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Original Article

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Melanocytes and melanin represent a first line of innate immunity against *Candida albicans*

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Abstract

Melanocytes are dendritic cells located in the skin and mucosae that synthesize melanin. Some infections induce hypo- or hyperpigmentation, which is associated with the activation of Toll-like receptors (TLRs), especially TLR4. *Candida albicans* is an opportunist pathogen that can switch between blastoconidia and hyphae forms; the latter is associated with invasion. Our objectives in this study were to ascertain whether *C. albicans* induces pigmentation in melanocytes and whether this process is dependent on TLR activation, as well as relating this with the antifungal activity of melanin as a first line of innate immunity against fungal infections. Normal human melanocytes were stimulated with *C. albicans* supernatants or with crude extracts of the blastoconidia or hyphae forms, and pigmentation and TLR2/TLR4 expression were measured. Expression of the melanosomal antigens Melan-A and gp100 was examined for any correlation with increased melanin levels or antifungal activity in melanocyte lysates. Melanosomal antigens were induced earlier than cell pigmentation, and hyphae induced stronger melanization than blastoconidia. Notably, when melanocytes were stimulated with crude extracts of *C. albicans*, the cell surface expression of TLR2/TLR4 began at 48 h post-stimulation and peaked at 72 h. At this time, blastoconidia induced both TLR2 and TLR4 expression, whereas hyphae only induced TLR4 expression. Taken together, these results suggest that melanocytes play a key role in innate immune responses against *C. albicans* infections by recognizing

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pathogenic forms of *C. albicans* via TLR4, resulting in increased melanin content and inhibition of infection.

Key words: melanocytes, melanin, antifungal activity, *Candida*, Toll-like receptors.

Introduction

Melanocytes, dendritic cells of neuroectodermal origin, are a major component of the epidermis. Although they are located in the basilar layer, they extend their dendrites to make contact with other epidermal cells such as keratinocytes and Langerhans cells [1,2]. Melanocytes synthesize a pigment called melanin, and their main role is to protect the skin against ultraviolet (UV) radiation by transferring melanin (synthesized in specialist organelles called melanosomes) to keratinocytes [1–3]. Melan-A (formerly MART-1) and gp100 (or Pmel-17) are antigens involved in melanosome maturation. Melan-A, the concentration of which correlates with melanin content, is necessary for gp100 function. As a result, it plays an important role in regulating pigmentation [4].

The presence of melanocytes in organs other than the skin (and therefore not involved in protection against UV radiation) raises the question of whether their sole role is to be an UV scavenger. Comparative and developmental biological studies of melanization suggest a different scenario, that is, melanization is a very important component of the innate immune response [5]. In invertebrates, melanocytes modulate melanin production during infection; in humans, inflammation often leads to hypo- or hyperpigmentation [3,5]. Melanin (specifically eumelanin, or black melanin) has antimicrobial properties [1,5,6]. In addition, the activation of Toll-like receptors (TLRs) 1, 2, 4, 5, and 7, particularly TLR4, is associated with an increase in melanocyte pigmentation [6,7].

Candida albicans is an opportunistic pathogen that colonizes the skin and mucosae. An important virulence mechanism of *C. albicans* is its ability to transform from the yeast (blastoconidia) form into a hyphae, a process associated with tissue invasion [8]. TLR2 and TLR4 are involved in the differential recognition of blastoconidia and hyphae [9,10]. Candidiasis is responsible for more than 50% of all systemic fungal infections [11]. Although a recent study explored the role of nonprofessional cells such as epithelial cells in innate immune responses to *C. albicans* [12], few studies have examined the participation of melanocytes. Early findings suggest that *C. albicans* negatively regulates melanogenesis at the transcriptional level [13].

Here, we present evidence that TLR4-mediated innate immune responses induce the melanization of pathogenic forms of *C. albicans*, resulting in increased melanin content and reduced infectivity.

