THE ROLE OF THE RECRUITED NEUTROPHIL IN THE INNATE RESPONSE TO *ASPERGILLUS FUMIGATUS*

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Masters of Science

in

Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 2005

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ABSTRACT

Strong clinical and experimental evidence links qualitative and quantitative neutrophil deficiencies to fatal infections caused by *A. fumigatus*. Yet the role of the neutrophil in mediating the protection observed in normal hosts remains largely unknown. Recent studies indicate neutrophils from CXCR2$^{-/-}$ mice are unable to migrate toward chemokine gradients of KC and MIP-2, rendering these animals susceptible to fatal aspergillosis. Mice with a mutation in the gene encoding for the gp91^{phox} component of the NADPH oxidase lack the ability to generate the reactive oxygen metabolites used by phagocytes in killing microbial pathogens, and these mice are also susceptible to invasive pulmonary aspergillosis.

In this investigation of the innate response to *A. fumigatus*, CXCR2$^{-/-}$ and gp91$^{-/-}$ mice were used to mimic the qualitative and quantitative neutrophil defects that are known to predispose to invasive pulmonary aspergillosis, the most lethal form of *Aspergillus* diseases. By comparing the nature of the predisposition of these mice with the robust immunity observed in normal and immunocompetent mice, insight was gained on the involvement of this key phagocyte in the innate response. Several important parameters of the innate response in the lung were analyzed in this investigation, including (1) leukocyte recruitment and organism engagement, (2) organism killing mechanisms, (3) cytokine levels of bronchoalveolar lavage fluid, and (4) *in vitro* organism killing by leukocytes.

Following intratracheal challenge of *A. fumigatus* conidia a delay of 3 hours in neutrophil recruitment to lungs of CXCR2$^{-/-}$ animals was observed, allowing significant conidial germination and hyphal formation not seen in normal animals. In contrast, the gp91$^{phox-/-}$ mice recruited neutrophils normally but failed to inhibit conidial germination and hyphal proliferation, apparently as a result of their inability to generate the appropriate conidiacidal mediators. In normal mice inoculated conidia were rapidly sequestered within neutrophil aggregates involving a response by the phagocyte NADPH oxidase detected by formazan deposition.

These results suggest a previously undescribed role for neutrophils that conduct early inflammatory events following exposure to *A. fumigatus* conidia, involving first sequestration of ungerminated conidia within neutrophil complexes, and subsequently oxidant generation that prevents hyphal outgrowth.

CHAPTER 1

INTRODUCTION

The saprophytic fungus *Aspergillus fumigatus* is a ubiquitous member of the soil environment, where it survives on decaying organic material and is passively transmitted as an airborne particle by the wind. A consequence of its prevalence in nature, small size and ease of dispersion is frequent inoculation into the lower respiratory tract of humans. The mammalian immune system has evolved with this continual exposure and appears to have developed a highly effective strategy for coping with this fungus. This protection is thought to depend on the innate arm of the immune response. Two effector cells of this innate response, resident alveolar macrophages and recruited polymorphonuclear leukocytes (neutrophils), are the primary mediators of this protective response. The efficacy of this response is indicated by the fact that infections caused by this organism are exceedingly rare in immunocompetent hosts, despite daily exposure to the pathogen.

The incidence of infections caused by this opportunistic fungus is changing proportionately to increasing numbers of immunocompromised individuals. Immunocompromising circumstances that predispose to *A. fumigatus* vary from iatrogenic effects to acquired immunodeficiency syndrome (AIDS), and the accompanying infections caused by this organism are usually fatal. Bone marrow and organ transplant recipients, as well as those receiving heavy-dose chemotherapeutics comprise the group with greatest susceptibility to the most invasive and lethal form of the disease, invasive pulmonary aspergillosis (IPA). This brings light to the fact that medical

advances that render hosts susceptible to *Aspergillus*, such as immunosuppressive techniques, have outpaced development effective measures for avoiding or treating these infections. Consequently, the prognosis for IPA in patients is poor, and *A. fumigatus* is now recognized as the leading airborne fungal pathogen in immunocompromised people, with mortality rates as high 80-90% in some patient groups[75].

This thesis represents work begun in the spring of 2003 with the objective of investigating the innate response against *A. fumigatus*. Certain aspects of the literature pertaining to IPA pathogenesis, which are detailed herein, suggest that the model used to define the protective innate response may not entirely or accurately reflect the host response in the lung. These discrepancies formed the basis of the hypothesis for this investigation and guided the research objectives and experimental approach. A detailed *in vivo* and *in vitro* study of the role of the neutrophil in engaging the conidial form of *A. fumigatus* followed, and is described in this document.

A. fumigatus, the Organism

Taxonomy

Criteria for the taxonomic classification of the filamentous fungi are based upon morphological and physiological characterizations of the organism[31]. *A. fumigatus* is classified is in the Division *Eumycota*, which is characterized by septations delimiting their somatic structure, mycelial thalli and a cell wall structure that contains chitin and noncellulosic glucans. The result of an asexual fruiting event of *A. fumigatus* is the

production of the conidium, which is typical of organisms in the *Ascomycetes* class, and because the conidia produced are exposed and scattered loosely over the mycelium, the organism is further classified as belonging to the *Euascomycetidae* subclass. Currently, a sexual stage in the reproduction of this fungus has not been identified, however new information suggests a sexual stage may exist [31].

A. fumigatus is considered a thermotolerant mesophile, based on a relatively wide temperature range of growth (12-52°C), and the ability of the organism to survive temperatures up to 70°C [31]. The ability of *A. fumigatus* to grow rapidly at 37° C, which is a property not found in a number of environmental fungi, contributes to its ability to cause disease in mammals.

Ecology/Life Cycle

The filamentous fungus *A. fumigatus* is an environmental saprophyte, obtaining its requisite energy source and moisture in the organic debris within the soil. Under satisfactory growth conditions, mycelial development results in the formation of the conidiophore, upon which conidia are produced on the termini of raised sporophores, facilitating their efficient airborne dispersal. This process of conidiogenesis involves asexual replication, which distinguishes these fruiting structures as conidia instead of spores. The conidia, which measuring 2-3μm in diameter, are small among most fungal spore-like structures, and their small size and ease of dispersal impacts both their ecology and pathogenicity. Conidia contain melanin and other pigments [79], which give the mature fruiting mycelium of *A. fumigatus* a dark green color. Once dispersed, and when in a

suitable environment containing an appropriate carbohydrate source [61], transcriptional changes begin within the conidium. Such changes initiate the break of dormancy, thus heralding complex metabolic events that lead to germination. Formation of septated hyphae within the new mycelium marks completion of the life cycle.

Airborne dispersal and germination characteristics of the *A. fumigatus* conidium are necessary for establishing this organism in new ecological niches. This process is also a requirement in the development of invasive infections in the mammalian host, and therefore impacts the focus of this thesis, as it relates to the mammalian immune system. Certain cellular mechanisms involved in the crucial process of germination of *A. fumigatus* conidia have been studied [2,85], though many of the details associated with this event are not completely understood. Importantly, certain data are presented in this document that suggests the germination process itself may be a crucial target of the mammalian immune defense mechanism.

To better understand the germination processes in *A. fumigatus* that leads in some cases to invasive infections in mammals, comparison of specific genetic components of *A. fumigatus* to other fungi which are better understood has proven rewarding. Studies in the closely related organism *A. nidulans*[34,60] have illuminated several crucial events involving germination in *A. fumigatus* [59,85]. Molecular analyses of the *Aspergilli* have revealed germination processes that could potentially reveal important aspects of the earliest stages of germination. For example, initial events associated with germination begin with the activation stage when, in the presence of water, oxygen and CO_2, a carbohydrate source is recognized, internalized and metabolized. Activation of a rasA-

dependent signaling cascade results in the beginning of the isotropic growth phase with initiation of other metabolic events, including RNA and protein synthesis, respiration, water uptake and cell wall growth; which collectively give the first visible morphological index of germination. During this stage of germination, the swollen conidium has doubled in size, now measuring between 4-6μm in diameter. Polarization of cell wall material deposition at one side of the conidium marks the site of eventual germ tube formation. Hyphal extension, mitosis and cell wall production then produce the characteristic septated hyphal mycelium [60,78].

Virulence

In spite of the ability of this *A. fumigatus* to cause fulminate and usually fatal disease in humans and animals, attempts to identify virulence traits using single-gene mutants have not been rewarding [44]. The apparent absence of a single virulence factor identified is supported by several of its ecological characteristics that suggest it is not likely to have evolved such hereditary mechanisms. Further discounting an infectious role in the ecology of *A. fumigatus* is the evidence that no genetic distinction has been shown between a groups of environmental and medical isolates examined [13].

Several characteristics about *A. fumigatus* suggest it has evolved a life cycle that is entirely dependent upon a saprophytic rather than parasitic existence. For example, the organism lacks the ability to produce fruiting structures within tissues, preventing its transmission from one host to another by airborne dispersal mechanisms. Rare cases when conidia have been observed in tissue have been associated with instances where the

mycelium has invaded tissue that contains cavitaceous lesions, such as those found in tuberculosis. But even in such rare cases where fruiting *A. fumigatus* structures have been found, it is unlikely the conidia could efficiently be dispersed outside the host.

The properties of the dormant conidial cell wall that confer resistance to environmental stresses, such as desiccation and high levels of UV exposure, nevertheless offer resistance to some of the microbicidal mechanisms employed by the mammalian immune system. Hyphal expression of proteolytic enzymes and secondary metabolites such as gliotoxin, which have a number of pathogenic effects including inhibiting NADPH oxidase and induction of apoptosis [80] aid in hyphal proliferation and evasion of the immune system. However, these factors are likely to be strategies of evading environmental predation from amoeboid soil organisms, rather than components of the cellular immune response. Thus, the ability of the *A. fumigatus* to cause disease in mammals is likely due to certain characteristics that have made it a successful saprophytic soil microbe.

In sum, *A. fumigatus* does not appear to have relied upon the ability to evade the mammalian immune system, and yet several of it characteristics such as those listed above have neverthess made it a successful opportunistic pathogen. A study of the host-*A. fumigatus* interaction should therefore be directed toward understanding the resistance mechanisms in the mammalian host, which likely are the result of extensive evolutionary pressure, that contribute to the remarkable host resistance to *A. fumigatus* infection. The focus of this thesis is therefore directed toward a study of the natural mammalian immune system that protects from infections by this organism. This pursuit may eventually lead to

a better ability to protect humans with a variety of risk factors from devastating infections by this ubiquitous fungus.

Host Factors Contributing to Aspergillosis Immunity

Pathogenic Manifestations of Aspergillus fumigatus

A. fumigatus is recognized to cause three forms of disease that are distinct in their clinical presentation and etiology, and are determined primarily by the underlying conditions in the host immune system and lung competency. Aspergilloma and allergic bronchopulmonary aspergillosis are chronic conditions that arise as a result of other long-term pulmonary diseases. For example, aspergilloma arises when inhaled *A. fumigatus* conidia germinate to colonize an existing cavity in lung tissue that was the result of some previous lung parenchyma damage. These cavities, which are usually caused by turberculous or sarcoidosis, may show localized foci of impaired immune function because of their avascular and sometimes-fibrotic, condition. Consequently, *A. fumigatus* can form well-defined mycelia that are mixed with mucous and cellular debris [12]. The fungus ball that arises does not typically invade the cavity wall or margin, but can cause debilitating hemoptysis.

In allergic bronchopulmonary aspergillosis (ABPA), hyphae are confined to the bronchial lumen of asthmatic patients, which exhibit type-I and type-III hyperimmune responses to the *Aspergillus* antigens. Pathologically, ABPA is characterized by

eosinophilia in the blood and sputum, and bronchiole damage associated with hypersensitivity, including bronchospasms, edema, denudation and bronchiectasis [10].

The third and most deadly form of aspergillosis is invasive pulmonary aspergillosis (IPA), a disease almost exclusively found in immune suppressed patients [27]. This aggressive infection is characterized by hyphal invasion of the lung parenchyma and hemorrhagic bronchopneumonia. In advanced stages angioinvasive IPA invades the pulmonary arteries resulting systemic dissemination. Extrapulmonary foci can be present in the brain, GI tract, heart, liver, spleen and thyroid [11]. Invasive forms of aspergillosis are usually spread from a primary pulmonary infection, though some information suggests IA in tissues involving the central nervous system, can occur without an obvious source of primary infection [81].

Immune Defects Associated with Invasive Aspergillosis

A variety of immunocompromising conditions are believed to predispose to the three types of aspergillosis 44,48,83. The aspergillosis diseases are a consequence of increasingly sophisticated medical treatments that rely on direct immunosuppression such as transplantation, and also current treatments for other conditions that now extend lives of individuals which often died before infection by *A. fumigatus* was likely. The emergence of new immune suppressive diseases such as HIV-AIDS, coupled with greater frequencies of certain types of cancer, have further increased the numbers of invasive *A. fumigatus* infections [3,21,46]. Due to the varied and complex mechanisms of immune suppression, and therefore predisposing factors for aspergillosis, *A. fumigatus* is an now

considered the leading cause of airborne fungal infections in immunosuppressed patients [44,76]. Additional medical advancements and new therapeutic regimens are expected to further extend this problem.

Conditions now recognized to predispose to invasive *Aspergillus* susceptibility can be categorized broadly as: 1) multifactorial complications in innate immune responses (as in AIDS and some hematologic disorders which involve multiple primary immune dysregulation events, not primarily impacting neutrophils [3,3,15,18,32]), and 2) direct defects in neutrophil functions owing to either qualitative or quantitative mechanistic insufficiency. Examples of conditions associated with immune suppression involving such neutrophil defects include corticosteroid therapy [30], myeloablation, and chronic granulomatous disease (CGD). At this time, CGD is the only known congenital defect associated with aspergillosis [40,56,84], and is therefore a disease which may provide significant information about the natural innate immunity to infection by this organism.

Susceptibility to aspergillosis due to purely alveolar macrophage (AM) and not neutrophil defects have not been shown, though most factors known to impact neutrophil function are likely to also impair AM functions, complicating the ability to delineate the roles of neutrophils and AM in aspergillosis immunity. Nevertheless, neutropenia has been recognized for decades as the most important acquired risk factor for invasive aspergillosis in humans [76], and several of these same causes of neutropenia in humans have been shown sufficient to render animal models vulnerable to IPA [38,55].

