Candida albicans induces arginine biosynthetic genes in response to host-derived reactive oxygen species

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Abstract

The interaction of Candida albicans with phagoctyes of the host’s innate immune system is highly dynamic, the outcome of which directly impacts the progression of infection. While the switch to hyphal growth within the macrophage is the most obvious physiological response, much of the genetic response reflects nutrient starvation - translational repression and induction of alternative carbon metabolism. Changes in amino acid metabolism are not seen, with the striking exception of arginine biosynthesis, which is upregulated in its entirety during co-culture with macrophages. Using single cell reporters, we show here that arginine biosynthetic genes are induced specifically in phagocytosed cells. This induction is lower in magnitude than during arginine starvation in vitro, and is not driven by an arginine deficiency within the phagocyte but instead by exposure to reactive oxygen species. Curiously, these genes are induced in a narrow window of sublethal ROS concentrations. C. albicans cells phagocytosed by primary macrophages deficient in the gp91phox subunit of the phagocyte oxidase do not express the ARG pathway, indicating that the induction is dependent on the phagocyte oxidative burst. C. albicans arg pathway mutants are retarded in germ-tube and hyphal formation within macrophages, but are not notably more sensitive to ROS. We also find that the ARG pathway is not regulated by the general amino acid control response, but by transcriptional regulators similar to the Saccharomyces cerevisiae ArgR complex. In summary, phagocytosis induces this single amino acid biosynthetic pathway in an ROS-dependent manner.
Introduction

Candida albicans is the most prominent fungal component of the human microbiome, residing within multiple body sites including the skin, oral cavity, gastrointestinal tract, and vagina. C. albicans causes a spectrum of infections in otherwise healthy individuals with or without predisposing risk factors, such as vulvovaginal candidiasis, oropharyngeal thrush, and various cutaneous infections. In individuals with compromised immunity, disseminated candidiasis may result in severe disease with a mortality approaching 40%, a rate that has not changed in decades (6, 52). An increasing incidence of hospital-acquired disseminated candidiasis has been observed in recent years as the population of susceptible patients with impaired immune systems has risen. As a successful fungal pathogen, C. albicans possesses multiple virulence attributes including filamentous growth, biofilm formation, numerous secreted hydrolases, and the ability to sense and adapt to numerous environmental changes within the host in order to facilitate establishment of an infection (reviewed in (13)).

Invasive candidemia is prevented by the collective efforts of the innate immune system. Protective physical barriers such as epithelial integrity are the first line of defense against invading Candida. Macrophages and neutrophils, phagocytes that will internalize C. albicans upon recognition, are also key anti-fungal effectors. Following phagocytosis, a strong respiratory burst results in the production of antimicrobial reactive oxygen species (ROS) generated by the phagocyte NADPH oxidase and myeloperoxidase (MPO), nitric oxide (NO) generated by NOS2 (iNOS), and other reactive nitrogen species (RNS) (reviewed in (19). The contribution of this respiratory burst to the prevention of a C. albicans infection varies in different phagocytes and with different models of disease. Mice deficient in the gp91phox subunit of NADPH oxidase mimic the immune deficiencies observed in chronic granulomatous disease.
phagocytes from \(gp91^{phox-/-}/NOS2^{-/-}\) mice were able to kill \(C.\ albicans\) just as effectively in vitro as cells from wild-type mice, even though these \(gp91^{phox-/-}/NOS2^{-/-}\) mice are more susceptible to a variety of bacterial and fungal infections including \(Candida\ guillermondii\) and \(C.\ albicans\) (7, 44). Neutrophil MPO is critical in host defense against pulmonary and intraperitoneal \(C.\ albicans\) infections (1, 4). In a zebrafish larval model, loss of host NADPH oxidase activity results in enhanced filamentous growth of \(C.\ albicans\), a critical virulence trait for this organism, and an increased susceptibility to infection (11). It seems clear that phagocyte-derived ROS are an important, but by no means the only, component of antifungal defenses in whole animals. While hyphal growth is critical for the escape of \(C.\ albicans\) from phagocytic cells like macrophages, numerous physiological changes are elicited upon recognition of phagocytes, and a striking diversity of means by which \(Candida\) is modulating immune function is just beginning to be revealed (reviewed in (14)).

Previously, transcript profiling experiments were performed to identify changes in \(C.\ albicans\) gene expression in response to macrophage phagocytosis (28). Upon internalization, \(C.\ albicans\) generates a rapid metabolic response by downregulating glycolysis genes while simultaneously upregulating genes involved in fatty acid utilization, the glyoxylate cycle, and gluconeogenesis. These alternative carbon utilization pathways are necessary for full virulence in the mouse tail vein injection model of disseminated candidiasis (9, 29, 37). While part of this reprogramming of transcription is broadly similar to changes in gene expression due to carbon starvation, hierarchal clustering identified a second set of genes whose regulation was independent of starvation. Genes belonging to this non-starvation cluster included those involved in oxidative stress response pathways, DNA damage repair pathways, and uptake and
utilization of peptides, metals, and other small molecules. The only metabolic gene pathway that did not partition with the starvation cluster but instead was part of the non-starvation, stress response cluster was the arginine (ARG) biosynthetic pathway where all but one gene was upregulated at least 3-fold (28). The ARG genes are also upregulated in cells phagocytosed by neutrophils, one of the few transcriptional responses shared by these two datasets (28, 40).

