

Role of Macrophages in Host Defense Against Aspergillosis and Strategies for Immune Augmentation

BRAHM H. SEGAL

Roswell Park Cancer Institute, Buffalo, New York, USA

Key Words. *Aspergillus* • Colony-stimulating factor • Macrophage • Review

Disclosure: B.H.S. has acted as a consultant to Pfizer, Berlex, ViraCor, and Enzon, and has received speaking honoraria from Merck and Pfizer.

ABSTRACT

Invasive aspergillosis is a major cause of morbidity and mortality in highly immunocompromised patients with cancer. Alveolar macrophages ingest inhaled conidia (spores). Through pathogen recognition receptors that ligate fungal cell wall motifs, macrophages are able to coordinate the inflammatory response to *Aspergillus* species. Macrophages and dendritic cells play an important role in regulating the balance between the

proinflammatory and anti-inflammatory cytokine responses that are required for recruitment and activation of neutrophils, and in augmenting or attenuating cellular immunity. Macrophages are therefore a target for immune augmentation strategies that include administration of cytokines, colony-stimulating factors, and pathogen recognition receptor ligands. *The Oncologist* 2007;12(suppl 2):7–13

INTRODUCTION

Patients with hematologic malignancies encompass a broad range of immunocompromised states. Important differences in both the degree and nature of the immunocompromise exist among different patients. The major host deficits that predispose to opportunistic mold infections are prolonged neutropenia and graft-versus-host disease (GVHD) [1, 2]. In neutropenic patients, the degree and duration of neutropenia predict the risk for life-threatening infections. Among allogeneic hematopoietic stem cell transplant (HSCT) recipients, the early period of risk for infections corresponds to neutropenia following the conditioning regimen and later periods correspond to the intensity of immunosuppressive therapy required to control GVHD. Several studies have reported the predominance of invasive aspergillosis cases occurring in the postengraftment rather than in the neutropenic period in allogeneic HSCT recipients [3–10], with immunosuppressive therapy for GVHD and T-cell depletion being principal risk factors. In severe GVHD,

global immune impairment occurs that affects both innate phagocyte function and antigen-specific immunity.

Most of the efforts related to immune augmentation against invasive aspergillosis have focused on increasing the number of circulating neutrophils in neutropenic patients through the use of colony-stimulating factors (CSFs) and granulocyte transfusions. Substantial knowledge has been gained regarding the role of macrophages in coordinating the inflammatory response to fungal pathogens [11]. Studies in animal models of invasive aspergillosis have identified both macrophages and dendritic cells (DCs) as promising targets for immunotherapy (Table 1).

COOPERATIVE ROLE OF MACROPHAGES AND NEUTROPHILS IN DEFENSE AGAINST ASPERGILLOSIS

Aspergillus species are ubiquitous soil inhabitants whose conidia (spores) we inhale on a regular basis, and are normally harmless to immunocompetent individuals. Respira-

Correspondence: Brahm H. Segal, M.D., Division of Infectious Diseases, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, New York 14263, USA. Telephone: 716-845-5721; Fax: 716-845-5777; e-mail: brahm.segal@roswellpark.org Received May 22, 2007; accepted for publication July 20, 2007. ©AlphaMed Press 1083-7159/2007/\$30.00/0 doi: 10.1634/theoncologist.12-S2-7

Table 1. Goals and strategies for augmentation of the immune response to fungal infections

| Goal | Strategies |
|---|---|
| Increase in neutrophil number | CSFs (G-CSF and GM-CSF); granulocyte transfusions; myeloid progenitors (common myeloid progenitors, granulocyte-monocyte progenitors); thymosin- α 1 |
| Activation of neutrophils | CSFs (G-CSF and GM-CSF); cytokines (e.g., IFN- γ); chemokines; TLR activation |
| Activation of macrophages and dendritic cells | CSFs (M-CSF and GM-CSF); cytokines (e.g., IFN- γ); TLR activation |
| Heightened cellular immunity | Cytokines (e.g., IFN- γ); TLR activation; pentraxin 3; thymosin- α 1; vaccines |
| Heightened humoral immunity | Vaccines; antibody administration (e.g., monoclonal antibody 18B7 for <i>Cryptococcus neoformans</i>) |