Materials and methods

TLR ligands, *C. albicans* supernatants, and crude extracts

Lipopolysaccharide (LPS) from *Escherichia coli* and lipoteichoic acid (LTA) from *Staphylococcus aureus* (both from Sigma-Aldrich, St. Louis, MO, USA) were used at 100 ng/ml and 10 μg/ml, respectively. *Candida albicans* (American Type Culture Collection [ATCC] 14053, Manassas, VA, USA) was grown for 24 h at 37°C in Sabouraud glucose broth (BD-Difco, Sparks, MD, USA; 30 g/L). Cells were pelleted by centrifugation at 4000 *g* for 10 min at 4°C. The supernatant was then filtered through a 10-KDa membrane filter (Amicon-Ultra-0.5 mL, Merck-Millipore Corporation, Billerica, MA, USA) and heated at 100°C for 20 min. Crude extracts of *C. albicans* were prepared by resuspending *C. albicans* (1 ml of 0.5 McFarland inoculum) in 49 ml of yeast extract peptone dextrose (YPD) medium from BD-Difco (Sparks, MD, USA) (to obtain 100% blastoconidia) or synthetic Lee's medium broth (to obtain 100% hyphae) [14] and grown for 3 h at 30°C and 37°C, respectively. Cell pellets were obtained by centrifugation at 4000 *g* for 10 min, washed twice in phosphate-buffered saline (PBS) from Life Technologies Corporation (Carlsbad, CA, USA), suspended in 1 ml of PBS, and then lysed by agitation with glass beads for 30 min. Finally, the cells were heated to 100°C for 1 h followed by centrifugation at 16c000 *g* for 10 min at 4°C. The soluble fraction was used in all subsequent assays adjusting proteins at a final 200 μg/ml, measured in a VITROS 5600 analyzer (Ortho Clinical Diagnostics, Rochester, NY, USA).

Measurement of melanin content

Normal human melanocytes (between passages 4 and 8; Invitrogen) were plated at a density of 1–5 × 10^6 cells per well in Medium 254 containing human melanocyte growth supplement (GIBCO) and then stimulated with filtered (0.22 μm) and heated *C. albicans* supernatant for 24, 48, or 72 h. Filtered Sabouraud glucose broth was used as a vehicle and LTA and LPS (at 100 ng/ml and 10 μg/ml, respectively) were used as positive controls. After incubation, the cells were trypsinized, counted, and spun down. The pellets were resuspended in sodium hydroxide for 90 min at 37°C according to a protocol described by Ahn et al. (7). Finally, the cell lysates were loaded into a microplate

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reader (Lab-Tec, Quinta Normal, Santiago, Chile) and the optical density was measured at 485 nm. To calculate the melanin concentration in the samples, a standard curve was prepared using known concentrations of synthetic melanin (Sigma-Aldrich, St. Louis, MO, USA). To assess differences between blastoconidia and hyphae, melanocytes were stimulated with crude extracts of both forms. In this case, PBS was used as a vehicle.

Melanosome antigens and measurement of TLR2 and TLR4 expression

Melanocytes (1–5 $\times$ 10^6 cells/well) were stimulated with crude extracts of blastoconidia or hyphae for 0, 24, 48, 72, or 96 h. Cells were then collected and analyzed by flow cytometry. Briefly, cells were detached by scraping and centrifuged at 3000 *g* for 5 min. The supernatants were discarded and the cells were resuspended in wash solution (1% PBS/3% fetal bovine serum [FBS], GIBCO, Life Technologies Corporation, Carlsbad, CA, USA). To examine the expression of TLR2 and TLR4 genes, total RNA was extracted with TRIzol Reagent (Life Technologies Corporation, Carlsbad, CA, USA), according to the manufacturer's instructions. TLR2 and TLR4 mRNA expression was measured using Taqman primers and probes (Applied Biosystems) in a StepOne thermal cycler (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA), according to the manufacturer's recommendations. Cells were incubated with mouse anti-human Melan-A, mouse anti-human gp100 (Abcam, Cambridge, MA, USA), or mouse anti-human TLR2 and TLR4 (e-Bioscience, San Diego, CA, USA) and analyzed by flow cytometry (FACSCalibur flow cytometer; BD Biosciences, San José, CA, USA).

