Immunological and histopathological characterization of cutaneous candidiasis

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Chronic mucocutaneous candidiasis constitutes a heterogeneous group of syndromes, characterized by non-invasive infection of the skin, nails and mucosal membranes by the fungus Candida spp. Although symptoms are heterogeneous, in all cases there is a reduction in protective cytokines, favouring the development of disease. The normal role of cytokines in skin lesions is not well understood. The present study aimed to investigate the progression of disease, understand specific cellular and molecular components involved in immunity to Candida albicans and determine the balance between pro- and anti-inflammatory cytokines over the course of cutaneous infection in immunocompetent mice. BALB/c mice (five per group) were inoculated with $5 \times 10^6$ C. albicans pseudohyphae in the deep dermis of the paw and analysed over 1–14 days post-infection. The contralateral paws were used for negative controls. Haematoxylin and eosin staining of skin sections during C. albicans infection was performed to analyse structural modifications to the epidermis such as hyperplasia, and infiltration of neutrophils and fibroblasts in the dermis. The cytokine populations were determined by capture ELISA using popliteal lymph node tissue. Pro-inflammatory cytokines (IL-6, TNF-α, IL-12, IFN-γ and IL-17) were detected at significant levels during the initial phase of cutaneous infection and correlated with the rapid elimination of C. albicans. Anti-inflammatory cytokines (IL-13, IL-4, IL-10 and transforming growth factor-β) were detected on day 4 post-infection, and prevented exacerbation of inflammation and participated in healing of lesions. Thus, a balance between pro- and anti-inflammatory cytokines was fundamental for the resolution of infection. Importantly, these findings broaden our understanding of the immune mechanisms involved in chronic cutaneous candidiasis.

INTRODUCTION

Candida albicans is a commensal organism found on human skin and in mucous membranes, and causes candidiasis only if the normal host–pathogen balance is disrupted. Chronic mucocutaneous candidiasis is caused by the selective inability of a patient to clear a Candida infection, resulting in persistent debilitating inflammation of the skin, nails and mucous membranes (Kirkpatrick, 2001). A variety of clinical conditions that impair immune function, such as infection by human immunodeficiency virus or the use of corticosteroids, favour the development of chronic mucocutaneous candidiasis; the disease may also be associated with endocrinopathies or genetic defects in the immune system (Chehimi et al., 2001; Collins et al., 2006).

A more critical parameter in the pathogenesis of chronic cutaneous candidiasis might be the cytokine secretion of...
T-cell subtypes. Numerous studies have described altered cytokine production with reduced production of type 1 cytokines, such as IFN-γ, IL-12 and IL-2, and increased secretion of IL-10 or IL-4 and IL-5 (Kobrsynki et al., 1996; Lilic et al., 2003; van der Graaf et al., 2003; Eyerich et al., 2007). In T-helper 1 (Th1) responses, IL-12 and IFN-γ production are essential for controlling C. albicans infections, whereas Th2 cytokines are associated with increased susceptibility to infection (Cenci et al., 1998; Romani, 2004). Th17 cells also play a role in host defense against C. albicans infection (Conti et al., 2009). Recently, our group verified that IL-17 stimulates neutrophil accumulation and function (Custodio et al., 2011; de Carvalho et al., 2012). According to findings by Eyerich et al. (2008), patients with chronic mucocutaneous candidiasis exhibit reduced production of Th17-associated cytokines IL-17 and IL-22. Moreover, IL-17A-deficient mice demonstrate delayed healing and decreased IL-17A production following skin infection with C. albicans, compared with WT mice (Kagami et al., 2010).

Due to the dysfunction of the cells involved in the immune response against C. albicans in patients with chronic cutaneous candidiasis leading to dysregulation of cytokine production, we hypothesized that in immunocompetent mice, inflammatory cytokines and lesion healing play a role in the resolution of cutaneous C. albicans infection, thus preventing chronic or recurrent cutaneous candidiasis. An understanding of immune responses in normal conditions could favor clinical interventions in patients with disturbed immunomechanisms.

