Original Article

Candida parapsilosis complex induces local inflammatory cytokines in immunocompetent mice

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Abstract

Despite the increasing incidence of the Candida parapsilosis complex in the clinical setting and high mortality rates associated with disseminated infection, the host-fungus interactions regarding Candida parapsilosis sensu stricto and the closely related species C. orthopsilosis and C. metapsilosis remains blurred. In this study, we analyzed inflammatory cytokines levels and histopathology as well as fungal burden in spleen, kidney and lung of mice infected with six strains of the “psilosis” group with different enzymatic profiles. Strong interleukin 22 (IL-22) and tumor necrosis factor α (TNF-α) responses were observed in analyzed organs from infected mice (P < .0001) regardless of the species and enzymatic profile. TNF-α and IL-22 levels were related with spleen inflammation and fungal load. Fungal cells were detected only in spleen and kidney of infected mice, especially by day 2 post-challenge. The kidney showed glomerular retraction and partial destruction of renal tubules. Our data suggest that a strong inflammatory response, mainly of IL-22 and TNF-α, could be involved in Candida parapsilosis complex infection control.

Key words: Candida parapsilosis complex, cytokines response, IL-22, TNF-α, IFN-γ, IL-17A.

Introduction

Candida parapsilosis sensu stricto has been recognized as one of the most common Candida species that originate candidemia [¹,²]. Along with Candida orthopsilosis and Candida metapsilosis, these three phenotypically indistinguishable cryptic yeasts belong to the so-called psilosis group [³]. The administration of parenteral hyperalimentation solutions, as well as the use of intravascular devices and...
Table 1. Enzymatic activities of the strains used to infect BALB/c mice though tail injection of \(1.5 \times 10^7\) CFU/mouse inocula.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Clinical origin</th>
<th>Aspartyl proteinase</th>
<th>Phospholipase</th>
<th>Esterase</th>
<th>Hemolysin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida parapsilosis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c/c 105 Peritoneal fluid</td>
<td></td>
<td>−</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>H-124 Blood</td>
<td></td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><em>Candida orthopsilosis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP-179 Blood</td>
<td></td>
<td>−</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>H-152 Blood</td>
<td></td>
<td>+++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><em>Candida metapsilosis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-18 Skin</td>
<td></td>
<td>++++</td>
<td>+++</td>
<td>−</td>
<td>+++</td>
</tr>
<tr>
<td>ATCC 96144 Skin</td>
<td></td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*a* Semiquantitative estimation according to the Pz index (colony diameter/total diameter of the colony plus precipitation or halo zone). Very strong (+++), strong (++), mild (+), weak (+), and negative (−) enzymatic activity.

prosthetic materials represent important risk factors that could lead to infection by these opportunistic pathogens in susceptible hosts [2,4].

The incidence of *C. parapsilosis* species complex has unacceptably increased in recent decades, a fact that has motivated many studies regarding their epidemiology [4,5], antifungal susceptibility [6], virulence [7], and pathogenesis [8], even in nonmurine novel infection models [9]. Otherwise, the model organism *Candida albicans* has led some progress in the field of host-fungus interactions. In this sense, the immune response elicited by *C. albicans* infection is driven by inflammatory mediators, particularly inflammasome-derived interleukin 1β (IL-1β), and is characterized by the production of interferon γ (IFN-γ) from Th1 cells and interleukin 17A (IL-17A) from Th17 cells [10,11]. In disseminated *C. albicans* infection, Th1 cells are associated with protection from disease, while a predominance of Th2 cells promotes susceptibility [11]. However, a comprehensive tissue-specific analysis of the cytokine levels participating in the anti-*Candida* host defense to disseminated disease is lacking and there is a large gap in the current knowledge of the immunity against infection by *C. parapsilosis* sensu stricto, *C. orthopsilosis* and *C. metapsilosis*.

Recent evidence suggests that the three species of the “psilosis” group possess a similar pathogenic potential in disseminated candidiasis regardless of their particular in *vitro* enzymatic profiles [8]. The aim of this study was to analyze the levels of inflammatory cytokines in three organs (spleen, kidney, and lung) in immunocompetent mice infected with six strains of the psilosis group with different enzymatic profiles.

**Materials and methods**

**Ethics statement**

Murine experiments were performed with the approval of the Ethics and Research Committee of the School of Medicine of the Universidad Autónoma de Nuevo León (registration code: MB12-002). The animal sacrifices were carried out by cervical dislocation, and all efforts were made to minimize suffering. The experimental protocol was designed in conformity with the International Review Board regulations, following the recommendations of the Guidelines for the Care and Use of Laboratory Animals, and in agreement with Good Laboratory Practices. Care, maintenance, and handling of the animals were in accordance with the Mexican regulations for animal experimentation (NOM-062-ZOO-1999).

