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Response of Cytokines and Hydrogen Peroxide to *Sporothrix schenckii* Exoantigen in Systemic Experimental Infection

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Abstract The response of hydrogen peroxide (H_2O_2) and cytokines during an experimental sporotrichosis in male Swiss mice was assessed over a period of 10 weeks by monitoring macrophage activation challenged with exoantigen (ExoAg) from the fungus *Sporothrix schenckii*. The studied endpoints were: H_2O_2 production, fungal burden at spleen, apoptosis in peritoneal macrophages, and IL-1β, IL-6, IL-2, IL-10 production. During the two first weeks of infection was observed low burden of yeast in spleen and high response of H_2O_2, IL-2, and IL-1β. The weeks of highest fungal burden (fourth–sixth) coincided with major apoptosis in peritoneal macrophages, normal production of IL-6 and lower production of H_2O_2, IL-2, and IL-1β, suggesting a role for these three last in the early control of infection. On the other hand, IL-1β (but not IL-6) was recovered since the sixth week, suggesting a possible role in the late phase of infection, contributing to the fungal clearance in conjunction with the specific mechanisms. The IL-10 was elevated until the sixth, principally in the second week. These results evidences that ExoAg is involved in the host immune modulation, influencing the *S. Schenckii* virulence, and its role is related with the time of the infection in the model used.

Keywords *S. schenckii* · Exoantigen · Macrophage · Hydrogen peroxide · Pro-inflammatory cytokines · Apoptosis

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Introduction

Sporotrichosis is a mycosis that affects humans and animals, caused by the traumatic inoculation of the dimorphic fungus *Sporothrix schenckii*, recently cleared as component of a complex related to several cryptic infectious species (i.e., *S. albicans, S. brasiliensis, S. globosa, S. luriei, S. mexicana, and Sporothrix schenckii* sensu stricto) by molecular and phenotypic studies [1]. The disease is considered an emerging health problem, classically characterized by lesions of cutaneous and subcutaneous tissues with regional lymphocutaneous dissemination, although,

pulmonary and systemic infections may also occur [2].The extent of the disease varies with size and depth of the initial inoculum, and with the immune status of the host [3]. The observation that sporotrichosis is more severe, and usually disseminated, in nude mice [4] and in patients with acquired immune deficiency syndrome suggests that T cell-mediated immunity is important to limit the extent of infection [5].

Macrophages are important effectors cells in the immune response against different fungus, mediating phagocytosis and the release of toxic agents, such as reactive oxygen and nitrogen intermediates [6]. The pathogen recognition and activation of macrophages largely depends on a family of pattern recognition receptor (PRRs) as Toll-like receptors (TLRs), which are regarded the major innate recognition system for microbial invaders in vertebrates and they are involved in the recognition and immunity against fungal infection [7]. Recently, our group reported that TLR-2 [8, 9], TLR-4 [10, 11] and intracellular caspase 1 inflammasome [12] are key molecules involved in macrophages activation during the innate immune response to *S. schenckii* infection. In these and other studies, were evaluated the stimulation of macrophages by major components of the fungal wall cells. However, in a pioneer in vitro experiment developed in our laboratory was observed that other component released in the *S. schenckii* culture, named exoantigen (ExoAg), a complex mixture containing the peptidorhamnomannan, composed by 33.5 % rhamnose, 57 % mannose, and 14.2 % protein, was able of inhibit the phagocytic process with very low liberation of NO and TNF-alpha in macrophage cultures in comparison with components of the fungal wall [13]. This intriguing result supports a possible role of ExoAg in the virulence of *S. schenckii* [14]. In this way, Fernandes et al. [15] reported a possible association between virulence profile, ExoAg secretion, and immunogenicity of different *S. schenckii* isolates. However, even is insufficiently known the role of ExoAg released for this fungi during the infectious process.

