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Host Immune Responses Against Pulmonary Fungal Pathogens

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1. Introduction

The lungs are a gateway for numerous airborne pathogens that are ubiquitous in our environment. Among these potential pathogens are fungi that can be found in the soil, bird excreta, air ducts, and many other places where their contact is unavoidable. Exposure to these fungal pathogens oftentimes goes unnoticed due to the activation of our robust immune systems which sequester and control these microbes before significant damage occurs. Still, there are many situations in which host immunity becomes compromised providing an opportunity for typically innocuous fungal organisms to become established and cause disease or for dormant infections to reawaken. Also, in certain cases disease may be exacerbated due to an over exuberant immune response. In this chapter, we will review the main aspects of innate and adaptive immune responses against pulmonary fungal pathogens. We will also discuss the potential for vaccines to prevent pulmonary fungal infections.

2. Introduction to pulmonary fungal infections

Pulmonary fungal infections can be grouped into primary fungal pathogens and opportunistic fungal pathogens. Those organisms that can cause disease in immune competent hosts are considered primary pathogens including *Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasilensis, and Blastomyces dermatiditis*. All of the primary pulmonary fungal pathogens are endemic to the United States and/or Central & South America. *Histoplasma* and *Blastomyces* are endemic to the Ohio River & Mississippi River Valleys of the United States and also to certain regions of Central and South America (Klein et al., 1986; Deepe, 2000). *Coccidioides* is prevalent in the desert southwest United States (Fisher et al., 2007), and *Paracoccidioides* is endemic in Central and South America, particularly in Brazil (Franco, 1987; Franco et al., 1989; Brummer et al., 1993). These

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infections are acquired by inhalation of fungi from contaminated soil, and severity of disease generally correlates with the amount of exposure to the pathogen.

Examples of organisms that are opportunistic pulmonary fungal pathogens include *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Pneumocystis*, and *Rhizopus*. *C. neoformans* is found ubiquitously in the soil, usually in soil contaminated with pigeon guano (Perfect and Casadevall, 2002). These fungi primarily cause disease in individuals with compromised immune systems. Most cryptococcal infections are asymptomatic, and the organism typically causes disease in immune compromised patients, such as AIDS patients, solid organ transplant patients on immune-suppressive drugs, or patients receiving chemotherapy (Levitz, 1991; Mitchell and Perfect, 1995; Singh et al., 1997; Shoham and Levitz, 2005). *A. fumigatus* is ubiquitously found in the environment, and is normally found in association with decaying wood and plant matter (Deacon et al., 2009). However, *A. fumigatus* can cause severe respiratory infections in cases of massive exposure or immune deficiency, such as neutropenia, due to chemotherapy, AIDS, or bone marrow transplant therapy (Denning, 1996; Almyroudis et al., 2005; Magill et al., 2008). *Pneumocystis* infection is acquired by inhalation of organisms from a yet unknown source (Keely et al., 1995; reviewed in Kelly and Shellito, 2010). Most *Pneumocystis* infections occur in immunosuppressed individuals due to either HIV or chronic obstructive pulmonary disease (Leigh et al., 1993; Nevez et al., 1999; Huang et al., 2003; Calderon et al., 1996; Morris et al., 2004; Norris et al., 2006; Davis et al., 2008; Morris et al., 2008a; Morris et al., 2008b; Kling et al., 2009). Similarly, infection with *Rhizopus* typically occurs in individuals who are immune compromised, such as organ transplant recipients (Kontoyiannis, 2010; Pappas et al., 2010).

3. Innate immune responses against pulmonary fungal pathogens

3.1 Phagocyte interactions with pulmonary fungal pathogens

Cells of the innate immune system such as dendritic cells (DCs) and macrophages residing in the lungs/airways are the first line of defense against pulmonary fungal pathogens. Although these innate cells cannot completely eliminate many fungal pathogens, they are involved in uptake and degradation of fungi and processing of antigens derived from these pathogens. In contrast, neutrophils which are also phagocytic and can be fungistatic are unable to present antigen. Based on the subset of receptors involved and signaling pathways triggered by these receptors, the innate immune system will trigger different types of early responses and subsequently translate these signals to mount different types of adaptive responses.

H. capsulatum can initially be engulfed by macrophages, DCs, and neutrophils (reviewed in (Deepe, 2005)), however, *H. capsulatum* recognition by different receptors results in different fates (Gomez et al., 2008). DCs recognize *H. capsulatum* by VLA-5, by interaction with an unknown receptor, which results in uptake, killing, and antigen presentation (Gildea et al., 2001; Gomez et al., 2008). Human DCs exert their antifungal activity via phagolysosomal fusion. The addition of suramin (which blocks phagolysosomal fusion) inhibits DC fungicidal activity, but inhibition of lysosomal acidification and inhibition of respiratory burst has no effect (Gildea et al., 2005). In contrast to DCs, macrophages recognize the *H. capsulatum* surface molecule heat-shock protein 60 (HSP 60) by LFA-1 (CD11a/CD18), complement receptor 3 (CD11b/CD18), and complement receptor 4 (CD11c/CD18) and this recognition leads to uptake and intracellular replication (Kimberlin et al., 1981; Bullock and Wright, 1987; Long et al., 2003; Gomez et al., 2008; Lin et al., 2010). However, activated

macrophages can halt intracellular replication (Wu-Hsieh and Howard, 1984; Wu-Hsieh et al., 1984). *H. capsulatum* can avoid the macrophage lysosomal environment by preventing phagolysosomal fusion (Newman et al., 1997; Strasser et al., 1999) or by alkalinizing the pH of the phagolysosome (Eissenberg and Goldman, 1988; Eissenberg et al., 1988; Eissenberg et al., 1993). Macrophages infected with *H. capsulatum* and activated with GM-CSF decrease available iron and zinc, while infected macrophages without GM-CSF do not. Further, chelation of zinc inhibits yeast replication; therefore zinc deprivation may be used by macrophages in host defense against *H. capsulatum* (Winters et al., 2010). Neutrophil phagocytosis of *H. capsulatum* requires opsonization with either antibody or complement (Brummer et al., 1991; Kurita et al., 1991a; Kurita et al., 1991b; Newman et al., 1993). Neutrophil uptake of *H. capsulatum* is fungistatic, as opposed to macrophages (permissive growth & replication) and DCs (fungicidal) (reviewed in Deepe, 2005). A lack of neutrophils causes a non-lethal infection to become a lethal infection (Zhou et al., 1998).

Immature DCs bind spherules of *Coccidioides* in a time and temperature-dependent manner, and binding is blocked by mannan, suggesting that mannose receptor (MR) is involved in this interaction (Dionne et al., 2006). Spherules of *Coccidioides* stimulate DC functional maturation, evidenced by decreased endocytic capacity and stimulation of allogeneic peripheral blood mononuclear cell activation (Dionne et al., 2006). Further studies showed that a DC-based *Coccidioides* vaccine had adjuvant properties and activated protective immune responses in mice (Awasthi, 2007). Although macrophages can ingest *Coccidioides*; earlier studies suggested that they are not able to kill the arthroconidia (Kashkin et al., 1977; Beaman et al., 1981, 1983; Beaman and Holmberg, 1980b, 1980a). Studies demonstrated that monocytes derived from human peripheral blood were able to kill *Coccidioides* (Ampel and Galgiani, 1991). Neutrophils are the earliest cell type to infiltrate upon pulmonary infection with *Coccidioides* arthroconidia (Savage and Madin, 1968). Phagocytosis by neutrophils is enhanced by the addition of immune serum (Drutz and Huppert, 1983; Wegner et al., 1972; Frey and Drutz, 1986). Uptake of *Coccidioides* arthroconidia by neutrophils induces a respiratory burst (Frey and Drutz, 1986), but less than 20% of the arthroconidia are killed (Frey and Drutz, 1986; Beaman and Holmberg, 1980b; Drutz and Huppert, 1983). The spherule form of *Coccidioides* cannot be phagocytosed by or killed by neutrophils (Frey and Drutz, 1986; Galgiani, 1986), but rupture of the spherule leads to an influx of neutrophils (Frey and Drutz, 1986).

P. brasiliensis can be phagocytosed by immature DCs, and uptake is significantly decreased with the addition of mannan, suggesting that MR is the primary receptor for *P. brasiliensis* on DCs (Ferreira et al., 2004). After DC uptake of *P. brasiliensis*, the fungal organisms survive and multiply intracellularly rather than being killed (Ferreira et al., 2004). Following *in vitro* culture of *P. brasiliensis* or the major surface antigen gp43 with DCs, major histocompatibility complex (MHC) II is downregulated as is the production of interleukin (IL)-12 and tumor necrosis factor (TNF)-α (Ferreira et al., 2004). However, *in vivo* studies showed that DC interaction with *P. brasiliensis* results in modification of DC receptor expression, including upregulation of CCR7, CD103, and MHC II and also induces migration of both pulmonary and bone marrow-derived DCs. DCs are also able to activate T helper cell responses in the draining lymph nodes following interaction with *P. brasiliensis* (Silvana dos Santos et al., 2011). Alveolar macrophages adhere to and internalize *Paracoccidioides* using the organism's phospholipase B, which also serves to downregulate macrophage activation (Soares et al., 2010). During pulmonary infection with *P. brasiliensis*, a shift in macrophage activation occurs, which is characterized by an increase in IL-1, TNF-α,

