Effector Mechanisms of Innate Leukocytes in Neutropenic Invasive Aspergillosis

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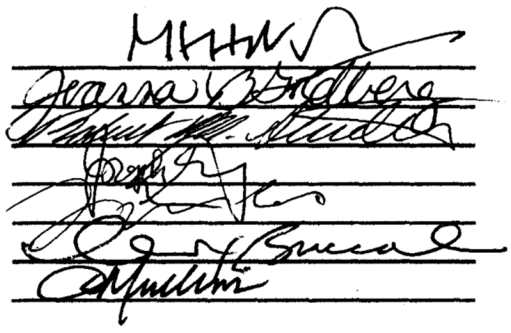

Abstract

Healthy hosts have evolved multiple layers of innate immune responses that readily clear inhaled *Aspergillus* conidia without the development of disease or acquired immunity to this organism. Defects in neutrophil function and numbers are considered to be the most pervasive risk factor for the development of the severe fungal infection, invasive aspergillosis. However, most patients with this infection exhibit complicated immune defects and the problem can extend beyond neutrophil deficiency. The current work supports the hypothesis that NK and dendritic cells are critical components of innate anti-fungal defenses in neutropenic hosts following pulmonary exposure to *Aspergillus*

In the lungs of neutropenic mice with invasive aspergillosis, NK cells mediate their protective effect by acting as the major source of IFN-γ during early infection. The absence of NK cells or IFN-γ contributed equally to increased fungal susceptibility and worsen outcome in neutropenic mice challenged with *Aspergillus*. NK-derived IFN-γ is an important anti-fungal defense mechanism as it led to enhanced antimicrobial effects of resident and recruited myeloid cells, and augmented the production of lung IFN-inducible chemokines that may mediate further recruitment of effector leukocytes.

A marked accumulation of immature lung myeloid DCs occurs in the lungs of neutropenic mice following *Aspergillus* challenge. This was associated with an augmented influx of monocytes from the blood to the lung and at the same time, reduced efflux of DCs to the draining lymph nodes. The rapid recruitment of DCs to the lung was attributable to greatly elevated lung TNF expression, resulting in lung expression of CCL2 and CCL20 which, in turn, mediated recruitment of TNF-producing myeloid DC. This important positive-feedback loop in the lung of neutropenic mice provided a protective and beneficial during the early phase of invasive aspergillosis since depletion of the cells resulted in a marked increased in the lung fungal burden. *In vitro* studies demonstrate that DC maturation require neutrophil contact and DC-SIGN expression. These data show that neutrophils may play an important in modulating the host dendritic cell response to *Aspergillus*.

For my family:

Dad, Mom, Jane and Ann; with love and much thanks.

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I offer my deepest and heartfelt gratitude to my family for the unconditional support they have provided for years. They have given me the strength and motivation to go that extra distance to ensure I perform my research at the highest level.

List of Abbreviations

ABPA - allergic bronochopulmonary aspergillosis

A.fumigatus - Aspergillus fumigatus

Ag - antigen

β-glucan - beta glucan

BMDC - bone-marrow derived dendritic cell

CCL - chemokine (C-C motif) ligand

CCR - chemokine (C-C motif) receptor

CD - cluster of differentiation

CFSE - carboxy-fluorescein diacetate, succinimidyl ester

CGD - chronic granulomatous disease

CXCL - chemokine (C-X-C motif) ligand

CXCR - chemokine (C-X-C motif) receptor

CX3CR - chemokine (C-X3-C motif) receptor

DCs - dendritic cells

DC-SIGN - DC-specific intercellular adhesion molecule-3-grabbing nonintegrin

DT - diphtheria toxin

ELISA - enzyme-linked immunosorbent assay

FITC - fluorescein isothiocyanate

FSC - forward scatter

GFP - green fluorescent protein

GR-1 - granulocyte differentiation antigen 1

IFN-γ - interferon-gamma

Ig - immunoglobulin

IL - interleukin

IP - intraperitoneal

IT - intratracheal

IV - intravenous

LD - lethal dose

mAb - monoclonal antibody

Mac-1 - macrophage 1

MDSCs - myeloid-derived suppressor cells

MHC - major histocompatability complex

MyD88 - myeloid differentiation primary response gene (88)

NK cell - natural killer cell

NKT cell - natural killer T cell

PARs - protease activated receptors

PBMC - peripheral blood mononuclear cell

PBS - phosphate buffered saline

PMN - polymorphonuclear

RAG-1 -recombinase activating gene -1

ROS - reactive oxygen species

SEM - standard error mean

SSC - side scatter

Th cell - T helper cell

TLR- toll-like receptor

TW - transwell

TNF-α - tumor necrosis factor alpha

VCAM - vascular cell adhesion molecule

WT - wild type

Chapter I Introduction

Innate Immunity to Aspergillosis

Aspergillus species are among the most common molds encountered by humans and are the etiologic agents for a remarkably diverse set of human diseases. With the exception of diseases caused by *Aspergillus*-derived mycotoxins, the host's response during its encounter with this microorganism is the key determinant in whether the host clears the microorganism without developing disease or whether the host is colonized by the microorganism, is infected by it, or develops a hypersensitivity illness as a result of the encounter. Despite constant exposure to *Aspergillus* conidia, it is remarkable that most humans do not develop any illness attributable to these organisms and have no evidence of antibody- or cell-mediated acquired immunity to this organism (1, 2). This suggests that, for most healthy humans, innate immunity is sufficient to clear the organism before acquired immunity is called upon.

The usual niche of *Aspergillus* species is in soil and decaying biomass. *Aspergillus* species grow as multicellular branching hyphae and reproduce asexually by means of aerial conidiophores; most *Aspergillus* species do not have a recognized teleomorph (sexual form). The reproductive spores, known as conidia, are produced in very large numbers and, by virtue of their small size and

hydrophobic exterior, remain airborne for hours once released. Resting conidia are metabolically quiescent and can remain viable for months. The development of new colonies begins with swelling of the conidia within hours of arriving in a permissive environment and is followed by the germination and subsequent elongation of hyphae.

The mean concentration of *Aspergillus* conidia in air is 0.2 to 15 conidia/m^3 according to different studies and is up to 10^6 conidia/m^3 in some agricultural settings (3). As a result, humans routinely inhale hundreds of conidia daily. Despite this constant exposure, it is remarkable that most humans do not develop any illness attributable to these organisms. Most human diseases caused by *Aspergillus* species begin in the respiratory tract. Among the >180 recognized *Aspergillus* species, *A. fumigatus* remains the most common cause of human disease; however, other species including *A. flavus*, *A. niger*, *A. nidulans*, and *A. terreus* are increasingly frequent causes of disease (4). The human diseases caused by these organisms are extraordinarily diverse and have been considered under three categories (Table 1): invasive infections, which are characterized by the growth of hyphae within tissues; infections caused by the colonization of mucosal surfaces without invasion into tissue; and hypersensitivity diseases, which are defined as diseases caused by the immune response of the host. Thus, while in the vast majority of healthy hosts the microorganism is

Table 1 Categories of human diseases caused by *Aspergillus* species

Category	Example(s) of specific diseases	Defect in host defense	Animal model
No disease		Healthy host	Intrapulmonary challenge of wild-type mice with conidia
Invasive infection	Invasive pulmonary aspergillosis, invasive rhinosinusitis, invasive tracheobronchial aspergillosis, and chronic cavitary pulmonary aspergillosis	Impaired cell-mediated immunity (including cell-mediated innate immunity)	Intrapulmonary challenge of immunocompromised animals with conidia (antibody-mediated neutrophil depletion, chemotherapeutic drugs, corticosteroids)
Colonization	Pulmonary mycetoma in preexisting lung cavities, asymptomatic (e.g., in bronchiectasis, chronic obstructive pulmonary disease	Impaired mucosal immunity	No established model to date
Hypersensitivity	Asthma, allergic bronchopulmonary aspergillosis, allergic sinusitis, and hypersensitivity pneumonitis (e.g., malt worker's lung)	Misdirected acquired immunity	Intrapulmonary challenge of sensitized mice with conidia

Data from ref (5)

cleared without causing any disease, the encounter between *Aspergillus* and the host can result in a broad range of diseases. Consistent with the damage-response framework of microbial pathogenesis (6, 7), colonization is distinct from commensalism and describes a form of infection that can result in continued low-level damage to the host and not a state of benign coexistence. The key determinant of the pathogenicity of *Aspergillus* species, and the reason for the diversity of host outcomes, is hypothesized to be the nature of the immune response of the host (6, 7) (Table 1), and the diseases may be conceptualized as corresponding to points along a spectrum of abnormal immune responses of the host (Fig. 1).

From an immunological perspective, the defense against inhaled conidia begins in the physical barriers of the respiratory tract (Fig. 2). These include the nasal turbinates and the branching pattern of the bronchial tree, which results in a highly turbulent airflow that deposits most inhaled particles against the airway surface fluid, allowing for their removal by the ciliary action of the respiratory epithelium. This constitutes a major mechanism of antimicrobial defense in the lungs (8). The small size of resting *Aspergillus* conidia (2 to 5 μm in diameter) allows some of the inhaled spores to avoid this defense mechanism and arrive in the respiratory zone of the lung, beyond the ciliated epithelium. Not only is the airway lining a passive means of trapping inhaled particulates in mucus, but it

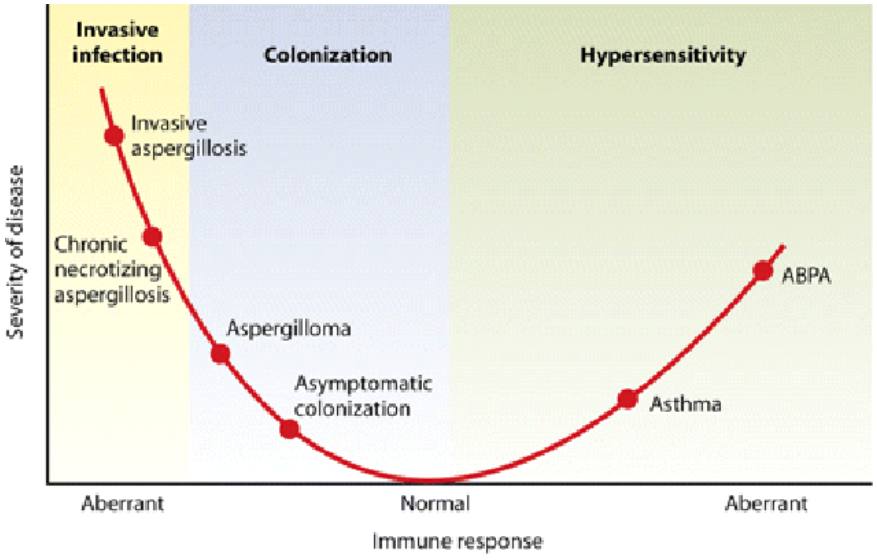

Invasive infection
Colonization
Hypersensitivity
Invasive aspergillosis
Chronic necrotizing aspergillosis
Aspergilloma
Asymptomatic colonization
Asthma
ABPA
Severity of disease
Aberrant
Normal
Aberrant
Immune response

Figure 1

Diagrammatic representation of diseases attributed to *Aspergillus* species as a function of the host's immune response.

Data from Ref **(5)**

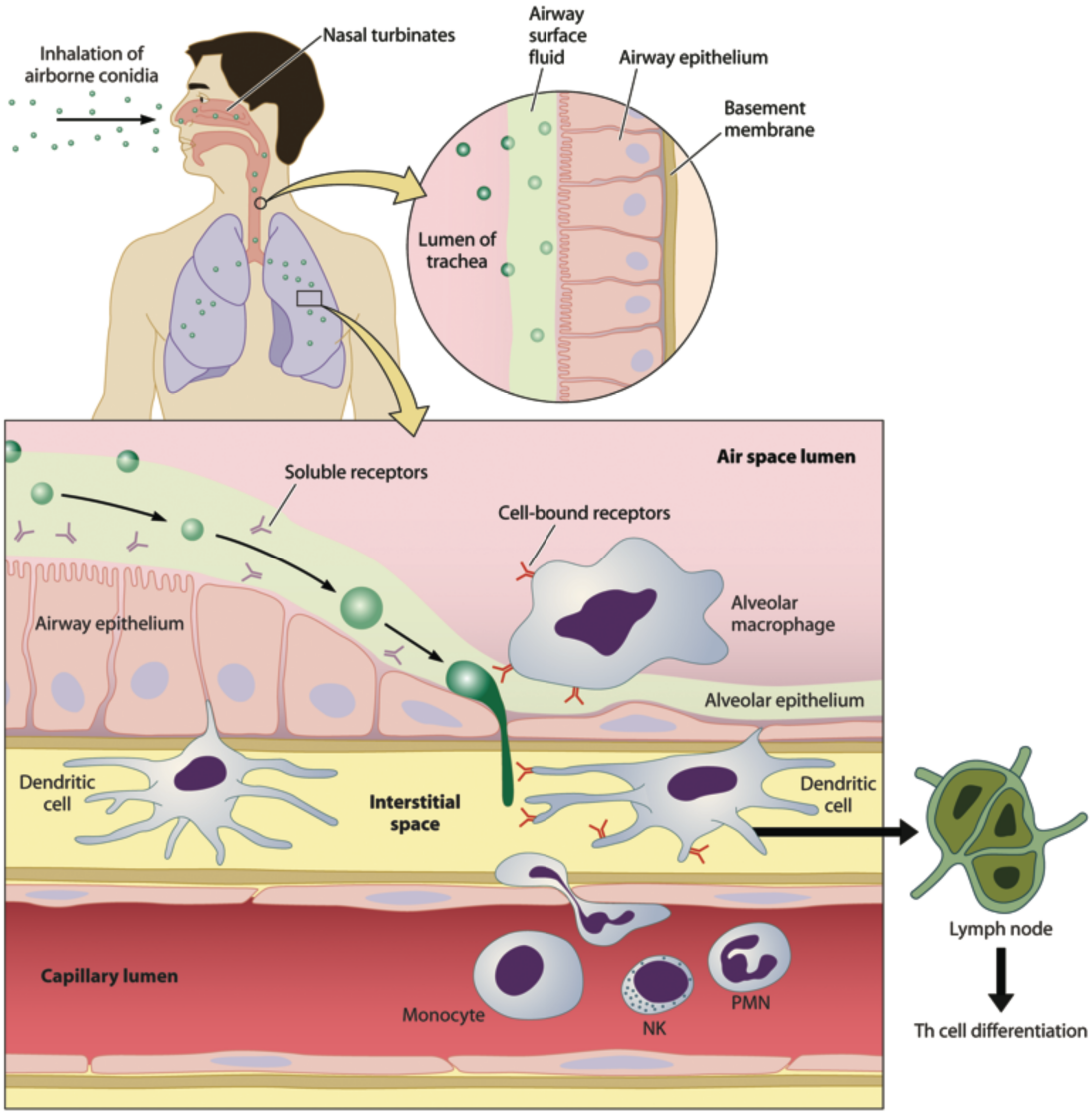

Inhalation of airborne conidia
Nasal turbinates
Airway surface fluid
Airway epithelium
Basement membrane
Lumen of trachea
Air space lumen
Soluble receptors
Cell-bound receptors
Alveolar macrophage
Airway epithelium
Alveolar epithelium
Dendritic cell
Interstitial space
Dendritic cell
Lymph node
Capillary lumen
Monocyte
NK
PMN
Th cell differentiation

Figure 2

Schematic representation of components of the host response to inhaled *Aspergillus* conidia.

Data from Ref **(5)**

also contains a rich array of soluble pathogen recognition receptors and microbicidal peptides. The recognition of *Aspergillus* species by the host is achieved by means of these soluble pattern recognition molecules as well as cell-bound receptors. The next step in defense against *Aspergillus* species is the activation of the effector mechanisms of innate immunity; these include the antimicrobial mechanisms of resident lung leukocytes such as alveolar macrophages and dendritic cells, recruitment of other leukocytes, and activation of recruited leukocytes after their arrival at the site of infection. Coincident with this, resting conidia become swollen within 4 to 5 hours of arrival in the lungs and, if not cleared, germinate and form hyphae within 12 to 15 hours of arrival. The hyphal forms invade the adjacent lung tissue, causing pneumonia, and often disseminate to other organs, most commonly the contralateral lung and the brain. If the organism has not been cleared, antigen presentation and clonal proliferation of *Aspergillus*-specific T-cell over the ensuing days result in the initiation of acquired immunity against the organism.

A key concept in the study of immune responses to *Aspergillus* is that the susceptibilities of the host determine the morphological form, antigenic structure, and physical location of the fungus. In healthy hosts inhaling small numbers of conidia (a circumstance encountered by all humans every day), conidia are successfully cleared by epithelial mucociliary defense mechanisms, and the occasional conidia reaching the alveoli may be dealt with by resident phagocytes

without an initiation of the recruitment cascade. In a host with impaired mucosal defenses, such as patients with bronchiectasis or preexisting lung cavities (which are lined with metaplastic epithelial cells), the conidia germinate and form hyphae on the luminal side of the abnormal mucosal surface and initiate a robust inflammatory response centered on the airway. In contrast, invasive infection, by definition, involves the invasion of the hyphae into the lung parenchyma. In hypersensitivity diseases, aberrant acquired immune responses are directed at *Aspergillus* antigens contained in, or elaborated by, conidia (for example, in a subset of patients with asthma and hypersensitivity pneumonitis) or colonizing hyphae (for example, in allergic bronchopulmonary aspergillosis).

Recognition of *Aspergillus* Species By The Host

The recognition of *Aspergillus* conidia and hyphae occurs via a number of soluble and cell-associated microbial pattern recognition receptors. Conidial maturation triggers a profound morphological change that involves the loss of the proteinaceous hydrophobic layer and exposure of the inner cell wall (9, 10). This cell wall is composed mainly of polysaccharides consisting of β-glucan, mannan, chitin, and galactomannan (11). The morphological state of *Aspergillus* is critical to its recognition by the host: the binding and ingestion of resting conidia, for example, induce very little inflammatory response (12-14), and optimal CD4+ T-cell responses appear to occur only in response to live conidia (15). Antifungal drugs that target and modulate fungal wall components have also been shown to alter inflammatory responses: *in vitro* studies show that the targeting of the synthesis of β-(1,3)-glucan with echinocandins results in increased β-glucan exposure at the tips of *Aspergillus* hyphae that is associated with higher levels of tumor necrosis factor (TNF) and CXCL2 secretion by bone marrow-derived macrophages (13) and increased neutrophil-mediated hyphal damage (16).

1. Soluble Receptors

Pulmonary collectins are a family of C-type lectins that include lung surfactant proteins A and D and mannan-binding lectin. These soluble receptors serve as opsonins for many microorganisms and have been shown to bind *A. fumigatus* conidial carbohydrate structures in a calcium-dependent manner (17-20).

Surfactant proteins A and D are essential for both normal lung function and host defense (21-23). Surfactant proteins A and D promote the agglutination of conidia and their binding to neutrophils and alveolar macrophages and enhance the phagocytosis and killing of conidia by neutrophils (18). Study of the *in vivo* role of these receptors in animal models has been complicated by the baseline derangement of alveolar macrophages and type II alveolar epithelial cells observed in surfactant protein A, and especially surfactant protein D, gene knockout animals (24). However, the administration of exogenous surfactant protein D to wild-type mice provides protection in a corticosteroid-induced model of invasive aspergillosis (19). Patients with allergic bronchopulmonary aspergillosis have higher serum concentrations of surfactant protein D, and in a mouse model of pulmonary hypersensitivity to *Aspergillus* species, there was a parallel marked induction in the expression of surfactant protein D (but not surfactant protein A) in the lungs that was mediated by interleukin-4 (IL-4) and IL-13 (25-27). Finally, the administration of exogenous surfactant proteins A and D to mice with pulmonary hyper- sensitivity to *Aspergillus* results in an attenuated obstructive defect, airway pathology, Th2 cytokines, and lung histamine release (28, 29).

There is substantial evidence for an involvement of several components of the complement cascade in response to *Aspergillus*. The binding of C3 to

A. fumigatus conidia and hyphae led to the activation of the complement alternative pathway (30, 31). In contrast, mannan-binding lectin promotes the activation of the lectin complement pathway via C4bC2a (32) and results in a dose-dependent deposition of complement on conidia and hyphae (20, 30-33). The incubation of *Aspergillus* conidia with healthy human serum also activates the alternative pathway via the mannan-binding lectin C2 bypass mechanism (33). Complement activation may be influenced by the antigenic structure of *A. fumigatus* strains, as clinical strains isolated from patients with invasive aspergillosis induced a stronger activation of the alternative pathway than did environmental strains (33). In the context of *in vivo* animal models, mannan-binding lectin is not necessary for antifungal defense in immunocompetent hosts, since mannan-binding lectin gene knockout mice are not susceptible to invasive aspergillosis (34). However, the administration of exogenous mannan-binding lectin to corticosteroid-treated mice with invasive aspergillosis resulted in improved survival and reduced lung fungal burden in infected mice. This improved outcome was associated with enhanced production of tumor necrosis factor (TNF-α) and gamma interferon (IFN-γ) and reduced production of IL-10 by cultured splenocytes of infected animals (32). Resting *Aspergillus* species are capable of binding several complement regulatory proteins including factor H and plasminogen, thereby inhibiting the activation of the complement cascade (35). In this context, a mutated form of the plasminogen gene was associated with susceptibility to invasive aspergillosis in immunocompromised mice, and a similar

single-nucleotide polymorphism in human plasminogen predisposes hematopoietic stem cell recipients to invasive aspergillosis (36).

Pentraxin-3 belongs to the family of long pentraxins and is secreted as a multimeric protein by a variety of cells in response to inflammatory mediators (37-41). The presence of conidia can rapidly promote the production of pentraxin-3 in mononuclear phagocytes and dendritic cells (42). This soluble receptor binds galactomannan on *Aspergillus* conidia and facilitates recognition by effector cells. The critical role of pentraxin-3 in host antifungal defense was demonstrated in a series of *in vitro* and *in vivo* experiments (42, 43). *In vitro*, pentraxin-3-deficient alveolar macrophages and neutrophils had reduced phagocytic and conidiocidal activities, and pentraxin-3-deficient dendritic cells had defective IL-12 production and upregulation of major histocompatibility complex class II and CD86 in response to the fungus, whereas the addition of exogenous pentraxin-3 restored the antifungal effector activities and responses to *Aspergillus* conidia in gene-deficient cells. *In vivo*, otherwise immunocompetent pentraxin-3-deficient mice were highly susceptible to invasive aspergillosis. This was associated with a concomitant increase in fungal load and IL-4 levels but a decrease in IFN-γ levels in the lungs. Both the systemic and local administration of exogenous pentraxin-3 resulted in improved outcomes for these animals. In addition, the adoptive transfer of wild-type neutrophils was

sufficient to decrease fungal growth in pentraxin-3-deficient animals challenged with *A. fumigatus* (43).

2. Cell- Bound Receptors

Mammalian Toll-like receptors (TLRs) are a family of structurally conserved receptors that recognize and mediate cellular responses to conserved pathogen-associated molecular patterns. The adaptor molecule MyD88 is a major (but not exclusive) signaling mechanism of the TLRs that induce the production of an array of inflammatory cytokines and reactive oxygen species. A number of studies have examined the role of specific TLRs in mediating the recognition of *A. fumigatus*.

Several *in vitro* studies have examined the role of TLR2 and TLR4 in the detection of *Aspergillus* species by leukocytes (14, 44-52). While those studies may appear to yield conflicting results at first glance, a detailed comparison of the experimental approaches shows that the discordant results are likely related to the use of different host cells, different *Aspergillus* morphotypes, and different measures of the host response (Table 2).

In the *in vivo* setting, otherwise immunocompetent mice that are genetically deficient in TLR2, TLR4, IL-1R1, or MyD88 are not susceptible to invasive

Table 2 ***In vitro*** **studies of the role of TLR2 and TLR4 in the response of primary leukocytes to** ***A.fumigatus***

Host cell	*Aspergillus* morphotype	Effect(s)	Ref
Human adherent PBMC (monocyte)	Ethanol-killed serum-opsonized hyphae	TLR4 but not TLR2 required for TNF response	(52)
Human PBMC	Heat-killed conidia and hyphae (nonopsonized)	TLR2 but not TLR4 required for TNF response to both conidia and hyphae	(51)
Mouse-resident peritoneal macrophages	Heat-killed conidia and hyphae (nonopsonized)	TLR4 required for TNF and IL-1α/β response to conidia but not hyphae TLR2 required for TNF and IL-1α/β response to both conidia and hyphae TLR2 required for IL-10 response to hyphae but not conidia	
Mouse-elicited peritoneal macrophages	Live resting conidia, heat-killed swollen conidia and hyphae (nonopsonized)	MyD88 required for TNF response to all TLR2 required for TNF response to resting conidia and hyphae CD14 not required	(48)
Mouse-elicited peritoneal macrophages	Ethanol-killed serum-opsonized hyphae (nonopsonized)	TLR2 and TLR4 required for TNF response to conidia and hyphae and MIP-2/CXCL2 response to hyphae	(50)
Mouse bone marrow derived macrophages	Heat-killed conidia and hyphae (nonopsonized)	MyD88 not required for phagocytosis or killing of conidia or TNF response to hyphae	(49)
Mouse alveolar macrophages	Conidia (nonopsonized)	TLR2 required for TNF response	(44)
Mouse alveolar macrophages	Heat-killed conidia (nonopsonized)	TLR2 required for TNF response but not CCL3/MIP-1α, CXCL2/MIP-2, IL-1α/β, IL-6, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor responses	(14)
Mouse alveolar macrophages	Conidia (nonopsonized)	TLR2, TLR4, or MyD88 not required for phosphorylation of ERK or p38 mitogen-activated protein kinases	(47)
Mouse-elicited peritoneal macrophages	Conidia and hyphae (nonopsonized)	TLR2 required for conidial but not hyphal killing TLR4 required for conidial and hyphal killing	(46)
Mouse lung dendritic cells	Conidia with amphotericin (nonopsonized)	TLR2 absence resulted in greater IL-12p70 and reduced IL-10 production levels TLR4 or MyD88 absence resulted in reduced IL-12p70 and greater IL-10 production levels	(45)

Data from Ref (5)

aspergillosis when challenged with conidia via the respiratory tract (45, 47, 53): these mice display lung histologies and cytokine production that are comparable to those of wild-type mice. Nevertheless, TLR signaling via MyD88 appears to be necessary for the early inflammatory responses to *Aspergillus* species in immunocompetent hosts (54): in the absence of MyD88, there were fewer natural killer (NK) cells and higher fungal burdens in the infected lung within 24 h of fungal challenge. In addition, MyD88-mediated signaling was important for the subsequent development of protective adaptive responses (45, 55)). In contrast to immunocompetent animals, mice with cyclophosphamide-induced immunosuppression require TLR4 and MyD88 for optimal host defense against invasive aspergillosis (45): TLR4 and MyD88 deficiencies each led to significantly lower survival rates, higher lung fungal contents, higher numbers of IL-4-producing but lower numbers of IFN-γ-producing CD4 T cells in thoracic lymph nodes, and, in MyD88-deficient hosts, reduced lung TNF levels (45).

The consequences of the absence of TLR2 in *in vivo* infection are more complex: in cyclophosphamide-treated mice, a TLR2 deficiency did not influence survival but resulted in an increased lung fungal content. This was associated with higher numbers of lung IL-4-producing CD4 T cells in thoracic lymph nodes but also with higher lung TNF levels (45). These findings are in contrast to findings using a model of invasive aspergillosis in mice immunosuppressed with vinblastine, in

which *Tlr2-/-* animals had higher mortality rates and significantly lower levels of lung TNF than did wild-type mice (44).

TLR9 can initiate immune responses to *Aspergillus* species via the recognition of fungal unmethylated CpG DNA in murine bone marrow-derived dendritic cells and human plasmacytoid dendritic cells (56). Surprisingly, TLR9-deficient mouse-elicited peritoneal neutrophils have a greater ability to kill *Aspergillus* conidia and hyphae (46), and TLR9-deficient mouse lung dendritic cells produce less IL-12p70 and more IL-10 in response to conidia (45). However, the role of TLR9 in the context of *in vivo* defense against *Aspergillus* species appears to be complex: in the setting of immunosuppression with cyclophosphamide or antibody-mediated neutrophil depletion, TLR9-deficient mice survive longer and have significantly lower fungal burdens than wild-type mice following challenges with *A. fumigatus* conidia (45, 46, 57), suggesting the involvement of TLR9 signaling in an immunoregulatory mechanism that ultimately benefits *Aspergillus* species and may be mediated by neutrophils. In the context of a model of airway hypersensitivity to *Aspergillus*, however, the absence of TLR9 led to lower levels of methacholine-induced airway hyperreactivity but promoted fungal growth in the lung associated with reduced lung dectin-1 expression levels; this is remarkable since wild-type mice sensitized to *Aspergillus* species do not develop invasive diseases following the administration of even large inocula in the setting of neutrophil depletion (58, 59). It remains to be established whether this effect is due to a failure of TLR9-deficient mice to develop acquired immunity to

Aspergillus species during the sensitization protocol or whether this finding is due to the absence of a TLR9-mediated recognition of *Aspergillus* species during secondary challenge with intratracheal conidia.