Innate and Acquired Responses.

Invasive pulmonary aspergillosis is almost universally fatal in mammals. Since A. fumigatus conidia are inhaled daily into the lungs of all mammals, survival of the individual depends on an immune response capable of neutralizing A. fumigatus conidia upon the first encounter, even in the newborn or otherwise naive individual. Therefore, the elegant natural immunity that protects from infections with A. fumigatus was likely to have evolved in response to strong evolutionary pressure driving an innate immune response. At this time, both experimental and clinical evidence supports the view that innate immunity is primarily responsible for the protection from invasive aspergillosis in healthy individuals 37.

Several facets of the innate response are believed to be important in protection from IPA. For example, anatomical barriers, certain humoral components, and pulmonary phagocytic cells have all been shown to play a role in responding to conidia infiltration into the lung [22-24,27,43,48,73,82]. It appears that these same mechanisms, though not completely understood, function efficiently to protect humans in spite of sometimes heavy doses [4]. While susceptibility to the organism has been associated with $CD4^+$ T cell type1/type 2-cytokine dysregulation [14,15,51,52,69], these cytokine and chemokine profiles probably are important to the extent that they support a proinflammatory immune response with phagocytic cells most directly involved.

Anatomical and Humoral Components

Inhaled airborne conidia are first engaged by the nasal turbinates, which cause airflow turbulence and lead to conidial deposition and impaction on the nasal mucosa, where they are neutralized by poorly understood mechanisms and eliminated in secretions. Conidia that continue through the trachea and into the lower respiratory tract may be deposited upon columnar epithelial cells and cleared through the muco-cilliary escalator activity of the bronchioles. Epithelial cells lining this area of the lung are known to phagocytose conidia, and deposition of conidia upon this tissue has been investigated as a source of infection [63,80]. Conidia penetrating further into the alveolar space encounter yet a different tissue milieu including surfactant proteins A and D, which are produced by type-I pneumocytes and line the surface of the alveoli [1]. These surfactant proteins are believed to carry out a homeostatic and protective role. These proteins interact with the carbohydrate structure of the cell wall of conidia, via the carbohydrate recognition domain, and agglutinate the conidia *in vitro* [47].

During exposure of high doses of conidia to the alveolar compartment, a purulent inflammatory response occurs, in which the inflamed tissue vessels may allow not only recruited immune leukocytes, but serum components as well. Such humoral immune components may include antibody and complement which might play a role in the immune defense of *A. fumigatus*. At this time, comprehensive evidence that either antibody or complement are important in the innate response to *A. fumigatus* conidia has not been firmly established. However, interaction of conidia with complement and

specific antibodies activated through the alternative pathway and bound C3 *in vitro,* has been investigated [42].

Possible support of a role of complement in the innate response to *A. fumigatus* conidia was shown in C5-deficient mice with increased susceptibility to airborne organism challenge [36]. In contrast to an apparent innate protective role for complement against *C. albicans* [33], *A. fumigatus* may actually utilize complement to protect itself from the immune system [65]. However, during exposure of low numbers of conidia to the lung, as is believed to occur in immune suppressed patients prior to the onset of IPA, it is less likely that complement is found in the alveolar space. Therefore, the relative contribution of complement in aspergillosis immunity is still disputed.

Antibody responses that mediate protection from aspergillosis have also been investigated [37,62], but the paucity of immunization studies and conflicting data have not revealed a clear involvement of antibody-mediated resistance to *A. fumigatus* infections [43]. Neutrophils are known to attach to hyphae and degranulate in the absence of serum [25], suggesting the natural immune response may not require serum components including antibody. Furthermore, the cellular inflammatory response in the lung consists primarily of mononuclear and neutrophilic phagocytes, and does not appear to include antibody-secreting B lymphocytes [20]. Adoptive transfer of immune serum failed to protect from intravenous challenge of *A. fumigatus* conidia, and the peritoneal and splenic macrophages of these mice had similar phagocytosis indexes and H_2O_2 production as those not receiving the immune sera [20]. Collectively these findings indicate that the

phagocyte response to *A. fumigatus* conidia acts independently of *A. fumigatus*-specific antibody.

In contrast to most studies that have investigated the involvement of serum antibodies in aspergillosis immunity, increase in *A. fumigatus*-specific IgA was observed in the lungs of patients with active IPA. However, the ability of IgA antibodies to protect in subsequent challenges or prior to prevent disease has not been determined.

Little is known of the involvement of various T cell subsets in the lung in response to *A. fumigatus* conidial challenge. T cell types found in the lung include γδ T cells and $CD4^-/CD8^-$ T cells, which, unlike other tissues, constitute a large portion of T cells in this location. Both $CD4^+$ and $CD8^+$ T cells have also been studied in the lung. The role of these cells in response to *A. fumigatus* in the lung is largely unknown [19]. Furthermore, a significant amount of information remains unknown regarding antigen-specific effector mechanisms of the host pulmonary system in response to *A. fumigatus*.

Cytokines and Chemokines

Cytokines are known to play key roles in modulating phagocyte responses in the lung, and the predisposition to aspergillosis in AIDS patients is generally attributed to the failure to manage the appropriate balance of proinflammatory and anti-inflammatory cytokines and chemokines [68], rather than a reduction in the numbers of phagocytic cells.

The innate response to *A. fumigatus* follows the classic inflammatory model where subsequent to macrophage engagement of the pathogen, neutrophils are recruited and activated by the chemokines and cytokines released by macrophages and endothelial

cells. These signaling molecules therefore constitute an essential cellular communication system to generate and maintain this response, keep it appropriately focused to the specific area in the lung, and ultimately to resolve the inflammatory response. Throughout the progression of these immune mechanisms, the delicate balance between adequate organism destruction and reduction of inflammation must be achieved to minimize damage to host tissue. The significance of these events in generating a protective response is represented by the numerous reports investigating the salient cytokine and chemokine mediators of these events. Studies indicate the critical phagocyte involvement is supported by a Th1 cytokine profile [14,15,18,50,51,53,69], including IFN-γ [18,77], IL-2, IL-12 [70], M-CSF [29], GM-CSF [9], and TNF-α [52].

Based on the dependence on the recruited neutrophil for protective immunity to the infectious challenge of *A. fumigatus*, it is not surprising that the pro-inflammatory chemokines and cytokines that mediate neutrophil recruitment and activation have been shown to be essential for fungal clearance. The pro-inflammatory cytokines that have been determined to have a role in the neutrophil involvement include granulocyte-macrophage colony stimulating factor (GM-CSF)[41,74], tumor necrosis factor-α (TNF-α) [26,52,52,66,74] interferon-γ (IFN-γ) [26,66] interleukin-6 (IL-6) [26], IL-1β [26,41], IL-12 [7] and IL-18 [7]. Several of these same pro-inflammatory chemokines have similarly been reported to have a role in protective immunity in the response to *A. fumigatus* conidia. These include macrophage inflammatory protein-1α (MIP-1α) and macrophage chemotactic protein-1 (MCP-1), CC chemokines that recruit and activate monocytes [50,74], and IL-8 and growth

related oncogene (GRO), which are ELR+ CXC chemokines (with mouse analogs MIP-2 and KC) [51,53,57,74].

Of these immune signaling molecules, TNF-α and the ELR+ CXC chemokine MIP-2 appear to be among the most essential in aspergillosis immunity, due to the severity of resulting infections when either the receptor of these molecules are knocked out or immunodepleted, or when the ligand itself is immunodepleted [51,74]. These proteins are both produced rapidly by AM in response to *A. fumigatus,* and both recruit and activate neutrophils to similar degrees.

TNF-α has a broad range of effects in the inflammatory response, as it affects the recruitment of neutrophils by inducing E-selectin, P-selectin and intracellular adhesion molecule 1 expression on the luminal surface of the venous endothelium [8,41]. TNF-α also induces the production of MIP-2 from AM in an autocrine and paracrine manner [8]. The protective effect of TNF-α is due in part to induction of MIP-2 by AM, which then signal via CXCR2 for PMN recruitment to the lung [7,57,66]. IL-1β and TNF-α have known to be capable of up-regulating the production of chemokines by AM.

Production of pro-inflammatory cytokines was equivalent from macrophages stimulated with *A. fumigatus* and other species of the aspergilli such as *A. niger*, suggesting a generalized response to a variety of organisms within this genus. This also indicates that the pathogenesis of *A. fumigatus* does not rely on immune-evasive mechanisms [54]. In support of the importance of proinflammatory signaling response in aspergillosis immunity, isolated AM from rats produce MIP-1α, MIP-2, KC and TNF-α when stimulated with *A. fumigatus* in vitro, similar to the production of these same

signaling molecules in the lung. Macrophages from dexamethasone-treated mice have significantly reduced the ability to produce cytokines in response to *A. fumigatus* conidia [77], which suggests a mechanism by which certain anti-inflammatory medications predisposes toward infections by *A. fumigatus*.

Phagocytes

General Phagocyte Biology. The cellular components of the protective innate response from aspergillosis involve primarily resident AM and recruited neutrophils. This conclusion is derived from extensive evidence supporting the predominance of these two phagocytes in response A. fumigatus conidia coupled with the absence of lymphocytes in the inflammatory response and the apparent independence on antibody.

The AM is the resident phagocyte of the pulmonary system and is therefore constitutively present where *A. fumigatus* conidia are likely deposited following inhalation. The AM is a professional phagocyte which is terminally differentiated from the blood monocytes which have undergone functional and morphological maturation during their emigration into the tissue from the bloodstream. While they are capable of antigen presentation, the primary role of AM in the lung is two-fold: they serve as phagocytic sentinels in the lower respiratory system where they are positioned to rapidly engulf and neutralize inhaled pathogens and debris, and they also act as chemical signaling sources, orchestrating the appropriate cytokine and chemokine production in response to various types of inflammatory challenges [19,57].

AM recognize *A. fumigatus* conidia by toll-like receptors (TLRs) 2 and 4, as well as other pathogen-associated molecular patterns on the surface of *A. fumigatus* conidia, which have yet to be determined [6,54,58]. The importance of these recognition contacts are indicated by studies of TLR2 and TLR4-deficient mice, which have strongly impaired TNF-α, NO, IL-6 and MIP-2 production, resulting in reduced neutrophil recruitment to the peritoneum following *A. fumigatus* challenge [54] that corresponds to increased mortality [5]. Recognition of the conidium by AM also occurs by a lectin-like binding of the carbohydrate cell surface with the mannosyl-fucosyl receptor of macrophages. Phagocytosis and neutralization of the conidia follows these recognition events, which then leads to phagocytosis of the organisms in a membrane-bound intracellular vesicle. Acidification and phagolysosomal maturation follows, in which the engulfed conidium is exposed to numerous antimicrobial factors, possibly including reactive oxygen intermediates produced by the NADPH oxidase system [39,56], as well as nitric oxide, antimicrobial peptides, and other antimicrobial factors [43,64].

Neutrophils are granulocytic leukocytes that comprise the largest percentage of the leukocyte population (~65%), with 55% of the bone marrow devoted to their development. Production of neutrophils in the bone marrow takes approximately five days. In contrast to the longevity of the AM, neutrophils have a half life of about 6 hours in circulation and a few days in tissues. Also unlike the AM, neutrophils are absent from the normal uninflamed lung. However, the close proximity of the microvasculature of the lung with lumen of the alveoli allows rapid response and recruitment of neutrophils following the appropriate inflammatory signaling. Extravasation of neutrophils into

various types of tissue has been shown to be dependent in part to the ELR+ CXC chemokines [51,53,74], which are released by the AM immediately following recognition of *A. fumigatus* conidia.

In addition to some of the same microbicidal and phagocytic mechanisms as AM, neutrophils are also able to fuse their microbicidal granules with the plasma membrane, thereby depositing their antimicrobial constituents into the extracellular milieu. This process, called degranulation or frustrated phagocytosis when surfaces too large for engulfment are encountered, enables the cell to neutralize pathogens of various sizes. Degranulation therefore has the benefit of extracellular microbial neutralization, but the pathological consequence can include increased damage to host tissue in the vicinity of the event.

Phagocyte Involvement in Aspergillosis Immunity.. Several lines of evidence indicate AM contribute to the natural immunity to aspergillosis [43,71], yet the mechanisms used by AM in this response have not been completely elucidated by *in vivo* methods. The importance of neutrophils in aspergillosis immunity has nevertheless been shown by the fact that immune depletion of neutrophils in mice using monoclonal antibody RB6 [28,51] or cyclophosphamide [14,52] predisposes invasive infection in mice in which identical inoculations in untreated mice did not cause infections [16].

Several of the innate resistance mechanisms that prevent infections by *A. fumigatus* may function prior to the formation of hyphae as a result of conidial germination [86]. The degree to which neutrophils contribute to this process has not been

fully revealed, in spite of the apparent importance of neutrophils in the response. Interestingly, it has been shown that neutrophils do not efficiently kill resting conidia *in vitro* [45].

Involvement of the microbicidal products such as reactive oxygen species (ROS) produced by the NADPH oxidase in conidial killing by the AM is disputed in the literature. Several reports indicate that AM do not rely on ROS in this antifungal response. This includes AM-mediated killing in anaerobic conditions [43], and conidial killing by AM from gp91$^{phox-/-}$ mice [55] that equaled that of conidial killing from normal mice. In contrast, ROS have been implicated in macrophage-mediated conidial killing by the detection of ROS, via luminol-HRP chemiluminescence, in macrophages with intracellular conidia, and a reduced *in vitro* killing effect using macrophages from p47$^{phox-/-}$ mice [64]. While the exact conidiacidal mechanism of the AM remains unclear, it is known that AM maturation in the lung is necessary for this response because peripheral blood monocytes and peritoneal macrophages show greatly reduced conidiacidal activity compared to alveolar macrophages [73].

