Central European Journal of **Biology**

Trichophyton rubrum Manipulates the Innate Immune Functions of Human Keratinocytes

Communication

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Abstract: Evasion or subversion of host immune responses have been shown for a variety of microorganisms, and this might be the case for *Trichophyton rubrum*, the most common pathogenic fungus causing chronic dermatophytosis in humans. Keratinocytes, the main epidermal cells, have important roles as a first defense against microbial challenges in local immune reactions. Epidermal keratinocytes express several Toll-like receptors and produce host defense peptides, cytokines and chemokines in response to various stimuli. We analyzed the expression of Toll-Like receptor TLR2, TLR4, TLR6, and Human Beta Defensin (HBD)-1, HBD-2, Interleukin IL-1β and IL-8 production, when exposing primary keratinocyte cultures to *T. rubrum*. We observed changes in size and granularity of keratinocytes stimulated with either whole conidia or conidial homogenates compared to other treatments. Intact conidia decreased keratinocytes' TLR2 and TLR6 expression without affecting that of TLR4, while conidial homogenates increased the expression of these three receptors. Interestingly, whole conidia decreased HBD-1 and HBD-2 production, whereas conidial homogenate increased it. No changes were observed in IL-1β and IL-8 production after stimulation with conidia or conidial homogenate. CONCLUSIONS. Our results suggest that: 1) Keratinocytes can recognize and respond to cell wall components of *T. rubrum*; 2) Viable intact conidia inhibit TLR-2 and TLR6 expression and decrease HBD-1 and HBD-2 production; 3) Conidial homogenate from *T. rubrum* increases the expression of TLR2, TLR4 and TLR6 and induces HBD-1 and HBD-2 production; 4) Therefore, innate immune functions of keratinocytes as the first level of local skin immunity are apparently manipulated by *T. rubrum*, likely to ensure its establishment, persistence and survival.

Keywords: Innate immunity • Manipulation • T. rubrum • Keratinocytes • TLR expression • HBDs • Fungal infection • Skin • Local immunity

1. Introduction

Trichophyton rubrum is the main filamentous fungus causing 90% of dermatophytosis, affecting the epidermis in humans. Tropism of the fungus is facilitated by the ability of *T. rubrum* to degrade keratin and use it as a nutrient source [1-3]. The clinical signs of infected patients are mild, and it has been proposed that the severity of the infection varies among hosts, with a spectrum from soft to hard lesions. The latter are known as "moccasin-type tinea". Nevertheless the factors of the fungus and of the host that determine the severity of infection are not well elucidated [4-6]. This tinea is the most common infection worldwide [4], raising an often overlooked issue: why is it that some patients present the chronic infection, despite a well developed immune system? [7,8].

It has been proposed that *T. rubrum* may have developed evasion mechanisms to escape or even to suppress the host immune responses [9-11]. It is well known that patients that resolve this infection do so using cellular immunity (Th1-type) as the main resource

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controlling the fungus [12]. However, in patients with chronic infection, it has been reported that their peripheral blood mononuclear cells do not proliferate efficiently with antigens from *Candida albicans* and tetanus toxoid, if they are previously exposed to mannan of the cell wall of *T. rubrum* [13]. The latter demonstrates that cellular immunity is indeed affected in some patients. Besides, in another experiment it was shown that the interaction of conidia of *T. rubrum* with macrophages induces the production of TNF-α and IL-10 but not of IL-12 nor of nitric oxide, with the concomitant decrease of costimulatory molecules and inhibition of phagocytosis. Finally, when conidia are ingested by macrophages, they are unable to digest the conidia, which grow inside and develop into hyphae until they disrupt the macrophage membrane [9].

The immune function of the keratinocytes is based on the pattern-recognition theory proposed by Janeway over 20 years ago that has served as a conceptual framework to unravel the functioning of the innate immune system [14,15]. It is now well appreciated that the innate immune system senses a great variety of microbial components called pathogen associated molecular patterns (PAMPs), particularly by means of the pattern recognition receptors (PRRs).

It is also established that activation of PRRs functions as a bridge inducing the adaptive immune responses. Amongst the PRRs, the best studied so far are the Toll-like receptors (TLRs). Epidermal keratinocytes express several TLRs, located either on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6) or in the endosomes (TLR3, and TLR9) [16]. Microbial components recognized by TLRs include lipopolysaccharides (LPS), peptidoglycans, flagellin, nucleic acids and mannans, among others. The ligation of TLRs by PAMPS leads to activation of host signaling pathways, triggering proinflammatory cytokines, chemokines and host defense peptides. Although these mechanisms are very complex, many pathogens have evolved a variety of ways to manipulate or evade it in order to survive in the host [10,11,17].

