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Cryptococcus neoformans modulates extracellular killing by neutrophils

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We recently established a key role for host sphingomyelin synthase (SMS) in regulating the killing activity of neutrophils against *Cryptococcus neoformans*. In this paper, we studied the effect of *C. neoformans* on the killing activity of neutrophils and whether SMS would still be a player against *C. neoformans* in immunocompromised mice lacking T and natural killer (NK) cells (Tgε26 mice). To this end, we analyzed whether *C. neoformans* would have any effect on neutrophil survival and killing *in vitro* and *in vivo*. We show that unlike *Candida albicans*, neither the presence nor the capsule size of *C. neoformans* cells have any effect on neutrophil viability. Interestingly, melanized *C. neoformans* cells totally abrogated the killing activity of neutrophils. We monitored how exposure of neutrophils to *C. neoformans* cells would interfere with any further killing activity of the conditioned medium and found that pre-incubation with live but not "heat-killed" fungal cells significantly inhibits further killing activity of the medium. We then studied whether activation of SMS at the site of *C. neoformans* infection is dependent on T and NK cells. Using matrix-assisted laser desorption–ionization tissue imaging in infected lung we found that similar to previous observations in the isogenic wild-type CBA/J mice, SM 16:0 levels are significantly elevated at the site of infection in mice lacking T and NK cells, but only at early time points. This study highlights that *C. neoformans* may negatively regulate the killing activity of neutrophils and that SMS activation in neutrophils appears to be partially independent of T and/or NK cells.

Keywords: neutrophils, sphingolipid, sphingomyelin, immunodeficient mice, fungal infection, *Cryptococcus neoformans*

INTRODUCTION

The fact that an infection caused by *Cryptococcus neoformans* still cannot be fully contained by the host is starkly evident. Almost all reviews on host defense against the ubiquitous environmental fungus emphasize the role of cell-mediated immunity (CMI), which is critical for containment of fungal cells through the activation of macrophages and neutrophils, resulting in granuloma formation (Huffnagle et al., 1991a,b; Goldman et al., 1994). While there are several studies elucidating the role and mechanisms by which alveolar macrophages control a *C. neoformans* infection (Feldmesser et al., 2001; Luberto et al., 2003; Fan et al., 2005; Shao et al., 2005; Alvarez and Casadevall, 2006; Ma et al., 2006; Rittershaus et al., 2006; Garcia et al., 2008; Stano et al., 2009), very little is known on the mechanisms by which neutrophils neutralize *C. neoformans*. Neutrophils are more effective killers than macrophages against fungi (Diamond et al., 1972; Miller and Mitchell, 1991) and they possess the innate ability to kill microbes without a CMI-mediated activation. We have recently shown that host sphingomyelin synthase (SMS) plays a pivotal role in the regulation of the killing activity of neutrophils against *C. neoformans* through a diacylglycerol-protein kinase D (DAG-PKD) dependent mechanism (Qureshi et al., 2010). In order to further understand the cryptococcal–neutrophil interaction, we have undertaken additional studies utilizing the HL-60 cell line model system and a mouse model lacking T and natural killer (NK) cells (Tgε26) to gain a better understanding of this important relationship in protecting the host.

MATERIALS AND METHODS

MATERIALS, STRAINS, AND GROWING MEDIA

Cryptococcus neoformans variety *grubii* serotype A strain H99 (WT), *Candida albicans* strain A39, and *Saccharomyces cerevisiae* JK9-3dα (WT) were routinely grown in yeast extract/peptone/2% dextrose-rich (YPD-rich) medium. *C. neoformans* Δ*gsc1* and Δ*isc1* mutant strains were created previously in our laboratory (Heung et al., 2005; Rittershaus et al., 2006; Shea et al., 2006). *C. neoformans GAL7::IPC1* strain was created from M001, an *ade2* isogenic strain derivative of H99, as described previously (Luberto et al., 2001). Melanized strain H99 was grown on BD BBL™Stacker™bird seed agar plates (cat. 297875). HL-60 cells (ATCC® CCL-240™) were cultured at 37°C, 5% CO_2 in RPMI 1640, supplemented with L-glutamine, 20% heat-inactivated FBS,

and 1% penicillin and streptomycin. RPMI 1640 medium, FBS, and penicillin–streptomycin were from Gibco/Invitrogen; pooled human serum, retinoic acid, and DMSO were from Sigma.

HL-60 VIABILITY ASSAY

To quantify the effect of *C. neoformans*, *C. albicans*, and *S. cerevisiae* on neutrophils, HL-60 cells were differentiated by incubation in the presence of 1.3% (v/v) DMSO and 2.5 μM retinoic acid for 72 h in growth medium. Cells were then washed with RPMI serum-free medium and 8×10^4 granulocytes/ml re-suspended in fresh RPMI containing 10% pooled human serum. These were co-cultured with 4×10^3 *C. neoformans*, *C. albicans*, or *S. cerevisiae* (20:1 ratio HL-60:yeast)/ml for 24 h at 37°C. At time points specified in the Section "Results," HL-60 cells were counted using trypan blue to determine their viability.

HL-60 MEDIUM KILLING ASSAY

To quantify the effect of HL-60 medium on *C. neoformans*, the killing assay described by Spellberg et al. (2007) was used. HL-60 cells were differentiated by incubation in the presence of 1.3% (v/v) DMSO and 2.5 μM retinoic acid for 72 h in growth medium. Cells were then washed with RPMI serum-free and 8×10^4 neutrophils/ml re-suspended in fresh RPMI containing 10% pooled human serum for 1, 2, 4, 6, 8, and 16 h. Conditioned media (900 μl) were then collected by centrifugation and 4×10^3 cells/ml *C. neoformans* (20:1 ratio HL-60:*C. neoformans*) incubated for 4 h at 37°C. At the end of the incubation, the medium was serially diluted and streaked onto YPD agar, and the plates incubated for 48 h at 30°C. CFUs were counted to assess killing of *C. neoformans* compared with control cultures of *C. neoformans* alone with no HL-60 medium. In order to check the effect of pre-incubating HL-60 cells with "heat-killed" *C. neoformans*, 4×10^3 *C. neoformans* cells/ml were boiled at 100°C for 10 min prior to addition to HL-60.