Antibodies mediated blocking of TLRs

To probe the participation of TLR2 and/or TLR4 in melanization, melanocytes at a density of 1–5 $\times$ 10^6 cells per well were stimulated with crude extracts of blastoconidia and hyphae in the absence or presence of anti-TLR antibodies. Anti-human CD284 (TLR4) and anti-human CD282 (E-biosciences, San José, CA, USA) were used in this experiment. Anti-mouse immunoglobulin G (IgG), IgG2a kappa (E-biosciences, San José, CA, USA), was used as the isotype control. Briefly, control and stimulated cells were incubated without and with the antibodies at a dilution of 1:1000 in PBS/3% FBS for 2 h at 37°C. Then, cells were washed in PBS and trypsinized, counted, and spun down. The pellets were resuspended in sodium hydroxide for 90 min at 37°C, as described previously. Optical density of melanin was measured at 485 nm.

Antifungal activity of melanin

Antifungal susceptibility testing was performed using the microdilution broth method published in the European Committee for Antimicrobial Susceptibility Testing 7.1 definitive document, with some modifications [15]. Briefly, synthetic melanin was dissolved in dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St. Louis, MO, USA) to obtain a stock solution. This solution was diluted and then added to culture medium to yield a final concentration of 2500 μg/ml and 1% of DMSO for testing *C. albicans* (ATCC strain 14053).

Antifungal activity of melanocyte lysates

Nonstimulated and *Candida*-stimulated melanocytes (crude extracts) were grown for 72 h at a density of 1–5 $\times$ 10^6 cells per well, trypsinized, washed twice in PBS, and resuspended in PBS containing 10% DMSO. Next, melanocytes were lysed by agitation with glass beads. Five hundred microliters of the melanocyte lysates were mixed with 10 μl *C. albicans* (10^4 colony-forming units/m) and incubated at 37°C for 30 min. The yeast was then plated onto Sabouraud agar and incubated for 18–24 h at 37°C.

Statistical analysis

The differences between groups were analyzed using the Student *t* test, analysis of variance, and post hoc test. The results were considered statistically significant at a P value <0.05.

Results

C. *albicans* induces melanocyte melanization

To obtain an *in vitro* melanization pattern, normal human melanocytes were stimulated with two TLR ligands, LTA or LPS, and the results compared with those obtained for melanocytes stimulated with *C. albicans* supernatant. Stimulation with LPS led to greater increases in melanocyte melanin content than stimulation with LTA, particularly at 72 h (Fig. 1A). Since YPD medium could stimulate pigmentation by itself, it could have been a factor in the observation that the *C. albicans* supernatant was more stimulatory. The following experiments were performed using crude extracts of *C. albicans* blastoconidia and hyphae that were previously washed in PBS and suspended in the same buffer. Consistent results were obtained with the crude extracts of blastoconidia and hyphae at 72 h, although the hyphae crude extract induced stronger melanization (similar to that induced by LPS; Fig. 1B). The melanin concentration in the melanocytes was calculated by reference to a standard curve (see Materials and methods section).