We thus inquired about mechanisms concerning the local innate immune response. To investigate whether *T. rubrum* alters the innate immune functions of human keratinocytes, we evaluated the expression of TLR2, TLR4, and TLR6. Furthermore, we examined the production of Human Beta Defensin 1 (HBD-1), Human Beta Defensin 2 (HBD-2), Interleukin-1β (IL-1β) and Interleukin-8 (IL-8) in keratinocytes cultured with whole conidia or with conidial homogenate of *T. rubrum*. We found that *T. rubrum* has evolved the ability to somehow manipulate the response of keratinocytes by inhibiting TLR expression and HBD production: viable conidia of *T. rubrum* are required to inhibit TLR expression and inhibit HBD production. On the other hand keratinocytes do respond when in contact with conidial homogenate of *T. rubrum*. In the latter case we see increased expression of TLR2, TLR4, TLR6 and induced production of HBD-1 and HBD-2.

2. Experimental Procedures

2.1 Cell culture

Primary cultures of human keratinocytes were established from surgical specimens of healthy abdominal human skin. Verbal consent for obtaining samples was approved after the protocol was evaluated by the ethical committee of the Institute of Reconstructive Surgery of Jalisco. Skin pieces of 1cm^2 were exposed to dispase 5 U/ml (Sigma-Aldrich, St Louis, MO) overnight at 4°C. The epidermis was then mechanically removed from the dermal layer, homogenized by repeated aspirations with Pasteur pipettes, and incubated in ethylenediamine tetraacetic acid (EDTA) trypsin 0.25% (Sigma-Aldrich) for 10 min at 37°C. Cells were washed three times in serum free medium for keratinocytes (Gibco, Life Technologies, Maryland, USA), stained for viability with 0.5% trypan blue (Sigma), and seeded into 75 cm^2 culture flasks (Corning, NY, USA) with keratinocyte SFM. Cells were kept at 37°C and 5% CO_2.

2.2 Fungus culture, conidia isolation and conidial homogenate preparation

We used a *T. rubrum* strain obtained from the Dermatological Institute of Jalisco, Jalisco, Mexico; this strain was isolated from a patient with athlete´s foot. The strain identification was performed by experts in the Laboratory of Mycology of the Institute. Morphologically, is a filamentous fungus, with abundant microconidia; growth in sabouraud agar shows white, cottony-looking colonies with reddish reverse. The dermatophyte was cultured in dextrose sabouraud agar (Dibco, México D.F., México) for 15 days at 25°C. Microconidia were collected from the agar and transferred to sterile double-distilled water. After filtration the suspension was titrated with a haemocytometer. Conidial homogenate was prepared by sonication with a modification of the Araujo *et al.* method [18].

2.3 Stimulation assay

For stimulation, keratinocytes were cultured in six-well plates. The keratinocytes were either unstimulated or

stimulated with 100 µg/ml LPS (Lipopolysaccharides from *Escherichia coli* O127:B8 (Sigma)), 100 µg/ml LTA (Lipoteichoic acid from *Staphylococcus aureus* (Sigma)), *T. rubrum* microconidia at a fungal cell/human keratinocyte ratio of 10:1, or with conidial homogenate of the same amount of conidia, for 6, 18, 24 or 48 hours, as indicated in each determination (based on [19,20]).

2.4 Flow cytometry

After 6 h of stimulation, cells were stained with Alexa 488-labelled anti-TLR2, Alexa 647-labelled anti-TLR4 (eBioscience) or FITC-labelled anti-TLR6 (Imgenex) and their respective isotype controls. Cells were acquired in an EPICS XL flow cytometer (Beckman Coulter) and data were analyzed using WinMDI software.

2.5 Immunofluorescence staining

The keratinocytes were seeded onto Laboratory-Tek tissue culture chamber slides for 24 h. The cells were then washed with Hank's Balance Salt Solution (Invitrogen-Gibco) and stimulated as described above. The slides were then fixed in 4% paraformaldehyde for 10 min and washed with 10X Tris Buffered Saline (TBS) three times for 5 min. Fixed cells were incubated in a blocking buffer of 5% BSA (Sigma) and 0.1% Triton X-100 in PBS for 30 min at room temperature. Cells were then incubated with the following dilutions of primary antibodies overnight at 4°C: primary polyclonal goat anti human HBD-1 (1:50) and polyclonal goat anti human HBD-2 (1:80) or goat normal serum (negative control). The secondary antibodies that were conjugated to FITC were diluted 1:200 in 5% BSA-PBS and incubated for 1 h at room temperature. Coverslips were washed three times in PBS for 5 min, mounted and analyzed using a fluorescence microscope (Olympus BX 51).

