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Activation of the neutrophil NADPH oxidase by *Aspergillus fumigatus*

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Upon infection of the respiratory system with the fungus *Aspergillus fumigatus* various leukoctytes, in particular neutrophils, are recruited to the lung to mount an immune response. Neutrophils respond by both phagocytosing conidia and mediating extracellular killing of germinated, invasive hyphae. Of paramount importance to an appropriate immune response is the neutrophil NADPH oxidase enzyme, which mediates the production of various reactive oxygen species (ROS). This is evidenced by the acute sensitivity of both oxidase-deficient humans and mice to invasive aspergillosis. Herein we briefly review the mechanisms and functions of oxidase activation and discuss our recent work identifying at least some of the important players in hyphal-induced oxidase activation and neutrophil function. Among these we define the phosphoinositide 3-kinase enzyme and the regulatory protein Vav to be of critical importance and allude to a kinase-independent role for Syk.

Keywords: reactive oxygen species; phosphoinositide 3-kinase; β_2-integrin; antifungal immunity

Aspergillus fumigatus is a saprophytic fungus whose air-borne spores (conidia) are ubiquitous in the environment and are inhaled daily by humans. Despite its unusualness among fungi in its ability to withstand the relatively high temperatures within the human body, most individuals do not incur noticeable infections during their lifetime. However, certain groups of human patients frequently present with often life-threatening invasive aspergillosis (IA), wherein inhaled conidia germinate and grow into proinflammatory, tissue-damaging hyphae. Such patient groups include those with lymphoma, leukemia or those having undergone bone-marrow transplantation. A common factor among these groups, either as a direct result of the disease itself or as a therapy-induced side effect, is a dramatic fall in the number of neutrophils in the body, known as neutropenia.[1,2]

Neutrophils are key effector cells in the immune response against *A. fumigatus*

Among white-blood cells of the hematopoietic system neutrophils belong to the granulocyte cell family, together with basophils and eosinophils, which are characterized by the presence of granules in their cytoplasm. Neutrophils are the most abundant of these cell types in mammals and are usually the first cell type recruited to sites of infection or injury. Although their life span within the circulatory system may be longer than previously estimated (approximately five days *in vivo* by recent estimates,[3] compared to 6–10 hours *ex vivo*), their numbers can increase up to 10-fold upon infection. The importance of this cell type to an appropriate immune response upon infection with *A. fumigatus* is evident from increased susceptibility of mice treated with neutralizing antibodies against the neutrophil recruiting cytokines MIP-2 or GM-CSF.[4] Moreover, mice that are depleted of neutrophils by means of a specific antibody also display a greatly increased mortality.[5] Among the mechanisms by which neutrophils respond to and eliminate pathogens is the NADPH oxidase Nox2. Upon cell activation this transmembrane enzyme complex mediates the generation of various reactive oxygen species (ROS), some of which are antimicrobicidal (more below). Human chronic granulomatous deficiency (CGD) patients, who carry

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inborn mutations in components of the oxidase, frequently present with IA, highlighting the importance of this enzyme in the neutrophil response.[6] Although the NADPH oxidase complex is found in other cell types, such as macrophages, the importance of the oxidase outside of neutrophils with respect to an anti-*Aspergillus* function is controversial, with evidence that oxidase-deficient macrophages can eliminate conidia effectively, at least *ex vivo*.[7]

Neutrophils commonly respond to pathogens by phagocytosing them, followed by maturation of the pathogen-containing phagosome through fusion with their intracellular granules. These granules are classified as primary/azurophilic granules (containing the serine proteases neutrophil elastase and cathepsin G together with myeloperoxidase (MPO)), secondary/specific granules (containing lactoferrin, lysozyme and MPO), and tertiary granules (containing gelatinase).[8] Neutrophils respond to *A. fumigatus* by phagocytosing both dormant conidia and germlings. While dormant conidia appear to be remarkably resistant to killing inside the phagosome, upon swelling and attempted germination they become more susceptible.[9] Hyphal filaments of the fungus are too large to be phagocytosed, so neutrophils attach themselves along the hyphal surface and apparently secrete their granule contents onto the hypha. It has been shown that neutrophils can mediate efficient killing of hyphae by this means.[10] Recent work has demonstrated that neutrophil extracellular traps (NETs) can be elicited by exposure to *A. fumigatus*, though there remains little evidence that this is an effective antifungal mode of action.[11] The fact that CGD patients do not all readily succumb to IA in the face of daily exposure to *A. fumigatus* spores suggests that there may be a means by which neutrophils may deal with conidia in a neutrophil oxidase-indendent manner. For very low inocula it is thought that this may be by means of elimination by macrophages or via lactoferrin-mediated iron sequestration in neutrophils, as a means to prevent conidial growth.[12]

