

Immune Response Against *Sporothrix schenckii* in TLR-4-Deficient Mice

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Abstract For many fungal diseases, macrophages are the major cell population implicated in host protection, primarily by their ability to eliminate the invading fungal pathogen through phagocytosis. In sporotrichosis, this remains true, because of macrophages' ability to recognize *Sporothrix schenckii* through specific receptors for some of the fungus' cellular surface constituents. Further confirmation for macrophages' pivotal role in fungal diseases came with the identification of toll-like receptors, and the subsequent numerous associations found between TLR-4 deficiency and host susceptibility to diverse fungal pathogens. Involvement of TLR-4 in immune response against sporotrichosis has been conducted to investigate how TLR-4 signaling could affect inflammatory response development through evaluation of H₂O₂ production and IL-1 β , IL-6 and TGF- β release during the course of *S. schenckii* infection on TLR-4-deficient mice. The results showed that macrophages are largely dependent on TLR-4 for inflammatory activation and that in the absence of TLR-4 signaling, increased TGF- β release may be one of the contributing factors for the abrogated inflammatory

activation of peritoneal exudate cells during mice sporotrichosis.

Keywords *Sporothrix schenckii* · TLR-4 · TGF- β · Cytokines · Macrophages

Introduction

Sporotrichosis is a fungal disease caused by thermophilic fungus *Sporothrix schenckii*, commonly recovered from soil and plants, besides many other natural sources and from wild and domestic animals like cats, which have been implicated in a recent outburst of this infection in Rio de Janeiro, Brazil [1]. Thus, it is considered an occupational disease among gardeners, horticulturists, florists, miners and other related occupations in which workers are exposed to contaminated material and high incidence of injuries to the skin, both necessary for establishment of infection [2]. Sporotrichosis most commonly occurs in lymph-cutaneous form, which compromises skin, subcutaneous tissues and regional lymphatic vessels. Disseminated and systemic forms of the disease are less frequent and generally linked to immune-compromising conditions [3], as denoted by various reports of *S. schenckii* meningitis in HIV-infected individuals [4–6].

Macrophages are probably the most important immune cells for containing and terminating this

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infection, in part, because of their ability to recognize *S. schenckii* through specific receptors for some of the fungus' cellular surface constituents, which take part in fungus-phagocyte adherence, so that phagocytosis can occur [7–9]. Besides that, a role for macrophage-released cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), and the characteristic cellular immune response depression during the course of infection in a murine sporotrichosis model have already been shown [10–12]. Further on, cytokines induced during experimental sporotrichosis present a pattern that is characteristic of a strong Th1 response coinciding with the infection's worsening period [13].

Further evidence for macrophages' important role in sporotrichosis and other fungal diseases came with the identification of "Toll-like receptors" (TLRs), regarded as essential tools for directing the immune response after the first contact with the pathogen. These are transmembrane receptors mainly expressed on macrophages, where they act as PRRs ("pattern recognition receptors") that evolved to recognize highly conserved and unique bacterial metabolism products, known as PAMPs ("pathogen-associated molecular patterns") [14]. There is a variety of TLRs able to recognize a wide repertoire of microbial products, and we now know that TLR-4 is capable of binding mannans, which are structures shared by most fungal cell walls [15], leading primarily to the onset of an inflammatory response. Also, there have been various reports showing a role for this receptor in pathogen recognition and immune response triggering for several fungi such as *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Pneumocystis jirovecii* [16–18].

Research aiming to elucidate TLR-4 involvement in immune response to sporotrichosis has been conducted by our group in an animal model using C3H/HeJ and C3H/HePas mice [19, 20], which have defective or normal TLR-4 expression, respectively. The results obtained until now show that upon stimulation with a *S. schenckii* lipid extract, peritoneal macrophages from TLR-4-deficient mice release reduced levels of both pro- and anti-inflammatory mediators, affecting disease progression. We now seek to further clarify this phenomenon through assessment of the regulatory cytokine TGF- β along with other pro-inflammatory mediators under TLR-4 deficiency on C3H/HeJ mice.

Materials and Methods

Animals

Male, 6- to 10-week-old TLR4-deficient mice, strain C3H/HeJ, were obtained from the Animal House at the School of Pharmaceutical Sciences, UNESP (Araraquara, SP, Brazil), and the control mice, C3H/HePas, were purchased from the University of São Paulo (São Paulo, SP, Brazil) and maintained in the above-mentioned Animal House. Procedures involving animals and their care were conducted in conformity with rules laid down by the Research Ethics Committee (#43/2005), of the School of Pharmaceutical Sciences, UNESP, Araraquara, SP, Brazil.