In this study, we demonstrate that the arginine biosynthetic pathway genes are induced specifically in phagocytosed cells. ARG pathway genes are also induced by multiple oxidative stress-inducing agents in vitro and expression of ARG genes in phagocytosed cells is dependent on the macrophage oxidative burst as this does not occur when the macrophages lack the NADPH oxidase. *C. albicans* strains containing a deletion of either *ARG1* or *ARG3* have no obvious sensitivity to ROS in vitro, but possess hyphal morphogenesis delays within macrophages. We have identified homologs of the *S. cerevisiae* Arginine Regulatory (ArgR) complex (Arg80p, Arg81p, Arg82p and Mcm1p) and find that, while broadly similar, *C. albicans* lacks an ARG80 homolog but has duplicated ARG81. ArgR is primarily a repressor of gene expression, as in *S. cerevisiae* (15). The *C. albicans* ARG genes appear to be independent of the general amino acid control response, in which starvation for any one amino acid activates multiple biosynthetic pathways via the Gcn4p transcription factor in both yeast (reviewed in (23) and *C. albicans* (46). Gcn4p also targets ArgR to ARG promoters as both a positive and negative regulator in yeast (53), but we show that it serves mostly as a repressor of *C. albicans* ARG loci. Taken together, these data suggest that in response to the oxidative burst generated by macrophages following phagocytosis, *C. albicans* specifically induces arginine biosynthetic pathway genes to promote filamentous growth within macrophages.
Materials and Methods

Strains and media

*C. albicans* strains and plasmids are listed in Supplemental Tables 1 and 2, respectively. Strains were propagated in standard conditions in YPD (1% yeast extract, 2% peptone, 2% glucose) or YNB (0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% glucose) (43). Amino acids were added to YNB where indicated. Macrophages and co-cultures were grown in RPMI with glutamine and HEPES.

Construction of *C. albicans* reporter and deletion strains:

To construct the reporter strains, the *HIS1* gene from plasmid Cip20 (17) was incorporated into plasmids pGFP and pACT1-GFP (8) to generate pCJ1 and pCJ2, respectively. 1000 bp of the promoter region of *ARG1* (pCJ5) and *ARG3* (pCJ4), 674bp of *LYS1* and 500bp *LEU2* were PCR amplified from genomic DNA and individually cloned into plasmid pCJ1 immediately 5' of the translational start of GFP. The resulting constructs as well as pCJ1 and pCJ2 plasmids were linearized with *Stu*I and used to transform *C. albicans* RM1000 (ura3/ura3 his1/his1) by electroporation. Integration at the *RPS10* locus was confirmed by PCR. Additionally, pCJ5 (*ARG1-GFP*) and pCJ4 (*ARG3-GFP*) were similarly integrated into *gcn4Δ*, *arg81Δ*, and *arg83Δ C. albicans* mutant strains derived from mutant libraries generated by Sanglard and colleagues (49). The *ADH1p-γCherry* plasmid (11, 25) was linearized with *SalI* and transformed by electroporation into the *ARG1, ARG3, ACT1*, and promoterless GFP reporter strains. Integration at the *ADH1* locus was confirmed by PCR. Strains and plasmids are described in Supplemental Tables 1 and 2, respectively.

Mutations in *ARG1* and *ARG3* were generated using the SAT-flipper method (39) as described previously (50). Complementation of each mutant strain was achieved by cloning the
open reading frames of *ARG1* and *ARG3* immediately adjacent to the first FRT sequence within the FRT-SAT1-FLP-FRT cassette. This linearized construct was used to transform *arg1*Δ and *arg3*Δ mutants to nourseothricin resistance. Complementation was confirmed by both PCR and rescue of arginine auxotrophy (Fig. S4). A SAT1-marked plasmid (pHZ130) encoding a constitutively expressed GFP was generated by replacing the *URA3* gene in pACT1-GFP between the *Bam*HI and *Sac*I sites with a PCR fragment containing the nourseothricin resistance gene from pSFS1 (39). Plasmid pHZ130 was linearized with *Stu*I and used to transform wild-type, *arg1*Δ, and *arg3*Δ cells to nourseothricin resistance, confirming integration at the *RPS10* locus by PCR.

**Fluorescence microscopy**

To test arginine-dependent expression of the *ARG1*-GFP and *ARG3*-GFP reporter constructs in vitro, overnight YPD cultures were diluted 1:100 in fresh YPD or YNB and grown for 4 hours at 30°C. 5µl of each culture were used for microscopy using an Olympus IX81–ZDC confocal inverted microscope and the SlideBook 5.0 digital microscopy software (Intelligent Imaging Innovations, Inc.). For the *in vitro* assays using YNB supplemented with different amino acids, YPD overnight cultures were diluted 1:100 in fresh YPD or YNB supplemented with 20, 100, and 200 µg/ml of L-arginine, or 200µg/ml of L-lysine or L-leucine, and grown for 4 hours at 30°C.

