Genes Differentially Expressed in Conidia and Hyphae of Aspergillus fumigatus upon Exposure to Human Neutrophils

Janyce A. Sugui1, H. Stanley Kim2, Kol A. Zarember3, Yun C. Chang1, John I. Gallin3, Willian C. Nierman4, Kyung J. Kwon-Chung1,*

1 Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, United States of America, 2 Department of Medicine, College of Medicine, Korea University, Anam-Dong, Seongbuk-Gu, Seoul, Korea, 3 Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, United States of America, 4 J. Craig Venter Institute, Rockville, Maryland, United States of America

Abstract

Background: Aspergillus fumigatus is the most common etiologic agent of invasive aspergillosis in immunocompromised patients. Several studies have addressed the mechanism involved in host defense but only few have investigated the pathogen’s response to attack by the host cells. To our knowledge, this is the first study that investigates the genes differentially expressed in conidia vs hyphae of A. fumigatus in response to neutrophils from healthy donors as well as from those with chronic granulomatous disease (CGD) which are defective in the production of reactive oxygen species.

Methodology/Principal Findings: Transcriptional profiles of conidia and hyphae exposed to neutrophils, either from normal donors or from CGD patients, were obtained by using the genome-wide microarray. Upon exposure to either normal or CGD neutrophils, 244 genes were up-regulated in conidia but not in hyphae. Several of these genes are involved in the degradation of fatty acids, peroxisome function and the glyoxylate cycle which suggests that conidia exposed to neutrophils reprogram their metabolism to adjust to the host environment. In addition, the mRNA levels of four genes encoding proteins putatively involved in iron/copper assimilation were found to be higher in conidia and hyphae exposed to normal neutrophils compared to those exposed to CGD neutrophils. Deletants in several of the differentially expressed genes showed phenotypes related to the proposed functions, i.e. deletants of genes involved in fatty acid catabolism showed defective growth on fatty acids and the deletants of iron/copper assimilation showed higher sensitivity to the oxidative agent menadione. None of these deletants, however, showed reduced resistance to neutrophil attack.

Conclusion: This work reveals the complex response of the fungus to leukocytes, one of the major host factors involved in antifungal defense, and identifies fungal genes that may be involved in establishing or prolonging infections in humans.

Introduction

Invasive aspergillosis (IA) is one of the leading causes of mortality in immunocompromised patients, with Aspergillus fumigatus being the most frequent etiologic agent [1,2]. IA usually develops after germination of the inhaled conidia, which then grow into mycelia in the lungs. In immunocompetent individuals, both phagocytes, alveolar macrophages and neutrophils, arrest conidial growth [3,4] whereas defenses against hyphae depend on neutrophils. Neutrophils are recruited to the site of infection to phagocytize and kill pathogens. Phagocytosis can trigger the production of cytotoxic reactive oxygen species (ROS) by the enzymatic complex NADPH oxidase and the fusion of cytoplasmic granules with vacuoles containing pathogens. Mutation in any of the four components of the NADPH oxidase complex, as is the case in patients with chronic granulomatous disease (CGD), leads to deficient production of superoxide ions, hydrogen peroxide, hydroxyl anions and hypohalous acid, resulting in increased predisposition to recurrent bacterial and fungal infections [5,6,7]. Both, clinical evidence and in vitro data, show that neutrophils from CGD patients, unlike neutrophils from healthy individuals, are not able to damage A. fumigatus hyphae, suggesting a dependency on ROS [8]. In contrast, CGD neutrophils are as efficient as normal neutrophils in inhibiting growth of A. fumigatus conidia indicating that the growth arrest occurs in an ROS-independent manner [4].

While much progress has been made in understanding how the host perceives fungal invasion and mobilizes the immune response, very little attention has been given to how Aspergillus alters itself in response to host attack. Such information could lead to the...
identification of metabolic pathways essential for pathogenesis and the design of novel targeted antifungal drugs to counter this pathogen. Although a few genes, such as αβC involved in melanin synthesis [9,10], gldP involved in the biosynthetic pathway of gliotoxin [11,12] and locA a regulatory protein of secondary metabolites synthesis [13], have been shown to play a role in the virulence of A. fumigatus, little is known about the metabolic requirements or the compensatory defense mechanisms that enable survival of the fungus inside the host.

Using genome-wide microarray, we aimed to identify genes differentially expressed in A. fumigatus conidia and hyphae following exposure to human neutrophils from either healthy individuals (normal) or CGD patients. Our findings indicate that oxidative stress responses, reductive iron assimilation and metabolic reprogramming are all mechanisms employed by A. fumigatus to attempt survive within the host environment.

Materials and Methods

Strains

A. fumigatus clinical isolate B-5233 was maintained on Aspergillus minimal medium [10]. Conidia from 7 day-old cultures were harvested with 0.01% Tween-20 in PBS and washed twice with water. Unless specified, the conidial suspensions were prepared in RPMI/HEPES [RPMI 1640 buffered with 25 mM HEPES, pH 7.4] and incubations were at 37°C with 5% CO₂.

Neutrophils

All human blood samples used in this study were collected after obtaining informed written consent from normal subjects (Protocol 99-CC-0168 approved by National Institutes of Health Internal Review Board) and patients with CGD (Protocol 93-I-0119 approved by National Institutes of Health Internal Review Board). Patients with clinically apparent infectious diseases were excluded. Blood was anticoagulated using acid citrate dextrose and the neutrophils were purified as described previously [4]. The neutrophil preparations were typically about 95% pure.

Viability of conidia

The neutral red dye was used to assess conidial viability upon exposure to neutrophils. A microscopic assay was developed to determine the viability of individual conidia internalized by host cells. Neutral red, a vital dye that requires cellular energy for active uptake was used as a viability indicator [14,15,16,17,18]. First, however, control experiments were performed to ascertain the viability of conidia that displayed different staining patterns of the neutral red dye. The first control was performed to determine whether conidia that were treated with high temperature were still viable. Conidia were incubated in RPMI/HEPES for 4 h before incubation at 100°C for 20 min. Heat-treated conidia failed to germinate or develop into hyphae upon further incubation in fresh RPMI/HEPES at 37°C, indicating that they were non-viable. The second control was performed to characterize the patterns of neutral red staining in conidia incubated at 100°C. Conidia were heat-treated as mentioned above and the neutral red dye (Matheson Coleman & Bell, USA) was added to a final concentration of 0.003%. The stained conidia were observed by bright-field microscopy. In the untreated control, conidia were able to compartmentalize the dye (Fig. 1A, black arrows). In the heat-treated conidia (Fig. 1C and D) two different patterns were observed: the conidia were either unstained showing clear cytoplasm or the dye was homogeneously distributed throughout the conidial cytoplasm without any evidence of compartmentalization. Based on these observations, the conidia were scored as viable only when they were able to compartmentalize the dye. The ratios of neutrophil to conidia tested were 0:1, 0:1:1, 0:5:1 and 1:1. 300 µl of conidial suspension (2 × 10⁶ conidia/ml) was added to the wells of 4-well chambered cover glass slides (Lab-Tek, USA) and incubated for 4 h prior to exposure to neutrophils. 175 µl of RPMI/HEPES containing the neutrophils required for each ratio was mixed with 25 µl of pooled human plasma from citrated human blood (BioReclamation, USA), added to the chamber wells and incubated for additional 2 h. Because neutrophils can release DNA when activated [19], 100 µl of bovine DNase I (Sigma) [10 U/ml in 10 mM Tris pH 7.5, 2.5 mM MgCl₂, 0.5 mM CaCl₂] was added to the chamber wells to prevent any unknown interaction of dye and DNA. After 20 min incubation with DNase I at room temperature, neutral red (0.003%) was added and the samples observed by bright-field microscopy to score the viability of conidia within neutrophils.

