Contact-dependent Immune Response by Macrophages

Bryan Prahl
Undergraduate Student, Applied Science - Biotechnology

Abstract

Infections caused by *Aspergillus fumigatus* are a significant cause of death in immune-compromised humans. Alveolar macrophages (AM) comprise an important line of immune defense in the lung and help prevent infections resulting from inhalation of *A. fumigatus* conidia. Despite significant study in this area, details about how AM engage *A. fumigatus* are not completely understood. A better description about how A. fumigatus conidia are phagocytosed by AM is needed to better understand the innate resistance of healthy individuals to various airborne infections. The focus of this study was to determine whether the ability of AM to phagocytose *A. fumigatus* conidia is dependent upon attachment to a surface. We began by showing that AM supported on the epithelial cells in the alveolar space of the lung phagocytose conidia. We next compared phagocytosis of *A. fumigatus* conidia in tissue culture macrophages bound on a plastic substrate to that of macrophages when free in solution. Our results show a loss of phagocytosis by macrophages when detached from a physical support. Monocyte-derived macrophages show a corresponding contact-dependent production of microbicidal reactive oxygen species (ROS), known to be crucial in the resistance to *A. fumigatus* infections within the lungs. Our results suggest macrophages require physical contact with a surface to efficiently phagocytose *A. fumigatus* conidia. Further studies in this area may help provide a better understanding of the inflammatory response of AM that could be used to enhance defense mechanisms in humans at risk of infection.

Keywords: Aspergillus, host defense, immunology, alveolar macrophage

A. fumigatus is a filamentous soil fungus that reproduces asexually through the production of small spore-like structures called conidia that are typically 3μM in diameter. The small size of the conidia enables efficient deposition into alveolar spaces of the lung, which results in deposition of several hundred conidia per day under normal conditions. Individuals involved in certain agricultural activities may be exposed to far greater numbers of conidia. Due to a normal immune response in the lung, infections by this organism are rare. However, increasingly sophisticated healthcare practices such as bone marrow and stem cell transplants have led to greater numbers of patients with compromised immune systems. *A. fumigatus* causes a life-threatening disease in patients with a variety of defects in the phagocyte inflammatory response (Ampel, 1996; Maertens, Vrebos, & Boogaerts, 2001; Dykewicz, 2001; Walsh & Groll, 1999) and is now the leading airborne fungal pathogen in immune-compromised individuals (Stevens et al., 2000; Latge, 2001). Despite modern standards in health care, invasive pulmonary aspergillosis is still associated with fatality rates near 80%.

Most conidia inhaled into the lung must be phagocytosed before they can be killed by the immune system. Phagocytosis of conidia can be carried out by alveolar macrophages, yet the conditions necessary for this event are not well understood. Our study seeks a better understanding of the conditions leading to phagocytosis of conidia by macrophages and investigated this process in both *in vivo* and *in vitro* settings.

In the current study, the hypothesis that phagocytosis of *A. fumigatus* conidia requires anchoring of the macrophage to a suitable physical support was tested. An understanding of this response is important because little experimental data exists to show whether phagocytosis of macrophages is impacted by their ability to coordinate both the pathogen and substratum simultaneously. Our results indicate that when macrophages are removed from their support, they show a loss in ability to phagocytose *A. fumigatus* conidia. Further studies are needed to determine whether this loss of function is related to direct physical changes in the cytoskeletal structure of macrophages or, indirectly, through induced alterations in metabolic function.

Material and Methods

Unless otherwise indicated, reagents and chemicals used in this study were obtained from either Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Microscopic examinations were performed on a Zeiss Axioscope 2-Plus microscope and imaging system using Zeiss Axiovision version 4.5 software.

Preparation of A. fumigatus Conidia

A. fumigatus was obtained from clinical isolate #13073 at the American Type Culture Collection (Manassas, VA). For flow cytometry and fluorescence microscopy, a congenic strain that expresses green fluorescent protein was used (Wasylnka & Moore, 2002). Conidia from both strains were grown at 37° C for five days on a Sabouraud Dextrose agar slant and collected as previously described (Bonnett, Cornish, Harmsen, & Burritt, 2006). Enumeration of conidia was done by hemocytometer.

