ATG Genes Influence the Virulence of *Cryptococcus neoformans* through Contributions beyond Core Autophagy Functions

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ABSTRACT The process of autophagy is conserved among all eukaryotes from yeast to humans and is mainly responsible for bulk degradation of cellular contents and nutrient recycling during starvation. Autophagy has been suggested to play a role in the pathogenesis of the opportunistic human fungal pathogen *Cryptococcus neoformans*, potentially through a contribution to the export of virulence factors. In this study, we showed that deletion of each of the *ATG1*, *ATG7*, *ATG8*, and *ATG9* genes in *C. neoformans* leads to autophagy-related phenotypes, including impaired amino acid homeostasis under nitrogen starvation. In addition, the *atgΔ* mutants were hypersensitive to inhibition of the ubiquitin-proteasome system, a finding consistent with a role in amino acid homeostasis. Although each *atgΔ* mutant was not markedly impaired in virulence factor production *in vitro*, we found that all four *ATG* genes contribute to *C. neoformans* virulence in a murine inhalation model of cryptococcosis. Interestingly, these mutants displayed significant differences in their ability to promote disease development. A more detailed investigation of virulence for the *atg1Δ* and *atg8Δ* mutants revealed that both strains stimulated an exaggerated host immune response, which, in turn, contributed to disease severity. Overall, our results suggest that different *ATG* genes are involved in nonautophagic functions and contribute to *C. neoformans* virulence beyond their core functions in autophagy.

KEYWORDS cryptococcosis, amino acid homeostasis, fungal pathogenesis, medical mycology, mouse model

Cryptococcus neoformans is an opportunistic human fungal pathogen that causes almost 300,000 infections and ~200,000 deaths per year globally, predominately in the HIV/AIDS population in Sub-Saharan Africa (1, 2). Cryptococcal infections generally start with inhalation of yeast cells or spores that colonize the lung to cause pulmonary infection. Subsequent dissemination can occur to systemic organs, including a predilection of the fungus to cross the blood-brain barrier and cause life-threatening meningitis. The ability of *C. neoformans* to cause disease depends to a large extent on three major virulence factors, including production of a polysaccharide capsule, deposition of the cell wall-associated pigment melanin, and ability to proliferate at the mammalian body temperature of 37°C (3). The *C. neoformans* capsule is composed of two polysaccharides, glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal), with immunosuppressive properties such as activation of the alternative complement pathway, depletion of complement, inhibition of phagocytosis, induction of immune unresponsiveness, and inhibition of neutrophil migration (4–6). Cell wall-associated melanin protects cryptococcal cells from phagocytosis and oxidative killing by macrophages (7–9).

The export of virulence-related factors, such as capsule polysaccharide and melanin, to the cell surface is poorly understood but may involve unconventional and conven-

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tional secretion mechanisms. Autophagy could potentially contribute to unconventional secretion through a process termed exophagy (10, 11). In general, autophagy is a process conserved among eukaryotic organisms from yeasts to humans, and the main functions of autophagy are to maintain cellular homeostasis through nutrient and organelle recycling during starvation. There are different types of autophagy, including primarily nonselective macroautophagy (referred as autophagy here) and selective autophagy such as mitophagy (targeting mitochondria), pexophagy (targeting peroxisomes), and xenophagy (targeting intracellular bacteria and viruses). The steps in autophagy include induction, nucleation of the phagophore to engulf cargo, expansion of the phagophore, completion of the autophagosome, docking and fusion with the vacuole, degradation of the cargo, and recycling of macromolecules (12). The biogenesis of the autophagosome is central to all types of autophagy processes (12). The Atg1 complex (including Atg1, Atg13, and the Atg17-Atg31-Atg29 scaffolding subcomplex) is required for the induction of autophagy at the phagophore assembly site (PAS), a single perivacuolar site that is proximal to the vacuole. Atg9 is the only transmembrane protein in the core autophagic machinery and has a proposed function in membrane delivery to the expanding phagophore. Atg9 is located at the PAS, the endoplasmic reticulum (ER), the Golgi complex, and peripheral structures proximal to the mitochondrial reticulum. There are two conjugation systems required for phagophore expansion involving the Atg8 ubiquitin-like protein (and Atg3, Atg4, and Atg7) and the Atg12 ubiquitin-like protein (and Atg5, Atg7, Atg10, and Atg16). Atg8 also plays a role in cargo recruitment (13). Notably, both the Atg8 and the Atg12 conjugation systems require Atg7, an E1-like ubiquitin activation enzyme, for activation (12). For exophagy, the autophagosome is thought to dock with endosomes and/or multivesicular bodies for eventual fusion with the plasma membrane to deliver materials outside the cell (10, 11).

The importance of autophagy in host cells during *C. neoformans* infection has been demonstrated in *Drosophila* S2 cells (14), murine macrophage-like J774.16 cells (15), murine bone marrow derived macrophages, and *in vivo* in C57BL/6 mice (15). On the other hand, direct and indirect evidence suggested that autophagy also plays a role in virulence of *C. neoformans*. For example, a transcriptome study showed that the putative *C. neoformans* autophagy genes *ATG3* and *ATG9* are upregulated after engulfment by murine macrophages (16), thus hinting at their involvement in pathogenesis. Another study demonstrated that deletion of the *C. neoformans* gene *VPS34*, encoding a phosphatidylinositol 3-kinase (PI3K) homologue that functions as an upstream signaling protein for autophagy, led to an autophagy defect and attenuated virulence in mouse models of cryptococcal disease (17). In the same study, an *ATG8* RNA interference (RNAi) knockdown strain of *C. neoformans* showed attenuated virulence in both intranasal and intravenous mouse infection models (17). Furthermore, the *ATG7* gene has recently been shown to play a role in *C. neoformans* virulence (18).

While these studies provided insights into the contributions of the *ATG7* and *ATG8* autophagy genes to *C. neoformans* virulence, we were interested in further defining the roles of different steps in autophagy in nutritional aspects of amino acid homeostasis, in the secretion of virulence factors, and in virulence in mice. We therefore examined the functions of four *ATG* genes representing steps in induction (*ATG1*), nucleation of the phagophore (*ATG9*), and expansion of the phagophore (*ATG7* and *ATG8*). *ATG1* and *ATG9* have not been studied before in *C. neoformans* to our knowledge, and we included the two previously studied genes, *ATG7* and *ATG8*, for comparison and more detailed analyses. We found that each of the four *ATG* genes was required for autophagy-related phenotypes. Surprisingly, when tested in a murine inhalation model of cryptococcosis, *atgΔ* mutants displayed different levels of virulence and caused different disease progression profiles in infected mice. These results suggest that *C. neoformans* virulence may not be completely dependent on core autophagy functions. In support of this idea, we found that *ATG1*, *ATG7*, *ATG8*, and *ATG9* each make different contributions to the virulence of *C. neoformans*.

TABLE 1 Identification of orthologues of *ATG* genes in *C. neoformans*

<i>C. neoformans</i>				<i>S. cerevisiae</i>			
<i>ATG</i> gene	H99 gene	Protein encoded	Reference	protein homologue	E value	% identity	% similarity
<i>ATG1</i>	CNAG_05005	ULK protein kinase	19	YGL180W	1.00E−85	46	61
<i>ATG7</i>	CNAG_04538	Ubiquitin-like modifier acting enzyme E1	18	YHR171W	2.00E−140	39	57
<i>ATG8</i>	CNAG_00816	Ubiquitin-like protein	17	YBL078C	7.00E−66	78	90
<i>ATG9</i>	CNAG_01445	Integral membrane protein	16	YDL149W	4.00E−158	39	60

RESULTS

The *ATG1*, *ATG7*, *ATG8*, and *ATG9* genes are required for autophagy in *C. neoformans*. Initially, we identified orthologues of the *ATG1*, *ATG7*, *ATG8*, and *ATG9* genes from the H99 strain of *Cryptococcus neoformans* var. *grubii* by BLASTp with the proteins from *Saccharomyces cerevisiae* S288c as queries (Table 1). Each candidate *ATG* gene was then tested by reciprocal BLASTp against the *S. cerevisiae* S288c genome to ensure that the most similar sequence was that of the *S. cerevisiae* inquiry gene. The *ATG1*, *ATG7*, and *ATG8* genes from *C. neoformans* were also previously identified by other groups (17–19). Single gene deletion mutants were constructed in *C. neoformans* var. *grubii* strain H99 (see Fig. S1 in the supplemental material).