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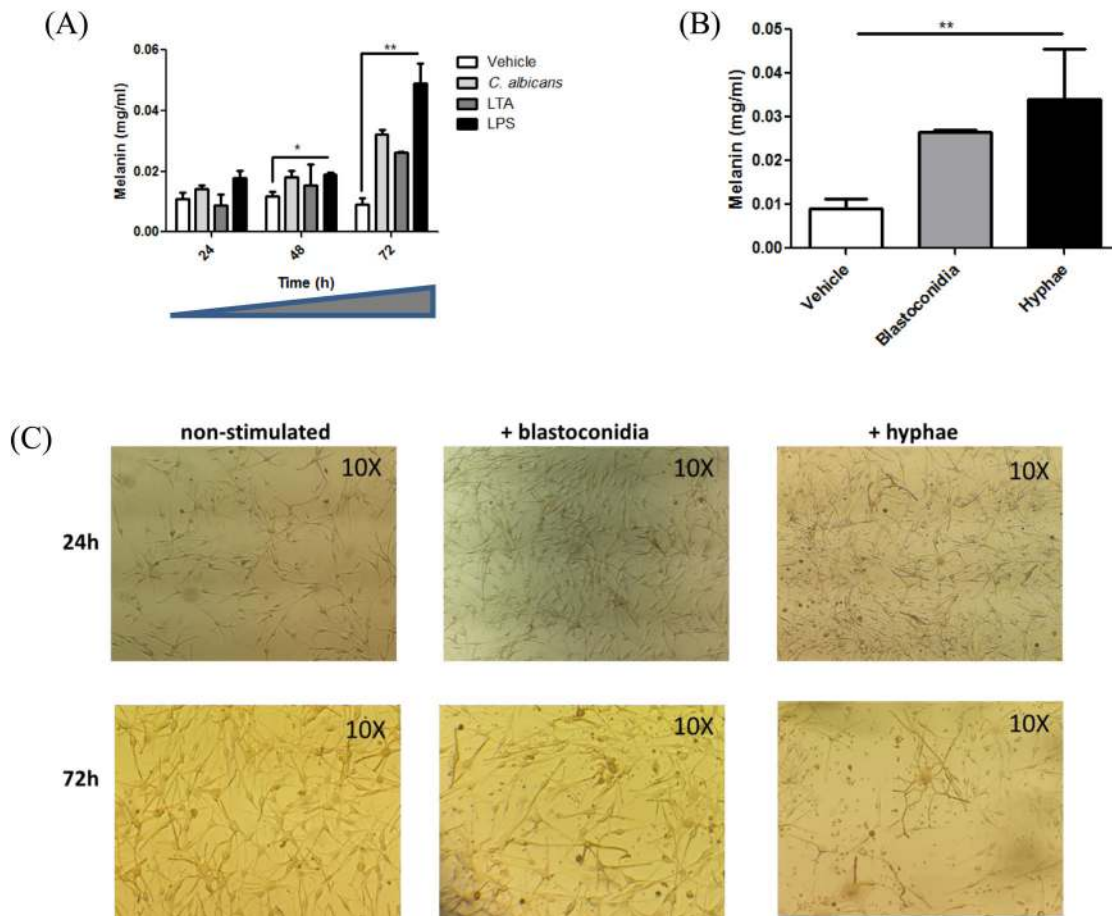

Figure 1. *Candida albicans* melanocyte melanization. (A) The melanin content of melanocytes increases over time (24, 48, and 72 h post-stimulation) with vehicle (yeast extract peptone dextrose broth), *C. albicans* supernatant, lipoteichoic acid (LTA), or lipopolysaccharide (LPS). (B) Crude blastoconidia or hyphae extracts of *C. albicans* with vehicle (phosphate-buffered saline) at 72 h post-stimulation. (C) Optical microscopy images of melanization in melanocytes stimulated with blastoconidia and hyphae extracts of *C. albicans* at 24 and 72 h post-stimulation. This figure shows an increase in size and hyperpigmentation in melanocytes stimulated with hyphae extracts (10×). Results are representative of three independent experiments. ∗, $P < 0.05$; ∗∗, $P < 0.01$. Gray triangle indicates an increase in the melanin content. This Figure is reproduced in color in the online version of *Medical Mycology*.

Crude extracts of *C. albicans* induce the expression of melanosome markers

To track the genesis of melanin induction caused by *C. albicans*, we examined the expression of major melanosome markers over time. When melanocytes were stimulated with crude extracts of *C. albicans*, expression of Melan-A and gp100 was induced at 24 h. This expression decreased at 48 h and 72 h as the melanin content of the melanocytes increased (Figs. 1A, 2A). Hyphae induced higher expression of gp100 than blastoconidia (Fig. 2B).

C. *albicans* induces TLR expression

At first glance, it appeared as if the *C. albicans* (both the blastoconidia and hyphae forms) induced differential expression of TLRs and melanin production by melanocytes. When melanocytes were stimulated with crude extracts of *C. albicans* at 24 h, we found that blastoconidia induced greater expression of TLR2 mRNA than hyphae (Fig. 3A), hyphae induced greater expression of TLR4 mRNA than blastoconidia (Fig. 3B), and the expression of TLR2 mRNA was higher than that of TLR4 mRNA. At the protein level, 20% of melanocytes constitutively expressed TLR2, which increased upon stimulation by blastoconidia (Fig. 3C), but was not time dependent. On the other hand, TLR2 expression did not increase upon stimulation by hyphae extracts (Figs. 3C and 3D). Nonstimulated melanocytes did not express TLR4, although a higher percentage of cells expressed TLR4 upon stimulation with blastoconidia than with hyphae (Figs. 3E and 3F). Minimal levels of TLR4 expression were observed upon stimulation with hyphae; however, this increased over time, and maximal levels were attained after 96 h of stimulation (Fig. 3E and 3F).