To normalize the fluorescent signal across different strains and experiments, we calculated the ratio between the background subtracted fluorescence intensity of GFP to the constitutively-expressed yCherry obtained using appropriate filter sets using Slidebook software, as described (11). A cell-free portion of each field was used as background, except in the BMDM experiments in which the entire field was used as background to compensate for a low-
level autofluorescence in the macrophages. At least 50 cells were counted for each reporter strain and each localization (intracellular versus extracellular, for instance).

For cocultures with J774.A, RAW264.7, or bone marrow-derived macrophages, the cells were seeded onto coverslips in 12-well plates (5 × 10^6 cells/ml) at least 2 hours prior to initiating the co-cultures. *C. albicans* cells harboring the desired GFP reporter were grown overnight in YPD, diluted 1:100 in fresh YPD and grown for 4 hours at 30°C. Cells were then washed once with water, resuspended in PBS, counted and incubated with the macrophages at a 1:1 ratio. The co-cultures were incubated at 37°C for 1 hour, and fixed with 4% paraformaldehyde. Fixed cells were stored at 4°C. Prior to imaging cells were washed with PBS twice and stained with 35 μg/ml calcofluor white for 30 seconds prior to imaging.

**Gene expression following exposure to ROS**

*ARG3-GFP* cells grown overnight in YPD were diluted 1:100 in fresh YPD and grown for 4 hours at 30°C. Cells were washed once with water and resuspended in PBS. 1 × 10^6 cells were inoculated onto coverslips on 12-well plates containing 2 ml of RPMI media containing hydrogen peroxide (0-5.0 mM). Cells were incubated at 37°C for 1 hour. Coverslips containing the attached cells were used for microscopy.

For Northern analysis, *C. albicans* cells grown overnight in YPD were diluted in fresh YPD to an O.D600 of 0.25 and grown to an O.D600 of 1.0. Cells were exposed to the indicated concentration of hydrogen peroxide, menadione, or tert-butyl hydroperoxide, washed and transferred to YNB media for 15 minutes at 30°C. RNA was isolated from each sample using the hot acidic phenol method (5). A total of 10 ng of RNA was loaded and run on a 1% MOPS/formaldehyde agarose gel, transferred to nylon membranes, and hybridized with randomly labeled probes specific to each gene, using standard protocols (5). Blots were detected
Growth assays under oxidative stress conditions

For assays on solid media, indicated strains were grown overnight in YPD, washed once with sterile water, diluted to an OD₆₀₀ of 0.1, and serially diluted 5-fold in 96-well plates. Cells were transferred to YPD plates containing the indicated concentrations of either H₂O₂ or menadione using a multi-pin replicating tool, and incubated at 30°C for up to 3 days. To assess toxicity to acute exposures to H₂O₂, indicated strains were grown overnight in YPD, washed once with sterile water, diluted to an OD₆₀₀ of 0.1 in fresh YPD containing 0-20mM H₂O₂ and cultured at 30°C for 1 hr. Cells were diluted and plated onto YPD plates, and colony forming units (CFUs) were determined after 24hr growth at 30°C.

Isolation of bone marrow-derived macrophages

BMDMs were isolated as described (42); briefly leg bones from sacrificed ICR mice were collected and bone marrow flushed with IMDM + 10%FBS + Pen/Strep using a 27-gauge needle and syringe. The suspension was centrifuged at 1000rpm for 5min, supernatant removed, and cell pellet resuspended in ACK buffer for 5min at room temperature to lyse red blood cells. Following centrifugation and removal of the supernatant as described above, the cell pellet was resuspended in PBS and loaded into a 1mL syringe containing a 27-gauge needle. These cells were flushed directly into 25mL IMDM + 10%FBS + Pen/Strep + 10ng/mL mouse GM-CSF, resuspended thoroughly and distributed evenly across each well of a 6-well plate. Cells were incubated at 37°C/5%CO₂ for 7 days with supplementation of fresh media containing 10ng/mL GM-CSF every second day. Following the 7 day incubation, cells were harvested, counted, and replated in the absence of GM-CSF for 24 hours before initiating the co-culture as described.
Results

Construction and validation of ARG1-GFP and ARG3-GFP reporter strains

Microarray analysis of transcriptional changes following phagocytosis of C. albicans by mammalian macrophages indicated a unique regulation of genes of the arginine biosynthetic pathway: seven of the eight genes were induced, while no other amino acid biosynthetic genes were differentially regulated (the sole uninduced gene, ARG2, is regulated largely post-translationally in S. cerevisiae; ref. (51). Moreover, hierarchical clustering grouped the ARG pathway with stress response genes and not with the large set of genes responsive to carbon starvation (28). To confirm the induction of the ARG genes upon phagocytosis, transcriptional reporters were constructed in which the predicted 5' promoter regions of ARG1 and ARG3 were fused to GFP. These two genes were chosen as they both showed the highest fold induction upon phagocytosis according to the microarray data (34.3- and 27.4-fold respectively). These single-cell reporters were initially tested in vitro and, as expected ARG3-GFP cells grown in media lacking amino acids (YNB) were strongly fluorescent, while the cells grown in complete media (YPD) were not fluorescent (Fig. 1A). Using Northern blotting, we confirmed that GFP fluorescence from these reporters is representative of endogenous gene expression (data not shown).