The aim of this study was to investigate the dynamic in the production of hydrogen peroxide (H_2O_2) IL-1β, IL-6 by macrophages, and IL-2, IL-10 by lymphocytes, during a murine infection by *S. schenckii* and the effect of ExoAg in this process.

Materials and Methods

Animals

Male Swiss mice (6–8 weeks old, weighing 18–25 g) were obtained from the Animal House at the School of Pharmaceutical Sciences, UNESP (Araraquara, SP, Brazil), and maintained under specific pathogen-free conditions, with water and food available ad libitum in proper Animal Houses. At least five animals were used for each experiment. All procedures were approved by the Ethics in Research Committee of the São Paulo State University - UNESP and are in accordance with the National Institutes of Health Animal Care Guidelines (CEP/FCF/CAr n° 14/2009).

Microorganisms and Culture Conditions

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 sporotrichosis and phenotypically characterized at the Mycology Section of the Department of Dermatology, Columbia University, New York, NY. The fungus was cultured at 37 °C for 8 days in brain–heart infusion broth (DIFCO Laboratories, Detroit, MI) with constant rotary shaking at 150 cycles/min, resulting in a suspension of yeast cells.

Exoantigen (ExoAg) Derivation

The fungus cultured as described above was submitted to UV radiation for 1 h. This culture was shaken at 37 °C for 24 h and then UV-irradiated again for 1 h. After this procedure, merthiolate was added to the culture medium at 1/5000 concentration, and the culture was frozen at −20 °C for 48 h. Next, culture sterility was tested by the Sabouraud agar test, and the culture was filtered and concentrated 50–100 times in a concentrator (Amicon 8050, Danvers, MA). Protein was measured by the Lowry method, 1951 [16], and the protein composition was confirmed by Polyacrylamide Gel.

Polyacrylamide Gel Electrophoresis (PAGE) of ExoAg from *S. schenckii*

The one-dimensional gel electrophoresis was performed basically as described by Laemmli in 1970, with minor modifications. The antigen samples (containing 50 µg of protein) were resuspended in Line Marker Reducing sample buffer (Thermo Fisher Scientific, USA) and boiled for 5 min. Following, the protein samples were electrophoresed on 10 % polyacrylamide mini gel using a vertical slab gel apparatus (Thermo Fisher Scientific, USA) at a constant voltage of 100 V for 2 h. As standards, the PageRuler Plus prestained protein ladder (Thermo Fisher Scientific, USA) was used. The protein bands were visualized using silver staining.

Infection Method

The animal infection with *S. schenckii* was carried out with a yeast suspension in phosphate-buffered saline (PBS, pH 7.4), containing 10^7 cells/mL. Each animal was inoculated intraperitoneally with 100 µL of this suspension in the experimental group, while animals in the control group were injected with PBS. Mice were euthanized in a CO_2 chamber at different weeks after infection, and peritoneal cells of infected animals and control group were collected and re-stimulated in vitro with the Exo extract of *S. schenckii* yeast form. The groups were studied over a period of 10 weeks of infection.

Peritoneal Macrophages and Cells from Lymph Nodes

Thioglycollate-elicited peritoneal exudate cells (PECs) were harvested from Swiss mice using 500 µL of sterile phosphate-buffered saline (PBS), pH 7.4. The cells were washed twice by centrifugation at $200 \times g$ for 5 min at 4 °C and resuspended in appropriate medium for each test.

Cells from lymph nodes were also 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. The cells were then separated from the supernatant by centrifugation at $300 \times g$ for 5 min at 4 °C, washed once with 3 mL of RPMI complete medium and then resuspended in 1 mL of the same medium. Cell concentration was determined by microscopy using the Trypan blue exclusion test

and then they were adjusted to 5×10^6 cells/mL in RPMI complete medium.

Determination of Fungal Burden

The spleen was removed aseptically and macerated with sterile PBS. A 200 µL aliquot of this suspension was spread-plated on Sabouraud Agar medium in Petri dishes. Serial dilutions of 10^{-1} and 10^{-2} were also prepared and plated. The material was incubated for 5–7 days at room temperature, and the number of colonies formed on each plate (CFU) was counted, and the mean of viable fungi was then calculated for each group.