Challenge of A. fumigatus with neutrophils

Experiments with conidia: To ensure that the RNA was isolated prior to emergence of the germ tube, conidial incubation was limited to 4 h. 1×10⁶ conidia suspended in 10 ml of RPMI/HEPES were inoculated into Petri plates and allowed to swell for 2.5 h prior to addition of neutrophils. 4.25 ml of RPMI/HEPES containing 2.5×10⁶ neutrophils was mixed with 0.75 ml of pooled human plasma and added to the plates. The plates were then incubated for an additional 1.5 h. Conidia and neutrophils were harvested in 1% Igepal-CA-630 (Sigma) to lyse the neutrophils and centrifuged at 12000 g, at 4°C for 5 min. Conidia were collected, frozen and lyophilized. Three biological replicates with neutrophils from normal as well as CGD neutrophils were carried out. In each biological replicate, the neutrophils were from a single donor. The biological replicates with normal and CGD neutrophils are referred as N1-N3 and C1-C3, respectively. Experiments with hyphae: 5×10⁶ conidia suspended in 10 ml of RPMI/HEPES were inoculated into Petri plates and incubated for 8 h prior to addition of neutrophils. After 8 h of incubation, a majority of the conidia had germinated and formed hyphal filaments. 4.25 ml of RPMI/HEPES containing 5×10⁶ neutrophils (a ratio of 1:1 of neutrophil to conidia) was mixed with 0.75 ml of pooled human plasma and added to the plates, which were then incubated for additional 75 min. Previously we have shown that 2 h of interaction between A. fumigatus hyphae (strain B-5233) and neutrophils was sufficient to cause significant hyphal damage [4] which was not apparent at 75 min interaction (data not shown). Therefore, to avoid excessive hyphal damage and yet allow enough time for the fungal cells to respond to neutrophils, the interaction was fixed at 75 min. Hyphal cells were harvested as described above. Four biological replicates with normal neutrophils and three biological replicates with CGD neutrophils were carried out. In each biological replicate, the neutrophils were from a single donor. The biological replicates with normal and CGD neutrophils are referred as N4-N7 and C4-C6, respectively. As a reference control, conidia or hyphae were incubated with plasma without neutrophils. RNA was isolated from the fungal cells using Trizol reagent (Invitrogen, USA) and purified with the RNAeasy kit (Qiagen, USA). The RNA samples were used for microarray analysis and quantitative real-time PCR (qRT-PCR) assays. For the qRT-PCR assays, the RNA was treated with Turbo DNase (Ambion, USA) according to the manufacturer’s protocol.

Transcriptome profiling with the whole genome microarray

To identify the A. fumigatus genes that are differentially expressed in response to human neutrophils, we used A. fumigatus strain
Af293 DNA amplicon microarrays containing 9,516 genes [20]. Hyphae and conidia, representing the two developmental stages of the fungus, were examined separately in this study. RNA from hyphae or conidia incubated in plasma in RPMI/HEPES without neutrophils was used as reference, and was co-hybridized with the query samples obtained from the corresponding sets incubated with neutrophils. Each biological replicate was carried out with neutrophils obtained from a different donor, and the RNA samples associated with each neutrophil source was paired separately with a corresponding reference sample prepared at the same time. RNA samples were labeled with Cy3 or Cy5 fluorescent dye and the collection of 13 hybridizations (Table 1) was replicated with dye labels reversed, except for samples N6 and C4. The labeling reactions with RNA and the hybridizations were performed according to protocols described in the J. Craig Venter Institute (JCVI) standard operating procedures [http://pfgrc.jcvi.org/index.php/microarray/protocols.html]. Hybridized slides were scanned using the Axon GenePix 4000B microarray scanner and the TIFF images generated were analyzed using the JCVI Spotfinder program [http://www.pfgrc.tigr.org/tools.shtml], JCVI, Rockville, MD) to obtain relative levels of transcript. Data was normalized using a local regression technique LOWESS (LOcally WEighted Scatterplot Smoothing) for hybridizations using the software MIDAS [http://www.pfgrc.tigr.org/tools.shtml], JCVI, Rockville, MD). The resulting data was averaged from triplicate copies of the genes printed on each array and from duplicate flip-dye arrays for each experiment, taking a total of 6 intensity data points for each gene (Accession number pending).

Figure 1. Effect of neutrophils on conidial viability. Conidial viability was monitored using the neutral red dye. (A–D) Control experiments to determine conidial viability based on patterns of staining with neutral red dye. (A) Conidia were incubated for 4 h at 37°C before the neutral red dye was added. Observation by bright field microscopy showed that conidia were able to take up and compartmentalize the dye (black arrows). (B) The same sample after 16 h showing extensive hyphal growth. (C and D) Conidia were incubated for 4 h at 37°C, heat-treated at 100°C for 20 min and stained with the dye. The conidia were not viable after the heat treatment. Some conidia stained faintly (C) while others stained deeply without compartmentalization of the dye (D). (E) Conidia exposed to neutrophils. Conidia were incubated for 4 h at 37°C, exposed to CGD neutrophils (0.5:1 neutrophil to conidia) for 2 h and stained with neutral red. Non-viable conidia appeared as entirely red (white arrows) similar to the heat treated conidia observed in (D), whereas viable conidia showed the compartmentalization of the dye (black arrows) as seen in the control (A). The magnification of the figures (A–E) is the same. Magnification bar of the inset in E is 10 μm. The conidia shown in C and D are smaller than those shown in A due to shrinkage caused by heat-treatment. Further incubation for 2 h showed conidia that compartmentalized the dye were able to germinate and develop hyphal filament (inset in E, black arrowhead). (F) Conidia were challenged with normal (gray bars) or CGD (white bars) neutrophils at different ratios and the viability was monitored using neutral red after 2 h exposure. Data presented are mean% viability ± SD from four experiments using different donors.

doi:10.1371/journal.pone.0002655.g001

A. fumigatus and neutrophils
Genes were grouped based on their expression vectors using the k-means algorithm, and selected groups of interest were again organized for similar expression patterns using Euclidean distance and hierarchical clustering with the average linkage clustering method JCVI MeV (<http://www.pfgrc.tigr.org/tools.shtml>, JCVI, Rockville, MD). The Significance Analysis of Microarrays (SAM) [21] method was used to identify genes differentially expressed between fungal cells exposed to normal vs CGD neutrophils.

**Gene expression under glucose-limited conditions**

RNA was isolated from swollen conidia that were incubated in media G⁺ alone [RPMI 1640 without glucose, buffered with 25 mM HEPES, pH 7.4] or G⁻ supplemented with either 10 mg/ml glucose (G⁺) or 10 µl/ml of a fatty acid mixture (FA) containing linoleic, oleic, lauric and arachidonic acids (Sigma). 10⁶ conidia were suspended in 10 ml of RPMI/HEPES, containing linoleic, oleic, myristic, lauric and arachidonic acids (Sigma). 10⁶ conidia were suspended in 10 ml of RPMI/HEPES, inoculated into a filtration unit [50 mm diameter filter with pore size 0.2 µm (Nalgene, USA)] and incubated for 2.5 h to allow conidial swelling. Since the filtration unit was not connected to a vacuum system, filtration did not occur during the 2.5 h incubation. The unit was then connected to a vacuum system to filter the media. The membrane was washed with 100 ml of water and the unit disconnected from the vacuum system. Ten milliliters of G⁺, G⁻ or FA was added to the filter and incubated for additional 1.5 h before conidia were harvested to isolate the RNA described as above.