Microorganisms and Culture Conditions

Sporothrix 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 at the Mycology Section of the Department of Dermatology, Columbia University, 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.

Lipid Extraction

Lipid extract (LeY) was prepared in the Laboratory of Fungal Surface Structures, Mycology Section, Department of Microbiology and Parasitology/Institute of Biomedical Science/Federal Fluminense University (UFF, RJ, Brasil) by Dr. Diana Bridon da Graça Sgarbi. Lipids were extracted from *S. schenckii* cultures grown at 37°C (yeast cells). The samples were subjected to lipid extraction with organic solvents, and the solutions were fractionated by chromatographic techniques (column chromatography and thin-layer chromatography on silica gel and paper chromatography).

Exoantigen Preparation

Fungus was cultured as described above, and the fungus culture was submitted to UV radiation for 1 h. This culture was maintained at 37°C for 24 h and then

resubmitted to UV radiation for 1 h as before. After this procedure, merthiolate was added to the culture medium at 1/5000 (v/v) concentration. The culture thus prepared 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) (*Exo*) [9]. Protein measurement was carried out by the method of Lowry et al. [21].

Infection Method

For *S. schenckii* infection, a yeast suspension in phosphate-buffered saline (PBS, pH 7.4), containing 10^7 cells/ml, was prepared. Each animal was inoculated intraperitoneally with 0.10 ml of this suspension in the experimental group, while animals in the control group were injected with 0.10 ml of PBS alone. Mice were killed at different times after infection, and peritoneal cells of infected animals and control group were collected and stimulated in vitro with the lipid extract of the *S. schenckii* yeast form.

Peritoneal Macrophages

Thioglycollate-elicited peritoneal exudate cells (PECs) were harvested from C3H/HeJ and C3H/HePas mice in 5.0 ml of sterile PBS (pH 7.4). The cells were washed twice by centrifugation at $200\times g$ for 5 min and resuspended in appropriate medium for each test.

Hydrogen Peroxide Assay

The PECs were resuspended in phosphate buffer pH 7.0 at a concentration of 2×10^5 cells/ml and exposed to phorbol 12-myristate 13-acetate (PMA), LeY, Exo or phosphate buffer alone for 2 h at 37°C in an atmosphere containing 5% CO_2 (Forma Scientific, Marietta, OH). Hydrogen peroxide levels were measured in cell culture supernatants by colorimetric reaction with phenol red [22]. Absorbance was read at 620 nm on a microplate reader (Multiskan Ascent, Labsystems), and H_2O_2 levels were calculated from a standard curve. The results were expressed in nmols/ 2×10^5 cells.

Cytokine Assays

The PECs were resuspended in RPMI-1640 Complete medium (RPMI-1640-C) at a concentration of 5×10^6

cells/ml, and adherent cells were obtained by incubation for 1 h at 37°C in an atmosphere containing 5% CO_2 (Forma Scientific, Marietta, OH). Non-adherent cells were removed by washing, and adherent cells were incubated with bacterial lipopolysaccharide (*Escherichia coli* 0111 B) (LPS), LeY, Exo or RPMI-1640-C medium. The levels of the cytokines IL- 1β , IL-6 and TGF- β in culture supernatants were determined by Enzyme-Linked Immunosorbent Assay (ELISA) (OptEIA; BD Biosciences, San Diego, CA) and performed in 96-well microplates, according to the manufacturer's instructions. Absorbance was read at 450 nm, within 30 min of stopping the reaction, on a microplate reader (Multiskan Ascent, Labsystems), and cytokine concentrations were calculated from a curve of known concentrations of each cytokine standard. The results were expressed in pg/ml.

Statistical Analysis

The Tukey's test (Prism Software, San Diego) was used to determine the statistical significance of differences between experimental groups. Significance was declared at $P < 0.05$. Data reported are representative of three independent experiments and are presented as the mean \pm SD of quadruplicate or triplicate observations. In vivo groups consisted of four to six animals.

Results

Hydrogen Peroxide

Oxidative burst was assessed through H_2O_2 release (Fig. 1) in the supernatants from PECs obtained from control or *S. schenckii*-infected C3H/HePas and C3H/HeJ mice and later cultured in the presence of PMA (positive control), LeY, Exo or culture medium alone (negative control). As expected, PMA induced H_2O_2 release in both strains at all time points ($P < 0.001$ compared to cells exposed to medium alone), although it was markedly increased in C3H/HePas compared to deficient mice ($P < 0.01$ at all time points). LeY stimulation was able to induce H_2O_2 only in infected wild-type mice ($P < 0.01$).

