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Altered distribution of peripheral blood dendritic cell subsets in patients with pulmonary paracoccidioidomycosis

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ABSTRACT

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by fungi from the genus *Paracoccidioides* in Latin America. PCM-patients (PCM-p) are classified as having acute/subacute or chronic (CF) clinical forms. CF is responsible for 75%–90% of all cases, affects mainly adults over 30 years old and the clinical manifestation are associated mainly with lungs and mucosa of upper airdigestive tract. In addition, the CF patients exhibit fibrosis of the lungs, oral mucous membranes and adrenals, and pulmonary emphysema. Consequently, CF PCM-p with active disease, as well as those that have been apparently cured, seem to be an interesting model for studies aiming to understand the long-term host-fungi relationship and hypoxia. Dendritic cells (DCs) constitute a system that serve as a major link between innate and adaptive immunity composed of several subpopulations of cells including two main subsets: myeloid (mDCs) and plasmacytoid (pDCs). The present study aimed to access the distribution of PBDC subsets of CF PCM-p who were not treated (NT) or treated (apparently cured – AC). CF PCM-p were categorized into two groups, consisting of 9 NTs and 9 ACs. Twenty-one healthy individuals were used as the control group. The determination of the PBDC subsets was performed by FACS (fluorescence-activated cell sorting) and the dosage of serum TNF-α, IL1β, IL-18, CCL3, IL-10 and basic fibroblast growth factor (bFGF) by ELISA (enzyme-linked immunosorbent assay). A high count and percentage of mDCs was observed before treatment, along with a low count of pDCs in treated patients. Furthermore, the mDC:pDC ratio and serum levels of TNF-α was higher in both of the PCM-p groups than in the control group. In conclusion, our findings demonstrated that active PCM influences the distribution of mDCs and pDCs, and after treatment, PCM-p retained a lower count of pDCs associated with pro-inflammatory profile. Therefore, we identified new evidences of persistent immunological abnormalities in PCM-p after treatment. Even these patients showing fungal clearance after successful antifungal treatment; the hypoxia, triggered by the persistent pulmonary sequelae, possibly continues to interfere in the immune response.

1. Introduction

Paracoccidioidomycosis (PCM) is the main endemic systemic mycosis in Latin America, where it is confined, and it is caused by thermodimorphic fungi from the genus *Paracoccidioides* (Shikanai-Yasuda et al., 2006; Teixeira et al., 2009). PCM-patients (PCM-p) exhibit an impaired antigen-specific cellular immune response, and the disease presents as two main distinct clinical forms, acute/subacute (AF) and chronic (CF) (Benard et al., 1996; Mendes, 1994).

AF affects young individuals and has clinical manifestations that involve the lymph nodes, liver, spleen and bone marrow. CF is the most prevalent clinical form, usually affects male adults older than 30 years, and shows a predominant pulmonary and mucocutaneous involvement. In this clinical form, the disease is usually caused by reactivation of latent foci that remained in the host for an unknown amount of time, which may be as long as 30 years (Mendes, 1994); thus, the host has already organized an adaptive immune response against the genus *Paracoccidioides* that was efficacious for some time. In addition, sequelae such as pulmonary fibrosis (PF) and emphysema (EMPH) are a hallmark in CF; despite they have been commonly identified during the

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diagnosis, there is no treatment and these conditions trigger serious economic, social and psychological problems (Mendes, 1994). Itraconazole is the first choice for PCM treatment; although this azole is efficacious and safe, the duration of the therapy is prolonged, sometimes reaching more than two years (Cavalcante et al., 2014).

Dendritic cells (DCs) are involved in linking innate immunity to adaptive immunity, are responsible for the antigen presentation to T lymphocytes, trigger the polarization of the adaptive immune response and induce immune tolerance and/or anergy (Kapsenberg, 2003). Several reports have shown the importance of these cells in experimental PCM models (Almeida and Lopes, 2001; Ferreira and Almeida, 2006; Ferreira et al., 2011, 2007; Magalhães et al., 2012; Pina et al., 2013; Silvana dos Santos et al., 2011; Tavares et al., 2012), but few studies have been performed in patients. Some researchers have demonstrated an altered distribution of DCs in the skin and/or mucocutaneous lesions from PCM-p (Gimenez et al., 1987; Kaminagakura et al., 2007; Pagliari and Sotto, 2003), and others have found that monocyte-derived DCs of treated PCM-p show a higher expression of HLA-DR, DC-SIGN, CD86 and higher production of IL-12p40 than patients with active disease and healthy individuals (Sato et al., 2011). Recently, Fernandes et al. (2015) showed that *P. brasiliensis* yeast cells impair the maturation of monocyte-derived DCs of normal individuals. Although these reports using monocyte-derived cells have shown important aspects of immunobiology of DCs in PCM, there is a lack of studies evaluating freshly isolated DCs from PCM-p.