Each single deletion mutant (*atg1Δ*, *atg7Δ*, *atg8Δ*, and *atg9Δ*) showed the expected autophagy phenotype of impaired survival upon nitrogen starvation (Fig. 1A to D). That is, the wild-type (WT) strain was able to undergo 1 or 2 rounds of replication after

FIG 1 Autophagy genes are required for survival during nitrogen starvation by *C. neoformans*. The WT strain H99, *atgΔ* mutants, and complementation strains (*atgΔ::ATG*) were transferred from rich YPD medium to minimal medium without a nitrogen source (MM-N). CFU were measured every 48 h and presented as relative CFU compared to that at time zero. (A to D) Representative starvation survival curves for the *atg1Δ*, *atg7Δ*, *atg8Δ*, and *atg9Δ* mutants, respectively. (E) Survival rate of each strain at 240 h under the conditions used for panels A to D. (F) Percentage of cells containing autophagic body-like cells inside the vacuole after 3 h of incubation in MM-N with PMSF and nocodazole. Experiments were repeated three times independently ($n = 3$). Error bars represent SEMs. Asterisks above each column depict statistical significance compared to the WT (H99) ($P < 0.05$, unpaired *t* test).

transfer from a nutrient-rich medium (YPD; see Materials and Methods for a description) to a nitrogen-free medium (YNB-N; see Materials and Methods) and remained viable for a prolonged period (>240 h). In contrast, less than 10% of the cells for the *atg1Δ*, *atg7Δ*, *atg8Δ*, and *atg9Δ* mutants were viable at 240 h after transfer to YNB-N (Fig. 1E). The survival of the mutants during starvation was restored to the WT level when each gene was reintroduced into the respective mutants either at the native locus (for *atg7Δ*) or at the genomic safe haven locus (for *atg1Δ*, *atg8Δ*, and *atg9Δ*) (20) (Fig. 1). Additionally, we observed that upon nitrogen starvation (in MM-N; see Materials and Methods) for 3 h in the presence of phenylmethylsulfonyl fluoride (PMSF) and nocodazole, $40.4\% \pm 3.0\%$ (mean $\pm$ standard error of the mean [SEM]) of the cells of the WT strain had accumulated autophagic body (AB)-like vesicles in the vacuole. In contrast, less than 4% of the cells of the *atg1Δ*, *atg7Δ*, *atg8Δ*, and *atg9Δ* mutants had accumulated AB-like vesicles (Fig. 1F; see also Fig. S2), and the level was significantly less than found for the WT strain (unpaired *t* test, $P < 0.05$; $n = 3$). The *atg1Δ::ATG1*, *atg7Δ::ATG7*, *atg8Δ::ATG8*, and *atg9Δ::ATG9* complementation strains had $28.1\% \pm 1.3\%$, $30.9\% \pm 4.5\%$, $22.2\% \pm 2.1\%$, and $19.0\% \pm 2.0\%$ of cells accumulating AB-like vesicles inside vacuoles, respectively, and these levels were significantly greater than for each respective mutant (unpaired *t* test, $P < 0.05$; $n = 3$). We did note that the levels for the complementation strains were still less than for the WT (unpaired *t* test, $P < 0.05$; $n = 3$), suggesting partial complementation.

The *atgΔ* deletion mutants are impaired in amino acid homeostasis. We extended our analysis of the sensitivity of the *atgΔ* mutants to starvation by examining the response to amino acids as a source of nitrogen. We chose the *atg8Δ* mutant as the representative for the other *atgΔ* mutants (Fig. 2A), and phenotypic differences observed with the *atg8Δ* mutant were confirmed for the other *atgΔ* mutants in comparison with the WT and complementation strains (Fig. S3). We first examined the ability of the strains to use individual L-amino acids as sole nitrogen sources by monitoring the growth of the WT strain and the *atg8Δ* mutant in 96-well plates (Fig. 2A). Consistent with the results of a recent study (21), we found that asparagine, arginine, glutamine, glutamate, alanine, proline, aspartate, and serine each supported robust proliferation of both the WT and *atg8Δ* strains equally well, allowing growth to reach stationary phase within 48 h. Hence, these are defined as “preferred” amino acids. In media with glycine, the *atg8Δ* mutant grew as well as the WT but reached stationary phase earlier and with a lower final cell density. In media with leucine, tryptophan, lysine, or phenylalanine, the *atg8Δ* mutant had a longer lag phase than the WT strain, but it had a growth rate similar to that of the WT in the exponential phase of growth (Fig. 2A). The same phenotypes were observed with the *atg1Δ*, *atg7Δ*, and *atg9Δ* mutants (Fig. S3). The WT strain grew moderately well in media with isoleucine, threonine, or valine, whereas the *atg8Δ* mutant had a longer lag phase and a notably lower growth rate. The *atg1Δ*, *atg7Δ*, and *atg9Δ* mutants displayed growth patterns similar to those of the *atg8Δ* mutant with these amino acids. In addition, the complementation strains showed the same patterns of growth as the WT strain in media with isoleucine, threonine, or valine (Fig. S3). Methionine supported robust growth for the WT strain but not for the *atg8Δ* mutant after 72 h of incubation (Fig. 2A). Extended incubation (120 h) showed that the *atg7Δ*, *atg8Δ*, and *atg9Δ* mutants were able to utilize methionine after a long lag phase, whereas the *atg1Δ* mutant had little growth (Fig. S3). Cysteine and histidine poorly supported the growth of the WT strain, and growth was even worse for the *atgΔ* mutants on these amino acids (Fig. 2A; see also Fig. S3). Overall, our studies revealed that autophagy affects the ability of *C. neoformans* to use “nonpreferred” amino acids as sole nitrogen sources, including glycine, phenylalanine, leucine, isoleucine, tryptophan, valine, threonine, and methionine. The influence of autophagy occurred at two levels: (i) the *atgΔ* mutants showed a longer lag phase than the WT before entering exponential growth, and (ii) the growth rate of the *atgΔ* mutants was lower during the exponential phase of growth.

FIG 2 Autophagy and the ubiquitin proteasome pathway are required for amino acid homeostasis in *C. neoformans*. For all experiments, *C. neoformans* cells were transferred from an overnight YPD culture into MM with or without supplementary amino acids or drugs at 10^6 CFU/ml and incubated for 48 h at 30°C. (A) A survey of WT and *atg8Δ* strains for the use of different L-amino acids as sole nitrogen sources in MM. Growth was monitored in 96-well plates. (B) *atgΔ* mutants are supersensitive to the proteasome inhibitor bortezomib (BTZ; 50 μ g/ml). Data are presented as the fold change in CFU per milliliter at 48 h versus the initial count. Each experiment was performed at least three times ($n = 3$) independently. Error bars represent SEMs. Asterisks show statistical significance (unpaired *t* test) between columns: **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. ns, not significant.

We also observed that the WT and complementation strains produced a pink water-soluble pigment on tryptophan; this pigment was not observed for the *atgΔ* mutants after 48 h of incubation (Fig. S4). The absorbance spectra of the culture supernatant matched the spectrum of tryptophol, a tryptophan-derived pigment previously reported as a quorum-sensing molecule in *S. cerevisiae*, *C. neoformans*, and *Candida glabrata*, with an absorbance maximum at 535 nm (Fig. S4) (22–24). Further examination revealed that *C. neoformans* produced tryptophol only in the stationary phase of growth (Fig. S4). That is, the WT and complementation strains were in the stationary phase at 48 h and when tryptophol was produced. The growth of the *atgΔ* mutants on tryptophan was slower than that of the WT and complementation strains (Fig. 2A, S3, and S4); the *atgΔ* mutants were just approaching stationary phase at 48 h and had not produced tryptophol. Extended incubation in MM plus tryptophan allowed all *atgΔ* mutants to produce tryptophol to levels similar to those of the WT and the complementation strains (Fig. S4). Hence, autophagy affected tryptophan utilization and consequently delayed the production of tryptophol.

Both autophagy and the ubiquitin-proteasome pathway (UPP) contribute to amino acid homeostasis (25–27). We therefore hypothesized that autophagy mutants would be hypersensitive to the proteasome inhibitor bortezomib (BTZ) in the absence of a nitrogen source. When challenged with 50 μ g/ml of BTZ for 48 h in MM-N medium, the WT strain showed a consistent but nonsignificant decrease in CFU, whereas the proliferation of the *atgΔ* mutants was reduced by 90% relative to the untreated control (Fig. 2B). This result suggests that autophagy may largely compensate for the loss of UPP function in maintaining cellular homeostasis under nitrogen restriction conditions.