2.6 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-1β and IL-8 in the cell culture supernatants were determined by ELISA. Ready-SET-Go (eBioscience) was used for IL-1β. Quantikine (R&D Systems, Minneapolis, US) was used for IL-8. The assays were performed according to the manufacturer's instructions. Results from two representative experiments are presented as the means ± SEM of triplicate cytokine measurements.

2.7 Statistical analysis

Data are expressed as mean ± SEM. Kruskal-Wallis test for TLR expression and one-way ANOVA test for ELISA data with Dunn´s multiple comparison post-hoc test were applied (GraphPad Prism 5.0; GraphPad Software Inc., San Diego, California, USA). P<0.05 was considered to be significant.

3. Results

3.1 *Trichophyton rubrum* causes changes in the size and cytoplasmic complexity and increases the number of keratinocytes in cultures

Keratinocyte cultures were left unstimulated or stimulated with LPS, LTA, conidia or conidial homogenate of *T. rubrum,* and keratinocyte viability was evaluated by trypan blue exclusion. These five different treatments did not affect viability (data not shown). In the initial flow cytometry analysis, we found that keratinocytes treated with whole conidia and with conidial homogenate of *T. rubrum* increased in number (Figure 1A). Likewise, we found that these keratinocytes showed larger sizes (as shown by forward scatter, FSC) and an increased cytoplasmic complexity (as shown by side scatter, SSC) compared with control cells (Figure 1B).

3.2 Expression of TLR2, TLR4 and TLR6 on keratinocytes *in vitro*

We found that TLR2 was expressed by 15% of unstimulated keratinocytes, by 5% of LPS stimulated keratinocytes, by 16% of LTA stimulated keratinocytes, by 7% of keratinocytes stimulated with conidia of *T. rubrum* and by 28% of keratinocytes stimulated with conidial homogenate (Figure 2A). We found TLR4 in 3% of unstimulated keratinocytes, in 8% of LPS stimulated keratinocytes, in 7% of LTA stimulated keratinocytes, in 5% of keratinocytes stimulated with conidia of *T. rubrum* and in 42% of keratinocytes stimulated with conidial homogenate (Figure 2B). Finally, we found that TLR6 was expressed in 23% of unstimulated keratinocytes, in 12% of LPS stimulated keratinocytes, in 18% of LTA stimulated keratinocytes, in 14% of keratinocytes stimulated with conidia of *T. rubrum* and in 35% of keratinocytes stimulated with conidial homogenate (Figure 2C). Significant differences were found between TR conidia and conidial homogenate for the three receptors.

