment with restriction enzymes, our data show a decrease in the cytokine response rather than complete abrogation of the response. One line of investigation should determine whether other nucleic acid receptors, such as DAI or RIG-I, detect and respond to *A. fumigatus* DNA and/or RNA. One approach to accomplish this is to determine whether *A. fumigatus* DNA or RNA stimulates a cytokine response following transfection into the cytoplasm of BMDCs. The responses of cells deficient in DAI, RIG-I or MDA-5 should be compared with those of WT cells. If any of these receptors play a role in the detection of *A. fumigatus* nucleic acids, one would expect a decrease of the cytokine response in cells that lack DAI, RIG-I or MDA-5. Since it is possible for TLR9 responses to overwhelm the system, another approach would include analyzing the response of mice deficient in TLR9 and each of the cytoplasmic nucleic acid receptors.

A second line of investigation should determine whether the inflammasome plays a role in the detection of fungal DNA. These molecular platforms or inflammasomes become activated upon cellular infection or stress. Activation of the inflammasomes results in the maturation of proinflammatory cytokines such as IL-1β, engaging innate immune responses (Franchi et al. 2010; Schroder and Tschopp 2010). Recent work has suggested that pathogenic DNA could induce a response mediated by the Nalp3, NLCR3 and AIM2 inflammasomes (Malireddi et al. 2010; Rathinam et al. 2010; Warren et al. 2010). It would be interesting to test whether BMDC from mice deficient in Nalp3, Nlcr3 or AIM2 inflammasomes show a difference in IL-1β and IL-18in response to *A.*

fumigatus DNA. Understanding the role of other nucleic acid receptors in the response to fungal DNA will provide further insights into the host response to fungal PAMPs.

Another line of research arises from Chapter III. The data show that addition of exogenous Zn^{++} partially restores *A. fumigatus* growth in the presence of pDCs; furthermore, we found by western blot that pDCs contain calprotectin. However, the data presented in this thesis does not establish whether calprotectin is responsible for the fungistatic effect of pDCs. One way to analyze whether calprotectin mediates the fungistatic activity would involve depleting calprotectin from pDC lysates by immunoprecipitation. Following the depletion, *A. fumigatus* hyphae would be incubated with the lysates and the metabolic activity of the hyphae easured by XTT following the incubation. Another way of analyzing whether calprotectin in pDCs is responsible for the observed antifungal effect would be by confocal microscopy. Calprotectin has been shown to be part of NETs; however, whether pDCs produce NET-like structures has not been investigated. One line could analyze whether pDCs generate NETs-like structures, and if pDCs undergo a NETosis-like mechanism.

Our data show for the first time that pDCs recognize and induce an antifungal response against *A. fumigatus*. However, we did not investigate which receptor is responsible for the observed response. Previous investigators have shown that murine pDCs contain the mannan-binding receptor dectin-2, and that this receptor could induce a response against *A. fumigatus* (Barrett et al. 2009; Graham and Brown 2009). While it is possible that dectin-2 is the receptor responsible for the observed antifungal response,

future work should concentrate on answering a few questions: 1) Do human pDCs express Dectin-2?; 2) If we block or inhibit expression of the receptor, is there a decrease in cytokine production and antifungal activity upon infection with *A. fumigatus*?; and 3) What signaling pathway or pathways is Dectin-2 activating following infection with *A. fumigatus*? Even though the future work proposed in this paragraph concentrates on Dectin-2, it is possible for other C-type lectins such as MR, dectin-1 and DC-SIGN to be involved in the detection of *A. fumigatus* by pDCs. Whether these receptors are present on human pDCs and whether they play a role in the detection of the mold should also be investigated.

Multiple questions remain unanswered about the interactions between mature pDCs and *A. fumigatus*. While we observed that pDCs secrete cytokines in response to live *A. fumigatus*, the project was abandoned. In order to understand the interactions between mature pDCs and *A. fumigatus*, future work should determine whether mature pDCs play an antifungal role against the mold. Assays similar to those performed in Chapter III, such as XTT and LDH, could be used to study the interaction. In addition, our results show that *A. fumigatus* secrete a putative immunostimulatory factor. We know that mature pDCs secrete cytokines in response to *A. fumigatus* independent of contact, and we found that during fungal growth the mold secretes carbohydrate and proteins into the environment; however, the specific fungal component or factor that is inducing a response in pDCs remains unidentified. *A. fumigatus* releases a number of bioactive proteins, carbohydrates and lipids that could be immunostimulatory. One way to determine the nature of the factor could be to treat fungal supernatants with proteases,