These reporter strains were engineered to constitutively co-express a second fluorescent marker, yCherry, integrated into the ADH1 locus to allow normalization and quantitation of the GFP signal. The background subtracted fluorescence intensity was calculated for both reporters and converted into a ratio of green:red (11). As expected, we saw very strong fluorescence from the ACT1-GFP control, and thus a high GFP:yCherry ratio (Fig. 1B). Expression of both the
ARG1-GFP and ARG3-GFP constructs was dependent on the presence of arginine, with much higher ratios in arginine-deficient minimal YNB medium than in rich medium (Fig. 1B). The fluorescence ratios can be used to calculate fold-induction and the absence of arginine induces the ARG1 and ARG3 promoters 48- and 122-fold, respectively, while the controls show little difference between conditions.

Our initial ARG1-GFP reporter strain was not fluorescent in any conditions as a result of an apparent annotation error for this gene: the originally assigned ATG codon appends 77 amino acids to the amino terminus of the protein that are not homologous to the amino termini of Arg1p homologs of closely related species (Fig. S1). An ARG1-GFP reporter constructed using a second in frame ATG codon 231 nt downstream was fluorescent in the absence of arginine (Figs. 1 and S1). Subsequent 5′-RACE analysis identified the primary transcriptional start site to be 187 bp 3′ to the annotated start codon (and 44 bp 5′ to the second ATG codon; Fig. S1). Thus, translation of Arg1p likely begins at the downstream start codon.

To determine whether the promoters are induced by arginine deficiency specifically or by a more general amino acid starvation, reporter strains were grown in YNB supplemented with L-arginine (0-200µg/ml), L-lysine, or L-leucine. Cells grown in YNB supplemented with >100µg/ml arginine were not fluorescent (Fig. S2). Conversely, ARG1 and ARG3 were highly expressed in cells grown in YNB supplemented with 200µg/ml of either L-lysine or L-leucine, as assayed by both reporter fluorescence and Northern blotting. These results indicate that ARG1 and ARG3 respond specifically to arginine deprivation, and that general starvation for other amino acids does not induce ARG gene expression. As discussed below, this implies that these ARG genes lie outside the general amino acid control response in C. albicans.
**ARG genes are induced specifically in phagocytosed cells**

We next used our single cell reporters to test whether induction of the ARG genes is specific to phagocytosed *C. albicans* cells. After co-incubation of reporter strains with the macrophage cell line RAW264.7 for one hour, cells were fixed and stained with Calcofluor White (CW), a membrane-impermeant fluorescent compound that binds to chitin present in the cell wall of *C. albicans*, to discriminate between intracellular (CW negative) and extracellular (CW positive) fungal cells. As seen in Fig. 2A, phagocytosed ARG3-GFP reporter cells are substantially more likely to be fluorescent than extracellular ones, while the fluorescence of control reporter strains (*ACT1-GFP*, promoterless-GFP) were unchanged based on localization. A representative image from all three fluorescent channels is shown in Fig. 2B. Specific induction of the ARG3-GFP reporter in phagocytosed cells can also be seen in time-lapse movies (Supplemental Movie 1).

Fluorescence intensity was quantified as described above for cells that we inside macrophages, outside, or grown in media alone (Fig. 2C). This data shows that the GFP fluorescence from the ARG1-GFP or ARG3-GFP reporters, while lower than for the constitutive *ACT1-GFP*, is 1.8-2.0-fold higher in phagocytosed cells than in cells grown in media alone. A small induction is seen in extracellular *C. albicans* cells; for reasons described below, we believe that this results from the oxidative burst generating extracellular ROS. Thus we conclude that there is a modest but specific induction of ARG promoters following engulfment by macrophages.

To further examine ARG gene induction after phagocytosis, we turned to an in vivo model using zebrafish in which *C. albicans* cells can be visualized within macrophages in the context of a living animal (11). ARG3-GFP fluorescence intensity (measured as a ratio to a
constitutively expressed dTomato) was similar between intracellular and extracellular C. albicans at 2 hours post-infection (hpi), but GFP expression increased over time such that the ratio was significantly higher in intracellular cells at 4 and 24 hpi (Fig. S3). Thus, phagocytosis by macrophages induced ARG3 expression in this live zebrafish model.