IL- 1β

IL- 1β release (Fig. 2) was assayed in the supernatants from PECs obtained from control or *S. schenckii*-

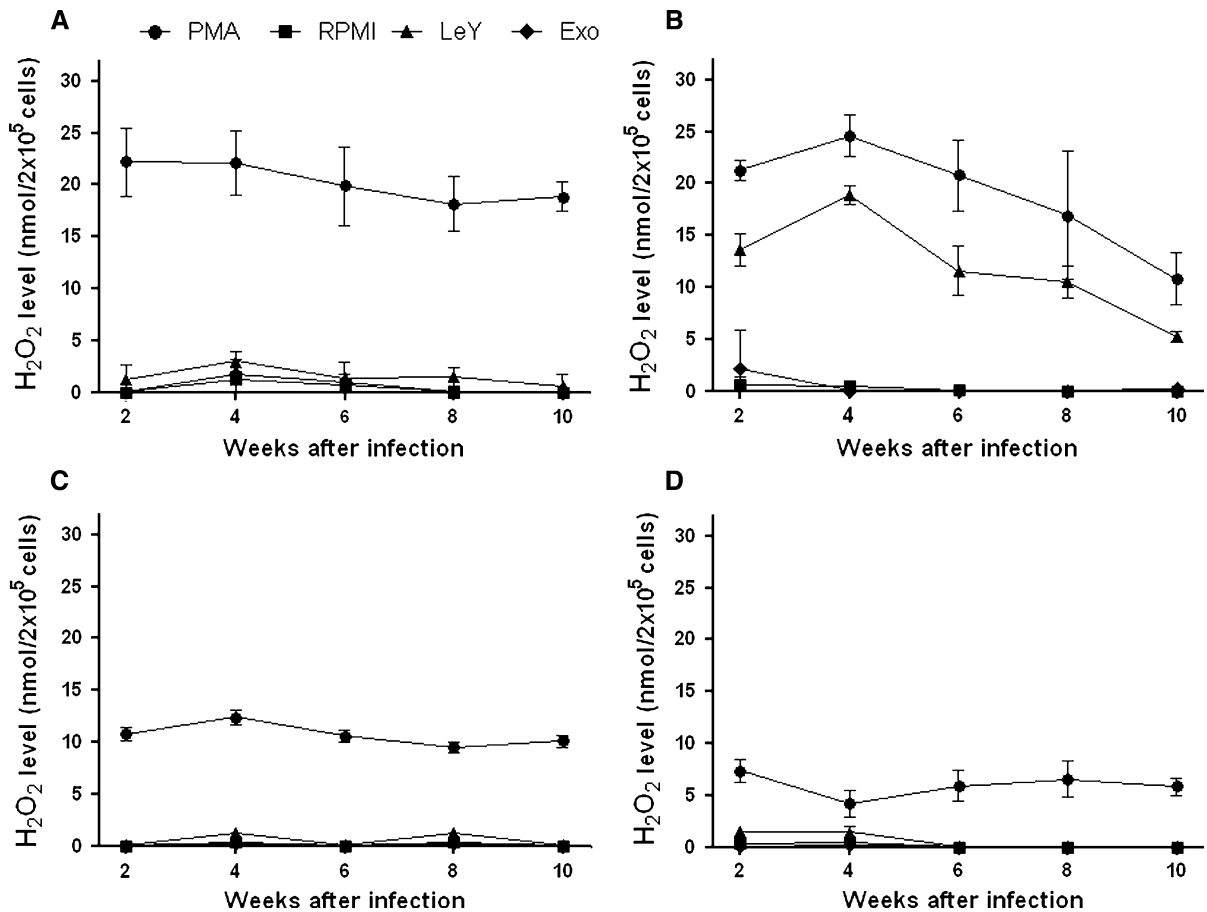


Fig. 1 Hydrogen peroxide measurement in supernatants from PECs obtained from control or *S. schenckii*-infected mice deficient or not for TLR-4. PECs were exposed ex vivo to PMA (positive control), *S. schenckii* yeast lipid extract (LeY), *S. schenckii* exoantigen (Exo) or RPMI medium only (negative control). **a** Control C3H/HePas mice; **b** infected C3H/HePas

mice; **c** control C3H/HeJ mice; **d** infected C3H/HeJ mice. PMA-induced H_2O_2 release in all situations. PMA-induced H_2O_2 release was elevated in C3H/HePas compared to TLR-4-deficient mice ($P < 0.01$ at all time points). LeY-s H_2O_2 release was increased in infected wild-type mice ($P < 0.01$) when compared to RPMI-stimulated release from those mice

infected C3H/HePas and C3H/HeJ mice and later exposed to LPS (positive control), LeY, Exo or culture medium alone. As expected, IL-1 β release was more evident in supernatants from PECs obtained from infected wild-type mice exposed to LPS ($P < 0.001$ at all time points) or LeY ($P < 0.001$ on 2nd, 6th, 8th and 10th weeks of study) compared to deficient mice. C3H/HeJ mice showed reduced release of this cytokine in response to LeY at the 6th and 8th weeks post-infection ($P < 0.001$ comparing to all other weeks), whereas C3H/HePas mice showed a proportional reduction at the 4th and 6th weeks ($P < 0.01$, comparing to all other weeks). As it can be seen in Fig. 2, Exo was not able to modulate IL-1 β release.