and IL-6 (Silva et al., 2011). *P. brasilensis* can proliferate within macrophages, but macrophage activation inhibits its growth (Brummer et al., 1988; Cano et al., 1994). Further, macrophages activated by interferon (IFN)-γ can kill *P. brasiliensis* (Cano et al., 1994; Gonzalez et al., 2000). *In vitro* stimulation of human monocytes and neutrophils with *Paracoccidiodes* yeast showed downregulation of toll-like receptor (TLR)2, TLR4, and dectin-1 on the surface of these cells. In addition, yeast cells induced the production of pro-inflammatory cytokines such as TNF-α (Bonfim et al., 2009). Mice lacking TLR2 had a less severe pulmonary infection than wild-type (WT) mice and had decreased nitric oxide (NO) production. However, despite the differences in infection, both $TLR2^{-/-}$ mice and WT mice had similar rates of survival and similar pulmonary inflammatory responses (Loures et al., 2009). Further, TLR2 deficiency skewed the adaptive response towards a T helper (Th)17 phenotype and caused a decrease in T regulatory cells. Increased neutrophils and eosinophils migrate to the lungs of mice susceptible to *P. brasiliensis*, (Cano et al., 1995), and this influx affects the disease outcome and the adaptive response induced to infection. In susceptible individuals recovered from *Paracoccidiodes*, neutrophils are able to phagocytose the organism, but this leads to degeneration of the neutrophils. These data suggest that susceptible individuals have an inherent neutrophil deficiency (Dias et al., 2008). Further, neutrophils from patients with *P. brasiliensis* have a digestive defect against the fungus (Goihman-Yahr et al., 1980), and also have a killing defect against the fungus (Goihman-Yahr et al., 1985; Goihman-Yahr et al., 1992).

B. dermatiditis interaction with DCs causes efficient upregulation of antigen presentation and costimulatory molecules and induces production of IL-12 and TNF-α (Wuthrich et al., 2006). DCs can activate $CD8^+$ T cells in the absence of $CD4^+$ T cells, and the yeast alone is a sufficient inflammatory stimulus that can directly induce maturation of DCs and can induce production of TNF-α, IL-1β, and IL-12 (Wuthrich et al., 2006). Monocyte-derived dendritic cells can associate with yeast in the lung and transport them to the draining lymph nodes, but fail to present antigen to $CD4^+$ T cells, however dermal DCs are capable of antigen presentation (Ersland et al., 2010). During *B. dermatiditis* infection, alveolar macrophages are only modestly able to ingest and kill the yeast form of the organism (Bradsher et al., 1987). Murine macrophages are only able to kill less than 5% of yeast (Brummer and Stevens, 1987; Brummer et al., 1988), and had a 25-30% reduction in respiratory burst compared to the respiratory burst induced by zymosan. The *Blastomyces* adhesion 1 (*BAD1*) molecule on the *Blastomyces* yeast surface is responsible for binding to CD11b/CD18 and CD14 on the macrophage surface and subsequent entry (Klein et al., 1993; Newman et al., 1995). Neutrophils rapidly infiltrate to pulmonary tissues following infection, and are responsible for the formation of pyogranulomatous lesions. Conidia are rapidly phagocytosed by neutrophils, but killing of conidia is inefficient. Similar to macrophages, the neutrophil respiratory burst induced by conidia is only 70% of that induced by zymosan (Drutz and Frey, 1985). In addition, yeasts are even more difficult for the neutrophils to phagocytose & kill than conidia (Drutz and Frey, 1985).

Pneumocystis interacts with DCs in vitro by MR, (Kobayashi et al., 2007) but the interaction does not lead to an increase in maturation markers such as MHC II, CD40, CD54, CD80, or CD86. Additionally, this interaction induces the production of IL-4 but not IL-12p40, IL-10, TNF-α, or IL-6 (Kobayashi et al., 2007). However, *Pneumocystis* cell wall β-glucans have the ability to induce costimulatory molecule upregulation on DCs, such as MHC II, CD80, CD86, and CD40. These DCs interacted with β-glucans from *Pneumocystis* via dectin-1, and

co-stimulatory molecule expression and Th1-type cytokine secretion by β-glucan stimulated DCs was regulated by Fas-Fas ligand interaction (Carmona et al., 2006). *In vivo* administration of DCs pulsed with *Pneumocystis* induced specific T cell responses and release of IL-4 as well as specific IgG_1, IgG_{2a}, and IgG_{2b} production (Kobayashi et al., 2007). Alveolar macrophages have been shown to directly kill both *Pneumocystis* trophozoites and cysts (Fleury et al., 1985; reviewed in Kelly and Shellito, 2010). Specifically, alternatively-activated macrophages (aaMac) are important effector cells against *Pneumocystis*, and aaMac is enhanced by IL-33 (Nelson et al., 2011). In addition, *Pneumocystis* infection causes changes in gene expression of alveolar macrophages that included upregulation of genes involved in antigen presentation and antimicrobial peptides, but downregulation of genes involved in phagocytosis and uptake (Cheng et al., 2010). *Pneumocystis* major surface glycoprotein (MSG), which is a heavily glycosylated surface antigen, is recognized by MR on alveolar macrophages (Ezekowitz et al., 1991). *Pneumocystis* infection in HIV^+ patients induces shedding of the MR, which results in reduced alveolar macrophage phagocytosis of the microbe (Koziel et al., 1998; Fraser et al., 2000). Neutrophils can also interact with *Pneumocystis*, but the presence of neutrophils is correlated with inflammation and increased severity of disease (reviewed in Kelly and Shellito, 2010).

Inhaled *A. fumigatus* conidia are first encountered by alveolar macrophages and neutrophils (reviewed in Hasenberg et al., 2011). Following uptake of *Aspergillus* by phagocytes, the organism enters the phagosome and killing occurs following phagosomal fusion with lysosomes, (Ibrahim-Granet et al., 2003). In the absence or impairment of phagocytic cells, there are dramatic increases in invasive *Aspergillus* infections (Latge, 1999). β-1,3 glucans of *Aspergillus* swollen conidia and hyphae are recognized by dectin-1 on the surface of alveolar macrophages, monocytes, and neutrophils (Taylor et al., 2002; Taylor et al., 2007). This recognition of *Aspergillus* leads to phagocytosis and the production of cytokines such as TNF-α, IL-6, and IL-18 (Gersuk et al., 2006). In addition, *Aspergillus* can be recognized by TLRs, predominantly TLR2 and TLR4 (Wang et al., 2001; Mambula et al., 2002; Netea et al., 2002; Meier et al., 2003; Netea et al., 2003; Bellocchio et al., 2004; Bochud et al., 2008), but phagocytosis and uptake are not due to recognition by TLR2, TLR4, TLR9, or MyD88 (Bellocchio et al., 2004). More recent data also points to recognition of *Aspergillus* unmethylated DNA by TLR9 (Ramirez-Ortiz et al., 2008; Ramaprakash et al., 2009). Further, TLR9 is actively recruited to the *Aspergillus* phagosome and requires the N-terminal proteolytic cleavage domain for proper intracellular trafficking (Kasperkovitz et al., 2010).

DCs bind and internalize *A. fumigatus* through DC-SIGN, and this binding triggers DC maturation (Serrano-Gomez et al., 2004). Mouse DCs can internalize conidia of *A. fumigatus* using MR and a C-type lectin receptor as well as FcγR (Bozza et al., 2002). Upon exposure of DCs to *A. fumigatus*, DCs upregulate HLA-DR, CD80 and CD86 (Grazziutti et al., 2001; Bozza et al., 2002). Following *A. fumigatus* infection, DCs can release the chemokine CXCL8, which promotes migration of PMNs, can upregulate CCL19, which is important in migration of $CCR7^+$ naïve T cells and mature DCs to lymph nodes, and can release soluble factors that increase CD11b and CD18 on PMNs (Gafa et al., 2007). DC phagocytosis of *A. fumigatus* conidia and hyphae occur by different means and through different receptors; conidia are phagocytosed by coiling phagocytosis and hyphae are phagocytosed by zipper-type phagocytosis (Bozza et al., 2002). *A. fumigatus* killing by DCs is dependent on phagolysosomal fusion and a reduction in pH (Ibrahim-Granet et al., 2003). Plasmacytoid DCs (pDCs) have the ability to spread over *A. fumigatus* hyphae and inhibit their growth

(Ramirez-Ortiz et al., 2011), and antifungal activity does not require direct cell contact. Following interaction of pDCs with *Aspergillus*, pDCs release pro-inflammatory cytokines, such as IFN-α and TNF-α, and these are produced via a TLR9-independent mechanism (Ramirez-Ortiz et al., 2011). During the early stages of *Aspergillus* infection, alternatively activated macrophages are recruited to the lung and are important in host defense (Bhatia et al., 2011). Studies examining the interaction of *Aspergillus* conidia with alveolar macrophages showed that infectivity and inhibition of macrophage killing by the fungus were due to the presence of a siderophore system that allows the fungus to acquire iron (Schrettl et al., 2010). In neutropenic mice, inflammatory DCs are recruited to the lungs during *Aspergillus* infection, and this recruitment is dependent on the absence of neutrophils (Park et al., 2010). This accumulation led to increased TNF-α, CCL2, and CCL20, which resulted in further recruitment of inflammatory DCs. Neutrophils, when incubated with *A. fumigatus* hyphae, form neutrophil extracellular traps (NETs), which are antifungal, but mostly act in a fungistatic manner to limit spread of the hyphae (Bruns et al., 2010; Hasenberg et al., 2011).