**Quantitative Real Time-PCR (qRT-PCR)**

The RNA was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, USA) according to manufacturer’s instructions. 5 µl of the first-strand cDNA reaction (corresponding to 25 ng of initial RNA used for cDNA synthesis) was added to 20 µl of the qRT-PCR mix [12.5 µl of TaqMan® Universal PCR Master Mix (Roche Molecular Systems, CA, USA), 2.25 µl of 10 µM Forward and Reverse primers each, 0.0625 µl of 10 µM probe and 2.93 µl of water]. The reaction was performed in an ABI Prism® 7700 Sequence Detection System. Total RNA was used as negative control. The genes glyceraldehyde 3-phosphate dehydrogenase (gpdA) and tubulin 2 (tub2) were amplified as endogenous control to standardize the amount of sample added to the reaction mix. Relative quantitation of gene expression was performed using the relative standard curve method. Results were expressed as relative fold-change. In the experiments with neutrophils the fold-change refers to the ratio between fungal cells exposed to neutrophils and non-exposed conidia, after normalization with gpdA. In the experiments with glucose-limited conditions, the fold-change refers to the ratio between conidia incubated in media G⁺ or FA and conidia incubated in media G⁻, after normalization with tub2. The gene tub2 was chosen as normalization factor because the expression of gpdA could be directly affected by the glucose concentration in the media tested. Comparisons among the tested strains showed that the target genes had similar basal levels in media G⁺. The sequences of the primers and probes used for the qRT-PCR are listed on Table 2.

**Deletion of farA** (Afu4g03960) and **farB** (Afu8g0413 and Afu1g00410)

**Deletion of farA**: a vector was constructed by inserting the hygromycin resistance cassette between a 1,010-bp fragment located upstream and a 496-bp fragment located downstream of the coding region of the farA gene. **Deletion of farB**: because the flanking regions adjacent to the two farB genes, farB1 and farB2, are identical, a deletion vector was built in such a manner that the two genes could be deleted by homologous integration of the vector at both loci simultaneously. The vector was constructed by insertion of the hygromycin resistance cassette between a 910-bp fragment located upstream and a 975-bp fragment located downstream of the coding region of the genes farB. The deletion constructs were then cloned into the pDHt/SK2 vector [22]. The deletion vector was integrated into the B-5233 genome via Agrobacterium tumefaciens-mediated transformation [22]. Southern hybridization identified two independent transformants (referred as JSA1 and JSA2) with homologous gene replacement resulting in deletion of farA. Likewise, two independent transformants (referred as JSB1 and JSB2) with homologous integration of the deletion vector in the farB1 as well as farB2 locus resulting in simultaneous deletion of both farB genes (data not shown) were identified.

**Growth assays**

To evaluate whether deletion of farA or farB affected the growth of *A. fumigatus*, conidia were incubated on yeast nitrogen base, YNB (Difco, MD, USA) broth, supplemented either with 10 mg/ml glucose (YNB/G), 50 µl/ml FA (YNB/FA) or 0.26 M glycerol (YNB/GLY). 2 x 10⁴ conidia were inoculated into 200 µl of YNB/G, YNB/FA or YNB/GLY and the conidial suspension was added to the wells of an 8-wells chambered cover glass slide. The slide was incubated for 24 h before visualization under bright-field microscopy.

**Deletion of the genes encoding a copper transporter** (Afu6g02810), a metalloreductase (Afu6g02820) and a putative GPI-anchored protein (Afu6g02800)

Since these three genes are located adjacent to each other on chromosome 6, a deletion vector was constructed to delete all three genes at once. The vector was constructed by insertion of the hygromycin resistance cassette between a 950-bp fragment and a 520-bp fragment flanking the coding regions of the first gene Afu6g02800 and the third gene Afu6g02820, respectively. The deletion constructs were then cloned into the pDHt/SK2 vector...
Cryptococcus neoformans identification of lysosomes and vesicles in fungal species such as viability indicator in mammalian cells [14,15] and for the process requiring cellular energy. This dye has been used as a cell neutrophils. Neutral red is a weak cationic dye that penetrates cells to monitor changes in the viability of individual conidia exposed to neutrophils. In this study, we used a dye, neutral red, to assess the efficacy [4]. Growth inhibition was assayed by monitoring the presence of either normal or CGD neutrophils. Conidia were exposed to neutrophils from normal or CGD donors and showed that conidial viability decreased in a neutrophil dose-dependent manner and that neutrophils from normal and CGD donors were equally capable of reducing conidial viability (Fig. 1F). This new method of scoring Aspergillus viability is consistent with previous findings based on metabolic activity of the fungus [4].

Genes up-regulated in conidia upon exposure to neutrophils

Since CGD neutrophils inhibited conidial growth as efficiently as normal neutrophils, we first focused on the identification of common genes that showed alterations of expression in the presence of either normal or CGD neutrophils. Conidia were exposed to neutrophils from normal or CGD donors and microarray analysis was carried out. To analyze the microarray data, genes were grouped based on their expression vectors using the k-means algorithm. The selected groups of genes were again organized for similar expression patterns using Euclidean distance and hierarchical clustering with the average linkage clustering method of JCVI MEV (see Materials and Methods). One of the most significant findings from the Clustering analysis was the identification of 244 genes up-regulated in conidia upon exposure to neutrophils [Fig. 2A, Table S1]. Since most of these genes were up-regulated in conidia but not in hyphae, it is likely that these genes are part of a conidial-specific response. A general classification, based on the annotated functions, showed that the major groups consisted of genes involved in transport, regulation of transcription, metabolism of molecules with 1–3 carbons and peroxisomal proteins (Fig. 2B). Many of these genes are involved in the beta-oxidation of fatty acids, acetate metabolism, glyoxylate cycle and peroxisome biogenesis, suggesting a reprogramming of peroxisomal proteins (Fig. 2B).

Results

Effect of neutrophils on conidial viability

We have shown previously that neutrophils from CGD and normal donors inhibit the growth of A. fumigatus conidia with equal efficacy [4]. Growth inhibition was assayed by monitoring the metabolic activity of mycelia that developed after the conidia were exposed to neutrophils. In this study, we used a dye, neutral red, to monitor changes in the viability of individual conidia exposed to neutrophils. Neutral red is a weak cationic dye that penetrates cells by nonionic diffusion and is compartmentalized by living cells in a process requiring cellular energy. This dye has been used as a cell viability indicator in mammalian cells [14,15] and for the identification of lysosomes and vesicles in fungal species such as Cryptococcus neoformans, Colletotrichum graminicola and Botrytis cinerea [16,17,18]. In control experiments, we observed that conidia capable of compartmentalizing the dye were able to germinate and develop into hyphae (Fig. 1A,B) whereas conidia that failed to compartmentalize the dye did not germinate (Fig. 1 C,D and data not shown). Figure 1E shows a micrograph of neutral red stained conidia exposed to CGD neutrophils. Assays with normal neutrophils showed similar results (data not shown). Germinating conidia, while phagocytized by neutrophils, showed compartmentalized neutral red whereas homogeneously stained conidia remained ungerminated (Fig. 1E inset). Based on these observations we scored the viability of conidia exposed to neutrophils and showed that conidial viability decreased in a neutrophil dose-dependent manner and that neutrophils from normal and CGD donors were equally capable of reducing conidial viability (Fig. 1F). This new method of scoring Aspergillus viability is consistent with previous findings based on metabolic activity of the fungus [4].