EFFECT OF PRE-INCUBATION WITH *C. NEOFORMANS* ON HL-60D MEDIUM KILLING ACTIVITY

In order to examine the effect of pre-incubating HL-60 cells with *C. neoformans* prior to removing the conditioned HL-60 medium for further experimentation, 8×10^4 neutrophils/ml were incubated with 4×10^3 *C. neoformans* H99 cells/ml for 1, 2, 4, and 6 h. At the specified time points, a portion of the cells were serially diluted and streaked onto YPD agar, and incubated for 48 h at 30°C. CFUs were counted to assess killing of *C. neoformans* at the various time points. At the same time, the remainder of the cells was centrifuged and the conditioned medium (800 μl) transferred to fresh wells. About 4×10^3 fresh *C. neoformans* cells/ml were then added and allowed to incubate for a further 4 h at 37°C. At the end of the incubation, the medium was serially diluted and streaked onto YPD agar, and incubated for 48 h at 30°C. CFUs were counted to assess killing of *C. neoformans* compared with control cultures of *C. neoformans* alone with no HL-60 medium.

EFFECT OF CAPSULE ON KILLING OF *C. NEOFORMANS* BY DIFFERENTIATED HL-60 (HL-60D)

Cryptococcus neoformans WT H99 and Δ*cap59* were used for capsule growth experiments. The yeast cells were grown overnight in 10 ml YPD at 30°C with shaking, collected in the logarithmic phase of growth by centrifugation (10 min at 3000 rpm at room temperature), washed twice with phosphate-buffered saline (PBS), and then incubated at a cell density of 5×10^6 cells/ml in the capsule growth inducing medium (DMEM buffered with 25 mM HEPES pH 7.2) overnight at 37°C, 5% CO_2. HL-60D cells were washed with RPMI and 8×10^4 cells/ml re-suspended in fresh RPMI supplemented with 10% pooled human serum and incubated for 6 h at 37°C, 5% CO_2. Conditioned media (900 μl) were then collected by centrifugation and 4×10^3 cells/ml *C. neoformans* H99 or Δ*cap59* added and allowed to incubate for 4 h at 37°C. At the end of the incubation, the medium was serially diluted and streaked onto YPD agar, and incubated for 48 h at 30°C. CFUs were counted to assess killing of *C. neoformans* compared with control cultures of *C. neoformans* alone with no HL-60 medium.

EFFECT OF MELANIN ON KILLING OF *C. NEOFORMANS* BY HL-60D MEDIUM

Following differentiation, HL-60D cells were washed with RPMI and 8×10^4 cells/ml re-suspended in fresh RPMI supplemented with 10% pooled human serum and incubated for 6 h at 37°C, 5% CO_2. Conditioned media (900 μl) were then collected by centrifugation and 4×10^3 melanized or non-melanized *C. neoformans* cells/ml added and allowed to incubate for 4 h at 37°C. At the end of the incubation, the medium was serially diluted and streaked onto YPD agar, and incubated for 48 h at 30°C. CFUs were counted to assess killing of melanized vs non-melanized *C. neoformans*.

NEUTROPHIL KILLING ASSAY

Approximately 1.6×10^5 freshly isolated murine neutrophils/ml were incubated in 124 ml sterile filtered PBS supplemented with 10% pooled mouse serum for 4 h in the presence of 9.92×10^4 *C. neoformans* cells/ml (10 μl used; 20:1 ratio murine neutrophils:*C. neoformans*). At the end of the incubation, the cultures were serially diluted and streaked onto YPD agar, and incubated for 48 h at 30°C. CFUs were counted to assess killing of *C. neoformans* compared with control cultures of *C. neoformans* alone with no neutrophils. Murine neutrophils were obtained from Tgε26 mice using the method of Shimizu et al. (2001) with the following modifications. Following harvest of the cells and a single wash with PBS, the collected cells were re-suspended in 4 ml PBS and neutrophils separated from mononuclear cells using 3 ml Ficoll-Paque™PREMIUM (GE Healthcare, cat. 17-5442-02). The mixture was centrifuged at $400 \times g$ for 40 min at 20°C. The resulting pellet was collected and red blood cells were lysed with 5 ml ice-cold water in ice for 1 min. Cold PBS (10 ml) was then added and the mixture centrifuged at $400 \times g$ for 10 min at 4°C. The resulting pellet was then re-suspended in 500 μl PBS. Mouse sera were obtained from Tgε26 mice.

ANIMAL STUDIES

Four- to six-week-old Tgε26 mice from the Animal Core Facility, Medical University of South Carolina, Charleston, were used for this study. Mice were anesthetized by intraperitoneal injection of 60 μl of a xylazine–ketamine mixture containing 5 mg xylazine and 95 mg ketamine per kg of body weight. The wild-type strain (H99) of *C. neoformans* was grown in YPD medium for 24 h at 30°C. The fungal cells were harvested, washed three times in PBS,

and re-suspended in PBS at a concentration of 2.5×10^7 cells/ml. Mice were infected intranasally with 20 µl containing 5×10^5 cells. Mice were fed *ad libitum* and monitored by inspection twice a day. Mice that appeared moribund or in pain were sacrificed using CO_2 inhalation followed by cervical dislocation. All animal procedures were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee and followed the guidelines of the American Veterinary Medical Association.

TISSUE SECTIONING AND SAMPLE PREPARATION

At 6, 12, and 15 days post-infection, mice were euthanized and lungs were harvested and flash-frozen in dry-ice/ethanol, then stored at −80°C until ready for use. The organ was attached to the cryostat sample stage using a small bead of optimal cutting temperature compound (OCT) at the base of the tissue only. The tissue was sectioned to a thickness of 30 µm at a temperature of −26°C using a cryotome (Microm HM 550, Walldorf, Germany). The sections were thaw-mounted onto conductive indium tin oxide (ITO) coated conductive glass slides for mass spectral analysis (Bruker Daltonics, Billerica, MA, USA). The resistance of the ITO coated microscope slide was 40 Ω over a distance of 1 cm. The tissue was allowed to warm on the microscope slides for 10 s before refreezing and storage at −80°C. For mass spectrometric analysis, the tissue sections were removed from the freezer and placed in a dessicator for 30 min prior to matrix deposition. A freshly prepared solution of 2,5-dihydroxybenzoic acid (DHB; Sigma; 40 mg/ml in 70% ethanol), was applied to the tissue by repeated cycles using a thin layer chromatography (TLC) sprayer. Each spray cycle was followed by 45–60 s of drying time, and the cycle repeated until an even coverage of matrix across the entire tissue was achieved.