Flow Cytometry Analysis of Apoptotic Cells

The PECs were resuspended in RPMI 1640-C at a concentration of 2×10^6 cells/mL, and the adherent cells were obtained by a 1-h incubation at 37 C in an atmosphere with 5 % CO_2 (Forma Scientific). After this period, FLICA reagent (FAM-YVAD-fmk FLI-CATM Kit, Immunochemistry Technologies, Bloomington, MN, USA) was used to determine the percentage of apoptotic cells by flow cytometry, according to the manufacturer's instructions. The percentage of apoptotic cells was detected by flow cytometry with a FACSCanto flow cytometer and FACSDiva software (BD Biosciences, San Jose, CA, USA).

Hydrogen Peroxide Measurement

PECs (2×10^6 cells/mL), harvested as described above, were suspended in a solution containing 140 mM NaCl, 10 mM potassium phosphate buffer, pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red, and 0.01 mg/mL type II horseradish peroxidase. Aliquots of 100 µL of this suspension were added to the wells of a 96-well flat-bottomed tissue-culture plate and supplemented with 50 µl of 47.40 µg/mL ExoAg or 2×10^{-5} M of phorbol myristate acetate (PMA, Sigma, St. Louis, MO) or phosphate-buffered saline (PBS) for control wells. The cells were incubated for 1 h at 37 °C in a mixture of 95 % air and 5 % CO_2. The reaction was interrupted with 50 µl of 4 N NaOH, and the samples were read at 620 nm against a blank containing phenol red solution and 4 N NaOH. The results were expressed as nmols of H_2O_2 per 2×10^5

cells, calibrated against solutions of known H_2O_2 concentration. At least four experiments were used for the test [17, 18].

Cytokine Detection in Culture Supernatant

PECs and cells from lymph nodes (5×10^6 cells/mL), harvested as described above, were cultured overnight in RPMI 1640-C (with 100 U/mL penicillin, 100 µg/mL streptomycin, 5×10^{-2} M mercaptoethanol and 5 % inactivated fetal calf serum, designated RPMI 1640-complete (C) medium) at 37 °C under 5 % CO_2. The culture media contained 47.40 µg/mL ExoAg, 10 µg/mL LPS (positive control for PEC) or 0.25 µg/mL Concanavalin A (positive control for lymph nodes, and RPMI 1640-C (negative control), to stimulate production of cytokines IL-6 and IL-1β by PEC and IL-2 and IL-10 by lymph nodes. Cytokines released into the supernatant of these cell cultures were quantified by enzyme-linked immunosorbent assay (ELISA) performed in 96-well plates according to manufacturer guidance (Kit DuoSet®, R&D Systems, Minneapolis, MN). Absorbance was read at 450 nm on a Multiskan Ascent ELISA microplate reader (Thermo Labsystems, Finland). The results were expressed in picograms/mL.

Statistical Analysis

Tukey's test was used for statistical comparison between experimental groups. Significance was declared at $p < 0.001$. Data reported are representative of three independent experiments and are presented as the mean ± SD of quadruplicate or triplicate observations ($n = 12$). Data were analyzed statistically by the program GraphPad InStat.

Results

Polyacrylamide Gel Electrophoresis (PAGE) of ExoAg from *S. schenckii*

Figure 1 shows the profile of proteins secreted as part of the ExoAg released by the fungus in the culture. The gels show several protein bands with different molecular weights ranging in size from 250 to 10 kDa.