3.3 HBD-1 and HBD-2 production by keratinocytes

The immunofluorescence staining revealed the presence of HBD-1 in unstimulated keratinocytes, apparently in a constitutive manner. Whereas LPS did not affect its synthesis, stimulation with *T. rubrum* conidia inhibited it. However, culture with conidial homogenate did not inhibit HBD-1, and it was detected as in unstimulated cells (Figure 3). HBD-2 is not produced by unstimulated keratinocytes, and LPS was the best inducer of its synthesis. Stimulation with *T. rubrum* conidia inhibits its production, and when keratinocytes were cultured with conidial homogenate HBD-2 was induced as with LPS (Figure 4).
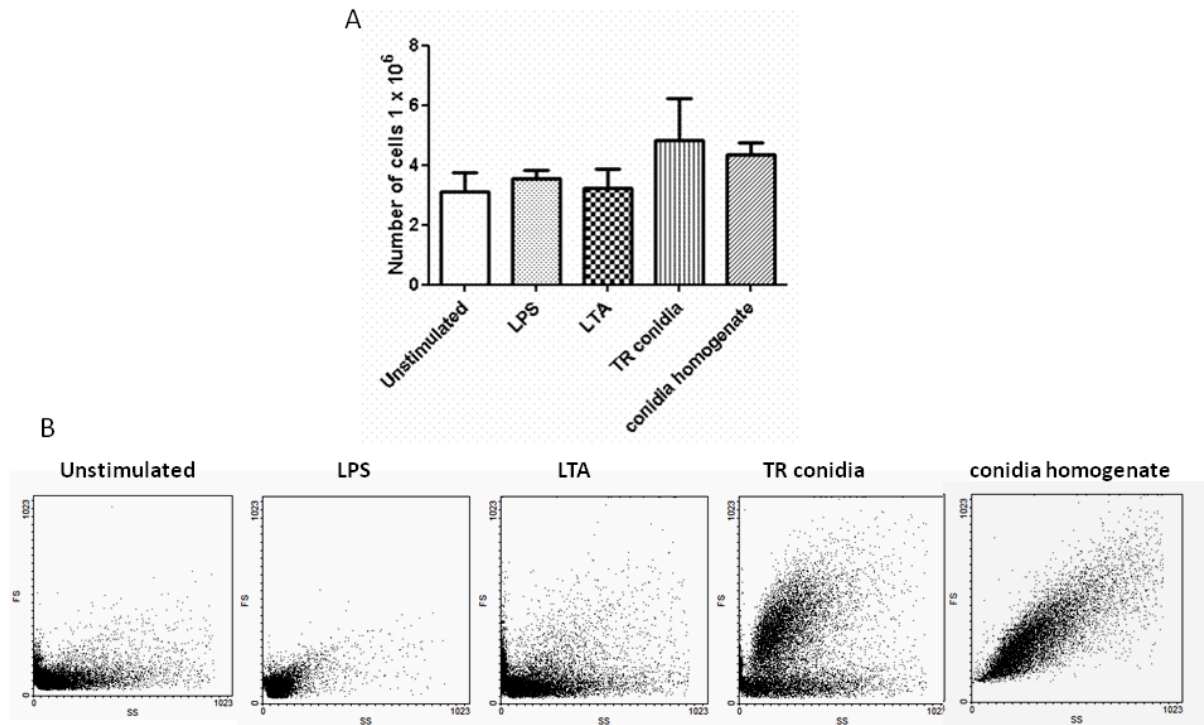
Figure 1. *T. rubrum* induces increased cell numbers and changes in size and cytoplasmic complexity of keratinocytes. (A) Keratinocytes were left unstimulated or stimulated with LPS, LTA, *T. rubrum* conidia (TR) or conidial homogenate of *T. rubrum* for 6 h. Keratinocytes were quantified using a haemocytometer. Results are shown as mean ± SEM of seven experiments with different donors. (B) After stimulation, size and cytoplasmic complexity properties of keratinocytes were analyzed by flow cytometry. Both conidia and conidial homogenate triggered an increase in size and cytoplasmic complexity in keratinocytes. Dot plots shown are representatives of one of seven experiments with different donors.
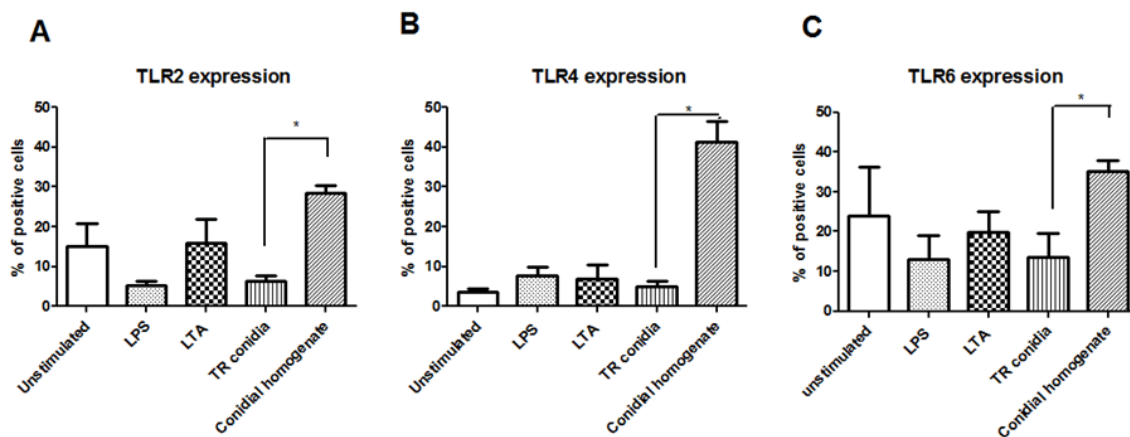

