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Dendritic cells pulsed with *Pythium insidiosum* (1,3)(1,6)-β-glucan, Heat-inactivated zoospores and immunotherapy prime naïve T cells to Th1 differentiation *in vitro*

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ABSTRACT

Pythiosis is a life-threatening disease caused by the fungus-like microorganism *Pythium insidiosum* that can lead to death if not treated. Since *P. insidiosum* has particular cell wall characteristics, pythiosis is difficult to treat, as it does not respond well to traditional antifungal drugs. In our study, we investigated a new immunotherapeutic approach with potential use in treatment and in the acquisition of immunity against pythiosis. Dendritic cells from both human and mouse, pulsed with *P. insidiosum* heat-inactivated zoospore, (1,3)(1,6)-β-glucan and the immunotherapeutic PitiumVac® efficiently induced naïve T cell differentiation in a Th1 phenotype by the activation of specific Th1 cytokine production *in vitro*. Heat-inactivated zoospores showed the greatest Th1 response among the tested groups, with a significant increase in IL-6 and IFN-γ production in human cells. In mice cells, we also observed a Th17 pathway induction, with an increase on the IL-17A levels in lymphocytes cultured with β-glucan pulsed DCs. These results suggest a potential use of DCs pulsed with *P. insidiosum* antigens as a new therapeutic strategy in the treatment and acquisition of immunity against pythiosis.

1. Introduction

Pythiosis is a severe disease, which can lead to death if not treated, caused by *Pythium insidiosum*, a zoosporic fungus-like microorganism belonging to the kingdom Straminipila, phylum Oomycota (Beakes et al., 2012). This infectious disease affects mainly mammals, and it is mostly recorded in horses and humans. Pythiosis can occur in different forms, the most common are cutaneous and subcutaneous, vascular, ocular, gastrointestinal and a systemic form, which is rarely seen (Gaastra et al., 2010; Krajaejun et al., 2006; Thitithanyanont et al., 1998; Wanachiwanawin et al., 2004). Since pythiosis treatment with antifungal drugs is difficult due to the cell wall characteristics of *P. insidiosum* (lack of ergosterol), the most effective treatment strategy is surgical removal and immunotherapy, together or independently (Gaastra et al., 2010; Sudjaritruk and Sirisanthana, 2011). Immunotherapeutic approach has an efficacy of approximately 80% in equine pythiosis (Mendoza and Newton, 2005) and only 50% in human cases (Krajaejun et al., 2006); therefore, it is important to investigate a vaccine that provides a better rate of cure together with immunity against pythiosis, because immunotherapy successfully treats the disease but does not provide immunity to new infections (Santos et al., 2011).

Dendritic cells (DCs) are the most effective antigen-presenting cells (APC) and play a key role in the regulation of innate and adaptive immunity (Walsh and Mills, 2013), which is crucial for antifungal defense. DCs are capable of taking up and processing antigens for presentation by major histocompatibility complex (MHC) to naïve T cells, driving naïve $CD4^+$ T cell differentiation into a T helper (Th) phenotype (Roy and Klein, 2012; Steinman, 2012). Cytokines and other mediators play an essential role in this process and may determine the type of effector response that is generated by the pathogen (Bozza et al., 2004; Romani, 2011). IFN-γ potently induces Th1 differentiation, and IL-4 is important for the induction of Th2 differentiation. Differentiation into Th17 cells requires the presence of IL-6, whereas the presence of IL-10 indicates Treg response with a suppressor function (Romani and Puccetti, 2006). The Th subtypes that correlate best with protection against fungi are Th1 and Th17 (Romani, 2011).

Because DCs provide an interface between innate and adaptive

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immunity, they can serve as a unique vehicle for vaccination (Bozza et al., 2004). The induction of strong cellular immunity via the appropriate activation of DCs is the first step in the host's resistance to fungi (Romani, 2011). In animal models, DCs primed with fungi *ex vivo* promote antifungal immunity in naive mice (Bacci et al., 2002; Magalhaes et al., 2012; Ueno et al., 2015). Similarly, DCs transfected with fungal RNA *ex vivo* express fungal proteins on their surface and promote the development of protective T cell responses (Bacci et al., 2002). The interaction of human DCs with *P. insidiosum* and its consequences in the immune defense against this pathogen have not been studied previously. In this study, we investigated the *in vitro* ability of DCs pulsed with three different *P. insidiosum* antigens in lymphocytes response through analysis of the profile of cytokines released after pulsed DCs contact with lymphocytes.

2. Materials and methods

2.1. Microorganism and culture conditions

A Brazilian *P. insidiosum* equine isolate was used in this study. The clinical strain Pi-290 was isolated in the Laboratório de Pesquisas Micológicas (LAPEMI) of the Universidade Federal de Santa Maria (UFSM) and was previously genotyped and registered with GenBank access number KJ176713 (Jesus et al., 2015). The strain was grown on corn meal agar (CMA, Himedia®) at 37 °C for 48 h before experiments. Zoosporogenesis was induced as described (Santurio et al., 2003). Zoospores were counted using a Neubauer chamber.

