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Optimal clearance of *Sporothrix schenckii* requires an intact Th17 response in a mouse model of systemic infection

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ABSTRACT

The discovery of Th17 cells, along with many other Th cell subsets in the recent years, has expanded the Th1/Th2 paradigm that had persisted since its proposition by Mosmann in 1986. Defined by the characteristic expression of the transcription factor retinoic-related orphan receptor γt (RORγt) and production of IL-17A (IL-17), Th17 cells are powerful inducers of tissue inflammation with a recognized role against extracellular bacteria and fungi. Despite this, the interest in their study came from the pivotal role they play in the development and maintenance of major chronic inflammatory conditions such as multiple sclerosis, rheumatoid arthritis and Crohn's disease, hence they have been the target of promising new anti-Th17 therapies. Accordingly, the identification of opportunistic pathogens whose clearance relies on the Th17 response is of huge prophylactic importance. As shown here for the first time, this applies to *Sporothrix schenckii*, a thermo-dimorphic fungus and the causative agent of sporotrichosis. Our results show that both Th17 and Th1/Th17 mixed cells are developed during the *S. schenckii* systemic mice infection, which also leads to augmented production of IL-17 and IL-22. Also, by using an antibody-mediated IL-23 depletion model, we further demonstrate that optimal fungal clearance, but not survival, depends on an intact Th17 response.

Introduction

Sporotrichosis is an acute or chronic subcutaneous mycosis affecting humans and other mammals, caused by the *Sporothrix schenckii* species complex, including *Sporothrix albicans*, *Sporothrix brasiliensis*, *Sporothrix globosa*, *Sporothrix luriei*, *Sporothrix mexicana* and *S. schenckii sensu strictu* (Oliveira et al., 2014). The disease follows the traumatic inoculation of the fungus through injuries containing soil, inhalation of conidia or zoonotic transmission, especially from cat scratches, affecting immunocompromised individuals in an opportunistic fashion (Aung et al., 2013; Barros et al., 2011; López-Romero et al., 2011). In several countries sporotrichosis is regarded as an emerging opportunistic infection, one that's often neglected (Freitas et al., 2014; Rodrigues et al., 2013). Specifically in Brazil, the last two decades saw a surge in feline and canine cases, mainly in the south and southeast regions of the country (Pereira et al., 2014; Madrid et al., 2012; Nobre et al., 2001; Schubach et al., 2004, 2006). The infection's outcome and clinical manifestation is highly dependent on the immune status of the host: while immunocompetent individuals usually develop localized cutaneous forms, immunocompromised patients, including those infected with HIV, are predominantly affected by disseminated and systemic forms (Carlos et al., 2009). Our research group has been exploring the *S. schenckii* host-pathogen interaction for quite some time and has previously shown that cell-surface

Abbreviations: RORγt, retinoic-related orphan receptor γt; T-bet, T-box expressed in T cells; GATA-3, GATA-binding protein 3; Foxp3, forkhead box P3; HKss, heat-killed *S. schenckii* yeast; IL-23R, IL-23 receptor; DCs, dendritic cells; CLRs, C-type lectin family of receptors; i.p., intraperitoneal; ILC, innate lymphoid cell; iNKT, invariant NKT; LTi, lymphoid-tissue inducer.

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antigens present on the cell-wall of *S. schenckii* are employed as an escape mechanism against host's macrophages (Carlos et al., 2003). We have also assessed the role played by toll-like receptors (TLRs) -2 and -4 in this fungus' innate immune recognition (Negrini et al., 2013, 2014; Sassá et al., 2012). Moreover, we have recently shown the involvement of nod-like receptors (NLRs) in fungal recognition through the inflammasome-dependent activation of caspase-1 (Gonçalves et al., 2014).

T helper (Th) cells are important orchestrators of the adaptive immune response. They can be subdivided into an ever growing number of subsets, two of which, namely Th1 and Th2 cells, have been known for a long time since their proposition by Mosmann et al. (1986). For over two decades we have known that cellular responses are developed during the *S. schenckii* mice infection (Carlos et al., 1992) and, more recently, that Th1 and Th2 responses are elicited in an antigen-specific manner against the fungus' cell-wall antigens (Maia et al., 2006). Since then, two other major subsets were described: Treg cells, which exert various immunosuppressive functions, and Th17 cells, named after the signature production of IL-17A (IL-17) (Nakayamada et al., 2012; Zhu and Paul, 2010). Th17 cells are characterized by expression of the master transcription factor retinoic-related orphan receptor γt (RORγt) and are able to produce, besides IL-17, also IL-17F, IL-22 and IL-21 (Huang et al., 2012). The combination of TGF-β1 plus IL-6 or IL-21 initiates Th17 cell differentiation, while IL-23 acts later on the commitment program to further stabilize the phenotype. Also, dendritic cells (DCs) are especially suited for promoting Th17 cell development, especially in response to fungal pathogens through the C-type lectin family of receptors (CLRs), whose triggering favors production of Th17-polarizing cytokines such as IL-23 (Huang et al., 2012; McGeachy and McSorley, 2012; Vautier et al., 2010). Accordingly, our group has previously shown that DCs recognition of *S. schenckii* antigens leads to development of a Th1/Th17 response *in vitro* (Verdan et al., 2012).

