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Article

Sporothrix schenckii cell wall proteins-stimulated BMDCs are able to induce a Th1-prone cytokine profile *in vitro*

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Abstract: Sporotrichosis is a subcutaneous mycosis affecting humans and other animals that can be transmitted a zoonosis with cats as the main vector. The conventional anti-fungal therapy is especially inefficient in immunocompromised patients, who tend to develop the most severe forms of the disease, thus prompting the search for alternative therapies. Given their antigen-presenting properties, dendritic cells (DCs) have been used in both prophylactic and therapeutic vaccination strategies. Hence, this study aims to assess the use of DCs as a prophylactic tool in sporotrichosis by evaluating the immune profile induced by *Sporothrix schenckii* cell wall proteins (SsCWP)-stimulated bone-marrow-derived DCs (BMDCs). Mouse BMDCs were stimulated with SsCWP for 24 hours and analyzed for the surface expression of co-stimulatory molecules and TLR-4, as well as the secretion of proinflammatory cytokines and IL-10. Following that, activated BMDCs were cocultured with splenocytes for 72 hours and had the same cytokines measured in the supernatant. SsCWP-stimulated BMDCs showed higher expression of CD80, CD86, and CD40, but not TLR-4, and higher secretion of IL-6, IL-17A, and TNF. On the other hand, higher levels of IFN-γ, IL-10, and IL-2 were found in the supernatants of the coculture as compared with the BMDCs alone; TNF secretion was almost completely abrogated, whereas IL-6 was only partially inhibited and IL-17A was unaffected. Our results thus suggest SsCWP-stimulated BMDCs are able to induce a Th1-prone cytokine profile, known to be protective against other fungal diseases. This result could lead to evaluate the development of prophylactic and/or therapeutic DC-based tools against sporotrichosis.

Keywords: *Sporothrix schenckii*; bone-marrow-derived dendritic cells; vaccine; sporotrichosis

1. Introduction

Sporotrichosis is a fungal infection affecting humans and other animals caused by different *Sporothrix* species [1]. The infection begins upon accidental inoculation of its causal agent in the skin or, more commonly in Brazil in the last years, through zoonotic transmission by cats infected with the fungus [2]. Sporotrichosis is a worldwide distributed mycosis, although it shows a higher prevalence in tropical and subtropical regions [3,4]. Over the years, many studies have attempted to develop vaccines against human- and animal-infecting opportunistic and endemic fungi [5-7].

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It is known that several proteins located in the cell wall of *S. schenckii* are important humoral and cellular immune response inducers and are, therefore, potential candidates for diagnostic applications and as a tool in the study of vaccines to prevent sporotrichosis [8]. Pathogen-associated molecular patterns (PAMPs) have been extensively studied because of their importance in host defense against microbes and there has been growing recognition of the potential benefit of using PAMPs in vaccine development [9]. PAMPs deliver a "danger" signal to dendritic cells (DCs), resulting in DC activation and secretion of cytokines/chemokines, migration, maturation, antigen presentation, and costimulatory molecule expression. This, in turn, impacts B- and T-cell responses to antigens co-delivered with PAMPs [10,11].

The last decade saw a rapid progress in the comprehension of DC biology concomitantly to the development of obtaining and culture methods for blood- and bone-marrow-derived DCs (BMDCs), which opened the path for DC vaccine development [12]. DCs present great potential to be used as adjuvants in both prophylactic and therapeutic vaccine formulations [13,14]. Many studies show that several immune responses are critically controlled by DCs, which are potent and widely distributed antigen-presenting cells (APCs), besides being unique in their prominent role in the activation, polarization and regulation of adaptive immune responses [15]. DCs are highly capable to recognize fungi associated data and translate it into different T cells responses both *in vivo* and *in vitro* [16]. Despite the many studies showing the use of DCs as tools for the development of vaccines against different fungi [13,17], this has been approached in sporotrichosis using complete fungal cells and exoantigens [18]. Our group reported that cell wall proteins extracted from *S. schenckii* (SsCWP), are able to induce a protective immune response against this fungi [19,20]. The present study assessed the immune response pattern induced in BMDCs when these cells were stimulated with SsCWP, as well as that produced by co-cultivation of SsCWP-stimulated BMDCs with mouse splenocytes. This is a first stage before evaluating whether stimulated DC are able to induce a therapeutic effect in models of murine sporotrichosis.