Inhibition of the proteasome in *S. cerevisiae* is known to cause cell death due to a failure to maintain amino acid homeostasis, and the deleterious effect of proteasome inhibition is rescued by amino acid supplementation (28). In light of this result, we tested whether individual L-amino acids could rescue proteasome inhibition for the WT strain and the *atgΔ* mutants of *C. neoformans*. When challenged with BTZ (50 μ g/ml), the ability of *C. neoformans* to proliferate varied with nitrogen source. In general, the *atg8Δ* mutant was more susceptible to BTZ than the WT in the presence of most amino acids. However, several amino acids were able to effectively rescue the proliferation of both the WT and *atg8Δ* strains (>50% of the proliferation observed in the absence of BTZ); these included arginine, asparagine, glutamine, glycine, and proline. Aspartic acid and glutamate moderately rescued the proliferation of the WT and *atg8Δ* strains (30 to 50%), while alanine, tryptophan, serine, leucine, phenylalanine, and valine minimally rescued WT from BTZ challenge and completely failed to do so for the *atg8Δ* mutant (Table 2), suggesting a role for the proteasome in the use of these amino acids as the sole nitrogen source. Lysine was able to rescue the WT strain from BTZ inhibition (40.2% $\pm$ 3.0% of the untreated control) but did not rescue the *atg8Δ* mutant (2.6% $\pm$ 1.1%), indicating that both autophagy and the proteasome play a role in proliferation with lysine. Methionine, which did not support the growth of the *atg8Δ* mutant over 48 h of incubation, could not rescue WT cells from BTZ challenge, suggesting that both autophagy and the proteasome pathway were required for effective use of methionine during the period. We did not determine the ability of cysteine and histidine to rescue *C. neoformans* from BTZ challenge because both amino acids did not serve as effective nitrogen sources. Overall, our results suggest that autophagy and the UPP both contribute to amino acid homeostasis in response to different amino acids. Moreover, the UPP was required for the use of alanine, tryptophan, serine, leucine, isoleucine, methionine, phenylalanine, and valine as sole nitrogen sources.

Loss of ATG7 impairs growth, alters cell morphology, and influences cell wall integrity. We next evaluated the effects of deleting individual ATG genes on growth and the *in vitro* production of three major *C. neoformans* virulence factors. Each of the *atgΔ* mutants was able to produce a polysaccharide capsule of a size similar to that of the WT strain (Fig. 3A and B). The *atg1Δ*, *atg8Δ*, and *atg9Δ* mutants were not different from the WT in growth at 30°C and 37°C or in the production of melanin (Fig. 3C). However, the *atg7Δ* mutant had a slight growth defect at both 30°C and 37°C and produced less melanin than the WT strain after 48 h of incubation. Further incubation allowed the *atg7Δ* mutant to

TABLE 2 Influence of different amino acids or ammonium sulfate as sole nitrogen sources to support the growth of the WT *C. neoformans* strain and the *atg8Δ* mutant^a

Sole N-source	Growth (CFU fold change 48 hr/ 0 hr)		Inhibition by bortezomib (% growth of without BTZ)	
	H99	<i>atg8Δ</i>	H99 + BTZ (%)	<i>atg8Δ</i> + BTZ (%)
NH ₄ ⁺	117.9 ± 42.1	82.7 ± 25.8	66.3 ± 6.4	75.4 ± 20.0
ARG	130.9 ± 6.0	140.3 ± 3.6	96.0 ± 6.7	101.0 ± 3.8
ASN	140.5 ± 13.5	120.2 ± 5.9	105.6 ± 15.5	110.9 ± 9.0
ASP	172.6 ± 29.7	163.1 ± 7.9	46.1 ± 12.6	39.2 ± 16.3
GLN	132.3 ± 4.2	114.7 ± 3.5	75.8 ± 11.3	87.4 ± 9.7
GLU	127.2 ± 6.3	138.3 ± 3.1	52.6 ± 13.5	31.5 ± 15.5
ALA	111.5 ± 23.9	141.1 ± 33.2	11.8 ± 3.0	2.0 ± 0.0
TRP	117.9 ± 9.9	98.7 ± 7.0	1.4 ± 0.2	1.1 ± 0.2
GLY	83.4 ± 45.1	74.9 ± 8.5	64.8 ± 20.2	79.3 ± 19.2
PRO	113.1 ± 17.9	104.0 ± 10.9	58.8 ± 5.0	31.4 ± 9.8
SER	110.0 ± 9.1	82.0 ± 33.4	8.0 ± 2.6	1.3 ± 0.2
LYS	104.3 ± 29.0	124.1 ± 21.9	40.2 ± 3.0	2.6 ± 1.1
ILE	82.7 ± 13.6	18.1 ± 16.2	3.9 ± 0.4	3.7 ± 0.5
LEU	74.7 ± 16.5	83.0 ± 47.6	5.9 ± 0.6	1.1 ± 0.2
PHE	63.5 ± 4.3	60.1 ± 3.1	5.8 ± 3.0	1.8 ± 0.4
VAL	50.8 ± 2.7	15.2 ± 4.7	10.3 ± 1.7	7.0 ± 0.5
MET	45.0 ± 13.0	2.6 ± 0.9	9.5 ± 2.8	3.6 ± 3.7
THR	21.2 ± 7.1	2.7 ± 0.5	36.2 ± 4.2	45.6 ± 11.6
CYS	10.4 ± 0.3	5.4 ± 1.2	N.D	N.D
HIS	5.3 ± 2.4	1.6 ± 0.4	N.D	N.D

^aThe results are presented as fold change in CFU over 48 h in MM for WT strain H99 and the *atg8Δ* mutant. For the proteasome inhibitor bortezomib (BTZ), the results are presented as the percentage of growth relative to that of cultures without BTZ. Data are presented as means ± SEMs from three biological replicates. Different colors indicate amino acids capable of supporting growth ≥80-fold (green), 15- to 80-fold (orange), and ≤15-fold (red) or capable of rescuing cells from BTZ challenge as follows: ≥50% without BTZ (green), 15 to 50% (orange), and ≤15% (red). N.D, not determined.

produce the same level of pigmentation as the WT cells (data not shown), suggesting that the reduced melanin production by the mutant was due to impaired growth. Interestingly, the two different approaches for complementation of the *atg7Δ* mutation gave different results. When the wild-type *ATG7* gene with its native promoter (~1 kb upstream of the *ATG7* start codon) was inserted in the genomic safe haven locus (20) [*atg7Δ::ATG7* (SH)], it complemented the survival phenotype upon nitrogen starvation (data not shown) but not the growth defect (Fig. 4A) or melanin production (Fig. 3C). In contrast, complementation at the native locus [*atg7Δ::ATG7* (NL)] corrected all observed defects (Fig. 3C). We hypothesize that the ~1-kb region upstream of *ATG7* was not sufficient to include all the regulatory elements for full expression, thus leading to partial complementation. The *atg7Δ::ATG7* (NL) complementation strain was therefore employed for the rest of the study, unless otherwise stated.

We next examined the poor growth of the *atg7Δ* mutant in more detail and found by microscopic examination that a small population of *atg7Δ* cells had an abnormal shape (Fig. 4). These cells appeared unable to complete cytokinesis, but the “daughter cell” continued to generate new buds, resulting in cells with elongated, pseudohypha-

FIG 3 The production of virulence factors by *C. neoformans* is not influenced by *atgΔ* mutations. Capsule formation (A) and cell size (B) were not affected by deletion of the *ATG* genes. Overnight YPD cultures were washed three times in low-iron medium before transfer to low-iron capsule-inducing medium. Cell size and capsule thickness were measured after 48 h of incubation at 30°C. Experiments were performed three times. For each experiment, at least 30 cells were measured and the averages were used as one biological replicate. The mean and SEMs from three biological replicates ($n = 3$) are plotted on the graph. (C) Spot assays showing growth of serial dilution of cells on YPD and L-DOPA medium after incubation at 30°C or 37°C. Pictures were taken after 48 h of incubation. *C. neoformans atg1Δ*, *atg8Δ*, and *atg9Δ* mutants were not different from the WT strain in growing at 37°C or in melanin production, whereas the *atg7Δ* mutant showed a minor growth defect at 30°C and reduced melanin production after 48 h of incubation. Safe haven (SH) complementation mutation and native locus (NL) complementation of *atg7Δ* showed different results. See the text for details.

like shapes (Fig. 4B). The *atg7Δ* cells with abnormal shape were alive but divided at a much lower rate than normal cells, as shown by time-lapse microscopy over a 12-h period (Fig. 4B). In addition, there were cells of normal shape but of slightly larger size in the *atg7Δ* mutant population that did not replicate over the 12-h period (Fig. 4B, arrows); these cells may have been alive but in a state of metabolic inactivity or cell cycle arrest. Indeed, staining with trypan blue revealed no difference in the percentage of live/dead cells among the *atgΔ* mutants and the WT (data not shown). Notably, the proliferation and morphological phenotypes were unique to the *atg7Δ* mutant and not seen for the other *atgΔ* mutants.

