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ARTICLE

Autophagy is redundant for the host defense against systemic *Candida albicans* infections

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Abstract Autophagy has been demonstrated to play an important role in the immunity against intracellular pathogens, but very little is known about its role in the host defense against fungal pathogens such as *Candida albicans*. Therefore, the role of autophagy for the host defense against *C. albicans* was assessed by complementary approaches using mice defective in autophagy, as well as immunological and genetic studies in humans. Although *C. albicans* induced LC3-II formation in macrophages, myeloid cell-specific ATG7$^{-/-}$ mice with defects in autophagy did not display an increased susceptibility to disseminated candidiasis. In in vitro experiments in human blood mononuclear cells, blocking autophagy modulated cytokine production induced by lipopolysaccharide, but not by *C. albicans*. Furthermore, autophagy modulation in human monocytes did not influence the phagocytosis and killing of *C. albicans*. Finally, 18 single-nucleotide polymorphisms in 13 autophagy genes were not associated with susceptibility to candidemia or clinical outcome of disease in a large cohort of patients, and there was no correlation between these genetic variants and cytokine production in either candidemia patients or healthy controls. Based on these complementary in vitro and in vivo studies, it can be concluded that autophagy is redundant for the host response against systemic infections with *C. albicans*.

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Introduction

Candida albicans is a commensal fungus that colonizes the gastrointestinal tract, skin, and mucosa of more than 50 % of healthy individuals. *Candida* colonization does not cause disease in healthy individuals, but in patients in whom the immune system is compromised, *Candida* can cause both mucosal and systemic disease, the latter with a mortality rate reaching up to 30–40 % [1]. *C. albicans* is recognized by the innate immune system through pathogen recognition receptors (PRRs) such as C-type lectin receptors or Toll-like receptors (TLRs) that interact with pathogen-associated molecular patterns on the *Candida* cell wall. *Candida* mannans are recognized by the macrophage mannose receptor and Dectin-2 [2, 3], Dectin-1 recognizes β-glucan [4], while DC-SIGN (CD209) recognizes fucose and mannose/mannan residues [5]. Furthermore, TLRs such as TLR2 [6] and TLR4 [7] also play an important role in the recognition of *C. albicans*. These interactions between *C. albicans* and the immune system lead to phagocytosis of the fungus [8] and the induction of proinflammatory cytokines, further promoting clearance of the infection [9].

In addition to these well-known effects of PRR engagement, recent studies have shown that TLRs can also engage autophagy proteins [10], and this, in turn, modulates the inflammatory reaction against pathogens [11–13]. Autophagy is an essential process for cell survival that allows the cell to efficiently regulate its biomass via the degradation of individual proteins (chaperone-mediated autophagy), cytosolic content, and whole-cell organelles (macroautophagy) [14]. Autophagy is characterized by the formation of a double-membrane vesicle, the autophagosome, which engulfs the cytosolic content to be degraded [15]. Subsequent fusion of the autophagosome with the lysosome and the breakdown of the inner membrane expose the content to hydrolases.

In addition to its role in cell homeostasis, autophagy has also been linked to the host defense against viruses [16] and to the processing of invading pathogens (xenophagy) [17, 18]. Inactivation of autophagy genes increases the replication of intracellular pathogens: Singh et al. demonstrated that, in mice, *IRGM* induces autophagy to eliminate intracellular *Mycobacterium tuberculosis* [19]; Zhao et al. demonstrated that ATG5$^{-/-}$ mice have decreased resistance to the intracellular bacterium *Listeria monocytogenes* and the protozoan *Toxoplasma gondii* [20]. Interestingly, *ATG5* and *ATG10* have been implicated in the defense against fungi in plants [21].

Little is known about whether autophagy is also involved in the immune response against fungal infections in mammals. Since several of the PRRs involved in the recognition of *C. albicans* have been demonstrated to induce autophagy, such as recruitment of the autophagosome marker LC3-II by TLR2 and TLR4 [10, 22], it is rational to hypothesize that autophagy might play an important role in the anti-*Candida* host immune response. In this study, the role of autophagy in the immune response against *C. albicans* was investigated using mouse knockout models, as well as human genetic association studies and in vitro experiments. We could not identify a major role for autophagy in anti-*Candida* host defense.