Menadione treatment

To assay the effect of the oxidative agent menadione, 200 μl of 106 conidia/ml was added to the wells of an 8-wells chambered cover glass slide and incubated for 8 h to allow germination and development of hyphal filaments. Menadione sodium bisulfite (Sigma), 40 μg/ml, was then added to the samples. The control wells received media instead of menadione. The slides were incubated for additional 24 h before visualization by bright-field microscopy (Zeiss Axiovert, Axiovison 4.0 software).

Table 2. Oligonucleotides used for the qRT-PCR assays.

| Locus ID/Gene or Function | Probe | Primer Forward | Primer Reverse |
|---------------------------|-------|----------------|----------------|
| Afu4g09240/gpdA            | CCCCCAGGACGCCAGCAGC | TTTGGTCAGCAGTACGTTAC | TCCACAGGACAGATTGCA |
| Afu4g13510/isocitrate lyase AcuD (ic) | TGTGCTCTACCCAACTCAAAGCAGCAGCAGTAC | CGCCGATCAACCCGTTCTAC | TCGAGCTGACGTTGCA |
| Afu4g07740/peroxisomal biogenesis factor (pex11) | AAGCTCTACCGCCGCCAGG | TGGAGTCGATGGCTGTTAAG | CGAGCGGACCCGAGGAC |
| Afu3g02270/myeloid catalase (cat1) | CAGGAGCCGGATTTGAGG | TTCGCCGTATTGAGGACAGCT | CGGAGCTGAGGAGGAC |
| Afu8g04130/C6 transcription factor Ctf1B (farB1) | GCCCGCGTGATATCCACCGTAC | GGTGCTGATTGATACTGAA | ACGCGCGTCAGGTGTTT |
| Afu4g03900/multifunctional beta-oxidation protein (mpf) | TTGGACCGAATCGACCGGAGTTAG | GATGTCGATGGCTGTTAAG | TCCACAGGACAGATTGCA |
| Afu3g09600/C6 transcription factor Ctf1A (farA) | CCGGGCTGATATCCACCGTAC | GGTGCTGATTGATACTGAA | ACGCGCGTCAGGTGTTT |

doi:10.1371 journal.pone.0002655.t002
NAD-dependent formate dehydrogenase, is one of the genes that showed the highest increases in mRNA levels (average of 4-fold increase compared to control). The *icl* and *pex11* genes encode isocitrate lyase and the peroxisome biogenesis factor, respectively, which are putatively involved in fatty acid catabolism (Table 3). The qRT-PCR data confirmed that these genes were highly up-regulated only in conidia upon exposure to either normal or CGD neutrophils, (Fig. 3A–C).

Hynes and collaborators have reported that fatty acid catabolism and peroxisomal function are both regulated by the genes *farA* and *farB* in *A. nidulans* [23]. Interestingly, our array data showed that two genes predicted to encode FarB (*farB1* and *farB2*) had their expression increased in conidia during the challenge with neutrophils (Table 3). Although the *facA* gene was not included in the array design, qRT-PCR assays with RNA isolated from neutrophil-exposed conidia showed that the mRNA levels of this gene increased in response to exposure to either normal or CGD neutrophils, (Fig. 3A–C).

To examine the potential role of fatty acid catabolism in the survival of *A. fumigatus* conidia, the genes *farA, farB1* and *farB2* were deleted. The deletants JSA1, JSA2, JSB1 and JSB2 were selected for the experiments. JSA1 and JSA2 are independent mutants generated by deletion of *farA*. JSB1 and JSB2 are independent mutants generated by double deletion of the genes *farB1* and *farB2*. qRT-PCR assays were performed to evaluate whether the deletion of *farA* and/or *farB* affected the transcriptional levels of *mfp*, *pex11* and *icl*. Germinating conidia from JSA1 and JSB1 were incubated in G−, G+ or FA before RNA was isolated. Similar to the observed with B-5233, conidia of both JSA1 and JSB1 exhibited higher mRNA levels of *mfp*, *pex11* and *icl* upon incubation in G− compared to G+ (Fig. 4B, G+/G−). The results suggest that the expression of the genes *farA*, *farB*, *mfp*, *pex11* and *icl* is induced under glucose-limited conditions as well as in the presence of fatty acids.

To examine the potential role of fatty acid catabolism in the survival of *A. fumigatus* conidia, the genes *farA, farB1* and *farB2* were deleted. The deletants JSA1, JSA2, JSB1 and JSB2 were selected for the experiments. JSA1 and JSA2 are independent mutants generated by deletion of *farA*. JSB1 and JSB2 are independent mutants generated by double deletion of the genes *farB1* and *farB2*. qRT-PCR assays were performed to evaluate whether the deletion of *farA* and/or *farB* affected the transcriptional levels of *mfp*, *pex11* and *icl*. Germinating conidia from JSA1 and JSB1 were incubated in G−, G+ or FA before RNA was isolated. Similar to the observed with B-5233, conidia of both JSA1 and JSB1 exhibited higher mRNA levels of *mfp*, *pex11* and *icl* upon incubation in G− compared to G+ (Fig. 4B, G+/G−). Incubation of conidia from JSA1 in FA, however, did not result in an increase of mRNA levels for these genes equivalent to that observed in B-5233, i. e., the levels of *mfp*...
and \textit{pex11} were not significantly higher upon incubation in FA compared to G\textsuperscript{2}. Although the mRNA level of \textit{icl} showed some increase, it was still to a lesser extent than in B-5233. In the case of JSB1 conidia, the pattern of up-regulation was similar to that in B-5233 (Fig. 4B, FA/G\textsuperscript{2}). Considering that FA is a mixture of fatty acids with chain-lengths from C\textsubscript{12}–C\textsubscript{20}, these results suggest that the gene \textit{farA} plays a major role in the beta-oxidation of long-chain fatty acids. Similar patterns, supporting these findings, were observed with the mutant JSA2 (data not shown).