Figure 2. Conidia stimulation of keratinocytes decreases TLR2 and TLR6 expression while conidial homogenate increases the expression of three TLRs (TLR 2, TLR 4 and TLR 6). Keratinocytes were either unstimulated or stimulated with LPS, LTA , *T. rubrum* conidia (TR) or conidia homogenate of *T. rubrum* for 6 h and the percentage of positive cells to TLR 2 (A), TLR 6 (B) and TLR 4 (C) was then evaluated by flow cytometry. Data are expressed as mean ± SEM of seven experiments performed each with a different donor. Asterisks (*) indicate significant differences, $P < 0.05$.
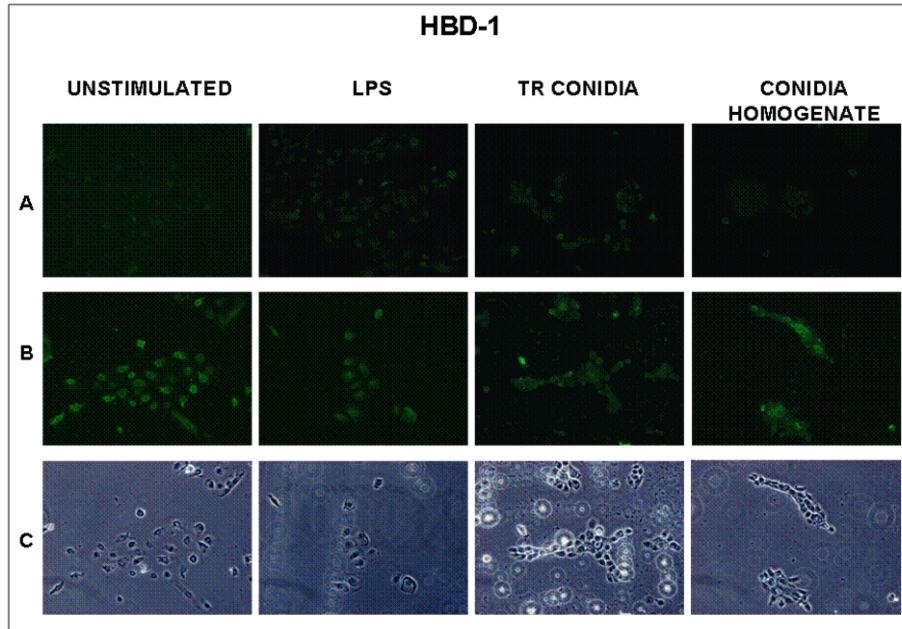
Figure 3. Keratinocytes stimulated by conidia decrease HBD-1 production whereas keratinocytes stimulated by conidial homogenate seem to restore it. Detection of HBD-1 by immunofluorescence was made after 18 h of stimulation. Keratinocytes were grown on glass slides and were either unstimulated or stimulated with LPS, *T. rubrum* conidia (TR) or conidial homogenate of *T. rubrum*. After stimulation, cells were labeled with goat normal serum as a negative control (A) or with a primary polyclonal goat anti-HBD-1 (dilution 1:50) (B) followed by FITC-labeled bovine anti-goat secondary antibody (dilution 1:200). C shows the phase contrast image of keratinocytes stained with anti-HBD-1.
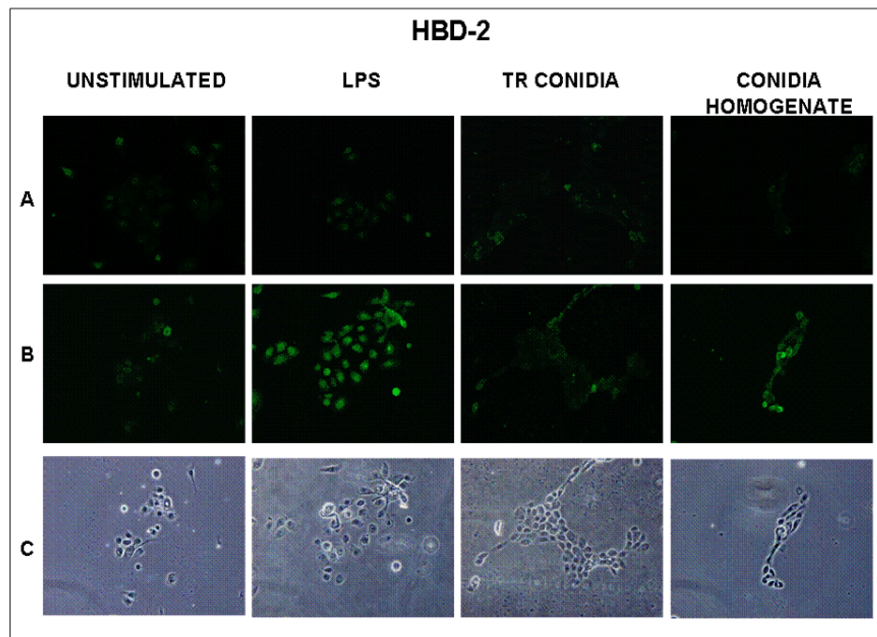

Figure 4. Keratinocytes cultured with conidia decrease HBD-2 production while conidial homogenates induce it. Detection of HBD-2 by immunochemistry was performed after 18 h of stimulation. Keratinocytes were grown on glass slides and were either left unstimulated or stimulated with LPS, *T. rubrum* conidia (TR) or conidial homogenate of *T. rubrum*. After stimulation, cells were incubated with goat normal serum as a negative control (A) or with a primary polyclonal goat anti-HBD-2 (dilution 1:80) (B), followed by FITC-labeled bovine anti-goat secondary antibody (dilution 1:200). C shows the phase contrast image of keratinocytes stained with anti-HBD-2.