Analysis of Cell Culture Macrophages

Phagocytosis of *A. fumigatus* conidia was examined in the J774 mouse macrophage cell line (J774A.1, ATCC #TIB-67). This continuous cell line has been studied extensively as a model of several types of macrophages in the body. Cells were grown in Dulbecco's Modified Eagle Medium with 10% fetal calf serum (DMEM10) at 37° C in 5% CO_2. When cell monolayers were greater than 50% confluent, cells were prepared by one of two methods to compare phagocytosis between adherent and detached cells. Adherent J774 cells were combined directly in situ with a 3:1 ratio (conidia to cells) of *A. fumigatus* conidia and incubated for 1 hour at 37° C in 5% CO_2, then scraped with a sterile tissue culture scraper and resuspended in Hank's Balanced Salt Solution (HBSS). Detached cells were scraped first, suspended in HBSS, and otherwise exposed to *A. fumigatus* conidia as described above. Conidia for these experiments were harvested from the strain of *A. fumigatus* conidia expressing green fluorescent protein to enable analysis by both fluorescent microscopy and flow cytometry. J774 cells exposed to conidia either before or after removal from the culture

container were then filtered through fine nylon mesh to remove cell clumps and examined on a flow cytometer as described below.

Live Animal Studies

All manipulations of animals were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Stout. Female eight-week-old C57Bl/6 mice were obtained from Harlan Laboratories (Madison, WI) and maintained in specific pathogen-free housing in microisolator cages in an environment of filtered air and given food and water *ad libitum.* Immune competent mice were inoculated intrapharyngeally as previously described (Cornish et al., 2008), using 40µl HBSS containing 5 x 10^6 conidia per animal following brief isofluorane inhalation. Following inoculation, animals were returned to their cages for specified times before being euthanized by an overdose of isofluorane. Bronchoalveolar lavage fluid (BALF) was collected from each mouse in 10mL ice-cold HBSS containing 3 mM ethylenediamenetetraaceticacid(EDTA)asdescribed(Cornishetal.,2008).

Flow Cytometry

Flow cytometric analyses were performed on a Millipore EasyCyte 5 flow cytometer using Millipore InCyte guavaSoft version 2.2.3 software. Cell counts in tissue culture and BALF samples were determined to ensure the cell densities were within tolerance ($5x10^4$-$5\ x10^5$ cells/ml) as specified by the instrument manufacturer. BALF from both a naïve mouse and inoculated mouse were then used to set the instrument gains and fluorescence channel compensation. Threshold values (in forward scatter) were sometimes increased to accommodate high background counts of pulmonary exosomes.

Results

AM Engulfed Conidia In vivo

Phagocytosis of conidia in macrophages was first examined in AM within the lungs of mice. Following a 6 h *in vivo* incubation after instillation of 5 x 10^6 conidia per mouse, mice were sacrificed and lung lavages performed as we have described previously. BALF showed evidence of conidial inoculation and of phagocytosis of

conidia in AM by light and fluorescence microscopy. Both AM and exosomes from the lungs of naïve and inoculated mice were evident in the BALF samples when examined by light microscopy of BALF.

Figure 1

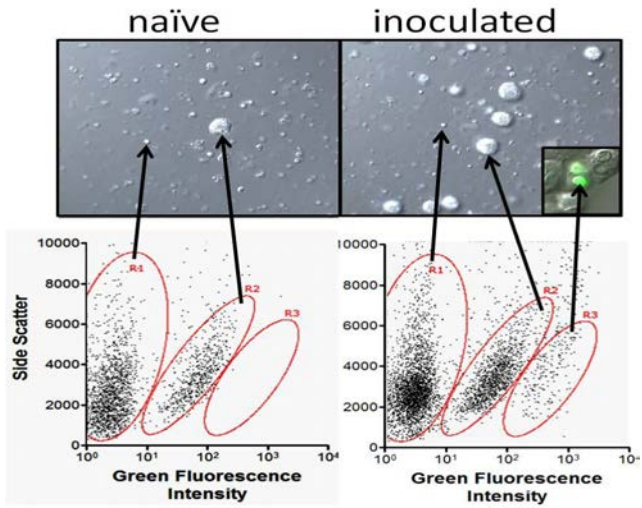

Figure 1: AM show phagocytosis of *A. fumigatus* conidia *in vivo* when examined by microscopy and flow cytometry. Photomicrographs are included in the top panel to identify the particulate material (exosomes, AM, and AM containing fluorescent conidia) in the BALF from both naïve mice and those 6 h after instillation of 5 x 10^6 conidia. Side scatter vs. green fluorescence profiles were obtained by flow cytometry to segregate the three populations of events seen in flow cytometry: region 1 (R1) contains exosomes, region 2 (R2) contains AM, and region 3 (R3) contains AM with phagocytosed fluorescent conidia. The increase in events in region 3 confirms phagocytosis of conidia in AM when incubated in the lungs of mice.