2. Materials and methods

2.1. Animals

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), Universidade de Campinas (UNICAMP), São Paulo, Brazil. Animals were housed in individually ventilated cages in an ambient with controlled temperature and 12-hour light/dark cycles. Water and food were offered *ad libitum*. All procedures were approved by the Institutional Ethics Committee (Protocol CEP/FCF/CAR no. 04/2014) and were in accordance with the National Institutes of Health Animal Care Guidelines.

2.2. Microorganism and culture conditions

Sporothrix schenckii (ATCC® 16345™) isolated from a case of human lung infection (Baltimore, MD), was kindly provided by the Department of Microbiology, Reference Materials Laboratory of the Oswaldo Cruz Foundation, National Institute of Quality Control in Health, Rio de Janeiro, Brazil. The fungus is kept in its filamentous phase in Mycosel™ agar at room temperature. The yeast form used in the experiments was obtained by transferring a small fragment of the mycelium into BHI (Brain Heart Infusion, Difco) broth and culturing it at 37 °C under constant stirring of 150 rpm/min for 7 days. After that, 2×10^7 yeast cells were transferred to a fresh medium and cultured for 5 more days in the same conditions in order to achieve a virtually 100% mycelium-to-yeast conversion in a logarithmically growing culture.

2.3. Extraction of the SsCWP

Extraction of the SsCWP was performed as previously described by Portuondo and colleagues (2016), with minor modifications [19]. Briefly, yeast cells collected from logarithmically growing cultures were washed with cold 25 mM Tris-HCl, pH 8.5. The yeasts were then incubated with the

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dithiothreitol (DTT)-based protein extraction buffer (2 mM DTT, 1 mM phenylmethyl sulfonyl fluoride, and 5 mM EDTA in This/HCl buffer) for 3 hours at 4 °C under mild agitation. The SsCWP-containing supernatant was collected, filtered through a 0.20 µm nitrocellulose membrane (Millipore) and dialyzed against distilled water at 4 °C for 48 hours with water changes every 12 hour and then concentrated 100 times using the Amicon® vacuum system (Milipore) according to the manufacturer's instructions. The protein extract was aliquoted and stored at -20 °C and protein concentration was measured by the BCA assay (Pierce), also according to the manufacturer's instructions.

2.4. BMDCs generation

After euthanasia, bone marrow precursor cells were extracted from the femur and tibia of Balb/c mice and resuspended in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 5 mM 2-mercaptoethanol and 1 mM sodium pyruvate (R-10). Cell concentration was adjusted to 2×10^6/mL in R-10, supplemented with 40 ng/mL murine recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech), distributed in cultivation bottles (10 mL) and incubated in humidified air at 37 ºC with 5% CO_2. On days 3 and 6 the non-adherent cells were collected, washed and resuspended in R-10 containing 20 ng/mL GM-CSF. On day 8, non-adherent cells were collected, washed and adjusted to 1×10^6 cells/mL in R-10. An aliquot of this suspension was analyzed for expression of the myeloid DC markers CD11c and MHC-II by flow cytometry. After that, 10^7 BMDCs in 10 mL of R-10 were transferred to new culture bottles, stimulated or not with 50 µg/mL SsCWP for 24 hours and analyzed for expression of CD83 and the co-stimulatory molecules CD86, CD80, CD40, and TLR4 by flow cytometry. The supernatant was collected and stored at -80 °C for later measurement of cytokines.