MALDI mass spectrometry SM imaging

Matrix-assisted laser desorption–ionization (MALDI) mass spectral analysis was carried out using a reflector time-of-flight mass spectrometer (Bruker Autoflex III TOF-TOF, Bruker Daltonik, Bremen, Germany) operating in positive ion mode with a +20-kV accelerating potential. The laser beam size was set to medium, and operated at 200 Hz. Using Bruker Peptide Standard 1 (Bruker Daltonik, Bremen, Germany), a linear, external calibration was applied to the instrument before data collection. Mass spectral data sets were acquired over each whole mouse lung using flexImaging™-software (Bruker Daltonik, Bremen, Germany) in the mass range of *m/z* 500–1200 with a raster step size of 100 µm and 250 laser shots per spectrum. After data acquisition, molecular images were reconstituted using flexImaging™software. Each data set consists of approximately 4000 individual sampling locations, each representing one pixel in the resultant image. Data was normalized using flexImaging™software, and each *m/z* signal plotted ± 0.5 mass units. For display purposes, signals between sampling locations were interpolated and pixel intensities were scaled to utilize the entire dynamic range. Tandem mass spectrometry was used to identify signals detected in the MALDI imaging data sets. Lipids to be identified were extracted from mouse lung tissue by homogenization of the tissue in 70% ethanol. Samples were centrifuged at 45,000 rpm for 30 min at 4°C using a Beckman Optima TL Ultracentrifuge with a TLA45 rotor (Beckman Coulter, Inc., Fullerton, CA, USA), and the supernatant containing extracted lipids removed. Samples were concentrated using a speed-vac (Labconco, Kansas City, MO, USA), and spotted on a MALDI plate using 40 mg/ml DHB in 70% ethanol. Standard solutions of known lipids were also spotted in a similar manner. A timed ion gate was used for precursor ion selection and the fragments generated were further accelerated with 19 kV in the LIFT cell, and detected following passage through the reflectron. No CID gas was used for fragmentation of the precursor ions. Signals in the MALDI tissue imaging data set were identified based on matching fragmentation spectra of lipids extracted from the tissue and prepared lipid standard solutions.

SM DETERMINATION

For lipid analysis in lung tissues, lungs from two mice from each time point were homogenized in 5 ml homogenization buffer consisting of 0.25 M sucrose, 0.5 mM EDTA, 25 mM KCl, and 50 mM Tris–HCl at pH 7.4. Then, 1 mg of homogenate was used for the mass spectrometry analysis at the Medical University of South Carolina Lipidomics Facility whereas an aliquot was used for the quantitation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Qureshi et al., 2010) by Western blot using the LabWorks Image Acquisition and Analysis software from UVP BioImaging Systems, version 4.5. Lipids were extracted and analyzed using established protocols in the facility (Bielawski et al., 2006, 2009).

Statistics

All experiments were performed at least in triplicate. Statistical analyses of the data were performed using Student's *t*-test, and $P < 0.05$ was considered statistically significant.

RESULTS

CRYPTOCOCCUS NEOFORMANS DOES NOT AFFECT THE VIABILITY OF HL-60D CELLS

To determine the fate of HL-60 granulocytes exposed to *C. neoformans*, the viability of the granulocytes was determined at 1, 2, 3, 4, 5, 6, and 24 h following co-incubation with *C. neoformans* cells at a ratio of 20:1 (HL-60D:*C. neoformans*). As a comparison, *C. albicans,* which is known to affect viability (Mullick et al., 2004), was used as a positive control. **Figure 1** shows that *C. albicans* induced a significant level of mortality starting at 2 h in the HL-60 cells whereas *C. neoformans* did not. In contrast to *C. albicans*, but similar to *C. neoformans*, *S. cerevisiae* does not elicit a mortality response in HL-60.

KILLING ACTIVITY OF NEUTROPHILS IS SOLELY DUE TO SECRETED FACTORS

In order to determine the optimal time point for incubation of differentiated HL-60 cells in fresh medium prior to exposure to *C. neoformans*, following differentiation 8×10^4 granulocytes/ml were incubated in fresh RPMI medium containing 10% human serum for 1, 2, 4, 6, 8, and 16 h. The medium was then collected by centrifugation at 3000 rpm for 10 min and 4×10^3 *C. neoformans* cells/ml added and incubated for 4 h at 37°C. **Figure 2** shows that the percentage killing of *C. neoformans* was greatest at 6 h of incubation of differentiated HL-60 (HL-60D) in fresh medium and that the killing effect diminishes after this time. However the

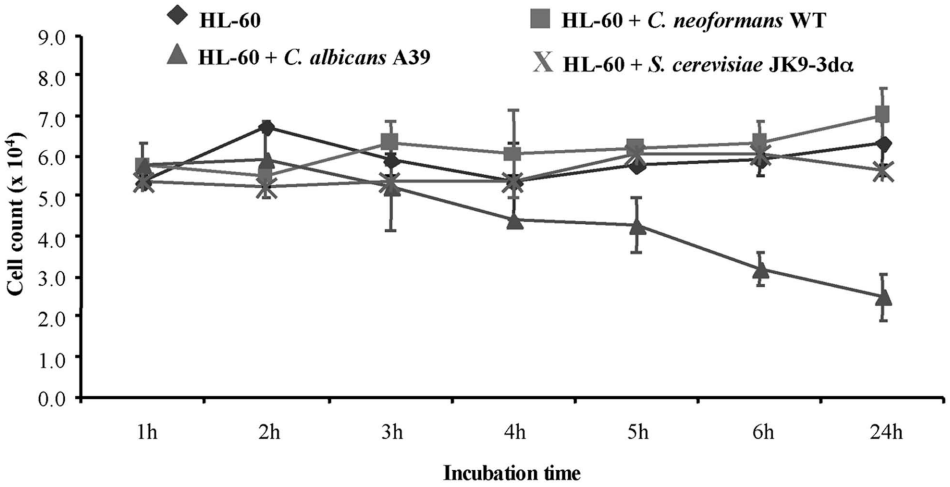

FIGURE 1 | *Cryptococcus neoformans* does not affect HL-60D viability. To quantify the effect of co-incubation, differentiated HL-60 cells (HL-60D) were incubated either alone or with *C. neoformans* WT (H99), *C. albicans* A39, or *S. cerevisiae* JK9-3dα. *C. albicans* affects viability of HL-60 cells whereas *S. cerevisiae* or *C. neoformans* do not.