Fig. 1 Protein profile of exoantigen (ExoAg) from *S. schenckii*. *S. schenckii* strain 1099–18 was cultured at 37 °C for 8 days in brain–heart infusion broth. ExoAg pattern was obtained by one-dimensional gel electrophoresis (*lane 2*) compared with and the molecular masses (in kDa) of standard proteins (*lane 1*)
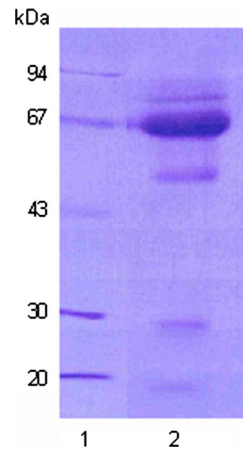
Determination of Fungal Burden

Interestingly, in this experiment while the H_2O_2 was elevated in the two first weeks of infection, the fungal burden in the spleen was diminished. Nevertheless, after that, it was found a pathogen's survival enhancing during the 4–6 weeks of infection, when a significant number of *S. schenckii* colonies were recovered (Fig. 2). However, the fungus recovered from the spleen was reduced significantly in the late phase of *S. schenckii* infection (eighth–tenth weeks).

Determination of Apoptosis in PECs

The apoptosis percentage was elevated in infected animals when compared with uninfected animals by all weeks of study. However, between the fourth and
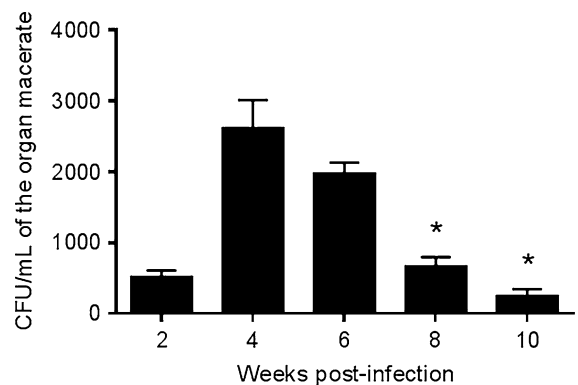

Fig. 2 Fungal burden (CFU/mL). Culture of microorganisms from spleen of animals infected through 2, 4, 6, 8 and 10 weeks. *Vertical bars* represent Mean ± SD; *$p < 0.001$ when compared to the fourth and sixth weeks (Tukey's test)
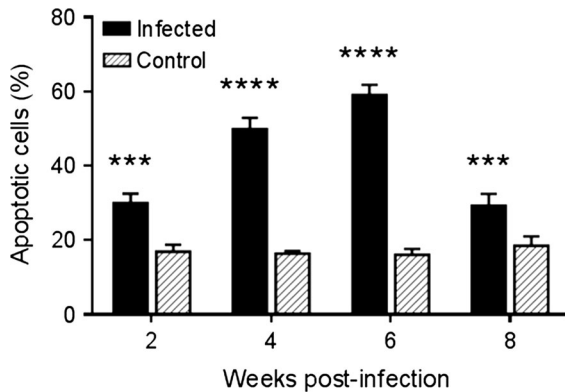
Fig. 3 Apoptosis in PECs from infected and uninfected with the fungus *S. schenckii*. In peritoneal exudate cells, after adhesion, of infected and uninfected mice the apoptosis was quantified by flow cytometry technique by determining FLICA and propidium iodide (PI). These results show values of three independent experiments. The results were expressed as percentage of cells undergoing apoptosis as mean ± SD of five animals each week of infection (***$p < 0.001$, ****$p < 0.0001$ when compared to uninfected mice)

sixth weeks of infection were observed significantly higher percent of apoptosis in comparison with the others weeks (Fig. 3).

Hydrogen Peroxide Measurement

In an attempt to understand the immune response during sporotrichosis, and to know the role of ExoAg released by *S. schenckii*, it was investigated the oxidative burst through monitoring of H_2O_2 production by PECs from infected or not infected animals stimulated by ExoAg. PECs from uninfected animals cultured and stimulated with PMA, but not with ExoAg, produced H_2O_2 throughout the studied period. On the other hand, both PMA and ExoAg stimulated H_2O_2 production by macrophages from infected animals in the second week post-infection. After that, ExoAg was unable to stimulate the H_2O_2 production, suggesting that the oxidative burst is stimulated by the ExoAg in the initial phase of *S. schenckii* infection with a withdrawal of this innate defensive mechanism in late stages of sporotrichosis (Fig. 4).