Because of different phenotypic and functional properties, peripheral blood DCs are subdivided into the following two main populations: myeloid (mDCs) and plasmacytoid (pDCs) DCs (Ziegler-Heitbrock et al., 2010). mDCs originate from myeloid precursors, express a high amount of β2 integrin (CD11c) and secrete IL-12, inducing a T helper type 1 (Th1) immune response that is crucial in immunity against intracellular pathogens (MacDonald et al., 2002; Osugi et al., 2002). pDCs produce large amounts of type I interferon (IFN-I), inducing Th2 response; they have been associated with viral immunity and the induction of immunological tolerance (Colonna et al., 2004). In the mucosal tissues, mDCs (conventional DCs) are predominantly found in the underlying tissue and express CD1c or CD141 on surface and the transcription factor IRF4 in the lung; pDCs exist expressing CD103 and are associated with the pulmonary epithelium (review in (Cook and MacDonald, 2016))

A recent report showed that B10.A mice, a mouse strain susceptible to *P. brasiliensis*, have a higher percentage of pulmonary mDCs and a lower percentage of pDCs than A/J mice, the resistant strain (Pina et al., 2013). Moreover, while the *P. brasiliensis*-induced mDCs of susceptible mice triggered high production of pro-inflammatory cytokines associated with intense initial tissue damage and the development of a specifically impaired adaptive immune response, the *P. brasiliensis*-induced pDCs of resistant mice produced large amounts of TGF-β1 associated with initial permissive fungal growth but followed by an efficient cellular-mediated immune response and subsequent fungal clearance (Pina et al., 2013).

Considering that: 1) the distribution and role of subsets in PCM patients remain to be evaluated; 2) CF PCM patients with active disease and those who were apparently cured seems to be an interesting model for studies aiming to understand the long-term host-fungus relationship as well as hypoxia; the present study aimed to access the distribution of peripheral blood DC (PBDC) subsets in CF PCM-p with active disease, either before treatment or after being apparently cured.

2. Methods

2.1. Patients

Eighteen CF PCM patients (PCM-p) from the Tropical Diseases Ward and Outpatient Clinic for Paracoccidioidomycosis at the University Hospital, Faculdade de Medicina de Botucatu (FMB), UNESP–Univ. Estadual Paulista, Botucatu, SP, Brazil, were studied. Cases with clinical manifestations that were compatible with PCM were considered either confirmed or probable [13]. Cases were considered confirmed when the typical *Paracoccidioides* genus yeast forms were identified in the clinical specimens and considered probable when only serum-specific antibodies were detected using a double agar gel immunodiffusion test (DID). All patients had pulmonary involvement and were classified as having clinical CF. Radiologic evaluation of chest injuries by CTScan was performed in 12 patients (Supp Table S1 in the online version at DOI: http://dx.doi.org/10.1016/j.actatropica.2017.06.007). Patients who exhibited neoplasia, inflammation, other infectious diseases or pregnancy were not enrolled.

2.2. Ethics statement

This study was approved by the Research Ethics Committee of FMB-UNESP (#3145/2009). After being informed of the study, written informed consent was obtained from all participating adults.

2.3. Experimental design

PCM-p were categorized into the following two groups: the non-treated (NT) group, consisting of 9 newly diagnosed patients, and the apparent cure (AC) group, consisting of 9 PCM-p who did not show any signs or symptoms and had a normal erythrocyte sedimentation rate (ESR), negative serology, and at least 2 full years of non-treatment after complete antifungal therapy, which are all characteristics of an apparent cure [6]. The groups were homogeneous as to gender, age, degree of severity, specific antibody serum levels at admission and antifungal treatment (Table 1). Twenty-one age- and gender-matched healthy individuals were selected among blood donors from the same geographical area to constitute the control group (CG) (Table 1).

2.4. Determination of the peripheral blood (PB) dendritic cell (DC) subsets

Venous blood was collected in Vacutainer tubes (BD, Becton Dickinson, Franklin Lakes, NJ, USA) containing EDTA anticoagulant. Whole blood (100 μl) was added to polystyrene tubes containing the following monoclonal antibodies: AlexaFluor® 488-conjugated lineage cocktail (mouse anti-human CD3, CD14, CD19, CD20, CD56); phycoerythrin (PE)-conjugated mouse anti-human CD123; peridinin chlorophyll protein complex (PerCP)-conjugated mouse anti-human HLA-DR; and allophycocyanin (APC)-conjugated mouse anti-human CD11c, all of which were purchased from BioLegend (San Diego, CA, USA). The tubes were incubated with the conjugated antibodies for 20 min at 4 °C. Next, the erythrocytes were lysed by resuspension in FACS lysing solution. The cells were washed with BD Pharmingen™ staining buffer and analyzed using a FACSCalibur (BD). Absolute counts number of DCs subsets was based on WBC. The data were analyzed using the FlowJo software (Tree Star Inc., USA). The normal range of the percentage of total DCs and the mDC/pDC ratio used in this study was based on a study performed by Riccardi et al. (2011).

2.5. Dosage of serum mediators

Serum levels of TNF-α, IL-1β, IL-18, CCL3 and bFGF were quantified the DuoSet@ ELISA Development kit (R & D systems, Minneapolis, MI, USA) according to the manufacturer's instructions.

2.6. Statistical analysis

The statistical tests were performed using the GraphPad v.5.00 software (GraphPad Software Inc., San Diego, CA, USA), and the significance was defined at $p \leq 0.05$ for all of the analyses. The homogeneity of the NT, AC, and control groups was determined by Mann-Whitney *U* test, Fisher's exact test, and Kruskal-Wallis test. The