Materials and methods

Study population

To investigate the correlation between autophagy and candidemia, 338 adult candidemia patients (positive blood culture) and 351 healthy controls were enrolled in a study between January 2003 and January 2009 [23]. The candidemia study was approved by the Institutional Review Boards from the Duke University Hospital (Durham, NC, USA) and the Radboud university medical center (Nijmegen, The Netherlands). Participants were included after giving written informed consent, with the exception of patients who were no longer hospitalized or died before a positive blood culture report was made. To investigate the link between autophagy and cytokine production, 67 healthy individuals donated blood. The age of the patients ranged from 23 to 73 years and 77 % was male. Blood was collected by venipuncture into 10-ml EDTA syringes (Monoject, 's-Hertogenbosch, The Netherlands). The study with the healthy blood donors was approved by the Ethical Committee of the Radboud university medical center (Nijmegen, The Netherlands). Participants were included after giving written informed consent. The studies were performed in accordance with the Declaration of Helsinki.

Mice

The LysM-Cre^{+} or LysM-Cre^{-}ATG7$^{flox/flox}$ GFP-LC3^{+} (the conditional ATG7$^{flox/flox}$ mice is a kind gift from Masaaki Komatsu, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) was described previously (kindly provided by Douglas R. Green, St. Jude Children's Research Hospital, Memphis, TN, USA). All mice were housed in a pathogen-free facility. The animal study was approved by the Animal Care and Use Committee from the St. Jude Children's Research Hospital (protocol 482-100097-10/11). The study was performed in accordance with the guidelines set by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Microorganism

Candida yeast [American Type Culture Collection (ATCC) MYA-3573 (UC820)], a strain well described elsewhere [24],

were grown overnight in Sabouraud broth at 37 °C. Cells were harvested by centrifugation, washed twice, and resuspended in culture medium. *C. albicans* yeasts or hyphae were heat-killed for 1 h at 100 °C.

Macrophage differentiation, stimulation, and Western blotting

Bone marrow-derived macrophages (BMDMs) were differentiated from the total cells isolated from the femurs of 6–10-week-old mice by using supernatant from L929 cells as the differentiation medium. BMDM cells in 12-well tissue culture plates (5×10^5/well) were infected or treated with various *Candida* ligands [live *Candida* yeast form (moi 5), heat-killed *Candida* yeast form (moi 5), live *Candida* hyphae form, and heat-killed *Candida* hyphae form) for 8 h. The cells were lysed in RIPA lysis buffer supplemented with complete protease inhibitor mixture (Roche) and PhosSTOP (Roche). The whole-cell lysates were separated on 15 % SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5 % non-fat milk and incubated overnight with primary antibody at 4 °C and for 45 min with secondary HRP-tagged antibody at room temperature. The membranes were developed with SuperSignal West Femto Chemiluminescent Substrate (Pierce).

Candida albicans infection model

The WT (LysM-Cre$^-$ ATG7$^{f/f}$ GFP-LC3$^+$) and ATG7$^{-/-}$ (LysM-Cre$^+$ ATG7$^{f/f}$ GFP-LC3$^+$) mice were injected intravenously with the inoculum of *C. albicans* blastoconidia [1×10^6 colony-forming units (CFU)/mouse] in a 100-μl volume of sterile pyrogen-free phosphate-buffered saline (PBS). Survival was assessed daily for 30 days. For assessing fungal burden, subgroups of 5–10 mice were humanely terminated on days 3 or 7 of infection. To measure the fungal burden, the kidneys of the sacrificed animals were removed aseptically and homogenized in sterile PBS using a tissue grinder. The CFU values of the viable *Candida* from the kidney homogenates were measured by plating serial dilutions on Sabouraud dextrose agar plates (50 μg/ml of gentamicin), as described previously.

Fluorescence microscopy

HeLa cells were transfected with a plasmid containing GFP-LC3 (kindly provided by Dr. T Yoshimori, Osaka, Japan) using the transfection medium Fugene 6 (Roche), according to the manufacturer's instructions. GFP-LC3+ HeLa cells were grown and stimulated on coverslips (19-mm diameter) in 12-well plates. Cells were fixed with 2 % paraformaldehyde for 15 min at room temperature and permeabilized for 10 min with cold methanol (100 %). After washing with PBS (three times), the coverslips were mounted onto glass slides with Vectashield+ DAPI and analyzed on a fluorescence microscope.

