

## **Immune Mechanism in Lung Prevents Fungal Infection**

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Invasive fungal infections are an increasingly common, life-threatening disease in hospitalized patients with complex health problems (Ampel, 1996; Maertens, Vreboos, & Boogaerts, 2001; Dykewicz, 2001; Walsh & Groll, 1999). In recent decades, *A. fumigatus* has become the leading airborne fungal pathogen in immune-compromised individuals (Stevens et al., 2000; Latge, 2001). Since 1978, the increase in aspergillosis has exceeded all other opportunistic mycoses (Groll et al., 1996). Invasive disease from *A. fumigatus* is now responsible for 4% of fatalities in tertiary care facilities and teaching hospitals (Vogeser, Wanders, Haas, & Ruckdeschel, 1999; Johnston, Jr., 2001).

Alveolar macrophages (AM) are the resident immune cells in the airway, which puts them at the front line in defending the host against airborne infectious agents. Despite extensive study of this cell, the mechanism by which they neutralize fungal pathogens is not completely understood. Recent published information about host defense mechanisms that protect from invasive aspergillosis continues to identify complexities preventing a clear understanding about the mechanisms by which leukocytes kill the aspergilli (Henriet et al., 2010).

In normal individuals, exposure to large numbers of *A. fumigatus* conidia does not lead to infection, due to the robust natural resistance to this organism. By contrast, clinical evidence indicates that invasive aspergillosis can occur in immune-suppressed humans even when following inhalation of low numbers of conidia. During low-level exposures in humans, AM rather than neutrophils are the primary phagocytes responding to conidia in the lung. Thus, infections by *A. fumigatus* in immune-suppressed individuals are probably due to defective responses by AM, since neutrophils are not generally recruited in this situation. This identifies a need to better describe the conidiacidal activity in AM which provides immune surveillance in the lung.

Published information from our laboratory provided evidence that unlike neutrophils, anticonidial defenses in AM are independent of the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (Bonnett, Cornish, Harmsen, & Burritt, 2006; Cornish et al., 2008). Moreover, AM phagocyte NADPH oxidase activity is vastly diminished relative to neutrophils under all conditions. We also previously reported that AM responding to conidia do not show increased transcription of genes associated with antimicrobial defense mechanisms, such as oxidant generation, phagolysosomal acidification, or iron sequestration, though other studies have indicated these may be important in this resistance (Ibrahim-Granet et al., 2003; Zarembler, Sugui, Chang, Kwon-Chung, & Gallin, 2007; Philippe et al., 2003). We are investigating the possibility that AM use nutrient starvation to suppress conidial germination, and therefore, reduce the threat of invasive aspergillosis in the lungs of humans without the release of potent inflammatory mediators.

In the current study, we examined *A. fumigatus* conidia phagocytosed in macrophages for evidence to describe the conditions inside the macrophage that prevent germination. Our study utilizes *A. fumigatus* conidia as a molecular probe of the internal conditions of the macrophage that prevents infections in the normal individual. Our results support the view that nutrient deprivation suppresses conidial germination in the phagolysosome of the AM.

### **Material and Methods**

Unless otherwise stated, all reagents and chemicals used in this study were obtained from either Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). All microscopic examinations were performed on a Zeiss Axioscope 2-Plus microscope and imaging system using Zeiss Axiovision version 4.5 software. Statistical analysis was performed for analyses using the Student's t-test.

#### **Preparation of *A. fumigatus* Conidia**

*A. fumigatus* used in these studies was obtained from clinical isolate #13073 at the American Type Culture Collection (Manassas, VA). In some analyses, a congenic strain that expresses green fluorescent protein was used, (Wasylnka & Moore, 2002)<sup>1</sup>. Conidia from both strains were grown at 37° C for 5 days on a Sabouraud Dextrose agar slant and collected as we previously described (Bonnett et al., 2006). Enumeration of conidia was done by hemocytometer. Minimal medium was prepared at 71 mM NaNO<sub>3</sub>, 7 mM KCl, 2 mM MgSO<sub>4</sub>, 11 mM KH<sub>2</sub>PO<sub>4</sub>, 56 mM glucose, and 2 ml Hunter's trace metals/liter.