^aMost of the listed strategies are experimental and have not been evaluated in patients. Abbreviations: CSFs, colony-stimulating factors; IFN- γ , interferon- γ ; TLR, toll-like receptor. From Segal BH, Kwon-Chung J, Walsh TJ et al. Immunotherapy for fungal infections. *Clin Infect Dis* 2006;42:507–515, with permission. ©2006 by the Infectious Diseases Society of America. All rights reserved.

tory mucosal epithelial cells serve as an anatomic barrier to parenchymal invasion, promote mucociliary clearance, and ingest inhaled conidia [12]. Alveolar macrophages (AMs) constitute the first line of phagocytic host defense against inhaled conidia [13]. Peripheral blood monocytes and neutrophils are subsequently recruited to sites of infection. Following germination (transformation from conidia to hyphae), neutrophils are the dominant host defense arm against hyphae, the tissue-invasive form of molds [13]. Thus, a cooperative early innate immune response occurs that is mediated by macrophages and neutrophils in which macrophages target inhaled conidia and neutrophils damage hyphae, preventing parenchymal invasion. Monocytes also have antifungal activity against *Aspergillus fumigatus* hyphae in vitro, an effect that has been enhanced by GM-CSF and interferon- γ (IFN- γ) [14]. Both macrophages and neutrophils are important targets for immune augmentation.

MACROPHAGES COORDINATE THE EARLY INFLAMMATORY RESPONSE TO INHALED MOLDS

In addition to ingesting inhaled conidia, AMs play a key role in orchestrating the inflammatory response. Pathogen recognition receptors (PRRs) recognize specific fungal cell wall motifs displayed during the conidial and hyphal stage and produce cytokines and chemokines that stimulate neutrophil recruitment and subsequent antigen-specific immunity. Recent studies have demonstrated the key role

of PRRs in regulating innate and antigen-dependent immunity in response to fungal infections [15, 16]. There are several classes of innate PRRs that recognize fungal motifs. Examples include toll-like receptors (TLRs), dectin-1, pentraxins, collectins, surfactant protein A (SP-A), SP-D, and mannose-binding lectin [17], classical C-type lectins, and lactosylceramide [18, 19]. Manipulation of responses of lung macrophages and DCs is a promising approach to augmenting antifungal immunity [11, 15]. The review focuses on TLRs and dectin-1 as key PRRs that recognize *Aspergillus* motifs and coordinate inflammatory responses.

TLRs

TLRs are a conserved family of receptors that recognize common protein and DNA pattern motifs present on microbial pathogens, and initiate signaling events related to cytokine production and T-cell and DC maturation. During the phagocytosis of pathogens, TLRs recognize pathogen-specific motifs within the vacuole, distinguish among pathogens, and trigger an inflammatory response appropriate to defense against the specific organism [20, 21]. TLRs have homology to interleukin-1 type R1 (IL-1R1) and share a similar signaling cascade leading to activation of nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinases, a process that mediates gene expression and regulation of inflammatory responses. TLR-dependent antifungal pathways are highly conserved in nature, as demonstrated by their presence in *Drosophila* species [22, 23].

TLRs recognize motifs on fungal pathogens including *Candida* [24], *Cryptococcus* [25], and *Aspergillus* species, and regulate the induced inflammatory responses. TLR4-defective mice are more susceptible to *Candida albicans* infection, and this is associated with impaired chemokine expression and neutrophil recruitment [24]. Netea et al. [26] reported that *A. fumigatus* conidia, but not hyphae, stimulated macrophages to produce the proinflammatory cytokines tumor necrosis factor- α (TNF- α) and IL-1 in a TLR4-dependent fashion. In contrast, *A. fumigatus* hyphae, but not conidia, stimulated production of the anti-inflammatory cytokine IL-10 through TLR2-dependent mechanisms. This switch from proinflammatory to anti-inflammatory signals during germination may help *Aspergillus* evade host defenses. Wang et al. [27] reported that TLR4, but not TLR2, mediated activation of human monocytes by *A. fumigatus* hyphae. Other investigators have found that both TLR2 and TLR4 recognize *A. fumigatus* hyphae, stimulate proinflammatory cytokines in effector cells, and stimulate neutrophil recruitment [28, 29].