Regulation of the ARG pathway by Gcn4p and ArgR

In S. cerevisiae, the ARG pathway is under complex and partially overlapping regulation by Gcn4p and the ArgR complex. Gcn4p is the central transcription factor of the general amino acid control response that, upon starvation for a single amino acid, upregulates a large number of genes for synthesis of multiple amino acids (reviewed in (23)). A functional homologue of ScGcn4p has been identified in C. albicans (46). To test if this general amino acid control pathway regulates the ARG genes in vitro or in vivo, we integrated the ARG3-GFP and ADH1-yCherry reporters into a gcn4Δ strain obtained from a knockout library constructed by Sanglard and colleagues (49). ARG3 was modestly derepressed in arginine-replete conditions (YPD) and further induced upon arginine starvation (YNB; Fig. 3A, left panels), although quantitation of the fluorescence ratios demonstrated that the maximal expression in the gcn4Δ mutant was only 42% that in the wild-type strain (Fig. 3B). Together, these two effects result in a 4.3-fold induction by arginine depletion in the gcn4Δ mutant versus 122-fold in the wild-type strain. Northern analysis confirmed the derepression in vitro, which was particularly notable for ARG4 and ARG5,6 (Fig. 3C). Derepression was more apparent in macrophage cocultures in which strong fluorescence was observed in cells regardless of location (Fig. 3A,D). With the higher basal level of expression, the induction in phagocytosed cells is lost (Fig. 3E). Thus, Gcn4p appears to primarily repress ARG gene expression, perhaps by recruiting ArgR, as suggested in yeast (15).
The second transcriptional regulator, ArgR, is in yeast a complex of three DNA binding proteins, Mcm1p, Arg80p, and Arg81p, and an inositol phosphate kinase, Arg82p (10, 18, 31, 41). *C. albicans* has close homologs of Mcm1p, Arg81p and Arg82p (called Ipk2p), but not Arg80p, which in yeast is both adjacent and highly homologous to Mcm1p, presumably the product of a tandem duplication. Instead, *C. albicans* has a second protein homologous to Arg81p, annotated as Arg83p. We identified arg81Δ and arg83Δ deletion strains in mutant libraries (49) and transformed both using the ARG3-GFP and ADH1-yCherry reporters. This reporter was strongly derepressed in the arg81Δ strain in the presence of arginine, and was not further induced by arginine depletion or by phagocytosis (Fig. 3A-E). In contrast, the arg83Δ mutant showed normal repression of ARG3 in the presence of arginine, and an induction in its absence both in vitro and in vivo.

**ARG genes are induced by reactive oxygen species**

Why might phagocytosed cells specifically and uniquely upregulate the ARG pathway? We considered that the phagolysosome might be deficient in arginine; while it seemed unlikely that this environment would be devoid of one specific amino acid, arginine is the substrate for the inducible nitric oxide synthase (iNOS or NOS2), which generates NO within the phagolysosome. RPMI contains 300 µg/ml and supplementation up to 1 mg/ml did not affect ARG gene expression in phagocytosed cells (data not shown). Next, we considered that the ARG pathway is induced to generate polyamines, which have been shown be required for the *C. albicans* yeast-to-hyphal switch (22, 48), but the gene encoding the rate-limiting step of polyamine biosynthesis, ornithine decarboxylase (*SPE1*), is not induced by phagocytosis (28). An alternative possibility was suggested by a recent RNA-seq analysis of the *C. albicans*
transcriptome in several host-relevant conditions by Bruno, et al. (12). The ARG pathway is induced by moderate concentrations of hydrogen peroxide, which mimics the reactive burst of macrophages.

Northern analysis was initially used to assess whether the ARG pathway responds to ROS in vitro. The ARG1 message is expressed in arginine-replete conditions containing moderate concentrations (0.3-1.0 mM) of hydrogen peroxide but expression is not detected at higher concentrations (>2 mM; Fig. 4A); this is consistent with the RNA-seq analysis by Bruno, et al. (12). ARG1 is also induced by concentrations of tert-butyl hydroperoxide (t-BOOH; Fig. 4B) and menadione (data not shown). In each case, the mRNA abundance for the gene encoding catalase (CAT1) continues to increase with the ROS concentration, indicating that the lack of ARG1 expression is not due to cell death from toxic ROS levels. These results were confirmed using the ARG3-GFP strain, which again showed induction only at moderate concentrations of ROS (Fig. 4C).

Because mRNA levels were increased for both ARG1 and ARG3 following H2O2 exposure, arginine biosynthesis might be necessary to counter the effects of prolonged exposures to oxidative stress. To test this possibility, we generated strains lacking either ARG1 or ARG3, along with complemented controls (50). As expected, these strains are arginine auxotrophs (Fig. S4). We plated serial dilutions of arg1Δ and arg3Δ cells on plates containing increasing concentrations of either H2O2 or the superoxide anion (O2·-) generating compound menadione and allowed cells to grow for up to 3 days using the known ROS-sensitive hog1Δ mutant as a control (45) and found no alterations in growth relative to the wild-type control at any H2O2 concentration (Fig S5). arg1Δ and arg3Δ mutants also did not increase the sensitivity to acute ROS toxicity as measured by the decrease in colony forming units after short-term exposure to...
higher concentrations of ROS (up to 20 mM, data not shown). Thus, there are no obvious in vitro ROS-related phenotypes for disruption of the ARG pathway.

During co-culture of C. albicans with macrophages, cells respond to one or more as-yet-unknown hyphal inducing signals to initiate germ tube formation, eventually rupturing the phagocyte. Arginine is catabolized into urea and ornithine by the cytosolic arginase Car1p and urea is further degraded into CO₂ and NH₄⁺ by the urea amidolyase Dur1,2p. Others have proposed that this CO₂ is a trigger for hyphal formation and shown that both dur1,2Δ and arg4Δ mutations reduce hyphal growth in the macrophage (21). Similarly, the argIΔ and arg3Δ mutants germinated more slowly than controls within the macrophage and generated shorter germ tubes. Cellular morphology of phagocytosed cells was quantified by counting and nearly twice as many cells remained in the yeast form after two hours (26% for the wild-type versus 48% for argIΔ and 43% for arg3Δ; Fig. 5). Hyphal elongation was also delayed, with more cells remaining in the germ tube phase in the mutants. Nevertheless, at longer timepoints there was no significant difference in fungal-induced macrophage damage, as assayed by lactate dehydrogenase release (data not shown). We tested the mutants in a mouse model of systemic candidiasis and found no alterations in virulence for either strain, as has been reported for arg4Δ mutants (36). Thus, despite the induction of these genes, disruption of the arginine biosynthetic pathway confers only minor alterations of the host-pathogen interaction.