IL-6

IL-6 release (Fig. 3) was assayed in the supernatants from PECs obtained from control or *S. schenckii*-infected C3H/HePas and C3H/HeJ mice and later exposed to LPS, LeY, Exo or culture medium alone. LPS-stimulated IL-6 release was evident on both mouse strains, as expected, although IL-6 levels were markedly more elevated on C3H/HePas than on C3H/HeJ mice ($P < 0.001$ at all time points). On the other hand, LeY-stimulated release was statistically significant when compared to RPMI or Exo ($P < 0.001$ on all comparisons, at all time points), but it was similar for both mouse strains ($P > 0.05$). On 8th week post-

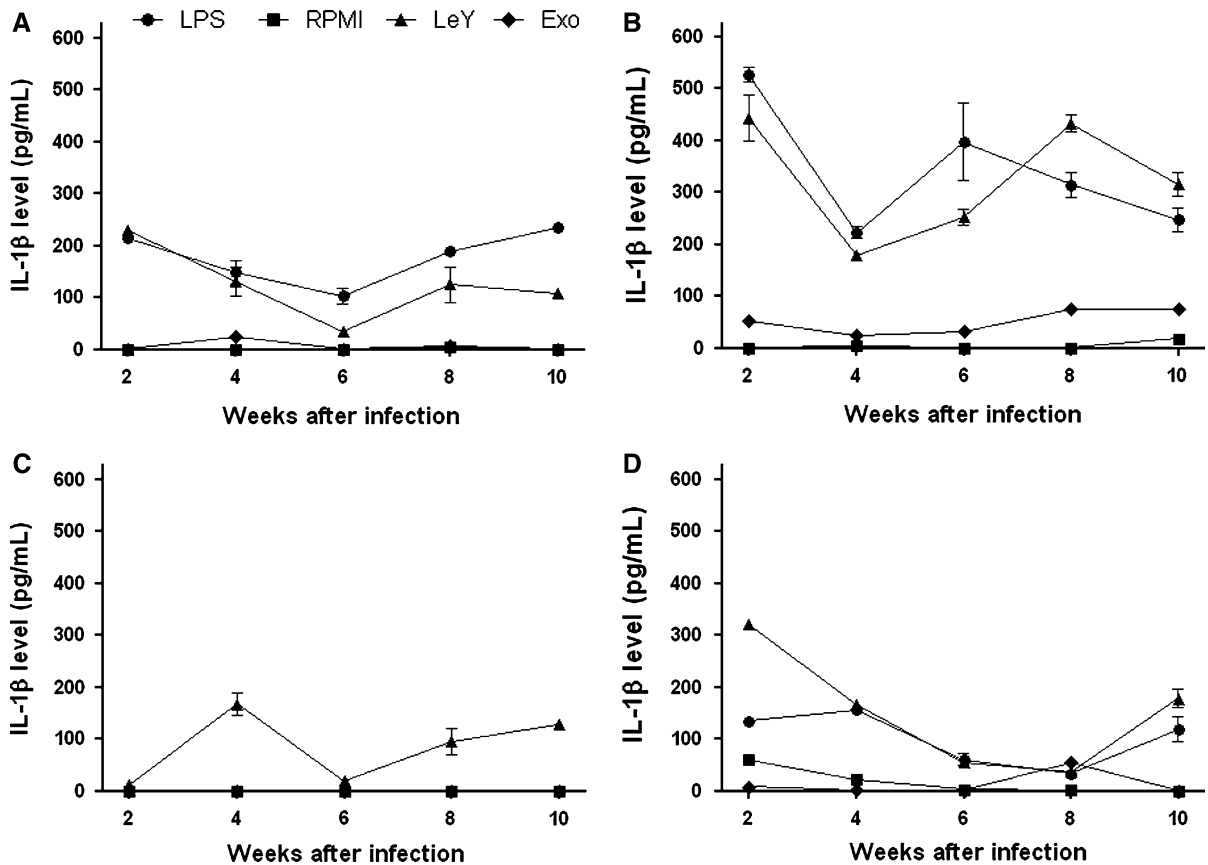


Fig. 2 IL-1 β quantification in supernatants from PECs obtained from control or *S. schenckii*-infected mice deficient or not for TLR-4. PECs were exposed ex vivo to LPS (positive control), *S. schenckii* yeast lipid extract (LeY), *S. schenckii* exoantigen (Exo) or RPMI medium only (negative control). **a** Control C3H/HePas mice; **b** infected C3H/HePas mice;