Phagocytosis and killing assays

Phagocytosis and killing was performed as described previously [24, 25]. In short, 5×10^5 peripheral blood mononuclear cells (PBMCs) (in a volume of 100 μl) were put in a flat-bottom well. The plate was incubated at 37 °C for 1 h, to allow the monocytes to adhere to the plastic surface, in the absence or presence of 3MA (10 mM). Thereafter, the supernatant was removed, and the monolayer was rinsed with modified Eagle's medium (MEM). 200 μl of live *C. albicans* (5×10^4/ml) in MEM, 2.5 % serum was added, and the plate was incubated for 15 min to allow phagocytosis of the yeast. The supernatant was removed, and the monolayer was rinsed with MEM. 200 μl of MEM/Sabouraud was added to the monolayer, after which the plate was incubated at 37 °C for 2 h and 45 min to allow intracellular killing of the yeast. After this incubation period, the monocytes were lysed. Both the supernatants with non-phagocytosed *Candida* and the lysed monocyte suspension with non-killed *Candida* were directly plated in duplicate in two different dilutions on agar plates. These plates were cultured for 24 h, after which the number of colonies was counted. The percentage of phagocytosed and killed *Candida* was calculated.

PBMCs isolation

The separation and stimulation of PBMCs was performed as described previously [26]. Briefly, the PBMC fraction was obtained by density centrifugation of diluted blood (one part blood to one part pyrogen-free saline) over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMCs were washed twice in saline and suspended in culture medium. The cells were counted in a Coulter counter (Coulter Electronics, Buckinghamshire, England) and their number was adjusted to 5×10^6/ml.

Cell stimulation

A total of 5×10^5 human PBMCs in a 100-ml volume of RPMI was added to round-bottom 96-well plates (Greiner). Cells were stimulated with live *C. albicans* UC820 (1×10^4/ml) or *E. coli*-derived lipopolysaccharide (LPS) (*E. coli O55:B5* LPS, Sigma Chemical Co.), in the absence or presence of 3MA (Sigma). After 24 h, supernatants were stored at −20 °C. IL-1β, IL-8, and IL-10 was measured in cell culture supernatants using an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, MN, USA and Sanquin, Amsterdam, The Netherlands).

Autophagy gene SNP genotyping

Genomic DNA was isolated from EDTA blood of patients, matched controls, and a cohort of healthy volunteers using

standard methods, and 5 ng of DNA was used for genotyping. We selected 18 single-nucleotide polymorphisms (SNPs) from 13 autophagy-related genes (Table 1). Multiplex assays were designed using Mass ARRAY Designer Software

Table 1 Single-nucleotide polymorphisms (SNPs) in autophagy genes are not correlated with susceptibility to candidemia

Gene	SNP (AA change)	Genotype	Matched controls, *n* (%)	Patients, *n* (%)	χ^2	*p*-Value
ATG10	rs1864183	GG	92 (39)	109 (40)	0.561	0.755
	(Thr→Met)	GA	103 (43)	111 (41)		
		AA	42 (18)	54 (20)		
	rs3734114	TT	156 (66)	191 (69)	1.521	0.467
	(Pro→Ser)	TC	71 (30)	79 (29)		
		CC	8 (3)	5 (2)		
ATG16L1	rs2241880	TT	77 (32)	86 (31)	0.743	0.690
	(Thr→Ala)	TC	107 (45)	134 (49)		
		CC	53 (22)	55 (20)		
ATG16L2	rs11235604 (Arg→Trp)	CC	237 (100)	275 (100)	–	–
ATG2A	rs77228473 (Glu→Asp)	CC	237 (100)	275 (100)	–	–
	rs77833427	CC	234 (99)	273 (99)	0.024	0.878
	(Arg→His)	CT	2 (1)	2 (1)		
ATG2B	rs3759601	CC	93 (39)	127 (46)	2.795	0.247
	(Gin→Glu)	CG	105 (44)	112 (41)		
		GG	39 (16)	36 (13)		
	rs74719094	TT	237 (100)	272 (99)	2.601	0.107
	(Arg→Ser)	TG	0 (0)	3 (1)		
	rs9323945	CC	228 (98)	270 (99)	0.886	0.347
	(Asn→Asp)	CT	5 (2)	3 (1)		
ATG5	rs2245214	GG	39 (18)	40 (19)	0.740	0.691
	(Intron)	GC	104 (49)	93 (45)		
		CC	70 (33)	75 (36)		
ATG9B	rs61733329	CC	237 (100)	273 (99)	1.730	0.188
	(Gly→Ser)	CT	0 (0)	2 (1)		
EREG	rs34477425 (Arg→Gly)	AA	237 (100)	275 (100)	–	–
IRGM	rs4958847	AA	15 (6)	24 (9)	1.334	0.513
	(Intron)	AG	77 (32)	81 (30)		
		GG	145 (61)	168 (61)		
	rs72553867	CC	221 (93)	257 (93)	1.170	0.557
	(Thr→Lys)	CA	15 (6)	18 (7)		
		AA	1 (0)	0 (0)		
LAMP1	rs9577229	CC	215 (96)	241 (91)	4.911	0.086
	(Ala→Val)	CT	9 (4)	24 (9)		
		TT	1 (0)	1 (0)		
LAMP3	rs482912	AA	43 (18)	54 (20)	0.670	0.715
	(Ile→Val)	AG	94 (40)	114 (42)		
		GG	100 (42)	106 (39)		
P2RX7	rs2393799	TT	48 (20)	48 (17)	0.664	0.717
	(Upstream)	TC	90 (38)	107 (39)		
		CC	99 (42)	120 (44)		
WIPI1	rs883541	AA	153 (65)	176 (64)	0.004	0.998
	(Thr→Ile)	AG	76 (32)	88 (32)		
		GG	8 (3)	9 (3)		