Owning primarily, but not exclusively, to IL-17 pro-inflammatory actions, which include neutrophil and Th1 cell recruitment and induction of pro-inflammatory cytokines production by epithelial cells, Th17 cells have a key role in host defense against extracellular bacteria and fungi, showing both protective and deleterious effects (Cua and Tato, 2010; Hernández-Santos and Gaffen, 2012; van de Veerdonk et al., 2009). However, a great part of our current understanding of the fungi-Th17 response relationship comes from studies of a narrow sample of pathogens, especially from *Candida albicans*. Specifically in regard to the mechanisms and relative importance of Th17 responses, this sample is even narrower, forcing dangerous generalizations when making therapeutic choices. Furthermore, Th17 cells and their associated cytokines have been implicated in numerous autoimmune and inflammatory diseases (Singh et al., 2014; Zambrano-Zaragoza et al., 2014), sparking interest in novel therapeutic approaches specifically targeted against Th17 responses, ranging from blockade of the Th17 differentiation and expansion to the specific neutralization of its effector cytokines (Gaffen et al., 2014; Maddur et al., 2012; Miossec and Kolls, 2012; Toussirot, 2012). However, despite promising results for some diseases, a common problem regards the increased susceptibility to opportunistic infections, commonly by fungal pathogens. Therefore, further understanding how Th17 responses shape the outcome of opportunistic fungal infections like sporotrichosis has major clinical applications. By using a murine model of systemic infection, this is, to our knowledge, the first study to assess the development and relative importance of the Th17 response against *S. schenckii*, providing valuable insights into the immune mechanisms triggered by sporotrichosis and paving the road to future preventive and curative therapies.

Materials and methods

Microorganism and culture conditions

S. schenckii ATCC 16345, originally obtained from a human case of diffuse lung infection (Baltimore, MD) and kindly provided by the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil), was used for all experiments. For mice infection and heat-killed *S. schenckii* yeast (HKss) preparation, a piece of the fungal mycelium grown on Mycosel (BD Biosciences) agar tubes was transferred to an Erlenmeyer flask containing 100 mL of brain-heart infusion broth (Difco) and then cultured for 7 days at 37 °C with constant shaking at 150 rpm. After that, an aliquot containing 2×10^7 yeast cells was transferred to a fresh medium and cultured for 5 more days at the same conditions in order to achieve a virtually 100% mycelium-to-yeast conversion in a logarithmically growing culture.

Preparation of the heat-killed S. schenckii yeast

HKss cells were prepared from the same 5-day-old culture of the fungus in brain-heart infusion broth used for animal infection in each respective experiment. Yeast cells were separated from the supernatant by centrifugation at 200 × *g* for 5 min at room temperature, washed twice with 8 mL of sterile PBS, pH 7.4 (hereafter, PBS), and then resuspended and adjusted to 2.5×10^8 yeast cells/mL in PBS. After that, three to four 1.5 mL aliquots of this suspension were transferred to disposable sterile 15 mL conical tubes, incubated for 1 h in a 60 °C water bath and then stored at 2–8 °C until use. A working suspension was obtained by making a 1/10 dilution of the stock suspension in RPMI complete medium (defined as the base RPMI-1640 medium containing 20 μM of 2β-mercaptoethanol, 100 U/mL of penicillin and streptomycin, 2 mM of L-glutamine and 5% fetal calf serum). As a control for the efficiency of the heat-killing process, 100 μL aliquots from each tube were inoculated on Mycosel agar plates and checked for CFU growth before use.

Animals and experimental design

Male BALB/c mice, 5–7 weeks old at the time of inoculation, were purchased from "Centro Multidisciplinar para Investigação Biológica na Área da Ciência de Animais de Laboratório" (CEMIB), UNICAMP University (Brazil). Animals were housed in individually ventilated cages in an ambient with controlled temperature and 12-h light/dark cycles. Water and food were offered *ad libitum*. For each independent experiment, only age-matched animals were used across all groups. Animals were intraperitoneally inoculated with 10^6 *S. schenckii* yeast cells in PBS or with an equal volume of PBS alone. Alternatively, for the IL-23 depletion experiments, mice were inoculated with sterile PBS ("control") or with 10^6 *S. schenckii* yeast cells in PBS alone ("PBS") or added with 10 μg of an anti-IL-23p19 neutralizing mAb (clone G23-8, eBiosciences) ("anti-IL-23") or its isotype control ("iso"). For these experiments, mice continued to be treated through the intraperitoneal (i.p.) route with 10 μg of the anti-IL-23p19 mAb, its isotype control or PBS alone on alternate days, from the day of the infection until 2 days prior to animal sacrifice on days 10 or 28 post-infection. Effectiveness of the treatment was assessed by measuring the *ex vivo* release of IL-17 and IL-22 in 24-h culture supernatants from total splenocytes challenged with HKss. Assessment of the systemic fungal load was performed by counting the CFU grown on Mycosel agar plates after the spread-plating of a previously determined dilution of the spleen macerate, collected before the red cell lysis treatment. All animal procedures were performed according to the guidelines of the Brazilian College of Animal Experimentation (COBEA) and were

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