Fluorescence microscopy was used to demonstrate green fluorescence of conidia associated with some AM (inset image from inoculated mouse). Dot plot flow cytometry profiles depicting side scatter vs. green fluorescence for BALF samples from animals revealed both abundant numbers of exosomes in region 1 (R1) and AM in region 2 (R2). The stronger overall green fluorescence of AM relative to exosomes is due to intrinsic autoflourescence. In inoculated animals, the strongest green fluorescence of AM corresponded to

those cells which had phagocytosed *A. fumigatus* conidia containing green fluorescent protein; they appeared in flow cytometry tracings in region 3 (R3), with photographic evidence of fluorescent conidia associated with AM (inset, though color was not reproduced for the publication). These results confirm the fluorescent conidia were being phagocytosed in the lungs of mice by AM while in association with epithelial cells upon which they are typically found.

Figure 2

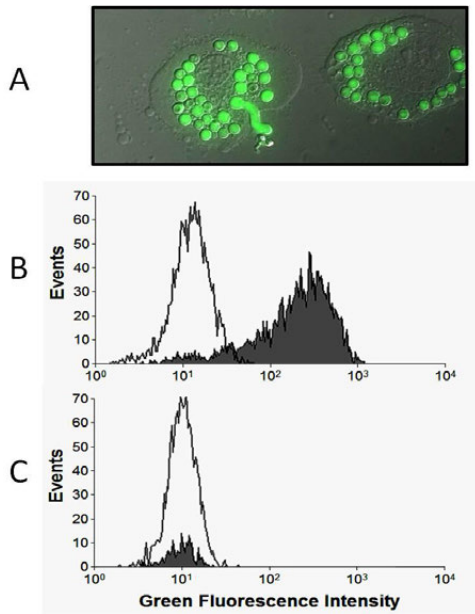

Figure 2: Detachment of J774 cells show differential loss of phagocytosis of fluorescent *A. fumigatus* conidia. A: The Photomicrograph of J774 cells attached to a plastic culture container surface shows engulfed fluorescent *A. fumigatus* conidia (green color not evident in black and white presentation), which surround the nuclei of the cells. B: Following phagocytosis of fluorescent conidia into bound J774 cells, flow cytometry shows a strong shift of fluorescence (shaded histogram) relative to J774 cells not exposed to conidia (unshaded histogram). C: When J774 cells were detached from the culture container prior to exposure to conidia, they do not show evidence of phagocytosis, as evidence by the lack of fluorescence increase in the shaded histogram relative to the unshaded control showing unbound J774 cells not exposed to conidia.

J774 Cells Require Attachment for Phagocytosis In Vitro

To further characterize phagocytosis of conidia in macrophages with respect to surface attachment, phagocytosis of conidia was examined in the murine J774 macrophage line. The results first validated phagocytosis of conidia in these cells when attached to the surface of the tissue culture container using fluorescence microscopy.

The photomicrograph shown in Figure 2A identifies the green fluorescent conidia engulfed in perinuclear spaces of the attached J774 cells. Phagocytosis of conidia was then compared between attached J774 cells to those that had been first dislodged by scraping. The results indicated the scraping process did not significantly damage the cells, reduce their viability, or result in aggregated cell forms (data not shown).

Flow cytometry was used to compare the green fluorescence of J774 cells in the absence of conidia as a negative control to that produced by J774 cells exposed to fluorescent conidia. First, fluorescent conidia were coincubated with adherent J774 cells prior to analysis by flow cytometry (Figure 2B). The experiment was then repeated using J774 cells that had been detached from the tissue culture surface prior to exposure to conidia (Figure 2C). For both adherent and detached J774 cells, gated overlay histograms were used, where J774 cells not exposed to conidia are unshaded, and histograms representing J774 cells exposed to conidia are shaded. Our results indicate that when J774 cells were attached to the tissue culture flask surface, they abundantly phagocytosed conidia, whereas those detached before exposure to conidia did not show a measureable increase in fluorescence.