A negative regulator of TLR-receptor signaling, Toll IL-1R8 (alternative name, immunoglobulin IL-1-related receptor), has been studied using immunocompetent mice challenged with intrapulmonary conidia. The absence of Toll IL-1R8 resulted in reduced survival rates and increased levels of lung fungal growth that were associated with elevated lung IL-17 and IFN-γ levels but lower IL-10 and Foxp3 transcript levels a week after infection, suggesting that the absence of this regulatory process results in the detrimental activation of Th1 and Th17 immunity (53). Another group of cell-bound G-protein-coupled cell surface receptors, the protease-activated receptors (PARs), has recently been shown to influence *in vivo* responses to *Aspergillus* species (60). Cyclophosphamide-treated mice deficient in PAR2 or treated with a PAR2 antagonist displayed higher lung oxidative burst and MMP-9 activities, higher lung TNF protein levels, and lower lung IL-10 levels after challenge with *Aspergillus* conidia (60). Consistent with this, the transgenic expression of PAR2 and treatment with a PAR2 agonist had the reverse effects (60), suggesting that PAR2 signaling attenuates responses in the context of invasive aspergillosis.

Several polymorphisms of human TLRs have been associated with an increased risk of invasive aspergillosis in susceptible hosts. A haplotype of TLR4, which consists of two single-nucleotide polymorphisms within the coding region of the gene that are associated with hyporesponsiveness to lipopolysaccharide, resulted in a hazard ratio of 2 to 4 for invasive aspergillosis in allogeneic hematopoietic stem cell transplant recipients when the polymorphism was present in the donor (61). This polymorphism was also associated with an increased risk of chronic necrotizing aspergillosis in a separate cohort (62). Another study linked polymorphisms in TLR1 and TLR6 in recipients of allogeneic hematopoietic stem cell transplantation to risk of invasive aspergillosis (63), and a polymorphism in TLR9 was associated with allergic bronchopulmonary aspergillosis (62). These observations provide indirect evidence of the relevance of TLRs in human aspergillosis.

Dectin-1 is a C-type lectin-like receptor that was initially identified as being a dendritic cell receptor (64, 65). Dectin-1 is a major receptor for fungal β-glucans (66) and is widely expressed in myeloid leukocytes including macrophages, neutrophils, and dendritic cells (67-69). Studies have demonstrated a stage-specific activation of dectin-1 in response to *Aspergillus* species and provide a mechanism in which the host inflammatory response is triggered only in the presence of swollen conidia (12, 14, 70, 71). The protective role of dectin-1 was demonstrated using immunocompetent mice challenged with *A. fumigatus*, in

which a blockade of dectin-1 reduced production of inflammatory cytokines and increased lung fungal burden (14, 72). In an immunosuppressed model of invasive aspergillosis, the administration of a synthetic dectin-1–Fc receptor fusion protein resulted in delayed mortality through a mechanism that involved enhanced conidial killing by alveolar macrophages (73). In immunocompetent mice, this enhanced susceptibility was associated with reduced lung neutrophil accumulation and a failure to induce the expression of IL-23 and IL-17 in the lungs in the first 24 h after infection (72).

Dendritic cell-specific ICAM-3-grabbing nonintegrin (DC- SIGN) is another C-type lectin present on surfaces of dendritic cells and some macrophages with specificity for high mannose moieties (74, 75). Human lung dendritic cells and alveolar macrophages bind *Aspergillus* conidia via the interaction of DC-SIGN with fungal galactomannan (76). While the binding and ingestion of conidia appear to be influenced by the cell surface expression of DC-SIGN, the precise contribution of this interaction to the host antifungal response is yet to be fully elucidated.

3. Cytokine Signaling of Recognition

Pathogen recognition via soluble and cell-bound microbial pattern recognition receptors is quickly followed by the afferent limb of pathogen recognition, which consists of the elaboration of an initial group of cytokines including TNF and

members of the IL-1 family. Among members of the IL-1 family, IL-1β is induced in alveolar macrophages in response to *Aspergillus* antigens and in peripheral blood monocytes in response to *Aspergillus* conidia and hyphae in *in vitro* studies (77, 78). In animal models, IL-1β is induced in mice with chronic glaucomatous disease and invasive aspergillosis, and IL-18 is induced in the lungs of immunocompetent mice and sensitized mice challenged with intrapulmonary conidia (79-81). The precise mechanism of action of these ligands in host defense against *Aspergillus* species has not been evaluated in detail; although the neutralization of IL-18 alone did not affect lung fungal killing in immunocompetent mice, the neutralization of both IL-18 and TNF did result in greater lung fungal viability (80). In mice with airway allergy to *Aspergillus*, however, the immunoneutralization of IL-18 has been shown to result in a prolonged retention of *Aspergillus* in the airways, lower TLR2 expression levels, and greater airway remodeling (81), suggesting that this innate immune mechanism is relevant to pathology in the context of acquired hypersensitivity responses to *Aspergillus*.

TNF is a 17-kDa protein that is secreted predominantly by cells of myeloid lineage, including alveolar macrophages, dendritic cells, recruited monocytes/ macrophages, and neutrophils. TNF is markedly induced when cells of the monocyte/macrophage lineage are coincubated with *Aspergillus* antigens or fungal elements and is markedly induced in the lungs of both immunocompetent

and immunocompromised mice after intrapulmonary challenge with conidia (80, 82, 83). In both immunocompetent animals and immunocompromised mice treated with cyclophosphamide, immunoneutralization of TNF results in an impaired fungal clearance and increased mortality that were associated with lower lung levels of several chemokines (CXCL1/GRO-α/KC, CXCL2/ macrophage inflammatory protein 2 [MIP-2], CCL2/monocyte chemoattractant protein 1 [MCP-1], and CCL3/MIP-1α) and lower levels of recruitment of neutrophils to the lungs (80, 84). Conversely, the pretreatment of immunocompromised animals with a TNF agonist resulted in markedly attenuated infection (82). The importance of TNF in the defense against *Aspergillus* in humans has since been supported by documentation that otherwise immunocompetent patients treated with TNF antagonists are susceptible to invasive aspergillosis (85-87).

Recruitment of Leukocytes To Site of Infection

Leukocyte recruitment is a complex and multistep process that begins with the interaction of circulating leukocytes and endothelial surface adhesion molecules, leading to the rolling and adherence of leukocytes, followed by the extravasation of the leukocytes into the extravascular space and finally directional homing to the site of inflammation. Among classes of molecules involved in these processes, *Aspergillus* hyphae have been shown to induce endothelial cells to generate the cell adhesion molecules E-selectin and vascular cell adhesion molecule-1 (VCAM-1) both *in vitro* and in models of invasive aspergillosis in mice treated with cyclophosphamide and also mice treated with corticosteroids (88).

Among the many classes of mediators involved in this process, several chemokine ligands and receptors have been examined in the context of innate defenses against *Aspergillus* species. Chemokine ligands are a superfamily of 8- to 14-kDa structurally related polypeptides that are divided into CC, CXC, C, and CX3C families based on the sequence of cysteine residues near the amino terminus. The chemokine receptors belong to the family of seven-transmembrane G-protein-coupled molecules. Unlike most cytokines, which are the products of primarily leukocytes, diverse cell types are capable of producing chemokines. In response of *Aspergillus* conidia and hyphae, for example, macrophages,

dendritic cells, alveolar and bronchial epithelial cells, and endothelial cells have been shown to generate these ligands (13, 68, 70, 88-92).

1. Recruitment of Neutrophils

A subset of the CXC family of chemokine ligands is defined by the presence of a glutamic acid-leucine-arginine (ELR) motif immediately downstream of the CXC sequence. These ELR-containing CXC chemokine ligands are critical for the recruitment of neutrophils in many models. Importantly, human and mouse ELR-containing CXC chemokine ligands are not precise structural homologues, complicating the application of experimental animal data to human disease: human ligands (CXCL1/GROα, CXCL2/GROβ, CXCL3/GROγ, CXCL5/ENA-78, CXCL6/GCP-2, CXCL7/NAP-2, and CXCL8/IL-8) can signal via two receptors, CXCR1 and CXCR2. Mouse ligands (CXCL1/KC and CXCL2/MIP-2, CXCL5/LIX, CXCL6/GCP-2, and CXCL15/lungkine) all signal via a single receptor, CXCR2 (93). In wild-type mice challenged with large intratracheal inocula of *Aspergillus* conidia, there is a marked induction of the ELR-containing CXC chemokine ligands CXCL1/KC and CXCL2/MIP-2 that was associated with a rapid recruitment of neutrophils to the lungs (84). In these animals, the immunoneutralization of CXCR2 resulted in a marked impairment of neutrophil influx to the site of infection in immunocompetent animals, resulting in severe invasive aspergillosis with nearly 100% mortality (84); similarly, CXCR2-deficient animals challenged with intrapulmonary conidia had an impaired recruitment of

neutrophils to the lungs associated with conidial germination in the lungs (93). Conversely, the transient overexpression of CXCL1/KC in the lungs of immunocompromised mice resulted in lower mortality rates and lower lung fungal contents, even when the transgenic expression of the ligand began after the infection had been established (94). Unexpectedly, the transgenic expression of CXCL1/KC also resulted in a greater accumulation of monocytes/macrophages at the site of infection that was associated with a greater local expression of IFN-γ and IL-12p70, suggesting that the increased numbers of lung neutrophils in transgenic animals exerted a beneficial immunomodulatory effect in addition to direct neutrophil-mediated fungal killing.

2. *Recruitment of Mononuclear Cells*

The CXCR3 chemokine ligands CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC represent a unique group of ELR-negative CXC chemokines that are potently induced by both type I and type II IFNs. The role of these chemokines in the host defense against *Aspergillus* has not been studied in detail. However, a single-nucleotide polymorphism associated with reduced levels of expression of CXCL10/IP-10 has been associated with an increased risk of invasive aspergillosis in allogeneic stem cell transplant recipients, providing a potential relevance of these chemokines to human infection (95).

The CC chemokine ligand CCL3/MIP-1α signals via two receptors, CCR1 and CCR5. In mice with antibody-mediated neutrophil depletion and invasive aspergillosis, CCL3/MIP-1α was markedly induced in the lungs, and its neutralization resulted in increased lung fungal content and increased mortality that was associated with reduced numbers of lung monocytes/macrophages (96). Interestingly, immunocompetent CCR1-deficient mice inoculated with intravenous *Aspergillus* conidia were reported to have an increased susceptibility to the infection that was associated with an impaired trafficking and proliferation of myeloid cells (97), suggesting that a similar mechanism may be operational in the lungs of neutrophil-depleted mice.

CCR6 is a chemokine receptor for the ligand CCL20/MIP-3α as well as several members of the β-defensin family and is expressed on immature dendritic cells, mature B cells, and memory T cells. In the context of mice with antibody-mediated neutrophil depletion and invasive aspergillosis, myeloid dendritic cells were the major population of CCR6-expressing cells in the lungs, and their accumulation in the lungs corresponded to the local induction of the ligand CCL20. Neutropenic mice deficient in CCR6 had worsened survival and greater lung fungal burden associated with fewer lung dendritic cells and recruited monocytes/macrophages; similarly, the neutralization of CCL20 resulted in impaired lung fungal clearance and impaired recruitment of dendritic cells to the lungs during the early phase of infection (98).

CCR7 is the chemokine receptor for the ligands CCL19 and CCL21, which are constitutively expressed in secondary lymphoid organs. The receptor is expressed on both naive and memory T cells, B cells and mature dendritic cells, facilitating their retention and recruitment to the secondary lymphoid organs (99-101). In mice with antibody-mediated neutrophil depletion and invasive aspergillosis, the absence of CCR7 led to an overall better outcome with enhanced survival and decreased lung fungal burden (102). Interestingly, the adoptive transfer of labeled CCR7-deficient bone-marrow-derived dendritic cells (BMDCs) to neutropenic wild-type mice led to a further decrease in lung fungal growth that was associated with a significantly higher recruitment of dendritic cells to the lungs, suggesting that enhanced dendritic cells recruitment to the lung may have a direct beneficial effect during invasive aspergillosis (102).

CCL2/MCP-1 is another CC chemokine ligand that is markedly induced in the lungs of both immunocompetent mice and mice with antibody-mediated neutrophil depletion challenged with intratracheal *A. fumigatus* conidia (103, 104). The neutralization of CCL2/MCP-1 in neutrophil-depleted mice resulted in an increased severity of infection that was associated with a markedly reduced accumulation of classical NK cells in the lungs but surprisingly did not affect other leukocyte subsets. The transfer of labeled NK cells to infected mice resulted in their accumulation in the lungs, but this effect was attenuated with CCL2/MCP-1

neutralization, indicating that this ligand is necessary for the influx of these cells to the lungs. Moreover, the expression of CCR2, the only known receptor for CCL2/MCP-1 on NK cells, was also shown to be necessary for the lung influx of NK cells in invasive pulmonary aspergillosis (103). This effect appeared not to represent a direct single-ligand–single-receptor interaction, however, since the neutralization of CCL2 in CCR2-deficient mice resulted in further decreases in lung NK cell influx compared to the absence of CCL2 or CCR2 alone (103).

In a recent study, CCR2 has been shown to mediate the influx of LY6C-expressing inflammatory monocytes to the lung following respiratory challenges in immunocompetent mice (105). The depletion of $CCR2^{+}$ cells resulted in higher lung fungal burden in challenged mice and more interestingly, the absence of lung monocytes impaired the priming and proliferation of *A.fumigatus*-specific CD4 T cell responses(105). The absence of CCR2 appears to affect T cell responses by reducing antigen transport to the draining lymph nodes by monocyte-derived $CD11b^{+}$ dendritic cells and while it remains unclear whether these dendritic cells are directly involved in the priming of naive T cells, CCR2 signaling may be required for optimal development of adaptive antifungal defenses in immunocompetent mice exposed *Aspergillus* (105).

CCR4 is a CC chemokine receptor that binds several ligands including CCL2/ MCP-1, CCL17/TARC, and CCL22/MDC. All of these ligands are induced in the

lungs of neutrophil-depleted mice with invasive aspergillosis (106). Surprisingly, the neutralization of CCL17 or deficiency of CCR4 resulted in greater protection from invasive aspergillosis. The precise cellular mechanism of this effect is not yet clear but appears to involve a local immunosuppressive effect, since CCR4-deficient mice had higher lung IL-12 and CCL2 levels that were associated with greater numbers of macrophages and dendritic cells in the lungs (106). Interestingly, despite higher lung CCL2 levels, CCR4-deficient mice had fewer lung NK cells, suggesting that intact CCR4 signaling may be required for optimal NK cell recruitment.

Cytokines in Innate Leukocyte Activation

The activation of both the resident and recruited leukocytes is, to a large extent, mediated by a diverse set of cytokines that act via autocrine and paracrine effects. The importance of these molecules in the defense against *Aspergillus* species was first noted when the susceptibility of inbred mouse strains was found to correlate with patterns of cytokine production: the production of T-helper1 (Th1) cytokines IFN-γ and IL-12p70 correlating with improved outcomes and the production of IL-4 (a T-helper2 cytokine) associated with a more severe infection (163, 164). The precise role of the T-helper17 cytokines IL-23 and IL-17 in the host response to *Aspergillus* species is not yet clearly defined. Despite the “Th” designation of these cytokines, note that the host defense mechanisms under discussion here occur within the first days after *Aspergillus* challenge and represent cytokines released from leukocytes in the context of innate immunity.

In the context of early infection, IFN-γ, IL-12, and IL-18 are induced in the lungs of immunocompetent animals in the first 48 h after *Aspergillus* challenge (80, 163). The administration of neutralizing cytokine-specific antibodies to IL-12p70 or IFN-γ, however, did not influence the clearance of *Aspergillus* from the lungs of immunocompetent mice (80). In contrast, IFN-γ or IL-12p40 knockout animals (the latter deficient in both IL-12p70 and IL-23) that are treated with cyclophosphamide are more susceptible to invasive aspergillosis (165).

Consistent with this, the exogenous administration of IFN-γ results in improved outcomes of experimental infection for mice treated with corticosteroids or cyclophosphamide (166, 167). The cellular source of IFN-γ in late infection and in immunized mice is recognized as *Aspergillus*-specific CD4 T cells in both immunocompetent and cyclophosphamide-treated mice (15, 55, 164). The cellular source of IL-12p70 during *in vivo* infection is not established, although cultured human and mouse dendritic cells produce this cytokine when exposed to *Aspergillus* conidia *in vitro* (76, 168, 169), and the transfer of cultured dendritic cells with transgenic expression of IL-12p35 and IL-12p40 to immunocompromised mice with invasive aspergillosis results in an attenuated infection (170).

With regard to Th2 phenotype cytokines, IL-4-deficient cyclophosphamide-treated mice with invasive aspergillosis have reduced mortality and lower lung fungal contents on day 1 of infection than wild-type mice, which are associated with increased lung levels of IL-12p70 and IFN-γ. In contrast, IL-5 deficiency had no effect on lung fungal content or survival (165). Additionally, cyclophosphamide-treated IL-4 knockout mice became more susceptible to infection when they were treated with a neutralizing antibody against IL-12p70 (165). These results indicate that in the first day of infection in immunocompromised mice, IL-4, but not IL-5, down regulates the Th1 phenotype in wild-type mice and contributes to the increased severity of infection. The

cellular source of IL-4 during *in vivo* infection has again not been determined, but cultured dendritic cells express IL-4 in response to *Aspergillus* hyphae or proteases (168, 171).

Similar to IL-4, cyclophosphamide-treated IL-10-deficient mice with invasive pulmonary aspergillosis showed enhanced antifungal inflammatory responses, Th1 cytokine production, and reduced severity of infection (172) Immunocompetent IL- 10-deficient animals were similarly resistant to infection when given intravenous conidia (173). Consistent with this, the presence of a human IL-10 promoter polymorphism resulting in reduced IL-10 expression levels in recipients of allogeneic bone marrow transplantation was associated with a reduced incidence of invasive aspergillosis (174, 175). IL-6 deficiency in both immunocompetent mice and animals treated with corticosteroids resulted in greater lung fungal contents and reduced survival associated with the impaired conidiocidal activity of lung phagocytes, which was restored after the addition of recombinant IL-6 (176). The cellular sources of IL-10 and IL-6 in invasive aspergillosis remain to be established.

IL-23 is a heterodimer of IL-12p40 and a p19 protein and is important in promoting and maintaining the Th17 phenotype. IL-23-deficient ($p19^{-/-}$) mice or animals with an antibody-mediated blockade of IL-23 or IL-17 in the setting of infection with *Aspergillus* species were reported to have a reduced lung fungal

burden; similarly, the neutralization of IL-17 resulted in a greater clearance of *A. fumigatus* from the lungs in gp46phox-deficient animals (177, 178). Recent work by another group, however, indicated that the early neutralization of IL-17 resulted in lower lung fungal contents (as determined by fungal RNA) in the first 48 h after intrapulmonary challenge with *A. fumigatus* (72). The reason for these potentially discrepant reports may relate to experimental conditions, including differences in the genetic backgrounds of the mouse strains examined (C57BL/6 versus 129/SvEv), the timing of measurements of lung fungal content (day 3 versus days 1 to 2), or, conceivably, the methods employed to quantify tissue fungal content (lung chitin content versus lung fungal RNA content by quantitative PCR) in the two studies (72, 178). Human monocyte-derived dendritic cells have been shown to generate both IL-12p70 and IL-23 when incubated with *Aspergillus* conidia (169). Mice lacking IL-23p19 or with an antibody-mediated blockade of IL-23 or IL-17 in the setting of infection with *Aspergillus* species displayed higher levels of lung IL-12p70 production and IFN-γ-producing $CD4^+$ T cells (178). During heightened fungal growth, dendritic cells were shown to produce IL-23, acting possibly as a positive-feedback loop for further IL-23 production, and neutrophil fungicidal activity, at least *in vitro*, was impaired in the presence of IL-23 and IL-17 (178). These observations suggest that the development of Th17 immunity inhibits the development of protective Th1 immunity. However, the blockade of IL-23 via neutralizing antibody in IFN-γ-deficient mice with invasive aspergillosis resulted in a further increased fungal

burden (178), suggesting that in the absence of effective Th1 immunity, the IL-23/ IL-17 pathway is protective against *Aspergillus* infection.

Innate Effector Mechanisms

1. Alveolar Macrophages

Alveolar macrophages are the major resident leukocytes in the lung and provide an early line of defense against inhaled conidia that have reached the alveoli (107). With a variety of soluble and surface pathogen recognition receptors at their disposal, alveolar macrophages can quickly adhere to and ingest conidia entering the alveolar space (108). Phagocytosis and the secretion of proinflammatory cytokines by alveolar macrophages help to eliminate conidia and restrict the initial spread of microorganisms in the alveoli. Alveolar macrophages are sufficient to overcome small inocula of *Aspergillus* conidia, as demonstrated in a murine model of invasive aspergillosis (109). However, larger challenges of *Aspergillus* conidia evidently overwhelm the capacities of local defenses, necessitating the recruitment of other effector leukocytes. This may be due to the relatively slow killing of conidia by alveolar macrophages: in *in vitro* studies, conidial killing by alveolar macrophages was delayed 3 to 6 h after phagocytosis (110), corresponding to the time when conidia become swollen.

The biochemical and molecular mechanisms for the killing of phagocytosed conidia have been studied most thoroughly in the context of chronic granulomatous disease. Chronic granulomatous disease results from inherited mutations in any of the four components of the NADP (NADPH) oxidase complex

and results in an impaired ability to generate reactive oxygen species and a consequent susceptibility to several infections, most notably invasive aspergillosis (111). Most available evidence suggests that alveolar macrophages can kill conidia via nonoxidative mechanisms: rabbit alveolar macrophages were able to kill *Aspergillus* conidia under anaerobic conditions (110), and human blood monocytes cultured for 10 days (which have a reduced capacity to generate reactive oxygen intermediates (112)) were also able to kill fungal conidia as effectively as human blood monocytes after 2 days of *in vitro* culture (which have intact reactive oxygen intermediate production (110)). Macrophage colony-stimulating factor-induced *Aspergillus* hyphal damage was observed in conjunction with enhanced superoxide anion production in both human monocyte-derived macrophages and rabbit alveolar macrophages (113). In addition, alveolar macrophages from mice deficient in gp91phox (analogous to human X-linked chronic granulomatous disease) inhibited conidium germination as efficiently as wild-type alveolar macrophages (79, 93, 114). In contrast, alveolar macrophages from mice lacking p47phox, another component of the NADPH oxidase complex and mimicking an autosomal recessive form of human chronic granulomatous disease, have been reported to phagocytose conidia normally but are unable to kill them (115). The explanation for this discrepancy may relate to methodological issues or differences in macrophage function in different forms of chronic granulomatous disease.

Several *in vitro* studies suggested that reactive nitrogen intermediates may also not be necessary for antifungal defense in alveolar macrophages: although IFN-γ-treated alveolar macrophages were shown to have higher levels of nitric oxide production, which was associated with higher rates of killing of *Aspergillus* conidia (116), murine alveolar macrophages did not produce nitric oxide in response to *Aspergillus* conidia (117), and the presence of a competitive inhibitor of nitric oxide synthase did not inhibit conidial killing by human or murine alveolar macrophages (118). In addition, alveolar macrophages from mice deficient in the inducible form of nitric oxide synthase killed conidia as effectively as wild-type alveolar macrophages (115).

Corticosteroid treatment was shown to significantly impair the capacity of killing of conidia by alveolar macrophages. While there was not a significant difference observed with regard to internalizing conidia, alveolar macrophages from corticosteroid-treated mice had more growing fungus, and this was associated with reduced reactive oxygen species production (115). The altered production of reactive oxygen intermediates may be one contributing factor for the increased susceptibility and development of invasive aspergillosis in corticosteroid-treated mice.

2. *Neutrophils*

The duration and extent of neutropenia as well as qualitative defects in neutrophil function are the best-described risk factors for invasive aspergillosis. Defects in neutrophil number and function have long been recognized as being the most pervasive risk factors for the development of invasive aspergillosis in diverse populations of patients including bone marrow recipients (119), patients receiving cytotoxic chemotherapy (120), and patients with chronic granulomatous disease (121) or human immunodeficiency virus (122, 123). Recruited neutrophils were initially thought to act exclusively on hyphae while resident alveolar macrophages killed resting and swollen conidia (107). While neutrophils remain responsible primarily for hyphal killing, they have been shown to have an essential role in killing germinating conidia (30, 93, 124, 125). In contrast to the delayed killing mediated by macrophages, fungal damage and killing by neutrophils are immediate and very rapid (126, 127). Neutrophils bind and internalize swollen conidia to trigger respiratory burst and degranulation (24, 128). The size of the hyphae prevents phagocytosis by neutrophils, but contact between neutrophils and hyphae can induce both oxidative and nonoxidative mechanisms to mediate hyphal damage (126, 127).

The importance of an oxidative killing mechanism in neutrophils is thought to be a major contributor to the susceptibility of patients with chronic granulomatous disease to invasive aspergillosis. Otherwise normal gp91phox-deficient mice are highly susceptible to invasive aspergillosis (79, 93, 129). Since gp91phox-

deficient alveolar macrophages can efficiently inhibit growth of *Aspergillus* conidia as well as wild-type alveolar macrophages but neutrophils from gp91phox-deficient mice or chronic granulomatous disease patients have impaired fungicidal activity *in vitro*, the generation of reactive oxygen species by neutrophils is considered to be the major antifungal mechanism lacking in chronic granulomatous disease. Another major neutrophil effector mechanism is myeloperoxidase, an enzyme stored in neutrophil azurophilic granules that, when released in the context of the oxidative burst, catalyzes the reaction of hydrogen peroxide and chloride anion to generate hydrochlorous acid (130). Otherwise immunocompetent myeloperoxidase-deficient mice are also susceptible to invasive aspergillosis but less so than gp91phox-deficient animals (129). *In vitro* studies show that neutrophils from mice with a gp91phox deficiency as well as humans with chronic granulomatous disease or myeloperoxidase deficiency are unable to kill hyphae (93, 125, 126, 131). The defect in oxidative killing of *Aspergillus* species by neutrophils from patients with chronic granulomatous disease or myeloperoxidase deficiency can be corrected when as few as 1 normal neutrophil is added to 15 neutrophils from mutant hosts (132), demonstrating cooperation between neutrophils in oxidative killing. In this context, the *in vivo* neutrophil oxidative burst in response to *Aspergillus* infection appears to occur in the context of intra-alveolar neutrophil aggregates (93). Granulocyte colony-stimulating factor and IFN-γ enhance the neutrophil oxidative response and their ability to kill hyphae, including neutrophils from patients

treated with corticosteroids (123, 127, 133). IFN-γ also improves the oxidative response of neutrophils from patients with chronic granulomatous disease, and the prophylactic administration of IFN-γ to patients with chronic granulomatous disease results in a reduction in serious infections, including invasive aspergillosis (134-136); consistent with this, neutrophils from patients with chronic granulomatous disease who were treated with IFN-γ demonstrated an enhanced ability to kill *Aspergillus* hyphae *ex vivo* (137).

There is also *in vitro* evidence to suggest that neutrophils can mediate antifungal activities via nonoxidative mechanisms. Purified neutrophil defensins, which are stored in the primary granule, also have fungicidal activities (138). In addition, neutrophils release lactoferrin from their secondary granules as part of their degranulation when interacting with *Aspergillus* conidia (125, 128). A recent study identified neutrophil lactoferrin sequestration of iron as an important contributor to inhibiting *Aspergillus* conidial growth (124). Cell-free supernatants of degranulated neutrophils from both healthy donors and chronic granulomatous disease patients, which had abundant lactoferrin, were capable of suppressing conidium growth, whereas the presence of ferritin, a soluble iron source, abolished the growth-inhibitory effect on conidia (124). Pentraxins, which have been shown to be critical for mediating resistance to invasive aspergillosis, as discussed above, are also stored in secondary granules and localize to the neutrophil extracellular traps upon neutrophil activation (43).

3. Natural Killer Cells

NK cells were first described as a subset of mouse splenic lymphocytes with spontaneous cytotoxicity against virally infected cells (139, 140). Unlike classical T and B cells, NK cells do not require clonal proliferation before they can respond to antigens; as a result, they can be deployed rapidly as part of the innate effector response. Most of the literature on NK cells has concentrated on cells obtained from the mouse spleen and human peripheral blood, where the cells are easily accessible in relatively large numbers, but NK cells have a broad tissue distribution (141, 142), and after the spleen, the lungs contain the largest number of tissue NK cells in experimental animals (141-144). In the uninflamed lung, the majority of NK cells are located in the vascular and interstitial compartments of the lung and therefore in close proximity to any inhaled microorganisms (144, 145)

In mice with antibody-mediated neutrophil depletion and invasive pulmonary aspergillosis, the additional depletion of NK cells results in worsened outcomes of infection (103).

4. Recruited Monocytes/Macrophages

Peripheral blood monocytes are a heterogeneous population of myeloid cells that contain the precursors of tissue macrophage and dendritic cells(146). Upon

interaction with *Aspergillus* conidia, human peripheral blood monocytes undergo profound changes in their expression of hundreds of genes (89, 147). Human monocytes are capable of ingesting and killing conidia and of inducing damage to *Aspergillus* hyphae (92, 113, 148, 149), and this killing can be enhanced in the presence of granulocyte-macrophage colony-stimulating factor, IFN-γ, and fungicidal drugs (150-153). In the context of neutropenic mice with invasive aspergillosis, these inflammatory mononuclear cells appear in the lungs within hours of the onset of infection (98), but their *in vivo* role in the defense against invasive aspergillosis in the setting of immunosuppression has not been directly examined to date. However, in immunocompetent mice challenged with *Aspergillus*, there is an influx of $CCR2^{+}LY6C^{+}$ inflammatory monocytes which has been shown to contribute to fungal clearance in the lung by promoting the development of *A.fumigatus*-specific T cell response.(105).