| Locus ID | Aspergillus fumigatus | Homologous genes in Aspergillus nidulans |
|----------|-----------------------|-----------------------------------------|
| Predicted function | (accession/gene) | Beta oxidation of fatty acids: |
| Afu8g04130 | C6 transcription factor | AN1425.2/farB1 |
| Afu1g00410 | C6 transcription factor | AN1425.2/farB2 |
| Afu1g14850 | acyl-coA dehydrogenase | AN0824.2 |
| Afu1g15170 | acyl-coA thioesterase II | AN0868.2 |
| Afu7g06100 | acyl-coA dehydrogenase family | AN6752.2 |
| Afu7g06090 | fatty-acyl coA oxidase (Pox1) | AN6752.2 |
| Afu4g10950 | 3-ketoacyl-coA thiolase peroxisomal A precursor | AN5646.2 |
| Afu2g10920 | enoyl-CoA hydratase/isomerase family protein | AN5816.2/echA |
| Acetate metabolism: |
| Afu1g13510 | C6 transcription factor (FabC/CatB) | AN0689.2/facB |
| Afu4g11080 | acetyl-coenzyme A synthetase FacA | AN5626.2/facA |
| Afug614100 | mitochondrial carnitine:acyl carnitine carrier | AN5356.2/acuH |
| Afu1g12340 | carnitine acetyl transferase | AN1059.2/facC |
| Afu2g12530 | carnitine acetyl transferase | AN6279.2/acuL |
| Glyoxylate cycle: |
| Afu4g13510 | isocitrate lyase AcuD | AN5634.2/acuD |
| Afu6g02860 | isocitrate lyase | AN8755.2 |
| Citric acid cycle: |
| Afug612930 | aconitate hydratase, mitochondrial | AN5525.2 |
| Afug627910 | succinate dehydrogenase subunit Sdh1 | AN2916.2 |
| Afug421010 | 2-oxo acid dehydrogenases acyltransferase | AN3639.2 |
| Afug510370 | iron-sulfur protein subunit of succinate Sdh2 | AN2332.2 |
| Afu1g15590 | succinate dehydrogenase subunit Cyb5 | AN0896.2 |
| Afug605210 | malate dehydrogenase, NAD-dependent | AN4699.2 |
| Afug4g00290 | succinyl-CoA synthetase beta subunit | AN7000.2 |
| Peroxisomal: |
| Afug6g07740 | Peroxisome biogenesis factor (Pex11) | AN1921.2 |
| Afug6g04310 | Peroxisome membrane protein Pmp47 | AN4820.2 |
| Afug5g0780 | Peroxisome membrane protein (PmpP24) | AN1483.2 |
| Afug6g03560 | Peroxisome biosynthesis protein (Peroxin-2) | AN4056.2 |
| Afug4g04780 | Peroxisome ABC transporter (Pxa1) | AN4262.2 |
| Afug7g04260 | Peroxisome biosynthesis protein (Peroxin-10) | AN5681.2 |
| Afug2g10150 | Peroxisome biosynthesis protein (Pox1/Peroxin-1) | AN5991.2 |

doi:10.1371/journal.pone.0002655.t003

**Figure 3. Expression level of genes differentially regulated between conidia and hyphae.** The relative fold-change in the mRNA levels of the genes \textit{fdh} encoding an NAD-dependent formate dehydrogenase (A), \textit{icl} encoding an isocitrate lyase (B) and \textit{pex11} encoding a peroxisomal biogenesis factor (C) were obtained by qRT-PCR. The assays were carried out with RNA isolated from hyphae and conidia exposed to neutrophils from two normal (grey bars) or two CGD (white bars) donors. Each bar represents a replicate carried out with neutrophils from a single donor (±SD of each qRT-PCR replicate). The relative fold-change represents the log2 ratio between fungal cells exposed to neutrophils and fungal cells without neutrophil challenge.

doi:10.1371/journal.pone.0002655.g003
Deletant strains of the fungus *A. fumigatus* were exposed to neutrophils and their transcriptome profiles were compared to wild type *A. fumigatus*. Genes differentially expressed in hyphae upon exposure to neutrophils were identified using qRT-PCR. The expression level of the genes farA, farB, mfp, pex11 and icl in the strains B-5233, JSA1 and JSB1 were compared to wild type strain B-5233. The profiles were compared using SAM with a median false discovery rate (FDR) of < 0.01%. Table 4 lists the genes differentially expressed in hyphae exposed to normal versus CGD neutrophils which showed an average relative fold-change ≥2. As expected, hyphae treated with normal neutrophils showed up-regulation of two genes encoding enzymes associated with oxidative stress responses, manganese superoxide dismutase (Afu1g14550) and catalase Cat1 (Afu3g02270). The cat1 gene, together with cat2, has previously been described as mycelial catalase [26,27]. Because the array data had missing values for cat2 in three of the experiments, this gene was not included in the SAM results. A further examination of the array data prior to SAM analysis suggested that this gene showed a trend of up-regulation in hyphae exposed to normal neutrophils (Table 5). qRT-PCR assays confirmed that exposure to normal, but not CGD neutrophils caused an increase in the mRNA levels of cat2 as well as cat1 (Fig. 6A). Furthermore, a closer survey of the array data revealed that genes encoding glutathione peroxidase and thioredoxin reductase, both typically associated with oxidative stress, showed a trend of higher expression in hyphae exposed to normal neutrophils compared to CGD neutrophils (Table 5). The increase in the mRNA levels of genes associated with oxidative stress supports the notion that fungal hyphae are responding to ROS generated by normal neutrophils.

Interestingly, four genes that are predicted to be involved in iron/copper assimilation were also differentially regulated. These genes encode two copper transporters (Afu2g03730 and Afu6g02810), one metallor ductase (Afu6g02820) and one ferric-chelate reductase (Afu6g13750) (Table 4). The genes encoding the copper transporter (Afu6g02810) and the metallor ductase (Afu6g02820) are located on chromosome 6 adjacent to the gene encoding a putative GPI-anchored protein (Afu6g02800) that was also differentially regulated (Table 4). Using qRT-PCR, we confirmed that the mRNA levels of these five genes were higher in hyphae exposed to normal versus CGD neutrophils.
in hyphae exposed to normal neutrophils compared to CGD neutrophils (Fig. 6A). To evaluate whether some of these genes were relevant during interaction with neutrophils, we chose to delete the three genes that are located adjacent to each other on chromosome 6 (Afu6g02800-02820). Two independent strains, T9 and T10, containing a deletion of the three genes were selected for the experiments. Since one of the hypotheses proposes that these genes are involved in the resistance to oxidative stress, T9 and T10 were assayed with the oxidizing agents hydrogen peroxide and menadione. Menadione, a chemical that generates superoxide ion through enzymatic redox cycling, has been previously used to assess oxidative stress response in *A. fumigatus* and *Sacharomyces cerevisiae* [28,29]. The mutant strains did not show an increased susceptibility to hydrogen peroxide (data not shown). However, in the presence of menadione, T9 and T10 were no longer able to germinate and develop mycelia while B-5233 grew robustly (Fig. 7). The defective growth of T9 and T10 suggests that one or more of the deleted genes are likely to be involved in resistance to menadione. However, in the interaction assays with normal neutrophils the mutant strains did not show an increased susceptibility to the phagocytes (data not shown). Our findings suggest that although these three genes are important for the response to the oxidative agent menadione, they are not essential for resistance to neutrophils. Although we were able to identify, by the SAM method, the genes whose expression levels were significantly higher in hyphae exposed to normal neutrophils relative to CGD neutrophils, we did not detect any genes whose expression levels were significantly lower (fold-change ≥2) in hyphae exposed to normal neutrophils.

**Genes differentially expressed in conidia upon exposure to normal vs CGD neutrophils**

To identify genes differentially expressed in conidia we used the SAM method to analyze the transcription profiles of conidia exposed to normal versus CGD neutrophils. Using a FDR of < 0.01% and a relative fold-change ≥2, we identified a group of 8 genes that showed differential regulation (Table 6). Interestingly, the gene encoding the high-affinity copper transporter (Afu2g03730), which was identified as differentially regulated in hyphae, was also included in this group. To test whether the other 3 genes related to iron/copper assimilation and the gene encoding the GPI-anchored protein were also differentially regulated in conidia, qRT-PCR assays were performed. Although differences in the relative fold-change were smaller than those observed in hyphae, all five genes showed higher mRNA levels upon exposure to normal neutrophils compared to CGD neutrophils (Fig. 6B). In addition, genes encoding the glutathione peroxidase and the

---

**Figure 5. Growth comparison of the wild type strain B-5233 with the strains JSA1 and JSB1.** JSA1 and JSB1 are mutant strains with deletion of the genes farA and farB, respectively. Conidia from B-5233 (A, D, G), JSA1 (B, E, H) and JSB1 (C, F, I) were inoculated in media supplemented with glucose (A–C), fatty acid mixture FA (D–F) or glycerol (G–I). After incubation for 24 h the samples were observed by bright-field microscopy. Magnification in F applies to all panels.

doi:10.1371/journal.pone.0002655.g005
thioredoxin reductase, identified as differentially expressed in hyphae, were also found to be differentially regulated in conidia (Tables 5 and 6). A closer examination of the array data showed that the genes $cat1$ and $cat2$ and two manganese superoxide dismutase genes were also up-regulated in conidia exposed to normal as well as CGD neutrophils (Table 5). qRT-PCR assays showed that although the mRNA levels of $cat1$ and $cat2$ increased in response to both types of neutrophils, the levels were lower in conidia exposed to CGD neutrophils than to normal neutrophils (Fig. 6B).