ARG induction in macrophages is at least partly ROS-dependent

To test whether ARG induction is due to phagocyte-derived ROS, we turned to primary bone marrow-derived macrophages (BMDMs). Both the ARG1-GFP and ARG3-GFP are induced in cells phagocytosed by BMDMs isolated from wild-type mice relative to media-grown
cells. In contrast to the cultured macrophages, however, there was a less significant difference between phagocytosed and non-phagocytosed cells. The magnitude of the ROS burst from these primary cells should be greater than cultured cells, so it is possible that even external cells are exposed to a greater oxidative stress than in our earlier experiments.

We next co-cultured our *C. albicans* reporter strains with BMDMs isolated from mutant mice lacking the gp91phox subunit of the phagocyte oxidase responsible for generating ROS in the macrophage (38). Though the overall phagocytic activity of these cells was similar to wild-type macrophages, the ARG1-GFP and ARG3-GFP reporters were induced just slightly in cells phagocytosed by the gp91phox-deficient macrophages (Figs. 6, 7). We noted a weak autofluorescence in some gp91phox-deficient macrophages that may account for some of this apparent induction. Thus, induction of the ARG genes results primarily from exposure of cells to ROS generated by the phagocyte’s oxidative burst.

**Discussion**

We demonstrate in this work that the genes encoding the arginine biosynthetic pathway are induced specifically in cells phagocytosed by either primary or cultured macrophages and that this induction results from exposure to moderate concentrations of phagocyte-derived ROS. Induction is dependent on the NADPH-dependent phagocyte oxidase, as macrophages lacking the gp91phox subunit do not activate the ARG1-GFP and ARG3-GFP reporter constructs. In vitro, the ARG genes are also induced by ROS at a narrow range of concentrations, which agrees with RNA-seq analysis (12). As expected, they are also strongly expressed when arginine is depleted in vitro.
An obvious alternative hypothesis to explain ARG gene induction in phagocytosed cells is that the phagolysosome is devoid of arginine. However, since arginine is unique among the amino acid synthesis pathways, this would imply the phagolysosome was replete in at least most of the other 19 amino acids and this seems unlikely, though it is possible that consumption of arginine as the substrate for iNOS (NOS2)-dependent generation of NO could lead to such an asymmetry. The data from the gp91phox macrophages (which still express a functional iNOS), in which the ARG1-GFP and ARG3-GFP reporters are not significantly induced make this unlikely.

Mutation of ARG1 or ARG3 impairs hyphal morphogenesis in phagocytosed cells, but does not obviously alter sensitivity to ROS in vitro and does not affect systemic virulence, as is also the case with an arg4Δ mutant (36). Why, then, does this pathway respond to ROS? One possibility is that increased arginine biosynthesis protects against ROS. In mammals arginine has been indirectly linked to increased resistance to ROS by enhancing generation of NO, which then induces antioxidant defenses. Fungi lack NOS-like enzymes, however, so this would not seem to be a likely mechanism. Takagi and colleagues (34) identified a yeast N-acetyltransferase, Mpr1p, whose substrates are intermediates of the proline and arginine pathways and contributes to resistance to endogenously generated ROS. Mpr1p, which is missing from the S288c genome but found in other strains of *S. cerevisiae* (i.e., Σ1278b) and in other fungi including *C. albicans*, facilitates arginine production from proline intermediates, but the mechanism by which this is protective is unknown.

Alternatively, *C. albicans* cells may use moderate concentrations of ROS as a signal to activate anti-phagocyte mechanisms, for instance to promote hyphal growth inside the macrophage. Indeed, moderate ROS concentrations stimulate hyphal growth (16, 32). A role for arginine in hyphal induction was proposed by Nickerson and colleagues (21), who showed that...
C. albicans strains unable to synthesize (arg4Δ) or completely degrade (dur1,2Δ) arginine displayed fewer filaments within macrophages, findings consistent with the present study.

Arginine is degraded by the arginase Car1p to generate ornithine and urea. Urea is subsequently degraded by the urea amidolyase Dur1,2p to generate ammonia and carbon dioxide, both of which are potent signals for hyphal morphogenesis (26, 50). Thus, both studies suggest that arginine synthesis within macrophages contributes to hyphal induction. The dur1,2Δ mutant was recently shown to be attenuated in a whole animal model (33), in contrast to arg1Δ, arg3Δ, and arg4Δ mutants (this study and ref. 36), which would indicate that arginine is not limiting in vivo, but its degradation does contribute to pathogenesis. In vitro, in macrophage-like conditions (amino acid-rich and glucose-poor), C. albicans cells rapidly neutralize acidic conditions. CAR1 is upregulated during this process, which is significantly slowed in a dur1,2Δ mutant (50), linking this phenomenon with arginine metabolism.