TLRs on macrophages, neutrophils, and DCs govern the inflammatory response to *Aspergillus* infection [16, 30, 31]. Because they play a key role in innate and antigen-

specific immunity, the use of TLR ligands that stimulate multiple inflammatory pathways is a promising approach. Local delivery of CpG oligodeoxynucleotides (which signal through TLR9) and the Asp f 16 *Aspergillus* allergen has resulted in activation of airway DCs capable of inducing T-helper type 1 (T_H1) cell priming and resistance to the fungus [32]. Thymosin- $\alpha1$, a naturally occurring thymic peptide, has induced maturation and IL-12 production in DCs pulsed with *A. fumigatus*, an effect mediated by distinct TLRs [33]. In this study, thymosin- $\alpha1$ augmented T_H1 cell immunity against *A. fumigatus*, accelerated myeloid recovery in neutropenic mice, and was protective against *Aspergillus* challenge in murine bone marrow transplant recipients [33]. These studies provide a rationale to stimulate or inhibit specific classes of TLRs as a means of enhancing both innate and antigen-specific immunity to fungi.

DECTIN-1

Brown et al. [34–36] identified dectin-1 as a major innate immune recognition receptor and immunomodulator of β -glucans, which are ubiquitous fungal cell wall constituents. Dectin-1 is a natural killer cell receptor-like C-type lectin expressed at high levels within the pulmonary and gastrointestinal tracts at portals of pathogen entry, suggesting a potential role for pathogen recognition and host defense [18]. After binding to particulate glucans, dectin-1 stimulates the production of IL-12, IL-10, TNF- α , and macrophage inflammatory protein type 2 (MIP-2). It also induces ligand uptake through phagocytosis and stimulates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity [37]. NADPH oxidase is a critical host defense pathway; chronic granulomatous disease, an inherited disorder of the phagocyte NADPH oxidase, is associated with recurrent life-threatening bacterial and fungal infections [38–40].

Dectin-1 contains an immunoreceptor tyrosine-based activation motif in its cytoplasmic tail that is involved in cellular activation [37]. Dectin-1 collaborates with TLR2 to induce TNF- α and IL-12 following zymosan stimulation [37]. Dectin-1 can recognize β -glucan motifs on several fungal species including *Candida* species [34], *Pneumocystis carinii* [41], *Coccidioides* species [42], and *Aspergillus* species [18]. Based on the ability of dectin-1 to recognize immunomodulatory fungal cell wall products, facilitate phagocytosis and fungal killing, induce NADPH oxidase activation, and, in cooperation with TLRs, stimulate and regulate cytokine responses, dectin-1 has been posited to play a role in fungal recognition and antifungal immunity [18]. Definitive evidence for a role of dectin-1 in antifungal immunity will be derived from genetically engineered dectin-1^{-/-} mice.

Gantner et al. [37] demonstrated a cooperative interaction among dectin-1, TLRs, and NADPH oxidase activation in response to zymosan. During macrophage and DC recognition of zymosan (particulate β -glucan), both dectin-1 and TLR2 were recruited to phagosomes where dectin-1 binds to β -glucans and TLR2/CD14 recognize other components of the fungal cell wall. Dectin-1 enhanced TLR2-mediated activation of NF- κ B in response to zymosan. Dectin-1 and TLR2 cooperatively interacted in activation of macrophages and DCs following zymosan challenge and in mediating production of IL-12 and TNF- α . Additionally, dectin-1 was required for zymosan-mediated activation of NADPH oxidase, a response that was primed by TLR4 activation.

Fungal β -glucans act as a trigger for the induction of inflammatory responses in macrophages through their time-dependent exposure on the surface of germinating conidia [43–45]. Dectin-1 and TLRs permit macrophages to distinguish between *A. fumigatus* conidia and hyphae. Whereas conidia ingested by macrophages did not stimulate NADPH oxidase or an inflammatory response, early germinated hyphae stimulated NF- κ B, secretion of proinflammatory cytokines, and NADPH oxidase activation in human and murine macrophages [44]. Germination rendered fungal cell wall β -glucans accessible to dectin-1, and dectin-1 binding to germ tubes augmented TLR2-mediated stimulation of cytokines [44].