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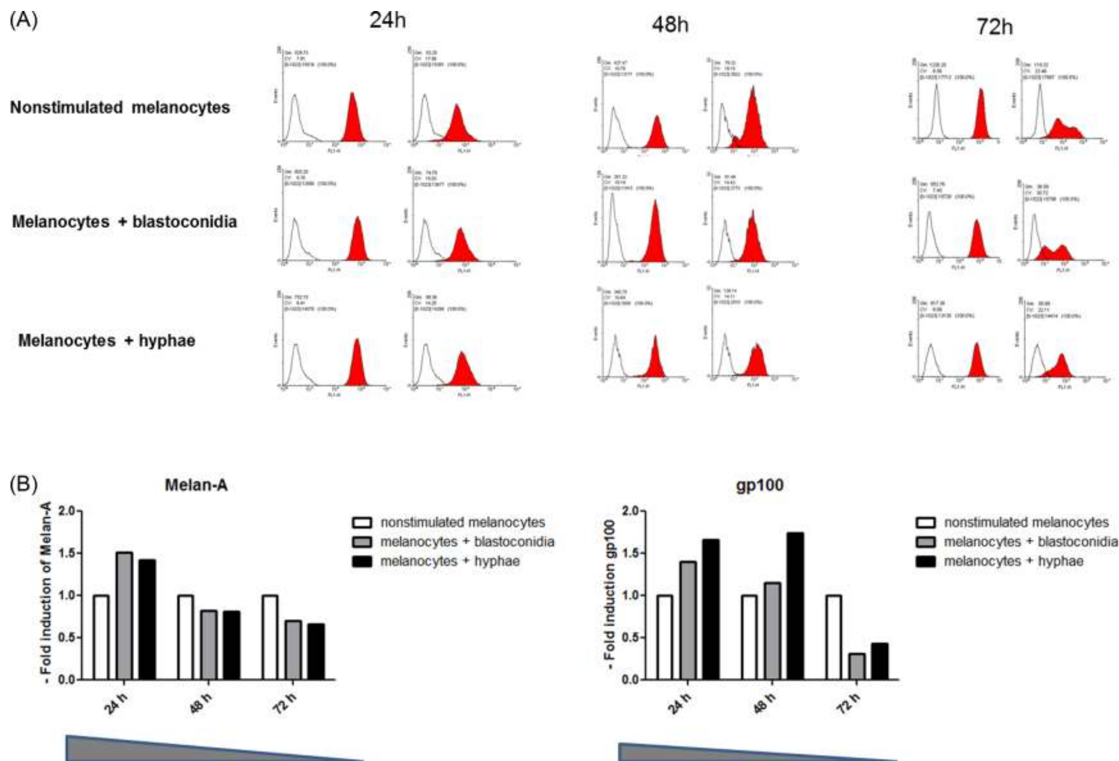

Figure 2. *Candida albicans* induces the expression of early melanosomal antigens (Melan-A and gp100) in melanocytes at 24, 48, and 72 h post-stimulation. (A) FACS plots showing the time course of antigen expression. x-axis: fluorescence level; y-axis: events number. (B) Fold of induction of Melan-A and gp100 relative to nonstimulated melanocytes. B, blastoconidia; H, hyphae. Gray triangles indicate a decrease in melanosomal antigens at 72 h of stimulation. This Figure is reproduced in color in the online version of *Medical Mycology*.

Anti-TLR4 antibodies block the melanization in hyphae extract–stimulated melanocytes

To study a possible role of TLRs in melanization induced by blastoconidia and hyphae extracts of *C. albicans*, cells were incubated with specific anti-TLR2 and anti-TLR4 antibodies. Only TLR4 was blocked post-stimulation with hyphae extracts but not with blastoconidia extracts (Fig. 3G).

Antifungal activity of melanin and melanocyte lysates

Synthetic melanin (at 0.03 mg/ml) inhibited *C. albicans* growth by 50% (Fig. 4A, B) and was fungicidal at concentrations >0.06 mg/ml (Fig. 4C). Melanocyte lysates inhibited the growth of *C. albicans*, with stimulated cells being more inhibitory than nonstimulated cells (Fig. 4D).