The pattern of expression in response to ROS is atypical: rather than increasing expression with increasing stress, the ARG genes are expressed only in a narrow range of sublethal concentrations, 0.3-1.0 mM H2O2. Inhibition of expression at higher concentrations cannot be from general toxicity of the ROS, because catalase (CAT1) expression remains very strong up to 5 mM.

In S. cerevisiae, the expression of at least some of the ARG genes is regulated by a complex called ArgR consisting of Mcm1p, Arg80p, Arg81p, and Arg82p (reviewed in (23). The first three of these are DNA binding proteins, with Arg80p a close homolog of the more general regulator Mcm1p. Gcn4p, which generally serves as an activator of transcription of other amino acid genes, recruits ArgR to ARG promoters, where it serves as a repressor in arginine replete conditions (53). This appears to be conserved in C. albicans with one significant
difference: the presence of arginine alone represses ARG gene transcription, even in the absence of other amino acids. Thus, despite the involvement of Gcn4p, the ARG pathway is largely outside the general control response system. *C. albicans* has a homolog of Mcm1p, which localizes to ARG promoters (27), though it lacks Arg80p, which appears to have been arisen in *S. cerevisiae* through a tandem duplication of Mcm1p. *C. albicans* has two Arg81p homologs, Arg81p, mutation of which derepresses expression in the presence of arginine, and Arg83p, for which we have not found a phenotype. Further study will be required to understand the function of the ArgR complex in response to different inputs in *C. albicans*.

Finally, the induction of the ARG genes may be the byproduct of an expanded role for ArgR or Mcm1p. Recently, an arg81Δ mutant was shown to be amongst the most defective mutants tested in a model of biofilm attachment (20). As there is no obvious connection between arginine biosynthesis and biofilm formation it is possible that ARG81 has acquired a new function in *C. albicans*. Rewiring transcription factor regulons has become a common theme in fungi, with proteins such as Gal4p, Rap1p, Tbf1p, and Mcm1p itself having different or additional functions in *C. albicans* than in *S. cerevisiae* (24, 30, 47). *C. albicans* Zap1p, a conserved regulator of zinc acquisition, has evolved a second function to control biofilm matrix production (35). A biofilm state is not conducive to escape from macrophages, so perhaps Arg81p is downregulated in such conditions, which would derepress ARG genes. Addressing this speculative proposal will require a more complete understanding of the regulons of the ArgR proteins.

**Acknowledgements**

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Recruitment of the ArgR/Mcm1p repressor is stimulated by the activator Gcn4p: a self-checking
**Figure Legends**

**Figure 1.** *ARG1* and *ARG3* promoters are induced in media lacking arginine. A) *ACT1p-GFP* (CJC26), promoterless GFP (CJC25), *ARG1p-GFP* (CJC28) and *ARG3p-GFP* (CJC29) strains were grown overnight in YPD and sub-cultured into fresh YPD or YNB for 4 hours at 30°C. Images shown are overlaid GFP and DIC pictures. “None” indicates a GFP reporter construct with no promoter. B) GFP fluorescence intensity was expressed as the average of the GFP:yCherry intensity ratios calculated from 50 cells per condition (YPD and YNB). Error bars represent the standard error. C) Fold inductions were calculated comparing each fluorescence intensity average to that shown by the same strain grown in YPD medium. Error bars represent the standard error.

**Figure 2.** *ARG1* and *ARG3* promoters are induced specifically in phagocytosed cells. A) *ACT1p-GFP* (CJC26), promoterless-GFP (CJC25; labeled as “none”), *ARG1p-GFP* (CJC28) and *ARG3p-GFP* (CJC29) were co-cultured for 1 hour with murine macrophages or grown in RPMI alone. Co-cultures were then fixed and stained with calcofluor white to distinguish intracellular cells before imaging. The images from left to right show calcofluor white, GFP fluorescence, and overlay of GFP and DIC pictures for both co-cultures and cells grown in RPMI media alone. B) Representative image of the *ARG3-GFP* reporter strain to show individually calcofluor white, GFP, and yCherry fluorescence pictures as well as a merged image of the three fluorophores. C) GFP fluorescence intensity was quantified as the average GFP:yCherry intensity ratio taken of 50 cells per condition (Media, External, and Internal) and expressed as the fold induction relative to strains grown in RPMI media. Error bars represent the standard error. *, P<0.05; **, P<0.001 (Student’s T-test).
Figure 3. Gcn4p and the ArgR complex regulate the ARG genes. A) Wild type (CJC29),
gen4Δ (CJC35), arg81Δ (CJC32), and arg83Δ (CJC33) C. albicans cells transformed with both
the ARG3p-GFP and ADH1p-yCherry reporter were grown in YPD or YNB media for four hours
at 30°C, co-cultured for 1 hour with murine macrophages, or grown in RPMI media alone. The
two columns on the left show GFP-DIC overlaid pictures for the in vitro experiment (YPD vs.
YNB). For the co-cultures, the images show calcofluor white fluorescence of extracellular C.
albicans cells and GFP fluorescence, individually. Overlay of GFP and DIC images are shown
for both co-cultures and cells grown in RPMI media only. B) GFP fluorescence intensity was
expressed as the average of the GFP:yCherry intensity ratio taken from 50 cells per condition
grown in vitro in arginine replete (YPD) or deficient (YNB) conditions. Error bars represent the
standard error. C) Wild type (SC5314), gen4Δ (DSY3233), arg81Δ (HZY28), and arg83Δ
(DSY3426-2) C. albicans cells were grown in YPD to an O.D600 of 1.0, washed and grown in
either YPD or YNB for 15 minutes. RNA was collected from each sample for northern blot
analysis with probes for the indicated genes. D) The GFP:yCherry ratio was calculated from
ARG3p-GFP-expressing cells grown in media alone or in cells either extracellular or intracellular
in macrophage co-cultures. At least 50 cells were counted for each localization. E) The fold-
induction, relative to media alone for each strain, is depicted for the data in panel D.