TNF- α

Ligation of PRRs by specific fungal cell wall motifs will result in cytokine and chemokine responses. In general, TLR ligation stimulates proinflammatory cytokine responses that lead to recruitment of inflammatory cells and augmentation of innate and cellular immunity. However, exceptions exist in which TLR2 may lead to an anti-inflammatory response. Following stimulation by a broad range of microbial products (e.g., endotoxin, CpG sequences), TLR activation typically causes robust TNF- α production by macrophages. Particularly in the context of existing immunocompromise (e.g., neutropenia or immunosuppressive regimens), TNF- α produced by AMs may have an important role in defending against pulmonary fungal infections.

Data from human and animal studies show that TNF- α has a protective role against aspergillosis. Because of confounding host factors, the degree that TNF- α inhibition predisposes to invasive aspergillosis is difficult to gauge in patients. Infliximab, an anti-TNF- α antibody, likely increases the risk for invasive aspergillosis in allogeneic HSCT recipients with refractory GVHD [46, 47]. Patients with rheumatologic disorders are generally at very low risk for invasive aspergillosis, and anti-TNF- α antibodies likely have a modest effect in increasing this risk [48].

TNF- α increases AM phagocytic activity against *A. fumigatus* conidia and augments the capacity of neutrophils to damage hyphae in vitro [49]. Mehrad et al. [50] showed that depletion of TNF- α in mice led to an increase in mortality in both normal and cyclophosphamide-treated animals challenged with *A. fumigatus*, and was associated with increased lung fungal burden. TNF- α is not directly chemotactic for neutrophils, but probably contributes to the chemotaxis of neutrophils via secondary mechanisms, including induction of neutrophil-chemotactic chemokines and induction of adhesion molecules [51, 52]. Depletion of TNF- α resulted in a reduced lung neutrophil influx in both normal and cyclophosphamide-treated animals, which occurred in association with a decrease in lung levels of the C-X-C chemokine MIP-2 and the C-C chemokines MIP-1 α and JE.

CSFs AND AUGMENTATION OF MACROPHAGE IMMUNITY

Normal myelopoiesis requires myeloid stem cells. Under the influence of stem cell factor, IL-3, and GM-CSF, these give rise to colony-forming units–granulocyte-macrophage. G-CSF acts at a later stage in concert with other growth factors to specifically drive granulopoiesis. Multiple randomized clinical trials of prophylactic recombinant human (rh) G-CSF and rhGM-CSF have shown the benefit of CSFs in reducing the time to neutrophil recovery and duration of fever and hospitalization in patients with acute myelogenous leukemia [53]. In one randomized study in patients receiving chemotherapy for acute myelogenous leukemia, prophylaxis with rhGM-CSF led to a lower frequency of fatal fungal infections compared with placebo and reduced overall early mortality [54]. However, no other randomized study of prophylactic CSFs has demonstrated a survival advantage compared with placebo. The American Society of Clinical Oncology has established authoritative guidelines related to the use of prophylactic CSFs in standard practice [55]. A gap in knowledge exists as to the role (if any) of CSFs as adjunctive therapy for established invasive fungal infections.

CSFs, in addition to augmenting leukocyte numbers, also augment phagocyte function. G-CSF, GM-CSF, and M-CSF increase the fungicidal activity of phagocytes in vitro against *Candida* and *Aspergillus* species [14, 56–58]. G-CSF influences survival, proliferation, and differentiation of all cells in the neutrophil lineage and augments the function of mature neutrophils. M-CSF increases phagocytosis, chemotaxis, and secondary cytokine production in monocytes and macrophages [59]. GM-CSF stimulates various neutrophil effector functions and prolongs neutrophil survival in vitro, increases antibody-dependent cytotoxicity of eosinophils, accelerates the proliferation of

the monocyte–macrophage system, and is a potent activator of monocytes and macrophages [59]. Some studies in vitro [60] and in animal models [61, 62] have shown that G-CSF and GM-CSF have additive antifungal activity when combined with antifungal agents. Clinical data on the use of adjunctive CSFs for invasive aspergillosis are sparse and no conclusions about efficacy can be made. However, given the importance of neutrophil recovery in determining the outcome of invasive aspergillosis, it is reasonable to use G-CSF or GM-CSF as adjunctive therapy for invasive aspergillosis in neutropenic patients.