Monocyte-derived Macrophages
Show Contact-dependent ROS production

Our previous results show that AM have almost imperceptible production of ROS from the NADPH oxidase (Cornish et al., 2008), despite the essential role of this event in defense against aspergillosis (Segal et al., 1998). However, different types of macrophages are known to produce ROS in response to some soluble and particulate triggers of the NADPH oxidase. Therefore, we examined monocyte-

derived macrophages for liberation of ROS following exposure to 100 nM phorbol mysristate acetate (PMA) as we have previously described (Cornish et al., 2008). When 1 x 10^5 macrophages were adhered to the surface of a white luminometry plate, exposure to 100 nM PMA resulted in reproducible and measurable production of ROS with a signal maximum at about 12 minutes when measured by MCLA-dependent luminometry.

Figure 3

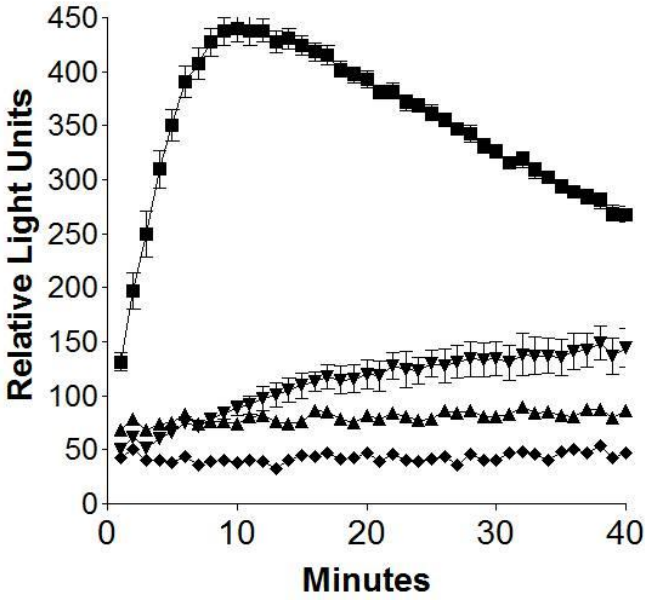

Figure 3: Monocyte-derived macrophages show a loss of ROS production if unattached to the substrate in MCLA-dependent luminometry. Liberation of ROS as superoxide was measured as relative light units from adherent monocyte-derived macrophages following exposure to 100 nm PMA (■). This amount of signal is reduced when examined prior to attachment of cells to the substrate (▼). Controls for this reaction are provided by adherent cells in the presence of 310 U/ml SOD (▲), and by the reagent control (♦). Error bars show standard error of the mean, and P values < 0.01 support the significant differences in results when comparing adherent vs. non adherent cells.

Inclusion of superoxide dismutase abolished this signal, demonstrating specificity of this reaction as superoxide production. When an identical number of cells were tested for ROS production prior to attachment, the

signal was abrogated and delayed, supporting the view that ROS release and phagocytosis requires the macrophage to be in contact with a support.

Discussion

We investigated the process of phagocytosis in macrophages, which is a first line of immune defense in tissues of the body including the lung. Several different types of leukocytes provide resistance from infection in the lung, which is regularly exposed to numerous airborne organisms. Our analyses included examination of AM within the lungs of mice, a macrophage cell line, and macrophages derived from monocytes. This combination of macrophage types examined offers different views that provide a greater overall understanding of the process of phagocytosis, which is a requirement of all macrophages.

Flow cytometry and fluorescent microscopy was used to show the phagocytic capability of AM in the lungs of mice. This provided an important positive control to validate our methods of analysis, confirming that AM phagocytose fungal conidia in the lungs of mice, as previously shown (Latge, 1999). The examination we utilized for phagocytosis in AM did not identify the percentage of conidia phagocytosed or the relative number of AM participating in this event, though these parameters would undoubtedly be influenced by both the dosage of conidia and the time of incubation.