Neutrophils which have been recruited into the lung, however, are known to rely on the reactive oxygen metabolites for anti-hyphal activity *in vitro* [23]. Neutrophil-mediated hyphal damage is detectable after a two hour incubation of *A. fumigatus* hyphae with neutrophils and is unaffected by the presence of serum and cationic inhibitors [25], but is inhibited by catalase [23]. Singlet oxygen is also implicated in hyphal damage by the finding that photoactivated rose bengal induced comparable hyphal damage as that mediated by the neutrophils [23]. These and other experiments confirmed the ability of

neutrophils to adhere and degranulate in the presence of *A. fumigatus* hyphae, thus mediating appreciable hyphal damage.

Early studies of the cellular engagement of conidia by the pulmonary phagocytes showed that neutrophils failed to show effective conidial killing compared to the macrophages [45,72]. Surprisingly, mechanisms of engagement of conidia by neutrophils have remained largely uninvestigated, and therefore the suggested role of the neutrophil has remained restricted to an anti-hyphal response. The possibility that neutrophils do in fact engage and destroy conidia has received surprisingly little attention. For this reason, efforts have been taken as described in this thesis to investigate evidence that neutrophils do in fact contact conidia in a unique manner, and in doing have revealed evidence of their use of the NADPH oxidase in this response (Chapter 2).

Though less studied than neutrophils and AM, other investigations have examined the roles of monocytes [24], lymphocytes [18,35,49,67] and platelets [17], which may contribute to the protective immunity to *A. fumigatus* as well. However, at this time, evidence for protective roles of these components in aspergillosis immunity is much less compelling than that of the phagocytes.

<u>Dogma Regarding Phagocytes in Aspergillosis Immunity</u>. A model of the innate response has been defined which describes a regimented phagocyte response in which the AM engulf and kill the conidial stage of the fungus and recruited neutrophils engage and destroy the emerging hyphal forms that have escaped killing by the AM [72]. Widespread acceptance of the model of AM engaging conidia and neutrophils engaging hyphae in the

immune response to *A. fumigatus* is seen in most new reports describing phagocyte mechanisms in aspergillosis immunity.

Shortcoming of Current Dogma in Aspergillosis Immunity. Although it is commonly accepted that immunity to *A. fumigatus* is mediated by the engagement of conidia by AM and hyphae by neutrophils, these views are not supported by compelling *in vivo* evidence, and fail to explain the clinical manifestations and predisposing conditions of the disease. Therefore, despite broad acceptance of this model, many of the experimental findings essential to the model were based on data obtained from systems that may not represent the pertinent immune mechanisms in the lung, where this immune response occurs.

In the normal host hyphal forms of the fungus are not found, or are not found in numbers that support the copious recruitment of neutrophils following high dose exposure. Rather, hyphae are found only in circumstances of qualitative or qualitative neutrophil defects, which are also primary predisposing factors for IPA. Further, AM from CGD mice have been shown to kill conidia as efficiently as AM from normal animals, yet these mice are extremely susceptible to low dose exposure of *A. fumigatus*, implicating the involvement of neutrophils even in low dose exposures.

New Model of Aspergillosis Immunity

The data presented in this thesis was obtained with the purpose of providing a better understanding about the role of neutrophils in the innate immune response

following the inhalation of *A. fumigatus* conidia. These findings propose alternative explanations compared with those predominating the literature regarding the role of the recruited neutrophil in engaging inhaled *A. fumigatus*, and support a revised model of the innate response to this fungus that suggest: 1) protective immunity to *A. fumigatus* depends on preventing germination of inhaled conidia rather than arresting hyphal outgrowth, 2) conidiacidal activity is mediated by both the resident alveolar macrophage (through ROS-independent mechanisms) and recruited neutrophils (through aggregate formation and ROS-dependent mechanisms).

This model, which is based predominantly on the *in vivo* and *ex vivo* experiments described in this thesis, offers several contributions to the understanding of the protective immunity to invasive infections caused by *Aspergillus*. Such contributions include:

(a) evidence supporting the importance of the early recruitment of neutrophils, which we show is a mechanism essential to prevent conidial germination,

(b) support for a previously unidentified neutrophil aggregation event in response to *A. fumigatus* which elicits reactive oxidants by the NADPH oxidase,

(c) a better understanding of the role of the innate immune response to *A. fumigatus* that prevents rather than arrests focal infections within the lung.

Each of these contributions is consistent with the low frequency of disease in normal hosts, and with the lack of experimental data that supports existing dogma about

the immune response to *A. fumigatus*, which is based on the assumption the primary role of neutrophils is to destroy hyphal form of the organism.

Thesis Statement and Hypothesis

According to current view about immunity to *A. fumigatus*, natural resistance that prevents invasive aspergillosis is explained by the important role of AM early after exposure, which are believed to destroy the inhaled conidia, while neutrophils engage and neutralize hyphal forms of the fungus that develop from any conidia that escape neutralization by AM. However, documentation of hyphal development in the lungs of normal animals and humans is lacking, even in spite of very heavy inoculum doses.

The lack of evidence that neutrophils primary engage hyphal forms in the defense against *A. fumigatus* infections raises the question about the role of the neutrophil in this immune response, which is nevertheless recognized to be a critical in preventing infection. Because of these conflicting observations, an understanding of the phagocyte response in this disease is currently lacking. Therefore, the hypothesis upon which this thesis is based is that the essential mechanism of neutrophils in preventing invasive disease caused by *A. fumigatus* is to neutralize conidia prior to germination rather than to kill hyphal forms of the organism. This information is important to identify the natural mechanisms of resistance to this pathogen in order to provide this same immunity in humans predisposed to life-threatening infections by this organism.

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CHAPTER 2

NEUTROPHIL SUPPRESSION OF CONIDIAL GERMINATION

Abstract

Several types of polymorphonuclear neutrophil (PMN) deficiency predispose to fatal *A. fumigatus* infection. Increased susceptibility to aspergillosis also occurs when there is delayed PMN recruitment in CXCR2$^{-/-}$ mice or a defect in NADPH oxidase in gp91$^{phox-/-}$ mice. In order to study the early defensive role of neutrophils, with particular reference to their antimicrobial oxidant activity, normal, CXCR2$^{-/-}$ and gp91$^{phox\ -/-}$ mice were challenged with intratracheal *A. fumigatus* conidia. In normal mice, germination was arrested by early enclosure of conidia within PMN aggregates where NADPH oxidase activity was detected with formazan staining. Swollen conidia were present in the aggregates suggesting that arrest occurred after activation of germination. Delayed neutrophil recruitment in CXCR2$^{-/-}$ mice allowed conidial germination before formation of oxidase-active aggregates. In gp91$^{phox-/-}$ mice, PMN devoid of NADPH-oxidase activity, like normal PMN, rapidly aggregated around conidia but failed to inhibit germination. Normal murine and human PMN also formed oxidase-active aggregates with conidia *in vitro*. These results provide a newly-described mechanism by which PMN prevent aspergillosis by rapidly sequestering conidia within cell aggregates causing early arrest of germination due to their oxidant activity. Our results provide a better understanding of the robust natural immunity to *A. fumigatus*.

Introduction

In recent decades there has been an increasing number of patients with immune suppressive disorders, with an associated increase of invasive and often fatal *Aspergillus fumigatus* infections [6,17,38]. *A. fumigatus* is now recognized as the leading airborne fungal pathogen in immune compromised people [14,36]. New management strategies to prevent aspergillosis are urgently needed, especially for patients with complex health problems.

A prevailing model of immunity to *A. fumigatus* is based on a two-tiered phagocyte response, whereby resident AM phagocytose and subsequently digest inhaled conidia, whilst recruited PMN have a back up role to destroy hyphae arising from conidia which have escaped destruction by alveolar macrophages (AM) [5,9,23,33]. Thus most studies have focused on the importance of the AM in aspergillosis immunity, but in patients suffering from several types of PMN deficiencies who have apparently normal AM function, there is a marked susceptibility to invasive pulmonary aspergillosis (IPA). Similarly, PMN deficiencies alone are sufficient to establish IPA in animal models of aspergillosis [2,10,25,25,37]. The key importance of PMN in immunity to aspergillosis is supported by the finding that in the chronic granulomatous disease (CGD) mouse model, produced by targeted disruption of genes encoding components of NADPH-oxidase, there is a susceptibility to aspergillosis despite non-oxidative killing of conidia by AM [10,25,34].

Despite life-long exposure to *A. fumigatus*, very low morbidity is seen in immunocompetent individuals, indicating that a rapid and effective resistance to

aspergillosis must have evolved. In the mouse lung, conidial germination to form hyphae is not usually observed when there is normal PMN function [5,19]. These observations indicate that in immune competent hosts, PMN may protect against aspergillosis by preventing conidial germination. We therefore hypothesize that the protective innate response to *A. fumigatus* involves rapid recruitment of PMN, not primarily for engaging hyphae, as previously suggested, but rather to prevent conidial germination by release of oxygen radicals. Arrest of germination would provide an elegant mechanism for avoiding the fatal consequences of subsequent hyphal proliferation, tissue invasion, and fulminate dissemination.

To test our hypothesis and to better understand the role of the PMN in aspergillosis immunity, we compared the pulmonary immune response to *A. fumigatus* conidia in normal mice, which are resistant to IPA, with that in CXCR2$^{-/-}$ and gp91$^{phox\ -/-}$ mice, both of which are susceptible to fatal IPA [20,22,25]. This susceptibility in CXCR2$^{-/-}$ mice is due to their only known defect, namely failure of PMN to migrate towards the chief PMN-recruiting chemokines MIP-2 and KC [1,20]. The gp91$^{phox\ -/-}$ mouse model is susceptible to IPA because of its inability to generate reactive oxygen species (ROS) by the NADPH oxidase system [25]. The gp91$^{phox-/-}$ mouse is therefore a good model for CGD patients, who are particularly susceptible to aspergillosis.

Results are presented that support our hypothesis and show that in normal mice following intratracheal challenge with *A. fumigatus* conidia, there is rapid sequestration of conidia within PMN aggregates that produce ROS by NADPH-oxidase, arresting conidial germination. However, with the same challenge, germination occurs in CXCR2$^{-/-}$

mice, which show a 3 hour (h) delay in PMN recruitment, and also in gp91$^{phox\ -/-}$ mice, which do not produce ROS by NADPH-oxidase. These findings contest a widely-accepted model of the innate response to *A. fumigatus* in which the prime roles of AM and recruited PMN are to destroy conidia and hyphae, respectively. They indicate instead that in normal mice PMN, like AM, engage conidia and arrest germination and that susceptibility to aspergillosis can be related to delay of PMN recruitment and aggregation, or failure to produce ROS.

Results

Delayed Neutrophil Response in CXCR2 $^{-/-}$ Mice

To investigate phagocyte recruitment into the lungs of normal and CXCR2$^{-/-}$ mice 0-48 h after inoculation with *A. fumigatus,* conidial and differential leukocyte counts were determined in bronchoalveolar lavage fluid (BALF). A delay in PMN recruitment in CXCR2$^{-/-}$ mice was observed at 3 and 6 h, when 6 fold fewer PMN were found in BALF compared to normal animals ($P<0.005$) (Figure 1A). However by 9 h, PMN numbers in these mice were indistinguishable from that in inoculated normal mice, despite the inability of CXCR2$^{-/-}$ mice to respond to the chemokines MIP-2α and KC. When comparisons were made at 24 h with mice which had received HBSS without conidia, there was 183 fold ($P<0.001$) more PMN in BALF from normal mice exposed to conidia, whilst in CXCR2$^{-/-}$ mice the corresponding difference was 232 fold ($P<0.05$). In contrast to the significant PMN recruitment over the first 24 h, CXCR2$^{-/-}$ and normal mice showed

minimal changes in the number of AM in BALF in response to conidia (Figure 1B), and at 24 h numbers of AM were indistinguishable from those in mice receiving only HBSS ($1.4 \pm 0.1 \times 10^5$, and $1.3 \pm 0.4 \times 10^5$, respectively). However, by 48 h there was 3.5 and 2.7 fold more AM than at time zero in CXCR2$^{-/-}$ and normal mice, respectively ($P<0.001$). These results were confirmed by two further experiments (data not shown).

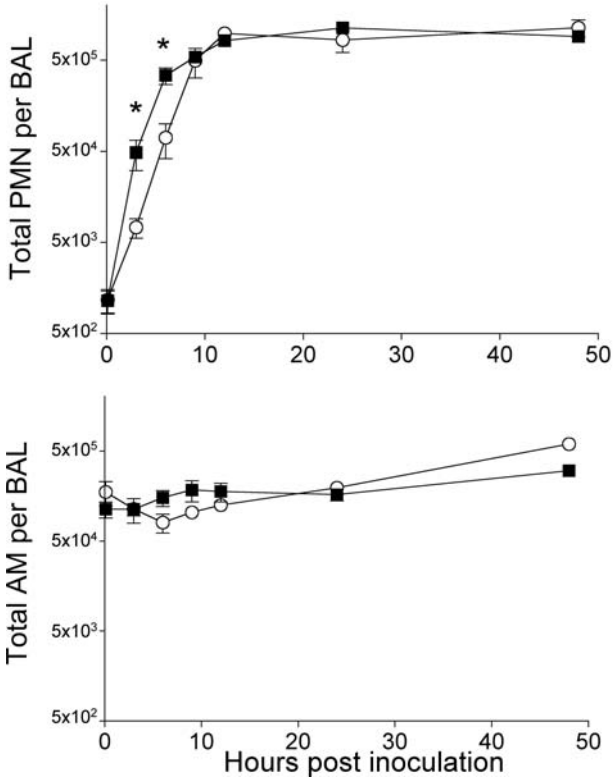

Figure 1. Total PMN (A) and AM (B) numbers in BALF following intratracheal inoculation of mice with *A. fumigatus* conidia. Normal (■) and CXCR2$^{-/-}$ (○) mice were inoculated with 3×10^7 conidia, euthanized and 5 ml BALF collected for cell counts and Wright staining for differential leukocyte analysis. The average total number of PMN (A) and AM (B) for groups of 5 mice per time point are shown ±SE (* indicates $P<0.005$).