Figure 4. ARG1 and ARG3 are induced by reactive oxygen species. A) Wild type (SC5314)
C. albicans cells were grown in YPD to an O.D600 of 1.0, and transferred to YNB or YPD
supplemented with the indicated concentration of H2O2 for 15 minutes. RNA was collected from
each sample and Northern blots were probed for ARG1, CAT1 and ACT1. RNA from an arg1Δ
strain grown in YNB was used as a control for the ARG1 probe specificity. B) Wild type C.
*albicans* (SC5314) cells were grown in YPD to an O.D₆₀₀ of 1.0, and transferred to YNB or to YPD supplemented with the indicated concentration of tert-butyl hydroperoxide for 15 minutes and Northern analysis was performed as in panel (A). C) *ARG3p*-GFP (CJC5) cells were grown in RPMI supplemented with different concentrations of H₂O₂ for one hour. The images shown are GFP-DIC overlays. The numbers on each image represent the concentration of H₂O₂ in mM for each sample. “C/M” is a one-hour co-culture with macrophages as a comparison.

**Figure 5. Mutants in ARG genes have abnormal germ-tube dynamics following macrophage phagocytosis.** A) Wild-type (RGC1), *arg1Δ* (JRC47), and *arg3Δ* (JRC42) strains, each expressing an *ACT1*-GFP construct were co-cultured with RAW264.7 macrophages (1:1 Candida:macrophage ratio) for 2 hr prior to chemical fixation with paraformaldehyde. To distinguish fungal cells that had been phagocytosed from extracellular fungi, fixed co-cultures were stained with calcofluor white prior to imaging. White arrows highlight *arg* mutants that have not initiated germ-tube formation. White arrowheads highlight *arg* mutants containing very short filaments. B) Quantitation of cellular morphologies observed for phagocytosed GFP-SC5314, GFP-*arg1ΔΔ*, and GFP-*arg3ΔΔ*. For each strain, the morphologies of at least 150 phagocytosed cells were determined. The Fisher’s exact test (two-tailed) was used to compare the distribution of yeast, germtube, and hyphal morphologies between GFP-SC5314 and GFP-*arg* strains (p < 0.0001 for comparisons between SC5314 and each *arg* mutant).

**Figure 6. Induction of ARG genes is partially dependent on ROS.** *ACT1p*-GFP (CJC26), promoterless GFP (CJC25), *ARG1p*-GFP (CJC28) and *ARG3p*-GFP (CJC29) cells were co-cultured with BMDMs from A) wild type mice or B) gp91phox⁻/⁻ mice for 1 hour. As a control,
cells were also grown in RPMI media alone. Images show calcofluor white, GFP, and GFP-DIC overlay images for both the co-cultures and media alone.

Figure 7. Quantification of ARG genes induction after phagocytosis by wild type and gp91phox-/- BMDM. GFP fluorescence intensity was calculated as the average of the GFP:yCherry intensity ratio taken from 50 cells per condition. The GFP fold induction was calculated based on the average GFP:yCherry ratio (n=50 cells) for both intracellular and extracellular cells, relative to media controls, in A) WT BMDM and B) gp91phox-/- BMDM. Error bars represent the standard error. *, P<0.01; ns, not significant (p>0.05); Student’s T-test.
### A.

C. albicans / Macrophage co-cultures

<table>
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<tr>
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<th>Calcofluor white</th>
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### B.

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### C.

![Bar graph showing fold induction for ACT1p, none, and ARG1p](image_url)

- Media
- External
- Internal

- **p < 0.05
- *p < 0.01
### Table A

<table>
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<tr>
<th></th>
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| WT       | ![Image](YPD_WT)
| gcnaΔ   | ![Image](YPD_gcn4Δ)
| arg81Δ  | ![Image](YPD_arg81Δ)
| arg83Δ  | ![Image](YPD_arg83Δ) |

### Graph B

![GFP:YCherry Ratio](graph)

### Graph C

![RNA gel](gel)

### Graph D

![GFP:YCherry Ratio](graph)

### Graph E

![Fold Induction](graph)
### A. C. albicans / WT BMDM co-cultures vs. RPMI

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### B. C. albicans / gp91 phox−/− BMDM co-cultures vs. RPMI

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