In vitro studies of DCs with *C. neoformans* have shown that DCs are involved in detection, binding, phagocytosis, processing, antigen presentation, T cell activation, and killing of the organism (Bauman et al., 2000; Bauman et al., 2003; Wozniak et al., 2006; Wozniak and Levitz, 2008). DCs isolated from infected lungs presented cryptococcal mannoprotein (MP) to MP-specific T cells and induced T cell activation ex vivo (Wozniak et al., 2006). Depletion of DCs abrogated the T cell response (Mansour et al., 2006). Furthermore, DC phagocytosis of mannoprotein (MP) in the presence of the appropriate adjuvant induces production of Th1-type cytokines (Dan et al., 2008). Additional studies revealed that the interaction of *C. neoformans* with DCs, but not macrophages, induced the production of IL-12 and IL-23, two cytokines associated with protection against cryptococcosis (Kleinschek et al., 2010). Phagocytosis of encapsulated *C. neoformans* by DCs requires opsonization with either anti-capsular antibody or complement, and the combination of these has an additive effect (Kelly et al., 2005). Also, both murine and human DCs are able to kill *C. neoformans*, by both oxidative and non-oxidative mechanisms (Kelly et al., 2005). Recognition and uptake of acapsular *C. neoformans* strains by DCs requires MR and FcγR II (Syme et al., 2002). TLR2 and TLR4 are not important in uptake of *C. neoformans* or activation of DCs by the fungus (Nakamura et al., 2006). DCs stimulated with DNA from *C. neoformans* release IL-12p40 and express CD40, a costimulatory molecule associated with DC maturation, and thus was tied to recognition by TLR9 (Nakamura et al., 2008). Upon infection with *C. neoformans*, CCR2-deficient mice, which are impaired in trafficking of monocyte-derived DCs, developed a non-protective Th2-type immune response and persistent infection, and had reduced DC recruitment, bronchovascular collagen deposition, and increased IL-4 production (Osterholzer et al., 2008). *C. neoformans* can also be phagocytosed by macrophages (Levitz et al., 1999; Del Poeta, 2004). Macrophage phenotypes are associated with differential immune responses against *C. neoformans*. Protection against infection is associated with the presence of classically-activated macrophages (caMac) (Zhang et al., 2009; Hardison et al., 2010a; Hardison et al., 2010b), while disease progression is associated with the presence of alternatively activated macrophages (aaMac) (Arora et al., 2005; Muller et al., 2007; Arora et al., 2011; Chen et al., 2007; Guerrero et al., 2010). Also, macrophages can serve as a site of replication of *C. neoformans* (Tucker and Casadevall, 2002). Intracellular replication rates within macrophages correlated to virulence for *C. neoformans* strains (Voelz et al., 2009). In addition to replication, yeasts can be expulsed from macrophages by a non-lytic mechanism

that leaves both *C. neoformans* and macrophages intact and capable of replication and growth (Alvarez and Casadevall, 2006; Ma et al., 2006; Alvarez and Casadevall, 2007; Johnston and May, 2010). *C. neoformans* can also be phagocytosed by activated neutrophils (Kozel et al., 1987). The capsule of *C. neoformans* induces neutrophils to release proinflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNF-α (Retini et al., 1996). Neutrophils can kill *C. neoformans* by non-oxidative mechanisms, including neutrophil defensins and calprotectin (Mambula et al., 2000). Interestingly, induction of neutropenia in mouse models of infection reduces their susceptibility to infection (Mednick et al., 2003).

Although innate immune responses against *Rhizopus,* the main causative agent of mucormycosis, have not yet been fully characterized, recent work has shown that *Rhizopus* can trigger a common innate sensing pathway in DCs that leads to the production of IL-23 and drives Th17-type responses (Chamilos et al., 2010). This is due to interaction of dectin -1 with β-glucans on the surface of *Rhizopus* hyphae.

3.2 NK cell activity

Another innate immune response to pulmonary fungal pathogens is due to recognition and action by natural killer (NK) cells. NK cells were thought to act primarily against viruses and tumors, but more recent studies have shown that NK cells have a wide variety of functions against bacteria, fungi, and parasites (Newman and Riley, 2007).

In *H. capsulatum* infection, there is little evidence of a protective role for NK cells. While beige mice (lacking functional NK cells) are more susceptible to *H. capsulatum* infection, T cells play a greater role in controlling infection (Patino et al., 1987). In studies evaluating both beige mice and mice depleted of NK cells, beige mice were still more susceptible to infection, while mice depleted of NK cells were no more susceptible to infection than WT mice, therefore indicating no major role for NK cells in protection (Suchyta et al., 1988). However, mice deficient in perforin, a major component of NK cell anti-microbial activity, had accelerated mortality and increased fungal burden (Zhou et al., 2001). Infection with *Coccidioides* during depletion of NK cells leads to increased susceptibility to infection (Petkus and Baum, 1987). Furthermore, NK cells have a direct cytotoxic effect on *Coccidioides* young spherule and endospore cells (Petkus and Baum, 1987). In *Paracoccidioides,* studies have shown increased NK cell activity in infected hamsters compared to uninfected controls. Impaired NK cell activity was associated with a decrease in cell-mediated immunity (CMI) and an increase in histopathologic lesions. However, after initial activation, NK cells alone were not able to control dissemination of *Paracoccidiodes* (Peracoli et al., 1995). *In vitro* NK cell activity correlated with growth inhibition of *Paracoccidiodes* yeast (Jimenez and Murphy, 1984).

In neutropenic mice with *A. fumigatus* infection, NK cells are the major cell type responsible for the production of IFN-γ early in the infection. Additionally, depletion of NK cells reduces IFN-γ levels and caused increased pulmonary fungal load (Park et al., 2009). NK cells have direct anti-fungal activity against hyphae but not against resting conidia (Schmidt et al., 2011). Killing is due to production of mediators by NK cells, including perforin. However, *A. fumigatus* can also down-regulate some cytokines induced by the NK cells, including IFN-γ and GM-CSF (Schmidt et al., 2011). In addition, recruitment of NK cells to the lung during *A. fumigatus* infection by the chemokine MCP-1 is required for optimal clearance of the organism from the lungs (Morrison et al., 2003). During *Pneumocystis* infection in SCID mice (lacking T and B cells), NK cells were responsible for production of

cytokines such as IFN-γ, TNF-α, TNF-β, IL-10, and IL-12 (Warschkau et al., 1998). Recent studies have shown that NK cells are recruited to the lung during *Pneumocystis* infection and are important in fungal clearance of murine PCP (M. Kelly and J. Shellito, personal communication). Further, combined depletion of NK and $CD4^+$ T cells resulted in increased pulmonary fungal burden compared to individual depletion of each subset. In vitro, NK cells have direct microbicidal activity against *Pneumocystis*, and this anti-fungal activity is significantly enhanced in the presence of $CD4^+$ T cells, suggesting that both cell types are necessary for a protective response against *Pneumocystis* infection (M. Kelly and J. Shellito, personal communication). Early studies showed that NK cells can directly kill *C. neoformans* (Murphy and McDaniel, 1982). Further, IFN-γ production by NK cells enhances elimination of the fungus in murine models (Kawakami et al., 2001a; Kawakami et al., 2001b; Kawakami et al., 2001c). Depletion of NK cells using anti-asialo GM antibody resulted in increased fungal burden in mice (Hidore et al., 1991a; Hidore et al., 1991b). Increased fungal burden was seen in beige mice compared to wild-type mice, and in mice depleted of $NK1.1^+$ NK cells, fungal burden was also increased compared to controls (Lipscomb et al., 1987; Salkowski and Balish, 1991). Human NK cells kill *C. neoformans* (Levitz and Dupont, 1993), and this killing is enhanced in the presence of anti-cryptococcal antibodies (Miller et al., 1990). Binding of NK cells is required for cryptococcal killing, and requires disulfide bonds and is dependent on magnesium (Nabavi and Murphy, 1985; Hidore and Murphy, 1989; Murphy et al., 1991). Killing of *C. neoformans* is due to perforin interaction with the organism (Ma et al., 2004; Marr et al., 2009). In summary, NK cells act as accessory cells in antifungal host defenses contributing to clearance of fungi by a variety of mechanisms.

3.3 Gamma/delta T cell activity

The role of gamma delta (γδ) T cells during the immune response to pulmonary fungal pathogens is diverse. During infection with *Cryptococcus*, mice genetically deficient or depleted in γδ T cells have reduced fungal burden compared to controls. Further, mice lacking γδ T cells had increased levels of IFN-γ and decreased levels of TGF-β compared to controls, therefore suggesting that γδ T cells are detrimental to protective immunity during cryptococcal infection (Uezu et al., 2004). In *Pneumocystis* pneumonia, $CD4^+$ T cells are necessary for protection against infection, but γδ cells are known to infiltrate into the lung during pneumonia (Kagi et al., 1993; Agostini et al., 1995; Steele et al., 2002). However, resolution of pulmonary *Pneumocystis* infection is augmented in γδ T cell–deficient mice, (Steele et al., 2002), suggesting that these cells are detrimental to clearance of the organism. Further, the absence of γδ T cells led to an increase in recruitment of $CD8^+$ T cells and production of cytokines such as IFN-γ. Complete lack of all T cell subsets (αβ and γδ) during *Pneumocystis* infection led to lethal consequences (Hanano and Kaufmann, 1999). Thus, γδ T cells have either a limited role in antifungal protection or are detrimental to antifungal host defenses.