Dendritic cells have a primary role in the surveillance of pathogens at the mucosal surfaces and have the ability to shape the subsequent development of T-cell response to the organisms. *In vivo* and *in vitro* studies have demonstrated that dendritic cells internalize both *Aspergillus* conidia and hyphae and transport them from the airways to draining lymph nodes *(105, 154)*. The internalization of conidia and hyphae by dendritic cells involves distinct phagocytic mechanisms and pathogen recognition receptors, and this translates into qualitatively different $CD4^{+}$ T-helper-cell responses. Dendritic cells ingest conidia through coiling

phagocytosis, in which the extension of unilateral pseudopods rotates around the pathogen to form self-apposed pseudopod layers. This mechanism involves the ligation of the mannose receptor DC-SIGN and complement receptor 3, and leads to the priming of Th1 responses in the draining lymph node and spleen in mice *(154)*. Alternatively, the internalization of hyphae occurs via the Fc receptor and complement receptor 3-mediated phagocytosis, resulting in a “zipper phagocytosis” that requires attachment through receptor-ligand binding, and engulfment follows the contour of the microorganisms. In contrast to the ingestion of conidia, hyphal phagocytosis by lung dendritic cells results in the production of IL-4 and IL-10 *in vitro* and the generation of IL-4-producing $CD4^+$ T cells in the spleen and mediastinal lymph nodes *in vivo (154)*.

In both immunocompetent and neutropenic mice, there is a rapid expansion of $CD11b^+$ dendritic cells but not alveolar macrophages or $CD103^+$ dendritic cells in the lung following a respiratory challenge of *Aspergillus* (98, 102, 105). While their presence is associated with reduced mortality and fungal burden (98, 102, 105), their role in the host’s innate response to the fungus has not been directly examined.

5. Other Cells

Several groups have documented the *in vitro* antimicrobial activity of human platelets against *Aspergillus* species. Platelets bind plasma-opsonized hyphae and degranulate (155). The interaction of platelets with hyphae results in reduced

hyphal galactomannan release, impaired hyphal elongation, and loss of hyphal wall integrity, and these effects were inhibited when granule exocytosis was blocked (155, 156). Serotonin, a component of platelet granules, has also been found to kill both conidia and hyphae and to induce damage to fungal cell membranes (157, 158).

The involvement of airway and alveolar epithelial cells in the recognition of *Aspergillus* species has received less attention, although these cells are clearly the first cells to encounter the inhaled organism. In both immunocompetent and neutrophil-depleted mice, ciliated airway epithelial cells can ingest fungal elements (Fig. 3). Similarly, human nasal ciliated epithelial cells phagocytose and kill conidia *in vitro* (159). A human alveolar epithelial cell line (A549) can bind both *Aspergillus* conidia and hyphae, ingest conidia, and generate IL-6 and CXCL8 in response to them (160, 161). Recent studies of a human bronchial epithelial cell line (BEAS-2B) have also demonstrated a time-dependent synthesis of CXCL8 in response to germinated *Aspergillus* elements (swollen conidia, hyphae, or both) but not resting conidia (162). Interestingly, the epithelial release of CXCL8 was dependent on NF-κB activation but was independent of the TLR-MyD88 pathway, indicating redundant pathways for epithelial recognition and responses to *Aspergillus* species (162).

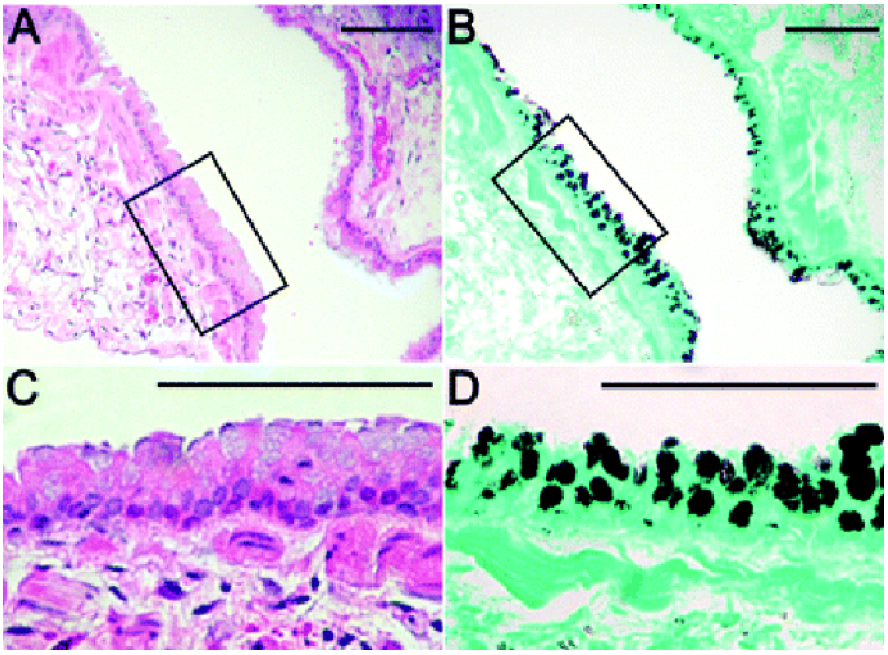

A
B
C
D

Figure 3

Association of *Aspergillus* species with bronchial epithelial cells *in vivo*. Shown is representative lung histology from neutropenic mice 3 days after intratracheal challenge with a sublethal inoculum of *Aspergillus* conidia, with sequential sections stained with hematoxylin and eosin to show mammalian cell morphology (A and C) and Grocott's methenamine silver to demonstrate fungal elements (B and D). (A and B) Extensive association of fungal elements with airway lining. (C and D) Higher-power images of the boxed areas in A and B showing that most of the fungal material appears to be intracellular. All scale bars are 20 μm; original magnifications, x100 (A and B) and x400 (C and D).

Data from Ref **(5)**

Murine Model of Neutropenic Invasive Aspergillosis

The immune mechanisms operating in immunocompromised patients against tissue-invading hyphae are quite different from those employed by normal hosts to clear conidia from the lumen of airways and alveoli: conidia and hyphae are antigenically distinct (179), are encountered in different tissue compartments (180, 181), and elicit disparate immune responses (45). The best defined defense innate mechanisms of immunocompromised host to invading *Aspergillus* hyphae involves neutrophils. As previously mentioned, clinical studies have shown that defects in neutrophil number and function are the most pervasive risk factor for the development of invasive aspergillosis in diverse populations of patients (119-123). Profound and prolonged neutropenia has been shown to correlate with the greatest increased risk for pulmonary invasive aspergillosis (120, 182). Since selective depletion of neutrophils establishes an invasive infection without affecting other components of the immune system and since recovery of neutrophils often occurs in patients with invasive aspergillosis, a murine model of invasive aspergillosis with transient antibody-mediated neutrophil depletion had been developed and thoroughly characterized to be used to study the host's innate response to *Aspergillus* (82, 84, 94, 96, 98, 103).

There are currently no genetic approaches to achieve neutropenia in experimental models. For this model, neutropenia is induced with a complement-

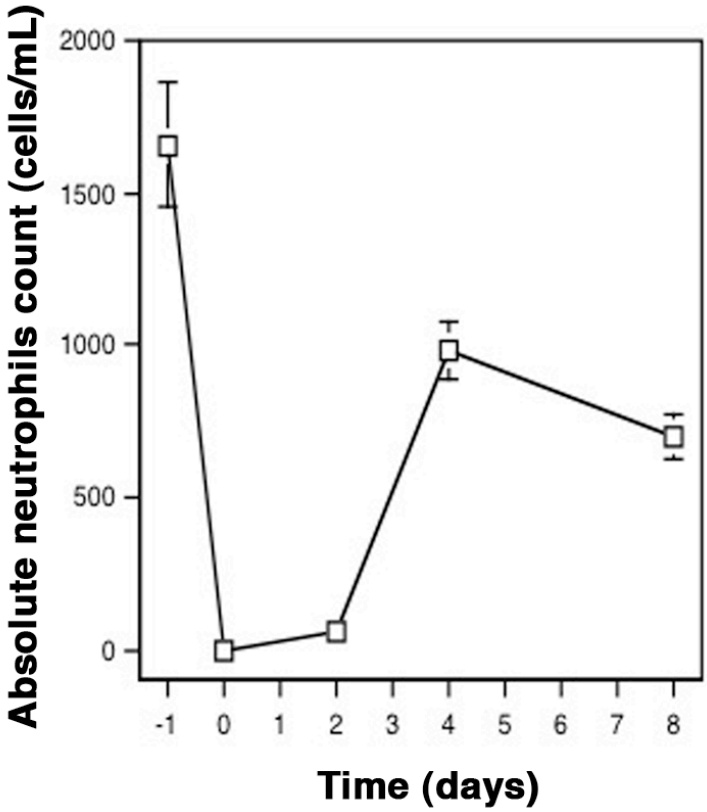
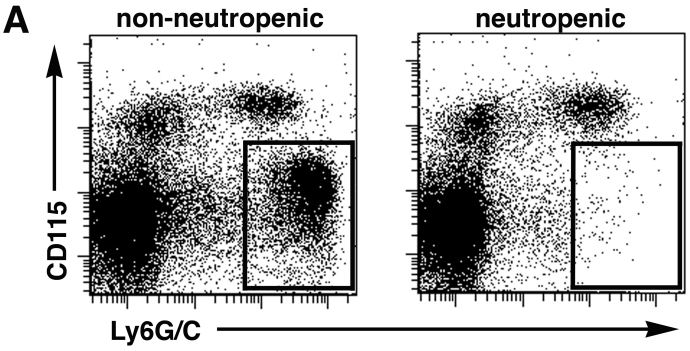

Absolute neutrophils count (cells/mL)
2000
1500
1000
500
0
-1
0
1
2
3
4
5
6
7
8
Time (days)

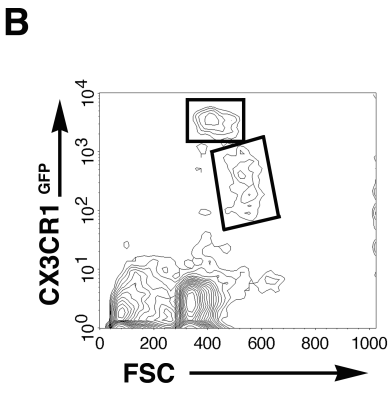
Figure 4

Peripheral neutrophil analysis. Absolute neutrophil count over time in uninfected mice after administration of monoclonal anti-neutrophil antibody (Gr-1; RB6-8C5). Concentration of blood neutrophils (per mL) were calculated from total leukocyte counts and differential counts from peripheral smears. n=4 per timepoint.

Data from unpublished observations (Mehrad Lab)

fixing rat anti-mouse monoclonal antibody GR-1 (clone RB6-8C5). A single intraperitoneal administration of this monoclonal antibody (mAb) results in a rapid drop in absolute peripheral neutrophil count that lasts for 2-3 days (Fig 4). The circulating neutrophil count returns by day 5. Inherent to antibody-mediated approaches are concerns over specificity of neutrophil depletion and potential “off target” immune complex-mediated effects (183). Gr-1 reacts with 2 cell surface markers: Ly-6G, an antigen expressed by neutrophils, inflammatory monocytes (184), subset of myeloid-derived suppressor cells (MDSCs) (185, 186) and, with 10-to 100-fold lower affinity, Ly-6C, an antigen expressed by multiple leukocyte subsets [(187) and our unpublished observations]. While administration of large quantities of Gr-1 can deplete Ly-6C- and LY6G-expressing cells, the administration of titrated doses results in neutropenia without detectable effect on the number of monocyte (Fig 5) and dendritic cell (Fig 6) subsets in both naive and infected animals. In addition, the administration of isotype control Ab does not appear to influence leukocyte numbers [Fig 5-6 and (59, 98, 188)]. The issue regarding the depletion of MDSCs still remains and is complicated by the fact that currently it is difficult to distinguish the granulocytic MDSC from neutrophils. These MDSCs have co-expression of CD11b, LY6G, and LY6C (189) and no other unique surface marker to separate them from neutrophils by flow cytometry . In this model, the mice are given an intratracheal inoculation of *Aspergillus* conidia 1 day after mAb administration results in an acute infection


A
non-neutropenic
neutropenic
CD115
Ly6G/C


B
CX3CR1 GFP
FSC
0
200
400
600
800
1000

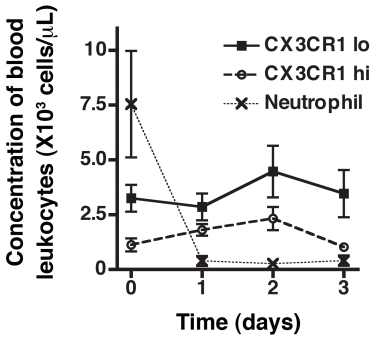

Concentration of blood leukocytes (X10³ cells/μL)
CX3CR1 lo
CX3CR1 hi
Neutrophil
10
7.5
5.0
2.5
0
0
1
2
3
Time (days)

Figure 5

Blood monocyte analysis in non-neutropenic and neutropenic mice. Representative flow cytometry plots in mice with administration of the Gr-1 mAb (anti-Ly-6G/C ; clone RB6-8C5) or isotype control Ab on day 3 of infection, representative data from 2 experiments, n = 4 mice per group. (B) Representative flow cytometry plot and quantitative data in uninfected CX3CR1 GFP/+ mice. CX3CR1 monocytes subsets and neutrophils were enumerated following administration of Gr-1 mAb or control antibody on day -1. Data shown represent mean +/- SEM of n = 4 mice per group.

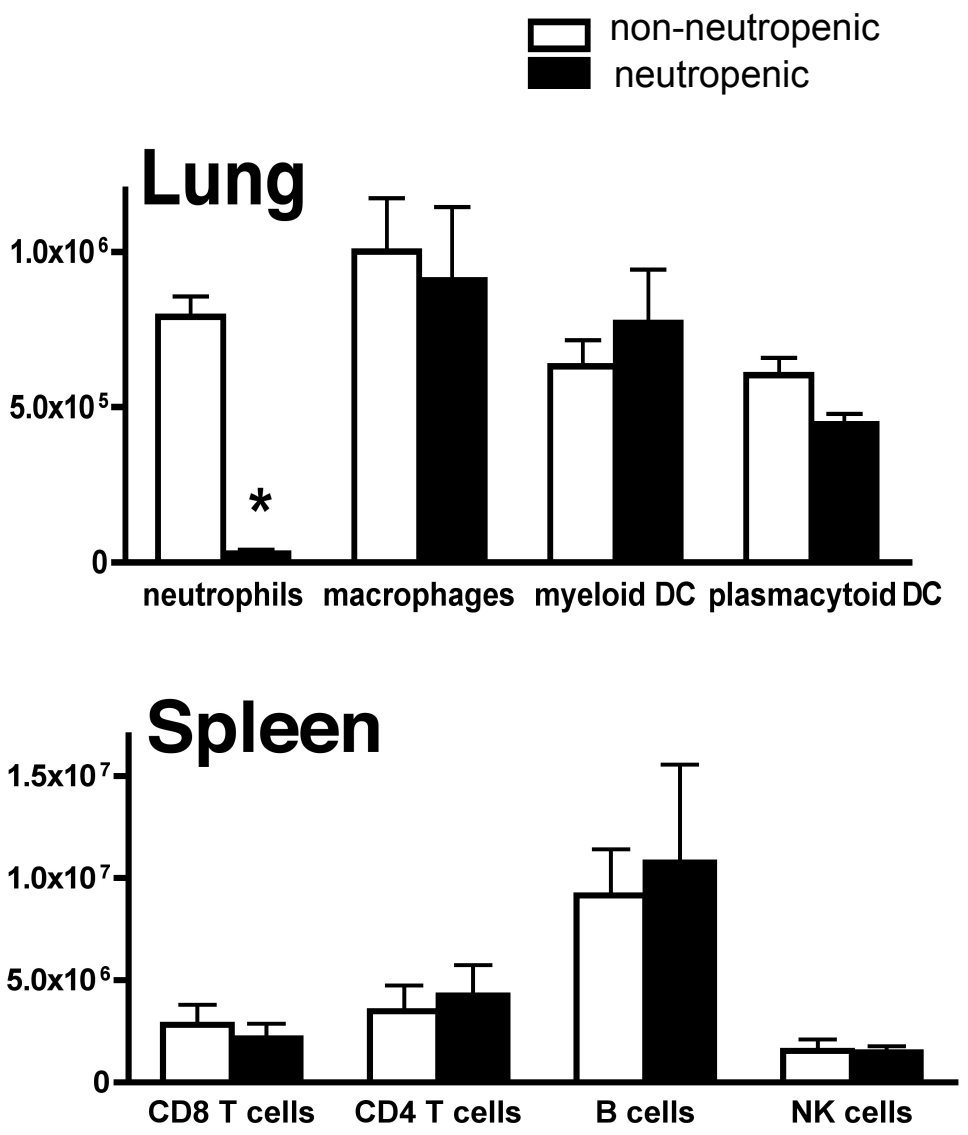
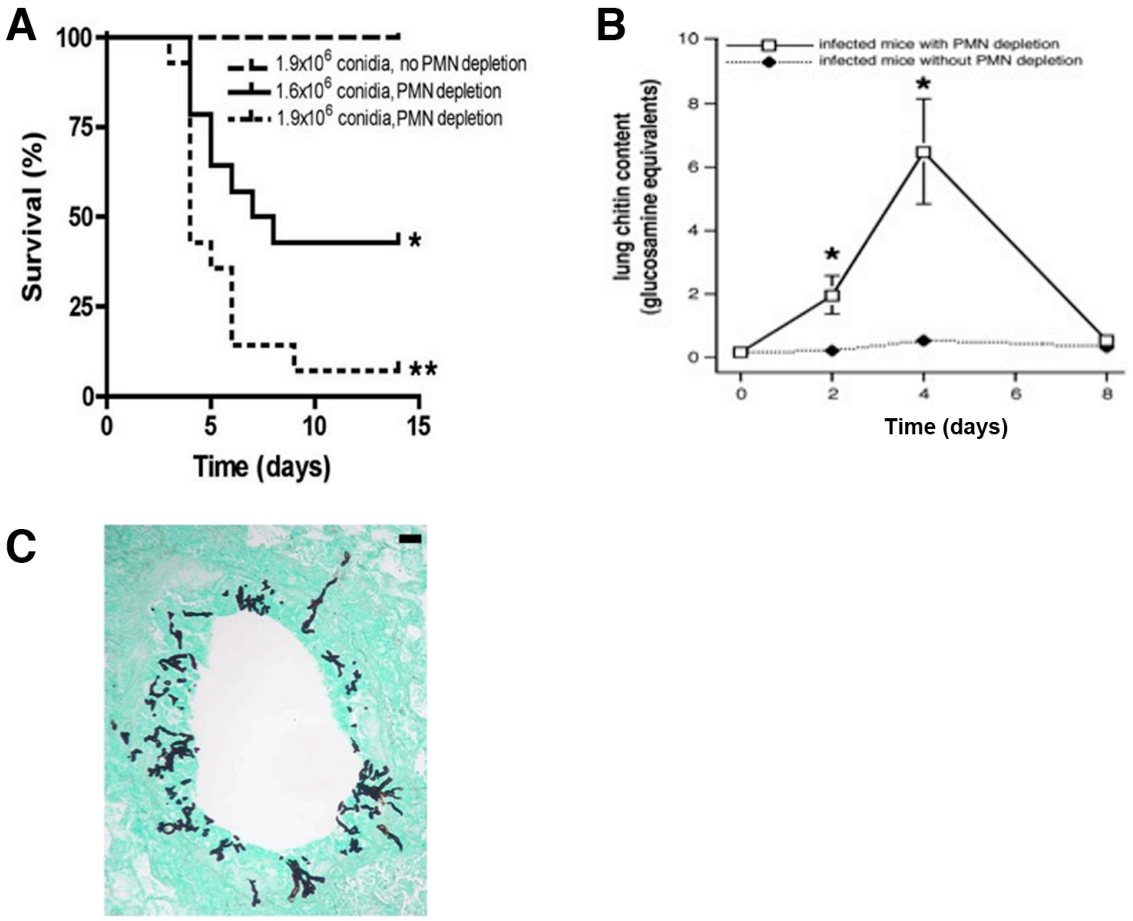

non-neutropenic
neutropenic
Lung
1.0x10 6
5.0x10 5
0
*
neutrophils
macrophages
myeloid DC
plasmacytoid DC
Spleen
1.5x10 7
1.0x10 7
5.0x10 6
0
CD8 T cells
CD4 T cells
B cells
NK cells

Figure 6

Lung and spleen leukocyte analysis in neutropenic and non-neutropenic mice. Number of leukocyte subsets of uninfected mice on day 1 after administration of Gr-1 or isotype control mAb. Absolute numbers of lung and spleen leukocytes were calculated after identification by flow cytometry. Mean ± SEM; n=4-6 per group. *, p,0.05 compared to isotype control group.

and characterized by a dose-dependent mortality (Fig 7A). Maximal fungal burden (as measured by chitin content) was found to be on day 4 of infection in neutropenic mice, with clearance of fungi from lungs in all mice that survive out to day 8 (Fig 7B). The observed lung histological pattern of fungal invasion from the airway into tissue is very similar to human infections (Fig 7C).

In humans, neutropenia is typically the result of treatment with cytotoxic drugs that influence both the number and function of multiple, often incompletely defined, lineages of cells in addition to neutrophils. In experimental models that seek to examine the consequences of neutropenia, the means of depleting neutrophils is an important methodological consideration. While it may be seem more suitable to develop an animal model using a clinically relevant route of immunosuppression through the use of corticosteroids and cytotoxic drugs, the wide-spread effect of the drugs on the immune system makes understanding the mechanism of host defense complex and difficult. Animals treated with cyclophosphamide are depleted of multiple lymphocyte subsets in addition to neutrophils, and animals treated with corticosteroids have normal numbers ofneutrophils but multiple defects in antimicrobial immune responses (190, 191).



Data from unpublished observation (Mehrad Lab)

Figure 7

Outcome of infection in mice with invasive aspergillosis. (A)Survival study of neutropenic and non-neutropenic mice challenged with *Aspergillus* conidia. n=15 per group, *,** $p<0.05$ compared to group without PMN depletion. (B) Measurement of lung chitin content as a surrogate for fungal burden. n=6 per group per timepoint; *, $p<0.05$ compared to non-neutrophil depleted mice. (C) Representative Grocott's methenamine silver (to demonstrate fungal elements) stain 3 days after onset of infection. Original magnification were 100x.

Rationale and Research Goals

Invasive aspergillosis is an increasingly common fungal infection with a poor prognosis in immunocompromised patients(192). Several factors contribute to the difficulty in managing this fungal infection. First, the growing incidence of invasive aspergillosis is attributed to the expanding number of immunocompromised patients. An autopsy study over a 12 year period documented a 132% increase in this diagnosis (193) while another center reported a three-fold increase in annual incidence between 1990-98 (194). The overall incidence of invasive aspergillosis is reported at 12.4 cases/million/year in the US. Second, invasive aspergillosis is difficult to diagnose and available therapy is limited by a sub-optimal response rate (195). Due to the rapid progression (1 to 2 weeks from onset to death) and severity of this infection, clinicians treat patients before the diagnosis is confirmed (192). Waiting for a confirmed diagnosis runs the risk of untreatable invasive aspergillosis, since fungal burden can reach a level too high for antifungal therapy. Finally, the crude mortality rate of invasive aspergillosis remains high. The annual death rate from invasive aspergillosis has increased in the US to nearly 1 in 100,000 population (196). In the latest systemic review of literature, the case-fatality rate of invasive aspergillosis was 58% (197). In recent studies of large samples, a majority of patients died from the infection while receiving appropriate treatment (198-200).

As previously described, innate immunity can be viewed as a multi-level system of defense, consisting of physical barriers to impede microbial entry; soluble and cell-bound pattern-recognition molecules that mediate microbial recognition; and an array of anti-microbial effector mechanisms, which include leukocyte recruitment and activation and numerous microbicidal activities. The majority of work involved in understanding the innate immune response to *Aspergillus* has been focused on host recognition of the fungus. Studies that have examined innate effector functions have mostly involved the roles of neutrophils and alveolar macrophages in this infection. The effector mechanisms of other innate leukocytes remains unclear. There is an increasing number of nonneutropenic immunocompromised patients with invasive aspergillosis that underlines the importance of nonneutrophil defense mechanisms in preventing this infection. Previous work have shown that NK and dendritic cells are important in host defense against invasive aspergillosis (98, 103). This dissertation is designed to define and understand the effector functions of these cells in the setting of neutropenic invasive pulmonary aspergillosis.

Chapter 2 Early NK Cell-derived IFN-γ Is Essential to Host Defense in Neutropenic Invasive Aspergillosis

Abstract

NK cells have previously been found to be important in host defense against invasvie aspergillosis, but the mechanism of this effect is not known. We hypothesized that NK cells mediate their protective effect in invasive aspergillosis by acting as the major source of IFN-γ during early infection. We found that, in the lungs of neutropenic mice with invasive aspergillosis, NK cells were the major population of cells capable of generating IFN-γ during early infection. Depletion of NK cells resulted in reduced lung IFN-γ levels and increased lung fungal load that was independent of T and B cell subsets. Depletion of NK cells and absence of IFN-γ resulted in a similar increase in susceptibility to the infection, but depletion of NK cells in IFN-γ-deficient hosts did not result in further increase in severity of the infection. NK cell-derived IFN-γ caused enhanced macrophage antimicrobial effects *in vitro* and also resulted in greater expression of IFN-inducible chemokines in the lungs. Finally, transfer of activated NK cells from wild-type, but not IFN-γ-deficient hosts, resulted in greater pathogen clearance from the lungs of both IFN-γ-deficient and wild-type recipients. Taken together, these data indicate that NK cells are the main source of early IFN-γ in the lungs

in neutropenic invasive aspergillosis, and this is an important mechanism in the defense against this infection.

Introduction

Invasive aspergillosis is among the most common human fungal infections and is often characterized by a poor outcome, even with appropriate antimicrobial therapy (201). Normal hosts uniformly eliminate inhaled *Aspergillus* conidia without developing disease, but in immunocompromised hosts, the conidia can germinate to form multicellular filaments, known as hyphae, that penetrate the respiratory epithelium and produce a severe pneumonia, invasive pulmonary aspergillosis (202). Despite advances in antifungal therapy, invasive aspergillosis continues to carry a poor prognosis and there is a pressing need to better understand the host defense mechanisms in this infection.

While the key risk factors that predispose the host to the development of invasive aspergillosis has primarily involved neutrophils, the immunological defects in patients with invasive aspergillosis are complex and can extend beyond neutrophil deficiency. The increasing proportion of nonneutropenic immunocompromised patients succumbing to invasive aspergillosis (119, 194, 200) underscores the importance of understanding other cellular mechanisms of host defense in this infection. In the context of animal models, the importance of Th1-acquired immunity is established as being critical to protective responses in invasive aspergillosis (163-165). We and others have previously observed the early expression of IFN-γ in the lungs of immunocompetent and neutropenic mice

challenged with the microorganism (80, 94). Importantly, while the cellular source of IFN-γ during late infection is CD4 T cells (15, 55, 164), the cellular source of IFN-γ early in the course of invasive aspergillosis is unknown.

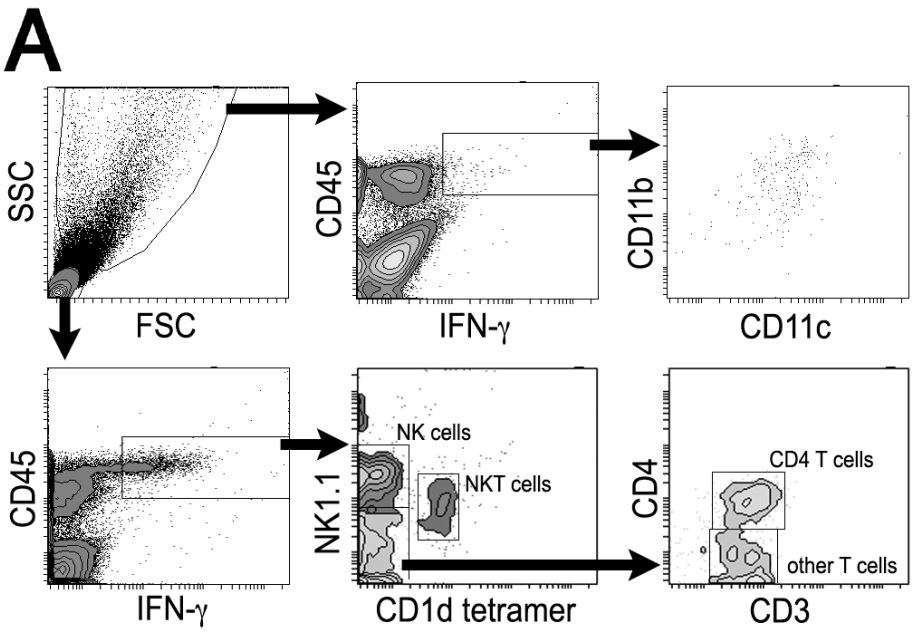
We have previously found that, in the context of early innate immunity, NK cells are essential to immune-mediated defense against invasive aspergillosis in neutropenic mice (103). NK cell-derived IFN-γ has been shown to be required for successful elimination of many viral (203-205), bacterial (206, 207), and protozoal (208, 209) pathogens, as well as the yeast Cryptococcus (210-212). We therefore tested the hypothesis that NK cells mediate their protective effect in invasive aspergillosis by acting as the major source of IFN-γ during early infection.