The association between autophagy SNPs and candidemia susceptibility was assessed using the Chi-squared test or Fisher's exact test, as appropriate

(Sequenom) and genotypes were determined using Sequenom matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) according to the manufacturer's instructions (Sequenom Inc., San Diego, CA, USA). Briefly, the SNP region was amplified by a locus-specific polymerase chain reaction (PCR) assay. After amplification, a single base extension from a primer adjacent to the SNP was performed to introduce mass differences between alleles. This was followed by salt removal and product spotting onto a target chip with 384 patches containing matrix. MALDI-TOF MS was then used to detect mass differences and genotypes were assigned in real-time using Typer 4 software (Sequenom Inc., San Diego, CA, USA). As quality control, 5 % of samples were genotyped in duplicate and each 384-well plate also contained at least eight positive and eight negative controls; no inconsistencies were observed. DNA samples of which SNPs failed were excluded from the analyses. Variants with call rates below 90 % were also excluded from further analyses ($n=0$).

Statistics

Data were analyzed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) and SAS (version 9.3, SAS Institute, Cary, NC, USA). All statistical analyses were two-sided and $p<0.05$ was considered to be statistically significant (*). The survival was analyzed using the log-rank Mantel–Cox test, and the CFUs were analyzed with the Student's t-test. Differences in cytokine production were analyzed using the Wilcoxon signed-rank test. The Hardy–Weinberg equilibrium (HWE) was checked for each SNP using the program HWE Version 1.10 (Rockefeller University, New York, NY, USA). The associations between autophagy SNPs and candidemia susceptibility and clinical outcome of disease (30-day survival, persistent disease, and disseminated disease) were assessed using the Chi-squared test or Fisher's exact test, as appropriate. With the application of the Bonferroni correction for multiple testing, $p<0.003$ and $p<0.001$ were considered to be statistically significant, respectively.

Results

Candida albicans induces LC3-II shift in mouse BMDMs

Two forms of LC3 exist; LC3-I is located in the cytoplasm, while LC3-II is a processed form of LC3, which is associated with the (auto)phagosome membrane. Mouse BMDMs were stimulated with live and heat-killed *C. albicans* yeasts and hyphae. LC3-I and LC3-II were measured using Western blot. All forms, except heat-killed *C. albicans* yeasts, induced a strong upregulation of LC3-II expression (Fig. 1).

ATG7 is redundant for host defense against systemic *Candida albicans* infection

Because *C. albicans* induced autophagy in murine macrophages, we studied the effects of autophagy deficiency in myeloid cells on the outcome of systemic candidiasis. Although ATG7$^{-/-}$ KO mice appeared to have a slightly lower survival rate compared to WT mice, the difference was small and did not reach statistical significance (Fig. 2a). Furthermore, there were no differences in the fungal burdens in the kidneys, the target organ of disseminated candidiasis, 3 or 7 days after *C. albicans* infection of wild-type or autophagy-defective mice (Fig. 2b).

Candida albicans induces LC3-II shift in HeLa cells

Although autophagy does not seem to have a major impact on the outcome of murine candidiasis, different effects may be seen in humans. To investigate whether autophagy is induced upon *Candida* stimulation in human cells, we transfected HeLa cells with GFP-LC3 and stimulated them with heat-killed *C. albicans* yeast. LC3-II was analyzed using immunofluorescence microscopy. Heat-killed *C. albicans* yeasts induced a strong upregulation of LC3-II expression, demonstrating the induction of autophagy (Fig. 3a). The induction of LC3-II was reverted in the presence of the autophagy inhibitors 3MA and wortmannin (Fig. 3b, c).