Cytokine Detection in Culture Supernatant

The ability of PECs to produce IL-6 and IL-1β was assayed too. Cells from uninfected animals (control group) produced IL-6 throughout the period, against LPS or ExoAg stimuli, while macrophages from infected animals produced IL-6 throughout the studied period; however, this production was less pronounced before the fourth and after the sixth week of infection (Fig. 5a). In contrast, in the infected mice, IL-1β was falling since the second week until a lower concentration at sixth week. After that it was observed a slight increment in the subsequent weeks, while in non-infected mice, there were not observed significant changes in IL-1β production during the evaluated time (Fig. 5b).

On the other hand, both IL-2 and IL-10 secreted by lymphocytes were initially elevated in the second week (Fig. 6a and b, respectively). After that, they were decreasing their production in the ulterior weeks in infected animals when stimulated by the ExoAg. Interestingly, ExoAg stimulated the IL-10 production more that ConA in the second week and sixth weeks (Fig. 6b). In non-infected mice, there were not also observed changes in the IL-2 and IL-10 regarding to the evaluated period.

Discussion

S. schenckii is a complex of environmental pathogenic and opportunistic fungi that affect humans and different domestic animals causing subcutaneous or rarely systemic infections. This pathogen has diverse virulence factor facilitating the environmental resistance and the host invasion [19]. ExoAg is a soluble component released in the culture media of *S. schenckii* [14, 20]. Although there are evidences of a possible role in the fungal virulence [14], even little is known about their role in the modulation of the immunity during the infectious process.