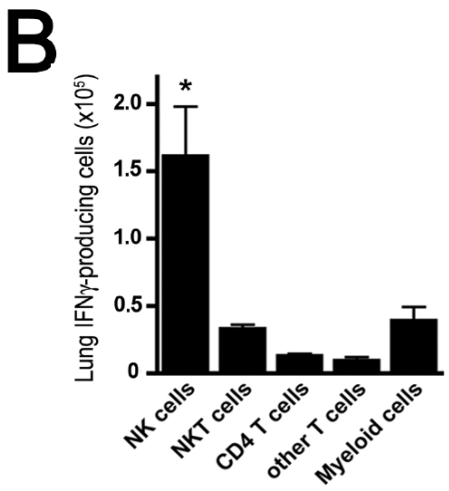
Results

NK cells as the major cellular source of lung IFN-γ in early invasive aspergillosis

Given that early expression of IFN-γ in the lungs in response to *Aspergillus* has previously been documented (80, 94), we began by examining lungs of neutropenic mice with invasive aspergillosis for cells capable of IFN-γ production (Figure 1A-B). We found lung NK cells to be the largest population of IFN-γ-producing cells in the context of the infected lungs, with NKT cells, other T cell subsets, and myeloid cells making up much smaller numbers of IFN-γ-producing cells. We next examined the effect of depletion of NK cells on lung IFN-γ levels during the infection. In the context of neutropenia, depletion of NK cells with anti-NK1.1 mAb (clone pk136) resulted in reduction in lung levels of IFN-γ in the first 2 days of the infection (Figure 1C).

The NK1.1 Ag is also expressed, at a lower density, on a subset of NKT cells. We sought to ascertain that the observed effect of anti-NK1.1 was not related to an effect on lung NKT cells. Depletion of NK cells in WT mice with invasive aspergillosis using anti-asialo GM1 (which does not influence NKT cell populations) resulted in comparable reduction of lung IFN-γ levels to the effect achieved with anti-NK1.1 (Figure 2A). Similarly, depletion of classical NK cells in neutropenic RAG-deficient animals, which lack T cell subsets including NKT cells, also resulted in reduction in lung IFN-γ levels (Figure 2A). Interestingly,


A
SSC
FSC
CD45
IFN-γ
CD11b
CD11c
CD45
IFN-γ
NK1.1
CD1d tetramer
NK cells
NKT cells
CD4
CD3
CD4 T cells
other T cells


B
Lung IFNγ-producing cells (x10⁵)
2.0
1.5
1.0
0.5
0
*
NK cells
NKT cells
CD4 T cells
other T cells
Myeloid cells

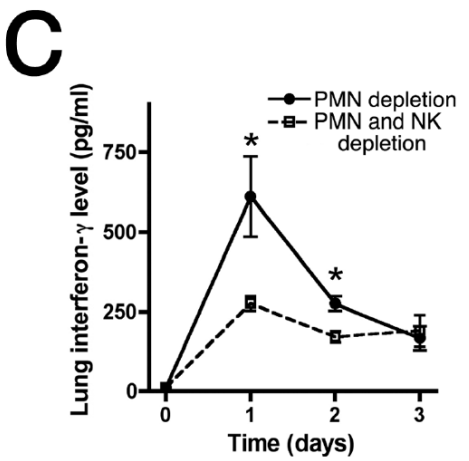

C
PMN depletion
PMN and NK depletion
Lung interferon-γ level (pg/ml)
750
500
250
0
*
*
0
1
2
3
Time (days)

Figure 1.

Cellular source of lung IFN-γ in neutropenic mice with invasive aspergillosis. (A-B) Gating scheme and summary data of flow cytometry of lung single cell suspensions on day 1 of infection. Data are representative of four mice per group. (C) Lung IFN-γ protein level in lung homogenates at various time points after onset of infection as measured by ELISA. Both groups were neutrophil-depleted; NK cell depletion was achieved with anti-NK1.1. Day 0 represents uninfected animals. n = 10–12 mice/group in two separate experiments. *, $p < 0.05$ compared with other groups.

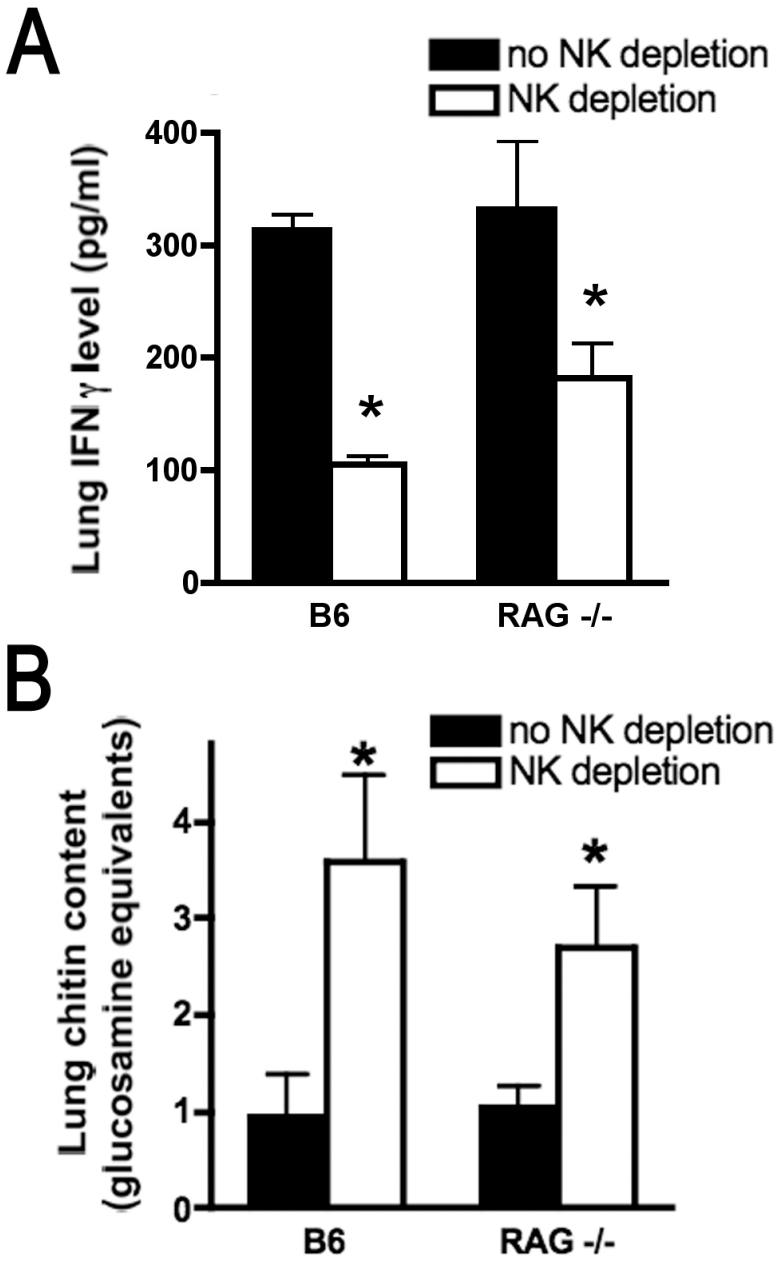

A
no NK depletion
NK depletion
Lung IFNγ level (pg/ml)
400
300
200
100
0
*
*
B6
RAG -/-
B
no NK depletion
NK depletion
Lung chitin content
(glucosamine equivalents)
4
3
2
1
0
*
*
B6
RAG -/-

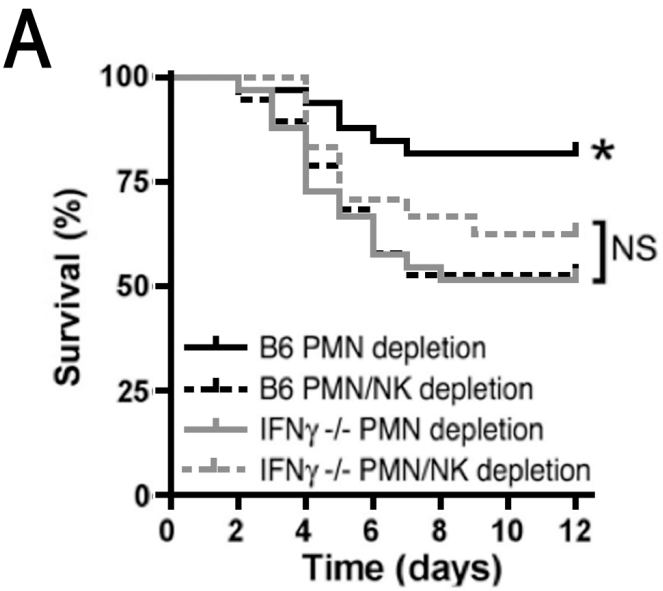
Figure 2.

Effect of NK cell depletion on lung IFN-γ and fungal content in WT and RAG-1 deficient mice. Lung IFN-γ levels (A) and chitin content (B) were measured on days 1 and 3 of infection, respectively. All groups were neutrophil depleted; NK cell depletion was achieved with anti-asialo-GM1. n = 6 mice/group; *, $p < 0.05$ compared with mice of the same genetic background without NK depletion.

lung IFN-γ levels were markedly reduced but remained detectable in the lungs of RAG-deficient animals with NK cell depletion, suggesting a nonlymphocyte source for IFN-γ in this context. Depletion of NK cells resulted in a parallel increase in lung chitin content on day 3 of infection in both WT and RAG-1-deficient mice with neutropenia (Figure 2B), indicating that, during early infection, NK cells are necessary for host defense and their presence correlated with lung IFN-γ content. In addition, the lung fungal content did not differ significantly between neutropenic WT and RAG-1-deficient animals, suggesting that, at this early point in the infection, clearance of the microorganism is independent of acquired immunity.

NK cell-derived IFN-γ exerts a protective role in invasive aspergillosis

To specifically link NK cell-derived IFN-γ to host defense during invasive aspergillosis, we next compared the effect of NK cell depletion on the severity of infection in WT and IFN-γ-deficient mice. As expected, an LD20 inoculum of *Aspergillus* conidia for neutropenic WT animals resulted in higher mortality in WT mice with depletion of both neutrophils and NK cells. Mortality from the infection in WT mice with NK cell depletion was comparable to the increased mortality observed in neutropenic IFN-γ-deficient animals. Furthermore, depletion of NK cells in neutropenic IFN-γ-deficient mice did not result in further increase in mortality (Figure 3A). Similar results were found when lung fungal content of these groups were compared: On day 3 of infection, depletion of NK cells,


A
100
75
50
25
0
Survival (%)
0
2
4
6
8
10
12
Time (days)
*
NS
B6 PMN depletion
B6 PMN/NK depletion
IFNγ -/- PMN depletion
IFNγ -/- PMN/NK depletion

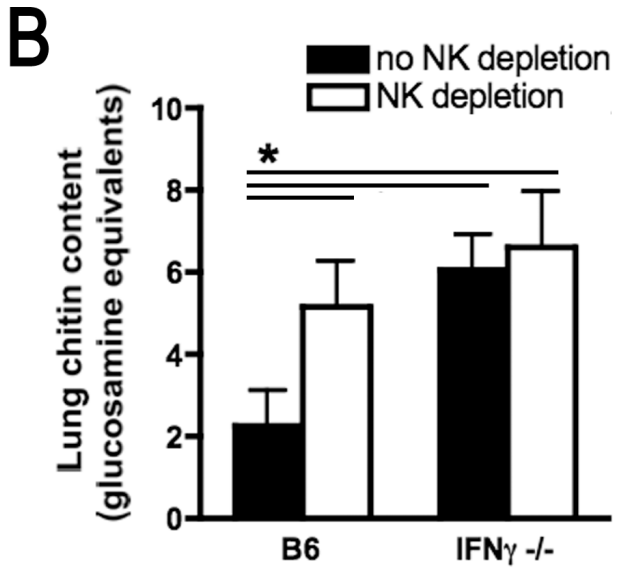

B
no NK depletion
NK depletion
Lung chitin content
(glucosamine equivalents)
10
8
6
4
2
0
*
B6
IFNγ -/-

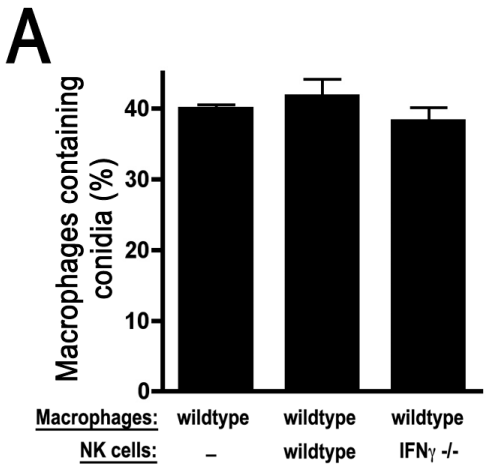
Figure 3.

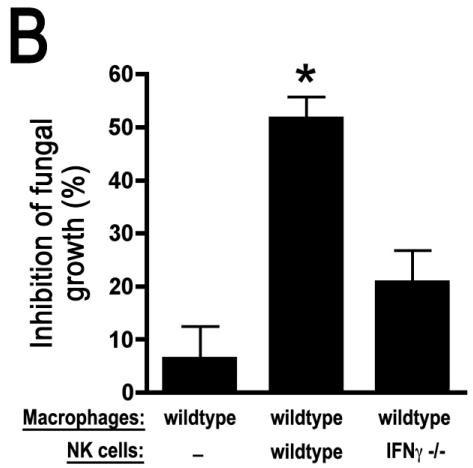
Role of NK cell-derived IFN-γ in outcome of neutropenic invasive aspergillosis. (A) Survival study comparing neutropenic WT and IFN-γ-deficient mice with invasive aspergillosis with or without NK cell depletion after infection with an LD20 inoculum for neutropenic WT mice. n = 24–35 mice/group, pooled from three experiments. (B) Lung chitin content on day 3 on infection. n = 14–18 mice/group, pooled from three experiments. *, $p < 0.05$ compared with each of the other experimental groups.

absence of IFN-γ, or depletion of NK cells in IFN-γ-deficient hosts each resulted in a comparable 2- to 3-fold increase in lung fungal content as compared with WT neutropenic mice (Figure 3B). These data indicate that, in the setting of IFN-γ deficiency, NK cells do not contribute measurably to host defense against this infection, and that, in the absence of NK cells, generation of IFN-γ by other cellular sources does not measurably affect host responses in this model. These observations support the hypothesis that NK cell-derived IFN-γ mediates host defense in this infection.

Antimicrobial mechanisms of NK cell-derived IFN-γ

To assess the mechanism by which NK cell-derived IFN-γ mediates antimicrobial effects, we examined the *in vitro* interaction of NK cells, macrophages, and *Aspergillus* elements. Resident alveolar macrophages are critical to early defense of the lung against many microorganisms, including *Aspergillus* (47, 114, 115). We therefore first examined the effect of NK cells on the efficiency of alveolar macrophage phagocytosis of conidia. Coincubation of alveolar macrophages with either WT or IFN-γ-deficient NK cells was found to have little effect on the ability of alveolar macrophages to ingest conidia (Figure 4A). When incubation periods were extended to 16 hours to allow conidial germination and formation of hyphae, however, alveolar macrophages incubated with WT NK cells induced 2- to 8-fold greater fungal killing as compared with macrophages alone


A
Macrophages containing conidia (%)
0
10
20
30
40
Macrophages: wildtype wildtype wildtype
NK cells: – wildtype IFNγ -/-


B
Inhibition of fungal growth (%)
0
10
20
30
40
50
60
*
Macrophages: wildtype wildtype wildtype
NK cells: – wildtype IFNγ -/-

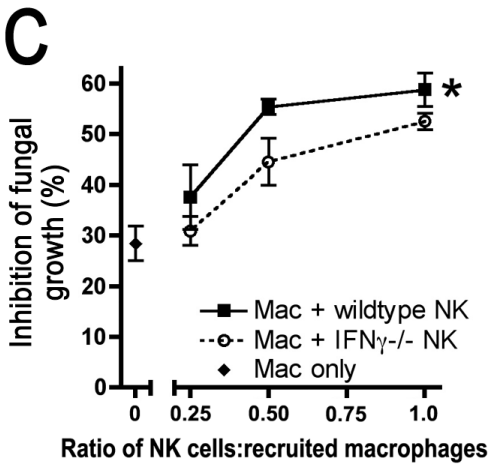

C
Inhibition of fungal growth (%)
0
10
20
30
40
50
60
*
Mac + wildtype NK
Mac + IFNγ-/- NK
Mac only
0
0.25
0.50
0.75
1.0
Ratio of NK cells:recruited macrophages

Figure 4.

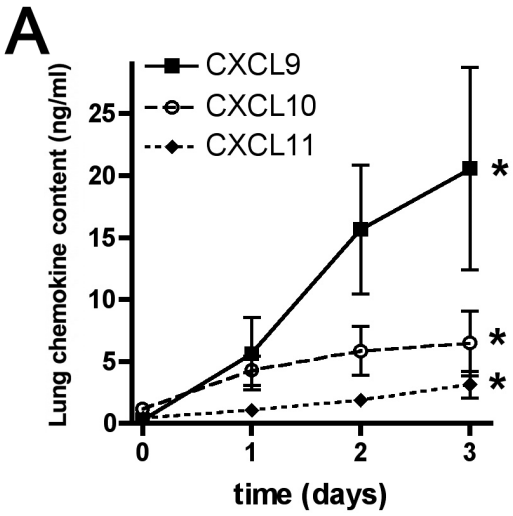
Effect of NK cell-derived IFN-γ on macrophage antimicrobial functions *in vitro*. (A-B) Effect of NK cells on alveolar macrophage phagocytosis and killing of *Aspergillus* elements. Macrophages with or without NK cells from various donors were co-cultured for 3 h before addition of conidia. Phagocytosis was determined after 1 h and fungal growth was measured after 16 h. *, $p < 0.05$ compared with each of the other experimental groups. (C) Effect of NK cells on inflammatory macrophage killing of *Aspergillus* hyphae. Macrophages from WT mice and activated NK cells from various donors were incubated at the indicated ratios with preformed hyphae. *, $p < 0.05$ compared with macrophages alone and macrophages incubated with IFN-γ-deficient NK cells.

or macrophages incubated with IFN-γ-deficient NK cells (Figure 4B). These data indicate that, in the confines of this *in vitro* coculture system, NK cell-derived IFN-γ results in inhibition of hyphal growth from resting conidia.

In addition to resident alveolar macrophages, there is an influx of inflammatory monocyte/macrophages into the lungs during invasive aspergillosis. Based on our prior observations regarding the timing of influx of these cells to the lungs (98), we reasoned that recruited macrophages are likely to encounter preformed hyphae rather than conidia upon their arrival to the lungs. Although neutrophils constitute the primary defense against the hyphal form of *Aspergillus*, monocytic phagocytes are capable of hyphal killing and constitute an important line of defense in neutropenic hosts (92, 113). Because the design of the experiments depicted in Figure 4B did not differentiate between activity against resting conidia, swollen conidia, or hyphae, we next examined the capacity of NK cell-derived IFN-γ to mediate fungicidal activity against preformed hyphae in the presence of inflammatory monocyte/macrophages. Incubation of increasing numbers of both WT and IFN-γ-deficient NK cells with inflammatory macrophages resulted in measurable increases in hyphal killing, but this effect was significantly greater with WT NK cells (Figure 4C). In the absence of macrophages, however, activated NK cells only induced detectable hyphal killing when the number of NK cells exceeded the number of fungal cells (data not shown), a circumstance that is likely not relevant to the *in vivo* setting of infection.

Thus, these data indicate that NK cell-derived IFN-γ is required for optimal macrophage-mediated anti- fungal effects. In addition, IFN-γ-deficient NK cells were found to augment macrophage antifungal effects to a lesser degree; this effect did not result in improved fungal clearance *in vivo* (Figure 3).

In addition to direct immunostimulation of phagocytes, we sought to determine whether NK cell-derived IFN-γ could affect local expression of mediators during the infection. We therefore examined the role of NK cells in mediating expression of IFN-inducible CXC chemokines, CXCL9, CXCL10, and CXCL11. There was a marked induction of CXCL9, CXCL10, and CXCL11 in the lungs during early invasive aspergillosis, with greater induction of CXCL9 and CXCL10 as compared with CXCL11 (Figure 5A). Depletion of NK cells in this context resulted in significant reduction in expression of CXCL9 and CXCL10 in WT mice, indicating that NK cells are necessary for optimal expression of these CXC chemokines in the lungs during invasive aspergillosis (Figure 5B). As expected, IFN-γ-deficient mice had lower lung levels of CXCL9, CXCL10, and CXCL11 compared with WT animals; but lung levels of these chemokines in IFN-γ-deficient mice was not substantially affected by the depletion of NK cells (Figure 5B). This indicates that the effect of NK cells on the lung expression of CXCL9 and CXCL10 is dependent on IFN-γ, which is consistent with the hypothesis that this effect is attributable to NK cell-derived IFN-γ. Interestingly, lung levels of


A
CXCL9
CXCL10
CXCL11
Lung chemokine content (ng/ml)
25
20
15
10
5
0
0
1
2
3
time (days)
*
*
*

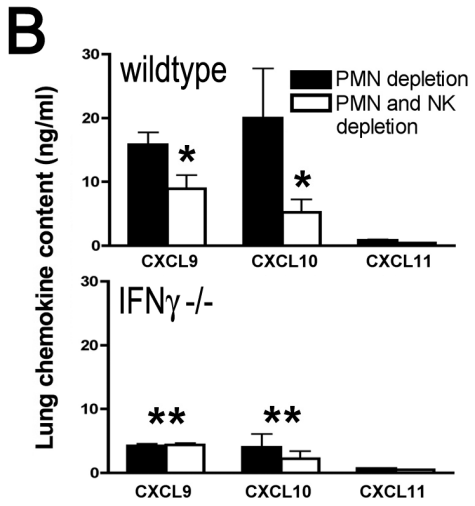

B
wildtype
PMN depletion
PMN and NK depletion
Lung chemokine content (ng/ml)
30
20
10
0
*
*
CXCL9
CXCL10
CXCL11
IFNγ -/-
30
20
10
0
**
**
CXCL9
CXCL10
CXCL11

Figure 5.

Protein levels of CXCL9, CXCL10, and CXCL11 in lung homogenates in neutropenic mice with invasive aspergillosis. (A) Lung chemokine levels at various time points after inoculation. Day 0 represents uninfected animals. Data represent mean ± SEM; n = 5– 6 mice per time point; *, $p < 0.05$ for increase over time for each chemokine. (B) Lung chemokine levels in neutropenic C57BL/6 and IFN-γ gene knockout mice on C57BL/6 background with or without NK depletion on day 3 of infection. Data represent mean ± SEM; n = 5–6 mice per group; *, $p < 0.05$ compared with no NK depletion; **, $p < 0.05$ compared with WT.

CXCL9 and CXCL10 were somewhat higher in WT NK cell-depleted mice as compared with IFN-γ deficient mice with NK depletion; this effect may be attributable to non-NK cells sources of IFN-γ in lungs of WT mice, as noted in Figure 1. Taken together, Figs. 4 and 5 suggest that NK cell-derived IFN-γ influences host defense during invasive aspergillosis by several mechanisms, including stimulation of phagocyte antifungal effects as well as induction of IFN-inducible CXC chemokines.

NK cell transfer mediates a protective effect in hosts with invasive aspergillosis

We next sought to determine whether the transfer of exogenous NK cells could improve the outcome of experimental invasive as- pergillosis. The transfer of activated NK cells from WT, but not IFN-γ-deficient, donors reversed the susceptibility of IFN-γ-deficient recipients to invasive aspergillosis (Figure 6A). Interestingly, the transfer of these highly activated WT NK cells to IFN-γ-deficient hosts resulted in lower lung fungal content as compared with infected WT mice that did not receive cell transfer (Figure 6A). This finding led us to examine the utility of NK cell transfer as a therapeutic strategy in WT mice inoculated with lethal (LD100) infectious inocula. We found that the transfer of WT, but not IFN-γ-deficient, NK cells to neutropenic recipients resulted in lower lung fungal burden in WT hosts (Figure 6B). Taken together, these data indicate that NK cells are the main source of early IFN-γ in neutropenic invasive aspergillosis, and this mechanism is an important mechanism in the early defense against this infection.

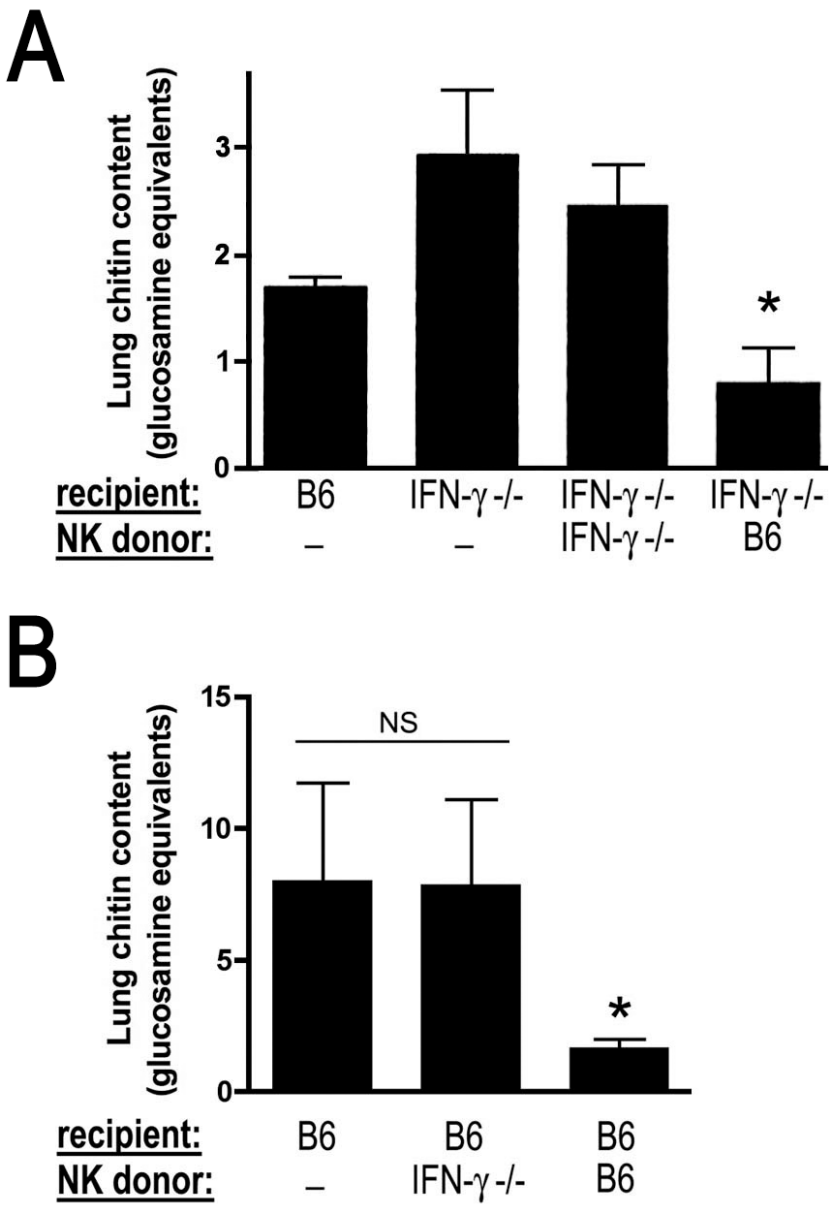

A
Lung chitin content
(glucosamine equivalents)
0
1
2
3
*
recipient:
NK donor:
B6
–
IFN-γ -/-
–
IFN-γ -/-
IFN-γ -/-
IFN-γ -/-
B6
B
Lung chitin content
(glucosamine equivalents)
0
5
10
15
NS
*
recipient:
NK donor:
B6
–
B6
IFN-γ -/-
B6
B6

Figure 6.

Effect of NK cell transfer on lung fungal clearance in neutropenic invasive aspergillosis. Lung chitin content on day 3 of invasive aspergillosis after infection with an LD20 (A) or LD100 (B) inocula for neutropenic mice after adoptive transfer of activated NK cells. Animals were given PBS, or activated NK cells from various donors on days 1 and 2 of infection. *, $p < 0.05$ compared with each of the other groups. Data shown represent mean ± SEM of n = 5–12 mice per group.

Discussion

The innate defenses against inhaled *Aspergillus* conidia include the physical barriers of the respiratory tract, recognition of the pathogen via cell-bound and soluble pattern-recognition receptors, and a complex array of cellular and soluble effector mechanisms. The cellular effector mechanisms have long been recognized to include myeloid cells, specifically neutrophils, recruited and resident monocyte/macrophages, and dendritic cells, but less in known about the role of innate lymphocytes in this infection. NK cells have been strongly implicated in early defenses against many infections (213), including defense against several viral and bacterial pathogens that cause pneumonia (214-217). The role of NK cells in pneumonia is compatible with the large numbers of resident NK cells found in normal lungs, which is only exceeded by the number of NK cells found in the spleen (141-143, 145). In addition, NK cells are capable of homing to inflamed tissues via several chemokine receptor-ligand interactions (141). We have previously found NK cells to be essential to early innate immunity in invasive aspergillosis in a neutropenic mouse model (103), but the mechanism of their contribution to host defense in this infection has not been defined.

NK cells were first described for their ability to mediate direct cytotoxicity against tumor cells, a finding that was subsequently extended to virally infected cells and transplanted cells. In addition to this cytotoxic effector function, NK cells

contribute to innate immunity and to the development of Th1 acquired immunity by mediating a potent regulatory effect via elaboration of several key cytokines, notably TNF-α and IFN-γ. In this context, the importance of Th1 immunity to defense against *Aspergillus* infection was initially noted when susceptibility of different inbred mouse strains to aspergillosis was found to correlate with their production of Th1 cytokines (163, 164). In mouse models that use cytotoxic drugs to mimic human immunosuppression, IFN-γ knockout animals are more susceptible to invasive aspergillosis (165). In contrast, vaccination with *Aspergillus* proteins with Th1-priming adjuvant (154), exogenous administration of IFN-γ (166, 167), or administration of Th1 T cells (218) or Ag-pulsed dendritic cells (168) result in improved outcome of experimental infection. In contrast, Th2-mediated immunity to *Aspergillus* results in a lesser degree of protection from infection as compared with a Th1 response (164, 165) and early evidence suggests that Th17 responses may also be less effective than the Th1 responses (178). Importantly, while the cellular source of IFN-γ in late infection and in immunized mice is recognized as *Aspergillus*-specific CD4 T cells (15, 55, 164), the cellular source of early IFN-γ in invasive aspergillosis is unknown. In the context of early infection, IFN-γ, IL-12, and IL-18 are induced in the lung of immunocompetent animals in the first 48 h after microbial challenge (80, 163). Similarly, human mononuclear cells express Th1 cytokines when exposed to *Aspergillus* conidia *in vitro* (87, 219-221).