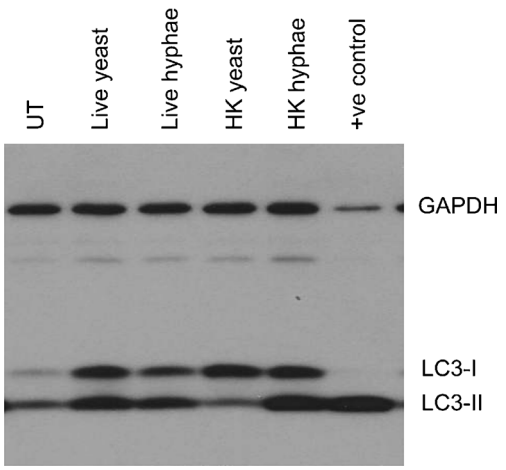

Fig. 1 *Candida albicans* induces LC3-II in mouse bone marrow-derived macrophages (BMDMs). Mouse BMDMs were stimulated with live and heat-killed (HK) *C. albicans* yeasts and hyphae. LC3-II was measured using Western blot (UT=unstimulated, +ve control=positive control)

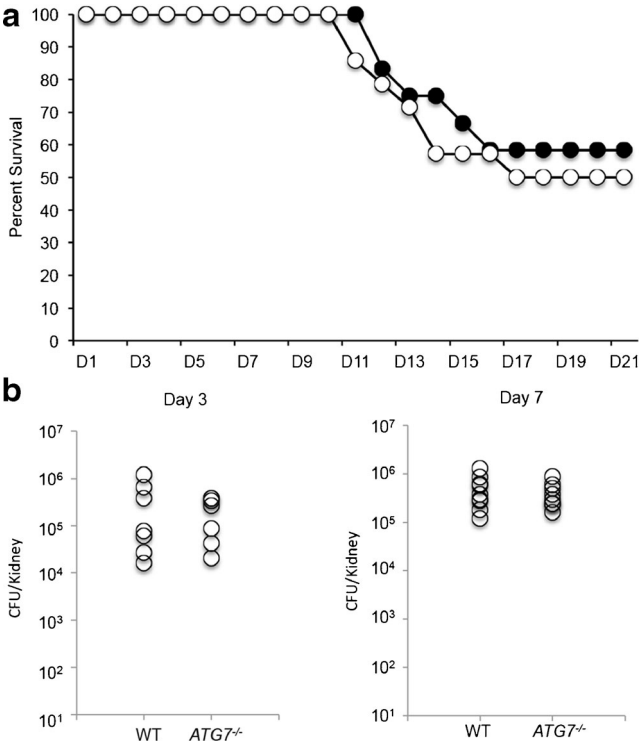

Fig. 2 No difference in survival between wild-type (WT) and ATG7$^{-/-}$ mice. **a** 13 WT (*open circles*) and 14 ATG7$^{-/-}$ (*filled circles*) mice were injected with live *Candida albicans* at day 1. Survival was monitored for 30 days. The survival was analyzed using the log-rank Mantel–Cox test. **b** After 3 and 7 days of infection, colony-forming units (CFUs) were counted in the kidneys ($n=7$ mice/group on day 3 and $n=9$ mice/group on day 7). The CFUs were analyzed with the Student's t-test