2.5. Flow cytometry

BMDCs were washed in PBS containing 1% bovine serum albumin (BSA) (Sigma) and then 1×10^6 cells were stained with the following anti-mouse mAbs (BD Biosciences): FITC anti-CD11c (clone HL3), APC anti-I-Ab (MHC-II) (clone AF6-120.1), PE anti-CD83 (clone Michel-19), PECy7 anti-CD86 (clone GL1), PE anti-CD80 (clone 16-10A1), PE anti-CD40 (clone 3/23), and PECy7 anti-CD284 (TLR4) (clone SA15-21, BioLegend). The events were acquired using a BD Accuri C6 flow cytometer (BD Biosciences) and analysed with the flow cytometer's proprietary software.

2.6. Splenocytes

After euthanasia, spleens were aseptically removed and passed through a 100 µm cell strainer into a Petri dish containing 2 mL of PBS with the aid of a syringe plunger. For red cell lysis, the resulting suspension was added with 6 mL of a 0.17 M ammonium chloride solution and then incubated on ice for 5 min. The splenocytes were then separated from the supernatant by centrifugation at 300 × g for 5 min at 4 °C, washed once with 3 mL of R-10 medium and then resuspended in 1mL of the same medium, counted using the Trypan blue exclusion test and then adjusted to the desired concentration.

2.7. Co-cultivation of BMDCs and splenocytes

To assess the inducing properties of the SsCWP-stimulated BMDCs, 10^5 activated BMDCs were co-cultured with splenocytes in a 1:5 or 1:10 ratio (BMDC:splenocytes) in a 96-well plate 72 hours at 37 °C with 5% CO_2. Splenocytes or SsCWP-stimulated BMDCs alone were used as controls. The plates were then centrifuged at 300 × g for 5 min at 4 °C and the supernatants were kept at -80 °C until cytokine determination.

2.8. Cytokine measurement

Cytokines were measured using the mouse Th1/Th2/Th17 cytokine cytometric bead array (CBA) kit (BD Biosciences), according to the manufacturer's instructions using a FACS LRS II Fortessa flow

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cytometer (BD Biosciences). The data were analyzed using FlowJo (Tree Star) and the results of cytokine concentration were expressed in pg/mL [21].

2.9. Statistical analysis

Statistical analysis was performed in GraphPad Prism ver. 6.01, by using two-way analysis of variance (ANOVA) with Tukey multiple comparisons test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Activation and cytokine profile of SsCWP-stimulated BMDCs

The protocol used for BMDC generation resulted in 69.2% and 76.7% of $CD11c^{+}MHCII^{+}$ cells, before and after stimulation with the SsCWP, respectively. The phenotypical analysis of BMDCs revealed higher expression of the maturation marker CD83, as well as the co-stimulating molecules CD80, CD86, and CD40, but not TLR4, after 24 hours (Fig. 1A and 1B). Measurement of the cytokines released in the supernatants of BMDC cultures revealed higher levels of IL-6, IL-17A, and TNF after stimulation with the SsCWP (Fig. 1C).