Conidial Germination Following Delayed Neutrophil Response

Phagocyte recruitment and conidial engagement in BALF are shown in representative photomicrographs for normal (Figure 2A-C) and CXCR2$^{-/-}$ (Figure 2D-F) animals 0, 6 and 9 hours following inoculation with *A. fumigatus* conidia. The 0 h time point showed the absence of PMN in both normal and CXCR2$^{-/-}$ mice (Figure 2A and 2D, respectively) and early engagement of some free conidia by the AM. By 6 h in the normal mouse (Figure 2B), discrete PMN aggregates containing conidia were evident in the BALF. In contrast, few recruited PMN had reached the lung of the CXCR2$^{-/-}$ mouse and germinated conidia were present (arrows, Figure 2E). At 9 h in the normal mouse, large PMN aggregates containing numerous conidia could be observed in the BALF (Figure 2C) whilst in CXCR2$^{-/-}$ mice, although aggregates were now visible, they were smaller than in normal mice and contained both conidia and hyphae (Figure 2F).

To quantify conidial association with recruited PMN and macrophages, the numbers of free and cell-associated conidia were counted in the BALF. Association of PMN with conidia (solid black bars, Figure 3A and B) was significantly greater in normal than CXCR2$^{-/-}$ animals at 3, 6, 9 and 24 h ($P<0.05$), presumably due to delayed recruitment of PMN to the lung. In normal animals, from 9-24 hours more conidia were associated with PMN than AM. No statistical differences between normal and CXCR2$^{-/-}$ mice were found for the proportion of conidia associated with AM at any time point (hatched bars, Figure 3A and B). At 3 h after inoculation in both normal and CXCR2$^{-/-}$ mice, 25% of the conidia were associated with AM, and these associations peaked at 6-9 hours.

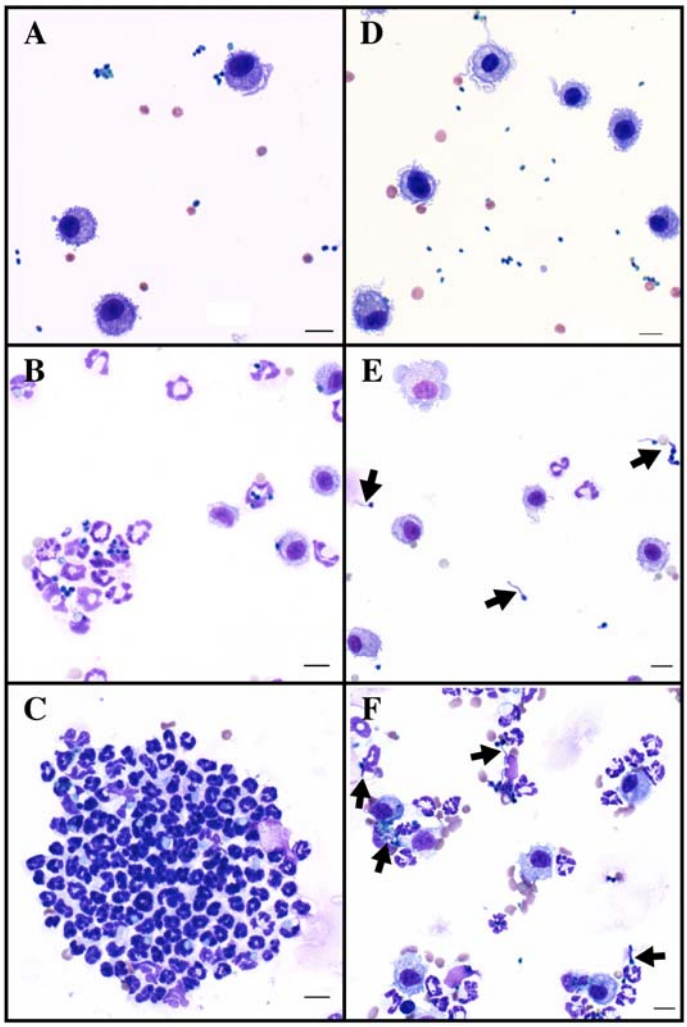

Figure 2. Leukocyte recruitment and conidial engagement in Wright-stained BALF. Cytospin slides were prepared from BALF of normal mice (A-C) and CXCR2$^{-/-}$ mice (D-F) and Wright-stained. PMN recruitment and conidial engagement is evident in normal mice at 6 and 9 h (B, C) but only at 9 h in CXCR2$^{-/-}$ mice (F). Germinating conidia in CXCR2$^{-/-}$ mice are indicated by arrows (E and F). Scale bars: 10μm

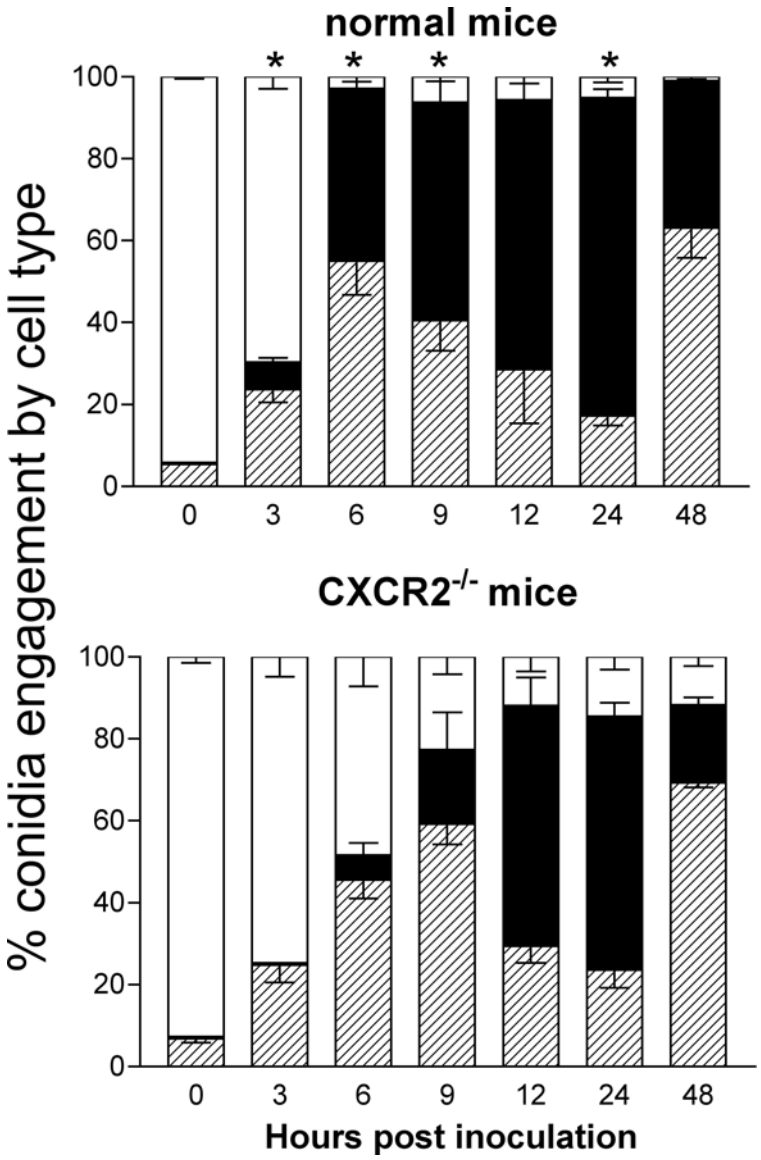

Figure 3. Conidial associations with pulmonary phagocytes in BALF samples. In both normal (top) and CXCR2$^{-/-}$ mice (bottom), BALF was Wright-stained and conidia categorized as either: unassociated with phagocytes (open bar); associated with PMN (black bar); or associated with AM (hatched bar) (n=200). Data points represent average percentage values ± SE for groups of 5 mice (* indicates significant difference between normal and CXCR2$^{-/-}$ PMN engagement of conidia, $P < 0.05$).

There was a marked difference in how AM and PMN engaged conidia. Solitary AM with greater than a dozen phagocytosed conidia were common in CXCR2$^{-/-}$, gp91$^{phox\ -/-}$ and normal mice (Figure 4A). In contrast, PMN engaged conidia by forming aggregates with them as shown in Figures 2B and 2C, and a higher magnification in Figure 4B. In normal mice, aggregates typically contained high concentrations of both resting and swollen conidia, whilst in aggregates from CXCR2$^{-/-}$ (Figure 4C) and gp91$^{phox\ -/-}$ mice (Figure 4D) hyphae were also present.

A consequence of the delayed neutrophil recruitment seen in CXCR2$^{-/-}$ mice was an increase in the percentage of germinated conidia observed in BALF, relative to normal mice, 6-48 h after inoculation with *A. fumigatus* conidia (Figure 5A). The percentage of germinated conidia in CXCR2$^{-/-}$ mice peaked at 6 h. At 48 h in both normal and CXCR2$^{-/-}$ mice the conidial forms of the fungus were scarce, however, in CXCR2$^{-/-}$ mice, the percentage that had germinated had increased. In gp91$^{phox\ -/-}$ mice, which lack a functional NADPH oxidase, germination was found in PMN aggregates but not in AM (Figure 5B).

Hyphae in Lung Sections of CXCR2$^{-/-}$ Mice

Lung sections stained with PAS indicated PMN recruitment and engagement of conidia similar to that observed in BALF. In normal mice, PMN aggregates containing numerous pink-staining conidia were present in the alveoli by 6 h (Figure 6A) whilst in CXCR2$^{-/-}$ mice at this time there was minimal PMN infiltration and hyphal development was evident (Figure 6B) suggestive of the onset of aspergillosis. GMS staining produced similar results (data not shown).

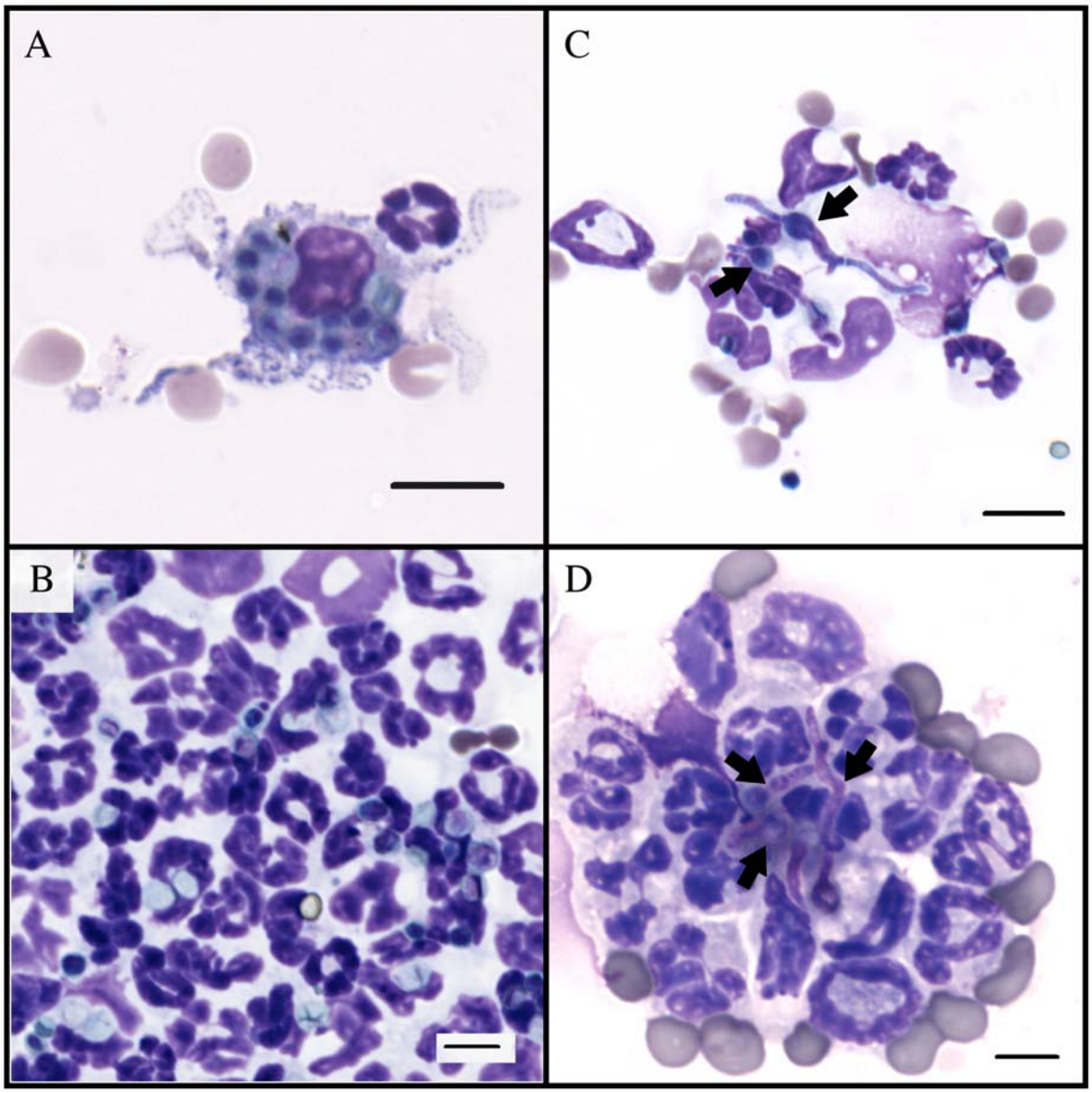

Figure 4. Pulmonary phagocyte association with *A. fumigatus* conidia in Wright stained BALF. Photomicrographs obtained from cells in BALF 6 hours after inoculation with conidia. (A) Individual AM from normal mouse with numerous engulfed conidia; (B) PMN aggregates from normal mouse containing numerous swollen and resting conidia, but no hyphae; (C) PMN aggregates from CXCR2$^{-/-}$ (D) and gp91$^{phox\ -/-}$ mice containing hyphae. Scale bars: 10μm