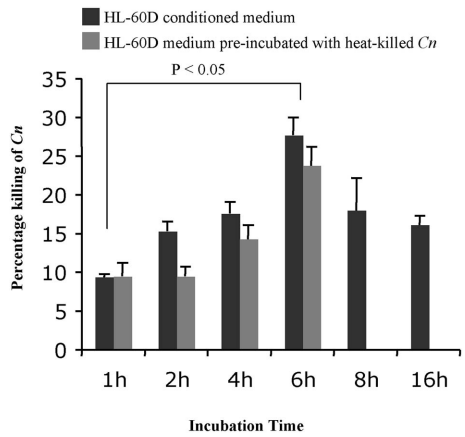

FIGURE 2 | Time course of the killing activity of neutrophils against *C. neoformans*. Six hours is the peak for the killing of HL-60D in fresh medium (10% HS + RPMI) prior to exposure to live *C. neoformans*. Incubation with heat-killed *C. neoformans* (up to 6 h) has no effect on the down-stream killing activity of the conditioned medium.

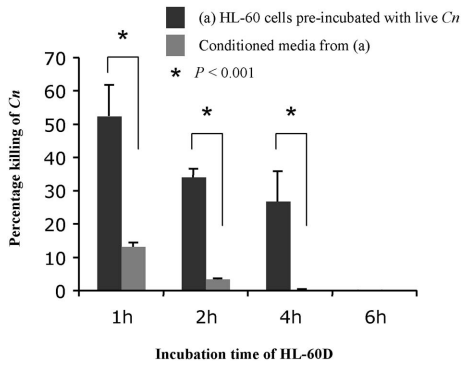

FIGURE 3 | Pre-incubation of HL-60D cells with *C. neoformans* significantly inhibits further killing activity of HL-60D medium. Pre-incubation with *C. neoformans* decreases any further killing of anti-cryptococcal factors in HL-60D medium.

presence of heat-killed *C. neoformans* has no effect on the down-stream killing activity of the conditioned medium. Together with the results shown in **Figure 1** in which viability of HL-60D at 6 h is not reduced, it appears that the killing of *C. neoformans* is solely due to secreted anti-cryptococcal factors (ACFs) by HL-60D.

PRE-INCUBATION OF DIFFERENTIATED HL-60 (HL-60D) CELLS WITH LIVE *C. NEOFORMANS* SIGNIFICANTLY INHIBITS ANY FURTHER KILLING ACTIVITY OF HL-60D MEDIUM

We next wished to test the effect of pre-incubation with *C. neoformans* on the killing ability of HL-60D medium (**Figure 3**). To this aim, following a 72-h incubation with 1.3% (v/v) DMSO and 2.5 μM retinoic acid as described above, 8×10^4 cells were washed, re-suspended in 1 ml fresh RPMI containing 10% pooled human serum and incubated with 4×10^3 *C. neoformans* cells for 1, 2, 4, and 6 h. At each time point, the killing activity was determined by counting CFUs and comparing to control cultures of *C. neoformans* alone with no HL-60 cells. At the same time, 800 μl of conditioned medium containing ACFs was collected by centrifugation at each time point and incubated with 4×10^3 fresh *C. neoformans* cells/ml for a further 4 h at 37°C. It was found that pre-incubation of HL-60D cells with *C. neoformans* decreases further killing ability of the ACFs in HL-60D medium.

EXTENT OF *C. NEOFORMANS* KILLING BY HL-60D IS NOT RELATED TO CAPSULE SIZE BUT IS RELATED TO MELANIN PRODUCTION

Since *C. neoformans* factors are able to neutralize the killing activity of the medium we sought to examine the effect of capsule

and melanin on the killing activity of the conditioned medium collected from HL-60D. *C. neoformans* produces a polysaccharide capsule, which consists of glucuronoxylomannan (GXM) and galactoxylomannan (GalXM), as well as mannoprotein (Bose et al., 2003). These components all occupy spatially separate and discrete regions in the capsule of *C. neoformans* (Jesus et al., 2010). Surprisingly the presence of capsule made no difference to the killing activity of HL-60D medium (**Figure 4A**), in line with previous findings suggesting that the extent to which different *C. neoformans* strains (capsular and acapsular) were killed by neutrophils was not consistently related to the size of the capsule or the entire cell (Miller and Mitchell, 1991). In order to determine the effect of HL-60D medium on the killing of melanized vs non-melanized *C. neoformans*, HL-60 granulocytes were incubated with melanized H99 for 4 h. Melanin has been shown to interfere with numerous host defense mechanisms, and it is well known that melanized *C. neoformans* cells are less susceptible to the toxic effects of microbicidal peptides than non-melanized cells (Doering et al., 1999). In addition, melanization protects *C. neoformans* against injury secondary to nitrogen or oxygen derived radical attack (Nosanchuk and Casadevall, 2003, 2006). In accordance with this, **Figure 4B** shows that HL-60D medium is unable to kill melanized *C. neoformans*, with no cell death observed suggesting that melanin totally protects *C. neoformans* from the killing activity of the conditioned medium collected from HL-60D.