3.4 Innate anti-fungal defenses by non-immune cells

While innate immune cells and components of the innate immune system are the predominant innate immune defenses, it has also been shown that unconventional cells, such as epithelial cells can also play a role in anti-fungal innate host responses. Airway epithelial cells are capable of uptake and processing of antigens and initiation of Th-type

immune responses (Gereke et al., 2009). *P. brasiliensis* interacts with and can be internalized by bronchial epithelial cells (Mendes-Giannini et al., 1994, and this internalization is due to activation of a tyrosine kinase pathway (Monteiro da Silva et al., 2007; Hanna et al., 2000). In addition, uptake of *P. brasiliensis* causes both cytoskeletal rearrangements as well as apoptosis of the epithelial cells (Mendes-Giannini et al., 2004). *C. neoformans* can bind to pulmonary epithelial cells by a mechanism believed to be due to carbohydrate moieties that can be a ligand for the yeast (Merkel and Scofield, 1997). *C. neoformans* interaction with bronchial epithelial cells causes the production of IL-8, but epithelial cells are also susceptible to damage by the organism (Guillot et al., 2008a). In *A. fumigatus* infection, conidia can be taken up by tracheal epithelial cells, alveolar type II cells, and endothelial cells (Paris et al., 1997). Further, cytokines such as IL-6 and IL-8 are released by epithelial cells *in vitro* following stimulation with *A. fumigatus* proteases (Borger et al., 1999) or by *A. fumigatus* hyphal fragments (Zhang et al., 2005) and nasal epithelium. And nasal epithelium can engulf *A. fumigatus* conidia (Botterel et al., 2008). Epithelial cells can also release antimicrobial peptides following stimulation with fungal organisms. Epithelial cells *in vitro* cultured with *A. fumigatus* conidia, swollen conidia, or hyphae produced large amounts of beta defensins (Alekseeva et al., 2009). Airway epithelial cells internalize *A. fumigatus* conidia, and a genome-wide analysis revealed differential gene expression in epithelial cells with conidia compared to cells without conidia. Genes that were upregulated with conidia included genes involved in repair and inflammation, such as matrix metalloproteinases and chemokines (Gomez et al., 2010). In *Pneumocystis*, several studies have shown that the organism interacts with pulmonary epithelial cells. The interaction of *Pneumocystis* with epithelial cells was shown to be one of the initial steps in infection (Lanken et al., 1980; Yoneda and Walzer, 1980; Long et al., 1986; Millard et al., 1990). The β-glucan from the *Pneumocystis* cell wall can stimulate pulmonary epithelial cells to produce IL-8, and the organism can induce the production of MCP-1 and ICAM -1 (Yu and Limper, 1997; Evans et al., 2005; Wang et al., 2007; Carmona et al., 2010). Interaction of *Pneumocystis* and alveolar epithelial cells also leads to the production of the chemokine MIP-2 following NF-κB signaling (Evans et al., 2005; Wang et al., 2005). These studies show that non-immune cells, such as epithelial cells, play a role in pulmonary anti-fungal immunity.

4. T cell and antibody mediated immune responses to fungal infections

4.1 Adaptive responses against pulmonary fungal pathogens

When challenged with pathogenic fungi, the adaptive immune system is capable of mounting an effective response against most fungal species to eliminate fungal infections and maintain immunological memory that prevents their reoccurrence. However, fungi are ubiquitous in the host's environment including the saprophytes and opportunists that survive on the host's body surfaces and thus, the adaptive immune system is constantly challenged by fungal antigens. Excessive response to these antigens could lead to allergic responses or other types of immunopathological reactions. The balance between day-to-day fungal antigen exposure and the immune system is thought to lead to a homeostatic state defined as protective tolerance. Protective tolerance allows the host to keep possible fungal pathogens "in check" while preserving integrity of the natural barriers, which are potential portals for fungal infections (Romani and Puccetti, 2008; de Luca et al., 2010a; Littman and Rudensky, 2010).

T cells are responsible for orchestration of adaptive immune responses and T cell derived signals produced in response to specific antigens lead to targeted expansion, recruitment, activation of leukocytes, and regulation of B cell antibody responses. T cells also serve as a pool of immunological memory. Additionally, T cells have been shown to directly act as the fungicidal effector cells and serve as regulators of inflammatory responses, contributing to the development and maintenance of the protective tolerance. These regulatory mechanisms are designed to limit the damage that the host immune system can inflict on host tissues during incorrect and/or excessive host responses. Thus, properly functioning T cells are responsible for building up protective immunity against fungal pathogens and play an essential role in maintaining normal homeostasis of the immune system in the context of normal presence of fungal antigens.

4.2 $CD4^+$ and $CD8^+$ T cell mediated immunity

The importance of T cells in antifungal protection is well documented. T-cell deficient individuals show diminished resistance to fungal infections including coccidiomycosis (Kappe et al., 1998) cryptococcosis (Kovacs et al., 1985; Chuck and Sande, 1989; Spitzer et al., 1993; Jarvis and Harrison, 2007), histoplamosis (Odio et al., 1999), pneumocystis pneumonia (Kelly and Shellito), *Paracoccidioides* (Bava et al., 1991; Brummer et al., 1993), as well as pulmonary aspergillosis (Mylonakis et al., 1998). Likewise, laboratory studies have shown a strong contribution and/or requirement of T cells for protection against most pathogenic fungi such as *Coccidioides* (Fierer et al., 2006), *Cryptococcus* (Lim and Murphy, 1980; Mody et al., 1990; Huffnagle et al., 1991; Huffnagle and Lipscomb, 1992; Mody et al., 1994), *Pneumocystis* (Harmsen and Stankiewicz, 1990), *Histoplasma* (Deepe et al., 1984), *Paracoccidioides* (Cano et al., 2000) and *Aspergillus* (Cenci et al., 1997). These epidemiological and experimental studies have established that T cells are an important component of the antifungal host resistance.

Both subsets of T lymphocytes, $CD4^+$ and $CD8^+$ cells, are involved in antifungal host defenses. $CD4^+$ T cells classically represent the T helper cell population. The T helper function was defined by MHC II restricted antigen specific activation of B-cell clones needed for the generation of specific antibodies. The $CD4^+$ cell function in cell-mediated immunity (CMI) likewise requires antigen presenting cells and MHC II restricted antigen presentation. Presentation of antigen to the reactive T cells by dendritic cells and/or macrophages results in cytokine production. Through generation of different cytokine spectra, $CD4^+$ T cells orchestrate recruitment and activation of various leukocyte subsets. The cytokines produced by the effector T cells are essential for macrophage fungicidal function and granuloma formation, but also may support chronic inflammation and immunopathology (Arora et al., 2005; Chen et al., 2008; Jain et al., 2009; Zhang et al., 2009). Thus, cytokine induction by differentially polarized T-cell lineages is the major determinant for fungicidal potential of distal effector cells. Although the effector $CD4^+$ cell function relies predominantly on cytokine production, $CD4^+$ T-cells are capable of fungal killing via direct cell contact. At least in some biological circumstances, the direct fungicidal effect of $CD4^+$ T cells relies on granulysin as the fungicidal mediator (Zheng et al., 2007; Zheng et al., 2008).

In contrast with $CD4^+$ T cells, $CD8^+$ T cells are classically viewed as cytotoxic lymphocytes. These cells respond to antigen presentation in the context of MHC I, to enable their cytotoxic machinery. Such cytotoxic responses are particularly crucial in responses to viral infection and tumor cells, leading to elimination of the virally infected or tumor-transformed cells by

cytotoxic lymphocytes. $CD8^+$ cells also play an important role in host defenses to bacterial, parasitic and fungal infections (Oykhman and Mody, 2010). Numerous studies showed that $CD8^+$ T cells significantly contribute to protection against *Cryptococcus, Pneumocystis, Histoplasma* and *Blastomyces*, even in the absence of $CD4^+$ T cells. Depending on the type and virulence of the fungal pathogen, $CD8^+$ cells could afford either partial or a complete protection against the major fungal pathogens in experimental models. In this context, $CD8^+$ T cells could induce all the protective effector functions of $CD4^+$ T cells including production of protective cytokines. Another important aspect of $CD8^+$ T cell effector function is the direct fungicidal effect of $CD8^+$ T cells. Such direct fungicidal activity of $CD8^+$ T cells have been demonstrated for *C. neoformans* (Ma et al., 2002). The killing of *C. neoformans* requires direct cell contact; it is enhanced by IL-15 and is thought to be mediated by granulysin. The direct cytotoxic effects are most pronounced when lymphocytes from fungus-immunized mice are used, however, a relatively high rate of binding of T cells to the fungus suggests that these cytotoxic mechanisms are innate rather than adaptive.