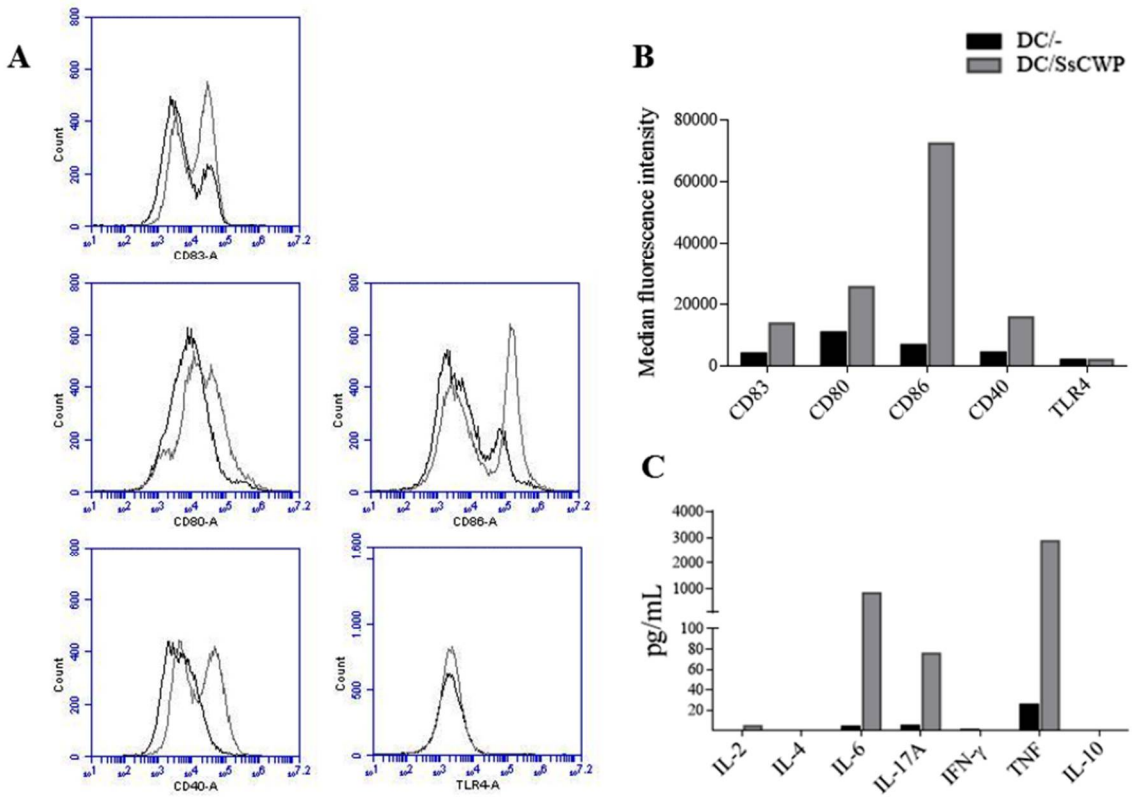

Figure 1. Phenotypical analysis of stimulated BMDCs. (A) Histograms. (B) Cell surface expression of indicated markers on BMDCs. (C) Cytokine concentration in the culture supernatant of BMDCs. The data is from a single culture and the expression of markers is presented as their median fluorescence intensity (MFI). DC/-: unstimulated BMDCs (black line); DC/SsCWP: SsCWP-stimulated BMDCs (grey line).

3.2. Cytokine profile induced by BMDCs upon co-cultivation with splenocytes

To assess the value of SsCWP-stimulated BMDCs as a vaccine adjuvant, the ability of these cells to activate T cells was tested *in vitro* by co-cultivation with total splenocytes for 72 hours and then

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measuring the cytokines released in the co-culture supernatant. Our results show that in both 1:5 and 1:10 (DC:splenocyte) ratios, SsCWP-stimulated BMDCs were able to induce a significant increase in the release of IL-2 (Fig. 2A) and IFN-γ (Fig. 2D), but not IL-4 (Fig. 2B) or IL-17A (Fig. 2C), as compared with unstimulated BMDCs; IL-10 was only significantly induced in the 1:5 ratio (Fig. 2G). As before, BMDCs released relatively high TNF amounts when cultured alone, but showed an unexpected, almost complete inhibition of this cytokine upon co-cultivation with splenocytes (Fig. 2F). Conversely, IL-6 levels were mostly maintained in the co-culture, although significantly decreasing in the 1:10 ratio as compared with SsCWP-stimulated BMDCs alone or the 1:5 ratio (Fig. 2E).