Assembly and activation of the neutrophil NADPH oxidase

In resting neutrophils, the oxidase is partitioned into the membrane-resident flavocytochrome subunits gp91phox and p22phox and the cytosolic components p67phox, p47phox, p40phox, and the small GTP-binding protein Rac. Genetic deletion of either gp91phox or Rac2 has been shown to render mice more susceptible to fungal infection.[7,13] Upon stimulation of the cell, with either soluble agonists or particulate stimuli such as bacteria and fungi, a complex series of events mediates the assembly of the oxidase on a designated membrane. For some time it was thought that this assembly occurred on the plamsa membrane itself or plasma membrane-derived phagosomal membrane, though it is now accepted that assembly occurs on at least some of the intracellular granule membranes.[14] Among the events required for assembly of a functional oxidase are phosphorylation of p47phox on a number of residues, thereby triggering a conformation change in the protein and permitting interaction of p47phox with p22phox.[15] In addition, Rac undergoes activation by means of exchange of the nucleotide GTP for GDP through the action of various guanine nucleotide exchange factor (GEF) proteins, together with loss of binding to its guanine nucleotide dissociation inhibitor (GDI), thereby exposing a lipid-modified membrane-inserting protein tail. Membrane localized GTP-Rac then binds to the TPR domain of p67phox permitting the recruitment of the p67phox/p40phox heterodimer.[15] In addition to these events, critical to the assembly and activation of the oxidase is the generation of specific lipid species in the target membrane.[16] These lipids, collectively termed phosphoinositides, exhibit differential phosphorylation of the various positions of the inositol ring of phosphatidylinositol, and thereby recruit-specific effector proteins. Of importance here is phosphorylation of the dual-phosphorylated lipid $PI(4,5)P_2$ to $PI(3,4,5)P_3$. This latter lipid acts to recruit and activate proteins containing pleckstrin homology (PH) domains, notably certain GEF proteins for Rac. $PI(3,4,5)P_3$ is formed by the action of class I phosphoinositide 3-kinases (PI3Ks), of which there are four members (PI3Kα, β, γ, and δ) expressed in neutrophils.[17,18] Class I PI3Ks are involved in diverse cellular functions with some of those pertinent to neutrophil function including oxidase activation and actin polymerization, the latter being important in the morphological changes required for neutrophil movement toward attractant stimuli or across various substrata.

Once assembled, the oxidase can now accept electrons from NADPH and transfer them across the membrane to molecular oxygen, thereby generating

superoxide. The mechanism by which superoxide and its ROS derivatives actually mediate their anti-*Aspergillus* effects has been the subject of controversy.[8] Biochemical experiments over 30 years ago showed that certain ROS, in particular those catalyzed by MPO, such as various hypohalous acids, could damage hyphae.[10] In support of this, MPO-deficient mice exhibit a fairly specific susceptibility to *A. fumigatus* infection.[19] Indeed, exogenous addition of the MPO substrate hydrogen peroxide to oxidase-deficient neutrophils corrects their defect.[20] It had been proposed that the electrogenic generation of ROS on one side of the membrane was coupled to compensatory ion transport and resulting activation of serine proteases such as elastase and cathepsin G.[21] However, neutrophils deficient in the specific ion pump or in the enzyme required for the maturation of serine proteases do not exhibit increased susceptibility to infection.[22,23] Thus, it remains likely that certain ROS species are the main means by which neutrophils damage and kill *A. fumigatus*.

What are the signaling proteins involved in the neutrophil response to hyphae?

In order to mount a destructive ROS response against *A. fumigatus* neutrophils must firstly recognize the pathogen and then coordinate intracellular molecular signaling pathways to assemble a functional oxidase. However, the mechanisms by which neutrophils do this, at least in the case of *A. fumigatus*, are not well understood. Neutrophils express various receptors on their surface, in common with other phagocytic cells, and have been shown to utilize at least some of these for microbial recognition, phagocytosis, and oxidase activation, e.g., Fc receptors and integrins for antibody and complement opsonized targets respectively.[24] However, in contrast to dendritic cells and macrophages, the identity and function of many of the pathogen-related receptors expressed on neutrophils, such as those of the Toll-like receptor family and C-type lectin family, remain understudied. This remains so in part due to the technical difficulty of working with neutrophils because of their short life-span *ex vivo* compared to other immune cell types. Nevertheless, what has been shown is that mice deficient in the cell-surface receptor Dectin-1 are more susceptible to infection with *A. fumigatus* and neutrophils from these mice have an attenuated ROS response to swollen conidia.[25] Dectin-1 is a receptor for the carbohydrate β-1,3-glucan, which is exposed on conidia only after germination commences with conidial swelling and appears to be masked again in hyphae.[26,27] It is unknown what cellular receptor is required for phagocytosis of dormant conidia and it is unclear as to whether Dectin-1 can recognize the hyphal form. It has been noted that Dectin-1 may only play a minor role in the response to *A. fumigatus* in humans since Dectin-1 deficiency is a susceptibility factor for aspergillosis only in patients already at high risk of infection.[28] Thus, other receptors must be involved. We have attempted to begin to work out the signaling pathways by which neutrophils respond to *A. fumigatus*, at least for the hyphal form against which neutrophils play a unique role. By making