Host–fungus interactions are complex and dynamic, nd, once the fungi invade host cell, a series immunological mechanisms are activated, where the macrophages and the Th17 response are involved in the early fungal elimination [21, 22]. Activated macrophages release pro-inflammatory cytokines such as IL-1β and IL-6 and produce nitrogen and oxygen reactive species, such as nitric oxide (NO) and H_2O_2, that together participate in the elimination of the invading pathogen [14], but also these innate mechanisms can produce deleterious effects if they are strongly activated [23–26]. Thus, other cytokines of the specific immunity can participate in the control of
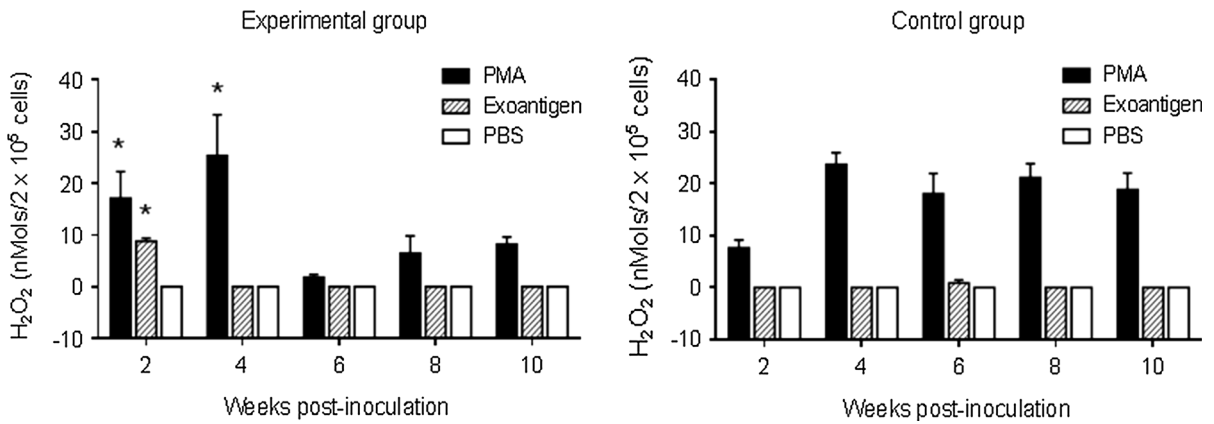
Fig. 4 Reactive oxygen intermediate induced during *S. schenckii* infection. Peritoneal exudate cells (PECs) (2×10^6 cells/mL) were cultured with exoantigen (ExoAg), PMA or PBS. After 1 h incubation at 37° C in a mixture of 95 % air and 5 % CO_2, the reaction was interrupted with 50 μL of 4 N NaOH, and the absorption at 620 nm was measured with an automated microplate reader. Results are reported as the mean ± SD of triplicate observations. The level of significance was set at $p < 0.005$ when H_2O_2 production by infected animals treated with ExoAg was compared with infected positive controls (PMA treated). Mean ± SD; *$p < 0.001$ (Tukey's test)
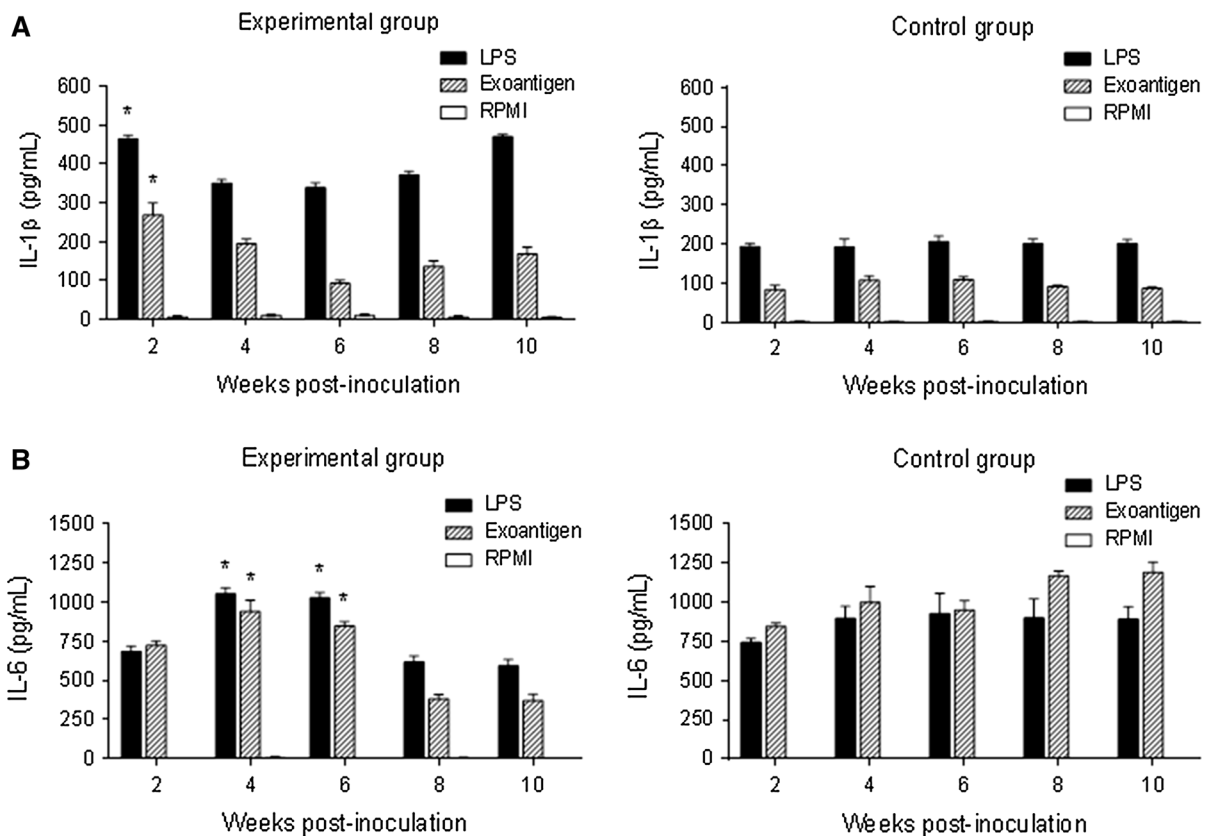