IFN-γ mediates diverse and complex effects in the context of antimicrobial immunity, including activation of oxidative and non-oxidative microbicidal mechanisms in phagocytes, enhancement of Ag presentation, and reciprocal induction of IL-12 leading to the development of Th1-acquired immunity (222). In addition, IFN-γ mediates influx of effector leukocytes to sites of infection via induction of adhesion molecules and expression of several local mediators. Notable among these are the IFN-inducible CXC chemokines, CXCL9, CXCL10, and CXCL11, which mediate the influx of Th1 effector cells via their interaction with their common receptor CXCR3 (223, 224). These ligands are potently induced by both type-1 and type-2 IFNs in diverse biological settings, and their induction is further potentiated by exposure to TNF (225-227). We found that NK cell-derived IFN-γ both augments the antimicrobial effects of macrophages against *Aspergillus* and also had a marked effect on lung expression of CXCL9 and CXCL10; we speculate that the induction of these chemokines is further promoted by high levels of lung TNF in this infection (82). Interestingly, these chemokine ligands are capable of inducing the influx of activated NK cells and Th1 CD4 T cells (228, 229), and may therefore be a component of a beneficial positive feedback cycle in this infection.

In summary, our data provide evidence for a protective role of NK cell-derived IFN-γ in the early phase of infection in neutropenic hosts with invasive aspergillosis. This mechanism appears to boost the antimicrobial responses of resident and recruited myeloid cells against the pathogen and augment the

expression of IFN-inducible chemokines that may, in turn, mediate further recruitment of effector leukocytes. The recognition of NK cells as the early cellular source of IFN-γ is likely to be relevant to human invasive aspergillosis for several reasons. First, human invasive aspergillosis typically occurs in patients with impaired T cell responses and can follow a rapid course (230, 231); cellular sources of IFN-γ other than clonally expanded CD4 T cells may therefore be particularly relevant in host defense in this setting. Second, NK cell-derived IFN-γ during the effector phase of innate immunity has been shown to promote the development of Th1 immunity by priming CD4 Th cells in other experimental systems (232, 233); this effect may be relevant in the development of protective immunity in invasive aspergillosis. Finally, transfusion of cultured autologous NK cells has been used therapeutically in human clinical trials for the treatment of several cancers, in some of which the transfer of NK cells resulted in enhanced expression of IFN-γ in the plasma (234). Administration of exogenous IFN-γ has been used successfully as adjunctive therapy in human invasive aspergillosis in case-reports (235-237); therapeutic infusion of NK cells has the potential to allow for targeting the IFN-γ response to the tissue microniche of infection.

Methods

Animals

Wild-type (WT) C57BL/6 mice, mice with targeted deletion of IFN-γ or the RAG-1 genes (all on C57BL/6 background) were purchased from The Jackson Laboratory. Age- and gender-matched 6- to 14-wk old animals were used in the experiments. All animals were maintained under pathogen-free conditions and in compliance with institutional animal care regulations.

In vivo procedures

We used a previously characterized model of invasive pulmonary aspergillosis in transiently neutropenic mice (84, 94, 96, 98, 103). Depletion of neutrophils was achieved using a single i.p. injection of a mAb (anti-Gr-1; clone RB6-8C5) 1 day before intratracheal challenge with *Aspergillus fumigatus* conidia. As described previously, this resulted in peripheral blood neutropenia (absolute circulating neutrophil count <50 cells/μl) on days 1 and 3 after injection in both infected and uninfected mice, with a return of peripheral counts to pretreatment levels (>1000 cells/μl) by day 5 (59, 188). In uninfected animals, administration of the mAb did not influence the number of nonneutrophil peripheral blood leukocytes, nor lung or spleen lymphocyte or DC subsets, including plasmacytoid DCs (98). *A. fumigatus* (strain 13073, American Type Culture Collection) conidia were collected in 0.1% Tween 80 in PBS from 7- to 14-day-old cultures on

Sabouraud's dextrose agar plates, filtered and counted under a hemacytometer, and administered in inocula ranging from 2 to 5 X 10^6 conidia per mouse in various experiments. At designated time points, animals were euthanized by CO_2 asphyxiation, and whole lungs were removed and processed as described (59, 94, 98, 103). NK cells were depleted using a mAb (PK136, anti-NK1.1) or anti-asialoGM1 (WAKO). Intraperitoneal administration of 200 μg of anti-NK1.1 or 25 μl of anti-asialoGM1 2 days before *A. fumigatus* challenge resulted in depletion of NK cells for >5 days, as previously described (103, 238, 239).

Identification of leukocyte subsets

Leukocyte-enriched whole lung single-cell suspensions were prepared as described (59, 94, 98, 103). The following reagents were used to label cells (from BD Biosciences, unless otherwise noted): α-Gal-Cer-loaded or control FITC-labeled CD1d tetramers (National Institute of Allergy and Infectious Disease Tetramer Facility), anti-CD3-allophycocyanin (clone 17A2), anti-CD4-Pacific Blue (GK1.5), anti-CD45-peridinin chlorophyll A protein (30-F11), anti-IFN-γ-PE (XMG1.2), and anti- NK1.1-biotin (PK136) or respective isotype controls. To determine the lung cells capable of producing IFN-γ, lung cell suspensions were incubated with brefeldin A (10 ng/ml), PMA (10 ng/ml), and ionomycin (100 ng/ml) in RPMI 1640 with 5% FCS for 4 h and intracellular stain- ing was detected using a commercial kit (Cytofix/Cytoperm, BD Biosciences). Samples were analyzed on a FACS Canto II instrument using Diva software (all from BD Biosciences). The

absolute number of each leukocyte subset was determined as the product of the percentage of the cell type and the total number of cells in the sample, as determined under a hemacytometer.

NK cell culture and in vitro studies

Activated NK cells were prepared as described (103). In brief, splenocytes were cultured with mIL-12 (1 ng/ml) and mIL-18 (100 ng/ml) for 5 days and negatively selected by immunomagnetic depletion of $CD5^+$, $Ly\text{-}6G^+$, $TER\text{-}119^+$, $CD22^+$, and $F4/80^+$ cells according to the manufacturer's instructions (Stem Cell Technologies), resulting in >95% purity of NK cells. The resulting cells were highly activated lymphoblasts, spontaneously pro- duced large amounts of IFN-γ and perforin and exhibited potent natural cytotoxicity against NK-sensitive targets, as previously shown (240-243). In adoptive transfer experiments, 2×10^6 NK cells in 100 µl PBS were delivered i.v.

For co-culture experiments, resident alveolar macrophages or thioglycollate-elicited inflammatory peritoneal macrophages were obtained from naive animals as described (244, 245) and phagocytosis and hyphal damage were assessed with minor modification of published protocols (98, 127, 131, 246, 247). Macrophages and various numbers of NK cells were cultured in RPMI 1640 with 5% FCS in U-bottom 96-well plates at 37°C in 5% CO_2 for 3 h before addition of fungal elements. To assess rates of phagocytosis, freshly harvested conidia were

first conjugated to FITC, washed, and uniform staining verified by epifluorescent microscopy before addition to leukocytes. In different experiments, 2–4 X 10^5 macrophages were added per well and the ratio of macrophages: NK cells: conidia was 2:1:10. Phagocytosis was halted after 1 h of incubation by addition of cytochalasin D (final concentration 4 μg/ml; Sigma-Aldrich) and extracellular fluores- cence quenched in trypan blue (250 μg/ml in PBS) before flow cytometric analysis. In preliminary studies, no further increase in phagocytosis was noted after 1 h (our unpublished observation). In some experiments, hyphal viability was assessed after conidia were co-incubated with leukocytes for 16 h. In other experiments, hyphae were prepared by incubating fresh *A. fumigatus* conidia in RPMI 1640 at 1.5 X 10^6 conidia/ml at 37°C for 18 h; the resulting hyphae were then incubated with leukocytes (4 X 10^5 macrophages and various numbers of NK cells per well) for a further 2 h before quantification of hyphal viability.

To measure hyphal viability, leukocytes were lysed hypotonically and remaining hyphae were then incubated for 60 min in 100 μl of a solution containing 0.5 mg/ml 2,3-bis-2-methoxy-4- nitro-5-sulfophenyl-2H-tetrazolium-5-carboxanilide (XTT) and 40 μg/ml coenzyme Q (both from Sigma-Aldrich), and absorbance was determined at 450 nm. Hyphal killing was expressed as percent reduction in viability as compared with hyphae incubated without leukocytes.

Cytokine and chitin assays

Molds, including *Aspergillus* species, grow as multicellular hyphae that do not form distinct reproductive structures in infected tissues. We therefore used a previously characterized assay for chitin, a carbohydrate component of hyphal wall absent from conidia and from mammalian tissues, to quantify the burden of hyphae in infected lungs, as detailed previously (84, 94, 96, 103). In brief, homogenized organs were boiled in sodium lauryl sulfate and then heated in KOH (120% w/v) at 121°C for 60 min. Samples were then washed in ethanol, and $NaNO_2$ (5% w/v), $KHSO_4$ (5% w/v), ammonium sulfamate (12.5% w/v), and 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (5 mg/ml) were added sequentially. Samples were boiled and OD650 nm was measured after addition of FeCl3.6H2O (0.83% w/v). Chitin content was expressed as the ratio to glucosamine control. We have previously shown that lung chitin content on day 3 of infection, as measured by this protocol, correlates with mortality from the infection and histological severity of the infection in this model (96).

ELISA was performed on filtered supernatants of lung homogenates using complementary Ab pairs against murine IFN-γ, CXCL9, CXCL10, and CXCL11 (all from BD Biosciences) according to manufacturer's instructions.

Statistical analysis

Data were analyzed on a Macintosh Powerbook G4 computer using Prism statistical package (v.4.0a, GraphPad Software). Survival data were compared

using Fischer's exact test. All other data were expressed as mean ± SEM. Values between two groups over multiple times were compared with 2-way ANOVA, comparisons between two groups at a single time were performed with unpaired two-tailed Mann-Whitney (nonparametric) test, and comparisons between multiple groups at a single time were compared using the Kruskal-Wallis test with Bonferroni posttest. Probability values were considered statistically significant if they were <0.05.

Chapter 3 Neutropenia shapes the lung dendritic cell response to an opportunistic mold

Abstract

Neutropenic hosts are susceptible to many infections but our understanding of specific defense mechanisms in neutropenic infections is limited. Invasive aspergillosis, a prototypic opportunistic infection in neutropenia, results in accumulation of DCs in the lungs. Given recent data indicating that neutrophils can modulate immune responses independent of direct microbial killing, we hypothesized that neutrophils direct the response to *Aspergillus* by dictating the migration and phenotype of lung DCs. Myeloid DCs, but not other DC subsets, were found to accumulate in the lungs of neutropenic mice challenged with killed or live-attenuated *Aspergillus* as compared to non-neutropenic hosts. This was associated with failure of neutrophil-mediated DC maturation and efflux to lymph nodes. In addition, neutropenia markedly augmented the influx of DCs from the blood to the lungs. This was attributable to greatly elevated lung TNF expression, resulting in lung expression of CCL2 and CCL20 which, in turn, mediated recruitment of TNF-producing myeloid DC. Finally, in the context of neutropenic invasive aspergillosis, DC depletion resulted in impaired fungal clearance. These observations identify a novel defense

mechanism in neutropenic infections that is the result of alterations in DC traffic and phenotype attributable to immunomodulatory functions of neutrophils.

Introduction

Neutrophils are among the first lines of defense against microbial infections and their infiltration into tissue is the histological hallmark of acute inflammation. Neutropenia, defined as reduced concentration of circulating neutrophils in the blood, is a common clinical problem that frequently complicates cytotoxic chemotherapy, transplantation and hematologic malignancies. Neutropenic hosts are dramatically more susceptible to many bacterial and fungal infections, but our understanding of immunologic mechanisms in the context of neutropenic infections is limited. In the conventional view, neutrophils are considered as non-specific killers of pathogens that induce substantial tissue inflammation and, as a side-effect, also cause damage to host cells. According to this paradigm, the susceptibility of neutropenic hosts to infection is a consequence of the mere absence of direct neutrophil-mediated microbial killing in infected tissues. On the other hand, more recent data indicate that neutrophils are also capable of modulating the immune response independent of their direct microbicidal activity (248), but the relevance of this function to infections in neutropenic hosts is not clear.

Invasive aspergillosis, a severe infection caused by common environmental molds of the *Aspergillus* species, is a prototypic opportunistic infection of neutropenic hosts (5). *Aspergillus* conidia are ubiquitous in air and, when inhaled,

can bypass the physical defenses of the respiratory tract and reach beyond the ciliated respiratory epithelium, where they become swollen and metabolically active. In normal hosts, swollen conidia are cleared without causing infection but in immunocompromised patients, they germinate to form hyphae that penetrate the lung epithelium and cause pneumonia (5). While immunocompromised hosts without neutropenia can also develop invasive aspergillosis, the severity and duration of neutropenia, as well as qualitative defects in neutrophil function, are the best characterized clinical risk factors for the development of this infection. Neutrophils kill swollen conidia and hyphae *in vitro* and their recruitment and antimicrobial mechanisms have been implicated in defense against invasive aspergillosis is animal models (5). Thus, host responses to *Aspergillus* are a clinically relevant setting to assess the effect of neutropenia on local immune mechanisms.

Dendritic cells have been shown to play an important role in shaping the development of T cell responses to *A. fumigatus* (168). DCs can distinguish both conidial and hyphal forms of *Aspergillus* and are known to transport *Aspergillus* elements from the lungs to secondary lymphoid organs (105, 154). Depending on the *Aspergillus* form encountered, DCs can initiate qualitatively different CD4+ T cell responses (154, 249). Myeloid DCs ($CD11b^{hi}$, CD11c+) are a subset of lung dendritic cells involved in inflammatory responses (250) and have been shown to quickly expand in the lung following a respiratory challenge of *Aspergillus* (98, 102, 105). We previously reported an unexpectedly large accumulation of

myeloid DCs in the lungs of neutropenic mice with invasive aspergillosis that was, in part, dependent on the interaction of the chemokine ligand-receptor pair, CCL20-CCR6 (98). More recently, efflux of mature myeloid DCs from the lungs to mediastinal lymph nodes in the context of invasive aspergillosis was shown to be dependent on the chemokine receptor CCR7 (102).

Since such large numbers of myeloid DCs are not observed in other models of pulmonary inflammation(105), we posited that the absence of neutrophils from infected tissues alters the local inflammatory environment independent of neutrophil-mediated microbial killing and, as a result, modulates the behavior of lung DCs. We therefore sought to test the hypothesis that neutrophils direct the host response to *Aspergillus* by dictating the migration and phenotype of lung DCs.

Results

Absence of neutrophils results in accumulation of DCs in the lungs in humans and mice in response to Aspergillus

Given our prior observation of accumulation of myeloid DCs in the lungs of neutropenic mice with invasive aspergillosis (98), we began by examining whether this phenomenon is observed in humans. Similar to the findings in the animal model, neutropenia was associated with a substantial increase in the number of DCs in the airways and alveolar spaces of patients with invasive aspergillosis compared to the number of DCs found in normal lungs and in non-neutropenic invasive aspergillosis (Figure 1-2).

To examine whether neutrophils were directly responsible for the observed effect on lung DCs, we compared the effect of antibody-mediated neutrophil depletion on the number of lung DCs after intra-tracheal challenge with *Aspergillus*. We reasoned that *Aspergillus* conidia germinate into hyphae in the lungs of neutropenic but are killed before forming hyphae in normal (non-neutropenic) mice (104); as a result, the antigens encountered by neutropenic hosts challenged with live conidia are very different from those encountered by normal hosts. We therefore challenged the animals with killed fungal elements in order to retain a comparable antigenic stimulus in the two groups. Lung DC accumulation was observed after challenge

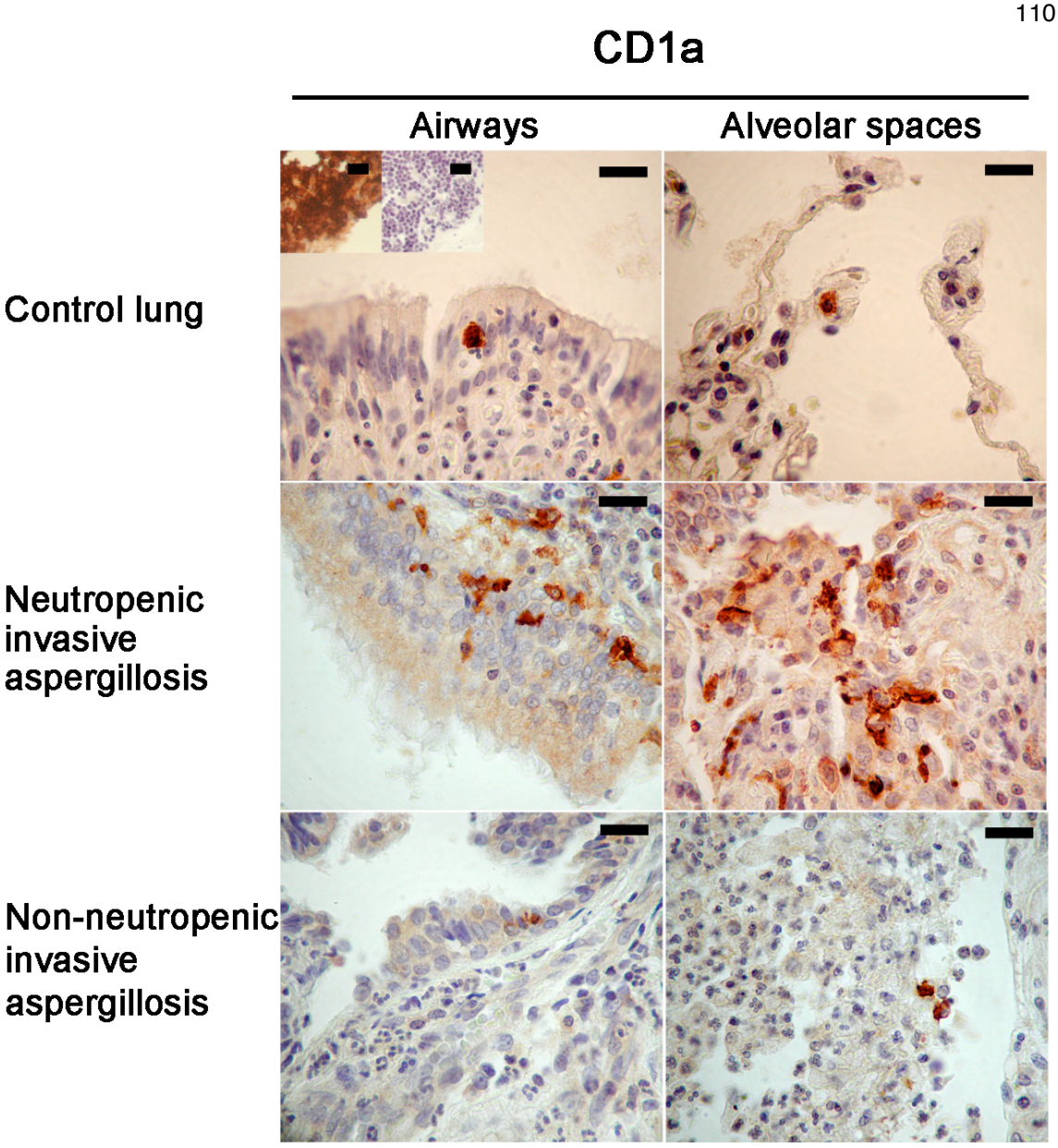

CD1a
Airways
Alveolar spaces
Control lung
Neutropenic invasive aspergillosis
Non-neutropenic invasive aspergillosis

Figure 1.

Accumulation of lung DC in neutropenic patients with invasive aspergillosis. Representative photomicrographs of the immunolocalization of CD1a in lung tissue in normal lung (top panels), neutropenic invasive aspergillosis (middle panels) and non- neutropenic invasive aspergillosis (bottom panels). Insets show control thymic tissue labeled with CD1a (left) or negative control Ab (right). All scale bars are 20 μm; original magnifications were 400X for all the panels.

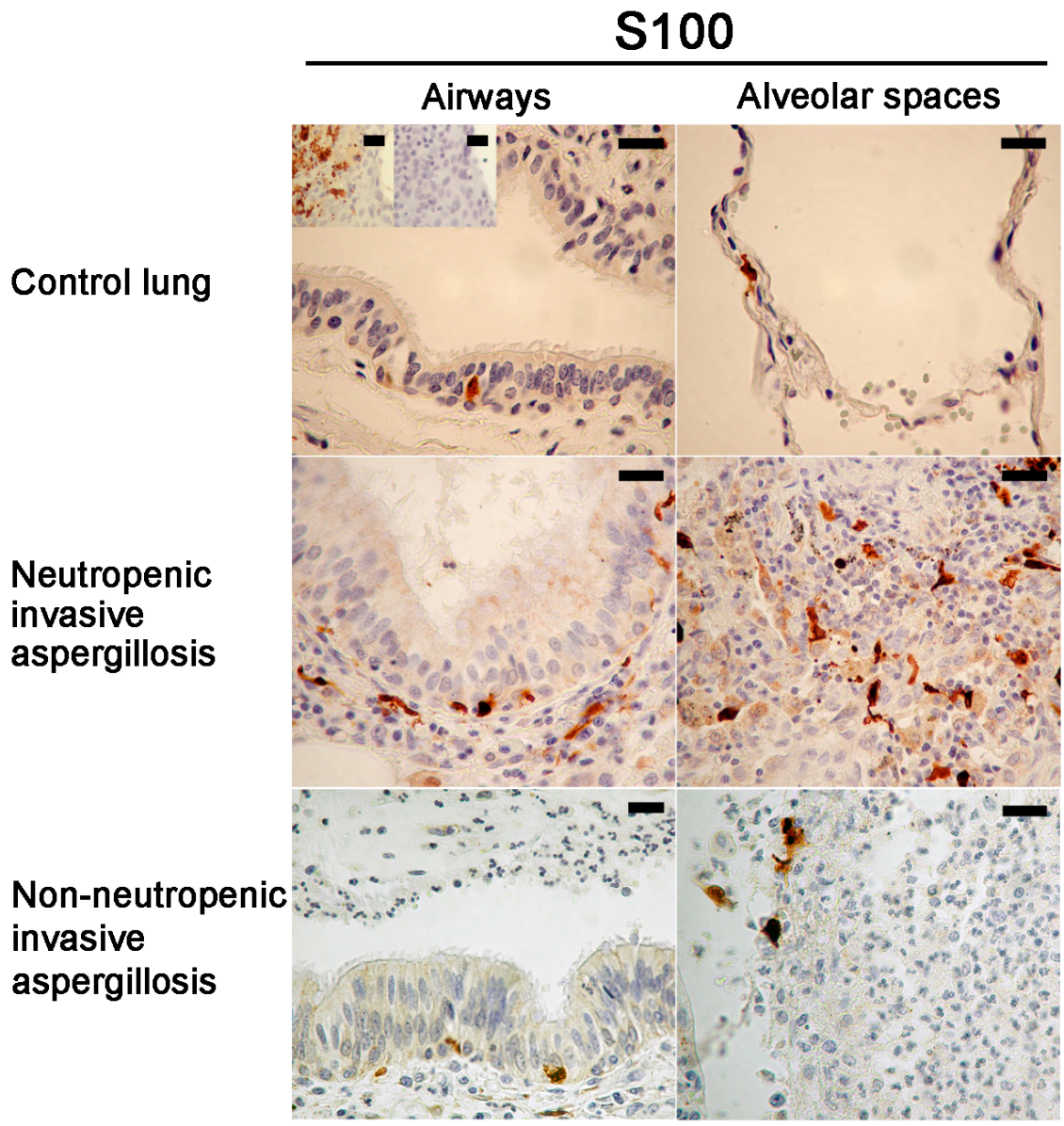

S100
Airways
Alveolar spaces
Control lung
Neutropenic invasive aspergillosis
Non-neutropenic invasive aspergillosis

Figure 2.

Accumulation of lung DC in neutropenic patients with invasive aspergillosis. Representative photomicrographs of the immunolocalization of S100 in lung tissue in normal lung (top panels), neutropenic invasive aspergillosis (middle panels) and non-neutropenic invasive aspergillosis (bottom panels). Insets show control thymic tissue labeled with S100 (left) or negative control Ab (right). All scale bars are 20 μm; original magnifications were 400X for all the panels.

with both swollen conidia and hyphae, but was more prominent after hyphal challenge, with a 4-fold increase in the number of myeloid DC in the lungs in neutropenic mice as compared to the non-neutropenic mice 3 days after challenge with hyphae (Figure 3A-B; Figure 4A). In contrast, depletion of neutrophils had no effect on the number of airway DC or lung plasmacytoid DC after challenge with hyphae (Figure 3C-D). We performed additional experiments to ensure that the observed effect was not an epiphenomenon related to the experimental conditions. We found a similar increase in lung myeloid DC after challenge with killed hyphae when neutropenia was induced using an alternative mAb (Figure 4B). Similar results were found when animals were challenged with live hyphae from an attenuated strain of *Aspergillus* that is growth impaired at body temperatures regardless of the presence of absence of neutrophil (251) (Figure 4C).

Accumulation of myeloid DCs in the lungs of neutropenic mice is beneficial during the early phase of invasive aspergillosis

We next sought to determine the contribution of the accumulated lung DC to host defense in mice with neutropenic invasive aspergillosis, using short-term ablation of DC in transgenic mice expressing the simian diphtheria toxin receptor driven by the mouse CD11c promoter (252). We confirmed that administration of diphtheria toxin to transgenic mice with invasive aspergillosis resulted in depletion of myeloid DC, but did not affect other DC subsets, resident lung

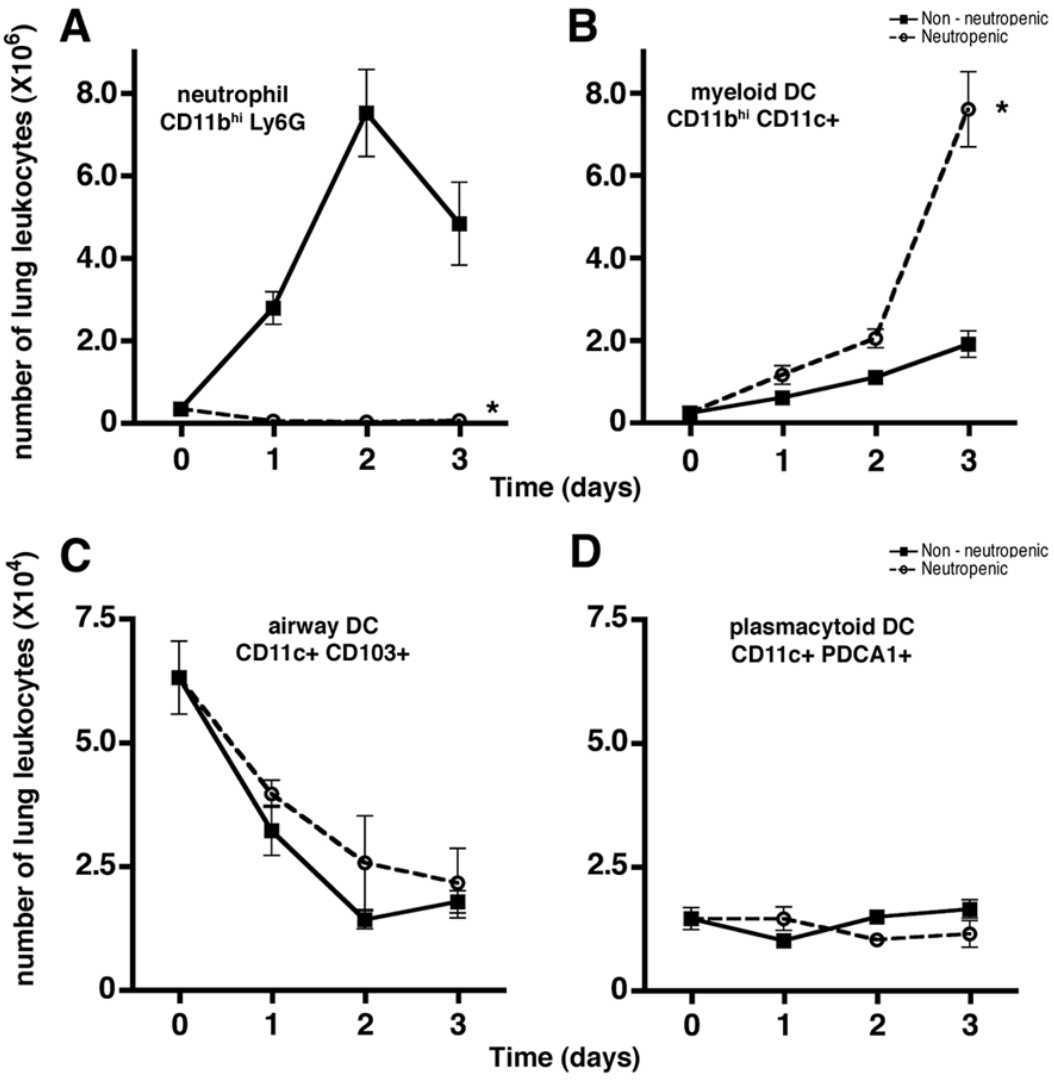

A
neutrophil
CD11b^hi Ly6G
number of lung leukocytes (X10^6)
8.0
6.0
4.0
2.0
0
*
0
1
2
3
Time (days)
B
Non - neutropenic
Neutropenic
myeloid DC
CD11b^hi CD11c+
*
C
airway DC
CD11c+ CD103+
number of lung leukocytes (X10^4)
7.5
5.0
2.5
0
D
plasmacytoid DC
CD11c+ PDCA1+

Figure 3.