### **Live Animal Studies**

Female 8-week-old C57Bl/6 mice were obtained from Harlan Laboratories (Madison, WI). Animals were maintained in specific pathogen-free housing in microisolator cages in an environment of filtered air and given food and water *ad libitum*. Mice were not immune-suppressed prior to the studies. Mice were inoculated intrapharyngeally as we previously described (Cornish et al., 2008), using 40µl HBSS containing 10<sup>6</sup> conidia per animal following brief isoflurane inhalation. Following inoculation, animals were returned to their cages for specified times before being euthanized by overdose of isoflurane. BAL fluid was collected in 10mL per sample in ice-cold HBSS containing 3 mM EDTA as described in Cornish et al. (2008). All manipulations in animals were approved by the Institutional Review Board for the Institutional Animal Care and Use Committee at the University of Wisconsin-Stout.

### **Coverslip Cultures of J774 Cells**

In some experiments, the J774 mouse macrophage cell line (J774A.1, ATCC #TIB-67) was utilized to examine the ability of this phagocyte to suppress germination of *A. fumigatus*

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<sup>1</sup> The strain of *A. fumigatus* expressing the green fluorescent protein was kindly provided by Dr. Margo Moore (Simon Fraser University, British Columbia, Canada).

conidia. J774 cells were seeded onto steam-sterilized coverslips in the bottom of 6-well cluster tissue culture plates containing 3 ml of DMEM with 10% fetal calf serum (DMEM10).

Incubations of J774 cells were carried out at 37° C in 5% CO<sub>2</sub>. When cell monolayers were approximately 70% confluent, 2 x 10<sup>5</sup> *A. fumigatus* conidia were introduced into individual wells, which were returned to incubation conditions between 4 and 24 h. Examination of coverslips was done by inverting the coverslip onto a glass slide so that cells were between the slide and coverslip, then visualizing the macrophages and conidia by microscopy. In this way, cells did not require manipulation to dislodge them from the substrate prior to examination by microscopy. Following examination of wet mounts, the percent germination was calculated for conidia that were confirmed to be internalized within macrophages. This was verified by examining the various focal planes of the cells in the wet mounts. In some cases, congenic *A. fumigatus* conidia expressing green fluorescent protein were used to enhance the visual contrast with the macrophage.

### **Fungal Transcriptional Analysis**

Conidial RNA was extracted by 0.1mm of zirconium/silica beads (BioSpec, 11079101Z) in a 1.5mL screw top microfuge tube using a bead-beating method. The tubes were run at speed 4 for 90 seconds on the FastPrep 120 from Thermo Electron Corporation. RNA from the supernatant following a centrifugation at 4000 RCF for 3 minutes to pellet conidial debris was extracted for RNA purification through Promega's SV Total RNA Isolation System (Z3100, Promega, Fitchberg, WI). RNA was concentrated using the CentriVap Concentrator from Labconco (Kansas City, MO). To minimize the contamination of genomic DNA, all samples were treated with DNase (Promega's RQ1 RNase-Free DNase, M6101). RNA samples were then amplified using a qRT-PCR Master Mix (Fisher Scientific, AB4100A) on a Bio Rad CFX96

Real-Time System. Primers and probes were designed<sup>2</sup> for isocitrate lyase (ICL), alpha-ketoglutarate dehydrogenase (AKD), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Sequences of the primers are shown in Table 1. RNA values from free conidia and those bound inside macrophages were compared, as described in the results.

**Table 1:** Sequences of primers used in the studies described.

| Gene  | Forward Primer            | Reverse Primer           | Probe  |
|-------|---------------------------|--------------------------|--|
| ICL   | 5'-CTGCCTAACATCGACTAC-3'  | 5'-CTCTCGATGAACAGCTTG-3' | 5'-/56-FAM/TCATAACAG/ZEN/CAGTGAGACCACCA/3IBkFQ/-3' |
| AKG   | 5'-CTGACAAATCATCTCAAGG-3' | 5'-CCCATAGTGGTCAAGTTC-3' | 5'-/5HEX/TGTTGTAACCGAATGCCTCAGC/3IABkFQ/-3'        |
| GAPDH | 5'-GTACCATTGAGACCTACG-3'  | 5'-GGTGAAAACACCAGTAGA-3' | 5'-/5TET/TCAACGGCAAGAAGATTCGCTT/3IABkFQ/-3'        |