Fig. 5 IL-1β (**a**) and IL-6 (**b**) production by peritoneal macrophages of Swiss mice infected with *S. schenckii*. Peritoneal macrophage culture supernatants were obtained after 24-h stimulation with exoantigen (ExoAg) from *S. schenckii*, LPS or culture medium, and used in the IL-6, and IL-1β assay by ELISA at 450 nm. Results are reported as the Mean ± SD; *$p < 0.005$ regarding the lower data of the series (Tukey's test)
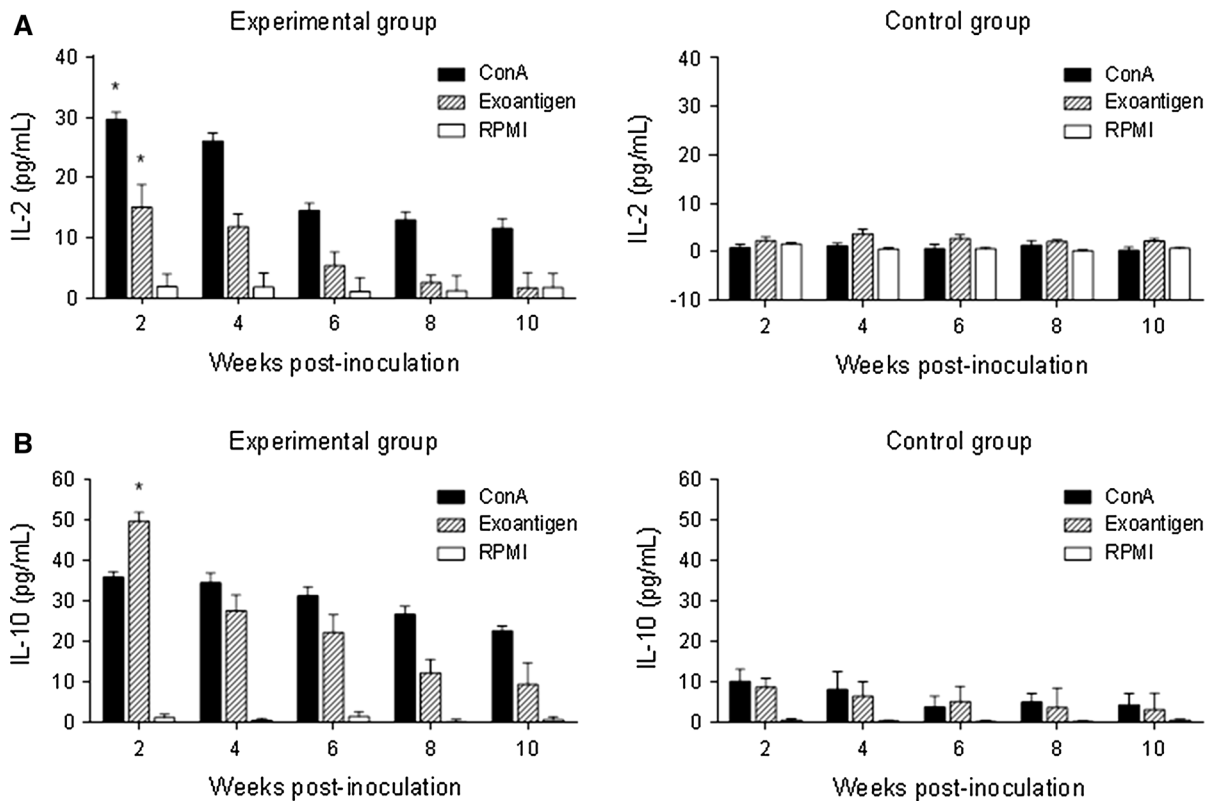
Fig. 6 IL-2 (**a**), and IL-10 (**b**) production in lymph nodes of Swiss mice infected with *S. schenckii*. Lymph nodes cells culture supernatants were obtained after 24-h stimulation with exoantigen (ExoAg) from *S. schenckii*, concanavalin A (ConA) or RPMI-1640-C culture medium, and used in the IL-2, and IL-10 assay by ELISA at 450 nm. Results are reported as the Mean $\pm$ SD; $*p < 0.005$ regarding the lower data of the series (Tukey's test)