infection, LeY-stimulated IL-6 release in the supernatants from PECs obtained from TLR-4-deficient mice was diminished, compared to the 2nd, 4th and 6th weeks ($P < 0.05$). Exo could not induce this cytokine release in any of the mice strains.

TGF- β

TGF- β release (Fig. 4) was assayed in the supernatants from PECs obtained from control or *S. schenckii*-infected C3H/HePas and C3H/HeJ mice and later exposed to LPS, LeY, Exo or culture medium alone. Differently from what was observed so far, TGF- β release was markedly increased in culture supernatants from PECs obtained from infected deficient mice exposed to LeY ($P < 0.001$ at all time points), LPS ($P < 0.001$ at all time points) or Exo ($P < 0.001$ at the

c control C3H/HeJ mice; **d** infected C3H/HeJ mice. LPS-stimulated IL-1 β release in all situations ($P < 0.001$). LeY-stimulated IL-1 β release was more elevated in wild-type mice when compared to TLR-4-deficient mice ($P < 0.001$ at the 2nd, 6th, 8th and 10th weeks of study)

2nd, 4th, 8th and 10th weeks of study) compared to the wild-type strain. Unexpectedly, LeY-stimulated TGF- β release was even greater than that due to LPS stimulation on the former strain ($P < 0.001$ throughout the course of infection). Even though only infected C3H/HeJ mice were able to release TGF- β at detectable levels, a pattern similar to that observed for IL-1 β and IL-6 arises, with diminished release at the 4th and 6th weeks post-infection ($P < 0.01$ compared to all other weeks).

Discussion

There's a growing interest in TLRs and their role at determining the following immune response after the host's first contact with an invading pathogen. These