Discussion

The patterns of gene expression in fungal pathogens during interaction with host immune cells have been studied in several

Table 4. Genes differentially expressed in hyphae exposed to normal vs CGD neutrophils

| Locus ID     | Predicted function                      | log2 relative fold-change* |
|--------------|-----------------------------------------|----------------------------|
| Afu1g14550   | Manganese superoxide dismutase           | 4                          |
| Afu2g03730   | High affinity copper transporter         | 6                          |
| Afu3g02260   | Hypothetical protein                    | 4                          |
| Afu3g02270   | Catalase 1 (Cat1)                       | 4                          |
| Afu5g02270   | Fungal specific transcription factor     | 2.5                        |
| Afu6g02800   | GPI anchored protein, putative           | 11                         |
| Afu6g02810   | Low-affinity copper transporter, putative| 8                          |
| Afu6g02820   | Metalloreducease                         | 3                          |
| Afu6g13750   | Ferric-chelate reductase                | 4                          |
| Afu6g13760   | Alpha-1,2-mannosidase subfamily         | 4                          |

Table 5. Relative expression of representative genes predicted to be involved in oxidative stress on fungal cells exposed to neutrophils.

| Locus ID     | Predicted function                      | Hyphae | Conidia |
|--------------|-----------------------------------------|--------|--------|
|              |                                         | N1     | N2     | N3     | C1     | C2     | C3     | N4     | N5     | N6     | N7     | C4     | C5     | C6     |
| Oxidative Stress |                                       |        |        |        |        |        |        |        |        |        |        |        |        |        |
| Afu3g12270   | glutathione peroxidase                  | 0.3    | 1.7    | 1.2    | 0.1   | 0.0   | 1.5   | 1.9   | 2.1   | 1.9   | 0.7   | 0.4   | 0.5   |
| Afu4g12990   | thioredoxin reductase                   | 0.6    | 1.2    | 1.9    | 0.1   | 0.0   | 1.0   | 1.3   | 1.2   | 1.2   | 0.5   | 0.3   | 0.4   |
| Afu4g11580   | Mn-superoxide dismutase                 | −0.1   | 0.1    | 0.0    | −0.1  | 0.0   | 1.2   | 1.1   | 1.1   | 1.3   | 1.0   | 0.9   | 1.4   |
| Afu1g14550   | Mn-superoxide dismutase                 | 1.5    | 2.6    | 2.2    | nd    | 0.0   | 2.7   | 3.7   | 2.6   | 3.8   | 1.9   | 0.4   | 2.3   |
| Afu3g02270   | catalase (Cat1)                         | 1.4    | 2.0    | 2.1    | −0.4  | nd    | 2.2   | 3.3   | 2.9   | 2.9   | 0.8   | nd    | 0.2   |
| Afu6g01670   | catalase (Cat2)                         | 0.3    | 2.0    | 2.1    | nd    | nd    | 2.6   | 3.4   | 3.2   | 3.0   | 1.9   | 1.7   | 2.2   |

Table 4: List contains the genes with statistically significant changes in expression (SAM) and fold-changes ≥2.

Table 5: Data are presented as the log2 of the average differences in the gene expression in hyphae exposed to normal neutrophils relative to CGD neutrophils.

Figure 6. Expression levels of the genes encoding catalase Cat1, catalase Cat2, GPI-anchored protein, low-affinity copper transporter, metalloreducease, ferric reductase and high-affinity copper transporter in hyphae (A) and conidia (B) exposed to human neutrophils. qRT-PCR was performed with RNA from hyphae/conidia exposed to neutrophils from normal (grey bars) or CGD (white bars) donors. Each bar represents a replicate carried out with neutrophils from a single donor. Two normal and two CGD donors were used in each assay. The relative fold-change represents the log2 ratio between hyphae/conidia exposed to neutrophils and those without neutrophil challenge. Error bars represents standard deviation of qRT-PCR. The legend for the hyphae is the same as that for conidia.

doi:10.1371/journal.pone.0002655.g006
species such as Candida albicans and Paracoccidioides brasiliensis [30,31,32]. Studies with C. albicans that were challenged with immune cells from human blood suggested that neutrophils induced gene expression more strongly than other cells [33]. In the present study, we have compared the transcriptome profiles of conidia and hyphae of A. fumigatus challenged with neutrophils from healthy donors and CGD patients. By exposing A. fumigatus to neutrophils from these two different types of donors, we were able to compare the response of the fungus between host cells that are capable of producing ROS with cells that are defective in ROS production. This system was suitable for identification of fungal genes that are up-regulated specifically in response to the ROS producing host cells.

The relatively recent appreciation of the important role of neutrophils in host defense against both conidia and hyphae of A. fumigatus prompted the present study. Bonnett and colleagues have shown that early recruitment and aggregation of oxidase-positive neutrophils, in addition to the phagocytic activity of alveolar macrophages, was essential in preventing conidial germination in the lungs of mice exposed to large numbers of A. fumigatus spores [3]. Furthermore, neutrophils from normal as well as CGD donors were shown to arrest conidial growth with equal efficiency and that lactoferrin, a major protein of neutrophil granules, contributes to reducing conidial growth [4]. Our findings suggest that upon internalization by neutrophils, conidia respond to changes in the environment by increasing the mRNA levels of genes putatively involved in the catabolism of fatty acids. The degradation of fatty acids is not a well described process in A. fumigatus. In Aspergillus nidulans, however, it has been described that the beta-oxidation of fatty acids occurs in peroxisomes as well as in mitochondria [34]. Two enzymes, the enoyl-CoA hydratase and the multifunctional

Table 6. Genes differentially expressed in conidia in response to normal vs CGD neutrophils.

| Locus ID     | Predicted function                  | log2 relative fold-change$^2$ |
|--------------|-------------------------------------|-------------------------------|
| Afu2g03730   | high affinity copper transporter    | 2                             |
| Afu3g02260   | hypothetical protein                | 3                             |
| Afu4g10930   | N-acetyltransferase superfamily     | 2.5                           |
| Afu3g12270   | glutathione peroxidase family protein | 2.5                       |
| Afu2g04060   | NADH-flavin oxidoreductase          | 2                             |
| Afu4g12990   | thioredoxin reductase               | 3                             |
| Afu5g02020   | aldehyde reductase (GIO), putative  | 3                             |
| Afu5g09910   | p-nitroreductase family             | 3                             |

$^1$: List contains the genes with statistically significant changes in expression (SAM) and fold-changes ≥ 2.