the inflammatory response such as IL-10, that inhibit the production of inflammatory cytokines by dendritic cells, macrophages, and T cells and indirectly IL-2 required for maintenance of regulatory T cells [27].

Our results reveal a strong production of H_2O_2 in the second week associated with low fungal burden in the spleen. Interestingly in the following four weeks, the endpoints are inverted with a drastic depression of H_2O_2 and a pick of fungal burden with a subsequent depletion after the fourth week. This pattern was associated with an increment in apoptotic peritoneal macrophages during the period of greatest fungal burden, and this seems to be due to the infectious process.

These results also reinforces the data from previous studies in our laboratory, in which similar pattern in the production of H_2O_2 was observed in cells from TLR-4-deficient mice cultured with ExoAg, in the same period [11].

The ExoAg is continuously released throughout the infectious process [13]. Its ability to strongly stimulate the oxidative burst in the early phase of infection with ulterior withdrawal of H_2O_2 production, associated with the immunosuppressive activity of H_2O_2 for adaptive immunity [23, 24], may together facilities the spread of the fungus in the host organism if they are not completely eliminated during the first 2 week of infection. Fernandes et al. [25] used another model of sporotrichosis, inoculating intravenously 5×10^6 yeast and demonstrated that NO cause immunosuppression and it high production is detrimental in the defense against *S. schenckii*. Thus, H_2O_2 could be decisive in the early phase of infection, but detrimental in later phases. Certainly, excessive production of NO and H_2O_2, if they are unable to control de infection, can produce deleterious effects invoking a delay in developing acquired immunity through T cell unresponsiveness, favoring the microbial dissemination [27].

The reduction in IL-1β production by infected animal cells may also contribute to the impairment of fungal elimination. These results suggest that IL-1β production, but not IL-6, could have important role in the late phase of *S. schenckii* infection, contributing to the clearance of disease. In this way, Maia et al. [28] presented evidences of IFN-γ production in the same period on *S. schenckii* infection, as evidence that a Th1 response is elicited in the late phase of infection and could be also responsible for the low fungal burden in this period. Other characteristic detected in this study was the presence of elevated of IL-2 that can be involved in the stimulation of the adaptive immune response, but also in the control of excessive inflammatory response mediated by lymphocytes T regulatory (T reg). In addition, more evident was the high production of the anti-inflammatory IL-10, which can be associated with the apoptotic process induced by the fungal infection, as was observed in a murine model of fungal infection by *Candida albicans* [29]. This tendency to a transitory immunosuppressive profile observed between the fourth and sixth week may have favored the fungal load detected during this period.

In summary, our data suggest that IL-1β, IL-2, and H_2O_2 may be important during the first weeks of *S schenckii* infection, but after that, they are ineffective and even may favor the fungal dissemination due to the H_2O_2 immunosuppressive activity. The apoptosis associated with the infection can also be involved in the continue production of IL-10, contributing to the later transitory immune-suppressive setting. On the other hand, IL-1β (but not IL-6) may have important roles in the late phase of *S. schenckii* infection, contributing to the fungal clearance in conjunction with the specific mechanisms. Thus, the protective or deleterious role of these immune mediators, in vivo is complex, involving different factors such as: the time of the infection, the balance between pro and anti-inflammatory and other mechanisms that even need to be unraveled. Our data also highlight a role of the ExoAg as an important fungal component directly involved in the host immune modulation, influencing the *S. schenckii* virulence.

Compliance with Ethical Standards

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