glycosidases or lipases. Following treatment, supernatants could be used to stimulate mature pDCs and measure the cytokine response to the fungal supernatants. Using the interaction of immature pDCs with *A. fumigatus* as groundwork, it is possible for *A. fumigatus* to secrete gliotoxin or other secondary metabolites that could induce a cytokine response of mature pDCs. In our assay, we usually stimulate the pDCs with conidia which germinate into hyphae within the incubation period. Furthermore, gliotoxin has been shown to be secreted during hyphal growth (Bok et al. 2005; Chiang et al. 2008). Future work should analyze whether *A. fumigatus* secrete gliotoxin when incubated with mature pDCs, and whether the observed cytokine response mediated by the mature pDCs is induced by the secondary metabolite.

The role of pDCs and IFNs during infections due to non-viral pathogens remains a fairly new area of investigation. Over the past few years, multiple reports have evaluated the role of these cells during bacterial infection; however, the role of pDCs during fungal infections remains largely unexplored. My work provides evidence that pDCs contribute to the antifungal responses to *A. fumigatus*; however, additional work is required in order to better understand the full role of pDCs during fungal infections.

Concluding Remarks: Interactions between pDCs and *A. fumigatus*

The data presented in this dissertation suggest a putative role for pDCs in host defenses against *A. fumigatus* infection. Although pDCs are capable of detecting "free" *A. fumigatus* DNA, during infection pDCs are most likely to encounter live fungi rather than naked DNA. Not only are pDCs present at low concentrations in tissues, but human serum contains endonucleases that could digest any DNA released by the fungus. However, the possibility of *A. fumigatus* DNA reaching TLR9-rich compartments in pDCs cannot be completely eliminated. *A. fumigatus* DNA has been found in the sera of patients with IA (Chamilos and Kontoyiannis 2006), and there is a possibility that the fungal DNA could be acting as a PAMP. Furthermore, it is possible that *A. fumigatus* DNA could be interacting with other DNA receptors such as DAI and the NOD-like receptors. Nonetheless, we found that pDCs are capable of secreting cytokines in response to *A. fumigatus* hyphae independent of TLR9 activation. The data suggest a seemingly complex host-pathogen interaction.

The following model is proposed for the interactions between *A. fumigatus* and pDCs (Figure 5.1): inhaled conidia in the alveoli that are not eliminated by alveolar macrophages, monocytes and neutrophils germinate into hyphae. Professional phagocytes kill the hyphae via oxidative and non-oxidative mechanisms that may release fungal factors, such as DNA (alone or associated with fungal proteins/carbohydrates). These secreted or released factors could be taken up or phagocytosed by other phagocytes and antigen presenting cells such as pDCs. In the case of fungal DNA, in the endosomes

of pDCs, it is capable of stimulating a proinflammatory response. In addition, circulating pDCs in the blood are capable of recognizing *A. fumigatus* hyphae and establishing an antifungal response. Although our results are from an *in-vitro* system, human pDCs spread over the hyphae and are capable of delaying fungal growth. Following recognition of *A. fumigatus*, pDCs undergo cell death. This results in the release of Zn^{2+} binding proteins such as calprotectin, which has been shown to inhibit fungal growth by mechanisms that involve nutritional deprivation of the essential metal. While the mechanism of pDC cell death remains to be further analyzed, it appears to be mediated in part by fungal mycotoxins released during hyphal growth. As a result of incubation with *A. fumigatus*, pDCs are dying by an apoptosis or pyroptosis mediated mechanism. In addition, pDCs secrete IFNα and TNFα in response to hyphae. The fact that pDCs are capable of secreting cytokines while undergoing cell death suggest that they are dying by a pyroptosis-like mechanism.

The interactions between immune cells and *A. fumigatus* are of extreme importance, as these interactions are responsible for the outcome of the disease. A deregulation between Th1 and Th2 responses will lead to protective or nonprotective responses, respectively. While engagement of TLR9 during *A. fumigatus* may be detrimental to the host rather than the pathogen, we show a novel mechanism of *A. fumigatus* detection by TLR9-rich pDCs. The mechanism of cytokine secretion by pDCs is independent of TLR9 activation. While further experimentation is required in order to determine whether pDCs induce a protective or non-protective response in vivo, we show that pDCs mediate fungistatic activity against *A. fumigatus* in vitro. Furthermore, upon

fungal stimulation, pDCs secrete cytokines which can recruit and activate other immune cells to come into the site of infection and eliminate the hyphae.