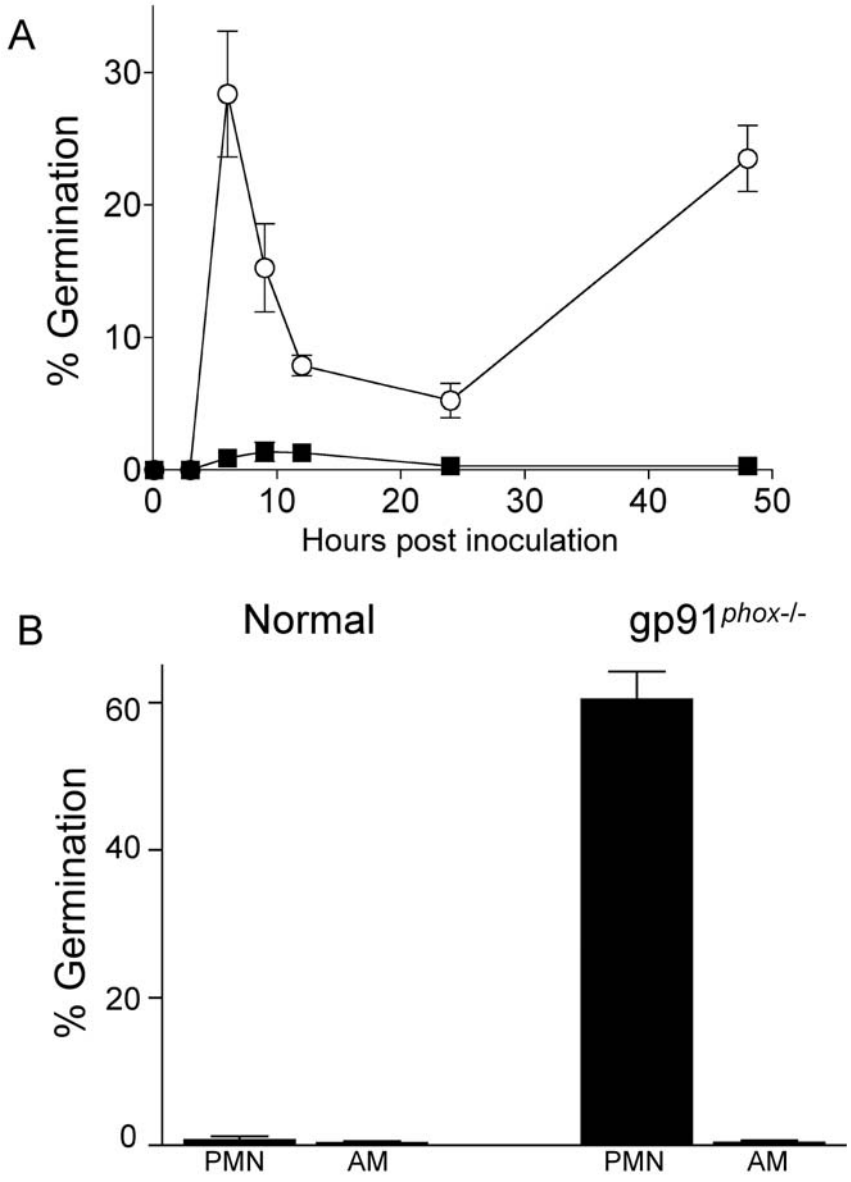

Figure 5. Quantitative analysis of germinating conidia in BALF. (A) The delayed PMN recruitment in CXCR2$^{-/-}$ mice (○) resulted in significantly higher percentages of germinated conidia than those found in normal mice (■) ($P<0.05$). Means ± SE (n=200). (B) PMN from gp91$^{phox\ -/-}$ mice were incapable of preventing germination. Means ± SE (n=200). AM from gp91$^{phox\ -/-}$ mice and AM and PMN from normal mice were associated with very few germinating conidia.

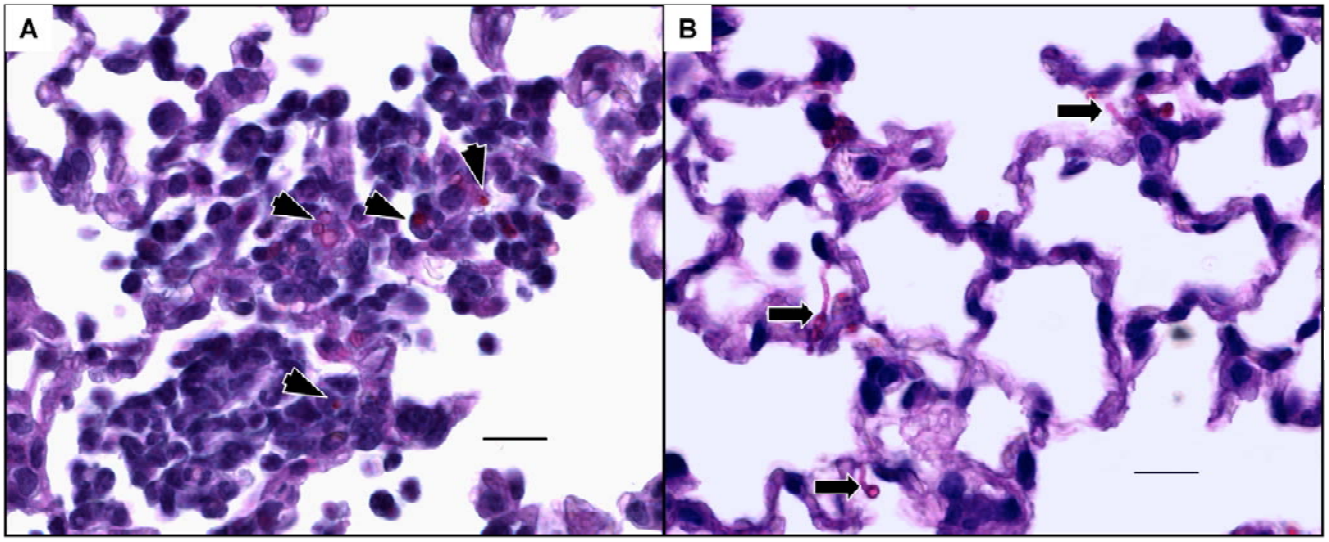

Figure 6. Pathological changes in the lungs of normal and CXCR2$^{-/-}$ mice 6 hours following inoculation with conidia of *A. fumigatus*. (A) Lung sections taken 6 hours following inoculation of normal mice with conidia and stained with PAS showed PMN accumulation around conidia (arrowheads). (B) Absence of PMN and presence of hyphae (arrows) were evident in CXCR2$^{-/-}$ mice. Scale bars: 20μm.

Cytokine Profiles of BALF

Levels of cytokines in BALF immediately after inoculation with *A. fumigatus* conidia were low (Figure 7): 142±35 pg/ml for TNF-α, and <10 pg/ml for IL-6, and four other cytokines, IL-10, IL-12 MCP-1 and IFN-γ. In normal mice, 3 hours after exposure to conidia there were 3.5 and 47 fold increases in the concentrations of TNF-α and IL-6, respectively, relative to time 0 ($P<0.05$). Concentrations of these cytokines in BALF of normal and CXCR2$^{-/-}$ mice were not significantly different immediately or 3 h after exposure to conidia. However, at 6, 12, 24 and 48 h, TNF-α and IL-6 levels were higher in CXCR2$^{-/-}$ mice than normal mice with increases in TNF-α being most marked ($P<0.05$). Concentrations of the other 4 cytokines were low or undetectable in both normal and CXCR2$^{-/-}$ mice from 0-12 h. However, in CXCR2$^{-/-}$ mice between 12 and 24 h, levels of IFN-γ, IL-10 and IL-12, but not MCP-1, increased significantly ($P <0.05$) and at 24 h, were significantly higher than in normal mice. At 48 h, only levels of IL-12 were significantly higher in CXCR2$^{-/-}$ mice compared to normal mice (data not shown).

ROS in PMN Aggregates

To examine NADPH oxidase activity of PMN and AM *in vivo*, BALF samples were obtained from gp91$^{phox\ -/-}$, CXCR2$^{-/-}$ and normal mice 9 h after inoculation. They were then probed with nitroblue tetrazolium (NBT), which results in the conversion of the soluble yellow NBT to insoluble blue formazan at the site of oxidant production [12].

Low background levels of formazan deposition were found on phagocytes that were not in association with conidia. There was marked formazan deposition on PMN-

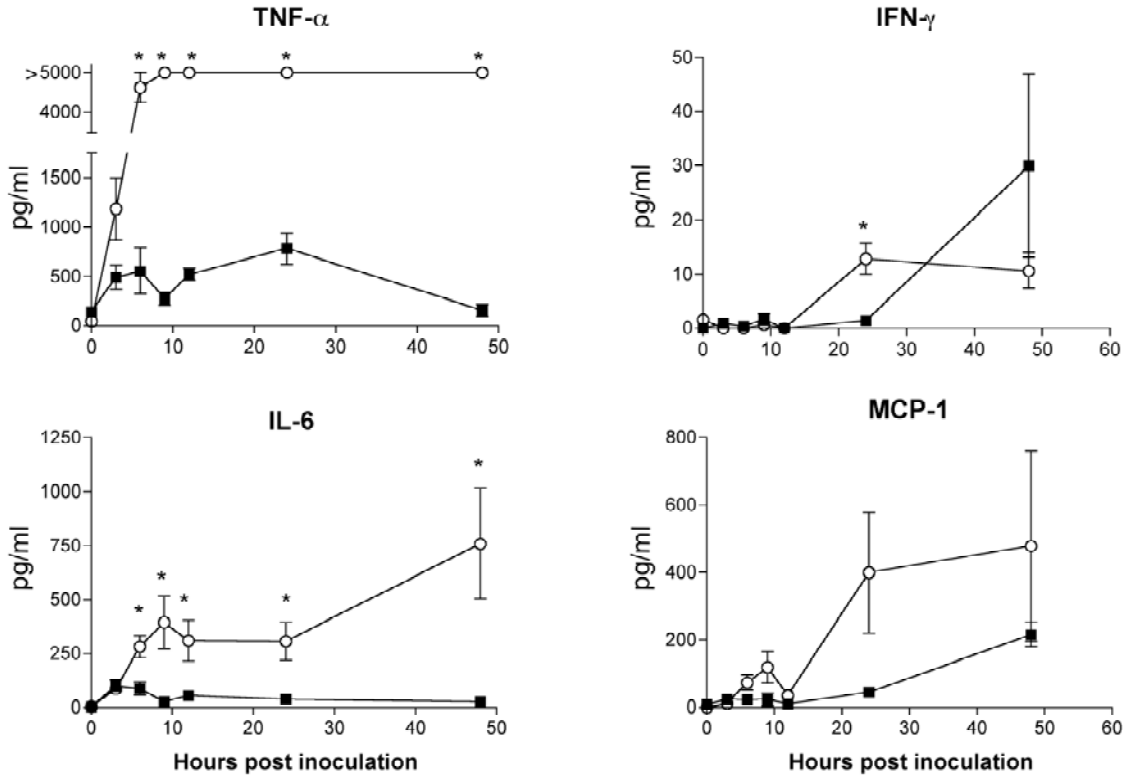

Figure 7. Analysis of cytokines in BALF in normal and CXCR2$^{-/-}$ animals following inoculation with conidia of *A. fumigatus*. Data points represent average percentage values (± SE) for groups of 5 mice obtained using a proinflammatory cytokine assay (* indicates a significant difference between normal and CXCR2$^{-/-}$ cytokine concentrations, P <0.05).

conidial aggregates in BALF obtained from normal and CXCR2$^{-/-}$ mice, but not in those from gp91$^{phox\ -/-}$ mice (Figure 8A and B, and C, respectively). Thus, normal and CXCR2$^{-/-}$ mice produce aggregates capable of generating reactive oxidants but despite this,

germination occurred in the CXCR2$^{-/-}$ mice (Figure 5A) presumably during the delay in PMN recruitment. Very rarely was formazan deposition found around conidia within AM, and then only in modest amounts. The intensity of the signal in AM may have been impaired by conidial inhibitors of AM-derived oxidants as suggested [24]. Since these experiments did not allow quantitative comparisons to be made between normal and CXCR2$^{-/-}$ mice regarding their oxidant-generating capacity, cytochrome c reduction by LPS-recruited PMN was also studied. BALF collected 9 hours following aerosol exposure to LPS contained cell concentrations and PMN:AM ratios (about 5:1 respectively) essentially the same as those seen 9 hours following inoculation with conidia (data not shown). However, using double blind examination of cytospin slides (n=14) under 200x magnification (which did not allow fungal elements to be identified), no neutrophil aggregates were found in BALF following inhalation of LPS but were consistently found following inhalation of conidia. LPS-recruited phagocytes from CXCR2$^{-/-}$ and normal mice showed indistinguishable superoxide production, whereas negligible amounts were found in mice lacking the gp91^{phox} gene required for oxidase activity (Figure 9). AM from normal mice were found to produce insignificant levels of superoxide by this method (data not shown).