Different autophagy mutants display difference in virulence. Previous studies indicated that autophagy is important for virulence in *C. neoformans*. For example, Hu et al. (17) showed that a deletion mutant of *PI3K*, an autophagy upstream signaling protein was defective in autophagy and virulence when using intravenous and inhalation models of cryptococcosis in CBA/J mice. In addition, Hu et al. (17) used RNA interference to knock down expression of *ATG8* (*iATG8*) and found attenuated virulence for the strain in CBA/J mice. However, the *iATG8* strain still had 20% of the expression level of the *ATG8* gene. A recent study with an *atg7Δ* mutant also revealed attenuated virulence in a *Galleria* (invertebrate) infection model (18). To extend these analyses, we assessed the ability of each of our autophagy mutants to survive and replicate in host macrophages and to cause disease in a mouse model of cryptococcosis. Among the *atgΔ* mutants, only the *atg7Δ* mutant showed significantly reduced intracellular replication during a 24-h incubation with the J774.A1 murine macrophage-like cell line,

FIG 4 The growth defect of an *atg7Δ* mutant is due to a subpopulation of cells with abnormal cell shape. (A) Growth curves of the WT strain, an *atg7Δ* mutant, and two different complementation strains [*atg7Δ::ATG7* (SH) and *atg7Δ::ATG7* (NL)] restored growth, whereas complementation at the safe haven only partially restored growth. (B to G) Time-lapse microscopy of cells of the *atg7Δ* mutant (B to E) and the WT strain (F and G) grown on a YPD agarose pad for 12 or 16 h after transfer from an overnight YPD culture. Panels D and E are magnified views of the red rectangular regions in panels B and C, respectively. The abnormally shaped, elongated cells are only seen in the *atg7Δ* mutant. There were also normal shaped, larger cells in the *atg7Δ* mutant population that were not replicating (red arrow in panels B and C).

FIG 5 Analysis of the survival of *atgΔ* mutants in macrophages and virulence in a mammalian host. (A) Intracellular replication of the WT strain, the *atgΔ* mutants, and complementation strains in murine macrophage-like J774A.1 cells. Only the *atg7Δ* mutant had a defect in intracellular replication within the cells (*, $P < 0.05$, unpaired *t* test). The experiments were repeated 5 times, and error bars represent SEMs. (B) The *atg1Δ* mutant showed hypervirulence in a BALB/c mouse model of cryptococcosis. (C) The *atg7Δ* mutant showed attenuated virulence in BALB/c mice. (D) The *atg8Δ* mutant showed attenuated virulence in a BALB/c mouse inhalation model of cryptococcosis. (E) The *atg9Δ* mutant showed attenuated virulence in a BALB/c mouse inhalation model of cryptococcosis. For each strain, 10 female BALB/c mice were infected. Each mouse was inoculated with 2×10^5 fungal cells intranasally, monitored daily, and euthanized when weight loss reached 85% of the initial weight. Asterisks beside each curve indicate statistical significance (log rank test) between each strain and the WT: *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

compared with the WT strain. The *atg1Δ*, *atg8Δ*, and *atg9Δ* mutants did not differ from the WT (Fig. 5A). Mutants with survival defects in interactions with macrophages generally show attenuated virulence in mouse models of cryptococcosis (29–32). We therefore infected BALB/c mice with the WT strain, each of the *atgΔ* mutants, or the respective complementation strains via intranasal inhalation and monitored the disease progression daily to test the importance of autophagy in the virulence of *C. neoformans* (Fig. 5B to D). Even though *ATG7* and *ATG8* have been implicated in *C. neoformans* virulence (17, 18), we included the *atg7Δ* and *atg8Δ* mutants in our virulence assays, hence extending the evaluation of *atg7Δ* virulence beyond the use of an invertebrate model to a mouse model and further evaluating the role of *Atg8* with a complete deletion mutant compared with the RNAi knockdown approach.

Interestingly, the four *atgΔ* mutants showed levels of virulence in the murine inhalation model of cryptococcosis different from that of the WT. Mice infected with the WT strain showed 100% mortality between 15 and 20 days postinfection (dpi). Unexpectedly, mice infected with the *atg1Δ* mutant showed 100% mortality after 7 dpi, and this was ~8 days earlier than with the WT ($P < 0.0001$, log rank test) (Fig. 5B). Mice infected with *atg1Δ::ATG1* showed a 100% mortality rate after 16 dpi, 1 day later than the WT ($P < 0.05$, log rank test), showing full complementation (Fig. 5B). Mice infected with the *atg1Δ* mutant had significantly lower numbers of fungal CFU than mice infected with the WT or complementation strain in all systemic organs tested, including

the lungs, brain, blood, kidneys, liver, and spleen (Fig. 6A), suggesting that damage due to *atg1Δ* cells contributed little to the mortality of the mice.

Among 10 mice infected with the *atg7Δ* mutant, only 8 reached mortality between 27 and 44 dpi, and the remaining 2 survived until the end of the experiment (54 dpi) (Fig. 5C). Mice infected with the *atg7Δ::ATG7* mutant showed 100% mortality after 21 dpi, 1 day later than the WT-infected mice ($P < 0.0001$, log rank test), showing a partial complementation (Fig. 5C). Fungal-load analyses revealed variability among the *atg7Δ* mutant-infected mice. The two mice that survived had no detectable fungal CFU, indicating complete clearance of the infection. Among the remaining eight mice, one had fungal CFU in the lung only ($\sim 10^5$ CFU/g of lung), and this was $\sim 1,000$ -fold less than the other seven mice, which all had fungal loads similar to those of the WT-infected mice (Fig. 6B).

Mice infected with the *atg8Δ* mutant had a 60% mortality rate after 30 dpi, and the remaining 40% survived to end of the experiment (41 dpi) (Fig. 5D); this outcome was significantly different from that of WT-infected mice ($P < 0.0001$, log rank test). The *atg8Δ::ATG8* mutant-infected mice showed 100% mortality after 21 dpi, with no statistical difference from WT-infected mice ($P > 0.05$, log rank test) (Fig. 5D). Two of the four *atg8Δ*-infected mice that survived to the end of the experiment had completely cleared the infection, while the other two only had fungal loads in the lung between 10^2 and 10^4 CFU/g. This level was significantly lower than observed in the other six mice, which all had fungal loads in the systemic organs similar to those of the mice infected with the WT or the complementation strains (Fig. 6C).

Mice infected with the *atg9Δ* mutant reached 100% mortality after 27 dpi, 12 days later than WT-infected mice ($P < 0.0001$, log rank test) (Fig. 5E). A partial complementation was achieved when the WT *ATG9* gene was reintroduced into the mutant, with *atg9Δ::ATG9* mutant-infected mice reaching 100% mortality at 18 dpi, 3 days later than mice infected with the WT ($P < 0.001$, Log rank test) (Fig. 5E). Fungal-load analysis revealed that mice infected with the *atg9Δ* mutant had more CFU in the lungs, liver, and spleen than mice infected with the WT strain (Fig. 6D).

Overall, our results showed that each *ATG* gene contributed to virulence differently in BALB/c mice, and these findings suggest that nonautophagic functions associated with each *ATG* gene might contribute to virulence in addition to the roles of the genes in autophagy.

Both the *atg1Δ* and *atg8Δ* mutants stimulate a strong immune response in BALB/c mice. The difference in virulence among the *atgΔ* mutants was also evident when we examined disease progression by monitoring mouse weight loss. Mice infected with WT strain H99 lost weight gradually, over a period of 15 days, whereas the *atg1Δ* mutant-infected mice showed a much more rapid weight loss and had to be sacrificed at 7 dpi, when the humane endpoint was reached. Similarly, *atg8Δ* mutant-infected mice showed a greater weight loss than WT-infected mice, with maximal loss at 7 dpi but with subsequent recovery as the mice began to gain weight. In contrast, no weight loss was observed in mice infected with the *atg7Δ* or *atg9Δ* mutant (Fig. 7). These observations led us to hypothesize that both the *atg1Δ* and *atg8Δ* mutants stimulated potent immune responses in the BALB/c mice and that this response might not only kill fungal cells but also contribute to host damage and weight loss.

To test this hypothesis, we first measured the host response at 6 dpi by examining cytokine production in pulmonary tissue upon infection with the WT, *atg1Δ*, or *atg8Δ* strain compared with responses in mice treated with phosphate-buffered saline (PBS) (Fig. 8A). We found that *C. neoformans* infection stimulated tumor necrosis factor alpha (TNF- α), gamma interferon (IFN- γ), interleukin 6 (IL-6), monocyte chemoattractant protein 1 (MCP-1), and IL-12p70 production in the lungs of the mice, but not the production of IL-10, at 6 dpi (Fig. 8A), a finding consistent with the fact that BALB/c mice clear *C. neoformans* infection via a Th1-skewed immune response (33). Notably, mice infected with the *atg1Δ* or *atg8Δ* mutant showed a significantly higher level of TNF- α production than mice infected with the WT strain (Fig. 8A). In addition, mice that had a greater weight loss upon infection with the *atg1Δ* mutant had higher levels of

FIG 6 Analyses of fungal burden in systemic organs (lungs, brain, kidneys, liver, and spleen) and blood at the end of the virulence assays performed for Fig. 5. (A) The *atg1*Δ mutant showed significant lower fungal burden systemically. (B) *atg7*Δ mutant infection caused (Continued on next page)

FIG 7 Average daily mouse weight to indicate disease progression in the first 20 dpi. Mouse weight is presented as weight relative to the initial weight of each mouse. Significant weight loss between 3 dpi and 7 dpi (purple vertical lines) was observed for mice infected with the *atg1Δ* (red) and *atg8Δ* (green) mutants. A black horizontal dotted line indicates the threshold for animal euthanasia at 85% of the initial weight. At 8 dpi, mice infected with the *atg8Δ* mutant started to recover. No weight loss was observed for mice infected with the *atg7Δ* or *atg9Δ* mutant. The error bars represent SEMs from all surviving mice ($n = 10$).