4.3 Immune polarization in antifungal host defenses

T helper cell subsets characterized by differential cytokine production by differentially programmed T-cell lineages were initially defined as Th1 and Th2 (Mosmann et al., 1986; Cherwinski et al., 1987). The types of immune responses driven by each of these cell lineages are described as Th1 and Th2 immune responses, generate different types of immune effector responses, and show different spectra of effectiveness against different classes of pathogens. For effective control/clearance of the majority of fungal pathogens, Th1 is the required type of the immune response. The Th1 response is promoted by IL-12, IFN-γ, and TNF-α. The two latter cytokines are also the major products of Th1 helper T cells (Cherwinski et al., 1987). Th1-type T-cells are responsible for the delayed-type hypersensitivity (DTH) reactions and CMI associated with vigorous proinflammatory responses and granuloma formation (Cher and Mosmann, 1987) and induction of IgG2a class antibodies in B cells (Stevens et al., 1988). The Th2 immune response is characterized by T-cell production of IL-4, IL-5, IL-9, IL-10, and IL-13 (Cherwinski et al., 1987), IgG1 and IgE antibody production by B cells (Stevens et al., 1988) and the presence of eosinophilic inflammation (Huffnagle et al., 1994; Cenci et al., 1999; Olszewski et al., 2001). The Th1 and Th2 responses counter-regulate each other predominantly via an IL-4/IFN-γ negative feedback loop (Fernandez-Botran et al., 1988; Gajewski and Fitch, 1988); however other cytokines can be also involved in Th1/Th2 regulation. The oversimplified Th1/Th2 paradigm has further evolved as new T cell lineages were defined. Th17 and regulatory type T cells (Treg), are T-cell lineages that are distinct from Th1 and Th2 cells that possess distinct functions in host defenses. Th17 cells are generated following the priming with IL-6 and TGF-β and sustained by the presence of IL-23. Th17 cells classically produce IL-17 and IL-22, however, a subset of Th17 cells can produce IFN-γ. Regulatory T-cells are thought to be responsible for tolerance that prevents auto-immune diseases and to contribute to resolution of inflammatory responses. These effects of Tregs are thought to be mediated by anti-inflammatory cytokines IL-10 and TGF-β, which are signature cytokines for Treg cells. New Th-cell lineages continue to be described including Th22 (Eyerich et al., 2009; Fujita et al., 2009) and Th9 (Soroosh and Doherty, 2009). Just like $CD4^+$ effector T-cells, $CD8^+$ T cells can also display a polarization pattern and thus can be an important source of the polarizing cytokines. Thus, both $CD4^+$ and $CD8^+$ T cells contribute to the cytokine balance during the immune response (Huffnagle et al., 1994).

4.4 Th1/2/17/22 cytokine responses

The protective role of Th1 along with the requirement of type 1 cytokines for fungal clearance have been demonstrated in models of cryptococcosis (Huffnagle et al., 1994; Kawakami et al., 1997; Blackstock et al., 1999; Abe et al., 2000; Traynor et al., 2000; Olszewski et al., 2001; Herring et al., 2002; Arora et al., 2005; Hernandez et al., 2005; Lindell et al., 2006; Wormley et al., 2007; Chen et al., 2008; Guillot et al., 2008b; Jain et al., 2009; Wozniak et al., 2009; Zhang et al., 2009), *Coccidioid*es (Silva and Benitez, 2005), *Paracocidioides* (Cano et al., 1998), *Histoplama* (Zhou et al., 1995; Deepe and Gibbons, 2006) and *Blastomyces* (Brummer et al., 2006) infections. Th1 skewing is beneficial for clearance of *Aspergillus* (Cenci et al., 1997), although clearance of the filamentous fungi is mainly a domain of the innate immune system. The Th1 cytokine environment promotes clearance of fungi by supporting the classical activation of macrophages (Mantovani et al., 2001). Pathogenic fungi possess mechanisms that interfere with their recognition by macrophages. These fungi can survive within macrophage unless additional "external" stimulation occurs to activate fungicidal mechanisms. Such stimulation can be provided by Th1-type cytokines, especially IFN-γ (Arora et al., 2005; Hardison et al.). In the context of a Th1 immune response, macrophages become classically activated and abundantly generate fungicidal molecules such as nitric oxide produced by nitric oxide synthase, an enzyme that utilizes L-arginine. Importance of classical macrophage activation and production of nitric oxide for fungal clearance has been demonstrated for *Blastomyces* (Brummer et al., 2005; Kethineni et al., 2006), *Cryptococcus* (Granger et al., 1990; Alspaugh and Granger, 1991; Rivera et al., 2002; Arora et al., 2005; Zhang et al.; Hardison et al.), *Histoplasma* (Zhou et al., 1995; Allendoerfer and Deepe, 1998; Allen and Deepe, 2006) and *Paracocidiodes* (Moreira et al.; Pinzan et al.) infections. The deficiencies in cytokines that support classical activation of macrophages GM-CSF, IFN-γ, TNF-α, IL-12 are generally associated with the development of progressive fungal infection (Romani et al., 1994; Kawakami et al., 1999; Rayhane et al., 1999; Herring et al., 2005; Deepe and Gibbons, 2006) consistent with the general conclusion that Th1-type immune responses and type 1 cytokines are most optimal for resistance against fungal infections.

Unlike Th1-type responses, the Th2 response is non-protective and frequently results in pathological responses to fungal challenges. For most fungal species, Th2 responses and type 2 cytokines decrease clearance of fungus. This is attributed to: 1) a suppression of protective Th1 responses due to a mutual counterregulatory feedback loop (Cenci et al., 1999) and 2) a generation of alternatively activated macrophages that can harbor fungal organisms (Arora et al., 2005; Jain et al., 2009; Osterholzer et al., 2009a; Zhang et al., 2009). Th2 cytokines such as IL-4, IL-13 are the major trigger of alternative activation of macrophages (Arora et al., 2005; Jain et al., 2009; Zhang et al., 2009). These alternatively activated macrophages do not express nitric oxide synthase but induce arginase which metabolizes L-arginine without yielding fungicidal nitric oxide. In the Th2 biased experimental models of fungal infections, intracellular survival of fungus within macrophages parallels high induction of alternatively activated macrophage markers (Arora et al., 2005; Zhang et al., 2009; Hardison et al.).

Increased production of Th2-type cytokines has been associated with increased susceptibility to *Cryptococcus* (Arora et al., 2005; Zhang et al., 2009; Hardison et al.) and *Paracoccidioides* (Ruas et al., 2009) infections and to invasive pulmonary aspergillosis (Cenci et al., 1997; Cenci et al., 1999). Fungus-triggered Th2-type responses in the respiratory

system may also lead to allergic diseases such rhinitis/sinusitis, asthma, allergic bronchopulmonary mycosis. Th2 type responses are highly detrimental to the respiratory system by promoting mucus hypersecretion/goblet cell metaplasia, eosinophilic inflammation, and peribronchial fibrosis all of which contribute to impaired airway function. Cytokines IL-4, IL-5 and IL-13 are the major triggers of these pathologies as increased expression of these cytokines can be reproduced along with the allergic symptoms in the lungs challenged with fungi or their antigenic components (Blease et al., 2000; Arora et al., 2005; Jain et al., 2009; Zhang et al., 2009). Some of the fungal antigens can directly promote Th2 skewing. The secreted protein fraction from *Aspergillus fumigatus* promotes Th2 bias of the immune response (Bozza et al., 2009). Th2 pathologies are also found in mouse models of *C. neoformans* infections (Abe et al., 2000; Jain et al., 2009; Osterholzer et al., 2009b) (Figure 1). Expression of enzymes phospholipase B and urease by *C. neoformans* promote Th2 bias in the infected mice (Noverr et al., 2003; Osterholzer et al., 2009b). While Th2-biased responses are clearly undesirable in most types of fungal infections the exception is *Pneumocystis* infection, in which the Th2 response can contribute to fungal clearance (Shellito et al., 2000; McKinley et al., 2006; Hu et al., 2009).

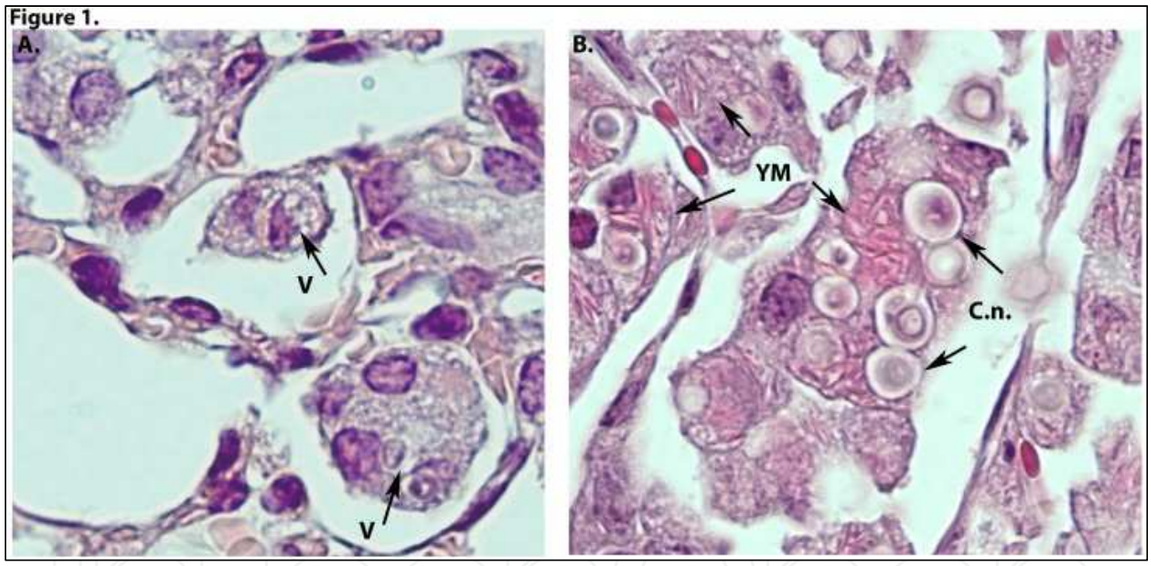

Fig. 1. Classical versus alternative activation of macrophages during pulmonary infection with *C. neoformans*. A) Classically activated macrophages upregulate fungicidal mechanisms that eliminate ingested fungi. Note that ingested intracellular organisms show signs of degradation. B) Alternatively activated macrophages (AAM) harbor the ingested fungi. Note the abundant capsule formation (evidence of fungal metabolic activity) and dividing organisms (evidence of intracellular growth) within AAM. Alternative activation of macrophages is associated with crystallization of chitinase family proteins YM1 and YM2, a hallmark of AAM-induced pathology. **V**-vacuoles with the remnants of destroyed organisms, **YM**- YM1/YM2 crystals, **C.n.** – intact cryptococcal organisms.

