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Dendritic cell are able to differentially recognize *Sporothrix schenckii* antigens and promote Th1/Th17 response *in vitro*

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ABSTRACT

Sporotrichosis is a disease caused by the dimorphic fungus *Sporothrix schenckii*. The main clinical manifestations occur in the skin, however the number of systemic and visceral cases has increased, especially in immunocompromised patients. Dendritic cells (DCs) are highly capable to recognize the fungus associated data and translate it into differential T cells responses both *in vivo* and *in vitro*. Although, the mechanisms involved in the interaction between DCs and *S. schenckii* are not fully elucidated. The present study investigated the phenotypic and functional changes in bone marrow dendritic cells (BMDCs) stimulated *in vitro* with the yeast form of *S. schenckii* or exoantigen (ExoAg) and its ability to trigger a cellular immune response *in vitro*. Our results demonstrated that the live yeast of *S. schenckii* and its exoantigen, at a higher dose, were able to activate BMDCs and made them capable of triggering T cell responses *in vitro*. Whereas the yeast group promoted more pronounced IFN-γ production rather than IL-17, the Exo100 group generated similar production of both cytokines. The exoantigen stimulus suggests a capability to deviate the immune response from an effector Th1 to an inflammatory Th17 response. Interestingly, only the Exo100 group promoted the production of IL-6 and a significant increase of TGF-β, in addition to IL-23 production. Interestingly, only Exo100 group was capable to promote the production of IL-6 and a significant increase on TGF-β, in addition with IL-23 detection. Our results demonstrated the plasticity of DCs in translating the data associated with the fungus *S. schenckii* and ExoAg into differential T cell responses *in vitro*. The possibility of using ex vivo-generated DCs as vaccinal and therapeutic tools for sporotrichosis is a challenge for the future.

Introduction

Sporothrix schenckii is a thermally dimorphic fungus that causes sporotrichosis, a subacute or chronic mycosis characterized by nodular lesions in cutaneous or subcutaneous tissues and associated with localized lymphangitis and lymphadenopathy (Rafal and Rasmussen 1991). Sporotrichosis is the most common subcutaneous mycosis in South America, and more recently, Japan and North America are also considered endemic areas (Barros et al. 2004). The extent of disease varies according to the immune status of the host. As sporotrichosis is more severe in nude mice (Dickerson et al. 1983) and in patients with acquired immune deficiency syndrome, T-cell-mediated immunity appears to have a central role in limiting the progression of infection (Aarestrup et al. 2001; Losman and Cavanaugh 2004).

A peptide-polysaccharide that is easily extracted from the fungus, exoantigen (ExoAg), has been identified as an important virulence factor for sporotrichosis (Nascimento et al. 2008; Teixeira et al. 2009). One of the most studied *S. schenckii* antigens, a 70 kDa protein fraction, was recently described by two different groups as a putative adhesion protein (Nascimento et al. 2008; Ruiz-Baca et al. 2009). Moreover, Carlos and co-workers have demonstrated ExoAg mitogenic activity in lymphocyte culture (Carlos et al. 1999).

Pathogens present highly conserved molecular signatures known as pathogen associated molecular patterns (PAMPs). Using a vast repertoire of pathogen recognition receptors (PRR), dendritic cells (DCs) are able to translate the pathogen-associated signature, leading to profound phenotypic and functional changes and activated status. Activated status is defined by high levels of MHC, high levels of the co-stimulatory molecules CD80 and CD86, and cytokine production, which enables the cells to present antigens to T cells and promotes T cell differentiation and expansion (Joffre et al. 2009).

Dendritic cells play a key role in regulating the balance between the pro- and anti-inflammatory responses that augment or

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attenuate cellular immunity (Segal 2007). The cytokine profile released by DCs can initiate cellular immunity through different pathways. Each pathway orchestrates specific responses through different cytokine profiles and effector cells. The best characterized pathways are Th1 and Th2, but recently, a new pathway, Th17, has been described (Zhu et al. 2010).

Recent data demonstrated that DCs are able to translate fungus-associated molecular signatures and coordinate an inflammatory response protecting against fungal infection (d'Ostiani et al. 2000; Bacci et al. 2002; Bozza et al. 2003). The acquired cellular response to *S. schenckii* antigens is associated with the immune mechanisms that control the infection, although the specific role of DCs in sporotrichosis is not fully understood. Uenotsuchi et al. demonstrated that human monocyte-derived DCs show differential cellular responses to *S. schenckii* fungus depending on the source of the fungal sample, specifically if it is derived from patients with visceral or cutaneous infections. An immature DC stimulated with the cutaneous fungal infection more potently activated a Th1 response compared to DCs stimulated with the visceral fungal infection (Uenotsuchi et al. 2006).

Our study investigated the phenotypic and functional changes in bone marrow dendritic cells (BMDCs) stimulated *in vitro* with the yeast form of *S. schenckii* or ExoAg and its ability to trigger a cellular immune response *in vitro*.

Materials and methods

Animals

Female 6–12-week-old C57BL/6 mice were purchased from the Multidisciplinary Center for Biological Research (CEMIB), University of Campinas (UNICAMP), São Paulo, Brazil. The mice were housed in specific pathogen-free (SPF) conditions in our animal facility. All procedures were approved by the Institutional Ethics Committee (Protocol CEP/FCF/CAR no. 10/2009) and were in accordance with the National Institutes of Health Animal Care Guidelines.