IFN- γ and IL-6 in lung tissue than did mice infected with WT (Fig. 8A). Histology revealed pathological differences between the mice infected with the WT strain versus the *atg1Δ* and *atg8Δ* mutants (Fig. 8B). First, the lungs from WT-infected mice showed a universal presence of fungal cells (stained in magenta by mucicarmine) throughout, whereas fungal cells of the *atg1Δ* or *atg8Δ* mutant were less frequently observed. Second, the *atg1Δ* or *atg8Δ* cells in the lung tissue were surrounded by an infiltration of host immune cells forming granuloma-like structures; these were sporadically found throughout the lung whereas the rest of the lung resembled uninfected tissue. In comparison, an infiltration of immune cells in WT-infected lungs was apparent only in the vicinity of airways and blood vessels (Fig. 8B). The analyses of fungal loads at 6 dpi were consistent with the histological findings that fungi were significantly more abundant in the lung tissue of mice infected with the WT strain than in mice infected with the *atg1Δ* or *atg8Δ* mutant (Fig. 8C).

To determine the types of immune cells responsible for the immune responses stimulated by the *atg1Δ* and *atg8Δ* mutants, we used multiparameter flow cytometry (FC) to compare immune cell populations in the lungs of the infected mice and PBS-treated controls on 6 dpi (Fig. 9). All *C. neoformans* strains stimulated an immune response in the lungs, as reflected by the increased number of total CD45⁺ immune cells, including Siglec F⁺ eosinophils, CD11b⁺ Ly6C⁺ monocytes, Ly6G⁺ neutrophils (PMNs), CD49b⁺ natural killer (NK) cells, and CD4⁺ and CD8⁺ T cells, as well as depletion of CD11c⁺ Siglec F⁺ alveolar macrophages (AMs) (Fig. 10). In addition, the lungs of the BALB/c mice infected with the *atg1Δ* or *atg8Δ* mutant had significantly higher numbers of CD45⁺ cells, monocytes, and PMNs than did those of the mice infected with the WT strain. Mice infected with the *atg8Δ* mutant also had more eosinophils, NK cells, and CD4⁺ and CD8⁺ T cells in the lung than WT-infected mice. Because TNF- α and IFN- γ were found at higher levels in mice infected with the mutants, we further examined the immune cells that produce these cytokines. Mice infected with the *atg1Δ* or *atg8Δ* mutant had significantly higher numbers of TNF- α -producing PMNs,

FIG 6 Legend (Continued)

variable fungal loads throughout mouse systemic organs and blood and significantly lower fungal burden in the lungs than the WT. (C) *atg8Δ* mutant-infected mice had variable fungal burdens in systemic organs and blood, with four mice almost clearing infection. (D) *atg9Δ* mutant infection caused slightly higher fungal burdens than the WT in the lungs and liver. Asterisks indicate statistical significance (Mann-Whitney test, $n = 10$) between different strains: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

FIG 8 Immune responses of BALB/c mice upon lung infection with the WT strain and an *atg8Δ* mutant. Three independent experiments were performed. The first experiment included H99, the *atg1Δ* mutant, and PBS, the second experiment included H99, the *atg8Δ* mutant, and PBS, and the third experiment included H99, the *atg1Δ* and *atg8Δ* mutants, and PBS. Five mice were used for each strain in each experiment. Data were pooled, resulting in 15 mice for both the WT and PBS and 10 mice for the *atg1Δ* and *atg8Δ* mutants. (A) Lung cytokine profiles on 6 dpi for BALB/c mice infected with the *atg1Δ* mutants or the WT strain or treated with PBS. Cytokines were released from lung tissue with a mixer mill and measured using a CBA bead array mouse inflammation kit. Significantly higher levels of TNF- α were produced by mice infected with the *atg1Δ* and *atg8Δ* mutants than by WT-infected mice ($P < 0.05$, Mann-Whitney test). Mice infected with the *atg1Δ* mutant also had significantly higher levels of IFN- γ and IL-6. (B) Representative histology micrograph of lung tissue from mice infected with the WT or the *atg1Δ* or *atg8Δ* mutant or treated with PBS (controls). Yellow arrowheads mark *C. neoformans* cells. In Mayer's mucicarmine-stained tissue, *C. neoformans* cell walls are (Continued on next page)

A. Total live cells from the lung **B. Lung CD45⁺ cells**

FIG 9 Multiparameter flow cytometer analysis of immune cell populations of BALB/c mice upon lung infection with WT and the *atg8Δ* mutant compared with PBS controls. Sequential gating strategies of multiparameter flow cytometer analysis of immune cell populations isolated from lungs of mice infected with the WT or the *atg1Δ* or *atg8Δ* mutant or treated with PBS are shown. (A) Representative flow cytometry plots showing gating for CD45⁺ leukocytes from total live cells in the lungs of mice treated with PBS or infected with H99 or the *atg8Δ* or *atg1Δ* mutant. (B) Representative flow cytometry plots showing gating of CD45⁺ AMs, eosinophils, Ly6C monocytes, PMNs, CD4 and CD8 T cells, and CD49b⁺ NK cells in the lungs of mice treated with PBS or infected with H99 or the *atg8Δ* mutant. Eosin, eosinophil; AMΦ, alveolar macrophage; Mono, monocyte; PMN: neutrophil, NK: natural killer cell. Numbers in each plot indicate percent population.

TNF- α -producing monocytes, and IFN- γ -producing CD4⁺ T cells than did WT-infected mice (Fig. 10). Mice infected with the *atg8Δ* mutant also had more TNF- α -producing AMs, IFN- γ -producing NK cells, and IFN- γ -producing CD8⁺ T cells. Overall, the results from cytokine production, immune cell profiling, histology, and fungal-load analyses supported the hypothesis that the *atg1Δ* and *atg8Δ* mutants stimulated a stronger immune response than the WT in BALB/c mice, which, in turn, helped clear *atg1Δ* and *atg8Δ* fungal cells from infected tissue.

DISCUSSION

In this study, we demonstrated that four *ATG* genes encoding key components for early induction and phagophore assembly are required for hallmarks of autophagy in *C. neoformans*, including survival in response to starvation (nutrient recycling) and maintenance of amino acid homeostasis. The relative sensitivities of autophagy mutants to survival upon nutrient limitation differ between fungal species. For example, deletion of the *ATG9* gene led to supersensitivity to nitrogen starvation in *S. cerevisiae*,

FIG 8 Legend (Continued)

stained in magenta, whereas the lung tissue is yellow-brown color. In H&E-stained tissue, *C. neoformans* cell walls are stained pink, with capsule nonstained, displaying as a clear halo around the fungal cell. The WT showed ubiquitous colonization of the lung, whereas *atg1Δ* and *atg8Δ* cells were only sporadically found in the lung tissue, surrounded by infiltration of host immune cells. (C) Lung fungal loads of BALB/c mice infected with the WT or the *atg1Δ* or *atg8Δ* mutant or treated with PBS. At 6 dpi, both the *atg1Δ* and *atg8Δ* mutants yielded significant lower numbers of fungal cells than the WT in mouse lungs. Error bars represent SEMs. Asterisks above each column indicate statistical significance (Mann-Whitney test) between different each strain and the WT or between the mutants: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

FIG 10 Immune cell profiles from the left lobe of the lungs of BALB/c mice infected with the WT or the *atg1Δ* or *atg8Δ* mutant or treated with PBS at 6 dpi. Three independent experiments were performed. The first experiment included H99, the *atg1Δ* mutant, and PBS, the second experiment included H99, the *atg8Δ* mutant, and PBS, and the third experiment included H99, the *atg1Δ* and *atg8Δ* mutants, and PBS. Data were pooled, resulting in 15 mice for both the WT and PBS and 10 mice for the *atg1Δ* and *atg8Δ* mutants. Graph summarizing the numbers of different leukocyte populations and their TNF- α or IFN- γ production in the lungs of mice infected with different *C. neoformans* strains as gated in Fig. 9. Error bars represent SEMs. Asterisks above each column indicate statistical significance (Mann-Whitney test) between different each strain and the WT or between the mutants: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

with the mutant unable to survive past 3 days (34). In contrast, it took much longer (~20 days) for 90% of the *atg9Δ* mutant cells of the fungal pathogen *Candida albicans* to die in nitrogen-free medium (35). For another fungal pathogen, *Aspergillus fumigatus*, cells of the Δ *Afatg1* mutant were not able to grow on starvation plates but remained viable after 14 days of incubation (36). In this context, our *C. neoformans atgΔ* mutants were more similar in sensitivity to nitrogen starvation to the *C. albicans atg9Δ* and *A. fumigatus ΔAfatg1* mutants than the *S. cerevisiae atg9Δ* mutant. As pathogens, *C.*

albicans, *A. fumigatus*, and *C. neoformans* are all capable of surviving under the nutrient-limited condition of mammalian hosts and in different environmental niches. It is therefore possible that these species have evolved mechanisms in addition to autophagy to withstand nitrogen starvation better than *S. cerevisiae*. In this regard, it is interesting that genes for autophagy appear to be dispensable for virulence in both *C. albicans* and *A. fumigatus* (35, 36) but generally contribute to virulence in *C. neoformans*, as shown by our studies and those of others (17, 18).