2.2. Ethics and sampling

The research protocols were approved by the Ethics Committee of Universidade Federal de Santa Maria and the participants signed informed consent forms. All the blood venous specimens were collected from a healthy voluntary donor using EDTA Vacutainer tubes (BD Diagnostics, Plymouth, UK). Experiments using animal material were also approved by the Universidade Federal de Santa Maria Animal Care and Use Committee (CEUA/UFSM) and this study was carried out in accordance with international recommendations for animal care.

2.3. Human peripheral blood mononuclear cells (PBMCs) isolation

PBMCs from two healthy donors were isolated from whole peripheral blood by density gradient centrifugation using Histopaque-1077 (Sigma–Aldrich, St Louis, USA). Briefly, after centrifugation, the cell pellets were washed with Phosphate Buffered Saline (PBS) pH 7.4 and resuspended in the RPMI 1460 medium (Sigma–Aldrich, St Louis, USA) supplemented with 10% Fetal Bovine Serum (Vitrocell, Campinas, Brazil) and 100 U/mL penicillin and 100 µg/mL streptomycin (from now on called Complete Growth Medium – CGM).

2.4. Generation of human monocyte-derived dendritic cells (DCs)

DCs were prepared as previously described (Chapuis et al., 1997). Monocytes were isolated by the plastic adhesion method, where PBMCs were plated in 24-well culture plates at a cell density of 10^7 cells/mL and incubated for 2 h at 37 °C in a 5% humidified CO_2 atmosphere. After monocytes adhesion, cells were washed three times with PBS to avoid lymphocyte contamination. DCs were generated by culturing monocytes with CGM supplemented with 50 ng/mL human recombinant Granulocyte-macrophage colony-stimulating factor (GM-CSF, Sigma–Aldrich, St Louis, USA) and 50 ng/mL of human recombinant IL-4 (Sigma–Aldrich, St Louis, USA) for 10 days and the medium was refreshed at days 3 and 6. DC generation was observed by inverted microscopy, and at day 10, the cells presented the typical morphology of immature DCs. The survival rate was evaluated by the trypan blue dye exclusion method to perform *in vitro* experiments.

2.5. Mice spleen monocytes isolation and DCs generation

Leucocytes from the spleens of three mice were used in the experiments. After leucocytes isolation, monocytes were isolated by the plastic adhesion method as described above for human monocytes isolation. Monocytes were washed three times with PBS and the DCs generation was performed using mouse recombinant GM-CSF and IL-4 (Sigma–Aldrich, St Louis, USA). After 10 days, typical DCs morphology was observed by inverted microscopy and cells were used for *in vitro* experiments.

2.6. Antigen preparation

Three different antigens were used in this study to assess DCs' *in vitro* potential to stimulate lymphocyte proliferation and cytokine production. The immunotherapeutic PitiumVac® was produced by Laboratório de Pesquisas Micológicas (LAPEMI) at the Universidade Federal de Santa Maria (UFSM) and it is composed of inactivated and macerated hyphae (Santurio et al., 2003). *P. insidiosum* cell wall (1,3)(1,6)-β-glucan was the same extracted and analyzed by Tondolo et al. (2017). Briefly, the process relies on induced autolysis followed by treatment with hot water and organic solvent, homogenization, enzymatic treatment with protease and lyophilization. The heat-inactivated (HI) zoospores consisted of *P. insidiosum* zoospores prepared as described above and inactivated by heating at 120 °C in an autoclave for 5 min. All antigens were diluted in CGM.

2.7. Human and mice DC pulsing and lymphocyte co-culture

Immature DCs were washed with RPMI 1460 medium before being resuspended in CGM and plated in 96-well microplates at 10^6 cells/mL to be primed with the antigen solutions. PitiumVac® and (1,3)(1,6)-β-glucan were both diluted in CGM and were adjusted to 100 µg/mL and 400 µg/mL based on previous observations (data not shown). The concentration of HI-zoospores was adjusted to 2×10^4 and 5×10^4 in CGM. In addition, a control group was primed with 1 µg/mL LPS (Sigma–Aldrich, St Louis, USA), representing a positive control. After treatments, DCs were incubated for 2 h at 37 °C in a 5% humidified CO_2 atmosphere to allow antigen internalization by cells (Bacci et al., 2002). To assess the tendency of the antigens to induce a T helper response by lymphocytes, PBMCs prepared as described above were counted by the trypan blue dye exclusion method and co-cultured at 10^6 cells/mL with the different pulsed DCs groups (at 1:1 ratio) for 24 h. In the same manner, mice lymphocytes were added to mice primed DCs. Subsequently, after 24 h incubation time, the plates were centrifuged at 1500 rpm for 5 min and the supernatant was collected and stored at −80 °C until cytokine analysis.

2.8. Cell proliferation

The proliferative rate of both human and mice lymphocytes stimulated with pulsed DCs was measured after 72 h of incubation at 37 °C in a 5% humidified CO_2 atmosphere using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). Briefly, 4 h before the end of incubation, 20 µL of MTT (5 mg/mL) were added to each well. The plates were centrifuged at 1500 rpm for 5 min at room temperature and the supernatant was carefully removed before 200 µL of dimethyl sulfoxide (DMSO) were added into each well. The absorbance was measured at 590 nm in a plate reader (Bio-Rad Laboratories, Hercules, CA). The cell proliferation observed in each treatment was expressed as the mean of each group optical density.

2.9. Cytokine detection

The levels of the cytokines IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A were detected using a Cytometric Bead Array (CBA) human