### In Vitro Nutrient Depletion in Growing Conidia

10<sup>7</sup> conidia were incubated at 37° C for the indicated time in DMEM-10 with 5.5 mM glucose at 37° C in 5% CO<sub>2</sub>, then pelleted at 2000 RCF for 3 minutes at 4° C and washed twice in Bio-Whittaker Hank's Balanced Salt Solution without glucose, then suspended in the wash medium. Incubations were then continued at 37° C for up to four additional hours prior to examination by microscopy and enumeration of conidial diameters. For each condition, 300 conidial diameter measurements were collected by microscopy and image analysis and then displayed using Graphpad Prism<sup>3</sup> software.

## Results

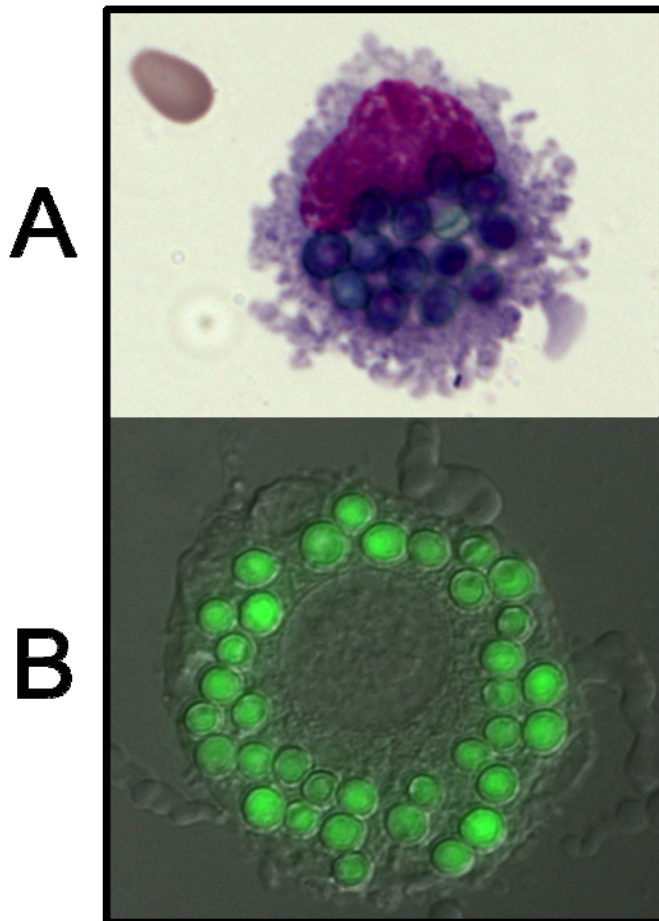
### Inhibition of Conidial Germination in J774 Cells

We previously showed that *A. fumigatus* conidia inoculated into the lungs of mice resulted in phagocytosis of the conidia and inhibition of conidial germination in normal BALB/c,

<sup>2</sup> Primers and probes were designed and analyzed using Beacon Designer Software ([http://www.premierbiosoft.com/molecular\\_beacons/index.html](http://www.premierbiosoft.com/molecular_beacons/index.html)).

<sup>3</sup> Graphical analysis was provided by Graphpad Prism software (<http://www.graphpad.com/prism/prism.htm>).

as well as in immune deficient CXCR2<sup>-/-</sup> and gp91<sup>phox</sup><sup>-/-</sup> mice (Cornish et al., 2008). We extended those studies in female 8-week-old C57Bl/6 mice by confirming that conidial germination is not evident within AM at additional time points up to 24 h, including the representative time of 12 h (Figure 1A). This result suggested that AM from mice have a mechanism of preventing fungal growth that is not dependent upon either the NADPH oxidase or neutrophil recruitment and remains evident at least 24 hours. We examined this effect of macrophages on *A. fumigatus* conidia more closely using the J774 mouse macrophage cell line, which suppressed conidial germination similar to that of AM. Our data indicate that conidia were phagocytosed rapidly (90% within 90 minutes, data not shown), demonstrated <5% germination for up to 24 hours, and showed <5% germination for up to 24 hours. At 12 h, following exposure of conidia to J774 cells, engulfed conidia have swollen but do not germinate (Figure 1B).