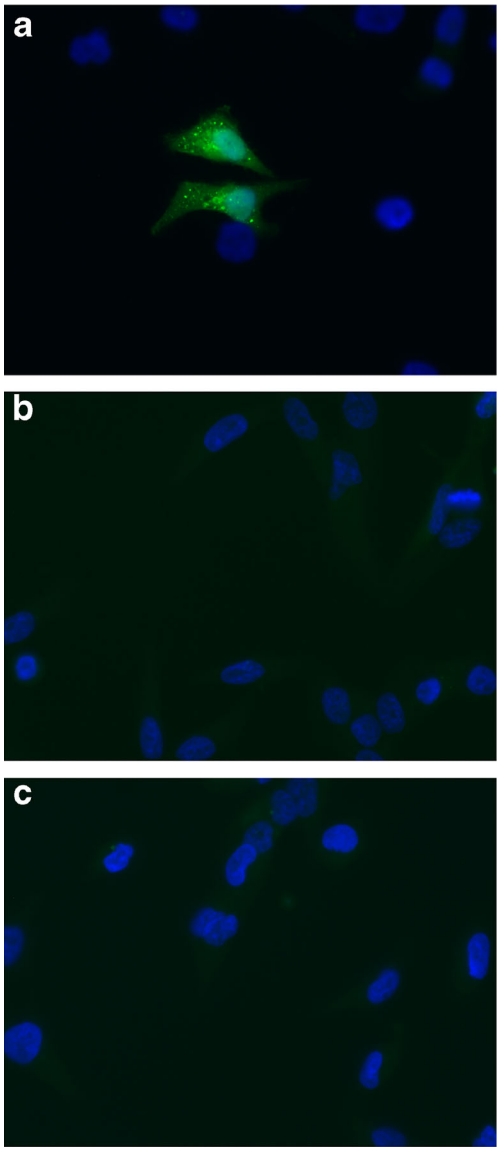

Fig. 3 *Candida*-induced LC3-II in GFP-LC3 transfected HeLa cells. **a** Fluorescence microscopy image (40×) showing LC3-II induction upon heat-killed *C. albicans* stimulation in GFP-LC3 transfected HeLa cells. In the presence of the autophagy inhibitors 3MA (**b**) and wortmannin (**c**), LC3-II is no longer induced, demonstrating the specificity of the assay

Inhibition of autophagy does not influence the phagocytosis and killing capacity of monocytes, or their cytokine production upon stimulation with *C. albicans*

In the next set of experiments, we investigated the importance of autophagy for the human anti-*Candida* host response. Firstly, we investigated whether autophagy is important for the phagocytosis and killing of live *C. albicans* by human monocytes. Freshly isolated human primary monocytes were stimulated with live *C. albicans* in the absence or presence of 3MA. Blocking autophagy with 3MA did not affect the capacity of human monocytes to phagocytose and kill *C. albicans* (Fig. 4a).

In a subsequent set of experiments, human PBMCs were stimulated with live *C. albicans* or *E. coli*-derived LPS, in the absence or presence of the autophagy inhibitor 3MA. While 3MA strongly increased LPS-induced IL-1β production, the increase in IL-1β production in *C. albicans*-stimulated cells was more modest and did not reach statistical significance (Fig. 4b). Stimulation of other proinflammatory cytokines such as TNF and IL-6 by *C. albicans* was not influenced by the modulation of autophagy (not shown).

SNPs in autophagy genes are not associated with candidemia

In order to further assess the importance of autophagy in the anti-*Candida* host defense in humans, we investigated whether polymorphisms in autophagy genes were correlated to susceptibility with systemic candidiasis. Although 18 SNPs in 13 different autophagy genes were studied, chosen based on their likelihood to influence the autophagy process, none of them were significantly associated with susceptibility to disseminated candidiasis (Table 1). Furthermore, none of the 18 SNPs studied were correlated with circulating cytokine concentrations in patients with candidemia or clinical outcome of disease (data not shown).

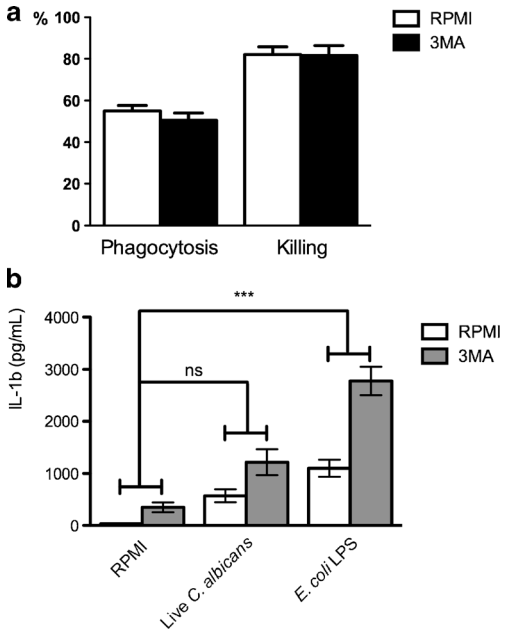

Fig. 4 Blocking autophagy does not inhibit the phagocytosis and killing capacity of human monocytes, nor the *C. albicans*-induced cytokine response. **a** Freshly isolated monocytes were stimulated with live *C. albicans*. The amount of phagocytosed and killed *Candida* was determined after 15 min and 3 h, respectively. The *bars* represent the mean±standard error of the mean (SEM) of four healthy volunteers. **b** Human peripheral blood mononuclear cells (PBMCs) were stimulated for 24 h with live *C. albicans* or *Escherichia coli*-derived lipopolysaccharide (LPS), in the absence or presence of 3MA. The concentration of IL-1β was measured in cell culture supernatants using enzyme-linked immunosorbent assay (ELISA). The *bars* represent the mean±SEM of 19 healthy volunteers. Differences in cytokine production were analyzed using the Wilcoxon signed-rank test (***$p<0.001$)

SNPs in autophagy genes do not correlate with *Candida*-induced cytokine production

Finally, we investigated whether the same SNPs in autophagy genes influenced *Candida*-induced cytokine production in PBMCs isolated from healthy volunteers. There were no statistically significant associations between the autophagy genotypes and *Candida*-induced cytokine production. A small number of these SNPs showed a tendency to influence cytokine production, but the associations were not statistically significant (Fig. 5).