The effects of Th17 responses and the IL-17 cytokine family in anti-fungal host responses may be protective or non-protective depending on fungal species and sites of infection (Figure 2). Thus, Th17 responses may be beneficial for some types of fungal infections or

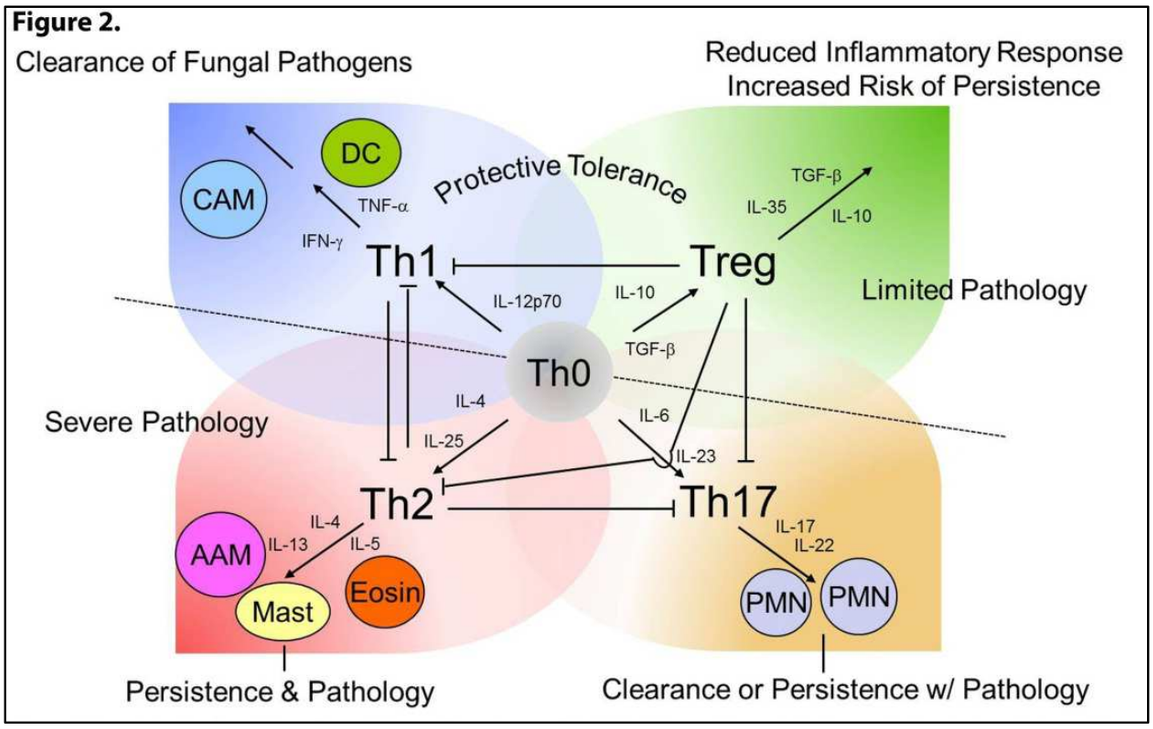

Fig. 2. Th polarization in antifungal host defenses. The outcome of Th1, Th2, Th17, and Treg polarization results from balance between Th lineages which can mutually regulate each other via cytokine feedback loops. Resultant outcome can either promote clearance of the fungal infection or result in persistent infection and limited or severe pathology. Th1 response promotes control of most fungal infections; Th2 leads to severe pathology and fungal persistence; Th17 may support clearance or persistence of different fungal infections, but may promote chronic neutrophilic inflammation. Treg may limit pathology by promoting resolution of the inflammatory response, but may increase the risk of persistence. Correct balance between Th1 and Treg is thought to support protective tolerance.

exhibit detrimental effects. In the *H. capsulatum* infection model, IL-17 neutralization increases pulmonary fungal burden in connection with increased Treg numbers, suggesting that Th17 is beneficial for clearance of *Histoplasma* (Kroetz and Deepe, 2010). Th17 responses also contribute to anticryptococcal protection and the development of the protective inflammatory response in *C. neoformans* infected lungs (Zhang et al., 2009; Hardison et al., 2010b; Wozniak et al., 2011a).

The IL-23/IL-17 axis contributes to clearance of *Pneumocystis* (Rudner et al., 2007). However, in IFN-γ deficient mice infected with *Pneumocystis* the development of strong Th17 response is detrimental, suggesting that a balance between IFN-γ and IL-17 is needed for optimal clearance of *Pneumocystis* (Hu et al., 2009). At other mucosal sites, the effects of Th17 are variable. Th17 cells and IL-17 receptor signaling are required for mucosal host defenses in oral candidiasis (Conti et al., 2009); whereas Th17 impairs antifungal resistance and promotes inflammation in gastric infection model (Zelante et al., 2007). Th17 responses impair antifungal resistance and promotes inflammatory damage in the lungs of mice infected with *Aspergillus* (Zelante et al., 2007; Bozza et al., 2009; D'Angelo et al., 2009).

Overall, the Th17 response may have beneficial effects for clearance of some fungal pathogens; however it also has high potential to produce undesirable effects, including inflammatory damage.

Excessive immune reaction and uncontrolled inflammation can result in serious damage of the inflicted organs and tissues. Anti-inflammatory or regulatory cytokines such as IL-10 and TGF-β are an important part of the balance which prevents over exuberant inflammation during acute responses. These cytokines are also thought to be important components of resolution and tissue repair that occurs after elimination of the pathogen. Regulatory T cells are important sources of these cytokines and their role in inflammatory diseases and in the maintenance of healthy tissue homeostasis becomes increasingly appreciated (Romani and Puccetti, 2008; De Luca et al., 2010b; Littman and Rudensky, 2010). The importance of balance between pro-inflammatory processes and Treg cell regulation has recently been demonstrated in models of *Pneumocystis* (McKinley et al., 2006) and *Histoplasma* infections (Kroetz and Deepe). The excessive/damaging inflammatory reaction can be exemplified by immune reconstitution inflammatory syndrome (IRIS). IRIS is characterized by uncontrolled inflammatory responses with high induction of IFN-γ, TNF-α and other pro-inflammatory cytokines (Mori and Levin, 2009). Overproduction of these cytokines, rather than having protective effects, contributes to tissue injury that leads to worsening of the patient condition and high mortality (Mori and Levin, 2009). Interestingly, occurrence of IRIS in HIV patients who undergo antiretroviral therapy is particularly high in patients with C*ryptococcus* and *Pneumocystis* infections (Singh et al., 2005; Singh and Perfect, 2007; Murdoch et al., 2008). The mechanism of inadequate inflammatory response in IRIS is not understood, however it has been proposed that the regulatory mechanisms that control the inflammation, including Tregs are not sufficiently mobilized to put a break on this inflammatory response (McKinley et al., 2006; Shankar et al., 2008). In fact, patients with IRIS showed reduced suppressor function and diminished secretion of anti-inflammatory IL-10 by Tregs in one of the studies (Seddiki et al., 2009). Tregs are critical for maintaining the proper homeostasis in the GI track, and such mechanisms of protective tolerance are likely to be critical in the respiratory tract which is constantly exposed to inhaled fungal antigens. Insufficiency of the regulatory mechanisms most likely contributes to the development of allergic diseases. Thus, Tregs cells are important for maintaining balance between appropriate clearance rate and the inflammatory tissue damage. Such balance can be disturbed and the excessive Treg function may promote fungal persistence. A detrimental role of IL-10 has been demonstrated in cryptococcal infection models (Blackstock et al., 1999; Arora et al., 2005). Future studies will be needed to evaluate the possible role of Tregs in fungal infections, especially in the patients who develop mycoses without apparent immunodeficiency.

The polarization of T cells to Th1, Th2, Th17 and Treg lineages is important for the development of protective immunity, protective tolerance, chronic/allergic syndromes, or overwhelming allergic reactions. The proper balance maintained by the mutual regulation between these arms of the immune system is necessary to optimize clearance and minimize inflammatory damage to the infected tissues in the context of fungal infection. Our present understanding of these responses evolved from an oversimplified polarized Th1/Th2 paradigm to a broader understanding of mutual regulation ongoing during the immune process. Recent studies show that the Th1, Th2, Th17 responses co-exist in a fungus infected

lungs and the balance of cytokine production alters during different time points in a chronic fungal infection (Arora et al., 2011). Modulation of these responses can be achieved experimentally and therapeutically by use of cytokines and vaccination with different fractions of fungal antigens resulting in the induction of the proper and protective Th-cell polarization.

4.5 Vaccine-induced therapies targeting cellular mediated immunity

Currently, there are no standardized vaccines available for the prevention of fungal diseases in humans (as discussed earlier). A preponderance of evidence points to the development of cell-mediated immune responses, principally by Th1–type $CD4^+$ T cells, as the predominant host defense mechanism against primary and opportunistic pulmonary fungal pathogens (Cutler et al., 2007). Further, ablation or neutralization of several Th1-type cytokines renders mice more susceptible to experimental infection with a number of fungal pathogens. Consequently, there has been great interest in identifying antigens that elicit protective CMI against fungal infections; some of which will be discussed herein.

Vaccination with native or recombinant Hsp60 from *H. capsulatum* or a domain within Hsp60 conferred protection in mice given a sub-lethal challenge with yeast cells and prolonged survival in mice given a lethal challenge (Gomez et al., 1995; Deepe and Gibbons, 2002). Protection was $CD4^+$ T cell dependent and associated with the induction of IFN-γ, IL-12 and, surprisingly, the Th2-type cytokine IL-10 (Deepe and Gibbons, 2002; Scheckelhoff and Deepe, 2005). A similar vaccination strategy in mice using Hsp70 did not induce robust IL-12 or IFN-γ responses and protection against subsequent challenge with live yeast. Neutralization of IL-12 or IFN-γ abolished the protective efficacy of the Hsp60 vaccine in mice further highlighting the importance of these Th1-type cytokines in the induction of protection against *H. capsulatum*.