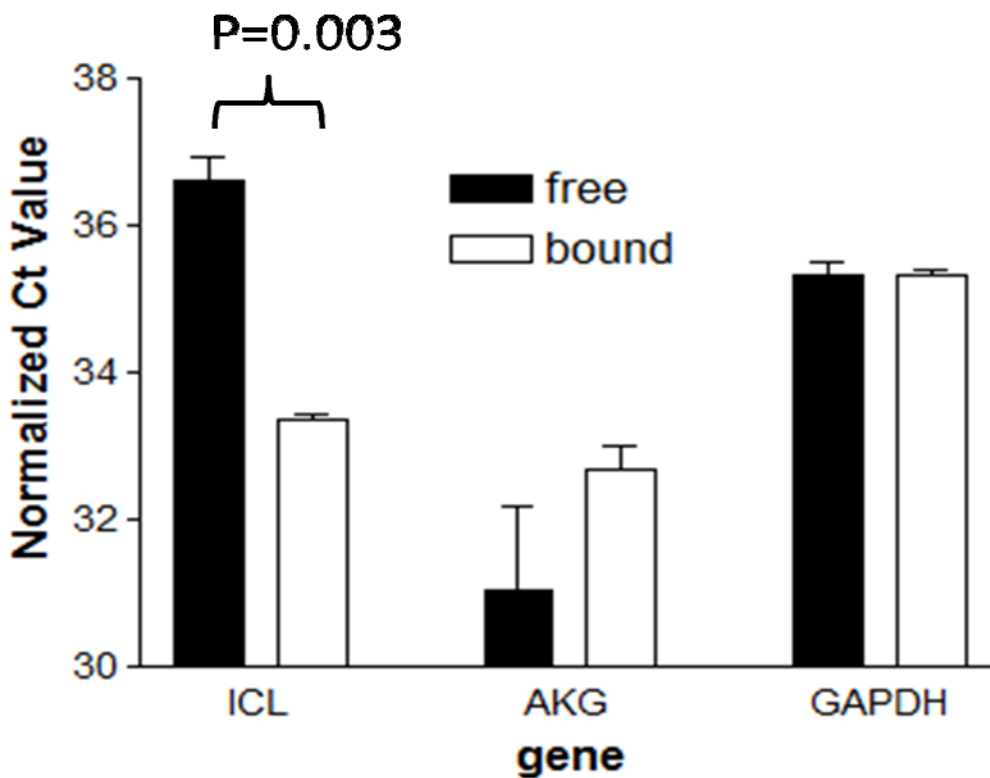
**Figure 1. Conidia are phagocytosed in AM from mice and J774 cells, but do not germinate.** Photomicrographs are provided for conidia within an AM from a C57Bl/6 mouse (A) and conidia in a J774 cell (B) 12 h after exposure to resting conidia of *A. fumigatus*. The degree to which AM and J774 cells suppress conidial germination is similar. The persistent fluorescence of the conidia in J774 cells from the constitutively expressed green fluorescent protein at 12 h suggests that even though the conidia are not germinating, they are still viable (Bonnert et al., 2006).

### **Relation of Conidia Germination to Glucose Concentration**

To determine whether a minimum concentration of glucose is needed to support conidial germination, minimal agar medium containing increasing glucose concentrations (between 0 and 8 mM) was prepared and inoculated with  $10^4$  *A. fumigatus* conidia. After 3 days of incubation at 37° C, abundant growth was observed on media containing 8 and 4 mM glucose, but below 2 mM, the germination rate was dependent upon both time and glucose concentration with no germination at 0 mM glucose (data not shown).