$^2$: Data are presented as the log2 of the average differences in the gene expression in conidia exposed to normal neutrophils relative to CGD neutrophils.

doi:10.1371/journal.pone.0002655.t006
protein FoxA, which catalyze similar steps in the beta-oxidation of fatty acids are located in distinct organelles in *A. nidulans*. The enzyme enoyl-coA hydratase is localized in mitochondria whereas FoxA is in peroxisomes [34]. Considering that *A. fumigatus* conidia up-regulate genes encoding an enoyl-coA hydratase and a multifunctional protein Mfp, which are homologous to the genes encoding the *A. nidulans* enoyl-coA hydratase and FoxA, it is plausible that the degradation of fatty acid in conidia internalized by neutrophils occurs in the same way as in *A. nidulans*, i.e., the fatty acids are degraded in mitochondria as well as in peroxisomes. Other genes found to be up-regulated in *A. fumigatus* conidia upon exposure to neutrophils show homology to *A. nidulans* genes (Table 3) whose function has been characterized as being involved in acetate metabolism and the glyoxylate cycle. This observation reinforces the hypothesis that the genes up-regulated in *A. fumigatus* conidia are involved in fatty acid catabolism.

Based on the transcriptome changes, we hereby propose a putative pathway for carbon metabolism in conidia exposed to neutrophils (Fig. 8). The beta-oxidation of fatty acids occurs in cycles that release acetyl units. The acetyl units can either enter the glyoxylate cycle and/or be transported to the mitochondria. An increase in the expression of the isocitrate lyase gene, a key enzyme of the glyoxylate cycle, and genes that encode acetyl-carnitine transferases that transport acetyl units into mitochondria, suggests that both mechanisms may take place in conidia upon phagocytosis by neutrophils. The succinate produced in the glyoxylate cycle can subsequently be transported to the mitochondria to replenish the citric acid cycle. The up-regulation of the gene encoding a putative succinate-fumarate carrier (ACR1) suggests that the enhanced transport of succinate to mitochondria may also occur. A similar mechanism has been described in *S. cerevisiae* where acetyl-coA enters the peroxisomal glyoxylate cycle to produce succinate, which is then transported to the mitochondria via the succinate-fumarate transporter Acr1p [35,36]. Multiple enzymatic steps can transform the co-transported fumarate into phosphoenolpyruvate, which then can be used for gluconeogenesis. Interestingly, our array data shows that one of the conidial genes exhibiting the highest transcriptional increase upon exposure to neutrophils encodes a formate dehydrogenase. It has been suggested that formate is an indirect product of the glyoxylate cycle and that formate dehydrogenase, Fdh1p, may be involved in the detoxification of ROS. The role of catalases in neutrophil attack. To further investigate the molecular mechanisms associated with the response of *A. fumigatus* to neutrophils, we searched for the genes differentially expressed in conidia and hyphae upon exposure to neutrophils. Our findings showed that genes encoding the catalases cat1 and cat2, a manganese superoxide dismutase (Afu1g14550), a glutathione peroxidase (Afu3g12270) and a thioredoxin reductase (Afu4g12990) were up-regulated in hyphae exposed to normal, but not CGD neutrophils (Fig. 6 and Table 5). In contrast, the mRNA levels of both cat1 and cat2 and two manganese superoxide dismutases (Afu1g11580 and Afu1g14550) were increased in conidia exposed to normal as well as CGD neutrophils (Fig. 6 and Table 5). An explanation to this difference may be that up-regulation of the two catalases and one of the manganese superoxide dismutases is a hyphal response specific to normal neutrophils whereas in conidia these genes are up-regulated as part of a general stress response. Another possibility is that since neutrophils from individuals with CGD are not always completely defective in producing ROS [6,41], even small amounts of ROS may be sufficient to elicit an oxidative stress response in conidia phagocytized by neutrophils and sequestered inside vacuoles. Hyphae, however, are larger structures and are not completely engulfed by neutrophils; and therefore, higher concentrations of ROS would be required to elicit a similar response. Similar profiles of gene expression were observed in *C. albicans* exposed to neutrophils [33]. Genes encoding enzymes with catalytic activity such as catalase, glutathione-peroxidase, thioredoxin reductase and superoxide dismutase were found to be up-regulated in *C. albicans* upon interaction with neutrophils. Fradin et al [33] suggested that these genes are likely to be involved in the detoxification of ROS. The role of catalases in *A. fumigatus* pathobiology has been assessed in other studies and it was shown that these enzymes are not essential for fungal virulence [27,42]. Another study using CGD mice showed that catalases are not required for virulence of *A. nidulans*, the second most common *Aspergillus* species to cause infection in CGD patients [43].

The up-regulation of the metalloreductase and the ferric reductase genes suggests an activation of the reductive system of
Figure 8. Proposed pathway for utilization of fatty acids in conidia exposed to neutrophils. The pathway was proposed based on the genes that were up-regulated in conidia. The locus ID and the predicted functions of these genes are listed (bold). The fatty acid molecules are degraded via beta-oxidation to produce acetyl-coA units. Two molecules of acetyl-coA are used in the glyoxylate cycle. The malate and oxaloacetate produced in the glyoxylate cycle can participate in gluconeogenesis and the succinate can be used to replenish the citric acid cycle. Alternatively, the acetyl-coA can be transported to mitochondria as acetyl-carnitine and enter the citric acid cycle. The fumarate produced in the citric acid cycle can then be used for gluconeogenesis. *Propionyl-coA is a secondary product of beta-oxidation of fatty acids with even numbers of carbon.
doi:10.1371/journal.pone.0002655.g008
iron transport. The gene encoding the metalloreductase is predicted to have oxidoreductase activity and is involved in the transport of transition metal ions like iron. In the S. cerevisiae model of high-affinity reductive iron transport, the ferric ion is reduced to ferrous ions by the ferric reductases Fet1p and Fet2p [44,45,46,47]. The ferrous ions are then reoxidized by the copper-dependent oxidase Feo3p and transported into the cytoplasm by the iron permease Frt1p or transported directly by the low-affinity Feo4p. The Feo3p catalytic activity depends on copper ions, which are transported into the cells via Ctr1p [48,49].

Our array data did not show differential mRNA levels of genes predicted to encode typical copper-dependent oxidases or iron permeases, however, two putative copper transporters were found to be up-regulated. It is conceivable that the up-regulation of these genes is related to the requirement of copper to activate oxidases involved in iron assimilation. In light of the fact that lactotetraen released by neutrophils reduced conidial growth via iron-chelation [4] it is possible that an increase in the mRNA levels of genes involved in iron/copper transport reflect an attempt of the fungal cells to augment iron assimilation. However, experiments with the deletion mutants T9 and T10, both lacking the genes encoding a metalloreductase, a copper transporter and a GPI-anchored protein (Afu6g02800-02820), showed no reduction in growth on iron-limited media (data not shown), suggesting that these genes are not essential for iron assimilation. In fact, it has been reported that assimilation of iron by A. fumigatus occurs mainly through siderophores and not reductive systems [50,51,52]. Since the up-regulation of these genes is more pronounced in fungal cells exposed to normal neutrophils, such as the ones encoding the fungal specific oxidase Fet3p and transported into the cytoplasm by the iron permease Ftr1p or transported directly by the low-affinity Feo4p, the Fet3p catalytic activity depends on copper ions, which are transported into the cells via Ctr1p [48,49].

Characterization of other genes that showed significant differential expression in conidia and hyphae in response to human neutrophils, such as the ones encoding the fungal specific transcription factor (Afu5g02270) and a hypothetical protein (Afu5g02260) may provide important clues as to their roles in A. fumigatus pathogenesis.

Supporting Information

Table S1 List of the genes up-regulated in conidia shown in Figure 2

Acknowledgments

We thank C. M. McClelland, H. F. Tsai and A. Varma for critically reviewing the manuscript.