Effect of neutrophils on the number of lung DCs in response to *Aspergillus*. Number of lung neutrophil (A), myeloid DC (B), airway DC (C) and plasmacytoid DC (D) are shown at various times after challenge with killed *Aspergillus* hyphae in neutropenic (anti-Ly6G/C; clone RB6-8C5) and non-neutropenic (isotype control) mice. Data shown represent mean ± SEM; n = 20 mice per group per time point pooled from 4 independent experiments for panels A-B and n = 8 mice per group per time point pooled from 2 independent experiments for panels C-D. Time 0 represents unchallenged animals; *, $p < 0.05$ comparing trend between the two groups over time.

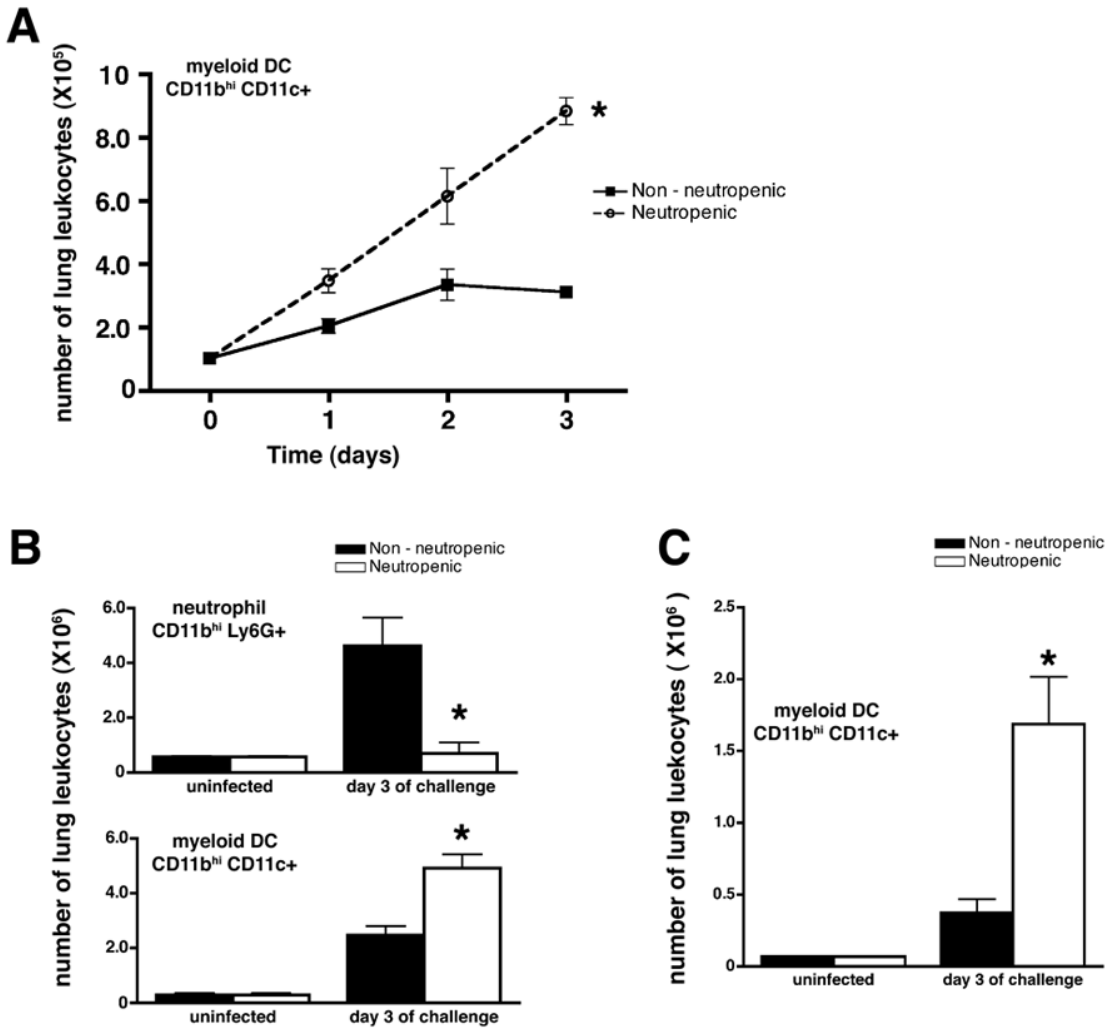

A
myeloid DC
CD11b^hi CD11c+
number of lung leukocytes (X10^5)
10
8.0
6.0
4.0
2.0
0
0
1
2
3
Time (days)
*
Non - neutropenic
Neutropenic
B
Non - neutropenic
Neutropenic
neutrophil
CD11b^hi Ly6G+
number of lung leukocytes (X10^6)
6.0
4.0
2.0
0
*
uninfected
day 3 of challenge
myeloid DC
CD11b^hi CD11c+
6.0
4.0
2.0
0
*
uninfected
day 3 of challenge
C
Non - neutropenic
Neutropenic
number of lung luekocytes (X10^6)
2.5
2.0
1.5
1.0
0.5
0
myeloid DC
CD11b^hi CD11c+
*
uninfected
day 3 of challenge

Figure 4.

Effect of neutrophils on the number of lung DCs in response to *Aspergillus*. (A) Number of lung myeloid DC at various times after challenge with killed *Aspergillus* swollen conidia in neutropenic (anti-Ly6G/C; clone RB6-8C5) and non-neutropenic (isotype control) mice. (B) Number of lung neutrophils (top graph) and myeloid DC (bottom graph) in neutropenic and non-neutropenic mice after challenge with killed *Aspergillus* hyphae, using an alternative Ab (anti-Ly-6G, clone 1A8) to induce neutropenia. (C) Number of lung myeloid DC after challenge with live hyphae from an attenuated thermo-sensitive mutant strain of *Aspergillus* in neutropenic (anti-Ly6G/C; clone RB6-8C5) and non-neutropenic (isotype control) mice. In all panels, data shown represent mean ± SEM; n = 4 for each group, representative data from 2 experiments. *, $p < 0.05$ comparing trend between the two groups over time.

macrophages, or recruited monocytes in the infected lungs (Figure 5A-B). Depletion of myeloid DC in the context of neutropenic invasive aspergillosis resulted in a >7-fold increase in lung fungal burden on day 3 of infection as compared to neutropenic wildtype mice (Figure 5C), thus providing evidence for a protective role of lung myeloid DCs in neutropenic hosts with invasive aspergillosis.

Neutrophil depletion causes reduced efflux of myeloid DCs from the lungs to mediastinal lymph nodes following challenge with Aspergillus

To determine the potential factors contributing to the marked accumulation of DCs in the lung, we reasoned that the number of leukocytes in the lung represents a dynamic balance between the arrival and departure of the cells. The observed increase in the number of lung DC may represent an increase in influx or local differentiation on the one hand, or a decrease in efflux or apoptosis on the other. Since neutrophils have previously been shown to mediate maturation of DC (which may, in turn, results in their efflux), we began by examining the maturation phenotype of lung myeloid DCs in lungs of neutropenic mice challenged with *Aspergillus.* DCs in lungs of neutropenic hosts were found to express lower levels of the co-stimulatory molecules CD86 and CD40 as compared to the DC from non-neutropenic mice, consistent with an immature phenotype (Figure 6).

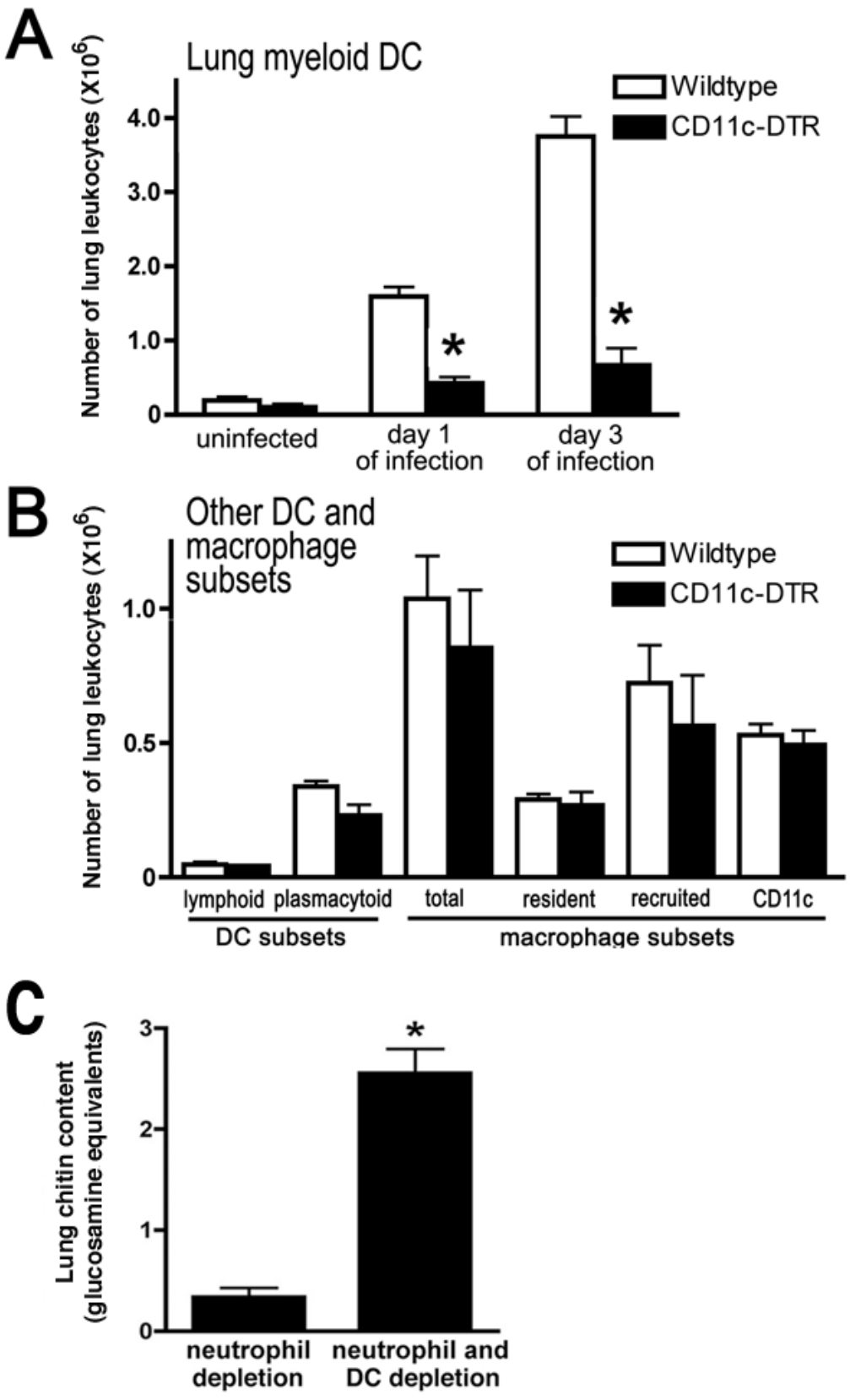

A
Lung myeloid DC
Number of lung leukocytes (X10^6)
Wildtype
CD11c-DTR
4.0
3.0
2.0
1.0
0
*
*
uninfected
day 1 of infection
day 3 of infection
B
Other DC and macrophage subsets
Number of lung leukocytes (X10^6)
Wildtype
CD11c-DTR
1.0
0.5
0
lymphoid
plasmacytoid
total
resident
recruited
CD11c
DC subsets
macrophage subsets
C
Lung chitin content (glucosamine equivalents)
3
2
1
0
*
neutrophil depletion
neutrophil and DC depletion

Figure 5.

Effect of DC depletion in neutropenic invasive aspergillosis. Mice were neutrophil depleted with GR-1 mAb prior to receiving an intratracheal challenge of live *Aspergillus* conidia. (A) Lung myeloid DC in CD11c-DTR and wildtype mice treated with diphtheria toxin on the day before the measurements were obtained. (B) Other lung DC and macrophage subsets in mice on day 1 of infection. The following surface markers were used for identification among CD45-positive cells: lymphoid DC, $CD8^+$ $CD11c^+$; plasmacytoid DC, $B220^+$ $CD11c^+$; total macrophages, $F4/80^+$; resident macrophages, F4/80-hi autofluorescence-hi; recruited monocyte/macrophages, F4/80-mid autofluorescence-low. There is overlap between the $CD11c^+$ and resident macrophage populations. (C) Lung chitin content on Day 3 of infection. Data represents mean ± SEM; n = 5-6 for each group; *, $p < 0.05$ compared with neutropenic wild-type mice

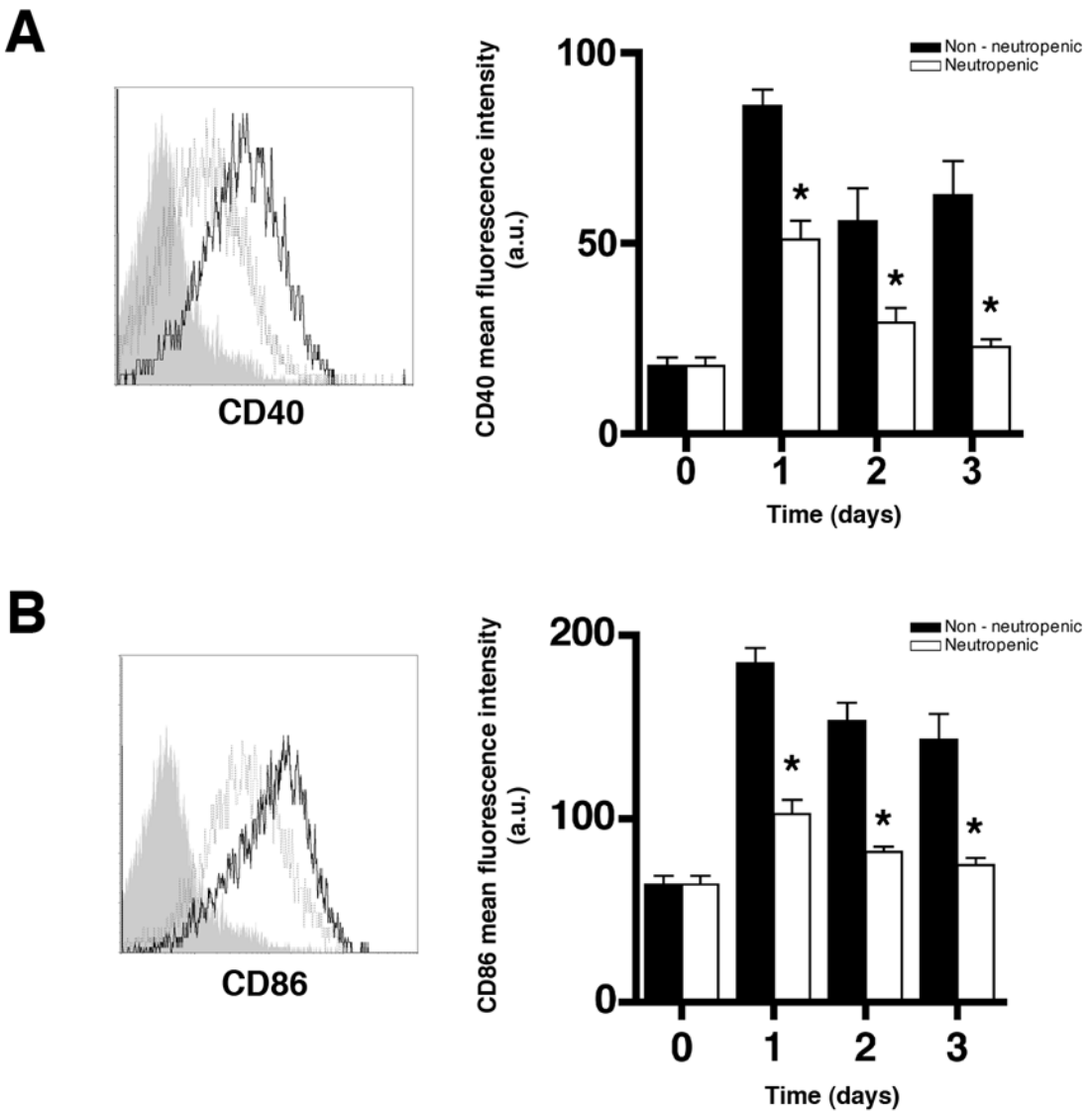
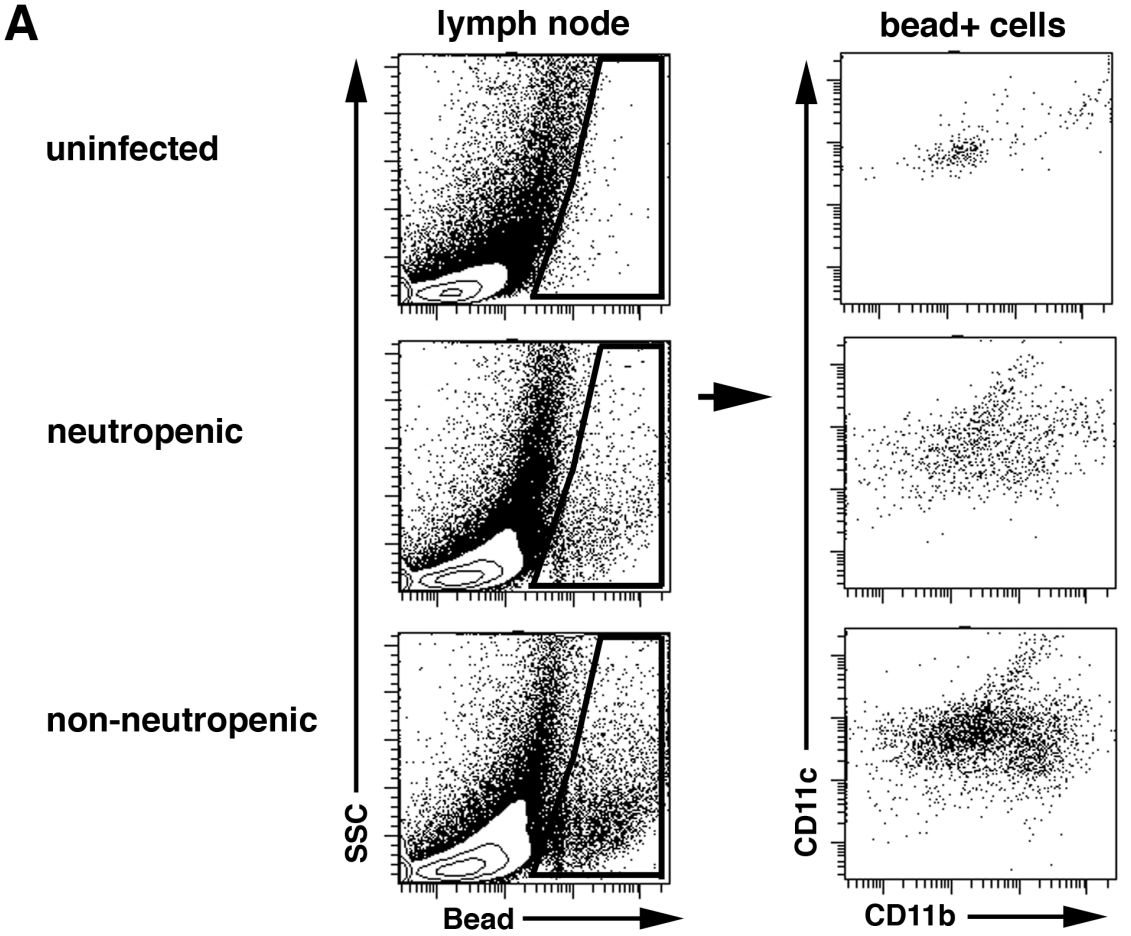

A
CD40
CD40 mean fluorescence intensity (a.u.)
100
50
0
Non - neutropenic
Neutropenic
*
*
*
0
1
2
3
Time (days)
B
CD86
CD86 mean fluorescence intensity (a.u.)
200
100
0
Non - neutropenic
Neutropenic
*
*
*
0
1
2
3
Time (days)

Figure 6.

Effect of neutrophils on maturation of lung myeloid DC *in vivo*. Panels show representative flow cytometry histograms and MFI of CD40 (A) and CD86 (B) gated on $CD45^+$ $CD11b^+$ $CD11c^+$ lung myeloid DC at various times after challenge with killed *Aspergillus* hyphae in neutropenic (anti-Gr1, clone RB6-8C5) and non-neutropenic mice (isotype control). Shaded histograms represents isotype control Ab, grey dotted line denotes neutropenic mice, and solid black line denotes non-neutropenic mice. Data shown in bar graphs represent mean ± SEM; n = 4 per group per time point, representative data from 3 experiments. *, $p < 0.05$ comparing trend between neutropenic and non-neutropenic challenged mice. but that the efflux of lung DCs to draining lymph nodes in response to A. fumigatus is dependent on neutrophils.

We next hypothesized that an impairment of efflux is the mechanism of DC accumulation in the lungs of neutropenic mice challenged with *A. fumigatus* and sought to track the migration of DCs from the lungs to the draining mediastinal lymph nodes by labeling lung phagocytes with intratracheally administered fluorescent latex beads. Analysis of lung cell suspension in animals with and without neutrophil depletion revealed comparable numbers of bead-labeled DCs and resident macrophages (data not shown). Examination of the mediastinal lymph nodes showed that, as compared to animals challenged with latex beads alone, challenge with *A. fumigatus* resulted in migration bead-associated MHC II^+ $CD11b^+$ $CD11c^+$ myeloid DC from the lungs to the lymph nodes (Figure 7). In contrast, the efflux of myeloid DC from the lungs to draining lymph nodes was greatly diminished in neutropenic hosts challenged with *A. fumigatus* (Figure 7). We found no difference in the proportion of apoptotic lung myeloid DC in broncholaveolar space of neutropenic and non-neutropenic mice 3 days after challenge with *Aspergillus* (7.64 ± 0.97% in neutropenic and 10.80 ± 4.39% in non-neutropenic animals challenged with *Aspergillus*, n = 8 mice per group pooled from 2 experiments, $p = 0.2345$). Taken together, these data suggest that neutropenia does not influence the rate of apoptosis of lung DCs, Given the immature phenotype of accumulated lung DCs in neutropenic animals challenged with *Aspergillus* (Figure 6), we sought to determine whether defective neutrophil-mediated DC maturation is responsible for the impaired efflux of lung DCs to mediastinal lymph nodes in neutropenic hosts. To assess whether neutrophils in


A
lymph node
bead+ cells
uninfected
neutropenic
non-neutropenic
SSC
Bead
CD11c
CD11b

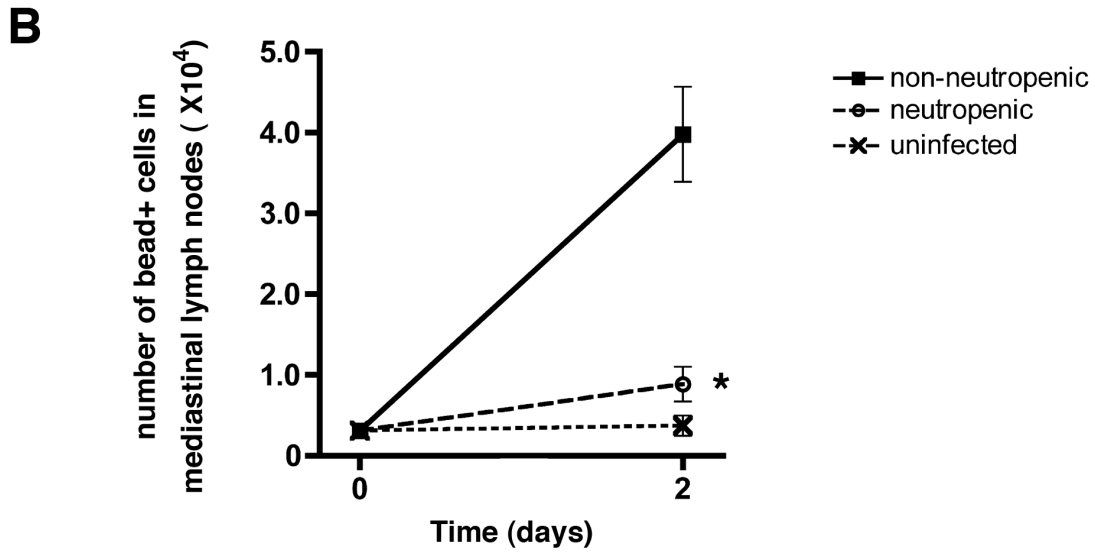

B
number of bead+ cells in mediastinal lymph nodes (X10^4)
5.0
4.0
3.0
2.0
1.0
0
0
2
Time (days)
*
non-neutropenic
neutropenic
uninfected

Figure 7.

Role of neutrophils in migration of lung myeloid DC to mediastinal lymph nodes in response to *Aspergillus* challenge. Neutropenic (anti-Gr1, clone RB6-8C5) and non-neutropenic mice (isotype control) were challenged intratracheally with killed *Aspergillus* hyphae mixed with FITC-labeled beads; uninfected control mice were challenged with intratracheal FITC-labeled beads only. (A) Representative flow cytometry plots and gating strategy of lymph node cell suspensions. All plots were initially are gated on MHC II+ cells. (B) Summary data represented as mean ± SEM of bead-associated cells in mediastinal lymph nodes; n = 8 per group per time point pooled from 2 independent experiments. *, $p < 0.05$ comparing trend between neutropenic and non-neutropenic challenged mice.

fact mediate DC maturation in response to *Aspergillus*, we cultured immature DCs with hyphae in the presence or absence of neutrophils (Figure 8). Cultured immature myeloid DCs expressed low levels of CD86 and CD40, and this phenotype was not affected when immature DCs were incubated in the presence of *Aspergillus* hyphae or resting neutrophils. In contrast, maturation of DCs was greatly enhanced when DCs were incubated with both hyphae and neutrophils (Figure 8) and was further enhanced with increasing the number of activated neutrophils added to the co-culture (data not shown). The maturation of DCs in response to hyphae required contact between DCs and neutrophils, since transwell separation of DCs from neutrophils and hyphae abrogated DC maturation. Ab-neutralization studies showed that DC maturation was dependent on the receptor DC-SIGN (Figure 8) but was independent of the beta-glucan receptor dectin-1, IL-12p70 or TNF (data not shown). Within the limits of this *in vitro* co-culture system, these data indicate that activated neutrophils can induce maturation of myeloid DC through cell-to-cell contact and DC-SIGN during responses to *Aspergillus*. *Neutropenia results in increased influx and differentiation of myeloid DCs from the blood to the lungs following challenge with Aspergillus*

While a disruption of DC efflux from the lung was a contributing factor in the accumulation of these cells in the lungs of neutropenic hosts, we next examined whether influx and differentiation of DCs had any role. To directly examine DC influx and differentiation in the lung, we began by tracing the fate of peripheral

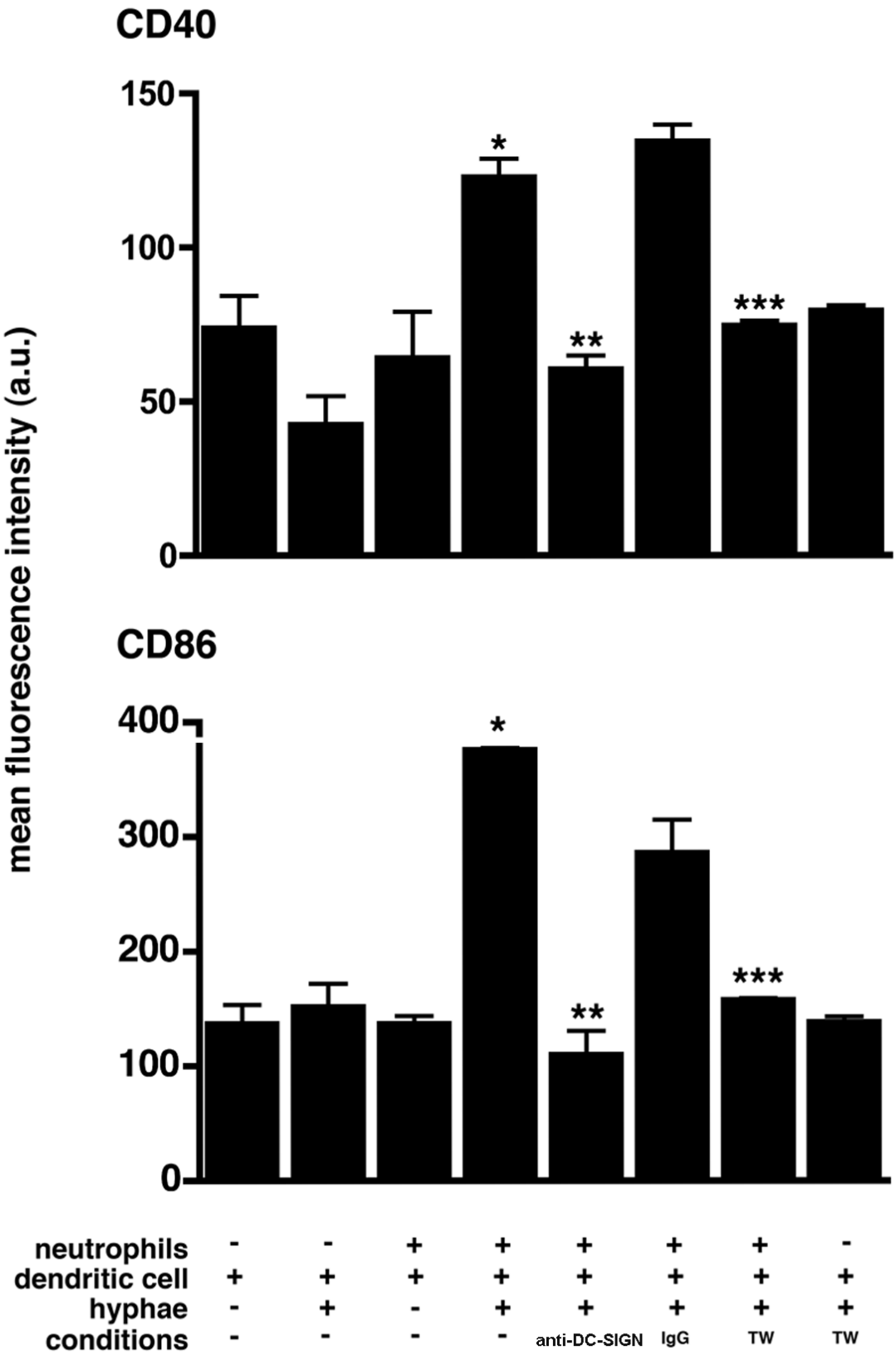

CD40
150
100
50
0
*
**
***
CD86
400
300
200
100
0
*
**
***
mean fluorescence intensity (a.u.)
neutrophils - - + + + + + -
dendritic cell + + + + + + + +
hyphae - + - + + + + +
conditions - - - - anti-DC-SIGN IgG TW TW

Figure 8.