Amino acids and the acquisition of nitrogen are important for virulence in *C. neoformans* (21, 37–40), and amino acids differed in their support of growth as sole nitrogen sources. We found that the WT strain and the *atgΔ* mutants of *C. neoformans* were able to grow equally well when transferred from a rich medium (YPD) to media with preferred amino acids, presumably because the cells were induced for uptake and use of the amino acids. In contrast, the poor growth of the *atgΔ* mutants in nonpreferred amino acid medium likely reflected a combination of nitrogen catabolite repression of the use of nonpreferred amino acids due to pregrowth in YPD and the requirement for autophagy to recycle nutrients in coordination with the expression of functions for amino acid uptake and synthesis (41). Methionine is of particular interest among the nonpreferred amino acids for several reasons. First, previous studies demonstrated that methionine metabolism is important for virulence in *C. neoformans*. For example, a methionine auxotroph is defective in virulence factor production in *C. neoformans* (42, 43), and methionine biosynthesis appears to be essential for virulence (44), suggesting that methionine availability may be limiting in vertebrate hosts. Second, methionine is a signaling molecule in both *S. cerevisiae* and *C. neoformans*. For example, methionine acts as an amino acid sufficiency signal in *S. cerevisiae* and inhibits autophagy through methylation of protein phosphatase 2A (PP2A) (45, 46). In *C. neoformans*, methionine is the only amino acid tested that triggers internalization of the G protein-coupled receptor Gpr4 and methionine activates the cAMP-protein kinase A (PKA) pathway (47). Third, methionine is also important for the synthesis of S-adenosylmethionine (SAM), the major methyl group donor in cells. Finally, methionine plays an important role in the initiation of protein synthesis. Given our finding that the use of methionine by *C. neoformans* was most affected by an autophagy defect among all amino acids, further investigation is needed to examine the contributions of methionine to sensing and virulence in the host.

We also examined the connection between autophagy and amino acid homeostasis in more detail by inhibiting the UPP with BTZ in the *atgΔ* mutants. This approach revealed that both degradation pathways contribute to *C. neoformans* survival under nitrogen limiting conditions and that autophagy is the main player in nutrient recycling in WT cells. This is consistent with findings that autophagy is upregulated to compensate for the decreased activity of the UPP (reviewed in reference 25).

Our virulence assays using a murine inhalation model of cryptococcosis with BALB/c mice and four *atgΔ* mutants in parallel revealed interesting differences among these mutants. Although the *atg7Δ*, *atg8Δ*, and *atg9Δ* mutants all showed attenuated virulence in the model, the progression of disease suggested different interactions between these mutants and the host. As expected, deletion of *ATG7* led to attenuated virulence in the mouse model. In particular, the slower growth of the *atg7Δ* mutant *in vitro* could contribute to the attenuated virulence in the mouse model; however, it is also possible that the small percentage of abnormally shaped cells formed by the *atg7Δ* mutant influenced virulence. As shown by Oliveira et al., there were more enlarged, abnormally shaped *atg7Δ* cells than WT cells recovered from bronchoalveolar lavage (BAL) fluid at 3 dpi (18). Upon arrival to the lung alveolar space, *C. neoformans* encounters the first line of host defense, the AMs, which phagocytose the cryptococcal cells (48). There is convincing evidence that *C. neoformans* uses macrophages to disseminate to systemic organs and to cross the blood-brain barrier (49–53). However, because of impaired cytokinesis, the elongated and enlarged cells of the *atg7Δ* mutant might be too large to be engulfed by AMs, similar to the normal titan cells that are inefficiently engulfed by host immune cells (54). As a result, we speculate that the

abnormally shaped cells might be retained in the alveolar space, as supported by the fungal loads recovered by BAL (18). Although titan cell formation enhances *C. neoformans* virulence by promoting dissemination of normal-size cells (55), this effect was not observed in mice infected with the *atg7Δ* mutant, highlighting the virulence defect of these cells. In addition, impaired intracellular replication within macrophage could further contribute to the virulence defect of the *atg7Δ* mutant. Overall, our results are consistent with the findings by Oliveira et al. that the WT strain caused significantly more lung colonization than the *atg7Δ* mutant (18). However, one of the main differences between our study and that of Oliveira et al. was that our *atg7Δ* mutant did not show enhanced melanin production. We noted that our H99 strain was fully capable of producing melanin within 48 h, unlike the H99 strain used by Oliveira et al. Microevolution of virulent H99 strains under laboratories has been well documented (56), and we suspect that the H99 isolate used by Oliveira et al. might have had delayed melanin formation.

Although the *atg8Δ* mutant showed attenuated virulence similar to that of the *atg7Δ* mutant in the mice survival assays, the disease progressions of mice infected with the two mutants were quite different. Indeed, the *atg8Δ* mutant showed similarity to the hypervirulent *atg1Δ* mutant in BALB/c mice, causing rapid weight loss and stimulating a stronger immune response than the WT. Both the *atg1Δ* and *atg8Δ* mutants stimulated an immune response at 6 dpi in the lung, with increased TNF- α production by the PMNs and monocytes and a greater number of these cells, as well as IFN- γ -producing CD4⁺ T cells, than in WT-infected mice, indicating both innate and adaptive immune activation. Our findings and the role of the identified immune cells in host response to *C. neoformans* are largely congruent. The elevated number of IFN- γ -producing CD4⁺ T cells indicated a typical Th1 response, which is known to promote fungal clearance (reviewed in references 3 and 57). Th1-type cytokines such as IL-12, IFN- γ , and TNF- α recruit phagocytes to the site of infection and activate macrophages (3, 57), which were all observed in the *atg1Δ*- and *atg8Δ*-infected mice. Infiltration of PMNs is known to be important for generating an early protective response against pulmonary *C. neoformans* infection (58, 59). While we monitored TNF- α production from monocytes and PMNs, we were unable to directly examine dendritic cells (DCs) and other monocyte-derived macrophage populations. However, monocyte-derived macrophages were within the CD11b⁺ gate, and dendritic cells within the CD11c⁺ gate contributed less TNF- α than the monocytes or PMNs (data not shown). DCs are antigen-presenting cells (APCs) acting at the interface of innate and adaptive immunity. Upon recognition of *C. neoformans*, DCs phagocytose the pathogen and their antigenic molecules and undergo maturation, and in the presence of IFN- γ , maturation results in IL-12-producing DCs, promoting Th1-biased responses (3, 58). Macrophages are also polarized toward M1 activation, generating fungicidal reactive oxygen and nitrogen species (60). Both the *atg1Δ* and *atg8Δ* mutants generated a stronger Th1 response than the WT and had significantly less colonization of the lung than WT cells. Hence, the rapid weight loss of *atg1Δ* and *atg8Δ* mutant-infected mice during days 3 to 7 postinfection was not due to an increased fungal load; instead, it may have been related to the increased Th1 immune response. While our study shows the induction of a Th1 response in the *atg1Δ* and *atg8Δ* mutant-infected mice, we did not examine the production of IL-23, IL-17, or IL-4 and so cannot exclude the possibility that a Th17 or Th2 response could also be made. However, since IFN- γ suppress a Th2 response (61), this possibility is less likely.