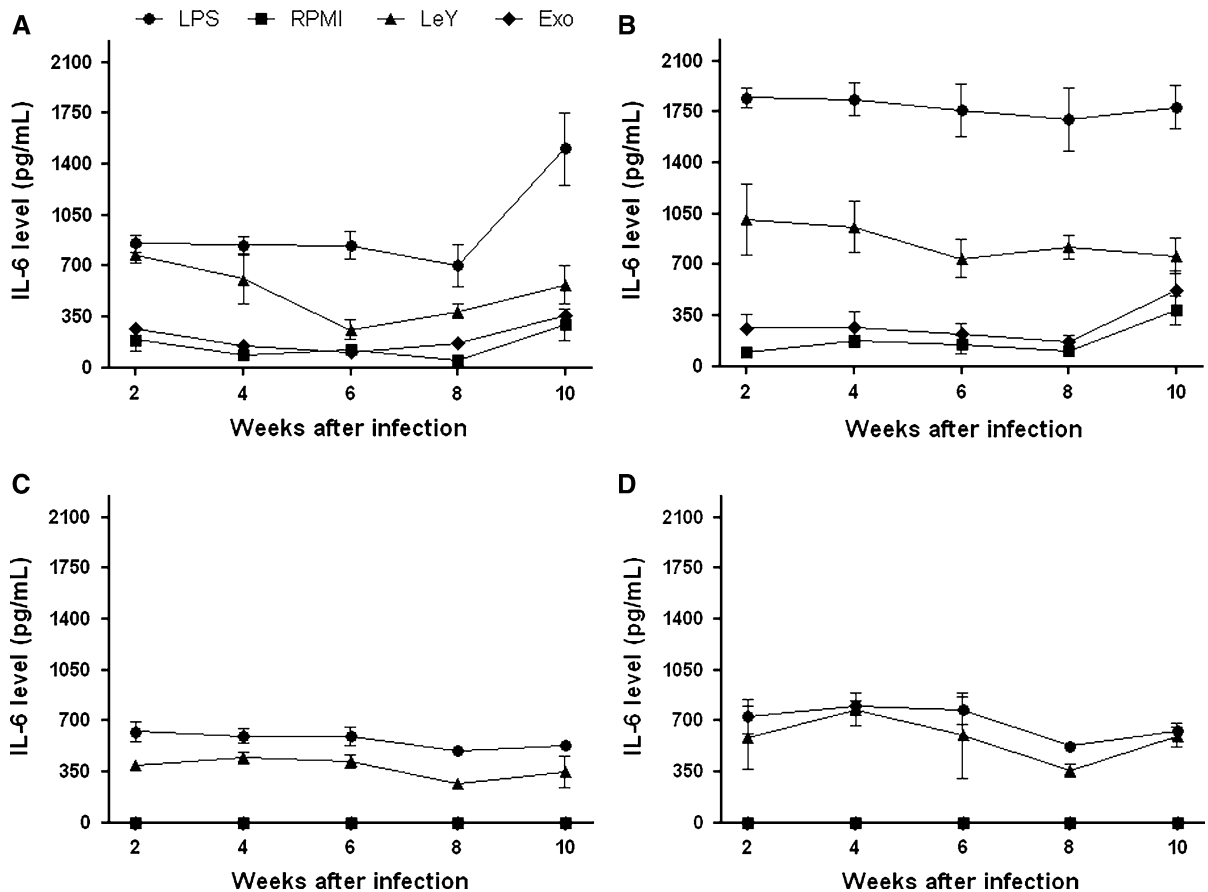


Fig. 3 IL-6 quantification in supernatants from PECs obtained from control or *S. schenckii*-infected mice deficient or not for TLR-4. PECs were exposed in vitro to LPS (positive control), *S. schenckii* yeast lipid extract (LeY), *S. schenckii* exoantigen (Exo) or RPMI medium only (negative control). **a** Control C3H/HePas mice; **b** infected C3H/HePas mice; **c** control C3H/HeJ

mice; **d** infected C3H/HeJ mice. LPS-induced IL-6 release was evident throughout the studied period ($P < 0.05$). IL-6 levels were more elevated in C3H/HePas when compared to C3H/HeJ mice ($P < 0.001$ throughout the study). LeY-stimulated release was significant when compared to RPMI in both mouse strains ($P < 0.001$ on all comparisons)

receptors are transmembrane complexes primarily expressed on macrophages, in which they work as PRRs (“pathogen recognition receptors”) that evolved to recognize highly conserved products unique to microbial metabolism, known as PAMPs (pathogen-associated molecular patterns) [23, 24]. Although there are over a dozen different TLRs, the one most commonly associated with the triggering of efficient Th1 anti-fungal responses is TLR-4, which is capable of recognizing mannan structures shared by most fungal cell walls [15].

We have previously shown that TLR-4-deficient mice (C3H/HeJ) release only basal levels of a series of pro-inflammatory mediators and present low PECs apoptosis rate during *S. schenckii* experimental

infection. On the other hand, mice bearing the wild-type TLR-4 (C3H/HePas) release much greater quantities of the same mediators at similar conditions, besides presenting much higher PECs apoptosis rate [20]. In consideration of that, we decided to expand from the previous study and to investigate how the absence of TLR-4 signaling could limit the development of the inflammatory response. We have so evaluated H_2O_2 production and IL-1 β , IL-6 and TGF- β release during *S. schenckii* infection on TLR-4-deficient mice. As demonstrated by our results, H_2O_2 production in response to PMA as well as IL-6 and IL-1 β in response to LPS on wild-type mice is significantly increased when compared to TLR-4-deficient mice. Together, these results show that macrophages’ activation toward the