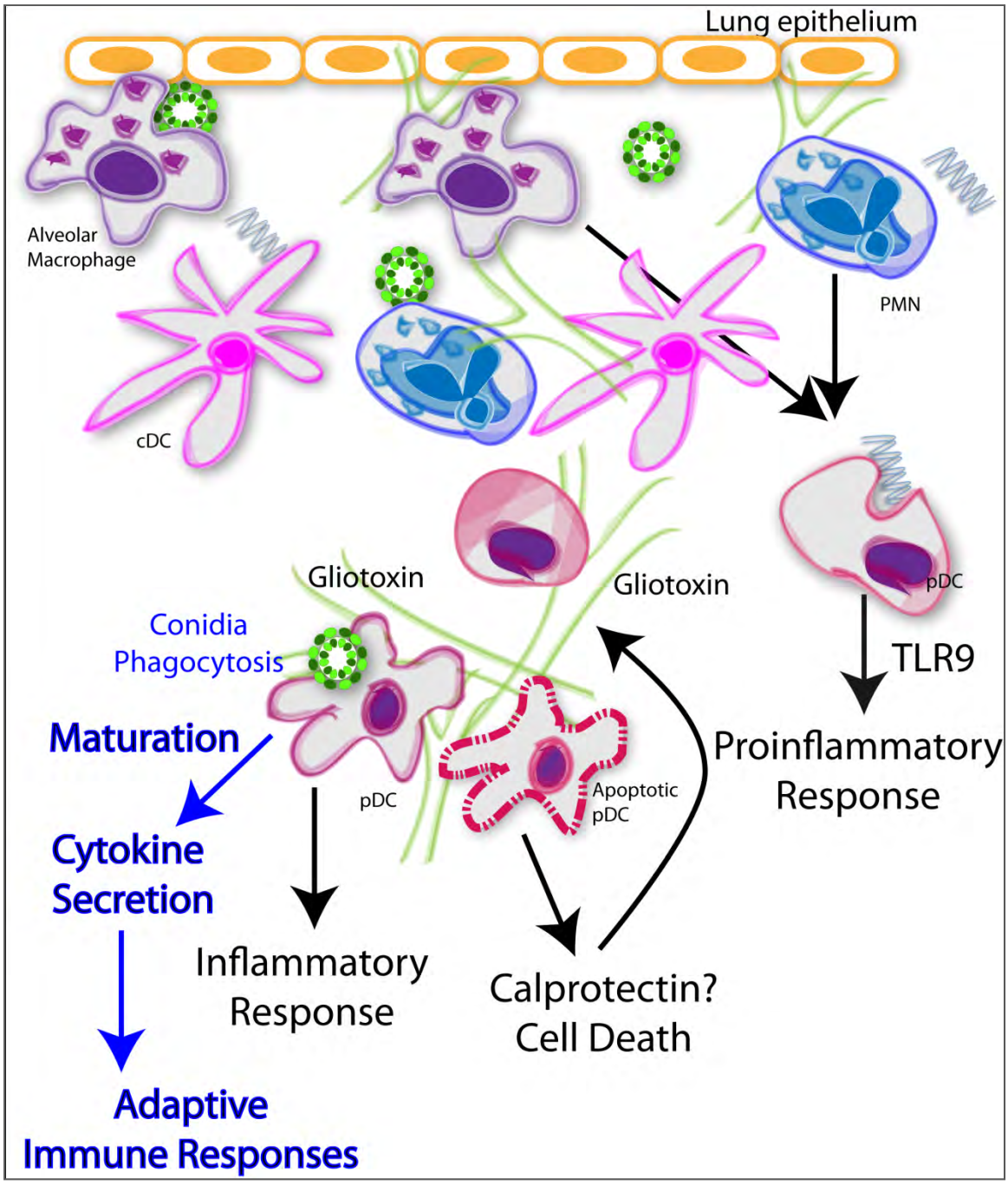
Lung epithelium
Alveolar Macrophage
PMN
cDC
Gliotoxin
Gliotoxin
pDC
TLR9
Conidia Phagocytosis
Maturation
Proinflammatory Response
pDC
Apoptotic pDC
Cytokine Secretion
Inflammatory Response
Calprotectin? Cell Death
Adaptive Immune Responses

Figure 5.1. Proposed model: Interactions between pDCs and *A. fumigatus*

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