Similarly, vaccination of mice with recombinant Hsp60 derived from *P. brasiliensis* elicited protection against a lethal intranasal challenge with yeast (de Bastos Ascenco Soares et al., 2008). The protective effect of *P. basiliensis* Hsp60 was abrogated following the depletion of $CD4^+$ T cells or neutralization of IFN-γ; similar to that observed for Hsp60 from *H. capsulatum* (Scheckelhoff and Deepe, 2005; de Bastos Ascenco Soares et al., 2008). However, IL-10 was not produced following antigen stimulation of splenocytes obtained from *P. basiliensis* Hsp60 immunized mice. While the efficacy of vaccination with forms of Hsp60 from *H. capsulatum* and *P. brasiliensis* are encouraging, immunization with recombinant Hsp60 derived from *C. immitis* resulted in predominantly Th2 cytokine responses and little protection against a subsequent intraperitoneal challenge (Li et al., 2001). Thus, the induction of Th1-type immune responses in the lungs appears critical for the development of protection following immunization with Hsp60.

Evaluation of live attenuated, recombinant, and DNA vaccines of *C. immitis* in murine models have also highlighted the importance of Th1-type cytokine production, particularly IFN-γ, in protection against this microbe (reviewed in (Cole et al., 2004; Cox and Magee, 2004; Xue et al., 2009)). Mice immunized with recombinant aspartyl protease (Pep1), alpha-mannosidase (Amn1), or phospholipase B (Plb) individually or together as amultivalent vaccine experienced a significant reduction in fungal burden and prolonged survival against a lethal pulmonary challenge with C. *posadasii* arthroconidia compared to controls (Tarcha et

al., 2006). Approximately 85% of mice immunized with the multivalent recombinant vaccine survived to day 90 post-inoculation. Similarly, immunization of mice with two recombinant antigens, *Coccidiodes*-specific antigen (CSA) and the proline-rich cell wall protein Ag2/PRA, either as a mixture of two separately expressed proteins or as a single chimeric expression product was shown to protect mice from a lethal intranasal infection with C. *posadasii* (Shubitz et al., 2006). The protection observed with each vaccination strategy was associated with robust IFN-γ responses in protected mice, again showing the importance of Th1-type cytokines during protective host responses. Further, these studies highlighted the utility of a multivalent vaccination strategy that potentially evokes protective responses towards a broader set of T-cell epitopes.

The importance of $CD4^+$ T cells and the generation of Th1-type responses towards eliciting protection against pulmonary fungal pathogens are also observed in vaccination models using a live *C. neoformans* strain engineered to express IFN-γ (Wormley et al., 2007) (Wozniak et al., 2009) (Young et al., 2009), a live attenuated strain of *B. dermatitidis* (Wuthrich et al., 2000), and recombinant *A. fumigatus* protein Asp f3 (Diaz-Arevalo et al., 2011). Immunization with recombinant Asp f3 of *A. fumigatus* protected cortisone acetate immune suppressed mice from an experimental pulmonary infection with *A. fumigatus* conidia (Diaz-Arevalo et al., 2011). The protection was dependent on $CD4^+$ T cells as their depletion reduced the survival of vaccinated mice and adoptive transfer of Aspf3 primed $CD4^+$ T cells into non-vaccinated mice enhanced their survival against experimentally induced pulmonary aspergillosis. Generation of sterilizing immunity in mice following pulmonary immunization with a *C. neoformans* strain engineered to express murine IFN-γ, designated H99γ, was shown to require the induction of Th1-type cell-mediated immune responses (Wozniak et al., 2009). Interestingly, B-cell deficient mice immunized with H99γ were protected from a subsequent lethal pulmonary challenge with WT *C. neoformans* (Wozniak et al., 2009; Young et al., 2009; Wozniak et al., 2011b). Also, vaccination of mice with an attenuated strain of *B. dermatitidis* containing a targeted deletion in the *BAD1* locus resulted in prolonged survival that was chiefly mediated by IFN-γ and TNF-α production by $CD4^+$ T cells (Wuthrich et al., 2000; Wuthrich et al., 2002). Although these studies show that Th1-type $CD4^+$ T cell responses are required for optimal host responses against these pulmonary pathogens, studies in *H. capsulatum*, *B. dermititidis*, and *C. neoformans* highlight the inherent plasticity of the host response against pulmonary fungal pathogens. That is that some elements of the immune response can compensate for the loss of other components.

Vaccine-induced immunity against *B. dermatitidis* was shown be mediated by $CD4^+$ α/β T cell production of TNF-α and IFN-γ (Wuthrich et al., 2002). Moreover, the initiation, but not maintenance, of protective memory responses to *B dermatitidis* required IL-12 production (Wuthrich et al., 2005). However, vaccine-induced immunity could be elicited and expressed in IFN-γ and TNF-α deficient mice. The reciprocal cytokine or the presence of GM-CSF was shown to compensate for the loss of IFN-γ and TNF-α showing some plasticity in the vaccine-induced host response to *Blastomyces* (Wuthrich et al., 2002). Furthermore, a role for Th17 cells in vaccine-induced protection against multiple pulmonary fungal pathogens has been shown (Wuthrich et al., 2011). Specifically, protection afforded by vaccination against *C. posadassi*, *H. capsulatum*, and *B. dermatitidis* was observed to be dependent on IL-17. In fact, IL-17 was shown to be indispensable since vaccinated IL-17A or IL-17RA deficient mice showed impaired anti-fungal resistance despite having normal Th1-type cytokine expression. In contrast, IL-17A was shown to contribute to but ultimately be dispensable for

protection against experimental pulmonary cryptococcosis in *C. neoformans* strain H99γ vaccinated mice (Hardison et al., 2010b; Wozniak et al., 2011a). Still, it appears imperative that vaccines strategies to prevent pulmonary mycoses be evaluated for their capacity to induce Th1 and Th17-type cytokine responses.

The induction of T cell-mediated immune responses is critical for optimal protection against pulmonary fungal pathogens (Cutler et al., 2007). Consequently, it may seem counterintuitive to suggest that vaccines designed to prevent fungal infections in patients with T cell deficiencies is possible. Nonetheless, vaccination studies using experimental models of *H. capsulatum* (Deepe and Gibbons, 2002), *P. brasiliensis* (de Bastos Ascenco Soares et al., 2008), *H. capsulatum* (Wuthrich et al., 2003), *B. dermatitidis* (Wuthrich et al., 2002; Wuthrich et al., 2003), *C. immitis* (Fierer et al., 2006), *and C. neoformans* (Wozniak et al., 2011b) have indicated that vaccine-induced protective immune responses can be elicited in immune deficient hosts. Cumulatively, the studies show that the presence of $CD4^+$ or $CD8^+$ T cells is essential for the induction (the period following vaccination) and expression (immune response following challenge) phases of the protective immune response. Protection is not induced in mice that are T cell deficient or depleted of both $CD4^+$ and $CD8^+$ T cell populations. Further, protection is lost in vaccinated mice following deletion of both T cell subsets. Interestingly, 80% of mice vaccinated with *C. neoformans* strain H99γ and subsequently depleted of both $CD4^+$ and $CD8^+$ T cells were protected from a lethal pulmonary challenge with WT *C. neoformans* (Wozniak et al., 2011b). These studies highlight dynamic compensatory mechanisms that mediate vaccine-induced protection during both the induction and expression phases of the anti-fungal immune response. Altogether, the results demonstrating the plasticity of the vaccine-induced immune response to pulmonary fungal pathogens are particularly exciting as they highlight the potential for inducing protection in immune competent and immune compromised hosts.

4.6 Antibody-mediated immunity and therapeutics

The contribution of antibody mediated immunity (AMI) towards protecting individuals against pulmonary fungal infections remains uncertain. Individuals with humoral deficiencies such as autosomal hyper-IgM syndrome and IgA deficiency do not exhibit an increased susceptibility to fungal infections (reviewed in Antachopoulos, 2007, 2010). In contrast, patients with X-linked hyper IgM syndrome and common-variable immunodeficiency which are often accompanied by defects in T CMI have a higher risk of developing pulmonary and invasive fungal infections like cryptococcosis and histoplasmosis. The efficacious role for antibodies during the host immune responses against fungi is like that observed against bacterial and viral pathogens. Antibodies produced in response to fungal infection serve as opsonins to promote phagocytosis, participate in antibody-dependent cellular cytotoxicity, augment Th1-type polarization, help to eliminate immunosuppressive polysaccharide antigen from serum and tissues, inhibit biofilm formation, have direct antifungal activity, and modulate the immune response to prevent host damage (reviewed in Alvarez and Casadevall, 2007; reviewed in Zaragoza and Casadevall, 2004).