To determine whether human PMN respond to *A. fumigatus* conidia in a manner similar to that observed for murine alveolar PMN, human peripheral blood was collected

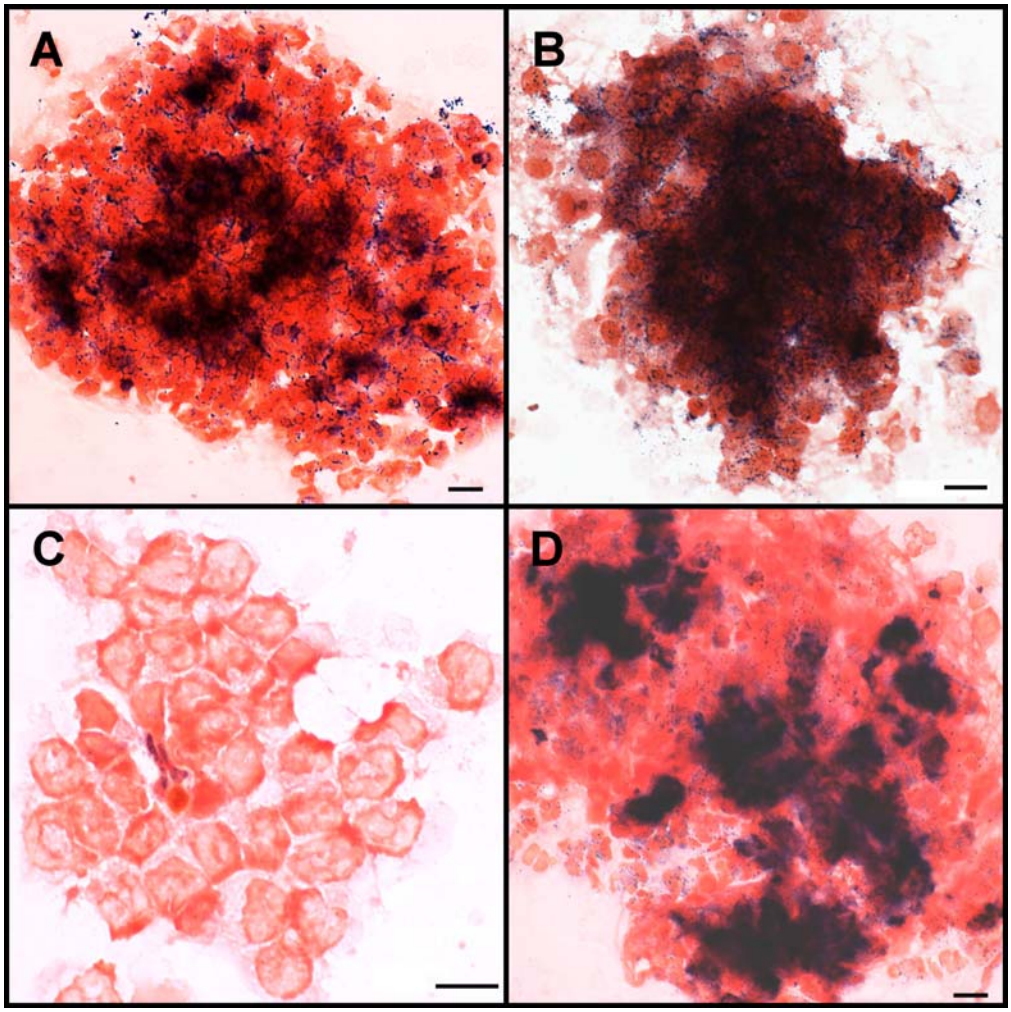

Figure 8. Formazan deposition on leukocytes of BALF. Conidia-PMN aggregates stained for oxidase activity (shown in blue): A) normal, B) $CXCR2^{-/-}$ and C) $gp91^{phox-/-}$ mice, 6 hours following inoculation with conidia, and D) human peripheral blood PMN incubated *in vitro* with conidia. Scale bars: 10μm.

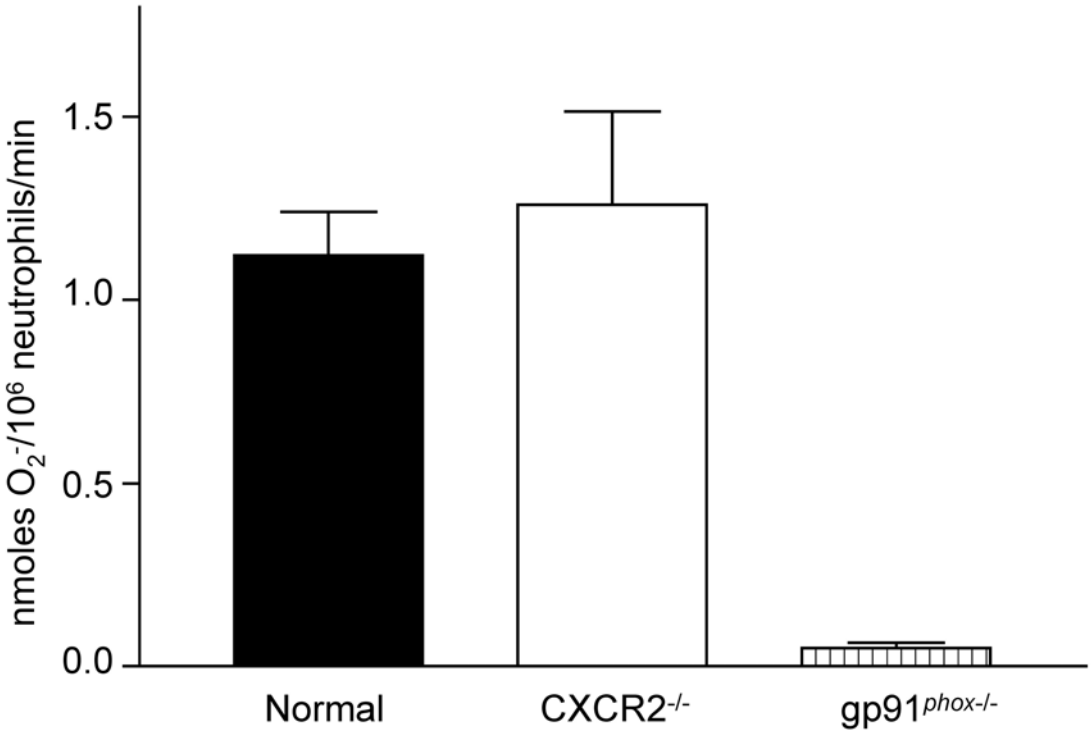

Figure 9. Superoxide generation by LPS-recruited PMN from normal, CXCR2$^{-/-}$ and gp91$^{phox-/-}$ mice assessed by PMA-stimulated cytochrome *c* reduction. Average values for cytochrome *c* reduction (±SE) are based on two kinetic analyses per animal, with at least 4 animals per group, with gp91$^{phox-/-}$ mice used as a negative control. from donors and PMN were prepared and reacted with *A. fumigatus* conidia. It was found that human PMN (Figure 8D), and LPS recruited murine PMN, formed oxidase-active aggregates around conidia *in vitro* (n = 6, data not shown).

Since germination of conidia occurred in CXCR2$^{-/-}$ mice in the presence of competent PMN NADPH-oxidase activity, and could only be linked to delayed PMN arrival, PMN recruitment and conidial germination was examined in gp91^{phox}-deficient mice 6h after inoculation. PMN recruitment and aggregation in gp91^{phox} animals was similar to that seen in normal animals (data not shown), but well developed hyphae were often found emerging form such PMN aggregates (Figure 4D). Thus in gp91$^{phox-/-}$ mice, there was a clear relationship between lack of PMN NADPH oxidase activity and

conidial germination. Importantly, despite inability to generate ROS by NADPH oxidase, no intracellular germination was observed in AM of gp91$^{phox\ -/-}$ mice (Figure 5B). This supports previous reports indicating that AM are capable of killing *A. fumigatus* conidia independently of NADPH oxidase [25,34].

Discussion

Important roles for PMN and NADPH oxidase function in immunity to *A. fumigatus* are indicated by the susceptibility to IPA of individuals with neutropenia [29] and also for those suffering CGD, an inherited disease involving a defective phagocyte NADPH oxidase [11,25]. A commonly accepted view is that the main role of PMN in the protective innate response to *A. fumigatus* is to engage hyphal elements that have escaped killing by the resident alveolar macrophages [33]. However, experimental evidence identifying the extent and nature of PMN involvement as well as NADPH oxidase function in aspergillosis immunity is contradictory [13,14,25,31,34].

One possible mechanism in the mammalian immune system for preventing IPA is through PMN-mediated inhibition of conidial germination [37], which would account for the broad lack of hyphal formation and *A. fumigatus* infections in immunocompetent humans and animals. This mechanism is supported by the present study which compared the protective innate response of normal mice with those of CXCR2$^{-/-}$ and gp91$^{phox\ -/-}$ mice, both of which are susceptible to fatal IPA [20,28]. The protective immunity observed in normal mice was associated with rapid PMN recruitment to the lung following exposure

to conidia, the formation of oxidase active PMN aggregates around conidia, and arrest of conidial germination. Aggregation was not due to lavage with HBSS *per se* since LPS-induced PMN recruitment was not associated with formation of PMN aggregates even though when PMN from the BALF were subsequently exposed to conidia *in vitro* they formed oxidase active aggregates with the fungus. In normal mice there was no significant concurrent recruitment of blood monocytes to the lung following inhalation of conidia, indicating that resident AM, together with recruited PMN, were capable of handling the relatively heavy inoculum used in this study.

Both CXCR2$^{-/-}$ mice and mice with immunoneutralized CXCR2 are known to be susceptible to fatal aspergillosis [20,35]. Our data confirm that CXCR2$^{-/-}$ mice demonstrate a significant delay in PMN recruitment, and show that the delay allows profuse conidial germination and hyphal development, an early pre-requisite for invasive aspergillosis, despite subsequent production of oxidase-active aggregates. A similar association of delayed PMN response with germination and hyphal proliferation has been found in a corticosteroid-induced model of aspergillosis [5], although other effects of corticosteroids [8], such as suppression of oxidant generation, cannot be excluded as contributing to susceptibility. The similarity of NADPH oxidase activity in normal and CXCR2$^{-/-}$ mice, shown both by cytochrome c reduction and formazan staining, indicates the competency of CXCR2$^{-/-}$ leukocytes with respect to oxidant generation. Early PMN recruitment and conidial engagement thus appear to be decisive elements in preventing aspergillosis by blocking conidial germination. The data clearly shows that AM alone cannot prevent germination from occurring.

Susceptibility to IPA has been reported in gp91$^{phox\ -/-}$ mice following administration of an inoculum containing only 52 conidia [25]. The severe susceptibility to IPA of gp91$^{phox-/-}$ mice, which lack NADPH oxidase activity, has lead to their use as a model of human CGD [11,25]. Our data confirmed that following instillation of conidia, germination occurs in gp91$^{phox\ -/-}$ mice, and showed additionally that hyphae developed within PMN aggregates by 6 h. Our data also indicated that aggregates in the gp91$^{phox-/-}$ mice, unlike those from normal mice where germination was arrested, were devoid of formazan deposition. Because of recent interest in the role of AM NADPH oxidase in killing *A. fumigatus* conidia [30], we also examined the ability of AM to inhibit germination in gp91$^{phox-/-}$ mice. We were unable to detect significant ROS production in AM of any mouse models examined in this study, but also found that after phagocytosis of conidia they very rarely contained germinating conidia, confirming previous reports that AM possess oxidant-independent anti-fungal activity [25,34]. Such activity was, however, by itself unable to prevent hyphal development in the lungs. We therefore suggest that in human CGD patients, failure of PMN, rather than failure of AM to produce ROS may be most important in susceptibility to IPA.

It has been shown that neutralization of TNF-α leads to susceptibility to IPA [21], and that the protective effect of TNF-α is due in part to induction of MIP-2 by AM [27], which then signals via CXCR2 for PMN recruitment to the lungs. We examined the concentration of TNF-α in BALF following inoculation with *A. fumigatus* conidia. In normal mice, we found a link between an early increase in TNF-α levels, PMN recruitment and absence of hyphae. For example, PMN recruitment to the lungs 3 hours

after inoculation with *A. fumigatus* conidia was associated with a 3-fold ($P<0.05$) increase in TNF-α in the BALF compared to the 0 hour time point, and arrest of germination. However, at this time interval in CXCR2$^{-/-}$ mice, there was a 27-fold increase in TNF-α, whilst the number of PMN in the BALF was reduced in comparison to normal animals. TNF-α levels remained significantly higher in CXCR2$^{-/-}$ than in normal mice for the remainder of the 48 hours after exposure to conidia, which corresponded to presence of hyphae in the lungs. It is thus possible that the higher levels of TNF-α found in the CXCR2$^{-/-}$ mice is indicative of a differential phagocyte response to conidia and hyphae, since macrophage cell lines produce more TNF-α when exposed to the hyphal rather than the yeast form of *Candida albicans* [7]. The association of increased TNF-α levels in CXCR2$^{-/-}$ mice with the presence of hyphae suggests these increased levels can be used as an indicator of aspergillosis progression [5]. IL-6 also has a role in PMN recruitment and levels in BALF were increased early following exposure of the lungs to conidia, with highest levels in CXCR2$^{-/-}$ mice.

Lack of early monocyte recruitment to the lung after exposure to conidia correlated with low or undetectable BALF levels of the macrophage-activating cytokines IFN-γ and IL-12, the macrophage recruiting chemokine MCP-1, and IL-10, which is an immunosuppressant and anti-inflammatory cytokine, from 0 to 12 h. Thereafter, increases in IFN-γ, IL-10 and IL-12 occurred 21 and 18 h after increases in levels of TNF-_ and IL-6, respectively. They were then higher in CXCR2$^{-/-}$ mice compared to normal mice, possibly due to the presence of hyphae as explained earlier. The cytokine data, together with the observed stability in the number of AM in BALF for up to 48 h following

inhalation of conidia, indicates that the cytokine response in the early stages following exposure to *A. fumigatus* conidia is directed toward recruitment and activation of PMN rather than AM.

Previous *in vitro* studies suggest that resting conidia are particularly resistant to PMN-derived antimicrobials [15,16]. Formation of PMN aggregates may be a mechanism to overcome this resistance, by generating sufficiently high local concentrations of antimicrobial factors such as ROS to kill the sequestered conidia. Our observations that both resting and swollen conidia are found within PMN aggregates (as shown in Figure 4B) indicates both forms are engaged by PMN, although the swollen forms may be more susceptible to killing [15,16].

Of clinical interest was the observation that human PMN suspended in HBSS aggregated around conidia within 30 minutes and similarly showed formazan deposition due to oxidant release. Thus, PMN aggregation is neither specific to BALF samples nor to mice, but may be a conserved antifungal response which we propose is vital for innate immunity to *A. fumigatus*.

Our data support a model of aspergillosis immunity whereby AM rapidly engulf some conidia and secrete proinflammatory cytokines to recruit PMN that then play a key role in preventing germination by formation of oxidase-active aggregates with conidia. It appears that it is essential for this sequence of events to occur within 6 h following conidial exposure to prevent hyphal emergence. If this PMN response is not developed within this time frame, ensuing conidial germination greatly increases risk of IPA.

In summary, this study provides the first *in vivo* evidence to indicate that: 1) when conidia reach the lungs in immune competent mice, early cytokine secretion promotes PMN recruitment, activation and formation of PMN aggregates with conidia that in turn arrest germination before swollen conidia can develop into hyphae, 2) production of oxidants by the NADPH oxidase of PMN within aggregates is a major mechanism for protecting the lung against aspergillosis. Future studies will be needed to better define the relationship between PMN and AM in aspergillosis immunity, to describe the mechanisms whereby PMN aggregates are formed, and to find out how conidia accumulate within such aggregates.