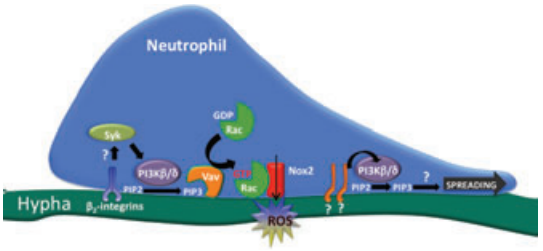

Figure 1. Overview of the signaling mechanisms by which neutrophils respond to hyphae. Neutrophils respond to hyphae by a number of means, including generation of reactive oxygen species (ROS) and spreading along the hyphae so as to maximize the surface area of interaction. The ROS response, mediated by NADPH oxidase (Nox2) activation, is initiated by recognition of hyphae by β_2-integrins on the neutrophil cell surface. Although the precise mechanisms remain unclear this somehow recruits the kinase Syk, which in turn recruits PI3K β and δ isoforms either directly,[38] or indirectly. Activated PI3Ks catalyze the synthesis of PI(3,4,5)P3 (PIP3) from PIP2 in the plasma membrane, which in turn recruits the exchange factor (GEF) Vav, part of whose function is to switch GTP for GDP on Rac, thereby activating it. It has been shown that Vav can interact with Syk directly[39] and also the oxidase subunit p67phox,[40] so all of these proteins may form a large juxta-membrane signaling complex. Activated Rac, together with numerous other events required for oxidase assembly (discussed in the main text), can then aid in activation of the oxidase, resulting in the generation of ROS outside of the neutrophil onto the hyphae. Our observations suggest that the vast majority of ROS produced is directed at the hyphal filament and not simply generated from the entire plasma membrane (data not shown). Concomitant with this are the changes in cell morphology required for spreading of the neutrophil along the hypha. While β_2-integrins may play a minor role here, another as-yet unidentified receptor(s) is required. Nevertheless, this receptor(s) still couples to activation of PI3K β and δ, though the other signaling events that occur remain unknown for now.

use of commercially available lipid and protein kinase inhibitors together with neutrophils purified from genetically targeted mice we have begun to determine which signaling proteins are involved in the neutrophil response to hyphae. We found that lipid products of class I PI3Ks were generated at the neutrophil membrane in contact with the hypal filament, that PI3K β and δ subunits played overlapping, partially redundant roles in not only oxidase activation but also in the morphological changes required for spreading of the neutrophil along the hyphal surface. In addition, the PI3K effector protein Vav (a GEF for Rac) and the kinase Syk were essential for oxidase activation (Fig. 1). Upstream of these components it was found, somewhat surprisingly, that Dectin-1 only played a very minor role in neutrophil responses. However, neutrophils from mice deficient in the multifunctional β_2-integrin receptor family had markedly impaired oxidase activation, though not neutrophil spreading.[29] It is likely that it is the β_2-integrin isoform Mac-1 that is important in this respect, as previous work has shown that this isoform can recognize various glucose-based carbohydrates.[30] However, the identity of the ligand(s) for integrins on hyphae remains unknown. There were two findings within this work that for now remain unexplained and require further attention. Firstly, what is the precise molecular signaling that links β_2-integrins specifically to oxidase activation and, secondly, what are the other neutrophil receptors involved, in particular for PI3K activation?