**METHODS**

**Fungal culture conditions strain.** C. albicans strain 577 was isolated from the skin of a patient with mucocutaneous candidiasis and kindly provided by Dr Luiz Rodolfo Travassos (UNIFESP, São Paulo, Brazil). Fungal cells were grown in YPD medium (1 % yeast extract, 2 % peptone, 2 % glucose) at 28 °C for 24 h. To generate pseudohyphae, the cells collected by centrifugation were re-suspended in YPD plus 10 % FBS at a concentration of 5 × 10^6/ml and incubated at 37 °C for 2 h, as described by Kagami et al. (2010). Microscopic examination confirmed that 85.7 ± 3.8 % of these cells converted to pseudohyphae.

**Cutaneous infection model.** The preliminary results of cutaneous candidiasis in BALB/c mice were presented at the International Congress of Immunology (Campois et al., 2013). Female BALB/c mice were obtained from the State University of Maringá and were housed at five animals per cage in a temperature- and humidity-controlled room with a 12 h light/dark cycle, with sterilized water and food ad libitum. Five mice per group were inoculated with 5 × 10^6 C. albicans pseudohyphae in the deep dermis of the hind paw, and PBS solution was inoculated into the contralateral paw as a negative control. Mice were killed 0, 1, 4, 7 and 14 days after infection. Skin injured by infection was collected for histopathological analysis. The area of inflammation was evaluated daily with a paquimeter, and the mice were assessed daily for the presence of nodules, ulcerations, erythema and a crust daily. Photographs were taken before the mice were killed. Disease was scored positively if any one of the four clinical features was present.

**Ethical approval.** All experiments were approved by the Animal Research Ethics board from the State University of Londrina, Brazil (Approval no. 188/12).

**Histopathological analysis.** The collected hind paw tissues were fixed in 10 % formalin for 24 h and then submitted to a histological processing facility for paraffin embedding. Serial 7 µm sections were stained with haematoxylin and eosin (H&E) for histopathological analysis. Twenty microscopic fields from each control and infected hind paw were captured using a high-resolution camera attached to a light microscope (× 20 objective). Five measurements of epidermal thickness from each image were obtained using the software Motic Images Plus, version 2.0. These measurements included tissues from the stratum basale to the stratum granulosum of the epidermis. The number of cell layers in the epidermis of each sample was also counted in these images. The number of cell layers of the strata granulosum and spinosum was counted in two different sites of each image to improve the reliability of the evaluations. The total number of neutrophils and fibroblasts was counted in 1.26 mm² of connective tissue from the dermis of each control and infected hind paw.

**Fungal burden assays and cytokine analysis.** Groups of five mice each were inoculated with C. albicans pseudohyphae (5 × 10^6 ml⁻¹ in PBS), and after 1, 4, 7 and 14 days, the sites of infected skin on the hind paw and the popliteal lymph nodes were collected, weighed and macerated. Homogenates were diluted and 100 µl aliquots were plated onto Sabouraud dextrose agar to allow Candida colony growth. The plates were incubated for 24 h at 37 °C and the number of c.f.u. (mg tissue)⁻¹ was calculated. To determine the concentrations of pro-inflammatory (IL-6, TNF-α, IL-12, IFN-γ and IL-17) and anti-inflammatory (IL-13, IL-4, IL-10 and transforming growth factor-β (TGF-β)) cytokines, the supernatants of the homogenates were submitted to capture ELISA (eBioscience). The cytokine quantification assays were performed in accordance with the manufacturer’s instructions.

**Statistical analysis.** For analysis of epidermal thickness, the data were analysed according to tissue type. As a non-normal distribution, data were compared among groups using the Mann–Whitney test with a 5 % significance level. Tests were performed using the software BioEstat, version 5.0. For cytokine analysis, the differences between groups were analysed using an unpaired Student’s t-test, except when the time post-infection (p.i.) was a variable, where one-way ANOVA was applied. P < 0.05 was considered statistically significant.

**RESULTS**

**Epidermal hyperplasia and hypertrophy induced by C. albicans infection.**

Histological analysis of the infected hind paw revealed epidermal hyperplasia beginning on day 1 p.i., reaching a maximum on day 7 and decreasing up to day 14 (Fig. 1). Qualitative analysis of the microscopic images revealed that infection caused hypertrophy of epidermal cells, nodules, ulceration, erythema and occasionally a crust, which were most prominent on day 7 (Fig. 1). The contralateral paw inoculated with PBS showed similar features to those of uninfected control mice (data not shown).