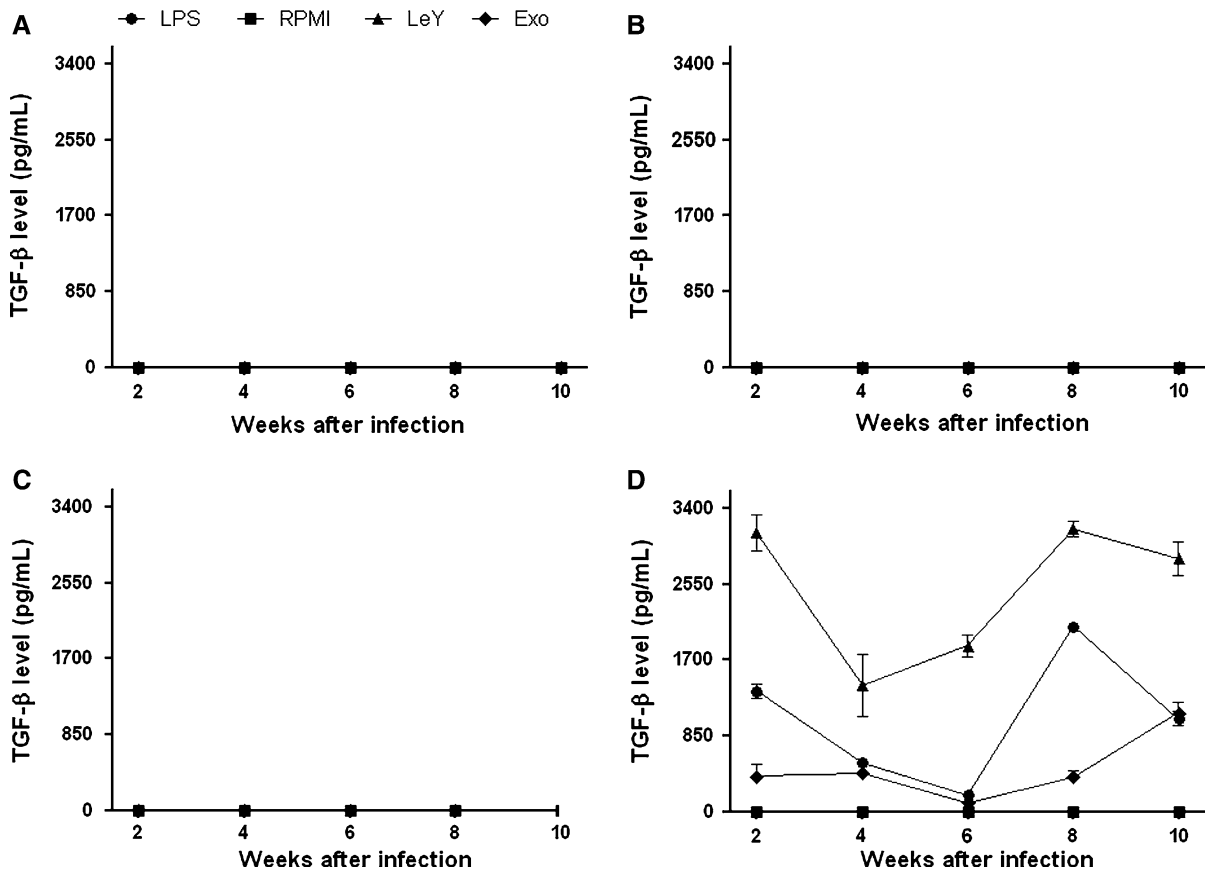


Fig. 4 TGF- β quantification in supernatants from PECs obtained from control or *S. schenckii*-infected mice deficient or not for TLR-4. PECs were exposed in vitro to LPS (positive control), *S. schenckii* yeast lipid extract (LeY), *S. schenckii* exoantigen (Exo) or RPMI medium only (negative control). **a** Control C3H/HePas mice; **b** infected C3H/HePas mice;

c control C3H/HeJ mice; **d** infected C3H/HeJ mice. PECs from C3H/HeJ infected mice exposed to LPS ($P < 0.001$ at all time points), LeY ($P < 0.001$ at all time points) or Exo ($P < 0.001$ at the 2nd, 4th, 8th and 10th weeks of study) showed increased release of this cytokine compared to wild-type mice

production and release of pro-inflammatory mediators during infection is largely dependent on TLR-4, such as seen on infection by *Paracoccidioides brasiliensis* [25, 26]. Also, various corroborating studies showed an important role for TLR-4 in protection against mycosis by *Candida albicans* [27, 28], *Aspergillus fumigatus* [29, 30] and *Pneumocystis jirovecii* [31]. However, not all fungal infections are adequately handled by TLR-4, as exemplified by *Cryptococcus neoformans* infections [32, 33].

In order to verify for antigen specificity of these responses during the infection, the release of the aforementioned cytokines were tested in response to two distinct *S. schenckii* antigen preparations: the fungus' yeast lipid extract (LeY) and its exoantigen (Exo). For H₂O₂ production, only PECs from wild-

type mice responded to LeY, but not to Exo stimulation, whereas TLR-4-deficient mice did not show any response to none of the antigens, which suggests that LeY-stimulated H₂O₂ production seems fully dependent on TLR-4. In regard to IL-1 β , PECs from infected mice of both strains were able to release increased levels of this cytokine when stimulated with LeY. However, when cells were stimulated with Exo, only wild-type mice showed increased IL-1 β release. In any case, wild-type mice released markedly higher levels of this cytokine, indicating that TLR-4 signaling is necessary for maximum IL-1 β release. Similar results were observed for IL-6, which was increased in infected wild-type mice when compared to TLR-4-deficient mice upon stimulation of PECs with LPS, LeY or Exo. It seems that in our model, IL-6 release is

TLR-4 dependent, and both fungal antigens tested rely on this receptor for maximum IL-6 induction. Additionally, LeY was able to induce IL-1 β and IL-6 release even on control uninfected mice of both strains, pointing toward an intrinsic potential disregarded of previous priming of cells by infection with *S. schenckii*.