Effect of neutrophils on maturation of myeloid DC in response to *Aspergillus in vitro*. Expression of CD40 (top panel) and CD86 (bottom panel) on immature bone marrow-derived DC was assessed by flow cytometry after overnight culture with resting neutrophils and *A. fumigatus* hyphae. Each culture conditions were performed in triplicates, representative date from two independent studies. anti-DC-SIGN, blocking mAb against DC-SIGN; IgG, respective isotype control mAb; TW, transwell system with DC in the lower chamber and hyphae and neutrophils or hyphae alone in the upper chamber. *, $p < 0.05$ compared to DC incubated alone, DC+neutrophils, or DC+hyphae; **, $p < 0.05$ compared to DC+neutrophils+hyphae or DC+neutrophils+hyphae+control Ig; ***, $p < 0.05$ compared to DC+neutrophils+hyphae.

blood monocytes labeled in the circulation with intravenously delivered fluorescent latex beads. We confirmed that after i.v. delivery of beads, bead-labeled peripheral blood leukocytes consisted of monocytes, as determined by surface expression of CD11b and CD115 (Figure 9), similar to prior reports (253). We found that few bead-positive cells arrived in the lungs of unchallenged mice after i.v. administration of labeled beads (Figure 10). In addition, we found that, within hours after i.v. delivery of beads, >2-fold more bead-positive cells arrived in the lungs of neutropenic mice challenged with *Aspergillus* as compared to non-neutropenic mice. The bead-positive cells arriving in the lungs of neutropenic mice consisted mostly of $CD11b^{hi}$ $CD11c^{+}$ myeloid DC as early as 1hr after administration of beads, with smaller numbers of $CD11b^{hi}$ $CD11c^{-}$ inflammatory monocytic cells; in contrast, bead-positive cells arriving in the lungs of non-neutropenic mice consisted almost entirely of inflammatory monocytic cells (Figure 10). The rapidity of appearance of bead-positive DC in the lungs of neutropenic mice suggested the possibility of selection of small numbers of circulating DCs from the blood in addition to in situ differentiation from recruited DC precursors in the lungs. To examine whether the recruitment of circulating cells was influenced by neutropenia, we intravenously delivered cultured and fluorochrome-labeled immature myeloid DC and tracked their arrival in the lungs after intrapulmonary challenge with killed *Aspergillus* hyphae. On day 2 following challenge, we found ~2- fold higher numbers of transferred DC in the lungs of neutropenic as compared to non-neutropenic animals (Figure11). Taken together,

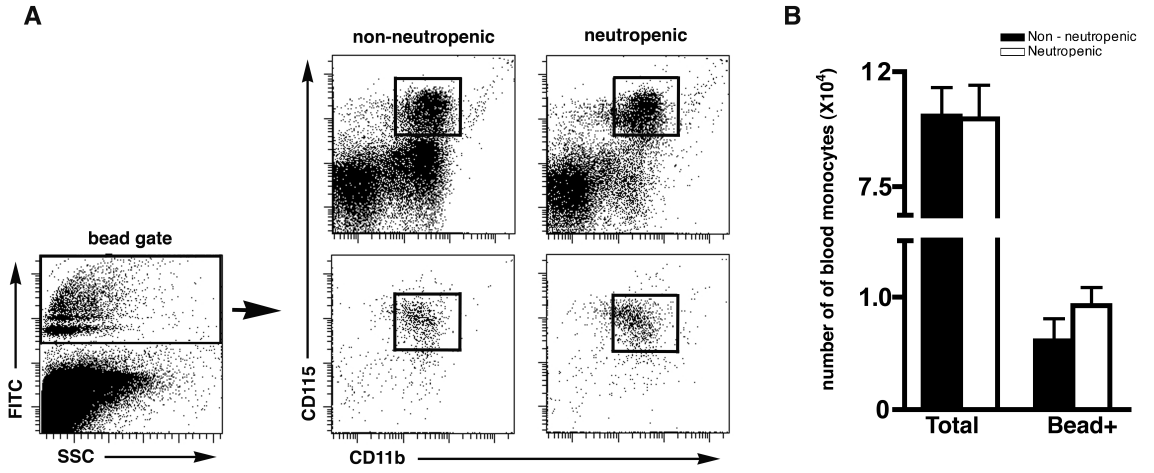

A
non-neutropenic
neutropenic
bead gate
FITC
SSC
CD115
CD11b
B
Non - neutropenic
Neutropenic
number of of blood monocytes (X10⁴)
12
7.5
1.0
0
Total
Bead+

Figure 9.

Blood monocyte analysis in neutropenic and non- neutropenic mice. (A) Representative flow cytometry plots and (b) quantitative data after labeling of endogenous monocytes with $FITC^+$ beads in neutropenic (anti-Ly6G/C; clone RB6-8C5) and non-neutropenic (isotype control) mice on day 3 following challenge with killed *Aspergillus* hyphae. Top panel shows total monocytes based on CD115 and CD11b expression. Bottom panels show the same population gated on $FITC^+$ beads. Representative data from 2 experiments, n = 4 mice per group.

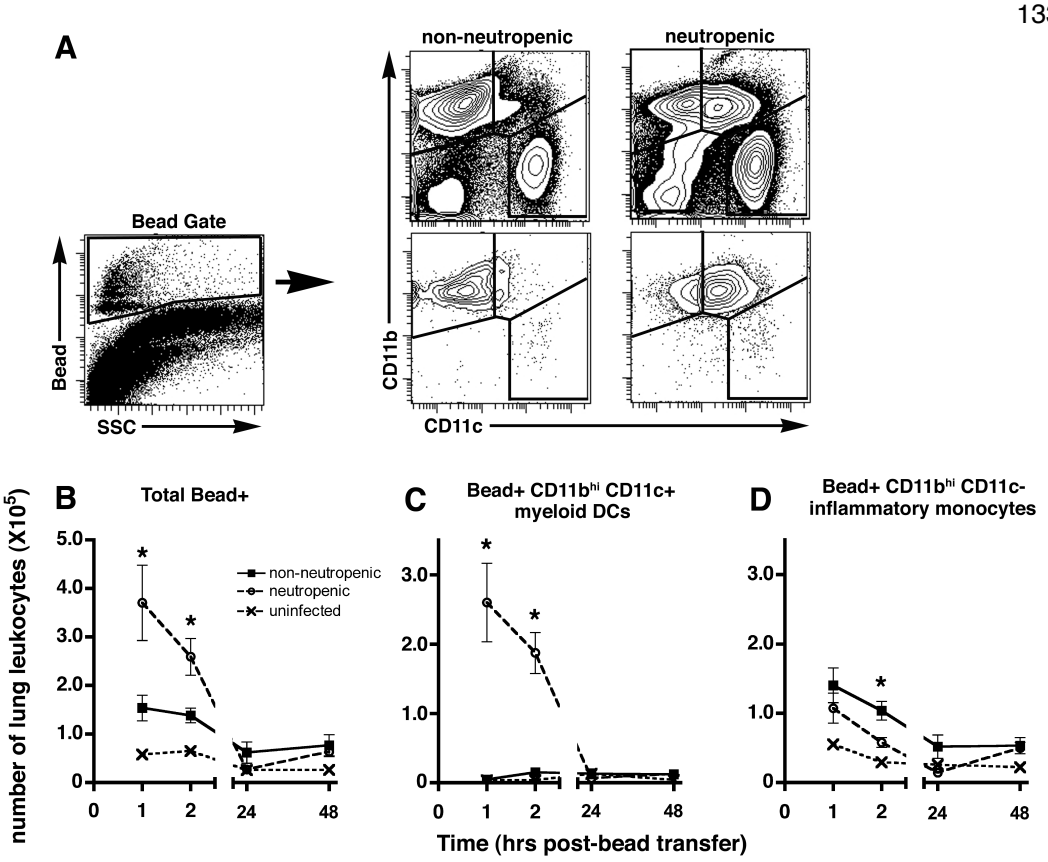

A
non-neutropenic
neutropenic
Bead Gate
Bead
SSC
CD11b
CD11c
B
Total Bead+
C
Bead+ CD11b^hi CD11c+
myeloid DCs
D
Bead+ CD11b^hi CD11c-
inflammatory monocytes
number of lung leukocytes (X10^5)
non-neutropenic
neutropenic
uninfected
Time (hrs post-bead transfer)

Figure 10

Effect of neutrophils on DC influx to the lung in response to *Aspergillus*. Neutropenic (anti-Ly6G/C; clone RB6-8C5) and non-neutropenic (isotype control) mice were challenged with killed *Aspergillus* hyphae 24 hours before i.v. administration of FITC-labeled latex beads. (A) Representative flow cytometry plots and gating strategy of whole lung single cell suspensions gated on $CD45^+$ cells, stained for CD11b and CD11c. The bottom flow cytometry plots show the same populations gated on FITC+ beads. (B-D) Bead-associated cell subsets in the lungs were identified based on surface expression of CD11b and CD11c. Day 0 represents unchallenged mice. Data shown represent mean ± SEM; n = 5 mice per group per time point. *, $p < 0.05$ comparing trend between neutropenic and non-neutropenic challenged mice

these results suggest that in the context of host response to *Aspergillus*, neutropenia results in enhanced influx of DCs, DC precursors, or both, to the lungs.

Absence of neutrophils results in increased local production of inflammatory cytokines in response to Aspergillus

Since neutropenia appeared to cause a much greater influx of DC (or their precursors) to the lungs after challenge with killed *Aspergillus*, we next examined the mechanism for this effect. Since TNF is a critical proximal signal for innate defenses against *Aspergillus* and has been shown to be important for the recruitment of inflammatory leukocytes in the context of this infection (82), we next examined the level of TNF in whole lung homogenates of neutropenic and non-neutropenic mice. Administration of killed *Aspergillus* hyphae resulted in a rapid and marked induction of lung TNF in the lungs of neutropenic but not non-neutropenic mice (Figure 12). CCL2 and CCL20, previously shown to be strongly induced by TNF and required for recruitment of DCs and monocytes (98, 103), were also markedly induced in the lungs of neutropenic mice in response to *Aspergillus* hyphae. We then sought to establish the cellular sources of the elevated lung TNF in neutropenic mice. As expected, we found ~2-fold greater number of TNF-producing cells in the lungs of neutropenic, as compared to non-neutropenic, mice challenged with *Aspergillus* hyphae (Figure 13). The TNF-producing cells consisted of approximately equal numbers of $CD11b^{hi}$ CD11c+

A

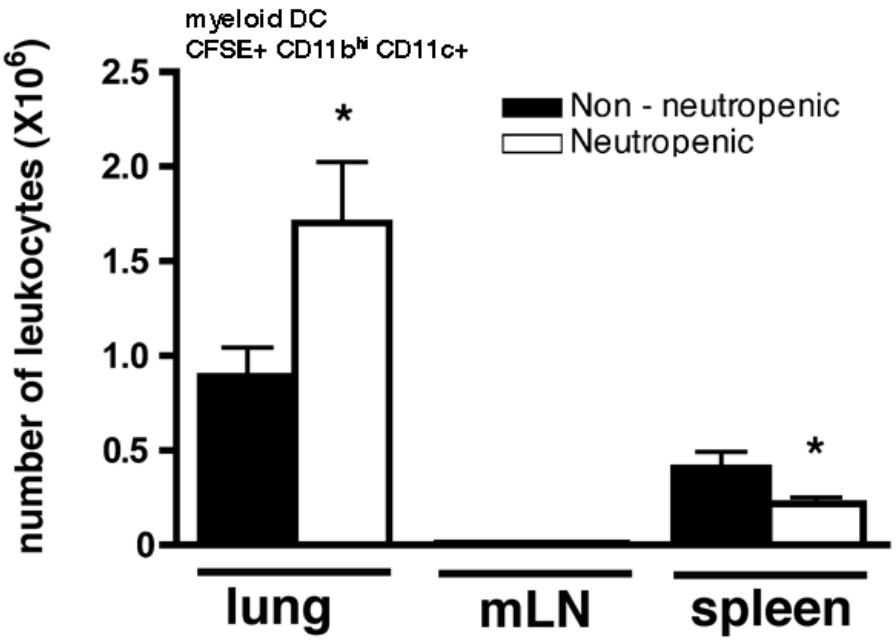

myeloid DC
CFSE+ CD11b^hi CD11c+
number of leukocytes (X10^6)
2.5
2.0
1.5
1.0
0.5
0
*
*
Non - neutropenic
Neutropenic
lung
mLN
spleen

Figure 11

Effect of neutrophils on myeloid dendritic cell influx into the lungs in response *Aspergillus*. Immature bone-marrow derived-dendritic cell (GM-CSF cultured and CD11c+ positively enriched) were labeled with a vital fluorchrome CFSE (CarboxyFluoroscein Succinimidyl Ester), transferred intravenously into neutropenic (anti-Gr1, clone RB6-8C5) and non-neutropenic (isotype control) mice on day 2 after challenge with ethanol-killed *Apergillus* hyphae. The CFSE labeled cells were enumerated in the lung, lymph node and spleen after 4 hours by flow cytometry. n=8 mice per group per time point, pooled results of 2 experiments. *, p = 0.038 compared to the non-neutropenic group

myeloid DCs and CD11b^{hi} CD11c^{-} inflammatory monocytic cells early after challenge with *Aspergillus* in both groups, but mostly of CD11b^{hi} CD11c^{+} myeloid DCs in neutropenic mice late after challenge. Resident macrophages (identified as CD11b$^{lo/-}$ CD11c^{+} cells) were a minor population of TNF- producing cells in both groups (Figure 13).

Given that TNF appeared to be produced by lung DCs in neutropenic hosts and could also induce their influx via induction of CCL2 and CCL20, we hypothesized a causal series of events: that in the context of neutropenic hosts challenged with *Aspergillus*, DC-derived TNF results in induction of CCL2 and CCL20, which in turn results in further recruitment of TNF-producing DCs to the lungs. To test this hypothesis, we examined the effect of Ab-mediated neutralization of TNF on lung

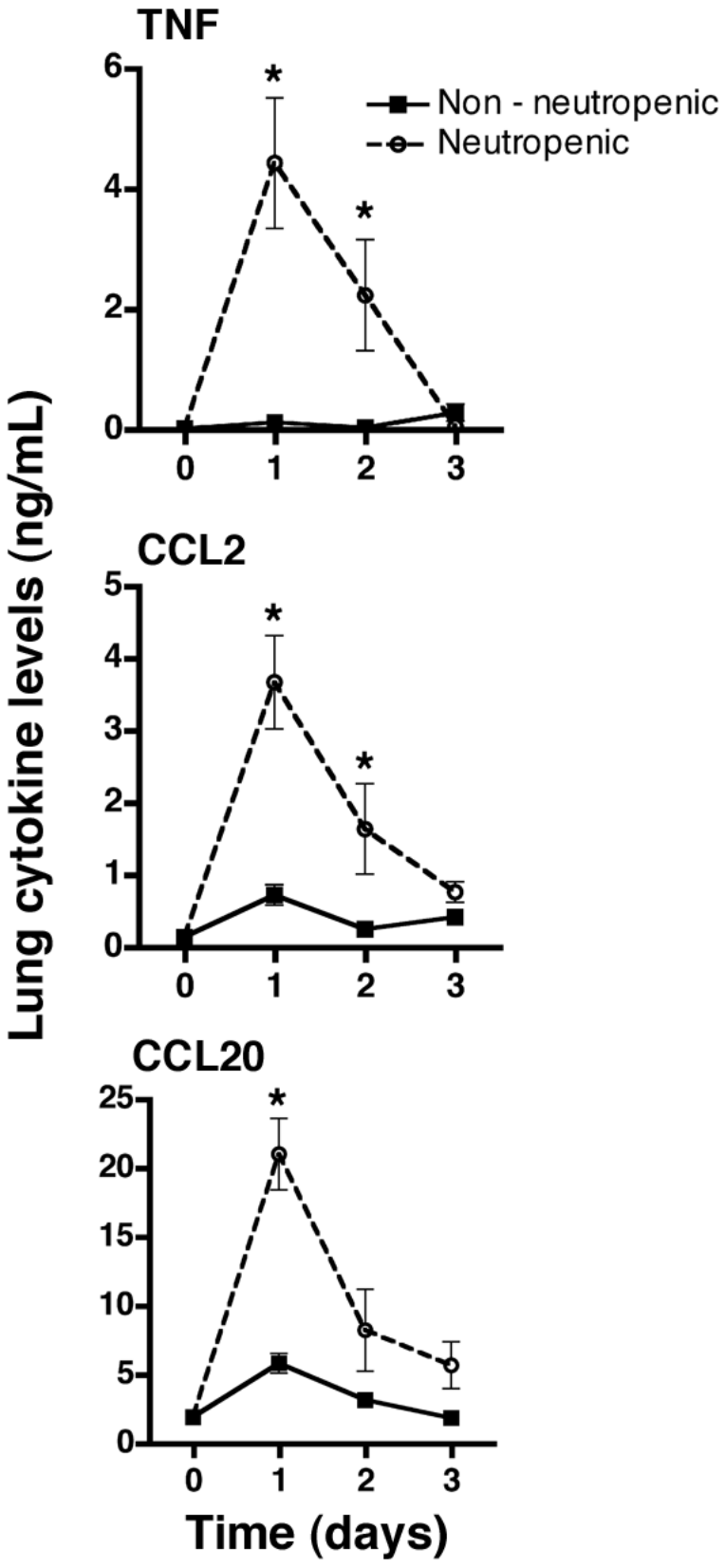

TNF
Non - neutropenic
Neutropenic
CCL2
CCL20
Lung cytokine levels (ng/mL)
Time (days)

Figure 12.

Protein levels of TNF, CCL2, and CCL20 in whole lung homogenates in mice challenged with *Aspergillus*. Cytokine levels in neutropenic (anti-Ly6G/C; clone RB6-8C5) and non-neutropenic (isotype control) mice were measured at various times following challenge with killed hyphae. Day 0 represents unchallenged mice. Data represent mean ± SEM of pooled data from 2 experiments; n = 6-8 mice per group per time point. *, $p < 0.05$ comparing trend between the two groups over time

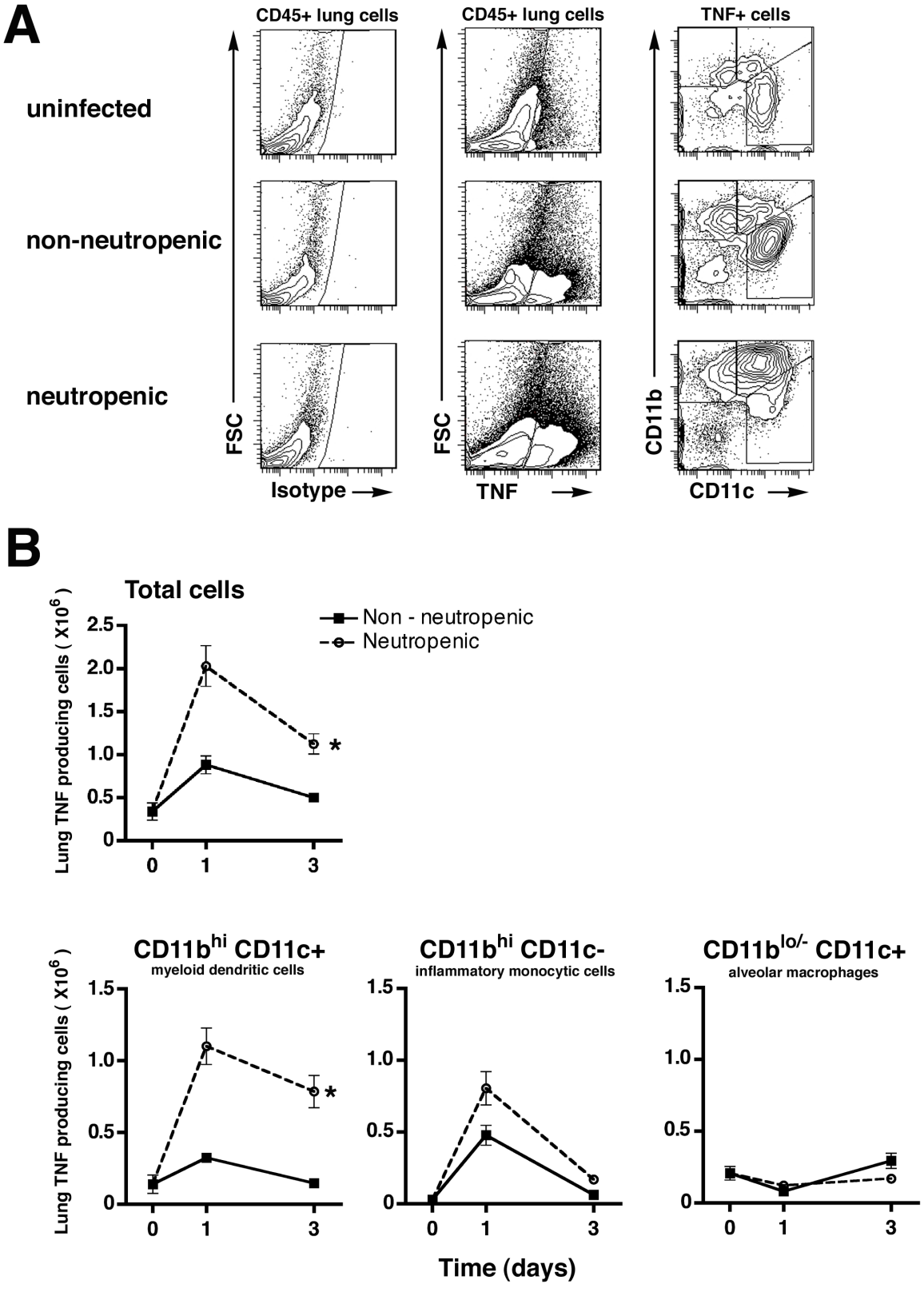

A
CD45+ lung cells
CD45+ lung cells
TNF+ cells
uninfected
non-neutropenic
neutropenic
FSC
Isotype
FSC
TNF
CD11b
CD11c
B
Total cells
Non - neutropenic
Neutropenic
Lung TNF producing cells (X10^6)
2.5
2.0
1.5
1.0
0.5
0
0
1
3
*
CD11b^hi CD11c+
myeloid dendritic cells
CD11b^hi CD11c-
inflammatory monocytic cells
CD11b^lo/- CD11c+
alveolar macrophages
Time (days)

Figure 13.

Cellular source of lung TNF in mice challenged with *Aspergillus*. (A) Representative flow cytometry plots and gating strategy of whole lung single cell suspensions in neutropenic (anti-Ly6G/C; clone RB6-8C5) and non-neutropenic (isotype control) mice after challenge with killed hyphae. Panels were initially gated on $CD45^+$ cells. (B) TNF-expressing cell subsets in the lungs were identified based on surface expression of CD11b and CD11c. Day 0 represents unchallenged mice. Data represents mean ± SEM; n = 6 mice per group per time point. *, $p < 0.05$ comparing trend between the two groups over time.

expression of CCL2 and CCL20, and accumulation of myeloid DCs in the lungs. Neutralization of TNF in neutropenic mice led to a marked reduction in both lung CCL20 and CCL2 levels, and resulted in reduction of the number of lung myeloid DCs comparable to non-neutropenic animals (Figure 14). Together with prior data supporting the role of CCL20 and its receptor, CCR6, in recruitment of myeloid DCs to the lungs (98), these data provide evidence for a positive-feedback loop in the lungs of neutropenic hosts challenged with *Aspergillus*, that results in accumulation of large numbers of immature TNF-producing myeloid DCs.

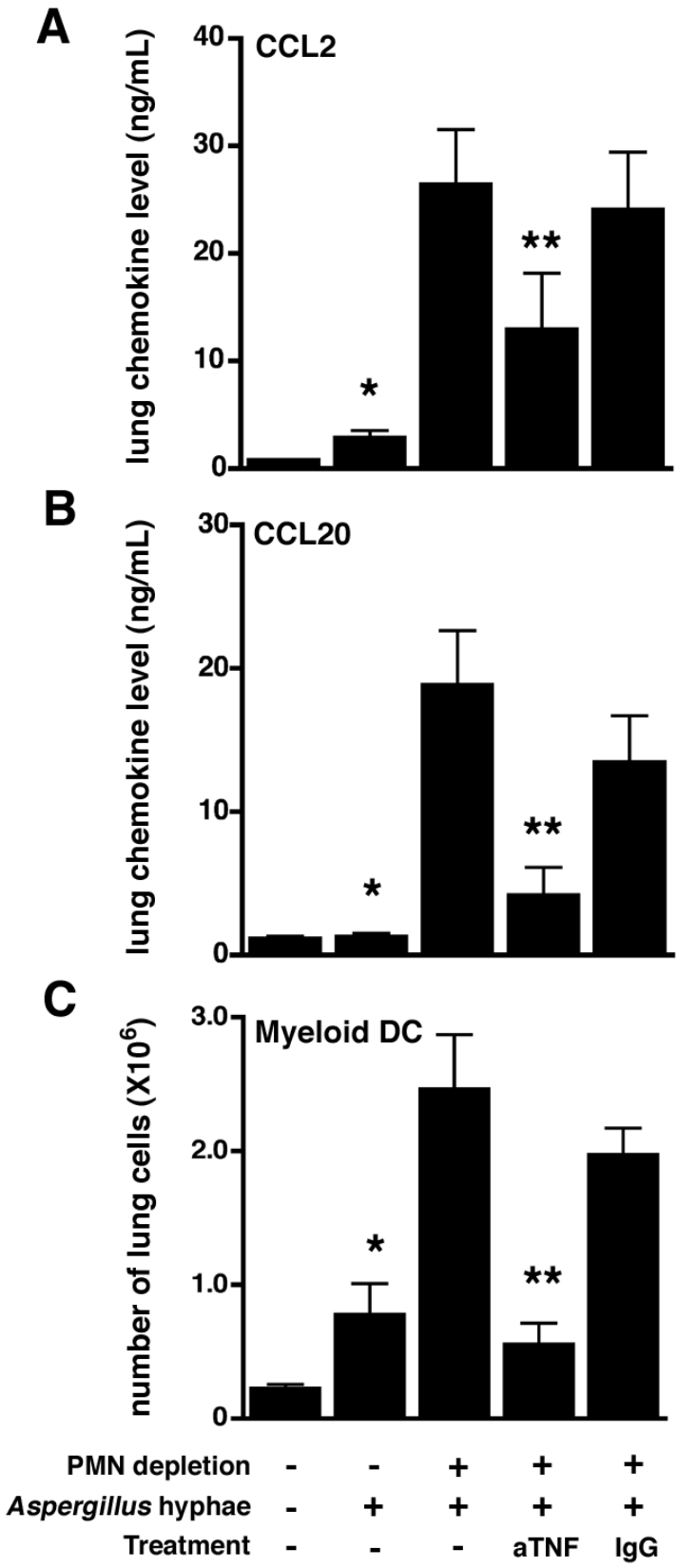

A
CCL2
lung chemokine level (ng/mL)
40
30
20
10
0
*
**
B
CCL20
lung chemokine level (ng/mL)
30
20
10
0
*
**
C
Myeloid DC
number of lung cells (X10^6)
3.0
2.0
1.0
0
*
**
PMN depletion - - + + +
Aspergillus hyphae - + + + +
Treatment - - - aTNF IgG

Figure 14.

Effect of TNF neutralization on lung chemokine levels and lung myeloid DC number in response to *Aspergillus*. Neutropenic (anti-Ly6G/C; clone RB6-8C5) and non-neutropenic (isotype control) mice were challenged with killed *Aspergillus* hyphae.(A-B) Protein levels of CCL2 and CCL20 in whole lung homogenates were measured 1 day after hyphae challenge. (C) Number of lung myeloid DC in whole lung single cell suspensions on day 3 after hyphal challenge. *, $p < 0.05$ compared with unchallenged animals; **, $p < 0.05$ compared with animals receiving control IgG and challenged mice not receiving any treatment. Data shown represent mean ± SEM; n = 5-6 for each group at each time point, representative data from 2 experiments.