3.4 IL-1β and IL-8 production by keratinocytes

In supernatants of keratinocyte cultured for 24 h (Figure 5A), we found that LPS was the best stimulus for IL-1β production (48 pg/ml) followed by conidia of *T. rubrum* (18 pg/ml) in comparison to unstimulated keratinocytes (10 pg/ml). Instead, LTA and conidial homogenate did not affect IL-1β production. On the other hand, after 48 h of stimulation, LTA was a better inducer than LPS (32 pg/ml and 10 pg/ml, respectively). IL-1β induced by conidia decreased to 12 pg/ml and conidial homogenate stimulation induced a slight increase to 14 pg/ml (Figure 5A). Concerning IL-8 production, we found that this chemokine is produced by unstimulated keratinocytes (2800 pg/ml) and at 24 h of stimulation we did not observe any change with any of the stimuli used (Figure 5B). Nevertheless, after 48 h of stimulation LPS was the best inducer (4100 pg/ml), followed by LTA (3300 pg/ml). In contrast, the production of IL-8 after conidia and conidial homogenate stimulation remained close to 3000 pg/ml (Figure 5B).

4. Discussion

Microbial cell walls constitute a frontier between these cells and their microenvironment, and therefore have several key functions, including molecular recognition, cell adhesion and aggregation, among others. Understanding these functions requires elucidation of the molecular architecture of microbial cell walls [21]. The cell wall of *Trichophyton* contains β-glucan, β-1,2-mannosides, chitin, phospholipomannan, glucuronoxylomannan, mannan, and galactomannan [21-23]. Conceivably, all these components of *T. rubrum* might interact with the first line of defense of the host, the keratinocytes, on the other frontier of this interplay.

In order to confirm the observation that keratinocytes increased their size and cytoplasmic complexity in response to the cell wall components of *T. rubrum*, and to rule out the possibility that we were looking at the conidia of *T. rubrum* rather than keratinocytes in the dot plot (Figure 1), we analyzed a conidia suspension alone by flow cytometry. We found that the amount of conidia used for the stimulation was almost undetectable, thus we proceeded to introduce ten times that amount. We observed that conidia had clearly different FSC and SSC properties than those of keratinocytes stimulated with conidia, thus confirming that we were looking indeed at the keratinocytes and not at the conidia. This is in agreement with the fact that conidia are several times smaller than keratinocytes (Figure 6).

On the other hand, to rule out that what we were detecting in the flow cytometer were keratinocyte-conidia agglomerates, we stimulated keratinocytes with a conidial homogenate, and found that the FSC and SSC properties of these keratinocytes were also different from those of conidia alone and of keratinocytes without stimuli (Figure 1B). These findings suggest that in response to *T. rubrum* antigenic stimuli, keratinocyte apparently increased their numbers and differentiated, a phenomenon that is usually attributed to immune cells like lymphocytes. We believe that this process may be due to the activation of signaling pathways that regulate proliferation and differentiation in keratinocytes
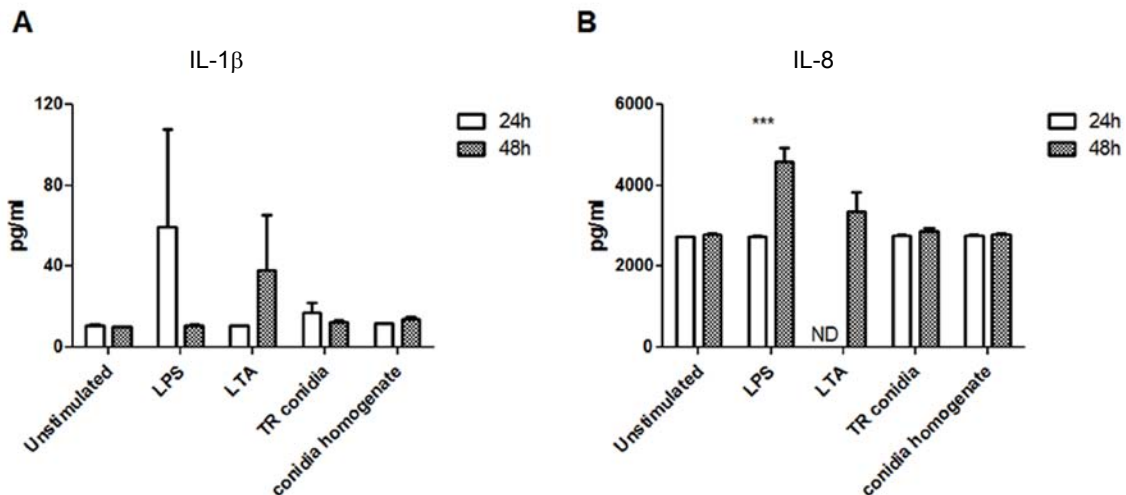