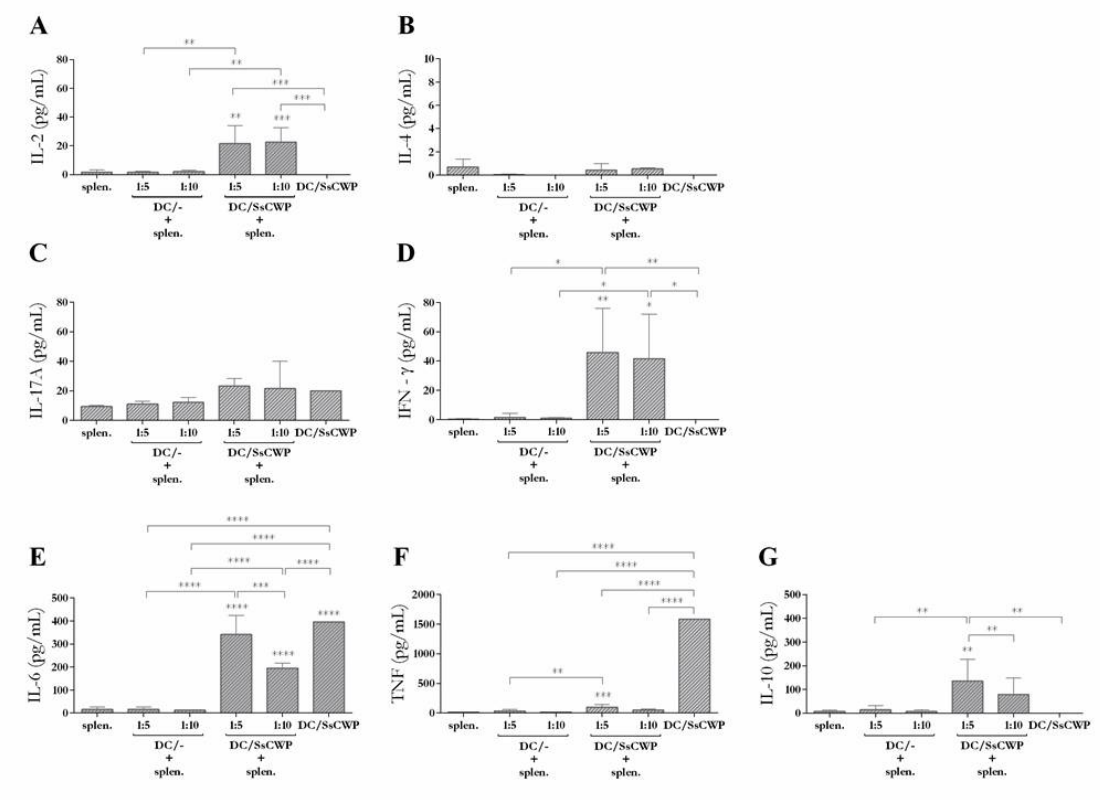

Figure 2. Measurement of cytokines released in the BMDC:splenocyte co-culture supernatant. Values are presented as the mean ± SD of 4 animals. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$) as compared with splenocytes alone ("splen.") or as indicated.

4. Discussion

The use of DCs in vaccination is promising as they lie in the intersection between innate and adaptive immunity, uniquely able to capture and process antigens for presentation to T cells through MHC class II molecules. DCs recognize fungi through a wide range of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), located both extra and intracellularly. Fungi recognition results in cytokine release and surface expression of the co-stimulating molecules CD80, CD86 and CD40 by DCs, which are necessary to direct the differentiation of naive $CD4^+$ T cells into a T helper phenotype [22]. As it is already known, the activation and expansion of T cells and their acquisition of effector functions are key to the development of adaptive immune responses, with a substantial part of T cell proliferation and differentiation being traced back to the initial encounter of DCs with a given antigen [23]. For decades, a variety of cell wall proteins from many different pathogenic fungi have been evaluated in mouse models of vaccination for assessment of their immunogenicity, safety and protection-affording potential [6]. Although very few clinical trials have been performed in humans, a growing number of antifungal vaccine candidates are being evaluated in preclinical studies, as part of the renewed interest in the potential use of vaccines, replacing or associated with chemotherapy, to reduce antifungal drugs use and consequently limit drug resistance and toxicity