In total, the results point to the necessity of TLR-4 for macrophages inflammatory activation, which is seriously impaired in the absence of this receptor's signaling. The main question lies in whether the absence of a functioning TLR-4 directly inhibits release of IL-1 β , IL-6 and H₂O₂ through lack of signals for their production or whether it does that indirectly, through modulation of the macrophage's activation profile or maybe by a combination of both mechanisms. To tackle that, we have evaluated the release of the regulatory cytokine TGF- β by PECs from both mouse strains during *S. schenckii* infection. Surprisingly, TGF- β release by PECs from TLR-4-deficient mice was markedly increased when compared to wild-type animals in response to any of the tested antigens. Indeed, PECs from wild-type mice could not release detectable levels of TGF- β in response to neither fungal antigens tested nor LPS. Taken together, our results suggest that, in the absence of TLR-4 signaling, increased TGF- β release may be one of the contributing factors for the abrogated inflammatory activation of PECs during mice sporotrichosis. Concordant results from Loures et al. [26] showed that increased TGF- β levels along with impaired release of pro-inflammatory mediators arise in C3H/HeJ mice infected with *P. brasiliensis*.

Also, by evaluating infection's evolution during the course of the 10 weeks, it was possible to spot various differences in cytokines release by both mouse strains, although some of these differences were clearer on one strain over the other. This is the case with IL-6, which kept high and almost invariable levels on infected wild-type mice throughout the studied period but showed a significant decrease at the 8th week on the TLR-4-deficient animals. In a similar fashion, IL-1 β release on TLR-4-deficient mice was also decreased during the 6th and 8th weeks in response to LPS or LeY, but not to Exo. Comparatively, wild-type mice presented an earlier decrease in IL-1 β release, during the 4th and 6th weeks post-infection, underlining the important role played by TLR-4 on immune response progression in this animal model. Regarding TGF- β ,

all the tested antigens showed significant reduction in this cytokine's release at the 6th week post-infection on TLR-4-deficient mice, the only ones whose PECs released detectable levels of this cytokine.

It is a known fact that TGF- β responses are commonly triggered by apoptotic events in the micro-environment as a result of mononuclear phagocytes interaction with apoptotic cells, serving as a mean to end the inflammatory response [34]. In face of the increased TGF- β release by PECs from TLR-4-deficient mice shown here along with the demonstration by early data that few cells are undergoing apoptosis in this condition [20], it can be suggested that this cytokine's release is not linked to apoptosis occurrence in the microenvironment, thus possibly corresponding to a direct effect of the fungus and its products over PECs. The former is in accordance with what was observed on wild-type mice, on which the high apoptosis rates are not followed by significant TGF- β release.

The similarity between the immune responses analyzed here in face of LPS or LeY stimulation led us to reflect about these antigens' composition and their interaction with TLR-4. LPS is a well-known TLR-4 ligand, composed of a hydrophobic portion A, hydrophilic polysaccharides and antigen O. It is known that the lipidic portion A corresponds to conserved molecular pattern of LPS, and it is the major inductor of biological responses linked to it [35, 36]. As LeY is mostly a lipidic antigen, it is possible that it interacts with TLR-4 in a similar fashion as LPS, justifying the similar pattern of immune responses generated. However, LeY seems to trigger other signaling pathways or engage different receptors, as it was able to induce greater TGF- β release than LPS. Also, engagement of different receptors is probably the cause for some of the low but significant release of the different mediators in TLR-4-deficient mice upon stimulation with PMA, LPS or LeY. These additional receptors could include other TLRs like TLR-2 and the C-type lectin receptors dectin-1 and macrophage mannose receptor (MR) [15]. Indeed, *S. schenckii* exoantigen is composed in part by mannans and β -1,3-glucans [37], structures known to be, respectively, recognized by the MR and dectin-1 [15], but even so it could not induce significant release of most mediators in the absence of TLR-4 signaling (aside from TGF- β , which is an odd case and was not significantly released in wild-type mice). Therefore, it is unlikely that engagement of these two receptors could be the main

trigger for some mediators' release in the absence of TLR-4 signaling. It is clear, then, that additional research is needed in order to evaluate the extent to which other PRRs contribute to *S. schenckii* recognition and how they interact with one another in different microenvironment conditions.

In conclusion, our data show that TLR-4 is important for the development of the inflammatory response during *S. schenckii* infection and also that some of the observed alterations in the absence of its signaling may be due to the effects of a TGF- β response, which arises only in this scenario.

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