Materials and Methods

Conidia Preparation, Chemicals and Reagents

A clinical isolate (ATCC #13073) of *A. fumigatus* was obtained from ATCC and reconstituted according to the supplier's protocol. Conidia were grown at 37° C for five days on a Sabouraud Dextrose agar slant, collected in 0.025% Tween 20 by gentle rocking [4], filtered through tissue paper to remove hyphal forms, counted by hemocytometer, then adjusted to 3×10^8 conidia/ml in Hanks Balanced Salt Solution (HBSS, BioWhittaker #10-547F). Wright stain (Diff-Quik #B4132-1A) was obtained from Dade Bering (Newark, DE) and other reagents were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise indicated.

Animals and animal handling:

CXCR2$^{-/-}$ and gp91$^{phox-/-}$ were obtained from Jackson Labs and reared at the Animal Resource Center at Montana State University. CXCR2$^{-/-}$ and gp91$^{phox-/-}$ mice are based on Balb/c and C57Bl/6J backgrounds, respectively. Normal Balb/c animals were used for comparison. All animal manipulations were approved by the institutional internal review board for IACUC. Animals were used at 8 to 13 weeks of age, housed in microisolator cages and provided with water and chow *ad libitum*. Following brief isofluorane inhalation, mice were inoculated with 3 x 10^7 conidia in 100µl HBSS intratracheally (by oral access) for a time course analysis, or in 40µl HBSS intranasally (for oxidase-activity assays). Control animals received equal volumes of HBSS alone. Mice were returned to the cages for specified times following inoculation (0, 3, 6, 9, 12, 24, and 48 hours), then euthanized by lethal i.p. injection of sodium pentobarbital. Following euthanasia, BAL was performed by perfusion of lungs with five 1 ml volumes of ice cold HBSS supplemented with 3 mM EDTA, and the lungs removed for histology. Lungs were routinely stained with Hematoxylin and Eosin, and Periodic Acid-Schiff and some lungs were also stained for fungal elements with Gomori's methanamine-silver (GMS). Animal groups were: normal inoculated mice (n=5 for each of 7 time points), CXCR2$^{-/-}$ inoculated mice (n=5 for each of 7 time points), gp91$^{phox-/-}$ mice (n=5, for 6 and 9 h time points) and normal mice exposed to HBSS (n=3, 24 h time point only).

Cell Counts and Evaluation of Host-Pathogen Interactions

Immediately after bronchoalveolar lavage, live cell counts were obtained by hemocytometer using trypan blue exclusion. One hundred μl of each lavage sample was then subjected to cytospin preparation and Wright stained to quantify leukocyte subsets recruitment to the lung and conidial germination (n = 200 cells). Ungerminated conidia (n=200) were also categorized as either: 1) free and extracellular (not obviously associated with immune cells), 2) phagocytosed by AM, or 3) associated with PMN. Microscopic examinations were performed on a Zeiss Axioscope 2-Plus microscope and imaging system using Zeiss Axoivision version 4.1 software.

Cytokine Analysis of BALF Samples

Cytokine concentrations (TNF-α, IL-6, IL-10, IL-12, IFN-γ, MCP-1) were determined from BALF supernatants, which had been frozen at -20° C for 1-2 weeks and thawed, using the Mouse inflammation kit (BD Biosciences, cat. #552364, San Diego, CA).

Superoxide Generation by BALF Leukocytes

Detection of phagocyte-derived superoxide was performed on cells from fresh lavage combined with an equal volume of DPBS (Sigma #D-5773) in the presence of 500 μM nitroblue tetrazolium (NBT, Sigma #N-6876) and incubated for 30 minutes at 37° C [32]. Slides were prepared by cytospin and counterstained with safranin.

Superoxide Generation by LPS-elicited Phagocytes

Pulmonary phagocyte recruitment was elicited in the lungs of mice by exposing them to nebulized lipopolysaccharide from *E. coli* (LPS, 50 μg/ml, Sigma #EC-9637) in an aerosolization chamber for 20 minutes. Mice were then returned to cages for 9 hours prior to BALF collection as described above. Cells in the BALF were counted and then pelleted at 850 x g for 10 minutes at 4° C and resuspended at 5 x 10^7 cells per ml in HBSS. Ten μl of the suspension (5 x 10^5 cells) were combined with 200 μl of 200 μM cytochrome *c* (Sigma #C-7752) in assay buffer (47 mM NaH_2PO_4, 18 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM EGTA, and 2 mM NaN_3) in individual wells of a 96 well plate [3], then activated by adjusting the solution in each well to 910 nM phorbol myristate acetate (PMA, Sigma #P-8139) [26]. The superoxide generation rate was then measured as the reduction of cytochrome *c* over the time period where there was maximal oxidase activity, and the signal was quantified using $\Delta\varepsilon_{550} = 21$ (cm mM)$^{-1}$ [18], blanked against representative wells containing 310 U/ml superoxide dismutase (Sigma #S-2515).

In Vitro Interactions Between PMN with Conidia

Human peripheral blood was collected from donors according to NIH guidelines, and PMN separated from citrated blood using Mono-Poly reagent (ICN cat #16-980-49) according to the manufacturer's specifications. PMN were incubated at 37°C in HBSS for 30 minutes with an equal number of conidia, then processed as described above using NBT to detect superoxide generation in murine BALF leukocytes following conidial

inoculation. LPS recruited murine neutrophils were similarly incubated with conidia *in vitro*.

Statistical Analysis

Means are given ± SE. Two-tailed t-tests assuming unequal variances were used to test for significance of differences between means.

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CHAPTER 3

CONCLUSIONS

In Vitro Measure of Conidial Killing

Observation and Problem

Our *in vivo* results suggest that neutrophils are able to kill *A. fumigatus* conidia. *In vitro* attempts to confirm these results using phagocytes from mouse lungs were unconvincing because of a lack of reproducibility. This observation indicates that a variety of factors exist in the lung which are difficult to mimic in culture. Published evidence illustrating the inconsistencies of *in vitro* conidial killing confirmed our observation [8]. These experiments indicated the necessity for robust and reproducible means of measuring, *in vitro*, the conidiacidal activity of phagocytes and chemotherapeutic agents.

Fundamental to the analysis of fungicidal mechanisms by *in vitro* methods is standardizing the measured output. The colony forming unit (CFU) assays are a common approach to measuring fungicidal effects of various forms of organisms including *A. fumigatus* [9-11]. An advantage in testing the CFU as an output is that such an assay, when correctly performed, gives meaningful information about the ability of the system to kill the fungus, which is often of importance in immunologic studies. *In vitro* techniques have been shown in some situations shown to be reliable. These include when *A. fumigatus* conidiacidal effects are measured prior to conidial germination, and when antimicrobial

chemotherapeutics tested *in vitro* [13]. However, utilization of phagocytes that deliver the fungicidal mechanisms is dependent upon providing standardized sources and capabilities of the phagocytic cells themselves, which appears to present a complex problem.

The difficulties of the *in vitro* assays to show phagocyte-mediated fungal killing data similar to those we encountered may have contributed to what appears to be conflicting data in the literature [4,5]. Thus, arriving at a consensus about the conidiacidal ability of phagocytes, and to be sure, a mechanism of a particular phagocyte type in this response, might be attributed in part to the lack of *in vitro* corroboration of the observations made *in vivo*. The heterogeneity in these studies is likely a result of different experimental techniques, varying sources of phagocytes, qualities of cells collected, various media within which reactions were tested, quantification methods, incubation times, and characteristics of the organism that obscure more conventional means of obtaining data of organism killing.

One concern about fungicidal effects using colony forming assays is that exaggerated counts can be obtained when hyphal structures fragment. The fragments can give multiple counted events that overestimate the amount of pathogen. Therefore, this type of study involves a difficult problem in quantifying of fungicidal effects upon the mycelial form of the fungus. Such observations underscore the importance of establishing careful quantification of the organism input, and of equal importance, a standardization of the colony forming unit.

Measurement of Conidiacidal Effects

Clearly, difficulties exist in obtaining reliable output numbers when fungal hyphae are measured as mentioned above. However, observing the fungicidal effects upon the conidial form of *A. fumigatus* is both easier and more reliable. This fact is pertinent in the studies described in this thesis, because the focus of the thesis has been devoted to the handling of the conidium by the immune system, rather than the hyphal form of the fungus.

Viability stains such as propidium iodide and FUN-1 [1,7] also offer advantages in the measurement of conidiacidal activity, particularly in high-throughput population surveys by flow cytometry analysis. However, cross-reactivity with hyphae, as observed with PI, and difficulty of delivery for other fluorescent dyes such as FUN-1 intracellularly impart some limitations with these approaches, especially when dealing with aggregates of leukocytes similar to those we observed both *in vivo* and *in vitro* (Chapter 2).

In Vitro Measurements of Conidial Germination

Observing inhibition of germination, rather than conidial growth on solid medium as a measure of killing, offers utility in evaluating immune pressure on the fungus, because it does not require isolation and recovery of intracellular organism, and presents lower variability within sample groups [6,12]. Since scoring outputs are obtained by microscopy rather than by culture, this method is suitable for examination of the conidial form of the fungus. Therefore, controls must be utilized that accurately measure the

degree of germination in samples of conidia that are not exposed to pressures from the immune system, and such values must then be related to such test values. This practice has the advantage of correcting the values obtained for variations in batches of conidia used, although the germination event is often unambiguous by microscopy. One drawback of scoring conidial germination is that it is not possible to differentiate inhibition of germination from conidial dormancy and conidial death.

Novel Approach to Measure Conidiacidal Effect of Phagocytes

In light of encountered difficulties that conventional methods assaying fungicidal mechanisms by in vitro methods have, a new approach to obtain *in vitro* measurements of *A. fumigatus* killing was undertaken using a modified isolate of *A. fumigatus* bearing a fluorescent marker showing sensitivity to phagocyte-derived microbicidal products. A strain of *A. fumigatus* that constitutively expresses green fluorescent protein (GFP) in both its conidial and hyphal stages was obtained from David S. Askew[2], of the University of Cincinnati. Human peripheral blood experiments with this strain of *A. fumigatus* (*Af*-GFP) indicated fluorescence intensities of the conidia were reduced when engaged by neutrophils. These preliminary results suggested the GFP signal might be used as an endogenous marker of conidial viability.

Our previous studies indicated that hydrogen peroxide (H_2O_2) and hypochlorous acid ($HOCl^-$) are potent mediators of conidial killing (data not shown), and both are components of the neutrophil antimicrobial function. To test this hypothesis that GFP fluorescence could be used as a marker of conidial viability, concentration-dependence of

certain oxidant solutions were performed to simultaneously examine fluorescence and CFU measurements, both as a function of exposure to concentrations of certain oxidant solutions.

A Fluorescence-based Method of Determining *In Vitro* Conidial Killing

Af-GFP (1.4 x10^7) were incubated with serial three-fold dilutions of H_2O_2 (9%-0.1%) and water in 200 μl. After 10 minutes, 100 μl of the mixture was aliquoted and subjected to serial dilution for CFU counts. The reaction was stopped by the addition of 3 ml dH_2O to each tube. Triplicates samples were then analyzed by FACS and the mean fluorescence index (MFI) of each sample population was obtained. To determine the viability of the conidia, 10^{-3} and 10^{-4} dilutions of each sample were made and 3-100 μl aliquots per sample were mixed with 5 ml LB soft agar and inoculated on LB agar plates. The plates were then incubated at 37°C for 36 hours, until colonies could clearly be counted on the control sample which had been exposed to water only. Viability was determined as the percent CFU in the oxidant-treated sample relative to the control treatment. Plates were incubated an additional length of time (3 days) to confirm that damaged organisms did not recover more slowly. Similarly, experiments examining *Af*-GFP conidia exposed to H_2O_2 conidia were also incubated with $HOCl^-$ for ten minutes at three-fold serial dilutions starting from 0.9mM to 0.001mM. Aliquots were then examined as described above.

Results.The data relating percent killing by both $HOCl^-$ and H_2O_2 to fluorescence intensity indicate a strong correlation of the GFP signal to viability (Figures 10A and B). Conidia exposed to dH_2O-only showed no killing (Figure 10A, black bar, measured on the left axis) and high MFI values of 75 (green bar, right Y-axis). In contrast, conidia subjected to 9% H_2O_2 showed 100% killing and only background MFI values. More dramatic results were obtained by examining the effect of $HOCl^-$ exposure on conidial viability and fluorescence (Figure 10B), with the correlation of GFP signal (low MFI) to killing clearly visible. While triplicate MFI values were not measured in this experiment, the histogram of the fluorescence signal measured in the FL1 channel of the flow cytometer (Figure 10C) gives the values for the MFI for all the samples. The well-demarcated minimum killing concentration of 0.01 mM $HOCl^-$ observed in Figure 10B identifies a defined endpoint in the GFP signal as well. This histogram also illustrates the biphasic nature of the GFP expression in the sample population, where some conidia are brighter than others. The lack of effect of $HOCl^-$ at the lower concentration was also observed. These findings indicate that there may be some residual low level GFP signal after the conidium is dead. More importantly, these data indicate that absence of GFP is clearly correlated with the death of the conidium, which is supportive of the utility of relying on the fluorescence as a marker of cell killing.