**Mice**

Male BALB/c mice, 5 weeks old (22–24 g weight) were purchased from Harlan (Harlan Mexico, S.A. de C.V., Mexico). A total of 132 animals were used; these were housed in ventilated cages of five mice each under specific pathogen-free conditions at the Animal Facility of the Department of Microbiology. All mice were given sterile water and Purina rodent food *ad libitum* and were monitored daily for 15 days. The day/night cycle was 12 h/12 h. Before use, the animals were allowed to acclimatize for 5 days.

**Fungal strains**

The strains used in this study were previously utilized in our laboratory [8]: *C. parapsilosis* sensu stricto (c/c 105 and H-124), *C. orthopsilosis* (HP-179 and H-152), and *C. metapsilosis* (MEX-18 and ATCC-96144). Details regarding the enzymatic activities of the strains are shown in Table 1. The strains were stored as suspensions in sterile distilled water at room temperature and cultured for 48 h on Sabouraud-dextrose agar (SDA) slants (Difco, Detroit, MI, USA) at 37°C before use.
Experimental infection

For inocula preparation, the six strains were passaged at least twice on SDA plates to check purity and viability of the cultures. After 48 h of incubation at 37°C yeast cells were harvested, washed twice in sterile saline, quantified with a hemocytometer, and adjusted to the desired concentration. To corroborate the yeast cell counts, serial fold dilutions were cultured on SDA at 37°C for 48 h. Systemic candidiasis was induced in mice by injecting an inoculum of 1.5 × 10⁷ CFU/mouse through the lateral tail vein in 200 µl of a yeast suspension in groups of 20 animals per strain. Three uninfected mice to which sterile saline were intravenously administered were used as controls per experimental day. No immunosuppressive scheme was used.

Kidneys are the target organ of murine disseminated candidiasis and therefore one of the most studied tissues in various works [12–14], so we considered analyzing both fungal tissue burden and inflammatory cytokine levels locally expressed in this organ and in the spleen and lungs. The fungal tissue burden determinations were previously published [8]. Briefly, five mice per strain were humanely killed at days 2, 5, 10, and 15 post-challenge. Three infected mice to which sterile saline were intravenously administered were used as controls per experimental day. No immunosuppressive scheme was used.

Results

Inflammatory cytokines were detected since early stage of infection

Cytokine assays

Four mouse cytokines were assayed in supernatants from tissue homogenates. The cytokines measured were: TNF-α, IFN-γ, IL-17A, and IL-22. Determinations were performed by sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocol for TNF-α, IFN-γ (PeproTech, Mexico City, Mexico), IL-17A, and IL-22 (eBioscience, San Diego, CA, USA), using the iMark microplate reader (Bio-Rad Inc., Hercules, CA, USA). The quantitation ranges were 32–2000 pg/ml, 16–2000 pg/ml, 4–500 pg/ml, and 8–1000 pg/ml for TNF-α, IFN-γ, IL-17A, and IL-22, respectively. Each sample was analyzed twice.

Histology

For histopathological analysis, half of each organ was fixed with 10% buffered formalin. Samples were dehydrated, paraffin embedded, and sliced into 5-µm sections, which were then stained with periodic acid-Schiff (PAS) and examined in a blinded fashion by light microscopy.

Statistical analysis

The Kruskal-Wallis and Dunn’s multiple comparison tests were applied to verify statistical significance among experimental groups. Pearson’s correlations between splenic inflammation and fungal load with local cytokine levels were additionally made. Calculations and graphics were performed using GraphPad Prism version 5.03 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). P values ≤ .05 were considered significant.
Figure 1. Inflammatory cytokines were detected since early stage of infection. Graphs show TNF-α locally expressed in kidney (A), lung (B), and spleen (C). The data represent the means of five mice per group ± SD. Asterisks represent significant differences compared to controls. TNF-α levels in kidney were found > 5–10-fold compared with lung or spleen.

at 2, 5 and 10 days post-infection. In lung, C. parapsilosis sensu stricto c/c 105 and H124 strains were the most potent inducers of IFN-γ at 2 and 5 days post-infection, respectively; however, in the next days it was reduced (Fig. 3B). IL-17A production was downregulated in the kidneys and lungs of mice infected with C. parapsilosis complex strains at 2, 5, and 10 days, respectively (Fig. 4A–B). In spleen, C. parapsilosis sensu stricto H124 strain induced high levels of IL-17A at 5 and 10 days post-infection (Fig. 4C).