### **Transcriptional Analysis of Conidia Phagocytosed in J774 Cells**

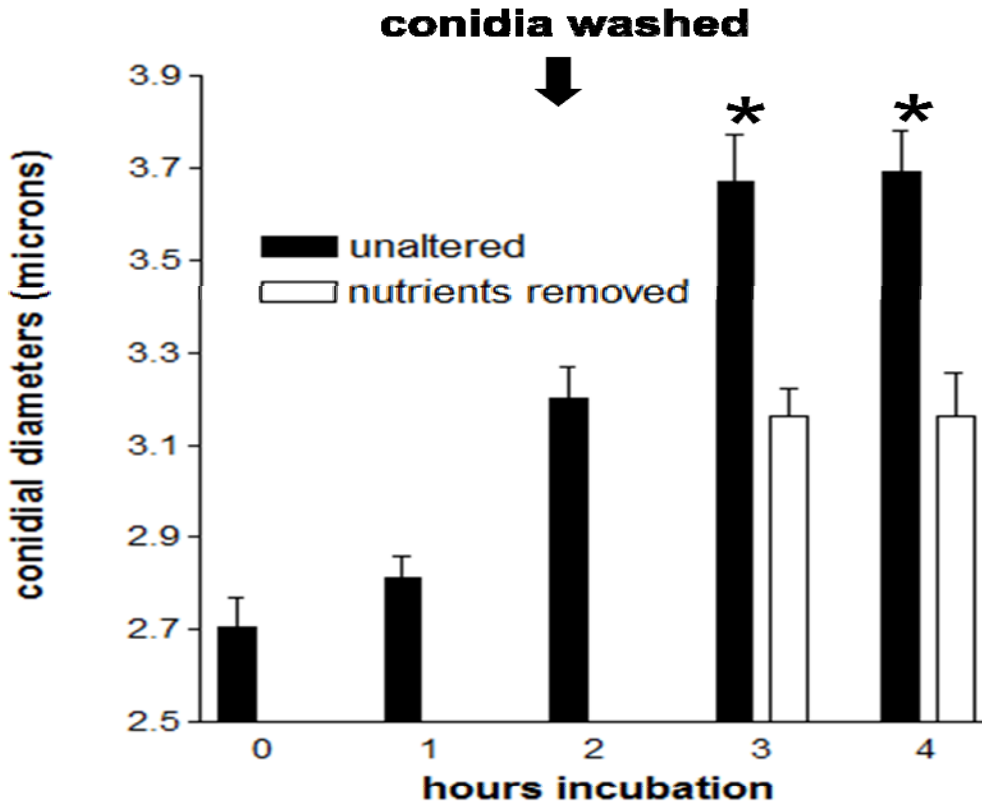
We next examined transcriptional profiles of conidia phagocytosed for 4 h in J774 cells. These studies were performed to determine whether engulfed (bound) conidia showed transcriptional evidence of nutrient deprivation that could account for the impaired intracellular germination. The abundance of mRNA transcripts corresponding to the ICL and AGD genes was determined using real time quantitative PCR, as Ct values were determined in triplicate. Bound conidial RNA that had been extracted from J774 cells was compared to that extracted from an equal number of free conidia incubated in RPMI-1640 medium without J774 macrophages. Values for both free and bound conidia were normalized to the abundance of transcripts for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used as a housekeeping reference gene (Figure 2). Statistical significance of groups shown identified  $P=0.003$ .



**Figure 2: Transcriptional responses of conidia phagocytosed in J774 cells supports nutrient deprivation.** A: Four hours following introduction of *A. fumigatus* conidia to J774 cells, fungal mRNA collected from the conidia showed increased abundance of transcripts for ICL but not AKD, as identified by decreasing Ct values for ICL. B: When compared to the GAPDH reference gene, the elevation in ICL expression was found to correspond to a 32-fold increase, which was significantly greater than ICL expression in conidia incubated in medium without J774 cells ( $P= 0.003$ ).

### Reduction in Nutrient Concentration in Actively-Developing Conidia

We next determined whether removal of nutrients from the culture medium containing actively developing conidia could halt further fungal development and germination. The effect of nutrient depletion in this experiment was determined by measuring conidia diameters using microscopy and imaging software. Our results showed that depletion of nutrients in actively growing cultures of conidia indeed causes an immediate blockade to further development (Figure 3).