Figure 5. IL-1β and IL-8 secretion by keratinocytes showed no apparent alterations under *T. rubrum* stimulation. Keratinocytes were stimulated as indicated; IL-1β (A) and IL-8 (B) were quantified in the supernatant by ELISA. ND, not determined. Asterisks (***) indicate significant differences, P<0.001 (LPS 24h *vs.* LPS 48h).
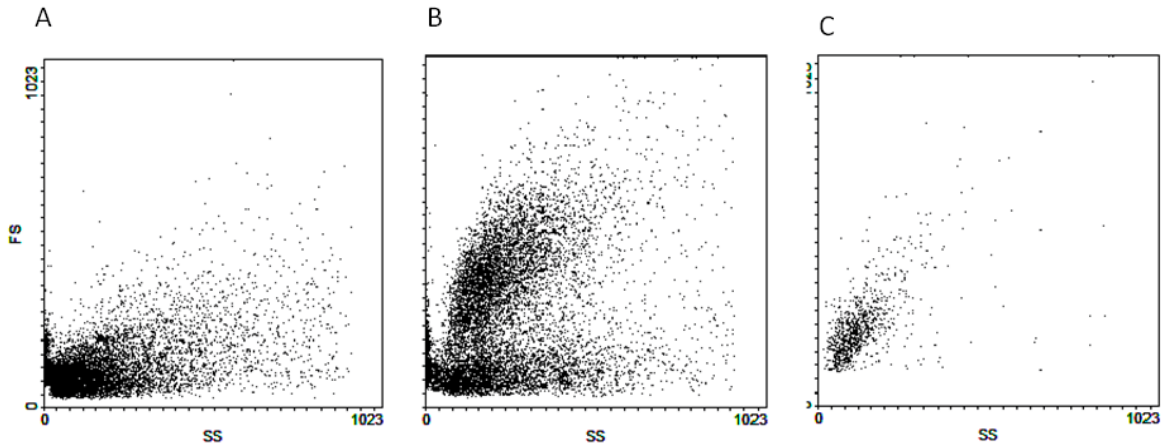
Figure 6. *T. rubrum* conidia and keratinocytes have different FSC and SSC properties. To rule out that in the dot plot we were detecting *T. rubrum* conidia rather than keratinocytes, we compared both unstimulated keratinocytes (A) and keratinocytes stimulated with *T. rubrum* conidia (B) with a suspension of conidia alone (C) in the flow cytometer. We observed that size (FSC) and granularity (SSC) properties in all samples are different.

such as p38-MAPK. In the infection process *in vivo* there is no production of microconidia; therefore, there is a possibility that the cell wall structures of hyphae and conidia is different. However, Jensen, *et al.* [24] reported that in skin of patients with *T. rubrum* infection, the keratinocytes showed more proliferation and differentiation markers than did keratinocytes derived from skin of healthy donors. These data indicate that keratinocytes proliferate and differentiate in response to *T. rubrum in vivo*; therefore we believe that key structural components that trigger this process are present in both hyphae and conidia. Our first question about this was whether this response would facilitate the elimination of the fungus by the keratinocytes through host defense peptide production, among other mechanisms. Another possibility was whether this response would facilitate fungal growth, since differentiated keratinocytes are the main source of keratin, the food of these dermatophytes.

The next question that we addressed was whether TLR2, TLR4 or TLR6 were induced in keratinocytes after *T. rubrum* stimulation, and if any of these receptors was involved in the response to cell wall components of *T. rubrum*. We found that expression of TLR2 and TLR6 decreased notably when keratinocytes were stimulated with whole conidia. In contrast, conidial homogenate increased their expression, suggesting that conidia of *T. rubrum* inhibited TLR2 and TLR6, thus preventing the activation of immune responses, likely to ensure the survival of the fungi. Regarding the mechanisms inhibiting the expression of TLR2 and TLR6, a possibility is that these receptors are not detected because they have been degraded by enzymes released by the fungi. In fact it has been widely described that proteolytic

enzymes are over-expressed by *T. rubrum* during the infection process [25]. This also happens in *Drosophila melanogaster*: it has been reported that some pathogenic fungi release proteases to degrade the *Drosophila* PRR, GNBP3, thus avoiding detection of the fungi [10]. This escape mechanism might be added to the list of adverse effects that *T. rubrum* can cause on other immune cells. For example, *T. rubrum* inhibits the phagocytic and cytokine secretion capabilities in macrophages [9], and a mannan of *T. rubrum* inhibits the anti-CD3 induced proliferation in lymphocytes [13].