Discussion

In this study, we investigated the role of autophagy in the anti-*Candida* host immune response. Using complementary immunological and genetic approaches in both mice and humans, we show that autophagy is redundant for the systemic host defense against *C. albicans*.

C. albicans is recognized by PRRs on the surface of the innate immune cells, and recent studies have shown that the engagement of PRR receptors can induce autophagy. Huang and Brumell demonstrated that LC3-II is recruited to the phagosome upon zymosan stimulation, through a Dectin-1-dependent pathway [27]. Indeed, both *C. albicans* and the Dectin-1 ligand β-glucan induce LC3 lipidation [28]. Furthermore, Nicola et al. also demonstrated that *Cryptococcus neoformans* can induce LC3 recruitment to the phagosome, although to a lesser extent than *C. albicans* [29]. LC3 lipidation does not necessarily implicate autophagy activation, but could also be a sign of LC3-associated phagocytosis (LAP) [30]. Here, we confirm the LC3-inducing activity of *C. albicans* by demonstrating that *Candida* stimulation was able to induce LC3-II in mouse BMDMs. Altogether, these data demonstrate that fungi could induce the process of autophagy, and this prompted us to investigate its role in host defense against disseminated candidiasis.

Surprisingly, however, mice with a specific deletion of the autophagy gene ATG7 in their myeloid cells did not display an increased mortality due to disseminated candidiasis. Furthermore, there was no difference in the fungal burden in the kidneys between wild-type and ATG7$^{-/-}$ mice. These data are paralleled by those of Nicola and colleagues, who also failed to find any difference in the survival between wild-type and conditional ATG5$^{-/-}$ mice infected with *C. neoformans* [29]. Although the authors reported that ATG5$^{-/-}$ mice die slightly sooner compared to wild-type mice upon infection with *C. albicans*, this difference was small (2 days), and they did not replicate this finding. Furthermore, the effect on *Candida* burdens in the kidneys was not reported [29]. In short, although both Nicola et al. and our group show that LC3-II can be induced upon fungal stimulation, the absence of autophagy has no major effect in in vivo infection models.

Because the immune system of mice can differ substantially from that of humans [31], we next investigated the potential role of autophagy in the immune response against *C. albicans* in humans. Similarly to what we have seen in mouse cells, we observed that LC3-II activation was induced upon *Candida* stimulation in the human HeLa cell line. Due to the fact that autophagy modulates inflammation induced by TLR ligands in human cells [32–34], we also tested whether anti-*Candida* immune responses are modulated by autophagy. Firstly, blocking autophagy with pharmacological inhibitors did not affect important aspects of the *Candida*-induced immune response, such as phagocytosis and killing of the fungus, or *Candida*-induced cytokine production. Of note, the inhibitors we used here are not completely specific. 3MA has been demonstrated to also be able to actually induce autophagy in some specific situations, and to influence cell survival through AKT1 [35]. Fortunately, similar results were obtained with 3MA and wortmannin. More importantly, Ma et al. previously demonstrated that the phagocytosis and killing of *C. albicans* is unaffected in LC3β-deficient mouse bone marrow-derived cells (BMDCs) [28]. The same is true