**Quantification of cell layers in the strata granulosum and spinosum.**

Epidermal hyperplasia was evaluated by analysing 100 images of skin lesions per group of mice, taken with a Nikon digital camera coupled to a light microscope. The number of cell layers of the strata granulosum and spinosum...
was counted in two different sites of each microscopic field. Infection provoked an epidermal hyperplasia characterized by a significant increase in the number of cell layers in the strata spinosum (Fig. 2a) and granulosum (Fig. 2b). The stratum spinosum underwent a dramatic increase in cell layers between days 4 and 7 p.i. relative to the rest of the course of infection, although the numbers of cell layers remained elevated up to day 14 p.i. ($P < 0.05$). Observations of the stratum granulosum revealed a significant increase in the number of cell layers beginning only from day 4 p.i., with numbers remaining elevated up to day 14 ($P < 0.05$). Moreover, hypertrophy of the epidermal cells was observed after 4 days of infection, contributing to an increased thickness of the epidermis.

**Inflammation caused by C. albicans**

Paw thickness (mm) was evaluated daily with a paquimeter, and the inflammatory area was measured as the difference between the thicknesses of the infected paw and control paw. The peak of the inflammatory area occurred on day 6 of infection ($4.36 \pm 0.59$ mm), persisting until day 9 ($4.28 \pm 0.51$ mm), and had reduced significantly in size by day 14 (Fig. 2c). Thus, we observed that *C. albicans* caused significant thickening of the epidermis and induced an inflammatory process in the infected skin. In the contralateral paw, no inflammatory process was observed due to PBS injection alone.

**Analysis of neutrophil and fibroblast infiltration**

Neutrophils migrated to the site of infection on day 1 p.i. and reached their peak on day 4 p.i. The high population density of neutrophils started to decrease from day 7 p.i., but remained high (40% of the peak) up to day 14. In contrast, the population density of fibroblasts increased progressively up to day 14 p.i. (Table 1).

**Cytokine analysis over the course of cutaneous infection by C. albicans**

Dysfunction of the cells involved in the immune response against *C. albicans* in patients with mucocutaneous candidiasis causes dysregulation in cytokine production. Thus, we evaluated the participation of pro- and anti-inflammatory cytokines in our mouse model of cutaneous candidiasis, which had neither metabolic nor immunodeficiency caused by genetic disorders.

![Fig. 1. Histopathological analysis of skin following C. albicans infection. Five mice per group received an inoculum of $5 \times 10^6$ *C. albicans* pseudohyphae in the deep dermis of the hind paw. The mice were killed, and infected skin was collected for histopathological analysis. H&E staining of skin sections during *C. albicans* infection is shown on the right. Note the epidermal hyperplasia and hypertrophy induced by *C. albicans* infection observed at days 4 and 7 p.i.](image-url)
**Table 1. Analysis of neutrophil and fibroblast migration into the dermis during C. albicans infection**

Five mice per group were inoculated with $5 \times 10^6$ C. albicans pseudohyphae in the deep dermis of the hind paw. H&E staining of mice skin during C. albicans infection was performed to analyse the migration of neutrophils and fibroblasts into the dermis. Values are reported as the mean ± SD.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Control</th>
<th>Time (days p.i.)</th>
</tr>
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<tbody>
<tr>
<td>Neutrophil</td>
<td>28 ± 2</td>
<td>245 ± 21*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>351 ± 62*</td>
</tr>
<tr>
<td></td>
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<td>314 ± 11*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>107 ± 9*</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>135 ± 9</td>
<td>107 ± 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>173 ± 21*</td>
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<tr>
<td></td>
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<td>242 ± 11*</td>
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<td></td>
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<td>549 ± 16*</td>
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*Significantly different from the control group ($P<0.001$).

Analysing the pro-inflammatory cytokines of innate immunity in the popliteal lymph node of infected mice, we verified that IL-6 was produced on days 1 and 4 to sufficient levels to stimulate neutrophil influx to the site of infection (Fig. 3a). A significant increase in both TNF-α and IL-12 production was observed on days 1 and 4 p.i. (Fig. 3b, c). Over the course of the infection, an adaptive immune response was also observed, with increased levels of IFN-γ and IL-17 in the popliteal lymph nodes (Fig. 4a, b). All contralateral control paws showed low levels of these cytokines, or in concentrations below the threshold of detection.