A study of prophylactic rhM-CSF in experimental pulmonary aspergillosis sheds light on the potential for augmentation of macrophage recruitment and function in host defense against *Aspergillus* infection [63]. Though rhM-CSF had no benefit when administered after pulmonary aspergillosis was established in neutropenic rabbits, prophylactic rhM-CSF starting 3 days prior to *A. fumigatus* challenge led to longer survival and less pulmonary injury compared with controls. Rabbits treated with rhM-CSF had greater numbers of AMs, and harvested AMs had greater cytoplasmic volume and more periodic acid-Schiff stain–positive granules (reflecting activation) than controls. In addition, AMs from rhM-CSF–treated animals were more effective at phagocytosis of *Aspergillus* conidia ex vivo. These data are consistent with the fact that AMs function as the first line of host defense against *Aspergillus* infection by phagocytosing and destroying inhaled conidia, whereas neutrophils are targeted against the hyphal (invasive) stage. By augmenting AM numbers and function, prophylactic rhM-CSF is an attractive candidate for the prevention of invasive fungal infection.

Another gap in knowledge is whether CSFs are safe and effective as either prophylaxis or adjunctive therapy in non-leukopenic patients with severe impairment in phagocyte function. Intensive immunosuppressive corticosteroid-based regimens for GVHD cause global impairment of phagocyte effector functions and disable reconstitution of antigen-specific immunity, though circulating neutrophil counts are generally normal. In theory, GM-CSF augments both macrophage and neutrophil functions that may be beneficial in severely immunocompromised non-neutropenic patients with invasive aspergillosis. There are no data to support the use of prophylactic CSFs in non-neutropenic patients, and they should not be used as prophylaxis in this setting outside a clinical trial.

RECOMBINANT IFN- γ

Interferons are immune modulators that regulate the expression of numerous genes that mediate inflammation. Exposure to various pathogens can stimulate at least two

patterns of cytokine production by CD4⁺ T cells. T_H1 cells are defined by production of IFN- γ , lymphotoxin, and IL-2, and T_H2 cells by production of IL-4, IL-5, and IL-13. Several laboratories have shown that IFN- γ augments the antifungal activity of effector cells (macrophages and neutrophils). Studies in vitro, in animal models [64], and in humans (limited patient data) provide a rationale for using adjunctive IFN- γ for invasive aspergillosis. Roilides et al. [56] reported that rhIFN- γ augmented the human neutrophil oxidative response and killing of *A. fumigatus* hyphae in vitro, and acted additively with G-CSF. It prevented corticosteroid-mediated suppression of neutrophil killing of hyphae [65]. In another study, rhIFN- γ also enhanced killing of *A. fumigatus* hyphae by human monocytes [14]. In addition to augmenting the function of phagocytes, IFN- γ is the signature cytokine for type 1 cellular immunity. Augmentation of cellular immunity has been protective in experimental aspergillosis [66].

Dignani et al. [67] reported successful outcomes using rhIFN- γ paired with CSFs in four patients with leukemia and refractory fungal disease. One concern about rhIFN- γ in allogeneic HSCT recipients is the potential for worsening GVHD. Though preliminary results suggest that rhIFN- γ is safe in allogeneic HSCT recipients [68, 69], no conclusions about efficacy can be made. It was dis-

appointing that a randomized trial evaluating rhIFN- γ as adjunctive therapy for invasive aspergillosis was prematurely terminated before any patient was enrolled and before institutional review board approval at most of the study sites.

CONCLUSIONS

AMs play an important role in host defense against aspergillosis. They ingest inhaled conidia and, through the signaling mediated by PRRs, have an important role in coordinating innate and antigen-driven immunity. AMs are targets for strategies for immunotherapy that include PRR ligands, recombinant cytokines, and CSFs. Animal models are required to delineate the importance of specific host defense pathways and the effect of experimental manipulation on the inflammatory response to *Aspergillus* challenge. In addition, it is an important research priority to evaluate existing immune augmentation strategies (e.g., CSFs, IFN- γ) as adjunctive therapy for established invasive fungal infections. Given that invasive aspergillosis is an uncommon disease and patients with aspergillosis are heterogeneous with regard to the predominant host defense deficits (e.g., neutropenia, GVHD), significant challenges exist in designing a trial aimed at showing the benefit of immunotherapy [11].

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Oncologist 2007;12;7-13

DOI: 10.1634/theoncologist.12-S2-7

This information is current as of September 5, 2010

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