Spleen inflammation is paralleled with local TNF-α and IL-22 levels

The splenomegaly was measured throughout the infection with a vernier, and the inflammation score was calculated using the ellipsoid equation. Mice infected with C. parapsilosis complex strains exhibited spleen inflammation, and this was in accord with TNF-α and IL-22 levels, especially with C. parapsilosis sensu stricto and C. metapsilosis strains.

TNF-α and IL-22 could control fungal load

TNF-α and IL-22 are essential cytokines in the control of fungal infections. TNF-α induces oxidative and nitrosative distress in the phagolysosome microenvironment and contribute to the inflammatory process [15]. On the other hand, IL-22 limits fungal growth due to induction of antimicrobial peptides [16]. Thus, we questioned if the levels of TNF-α and IL-22 are related with the decrease in fungal load. These analyses demonstrated that both cytokines increase in kidney from mice infected with most C. parapsilosis complex strains, showing > 1 log reduction in fungal load. C. orthopsilosis H-152 (a biofilm former strain) was the lowest affected of the studied strains in the kidney, showing < 0.5 log reduction. In spleen and lung, fungal load was reduced > 2 log in most C. parapsilosis complex strains, where high...
levels of TNF-α and IL-22 were presented (data not shown); however, when a correlation was analyzed it was not significant.

The kidney is the most affected organ during *C. parapsilosis* complex infection

During the course of the infections, histological alterations in spleen and lung were not found compared with control mice. However, in the spleen, the presence of isolated yeasts near the cells of the Billroth cords and in the red pulp sinusoids were evident, especially at day 2 post-challenge. There was no evidence of fungal structures in lungs of infected mice. On the other hand, abundant yeast cells at day 2 were detected in the kidney after systemic infection, mainly in the renal interstitium, and these tended to gradually decrease by day 15. In addition, the presence of acute inflammatory infiltrate foci surrounding yeasts in kidney was evident at day 5 and 10 post-challenge (Table 2). Unlike the spleen and lung, the kidney of infected mice showed some histological alterations, glomerular retraction, and destruction of renal tubules, which were the principal findings compared with uninfected controls, and these were detected since day 2 post-challenge (Fig. 5 and Table 2). Importantly, a particular histopathological pattern associated to a specific species or enzymatic profile among the analyzed strains was not found.

**Discussion**

Diverse animal models of infection have been developed to investigate the different clinical forms of candidiasis, being murine models the gold standard to study pathogenesis as well as efficacy of antifungal agents alone or in multiple combinations [9]. The murine intravenous (IV) challenge model mimics human bloodstream-derived
Inflammatory cytokines were detected since early stage of infection. Graphs show IFN-γ locally expressed in kidney (A), lung (B), and spleen (C). The data represent the means of five mice per group ± SD. Asterisks represent significant differences compared to controls. IFN-γ was down-regulated in lung and spleen with respect to kidney.

candidiasis, and due to its highly reproducibility it remains as the most common long-standing model used to investigate C. albicans virulence and to determine particular aspects of host-fungus interactions [17–19]. During the systemic infection, the bloodstream and the majority of organs (spleen, lungs, and liver) are gradually cleared of the pathogen, but this scenario is quite different in the kidney, the principal target organ for IV challenge in the mouse, in which the increase of fungal burden is accompanied by increasing levels of renal cytokines and chemokines [13,14], with an existing correlation between the increased renal cytokine levels and the lesion severity with the consequent infection outcome [14,19]. Yet, the cellular and molecular factors that determine differential organ-specific control of Candida remain largely unknown.

During pathogenic C. albicans infection, the inflammatory mediators of the immune response are driven by Th1 and the recently implicated involvement of Th17 cells. The stimulated Th1 lymphocytes produce IFN-γ, which contributes to anti-Candida host defense by inducing nitric oxide (NO) production by macrophages [20]. Moreover, Th17 cells are characterized by the production of IL-17A, IL-17F, IL-21, and IL-22 [21]. IL-17A interconnect lymphoid and myeloid host defense [22] through the infiltration induction of neutrophilic granulocytes at the site of infection and activation of macrophages [21]. Meanwhile, IL-22 is a member of the IL-10 family of cytokines, which has been confounded by data suggesting both pro- and anti-inflammatory functions. However, recent evidence suggests that this cytokine accounts for innate resistance to candidiasis at the early stages of infection in the kidney and gut by critically controlling initial fungal growth and epithelial homeostasis in the relative absence of Th1 immunity [23]. Interestingly, current findings suggests that IL-22 and TNF-α represent a potent synergistic cytokine combination for skin immunity, efficiently conserving epidermal barrier integrity in a skin infection model compared with IFN-γ, IL-17, IL-22, or TNF-α alone [24]. In disseminated C. albicans infection, Th1 cells are associated with protection from disease, while a predominance of Th2 cells promotes susceptibility [11]. Finally, the balance in innate responses between fungicidal (beneficial) immunity and immunoregulatory (detrimental) compensative mechanisms determines the scope of tissue damage in fungal infections [16].