Counts of c.f.u. (mg tissue)$^{-1}$ obtained over the course of infection confirmed that the fungal burden was at its maximum on day 1 p.i., reducing significantly by day 4. By day 7, the pathogen was no longer detected at the site of infection. The number of c.f.u. in popliteal lymph node tissue was significantly lower compared with the hind paw for all the time points analysed (Fig. 5). In contralateral paws that received PBS injections, no pathogen was detected.

The levels of anti-inflammatory cytokines were determined, and a significant increase in IL-13, IL-4 and IL-10 was observed on day 4. TGF-β levels were increased on day 4, and higher levels were measured on day 14 p.i. (Fig. 6a-d). Lower undetectable levels of anti-inflammatory cytokines were measured in the contralateral control paws (data not shown).

**DISCUSSION**

Most studies of chronic cutaneous candidiasis use cells from the peripheral blood of human patients. Thus, the mechanisms that favour the development of this non-invasive infection are not fully understood.

We examined the paws of mice following infection with C. albicans for structural modifications in the epidermis and dermis and changes in cytokine production by the popliteal lymph node during the inflammatory and healing process. The choice was very successful due to the proximity of the popliteal lymph node to the site of infection, thus permitting a greater understanding of the network of cytokines that participate in inflammation and tissue

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Fig. 2. Quantification of epidermal hyperplasia and analysis of paw thickness. (a, b) Quantification of cell layers in the strata spinosum (a) and granulosum (b). (c) Analysis of paw thickness. The extent of the inflammatory area was evaluated daily with a paquimeter. The data were compared between groups using the Mann–Whitney test with a 5 % significance level. *$P<0.05$.
repair. As patients with cutaneous candidiasis present with dysfunctions in Th1 and Th17 cells, and as a consequence suffer persistent recurrent or chronic fungal infections (Kobrynski et al., 1996; Lilic et al., 2003; van der Graaf et al., 2003; Eyerich et al., 2007, 2008), special attention was given to the cytokines produced by these cells in this study.

During the early phase of infection, an increase in inflammatory exudates was verified by an increased extent of the inflammatory area and hyperplasia of the epidermis. Moreover, the number of cell layers of the stratum spinosum increased significantly during C. albicans infections, and hypertrophy of epidermal cells was observed beginning at 4 days p.i. These results suggested expansion of the epidermis, functioning as a biological barrier to contain both the infection and the inflammatory exudate. Therefore, both epidermal hyperplasia and hypertrophy were important for prevention of epithelial disruption, maintaining the barrier against antigens from the external environment.

In the control paws of infected mice, the analysis of the epidermis and the dermis revealed features similar to uninfected mice (data not shown), demonstrating that in our experimental conditions the candidiasis was non-invasive.
Introduction of *C. albicans* induced the production of inflammatory cytokines, such as IL-6, TNF-α and IL-12. Cytokines, such as IL-6 and TNF-α, certainly participate in the accumulation and activity of neutrophils in the dermis, as verified in this study. Maximum levels of IL-6 and TNF-α were detected on day 4 of infection, contributing to clearance of the pathogen, which was probably complete by day 7 or, at the very least, no longer present at detectable levels. As has been reported previously, patients with chronic mucocutaneous candidiasis may possess a defect in the IL-12 and IL-23 receptor signalling pathways, predisposing such patients to this infection (van de Veerdonk *et al.*, 2011). In our animal model, the same pathways were probably involved, as indicated by the increased production of IL-6 and IL-12. Thus, our model can be used for future studies to determine the impact of induced disorders of these pathways.

Dendritic cells in the skin probably processed *C. albicans* at the site of infection, and then migrated to the popliteal lymph node where they activated naive CD4⁺ Th-precursor cells. These precursor cells differentiated into effector Th cells of the subtype Th1, which produced IFN-γ. We detected an increase in IL-12 production, which corroborates our conclusion of Th1 subtype differentiation, as has been described by Ashman *et al.* (2011). These observations do not exclude the possibility of natural killer (NK) cells participating in the initial phase of infection.

IL-17 production was observed on day 1 p.i., suggesting activation of cells of the innate immune response system, including NK cells and γδ T cells. A significant increase in IL-17 production was observed on day 4, which could

**Fig. 5.** Recovery of *C. albicans* from the hind paw and popliteal lymph node. Mice were inoculated with $5 \times 10^6$ *C. albicans* pseudohyphae in the deep dermis of the hind paw. Fungal burden was analysed by plating macerated skin tissue and popliteal lymph node onto YPD growth medium. Following incubation at 37 °C for 24 h, the c.f.u. were counted. Data are reported as the mean of c.f.u. ± SEM mg⁻¹ of tissue from five experiments. Statistical significance was calculated using Student’s *t*-test (*P < 0.05).