Author Contributions

Conceived and designed the experiments: JK HK WN JS KZ YC. Performed the experiments: HK JS KZ. Analyzed the data: JK KK WN JS KZ YC. Contributed reagents/materials/analysis tools: JK KK WN JS KZ. Wrote the paper: KK HK JS.

References

1. Munoz P, Guinea J, Bouza E (2006) Update on invasive aspergillosis: clinical and diagnostic aspects. Clin Microbiol Infect 12: 24–39.
2. Deming DW, Anderson MJ, Turner G, Latge JP, Bennett JW (2002) Sequencing the Aspergillus fumigatus genome. Lancet Infect Dis 2: 251–253.
3. Bennett CR, Cornish EJ, Harmson AG, Burritt JB (2006) Early neutrophil recruitment and aggregation in the murine lung inhibit germination of Aspergillus fumigatus conidia. Infect Immun 74: 6529–6539.
4. Zarember KA, Sugui JA, Chang YC, Kwon-Chung KJ, Gallin J (2007) Human Polyomaviral-Like DNA Containing a Gliotoxin Gene Confers Resistance to Neutrophil Killing. J Infect Dis 196: 479–486.
5. Sugui JA, Pardo J, Chang YC, Zarember KA, Nardone G, et al. (2007) Gliotoxin is a virulence factor of Aspergillus fumigatus: glp deletion attenuates virulence in mice immunosuppressed with hydrocortisone. Environ Toxicol 2101–2103.
6. Zarember KA, Chang YC, Washburn RG, Wheeler NH, Kwon-Chung KJ (1998) The developmentally regulated aft1 gene of Aspergillus fumigatus: its role in modulation of conidial morphology and virulence. J Bacteriol 180: 3031–3038.
7. Segal BH, Lezo TJ, Gallin JI, Malech HL, Holland SM (2000) Genetic, biochemical, and clinical features of chronic granulomatous disease. Medicine (Baltimore) 79: 170–200.
8. Zarember KA, Chang YC, Kwon-Chung KJ, Gallin J (2004) The developmentally regulated aft2 gene of Aspergillus fumigatus: its role in modulation of conidial morphology and virulence. J Infect Dis 196: 479–486.
16. Mason DL, Wilson CL (1979) Cytochemical and biochemical identification of lysosomes in Cryptococcus neoformans. Mycopathologia 68: 183–190.
17. Schadeck RJ, Randi MA, de Freitas Bucí D, Leite B (2003) Vascular system of ungerminated Colletotrichum gloeosporioides conidia: convergence of autophagic and endocytic pathways. FEMS Microbiol Lett 218: 277–283.
18. Weber RW, Wakley GE, Pitt D (1999) Histochemical and ultrastructural characterization of vacuoles and spherosomes as components of the lytic system in hyphae of the fungus Botrytis cinerea. Histochem J 31: 293–301.
19. Urban CF, Rechard U, Brinkmann V, Zychlinsky A (2006) Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. Cell Microbiol 8: 669–676.
20. Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, et al. (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. Nature 438: 1154–1156.
21. Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 98: 5116–5121.
22. Sugui JA, Chang YC, Kwon-Chung KJ (2005) Agrobacterium tumefaciens-mediated transformation of Aspergillus fumigatus: an efficient tool for insertional mutagenesis and targeted gene disruption. Appl Environ Microbiol 71: 1795–1802.
23. Hynes MJ, Murray SL, Duncan A, Khew GS, Davis MA (2006) Regulatory genes controlling fatty acid catabolism and peroxisomal functions in the filamentous fungus Aspergillus nidulans. Eukaryot Cell 5: 794–805.
24. Gurevitz A, Ronensten BE (2006) The biochemistry of oleate induction: transcriptional upregulation and peroxisome proliferation. Biochim Biophys Acta 1763: 1392–1402.
25. Kionka C, Kunau WH (1985) Inducible beta-oxidation pathway in Neurospora crassa. J Bacteriol 161: 153–157.
26. Hearn VM, Wilson EV, Mackenzie DW (1992) Analysis of Aspergillus fumigatus catalases possessing antigenic activity. J Med Microbiol 36: 61–67.
27. Paris S, Wysong D, Debeaupuis JP, Shibuya K, Philippe B, et al. (2003) Catalases of Aspergillus fumigatus. Infect Immun 71: 3551–3562.
28. Yamamoto A, Ueda J, Yamamoto N, Hashikawa N, Sakurai H (2007) Role of heat shock transcription factor in Aspergillus fumigatus. Mol Genet Genom 275: 5493–5503.
29. Schadeck RJ, Randi MA, de Freitas Bucí D, Leite B (2003) Characterization of vacuoles and spherosomes as components of the lytic system in hyphae of the fungus Botrytis cinerea. Histochem J 31: 293–301.
30. Urban CF, Rechard U, Brinkmann V, Zychlinsky A (2006) Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. Cell Microbiol 8: 669–676.
31. Tavares AH, Silva SS, Dantas A, Campos EG, Andrade RV, et al. (2007) Early virulence of catalase-deficient Aspergillus nidulans in p47(phox)-/- mice. Implications for fungal pathogenicity and host defense in chronic granulomatous disease. J Clin Invest 110: 163–1650.
32. Yun CW, Bauler M, Moore RE, Klinepa PE, Philpott CC (2001) The role of the FKE family of plasma membrane reductases in the uptake of siderophore-iron in Saccharomyces cerevisiae. J Biol Chem 276: 10210–10223.
33. Dancis A, Klausner RD, Hemejus AB, Barrocanal JG (1996) Genetic evidence that ferric reductase is required for iron uptake in Saccharomyces cerevisiae. Mol Cell Biol 15: 2294–2301.
34. Anderson CJ, Lesuisse E, Dancis A, Roman D, Labbe P, et al. (1992) Ferric iron reduction and iron assimilation in Saccharomyces cerevisiae. J Inorg Biochem 47: 249–255.
35. Georgatou E, Alexandraki D (1994) Two distinctly regulated genes are required for ferric reduction, the first step of iron uptake in Saccharomyces cerevisiae. Mol Cell Biol 14: 3065–3073.
36. Askwith C, Eide D, Van Ho A, Bernard PS, Li L, et al. (1994) The FET3 gene of S. cerevisiae encodes a multicopper oxidase required for ferrous iron uptake. Cell 76: 403–410.
37. Dancis A, Yuan DS, Hade D, Askwith C, Eide D, et al. (1994) Molecular characterization of a copper transport protein in S. cerevisiae: an unexpected role for copper in iron transport. Cell 76: 393–402.
38. Schrettl M, Biguell E, Kraig C, Joehl C, Rogers T, et al. (2004) Siderophore biosynthesis but not reductive iron assimilation is essential for Aspergillus fumigatus virulence. J Exp Med 200: 1213–1219.
39. Wasilnka JA, Hissin AH, Wan AN, Moore MM (2005) Intracellular and extracellular growth of Aspergillus fumigatus. Med Mycol 43 Suppl: 1: S27–30.
40. Hissin AH, Wan AN, Warwas ML, Pinto LJ, Moore MM (2005) The Aspergillus fumigatus siderophore biosynthetic gene sifA, encoding L-ornithine N5-oxygenase, is required for virulence. Infect Immun 73: 5493–5503.
41. Blaesse PL, Lesuisse E, Camadro JM (2003) Aft2p, a novel iron-regulated transcription activator that modulates, with Aft1p, intracellular iron use and resistance to oxidative stress in yeast. J Biol Chem 287: 34221–34226.