The tissue specificity of mouse cytokine host responses in hematogenously C. albicans infection was previously demonstrated by Spellberg et al. [25] They examined the relationship between host survival and local immune responses in kidney and spleen during candidiasis with different inocula, concluding that this pathogen induced type 2 splenocyte responses with both fatal and nonfatal inocula. Conversely, the nature of immune polarization in the
Figure 4. Inflammatory cytokines were detected since early stage of infection. Graphs show IL-17A locally expressed in kidney (A), lung (B), and spleen (C). The data represent the means of five mice per group ± SD. Asterisks represent significant differences compared to controls. IL-17A was down-regulated in kidneys and lungs at days 2, 5, and 10 post-challenge.

Kidney correlated with host survival, with IFN-γ-dominant type 1 responses causing no or low mortality and type 2 or IL-10-dominant responses during 100% fatal infection. Subsequently, MacCallum et al. [14] analyzed cytokine and chemokine levels in infected organs elucidating organ-specific responses, with high cytokine and chemokine levels in infected kidneys but reduced responses in the spleen. They concluded that keratinocyte-derived chemokine is an important early mediator of overall outcome and correlate along with IL-6 and MIP-1β with lesion severity, whereas GM-CSF and IL-10 showed inverse correlations with histological damage. These differences were also reflected at the transcriptional level, with differential expression of cytokine genes in both organs [26]. Since our interest was focused in local responses to infection, the experimental design we adopted did not include measurement of serum cytokine levels. Of the assayed organs in this study, the kidney showed the highest fungal load, as well as pro-inflammatory cytokine levels, and it was the only tissue that showed histological alterations due to the systemic infection (Fig. 7). Recently, Lionakis et al. [27] characterized the immune cell populations in infected organs during the progression of disseminated candidiasis in a mouse model. They found neutrophils accumulated in all infected organs, but a delay in their appearance in the kidneys, leaving these organs unprotected during the first 24 h post-challenge. Further increases in neutrophils occurred in these organs as disease progressed. The kidney inflammation could be explained in part by the CCR1 neutrophils, which play a pathogenic role in invasive candidiasis mediating renal immunopathology via excessive neutrophil recruitment from the blood into the kidney [12]. The role of IL-22 in neutrophil recruitment in peripheral tissue has been already reported in murine cytomegalovirus [28]. We found downregulation of IFN-γ production due to C. parapsilosis complex infection, agreeing with Carvalho et al., who also reported this finding.
in some patients with recurrent vaginal candidiasis (RVC) [29]; this could probably be coupled to the absence of IL-6 in response to the fungus on epithelial surfaces [30]. On the other hand, high levels of IL-22 found in kidney could be involved in neutrophil recruitment in this tissue. In lung and spleen were not observed neutrophil indeed IL-22 increment during C. parapsilosis complex infection, it may be to absent or low number of yeasts in these tissues. Finally, a significant correlation between splenic inflammation and fungal load with local cytokine levels was not found.

Overall, this study demonstrates that male BALB/c mice experimentally infected with strains of the “psilosis” group exhibiting different in vitro enzymatic profiles irrespectively induce a pronounced inflammatory response that is essential for protective antifungal immunity and drive pathology during disseminated infection, principally in the kidney. Although a correlation between cytokine levels and enzymatic activity of the strains was not found, the role of these enzymes in systemic disease caused by the C. parapsilosis sensu lato group should not be underestimated. In this regard, further studies with more characterized strains, as well as homozygous mutants for the

**Table 2. Histopathological findings in kidney of mice infected with six strains of Candida parasilopsis complex.**

<table>
<thead>
<tr>
<th>Finding</th>
<th>Day post-infection (dpi)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Glomerular retraction</td>
<td>−</td>
</tr>
<tr>
<td>Neutrophil infiltration</td>
<td>−</td>
</tr>
<tr>
<td>Destruction of renal tubules</td>
<td>−</td>
</tr>
</tbody>
</table>

Note: Values: − Absent, −+ Variable, + Present, ++ Moderate, +++ Abundant.
genes involved in each enzyme, become essential in order to elucidate their particular contribution to the pathogenesis by these opportunistic pathogens. In general, this knowledge has implications in the development of novel immunotherapy strategies, which aims improve host defense against C. parapsilosis complex, providing a solution to prevent and reduce the high mortality rates of the invasive candidiasis.

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Declaration of interest

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

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