**Fig. 6.** Anti-inflammatory cytokine analysis during the infectious process. Five mice per group were inoculated with $5 \times 10^6$ *C. albicans* pseudohyphae in the deep dermis of the hind paw. The concentrations of anti-inflammatory cytokines in supernatants of macerated popliteal lymph node were determined by capture ELISA: IL-13 (a); IL-4 (b); IL-10 (c); TGF-β (d). Assays were performed in duplicate and the results represent the mean ± SEM of five independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.
correspond to the activation of the Th17 subset of CD4+ lymphocytes, as verified by our observations of a reduction in the number of c.f.u. from samples of injured skin and an increased recruitment of neutrophils to the site of infection. Yu and Gaffen (2008) described IL-17 as a novel inflammatory cytokine that bridges the innate and adaptive immune responses. We have demonstrated previously that a significant increase in IL-17 levels can be considered to be protective against candidiasis, due to increased phagocytic and fungicidal activities of macrophages and neutrophils over the course of infection by C. albicans (Custodio et al., 2011).

It has been verified that patients with chronic mucocutaneous candidiasis exhibit reduced production of Th17-associated cytokines such as IL-17. Mice deficient in IL-17A due to mutations in the cytokines genes (IL-17A and IL-17F) demonstrated delayed healing of lesions after skin infection with C. albicans, compared with WT mice (Kagami et al., 2010). These findings were corroborated by our results.

IL-13 shares numerous biological activities with IL-4. Both cytokines reduce the production of IL-6, TNF-α and other pro-inflammatory mediators produced by M1 macrophages, and both induce differentiation of M2 macrophages by alternative activation. In addition, they upregulate the expression of mannose receptor and dectin-1 (Gordon, 2003). In our study, the levels of both cytokines were increased on day 4 p.i., concurrent with the inflammatory response and pathogen elimination. M2 macrophages produce anti-inflammatory cytokine IL-10 and are involved in the resolution of inflammation, tissue repair and wound healing. Moreover, IL-13 induces tissue fibrosis by a TGF-β-independent mechanism (Kaviratne et al., 2004), or by selectively stimulating and activating TGF-β 1 (Fichtner-Feil et al., 2006). In our experiments, IL-13 levels increased significantly at 1 and 4 days p.i. This result suggests that IL-13 is involved with an early fibrotic process, with increased levels due to an inflammatory process caused by Candida infection that is independent of TGF-β. In addition, IL-13 also could be stimulating the TGF-β production, which increased in the late phases of infection.

TGF-β production is necessary for the proliferation of fibroblasts, and we observed increased levels from day 4 p.i. onward, with a significant increase on day 14. These levels correlated with the increase in fibroblasts we observed, which led to the healing of lesions provoked by C. albicans infection. TGF-β is also released by macrophages, platelets and fibroblasts, and is found at increased levels in acute wounds (Eming et al., 2007; Barrientos et al., 2008; Kim et al., 2013).

In the late phase of infection, a balance of pro-inflammatory cytokines such as IL-4, IL-13, IL-10 and TGF-β was verified. These cytokines prevent exacerbation of the inflammatory process and allow tissue repair mechanisms, such as fibrosis, to take place in healing lesions caused by the pathogen. Thus, our results indicated that cutaneous infection by C. albicans induced the production of pro-inflammatory cytokines of both the innate and adaptive responses, which were able to eliminate the pathogen, followed by anti-inflammatory cytokines that correlated with lesion healing. These findings are in support of our initial hypothesis that a balance between these systems is necessary to prevent chronic cutaneous candidiasis.

CONCLUSION

Our model of C. albicans infection in cutaneous tissues provides an expanded understanding of host defence mechanisms by analysing paw symptomatology and the histopathological development of infection. Additionally, we were able to investigate the effect of cytokines at the site of infection that were produced in the adjacent lymph node (popliteal). This approach will enable future studies of systemic disorders that predispose an individual to cutaneous candidiasis, such as endocrinopathies and genetic disorders, adding new insights to the clinical interventions that can be used to overcome this disease.

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