Discussion

These results suggest that melanocytes are not only melanin factories but they apparently play a key role in the innate immune response against *C. albicans* infections by recognizing both commensal and pathogenic forms of *C. albicans* and reacting by regulating their melanin content. This process inhibits the growth of *C. albicans*, particularly when melanocytes are stimulated by the hyphae form. Consistent with this, an earlier study of the etiology of depigmentation in response to some fungal infections suggested that *C. albicans* negatively regulates melanogenesis at the transcription level [13]. Following the publication of epidemiological data that showed that persons with darker skin have increased resistance to *Trichophyton mentagrophytes* and *C. albicans* infections [5], we examined the hypothesis that pathogenic forms of *C. albicans* induce melanocyte pigmentation as a first line of innate immunity against fungal infections.

Melanin synthesis by mammalian melanocytes is regulated at the genetic, biochemical, and environmental levels [2]. Melanocytes induce the expression of early melanosomal antigens, which are related to melanosome maturation and the regulation of melanization [4,16]. Here, we showed that Melan-A and gp100 were induced after 24 h of stimulation by *C. albicans* extracts, suggesting that they play a role in melanin synthesis. Whereas the expression of Melan-A decreased at 48 h post-stimulation, that of gp100 decreased at 72 h. This hierarchical expression pattern suggests that Melan-A is required for gp100 function [4]. Melanosomal antigen expression disappeared after 72 h of stimulation, whereas the level of pigmentation peaked at this time,

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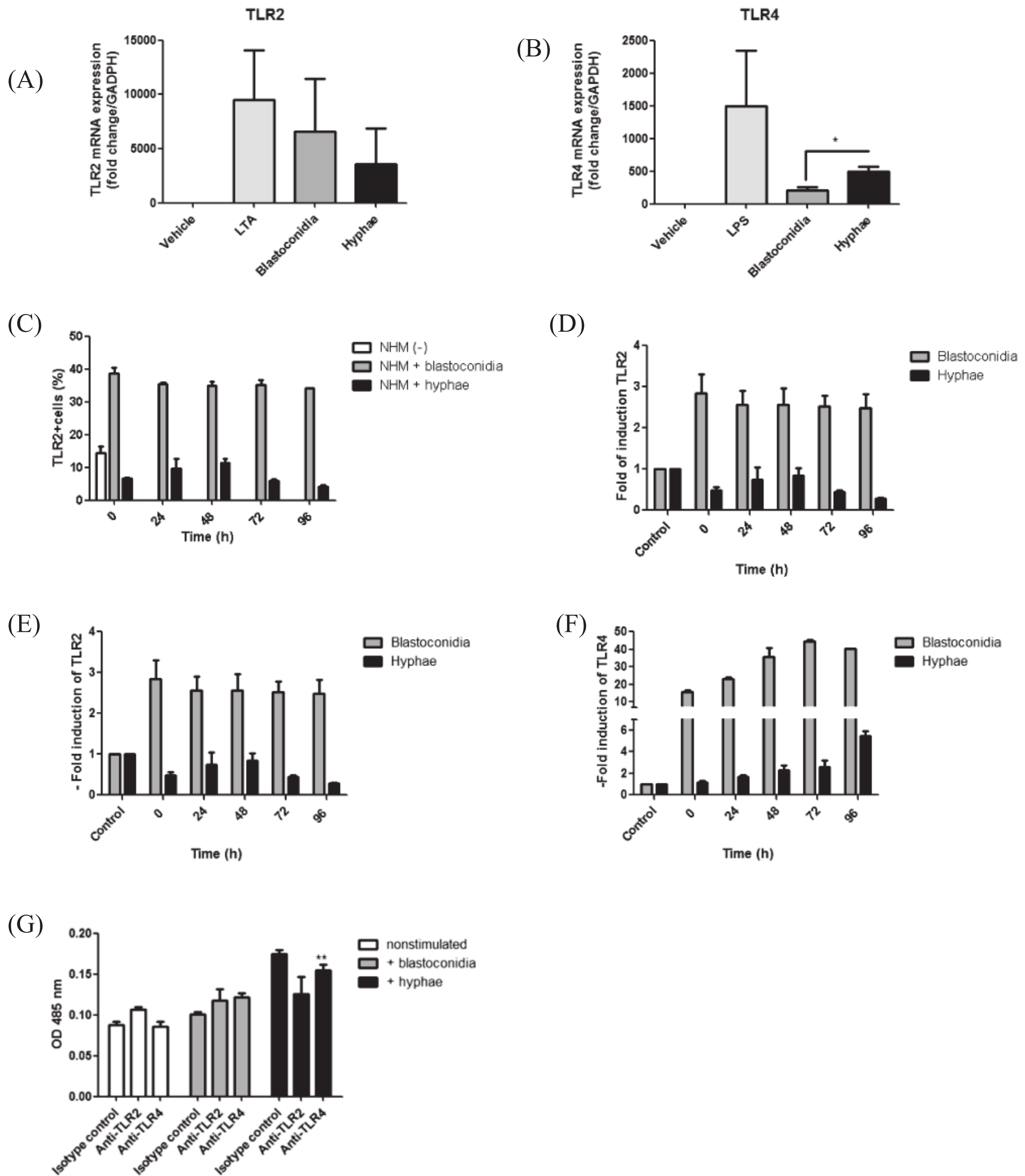