FUNGAL INOSITOL SPHINGOLIPIDS AND GLUCOSYLCERAMIDE CONTENT AFFECT *C. NEOFORMANS* SUSCEPTIBILITY TO NEUTROPHILS

Fungal inositol containing sphingolipids have been implicated in the regulation of the sensitivity of *C. neoformans* to oxidative and nitrosative stresses (Shea et al., 2006). At the same time, glucosylceramide is essential for fungal growth in host extracellular environments (Rittershaus et al., 2006). Interestingly, intracellular growth is regulated by a different sphingolipid (phytoceramide C26) which is mainly produce by Isc1. We therefore wished to investigate whether the HL-60D medium killing of *C. neoformans* correlated with the amount of complex sphingolipids present in the H99 strain. In order to test this, we used *C. neoformans* WT H99, Δ*gcs1*, *GAL7::IPC1*, and Δ*isc1* mutant strains, each having a different "fitness" compared to the WT strain. The Δ*gcs1* mutant stain was included in the study as it shows no growth defect in the intracellular environment, compared to Δisc1 which grows poorly at low pH and is susceptible to intracellular compounds such as nitric oxide and hydrogen peroxide. Following incubation of these strains for 4 h with HL-60D medium, they were streaked onto YPD agar plates and CFUs counted after 48 h incubation at 30°C. We found that the *GAL7::IPC1* cells, in which the level of inositol sphingolipids compared to the WT is decreased (Luberto et al., 2001), were significantly less susceptible to the killing activity of HL-60D medium compared to WT cells (**Figure 5**). Similarly, the glucosylceramide-lacking strain (Δgcs1) was slightly more resistant (although not significant) to the killing activity of the

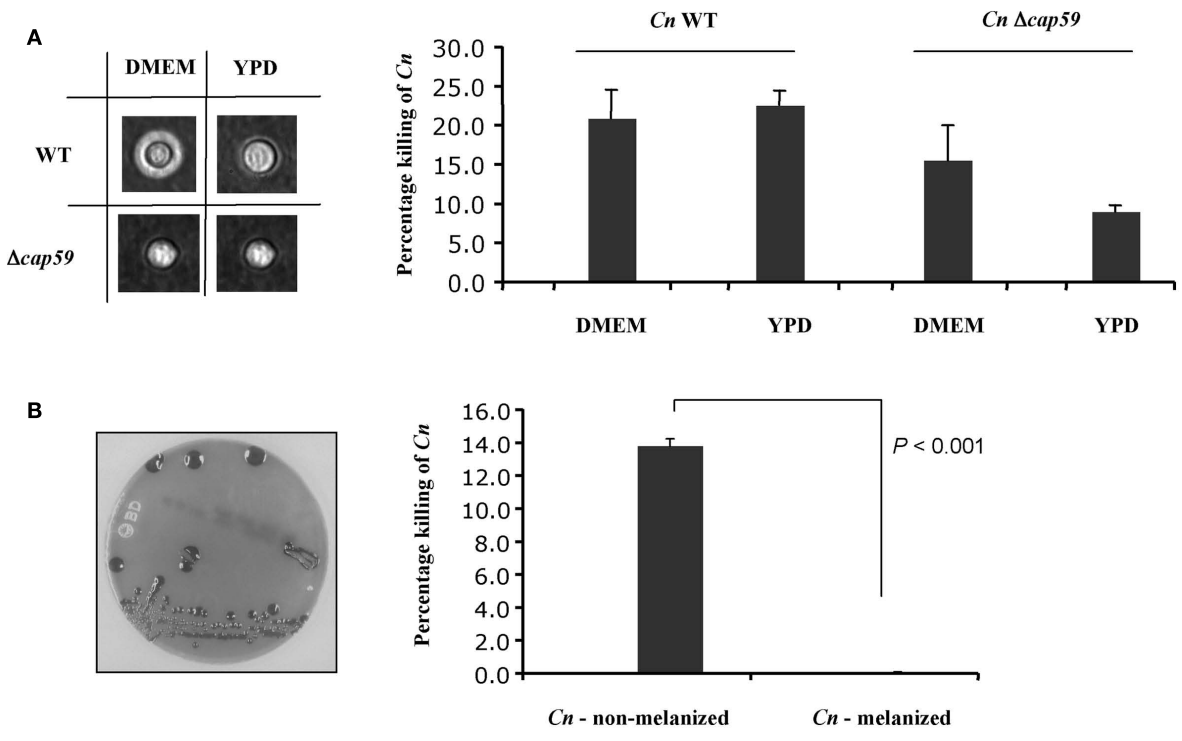

FIGURE 4 | Effect of *C. neoformans* capsule and melanin on neutrophils killing activity. (A) Capsule size does not affect HL-60D killing ability. The acapsular strain Δ*cap59* has no capsule, but is more resistant to killing by HL-60D, presumably due to its thicker cell wall. **(B)** Non-melanized *C. neoformans* is killed by HL-60D whereas melanin confers resistance against anti-cryptococcal factors.

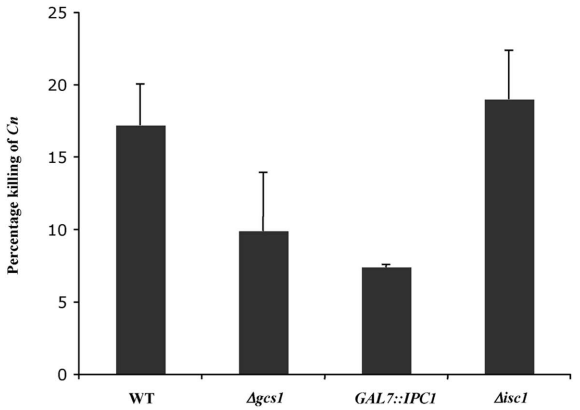

FIGURE 5 | Fungal inositol sphingolipids and glucosylceramide content affect *C. neoformans* susceptibility to neutrophils. Decrease in inositol sphingolipids (GAL7::IPC1) compared to WT seems to confer resistance to killing. Lack of glucosylceramide (Δ*gcs1*) is not significantly more resistant to killing. Δ*isc1* accumulates inositol sphingolipids and lacks phytoceramide C26 but it is as sensitive to killing as WT.