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[7]. In the study presented here, we observed that the SsCWP are able to promote BMDCs maturation, as well as activation, as seen by the increased expression of CD83 and CD80, CD86 and CD40 post-stimulation, respectively. This leads us to believe that it is possible to use DCs as a vaccine for sporotrichosis. Other studies using fungal antigens as DC activators showed their capacity to promote the maturation of these cells, as seen by increased expression of MHC class II and co-stimulatory molecules involved in antigen presentation and T cell activation [24,25].

Regarding cytokine production by the SsCWP-stimulated BMDCs, our data showed increased production of IL-6, IL-17A, and TNF, suggesting that SsCWP-stimulated BMDCs could induce, *in vivo*, a Th17 pattern inflammatory response. Contrary to our expectations, however, these BMDCs induced a predominantly Th1 cytokine profile, as noted by increased IL-2 and IFN-γ and only basal levels of IL-17A and IL-4, when cocultured with splenocytes. It has been previously indicated that granuloma formation in sporotrichosis may be associated with a Th1 response in the skin lesions, as evidenced by the local detection of IFN-γ plus the fact that *S. schenckii* of cutaneous origin is a more potent inducer of Th1-prone DC activation than that of visceral origin [26]. Traditionally, responses mediated by IFN-γ-producing Th1 cells are considered to be responsible for conferring protection against fungi, while IL-4-mediated Th2 responses lead to increased susceptibility [27]. Furthermore, a previous study performed in our lab showed that *S. schenckii*-primed BMDCs were able to promote a Th1 and Th17-biased response when cocultured with splenocytes extracted from mice that had been previously challenged intraperitoneally with *S. schenckii*, as observed by increased IFN-γ and decreased IL-17A release [18]. Other study reported that DCs are able to phagocytose *S. schenckii* and to induce a Th1-prone cytokine profile, as well as to induce the proliferation of T lymphocytes that had been pre-sensitized with *S. schenckii* [28]. Lastly, TNF release was abrogated whereas IL-10 was induced when SsCWP-stimulated BMDCs were co-cultivated with splenocytes. As IL-10 can directly inhibit IL-2, TNF-α and IL-5 production [29], it seems this pathway could be responsible, at least partially, for this finding.

Although very few clinical trials have been performed in humans, a growing number of antifungal vaccine candidates are being evaluated in preclinical studies as part of the renewed interest in the potential use of vaccines, replacing or associated with chemotherapy, to reduce the use of antifungal drugs and consequently limit drug resistance and toxicity [7]. Moreover, one of the most interesting aspects is to achieve an adequate efficacy/toxicity balance of the vaccines and adjuvants frequently causes adverse reactions [30]. For this reason, the search for alternatives using molecular modulators seems to be a promising way [31]. Since activated DCs may be more efficient than nonspecific commercial adjuvants, we propose that SsCWP-stimulated BMDCs could represent a potential therapeutic tool for sporotrichosis management. New studies of immunogenicity and protection are needed to confirm the true usefulness of this proposal.

5. Conclusions

In summary, our results indicate that SsCWP are able to activate BMDCs to acquire an activated phenotype that promote a Th1 bias, which leads us to believe that a SsCWP-stimulated BMDCs-based vaccine could be evaluated as a potential tool for sporotrichosis immunotherapy.

Author Contributions: C.C., A.B.-D., and I.Z.C. conceived and designed the experiments; C.C., LSF., I.P., MLL., and DLP performed the experiments, C.C., LSF., A.B.-D., and I.Z.C. analyzed the data; C.C. wrote the paper, all the author revised and approved the manuscript.

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Conflicts of Interests: The authors declare no commercial or financial conflict of interest.

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