Most studies showing the efficacy of AMI against pulmonary fungal pathogens has involved experimental models of PcP and cryptococcosis. The polysaccharide capsule of *Cryptococcus*, its main virulence determinant, is predominantly comprised of the polysaccharides glucuronoxylomannan (GXM) and galactoxylomannan (GalXM) and to a

much lesser extent, <1%, mannoproteins (MP) (reviewed in Zaragoza et al., 2009). Conjugate vaccines consisting of GXM combined to either tetanus toxoid (TT) or *Pseudomonas aeruginosa* exoprotein A (rEPA) induce high antibody titers (Devi et al., 1991; Casadevall et al., 1992), enhanced antifungal activity of murine and human phagocytes (Mukherjee et al., 1995c; Zhong and Pirofski, 1996, 1998) and conferred some protection against cryptococcosis in mice (Devi, 1996; Fleuridor et al., 1998; Nussbaum et al., 1999). Unfortunately, the profound suppressive effects on immune responses induced by cryptococcal polysaccharides and the highly variable immune responses observed in response to the intact GXM portion of the conjugate vaccine renders it an unlikely choice for future vaccine development (reviewed in Zaragoza et al., 2009; reviewed in Pirofski, 2001). A strategy using small peptide mimotopes (peptides which are able to induce antibodies that are capable of binding to the native antigen when administered as an immunogen) that mimic defined GXM epitopes was attempted to elicit protective antibody responses where using total GXM was unsuccessful. Zhang et al. described a peptide mimetic (P13) of GXM that was recognized by human anti-GXM antibodies (Zhang et al., 1997) and showed that vaccination with P13-protein conjugates in mice resulted in prolonged survival after a lethal *C. neoformans* challenge compared to controls (Fleuridor et al., 2001) or following establishment of a chronic infection (Datta et al., 2008).

Casadevall et al. developed a murine monoclonal antibody (MAb), 18B7, to *C. neoformans* polysaccharide that underwent phase I clinical studies in HIV^+ patients with cryptococcal antigenemia (Casadevall et al., 1998). A modest reduction in serum cryptococcal antigen titers was observed in patients receiving singular doses of 1 and 2 mg/kg up to 10 weeks post-treatment before returning to baseline (Casadevall et al., 1998). To date, no follow-up clinical studies have been published. A new approach using MAb 18B7 currently being investigated in mice involves conjugation of the MAb to the therapeutic radioisotopes ^{188}Rhenium or ^{213}Bismuth (Dadachova et al., 2003; Bryan et al., 2010). Studies have shown that administration of radiolabeled MAb 18B7 to lethally infected mice results in prolonged survival, reduced organ fungal burden, and was a more effective therapy compared to mice treated with amphotericin B. Radioimmunotherapy can be applied using MAb derived against multiple pulmonary fungal pathogens and thus may evolve into an attractive option for the treatment of other pulmonary mycoses. Lastly, while most studies have examined passive administration with antibodies targeting *C. neoformans* polysaccharide, other cryptococcal targets for passive antibody therapy under experimental investigation include melanin (Rosas et al., 2001), β-glucan (Rachini et al., 2007), heat shock protein (HSP) 90 (Nooney et al., 2005) and glucosylceramide (Rodrigues et al., 2007).

Mice deficient in B cells, either due to a targeted disruption of the IgM constant region (μMT mice) or using depletion antibodies, are more susceptible to PcP infection (Harmsen and Stankiewicz, 1991; Marcotte et al., 1996) (Lund et al., 2003; Lund et al., 2006). These studies showed that B cells were able to provide protection against PcP not only by producing Ab but also by amplifying the $CD4^+$ T cell-mediated immune response. Passive administration of an IgM mAb shown to be directed against a surface antigen present on rat-, rabbit-, ferret-, and human-derived *P. carinii* induced partial protection against PcP in animal models (Gigliotti and Hughes, 1988). Subsequent studies showed that the passive administration of mAbs recognizing kexin-like molecule (KEX1) via the intranasal route prior to experimentally induced PcP resulted in a significant reduction in pulmonary fungal burden (~99%) (Gigliotti et al., 2002).

Contrasting studies have shown that B cell deficient μMT mice have lower pulmonary fungal burden following intranasal infection with *A. fumigatus* (Montagnoli et al., 2003) or *B. dermatitidis* (Wuthrich et al., 2000) and are not more susceptible to experimental pulmonary histoplasmosis infection (Allendorfer et al., 1999) compared to WT controls. Also, passive transfer of polyclonal serum or MAbs obtained from *A. fumigatus*, *B. dermatitidis*, or *C. immttis* vaccinated mice did not enhance protection against a subsequent intranasal challenge with these pathogens (Kong et al., 1965; Beaman et al., 1977; Frosco et al., 1994; Wuthrich et al., 2000; Beaman et al., 1979). The role of antibodies in the host defense against fungal infection remains controversial because of its complexity. The current consensus is that antifungal antibodies can mediate protective, nonprotective, or disease-enhancing effects on host defenses during infection (Mukherjee et al., 1995a; Mukherjee et al., 1995b; Yuan et al., 1998a). Thus, resistance to disease may depend upon the proportion of protective antifungal antibodies produced during infection. In support of this concept, non-protective and protective MAbs to *C. neoformans* has been described (Mukherjee et al., 1995a; Maitta et al., 2004). Also, Nosanchuk et al. demonstrated that mice passively administered MAbs targeting a histone H2B-like protein on the surface of *H. capsulatum* before infection experienced a reduction in fungal burden and prolonged survival (Nosanchuk et al., 2003). These studies were somewhat surprising in light of previous studies showing no increased susceptibility to experimental histoplasmosis infection in B cell deficient mice (Allendorfer et al., 1999). Studies in *C. neoformans* has shown that the efficacy of MAbs appears to be dependent on several variables including host genetics (Rivera and Casadevall, 2005), Ab isotype (Yuan et al., 1995; Yuan et al., 1998b), T cell function (Yuan et al., 1997), and the presence of Th1- and Th2-related cytokines (Beenhouwer et al., 2001). AMI during the protective response to pulmonary fungal pathogens is broad and divergent, but it is clear that specific antibodies are efficacious for the host in the resolution of infection.

Studies also support the potential of using antibodies that target antigens common among multiple fungi to mediate cross-protection. Passive immunization using anti β-glucan MAbs or vaccination with β-glucan (laminarin) conjugated with the genetically-inactivated diphtheria toxin CRM197 (Lam-CRM vaccine) has been shown to confer protection against *C. neoformans, C. albicans and A. fumigatus* (Torosantucci et al., 2005; Rachini et al., 2007). Since β-glucans are found in the cell wall of fungi, the efficacy of anti-β-glucan antibodies can be very broad. Cenci et al. used a killer anti-idiotype MAb reacting to a yeast killer toxin to protect mice from a lethal *A. fumigatus* challenge during experimental bone marrow transplantation (Cenci and Romani and 2375). Killer toxin is also expressed by multiple fungal species. Mycograb (NeuTec Pharman plc.), a recombinant antibody targeting an epitope within the HSP90 of *Candida albicans* that is conserved with the corresponding protein in *C. neoformans*, has been shown to act in adjunct with amphotericin B against multiple *Candida* species and *C. neoformans*. Altogether, these studies highlight the possibility that antibodies targeting "universal' antigens common to multiple fungal species such as β-glucans, killer toxins, or Hsps may extend protection to multiple disparate fungal pathogens. Casadevall and Pirofski has published an elegant commentary on the emergence of cross-protective targets for fungi (Casadevall and Pirofski, 2007).

5. Conclusion

The principal route of entry for several of the primary and opportunistic fungal pathogens is via inhalation of infectious propagules into the lungs. Consequently, exposure to these fungi

is unavoidable. Nevertheless, most encounters are asymptomatic due to the quick resolution of the fungi by resident effector cells within the lung. On those occasions that the fungal insult cannot be quickly eradicated, T cells, predominantly $CD4^+$ T cells, preside over orchestrating the adaptive responses and provide help for antibody production. T cell responses are also influenced by cytokine production by innate effector cells. Nonetheless, T cells mediate various cellular responses at the sites of infection and are ultimately responsible for either resolution or pathological reactions associated with these infections. Furthermore, T cells are important players in homeostasis and protecting integrity of natural barriers. Recent advances in experimental animal models support the premise that anti-fungal vaccines may be effective in immune compromised hosts. The efficacy of anti-fungal vaccines in immune compromised populations is undoubtedly due to the inherent plasticity of host immunity. Altogether, it is clear that immune responses to pulmonary fungal infections are as diverse as the fungi themselves but that significant ground has been made towards its understanding.

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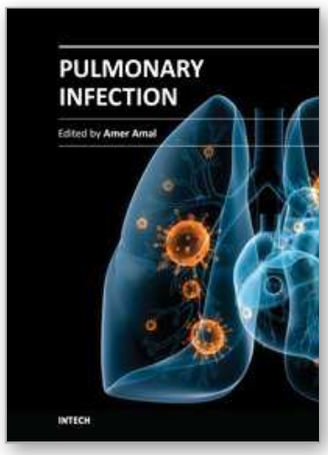

Pulmonary Infection
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Pulmonary infections are notorious in causing considerable morbidity and mortality. Caused by bacteria, viruses or fungi, respiratory infections require distinct knowledge of recent advances in pathogenesis. Progress in the understanding of immunopathogenesis of Acinetobacter baumannii infection will explain how an atypical organism establishes infection. The chapter regarding pulmonary nontuberculous mycobacterial infections in the State of Para depicts a unique study in an endemic region for tuberculosis in North of Brazil. The diagnosis and treatment of latent tuberculosis is a formidable challenge. Thus, new developments in diagnosis and treatment of latent tuberculosis are included in this book. Challenging in their diagnosis, nontuberculous mycobacterial pulmonary diseases require special education for management. The problems of respiratory infections in the immunocompromised host are increasing in numbers and in resilience to treatment. Therefore, the chapter describing the host immune responses against pulmonary fungal pathogens comes as a necessary section in this book. The insight brought forth from this book can be valuable for both clinicians and scientists.

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