Figure 3. Melanocyte expression of Toll-like receptors (TLRs) 2 and 4 is induced by stimulation with *Candida albicans* crude extracts (blastoconidia and hyphae). (A) TLR2 induction at the mRNA level. (B) TLR4 induction at the mRNA level. (C) TLR2 induction at the protein level (assessed by FACS). (D) Fold induction of TLR2 relative to control (assessed by FACS). (E) TLR4 induction at the protein level (assessed by FACS). (F) Fold induction of TLR4 compared to nonstimulated melanocytes (assessed by FACS). (G) Melanization reduction by antibody-mediated blocking of TLT4. Control, nonstimulated melanocytes; OD, optical density; GADPH, glyceraldehyde 3-phosphate dehydrogenase.

probably due to the cessation of early-phase melanosome maturation.

An interesting finding was that melanocytes induced the expression of TLR2 and TLR4 upon stimulation by *C. albicans* and that this expression was different after exposure to blastoconidia and hyphae (the pathogenic form). Thus, while blastoconidia induced higher expression of TLR2 mRNA, hyphae induced higher expression of TLR4

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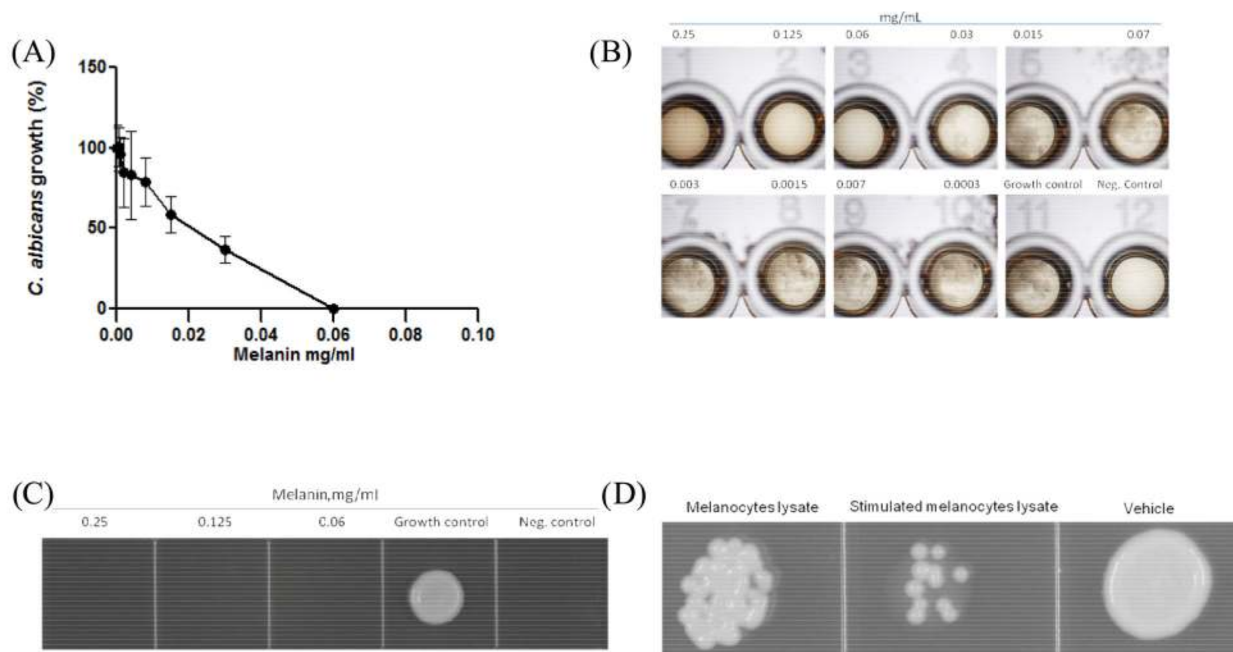