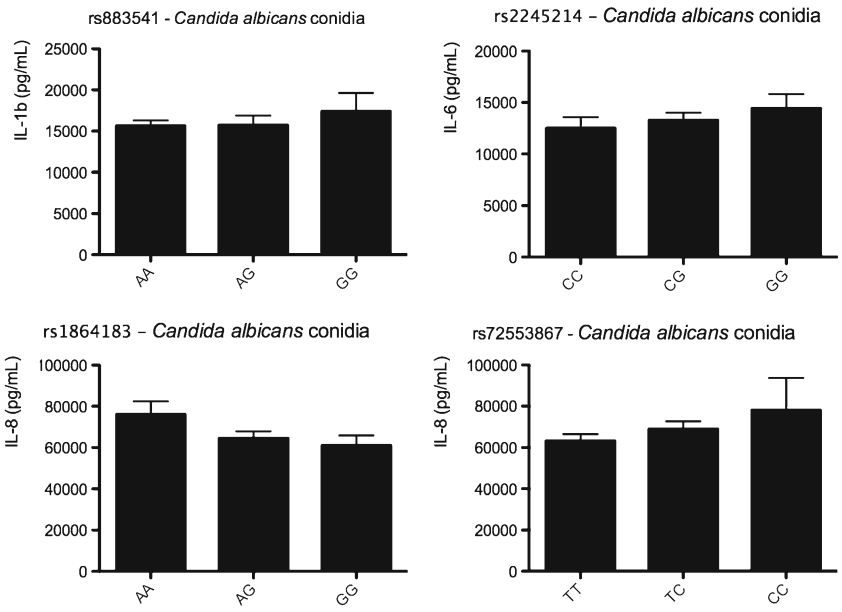

Fig. 5 No significant correlation between cytokine production and genotype in healthy volunteers. PBMCs of healthy volunteers were stimulated with heat-killed *C. albicans* conidia for 24 h. Cytokines were measured in cell culture supernatants using ELISA. Data are presented as the mean±95 % confidence interval (CI)

for *Salmonella enterica*, which can be recognized by autophagy machinery in the absence of LC3 recruitment [36].

Secondly, the (mostly) non-synonymous SNPs in autophagy genes (*ATG10*, *ATG16L1*, *ATG16L2*, *ATG2A*, *ATG2B*, *ATG5*, *ATG9B*, *EREG*, *IRGM*, *LAMP1*, *LAMP3*, *P2RX7*, and *WIPI1*) did not influence susceptibility to candidemia, nor did they influence serum cytokine levels in the patients or the clinical outcome of disease. However, several of these SNPs have been demonstrated to be associated with immune function. For example, rs2241880 in *ATG16L1* influences IL-1β and IL-6 production upon NOD2 stimulation [34], and rs72553867 in *IRGM* has been associated with inflammatory bowel disease [37]. The lack of association here could be explained by the fact that systemic candidiasis is a relatively rare disease, with a population frequency of 6:100,000 [38]. However, the genetic association study presented here has been performed in the largest cohort available to date. With the current sample size, we should be able to detect differences in proportions from 9 % and higher with a power of 80 % [39]. So, although we cannot fully exclude that these polymorphisms do influence the susceptibility to candidiasis, at least we can conclude that these effects, if existent, are very small. While several PRR and cytokine polymorphisms have been shown to be associated with susceptibility to candidemia [40, 41], the fact that none of the autophagy SNPs is associated with an increased susceptibility or severity of candidemia is another argument for a redundant role of autophagy for the systemic anti-*Candida* host defense in humans. Thirdly, we have identified no correlation between genetic variants in autophagy genes and ex vivo cytokine production by PBMCs of healthy controls. In line with this, Ma et al. demonstrated that cytokine production was normal in LC3β-deficient cells that completely lack functional autophagy [28].

Despite these complementary data demonstrating that autophagy does not play a central role for the systemic host defense against *Candida* spp., we cannot exclude a role of autophagy in other anti-*Candida* host defense mechanisms, e.g., mucosal antifungal defense. In order to prevent lysosomal degradation, *Candida* actively stimulates the recycling of LAMP-1 from the phagosome [42], an important protein involved in chaperone-mediated autophagy [43]. The C-type lectin receptor Dectin-1 is crucial for the recognition of β-glucans from *Candida* [44, 45], and defects in Dectin-1 have been previously shown to be associated with mucosal and skin *Candida* infections, but not systemic candidiasis [23, 46, 47]. While Dectin-1-dependent mechanisms induce autophagy [27], interestingly, it has also been demonstrated that the autophagy protein Rubicon can bind CARD9, dampening the signaling downstream of Dectin-1 [48]. Furthermore, Ma et al. showed that the recruitment of MHCII to the phagosome was reduced in LC3β-deficient cells, demonstrating that autophagy-related proteins may play a role in enhancing antigen presentation and adaptive immune responses [28]. Indeed, adaptive Th17 and Th1 responses are known to play an important role, especially for mucosal antifungal infections, as demonstrated in STAT1 mutations and STAT3 deficiency syndromes characterized by defective Th17 responses and chronic mucocutaneous candidiasis [49–51]. Taking into consideration this entire body of information, autophagy induction by *Candida* through Dectin-1-dependent mechanisms may play a role in the modulation of adaptive Th17 responses and mucosal antifungal defense, but this hypothesis remains to be demonstrated.

In conclusion, although *C. albicans* can induce LC3-II in both mice and human cells, the consequences at the level of phagocytosis, killing, and cytokine induction are limited, and autophagy is redundant for the host defense against systemic candidiasis. However, this does not exclude that autophagy could play a role in the mucosal anti-*Candida* immune response through antigen presentation and/or T-helper cell activation, and future studies are warranted to assess this possibility.

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Conflict of interest The authors declare that they have no conflict of interest.

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