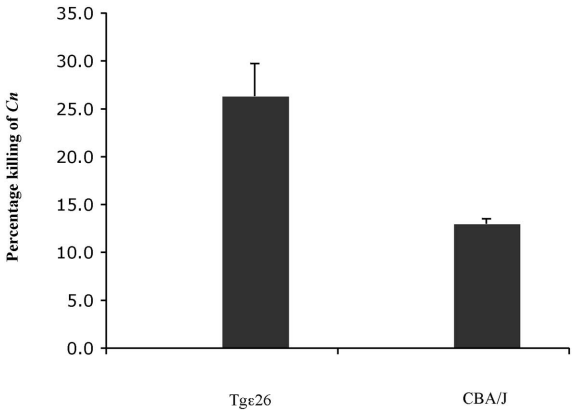

FIGURE 6 | Effect of freshly isolated murine neutrophils from immunocompromised host on *C. neoformans*. Neutrophils from both immunocompromised Tgε26 mice as well as immunocompetant CBA/J mice display the ability to kill *C. neoformans,* even though Tgε26 mice lack T and NK cells.

medium. Interestingly, the Δisc1 mutant which accumulates inositol sphingolipids (Henry et al., 2011) was as sensitive to the killing as the wild-type (**Figure 5**).

NEUTROPHILS FROM IMMUNOCOMPROMISED Tgε26 MICE KILL *C. NEOFORMANS*

Since cryptococcosis is an opportunistic fungal disease often seen in patients with impaired cellular immunity such as acquired immunodeficiency syndrome (AIDS), and since the host defense is regulated by CMI (Lim and Murphy, 1980) where CD4+ T cells play a central role in limiting infection (Hill and Harmsen, 1991; Huffnagle et al., 1991a; Kawakami, 2002; Uezu et al., 2004), we were interested in studying whether neutrophils from an immunocompromised host lacking NK and T cells would still be able to kill *C. neoformans*. We had previously observed that neutrophils obtained from immunocompetent mice having a fully functional immune system were able to kill *C. neoformans* (Qureshi et al., 2010). To this aim, we employed fresh murine neutrophils from Tgε26 mice, and carried out the killing assay as described in the Section "Materials and Methods". It was found that these mouse neutrophils kill *C. neoformans* even though they lack NK and T cells (**Figure 6**).

SPHINGOMYELIN 16:0 IN LUNGS OF MICE LACKING NK AND T CELLS IS UPREGULATED WHEN MICE ARE INFECTED WITH *C. NEOFORMANS*

Matrix-assisted laser desorption/ionization-mass spectrometric imaging (MALDI–MSI) allows the visualization of the spatial distribution of specific molecules according to their *m/z* ratio within thin sections of tissue. The identification of the imaged lipid was accomplished by MALDI tandem mass spectrometry where fragmentation patterns were compared to those of sphingomyelin standards (see Materials and Methods). In our previous studies we showed that killing activity of neutrophils is mediated by SMS activity, and that there is a differential distribution of SM species as observed by MALDI–MSI. It is known that infection by *C. neoformans* mainly occurs in immunodeficient subjects, particularly those in which T cells count is drastically low (e.g., HIV positive subjects; Kovacs et al., 1985; King and Dewitt, 2010). Since we found that neutrophils isolated from the Tgε26 mice are able to kill *C. neoformans* very efficiently (**Figure 6**), and in previous studies we found that this killing ability is mediated by SMS activity (Qureshi et al., 2010), it was important to determine whether SMS activity would also have a role against *C. neoformans* under conditions of T cell deficiency. Thus, we examined the distribution of SM (as a read-out of SMS activity) species in *C. neoformans*-infected vs un-infected lung of Tgε26 mice. Overall, we found that SM 16:0 was distributed more homogenously in Tgε26 compared to CBA/J infected lungs (**Figure 7**). However, SM 16:0 was still clustered around the site of *C. neoformans* infection where an intense neutrophil infiltration was observed especially at day 15 of infection, although it was much less organized than what we observed in CBA/J lungs (**Figure 7**).

LIPID ANALYSIS BY HPLC-MS/MS

Since we found that SM 16:0 was elevated in the lungs of Tgε26 mice, we wondered whether any other subspecies of SM not observed by MALDI–MSI would also be elevated. To address this question, we examined the level of different species of SM, such as 16:0, 18:0, 18:1, 20:0, 20:1, 22:0, 22:1, 24:0, and 24:1 in lung of Tgε26 infected mice. As expected, we found that among the measured species, SM 16:0 and 24:1 were the most abundant. Indeed, these two species have been shown to be the most abundant in many cellular types and tissues (Fitzgerald et al., 1995; Venable et al., 1995; Bielawska et al., 2000; Merrill et al., 2005; Kono et al., 2006; Valsecchi et al., 2007; Jin et al., 2008a). Importantly, LC–MS results showed that SM 16:0 was significantly elevated at day 12 of infection compared to un-infected mice (**Figure 7**) but not at a later time point, as we observed in CBA/J mice. These results suggest that the production of this sphingolipid might be independent of T cell activation in immunocompromised mice but only at early time points of infection. In fact, the basal levels of SM 16:0 in Tgε26 un-infected lung (**Figure 8**) was similar to the levels