Despite the similarities, there were also differences between *atg1Δ* and *atg8Δ* mutant-infected mice. At 6 dpi *atg1Δ* mutant-infected mice had accumulated more of the cytokines TNF- α and IL-6, and these mice showed more rapid weight loss than the mice infected with the *atg8Δ* mutant. While mice infected with either mutant showed approximately equal numbers of leukocytes in the infected lungs, there were some differences within the leukocyte populations. Lungs of *atg8Δ* mutant-infected mice at 6 dpi had similar numbers of TNF- α -producing PMNs and IFN- γ -producing CD4⁺ T cells yet had more TNF- α -producing monocytes, TNF- α -producing AMs, IFN- γ -producing

CD8⁺ T cells, and NK cells. These mice also had lower fungal burdens than *atg1Δ* mutant-infected mice, raising the possibility that the immune response raised against the *atg8Δ* mutant maybe more effective than that raised in response to the *atg1Δ* mutant. AMs are known to play an important role in the host response to *C. neoformans* infection (48), and NK cells can also kill *Cryptococcus* (62–64). A recent study identified the fungal cell wall component β -1,3-glucan as the ligand triggering NK cell cytotoxic killing of *C. neoformans* through activating receptor NKp-30 (65). It will be of interest to know if the WT and *atg1Δ* and *atg8Δ* mutants interact differently with NK cells and whether this might be due to differences in their cell surface exposure of β -1,3-glucan. Furthermore, our study provided only a snapshot of the immune response and fungal burden at 6 dpi. Given the dynamic nature of microbe-host interactions, a full time course after infection may provide additional insights. In *Cryptococcus*-host interactions, there have been several different scenarios where the host immune response had a negative impact on the host. Although rare, similar weight loss followed by recovery has been reported for A/J mice infected with *pbx1Δ* or *pbx2Δ* mutants of *C. neoformans* (66). Also, a clinical study showed that HIV-positive patients with cryptococcal infection developed immune reconstitution inflammatory syndrome after antiretroviral therapy (67). A recent study using a mouse model explicitly demonstrated that CD4⁺ T cells act as a “double-edged sword” in both killing fungal cells and causing significant immunopathology and mortality to the host during cryptococcal central nervous system infection (68, 69).

It is unclear why both the *atg1Δ* and *atg8Δ* mutants triggered a stronger PMN and Th1 response in BALB/c mice than the WT infection, although we speculate that changes in capsule and/or cell wall composition may have occurred in the mutants. It is known that the capsule has immune suppressive activities, and associated mannoproteins are immunogenic (70, 71). The Th1 response plays a critical role in killing *C. neoformans*, unlike the Th2 response (3, 57). Mannoproteins are known to induce a strong TNF- α -mediated Th1 response (72, 73), and fungal cell wall component β -1,3-glucan stimulates NK cell activities (65). Hence, it is possible that deletion of *ATG1* or *ATG8* somehow alters the composition and assembled structure of capsule or leads to the accumulation of immunogenic mannoproteins or fungal cell wall components on surface of fungal cells, which subsequently leads to the activation of more CD4⁺ Th1 cells, more M1 polarized macrophages, and a greater recruitment of PMNs. Effort to compare the capsule composition and complement of mannoproteins in the *atg1Δ*, *atg8Δ*, and WT strains to explore this possibility is certainly warranted. Alternatively, the *atg1Δ* and *atg8Δ* mutants might not be able to survive in AMs as well as the WT upon entry into the host, although there were no differences between the WT and the *atg1Δ* and *atg8Δ* mutants in intracellular replication in macrophage-like J774.A1 cells. Dead *atg1Δ* or *atg8Δ* cells and their antigens could be taken up by APCs, and *C. neoformans* antigens could be presented to naive T cells (74, 75) to activate T cells and induce their proliferation, resulting in a T cell-mediated immune response against the *atg1Δ* or *atg8Δ* mutant. This hypothesis may be tested by comparing the numbers of APCs and the degrees of T cell activation in the mediastinal lymph nodes, which drain the lung tissue after infection with WT or mutant *C. neoformans* strains.

Overall, our *in vitro* phenotypic studies and virulence assays with mice demonstrated that different genes for autophagy make different contributions to the virulence of *C. neoformans*. These results are in contrast to studies with other pathogenic fungi, including *C. albicans* and *A. fumigatus*, in which autophagy mutants were fully virulent (35, 36). Interestingly, individual *ATG* genes in *C. neoformans*, including *ATG1*, *ATG7*, and *ATG8*, appear to participate in other cellular processes in addition to autophagy. Reports of contributions of autophagy genes to nonautophagic functions are appearing (76), and it will therefore be important to determine whether the difference in virulence for the four *atgΔ* mutants of *C. neoformans* could be related to nonautophagic roles in morphogenesis and cell surface remodeling.

MATERIALS AND METHODS

Strains and growth conditions. *Cryptococcus neoformans* var. *grubii* strain H99 was used as the wild-type (WT) strain. Depending on the experiment, all strains were cultured at 30°C or 37°C with shaking at 150 rpm, in the following media: YPD (2% yeast extract, 1% peptone, 2% dextrose), YNB without nitrogen (YNB-N) (2% dextrose, 0.67 g · liter⁻¹ yeast nitrogen base without amino acid or ammonium sulfate), minimum medium without nitrogen (MM-N) (15.0 mM glucose, 10.0 mM MgSO₄, 29.4 mM K₂HPO₄, and 3.0 μM thiamine, pH 5.4) (77) supplemented with different amino acids (13 mM) or (NH₄)₂SO₄ (6.5 mM), low-iron capsule-inducing medium (CIM) (78) (0.5% dextrose, 0.4 g · liter⁻¹ dipotassium monohydrogen phosphate, 5 g · liter⁻¹ asparagine, 0.25 g · liter⁻¹ calcium chloride dehydrate, 0.4 mg · liter⁻¹ thiamine, 0.005 mg · liter⁻¹ cupric sulfate pentahydrate, 2 mg · liter⁻¹ zinc sulfate heptahydrate, 0.01 mg · liter⁻¹ manganese chloride tetrahydrate, 80 mg · liter⁻¹ magnesium sulfate heptahydrate, 0.46 mg · liter⁻¹ sodium molybdate, and 0.057 mg · liter⁻¹ boric acid). CIM was prepared using Chelex 100-treated NANOpure water. Solid media were supplemented with 1.5% agar. The following antibiotics were used for selection: 200 μg · ml⁻¹ Geneticin 418 and 200 μg · ml⁻¹ hygromycin B.

Construction of *atgΔ* mutants and complementation strains. Each of the *ATG1*, *ATG7*, *ATG8*, and *ATG9* genes was deleted from strain H99 (77) by replacing the open reading frame with a neomycin resistance cassette (Neo^r) from pJAF1 (79). Briefly, the left and right flanking regions of each gene were amplified using primer pairs LF-LR and RF-RR (Table S1), and the neomycin resistance cassette was amplified from pJAF1 (79) using primers M13F and M13R (Table S1). Note that both primers LR and RF have 5' end sequences complementary to primers M13R and M13F, respectively. These three fragments were joined by overlapping PCR using LF and RR primers. All PCRs were using Phusion Hi-fidelity polymerase by following the manufacturer's protocols (New England BioLabs). The final construct was biolistically transformed into H99 using the PDS-1000 particle delivery system (Bio-Rad). The resulting transformants were selected, purified, screened by PCR using *Taq* polymerase (New England BioLabs), and confirmed by Southern blotting (Fig. S1).

We constructed the complementation strains using the genomic safe haven approach (20). Each gene and its upstream promoter region (~1 kb) was amplified by PCR (Phusion polymerase) using primers with restriction enzyme recognition sites (Table S1) and subsequently subcloned into pSDMA58. The resulting plasmid was linearized by restriction digestion with *PacI* or *BaeI*, followed by biolistic transformation into the corresponding deletion mutant. Transformants were selected on YPD medium supplemented with both Geneticin 418 and hygromycin, purified, and confirmed by PCR (*Taq* polymerase). For the *atg7Δ* mutant only, where the genomic safe haven approach resulted in a partial complementation phenotype (see Results), *ATG7* complementation was also performed at the native locus using a split marker transformation approach (80). Briefly, the incomplete left part of the hygromycin resistance cassette (Hyg^r) was excised from pJAF15 (79) by restriction enzymes *SpeI* and *NotI*-HF and ligated into *SpeI*- and *NotI*-HF-doubly digested safe haven complementation plasmid pSDMA58-*ATG7*-Comp, resulting in pSDMA58+*ATG7*comp+HYGL. The *ATG7*compL+HYGL fragment was then excised with *PvuI*. Overlapping PCR was used to construct *ATG7*CompR+HYGR. *ATG7*CompR was PCR amplified from H99 genomic DNA using *atg7*Right_F and *atg7*Right_R, and the Hyg^r cassette4e was amplified using primers M13F and M13R. The resulting fragments were then used as the templates for PCR using primers HYG-R and *atg7*comp_R to give rise to *ATG7*compR+HYGR. *ATG7*compL+HYGL and *ATG7*CompR+HYGR fragments were mixed at equal molar ratios and biolistically transformed into the *atg7Δ* mutant. The resulting hygromycin-resistant and Geneticin 418-sensitive transformants were selected. Correct integration events were confirmed by PCR on both sides of the integration site.

Nitrogen starvation survival, bortezomib inhibition, and amino acid rescue assays. Nitrogen starvation survival, bortezomib inhibition, and amino acid rescue assays were carried out using methods previously described for *S. cerevisiae* (34). Briefly, cells of the WT, mutants, and complementation strains were grown in YPD broth overnight (16 to 18 h). Harvested cells were washed three times with PBS and used to inoculate media to reach a concentration of 10⁶ cells · ml⁻¹. YNB-N was used for the nitrogen starvation survival assay. For growth assays on amino acids, MM-N was used and supplemented with different L-amino acids (13 mM) or ammonium sulfate (6.5 mM). The proteasome inhibitor bortezomib (New England BioLabs) was used at 50 μg · ml⁻¹. Appropriate dilutions were plated on YPD agar plates at different times postinoculation for determination of CFU. Percent or fold change of the initial CFU was calculated. The experiments were repeated at least three times for each strain. For determination of the growth curve in 96-well plates, 200 μl of medium was used for each well. The plate was incubated in Infinite Tecan 200 PRO multimode microplate reader at 30°C. Optical density at 600 nm was read every hour immediately after orbital shaking for 10 min.