Fungal samples

S. schenckii, strain 1099-18, was kindly provided by Dr. Celuta Sales Alviano, Institute of Microbiology, Federal University of Rio de Janeiro, RJ, Brazil. This strain was isolated from a human case of cutaneous sporotrichosis at the Mycology Section of the Department of Dermatology, Columbia University, New York, NY, USA. The fungus was cultured at 37 °C for 7 days in brain heart infusion (BHI) broth (Difco) with constant orbital shaking at 150 cycles/min, resulting in a suspension of yeast cells.

Infection method

A yeast suspension was prepared containing 1×10^8 cells/mL in phosphate-buffered saline (PBS), pH 7.4. A volume of 0.1 mL was inoculated intraperitoneally into each animal.

Preparation of ExoAg

The ExoAg was obtained as described previously by Carlos and colleagues (Carlos et al. 2003) with minor modifications. The yeast phase fungal culture was subjected to UV radiation for 1 h. Then, the culture was shaken at 37 °C for 24 h and UV-irradiated again for 1 h. After this procedure, thimerosal (Synth) was added to the culture medium at a 1:5000 concentration, and then, the culture was shaken at 37 °C for 48 h. Next, the culture sterility was tested by the Sabouraud (Difco) agar test. The cellular suspension was centrifuged and the supernatant was filtered onto a cellulose ester membrane with 0.45 μm pores (Millipore) and concentrated from 50- to 100-fold using polyethylene glycol (Sigma). The protein concentration (9.5 mg/mL) was measured by the Lowry method (Lowry et al. 1951). The exoantigen was tested for cytotoxicity against murine cells and the higher dose (100 μg/mL) had a survival rate of more than 80% (Mosmann 1983).

Bone marrow preparation and generation of bone marrow-derived dendritic cells (BMDCs)

The femurs and tibiae of C57BL/6 mice were removed aseptically, disinfected in 70% ethanol for 2–5 min, and washed two times with RPMI-1640 (Sigma). Then, the ends of both bones were cut with scissors and the marrow was flushed with RPMI-1640 using a 10 mL syringe (BD Biosciences) with a 0.45 mm diameter needle. Clusters in the marrow cell suspension were disintegrated by vigorous pipetting. The bone marrow cell suspension was depleted of red blood cells (RBC) using ACK lysis buffer and washed with RPMI-1640. The bone marrow cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) (Sigma), 100 U/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), 50 μM β-mercaptoethanol (Sigma), 1 mM sodium pyruvate (Sigma), 30 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 ng/mL recombinant murine IL-4 (BD Biosciences). Two and four days after the initial culture, the cells were supplied with fresh medium supplemented with GM-CSF and IL-4. The morphological alterations during the differentiation process were assessed daily using an optical inverted microscope (Nikon Eclipse TS100). On day six, differentiated cells were analyzed phenotypically using flow cytometry to evaluate the differentiation efficiency and the maturation profile. Day six was established as the standard for stimulation and co-culture assays. A differentiation assay over nine days was performed to compare differentiation efficiencies using a similar protocol, in which GM-CSF and IL-4 was added on day zero, two, four, six and eight. On day six, the cells were washed, adjusted to 2×10^6/mL cells, and 0.5 mL per well was measured into 24-well plates (BD Biosciences) with fresh medium and one of the following treatments: yeast cells at a ratio of 1:1, ExoAg (10 or 100 μg/mL), LPS (1 μg/mL) (Sigma), or medium only for 24 h at 37 °C. For the live yeast stimulus, 2.5 μg/mL of amphotericin (Sigma) was added to the culture 2 h after the live yeast to prevent fungal overgrowth. At the end of the incubation, the supernatant was collected for cytokine measurement using ELISA, and all of the DCs were harvested, washed three times with PBS, and either phenotypically analyzed by flow cytometry or co-cultured in 96-well plates (BD Biosciences) at 1×10^5 final concentration per well. The DCs were co-cultured with primed T cells from C57BL/6 at a DC:T cell ratio of 1:9 for 72 h at 37 °C. Both cells were cultivated at a final concentration of 1×10^6 cells per well.

Flow cytometry analysis

The phenotypic changes of stimulated DCs were determined with mAbs specific for DC activation surface markers. We evaluated $(0.5–1) \times 10^6$ DCs diluted in PBS containing 2% fetal bovine serum (FBS) (Sigma) for the expression of CD11c, MHCII, CD80 and CD86 molecules. The unspecific binding sites were blocked for 40 min at 4 °C using Fc Block™ (BD Biosciences PharMingen), then the cells were washed for 10 min, centrifuged at $700 \times g$ at 4 °C and stained for 30 min at 4 °C. Before analysis, the cells were washed and resuspended in 500 μl of PBS with 2% FBS. All antibodies were analyzed against their specific isotype controls. The CD11c mAb (clone: HL3) Isotype Armenian Hamster IgG1 λ2 was labeled with fluorescein isothiocyanate (FITC). Phycoerythrin (PE)-labeled mAbs were also used, including the following: MHC II ($I\text{-}A^b$)