On the other hand, when keratinocytes were stimulated with conidial homogenate, we found an increased expression of TLR2, TLR4 and TLR6, suggesting that keratinocytes and other epithelial cells have the ability to recognize soluble products of this fungus cell wall components, which could be useful for immunization purposes. Other reports show, for instance, that inactivated hyphae of *Fusarium solani* increase the expression of TLR2 and TLR4 in corneal epithelial cells [26].

We conclude that viable and whole conidia are required by *T. rubrum* for TLR inhibition with the subsequent establishment of the evasion mechanisms described. Besides, we found that TLR4 expression shows the major increase after stimulation with the conidial homogenate compared with whole conidia stimulation, and that this is probably explained by the fact that the whole conidia might not expose the required antigens to properly stimulate surface TLR4.

Our study seems to be the first reporting that components of the cell wall from *T. rubrum* induce responses through TLRs on keratinocytes, and that

evasion mechanisms are present on the latter cells, although it remains to be examined which of the cell wall components of *T. rubrum* are the putative ligands for TLR2, TLR4 and TLR6. In this sense, for other fungi it has been reported that tumor necrosis factor (TNF) production in response to *C. albicans* phospholipomannan is TLR2-, TLR4- and TLR6-dependent, and that in the case of *C. neoformans*, the major capsular component glucuronoxylomannan activates TLR4-dependent NF-κB translocation *in vitro* [10,12,22,27,28].

Regarding the production of host defense antimicrobial peptides, our results show a decrease in HBD-1 and HBD-2 synthesis when keratinocytes were stimulated with conidia of *T. rubrum*. We think this might be a consequence of the decreased expression of TLRs since it has been reported that HBD gene transcription is regulated by TLR agonists, among others [29]. Interestingly, when keratinocytes were stimulated with the conidial homogenate, there was induction of TLR expression: HBD-1 production was restored and HBD-2 production was induced. In this regard it has been reported that aspartyl proteases of *Candida albicans* are crucial virulence factors that inhibit HBD expression [30].

Thus, it remains to be elucidated if HBD production is dependent on TLR2, TLR4 or TLR6 or even if the three receptors are activated in response to cell wall components of *T. rubrum*, since it has been reported that BDEF-2 (a murine homolog of HBD-2) is TLR2- and TLR4-dependent [31].

Interestingly, we did not observe an important increase in IL-1β or IL-8 production after stimulation with either conidia or conidial homogenate; this may suggest that *T. rubrum* does not induce their production. Indeed, other studies have failed to detect IL-1β after stimulation with several species of *Trichophyton* [32]. These findings suggest that this can be another escape mechanism of *T. rubrum.* There are reports that some other microorganism such as *Yersinia enterocolitica, C. albicans* and *Aspergillus fumigatus*, instead of leading to an inflammatory state through IL-1β, IL-8 or other cytokines, induce immunosupression through IL-10 [10]. Therefore it remains to be explored if *T. rubrum* induces IL-10 or TGF-β production.

One other consideration in our work is that we used only one strain of *T. rubrum*. It needs to be determined whether other strains show the same results.

Taken together our results suggest that *T. rubrum* induced increases in the numbers, size and cytoplasmic complexity of keratinocytes in primary cultures. Furthermore *T. rubrum* manipulates the innate immune functions of these cells through the inhibition of TLR expression and of the HBD synthesis when keratinocytes are in contact with the whole conidia. Nevertheless, it seems clear that keratinocytes have the ability to recognize cell wall components present in the conidial homogenate of the fungus, which seem adequate to activate TLR2, TLR4, or TLR6 and subsequently induce HBD-1 and HBD-2.

Elucidation of the mechanisms by which *T. rubrum* interferes with the immune functions of keratinocytes in the initial stage of innate immune response is basic to develop better therapeutic strategies to improve the innate and adaptive immune responses, and thus to improve the quality of life of infected patients. Our results provide evidence of the importance of keratinocytes in local innate immunity as effector cells to combat epidermal and dermal infections.

Acknowledgements

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