Discussion

The susceptibility of neutropenic hosts to life-threatening infections is axiomatic. As case in point, a large body of literature supports the role of neutrophils in host defense against *Aspergillus* species: neutrophils recognize *Aspergillus* elements using TLR and C-type lectin pathogen recognition receptors (46, 72) and induce damage against swollen conidia and hyphae via production of reactive oxygen species, release of antimicrobial granule contents, and elaboration of neutrophil extracellular traps (5, 254). Emerging evidence has recently defined an additional role for neutrophils in shaping and directing immune responses that is distinct from their direct antimicrobial properties. These immunoregulatory functions are diverse and include generation of chemotactic signals, mediating vascular permeability, directing DC and macrophage phenotype and suppressing T cell responses in different experimental settings (255). Notably however, these observations have been made either under *in vitro* conditions or in animal models that are not readily applicable to clinical infections in neutropenia; as such, the relevance of the immunomodulatory effects of neutrophils to opportunistic infections of neutropenic patients has not been clearly defined.

In the present manuscript, we demonstrate that neutropenia fundamentally alters the inflammatory environment of the lung in response to the opportunist mold, *Aspergillus*, resulting in dramatic changes in the traffic and phenotype of lung

myeloid DCs. Lung myeloid DCs are known to transport *Aspergillus* elements from the lungs to secondary lymphoid organs and initiate protective T cell-mediated immunity (154, 249). We previously reported an unexpectedly large accumulation of immature myeloid DCs in the lungs of neutropenic mice with invasive aspergillosis that was, in part, dependent on the interaction of the chemokine ligand-receptor pair, CCL20-CCR6 (98). More recently, efflux of mature myeloid DCs from the lungs to mediastinal lymph nodes in the context of invasive aspergillosis was shown to be dependent on the chemokine receptor CCR7 (102). In this context, our present findings of contact-dependent neutrophil-mediated maturation of myeloid DC via DC-SIGN is consistent with prior *in vitro* studies with human neutrophils and DCs (256-258), and provides one mechanism for accumulation of DCs in the lungs.

As a second mechanism for the observed accumulation of DCs in the lungs, we found greatly accelerated recruitment of DCs to the lungs in neutropenic mice, which contrasts with prior reports of neutrophil mediating the recruitment of DCs in other experimental systems (259-261). This enhanced recruitment was dependent on a positive- feedback cycle involving lung DC-derived TNF, driving the local production of the chemokine ligands CCL2 and CCL20, and resulting in further recruitment of TNF-producing DCs to the lungs. This positive-feedback loop provides an explanation for prior observations of high expression of these mediators in the lungs of neutropenic mice with invasive aspergillosis (82, 103).

In addition, this paradoxical finding of an enhanced inflammatory cytokine milieu in the absence of neutrophils is consistent with the recently described role of neutrophils in dampening immune responses (262).

Another interesting finding of the current work pertains to the specific role of the accumulated lung DCs in defense against neutropenic invasive aspergillosis. In prior studies, interruption of the CCL20-CCR6 axis resulted in reduced lung DC numbers and worsened outcome of infection (98); given the well-documented role of DCs in initiating T cell-mediated protective immunity, this observation may have been attributable to failure to mount acquired immunity. Absence of CCR7, which resulted in failure of efflux of lung DCs, however, was found to result in an unexpected improvement of the outcome of the infection (102), suggesting one of two possibilities: either that DC migration to mediastinal lymph nodes was detrimental (for example by initiating T regulatory responses) or that retaining activated immature DCs in the lung was beneficial. As an additional consideration, observations in CCR6- and CCR7-deficient mice could conceivably be attributable to expression of these receptors on cells other than DCs. In the context of this literature, we found that the depletion of myeloid DCs in neutropenic hosts resulted in substantial worsening of pathogen clearance at a very early phase of the infection, suggesting that the accumulation of immature myeloid DCs in the lungs of neutropenic hosts, rather than their maturation and

efflux to mediastinal lymph nodes, is the key protective mechanism. More broadly, these data support a model in which, in the absence of neutrophils, lung myeloid DCs abandon their maturation and migratory phenotype in favor of assuming a critical role in local innate defense of the lung against the fungus.

In summary, we report that neutropenia causes enhanced lung inflammatory response to the common environmental mold, *Aspergillus*, and is associated with augmented influx of immature TNF-producing myeloid DCs to the lungs. Concomitantly, DC maturation and efflux to the draining lymph nodes is inhibited, resulting in an accumulation of these cells in the lungs to the benefit of the host. These findings have several ramifications for future investigations: First, given the evidence for an immunosuppressive role for neutrophils, the mechanisms underlying this phenomenon is of great interest since they may constitute therapeutic targets. Second, we provide data in support of the paradigm that DCs, like other myeloid cells, can act as effector cells in the early phase of infection, suggesting a function that is independent of their antigen presentation properties. Finally, neutrophils have recently been shown to include phenotypically and functionally distinct sub-populations (263, 264) and a subset of myeloid-derived suppressor cells are indistinguishable from neutrophils on the basis of morphology and cell surface markers (189). These observations raise the possibility that the observed immunomodulatory effect is a property of a specialized subset of neutrophils.

Methods

Animals and in vivo procedures

Wildtype C57BL/6 mice, CCR7-gene deficient mice (265), and mice heterozygote for the CD11c-DTR transgene (252) (all on C57Bl/6 background) were purchased from Jackson Laboratories (Bar Harbor, Maine). Age- and gender-matched 6- to 8-week old animals were used in all experiments. All animals were were bred and maintained under pathogen-free conditions and in compliance with institutional animal care regulations at the University of Virginia animal care facility.

Neutrophil depletion was achieved with a single i.p. injection of 80μg of a monoclonal Ab (Gr-1, clone RB6-8C5) 1 day before an intratracheal challenge with *Aspergillus fumigatus*, as described (84). This resulted in peripheral blood neutropenia (absolute circulating neutrophil count less than 50 cells/μL) on days 1 and 3 after injection in both infected and uninfected mice, with a return of peripheral counts to pretreatment levels (>1000 cells/μL) by day 5 (59, 188). Administration of the mAb did not influence the number of non-neutrophil peripheral blood leukocytes, nor lung and spleen lymphocytes or DC subsets ((98)and Figure 8A-B). In some experiments, neutrophil depletion was achieved by i.p. administration of 200 μg of another mAb (clone 1A8); this resulted in peripheral blood neutropenia for 3-4 days, as previously described (266). All non-neutropenic mice received isotype control mAb before being fungal challenge.

For depletion of DCs, mice heterozygous for the CD11c-DTR transgene were injected with diphtheria toxin (Sigma-Aldrich, St Louis, MO), as described (252). In *in vivo* TNF neutralization experiments, animals received i.p. administration of 300 μg of anti-TNF or isotype control monoclonal antibodies (clones XT3.11 and HRPN, respectively; BioXcell, West Lebanon, NH) 1 day before *Aspergillus* challenge. For the day 3 time point, an additional i.p. administration of anti-TNF or control Ab was given 48h later for a total of 2 doses.

In experiments designed to track the movement of cells to and from the lungs, we used previously described protocols with minor modifications (253, 267, 268): In studies of cellular influx to the lungs, circulating blood monocytes were labeled with latex beads by i.v. administration of 100μl of a 1:10 dilution of 0.5μm yellow-green latex microspheres (Polysciences, Warrington, PA) 24 hrs after administration of *Aspergillus* hyphae. In studies of cellular efflux from the lungs to lymph nodes, phagocytic lungs cells were labeled with 30μl intratracheal injection of a 1:25 dilution of the latex microspheres, administered simultaneously with *Aspergillus* hyphae. In adoptive transfer experiments, 4-5x10^6 DC (prepared as described below) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen Life Technologies) and administrated via the lateral tail vein in 100μl saline on day 2 following *Aspergillus* challenge, as described (98).

Preparation and administration of A. fumigatus

We used a modification of a previously characterized mouse model of neutropenic invasive aspergillosis (84, 94, 96, 98, 103, 269). Briefly, A. *fumigatus* (strain 13073, American Type Culture Collection) conidia were collected in 0.1% Tween in PBS from 7- to 14- day old cultures on Sabouraud's dextrose agar plates, filtered through sterile gauze and counted under a hemacytometer. In some experiments, resting conidia were then grown in RPMI-1640 in a shaking 37°C incubator for 5 hours to obtain swollen conidia or 12 hours to obtain short hyphae; the resulting fungal forms were then killed by resuspension in 70% ethanol in sterile water for 48 hours. Viability of the resulting suspension was determined to be < 1 in $2.6x10^7$ cfu by serial dilution and culture. Fungal forms were administered intratracheally in inocula ranging from 2 to $5x10^6$ for swollen conidia and 6 to $9x10^5$ for hyphae in 30µl per mouse. In some experiments, mice were infected with mutant temperature-sensitive strain of *Aspergillus* that has been shown to have attenuated virulence due to impaired growth at 37°C but not at room temperature (251). The mutant organism was grown on minimal media agar plates with phleomycin at room temperature for 5 days before harvesting of conidia.

Histology

We obtained tissue blocks of surgical lung biopsy samples from 16 patients with the histological diagnosis of invasive mold infection who had *Aspergillus fumigatus* isolated from their respiratory secretion during that hospital admission

and obtained peripheral blood absolute neutrophil counts from the day of surgery. Histologically normal lungs were used as controls. Representative 4μm paraffin-embedded sections were deparaffinized in xylene and rehydrated through graded ethanol to water, subjected to heat-induced target retrieval (for S100 antigen only, Dako Target Retrieval solution, Dako North America, Carpinteria, CA), had endogenous peroxidase activity quenched (Dual Endogenous Enzyme Block, Dako), were labeled with Ab against S100 (Dako, code Z0311) or CD1a (Dako, code M3571) followed by incubation with the labeled polymer and 3-3' diaminobenzidine (DAB+) substrate chromogen. Slides were counterstained with hematoxylin and bluing reagent (Thermo Scientific Anatomical Pathology, Pittsburgh, PA), dehydrated through graded alcohol to xylene and coverslipped.

Identification of leukocyte subsets

At designated time points, animals were euthanized by CO_2 asphyxiation, the pulmonary vasculature was perfused, and whole lungs were removed and leukocyte- enriched lung single cell suspensions were prepared as previously described (59, 94, 98, 103, 269). In some experiments, bronchoalveolar lavage was performed, as described (94). Peripheral blood was collected from the right ventricle into heparinized tubes. Mediastinal lymph nodes were resected and incubated in digestion buffer before being passed through 70 μm cell strainers (BD Biosciences). The following antibodies were used to label cells for flow cytometry (from BD Biosciences, San Jose, CA, eBiosciences, San Diego, CA,

Miltenyi, Auburn, CA, or R&D Systems, Minneapolis, MN): anti-B220-pacific blue (clone RA3-6B2), anti-CD3e-pacific blue (clone 500-A2), anti-CD11b-allophycocyanin-Cy7 (clone M1/70), anti-CD11c-PE-Cy7 (clone HL3), CD40-PE (clone 3/23), anti-CD45-peridinin chlorophyll protein (clone 30-F11), CD80- FITC (clone 16-10A1), anti-CD86-PE (clone GL1), anti-CD103-biotin (clone 2E7), anti-CD115-PE (clone AFS98), anti-F4/80-PE (clone A31), anti-I-A/I-E-FITC, -biotin, allophycocyanin and -pacific blue (clone M5/114.15.2), anti-Ly-6C-FITC (clone AL-21), anti-Ly-6G-PE and -FITC (clone 1A8), anti-Ly-6G/C-PE and -pacific blue (clone RB6-8C5), anti-PDCA1-PE (clone JF05-IC2.4.1), and anti-TNF-PE (clone MP6-XT22). To determine the lung cells capable of producing TNF, lung suspensions were incubated with brefeldin A (10ng/mL), PMA (10ng/mL) and ionomycin (100ng/mL) in RPMI-1640 with 5% FBS for 5 hours and intracellular staining was detected using a commercial kit (Cytofix/Cytoperm, BD Biosciences). For apoptosis detections, cells were labeled with Annexin V, according to manufacturer's instructions (Annexin V:PE Apoptosis Detection Kit I, BD Biosciences). Samples were analyzed on a FACS Canto II instrument using Diva software (BD Biosciences). The absolute number of each leukocyte subset was determined as the product of the percentage of the cell type and the total number of cells in the sample, as determine under a hemocytometer or on an automated cell counter (Countess, Invitrogen, Carlsbad, CA).

Cytokine and chitin assays

Aspergillus fumigatus grows as multicellular branching hyphae and does not form distinct reproductive structures in infected tissues. We therefore used a previously characterized assay for chitin, a carbohydrate component of hyphal wall that is absent from mammalian tissues and conidia, to quantify the burden of hyphae in infected lungs, as detailed previously (84, 98, 103, 269). We have previously shown that lung chitin content on day 3 of infection, as measured by this protocol, correlates with mortality from the infection and histological severity of the infection in this model (84). TNF, CCL2 and CCL20 protein levels in filtered supernatant of lung homogenates were determined either using commercial ELISA kits (Duoset ELISA Development, R&D Systems) or multiplex bead array kits (Milliplex Map, Millipore, Billerica, MA), according to the manufacturer's instructions.

In vitro studies

Immature bone marrow-derived myeloid DCs were prepared as described previously (98). In brief, bone-marrow cells were cultured in 20ng/mL mGM-CSF for 5 days and positively enriched by immunomagnetic selection of CD11c+ cells according to the manufacturer's instructions (Miltenyi, Auburn, CA), resulting in >95% purity. Recovered cells were >95% viable by trypan blue exclusion and consistent with immature phenotype, with low expression (MFI) of CD86, CD40, CD80, and MHC class II molecules by flow cytometry.

For co-culture experiments, resting bone marrow neutrophils were obtained from naive animals as described (59, 188) and incubated alone or with ethanol-killed hyphae in RPMI-1640 with 5% FBS in 24-well plates (Corning, Corning, NY) at 37°C in 5% CO2 for 5 hours before the addition of DCs and antibodies; cells were then cultured for an additional 16h. The ratio of DC to hyphae to neutrophil was 1:1:10. For cultures performed in transwell plates, DCs were placed in the lower chambers while both ethanol-killed hyphae and neutrophils were added to the upper chambers. The blocking antibodies and isotype controls were used at the following concentrations (all from BD Biosciences, R&D Systems): 20μg/mL anti-CD209a/SIGNR1, 10μg/mL anti-TNF (MP6- XT22), 10μg/mL anti-IL12p70 (C17.8), and 10μg/mL anti-dectin1/CLEC7A .

Statistical analysis

Data were analyzed on a Macintosh Powerbook G4 computer using Prism statistical package (v.4.0a, Graphpad Software, San Diego, CA). Values between 2 groups over multiple times were compared with 2-way ANOVA, comparisons between 2 groups at a single time were performed with unpaired two-tailed Mann-Whitney (non-parametric) test, and comparisons between multiple groups at a single time were compared using the Kruskal-Wallis test with Bonferroni post-test. Probability values were considered statistically significant if they were less than 0.05.

Chapter IV. Future Directions

Role of DC-SIGN on DCs

Dendritic cells have been shown to have an important role in the induction of specific immunity to *Aspergillus* (154). Early studies demonstrated the functional plasticity of dendritic cells in response to *Aspergillus* conidia and hyphae. With a variety of pathogen recognition receptors at their disposal including DC-SIGN (14, 45, 70, 76), DCs can recognize both forms of *Aspergillus* and initiate qualitatively different CD4 T cells responses (154, 168, 249). Myeloid DCs ($CD11b^{hi}$, CD11c+) are a subset of lung dendritic cells derived from Ly-$6C^{hi}$ monocytes during inflammatory responses (250) and have been shown to quickly expand in the lung following a respiratory challenge of *Aspergillus* (98, 102, 105). While their presence have been shown to promote host defense in the early stages of the infection, it is unclear how they mediate this effect. In an immunocompetent model of invasive aspergillosis, the absence of monocytes led to a clear reduction in the number of myeloid dendritic cells and impaired fungal clearance by abolishing CD4 T cell priming (105). Our studies show that DC ablation leads to a marked increase in fungal burden by day 3 of infection in neutropenic mice and suggests in the setting of neutropenia, myeloid DCs may be involved in innate host defense possibly through direct microbicidal functions. The myeloid DCs in a neutropenic system may be equivalent to TipDCs [TNF-α

(tumor necrosis factor-α)-iNOS (inducible nitric oxide synthase)-producing DCs], a subpopulation of monocyte-derived DCs described to be involved in the innate defense mechanisms against bacterial and parasitic pathogens (270-274). A hallmark of TipDCs is the ability to produce both iNOS and TNF and they appear to be involved in innate immunity through the production of soluble mediators with microbicidal activity rather than in the development of a specific T-cell response.

While neutrophils and dendritic cells are located in different compartments in steady state, they colocalize at the site of inflammation during the early stages of the immune response. Number of studies demonstrate that cell interactions occur between neutrophils and dendritic cells. Direct contact involved a glycoslation-dependent interaction between the c-type lectin DC-SIGN on dendritic cells and the adhesion molecules CD11b or CECAM1 on PMNs (256, 257). The interaction subsequently led to the maturation of immature dendritic cells as demonstrated by the upregulation of costimulatory molecules CD86 and CD40 and the production of the activating cytokine IL-12 (257, 258, 275). In addition, TNF-α produced by activated neutrophils was shown to be essential for DC maturation (257, 261, 276). The current work supports these observations since in our *in vitro* system, we have also shown that dendritic cell maturation also requires PMN contact and through an interaction involving the DC-SIGN receptor. In addition, co-incubation of DCs with hyphae does not induce maturation. This

suggests that during an immune response, DC function and phenotype can be shaped by its specific interactions with other cells or pathogens (Fig 1). In the host response to *Aspergillus*, contact with neutrophils through the DC-SIGN receptor may be required for the induction of DC maturation and subsequent migration to the draining lymph nodes. However, in the absence of neutrophils, the interaction of DCs with *Aspergillus* via DC-SIGN may lead to the expansion of immature TNF-producing DCs. Future studies need to define what other surface receptors may be involved in the communication between neutrophils and dendritic cells. In addition, it is unknown what PMN-derived mediators are required for DC maturation.

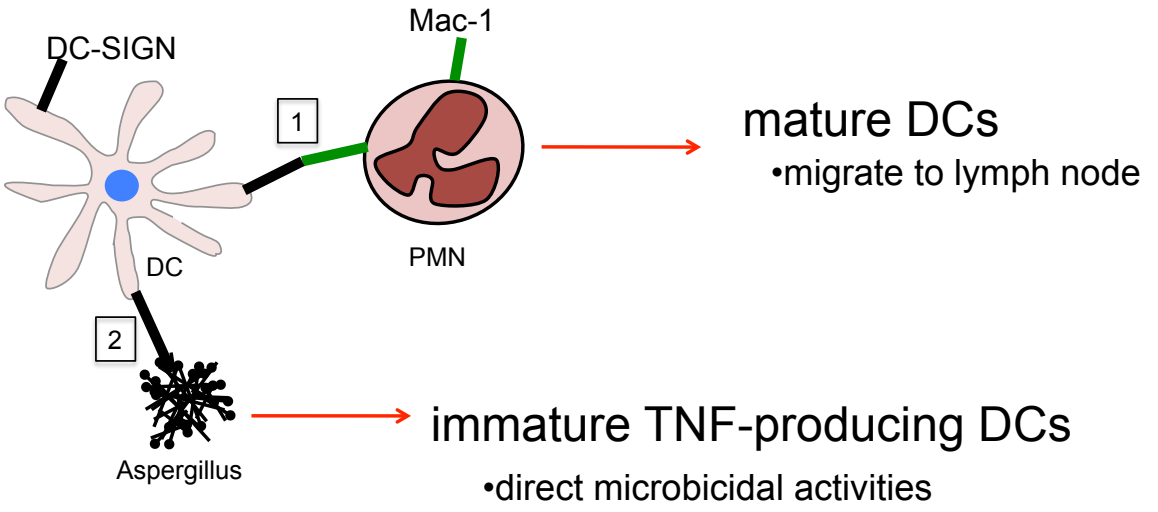


Figure 1

Schematic representation of the role of DC-SIGN in DC maturation and function

Neutrophil mediated anti-inflammatory response

In this murine system of invasive aspergillosis, the absence of neutrophils profoundly changes the lung environment by triggering a rapid influx of dendritic cells and promoting a pro-inflammatory response to *Aspergillus*. These observations raise the question: How are neutrophils shaping the dendritic cell response? The most recognized function of neutrophils is its ability to effectively kill pathogens and contain infections. Until recently, the study of neutrophils primarily focused on examining its direct antimicrobial functions including the ability to generate reactive oxygen species and the extracellular release of antimicrobial polypeptides. The ensuing inflammation from neutrophil-dependent tissue injury is essential to host defense as it is one of the major signals that launches the host's immune response but often viewed as a process that must ultimately be curbed by other leukocytes to prevent excessive host tissue damage. The idea that neutrophils may modulate the immune response or inflammation is a relatively new and under-appreciated concept. There is increasing evidence to support neutrophils mediating the immune response by interacting with a variety of cells of the immune system and influence the phenotype and functions of these cells (248, 255). Activated neutrophils produce and release a variety of chemokines to attract more neutrophils and to recruit other immune cells like T cells, monocytes, macrophages, and DCs (277-279). Antimicrobial peptides produced by neutrophils have chemotactic functions to

attract cells such as T cells and immature DCs (280, 281). Thus, neutrophils have the potential to orchestrate ongoing immune responses at the site of infection.

There are some evidence to suggest that nicotinamide adenine dinucleotide phosphate (NADPH)-derived reactive oxygen species (ROS) may be involved in anti-inflammatory responses. The NADPH oxidase is a multicomponent plasma membrane associated enzymes responsible for the production of ROS including superoxide anion (O_2), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-) and the core enzyme comprises five components: p40phox (PHagocyte OXidase), p47phox, p67phox, p22phox and gp91phox. (282). While this enzyme is found in a variety of cells of mesodermal origin, activated neutrophils are known to produce large amounts of ROS as part of a major anti-microbial defense mechanism.

The functional significance of NADPH-oxidase has been shown in studies of patients with chronic granulomatous disease (CGD), a rare, X-linked or autosomal-inherited disorder showing deficient ROS production as a result of a genetic defect in any one of the four components of the NADPH oxidase (282). Failure to produce an oxidative burst results increases susceptibility to severe and recurrent infections by various pathogens including *Aspergillus fumogatus*. Interestingly, CGD patients are also highly susceptible to developing

inflammatory granulomas without clinical evidence of infection (283). In fact, mouse models of X-linked (gp91phox-/-) and autosomal-recessive (p47phox-/-) CGD show similar observations: these mice are susceptibility to infections but also show exaggerated inflammatory reactions to heat-killed fungus, with increased leukocyte accumulation compared with wild-type animals and increased early production of the pro-inflammatory cytokines IL-1β and TNF-α (79). Indeed, following challenges with killed *Aspergillus, hyphae*, we found an increased accumulation of lung dendritic cells in both neutropenic and non-neutropenic CGD mice (Fig 2). This suggests that the enhanced inflammatory response we have observed in neutropenic mice following *Aspergillus* challenges may be due to the lack of ROS. Recently, ROS have been regarded as important intracellular signaling messengers inducing apoptosis (2, 284, 285). In addition, in a study involving acute lung injury induced by lipopolysaccharide (LPS), H_2O_2 demonstrated an anti-inflammatory effect and shown to be beneficial in this injury model. The persistence of inflammation could be linked to an endogenous defect and imbalance in the release of pro- and anti-inflammatory mediators in neutrophils. Future studies should involve examining the effects of ROS on neutrophils and dendritic cells in the host response to *Aspergillus*: PMN-derived ROS may have an important role in dampening the inflammatory response created by the myeloid DCs.

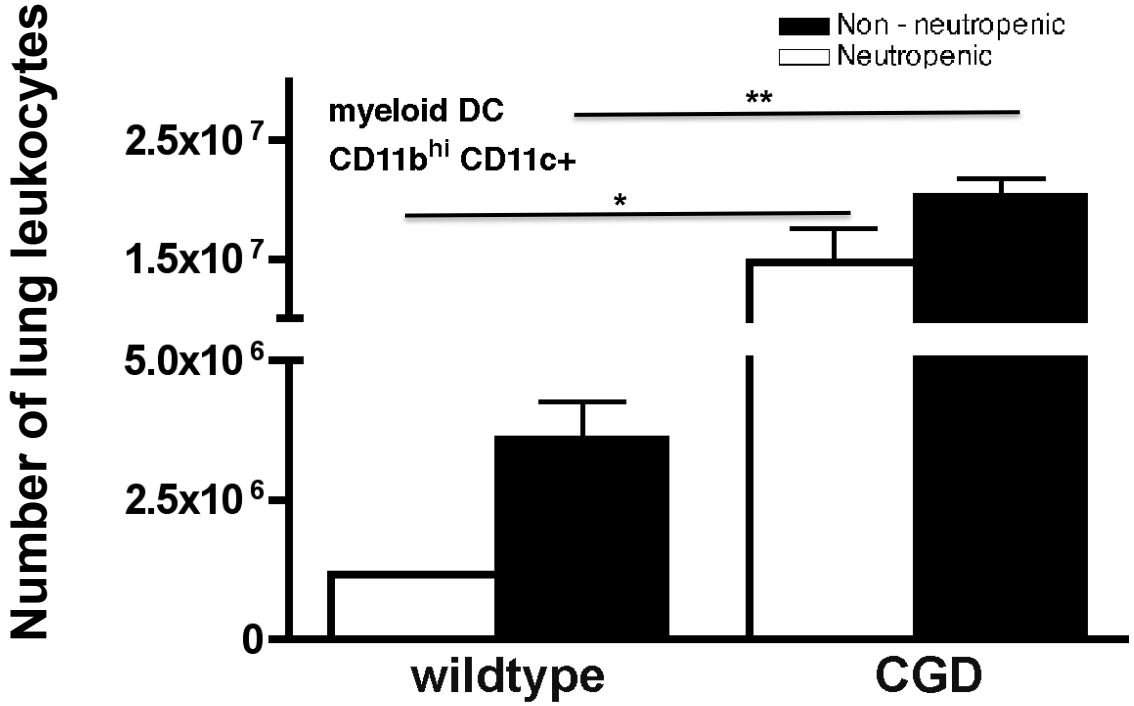

Non - neutropenic
Neutropenic
myeloid DC
CD11b^hi CD11c+
**
*
Number of lung leukocytes
2.5x10^7
1.5x10^7
5.0x10^6
2.5x10^6
0
wildtype
CGD

Figure 2

Effect of ROS production on the number of lung DCs in response to *Aspergillus*. Number of lung neutrophil myeloid DC are shown on day 3 after challenge with killed *Aspergillus* hyphae in neutropenic (anti-Ly6G/C; clone RB6-8C5) and non-neutropenic (isotype control) WT and CGD (gp91phox-/-) mice. Data shown represent mean ± SEM; n = 5 mice per group, representative data from 2 independent experiments *, $p < 0.05$ comparing trend between the neutropenic groups of WT mice and CGD mice, **, $p<0.05$ comparing trend between the non-neutropenic groups of WT and CGD mice.

DC and NK cell cross-talk

The differentiation of TNF-producing DC may also involve the presence of activated NK cells. In a study involving the model of infection by *Listeria monocytogenes*, the differentiation and function of TipDCs was shown to be dependent on the previous activation of splenic DCs and NK cells (286). IL-12 and IL-18 production by splenic DCs triggered IFN-γ production by NK cells. NK-derived IFN-γ was important for the differentiation of TipDCs from Ly-6C^{hi} monocytes (286). IL12 and IL-18 has been shown to be induced the lungs of mice following respiratory challenges of Aspergillus. (80, 163, 164). In addition, myeloid DCs exposed to *Aspergillus* conidia produce large amounts of IL12 *in vitro* (154, 168).

Both *in vitro* and *in vivo* evidence demonstrate cross talk between DC-NK cells (287-289). DC and NK cell interaction has been shown to be important for the activation of NK cells (290). DC-mediated NK cell activation is contact-dependent (290-292) and mediated by several cytokines including IL12 and IL18 (289, 291). The cellular sources of IL12 and IL18 have not been defined in the neutropenic model of invasive aspergillosis. Further studies are required to determine whether in the host response to *Aspergillus*, myeloid DCs are involved in NK cell activation and production of IFN-γ. Since NK cells are the primary source of IFN-

γ in this model, it will be interesting to examine whether these activated NK cells are then involved in the development of TNF-producing myeloid DCs.

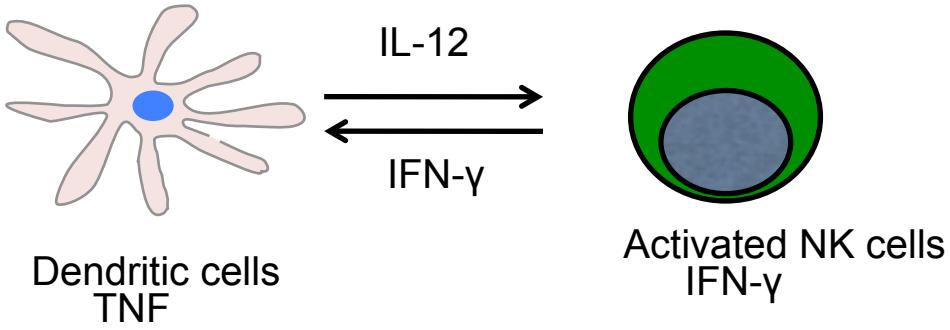


Figure 3 Schematic representation of DC-NK cell cross talk

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