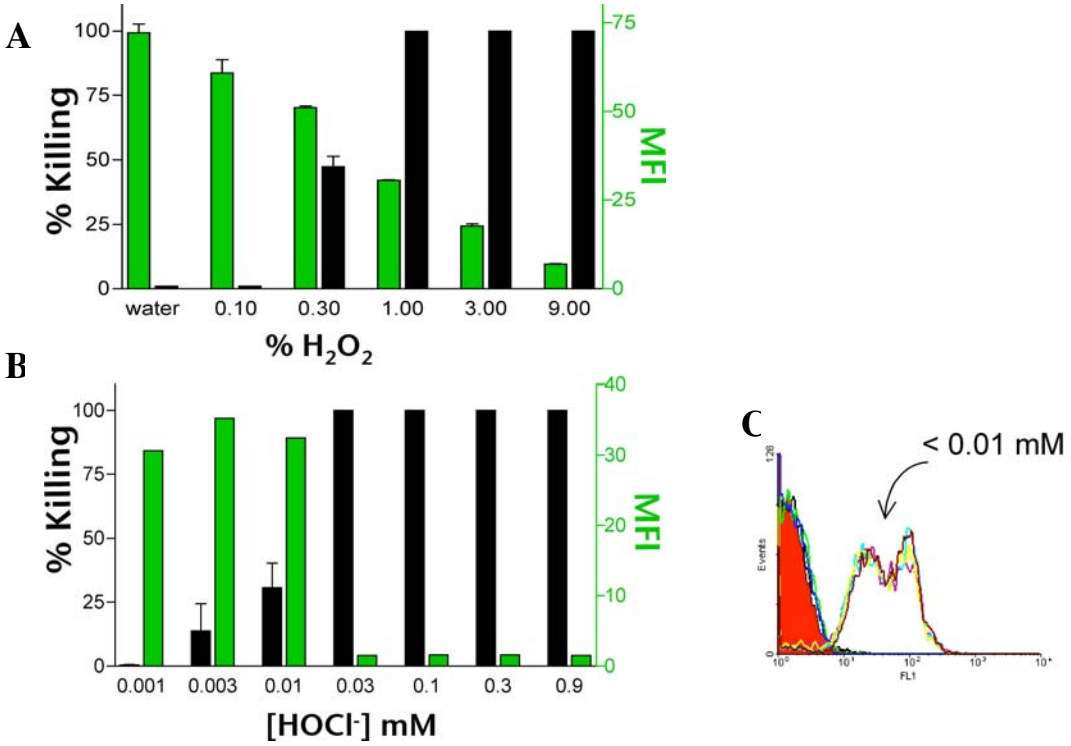

Figure 10. H_2O_2 and $HOCl^-$ mediated conidial killing and abolishment of GFP fluorescence in *Af*-GFP conidia. *Af*-GFP conidia were exposed to H_2O_2 and water (A) and $HOCl^-$ (B) for 10 minutes; the number of conidia that grew on triplicate plates relative to water-only treatment determined the % killed (black bars), indicated on the left Y-axis. Triplicate values of the mean fluorescent index (MFI; green bars) of each sample in the H_2O_2 treatment was determined by FACS analysis, and scored on the right Y-axis. Triplicate samples were not plated for the $HOCl^-$; instead, the histograms of the GFP signal for all the $HOCl^-$ samples are presented (C). Those samples positive for GFP are indicated by the <0.01 mM annotation, which indicates the uniformity of the histogram for those samples below 0.03 mM $HOCl^-$.

To determine if the phagocytes can produce similar effects of abolishing fluorescence of the GFP-labeled organisms, $3x10^7$ *Af*-GFP conidia were inoculated intranasally into four BALB/c mice. At 6, 9, 12 and 24 hours following inoculation,

BALF was collected and cytospins samples were prepared as described in Chapter 2. The amount of GFP-positive conidia within the neutrophil aggregates was counted and reported at the various time points (Figure 11A). By 6 h, approximately 45% of the *Af*-GFP conidia had lost their fluorescence, which did not change by 9 h. By 12 and 24 h, the neutrophil aggregates had further reduced the population of GFP-positive conidia to less than 25%. Representative images from each time point are shown in Figure 11B. Black arrowheads indicate several *Af*-GFP conidia that have lost their signal and the arrows identify conidia with GFP signal remaining.

These *ex vivo* results corroborate those describing the effects of H_2O_2 and $HOCl^-$ on abolishing the signal of *Af*-GFP conidia, which link fluorescence quenching and conidial killing. Based on this information, it may now be possible to verify the death of thee non-fluorescing conidia within the neutrophil aggregates. This method thus has the potential to serve as inhibition of germination for evaluating the result of the phagocyte effect on conidia as shown in Chapter 2. A further advantage of this method is that conidia within neutrophil aggregates can be evaluated independently of their recovery from such cells, a drawback in methods of evaluating these conidia by methods based on flow cytometry.

Though still not widely accepted or applied, the technique involving the GFP-labeled *A. fumigatus* has a number of promising applications in studying the effects of cell-specific engagement of conidia. The advantage of the possible endogenous marker of conidial viability is useful because it will now be possible to examine intracellular

conidia and conidia located within neutrophil aggregates to evaluate immune pressure on individual conidia.

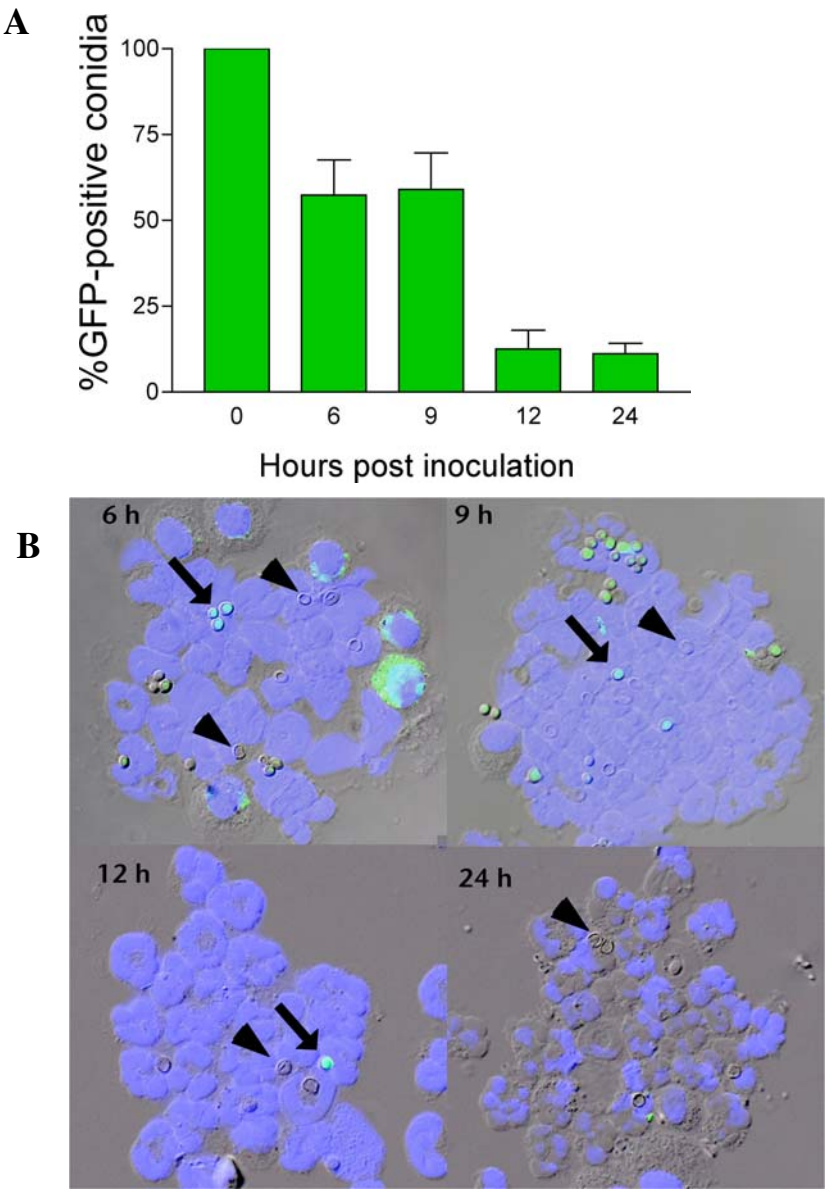

Figure 11. *In vivo* time course analysis of neutrophil-aggregate mediated abolishment of *Af*-GFP conidia. $3x10^7$ *Af*-GFP conidia were inoculated intranasally into four BALB/c mice, and the BALF was collected at 6, 9, 12 and 24 h. The percentage of conidia that were GFP positive within neutrophil aggregates was quantitated (A). Representative overlay-images (DIC, DAPI and FITZ channels) from each BALF are shown in (B), with GFP-positive conidia enumerated by arrow, and non-fluorescent conidia indicated by the arrowheads. Blue DAPI-containing mounting media was added for nuclear morphology.

While the use of the *Af*-GFP organism as an indicator of cell-mediated damage is still in its infancy, it appears to have promise. A reproducible and practical means of obtaining conidiacidal activity is needed and the use of this organism offers several benefits towards this end. Since the GFP signal is constitutively expressed in conidia and hyphae, then cell-mediated hyphal damage might also be measured in the same way. Tracking the diminishing signal of a single conidium also offers the possibility to measure the death of the conidium in 'real-time'. Moreover, having the endogenous marker enables the tracking of the fungus *in vivo*, without having to deliver exogenous labeling compounds to it and facilitates easy population based monitoring using flow cytometry.

Redefined Role of PMN in the Innate Response

The findings of this study offer significant deviations from the conventional model of the innate response to *A. fumigatus*, and introduce the following aspects of the role of the neutrophil in the protective response: (1) neutrophils engage conidia directly in the normal mouse, (2) neutrophils engage conidia and begin killing rapidly upon recruitment, (3) neutrophils use reactive oxygen species (ROS) to kill the conidia, and (4) neutrophils form ROS-rich aggregates in killing conidia.

These results observations of conidial germination in normal and immune-compromised mice support the following revised model of the innate response to *A. fumigatus*:

Protective immunity to *A. fumigatus* infection depends on preventing germination of inhaled conidia rather than arresting hyphal outgrowth. This conidiacidal activity is mediated by both the resident alveolar macrophage - through ROS-independent mechanisms, and recruited neutrophils through aggregate formation and ROS-dependent mechanisms.

Potential Importance of Aggregate Formation in PMN Biology

Subsequent experiments investigating the nature and specificity of the PMN aggregates that were observed in the mouse lung following inoculation with *A. fumigatus* conidia indicated that the aggregation event appears to be a generalized PMN response. Similar aggregates were observed in BALF taken from mice 9 h following inoculation with *Rhizopus oryzae* spores and also with 4 μm polystyrene beads. The findings of PMN aggregating in response to different fungi and inert particulates which were of similar size (2-5μm), indicate that this aggregation event is not specific to *A. fumigatus*, but potentially a generalized response by the recruited PMN to small particulate matter that finds its way into the lower respiratory tract. The open space of these compartments may allow formation of these aggregates, which may not be able form in other tissues.

Impact in *A. fumigatus* Arena

These findings support not only a revision of the way in which the innate response is thought to prevent germination and infection of the fungus, but also the importance of

the very early stages of conidial engagement by the phagocytes including neutrophils. This is important when considering the time stages in which patients at risk for *A. fumigatus* infections are monitored for the presence of this fungus. Also, the effect of corticosteroids on PMN may exacerbate the problem these neutropenic patients already have, thus shifting the balance towards susceptibility. These findings have potential clinical relevance because they provide substantial insight into the poorly-understood link between neutrophil deficiencies and predisposition to IPA.

The increase in TNF-α levels in the BALF, and its correlation with presence of hyphae may have significant clinical potential. Surrogate markers of disease progression are needed for early diagnosis, which is crucial in clinical management. Using TNF-α levels in BALF has previously been proposed as such a marker of disease progression [3]. The cytokine data presented in this thesis are supportive of this notion, and suggest TNF-α levels may offer rapid identification of tissue invasion by fungal infiltration in the lung.

Future Studies

The experimental results described in this manuscript establish the importance of the recruited neutrophil to engage and kill the conidial form of *A. fumigatus*. They also suggest this event is necessary to prevent hyphal proliferation and subsequent invasive pulmonary aspergillosis. However, the degree to which the experimental conditions relate to the nature of the susceptibility observed in human hosts is debatable on the grounds of the relatively high dose ($3x10^7$ conidia) used for the inoculations. While this is not an unreasonable exposure in certain occupational settings involving high exposure

environments, it is not expected that comparable doses would be seen in those hospitalized patients who are immunosuppressed and subsequently develop IPA. This raises the important question of whether the nature of the neutrophil-response described in this thesis is reserved for those circumstances of high exposures.

The extreme susceptibility of gp91$^{phox\ -/-}$ mice to *A. fumigatus* infection despite apparently normal macrophage killing strongly implicates the involvement of neutrophils under low-dose as well as high-dose exposure. Sequentially reduced conidial numbers in inoculations into normal mice followed by careful inspection of the BALF for neutrophil-aggregation events, may reveal the minimum level of exposure necessary to elicit the neutrophil recruitment and probably the aggregation event as well. Resolving the lower limits of exposure will become difficult however, due to the practical limitations of recovering conidia from the lung following inoculation with low ($<10^3$) numbers. Using the *Af*-GFP strain may be useful towards this end, in making it easier to detect organism-phagocyte contacts in the BALF and lung following low-dose exposure.

From a neutrophil biology standpoint, the formation of neutrophil aggregates in response to some level of conidia exposure may prove to be essential in aspergillosis immunity. How these events are coordinated, what adhesion and signaling molecules are involved that mediate the conidial-phagocyte and interphagocyte contacts, and which killing compounds (such as which reactive oxidants) are used are the pertinent and testable questions.

The use of the *Af*-GFP strain offers an opportunity for some new experimental approaches to some of the presently unanswered questions. For example, the timing of

AM-mediated conidial killing may be better answered with this strain. More experiments testing neutrophil aggregation in response to this strain may shed further light upon the nature of the aggregate mediated conidial killing. *In vitro* studies of the involvement of exogenous TNF-α in enhancing the conidiacidal potential of pulmonary recruited neutrophils, following LPS-stimulation and BAL, may improve the ability to carry out in vitro killing assays with greater reproducibility.

Some studies described in this thesis were carried out to examine neutrophil-mediated conidiacidal potential were carried out using unstimulated peripheral blood neutrophils. A more relevant *in vitro* killing assay can now be achieved using the *Af*-GFP strain to assess conidial killing, and recruited and activated alveolar neutrophils as the cell source. With these new additional approaches now available, important elements of the mechanisms involved such as cytokines, interactive molecules, and killing compounds, can now be studied more effectively.

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