Capsule size, cell body size, and microscopy. A total of 10⁶ cells from overnight YPD cultures of each strain were used to inoculate 3 ml of low-iron capsule-inducing medium (78). Cells were incubated at 30°C with shaking (150 rpm) for 48 h. Cell and capsule sizes were determined by India ink staining and differential interference contrast (DIC) microscopy using a Zeiss Axioplan 2 microscope. Cell body diameter and capsule thickness were measured using ImageJ software.

Assays for *in vitro* virulence factors and cell stress. The WT, mutant, and complementation strains were grown in YPD broth overnight (16 to 18 h). Harvested cells were washed three times in PBS. Tenfold serial dilutions of the WT, mutants, and the complementation strains were spotted onto agar plates and incubated at 30°C and/or 37°C for 2 days before photography. YPD agar medium was used to assess fungal growth at 37°C. To assess the ability of strains to produce melanin, 10-fold serial dilutions of the WT, mutants, and complementation strains were spotted onto L-DOPA agar plates (1 g · liter⁻¹ dextrose,

1 g · liter⁻¹ asparagine, 3 g · liter⁻¹ KH₂PO₄, 0.25 g · liter⁻¹ MgSO₄·7H₂O, 0.2 g · liter⁻¹ L-3,4-dihydroxyphenylalanine [Sigma; D9628], 1 mg · liter⁻¹ thiamine, 5 µg · liter⁻¹ biotin, 1.5% agar [pH 5.6].

Macrophage survival assay. The intracellular replication of WT and mutant strains in the J774A.1 mouse macrophage-like cell line (ATCC TIB-67) were determined as previously described (81). Briefly, the J774A.1 cell line was maintained at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 µg · ml⁻¹ penicillin-streptomycin, and 4 mM L-glutamine (Gibco). The cell line was used between passages 4 and 10 for the assays. Confluent monolayers of J774A.1 cells in 24-well plates (~2 × 10⁵ cells/well) were activated with 150 ng · ml⁻¹ phorbol myristate acetate (PMA) for 2 h prior to infection. Cells of the WT, mutants, and complementation strains were opsonized for 1 h with 1 µg · ml⁻¹ monoclonal antibody (MAb) 18B7 against GXM. Opsonized cells were then incubated with PMA-activated macrophages for 2 h at a multiplicity of infection (MOI) ratio of 1:1. Noninternalized fungal cells were washed off three times with 37°C PBS (Gibco). Wells were used for determination of the initial internalized fungal cells, or DMEM was added for continued coinoculation for 24 h at 37°C with 5% CO₂. To determine the number of internalized fungal cells, sterile distilled H₂O was added to each well to lyse the macrophages by incubation at 37°C for 0.5 h. Appropriate dilutions were plated on YPD agar for CFU counts. To determine the fungal intracellular replication at 24 h postinfection, extracellular fungal cells were washed off three times with 37°C PBS, followed by lysis of macrophages in sterile H₂O at 37°C for 0.5 h. Appropriate dilutions were plated on YPD agar for CFU counts.

Virulence of *atgΔ* mutants in a murine inhalation model of cryptococcosis. To assess the virulence of *C. neoformans* strains, a mouse survival assay with determination of fungal burden at the humane endpoint was executed as previously described (29, 31, 81). Virulence was assessed using female BALB/c mice (4 to 6 weeks old) from Charles River Laboratories (Ontario, Canada). Fungal cells were grown in 5 ml YPD at 30°C and washed three times with PBS (Gibco). Mice were anesthetized intraperitoneally with ketamine (80 mg · kg of body weight⁻¹) and xylazine (5.5 mg · kg⁻¹) and suspended on a silk thread by the superior incisors. A suspension of 2 × 10⁵ cells in 50 µl was slowly inoculated into the nares of the mice. The health status of the mice was monitored daily postinoculation. Mice were euthanized by CO₂ anoxia when their weight dropped below 85% of the initial weight. Fungal burdens of organs (lungs, brain, liver, spleen, and kidneys) and cardiac blood were assessed. The organs and blood were aseptically removed. Blood was retrieved from the heart using sterile syringes preincubated with 500 U of heparin. Organs were homogenized in 2 volumes of PBS using a Retsch MM301 mixer mill. The samples were serially diluted, plated on YPD containing chloramphenicol (30 µg · ml⁻¹), and incubated at 30°C for 2 days; CFU were then counted. The protocols for the virulence assays (A17-0117) and all animal experiments were approved by the University of British Columbia Committee on Animal Care.

Immune cell isolation. To assess host immune responses stimulated by *C. neoformans* infections, intratracheal instillation was performed on mice as previously described (82), and mice were euthanized at 6 dpi by isoflurane overdose. The whole lung was perfused with PBS via cardiac puncture of the right ventricle and the left lobe was harvested, minced, incubated in RPMI medium (with 0.7 mg · ml⁻¹ collagenase IV [Worthington, OH] and 50 µg · ml⁻¹ DNase I [Worthington]) for 1 h at 37°C, and passed through a 70-µm cell strainer to generate the lung homogenate. This was treated with red blood cell (RBC) lysis buffer, passed through a 35-µm cell strainer, and resuspended in FC buffer.

FC. Cells were incubated at 4°C with 2.4G2 tissue culture supernatant (TCS) for 20 min to block Fc receptors, washed with flow cytometry (FC) buffer (PBS, 2% bovine serum albumin [BSA], and 2 mM EDTA), labeled with MABs for 20 min at 4°C, washed twice in FC buffer, and resuspended in PBS containing LIVE/DEAD fixable aqua dead cell stain to label nonviable cells. Total cell numbers were counted using hemocytometer. For intracellular labeling, cells were fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature (RT), permeabilized with PBS, 2 mM EDTA, 0.1% saponin, and 1% BSA for 30 min at RT, blocked with 2.4G2, and then incubated with MABs against TNF-α and IFN-γ. The following MABs against mouse antigens were used for flow cytometry: CD4 (GK1.5), CD8α (53-6.7), CD11c (N418), CD11b (M1/70), CD45 (30-F11), CD49b (DX-5), T cell receptor β (TCRβ; H57-597), Ly6C (HK1.4), Ly6G (1A8), Siglec F (E50-2440 or 1RNM44N), TNF-α (MP6-XT22), and IFN-γ (XMG1.2). Antibodies were purchased from eBioscience, R&D Systems, BD Biosciences, or AbLab. Cells were processed on an LSR II (BD Biosciences) flow cytometer and analyzed using FlowJo software (Ashland, OR). Immune cell populations isolated from lungs of mice infected with the WT, *atg1Δ* mutant, or *atg8Δ* mutant or treated with PBS were first gated based on cell size and LIVE/DEAD staining, followed by sequential gating strategies of multiparameter flow cytometer analysis as shown in Fig. 9. A million cells were labeled for flow cytometry, and 10⁵ cells were acquired after gating by size, single cells, and viability.

Cytokine profiling. For cytokine profiling, excised lung pieces were collected in preweighed 500 µl PBS containing 2× cComplete, EDTA-free protease inhibitor cocktail (Roche). After total weight was determined, lungs were homogenized using a Retsch MM301 mixer mill. Supernatants of homogenates were collected and used for cytokine profiling using a BD cytometric bead array mouse inflammation kit (BD Biosciences).

Histology. Organs were harvested and fixed overnight in 37% formaldehyde. Samples were processed by Wax-it Histology Services (Vancouver, Canada) for paraffin embedding, sectioning, and staining with either hematoxylin and eosin (H&E) or Mayer's mucicarmine. Visualization of host tissues was performed using a Zeiss AxioSkop2 microscopy equipped with an AxioCam HRC camera.

Statistical analysis. All data are presented as means ± standard errors of the means (SEMs). Statistical analysis was performed using unpaired *t* tests for comparison of two groups *in vitro*, using Mann-Whitney test for *in vivo* experiments and using log rank test for mouse survival curve comparison.

Statistical tests were carried out using GraphPad Prism (La Jolla, CA) software. The replicates used were biological replicates. Results were considered significant at P values of ≤ 0.05 .

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00069-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, PDF file, 6.0 MB.

SUPPLEMENTAL FILE 3, PDF file, 4.6 MB.

SUPPLEMENTAL FILE 4, PDF file, 1.2 MB.

SUPPLEMENTAL FILE 5, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 6, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 7, PDF file, 0.3 MB.

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