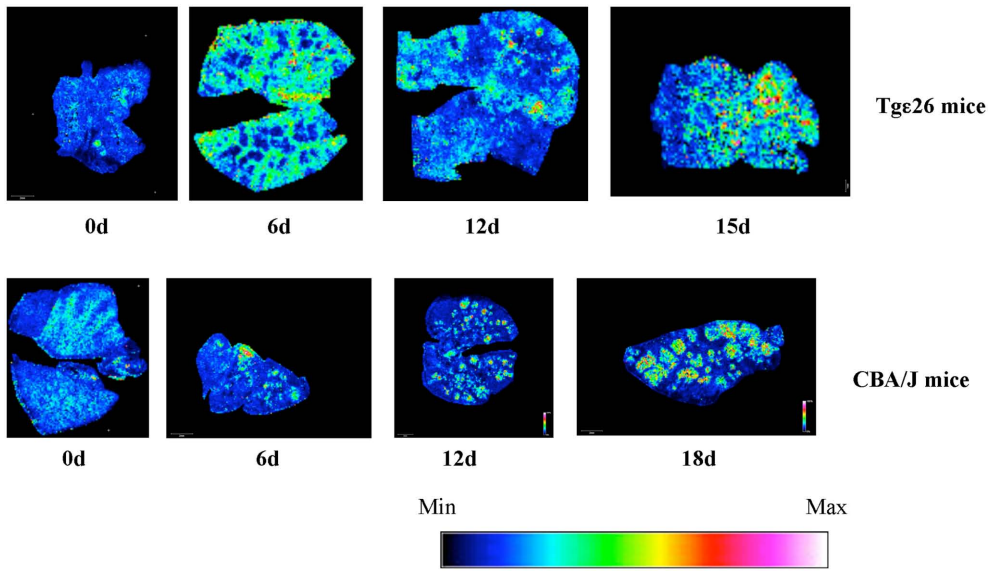

FIGURE 7 | Effect of *C. neoformans* infection on the level of SM 16:0 in lungs of mice lacking NK and T cells. Lungs of Tgε26 mice un-infected and infected with *C. neoformans* wild-type H99 strain were processed for MALDI tissue imaging. Sphingomyelin (SM) 16:0 species ($[M + Na]^+$ *m/z* 725) is distributed throughout the lungs at all timepoints. Min and Max, minimum and maximum intensity of SM 16:0 in the lung, respectively.

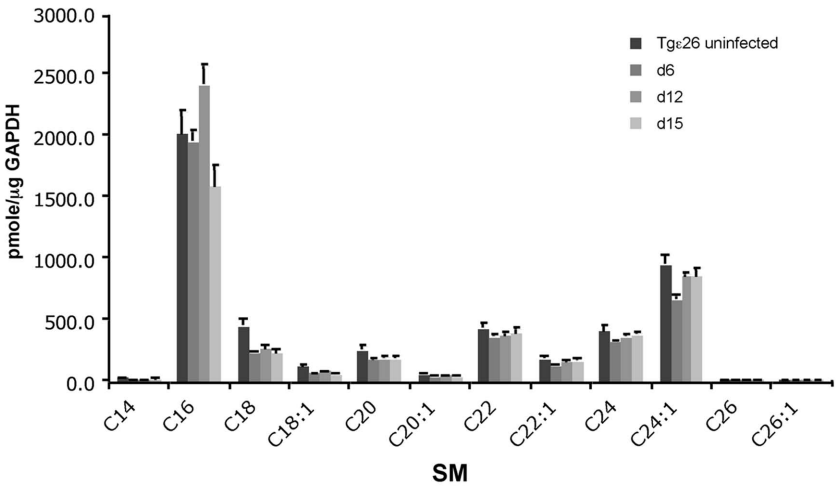

FIGURE 8 | Quantitative analysis of the lung sphingolipids upon *C. neoformans* infection. Liquid Chromatography–Mass Spectrometry (LC–MS/MS) analysis of immunocompromised mouse lungs infected with wild-type *C. neoformans* show that SM 16:0 is the major species elevated at the site of infection. Other SM species remain constant or are not detected.

found in CBA/J un-infected lung (Qureshi et al., 2010). We could not detect any SM 14:0, 26:0, or 26:1, suggesting that these species are either not produced by cells found in the mouse lung or that their levels are too low to be detected by MS.

DISCUSSION

In this investigation, the effect of pre-incubation of *C. neoformans* with HL-60 derived neutrophils has been studied. Our results show that pre-incubation of neutrophils with live but not heat-killed *C. neoformans* inhibits any further killing by secreted ACFs. Furthermore, activation of SMS in neutrophils, which is responsible for modulating their killing activity, appears to be partially independent of T and/or NK cell activation.

Neutrophils play important roles in host defense against all classes of infectious agents including *C. neoformans*. They constitute the second line of defense against pathogens after alveolar macrophages because, once an inflammatory response is initiated, neutrophils are the first cells to be recruited to the site of infection (Schleimer et al., 1989). The microbicidal arsenal of neutrophils includes the formation of reactive oxygen and nitrogen

species, hydrolytic enzymes, and antimicrobial peptides, all of which target microbes (Smith, 1994). Neutrophils can also kill extracellular pathogens via the formation of neutrophil extracellular traps (NETs), which have activity against *C. albicans* and *Aspergillus fumigatus* (Urban et al., 2006a,b; Bruns et al., 2010). Notably, neutrophils are more effective killers of *C. neoformans* than macrophages, and they possess the innate ability to kill microbes without a cell-mediated activation. So while it has long been appreciated that neutrophils kill *C. neoformans* in part via generation of fungicidal oxidants, non-oxidative mechanisms also make a significant contribution (Mambula et al., 2000). Interestingly, transfusion of human neutrophils significantly improved the survival of mice challenged with fungal organisms (Spellberg et al., 2005, 2007), suggesting that these cells are able to control the infection through their antifungal activity.

To gain a better understanding of the neutrophil–*C. neoformans* interaction, and specifically the interaction of ACFs secreted by differentiated HL-60 (HL-60D) cells into the medium, we turned our attention to several *in vitro* systems. Before studying the response of neutrophils upon *C. neoformans* challenge however, we verified that the viability of HL-60 derived neutrophils used in this study is not reduced upon exposure to *C. neoformans*, as is the case with *C. albicans* where viability in the HL-60 granulocytic population is significantly affected (Mullick et al., 2004) upon phagocytosis of *C. albicans* by neutrophils, which in turn induces the HL-60 derived neutrophils to undergo apoptosis. In the case of *C. neoformans*, we have shown that killing is induced through the release of ACFs instead of phagocytosis (Qureshi et al., 2010). This suggests that, in contrast to *C. albicans*, *C. neoformans* cells may have developed other mechanisms to protect themselves against the killing activity of neutrophils. Thus, we sought to determine whether *C. neoformans* would be able to neutralize the antifungal action of the medium.