J774 cells were also used because they are well characterized and can be manipulated for the purposes of our study. This cell type has been used extensively to model the activities of macrophages in tissue that engage and destroy fungal pathogens. However, we do not assume results obtained using this cell line are universally extrapolated to AM, which is the cell type most relevant to our study. Nevertheless, we were able to demonstrate extensive phagocytosis of conidia into J774 cells when attached to the plastic cell culture container surface, which may mimic the situation for AM being associated with the alveolar epithelial surface in the lung. When the J774 cells were dislodged from the surface of the tissue culture container, we observed an unexpected loss of their phagocytic capacity. The reason for this alteration is not known, but could reflect a number of adjustments in the cell. For example, since detachment could be

regarded as an unnatural situation for tissue macrophages, the cells may be undergoing a process to reestablish attachment necessary for their survival, thereby reducing their ability to carry out phagocytosis. Because macrophages are technically mobile rather than stationary cells, it is also possible that detachment is a normal and perhaps essential event at times. In this case, detachment may require temporary substitution of alternative processes involved in motility at the expense of phagocytosis. We did not observe obvious damage to cells due to scraping, which could also contribute to the loss of phagocytosis.

The observation that monocyte-derived macrophages produce ROS only when bound to a surface is intriguing. This same observation does not extend to neutrophils, which can produce abundant ROS when suspended in liquid (Dahlgren & Karlsson, 1999). It is tempting to hypothesize that neutrophils, which are generally suspended in blood or body fluids, are adapted to carry out phagocytosis and ROS production independently of a supporting structure, while macrophages reserve these activities to times of tissue association. Further studies are needed to understand the molecular basis for loss of phagocytosis and ROS production in detached macrophages.

The results presented offer a better understanding of the role of macrophages in immune defense. This information has relevance to several aspects of health care, since a number of types of drugs and medical therapies that reduce inflammation can impact phagocytosis. This event is required in several cell types with roles in tissue remodeling, wound healing, and fetal development. Additional studies are required to determine whether pharmacologic approaches to either enhance or suppress phagocytosis might alleviate symptoms in some types of diseases. It also remains to be determined whether transfusion of phagocytic leukocytes to patients with deficiencies in these cells could be made more effective by modulating the process of phagocytosis in the cells they receive.

References

Ampel, N. M. (1996). Emerging disease issues and fungal pathogens associated with HIV infection. *Emerging Infectious Diseases.*, *2*, 109-116.

Bonnett, C. R., Cornish, E. J., Harmsen, A. G., & Burritt, J. B. (2006). Early neutrophil recruitment and aggregation in the murine lung inhibit germination of Aspergillus fumigatus Conidia. *Infection and Immunity, 74*, 6528-6539.

Cornish, E. J., Hurtgen, B. J., McInnerney, K., Burritt, N. L., Taylor, R. M., Jarvis, J. N., . . . Burritt, J. B. (2008). Reduced Nicotinamide Adenine Dinucleotide Phosphate Oxidase-Independent Resistance to Aspergillus fumigatus in Alveolar Macrophages. *Journal of Immunology, 180*, 6854-6867.

Dahlgren, C., & Karlsson, A. (1999). Respiratory burst in human neutrophils. *Journal of Immunological Methods, 232*, 3-14.

Dykewicz, C. A. (2001). Hospital infection control in hematopoietic stem cell transplant recipients. *Emerging Infectious Disease., 7*, 263-267.

Latge, J. P. (1999). Aspergillus fumigatus and aspergillosis. *Clinical Microbiology Review., 12*, 310-350.

Latge, J. P. (2001). The pathobiology of Aspergillus fumigatus. *Trends in Microbiology, 9*, 382-389.

Maertens, J., Vrebos, M., & Boogaerts, M. (2001). Assessing risk factors for systemic fungal infections. *European Journal of Cancer Care (Engl.), 10*, 56-62.

Segal, B. H., DeCarlo, E. S., Kwon-Chung, K. J., Malech, H. L., Gallin, J. I., & Holland, S. M. (1998). Aspergillus nidulans infection in chronic granulomatous disease. *Medicine (Baltimore), 77*, 345-354.

Stevens, D. A., Kan, V. L., Judson, M. A., Morrison, V. A., Dummer, S., Denning, D. W., . . . , Pankey, G. A. (2000). Practice guidelines for diseases caused by Aspergillus. Infectious Diseases Society of America. *Clinical Infectious Diseases, 30*, 696-709.

Walsh, T. J., & Groll, A. H. (1999). Emerging fungal pathogens: Evolving challenges to immunocompromised patients for the twenty-first century. *Transplant Infectious Disease, 1*, 247-261.

Wasylnka, J. A., & Moore, M. M. (2002). Uptake of Aspergillus fumigatus conidia by phagocytic and nonphagocytic cells in vitro: Quantitation using strains expressing green fluorescent protein. *Infection and Immunity, 70*, 3156-3163.