Recent work has shown that β_2-integrins couple to downstream oxidase activation in neutrophils via two adaptor proteins, FcRγ and DAP12, that contain a classical Immunoreceptor tyrosine-based activation motif (ITAM).[31,32] Phosphorylation of these adaptor proteins recruits the kinase Syk, a protein that plays important roles in various aspects of the hematopoietic system.[33] Syk, in turn, phosphorylates a number of target proteins, including itself, to propagate the appropriate signals. However, although deletion of Syk abolished a neutrophil ROS response to hyphae, we found that deletion of both FcRγ and DAP12 had no effect on oxidase activation.[29] Furthermore, selective inhibition of Syk activity with a small molecule inhibitor was much less potent at inhibiting hyphal-induced ROS compared to immune complex-stimulated ROS (a known Syk-dependent response; see Fig. 2). To our knowledge this is the first hint of a kinase-independent role for

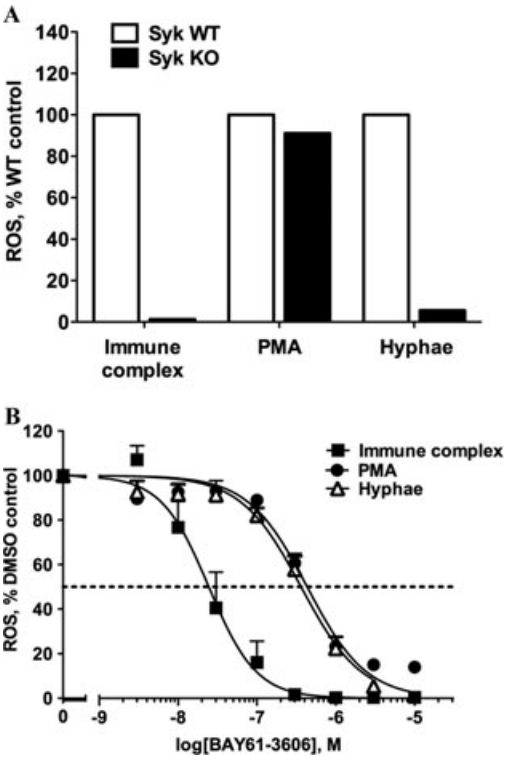

Figure 2. Syk protein but not kinase activity is required for hyphal-induced oxidase activation. Syk is a tyrosine kinase involved in relaying signals from receptors to the cell interior for various cell types of the hematopoietic system. Its importance is demonstrated by the embryonic lethality of mice deficient for the protein.[41] The specific involvement of Syk in hematopoietic cellular functions of interest can be interrogated by means of reconstitution of the hematopoietic system of lethally irradiated mice using stem cells from genetically deficient fetal livers.[42] This approach has shown that Syk is required for functional responses downstream of various receptors on neutrophils, such as antibody Fc receptors, certain C-type lectins, and β_2-integrins.[33] (A) We found that Syk-deficient neutrophils purified from reconstituted mice had a severely impaired ROS response to both a synthetic antigen-antibody immune complex, as expected, and hyphae. A Syk-independent stimulus (PMA) was unaffected by Syk deletion, evidence that a functional oxidase complex can be assembled (data are from a single experiment, representative of two). (B) The catalytic activity of Syk was investigated by means of a Syk-selective inhibitor (BAY61–3606) whose IC_{50} *in vitro* is around 7.5 nM.[43] Neutrophils were titrated with increasing concentrations of inhibitor and the ROS response to the same three stimuli measured by means of a luminol chemiluminesence assay (as in Boyle *et al.*). An inhibitory curve with an IC_{50} of approximately 25 nM was observed for the immune complex positive control. In contrast, an IC_{50} of 360 nM was observed for hyphae, similar to that for the Syk-independent stimulus PMA (430 nM), indicating that these are likely off-target effects of the inhibitor at these concentrations. (Error bars represent the SEM of three indepenent experiments, with neutrophils from two mice combined per experiment.)

Syk. Thus, either a novel paradigm for β_2-integrin-induced signaling exists or another, as yet unidentified, adaptor protein or integrin-coupled coreceptor is involved. The former may involve direct interaction of Syk with β_2-integrins, a concept shown thus far only in transfected cell lines,[34] whereas the latter is supported by our observation that, unlike oxidase activation, both the neutrophil spreading response and PI3K activation are refractory to integrin deletion, indicating that other receptor(s) are involved. Interestingly, preincubation of neutrophils with a soluble glucan preparation, in the form of laminarin, potently inhibited PI3K activation suggesting that there may be a non-Dectin-1 glucan receptor involved,[29] for example one for β-1,6-glucan, a potent neutrophil stimulus.[35]

While our own work has made some inroads into establishing what events occur inside neutrophils as they mount their response to *Aspergillus* much further work is clearly required to determine the precise signaling that occurs and establish the mechanisms by which neutrophils kill this fungus. It should not be forgotten that *A. fumigatus* has coevolved with its host organisms (thought to not necessarily be humans in this context but various water-fowl) over a significant period of time and as a result has evolved strategies to counteract the host immune response. For example, the secreted toxins gliotoxin and fumagillin exhibit antagonistic effects against neutrophil oxidase activation.[36,37] Thus, future studies investigating the immune response of neutrophils against the fungus should bear this in mind.

Conflicts of interest

The authors declare no conflicts of interest.

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