Figure 4. Both synthetic melanin and melanocyte lysates inhibit the growth of *Candida albicans*. (A) Graphic representation of *C. albicans* inhibition by synthetic melanin (assessed by spectrophotometry), expressed in growth percentage (%). (B) Visual inhibition of *C. albicans* growth by synthetic melanin in a broth microdilution test. (C) Fungicidal activity of synthetic melanin assessed onto culture plate. (D) Inhibitory activity of melanocyte lysate on *C. albicans* onto culture plate. Results are representative of three independent experiments. $*$, $P < 0.05$; $**$, $P < 0.01$. The *C. albicans* inocula contained 10^4 ufc/ml *C. albicans.* This Figure is reproduced in color in the online version of *Medical Mycology*.

mRNA (Fig. 3A, B). Previous reports describe differential TLR responses in other cell types in response to both forms of *C. albicans* [9,17]. At the protein level, TLR2 (but not TLR4) was constitutively expressed in 20% of melanocytes (Fig. 3A), but TLR2 expression increased upon stimulation with *C. albicans* blastoconidia but not upon stimulation with hyphae. This increase was consistent over time (Fig. 3C and D). TLR4 induction was lower than that of TLR2, and blastoconidia-induced TLR4 expression was stronger than that caused by hyphae. Unlike that of TLR2, TLR4 expression increased over time, reaching a peak at 72 h (blastoconidia) or 96 h (hyphae). This behavior reflects the melanogenesis, suggesting a role for TLR4 in this process [6,7].

To test the direct participation of TLRs, melanocytes were incubated with anti-TLR2 and anti-TLR4 antibodies, resulting in a reduction of melanization in hyphae extract–stimulated melanocytes in the presence of anti-TLR4 antibodies. This finding corroborates a role of TLR4 in melanogenesis induced by the pathogenic form of *C. albicans*. Although blastoconidia extracts induce melanization, hypha extracts induce a stronger pigmentation and additionally cause changes in the melanocytes morphology, which increases their size and melanin content (Fig. 1C). This could be related to TLR4 induction by hyphae; however, other receptors could be involved in melanogenesis; therefore, more studies are needed to dissect the specific mechanism.

Melanin not only protects against UV radiation, it also has antimicrobial properties. An important biological role of melanocytes, melanosomes, and melanin is to inhibit the growth of bacteria, fungi, and parasites, which may explain the presence of melanocytes at different sites within vertebrates [1,5] and the immunomodulatory properties of melanin from different sources [18]. In this study we demonstrated that synthetic melanin inhibited or killed *C. albicans* as effectively as melanocyte lysates, especially after *C. albicans* stimulation. This could be explained, at least in part, by the higher melanin content of stimulated cells. In addition, we noted that the minimal inhibitory concentration of melanin for *C. albicans* (0.03 mg/ml) was equivalent to the physiological concentration in hyphae extract–stimulated melanocytes (Fig. 1B), supporting the idea of a defensive role of melanization in *C. albicans* infections. While the mechanism underlying the inhibitory/cytotoxic properties of melanin is not fully understood, previous studies suggest that reactive quinone intermediates produced during melanin biosynthesis per se and/or hydrogen peroxide have antifungal properties [1]. Furthermore, melanin absorbs organic and inorganic compounds by acting as a “cationite.” For example, in the eye,

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melanin is able to bind bacteria-derived toxins such as botulinum A [1,5]. Thus, melanin could adsorb different compounds within the cell wall of *C. albicans* or directly disrupt the cell membrane. Futures studies will focus on gaining a better understanding of these mechanisms.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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