**Figure 3: Removal of carbohydrate from medium results in blocked conidial development.** Two hours following incubation at 37°C in DMEM10 containing 5.5 mM glucose, a subset of conidia was washed and resuspended in Hanks Balanced Salt Solution lacking carbohydrate. Further incubation without carbohydrate inhibits additional swelling and hyphal outgrowth, supporting the plausibility of nutrient deprivation functioning as mechanism to prevent *Aspergillus* infections in the lung. Asterisks identify statistically significant differences ( $P < 0.002$ ) in conidial diameters when comparing washed to unaltered conditions.

Progression in conidial diameters and germination was not restored within four hours when remaining in nutrient-free medium (data not shown). Statistical analysis was used to compare conidia in unaltered conditions to those where nutrients were removed, with P values for both comparisons  $< 0.002$ .

### Discussion

We have investigated nutrient deprivation as a means by which AM inhibit germination of *A. fumigatus* conidia. This immune mechanism has been implicated in neutrophils and

macrophages to pathogens other than *A. fumigatus* (Lorenz & Fink, 2002). Nutrient deprivation would be a logical means of immune surveillance for the lung against aspergillosis, since it could prevent disease with minimal damage to host tissue. This is important in the lung, where constant exposure to airborne particulates could trigger chronic inflammation and impaired lung function.

The goal of this study was to provide information about the conditions in the macrophage that suppress conidial germination. We collected conidia following incubation in viable macrophages, then examined their transcriptional changes using real time quantitative polymerase chain reaction (PCR). We compared the expression of two enzymes that regulate carbon fluxes within the mitochondria. When examined together, activity of these enzymes give clues to nutrient availability for the organism. During times of nutrient abundance for fungi, AKD is expressed and serves a key role in the Krebs cycle by directing carbon flow toward succinyl-CoA as CO<sub>2</sub> is produced and lost. When carbon sources are limiting, reducing power for the cell is maintained by shifting to an alternative metabolism that does not involve loss of carbon through CO<sub>2</sub> production. In fungi, this starvation response includes the glyoxylate cycle, where isocitrate lyase shunts carbon flow from isocitrate to glyoxylate. Thus, upregulation of isocitrate lyase functions as a molecular switch to ensure the production of glyoxyloate from isocitrate, and prevent the loss of carbon in the form of CO<sub>2</sub>.

By comparing the expression of ICL and AKD, we were able to evaluate nutrient availability inside the macrophage indirectly through conidial responses. Since both ICL and AKD are known to be regulated at the level of transcription, quantitative PCR is suitable for evaluating these pathways in the fungus. Our results confirmed that within four hours following phagocytosis in macrophages, conidia reprogram their metabolism toward a starvation response not seen when conidia develop in medium alone. Our conclusion was based on the fact that mRNA corresponding to ICL gene expression, associated with the starvation response, was

upregulated 32-fold with respect to that expressed under conditions of nutrient abundance in comparison to GAPDH expression as a molecular reference.

Our transcriptional information suggests phagocytosed conidia experience limited nutrient availability. However, they do not confirm that these conditions are sufficient for suppressing conidial germination, especially once conidial swelling had begun. We therefore examined this aspect of our hypothesis in conidia incubated in artificial media where nutrient concentrations could be controlled. We confirmed that development and germination of live conidia can be blocked by switching them from a medium containing 5.5 mM glucose, a normal concentration of glucose in blood, to one devoid of utilizable carbon. This situation presumably parallels blockade of germination for conidia phagocytosed in AM and provides further evidence to support the notion that nutrient deprivation is a plausible immune response in the lung that suppresses infection from airborne fungi. We cannot exclude the possibility that additional AM responses are involved in this anticonidial response. However, our results are consistent with the idea that nutrient depletion alone is sufficient for preventing morphological development of conidia inside the AM.

Our results offer new insight into the complex immune responses involved in pulmonary host defense and suggest a possible clinical application. Pharmacologic manipulation of nutrient access inside the phagolysosome in AM may reduce the chance of infections in immune compromised humans, though additional analyses are necessary to test that possibility. Further research will also be needed to reveal the eventual fate of conidia bound within AM. It remains to be determined whether conidia phagocytosed in AM are eventually killed as a result of starvation, or subsequently transported elsewhere to be destroyed by an unresolved fungicidal response.