Interestingly, secretion of the ACFs by HL-60 derived neutrophils into the medium, and subsequent killing of *C. neoformans*, is maximal at 6 h. However, co-incubation of HL-60D cells with *C. neoformans* shows maximal killing at 1 h (**Figure 3**), followed by steady-state killing at 2 and 4 h, and no killing at 6 h, at which time it is known that *C. neoformans* cells start doubling in RPMI (Miller and Mitchell, 1991). Therefore 4 h was chosen as the time point for *C. neoformans* killing by HL-60D medium. Surprisingly, when the medium of HL-60D cells is used for further killing of *C. neoformans*, after the preliminary *C. neoformans*–HL-60D co-incubation, percent killing is severely reduced, implying that co-incubation of HL-60D cells with *C. neoformans* decreases the killing ability of the ACF in the medium, either because some *C. neoformans* binds such factors and thus neutralizes them and/or because *C. neoformans* degrades such factors. Thus *C. neoformans* appears to play a pivotal role in the killing-degradation cycle of the ACFs. The presence of the pathogen induces neutrophils to kill *C. neoformans*, but degradation of the ACFs which are secreted into the medium by neutrophils is also driven by the presence of *C. neoformans*. This observation is supported by studies showing that *C. neoformans* is able to secrete a variety of proteins including proteases (Eigenheer et al., 2007). In order to better understand this interaction, we sought to explore specifically the effect of *C. neoformans* virulence factors upon the ACFs.

The antiphagocytic polysaccharide capsule and melanin are important virulence components of *C. neoformans*. The regulation of these virulence factors has been reviewed recently in several excellent works (Li and Mody, 2010; Alspaugh et al., 2011; Fox et al., 2011; Idnurm et al., 2011; Nielsen and Kwon-Chung, 2011; Pfaller et al., 2011). Interestingly, the presence of capsule made little to no difference on killing of *C. neoformans* by the ACF in HL-60D medium. Notably, the acapsular strain *C. neoformans* Δ*cap59* was more resistant to killing by ACF, regardless of growth conditions. This is likely due to the fact that despite lacking a polysaccharide capsule and the protective architecture conferred upon it by the main capsular components, *C. neoformans* Δ*cap59* has a thicker cell wall which may provide some resistance to killing. Melanin on the other hand confers excellent resistance to *C. neoformans* against ACFs in the HL-60D medium.

Inositol phosphoryl ceramide synthase 1 (Ipc1) is a fungal enzyme that transfers inositol phosphate from phosphatidylinositol (PI) to phytoceramide, producing IPC and diacylglycerol (DAG; Kuroda et al., 1999; Heidler and Radding, 2000). Once produced, IPC is metabolized by Isc1 (Dickson and Lester, 1999; Henry et al., 2011), and, by doing so, it regulates the levels of complex sphingolipids and very long chain phytoceramides in *C. neoformans* (Shea et al., 2006; Garcia et al., 2008; Tommasino et al., 2008; Henry et al., 2011). Thus, we wished to examine the effect of the modulation of these sphingolipids on killing under conditions where Ipc1 was downregulated or Isc1 deleted. Loss of Isc1 leads to an accumulation of complex sphingolipids and a specific depletion of phytoceramide C26, whereas no changes are observed with phytoceramide C18 or other subspecies (Garcia et al., 2008). Downregulation of Ipc1 (*GAL7::IPC1*, grown in glucose) shows less complex sphingolipids, no change in phytoceramide C26 and accumulation of C18 phytoceramides compared to the WT strain.

The observation that the *GAL7::IPC1* strain (which contains less inositol sphingolipids) is less susceptible to the killing activity of neutrophils would argue that the presence of complex sphingolipids may favor the action of the ACFs present in the medium. However, the fact that accumulation of complex sphingolipids (Δ*isc1*) does not render the cells more susceptible than the wild-type does not support this hypothesis. On the other hand, the increased resistance to killing by neutrophils may be due to accumulation of phytoceramide C18 (observed in the *GAL7::IPC1* strain). This hypothesis is supported by the results showing that the Δ*gcs1* strain is slightly more resistant and, in this strain, ceramide C18 accumulates (Rittershaus et al., 2006). Taken together with the results of the Δ*isc1* strain where no change in phytoceramide (or ceramide) C18 correlates with no change in susceptibility, this would support the hypothesis that phytoceramide C18 and ceramide C18 play a role in resistance.

In continuation of our work on the role of host SMS in the regulation of the killing activity of neutrophils against *C. neoformans*, we investigated whether neutrophils derived from immunocompromised mice would still kill *C. neoformans*. Neutrophils from transgenic epsilon 26 mice (Tgε26), deficient in NK cells and T cells, were used to explore the killing of *C. neoformans* WT. Macrophages, monocytes, and granulocytes are present and functional in these mice (Wang et al., 1994, 1997). NK cells constitute a major component of the innate immune system and are

responsible for release of cytoplasmic granules that kill pathogen cells, whereas T cells play a major role in CMI. The fact that neutrophils from Tgε26 mice were able to kill *C. neoformans* despite lacking T cells implies that activation of SMS in the host is partially independent of T and/or NK cells.

Finally, using MALDI–MS imaging of lung tissue from Tgε26 mice infected with *C. neoformans* WT we observed that SM 16:0 is found in the immunocompromised mouse lung similarly as in the immunocompetent lung, suggesting that NK and/or T cells are not necessary to produce a basal level of SM 16:0. When the lungs were infected with *C. neoformans* though, we found that SM 16:0 was elevated only at day 12 of the infection and its overall distribution was much less organized in the immunocompromised compared to the immunocompetent host, suggesting that T and/or NK cells may have a role in activating SMS in the lung, and coordinating the SMS response of neutrophils against *C. neoformans*, especially when the infection progresses. It has been suggested that sphingolipid-rich microdomains form platforms for the regulation and transduction of T cell receptors which cluster at the center of the T cell-antigen presenting cell interface during T cell activation. T cell clustering was shown to be impaired in an SMS knockdown cell line (Jurkat-SMS1/kd), indicating that SM and hence SMS may be important for full T cell activation (Jin et al., 2008b), and in the case where T cells are lacking, SMS will be somewhat impaired as one of its regulatory functions will be curtailed.

In conclusion, we have shown that pre-incubation of live but not heat-killed *C. neoformans* inhibits further killing activity of ACFs. Neither the presence of *C. neoformans* nor the capsule size has any effect on neutrophil viability but melanin confers resistance to *C. neoformans* against neutrophil killing. Finally SMS activation in neutrophils may be independent of T cell and/or NK cells activation.

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