### References

- Ampel, N. M. (1996). Emerging disease issues and fungal pathogens associated with HIV infection. *Emerging Infectious Diseases*, 2(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(12), 6528-6539. Retrieved from PM:16920786
- Cornish, E. J., Hurtgen, B. J., McInnerney, K., Burritt, N. L., Taylor, R. M., Jarvis, J. N. et al. (2008). Reduced Nicotinamide Adenine Dinucleotide Phosphate Oxidase-Independent Resistance to *Aspergillus fumigatus* in Alveolar Macrophages. *Journal of Immunology*, 180(10), 6854-6867. Retrieved from PM:18453606
- Dykewicz, C. A. (2001). Hospital infection control in hematopoietic stem cell transplant recipients. *Emerging Infectious Diseases*, 7(2), 263-267.
- Groll, A. H., Shah, P. M., Mentzel, C., Schneider, M., Just-Nuebling, G., & Huebner, K. (1996). Trends in the postmortem epidemiology of invasive fungal infections at a university hospital. *Journal of Infectious Diseases*, 33(1), 23-32.
- Henriet, S. S., Hermans, P. W., Verweij, P. E., Simonetti, E., Holland, S. M., Sugui, J. A. et al. (2010). Human leucocytes kill *Aspergillus nidulans* by ROS-independent mechanisms. *Infection and Immunity*, Retrieved from PM:21078850
- Ibrahim-Granet, O., Philippe, B., Boleti, H., Boisvieux-Ulrich, E., Grenet, D., Stern, M. et al. (2003). Phagocytosis and intracellular fate of *Aspergillus fumigatus* conidia in alveolar macrophages. *Infection and Immunity*, 71(2), 891-903. Retrieved from PM:12540571
- Johnston, R. B., Jr. (2001). Clinical aspects of chronic granulomatous disease. *Current Opinion in Hematology*, 8(1), 17-22.

- Latge, J. P. (2001). The pathobiology of *Aspergillus fumigatus*. *Trends in Microbiology*, 9(8), 382-389.
- Lorenz, M. C., & Fink, G. R. (2002). Life and death in a macrophage: role of the glyoxylate cycle in virulence. *Eukaryotic Cell*, 1(5), 657-662. Retrieved from PM:12455685
- Maertens, J., Vreboos, M., & Boogaerts, M. (2001). Assessing risk factors for systemic fungal infections. *European Journal of Cancer Care*, 10(1), 56-62.
- Philippe, B., Ibrahim-Granet, O., Prevost, M. C., Gougerot-Pocidalo, M. A., Sanchez, P. M., Van Der, M. A. et al. (2003). Killing of *Aspergillus fumigatus* by alveolar macrophages is mediated by reactive oxidant intermediates. *Infection and Immunity*, 71(6), 3034-3042. Retrieved from PM:12761080
- Stevens, D. A., Kan, V. L., Judson, M. A., Morrison, V. A., Dummer, S., Denning, D. W. et al. (2000). Practice guidelines for diseases caused by *Aspergillus*. Infectious Diseases Society of America. *Clinical Infectious Diseases*, 30(4), 696-709.
- Vogeser, M., Wanders, A., Haas, A., & Ruckdeschel, G. (1999). A four-year review of fatal aspergillosis. *European Journal of Clinical Microbiology and Infectious Diseases*, 18(1), 42-45.
- Walsh, T. J., & Groll, A. H. (1999). Emerging fungal pathogens: evolving challenges to immunocompromised patients for the twenty-first century. *Transplant Infectious Disease*, 1(4), 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.

Zarembek, K. A., Sugui, J. A., Chang, Y. C., Kwon-Chung, K. J., & Gallin, J. I. (2007). Human polymorphonuclear leukocytes inhibit *Aspergillus fumigatus* conidial growth by lactoferrin-mediated iron depletion. *Journal of Immunology*, 178(10), 6367-6373.